APLICACIÓ DE NOUS MATERIALS I FORMATS AMB PROPIETATS MILLORADES PER A L' ANÀLISI DE DROGUES

AITOR SORRIBES SORIANO

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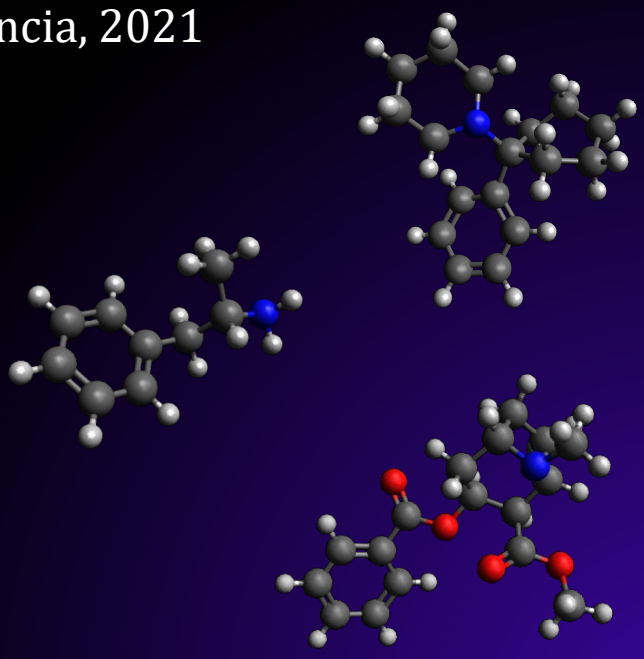

VNIVERSITAT DE VALÈNCIA Facultat de Química

TESI DOCTORAL

APLICACIÓ DE NOUS MATERIALS I FORMATS AMB PROPIETATS MILLORADES PER A L' ANÀLISI DE DROGUES

Aitor Sorribes Soriano

Programa de Doctorat en Química (R.D. 99/2011)

Directors:

José Manuel Herrero Martínez
Sergio Armenta Estrela
Francesc Albert Esteve Turrillas

València, maig 2021

VNIVERSITAT DE VALÈNCIA Facultat de Química

Universitat de València
Departament de Química Analítica
Campus de Burjassot-Paterna
46100 Burjassot, Espanya

El Dr. D. José Manuel Herrero Martínez, Catedràtic d'Universitat; el Dr. D. Sergio Armenta Estrela, Professor Titular d'Universitat i el Dr. D. Francesc A. Esteve Turrillas, Ajudant Doctor, del Departament de Química Analítica de la Universitat de València,

CERTIFIQUEN

Que D. Aitor Sorribes Soriano ha realitzat la present Tesi Doctoral titulada "Aplicació de nous materials i formats amb propietats millorades per a l'anàlisi de drogues" baix la seua direcció en el Departament de Química Analítica de la Universitat de València i autoritzen la seua presentació per a optar al Grau de Doctor en Química.

I per què conste, als efectes oportuns, signen la present a Burjassot, abril de 2021.

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Aquesta Tesi Doctoral ha sigut escrita sota la modalitat “compendi de publicacions” contemplada al Reglament de la Universitat de València de 29-11/2011 (ACGUV 266/2011). D’acord amb dita normativa, la Tesi Doctoral inclou en primer lloc una introducció general i a continuació un resum global de la temàtica, resultats i conclusions de tots els treballs compendiats. Tanmateix s’inclouen també els articles ja publicats, els quals corresponen en la seua majoria a revistes indexades. El doctorand ha contribuït substancialment a totes les etapes del desenvolupament de tots els articles, des de l’elaboració de la idea, recerca bibliogràfica, realització experimental, anàlisi i interpretació de les dades obtingudes, redacció i preparació del manuscrit, seguiment i correcció final del mateix d’acord a les recomanacions dels avaluadors. Tots els articles han estat escrits per Aitor Sorribes Soriano amb correccions i revisió final per part dels supervisors d’aquesta Tesi Doctoral.

Títol	*Any de publicació*	*Revista*	*Índex impacte*
Cocaine abuse determination by ion mobility spectrometry using molecular imprinting	*2017*	*Journal of Chromatography A*	*3.716*
Magnetic molecularly imprinted polymers for the selective determination of cocaine by ion mobility spectrometry	*2018*	*Journal of Chromatography A*	*3.858*
Trace analysis by ion mobility spectrometry: From conventional to smart sample preconcentration methods: a review	*2018*	*Analytica Chimica Acta*	*5.256*
Amphetamine-type stimulants analysis in oral fluid based on molecularly imprinting extraction	*2019*	*Analytica Chimica Acta*	*5.977*
Development of a molecularly imprinted monolithic polymer disk for agitation-extraction of ecgonine methyl ester from environmental water	*2019*	*Talanta*	*5.339*
Determination of the new psychoactive substance dichloropane in saliva by microextraction by packed sorbent – ion mobility spectrometry	*2019*	*Journal of Chromatography A*	*4.049*
Development of pipette-tip-based poly (methacrylic acid-co-ethylene glycol dimethacrylate) monolith for the extraction of drugs of abuse from oral fluid samples	*2019*	*Talanta*	*5.339*
Methylone determination in oral fluid using microextraction by packed sorbent coupled to ion mobility spectrometry	*2020*	*Microchemical Journal*	*3.594*
Tuning the selectivity of molecularly imprinted polymer extraction of arylcyclohexylamines: From class-selective to specific	*2020*	*Analytica Chimica Acta*	*5.977*
Molecularly imprinted polymer-based device for field collection of oral fluid samples for cocaine identification	*2020*	*Journal of Chromatography A*	*4.049*
Determination of third-generation synthetic cannabinoids in oral fluids	*2021*	*Journal of Analytical Toxicology*	*3.513*
Dual mixed-mode poly (vinylpyridine-co-methacrylic acid-co-ethylene glycol dimethacrylate)-based sorbent for acidic and basic drug extraction	*2021*	*Analytica Chimica Acta (Sent to publication)*	*5.977*

Aquesta Tesi Doctoral ha sigut realitzada gràcies a una beca predoctoral (ACIF-2017/386) concedida per la Conselleria d'Educació, Investigació, Cultura i Esport de la Generalitat Valenciana.

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ABREVIATURES / *ABBREVIATIONS*

α-PHP: α-Pirrolidinohexiofenona / *α-Pyrrolidinohexiophenone*

α-PVP: α-Pirrolidinopentiofenona / *α-Pyrrolidinopentiophenone*

ϒ-MPS: 3-(Trimetoxisilil)propil metacrilat */ (3-(Trimethoxysilil)propyl methacrylate*

µ-SPE: Micro-extracció en fase sòlida / *Micro-solid phase extraction*

2-DPMP: Desoxipipradol / *Desoxypipradrol*

2-FA: 2-Fluoroamfetamina / *2-Fluoroamphetamine*

2-FMA: 2-Fluorometamfetamina / *2-Fluoromethamphetamine*

2-oxo PCE: Descloro-N-etilketamina / *Deschloro-N-ethyl ketamine*

3,4-MDPHP: 3,4-Metilenedioxi-α-pirrolidinohexanofenona / *3,4-Methylenedioxy-α-pyrrolidinohexanophenone*

3-FA: 3-Fluoroamfetamina / *3-Fluoroamphetamine*

3-FEA: 3-Fluoroetamfetamina / *3-Fluoroethamphetamine*

3-FMC: 3-Fluorometacatinona / *3-Fluoromethcathinone*

3-MeO PCP: 3-Metoxifenciclidina / *3-Methoxyphencyclidine*

3-MMA: 3-Metilmetamfetamina / *3-Metilmethamphetamine*

3-MMC: 3-Metilmetacatinona / *3-Methylmethcathinone*

3-OH PCP: 3-Hidroxifenciclidina / *3-Hydroxyphencyclidine*

4-CEC: 4-Cloroetacatinona / *4-Chloroethacathinone*

4-CMC: 4-Clorometacatinona / *4-Chloromethcathinone*

4-FMA: 4-Fluorometamfetamina / *4-Fluoromethamphetamine*

4-MEC: 4-Metiletacatinona / *4-Methylethacathinone*

4-MeMABP: 4-Metilbufedrona / *4-Methylbuphedrone*

4VP: 4-Vinilpiridina / *4-Vinylpiridine*

5-EAPB: (1-(Benzofurà-5-il)-N-etilpropan-2-amina / *(1-(Benzofuran-5-yl)-N-ethylpropan-2-amine*

5-IT: 5-(2-Aminopropil)indol / *5-(2-Aminopropyl)indole*

5-MeO-AMT: 5-Metoxi-α-metiltriptamina / *5-Methoxy-α-methyltryptamine*

6-APB: 6-(2-Aminopropil)benzofurà / *6-(2-Aminopropyl)benzofuran*

AA: Àcid acrílic / *Acrylic acid*

AIBN: Azobisisobutironitril / *Azobisisobutyronitrile*

AIMS: Espectrometria de mobilitat iònica d'aspiració / *Aspiration ion mobility spectrometry*

APCI: Ionització química a pressió atmosfèrica / *Ambiental preasure chemical ionization*

BB: Tampó de càrrega / *Binding buffer*

BE: Benzoilecgonina / *Benzoylecgonine*

BZP: Benzilpiperazina / *Benzylpiperazine*

CBD: Cannabidiol / *Cannabidiol*

CBN: Cannabinol / *Cannabinol*

CE: Electroforesi capil·lar / *Capillary electrophoresis*

CNTs: Nanotubs de carboni / *Carbon nanotubes*

DAD: Detector de fila de diodes / *Diode array detector*

DLLE: Extracció líquid-líquid dispersiva / *Dispersive liquid-liquid extraction*

DMA: Analitzador de mobilitat diferencial / *Differential mobility analyzer*

DMT: Dimetilltriptamina / *Dimethyltriptamine*

DOC: 2,5-Dimetoxi-4-cloroamfetamina / *2,5-Dimethoxy-4-chloroamphetamine*

DSPE: Extracció en fase sòlida dispersiva / *Dispersive solid phase extraction*

DTIMS: Espectrometria de mobilitat iònica de temps de deriva / *Drift time ion mobility spectrometry*

DVB: Divinilbenzé / *Divinylbenzene*

EGDMA: Dimetacrilat d'etilenglicol / *Ethylene glycol dimethacrylate*

ESI: Ionització per electrosprai / *Electrospray ionization*

FAIMS: Espectrometria de mobilitat iònica de camp asimètric / *Field assymetric ion mobility spectrometry*

FID: Detector de ionització en flama / *Flame ionization detector*

FTIR: Espectroscòpia infraroja amb transformada de Fourier / *Fourier transform infrared spectroscopy*

GC: Cromatografia de gasos / *Gas chromatography*

GC-MS: Cromatografia de gasos acoblada a espectrometria de masses / *Gas chromatography - mass spectrometry*

GHB: Àcid ϒ-hidroxibutíric / *ϒ-Hydroxybutyric acid*

HEMA: 2-Hidroxietilmetacrilat / *2-Hydroxyethylmethacrylate*

IIP: Polímer de reconeixement iònic / *Ionic imprinted polymer*

IL: Líquid iònic / *Ionic liquid*

IMS: Espectrometria de mobilitat iònica / *Ion mobility spectrometry*

IR: Infraroig / *Infrared*

LC: Cromatografia líquida / *Liquid chromatography*

LC- UV: Cromatografia líquida amb detector ultraviolat / *Liquid chromatography ultraviolet detection*

LC-MS/MS : Cromatografia líquida acoblada a espectrometria de masses en tándem / *Liquid chromatography tandem mass spectrometry*

LFIA: Immunoassaig de flux lateral / *Lateral flow immunoassay*

LLE: Extracció líquid-líquid / *Liquid-liquid extraction*

LOD: Límit de detecció / *Limit of detection*

LOQ : Límit de quantificació / *Limit of quantification*

LSA: Amida d'àcid D-lisèrgic / D-Lysergic acid amine

LSD: Dietilamida de l'àcid D-lisèrgic / D-*Lysergic acid diethylamide*

MAA: Àcid metacrílic / *Methacrylic acid*

MAPA: Metacrilolamidofenilanalina / *Methacryloylamidophenylalanine*

MBI: 2- Mercaptobenzimidazol / *2- Mercaptobenzimidazole*

MDMA: Metilendioximetanfetamina / *Methylenedioxymethamphetamine*

MDPBP: 3 ', 4'-Metilendioxi-α-pirrolidinobutirofenona / *3',4'-Methylenedioxy-α-pyrrolidinobutyrophenone*

MDPHP: 3',4'-Metilenedioxi-α-pirrolidinohexiofenone / *3',4'-Methylenedioxy-α-pyrrolidinohexiophenone*

MDPPP: 3',4'-Metilendioxi-α-pirrolidinopropiofenona / *3',4'-Methylenedioxy-α-pyrrolidinopropiophenone*

MDPV: Metilendioxipirovalerona / *Methylenedioxypyrovalerone*

MDSPE: Extracció en fase sòlida dispersiva magnètica / *Magnetic dispersive solid phase microextraction*

MeOPP: p-Metoxifenilpiperazina / *p-Methoxyphenylpiperazine*

MEPS: Microextracció amb adsorbent empaquetat / *Microextraction by packed sorbent*

MIP: Polímer de reconeixement molecular / *Molecularly imprinted polymer*

MMIP: Polímer de reconeixement molecular magnètic / *Magnetic molecularly imprinted polymer*

MMS: Adsorbent de tipus mixt / *Mixed-mode sorbent*

MNP: Nanopartícula magnètica / *Magnetic nanoparticle*

MOF: Xarxa organometàl·lica / *Metal organic framework*

MS: Espectrometria de masses / M*ass spectrometry*

MSPE: Extracció en fase sòlida magnètica / *Magnetic solid phase extraction*

MWCNT: Nanotub de carboni multicapa / *Multi-walled carbon nanotube*

MXE: Metoxetamina / *Methoxethamine*

NIP: Polímer no empremtat / *Non-imprinted polymer*

NMR: Resonància magnètica nuclear / *Nuclear magnetic ressonance*

NPS: Noves substàncies psicoactives / *New psychoactive substances*

OLIMS: Espectrometria de mobilitat iònica de llaç obert / *Open loop ion mobility spectrometry*

PCP: Fenciclidina */ Phencyclidine*

PDMS: Polidimetilsiloxà / *Polydimethylsiloxane*

PE: Polietilé / *Polyethylene*

PEG-MMIP: Polímer de reconeixement molecular magnètica amb polietilenglicol / *Polyehtylenglycol magnetic molecularly imprinted polymer*

PP: Polipropilè / *Polypropylene*

PTFE: Politetrafluoroetilè / *Polytetrafluoroethylene*

RAM: Material d'accés restringit / *Restricted access material*

RDSE: Extracció adsorbent amb disc rotatori / *Rotating disk sorptive extraction*

SAX: Bescanvi aniònic fort / *Strong anion exchange*

SBSE: Extracció adsorbent amb vareta agitadora / *Stir bar sorptive extraction*

SCSE: Extracció adsorbent amb disc agitador / *Stir cake sorptive extraction*

SCX: Bescanvi catiònic fort / *Strong cation exchange*

SDME: Microextracció per gota simple / *Single drop microextraction*

SELEX: Evolució sistemàtica dels lligands mitjançant concentració exponencial / *Systematic evolution of ligands by exponential enrichment*

SEM: Microscòpia electrònica d'escombratge / *Scanning electron microscopy*

SPDE: Extracció en fase sòlida dinàmica / *Solid phase dynamic extraction*

SPE: Extracció en fase sòlida / Solid phase extraction

SPME: Microextracció en fase sòlida / *Solid phase microextraction*

SRSE: Extracció adsorbent amb vareta d'agitació / *Stir rod sorptive extraction*

SWCNT: Nanotubs de carboni d'una capa / S*ingle-walled carbon nanotubes*

THC: Tetrahidrocannabinol / *Tetrahydrocannabinol*

THC-COOH: 11-Nor-9-carboxitetrahidrocannabinol / *11-Nor-9-carboxy-tetrahydrocannabinol*

THC-OH: 11-Hidroxi- *δ* -9-tetrahidrocannabinol / *11-hydroxy-δ-9-tetrahydrocannabinol*

TIMS: Espectrometria de mobilitat iònica de trampa / *Trapped ion mobility spectrometry*

TWIMS: Espectrometria de mobilitat iònica d'ona viatgera / *Travelling wave ion mobility spectrometry*

UE / EU: Unió Europea / *European Union*

UHPLC-MS/MS: Cromatografia líquida d'alt rendiment acoblada a espectrometria de masses en tàndem / *Ultra high performance líquid chromatography tandem mass spectrometry*

UNODC: Oficina de les Nacions Unides contra les drogues i el crim organitzat / *United Nations Office on Drug and Crime*

UV: Ultravioleta / *Ultraviolet*

V-MMIP: Polímer de reconeixement molecular magnètic amb grups vinil / *Vinylized magnetic molecularly imprinted polymer*

WAX: Bescanvi aniònic feble / *Weak anion Exchange*

WCX: Bescanvi catiònic feble / *Weak cation Exchange*

TAULA DE CONTINGUTS / ***TABLE OF CONTENTS***

INTRODUCCIÓ

1. CONSUM DE DROGUES A LA SOCIETAT

Durant l'any 2018, 269 milions de persones van declarar haver consumit drogues en algun moment de l'any anterior; aquest fet suposa que el 5,3 % de la població adulta d'entre 15 i 64 anys havia consumit algun tipus de drogues [WDR 2019]. Aquesta tendència si és comparada amb dades del 2009 reflecteix un augment del consum generalitzat, ja que en aquest any el nombre de consumidors habituals o puntuals era de 210 milions de persones, la qual cosa suposa un augment d'un 30 % en només 9 anys. A més dels propis riscos que comporta el consum d'aquest tipus de substàncies, nombroses malalties com hepatitis C o virus d'immunodeficiència humana (VIH) estan íntimament relacionades amb el seu consum [WDR 2020]. Així doncs, el nombre de morts a tot el món per problemes relacionats amb el consum de drogues es va situar al 2017 en 585.000 morts, segons dades de la Oficina de les Nacions Unides Contra les Drogues i el Crim Organitzat (*United Nations Office on Drug and Crime, UNODC)* als seus *World Drug Report* del 2019 i 2020 [WDR 2019; WDR 2020]. Aquest mateix organisme, ha informat que durant el 2017 la producció, així com les confiscacions de cocaïna han experimentat un creixement notable, de la mateixa manera que ho ha fet el seu consum a països de l'Amèrica del Nord i Europa Occidental i Central. Tanmateix, el consum d'altres drogues com metamfetamina s'ha vist incrementat a regions d'Àsia Sud-oriental.

Segons dades del Ministeri de Sanitat del Govern Espanyol al voltant d'un terç de la població espanyola ha consumit algun tipus de substància il·lícita al llarg de la seua vida, mantenint-se aquest percentatge constant al llarg dels darrers anys. D'entre aquestes substàncies il·legals, el cànnabis i la cocaïna continuen estant al capdavant fent referència al seu consum. A més, el seu consum arreu de tot l'Estat Espanyol es situa en nivells considerablement elevats respecte d'altres països de la Unió Europea (UE). S'estima que al voltant del 12 % dels conductors d'automòbils han consumit alguna droga, seguint una tendència a l'alçada en els darrers anys el que suposa greus problemes de seguretat pública [Ministerio de Sanidad 2017]

Les principals vies d'introducció de drogues d'abús tradicionals com la cocaïna al mercat europeu han estat per via marítima a través de diferents rutes com la "Ruta del Nord o dels velers", la "Ruta Central o dels pesquers", la "Ruta Africana" i la "Ruta de la Mediterrània". A l'any 2016, les confiscacions de substàncies il·legals anaven de 15 tones de cocaïna, fins a les 346 tones d'èxtasi o

324 tones d'haixix, seguit de les confiscacions de plantes de cànnabis que es van situar en 724 tones [WDR 2019].

Un problema afegit al tràfic i consum de drogues d'abús el trobem en l'aparició de Noves Substàncies Psicoactives (NPS) que ha suposat una nova càrrega a les administracions públiques a l'hora de normativitzar-les. Les NPS són substàncies, habitualment derivades de drogues conegudes i àmpliament utilitzades i legislades que presenten canvis mínims en la seua estructura química, amb la intenció d'imitar i inclús potenciar els seus efectes sense que aquestes siguin reconegudes com substàncies il·legals per les autoritats. A dia d'avui, més de 60 països han donat resposta legal a la complicada situació a causa de l'aparició de les NPS als seus respectius territoris. D'entre les diferents respostes possibles als països que s'han vist més afectats per la distribució i han donat una resposta més efectiva cal destacar l'enfocament generalista de la seua regularització, sotmetent sota legislació famílies de compostos i en extensió tota aquella substància d'estructura similar que pogués aparèixer [UNODC 2018].

A diferència dels mètodes d'introducció de substàncies més tradicionals com la cocaïna, la principal via d'entrada de les NPS a l'Estat Espanyol és l'aèria; essent la seua principal via de comercialització i distribució a través de portals web on es poden adquirir fàcilment realitzant la majoria d'enviaments a través de paqueteria postal. A més, el seu consum no mostra una tendència regular en el temps, ja que aquest ve considerablement determinat per la situació socioeconòmica, observant un major consum a l'arribada de crisis econòmiques a causa del seu menor preu en el mercat. Així, després de la crisi de 2008 i coincidint amb un canvi en la legislació xinesa que regulava la venta de precursors, es va observar un repunt de la distribució de NPS com a conseqüència d'una manca de drogues més clàssiques com la metamfetamina [UNODC 2018].

2. CLASSIFICACIÓ DE DROGUES

Les drogues poden ser classificades de moltes maneres diferents, entre aquestes es poden fer distincions segons els seus usos, els seus efectes, el seu valor terapèutic o les seues estructures químiques. A aquest apartat les drogues seran separades en dos grans grups, d'una banda les drogues tradicionals com la cocaïna, amfetamina, heroïna o cànnabis entre altres i d'altra banda s'hi farà una classificació de les NPS. Dins de cadascun dels apartats es poden fer algunes classificacions més útils des d'un punt de vista pràctic com són segons la seua fiscalització o els seus efectes.

2.1. DROGUES TRADICIONALS

Des del punt de vista legal, una de les classificacions més interessants és la que es fa segons els usos mèdics, potencials efectes d'addició i riscos per a la salut que aquestes drogues puguin presentar. Segons el sistema de les Nacions Unides, 250 substàncies estan incloses a quatre llistats diferents que es van crear a la *Single Convenction on Narcotic Drugs (*Nova York, 1961); la *Convenction on Psychotropic Substances (*Viena, 1971) i la *Convenction against Illicit Traffic in Narcotic Drugs and Psychotropic Substances (*Viena, 1988) la qual, a més, incloïa el control de precursors.

Els narcòtics estan classificats segons els quatre llistats que es mostren a la Taula 1, establert a la convenció del 1961.

Taula 1. *Classificació de narcòtics segons la Convenction on Narcotic Drugs (Nova York, 1961).*

Llistat	Perillositat	Fiscalització	Exemples
I	Substàncies amb elevat risc d'addició	Molt estricta	Cànnabis i derivats, cocaïna, heroïna, metadona, morfina, opi
II	Substàncies amb usos mèdics i menor risc d'addició	Menys estricta	Codeïna, dihidrocodeïna, propiram
III	Preparats de substàncies del llistat II o de cocaïna	Indulgent	Mescles que contenen codeïna, dihidrocodeïna o propiram
IV	Substàncies del llistat I d'especial perillositat amb valor terapèutic limitat	Molt estricte	Cànnabis i resina de cànnabis, heroïna

D'altra banda les substàncies psicotròpiques van ser limitades a usos mèdics i científics a la convenció de 1971 i classificades en els quatre llistats que es mostren a la Taula 2.

Taula 2. *Classificació de substàncies psicotròpiques segons la Convenction on Psychotropic Substances (Viena, 1971).*

Llistat	Perillositat	Fiscalització	Exemples
I	Substàncies amb alta perillositat i addició que presenten nombrosos riscos per a la salut i pocs usos terapèutics	Molt estricte. Només permeses per a usos mèdics o científics	LSD, MDMA (èxtasi), mescalina, psilocibina, tetrahidrocannabinol
II	Substàncies que presenten risc d'addició i per a la salut amb limitat valor terapèutic	Menys estricte	Amfetamines i derivats d'amfetamines
III	Substàncies que presenten risc d'addició i per a la salut però amb cert valor terapèutic	Substàncies legalitzades per a usos mèdics	Barbitúrics
IV	Substàncies que presenten risc d'addició però problemes menors per a la salut amb gran valor terapèutic	Substàncies legalitzades per a usos mèdics	Tranquil·litzants, analgèsics, narcòtics com alobarbital, diazepam, lorazepam, temazepam, etc.

LSD: dietilamida d'àcid d-lisèrgic, MDMA: metilendioximetamfetmina

De la mateixa manera, com s'ha mencionat anteriorment les substàncies il·lícites també poden ser classificades en diferents grups segons els seus potencials efectes sobre el sistema nerviós així com els riscos associats al seu abús:

- Amfetamines i derivats: Les amfetamines i els seus derivats tenen un efecte principalment estimulant que es tradueix en sensació d'eufòria i una disminució de la sensació de fatiga. Entre els problemes que pot ocasionar el seu consum es troben una sèrie de complicacions cardiovasculars i hepàtiques així com hipertèrmia, trastorns psíquics com paranoia o al·lucinacions [Murray 1998]. Un derivat d'especial importància de les amfetamines és la metilendioximetamfetamina (MDMA) que també té efectes estimulants principalment que inclouen sensació de benestar i extraversió entre altres. Tot i que el seu consum no comporta amb massa freqüència sobredosis letals, l'abús perllongat pot provocar hipertensió o hipertèrmia [Butler et al. 2004].

- Bolets al·lucinògens i dietilamida d'àcid d-lisèrgic o LSD (de l'alemany *Lysergsäurediethylamid*): Estes drogues generen principalment al·lucinacions, i en dosis moderades provoquen una alteració lleugera de la realitat, mentre que a dosis altes poden suposar una modificació de l'estat de consciència. Entre els riscos que poden presentar es troben paranoia o ansietat, possibilitat d'aplegar a un estat de pànic, risc de patir algun dels seus efectes al cap d'un temps considerable després de l'últim consum (*flash-backs*), problemes mentals transitoris o permanents, així com els riscos propis associats a la pèrdua de consciència [Singh et al. 2019].

- Cocaïna: La cocaïna produeix els seus efectes d'addició principalment per la seua actuació al sistema límbic a aquelles regions del cervell que estan encarregades de regular sensacions com el plaer o la motivació. A curt termini, l'alliberament de dopamina que provoca el consum de cocaïna produeix una sensació d'eufòria i desig de continuar administrant dosi de la droga cosa que comporta seriosos riscs d'addició motivats per les alteracions produïdes al funcionament del cervell. A banda de generar efectes estimulants, sensació d'eufòria i seguretat, els riscos que comporta es troben associats a ansietat o paranoia, trastorns cardiovasculars greus, i també una falsa sensació de seguretat [Nestler et al. 2005; Singh et al. 2019].

- Cannabinoides: Aquest grup de substàncies, presenten efectes principalment depressors, relaxació, intensificació de les sensacions i en alguns tipus de marihuana poden provocar efectes al·lucinògens. Els riscos principalment que comporten venen associats a una disminució de l'atenció i la coordinació motora, problemes de memòria, hipotensió i atacs d'angoixa. Com que el seu consum va sovint associat al tabac, a llarg termini pot comportar problemes respiratoris i cardiovasculars així com càncers associats a la seua forma de consum com càncer de pulmó [Hall et al. 2009].

- Heroïna: Presenta efectes principalment depressors, relaxació, sensació de benestar i plaer. A més, comporta nombrosos riscos com possibilitat de sobredosi associada a la forta dependència que genera, síndrome d'abstinència si el seu consum és interromput sobtadament i un elevat risc de transmissió de malalties infeccioses associat a la seua forma d'abús [Chawarski et al. 2006].

- Ketamina: La ketamina és un al·lucinogen dissociatiu, a dosis baixes pot presentar efectes similars a l'abús d'alcohol i en incrementar la dosi pot conduir a efectes dissociatius. La ingesta excessiva de ketamina o derivats produeix moviments musculars incontrolats, possibilitat de desencadenar problemes mentals i una perillosa insensibilitat al dolor [Wei et al. 2020].

- Psicofàrmacs: Segons el fàrmac i dosi administrada els efectes són principalment ansiolítics o hipnòtics. Entre els riscos que comporten es troben somnolència o pèrdua de la coordinació motora, i a llarg termini provoquen dependència. El seu consum junt al d'altres drogues depressores provoquen una manca de capacitat de reacció i una reducció del ritme de la respiració [Longo et al. 2000].

2.2. NOVES SUBSTÀNCIES PSICOACTIVES

La definició que dona de les NPS la UNODC és literalment "substàncies d'abús, bé siguin de forma pura o en preparats que no són controlades per la Convenció de 1961 sobre narcòtics ni pel Conveni de 1971 sobre substàncies psicotròpiques, però que poden suposar una amenaça per a la salut pública" [UNODC 2018]. A banda d'aquesta definició, es pot englobar com NPS la major part de les substàncies que han sorgit des dels inicis de la dècada dels 2000. Tot i que aquesta darrera definició no fa referència a una descripció precisa de les mateixes, és la manera adient de fer una ràpida distinció entre el que es consideren drogues d'ús tradicional i les NPS [Peacock et al. 2019].

La millora en la transferència de coneixements, així com la globalització han permès portar a terme una difusió molt més ràpida i efectiva de les NPS per tot el món. Inicialment, les empreses tradicionals van ser imprescindibles per a la venta de NPS a nombrosos països; per exemple, d'entre les primeres NPS que es van detectar es poden trobar píndoles que contenien piperazines com la trifluorofenilmetilpiperazina que van ser trobades a Nova Zelanda a principis dels 2000 i posteriorment, al 2004, també van ser trobades a altres països europeus. Aquestes píndoles com a diferència principal respecte de les drogues tradicionals estaven produïdes per empreses de productes químics i eren distribuïdes de manera legal tant per diversos portals d'internet com a punts de venda minorista. De la mateixa manera, durant aquest anys a tota Europa així com altres regions es van trobar algunes catinones i cannabinoides sintètics en mescles d'herbes per a fumar [Peacock et al. 2019].

Entre les principals causes que van portar a fer un seguiment més rigorós sobre les NPS van estar que a més dels seus efectes, aquestes contribueixen a una falsa seguretat proporcionada per la seua legalitat, essent percebudes inicialment com drogues de menor perillositat o que no s'incomplia la llei amb el seu tràfic i consum. També el seu preu tendeix a ser més reduït que no pas el de les drogues tradicionals. Tanmateix, als inicis del seu sorgiment la majoria de NPS no eren detectades a les anàlisis rutinàries, motiu pel qual algunes persones que eren sotmeses a controls de drogues de manera regular, podien evadir-los mitjançant el seu consum en lloc del de les drogues ja prohibides [Peacock et al. 2019].

Com és evident segons les dades que es mostres a la Taula 1 i Taula 2, les NPS queden fora d'aquests llistats a causa de la seua recent introducció en el mercat il·legal i habitualment a la curta durada d'aquestes en el mateix. Per aquest motiu, la Unió Europea té un sistema propi per poder donar una resposta més ràpida sobre els riscos que presenten les NPS. Segons els procediments que s'estableixen al Reglament UE 2017/2101, les NPS són sotmeses a un sistema d'alerta primerenca que avalua els riscos, i aleshores la comissió pot presentar una proposta per sotmetre les substàncies a mesures de control. Una vegada la decisió entre en vigor, les autoritats dels diferents països que integren la UE tindran 6 mesos per sotmetre a control la substància [UE 2017/2101]

La detecció de NPS planteja grans reptes. No sols per l'elevat nombre d'aquestes que existeixen i la seua curta durada al mercat, si no perquè els rangs de concentració a les diverses matrius biològiques és parcial o totalment desconegut. La bibliografia en general, mostra casos d'algunes NPS en matrius com sang o orina per a casos d'intoxicacions agudes, mostres *post-mortem* o aigües residuals, però la falta d'estudis de metabolisme en humans fa molt complexa la seua interpretació. No obstant, a partir de les escasses dades disponibles a la literatura, es poden extraure que per exemple els cannabinoides sintètics poden ser fatals a concentracions tan baixes com entre 1 i 10 μg L^{-1} mentre que alguns derivats de les amfetamines tenen concentracions tòxiques a nivells de pocs mg L^{-1} [Graziano et al. 2019].

Pel que respecta als grups de NPS que s'hi troben actualment al mercat de les drogues, la classificació més comuna que es pot fer de les NPS és l'associada als seus efectes principals que poden ser ben diferenciats entre les diverses famílies, com es pot veure a la Figura 1. No obstant, una sèrie d'efectes i riscos generals es poden associar al consum de NPS. Els efectes secundaris del seu abús poden anar des de l'agitació, agressivitat, psicosis aguda o una imprevisible dependència que

pot ser en la majoria d'ocasions major a la que presenten les drogues tradicionals. El coneixement sobre els efectes secundaris, sobre els riscos així com la toxicitat de moltes de les NPS és molt limitat, si aquests factors són afegits a la manca de coneixement sobre la composició i puresa d'allò que moltes vegades és consumit, els riscos associats al consum de NPS són molt més elevats del que ho són el consum de drogues més conegudes, cosa que acaba provocant hospitalitzacions amb molta freqüència i nombrosos casos d'intoxicacions fatals [UNODC 2018].

Tanmateix, dins d'aquesta classificació general existeixen diversos grups o famílies de NPS que tenen semblances tant estructurals com en quant als seus efectes. A la Figura 1 es poden veure les diverses famílies que existeixen dins dels diversos grups de NPS classificades pels seus efectes, així com una estimació del percentatge que suposen d'entre totes les NPS. En general, es consideren estimulants aquelles substàncies que copien els efectes de l'amfetamina, la metamfetamina, el MDMA o la cocaïna. Dins de les NPS estimulants es poden trobar les conegudes catinones sintètiques, piperazines, piperidines o triptamines entre altres. Els cannabinoides sintètics són considerats com anàlegs dels cannabinoides tradicionals, amb un potencial efecte d'addició molt superior, d'ells es poden trobar diferents famílies com els aminoalquilindols, indazols o cannabinoides de tipus JWH. Les drogues psicodèliques són aquelles que provoquen efectes al·lucinògens de igual manera que ho fan el LSD o la psilocina, d'elles són destacables les fenetilamines així com algunes triptamines que també provoquen aquests efectes. Les dissociatives són aquelles que produeixen efectes similars a la ketamina, hi podem trobar exemples d'elles en les arilciclohexilamines o les diariletilamines. Els opioides sintètics són els que emulen els efectes de la morfina o l'heroïna, dels quals cal destacar per la seua rellevància i perillositat els anàlegs del fentanil. També els psicofàrmacs que actuen com sedants tenen els seus anàlegs entre les NPS, aquestes drogues són aquelles amb efectes hipnòtics i estructures semblants a les benzodiazepines. [Zowilska et al. 2018].

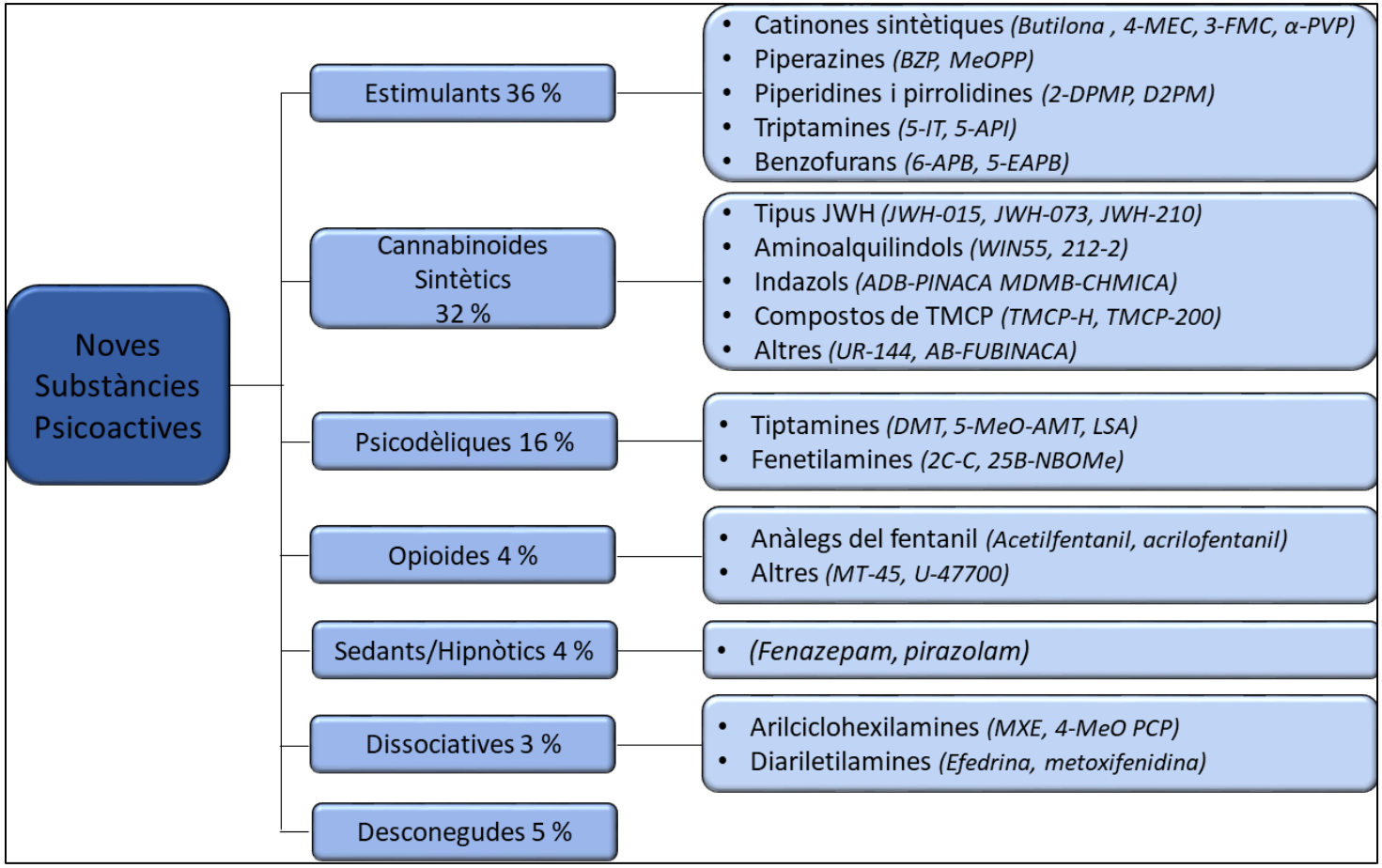


Figura 1. *Classificació de les NPS segons els seus efectes i families de cada grup amb exemples concrets.*

3. ANÀLISI DE DROGUES EN MATRIUS BIOLÒGIQUES

Tal i com es reflecteix a nombrosos estudis governamentals [EMCDDA 2020], l'abús de drogues és un comportament que es registra amb elevada freqüència a països arreu de tot el món, i aquest fet comporta seriosos riscos tant a nivell sanitari com social. Per aquests motius és fonamental disposar de metodologies d'anàlisi ràpides, econòmiques i fiables capaces de ser aplicades en diferents situacions com per exemple controls antidòping, controls a carreteres, anàlisi a centres sanitaris, en ambients ludicofestius o en altres casos per tal de minimitzar els riscos associats al seu consum.

3.1. TIPUS DE MOSTRES

Per tal de monitoritzar el consum de drogues s'han emprat al llarg dels anys nombroses matrius. Des de mostres biològiques com puguin ser sang, orina o saliva per a determinar el consum recent de substàncies psicoactives; cabells que permeten determinar el consum a llarg termini d'alguna substància o aigües residuals que permeten avaluar quantitativament el consum d'una o més substàncies a una gran població a partir del contingut dels metabòlits de les drogues que es troben en elles.

Cal destacar que els temps de detecció d'una substància a les diferents matrius biològiques es veuen afectats per nombroses variables com ara bé la dosi administrada, la via d'administració, el pH de la saliva o de l'orina de l'individu així com el propi metabolisme de cada subjecte [Vestraete 2004].

3.1.1. SANG, PLASMA I SÈRUM

La sang, així com altres matrius que s'obtenen a partir d'ella com el sèrum o el plasma, ha estat entre les matrius més utilitzades històricament per a realitzar anàlisi clínica i forense. La sang com a matriu presenta una elevada homogeneïtat que fa atractiu el seu ús per a aquest tipus d'anàlisi, però a més, el fet de que haja estat àmpliament utilitzada durant les darreres dècades deixa a disposició en la literatura, nombrosos mètodes per a l'anàlisi de substàncies il·lícites [Mali et al. 2011].

En el cas concret de les drogues, aquestes segons les seues propietats fisicoquímiques es troben en major o menor mesura metabolitzades en sang, però amb una quantitat notable de la molècula en la seua forma d'abús, és a dir, en general a la sang es pot trobar tant la droga com els seus principals metabòlits en percentatges considerables. Després de l'administració, la majoria poden ser

detectades durant un període que oscil·la generalment entre 1 i 2 dies segons la substància, la dosi i la freqüència de consum [Mali et al. 2011].

D'altra banda, la sang presenta alguns inconvenients com poden ser la seua elevada complexitat, però sobretot la seua difícil obtenció, que fa necessari la participació de personal sanitari per extraure-la, suposant a més mètodes invasius per a l'individu.

3.1.2. ORINA

D'entre tots els fluids biològics, l'orina és el que presenta un ús més estès i per tant ha estat la matriu més estudiada per a l'anàlisi toxicològica al llarg dels anys. El principal fet que fa de l'orina una matriu atractiva per a aquest tipus d'anàlisi és l'elevat volum que s'obté d'ella, així com l'elevada concentració dels anàlits que es poden trobar. No obstant a la pràctica, l'orina pot presentar diversos problemes per a realitzar anàlisi rutinàries o que requereixen una elevada fiabilitat per garantir la validesa de la prova. Entre aquests problemes cal destacar que tot i que és menys invasiva que la sang, presenta la dificultat per a la seua obtenció sota supervisió, la qual cosa pot derivar en la fàcil adulteració d'aquesta. A més, l'orina no presenta una relació directa entre la concentració de drogues i metabòlits presents en la mostra i la dosi consumida.[Saito et al. 2011].

L'orina presenta una major presència de metabòlits en general, tant que pot ser en el cas d'algunes drogues pràcticament impossible detectar la substància en la seua forma d'abús, com per exemple el tetrahidrocannabinol (THC) o la cocaïna (vegeu Taula 3) on podem trobar en una quantitat considerable els seus metabòlits en comparació a la quantitat de droga que s'hi troba com el 11-nor-9-carboxitetrahidrocannabinol (THC-COOH) o la benzoilecgonina (BE), respectivament. Per contra, en altres casos com l'amfetamina, el grau d'excreció en forma d'aquest anàlit pot anar del 30 a més del 70 % [Verstraete 2004]. Per aquest motiu, per a realitzar l'anàlisi i determinar el consum d'una droga, habitualment és necessari conèixer els metabòlits i disposar de patrons dels mateixos. Aquest fet presenta dos problemes derivats, el primer d'ells és que per a moltes de les NPS que poden haver sorgit no es coneixen els metabòlits i per tant es complica la identificació de la substància consumida. El segon problema és que en aquells casos on es produeixi un consum de dues o més substàncies d'estructura similar, és difícil conèixer quines drogues s'han consumit amb certesa [Saito et al. 2011].

3.1.3. SALIVA

La saliva presenta nombrosos avantatges per a l'anàlisi de drogues, que han fet que cada vegada més, haja anat guanyant popularitat com a matriu per a realitzar anàlisi toxicològica i clínica. Entre els seus avantatges, es troba la seua fàcil obtenció ja que no requereix de mètodes invasius. Es pot obtenir sota supervisió, cosa que fa quasi impossible la seua adulteració. A més, si considerem que el temps de detecció pot anar de pocs minuts fins a 72 hores, tal i com es pot observar a la Taula 3, segons el tipus de droga fa d'ella una matriu molt atractiva. A la saliva es pot observar que la droga, roman generalment mentre duren els efectes de l'abús a la majoria dels casos, cosa que la converteix en una matriu especialment útil per a la confirmació de presència de drogues *in-situ* en controls de carretera o circumstàncies similars. D'aquesta manera, alguns països com Austràlia o Bèlgica ja fan ús d'ella com a mètode confirmatori [Verstraete et al. 2011].

Cal tenir en compte, també que els volums de saliva disponibles en comparació a altres matrius és més menut. Aquest fet implica certes restriccions en els mètodes analítics com la necessitat de poder detectar un ventall de drogues més gran amb volums menors o disposar de límits de detecció baixos. Aquest fet cobra especial importància en drogues com els cànnabis que poden portar a una salivació reduïda i complicar el mostreig [Palmer et al. 2019].

Altre avantatge de la saliva és la baixa proporció respecte a la droga de metabòlits que presenta, especialment en els moments posteriors a l'administració, el que permet suplir algunes de les limitacions que puguin presentar altres matrius com l'orina. D'aquesta manera com es pot veure a la Taula 3, substàncies com els cànnabis mostren el seu principal component psicoactiu (THC) i no el seu metabòlit principal, el THC-COOH. Altres anàlits com la cocaïna, es troba majoritàriament en la saliva durant les primeres 12 h, i a continuació es mostra una major presència del seu metabòlit benzoilecgonina (BE) fins les següents 12 h. Altres drogues com l'amfetamina, tenen una presència reduïda de metabòlits a causa de la baixa taxa de degradació que te [Saito et al. 2011, Verstraete 2004].

Taula 3. *Dosis habitualment consumides i temps de detecció per a algunes de les drogues més habituals als diferents fluids biològics emprats per a l'anàlisi [Verstraete 2004].*

Substància	Dosi	Sang	Orina	Saliva
Amfetamina	10 – 30 mg	46 h	24 – 72 h	50 h
Metamfetamina	5 – 10 mg	48 h	24 – 60 h	72 h
MDMA i derivats	50 – 100 mg	24 h	24 – 72 h	24 h
Cànnabis	5 – 30 mg	5 h (THC) 36 h (THC-COOH)	87 h (THC-COOH)	34 h (THC)
Cocaïna	20 – 100 mg	12 h (Cocaïna) 48 h (BE)	48 – 72 h (BE)	12 h (Cocaïna) 12 – 24 h (BE)
Heroïna	10 – 2000 mg	20 h	11 – 54 h	0,5 – 8 h
GHB	*	5 h	12 h	5 h

**No especificat, BE: benzoilecgonina, GHB: àcid Ɣ-hidroxibutíric, MDMA: metilendioximetamfetamina, THC: tetrahidrocannabinol, THCCOOH: 11-nor-9-carboxi-delta-9-tetrahidrocannabinol.*

3.1.4. CABELLS

Els cabells com a matriu per a aquest tipus d'anàlisi presenten característiques particulars i que fan d'ells una matriu d'especial interès en alguns casos molt concrets. Durant la formació dels cabells les drogues consumides bé siga de forma esporàdica o no, queden atrapades a la seua estructura. D'aquesta manera romanen estables durant períodes superiors a 12 mesos en aquells talls del cabell que s'hagen format durant el període d'administració. Així doncs, durant el creixement del cabell si és constant, les drogues s'incorporen al cabell segons la concentració de droga en sang [Usman et al. 2019]. Per tant, en el cas dels cabells, el temps de detecció de qualsevol substància el determinarà la longitud del cabell. Aquesta particularitat fa d'ells una matriu interessant per a determinar l'abús d'una substància a llarg termini, o per a dur a terme l'anàlisi després d'un llarg període de temps des de l'administració, com per exemple anàlisi a víctimes d'agressions sexuals. D'altra banda és necessari un procediment de digestió adequat per tal d'extraure l'anàlit dels cabells sense que la seua estructura es veja alterada. A més en el cas d'una administració puntual d'una substància psicotròpica es fa necessari disposar de mètodes amb límits de detecció molt baixos [Koren et al. 2019].

3.1.5. ALTRES MATRIUS BIOLÒGIQUES

Altres matrius biològiques han estat estudiades i usades per a l'anàlisi toxicològica i clínica de drogues, tot i que a causa de la dificultat per obtenir-les o manca d'avantatges que presenten front a les quatre matrius ja esmentades anteriorment, el seu ús no està generalitzat i no hi ha massa bibliografia que descriga mètodes que facen ús d'elles:

- Meconi: El meconi, és el primer excrement dels nounats i està format principalment per mucosa, bilis i restes superficials de pell. El meconi ha estat usat sobretot per a establir l'exposició dels fetus durant l'embaràs a drogues o fàrmacs a través del consum per part de la persona gestant [Palmer et al. 2019].

- Ungles: El procediment de formació de les ungles és similar al dels cabells per tant els avantatges que comporta l'ús d'ungles per a l'anàlisi de substàncies il·lícites és similar. Així doncs, són de fàcil obtenció però abans de l'anàlisi és necessària una descontaminació de la mostra per tal d'eliminar possibles productes estètics emprats sobre la superfície de les ungles o altres possibles interferents [Mali et al. 2011].

- Llàgrimes: Tot i que les llàgrimes han estat avaluades com a matriu biològica per a l'anàlisi de drogues, la seua difícil obtenció deriva en resultats poc reproduïbles que fan d'elles una matriu poc estudiada i emprada [Capiau et al. 2016].

- Suor: Ha guanyat certa popularitat en els últims anys, però continua sense ser una matriu que destaque de manera especial pel seu interès en l'anàlisi toxicològica. La seua obtenció habitualment es fa a través d'adhesius en alguna zona del cos d'especial sudoració durant un cert període de temps, fet que assegura l'obtenció d'una quantitat suficient de mostra [De Giovanni et al. 2013].

- Contingut gàstric: Aquesta matriu ha estat especialment útil per a determinar l'abús de drogues *post-mortem,* si ha sigut per via oral, permetent així la identificació de morts per sobredosi en aquelles drogues que es consumeixen en forma de píndoles o similars [Joyce et al. 2018].

3.2. MÈTODES D'ANÀLISI

Habitualment, l'anàlisi de drogues en matrius biològiques està formada per dues etapes. En primer lloc és habitual portar a terme un escombratge que identifique ràpidament la substància o substàncies presents a la mostra i posteriorment es realitza una segona anàlisi amb instrumentació més sofisticada per confirmar la presència de les drogues i quantificar la seua concentració.

3.2.1. MÈTODES D'ESCOMBRATGE

L'anàlisi de drogues habitualment consta d'una primera etapa d'escombratge o *screening* que permet una ràpida identificació de les substàncies presents en la mostra a analitzar. Hi ha nombrosos mètodes d'escombratge que han sigut usats i que es poden classificar segons la seua precisió en la identificació, segons la seua portabilitat o el temps de resposta que ofereixen. Tot i que algunes tècniques com l'espectrofotometria UV poden estar usades per a l'anàlisi qualitativa de les drogues més consumides habitualment i tenen un cost relativament baix [Li et al 2012] la tendència que han seguit aquests mètodes de *screening* ha estat en l'ús de materials d'immunoafinitat per la seua bona selectivitat [Harper et al. 2017].

Existeixen tires reactives basades en immunoassajos que són usades principalment per a la determinació dels grups clàssics de drogues en mostres d'orina i permeten donar una resposta qualitativa molt ràpida, de l'ordre de segons o pocs minuts. Així aquestes tires van principalment enfocades a la reducció de riscos. Les anàlisis es poden realitzar fàcilment *in-situ* amb un preu que es troba al voltant d'1 a 5 € per tira pel que suposa una de les alternatives més barates [PharmaDrugtest].

Els immunoassajos també permeten determinar de manera qualitativa una sèrie determinada de drogues i/o metabòlits en pocs minuts emprant un lector, generalment òptic; el seu maneig és relativament senzill ja que en la majoria de casos no se'n requereix de personal especialitzat. Comparat amb altres instruments té costos relativament assequibles ja que després d'aconseguir el corresponent analitzador (5.000 – 22.000 €), el material necessari per a dur a terme les anàlisis presenta costos no massa elevats [Harper et al. 2017].

Un bon exemple d'aquests instruments és el Drugtest 5000 de Dräger (Lübeck, Alemanya), que està basat en un immunoassaig de flux lateral *(Lateral Flow Immunoassay, LFIA)* que han estat de gran utilitat per al diagnòstic en punts de control no només de drogues si no en nombroses altres aplicacions [Anfossi et al. 2019]. A l'Estat Espanyol, el Drugtest 5000 de Dräger està un dels més emprats en controls rutinaris de drogues per cossos com el de la Guàrdia Civil. A la Figura 2, on es pot veure un exemple d'aquest instrument s'observa com el material necessari per realitzar l'anàlisi consta del lector i d'un mostrejador que té al seu extrem un material esponjós comprimit que en entrar en contacte amb la saliva quan s'introdueix a la boca s'expandeix aconseguint absorbir tota la saliva possible. Aquesta etapa de mostreig habitualment dura uns 60 segons fins que l'indicador revela que la mostra ha sigut recollida satisfactòriament. Una vegada s'ha acabat la recol·lecció de la mostra, el material esponjós s'introdueix a l'equip junt amb un cartutx que conté el tampó que s'utilitza per arrossegar les drogues que es troben adsorbides sobre el material esponjós fins a les tires on s'hi troba el material d'immunoafinitat. Finalment, l'equip mostra els resultats a través del lector. Aquest equip, mostra valors de tall diferents per a les drogues més conegudes com són els opiacis (20 µg L^{-1}), amfetamines (50 µg L^{-1}), metamfetamina (35 µg L^{-1}), cocaïna (20 µg L^{-1}), cànnabis (5 µg L^{-1}) i benzodiazepines (15 µg L^{-1}) [López-Rivadulla et al. 2011].

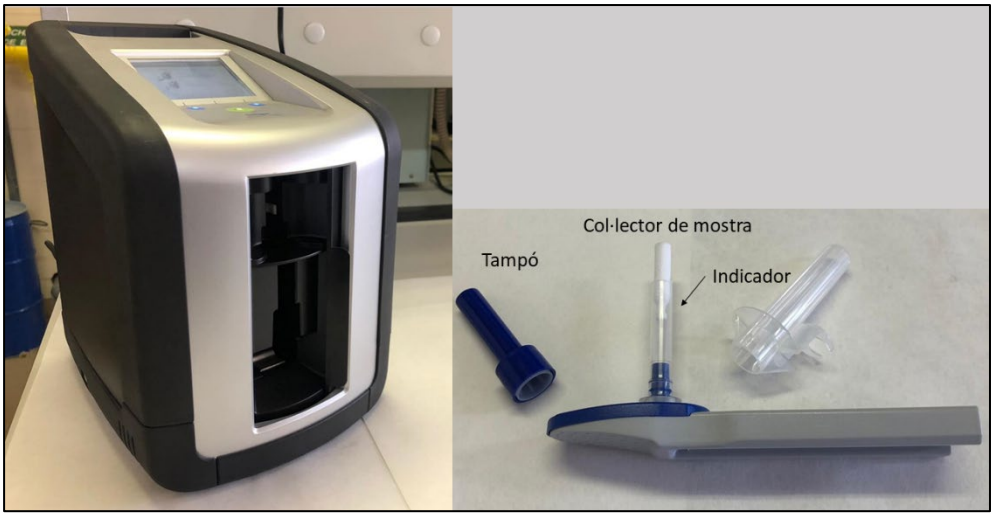


Figura 2. *Drugtest 5000 de Dräger (Lübeck, Alemanya).*

3.2.2. MÈTODES CONFIRMATORIS

Després de l'anàlisi inicial mitjançant una tècnica de *screening*, habitualment s'han emprat com a mètodes confirmatoris tècniques cromatogràfiques o afins a causa de la seua major fiabilitat i robustesa a l'hora de determinar de manera quantitativa el contingut de drogues en fluids biològics o en qualsevol altre tipus de matrius. Segons la base de dades Scopus d'Elsevier, el nombre d'articles publicats als últims 20 anys usant el nom de cadascuna de la tècniques analítiques que es mostra a la Figura 3 junt a "*drug analysis*" demostra que la cromatografia líquida *(Liquid Chromatography, LC)* acoblada a espectrometria de masses (MS) en la seua majoria, però també altres detectors més convencionals, han estat les tècniques més utilitzades per a la determinació de drogues, seguida per la cromatografia de gasos *(Gas Chromatography, GC)*, fonamentalment acoblada a MS. També, altres tècniques afins a la cromatografia com les tècniques electroforètiques acoblades a diversos detectors.

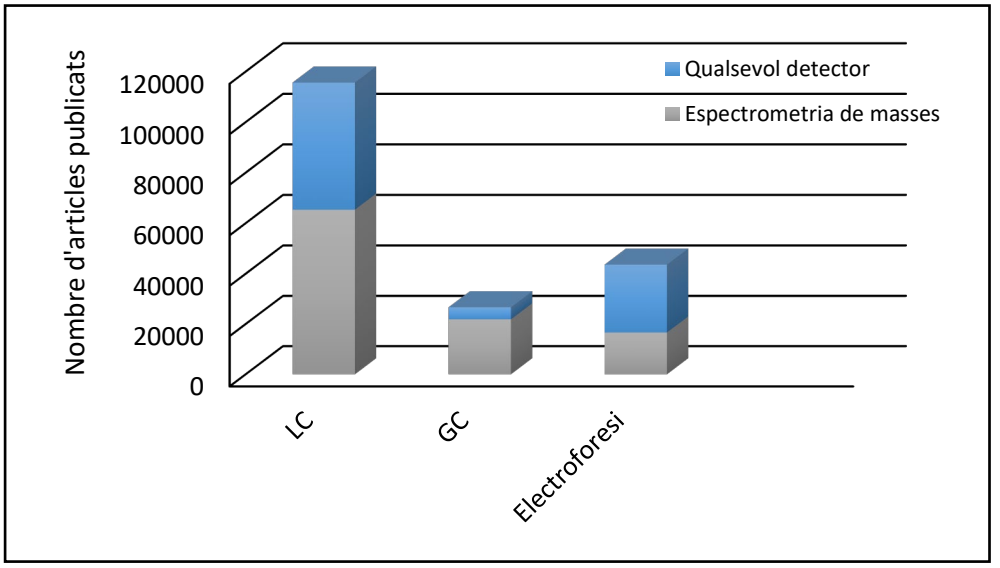


Figura 3. *Nombre d'artícles publicats a Scopus als darrers 20 anys per a la determinació de drogues emprant LC, GC i electroforesi amb detectors de masses o qualsevol altre tipus de detector.*

Tal i com es recull a la bibliografia, les tècniques cromatogràfiques acoblades a MS han estat àmpliament utilitzades per a l'anàlisi de drogues en qualsevol tipus de matriu, especialment des del seu desenvolupament i millora de les interfases entre el sistema cromatogràfic i l'analitzador de masses, de les característiques de les columnes, etc. Durant molts anys la GC-MS ha estat considerada la tècnica

preferida per a l'anàlisi confirmatòria per a la presència de drogues, però molts laboratoris han preferit fer ús de la LC-MS/MS per la seua selectivitat i sensibilitat en comparació a altres tècniques, especialment per a l'anàlisi de drogues en matrius complexes com les biològiques que a més, té intervals de concentracions lineals molt més grans que la majoria de tècniques analítiques existents [Snozek et al. 2019].

En primer lloc, la GC acobada a detector com de ionització en flama *(Flame Ionization Detector, FID)* ha estat una de les tècniques cromatogràfiques preferides per a l'anàlisi de compostos semivolàtils com ho són la majoria de drogues d'abús, no obstant des de la seua aparició, la GC-MS va ser una eina molt útil per a l'anàlisi toxicològica clínica i forense. Nombrosos estudis realitzats han descrit el desenvolupament de mètodes ràpids i amb capacitat de determinar simultàniament més d'un anàlit, tot i que en gran part d'ells, com es venia assenyalant en apartats posteriors, ha sigut indispensable una etapa d'extracció prèvia per a reduir l'efecte matriu així com fer ús de dissolvents compatibles amb la tècnica analítica. Per exemple, alguns d'aquests mètodes descriuen l'anàlisi de drogues de tipus amfetamina en mostres d'orina emprant com a etapa de tractament de mostra la microextracció mitjançant adsorbent compactat (*Microextractions by Packed Sorbent, MEPS)*, de la que es parlarà en més deteniment en apartats posteriors d'aquesta Tesi Doctoral [Malaca et al. 2019] , o l'anàlisi de cocaïna i els seus set metabòlits en orina després d'una extracció en fase sòlida *(Solid Phase Extraction, SPE)* [Fernández et al. 2019] o per a la determinació de cannabinoides en sèrum [Gottardo et al. 2019]. En ocasions, la GC-MS requereix de derivatització dels analits prèvia a l'anàlisi per augmentar la sensibilitat, a causa de la manca de volatilitat d'algunes drogues com la catinona, bufedrona, 4-metiltioamfetamina, d'α-pirrolidinopentifenona o la metilona, a la seua determinació en mostres de sang [Cláudia et al. 2019].

La LC-MS va ser ràpidament implementada per a l'anàlisi toxicològica i forense de substàncies il·lícites. Tot i l'elevada selectivitat d'aquesta tècnica, s'ha de tenir en compte els efectes matriu que es puguin tenir si l'anàlisi es porta a terme de manera directa, la qual cosa pot afectar de manera important a la quantificació dels anàlits, especialment quan la matriu és molt complexa com és el cas dels fluids biològics, en aquests casos és d'especial interès afegir un patró intern adequat per tal de minimitzar aquests efectes o recórrer a etapes de tractament de mostra. En aquest sentit, s'ha observat que el tipus de ionització per a la introducció dels anàlits a l'analitzador de masses és un punt crític en la importància de l'efecte matriu. La ionització per electrosprai *(Electrospray*

Ionization, ESI) i la ionització química a pressió atmosfèrica (*Ambiental Pressure Chemical Ionization, APCI)* han demostrat ser aquelles formes de ionització que provoquen major nombre d'interferències proporcionals [Castiglloni et al. 2008]. Algunes exemples que mostren l'aplicació d'aquesta tècnica per a l'anàlisi de drogues són la determinació mitjançant cromatografia líquida d'alt rendiment acoblada a espectrometria de masses en tàndem *(Ultra High Performance Liquid Chromatography tandem Mass Spectrometry, UHPLC-MS/MS)* cocaïna i els seus metabòlits en plasma després d'una microextracció en fase sòlida *(Solid Phase Microextraction, SPME)* amb un adsorbent genèric com el C18 [Feltraco-Lizot et al. 2019] o altres treballs han determinat simultàniament 29 cannabinoides sintètics i els seus metabòlits, amfetamines i cannabinoides naturals en sang [Ong et al. 2019].

Les tècniques electroforètiques acoblades a diversos detectors, entre ells analitzadors de masses, han estat emprades com a tècniques de separació alternatives a la cromatografia ja que permeten separacions més ràpides que la LC o són capaces de separar analits que habitualment no es poden analitzar per GC a causa de la seua inestabilitat tèrmica o reactivitat a altes temperatures com és el cas d'algunes drogues entre les quals és destacable el LSD pel seu alt punt d'ebullició. El conjunt de tècniques electroforètiques és poden definir com aquella agrupació de tècniques on la separació te lloc en una columna capil·lar buida (sense cap fase adsorbent) a partir de l'aplicació d'un camp elèctric, aquestes separacions són eficients i ràpides i poden separar-se anàlits de qualsevol natura, bé siguin ions positius, negatius o anàlits sense càrrega neta. D'entre les tècniques electroforètiques, l'electroforesi capil·lar *(Capilar Electrophoresi, CE)* ha estat una ferramenta molt útil per a l'anàlisi de drogues en fluids biològics [Thormann 2020]. Per exemple, a l'anàlisi de drogues s'ha usat recentment la CE per a la determinació de MDMA en saliva [Saar-Reismaa et al. 2019] o per a la determinació de psilocina i muscimol en orina [Poliwoda et al. 2020].

4. TRACTAMENT DE MOSTRA

Tot i les bones oportunitat que ofereixen les tècniques confirmatòries que s'han descrit anteriorment, per realitzar una segona anàlisi confirmatòria quantitativa de drogues en mostres biològiques, habitualment es requereix abans de l'ús de la tècnica analítica d'una etapa de preconcentració dels anàlits a determinar i d'una neteja (*clean-up)* de la mostra principalment a causa de la baixa concentració a la que és habitual trobar els anàlits i a la gran quantitat de

molècules orgàniques que poden interferir a l'anàlisi. El tractament de mostra, és habitual que sigui l'etapa de qualsevol procediment analític on major despesa de temps hi ha i on amb més freqüència s'introdueixen errors que poden portar a una mala determinació dels anàlits o manca de reproductibilitat [Namiesnik et al. 2000].

L'extracció líquid-líquid *(Liquid-Liquid Extraction, LLE)* en general ha estat un dels procediments més usats en nombrosos camps de la Química Analítica per al tractament de mostres de diferent origen: biològic, ambiental, industrial, etc. La LLE requereix l'ús d'un dissolvent, generalment orgànic, immiscible en aigua que permeta la partició de l'anàlit entre la fase immiscible i l'aquosa on presumiblement es troba inicialment l'anàlit. A més, acostuma a ser necessari canviar l'anàlit d'un estat carregat positiva o negativament quan es troba en la fase aquosa, a la seua forma neutra per augmentar la seua afinitat per la fase orgànica, de caràcter més apolar. En matrius com el plasma, anàlits com les drogues o els seus metabòlits solen trobar-se a nivell de traces, la qual cosa fa que sigui necessari diverses extraccions consecutives per tal d'afavorir un rendiment adequat. Aquest fet, afegit a la manca de rendiment que presenta la LLE en general, condueix a una reproductibilitat no sempre satisfactòria, a més d'una gran limitació quant a l'ús de dissolvents, que han de ser molt poc miscibles en aigua, cosa que habitualment comporta l'ús de dissolvents clorats, poc sostenibles per al medi ambient [Li et al. 2006].

Dins dels mètodes de LLE, es poden trobar a més de la forma clàssica diverses variants que han sorgit als darrers anys que permeten suplir alguns dels problemes que comporta l'ús d'aquest procediment. Un bon exemple, és la LLE dispersiva *(Dispersive Liquid-Liquid Extraction, DLLE)* (vegeu Figura 4) que incorpora l'ús d'un dissolvent miscible tant en la fase orgànica com en l'aquosa, cosa que permet dispersar en forma d'emulsió un volum menut del dissolvent extractant aconseguint un ràpid repartiment de l'anàlit entre les dues fases amb un volum d'extractant molt més menut del necessari en la LLE clàssica [Razaee et al. 2010]. Aquesta modalitat de LLE s'ha emprat per a la determinació de diverses drogues com mostren els treballs de Fernández i col·laboradors que van determinar 20 drogues incloent drogues clàssiques i NPS en saliva per a la seua posterior determinació per LC-MS/MS usant cloroform com a dissolvent extractant i metanol com a dispersant [Fernández et al. 2019], o com van demostrar els equips de Xu i Liu a la determinació de metamfetamina, tramadol i ketamina en orina amb detecció mitjançant GC-MS/MS [Xu et al. 2019].

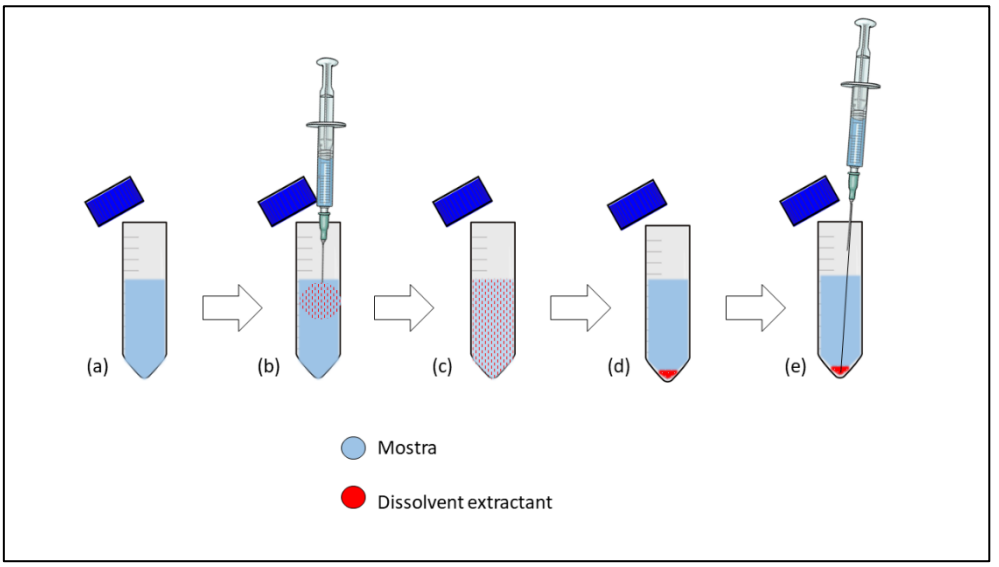


Figura 4. *Esquema bàsic d'una DLLE: (a) mostra, (b) introducció del dissolvent extractant i dissolvent dispersant, (c) dispersió del dissolvent extractant, (d) separació del dissolvent extractant i (e) extracció del dissolvent extractant.*

Altra de les modificacions que s'han proposat per a la LLE és el canvi de dissolvents orgànics immiscibles en aigua, pels anomenats líquids iònics *(Ionic Liquids, ILs)*. Els ILs poden ser definits com a sals amb una temperatura de fusió menor que el punt d'ebullició de l'aigua que actuen de forma anàloga a com es comportaria un dissolvent orgànic. Els principals avantatges que mostren són la seua elevada estabilitat tèrmica, reduïda pressió de vapor i la seua baixa inflamabilitat, però a més, els ILs inclús poden ser triats en funció de la seua afinitat per determinats grups funcionals, cosa que pot afegir al procediment d'extracció una determinada selectivitat [An et al. 2017]. Els ILs han sigut usats prèviament per a l'anàlisi de drogues com antidepressius en mostres d'aigua naturals [Ge et al. 2013] o cefalosporina en matrius biològiques [Wu et al. 2016], tot i que no existeix a la bibliografia un ampli ventall d'aplicacions que fan ús d'ells per a l'anàlisi toxicològica. Per exemple, l'equip d'Abujaber els va fer servir per a l'extracció de cortisona i cortisol present en mostres de saliva per a la seua determinació mitjançant cromatografia líquida amb detector ultraviolat *(Liquid Chromatography – Ultraviolet , LC-UV)* [Abujaber et al. 2019].

Més modificacions de LLE han sorgit, com per exemple aquella que es basa en l'ús d'una gota suspesa (*Single Drop Microextraction, SDME)*. Aquesta tècnica és basa en la suspensió d'una gota dins d'una mostra que generalment és de natura aquosa. La gota de pocs microlitres es troba a l'extrem d'una xeringa i acumula

l'anàlit que és transferit des de la dissolució aquosa mitjançant difusió passiva fins assolir l'equilibri. Posteriorment aquesta gota pot ser injectada directament a l'instrument de mesura. Amb aquest mètode de LLE es pot aconseguir estalviar fins a un 99 % del consum de dissolvent i les típiques etapes d'evaporació i redissolució de l'anàlit que venen lligades a la LLE convencional. El principal problema que aquesta tècnica presenta és la inestabilitat de la gota, cosa que pot provocar una reproductibilitat baixa. Pel que fa al dissolvent emprat en la SDME, és crucial que aquest no sigui molt volàtil per a que no s'evapore durant l'extracció, per açò entre els dissolvents més usats es poden trobar: alcohol isoamílic, undecà, nonà o octà [Kataoka 2010]. Alguns treballs han emprat prèviament la SDME per a la determinació de drogues com amfetamina [Choi et al. 2009] o psilocina i muscimol en orina [Poliwoda et al. 2020].

Però, a les darreres dècades, la SPE ha anat substituint la LLE progressivament per l'anàlisi toxicològica fins a disposar d'una gran quantitat de mètodes descrits a la bibliografia. La SPE fa ús d'una fase sòlida que actua com adsorbent dels anàlits sobre la seua superfície segons l'afinitat existent entre l'anàlit i la fase sòlida. Hi ha nombrosos tipus de fases adsorbents, la utilitat de la qual ve determinada segons les característiques fisicoquímiques de l'anàlit. La majoria dels adsorbents que es comercialitzen venen com a fases polimèriques, resines o de sílice compactades dins de cartutxos d'extracció.

A la bibliografia, es pot comprovar com la SPE ha estat una tècnica molt emprada per a l'anàlisi de drogues en un gran nombre de matrius. Mitjançant la cerca a la base de dades Scopus d'Elsevier de les paraules "SPE" i *"drug analysis" (anàlisi de drogues)* fins a 5.073 articles es troben publicats des de 1995, en els que es descriuen diversos protocols per a l'extracció de drogues il·lícites i/o legals que es troben a mostres de diferent natura, fent ús de fases sòlides de tipus molt variable. Com es pot veure a la Figura 5, la SPE va experimentar un augment considerable d'atenció en l'anàlisi de drogues a partir de principis del segle XXI i des d'aleshores, es manté com una important font d'estudis.

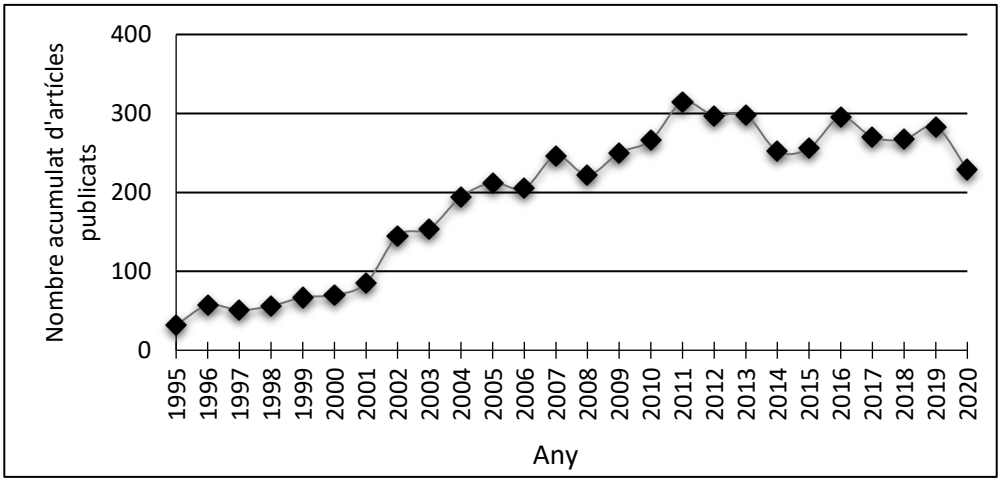


Figura 5. *Nombre d'articles publicats a Scopus des de 1995 fins a l'actualitat sobre SPE i anàlisi de drogues.*

La cerca bibliogràfica fa evident que la SPE és una de les metodologies més emprades actualment per realitzar tractament de mostra en quant anàlisi de drogues en matrius biològiques o aigües es refereix. Açò és a causa de la bona reproductibilitat que s'aconsegueix, les eficients extraccions i la bona capacitat que te per a concentrar anàlits així com eliminar gran part dels interferents que provenen de les complexes matrius. No obstant, tot i que habitualment es fa ús de columnes de SPE com a format per dur a terme el *clean-up*, una part important de recerca ha estat encaminada a obtenir nous formats o enfocaments per a realitzar l'extracció que permeta miniaturitzar l'anàlisi, fer-lo més senzill, ràpid, reduir les despeses o aproximar-se cada vegada cap a mètodes més sostenibles [Oliveira-Silveira et al. 2019].

4.1. EXTRACCIÓ EN FASE SÒLIDA CONVENCIONAL

El format més tradicional i més àmpliament utilitzat ha estat la SPE en cartutxos o columnes d'extracció. Les columnes són tubs de polipropilé (PP) o vidre, oberts per un extrem i acabat per l'altre extrem amb una obertura de diàmetre inferior a la de la columna. Dins de la columna hi ha una determinada quantitat de fase sòlida compresa entre dues frites, habitualment de polietilè (PE), per tal de retenir el material que es troba finament dividit en forma de partícules. Comercialment hi ha una gran varietat de columnes d'extracció amb fases sòlides de diferent natura, des de materials genèrics com sílice modificada amb C18 o altres materials polimèrics fins a materials de caràcter més selectiu com els MIPs o immunosorbents. A més, també es poden trobar de volums molt variables essent les més habituals d'entre 1 a 10 mL, tot i que existeixen fins a 150 mL.

Habitualment les columnes contenen entre 30 mg i 4 g de fase estacionària tot i que poden aplegar fins a 75 g per a les columnes de major grandària [Płotka-Wasylka et al. 2016].

En general, un procediment de SPE consta de les quatre etapes que es poden observar a la Figura 6: i) condicionament, on la fase adsorbent es prepara per a rebre els anàlits; (ii) càrrega, on s'introdueix la mostra que conté els anàlits i es fa passar a través de la fase adsorbent per a que l'anàlit i altres components de la matriu de característiques afins a la fase sòlida queden adsorbits sobre aquesta; (iii) rentat, on es fa passar un dissolvent amb força d'elució moderada per a que els compostos no desitjats siguin eluïts del adsorbent; (iv) elució, amb el volum mínim d'un dissolvent d'elevada força d'elució, s'extrauen els anàlits de la fase sòlida amb la intenció d'obtenir el màxim factor de preconcentració possible. Si és necessari el volum de dissolvent d'elució es pot evaporar i dissoldre de nou en un volum menor per tal d'aconseguir major concentració [Hennion et al. 1994].

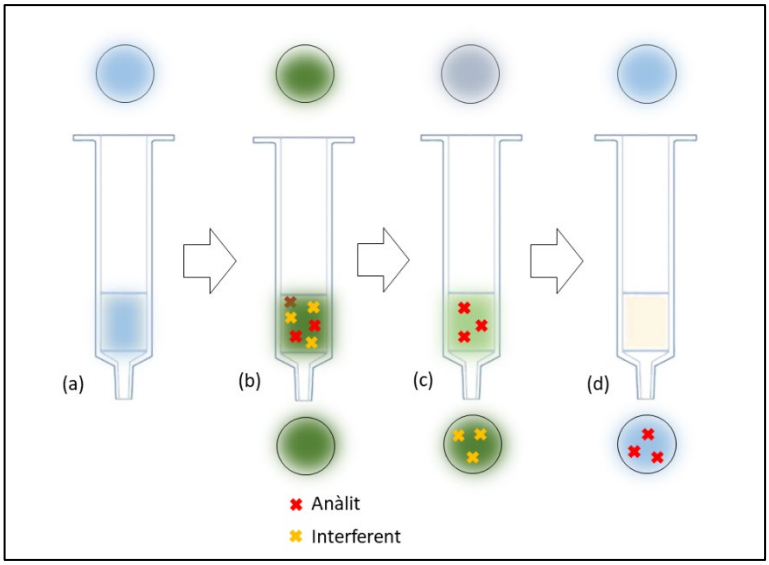


Figura 6. *Esquema bàsic de les etapes d'un procediment de SPE convencional: (a) condicionament, (b) càrrega, (c) rentat i (d) elució de l'anàlit.*

Per fer passar la mostra o els diferents dissolvents a través de la columna es pot aplicar pressió positiva (per exemple, amb una xeringa), centrifugar les columnes o connectar aquestes a un sistema de buit. També és possible quan la fase sòlida no està excessivament compactada deixar fluir la mostra o dissolvents per gravetat. El principal avantatge de l'ús de columnes és la gran quantitat de mètodes ja descrits que hi ha, així com el gran ventall de fases sòlides disponibles

comercialment o la facilitat per emprar una fase sòlida preparada al propi laboratori i la possibilitat de connectar columnes en sèrie si les necessitats de l'anàlisi ho requereixen [Lavén et al. 2009]. La SPE s'ha usat recentment en l'anàlisi d'aigües ambientals per a la determinació de 20 drogues d'abús incloent 15 NPS, tres drogues d'abús tradicionals (cocaïna, amfetamina i metamfetamina) i antidepressius [Peng et al. 2019]. Així com per a l'anàlisi dels metabòlits de 61 cannabinoides sintètics en orina seguit d'una determinació mitjançant LC-MS/MS [Gaunitz et al. 2020].

La µ-SPE *(Micro Solid Phase Extraction)*, que és una adaptació de la SPE però reduïda a volums inferiors a 1 mL, també s'ha utilitzat per a l'anàlisi d'amfetamina, metamfetamina, metilendioxiamfetamina, MDMA, cocaïna, benzoilecgonina, ketamina, fenciclidina *(Phencyclidine, PCP)*, psilocibina i mescalina a mostres d'orina i plasma, seguida d'una determinació per LC-MS/MS [Napoletano et al. 2012] o per a la determinació d'estimulants, al·lucinògens, ketamina i PCP en mostres de saliva [Sergi et al. 2010].

Altra de les variants de la SPE tradicional és l'anomenada SPE *on-line* que permet l'acoblament directe del procediment d'extracció a la tècnica analítica que es vaja a emprar. La SPE *on-line* presenta com avantatge principal que redueix al mínim la manipulació de les mostres, és per aquest motiu que permet minimitzar el treball al laboratori el que suposa menor despesa de costos i temps. Tanmateix, aquesta reduïda manipulació ajuda a que a la fi s'obtinguen resultats molt més exactes, precisos, i fiables. En aquest sentit, la SPE *on-line* ha estat descrita a diversos treballs per a l'anàlisi de drogues com el publicat per l'equip de López-García on es feia ús per a l'anàlisi de 37 drogues diferents en mostres d'aigua residual emprant LC-MS/MS [López-García et al. 2018] o per exemple per a l'equip d'Aqda que va fer ús de fibres poroses per a la µ-SPE de fàrmacs antiinflamatoris en mostres d'orina i plasma [Aqda et al. 2018].

4.2. EXTRACCIÓ EN FASE SÒLIDA DISPERSIVA

Una variant de la SPE tradicional que també ha estat emprada per la seua utilitat és l'anomenada extracció en fase sòlida dispersiva *(Dispersive Solid Phase Extraction, DSPE)*. Aquesta manca d'un suport sòlid on sigui retinguda la fase sòlida, fa necessari que s'afegeixi directament sobre la mostra. Una vegada addicionada, la dispersió s'afavoreix mitjançant agitació per tal de garantir una major homogeneïtat que permeti un repartiment efectiu dels anàlits des de la mostra a l'adsorbent, assolint l'equilibri més ràpidament. Quan el repartiment ha estat quantitatiu la fase sòlida es pot separar de la mostra mitjançant processos

mecànics com centrifugació o filtració, després els anàlits poden ser eluïts amb un dissolvent capaç de trencar les interaccions anàlit-adsorbent o realitzar la mesura mitjançant desorció tèrmica si l'instrument i l'adsorbent ho permeten. Aquesta tècnica d'extracció ha estat combinada amb diverses tècniques analítiques com bé la CE per a la determinació de quinolones en mostres de llet emprant un detector de fila de díodes *(Diode Array Detector, DAD)* [Ibarra et al. 2012] o combinada amb LC-UV per a la determinació de bisfenol A en mostres de llet [Reyes-Gallardo et al. 2016]. També s'ha emprat CE-MS per a la determinació de medicament antihelmíntic en mostres d'ou [Domínguez-Álvarez et al. 2013] o per a la determinació de sulfonamides en mostres de llet mitjançant LC-UV/UV [Gao et al. 2010].

Tot i que l'ús de la DSPE proporciona una reducció de temps considerable i és ben adaptable, el pas de filtració/centrifugació per a la separació de l'adsorbent pot suposar una etapa del procés tediosa. Per aquest motiu, una solució prometedora a l'ús dels tradicionals adsorbents per a la DSPE ha estat l'extracció en fase sòlida dispersiva magnètica *(Magnetic Dispersive Solid Phase Extraction, MDSPE)* que es mostra a la Figura 7. Aquesta tècnica va ser introduïda a l'anàlisi química per Šafaříkova el 1999 [Šafaříkova et al. 1999] després de que Robinson el 1973 publicara la primera separació realitzada mitjançant l'aplicació de forces magnètiques [Robinson et al. 1973]. Per a la MDSPE s'empren partícules magnètiques de Fe_3O_4 de petit diàmetre (de l'ordre de nanòmetres), tradicionalment cobertes de materials com òxids de sílice o alumini per tal d'augmentar la seua estabilitat. Als recobriments tradicionals de les nanopartícules magnètiques *(Magnetic Nanoparicles, MNPs)*, s'ha afegit als darrers anys l'ús de materials selectius immobilitzats sobre la superfície com ara bé aptàmers, emprats per exemple per a l'anàlisi d'oxitetraciclina o kanamicina en mostres de llet, mel o carn de porc [Liu et al. 2015], anticossos per a la determinació d' antibiòtics en mostres de llet o cabells [Font et al. 2008] o polímers de reconeixement molecular *(Molecularly Imprinted Polymers, MIPs)* per a la determinació de tizanidina en orina [Sheykhaghaeia et al. 2016].

L'aplicació de MNPs per a l'anàlisi ha estat demostrada en nombrosos camps, essent usades per a determinar anàlits de molt variable natura i en un nombrós número de matrius. Per exemple, aquestes s'han usat, recobertes de diversos materials, per a la determinació de molècules menudes com ocratoxina A en cereals com arròs o dacsa [Mashhadizadeh et al. 2013]. També s'han determinat hidrocarburs poliaromàtics en mostres ambientals d'aigua [Wang et al. 2013]. A més ha estat aplicada per a l'anàlisi de mostres més complexes com matrius biològiques. Per exemple, per a la determinació d'estrògens en plasma [Wang et al. 2012] i inclús per a la determinació del VIH tal i com va publicar l'equip de Chen [Chen et al. 2010]. La MSPE també ha estat usada per a la determinació de molècules de major grandària com proteïnes, veient reflectit aquest fet en les interesants aplicacions que han tingut a proteòmica [Li et al. 2013]. En l'àmbit de l'anàlisi toxicològica recentment s'han usat per a l'anàlisi de drogues com tramadol en mostres d'orina mitjançant LC-UV [Taghvimi et al. 2019], o l'anàlisi de cocaïna i metabòlits en mostres d'orina emprant LC-MS/MS [Sánchez-González et al. 2016].

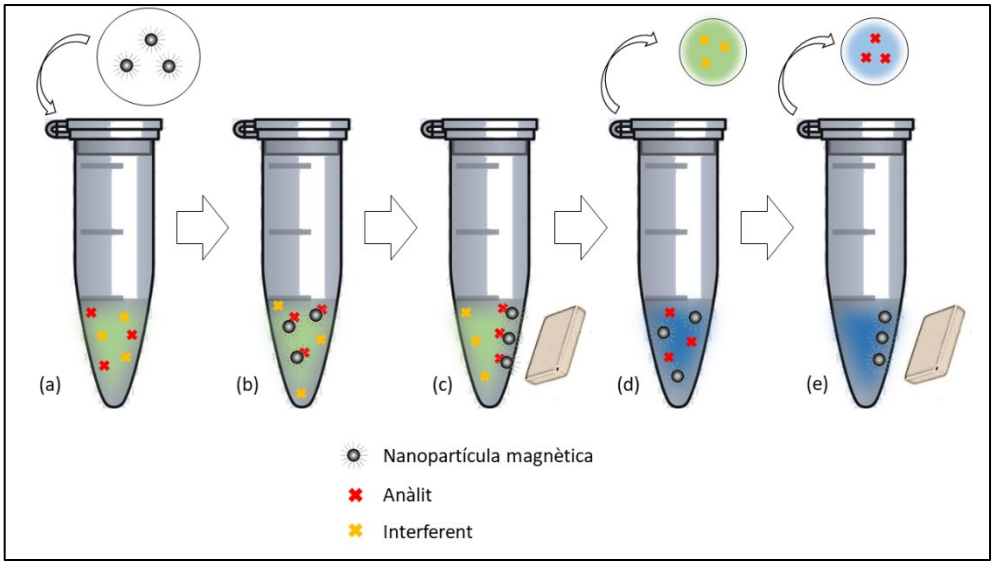


Figura 7. *Etapes de l'extracció en fase sòlida dispersiva magnètica: (a) addició de les MNPs, (b) adsorció de l'anàlit, (c) separació magnètica, (d) eliminació de la matriu i (e) elució de l'anàlit.*

4.3. MICROEXTRACCIÓ AMB ADSORBENT EMPAQUETAT

La MEPS és una adaptació directa de la SPE a un format miniaturitzat i automatitzable que permet disminuir considerablement els volums necessaris per dur a terme el tractament de mostra. Per tant, aquest enfocament, permet una adaptació de la majoria de protocols de SPE ajustant volums. A la MEPS es fa ús d'una xeringa de líquids, habitualment d'entre 100 i 250 µL de capacitat, que incorpora al final de l'èmbol la fase adsorbent adient. Les quantitats d'adsorbent que alberga la xeringa oscil·la al voltant de 1 mg, segons la fase i el fabricant, el que comparat amb la tradicional SPE, suposa una disminució de més de 100 vegades en molts casos [Abdel-Rehim 2010]. Aquest format (veure Figura 8) permet integrar el procediment d'extracció en un sol dispositiu, sense necessitar sistemes de buit, centrifugues o cap altre material, cosa que fa de l'extracció un procediment senzill i fàcil d'integrar en un braç robòtic. Si la xeringa s'adapta a un braç robòtic permet automatitzar completament el procés de tractament de mostra i de mesura en l'instrument de detecció triat (típicament GC o LC) [Moein et al. 2015].

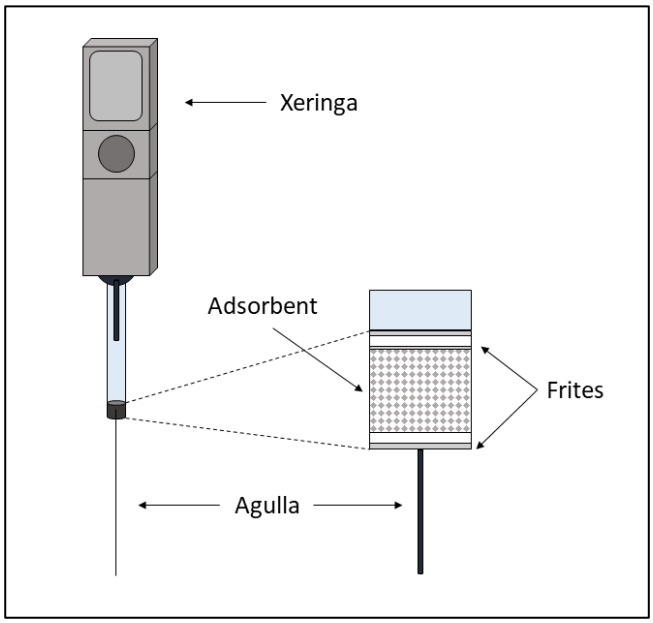


Figura 8. *Esquema d'una xeringa de microextracció mitjançant adsorbent empaquetat.*

A la bibliografia hi ha diversos articles que descriuen l'aplicació de MEPS per a la determinació de diverses drogues i fàrmacs en matrius biològiques com plasma i orina. Per exemple, Abdel-Rehim i col·laboradors van aplicar la MEPS per a la determinació de lidocaïna, mepivacaïna, bupivacaïna i ropivacaïna en plasma emprant com a fase estacionària sílice modificada amb C18, seguit de la posterior determinació dels compostos mitjançant GC-MS [Abdel-Rehim 2004]. De la mateixa manera es va usar per a la determinació d'anestèsics locals a plasma, emprant com fase estacionària un bescanviador de cations [Altun et al. 2004] o per a la determinació de metadona en mostres de plasma i orina emprant una fase estacionària de Sílica-C18 [El-Beqqali et al. 2007].

4.4. EXTRACCIÓ AMB PUNTES DE MICROPIPETA

Un format derivat de l'ús de les columnes però adaptat a volums molt més menuts és l'extracció mitjançant puntes de micropipeta (*pipette tips)* (vegeu Figura 9). Aquestes, són puntes de polipropilè de diferent grandària que poden contenir al seu interior la fase estacionària adequada per a l'anàlisi, bé sigui retinguda entre dues frites en forma de partícules o ancorada directament sobre les parets internes de la punta en forma de monòlit. En qualsevol cas, a causa de la limitada pressió que és capaç d'exercir la micropipeta, és necessari que el material sigui suficientment porós com per a permetre el flux de mostra i dissolvents. El principal avantatge de l'ús d'aquest format és la senzillesa del procediment d'extracció i la seua rapidesa, així com la seua fàcil automatització. L'ús de *pipette tips* permet reduir notablement el volum de dissolvent necessari, generant menor quantitat de residus. Actualment, hi ha disponibles nombroses micropipetes multicanal, que hi permeten acoblar-ne diverses puntes a la mateixa i realitzar de manera simultània tantes anàlisis com canals tingui la micropipeta. Front a tots aquests avantatges que suposa l'ús de les puntes, cal destacar també les dues principals limitacions: (a) la manca de fases sòlides disponibles, essent necessari en moltes ocasions preparar-les al laboratori abans d'usar-les, i (b) l'elevat volum de residus en forma de plàstic que genera l'ús de puntes d'un sol ús [Seidi et al. 2019].

Hi ha diverses *pipette tips* disponibles comercialment com per exemples les ZipTip o NuTip (Millipore, EEUU), Monotio (GL Science™ Inc., Japó) o les TopTip (Glygen Corp., Columbia, EEUU) amb diverses fases sòlides cadascuna. Per exemple les ZipTip estan composades de fases com C18, C4 o bescanvi catiònic fort *(Strong Cation Exchange, SCX)*. Les NuTip solen ser-hi monòlits que actuen com adsorbent recobrint la paret interna de la punta que mentre que en les

TopTip hi ha una major varietat d'adsorbents que van des de C18 o C8 fins a cel·lulosa, NH_2 o fases de ZrO_2 o TiO_2 [Seidi et al. 2019].

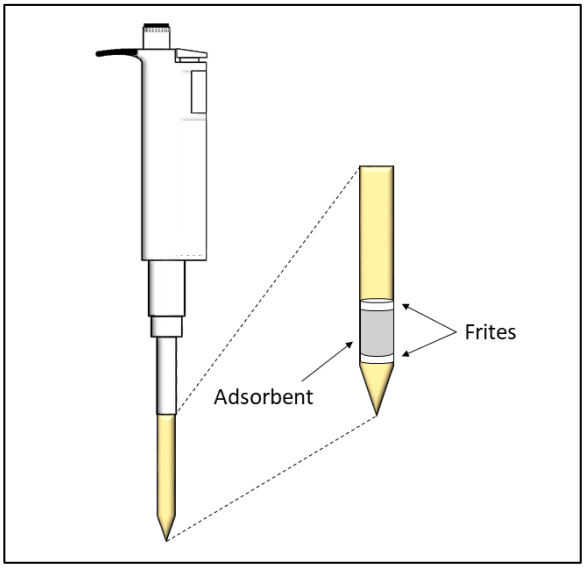


Figura 9. *Esquema bàsic d'una punta de micropipeta farcida d'una fase adsorbent.*

Les puntes de micropipeta farcides amb fase adsorbent s'han usat a diversos treballs combinades fonamentalment amb tècniques cromatogràfiques. Com per exemple, per a l'anàlisi de benzodiazepines, emprant un adsorbent preparat al laboratori copolimeritzant estiré i divinilbenzé (*Divinylbenzene, DVB*) en complements dietètics [Sun et al. 2020] així com per a l'anàlisi en mostres d'orina d'amfetamina, metamfetamina, metilendioxiamfetamina i MDMA mitjançant GC-MS/MS amb derivatització prèvia emprant àcid trifluoroacètic anhidre [Shi et al. 2020].

4.5. MICROEXTRACCIÓ EN FASE SÒLIDA

La SPME és una tècnica analítica de preparació de mostra que va ser introduïda per Pawlyszin i Arthur el 1990 [Arthur et al. 1990] amb la intenció d'eliminar dos dels majors inconvenients de la SPE tradicional: la necessitat de volums relativament grans de dissolvents orgànics i la despesa de temps requerida per a la SPE. Des d'aleshores, la SPME ha guanyat gran popularitat dins del camp de la Química Analítica. Ha estat usada per a una gran varietat d'anàlits bé de caràcter polar com apolars, en diferents tipus de matrius de més complexes a més simples. El desenvolupament d'aquesta tècnica ha permès que siga adaptada a instruments de separació i detecció que permeten automatitzar i miniaturitzar l'anàlisi mitjançant mètodes d'immersió o desorció tèrmica cosa que

en moltes ocasions suposa un estalvi important de dissolvents [Lashgari et al. 2019]. La SPME aconsegueix una separació i preconcentració en una sola etapa per a compostos volàtils i no volàtils en mostres complexes. A l'enfocament tradicional de la SPME el dispositiu està construït per una capa de sílice fosa recoberta per una fase adsorbent que actua com a fase estacionària. En aquesta tècnica, la fase extractant està exposada a la matriu de la mostra durant un temps determinat i després d'assolir l'equilibri els compostos adsorbits són analitzats col·locant la fibra en el port d'injecció d'un equip GC. Habitualment, el temps necessari per assolir l'equilibri depèn de les propietats fisicoquímiques dels anàlits. No obstant, hi ha diverses estratègies que permeten reduir el temps d'extracció, com per exemple l'agitació de la mostra, maximitzant la interfície mostra-*headspace*, escalfant la mostra o refredant la fibra [Jalili et al. 2020].

La SPME es pot portar a terme per tres mètodes diferents que inclouen *headspace,* immersió directa i protecció de membrana. En immersió directa, la fibra s'introdueix en la matriu de la mostra i els anàlits seran transferits directament de la mostra a la fase extractant. A *headspace* es prefereixen per a anàlits volàtils i mostres complexes com poden ser matrius biològiques, ací la fibra s'exposa a l'espai entre la mostra i la tapa del vial de manera que els anàlits volàtils i semi-volàtils queden en fase gas mentre que les molècules d'elevat pes molecular queden separades dissoltes a la mostra. Finalment, la SPME de membrana protegida pot ser usada per a l'anàlisi d'anàlits no volàtils o molècules d'elevat pes molecular, on l'adsorbent es protegit amb una membrana buida que impedeix la difusió de grans molècules a la fase extractant mentre al mateix temps s'afavoreix la transferència de massa [Jalili et al, 2020]. Recentment, un article de revisió de Gorynski recull les aplicacions més importants de la SPME per a l'anàlisi de drogues d'abús [Gorynski et al. 2019]

També hi podem trobar l'anomenada SPME *in-vivo*, que va sorgir com una ferramenta molt prometedora per a la presa de mostra i posterior anàlisi d'organismes vius que permet no retirar la mostra que s'hi vol prendre. Per exemple, la SPME *in-vivo* s'ha emprat per a prendre mostres de sang d'animals col·locant la fibra a l'interior de les venes d'animals a través d'un catèter permetent reduir en alguns casos l'ús de mètodes massa invasius [Bojko et al. 2014].

No obstant, cal destacar que la SPME compta també amb algunes limitacions que estan associades a la variació que pot haver-hi entre diverses fibres usades de la mateixa natura, el cost de la fibra o per la manca de recobriments que pot

haver-hi disponibles de manera comercial. Per tal del suplir alguna d'aquestes limitacions, han sorgit diverses variants de la SPME, com ara bé la SPME en tub (*In-tube SPME)* que empra una columna capil·lar recoberta al seu interior pel material adsorbent reemplaçant la tradicional fibra essent així menys fràgil i aconseguint una major capacitat de càrrega [Moliner-Martínez et al. 2015; Costa-Queiroz et al. 2019] *o l'extracció en fase sòlida dinàmica (Solid Phase Dynamic Extraction, SPDE)* on la xeringa farcida del material adsorbent en el seu interior, es introduïda en la mostra on s'hi realitza de manera repetida diversos cicles de càrrega/descàrrega per a que els anàlits es vagin concentrant sobre la fase sòlida [Sajid et al. 2019].

La SPME ha demostrat estar una eina útil per a l'anàlisi de drogues en fluids biològics. Per citar alguns exemples es poden esmentar els treballs de Fucci i el seu equip on es van determinar moltes drogues que incloïen algunes com metamfetamina, amfetamina, cocaïna, THC o MDMA en saliva mitjançant una SPME tant emprant *headspace* com immersió directa amb determinació per GC-MS [Fucci et al. 2003]. També, el treball desenvolupat per l'equip de Lima Feltrado Lizot, els quals van determinar cocaïna i els seus metabòlits en mostres de plasma emprant una SPME amb una fibra C18 i determinació mitjançant UHPLC-MS/MS [De Lima Feltraco Lizot et al. 2020].

4.6. EXTRACCIÓ ADSORBENT SOBRE BARRA AGITADORA

El 1999, l'equip de Baltussen va descriure l'aplicació d'un nou format per l'extracció d'analits de microcontaminants de mostres d'aigua [Baltussen et al. 1999]. L' extracció per adsorció sobre barra agitadora *(Stir Bar Sorptive Extraction, SBSE),* tradicionalment està basada en un agitador magnètic, típicament d'entre 10 i 40 mm de longitud, recobert de polidimetilsiloxà *(Polydimethylsyloxane, PDMS)* que actua com a fase adsorbent (vegeu Figura 10). L'ús de PDMS com a fase sòlida fa que aquesta tècnica d'extracció sigui especialment útil per a anàlits apolars, essent la major part de les interaccions entre anàlit i fase estacionària forces de Van der Waals o enllaços per pont d'hidrogen en aquells anàlits de caràcter lleugerament més polar. Mitjançant l'extracció amb SBSE, habitualment hi ha dos mètodes de desorció possibles: desorció tèrmica i elució mitjançant dissolvent [Nogueira et al. 2012]. El procediment usat per a l'extracció fa d'aquest un format considerablement senzill així com fàcil de fer automàtic que permet a més el tractament de grans volums de mostres. Les principals limitacions associades a la seua aplicació venen determinades pel PDMS que es fa servir com a fase adsorbent, ja que només dona bons resultats per a l'anàlisi d'anàlits

semivolàtils, tot i que aquestes limitacions han estat resoltes en nombroses ocasions gràcies a la derivatització de les espècies a analitzar. D'altra banda la manca de selectivitat d'aquest material fa complicada l'aplicació de la SBSE per a matrius prou complexes com puguin ser matrius biològiques [David et al. 2019].

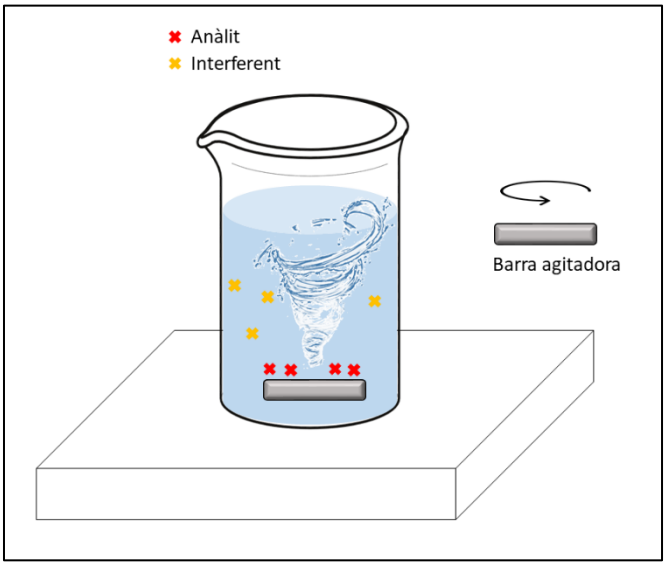


Figura 10. *Esquema d'una extracció amb barra agitadora.*

Recentment, s'han utilitzats enfocaments basats en la SBSE per a la determinació d'amfetamina i metamfetamina en mostres d'orina emprant una barra agitadora recoberta d' òxid de grafé [Taghvimi et al. 2019] o mitjançant la tecnologia d'empremta molecular per a la determinació de diclofenac en diverses mostres reals [Hashemi et al. 2019].

4.7. EXTRACCIÓ ADSORBENT AMB VARETA D'AGITACIÓ

Un inconvenient que presenta la SBSE és la manca de longevitat que presenta l'adsorbent a causa del contacte continuat entre l'agitador amb les parets del vas de precipitats o vial on es duu a terme l'extracció. Amb la finalitat de resoldre aquest problema, el 2011 es va desenvolupar l'anomenada extracción adsorbent amb vareta d'agitació (*Stir Rod Sorptive Extraction, SRSE).* Aquesta tècnica va ser proposada per a l'extracció d'hidrocarburs policíclics aromàtics de mostres ambientals [Luo et al. 2011]. El concepte de la SRSE és similar al de la SBSE, amb la diferència que en aquest cas la fase sòlida es troba suspesa en el medi d'extracció mitjançant una barra rotatòria vertical, evitant així el contacte amb les parets dels recipient i donant una major longevitat al material (vegeu Figura 11). Aquest format ha demostrat tenir una bona capacitat d'extracció i a més és relativament

fàcil de preparar l'adsorbent necessari segons el tipus d'anàlit a determinar i la matriu on es trobe. No obstant, la SRSE encara manca de recerca per fer sobre aquest format motiu pel qual la seua aplicació encara no ha estat provada en nombroses anàlisis. Per aquest motiu, la l'extracció pot tenir associada un efecte matriu que en mostres complexes pot ser de dimensions considerables i és necessari emprar unes rigoroses condicions d'extracció per a tenir una bona reproductibilitat dels resultats [Luo et al. 2010].

Tot i que aquesta modalitat no ha estat molt emprada per a l'anàlisi de drogues il·lícites en matrius biològiques, al 2011 l'equip de Luo va publicar un treball on es feia ús de la SRSE emprant un adsorbent de bescanvi aniònic per a l'anàlisi de fàrmacs antiinflamatoris com el ketoprofè, el fenbufè o l'ibuprofè en mostres d'aigües ambientals mitjançant LC-UV [Luo et al. 2011].

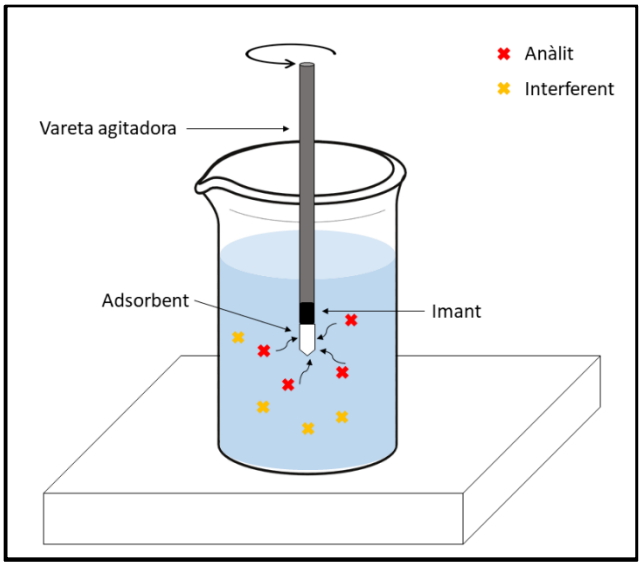


Figura 11. *Esquema d'una extracció adsorbent amb vareta agitadora.*

4.8. EXTRACCIÓ ADSORBENT AMB DISC AGITADOR

L' extracció adsorbent amb disc agitador *(Stir Cake Sorptive Extraction, SCSE)* (vegeu Figura 12) podria ser considerada també, una variant de la SBSE, ja que els principis en els que es basa són molt similars. En aquest cas la fase estacionària es deposita en forma de monòlit sobre una base circular de ferro protegida per una capa de vidre, que conté al seu interior una barra agitadora de manera que la SCSE pugui agitar la dissolució rotant sobre el seu centre. La major superfície de contacte entre la mostra i la fase estacionària, dona millors rendiments d'extracció i amb major reproductibilitat que al cas de la SBSE. Els procediments

d'extracció d'igual manera que anteriorment, són molt senzills i requereixen l'ús de pocs materials addicionals. Tot i que habitualment per a aquest tipus d'aplicació els materials adsorbents més utilitzats han estat polímers d'àcid 4-vinilbenzòic – DVB [Wang et al. 2014]. Aquest tipus de format fa senzill incorporar el material més adient per a cada anàlisis segons l'anàlit que es desitge analitzar. Per exemple, l'ús de MIPs ha estat emprat en aquest format com a fase adsorbent, per a la determinació de bisfenol A en mostres d'aigua [Lin et al. 2012]. A més, la manca de contacte entre la fase estacionària i les parets internes del recipient, dona una llarga durabilitat a l'adsorbent i fa que pugui ser utilitzat per a nombrosos anàlisis sense perdre la seua capacitat d'extracció. D'aquesta manera la SCSE presenta com a major limitació la necessitat d'alts temps d'extracció tot i ser menors que altres enfocaments com la SBSE [Huang et al. 2011].

Un format, molt similar a la SCSE el trobem en la extracció adsorbent amb disc rotatori *(Rotation Disc Sorptive Extraction, RDSE)*, on la principal diferència és el material sobre el que se suporta la fase estacionària, que en aquest cas és un disc de tefló que s'emplena de l'adsorbent usant la tècnica sol-gel per immobilitzar-lo sobre la seua superfície [Manzo et al. 2019].

La RDSE segons la literatura ha mostrat millors rendiments d'extracció i una bona reproductibilitat dels resultats, a més, aquesta tècnica també demostra la bona capacitat per al seu ús repetit, per tant ofereix avantatges molt similars a la SCSE. Per exemple, aquesta tècnica s'ha emprat per a l'anàlisi de contaminants emergents en aigües [Arismendis et al. 2019] però no hi ha aplicacions recents a l'anàlisi de drogues en matrius biològiques.

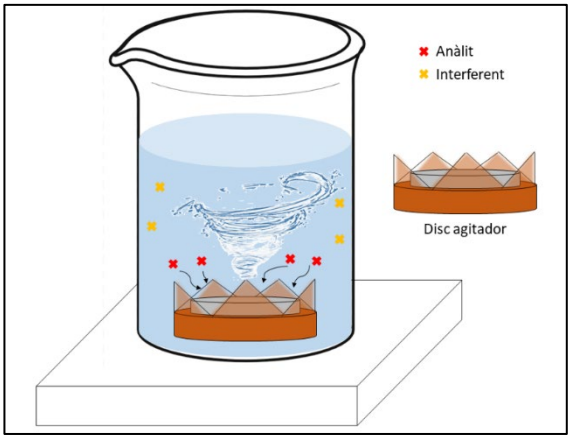


Figura 12. *Esquema d'una extracció adsorbent amb disc agitador.*

5. TIPUS DE FASES ADSORBENTS

Per a portar a terme una SPE en qualsevol de les seus modalitats que han sigut mencionades anteriorment, és necessari seleccionar un material adsorbent adequat per a l'anàlit o anàlits, així com per a la matrius en la que es troben presents. Actualment hi ha nombrosos materials que són capaços de retenir els anàlits bé siga mitjançant interaccions inespecífiques com per interaccions específiques. A aquest apartat es descriuen alguns dels materials que han tingut una major aplicació per a la SPE o materials que tenen bones perspectives de futur.

5.1. FASES GENÈRIQUES

En SPE, cal remarcar que l'extracció depèn de les característiques fisicoquímiques de l'anàlit, concretament de la seua hidrofobicitat i de les seues propietats àcid-base. Segons el tipus d'interacció amb la fase sòlida, les energies d'enllaç són molt variables, en general es pot establir que les forces de Van der Waals poden anar d'1 a 5 kJ mol^{-1}, entre 2 i 50 kJ mol^{-1} per a forces de cert caràcter polar, entre 50 i 500 kJ mol^{-1} per a forces iòniques i des de 200 fins a 1.000 kJ mol^{-1} per a enllaços covalents [Li et al. 2006]. Així atenent al tipus de matriu on es troben els anàlits i el tipus d'interacció que es formaran amb la fase sòlida, els materials genèrics més utilitzats per a la SPE es poden classificar de la forma següent:

- No polars: Aquest tipus de columnes són emprades per a matrius aquoses fonamentalment i entre les fases adsorbents més utilitzades que formen aquest tipus d'interacció amb l'anàlit es troben partícules de sílice modificades amb cadenes de carboni com C18, C8, C6, C4 o C2. D'entre aquestes fases sòlides, una de les que més popularitat té és la C18 la qual s'ha utilitzat per a anàlisi de drogues com ara clonazepam en mostres de saliva [Rojas et al. 2017] o per a la determinació de fluoxetina, sertralina, alprazolam, perfenazina, zolpidem i hidroxicina en sèrum humà mitjançant posterior anàlisi per LC [Wróblewski et al. 2017].

- Bescanvi catiònic: Les columnes de bescanvi catiònic són usades per a matrius aquoses on es produeix un intercanvi de cations entre la mostra i la fase estacionària. Entre els exemples de les més usades es trobenles d' àcid sulfònic com a SCX i les d'àcid carboxílics com a bescanvi feble *(Weak Cation Exchange, WCX)*. Les columnes de bescanvi catiònic s'han emprat prèviament per a

controls antidòping on 264 substàncies van ser determinades en orina mitjançant LC-MS [Kim et al. 2018]

- Bescanvi aniònic: Les columnes de bescanvi aniònic solen estar empaquetades amb amines quaternàries com a materials de bescanvi fort (*Strong Anion Exchange, SAX)* i amines primàries i secundàries o dietilaminopropil com a materials de bescanvi feble *(Weak Anion Exchange, WAX)*. Aquest tipus de fases també són usades per a matrius aquoses on l'anàlit es trobe en forma aniònica. Gràcies a l'ús de columnes de bescanvi aniònic s'han determinat diversos cannabinoides en mostres de plasma i sèrum emprant GC-MS [Gasse et al. 2016].

- Polars: Aquest tipus de fases són les úniques que es poden usar en matrius apolars com alguns dissolvents orgànics i estan pensades fonamentalment per a anàlits amb caràcter polar que experimenten major afinitat per la fase estacionària que pel dissolvent. Es solen usar com adsorbents polars aquells materials modificats amb grups funcionals com SN, Si, NH_3 o OH. Aquestes fases tenen poca aplicació a l'anàlisi toxicològica de drogues pel fet de no ser útils en mostres aquoses [Poole 2003].

5.2. MATERIALS DE TIPUS MIXT

Els materials genèrics típicament emprats per a la SPE no solen mostrar bones capacitats per a la retenció de compostos amb un marcat caràcter polar. Per aquest motiu, els investigadors han portat a terme durant les darreres dècades el desenvolupament de nous materials que presenten una major selectivitat en comparació als materials genèrics tradicionals i que siguin capaços de reconèixer anàlits de més diversa natura d'acord amb els grups funcionals presents. En aquest sentit, una bona alternativa que ofereix certa versatilitat a l'hora de determinar diferents substàncies la presenten els anomenats adsorbents de tipus mixt (*Mixed-Mode Sorbents, MMS)* [Fontanals et al. 2010].

Els MMS poden ser considerats com un híbrid entre els materials genèrics que proporcionen interaccions inespecífiques amb molècules apolars i materials de bescanvi iònic que presenten interaccions de major intensitat amb molècules de marcat caràcter polar. Per aquest motiu, els MMS habitualment es composen de dues fases: una hidròfoba que en nombroses ocasions és de natura polimèrica, que serà la que conforma l'esquelet del material i una segona fase de bescanvi iònic formada per grups ionitzables que permetran l'adsorció de ions de càrrega

contraria. Segons els tipus de grups iònics que formen els materials és possible classificar els MMS en quatre grups diferents: (i) SCX, (ii) SAX, (iii) WCX i (iv) WAX [Fontanals et al. 2020; [Plotka-Wasylka et al. 2017].

Pel que respecta a l'aplicació dels MMS a l'anàlisi de drogues en matrius com fluids biològics o aigües residuals, aquests materials han demostrat tenir bons resultats. Per exemple, l'equip de Jin i col·laboradors [Jin et al. 2018] va portar a terme l'anàlisi de diverses drogues amb característiques àcid-base diferents que incloïen quinidina, fenacetina, hidrocortisona, indometacina, propanolol, alprenolol, cloramfenicol, naproxé o àcid flufenamic en mostres de sèrum, emprant com a fase adsorbent un MMS que combinava WCX (Si-COOH) i WAX (-NH_2) junt a la fase reversa. Aquest estudi que feia ús de LC-UV com a tècnica analítica va aconseguir límits de detecció (*Limit of Detection, LOD)* inferiors a 2,3 μg L^{-1} i recuperacions que oscil·laven entre 90 i 100 % segons l'anàlit. Altres estudis han fet ús de columnes Oasis MAX (Waters) que contenien fases SAX a més de la fase inversa, per a l'anàlisi d'àcid ϒ-hidroxibutíric (GHB) en cabells analitzats per LC-MS, aconseguint recuperacions de 80 % i LODs de 0,33 ng mg^{-1} [Jageredo et al. 2015]. Al 2017, Fontanals i col·laboradors [Fontanals et al. 2017] van emprar com a MMS fases Oasis WCX per a l'anàlisi de tretze catinones diferents en mostres d'aigües residuals i aigües de riu mitjançant LC-HRMS obtenint LODs no superiors a 0,5 ng L^{-1} per a totes les catinones. Altres treballs han fet ús de diverses fases MMS que contenien SAX com HXLPP o SiO_2@DEAEMA-DVB funcionalitzat amb trietanolamina per a l'anàlisi de drogues com carbamazepina, hidrocortisona, amitriptilina, ketoprofé, naproxé o ibuprofè en mostres d'aigua residual obtenint en tots els casos recuperacions quantitatives i LODs inferiors a 0.1 μg L^{-1} [Bratowska et al. 2012; Huang et al. 2018].

5.3. IMMUNOSORBENTS

Els immunosorbents són materials que contenen anticossos immobilitzats sobre una fase sòlida. Aquests anticossos tenen una clara afinitat cap a un antigen que es tradueix en una interacció específica entre l'anticòs i l'anàlit quan aquests primers són usats com a fase adsorbent. Els anticossos són bàsicament glicoproteïnes produïdes per un cos o una cèl·lula com a resposta a la presència d'agents estranys o antígens. Per tant, una de les limitacions més importants dels anticossos és la seua forma d'obtenció, que requereix de l'ús d'animals vius o cultius cel·lulars, essent difícil la seua producció de manera sintètica. Habitualment a l'anàlisi química s'empren anticossos monoclonals o policlonals, tot i que en casos més especials també s'han usat anticossos alternatius com

autoanticossos, anticossos antidiotípics o fragments d'un anticòs [Augusto et al. 2018]. D'entre els dos primers les seues diferències són:

- Monoclonals: Són els que s'obtenen a partir de la producció d'una sola cèl·lula o un sistema immune que resulta en un únic tipus d'anticòs que mostra una bona especificitat i afinitat per un antigen determinat [Hage et al. 2005].

- Policlonals: Són els produïts per diverses cèl·lules a un sistema immune i són els que s'obtenen quan un animal és exposat a un antigen. En aquest cas, l'especificitat normalment depèn de l'interval de temps entre la immunització i el de l'extracció de l'anticòs de l'animal [Hage et al. 2005].

Una vegada s'han obtingut els anticossos necessaris per a reconèixer un antigen, si aquests es pretenen emprar en algun format d'SPE per a l'anàlisi química, serà necessari immobilitzar-lo sobre un suport sòlid (vegeu Figura 13). En aquest sentit es poden diferenciar dos enfocaments per aconseguir una immobilització eficient que permeti el seu ús:

- Covalent: La ruta més comú per a una immobilització covalent, és a partir dels grups amino primaris presents en els anticossos. Estos grups amino, poden ser usats per a l'ancoratge en suports que han estat prèviament activats amb reactius com *N,N*-carbonildimidazol, isotiocianat o bromur de cianogen tot i que les amines lliures també poden reaccionar amb suports que han estat tractats per formar grups epoxi o aldehid sobre la seua superfície. A causa de l'elevat nombre de grups amino present en els anticossos, la immobilització es pot donar per diversos punts diferents de l'anticòs fet que pot comportar una pèrdua de l'activitat del mateix a l'hora de reconèixer l'anàlit [Jung et al. 2008].

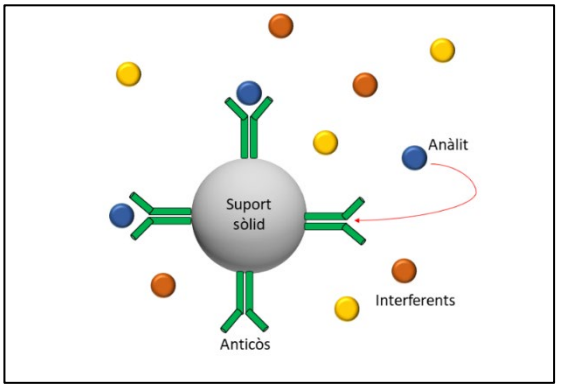


Figura 13. *Anticossos immobilitzats sobre la superficie d'un suport sòlid.*

- No covalent: En aquest cas, la immobilització consisteix en una adsorció sobre el suport sòlid basada en forces de Van der Waals, interaccions electrostàtiques o enllaços iònics, totes elles molt més febles que les interaccions covalents. Aquest enfocament, en principi, comporta habitualment problemes a causa d'una orientació molt més aleatòria dels anticossos immobilitzat. Per tractar de minimitzar aquest problema es solen modificar els anticossos emprant biotina. Si aquests anticossos modificats amb l'addició de biotina s'immobilitzen sobre alguns suports basats en avidina o estreptavidina es poden aconseguir ancoratges considerablement eficients. Cal tenir en compte que aquesta immobilització serà molt més sensible a l'ús de determinats dissolvents que no pas una immobilització covalent [Mejía-Manzano et al. 2016].

Els immunosorbents han mostrat bones capacitats per a l'anàlisi de molècules menudes en matrius complexes, a causa de l'elevada selectivitat que proporcionen els fan bons candidats per a l'anàlisi de drogues en fluids biològics. Tanmateix, la seua aplicació en format SPE no ha sigut molt extensa fins ara. L'elevada selectivitat que aquests proporcionen, habitualment els fa capaços d'aïllar l'anàlit de la resta de components d'una matriu complexa. Per exemple Vidal i col·laboradors [Vidal et al. 2016] han emprat anticossos immobilitzats sobre partícules magnètiques per a la determinació de cocaïna en mostres d'orina, saliva i sèrum mitjançant espectrofotometria, obtenint-se recuperacions quantitatives superiors al 86 % i amb LODs no superiors a 0,15 μg L^{-1}.

5.4. APTÀMERS

Els aptamers són cadenes d'oligonucleòtids d'una sola heura que habitualment contenen entre 20 i 100 bases. Aquestes cadenes d'ADN o ARN poden oferir respostes específiques front a determinades molècules orgàniques de manera molt similar a la que ho fan els anticossos. Els aptamers tenen una estructura tridimensional específica que aconsegueix crear plegaments que per la seua grandària i grups funcionals ofereixen selectivitat front a una molècula determinada. Aquestes molècules que poden mostrar afinitat per un aptàmer poden ser cations metàl·lics divalents, proteïna, cèl·lules o altres molècules orgàniques de xicoteta grandària (veure Figura 14) [Pichon et al. 2015].

Per a seleccionar l'aptàmer més adequat, en primer lloc s'ha de recorrer a l'anomenat mètode SELEX (*Systematic Evolution of Ligands by EXponential enrichment)*, que fa ús d'un banc de més de 10^{15} seqüències d'ADN o ARN del qual es van seleccionant aquelles cadenes que ofereixen habilitat per a reconèixer una

molècula diana o *target*. Una vegada s'ha seleccionat l'aptàmer, aquest es produeix de manera sintètica de manera senzilla [Darmostuk et al. 2015].

Els aptamers han estat usats per a nombrosos mètodes d'anàlisi, algun d'ells similars al ELISA, però substituint els anticossos per aquets lligands de reconeixement. També, aquests han estat emprats per a fer biosensors [Toh et al. 2015]. No obstant, el seu ús per a la preparació de fases estacionaries selectives per a SPE és relativament recent i poc explorat comparat a altres materials, però que fins ara mostra bones expectatives per l'aplicació a nombrosos tipus d'anàlisis [Toh et al. 2015].

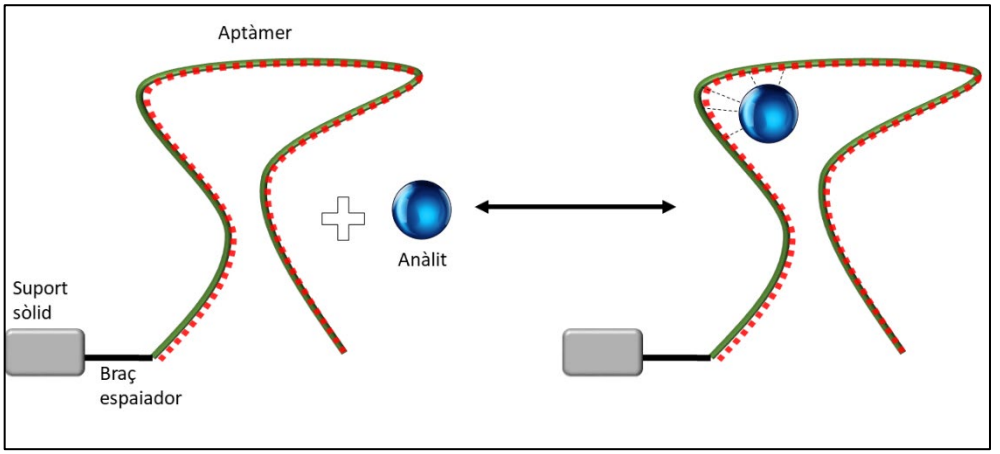


Figura 14. *Representació de l'afinitat que mostra un aptàmer immobilitzat sobre un suport sòlid.*

D'igual manera que amb els anticossos, els aptamers han de ser ancorats a suports sòlids que permeten la seua aplicació en SPE. En general, aquests suports han de ser químicament inerts, estables mecànicament i tenir una bona homogeneïtat pel que fa a la seua grandària de partícula i superfície. Aquests suports s'han de poder activar de manera senzilla per tal de permetre una unió efectiva entre les biomolècules i a més tenir una elevada grandària de partícula que permeti l'accés dels aptamers per poder ser ancorats a la superfície. A més, també han de ser hidrofílics per a evitar interaccions inespecífiques [Pichon 2019].

Gràcies a que els aptamers poden ser obtinguts mitjançant síntesi química, és fàcil introduir modificacions tant a l'extrem 5' com al 3' de la cadena d'oligonucleòtids que faciliti la seua immobilització. La modificació que s'hi vol introduir es selecciona segons el mètode i el suport que es vagin a triar per a la immobilització. Una de les modificacions més usades és la modificació amb grups terminals amino, ja que aquests permeten un mètode d'immobilització molt

similar al dels anticossos, amb la diferència que en aquest cas, els aptamers tindrien un ancoratge orientat d'una manera més específica que en el cas dels anticossos, ja que el grup amino estarà introduït específicament en un dels extrems de la cadena. A més, també poden ser introduïts braços espaiadors com C6, C12 o derivats d'etilenglicol que permeten mantenir les propietats de l'aptàmer quan aquest és ancorat sobre la superfície. Altres mètodes per a immobilitzar-los inclouen la introducció de grups tiol que afavoreixen l'ancoratge sobre superfícies d'or o emprant biotina per aconseguir una unió no covalent a partícules d'estraptavidina. Aquesta última immobilització no covalent, presenta alguns inconvenients d'igual manera que ocorre amb els anticossos com és la reduïda vida útil de l'adsorbent per l'ús de dissolvents orgànics que afecten a la interacció biotina-estreptavidina [Madru et al. 2011; Pichon 2019].

Pel que respecta als procediments SPE que involucren materials preparats amb aptamers com a adsorbents, la adequada retenció de l'anàlit ve donada per la mostra, la composició de la qual ha de ser tant semblant com sigui possible a la composició del tampó emprat durant la selecció de l'aptàmer. Aquest tampó anomenat tampó d'enllaç *(Binding Buffer, BB)* afecta considerablement a la conformació de l'aptàmer, cosa que és de vital importància per a un reconeixement efectiu de l'anàlit. A més de la presència de determinats cations en el BB, altres paràmetres que poden afectar considerablement al procediment d'extracció són el pH i la força iònica del mateix, així com la temperatura [Pichon 2019]. Per a l'elució de l'anàlit es poden adoptar diversos enfocaments com l'ús de mescles d'aigua i dissolvents orgànics i agents caotròpics com $NaClO_4$ o agents desnaturalitzants com l'EDTA. Altres estratègies també fan ús d'un augment de la temperatura o variacions del pH per a eluir l'anàlit. És comú que en finalitzar el procediment d'extracció els adsorbents que contenen aptamers siguin conservats en el seu corresponent BB a una temperatura de 4 °C [Pichon 2019].

A la bibliografia es descriuen diversos treballs on es fa ús de fases estacionàries que contenen aptamers com a adsorbent selectiu per a la determinació de diverses drogues. L'equip de Madru [Madru et al. 2009] va determinar cocaïna en plasma emprant un aptàmer modificat amb un grup amino que incloïa un braç espaiador C6. Altres treballs també han emprat aptamers en SPE per a la determinació de cocaïna i diclofenac en mostres d'aigua emprant la mateixa modificació que s'ha descrit anteriorment [Hu et al. 2011]. També s'ha emprat cel·lulosa com a suport per a la immobilització d'aptàmers i la determinació de codeïna en mostres d'orina, així com per a la determinació de metamfetamina en mostres de saliva i plasma, en tots dos casos emprant una

modificació amino d'un dels extrems de la cadena amb un braç espaiador C6 [Hashemian et al. 2014; Zargar et al. 2018]. Altres treballs han emprat capes de grafé per a la immobilització d'aptàmers modificats amb NH_2/C6 per a la determinació de trombina en plasma [Xu et al. 2017].

5.5. ALTRES MATERIALS

A banda dels materials dels que s'ha parlat anteriorment, existeixen alguns altres materials que tot i no haver estat molt estudiats en quant a la seua aplicació per a l'anàlisi de drogues però, presenten bones característiques i oportunitats per al seu desenvolupament en aquest sentit.

Els nanomaterials són aquells que presenten almenys en una direcció una grandària inferior a 100 nm, tot i que la definició de que pot ser nanomaterial encara no és clara i ben acceptada per tothom. D'entre els nanomaterials, els nanotubs de carboni (*Carbon NanoTubes, CNTs)* representen un grup de nanomaterials que tenen una geometria, així com propietats mecàniques, electròniques i químiques úniques. Els CNTs són cilndres formats per capes enrotllades de grafit que poden ser separats en dos grups: *Single-Walled Carbon Nanotubes (SWCNTs)* i *Multi-Walled Carbon Nanotubes (MWCNTs)* els quals estan formats per una única capa de grafit o per diverses superposades, respectivament. Els CNTs han estat emprats en diverses aplicacions com a portadors per al lliurament de fàrmacs, com a lubricants en la síntesi de medicaments, en enginyeria genètica com a conservants per la seua natura antioxidant, com a eina per al diagnòstic, per a catàlisi o com a biosensors [Varshney 2014]. Tot i el potencial d'aquest tipus de material per a l'anàlisi química, fins al moment no ha tingut una gran aplicació en particular per a l'anàlisi de drogues en matrius biològiques. Al 2011 l'equip de Bhadra [Bahdra et al. 2011] va publicar un treball on es feien ús de MWCNTs per l'anàlisi de diversos medicaments (ibuprofè, ciprofloxacina, acetaminofè fenofibrat i sulfadiazina) en mostres d'aigua, que van ser determinats mitjançant LC-UV amb LODs que en cap dels casos van superar els 4,5 $\mu g\ L^{-1}$.

Altres materials d'especial rellevància són les estructures organometàl·liques (*Metal-Organic Frameworks, MOFs)*. Els MOFs són estructures basades en la química de coordinació o formació de complexos, que estan constituïts per un catió metàl·lic i una sèrie de lligands orgànics. Aquestes estructures quan són preparades sota les condicions adequades donen lloc a estructures tridimensionals que poden ser funcionalitzades amb diferents grups. Un dels aspectes més atractius dels MOFs a l'hora d'usar-los en SPE és la seua elevada

àrea superficial junt a la senzilla modificació que se'ls pot aplicar, la seua senzilla síntesi així com una bona estabilitat [Rocío-Bautista et al. 2019]. Tot i que no estan dels materials més usats per a l'anàlisi de drogues en fluids biològics, alguns treballs han fet ús de MOFs per a la determinació, per exemple, de metadona, metamfetamina i tramadol en mostres de plasma i orina mitjançant CE-UV [Fakhari et al. 2017].

També els anomenats materials d'accés restringit *(Restricted Access Materials, RAMs)* tenen bones oportunitats per a l'anàlisi de drogues en mostres biològiques. Els RAMs són materials capaços d'extraure diversos tipus d'anàlits directament de matrius biològiques sense cap tractament previ, ja que aquests poden impedir l'accés de molècules grans com proteïnes a l'interior del material mitjançant un procediment d'exclusió per grandària [Sadílek et al. 2007]. Nombrosos estudis recents han descrit procediments per obtenir RAMs a partir de materials com polímers, CNTs o materials basats en sílice emprant grups hidrofílics així com la presència de porus accessibles només per a molècules de baix pes molecular. La capa hidrofílica evita per tant una unió irreversible de les proteïnes sobre la superfície del material adsorbent que ajudarà a donar-li major vida útil tot i que el paper vital en l'exclusió de macromolècules el juguen els porus que impedeixen l'accés [Dipe de Faria et al. 2017]. Aquest material, per aquestes característiques han demostrat les bones oportunitats que ofereixen per a l'anàlisi de molècules menudes com ho són les drogues en fluids biològics [Souverain et al. 2004; Cassiano et al. 2006].

5.6. POLÍMERS DE RECONEIXEMENT MOLECULAR

L'any 1931 Polyakov va observar que el gel de sílice preparat en presència de diferents dissolvents com benzè, toluè o xilè mostrava certa afinitat per aquests dissolvents front a d'altres que no havien estat presents durant la preparació [Polyakov 1931]. Aquest fet podria estar considerat l'inici de la síntesi de materials que mostraven reconeixement molecular. Fent ús de la teoria que explica l'aparició d'anticossos als sistemes biològics, Dickey va ser capaç d'explicar els resultats de Polyakov, a partir de diversos derivats del taronja de metilè, el qual s'usava per a l'obtenció de gel de sílice [Dickey 1955]. Més endavant, a la dècada dels 70, diversos treballs publicats demostraven la presència de llocs de reconeixement dins de polímers orgànics sintetitzats al laboratori [Wulff et al. 1972; Takagishy et al. 1972].

Des d'aleshores, els MIPs han esdevingut materials selectius de gran interès per al tractament de mostra [Choong et al. 2013], catàlisi [Muratsugu et al. 2020]

o fabricació de sensors [Lutfi-Yola et al. 2017] a causa de la seua bona resistència mecànica i durabilitat respecte d'altres materials selectius com anticossos [Qiao et al. 2006], essent capaços de romandre estables durant anys en condicions de conservació poc rigoroses. També han demostrat que en la majoria dels casos poden ser usats més d'una ocasió i en finalitzar el seu ús poden ser estabilitzats de manera senzilla. A més pel que respecta al cost, els MIPs poden ser fins 200 vegades més barats que altres materials selectius [Wackerling et al. 2016].

Els MIPs són materials sintètics i artificials dissenyats de manera adequada per tal d'obtenir llocs de reconeixement específic per a una molècula diana. Aquests llocs de reconeixement una vegada estan lliures són capaços de retenir de manera selectiva una molècula o una família de molècules estructuralment similars front a altres d'estructura variada que quedarien excloses. Els MIPs són obtinguts a partir de la polimerització de monòmers funcionals i agents entrecreuants en presència d'una molècula plantilla o *template*. Així, es forma una xarxa polimèrica tridimensional al voltant d'aquest *template*, donant lloc a les cavitats de reconeixement específiques. Finalitzada la polimerització cal extraure la molècula plantilla per deixar lliure els llocs de reconeixement que hi seran complementaris en grandària, forma i grups funcionals [Turiel et al. 2020].

La polimerització per radicals lliures és un dels mecanismes més importants per a l'obtenció de MIPs, especialment a nivell industrial a causa de la seua facilitat per escalar-la. En aquest mètode, els monòmers i agents entrecreuants es converteixen en polímers directament a partir de l'aplicació de temperatures suaus o de radiació UV. La polimerització pot ser portada a terme en dissolució o en bloc i és molt tolerant a la presència de grups funcionals en diversos monòmers així com a la possible presència d'impureses en els reactius. La polimerització radicalària es porta a terme en tres etapes: (i) iniciació, (ii) propagació i (iii) terminació.

En termes generals, la propagació és un procediment molt més ràpid que la iniciació, cosa que afavoreix el creixement polimèric donant lloc a unitats moleculars d'elevat pes molecular en contra de la formació de nombrosos nuclis que acabarien esdevenint-ne partícules de petita grandària. L'iniciador sol estar actiu durant tot el temps en que transcorre la polimerització, cosa que permet seguir el procés de polimerització en qualsevol instant. Els iniciadors habitualment s'afegeixen en quantitats no superiors a l'1% en pes o en mols i gràcies a ell la reacció es controla per l'acció de la temperatura, radiació o químicament [Cormack et al. 2004].

Segons la interacció entre el *template* i el MIP, hi ha diversos enfocaments de l'empremta molecular:

- No covalent. És el tipus d'empremta molecular més comú a causa de la seua senzillesa i bona eficàcia per a anàlits molt variables. Les interaccions no covalents comprenen una varietat de tipus d'enllaços que dependran dels grups funcionals de l'anàlit així com del monòmer en qüestió [Spivak et al. 2005]. Les interaccions àcid-base de Lewis són aquelles que comprenen l'atracció entre dos parts de les molècules de càrrega oposada que suposa una transferència parcial de la càrrega de la base a l'àcid. El tipus més important d'aquestes interaccions són els enllaços d'hidrogen que ocorren en aquelles molècules on l'àtom d'hidrogen va enllaçat de manera covalent a un àtom més electronegatiu i forma l'enllaç d'hidrogen amb altres molècules amb una part amb elevada densitat de càrrega [Grabowski 2016]. Les forces de Van der Waals engloben tres tipus de forces: les forces de Keesom, entre dos dipols permanents; les forces de Debye, entre un dipol permanent i un dipol induït; i les forces de London, entre dos dipols induïts, totes aquestes forces als MIPs habitualment actuen de manera complementaria però són de gran importància [Li et al. 2006]. Les interaccions hidròfobes són aquelles que apareixen entre molècules apolars en dissolvents polars a causa de la tendència de les molècules solvatades pel dissolvent d'agrupar-se per augmentar la seua estabilitat. Als MIPs que fan ús d'aquest tipus d'interaccions, l'exemple més destacable és ciclodextrina que forma llocs de reconeixement capaços de retenir molècules apolars. No obstant, el major problema d'aquest tipus d'interaccions són la seua manca de selectivitat [Kyzas et al. 2013].

- Covalent. Hi ha enllaços covalents adequats per a la síntesi de MIPs com els enllaços amino, tiol, diol, àcid borònic o bases de Schiff. L'enfocament covalent de l'empremta molecular te alguns avantatges com la presència de llocs de reconeixement homogenis i altament selectius. La principal limitació d'aquest enfocament és la necessitat d'enllaços covalents que siguin reversibles així com la seua preparació que sovint suposa polimeritzacions molt més complexes que en altres enfocaments [Shen et al. 2014].

- Semicovalent. Aquest enfocament consisteix en un enllaç covalent entre el *template* i el monòmer durant la formació del MIP i un posterior enllaç no covalent entre l'anàlit i els llocs de reconeixement [Qi et al. 2010].

- Coordinació metall-lligand. Aquest tipus de MIPs han guanyat popularitat recentment per la síntesi de polímers de reconeixement iònic (*Ion Imprinted Polymer, IIP*). Els IIP és basen en el mateix principi que la formació de llocs de reconeixement que als MIPs, però en aquest cas els monòmers funcionals són canviats per lligands orgànics que formen interaccions estables i selectives amb un metall [Branger et al. 2013]

5.6.1. SÍNTESI DE MIPs

La síntesi de MIPs, generalment és un procés senzill però que requereix una acurada selecció de reactius i condicions per tal d'obtenir un polímer amb les característiques apropiades, com una bona estabilitat mecànica, porositat suficient així com una selectivitat tan alta com sigui possible. Fonamentalment els reactius que s'han de considerar a la síntesi d'un MIP són: la pròpia molècula plantilla o *template,* el monòmer, a, l'agent entrecreuant o *cross-linker*, l'iniciador i finalment el dissolvent o porogen. Tanmateix, a banda de seleccionar els reactius i les seues quantitats és important controlar una sèrie de factors com la via de polimerització: tèrmica o UV, així com el seu temps [Yan et al. 2006].

- *Template*. El *template* o molècula plantilla és l'encarregada de formar el complex de prepolimerització amb el monòmer i que en finalitzar la síntesi deixarà els llocs de reconeixement per a l'adsorció de l'analit. A causa de que el polímer es forma al voltant del *template*, és necessari eliminar el *template* per alliberar els llocs de reconeixement. La selecció del *template* està limitada a l'anàlit que es vaja a analitzar posteriorment, per tant, a l'aplicació concreta del MIP. No obstant, aquesta molècula plantilla ha de complir una sèrie de requisits com són: estabilitat tèrmica o fotoquímica, segons les condicions en que es va a portar a terme la polimerització, i a més ha d'actuar de manera reversible amb el monòmer [Madikizela et al. 2018]. Quan el propi anàlit no sigui capaç de complir aquestes característiques és possible recórrer als anomenats *dummy-template* que són molècules similars a l'anàlit en grandària i grups funcionals que crearan llocs de reconeixement que seran selectius front a l'anàlit d'igual manera. Per exemple, l'equip de Marc [Marc et al. 2018] va sintetitzar un MIP emprant com a *template* 4,4'-dihidroxidifenil èter per a la determinació de polibromodifenils èters. Existeixen també, els MIPs *multi-template*, els quals es preparen en presència de dos o més *templates* per a la determinació simultània de dos o més anàlits. És fonamental en aquests casos

que els *templates* no mostren interacció entre ells o que sigui menyspreable en comparació a la que mostren amb els monòmers [Mdikizela et al. 2016].

- Monòmer. És un dels components de la mescla més importants, ja que és el responsable de la formació dels llocs de reconeixement a partir de la formació d'un complex estable i reversible amb el *template.* Per aquest motiu el monòmer ha de ser complementari en grups funcionals al *template*. L'elecció del monòmer adequat i la seua concentració afectarà a la formació dels llocs de reconeixement. En aquest sentit, generalment la proporció *template:*monòmer més emprada és 1:4, essent l'àcid metacrílic el monòmer més versàtil i usat per les seues característiques àcid-base, d'entre una gran varietat de monòmers que inclouen àcids carboxílics, àcids sulfònics o bases heteroatòmiques d'entre altres. A la Figura 15 és mostren exemples de les estructures d'alguns dels monòmers més populars com l'àcid metacrílic *Methacrylic Acid, MAA).* [Gué et al. 2017].

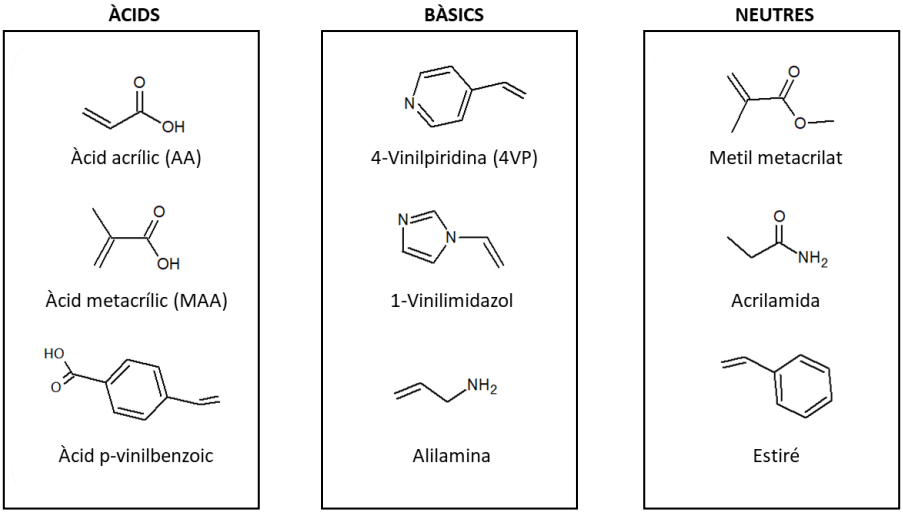


Figura 15. *Mònomers típicament emprats a la síntesi de MIPs.*

- <u>*Cross-linker.*</u> La funció principal de l'agent entrecreuant o *cross-linker* és garantir que els grups funcionals dels monòmers es troben en les posicions adequades per a un reconeixement específic i al mateix temps, mantenir la integritat estructural del polímer. D'aquesta manera, l'habilitat de reconeixement selectiu i les propietats mecàniques del MIP, dependran fortament de la natura i de la proporció del *cross-linker*. Generalment, s'accepta que una proporció adequada d'agent entrecreuant és del 80 % [Muhammed et al. 2012]. Hi ha una gran varietat de *cross-linkers*, les estructures d'alguns dels quals, com el dimetacrilat d'etilenglicol (*Ethylene Gylcol Dimethacrylate, EGDMA*) o el DVB que són dels més emprats es mostren a la Figura 16.

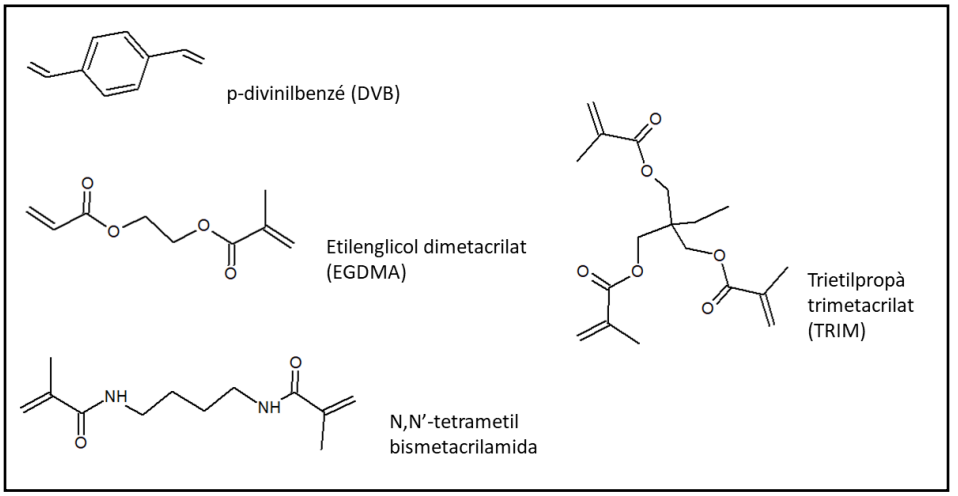


Figura 16. *Agents entrecreuants típicament usats a la síntesi de MIPs.*

- <u>Iniciador.</u> En general, els iniciadors són els encarregats de desencadenar la reacció de polimerització. Habitualment, alts percentatges d'iniciador donen polímers més rígids el que dona llocs de reconeixements més rígids també i una major especificitat. D'altra banda, percentatges reduïts d'iniciadors redueixen la temperatura a la que aplega la mescla de polimerització, cosa que es tradueix en la formació d'unes cavitats apropiades per al reconeixement de l'anàlit [Mijangos et al. 2006]. Cada iniciador es pot descompondre per vies diferents, bé siga tèrmicament o mitjançant l'aplicació de radiació UV, tot i que molts actuen sota les dues vies. A la Figura 17 es poden veure alguns exemples dels iniciadors més emprats.

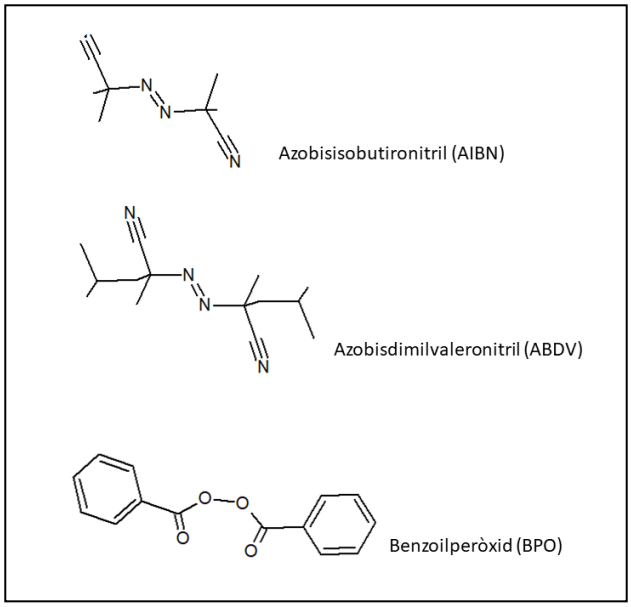


Figura 17. *Iniciadors típicament usats per a la síntesi de MIPs.*

- Dissolvent. Els polímers aconsegueixen estructures poroses amb una elevada àrea superficial i una capacitat de càrrega satisfactòria gràcies a l'ús de dissolvents porogènics. Generalment aquests dissolvents es seleccionen en base a l'experiència prèvia o segons les seues propietats fisicoquímiques, incloent el moment dipolar, el coeficient de partició o la solubilitat [Mansour et al. 2020]. A la majoria de casos es prefereixen dissolvents apròtics que siguen capaços de solvatar el complex de prepolimerització i no interferir entre les interaccions *template*-monòmer com poden ser l'acetonitril, el cloroform o el tolué.

- Temperatura. Per tal d'iniciar la reacció radicalària s'ha d'aplicar habitualment un augment de la temperatura, però també és imprescindible que el complex *template*-monòmer siga estable en les condicions de polimerització, per garantir la formació de llocs específics. En aquest sentit, la temperatura juga un paper fonamental i temperatures baixes que afavoreixen la complexació a causa d'una reducció de la influència de modes de vibració residuals, per aquest motiu es solen emprar les temperatures compreses entre 60 i 70 °C, que activen els iniciadors sense afectar al complex de prepolimerització [Piletska et al. 2009].

- Radiació UV. En presència d'un fotoiniciador, l'aplicació de llum UV (normalment 254 o 365 nm) provoca la seua descomposició radicalària desencadenant la reacció de polimerització. La quantitat de radicals produïts depenen tant del medi com de la natura de l'iniciador. Tanmateix, l'eficiència del fotoiniciador depén del seu propi espectre d'absorció així com de la seua compatibilitat amb l'espectre de la resta de reactius [Catalá-Icardo et al. 2020]

- Temps de polimerització. A priori, un temps llarg de polimerització porta a una major conversió de reactius que polimeritzen. Així, els MIPs que polimeritzen durant llargs períodes de temps esdevenen estructures més rígides que en conseqüència tindran cavitats millor definides i que aportaran una major especificitat. Però, també és cert que polímers excessivament rígids poden comportar problemes de transferència de massa i una cinètica massa lenta. Per tant, la polimerització deurà tindre un temps que garantisca les dues propietats del MIP [Piletska et al. 2009].

Segons el format d'extracció emprant MIPs que es requerisca obtindre hi han nombroses tècniques de polimerització que varien en el tipus de material obtingut i en les seues característiques. A continuació, s'exposen de manera breu les principals tècniques de polimerització.

En primer lloc, una de les més destacables ha estat la polimerització en bloc. En la polimerització en bloc, el polímer es forma com a una única unitat al voltant de les molècules de *template* i després de la polimerització és necessari eliminar-lo de la matriu polimèrica mitjançant tècniques de rentat, tal i com es pot veure a la Figura 18. Amb aquesta tècnica es forma un monòlit més o menys rígid segons les condicions. Posteriorment si en lloc d'obtenir un monòlit es desitja obtenir partícules de grandària petita, el polímer ha de ser triturat i seleccionar les partícules segons la seua grandària mitjançant un tamisat de les partícules molturades. En general, la polimerització en bloc és la tècnica preferida per a la síntesi de MIPs de molècules petites ja que teòricament l'adsorció de l'anàlit serà ràpida i reversible afavorint la seua reutilització [Ertürk et al. 2017]. Entre els principals desavantatges de la polimerització en bloc es troba la destrucció de llocs de reconeixement al triturar el polímer. No obstant, aquest desavantatge queda ben resolt si es te en compte les elevades àrees superficials que s'obtenen així com la senzillesa d'aquesta tècnica en comparació a la resta de tècniques de polimerització [Pérez-Moral et al. 2004].

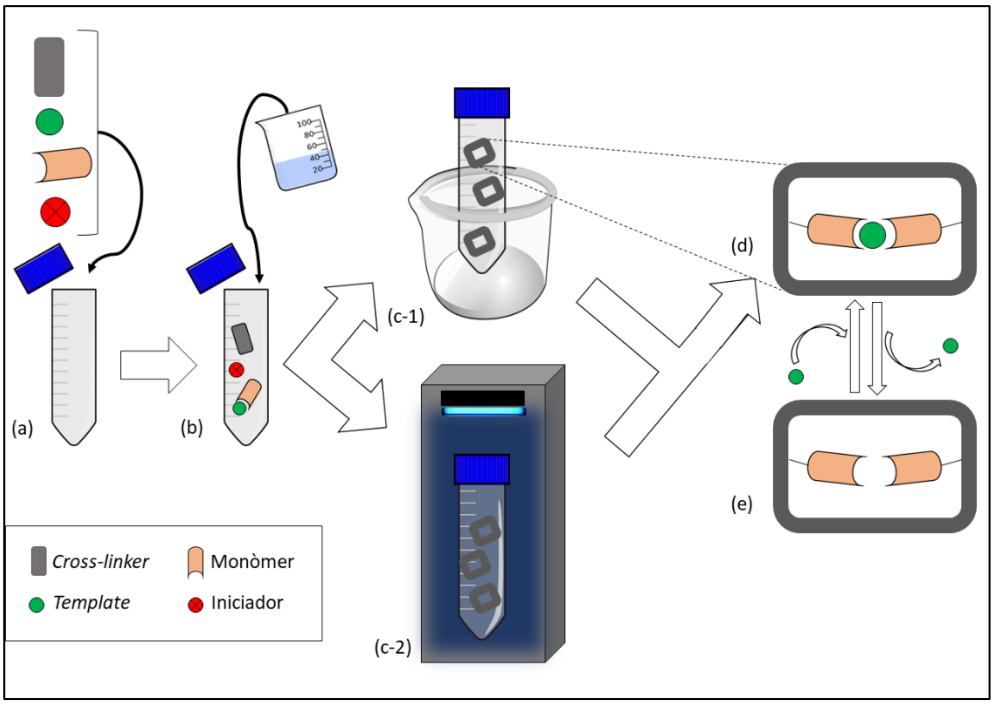


Figura 18. *Esquema de la síntesi d'un MIP mitjançant polimerització en bloc: (a) pesada de reactius, (b) addició del dissolvent, (c-1) polimerització per vía térmica; (c-2) polimerització mitjançant radiació UV, (d) obtenció del MIP amb el template a l'interior i (e) obtenció del MIP amb els llocs de reconeixement alliberats.*

En segon lloc, es troba la polimerització per suspensió. Aquesta ha estat una de les alternatives més comunes front a la polimerització en bloc. Mitjançant aquesta tècnica s'obtenen partícules esfèriques més homogènies sense la necessitat de triturar el polímer obtingut, evitant així la destrucció dels llocs de reconeixement. Per obtindre les partícules de MIP esfèriques s'han de seleccionar dues fases: una orgànica que conté l'iniciador, monòmer, agent entrecreuant, *template* i dissolvent porogènic i una segona fase aquosa. Ambdues fases són mesclades mitjançant agitació vigorosa i s'inicia la polimerització radicalària, donant com a resultat de la formació de petites gotes de la fase orgànica dispersa en la fase aquosa. Les partícules esfèriques de MIP obtingudes per aquest tipus de polimerització es situen al voltant de 6 i 40 µm de diàmetre [Pérez-Morat et al. 2004]. La principal limitació que troba aquesta tècnica és la possible migració del *template* de la fase orgànica a la fase aquosa, el que reduiria la capacitat de càrrega i l'eficàcia en la preparació del MIP [Prasad et al. 2017].

La polimerització per emulsió també ha estat una tècnica habitual en la preparació de MIPs. La polimerització per emulsió permet també l'obtenció directa de partícules esfèriques, d'uns 70 nm de diàmetre que no requereixen trituració, cosa que redueix la possibilitat de destruir llocs de reconeixement. Aquest tipus de polimerització dona esferes elevadament uniformes de manera ràpida i estable [Zhao et al. 2020]. Durant la polimerització per emulsió es formen miscel·les per l'acció d'un tensioactiu, com per exemple el Tween 20, que dona lloc a esferes encapsulades en una matriu aquosa que proveeixen el medi necessari per a la polimerització [Kujawska et al. 2018].

També n'és destacable la polimerització per precipitació que permet l'obtenció de partícules esfèriques de grandàries d'uns 700 nm [Pérez-Moral et al. 2004]. A diferència d'altres tècniques, la polimerització per precipitació no requereix l'addició d'un tensioactiu i en canvi fa ús de volums de dissolvent molt elevats. Els petits nuclis que es van formant van capturant oligòmers permetent la formació de petites esferes de MIP amb bons rendiments. A més, es pot controlar la grandària i la porositat a partir de les condicions de polimerització [Wang et al. 2003].

La polimerització per etapes és una de les tècniques de polimerització més complexes que te associades reaccions en condicions molt específiques, per contra, permet l'obtenció de partícules esfèriques uniformes que són preparades per tenir un diàmetre concret. La polimerització per etapes, sovint involucra alguna etapa de formació d'emulsions aquoses. Els MIPs obtinguts són especialment útils per a aplicacions cromatogràfiques [Nakamura et al. 2017].

Finalment, la polimerització per empremta sobre la superfície té aplicacions més concretes per a la preparació de MIPs de grans molècules. La cinètica d'adsorció dels anàlits sobre el MIP no sempre és suficientment ràpida, cosa que limita la seua aplicació per a l'anàlisi de molècules de gran grandària com proteïnes. En aquests casos, una alternativa emprada en l'actualitat és l'empremta sobre la superfície de partícules de MIP. Per aconseguir-ho, una partícula sòlida, com sílice modificada [Yilmaz et al. 2000] es fa servir com a com a suport sobre el qual els reactius necessaris per preparar el polímer polimeritzaran. Així doncs, posteriorment el *template* serà eliminat de la superfície i els llocs de reconeixement lliures del *template* quedaran sobre aquesta [Ding et al. 2014].

5.6.2. CARACTERITZACIÓ DE MIPs

Quan s'ha finalitzat la síntesi del MIP, en algunes ocasions pot ser interessant conèixer les seus principals característiques, per aquest motiu hi ha diverses tècniques que ens permeten conèixer-les des de diverses perspectives com per exemple la composició química dels polímers, la seua forma i propietats físiques o finalment una anàlisi més pràctica avaluant les seues capacitats per reconèixer els anàlits. Tal i com es recull a la Taula 4, Cormack i col·laboradors [Cormack et al. 2004] van resumir aquelles tècniques analítiques més comunament emprades per a la caracterització de MIPs una vegada són obtinguts.

En primer lloc hi ha casos en que pot ser interessant portar a terme una caracterització química dels MIPs. Aquesta caracterització fa referència sobretot a conèixer la seua composició elemental o els tipus d'enllaços que s'han format. En aquest sentit, les tècniques que més s'han emprat per a aquest tipus de caracterització han estat la microanàlisi elemental, per conèixer i semiquantificar la presència d'algun element concret, especialment en aquells casos en que el MIP pugui contenir heteroàtoms. De la mateixa manera, l'espectroscòpia infraroja amb transformada de Fourier *(Fourier Transform InfraRed, FTIR)* també pot proporcionar valuosa informació sobre la composició elemental del polímer preparat, així com de la formació d'enllaços que s'hi puguen produir entre els diferents reactius. Finalment, la ressonància magnètica nuclear *(Nuclear Magnetic Ressonance, NMR)* també ha estat emprada per donar informació sobre l'entorn en el que es troba el polímer.

És habitual, quan s'ha preparat per primera vegada un MIP, estudiar la seua morfologia per conèixer paràmetres tan importants com l'àrea superficial o la seua porositat. D'aquesta manera, pot ser interessant conèixer factors com la quantitat de dissolvent que el MIP és capaç d'adsorbir i d'aquesta manera fer una estimació del volum específic dels porus. Altres formes de caracterització molt més precises són la mesura de l'àrea superficial, del volum dels porus i el seu diàmetre així com la distribució de micro i mesoporus així com de macroporus, tots aquest paràmetres poden ser fàcilment obtinguts emprant isotermes d'adsorció/desorció de nitrogen. Altres tècniques permeten conèixer la grandària dels porus, com per exemples la porosimètria d'intrusió de mercuri que en aquest cas només serveix per caracteritzar els macroporus. Altra tècnica emprada en la caracterització morfològica és la cromatografia d'exclusió de grandària inversa la qual ajuda a mesurar l'estructura dels porus en estat de solvatació. Si cal destacar alguna tècnica de caracterització morfològica per la seua rellevància i extens ús

després de la preparació de MIPs és la microscòpia electrònica d'escombratge *(Scanning Electron Microscopy, SEM)* que dona una visió de l'estructura superficial del MIP i permet estimar la grandària dels macroporus així com la seua rugositat [Spivak et al. 2005].

Taula 4. *Principals tècniques usades per a la caracterització de MIPs [Cormack et al. 2004].*

Tipus	Paràmetre	Tècnica
Caracterització química	Percentatge d'algun element concret (si el MIP te heteroàtoms)	Microanàlisi elemental
	Mesura de la composició elemental i de la formació d'enllaços no covalents	FTIR
	Composició de la matriu polimèrica	NMR en estat sòlid
Caracterització morfològica	Volum específic dels porus	Consum de dissolvent
	Mesura de l'àrea superficial, volum dels porus, diàmetre dels porus i distribució	Isotermes d'adsorció/desorció de nitrogen
	Grandària de macroporus (volum i diàmetre)	Porosimètria d'intrusió de mercuri
	Estructura dels porus en estat de solvatació	Cromatografia d'exclusió de grandària inversa
	Estructura superficial del MIP. Macroporositat i rugositat de la superfície	SEM
Capacitat de reconeixement	Mesura de la selectivitat i capacitat d'extracció	Estudis de reconeixement

FTIR: espectroscòpia infraroja amb transformada de Fourier, MIP: polímer de reconeixement molecular, NMR: ressonància magnètica nuclear, SEM: microscòpia electrònica d'escombratge.

Finalment, una de les característiques més importants dels MIPs és la seua selectivitat, per açò és fonamental fer estudis de recuperació així com d'avaluació de selectivitat mitjançant la comparació amb polímers no empremtats *(Non-Imprinted Polymer, NIP)*, per tal d'obtenir una mesura de la seua eficàcia en l'extracció.

A la bibliografia hi ha nombrosos treballs que descriuen l'ús de les tècniques mencionades anteriorment per a la caracterització de MIPs. A mode d'exemple es pot citar el treball de Barahona i col·laboradors [Barahona et al. 2010] on van fer ús de SEM, isotermes d'adsorció/desorció i microanàlisi elemental per a la caracterització d'un MIP preparat emprant tiabendazol com a *template*. També s'han caracteritzat MIPs preparats per a la determinació d'atrazina emprant toluè i acetonitril com a dissolvent porogènic, emprant tècniques com la porosimetria d'intrusió de mercuri, la SEM o estudis de càrrega i reconeixement d'anàlits [Zhang et al. 2019]. De la mateixa manera, aquestes tècniques s'han emprat per a la caracterització de MIPs preparats en forma de monòlit, com l'equip d'Urban [Urban et al. 2008] va fer després de la preparació d'un monòlit en columnes capil·lars mitjançant cromatografia d'exclusió de grandària inversa i porosimetria de mercuri. Altres tècniques com la NMR han estat emprades també com s'ha mencionat anteriorment, per a la caracterització de MIPs per Samah [Samah et al. 2018] que la va usar junt a la SEM per a caracteritzar un MIP emprat posteriorment per a l'anàlisi de diclofenac en mostres d'aigua.

5.6.3. APLICACIÓ A L'ANÀLISI TOXICOLÒGICA

Els MIPs s'han usat en nombroses ocasions per a l'anàlisi toxicològica en diversos formats per al tractament de mostres. Tot i que el més emprat ha estat tradicionalment la SPE, als darrers anys han estat aplicats a altres formats que en ocasions presenten interessants avantatges front a la SPE. A la Taula 5, es mostren alguns dels més destacables treballs que han fet ús als darrers anys per a l'anàlisi de drogues emprant MIPs en diferents matrius.

Un grup important de drogues amb nombrosos derivats i metabòlits són els cannabinoides, incloent-hi els cannabinoides sintètics dels quals s'han sintetitzat diversos MIPs. Com exemples, es poden citar el treball de Nestic i col·laboradors [Nestic et al. 2013]per a la determinació de THC i els seus metabòlits en mostres d'orina, els quals obtingueren LODs inferiors a 2,5 ng mL^{-1} mitjançant determinació per GC-MS després d'una SPE emprant un MIP selectiu per a aquests anàlits. Tanmateix, el THC, THC-COOH i el 11-Hidroxi-delta-9-tetrahidrocannabinol (THC-OH) i altres cannabinoides com el Cannabidiol (CBD) i

Cannabinol (CBN) han estat determinats en diversos treballs, per LC-MS/MS en mostres d'orina, saliva i plasma fent ús d'una extracció SPE prèvia amb MIPs basats en EGDMA i DVB [Sánchez-González et al. 2017] o en àcid acrílic (AA) i EGDMA [Cela-Pérez et al. 2016]. D'altres treballs, han usat MIPs basats en metacrilolamidofenilanalina *(Methacryloylamidophenylalanine, MAPA)* com a monòmer i EGDMA o 2-hidroxietilmetacrilat *(2-Hydroxyethylmethacrylast, HEMA)* com a *cross-linkers* per a la determinació de cannabinoides sintètics com JWH-018 i JWH-073 mitjançant LC-MS/MS [Aknögüllü et al. 2020]

Altra droga popularment consumida és la cocaïna, no és estrany doncs, que el seu anàlisi desperte un gran interès i s'hagen publicat nombrosos treballs descrivint diverses metodologies per a la seua anàlisi. Recentment s'han emprat MIPs de cocaïna per a la seua anàlisi en mostres de plasma, directament amb el MIP, preparat emprant MAA com a monòmer, com a fase estacionària en nano-LC-UV [Bouvarel et al. 2020] o en altres formats com sobre paper per a la introducció directa a l'analitzador de masses per a ionització per esprai [Tavares et al. 2017].

Les benzodiazepines desperten gran interès també a causa del seu abús sota la manca de control mèdic que a sovint es produeix. Així, diazepam i altres benzodiazepines han estat analitzades en cabells o plasma fent ús de MIPs que empren MAA com a monòmer i EGDMA com agent entrecreuant, analitzant els extractes mitjançant LC-MS/MS [Ariffin et al. 2007] o directament per MS [Costa-Figuereido et al. 2011]. Tanmateix, s'han desenvolupat sensors basats en MIPs i nanopartícules d'or per a l'anàlisi de lorazepam en fàrmacs, plasma i orina [Razaer et al. 2014].

Altres formats d'extracció que han fet ús de MIPs per a la determinació de diverses drogues han estat, per exemple, l'extracció en "agulla farcida" de MIP fent ús de metamfetamina com a *template* i MAA com a monòmer per a la determinació de metamfetamina, amfetamina i MDMA en orina mitjançant GC-FID [Djozan et al. 2012]. Altre exemple és la deposició d'un MIP de MAA/EGDMA sobre elèctrodes per a la determinació de metilona [Couto et al. 2020] o la determinació de morfina en plasma i orina mitjançant SPE magnètica [Rahmani et al. 2018]. També s'han emprat MIPs basats en MAA i EGDMA per a la determinació de LSD després d'una SPE mitjançant LC-MS, obtenint-se un LOD després de l'extracció de 0.06 ng L^{-1} [Chapuis-Hugon et al. 2009].

Taula 5. *Drogues analitzades en les seues corresponents matrius emprant polímers de reconeixement molecular.*

Analit	Matriu	Format	Monòmer/*Cross-linker*	LOD	Tècnica analítica	Referència
THC i metabòlits	Orina	SPE	MAA/EGDMA	< 2,5 μg L^{-1}	GC-MS	[Nestic et al. 2013]
THC, THC-COOH, THC-OH	Plasma i orina	SPE	EGDMA / DVB	< 0,17 ng L^{-1}	LC-MS/MS	[Sánchez-González et al. 2017]
THC, THC-COOH, CBD, CBN	Orina i saliva	SPE	AA / EGDMA	< 0,75 μg L^{+1}	LC-MS/MS	[Cela-Pérez et al. 2016]
JWH-018, JWH-073 i metabòlits	Saliva artificial	MIP-Cristall de quars	MAPA/EGDMA-HEMA	< 0,29 ng L^{-1}	LC-MS/MS	[Akgönüllü et al. 2020]
Cocaïna	Plasma	Columna LC	MAA / TRIM-EGDMA	< 25,5 μg L^{-1}	Nano-LC-UV	[Bouvarel et al. 2020]
Cocaïna	Saliva	Sprai en paper	MAA / EGDMA	< 0,27 μg L^{-1}	MS	[Tavares et al. 2017]
Diazepam i metabòlits	Cabells	SPE	MAA / EGDMA	0,026 μg g^{-1}	LC-MS/MS	[Ariffin et al. 2007]
Diazepam, medazepam, nitrazepam, clordiazepòxid, clonazepam, midazolam	Plasma	SPE	MAA / EGDMA	< 3 μg L^{-1}	MS	[Costa-Figuereido et al. 2011]

Taula 5. *(Continuació)*

Analit	Matriu	Format	Monòmer/*Cross-linker*	LOD	Tècnica analítica	Referència
Lorazepam	Fàrmacs, orina i plasma	Sensor de nanopartícules d'or	Polipirrol	0,09 nmol L^{-1}	*	[Rezanei et al. 2012]
Metamfetamina, amfetamina i extàsi	Orina	En agulla	MAA / EGDMA	12 µg L^{-1}	GC-FID	[Djozan et al. 2012]
Metilona	Plasma i orina	Elèctrodes	MBI	1,1 µmol L^{-1}	*	[Couto et al. 2020]
Morfina	Plasma i orina	SPE magnètica	MAA / EGDMA	0,03 mg L^{-1}	LC-UV	[Rahmani et al. 2018]
LSD	Orina	SPE	MAA / EGDMA	0,06 ng L^{-1}	LC-UV	[Chapuis-Hugon et al. 2009]

**No usa técnica analítica*

AA: Àcid acrílic, CBD: Cannabidiol, CBN: Cannabinol, DVB: Divinilbenzé, EGDMA: Etilenglicoldimetacrilat, GC-FID: Cromatografia de fasos amb detector de ionització en flama, HEMA: 2-Hidroxietilmetacrilat, LC-MS/MS: Cromatografia liquida acoblada a espectrometria de masses en tàndem, LC-UV: Cromatografia líquida amb detector ultraviolat, LOD: límit de detecció, LSD: Àcid d-Lisèrgic, MAA: Àcid metacrílic, MAPA: Metacriloilamidofenilalanina, MBI: 2- Mercaptobenzimidazol, MS: Espectrometria de mases, SPE: Extracció en fase sòlida, THC: Tetrahidrocannabinol, THC-COOH: 11-Nor-9-carboxi-delta-9-tetrahidrocannabinol, THC-OH: 11-Hidroxi-delta-9-tetrahidrocannabinol, TRIM: Trimetilolpropà trimetacrilat

6. ESPECTROMETRIA DE MOBILITAT IÒNICA

6.1. HISTÒRIA I DESENVOLUPAMENT DE LA TÈCNICA

Als últims anys del segle XIX, Rutherford va aconseguir mesurar la mobilitat de ions produïts amb rajos X i caracteritzar-los a partir d'aquesta mateixa mobilitat [Rutherford 1897; Rutherford 1899]. Anys després, Lattey va demostrar la relació entre la mobilitat i els gasos neutres que s'introdueixen [Lattey 1910]. Les primeres dècades del segle XX van esdevenir una època de ràpid creixement dels coneixements sobre la cinètica dels ions i de totes aquelles variables que podien afectar-la. No obstant, a la dècada dels 30, l'interès de la mobilitat iònica va caure ràpidament a causa de l'aparició de l'espectrometria de masses que eliminava els problemes de la reactivitat com a conseqüència de no usar altes pressions [Eiceman et al. 2005].

Al 1948 Lovelock va desenvolupar el primer detector que tenia la capacitat de detectar compostos orgànics en vapors industrials a partir d'un camp elèctric lineal dins d'un tub de deriva [Lovelock et al. 1949]. Aquest fet junt als estudis que es van portar a la segona meitat del segle XX basats en estudis teòrics de la mobilitat iònica [Mason et al. 1958] i l'interès que es va despertar entre les forces armades per la mobilitat iònica i el seu potencial per a detectar agents de guerra química durant la Segona Guerra Mundial va donar lloc al naixement i les bases teòriques del que avui es coneix com espectrometria de mobilitat iònica *(Ion Mobility Spectrometry, IMS)*.

A la dècada dels 70, Cohen i Karasek van introduir el primer instrument d'IMS comercial acoblat a un espectròmetre de masses de quadrupol [Cohen et al. 1970]. Després l'IMS va ser acoblada a diversos analitzadors de masses per a usar-los com a detectors posteriorment a la separació per mobilitat o a GC per a variar la forma d'introducció de mostra i realitzar la separació cromatogràfica abans de la introducció a l'instrument d'IMS [Geuvremont et al. 1997; Gillig et al 1997. Henderson et al. 2000].

Des d'aleshores l'IMS ha esdevingut una tècnica valorada especialment en el camp de l'anàlisi de drogues i explosius a causa de la seua senzillesa i rapidesa en les anàlisis. De fet, és una tècnica mot comuna a llocs on es requereixen respostes ràpides com aeroports o altres punts de control [Johnson et al. 2007].

6.2. FONAMENT TEÒRIC

L'IMS es pot descriure com una tècnica analítica que es basa en la separació en fase gas dels ions de l'anàlit a pressió atmosfèrica sota la influència d'un camp elèctric. En IMS, a més de la influència del camp elèctric en la separació, és indispensable l'actuació d'un gas inert (també conegut com a *drift gas* en anglès) la fricció del qual amb l'anàlit determinarà la seua velocitat de deriva (v_d). D'aquesta manera, tots els ions quan es separen mitjançant IMS tenen una constant característica per a cadascun d'ells en determinades condicions de pressió i temperatura que s'anomena constant de mobilitat iònica, representada pel símbol K. Aquesta constant pot ser definida com una mesura de la fricció entre el ió i el gas inert, a partir del temps de deriva que es mostra en la separació per a cada ió en concret. A l'equació 1 es pot veure la definició matemàtica més comuna per a la K [Gabelica et al. 2018]

$$K = \frac{v_d}{E} = \frac{l}{t_d\, E} \qquad (1)$$

on l, representa la longitud del tub de deriva (cm), t_d el temps de deriva del ió (s) i E representa el camp elèctric aplicat (V cm^{-1}). Per tant, les unitats habituals per expressar la constant K són $cm^2\ V^{-1}\ s^{-1}$. Però aquesta constant, es troba influenciada per algunes variables com la densitat del gas de deriva, la temperatura així com la pressió atmosfèrica que és habitual que canvie lleugerament respecte de les condicions atmosfèriques normals. Per tant, a la pràctica és més comú trobar la constant de mobilitat reduïda, K_0, en la qual es normalitzen les condicions per tal de que el seu valor no depenga de les condicions estàndard (vegeu equació 2) [Gabelica et al. 2018].

$$K_0 = K \cdot \frac{N}{N_0} = K \cdot \frac{P}{P_0} \cdot \frac{T_0}{T} \qquad (2)$$

on N, P i T representen el número de densitat del gas de deriva, la pressió i la temperatura i el subíndex 0 indica les mateixes magnituds però en condicions normals; $2{,}687 \cdot 10^{25}\ m^{-3}$ per a N_0, 760 torr per a P_0 i 273,15 K per a T_0.

Quan la velocitat de deriva és molt més baixa que la velocitat pròpia del moviment de les partícules a una determinada temperatura, açò és, la velocitat tèrmica del ió, la constant es pot expressar tal i com es mostra a l'equació 3 [Revercomb et al. 1975].

$$K = \frac{3}{16} \sqrt{\frac{2\pi}{\mu\, k_B\, T}} \cdot \frac{z_e}{N\, \Omega} \qquad (3)$$

On μ representa la massa reduïda del parell gas-ió, z_e la càrrega del ió i Ω la secció de col·lisió entre el gas de deriva i el ió i la k_B representa la constant de Boltzmann.

L'IMS segons el tipus d'anàlit que es pretén determinar, treballa habitualment en dos modes diferents, el mode positiu especialment útil per a anàlits fàcilment protonables com amines, essent el mode emprat per a la determinació de drogues. Per altra banda, el mode negatiu és usat per a la determinació d'altres compostos com explosius o agents de guerra química ja que aquests tenen grups amb tendència a formar ions negatius com àcids carboxílics, nitrils o sulfamides [Kaur-Atwal et al. 2009].

No obstant, l'IMS planteja algunes limitacions inherents a la tècnica. Entre les principals limitacions que planteja l'IMS es troben la manca de resposta lineal, la reduïda selectivitat que pot presentar en la majoria d'ocasions, així com els problemes que poden vindre associats a la interacció del reactant/calibrant intern amb els components de la mostra. El calibrant intern és aquella substància que utilitza l'equip d'IMS com a referència per a establir la resta de K_0 de totes aquelles substàncies analitzades sense ser influenciades per la temperatura o pressió atmosfèrica. El reactant que pot ser o no la mateixa substància que el calibrant intern, és aquella espècie química de la qual fa ús l'instrument per generar els ions bé sigui en mode negatiu o positiu. Aquestes limitacions es poden traduir en diversos fets com [Hill et al. 1997; Márquez-Sillero et al. 2011]:

- Alguns compostos poden ser no detectats a causa de la seua baixa capacitat de formar ions en comparació a la que pugui tenir el calibrant intern o altres components de la mostra.

- L'aparició de falsos positius per la reduïda selectivitat de la tècnica. Ions de diferent massa, forma o grandària poden casualment presentar mobilitats molt semblants a aquella que presenta l'anàlit d'interès i en conseqüència donar un senyal que falsament és correspon amb el ió sota estudi.

- L'existència de ionització competitiva. Aquesta ionització ve determinada per l'afinitat protònica en el cas del mode positiu o l'electronegativitat si es tracta del mode negatiu. La presència d'altres molècules amb una afinitat protònica/electronegativitat molt superior a aquella que presenta l'anàlit, pot suposar una completa ionització de l'interferent impedint la formació del ió corresponent a l'analit, motiu pel qual aquest no mostrarà senyal o la mostrarà

de manera minimitzada. Altre factor que pot afectar a la ionització competitiva és la concentració de la mostra, una concentració massa elevada d'interferents tot i que aquests no presenten afinitat protònica/electronegativitat superior a l'anàlit però, pot afectar d'igual manera a la formació del ió esperat.

- La saturació de l'instrument, és altre problema molt comú associat a l'ús de l'IMS. La limitada capacitat de l'IMS per formar ions a causa de que no hi ha protons o electrons suficients per ionitzar totes les molècules. Comporta en ocasions que l'excés de mostra introduïda junt al fenomen de ionització competitiva, pot suposar que part dels anàlits no siguin capaces de ionitzar-se completament, i aquestes molècules queden dins l'equip contaminant-lo i provocant efecte memòria durant mesures posteriors, resultant complicat eliminar de manera ràpida el senyal de fons.

Totes aquestes limitacions de l'IMS fan necessari recórrer habitualment a alguns tractaments de mostra i/o dades per minimitzar-les. De manera molt comuna, abans de la determinació mitjançant IMS es realitzen tractaments de mostra tals com SPE, LLE o derivatització [Holopainen et al. 2012]. Però també altres estratègies han sigut emprades per solucionar les limitacions com per exemple l'acoblament de tècniques cromatogràfiques a l'IMS que permet separar components abans d'entrar a l'instrument o l'acoblament de l'IMS a analitzadors de masses [Wilkins et al. 2011]. La separació en IMS és causada per la col·lisió dels ions amb un gas neutre, per aquest motiu l'IMS pot ser acoblada amb gran senzillesa a fonts de ionització química a pressió atmosfèrica, cosa que aconsegueix LODs molt baixos, i a més, permet acoblar fàcilment amb GC, LC i MS afegint així una dimensió addicional a la separació, essent possible aplegar a separacions de fins a quatre dimensions en equips de doble cromatografia acoblats a IMS i MS en sèrie [Stephan et al. 2016]. Altres formes que s'han usat per augmentar la selectivitat ha sigut la introducció de gasos dopants com per exemple acetona o amoníac en mode positiu o clorurs en mode negatiu o gasos basats en cetones i compostos organofosforats per a aplicacions molt més concretes. Aquests gasos permeten una ionització més selectiva d'un anàlit determinat [Puton et al. 2008]; o recórrer a tractaments quimiomètrics per realitzar un estudi estadístic de les variables, permetent obtenir una major fiabilitat en els resultats [Bota et al. 2006, Szymanska et al. 2016].

6.3. TIPUS D'ESPECTROMETRIA DE MOBILITAT IÒNICA

L'IMS és una tècnica, com ja s'ha mencionat, basada en la mobilitat que ofereixen els ions en fase gas front a un camp elèctric extern. No obstant, hi ha diversos equips i modalitats de la tècnica que varien en la disposició dels seus components i en la forma de separar aquest ions.

- Temps de deriva. Una de les formes més comunes de l'IMS és la del temps de deriva *(Drift Time Ion Mobility Spectrometry, DTIMS)*. En aquesta modalitat els ions es mouen a través d'un camp uniforme en presència d'un gas inert que flueix al llarg d'una sèrie d'anells col·lectors que formen elèctrodes que creen el camp elèctric, tal i com es mostra a la Figura 19. Un gas portador arrossega la mostra vaporitzada i aquesta s'introdueix en la regió de ionització mentre el gas de deriva (nitrogen, heli o argó) s'introdueix des de l'altre extrem i viatja a contra direcció per tal d'eliminar tots els compostos no ionitzats fora de la regió de deriva. Una vegada formats els ions, aquests entren en forma de cúmuls a través d'una escletxa que queda oberta entre 50 i 200 µs. Els ions es mouen a una velocitat proporcional a K mentre els possibles guanys energètics són dissipats pel gas portador [Dodds et al. 2019].

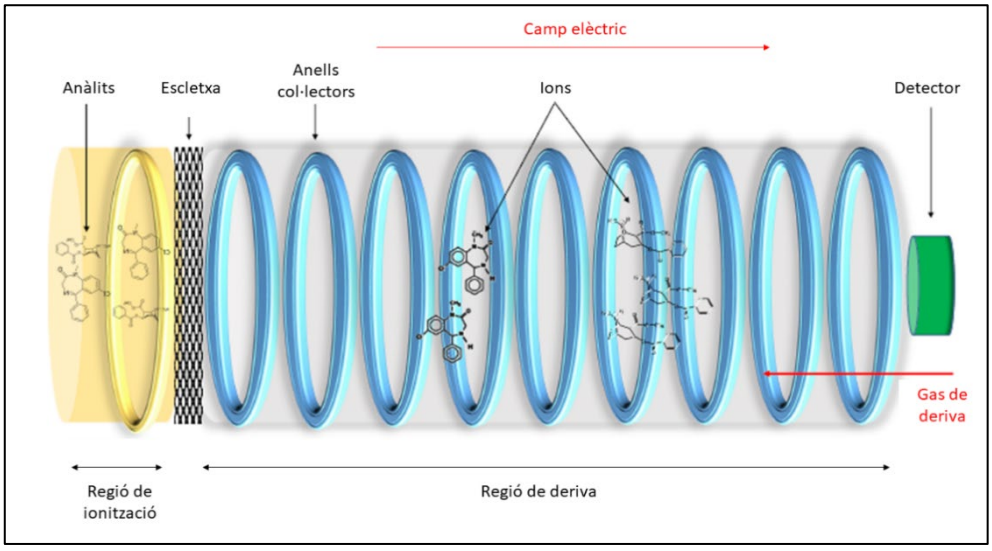


***Figura 19**. Esquema d'un instrument IMS de temps de deriva, DTIMS.*

- D'ona itinerant. A l'IMS d'ona itinerant *(Travelling Wave Ion Mobility Spectrometry, TWIMS)* els ions s'acumulen i s'alliberen de manera periòdica dins d'una cèl·lula de mobilitat on són separats segons la seua mobilitat a través d'una filera de transistors que produeixen impulsos de voltatge. La freqüència de les col·lisions entre els ions i el gas de deriva juga un paper fonamental permetent que alguns ions tinguin temps de deriva curts mentre que altres poden romandre períodes de temps superiors. Tot i que al igual que la DTIMS, el temps de deriva depèn de nombrosos factors com la càrrega del ió, la massa reduïda del ió i del gas de deriva així com la seua pròpia natura, pressió i temperatura [Knapman et al. 2010].

- Camp asimètric. A l'IMS de camp asimètric (*Field Asymmetric Ion Mobility Spectrometry, FAIMS)* els ions són separats mentre són transportats per un gas prop d'elèctrodes espaiats entre ells. A FAIMS, els ions s'exposen de manera alternada a camps elèctrics forts i febles que actuen en direcció perpendicular al flux del gas. Els voltatges són aplicats usant una ona de voltatge de freqüència asimètrica caracteritzada per una notable diferència en el voltatge de les polaritats negativa i positiva. La separació dels ions és el resultats de la diferència de mobilitat en els camps elèctrics fort i feble. Sota camps elèctrics forts la col·lisió ió-gas és molt més energètica que l'energia tèrmica quan el ió roman estàtic. Així la mobilitat iònica es veu afectada per aquests canvis d'energia en alguns casos tenint mobilitats molt diferents segons els gas de deriva emprat [Geuvremont 2004].

- De trampa. L'IMS de trampa *(Trapped Ion Mobility Spectrometry, TIMS)* comença en l'acumulació de ions per un període determinat de temps. Durant aquesta acumulació, una placa fixada en potencial de repulsió dirigeix els ions a l'embut d'entrada, els ions que entren a l'analitzador travessen el gradient de camp elèctric aplicat aplegant al límit on la força neta que actua sobre els ions és zero ja que és el resultat de la força exercida pel camp elèctric i el gas de deriva de manera similar a la DTIMS. Posteriorment els ions són eluïts mitjançant l'aplicació d'una rampa o decreixement del camp elèctric [Castellanos et al. 2014; Michelmann et al. 2015].

- De "llaç obert". A l'IMS de "llaç obert" (*Open Loop Ion Mobility Spectrometry OLIMS)* o d'aspiració *(Aspiration Ion Mobility Spectrometry, AIMS)* les molècules ionitzades i els grups de ions flueixen sota un camp elèctric a través

de la cèl·lula de mobilitat iònica. Aquesta cèl·lula conté huit parells d'elèctrodes que creen un camp elèctric que causa que els grups de ions impacten amb els elèctrodes segons la seua mobilitat. Així els ions amb elevada mobilitat impacten amb la primera placa del detector i els ions amb menor mobilitat són desplaçats pel corrent de gas més lentament a través del camp elèctric i aquests ions viatjaran major distància a les plaques del detector fins colpejar. Tanmateix, la polaritat del camp elèctric es converteix de manera periòdica de negatiu a positiu durant curts períodes permetent determinar tant ions positius com negatius en els 8 parells d'elèctrodes [Räsänen et al. 2010].

- Analitzadors de mobilitat diferencials. Els analitzadors de mobilitat diferencials *(Differential Mobility Analyzer, DMA)* són usats per a seleccionar i classificar partícules en base a la seua mobilitat i càrrega dins d'un flux d'aerosol. Les partícules poden ser comptabilitzades o detectades com a mesura de la distribució de la seua grandària. Els DMA actuen com una porta que permet únicament el pas en una banda determinada de mobilitat iònica i deixa passar la resta de l'aerosol. La seua funció es caracteritza per la dimensió de la seua porta de mobilitat o funció de transferència. La probabilitat de transmetre amb èxit una partícula d'una mobilitat iònica ve donada pels paràmetres operacionals del DMA [Stolzenburg 2018].

Entre totes les modalitats d'IMS que s'han exposat anteriorment es poden trobar diverses característiques comunes i altres en les que es diferencien notablement. Cada tècnica presenta uns avantatges i unes aplicacions més concretes ja que han anat sorgint en funció a les necessitats a partir de la DTIMS que és considerada la tècnica tradicional i la primera en desenvolupar-se. A mode de resum es poden establir línies generals com per exemple que totes les tècniques d'IMS treballen a temperatures ambientals mentre que no totes elles ho fan a pressió ambiental. Per exemple, mentre que la DTIMS, FAIMS i OLIMS treballen a pressió atmosfèrica al voltant de 1 bar, la TWIMS treballa a pressions més baixes que oscil·len entre 0,025 i 3 mbar, la TIMS a pressions d'entre 2,6 i 3,4 mbar i la DMA a pressions que van des de 80 mbar fins a 1 bar. De la mateixa manera, algunes de les tècniques com la DTIMS, l'OLIMS o la DMA treballen a camp elèctric uniforme essent d'intensitat baixa per a DTIMS i OLIMS ,i d'intensitat elevada per a la DMA, en el sentit oposat la TWIMS treballa amb camps elèctrics baixos no uniformes així com la FAIMS ho fa alternant camps d'intensitat baixa i elevada. Totes les tècniques han estat exitosament acoblades a

MS, mentre que només la DTIMS i FAIMS han estat acoblades a altres tècniques com per exemple tècniques cromatogràfiques [Cumeras et al. 2015].

6.4. APLICACIÓ A L'ANÀLISI DE DROGUES

Com s'ha comentat breument a apartats anteriors, l'IMS és una tècnica que ofereix bones possibilitats per a l'anàlisi de drogues en diverses matrius, essent necessari habitualment el tractament de mostra adequat si els anàlits es troben en matrius complexes. Tot i que aquesta Tesi Doctoral va encaminada a l'anàlisi de drogues en matrius biològiques, l'IMS s'ha emprat més bé per a l'anàlisi de substàncies psicotròpiques en matrius més simples com aire, material confiscat o altres superfícies. Per aquest motiu, es comentaran breument algunes d'aquestes aplicacions de l'IMS.

Per exemple, diversos treballs apunten a l'aplicació de l'IMS per a l'anàlisi de diverses drogues en material confiscat. Cal destacar el treball de Verkouten i col·laboradors [Verkouten et al. 2011] que van determinar drogues com metamfetamine, MDMA, cocaïna, heroïna, fentanil, hidrocodona, oxicodona o alprazolam emprant la DTIMS o el treball de Wu i col·laboradors [Wu et al. 2000] on es van determinar amfetamina, metamfetamina, fenciclidine *(Phencyclidine, PCP)*, morfina, cocaïna THC, LSD i heroïna emprant un acoblament ESI-IMS-MS. També substàncies d'especial perillositat com el fentanil i anàlegs d'aquest han estat determinats en material confiscat mitjançant DTIMS aconseguint LODs tan baixos com 1 ng [Sisco et al. 2017]. Al 2018, l'equip de Yanini [Yanini et al. 2018] va aconseguir fer una determinació d'una gran varietat de NPS en material confiscat emprant la DTIMS amb LODs que es trobaven entre 50 i 160 pg.

L'IMS també ha estat emprada per a la determinació de diversos cannabinoides com el THC, CBD, àcid tetrahidrocannbinòlic o àcid cannabidiòlic en mostres de marihuana emprant un acoblament ESI-IMS-MS [Hädener et al. 2018] o cannabinoides naturals en mostres de plantes emptrant DTIMS [Contreras et al. 2018], així com de diversos cannabinoides sintètics en mostres d'herbes que havien estat addicionades amb aquestes substàncies mitjançant DTIMS amb LODs que no superaven els 3,6 ng [Metternich et al. 2019].

Tanmateix l'IMS ha servit per a la determinació de drogues suspeses en aire com mostra el treball de Mohsen i el seu equip que va determinat THC, metamfetamina i amfetamina emprant FAIMS amb LODs inferiors a 65 µg L^{-1} [Mohsen et al. 2014]. Altres treballs que han determinat drogues com cocaïna en aire han permés obtindré límits de detecció de 0,15 ng emprant una DTIMS [Armenta et al. 2014]. Altres treballs han permés determinar drogues com heroïna

junt a cocaïna amb LODs inferiors a 164 ng [Dussy et al. 2008] o piperonal amb LODs de 600 ng [Guerra-Diaz et al. 2010] tots dos emprant DTIMS.

A banda de les aplicacions ja mencionades, l'IMS també ha estat aplicada a l'anàlisi de drogues en matrius biològiques tot i que la complexitat que aquestes presenten. En aquest sentit a la Taula 6 es fa un recull d'alguns dels treballs més destacables de l'anàlisi de drogues en mostres biològiques. Per exemple, al 2013 es va publicar un treball on es feia ús de la DTIMS com a tècnica d'elevada sensibilitat per a la determinació de cocaïna en mostres de secrecions nassals, portant a terme la confirmació de la seua presència per una tècnica de major selectivitat com la FTIR [Armenta et al. 2013]. Altres treballs, a més de l'anàlisi de cocaïna també han portat a terme l'anàlisi d'ecgonina metil èster que és un dels seus metabòlits principals en mostres de saliva emprant la DTIMS després d'una LLE aconseguint recuperacions superiors al 85 % i LODs que no superaven els 0,3 μg L^{-1} [Cocovi-Solberg et al. 2017]. També Lu i cil·laboradors [Ly et al. 2009] van publicar l'any 2009 un treball que feia úsde la DTIMS per a l'anàlisi de cocaïna i els seus metabòlits principals emprant com a matriu orina. En aquest cas, els autors van aplicar una SPE com a mètode de preparació de mostra, obtenint-se finalment LODs inferiors a 10 μg L^{-1}.

L'IMS també ha estat usada per a l'anàlisi de diverses benzodiazepines que incloïen prazepam, lorazepam, tetrazepam, clordiazepòxid, temazepam, diazepam, nitrazepam, oxazepam i medazepam en mostres de saliva. En aquest mètode, desenvolupat pel nostre grup de recerca [Armenta et al. 2011] es van obtenir recuperacions de fins a 104 % amb LODs que anaven de 2 a 59 μg L^{-1} segons l'anàlit.

Al 2008 Alizadeh i el seu equip [Alizadeh et al. 2008] van publicar un treball on es feia ús de la SPME emprant com a fase adsorbent polipirrol dopat amb dodecilsulfat per a l'extracció de metamfetamina i MDMA per als quals es van obtenir recuperacions de 92,4 i 97 %, respectivament emprant com a tècnica analítica la DTIMS. També s'ha determinat metamfetamina en sang i saliva emprant paper per a formar el sprai per a la seua introducció a l'IMS, en aquest treball per a la metamfetamina es va obtindré LODs inferiors a 1,5 μg L^{-1} per a les dues matrius considerades [Zargat et al. 2018]. Entre aquestes substàncies, el nostre grup d'investigació [Armenta et al. 2015] va publicar l'any 2015 un article on es va emprar la DTIMS per a la determinació de MDMA en mostres de saliva aconseguint LODs de 11 μg L^{-1}. Finalment, també cal mencionar un treball on es va fer ús de l'acoblament ESI-IMS-MS per a la determinació d'amfetamina,

metamfetamina i altres derivats en mostres d'orina obtenint-ne LODs inferiors a 7,5 μg L^{-1} [McCooeye et al. 2002].

Les NPS també han sigut determinades en mostres de fluids biològics emprant l'IMS tot i que hi ha menys treballs a la bibliografia al respecte. Per exemple, Peiró i col·laboradors [Peiró et al. 2016] van emprar la DTIMS per a la determinació de metilendioxipirovalerona *(methylenedioxypyrovalerone, MDPV)* que pertany a la família de les catinones sintètiques i va ser analitzada en mostres de saliva i secrecions nasals obtenint-se LODs inferiors a 22 μg L^{-1}. Altra NPS que ha estat analitzada mitjançant DTIMS és la 2C-C que és una droga de la família de les fenetilamines; en aquest cas és va portar a terme la determinació d'aquesta substància en mostres de saliva mitjançant una doble confirmació: sense i amb derivatització emprant acetona, en tots dos casos les recuperacions obtingudes van ser superiors al 83 % amb un LOD de 14 μg L^{-1} per a la determinació directa i 10 μg L^{-1} amb derivatització [Torres et al. 2017].

Taula 6. *Drogues analitzades mitjançant IMS en diversos fluids biològics.*

Anàlit	Matriu	Tipus d'IMS	LOD	Referència
Cocaïna	Secrecions nasal	DTIMS	*	[Armenta et al. 2013]
Cocaïna, ecgonina metil èster	Saliva	DTIMS	< 0,3 μg L^{-1}	[Cocovi-Solberg et al. 2017
Cocaïna i metabòlits	Orina	DTIMS	< 10 μg L^{-1}	[Lu et al. 2009]
Prazepam, lorazepam, tetrazepam, clordiazepòxid, temazepam, diazepam, nitrazepam, oxazepam, medazepam, clorazepat	Saliva	DTIMS	< 59 μg L^{-1}	[Armenta et al. 2011]
Metamfetamina, MDMA	Sèrum	DTIMS	< 8 μg L^{-1}	[Alizadeh et al. 2008]

Taula 6. *(Continuació)*

Anàlit	Matriu	Tipus d'IMS	LOD	Referència
Metamfetamina	Sang i saliva	PaperSpray-DTIMS	< 1,5 μg L^{-1}	[Zargar et al. 2018]
MDMA	Saliva	DTIMS	11 μg L^{-1}	[Armenta et al. 2015
Amfetamina, metamfetamina i derivats	Orina	ESI-FAIMS-MS	< 7,5 μg L^{-1}	[McCooeye et al. 2002]
MDPV	Saliva i secrecions nasals	DTIMS	22 μg L^{-1}	[Peiró et al. 2016]
2 C-C	Saliva	DTIMS	10 μg L^{-1}	[Torres et al. 2017]

** No especificat, DTIMS: Espectrometria de mobilitat iònica de temps de deriva, ESI: Ionització per electrosprai, FAIMS: Espectrometria de mobilitat iònica de camp asimètric, IMS: Espectrometria de mobilitat iònica, LOD: Límit de detecció, MDMA: Metildimetilmetamfetamina, MDPV: 3,4-Metilendioxipirovaleronea, MS: Espectrometria de masses.*

OBJECTIUS

AIMS

L'objectiu principal d'aquesta Tesi Doctoral és el desenvolupament de noves metodologies analítiques per a l'anàlisi de drogues il·lícites en mostres de saliva i altres matrius com mostres d'aigües residuals i ambientals. Aquestes metodologies estaran basades en l'obtenció de nous materials i formats capaços de retenir de manera selectiva un anàlit o famílies d'anàlits a les matrius esmentades, per a la seua posterior determinació per tècniques analítiques ràpides i sensibles com l'IMS o tècniques més selectives com GC-MS o UHPLC-MS/MS.

Per assolir aquest objectiu principal, aquesta Tesi Doctoral es divideix en tres objectius concrets. El primer és fer una revisió actualitzada de l'ús de diversos materials per al tractament de mostra i la posterior anàlisi per IMS. Aquests materials van des dels materials tradicionals de selectivitat limitada fins als materials selectius que avui en dia han estès el seu ús. Tanmateix, es realitza una revisió sobre l'ús a l'anàlisi forense en general d'aquests materials intel·ligents, no només per a la posterior determinació per IMS sinó per a qualsevol tècnica analítica incloent-hi el seu ús com a sensors.

El segon objectiu, planteja la preparació de nous materials genèrics però amb propietats millorades, així com formats que permeten l'automatització i/o miniaturització dels procediments per a l'anàlisi de drogues en saliva. En concret, s'estudia l'aplicació de la MEPS per a l'anàlisi de drogues de diferents famílies, però totes elles de recent aparició. D'igual manera, s'hi tracta d'obtenir un polímer amb propietats de material de tipus mixt, però adaptat a puntes de micropipeta amb la intenció de facilitar l'extracció de diverses drogues de mostres de saliva permetent la seua posterior determinació per una tècnica selectiva i molt sensible com l'UHPLC-MS/MS. Seguint l'aplicació dels materials mixtos, es planteja una síntesi que incorpore interaccions apolars així com bescanvi iònic de doble acció per a la determinació de drogues amb diferents propietats àcid-base.

L'últim dels tres objectius, es basa en l'aplicació de materials selectius, principalment MIPs, però també aptàmers en diversos formats d'extracció. Aquest materials, pensats per a la determinació de diverses drogues en saliva i altres matrius s'avaluen amb la finalitat de portar a terme una ràpida i sensible determinació per IMS. D'entre els MIPs que es proposen s'han emprat com a molècules plantilla cocaïna, metamfetamina o arilciclohexilamines, amb el propòsit d'avaluar algunes propietats importants com la selectivitat creuada que poden ajudar a estendre el seu ús a un gran nombre de molècules estructuralment similars. Considerant la importància de la cocaïna, també s'aplica l'extracció emprant MIPs en diferents formats que ajuden a millorar algunes de les limitacions

de la SPE convencional, per obtenir nous mètodes més senzills, ràpids, miniaturitzats i portàtils que permeten una anàlisi en camp del consum de cocaïna. Finalment, amb el motiu d'avaluar altres materials selectius de relativa recent aparició es proposa la immobilització d'aptàmers per a preparar oligosorbents. Els aptàmers, sorgits com a bona alternativa als anticossos, són immobilitzats en fases com safarosa activada amb bromur de cianogen o partícules magnètiques per a la posterior extracció mitjançant SPE o MDSPE de fluoroquinolones o metamfetamina. D'aquesta manera, l'últim estudi d'aquesta Tesi mostra resultats preliminars de l'extracció d'aquest anàlits emprant els oligosorbents, preparats a partir de la immobilització dels aptàmers.

The main aim of this Doctoral Thesis is the development of new analytical methodologies for the analysis of illicit substances in oral fluid and other samples such as natural water and wastewater. These methodologies are based on the achievement of new materials and approaches in order to retain selectively an analyte or a family of substances from the aforementioned matrices. After extraction, materials must allow a determination by simple and fast analytical techniques such as IMS or high-selective analytical techniques such as GC-MS or UHPLC-MS/MS.

For this purpose, this Doctoral Thesis is divided in three particular scopes. The first one is to do an updated bibliographic research of the use of materials for sample treatment prior to IMS determination. These materials include the most traditional ones that have limited selectivity till the most recent materials that provides a high selectivity towards target analytes. Likewise, a review about the use of smart materials for sample treatment in forensic analysis is done. Forensic application of smart materials includes both their use for determination by any analytical technique and their use as sensors.

The second scope raised is the preparation of new non-selective materials with improved properties as well as their implementation on new approaches to allow the automatization and miniaturization of the analytical procedures for drug analysis. Particularly, the application of MEPS to determine different NPS is done. Besides, it is proposed the synthesis of a polymeric material with mixed-mode properties. This polymer is adapted to pipette tips in order to make easier the extraction procedure for several drug determination in oral fluid by UHPLC-MS/MS. Following the studies of mixed-mode materials, it is proposed the preparation of materials that exhibit both non-polar interactions as well as ionic exchange behavior for determination of drugs with different acid-base properties.

The last scope is based on the application of selective materials, mainly MIPs and aptamers in different extraction approaches. These materials are focused on the extraction of several drugs in oral fluid and other matrices in order to perform fast and sensitive analysis by IMS. The developed MIPs use as template molecule for their synthesis several substances including cocaine, methamphetamine and arylcyclohexylamines. After MIP preparation, the studies are focused on the evaluation of some features such as cross-selectivity in order to extend their use for the extraction of numerous related compounds. Considering the relevance of cocaine, several extraction approaches are developed using molecular imprinting to enhance some limitations of the conventional SPE, achieving easier, faster,

miniaturized and portable new methodologies for cocaine analysis. Finally, in order to study more recent materials, it is proposed the immobilization of aptamers to prepare oligosorbents. Aptamers, which emerged as a promising alternative to antibodies, can be attached to solid supports such as CNBr-activated sepharose or magnetic beads for its application by SPE or MDSPE. In this project, fluoroquinolones and methamphetamine were used as target analytes to show the preliminary results of studies performed using these oligosorbents.

RESUM

SUMMARY

Aquesta Tesi Doctoral que porta per títol *"Aplicació de nous materials i formats amb propietats millorades per a l'anàlisi de drogues"* està estructurada en tres blocs principals, on s'aborda des de diferents punts de vista el problema de l'anàlisi de drogues i s'exposen els treballs publicats en els quals es pretén aplicar diferents materials amb propietats millorades per l'extracció de drogues en fluids biològics en diferents formats de SPE.

Com ja s'ha esmentat més profundament a la introducció, l'IMS és una tècnica basada en la separació en fase gas sota un camp elèctric feble a pressió atmosfèrica dels anàlits ionitzats. Inicialment aquesta tècnica va estar usada per agencies de defensa per a l'anàlisi d'explosius, drogues il·lícites i agents de guerra química. Aquests usos típics de l'IMS, són causats principalment pels avantatges que aquesta tècnica proporciona, com són la seua senzillesa operacional, rapidesa i bona sensibilitat. Els seus avantatges han conduït que l'IMS haja estat estesa a altres camps d'anàlisi com el farmacèutic, clínic, indústria petroquímica, ambiental o anàlisi d'aliments.

És conegut, que l'IMS presenta també algunes limitacions que poden entorpir la seua aplicació per a determinats tipus d'anàlisi com aquells que es realitzen a mostres més complexes. Aquestes limitacions són principalment, la manca de resposta lineal i sobretot la seua baixa selectivitat per la possible interacció del reactant amb la resta de components de la mostra. Tot açò, pot conduir que algunes anàlisis per IMS plantegen problemes com que l'anàlit no pugui ser detectat, que es detecten falsos positius a causa de l'errònia identificació d'un interferent com al propi anàlit o l'aparició de ionització competitiva que provoqui una ionització preferent d'altres components abans que l'anàlit.

Aquestes limitacions de l'IMS han estat enfocades des de diversos punts per tal de minimitzar o eliminar els seus efectes. Per exemple, la primera de les opcions per tal d'augmentar la seua selectivitat va ser l'acoblament de l'IMS a una separació prèvia mitjançant GC, però també s'ha emprat l'IMS com a tècnica de separació prèvia a una determinació mitjançant MS, aconseguint en ambdós casos augmentar la selectivitat i reduir l'efecte matriu. Altra estratègia interessant plantejada ha estat la introducció de gasos dopants que permeten eliminar interferències provocant la ionització més selectiva dels anàlits aconseguint a més interpretacions dels plasmagrames més còmodes. Un altre enfocament, és fer ús de les dades aportades després de l'anàlisi per IMS per fer un tractament dels resultats quimiomètric que permeti extraure informació no sols qualitativa sinó també quantitativa.

Tot i l'interès que tenen aquests enfocaments per resoldre les limitacions de l'IMS, els tractaments previs de mostra per eliminar efectes matriu i augmentar la

selectivitat continuen essent l'estratègia més utilitzada per la fàcil aplicació i versatilitat que proporcionen. Tècniques que van des de la LLE fins a la SPE, tenint en compte totes les seues respectives variants, han demostrat que són molt valuoses per al tractament de mostra i la posterior determinació dels anàlits mitjançant IMS.

Tècniques extractives com la SPE convencional, la SPME, SBSE o la MEPS emprant materials de caràcter genèric o no selectiu, com sílice modificada amb C18, PDMS, materials de tipus mixt o materials de carboni, han estat emprades per a l'extracció de nombrosos anàlits incloent-hi drogues, demostrant la seua efectivitat en moltes ocasions. No obstant, altres materials també han sorgit a les darreres dècades per tal d'oferir major selectivitat a aquest tipus de tractament, facilitant l'eliminació amb major eficàcia dels components de mostres complexes.

Els materials intel·ligents o *smart materials* en anglès, podrien ser definits com aquells materials que tenen la capacitat de respondre a estímuls externs d'una manera controlada o específica. En tractament de mostra, es podria entendre com material intel·ligent tot aquell que aporta una característica determinada millorada que els fa més adequats que la resta de materials o substàncies. D'aquesta manera, és interessant fer-hi una revisió bibliogràfica rigorosa de l'aplicació que han tingut aquests materials per a l'anàlisi forense en general i posant especial atenció a l'anàlisi de drogues, així com l'aplicació que hi han tingut per a la posterior determinació emprant com a tècnica analítica l'IMS (vegeu Figura 20).

En primer lloc, existeixen materials intel·ligents com per exemple ho són els IL, CNTs, RAMs o MOFs que presenten una selectivitat moderada front a determinats grups de molècules. Aquesta selectivitat moderada es deu a la possibilitat d'estar funcionalitzats amb determinats grups que ofereixen una resposta orientada cap a grups funcionals concrets, tot i que no responen a molècules de manera específica. En general, encara que alguns materials com els RAMs o els CNTs han estat emprats en alguna ocasió per a l'anàlisi forense i en concret per a l'anàlisi de drogues, actualment la seua aplicació per a la determinació de drogues per IMS és inexistent.

D'altra banda, considerant altres *smart materials* molt més selectius, trobem els immunosorbents. Aquests són materials produïts a partir de la immobilització d'anticossos sobre un suport sòlid. Els immunosorbents han demostrat tenir una gran aplicació per augmentar la selectivitat de mètodes que fan ús de l'IMS per l'elevada especificitat presentada pel complex anticòs-antigen. No obstant, l'aplicació d'immunosorbents en SPE per a l'anàlisi de drogues queda pràcticament limitada a l'anàlisi de fàrmacs com clenbuterol o salbutamol i

concretament emprant l'IMS com a tècnica, el cloramfenicol [Armenta et al. 2016]. També és cert que si en fem referència als immunoassajos, els anticossos han estat àmpliament usats per a la determinació ràpida (*screening*) de drogues en fluids biològics, demostrant les seues bones possibilitats per al reconeixement selectiu de drogues. Així, es poden trobar disponibles comercialment immunoassajos emprats per a la determinació de diverses drogues en matrius com orina, saliva, sang, cabells o suor [Payne-James et al. 2005].

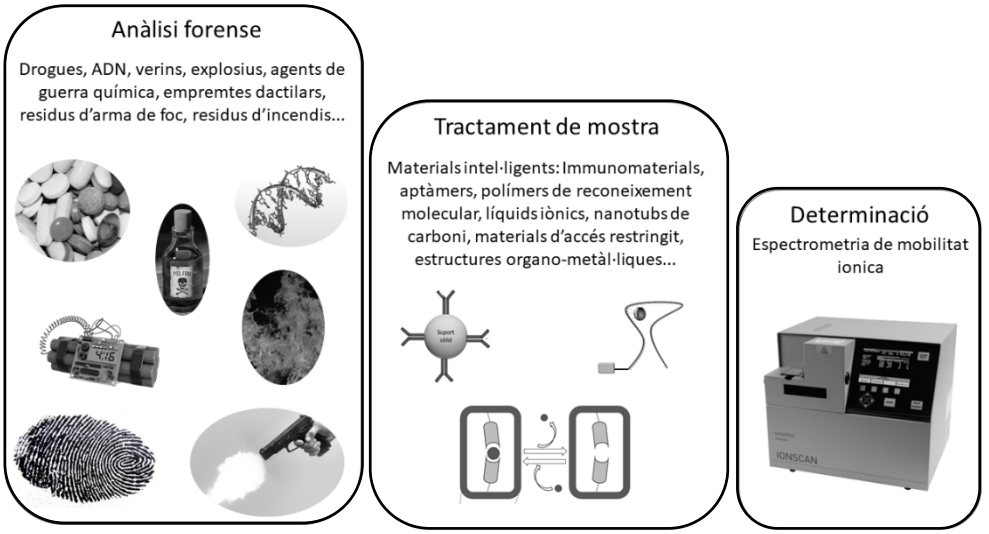


Figura 20. *Esquema dels tipus d'anàlisi forense i materials intel·ligents emprats.*

Amb un principi d'actuació semblant als immunosorbents, altres materials intel·ligents més nous usats han estat els aptàmers, seqüències d'una sola heura d'ADN o ARN que si són immobilitzades sobre un suport sòlid s'obtenen els anomenats oligosorbents. Tot i que la majoria de seqüencies d'oligonucleòtids identificades van dirigides a l'anàlisi de molècules grans com proteïnes, també existeixen un gran nombre d'aptàmers identificats per a l'anàlisi de molècules més menudes. Els oligosorbents són excel·lents candidats per a la separació selectiva d'anàlits, essent aquesta selectivitat comparable a la que ofereixen els anticossos. A més de tenir un cost més reduït, durant la seua preparació permeten introduir modificacions que milloren les seues propietats. Els aptàmers han estat emprats com a sensors per a la determinació de metamfetamina [Yarbakht et al. 2016] o cocaïna i els seus metabòlits en matrius biològiques [Guler et al. 2017]. Però també en format SPE hi ha exemples del seu ús, ja que han estat emprats entre altres per a la determinació per IMS de codeïna o acetamiprid en mostres d'orina [Zargar et al. 2017].

Tot i tenir en compte que tant anticossos com aptàmers presenten característiques molt atractives per a l'anàlisi de drogues, i concretament per al tractament de mostra i posterior anàlisi mitjançant IMS. Cal considerar també, un darrer grup de *smart materials* com ho són els MIPs. En comparació, els MIPs han tingut una major aplicació per a l'anàlisi de drogues, especialment emprats com a fase adsorbent en SPE. Els MIPs, preparats de manera sintètica, presenten cavitats selectives per reconèixer els anàlits per als quals han estat dissenyats, però a més, les seues característiques químiques i físiques, la seua versatilitat, estabilitat i la capacitat de reconeixement, els fan candidats a tenir molt en compte front als altres materials intel·ligents. Tot i que comercialment hi ha MIPs disponibles per a la determinació de clenbuterol [Peñuela-Pinto et al. 2017] o cloramfenicol [Armenta et al. 2016], la seua senzilla preparació ha fet que nombrosos d'ells hagen estat preparats en el propi laboratori per a la determinació de molècules com metamfetamina [Djozan et al. 2012], cocaïna [Thibert et al. 2014] o diazepam [Ariffin et al. 2007] en mostres biològiques. També, altres MIPs han estat preparats per a la determinació de cafeïna [Jafari et al. 2014] o ibuprofè [Alizadeh et al. 2014] mitjançant IMS en mostres com sèrum o plasma.

Sembla evident doncs, que l'IMS és una bona opció quan es vol fer una anàlisi d'un anàlit determinat sempre que sigui possible aïllar-lo de manera selectiva per tal d'evitar que altres components puguin interferir a l'anàlisi. En aquest sentit, materials com els MIPs, aptàmers o immunosorbents semblen tenir capacitats idònies per ser aplicats en el tractament de mostra previ. No obstant, en ocasions pot ser més interessant portar a terme anàlisis dirigides a la determinació d'un gran nombre de drogues en una matriu biològica front a la determinació selectiva d'una d'elles. En aquest darrer cas, seria més interessant recórrer a altres tipus de materials genèrics que no reaccionen de manera específica només front a una molècula o família d'elles, sinó que siguin capaços de reconèixer un gran nombre d'aquestes, considerant que per aquests casos, habitualment, s'hauria de recórrer a tècniques analítiques molt més selectives com la GC-MS o la LC-MS/MS.

Per aquest motiu, després de la recerca bibliogràfica realitzada, surt el primer bloc d'aquesta Tesi Doctoral que engloba els capítols 1 i 2. La resta dels capítols, separats en altres dos blocs, recullen els resultats que s'exposen en forma de publicació original dels dos enfocaments des dels quals s'ha abordat l'anàlisi de drogues. Al segon bloc es desenvolupa l'obtenció de materials i mètodes per a l'anàlisi de drogues emprant materials genèrics o amb selectivitat moderada. Mentre que el tercer bloc es centra en un desenvolupament de materials selectius i la seua posterior adaptació a nous formats d'extracció per a l'anàlisi de drogues d'una manera específica front a la resta de molècules.

Entre els materials genèrics trobem que uns dels que han tingut una major repercussió i ús des del desenvolupament de la SPE són les fases de sílice modificades amb C18 o C8. Aquestes fases han demostrat que serveixen de manera adequada per a retenir anàlits apolars o amb una part important apolar a la seua estructura quan es troben a matrius aquoses. Si bé és cert, que la seua inespecificitat pot conduir a la presència d'un elevat nombre d'interferents, és possible modificar l'etapa de rentat en l'extracció per tal d'aconseguir extractes suficientment purificats. Front a la SPE tradicional, la MEPS pot ser considerada una adaptació miniaturitzada quasi directa de la SPE, col·locant una xicoteta quantitat de fase adsorbent (aproximadament 1 mg) al final d'una agulla que pot ser acoblada a una xeringa manual o de manera automàtica a un braç robòtic. En general, una MEPS consta de les mateixes etapes que una SPE tradicional: (i) condicionament de l'adsorbent, (ii) càrrega de la mostra, (iii) etapa de rentat i (iv) elució de l'anàlit.

En aquest sentit, per a la determinació de grups variats de drogues com les NPS, resulta interessant prendre enfocaments genèrics per a la seua anàlisi, que permeta la determinació d'un gran nombre d'elles. Emprant la MEPS s'han desenvolupat tres mètodes similars però adaptats a l'anàlisi de diverses NPS. El dicloropà, una substància que simula els efectes de la cocaïna; la metilona considerada un anàleg del MDMA i cinc cannabinoides sintètics de tercera generació que inclouen 5F-ADB, MMB-CHMICA, THJ-2201 i CUMYL-4CN-BINACA (vegeu Figura 21). A l'estudi de les millors condicions d'extracció es van avaluar paràmetres com la fase adsorbent a utilitzar, l'etapa de càrrega incloent el seu pH i el nombre de cicles d'aspiració necessaris per aconseguir una retenció adequada, el dissolvent de rentat, així com el volum i nombre de cicles d'elució. Posteriorment per a la determinació de totes les NPS, es va portar a terme la validació del mètode avaluant paràmetres analítics bàsics com la linealitat, LODs, precisió o exactitud. Finalment, els mètodes desenvolupats per a cadascun dels anàlits van ser aplicats a l'anàlisi de mostres de saliva reals fortificades a diferents nivells de concentració amb les diverses NPS esmentades. L'anàlisi tant de dicloropà com de metilona va ser realitzat mitjançant IMS a causa de que és habitual que els consumidors de substàncies com aquestes no tinguin presents altres substàncies similars. No obstant, al cas dels cannabinoides sintètics, es prou comú observar com els productes que es comercialitzen contenen entre dos o tres cannabinoides sintètics diferents [Tai et al. 2014], per aquest motiu i amb l'objectiu de poder identificar correctament quins són els cannabinoides sintètics presents a cada mostra, s'ha optat per una tècnica de major selectivitat com la GC-MS. En aquest sentit, com a resultats del desenvolupament i aplicació de

metodologies de MEPS per a l'anàlisi de diverses NPS en mostres de saliva han sorgit els capítols 3,4 i 5 d'aquesta Tesi Doctoral.

La SPE tradicional també ha mostrat poder ser adaptada a altres formats. D'entre aquests un que presenta beneficis interessants a estudiar és l'extracció en puntes de micropipeta. En aquest cas, una fase adsorbent, bé en forma de partícules i continguda entre dues frites o bé en forma de monòlit es col·loca a l'interior d'una punta de micropipeta per realitzar l'extracció durant la mateixa etapa que es pipeteja. Aquest format no només facilita sinó que accelera notablement el procediment d'extracció, eliminant la necessitat de sistemes de buit per portar a terme l'extracció. Prenent l'enfocament de la síntesi d'un monòlit ja que proporcionen materials amb una major resistència, la síntesi d'un polímer basat en MAA i EGDMA va ser triada a partir de la recerca bibliogràfica on aquests materials han mostrat bones capacitat per a la retenció de molècules de caràcter bàsic, com ho són la majoria de les drogues. Per a la preparació del *poly (MAA-co-EGDMA)* ancorat sobre les parets internes de la punta de polipropilè, en primer lloc és necessària la modificació de les parets emprant agents com la benzofenona, amb una posterior funcionalització amb EGDMA, per tal de permetre la formació d'enllaços covalents entre la paret i el polímer format. Una vegada funcionalitzades les puntes, és necessari aconseguir un monòlit que tingui una adequada resistència mecànica així com una permeabilitat suficient que permeta el flux de dissolvents i mostra amb la pressió exercida per la micropipeta. Per aconseguir aquestes característiques, és necessari l'estudi de diverses proporcions de MAA i EGDMA, així com el volum i natura del dissolvent porogènic emprat per a la preparació del monòlit. Al Capítol 6 s'exposen els resultats obtinguts per a la preparació d'un polímer d'aquestes característiques.

Tanmateix, després de la preparació de les puntes, es mostra l'avaluació de les millors condicions de càrrega, de la capacitat de reutilització així com la precisió i exactitud, emprant una droga model per a l'estudi, l'α-pirrolidinopentiofenona *(α-Pyrrolidinopentiophenone, α-PVP)* (vegeu Figura 21). Posteriorment, s'hi va avaluar l'aplicació del mètode desenvolupat per a l'extracció de diverses drogues en fluids orals que incloïen 20 NPS diferents, així com drogues tradicionals com la cocaïna, la metamfetamina i el diazepam. A causa del gran nombre de drogues seleccionades, per avaluar l'efectivitat de les puntes farcides amb el monòlit, es va emprar com a tècnica analítica l'UHPLC-MS/MS per la seua elevada selectivitat.

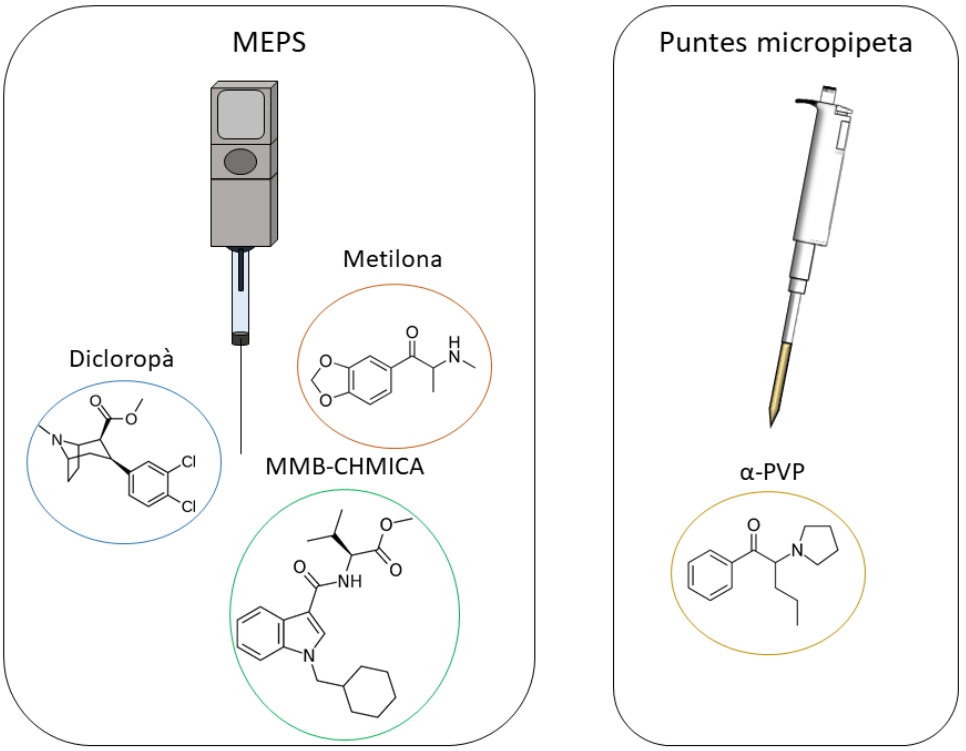


Figura 21. *Anàlits emprats per a l'extracció MEPS i puntes de micropipeta.*

Els materials basats en *poly (MAA-co-EGDMA)*, com queda demostrat a aquest treball tenen propietats de materials de tipus mixt. Els materials de tipus mixt són aquells que proporcionen característiques tant de bescanvi iònic com retencions mitjançant interaccions inespecífiques semblants a les que proporcionen les fases inverses. En aquest sentit, en concret l'EGDMA actua com a xarxa troncal que manté la integritat estructural del polímer, però també és capaç de formar interaccions hidròfobes amb els anàlits més apolars. D'altra banda, el MAA actua com a bescanviador de cations, ja que la càrrega negativa present al grup carboxílic a pH bàsic afavoreix les interaccions iòniques amb aquelles substàncies que es troben protonades a eixos mateixos pH. Per tant, el cas concret del *poly* (MAA-*co*-EGDMA) mostraria capacitats de WCX/fase inversa. No obstant, és interessant aprofundir en els materials de tipus mixt per buscar noves formes d'aquests que permeten augmentar la seua versatilitat i aprofitar les seues capacitats al màxim.

En aquest sentit, introduir altres monòmers funcionals com la 4-vinilpiridina *(4-Vinylpyridine, 4VP)* es pot aconseguir l'obtenció de materials que presenten característiques WCX si tenen àcids com el MAA, però també WAX causades per la presència de 4VP. A més, la 4VP està formada per un anell aromàtic heteroatòmic

que conté nitrogen, a banda de les interaccions iòniques que pugi formar aquest àtom de nitrogen quan es troba protonat, altres interaccions addicionals com les de tipus π-π poden ser afavorides. Si tenim en compte que algunes drogues tenen una natura àcida i que per altra part, la majoria de drogues contenen anells aromàtics a la seua estructura, la presència de 4VP pot ser útil per sintetitzar un polímer de tipus mixt de doble acció WCX/fase inversa, però també WAX/fase inversa permetent estendre el seu ús a l'extracció de pràcticament qualsevol droga.

En aquest sentit, la recerca de materials de tipus mixt amfòters por ser interessant per a la seua aplicació a l'anàlisi de drogues. Aquest materials permeten l'extracció segons el pH de càrrega de substàncies que presenten des d'un marcat caràcter àcid, fins substàncies amb caràcter bàsic sense oblidar-ne les molècules neutres que poden quedar retingudes gràcies a la part hidròfoba del material. Al Capítol 7 d'aquesta Tesi Doctoral, es planteja la síntesi d'un material de tipus mixt de doble acció WCX/WAX/fase inversa d'aquestes característiques, *poly (4VP-co-MAA-co-EGDMA)*, per a la determinació de diverses drogues àcides i bàsiques (vegeu Figura 22). Al treball s'hi posa interès en el desenvolupament d'un mètode emprant com a anàlits diverses drogues il·lícites que inclouen la 3-metilmetcatinona *(3-Methylmethcathinone, 3-MMC)* i cocaïna com a drogues bàsiques i el diazepam, essent aquesta última de natura àcida. El procediment per a l'anàlisi d'aquestes drogues, va ser acuradament desenvolupat, per ser després aplicat a l'anàlisi de mostres de saliva reals que contenien tant diazepam com cocaïna on es va emprar com a tècnica analítica per a la determinació l'UHPLC-MS/MS.

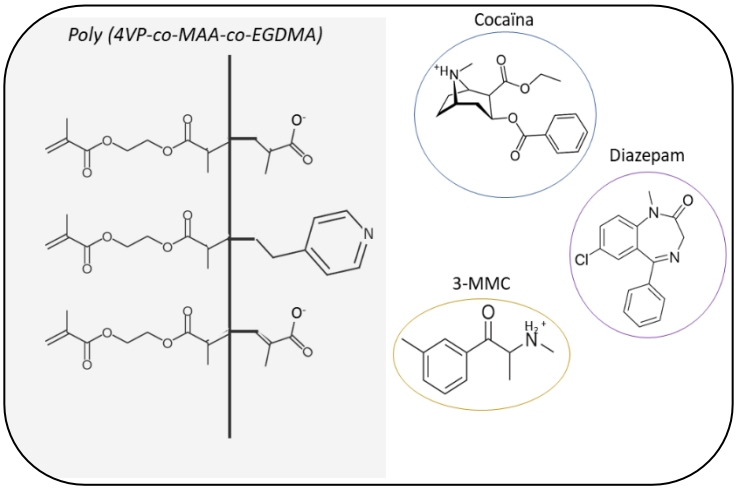


Figura 22. *Estructura bàsica del material de tipus mixt de doble acció poly (4VP-co-MAA-co-EGDMA) i drogues seleccionades.*

A banda de la síntesi de materials genèrics, al present projecte s'hi proposa l'obtenció de materials selectius de natura polimèrica com els MIPs. Per a la preparació dels MIP es requereix l'ús d'una molècula plantilla o *template* per tal de crear llocs de reconeixement selectius. En ser alliberades aquestes cavitats, després d'un rentat adequat del MIP, seran capaces de reconèixer i retenir de manera selectiva aquella molècula emprada com a *template* o molècules relacionades. Una de les tècniques de polimerització més habituals per preparar MIPs és la polimerització en bloc. Comparada amb altres tècniques de polimerització, ofereix evidents avantatges com són la seua senzillesa, bona tolerància a la xicoteta presència d'impureses en els reactius o la facilitat que proporciona aquesta tècnica per ser exportada a una producció a major escala, és per aquest motiu que fins al moment ha estat una de les tècniques preferides per a la preparació de MIPs. La polimerització en bloc consisteix en la pesada dels reactius necessaris per a la preparació del polímer que inclouen el *template* i monòmer, per formar els complexos de prepolimerització que posteriorment crearan els llocs de reconeixement; el *cross-linker* per donar integritat estructural al MIP i l'iniciador per desencadenar la reacció. Finalment, tots els reactius es dissolen en un dissolvent porogènic encarregat d'estabilitzar la mescla de prepolimerització (el complex *template*-monòmer) i donar una porositat adequada al polímer resultant.

Considerant que l'aplicació de l'IMS per a l'anàlisi de drogues en fluids biològics requereix d'un tractament de mostra suficientment selectiu, és interessant estudiar l'aplicació de materials com els MIP per a l'anàlisi de drogues en matrius biològiques. En aquest sentit, considerant en primer lloc que la cocaïna és una de les drogues més consumides i que hi ha precedents de MIPs que fan ús de cocaïna com a *template* demostrant la seua efectivitat per a la seua posterior extracció. A aquesta Tesi Doctoral es planteja la síntesi per polimerització en bloc d'un MIP emprant cocaïna com a *template* per a la determinació selectiva d'aquesta en mostres de saliva i la seua posterior determinació per una tècnica ràpida i sensible com l'IMS.

El MIP preparat per a la determinació de cocaïna, va ser adequadament rentat i tamisat per incorporar-lo a columnes de SPE de 1 mL de capacitat (vegeu Figura 23). Tanmateix, el MIP va ser caracteritzat morfològicament amb SEM, una de les tècniques més comunes per observar la superfície d'aquest tipus de materials. Per a la seua posterior aplicació, es van estudiar les millors condicions d'extracció que asseguraren una elevada eliminació d'interferents conservant la retenció de la cocaïna i es van avaluar els principals paràmetres analítics del mètode. Altres característiques d'elevada importància en els MIPs també van ser

avaluades, com ho són la capacitat de càrrega per estimar quina és la màxima quantitat de cocaïna que poden retenir; la capacitat de reutilització o el factor d'empremta. Aquest últim es va avaluar mitjançant la comparació de les recuperacions oferides pel MIP i pel NIP que deuria mostrar una baixa capacitat de retenció. Finalment, l'ús d'aquest MIP selectiu, va ser aplicat per a l'anàlisi de mostres de saliva reals de persones que havien consumit cocaïna. Els resultats de la recerca de l'aplicació d'aquest MIP es troben en el Capítol 8 d'aquesta Tesi.

Un dels objectius que es planteja a aquesta Tesi Doctoral, no és només l'aplicació de MIPs per a l'anàlisi de diverses drogues, sinó fer un estudi de les propietats i capacitats que aquests poden presentar. En aquest sentit, un paràmetre important dels MIPs és conèixer la seua selectivitat creuada. La selectivitat creuada d'un MIP, és la selectivitat que un MIP preparat emprant com a *template* una molècula determinada pot oferir cap a altres molècules d'estructura similar. Si bé en algunes ocasions, la selectivitat creuada que ofereixen materials selectius com els anticossos, aptàmers o els propis MIPs és definida com una propietat negativa, també és cert que pot ser usada com un avantatge en determinades ocasions. Des d'aquelles ocasions on hi és difícil aconseguir estàndards per usar-los com a molècula plantilla a la preparació d'un MIP fins a altres casos on pot ser interessant extraure molècules amb estructura molecular similar de manera selectiva emprant un únic MIP.

Per avaluar la selectivitat creuada que poden oferir alguns MIPs, es va prendre com a punt de partida, un MIP ja emprat en estudis anteriors [Djozan et al. 2012] que feia ús de metamfetamina com a *template* (vegeu Figura 23) per a la posterior determinació de metamfetamina, amfetamina i MDMA. Aquest MIP, va ser seleccionat per les possibilitats que semblava oferir per reconèixer molècules com l'amfetamina i MDMA, molècules amb elevada semblança estructural amb la metamfetamina. Aquest MIP, una vegada preparat, va ser caracteritzat mitjançant tècniques com la FTIR per determinar les interaccions *template*-monòmer, SEM o adsorció/desorció de nitrogen per fer un estudi més detallat de la seua àrea superficial i volum dels porus. Concretament per conèixer la grandària dels micro/meso porus que són aquells de menor grandària i que influeixen directament en el reconeixement molecular. Posteriorment, 32 NPS diferents, totes elles drogues de tipus amfetamina (amb semblances estructurals amb el *template)*, que incloïen derivats de l'amfetamina, catinones sintètiques o altres fenetilamines van ser analitzades emprant un procediment SPE amb el MIP preparat. De la mateixa manera s'hi van seleccionar altres substàncies, en aquest cas sense similituds estructurals amb la metamfetamina per avaluar si hi havia diferències significatives entre l'afinitat oferta pel MIP cap a les substàncies de

tipus amfetamina front l'altre grup de substàncies. Tanmateix, aquest MIP va ser emprat per extraure mostres de saliva fortificades amb amfetamina, amb la intenció d'avaluar la seua aplicació per a l'anàlisi de mostres reals bé sigui per determinacions més noves, ràpides i portàtils com la IMS o per tècniques analítiques més ben establertes i que gaudeixen de suficient reconeixement com l'UHPLC-MS/MS, resultats que van donar lloc al Capítol 9.

Avaluada la selectivitat creuada que ofereixen alguns MIPs i considerant que aquesta pot ser usada en benefici d'obtenir mètodes d'anàlisi més versàtils sense perdre la selectivitat que ofereixen els MIPs. És interessant continuar amb l'estudi de la selectivitat i com aquesta pot ser modificada segons el procediment d'extracció aplicat, tractant d'obtenir mètodes des de més selectius cap a famílies de drogues fins altres més específics cap a l'extracció d'una única molècula.

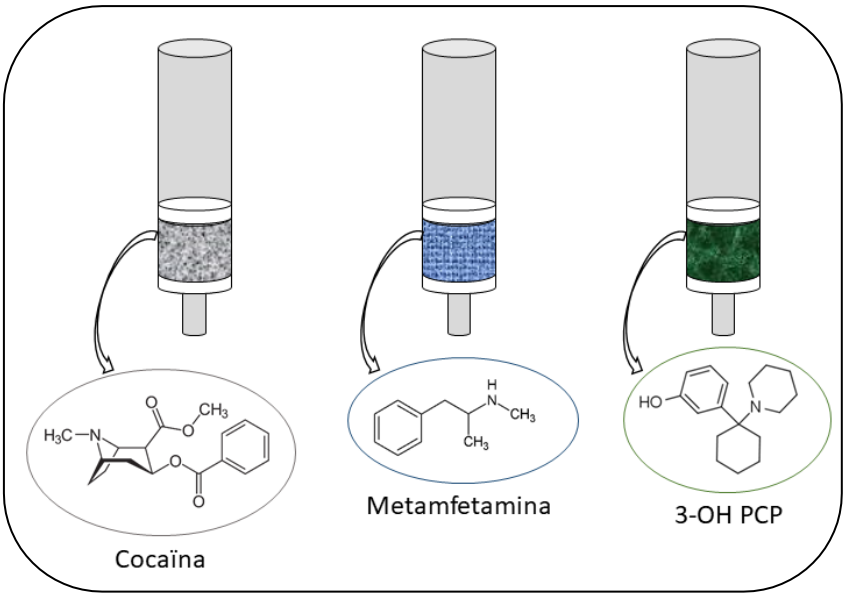


Figura 23. *Templates emprats per a la preparació de MIPs que van estar aplicats per a l'extracció de diverses drogues en format SPE convencional.*

Perseguint aquest objectiu, es va seleccionar com a molècula plantilla una NPS que no disposa de MIPs preparats per a la seua determinació a la bibliografia consultada. D'aquesta manera la 3-hidroxifenciclidina *(3- Hydroxyphencyclidine, 3-OH PCP)* va ser emprada per a la preparació d'un MIP mitjançant polimerització en bloc (vegeu Figura 23). La 3-OH PCP, és una arilciclohexilamina que pertany al mateix grup que la fenciclidina *(Phencyclidine, PCP)*, la 3-metoxifenciclidina *(3-Methoxy Phencyclidine, 3-MeO PCP)*, 3-metoxietilciclidine *(3-Methoxyethylcyclidine, 3-MeO PCE)*, metoxetamine *(Methoxetamine, MXE)*, descloro-n-etil ketamina *(Deschloro-N-ethyl ketamine, 2-oxo PCE)*,

descloroketamina i ketamina. Després d'obtenir el MIP mitjançant polimerització en bloc emprant aquesta substància com *template*, es van estudiar diversos procediments de SPE posant especial atenció a l'etapa de rentat. Principalment, l'objectiu a assolir, era l'obtenció d'un mètode capaç de determinar de manera específica la 3-OH PCP front un segon mètode on un rentat amb menor força d'elució permetera l'extracció selectiva de les arilciclohexilamines descrites anteriorment i que es mostren a la Figura 24. Finalment, per demostrar l'aplicabilitat d'aquest MIP en format SPE combinat amb l'IMS per a l'anàlisi de mostres de saliva reals. Es va realitzar l'anàlisi de mostres reals fortificades amb 3-OH PCP, resultats que es mostren detalladament al Capítol 10 d'aquesta Tesi Doctoral.

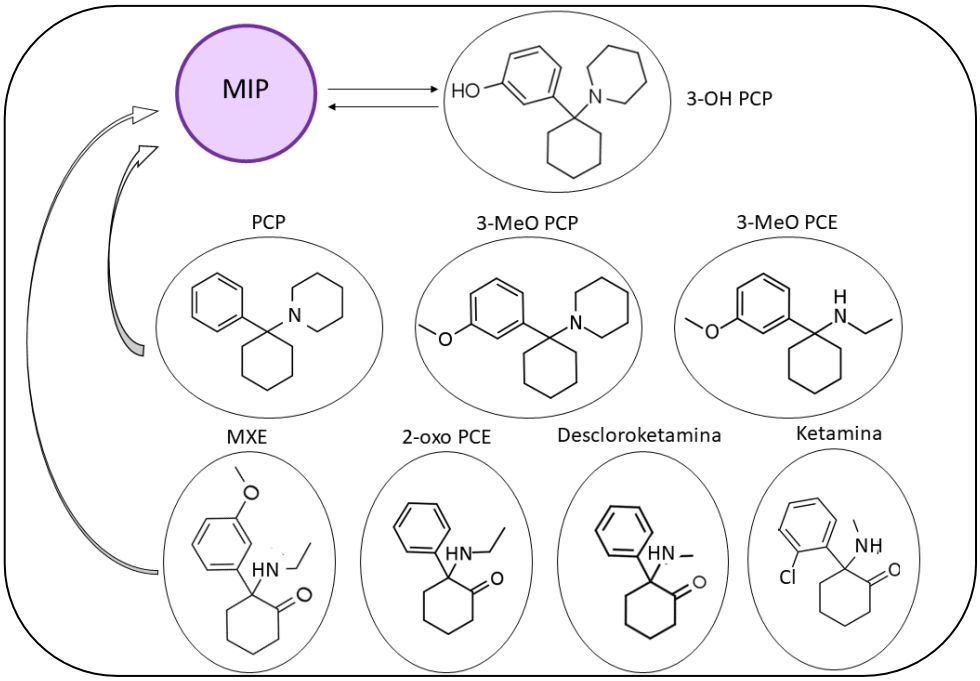


Figura 24. *Estructures de les arilciclohexilamines emprades per avaluar la selectivitat creuada del MIP.*

Una vegada estudiades les possibilitats que pot oferir conèixer i inclús modificar la selectivitat creuada d'alguns MIPs, cal destacar que un altre dels objectius marcats al llarg d'aquest projecte, ha estat l'adaptació dels materials i en concret si fem referència als materials selectius, els MIPs, a nous formats d'extracció (vegeu Figura 25). En aquesta línia, s'ha seleccionat la cocaïna com a molècula diana tenint en compte que és una de les drogues més consumides arreu de tot el món, i en particular als països del sud d'Europa. Així, s'ha seleccionat el MIP preparat i avaluat en treballs anteriors emprant cocaïna com a

template per desenvolupar alguns dels nous formats d'extracció proposats que permeten miniaturitzar, simplificar i/o fer portàtils aquests tipus d'anàlisis.

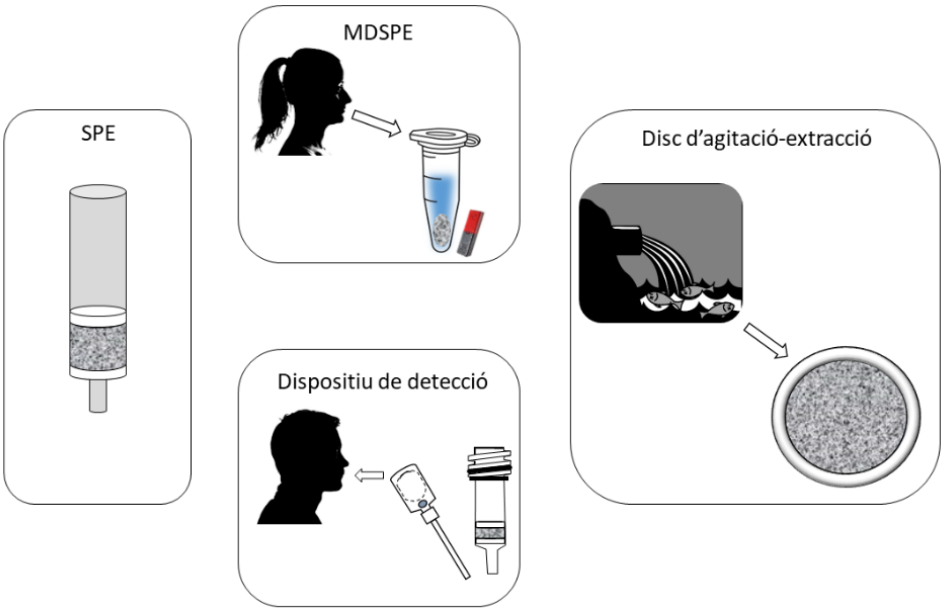


Figura 25. *Formats emprats per a l'extracció selectiva de cocaïna i els seus metabòlits mitjançant l'ús de MIPs i determinació per IMS.*

En primer lloc, es proposa l'obtenció d'un dispositiu econòmic i senzill que permeta aplicar un procediment ràpid i portàtil per a la identificació de l'abús de cocaïna a partir de mostres de saliva. El dispositiu proposat està format per una primera part que serveix per recol·lectar la mostra i que està basat en una bola de cotó amb un indicador d'humitat que indicarà quan el cotó ha absorbit la mostra necessària. La segona part fa ús d'un MIP preparat en presència de cocaïna com a *template* i que va ser avaluat en estudis previs (Capítol 8). Una vegada la mostra de saliva ha estat recollida, la cocaïna es transfereix al MIP junt a la resta de components de la saliva, emprant un tampó fosfat (pH 7,0) que permetrà que la cocaïna quedi adsorbida sobre la superfície del MIP, mentre que la resta de components de la saliva seran eliminats. Finalment, la cocaïna s'elueix amb un volum petit d'isopropanol per mesurar aquest extracte mitjançant IMS. Per avaluar la sensibilitat del mètode es van addicionar diverses mostres a 5,10 i 20 µg L^{-1}. La metodologia proposada va permetre l'anàlisi de cocaïna en saliva amb una concentració de tall de 20 µg L^{-1}, proporcionant un 5 % de falsos positius i un 0 % de falsos negatius. El procediment proposat va ser aplicat a l'anàlisi de saliva d'individus en ambients d'oci que van declarar haver consumit cocaïna, els

resultats del desenvolupament d'aquest dispositiu es mostren en major detall al Capítol 11.

El format descrit anteriorment, va fonamentalment dirigit cap a l'anàlisi semi quantitatiu *in-situ* de cocaïna. No obstant, en algunes ocasions és més interessant realitzar l'anàlisi al laboratori amb major precisió i exactitud tal com els que es fan emprant una SPE tradicional, però tractant de miniaturitzar l'anàlisi, ja que és habitual que els volums de mostra disponibles quan parlem de saliva siguin menuts. Altra proposta realitzada per tal d'aconseguir anàlisis més miniaturitzades és la substitució de la SPE tradicional per una DSPE. Tenint en compte que la DSPE habitualment requereix etapes de filtració o separació de l'adsorbent tedioses, modificar la DSPE per una MDSPE pot ajudar a facilitar aquesta separació mitjançant l'aplicació d'un camp magnètic. Amb l'objectiu d'avaluar l'aplicació de la MDSPE i com actuen els MIPs quan són combinats amb MNPs, es van preparar polímers de reconeixement molecular magnètic *(Magnetic Molecularly Imprinted Polymer, MMIP),* al Capítol 12 es mostren els resultats obtinguts després de la síntesi emprant la polimerització en bloc per capturar MNPs a l'interior d'un MIP preparat emprant la cocaïna com a molècula plantilla i amb la intenció de ser seleccionada com anàlit. A aquest treball, no només s'hi va estudiar un procediment d'extracció adequat per a la posterior aplicació a l'anàlisi de cocaïna en mostres de saliva, sinó que es van comparar dos mètodes per a la immobilització del MIP sobre les MNPs. La primera proposta, està basada en la prèvia modificació de les MNPs amb polietilenglicol *(Polyethyleneglycol, PEG)* que ajuda a que aquestes queden envoltades per una coberta hidròfila que afavoreix el posterior encapsulament de les MNPs dins del MIP, obtenint-ne els que s'han anomenat PEG-MMIP *(Polyethyleneglycol-Magnetic Molecularly Imprinted Polymer)*. L'altra proposta per immobilitzar els MIPs sobre les MNPs ha estat la modificació d'aquestes emprant un agent silinitzant com el 3-(trimetoxisilil)propil metacrilat *(3-(trimethoxysilil)propyl methacrylate, Y-MPS)* que deixa grups vinil a la superfície de les MNPs per enllaçar de manera covalent el MIP a la superfície de les pròpies MNPs. En aquest cas els polímers obtinguts van ser anomenats V-MMIP *(Vinyl-Magnetic Molecularly Imprinted Polymer).* Els resultats de la recerca d'aquest materials per a la seua posterior aplicació es veuen detalladament exposats al Capítol 12 d'aquesta Tesi Doctoral.

L'estudi del consum de cocaïna, no només s'estén a les mostren biològiques. Altres tipus de mostres com les aigües residuals o inclús naturals poden donar informació molt valuosa sobre el consum de diverses drogues com la cocaïna entre una determinada població. En aquest sentit, s'ha de tenir en compte que la determinació s'ha de portar a terme principalment a través dels metabòlits de les

substàncies a determinar, especialment aquelles que s'excreten majoritàriament metabolitzades com és el cas de la cocaïna. Cal afegir que el major problema de l'anàlisi de drogues en mostres d'aigües és la baixa concentració en la que es troben, essent fonamental disposar de mètodes que permeten obtenir grans factors de concentració per aconseguir assolir LODs acceptables. Per aquest motiu, entre els formats de la SPE que permeten el tractament de grans volums de mostra trobem exemples com la SBSE, si bé es cert que aquest format presenta problemes en quant a la facilitat de que la fase sòlida s'hi vaja desprenent del suport sòlid o els elevats temps d'extracció que sovint requereix per adsorbir quantitativament l'anàlit.

Front la SBSE, altre format d'extracció que ha estat usat prèviament és la SCSE. La SCSE permet resoldre algunes de les limitacions principals de la SBSE donant una major àrea de contacte entre l'adsorbent i la mostra i evita el contacte directe de l'adsorbent amb les parets del recipient on s'hi realitza l'extracció. Per això, al Capítol 13 s'hi proposa el desenvolupament d'un disc de politetrafluoroetilè *(Polytetrafluoroethylene, PTFE)* modificat que conté un MIP immobilitzat. Aquest MIP va estar preparat emprant com a *template* ecgonina metil èster, un dels metabòlits principals de la cocaïna. Aquest disc, incorpora dos imants que permeten una agitació rotatòria en vertical front a l'agitació horitzontal de la SCSE, permetent una extracció més eficient i ràpida. A més, el dispositiu desenvolupat, aconsegueix obtenir factors de preconcentració de fins 40 vegades, cosa que permet la determinació més fiable de l'anàlit gràcies als elevats volums de mostra que és capaç d'agitar. Aquest treball, presenta els resultats obtinguts durant el desenvolupament del mètode d'extracció, així com per a l'anàlisi de diverses mostres d'aigua recollides de diversos punts propers a la ciutat de València que inclouen aigües naturals i aigües residuals de depuradora. Les tècniques analítiques que s'avaluen per aquesta anàlisi han estat l'IMS com a tècnica ràpida per avaluar les mostres positives o negatives, mentre que l'UHPLC-MS/MS s'empra com a tècnica d'elevada sensibilitat per a la determinació quantitativa d'ecgonina metil èster.

Tot i que aquesta Tesi Doctoral està centrada en el desenvolupament de nous formats i materials, fonamentalment de natura polimèrica, aplicats a l'anàlisi de drogues, és destacable que hi ha més materials selectius que prometen bones expectatives de futur. En aquest sentit, els aptàmers presenten importants avantatges front als anticossos, com ha estat mencionat prèviament, i de moment els queda un llarg recorregut pel que fa a la recerca de les seues propietats i la seua posterior aplicació en SPE.

D'aquesta manera, s'han seleccionat aptàmers selectius front a fluoroquinolones [Reinemann et al. 2016] i front a metamfetamina [Zargar et al. 2018] prèviament descrits a altres articles de recerca per ser immobilitzats sobre safarosa activada amb bromur de cianogen, així com sobre partícules magnètiques. El Capítol 14, mostra els resultats preliminars obtinguts després de la immobilització d'aquests aptàmers sobre els suports sòlids proposats. La principal finalitat d'aquest estudi, està en mostrar l'aplicació dels aptàmers tant immobilitzats sobre la safarosa com sobre les partícules magnètiques per a l'anàlisi de fluoroquinolones i metamfetamina en mostres biològiques, comparant els formats d'extracció SPE convencional i MDSPE. No obstant, les recuperacions obtingudes són molt baixes, indicant una baixa interacció entre els anàlits i l'aptàmer. Per aquest motiu, és necessari aprofundir en la recerca d'aquests materials, buscar-ne les possibles causes de la manca d'afinitat que mostren cap als anàlits i estudiar les possibles solucions de cara a una futura aplicació d'aquest innovador tipus de material.

This Doctoral Thesis entitled "A*pplication of new materials and formats with improved properties for drug analysis*" is structured in three different sections addressed from different perspectives the drug analysis issue. It collects the published works developed in order to apply different materials with improved properties for drug extraction from biological fluids using different SPE approaches.

As it was deeply explained along introduction section, IMS is a technique based on gas phase separation of ionized analytes under weak electric field at atmospheric pressure. This technique was initially used by defense agencies for the analysis of explosives, illicit drugs and chemical warfare agents. Typical uses of IMS are mainly attributed to the advantages that this technique provides, such as its operational simplicity, speed and good sensitivity. These advantages have led to IMS being extended to numerous fields of analysis such as pharmaceutical, clinical, petrochemical, environmental or food analysis industries.

It is well-known that IMS has some limitations that can hinder its application for certain types of analysis such as those performed on more complex samples. These limitations are mainly the lack of linear response and low selectivity due to the possible interaction of reactant with the other components of the sample. These interactions can lead some problems in the analysis by IMS such as no-detection of the analyte, false positives detected due to an erroneous identification of an interfering compound or the presence of competitive ionization that causes preferential ionization of other components than target molecule.

These limitations of IMS have been approached from several perspectives in order to minimize or eliminate their effects. For example, the first approach in order to increase its selectivity was the coupling of the IMS to a pre-separation by GC, but IMS has also been used as separation technique prior to determination by MS, obtaining in both cases an increase of selectivity by mitigating the matrix effect. Another interesting strategy proposed has been the introduction of doping gases that allow to remove interferences because of the selective ionization of the analytes, obtaining plasmagrams easy to read. Another approach employed is to use the data provided after IMS analysis to perform a data treatment of the results by chemometric methodologies that allows to extract qualitative and quantitative information.

Despite the interest of these approaches to solve IMS limitations, sample treatment prior to determination to remove matrix effects and increase selectivity remains the most widely used strategy because of the easy application and versatility provided by them. LLE and SPE methodologies, including all their

respective versions, have demonstrated being very valuable tools for sample treatment and subsequent determination using IMS.

Extractive techniques such as conventional SPE, SPME, SBSE or MEPS using generic or non-selective materials, such as silica modified with C18, PDMS, mixed type materials or carbon materials, have been used for the extraction of numerous compounds including drugs. They have proved their effectiveness on many occasions. However, new materials have also emerged during last decades to offer greater selectivity to this type of sample treatment, allowing to remove more effectively non desired components from complex samples.

Smart materials could be defined as materials that have the ability to respond to external stimulus in a controlled or specific way. In sample treatment, smart material could be understood as each material that provides an improved certain characteristic that makes them more suitable than other materials or substances. Thus, it is interesting to make a rigorous bibliographic research of the application that these materials have shown for forensic analysis in general and particularly to drug analysis, as well as the application that they have reported for determination using IMS as analytical technique (see Figure 26).

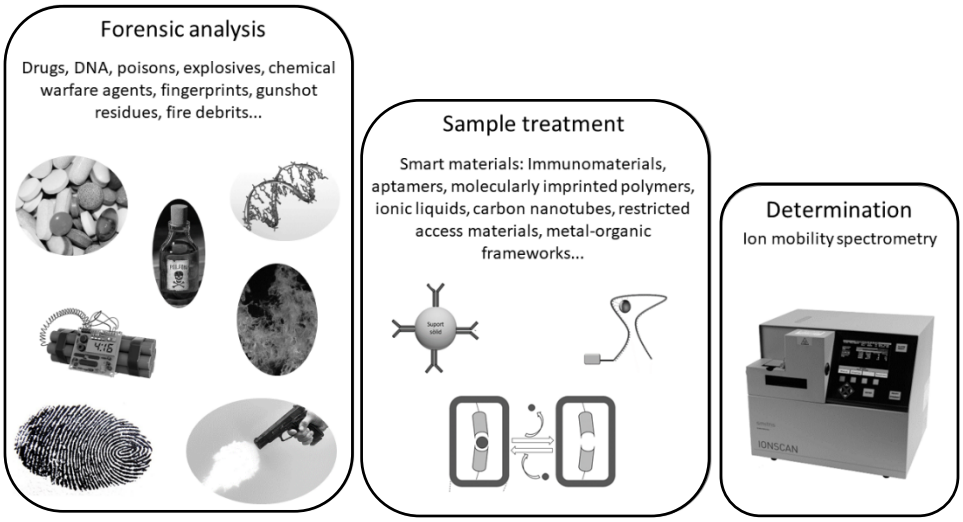


Figure 26. *Summary of forensic analysis and smart materials used.*

Firstly, there are smart materials such as ILs, CNTs, RAMs or MOFs that have moderate selectivity against certain groups of molecules. This moderate selectivity is due to the possibility of being functionalized with some functional groups that offer an oriented response towards specific functional groups, although they do not respond to molecules in a specific way. Some materials such as RAMs or CNTs have been used for forensic analysis and specifically for drug analysis, but their application for drug determination by IMS has not been investigated.

On the other hand, considering more selective smart materials, we can mention immunosorbents. They are materials produced by immobilization of antibodies onto a solid support. Immunosorbents have shown to have great application increasing the selectivity of methods using IMS due to the high specificity presented by antibody-antigen complex. However, application of immunosorbents in SPE for drug analysis is virtually limited to the analysis of drugs such as clenbuterol or salbutamol. Specifically, using IMS as analytical technique, chloramphenicol has also been determined using immunosorbents [Armenta et al. 2016]. Immunoassay, as one of the most employed approach of immunoaffinity materials, has been widely used for the fast screening of drugs in biological fluids, demonstrating the good possibilities of antibodies for selective drug recognition. Thus, commercially available immunoassays have been used for the determination of various drugs in matrices such as urine, saliva, blood, hair, or sweat [Payne-James et al. 2005].

New smart materials with similar interaction mechanism to immunosorbents called aptamers, have emerged in the last decade. They are single-stranded sequences of DNA or RNA that after their immobilization onto solid support, oligosorbents can be obtained. Even most of identified oligonucleotide sequences are aimed at the analysis of large molecules such as proteins; there are also a high number of aptamers identified for the analysis of small molecules. Oligosorbents are excellent candidates for selective separation of analytes. Selectivity promoted by oligosorbents is comparable to that offered by antibodies. In addition, they have lower cost and during their preparation they allow to introduce modifications to improve their properties. Aptamers have been used as sensors for methamphetamine determination [Yarbakht et al. 2016] or cocaine and its metabolites in biological matrices [Guler et al. 2017]. There are also examples of their use in SPE columns for the determination by IMS of codeine or acetamiprid in urine samples [Zargar et al. 2017].

Although, antibodies and aptamers have very attractive features for drug analysis and specifically for sample treatment and subsequent analysis by IMS, it must also be considered a last group of smart materials such as MIPs. In comparison, MIPs have greater application for the analysis of drugs, especially used as sorbent in SPE. MIPs, prepared in a synthetic way, have selective cavities to recognize the analytes for which they have been designed. Besides, their chemical and physical properties, their versatility, stability and ability to recognize, make them candidates to consider versus other smart materials. Although commercially available MIPs for determination of clenbuterol [Peñuela-Pinto et al. 2017] or chloramphenicol [Armenta et al. 2016] are availabe, its simple preparation has led to many of them have been prepared in-home for the determination of molecules such as methamphetamine [Djozan et al. 2012], cocaine [Thibert et al. 2014] or diazepam [Ariffin et al. 2007] in biological samples. Other MIPs have been prepared for the extraction of caffeine [Jafari et al. 2014] or ibuprofen [Alizadeh et al. 2014] by IMS determination in samples such as serum or plasma.

It seems logical, that IMS is a good choice when it is used to perform an analysis of only one analyte whenever it is possible to isolate it selectively to prevent interferences of other compounds. In this sense, materials such as MIPs, aptamers or immunosorbents seem to have suitable capacities to be applied for sample treatment prior to determination. However, certain cases could be more interesting to focus the analysis on the determination of a large number of drugs in a biological matrices versus the selective determination of one of them. Then, it would be more interesting to use other types of generic materials that do not interact specifically only with a molecule or family of them, but it is able to recognize a large number of them. Considering the use of generic materials, a selective analytical technique such as GC-MS or LC-MS/MS should be used.

For this reason, a bibliographic research was firstly carried out comprising the first section of this Doctoral Thesis and being composed by chapters 1 and 2. The rest of the chapters are separated into two additional sections, including the results presented as original publications resulting from the two approaches in which drug analysis has been addressed. The second section develops the production of materials and methods for drug analysis using generic materials or with moderate selectivity. The third section focuses on the development of selective materials and its subsequent adaptation to new extraction formats for the analysis of drugs in a specific way compared to other molecules.

Among the generic materials, we find that one of the most common materials used in the development of SPE have been the silica phases modified with C18 or C8. These phases have shown usefulness to retain non-polar compounds when they are found in aqueous matrices. Its non-specificity can lead to the presence of large number of interferents, although it is possible to modify the washing step of the extraction in order to obtain more purified extracts. Compared to the traditional SPE, MEPS can be considered an almost direct miniaturized adaptation of the SPE, by placing small amount of sorbent phase (approximately 1 mg) at the end of a needle that can be attached to a manual syringe or automatic mode to a robotic arm. In general, MEPS consists on the same procedure as a traditional SPE: (i) sorbent conditioning, (ii) sample loading, (iii) washing step, and (iv) elution of the analyte.

In this sense, for determination of various groups of drugs such as NPS, it is interesting to take generic approaches for their analysis, which would allow the determination of most of them. Using MEPS, three similar methods have been developed but adapted to the analysis of various NPS. Dichlorophane which is a substance that mimcs the effects of cocaine; methylone that is considered as an analogue of MDMA and five third-generation synthetic cannabinoids that include 5F-ADB, MMB-CHMICA, THJ-2201, and CUMYL-4CN-BINACA (see Figure 27). In order to study the best extraction conditions, certain parameters such as sorbent phase, loading step (including its pH), number of aspiration cycles required to achieve adequate retention and washing solvent were evaluated, as well as the volume and number of elution cycles. After that, for determination of all NPS, the validation of the method was carried out evaluating basic analytical features such as linearity, LODs, accuracy or precision. Finally, the developed method was applied to analysis of spiked field oral fluid samples at different concentration levels with the different NPS aforementioned. The analysis of dichlorophane and methylone was performed by IMS since it is quite common that their potential consumers do not abuse similar substances at the same time. However, in case of synthetic cannabinoids, it is quite common to observe how the commercial products contain two or three different synthetic cannabinoids [Tai et al. 2014]. For this reason and in order to be able to identify which are the synthetic cannabinoids present in each sample, a more selectivity technique such as GC-MS has been chosen. In this sense, the development and application of MEPS methodologies for the analysis of various NPS in oral fluid samples is shown in chapters 3, 4 and 5 of this Doctoral Thesis.

Also, traditional SPE has been adapted to other formats different from cartridge/syringe. One of this formats is micropipette tip extraction. In this case, a

sorbent phase, either in the form of particles and contained between two frits or in as a monolith is placed into a micropipette tip to perform the extraction. This format makes easier the extraction and it also increases significantly the speed of the extraction procedure, avoiding the need of vacuum systems to carry out the extraction. Taking into account the benefits of monolithic phases, the synthesis of a polymer based on EGDMA and functionalized with MAA was chosen from the bibliographic research, where these materials have shown good ability to the retention of basic molecules, as almost drugs. For the preparation of poly (MAA-co-EGDMA) anchored onto inner walls of the PP tip, it is first necessary to modify the inner walls of pipette tips using agents such as benzophenone, with subsequent functionalization with EGDMA to allow the formation of covalent bonds between tip walls and the monolith. Once the tips have been functionalized, it is necessary to obtain a monolith that has an appropriate mechanical strength as well as enough permeability to allow flow through solvents and sample. To achieve these characteristics, the study of various proportions of MAA and EGDMA, as well as the volume and nature of the porogenic solvent used for the preparation of the monolith was considered. Chapter 6 presents the results obtained for the preparation of this polymer in this format.

After tips preparation, the evaluation of the best loading conditions, reusability as well as the precision and accuracy using as target molecule for the study α-PVP were investigated (see Figure 27). Then, the application of developed method for the extraction of several drugs in oral fluids that included 20 different NPS as well as traditional drugs such as cocaine, methamphetamine and diazepam, was evaluated. Due to the large number of drugs selected, UHPLC-MS/MS was used as analytical technique to evaluate the effectiveness of the tips filled with the monolith due to its high selectivity.

Poly (MAA-co-EGDMA)-based materials has demonstrated in this work to have properties of mixed-mode materials. MMS are materials that provide ion exchange and retention characteristics through nonspecific interactions similar to those provide by reversed-phase. In this sense, EGDMA acts as backbone that ensures the structural integrity of the polymer and it is also able to create hydrophobic interactions with non-polar analytes. On the other hand, MAA acts as cation exchanger because the negative charge present in the carboxylic group at basic pH encourage ionic interactions with those substances that are protonated at the same pH. Therefore, the specific case of poly (MAA-co-EGDMA) would show WCX /

reversed-phase capabilities. However, it is interesting to study new MMS to look for new materials that allows to increase their versatility.

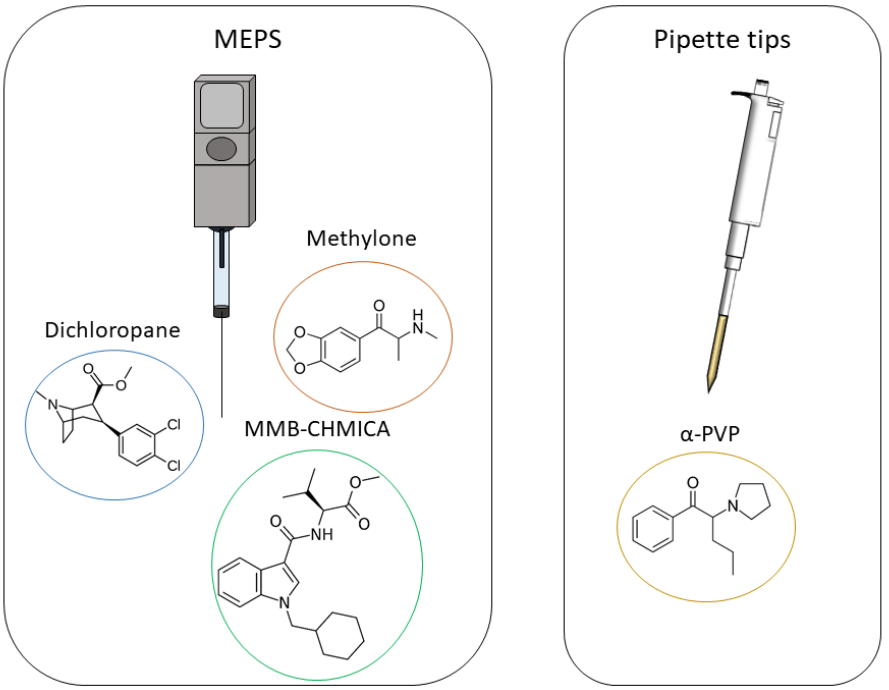


Figure 27. *Target analytes selected for the extraction using MEPs and pipette tips.*

In this sense, by introducing other functional monomers such as 4VP, it can be achieved the production of materials that have WCX characteristics if they have acids such as MAA, but also WAX caused by the presence of 4VP. In addition, 4VP contains a nitrogen atom into an heteroatom aromatic ring, hence, to ionic interactions that this nitrogen atom can form when it is protonated. Besides, other additional interactions such as π-π-type can be encouraged. Considering that few drugs have an acidic nature and that on the other hand, most drugs contain aromatic rings in their structure, the presence of 4VP may be useful to synthesize a double action mixed-mode polymer WCX / reversed-phase, but also WAX / reversed-phase allowing to extend its use for the extraction of almost all drugs.

In this sense, the research for amphoteric MMS may be interesting for their application to drug analysis. These materials allow the extraction according to the loading pH of substances that exhibit a marked acidic character, to substances with a basic character and also the neutral molecules that can be retained thanks to the hydrophobic part of the material. In Chapter 7 of this Doctoral Thesis, the synthesis

of a MMS with properties of WCX / WAX / reversed-phase, poly (4VP-co-MAA-co-EGDMA) was done for the determination of several drugs (see Figure 28). Cocaine and 3-MMC as basic drugs and diazepam as acidic. Procedure for the analysis of these drugs was carefully developed, to be applied for the analysis of field oral fluid samples containing both diazepam and cocaine, where UHPLC-MS/MS was used as analytical technique for determination.

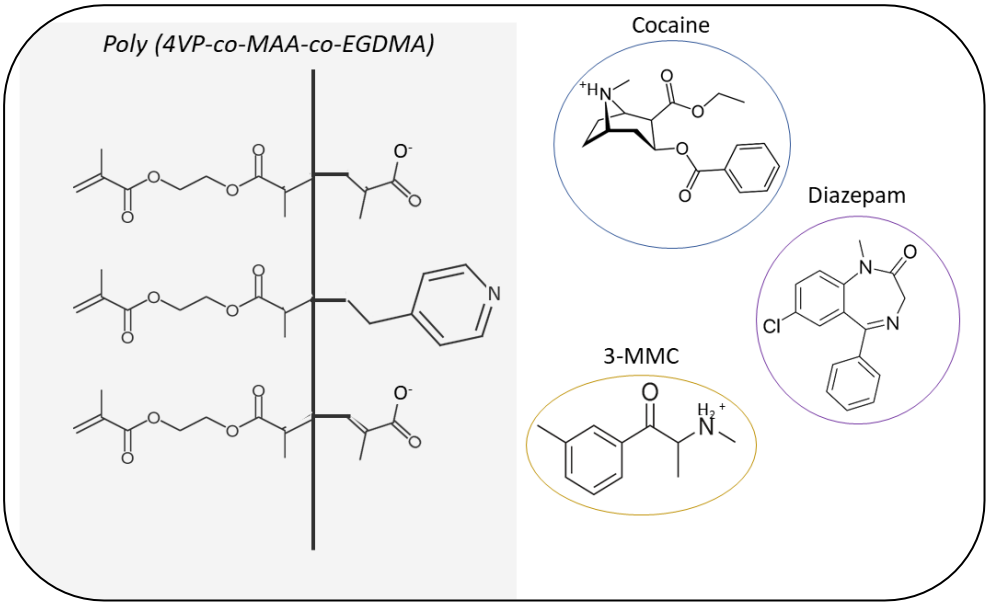


Figure 28. *Simplified structure of mixed-mode material based on poly (4VP-co-MAA-co-EGDMA) and selected drugs.*

In addition to the synthesis of generic materials, this project proposes the synthesis of selective materials of a polymeric nature such as MIPs. Preparation of MIPs requires the use of a template molecule to create selective recognition sites. When these cavities are released, after an appropriate washing of the MIP, they will be able to selectively recognize and retain that molecule used as template or structurally related molecules. One of the most common polymerization techniques to prepare MIPs is bulk polymerization. Compared with other polymerization techniques, it offers obvious advantages such as its simplicity, good tolerance to the small presence of impurities in the reagents or the ease that this technique provides to be exported to a larger scale production. For this reason, it has been one of the preferred techniques for the preparation of MIPs. Bulk polymerization consists of the weighing of the reagents necessary for preparation of the polymer including the template and monomer, to form the prepolymerization complexes that will later create the recognition sites; the cross-

linker to give structural integrity to the MIP and the initiator to trigger the reaction. Finally, all the reagents are solved in a porogenic solvent that must stabilize the prepolymerization mixture (the template-monomer complex) and giving a suitable porosity to the resulting polymer.

Considering that the application of IMS drug analysis in biological fluids requires a selective sample treatment, it is interesting to study the application of materials such as MIPs for drug analysis in biological matrices. In this sense, considering that cocaine is one of the most consumed drugs and that there are precedents of MIPs that have used cocaine as template, thus, demonstrating its effectiveness for extraction of cocaine. This Doctoral Thesis proposes the synthesis by bulk polymerization of a MIP using cocaine as template for its selective determination in oral fluid samples and its subsequent determination by rapid and sensitive technique such as IMS.

The prepared MIP for cocaine determination, was properly washed and sieved to be incorporated into 1 mL capacity SPE columns (see Figure 29). Then, MIP was morphologically characterized by SEM, one of the most common techniques used in characterization of materials. In order to guarantee the good extraction of cocaine, the best extraction conditions were studied to ensure high removal of interferences, while maintaining the retention of cocaine. Analytical features of the method were also evaluated. Other features of high importance in MIPs were also assessed, such as loading capacity to estimate the maximum amount of cocaine they can retain; reusability or the imprinting factor. Imprinting factor was assessed by comparing the recoveries offered by the MIP and the NIP, which should show low retention capacity. Finally, the use of this selective MIP was applied for the analysis of field oral fluid samples from people who had consumed cocaine. The results of the research for the application of this MIP can be found in Chapter 8 of this Thesis.

One of the scopes of this Doctoral Thesis is the application of MIPs for the analysis of several drugs, but also to make a study of the properties and capabilities that they can exhibit. In this sense, an important parameter of MIPs is their cross-selectivity. The cross-selectivity of a MIP is the selectivity that a MIP prepared using a molecule as template can offer to other related molecules with similar structure. While sometimes the cross-selectivity offered by selective materials such as antibodies, aptamers or MIPs is defined as negative property, it is possible to use it as an advantage on certain situations. For example, when it is difficult to achieve standards to use it as template in the preparation of a MIP to other cases where it

may be interesting to extract selectively molecules with similar molecular structure using a single MIP.

To assess the cross-selectivity that some MIPs may offer, a MIP already used in previous studies was taken as a starting point [Djozan et al. 2012]. This MIP used methamphetamine as template (see Figure 29) for the subsequent determination of methamphetamine, amphetamine and MDMA. This MIP was selected on the basis of recognition of molecules such as amphetamine and MDMA, molecules with a high structural resemblance to methamphetamine. After MIP preparation, MIP was characterized by techniques such as FTIR to evaluate template-monomer interactions and SEM or nitrogen adsorption/desorption measurements to get a more detailed study of its surface area and pore volume. The study of the size of the micro/mesopores is important since those with smaller size are directly involved in the molecular recognition. Hence, 32 different NPS, all of them amphetamine-type drugs (with structural similarities to the template), which included amphetamine derivatives, synthetic catinones or other phenethylamines were analyzed using an SPE procedure with prepared MIP. Similarly, other substances were selected, in this case without structural similarities to methamphetamine to assess that there were significant differences between the affinity offered by MIP towards amphetamine-type substances versus the other group of substances. Then, this MIP was used to extract amphetamine spiked oral fluid samples, with the intention to evaluate its application for the analysis of field samples by newer, faster and portable analytical techniques such as IMS or by using sophisticated techniques such as UHPLC-MS/MS. The results are shown in Chapter 9.

After evaluation of cross-selectivity offered by some MIPs and considering that this can be used to the benefit in order to obtain more versatile methods of analysis without losing the selectivity offered by MIPs, it is interesting to continue with the study of selectivity. It can be modified according to the extraction procedure applied, trying to obtain methods from more selective towards drug families to more specific towards the extraction of a single molecule.

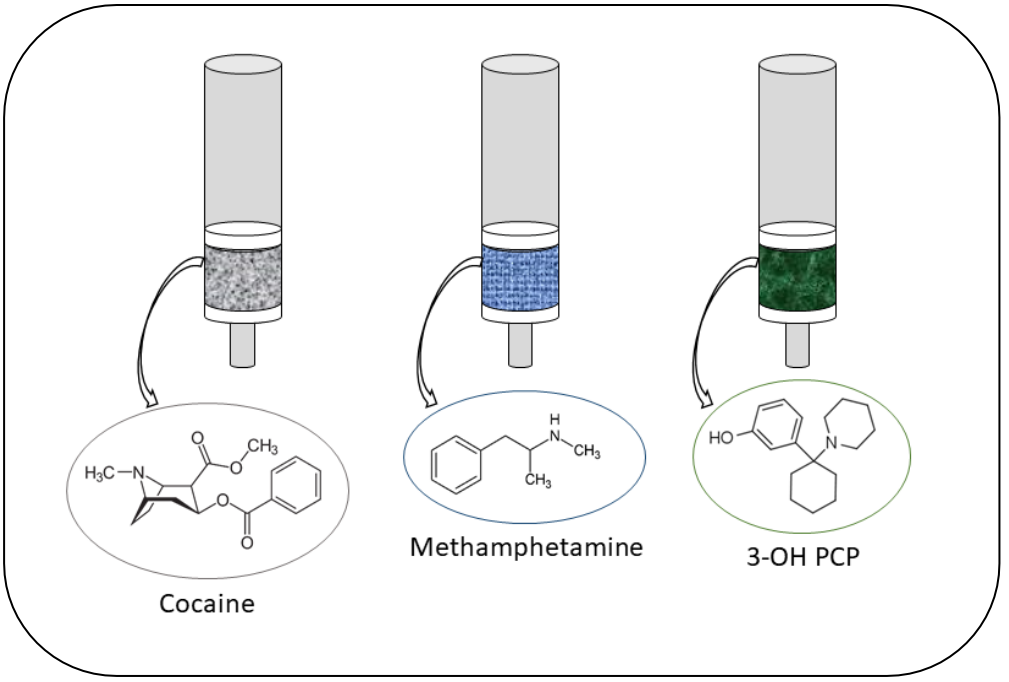


Figure 29. *Templates used for MIP production used in conventional SPE approach for drug determination.*

For this purpose, a NPS was selected as template molecule that does not have MIPs prepared for its determination in the consulted bibliography. Thus, 3-OH PCP was used for the preparation of a MIP by bulk polymerization (see Figure 29). 3-OH PCP is an arylcyclohexylamine that belongs to the same group as PCP, 3-MeO PCP, 3-MeO PCE, MXE, 2-oxo PCE, dechloroketamine and ketamine. After MIP preparation by bulk polymerization using this substance as template, several SPE procedures were studied with special attention to the washing step. The main goal to be achieved was to obtain a method able to extract specifically 3-OH PCP versus a second method where a washing with lower elution strength will allow the selective extraction of the arylcyclohexylamines described above, which are shown at Figure 30. Finally, to demonstrate the applicability of this MIP in SPE columns combined with IMS for the analysis of field oral fluid samples. The analysis of field samples spiked with 3-OH PCP was performed. These results are shown in detail in Chapter 10 of this Doctoral Thesis.

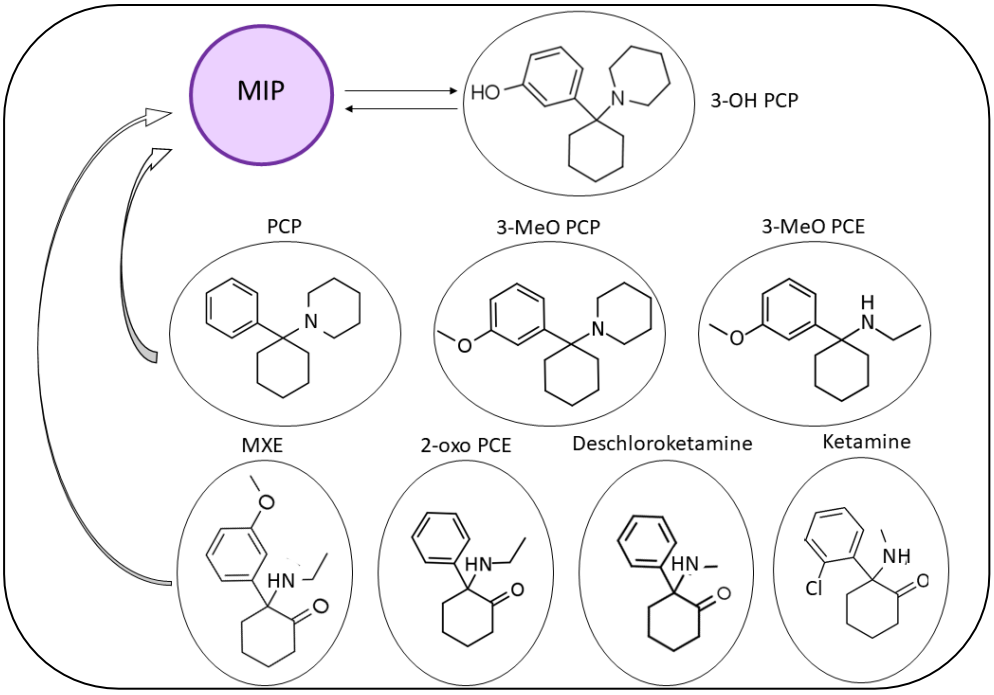


Figure 30. *Structures of arylcyclohexylamines used to study the cross-selectivity of the MIP.*

After considering the possibilities that some MIP cross-selectivity can offer and how to modify it, other of the scopes set throughout this thesis, has been the adaptation of MIP materials to new extraction formats (see Figure 31). Along these lines, cocaine has been selected as target molecule, since it is one of the most widely used drugs worldwide and in particular in the countries of southern Europe. Thus, the MIP prepared and evaluated in previous work has been selected using cocaine as template to develop some of the proposed new extraction formats that allow to achieve miniaturized, simplified and/or portable analysis.

For this purpose, an economical and simple device that allows a fast and portable procedure to be applied for the identification of cocaine abuse using oral fluid samples was developed. The proposed device consists of a first part that which is used to collect the sample and it is based on a cotton pad with a humidity indicator that will indicate when the cotton has absorbed the necessary sample. The second part uses a MIP prepared in the presence of cocaine as template and evaluated in previous studies (Chapter 8). When oral fluid sample has been collected, cocaine with other components of the oral fluid is transferred to the MIP using a phosphate buffer (pH 7.0), that will allow cocaine to be adsorbed on the surface of the MIP, while the remaining components of the saliva will be removed.

Finally, cocaine is eluted with a small volume of 2-propanol to measure the extract using IMS. To evaluate the sensitivity of the method several, samples were spiked to 5, 10 and 20 µg L^{-1}. The proposed methodology allowed the analysis of cocaine in oral fluid with a cut-off concentration of 20 µg L^{-1}, providing 5 % false positives and 0 % false negatives. The proposed procedure was applied to the oral fluid analysis from individuals who reported having consumed cocaine. The results of the development of this device are shown in more detail in Chapter 11.

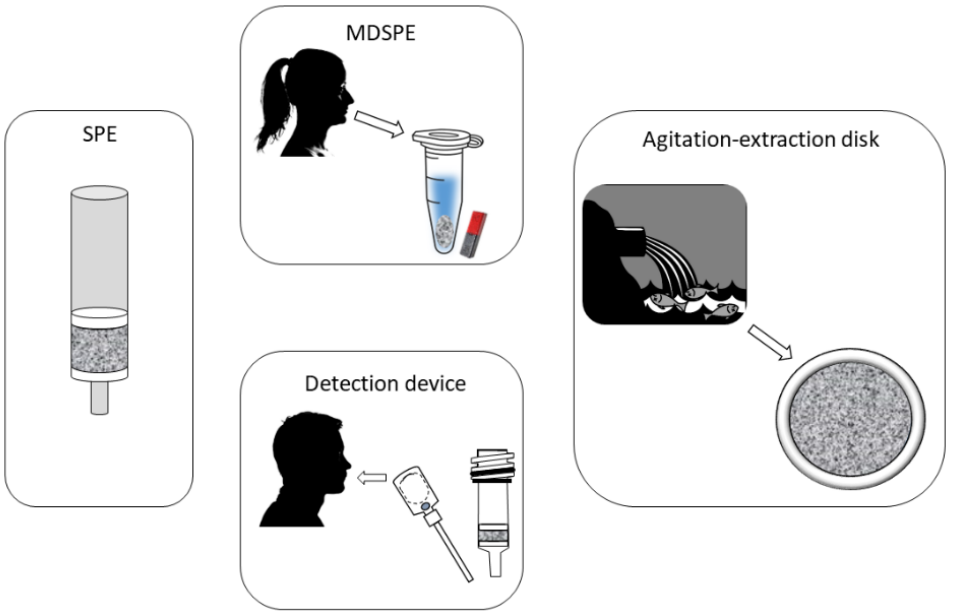


Figure 31. *Approaches used for the selective extraction of cocaine and their metabolites by MIP-IMS.*

Format described above is mainly aimed at the semi-quantitative and *in-situ* analysis of cocaine. However, sometimes it is more interesting to perform the analysis in laboratory with greater precision and accuracy such as analysis done using a traditional SPE but trying to miniaturize the analysis, because as it is common in oral fluid samples, the sample volume available is reduced. Another proposal made to achieve more miniaturized analysis is the replacement of the traditional SPE by a DSPE. However, DSPE typically requires tedious filtration or sorbent separation steps, which can be simplified by using MDSPE can facilitate this separation by direct application of a simple magnetic field. In order to evaluate the application of MDSPE, MMIPs were prepared. Chapter 12 shows the results obtained after the synthesis by bulk polymerization to capture MNPs inside a

prepared MIP using cocaine as template. In this work, an appropriate extraction procedure was studied for cocaine analysis in oral fluid samples. For this, two methods to immobilize the cocaine based-MIP onto MNPs were compared. The first proposal is based on the previous modification of the MNPs with PEG, which helps them to be surrounded by a hydrophilic cover that favors the encapsulation of the MNPs within the MIP, thus, obtaining the PEG-MMIP. The other method to immobilize MIPs onto MNPs was done by using a silinization agent such as ϒ-MPS, which leaves vinyl groups in the surface of the MNPs to covalently bind the MIP to the MNPs. In this case, polymers obtained were called V-MMIP. The results of the research of these material for their application are detailed in Chapter 12 of this Doctoral Thesis.

Studies about the consume of cocaine in a population is extended beyond biological samples. Other samples such as wastewater or natural water can provide very valuable information about the abuse of several drugs, such as cocaine, among a population. In this regard, it should be noted that determination should be carried out mainly by metabolites of the substances under study, especially if the studied drug is excreted mostly metabolized as cocaine. The biggest problem of drug analysis in water samples is the low concentration found of those subtracted, and it is essential to have methods that allow large concentration factors to achieve acceptable LODs. For this reason, among the formats of SPE that allow the treatment of large sample volumes, there are examples such as SBSE, although this approach shows problems due to the solid-phase can be detached easily from the solid support or usually requires high extraction times to achieve a quantitative sorption of the analyte.

Another extraction format that has been used previously is SCSE. The SCSE solves some of the main limitations of SBSE providing a greater contact area between sorbent and sample, it also avoids the direct contact of sorbent with the walls of the container, where the extraction is performed. Therefore, Chapter 13 proposes the development of a modified PTFE disk containing an immobilized MIP. This MIP was prepared using as template ecgonine methyl ester, one of the major metabolites of cocaine. This disk incorporates two magnets that allow a vertical rotational agitation against the horizontal agitation of traditional SCSE, allowing more efficient and faster extraction. In addition, the developed disk was able to obtain preconcentration factors up to 40 times, which allows the most reliable determination of the analyte due to the high sample volumes. This work presents the results obtained during the development of the extraction method as well as for the analysis of various water samples collected from various points near the city

of València that include natural water and wastewater treatment plant. Analytical techniques evaluated for this analysis have been IMS as fast technique to detect positive or negative samples, while UHPLC-MS/MS is used as high-sensitivity technique for quantitative determination of ecgonine methyl ester.

Although this Doctoral Thesis is focused on the development of new formats and materials, mainly of polymeric nature, applied to drug analysis, it must be highlighted that there are more selective materials that provide good future prospects. In this sense, aptamers have important advantages over antibodies and they have a long way to do in terms of researching and their subsequent application in SPE.

Selective aptamers against fluoroquinolones [Reinemann et al. 2016] and methamphetamine [Zargar et al. 2018] have been selected. They were previously described in other research articles. Thus, they were selected for their immobilization onto CNBr-activated sepharose as well as on magnetic particles. Chapter 14 shows preliminary results obtained after immobilization of these aptamers onto proposed solid supports. The main purpose of this study is to show the application of selected aptamers immobilized onto sepharose and magnetic particles for the analysis of fluoroquinolones and methamphetamine in biological samples, comparing conventional SPE and MDSPE formats. However, the recoveries obtained are very low, indicating a lack of interaction between the analytes and aptamers. For this reason, it is necessary to follow on the research of these materials in order to look for the possible causes of the lack of affinity that they show towards the analytes and to study the possible solutions for a future application of this innovative type of material.

METODOLOGIA I RESULTATS

METHODOLOGY AND RESULTS

Bloc 1. Rercerca bibliogràfica

Section 1. Bibliographic research

Capítol 1 */ Chapter 1. Trace analysis by ion mobility spectrometry: from conventional to smart sample preconcentration methods. A review.*

Analytica Chimica Acta, 1026 (2018) 37-50

Capítol 2 */ Chapter 2. Smart material for forensic analysis.*

Handbook of Smart Materials in Analytical Chemistry, ISBN: 9781119422594

Trace analysis by ion mobility spectrometry: from conventional to smart sample preconcentration methods. A review

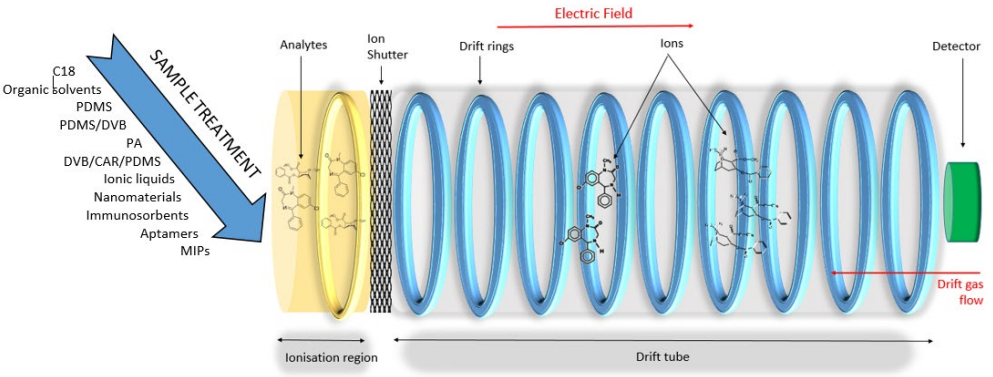
A. Sorribes-Soriano, M. de la Guardia, F.A. Esteve-Turrillas, S. Armenta

Analytical Chemistry Department, University of Valencia, 50th Dr. Moliner St., 46100 Burjassot, Spain

Abstract

Ion mobility spectrometry (IMS) is a rapid and high sensitive technique widely used in security and forensic areas. However, a lack of selectivity is usually observed in the analysis of complex samples due to the scarce resolution of the technique. The literature concerning the use of conventional and novel smart materials in the pretreatment and preconcentration of samples previous to IMS determinations has been critically reviewed. The most relevant strategies to enhance selectivity and sensitivity of IMS determinations have been widely discussed, based in the use of smart materials, as immunosorbents, aptamers, molecularly imprinted polymers (MIPs), ionic liquids (ILs) and nanomaterial. The observed trend is focused on the development of IMS analytical methods in combination of selective sample treatments in order to achieve quick, reliable, sensitive, and selective methods for the analysis of complex samples such as biological fluids, food, or environmental samples.

Graphical abstract



Keywords: *ion mobility spectrometry; sample preconcentration; analyte extraction; immunosorbents; aptamers; molecularly imprinted polymers; ionic liquids; nanomaterial*

1. Ion mobility spectrometry

Ion mobility spectrometry (IMS) is an analytical technique based on the gas phase separation of ionized analytes under a weak electric field at atmospheric pressure. Ion velocities (v_d) are directly related to the intensity of the applied electric field (ε) and the mobility constant (K) of target species through equation 1.

$$v_d = K\,\varepsilon \qquad (1)$$

Mobility constant (K) is characteristic of each analyte and is related to the mass, charge, size and shape of the ionized compounds. K is expressed in units of $cm^2\ V^{-1}\ s^{-1}$, and it is affected by properties of the drift gas, temperature and pressure through equation 2.

$$K = \frac{3e(2\pi)^{\frac{1}{2}}(1+\alpha)}{16N(\mu k T_{eff})^{\frac{1}{2}}\Omega_D(T_{eff})} \qquad (2)$$

where q is the charge on the ion, N is the number density of the drift gas, μ is the reduced mass of the analyte, k is Boltzmann's constant, T_{eff} is the effective temperature, and Ω_D is the average ion-neutral collisional cross section. Usually, K values are normalized to allow the comparison between data obtained by using different instruments, through the reduced mobility constant (K_0) as shown equation 3.

$$K_0 = K\frac{273}{T}\frac{P}{760} \qquad (3)$$

The science of ion formation after electric discharges in ambient air in the presence of different gases has been known since the end of the 19th century. This period has been named by some authors as the discover and innovation period (1895-1960) [1,2,3]. In the beginning of this period, P. Langevin deeply studied the motion of ions under an electric field [4,5]. It should be also highlighted the development of ion gates for the injection of ion pulses inside the drift tube. Ion gates with parallel wires was firstly described by Cravath [6] and van de Graaff [7] in 1929, and later improved in 1936 by Bradbury [8]. This period ends with the studies of Lovelock in 1948, who described a simple detector based on ionization to detect industrial organic vapour traces and the development of appropriate drift tubes using electrically isolated rings to obtain a linear electric field [9,10].

The second period of IMS evolution summarizes the development of the ion mobility spectrometry as analytical technique for the measurement of chemicals (1960-1990). In 1970, Cohen named the technique as plasma chromatography [11], and during this decade, Cohen and Karasek developed a huge number of

applications of the technique for the determination of organic compounds [12,13,14]. After the initial surge of work conducted utilizing IMS, it suffered a decline of interest in the late 1970s and early 1980s. This decline was attributed to a broad disenchantment from unmet expectations and misunderstanding of response characteristics [15]. However, a new cycle of interest began in the mid-1980s which has resulted in advances in all aspects of IMS including improvements in ionization methods, drift tube technologies, and the use of reactant ions to increase specificity [16].

Over the past decades, IMS has evolved into a reduced cost and powerful analytical technique for the detection of volatile and semi-volatile organic compounds at ambient pressures. Figure 32a shows the growth trend in the number of published papers between 1967 and September 2017, using data from the Scopus database of Elsevier B.V., regarding “ion mobility spectrometry” and “plasma chromatography”. As it can be seen, data indicates a clear change in the rate of IMS literature production from the beginning of the XXI century. Thus, the average rate of 11 papers per year from 1967 to 1999 moved to an average rate of 140 published papers per year from 2000 till today, being published in 2016 more than 250 papers. Figure 32b shows the main journals in which articles devoted to IMS were published during this period. Most of them are journals included in the Analytical Chemistry area, such as Analytical Chemistry, Analytica Chimica Acta, Talanta, Analyst, Journal of Chromatography A, and Analytical and Bioanalytical Chemistry. The rest of the journals are specialized in ion mobility spectrometry, as International Journal for Ion Mobility Spectrometry, or mass spectrometry, as Journal of the American Society for Mass Spectrometry, International Journal of Mass Spectrometry, and Rapid Communications in Mass Spectrometry.

The most employed IMS acquisition modes are; i) traditional/conventional drift time IMS (DTIMS), in which ions travel along a uniform electric field tube filled with a drift gas; ii) traveling wave IMS (TWIMS), a stacked-ring ion guide to which a travelling voltage wave is applied that is always hyphenated with a mass spectrometer; and iii) field asymmetric waveform IMS (FAIMS), which takes advantage of the differences in the mobility of ions in high electric fields. Nevertheless, other IMS modes, such as trapped IMS (TIMS), open loop IMS (OLIMS), transversal modulation IMS (TMIMS), and overtone mobility spectrometry (OMS) have been also reported. Additional details on the aforementioned IMS modes can be found in several review papers [17,18,19] and are far beyond the scope of this paper.

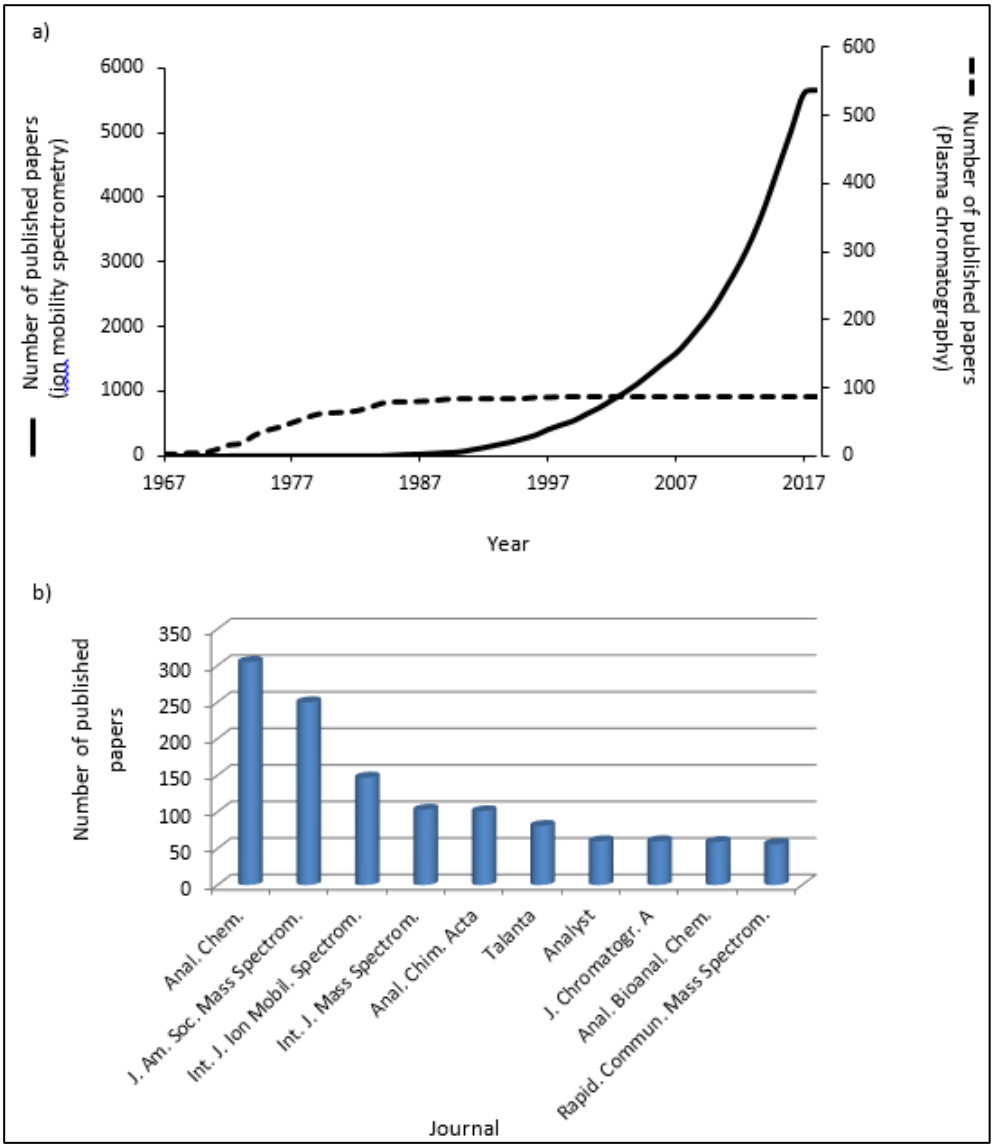


Figure 32. *Evolution of the literature on both, IMS and plasma chromatography, as a function of time (a), and regarding the journals in which papers were published (b).*

As it has been recently reviewed [17,20], a wide variety of devices can be used to introduce gas, liquid and/or solid samples into IMS instruments. The sample introduction system can be considered as a central component of an IMS due to the necessity to transform analytes present in liquid or solid samples into a gas phase in a precise and reproducible way. Permeation tubes, membrane-inlet systems, purge vessels and dilution glass flasks, headspace samplers, evaporation and thermal desorption units together with, solid phase micro-extraction (SPME)

and stir-bar sorptive extractors (SBSE) are the most commonly used approaches nowadays to improve analyte introduction in IMS, due to their efficacy, low cost and easy operation.

2. Trace analysis by ion mobility spectrometry

IMS was initially used by defence agencies for the analysis of explosives, illicit drugs, and chemical warfare agents with dedicated commercially available equipment. However, the analytical potential of IMS, particularly as regards operational speed and sensitivity, has extended its scope to other areas like pharmaceutical [21], food and feed [22,23], clinical [18], polymer and petrochemical industries [24], and environmental analysis [25,26].

Unfortunately, IMS presents some serious weaknesses, such as non-linear response, limited selectivity, and potential interactions of the reactant ions with sample components. Therefore, different situations occur in the analysis of trace compounds in complex samples by IMS because: i) individual components can be undetected, ii) false positive results can be generated by interferents, and iii) competitive ionization of preferentially ionized compounds can hinder detection of the analytes. Ionization in IMS depends on several factors that can change from run-to-run, including the temporal nature of various ion species in their competition, and collision driven atmospheric-pressure ionization process. Preferential ionization of non-targeted substances can produce severe interferences in trace detection capabilities via analyte masking. The distribution of ions in pure or mixed systems is governed by the proton affinity, ionization potential, electronegativity, and concentration of analytes, but also by further experimental factors, including drift tube temperature and humidity of the reactant ion source region. Thus, signal variability can be as high as 25 % in terms of relative standard deviation (RSD) of the obtained peak areas [27,28]. In addition, matrix effects during ionization can also bias the IMS quantitative results [29]. Moreover, others frequently found problems are associated to the relatively easiness of overloading of IMS instruments due to the limited number of available reactant ions, making the reaction chemistry of IMS unpredictable as the reservoir of charge could be depleted for high sample amounts [30]. IMS saturates, depending on the compound and the operating conditions, at low amounts ranging from few to hundreds of nanograms. Moreover, the excess of sample (non-ionized compounds) can result in the contamination of the instrument, resulting in memory effects and the impossibility to a fast recovery of the background level. In summary, due to the

very low concentrations involved in trace analysis, a sample pre-treatment step such as purification, separation, and enrichment of target analytes is usually required for the IMS analysis of complex samples.

One way of addressing the aforementioned limitations is to separate sample molecules using chromatography techniques before IMS analysis. IMS was first coupled to a GC in 1972 and, nowadays it has become a widely used environmental sensing instrument for volatile organic compounds screening [31]. Multi-capillary columns (MCC) have been coupled to IMS offering high flow rates as compared to normal GC columns and appropriate separation efficiencies [32]. In this sense, it should be commented that MCC typically operates at 30-80 ˚C and potentially high flow rates, being able to separate volatile organic compounds in few minutes. For this type of compounds, a preseparation method, such as MCC, could be enough for the determination of volatile organic compounds at ppb levels without any preconcentration step [33]. On the other hand, for the analysis of semi-volatile organic compounds at trace levels, the oven temperature of the GC should be increased to 250-270 ˚C, increasing at the same time the separation time till 15-20 minutes. This fact makes the use of a preconcentration procedure to be an interesting and highly recommendable alternative. Liquid chromatography (LC) [34,35] and supercritical fluid chromatography (SFC) [36] have been also employed as separation techniques coupled to IMS to avoid the inherent limitations of the technique.

On the other hand, IMS has also been coupled, as a previous separation device, to mass spectrometers (MS) [37,38]. Hyphenated methods have improved the ionization and separation of sample components in a mixture, increasing the overall selectivity of the method. However, these methods also increase the analysis time, the system complexity, and the cost of the instrumentation.

The use of chemometrics to improve the quantitative performance of IMS has been also widely explored. Several papers have demonstrated the use of various multivariate methods to extract either, qualitative or quantitative information, from IMS raw data [39,40]. Many chemometric techniques, including peak deconvolution techniques and multivariate calibration, have been applied to IMS spectra. Mixture analysis methods include simple to use interactive self-modelling mixture analysis (SIMPLISMA) and its recursive version (RSIMPLISMA) [41,42], as well as multivariate curve resolution (MCR) with alternating least squares (ALS) [43,44], stationary wavelet transform [45], and calibration methods include Partial Least Squares (PLS) regression, its modifications such as non-linear PLS [46,47] and

artificial neural networks (ANN) [48]. The uses of chemometrics, recent advances, and future prospects for the treatment of IMS data have recently been reviewed [49].

Another strategy to increase the selectivity of IMS measurements is the introduction of a dopant gas in the ionization chamber. The use of dopants provides several advantages, such as: i) remove the background interferences; ii) concentrate the charge reservoir in only one reactant ion, and iii) simplify the interpretation of the IMS spectrum. The increase in selectivity is based on the ionization of molecules with a proton affinity (in positive mode) or electronegativity (in negative mode) higher than that of the dopant gas. If sample interferents have proton affinities lower than that of the dopant gas (electronegativities in negative mode) would not be ionized and the interferences would be removed. The signal of those dopant molecules or reagent gases is also used to correct for small variations of pressure and temperature [50].

Moreover, the introduction of dopant gases could improve the separation of partially or completely overlapped peaks by selective reactions, thus, increasing the selectivity and/or sensitivity of IMS measurements. In those cases, dopant gases are named derivatization reagents. The use of derivatization reagents increases the analyte molecular volume and consequently, reduces its mobility, avoiding potential interferences by drift time [51,52]. The main advantages of this approach are those related to its simplicity and the potential portability for field measurements, using simple and fast *in situ* derivatization, which avoids tedious sample pre-treatment steps.

3. Conventional sample treatments

The most used strategies to avoid the inherent selectivity problems of IMS continues to be a conventional sample treatment, such as liquid phase microextraction (LPME), solid-phase extraction (SPE), solid-phase microextraction (SPME), and stir bar sorptive extraction (SBSE). These conventional strategies have been recently reviewed for the analysis of water [53].

Dispersive liquid-liquid microextraction (DLLME) has been previously used in IMS for the determination of pesticide residues [54,55], food contaminants [56], and mercury analysis [57] due to its advantages, such as reduced solvent consumption, low cost, short extraction times, lack of memory effects, and high enrichment ratios. DLLME employs the dispersion of a nonpolar solvent, called

extraction solvent, with a polar dispersive solvent, into an aqueous sample to ensure the efficient dispersal of the nonpolar solvent and to increase the extraction efficacy. The phase separation is performed by centrifugation, and the sedimentary phase is analysed by IMS. Several variants of this technique have been successfully developed such as the LPME for the analysis of abuse drugs in saliva [58,59], and hollow fibre liquid phase microextraction (HFLPME) for the analysis of pesticide residues in vegetables [60], artemisinin in plants [61], and therapeutic drugs in urine and plasma [62,63,64]. However, these methods are difficult to be automated and typically are not suitable for on-site rapid detection.

The use of SPE with conventional sorbents is a reliable approach to increase the sensitivity and selectivity of IMS determinations. However, SPE operational conditions must be studied carefully, such as the volume and type of solvent employed to wash and elute the target analytes from the sorbent and to avoid matrix interferences in the IMS analysis. The traditional octadecyl-silica adsorbent (C18) has been employed for the pre-concentration of traces of DDT and its metabolites from water samples [65], and cocaine from urine samples [66]. Conventional SPE cartridges, including C18, mixed mode, graphitized carbon, and HILIC sorbents, have been used for the extraction of endogenous metabolites and xenobiotics from biofluids and their later analysis by IMS-MS [67]. Mixed-mode cationic/reversed-phase sorbent has been successfully employed for the SPE of cocaine and ecgonine methyl ester from oral fluids in a lab-on-valve miniaturized system [68].

SPME is a non-solvent technique which integrates the sampling, extraction, enrichment, and injection in a single step, providing a high enrichment factor in a simple and environmentally friendly device. SPME has been thoroughly used in combination with IMS since the first precedent in 1997 [69]. SPME direct injection mode coupled to IMS has been used for the rapid determination of dichlorvos traces in tea drinks [70], chemical warfare agents and simulants in water [71], ephedrine in urine [72], parabens in pharmaceutical formulations [73], methyl tert-butyl ether in water [74], and BTEX, naphthalene, chlorinated alkenes and chlorinated benzenes in water [75], using classical sorbents, such as polydimethylsiloxane (PDMS), polydimethylsiloxane/divinylbenzene (PDMS/DVB), polyacrylate (PA), and DVB/Carboxen/PDMS (DVB/CAR/PDMS). Head space-SPME analysis of solid and liquid samples has been also used for the detection of piperonal [76], odour signatures of smokeless powders [77], volatile chemical markers of explosives [78,79], precursor and degradation products of chemical warfare agents [80], drugs like cocaine, MDMA and marijuana [81,82],

chlorophenols in water [83], and *Listeria monocytogenes* using exogenous volatile organic compound metabolites [84]. A planar SPME device has been also successfully coupled to IMS for the determination of illicit drugs and explosives [85,86,87]. Moreover, a prototype of SPME–IMS system has been developed for an effective combination of the extraction efficiency of SPME with the detection capability of hand held IMS systems [88].

Other extraction formats, such as SBSE and microextraction in packed syringe (MEPS), have been also used in combination with IMS for the analysis of trace levels of organic compounds. In this sense, an appropriate injection port was designed for the direct analysis of PDMS stir bars using IMS [89]. The capabilities of the injection port design were demonstrated through the determination of diazinon residues in agricultural wastewater, well water, and apple samples, offering great simplicity and high sensitivity. A MEPS-based method coupled to negative electrospray ionization-IMS was developed for the determination of 2,4-D, silvex, and haloxyfop herbicides in water [90]. After optimization of the pH, sample loading rate, nature and volume of elution solvent, and number of extraction cycles, the method provided appropriate sensitivity, recovery, reproducibility and enrichment factor values.

In spite of the extensive use of conventional sorbents for the extraction of target analytes from complex samples, it has been demonstrated that the achieved selectivity was not enough to avoid the inherent problems of IMS related to the competitive and collision driven atmospheric-pressure ionization processes. Thus, the preferential ionization of non-targeted substances, which can be found in samples at concentrations ten, hundred, or even thousand times higher than that of the analyte, may produce severe interferences in IMS determinations.

4. Smart sample treatment

Smart materials can be defined in a strict way as materials that present the ability to respond to an external stimulus in a specific way, this response being in a controlled manner. We extend this definition to tailored, task-specific, or designed materials suitable to incorporate unexpected advantages from the selectivity, sensibility or practical aspects of analytical determination. We have focussed our attention on compiling the literature published regarding the use of smart materials in the sample treatment of samples previous to IMS determinations. The main aim is not to make an exhaustive list of all the published papers in the topic, but to provide a critical and comprehensive description of the most relevant strategies, highlighting its novelties, importance, and relative merits. Figure 33 shows the number of published papers regarding IMS and the different smart preconcentration procedures.

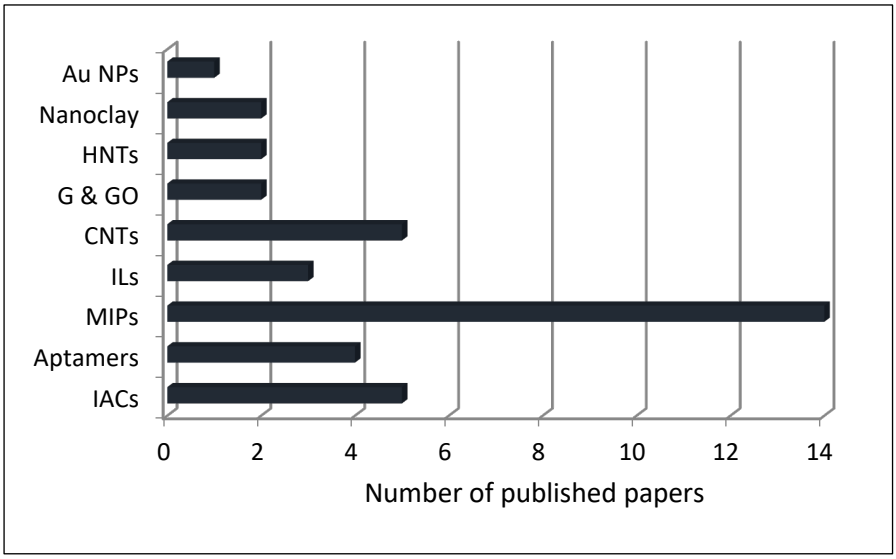


Figure 33. *Number of published papers dealing with IMS and different smart preconcentration procedures.*

4.1. Immunosorbents

An immunosorbent, also called immunoaffinity sorbent or immunoaffinity chromatography (IACs), is produced by immobilization of specific antibodies on a solid-support. The use of immunosorbents for the pretreatment of samples significantly improves selectivity of IMS methods because of the high specificity of antibody-antigen interactions. Immunosorbents are mainly employed using SPE approaches that allow the extraction, concentration, and clean-up of target analyte from complex matrices in a single step. Relatively large volumes of sample can be

loaded, which provides high enrichments factors. Additionally, specificity of the immunosorbent depends of that of the antibody and it can be tuneable to interact with a single analyte or to a family of compounds with similar chemical structure.

The extreme specificity provided by immunosorbents coupled to the high sensitivity of IMS measurements provides selective and sensitive methods for the determination of multiresidues in complex samples. A variety of immunosorbents are commercially available for several compound families such as: mycotoxins (aflatoxin, ochratoxin, zearalenone, fumonisin,…), β-agonists (clenbuterol, salbutamol,…), corticosteroids, stilbenes, growth promoters (zeranol, ractopamine,…), phenylurea herbicides, polycyclic aromatic hydrocarbons, and among others. Using commercial immunosorbents, the capability of IMS, for quantitative determinations of aflatoxins B1 and B2 in pistachio samples [91] and ochratoxin-A in licorice root [92], has been demonstrated. These approaches provided acceptable detection limits, good accuracies and appropriate recovery levels together with fast response, simplicity and portability of the IMS devices. Extraction of aflatoxins from pistachio was performed according to the AOAC method [32]. Briefly, ground pistachio samples were mixed with 2.5 g sodium chloride, 200 mL of methanol in water 80% (v/v), 100 mL of n-hexane. After purification of the extract, 5 µL of this solution was introduced into the injection port of the IMS. On the other hand, ground licorice root powder was added to a mixture of 30 mL sodium bicarbonate 0.1 M and methanol (9:1, v/v) which was then homogenized in an Ultrat-turrax homogenizer for 2 min. The extract containing ochratoxin was centrifuged and purified.

Alternatively, immunosorbents can be easily prepared from commercial antibodies and appropriate supports. Traditional supports are based on i) polysaccharides like agarose, sepharose, and cellulose; ii) polymers based in acrylamide, polymethacrylate derivatives, and polyethersulfone; and iii) alternatively, disks, fibers, and monolithic rods [93]. Following the aforementioned strategy, immunosorbents have been prepared with laboratory produced antibodies immobilized on activated sepharose beads for the SPE of three strobilurin fungicides in water and strawberry juice, the selective retention in stacked IAC columns of anilinopyrimidine fungicides in wine, and the separation of *Z* and *E* isomers of azoxystrobin fungicide [94]. The specificity of IAC columns coupled to the high sensitivity of IMS measurements made this combination really useful for rapid, selective, and sensitive determination of a variety of analytes in different samples.

Immunosorbent-based SPE in combination to DLLME has been also employed for the determination of chloramphenicol in water, milk, honey, and urine samples [95]. In this approach, the obtained limit of quantification was set at 0.1 μg L^{-1} which was lower than the minimum required performance limit established by the EU.

The main drawbacks of immunosorbents are those related to the limited range of commercially available sorbents, the high cost of antibody production, and the complex strategies for the production of selective antibodies against small molecules. Regarding the high cost of antibodies, the use of magnetic nanoparticles (MNPs) allows a considerably reduction in the antibody consumption per sample. In this sense, magnetic immunosorbents have been produced from specific monoclonal antibodies against anatoxin-a, characterized by microscopy and ATR-FTIR spectroscopy, and employed for the dispersive magnetic immunoaffinity (d-MagIA) extraction of anatoxin-a from water and IMS determination [96]. Limits of detection and quantification were set at 0.02 and 0.08 μg L^{-1}, respectively, with RSD values lower than 15 %.

A summary of the details and analytical features of the published papers regarding the immunosorbent-based SPE coupled to IMS determinations can be found in Table 7.

Table 7. *Application studies of ion mobility spectrometry using immunosorbents in the sample treatment step*

Extraction mode	Analyte	Sample	Ionization mode	Linear range (μg L^{-1})	LOD (μg L^{-1})	Recovery (%)	RSD (%)	Preconcentration time (min)	Ref
SPE	Aflatoxins B1 and B2	Pistacchio	CD	2-70 (ng)	0.25 ng	-	<10	-	[91]
	Ochratoxin A	Licorice root	CD	0.01-1 ng	0.01 ng	109-111	1 (n=3)	5	[92]
	Strobilurin fungicides	Water and strawberry juice	^{63}NI	50-500	3 -19	96-106 (water) 67-104 (strawberry)	2.6-4.6 (n=5)	30	[94]
	Anilinopyrimidine fungicides	Wine	^{63}Ni	10-200	3-5	82-123	3.0-5.1 (n=5)	30	[94]
	Chloramphenicol	Food and urine	^{63}Ni	1.3-80	0.4	88-116	2.1	10	[95]
mDSPE	Anatoxin-a	Water	^{63}Ni	20-150	0.02	91-115	15	15	[96]

Note: CD, corona discharge; LOD, limit of detection; mDSPE, magnetic dispersive solid phase extraction; RSD, relative standard deviation; SPE, solid phase extraction;

4.2. Aptamers

Aptamers are short single-stranded oligonucleotides that exhibit molecular recognition. The word aptamer derives from the Latin word "*aptus*", that means "to fit" [97]. The use of aptamers as tools in analytical chemistry has considerably increased due to the development of the in vitro iterative selection process called "systematic evolution of ligands by exponential enrichment" (SELEX) [98]. Aptamer based sorbents are one of the most appropriate candidates for selective separation of an analyte from a complex matrix. In comparison with antibodies, aptamers offer several advantages; such as the possibility to introduce chemical modifications, during their synthesis, to improve their stability, detection, or immobilization [99]. Aptamers have provided excellent selectivity and recovery factors and they are much cheaper than antibodies.

The combination of aptamer-based SPE with IMS has been developed for the analysis of tetracycline by IMS in human plasma and urine [100]. Anti-tetracycline aptamers were immobilized onto sepharose beads and packed into 1 mL SPE cartridges. Detection limits of 0.019 and 0.037 μg mL^{-1} were obtained with an extraction efficiency of 86 and 83 % for urine and plasma, respectively.

Other formats of aptamer-based extraction techniques have been reported in the literature. Aptamers have been immobilized into aldehyde-modified cellulose paper to perform thin-film microextraction (TFME) of codeine from urine samples and its analysis by IMS [101]. A similar approach was used for the determination of codeine and acetamiprid by IMS [102]. In this method, the modified paper is not only used for the selective extraction of the target analyte by immobilization of an aptamer, but also as an ionization source (paper spray ionization). MNPs have been conjugated with aptamers for the selective extraction of adenosine in urine samples followed by electrospray ionization–IMS [103]. The method provided a detection limit of 0.02 μg mL^{-1} with an extraction efficiency of 94 % and RSD of 4 % for the determination of adenosine at 0.25 μg mL^{-1} in urine. In summary, aptamers have been also employed as an effective strategy to improve the selectivity of the extraction methods and to compensate its weakness in IMS determinations.

Main details and analytical features of the published papers regarding methodologies that use aptamers in combination to IMS can be found in Table 8.

Table 8. *Applications of aptamer-based sample treatment coupled to ion mobility spectrometry.*

Extraction mode	Analyte	Sample	Ionization mode	Linear range (mg L^{-1})	LOD (mg L^{-1})	Recovery (%)	RSD (%)	Preconcentration time (min)	Ref
SPE	Tetracycline	Urine and human plasma	ESI	0.05-5.00 (urine) 0.10-5.00 (plasma)	19 (urine) 37 (plasma)	86.5 (urine) 82.8 (plasma)	5.9 (urine) 6.3 (plasma) (n=6)	-	[100]
TFME	Codeine	Urine	ESI	0.01-0.3	3.4	90.1	6.8 (n=3)	45	[101]
	Codeine	Urine	PSI	0.01-0.5	3.7	87.3-90.7	2.1 (n=3)	35	[102]
	Acetamiprid	Urine	PSI	0.005-0.3	1.8	86.5-101.7	5.6 (n=3)	35	[102]
mDSPE	Adenosine	Urine	ESI	0.05-5	0.02	94	4 (n=3)	45	[103]

Note: ESI, electrospray ionization; LOD, limit of detection; mDSPE, magnetic dispersive solid phase extraction; PSI, paper spray ionization; RSD, relative standard deviation; SPE, solid phase extraction; TFME, thin film microextraction

4.3. Molecularly imprinted polymers

Molecular imprinting technique was first proposed by Wulff and Sarhan to obtain molecularly imprinted polymers (MIPs) that were able of capturing target molecules [104]. Molecularly imprinting is a process by which selected functional monomers are self-assembled around a template molecule, and then, polymerized in the presence of a cross-linker. After removing the template molecule from the MIP, a cavity complementary in shape and chemical properties is generated in the polymer structure, and becomes available to bind specifically template molecules [105]. Different approaches have been used for MIP synthesis including: i) the formation of reversible covalent bonds between the monomers and the template; ii) non-covalent interactions such as ionic, hydrophobic, or hydrogen-bond interactions; and iii) the semi-covalent method which involves both processes abovementioned [106]. Several polymerization techniques have been used for the production of MIPs including bulk, precipitation, suspension, and emulsion polymerization [107]. The high versatility, chemical and physical stability, and molecular recognition capabilities made MIPs a perfect candidate for the use in combination with IMS determinations.

Commercial available MIPs cartridges for clenbuterol and chloramphenicol, obtained from Supelco (Bellefonte, PA, USA), were used for the determination of clenbuterol in water and urine samples [108], and chloramphenicol in water, milk, honey and urine samples [95].

Non-covalent synthesis of MIPs produced with methacrylic acid (MAA) as functional monomer, 2,2'-azobis-(2-isobutyronitrile) (AIBN) as initiating agent, ethylene glycol dimethacrylate (EDMA) as cross-linker, and dimethylformamide or acetonitrile as porogen, has been employed for the MIP-based SPE and analysis by IMS. Several applications have been developed such as the analysis of pioglitazone from cow plasma [109], phenazopyridine [110], metronidazole [111] and primidone [112] in pharmaceutical and serum samples, testosterone in human urine [113], caffeine and theophylline in tea and plasma [114], and cocaine in saliva [115]. For the analysis of solid pharmaceutical samples, tablets were finely powdered using a mortar, weighed and extracted with methanol:water (90:10) for 15 min in a an ultrasonic bath, filtered and analysed by IMS.

Using acrylic amide (AM), EDMA, azobisisoheptonitrile (ABVN), and acetonitrile a selective MIP was obtained by precipitation polymerization for the determination of nitrobenzene compounds by IMS [116], being found a detection limit of 0.1 mg L^{-1} for 2,4,6-trinitrotoluene. The MIP-SPE-IMS system provided similar

selectivity for trinitrotoluene and 2,4-dinitrotoluene and it was used for the analysis of industrial waste water and surface water. In another example, the authors used 4-vinylpyridine (4-VP), EDMA, AIBN, and acetonitrile for the synthesis of a salicylic acid selective MIP for its determination by IMS in human urine and blood plasma [117].

Molecularly imprinted fibres have been also prepared for SPME using MAA, EDMA, AIBN, and acetonitrile as polymerization mixture. Subsequently, 1 mL of the mixture was transferred into a small glass tube and both capillary ends were closed with two small pieces of soft rubber. Then, the filled capillaries were introduced in an oven and polymerization took place at 60 °C for 14 h. The polymeric monolith fibre was immersed in a 40 % hydrofluoric acid solution for 2 h to dissolve the glass capillary. Using these imprinted fibers, thidiazuron was determined by IMS in fruit and vegetable samples [118] with a limit of detection of 5 ng L^{-1} and intra- and inter-day precisions of 0.9 and 2 %, respectively.

Electrochemically controlled MIP-SPME of ibuprofen, based on nanostructure conducting molecular imprinted polypyrrole (Ppy), was synthetized for its analysis in biological and pharmaceutical samples using IMS [119]. For imprinted electropolymerization, an aqueous solution containing pyrrole and ibuprofen was prepared, obtaining the conducting MIP by electrodeposition on the surface of the platinum electrode. After that, for the formation of complementary cavities, the prepared Ppy-ibuprofen film was overoxidized in NaCl solution by application of a constant potential of +1.1 V for 10 min. Then a negative potential of −0.6 V was applied under stirring until the total release of accumulated drug. A similar approach was used for the determination of non-steroidal anti-inflammatory drugs using naproxen as template for the preparation of the conducting MIP [120].

MIPs compared to immunosorbents possess high physical robustness, strength, temperature and pressure resistance, and chemical inertness towards acids, bases, metal ions, and organic solvents. Regarding acquisition costs, MIPs have a low cost as compared to monoclonal antibodies employed in the production of immunosorbents, which require several phases like hapten design and synthesis, immunization of animals, production and clonation of hybridoma, and large scale production. Other advantage of MIPs is that they usually present a higher binding capacity than immunosorbents due to their enhanced number of active sites. Main characteristics and analytical features of the papers regarding IMS determination after a MIP-based sample teratment can be found in Table 9.

4.4. Ionic liquids

An accepted definition of ionic liquids (ILs) concerns a salt with a melting temperature below 100°C. If the melting temperature is close or below room temperature, they are named room-temperature ionic liquids (RTILs). In most cases, ILs are composed of an organic cation and an organic or inorganic anion. The main advantages of ILs are related to their high thermal stability, negligible vapour pressure, and non-flammability, in addition to varying viscosity, conductivity, and miscibility with different solvents. These characteristics can also be finely tuned to meet specific requirements by imparting different functional groups and/or varying the combinations of cations and anions in the ILs [121]. Aside from the aforementioned properties, ILs can also be engineered to exhibit exceptional selectivity toward specific groups of compounds, enabling the preconcentration of analytes in complex samples.

The use of ILs in single drop microextraction (SDME) approaches has been used for the extraction and preconcentration of 2,4,6-trichloroanisole in wines [122,123]. These papers propose a simple, cheap, fast, and sensitive method for the determination of 2,4,6-trichloroanisole by IMS using a 1-hexyl-3-methylimidazolium bis(trifluoromethylsulfonyl)-imide-based MCC column, to improve both, sensitivity and selectivity, of measurements. The good affinity of this IL to the haloanisole facilitates its preconcentration by means of headspace-single drop microextraction (HS-SDME) techniques. The combination of IL-HS-SDME and room-temperature gas chromatography (RTGC) has been also proposed to increase the selectivity of extraction procedures, improving IMS analysis [124]. The arrangement was evaluated using the determination of different halocompounds in water as model analytical problem. Table 10 summarizes the main analytical features of the papers devoted to IL-based extraction methods and IMS determinations.

Table 9. *Published papers regarding ion mobility spectrometry using molecular imprinted polymers in the sample treatment step.*

Extraction mode	Analyte	Sample	Ionization mode	Linear range (μg L^{-1})	LOD (μg L^{-1})	Recovery (%)	RSD (%)	Preconcentration time (min)	Ref
SPE	Clenbuterol	Urine	^{63}Ni	5-40	2	82-99	4.5 (n=5)	-	[108]
	Chloramphenicol	Food and urine	^{63}Ni	19-125	0.6	90-124	2.3 (n=5)	12	[95]
	Pioglitazone	Cow plasma	ESI	100-20000	20	91	6 (n=3)	50	[109]
	Phenazopyridine	Pharmaceutical and serum	ESI	1000-100000	0.2	91-110	-	20	[110]
	Metronidazole	Pharmaceutical and serum	CD	50-70000	4.4	89	6 (n=4)	120	[111]
	Primidone	Pharmaceutical and serum	ESI	20-2000	5.1	>90	2.4 (n=4)	40	[112]
	Testosterone	Urine	CD	10000-250000	0.9	97-109	10 (n=3)	15	[113]
	Caffeine and theophylline	Tea and plasma	ESI	100-50000	200-300	78-98	<6 (n=3)	70	[114]
	Cocaine	Saliva	^{63}Ni	60-500	18	81-100	6.1 (n=3)	25	[115]
	TNT	Surface water	^{63}Ni	0.5 - 50	0.1	90-105	6.8 (n=5)	30	[116]
	2,4-DNT	Surface water	^{63}Ni	0.1 - 10	0.05	98-103	5.14 (n=5)	30	[116]
	Salicylic acid	Urine and plasma	ESI	20-2000	8	84-95	<6	50	[117]
SPME	Thidiazuron	Fruit and vegetables	CD	0.01-20	0.005	90	4 (n=3)	30	[118]
	Ibuprofen	Pharmaceutical and serum	CD	5-100 100-1000	2.5	89-128	4.6 (n=5)	15	[119]
	NSAIDs	Human serum	CD	0.1-30	0.07-0.37	-	6 (n=5)	-	[120]

Note: CD, corona; DNT, dinitrotoluene; ESI, electrospray ionization; LOD, limit of detection; NSAIDs, non-steroidal anti-inflammatory drugs; RSD, relative standard deviation; SPE, solid phase extraction; SPME, solid-phase microextraction; TNT, trinitrotoluene.

Table 10. *Published papers regarding ion mobility spectrometry using ionic liquids and nanomaterial in the sample treatment step.*

Smart material	Extraction mode	Analyte	Sample	IMS device	Linear range (μg L^{-1})	LOD (μg L^{-1})	Recovery (%)	RSD (%)	Preconcentration time (min)	Ref
Ionic Liquids	HS-SDME	2,4,6-trichloroanisole	Wine	MCC-IMS	0.05-25 ng L^{-1}	0.01 ng L^{-1}	-	<6	30	[122]
		2,4,6-trichloroanisole	Wine and water	MCC-IMS	1-100 ng L^{-1}	0.66 ng L^{-1}	94-100	2.2 (n=5)	40	[123]
		Halocompounds	Water	RTGC-IMS	0.1-25 0.5-50	0.1-0.9	-	4.1-7.1 (n=5)	30	[124]
MWCNTs	dSPE	Pesticides	Water	PGD-IMS	5-200	0.01-0.8	79-118	-	20	[125]
HLB, C_{18} and MWCNTs	μSPE	Benzene, toluene, butyraldehyde, benzaldehyde, and tolualdehyde	Saliva	GC-IMS	250-2000	200-500	73-100	6-15 (n=4)	15	[126]
Fe_3O_4/Ppy/MWCNTs	mdSPE	Methacarbamol	Plasma	ESI-IMS	2-150	0.9	83	5.3 (n=3)	25	[127]
CNTs@SiO_2	SPME	Organophosphorous	Food and water	GC-CD-IMS	0.01-3.0	0.005-0.02	79-99	4-9 (n=3)	35	[129]
Ppy/GO	SPME	Creatinine	Urine and plasma	FV-IMS	600-500000	600-2600	92-110	-	7	[130]

G/polyaniline	SPME	NSAIDs	Urine and plasma	CD-IMS	0.1-30	0.04-0.05	70-95	12	25	[131]
MWCNTs-COOH/PDMS	SBSE	Triazine herbicides	Water and soil	PGD-IMS	0.05-10	0.006-0.015	86-104	6-9 (n=5)	120	[132]
HNTs-TiO_2	SPME	Parathion	Food and water	CD-IMS	0.1-25	0.03	85-97	6.3 (n=3)	25	[134]
HNTs modified	SPME	Diazinon, parathion and fenthion	Food and water	GC-CD-IMS	0.03-3	0.01-0.03	84-97	7-9 (n=3)	30	[135]
PPy/nanoclay	SPME	Diazinon and fenthion	Food and water	GC-CD-IMS	0.05-10	0.02-0.035	72-98	5-8	30	[138]
Sol-gel/nanoclay	SPME	Diazinon, parathion, fenthion and chlorpyrifos	Water	GC-CD-IMS	0.01-2.0	0.003-0.0012	86-104	6.4-8.4 (n=3)	60	[139]
Au NP–thiol silane film	SPME	Xylene and toluene	Breath	CD-IMS	1-32 16-112	0.07 8	85-95	3-5 (n=3)	15	[141]
		Acetone, acetaldehyde, and acetonitrile	Breath	CD-IMS	0.01-4.0 2300-400000 2.5-76	0.001 180 0.22	75-97	3-5 (n=3)	15	[142]

Note: C_{18}, octadecyl silica; CD, corona discharge; CNTs, carbon nanotubes; dSPE, dispersive solid phase extraction; ESI, electrospray ionization; FV, fast vaporization; G, graphene; GC, gas chromatography; GO, graphene oxide; HLB, hydrophilic-lipophilic balance polymeric reversed-phase sorbent; HD-SDME, head-space single drop microextraction; IMS, ion mobility spectrometry; LOD, limit of detection; MCC, multicapillary chromatography; mSPE, magnetic dispersive solid phase extraction; µSPE, mictro solid phase extraction; MWCNTs, multi-walled carbon nanotubes; NHTs, halloysite nanotubes; NP, nanoparticles; NSAIDs, non-steroidal anti-inflammatory drugs; PDMS, polydimethylsiloxane; PGD, pulsed glow discharge; Ppy, polypyrrole; RSD, relative standard deviation; RTGC, room temperature gas chromatography; SBSE, stir-bar sorptive extraction; SPME, solid-phase microextraction.

4.5. Nanomaterial

Nanomaterial is a material with at least one dimension below 100 nm, although currently the definition of what is "nano" is still under debate. The size of the particles could lead to a misinterpretation when aggregates of nanoparticles present external dimensions well beyond 100 nm, even when they have specific physicochemical properties characteristic of nanomaterial, due to their large specific surface area. So, nanomaterial can be also characterized by specific surface area higher than 60 $m^2 g^{-1}$. Nanomaterial can be a thin film or surface coating, wire, tube rod or particle. The main advantages of these nanoparticles are related to their small size with properties, which are generally not seen in their conventional bulk counterparts, offering exciting possibilities in analytical chemistry.

Carbon nanotubes (CNTs) represent an increasingly important group of nanomaterial with unique geometric, mechanical, electronic, and chemical properties. CNTs can be viewed as hollow cylinders formed by rolling graphite sheets. Since CNTs are derived from fullerenes, they are referred to as tubular fullerenes or bucky tubes. CNTs can be grouped into two main forms: single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs), being a hollow cylinder of a single or multiple graphite sheets, respectively. MWCNTs have been used in IMS determinations as reversed phase dispersive solid-phase extraction (dSPE) materials for the analysis of pesticides in water [125]. Extraction capacity and efficiency are increased because of the high surface area-to-volume ratio offered by these nanosized adsorbents. For pesticide determination in water, 10 mg of MWCNTs was dispersed into 10 mL sample in a centrifuge tube, and the mixture was shaken by vortex for 1 min. After centrifugation, the supernatant was discarded and 200 µL desorption solvent and 400 mg anhydrous Na_2SO_4 were added. The extract was shaken by vortex, filtered, centrifuged, and analysed by IMS. Under optimal pre-treatment conditions, the obtained enrichment factors of pesticides were from 5.4 to 48.7-fold. In a similar approach, a combination of hydrophilic-lipophilic balance polymeric reversed-phase, C_{18}, and MWCNT sorbents, packed inside a piece (3 × 1.5 cm^2) of Parafilm M®, was employed for the determination of benzene, toluene, butyraldehyde, benzaldehyde, and tolualdehyde in saliva samples obtained from smokers and non-smokers [126]. After the extraction step, the parafilm device was dried with paper tissue and directly analysed by HS-GC-IMS. The aforementioned set-up avoids any centrifugation or filtration step, simplifying the treatment procedure.

The use of magnetic-modified adsorbents in SPE procedures has been proposed to overcome the disadvantage of separation or filtration steps in conventional extraction procedures. Magnetic adsorbents can be easily removed from sample solution with the assistance of an external magnetic field, providing a smart, fast, and low cost extraction technique with high analyte enrichment factors. Different conducting polymers, such as Ppy-polyaniline, and their derivatives have been used in separation and extraction approaches because of their versatile properties, as large π-conjugated structure, polar functional groups, and ion exchange characteristics. In this sense, Fe_3O_4-MWCNTs, Fe_3O_4-Ppy and Fe_3O_4-nanoparticles-decorated Ppy/MWCNTs composites were used and compared as smart adsorbents for the pre-concentration of methocarbamol in human plasma prior to ESI-IMS determination [127]. The main advantages of Fe_3O_4-nanoparticles-decorated Ppy/MWCNTs are those related to high surface area, high porosity, and hydrophobicity of MWCNTs combined to the presence of hydrophilic functional groups from Ppy, showing better extraction efficiency than Fe_3O_4-Ppy and Fe_3O_4-MWCNTs for the analysis of polar compounds (log K_{ow} < 3).

Nanomaterial has been also used to modify polymers for enhanced SPME methodologies. CNTs are excellent adsorbent materials to be coated to SPME fibres due to their large surface area (typically > 1000 $m^2\ g^{-1}$), unique tubular structure, and strong π–π interactions [128]. Composites with metal oxides have been developed to enhance the physical and chemical characteristics of CNTs, such as those based on CNT–TiO_2 which provides a high surface area, large pore volume, structural stability, high thermal resistance, and tuneable pore size. CNTs@SiO_2 nanohybrids, fabricated using pristine CNTs, tetraethoxysilane, and glucose in a one-step hydrothermal process, were immobilized on the surface of a stainless-steel wire, using the electrospinning technique and polyvinyl alcohol as polymer, for the SPME extraction of organophosphorus pesticides and GC-IMS determination [129]. *-143-*

A polypyrrole/graphene oxide (Ppy/GO), coated on a platinum wire, was employed for the SPME extraction of creatinine from urine and plasma after derivatization, using a home-made fast vaporization SPME system [130]. Ppy/GO coating has a porous structure, with high specific surface area, and adsorption capacity, and as consequence, a high extraction capacity for the selected analytes was found. The excellent thermal stability of Ppy/GO coating allows the use of high desorption temperatures in the inlet of the IMS system up to 300°C. In a similar approach, an electromembrane-surrounded hexadecyltrimethylammonium bromide-doped graphene/polyaniline composite was coated to the SPME fibre for

the determination of ibuprofen and mefenamic acid in complex matrices by SPME-IMS [131]. The aforementioned home-made graphene/polyaniline composite is characterized by a high electrical conductivity, thermal stability, and surface area.

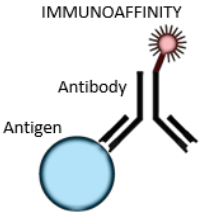
MWCNTs have been employed as coating of a SBSE system for the IMS determination of triazine herbicides in water and soil samples [132]. Powdered soil was mixed with 50 mL of ammonium formate and extracted for 1 h. After centrifugation, the supernatant was filtered and collected for further analysis. The MWCNTs-COOH/PDMS coated stir bars provided an adequate precision with RSD values ranging from 6.3 to 7.8 % in one batch and from 7.4 to 8.9 % among different batches. The approach included the development of an injection port with sealing device and a stir bar hold device, making the simultaneous desorption and detection of analytes.

Other nanoparticles widely employed for the SPME of traces determination by IMS from complex samples included the use of naturally available minerals like halloysite nanotubes (HNTs). HNTs are low cost materials with a $Al_2Si_2O_5(OH)_4 \cdot 2H_2O$ molecular structure and CNT-like morphology. HNTs are tube-shape of 10–15 nanostructure layers with high thermal and mechanical resistances, and high adsorption capacity. Moreover, its inner and outer surface can be easily modified chemically [133]. HNTs have been combined with TiO_2 by a sol–gel technique to chemically bond on a fused–silica SPME fibre, showing a high surface area, highly ordered pores, high durability, low cost, and environmental friendliness [134]. Parathion residues from celery, strawberry, apple, river water, and agricultural wastewater were determined by IMS using the developed HNTs-TiO_2 fibre. Organosilane compounds have been employed to modify both, the outer and inner, HNTs surfaces to increase the specific surface area of SPME fibres and to enhance the extraction capability of the material [135]. The inner diameter of the nanotubes was enhanced by sulfuric acid etching and the outer surface was hydroxylated with sodium hydroxide. After grafting by (3-aminopropyl)triethoxysilane, the modified HNTs were chemically coated on a fused-silica support by sol–gel process. The silanized HNTs fibre was used for the extraction of diazinon, parathion, and fenthion from wastewater and fruit samples.

Montmorillonite is a clay mineral with molecular structure of hydrated sodium calcium aluminium magnesium silicate hydroxide $(Na,Ca)_{0.33}(Al,Mg)_2(Si_4O_{10})(OH)_2 \cdot nH_2O$ with interesting properties such as high porosity, high adsorption capacity, high surface area, swelling behaviour, enhanced mechanical properties, and thermal stability [136]. Generally, montmorillonite is

chemically modified to reduce its polarity, being the most commonly used method a cation-exchange reaction with alkylammonium, phosphonium or imidazolium compounds [137]. A Ppy/montmorillonite nanocomposite has been coated on a Ni–Cr wire and it has been employed for the determination of diazinon and fenthion from cucumber, lettuce, apple, and water by SPME-IMS [138]. The obtained fibres provided a specific surface with high thermal stability and loading capacity, due to their non-smooth porous structure. A sol-gel/montmorillonite nanocomposite prepared by the sol-gel technique on a stainless steel wire was used for the SPME extraction of diazinon, parathion, fenthion, and chlorpyrifos from water samples and GC-IMS determination [139]. Montmorillonite nanoclay was modified by grafting a silica network between silicate layers, using well-controlled sol-gel procedures.

Gold nanoparticles (Au-NPs) have been used in the preparation of smart SPME fibres. Au-NPs possess intrinsic interesting properties such as: easiness in chemical modification, strength, biocompatibility, and chemical stability, among others [140]. Different ways have been used to prepare Au-NP-based functional coatings and films, such as the sol-gel method using 3-(trimethoxysilyl)-1-propanthiol (TPT) for the preparation of stainless steel wire fibres [141]. Precision of Au-NPs-TPT-based fibres was assessed for the analysis of xylene and toluene by SPME–IMS, with an obtained repeatability from 1.8 to 2.3 %, and reproducibility from 2.1 to 2.6 %, established as the RSD of ten successive assays of a single fibre and five assays with different fibres, respectively. A similar approach, using Au-NPs–thiol silane film/SPME fibre coupled to IMS, was used for the analysis of acetone, acetaldehyde, and acetonitrile in exhaled breath [142]. Table 10 summarizes the main analytical features of the literature devoted to the use of nanomaterials in the extraction of target analytes from samples and IMS determination.



ADVANTAGES

- High selectivity

DRAWBACKS

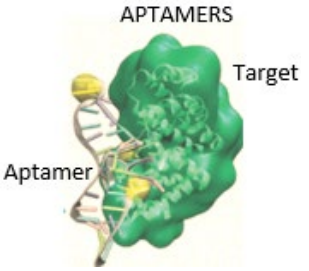
- High cost of antibody production
- Limited availability

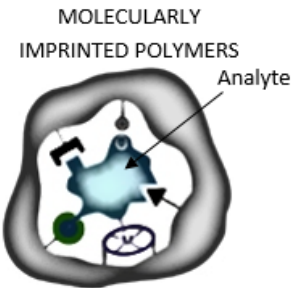
ADVANTAGES

- High selectivity
- Chance to improve their capabilities during the synthesis
- Cheaper than antibodies

DRAWBACKS

- Poor stability





ADVANTAGES

- High physical stability
- Chemical inertness
- Relative low cost
- High binding capacity

DRAWBACKS

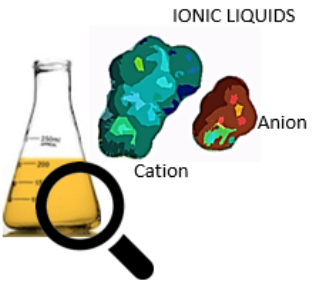
- Difficulty to large-scale production

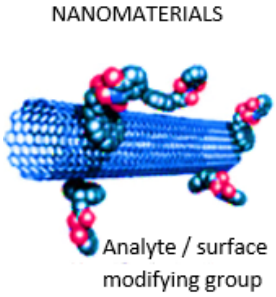
ADVANTAGES

- High thermal stability
- Negligible vapour pressure
- Non-flammability
- Selectivity towards specific groups

DRAWBACKS

- Reduced selectivity





ADVANTAGES

- Good geometric, mechanical, electronic and chemical properties
- High surface area-to volume

DRAWBACKS

- Reduced selectivity

Figure 34. *Main advantages and drawbacks of the main materials employed for sample treatment previous IMS determinations.*

5. Conclusions & future trends

The use of novel smart materials has become a major trend in the development of novel extraction procedures in the analytical chemistry of the 21st century. In this sense, we are absolutely convinced that the use of tailored and task-specific materials in the sample treatment of IMS methods has much to say in the development of future IMS applications with enhanced selectivity and sensitivity. The well-known lack of selectivity of IMS determination can be solved by the use of high specificity extraction procedures based on smart materials, such as: immunosorbents, aptamers, or MIPs. Additionally, the use of nanomaterial and ILs are mainly focussed in the improvement of method sensitivity, where IMS technique is usually hyphenated to previous or later separations systems as GC, HS or MS approaches. Figure 34 shows the main advantages and drawbacks that offer the smart materials evaluated in the present review.

However, there are further smart materials that have not been employed yet in the development of IMS-based methodologies, and there are still novel applications to discover. Among them, we want to highlight the potential use of restricted access materials (RAMs) and metal-organic frameworks (MOFs) as smart materials of high value for sample treatment in IMS analysis of biological samples to avoid matrix effects and enhance direct determinations.

The use of RAMs, alone or in combination with another high selective material, is focussed in reusability increase of sorbent materials, especially in the analysis of complex samples like biological fluids. RAMs are sorbents that possess the capability to extract low molecular weight target compounds directly from untreated biological fluids, avoiding the access of proteins and other macromolecules to the active sites by a size-exclusion process to the “active” bonded phase. Lifetime of RAMs treated solid phase materials is extremely high compared to that of conventional SPE sorbents, being this fact directly related to the cost of analysis. Despite that the price of RAM columns is usually higher than conventional SPE cartridges, the use of RAMs allow a sample throughput up to 2000 analyses with a single column without any change in recovery and separation performance, whereas commercial SPE cartridges are designed in general for a single use [143].

On the other hand, metal-organic frameworks (MOFs) are smart materials with a high potential use for sample treatment in analytical chemistry. MOFs are an emerging class of hybrid inorganic organic microporous crystalline materials self-

assembled from metal ions with organic linkers via coordination bonds that possess unusual properties; such as high surface area, uniform structured nanoscale cavities, and the availability of in-pore functionality and outer-surface modifications. The main advantages of MOFs as extraction sorbents are those derived from: i) enhanced selectivity by analyte size and/or analyte-net interactions; ii) high thermal stability (till 400 °C); iii) easy tuneable properties; iv) flexibility and dynamics of the network; and v) permeability of the channels and nanospace coordination.

Therefore, nowadays a new horizon has opened up before us. We have a large assortment of smart materials with unique and versatile properties to exploit in the development of high sensitive and selective methods based in IMS and it will influence the applicability of this relatively low cost technique.

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Conflict of interest

The authors declare no competing financial interest.

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Smart materials for forensic analysis

Aitor Sorribes-Soriano, Sergio Armenta

Department of Analytical Chemistry, University of Valencia, 50th Dr. Moliner St., 46100 Burjassot, Spain

Abstract

In this chapter, we will discuss the different types of smart phases used in the forensic analysis, including immunoaffinity chromatography (IACs) columns, aptamers, molecularly imprinted polymers (MIPs), restricted access materials (RAMs), metal-organic frameworks (MOFs), carbon based materials and other types of nanoparticles. The chapter will include the different applications of the smart phases in sample treatment and also active element of sensors.

Keywords: *pharmaceutical, clinical, forensic analysis, smart materials, molecularly imprinted polymers (MIPs), immunoaffinity chromatography (IACs) columns, aptamers, metal-organic frameworks (MOFs), restricted access materials (RAMs)*

1. Smart materials in forensic science

Forensic science can be defined as the application of analytical tools and techniques to prove the guilt or innocence of the defendant in criminal law by the discovery of evidence deemed relevant in the investigation of a legal proceeding. The Latin word "forensis" means "public discussion or debate", thus, the combination of both, forensics and science, provide the practical application of science to matters of debate, which in modern times translates to the law. Forensic sciences from a broad point of view include forensic chemistry, biology, anthropology, medicine, material science, engineering, computational forensics, and so on [1]. This chapter will be focused on forensic chemistry and analysis, and the required instrumental analytical methods. The most frequently encountered examples of forensic science applications are fingerprints [2] and DNA [3]. However, forensic analysis goes much beyond these well-known applications and often includes different analytical procedures such as vibrational spectroscopy, mass spectrometry, chromatography or electrochemistry [4]. The main sources of evidence analyzed nowadays are human or animal hair, fiber, paints and inks, human body fluids, physical objects, deposited trace materials, among others, being the target structures related to illicit and therapeutic drugs of forensic relevance, poisons and toxins, explosives and chemical warfare agents, DNA, gunshot residues, fire debris and accelerants and writing media and documents (see Figure 35).

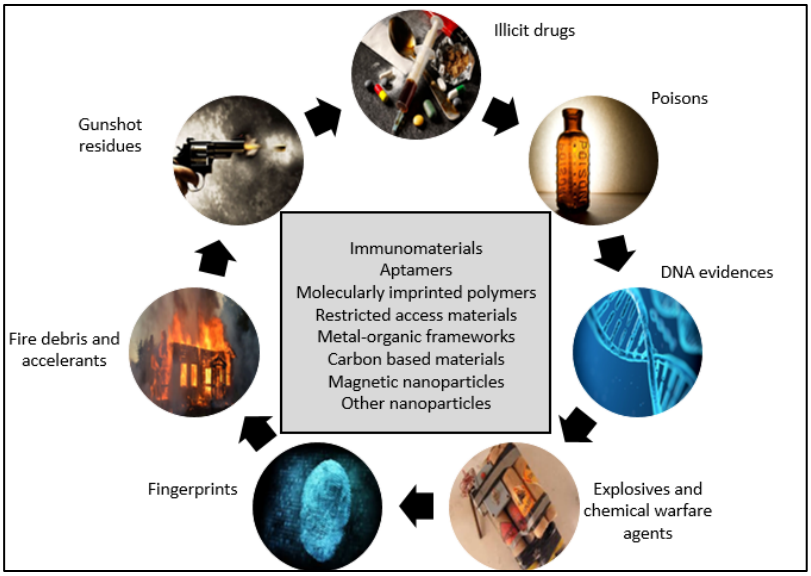

Figure 35. *Different areas of interest in which smart materials can be useful for forensic sciences.*

The analytical approach can be divided in several steps, including i) identification and definition of the problem, ii) design of the experimental plan/procedure iii) perform the experiments to obtain relevant data, iv) analysis of the experimental data and vi) proposal of solutions. Taking a closer look at the design of the experimental procedure part, it is easy to identify the different steps of an analytical procedure: i) sampling and weighing; ii) transport and storage (if necessary), iii) sample treatment including dissolution, extraction, clean-up, preconcentration, and so on, iv) analyte separation and v) detection and data treatment. Meanwhile the development of separation, detection and data treatment methodologies have been tremendous in the last 20 years, preliminary steps are the pending goals of today's analytical chemistry.

Traditional approach in most forensic applications implies the transport of the collected samples to a specialized laboratory for instrumental analysis. The increasing capabilities and discriminating power of actual analytical instrumentation and procedures has made possible to perform forensic investigations at ever smaller size scales with greater sensitivity and finer ranges of differentiation. As a result, the available literature regarding this topic has increased substantially in the last decades.

However, rapid, on-site analysis of the samples at the crime scene could be very beneficial for the investigation; thus, simple biosensors are finding their own place in forensic analysis [5]. Sensing devices can be miniaturized and assembled in wearable sensors [6]. New trends, especially in the sensor and sample treatment areas, are continuously appearing, some of them are short lived but others grow to become new standard tools. By definition a trend is a new, potentially useful approach explored, not fully established itself, and as such, it remains to be seen whether it will succeed in the transition to a well-established and accepted technique. One of these recent trends is the use of smart materials from an analytical point of view. Smart materials are defined as a material that presents the ability to respond to an external stimulus in a specific way, this response being both timely and in a controlled manner. Figure 36 shows in a schematized way the different smart materials that will be described during this chapter.

In this chapter we have focused our attention on compiling the literature published regarding smart materials for forensic analysis. Due to the vast number of different smart nanomaterials, all with its own specific properties, only few examples could be mentioned here to emphasize the principal advantages of such materials. Thus, the main aim is not to cite all the published papers in the topic, but

mention the most relevant papers, highlighting its novelties, contributions and importance for forensic analysis.

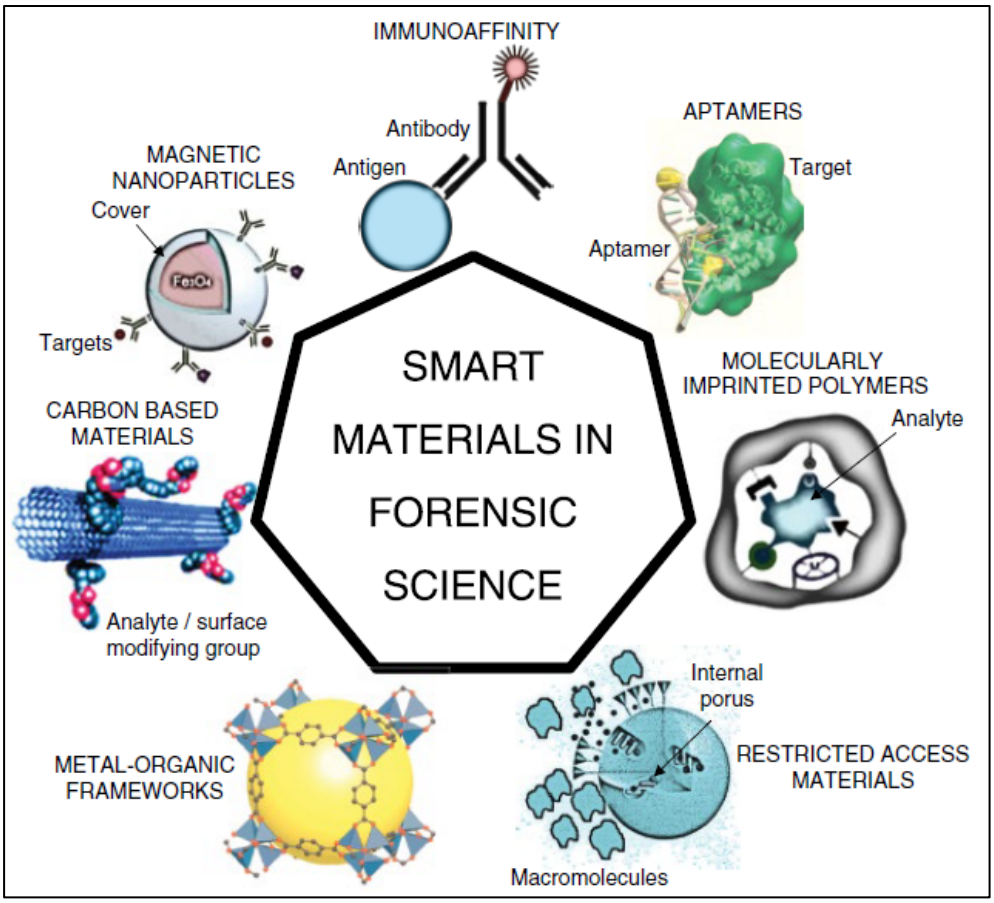

Figure 36. *Scheme of the different smart materials described throughout this chapter.*

2. Antibody-antigen interaction based materials

An immunosorbent (or immunoaffinity sorbent or immunoaffinity chromatography (IACs)) is prepared by immobilization of antibodies specific to the target analytes on a solid-support. Their capability is based on the high degree of molecular selectivity of the specific antibody-antigen (analyte) interaction. This type of materials has long been used for sample treatment in medicine, biology and food science, and more recently in environmental samples [7]. The main advantage of this smart material is that based on its high selectivity, which allows extraction,

concentration and clean-up from complex matrices in a single step, and from large sample volumes.

Specificity of the immunosorbent can be limited to only one compound, or to a family of compounds depending on the antibody production. As compounds of low-molar-mass (<1.000 Da) are unable to evoke an immune response in laboratory animals, they must be modified in an hapten, via the introduction of a functional group into the selected molecule, which can be linked to a carrier protein. To obtain antibodies with an appropriate specificity, the hapten design is fundamental.

Immunoassays were firstly used with forensic purposes in the early 1970s for the screening of certain drug classes in biological fluids. Since then, commercial immunoassays directed towards abused drugs are common tools for forensic toxicologists. Most of them are competitive, which means that an antigen, structurally related to the target compound, is conjugated to a labelling molecule and competes with the analyte of the sample for antibody binding. Immunoassays can be classified as heterogeneous and homogeneous, depending on the separation or not of the original sample from the final detection solution. Homogeneous immunoassays include enzyme immunoassay (EIA), fluorescent polarization immunoassay (FPIA) and kinetic interaction of microparticles in solution (KIMS) immunoassay. Heterogenous immunoassays include radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA).

Commercial immunoassays have been used with urine, blood, hair, saliva, sweat, tissue homogenates, blood stains and other physiological samples. The interaction of antigen-antibody has been also successfully used in solid phase extraction (SPE) treatments. A variety of immunosorbents are commercially available for mycotoxins (aflatoxin, ochratoxin, zearalenone, fumonisin,…), β-agonists (clenbuterol, salbutamol,…), corticosteroids, stilbenes, growth promoters (zeranol, ractopamine,…), phenylurea herbicides, polycyclic aromatic hydrocarbons among others. Alternatively, immunosorbents can be easily prepared from commercial antibodies and appropriate supports. There are several types of sorbents that can be used to immobilize the antibodies for use in IACs. Traditional supports are based on agarose, sepharose and cellulose or acrylamide polymers, polymethacrylate derivatives and polyethersulfone matrices. Alternative support materials that have recently been used in IAC are disks, fibers and monolithic rods [8J. Unique features offered by these newer support materials include their good mass-transfer and flow properties [9]. The main advantages of those materials are their low cost, good efficiency, mechanical stability, low nonspecific binding and an

easy modification for antibody attachment. However, they can be used under gravity flow and have slow mass-transfer properties. On the other hand, the most important drawbacks of the IACs application in forensic analysis are those based on the high cost, the limited range of commercially available immunosorbents and the difficulty of making antibodies selective to small molecules as well as the lack of expertise among forensic and analytical chemists with the procedures used to make specific antibodies.

Detection of biological evidences, such as saliva, sweat, blood, semen, among others, plays a critical role in forensic investigation for understanding the circumstances surrounding a crime scene and determining the presence or not of an individual in the scene. Biological samples are important because their components can often provide some type of information about a person-of-interest due to the DNA content which can be extracted and analyzed to provide information that can be imperative to a criminal investigation.

Many current methods for identification of human blood involve the detection of specific proteins in antigen–antibody interactions [10]. The precipitin test identifies human blood through the addition of the sample to anti-human serum. If a visible interaction occurs in vitro and a precipitin ring is formed, the blood is confirmed as being of human origin [11]. The Ouchterlony immunodiffusion test uses a similar method and was, until recently, the test of choice for bloodstain speciation [12]. Moreover, there are available immunochromatographic tests such as the ABAcard® HemaTrace®, which relies on the sample reacting with a mobile monoclonal anti-human antibody with a detection limit of 0.07 μg haemoglobin mL^{-1} [13].

Identification of other body fluids in crime scenes has been made after immunoassays on specific proteins, for instance Tamm-Horsfall protein and uroplakin III for urine [14], statherin for saliva and nasal fluids [15], dermcidin for sweat [16] and prostate-specific antigen and semenogelin for semen [17].

The presence of specific proteins has been also the probe of the damage of an organ by a bullet. In this sense, traces of S-100 proteins means brain damage [18], Tamm-Horsfall protein means kidney damage [19], liver-specific antigen for liver injuries [20], and so on.

Additionally to detect biological evidences, immunoassay based methodologies have been applied for the analysis of abuse drugs with forensic purposes. Those methods have excellent sensitivity and specificity. The most common immunoassays used in forensic toxicology includes: RIA, FPIA; KIMS and

ELISA and the substances commonly analyzed are amphetamines, barbiturates, benzodiazepines, cannabinoids, cocaine, methadone, opiates and tricyclic antidepressants among others [**21**]. However, in this section of the chapter, well-known immunoassay analytical techniques were not deeply described and we will focus on new materials or techniques based on antigen-antibody interactions in forensic science.

In this sense, a magnetic particles-based enzyme-linked immunoassay (mpEIA) method has been developed for the rapid and sensitive determination of cocaine in biological fluids [22]. Limits of detection were 0.09 ng mL^{-1} (urine), 0.15 ng mL^{-1} (saliva), and 0.06 ng mL^{-1} (human serum) cocaine. Cross-reactivity with benzoylecgonine, the main metabolite of cocaine, was only 1.6%. An immunoelectrochemical platform for biosensing of cocaine based on the combination of a benzoylecgonine antibody and poly-L-phenylalanine bearing electroactive macromonomer has been successfully developed [23]. Construction of the immunosensor is based on the modification of a glassy carbon electrode with poly-L-phenylalanine bearing electroactive macromonomer and the subsequent immobilization of the antibody on the polypeptide chains. Morphine has been determined using a solid phase sensor consisting of polymer beads coated with commercial monoclonal antibodies (MAbs) [24]. Fluorescein-conjugated morphine was used as the fluorescein-labeled hapten, reaching the binding steady state with the anti-morphine monoclonal antibody within minutes. The complex is effectively displaced by morphine and other opiates.

Lateral-flow immunoassay (LFIA) [25] developed on disposable membrane strip represents a powerful tool for rapid in situ detection of explosives, but it usually presents high detection limits and are suitable for qualitative or semiquantitative analyses. Very recently, a chemiluminescence based quantitative lateral flow immunoassay for on-field detection of 2,4,6-trinitrotoluene has been developed [26]. In this sense, a new amperometric immunosensor for 2,4,6-trinitrotoluene based on the working principle of competitive ELISA was developed and characterised. An electrodeposited nanogold substrate, functionalised by deposition of self-assembled monolayers of 2-aminoethanethiol and subsequently modified by immobilisation of polyamidoaminic dendrimers was used to retain a trinitrobenzene-ovalbumin. The immunosensor was tested and validated for the determination of 2,4,6-trinitrotoluene showing high selectivity with respect to other nitroaromatic compounds, a limit of detection of 4.8 ng mL^{-1} and a limit of quantitation of 6 ng mL^{-1} [27].

Other sensor based on the antigen-antibody interaction is constructed from ordered mesoporous silica nanoparticles loaded with a fluorescent indicator dye, sulforhodamine B, functionalized in the external surface with a suitable hapten and covered with a triacetone triperoxide selective polyclonal antibody to close the void openings. The sensor has been used as selective, sensitive and rapid sensor for explosive analysis [28].

Using mAbs against common epitopes of epsilon toxin and prototoxin, different highly sensitive immunoassay based methodologies were developed from sandwich enzyme immunoassays to immunochromatographic tests [29]. The limits of detection of those methods were in the pg mL^{-1} concentration range. These tests were evaluated for detection of epsilon toxin, the third most potent clostridial toxin after botulinum and tetanus toxins, in different matrices such as milk and tap water for biological threat detection, serum, stool and intestinal content for human or veterinary diagnostic purposes. A rapid and ultrasensitive method using a single-molecule array assay, employing high affinity single domain antibody (the heavy-chain-only antibody) was developed for the quantitative analysis of botulinum neurotoxin, one of the most poisonous substances ever known [30]. Botulinum neurotoxin is sandwiched between the heavy-chain-only antibody and a biotin-conjugated heavy-chain-only antibody and later labeled with streptavidin-β-galactosidase enzyme, forming an enzyme-labeled immunocomplex on the beads. The limits of detection were 200 fg mL^{-1} for serum and 1 pg mL^{-1} for urine.

Table 11 shows a summary of the characteristics of the immunoassay based methodologies described in this chapter with forensic purposes.

3. Aptamers

As it has been aforementioned in previous sections, an important analytical feature in sample treatment or in sensor development is that based on molecular recognition. In this sense, aptamers are short single-stranded oligonucleotides (DNA or RNA, typically 20–110 base pairs) that exhibit molecular recognition, thus, are capable of specifically binding a target molecule, and have exhibited affinity for several classes of molecules. The word aptamer derives from the Latin word "*aptus*", that means "to fit" [31]. It has long been known that certain RNA and DNA sequences exhibit recognition for small molecules [32], after a careful and appropriate selection from a random oligo-nucleotide sequences combinatorial library.

The use of aptamers as tools in analytical chemistry has considerably increased due to the development of the in vitro iterative selection process called “systematic evolution of ligands by exponential enrichment” (SELEX). SELEX method isolates and amplifies oligonucleotide sequences that present a high-affinity binding capability to a target molecule [33]. Most of the isolated sequences already identified are directed against large molecules such as peptides, proteins, or nucleic acids. However, a significant number of aptamers has also been selected for small molecules [34]. In comparison with antibodies, aptamers offer several advantages such as chemical modifications during their synthesis to improve their stability or to facilitate their detection or their immobilization [35].

Readers of this book can obtain more information of the aptamer based techniques and their applications in analytical chemistry in Chapter 8 (Volume I) of this handbook.

Immobilized aptamers have been predominantly developed for use within traditional ELISA, western blot, flow cytometry and lateral flow assays as replacements for expensive antibodies [36]. However, this chapter will be focused on the aptasensor based technology for forensic applications.

The main applications of aptamers in forensic science have been reviewed recently [37]. In this sense, optical aptasensors have been developed for the confirmation of body fluids presence in a crime scene. A fluorescent aptasensor against prostate specific antigen, a protease enzyme used in forensic analysis for the detection of seminal fluid, has been successfully developed [38]. Other examples can be found in the sensor developed by Wang *et al.* which involves the use of aptamer-functionalized upconverting phosphors. This sensor is initially quenched by the binding of carbon nanoparticles to DNA by π-π interactions. In the presence of thrombin, carbon nanoparticles dissociate from DNA, relieving FRET quenching effects and producing fluorescence [39].

Aptasensors have been also used for the analysis of drugs with forensic purposes. For instance, cocaine can be detected at picomolar levels by the displacement of 4-(4’-dimethylaminophenylazo) benzoic acid quencher-attached complementary oligonucleotides from carboxyfluorescein [40]. Examples of aptamers combined with other types of nanoparticles and materials can be found in the literature. For instance, a combinatorial platform by using quantum dots and gold nanoparticles as well as a functional aptamer which selectively recognizes cocaine and its metabolite benzoylecgonine was developed and applied to the analysis of urine samples [41]. Amphetamine derivatives have been also analyzed

using aptasensors [42], using single stranded aptamers to shield gold nanoparticles from salt-induced aggregation in order to determine methamphetamine and 3,4-methylenedioxy-methamphetamine (MDMA). Codeine was also determined electrochemically using an aptamer retained onto gold-mesoporous silica nanoparticles [43].

Aptamers have been also used for the detection of toxins such as ochratoxin A [44], arsenic compounds [45], ricin [46,47,48] and dimethyl methylphosphonate, a nerve agent simulant [49]. Detection of explosives has been also successfully performed by using aptasensors, using the example of 2,4,6-trinitrotoluene (TNT) as proof of concept [50].

Table 12 summarizes the analytical properties of the methodologies based on aptamer recognition with forensic purposes described in this chapter.

4. Molecularly imprinted polymers

Molecular imprinting technique was first proposed by Wulff and Sarhan to obtain molecularly imprinted polymers (MIPs) that were capable of capturing target molecule [51]. Molecularly imprinting is a process by which selected functional monomers are self-assembled around a template molecule, and polymerized in the presence of a cross-linker. After removing the template molecule from the MIP, a cavity complementary in shape and chemical properties is generated in the structure, and becomes available to bind template molecules (or closely related to the template) [52]. Different approaches have been used for MIP synthesis including the formation of reversible covalent bonds between the monomers and the template, by non-covalent interactions such as ionic, hydrophobic or hydrogen-bond interactions and the semi-covalent method which involves both processes mentioned [53]. Several polymerization techniques can be used for the production of MIPs including bulk, precipitation, suspension and emulsion polymerization [54]. For more information regarding MIPs synthesis, selection of monomer, template and cross-linker, and polymerization techniques, readers can visit Chapter 5 (Volume I) of this handbook.

Versatility, stability and molecular recognition capabilities converts MIPs in a perfect candidate for the use in forensic analysis. MIPs compared to antibodies have higher physical robustness, strength, resistance to elevated temperature and pressure and inertness towards acids, bases, metal ions and organic solvents. MIPs can be prepared in different physical shape and sizes while conferring them with

some multi-functional smart material capabilities, like magnetic, stimuli-responsive, fluorescence labelling, etc. These functions support many possible application areas in the field of forensic sciences.

However, MIPs prepared by the conventional technique have some disadvantages such as high diffusion barrier, low affinity binding, and low rate mass transfer. In recent years, surface imprinting (2D imprinting) has been used to prepare surface imprinted polymers (SIPs) with better accessibility to specific binding sites [55]. Some researchers have reported SIP composites based on Fe3O4 nanoparticles [56], Au nanoparticles [57], carbon nanotubes [58], graphene [59], quantum dots [60;61], carbon dots [62] and graphene quantum dots [63]. Many routes have been explored to develop these surface MIP composites, such as free radical polymerization, reversible addition fragmentation chain transfer polymerization and sol–gel method. Applications of MIPs combined with other materials to obtain luminescent nanocomposites (MIP-lum-NCs), have been recently reviewed [64]. Those materials are stable, robust and cheap with specific binding sites for recognition of target molecules such as proteins, drugs, pesticides and explosives. The exploration of luminescent and magnetic MIP-NCs with high-specificity affinity toward analytes has great significance for clinical diagnosis, environmental analysis and homeland security.

MIPs applications in forensic sciences have been recently discussed [65]. One area in which MIPs have been extensively used is the detection of illegal and therapeutic drugs of forensic relevance in biological samples. Using data from the Science Citation Index (SCI) database of the Institute for Scientific Information (ISI, Philadelphia, PA, USA), cocaine and morphine are the two most frequently substances of abuse determined using MIPs, followed by benzodiazepines, codeine, amphetamines, cannabinoids, heroin, ecstasy and lysergic acid diethylamide (see Figure 37). The MIP extraction of diazepam and other benzodiazepines from hair samples, reported a high recovery up to 93% with a good precision (RSD = 1.5%) and a limit of quantification of 0.14 ng mg^{-1} [66]. Benzodiazepine extraction performance of an imprinted SPE system was compared to that of a traditional one for 10-post-mortem scalp hair samples [67], concluding that MIP cartridges had a higher selectivity than the classical ones.

Cannabinoids like marijuana and hashish, the most commonly used illicit drug, have been also analyzed after a MIP treatment. For instance, a combination of MIP and GC-MS has been developed for simultaneous determination of tetrahydrocannabinol and its main metabolite in urine samples [68]. A micro-solid

extractor for cannabinoids for assessing plasma and urine analysis of marijuana abusers by the combination of MIPS with a HPLC-MS/MS system has been also developed [69]. The method provided limit of quantification values for plasma and urine samples in the ranges of 0.36–0.49 ng L^{-1} and 0.47-0.57 ng L^{-1}, respectively.

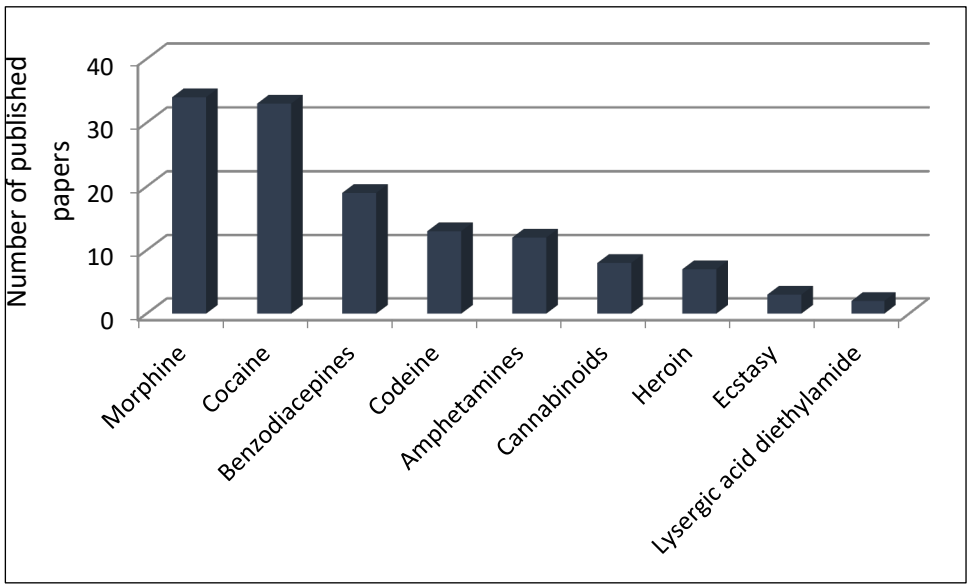

Figure 37. *Number of published papers in the Science Citation Index (SCI) database of the Institute for Scientific Information (ISI, Philadelphia, PA, USA) regarding molecularly imprinted polymers and drug analysis in biological fluids.*

Another class of the drugs often studied are stimulants like cocaine, amphetamine, and methamphetamine [70,71,72]. MIPs have been also used for the extraction of morphine from biological materials [73,74].

Other substances with relevant forensic interests analyzed by MIPs include theophylline and ephedrine [79], methylenedioxymethamphetamine (MDMA), flunitrazepam, γ-hydroxybutyrate and dissociative drugs like ketamine, phencyclidine (PCP) and its analogs, Salvia divinorum, and dextromethorphan [79] lysergic acid diethylamide (LSD), mescaline and psilocybin (magic mushrooms) [75].

Poisons including nicotine [76], cyanide [77], brucine [78], arsenic [79] and strychnine [80] were also intensively studied targets in the MIP literature. MIPs have been used for a simple DNA detection method recognizing a specific double-strand of DNA in MIP gel electrophoresis [81].

MIPs have already been utilized for the detection of hazardous materials such as explosives and chemical warfare agents, due to their strong mechanical strength, flexibility, long-time storage and low cost. MIP sensors, including electrochemical,

surface acoustic and optical sensors, for the detection of explosives and other hazardous materials have been reviewed [82]. A simple strategy was used to prepare mesoporous structured molecularly imprinted polymers capped carbon dots (M-MIPs@CDs) fluorescence sensor for highly sensitive and selective determination of TNT [83]. The as-prepared M-MIPs@CDs sensor, using periodic mesoporous silica as imprinting matrix, and amino-CDs directly as "functional monomer", exhibited excellent selectivity and sensitivity toward TNT with detection limit of 17 nM. Other example of determination of TNT is based on the development of an electrochemical sensor prepared from picric acid, as template at the surface of graphene polyaniline (PANI) nanocomposites [84].

Other explosive substances, such as triacetone triperoxide (TATP), have been also analyzed using MIP based sensors with electrochemical capabilities [85]. The molecular imprinting was performed via electropolymerisation onto a glassy carbon electrode surface by cyclic voltammetry from a solution of pyrrole functional monomer, TATP template and $LiClO_4$.

MIPs have been used for the determination of nitroaromatic compounds in water samples followed by ion mobility spectrometry detection [86]

MIPs have been used for the improvement of forensic evidence collection at the crime scene. In this sense, a MIP developed for the selective retention of diphenylamine, an organic compound coming from gunshot residues has been proposed [87]. MIPs were synthetized by solution radical polymerization, using 1-vinyl-2-pyrrolidone as monomer, and diphenylamine as imprinting and target molecule.

Besides, the use of MIPs has been applied to the determination of fire debrits as ethanol vapor in air prepared by mixing multi-walled carbon nanotube and nano-sized molecularly imprinted as recognition element, using that composite as a chemiresistor sensor [88]. A methacrylic acid and divinylbenzene based polymer were prepared using p-xylene or toluene as a solvent, that makes the MIP have more affinity for the absortion of this solvents, showing molecular recognition explained by the effect of the molecular imprinting [89].

MIPs have a large number of applications in forensic sciences because of their versatility, for this reason MIPs have been used for the determination of different warfare agents such as methylphosphonic acid [90] and ricin [91].

Table 13 provides the analytical features of the selected methodologies using molecular imprinting in forensic science, including drug, poison, DNA, explosives, fire debris and accelerants and warfare agents analysis.

5. Restricted access materials

One of the main problems of forensic toxicology is related to the determination of illicit and therapeutic drugs of forensic relevance in biological samples, mainly due to the presence of macromolecules and their incompatibility with most analytical techniques, clogging tubes and lowering analytical features. Other problem caused by proteins is the decrease of the adsorption capacities and selectivity by retention in the conventional solid extraction sorbents. Different strategies have been developed to solve this problem, such as protein precipitation and ultrafiltration procedures. Those procedures are quite fast and simple but losses of some analytes through co-precipitation or adsorption onto the proteins could happen.

Restricted access materials (RAMs) have been developed as an alternative to eliminate proteins and other macromolecules from biological samples. RAMs are sorbents that possess the capability to extract low molecular weight target compounds directly from untreated biological fluids, avoiding the access of proteins and other macromolecules by a size-exclusion process to the "active" bonded phase. Retention of analytes can be done by partition, ion exchange and/or adsorption [92].

Taking into consideration the main advantages of this type of smart materials compared to common SPE sorbents, many scientific publications aimed to modify conventional sorbents to obtain RAMs. These advantages include analyte recovery, and lifetime among others. Analyte recovery of RAMs is practically 100%, although partial losses of analytes have been observed in the analysis of drugs strongly bound to plasmatic proteins [93]. Lifetime of RAMs is extremely high compared to that of conventional SPE sorbents, being this fact directly related to the price of analysis. The price of RAM columns is usually higher than that of common SPE cartridges; however it is possible to perform till 2000 analyses without any change in recovery and separation performance, whereas commercial SPE cartridges are designed for a single use.

The main examples of RAMs are those based on the modification with hydrophilic groups of the surface of polymers, carbon nanotubes and silica-based

materials, as well as by the presence of small pores accessible only to low molecular weight molecules [94]. A chemical barrier due to the presence of hydrophilic groups, and a physical barrier characterized by presence of small size pores, avoids the irreversible binding of proteins to the sorbent.

For more information regarding RAMs types, modification and properties, readers can visit Chapter 13 (Volume I) of this book.

RAMs have been applied to the analysis of biological fluids such as hemolyzed blood, plasma, serum, cell culture and tissue homogenates for the determination of low-molecular weight compounds as drugs, xenobiotics, metabolites and so on. This was accomplished by using chemically and/or enzymatically tailored reversed-phase packing materials with restricted access properties. The bonded phase which exclusively covers the internal pore surface of a glyceryl-modified silica could be a butyryl-(C-4), capryloyl-(C-8) or stearoyl-(C-18) moiety [95]. Surface of the particles was modified with immobilized enzymes (lipase, esterase) which cleave the fatty acid esters exclusively at the outer surface, being the lifetime of a precolumn packed with these phases more than 200 injections of 500 µL plasma.

Another example of analysis of drugs after RAM based extraction is that of Pinto et al. [96] in which porous octadecylsilane particles were surface covered with bovine serum albumin (BSA) as a stationary phase to extract drugs from plasma samples by disposable pipette extraction for further analysis by liquid chromatography–tandem mass spectrometry (LC–MS/MS). The C18-BSA phase simultaneously excluded macromolecules by chemical diffusion barrier and enrichment of the interior phase with drug traces by sorption.

Moreover, RAMs have been also used in combination with other type of smart materials such as MIPs to obtain a new class of hybrid materials, called restricted access molecularly imprinted polymers (RAMIPs). The usefulness of these hybrid materials has been demonstrated in the analysis of tricyclic antidepressants from human plasma by MS [97]. RAMIP for amitriptyline was synthesized by the bulk method, using methacrylic acid as a functional monomer and glycidilmethacrylate as a hydrophilic co-monomer. The surface of the polymer was covered with BSA.

For more information regarding forensic applications of RAMs, readers can see the revision article of RAMs in bioanalysis [98]. These materials have been obtained by modifying the external surfaces of conventional sorbents such as polymers, carbon nanotubes, active carbon, and silica-based materials with hydrophilic groups, as well as by the presence of small pores accessible only to low molecular weight molecules.

RAMs have been used as trap or liquid-chromatography precolumn (with a C18 or porous graphitic carbon stationary phase) followed by the porous graphitized carbon analytical column and analyzed by MS. The method was applied to the analysis of untreated groundwater and drinking water samples, spiked with 20 ng of 2,4-dinitrotoluene, 2,6-diamino-6-nitrotoluene and pentaerythritol tetranitrate [99].

Supramolecular solvents (SUPRAs) have also been used as RAMs, due to their abilities to exclude proteins according to size or through precipitation with the solvents. In this sense, SUPRAs made up of inverted hexagonal aggregates of oleic acid to behave as a liquid with restricted access properties (SUPRAS-RAM) have been proven to be useful for the fusarium toxins determination in cereals by LC-electrospray ionization ion trap-MS (LC-ESI-IT-MS) [100]. SUPRAs are composed by inverted hexagonal aggregates of alkanols or alkyl carboxylic acids in tetrahydrofuran and water and present restricted access properties by both chemical and physical mechanisms [101]. This solvent removes or reduces ionization suppression and/or enhancement in the analysis of complex samples by MS. A SUPRAS-RAM made up of tetradecanoic acid reverse micelles is proposed as a wide-scope and low-cost strategy for the treatment of agrifood samples prior to ELISA [102]. The approach was assessed for the determination of ochratoxin A in wines and aflatoxin B1 in cereals.

RAMs have been also used to determine other possible contaminants. In this sense, oxidized carbon nanotubes were covered with layers of bovine serum albumin to result in so-called restricted-access carbon nanotubes (RACNTs) [103]. This material can extract Pb^{2+} ions directly from untreated human blood serum while excluding all the serum proteins. The RACNTs presented a protein exclusion capacity of almost 100% and a maximum Pb^{2+} adsorption capacity of 34.5 mg g^{-1}. A mini-column filled with RACNTs was used in an on-line solid phase extraction system coupled to a thermospray flame furnace atomic absorption spectrometry.

RAMs particles located inside HPLC chromatographic columns have been widely used, especially for the determination of different drugs such as barbiturates [104], cetacholamines [105], cocaine [106], methadone [107] and heroin [108] in biological matrices being capable to remove the macromolecules of these fluids.

Table 14 describes featured applications of restricted access materials in forensic sciences including drug, toxins and explosives.

6. Metal-organic frameworks

Metal-organic frameworks (MOFs) are an emerging class of hybrid inorganic organic microporous crystalline materials self-assembled from metal ions with organic linkers via coordination bonds that possess unusual properties such as high surface area, good thermal stability (200 to 400 °C), uniform structured nanoscale cavities, and the availability of in-pore functionality and outer-surface modifications. As a new class of crystalline porous materials, MOFs have attracted tremendous interest in different applications, including also extraction procedures [109]. The main advantages of MOFs as extraction elements are those derived from: i) enhanced selectivity and stability, being able to differentiate by size, and/or analyte-net interaction; ii) easy tuneability of the properties of the polymer; iii) flexibility and dynamics of the network and iv) permeability of the channels and nanospace coordination. For more information regarding MOFs as smart materials, the reader can refer to Chapter 15 (Volume I) of this book.

Magnetic MOFs, MIL-100(Fe) on mercaptoacetic acid-functionalized Fe_3O_4 nanoparticles, have been used for the selective extraction of neurotransmitters from urine and serum [110]. Mil-53(Fe) derived magnetic porous carbons showed appropriate sorbent efficiency for the magnetic SPE of hormones from urine samples [111]. The MOF MIL-101 was fabricated in a polyetheretherketone tube as micro-trapping device, and applied to sorptive extraction of naproxen and its metabolite in urine samples [112]. The remarkable water stability of the MIL-101 characterizes the material as being different from other moisture sensitive MOF. An in situ solvothermal growth method was used for the immobilization of MOF–ionic liquid functionalized graphene (MOF-5/ILG) composite on etched stainless steel wire for the analysis of chloramphenicol and thiamphenicol in different samples including urine and serum [113]. The obtained material combined the favorable attributes of both MOF and ILG, having high surface area (820 $m^2 g^{-1}$) and good adsorption capability. Under the optimum conditions, low limits of detection and good precision were achieved.

Magnetic Fe_3O_4@ZIF-8 core-shell nanoparticles were employed for effective adsorption of inorganic arsenic from urine samples [114]. The MNPs were then completely dissolved in hydrochloric acid prior to the determination of inorganic arsenic by hydride generation-atomic fluorescence spectrometry.

Additionally, luminescent MOF sensors have been widely used for explosive detection, mainly nitroaromatic and nitroaliphatic explosives [115]. That kind of material provides an effective fluorescence response in the vapor and/or liquid

phases, and there are so many opportunities to use it to the easy detection by a fluorometer or just by the naked eye in the case of colorimetric response.

Table 15 includes the main applications of the MOFs related to forensic sciences.

7. Carbon based nanomaterials

Carbon based nanomaterials have been extensively used in Forensic science. Applications of carbon-based materials previously reported in the literature include the use of nanodiamonds, peapods, nanofibers, and nanorings, being the most recent studies based on fullerenes and nanotubes [116].

Fullerenes are a large class of allotropes of carbon and have attracted considerable attention in different fields of science since their discovery in 1985.

Carbon nanotubes (CNTs) represent an increasingly important group of nanomaterials with unique geometric, mechanical, electronic, and chemical properties. CNTs can be viewed as a hollow cylinder formed by rolling graphite sheets. Since CNTs are derived from fullerenes, they are referred to as tubular fullerenes or bucky tubes. CNTs can be grouped into two main forms: the single-walled carbon nanotube (SWCNTs), which is a hollow cylinder of a graphite sheet, and the multi-walled carbon nanotube (MWCNTs). In this sense, a modified electrode using fullerene-functionalized carbon nanotubes and ionic liquid (IL, 1-butyl-3-methylimidazolium tetrafluoroborate) has been applied for the determination of diazepam in real samples including serum, urine, and tablets [117]. Morphine and codeine have been determined in urine samples of heroin addicts using MWCNTs modified SnO_2–Zn_2SnO_4 nanocomposites paste electrode [118]. CNTs have been utilized many times in electrochemistry since they were first used as an electrode in 1996 [119] and since then have shown to provide an improved electrochemical response over more traditional carbon substrates such as glassy carbon and diamond [120]. The majority of these electrochemical sensors appear to be directed toward biosensors rather than explosive sensors, due to the easily functionalized surface of the carbon; however, some studies have been reported utilizing CNTs for detection of explosives. In 2007, it was explored a capacitance-based sensor using SWCNTs for trace chemical vapors, including explosives, detection [121]. Flexible SWCNTs chemical sensors were developed in 2010 with a sensitivity of 8 ppb TNT at room temperature [122]. The response of

SWCNT sensors to 2,4-dinitrotoluene vapor was improved dramatically after decoration with single stranded-DNA [123].

Metal nanoparticles (Pt, Au, or Cu) together with MWCNT and SWCNT solubilized in Nafion have been used to form nanocomposites for electrochemical detection of TNT and several other nitroaromatics [124]. Eastwood and coworkers have used CNTs-supported Pd particles for reduction of nitroaromatic compounds followed by laser-induced fluorescence [125].

Silver-coated carbon nanotubes have been employed for benzoylecgonine detection, the main cocaine metabolite, in urine by surface enhanced Raman spectroscopy [126]. Detection was based on monitoring the vibrational changes occurring at a specific biointerface, a MAb, supported on the silver-coated carbon nanotubes.

Carbon dots are a new type of fluorescent carbon nanoparticles with good resistance to photobleaching, low toxicity, high chemical stability and photostability, excellent biocompatibility and low cost [127]. Carbon dots are surface passivated carbon nanoparticles by organic compounds or biomolecules to become strongly fluorescent in the visible and near infrared spectral range. N-rich carbon dots prepared via a microwave-assisted pyrolysis method have been successfully employed for the fluorescence and the electrochemical determination of TNT [128].

Carbon dots capped by molecularly imprinted polymers with mesoporous structures (M-MIPs@CDs) have been developed for the highly sensitive and selective determination of TNT with exhibited excellent selectivity and sensitivity with a detection limit of 17 nM [129].

Graphene is other material that with appropriate properties for analyte extraction in bioanalysis [130]. Graphene is a single carbon layer of graphite. It can also form related structures such as graphene nanowalls and graphene nanoribbons. Graphene contains of a single layer of carbon atoms where all are sp2-hybridized. The remarkable tendency of graphene nanoparticles to aggregate leads to a significant reduction of its surface area and consequently, adsorption efficiency. Graphene oxide is a chemically modified graphene which possesses a layered structure and negatively charged surface [131] capable of strong interactions with aromatic compounds. Graphene also showed promising potential in vapor phase explosive detection. PtPd concave nanocubes anchored on graphene nanoribbons (PtPd-rGONRs) were used to modify glassy carbon electrode to enhance current signals for TNT reduction, which is 4 and 12-fold higher than

rGONRs and bare glassy carbon electrode, respectively [132]. The PtPd-rGONRs showed excellent detection stability for the determination of TNT. Aromatic explosives in particular have strong adsorption on the graphene surface rendering this nanomaterial suitable in the selective determination of these species [133].

The use of graphene oxide for the detection of nitroaromatic explosives was extended for other species such as dinitrotoluene (DNT), dinitrobenzene (DNB) and trinitrobenzene (TNB), as well as TNT [134]. Reduced graphene oxide was used as the active material for explosive sensors [135] with a limit of detection of DNT at a concentration of 0.1 ppbv with an exposure time of 10 s.

CNTs [136] and, more recently, graphene [137] have been introduced as novel matrix solid-phase dispersion dispersing materials. However, the number of applications of graphene in matrix solid phase dispersion is not really big, probably due to its fragile structure, which difficulties sample architecture disruption.

All those carbon based materials can be combined with immunosorbents [138], aptamers [139], MIPs [140] and other nanoparticles to improve the inherent characteristic of the originals.

Carbon nanostructures showed great promise as immobilization platform in electrochemical DNA sensors due to its characteristic properties like fast electron transportation, high thermal conductivity, excellent mechanical flexibility, rapid electrode kinetics, ease of functionalization and large surface area [141]. MWCNTs functionalized with carboxylic acid groups along with polydopamine and AuNPs have been used to modify glassy carbon electrodes for DNA detection [142]. Oxidized SWCNT immobilized on gold electrode through a self-assembled monolayer of cystamine for DNA hybridization detection also has been reported [143]. Composite materials have been also used in DNA detection. A DNA sensor which used copper oxide nanowires/SWCNT nanocomposite as the immobilization platform was reported [144]. Other biosensor for DNA detection was based on WS2/MWCNT composite along with hybridization chain reaction amplification [145]. Additionally, there are numerous reports based on different nanocomposites containing graphene, graphene oxide and reduced graphene oxide used for the development of DNA sensors [146].

Table 16 describes the analytical properties of the selected applications of carbon based nanomaterials in forensic sciences.

8. Magnetic nanoparticles

Magnetic nanoparticles can be considered smart materials due to their interactions with magnetic fields and field gradients. This type of particles can be subdivided in those with a permanent magnetism (ferromagnetism) and those that can be attracted by a magnetic field without retaining residual magnetism after the elimination of the magnetic field (superparamagnetism). The main advantages of this type of particles when used as SPE sorbents are those related to the simplicity of operation and the reduction of the required analysis time. Emerging analytical techniques and new uses of conventional methods have begun to integrate magnetic nanoparticles to take advantage of the ability to magnetically induce motion, enhance signals, and switch behaviors [147]. For instance, dispersive-SPE (d-SPE) using magnetic sorbents in which the sorbent is added directly to the sample, is simple, rapid, and efficient compared to d-SPE with traditional sorbents.

Magnetic particles are available in a wide range of sizes (from nanoscale to microparticles), being the typical diameters from 1 to 100 nm [148]. The magnetic core is often composed of iron, nickel, cobalt or any of their oxides, and the most common material is magnetite (Fe_3O_4) [149]. Modification of the surface of magnetic nanoparticles with inorganic materials such as silica or modified silica [150,151,152], alumina [153] and graphene [154]; or organic compounds such as polypyrrole [155], polyaniline [156], MIPs [157], silane [158], chitosan [159], surfactants as sodium dodecyl sulphate (SDS)-coated [160], cetyl trimethylammonium bromide (CTAB) [161], and carbon-based nanocomposites [162,163], confers the material appropriate characteristics to be used as solid phase adsorbent for the extraction of abuse drugs from biological fluids and increases the durability of the sorbent preventing its oxidation.

Magnetic nanoparticles can also be modified employing other inorganic materials such as Au or Ag to enhance their surface plasmon resonance and, thus, their detection capabilities [164].

Explosives, such as TNT has been also analyzed using magnetic particles. For instance, fluorescein isothiocyanate (FITC) was conjugated to 1,6-hexanediamine (HDA)-capped iron oxide magnetic nanoparticles (FITC–HDA Fe_3O_4 MNPs) [165]. HDA ligands on the surface of Fe3O4 MNPs can bind TNT, resulting in quenching of the fluorescence at 519 nm. TNT can be also analyzed using a lignin modified hybrid microsphere, comprising poly(styrene-co-acrylic acid) core and magnetite (Fe3O4)/Au nanoparticle (NP) shell based on SERS and electrochemical detection methods [166]. Quenching of the luminescence of LaF_3:Ce_3+–Tb_3+ and Fe_3O_4

nanoparticle-codoped multifunctional nanospheres due to the presence of nitroaromatics into the solution was used for TNT detection [167]. SERS was used for TNT detection on the surface of self-assembled Ag NPs with ferromagnetic Fe3O4 microspheres, forming a hybrid SERS nanoprobe with both optical and magnetic properties [168]. Other study used a TNT-imprinted polymer shell created onto nano-sized Fe_3O_4 cores in order to construct the nano-sized magnetic MIP [169]. For this purpose, the surface of the synthesized magnetic nanoparticles was modified with methacrylic acid. The resulting magnetic nano-MIP particles were suspended in TNT solution and then collected on the surface of a carbon paste electrode via a permanent magnet, situated within the carbon paste electrode. TATP can be determined by acid hydrolysis into H_2O_2 at pH 3.6, in the presence of Fe_3O_4 MNPs to produce hydroxyl radicals from H2O2 [170]. The formed radicals converted the N,N-dimethyl-p-phenylenediamine (DMPD) probe to the colored DMPD+ radical cation and the absorbance measured at 554 nm.

Magnetic nanoparticles have been also used for the determination of toxins in biological fluids. In this sense, microcystin-LR (MC-LR) has been determined in blood plasma using gold coated magnetic nanoparticles functionalized with anti MC-LR antibody Fab' fragments by SERS [171]. Polyethylene glycol capped Fe3O4 nanoparticles were deposited onto indium tin oxide coated glass substrate and further functionalized with the MAbs specific to Vibrio cholerae toxin and bovine serum albumin for their determination by electrochemical methods [172].

A range of nanomaterials such as magnetic nanoparticles has been introduced as electrochemical labels in the sensor design to enhance the sensing performance of electrochemical DNA sensors [173]. Efficient isolation of DNA strands in complex media was achieved in a fast and efficient manner using silica or gold coated core/shell nanoparticles [174].

Table 17 summary the main analytical features of the selected procedures based on magnetic nanoparticles in forensic analysis.

Table 11. *Selected immunoassay procedures to identify several issues in forensic analysis.*

Material	Antigen (target molecule)	Issue to identify	Analytical technique	LOD	Ref
Body fluid / body fluid stain	Tamm- Horsfall protein, uroplakin III	Urine	ELISA	Dilution 1:640	14
	Statherin, amylase	Saliva and nasal fluid	ELISA	-	15
	Dermcidin	Sweat	Real time PCR, ELISA	10 µL sample	16
	Prostate-specific antigen, semenogelin	Semen	Dot-blot immunoassay	Dilution 1:6400	17
Stain on the weapon	S-100 protein	Injury of brain	Sandwich enzyme immunoassay	0.6 pg	18
	Tamm- Horsfall protein	Injury of kidney	Gold labeled immunoassay	-	19
	Liver-specific antigen	Injury of liver	Sandwich enzyme immunoassay	1 fmol/tube	20
Biological fluids	Cocaine	Drug abuse	Spectrophotometry	0.09 µg L^{-1} (urine) 0.15 µg L^{-1} (saliva) 0.06 µg L^{-1} (serum)	22
	Benzoyecgonine	Drug abuse	Electro-immunosensor	0.41 µM	23
	Morphine	Drug abuse	Fluoroimmunoassay	0.2 µg L^{-1}	24
Explosion residues	TNT	Presence of explosives	ELISA LFI	ng mL^{-1} 1 µg mL^{-1}	25
	TNT	Presence of explosives	Chemiluminescence-LFI	0.2 µg L^{-1}	26

	TNT	Presence of explosives	Competitive amperometric immunosensor	4.8 µg L^{-1}	27
	TATP	Presence of explosives	Fluorimetry	12.5 ppb	28
Biological fluids	Epsilon toxin	Presence of toxins	Sandwich enzyme immunoassays and immunochromatographic test	5 pg mL^{-1} (0.15 pM) and 100 pg mL^{-1} (3.5 pM)	29
	Botulinum neurotoxin	Presence of toxins	Single-molecule array	200 fg mL^{-1} (serum) 1 pg mL^{-1} (urine)	30

ELISA: enzyme-linked immunosorbent assay; PCR: polymerase chain reaction; LFI: Lateral flow immunoassay; TNT: 1,3,5-trinitrotoluene; TATP: triacetone triperoxide.

Table 12. *An overview of the main characteristics of the selected procedures based on aptamers in forensic sciences.*

	Analyte	Matrix	Method	LOD	Ref
Biological	Prostate Specific Antigen	Serum	Aptamer-functionalized MoS_2 nanosheet fluorescent biosensor	0.2 ng mL^{-1}	38
	Thrombin	Plasma	FRET	0.25 nM	39
Drugs	Cocaine	Serum	FRET	10 pM	40
	Cocaine and benzoylecgonine	Urine	Aptamer folding-based sensory device	0.138 nM and 1.66 µM	41
	Metamphetamine	Saliva	SPR	5 mM	42
	Codeine		Voltammetry	3 pM	43
Toxins and Poisons	Ochratoxin A	Wheat	Aptasensors with functional nanomaterials	20 nM	44
	As (III)	Water	Colorimetric and RS	0.6 µg L^{-1} (colorimetric) 0.77 µg L^{-1} (RS)	45
	Ricin	Urine and food	SERS and fluorescence	25 µg mL^{-1}- 10 mg mL^{-1}	46, 47, 48
Explosives	Dimethyl methylphosphonate	Aqueous matrices	Piezoresistive cantilever-based aptasensor	50 nM	49
	TNT	Soils	Fibre-optic biosensor	pM range	50

FRET: Fluorescence resonance energy transfer; SPR: Surface plasmon resonance; RS:Resonance scattering; SERS: Surface enhanced Raman scattering, TNT: 1,3,5-trinitrotoluene

Table 13. *Main features of the selected applications of molecularly imprinted polymers in drug, poison, DNA, explosive, gunshot residues, fire debrits and warfare agents analysis.*

	Material	Analyte	Matrix	Coupled device	LOD	Ref
Drugs	MAA and EDMA monolith	Diazepam	Hair	SPE - LC-MS-MS	0.09 μg L^{-1}	66,67
	AAm and EDMA pill	Cannabinoids	Plasma and Urine	SPE - LC-MS-MS	0.11-0.15 μg L^{-1} and 0.14-0.17 μg L^{-1}	69
	MAA and EDMA partices	Cocaine	Oral fluid	SPE - IMS	18 μg L^{-1}	70
	MAA and EDMA based monolith	Amphetamine	Urine	Inside-needle trap GC-FID	30 μg L^{-1}	71
	MAA and EDMA based monolith	Metamphetamine	Urine	Inside-needle GC-FID	12 μg L^{-1}	72
	Molecularly imprinted photonic hydrogels	Morphine	Biological samples	Optical response without instrument	1 ng L^{-1} and 0.1 μg L^{-1}	73,74
Poisons	Imprinted polymer coating	Nicotine	Human serum and urine	Quartz crystal-TSM sensor	$2.8x10^{-8}$ M	76
	MAA and EGDN polymer	Cyanide	Urine	SBSE - EI-MS	ND	77
	Imprinted polymer coating	Brucine	Serum	Electrochemical sensor	$2.1×10^{-7}$M	78

	MAA and EDMA based NPs	Arsenic	Biological fluids	Electrochemical impedance spectroscopy	$5.0x10^{-7}$ M	79
	MAA and EDMA	Strychnine	Nux-vomica	SPE	-	80
DNA	MIP gel as an electrophoretic matrix	Double strand DNA	Mixed DNA sample	GE	-	81
Explosives	Spin casted xerogels films	TNT	Artificial solutions	M-MIPs@CDs Fluorescence sensor	17 nM	83,84
	AAm and EDMA based particles	Nitroaromatic	Surface water	SPE - IMS	0.1 µg mL^{-1}	86
Gunshot residues	Polymeric microparticles	Diphenylamine	Gunshot residues	SPE - HPLC	ND	87
Fire debrits and accelerants	MWCNs-PMMA composites	Ethanol vapor	Air	Chemoresistor sensor	0.5 mg L^{-1}	88
	PMMA and DVB based particles	Xylene and toluene	Air	Quartz crystal microbalance	ND	89
Warfare agents	MMA, VP and EDMA based particles	Methylphosphonic acid	Natural water	Potenciometry based sensor	ND	90
	Molecularly imprinted silica particles	Ricin	Artificial solutions	Fluorescence spectrometer	ND	91

MAA: Methacrylic acid; EDMA: Ethylene glycol dimethacrylate; SPE: Solid-phase extraction; LC-MS-MS: Liquid chromatography tandem mass spectrometry; AAm: Acrylamide; IMS: Ion mobility spectrometry; GC-FID: Gas chromatograpy-flame ionization detector; TSM: thickness-shear mode; EGDN: Ethylene glycol dinitrate; SBSE: Stir-bar sorptive extraction; EI-MS: Electrospray ionization mass spectrometry; ND: Not determined; NP: nanoparticle; MIP: Molecularly imprinted polymer; TNT: 2,4,6-trinitrotoluene; HPLC: High performance liquid chromatography; MWCNs: Multi-walled carbon nanotubes; PMMA: Poly(methyl methacrylate); DVB: Divinyl benzene; VP: 4-Vinylpyridine; GE: Gel electrophoresis.

Table 14. *Use of restricted access material for the analysis of explosives, toxins, poisons and drugs.*

	Analyte	Matrix	Analytical technique	LOD	Ref
Explosives	2,4-dinitrotoluene 2,6-diamino-6-nitrotoluene Pentaerythritol tetranitrate	Drinking water and groundwater	MS	2.5 – 563 pg mL^{-1}	99
Toxins and poisons	Fusarium toxins	Cereals	LC-ESI-IT-MS	8-15 µg kg^{-1}	100
	Ochratoxin A Aflatoxin B1	Wine and cereals	ELISA	0.1-3.1 µg mL^{-1}	102
	Lead	Serum	HPLC-FD	2.1 µg L^{-1}	103
Drugs	Tricyclic antidepressants	Plasma	MS	4.5 µg L^{-1}	96
	Barbiturates	Human plasma and serum	HPLC	6.4 µg L^{-1}	104
	Catecholamines	Urine	HPLC	0.4-0.7 µg L^{-1}	105
	Cocaine	Plasma	HPLC	0.03 µg mL^{-1}	106
	Methadone	Serum	HPLC	3 ng mL^{-1}	107
	Heroin	Urine	HPLC	0.1-3 ng mL^{-1}	108

MS: Mass spectrometry; LC-ESI-IT-MS: Liquid chromatography coupled with electrospray ionization ion trap tandem mass spectrometry; ELISA: Enzyme-Linked Immuno Sorbent Assay; HPLC-FD: High performance liquid chromatography-fluorescence detection; HPLC: High performance liquid chromatography.

Table 15. *Selected applications of metal-organic frameworks with forensic purposes.*

MOF	Analyte	Matrix	Analytical technique	LOD	Ref
MIL-100 Fe	Dopamine, Epinephrine and norepinephrine	Human urine and serum	LC-UV	0.22-0.36 μg L^{-1}	110
MIL-53-C	Sex hormones	Water and human urine	LC-UV	5-10 ng L^{-1} (water) 100-300 ng L^{-1} (urine)	111
MIL-101	NAP and D-NAP	Human urine	LC-FD	34 ng L^{-1} and 11 ng L^{-1}	112
MOF-5/ILG	Chloramphenicol and Thiamphenicol	Milk, honey urine and serum samples	GC-FID	14.8-19.5 ng L^{-1}	113
Fe_3O_4@ZIF-8	As	Water and human urine	HG-AFS	3.0 ng L^{-1}	114

LC-UV: Liquid chromatography-ultraviolet detection; NAP: Naproxen; D-NAP: 6-O-desmethylated naproxen; LC-FD: High performance liquid chromatography- fluorescence detector; GC-FID: Gas chromatography – flame ionization detector; HG-AFS: Hydride generation atomic fluorescence spectrometry.

Table 16. *An overview of the main forensic analysis using carbon-based nanomaterials.*

	Analyte	Matrix	Material	Analytical technique	LOD	Ref
Drugs	Diazepam	Serum, urine and tablets	Modified electrode using fullerene-functionalized carbon nanotubes and ionic liquid	Voltammetry	87 nM	117
	Morphine and codeine	Urine	MWCNT modified SnO_2-Zn_2SnO_4 nanocomposites paste electrode	Voltammetry and chronoamperometry	0.009 µM	118
	Benzoylecgonine	Urine	Silver coated carbon nanotubes	SERS	-	126
Explosives	DNT	Chemical vapors	SWCNTs	Capacitance and conductance based detection	0.0013 µg L^{-1}	121
	TNT	Chemical vapors	SWCNTs	Chemoresistor and field-effect transistors	8 ppbv	122
	DNT	Chemical vapors	SWCNT	Metal oxide semiconductor sensor	ppm level	123
	TNT	Tap water, river water, and contaminated soil	MWCNTs and SWCNTs with metal nanoparticles	Electrochemical detection	1 µg L^{-1}	124
	Nitroaromatics	Vapors of landmines	Carbon nanotubes supported Pd particles	Laser-induced fluorescence	10^{-12} M	125

	TNT	Tap water	Carbon dots	Fluorescence and electrochemical determination	nM level	128
	TNT	Soils and water	Carbon dot capped by MIPs	Fluorescence sensor	17 nM	129
	TNT	Tap water and lake water	Graphene Nanoribbon-supported PtPd concave nanocubes	Electrochemical detection	0.8 µg L^{-1}	132
	DNT	Vapors	Reduced graphene oxide	Electrochemical sensor	0.1 ppbv	135

MWCNT: Multi-walled carbon nanotube; SERS: Surface enhanced Raman Spectroscopy; DNT: 2,4- dinitrotoluene; SWCNT: Single-walled carbon nanotube; TNT: 1,3,5-trinitrotoluene; MIP: Molecularly imprinted polymer;

Table 17. *Selected applications of magnetic nanoparticles in forensic analysis.*

	Analyte	Matrix	Material	Analytical technique	LOD	Ref
Drugs	Drugs of abuse	Urine	Silica modified Fe_3O_4	SPE-CE	20−50 ng mL^{-1}	150
	Diazepam	Plasma	RAM-C8-silica modified Fe_3O_4	LC-MS	0.003 µg mL^{-1}	151
	Illegal drugs	Urine	Silica modified Fe_3O_4	SPE-CZE	0.015 to 0.105 µg mL^{-1}	152
	Alendronate	Plasma and urine	Alumina modified Fe_3O_4	SPE-CE-FD	1.5 - 5 ng mL^{-1}	153
	Amphetamine and methadone	Urine	Graphene modified Fe_3O_4	SPE-LC	20-25 ng mL^{-1}	154
	Megestrol acetate and levonorgestrel	Urine	Polypyrrole modified Fe_3O_4	SPE-dLPME	0.03 ng mL^{-1}	155
	Benzodiazepines	Biological fluids	Polyaniline modified Fe_3O_4	dMSPE	0.2–2.0 µg L^{-1}	156
	Morphine	Urine	Imprinted polymer-supported on MWCNT-Fe_3O_4	Ultrasonic-assisted magnetic solid phase extraction	0.18-3.2 mg L^{-1}	157
	Morphine	Hair	Silane modified Fe_3O_4	LC-DAD	0.1 µg L^{-1}	158
	Flavonoids	Biological fluids	Chitosan modified Fe_3O_4	LC	0.5-1.0 ng mL^{-1}	159
	Opiod drugs	Urine	SDS coated Fe_3O_4	Supramolecular based magnetic NP solid-phase extraction	<0.27 ng mL^{-1}	160

	Benzodiazepines	Hair	CTAB coated Fe_3O_4	Superparamagnetic Fe_3O_4@SiO_2 core-shell composite NPs for hemimicelle SPE-LC	0.01-0.03 µg mL^{-1}	161
	Methamphetamine and ephedrine	Urine	Carbon based modified Fe_3O_4	SPE-LC	15-20 ng mL^{-1}	163
Explosives	TNT	Aqueous solutions	FITC-HDA capped iron oxide MNPs	Fluorescencce	37.2 nM	165
	TNT	Soils	Lignin modified hybrid microsphere comprising poly(stryrene-co-acrylic acid) core magnetic/AuNP	SERS and electrochemical detection	1 pM	166
	TNT	Aqueous solutions	LaF_3:Ce^{3+}-$Tb^{3+}$and Fe_3O_4 NP-codoped multifunctional nanospheres	Quenching of the luminescence	10.2 µg L^{-1}	167
	TNT	Ethanol solutions	AgNPs with ferromagnetic microspheres	SERS	10^{-7} M	168
	TNT	Water samples	TNT imprinted polymers shell onto nanosized Fe_3O_4	Voltammetry	0.5 nM	169
	TATP	Soils	Fe_3O_4 MNPs	Absorbance	0.47 mg L^{-1}	170
Toxins	Mycrocystin-LR	Blood plasma	Au coated MNPs functionalized with anti- mycrocystin-LR antibody	SERS	10 fM	171
Biological evidences	Vibrio cloreae and bovine serum albumina	Biological samples	PEG capped Fe_3O_4 NPs onto indium tin oxide coated glass	Electrochemical methods	0.5 µg L^{-1}	172

		functionalized with monoclonal antibody			
Escherichia coli DNA	River water	Fe_2O_3@Au core/shell NP-based sensor	Amperometry	5 cfu mL^{-1}	174

SPE: solid phase extraction; CE: capillary electrophoresis; RAM: restricted Access material; LC-MS: Liquid chromatography – mass spectrometry; CZE: Capillary zone electrophoresis; FD: Fluorescemnce detection; dLPME: dispersive liquid phase microextraction; DMSPE: dispersive magnetic solid phase extraction; MWCNT: Multi-walled carbon nanotubes; LC-DAD: liquid chromatography – diode array detection; SDS: sodium dodecyl sulphate; CTAB: Cetyl trimethylammonium bromide; NPs: Nanoparticles; TNT: 1,3,5-trinitrotoluene; FITC-HDA: Fluoresceine isothiocyanate conjugated to 1,6-hexanediamine; AuNP: Gold nanoparticle; AgNPs: Silver nanoparticles; SERS: Surface enhanced Raman spectroscopy; TATP: triacetone triperoxide; MNPs: Magnetic nanoparticles; PEG: Polyethylene glicol.

9. Conclusions and future trends

Smart nanomaterials have begun to be recognized as important components in forensic analysis since they clearly enhance the analytical performances in terms of selectivity, sensitivity and detection limits of the procedures. Their unique properties make smart nanomaterials interesting elements for signal amplification in the field of electrochemical DNA-based sensing.

Furthermore, the combination of different smart nanomaterials, each with its characteristics, as it has been demonstrated through the numerous examples of this chapter, to increase even more the performances of biosensors is a well-accepted and widely used strategy. The synergy of multifunctional materials and recognition elements improves the selectivity, stability, and reproducibility of the procedures, thus promoting the development of methodologies for assays and bioassays. One of their main potential applications in forensic sciences is the analysis of biological fluids and, in this sense, the capability of the developed procedures for “point-of-care” or “on-site” applications is highly desired.

Finally, we want to indicate that surely, other type of nanomaterials could have been included in this chapter, such as metal nanoparticles (Au, Ag,…), metal oxide nanomaterials silicon nanowires, quantum dots and so on, because they have been also used in forensic analysis. However, the need to summarize all the information on smart materials and forensic sciences in a book chapter has resulted in the exclusion of those nanomaterials from the body of the text.

In summary, forensic science will definitively benefits from the new developments in nanotechnology and material science as well as in custom engineering of bio/recognition components and with those the parallel advance of the progress of useful and reliable sensors and biosensors.

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Bloc 2. Materials genèrics

Section 2. Generic materials

Capítol 3 */ Chapter 3. Determination of the new psychoactive substance dichloropane in saliva by microextraction by packed sorbent – ion mobility spectrometry.*

Journal of Chromatography A, 1603 (2019) 61-66

Capítol 4 */ Chapter 4. Methylone determination in oral fluid using microextraction by packed sorbent coupled to ion mobility spectrometry.*

Microchemical Journal, 153 (2020) 104504

Capítol 5 / Chapter 5. *Determination of third generation synthetic cannabinoids in oral fluids.*

Journal of Analytical Toxicology, 45 (2021) 331-336

Capítol 6 / Chapter 6. *Development of pipette tip-based poly (methacrylic acid-co-ethylene glycol dimethacrylate) monolith for the extraction of drugs of abuse from oral fluid samples.*

Talanta, 205 (2019) 120158

Capítol 7 / Chapter 7. *Dual mixed-mode poly (vinylpyridine - co - methacrylic acid - co- ethylene glycol dimethacrylate)-based sorbent for acidic and basic drug extraction.*

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Determination of the new psychoactive substance dichloropane in saliva by microextraction by packed sorbent – ion mobility spectrometry

Aitor Sorribes-Soriano, Amparo Monedero, Francesc A. Esteve-Turrillas, Sergio Armenta

Department of Analytical Chemistry, University of Valencia, 50th Dr. Moliner St., 46100 Burjassot, Spain

Abstract

A simple procedure based on microextraction by packed sorbent (MEPS) has been proposed for the extraction of dichloropane in oral fluids and its determination by ion mobility spectrometry (IMS). Extraction conditions such as type of sorbent (octyl and octadecyl silica), sample pH, number of sample loadings, and elution volume were evaluated to obtain the most appropriate values. Dichloropane was extracted from saliva samples using C8 MEPS, loading with 100 µL sample (adjusted to pH 7) in 4 cycles, washing with 100 µL deionized water, and eluting with 50 µL 2-propanol in 10 cycles. The proposed MEPS procedure has been validated in terms of linearity, accuracy, and precision. A limit of detection of 30 µg L^{-1} was obtained for dichloropane determination in saliva. The analysis of field and synthetic saliva samples spiked with dichloropane at concentration levels from 250 to 750 µg L^{-1} provided relative recoveries between 85 and 110 %, using the proposed MEPS-IMS procedure. Field oral fluid samples were collected from healthy individuals, blind-spiked from 92 to 278 µg L^{-1}, and analysed by IMS and gas chromatography-mass spectrometry, being the results obtained from both methods statistically comparable. Thus, the proposed MEPS-IMS procedure involves a simple, sensitive, and accurate analysis of dichloropane in saliva.

Keywords: *dichloropane, new psychoactive substances, micro extraction by packed sorbent, ion mobility spectrometry, gas chromatography-mass spectrometry, saliva*

Introduction

New psychoactive substances (NPS) are defined by the United Nations Office on Drugs and Crime (UNODC) as substances of abuse which have similar effects to drugs under international control conventions, but not controlled yet [1]. Most NPS tries to mimic or increase physiological effects produced by classical controlled drugs such as cocaine, amphetamine, and Δ9-tetrahydrocannabinol. It is the case of the phenyltropanes, a family of chemical compounds structurally related to cocaine. The main feature differentiating phenyltropanes from cocaine is the lack of the ester functionality at the 3-position of the benzene, being the phenyl group directly attached to the tropane skeleton without further spacer group (see Figure 38). One example of those compounds is dichloropane, ((−)-2β-carbomethoxy-3β-(3,4-dichlorophenyl)tropane, also called RTI-111 or O-401, a stimulant substance synthesized to investigate the binding affinity of cocaine-like substances at monoamine transporters. The first seizure of this substance was reported to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) in late 2016 by the Swedish police, while another sample of dichloropane was collected by the Slovenian National Forensic Laboratory in the same year [2]. Up-to-date few studies exist regarding the pharmacology, metabolism, or toxicity of dichloropane in humans [3,4]. To the best of our knowledge, there is only one precedent of the analysis of dichloropane in biological fluids (serum, femoral blood and urine) for the determination of its uptake [5]. Dichloropane, its ester hydrolysis metabolite, and the *N*-demethylation product were detected and quantified using a liquid chromatography tandem mass spectrometry (LC-MS) procedure after solid-phase extraction (SPE) using mixed mode (C_8:strong cation exchanger) cartridges, with concentrations of few μg L^{-1}. This study concluded that unchanged dichloropane can be considered a suitable consumption marker in human body fluids.

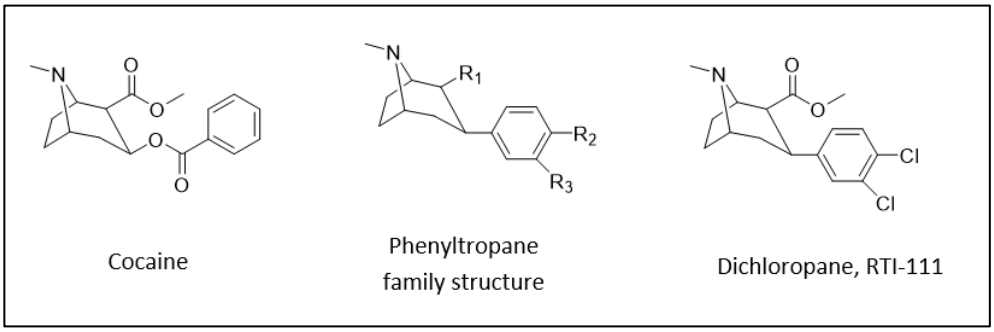

Figure 38. *Molecular structures of cocaine, phenyltropane and dichloropane.*

As it has been previously indicated, most of analytical methods for toxicological analysis of NPS in biological fluids require a preliminary sample treatment step, being SPE the most widely employed method, using hydrophobic and/or ion-exchange sorbents. To overcome the main drawbacks of SPE, novel miniaturized SPE techniques have been developed and applied for drug analysis in biological fluids, requiring less time and labour than multi-step procedures of SPE and allowing the integration of activities. Among them, it can be highlighted solid-phase microextraction (SPME), micro-SPE, stir bar sorptive extraction (SBSE), and microextraction by packed sorbent (MEPS) [6]. MEPS was introduced in 2004 as a simple, fast, and on-line sample-preparation technique based on a miniaturized SPE, with great advantages such as the low sample volume and solvent consumption [7]. In MEPS, a small amount of solid sorbent is packed between the barrel and the syringe needle, which may work manually of automatically. Different sorbents can be employed based on reversed phases, normal phases, mixed mode, or ion-exchange chemistries. MEPS has been previously employed for the extraction of benzodiazepines from beverages [8], and local anaesthetics [9], psychotropic drugs [10], antipsychotic drugs in human plasma [11], and NPS in oral fluids [12,13] being considered a suitable tool for quick sample preparation of biological matrices in forensic and clinical areas. However, in our knowledge MEPS has not been employed for the extraction of NPS in biological fluids yet.

Abuse drug consumption analysis is typically carried out in plasma, serum and urine. However, an increase on the use of drug tests addressed to oral fluid analysis instead of traditional fluids is observed, mainly due to the advantages provided by oral fluid sampling, regarding: i) easy and quick collection of sample; ii) non-invasive technique; iii) non-metabolized drugs are typically found; and iv) allow supervised sampling to avoid exchanges or adulterations [14]. Moreover, detection time windows of drugs in saliva are relatively similar than those in blood [15].

Ion mobility spectrometry (IMS) is an analytical technique based on the gas-phase separation of ionized analytes under an electric field at ambient pressure, which provide a quick and high sensitive determination of organic compounds in simple matrices. The use of IMS provides many advantages against techniques like MS, such as it operates at atmospheric pressure, do not require the use of gas cylinders or vacuum pumps, and it shows a high portable capacity for in-field analysis. However, the analysis of complex matrices like biological fluids by IMS is typically not feasible due to the presence of interferences and competitive ionization phenomena. Thus, it requires the use of chemometric tools for data treatment [16] or the previous extraction and clean-up of target analytes by using

assorted techniques such as liquid phase microextraction (LPME), SPE, SPME, and SBSE [17].

So, the main objective of this paper is the development of a fast, selective, and sensitive procedure for the determination of dichloropane in oral fluids based on IMS measurements after extraction by MEPS. Operational conditions have been evaluated such as: sample pH, number of loadings, elution solvent, and elution volume. The proposed MEPS-IMS method has been validated in terms of sensitivity, selectivity, accuracy, and precision. Synthetic and field saliva samples were blind-spiked with dichloropane and analysed by a reference methodology based in gas chromatography-mass spectrometry (GC-MS) in order to compare the obtained results.

Experimental Section

Material, reagents and samples

Dichloropane was kindly provided by the Unidad de Inspección de Farmacia y Control de Drogas (Valencia, Spain). Dichloropane working standard solutions were prepared in 2-propanol and kept at 4 ºC in amber glass vials. Cocaine-D_3 was obtained from Sigma (San Luis, MO, USA) and employed as internal standard for GC-MS determinations. All the organic solvents and buffer constituents used in this study were obtained from Scharlab (Barcelona, Spain). Buffer solutions were prepared at 0.1 M with acetate (pH 4), phosphate (pH 7 and 12), and ammonium (pH 9) salts.

MEPS was performed using an eVol XR digitally controlled positive displacement dispensing system, with 100 µL syringe, and a 22 gauge needle (0.72 mm outside diameter, 55.5 mm in length) from SGE Analytical Science (Victoria, Australia). The employed sorbent materials contained 4 mg silica with 45 µm mean particle size and 60 Å pore size, functionalized with octyl (C_8), and octadecyl (C_{18}) groups, obtained from SGE Analytical Science.

Synthetic saliva was prepared following the guidelines proposed by the Centre for Applied Science and Technology [18], using mucin from porcine stomach Type II from Merck (Darmstadt, Germany). Field saliva samples were obtained from volunteers who provided their consent after appropriate information about the study following the Ethical guidelines established by the University of Valencia (H1454687358321 – drug analysis in biofluids). Field samples were collected by spitting into an 1.5 mL Eppendorf tubes and stored at −20 ºC until analysis.

Saliva samples were spiked with different amounts of dichloropane using 10 µL concentrated standards prepared in 2-propanol.

MEPS procedure

All extraction steps were carried out using a 100 µL semiautomatic pipette working at the lowest plunger speed (10 µL s^{-1}) in order to increase the contact time between analyte and sorbent. A summary of the proposed MEPS method is shown in Figure SM1 of Supplementary Material. Sorbent conditioning was performed with 3x 100 µL 2-propanol and 2x 100 µL deionized water. Dichloropane extraction was carried out by loading 100 µL standard/sample solution, previously adjusted to pH 7 with a phosphate buffer, through the MEPS 4 consecutive times (4x 100 µL) using a 200 µL glass GC insert. Then, the sorbent was washed with 4x 100 µL deionized water and dried by passing 3x 100 µL air using the eVol XR digitally controlled positive displacement dispensing system with a 100 µL syringe. Finally, dichloropane was eluted with 50 µL 2-propanol using 10 cycles (10x 50 µL), being the final volume 50 µL.

Saliva samples were previously centrifuged at 5000 rpm for 5 min to separate solids from the liquid matrix and to avoid any syringe clogging. Sample pH was adjusted by adding 100 µL phosphate buffer (1 M, pH 7) previous to the MEPS extraction following the aforementioned procedure.

IMS procedure

Acquisitions were performed in an IONSCAN-LS IMS from Smiths Detection (Morristown, NJ, USA) equipped with a ^{63}Ni foil radioactive ionization source. IM station software (version 5.389) was used for acquisition and processing of data. Plasmagrams were acquired in positive ion mode using nicotinamide as internal calibrant. Plasmagrams were collected with a scan period of 25 ms, 60 number of segments per analysis, and 657 data points. A 300 mL min^{-1} counterflow dry air was introduced as drift gas at the end of the drift region. Drift tube conditions were 251 V cm^{-1} electric field strength, 1759 V total drift voltage, and a 7 cm length drift tube. Sample extract was placed on a PM 2.5 air monitoring polytetrafluoroethylene membrane (46.2 mm diameter, 40 µm thickness, and 2 µm pore) with a 0.38 mm polypropylene support ring, obtained from Whatman (Florham Park, NJ, USA), and thermally desorbed at 250 ºC for 30 s to transfer the analytes to the ionization and drift regions.

For IMS, a calibration curve was prepared with 1 mL dichloropane standard, from 100 to 1000 μg L^{-1} dichloropane in pH 7 phosphate buffer and extracted using the proposed MEPS procedure. Quantitation was based on the injection of 7 μL of the extracted solution in the IMS and the measurement of peak area of dichloropane on the average ion mobility spectrum of 4 s desorption time.

GC-MS procedure

The employed GC-MS system from Agilent Technologies (Palo Alto, CA, USA) consists of a 7890A GC system, equipped with an HP-5ms capillary column (30 m x 0.25 mm, 0.25 μm) and a 5975C inert XL EI/CI MSD triple axis single quadrupole detector. One μL of sample extract was injected in splitless mode at 250 °C, using 1 mL min^{-1} helium flow as carrier gas. Oven temperature program was 150 °C, held for 1 min, increased at a rate of 10 °C min^{-1} up to 250 °C, and finally held 5 min. Transfer line and ion source temperatures were 300 and 250 °C, respectively. Electron impact ionization was performed at a 70 eV electron voltage in selected ion monitoring (SIM) mode. Quantifier and qualifier selected ions were 82 and 96 m/z, for dichloropane and 85 and 185 m/z for the chromatographic standard (cocaine-D_3).

For GC-MS, a calibration curve was prepared with 1 mL dichloropane standard from 100 to 1000 μg L^{-1} dichloropane in pH 7 phosphate buffer and extracted using the proposed MEPS procedure. After extraction, cocaine-D_3 was spiked, as chromatographic standard, at 500 μg L^{-1} in both sample and standard extracts. Finally, 1 μL of those solutions was injected and analysed by GC-MS.

Results and discussion

Evaluation of IMS instrumental parameters

The obtained IMS plasmagram for a 500 μg L^{-1} dichloropane standard is shown in Figure 39. As it can be seen, the most intense peak at 9.57 ms drift time corresponded to nicotinamide signal, the IMS internal calibrant with a reduced mobility (K_0) of 1.860 cm^2 V^{-1} s^{-1}, which was used to correct temperature and pressure variations. Dichloropane showed a signal at 15.84 ms drift time (K_0 = 1.122 cm^2 V^{-1} s^{-1}), consistent with previously reported values [19], which corresponds to the analyte molecular ion peak. Thus, an alarm was generated in the IMS equipment to alert the presence of dichloropane in the analysed MEPS saliva extracts, using the following peak descriptors: (i) a K_0 value of 1.122, (ii) a peak drift

time variability value of 0.05 ms, (iii) a 1.5 peak amplitude, (iv) a 20 signal threshold value, and (v) a 200 µs full width value at the half-maximum amplitude of the peak.

The most relevant IMS parameters such as injection volume and desorption temperature were evaluated in order to reach the maximum sensitivity of the technique.

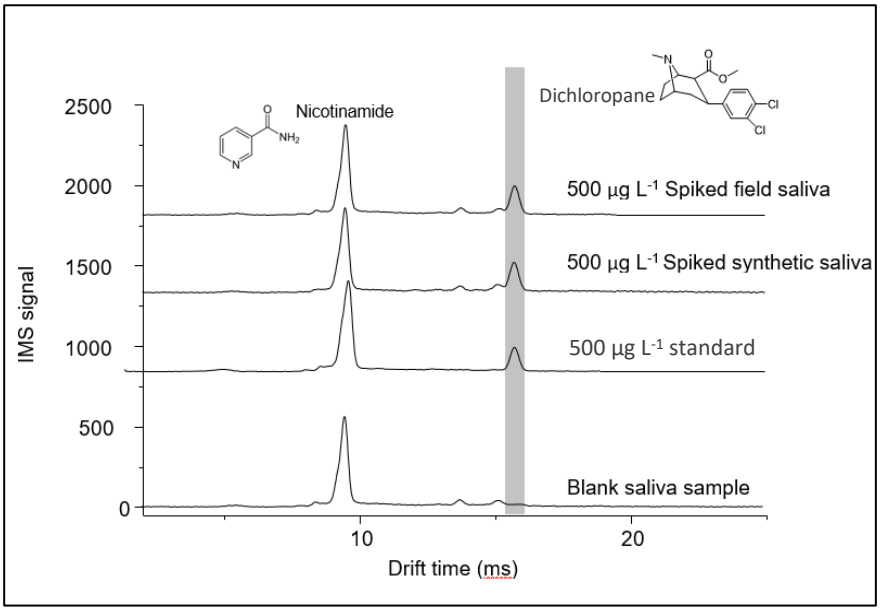

Figure 39. *Ion mobility spectrometry plasmagrams obtained for the microextraction by packed sorbent using C8 sorbent of a blank saliva sample (A), a dichloropane standard in pH 7 buffer (B), a spiked synthetic saliva (C), and a spiked field saliva sample (D). Dichloropane concentration of standard and spiked samples was 500 µg L^{-1}.*

Effect of injection volume was evaluated by the injection, from 1 to 10 µL, of a 250 µg L^{-1} dichloropane standard in 2-propanol. Figure 40A shows the obtained results and as it can be seen, IMS signals increase with the injection volume until to reach a plateau from 7 µL. In the same way, different desorption temperatures were evaluated, from 200 to 280 °C, using 7 µL of a 250 µg L^{-1} dichloropane standard in 2-propanol. Figure 40B indicates that the highest IMS signal was obtained at 250 °C not increasing at higher temperature values.

As it is well-known, 7 µL of the extraction phase introduces in the ionization source of the IMS a large amount of solvent molecules that could decrease proton transfer to analyte molecules in gas phase, thus, reducing analyte protonation. This effect would reduce, or even could lead to the disappearance, of the analyte signal in the IMS spectrum. Figure 40C shows the effect of drying time before the

introduction of the sample extract in the ionization region, from 1 to 7 s, on the analyte signal of a 250 µg L^{-1} dichloropane standard in 2-propanol. Based on the results, increasing drying time increased the analyte signal reaching a plateau at 3 s drying time. Thus, an injection volume of 7 µL, a desorption temperature of 250 °C, and 3 s drying time were selected and employed for further experiments.

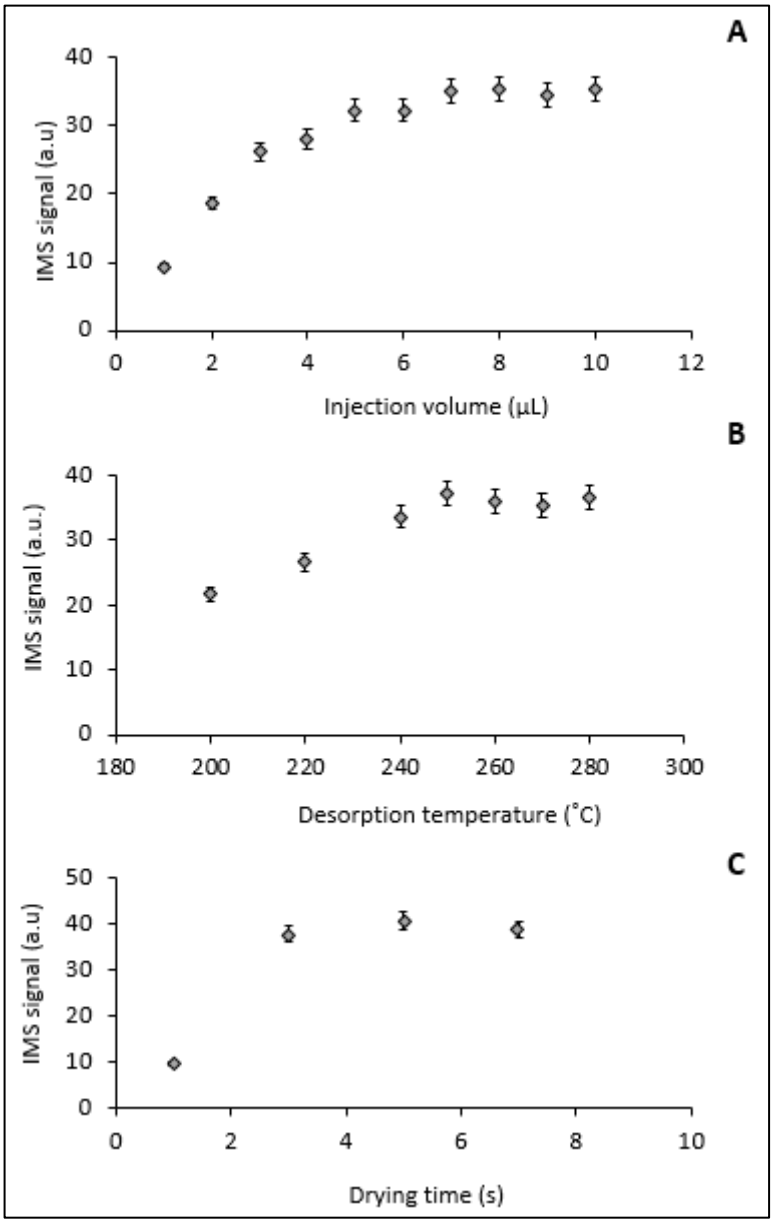

Figure 40. *Effect of injection volume (A), desorption temperature (B), and drying time (C) on the IMS signal of a 250 µg L^{-1} dichloropane standard in 2-propanol (n=3).*

Evaluation of MEPS conditions

Dichloropane is a 3',4' disubstituted phenyltropane compound with an ester group at the carbon 2 of the tropane ring. Dichloropane pK_a has not been reported in the scientific literature yet. However, pK_a of other similar compounds, such as cocaine (8.6) [20], scopolamine (7.6) [21], hyoscine (7.25) [22], and atropine (9.8) [23] have been previously described. Thus, dichloropane should be a weak basic drug with an approximate pK_a in the 7.2-9.8 range. Thus, the effect of sample pH on the extraction efficiency was assessed by the analysis of a 250 µg L^{-1} dichloropane standard prepared in different buffers at pH 4, 7, 9, and 12. Thus, 100 µL buffered standard were loaded 10 consecutive times by MEPS using the C_8 sorbent, washed with 100 µL deionized water (4x 100 µL), and eluted with 50 µL 2-propanol (10x 50 µL). Preliminary results showed a high recovery at pH 4 that decreased at pH values higher than 9. In spite of the initial hypothesis in which dichloropane extraction using C_8 or C_{18} solid sorbents should perform better at basic pH, IMS signal decreased when pH increased. A possible explanation can be the potential hydrolysis of the ester group present in dichloropane structure that may occur at alkaline pHs. To demonstrate this hypothesis, the analysis of a 250 µg L^{-1} dichloropane standard prepared in different buffers at pH 4, 7, 9, and 12, was incubated at different times, from 0 to 180 min, and then were analysed by the proposed procedure. Figure 41A shows the obtained results for dichloropane recovery as a function of the incubation time and pH. As it can be seen, for low incubation times, slightly high recoveries of dichloropane were obtained at high pH values; however, an increase of the incubation time reduces dichloropane recovery for basic solutions due to the hydrolysis of the ester group. Nevertheless, a pH 7 value was selected for further experiments in order to increase the robustness of the procedure

Other extraction conditions such as: nature and volume of elution solvent, nature of the solid sorbent, and number of loading cycles, were evaluated in order to increase the efficacy of MEPS.

Dichloromethane, acetonitrile, methanol and 2-propanol were evaluated as elution solvents to elute dichloropane from MEPS. 100 µL of a 250 µg L^{-1} dichloropane standard in water at pH 7 were placed in a GC insert and loaded 10 times by MEPS using C_8 and C_{18} sorbents. After a washing step with 100 µL deionized water (4x 100 µL), dichloropane was eluted using 50 µL of each aforementioned solvent (10x 50 µL) in independent experiments. The obtained IMS signals are shown in Figure SM2 of Supplementary Material. Recoveries were calculated by

comparison to a direct injection of a standard at the same level of concentration without any sample treatment. Dichloromethane and 2-propanol provided the highest elution of dichloropane with recoveries values of 75 ± 3 and 71 ± 4 %, respectively, while the other evaluated solvents provided recoveries lower than 55 %. The elution strength of dichloromethane and 2-propanol were quite similar, and thus, 2-propanol was selected due to its non-toxic and environmentally friendly character. A second extraction using 10 elution cycles with 50 µL 2-propanol was also performed to confirm that no more analyte was retained in the sorbent.

The effect of the nature of the solid sorbent on the dichloropane extraction was evaluated. Silica-based sorbents such as ion exchangers, C_2, C_8, and C_{18} are commercially available by the supplier. C_8 and C_{18} were selected for dichloropane extraction due to the wide use of this kind of sorbents. Thus, a calibration curve of dichloropane from 100 to 1000 µg L^{-1} prepared in pH 7 buffer was extracted by using both, C_8 and C_{18} sorbents, using the recommended MEPS procedure, and analysed by IMS. A similar sensitivity was achieved for both sorbents (see Table 18 and Figure SM3 of Supplementary Material for details), being slightly more sensitive and reproducible using the C_8 sorbents.

The interaction of the target analyte by MEPS requires enough contact time with the employed sorbent. Thus, several extraction cycles were performed for the extraction of 100 µL of a 250 µg L^{-1} dichloropane standard prepared in pH 7 buffer, passing the sample several times through the C_8 sorbent, to assess the number of cycles required to obtain the maximum extraction efficiency. After sample loading, C_8 sorbent was washed with 100 µL deionized water (4x 100µL), dried and eluted using 10 extraction cycles with 50 µL 2-propanol. Figure 41B shows the IMS signal obtained for different extraction cycles, from 1 to 10, of the aforementioned standard. As it can be seen, 4 extraction cycles provided the maximum extraction of dichloropane.

In order to increase the enrichment factor of the proposed procedure a reduction of the elution solvent volume was also proposed. Thus, 100 µL of a 250 µg L^{-1} dichloropane standard prepared in pH 7 buffer was extracted in four cycles by using C_8 sorbent, washed with deionized water (4x 100µL), dried and eluted with 25, 50, and 100 µL 2-propanol (10x each volume) in independent experiments. Quantitative recoveries were obtained for 50 and 100 µL 2-propanol (87 ± 4 and 100 ± 4 %, respectively), being the recovery obtained for 25 µL 2-propanol lower than 50%. Thus, a volume of 50 µL 2-propanol was selected for

further experiments. Thus, an elution volume of 50 µL was selected in order to have a pre-concentration factor of 2 to increase the sensitivity of the proposed approach.

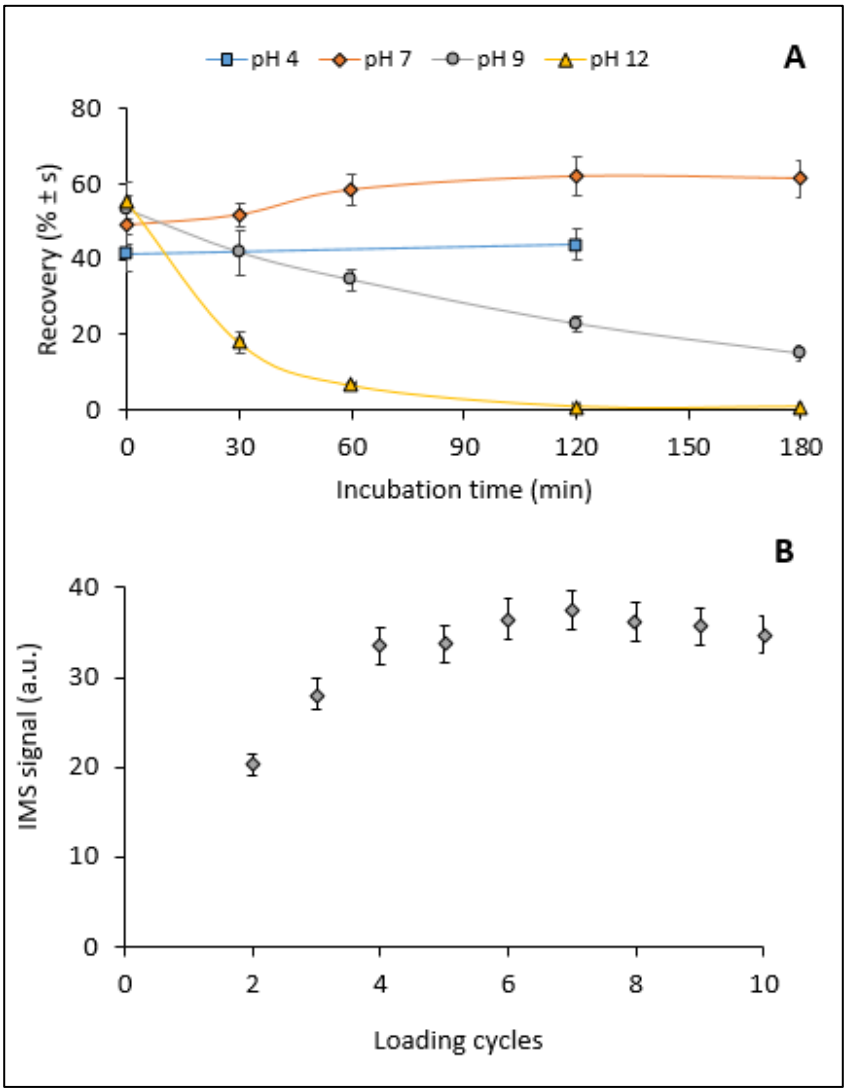

Figure 41. *Effect of sample pH at different incubation times (A) and number of loading cycles (B) on the IMS signal of a 250 µg L^{-1} dichloropane standard in 2-propanol (see the text for details).*

Application of MEPS-IMS for dichloropane determination in oral fluids

Analytical features of the proposed methodology were evaluated in terms of linearity, precision and limits of detection (LOD) and quantification (LOQ) as shown in Table 18. Calibration curves were prepared with dichloropane standards from 100 to 1000 µg L^{-1} prepared in a pH 7 buffer and extracted by C_8 based MEPS and

analysed by IMS. Linearity was assessed by the determination coefficient (R^2) of the curve obtained after the application of the recommended MEPS procedure. The obtained R^2 value was 0.999.

Precision was calculated as the relative standard deviation (RSD) of the signal obtained for three independent measurements of a 250 µg L^{-1} dichloropane standard in pH 7 buffer treated by the recommended MEPS procedure, being obtained a value of 8 %. LOD and LOQ values were calculated as three and ten times the standard deviation of the intercept divided by the slope of the calibration curve with values of 30 and 90 µg L^{-1}, respectively. LOD and LOQ values were confirmed by the analysis of dichloropane solutions prepared at these concentration levels, giving measurable signals.

Table 18. *Analytical parameters obtained for the determination of dichloropane by microextraction by packed sorbent using C_8 and C_{18} sorbents and ion mobility spectrometry determination.*

Parameter	C8	C18
Slope (L μg^{-1})	0.123 ± 0.002	0.101 ± 0.005
Intercept	-0.1 ± 0.9	5 ± 2
R^2	0.999	0.989
LOD (µg L^{-1})	30	40
LOQ (µg L^{-1})	90	130

The proposed MEPS method was applied to the IMS analysis of dichloropane residues in oral fluids. Synthetic and field saliva samples were spiked at 250, 500 and 750 µg L^{-1} dichloropane using 10 µL of a concentrated standard in 2-propanol, and analysed by the proposed MEPS procedure using C_8 as sorbent. Figure 39 shows the obtained IMS plasmagram for the synthetic and field saliva samples spiked at 500 µg L^{-1} dichloropane. As it can be seen, dichloropane can be easily identified and quantified by the proposed methodology, providing a clean plasmagram free of interferent signals. The obtained relative recoveries were quantitative for both evaluated fluids, ranging from 90 to 115 % and from 85 to 107 % for synthetic and field saliva samples, respectively (see Table 19).

Table 19. *Relative recoveries obtained for the analysis of water, synthetic saliva, and field saliva samples spiked at different levels of dichloropane by using microextraction by C_8 packed sorbent followed by ion mobility spectrometry determination.*

Sample	**[Spiked] (μg L^{-1} ± s)**	**[Found] (μg L^{-1} ± s)**	**Recovery (% ± s)**
Water	250	220 ± 30	87 ± 14
	500	460 ± 20	92 ± 4
	750	741 ± 6	99 ± 1
Synthetic saliva	250	260 ± 30	105 ± 12
	500	550 ± 15	110 ± 3
	750	675 ± 15	90 ± 2
Field saliva	250	240 ± 18	96 ± 7
	500	535 ± 10	107 ± 2
	750	640 ± 30	85 ± 4

Additionally, blind-spiked oral fluid samples were analysed by the proposed method. Oral fluids were taken from different healthy individuals after the consumption of potential interferents, such as nicotine and caffeine. The extracts obtained after MEPS of the aforementioned samples were injected in the IMS and then, the same extracts were spiked with cocaine-D_3 as chromatographic standard and measured by GC-MS. The results obtained for the 6 oral fluid samples are shown in Table 20. Those results were statistically comparable using a paired t-test with a 95 % of confidence level obtaining a value of 1.80 face to the critical value of 2.57.

Table 20. *Concentration found for the analysis of blind spiked oral fluid samples by ion mobility spectrometry (IMS) and gas chromatography-mass spectrometry (GC-MS) determination after microextraction by packed sorbent using C_8.*

Sample	**[Found] (μg L^{-1} ± s)**	
	IMS	**GC-MS**
1	131.0 ± 1.7	126 ± 6
2	218 ± 4	267 ± 13
3	120 ± 4	124 ± 6
4	155 ± 6	153 ± 8
5	222.9 ± 1.2	278 ± 14
6	93 ± 7	92 ± 5

Finally, Table 21 shows a comparison of the analytical performance for the analysis of dichloropane in saliva by MEPS using IMS and GC-MS as detection techniques. As it can be seen, IMS provides a slightly better sensitivity and high portable capacity.

Table 21. *Comparison of analytical performance for the analysis of dichloropane in saliva by MEPS and ion mobility spectrometry (IMS) and gas chromatography-mass spectrometry (GC-MS).*

Parameter	IMS	GC-MS
Limit of detection (μg L^{-1})	30	70
Limit of quantification (μg L^{-1})	90	200
Determination coefficient (R^2)	0.9972	0.9971
Precision (RSD, %)	6	4
Portable	High	Medium

Conclusions

MEPS has been employed for the extraction of dichloropane from oral fluid samples followed by IMS determination. The main parameters of the extraction procedure were studied for its validation and application to the analysis of oral fluid samples. The loading pH was studied by the preparation of standard solutions in water incubated at different pH (4, 7, 9, and 12), being observed a dichloropane degradation at basic pH; thus, the samples were buffered at pH 7. The elution volume was also evaluated to obtain the most appropriate enrichment factor with a quantitative recovery, being an elution with 10x 50 μL 2-propanol the selected conditions. The features of C_8 and C_{18} sorbents were also studied obtaining a better sensitivity by C_8 with a LOD of 30 μg L^{-1}. Finally, the proposed procedure was used for the analysis of blind-spiked field saliva samples and the obtained results by IMS were statistically comparable to those obtained by GC-MS, showing no significant differences.

Acknowledgements

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Conflict of Interest Disclosure

The authors declare no competing financial interest

Appendix A. Supplementary data

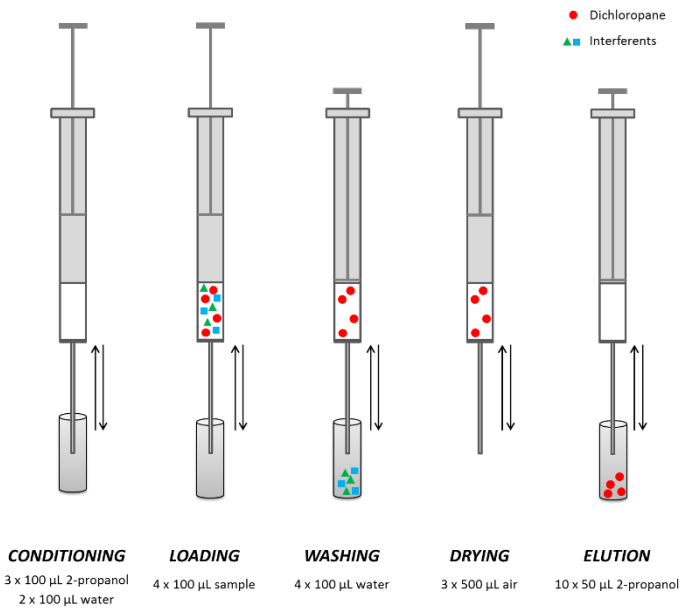

Figure SM1. *Proposed micro extraction by packed sorbent procedure.*

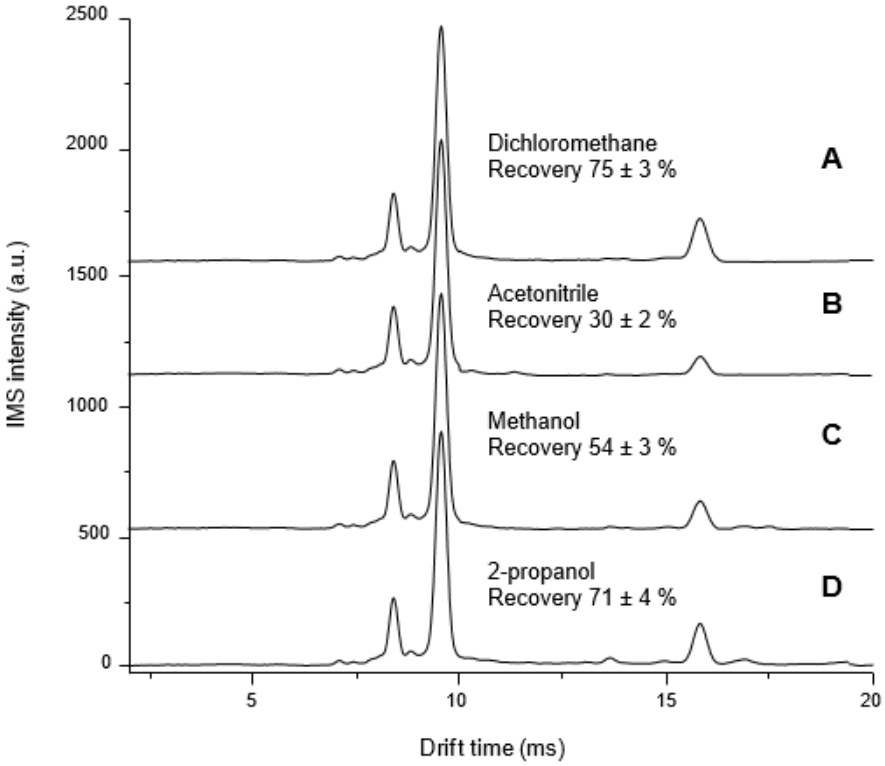

Figure SM2. *IMS plasmagrams obtained for the MEPS extraction of 100 µL of a 250 µg L^{-1} dichloropane standard in water at pH 7 (10x cycles) using C_8 sorbent, washed with 100 µL deionized water (4x cycles), and eluted with 50 µL (10x cycles) dichloromethane (A), acetonitrile (B), methanol (C) and 2-propanol (D).*

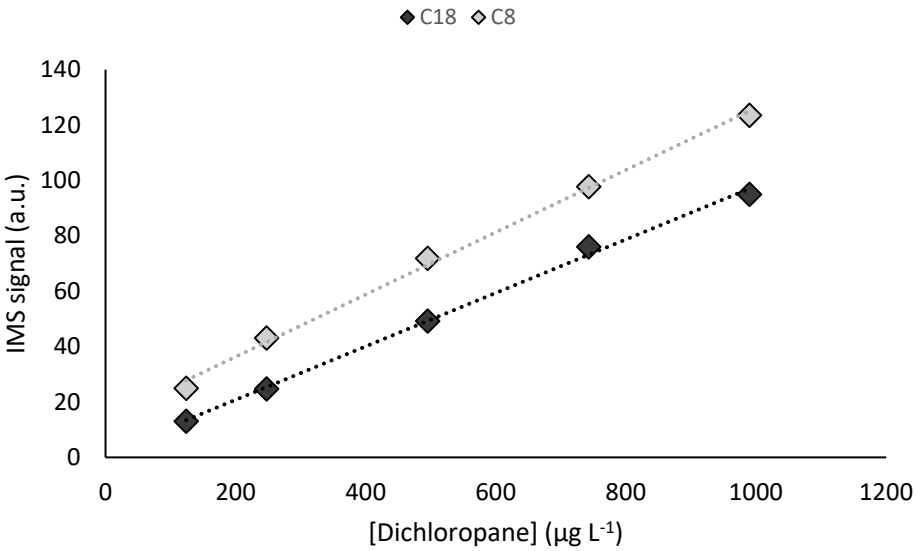

Figure SM3. *Calibration curves obtained from dichloropane standards from 100 to 1000 µg L^{-1} prepared in a pH 7 buffer and extracted by MEPS using C_8 (grey diamonds) and C_{18} (black diamonds) sorbents.*

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Methylone determination in oral fluid using microextraction by packed sorbent coupled to ion mobility spectrometry

A. Sorribes-Soriano, S. Sánchez-Martínez, R. Arráez-González, F.A. Esteve-Turrillas, S. Armenta

Department of Analytical Chemistry, Universitat de València, 50th Dr. Moliner St., 46100, Burjassot, Spain.

Abstract

A methodology based on microextraction by packed sorbent (MEPS) followed by ion mobility spectrometry (IMS) has been developed for the simple and automated determination of methylone in oral fluids. MEPS was carried out using a 100 µL eVol® XR hand-held automated analytical syringe filled with octadecyl silica sorbent. The effect of main experimental parameters of MEPS such as sample pH and ionic strength, number of aspirating/dispensing cycles, and elution solvent was studied to obtain a quantitative extraction of methylone. Oral fluid samples were spiked with methylone at different concentration levels from 50 to 400 µg L^{-1} and analyzed by the proposed MEPS-IMS procedure, giving quantitative recoveries from 78 to 91 % and precision values, estimated as the relative standard deviation, lower than 9 %. The proposed MEPS-IMS method was tested under extreme conditions using spiked field saliva samples of volunteers in different situations such as 15 minutes after drinking coffee and syrup, and smoking cigarettes and cannabis with appropriate results. Moreover, results obtained by IMS were statistically comparable to those obtained by ultra high performance liquid chromatography - tandem mass spectrometry.

Keywords: *methylone; oral fluid; microextraction by packed sorbent; ion mobility spectrometry; ultra high performance liquid chromatography - tandem mass spectrometry*

Introduction

New psychoactive substances (NPS) are chemical compounds with molecular structures similar to classical drugs, such as cocaine, amphetamine, or cannabinoids, but slightly modified in order to evade legal controls and possess unknown effects on consumers [1]. The European Centre for Drugs and Drug Addiction (EMCDDA) found more than 670 different NPS, between 2005 and 2017, including synthetic cathinones and cannabinoids, arylcyclohexylamines, phenetylamines, and opioids substances, among others [2]. Synthetic cathinones, which are typically sold as bath salts, legal highs, or research chemicals, are the second most frequently seized NPS in the black market as highlighted by the United Nations Office on Drugs and Crime (UNODC) [3]. Methylone is one of the most well-known synthetic cathinones being its structure closely related to that of 3,4-methylenedioxymethamphetamine (MDMA) with the β-keto group characteristic of cathinones [4]. This fact explain the effects of methylone that include empathy, openness, and increased energy and libido, but also harm effects related to neurological, cardiac, and psychiatric damages [5,6]. Their mechanism of action is based primarily on inhibiting the uptake of norepinephrine, serotonin and dopamine by monoamine neurotransmitter transporters. However, the full, metabolism in the human body is not yet completely understood [7]. Legal status of methylone is strongly dependent of the country mentioned. Canada lists methylone as amphetamine derivate [8]. In the UK, all cathinone derivatives, including mephedrone, methylone, and methedrone are Class B drugs under the Misuse of Drugs Act 1971 [9]. In the United States methylone was reclassified as Schedule I under the Controlled Substance Act in 2013 [10]. Methylone is a Schedule I controlled substance in Denmark, Ireland, Romania and Sweden [11].

Usually, the NPS analysis in biological matrices is carried out by solid phase extraction (SPE) or liquid-liquid extraction (LLE) followed by a chromatography determination coupled to mass spectrometry [12,13,14,15]. Alternative miniaturized extraction methods have been proposed. Attending to the need of faster and sensitive techniques, compatible with the Green Analytical Chemistry principles, solid phase microextraction (SPME), stir bar-sorptive extraction (SBSE), magnetic solid phase extraction (MSPE), or dispersive micro-solid phase extraction (DSPE) [16] were developed. In this sense, microextraction by packed sorbent (MEPS) is a SPE-based technique where a small amount of sorbent (0.5 – 4 mg) is packed at the end of a needle which is inserted in a semiautomatic syringe. The main advantages offered by MEPS are: automatable and programmable, high

reusability for biological matrices, minimum required amount of sample, and on-line coupling to detection systems [17].

Ion mobility spectrometry (IMS) is an analytical technique widely used for fast and direct detection of explosives, chemical warfare agents and drugs [18], which separates ions at atmospheric pressure under the influence of an electric field, according to their size and shape. The main benefits of IMS are related to its low cost, short response times (less than 1 min), simplicity of operation, and portability. Besides, IMS has high sensitivity and a moderate selectivity that can be easily improved with an appropriate sample treatment [19,20].

The main aim of this study is to develop a simple and automated procedure for the analysis of methylone in oral fluid samples by IMS after a MEPS sample treatment. For this purpose, the main operational parameters of MEPS extraction were studied such as: pH loading, ionic strength, loading and elution aspirating/dispensing cycles, washing solvent, and nature and volume of elution solvent. The proposed method was validated in terms of accuracy, precision, sensitivity, and selectivity. Finally, the developed MEPS-IMS procedure was applied for the analysis of spiked oral fluid samples in presence of interfering compounds and the results were compared to those obtained by ultra high performance liquid chromatography - tandem mass spectrometry (UHPLC-MS-MS).

Experimental section

Materials, reagents and samples

Methylone was kindly provided by the "Unidad de Inspección de Farmacia y Control de Drogas (Valencia, Spain). Methylone standard solution (1000 mg L^{-1}) was prepared in 2-propanol and working solutions were prepared by dilution in 2-propanol; all solutions were kept in an amber glass vials at 4 °C. Methylone-d_3, obtained from Merck (Darmstadt, Germany), was used as internal standard for UHPLC-MS-MS acquisitions.

MEPS was performed using an eVol XR digitally controlled positive displacement dispensing system from SGE Analytical Science (Victoria, Australia), with a 100 µL syringe, and a 22 gauge needle (0.72 mm outside diameter and 55.5 mm length), containing 4 mg of silica functionalized with octadecyl (C18) groups (45 µm particle size and 60 A pore size).

Organic solvents and buffer constituents were obtained from Scharlab (Barcelona, Spain). Acetate (pH 5), phosphate (pH 7 and 12) and carbonate (pH 9, 10, and 11) buffer solutions were prepared at 1 M concentration.

Oral fluid samples were obtained from volunteers who provided their consent after appropriate information about the study following the ethical guidelines established by the Ethical Committee on Experimental Investigation of the University of Valencia (H1454687358321 – drug analysis in biofluids). Field samples were collected by spitting approximately 0.5 mL oral fluid into 1.5 mL Eppendorf tubes and stored at -20 °C until analysis. 0.5 mL oral fluid samples were spiked with different amounts of methylone to obtain concentration ranging from 50 to 400 µg L^{-1} using 10 µL concentrated standard solution prepared in 2-propanol.

It should be highlighted that under no circumstances have the authors trafficked or provided illegal substances, aimed, promoted, facilitated, stimulated, or forced in any way the consuming of illegal substances during this study.

MEPS procedure

500 µL oral fluid were initially collected, spiked with different methylone concentrations from 50 to 400 µg L^{-1}, per triplicate, and centrifuged at 5000 rpm for 5 min to remove solids from the matrix and to avoid the clogging of syringe. Then, 90 µL of the liquid fraction was buffered by the addition of 10 µL of 1 M carbonate buffer at pH 9.0 to increase analyte retention. Extractions were performed using the semiautomatic pipette at the lowest plunger speed (10 µL s^{-1}). Conditioning of the sorbent was carried out with 100 µL of 2-propanol followed by 100 µL of water. Sample loading was carried out using 5 aspirating/dispensing cycles of the same 100 µL sample aliquot. Washing step was carried out with a single aspirating/dispensing cycle of 100 µL of 0.1 M carbonate buffer at pH 9.0. The sorbent was dried by using 2 x 100 µL air and methylone was eluted using 100 µL of 2-propanol using 5 aspirating/dispensing cycles.

IMS determination

Acquisitions were performed in an IONSCAN-LS IMS from Smith Detection (Morristown, NJ, USA) equipped with a ^{63}Ni foils radioactive ionization source. Data acquisition and processing were done with IM station software (version 5.389). Nicotinamide was used as internal calibrant to correct for temperature and pressure variations which could affect the drift time of analytes. Plasmagrams were

obtained with a scan period of 25 ms and 60 segments per analysis. Dry air, introduced at the end of the drift region, was employed as drift gas at 300 mL min^{-1}. Drift tube length was 7 cm and 251 V cm^{-1} electric field strength was used. 3 µL sample was placed on a polytetrafluoroethylene (PTFE) membrane and thermally desorbed at 260 °C for 30 s, being the analyte introduced in the ionization and drift regions. A calibration curve was prepared from 20 to 500 µg L^{-1} methylone prepared in 2-propanol.

UHPLC-MS-MS determination

A Waters Acquity UPLC™ system from Waters (Milford, MA, USA), equipped with an autosampler, a binary solvent delivery system, a C18 Waters (1.7 µm, 2.1×50 mm) column, and a triple quadrupole detector with a Z-spray electrospray ionization source, was employed for methylone determination. The flow rate was 0.4 mL min^{-1} and mobile phases were (A) 5mM formic acid in water and (B) acetonitrile. The gradient elution consisted initially in a 100 % of A and B was increased from 0 to 30% within 6 min, to 50% within 2 min, to 100% within 4 min, and finally an isocratic elution was maintained during 3 min. MS acquisitions were done using 3.5 kV capillary voltage, 120 °C source temperature, and 350 °C desolvation temperature. The multiple reaction monitoring (MRM) conditions were adjusted for methylone (m/z 208 > 190, 160) and methylone-d_3 (m/z 211 > 193, 163) as internal standard [21]. A calibration curve was prepared from 2 to 500 µg L^{-1} with methylone-d_3 at a concentration of 100 µg L^{-1}. Sample extracts obtained by the proposed MEPS procedure were also spiked with methylone-d_3 to a final concentration of 100 µg L^{-1}.

Results and discussion

Evaluation of analytical features

Plasmagrams obtained for a 250 µg L^{-1} methylone standard prepared in 2-propanol showed an intense peak at a drift time of 9.57 ms that corresponds to the internal calibrant (nicotinamide) with a reduced mobility (K_0) of 1.860 cm^2 V^{-1} s^{-1} and methylone signal arise at a drift time of 12.19 ms (see Figure 42), which corresponds to a K_0 value of 1.433 cm^2 V^{-1} s^{-1}. It should be mentioned that it is similar to previously reported K_0 values of methylone (1.435 cm^2 V^{-1} s^{-1}) [22,23,24].

The most important analytical features of IMS for methylone determination are summarized in Table 22. The obtained coefficient of determination (R^2) of a methylone calibration curve from 20 to 500 µg L^{-1} was 0.994, indicating an acceptable linearity. The obtained slope and intercept were 0.5217 and 19.586, respectively. The relative standard deviation (RSD) of five measurements of 20 µg L^{-1} methylone standard was 6 %. The limit of detection (LOD) and quantification (LOQ) values were calculated as 3 and 10 times, respectively, the standard deviation of a 20 µg L^{-1} methylone standard divided the slope of the calibration curve. The obtained LOD and LOQ for methylone determination by IMS were 4 and 14 µg L^{-1}, respectively.

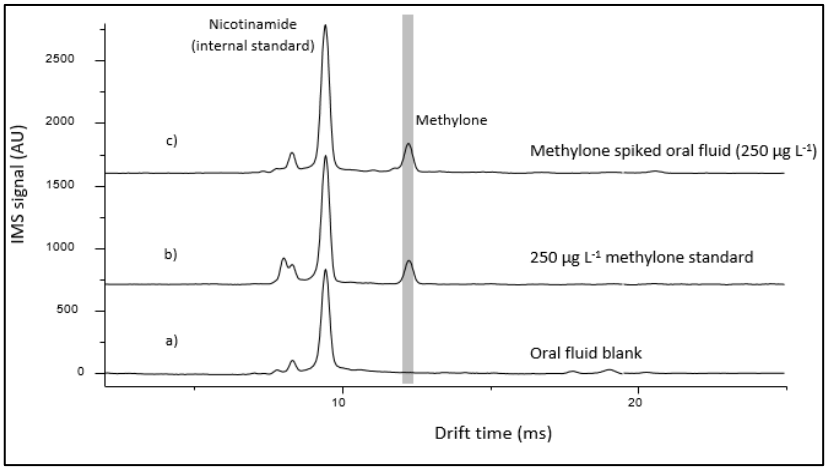

Figure 42. *Ion mobility spectrometry plasmagrams obtained for the direct analysis of a blank saliva sample (a), 250 µg L^{-1} methylone standard (b), and a blank saliva sample spiked at 250 µg L^{-1} methylone (c). Both oral fluid samples were MEPS extracted following the recommended procedure.*

Table 22. *Analytical features for the determination of methylone by of ion mobility spectrometry (IMS) and ultra performance liquid chromatography - tandem mass spectrometry (UPLC-MS-MS).*

Feature	IMS	UPLC-MS-MS
Linear Range (µg L^{-1})	14 - 500	2 - 500
$R^{2\,a}$	0.994	0.999
LOD (µg L^{-1}) [b]	4	0.6
LOQ (µg L^{-1}) [b]	14	2
RSD (%, n=5) [c]	6	5

[a] *Coefficient of determination.*
[b] *The limit of detection (LOD) and limit of quantification (LOQ) were calculated as 3 and 10 times, respectively, the standard deviation of a 20 µg L^{-1} methylone standard divided the slope of the calibration curve.*
[c] *Relative standard deviation of a 20 µg L^{-1} methylone standard for IMS and UPLC-MS-MS, respectively.*

Evaluation of MEPS condition

The main operational parameters of MEPS were evaluated by univariate studies using the recommended procedure with the variation of the target parameter. A conditioning step was carried out with 100 µL of 2-propanol and 100 µL of deionized water to remove potential interferences.

Sample pH is one of the most important parameters that may affect the extraction of target analytes from oral fluid using SPE-based techniques. pH values of oral fluid samples typically range between 6 and 7 [25], and it has been previously reported a pKa value for methylone of 7.74 [26]. Thus, different extractions were performed using 90 µL of a methylone standard at 250 µg L^{-1} prepared in water and adjusted by adding 10 µL of buffer solutions to pH values of 5.0, 7.0, 9.0, 10.0, 11.0 and 12.0. As it can be seen in Figure 43a, the use of acidic-neutral buffers provided recovery values lower than 36 % for methylone. In the case of basic buffers, the obtained methylone recoveries were higher than 83 %, with the exception of pH 12 that provide a reduced recovery (65 %), probably due to the potential hydrolysis of methylone in strong alkaline pH. So, the use of a pH 9.0 buffer was proposed for further experiments, giving a recovery value of 90 ± 2 %.

The effect of sample ionic strength on the extraction of methylone by MEPS was also studied by the addition of different concentrations of NaCl to a 250 µg L^{-1} methylone standard prepared at pH 9.0. NaCl concentrations of 0.1, 1.0 and 2.0 M were evaluated (Figure 43b), showing recovery values ranging from 68 to 86 %. Thus, effect of ionic strength is not significant for methylone extraction and no NaCl was added in further experiments.

The number of aspirating/dispensing cycles, from 1 to 25, required to quantitatively extract methylone from aqueous media was also evaluated using 100 µL of a 250 µg L^{-1} methylone standard prepared at pH 9. The obtained recovery for methylone increased with the number of aspirating/dispensing cycles reaching a plateau in 5 cycles (see Figure 43c). So, 5 aspirating/dispensing cycles were proposed for further experiments.

The use of pure organic solvents in the washing step provided a considerably elution of methylone from the C18 sorbent; so, mild conditions were employed to remove matrix interferences and retain methylone in the sorbent. Thus, blank oral fluid samples were spiked with methylone at 250 µg L^{-1} and loaded in the MEPS sorbent as aforementioned, then 100 µL of a 0.1 M carbonate buffer at pH 9.0 prepared in deionized water with different concentrations of 2-propanol (from 0 to 15 %) was evaluated as washing solution. Figure 43d shows that methylone

recovery decreases with the percentage of 2-propanol in the washing media, with values ranging from 87 to 36 %. Thus, washing step was carried out using 0.1 M carbonate buffer without the addition of 2-propanol.

Regarding the elution of methylone form the sorbent, the most important parameters to evaluate are the nature and volume of the elution solution, and the number of the employed aspirating/dispensing cycles. Pure organic solvents are typically employed as elution solution in order to break nonpolar analyte-sorbent interactions and quantitatively elute methylone. In this study 100 µL methanol, chloroform, acetonitrile, and 2-propanol were evaluated as elution solvents using 5 aspirating/dispensing cycles. The obtained recoveries for a 250 µg L^{-1} methylone standard prepared at pH 9.0, loaded and washed using the recommended MEPS conditions, were shown in Figure 43e. All the evaluated solvents provided quantitatively recoveries from 85 % for chloroform to 114 % for acetonitrile. 2-propanol was selected due to it provides a quantitative recovery of methylone (90 ± 3 %), it is considered an environmentally friendly solvent and also because of its compatibility with PTFE membranes of the thermal desorption system of the IMS instrument. Different elution volumes (50, 75, and 100) of 2-propanol, using 5 aspirating/dispensing cycles, were studied in order to obtain higher pre-concentration factor. Nevertheless, the use of 50 and 75 µL volumes provided low recoveries for methylone with values of 56.5 ± 0.4 and 61.8 ± 0.2 %, respectively. So, the use of 100 µL 2-propanol was proposed. Finally, the number of aspirating/dispensing cycles for elution step was also studied by using 2, 5, and 10 cycles, obtaining recoveries of 69 ± 8 %, 86 ± 9, and 87 ± 2 respectively. Thus, 5 aspirating/dispensing cycles of 100 µL of 2-propanol was selected as the recommended elution step to reach quantitative recoveries of methylone.

Figure 42 shows the obtained plasmagrams for the extraction of a blank oral fluid sample and an oral fluid spiked at 250 µg L^{-1} methylone concentration level. As it can be seen, the obtained signal was free of matrix interferents and it allowed a correct determination of methylone in oral fluid samples.

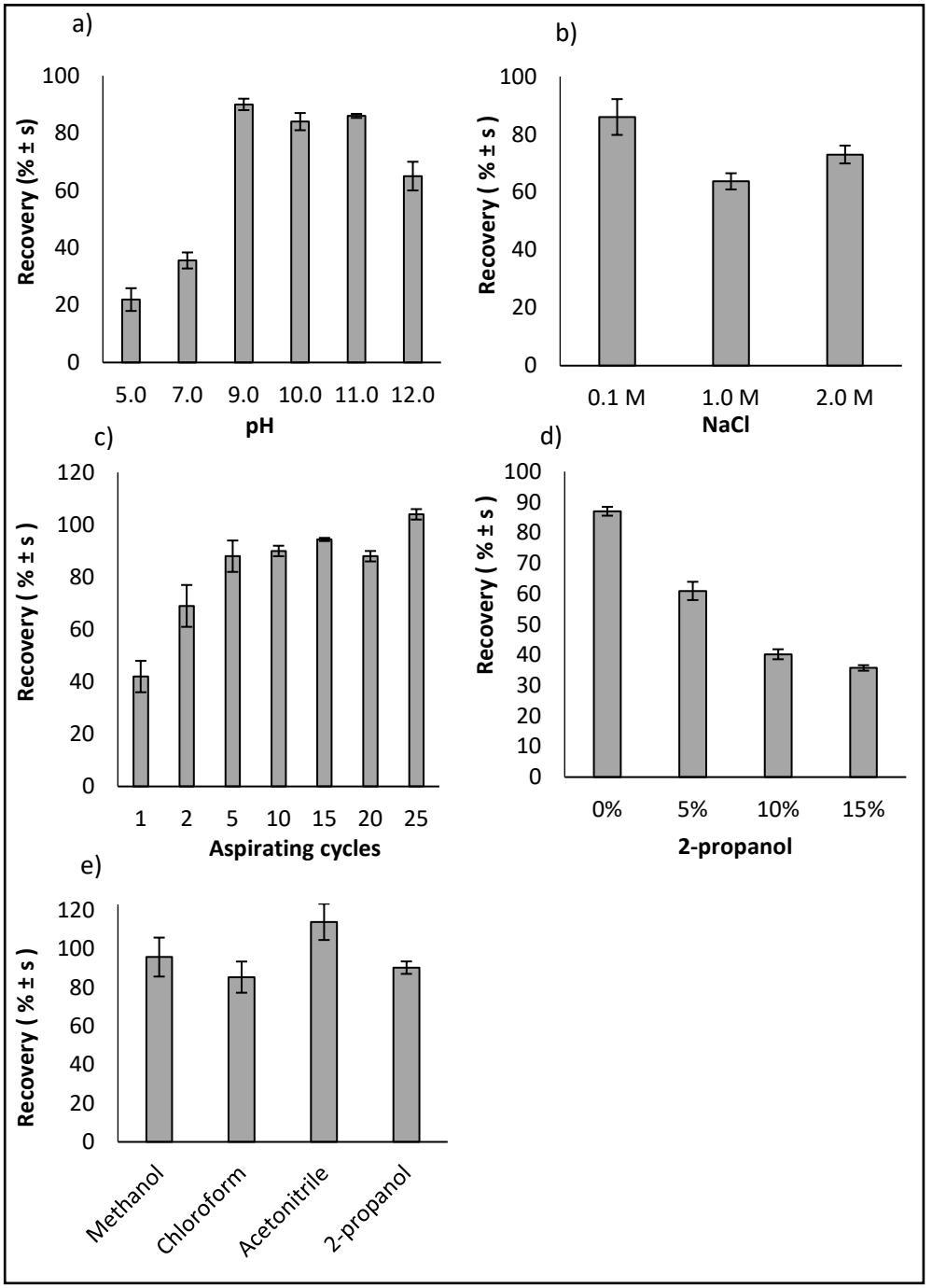

Figure 43. *Effect of sample pH (a), ionic strength (b), number of aspirating/dispensing cycles (c), and nature of washing (d) and elution (e) solution on the IMS signal of a 250 µg L^{-1} methylone standard in carbonate buffer (see the text for details).*

Determination of methylone in oral fluid samples

In previous studies of NPS in biological fluids, different concentration levels of methylone were found in blood (7 to 375 ng mL^{-1} with an average value of 89 ng mL^{-1}), in urine (1 to 91093 ng mL^{-1} with an average value of 6296 ng mL^{-1}) and in oral fluid (40 to 10027 ng mL^{-1} with an average value of 2445 ng mL^{-1}) [27]. Taking these data into consideration, the proposed MEPS procedure was used for the analysis of oral fluid samples, taken from volunteers, and spiked at different methylone concentration levels, from 50 to 400 µg L^{-1}. Table 23 shows the obtained recoveries for methylone that ranged from 78 to 91 % with a RSD value lower than 9 %.

Table 23. *Recoveries obtained for the analysis of blank field saliva samples spiked with different concentration of methylone by the proposed MEPS-IMS method.*

[Spiked] (µg L^{-1})	[Found] (µg L^{-1} ± s)	Recovery (% ± s)	RSD (%)
50	45 ± 3	90 ± 6	7
100	78 ± 6	78 ± 6	8
250	200 ± 20	81 ± 7	9
400	360 ± 20	91 ± 6	7

Selectivity of the proposed method was evaluated by the analysis of several blank oral fluid samples of volunteers in different situations such as 15 minutes after drinking coffee and syrup, and smoking cigarettes and cannabis. These samples were spiked with methylone at 250 µg L^{-1}, but additionally contained potential interfering compounds, such as caffeine, terbutaline, metoclopramide, nicotine, and tetrahydrocannabinol. Figure 44 shows the obtained plasmagrams for the aforementioned samples. Methylone was identified by the proposed method in all cases, providing recovery values from 58 to 83 % (see Table 24). As it can be seen, nicotine peak is present in oral fluids after smoking cigarettes and marihuana (drift time 11.1 ms and K_0 value of 1.59 $cm^2\ V^{-1}\ s^{-1}$) and pseudoephedrine peak is also detected in oral fluids after decongestant syrup administration (drift time 11.2 ms and K_0 value of 1.58 $cm^2\ V^{-1}\ s^{-1}$). It should be aforementioned that the lowest methylone recovery (58%) was obtained from the analysis of oral fluid after smoking, in which nicotine peak has a considerable intensity. It could be probably due to competitive ionization between nicotine and methylone in the ionization region of the IMS.

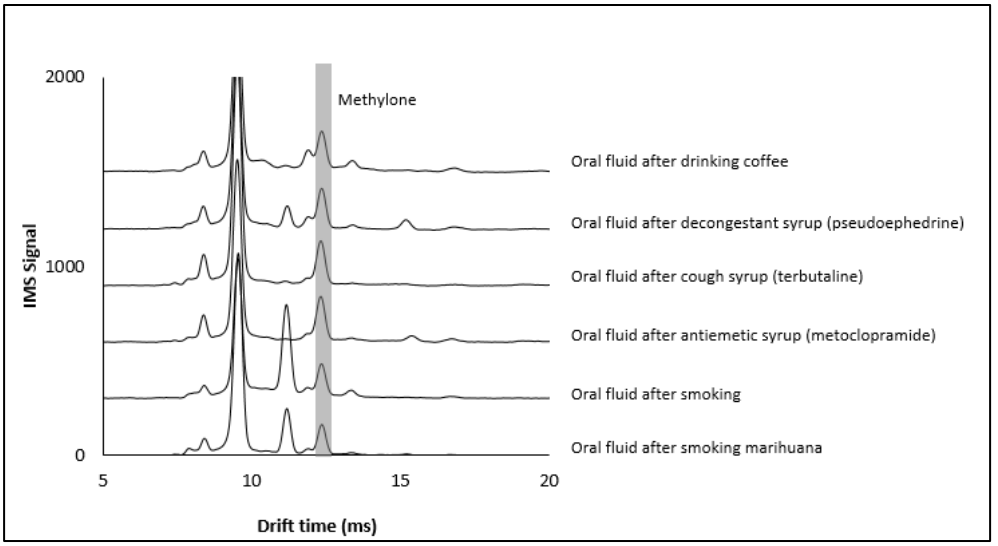

Figure 44. *Ion mobility spectrometry plasmagrams obtained for the microextraction by packed sorbent of a methylone spiked oral fluid (250 µg L^{-1}) in different situations.*

Table 24. *Recoveries obtained for blank oral fluid samples taken at different circumstances, containing potential interferents, spiked with 250 µg L^{-1} methylone and analyzed by the proposed MEPS-IMS method and the reference UPLC-MS-MS method.*

Sample	Situation	Interfering compound	[Found] (µg L^{-1} ± s)		
			IMS	UPLC-MS-MS	t_{calc}
1	Coffee drinking	Caffeine	180 ± 15	189 ± 18	0.941
2	Syrup drinking	Terbutaline	208 ± 13	212 ± 17	0.458
3	Syrup drinking	Metoclopramide	185 ± 10	193 ± 15	1.087
4	Cigarettes smoking	Nicotine	159 ± 10	204 ± 13	6.721
5	Cannabis smoking	Tetrahydrocannabinol	164 ± 21	193 ± 20	2.450

t_{tab} two tailed (gl=3+3-2, α=0.05) = 2.776

Moreover, MEPS extracts of aforementioned samples were also analysed by UPLC-MS-MS, considered as reference methodology in order to evaluate the method trueness (see Table 24). A paired samples Student's t-test was performed on the differences between results obtained for each methodologies using Equation 1.

$$t_{calculated} = \frac{|average|\sqrt{number\ of\ samples}}{standard\ deviation} \qquad \text{(Equation 1)}$$

The calculated *t*-value was 2.41, being lower than the tabulated one ($t_{(\alpha=0.05,\ fd=4)}$ = 3.18), which implies that there was no differences between the obtained results. On considering the results of each oral fluid individually, in Table 24 it can be observed the t-test results. As it can be seen, all the analysed samples provided statistically comparable results, except the oral fluid of a heavy smoker, suggesting that competitive ionization phenomena due to the presence of nicotine during the IMS acquisition of methylone may occur. Nevertheless, methylone was identified in field samples using the proposed method under extreme conditions and it was adequately quantified. In summary, the use of C18 cartridges provided enough selective extracts for the analysis of methylone by the proposed IMS methodology.

Conclusions

A simple and automated methodology based on MEPS-IMS was proposed for the determination of methylone in oral fluids. Operational MEPS conditions including pH loading, ionic strength, loading and elution aspirating/dispensing cycles, washing solvent, and nature and volume of elution solvent were studied. The proposed MEPS-IMS method provided an adequate sensitivity with LOD and LOQ values of 4 and 14 μg L^{-1}, respectively. Quantitative recoveries for methylone determination in oral fluid samples were obtained, ranging from 78 to 91 % with RSD values lower than 9 %. Selectivity of the method was assessed by the analysis of oral fluid samples spiked with methylone containing potential interfering compounds, such as caffeine, terbutaline, metoclopramide, nicotine, and tetrahydrocannabinol. In these conditions, methylone was identified in all the samples with recovery values ranging from 58 to 83 %, being the procedure appropriate for the determination of methylone in oral fluids.

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Conflicts of interest

The authors declare that have no competing financial interests in this investigation.

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Determination of third generation synthetic cannabinoids in oral fluids

Aitor Sorribes-Soriano, Josep Verdeguer, Agustín Pastor, Sergio Armenta, Francesc A. Esteve-Turrillas

Department of Analytical Chemistry, University of Valencia, Jeronim Muñoz Building, 50th Dr. Moliner St., 46100 Burjassot, Spain

Abstract

A procedure has been developed for the determination of third generation synthetic cannabinoids in oral fluid samples by using a semi-automated microextraction by packed sorbent (MEPS) procedure and gas chromatography-mass spectrometry (GC-MS) determination. Five synthetic cannabinoids were employed as model compounds 5F-ADB, MMB-CHMICA, THJ-2201, CUMYL-4CN-BINACA, and MDMB-CHMCZCA. The most adequate operative conditions for MEPS were evaluated giving quantitative recoveries, from 89 to 124 %, in synthetic and field saliva samples spiked with 125 and 250 μg L^{-1} of the studied cannabinoids, with the exception of MDMB-CHMCZCA in field saliva samples that provided slightly lower recoveries from 62 to 66 %. A high sensitivity was obtained for the proposed MEPS-GC-MS procedure with limits of detection from 10 to 20 μg L^{-1}. The obtained results demonstrate the high potential of MEPS-GC-MS combination for semi-automated, selective, and sensitive determination of synthetic cannabinoids in oral fluid samples.

Keywords: *new psychoactive substances; synthetic cannabinoids; microextraction by packed sorbent; gas chromatography-mass spectrometry; saliva*

Introduction

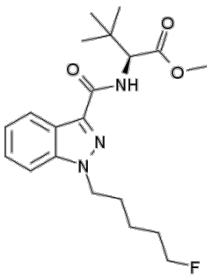
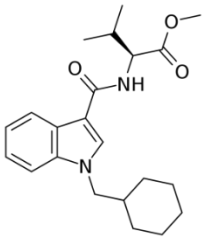
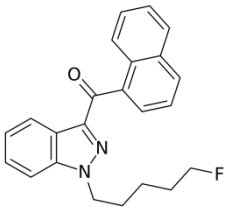
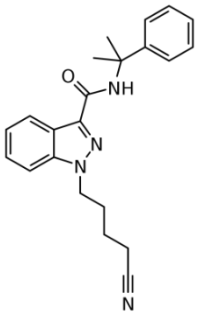
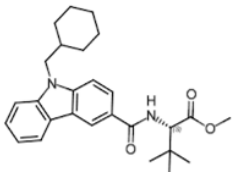
Synthetic cannabinoids are substances designed to mimic the desired effects of cannabis, acting on cannabinoid type-1 (CB1) and type-2 (CB2) receptors [1]. These substances, soaked into or sprayed onto plant material, are present in the market from 2004, being one of the main categories of substances reported as new psychoactive substances (NPS) at international markets by the United Nations Office on Drugs and Crime (UNODC) [2]. JWH-018 (1-pentyl-3-(1-naphthoyl)indole) was one of the first synthetic cannabinoids identified; since then, wide-ranging and chemically diverse substances have been synthesized to circumvent legislation. In fact, 250 synthetic cannabinoids have been reported by the UNODC Early Warning Advisory between 2009 and 2017 [3]. Currently, the third generation of synthetic cannabinoids is formed by a four sub-structures pattern (tail-core-link-ring) resembling JWH-018, with an indole, indazole, or carbazol core surrounded by different N-substituents like pentyl, 5-fluoropentyl, cyclohexylmethyl, and 4-cianobutyl groups, and 3-acyl-substituted by means of a methanone or carboxamide link with L-valinate, 3-methyl-L-valinate, adamantyl, naphtyl, or cumyl substituents [4]. Table 25 shows the molecular structure of the synthetic cannabinoids employed in this study. The prevalence of synthetic cannabinoids in the market is variable; however, substances like 5F-ADB and FUB-AMB show a continuous occurrence across several countries, being the most reported synthetic cannabinoids in both, 2017 and 2018 [5].

Psychological effects of synthetic cannabinoids are similar to those reported during intoxication with cannabis, but additional effects like distorted perceptions of time, hallucinations, and paranoia may also appear [6]. In fact, 52 deaths have been reported in different countries between 2016 and 2018 involving synthetic cannabinoids [5]. Consumption of synthetic cannabinoids is revealed by the analysis of these substances or their metabolites in biological fluids. Thus, the knowledge of metabolism pathways of synthetic cannabinoids is essential to focus their analysis in biological fluids. Urine is traditionally the most common biological fluid for drugs of abuse screening, but synthetic cannabinoids are quickly metabolized and parent compounds are rarely detected in urine [7]. Moreover, misidentification of synthetic cannabinoids with similar molecular structure may occur because of some of them provide the same metabolites in urine. Nevertheless, a more common occurrence of parent compounds has been observed in the analysis of synthetic cannabinoids in serum, blood, and oral fluids [8]. Furthermore, detection times are noticeably different depending on the target analyte and the analyzed fluid, being from hours to days for oral fluids, 1-2 days for blood, and 2-3 days for urine [9].

Even some studies have revealed that synthetic cannabinoids can be accumulated in adipose tissue for heavy users with half-lives in serum higher than 40 days [10]. Typical synthetic cannabinoids intake is in the low mg range; thus, very low concentrations are expected to be found in biological fluids, requiring the use of highly sensitive techniques. Regarding the analytes under study (see Table 25), parent compounds and their metabolites have been analysed by liquid chromatography-mass spectrometry (LC-MS) in different biological fluids, such as: 5F-ADB metabolites in urine [7,11,12,13,14,15] and in blood [16,17]; THJ-2201 in urine [18]; CUMYL-4CN-BINACA in urine [19,20,21] and post-mortem blood samples [22], and MDMB-CHMCZCA in urine [23]. Thus, in our knowledge there are no existing studies in the literature for the determination of the studied synthetic cannabinoids in oral fluids or saliva. Even, no precedents have been found in the literature for the determination of MMB-CHMICA in any biological fluid.

Analytical procedures for the determination of synthetic cannabinoids in oral fluids are mainly based on a conventional sample preparation step, such as liquid-liquid or solid-phase extraction (SPE), followed by a chromatographic analysis coupled to mass spectrometry detectors [9]. In this frame, the use of microextraction by packed sorbent (MEPS), a simple, fast, and miniaturized extraction procedure has shown a high potential for psychoactive compound analysis in liquid matrices [24]. The use of microscale volumes in MEPS greatly reduces both, sample and reagent consumption without compromising the extraction efficiency. MEPS has been previously employed in the determination of NPS in oral fluids in combination with different techniques such as: LC-MS [25,26] and ion mobility spectrometry [27,28]. Thus, the objective of the present study is the development of MEPS-based procedure for the determination of third generation synthetic cannabinoids in oral fluids. Working conditions for MEPS have been selected and the method has been validated in terms of linearity, selectivity, sensitivity, trueness, and precision.

Table 25. *Molecular structure, formula and molar mass (M) of the synthetic cannabinoids employed in this study.*

Abbreviation	Core	Molecular structure	Formula	M (g mol^{-1})
5F-ADB (or 5F-MDMB-PINACA)	Indazole		$C_{20}H_{28}FN_3O_3$	377.460
MMB-CHMICA	Indol		$C_{22}H_{30}N_2O_3$	370.493
THJ-2201 (or AM-2201 indazole)	Indazole		$C_{23}H_{21}FN_2O$	360.420
CUMYL-4CN-BINACA	Indazole		$C_{22}N_{24}N_4O$	360.461
MDMB-CHMCZCA	Carbazol		$C_{27}H_{34}N_2O_3$	434.570

Experimental

Apparatus and reagents

Analysis of synthetic cannabinoids was performed using an Agilent 7890A GC coupled to an Agilent 53975C inert XL EI/CI MSD with Triple-Axis single quadrupole detector and an Agilent HP-5 MS (30 m, 0.25 mm, 0.25 µm) capillary column, obtained from Agilent Technologies (Palo Alto, CA, USA).

Extraction of oral fluid samples by MEPS was performed using a SGE Analytical Science (Victoria, Australia) eVol XR digitally controlled positive displacement dispensing system, with a 100 µL syringe (22 gauge needle, 0.72 mm outside diameter, and 55.5 mm in length) working at the lowest plunger speed (10 µL s^{-1}) and an octadecyl (C_{18}) packed sorbent (4 mg silica with 45 µm mean particle size).

5F-ADB (methyl (s)-2-[1-(5-fluoropentyl)-1h-indazole-3-carboxamido]-3,3-dimethylbutanoate), MMB-CHMICA (methyl (n-{[1-(cyclohexylmethyl)-1h-indol-3-yl]carbonyl}-l-valinate)), THJ-2201 ([1-(5-fluoropentyl)-1h-indazol-3-yl](1-naphthyl)methanone), CUMYL-4CN-BINACA (1-(4-cyanobutyl)-n-(2-phenylpropan-2-yl)-1h-indazole-3-carboxamide), and MDMB-CHMCZCA (methyl (s)-2-(9- *-263-*
(cyclohexylmethyl)-9h-carbazole-3-carboxamido)-3,3-dimethylbutanoate)
standards were provided by the Unidad de Inspección de Farmacia y Control de Drogas (Valencia, Spain). Deuterated synthetic cannabinoids were not commercially available to be employed as internal standard for GC-MS acquisitions. Thus, triphenyl phosphate (TPP), provided by Sigma (St. Louis, MO, USA), was employed as internal standard. Standard working solutions were prepared in 2-propanol and stored at 4 ºC in amber glass vials. Methanol, 2-propanol, chloroform and buffer constituents were obtained from Scharlab (Barcelona, Spain). Buffer solutions were prepared at 0.1 and 2.5 M with sodium acetate (pH 5.0), di-potassium hydrogen phosphate (pH 7.0), and sodium carbonate (pH 9.0) salts.

Synthetic saliva was prepared following the Centre for Applied Science and Technology guidelines [29], using buffer salts and mucin from porcine stomach Type II, obtained from Merck (Darmstadt, Germany). Field oral fluid samples were obtained by expectoration into 1.5 mL Eppendorf tubes from volunteers who provided their consent after appropriate information about the study following the Ethical guidelines established by the University of Valencia (H1454687358321 – drug analysis in biofluids). Fifteen saliva samples from different volunteers were pooled in order to obtain representative field saliva sample and it was employed for recovery studies.

Extraction of oral fluid samples

Samples were previously centrifuged at 10000 rpm for 4 min to remove solids that may provide any syringe clogging. Sample pH was adjusted by adding 20 µL buffer (pH 7, 2.5 M) to 480 µL sample. Spiked samples were prepared using 455 µL blank saliva, 20 µL buffer (pH 7, 2.5 M), and 25 µL synthetic cannabinoid standard of 5 and 10 mg L^{-1} in 2-propanol.

Extraction of synthetic cannabinoids from oral fluid samples was carried out by MEPS using a C18 sorbent. Sorbent was previously conditioned with 100 µL 2-propanol plus 100 µL deionized water. 100 µL sample was loaded using 5 charge/discharge cycles, washed with 100 µL deionized water, dried with 100 µL clean air, and eluted with 50 µL 2-propanol, using 5 charge/discharge cycles. Before GC-MS determination 5 µL TPP at 5 mg L^{-1} were added to the MEPS extract.

GC-MS determination of synthetic cannabinoids

Synthetic cannabinoids were determined by GC-MS by the injection of 1 µL extract at 300 °C, in splitless mode, using 1 mL min^{-1} helium as carrier in constant flow mode. Oven temperature program was as follow: initial temperature 150 °C for 1 min, increased at a rate of 10 °C min^{-1} up to 300 °C, and held 10 min. Transfer line and ion source temperatures were 300 and 250 °C, respectively. Electron impact ionization was performed at 70 eV in selected ion monitoring (SIM) mode. Table 26 shows retention times and ions employed for SIM determination for each studied compound.

Table 26. *Analytical features for the determination of synthetic cannabinoids by the proposed method.*

Analyte	RT (min)	SIM (m/z)	R^2	LOD (µg L^{-1})	LOQ (µg L^{-1})	RSD (%)
TPP (IS)	9.49	326, 325	-	-	-	-
5F-ADB	12.01	233, 145	0.996	10	30	3.6
MMB-CHMICA	14.61	240, 256	0.999	10	30	4.8
THJ-2201	14.91	127, 271	0.991	20	60	7.8
CUMYL-4CN-BINACA	15.07	226, 345	0.990	20	60	8.9
MDMB-CHMCZCA	19.73	290, 179	0.996	20	60	6.8

Abbreviations: IS, internal standard; LOD, limit of detection; LOQ, limit of quantification; RSD, relative standard deviation; RT, retention time; SIM, selected ion monitoring.

A calibration curve was prepared from 60 to 1000 µg L^{-1} synthetic cannabinoids in 2-propanol, containing 500 µg L^{-1} TPP.

Results and discussion

Selection of extraction parameters

Extraction parameters for MEPS were evaluated for the extraction of synthetic cannabinoids from oral fluid samples. As in the case of standard SPE, the main parameters to evaluate were type of sorbent, pH of the loading step, and nature of elution solvent. However, because of the particularities of MEPS, which operates with volumes in the microliter scale, additional operative parameters must be evaluated such as: volume of sample and elution solvent, and number of charge/discharge cycles in loading and elution steps.

Commercially available sorbents for MEPS bins are amino-propyl silane for ion exchange purposes and C2, C8, and C18 functionalized silica for reverse phase extraction (30). Among these, C18 was selected due to it was typically employed for MEPS application for forensic drug analysis (24). A 100 µL syringe was selected in order to reduce the sample amount required to perform the analysis and to afford a slow flow of sample through the sorbent to enhance analyte/sorbent interactions.

The initial procedure for MEPS extraction was a pre-conditioning step with 100 µL 2-propanol and 100 µL deionized water, a loading step using 100 µL sample using a single charge/discharge cycle, a washing step with 100 µL deionized water, and a elution step with 100 µL using one charge/discharge cycle. These working conditions were evaluated by means of monoparametric studies by the evaluation of the effect of the selected parameter on the recovery obtained for a buffer solution spiked with 250 µg L^{-1} of the studied synthetic cannabinoids.

Loading pH value is not a critical parameter because of the evaluated synthetic cannabinoid do not show any protonation/deprotonation at physiological pH, and in our knowledge no pKa values have been reported for these compounds. Nevertheless, 0.1 M buffer solutions at pH 5.0, 7.0, and 9.0 were spiked and extracted by MEPS in order to evaluate the effect of pH. Figure 45A shows the effect of the pH on the obtained recoveries of the studied compounds, which ranged from 52 to 95 %. As it was expected, there were no significant changes in the obtained recovery values for the studied compounds at different pH. Thus, pH 7.0 was proposed for the loading step and it was employed for further experiments.

The number of charge/discharge cycles in the loading step, from 1 to 20, was also evaluated (see Figure 45B). The use of a single charge/discharge cycle provides low recoveries for the studied compounds (from 65 to 86 %) that reach a plateau at 5 cycles with quantitative recoveries (from 77 to 95 %). Thus, five charge/discharge cycles were selected in order to obtain high recoveries and low analysis time.

Methanol, 2-propanol, and chloroform were evaluated as elution solvents. Figure 45C shows the obtained results, showing a high dependence with the employed solvent. The use of chloroform provided a reduced recovery for THJ-2201 and MDMB-CHMCZA, and high variability in the results, with relative standard deviation (RSD) values from 11 to 23 %. Methanol is an adequate extraction solvent for the evaluated compounds, except for MMB-CHMICA and THJ-2201. The use of 2-propanol provided the best results with RSDs from 2 to 11 % and quantitative recoveries from 78 to 105 %. Thus, 2-propanol was selected for further experiments. The number of charge/discharge cycles was fixed to 5 in order to achieve the highest elution of the studied synthetic cannabinoids. The volume of 2-propanol was decreased to 75 and 50 µL in order to increase pre-concentration factors (see Figure 45D). As it can be seen, the use of 50 µL 2-propanol provided pre-concentration factors from 1.27 to 1.87 with quantitative recoveries for evaluated compounds. Thus, 50 µL 2-propanol, in five charge/discharge cycles, was proposed as elution conditions for additional experiments.

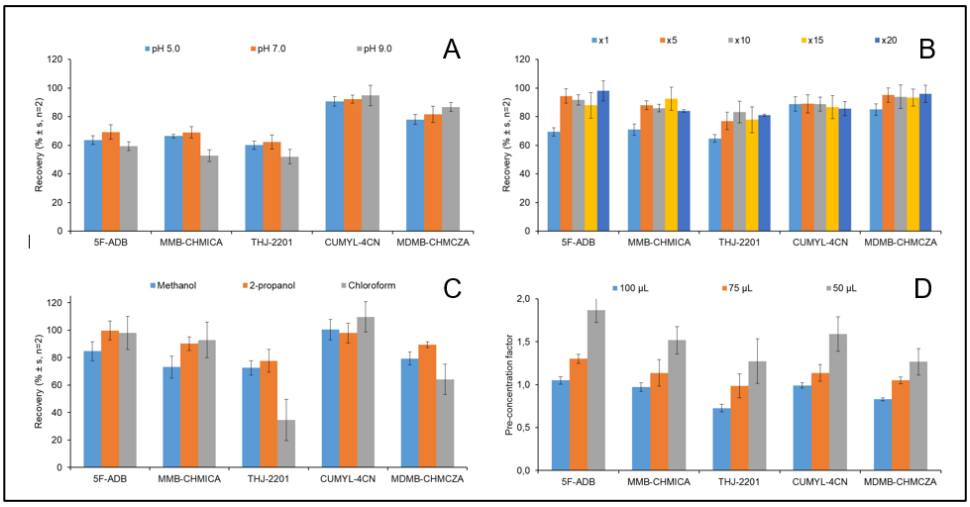


Figure 45. *Selection of extraction parameters for the analysis of synthetic cannabinoids in oral fluid samples using a buffer solution spiked with 250 µg L-1 of the studied synthetic cannabinoids (A, sample pH; B, number of loading cycles; C, elution solvent; and D, elution solvent volume). See the text for more details.*

Analytical features of the method

Figure 46 shows the chromatogram for a 250 µg L^{-1} synthetic cannabinoids standard measured by GC-MS. The five evaluated synthetic cannabinoids were adequately resolved by the chromatographic method and quantification was performed in SIM mode in order to avoid interferences with close retained peaks. The obtained chromatogram for a blank oral fluid spiked with 250 µg L^{-1} synthetic cannabinoids and analysed by the proposed MEPS-GC-MS methodology is also shown in Figure 46. As it can be seen, the analysis of oral fluids did not provide any additional peak or interferences, demonstrating the usefulness of the proposed extraction and determination procedures.

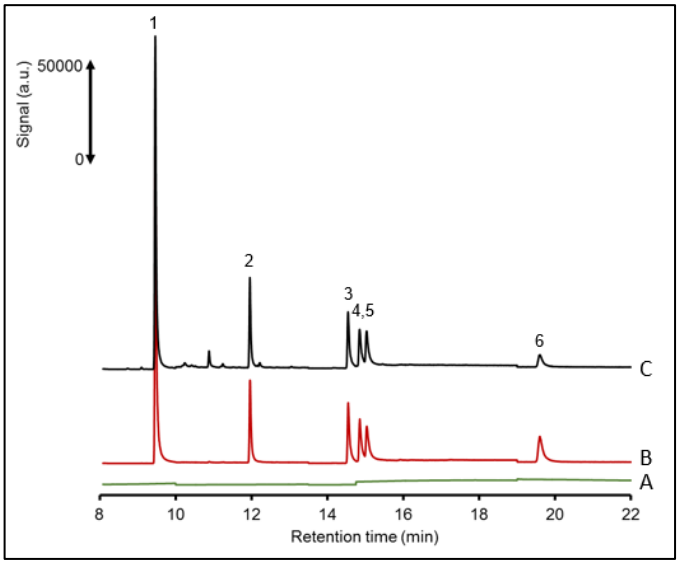


Figure 46. *GC-MS chromatograms obtained for (A) an oral fluid blank, (B) a 250 µg L^{-1} synthetic cannabinoids standard, and (C) an oral fluid blank spiked with 125 µg L^{-1} synthetic cannabinoids. Chromatograms have been shifted for clarification purposes. Note: 1. TPP (internal standard), 2. 5F-ADB, 3. MMB-CHMICA, 4. THJ-2201, 5. CUMYL-4CN-BINACA and 6. MDMB-CHMCZCA.*

Linearity of GC-MS acquisitions was established from 60 to 1000 µg L^{-1} synthetic cannabinoids standards with coefficients of determination (R^2) ranged from 0.990 to 0.999. Limits of detection (LOD) and quantification (LOQ) were calculated as 3 and 10 times, respectively, the standard deviation (n=3) of a 100 µg L^{-1} synthetic cannabinoid standard, divided by the slope of the calibration curve. The obtained LOD and LOQ values ranged from 10 to 20 µg L^{-1} and from 30 to 60 µg L^{-1}, respectively. Precision was established as the RSD (n=3) of a 100 µg L^{-1}

synthetic cannabinoid, with values ranging from 3.6 to 8.9 %. Individual values for each studied synthetic cannabinoid can be seen in Table 26.

Previous reports on the determination of synthetic cannabinoids in oral fluids established concentrations up to 40, 80, 381, 460, and 2036 μg L^{-1} for AB-FUBINACA, AM-2201, JWH-122, JWH-210, and JWH-018, respectively [31,32,33,34,35]. Thus, trueness of the method was established by the analysis of synthetic and field oral fluids spiked with synthetic cannabinoid at 125 and 250 μg L^{-1} concentration levels. Quantitative recoveries were obtained for all the synthetic cannabinoids evaluated (see Table 27), ranging from 90 to 124 % and from 89 to 103 %, for spiked synthetic and field oral fluids, respectively, with the exception of MDMB-CHMCZCA that provided low recoveries from 62 to 66 %. Thus, the proposed MEPS-GC-MS procedure provides a high extraction efficiency and adequate sensitivity for the determination of new generation synthetic cannabinoids in oral fluids.

Table 27. *Recoveries obtained for synthetic and field oral fluids spiked with synthetic cannabinoids and extracted by the proposed MEPS method.*

Analyte	Recovery (% ± s, n=3)			
	Synthetic		Field	
	125 μg L^{-1}	250 μg L^{-1}	125 μg L^{-1}	250 μg L^{-1}
5F-ADB	120 ± 7	124 ± 4	100 ± 2	102 ± 5
MMB-CHMICA	116 ± 10	121 ± 11	91 ± 6	103 ± 3
THJ-2201	110 ± 1	112 ± 9	89 ± 6	93 ± 1
CUMYL-4CN-BINACA	118 ± 16	121 ± 13	97 ± 11	104 ± 4
MDMB-CHMCZCA	108 ± 13	90 ± 9	62 ± 14	66 ± 9

Conclusions

The consumption of synthetic cannabinoids has increased in the last decade with a great social incidence due to the high number of undesirable episodes of intoxications and deaths reported in many countries. In this study a novel procedure has been developed for the determination of third generation synthetic cannabinoids in saliva based in MEPS extraction and GC-MS analysis. Experimental conditions for MEPS of the target analytes have been evaluated in order to obtain quantitative extraction recoveries with a high sensitivity with LOD values in the 10-20 μg L^{-1} range. The use of MEPS shows a high potential automation to perform routinely analysis of a high number of samples and a reduced consumption of solvents and reagents. Moreover, the analysis of oral fluid allows the identification

of the parent compounds instead of their respective metabolites, which reduces potential misidentification that often occurs in urine analysis.

Funding

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Development of pipette tip-based poly(methacrylic acid-co-ethylene glycol dimethacrylate) monolith for the extraction of drugs of abuse from oral fluid samples

A. Sorribes-Soriano, A. Valencia, F.A. Esteve-Turrillas, S. Armenta, J.M. Herrero-Martínez

Department of Analytical Chemistry, University of Valencia, 50th Dr. Moliner St., 46100 Burjassot, Spain

Abstract

In this work, a monolithic polymer based on poly(methacrylic acid-*co*-ethylene glycol dimethacrylate) (MAA-*co*-EDMA) was prepared inside 200 µL pipette tips for the extraction of drug of abuse from oral fluid samples. After an appropriate surface tip modification, several polymerization mixtures with different monomer/cross-linker ratios, and percentage of porogen were studied. The most appropriate monolith to easily flow organic solvents and oral fluid samples was prepared with a MAA/EDMA ratio of 8:92 wt/wt and dodecanol containing 10 wt% toluene, as porogenic solvent. Parameters affecting the extraction procedure were evaluated and the monolith was characterized in terms of binding capacity, reusability, and precision, using α-pyrrolidinovalerophenone as model compound. Cocaine, diazepam, methamphetamine and 20 new psychoactive substances were determined in oral fluids, using the synthesized poly(MAA-*co*-EDMA) monolith in-tip on an eight-channel micropipette extraction and ultra-high performance liquid chromatography tandem mass spectrometry. Appropriate recoveries were obtained, ranging from 64 to 115 %, with limit of detection values from 0.03 to 0.6 µg L^{-1}, and a high precision with relative standard deviation values lower than 10 % for all the evaluated drugs.

Graphical abstract

Keywords: *monolithic polymer; MAA-co-EDMA; pipette tip extraction; solid phase extraction; new psychoactive substances; α-PVP, UHPLC-MS/MS*

Introduction

According to the United Nations Office on Drugs and Crime (UNODC), about 275 million people worldwide consumed drugs at least once during 2016. Cannabis and cocaine continue to be the most consumed drugs around the world, opioids are considered the most harmful, accounting for 76 % of deaths where drug use disorders were implicated, and in recent years, hundreds of new psychoactive substances (NPS) have been synthesized and added to the established synthetic drug market for amphetamine-type substances [1].

Drug determination in biological fluids usually requires a sample treatment due to the low concentration of these substances and the complexity of the matrix. Sample treatment is typically based on liquid-liquid (LLE) [2,3] and solid-phase extraction (SPE) [4,5] approaches. Despite the wide use of classical SPE cartridge format, some shortcomings are present related to the large sorbent amount and high volumes of sample and solvent required. In this sense, novel SPE formats have emerged in the last years to miniaturize and simplify the extraction procedures, such as magnetic micro solid-phase dispersive extraction [6], in-tube solid-phase microextraction [7], and inside the channels of a microfluidic device [8]. One of the most promising improved SPE techniques is based on pipette tip-based SPE [9,10], due to its inherent advantages that include ease of use, miniaturization of the system, reduced sample amount, reduced solvent consumption, and high sample throughput using either multi-channel hand-held pipettes or robotic liquid handling systems [11].

Early, SPE disks or particles were introduced inside the pipette tips and they were confined using frits [12], which often caused severe plugging problems due to the small cross-sectional area and the presence of the discs and retaining frits [9]. This problem was overcome by the introduction of monolithic beds [13], with attractive features such as including frit-free construction, easy preparation with good control of porosity, and improved convective mass transfer which is desirable in extraction processes. Silica-based monolith pipette tips are commonly manufactured by sol-gel technology and are commercially available by different suppliers like Fisher Scientific [14], Merck [15], and GL Sciences [16], with different volume capacities from 0.1 to 200 µL, but only conventional sorbents are available such as octadecyl silica, octyl silica, HILIC, and ZrO_2, among others. However, the synthesis of silica monoliths includes a complicated post-treatment step, followed by a derivatization with the desired functional group. Moreover, the stability of silica-based monoliths is affected by extreme pH values (2-9) [17]. On the other

hand, organic polymer monoliths can be easily *in situ* synthesized by thermal or UV irradiation initiating the polymerization of a mixture of monomer, cross-linker, and porogen in a "mold". Since the monomers and cross-linkers available are variable, monoliths can be tailored with different functional groups for specific purposes [18]. Many of these materials are biocompatible and stable at extreme pH values (1-12). Thus, UV-initiated poly(butyl methacrylate-ethylene glycol dimethacrylate) porous polymer monoliths have been used for the extraction of ropivacaine [19], and pindolol and metoprolol [20], from plasma samples. Moreover, poly(methacrylic acid-*co*-ethylene glycol dimethacrylate) (poly(MAA-*co*-EDMA)) monolith has been commonly used as polymeric bed for its use as extraction sorbent. The main advantages of this polymer underlies in its hydrophobic polymer bone structure jointly with the acidic pendant groups, which offer the possibility of electrostatic interaction, being an ideal sorbent for basic analytes, which includes most of the psychoactive substances [21].

The aim of this study is the synthesis of a monolithic MAA-*co*-EDMA polymer inside polypropylene tips to perform pipette tip SPE of drugs from oral fluid samples. In this sense, several polymerization mixtures, with different monomer:cross-linker ratio, were evaluated to obtain the most appropriate, permeable, and mechanical resistant polymer. Finally, different psychoactive substances, including classical abused drugs and NPS were determined in oral fluids, using the synthesized poly(MAA-*co*-EDMA) monolith in-pipette tip as fast and simple extraction approach and ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) for analytical determinations.

Experimental section

Standards and reagents

Standards employed in this study were kindly provided by the laboratory of "Inspección de Farmacia y Control de Drogas" (Valencia, Spain). These standards included cocaine, diazepam, methamphetamine and the next 20 NPS: 2-fluoroamphetamine (2-FA), 3-fluoroamphetamine (3-FA), 2-fluoromethamphetamine(2-FMA), 4-fluoromethamphetamine (4-FMA), 3-fluoroethamphetamine (3-FEA), 3-methoxymethamphetamine (3-MMA), 6-(2-aminopropyl)benzofuran (6-APB), ethcathinone, 4-chloroethcathinone (4-CEC), 4-chloromethcathinone (4-CMC), 4-methylbuphedrone (4-MeMABP), α-pyrrolidinovalerophenone (α-PVP), α-pyrrolidinohexiophenone (α-PHP), 4'-methyl-α-pyrrolidinohexiophenone (MPHP), mexedrone, methylone, butylone, 3',4'-

methylenedioxy-α-pyrrolidinopropiophenone (MDPPP), 3'4'-methylenedioxy-α-dimethylamino-isovalerophenone (MDPBP), and 4-ethyl-2,5-dimetoxiphenethylamine (2C-E). Purity of all the standards used was higher than 95 wt%. Stock solutions were prepared at 1 g L^{-1} concentration level in methanol and kept at -20 °C in amber glass vials. Cocaine-d_3, (±)-amphetamine-d_5, and methylone-d_3, used as internal standard, were purchased from Sigma (Stenheim, Germany).

Cadmium-free polypropylene tips (200 µL), organic solvents, and buffer substances were purchased from Scharlab (Barcelona, Spain). The reagents used for the synthesis of the monolith, MAA, EDMA, 2,2'-azobis(2-methylpropionitrile) (AIBN), and benzophenone (BP) were obtained from Sigma.

Synthetic saliva was prepared following the guidelines of Centre for Applied Science and Technology (CAST) document "Preliminary Drug Testing Devices" [22]. Mucin from porcine stomach Type II was obtained from Sigma.

Oral fluid samples were obtained from volunteers, with ages ranging from 19 to 40 years old, who provided their consent after appropriate information about the study following the Ethical guidelines established by the University of Valencia (H1454687358321 – drug analysis in biofluids). It should be highlighted that under no circumstances the authors have trafficked or provided illegal substances, aimed, promoted, facilitated, or forced any way the consuming of illegal substances. Samples were collected by volunteer's stimulation, without the use of any special sampling device, in 1.5 mL Eppendorf tubes and stored at -4 °C until analysis.

Preparation of poly(MAA-co-EDMA) monolith in-tip

Surface of the internal walls of the polypropylene tips was modified by photografting with EDMA, adapting the procedure described in previous studies [23,24,25]. Pipette tips were sequentially washed with 200 µL methanol and acetone (five cycles for each solvent) to remove potential impurities and they were dried with a nitrogen stream for 1 min. Pipette tips were placed vertically in a silicone support (see Figure 47), to avoid the dropping of the reagents, and filled with 100 µL of 5 wt% deoxygenated BP in methanol. Surface tip modification was performed in a UV cross-linker (model CL1000) from UVP Inc. (Upland, CA, USA) equipped with UV lamps (5 x 8 W, 254 nm). Thus, tips were irradiated with UV light (1 J cm^{-2}) for 10 min and twice washed using 200 µL methanol and dried 1 min using a nitrogen stream. Pipette tips were placed again in the silicone support and filled with 100 µL of 15 wt% EDMA solution prepared in methanol irradiating again under

UV light for 10 min. Finally, pipette tips were washed twice with 200 µL methanol and dried q min with a nitrogen stream.

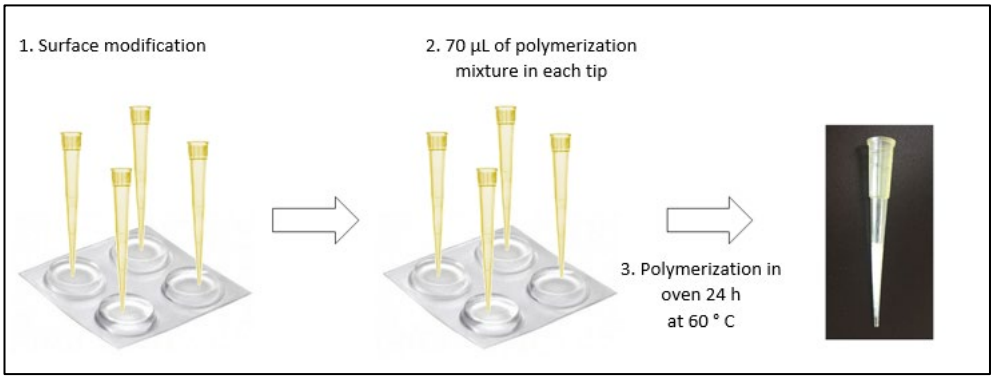

Figure 47. *Scheme of the modification surface pipette tips and synthesis procedure of monolith pipette tips.*

Polymerization mixture was prepared using MAA (17.6 mg) and EDMA (204.8 mg) dissolved in 90:10 wt/wt dodecanol:toluene (334 mg) in a vial. Then, initiator AIBN (5 mg) was added to previous solution and was purged with nitrogen for 10 min and sonicated for 10 more min. The tips were placed vertically in the silicone support and 70 µL of the polymerization mixture were introduced using a needle. Then, the top of the pipette tips were covered with parafilm and the tips were placed in an oven for 24 hour at 60 °C. When polymerization is finished, the resultant pipette tip monolith was sequentially washed with acetonitrile, methanol, and water to remove excess of reagents and dried for its use.

Monolith was characterized by scanning electron microscopy (SEM) using a S-4800 from Hitachi (Ibaraki, Japan), provided with a field emission gun and a back secondary electron detector. EMIP 3.0 software from Rontec (Normanton, UK) was employed for image data acquisition.

Monolith in-tip (micro)extraction procedure

Extraction of drugs was achieved using eight pipette tip monolith assembled in an 8-channels automatic micropipette. Monolith was previously conditioned by aspirating and dispensing (to waste) 200 µL 1 % acetic acid in methanol and 200 µL deionized water. 200 µL oral fluid sample was diluted with 200 µL phosphate buffer (0.1 M, pH 8.0), to reduce solution viscosity, and spiked with 10 µL internal standard mixture at 100 µg L^{-1}. Sample solution was 10 times aspirated and dispensed using a 200 µL volume, to load target analytes to the monolith, and it was washed once

with 200 µL 10 % 2-propanol in water and dried twice with 200 µL air. Finally, analytes were eluted from the pipette tip monolith with five aspirating/dispensing cycles using 100 µL 1% acetic acid in methanol and analysed by UHPLC-MS-MS.

UHPLC-MS-MS determination

A Waters Acquity UPLC™ system from Waters (Milford, MA, USA), equipped with an autosampler, a binary solvent delivery system, a C18WATERS (1.7 µm, 2.1 x 50 mm) column, and a triple quadrupole detector with a Z-spray electrospray ionization source, was employed for drug determination. The flow rate was 0.4 mL min^{-1} and the employed mobile phases were (A) 5 mM formic acid in water and (B) acetonitrile. The gradient elution consisted initially in a 100% of A and B was increased from 0 to 30 % within 6 min, to 50 % within 2 min, to 100 % within 4 min, and finally an isocratic elution was maintained during 3 min. MS acquisitions were done using 3.5 kV capillary voltage, 120 °C source temperature, and 350 °C desolvation temperature. The multiple reaction monitoring (MRM) conditions were adjusted for each analyte and internal standards.

Results and discussion

Synthesis of the poly(MAA-co-EDMA) monolith in pipette tips

Polypropylene tips were selected as starting material due to its mechanical resistance, high level of availability, low cost, easy modification of its surface to achieve an appropriate attachment of the polymer, and its UV radiation transparency that facilitates polymerization processes. The inner surface modification of pipette tips was done using a sequential two-step photografting approach. Firstly, BP is immobilized onto the inner surface by UV irradiation of polypropylene surface in contact with the BP solution. In the second step, the EDMA solution is added to the active substrate, and after UV irradiation the immobilized latent free radicals (from BP) are released thus initiating the graft polymerization. The resulting grafting of EDMA chains onto propylene surface, which will assure a proper anchorage to the monolith to the propylene inner wall. The effectiveness of the described modification process was demonstrated in previous studies [23,26].

The retention abilities of a polymer monolith are ruled to some extent by the functional monomer. Thus, due to the basic behaviour of selected analytes an acidic functional monomer (MAA) was selected in this study, to increase the analyte-

monomer interactions by electrostatic forces, ionic interaction, and even hydrogen bonds. On the other hand, a material with high permeability and porosity was required to flow at low pressures both, organic solvents and sample solutions; so, the first step of this study consisted on the attainment of a monolithic material with appropriate flow resistance, wettability, and adhesion to the pipette surface. In this sense, the choice of the appropriate porogen is essential to produce pores wide-enough to assure good flow-through properties of the monolithic polymer. In this sense, several studies focused on the synthesis of polymeric materials have suggested the use of dodecanol-toluene mixtures as efficient porogenic solvent [27,28]. Thus, dodecanol was initially selected for preparing different MAA-based materials (see conditions in Table 28). Figure 48A shows the SEM micrograph corresponding to a polymeric material prepared using this solvent as single porogen, where small globules with grainy appearance were evidenced. It suggests that this solvent is a good solvating solvent for the resulting polymer. Thus, a porogenic mixture with lower polarity, containing toluene and high percentage of dodecanol, is a more potent precipitant in the polymerization mixture and causes earlier onset of the polymer separation, giving as a result a polymer with larger globules [29,30,31]. Thus, the ratio of toluene and dodecanol was then studied. As shown in Figure 48B, the size of globules increased with the introduction of toluene in polymerization mixture. The investigation revealed that 10 wt % of this solvent in the porogenic mixture provided an appropriate balance between porous morphology and permeability. To obtain tips with rigid (or less fragile) monolithic structures, the ratio of the weight fractions of the monomers and the selected porogenic solvent was also investigated (from 80 to 50 wt %). As a result of this study, a monolith obtained using 60 wt % of porogen (see Figure 48C) gave a porous monolith strongly permeable against organic solvents and oral fluid samples and able to provide a satisfactory mechanical resistance.

In order to test the reproducibility of the synthesis procedure of polymeric monoliths inside of the pipette tips, several microextraction units (n = 8) were prepared using the proposed polymerization mixture and the resultant polymer was weighed. The obtained monolith mass ranged from 23 to 29 mg with a relative standard deviation (RSD) value of 8 %.

Table 28. *Monoliths prepared using different pre-polymerization mixtures and polymerization conditions.*

Monolith	Porogen	Monomer/porogen ratio (wt/wt)	MAA/EDMA[a] ratio (wt/wt)	Polymerization condition (time)
1	Dodecanol	20:80		
2	Dodecanol:toluene (90:10 wt/wt)	20:80		
3	Dodecanol:toluene (90:10 wt/wt)	30:70	8:92	Thermal (24 h)
4	Dodecanol:toluene (90:10 wt/wt)	40:60		
5	Dodecanol:toluene (90:10 wt/wt)	50:50		

[a] *EDMA, ethylene glycol dimethacrylate; MMA, methacrylic acid*

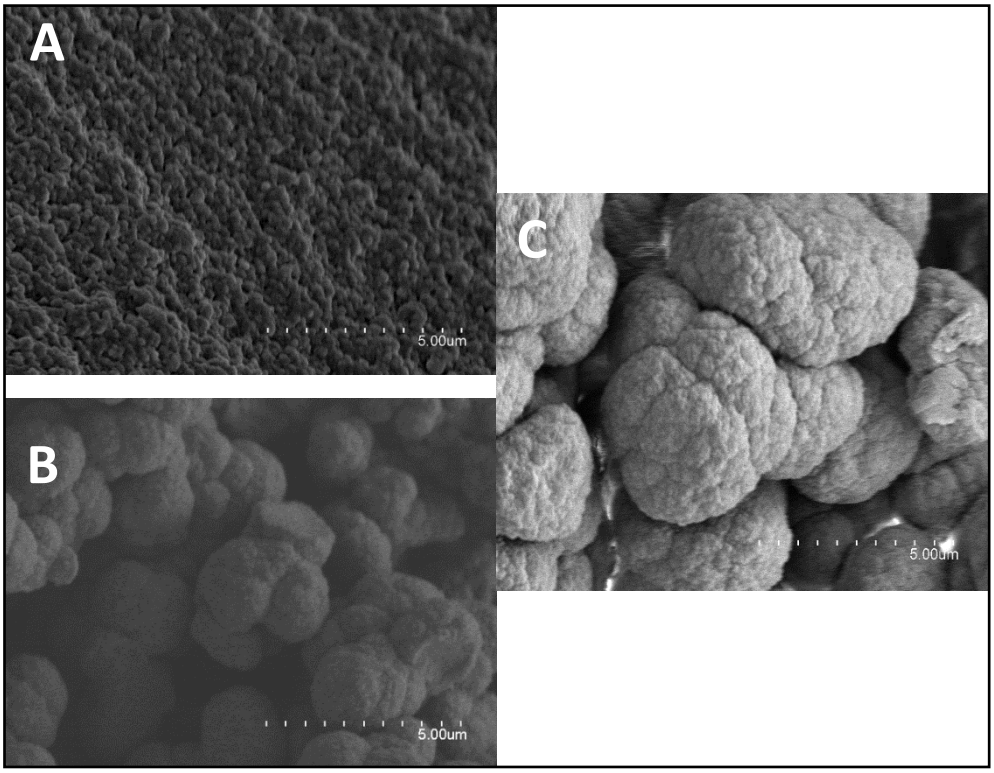

Figure 48. *SEM images at x20000 of the monoliths (A) 80 wt % of dodecanol, (B) 80 wt % of dodecanol:toluene (90:10 wt/wt), and (C) 60 wt % of dodecanol:toluene (90:10 wt/wt) as porogens.*

Study of poly(MAA-co-EDMA) monolith pipette tip extraction parameters

The effect of different variables of the extraction procedure, such as pH of sample, number of loading and elution cycles, washing solvent, and elution volume, among others, on the retention efficiency was evaluated, using α-PVP as model analyte due to it is the most used NPS of those evaluated in this study. Extraction parameters were studied using 200 µL of a synthetic saliva sample spiked with α-PVP at 100 µg L^{-1}. 1 % acetic acid in methanol was selected as elution solvent according to results from previous studies [32].

Thus, the poly(MAA-co-EDMA) monolith pipette tip was conditioned using 200 µL 1% acetic acid in methanol and 200 µL deionized water, previous to the evaluation of different extraction conditions.

The pH of sample solution has a great influence on the extraction efficiency, since it may weaken the interaction forces between the basic analytes and polymer, being pH of oral fluids between pH 6 and 7 [33]. Technical data for NPS is scarce and no pK_a values were established for many of the studied compounds. However, the studied NPS are primary and secondary aliphatic amines, and pyrrolidines; so, the expected pK_a of them may range between 7 and 11 values. The use of a basic pH in the loading step may promote the retention of the studied drugs on the monolith; however, many of the NPS evaluated were not soluble at pH higher than 9. Moreover, under extreme alkaline conditions, the hydrolysis of some drugs may occur [34]. Thus, the use of a buffer at pH 8 for the loading of drugs was selected for the following studies.

A critical parameter in the loading step is to promote the equilibrium between the monolith and the sample solution for achieving a high extraction recovery of target analytes. In this sense, the effect of the number of aspirating/dispensing cycles through the pipette tip in the loading step was evaluated using 200 µL of a synthetic saliva sample spiked with α-PVP at 100 µg L^{-1} diluted with 200 µL phosphate buffer (0.1M, pH 8.0), using 10 aspirating/dispensing cycles of 200 µL 1% acetic acid in methanol in the elution step. Sample dilution with buffer solution provides the right pH to promote drug retention on the monolith, but it also reduced viscosity of oral fluid samples. Figure 49A shows the α-PVP recovery values obtained from 1 to 12 aspirating/dispensing cycles. As it can be seen, a plateau after 10 aspirating/dispensing cycles was reached with a recovery of 82 ± 4 %; so, it was selected to improve the extraction of target analytes.

At this stage, the choice of the most appropriate washing solvent was considered in order to remove matrix compounds, but retaining target analytes, from the monolith. Several washing solutions were evaluated such as deionized water, 10 mM HCl, pH 8 phosphate buffer, and water solution containing a 10 % organic solvent like 2-propanol, acetonitrile, and acetone. Figure 49B shows the recoveries obtained for α-PVP, using the aforementioned procedure with an additional washing step with 200 µL different solutions in a single aspirating/dispensing cycle. It can be seen that acid conditions provided a slight decrease in the recovery (66 ± 2 %), probably due to the protonation of amine groups of analytes. Washing with water and pH 8 phosphate buffer provided no significant differences with quantitative α-PVP recoveries with values of 97 ± 3 and 104 ± 4 %, respectively. The addition of a 10 % organic solvent solution also provided quantitative recoveries for α-PVP, from 115 ± 6 to 107 ± 5 %; so, a washing step with 200 µL 10 % 2-propanol in water was proposed to have a higher elution capacity of matrix constituents with weak interactions with the monolith.

The number of aspirating/dispensing cycles of the elution step, using 200 µL 1 % acetic acid in methanol, was evaluated from 1 to 10 cycles (results not shown), achieving quantitative recoveries (80 ± 3 %) from 5 cycles. Then, elution volume was evaluated from 25 to 200 µL in order to increase the enrichment factor of the extraction. Figure 49C shows that low extraction efficiency was obtained by using volumes lower than 100 µL. Thus, an elution volume of 100 µL was selected, providing an enrichment factor value of 2.

The binding capacity of the produced monolith pipette tips was evaluated using 200 µL synthetic saliva spiked at 2 mg L^{-1} α-PVP. The obtained binding capacity was 5.3 µg g^{-1} polymer (152 ng per tip).

Reusability of the monolith pipette tip was evaluated, as a robustness indicator, by repetitive uses (n=15) of a same tip for the analysis of 200 µL synthetic saliva samples spiked at 100 µg L^{-1} α-PVP indicating that the produced monolithic pipette tips can be reused at least 15 times with an extraction yield higher than 80 %.

Moreover, reproducibility of the polymeric monolith in tips was evaluated by means of the analysis of the analysis of eight synthetic saliva samples spiked at 100 µg L^{-1} of α-PVP using a eight channels micropipette with eight different microextraction units. Average recovery of α-PVP was 107 % being the RSD of 12 %.

The proposed monolith pipette tip extraction procedure was applied for the analysis of field oral fluid samples spiked with α-PVP at different concentration levels. The recovery values obtained were 89 ± 12, 119 ± 10, 103 ± 9, and 82 ± 7 %, for 10, 100, 250, and 500 μg L^{-1}, respectively. From the results, it can be concluded that oral fluid matrix does not interfere the extraction of α-PVP using the developed monolithic polymer in tip.

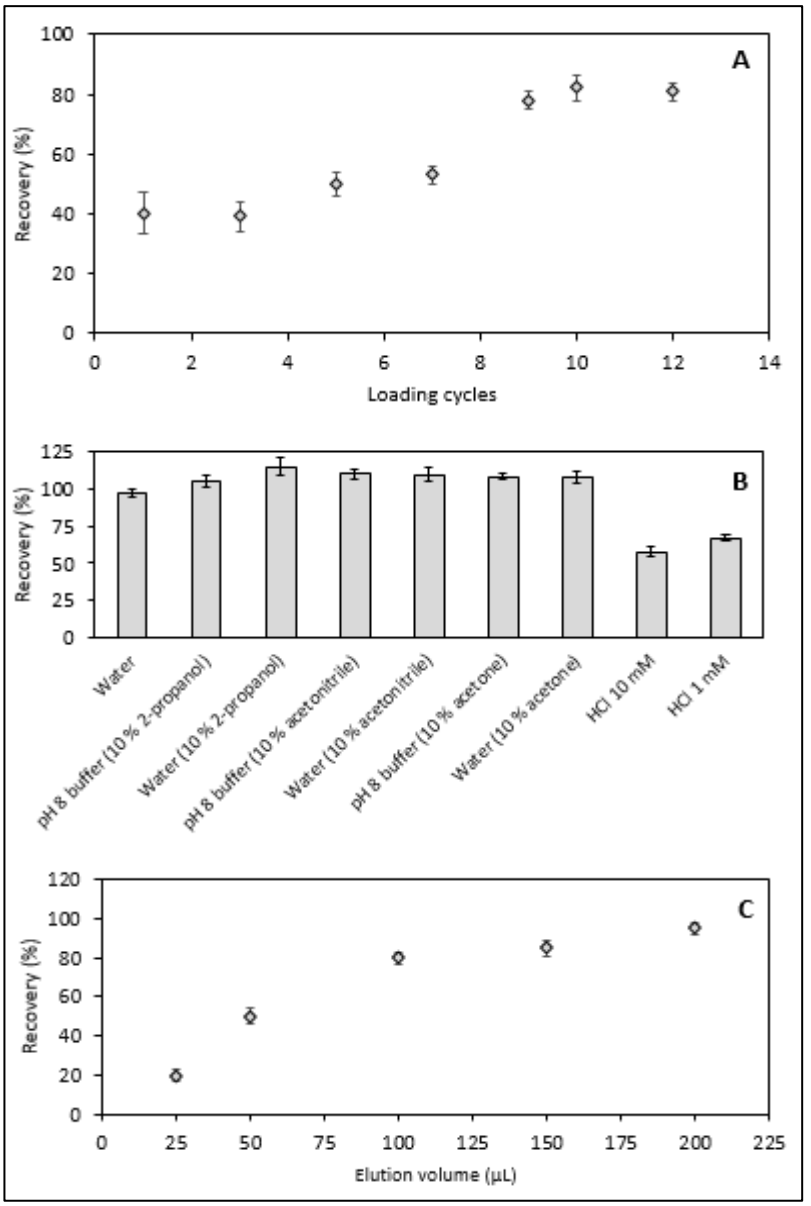

Figure 49. *Effect of (A) the number of 200 μL aspirating/dispensing cycles in loading step of sample, (B) type of washing solution, and (C) volume of elution solution (1 % acetic acid in methanol) on extraction recoveries of α-PVP at 100 μg L^{-1}.*

Analytical features for the analysis of drugs of abuse in oral fluids

Analytical determinations of the studied drugs were carried out by UHPLC-MS/MS. Table 29 shows the obtained retention time for each analyte, the employed MRM detector conditions, and the employed internal standard. The proposed methodology has been validated in terms of linearity, sensitivity, precision, and trueness. A summary with the analytical features for the determination of psychoactive substances in oral fluids are summarized in Table 29.

A calibration curve was prepared at six concentration levels from 1 to 100 µg L^{-1} prepared in methanol. The linearity of the UHPLC-MS/MS procedure was evaluated as the determination coefficients (r^2), using a 1/x weighted linear regression, with values higher than 0.99 in all cases. Moreover, all analyte standards showed residuals within ±20 %. Instrumental limit of detection was calculated as three times the standard deviation of the intercept divided by the slope of the calibration curve with values. Considering a 2-fold enrichment factor, the obtained limit of detection ranged, from 0.06 µg L^{-1} for 4-FMA and butylone, to 0.6 µg L^{-1} for methylone and 3-FEA. Figure 50 shows the obtained UHPLC-MS/MS chromatogram for the analysis of field saliva samples spiked at 20 µg L^{-1} with different psychoactive substances. Table 29 shows the obtained recoveries of the studied compounds in blank saliva samples of volunteers. Recovery values were acceptable (higher than 70 %) in all cases, except for mexedrone and methylone that showed recoveries of 64 and 67 %, respectively. The obtained quantitative recoveries demonstrate that the employed working conditions are adequate for the extraction of the evaluated drugs of abuse. Moreover, a high precision was observed with RSD values lower than 10 % for all the evaluated drugs.

Table 29. *Analytical features of the UHPLC-MS/MS procedure developed for the analysis of psychoactive substances in oral fluids.*

Substance	RT (min)[a]	MRM (m/z)[b]	IS[c]	LOD (μg L^{-1})[d]	Recovery (% ± s)[e]	RSD (%)[f]
Methylone	3.14	208 > 160	IS_1	0.6	67 ± 4	6.0
Methylone-d_3 (IS_1)[c]	3.14	211 > 163	-	-	-	-
Methamphetamine	3.46	150 > 91	IS_2	0.5	71 ± 3	4.2
Amphetamine-d_5 (IS_2)[c]	3.18	141 > 124	-	-	-	-
FA (2- and 3-isomer)	3.49	154 > 137	IS_2	0.10	74 ± 5	6.8
Ethcathinone	3.57	178 > 160	IS_1	0.15	72 ± 4	5.6
4-FMA	3.63	168 > 109	IS_2	0.06	71 ± 6	8.5
2-FMA	3.85	168 > 109	IS_2	0.12	103 ± 5	4.9
3-MMA	3.94	180 > 121	IS_2	0.08	78 ± 2	2.6
MDPPP	3.94	248 > 98	IS_1	0.15	115 ± 9	7.8
Butylone	4.03	222 > 204	IS_1	0.06	98 ± 6	6.1
3-FEA	4.15	182 > 109	IS_2	0.6	99 ± 8	8.1
4-CMC	4.17	198 > 145	IS_1	0.3	73 ± 4	5.5
Mexedrone	4.21	208 > 176	IS_1	0.3	64 ± 4	6.3
6-APB	4.23	176 > 131	IS_2	0.15	87 ± 6	6.9
4-MeMABP	4.24	192 > 145	IS_1	0.4	82 ± 3	3.7
4-CEC	4.48	212 > 159	IS_1	0.2	90 ± 4	4.4
MDPBP	4.88	250 > 202	IS_1	0.03	99 ± 5	5.1
α-PVP	4.92	232 > 91	IS_1	0.25	104 ± 8	7.7
Cocaine	5.16	304 > 182	IS_3	0.10	85 ± 7	8.2
Cocaine-d_3 (IS_3)[c]	5.16	307 > 185	-	-	-	-
α-PHP	5.74	246 > 91	IS_1	0.15	99 ± 3	3.0
2C-E	5.81	210 > 193	IS_2	0.35	107 ± 9	8.4
MPHP	6.72	260 > 105	IS_1	0.2	115 ± 5	4.3
Diazepam	8.77	285 > 154	IS_3	0.25	103 ± 7	6.8

[a] *Retention time.*

[b] *Multiple reaction monitoring transition.*

[c] *Used internal standard.*

[d] *Limit of detection.*

[e] *Recovery values obtained from field oral fluid samples spiked at 20 μg L^{-1} extracted by the proposed procedure.*

[f] *Relative standard deviation.*

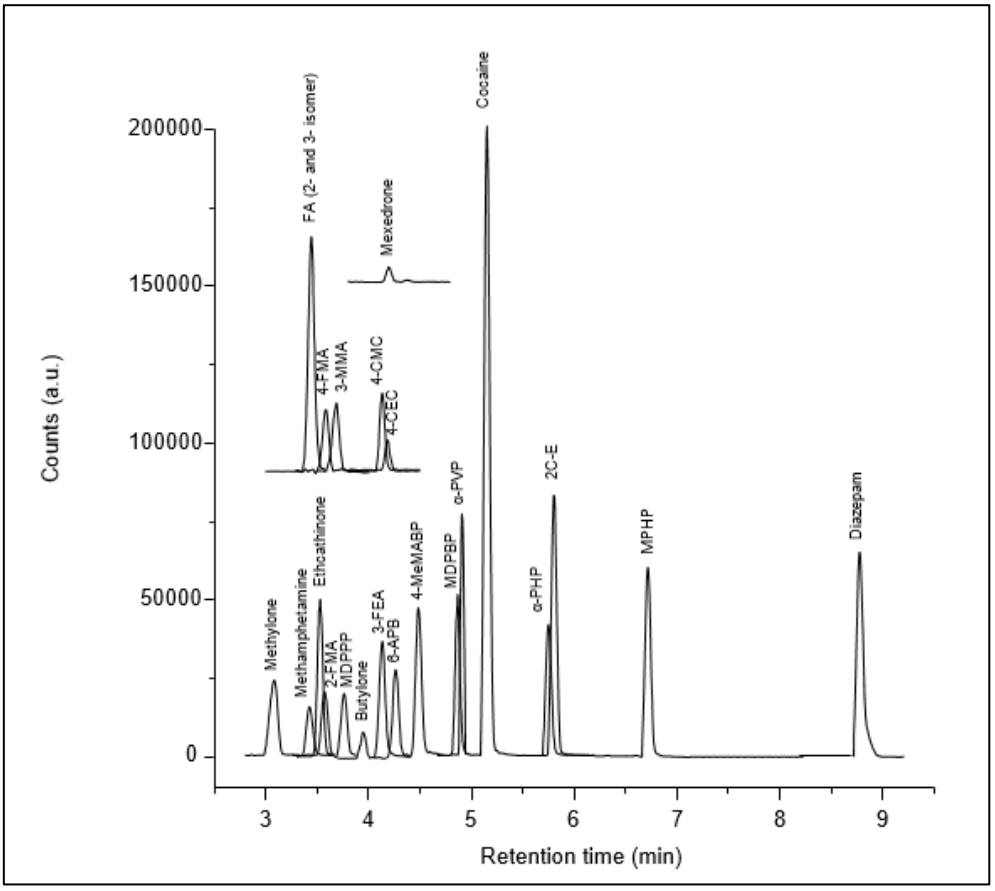

Figure 50. *UHPLC-MS/MS chromatogram obtained for the analysis of field saliva samples spiked at 20 µg L^{-1} with different psychoactive substances.*

Conclusions

A poly (MAA-co-EDMA) monolith was synthesized into 200 µL pipette tips for drugs of abuse extraction from oral fluid. Different polymerization mixtures including variables such as monomer:porogen ratio and porogenic solvent content were tested. The best results in terms of a permeability and physical resistance of the monolith were obtained with 40 wt% of monomers (8 wt% MAA and 92 wt% EDMA) and 60 wt% of porogen (90 wt% dodecanol and 10 wt% toluene). The different stages of the microSPE were also studied to obtain the best extraction conditions and the analytical features of the monolith were evaluated using α-PVP as target molecule. Thus, high recovery values (recovery values higher than 62 % in spiked-oral fluid samples), a satisfactory reusability (till 15 uses), and high binding capacity (152 ng per tip) were obtained. The applicability of the proposed method for the determination of 23 drugs of abuse, including cocaine, methamphetamine,

diazepam, amphetamine-type stimulants, and synthetic cathinones in oral fluid was confirmed, showing quantitative recoveries from 67 to 115 %. To the best of our knowledge, this is the first report dealing with polymer monolith in-tip and UPLC-MS/MS for the simultaneous determination of drugs of abuse in oral fluids. Under selected conditions, low detection limits (0.03 μg L^{-1}) and good precision were obtained for these analytes. The total volume of liquid solutions required for the proposed procedure was lower than 1 mL, which was lower than that required for conventional SPE cartridges (around 5-20 mL) [35], being a significant advance in the proposed miniaturized extraction process. Furthermore, the required volume of sample was 200 μL, being a great benefit because of the small volumes of oral fluids typically collected for drug consumption monitoring. Extraction procedure using monolith pipette tips required approximately 1 min, including conditioning, loading, washing, and elution steps. Moreover, a high sample throughput of 8 samples per minute can be achieved by serial extraction using an eight-channel micropipette. Therefore, the use of the developed polymer monolith pipette tip is strongly recommended for a rapid and quantitative extraction of drugs of abuse in oral fluids.

Conflict of interests

The authors declare that have no competing financial interests in this investigation.

Acknowledgments

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Dual mixed-mode poly (vinylpyridine-co-methacrylic acid-co-ethylene glycol dimethacrylate)-based sorbent for acidic and basic drug extraction

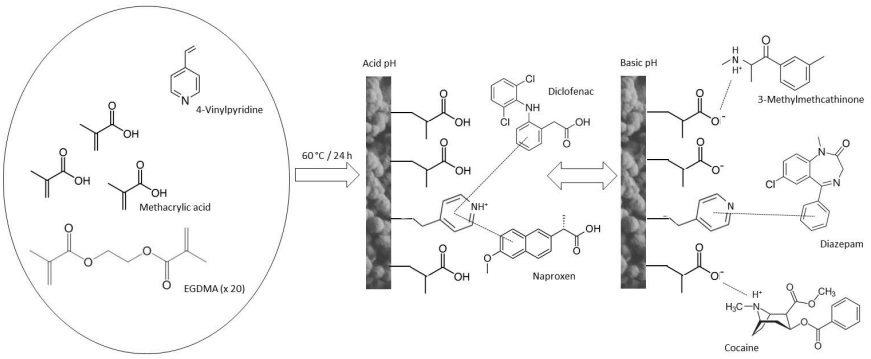
Aitor Sorribes-Soriano, Francesc A. Esteve-Turrillas, Sergio Armenta, José Manuel Herrero-Martínez

Department of Analytical Chemistry, Universitat de València, 50th Dr. Moliner St., 46100 Burjassot, Spain

Abstract

In this study, a dual mixed-mode polymer sorbent was prepared via one-step thermally initiated polymerization of 4-vinylpyridine (VP), methacrylic acid (MAA) and ethylene glycol dimethacrylate (EGDMA) for the solid-phase extraction (SPE) of basic and acidic drugs. The use of VP and MAA as ionizable functional monomers allowed the tailoring of ion-exchange and hydrophobic features of the polymer. The obtained polymer was characterized by Fourier-transform infrared spectroscopy and scanning electron microscopy. Next, the retention behavior of dual mixed-mode polymer towards basic and acidic drugs was investigated. Moreover, the practical capability of this novel material was tested for the extraction of relevant drugs such as cocaine, 3-methylmethcathinone, and diazepam in oral fluid samples. Recovery values (at different spiked levels in blank oral fluid samples), ranging from 69 to 99 %, and limits of detection (LODs), between 0.10 and 0.25 μg L^{-1}, were obtained.

Graphical abstract



Keywords: Dual mixed-mode polymeric sorbent; solid-phase extraction; drugs; oral fluids; cocaine; diazepam

Introduction

Since the 1990s, drug abuse has experienced a continuous growth every year, which represents an increased risk for public health. There are a lot of classical drugs worldwide consumed. Indeed, one of the most consumed drugs is cocaine, being one of the most stimulant drug taken at the European southwest [1]. Among other effects produced by this drug, the cocaine administration can increase behavioral signs of anxiety in fear-inducing situations [2]. These anxiogenics effects are minimized by pretreatment with different anxiolytic drugs, such as diazepam, a broad-spectrum benzodiazepine anxiolytic [3]. Additionally, the use of this prescripted drug used for sleep disorders or as an antidepressant has increased significantly during the last decades [4]. On the other hand, apart from established illicit drugs such as cocaine, the emergence of new psychoactive substances (NPS), such as synthetic cathinones, has led to an increase in hospitalizations and overdose deaths [5]. Taking into account the health, social and economic implications that these drugs pose, there is a need to develop analytical methods in biological matrices to assess and quantify the potential consumption of prescription and/or illicit drugs within the population. Despite the development of highly efficient analytical instrumentation, sample pretreatment prior to determination is crucial in these complex matrices in order to eliminate possible matrix effects and to preconcentrate analytes; thus, obtaining better detection limits and accurate results. Among pretreatment techniques, solid-phase extraction (SPE) has become one of the most popular techniques due to its high recovery, easy operation, and low solvent consumption [6].

The majority of drugs are weak acids and/or bases, and thus, knowledge of their dissociation constants in each case is essential to develop appropriate sample treatment steps for their isolation and enrichment. The proportion of drugs with an ionizable group has been estimated at 95% [7]. It has been also estimated that 75 % of drugs are weak bases, 20 % weak acids, and the rest contained non-ionics, ampholytes and alcohols. Consequently, the need of SPE sorbents with enhanced versatility and selectivity to retain drugs with different acid-base properties is highly convenient.

In this sense, conventional C_{18} or C_8 modified silica phases have been used for drug extraction followed by determination by liquid chromatography coupled to mass spectrometry (LC-MS) in biological samples [8,9]. However, these sorbents showed certain problems such as their instability at extreme pH as well its low ability to retain polar compounds and ionic drugs [6]. To solve these limitations,

hydrophilic lipophilic balanced reversed-phase (RP) sorbents such as Oasis HLB and Strata-X were developed to achieve greater retention of polar compounds in aqueous matrices [6,10]. Although these commercial single-mode sorbents have demonstrated to improve the capacity of extraction procedure, more efforts to remove interferences and gain selectivity, particularly in the analysis of complex matrix samples are required.

Mixed-mode polymeric sorbents have progressively emerged to enhance the selective extraction of analytes [11]. These materials are based on a carbon skeleton modified with ionic groups, resulting in a dual mechanism of retention: RP and ion-exchange (IEX) interactions, respectively. The main advantage of these sorbents over single-mode sorbents is their tunable selectivity by modifying the pH values of loading and eluting solutions, which makes more suitable to extract analytes from complex samples. Mixed-mode IEX sorbents can be classified in four groups: strong cation-exchange (SCX) and weak cation-exchange (WCX) using sulfonic or carboxylic acids groups, respectively, and strong anion-exchange (SAX) and weak anion-exchange (WAX) using quaternary amine groups and tertiary or secondary amine moieties, respectively. Mixed-mode IEX sorbents, in their commercial or home-made version, have been successfully applied to the extraction of drugs, including pharmaceutical and illicit drugs, in environmental, food and biological samples [12,13,14,15]. Despite the ion-selectivity exhibited by these mixed-mode sorbents, one drawback of their use is that any of these phases can be used to simultaneously extract both basic and acidic compounds. To overcome this limitation, several approaches have been proposed. A first approach is the combination of mixed-mode cation- and anion-exchange sorbents in tandem to retain acidic, basic and neutral compounds [16]. Alternatively, a multi-layer SPE can be prepared by combination of two or more different sorbents within the same cartridge [17]. Other strategy implies the preparation of organic polymers bearing amphoteric groups for its application as mixed-mode phases for the determination of acidic, basic, and neutral analytes in environmental water samples [18]. Despite these contributions, the incorporation of anionic and cationic exchangers simultaneously to prepare efficient dual mixed-mode SPE sorbents is a field relatively unexplored.

Indeed, a direct copolymerization of functional monomers with different charge could be used to produce efficient stationary phases with dual and switchable selectivity. This relatively simple approach would not require of additional functionalization steps to introduce ionic functionalities compared to

that of previously reported mixed-mode sorbents. Besides, to our knowledge, this strategy has not been applied to drug analysis in biological matrices as oral fluids.

In the present work, a one-pot synthesis has been explored to prepare dual mixed-mode sorbents for SPE extraction of acidic and basic drugs in oral fluid samples. A polymer composed by ionizable monomers 4-vinylpyridine (VP) and methacrylic acid (MAA) and ethylene glycol dimethacrylate (EGDMA) as cross-linker was synthesized. The appropriate combination of these monomers could be promising to adjust the distribution of charge and polarity on the surface of the stationary phase to isolate and preconcentrate acidic and basic drugs. Cocaine and 3-methylmethcathinone (3-MMC) with pK_a values of 8.9 and 8.7, respectively [2,3], diazepam, a benzodiazepine with a pK_a value of 3.4 [19], and naproxen and diclofenac, two non-steroidal anti-inflammatory drugs with pK_a values of 4.1 and 4.2, respectively [20,21] have been selected as basic and acidic model analytes to test the usefulness of the developed dual mixed-mode sorbent. Later on, the resulting sorbent was applied to the isolation and enrichment of cocaine and diazepam from oral fluid samples followed by their determination by UHPLC-MS/MS.

Experimental

Standards and reagents

3-MMC used in this study was kindly provided by the laboratory of "Inspección de Farmacia y Control de Drogas" (Valencia, Spain). Diazepam was purchased from Guinama (Valencia, Spain). Cocaine, cocaine-d_3, naproxen and diclofenac were obtained from Sigma (Stenheim, Germany). MAA, VP, EGDMA and azobisisobutyronitrile (AIBN) used for mixed-mode polymer preparation were purchased from Sigma. Organic solvents and buffer constituents employed in this study were obtained from Scharlau (Barcelona, Spain). Solutions at pH 2.0, 4.0, 7.0, and 10.0 were prepared using phosphate, acetate, phosphate, and carbonate buffers at 0.1 M concentration, respectively.

Stock solutions were prepared at 1000 mg L^{-1} concentration level in 2-propanol and kept at −20°C in amber glass vial.

Visiprep™ SPE Vacuum Manifold and 1 mL empty polypropylene SPE tubes with polyethylene frits (20 µm porosity) were obtained from Supelco (Bellefonte, PA, USA). ExtraBond® Drug cartridge (200 mg, 3 mL) from Scharlau were employed as reference sorbent.

Oral fluid samples were obtained from volunteers who after appropriate information consented to provide samples following the Ethical guidelines established by the Universitat de València (H1454687358321 – Drug analysis in biofluids). Volunteer were males and females with ages ranging from 18 to 45 years old. It should be highlighted that under any circumstances authors have trafficked, proportionated, facilitated or forced any volunteer to consume illegal substances. Samples were collected in an Eppendorf tube 1.5 mL of capacity and stored at -20°C until analysis.

Preparation of mixed-mode sorbent

Polymer was prepared by bulk polymerization of the appropriate amounts of ionizable monomers (VP and MAA) and EGDMA as crosslinker to obtain a molar ratio of (1:3:20) VP/MAA/EGDMA. Acetonitrile was chosen as solvent and it was added at 90 % (w/w). AIBN was selected as initiator and it was introduced in a 1 % (w/w) in the polymerization mixture. The solution was then vortexed during 1 min and a nitrogen stream was applied during 10 min to remove possible oxygen dissolved. Next, the vial containing the polymerization mixture was introduced in a thermal bath at 60 °C during 24 h. After polymerization, the resulting polymer (poly (VP-co-MAA-co-EGDMA)) was crushed and sieved to obtain a particle size of 100 µm.

Then, 25 mg of polymer were introduced into 1 mL polypropylene SPE cartridge embedded between two frits. Also, polymers composed of one functional monomer (poly (VP-co-EGDMA) and poly(MAA-co-EGDMA)) were prepared using the respective molar content indicated above by keeping constant the rest of other reagents. The polymers were subjected to the same protocol described above for the material synthesized with VP/MAA monomers.

Characterization of synthesized materials

The synthesized materials were characterized by Fourier-Transform Infrared Spectroscopy (FTIR) using a Tensor 27 spectrometer from Bruker (Bremen, Germany) with a DLaTGS detector, with a Dura Sample IR II attenuated total reflection (ATR) accessory for solid samples from Smiths Detection Inc. (Warrington, UK) and a nine-reflection diamond/ZnSe Dura Disk plate. To register the FTIR spectra, a small amount of ground polymer was placed onto the ATR crystal and it was pressed. Spectra were collected in the region from 4000 to 600 cm^{-1} with

a resolution of 4 cm^{-1} and 30 averaging scans. Background spectrum was acquired following the same measurement conditions

The synthesized mixed-mode sorbent was also characterized by using a S-4800 Hitachi scanning electron microscope (Ibaraki, Japan). It was provided with a field emission gun and a back secondary electron detector. EMIP 3.0 software from Rontec (Normanton, UK) was used for image data acquisition.

SPE procedure

The SPE protocol consisted on a previous activation of the cartridge using 1 mL of 2-propanol; equilibration with 1 mL phosphate buffer (0.1 M, pH 7.0). Sample pH was adjusted using 1 mL oral fluid and 0.5 mL phosphate buffer (0.1 M, pH 7.0) and it was loaded to the SPE column. Washing step was carried with 1 mL phosphate buffer (0.1 M, pH=7.0) prepared in water with a 5 % acetonitrile and the cartridge was dried for 10 min under vacuum. Finally, drugs were eluted

with 0.4 mL of 2-propanol and 10 µL of internal standard (10 µg L^{-1} cocaine-d3 in 2-propanol) were added before UHPLC-MS/MS analysis.

A commercial ExtraBond® Drug cartridge, specially developed for the extraction of drugs of abuse, was used as reference procedure. In this case, the analytes were extracted following the manufacturer recommendations for amphetamine and derivatives extraction [22]. Briefly, the cartridge was conditioned with 6 mL of methanol followed by 6 mL of sulfuric acid 0.1 M. Then, 0.5 mL of oral fluid spiked at 100 µg L^{-1} of each drug (adjusted using 2.5 mL of sulfuric acid 0.1 M) was percolated through the cartridge. Washing step was done with 3 mL phosphate buffer at pH 7.0, 3 mL sulfuric acid 0.1 M and 3 mL of methanol. Finally, analytes were eluted with 2x3 mL of ammonia aqueous solution 5 % (v/v), and the fraction was analyzed by UHPLC-MS/MS.

UHPLC-MS/MS analysis

A Water Acquity UPLC system from Waters (Milford, MA, USA) equipped with an autosampler, a binary solvent delivery system, a C18 Waters column (1.7 µm, 2.1 x 50 mm) column and a triple quadrupole detector with a Z-spray electrospray ionization source was employed for the analysis of drugs in oral samples. 5 µL injection volume was used and the mobile phase, at a flow rate of 0.4 mL min^{-1}, consisted of (A) 5 mM formic acid in water and (B) acetonitrile. The gradient started

at 100 % of A, followed by an increase to 30 % B at 6 min and to 50 % B at 8 min, and finally to 100 % at 15 min, which was held constant for further 3 min. MS acquisitions were done by a 3.5 kV capillary voltage, using 120 °C source temperature and 350 °C desolvation temperature. Multiple reaction monitoring (MRM) conditions were selected for each analyte and internal standard (see Table 30).

Table 30. *Analytical features for cocaine, 3-MMC, and diazepam determination by the proposed procedure.*

Parameter	Cocaine	Cocaine-d3	3-MMC	Diazepam
Retention time (min)	5.18	5.18	3.90	8.65
MRM	304 > 182	307 > 185	178 > 145	285 > 154
Linear range (µg L^{-1})	0.30 - 1000	-	0.65 - 1000	0.80 - 1000
Linearity (R^2)	0.9998	-	0.9994	0.9992
Slope (L μg^{-1})	4.296 ± 0.019	-	1.101 ± 0.006	1.016 ± 0.006
Intercept	- 0.1 ± 2.0	-	-0.1 ± 0.7	1.3 ± 0.7
LOD (µg L^{-1})	0.04	-	0.08	0.10
LOQ (µg L^{-1})	0.12	-	0.26	0.32
RSD (%)	5	-	7	9

Abbreviations: LOD, limit of detection; LOQ, limit of quantification; MRM, Multiple reaction monitoring; RSD, relative standard deviation.

Results and discussion

Selection of monomers

As it has been aforementioned in the Introduction section, VP and MAA have been selected as ionizable monomers for the synthesis of dual mixed-mode sorbent material. The choice of these functional monomers obeys to their particular (physicochemical) features. VP has weakly basic properties with a pK_a value of the monomer of 5.5, while the pK_a of polyvinylpyridine in aqueous medium is 4.0 [23]. It has been used to modulate the surface properties of hydrophobic polymers, being the resulting sorbents suitable for the extraction of acidic analytes in aqueous matrices [24,25]. The retention mechanisms of VP are mainly based on the existence of strong electrostatic interactions provided by the pyridyl group and the

acidic group of analytes. Additionally, this monomer can form π–π-interactions with solutes containing aromatic rings.

On the other hand, MAA, with a pK_a value 4.7, has been extensively used in the extraction of basic analytes owing to its capability to act both, as hydrogen bond and proton donor, and as hydrogen bond acceptor. Thus, MAA-based sorbents have demonstrated a good affinity for basic analytes through hydrogen bonding [26,27]. Recently, our group has developed poly (MAA-co-EGDMA) monoliths in-pipette tip for the extraction of basic drugs [28].

Polymer prepared with those monomers will be positively charged below 4.0 due to the VP moiety, being appropriate to interact to strong and weak acidic drugs with pK_a values between 2.0 and 4.0. The total charge of the polymer will be negative at pH higher than 4.7, due to the MAA moiety, favoring interactions with basic drugs with pK_a values around 8-10.

Considering that most of illegal drugs including cocaine, amphetamines and derivatives, cathinones and derivatives, opioids, tryptamines, piperazines, and arylcyclohexylamines, are basic compounds, the strongest interaction with the dual mixed-mode would correspond to the interactions of positively charged drug with negatively charged MAA at pH higher than 4.7. Additionally, VP would form π–π-interactions with those compounds, all containing aromatic rings. Thus, taking into consideration all those aspects, the ratio VP:MAA was fixed at 1:3 to synthesize the dual mixed-mode sorbent.

Characterization of sorbent

The dual mixed-mode material was prepared and characterized by FTIR in order to corroborate its successful preparation. For this purpose, two additional materials composed of only one functional monomer poly (VP-co-EGDMA) and poly (MAA-co-EGDMA) were prepared, both containing an 80 % wt of EGDMA as cross-linking agent and 20 % wt of functional monomer, keeping constant the rest of reagents. Figure 51A shows FTIR spectra of VP- and MAA-based polymers ("control polymers") and VP/MAA-based polymer. The spectrum of the dual mixed-mode material shows certain absorption peaks present in the spectra of control VP- and MAA-based polymers. Thus, the incorporation of VP into mixed-mode polymer was demonstrated by the peaks located at 1600 cm^{-1} (Figure 51B) and 820 cm^{-1} (Figure 51C), corresponding to the symmetric C=N stretching and C–H out of plane bending vibration of pyridine ring, respectively. Also, the spectrum of poly(VP-co-MAA-co-

EDMA) exhibited the characteristic bands of poly(MAA-co-EDGMA) due to MAA and EDGMA, such as 1294 and 1204 cm^{-1} attributed to the presence of C–O–C stretching in the MAA monomer, and 1697 cm^{-1} attributed to the ester functionality.

The morphology of mixed-mode polymer was investigated by SEM measurements. As shown in Figure 51D, the SEM image of this material shows the presence of globular-shaped particles with an approximate diameter of 1 µm and rough surface. This rugosity can be an indicative of the presence of meso and micropores, which benefits the sorbent retention properties, which will be described in detail below.

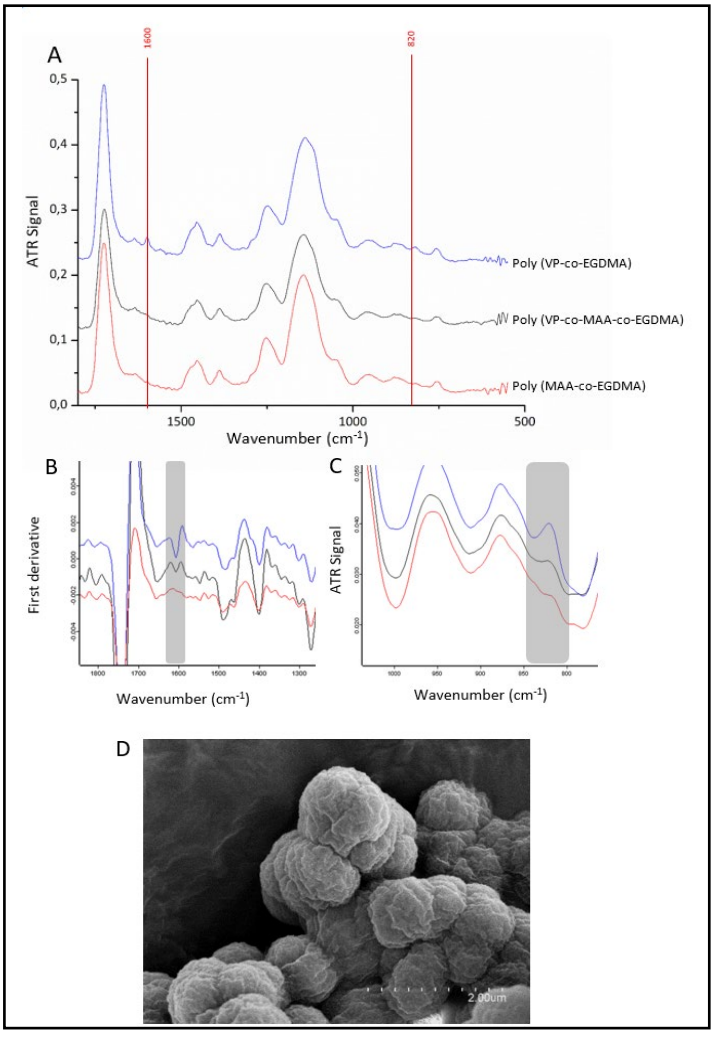


Figure 51. *(A) ATR-FTIR spectra of poly(MAA-co-EGDMA) (red line), poly(VP-co-EGDMA) (blue line), and poly(VP-co-MAA-co-EGDMA) (black line) materials; (B) zoom of first derivative ATR-FTIR spectra over 1800-1300 cm^{-1}; (C) zoom of ATR-FTIR spectra over 1000-800 cm^{-1}.*

Evaluation of sorbent against acidic and basic molecules

In order to test the usefulness of the dual mixed-mode material for the extraction of basic and acidic analytes, the five target molecules were percolated through the SPE sorbent at different pH values. For this purpose, 1.5 mL loading solution containing 100 µg L^{-1} of cocaine, 3-MMC, diazepam, naproxen, and diclofenac at different pH values (2.0, 4.0, 7.0, and 10.0) were investigated. Washing was carried out with 1 mL of water, and elution with 1 mL 2-propanol.

Figure 52 shows the recoveries obtained for the tested analytes as a function of the pH of the loading sample. As it can be seen in Figure 52, three different behaviors can be observed.

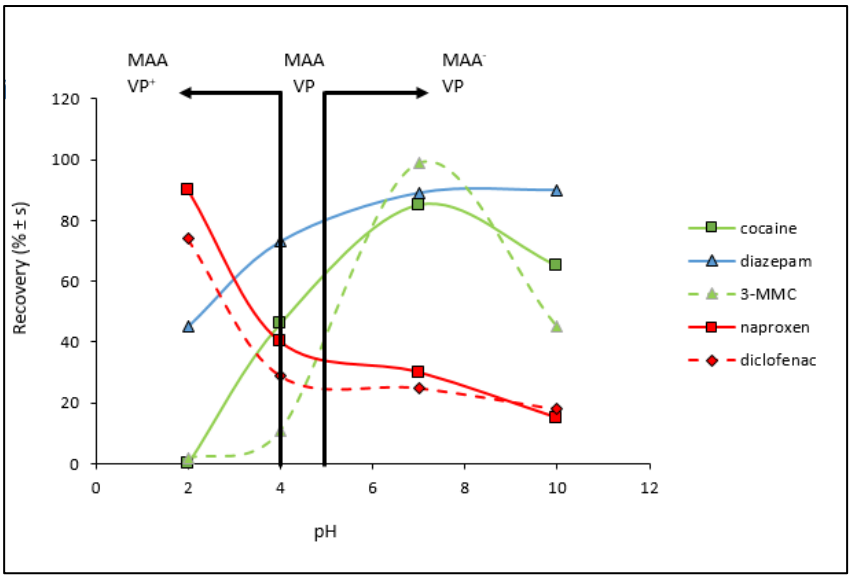


Figure 52. *Effect of loading pH solution on the recoveries of cocaine, 3-MMC, diazepam, naproxen and diclofenac.*

Cocaine and 3-MMC, with pK_a values of 8.9 and 8.7, respectively are protonated at acidic pH and, thus, have a strong interaction with the dual mixed-mode material at pH between 4.7 and 8.5. Below pH 4.7, the sorbent material will be no longer negatively charged and the strength of the interactions substantially decreased. On the other hand, at pH higher than that of pK_a values, analytes will be in its neutral form and the interactions with the sorbent material also decreased.

Retention of naproxen and diclofenac, two non-steroidal anti-inflammatory drugs with pk_a values of 4.1 and 4.2, reached a maximum at loading pH of 2.0, whereas increasing pH, retention values of those analytes decreased significantly. At pH values lower than 4.0, these compounds are in its neutral form, favoring

interactions such as hydrogen bonding (between carboxylic acid groups of naproxen and diclofenac and the MAA sites present on the polymer) and π- π stacking (between pyridyl groups and aromatic rings of these drugs). When the pH of loading solution increased above 4.0, an electrostatic repulsion between the negatively charged surface of the polymer due to MAA moieties and the anionic naproxen and diclofenac is produced, which explains the decrease observed in the extraction recoveries.

Finally, diazepam, a benzodiazepine with a pK_a value of 3.4, would be present in a neutral form and retention process will be done predominantly by hydrogen bonding interactions and π– π stacking.

In summary, all these results highlighted the significant advantage of dual mixed-mode sorbents for tailoring the retention of neutral and ionizable species. Thus, the possibility to switch the ion-exchange capability of sorbent towards basic or acidic compounds can be easily achieved by changing the pH of the loading solution. In the synthesized sorbent, the WAX features dominate at low pH, whereas the WCX behavior prevails at high pH.

Application of dual mixed-mode sorbent to the extraction of drugs in oral fluids

Once it has been demonstrated the dual mixed-mode character of the synthesized sorbent, its applicability to the simultaneous extraction of relevant drugs with different acid/base properties from oral fluid samples will be evaluated. For this purpose, cocaine, 3-MMC, and diazepam were selected as target compounds.

As it can be seen in Figure 52, the most appropriate pH of loading solution for a quantitative extraction of these drugs is pH 7.0. To evaluate the effect of different washing and elution solvents, oral fluid samples spiked at 100 μg L^{-1} with the three selected target drugs, adjusted to pH 7.0, were percolated through the polymeric sorbent.

Several washing solutions were investigated in order to increase the strength of the washing solution and to improve the clean-up of the sample extracts. Thus, 1 mL phosphate buffer (pH 7.0, 0.1 M) in water, and 2 propanol, acetone, and acetonitrile at 5 % (v/v) in water, were evaluated. Figure 53A shows the obtained results, giving the pH 7.0 phosphate buffer in 5 % acetonitrile the best recoveries (from 89 to 106 %). Higher concentrations of acetonitrile in the washing solution

were evaluated, but recoveries decreased with the percentage of acetonitrile. Hence, 1 mL phosphate buffer at pH 7.0 containing 5 % acetonitrile was selected as washing solution in the following experiments.

2-propanol was selected as eluting solvent due to its high elution strength and its lower toxicity compared to acetonitrile and methanol. The effect of eluent volume, from 1.0 to 0.2 mL 2-propanol, was investigated in order to obtain a higher enrichment factor (Figure 53B). The obtained recoveries for target analytes were quantitative up to 0.4 mL 2-propanol with recovery values in the 72-88 % level, and they decreased to 45-60 % using 0.2 mL. Thus, 0.4 mL 2-propanol was selected as elution volume, providing a 2.5-fold enrichment factor.

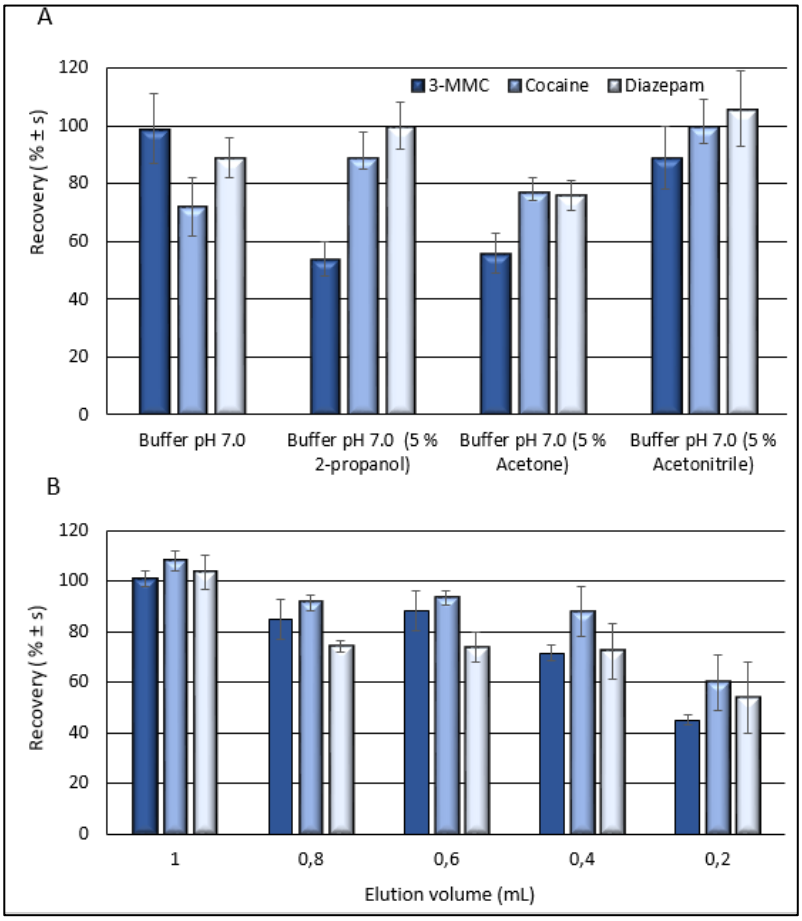


Figure 53. *Effect of (A) washing solution and (B) volume of elution on the recoveries of cocaine, 3-MMC and diazepam.*

The reusability of the of dual mixed-mode material was also investigated. For this purpose, oral fluid samples spiked with target compounds at 100 µg L^{-1} were repeatedly analyzed (n=10), using the same sorbent cartridge, providing quantitative recoveries higher than 80 % for all target analytes. Thus, the produced sorbent can be reused at least for ten consecutive extraction cycles.

The performance of the prepared dual mixed-mode sorbent was compared with that of commercially available ExtraBond® Drug SPE cartridges, a mixed phase nonpolar/silica-based cation exchange (C8/benzenesulfonic acid) specially manufactured for drug analysis. The procedure, recommended by the manufacturer [22], provided recoveries of 5, 39, and 9 % for cocaine, 3-MMC, and diazepam, respectively. Thus, ExtraBond® Drug procedure cannot be applied for the simultaneous extraction of the evaluated drugs in oral fluids, demonstrating the high performance of the proposed dual mixed-mode sorbent.

Analytical performance of the method

The analytical features of the method were established using the proposed methodology using dual mixed-mode SPE in combination with UHPLC-MS/MS (Table 30). Calibration curves were analyzed from 1 to 1000 µg L^{-1}, providing a good linearity (R^2 > 0.9998) for the three drugs investigated. The limits of detection (LOD) and quantification (LOQ) were calculated as three and ten times the standard deviation of the intercept divided by the slope of the calibration curve, considering the 2.5x pre-concentration factor, giving values from 0.04 to 0.10 µg L^{-1}, and from 0.12 to 0.32 µg L^{-1}, for LOD and LOQ, respectively. Precision was established as the relative standard deviation of three 100 µg L^{-1} standards (n = 3) , providing values of 5, 7 and 9 % for cocaine, 3-MMC, and diazepam, respectively. Method trueness was estimated as the recoveries obtained for the analysis of blank oral fluid samples spiked with the selected analytes at concentrations from 100 to 1000 µg L^{-1}. The obtained recoveries are shown in Table 31, with values ranging from 80 to 96 % for cocaine, from 72 to 98 % for 3-MMC, and from 69 to 91 % for diazepam.

Table 31. *Recoveries obtained for the analysis of blank oral fluids spiked with cocaine, 3-MMC, and diazepam determined by the proposed procedure.*

[Spiked] (μg L^{-1})	Recovery (% ± s, n=2)		
	Cocaine	**3-MMC**	**Diazepam**
100	99 ± 12	72 ± 10	69 ± 7
250	85 ± 13	79 ± 11	73 ± 15
500	80 ± 8	89 ± 11	88 ± 19
750	84 ± 9	98 ± 13	91 ± 8
1000	80 ± 9	96 ± 13	85 ± 11

Analysis of field samples

The developed procedure was applied for the analysis of oral fluid samples provided by drug consumers (n = 20). In the case of sample extract was out of the linear range, extracts were re-analyzed after an appropriate dilution. Table 32 shows the obtained results for the analysis of field samples. As it can be seen, 7 samples were positive in cocaine with concentrations from 54 μg L^{-1} to 25.7 mg L^{-1}, and 6 samples were positive in diazepam with concentrations from 5 to 139 μg L^{-1}. No positive samples were found for 3-MMC.

Table 32. *Concentration of the evaluated drugs found in field oral fluid samples.*

Sample	Analyte	Concentration (μg L^{-1} ± s)
1	Cocaine	5300 ± 300
2	Cocaine	25700 ± 1200
3	Cocaine	710 ± 30
4	Cocaine	6800 ± 500
5	Cocaine	54 ± 3
6	Cocaine	1760 ± 90
7	Cocaine	104 ± 8
8	Diazepam	5.5 ± 0.3
9	Diazepam	139 ± 6
10	Diazepam	129 ± 5
11	Diazepam	85 ± 3
12	Diazepam	83 ± 4
13	Diazepam	80 ± 3

Furthermore, the developed protocol was used to monitor diazepam concentration in oral fluid at different collection times after a single administration. For this purpose, a dose of 5 mg tablet was taken by a male volunteer who placed it under the tongue until its complete dissolution. Oral fluid samples were collected prior intake and at several times after drug intake, from 15 min to 6 h. Figure 54 shows the concentration of diazepam found in these samples, reaching a maximum of 749 µg L^{-1} at 15 min that significantly decreases with the time. In any case, diazepam concentration can be monitored with the proposed methodology even after several hours after intake, with residual concentration from 5.14 to 1.83 µg L^{-1} after 2 and 6 h intake, respectively. These levels of diazepam are in the same range of concentration than those observed in previous studies [29].

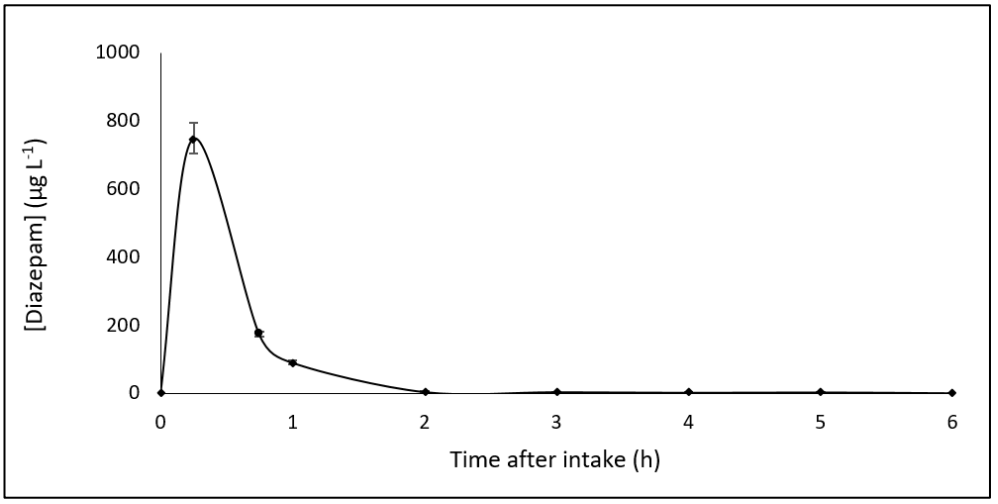


Figure 54. *Concentration of diazepam in a user oral fluid after an oral dose of 5 mg of active ingredient.*

Conclusions

A simple and practical method for the synthesis of a dual mixed-mode polymeric sorbent containing VP and MAA as ionizable monomers has been described. The use of VP and MAA as monomers provides a hydrophilic amphoteric polymer with ion-exchange sites available for the interaction of both, acid and basic analytes. The results revealed that ion-exchange, hydrogen bonding, and hydrophobic interactions were present in the retention process of target analytes. In particular, the ion-exchange contribution can be switched reversibly between anion- and cation-exchange by a simple control of the pH of loading solution. Finally, the practical applicability of the developed sorbent was evaluated for the

extraction of cocaine, 3-MMC, and diazepam in oral fluid samples followed their determination by UHPLC-MS/MS. The method showed excellent recoveries and LODs in the range of 0.12 - 0.32 μg L^{-1}, and exhibited better capability for these drugs than commercial sorbents (ExtraBond® Drug). Additionally, the results provided here afford valuable information for the development of new analyte-oriented sorbent design to face up challenging issues as enhanced selectivity in analytical applications.

CRediT authorship contribution statement

A. Sorribes-Soriano: Validation, Writing - original draft. **S. Armenta:** Conceptualization, Methodology, Validation, Writing - review & editing. **F.A. Esteve- Turrillas:** Conceptualization, Methodology, Validation, Writing - review & editing. **J.M. Herrero-Martínez:** Conceptualization, Methodology, Validation, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Bloc 3. Materials selectius

Section 3. Selective materials

***Capítol 8** / Chapter 8. Cocaine abuse determination by ion mobility spectrometry using molecular imprinting.*

Journal of Chromatography A, 1481 (2017) 23-30

***Capítol 9** / Chapter 9. Amphetamine-type stimulants analysis in oral fluid based on molecularly imprinting extraction.*

Analytica Chimica Acta, 1052 (2019) 73-83

***Capítol 10** / Chapter 10. Tuning the selectivity of molecularly imprinted polymer extraction of arylcyclohexylamines: from class-selective to specific.*

Analytica Chimica Acta, 1124 (2020) 94-103

***Capítol 11** / Chapter 11. Molecularly imprinted polymer-based device for field collection of oral fluid samples for cocaine identification.*

Journal of Chromatography A, 1633 (2020) 461629

***Capítol 12** / Chapter 12. Magnetic molecularly imprinted polymers for the selective determination of cocaine by ion mobility spectrometry.*

Journal of Chromatography A, 1545 (2018) 22-31

***Capítol 13** / Chapter 13. Development of a molecularly imprinted monolithic polymer disk for agitation-extraction of ecgonine methyl ester from environmental water.*

Talanta, 199 (2019) 388-395

***Capítol 14** / Chapter 14. Fluoroquinolone and methamphetamine oligosorbent preparation by aptamer immobilization.*

Not published

Cocaine abuse determination by ion mobility spectrometry using molecular imprinting

A. Sorribes-Soriano, F.A. Esteve-Turrillas, S. Armenta, M. de la Guardia,

J.M. Herrero-Martínez

Department of Analytical Chemistry, University of Valencia, 50th Dr. Moliner St., 46100 Burjassot, Spain

Abstract

A cocaine-based molecular imprinted polymer (MIP) has been produced by bulk polymerization and employed as selective solid-phase extraction support for the determination of cocaine in saliva samples by ion mobility spectrometry (IMS). The most appropriate conditions for washing and elution of cocaine from MIPs were studied and MIPs were characterized in terms of analyte binding capacity, reusability in water and saliva analysis, imprinting factor and selectivity were established and compared with non-imprinted polymers. The proposed MIP-IMS method provided a LOD of 18 µg L^{-1} and quantitative recoveries for blank saliva samples spiked from 75 to 500 µg L^{-1} cocaine. Oral fluid samples were collected from cocaine consumers and analysed by the proposed MIP-IMS methodology. Results, ranging from below the LOD to 51±2 mg L^{-1}, were statistically comparable to those obtained by a confirmatory gas chromatography-mass spectrometry method. Moreover, results were compared to a qualitative lateral flow immunoassay procedure providing similar classification of the samples. Thus, MIP-IMS can be considered an useful alternative that provided fast, selective and sensitive results with a cost affordable instrumentation that does not require skilled operators.

Keywords: *Cocaine, ion mobility spectrometry, molecular imprinted polymer, saliva*

Introduction

According to United Nations Office on Drugs and Crime World Drug Report 2015 (UNODC), the traffic of cocaine is done South America, mainly Bolivia, Peru and Colombia to North America and Europe being one of the most consumed drugs all around the world [1].

Determination of cocaine in biological fluids, mainly in urine and blood has relative importance in different areas such as toxicology in hospitals, traffic control, justice, drug treatment programs, and doping in professional sport [2]. The analysis of blood implies the application of invasive sampling and urine can be easily falsified; thus, there is a high interest on the use of alternative biological specimens. Oral fluid possesses as main advantages, in front of urine or blood, those related to the analysis of free drug concentrations and it is noninvasive. All this characteristics have provided oral fluids a level of scientific credibility to be considered in drug testing.

Usually, detection of cocaine involves a two-step procedure: i) an initial screening, normally using an immunoassay method, to classify samples in two groups, positives and negatives; and ii) a confirmatory method, generally performed by gas chromatography-mass spectrometry (GC–MS) [3], or liquid chromatography-mass spectrometry (LC-MS) [4] with limits of detection (LODs) in the low μg L^{-1} range.

The cutoff concentration used to classify positive/negative samples varies from 80 to 100 μg L^{-1} of cocaine (or its metabolites) and the main problem related to immunoassay methods is the relative proportion of false positive and false negative results obtained by using available commercial kits. On the other hand, chromatography methods usually involve multiple laborious steps such as extraction, preconcentration and clean-up [5].

Ion mobility spectrometry (IMS) is a fast and sensitive analytical technique based on the separation in the gas-phase of ionized analytes under an electric field at ambient pressure. However, the potential of IMS is reduced due to its limited selectivity and the analysis of complex samples must be done after a complex chemometric treatment [6,7] or after a preconcentration/clean-up based on assorted techniques like solid-phase extraction (SPE) [8], solid-phase microextraction (SPME) [9], or liquid-liquid microextraction (LLME) [10].

In recent years, the selectivity of sample treatments have been improved by using analyte-selective supports. Thus, immunoaffinity chromatography columns

have been used for the determination of toxins [11,12] and fungicides [13]; and molecularly imprinted polymers (MIPs) have been applied to the analysis of pharmaceuticals [14, 15, 16, 17] and nitrobenzenes [18]. The main advantage of MIPs in front of the other selective supports are related to their chemical inertness, long-term stability, possibility to synthetize large quantities, higher sorption capacity, and relatively low acquisition cost.

MIPs dedicated to the selective recognition of cocaine have already been synthesized using different types of functional monomers and solvents. For instance, selective retention of cocaine in MIPs has been previously studied. In this sense, methacrylic acid (MAA) and ethylene glycol dimethacrylate (EDMA) were prepared in chloroform to prepare cocaine specific MIP, being coated with mineral oil the MIP surface in order to improve its recognition properties in polar solvents [19]. In another study, computational techniques were applied to study different functional monomers in the synthesis of cocaine-based MIPs [20], obtaining that the best results were achieved using acrylamide and itaconic acid as monomers, in dimethylformamide as porogen. The potential of various monomers for the selective extraction of cocaine were also evaluated, showing MAA as monomer inacetonitrile as porogen and azo-bisisobutyronitrile as UV initiator, a high selectivity versus cocaine [21]. However, in our knowledge, there is no precedent on the use of MIPs for the determination of cocaine by IMS

The main objective of this study has been the development of a cocaine-based MIP for the determination of cocaine in oral fluids and its analysis by IMS. Thus, cocaine has been extracted from saliva through a MIP-SPE and the eluate directly injected into the IMS. The proposed MIP-IMS method was validated in terms of sensitivity, selectivity, trueness and precision. Cocaine user saliva samples were analyzed by the proposed methodology and by a reference procedure in order to evaluate the accuracy of results.

Experimental Section

Material, reagents and samples

Cocaine standard was kindly provided by the “Unidad de Inspección de Farmacia y Control de Drogas” from the Valencia Health Service Area. Cocaine standard solutions were prepared in methanol, containing 5% (v/v) acetic acid, and kept at –20 ºC in amber glass vials. All the solvents used in this study were HPLC grade or higher. Organic solvents and buffer constituents were obtained from

Scharlab (Barcelona, Spain). Methacrylic acid (MAA), ethylene dimethacrylate (EDMA) and azobisisobutyronitrile (AIBN) were provided by Sigma (Stenheim, Germany).

Visiprep™ SPE Vacuum Manifold and 1 mL empty polypropylene SPE tubes with polyethylene frits (20 µm porosity) were obtained from Supelco (Bellefonte, PA, USA).

Oral fluid samples were obtained from cocaine users who provided their consent after appropriate information. Cocaine consumers were males and females with ages ranging from 20 to 40 years old. Samples were collected at different times after abuse of the drug and stored at −4°C until their analysis. Cocaine-free saliva samples were collected from males and females with ages ranging from 25 to 40 years old. It should be highlighted that under no circumstances have the authors trafficked or provided illegal substances, aimed, promoted, facilitated, stimulated, or forced in any way the consuming of illegal substances.

Production of MIPs and NIPs

A MIP was prepared by bulk polymerization using cocaine as template, MAA as monomer, EDMA as cross-linker, acetonitrile as porogen, AIBN as initiator and, in a molar ratio of template/monomer/cross-linker ratio of 1:4:20 (0.0340 g/0.0344 g/0.3964 g). A non-imprinted polymer (NIP) was also prepared in the absence of template molecule in the polymerization mixture. The solution (placed in a vial) was sonicated for 5 min and purged with nitrogen for additional 10 min. The polymerization was carried out in a water bath at 60°C for 24 h. After polymerization, the glass vials were broken, and the resultant polymers were crushed in an agate mortar, dried in an oven at 80°C overnight, and sieved with a steel sieve with size $\leq$ 100 µm in order to obtain an homogeneous particle size.

The MIP particles were treated with a mixture of 5% (v/v) acetic acid in methanol in a Soxhlet extractor for 24 h to remove the template from polymer and were then rinsed with the same solution until no cocaine signal was obtained by IMS analysis. Soxhlet extraction was not performed on the NIP particles.

Characterization of MIPs and NIPs

SEM images of polymers were taken with a scanning electron microscope (S-4100, Hitachi, Ibaraki, Japan) provided with a field emission gun, a back secondary electron detector and an EMIP 3.0 image data acquisition system (Rontec, Normanton, UK).

MIP-SPE procedure

25 mg of each polymer (MIP or NIP) were introduced inside 1 mL empty polypropylene SPE tubes, between two 20 µm polyethylene frits, and it was equilibrated with 1 mL 5% (v/v) acetic acid in methanol and 1 mL deionized water under gravity flow. Saliva samples (500 µL if possible), previously diluted 1:5 (v/v) with ammonium/ammonia buffer pH=9.3, were loaded through the cartridges, and washed with 1 mL deionized water and 1 mL chloroform. Between each step of washing, a gentle vacuum was applied for complete drying using a SPE vacuum manifold. Finally, cocaine was eluted with 1 mL of 5% (v/v) acetic acid in methanol and measured by IMS. The same procedure was applied for cocaine extraction using NIPs in order to obtain the imprinting factor (IF). IF is used as a measure of the strength of interaction of the imprinted polymer towards the template molecule. It is obtained from the ratio of the retention factor (k') of MIP to NIP following:

$$IF = \frac{k'_{MIP}}{k'_{NIP}} \qquad \text{(Equation 1)}$$

where k'_{MIP} corresponds to the retention factor of MIPs, and k'_{NIP} denotes the retention factor of NIPs.

IMS procedure

An IONSCAN-LS from Smiths Detection (Morristown, NJ, USA) equipped with a ^{63}Ni foil radioactive ionization source was used to separate and identify the different compounds involved in this study. IM station software (version 5.389) was used for data acquisition and processing. Plasmagrams were acquired in positive ion mode using nicotinamide, with a reduced mobility, K_0, of 1.860 $cm^2\ V^{-1}\ s^{-1}$, as internal calibrant. The number of segments per analysis was 56, containing every plasmagram 779 data points. The shutter grid width was 0.2 ms (the value optimized by the manufacturer) and plasmagrams were collected with a scan period of 40 ms. A counterflow of dry air, set at 300 mL min^{-1}, was introduced as

drift gas at the end of the drift region. The electric field strength in the drift region was 251 V cm^{-1} with a total drift voltage of 1759 V and a drift tube length of 7 cm.

Thermal desorption from a polytetrafluoroethylene (PTFE) membrane was used for sample introduction. In this strategy, 8 µL of sample were placed onto the PTFE membrane and heated to vaporize the analyte, which was transferred to the ionization region. Before first analysis, PTFE membrane was introduced into the IMS instrument to remove any possible interference. Desorption, inlet and drift tube temperatures were adjusted to 260, 270 and 232°C, respectively. Using a 1 s post-dispense delay, the sample tray containing the PTFE membrane was inserted in the heated zone and the sample was held in this position for 60 s.

Lateral-flow immunoassay reference procedure

The reference methodology is based on the analysis of oral fluid samples using the Dräger DrugTest 5000 from Dräger (Madrid, Spain). The instrument comprises an on-site competitive lateral-flow immunoassay and an opto-electronic analyzer for the qualitative detection of abuse substances such as cocaine metabolites, opiates, amphetamines, methamphetamine, benzodiazepines and tetrahidrocannabinol in oral fluids.

Oral fluid was collected by direct absorption in the porous collector, which forms an integral part of the test cassette. The built-in indicator turns blue when sample collection is finished. After sampling, the analysis was made by placing both, the test cassette and the buffer cartridge, into the analyzer. The assays provided a cut-off concentration of 20 µg L^{-1} cocaine in oral fluid samples.

GC-MS reference procedure

An Agilent Technologies 7890A GC system (Palo alto, CA, USA), equipped with a Zebron ZB-5MS capillary column (30 m x 0.32 mm x 0.25 mm) and an Agilent Technologies 5975C inert XL EI/CI MSD triple axis single quadrupole detector was used for the cocaine chromatography determination. One µL of the MIP-SPE extract, after appropriate dilution if required, was injected in splitless mode at 250 °C, employing helium as carrier gas in constant flow mode of 1 mL min^{-1}. Oven temperature program was 150 °C, held for 1 min, increased at a rate of 10 °C min^{-1} up to 250 °C and finally held 5 min.

Transfer line and ion source temperatures were 300 and 250 °C, respectively, and an electron voltage of 70 eV was employed for electron impact ionization. Cocaine determination was performed in the selected ion monitoring (SIM) mode using the 182 and 82 m/z peaks as quantifier and qualifier ion, respectively.

Triphenyl phosphate has been used as internal standard in the GC-MS determination. All samples and standards were spiked to a final concentration of internal standard of 200 µg L^{-1}. Triphenyl phosphate was measured in the SIM mode using the 326 m/z peak.

Results and Discussion

Synthesis and characterization of the MIP and NIP polymers

Previous works [19, 20, 22] have revealed that using MAA, EDMA and acetonitrile and AIBN as functional monomer, cross-linker, porogen and initiator have led to the synthesis of MIPs with high selective recognition of cocaine either in organic or in aqueous media. However, to our knowledge, MIPs have never been applied in combination with IMS for the selective extraction of cocaine in saliva samples. Thus, MIPs and NIPs were prepared via bulk polymerization method using a non-covalent approach from the typical molar ratio of template/functional monomer/cross-linker of 1:4:20 [23]. The surface morphology and the particle size of both MIP and NIP were analyzed using SEM. As shown in Figure 55A, the MIP gave globules slightly higher than those found for the control polymer (NIP) (Figure 55B). It could be attributed to the formation of cavities during the synthesis process in MIP.

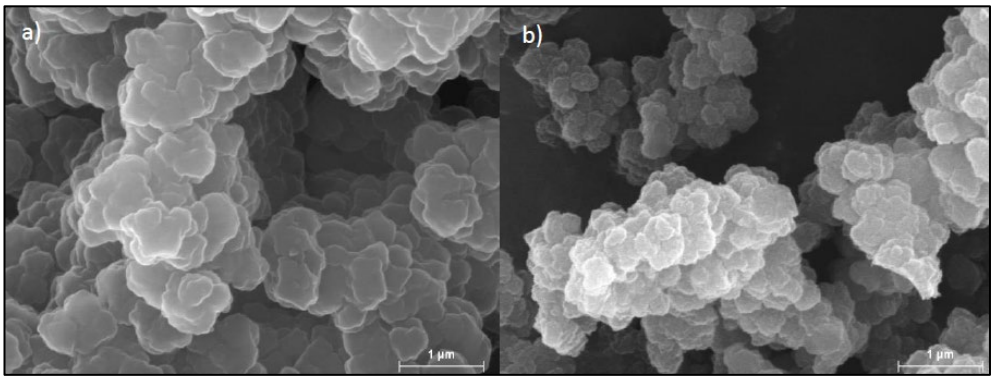

Figure 55. *SEM micrographs of MIP (a) and NIP (b) measured at 20000x.*

IMS plasmagrams

Ion mobility plasmagram of a 200 µg L^{-1} cocaine standard is depicted in Figure 56. The most intense peak in the plasmagrams is that of nicotinamide (K_0 = 1.860 cm^2 V^{-1} s^{-1}), used as internal calibration in the positive ionization mode to correct small variations in temperature and pressure. Cocaine plasmagram provides a peak at 15.07 ms drift time with a reduced mobility of 1.16 cm^2 V^{-1} s^{-1}, which is similar to previously reported values [24]. On the other hand, in negative samples cocaine peak is absent, and no peaks can be observed (see Figure 56). Moreover, it should be commented that the cocaine standard plasmagram shows a small unidentified peak around 12 ms, probably due to an impurity of the cocaine standard used through this study.

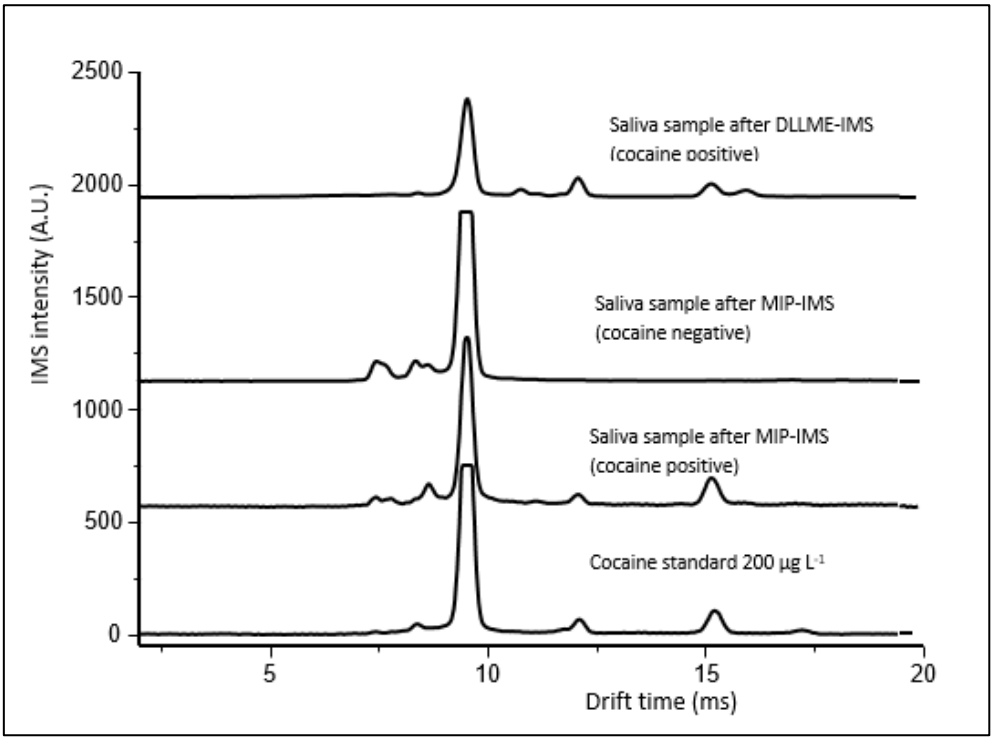

Figure 56. *IMS signals for a cocaine standard (200 µg L^{-1}), positive and negative oral fluid samples extracted using the recommended MIP-SPE procedure, and positive oral fluid sample extracted following a dispersive liquid-liquid microextraction (DLLME) procedure.*

The IMS software alerts the presence of cocaine when the obtained sample signal matched that of the standard using the following peak descriptors: (i) a K_0 value of 1.16, (ii) a variability value of the drift time of 50 μs, (iii) an amplitude of 1.5, (iv) a minimum IMS signal of 20, and (v) a full width value at the half-maximum amplitude of 200 μs.

Study of the IMS instrumental parameters affecting cocaine signal

To obtain the most appropriate conditions for the analysis of cocaine by IMS, different instrumental parameters such as desorber and inlet temperatures, and volume of sample, were carefully studied, using as analytical response the area of the cocaine peak of a cocaine standard of 200 μg L^{-1}.

The effect of desorber and inlet temperatures on the cocaine signal was evaluated in the range from 220 to 285 ^{o}C and from 225 to 290 ^{o}C, respectively. In all the cases, the inlet temperature would be set 5^{o}C higher than the desorber one to avoid possible condensation of cocaine through the gate. Cocaine signal increases by increasing the desorber and inlet temperature until a maximum value obtained at 255 and 260 ^{o}C in the desorber and inlet, respectively (See Figure 57A).

Injection volume was also evaluated in the range from 1 to 10 μL and, as it can be seen in Figure 57B, cocaine signal increases till achieve a maximum value at 8 μL, remaining constant at higher sample volumes. Thus, 8 μL injection volume was selected to provide maximum IMS signal and to avoid sample overloading

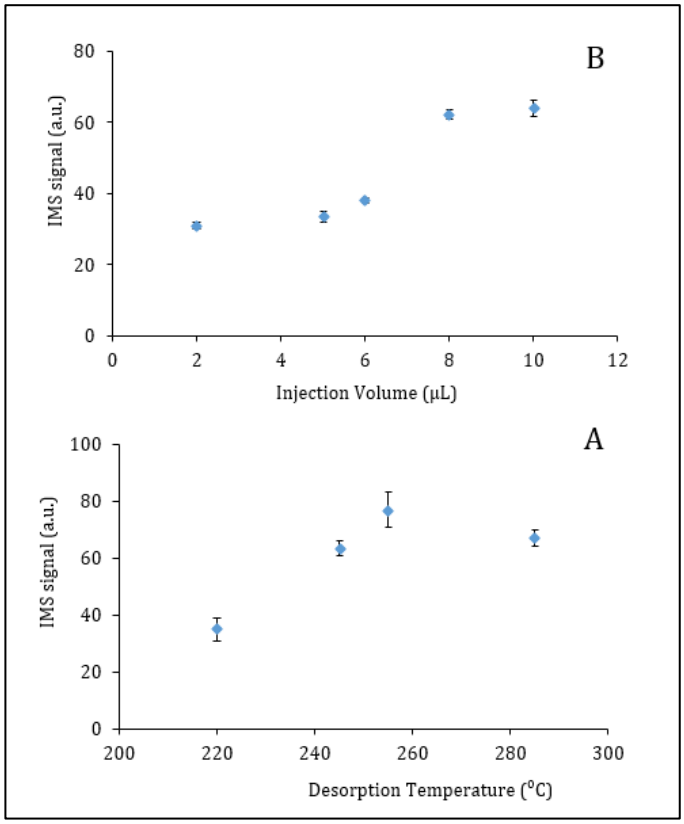

Figure 57. *Effect of desorption temperature (A) and injection volume (B) on the IMS signal of a 200 µg L^{-1} cocaine standard.*

Study of the MIP-SPE of cocaine from saliva

Sample pH was evaluated to assess appropriate interactions between the template and the polymer. Blank saliva samples spiked with 300 µg L^{-1} of cocaine were adjusted to different pH values using acetic acid/acetate, potassium dihydrogen phosphate/potassium hydrogen phosphate, and ammonium/ammonia buffer solutions of pHs 4.5, 7.5 and 9.3, respectively. Retention was performed at room temperature and a cleaning step, consisting of 1 mL water, a gentle vacuum for complete drying, and 1 mL chloroform, was applied. As eluting solvent 1 mL 5% (v/v) acetic acid in methanol was used. Recoveries obtained as a function of sample pH ranged from 48 ± 2 to 106 ± 5 %, obtaining better recoveries when working at high pH values, thus, a pH of 9.3 was selected.

The washing step is a key parameter to obtain, at the same time, maximum selectivity and recovery of cocaine in MIP extraction [25]. 1 mL of ammonium/ammonia pH 9.3 buffer solutions of 200 µg L^{-1} cocaine were loaded in

the SPE cartridges containing both, MIP and NIP particles. Water, methanol, acetonitrile, chloroform and different binary combinations of them were evaluated (see Figure 58) as washing solvents. As it can be seen, the combination of 1 mL water, a gentle vacuum for complete drying, and 1 mL chloroform showed suitable characteristics and selectivity for cocaine determinations by IMS. Applying the aforementioned washing step, 97 ± 2 % cocaine recovery was obtained, whereas only 40 ± 5 % cocaine recovery was provided using NIP-SPE column; which corresponds to an IF of 2.5.

To elute cocaine from the MIP cartridge acidic solutions are usually recommended due to the basic character of cocaine [26]. Different volumes of 5 % (v/v) acetic acid in methanol were studied as eluting solvent. 81 ± 2 % cocaine recovery was obtained when 0.5 mL elution solvent was used, whereas this value increases till 97 ± 2 % when 1 mL was employed.

To evaluate the reusability of the MIP-SPE columns, different water (n=13) and saliva (n=10) samples spiked with cocaine at 200 µg L^{-1} concentration level were repeatedly analyzed. For the analysis of water samples, the MIP demonstrated appropriate performance for at least eight uses, with recoveries higher than 80 % for cocaine (see Figure 59), while for saliva analysis the performance was appropriate for five consecutive uses. After that, a clogging of the column limited the reusability of the column with this type of samples.

To prove the selectivity of the developed MIP-SPE procedure, a saliva sample obtained from a cocaine abuser was analysed by the recommended procedure and the IMS signal was compared to that obtained after a LLME using chloroform as organic solvent (see Figure 56). As it can be seen, the IMS signal of the MIP extract was practically free of interferences, and only cocaine signal was present. On the other hand, in the LLME extract it can be observed the presence of three additional peaks with considerable intensities which can affect the cocaine signal by competitive ionization, interfering its determination in saliva samples.

In order to evaluate the selectivity of MIP cartridges, several compounds that can be found in saliva, such as codeine, nicotine, diazepam, benzocaine, doxylamine succinate and diphenhydramine chlorhydrate were studied. The previously described MIP-SPE procedure was applied to saliva samples spiked with cocaine and the aforementioned compounds (200 µg L^{-1}) on MIP and NIP cartridges. The recovery of cocaine in the presence of the interfering compounds in MIP cartridges was 85 ± 9 %, which was significantly higher than that obtained in the case of NIP

cartridges (49 ± 15 %). Concerning the studied interfering compounds, no significant differences were found on their retention in MIP and NIP cartridges.

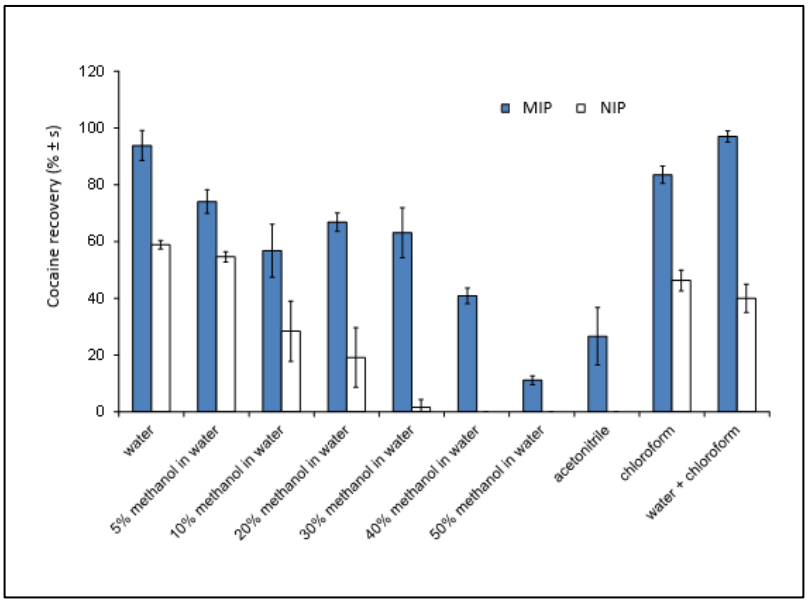

Figure 58. *Effect of the washing solvent composition on the efficiency of cocaine solid-phase extraction using MIP and NIP columns (1 mL of 200 µg L^{-1} cocaine in water were loaded onto the column, washed, and eluted with 1 mL of 5% (v/v) acetic acid in methanol).*

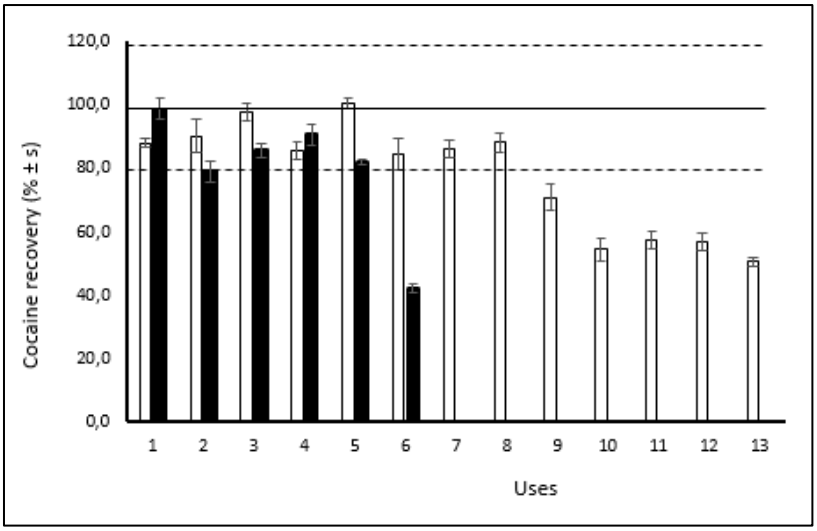

Figure 59. *Reusability of MIP columns after repetitive uses with 1 mL water spiked at 200 µg L^{-1} cocaine (white bars) and 0.5 mL blank saliva spiked at 200 µg L^{-1} cocaine (black bars).*

Additionally, the retention of cocaine metabolites including benzoylecgonine and ecgonine methyl ester in the MIP-SPE column was evaluated. For this purpose, 5 mL of an aqueous solution of both metabolites at 190 μg L^{-1} concentration level were loaded onto the MIP-SPE column and then the recommended extraction procedure was applied. IMS analysis of the extract concluded that neither benzoylecgonine nor ecgonine methyl ester were retained in the MIP-SPE column, demonstrating an excellent selectivity for cocaine determination. This fact could be explained by the presence of the methyl ester and benzoate groups in the cocaine to specifically bind to the MIP which is not the case of metabolites.

Analytical features

Table 33 shows the analytical features of the proposed methodology in terms of linearity, precision and limits of detection (LOD) and quantification (LOQ). Determination was based on peak area measurements on the average ion mobility spectrum of 4 s desorption time. Linearity was assessed from the determination coefficients (R^2) of cocaine calibration curves in the concentration range from 60 to 500 μg L^{-1}, being the R^2 value higher than 0.996.

Table 33. *Analytical figures of merit of cocaine determination in saliva by IMS using the proposed MIP-SPE procedure.*

Parameter	**Value**
Coefficient of determination (R^2)	0.996
Linear range (μg L^{-1})	60-500
RSD (%, n=3)	
Water at 60 μg L^{-1}	4.6
Water at 200 μg L^{-1}	4.0
Water at 500 μg L^{-1}	0.8
Saliva at 200 μg L^{-1}	6.1
LOD (μg L^{-1})	18
LOQ (μg L^{-1})	60
Analysis time per sample (min)	5.0
Sample throughput (h^{-1})	12

Precision of the measurements was calculated as the relative standard deviation (RSD) of the peak area values of three independent measurements of 60, 200 and 500 μg L^{-1} cocaine standard solution, being the RSD values of 4.6, 4.0 and 0.8 %, respectively. On the other hand, the RSD was also established in oral fluid samples to evaluate the precision of the complete procedure also including the MIP-SPE treatment. The RSD of an oral fluid sample spiked with cocaine at

200 μg L^{-1} and analysed per triplicate using three different columns by the recommended procedure was 6.1 %.

Additionally, RSD values intra- and inter-day ranged from 2 to 7 % and from 6 to 12 %, respectively.

LOD and LOQ were calculated as three and ten times, respectively, the standard deviation of the intercept divided by the slope of the calibration curve, being respectively 18 and 60 μg L^{-1}.

Recoveries of the developed procedure were calculated using blank oral fluid samples spiked at 75, 100, 200 and 500 μg L^{-1} cocaine. As it can be seen in Table 34, quantitative recoveries were obtained in all cases, ranging from 81 ± 2 to 100 ± 7 %.

Table 34. *Recovery values for cocaine determination in blank saliva samples spiked at different levels analyzed by IMS after MIP-SPE[a].*

[Spiked] (μg L^{-1})	[Found] (μg L^{-1} ± s)	Recovery (%)	% RSD (interday)	% RSD (intraday)
75	75 ± 5	100	12	7
100	97 ± 3	97	10	5
200	170 ± 11	85	9	6
500	405 ± 12	81	6	2

[a]In all the cases the volume of sample was 1 mL.

Compared with previously reported methodologies based on LC-MS for the quantitative determination of cocaine in urine and plasma [27, 28, 29], the LOD and LOQ values obtained by the proposed method are substantially higher. However, the main advantages of the MIP-IMS procedure are related to its fastness, ease of use and screening capability. Moreover, it should be indicated that the saliva/plasma ratio in unstimulated saliva is greater than 5 [30], thus, the obtained LOD and LOQ values are appropriate for the determination of cocaine abuse by using saliva samples.

Analysis of cocaine residues in saliva samples

A total of 19 oral fluid samples were collected from cocaine consumers. Saliva samples were analysed by the recommended MIP-IMS procedure and by two reference procedures. Saliva samples were directly analysed by an screening method, based in a competitive lateral flow immunoassay, and SPE-MIP extract was also analysed by a confirmatory GC-MS method.

It should be indicated that the sampled saliva volume was 500 µL when possible. Thus, the enrichment factor of the proposed procedure is 0.5. It should be mentioned that the enrichment factor could be increased by increasing the saliva sample volume from 500 to 1000 µL. However, in this case, the enrichment factor is not as important as the clean-up of the sample provided by the MIP, and due to the difficulty for sampling 1000 µL of saliva in cocaine consumers, 500 µL was selected as reference sample volume.

The obtained results for the three aforementioned methodologies are summarized in Table 35. The lateral flow immunoassay procedure detected 17 samples as cocaine positive and two as negative, in addition other illicit substances like amphetamine, methamphetamine, and benzodiazepine were also detected in some of them. The cocaine concentration range in users saliva determined by the proposed MIP-IMS method was from 0.27 to 51 mg L^{-1}, depending on the time elapsed from the cocaine consumption and the saliva sampling. It should be mentioned that concentrated samples required a dilution to accurately quantify cocaine by the IMS and GC-MS procedures. It should be mentioned that cocaine was detected in sample 3 by MIP-IMS as well as by lateral flow immunoassay, but the concentration of cocaine was lower than the LOQ of the MIP-IMS procedure. Similar results were obtained for the rest of positive samples analysed by IMS and GC-MS methods with bias values ranging from -21 to 21 %. Deming regression analysis between data found by MIP-IMS and MIP-GC-MS was used to evaluate the agreement between both sets of measurements. A determination coefficient of 0.999 was found, and the 95% confidence intervals for the slope (0.88, 1.13) and the intercept (-0.067, 0.099) included 1 and 0 values, respectively (see Figure 60). Therefore, we can conclude that the proposed MIP-IMS method provided results statistically comparable with those obtained by GC-MS, being an useful alternative that provided fast, selective and sensitive results in a cost affordable instrumentation that does not require skilled operators.

Table 35. *Analysis of cocaine users saliva samples. Results were obtained by the proposed MIP-IMS procedure and the lateral flow immunoassay (LF-IA) and GC-MS reference methods.*

Sample	Illicit substances detected	[Cocaine] (mg L^{-1} ± s, n=2)		Bias (%)
	LF-IA	MIP-IMS	MIP-GC-MS	
1	Cocaine	0.60 ± 0.03	0.60 ± 0.05	0
2	Cocaine	0.27 ± 0.06	0.21 ± 0.05	-21
3	Cocaine	Detected [a]		-
4	Cocaine	0.27 ± 0.04	0.28 ± 0.04	4
5	Negative	- [b]	0.038 ± 0.003	-
6	Negative	- [b]	0.047 ± 0.02	-
7	Cocaine	0.95 ± 0.03	0.87 ± 0.06	-8
8	Cocaine	0.465 ± 0.014	0.516 ± 0.005	11
9	Cocaine	0.75 ± 0.02	0.73 ± 0.01	-3
10	Cocaine Methamphetamine	0.41 ± 0.02	0.35 ± 0.01	-15
11	Cocaine	2.07 ± 0.10	2.00 ± 0.05	-3
12	Cocaine	0.35 ± 0.09	0.32 ± 0.02	-7
13	Cocaine	0.27 ± 0.02	0.26 ± 0.06	-4
14	Cocaine	0.89 ± 0.03	0.90 ± 0.03	1
15	Cocaine Amphetamine Methamphetamine	0.77 ± 0.06	0.93 ± 0.01	21
16	Cocaine Methamphetamine	0.44 ± 0.04	0.40 ± 0.03	-9
17	Cocaine	20 ± 2	20.4 ± 0.2	2
18	Cocaine Benzodiacepine	2.23 ± 0.08	1.8 ± 0.1	-19
19	Cocaine	51 ± 2	58 ± 1	14

[a] *Less than LOQ*

[b] *Less than LOD*

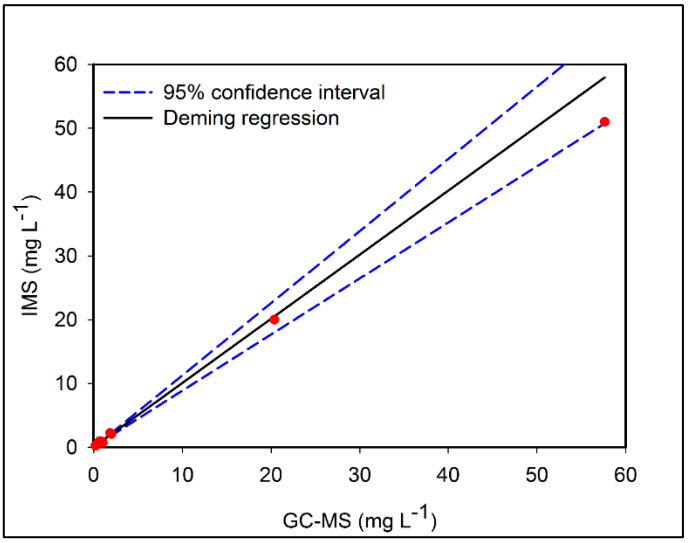

Figure 60. *Deming regression for the comparison of results obtained for cocaine users oral fluids extracted by MIPs and analyzed by IMS and GC-MS.*

Conclusions

In the present study, cocaine-based MIP and a NIP were prepared by bulk polymerization and employed as SPE to determine cocaine in saliva samples by IMS. After study of the SPE washing and eluting steps, MIPs were characterized in terms of analyte binding capacity of 0.83 mg cocaine per g of polymer, reusability in water and saliva analysis till 8 and 5 consecutive uses, an imprinting factor of 2.5 and a good selectivity in front of the main cocaine metabolites. The IMS procedure was validated in terms of linearity, precision, trueness, LOD and LOQ. Moreover, results obtained for cocaine abusers saliva samples analyzed by the proposed MIP-IMS method were comparable to those obtained by a qualitative lateral flow immunoassay procedure and a GC-MS reference procedure. Thus, it can be concluded that the recommended MIP-IMS procedure is a really interesting alternative to immunoassay procedures for the screening of cocaine in biological fluids and can be used to obtain quantitative results comparable with those obtained by the reference chromatography procedure.

Acknowledgements

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Conflict of Interest Disclosure

The authors declare no competing financial interest.

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Amphetamine-type stimulants analysis in oral fluid based on molecularly imprinting extraction

Aitor Sorribes-Soriano [a], Francesc A. Esteve-Turrillas [a], Sergio Armenta [a], Pedro Amorós [b], José Manuel Herrero-Martínez [a,]

[a] *Department of Analytical Chemistry, University of Valencia, 50th Dr. Moliner St., 46100 Burjassot, Spain*

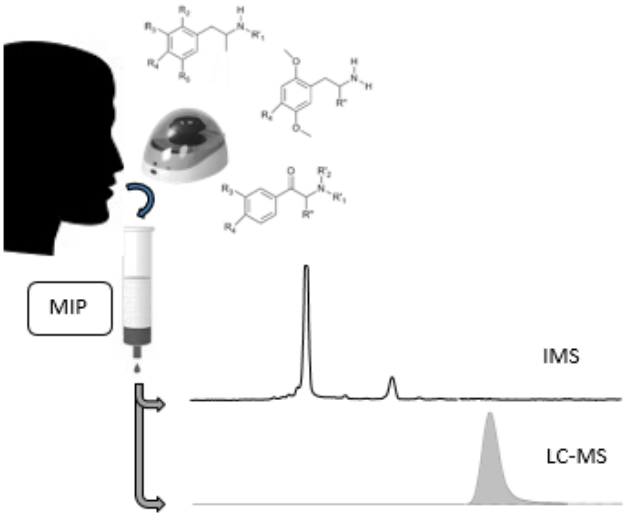
[b] *Institute of Material Science (ICMUV), University of Valencia, Catedrático José Beltrán 2, 46980 Paterna, Spain*

Abstract

A methamphetamine-based molecularly imprinted polymer (MIP) has been prepared by bulk polymerization to recognize new psychoactive substances (NPS) of the amphetamine, cathinones and 2C families in oral fluid samples, being the first precedent of a synthetized MIP for the extraction and preconcentration 32 NPS including amphetamine type substances and synthetic cathinones from oral fluids. Pre-polymerization complex and resulting materials were appropriately characterized by infrared spectroscopy, scanning electron microscopy, and nitrogen adsorption-desorption isotherms. Appropriateness of the material for the specific recognition of the target analytes was also evaluated through computational calculations and experimentally assessed by solid phase extraction (SPE). The most appropriate SPE conditions were evaluated and recoveries of 32 different NPS were obtained, ranging from 80 to 120 % with a relative standard deviation (RSD) in all cases lower than 12 %. Amphetamine-related NPS were analyzed by a fast and portable methodology based on ion mobility spectrometry (IMS) and a rearguard procedure based on ultra-high performance liquid chromatography coupled to mass spectrometry (UHPLC-MS) providing limit of detection values from 10 to 80 μg L^{-1} and from 0.03 to 1.3 μg L^{-1}, respectively. Oral fluid samples, containing different interferents like caffeine, fluticasone and cetirizine, were spiked with 300 μg L^{-1} amphetamine and subsequently analyzed, showing recoveries ranging from 81 to 115 % using both methodologies. Thus, this paper shows preliminary results to demonstrate the applicability of the developed procedure which could be used with minor modifications as screening technique in on-road drug analysis.

Keywords: *Molecularly imprinted polymer, new psychoactive substances, amphetamine, cathinone, oral fluid*

Graphical abstract



Introduction

The new psychoactive substances (NPS) phenomenon can be considered a truly global issue from 2009 onwards, with more than 100 countries involved from all regions in the world and over 20 tonnes seized in 2015. The NPS market is very dynamic; up to December 2017, more than 800 substances have been reported to the United Nations Office on Drugs and Crime (UNODC) early warning advisory on NPS by Governments, laboratories and partner organizations [1]. According to the UNODC report on amphetamine-type stimulants and NPS, synthetic cannabinoids constitute the largest category in terms of the number of different substances reported, followed by synthetic cathinones and phenethylamines [2]. Seizures of classical and new amphetamine-type stimulants (ATS) reached 191 tonnes in 2015. One of the most troubling aspects of NPS is that users are unaware of the content and the dosage of the psychoactive substances which turns into additional serious health risks. Generally, no scientific information is available to determine the effects of these products and consequently, fast and reliable detection of these ATS in biological fluids is frequently required in clinical and forensic cases.

Analytical methods for toxicological analysis of ATS in biological fluids are mainly based on gas (GC) [3] and liquid (LC) [4] chromatography coupled with mass spectrometry (MS). Most of these methods require a preliminary sample treatment step typically based on liquid-phase microextraction (LPME) [5] or solid-phase extraction (SPE) [6], being the later the most widely employed method, using hydrophobic and/or ion-exchange sorbents. SPE provides a non-selective extraction of the analytes, leading to co-extraction of endogenous compounds and other drugs. The use of specific and selective SPE sorbents reduces the co-extraction of interfering compounds, increases the mean lifetime of chromatographic columns, and decreases the limits of detection (LODs).

Molecular imprinting is a technique that creates specific recognition sites for a target molecule within a highly cross-linked synthetic polymer designed to mimic biological recognition pathways [7]. In a comparison study, several SPE sorbents including the common hydrophilic-lipophilic balanced (Oasis HLB), mixed-mode (Oasis MCX) and commercial molecularly imprinted polymers (MIPs) have been evaluated for the SPE of amphetamine and other abuse drug residues in wastewater [8], showing that MIPs rendered cleaner extracts with less matrix effect, and thus better sensitivity, trueness and precision. The aforementioned commercially available amphetamine-MIP sorbents have been also employed for the extraction of methamphetamine, amphetamine, and

methylenedioxyphenylalkylamine from human whole blood [9]. Methamphetamine, amphetamine, and ecstasy have been also determined by GC-FID in urine after inside-needle adsorption trap extraction [10] or MIP-based SPE along with simultaneous derivatization and dispersive liquid-liquid microextraction (DLLME) [11]. Moreover, a MIP solid-phase microextraction (SPME) fiber has been also fabricated for extraction and pre-concentration of methamphetamine from saliva samples [12]. All the aforementioned papers demonstrated that MIP can be considered a superior methodology compared to traditional extraction sorbents.

On the other hand, urine and blood are the most widely employed biological matrices for the analysis of suspected illegal narcotics. However, these techniques are quite invasive and metabolites, instead of parent compounds, are typically detected [13]. Moreover, oral fluid analysis allows the detection of short time consumption being useful in cases of driving under the influence of drugs [14]. In the case of ATS analysis, previous studies estimate a saliva/blood ratio from 2.9 to 7.1 [15]; thus, analysis of ATS in oral fluids seems to be the most relevant of the biological matrices.

The aim of this paper is the synthesis, characterization and evaluation of an ATS-selective MIP material using methamphetamine as template molecule, which can be used as solid sorbent in the SPE of ATS, synthetic cathinones and other phenetylamine related compounds from oral fluid samples. As far as we know, this is the first precedent of a synthetized MIP material for the extraction and preconcentration of amphetamine and other 32 ATS and synthetic cathinones from oral fluids. The selectivity of the MIP extraction combined with the sensitivity and speediness of the ion mobility spectrometry (IMS) provides an excellent analytical tool for the fast detection and identification of ATS in biological fluids that can be used as vanguard methodology in clinical and forensic cases. Automation of sample treatment by using a lab-on-valve miniaturized system integrating on-line disposable micro-solid phase extraction has been previously interfaced with IMS for the accurate and sensitive determination of cocaine and ecgonine methyl ester in oral fluids [16]. Thus, this paper shows preliminary results to demonstrate the applicability of the developed procedure which could be used with minor modifications as screening technique in on-road drug analysis.

On the other hand, in this work, the combination of the MIP extraction with an advanced rearguard analytical tool, such as ultra-high performance liquid chromatography-mass spectrometry (UHPLC-MS) was also addressed, which

unequivocally confirms the identity of the analyzed substance with improved sensitivity.

Experimental

Standards and reagents

NPS analyzed in this study (see Table 36) were kindly provided by the laboratory of the Inspección de Farmacia y Control de Drogas (Valencia, Spain) together with amphetamine and methamphetamine, employed for the MIP synthesis. All the studied compounds were provided in solid form with purities higher than 95%. The studied NPS include eight amphetamines (2-fluoroamphetamine, 3-fluoroamphetamine, 2-fluoromethamphetamine, 4-fluoromethamphetamine, 6-(2-aminopropyl)benzofuran, 3-methoxy-4-methylamphetamine, 3-fluoroethamphetamine, and 5-(2-ethylaminopropyl)benzofuran)), twenty cathinone derivatives (ethcathinone, 3-methylmethcathinone, 3-fluoromethcathinone, 4-methylethcathinone, 4-methylbuphedrone, 4-chloromethcathinone, methylone, mexedrone, butylone, α-pyrrolidinopentiophenone, α-pyrrolidinohexiophenone, 3',4'-methylenedioxy-α-pyrrolidinopropiophenone, 4-chloroethcathinone, N-ethylpentylone, 4'-methyl-α-pyrrolidinohexiophenone,4-chloro-α-pyrrolidinopentiophenone, methylenedioxypyrovalerone, 3',4'-tetramethylene-α-pyrrolidinopentiophenone, 1-phenyl-2-(pyrrolidin-1-yl)octan-1-one, and 3,4-methylenedioxy-α-pyrrolidinohexanophenone) and four compounds of the 2C family (4-ethyl-2,5-dimetoxiphenethylamine, 4-chloro-2,5-dimethoxyphenethylamine, 4-propyl-2,5-dimethoxyphenethylamine, 4-chloro-2,5-dimethoxyamphetamine). All drugs were dissolved at 1 g L^{-1} concentration in methanol and kept at -20 °C in amber glass vials.

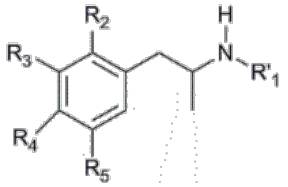
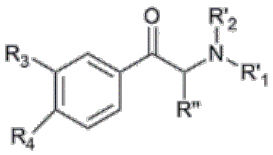
HPLC grade or higher organic solvents and buffer salts used in the study were obtained from Scharlab (Barcelona, Spain). Methacrylic acid (MAA), ethylene dimethacrylate (EDMA) and azobisisobutyronitrile (AIBN) used in the MIP synthesis were provided by Sigma (Stenheim, Germany).

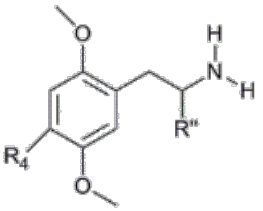
Visiprep™ SPE vacuum manifold and 1 mL empty polypropylene SPE tubes with polyethylene frits (20 µm porosity) were obtained from Supelco (Bellefonte, PA, USA). Synthetic saliva was prepared according to the recipe from the Centre for Applied Science and Technology (CAST) document “Preliminary Drug Testing Devices” [17]. Mucin from porcine stomach Type II was obtained from Sigma and

buffer constituent by Scharlab. Synthetic saliva was spiked with 300 µg L^{-1} amphetamine for its analysis.

Oral fluid samples were obtained by spitting technique from healthy individuals who had consumed fluticasone, caffeine and cetirizine, following ethical guidelines established by the University of Valencia (H1454687358321 – drug analysis in biofluids). Field samples were spiked with 300 µg L^{-1} amphetamine before to the analysis. Samples were analyzed immediately after sampling, thus ensuring the stability of drugs.

Table 36. *Structure and molecular weight (MW) of the new psychoactive substances analyzed in this study.*

Analyte	Structure			MW (g mol^{-1})
				
Amphetamine derivatives				
2-fluoroamphetamine (2-FA)	R_2=F			153.2
3-fluoroamphetamine (3-FA)	R_3=F			153.2
2-fluoromethamphetamine (2-FMA)	R_1'= methyl	R_2=F		167.2
4-fluoromethamphetamine (4-FMA)	R_1'= methyl	R_4=F		167.2
6-(2-aminopropyl)benzofuran (6-APB)	R_3,R_4=furan			175.2
3-methoxy-4-methylamphetamine (3-MMA)	R_1'=methyl	R_3=methoxy		179.3
3-fluoroethamphetamine (3-FEA)	R_1'=ethyl	R_3=F		181.2
5-(2-ethylaminopropyl)benzofuran (5-EAPB)	R_1'=ethyl	R_3,R_4=furan		203.3
				
Cathinone derivatives				
Ethcathinone	R_2'=ethyl	R"=methyl		177.3
3-methylmethcathinone (3-MMC)	R_2'=methyl	R_3=methyl	R"=methyl	177.3
3-fluoromethcathinone (3-FMC)	R_2'=methyl	R_3=F	R"=methyl	181.2
4-methylethcathinone (4-MEC)	R_2'=ethyl	R_4=methyl	R"=methyl	191.3
4-methylbuphedrone (4-MeMABP)	R_2'=methyl	R_4=methyl	R"=ethyl	191.3
4-chloromethcathinone (4-CMC)	R_2'=methyl	R_4=Cl	R"=methyl	197.7
Methylone	R_2'=methyl	R_3,R_4=methylenedioxy	R"=methyl	207.2
Mexedrone	R_2'=methyl	R_4=methyl	R"=-CH_2-O-CH_3	207.3
Butylone	R_2'=methyl	R_3,R_4=methylenedioxy	R"=ethyl	221.2
α-pyrrolidinopentiophenone (α-PVP)	R_1',R_2'=pyrrolidin	R"=n-propyl		231.3
α-pyrrolidinohexiophenone (α-PHP)	R_1',R_2'=pyrrolidin	R"=n-butyl		245.4
3',4'-methylenedioxy-α-pyrrolidinopropiophenone (MDPPP)	R_1',R_2'=pyrrolidin	R_3,R_4=methylenedioxy	R"=methyl	247.3
4-chloroethcathinone (4-CEC)	R_2'=ethyl	R_4=Cl	R"=methyl	248.2
N-ethylpentylone	R_2'=ethyl	R_3,R_4=methylenedioxy	R"= n-propyl	249.3

4'-methyl-α-pyrrolidinohexiophenone (MPHP)	R_1',R_2'=pyrrolidin	R_4=methyl	R"=n-butyl	259.4
4-chloro-α-pyrrolidinopentiophenone (4Cl-PVP)	R_1',R_2'=pyrrolidin	R_4=Cl	R"=n-propyl	265.8
Methylenedioxypyrovalerone (MDPV)	R_1',R_2'=pyrrolidin	R_3,R_4=methylenedioxy	R"= n-propyl	275.3
3',4'-tetramethylene-α-pyrrolidinopentiophenone (TH-PVP)	R_1',R_2'=pyrrolidin	R_3,R_4=cyclohexane	R"= n-propyl	285.4
1-phenyl-2-(pyrrolidin-1-yl)octan-1-one (PV9)	R_1',R_2'=pyrrolidin	R"= n-hexyl		309.9
3,4-methylenedioxy-α-pyrrolidinohexanophenone (3,4-MDPHP)	R_1',R_2'=pyrrolidin	R_3,R_4=methylenedioxy	R"= n-butyl	325.8
2C-family				
4-ethyl-2,5-dimetoxiphenethylamine (2C-E)	R_4=ethyl	R"=H		209.3
4-chloro-2,5-dimethoxyphenethylamine (2C-C)	R_4=Cl	R"=H		215.7
4-propyl-2,5-dimethoxyphenethylamine (2C-P)	R_4=n-propyl	R"=H		223.3
4-chloro-2,5-dimethoxyamphetamine (DOC)	R_4=Cl	R"=methyl		229.7

Characterization of the methamphetamine-MAA pre-polymerization complex

Interactions between template and monomer were studied by Fourier-transform infrared (FTIR) spectroscopy, using a Tensor 27 spectrometer from Bruker (Bremen, Germany). It was constituted by a DLaTGS detector and a Dura Sample IR II attenuated total reflection (ATR) accessory for liquid samples from Smiths Detection Inc. (Warrington, UK) equipped with a nine-reflection diamond/ZnSe Dura Disk plate.

Measurements were done by dropping 5 µL template, monomer and template-monomer solutions in acetonitrile on the ATR crystal surface and letting the solvent to evaporate with the help of a warm air flow. Different template:monomer molar ratios from 1:4 to 1:18 were studied. Once the solvent was evaporated, dry film spectrum was collected in the region between 4000 and 550 cm^{-1}, with a resolution of 4 cm^{-1} and 15 averaging scans. Background spectrum was acquired following the same measurement conditions.

Imprinting polymer synthesis

A scheme of the MIP synthesis procedure including the whole developed analytical procedure is shown in Figure 61. MIP was prepared by bulk polymerization using methamphetamine as template. For this purpose 0.17 mmol methamphetamine and 3 mmol MAA were dissolved in 2 mL of acetonitrile into a glass vial. The solution was kept for 1 hour to allow the formation of the complex between the template and the monomer. Meanwhile, 12 mmol EDMA and 0.03 mmol AIBN were dissolved in 2 mL of acetonitrile and mixed with the aforementioned template-monomer solution. Then the solution was sonicated for 10 min and purged with a nitrogen flow for additional 10 min. Thermal polymerization was made at 60 °C for 24 h. After polymerization, the MIP was crashed and sieved to 250 µm. MIP was washed with 10 % (v/v) acetic acid in methanol overnight by Soxhlet extraction to quantitatively elute methamphetamine from the MIP. Then, 25 mg MIP were placed in a 1 mL polypropylene cartridge, between two 20 µm polyethylene frits, and washed again with 5 mL 10 % (v/v) acetic acid in methanol. Additionally, a non-imprinted polymer (NIP) was prepared using similar conditions without the presence of the template.

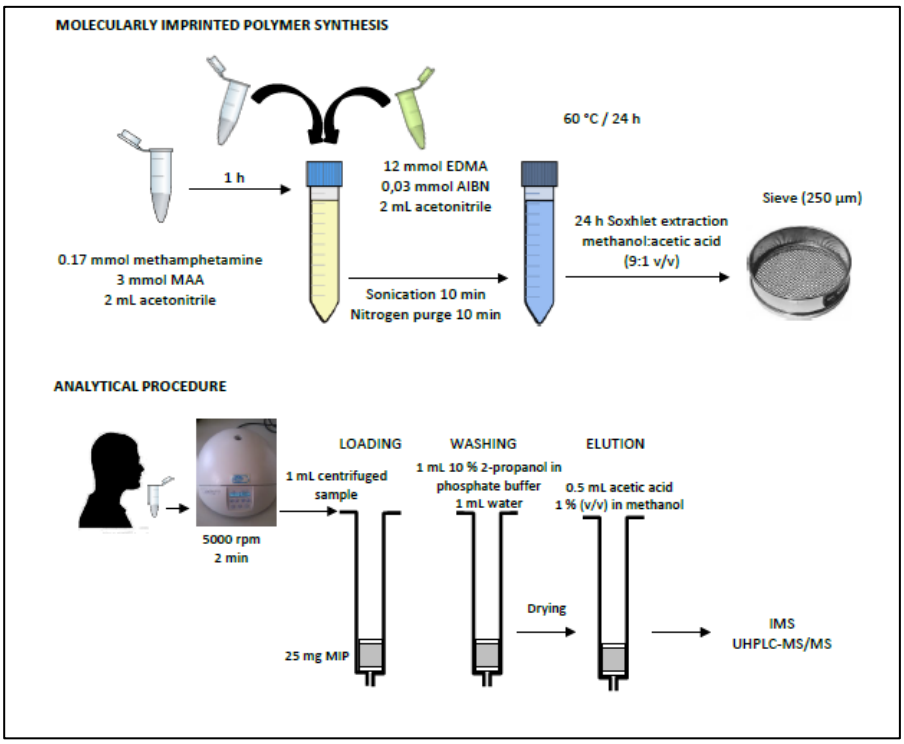


Figure 61. *Scheme of the imprinting polymer synthesis including the whole developed analytical procedure.*

Characterization of the synthesized MIP

Surface characterization of MIP and NIP was made by microscopy using a S-4800 scanning electron microscope from Hitachi (Ibaraki, Japan), provided with a field emission gun and a back secondary electron detector. EMIP 3.0 software from Rontec (Normanton, UK) was employed for image data acquisition.

Surface area and pore size values were calculated by nitrogen adsorption–desorption isotherms at -196 °C recorded on a Micromeritics (Norcross, GA, USA) ASAP-2020 automated instrument. Samples were degassed at 80 °C and 10^{-6} Torr before analysis. The total pore volume was calculated by converting the amount of nitrogen adsorbed at a relative pressure of about 0.99 to the volume of liquid adsorbate. Surface areas were evaluated using the Brunauer–Emmett–Teller (BET) method and the pore diameter and pore size distribution were calculated using the Barrett–Joyner–Halenda (BJH) analysis of the adsorption branch.

Estimation of the molecular size of the analyzed NPS

The different stable conformations of each studied NPS were established through classic mechanics equations using the Gabedit graphical interface [18] with stochastic dynamics via verlet velocity algorithm (till 3000 K). Each stable conformation was optimized by using Orca version 4.0.1 software from Max-Planck Institut for Chemical Energy Conversion (Mülheim an der Ruhr, Germany) and the semiempirical PM3 method, considering that the analyte is placed inside a hole and water dielectric constant is out to stabilize charges distribution. Energy of optimized structures was calculated using Molcas 8.2 software from Lund University (Lund, Sweden) and DFT method, B3LYP functional and 6-34g base. Population probability of each conformation was calculated according to the Boltzmann distribution. Different conformers were optimized, allowing to calculate the longest z distance and the longest perpendicular axes (x and y).

SPE procedure

MIP cartridges were equilibrated using 1 mL acetic acid 1 % (v/v) in methanol using a SPE vacuum manifold at 2 mL min^{-1} flow rate, followed by 1 mL deionized water to remove the excess of organic solvent. Oral fluid sample was centrifuged at 5000 rpm for 2 min and 1 mL sample volume was loaded onto the column. MIP column was washed with 1 mL of solution 10 % 2-propanol in phosphate buffer (0.1 M, pH 9), followed by 1 mL deionized water. Cartridges were dried in the

vacuum manifold, and then 0.5 mL acetic acid 1 % (v/v) in methanol was used for the elution of ATS.

Analysis using portable IMS instrumentation

An IOSCAN-LS from Smiths Detection (Morristown, NJ, USA) with a ^{63}Ni foil radioactive ionization source was used to separate and identify the different substances involves in this study. IM station software (version 5.389) from Smiths Detection was used to acquire and process data. Positive ion mode was used with nicotinamide, with a reduced mobility constant (K_0) of 1.860 $cm^2 V^{-1} s^{-1}$, as internal calibrant. A 25 ms scan period using a 0.2 ms shutter grid width, 60 segments per analysis, and 479 data points conditions were employed for plasmagram acquisition. A counterflow dry air at 300 mL min^{-1} was used as a drift gas. The electric field strength in the drift region was 251 V cm^{-1}, with 1759 V total drift voltage, and a 7 cm drift tube length.

The extract of the sample was introduced by thermal desorption onto a

polytetrafluoroethylene (PTFE) membrane. Hence, 1 µL sample extract was spiked with 2 µL 2-propanol and placed onto the PTFE membrane, using a 1 s post-dispense delay to introduce the sample in the heated zone. Desorption, inlet, and drift tube temperatures were adjusted to 255, 260 and 237 °C, respectively. After 30 s of heating, the membrane was removed from the heating zone.

UHPLC–MS/MS analysis

An UHPLC coupled to a triple quadrupole MS equipped with a Z-spray electrospray ionization source was employed as rearguard methodology for the analysis of ATS in oral samples. UHPLC was performed on a Waters Acquity System from Waters (Milford, MA, USA), equipped with a binary solvent delivery system, an autosampler and a BEH C18 (1.7 µm, 2.1 × 50 mm) column. A 5 µL injection volume was employed and the mobile phase, at a flow rate of 0.4 mL min^{-1}, consisted of (A) 0.1 % (v/v) formic acid in water and (B) methanol. The gradient started at 5% of B, and it was linearly increased to 95 % in 4 min, and then kept for 1 min. Acquisitions were performed at 3.5 kV capillary voltage, 120 °C source temperature and 350 °C desolvation temperature.

Evaluation of the green character of the developed procedure

The proposed procedure for the determination of ATS in oral fluids has been evaluated in terms of Green Analytical Chemistry using the previously proposed Analytical Eco-scale [19] a semiquantitative green metric tool. The greenness of the proposed analytical method was assessed by assignation of penalty points to parameters of an analytical process that are not in agreement with the ideal green analysis.

Results and discussion

Formation of the pre-polymerization complex

A key step in the MIP synthesis using the non-covalent approach is the formation of the pre-polymerization complex between template and monomer. It is widely accepted that stronger interactions between the template and monomers lead to more stable host-guest complex prior to polymerization [20], and consequently a better imprinting efficiency of the resulted polymers is obtained. Thus, different solutions of template (methamphetamine), MAA, and template:MAA at different molar ratios (from 1:4 to 1:18) were prepared in acetonitrile as porogenic solvent to study the mechanism of interaction between both species. It must be indicated that methamphetamine was partially soluble in acetonitrile, but it was completely dissolved in this solvent in presence of MAA, which may confirm the effective interaction between the monomer and the template in the pre-polymerization mixture.

Indeed, the formation of hydrogen bonds between template and MAA can be readily identifiable using FTIR since the stretching frequency of the hydroxyl or amino groups (hydrogen bond donors) and carbonyl group (hydrogen bond acceptor) are displaced. As it can be seen in Figure 62, the band associated with the NH stretching vibration around 3500 cm^{-1}, present in the methamphetamine IR spectrum, disappears when methamphetamine and MAA are mixed together, particularly, at template:monomer ratios above 1:10. On the other hand, the O-C stretching vibration of carboxylic acids, present in the MAA IR spectrum at 1175 cm^{-1}, substantially decreased its intensity, became broaden and vibrate at lower frequencies when MAA is mixed with methamphetamine, mainly due to the formation of hydrogen bonding between monomer and template. A similar effect happened with the OH bending out-of-plane, appearing at 780 cm^{-1} in the spectra of MAA.

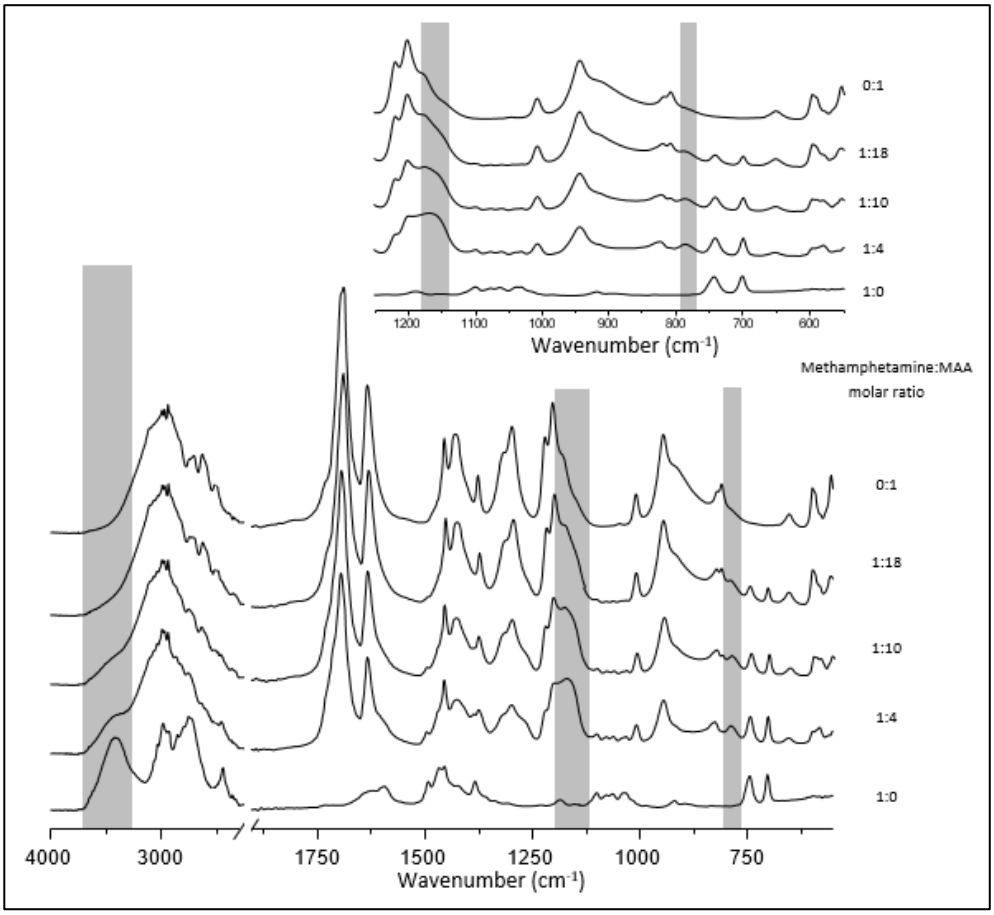


Figure 62. *ATR-FT-IR spectra of methamphetamine, methacrylic acid (MAA), and mixtures at different molar ratios. Inset: zoom of the 1250-650 cm^{-1} spectra.*

To summarize, H-bonding interaction between the template and monomer can cause that vibrational modes of involved atoms to broaden and shift at lower frequencies than before. A ratio template:monomer of 1:18 was suggested to provide appropriate permeability, rigidity and shape of the cavities, following results of a previous study [10] although, an excess of monomer could reduce the number of specific interactions. On the other hand, a template:cross linker ratio of 1:70 was selected, due to it is well-known that rigidity in the polymer network helps to ensure a cavity that is complementary in shape as well as functionality to the template [21].

MIP characterization

The surface morphology and the particle size of both MIP and NIP, prepared via bulk polymerization method using a non-covalent approach, were analyzed using scanning electron microscopy. As shown in Figure 63, the MIP gave globules slightly lower than those found for the control polymer (NIP). These differences could be attributed to the influence of the template on the particle growth during the polymerization process, and consequently in the polymer microstructure. In addition, the surface of MIP globules was more rough than that of NIPs, which translates into a higher surface area, thus facilitating the mass transfer rate and the recognition ability of template molecule.

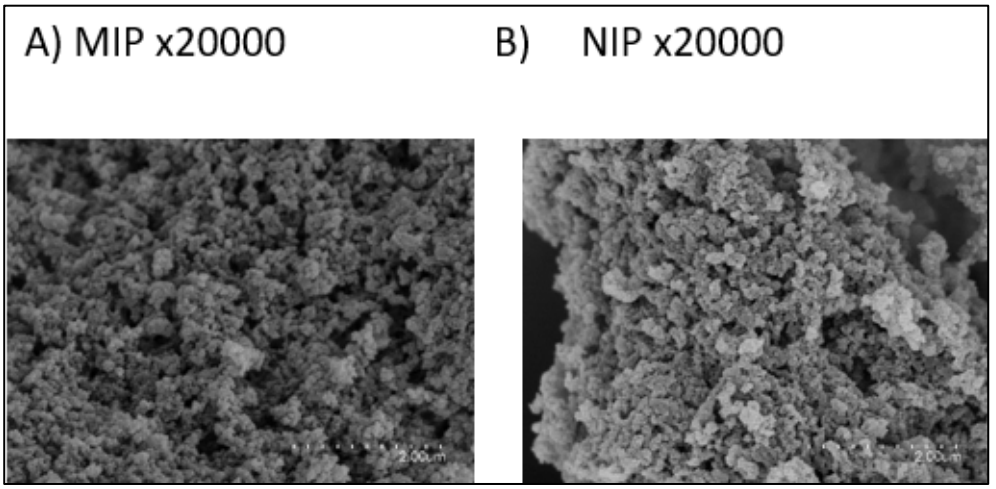


Figure 63. *SEM images for A) NIP and B) MIP polymers at x20000.*

Nitrogen adsorption-desorption plots and pore size distribution of NIP and MIP are shown in Figure 64. The results showed that the specific surface area of MIPs (393.0 ± 1.4 $m^2 g^{-1}$) was greater than that of NIPs (182.8 ± 0.3 $m^2 g^{-1}$). On the other hand, the BJH pore volume of the MIP is 0.58 $cm^3 g^{-1}$ while that of the NIP is 0.85 $cm^3 g^{-1}$. Moreover, pores around 2 nm diameter (in the border between micro and mesopores) were observed in MIPs, while only residual micropores (practically negligible) were observed in NIP (see Figure 64). This indicates that the template confined the shrinkage of the mesoporous voids effectively in the process of the polymerization and the synthesized MIPs had a more regular pore structure.

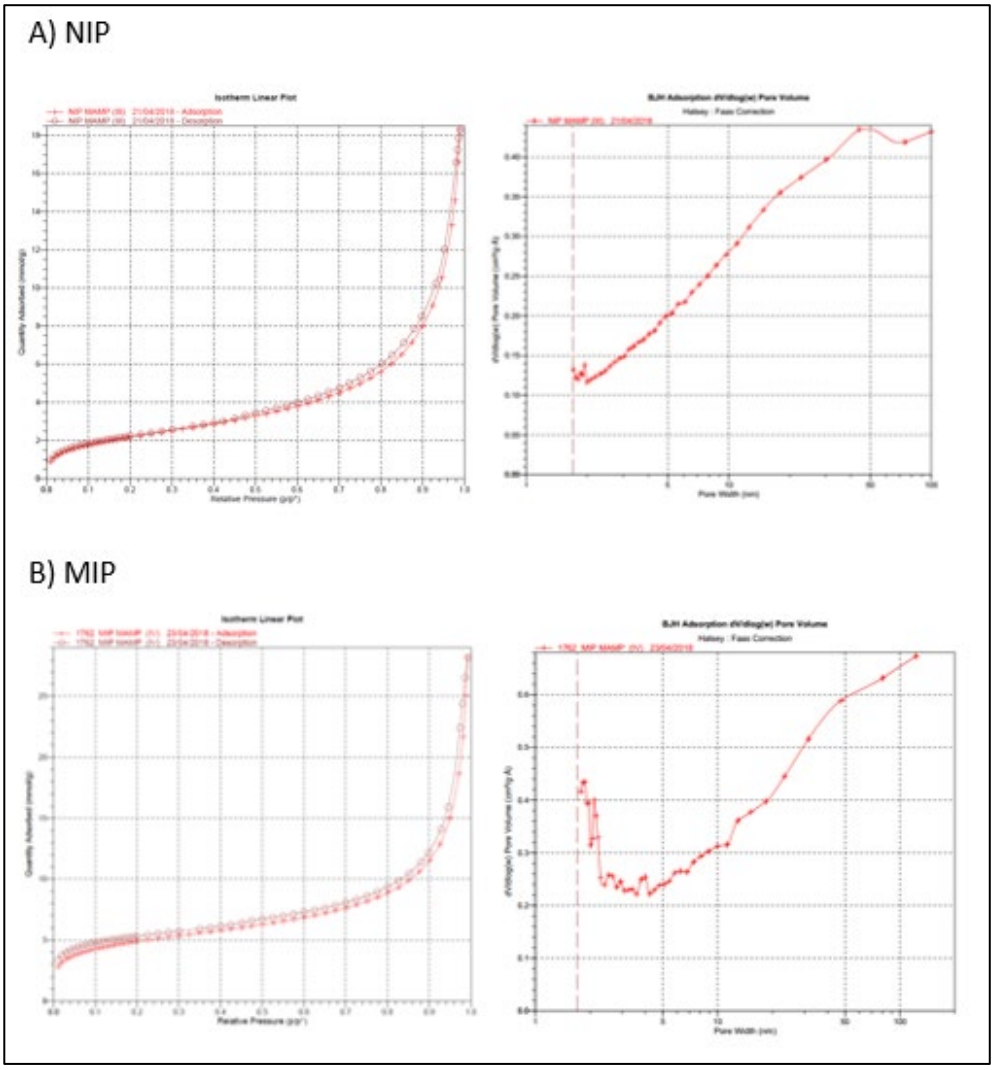


Figure 64. *Nitrogen adsorption-desorption plots and BJH pore size distribution for A) NIP and B) MIP polymers.*

In this study, the effectiveness of the MIP for the extraction of different NPS from oral fluids was evaluated. ATS present a phenethylamine core with a methyl group in the aliphatic chain and different substituents in both, the aromatic ring and the amine. Cathinones derivatives are β-keto-amphetamines with different substituents in the aromatic ring, the aliphatic ring, and the amine; while 2C family phenethylamines contains two methoxy groups on the aromatic ring at positions 2 and 5. On the other hand, size and spatial distribution of methamphetamine, template used during the MIP synthesis, differ from that of the aforementioned compounds and it may provide diverse interactions with the methamphetamine-modelled holes of the MIP. The size and maximum area of some representative

examples of the evaluated NPS were theoretically estimated, as shown in Table 37, to predict if those molecules can access to the template-modelled holes of the MIP. The longest distance z ranged from 6.73 to 14.29 Å, while x and y distances ranged from 2.92 to 7.22 Å and from 2.90 to 7.39 Å, respectively. Slight differences were observed between the studied drugs with maximum areas from 31.1 $Å^2$ for 2-FMA to 87.6 $Å^2$ for PV9; but in all the cases, NPS had an appropriate size to access and interact with MIP micropores.

Table 37. *Theoretical conformational study of selected amphetamine-type stimulants.*

Analyte	Conformation	Population	Axis (Å)			Maximum area ($Å^2$)
			Z	X	Y	
2-FA	1	0.46	8.12	4.43	2.92	36.0
	2	0.19	7.84	3.92	4.58	35.9
3-FA	1	0.51	8.11	4.30	2.90	34.9
	2	0.12	8.12	3.49	3.90	31.7
	3	0.19	7.84	4.39	3.59	34.4
2-FMA	1	0.15	8.35	3.94	4.38	36.6
	2	0.59	8.34	4.19	2.90	34.9
	3	0.14	8.09	3.84	3.63	31.1
4-FMA	1	0.30	7.75	5.30	3.81	41.1
	2	0.12	8.12	4.71	4.06	38.2
	3	0.13	7.98	4.62	4.42	36.9
	4	0.43	9.06	4.27	4.47	40.5
6-APB	1	0.57	6.73	4.82	4.60	32.4
	2	0.18	9.02	4.66	3.68	42.0
	3	0.25	8.65	2.92	4.57	39.5
3-MMA	1	0.24	8.71	3.68	5.85	50.9
	2	0.41	8.08	6.10	4.20	49.3
	3	0.12	9.24	4.94	4.44	45.6
5-EAPB	1	0.62	10.84	4.34	4.19	47.0
	2	0.38	11.36	3.28	4.62	52.5
PV9	1	0.78	14.29	4.49	6.13	87.6
	2	0.14	12.60	6.27	6.30	79.4
2C-E	1	0.36	8.93	4.60	7.39	66.0
	2	0.40	9.79	7.22	4.19	70.7
DOC	1	0.46	8.98	6.17	5.64	61.6
	2	0.36	8.80	5.74	4.72	50.5

Evaluation of the MIP-SPE extraction conditions

Extraction conditions were evaluated taking as starting point those obtained from previous studies performed with MIPs synthesized by using a similar synthetic route [16]. Sample pH is a critical factor to be considered during SPE loading step, because of amphetamines interact through hydrogen bonds and/or electrostatic interactions with the MIP. This interaction depends on the pH of sample solution and the pK_a values of analytes, which range from 9 to 10 approximately [22]. Synthetic saliva showed a pH of 7.2 and field oral samples typically provides a pH between 6 and 7 [23]; consequently, the amino group of ATS is protonated and accordingly, the interaction should be reduced. However, an alkaline pH cannot be used in the loading step because solubility of several NPS substantially decreases according to pH. Taking into account these considerations, no buffer was added to the oral fluid sample and it was directly extracted by the produced MIP.

Amphetamine was used as target molecule for recovery studies in order to evaluate the most appropriate SPE conditions. Thus, 1 mL amphetamine standard solution of 300 μg L^{-1} in water was loaded onto the MIP-SPE cartridge after the appropriate conditioning. The use of 0.1 M HCl as washing solution provided a non-quantitative extraction of amphetamine, indicating that a basic pH must be kept during the washing step. In this sense, different washing solvents were evaluated, based on 0.1 M phosphate buffer at pH 9 in different media such as 5 % (v/v) methanol in water, 10 % (v/v) acetonitrile, and from 5 to 20 % (v/v) 2-propanol in water. In all cases, buffer residues were removed from the MIP using 1 mL water, then the cartridges were dried using vacuum, and amphetamine was eluted with 1 mL of 1 % (v/v) acetic acid in methanol. Figure 65 shows that phosphate buffer in 5 % (v/v) methanol in water partially eluted amphetamine from the MIP obtaining a recovery percentage of 66 %, while recoveries obtained after washing the MIP with phosphate buffer prepared in 10 % (v/v) acetonitrile in water and 10 % (v/v) 2-propanol in water were higher than 80 %. In addition, different concentrations of 2-propanol in water from 5 to 20 % (v/v) were also evaluated as washing solutions. As it can be seen in Figure 65, phosphate buffer in 5 % (v/v) 2-propanol in water provided an amphetamine recovery of 96 % from the MIP, but recoveries were lower than 65% for buffer prepared in 15 and 20 % (v/v) 2-propanol in water. Thus, phosphate buffer prepared in 10 % (v/v) 2-propanol in water was selected as washing solvent for further experiments providing a quantitative recovery for amphetamine. Once the SPE protocol was established, the imprinting factor was calculated as the ratio of analyte retained in the MIP versus the analyte retained in the NIP, achieving a value of 2.2.

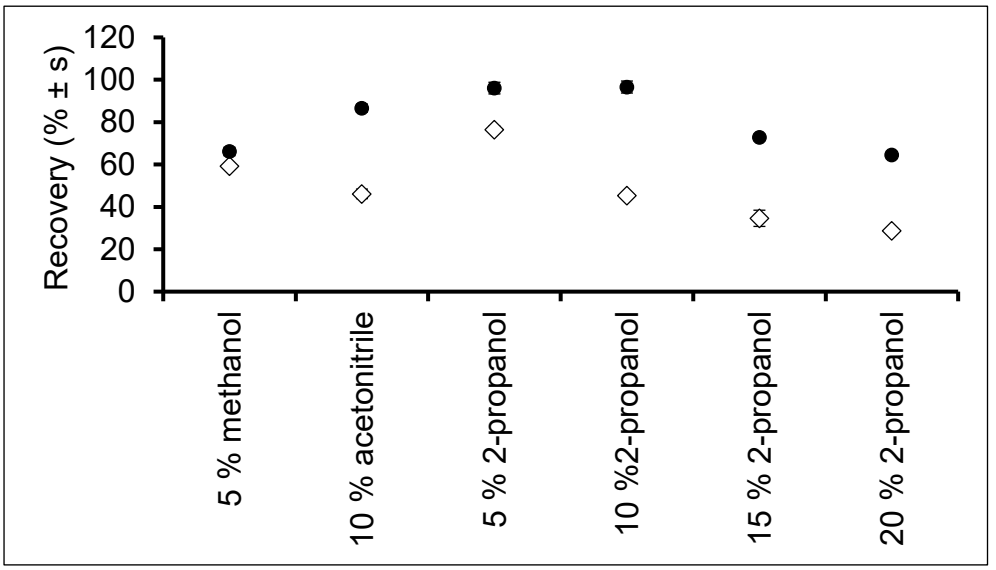


Figure 65. *Effect of the organic solvent content in the 0.1 M phosphate buffer (pH 9) solution employed as washing step in the SPE of 1 mL amphetamine water solution at 300 µg L^{-1} using NIP (white diamonds) and MIP (black circles)(see text for additional details).*

In order to increase the pre-concentration factor of the methodology, different volumes of 1 % (v/v) acetic acid in methanol were studied as eluting solvent using water spiked at 300 µg L^{-1} amphetamine and the aforementioned washing procedure. Amphetamine recoveries of 55 ± 8, 103 ± 14, and 107 ± 11 % were obtained for 0.3, 0.5 and 1.0 mL elution solvent, respectively. Thus, 0.5 mL was selected as the most appropriate elution volume because of it provided a 2-bold pre-concentration factor.

Next, the MIP column, containing 25 mg polymer, was loaded with 1 mL of a 10 mg L^{-1} amphetamine standard in water and extracted following the recommended procedure to calculate the maximum binding capacity of the sorbent. The column was able to retain till 3.0 ± 0.5 µg of amphetamine, which corresponded to 120 ± 20 µg amphetamine per gram of polymer. This capacity value was high enough for trace analyses of ATS in biological matrices.

Assessment of the effectiveness of the MIP for the NPS extraction

Typical concentrations of NPS in positive oral fluid samples, including ethylone, methylone, α-PVP, butylone, 4-fluoroamphetamine and dimethylone, range from 329 to 2445 µg L^{-1} [24]. Thus, synthetic saliva was spiked at concentration levels

from 350 to 900 µg L^{-1} and extracted by the proposed MIP-SPE procedure in order to evaluate the effectiveness of the synthesized MIP. The obtained recoveries (n=3) are shown in Figure 66. NPS were quantitatively extracted using the MIP-SPE procedure, with recovery values ranging from 82 to 120 % for amphetamine derivatives, from 80 to 117 % for synthetic cathinone derivatives, and from 80 to 113 % for 2C-family compounds. In all cases, relative standard deviation (RSD) values were lower than 12 %.

Selectivity of the synthetized MIPs was also evaluated by the analysis of synthetic saliva spiked with other drugs, such as nicotine, benzocaine, lidocaine, levamisole, diphenhydramine, codeine, and phenacetine. The obtained recovery values can be found in Figure 66, being lower than 56 %, indicating a weak interaction of non-amphetamine drugs with the MIP due to a lack in structural similarity with the template molecule, with the exception of lidocaine that showed a 68 % recovery due to it has a structure relatively similar to amphetamine. Analogous studies were performed with NIP, giving imprinting factors in the range of 0.8 to 1.2 for the aforementioned traditional drugs, which indicated that there was no specific recognition of these substances by the MIP.

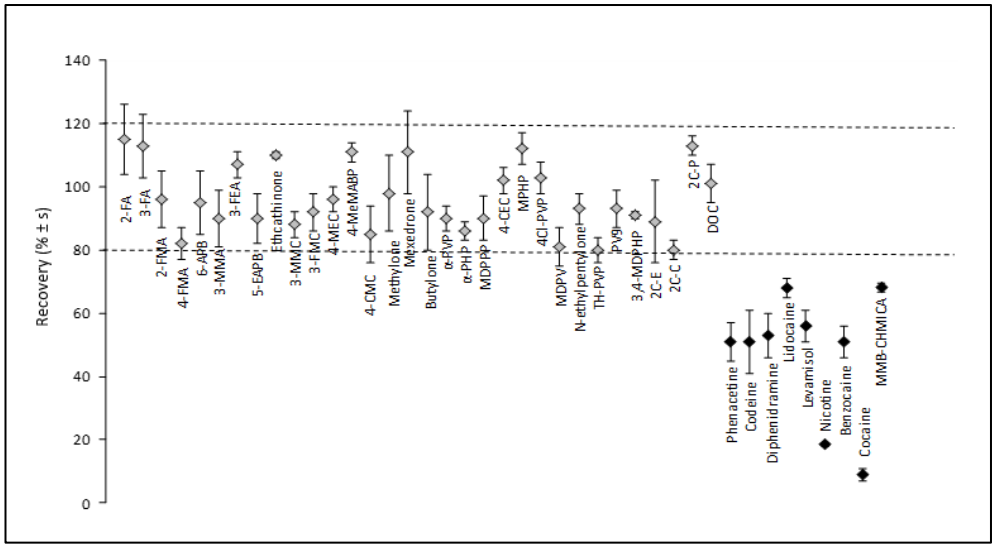


Figure 66. *Recoveries (n=3) obtained for the analysis of amphetamine-type stimulants (grey diamond) and conventional drugs (black diamond) in synthetic saliva spiked at 350 to 900 ng mL^{-1} concentration level and extracted by the proposed MIP-SPE procedure.*

Reusability of the MIP columns was also evaluated by the repetitive analysis (n = 6) of a synthetic saliva sample spiked with 300 μg L^{-1} amphetamine using a unique MIP cartridge. The MIP demonstrated an appropriate performance for at least five uses, with amphetamine recoveries higher than 80 %.

Analysis of NPS in oral fluid

Sample extracts were analyzed by a fast and portable methodology based on IMS in order to provide high sensitive results in few seconds and by a confirmatory technique based on UHPLC-MS/MS. Analytical features of both methodologies were evaluated in terms of sensitivity with LOD values for oral fluid analysis ranged from 10 to 80 μg L^{-1} for IMS and from 0.03 to 1.3 μg L^{-1} for UHPLC-MS (see Table 38). Synthetic saliva samples spiked at 300 and 50 μg L^{-1} amphetamine were extracted following the recommended procedure and analyzed by IMS and UHPLC-MS obtaining recovery values of 99 ± 6 and 79 ± 4 % for IMS, and 84 ± 5 and 112 ± 9 % for UHPLC-MS, respectively.

Moreover, oral fluid samples, obtained from volunteers whom consumed fluticasone, caffeine, and cetirizine were spiked with 300 μg L^{-1} amphetamine, extracted following the proposed procedure and analyzed by both, IMS and UHPLC-MS techniques. The amphetamine concentration found by IMS in oral fluids containing fluticasone, caffeine, and cetirizine were 297 ± 6, 346 ± 18, and 312 ± 9 μg L^{-1}, respectively. Amphetamine concentrations of 249 ± 12, 323 ± 16, and 243 ± 9 μg L^{-1} were found for the aforementioned samples by UHPLC-MS, respectively. Thus, it confirms the quantitative recoveries from 99 to 115 % for IMS and from 81 to 108 % for UHPLC-MS, even in the analysis of field samples with high contents of interferents.

Evaluation of the green character of the developed procedure

Green character of the developed procedure has been evaluated using the Analytical Eco-scale. Sample treatment contributed with a total of 8 penalty points (1x2 for acetic acid, 1x3 for methanol, 1x2 for 2-propanol and 1x1 for phosphate buffer). Zero penalty points were assigned to the IMS procedure due to energy consumption, lower than 0.1 kWh, and absence of harmful emissions, since the IMS instrument incorporates a carbon filter to trap analyzed compounds. However, the chromatographic instrumental setup requires an energy consumption of less than 1.5 kWh per sample, being applied one penalty point. 3 penalty point scores per

sample were assigned to both analytical methods for waste generation. Additional 6 penalty points per sample were assigned to the UHPLC-MS procedure due to reagent and solvent consumption (mobile phase).

In summary, a total of 89 points in the Eco-Scale characterized the IMS methodology, being the UHPLC-MS one characterized by a total score of 85 points.

Table 38. *Analytical figures of merit for ion mobility spectrometry (IMS) and ultra-high performance liquid chromatography-mass spectrometry (UHPLC-MS) determination of the studied amphetamine-type stimulants.*

Analyte	IMS		UHPLC-MS	
	K_0 ($cm^2 V^{-1} s^{-1}$)	LOD ($\mu g L^{-1}$)	RT (min)	LOD ($\mu g L^{-1}$)
Amphetamine	1.668	15	4.55	0.5
2-FA	1.605	19	4.80	0.3
3-FA	1.605	21	5.65	0.2
2-FMA	1.595	26	4.82	0.4
4-FMA	1.595	28	7.34	0.13
6-APB	1.496	43	5.21	0.06
3-MMA	1.487	37	5.24	0.16
3-FEA	1.515	38	5.97	1.3
5-EAPB	1.419	30	5.49	0.8
Ethcathinone	1.519	15	4.82	0.3
3-MMC	1.497	37	5.05	0.9
3-FMC	1.530	18	7.52	0.03
4-MEC	1.447	25	7.29	0.07
4-MeMABP	1.447	32	5.46	0.8
4-CMC	1.458	45	5.24	0.7
Methylone	1.435	17	5.35	1.1
Mexedrone	1.396	25	5.21	0.6
Butylone	1.382	12	4.92	0.12
α-PVP	1.306	26	6.24	0.4
α-PHP	1.264	80	5.85	0.3
MDPPP	1.295	25	4.76	0.3
4-CEC	1.409	65	5.37	0.4
N-ethylpentylone	1.277	55	5.56	0.08
MPHP	1.193	38	6.24	0.4
4Cl-PVP	1.223	40	7.41	0.15
MDPV	1.199	21	6.91	0.07
TH-PVP	1.116	50	6.54	1.0
PV9	1.151	35	6.52	0.6
3,4-MDPHP	1.160	45	8.07	0.06
2C-E	1.326	17	6.13	0.7
2C-C	1.358	10	5.58	0.10
2C-P	1.267	15	5.94	0.4
DOC	1.321	12	5.67	0.3

Abbreviations: K_0, reduced mobility constant; LOD, limit of detection; RT, retention time.

Conclusions

A MIP has been synthesized by bulk polymerization using methamphetamine as template for its use in the selective extraction of amphetamine and other thirty ATS with similar molecular structure. The developed MIP has been prepared and characterized by FTIR, SEM and adsorption isotherm techniques. The presence of pores of around 2 nm diameter in the MIP indicated the formation of specific holes that interacts specifically with the studied ATS due not only for its molecular size, but the position of main functional groups too. The proposed MIP-SPE procedure has been employed for the analysis of ATS in both synthetic and field saliva, providing a quantitative and selective extraction from samples. In addition, the synthesis of our material is simpler and cheaper than the commercial MIP sorbents [9-12] or even consumable materials of some sorbents reported [8]. Beside this, the acceptable reusability of our sorbent combined with the possibility of fabricating several SPE cartridges (ca. 50) from the bulk MIP material, undoubtedly made this protocol economically attractive.

Moreover, although the sample treatment was performed manually (off-line), it can be easily automated using a lab-on-valve approach integrating on-line disposable micro-solid phase extraction interfaced with IMS similar to that employed for cocaine determination [16]. Furthermore, the analysis of ATS was carried out by both, vanguard IMS and rearguard UHPLC-MS approaches. The comparison of the analytical features obtained for each technique has revealed the high utility of the joint use of the two techniques, IMS for a quick and quantitative screening analysis and UHPLC-MS as a confirmation technique.

The main advantages offered by the proposed procedure compared to the available immunoassay screening test is the capability to detect/determine ATS compounds (not only amphetamine and methamphetamine), synthetic cathinones and other related compounds, increasing the selectivity and reducing at the same time the probability of false positive and negative results and, above all, the capability of the methodology to be adapted for the detection/determination of upcoming next generation of ATS compounds without the need to obtain new antibodies.

Conflict of interests

The authors declare that have no competing financial interests in this investigation.

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Tuning the selectivity of molecularly imprinted polymer extraction of arylcyclohexylamines: from class-selective to specific

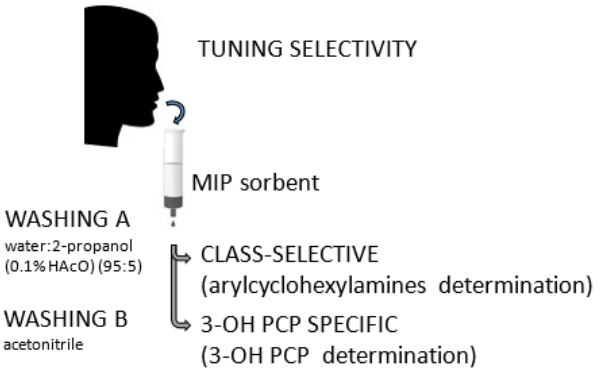
A. Sorribes-Soriano, S. Armenta, F.A. Esteve- Turrillas, J.M. Herrero-Martínez

Department of Analytical Chemistry, University of Valencia, 50th Dr. Moliner St., 46100, Burjassot, Spain

Abstract

A molecularly imprinted polymer (MIP) has been prepared in presence of 3-hydroxy phencyclidine (3-OH PCP) as template by bulk polymerization using *N,N*-dimethylformamide, as porogenic solvent, for the selective solid-phase extraction (SPE) of arylcyclohexylamines from oral fluids. Experimental variables of the extraction procedure have been studied in order to increase both, extraction recovery of 3-OH PCP, used as model analyte, and imprinting factor. By modifying the composition of the washing solvent, the selectivity of the MIP extraction procedure can be tuned, moving from an arylcyclohexylamine selective method to a 3-OH PCP specific method. The applicability of the synthesized MIP was evaluated by the analysis of oral fluids spiked with 3-OH PCP at different concentration levels, extracted using both recommended SPE procedures and analysed by ion mobility spectrometry. Recovery values ranging from 70 to 101 % and a limit of detection of 15 μg L^{-1} were obtained.

Graphical abstract



Keywords: *3-hydroxy phencyclidine; new psychoactive substances; oral fluid; molecularly imprinted polymer; ion mobility spectrometry*

Introduction

Arylcyclohexylamines are a class of psychoactive substances that typically produce dissociation, anaesthesia, and hallucinogenic effects. Ketamine and phencyclidine (PCP) are, probably, the most renowned arylcyclohexylamines, and both are drugs of abuse and controlled substances [1,2]. PCP (1-(1-phenylcyclohexyl)piperidine) was sold until 1967 as an anaesthetic in the United States. However, it was withdrawn due to its intense negative psychological effects [2], starting its use as recreational drug in the mid-1960s. Ketamine (2-(2-chlorophenyl)-2-(methylamino)cyclohexanone) is employed as anaesthetic; however, its use as recreational drug has been reported in many countries. Ketamine and PCP are included in the lists of controlled substances [3,4,5,6]. Many phencyclidine and ketamine analogues have been reported since 1969, including in the list of illegal arylcyclohexylamines the following analogues: eticyclidine (PCE), rolicyclidine (PCPy), tenocyclidine (TCP), and methoxetamine (MXE) [7].

Recently, a new generation of phencyclidine and ketamine derivatives has appeared in the new psychoactive substances market [8,9,10]. The list includes substances such as; 3-methoxyeticyclidine (3-MeO PCE), 3-methoxyphencyclidine (3-MeO PCP), 4-methoxyphencyclidine (4-MeO PCP), 3-hydroxyphencyclidine (3-OH PCP), and deschloroketamine, among others. The effects of these compounds are diverse: 3-MeO PCP is a dissociative hallucinogenic drug that binds to *N*-methyl-D-aspartate receptors more effectively than PCP, ketamine, or MXE; 4-MeO PCP induces euphoric and hallucinogenic effects, dizziness, confusion, psychomotor agitation, and cognitive impairment [11]; and 3-OH PCP, firstly reported in 1978 by Kamir et al. [12], causes euphoria, hallucinations, dissociative effects and muscle pain and flu symptoms assignable to a possible toxicity of 3-OH PCP.

Determination of arylcyclohexylamines in biological samples implies the use of gas chromatography (GC-MS) or liquid chromatography (LC-MS) coupled to tandem mass spectrometry after an appropriate sample treatment such as liquid- liquid extraction [13], solid- phase extraction [14,15,16], microextraction by packed sorbent [17], or molecularly imprinted polymer (MIP) extraction [18]. MIPs are synthetic materials complementary in shape and functional groups to a template molecule, which are able to selectively recognize a compound or a family of compounds with molecular structure similarities. For MIP synthesis, template molecule is mixed with a monomer in the presence of a porogenic solvent to create a complex template-monomer. After that, a cross-linker and an initiator are added

to the mixture to initiate the co-polymerization of monomer and cross-linker, obtaining a polymer with specific cavities for the analyte [19]. These specific cavities can interact with structurally related molecules, being named cross-reactivity to the ability of MIPs to interact with other molecules different than the template [20]. It has been previously reported that cross-reactivity can be used in a positive sense [21,22] to synthetize class-selective MIPs or to use "dummy molecules" as templates. Moreover, cross-reactivity can be tuned or modulated by modifying the washing step to remove the non-specifically bound compounds from the polymer before elution step [23].

In summary, the main aim of this study is: i) to obtain a class-selective MIP to effectively extract arylcyclohexylamines from biological samples, ii) modulate the selectivity of the MIP by studying the effect of different washing solvents to specifically isolate the target analyte from other analogues, and iii) validate an analytical procedure for the analysis of a PCP analogue, 3-OH PCP, in oral fluid samples. To our knowledge, there is no precedent for the analysis of the PCP analogue 3-OH PCP in oral fluids using MIPs.

Experimental

Standards and reagents

PCP, 3-OH PCP, 3-MeO PCP, 3-MeO PCE, ketamine, deschloroketamine, deschloro-*N*-ethylketamine, and MXE were provided by Merck KGaA (Darmstadt, Germany) and the laboratory of the Inspección de Farmacia y Control de Drogas (Valencia, Spain). Figure 67 shows the chemical structures of the studied arylcyclohexylamines.

Acetonitrile, 2-propanol, and *N,N*-dimethylformamide, HPLC grade, acetic acid, and buffer constituents were provided by Scharlab (Barcelona, Spain). Methacrylic acid (MAA), ethylene glycol dimethacrylate (EGDMA), and azobisisobutyronitrile (AIBN) used for MIP synthesis were purchased from Merck.

Visiprep™SPE vacuum manifold and 1 mL empty poly-propylene SPE tubes with polyethylene frits (20 µm porosity) were obtained from Supelco (Bellefonte, USA).

Synthetic oral fluid was prepared following the guidelines of Centre for Applied Sciences and Technologies (CAST) in the document "Preliminary Drug Testing Devices" [24]. Mucin from porcine stomach Type II was obtained from Merck.

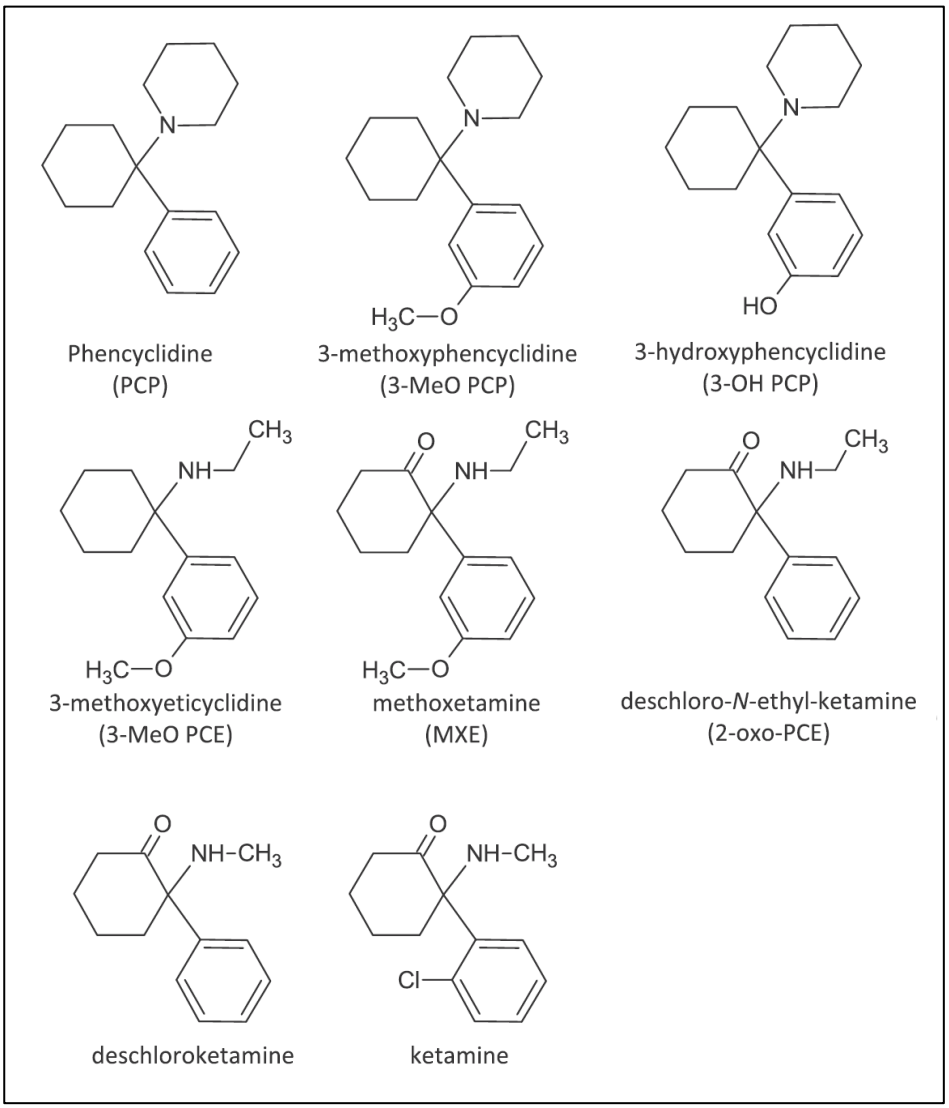


Figure 67. *Molecular structures of the evaluated arylcyclohexylamines.*

MIP synthesis

MIP was prepared in a ratio 1:4:20 (template/monomer/cross-linker) by bulk polymerization strategy. Firstly, 5.0 mg 3-OH PCP was introduced in a glass vial with 7.1 mg of MAA and both components were dissolved in 130 µL of *N,N*-dimethylformamide. The obtained solution was kept for 1 hour in darkness at room temperature to allow template-monomer complex formation. EGDMA cross-linker (81.3 mg) and AIBN initiator (2.5 mg) were added to the template-monomer

solution, and it was sonicated for 5 min and purged with a N_2 stream for 1 min. After that, polymerization mixture was introduced in a water bath at 60 °C for 24 h.

The obtained MIP was crushed and sieved to obtain a particle size under 250 µm. Empty 1 mL polypropylene SPE tubes were filled with 25 mg MIP, embedded between two frits. Each SPE tube was washed with methanol containing 5 % of acetic acid until no template was detected in the extract by ion mobility spectrometry (IMS).

Non-imprinted polymer (NIP) was also prepared following the aforementioned procedure for MIP synthesis without any template addition.

MIP characterization

MIP and NIP were characterized by scanning electron microscopy (SEM) after a previous grafting over an Au-Pt layer to increase conductivity. The morphological characterization was done by using a S-4800 scanning electron microscope from Hitachi (Ibaraki, Japan) provided with an emission field gun, a secondary electron detector, and an EMIP 3.0 acquisition data system from Rontec (Normanton, UK).

Extraction procedure

Two extraction procedures were developed in this study. The first procedure, for the class-selective determination of arylcyclohexylamine compounds, implies cartridge conditioning with 1 mL of a mixture 2-propanol:acetic acid (95:5, v/v) and 1 mL deionized water. 1 mL sample was mixed with 0.5 mL phosphate buffer (0.1 M, pH 7.0) and percolated through the cartridge at a 2 mL min^{-1} flow rate. Washing step was performed with 1 mL deionized water:2-propanol (95:5 v/v) containing 0.1 % acetic acid. Finally, arylcyclohexylamines were eluted using 1 mL 2-propanol containing 5 % acetic acid.

In the second procedure, for the specific extraction of 3-OH PCP, 2 mL acetonitrile was used as washing solvent.

IMS analysis

Arylcyclohexylamines involved in this study were determined by IMS using an IONSCAN-LS from Smiths Detection (Morristown, NJ, USA) with a ^{63}Ni foil radioactive source. Data acquisition and processing was done by IMstation

software (version 5.389) from Smiths Detection. Positive ion mode was selected for measurements, using nicotinamide as internal calibrant with a reduced mobility constant (K_0) of 1.861 $cm^2V^{-1}s^{-1}$. Plasmagram acquisition was made using 30 ms scan period and a 0.2 ms shutter grid width. A counter flow of dry air at 0.265 L min^{-1} was used as drift gas and drift region electric field was set at 251 V cm^{-1}.

3 µL sample extract was introduced by thermal desorption from a polytetrafluoroethylene (PTFE) membrane using 1 s post-dispense delay. Temperatures were adjusted to 255, 260, and 237 °C for desorption, inlet, and drift tube, respectively.

Geometry optimization and energy calculations

The three-dimensional structure of the monomer, template, and monomer-template complexes were built up using AVOGADRO programme [25] and preoptimized by MMFF94 [26] force field applying its implementation in the Avogadro programme [27].The molecular geometries and the hydrogen bond interactions among the arylcyclohexylamines with MAA were optimized by using density functional theory (DFT) at the B3LYP/6-31+G(d,p)//B3LYP/6-31G(d) level [28]. The single point energies of each optimized conformations were calculated by using B3LYP/6-31+G(d,p) basis set. The energy values were calculated by using equation 1.

$$E = E(\text{template} - \text{monomer}) - E(\text{template}) - \Sigma E(\text{monomer}) \quad \text{(Eq. 1)}$$

Results and discussion

Preparation conditions for MIP synthesis

As it has been mentioned in the Introduction section, there is a precedent of synthesis of MIPs for ketamine and norketamine extraction from hair samples [18]. In that paper, a MIP was prepared by free radical polymerization using (±)-ketamine base as template, MAA as functional monomer, and EGDMA as crosslinker (in a ratio of 1:3:15), and toluene as porogen. Using this information as starting point, different mixtures of template, MAA, EGDMA, and porogen were evaluated. 3-OH PCP and deschloroketamine were employed as templates, both in its hydrochloride form, which is the form normally found in illicit preparations. On the other hand, toluene, chloroform, acetonitrile, and *N,N*-dimethylformamide were also evaluated as porogenic solvents. However, the templates considered were not soluble in the

first three solvents, even in the presence of MAA and, as consequence, MIPs did not polymerize. Additionally, a mixture of deschloroketamine with MAA, EGDMA, and AIBN using *N,N*-dimethylformamide as porogen did not polymerize adequately, giving a small amount of material after polymerization (24 h at 60 °C). On the other hand, the mixture of 3-OH PCP, MAA, EGDMA, AIBN, and *N,N*-dimethylformamide provided 75 mg of polymer after treatment at 60 °C during 24 h and it was used for subsequent experiments.

MIP characterization

The morphology and particle size of synthesized MIP and NIP were characterized using SEM and the results were depicted in Figure 68. Since bulk polymerization has been carried out, and the MIP was crushed and sieved, irregular particles, with size between 50 and 250 µm, were obtained. Polymer particles are composed of small and agglomerated nodules. The size of these nodules was determined to be in the nano range (MIP 60 ± 4 nm and NIP 83 ± 6 nm, n = 5), measured using the EMIP 3.0 acquisition and data manipulation software. Nodules of MIP were lower than NIP, suggesting that the template compound has an important influence on the material growth during the polymerization. It could be explained by strong interactions between functional monomer and template, which are responsible in assembling the components tightly around the nucleation points, thus making the MIP particles smaller [29,30]. Moreover, SEM images of Figure 68 shows the presence of flow-through pores in the network skeleton, which provide appropriate permeabilitywith low resistance to flow, which ishighly adequate for solid-phase extraction purposes.

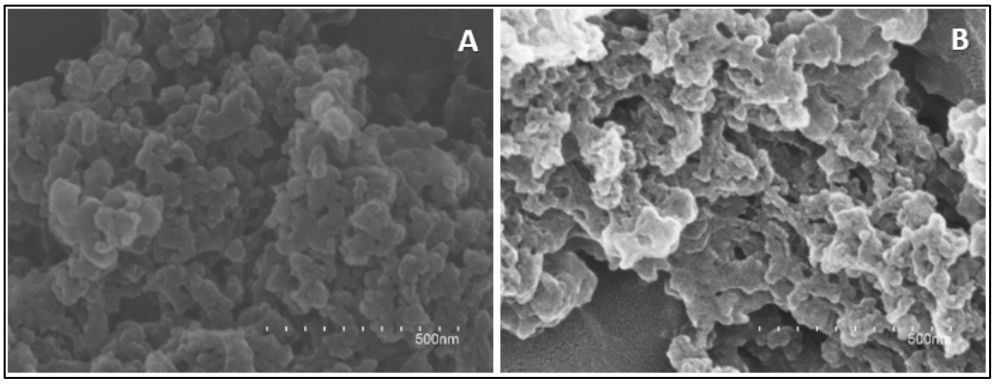


Figure 68. *SEM images of MIP (A) and NIP (B) at x50,000 magnification.*

Geometry optimization and energy calculations

If excess mol of functional monomer are used, only specific number of mol reacts with template and the rest remain as excess leads to non-specific binding sites and hence decreases the binding capacity. Thus, the number of moles of functional monomer that interacts with one mol of template was firstly optimized. From the results, it can be concluded that increasing the ratio of monomer from 1:1 to 1:4, the binding energy increases from -68.9 KJ mol-1 to -300 KJ mol-1, which translates into the formation of the most stable complex. However, only two molecules of MAA present interactions through hydrogen bonding with the molecule of 3-OH PCP, being the interaction of the other two molecules of MAA less selective. Figure 69 shows the optimized geometry of the interaction of 3-OH PCP with two molecules of MAA. As it can be seen, the complex is stabilized through the presence of three hydrogen bonding interactions The binding energy of the complex template:monomer 1:2 was -200 KJ mol^{-1}. Thus, in order to compare with the other arylcyclohexylamines only the complex analyte:MAA 1:2 will be considered. As it can be seen in Table 39, the highest binding energy is that provided by 3-OH PCP, followed by the most similar class members, PCP and 3-MeO PCP. The other members of the arylcyclohexylamines family show a binding energy lower than -150 KJ mol^{-1}. Those differences in the binding energy of the different members of the family suggest that selectivity of the procedure can be tuned by modification of the elution strength of the washing solvent.

Table 39. *Binding energy calculated for the pre-polymerization complex analyte:methacrylic acid (in a ratio 1:2) for the studied arylcyclohexylamines.*

Analyte	**Binding energy (kJ mol^{-1})**
3-OH PCP	-200.0
PCP	-186.2
3-MeO PCP	-172.0
3-MeO PCE	-110.0
MXE	-144.4
2-oxo PCE	-140.9
Deschloroketamine	-150.0
Ketamine	-136.8

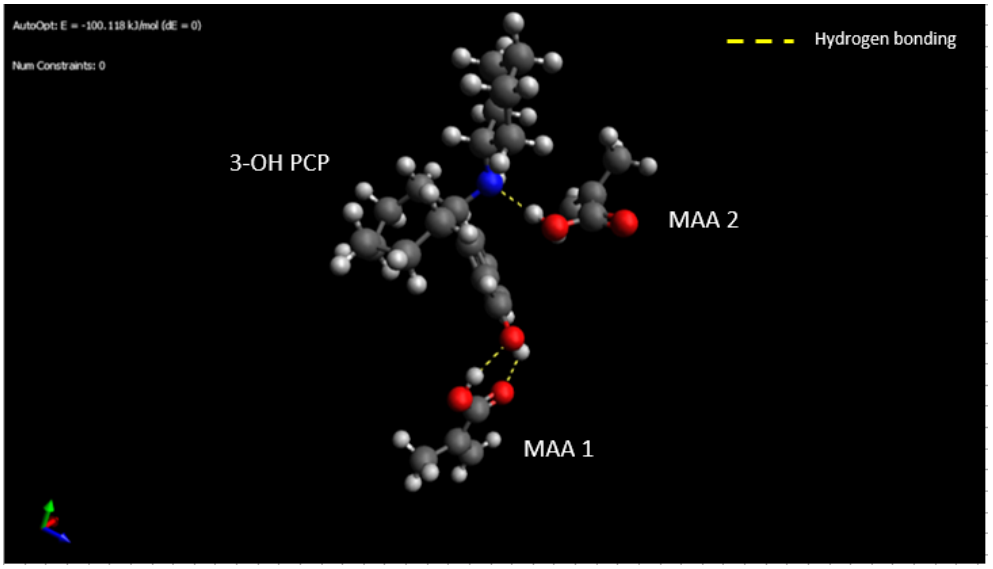


Figure 69. *Optimized geometry of the complex formed by 3-OH PCP with two molecules of MAA. Color code for the atoms: oxygen, red; nitrogen, blue; carbon, grey; hydrogen, white.*

Evaluation of the MIP extraction conditions

A SPE procedure was developed for the retention of arylcyclohexylamines in oral fluids using the synthesized MIP. Thus, the main SPE conditions were investigated including pH of sample, and washing and elution solutions. 3-OH PCP was used (at a concentration level of 250 µg L^{-1}), as target molecule, for recovery studies in order to evaluate the most appropriate SPE conditions.

pH of oral fluids typically ranges pH values between 5.5 and 7.5 [31]. At these pH values, MAA is deprotonated (pKa value of 4.7) [32], acting as hydrogen acceptor, while protonated arylcyclohexylamines (weak bases with pKa values between 7.5 and 8.5) [33] are hydrogen donors. Thus, the effect of sample pH on the MIP extraction of 3-OH PCP was evaluated from pH 5.0 to 8.0, using 1 mL of 250 µg L^{-1} concentration level 3-OH PCP spiked oral fluid and the addition of 0.5 mL different buffers. In these experiments, 1 mL deionized water was employed as washing solution and 1 mL 2-propanol containing 5 % acetic acid was used to elute 3-OH PCP for IMS analysis. As it can be seen in Figure S1 of the supplementary material, quantitative extraction values of 3-OH PCP were obtained in all cases, with

recoveries from 85 to 105 %, thus confirming that pH values from 5.0 to 8.0 did not affect to the 3-OH PCP retention in the MIP cartridge.

Different washing solutions were evaluated, such as 1 mL deionized water, and water:2-propanol and water:2-propanol:acetic acid mixtures, in order to minimize unspecific interactions and to achieve an appropriate imprinting factor. Imprinting factor is the measurement of the strength of selective interactions of the MIP towards the template molecule and it can be calculated as the ratio between the obtained recoveries for MIP and NIP. As it can be seen in Figure 70A, the use of water and water:2-propanol (50:50 v/v) provided similar recovery values for the MIP and NIP extraction, which implies imprinting factor values close to 1. Thus, both washing solutions were discarded due to unspecific interactions of both polymers with the target analyte. Then, the percentage of 2-propanol in the washing solution was increased until 75 % leading to an increase in the imprinting factor to 1.6. However, the use this solution was discarded since the recovery value decreased to 40 %. The use of different water:2-propanol mixtures (from 100:0 to 50:50 v/v) with 0.1 % acetic acid were evaluated (Figure 70A). The addition of acetic acid increases the elution strength of the washing solution; since this acid protonates the MAA carboxylic group of the polymer, breaking non-specific interactions in NIP and, as consequence, increasing the imprinting factor. Water:2-propanol mixtures of 75:25 v/v and 50:50 v/v, containing 0.1 % acetic acid, provided imprinting factors of 2.3 and 1.7, respectively; however, non-quantitative MIP extraction of 3-OH PCP was obtained in both cases (43 and 25 %, respectively). The most appropriate recovery (95 %) and imprinting factor (1.6) values were obtained using a water:2-propanol (95:5 v/v) mixture containing 0.1 % acetic acid as washing solvent. The use of a higher content of acetic acid in the washing solution (0.2 and 0.3 %) provided similar imprinting factors; however, reduced 3-OH PCP recoveries for MIP below 80 % were obtained.

On the other hand, the use of an aprotic polar solvent, such as acetonitrile, was also evaluated as washing solvent, providing a high imprinting factor of 2.4; but a recovery of 62 % 3-OH PCP was found. Thus, taking into account both high recovery values and imprinting factor, the use of water:2-propanol (95:5 v/v) with 0.1 % acetic acid and acetonitrile were selected as washing solvents for further studies.

In order to assess complete elution of arylcyclohexylamines, 1 mL of 2-propanol containing 5 % acetic acid was employed as eluting solvent. As mentioned above, acetic acid protonates MAA and breaks hydrogen bonding with arylcyclohexylamines, which were eluted by an organic solvent with the

appropriate elution strength. Methanol and 2-propanol were also tested as elution solvents. Similar results were obtained using both organic solvents, and 2-propanol was selected, considering its higher compatibility with IMS determination and lower toxicity compared to methanol.

Reusability of the MIP was evaluated by sequential analysis (n=10) of spiked synthetic oral fluid using a single MIP cartridge. The obtained recoveries were higher than 80 % for all experiments, showing a good reusability of the produced MIPs.

Maximum loading capacity of the polymer was evaluated by the analysis of 1 mL blank oral fluid sample spiked with 5 mg L^{-1} 3-OH PCP and analyzed by the proposed procedure using MIP sorbent. The amount of 3-OH PCP recovered, analyzed after the appropriate dilution of the extract to fit within the linear range of IMS, was 2.48 ± 0.13 μg, Considering, that 25 mg of MIP were placed in each cartridge, the loading capacity of MIP was estimated at 99 ± 6 μg per gram of polymer what shows a similar binding capacity than ketamine-MIP describe at the literature which have a binding capacity of 130 μg per gram of polymer [18].

Tuning selectivity of MIP extraction

As it has been aforementioned after molecular modelling study, the differences of the binding energy of the evaluated class members suggest that selectivity can be tuned modified the elution strength of the washing solvent. Cross-reactivity of synthesized MIP was evaluated by using the proposed procedure and water:2-propanol (95:5 v/v) with 0.1 % acetic acid, as washing solvent, for the analysis of other arylcyclohexylamines, such as: PCP, 3-MeO PCP, 3-MeO PCE, MXE, 2-oxo PCE, deschloroketamine, and ketamine. Recovery values of synthetic saliva individually spiked with the aforementioned compounds at 250 μg L^{-1} concentration level are shown in Figure 70B. The obtained recovery values were from 67 to 98 %; thus, it can be concluded that the developed procedure using water:2-propanol (95:5 v/v) with 0.1 % acetic acid, as washing solution, provided appropriate recovery values for the determination of arylcyclohexylamines in oral fluids.

Nevertheless, in other cases a more selective method can be required. In such cases, considering that analyte-MIP interactions are based on hydrogen bonding, the use of an aprotic polar solvent with moderate-high elution strength may drastically reduce the unspecific interactions of analogue molecules with the MIP maintaining unaltered the template specific ones. Figure 70C shows the obtained

results using acetonitrile as washing solvent. As it can be seen, significant differences can be observed between 3-OH PCP and the rest of arylcyclohexylamines evaluated. The obtained recovery for 3-OH PCP was 62 %; however, for the rest of evaluated arylcyclohexylamines was below 24 %.

On the other hand, the imprinting factors obtained for the different evaluated arylcyclohexylamines ranged from 2.5 to 1.1, for 3-OH PCP and PCP, and deschloroketamine, respectively. It should be aforementioned that it was not possible to obtain the imprinting factors of 3-MeO PCE, MXE, deschloro-*N*-ethyl-ketamine, and ketamine because concentration of those analytes in the extract was below the limit of quantification. Thus, the procedure using acetonitrile as washing step can be considered a 3-OH PCP specific method for the analysis of oral fluids taking into consideration the obtained recovery and the imprinting factors.

Determination of 3-OH PCP in oral fluids

Figure 71 shows the signals obtained for the IMS analysis of the studied arylcyclohexylamines. As it can be seen, the most intense peak around 9.5 ms drift time corresponds to nicotinamide, used in positive ionization acquisitions, as internal standard, to correct variations in temperature and pressure. The peaks located between 12.209 and 14.779 ms drift time corresponded to arylcyclohexylamines, with K_0 values between 1.179 and 1.436 $cm^2V^{-1}s^{-1}$. The signal of 3-OH PCP was observed at 14.240 ms corresponding to a K_0 of 1.226 $cm^2V^{-1}s^{-1}$ and the area of this peak was used as analytical response for the determination of 3-OH PCP in oral fluids by the proposed SPE-IMS procedure using the synthetized MIP.

Analytical features, including precision, accuracy, sensitivity, and linearity of the methodology, were evaluated for 3-OH PCP (see Table 40). Standards from 50 to 600 µg L^{-1} 3-OH PCP, prepared in 2-propanol containing 5 % acetic acid, were measured 3 times, obtaining in all cases a relative standard deviation (RSD) lower than 2 %. Linearity of the calibration curve (R^2) was 0.999. Limits of detection and quantification, established as 3 and 10 times the standard deviation of the intercept divided by the slope of the calibration curve, respectively, were 15 and 50 µg L^{-1}, respectively.

Table 40. *Analytical features of 3-OH PCP determination by IMS.*

Analytical feature	Value
Linear range (μg L^{-1})	50 – 600
Linearity (R^2)	0.999
Slope (L μg^{-1})	0.101 ± 0.002
Intercept (μg L^{-1})	1.7 ± 0.8
RSD (%, n = 3)	2
LOD (μg L^{-1})	15
LOQ (μg L^{-1})	50

Method accuracy was estimated as the recoveries obtained for the analysis of water, synthetic saliva, and pooled human oral fluids spiked with 3-OH PCP at different concentrations levels (250 and 500 μg L^{-1}) using both proposed SPE procedures, the class selective method that used water:2-propanol (95:5) with 0.1 % acetic acid as washing solvent; and the specific method that used acetonitrile as washing solvent. The obtained recoveries were satisfactory (see Table 41), ranging from 82 to 101 % for the class selective method, and from 71 to 73 % for the specific method Those obtained recoveries demonstrate an appropriate molecular recognition of the 3-OH PCP by the MIP and the presence of non-covalent specific interactions unbreakable by the washing solvent in both, class-selective and specific method.

Table 41. *Concentration and recoveries obtained for the determination of 3-OH PCP in water, synthetic saliva, and pooled human oral fluids by the proposed procedure using water:2-propanol (95:5 v/v) with 0.1 % acetic acid (class-selective method) and acetonitrile (specific method) as washing solvents.*

Sample	[Spiked] (μg L^{-1})	Recovery (% ± s, n=3)	
		Class-selective method	Specific method
Water	250	90 ± 5	62 ± 2
	500	83 ± 4	61.4 ± 1.7
Synthetic saliva	250	92 ± 2	63 ± 10
	500	121 ± 12	77 ± 6
Human oral fluids	250	101 ± 8	73 ± 6
	500	82 ± 5	71 ± 4

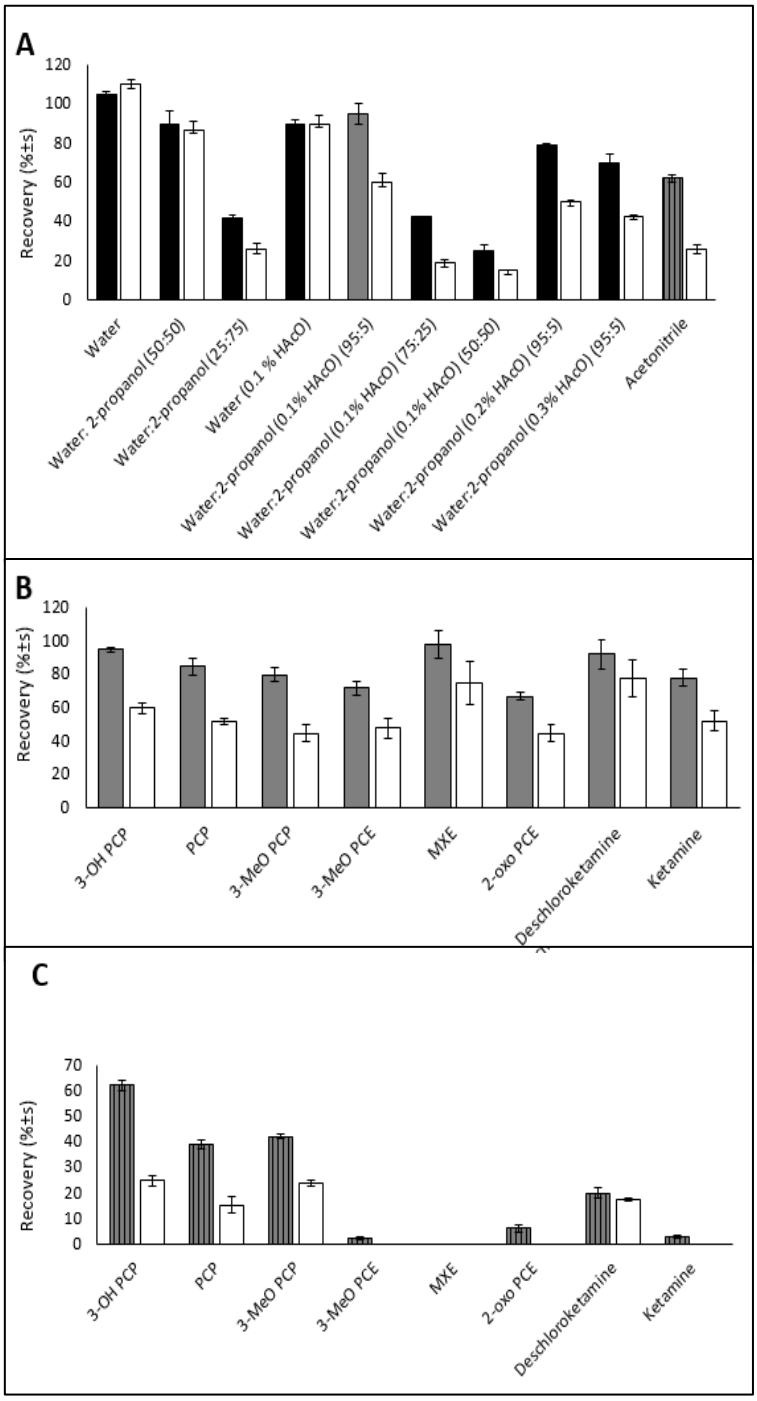


Figure 70. *Recoveries obtained for 250 µg L^{-1} of 3-OH PCP in water extracted by using MIP (black bars) and NIP (white bars) washed with different solutions (A). Recoveries obtained for 250 µg L^{-1} of arylcyclohexylamines in water using MIP extraction after a washing step consisting of 1 mL of water:2-propanol (95:5 v/v) containing 0.1 % acetic acid (B) and acetonitrile (C).*

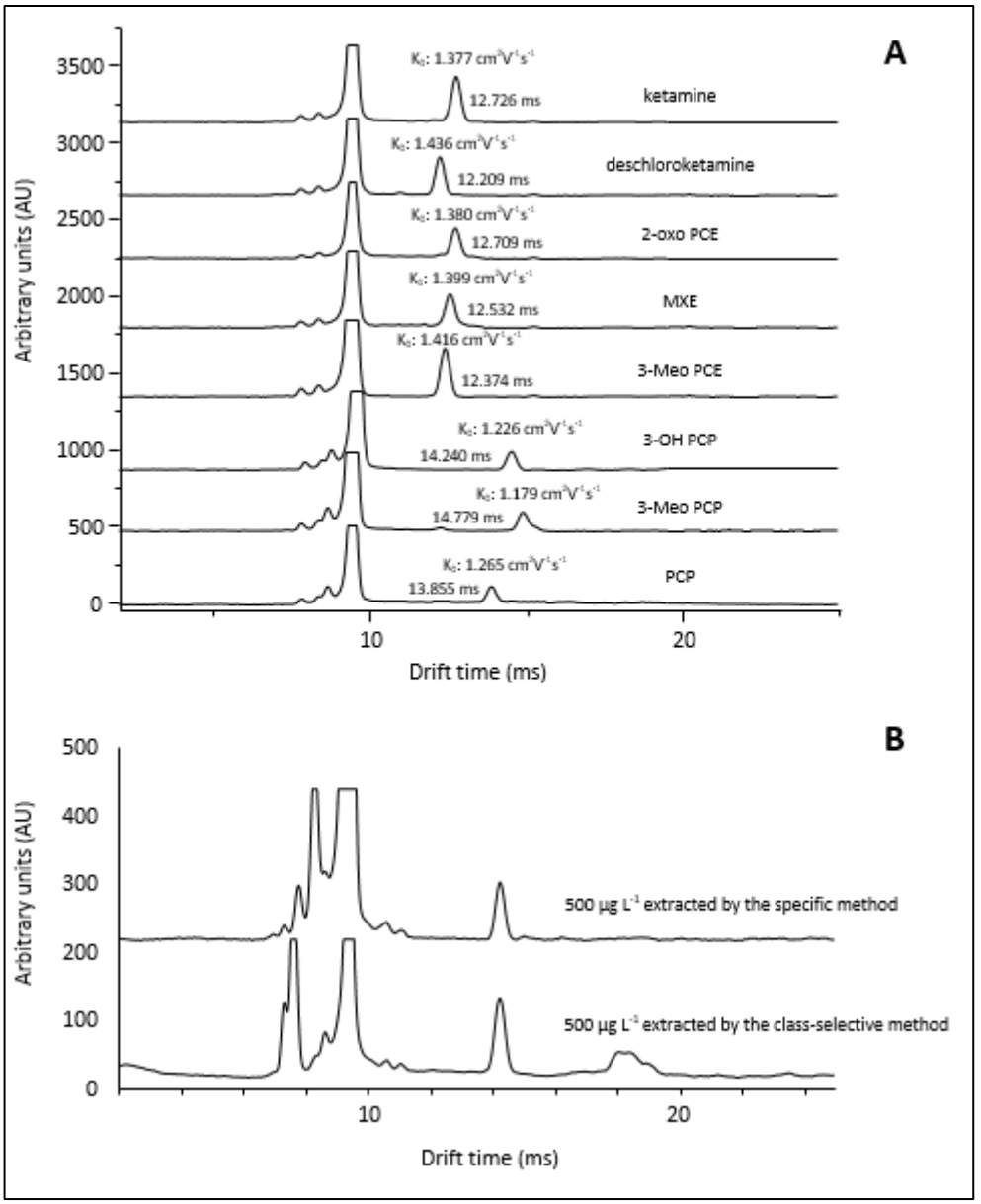


Figure 71. *IMS signals obtained for the studied arylcyclohexylamines standards at a concentration level of 250 µg L^{-1} (A), and for a pooled human oral fluids sample spiked with 500 µg L^{-1} 3-OH-PCP extracted by the specific and class-selective methods (B).*

Comparison with other sample treatment methods

Determination of arylcyclohexylamines in biological fluids has been carried out by using different extraction approaches. Table 42 summarizes extraction conditions and the analytical performance of relevant examples found in the literature for the determination of arylcyclohexylamines in biological fluids. As it can be seen, PCP, 3-MeO PCP, ketamine, and MXE are the most analyzed arylcyclohexylamines compounds, and to our best knowledge, no studies have been developed for the determination of 3-OH PCP yet. Diverse extraction techniques have been employed such as liquid-liquid extraction, dispersive liquid-liquid extraction, solid-phase extraction and microextraction by packed sorbent. The obtained limits of detection range from 0.04 to 50 µg L^{-1}, and recovery studies from 33 to 100 %. Our study provides an analytical methodology with a sensitivity in the same order than the evaluated methodologies. Regarding extraction efficacy, the obtained recoveries were up to 71 %compared to the other extraction approaches. Moreover, the developed methodology proposed the use of IMS for the determination of arylcyclohexylamines extracts, while most of the studies shown in Table 42 employ LC-MS/MS techniques. IMS provides high advantages in comparison to LC-MS/MS techniques, such as: ease of use, mechanical robustness, portability, non-specialized operators, and moderate cost [42].

Conclusions

A MIP has been synthesized by bulk polymerization using 3-OH PCP as template for its use in the selective extraction of arylcyclohexylamines from oral fluid samples. The developed MIP has been characterized by SEM. The effect of experimental variables, such as sample pH, and washing and elution solvents, on the extraction recovery of 3-OH PCP from water has been evaluated using the synthesized MIP as SPE sorbent. Selectivity of the MIP extraction procedure can be tuned by modifying the composition of the washing solvent, moving from a class selective method for arylcyclohexylamines, to a more specific method for 3-OH PCP. Beside this, the acceptable reusability of our sorbent combined with the possibility of fabricating several SPE cartridges from the bulk MIP material, undoubtedly made this protocol economically attractive. Finally, to evaluate the applicability of synthetized MIPs in complex matrices, synthetic saliva was spiked with 3-OH PCP at different concentration levels, extracted using the recommended procedures and analysed by IMS. Results demonstrated that the procedure can be successfully applied for the analysis of 3-OH PCP in oral fluid samples.

Table 42. *Examples of different extraction approaches for the determination of arylcyclohexylamines in biological samples.*

Analytes	Matrix	Sample treatment	LOD (μg L^{-1})	Recovery (%)	RSD (%)	Determination	Ref
3-MeO PCP, 4-MeO PCP, MXE, norketamine	Blood	Deproteination	0.3	95	11	LC-MS/MS	34
3-MeO PCE, 3-MeO PCP, MXE	Blood, urine, vitreus humor	LLE (1-chlorobutane, H_2SO_4 0.05 M)	50	87	-	LC-MS	13
PCP	Hair	DLLME (chloroform)	1	100	11	LC-MS	35
Ketamine	Blood, urine	DLLME (chloroform)	10	100	10	GC-MS	36
Ketamine	Urine	DLLME (chloroform)	0.5	55	-	LC-MS/MS	37
PCP, 3-MeO PCP, 4-MeO PCP, 3-MeO PCE, ketamine	Urine	SPE (RP/SCX)	1.2	50	15	LC-MS/MS	38
PCP, MXE	Serum	SPE (RP/SCX)	1.5	51	19	LC-MS/MS	39
MXE	Oral fluid	MEPS (C18 bin)	0.04	33	15	LC-MS/MS	40
Ketamine	Oral fluid	MEPS (C18 bin)	15	83	-	DESI-HRMS	41
Ketamine, Nor-ketamine	Hair	MISPE	0.1 [a]	86	5	LC-MS/MS	18
3-OH PCP	Oral fluid	SPE (MIP)	15	70	2	IMS	This study

Abbreviations: DESI-HRMS, desorption electrospray ionization-high resolution mass spectrometry; DLLME, dispersive liquid-liquid microextraction; GC, gas chromatography; IMS, ion mobility spectrometry; LLE, liquid-liquid extraction; LC, liquid chromatography; LOD, limit of detection; MEPS, microextraction by packed sorbent; MIP, molecular imprinting polymer; MS, mass spectrometry; RP, reverse phase; RSD, precision set as relative standard deviation; SCX, strong cation eschange; SPE, solid-phase extraction.

[a] LOD expressed as ng g^{-1}.

Conflict of interests

The authors declare no competing financial interest.

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Supplementary material

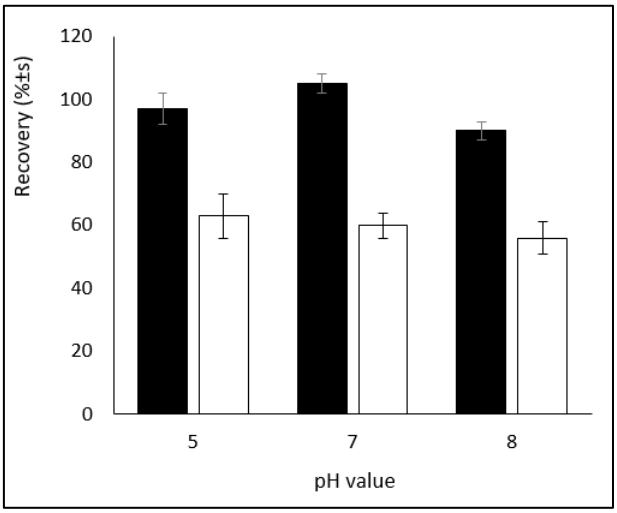

Figure S1. *Effect of the pH value on the extraction recovery of an oral fluid spiked with 250 µg L^{-1} of 3-OH PCP (MIP: black bars; NIP: white bars).*

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Molecularly imprinted polymer-based device for field collection of oral fluid samples for cocaine identification

A. Sorribes-Soriano, J.M. Herrero-Martínez, F.A. Esteve-Turrillas, S. Armenta

Department of Analytical Chemistry, University of Valencia, 50th Dr. Moliner St., 46100 Burjassot, Spain

Abstract

In this paper, a low–cost, rapid, easy, and potentially portable tool for the identification of cocaine and its semi-quantitative determination in oral fluid has been proposed. A field collection device has been designed, based on a cotton pad with an indicator and a molecularly imprinted polymer (MIP) sorbent, to selective retain cocaine from oral fluid components. After sample collection, cocaine is transferred by using phosphate buffer to the MIP and then eluted with 2-propanol. The obtained extract is analysed by ion mobility spectrometry (IMS), providing a cut-off value of 20 μg L^{-1} that identifies 100 % true-positive and 95 % true-negative samples. The MIP-IMS procedure has been validated by the analysis of oral fluid samples, collected from cocaine users at recreation environments, by comparing the results with lateral flow immunoassay and chromatographic reference methods. Thus, the proposed methodology allows a simple and fast cocaine identification that can be carried out in field by non-specialized personnel, such as health personnel, law enforcement bodies, and customs staff.

***Keywords**: Cocaine; field collection device; molecularly imprinted polymer; ion mobility spectrometry; oral fluid*

Introduction

According to data reported by the Division for Policy Analysis and Public Affairs of the United Nations Office on Drugs and Crime (UNODC) in 2017, 271 million people, which accounts as 5.5 % of the global population, used drugs, being this value 30 % higher than that reported in 2009 [1]. The most commonly used drug worldwide continues to be cannabis, with an estimated 188 million users, followed by opioids (53.4 million people worldwide, among those people, 29.2 million used heroin and opium) and cocaine (18.1 million people worldwide). Amount of seized cocaine increased in 2017 to 1,275 tons, the largest quantity ever reported.

Current reference analytical techniques for the analysis of psychoactive substances in biological fluids involve a combination of gas or liquid chromatography coupled to mass spectrometry (GC-MS and LC-MS, respectively) [2]. These procedures are non-portable, time-consuming, require skilled personnel and have high acquisition, maintenance, and operation costs. In the same time, there is a high-demand for rapid on-site testing.

Detection of psychoactive substances in oral fluid using field devices provides several advantages, such as real-time analysis and simple, easy handling, and non-invasive collection procedures, which resulted in an increase of its strength and applicability in roadside testing, hospitals, and treatment centres [3,4] . The use of oral fluid as biological matrix also minimizes the risk of adulterating or substituting the sample, because of its collection can be directly supervised [5]. Additionally, oral fluid is a simpler matrix compared to urine or plasma, and the parent drug can be found instead of its metabolites. Moreover, the presence of the drug can be directly related to a recent consumption and possible impairment than its detection in urine or other biological matrices [6]. With regard to cocaine, the correlation between oral fluid and blood cocaine concentration is still not well established and depends upon the administration route [7]. An average oral fluid:blood ratio of 22 (range 4-119) was reported in a sample of people driving under the influence of this drug [8], and a similar oral fluid:blood ratio value (17) was also obtained by Langel and collaborators [9]. After subcutaneous administration, the correlation between oral fluid and blood concentrations was significant, although no correlation with clinical symptoms was described [10]. In other study, it was demonstrated that saliva:plasma ratios were generally greater than 1, and there was evidence of moderate to extreme contamination of saliva by cocaine immediately following intranasal and smoked routes of administration. However, contamination of the oral cavity and saliva cleared rapidly and samples obtained 2 h after dosing appeared to be free of contamination [11].

Lateral flow immunoassay (LFA)-based procedures are the most commonly employed on-site tests for cocaine detection due to the non-sophisticated nature, low cost, easy operation, and small size. Most of these LFA sensors are developed in combination with optical detectors [12] to avoid the main drawback of the LFA based on-site drug tests, subjective limitation of positive/negative judgment. This combination of LFA and optical detectors (most detector manufacturers supply their own test strips) enhances not only the measurement objectivity but also increases acquisition and operational cost of the LFA sensors.

Ion mobility spectrometry (IMS) is an analytical technique that separates generated ions under a weak electric field under atmospheric pressure [13]. IMS has been traditionally used for the detection and identification of illegal drugs in custom scenarios for more than 30 years [14]. On the other hand, the applications of IMS for the determination of illicit drugs in biological samples are increasing for the last years [15]. The most used strategy to avoid the inherent selectivity problems of IMS is the addition of a previous sample treatment step, such as liquid-liquid extraction (LLE), solid-phase extraction (SPE), solid-phase microextraction (SPME), and stir bar sorptive extraction (SBSE) [16]. The selectivity of sample treatments has been improved recently by using analyte-selective supports such as immunosorbents [17], aptamers [18], and molecularly imprinted polymers (MIPs) [19]. Compared to aptamers and immunosorbents, MIPs offer higher chemical inertness, long-term stability, and relatively low cost. Indeed, determination of cocaine in oral fluid by IMS after MIP extraction has been previously reported [20,21,22].

The main purpose of the developed procedure is to provide a rapid, easy, and potentially portable tool for the detection of cocaine and its semi-quantitative determination in oral fluid. The designed device is focused on a simplification of the methodology in order to be used in field conditions by law enforcement or custom officers. The effectiveness of the proposed device was compared with reference methods based on LFA and GC-MS for the detection of cocaine in oral fluid samples taken from cocaine users.

Experimental

Material, reagents and samples

Cocaine and cocaine-D3 standards were provided by Merck KGaA (Darmstadt, Germany, www.merckgroup.com). Cocaine standard solutions were prepared in 2-propanol, and kept at –20 ºC in amber glass vials until their use. All the solvents

used in this study were HPLC grade. Organic solvents and buffer constituents were obtained from Scharlab (Barcelona, Spain, www.scharlab.com). Methacrylic acid (MAA), ethylene glycol dimethacrylate (EDMA) and azobisisobutyronitrile (AIBN) were provided by Sigma (Steinheim, Germany, www.sigmaaldrich.com). Polyethylene frits (20 µm porosity) were obtained from Supelco (Bellefonte, PA, USA). Buffer solutions were prepared at 0.1 M concentration using acetate, phosphate, and carbonate buffers in the pH range of 3.0–12.0. Sampling component drierite was purchased from Acros Oganics (Geel, Belgium) and cotton pad was obtained from Sigma.

Oral fluid samples were obtained from cocaine consumers and non-consumers after freely-given informed consent. Cocaine consumers were 12 males and 8 females with ages ranging from 19 to 38 years old. Oral fluid was sampled at different times (from 10 to 120 min) after cocaine abuse. Two aliquots of oral fluid were sampled, one using the part A of the proposed device, and the other by spitting inside a 1.5 mL polypropylene Eppendorf tube for reference procedures analysis. Samples were immediately analysed or stored in the device at −20°C until their analysis. Cocaine-free oral fluid were collected from males and females with ages ranging from 19 to 66 years old and were immediately analysed or stored in the device at −20°C until their analysis. It should be highlighted that under no circumstances have the authors trafficked or provided illegal substances, aimed, promoted, facilitated, stimulated, or forced in any way the consuming of illegal substances.

Synthesis of MIPs

A cocaine-based MIP was prepared following the procedure previously described [19]. The method used cocaine as template, MAA as monomer, EDMA as cross-linker, acetonitrile as porogen, and AIBN as initiator in template/monomer/cross-linker molar ratio of 1:4:20. The solution was sonicated for 5 min and purged with nitrogen for 10 min. The polymerization was carried out in a water bath at 60°C for 24 h. After polymerization, the resultant polymers were crushed, dried at 80°C overnight, and sieved (≤ 100 µm). Cocaine was removed from the MIP by Soxhlet extraction for 24 h using a mixture of 5 % (v/v) acetic acid in methanol.

Sampling procedure using the developed device

The developed device comprised of two independent parts (A and B) depicted in Figure 72. Thus, oral fluid sampling is performed by the part A of the device, while sample treatment, clean-up and preconcentration are done by the MIP placed in the part B.

Part A has been specially developed to allow an easy and comfortable sampling procedure. It is a polypropylene hollow cylinder of 2.7 cm length and 1.0 cm internal diameter with a female Luer connection in one side connected to a 4.2 cm plastic handle. A 50 mg cotton pad is placed inside the plastic cylinder, with a drierite indicator impregnated with cobalt chloride in the back side.

Part B is a 2.7 cm length and 0.8 cm internal diameter polypropylene hollow cylinder in which 15 mg cocaine based MIP has been introduced between two polyethylene frits (20 µm pore size) in order to perform the selective clean-up of sample.

The proposed procedure is comprised of four sequential steps: 1) Sampling step: volunteers place the open part A of the plastic cylinder inside the mouth or under the tongue, till the drierite indicator changes its colour from blue to pink (320 ± 30 µg oral fluid is collected); 2) Assembly step: the plastic handle is removed and part A of the device is connected to part B by using an acrylonitrile O-ring and a self-closing plastic washer; 3) Loading/washing step: 4 mL phosphate buffer (0.1 M, pH 7) at an approximate flow of 5 drops per second is passed through the sorbent using a 5 mL single-use prefilled syringe to load cocaine onto the MIP and to wash oral fluid matrix; and 4) Elution step: part A is removed and a plastic threaded fitting with a female Luer connection in one side is connected to part B, to pass 0.3 mL 2-propanol to elute cocaine from the MIP, using a 1 mL single-use prefilled syringe. Then, the obtained extract is directly injected in the IMS or stored at 4°C for a later analysis.

Spiked samples were prepared in three steps, i) sampling of non-user oral fluid, using the part A of the device as explained in the experimental section; ii) direct injection of 10 µL cocaine standard prepared in 2-propanol inside the cotton pad; and iii) storage of part A of the device 24 h at 4 °C, in order to allow the interaction of oral fluid with the spiked cocaine.

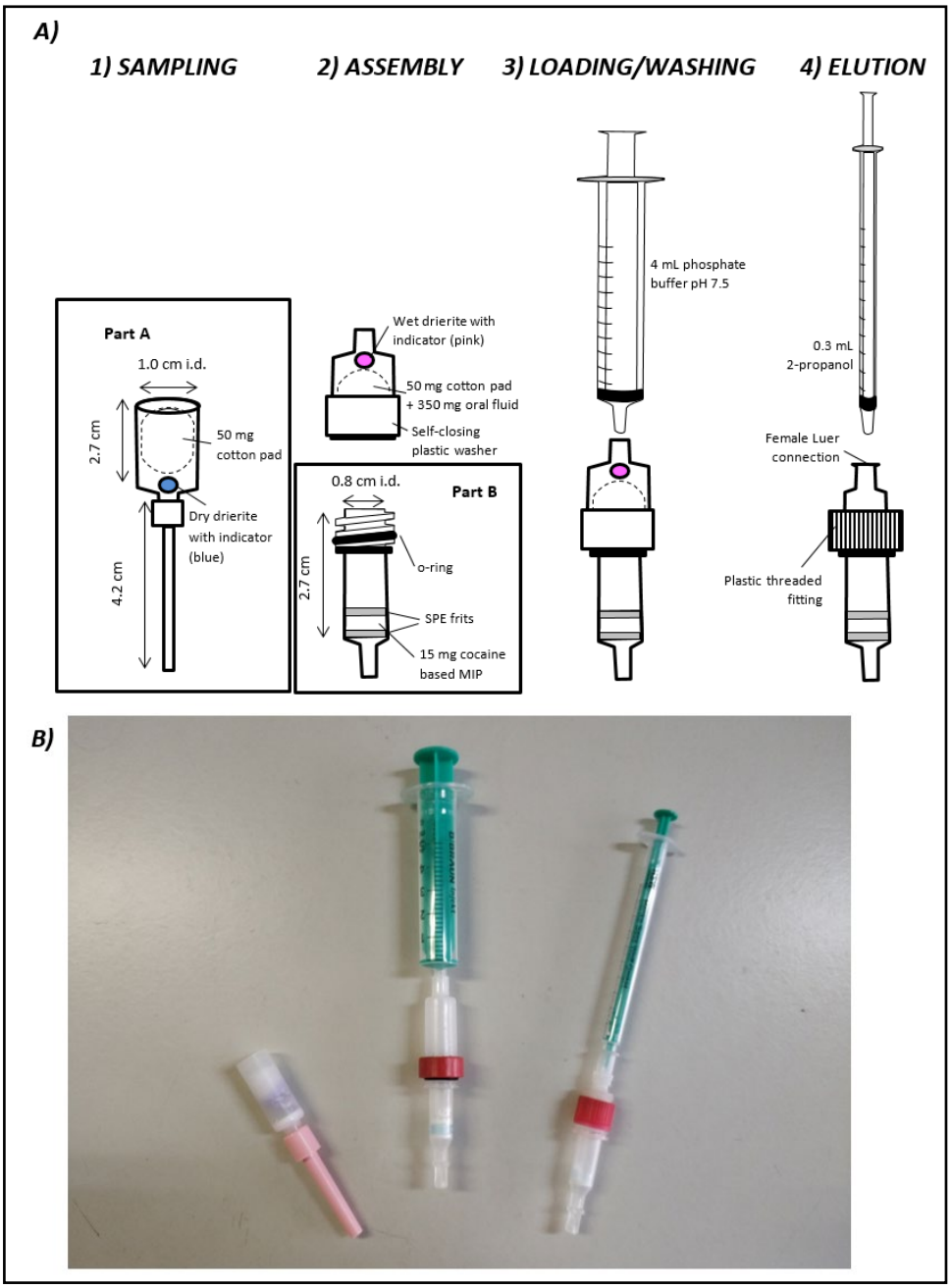

Figure 72. *A) Scheme of the device in the different steps of the extraction procedure. B) Photograph of the whole device in the sampling, loading, and elution steps (from left to right).*

IMS procedure

An IONSCAN-LS from Smiths Detection (Morristown, NJ, USA), equipped with a ^{63}Ni foil radioactive ionization source, was used for cocaine detection of extracts. Plasmagrams were acquired in positive ion mode using nicotinamide as internal calibrant (K_0=1.860 $cm^2\ V^{-1}\ s^{-1}$). Plasmagrams were obtained with a counterflow of dry air, set at 300 mL min^{-1}, a scan period of 40 ms, and a shutter grid width of 0.2 ms. A total drift voltage of 1759 V was applied in the drift tube region (7 cm), being the electric field strength in the drift region 251 V cm^{-1}.

Thermal desorption was used for sample introduction. 3 µL sample extract was dispensed onto a PTFE membrane, whereas desorption, inlet, and drift tube temperatures were adjusted to 260, 270, and 232 °C, respectively. The PTFE membrane remains in the heated zone during 30 s.

Lateral-flow immunoassay reference procedure

The LFA reference methodology employed for the analysis of oral fluid is the Dräger DrugTest 5000 6-Panel Test Kit from Dräger (Madrid, Spain). The instrument uses an opto-electronic analyzer for the qualitative detection of abuse substances, such as cocaine, opiates, amphetamines, methamphetamine, benzodiazepines, and tetrahydrocannabinol in oral fluid. Sample was collected by rubbing the porous collector against the tongue and the inside part of the cheek, until the indicator turns blue. The procedure is followed by placing the test cassette and the buffer cartridge, into the analyser. The assays provided a cut-off concentration of 20 µg L^{-1} cocaine in oral fluid samples.

LLE-GC-MS reference procedure

GC-MS reference method was adapted from previous studies [23,24]. Several drops of 1M $NaHCO_3$:K_2CO_3 buffer were added to 300 µL oral fluid to reach a pH of approximately 9–10. Sample was extracted with 300 µL chloroform, containing 200 µg L^{-1} cocaine-D3, used as internal standard. The mixture was shaken 2 min by vortex and centrifuged for 5 min at 3500 rpm. Chloroform phase was taken and 1 µL of this solution was measured by GC-MS.

An Agilent Technologies 7890A GC system (Palo alto, CA, USA), equipped with a HP-5MS capillary column (30 m x 0.25 mm x 0.25 mm) and an Agilent Technologies 5975C inert XL EI/CI MSD triple axis single quadrupole detector was used for

cocaine determinations of oral fluid extracts. Injection was performed in splitless mode at 250 °C, using 1 mL min^{-1} helium as carrier gas. Oven temperature program was 150 °C for 1 min, up to 250 °C with a rate of 10 °C min^{-1}, and kept for 5 min. Transfer line and ion source temperatures were 300 and 250 °C, respectively. Selected ion monitoring (SIM) was used for MS acquisition with 182.1 and 82.1 m/z ions for cocaine, and 185.1 and 85.1 m/z ions for cocaine-D3. A calibration curve was prepared from 50 to 1000 µg L^{-1} with cocaine standards prepared in chloroform, with a 200 µg L^{-1} fixed concentration of internal standard.

The method was validated in terms of repeatability, linearity, limit of detection, and accuracy (see supplementary material).

Results and discussion

Evaluation of experimental conditions

Previous studies have demonstrated the great performance of cocaine-MIP for cocaine extraction from oral fluid samples. However, those studies have been focused on the analysis in laboratory with specialized instrumentation and trained personnel. Cocaine extraction by SPE procedure [18] described a conventional SPE including typical steps (conditioning, loading, washing, and elution), using a vacuum system and giving a moderate enrichment factor. Likewise, cocaine extraction by magnetic-SPE [19] described a similar four-step process, although the use of magnetic materials allowed a miniaturization of the process and avoided the requirement of vacuum systems, achieving a better sensitivity. The novelty of the proposed procedure consists in the development of a swab sampling device based on a simplified SPE procedure, using the aforementioned synthesized MIP, to be employed as field collector of oral fluid samples for cocaine analysis. The proposed design integrates sampler collector and selective material. Besides, the format of the device provides a reduction of conventional SPE steps to only two (i) transferring of sample from the cotton pad to the MIP sorbent, merging conditioning, loading and washing step; and (ii) the elution of cocaine. Part A of the device, containing cotton pad, must be introduced into the mouth or under the tongue of the user to sample a fixed aliquot of oral fluid. Both steps of the extraction are carried out with the required solutions using pre-filled syringes in order to avoid solvent manipulation by the operator, increasing precision of the procedure and safety of the operator. Thus, the described procedure is a practical application of MIP for the detection of cocaine in field conditions.

The amount of oral fluid sampled is directly related to the amount of cotton in the part A of the device. Thus part A of the device was filled with 25, 50, 100, and 200 mg cotton, and the amount of oral fluid sampled was calculated by weighing the device, being 175 ± 18, 320 ± 30, 620 ± 40, and 1340 ± 70 µL, respectively, by considering 1 g mL^{-1} as the average density of oral fluid, and using oral fluid from different volunteers (n=10). In order to select the most appropriate amount of cotton in the device, blank oral fluid spiked with 300 µg L^{-1} cocaine were loaded onto the part A of the device filled with 25, 50, 100, and 200 mg cotton, respectively. 4 mL phosphate buffer (0.1 M, pH 7) at an approximate flow of 5 drops per second was passed through the cotton, loading cocaine onto the MIP. Finally, 0.3 mL 2-propanol eluted cocaine from the MIP, and the obtained extract was injected in the IMS to calculate cocaine recovery. Cocaine recovery decreased with an increase of the cotton amount from 68±3 to 7±2 %. Thus, 50 mg cotton was used in the part A of the device to increase recovery of cocaine and sensitivity of the methodology.

Different transferring solutions were evaluated to transfer the oral fluid from the cotton to the MIP (from part A to part B). The pH of oral fluid typically ranges from slightly acidic to slightly basic media, with pH values from 5.5 to 7.9 [25]. The effect of transferring solution pH on the MIP extraction of cocaine was evaluated using 5 mL of different 0.1 M buffer solutions, with pH values from pH 3.0 to 12.0, to transfer a blank oral fluid spiked with 300 µg L^{-1} cocaine. Figure 73A shows the obtained results, achieving the maximum cocaine recovery of 52.6 ± 6 % at pH 7.0, and it decreased with the acidity and basicity of the transferring solution. It is well-known that using MAA as monomer, there is an adverse effect, under mild acidic conditions, on binding of the target molecules to the MIP layer [26]. This can be explained by the protonation of the MAA (pK_a value of 4.7) [27] and cocaine (pK_a value of 8.6) [28] and the need of a hydrogen bond donor and an acceptor to form hydrogen bonding between cocaine and the MIP surface. Below pH 4.7, MAA is mostly in the neutral form, and cocaine is remained in its protonated form, being both hydrogen donors. Between pH 4.7 and 8.6, MAA would act as acceptor, in the deprotonated state, while cocaine would be hydrogen donor, in the protonated state. From this finding, we might infer that retention of cocaine by MIP can be explained by electrostatic interaction as well as by hydrogen bonding between nitrogen from cocaine and carboxylic acid group of the MAA, To ensure that both, monomer and analyte are in the appropriate form, oral fluid sample was transferred from the cotton to the MIP using a pH 7.0 phosphate buffer solution.

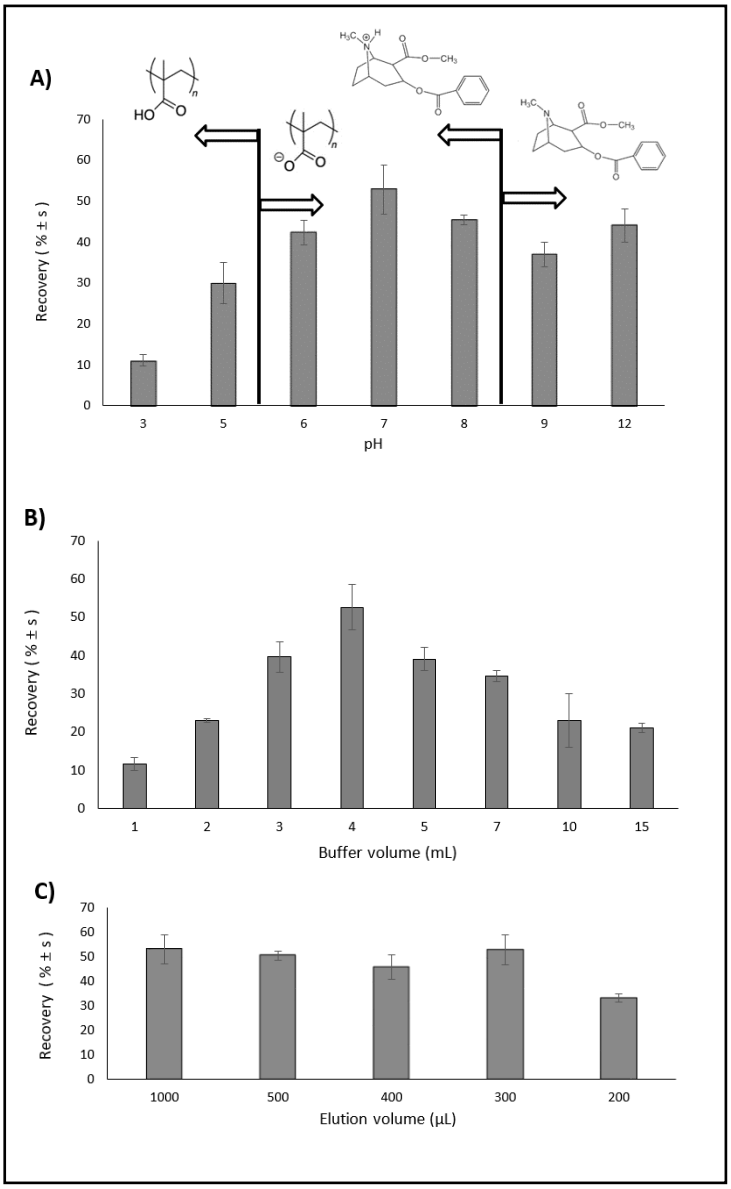

Figure 73. *Evaluation of different extraction conditions using an oral fluid sample spiked at 300 µg L^{-1}: A) 0.1 M phosphate buffer at several pH values used as transferring solution, B) phosphate buffer (0.1 M, pH 7) volume used for transferring, and C) 2-propanol volume used for elution of cocaine.*

The effect of the volume of transferring solution (from 1 to 15 mL phosphate buffer 0.1M at pH 7.0) on the cocaine recovery was also evaluated by using a blank oral fluid sample spiked with cocaine at 300 μg L^{-1}. Figure 73B shows that with increasing volume of transferring solution up to 4 mL, an increase in the cocaine retention in the MIP was observed reaching a maximum value of 53 ± 6 %. However, the use of volumes higher than 5 mL caused a partial elution of cocaine from the MIP, giving recoveries lower than 40 %. Thus, 4 mL transferring solution was employed for further studies.

The elution of cocaine retained in the MIP was carried out using 2-propanol, based on the results of previous studies [18,19]. Elution solvent volumes ranging from 0.2 to 1.0 mL 2-propanol were tested using blank oral fluid samples spiked with 300 μg L^{-1} cocaine in order to obtain the best enrichment factor. Figure 73C shows that the obtained cocaine recovery was nearly constant with values *ca*. 50 % using elution volumes from 0.3 to 1.0 mL and it decreased to 33.2 ± 1.8 % using 0.2 mL. Thus, 0.3 mL of 2-propanol was selected as the most appropriate elution volume. Considering the amount of sample taken by the device (320 μL), the resulting enrichment factor was 1.07.

The cocaine recovery of the whole process for cocaine determination in oral fluid using the proposed sample collector-MIP approach was 53 %. The main losses were due to an irreversible retention of cocaine in the cotton pad (24 %), calculated by the direct MIP extraction of a cocaine standard prepared in 4 mL phosphate buffer (0.1 M, pH 7), and to the non-optimal SPE conditions employed (23 %).

Method performance validation

The proposed procedure has been validated in terms of linearity, precision, limits of detection (LOD) and quantification (LOQ), sensitivity, and specificity. A summary with the validation parameters for the determination of cocaine in oral fluid by the developed device are summarized in Table 43. Calibration curve was at six concentration levels, from 6 to 400 μg L^{-1} cocaine prepared in 2-propanol. Linearity of the IMS procedure was evaluated as the determination coefficient (r^2) with an obtained value higher than 0.99. Precision of the method was 5.1 %, evaluated as the relative standard deviation of a 10 μg L^{-1} cocaine standard (n=10). LOD and LOQ values were calculated as three and ten times the standard deviation of the intercept divided by the slope of the calibration curve, obtaining values of 3 and 10 μg L^{-1}, respectively. These values were obtained by considering the

enrichment factor of the method (1.07) and an average extraction recovery of 53 %.

Table 43. *Analytical features of the developed sensor for cocaine determination in oral fluids.*

Parameter	Value
Linear range	6 – 400 μg L^{-1}
Determination coefficient (r^2)	0.997
Cut-off value	20 μg L^{-1}
Relative standard deviation	5.1 %
True-positive detection (n = 20)	100 %
True-negative detection (n = 20)	95 %
Maximum loading capacity	130 mg L^{-1}

The cut-off value of the methodology was determined as the lowest concentration of cocaine for which the developed method is able to correctly classify samples as positives or negatives. To obtain the cut-off value of the proposed method, 20 blank oral fluid samples collected immediately after consumption of several potential interfering compounds (such as coffee, sweets, tobacco and/or pharmaceuticals) were analysed by the recommended procedure with and without cocaine spiked at 5, 10, and 20 μg L^{-1} concentration levels. Sensitivity of the device, defined as the proportion of true drug-positive cases correctly identified, was calculated at the three different concentration levels. Percentages of true-positive samples correctly identified were 50, 65, and 100 % for 5, 10, and 20 μg L^{-1}, respectively. Specificity, defined as the proportion of true drug-negative cases correctly identified by the device was evaluated by analysing the same 20 blank oral fluid samples. Percentage of true-negative samples correctly identified was 95 %, being the false alarm rate of 5 %. In summary, considering the rate of true-positive and true-negative samples at the three different concentration values, a cut-off of 20 μg L^{-1} was established for the developed device.

Maximum loading capacity of the device was evaluated by the analysis of a blank oral fluid sample spiked with 500 mg L^{-1} cocaine, using the proposed procedure, in order to saturate the specific recognition sites of MIP. The amount of cocaine extracted was 42 μg, analysed after the appropriate dilution of the extract to fit within the linear calibration range of IMS. Thus, the maximum concentration of cocaine in the sample that can be extracted by the device was 130 mg L^{-1}.

To evaluate the behaviour of the proposed method against different concentrations of cocaine in oral fluid, blank oral fluid samples (n = 3) were spiked

at 0.05, 0.1, 0.2, 0.4 μg L^{-1} and 52 mg L^{-1}, obtaining recoveries of 45.3 ± 1.8, 53 ± 6, 46 ± 4, 47 ± 3 and 41 ± 4%, respectively.

Stability of samples

It has been previously reported that cocaine hydrolyses to benzoylecgonine in aqueous solutions or biological fluids at pH values higher than 5.5 [29]. In that study, it was found that unstimulated oral fluid specimens can be stored in various containers for as long as four days refrigerated.

A study was carried out to evaluate the stability of samples in the developed collector device. Thus, 8 blank oral fluid samples were collected with part A of the device, spiked at cut-off concentration level (20 μg L^{-1} cocaine), and transferred to the MIP of the part B of the device. Samples were stored in part B of the device at room temperature, and two replicates were eluted and analysed by IMS every 7 days. In all cases, the peak area obtained was practically the same (RSD < 15 %), along the duration of the experiment (a total time of 28 days), which suggests that no degradation exists when cocaine-positive samples were stored into the MIP. -401-

Analysis of cocaine users oral fluid

Twenty volunteers coming from a recreational environment were submitted to analysis after cocaine abuse. Individuals, males and females with ages ranging from 19 to 38 years old, declared the approximated time passed after sniffing of a non-controlled cocaine dose, and provided two oral fluid aliquots, one collected using the part A of the proposed device for MIP-IMS analysis, and the second by spitting in an Eppendorf vial for LFA and LLE-GC-MS reference analysis. Table 44 shows the information of the anonymous volunteers and the found concentrations of cocaine in oral fluid samples analysed by the proposed MIP-IMS method and those obtained with reference protocols. Those samples that exceed the linear range were properly diluted after extraction and re-analysed by the instrumental technique. As it can be seen, the concentration of cocaine in oral fluid directly depends on the time from the abuse, with cocaine concentrations ranging from 0.19 to 78.6 mg L^{-1}, obtained by the proposed MIP-IMS method. Figure 74 shows IMS plasmagrams corresponding to the samples 7 and 10 (plasmagrams C, D E and F), where the 100x represent the zoom made. As it can be seen, MIP extraction is able to remove matrix components and allows the determination of cocaine.

In parallel, after sample collection by the proposed methodology, oral fluid of all the volunteers were analysed by Dräger DrugTest 5000 (with a cut-off value of 20 µg L^{-1}). This study was done to verify the positive results of cocaine as well as to investigate the possible presence of other illicit substances that can play as interfering compounds. As it can be seen in Table 44, all the samples analysed were positive by the LFA, which was in accordance to MIP-IMS procedure. It should be highlighted that no sample showed positive results for other illicit substances than cocaine identified by the Dräger DrugTest 5000 (amphetamines, benzodiazepines, Δ^9-tetrahydrocannabinol, methamphetamines, and opiates). Moreover, ten oral fluid samples were also analysed by a reference LLE-GC-MS procedure in order to evaluate method agreement. Table 44 shows the obtained results, which ranged from 0.17 to 54.6 µg L^{-1}. As it can be seen, the results provided by the developed method, after recalculation taking into consideration the recoveries of the procedure, are similar to those reported by the GC-MS reference method.

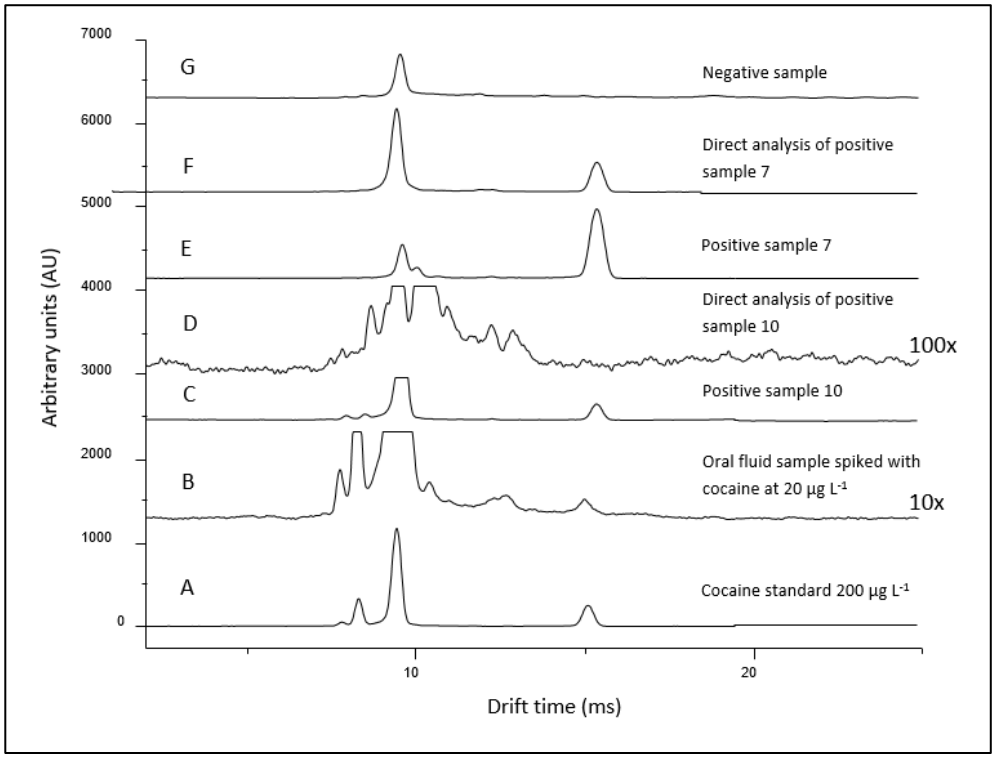

Figure 74. *IMS plasmagrams obtained for a (A) 200 µg L^{-1} cocaine standard, (B) oral fluid sample spiked at 20 µg L^{-1}, (C, D, E and F) two different positive oral fluid samples analysed directly and after extraction, and a (G) blank oral-fluid sample after extraction*

Table 44. *Information about cocaine users under study, approximate time declared after cocaine abuse, and results of sampled oral fluids analyzed by lateral flow immunoassay (LFA), reference chromatography procedure (LLE-GC-MS), and the proposed procedure by MIP-IMS.*

User	Age	Gender	Time (min)	LFA (n = 1)	[Found] (mg L^{-1} ± s, n = 2)	
					LLE-GC-MS	MIP-IMS
1	26	Male	20	Positive	-[a]	23.9 ± 1.7
2	19	Male	40	Positive	-[a]	0.626 ± 0.002
3	25	Female	120	Positive	-[a]	0.327 ± 0.003
4	24	Male	15	Positive	-[a]	18 ± 3
5	20	Male	30	Positive	-[a]	0.24 ± 0.04
6	22	Female	90	Positive	-[a]	0.417 ± 0.010
7	26	Male	15	Positive	-[a]	78.6 ± 0.2
8	26	Male	15	Positive	-[a]	43.4 ± 1.3
9	24	Female	180	Positive	-[a]	0.1940 ± 0.0012
10	25	Male	90	Positive	-[a]	0.296 ± 0.018
11	24	Female	120	Positive	0.171 ± 0.009	0.4 ± 0.02
12	23	Female	20	Positive	9.4 ± 0.5	9.4 ± 0.2
13	38	Male	15	Positive	23.8 ± 1.2	22.7 ± 1.2
14	25	Female	15	Positive	31.6 ± 1.4	34 ± 2
15	31	Male	15	Positive	43.7 ± 1.9	63 ± 2
16	28	Male	30	Positive	8.6 ± 0.4	12.3 ± 0.6
17	24	Male	20	Positive	8.8 ± 0.4	10.0 ± 1.0
18	26	Female	45	Positive	1.70 ± 0.08	1.810 ± 0.019
19	25	Male	60	Positive	1.04 ± 0.05	1.75 ± 0.12
20	32	Female	15	Positive	54.6 ± 2.7	78 ± 2

[a] *Not measured*

Conclusions

A methodology has been developed and validated for the determination of cocaine in oral fluid for IMS determination, using a field sample collector specifically designed for the sampling of a fixed amount of oral fluid and its later extraction in a MIP sorbent.

The developed sampling device is the first of this type that includes oral fluid sampling with a cotton swab and sample treatment with in-house developed MIP sorbent. Moreover, the obtained extract can be directly analyzed by the most usual analytical instrumentation such as LC-MS, GC-MS, MS (portable or benchtop) and IMS (benchtop, portable or handheld) among others. This sampling step can be

completely performed in the field by non-expert operators, and samples can be stored onto the MIP sorbent at room temperature for at least 4 weeks.

IMS was proposed for the determination of cocaine in the extract, due to the high sensitivity, moderate selectivity, and speed of the analysis. This study was carried out on a bench-top IMS in the laboratory. However, it can be considered a semiportable instrument able to carry out screening studies in the field. IMS instrument is stable enough to be used in a desktop, table or any horizontal surface being its power requirements 100-240V AC, 50-60Hz, but it can be operated with an external Lithium-ion battery kit.

Extraction conditions were modified from previous studies [18] to provide a procedure as simple as possible by reducing the number of SPE steps and using pre-filled syringes that contained the required solutions, in order to make the procedures fast and accessible to non-expert personnel. The evaluation of instrumental parameters offered a LOD and LOQ of 3 and 10 μg L^{-1}, respectively, and a cut-off value of 20 μg L^{-1} (identification of 100 % true-positive and 95 % true-negative samples). Loading capacity of MIP allowed the determination of cocaine in oral fluid samples until 130 mg L^{-1}. The performance of the proposed MIP-IMS method has been evaluated by the analysis of oral fluid samples, obtained from cocaine users, measured also by two reference procedures with comparable results.

The total analysis time is lower than 5 min, including sample collection, transfer to the MIP, and IMS measurement, which is substantially shorter than that of commercial LFA sensors (10–15 min). Production cost of reagents and consumable materials used to prepare MIP-based device are more available and significantly cheaper than antibody-based devices. Furthermore, the template can be reused after the washing step during MIP preparation, in order to reduce costs of large scale production of MIPs [30]. All these features, combined with the moderate price of portable or handheld IMS equipments, provide an appropriate alternative to actual cocaine sensors. In this sense, future studies are focussed on the use of blended specific MIPs for the analysis of different drugs or even the production of a multi-template MIP for the simultaneous analysis of several drug families.

Conflict of interest disclosure

The authors declare that have no competing financial interests in this investigation.

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Supplementary material

LLE-GC-MS validation

The GC-MS procedure has been validated in terms of linearity, precision, limits of detection (LOD) and quantification (LOQ), and accuracy. A calibration curve was prepared in chloroform at six concentration levels, from 50 to 1000 µg L-1 cocaine. Linearity of the procedure was evaluated as the determination coefficient (r2) with an obtained value higher than 0.999. Precision of the method was lower than 2 %, evaluated as the relative standard deviation of a 200 µg L-1 cocaine standard (n=10). Instrumental LOD and LOQ values were calculated as three and ten times the standard deviation of the intercept divided by the slope of the calibration curve, obtaining values of 17 and 50 µg L−1, respectively.

To evaluate the behaviour of the proposed method against different concentrations of cocaine in oral fluid, blank oral fluid samples were spiked at 0.175, 0.5, 0.75 and 50 mg L−1, obtaining recoveries of 82 ± 5, 93 ± 2, 97 ± 2 and 89 ± 10 %, respectively.

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Magnetic molecularly imprinted polymers for the selective determination of cocaine by ion mobility spectrometry

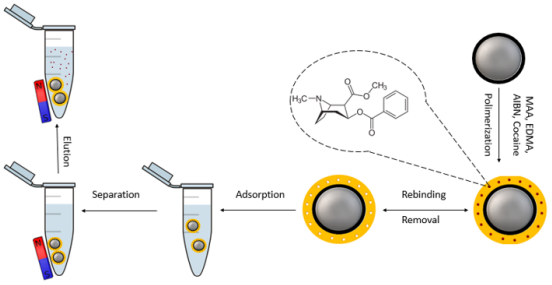
Aitor Sorribes-Soriano, Francesc Albert Esteve-Turrillas, Sergio Armenta, Ana Montoya, José Manuel Herrero-Martínez, Miguel de la Guardia

Department of Analytical Chemistry, University of Valencia, 50th Dr. Moliner St., 46100 Burjassot, Spain

Abstract

Magnetic molecularly imprinted polymers (MMIPs) were prepared for cocaine recognition by bulk polymerization in the presence of magnetic nanoparticles (MNPs). Two reagents (polyethylene glycol (PEG) and 3-(trimethoxysilyl)propyl methacrylate (V)) were used for MNPs modification. MMIPs were characterized and compared in terms of loading capacity, reusability, accuracy and precision for the extraction of cocaine from saliva samples. It was observed that V-MMIPs gave higher physical stability than PEG-MMIPs. Thus, V-MMIP were used for the analysis of cocaine users saliva. The developed procedure based on ion mobility spectrometry (IMS) provided limits of detection and quantification of 4 and 14 μg L^{-1}, respectively, and recoveries in cocaine free saliva samples spiked at 80, 270 and 560 μg L^{-1} ranging from 80 to 99 %. Results found by the proposed method were statistically comparable to those obtained by two reference procedures; a lateral flow immunoassay and an ultra-high performance liquid chromatography coupled with tandem mass spectrometry. Therefore, MMIP-IMS can be considered as a fast, selective and sensitive alternative to reference methods with affordable cost avoiding the requirement of skilled operator.

Graphical abstract



Keywords *Cocaine, ion mobility spectrometry, magnetic nanoparticles, magnetic molecularly imprinted polymer, saliva*

Introduction

Cocaine is an alkaloid obtained from coca leaves, being the most popular abuse drug in Europe after cannabis. Cocaine is a potent central nervous system stimulant that induces a state of high alertness and euphoria [1]. According to the United Nations Office on Drugs and Crime, in 2015 the coca brush cultivate was increased (mainly in Colombia), reaching 1.125 tones worldwide [2].

Cocaine doses range from 0.2 to 4 mg Kg^{-1} being rapidly absorbed producing their effects in 30 s after nasal or intravenous administration, and only 5 s if it is smoked [3,4]. Blood and urine are the most employed biological matrices analyzed to detect cocaine consumption [5]. However, they are invasive sampling techniques in which metabolites are present instead of the parent compound. Thus, alternative biological fluids are preferred for abuse drugs screening. In this sense, the analysis of cocaine in oral fluids is not considered as invasive sampling [6] and its main advantages are the ease to sampling and the presence of unmetabolized cocaine [7]. Most cocaine is metabolized and excreted in 12-24 h after consume, but a prolonged occurrence of cocaine in saliva and urine was observed in chronic cocaine addicts for 5-10 days during abstinence [8]. Cocaine can be detected in saliva till 2-10 days after consumption [9]; consequently, it is an appropriate matrix to detect cocaine consumption.

Usually, quantitative methods to analyze drugs have been performed by gas chromatography - mass spectrometry (GC-MS) [10], liquid chromatography - mass spectrometry (LC-MS) [11] or liquid chromatography with ultraviolet detection (LC/UV) [12]. A promising alternative for the analysis of cocaine is ion mobility spectrometry (IMS), which is based on the gas phase separation of ionized compounds under an electric field at ambient pressure. IMS provides a robust, fast and sensitive analysis of cocaine with additional advantages like ease of use, a relatively low cost of instrumentation and a minimal required maintenance [13]. However, IMS presents a reduced selectivity, being observed interferences and matrix effects in the analysis of complex samples such as saliva. This drawback can be reduced or avoided by the use of a selective/specific sample treatment such as: immunosorbents [14] or molecular imprinted polymers (MIPs) [15].

Cocaine extraction from biological matrices is usually carried out by liquid-liquid extraction (LLE) [16], solid-phase microextraction (SPME) [17] and mainly solid-phase extraction (SPE) [18]. Conventional SPE provides some disadvantages such as long extraction times, requires the use of vacuum manifolds, high solvent consumption and the need of a high amount of sample.

The use of MIPs for the selective extraction of cocaine from biological fluids has been previously proposed as an alternative to conventional sorbents in SPE [19,20]. Indeed, alternative extraction methods based on porous membrane protected MIPs for micro-SPE have been developed to avoid the aforementioned drawbacks of conventional methodologies for the analysis of cocaine in plasma [21] and urine [22]. In both cases, MIP beads (50 mg) were loaded inside a cone-shaped device made of a polypropylene membrane. Other MIP based approaches for the selective extraction of cocaine are based on paper spray MS/MS, where the paper was modified with MIPs to create a specific site for cocaine analysis in oral fluid [23] and a fluorescence sensor for cocaine determination prepared by anchoring a selective MIP for cocaine on the surface of polyethylene glycol (PEG) modified Mn-doped ZnS quantum dots (QDs) [24].

Dispersive SPE reduces extraction times but it needs centrifugation or filtration steps to separate the solid phase from the liquid sample [25]. Recently, the development of dispersive magnetic SPE methods allows the separation of the solid phase by a magnet, providing satisfactory benefits as ease of use, quickness and a deep reduction in the required sample amount and solvent consumption due to the miniaturization of the procedure. In this sense, magnetic MIPs (MMIPs) have been used for the selective extraction of triazines in environmental samples [26] rhodamine B in wine [27], estrogens in meat and fish [28], and cocaine in urine and plasma [29, 30].

Magnetic composites are commonly prepared by embedding magnetic nanoparticles (MNPs) (commonly based on Fe_3O_4 core) within a polymer. Before MIP synthesis, magnetite is usually surface-modified with several reagents such as oleic acid [26,27,31], PEG [26,29,30] or silanizing agents [32,33,34], among others. In particular, the PEG coating, based on a non-covalent interaction between Fe3O4 surface and hydroxyl groups of PEG [35], has been used to prevent MNPs agglomeration and to make them biocompatible. In fact, PEGylated MNPs have been used in the preparation of MMIPs for cocaine extraction [29, 30]. However, the presence of weak electrostatic interactions between PEG coating and polymer could lead to a release of polymeric material from the magnetic beads after several uses.

On the other hand, silanizing agents have been studied as a covalent anchor group suitable for modifying MNPs [36]. Besides, the use of certain silane agents (*e.g.* 3-methacryloxypropyltrimethoxysilane, V-MPS) provides a terminal double bond, which can react with other vinylic monomers during the MIP polymerization

procedure. It ensures homogeneous embedding of MNPs [36] and produces resistant and stable materials.

In spite of the use of modified MNPs in MIP preparation, there is little research about the effect of MNP modifier used, its content in the polymeric matrix and its influence on the extraction performance of magnetic MIPs. To our knowledge, there is not any precedent of the application of MMIPs for the analysis of cocaine in saliva samples.

The main aim of this study is the synthesis of MMIPs for the fast extraction of cocaine from oral fluid samples to be analyzed by IMS. In order to achieve a better understanding of the important role of modified MNPs in the extraction properties of magnetic composites, two types of MNPs (coated with PEG or V–MPS) were prepared before its incorporation into the polymerization mixture for preparation of MIP. The resulting MMIPs (namely, PEG-MMIPs and V-MMIPs, respectively) were produced and their analytical features compared in terms of sensitivity, loading capacity, and reusability. Field saliva samples, obtained from cocaine consumers, were also analyzed by the proposed method and results found were compared to reference methodologies.

The innovative character of the present study is based on the high versatility and molecular recognition capabilities of MIPs in combination with IMS determination and the improvement of the preconcentration and separation steps through the use of MMIPS to provide a simple and fast analytical procedure for the analysis of trace level compounds in complex biological samples.

Experimental

Material, reagents and samples

Cocaine standard was kindly provided by the "Unidad de Inspección de Farmacia y Control de Drogas" from Valencia Health Service Area. Cocaine standard solutions were prepared in methanol, containing 5 % (v/v) acetic acid, and kept at -20 °C in amber glass vials. All the solvents used in this study were HPLC grade or higher. Organic solvents and buffer constituents were obtained from Scharlab (Barcelona, Spain, www.scharlab.com). Methacrylic acid (MAA), ethylene glycol dimethacrylate (EDMA), azobisisobutyronitrile (AIBN) and V-MPS were provided by Sigma (Steinheim, Germany, www.sigmaaldrich.com). MagRack™ 6 magnetic rack for 1.5 mL microcentrifuge tubes and PEG 6000 were provided by Merck KGaA (Darmstadt, Germany, www.sigmaaldrich.com). Iron (III) chloride 6-hydrate and

iron (II) chloride 4-hydrate were provided by Panreac (Barcelona, Spain, www.panreac.es).

Oral fluid samples were obtained from cocaine users who provided their consent after appropriate information. Cocaine consumers were males with ages ranging from 19 to 30 who have abused between 1 or 3 h before sampling. Samples were collected and stored at -4 °C until their analysis. Cocaine-free saliva samples were collected from males and females with ages ranging from 22 to 25. It should be highlighted that under no circumstances the authors have trafficked or provided illegal substances, aimed, promoted, facilitated, or forced any way the consuming of illegal substances.

Preparation of bare and coated MNPs

The synthesis of MNPs was conducted by co-precipitation according to the previous work [33, 37]. Briefly, the MNPs were prepared by mixing 1.11 g of $FeCl_2 \cdot 4H_2O$ and 3.03 g of $FeCl_3 \cdot 6H_2O$, with 180 mL water in a reflux system, setting the temperature at 50 °C. When temperature was reached, 12.5 mL of ammonia (32 %) were added under vigorous stirring. After 30 min reaction time, the temperature was then increased until 90 °C and stirred for additional 30 min. All synthesis steps were carried out under N_2 atmosphere. The obtained precipitate was separated using an external magnet, and it was washed with water and ethanol. Finally, the MNPs were dried for 12 h at 80 °C.

The MNPs coated with PEG were produced, by mixing 1 g of MNPs and 5 g of PEG 6000 in 15 mL water. The mixture was sonicated for 20 min, and centrifuged at 3000 rpm for 20 min. The obtained precipitate was dried at 60 °C during 12 h [29,30].

To obtain the MNPs coated with vinyl groups (V-MNPs), 50 mg of nanoparticles were mixed with 4 mL of V-MPS in a water:ethanol (1:1, v/v) solution. The mixture was held at 40 °C in a thermal bath for 12 h under N_2 atmosphere. The obtained V-MNPs were washed with ethanol and dried at 60 °C for 12 h [36].

Preparation of MMIPs

MMIPs were synthesized by bulk polymerization as follows. A mixture of cocaine (template), MAA (monomer), EDMA (cross-linker), AIBN (initiator) and modified MNPs was prepared in acetonitrile (porogen). The molar ratio

template/monomer/cross-linker was 1:4:20 [37], and different amounts of MNPs were added to the polymerization mixture to give a final content ranging from 0.5 to 2 wt%.The slurry was sonicated for 5 min and purged with nitrogen for additional 10 min. The mixture was placed in a vial and shaken during 20 h using a twister. After that, the vial was put in a thermal bath at 60 °C during 24 h under magnetic stirring mode until the synthesis was finished. After polymerization, the MMIPs were crushed in an agata mortar, dried at 80 °C overnight, and sieved with a 425 µm steel sieve in order to obtain an homogeneous particle size. MMIP particles were treated with a mixture of 10 % (v/v) acetic acid in methanol in a Soxhlet extractor for 24 h to remove the template from the polymer. After that, the particles were rinsed again with 10 % (v/v) acetic acid in methanol and analyzed by IMS to check the absence of cocaine. The procedure adopted for the preparation of MMIPs is illustrated in Figure 75. Magnetic non-imprinted polymers (MNIPs) were also prepared as above but in the absence of cocaine in the polymerization mixture to evaluate unspecific interactions of the polymer.

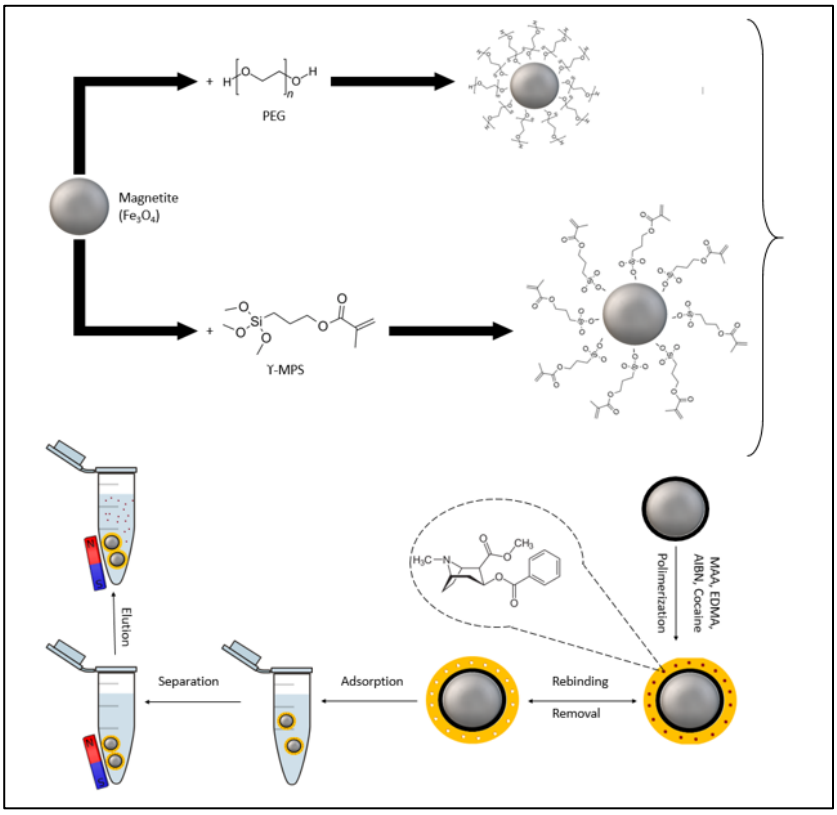


Figure 75. *Schematic diagram for the preparation of MMIPs using MNPs modified with PEG and V-MPS.*

Characterization of magnetic materials

Images of the MNPs were taken by a JEM-1010 transmission electron microscope from JEOL (Ibaraki, Japan) with a digital camera AMT RX80 (8Mpx), and the surface analysis of magnetic composite materials was performed by a S-4800 scanning electron microscope from Hitachi (Ibaraki, Japan) provided with a field emission gun and a back secondary electron detector. EMIP 3.0 image data acquisition system was obtained from Rontec (Normanton, UK).

Magnetic properties were measured with a Quantum Design MPMS-XL 5 SQUID magnetometer. Vibrating sample magnetometry (VSM) data were taken at 300 K in the range from −15,000 Oe (−1.5 T) to +15,000 Oe (+1.5 T). The magnetization saturation value (M_{sat}) was the maximum magnetization observed and was taken at 15,000 Oe.

Extraction procedure

The procedure for cocaine extraction from saliva samples was adapted from that of Sorribes-Soriano et al. [37]. Five milligrams of each polymer (PEG-MMIP, V-MMIP, PEG-MNIP or V-MNIP) were introduced inside 1 mL glass vial and were equilibrated with 1 mL of 5 % (v/v) acetic acid in methanol and 0.5 mL of deionized water. Saliva samples (500 µL), diluted with 100 µL 0.3 M ammonium/ammonia buffer pH 9, were added to the MMIPs and stirred for 10 min. After that, the MMIPs were washed during 1 minute with 500 µL deionized water and 500 µL chloroform. Finally, retained cocaine was eluted with 0.2 mL of 5 % (v/v) acetic acid in methanol for 5 min and directly measured by IMS.

IMS procedure

An IOSCAN-LS from Smiths Detection (Morristown, NJ, USA) equipped with a ^{63}Ni foil radioactive ionization source was used for the identification and determination of cocaine. The software used for data acquisition was IM station software (version 5.389). Plasmagrams were acquired in positive ion mode using nicotinamide, with a reduced mobility (K_0) of 1.860 $cm^2\ V^{-1}\ s^{-1}$, as internal calibrant. The number of segments per analysis was 56, and each plasmagram contained 779 data points. The shutter grid width was 0.2 s (the value optimized by the manufacturer) and the scan period used to collect plasmagrams was 40 ms. A counterflow of dry air, set at 300 mL min^{-1}, was introduced as drift gas at the end

of the drift region. The electric field strength in the drift region was 251 V cm^{-1} with a total drift voltage of 1759 V and a drift tube length of 7 cm.

Sample introduction was based on thermal desorption from a polytetrafluoroethylene (PTFE) membrane. The volume of sample was 8 µL which was placed onto the PTFE membrane and heated to vaporize the analyte for 60 s, using a 1 s post-dispense delay. Desorption, inlet and drift tube temperature was adjusted at 260, 270 and 232 °C, respectively.

An alarm was configured in the IMS instrument to alert the presence of cocaine in the analyzed samples, using the following peak descriptors: (i) a K_0 value of 1.16, (ii) a 50 µs peak drift time variability, (iii) a 1.5 peak amplitude, (iv) a 20 signal threshold, and (v) a 200 µs full width at the half-maximum amplitude of the peak.

Lateral-flow immunoassay reference procedure

A DrugTest 5000 from Dräger (Madrid, Spain) was employed for the lateral-flow immunoassay screening of cocaine in saliva samples. A porous collector was placed into the saliva sample until the indicator turned blue. Then, the cassette, which contains the porous collector and the buffer cartridge, was introduced in the DrugTest 5000 analyzer. The procedure provided a cut-off concentration of 20 µg L^{-1} cocaine in oral fluid samples and involved 9 min of analysis time.

UHPLC-MS-MS reference procedure

Ultra-high performance liquid chromatography (UHPLC) coupled to a triple quadrupole mass spectrometer (MS) was also employed as reference methodology for the analysis of cocaine in field oral samples. UHPLC was performed on a Waters Acquity UPLC System from Waters (Milford, MA, USA), equipped with a binary solvent delivery system, an autosampler and a BEH C18 (1.7 µm, 2.1 × 50 mm) column. An injection volume of 5 µL was employed. The mobile phase consisted of (A) 0.1% (v/v) formic acid in water and (B) methanol. The gradient started at 5% of B, and it was linearly increased to 95% in 4 min, and then kept 1 min at a flow rate of 0.4 mL min^{-1}. The retention time found for cocaine was 2.77 min. MS acquisitions were performed in a Waters Acquity triple quadrupole mass spectrometry detector, equipped with a Z-spray electrospray ionization source (ESI), with 3.5 kV capillary voltage, 120 °C source temperature and 350 °C desolvation temperature. Cocaine determination was performed in ESI+ mode using 304 m/z as parent ion and 182 and 82 m/z as daughter ions.

Quality control and quality assurance (QC/QA)

The application of QA/QC procedures has been carried out in order to provide accurate, precise, and traceable results. Saliva blanks, from non-consumer volunteers, were analyzed in order to check the absence of cocaine interferences during the saliva sampling, transport, storage, and analytical determination. Moreover, several water blanks were treated and analyzed in the same way than samples to check the absence of any potential release of cocaine template from the MIP.

Trueness of the methodology was evaluated by comparison of the results of cocaine determination in field samples by the proposed method and two reference procedures, an screening method based on the direct determination of saliva by a lateral flow immunoassay, and a quantitative method based on the analysis of the obtained V-MMIP extract by UHPLC-MS-MS. A paired *t*-test for comparison of two analytical methodologies using Eq. 1 has been applied:

$$t_{calc} = \frac{d\sqrt{n}}{sd} \qquad (1)$$

where d is the average of the differences between the results of each method, n the number of analyzed samples and sd the standard deviation of the differences [38]. This test was performed using Excel spreadsheet software.

Moreover, trueness of the method was evaluated by the determination of recoveries of a blank saliva sample spiked at 80, 270 and 560 μg L^{-1} cocaine extracted by the proposed procedure by using PEG-MMIPs and V-MMIPs.

Repeatability and reproducibility of the proposed procedure was evaluated in terms of relative standard deviation (RSD) at different cocaine concentration levels. The obtained precision for cocaine determination of saliva samples was assessed with a blank saliva sample spiked with cocaine at different concentrations and analyzed by the recommended procedure using the proposed protocol.

Limits of detection (LOD) and quantification (LOQ) were calculated as three and ten times, respectively, the standard deviation of the intercept divided by the slope of the calibration curve.

Evaluation of method greenness

The proposed MMIP-IMS procedure and the reference methodologies have been evaluated in terms of Green Analytical Chemistry using the previously

proposed Green Certificate [39]. The greenness of our analytical method was assessed as a function of the amounts of reagents and wastes by assignation of penalty points determined by mathematical expressions. This Green Certificate classifies the analytical methods onto a scale from A to G, taking into account also the energy consumption.

Results and discussion

MMIPs preparation and characterization

In a previous work, MIPs were prepared using MAA as monomer, cocaine as template, EDMA as cross-linker, AIBN as initiator and acetonitrile as porogen, showing an excellent selectivity for the analysis of cocaine in oral fluids in front another substances such as diazepam, nicotine, benzocaine, doxylamine succinate, codeine, diphenhydramine chlorhydrate and the main metabolites of the cocaine [37]. In this case, the synthesis of magnetic MIPs is proposed in order to reduce the time and sophistication of the analysis, and increase the preconcentration factors.

For this purpose, the aforementioned reagents (with a template/monomer/cross-linker ratio of 1:4:20) were selected for the preparation of MMIPs containing two types of MNPs modified with different reagents (PEG or V-MPS).

MNPs were firstly obtained by a co-precipitation method, and were subsequently coated with PEG [29,30] and vinyl groups [36], as described in Section 2.2 to avoid their agglomeration. The morphology of these modified MNPs was characterized by TEM (Figure S1). PEG-MNP particles were mostly spherical in shape and have particle diameters ranging from 8 to 15 nm (see Figure S1A), whereas the V-MNPs (see Figure S1B) also showed spherical forms, with primary particle sizes around 12 nm. After modification (coating) of Fe_3O_4 surface, the resulting V-MNPs showed less aggregation degree than PEG-MNPs.

Then, scanning electron microscopy (SEM) images from MMIP beads prepared in the presence of different contents (0.5 and 2.0 wt%) of modified MNPs were taken. The magnetic composites prepared at 0.5 wt% MNPs (see Figures 76A and 76C) showed the presence of a few modified MNPs protruding from the polymer surface (bright spots in Figures 76A and 76C), being this fact more evident in those prepared using PEG-MNPs. The lower encapsulation capability of PEG-MNPs in front of V-MNPs is due to the absence of vinyl groups, which are able to react (copolymerize) with other monomers (MAA) to produce a MIP network.

On the other hand, on increasing the MNP content from 0.5 to 2 w/w %, it was observed an increased proportion of modified Fe_3O_4 nanoparticles (see Figures 76B and 2D), being again remarkable for PEG-MNPs, which suggests an unsuitable embedding (covering) of MNPs by the MIP shell. In any case, a decrease in polymer shell was evidenced. These results can be explained by a decrease of the thickness of polymeric network on the MNP surface. This reduction of thickness could be due to the dilution produced in the polymerization mixture by the presence of large contents of MNPs. Another possible effect to be considered is that an excessive load of MNPs can favour the formation of aggregates, which can hinder the motion of growing polymer chains, giving as a result the formation of a narrow polymer layer during polymerization, which could be easily broken by clusters of MNPs.

Additionally, for both types of MMIPs, the extraction efficiency decreased with increasing percentages of embedded MNP particles (data not shown). At sight of these results, MMIPs prepared with 0.5 wt% (either PEG- or V-MNPs) were selected for further studies.

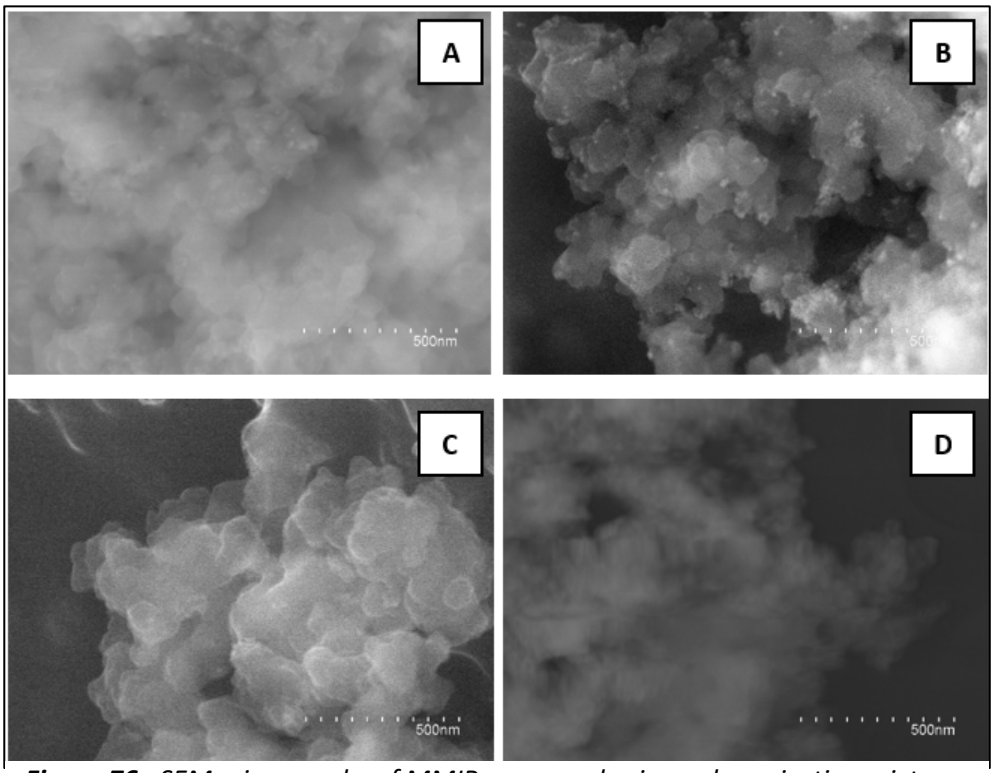


Figure 76. *SEM micrographs of MMIPs prepared using polymerization mixtures containing PEG-MNPs (0.5 and 2 wt %, traces A and B, respectively) and V-MNPs (0.5 and 2 wt %, traces C and D, respectively). The images were obtained at 20,000x magnification.*

The magnetic properties of the Fe_3O_4 nanoparticles and MMIPs were characterized by VSM at room temperature and the results are shown in Figure S2. The saturation magnetization value of the MMIPs was about 20 emu g^{-1}, which is smaller than that of Fe_3O_4 nanoparticles (60 emu g^{-1}). The decrease of the saturation value could be attributed to the existence of MIPs onto the surface of Fe_3O_4 nanoparticles because the polymer does not contribute to the overall magnetization. This decrease in magnetization is comparable to other magnetic composite materials [40]. The typical characteristics of superparamagnetic behavior were observed in MMIPs, enabling the separation of the sorbent from the suspension easily under the external magnetic field and redispersed rapidly without the external magnetic field (see Figure S3).

Study of the extraction conditions

Extraction conditions were adapted from previous studies performed with similar MIPs [37]. Sample was conditioned by adding 0.3 M ammonium/ammonia buffer at pH 9, before the loading step. Matrix interferences were removed by washing with deionized water and chloroform, and cocaine was eluted with 0.2 mL 5 % (v/v) acetic acid in methanol.

Extractions were then carried out in 1 mL glass vials and the most appropriate amount of MMIP was chosen. Different amounts of MMIPs (5, 10 and 15 mg) were introduced in different glass tubes containing 1 mL water that were stirred for 1 min and placed in the MagRack device (see Figure S3). When an external magnetic field was applied, the use of 10 and 15 mg of MMIPs gave turbid solutions, whereas 5 mg provided a clear and transparent solution, being this amount selected for the following experiments. Both assayed materials, PEG-MMIP and V-MMIP, provided similar recovery results. A volume of 500 µL of liquid phase was then selected in order to obtain an adequate dispersion of the 5 mg MMIPs during the washing steps and to achieve a clear, fast and simple magnetic separation. Thus, 500 µL of sample was mixed with the MMIP particles in the loading step, and the elution volume was fixed at 200 µL. This volume is the lowest one that can be separated properly from the solid phase to obtain appropriate preconcentration factors.

Extraction time was evaluated using 500 µL blank saliva spiked at 250 µg L^{-1} cocaine level and 5 mg V-MMIP following the above procedure and vortex shaking from 2 to 15 min. The effect of this variable on the cocaine recovery is shown in Figure 77A. From these data, it can be seen that 10 min provided a quantitative extraction of cocaine from saliva samples. The effect of loading time on cocaine

extraction by MNIPs was also evaluated (see Figure 77A). As it can be seen, no cocaine signal was obtained from MNIP extract at 10 min loading time, being necessary, at least, 15 min loading time to obtain a recovery of 5 % approximately of cocaine. This recovery increased till 10 % after 30 minutes loading time.

The effect of elution time (from 1 to 10 min) was also assessed using 200 µL 5 % (v/v) acetic acid in methanol as elution solvent. Figure 77B shows that an elution time of 5 minutes provides the complete elution of cocaine from the MMIP beads.

The loading capacity of magnetic MIPs was calculated by applying the recommended protocol (5 mg of each MMIP) to 0.5 mL of cocaine aqueous standards of increasing concentrations (up to 2 mg L^{-1}). The loading capacity of the synthesized materials was 0.124 mg and 0.102 mg per g of PEG-MMIP and V-MMIP, respectively. These values found were higher than those reported using similar MMIPs [29,30]. Unspecific interactions were also evaluated using the aforementioned procedure with V-MNIP and PEG-MNIP and no cocaine signals were detected for 10 minutes loading time, indicating the high specificity of the MMIPs employed. The saturated amount of MNIPs was 0.004 and 0.009 mg per g of PEG-MNIP and V-MNIP, respectively. It has been calculated using 15 minutes of sample loading time (see Figure 77A).

The selectivity of the polymer was evaluated using analogues (benzoylecgonine and ecgonine methyl ester, the major cocaine metabolites) and non-analogues (benzocaine and tetracaine, two organic substances commonly used as cutting agents during drug manipulation) as interfering substances. To evaluate the amount of benzoylecgonine and ecgonine methyl ester retained onto the MMIP using the developed procedure, a calibration line from 50 to 2000 µg L^{-1} of for the two cocaine metabolites were prepared and measured by IMS. Benzoylecgonine and ecgonine methyl esters show peaks at 15.04 and 11.95 ms drift time with K_0 values of 1.19 and 1.48 $cm^2\ V^{-1}\ s^{-1}$, respectively. These signals of cocaine metabolites were not observed when 500 µL blank saliva, spiked at 250 µg L^{-1} metabolite concentration, was analyzed.

Benzocaine and tetracaine standards were analyzed by IMS, showing peaks at 14.60 and 15.25 ms drift time with K_0 values of 1.21 and 1.15 $cm^2\ V^{-1}\ s^{-1}$, respectively. From these signals, the corresponding calibration lines, from 50 to 1000 µg L^{-1}, were prepared. The analysis of 500 µL blank saliva, spiked at 250 µg L^{-1} interferent concentration, using the proposed procedure, provided no signals at the benzocaine and tetracaine characteristic drift times.

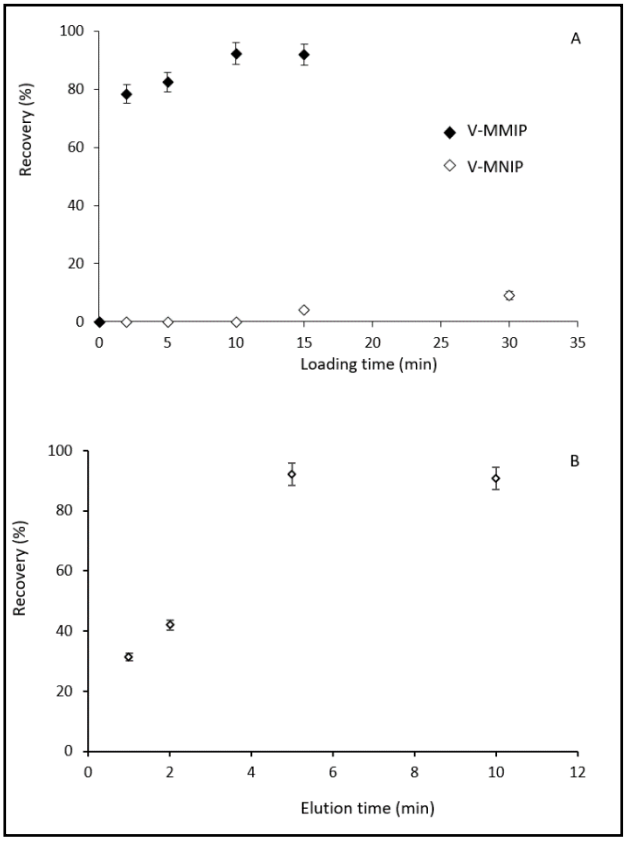


Figure 77. *Effect of the V-MMIP and V-MNIP loading time (A) and elution time (B) on cocaine recovery (mean ± SD, n= 3).*

Moreover, recoveries of cocaine in 500 µL blank saliva spiked at 250 µg L^{-1} cocaine and also 250 µg L^{-1} interfering compounds were calculated by the proposed procedure. Cocaine recoveries ranging from 80 to 97 % were obtained using, both, PEG-MNIP and V-MNIP sorbents, respectively.

Reusability of the MMIPs was evaluated with the repeated analysis of blank saliva samples (n=3) spiked with cocaine at 250 µg L^{-1} concentration level using the proposed procedure. Figure 78 shows the recoveries obtained for three consecutive cycles of sorption/desorption with PEG-MMIP and V-MMIP. As it can be seen, MMIPs are not reusable since the second use produced recoveries lower than 50% for both evaluated MMIPs. Further, SEM micrographs of MMIPs taken after one use by using the rinsing protocol described in Section 2.5 were done (Figure S4). V-MNPs showed (see Figure S4B) a high physical resistance to the solvent treatment and no modifications (absence of MNP protuberances) was observed in the MMIP surface after its use due to the covalent bond between MIP and vinylized MNPs. However, in the case of PEG-MMIPs, after one single use

(Figure S4A), a high number of MNPs located onto the polymer surface were evidenced, probably due to the weak binding between these particles and polymer. Several reasons were hypothesized to explain the decrease in extraction efficiency for both MMIPs. A potential explanation might be attributed to slight changes in imprinting sites during the repeatedly washing, which could explain the low rebinding capacity of template (decrease in extraction efficiency) after regeneration. Also, other possible reason could be ascribed to a deterioration of magnetic properties in the small amount of sorbent used after washing. Although in this case, it is not possible to reuse the sorbent. However, just 5 mg of sorbent are required per sample (0.5 mL), and consequently, the sorbent reusability cannot be considered as a critical step for the implementation of this method. At this point, reproducibility of the MMIP synthesis process was evaluated by comparison of the cocaine recoveries of spiked blank saliva samples at 250 µg L^{-1} concentration level using MMIP of three different batches, prepared from June 2017 to January 2018. Cocaine recoveries from 90 to 95%, from 80 to 97%, and from 80 to 95% were obtained using MMIPs of the three different batches, thus evidencing the satisfactory reproducibility of the synthesis process.

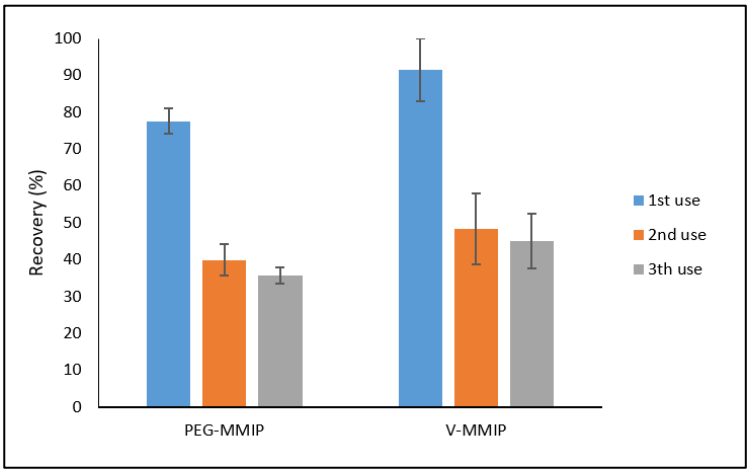


Figure 78. *Comparative reusability of the PEG-MMIPs and V-MMIPs after repetitive uses for 0.2 mL of a blank saliva sample spiked at 250 µg L^{-1} cocaine.*

IMS plasmagrams

Ion mobility plasmagram of a 300 µg L^{-1} cocaine standard is depicted in Figure 79. The most intense peak in the plasmagram corresponds to nicotinamide, the internal standard used in positive ionization acquisitions to correct variations in temperature and pressure. Cocaine shows a peak at 15.07 ms drift time with a K_0 of 1.16 $cm^2\ V^{-1}\ s^{-1}$ as previously reported [13, 18, 37]. Cocaine determination was based in the peak area measurement on the average ion mobility spectrum for 4 s desorption time.

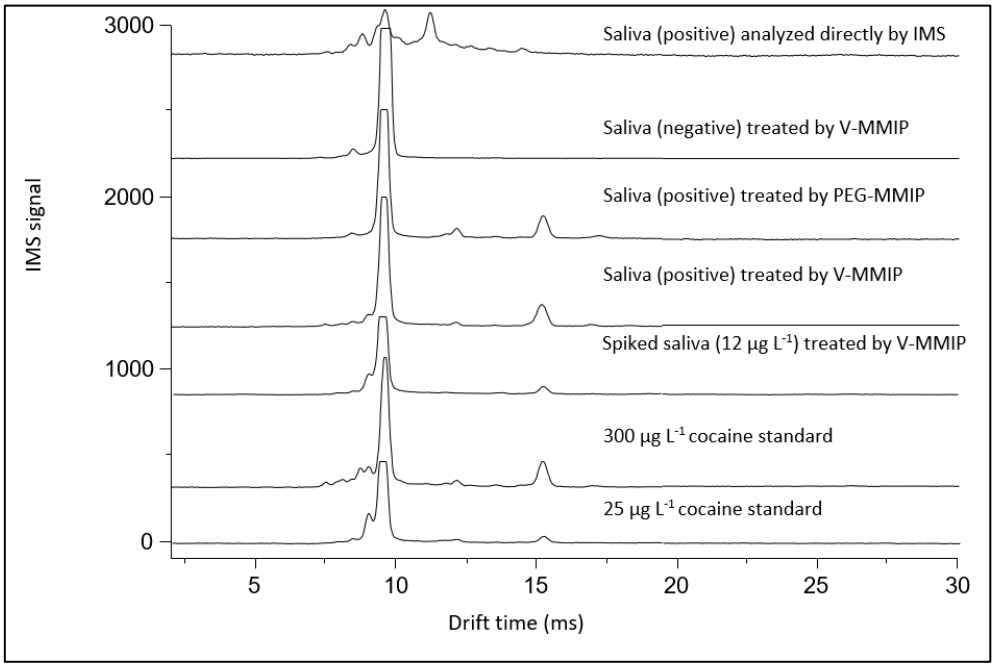


Figure 79. *IMS signals for: From bottom to top; cocaine standard (25 and 300 µg L^{-1}), blank saliva sample spiked at 12 µg L^{-1}, positive and blank saliva samples after V-MMIP and PEG-MMIP treatment, and a cocaine positive sample directly analyzed by IMS.*

Analytical features

Analytical features of the proposed methodology were evaluated in terms of linearity, precision and limits of detection (LOD) and quantification (LOQ) as shown in Table 45. Calibration curves were prepared with cocaine standards from 35 to 560 µg L^{-1} prepared in 5 % (v/v) acetic acid in methanol. Linearity was assessed by the determination coefficient (R^2) of the curve, obtaining values higher than 0.998. Precision was calculated as the relative standard deviation (RSD) of peak area

values of three independent measurements of 40, 100 and 520 μg L^{-1} cocaine standards, being the RSD values 0.6, 2.5 and 2.5 %, respectively. LOD and LOQ were 4 and 14 μg L^{-1}, respectively. LOQ in sample was also evaluated, giving 6 μg L^{-1} using a preconcentration factor of 2.5. Trueness was evaluated as described in section QC/QA. Recoveries can be seen in Table 46 with values ranging from 80 to 99 % for PEG-MMIPs, and from 80 to 95 % for V-MMIPs, being in all the cases appropriate values for cocaine determination in saliva samples. The obtained precision for cocaine determination in saliva samples using the recommended protocol with V-MMIP and PEG-MMIP gave RSD values of 5 and 13 %, respectively. Recovery experiments were also performed on blank saliva samples spiked close to cocaine LOQ level 10 μg L^{-1}, providing values of 114 ± 20 %.

Table 45. *Analytical features for the determination of cocaine by IMS.*

Parameter	**Value**
Coefficient of determination (R^2)	0.998
Linear range (μg L^{-1})	35-560
RSD intraday (%, n = 3)	
40 μg L^{-1}	0.6
100 μg L^{-1}	2.5
520 μg L^{-1}	2.5
LOD (μg L^{-1})	4
LOQ (μg L^{-1})	14
LOQ in sample (μg L^{-1})[a]	6
Total analysis time per sample (min)	16

[a]. *Pre-concentration factor of 2.5.*

Table 46. *Recoveries obtained for blank saliva samples, spiked at different cocaine concentration, extracted with V-MMIP and PEG-MMIP and analysed by IMS.*

[Cocaine] (μg L^{-1})	**Recovery (%)**		**% RSD (interday)**	
	V-MMIP	PEG-MMIP	V-MMIP	PEG-MMIP
80	93 ± 12	99 ± 10	13	10
270	95 ± 10	80 ± 4	11	5
560	88 ± 7	80 ± 5	8	6

Figure 79 shows the IMS plasmagrams obtained by a cocaine standard of 300 μg L^{-1} concentration level, a cocaine free saliva sample extracted using the developed procedure, two blank saliva samples spiked at 250 μg L^{-1} cocaine and extracted using PEG-MMIPs and V-MMIPs and a blank saliva sample spiked at 250 μg L^{-1} cocaine without sample treatment. As it can be seen, both MMIPs provided clean extracts with only the cocaine peak, demonstrating an excellent selectivity of the synthesized materials. Nevertheless, V-MMIP provided a little bit cleaner IMS plasmagram than that of PEG-MMIP, which showed some small interference peaks, close to the peak of the internal calibrant, probably due to small amounts of polymer released from the magnetic material. So, in spite of the fact that both materials provided adequate analytical parameters, V-MMIP was selected for the analysis of field samples.

Analysis of cocaine residues in saliva samples

A total of 7 oral fluid samples were collected from volunteer cocaine consumers. Saliva samples were analyzed by the proposed methodology using V-MMIP extraction followed by IMS determination. Two reference methods were employed to compare the obtained results: i) an screening method based in the direct determination of saliva by a lateral flow immunoassay, and ii) a quantitative method based in the analysis of the obtained V-MMIP extract by UHPLC-MS-MS. The obtained results for field samples analyzed by the three aforementioned methods are given in Table 47. Cocaine was detected by lateral flow immunoassay in all analyzed samples, as well benzodiazepines in sample 3 and cannabis in sample 4, 5, 6 and 7. No false negatives were obtained using the proposed procedure.

Concentrations of cocaine in samples ranged from 298 to 760 μg L^{-1}, analyzed by the proposed procedure. It should be mentioned that some sample extracts required a dilution to fit the linear range of IMS. Similar results were obtained by IMS and UHPLC-MS methods with bias values ranging from -22 to 23 %. Results were compared statistically by the application of *t*-test for two paired sample, obtaining a t value of 0.30 in front of the tabulated one (2.45), thus, it can be concluded that the two methods provide statistically comparable values.

Table 47. *Analysis of cocaine in consumer saliva samples by lateral-flow immunoassay (LF-IA) and extracted using V-MMIPs and analyzed by IMS and UHPLC-MS-MS.*

Sample	Illicit substances detected	[Cocaine] (μg L^{-1} ± s)		Bias (%)
	LF-IA	V-MMIP-IMS	V-MMIP-UHPLC-MS	
1	Cocaine	350 ± 40	329 ± 5	7
2	Cocaine	367 ± 15	354 ± 5	4
3	Cocaine Benzodiazepines	298 ± 8	383 ± 6	-22
4	Cocaine Cannabis	350 ± 50	361 ± 5	-4
5	Cocaine Cannabis	315 ± 16	358 ± 5	-12
6	Cocaine Cannabis	760 ± 20	615 ± 9	23
7	Cocaine Cannabis	547 ± 13	528 ± 8	3

Evaluation of method greenness

A comparison between the developed MMIP-IMS methodology and the reference procedures based on LFIA and MMIP-UHPLC-MS was performed in terms of Green Analytical Chemistry using the Green Certificate (see Figure 80). Zero penalty points were assigned due to energy consumption, lower than 0.1 kWh, and absence of harmful emissions [41], since the IMS instrument incorporates a carbon filter to trap analyzed compounds. 3 penalty point scores per sample were assigned to IMS due to reagent consumption and waste generation per sample. Thus, a total of 94 points in the Eco-Scale characterized the IMS developed methodology.

On the other hand, a total of 89 Eco-Scale points were assigned for the UHPLC-MS reference procedure. As well as IMS methodology, UHPLC-MS provided zero penalty points due to the absence of harmful environmental emissions. However, the chromatographic instrumental setup requires an energy consumption of less than 1.5 kWh per sample [41], which is higher than the IMS one, 0.0096 kWh per sample. Additional 5 penalty points per sample were assigned due to reagent and solvent consumption, and waste generation. On the other hand, the LFIA procedure presented a total of 98 Eco-Scale points, being penalized with 2 points due to the

waste generation. However, the qualitative nature of the procedure and the possibilities of false positive and negative results are the main drawbacks of LFIA.

	IMS	LFIA	UHPLC-MS
	Penalty points	Penalty points	Penalty points
Reagents	3	0	5
Instrumental	0	0	1
Occupational hazard	0	0	0
Waste	3	2	5
	Σ 6	Σ 2	Σ11
Total score	94	98	89

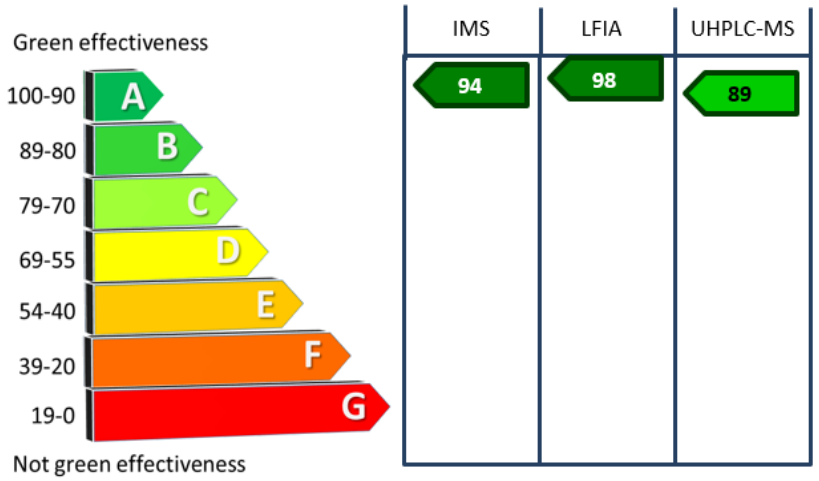


Figure 80. *Green evaluation; penalty points per sample and Green Certificate of the proposed MMIP-IMS method compared with the reference procedures.*

Table 48 summarizes the main analytical features of selected precedents regarding MIP extraction of cocaine from biological fluids. Obviously, the methodologies based on LC-MS/MS provided very low LOQ values, in the ppt range. However, the main drawbacks of those procedures are the acquisition cost of the analytical instrumentation and maintenance, necessity of trained personnel and weight and power requirements, which impossibilities the portability of the instrumentation. On the other hand, procedures based on direct measurements by fluorescence and MS/MS provided LOQ values in the ppb range similar to the LOQ value provided by this study with similar measurement times of the order of few seconds. However, these procedures employed a mass of sorbent significantly higher than that used in the proposed procedure.

Table 48. Comparison of the analytical features of selected published studies regarding MIP extraction of cocaine from biological fluids.

Sorbent material	*Sample*	*Amount of sorbent (mg)*	*Analytical method*	*LOQ (μg L^{-1})[a]*	*Recovery (%)*	*RSD (%)*	*Measurement time (min)[b]*	*Ref*
MIP-SPE	Urine and serum	25	LC-MS	0.5	92-96	-	15	[19]
MIP-SPE	Hair	25	LC-MS	0.07 ng mg^{-1}	89 ± 7	-	7	[20]
MIP-μ-SPE	Plasma	50	LC-MS/MS	0.2	94-101	5-10	5	[21]
MIP-μ-SPE	Urine	50	LC-MS/MS	0.081	92-101	2-8	8.5	[22]
Paper spray-MIP	Saliva	-	MS/MS	1	100-105	0.5-5	0.5	[23]
MIP – Mn-Doped ZnS QDs	Urine	100	Fluorescence	250	97-104	2-12	1	[24]
MMIP	Urine	50	LC-MS/MS	0.41	88-101	3-7	8.5	[29]
MMIP	Plasma	50	LC-MS/MS	0.043	91-100	1-7	5	[30]
MMIP	Saliva	5	IMS	6	88-95	8-13	0.5	This study

[a].LOQ for sample analysis (considering appropriate preconcentration factors).

[b]. Measurement time does not include the time needed in the sample treatment.

Abbreviations: IMS, ion mobility spectrometry; LC, liquid chromatography; LOQ, limit of quantification; MIP, molecular imprinting polymer; MMIP, magnetic MIP; MS, mass spectrometry; QD, quantum dot; RSD, relative standard deviation; SPE, solid-phase extraction.

Conclusions

In this study, MMIPs were prepared by bulk polymerization using modified magnetite nanoparticles as magnetic core. Dispersive magnetic SPE allowed the preconcentration and clean-up of cocaine, providing satisfactory benefits as ease of use, quickness and a deep reduction in the required solvent consumption due to the miniaturization of the procedure. Different surface modifiers (PEG or V-MPS) were tested for modification of the MNPs, which is a crucial procedure for further encapsulation. The features of the PEG-MMIPs and V-MMIPs were also compared for the extraction of cocaine from oral fluids and its later determination by IMS. Loading capacities of 0.124 and 0.102 mg per gram of polymer were obtained for PEG-MMIP and V-MMIP, respectively. The obtained LOD and LOQ were 10 and 35 $\mu g\ L^{-1}$, respectively, and quantitative recoveries from 80 to 95 % were obtained for the analysis of saliva samples spiked with cocaine.

The selected procedure was applied for the analysis of saliva of cocaine consumers, comparing the results with two reference methodologies obtaining statistically comparable results. Thus, it can be concluded that the proposed procedure combines the simple and selective extraction of cocaine from saliva samples with the fast and high sensitivity of IMS determination showing results comparable to reference methods.

Conflict of interest disclosure

The authors declare that have no competing financial interests in this investigation.

Acknowledgements

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Supplementary material

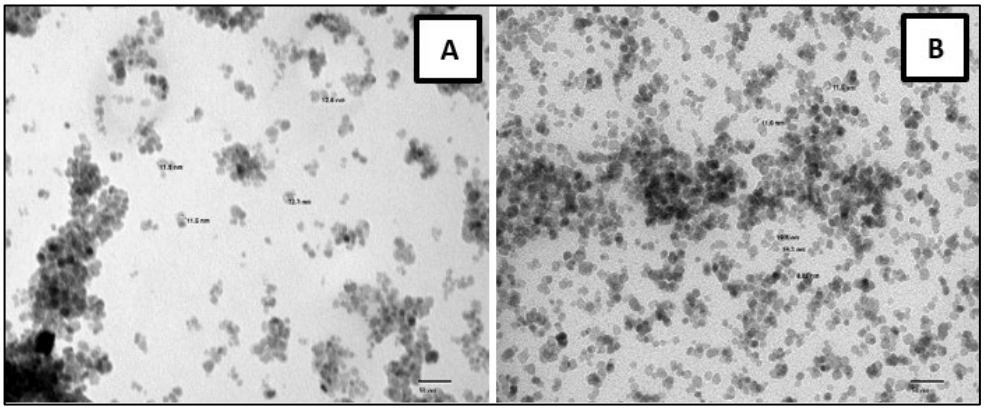

Figure S1. *TEM images of PEG-MNPs (A) and V-MNPs (B) obtained at 50,000x magnification.*

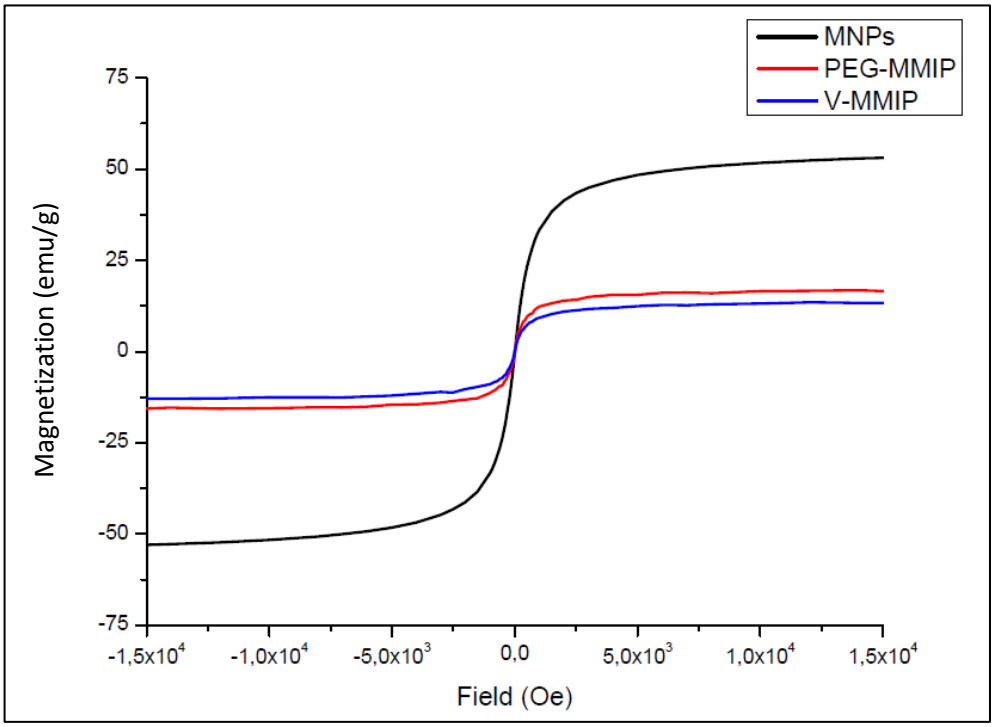

Figure S2. *Magnetization data at 300 K for MNPs (black), PEG-MMIP (red) and V-MMIPs (blue) from -15000 Oe to 15000 Oe.*

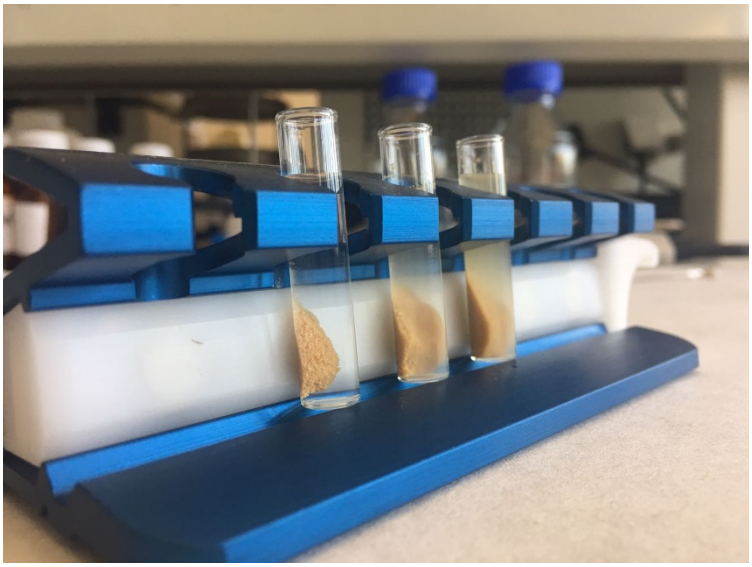

Figure S3. *Magnetic separation of different amounts of MMIP: 5, 10 and 15 mg (from left to right).*

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Development of a molecularly imprinted monolithic polymer disk for agitation-extraction of ecgonine methyl ester from environmental water

A. Sorribes-Soriano, R. Arráez-González, F.A. Esteve-Turrillas, S. Armenta, J.M. Herrero-Martínez

Department of Analytical Chemistry, University of Valencia, 50th Dr. Moliner St., 46100 Burjassot, Spain

Abstract

In this study, a new extraction approach based on rotating molecularly imprinted polymer (MIP) disks was developed. The preparation procedure of MIP-disk is simple. Firstly, in order to immobilize MIP onto the surface of polytetrafluoroethylene (PTFE) disk, previous modification and vinylization steps of this fluoropolymer were conducted. Then, MIP synthesis was done by in situ polymerization. The resulting MIP was characterized by Fourier-transform infrared spectroscopy and scanning electron microscopy. Afterwards, two ring magnets were placed in the sides of the MIP-disk to integrate the stirring and preconcentration of sample in just one step. To demonstrate the feasibility of this novel extraction system, the selective extraction of ecgonine methyl ester (EME) from water samples was performed. Extraction conditions were also evaluated and the extracts were analyzed by ion mobility spectrometry and by ultrahigh performance liquid chromatography-tandem mass spectrometry, allowing limits of detection of 13 and 75 ng L^{-1}, respectively. Field surface water and wastewater were analyzed using the proposed methodology, being a good alternative for the fast and potentially portable methodology for in-field screening analysis.

Graphical abstract

Keywords: *magnetic PTFE disks, vinylization, molecular imprinting, cocaine, ecgonine methyl ester, environmental water.*

Introduction

Despite the growing advance in terms of selectivity and sensitivity achieved by instrumental analytical techniques, sample preparation remains a critical issue in the whole analytical procedure; particularly in complex samples, where analytes are found at very low concentrations and a number of unknown interferents are present. Indeed, extraction techniques continue being the focus of intensive research (with novel advances in automation, miniaturization, and simplification of sample treatment) as evidenced by numerous and recent reviews on this subject [1,2]. Within these extraction techniques, solid sorbent materials used in different extraction technologies such as solid-phase extraction (SPE), solid-phase microextraction (SPME) and stir bar sorptive extraction (SBSE) have been widely described. Some of these extraction modes have enhanced their extraction yield by improving kinetic aspects; however, in general, an enhancement of sorption (or sorbent) selectivity is required to increase their extraction performance. An effective way to address this higher selectivity is by using molecularly imprinted polymers (MIPs) as solid phases [3].

MIPs are synthetic materials that mimic the interaction between antigen-antibody, prepared in presence of a template, which is usually the target molecule. Given its advantages of high selectivity, easy preparation, and low cost, these polymeric networks have been extensively investigated as molecular recognition media in SPE [3], SPME and SBSE [4,5]. To overcome inherent limitations of SBSE and SPME procedures, stir cake sorptive extraction (SCSE) [6] and rotating disk sorptive extraction (RDSE) [7] modes were developed. In particular, SCSE is based on the synthesis of a monolithic "cake" placed in a homemade holder with a protected iron wire. This extraction mode has been used for the extraction of inorganic anions in water [8], preservatives in juice and soft drinks [9], benzimidazole in water, honey and milk [10] or estrogens in surface water [11]. In spite of these good features, to our knowledge, there is no study related to the use of MIPs in SCSE and only one work (using MIP particles) in RDSE [12] have been described.

On the other hand, the use of drugs of abuse is increasing worldwide and causes serious social and environmental problems [13]. The main source of environmental contamination by illicit drugs is human consumption. The first multiresidue analytical method to detect illicit drugs in urban untreated wastewater was set up in 2006 [14] to measure the most widely used classes of illicit drugs in Italy and Switzerland. Wastewater analysis has been also used to

estimate drug consumption in several case studies [15]. The use of environmental analytical data to estimate levels of community consumption of illicit drugs, complementing epidemiological studies [16] is increasing and different European cities have published wastewater monitoring studies [17].

Cocaine is one of the most widely consumed drug worldwide, according to the World Drug Report of 2018 presented by United Nation Office of Drug and Crime (UNODC), the annual global production of pure cocaine was estimated in 1.410 tons [18]. Data provided by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) [19] estimate that, in the last year, 2.3 million young adults (between 15 and 34 years in EU-28) consumed cocaine. This drug is metabolized quickly in its metabolites benzoylecgonine and ecgonine methyl ester (EME) representing a 45 and 40 % of the total drug consumed, respectively, and they are found in urine at higher concentration than cocaine [20,21]. Although the stability of benzoylecgonine in wastewater is higher than that of EME [22], both cocaine metabolites have been measured and the results used to calculate the daily loads (g day^{-1}) in different monitoring campaigns [19,23].

Analytical methods for the analysis of abuse drugs are based on liquid chromatography–mass spectrometry (LC-MS/MS) using a triple quadrupole detector after solid-phase extraction (SPE) [21,22,24,25,26]. Different types of SPE cartridges have been tested [15, 28], being the most employed sorbents either hydrophilic-lipophilic balanced (Oasis HLB) reversed-phases [27] or mixed-mode (with a cation exchange, MCX) phases [28]. Recently, González-Mariño et al. [29] evaluated a commercial amphetamine MIP as well as Oasis HLB and MCX sorbents for the extraction of amphetamine drugs. MIPs have been also used as solid sorbents for the extraction of cocaine employing different alternative approaches including SPE in cartridges [30], magnetic dispersive SPE [31,32], porous membrane-protected μ-SPE [33], among others.

In the present study, a novel extraction SCSE design based on the use of polytetrafluoroethylene (PTFE) disks containing monolithic MIP cakes is developed. Thus, laboratory-made extraction units and MIPs were simply prepared. Thus, the developed extraction device exhibited an extraction phase with a high surface area-to-volume ratio, and it can be magnetically stirred (along the vertical axis) at higher rates without damaging the phase. A proof-of-concept application of this novel system is presented for the extraction of EME from water samples and its determination by ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) and ion mobility spectrometry (IMS).

Experimental

Chemicals and materials

EME (1 g L^{-1} in acetonitrile (ACN)), cocaine (1 g L^{-1} in ACN), benzoylecgonine (1 g L^{-1} in methanol), EME-D_3 (100 mg L^{-1} in ACN), cocaine-D_3 (100 mg L^{-1} in ACN), and benzoylecgonine-D_3 (100 mg L^{-1} in methanol) standards were purchased from Sigma (Steinheim, Germany).

Custom made PTFE disks (4.8 mm height and 40.3 mm diameter) with a circular edge (2.6 mm height and 26.4/25.3 mm outer/inner diameter) in one of the surfaces were provided by Suministros Industriales Aramo S.L. (Valencia, Spain). A scheme of the employed PTFE disks is shown in Figure 81A. PTFE-coated neodymium ring magnets (5 mm height and 44/30 mm outer/inner diameter) used to insert the treated MIP-disk were obtained from (SM Magnetics, Pelham, AL, USA) with an axial direction of magnetization (north and south poles were located on the top and bottom flat circular surfaces).

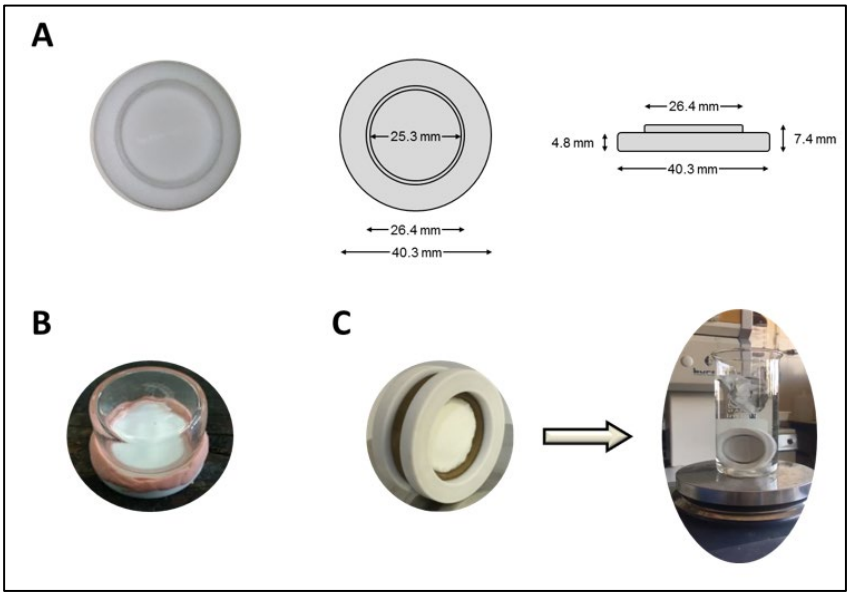


Figure 81. *A) Scheme and dimensions of the employed polytetrafluoroethylene (PTFE) disks, B) polymerization assembling, and C) sampling assembling.*

Sodium naphthalene solution (FluoroEtch®) for the treatment of PTFE disk surface was provided by Acton Technologies (County Limerick, Ireland). Glycidyl methacrylate (GMA), triethylamine, *N,N*-dimethylformamide (DMF), methacrylic acid (MAA), ethylene glycol dimethacrylate (EDMA), and azobisisobutyronitrile (AIBN) were provided by Sigma.

Organic solvents and buffer constituents used in this study were HPLC grade or higher provided by Scharlau (Barcelona, Spain).

Water samples were collected in different sampling sites close to Valencia (Spain): Port of Valencia, Almenara pond, Marjal dels Moros lagoon, Puçol and Llíria irrigation ditches, and from the inlet of Valencia sewage plant. Samples were collected in different 1 L glass containers without any preservative addition and kept at −4 °C until analysis.

Preparation of MIP-disks

PTFE surface of the disks was activated by immersion in 20 mL FluoroEtch® solution heated at 60 °C under nitrogen stream for 10 min following the operational procedure recommended by the supplier [34]. Modified disks were sequentially washed with 30 mL water and 30 mL methanol for 60 s and then dried at 60 °C for 2 hours.

Surface of treated disks was vinylized according to the literature [35] in order to provide the appropriate anchorage of the MIP over disk surface. Vinylization solution was made with GMA 2 M in DMF, containing triethylamine 5 mM, and adjusted to pH 8 with HCl. Then, disks were immersed in 10 mL vinylization solution for 2 h under stirring at 60 °C. Vinylized disks were then washed with 20 mL acetone and dried at 60 °C for 2 h.

Polymerization mixture was prepared with 13.6 mg EME and 209 mg MAA in 2 mL ACN and kept at room temperature for 1 h. Then, 2.378 g of EDMA and 120 mg of AIBN in 2 mL ACN were mixed with the former solution. The mixture was purged by a nitrogen stream for 10 min and sonicated for 10 min. MIP polymerization was carried out inside the circular edge present in the surface of the disk using a close-fitting glass beaker externally sealed with modelling clay (see Figure 81B for additional details), using 1 mL of the polymerization mixture. Thermal polymerization was performed at 60 °C for 24 h. After that, template was removed washing the MIP-disk ten times with 100 mL 10 % acetic acid in methanol and intense stirring for 1 h (each washing) till template was completely removed from the polymer. Complete removal of the template was assessed by LC-MS/MS. Non-imprinted (NIP) disks were prepared following the aforementioned procedure without the template. MIP- and NIP-disks were stored at room temperature completely immersed in methanol to avoid polymer cracking.

MIP-disks characterization

Characterization of the disk surface after each treatment was monitored by Fourier-transform infrared (FTIR) spectroscopy, using a Tensor 27 spectrometer with a DLaTGS detector from Bruker (Bremen, Germany) and a Dura Sample IR II attenuated total reflection (ATR) accessory for liquid samples from Smiths Detection Inc. (Warrington, UK) equipped with a nine-reflection diamond/ZnSe Dura Disk plate. The disk was placed on the ATR crystal surface and the spectra were collected in the region between 4000 and 550 cm^{-1} with a resolution of 4 cm^{-1} and averaging 50 scans.

MIP was characterized by scanning electron microscope (SEM) using a S-4800 SEM provided with a field emission gun and a back secondary electron detector from Hitachi (Ibaraki, Japan). EMIP 3.0 software from Rontec (Normanton, UK) was employed for image data acquisition.

Extraction procedure

MIP-disk was placed between two PTFE-coated ring magnets in order to keep the disk safe and to stir the solution, as shown in Figure 81C. MIP-disk was previously conditioned by stirring 200 mL deionized water for 5 min using a 250 mL tall glass beaker and a magnetic stirrer. The pH of 200 mL sample was adjusted to pH 10 by addition of 10 mL carbonate buffer 0.1 M, and then the MIP-disk was introduced inside the sample solution and stirred for 30 min. After sample extraction, MIP-disk was washed with 50 mL deionized water for 1 min and magnets were removed to the disk. MIP-disk was softly dried with a paper tissue and submerged upside down in a 100 mL glass baker containing 5 mL acetic acid 1 % in methanol for 30 min to elute EME.

IMS analysis

An Ionscan-LS from Smiths Detection (Morristown, NJ, USA), using a ^{63}Ni foil as radioactive ionization source, was used for a fast screening determination of EME. IM station software (version 5.389) from Smiths Detection was used to acquire and process data. Nicotinamide, with a reduced mobility constant (K_0) of 1.860 $cm^2\ V^{-1}\ s^{-1}$, was used as internal calibrant in positive mode. A 25 ms scan period using a 0.2 ms shutter grid width, 60 segments per analysis and 479 data points conditions were employed for plasmagram acquisition. A counterflow dry air at 300 mL min^{-1} was used as a drift gas. The electric field strength in the drift region

was 251 V cm^{-1}, with 1759 V total drift voltage, and 7 cm drift tube length. Desorption, inlet and drift tube temperatures were adjusted to 285, 290 and 237 °C, respectively. 10 µL sample extract was thermally desorbed for 60 s over a PTFE membrane. A calibration curve was prepared at EME concentrations from 10 to 200 µg L^{-1} prepared in acetic acid 1 % in methanol.

UHPLC-MS/MS analysis

UHPLC was employed for EME determination using a Waters Acquity System from Waters (Milford, MA, USA), equipped with a binary solvent delivery system, an autosampler and a Waters Acquity UPLC® BEH HILIC (1.7 µm, 2.1 x 100 mm) column. A 5 µL injection volume was employed and the mobile phase, at a flow rate of 0.4 mL min^{-1}, consisted of (A) 5 mM of ammonium formate in water and (B) 5 mM of ammonium formate in ACN. The isocratic elution was performed using 70 % of B during 6 min. Acquisitions were performed in positive mode at 3.5 kV capillary voltage, 120 °C source temperature and 450 °C desolvation temperature. The precursor and product ions used for EME determination were m/z 200 and 182, respectively, using a 18 V collision energy. In this study, EME-D_3 was used as internal standard using m/z 203 and 185 as precursor and product daughter ions, respectively, using a 18 V collision energy. A calibration curve was prepared from 2 to 500 µg L^{-1} EME prepared in acetic acid 1 % in methanol and containing 20 µg L^{-1} EME-D_3. Sample extracts were spiked with EME-D_3 at a final concentration of 20 µg L^{-1}.

Additionally, selectivity studies were performed using cocaine and benzoylecgonine standards prepared in the same conditions than aforementioned, using cocaine-D_3 and benzoylecgonine-D_3 as internal standards, respectively.

Results and discussion

Disk surface modification, preparation of MIP-disk and characterization

First, PTFE disks were treated with FluoroEtch® (as etchant reagent) to modify their surface through the conversion of C-F bonds into C-H, C-OH, and -COOH functional groups, obtaining in this manner disk surfaces with decreased hydrophobicity [35]. Besides, the introduction of -OH groups increases the reactivity of PTFE-based material and favours its methacryloylation to obtain a vinylized surface. This step was (or can be) achieved through the reaction of

epoxide group of GMA [34], leaving free its vinyl group for the later covalent binding of MIP during the polymerization step to the PTFE surface. Figure 82 shows images of disk surface and their ATR-FTIR spectra throughout the modification, vinylization, and polymerization steps. ATR-FTIR spectrum of untreated PTFE disk showed two characteristic bands between 1200-1000 cm^{-1}, corresponding to C-F bonds. A dark browning of disk surface was observed after FluoroEtch® treatment, and although the C-F bands are still present in the ATR-FTIR spectrum, it can be observed the presence of a characteristic band around 3500 cm^{-1}, corresponding to OH vibrations and close 1900 cm^{-1} attributed to the presence of carboxylic groups [36]. The vinylization step introduced by the attachment of GMA to PTFE surface was confirmed by ATR-FTIR by an increase in the signals provided for OH and carbonyl vibrations (around 3500 and 1800 cm^{-1}, respectively), jointly with the onset of absorption bands at 2000-1600 cm^{-1} attributable to vinyl groups.

After MIP polymerization, the bands obtained were completely attributed to MAA-*co*-EDMA-based polymeric material (1800 cm^{-1} for carbonyl group, CH_2 bending vibrations around 1405-1465 and C-O-C vibrations in esters around 1240 and 1010-1040 approximately), while the C-F characteristics bands of disk surface were not observed.

The obtained MIP- and NIP-disks by using the proposed synthesis procedure using 1 mL of the polymerization mixture provided a polymer disk of 502 mm^2 surface area and 1.60 ± 10 mm thickness, with an average weight of 384 ± 17 mg (n=10). Higher volumes of polymerization mixture were not evaluated in order to prevent spillage of the solution.

Polymeric material was removed from the MIP-and NIP-disks surface and then characterized by SEM. Figure 83 shows that the synthesized polymers have a globular morphology with high porosity. Moreover, MIP material had bigger globules than NIP, probably due to the absence of the template during the polymerization step. Thus, the porous structure of MIP was beneficial for analyte adsorption and mass transfer.

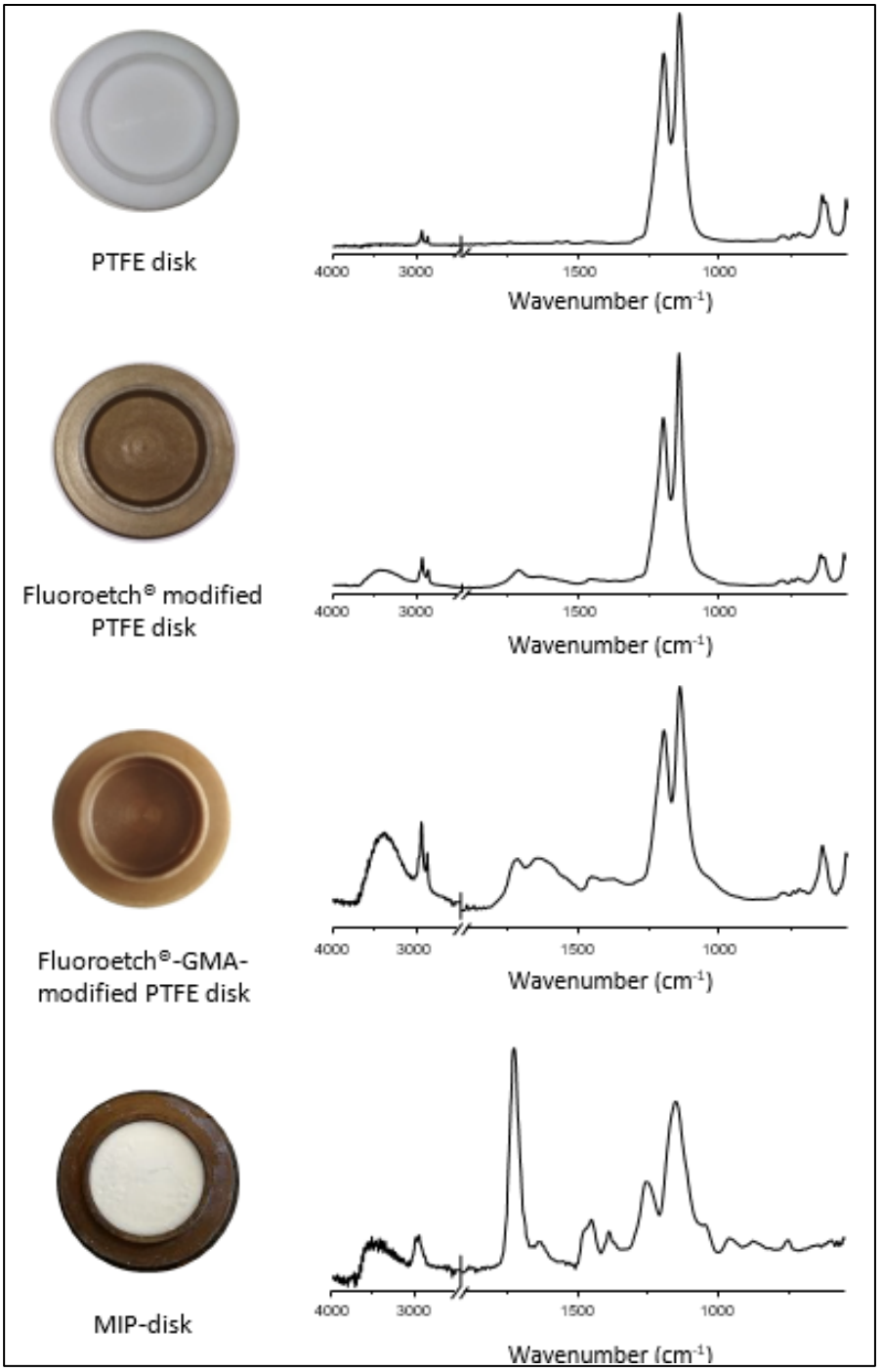


Figure 82. *External appearance and attenuated total reflectance-Fourier-transform infrared (FTIR-ATR) spectra for polytetrafluoroethylene (PTFE) disks after each modification step.*

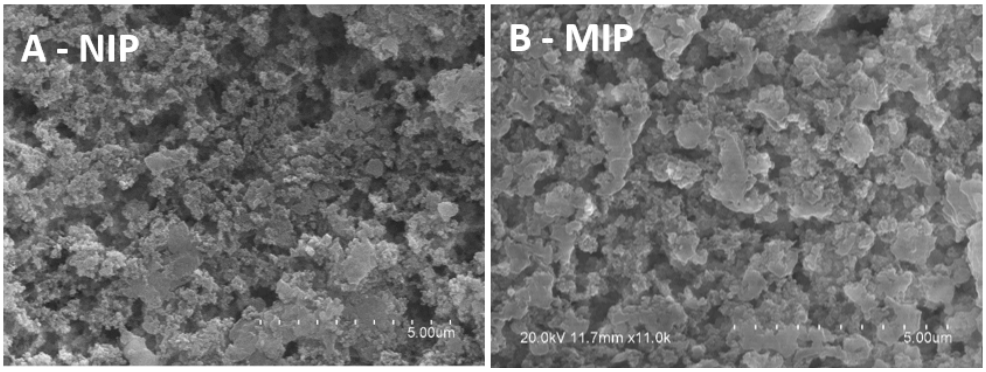


Figure 83. *Scanning electron microscopy (SEM) images obtained for the produced A) non-imprinted (NIP) and B) molecularly imprinted (MIP) polymers.*

Study of extraction conditions

Then, the developed extraction unit was applied to the extraction of EME in water samples. For this purpose, several extraction parameters (sample pH value, adsorption and desorption time, and so on) were studied using blank samples spiked with the target analyte at 2 µg L^{-1} concentration level using UHPLC-MS/MS. Sample volume was also fixed at 200 mL using a 250 mL tall glass beaker, in order to assure the complete immersion of the disk into the water sample. Stirring speed was adjusted to provide a gentle turbulence with an approximate 2 cm depth vortex. In any case, the MIP-monolithic disk in this novel SCSE format did not contact the wall of vessel during stirring.

Sample pH and extraction time were evaluated for the adsorption step by using MIP-disks. Spiked water samples were adjusted at pH 5, 10, and 12 using a 0.01 M buffer solution and analyzed by the proposed procedure using as adsorption and desorption times 90 and 60 min, respectively. Figure 84A shows the obtained results, being pH 10 the experimental condition that provided the highest recovery. EME has a pK_a around 9 that means at pH higher than 10 it was in its neutral form allowing the interaction between EME and the acid group of MAA by hydrogen bonds. A decrease of signal was observed at pH 12 probably due to a partial hydrolysis of EME. According to these results and to obtain stable extraction efficiencies, pH 10 was selected for subsequent experiments.

Additionally, different extraction times were evaluated for the adsorption step in order to obtain quantitative recoveries of EME. Figure 84B shows the effect of 15, 30, 60, and 90 min adsorption times on the recovery obtained for blank samples spiked at 2 µg L^{-1} concentration level using the aforementioned extraction

conditions and an desorption time of 30 min. A quantitative extraction was obtained for extraction times higher than 30 min, with recoveries values ranging from 90 to 114 %. Salting out effect was also evaluated using the recommended procedure with the addition of a 1 % NaCl; however, no significant changes were observed in the obtained recovery.

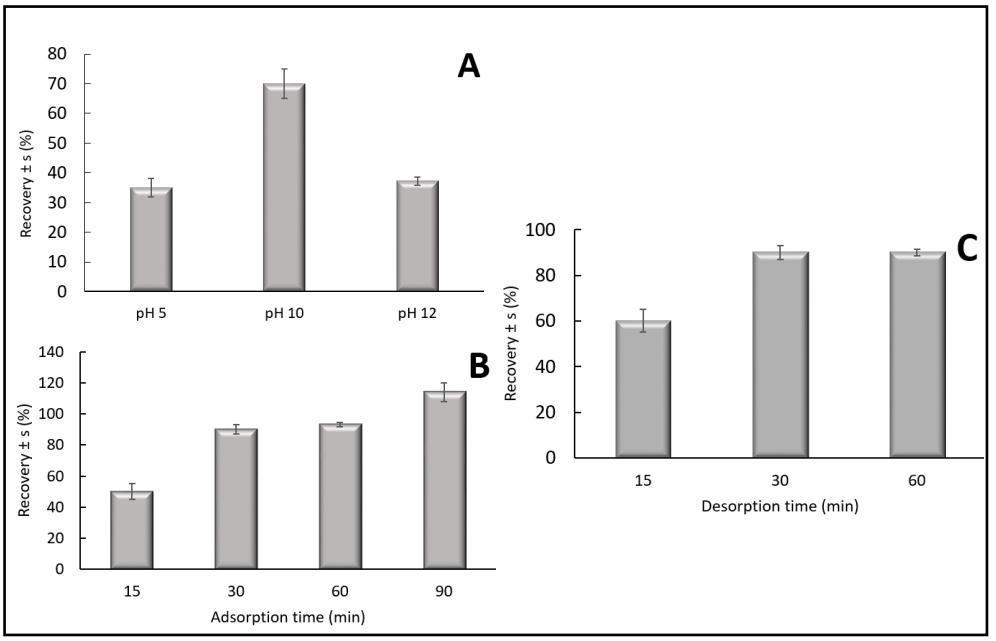


Figure 84. *Effect of A) loading (or adsorption) pH, B) loading (or adsorption) time, and C) elution (or desorption) time on the recoveries obtained (using the proposed methodology) using water spiked with 2 µg L^{-1} EME (see text for further details).*

Washing step was based on a quick rinsing of MIP-disk with 50 mL deionized water for 1 min and then external magnets were disassembled from the disk. No interferences or ionization enhancement or suppression troubles were observed in UHPLC-MS/MS determinations by using these mild washing conditions; consequently, a more intense washing step was not required.

Desorption of EME was carried out using acetic acid 1 % in methanol as shown in the literature [37]. The employed elution volume was set at 5 mL, the minimal volume that allowed a complete immersion of the upside down disassembled MIP-disk. No stirring or shacking was proposed in order to avoid any MIP rupture (or possible losses of MIP material), and the desorption time of the studied analyte was investigated ranging from 15 to 60 min using the aforementioned selected conditions. Figure 84 C shows that an elution time of 30 min provided quantitative results (90 %), and it was selected for further studies.

Study of MIP-disk features

The produced MIP-disks were characterized in terms of imprinting factor, binding capacity, and selectivity. The imprinting factor indicates the ratio of specific and generic interactions of the analyte with the polymer and it was calculated experimentally. 200 mL water were adjusted to pH 10, spiked at 2 µg L^{-1} EME, and extracted by the recommended procedure using NIP- and MIP-disks in independent experiments. The obtained recovery values were 3.5 ± 1.2 % and 88 ± 4 %, respectively, having the produced MIPs an imprinting factor value of 25, giving a high selectivity in the extraction of target analyte.

The binding capacity is the maximum amount of analyte that MIP can retain and it is typically associated with the template/monomer ratio or the porosity of the polymer. Thus, 200 mL water was adjusted to pH 10, spiked at 5 mg L^{-1} of EME, and extracted by the proposed procedure using MIP-disks. The obtained maximum binding capacity was set at 6.68 µg EME per disk. Taking into account the average disk weight (384 ± 17 mg), the maximum binding capacity of the polymer was 17.7 µg g^{-1}, high enough for the analysis of EME in water samples.

Selectivity of the MIP was also evaluated by the extraction of two related compounds with a close molecular structure, such as cocaine and benzoylecgonine. Thus, 200 mL blank water samples spiked at 3.75 µg L^{-1} cocaine and benzoylecgonine were extracted by the recommended procedure. The obtained recovery for cocaine was 58 ± 4 %, whereas the concentration found for benzoylecgonine was below the LOD. The structures of cocaine and EME differ in the benzoyl moiety (hydroxyl versus benzoyl group). This substitution does not modifies substantially the chemical properties of both compounds, being weak bases with pKa values around 8.6-9.2. On the other hand, the structures of cocaine and benzoylecgonine differ in the substitution of the carboxylic moiety. And compared to EME, the substance used as template in the MIP synthesis, the structures differ in both, the methyl ester and benzoyl moiety. This change causes that benzoylecgonine would be an amphoteric compound with two pKa values; one at 2.1 and the second at 11.4, respectively. Probably, this difference implies that MIP-disks may co-extract small amounts of cocaine, which do not affect to neither, IMS or UHPLC-MS/MS measurements, but do not extract benzoylecgonine.

Table 49. *Molecular structure, ion mobility spectrometry reduced mobility constant (K_0), UHPLC-MS/MS retention time (RT) and extraction recovery.*

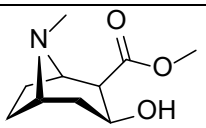
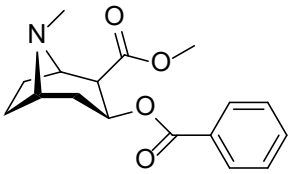
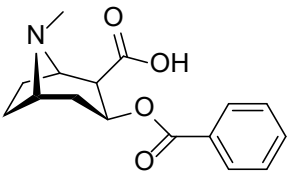
Compound	Molecular structure	K_0 (cm^2 V^{-1} s^{-1})	RT (min)	Extraction recovery (%)
Ecgonine methyl ester		1.48	1.79	100
Cocaine		1.16	1.17	58
Benzoilecgonine		1.19	0.98	0

Figure 85A shows the obtained plasmagram for a 100 µg L^{-1} EME standard, and a blank water sample unspiked and spiked at 2.5 µg L^{-1} extracted by using the proposed protocol. As it can be seen, EME standard can be easily identified by IMS without the presence of any interference from the sample. Also, multiple reaction monitoring (MRM) ion chromatograms for 25 µg L^{-1} EME standard and its corresponding tri-deuterated form (internal standard) were obtained (see Figure 85B) for standard solutions and environmental water samples.

Also, the batch-to-batch reproducibility of MIP-disks was evaluated by extraction of a blank water sample spiked at 2.5 µg L^{-1} of EME standard, achieving RSD (n = 3) values for extraction yields below 12%

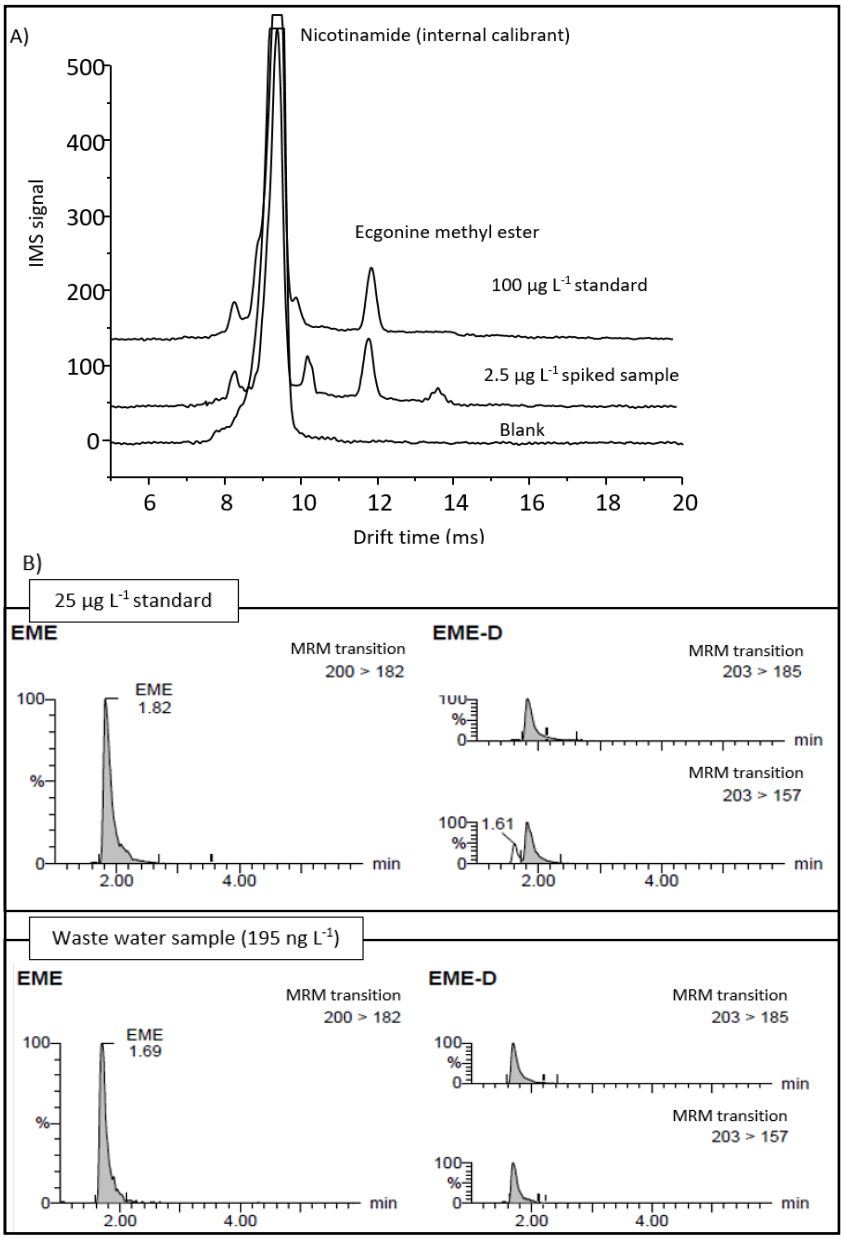


Figure 85. *A) Ion mobility spectrometry plasmagram obtained for an EME standard of 100 µg L^{-1} in methanol, a blank water sample and the same sample spiked at 2.5 µg L^{-1} extracted by using the proposed procedure using MIP-disks and B) Multiple reaction monitoring.*

Analytical features of IMS and UHPLC-MS/MS procedures

Next, methodologies for the analysis of EME via MIP-disk coupled to IMS or UHPLC-MS/MS were developed. These techniques were compared in terms of linearity, precision and LOD and limit of quantification (LOQ) values.

IMS analysis provided a fast response in less than 1 min. The obtained drift time for EME was 11.8 ms drift time that corresponded to a K_0 of 1.48 $cm^2 V^{-1} s^{-1}$. A linear response was obtained from 10 to 200 µg L^{-1} EME with a correlation coefficient (R^2) of 0.9992. Instrumental LOD and LOQ were calculated as three and ten times, respectively, the standard deviation of the intercept divided by the slope of the calibration curve. The obtained instrumental LOD and LOQ values were 3 and 10 µg L^{-1}, respectively. Thus, considering a 40 times pre-concentration factor for the MIP-disk extraction (200 mL sample in 5 mL extract), LOD and LOQ values were set as 75 and 250 ng L^{-1}. Precision was set as the relative standard deviation (RSD) of a 100 µg L^{-1} EME prepared in 1 % acetic acid in methanol with a value of 7 % (n=5).

UHPLC-MS/MS analysis provided a chromatographic peak at 1.79 min retention time with a runtime between samples of 6 min. Calibration curve was linear from 2 to 500 µg L^{-1} with a R^2 value of 0.9998 and a precision of 5 % (n=5) was obtained. LOD and LOQ were calculated as aforementioned, providing values of 0.5 and 1.8 µg L^{-1} EME for UHPLC-MS/MS determinations, respectively, and 13 and 45 ng L^{-1} EME for water analysis, respectively.

Application of MIP-disk to extract EME from water samples

Surface water and wastewater samples were obtained from different locations in the Comunitat Valenciana (Spain) and extracted by the proposed MIP-disk extraction methodology. Sample extracts were analyzed by both IMS and UHPLC-MS/MS techniques (results shown in Table 50). EME was found in 5 of the 6 analyzed samples at very low concentrations, ranging from 67 to 195 ng L^{-1} by UHPLC-MS/MS. All analyzed samples have an EME concentration lower than the LOQ established by IMS. Nevertheless, EME was detected in two samples by IMS with concentration between LOD and LOQ.

Table 50. *Ecgonine methyl ester concentration found in field samples analyzed by the developed MIP-disk extraction analyzed by IMS and UHPLC-MS/MS.*

Sample	Location	[Found] (ng L^{-1} ± s, n=2)	
		IMS	UHPLC-MS/MS
1	Port of Valencia	- [a]	98 ± 8
2	Pond (Almenara)	- [a]	< LOQ
3	Lagoon (Marjal dels moros)	- [a]	67 ± 5
4	Irrigation ditch (Puçol)	< LOQ	143 ± 10
5	Irrigation ditch (Llíria)	- [a]	- [a]
6	Urban wastewater	< LOQ	195 ± 13

[a] *Not detected.*

Conclusions

A novel extraction device based on a SCSE approach has been designed in order to allow the stirring of monolithic MIP cakes in PTFE disks during the extraction protocol. The construction of this device was quite simple and cost-effective. Thus, after a proper modification of disks to allow a robust anchorage of monolithic MIPs to PTFE surface, and subsequent synthesis of MIPs, two magnets were assembled to the PTFE disks. These novel systems improved the stirring performance which significantly favours a fast mass exchange compared to the traditional cylindrical iron wire or magnetic bar design in SCSE and RDSE holders. It can be explained taking into account both to the geometry of the stirrer as well as the use of a magnetic material with a stronger magnetic transmission force compared to standard ferrite used in these microextraction techniques.

Further, experimental conditions in the proposed configuration were evaluated in order to obtain quantitative recoveries of EME in high volumes of aqueous samples. Thus, factors such as loading pH, loading time and elution time were optimized in order to reduce extraction time. Other aspects including the imprinting factor (25), binding capacity (17.7 µg g^{-1}), and selectivity were also evaluated, thus demonstrating the high effectiveness of the proposed extraction methodology for surface water and wastewater analysis. Two analytical techniques were proposed for the determination of EME in extracts, being UHPLC-MS/MS more sensitive (LOD of 13 ng L^{-1}) than IMS (LOD of 75 ng L^{-1}). In any case, the use of IMS can be proposed as quick screening method with an analysis time less than 1 min, while UHPLC-MS/MS is only proposed as rearguard confirmatory tool. Ultimately, the successful results obtained suggest that the proposed unit extraction in combination with MIP technology can provide a simple, effective and selective extraction approach in sample treatment of complex liquid samples,

which undoubtedly opens a new promising area in extraction/stirring integrated techniques.

Conflict of interests

The authors declare that have no competing financial interests in this investigation.

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Supplementary material

Video S1. Molecularly imprinted monolithic polymer disk for agitation-extraction of ecgonine methyl ester from environmental water.

(Access to the video)

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FLUOROQUINOLONE AND METHAMPHETAMINE OLIGOSORBENT PREPARATION BY APTAMER IMMOBILIZATION

A. Sorribes-Soriano[a,b], V. Pichon[b], J.M. Herrero-Martínez[a], F.A. Esteve-Turrillas[a], S. Armenta[a]

[a]*Department of Analytical Chemistry, Universitat de València, 50th Dr. Moliner St., 46100 Burjassot, Spain*

[b]*Department of Analytical and Bioanalytical Sciences and Miniaturization (LSABM), ESPCI ParisTech, 10 rue Vauquelin, 75231 Paris, France*

Abstract

Solid-phase extraction (SPE) is one of the most extended analytical sample treatments due to its high extraction efficiency, simplicity and versatility. During the last decades, numerous selective sorbents have emerged in order to achieve the isolation of the target molecules from complex matrices containing a high number of interfering compounds. In this sense, aptamers have demonstrated their good affinity towards certain molecules and they have been applied for the extraction of compounds following the guidelines opened by immunosorbents. The study presented in these pages is focused on the immobilization of aptamers previously described for fluoroquinolones and methamphetamine determination. The solid supports selected were CNBr-activated sepharose and magnetic beads to compare the difference in performance between SPE and magnetic dispersive solid phase extraction (MDSPE). The parameters to evaluate were the selectivity of the prepared oligosorbents and the main analytical features for their next application to the analysis of field biological samples. However, even after achieving acceptable grafting yields (from 12.3 to 36.6 %), the selected aptamers did not show acceptable retention of the target molecules.

Keywords: *Aptamer, Oligosorbent, Fluoroquinolones, Methamphetamine, Solid-Phase Extraction, Magnetic Dispersive Solid-Phase Extraction*

Introduction

Quinolones are synthetic antimicrobial drugs able to inhibit the DNA synthesis of some bacteria. They are active via oral intake, have a high distribution volume and are generally well tolerated. These reasons make quinolones a widely used family of drugs for urinary infection treatment in veterinary and human medicine [Hooper et al. 1993]. When quinolones have the presence of a fluorine atom in their structure they are usually named as fluoroquinolones. Last decades, fluoroquinolones research has promoted an increase in their use to heal different kind of infectious diseases and not only urinary bacterias. Therapeutic drug monitoring has important applications when drugs under study have narrow concentration range where they have therapeutic effects, when they are used chronically or when they have dangerous effects in case of overdose. Particularly, in case of fluoroquinolones, some clinical and molecular pharmacological studies have demonstrated a higher relationship between their clinical effects and their concentration in plasma compared to the relationship between pharmacological and administrated dose [Carlucci et al. 1998].

Methamphetamine is a stimulant with an intense effect on nervous system and brain, even when it is consumed at low doses. Usually, methamphetamine is abused via oral intake, aspirated, injected or smoked and it can cause convulsions and promote an increase of corporal temperature until fatal levels. Commonly, methamphetamine consumers develop violent behaviors, anxiety, sleep disorders, paranoia and a strong psychological dependence to the substance. Methamphetamine is easily produced in the illegal market, for this reason it is easy and cheap to purchase this drug. This facts makes that it becomes in one of the most abused drugs for users that consume stimulants [Svorc et al. 2014].

Both, legal and illicit drugs require suitable analytical method for their determination in biological fluids for medical and legal matters. Due to the improvement of analytical instrumentation and technology, more analytes can be easily determined by direct injection by liquid chromatography coupled to mass spectrometry (LC-MS) [Kalmár et al. 2013]. Nevertheless, there are still some challenges for determination of those analytes that are found at low concentration level in complex matrices since it is difficult to achieve the required sensitivity [Zhang et al. 2009]. In those cases, it is essential to introduce a sample treatment prior to analytical determination in order to remove matrix effects and to improve LODs. Sample treatment approaches usually involve analyte isolation and preconcentration as well as interfering compounds elimination [Pan et al. 2013]. Particularly, sample treatment is necessary for biological matrices because they are complex and there can be found a lot of potential interfering compounds. One of

the most useful and extended sample treatment is the solid phase extraction (SPE) because of the high recoveries, easy performance and appropriate selectivity if suitable sorbent is selected to extract target molecules [He et al. 2013].

Aptamers offer high selectivity in SPE. They are able to achieve an appropriate analyte isolation when they are immobilized onto solid supports [De Girolamo et al. 2011]. Aptamers are single-stranded oligonucleotide chains that can recognize selectively a target molecule with high affinity and specificity, comparable to that of antibodies. They can be produced to show affinity towards numerous analytes such as divalent metallic ions, small organic molecules, proteins or cells [Plotka-Wasylka et al. 2016]. The aptamers that reveal affinity to a target molecule are selected from libraries that contains up to 10^{15} oligonucleotides sequences using an iterative *in-vitro* procedure based on selection and amplification of the aptamers. This iterative procedure is known as "Systematic Evolution of Ligands by Exponential Enrichment" (SELEX). SELEX is done in a buffer media, specific in each case, named selection or binding buffer (BB). The BB ensure the best disposal of the aptamers to recognize the target molecule [Madru et al. 2009].

Once a suitable sequence is identified, it can be easily prepared via chemical synthesis, avoiding the use of animals as are required for antibodies production. Aptamer synthesis allows to introduce some modifications in the sequence to enhance the stability, specificity or immobilization of the aptamer. Nowadays, aptamer application to SPE is not a very explored field compared to other sorbents, however, selectivity offered by them ensure a promising tool for future research trends as SPE sorbents. Immobilization of aptamers onto solid supports to create oligosorbents can be adapted easily from the strategies developed for antibody immobilization. The selected strategy depends strongly of the nature of functional groups placed at the end of the oligonucleotides sequence. Generally, there are three functional groups commercially available in aptamers modification: amino, thiol and biotin. Those modifications, typically applied for other purposes, makes the immobilization step easy to develop. [Madru et al. 2011].

The main aim of this study is to immobilize aptamers onto a solid support to develop oligosorbents for SPE and magnetic dispersive solid-phase extraction (MDSPE) of fluoroquinolones and methamphetamine from biological matrices. Oligosorbents were prepared in two different solid supports, activated sepharose and magnetic beads, in order to select the one that offers better capacities for drug determination. After immobilization, the prepared oligosorbents will be also studied in terms of selectivity toward different related compounds to evaluate the cross-selectivity offered. Finally, if they offer good recoveries values and capacities

they will be applied for extraction of fluoroquinolones and methamphetamine from field biological samples.

Experimental

Materials and reagents

Aptamers selective towards fluoroquinolones FQ1 *(5'-ATA-CCA-GCT-TAT-TCA-ATT-GCA-GGG-TAT-CTG-AGG-CTT-GAT-CTA-CTA-AAT-GTC-GTG-GGG-CAT-TGC-TAT-TGG-CGT-TGA-TAC-GTA-CAA-TCG-TAA-TCA-GTT-AG-3')* and FQ2 *(5'-ATA-CCA-GCT-TAT-TCA-ATT-AGT-TGT-GTA-TTG-AGG-TTT-GAT-CTA-GGC-ATA-GTC-AAC-AGA-GCA-CGA-TCG-ATC-TGG-CTT-GTT-CTA-CAA-TCG-TAA-TCA-GTT-AG-3')* were purchased from Eurogentec (Seraing, Belgium). Aptamer selective to methamphetamine, MA *(5'-ACG-GTT-GCA-AGT-GGG-ACT-CTG-GTA-GGC-TGG-GTT-AAT-TTG-G-3')* was prepared by IDT (Leuven, Belgium). All aptamers employed in this study were modificated introducing amino groups in the extreme 5' with spacer arms C12 for FQ1 and FQ2 and C6 for MA. Table 51 summarizes the most important specifications of the aptamers aforementioned.

Magnetic beads (MB) LOABeads™ AffiActive were acquired from Labonabead (Uppsala, Sweden). Two different CNBr-activated sepharose were purchased from GE Healthcare (Madrid, Spain) and from Sigma (Saint-Quentin Fallavier, France).

Fluoroquinolones solid standards used in the study were marbofloxacin, ofloxacin, levofloxacin, pefloxacin, ciprofloxacin, enrofloxacin and moxifloxacin and they were provided by Sigma (Saint-Quentin Fallavier, France). A stock solution of each fluoroquinolone at 1000 mg L^{-1} was prepared in acetonitrile. Methamphetamine stock solution, prepared in acetonitrile at 1000 mg L^{-1} was purchased from Sigma (Stenheim, Germany). All buffer constituents and organic solvents were obtained from Scharlab (Barcelona, Spain).

Instrumentation

An Agilent 1200 series (Agilent Technology, Massy, France) RRLC (Rapid Resolution LC) system equipped with a binary pump, an autosampler, and a diode array detector controlled by Chemstation software was used for LC-UV acquisitions. Ofloxacin was quantified using a Waters SymmetryShield RP18 column (150 × 2.1 mm i.d., 5 µm, Waters, Saint-Quentin-en-Yvelines, France) maintained at 35 °C. Gradient elution mode was chosen using as mobile phase 50 mM citrate buffer pH 2.7 and acetonitrile. The organic solvent content was increased from 10 % to 50 % in 30 min using a flow rate of 0.2 mL min^{-1}. The detection of ofloxacin was done at 300 nm.

Table 51. *Specifications of aptamers selective towards fluoroquinlones (FQ1 and FQ2) and methamphetamine (MA).*

Name	FQ1
Sequence	*5'-/5AmMC12/-ATA-CCA-GCT-TAT-TCA-ATT-GCA-GGG-TAT-CTG-AGG-CTT-GAT-CTA-CTA-AAT-GTC-GTG-GGG-CAT-TGC-TAT-TGG-CGT-TGA-TAC-GTA-CAA-TCG-TAA-TCA-GTT-AG-3'*
Number of bases	98
Molecular weight (g mol^{-1})	30583.1
Selection buffer (SELEX)	100 mM NaCl, 20 mM Tris-HCl pH 7.6, 2 mM $MgCl_2$ and 1 mM $CaCl_2$
Name	FQ2
Sequence	*5'-/5AmMC12/-ATA-CCA-GCT-TAT-TCA-ATT-AGT-TGT-GTA-TTG-AGG-TTT-GAT-CTA-GGC-ATA-GTC-AAC-AGA-GCA-CGA-TCG-ATC-TGG-CTT-GTT-CTA-CAA-TCG-TAA-TCA-GTT-AG-3'*
Number of bases	98
Molecular weight (g mol^{-1})	30262.8
Selection buffer (SELEX)	100 mM NaCl, 20 mM Tris-HCl pH 7.6, 2 mM $MgCl_2$ and 1 mM $CaCl_2$
Name	MA
Sequence	*5'-/5AmMC6/-ACG-GTT-GCA-AGT-GGG-ACT-CTG-GTA-GGC-TGG-GTT-AAT-TTG-G-3'*
Number of bases	40
Molecular weight (g mol^{-1})	12673.3
Selection buffer (SELEX)	200 mM NaCl, 20 mM Tris-HCl pH 7.4 and 5 mM $MgCl_2$

An IONSCAN-LS from Smiths Detection (Morristown, NJ, USA) with a ^{63}Ni foil radioactive ionization source was used to separate and identify the methamphetamine involved in this study. IM station software (version 5.389) from Smiths Detection was used to acquire and process data. Positive ion mode was used with nicotinamide as internal calibrant, with a reduced mobility constant (K_0) of 1.860 $cm^2\ V^{-1}\ s^{-1}$. A 25 ms scan period using a 0.2 ms shutter grid width, 60 segments per analysis, and 479 data points conditions were employed for plasmagram acquisition. A counter flow dry air at 275 mL min^{-1} was used as a drift

gas. The electric field strength in the drift region was 251 V cm $^{-1}$, with 1759 V total drift voltage, and a 7 cm drift tube length. The extract of the sample was introduced by thermal desorption onto a polytetrafluoroethylene (PTFE) membrane. Hence, 3 µL sample extract was placed onto the PTFE membrane, using a 1 s post-dispense delay to introduce the sample in the heated zone. Desorption, inlet, and drift tube temperatures were adjusted to 255, 260 and 237 °C, respectively. After 30 s of heating, the membrane was removed from the heating zone.

Aptamer grafting yield was estimated after concentration measurement in the different washing fractions after immobilization. Concentration was measured with a NanoDrop™ 1000 spectrophotometer (Thermo Scientific, Waltham, USA) using single stranded-DNA function and the respective buffer as a blank. The concentration was calculated based on the Lambert-Beer equation using an extinction coefficient of 33 cm mL^{-1}. The amount of immobilized aptamer was calculated via subtraction of the amount of aptamers remaining in solution after immobilization, including the aptamers found in the washing fraction.

Determination of grafting yield was also done by HPLC-UV using ion pairing. For this purpose, 25 µL of the recovered fractions after immobilization were measured using a Waters Atlantis C18 column (100 x 3 mm, 3 µm) with a column temperature set at 40 °C. The elution was performed in isocratic mode employing as mobile phase a mixture 0.1 M triethanolammonium (pH 7.0): acetonitrile (85:15, v/v) with a flow rate of 0.5 mL min $^{-1}$. The wavelength was set at 260 nm for DNA determination.

Immobilization procedure

Three different immobilization approaches were used in this study. Procedure A and procedure B were used for amino-modified aptamer grafting onto CNBr-activated sepharose and the procedure C was used to immobilize the aptamers to magnetic beads. The three applied procedures are described in more detail below.

Procedure A: An aptamer solution (1 g L^{-1}) was prepared in 200 mM Na_2HPO_4 and 5 mM $MgCl_2$, then it was heated at 75 °C during 5 min and equilibrated at room temperature during 30 min to renature it. In parallel, 35 mg of CNBr-activated sepharose were placed in an Eppendorf tube and it was washed six times with 1 mL HCl 1 mM and twice with distilled water. Then, an additional washing with 175 µL of solution containing 200 mM Na_2HPO_4 and 5 mM $MgCl_2$ was done. Aptamer solution was mixed with CNBr-activated sepharose overnight using rotatory mixer. The resulting oligosorbents were introduced into a 1 mL SPE cartridge and washed with 3 mL of 200 mM Na_2HPO_4. Washing fractions were collected for further

analysis. In order to block the remaining active groups of CNBr-activated sepharose, 0.1 M Tris solution was flushed during 2 h at room temperature. Finally, the obtained oligosorbent was washed 3 cycles using alternatively 3 mL acetate solution (0.1 M acetate + 0.5 M NaCl, pH 4.0) and 3 mL Trizma buffer (0.1 M Tris + 0.5 M NaCl, pH 8.0) to remove the non-covalently adsorbed aptamer.

Procedure B: In this immobilization strategy, aptamer solution (0.5 g L^{-1}) was prepared in 0.1 M $NaHCO_3$ pH (8.3) and 0.5 M NaCl. In parallel, 0.15 g CNBr-activated sepharose were introduced into a 3 mL SPE cartridge and washed using 2 mL HCl 1 mM. Then, additional 30 mL HCl 1 mM were flushed through the cartridge followed by 5 mL coupling buffer (0.1 M $NaHCO_3$ + 0.5 M NaCl, pH 8.3). Then, the aptamer solution was added to cartridge containing CNBr-activated sepharose and it was covered by the top and bottom. Aptamer solution and CNBr-activated sepharose were mixed for 1 h at room temperature. After 1 h coupling time, sorbent was washed using 5 mL coupling buffer. The coupling buffer used for washing step was kept for further analysis. In order to block the remaining active groups, 0.1 M Trizma solution pH 8.0 was employed to suspend the sorbent and it was agitated during 2 h. Finally, the oligosorbents prepared were washed three times alternatively using 5 mL of acetate solution (0.1 M sodium acetate + 0.5 M NaCl, pH 4.0) and 5 mL of Tris solution (0.1 M Tris-HCl + 0.5 M NaCl, pH 8.0) to remove the non-bonded aptamer.

Procedure C: MB solution contains 10 % of magnetic particles suspended in phosphate buffer saline (PBS) in the presence of 20 % ethanol. 0.2 mL MB suspension were placed in an Eppendorf tube and the supernatant was removed. Then, MB were dispersed in 0.2 mL PBS solution containing 0.1 % Tween 20. PBS-Tween 20 solution was removed and MB were dispersed again in PBS-Tween 20 by adding 10 µL activation buffer. After 15 min the supernatant was removed and the MB were washed three times with 0.2 mL PBS. Then, 0.2 mL of the aptamers solution were added to the MB and they were dispersed. In this strategy, aptamer solution was prepared at 100 µM in PBS and renatured during 10 min at 95°C followed by 10 min at 4°C. The MB and aptamer solution were slightly stirred during 8 hours and the supernatant was kept for further analysis. Then, MB were washed three times with PBS solution. The washing fractions were kept and analyzed by Nanodrop™. Finally, MB were dispersed in 0.2 mL of PBS and 20 µL blocking buffer were added and shaken 45 min to block the remaining active groups. Finally, MB were washed with PBS and they were kept in 0.2 mL of PBS at 4 °C until their use.

Results and discussion

Analytical features of instrumental methods

Fluoroquinolones selected for this study: marbofloxacin, ofloxacin, levofloxacin, pefloxacin, ciprofloxacin, enrofloxacin, and moxifloxacin were analyzed by high performance liquid chromatography with ultraviolet detector (HPLC-UV). As it can be seen in Table 52, LODs obtained ranged from 11 to 40 μg L^{-1}. All fluoroquinolones were prepared from a stock solution by dilution in water:acetonitrile (90:10, v/v) in a calibration range from limit of quantification (LOQ) to 1000 μg L^{-1} achieving coefficient of determination (R^2) up to 0.991. Wavelenghts used for fluroquinolones determination were 300 nm for marboflloxacin, ofloxacin, levofloxacin and moxifloxacin; and 280 nm for pefloxacin, ciprofloxacin and enrofloxacin.

Table 52. *Analytical features of fluoroquinolones used in this study by HPLC-UV.*

Analyte	Retention time (min)	Wavelenght (nm)	LOD (μg L^{-1})	LOQ (μg L^{-1})	Linear range (μg L^{-1})	Linearity (R^2)
Marbofloxacin	9.8	300	40	130	130-1000	0.991
Ofloxacin	12.9	300	17	60	60-1000	0.9992
Levofloxacin	13.2	300	30	110	110-1000	0.994
Pefloxacin	14.4	280	30	100	100-1000	0.998
Ciprofloxacin	15.3	280	11	40	40-1000	0.993
Enrofloxacin	24.2	280	40	130	130-1000	0.996
Moxifloxacin	33.4	300	31	110	110-1000	0.9990

Methamphetamine was analyzed by IMS. After an appropriate dilution of stock solution in 2-propanol, methamphetamine standards were injected providing a drift time of 10.778 ms, corresponding to a K_0 value of 1.638 $cm^2 V^{-1} s^{-1}$. IMS provided a LOD and LOQ for methamphetamine of 9 and 30 μg L^{-1}, respectively. Thus, calibration curve was prepared from 30 to 250 μg L^{-1} with a coefficient of determination of linearity of 0.998 (R^2) (see Table 53).

Table 53. *Analytical features of methamphetamine determination by IMS.*

Parameter	Value
K_0 (cm^2 V^{-1} s^{-1})	1.638
Drift time (ms)	10.778
LOD (µg L^{-1})	9
LOQ (µg L^{-1})	30
Linear range (µg L^{-1})	30-250
Linearity (R^2)	0.998

Fluoroquinolones aptamer immobilization

FQ1 and FQ2 aptamers were selected from previous studies reported by Reinemann and coworkers who used these aptamer sequences for water monitoring of quinolones. Aptamers were selected after 8 rounds of SELEX procedure using ofloxacin as target molecule [Reinemann et al. 2016].

FQ1 and FQ2 were immobilized onto CNBr-activated sepharose following the procedure A, previously used for the immobilization of aptamers selective towards ochratoxin A [Hadj-Ali et al. 2014]. After the analysis of the washing fractions containing the non-bonded aptamers by ion pairing LC-UV method, 34 µg FQ1 were immobilized onto the CNBr-activated sepharose and 20 µg FQ2. These amounts of immobilized aptamers correspond to a grafting yield of 22.5 % and 13.3 % of FQ1 and FQ2 (see Figure 86), respectively.

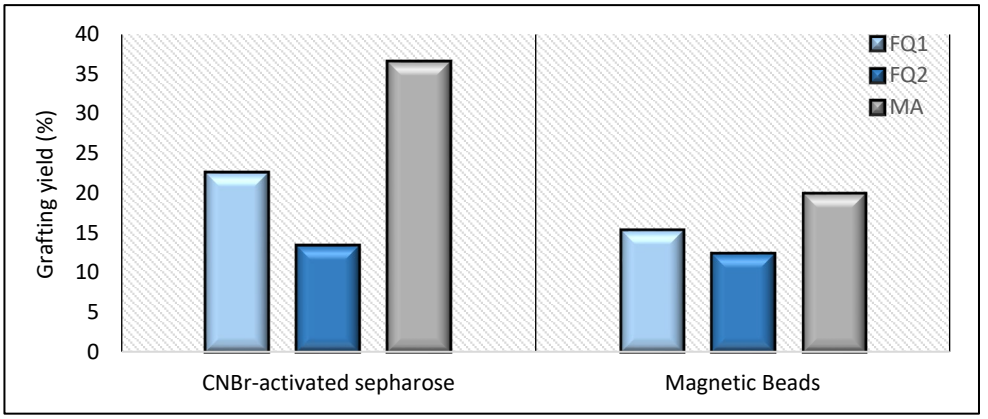

Figure 86. *Grafting yield of FQ1, FQ2 and MA aptamers achieved onto CNBr-activated sepharose and magnetic beads.*

In order to study the selectivity and binding capacity of the prepared oligosorbents, different extraction procedures were performed. All of them used as conditioning, loading and washing solvent the binding buffer for fluoroquinolones (BBFQ). BBFQ is the buffer used for aptamers selection via SELEX which ensures the suitable aptamers disposition to recognize the target molecule. BBFQ contained 100 mM NaCl, 20 mM Tris-HCl pH 7.6, 2 mM $MgCl_2$ and 1 mM $CaCl_2$ in water. For the conditioning step (to prepare the oligosorbent to adsorb the target molecule) 1 mL BBFQ was used. Loading solutions were prepared containing 40 ng ofloxacin for FQ1 and 24 ng for FQ2 in different volumes of BBFQ. Loading volumes were evaluated between 0.2 to 1 mL in order to establish the best loading volume to avoid overcome the breakthrough volume. BBFQ was used also as washing solvent and in the same way, different volumes (from 0.1 to 1 mL) were tested. Concerning to elution solvent a mixture acetonitrile:water (40:60, v/v) was used at different volumes (0.2 - 1 mL). All the extraction procedures evaluated were done at 4 °C and room temperature to verify the influence of temperature in the extraction. All the extractions performed reveal a low affinity of the oligosorbents towards ofloxacin. In all of the studied cases, the ofloxacin recovered in the elution fraction was under LOQ, while the most part of the loaded ofloxacin was obtained in the loading and washing fractions.

For example, as shown in Figure 87A, 1 mL as conditioning step, 1 mL as loading volume, 1 mL for washing and an elution performed using 0.4 mL at room temperature, the recoveries obtained in the loading solution were 56 and 64 % for FQ1 and FQ2, respectively, while recoveries for washing fraction were 16 and 13 % for FQ1 and FQ2, respectively. In the same extraction procedure, elution fraction reveals no analyte presence. The best recoveries profiles for those aptamers immobilized onto CNBr-activated sepharose were performing a extraction at 4 °C using 1 mL of BBFQ as conditioning, 0.1 mL as loading volume, 0.1 mL BBQF for washing and elution in 0.2 mL of acetonitrile:water (40:60, v/v). As it can be seen at Figure 87B, low recoveries of 43 and 36 % in the elution fraction for FQ1 and FQ2 were found. The low volumes used for loading and washing step demonstrate that even when sample is percolated with reduced volumes, the oligosorbents did not show affinity toward ofloxacin.

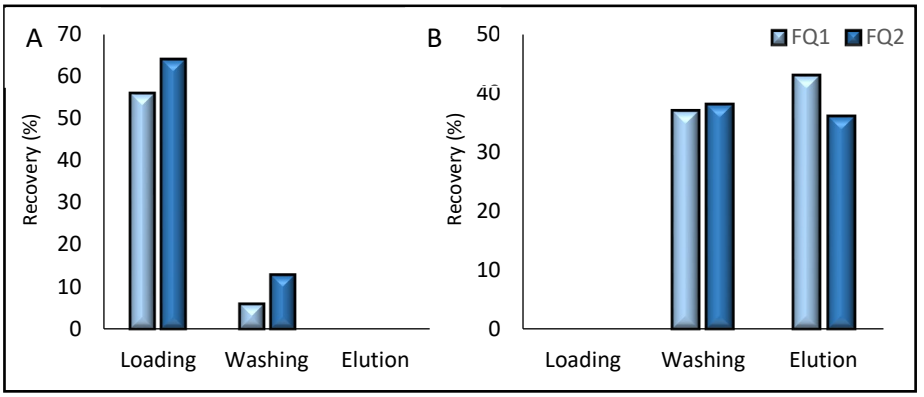

Figure 87. *Recoveries obtained for FQ1 and FQ2 using different extraction conditions: (A) 1 mL conditioning, 1 mL loading, 1 mL washing and 0.4 mL elution at room temperature, (B) 1 mL conditioning, 0.1 mL loading, 0.1 mL washing and 0.2 mL elution at 4 °C.*

The same procedures were repeated for the extraction of marbofloxacin, levofloxacin, pefloxacin, ciprofloxacin, enrofloxacin and moxifloxacin in order to verify the poor affinity of oligosorbents to all fluoroquinolones as well as ofloxacin. Recoveries obtained for them provided similar elution profile than that found for ofloxacin with a lack of presence of analytes in the elution fraction.

Considering the low extraction capacity of oligosorbents prepared using CNBr-activated sepharose as solid support, MB were used as immobilization support. FQ1 and FQ2 were immobilized onto MB following the procedure C, previously described by Pero-Gascon and coworkers [Pero-Gascon et al. 2020]. The grafting yield was calculated hereon by measuring PBS fractions collected after immobilization. Those washing fractions were analyzed by Nanodrop™. This strategy achieves to immobilize 24 µg FQ1 and 19 µg FQ2. The amount of immobilized aptamers entails grafting yields of 15.3 and 12.3 % for FQ1 and FQ2 (see Figure 86). BBFQ was used following typical procedure of MDSPE: conditioning (0.3 mL), loading (0.2 mL) and washing (0.2 mL) solvent and acetonitrile:water (40:60, v/v) was used as elution solvent. The extraction proposed using MB is MDSPE, on this account a magnet was used to separate the MB from the sample or solvent. In MDSPE, a critical parameter is the agitation time of the loading step to ensure and appropriate mass transfer from the loading solution to the sorbent. Loading extraction times of 5, 15, 30 and 60 min were evaluated using as loading solution 0.2 mL at 140 and 112 µg L^{-1} ofloxacin prepared in BBFQ for FQ1 and FQ2, respectively. The studies were done at 4 °C and room temperature. Similar results for aptamers immobilized onto MB than CNBr-activated sepharose were obtained, where ofloxacin was eluted during loading and washing steps. Besides, in all

performed studies, the content of ofloxacin obtained in the elution fractior was under LOQ.

Methamphetamine aptamer immobilization

MA was previously used for methamphetamine determination in saliva and plasma by paperspray ionization – IMS by Zargar and coworkers [Zargar et al. 2018] after SELEX procedure described by Ebrahimi and coworkers [Ebrahimi et al. 2013]. This aptamer was selected to be immobilized onto CNBr-activated sepharose and MB.

Taking into account to the low efficiency of the protocol described to immobilize FQ aptamers onto CNBr-activated sepharose, procedure B was used for MA immobilization onto this support. This procedure was previously reported for antibodies immobilization developed by our group [Orellana-Silla et al. 2018]. Due to amino group of the aptamers, its attachment onto the CNBr-activated sepharose was similar to that found for of antibodies immobilization, where an amino nucleophilic group reacts to the cyanate ester groups under mild conditions. After MA immobilization, washing fractions of the non-bonded aptamers were measured by Nanodrop™. The amount of MA grafted onto sepharose was calculated by difference of the total amount of aptamers and the non-bonded aptamers recovered. Thus, according to the 192 µg of aptamer immobilized, the grafting yield was estimated to be 36.6 % (see Figure 86).

In parallel, MA was immobilized onto MB following the procedure C. Washing PBS fractions were collected to remove the non-bonded aptamers and they were measured by Nanodrop™ to evaluate the grafting yield. Thus, the amount of MA immobilized onto 0.2 mL of the MB suspension was 28 µg, which represents a grafting yield of 20 % of the total aptamers introduced (see Figure 86).

In order to evaluate the extraction capacity of both prepared oligosorbents towards methamphetamine, different extraction procedures were evaluated in SPE as well as MDSPE. The first binding buffer for methamphetamine (BBMA1) evaluated was the selection buffer for the SELEX procedure, which contains 200 mM NaCl, 20 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$ [Ebrahimi et al. 2013]. For the oligosorbent prepared using sepharose as solid support, 226 ng methamphetamine were used in the loading step in different volumes of BBMA1. After conditioning of SPE cartridge with 1 mL BBMA1, loading solution was percolated using different volumes (0.2 - 1 mL). Similarly, washing volumes were evaluated between 0.1 and 1 mL. The elution was performed using 1 mL of 2-propanol. MDSPE was also evaluated used MA immobilized onto MB. For this purpose, 34 ng of

methamphetamine used in loading step (0.2 mL) were percolated after conditioning of sorbent using BBMA1 (0.2 mL). Different loading times were evaluated including 5, 15, 30 and 60 min to guarantee the mass transfer to the sorbent. BBMA1 was used as washing solvent (0.2 mL) and 0.2 mL of 2-propanol were used for methamphetamine elution. All the studies were performed at room temperature and 4 °C.

In the study published by Zargar and coworkers, another binding buffer was employed for loading methamphetamine to the immobilized aptamer. This binding buffer (BBMA2) was constituted by 20 mM NaCl, 5 mM Tris HCl pH 7.4, and 10 mM $MgCl_2$ [Zargar et al. 2018]. Hence, the extraction procedure performed using BBMA1 were repeated after changing by BBMA2.

All the studies performed revealed an elution of methamphetamine during loading (58-70 %) and washing (29 – 38 %), with concentrations below LOD in the elution fraction. Thus, non-retention of methamphetamine was demonstrated as well as FQ1 and FQ2. In MA immobilized onto CNBr-activated sepharose as well as MB, recoveries of the elution fraction were negligible, showing poor affinity of the oligosorbents toward methamphetamine.

Analysis of results

The obtained results have demonstrated a lack of affinity of the prepared oligosorbents towards target molecules. Even using the supposed best conditions to ensure the recognition, that is using the binding buffer as media, the oligosorbents have shown a lack of affinity. Two reasons could explain this fact.

The first reason to explain the lack of retention is the negligible presence of active aptamers bonded to the solid support. Even if it is considered the low grafting yield of aptamers onto the CNBr-activated sepharose with values of 22.5, 13.3 and 36.6 % for FQ1, FQ2 and MA, respectively, the binding capacities of aptamers if we supposed that the total amount of active aptamers bonded is a 10 % must be 0.556, 0.330 and 1.70 nmol of target molecule per gram of oligosorbent for FQ1, FQ2 and MA, respectively. These binding capacities were in the range of normal values assumed for oligosorbents prepared using sepharose as solid support (typically 0.26 – 69 nmol g^{-1}) [Pichon et al. 2020]. To calculate the binding capacity is common to assume that 35 mg of dry sepharose are equally to 200 mg of oligosorbent. But, this reasoning to assume a low ratio of active aptamers can be difficult to explain, usually percentages ranging from 19 to 37 % of active aptamers regarding the total of immobilized aptamers are found using those immobilization procedures [Pichon et al. 2020].

In the same way, MB immobilization procedure achieved grafting yields of 15.3, 12.3 and 20 % for FQ1, FQ2 and MA, respectively. These immobilization yields if it is assumed that only a 10 % of the aptamers immobilized are functional must reveal binding capacities of 0.134, 0.106 and 0.382 nmol g^{-1}. If the obtained values are compared with values reported in literature (0.094 - 0.236 nmol g^{-1}) [Kölkpinar et al. 2011], it can be interpreted that less active aptamers are immobilized onto MB.

Another reason to explain the lack of selectivity towards the target molecules could be imputable to the denaturation of the immobilized aptamers onto the solid supports. Non-controlled factor presents in the BB selected for analyte extraction could interrupt the linkage between aptamers-target that need a deep study of the SPE/MDSPE conditions.

Conclusions

Different aptamers selective to fluoroquinolones and methamphetamine have been selected from bibliography in order to immobilize them onto CNBr-activated sepharose and MB. Aptamers were prepared by introducing an amino group to the 5' side and an spacer arm C12/C6. Different immobilization procedures were carried out following the guidelines described by previous reports to immobilize amino groups onto the aforementioned solid supports. Even considering the promising results offered by oligosorbents for the SPE/DSPE of complex matrices such as biological fluids, the binding assays of the prepared oligosorbents have demonstrated a lack of affinity towards the selected target molecules. The grafting yields obtained in both solid supports (12.3 – 36.6 %) and the ratio of active aptamers from the total of immobilized usually found for reported oligosorbents should reveal functional aptamers for the extraction of fluoroquinolones and methamphetamine. However, due to unidentified reasons the prepared oligosorbents have not able to retain selected target molecules. Thus, a deep study of the immobilization procedure or extraction conditions must be performed in future studies to guarantee the presence of functional aptamers grafted onto the solid supports to recognize the target molecules and perform the planned selectivity assays and their application to field biological samples.

Comments

It must be highlighted that results of present study are not published in any cientific journal and it shows the preliminary results of different studies performed between "l'École supérieure de physique et de chimie industrielles de la ville de Paris" during an internship and the "Universitat de València"

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DISCUSSIÓ DE RESULTATS

RESULTS
DISCUSSION

Al llarg d'aquest apartat es mostraran i s'analitzaran els resultats més importants de la recerca portada a terme al llarg d'aquesta Tesi Doctoral. D'aquesta manera s'extrauran les observacions més rellevants de la recerca bibliogràfica realitzada al llarg de Bloc 1 i es mostraran les qualitats i avantatges més destacables dels materials tant de caràcter genèric com selectiu així com dels nous formats desenvolupats al llarg dels Blocs 2 i 3.

Al primer bloc es fa un recull de l'aplicació de materials intel·ligents per a l'anàlisi de mostres de diferent natura (biològiques, ambientals, medicaments, aliments...) mitjançant IMS, així com també s'aprofundeix en l'aplicació que han tingut els materials intel·ligents en general per a l'anàlisi forense, incloent-hi l'anàlisi de drogues.

En general, es pot afirmar que la limitada selectivitat que proporciona l'IMS pot ser ben resolta amb l'aplicació de materials intel·ligents com els MIPs, immunosorbents o aptàmers que permeten aïllar de manera selectiva l'anàlit abans de la seua determinació. Altres materials, com els ILs o CNTs per al tractament previ de la mostra si bé no aporten una gran selectivitat, permeten eliminar la major part dels interferents presents a la mostra contribuint d'aquesta manera a millorar la sensibilitat del mètode. Tot i les bones perspectives de futur que proporcionen i els resultats satisfactoris que ha demostrat tenir la combinació material intel·ligent-IMS, el seu desenvolupament encara te un llarg camí a recórrer en quant a l'àmbit de la recerca. A causa del reduït nombre d'estudis que fan servir IMS com a tècnica analítica en comparació a altres tècniques com les cromatogràfiques, encara hi és necessari fer una major recerca en aquest sentit. Entre els materials intel·ligents que han estat emprats per combinar-los amb una determinació per IMS es poden diferenciar dos grups principals.

D'una banda els immunosorbents, aptàmers i MIPs són el grup de materials que ofereixen una major selectivitat essent capaços de reconèixer famílies de molècules i en molts casos una elevada especificitat cap a una única molècula determinada. Per la seua banda, els immunosorbents presenten com a principal avantatge la seua excel·lent selectivitat, a sovint superior a la d'altres materials selectius. No obstant, també presenten determinats punts febles com són la seua limitada disponibilitat al mercat, l'elevada dificultat d'obtenció a causa de que habitualment requereixen mètodes invasius per a determinats éssers vius o la seua limitada estabilitat, ja que poden ser desnaturalitzats amb relativa facilitat si són sotmesos a temperatures massa elevades, pH extrems o a l'acció de determinats dissolvents. Els aptàmers han demostrat tenir una gran quantitat d'avantatges que inclouen una molt bona selectivitat, comparable amb la que es pot obtenir amb els immunosorbents. La seua senzilla síntesi química els fa més

fàcils d'obtenir en comparació als anticossos i a més contempla la capacitat d'introduir modificacions que permeten facilitar la seua immobilització i fer-la més efectiva. La principal limitació que presenten els aptàmers es troba relacionada amb la seua estabilitat. Si bé aquests materials són estables, per tal de conservar la seua estructura i que aquesta siga funcional durant l'extracció, la mostra ha de trobar-se en un medi determinat que no modifique la capacitat de reconèixer l'anàlit. Tanmateix, les condicions de conservació han de ser ben controlades per evitar un malbaratament de l'aptàmer. Finalment, en aquest grup de materials, els MIPs presenten algunes característiques úniques per la seua natura química com ho són la gran estabilitat mecànica i inèrcia química que permeten que les condicions de conservació i ús no siguin massa rigoroses. Altre avantatge és que en comparació, els MIPs són més senzills de preparar, podent ser preparats fàcilment inclús al laboratori i per tant menys costosos d'obtenir. Si bé és cert que habitualment la seua selectivitat no és tan elevada com la d'anticossos o aptàmers, és comú observar que presenten una capacitat de càrrega prou més elevada.

D'altra banda, el segon grup de materials que es recull a aquest primer bloc són els ILs així com alguns nanomaterials, entre els que s'hi destaquen especialment els CNTs. Aquests presenten una menor selectivitat cap a molècules determinades però permeten eliminar en gran mesura els interferents de la matriu augmentant així la sensibilitat del mètode analític. Així, pot ser necessari que la selectivitat no aconseguida per aquests materials pugui ser compensada si es recorre a separacions cromatogràfiques prèvies a la determinació per IMS o una determinació mitjançant MS després de la separació per IMS. En aquest sentit, cal destacar que els principals punts forts dels ILs es troben associats a l'elevada estabilitat tèrmica i reduïda pressió de vapor que proporcionen junt a la seua baixa inflamabilitat. A més, els líquids iònics són capaços d'interaccionar de manera preferent segons els grups funcionals dels anàlits. Tanmateix, els CNTs en general destaquen per les seues bones propietats mecàniques, químiques i electròniques que els fan uns candidats ideals per a ser emprats com a suports en el desenvolupament de sensors, però presenten una selectivitat molt baixa comparada amb tots els materials descrits anteriorment si hi són aplicats per a tractaments de mostra previs.

Altres materials que per les seues característiques poden ser associats al grup de materials intel·ligents, però amb menor selectivitat que MIPs, aptàmers o immunomaterials, són els RAMs, que permeten eliminar els efectes matriu a partir de l'exclusió de molècules de gran grandària. Els RAMs a més permeten la seua combinació amb altres materials selectius com els MIPs aconseguint amb la

combinació d'ambdós una gran selectivitat i una major capacitat de reutilització del material. També els MOFs presenten unes qualitats atractives per la seua elevada àrea superficial i possibilitat de funcionalitzar-los amb determinats grups, motiu pel qual es consideren materials amb una elevada capacitat de càrrega i moderada selectivitat.

Un aspecte important a destacar, és que entre els materials selectius dels que s'ha parlat, la seua aplicació per a l'anàlisi forense i en concret per a la determinació de drogues en fluids biològics ha estat majoritàriament en forma de sensors en el cas dels aptàmers i d'immunoassajos en el cas dels materials d'immunoafinitat. Per exemple, entre els immunoassajos, alguns han estat desenvolupats per a l'anàlisi de cocaïna en mostres com orina, saliva o sèrum aconseguint LODs d'entre 0,06 i 0,09 μg L^{-1} [Vidal et al. 2016]. També, els metabòlits de la cocaïna com la benzoilecgonina mitjançant l'ús d'un electroimmunosensor amb LODs de 0,41 μM [Yilmaz-Sengel et al. 2017] o altres drogues com morfina emprant un fluoroimmunoassaig en mostres d'orina on es van aconseguir LODs 0,2 μg L^{-1}, han estat descrits [Eldefrawi et al. 2000]. D'entre els aptàmers s'han emprat sensors per a la determinació de cocaïna i benzoilecgonina en mostres d'orina aconseguint LODs de 0,138 nM i 1,66 μM, respectivament [Guler et al. 2017].

Els materials genèrics com la sílice modificada amb C18 o C8, ofereixen una gran versatilitat a causa del tipus d'interaccions inespecífiques que promouen amb tots aquells anàlits que disposen d'una part apolar. És per aquest motiu que al projecte que s'engloba en aquesta Tesi Doctoral s'ha començat per avaluar l'aplicació d'aquest materials adsorbents, ja ben establerts, en un format de relatiu desenvolupament recent com és la MEPS. Els principals avantatges de la MEPS venen donats per la xicoteta quantitat d'adsorbent que fan servir i per la facilitat d'acoblar-la a automostrejadors o instruments semi-automàtics, cosa que dona lloc a mètodes d'extracció miniaturitzats i sovint automatitzats.

Si bé és cert, que drogues tradicionals com la cocaïna, amfetamina o diazepam ja disposen de nombrosos mètodes miniaturitzats per a la seua anàlisi en nombrosos tipus de matrius incloent-hi fluids biològics, altres drogues com les NPS de més recent aparició tenen un buit pel que respecta a mètodes d'anàlisi. L'aplicació de la MEPS emprant materials genèrics com a fases adsorbents pot suposar un interessant avançament en els mètodes que actualment es descriuen per a l'anàlisi d'aquestes drogues especialment en aquells laboratoris que disposen d'una limitada varietat de fases adsorbents per ser-hi emprades segons la situació.

Als Capítols 3, 4 i 5, s'han descrit mètodes diferents emprant la MEPS per a la l'extracció de diverses drogues englobades entre les NPS. Dicloropà i metilona van ser determinades per IMS, mentre que 5F-ADB, MMB-CHMICA, THJ-2201, CUMYL-4CN-BINACA i MDMB-CHMCZCA van ser analitzades per GC-MS, després de la MEPS. A més de la velocitat que acostuma a oferir l'IMS, tant per al dicloropà com per a la metilona es van obtenir bones sensibilitats amb LODs de 30 i 4 μg L^{-1}, respectivament. D'altra banda, per a la determinació dels cannabinoides sintètics es va emprar GC-MS com a tècnica analítica per evitar fenòmens de ionització competitiva o de coelució que farien difícil identificar o quantificar amb exactitud les substàncies consumides. En aquest cas, la combinació MEPS-GC-MS va presentar una bona separació dels anàlits i una sensibilitat comparable a la obtinguda per a dicloropà i metilona en IMS, amb LODs per als cinc cannabinoides estudiats d'entre 10 i 20 μg L^{-1}.

A l'hora de determinar qualsevol anàlit després d'un procediment de SPE o similar és de vital importància conèixer les millors condicions d'extracció per a cadascun dels anàlits a determinar. És per aquest motiu, que part important dels treballs enfocats a la determinació de les NPS en mostres de saliva ha estat dirigit al desenvolupament del mètode d'extracció per tal d'obtenir les millors condicions. Les fases sòlides seleccionades van ser de C18 i C8 a causa de les interaccions apolars que ofereixen quan les molècules estan en el seu estat neutre. En aquest sentit, no només la fase adsorbent seleccionada ha de ser considerada, sinó també el pH al que la mostra és carregada s'ha de controlar per garantir que la molècula es trobe en estat neutre i tingui suficient afinitat per l'adsorbent per quedar retinguda. Per a l'extracció del dicloropà, la fase sòlida seleccionada va ser la C8 després de realitzar un estudi on s'hi van observar diferències menudes pel que fa a la sensibilitat oferida per C8 i C18, amb pendents de rectes de calibratge emprant els dos adsorbents de 0,123 ± 0,002 i 0,101 ± 0,005 L μg^{1}, respectivament. No obstant, per a la resta d'anàlits, s'hi va seleccionar la fase C18 directament per la seua major apolaritat i consonància amb allò observat a la bibliografia pel que respecta a l'ús d'aquestes fases [Montesano et al. 2017].

Una vegada seleccionada la fase adsorbent, com s'ha esmentat anteriorment, entre les etapes crítiques per a l'extracció, s'hi troben els pH de les dissolucions de càrrega i rentat. Però no només els pH són importants, també s'ha de garantir una adequada transferència de massa de la dissolució de càrrega cap a l'adsorbent. En aquest sentit va ser necessari estudiar els diversos cicles d'aspiració de la dissolució de càrrega i la d'elució. També, s'hi va estudiar el volum necessari d'elució, per garantir una adequada retenció i posterior elució de l'anàlit. A la

Taula 54, es poden observar els procediments d'extracció resultants després dels corresponents estudis per a la determinació tant del dicloropà i la metilona com dels cannabinoides sintètics.

Taula 54. *Procediments de MEPS aplicats per a l'extracció de dicloropà, metilona i dels cinc cannabinoides sintètics (5F-ADB, MMB-CHMICA, THJ-2201, CUMYL-4CN-BINACA i MDMB-CHMCZCA).*

	Dicloropà	**Metilona**	**Cannabinoides sintètics**
Adsorbent	C8	C18	C18
Condicionament	100 µL 2-propanol (x3) 100 µL aigua (x2)	100 µL 2-propanol (x1) 100 µL aigua (x1)	100 µL 2-propanol (x1) 100 µL aigua (x1)
Càrrega	90 µL mostra + 10 µL tampó fosfat (pH 7,0 - 1 M) (x4)	90 µL mostra + 10 µL tampó carbonat (pH 9,0 - 1 M) (x5)	96 µL mostra + 4 µL tampó fosfat (pH 7,0 - 2,5 M) (x5)
Rentat	100 µL aigua (x1)	100 µL tampó carbonat (pH 9,0 - 0,1 M) (x1)	100 µL aigua (x1)
Elució	50 µL 2-propanol (x10)	100 µL 2-propanol (x5)	50 µL 2-propanol (x5)

Després d'obtenir les millors condicions d'extracció per a cadascun dels anàlits, els mètodes hi van ser aplicats per a la determinació de mostres de saliva reals que van ser fortificades prèviament a diferents nivells de concentració. Tal i com es pot veure a la Figura 88, s'hi van obtenir recuperacions mitjanes de 96 ± 11 % per al diclopà a nivells de concentració d'entre 250 i 750 µg L^{-1}. Per a les mostres de saliva que contenien metilona, addicionades entre 50 i 400 µg L^{-1}, les recuperacions mitjanes que es van observar van ser de 85 ± 6 %. Tanmateix, diverses mostres de saliva van ser addicionades amb els cinc cannabinoides sintètics a concentracions de 125 i 250 µg L^{-1}, mostrant recuperacions mitjanes de 101 ± 2, 97 ± 8, 91 ± 3, 101 ± 5 i 64 ± 3 % per al 5F-ADB, MMB-CHMICA, THJ-2201, CUMYL-4CN-BNACA i MDMB-CHMCZCA, respectivament.

Amb aquests resultats, la MEPS va demostrar ser una tècnica d'extracció fàcilment adaptable d'una SPE, però en la qual es va aconseguir reduir volums de

l'ordre de mL, típicament emprats en procediments de SPE convencionals fins emprar volums no superiors a 100 µL en cada etapa del procediment d'extracció.

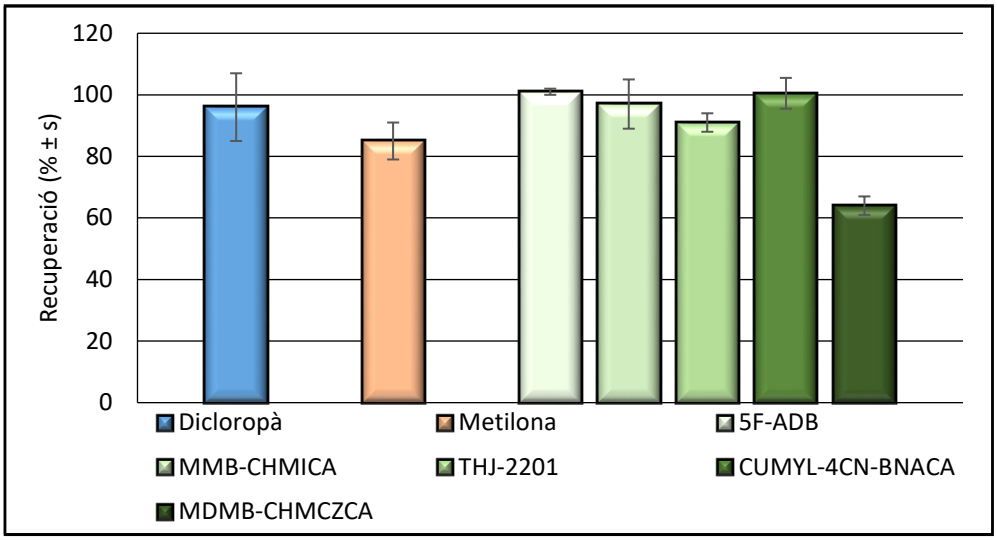


Figura 88. *Recuperacions obtingudes després d'una MEPS per al dicloropà, metilona i els cinc cannabinoides sintètics de tercera generació en mostres de saliva a nivells de concentració d'entre 50 i 750 µg* L^{-1}*.*

D'entre els materials considerats com materials genèrics hi podem trobar aquells com els que s'han emprat per a la MEPS que proporcionen interaccions inespecífiques i per tant només són capaços de retenir sobre ells aquelles molècules amb un marcat caràcter apolar. Aquest materials descarten automàticament totes aquelles molècules que presenten algun tipus de càrrega o grups ionitzats. També cal destacar que algunes limitacions que presenten són els baixos factors de preconcentració que aconsegueixen oferir. Per aquest motiu, diversos materials típicament reconeguts com materials de tipus mixt han sorgit des del desenvolupament de la SPE per tal d'afavorir les retencions entre la fase adsorbent bé sigui mitjançant interaccions hidròfobes produïdes per part de la fase sòlida, o mitjançant mecanismes de bescanvi iònic produïts per determinats grups funcionals amb els que es poden funcionalitzar aquestes fases sòlides.

En aquest sentit, a la Tesi es va plantejar la síntesi d'un material de tipus mixt basat en MAA i EGDMA per a la determinació de drogues en saliva. A més, dins d'altre dels objectius d'aquesta Tesi Doctoral el material preparat va ser adaptat a un nou format d'extracció en SPE com ho és l'extracció en puntes de micropipeta. Per aquest motiu, el polímer va ser preparat en forma de monòlit ancorat sobre les parets internes de puntes de micropipeta de PP amb una capacitat de 200 µL.

Per a la preparació de les puntes en primer lloc va ser fonamental la funcionalització de les seues parets internes. Aquesta funcionalització es va portar

a terme emprant benzofenona i un posterior tractament amb EGDMA tal i com es descriu detalladament al Capítol 6 per permetre l'adequada unió covalent entre la superfície interna de la punta i el monòlit format. Després d'avaluar diverses mescles de polimerització on es van emprar dissolvents porogènics de diferent natura, així com diverses proporcions d'aquests es va triar com a millor mescla de polimerització la que contenia un 40 % de monòmers respecte un 60 % de dissolvent porogènic, el qual va ser dodecanol:toluè (9:1, v/v). Pel que fa als monòmers, la mescla d'ells estava constituïda per un 8 % (m/m) de MAA i un 92 % (m/m) d'EGDMA. A la mescla de polimerització se li van afegir 5 mg d'AIBN per iniciar la reacció radicalària. A la Figura 89, es pot veure un esquema simplificat del procediment seguit per a la síntesi de les puntes de micropipeta.

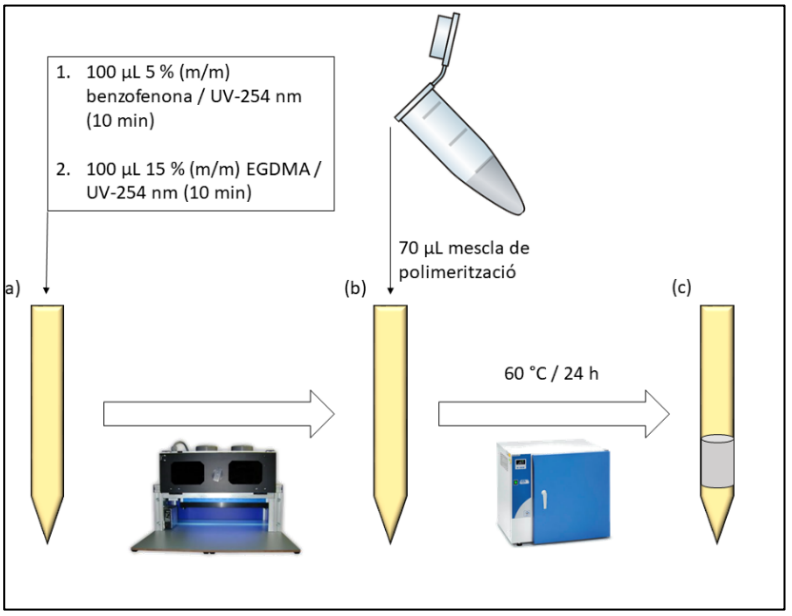


Figura 89. *Esquema bàsic del prcediment seguit per a la preparació de les puntes de micropipeta: (a) funcionalització de les parets internes de la punta, (b) introducció de la mescla de polimerització i (c) obtenció del polímer ancorat sobre la punta*

En aquest estudi de l'ús de *poly (MAA-co-EGDMA)* en puntes de micropipeta, es va seleccionar com a droga model per a desenvolupar el procediment d'extracció l'α-PVP de la mateixa manera, i amb aquesta droga es van avaluar les característiques principals del mètode. Després d'obtenir recuperacions quantitatives (107 ± 12 %) per a mostres de saliva addicionades amb 100 µg L^{-1} d'α-PVP, es van estimar la capacitat de càrrega (152 ng per punta) i la seua capacitat de reutilització (15 usos per punta). Posteriorment, aplicant el mètode desenvolupat per a l'α-PVP es van extraure 23 drogues diferents que incloïen NPS

i drogues tradicionals afegint metilona-d_3, amfetamina-d_5 i cocaïna-d_3 com a patrons interns, emprant com a tècnica analítica per a la determinació l'UHPLC-MS/MS. A la Figura 90 es mostren les recuperacions obtingudes per a totes les drogues analitzades en aquest estudi on es poden veure recuperacions quantitatives (superiors al 80 %) per a la 2-fluorometamfetamina *(2-fluoromethamphetamine, 2-FMA)*, la 3'4'-metilendioxi-α-dimetilaminoisovalerofenona *(3'4'-methylenedioxy-α-dimethylamino-isovalerophenone, MDPBP)*, butilona, 3-fluoroetamfetamina *(3-fluoroethamphetamine, 3-FEA)*, 6-(2-aminopropil)benzofurà *(6-(2-aminopropyl)benzofuran, 6-APB)*, 4-metilbufedrona *(4-methylbuphedrone, 4-MeMABP)*, 4-cloroetcatinone *(4-chlorethcathinone, 4-CEC)*, 3',4'-metilendioxi- α – pirrolidinopropiofenona *(3',4'-methylenedioxy-α-pyrrolidinopropiophenone, MDPPP)*, α-PVP, cocaïna, α-pirrolidinohexiofenona *(α-Pyrrolidinohexiophenone, α-PHP)*, 2,5-dimetoxi-4-etilfenetilamina *(2,5-dimetoxi-4-ethylphenethylamine, 2C-E)*, 4'-metil-α-pirrolidinohexiofenona *(4'-methyl-α-pyrrolidinohexiophenone, MPHP)* i diazepam. Per a la resta de substàncies analitzades es van obtenir recuperacions superiors al 60 % cosa que demostra una adequada afinitat del monòlit front a les diferents drogues estudiades. A més de les satisfactòries recuperacions obtingudes per als anàlits seleccionats, els LODs del mètode anaven des de 0,03 μg L^{-1} per a la MDPBP fins a 0,6 μg L^{-1} per a la metilona o la 3-FEA.

A partir dels resultats obtinguts amb el nou format de les puntes de micropipeta farcides amb *poly (MAA-co-EGDMA)*, un monòlit de tipus mixt, s'hi va continuar amb la recerca dels materials de tipus mixt, però cap a materials més versàtils que permeteren una selectivitat adreçada al tipus de drogues que s'hi vulguin determinar. En aquest sentit s'han descrit a la bibliografia formes de desenvolupar materials de tipus mixt de bescanvi aniònic i catiònic. No obstant, la majoria d'aquests mètodes fan ús de mescles dels materials en capes SAX/WAX i SCX/WCX o requereixen mètodes de síntesi llargs dels materials amb característiques amfòteres.

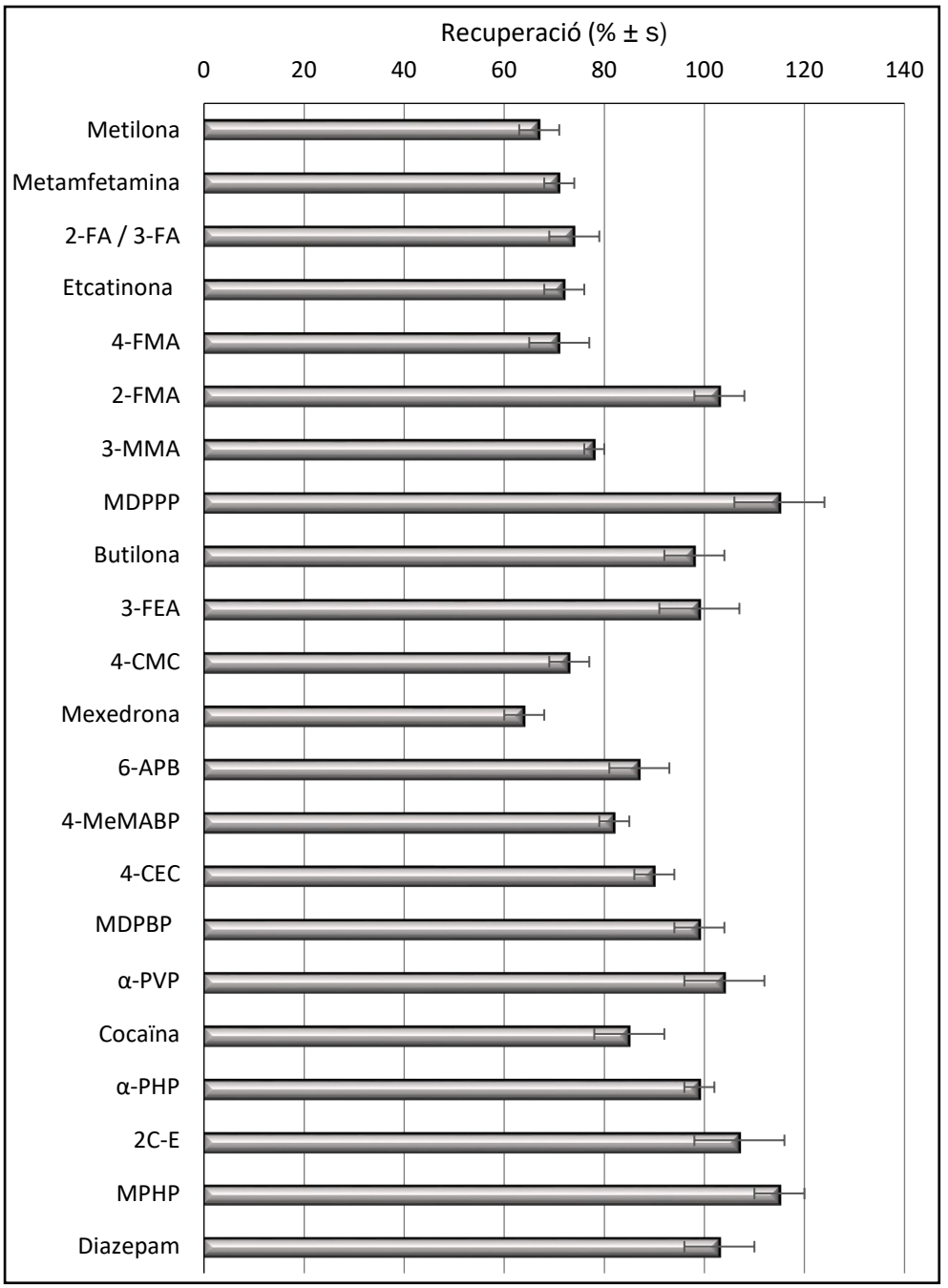
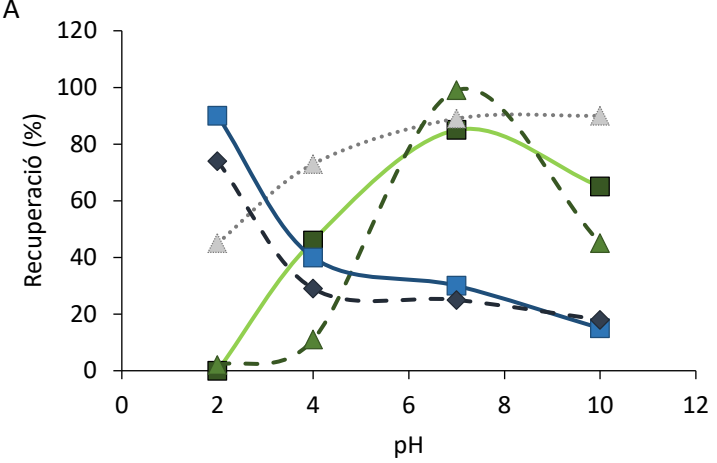


Figura 90. *Recuperacions obtingudes per a cada droga, després de l'extracció emprant les puntes de micropipeta farcides amb poly (MAA-co-EGDMA).*

Al Capítol 7 d'aquesta tesi, s'ha descrit un mètode senzill i pràctic per aconseguir la síntesi d'un material MMS que contenia com a monòmers funcionals 4VP i MAA. També contenia EGDMA com a comonòmer capaç de formar interaccions hidròfobes. Tant la 4VP com el MAA aconsegueixen augmentar la hidrofília del polímer preparat, mostrant a més un comportament amfotèric que aconsegueix retenir tant anàlits àcids com bàsics segons el pH emprat. En aquest sentit, a la Figura 91 es mostra el comportament que tenen els diversos anàlits seleccionats en funció del pH pel que respecta a la seua afinitat cap al material preparat. Els anàlits seleccionats per als estudis de pH van ser cocaïna i 3-metilmetcatinona *(3-metylmethcathinone, 3-MMC)* com a anàlits bàsics amb capacitat de formar molècules carregades positivament a pH sota el seu pK_a. D'altra banda, diazepam, naproxè i diclofenac van ser seleccionades com a drogues amb caràcter àcid. En el cas del diazepam, és capaç de formar ions carregats positivament sota el seu pK_a, mentre que naproxè i diclofenac formen anions a pH superior a la seua constant de dissociació.

A la Figura 91A es pot veure com tant la cocaïna com el 3-MMC mostren una bona afinitat cap al material polimèric a pH entre 6 i 8, aquest fet es deu a que les dos substàncies es troben carregades positivament (a causa de la protonació del grup amino) mentre que l'àcid carboxílic del MAA es troba desprotonat creant interaccions iòniques de considerable força. Per contra al comportament d'aquestes dos drogues de caràcter bàsic, es troba el comportament del naproxè i el diclofenac, que mostren major afinitat front al *poly (4VP-co-MAA-co-EGDMA)* a pH àcid. Aquests pHs afavoreixen que els dos fàrmacs es troben en la seua forma protonada (sense càrrega neta), mentre que tant el MAA com la 4VP s'hi troben també protonats. La manca de repulsions iòniques, l'existència d'interaccions de tipus π-π així com l'existència de mecanismes de fase inversa afavoreixen una elevada afinitat d'aquest tipus d'anàlits a pH àcids. Finalment, el diazepam tot i ser un anàlit amb característiques àcides mostra un comportament semblant a la cocaïna i al 3-MMC però amb una influència menys acusada per la variació de pH. El diazepam tot i ser un anàlit àcid te un grup amino protonable a pH inferiors a 3,4. Per aquest motiu, a pH neutres i bàsics el diazepam s'hi troba sense càrrega neta d'igual manera que la 4VP, predominant en aquests cas els mecanismes de fase inversa junt a interaccions π-π entre l'anell aromàtic del diazepam i el de la 4VP.

A



B

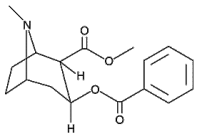
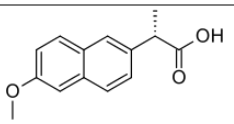
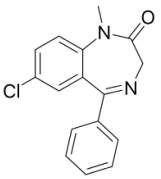
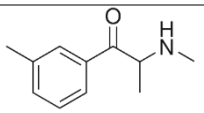
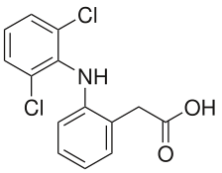
Cocaïna		8.9
3-MMC		8.7
Diazepam		3.4
Naproxè		4.1
Diclofenac		4.2

Figura 91. *(A) Recuperació obtinguda amb el poly (4VP-co-MAA-co-EGDMA) per a la cocaïna, 3-MMC, diazepam, naproxè i diclofenac a diferents pH. (B) Estructura i pK_a de les diferents substàncies involucrades en aquest estudi.*

El material desenvolupat, seguint l'àmbit d'aplicació dels resultats obtinguts al llarg d'aquesta Tesi Doctoral, va ser aplicat per a l'anàlisi de mostres de saliva que contenien cocaïna, 3-MMC i diazepam mitjançant UHPLC-MS/MS després de la corresponent extracció SPE. El mètode desenvolupat va mostrar recuperacions quantitatives per a la determinació de cocaïna (80 – 96 %), 3-MMC (72 – 98 %) i diazepam (69 – 91 %) a nivells de concentració d'entre 100 i 1000 µg L^{-1} , amb LODs d'entre 0,12 – 0,32 µg L^{-1}. Cal destacar que aquest material tot i haver estat aplicat per a la determinació d'aquestes tres substàncies presenta importants avantatges i prometedores aplicacions per a l'anàlisi d'un major nombre d'anàlits incloent àcids més forts que els que es presenten a aquest treball, aconseguint una important millora en la recerca de materials genèrics amb una selectivitat més adreçada segons l'anàlisi a realitzar.

Altre dels objectius que s'ha plantejat al llarg del desenvolupament d'aquesta Tesi Doctoral és l'obtenció de materials selectius i la seua posterior adaptació a nous formats d'extracció. En aquest sentit, tal i com s'ha esmentat als primers capítols, d'entre els materials selectius, els MIPs són aquells que gaudeixen d'una major aplicació en SPE per a la determinació d'anàlits en matrius complexes. A més, la fàcil preparació dels MIPs fa que sigui possible la seua preparació al laboratori per a la posterior aplicació a l'extracció de drogues de fluids biològics i determinació mitjançant IMS, aconseguint mètodes senzills d'anàlisi emprant instrumentació més econòmica, senzilla i ràpida que les tècniques cromatogràfiques.

Al Capítol 8 d'aquesta Tesi Doctoral s'ha preparat mitjançant polimerització en bloc un MIP emprant cocaïna com a *template*, MAA com a monòmer i EGDMA com a *cross-linker.* La polimerització en bloc requereix, si el MIP obtingut va a ser emprat en format SPE en columna, d'una trituració i posterior tamisat del material preparat. En aquest sentit, el MIP va ser tamisat a grandàries de partícula de 100 µm i posteriorment va ser rentat emprant una extracció Soxhlet per aconseguir alliberar els llocs de reconeixement específics cap a la cocaïna. Al treball s'hi van estudiar les millors condicions per a l'extracció de cocaïna de mostres de saliva per a la seua posterior determinació per IMS. Els resultats mostraven recuperacions mitjanes de cocaïna satisfactòries (91 ± 9 %) per a nivells de concentració d'entre 75 i 500 µg L^{-1}, amb LODs de 18 µg L^{-1}. Tanmateix, diversos paràmetres importants pel que respecta a les capacitats del MIP van ser avaluats. Aquest paràmetres incloïen la capacitat de càrrega del MIP, estimada en 0,83 ng de cocaïna per gram de MIP així com la capacitat de reutilitzar cadascuna de les columnes preparades que va ser de 5 usos. Per tal de comparar les retencions específiques i inespecífiques, s'hi va emprar el NIP, obtenint-se un factor

d'empremta de 2,5 cosa que demostrava una satisfactòria selectivitat del MIP preparat.

Considerant els bons resultats que s'havien obtingut emprant aquest MIP combinat amb l'IMS, el procediment d'extracció va ser aplicat per a l'anàlisi de mostres de saliva de diferents individus que havien declarat haver consumit cocaïna i en alguns casos altres substàncies. Les mostres van ser analitzades mitjançant LFIA (DrugTest 5000 de Dräger) per determinar les mostres positives i després de la SPE emprant el MIP, els extractes obtinguts van ser analitzats mitjançant IMS com a tècnica d'avantguarda i posteriorment per GC-MS, emprada com a tècnica de referència. Els resultats obtinguts mitjançant IMS i GC-MS van ser comparats estadísticament mostrant resultats comparables. A la Figura 92 es poden veure els plasmagrames obtinguts per a la determinació de cocaïna en mostres de saliva després de la SPE tant d'una mostra positiva com d'una negativa, on la senyal més intensa correspon al pic de nicotinamida, emprada com a calibrant intern. Tanmateix, a la Figura 92 s'hi mostren els plasmagrames d'un patró de cocaïna i d'una mostra contenint cocaïna després d'una DLLE emprant cloroform com a dissolvent extractant. A la Figura 92 s'hi pot veure com la mateixa mostra extreta pels dos mètodes proporciona un plasmagrama amb menor presència de substàncies ionitzables al cas de la SPE que al cas de la DLLE, on un major nombre de potencials interferents és extret afavorint la possibilitat de que hi aparega ionització competitiva.

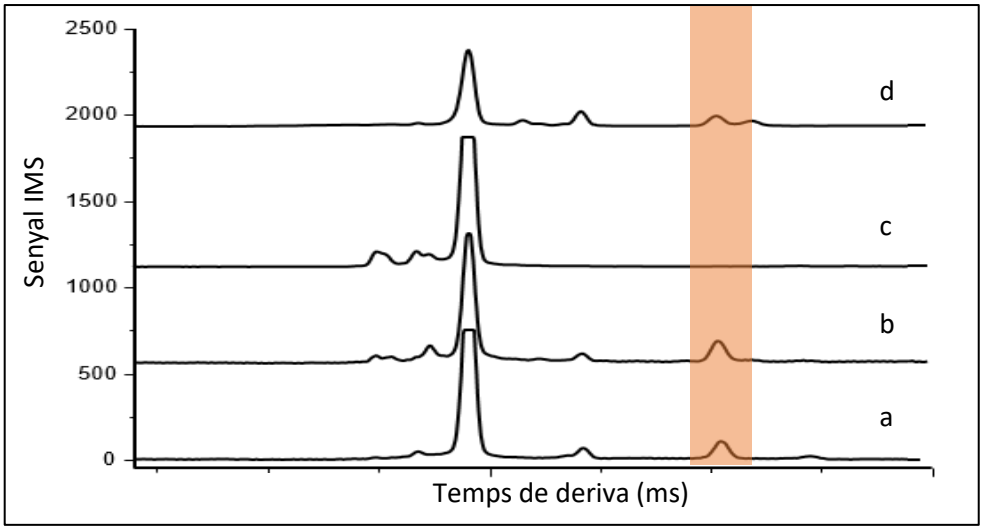


Figura 92. *Plasmagrames obtinguts per IMS de (a) patró de cocaïna de 200 µg L^{-1}, (b) mostra de saliva positiva en cocaïna després de la SPE emprant el MIP, (c) mostra de saliva negativa en cocaïna després de la SPE emprant el MIP i (d) mostra de saliva positiva en cocaïna després d'una DLLE.*

La preparació de MIPs mitjançant polimerització en bloc, ha demostrat ser una estratègia senzilla, ràpida i efectiva per obtenir materials que mostren una selectivitat adequada front als anàlits que han estat emprats com *template* durant la síntesi. No obstant, alguns MIPs mostren el fenomen de la selectivitat creuada que s'hi observa quan el MIP és capaç de reconèixer selectivament també aquelles molècules que guarden determinades semblances amb la molècula usada com a plantilla durant la síntesi.

Per tal d'avaluar la selectivitat creuada que poden presentar els MIPs, s'hi va preparar un MIP emprant metamfetamina com a *template*. Una vegada preparat aquest polímer, es va desenvolupar un procediment de SPE que consistia de les següents etapes: i) condicionament de la columna (1 mL metanol contenint 1 % d'àcid acètic + 1 mL d'aigua desionitzada), ii) la càrrega d'1 mL de mostra (prèviament centrifugada durant 2 min a 5000 rpm), iii) un rentat (emprant 1 mL de tampó fosfat 0,1 M de pH 9,0 contenint 10 % de 2-propanol) i iv) l'elució per a la qual es van emprar 0,5 mL de metanol contenint 1 % d'àcid acètic. Aquest procediment d'extracció es va estudiar emprant amfetamina com a molècula *target*, per a la qual s'hi van obtenir recuperacions d'entre 99 i 115 % per IMS i d'entre 81 i 108 % per UHPLC-MS/MS en mostres de saliva addicionades a nivells de concentració de 300 μg L^{-1}.

Emprant el procediment desenvolupat per a l'extracció d'amfetamina es va portar a terme l'extracció de 32 NPS diferents que incloïen els grups dels derivats de l'amfetamina, catinones sintètiques i fenetilamines. Tanmateix, es va realitzar l'extracció d'altres 9 substàncies emprades com a control que no tenien semblances estructurals amb la metamfetamina que incloïen fenacetina, codeïna, difenidramina, lidocaïna, levamisol, nicotina, benzocaïna, cocaïna i MMB-CHMICA. Les recuperacions obtingudes per a les substàncies semblants a la metamfetamina, com es pot veure a la Figura 93 són quantitatives, trobant-se en valors d'entre el 80 i 120 % amb una recuperació mitjana entre les 32 NPS de 96 ± 12 %. Contràriament, la resta de substàncies seleccionades com a control per avaluar la selectivitat creuada van mostrar recuperacions considerablement més baixes, amb una recuperació mitjana d'entre totes elles de 46 ± 19 %. El mètode preparat per a l'extracció d'aquestes NPS, a banda de les bones recuperacions va oferir LODs satisfactoris d'entre 10 i 80 μg L^{-1} per IMS i d'entre 0,03 i 1,1 μg L^{-1} per UHPLC-MS/MS.

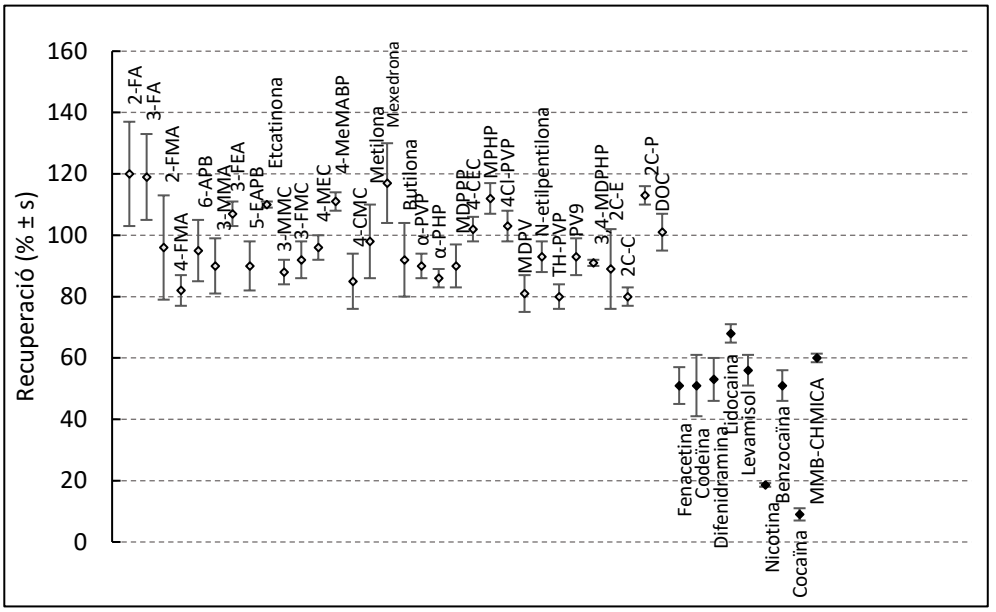


Figura 93. *Recuperacions obtingudes per a les 41 substàncies emprades per estudiar la selectivitat creuada del MIP preparat emprant metamfetamina com a template mitjançant SPE.*

El MIP preparat va ser caracteritzat morfològicament emprant SEM per observar les característiques de la seua superfície. La caracterització morfològica va ser complementada amb la mesura de les àrees superficials tant del MIP com del NIP emprant la tècnica d'adsorció i desorció de nitrogen. La mesura d'aquest paràmetre va permetre estimar una àrea superficial de 393 ± 4 m^2 g^{-1} per al MIP i de 182,8 ± 0,3 m^2 g^{-1} per al NIP, on s'hi observa una major àrea superficial en el cas del MIP que permet un repartiment més efectiu dels anàlits entre la mostra i la fase adsorbent. De la mateixa manera, la tècnica d'adsorció i desorció de nitrogen va permetre estimar el diàmetre dels micro/mesoporus de 2 nm al cas del MIP, mentre que al NIP la presència de micro/mesoporus era negligible. Aquestes dades van ser emprades per comparar la grandària dels micro/mesoporus amb la grandària de diverses drogues que van ser estimades mitjançant càlculs computacionals emprant el software Orca (versió 4.0.1) de l'institut Max-Planck (Mülheim an der Ruhr, Alemanya) i el mètode semiempíric PM3. Aquestes substàncies van ser la 2-fluoroamfetamina *(2-fluoroamphetamine, 2-FA)*, 3-fluoroamfetamina *(3-fluoroamphetamine, 3-FA)*, 2-fluorometamfetamina *(2-fluoromethamphetamine, 2-FMA)*, 4-fluorometamfetamina *(4-fluoromethamphetamine, 4-FMA)*, 6-APB, 3-metilmetamfetamina *(3-methylmethamphetamine, 3-MMA),* N-etil-α-metil-5-benzofuranoetanamina *(N-ethyl-α-methyl-5-benzofuranethanamine, 5-EAPB),*

4'-fluoro-α-pirrolidinooctanofenona *(4'-fluoro-α-pyrrolidinooctanophenone, PV9),* 2C-E i 2,5-dimetoxi-4-cloroanfetamina *(2,5-dimethoxy-4-chloroamphetamine, DOC)* les quals van mostrar, segons les estimacions semiempíriques, que la seua grandària era inferior a 87,6 $Å^2$ essent per tant capaces totes elles d'accedir a l'interior dels micro/mesoporus.

Observant que efectivament alguns MIPs són capaços d'oferir selectivitat creuada un plantejament interessant pot ser la modificació d'aquesta selectivitat segons el procediment d'extracció emprat. D'aquesta manera, al Capítol 10 s'hi va preparar un MIP mitjançant polimerització en bloc emprant 3-OH PCP com a *template*. El MIP va ser preparat emprant MAA i EGDMA com a monòmer i *cross-linker,* respectivament en un ratio de 1:4:20 (*template:*monòmer:*cross-linker*), i com dissolvent porogènic N,N-dimetilformamida. Una vegada obtingut el MIP, es van estudiar les millors condicions d'extracció posant especial importància a l'etapa de rentat per determinar de manera selectiva diverses arilciclohexilamines, (PCP, 3-MeO PCP, 3-MeO PCE, MXE, 2-oxo PCE, descloroketamina i ketamina). A la Taula 55 es poden veure els procediments adoptats per a la determinació selectiva de les diverses arilciclohexilamines així com el procediment específic per a la determinació de 3-OH PCP.

Taula 55. *Procediments emprats per a l'extracció selectiva d'arilciclohexilamines i per a l'extracció específica de 3-OH PCP en SPE convencional.*

	Arilciclohexilamines	**3-OH PCP**
Condicionament	1 mL 2-propanol (contenint 5 % d'àcid acètic) 1 mL aigua desionitzada	1 mL 2-propanol (contenint 5 % d'àcid acètic) 1 mL d'aigua desionitzada
Càrrega	1 mL mostra + 0,5 mL tampó fosfat (pH 7,0 - 0,1 M)	1 mL mostra + 0,5 mL tampó fosfat (pH 7,0 - 0,1 M)
Rentat	1 mL aigua desionitzada (contenint 5 % de 2-propanol i 0,1 % d'àcid acètic)	2 mL acetonitril
Elució	1 mL 2-propanol (contenint 5 % d'àcid acètic)	1 mL 2-propanol (contenint 5 % d'àcid acètic)

Emprant el procediment selectiu es va demostrar una bona afinitat del MIP cap a les arilciclohexilamines, amb percentatges de recuperació d'entre el 67 i 98 % per a totes elles després d'una SPE de patrons preparats en aigua a un nivell de concentració de 250 µg L^{-1}. D'altra banda, el procediment que feia ús d'acetonitril com a dissolvent de rentat va mostrar recuperacions del 62 % en patrons de 3-OH PCP preparats en aigua a 250 µg L^{-1} mentre que la resta d'arilciclohexilamines van donar recuperacions sota el 42 %, demostrant una afinitat major del MIP per aquella molècula que havia estat emprada com a *template.*

Emprant el mètode específic per a l'extracció de 3-OH PCP es van analitzar diverses mostres de saliva addicionades a 250 i 500 µg L^{-1} mitjançant determinació per IMS, on s'hi van obtenir recuperacions mitjanes de 72,0 ± 1,4 % mentre que per al mètode indicat per a l'extracció d'arilciclohexilamines les recuperacions obtingudes per a les mateixes mostres van ser de 92 ± 13 %. A la Figura 94, s'hi poden observar els plasmagrames obtinguts després de l'extracció d'una mostra de saliva fortificada amb 500 µg L^{-1} emprant el mètode més específic i el mètode selectiu per a la determinació d'arilciclohexilamines. Tal i com es mostra a la Figura 94, el mètode específic per al 3-OH PCP mostra un plasmagrama amb un únic pic de gran intensitat, mentre que el mètode selectiu per a la determinació d'arilciclohexilamines presenta major presència d'altres compostos ionitzables, tot i que ambdós plasmagrames proporcionen senyals comparables de 3-OH PCP.

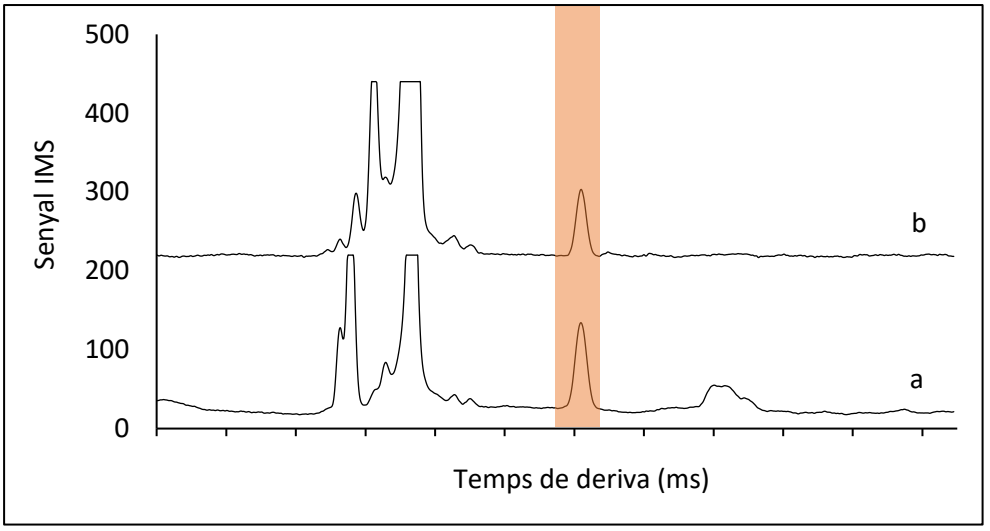


Figura 94. *Plasmagrames obtiguts mitjançant IMS per a mostres addicionades amb 3-OH PCP emprant el procediment selectiu per a totes les arilciclohexilamines (a) i el procediment específic per al 3-OH PCP (b).*

Fins a aquest punt tots els estudis emprant els MIPs s'hi van portar a terme emprant un format tradicional de SPE en forma de cartutx. Cal destacar, que la SPE convencional és una forma d'extracció ben establida i que presenta pocs problemes a l'hora de provar nous materials i desenvolupar nous procediments, per aquest motiu, els primers estudis d'aquesta Tesi Doctoral on s'han emprat MIPs es van portar a terme en el format de SPE en columna. No obstant, també és cert que la SPE en columna presenta algunes limitacions o inconvenients. D'una banda, l'extracció mitjançant SPE habitualment suposa mètodes de diverses etapes (condicionament, càrrega, rentat, assecat i elució) que poden provocar temps de tractament de mostra més llargs. De la mateixa manera, necessiten ser desenvolupats habitualment a un laboratori ja que s'hi necessiten sistemes de buit o requereixen d'ús considerable de dissolvents. Habitualment, aquests volums de mostra i dissolvents comporten una limitada capacitat de preconcentrar. En aquest sentit han sorgit diversos formats o variants de la SPE que aconsegueixen resoldre alguns d'aquests problemes. Als treballs que s'hi presenten a continuació, es posa especial interès en la resolució d'aquesta manca de portabilitat de la SPE, la reducció del consum de dissolvents així com millorar la capacitat de preconcentració front a volums grans de mostra.

A partir dels bons resultats que va oferir el MIP per a la determinació de cocaïna avaluat al Capítol 8, i tenint en compte que la cocaïna és una droga d'especial rellevància i que la seua anàlisi te un interès particular, al Capítol 11 s'hi va desenvolupar un dispositiu de mostreig i tractament de mostra per a la determinació de cocaïna en mostres de saliva. Aquest dispositiu es va preparar amb l'objectiu d'incorporar aquestes dues etapes bàsiques de qualsevol anàlisi abans de la determinació en un sol dispositiu per tal de poder realitzar l'anàlisi *in-situ*. A la Figura 95, s'hi poden observar les diferents parts de les quals està composat el dispositiu proposat. En primer lloc, té la part del mostrejador formada per una bola de cotó que contenia a l'extrem un indicador d'humitat. La finalitat d'aquest mostrejador és que en ser introduït a la boca, el cotó adsorbisca la màxima quantitat de saliva possible fins que l'indicador canvie de color indicant que el cotó ha estat completament mullat. Després d'estudiar la massa de cotó més adequada per realitzar el mostreig (50 mg) s'hi va estimar que la quantitat de saliva que era capaç d'absorbir era de 320 ± 30 µL (considerant una densitat de saliva d' 1 g mL^{-1}). La segona part de la que es forma el dispositiu incloïa el MIP selectiu per a la determinació de cocaïna. El principal avantatge d'aquest sistema és que el material selectiu no està en contacte directament amb el mostrejador, però la transferència de la mostra des del col·lector fins al MIP es pot realitzar de manera senzilla.

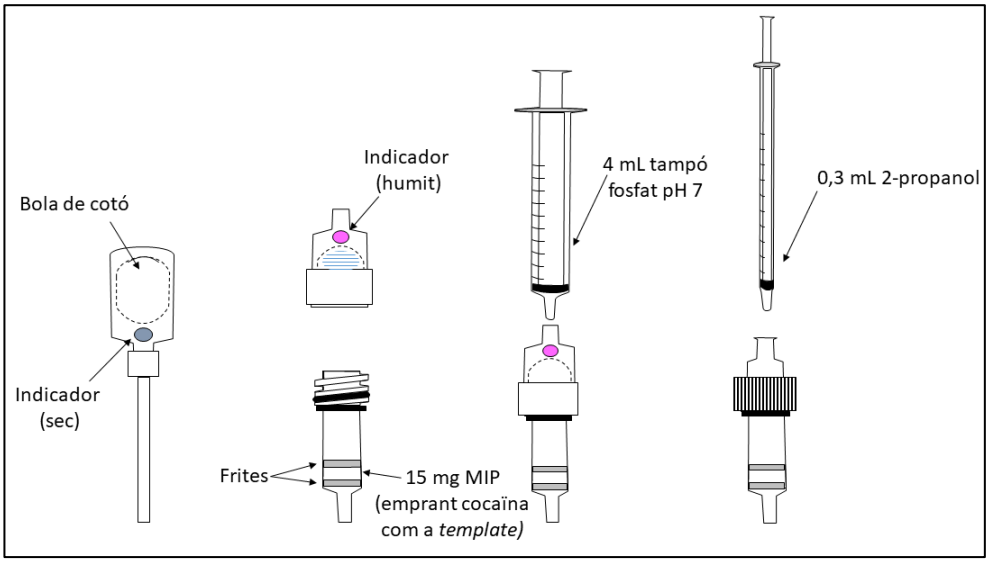


Figura 95. *Esquema de les parts del dispositiu desenvolupat per a l'extracció in-situ de cocaïna en saliva.*

Per portar a terme la transferència de la mostra des del col·lector fins al MIP, s'hi va emprar una única xeringa precarregada amb 4 mL de tampó fosfat 0,1 M de pH 7,0. Aquesta etapa suposa la incorporació de les típiques etapes de condicionament, càrrega i rentat d'una SPE en una única etapa, eliminant la necessitat de sistemes de buit. Amb aquest tampó de transferència s'hi va aconseguir arrossegar de manera semi quantitativa la cocaïna present en la saliva adsorbida sobre el cotó, al mateix temps que s'eluïen la resta de components de la matriu. Aquesta cocaïna retinguda sobre el MIP va ser eluida emprant una segona xeringa precarregada amb 0,3 mL de 2-propanol, extracte que va ser recollit i analitzat mitjançant IMS. L'ús de l'IMS va permetre determinar la concentració de cocaïna de manera sensible, ràpida i sense necessitat de personal especialitzat. A més, presenta un gran potencial de portabilitat, essent possible realitzar les anàlisis *in-situ*, resolent la seua manca de selectivitat gràcies al MIP.

El valor de la concentració de tall per a la determinació de cocaïna va ser estimat en 20 µg L^{-1} ja que va ser el valor amb el qual 20 mostres de saliva, que no contenien cocaïna inicialment, addicionades a aquest nivell de concentració van mostrar un 100 % d'encert en identificar mostres positives i de la mateixa manera un 95 % d'encert identificant mostres negatives. A més, la capacitat de càrrega del dispositiu va ser estimada en 130 mg L^{-1} considerant la quantitat de mostra adsorbida pel mostrejador, així com les recuperacions que va mostrar el dispositiu per a mostres de saliva addicionades amb cocaïna a 100 µg L^{-1} (53 ± 6 %).

Finalment, el dispositiu desenvolupat va ser emprat per a l'anàlisi de 20 mostres de saliva d'individus amb edats compreses entre 19 i 38 anys que havien declarat haver consumit una dosi no controlada de cocaïna entre 15 i 180 min abans de la presa de mostra. Després de realitzar el mostreig, en una durada inferior a 5 min, s'hi van observar resultats positius per a totes les mostres, essent comparables amb els resultats oferts mitjançant LFIA (DrugTest 5000 de Dräger) que va ser usat com a mètode de referència per comprovar la presència o no de cocaïna.

Altra de les limitacions que s'ha plantejat de la SPE era la manca de capacitat de preconcentració en aquelles ocasions on es disposa de volums de mostra menuts i es necessiten volums d'extracció relativament elevats, així com la necessitat de sistemes de buit per portar a terme l'extracció habitualment. En particular, el procediment SPE descrit per a la determinació de cocaïna en mostres de saliva (Capítol 8) planteja aquests dos inconvenients. Per aquest motiu, al Capítol 12 s'estudia la preparació de MMIPs mitjançant la polimerització en bloc emprant MNPs modificades superficialment per tal d'ancorar el polímer sobre les MNPs.

Tal i com es mostra a la Figura 96, van ser preparats dos MMIPs diferents. Els PEG-MMIP i els V-MMIP, plantegen com a única diferència el procediment d'ancoratge del MIP sobre les nanopartícules. A partir de nanopartícules de magnetita no modificades, s'hi van preparar MNPs funcionalitzades per tal d'aconseguir una adequada immobilització dels MIPs. En primer lloc, es va emprar PEG per tal de crear un recobriment hidròfil sobre les MNPs que afavorirà l'encapsulament de les mateixes durant l'etapa de polimerització. En segon lloc, altre grup de MNPs van ser modificades emprant γ-MPS, aquest agent va aconseguir crear un recobriment sobre les MNPs funcionalitzant l'exterior amb grups vinil que van permetre una unió covalent de les MNPs amb el MIP sintetitzat posteriorment. Per a la preparació del MIP s'hi van emprar les mateixes condicions que al Capítol 8, a partir de cocaïna com a *template* i MAA com a monòmer, l'EGDMA va ser emprat com agent entrecreuant per portar a terme la polimerització en bloc. La mescla de polimerització va ser dissolta en acetonitril que va estar usat com dissolvent porogènic i l'AIBN es va emprar per desencadenar la reacció radicalària.

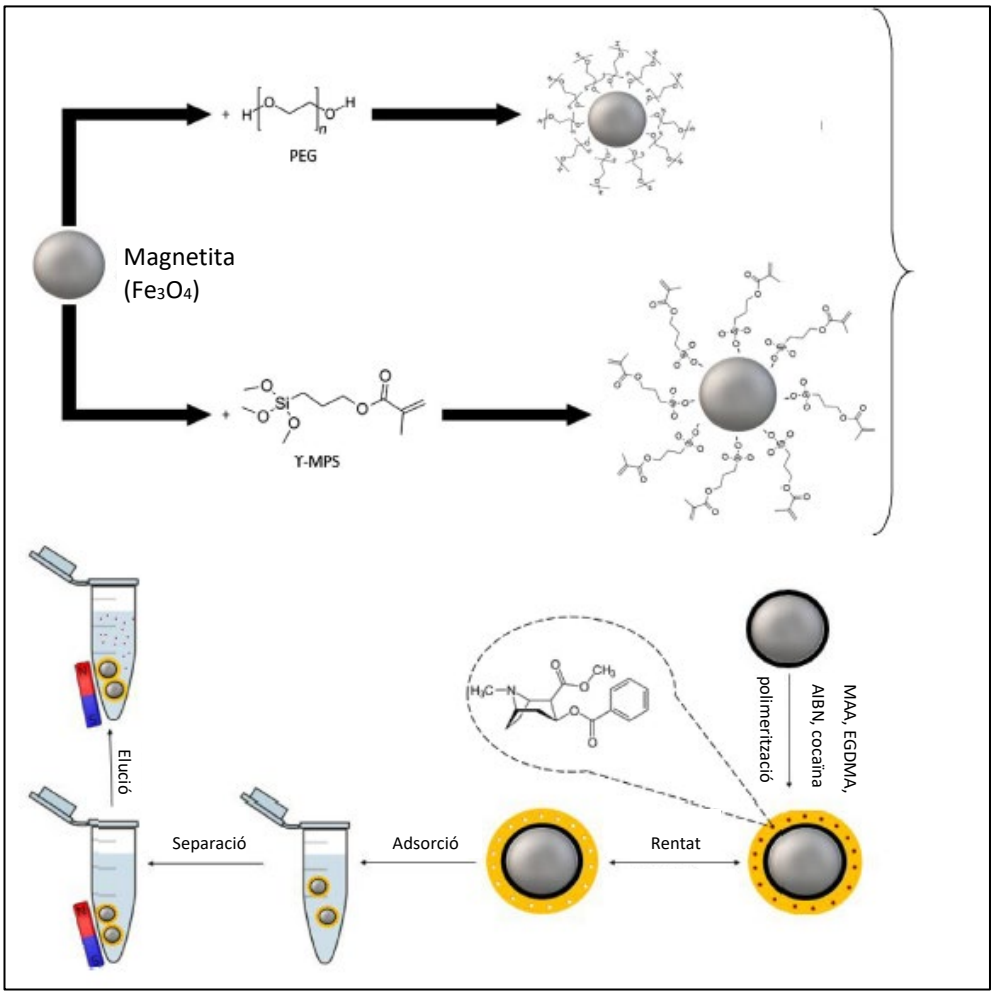


Figura 96. *Procediment de preparació i ús dels MMIPs .*

Després de la preparació del PEG-MMIP i V-MMIP, s'hi van estudiar les millors condicions d'extracció per a la posterior determinació de cocaïna per IMS. El procediment desenvolupat va ser adaptat a partir de la SPE per a l'extracció de cocaïna i va consistir en l'addició de 5 mg de MMIP sobre 1 mL de metanol contenint 5 % d'àcid acètic per a l'activació de l'adsorbent. Després d'eliminar el sobrenedant, s'hi van afegir 0,5 mL d'aigua desionitzada per eliminar l'excés de metanol, eliminant-ne el sobrenedant. La càrrega s'hi va realitzar afegint 0,5 mL de mostra i 0,1 mL de tampó amoni de pH 9,0 agitant 10 min per garantir una transferència de massa quantitativa. El rentat es va realitzar en dues etapes, un primer rentat emprant 0,5 mL d'aigua i un segon amb 0,5 mL de cloroform. Finalment 0,2 mL de metanol contenint 5 % d'àcid acètic van ser usats per eluir la cocaïna i ser injectats a l'IMS. Les característiques de PEG-MMIP i V-MMIP van ser avaluades i comparades entre elles per a l'extracció de cocaïna en mostres de

saliva per a la posterior determinació per IMS. D'aquesta manera es van obtenir recuperacions mitjanes de 86 ± 11 % per al PEG-MMIP i 92 ± 4 % per al V-MMIP després de l'extracció de mostres de saliva fortificades a nivells de concentració d'entre 80 i 560 µg L^{-1}. També altre paràmetre com la capacitat de càrrega va ser avaluat per a ambdós materials, les quals van ser estimades en 0,124 i 0,102 mg de cocaïna per gram de PEG-MMIP i el V-MMIP, respectivament. Cal destacar, que s'hi va observar que el V-MMIP ofereix una major estabilitat que el PEG-MMIP a l'hora de realitzar les extraccions. Aquesta estabilitat s'hi va fer evident fonamentalment a causa del despreniment de part del PEG-MMIP durant l'extracció. Ací part del polímer es va separar de les MNPs, deixant-les al descobert mentre que al cas del V-MMIP romanen sense separar-se. Aquest fet es pot comprovar a la Figura 97, on es veu que mentre als V-MMIP les imatges SEM abans i després de l'extracció no ofereixen canvis significatius pel que fa a la forma del polímer, mentre que al PEG-MMIP després d'un ús s'observa una major presència de MNPs (en forma de punts blancs a la imatge) que demostren que aquestes han quedat al descobert.

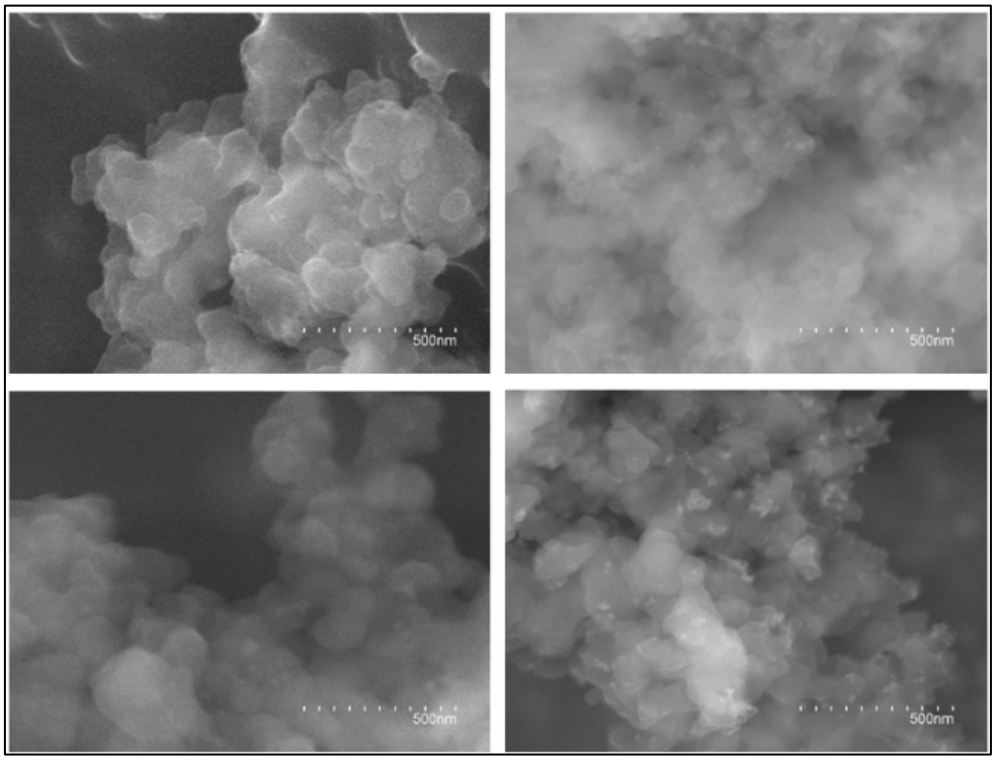


Figura 97. *Imatges SEM obtingudes a 20.000x magnificacions del V-MMIP (dalt esquerra) i PEG-MMIP (dalt dreta) abans d'una extracció i del V-MMIP (baix esquerra) i PEG-MMIP (baix dreta) després d'una extracció amb les condicions de treball.*

Finalment, el procediment desenvolupat que va aconseguir factors de preconcentració superiors a la SPE (2,5) i LODs de 6 µg L^{-1} , emprant els V-MMIP, va ser aplicat per a l'anàlisi de 7 mostres de saliva de persones que havien declarat haver consumit cocaïna i els resultats obtinguts van ser comparats amb dos mètodes de referència. En primer lloc totes les mostres avaluades van ser analitzades prèviament amb un LFIA (DrugTest 5000 de Dräger) per determinar el nombre de mostres positives i negatives. Tanmateix, totes les mostres van ser analitzades amb un segon mètode confirmatori de gran sensibilitat com la UHPLC-MS/MS obtenint resultats estadísticament comparables amb el mètode proposat i el mètode confirmatori.

La concentració de drogues en mostres d'aigües residuals i naturals, és un dels millors paràmetres epidemiològics per conèixer el consum dins d'una població d'una o diverses substàncies. La SPE en ocasions ha mostrat ser un enfocament efectiu per la preconcentració d'aquest tipus de mostres. No obstant, els grans volums que es requereixen per tal d'aconseguir elevats factors de preconcentració comporta temps d'anàlisi molt llarg a causa de l'elevat temps de càrrega per garantir una retenció adequada de l'anàlit. En aquest sentit, al Capítol 13 s'ha presentat la preparació d'una fase adsorbent en forma de monòlit sobre un disc de PTFE per tal de retenir l'anàlit d'una manera senzilla i sense emprar temps d'anàlisi massa elevats. En concret, la fase adsorbent que s'empra al treball és un MIP preparat emprant ecgonina metil èster com a *template* que és un dels metabòlits principals de la cocaïna. La resta de reactius emprats per a la preparació del MIP van estar MAA com a monòmer, EGDMA com a *cross-linker*, AIBN com a iniciador i acetonitril que es va usar com dissolvent porogènic .

En primer lloc, abans de la síntesi del MIP sobre el disc de tefló, aquest va haver de ser modificat per funcionalitzar la seua superfície i permetre l'ancoratge efectiu del MIP sobre el mateix. Després d'aquesta funcionalització que s'hi descriu detalladament al Capítol 13, es va preparar el MIP corresponent per via tèrmica, obtenint un monòlit correctament immobilitzat i resistent a l'agitació (vegeu Figura 98). Per permetre l'agitació, s'hi van col·locar dos imants sobre les parets externes del disc.

El disc preparat que va permetre l'agitació i extracció en una mateixa etapa de volums de mostra molt elevats (200 mL) va ser avaluat per obtenir les millors condicions d'extracció i conèixer els paràmetres més importants. D'aquesta manera es va estudiar la influència de factors com el pH de la mostra o els temps de càrrega i elució. Finalment s'hi van obtenir recuperacions quantitatives d'ecgonina metil èster (100 %) a un nivell de concentració de 2 µg L^{-1}. Tanmateix, es va avaluar l'afinitat del MIP preparat front a dos molècules relacionades, com

són la cocaïna (58 % de recuperació) i la benzoilecgonina (0 % de recuperació) observant una bona selectivitat del MIP cap a l'ecgonina metil èster i en menor mesura cap a la cocaïna, però sense afinitat cap a l'altre metabòlit principal de la cocaïna. També altres característiques més importants del MIP van ser avaluades. D'entre elles la capacitat de càrrega es va estimar en 6,68 µg d'ecgonina metil èster per disc o el factor d'empremta amb un valor de 25, cosa que demostrava la gran selectivitat del MIP front altres molècules no relacionades.

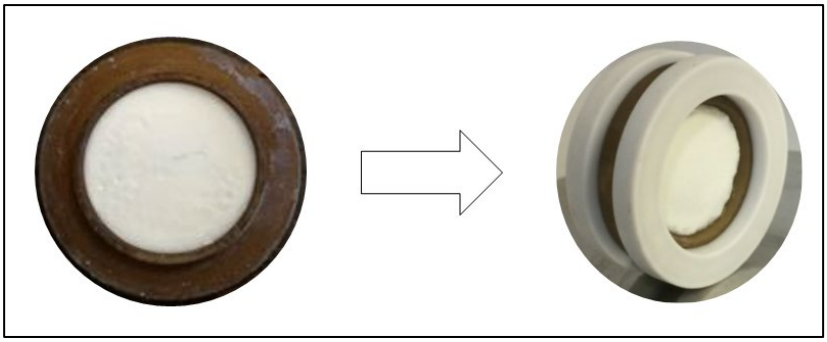


Figura 98. *Imatges del disc d'agitació-extracció preparat sense (esquerra) i amb (dreta) els imants incorporats.*

Una vegada demostrada la bona capacitat del MIP preparat en format de disc per a l'extracció d'ecgonina metil èster, va ser aplicat per a l'anàlisi de mostres d'aigües superficials i aigües de depuradora emprant dues tècniques analítiques diferents. L'IMS avaluada com a tècnica de *screening,* de menor sensibilitat ja que presentava un LOD i LOQ de 75 i 250 ng L^{-1}, respectivament, i l'UHPLC-MS/MS emprada com a tècnica confirmatòria, d'elevada sensibilitat, amb un LOD i LOQ de 13 i 45 ng L^{-1}, respectivament. A la Taula 56 es mostren els resultats obtinguts per a l'anàlisi de les 6 mostres recollides de diferents àrees properes a la ciutat de València, on s'hi pot observar que va permetre la identificació d'ecgonina metil èster en dos de les mostres. D'altra banda l'UHPLC-MS/MS va permetre la quantificació de quasi totes les mostres, observant concentracions molt menudes de l'anàlit en les mostres recollides essent indetectable en una d'elles (mostra 5) i no quantificable en altra (mostra 2).

Taula 56. *Resultats obtinguts per IMS i UHPLC-MS/MS per a la determinació d'ecgonina metil èster en diferents mostres d'aigua després de l'extracció emprant el disc amb el MIP immobilitzat.*

Mostra	Localització	Concentració (ng L^{-1} ± s)	
		IMS	UHPLC-MS/MS
1	Port de València	< LOD	98 ± 8
2	Estanc (Almenara)	< LOD	< LOQ
3	Llacuna (Marjal dels Moros)	< LOD	67 ± 5
4	Sèquia (Puçol)	< LOQ	143 ± 10
5	Sèquia (Llíria)	< LOD	< LOD
6	Aigua de depuradora	< LOQ	195 ± 13

IMS: espectrometria de mobilitat iònica, LOD: límit de detecció, LOQ: límit de quantificació, UHPLC-MS/MS: cromatografia líquida d'alta resolució acoblada a espectrometria de masses en tàndem.

Considerant els bons resultats oferts en general pels MIPs com materials selectius, és interessant posar el focus d'atenció en uns materials que més recentment han començat a estar aplicats per a la SPE. Els aptàmers són candidats excel·lents per poder substituir els anticossos. Amb preus sovint inferiors, una major estabilitat i la possibilitat d'introduir modificacions durant la seua síntesi que aporten una estabilitat extra o milloren la immobilització, els aptàmers ofereixen selectivitats i capacitat de càrrega comparables als anticossos.

En aquest sentit, al darrer capítol (Capítol 14) d'aquesta Tesi Doctoral s'hi presenten els resultats d'estudis preliminars de la immobilització de tres aptàmers diferents sobre safarosa activada amb bromur de cianogen i sobre partícules magnètiques. Els aptàmers seleccionats van ser FQ1 i FQ2, selectius per a la determinació de fluoroquinolones i l'aptàmer MA selectiu per a la determinació de metamfetamina. Els aptàmers van ser immobilitzats sobre la safarosa amb l'objectiu d'estudiar la selectivitat creuada que oferien emprant un SPE convencional, mentre que la immobilització sobre les partícules magnètiques anava adreçada a l'extracció mitjançant una MDSPE. Els aptàmers emprats al llarg d'aquest estudi van ser modificats amb la introducció d'un grup amino a l'extrem 5' i un braç espaiador de C12 per als aptàmers FQ1 i FQ2. L'aptàmer MA va ser modificat també introduint un grup amino a l'extrem 5' i un braç espaiador C6.

Després d'assajar diversos procediments d'immobilització que es descriuen detalladament al Capítol 14, es van obtenir rendiments d'immobilització d'entre 12,3 i 36,6 % considerant els dos tipus de suports esmentats. Tot i que el percentatge d'aptàmers immobilitzats suposava valors comparables als que s'obtenen habitualment en aquests procediments, els oligosorbents preparats van mostrar una completa manca d'afinitat pels anàlits corresponents, amb recuperacions sota el LOD en la majoria de les condicions de càrrega i rentat avaluades, demostrant una baixa interacció anàlit-aptàmer. D'aquesta manera, dos factors poden ser els condicionants d'aquesta manca de reconeixement cap als anàlits. El primer factor pot ser una baixa proporció d'aptàmers actius o funcionals d'entre tots els que han estat immobilitzats, que no tinguen la capacitat de reconèixer els anàlits. La segona possible causa d'aquesta manca d'afinitat és la desnaturalització de les cadenes d'oligonucleòtids, per causes desconegudes i que provoquen la pèrdua de l'estructura adequada de la seqüencia per retenir els anàlits. En aquest sentit, cal continuar treballant en aquests possibles factors per tal d'avaluar l'aplicació dels oligosorbents a l'extracció de drogues en matrius biològiques com saliva per a la seua posterior determinació mitjançant tècniques ràpides i sensibles com l'IMS.

In this section, the most important results of the research carried out during this Doctoral Thesis will be shown and analyzed. In this way, the most relevant findings of the bibliographic research done in the first section will be extracted, followed by the most remarkable advantages of generic and selective materials and the new formats developed in sections 2 and 3.

Section 1 includes a collection of the published papers about application of smart materials for the analysis of samples from different nature (biological, environmental, drugs, food, etc) using IMS, as well as the the review of application of smart materials for forensic analysis, including drug analysis.

In general, the limited selectivity provided by IMS can be well solved by smart material application such as MIPs, immunosorbents or aptamers that allow a selective isolation of the analyte prior to its determination. Other materials, such as ILs or CNTs for sample treatment, although they do not provide great selectivity, they are able to remove most of interfering compounds from the sample, thus, contributing to improve sensitivity of the method. Despite good prospects for the future and the satisfactory results that smart material-IMS combination has shown, its development is still growing. Due to the reduced number of studies using IMS as an analytical technique compared to other techniques such as chromatography, more research is still needed. Among the smart materials that have been used in combination with an IMS determination, two main groups can be differentiated.

On the one hand, immunosorbents, aptamers and MIPs are a group of smart materials that offer a large selectivity. They are able to recognize families of molecules. They provide in many cases high specificity towards a single specific molecule. The main advantage of immunosorbents is their excellent selectivity, often higher than other selective materials. However, they also have certain weaknesses such as their limited availability in the market, high difficulty to obtain them, particularly for antibodies which require invasive methods using living organisms as host. Antibodies, also suffer from, since they can be denatured with relative ease if they are subjected to high temperatures, extreme pH or to the action of some organic solvents. Aptamers have shown numerous advantages, including very good selectivity, comparable to that obtained with immunosorbents. They have a simple chemical synthesis compared to antibodies, and they also includes the ability to introduce modifications easily to facilitate their posterior immobilization, and make them more effective. The main limitation of aptamers is related to their stability. Even if these materials are

stable, in order to preserve their structure and make them functional during extraction, the sample must be in a specific medium that did not change the ability to recognize the analyte. Likewise, storage conditions must be well-controlled to avoid degradation of aptamer. Finally, in this group of materials are found MIPs, which have some unique characteristics due to their chemical nature, such as the great mechanical and chemical stability that allow non stricto conditions of conservation and use. Other advantage of MIPs is their easy preparation compared to immunosorbents, and they can be easily prepared in the laboratory. Thus, they are usually less expensive than antibodies and aptamers. However, it is true that their selectivity is usually lower than antibodies or aptamers, although they exhibit higher loading capacity.

On the other hand, the second group of materials included in this first section are ILs as well as some nanomaterials such as CNTs. They have a lower selectivity towards target molecules, but they allow to remove a large number of interfering compounds from the matrix, thus increasing the sensitivity of the analytical method. Thus, it may be necessary that selectivity not achieved by these materials can be overcome by using chromatographic separations prior to IMS determination or MS determination after IMS separation. In this sense, it should be mentioned that the main strengths of ILs are associated with the high thermal stability, low vapor pressure and their low flammability. In addition, ILs are able to interact preferentially according to the functional groups of analytes. Likewise, CNTs in general stand out for their good mechanical, chemical and electronic properties that make them ideal candidates to be used as supports in the development of sensors. However, they have very low selectivity compared to aforementioned materials when they are applied to sample treatment.

Also, other materials can be associated to smart materials group, even if they exhibit less selectivity than MIPs, aptamers or immunomaterials. These material are RAMs, which allow the removal of matrix effects by exclusion of large molecules from the sample. The combination of RAMs with other selective materials (such as MIPs) provides a high selectivity and greater reusability of the resulting material. Also, MOFs have attractive qualities due to their high surface area and the possibility of functionalizing them with certain groups. For this reason, they are considered materials with a high loading capacity and moderate selectivity.

An important aspect to highlight is the application of selective materials for forensic analysis and in particular for the determination of drugs in biological

fluids. This aplication has been mostly done as sensors, in the case of aptamers, and immunoassays for immunoaffinity materials. For example, immunoassays have been developed for the analysis of cocaine in samples such as urine, saliva or serum achieving LODs of between 0.06 and 0.09 μg L^{-1} [Vidal et al. 2016]. Also, metabolites of cocaine such as benzoylecgonine have been analyzed using an electroimmunosensor, leading 0.41 μM LODs [Yilmaz-Sengel et al. 2017]. Other drugs such as morphine using fluoroimmunoassay in urine samples (with LODs of 0.2 μg L^{-1}) have been developed [Eldefrawi et al. 2000]. Besides, aptamers based sensors have been used for determination of cocaine and benzoylecgonine in urine samples, achieving LODs of 0.138 nM and 1.66 μM, respectively [Guler et al. 2017].

Generic materials such as C18 or C8-modified silica, offer great versatility due to the of non-specific interactions promoted with all compounds that show a non-polar structure. For this reason, the present PhD project has begun to evaluate the application of these sorbent materials, in a format of relatively recent development such as MEPS. The main advantages of MEPS are the small amount of sorbent used, and the ease to attach it to autosamplers or semi-automatic instruments, which leads to miniaturization extraction methods.

Traditional drugs such as cocaine, amphetamine or diazepam have been analyzed usinf several miniaturized methods in different matrices including biological fluids. However, other drugs such as the recent NPS still need new methodologies for their analysis. The application of MEPS using generic materials as sorbent is an interesting advance in the methods currently described for the analysis of these drugs.

Chapters 3, 4 and 5 describe different methods using MEPS for the extraction of several drugs included into the group of NPS. After MEPS treatment, dichlorophane and methylone were determined by IMS and 5F-ADB, MMB-CHMICA, THJ-2201, CUMYL-4CN-BINACA, and MDMB-CHMCZCA were analyzed by GC-MS. Apart from the fast determination by IMS, dichlorophane and methylone analysis revealed a good sensitivity, with LODs of 30 and 4 μg L^{-1}, respectively. On the other hand, GC-MS was used as analytical technique to determine synthetic cannabinoids to avoid competitive ionization or coelution of analytes, that would make it difficult to accurately identify or quantify the consumed substances. In this case, the MEPS-GC-MS combination presented a good separation of analytes and sensitivity comparable to that obtained for dichloropane and methylone by

IMS. The LODs for the five cannabinoids studied were comprised between 10 and 20 μg L^{-1} .

To perform the determination of analytes after SPE or similar procedure, it is crucial to know the best extraction conditions for each analyte to be determined. For this reason, an important part of the work is focused on the determination of NPS in oral fluid samples. The best conditions of extraction method were properly studied. The solid phases selected were C18 and C8 due to the non-polar interactions that they offer when the molecules are in their neutral state. In this regard, apart from the selected sorbent phase other conditions such as the pH of sample must be controlled to ensure that target molecules are in neutral state, and they have enough affinity to be retained into them. For the extraction of dichloropane, the solid phase selected was C8, after a preliminary study where slightly differences were observed in terms of the sensitivity between C8 and C18. The calibration slopes obtained using the two sorbents were 0.123 ± 0.002 and 0.101 ± 0.005 L μg^{-1} for C8 and C18, respectively. However, the rest of the analytes were extracted using C18 phase, which was selected directly for its larger hydrophobicity which was consisten with the studies reported in the literature [Montesano et al. 2017].

Once sorbent phase has been selected, critical factors to be considered, such as pH of loading and washing solutions. Also, proper mass transfer of the sample solution to the sorbent must be ensured. In this sense, it was necessary to study several aspiration cycles of loading and elution solution, as well as the necessary volume of elution to ensure adequate retention and subsequent elution of the analyte. Table 57 summarizes the proposed extraction procedures for the determination of dichloropane, methylone and synthetic cannabinoids.

After the evaluation of the best extraction conditions for each analyte, the methods were applied to determine field oral fluid samples previously fortified at different concentration levels. As it can be seen in Figure 99, average recoveries of 96 ± 11% for dichloropane were obtained at concentration levels between 250 and 750 μg L^{-1}. Oral fluid samples spiked with methylone between 50 and 400 μg L^{-1} gave recoveries of 85 ± 6%. Following the same strategy, several oral fluid samples were spiked with the five synthetic cannabinoids at concentrations of 125 and 250 μg L^{-1}, showing mean recoveries of 101 ± 2, 97 ± 8, 91 ± 3, 101 ± 5 and 64 ± 3% for 5F-ADB, MMB-CHMICA, THJ-2201, CUMYL-4CN-BNACA and MDMB-CHMCZCA, respectively.

Table 57. *MEPS procedure applied for the extraction of dichloropane, methylone and five synthetic cannabinoids (5F-ADB, MMB-CHMICA, THJ-2201, CUMYL-4CN-BINACA i MDMB-CHMCZCA).*

	Dichloropane	**Methylone**	**Synthetic cannabinoids**
Sorbent	C8	C18	C18
Conditioning	100 µL 2-propanol (x3) 100 µL water (x2)	100 µL 2-propanol (x1) 100 µL water (x1)	100 µL 2-propanol (x1) 100 µL water (x1)
Loading	90 µL sample + 10 µL phosphate buffer (pH 7,0 - 1 M) (x4)	90 µL sample + 10 µL carbonate buffer (pH 9,0 - 1 M) (x5)	96 µL sample + 4 µL phosphate buffer (pH 7,0 - 2,5 M) (x5)
Washing	100 µL water (x1)	100 µL carbonate buffer (pH 9,0 - 0,1 M) (x1)	100 µL water(x1)
Elution	50 µL 2-propanol (x10)	100 µL 2-propanol (x5)	50 µL 2-propanol (x5)

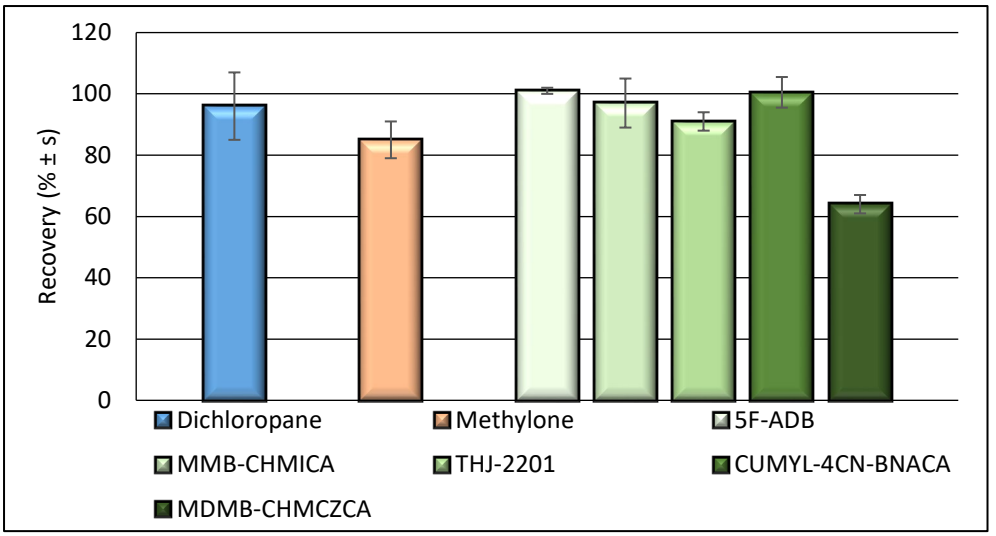


Figure 99. *Recoveries obtained after MEPS for dichloropane, methylone and the third generation synthetic cannabinoids in oral fluid samples spiked at concentration levels between 50 and 750 µg L^{-1}.*

Additionally, MEPS allows the possibility to reduce the volumes of tested drom mL (used in conventional SPE procedure) to below 100 µL for each stage of the extraction procedure.

Typical generic materials used for MEPS provides hydrophobic interactions and they are able to retain only those molecules with a remarkable non-polar behavior. These materials automatically discard all those molecules that have charge or ionic groups. It should also be mentioned that some of the limitations are the low preconcentration factors that they offer. For this reason, several materials typically recognized as mixed-mode materials in SPE have emerged in order to promote retentions between the sorbent phase by hydrophobic interactions or through iònic exchange mechanisms. Hydrophobic interactions are typically produced by the backbone of the MMS, while ionic interactions are produced by certain functional groups introduced in the solid-phase.

In this sense, this PhD project proposes the synthesis of a MMS based on MAA and EGDMA for drug determination in oral fluid samples. In addition, the prepared material was adapted to new extraction format in SPE such as micropipette tip extraction. For this reason, the polymer was prepared as monolith anchored onto the inner walls of PP micropipette tips with a capacity of 200 µL.

For the preparation of the tips, a functionalization of the internal walls of the tips was fundamental. This functionalization was carried out using benzophenone and a subsequent treatment with EGDMA as described in detail in Chapter 6 to allow a covalent bonding between the tip and the prepared monolith. After the evaluation of several polymerization mixtures, using different porogenic solvents and monomer/porogen ratios, a mixture containing 40 % of monomers and 60 % of porogenic solvent was selected. Porogenic solvent chosen was dodecanol:toluene (9:1, v/v). The monomers of the mixture consisted of 8 % (w/w) MAA and 92 % (w/w) EGDMA. 5 mg of AIBN were added to the polymerization mixture to initiate the free radical reaction. Figure 100 shows a simplified scheme of the preparation of micropipette tips.

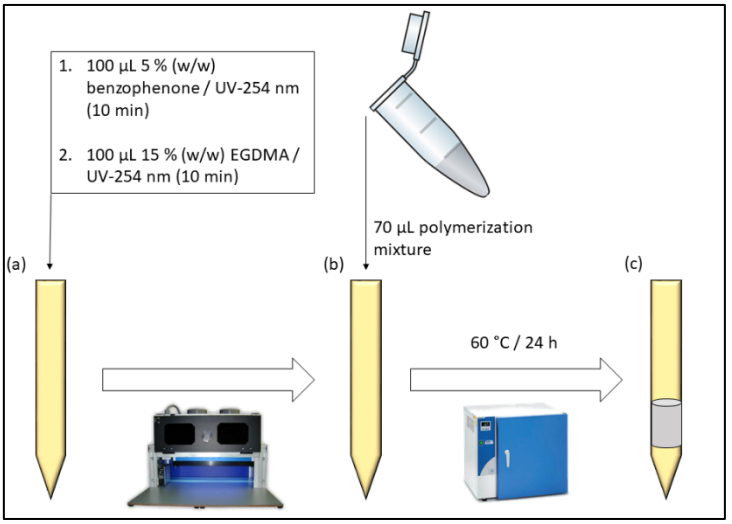


Figure 100. *Scheme of the procedure followed to prepare micropipette tips: (a) functionalitation of the tip, (b) introduction of polymerization mixture and (c) obtention of the monolith anchored to the tip.*

In this study, the use of poly (MAA-co-EGDMA) in micropipette tips, α-PVP was selected as model drug to develop an extraction procedure, the figures of merits of the developed protocol were: quantitative recoveries (107 ± 12%) for oral fluid samples spiked with 100 µg L^{-1} of α-PVP, satisfactory loading capacity (152 ng per tip) and reusability (15 uses per tip). The developed method for α-PVP was applied for the extraction of 23 different drugs including NPS and traditional drugs. The extraction was done by adding methylone-d_3, amphetamine-d_5 and cocaine-d_3 as internal standards, using UHPLC-MS / MS as analytical technique for determination. Figure 101 shows quantitative recoveries obtained for the analyzed drugs in this study (higher than 80%) for 2-FMA, MDPBP, butylone, 3-FEA, 6-APB, 4-MeMABP, 4-CEC, MDPPP, α-PVP, cocaine, α-PHP, 2C-E, MPHP and diazepam. Other substances analyzed provided recoveries higher than 60 %, which demonstrates an adequate affinity of the monolith towards the different drugs studied. Furthermore, the LODs of the method ranged from 0.03 µg L^{-1} for MDPBP to 0.6 µg L^{-1} for methylone or 3-FEA.

Based on the results obtained with the new micropipette tip filled with poly (MAA-co-EGDMA), that it can be considered as mixed-mode monolith, the research of MMS continued but trying to expand the application to more drugs. In this sense, the bibliography describes mixed-mode materials with anionic and cationic exchange behavior. However, most of these methods use mixtures of SAX / WAX and SCX / WCX layered materials or require tedious methods for synthesizing materials with amphoteric features.

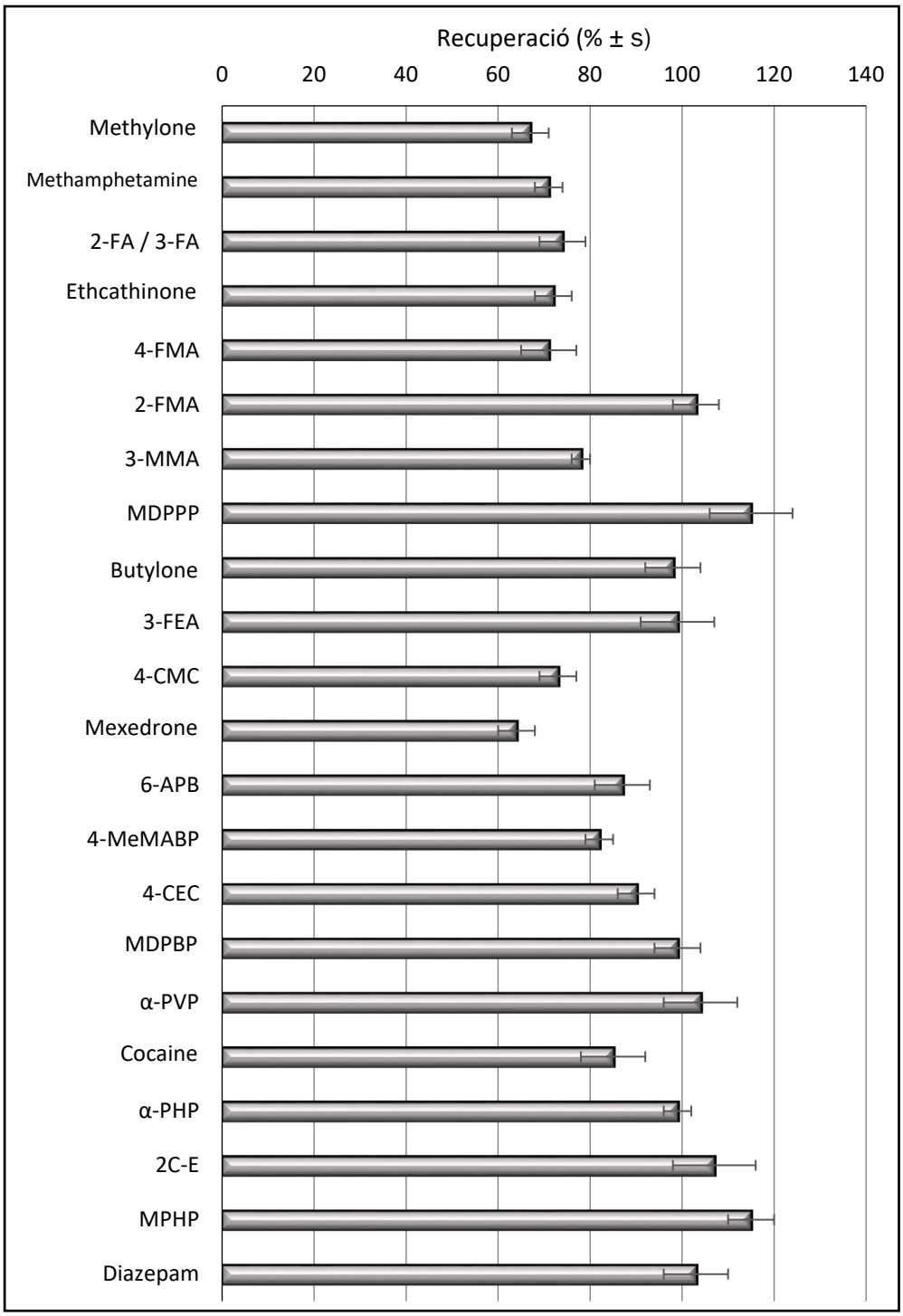
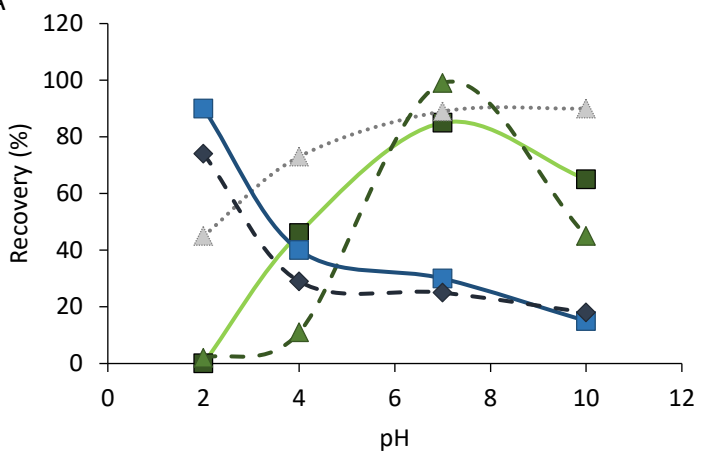


Figure 101. *Recoveries obtained for each analyzed drug after poly (MAA-co-EGDMA) pipette tip extraction.*

In Chapter 7 of this PhD Thesis, a simple and practical method to obtain aMMS containing 4VP and MAA functional monomers has been described. It also contained EGDMA as a co-monomer able to create hydrophobic interactions. Both, 4VP and MAA increase hydrophilicity of the prepared polymer, showing an amphoteric behavior that encourage to retain both acidic and basic analytes depending on the selected pH. Figure 102 shows the behavior of the target analytes for the prepared material as a function of pH. The tested compounds were cocaine and 3-MMC as basic analytes with the ability to form positively charged molecules at pH below their pK_a. On the other hand, diazepam, naproxen and diclofenac were selected as acidic drugs. In the case of diazepam, it was able to form positively charged ions under its pK_a, while naproxen and diclofenac form anions at a pH higher than their dissociation constants.

Figure 102A shows how cocaine and 3-MMC exhibit good affinity for the polymeric material at pH between 6 and 8. This is due to the fact that both substances are positively charged (due to protonation of the amino group), while the carboxylic acid of MAA is deprotonated creating ionic interactions of considerable strength. In contrast to the behavior of these two basic drugs, it is found the behavior of naproxen and diclofenac, which show greater affinity for poly (4VP-co-MAA-co-EGDMA) at acidic pH. This pH favors that the two drugs are in their protonated form (without charge), while MAA and 4VP are also protonated. The lack of ionic repulsions, the existence of π-π interactions as well as the existence of reversed-phase mechanisms encourage a high affinity of this type of analytes at acidic pH. On the other hand, diazepam shows a behavior similar to cocaine and 3-MMC, but with a less influence of pH variation. Despite, diazepam is being an acid analyte, it has a protonable amino group at pH below 3.4. For this reason, at neutral and basic pH, diazepam is uncharged in the same way as 4VP, predominating in these cases, the reversed-phase mechanisms and π-π interactions between the aromatic ring of diazepam and the 4VP.

A



B

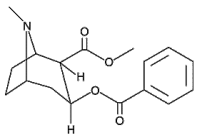
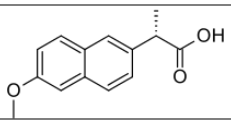
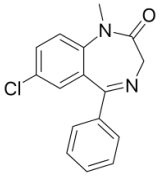
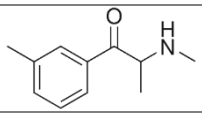
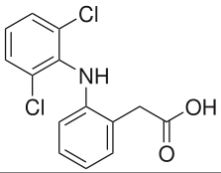
Cocaine		8.9
3-MMC		8.7
Diazepam		3.4
Naproxen		4.1
Diclofenac		4.2

Figure 102. *(A) Recoveries obtained with poly (4VP-co-MAA-co-EGDMA) for cocaine, 3-MMC, diazepam, naproxen and diclofenac at differents pH. (B) Structure and pK_a of different substances involved in this study.*

The developed material, following the aim of this Doctoral Thesis, was applied for the analysis of oral fluid samples containing cocaine, 3-MMC and diazepam using UHPLC-MS/MS after SPE. The developed method showed quantitative recoveries for the determination of cocaine (80 - 96%), 3-MMC (72 - 98%) and diazepam (69 - 91%) at concentration levels between 100 and 1000 µg L^{-1}, with LODs between 0.12 - 0.32 µg L^{-1}. It should be noted that this material has important advantages and promising applications for the analysis of large number of analytes including stronger acids than those presented in this work. Thus, these materials would achieve significant improvement in the search for generic materials with higher selectivity according to the requirements of the analysis.

Another objective that has been set throughout the development of this Doctoral Thesis is the preparation of selective materials and their subsequent adaptation to new extraction formats. In this sense, as it has been mentioned in the first section, among the selective materials, MIPs have greater application in SPE for the determination of analytes in complex matrices. In addition, the easy preparation of MIPs makes it possible to prepare them in home for its application to the extraction of drugs from biological fluids and determination using IMS, achieving simple methods of analysis using cheaper, simpler and faster instrumentation than chromatographic techniques.

In Chapter 8 of this Doctoral Thesis, a MIP has been prepared by bulk polymerization using cocaine as template, MAA as monomer and EGDMA as cross-linker. The MIP was prepared by bulk polymerization. The MIP was crushed and sieved to particle sized of 100 µm, and subsequently washed using a Soxhlet extraction to achieve the release of specific recognition sites towards cocaine. The resulting MIP particles were placed into a SPE cartridge. The best conditions for the extraction of cocaine from oral fluid samples were studied for later determination by IMS. The results showed satisfactory average cocaine recoveries (91 ± 9 %) for concentration levels between 75 and 500 µg L^{-1}, with LODs of 18 µg L^{-1}. However, different important parameters were assessed such as loading capacity of the MIP, estimated at 0.83 ng of cocaine per gram of MIP as well as the ability to reuse each of the prepared columns (5 re-uses). In order to compare the specific and non-specific retentions, a NIP was used, obtaining an imprinting factor of 2.5, which demonstrated a satisfactory selectivity of the prepared MIP.

Considering the good results obtained using this cocaine based-MIP combined with the IMS, the proposed extraction procedure was applied for the analysis of oral fluid samples from different individuals, who declared an abuse of cocaine and in some cases other substances. Samples were analyzed using LFIA (Dräger's DrugTest 5000) to determine the positive samples and also by SPE using the MIP, the extracts obtained were analyzed using IMS as vanguard technique and later by GC-MS, used as to reference technique. Results obtained by IMS and GC-MS were statistically compared. Figure 103 shows the plasmagrams obtained for the determination of cocaine in oral fluid samples after SPE for positive and a negative samples, where the most intense signal corresponds to the nicotinamide peak, used as internal calibrant. Figure 103 also shows the plasmagrams of a cocaine standard and a sample containing cocaine after DLLE using chloroform as the extracting solvent. As it can be seen, the same sample extracted by the two methods provides a plasmagram with a lower presence of ionizable substances in the case of SPE than in the case of DLLE, where a greater number of interfering compounds is extracted favoring the possibility of competitive ionization.

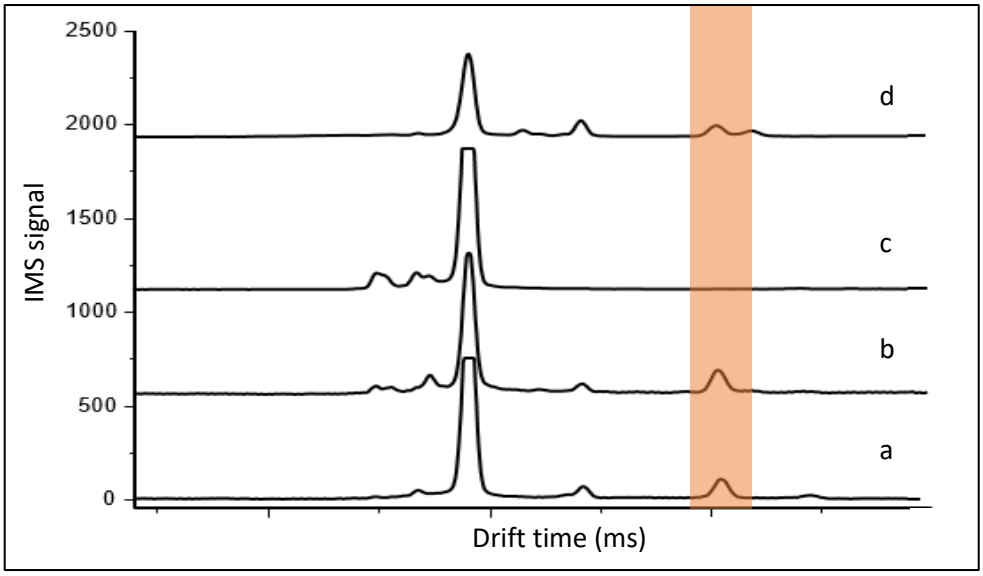


Figure 103. *Plasmagrams obtained by IMS for (a) cocaine standard of 200 µg L^{-1}, (b) positive-cocaine oral fluid sample after SPE using MIP, (c) negative-cocaine oral fluid sample after SPE using MIP (d) positive-cocaine oral fluid sample after DLLE.*

Preparation of MIPs by bulk polymerization has proven to be a simple, fast and effective strategy to obtain materials that show adequate selectivity against the analytes that have been used as template during the synthesis. However, some MIPs show cross-selectivity. Cross-selectivity is observed when a MIP is able to selectively recognize also those molecules that have certain similarities to the molecule used as template during the synthesis.

In order to assess the cross-selectivity that MIPs may present, a MIP was prepared using methamphetamine as template. Once this polymer was prepared, an SPE procedure was developed consisting in: i) column conditioning (1 mL methanol containing 1 % acetic acid + 1 mL deionized water), ii) loading 1 mL of sample (previously centrifuged for 2 min at 5000 rpm), iii) washing (using 1 mL 0.1 M phosphate buffer pH 9.0 containing 10 % 2-propanol) and iv) elution for which 0.5 mL of methanol containing 1 % acetic acid were used. This extraction procedure was studied using amphetamine as target molecule, achieving recoveries between 99 and 115 % by IMS and between 81 and 108 % by UHPLC-MS/MS in oral fluids samples spiked at concentration levels of 300 μg L^{-1}.

Using the developed procedure for amphetamine extraction, 32 different NPS including groups of amphetamine derivatives, synthetic catinones and phenethylamines were extracted. Likewise, 9 other substances used as control that did not have structural similarities to methamphetamine were extracted, including phenacetin, codeine, diphenhydramine, lidocaine, levamisole, nicotine, benzocaine, cocaine, and MMB-CHMICA. Recoveries obtained for those substances similar to methamphetamine, as it can be seen in Figure 104, are quantitative, with values between 80 and 120% with an average recovery of 96 ± 12 %. In contrast, the other substances selected as control to assess cross-selectivity showed considerably lower recoveries, with an average recovery of 46 ± 19 %. The proposed method for the extraction of these NPS, apart from the good recoveries, it offered satisfactory LODs of between 10 and 80 μg L^{-1} for IMS and between 0.03 and 1.1 μg L^{-1} for UHPLC-MS/MS.

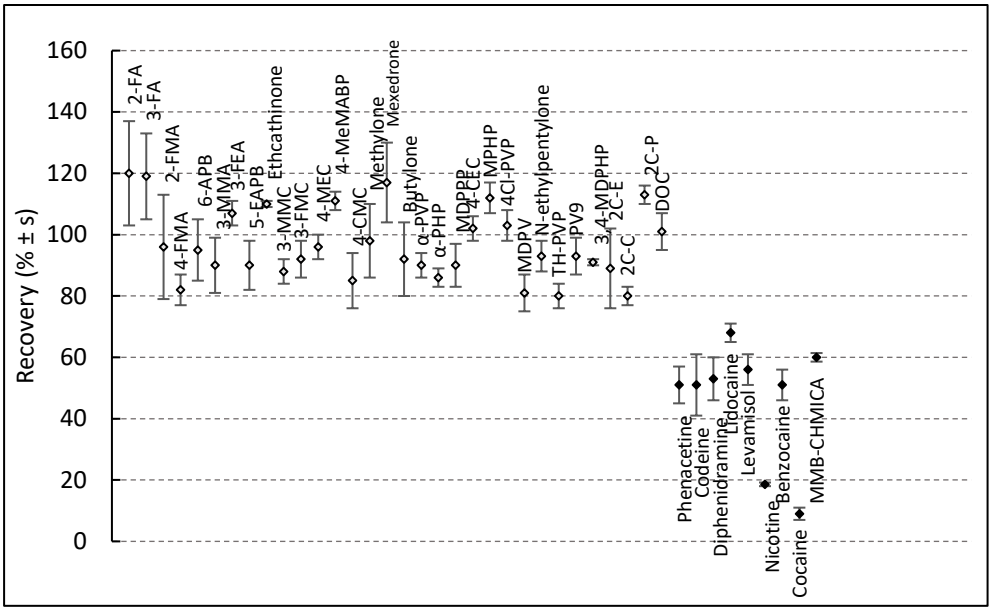


Figure 104. *Recoveries obtained for the 41 substances used to evaluate the cross-selectivity of methamphetamine based-MIP after SPE extraction.*

The prepared MIP was morphologically characterized using SEM and by the measurement of the surface area using nitrogen adsorption / desorption technique. The results of these measurements gave a surface area of 393 ± 4 m^2 g^{-1} for the MIP and 182.8 ± 0.3 m^2 g^{-1} for the NIP, where higher surface area is observed in the case of MIP which allows a more effective distribution of the analytes between the sample and the sorbent phase. The nitrogen adsorption / desorption technique made also possible to estimate the diameter of the micro / mesopores of materials being 2 nm in the case of MIP, while in the NIP the presence of micro / mesopores was negligible. These data were used to compare the size of micro / mesopores with the size of various drugs that were estimated using computational calculations using the Orca software (version 4.0.1) of the Max-Planck Institute (Mülheim an der Ruhr, Germany) and the PM3 semiempirical method. These substances were 2-FA, 3-FA, 2-FMA, 4-FMA, 3-MMA, 5- EAPB, 2C-E and DOC which showed a size lower than 87.6 $Å^2$, therefore they were able to access into micro / mesopores of MIP.

Some MIPs are able to offer cross-selectivity. Thus, an interesting approach is the modification of this selectivity according to the extraction procedure employed. In Chapter 10, a MIP was prepared by bulk polymerization using 3-OH PCP as template. Thus, a MIP was prepared using MAA and EGDMA as monomer and cross-linker, respectively in a ratio of 1:4:20 (template:monomer:cross-linker),

and as porogenic solvent N, N-dimethylformamide. Once the MIP was synthesized, the best extraction conditions were studied with special attention on the washing step to selectively determine several arylcyclohexylamines, (PCP, 3-MeO PCP, 3-MeO PCE, MXE, 2-oxo PCE, dechloroketamine and ketamine). Table 58 shows the procedures followed for the selective determination of selected arylcyclohexylamines as well as the specific procedure for the determination of 3-OH PCP.

Table 58. *SPE procedures followed for the selective extraction of arylcyclohexylamines and the specific extraction of 3-OH PCP.*

	Arylcyclohexylamines	**3-OH PCP**
Conditioning	1 mL 2-propanol (containing 5 % acetic acid) 1 mL water	1 mL 2-propanol (containing 5 % acetic acid) 1 mL water
Loading	1 mL sample + 0,5 mL phosphate buffer (pH 7,0 - 0,1 M)	1 mL sample + 0,5 mL phosphate buffer (pH 7,0 - 0,1 M)
Washing	1 mL water (containing 5 % 2-propanol and 0,1 % acetic acid)	2 mL acetonitrile
Elution	1 mL 2-propanol (containing 5 % acetic acid)	1 mL 2-propanol (containing 5 % acetic acid)

On the one hand, using the selective procedure, good affinity of the MIP towards arylcyclohexylamines was demonstrated, with recovery percentages between 67 and 98 % for all of them after an SPE of standards prepared in water at a concentration level of 250 µg L^{-1}. On the other hand, the procedure using acetonitrile as a washing solvent showed recoveries of 62 % in standards of 3-OH PCP prepared in water at 250 µg L^{-1} while the rest of arylcyclohexylamines gave recoveries below 42 %. This, demonstrated higher affinity of the MIP towards that molecule used as template.

The specific method for the extraction of 3-OH PCP was applied for different oral fluid samples spiked at 250 and 500 µg L^{-1} followed by determination by IMS. Average recoveries of 72.0 ± 1.4 % were obtained, while the method specified for the extraction of arylcyclohexylamines gave recoveries of 92 ± 13 % for the same samples. Figure 105 shows the plasmagrams obtained after extraction of oral fluid samples spiked at 500 µg L^{-1} using the most specific method and the selective method for the determination of arylcyclohexylamines. As it is shown at Figure

105, the specific method for 3-OH PCP provided a single-peak, whereas while the selective method for the determination of arylcyclohexylamines shows the presence of other ionizable compounds, although both plasmagrams provided comparable signals of 3-OH PCP.

Up to this point, all studies using MIPs were conducted using a traditional SPE format. It should be noted that the conventional SPE (in cartridge or column) is a well-established format of extraction and it is the first choice when new materials and new procedures are being developed. For this reason, the first studies of this Thesis Doctoral were carried out in a SPE cartridge. However, it is also true that this format has some limitations or drawbacks. On the one hand, SPE extraction usually involves multi-stage methods (conditioning, loading, washing, drying, and elution) that can result in long sample treatment times. Similarly, they need to be developed in a laboratory on a regular basis as vacuum systems are required or require considerable use of solvents. Usually, the sample volumes and solvents have a limited ability to preconcentrate. In this sense, various formats of SPE have emerged to solve some of these problems. The work presented below is of particular interest in resolving this lack of portability of convenctional SPE, reducing solvent consumption as well as improving the preconcentration capacity using large sample volumes.

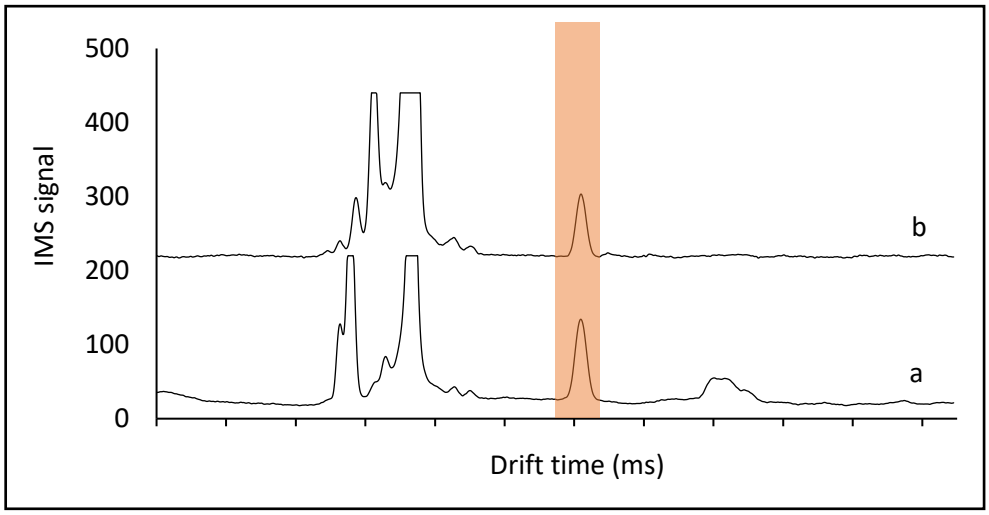


Figure 105. *Plasmagrams obtained by IMS for 3-OH PCP spiked samples using the selective procedure to arylcyclohexylamines (a) and the specific method to 3-OH PCP (b).*

Based on the satisfactory results offered by MIP synthesized for the determination of cocaine evaluated in Chapter 8, and taking into account that cocaine is a drug of special importance, its analysis has a relevant interest. Thus, in Chapter 11 a sampling and sample treatment device for the determination of cocaine in oral fluid samples is developed. This device was prepared with the scope of incorporating these two basic stages of any analysis before determination into a single device in order to be able to perform the *in-situ* analysis. Figure 106 shows the different parts that compose the proposed device. First, the sampler is formed by a cotton pad containing a wet indicator at the end. The purpose of this sampler is that when it is introduced into the mouth, the cotton adsorbs as much oral fluid as is possible until the indicator changes color to reveal that it has been completely wet. After studying the most suitable cotton mass for sampling (50 mg), it was estimated that the amount of oral fluid that it was able to absorb was 320 ± 30 µL (considering a saliva density of 1 g mL^{-1}). The second part of the device is formed by the selective MIP for the determination of cocaine. The main advantage of this system is that the selective material is not in direct contact with the sampler, but the transfer of the sample from the collector to the MIP can be easily done.

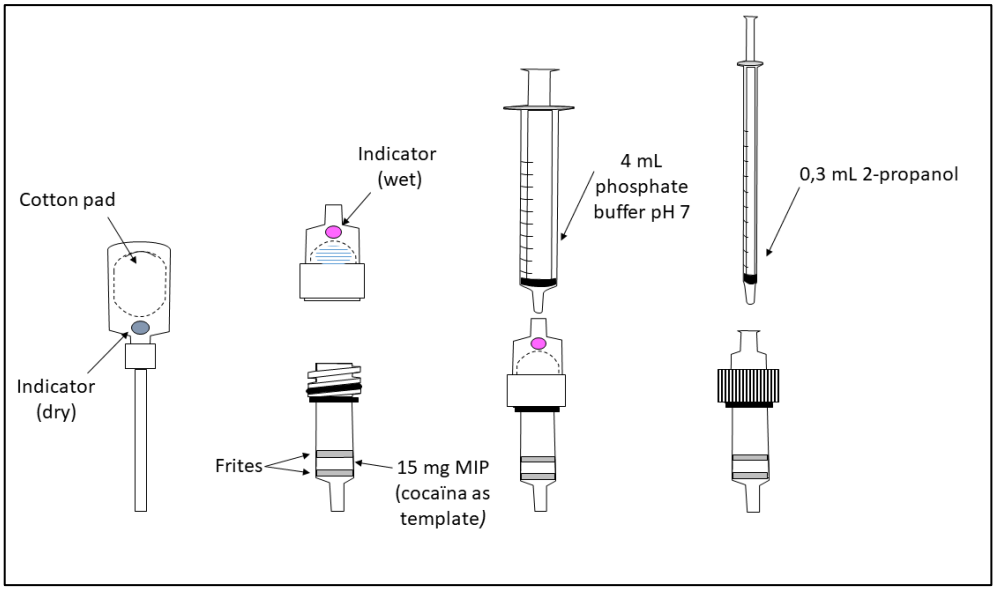


Figure 106. *Scheme of the different parts of device for in-situ extraction of cocaine.*

To transfer the sample from the collector to the MIP, a single pre-filled syringe with 4 mL of 0.1 M phosphate buffer pH 7.0 was used. This step involves the incorporation of the typical steps of conditioning, loading and washing of typical SPE in a single step, removing the need for vacuum systems. With this transfer buffer, cocaine of the oral fluid adsorbed onto cotton was semi-quantitatively dragged, while the other components of the matrix were eluted. The cocaine retained on the MIP was eluted using a second syringe pre-filled with 0.3 mL of 2-propanol being the collected extract analyzed using IMS. The use of IMS made possible to determine concentration of cocaine with enough sensitivity, quickly and without the need for specialized personnel. In addition, it has great potential for portability being possible to perform *in-situ* analyzes.

Cut-off concentration for determination of cocaine was estimated at 20 μg L^{-1}, since it was the value at which 20 saliva samples, which did not contain cocaine previously, were spiked to this concentration level showing 100% accuracy in identifying positive samples and in the same way 95% accuracy in identifying negative samples. In addition, loading capacity of the device was estimated at 130 mg L^{-1} considering the amount of sample adsorbed by the sampler as well as the recoveries shown by the device for saliva samples spiked with cocaine at 100 μg L^{-1} (53 ± 6 %).

The developed device was tested for the analysis of 20 saliva samples from individuals age between 19 and 38 years who had declared an abuse of an uncontrolled dose of cocaine between 15 and 180 min before sampling. After sampling analysis (performed in less than 5 min), positive results were observed for all samples. These results were comparable to those found by LFIA (Dräger DrugTest 5000), which was used as reference method to check the presence of cocaine.

Other of the limitations of SPE was the lack of preconcentration capacity in those situations where reduced sample volumes are available and relatively high extraction volumes are needed as well as the need for vacuum systems to carry out the extraction. Particularly, SPE procedure described for the determination of cocaine in oral fluid samples (Chapter 8) raises these two drawbacks. For this reason, Chapter 12 studies the preparation of MMIPs by bulk polymerization using surface-modified MNPs to anchor the polymer onto the MNPs.

As is shown in Figure 107, two different MMIPs were prepared. The only difference between the PEG-MMIP and the V-MMIP is the procedure for anchoring the MIP to the surface of nanoparticles. Non-modified magnetite

nanoparticles were synthezised and subsequently functionalized in order to achieve appropiate immobilization of MIPs. First, PEG was used to create hydrophilic coating on the MNPs that will favor their encapsulation during the polymerization step. Another approach was the modification of MNPs using γ-MPS. This agent produces a MNPs surface functionalitation with vinyl groups that allowed a covalent bonding of the MIP. The conditions to prepare the MIP were those indicated in Chapter 8.

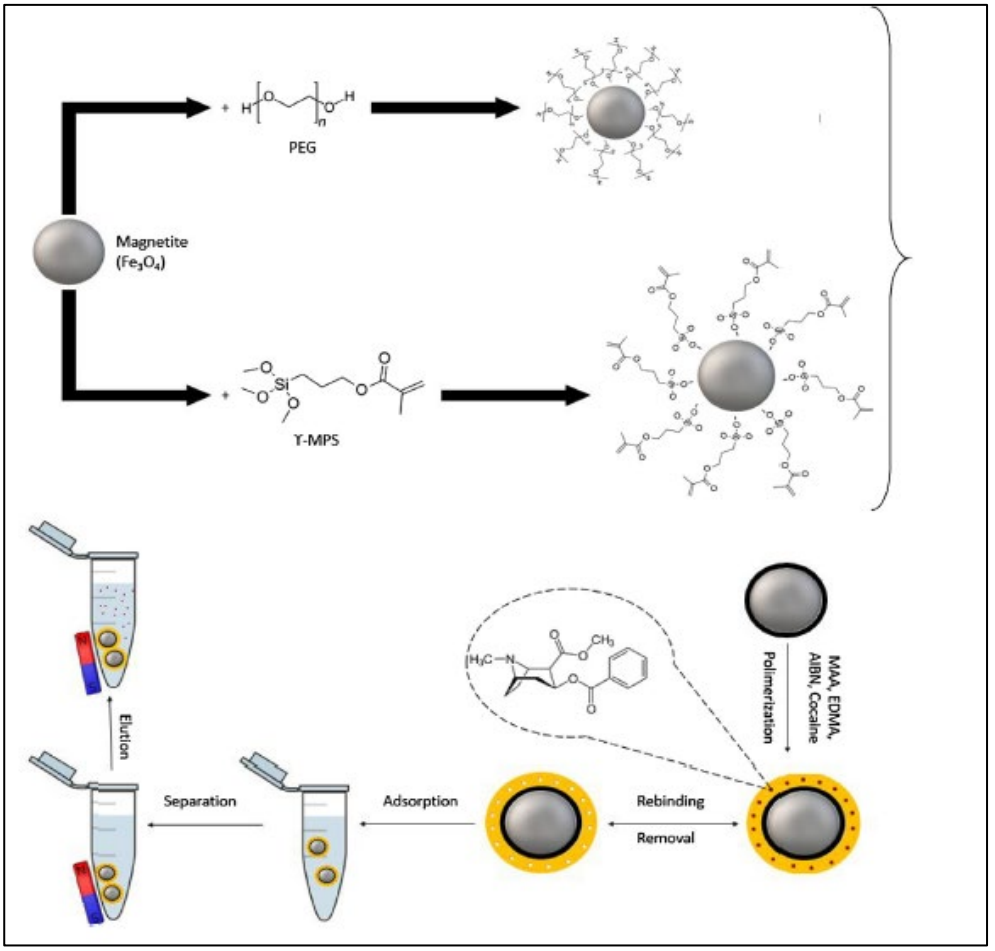


Figure 107. *Preparation of MMIPs and extraction procedure developed.*

After the preparation of PEG-MMIP and V-MMIP, the best extraction conditions for the subsequent determination of cocaine by IMS were studied. The developed consisted in the addition of 5 mg of MMIP in 1 mL of methanol containing 5 % acetic acid for the activation of the sorbent. Supernatant was removed and then 0.5 mL of deionized water was added to remove excess

methanol. Loading step was performed by adding 0.5 mL of sample and 0.1 mL pH 9.0 ammonium buffer and subjected to 10 min of stirring to ensure a quantitative mass transfer. The washing step was performed in two stages, a first washing using 0.5 mL of water and a second with 0.5 mL of chloroform. Finally, 0.2 mL of methanol containing 5 % acetic acid was used to elute the cocaine and injected into the IMS. The features of PEG-MMIP and V-MMIP were evaluated and compared with each other for cocaine extraction in oral fluid samples and subsequent determination by IMS. Thus, average recoveries of 86 ± 11% for PEG-MMIP and 92 ± 4% for V-MMIP were obtained after extraction of spiked oral fluid samples at concentration levels between 80 and 560 μg L^{-1}. Also, the loading capacity was evaluated for both materials, which was estimated at 0.124 and 0.102 mg of cocaine per gram of PEG-MMIP and V-MMIP, respectively. It should be mentioned that it was observed that the V-MMIP offers greater stability than the PEG-MMIP when performing extractions. It can be explained by the detachment of part of the PEG-MMIP during the extraction. Thus, part of the polymer in PEG-MMIP was separated from the MNPs as could be observed by SEM while in the case of the V-MMIP they remain stable. It can be seen in Figure 108, where the SEM images before and after extraction, for V-MMIPs did not offer significant changes in terms of polymer shape, whereas the PEG-MMIPs after one use, showed the presence of bare MNPs (white dots in the image).

The developed procedure achieved higher preconcentration factors than SPE (2.5) and LODs of 6 μg L^{-1} using V-MMIP. The protocol was applied for the analysis of 7 oral fluid samples from people who had consumed cocaine and results obtained were compared with two reference methods. First, all the evaluated samples were previously analyzed with an LFIA (Dräger's DrugTest 5000) to determine the number of positive and negative samples. Then, all samples were analyzed with a second high-sensitivity confirmatory method such as the UHPLC-MS/MS, obtaining results statistically comparable to the proposed method and the confirmatory method.

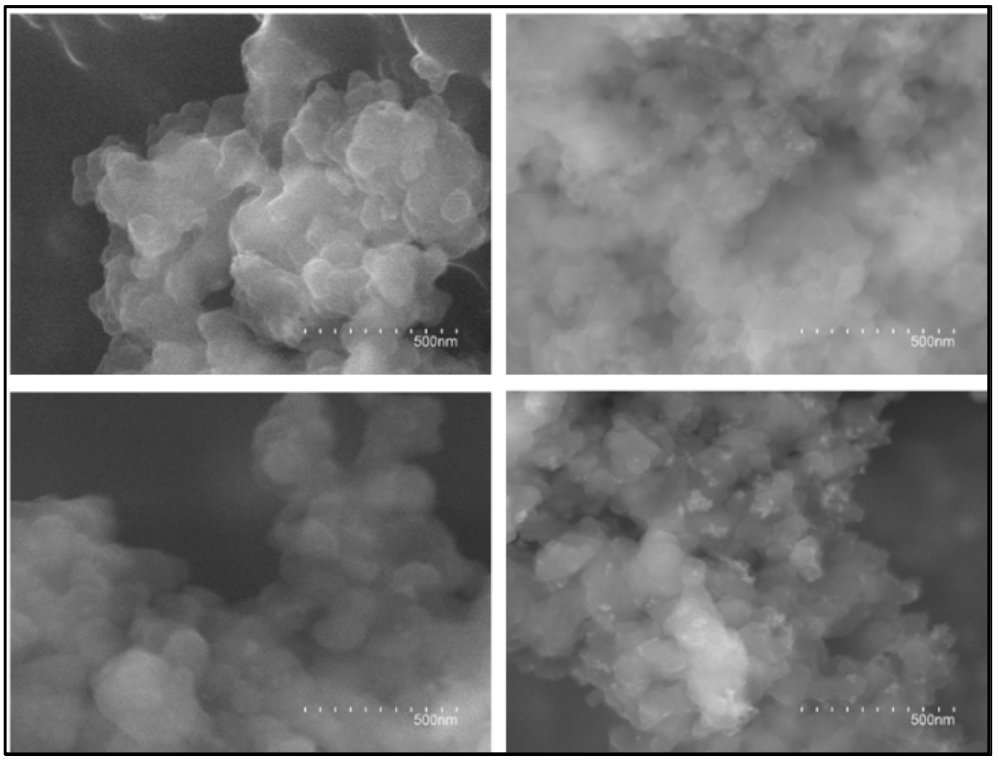


Figure 108. *SEM images obtained at 20.000x magnifications of V-MMIP (up left) and PEG-MMIP (up right) prior to their use, andl V-MMIP (down left) and PEG-MMIP (down right) after an extraction under proposed procedure.*

Concentration of drugs in wastewater and natural water samples is one of the best epidemiological parameters to know the consumption within a population. SPE has proved to be an effective approach for the preconcentration of such samples. However, the large volumes required to achieve high preconcentration factors lead to very long analysis times due to the high loading time to ensure adequate retention of the analyte. In this regard, Chapter 13 presents the preparation of a sorbent as monolith onto a PTFE disk in order to retain the analyte in a simple manner and without using high sample preparation times. Specifically, the sorbent used in this paper is a MIP prepared using ecgonine methyl ester as template, which is one of the main metabolites of cocaine. The other reagents used for the preparation of the MIP were MAA as monomer, EGDMA as cross-linker, AIBN as initiator and acetonitrile, which was used as porogenic solvent.

First of all, before the synthesis of MIP onto PTFE disk, it was modified to allow the effective anchoring of the MIP on its surface. After this functionalization, which is described in detail in Chapter 13, the corresponding

MIP was prepared thermally, obtaining a proper immobilized and agitation-resistant monolithic bed (see Figure 109). To allow agitation, two magnets were placed on the outer walls of the disk.

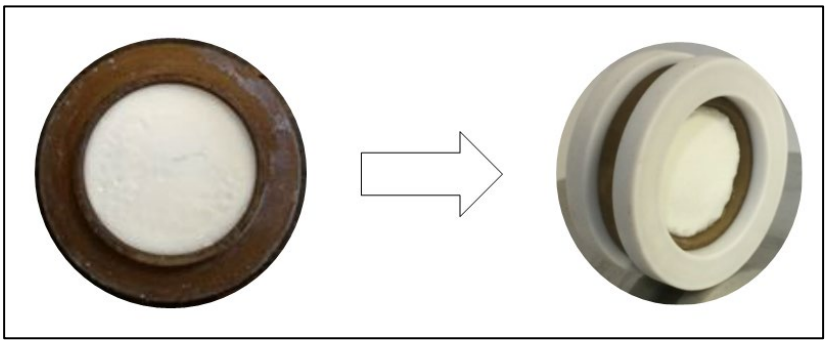


Figure 109. *Images of the agitation-extraction MIP disk prepared without (left) and with magnets (right).*

The prepared disk allowed agitation and extraction, during the same step, of high sample volumes (200 mL). The best extraction conditions were investigated including the influence of factors such as pH of sample or the loading and elution times. Under studied conditions, quantitative recoveries of ecgonine methyl ester (100 %) were obtained at concentration level of 2 µg L^{-1}. Affinity of the prepared MIP against two related molecules was evaluated, such as cocaine (58 % recovery) and benzoylecgonine (0 % recovery), giving a good selectivity of the MIP towards the ecgonine methyl ester and to cocaine, but without affinity towards the other major metabolite of cocaine. Some of the most important features of the MIP were also evaluated. For instance, loading capacity was estimated at 6.68 µg of ecgonine methyl ester per disk, and imprinting factor with a value of 25 was found, which demonstrated the high selectivity of MIP towards cocaine agains other related compounds.

Once the good capacity of the prepared MIP in disk format for the extraction of ecgonine methyl ester was demonstrated, it was applied for the analysis of surface water and sewage water samples using two different analytical techniques. IMS was evaluated as lower sensitivity screening technique with a LOD and LOQ of 75 and 250 ng L^{-1}, respectively. On the other hand, UHPLC-MS/MS was used as confirmatory technique of high sensitivity with an LOD and LOQ of 13 and 45 ng L^{-1}, respectively. Table 59 shows the results obtained for the analysis of 6 samples collected from different areas near the city of València. As it can be seen, the presence of ecgonine methyl ester was found in two samples. On the other hand, UHPLC-MS/MS allowed the quantification of almost all samples,

giving very small concentrations of the analyte in the samples collected being undetectable in one of them (sample 5) and below LOD in another sample (sample 2).

Table 59. *Obtained results by IMS and UHPLC-MS/MS for econine methyl ester determination in different water samples after extraction using the agitation-extraction MIP-disk .*

Sample	Location	Concentration (ng L^{-1}± s)	
		IMS	UHPLC-MS/MS
1	Port of València	< LOD	98 ± 8
2	Pond (Almenara)	< LOD	< LOQ
3	Lagoon (Marjal dels Moros)	< LOD	67 ± 5
4	Irrigation ditch (Puçol)	< LOQ	143 ± 10
5	Irrigation ditch (Llíria)	< LOD	< LOD
6	Urban wastewater	< LOQ	195 ± 13

IMS: ion mobility spectrometry, LOD: limit of detection, LOQ: limit of quantification, UHPLC-MS/MS: ultra high performance liquid chromatography tandem mass spectrometry..

On the bassis of the good results shown by MIPs as selective materials, it is interesting to focus on materials that have more recently application in sample treatment. Aptamers are excellent candidates to replace antibodies. They have often lower prices, greater stability and the ability to make modifications during synthesis, which provides extra stability or improved immobilization. Aptamers offer selectivity and loading capacity comparable to antibodies.

In this sense, the last chapter (Chapter 14) of this Doctoral Thesis presented the results of preliminary studies of the immobilization of three different aptamers onto CNBr-activated saphanose and magnetic particles. Aptamers selected were FQ1 and FQ2, selective for determination of fluoroquinolones and MA, selective for determination of methamphetamine. Aptamers were immobilized onto sepharose with the scope of studying their cross-selectivity using a conventional SPE procedure while the immobilization onto the magnetic particles was aimed at the extraction by a MDSPE procedure. Aptamers used throughout this study were modified with the introduction of an amino group at the 5 ’ end and a C12 spacer arm for the FQ1 and FQ2 aptamers. The MA aptamer

was also modified by introducing an amino group at the 5 'end and a C6 spacer arm. After testing various immobilization procedures, which are described in detail in Chapter 14, immobilization yields between 12.3 and 36.6% were obtained considering the two types of supports mentioned. Although the percentage of immobilized aptamers was comparable to the percentage usually obtained in these procedures, the prepared oligosorbents showed a complete lack of affinity to the corresponding analytes, with recoveries under LOD in most loading and washing conditions evaluated. Thus, it demonstrated a low analyte-aptamer interaction. Two factors may explain this lack of recognition towards analytes. The first factor may be a low proportion of active or functional aptamers immobilized and consequently they do not have the ability to recognize the analytes. The second possibility is the denaturation of the oligonucleotide chains due to unknown reasons that produce the lost of appropriate structure to retain analytes. In this sense, it is necessary to continue working on these possible factors in order to evaluate the application of oligosorbents to the extraction of drugs in biological matrices such as saliva for later determination using rapid and sensitive techniques such as IMS.

CONCLUSIONS

CONCLUSIONS

Com a conclusions dels estudis portats a terme al llarg de la realització d'aquesta Tesi Doctoral, s'hi pot extraure que l'aplicació de materials intel·ligents per a l'anàlisi forense en general ha demostrat tenir molt bones capacitats per tal de determinar de manera selectiva els anàlits. D'aquests casos és especialment destacable l'anàlisi de drogues per la seua importància a la societat actual i els problemes que comporta el consum de drogues en nombrosos àmbits. En aquest sentit, l'IMS és una tècnica prometedora per a l'anàlisi ràpid de drogues per la seua elevada sensibilitat i rapidesa però amb un ús limitat causat per la seua baixa selectivitat. La combinació de tractaments de mostra emprant materials intel·ligents amb l'IMS ha demostrat que tot i ser un camp relativament inexplorat és una bona solució per tal d'eliminar potencials interferents i aconseguir resoldre la principal limitació de l'IMS en aquelles mostres on les drogues es troben en matrius complexes com ho són els fluids biològics.

L'anàlisi de drogues, pot prendre dues vessants diferents. En algunes ocasions és interessant disposar de metodologies versàtils que permeten una determinació senzilla d'un gran nombre de substàncies. En aquests casos serà fonamental recórrer a tècniques analítiques d'elevada sensibilitat i selectivitat com ho són la GC-MS o l'UHPLC-MS/MS. En aquest sentit, com que la selectivitat durant l'etapa de tractament de mostra no suposa un element crític es poden emprar materials de caràcter més genèric com ho són les tradicionals fases de sílice modificada amb C18 o C8 o materials de tipus mixt. Al llarg d'aquesta memòria s'ha exposat materials com aquests i s'han adaptat a nous formats d'extracció miniaturitzats que permeten una fàcil adaptació de tècniques com la SPE. Un exemple és l'adaptació de la MEPS per a l'anàlisi de dicloropà, metilona, 5F-ADB, MMB-CHMICA, THJ-2201 i CUMYL-4CN-BINACA totes elles considerades NPS de diverses famílies. Un altre exemple és la preparació de puntes de micropipeta farcides de *poly(MAA-co-EGDMA)* per a l'extracció de drogues, les quals van mostrar resultats satisfactoris. També, el desenvolupament de nous materials polimèrics que combinen els avantatges dels materials de bescanvi iònic amb les interaccions hidrofòbiques, és altament desitjable com per exemple, propietats WAX-WCX en un mateix adsorbent, cosa que permet estendre l'aplicació d'aquest material a quasi qualsevol substància bé tinga propietats àcides, bàsiques o neutres.

L'altra perspectiva per a l'anàlisi de drogues és enfocar-la cap a tractaments de mostra més selectius que permeten una determinació molt més ràpida amb tècniques analítiques com l'IMS. En aquest sentit, s'han estudiat les propietats dels MIPs per tal de ser aplicats a la posterior anàlisi de drogues en mostres de saliva. Al llarg d'aquesta Tesi Doctoral s'ha posat especial rellevància a l'estudi de la selectivitat creuada que alguns MIPs poden oferir cap a molècules estructuralment

similars. Les conclusions que s'hi poden extraure és que per exemple el MIP que es va preparar emprant metamfetamina com a *template* va demostrar tenir una molt bona selectivitat cap a totes aquelles NPS de tipus amfetamina que s'hi van estudiar. Però també, el MIP preparat emprant 3-OH PCP com a *template* va oferir selectivitat creuada que podia ser modificada segons l'etapa de rentat cap a la determinació específica del 3-OH PCP o cap a la determinació selectiva d'un nombre major d'arilciclohexilamines.

Els darrers capítols d'aquest projecte es centren en l'adaptació dels MIPs a nous formats d'extracció. Tots els materials desenvolupats van aconseguir mostrar avantatges front a la SPE convencional. En el cas del dispositiu per a la determinació *in-situ* de cocaïna s'hi va aconseguir desenvolupar un únic dispositiu, que amb un procediment senzill permet una extracció ràpida i semi-quantitativa de cocaïna en mostres de saliva. Els MMIPs, van demostrar aconseguir millors factors de preconcentració que la SPE i a més la MDSPE va aconseguir eliminar la necessitat de sistemes de buit i reduir els volums de mostra i dissolvents per a portar a terme l'extracció. Finalment, el disc d'agitació-extracció va mostrar poder assolir factors de preconcentració de fins a 40 vegades, cosa que en combinació amb una tècnica d'elevada sensibilitat com l'UHPLC-MS/MS va permetre la determinació d'ecgonina metil èster en mostres d'aigües on la concentració d'aquest anàlit es presenta a nivell de traces.

Finalment, aquesta Tesi Doctoral també serveix com a punt de partida per al desenvolupament de noves metodologies basades en l'extracció emprant oligosorbents. En aquest sentit, no s'han aconseguit bons resultats als estudis preliminars portats a terme, motiu pel qual és necessari continuar l'estudi amb major profunditat per resoldre els problemes que s'han presentat i que de moment han mostrat una baixa afinitat de l'oligosorbent cap als anàlits.

As general conclusions of studies done along this Doctoral Thesis it can be mentioned that smart materials have been applied satisfactorily to forensic analysis. They have good properties and they allow to determine selectively numerous analytes. Drug analysis is one of the most remarkable fields on forensic analysis due to its importance on the society and the numerous risks associated with drug abuse. In this sense, IMS is a powerful technique for drug analysis due to its quickness, simplicity and sensitivity. However, IMS shows an important drawback due to its limited selectivity. Sample treatment using smart materials combined to IMS for analytical determination is relatively unexplored, but it promises to be an interesting solution to remove interfering compounds and to solve the limited IMS selectivity for drug analysis in biological fluids.

Drug analysis can be addressed from two approaches. Sometimes it is interesting to obtain versatile methodologies to determine a high number of substances. Then, it is fundamental to use sensitive and selective analytical techniques such as GC-MS or UHPLC-MS/MS. In this sense, selectivity of the sample treatment is not a critical step, and hence, non-selective materials can be used such as C18 modified silica or mixed-mode materials. Along this dissertation, it has been described how non-selective materials have been adapted to miniaturized SPE methods. For example, MEPS has been used for NPS determination in oral fluid including dichloropane, methylone, 5F-ADB, MMB-CHMICA, THJ-2201 and CUMYL-4CN-BINACA. Besides, a mixed-mode material based on poly (MAA-co-EGDMA), has been used anchored to pipette tips to determine satisfactorily several illicit substances. Thus, new polymeric materials have proved their good properties due to the combination of ionic exchange and hidrophobic interactions. The resulting material can exhibit simultaneously properties of WAX-WCX in only sorbent. For this reason, this material results suitable for determination of numerous substances including acidic, basic and neutral compounds.

The other approach adopted for drug analysis is focused on the use of selective sample treatment. When selectivity of sample treatment is improved, simple and fast analytical techniques as IMS can be employed for drug determination. In this sense, MIPs have been studied for its subsequent application to drug analysis in oral fluid samples. One of the most studied parameters of these imprinted materials has been cross-selectivity. As an example, a prepared methamphetamine based-MIP exhibited a good selectivity towards all amphetamine-type NPS studied. Besides, 3-OH PCP based-MIP offered selectivity towards arylcyclohexylamines. Its selectivity was modified by tailoring the washing step, hence, two SPE procedures were obtained. One procedure showed specificity toward 3-OH PCP, while the second one provided good recoveries for all studied arylcyclohexylamines.

Last chapters of this PhD Thesis were focused on the adaptation of MIPs to new extraction approaches. The developed materials demonstrated benefits against traditional SPE formats. MIP-based device for *in-situ* determination of cocaine allowed to integrate in only one device a simple, fast and semi quantitative method for cocaine determination. On the other hand, MMIPs achieved better preconcentration factors than conventional SPE. Besides, MDSPE achieved to remove vacuum systems and to decrease sample/solvent volume used in the extraction procedure. Moreover, agitation-extraction disk showed preconcentration factors up to 40. Thus, the combination of disk extraction and UHPLC-MS/MS, allowed the determination of ecgonine methyl ester at traces level in natural and wastewater samples.

Finally, this Doctoral Thesis opens the wat to the development of new extraction methodologies based on the use of oligosorbents. Although, preliminary results were not satisfactory, further studies are needed in order to find the possible causes of the lack of affinity exhibited by oligosorbents toward target analytes.

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ANNEXOS

ANNEX

Annex A. Publicacions derivades de la Tesi Doctoral

Annex A. Published papers from the Doctoral Thesis

Analytica Chimica Acta 1026 (2018) 37–50

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Review

Trace analysis by ion mobility spectrometry: From conventional to smart sample preconcentration methods. A review

A. Sorribes-Soriano, M. de la Guardia, F.A. Esteve-Turrillas, S. Armenta*

Analytical Chemistry Department, University of Valencia, 50th Dr. Moliner St., 46100, Burjassot, Spain

Journal of Chromatography A, 1603 (2019) 61–66

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Determination of the new psychoactive substance dichloropane in saliva by microextraction by packed sorbent – Ion mobility spectrometry

Aitor Sorribes-Soriano, Amparo Monedero, Francesc A. Esteve-Turrillas*, Sergio Armenta

Department of Analytical Chemistry, University of Valencia, 50thDr. Moliner St., 46100 Burjassot, Spain

Microchemical Journal 153 (2020) 104504

Contents lists available at ScienceDirect

Microchemical Journal

journal homepage: www.elsevier.com/locate/microc

Methylone determination in oral fluid using microextraction by packed sorbent coupled to ion mobility spectrometry

A. Sorribes-Soriano, S. Sánchez-Martínez, R. Arráez-González, F.A. Esteve-Turrillas, S. Armenta*

Department of Analytical Chemistry, Universitat de València, 50th Dr. Moliner St., 46100 Burjassot, Spain

Journal of Analytical Toxicology, 2020;00:1–6
doi: 10.1093/jat/bkaa091
Article

OXFORD

Article

Determination of Third-Generation Synthetic Cannabinoids in Oral Fluids

Aitor Sorribes-Soriano, Josep Verdeguer, Agustín Pastor, Sergio Armenta and Francesc A. Esteve-Turrillas*

Department of Analytical Chemistry, University of Valencia, Jeronim Muñoz Building, 50th Dr. Moliner St., 46100 Burjassot, Spain

*Author to whom correspondence should be addressed. Email: francesc.a.esteve@uv.es

Talanta 205 (2019) 120158

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

ELSEVIER

Development of pipette tip-based poly(methacrylic acid-*co*-ethylene glycol dimethacrylate) monolith for the extraction of drugs of abuse from oral fluid samples

A. Sorribes-Soriano, A. Valencia, F.A. Esteve-Turrillas*, S. Armenta, J.M. Herrero-Martínez**

Department of Analytical Chemistry, University of Valencia, 50th Dr. Moliner St., 46100, Burjassot, Spain

Journal of Chromatography A, 1481 (2017) 23–30

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

ELSEVIER

Cocaine abuse determination by ion mobility spectrometry using molecular imprinting

A. Sorribes-Soriano, F.A. Esteve-Turrillas, S. Armenta*, M. de la Guardia, J.M. Herrero-Martínez*

Department of Analytical Chemistry, University of Valencia, 50th Dr. Moliner St., 46100 Burjassot, Spain

Analytica Chimica Acta 1052 (2019) 73–83

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Amphetamine-type stimulants analysis in oral fluid based on molecularly imprinting extraction

Aitor Sorribes-Soriano [a], Francesc A. Esteve-Turrillas [a], Sergio Armenta [a, *], Pedro Amorós [b], José Manuel Herrero-Martínez [a, **]

[a] Department of Analytical Chemistry, University of Valencia, 50th Dr. Moliner St., 46100, Burjassot, Spain
[b] Institute of Material Science (ICMUV), University of Valencia, Catedrático José Beltrán 2, 46980, Paterna, Spain

Analytica Chimica Acta 1124 (2020) 94–103

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Tuning the selectivity of molecularly imprinted polymer extraction of arylcyclohexylamines: From class-selective to specific

A. Sorribes-Soriano , S. Armenta *, F.A. Esteve- Turrillas , J.M. Herrero-Martínez

Department of Analytical Chemistry, University of Valencia, 50th Dr. Moliner St., 46100, Burjassot, Spain

Journal of Chromatography A 1633 (2020) 461629

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Molecularly imprinted polymer-based device for field collection of oral fluid samples for cocaine identification

A. Sorribes-Soriano, J.M. Herrero-Martínez, F.A. Esteve-Turrillas, S. Armenta*

Department of Analytical Chemistry, University of Valencia, 50th Dr. Moliner St., 46100 Burjassot, Spain

Journal of Chromatography A, 1545 (2018) 22–31

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Magnetic molecularly imprinted polymers for the selective determination of cocaine by ion mobility spectrometry☆

Aitor Sorribes-Soriano, Francesc Albert Esteve-Turrillas, Sergio Armenta*, Ana Montoya, José Manuel Herrero-Martínez*, Miguel de la Guardia

Department of Analytical Chemistry, University of Valencia, 50th Dr. Moliner St., 46100 Burjassot, Spain

Talanta 199 (2019) 388–395

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Development of a molecularly imprinted monolithic polymer disk for agitation-extraction of ecgonine methyl ester from environmental water

A. Sorribes-Soriano, R. Arráez-González, F.A. Esteve-Turrillas, S. Armenta*, J.M. Herrero-Martínez*

Department of Analytical Chemistry, University of Valencia, 50th Dr. Moliner St., 46100 Burjassot, Spain

Annex B.
Publicacions relacionades amb la Tesi Doctoral

Annex B.
Published papers related to the Doctoral Thesis

Microchemical Journal 144 (2019) 195–202

Contents lists available at ScienceDirect

Microchemical Journal

journal homepage: www.elsevier.com/locate/microc

Uptake and translocation monitoring of imidacloprid to chili and tomato plants by molecularly imprinting extraction - ion mobility spectrometry

M.M. Aria[a,b], A. Sorribes-Soriano[a], M.T. Jafari[c], F. Nourbakhsh[b], F.A. Esteve-Turrillas[a], S. Armenta[a,*], J.M. Herrero-Martínez[a], M. de la Guardia[a]

[a] Department of Analytical Chemistry, University of Valencia, 50th Dr. Moliner St., 46100 Burjassot, Spain
[b] Department of Soil Science, Isfahan University of Technology, Isfahan 84156-83111, Iran
[c] Department of Chemistry, Isfahan University of Technology, Isfahan 84156-83111, Iran

ARTICLE INFO

Keywords:
Pesticide uptake
Translocation
Imidacloprid
Plants
Molecularly imprinted polymer
Ion mobility spectrometry

ABSTRACT

The degradation of imidacloprid in soil and its uptake and translocation to chili and tomato plants was evaluated, as a proof of concept, of the possibilities of the combination of molecularly imprinted polymers (MIPs) and ion mobility spectrometry (IMS) for a fast and sensitive bioprocesses monitoring tool. To do it, a method based on the selective extraction of imidacloprid from soil and plant materials was developed. In the selected conditions, the MIP-IMS procedure provided a recovery of imidacloprid in soil and plant samples from 102 to 114%, for spiked concentration levels from 0.2 to 2.0 $\mu g\,g^{-1}$. Precision of the methodology, expressed as the relative standard deviation (RSD) of a 100 and 1000 $\mu g\,L^{-1}$ imidacloprid standard solution was 11 and 6%, respectively, being the RSD for the analysis of a soil sample spiked at a concentration level of 1 $\mu g\,g^{-1}$ of 11% ($n = 4$). Limits of detection and quantification of 0.03 and 0.10 $\mu g\,g^{-1}$ in the solid sample were also obtained, respectively. Regarding imidacloprid degradation, this study evidenced that the process follows a first order kinetics with a half-life between 39 and 45 days in soil, being necessary a growing period of 33 days before pesticide detection in stems and leaves.

Annex C. Pòsters presentats a congressos

Annex C. Presented poster communications

1. MAGNETIC MOLECULARLY IMPRINTED POLYMERS FOR COCAINE DETERMINATION BY ION MOBILITY SPECTROMETRY

F.A. Esteve-Turrillas, A. Sorribes-Soriano, S. Armenta, M. de la Guardia, J.M. Herrero-Martínez

19th International Symposium on Advances in Extraction Technologies, 2017 (Santiago de Compostela, Espanya)

2. SELECTIVITY EVALUATION OF METHAMPHETAMINE BASED MOLECULARLY IMPRINTED POLYMER

A. Sorribes-Soriano, S. Armenta, F.A. Esteve-Turrillas, J.M. Herrero-Martínez

X Iberian Spectroscopy Conference / XXVI National Spectroscopy Meeting, 2018 (Lisboa, Portugal)

3. MOLECULARLY-IMPRINTED POLYMER PIPETTE TIP EXTRACTION OF AMPHETAMINE-TYPE SUBSTANCES FROM ORAL FLUIDS

A. Valencia, A. Sorribes-Soriano, J.M. Herrero-Martínez, S. Armenta, F.A. Esteve-Turrillas

XVIII Meeting of the Spanish Society of Chromatography and Related Techniques, 2018 (Granada, Espanya)

4. ION MOBILITY SPECTROMETRY DETERMINATION OF DICHLOROPANE IN ORAL FLUID BY MICROEXTRACTION BY PACKED SORBENT

A. Monedero, A. Sorribes-Soriano, F.A. Esteve-Turrillas, S. Armenta

XVIII Meeting of the Spanish Society of Chromatography and Related Techniques, 2018 (Granada, Espanya)

5. MOLECULARLY-IMPRINTED POLYMER-STIR CAKE SORPTIVE EXTRACTION OF ECGONINE METHYL ESTER FROM WATER AND BIOLOGICAL SAMPLES FOR THE EVALUATION OF COCAINE ABUSE

R. Arraez, A. Sorribes-Soriano, J.M. Herrero-Martínez, S. Armenta, F.A. Esteve-Turrillas

XVIII Meeting of the Spanish Society of Chromatography and Related Techniques, 2018 (Granada, Espanya)

6. DETERMINATION OF METHYLONE IN ORAL FLUID BY MICROEXTRACTION BY PACKED SORBENT AND ION MOBILITY SPECTROEMTRY

S. Sánchez-Martínez, A. Sorribes-Soriano, S. Armenta, F.A. Esteve-Turrillas,

XXII Reunión de la Sociedad Española de Química Analítica, 2019 (Valladolid, Espanya)

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Magnetic molecularly imprinted polymers for cocaine determination by ion mobility spectrometry

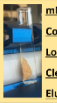
Aitor Sorribes-Soriano, Sergio Armenta, Francesc A. Esteve-Turrillas,* Miguel de la Guardia, José M. Herrero-Martínez

*Department of Analytical Chemistry. University of Valencia. 50th Dr. Moliner St.. 46100 Burjassot. Spain. (*e-mail address Francesc.A.Esteve@uv.es)*

Introduction

According to United Nations Office on Drugs and Crime World Drug Report 2015 (UNODC), cocaine is one of the most consumed drugs all around the world. Determination of cocaine in saliva, urine and blood has relative importance in different areas such as toxicology, traffic control, justice, drug treatment programs and doping. The main aim of this study is the development of a rapid procedure for the determination of cocaine in saliva samples at trace levels. IMS measurement of complex samples complicate the interpretation of IMS spectra and usually interfere in the target analyte determination. Thus, magnetic molecularly imprinted polymers (mMIPs) has been produced to be used as a cocaine specific sample extraction method previous to the IMS analysis of saliva samples. Vinylized and polyethylene glycol coated magnetite nanoparticles have been recoated with MIP at 0,5 and 2,0 % ratio and fully characterized. The proposed mMIP-IMS method was validated in terms of sensitivity, selectivity, trueness and precision. Cocaine user saliva samples were analyzed by the proposed methodology and by a reference procedure in order to evaluate the accuracy of results.

Experimental



mMIP amount: 500 mg

Conditioning: 1 mL acetic acid (5% v/v) in methanol + 0,5 mL water

Loading: 0,5 mL saliva diluted in 0,3 mL ammonium buffer (pH 9,3)

Cleaning: 0,5 mL water + 0,5 mL $CHCl_3$

Elution: 0,2 mL acetic acid (5% v/v) in methanol

mMIP-IMS

8 µL $V_{injection}$

$T_{Desorption}$ = 260°C
T_{inlet} = 270°C
$T_{drift\ tube}$ =232°C
Counterflow of dry air: 300 mL min^{-1}
Electric field strength: 252 V cm^{-1}

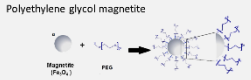
UHPLC-MS-MS

Column: BEH C_{18} (1.7 µm, 2.1 × 50 mm)
Mobile phase (A) 0.1 % (v/v) formic acid in water (B) methanol
5% of B linearly increased to 95% in 4 min maintained 1 min at a flow rate of 0.4 µL min^{-1}
Retention time: 2.77 min
ESI+: parent ion 304 m/z → 182 m/z
25 V cone energy, 15 and 25 eV collision energy

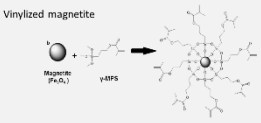
Results

Synthesis of mMIPs

Polyethylene glycol magnetite



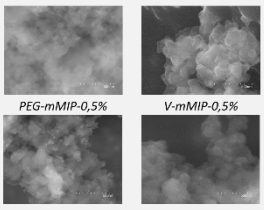
Vinylized magnetite



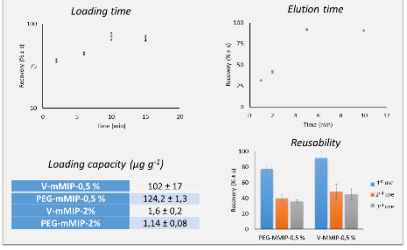
Bulk polymerization method (60°C – 24 h)

	[Reagent] (mmol)	Amount (g)
mMIP-0.5 %	Cocaine	0.10	0.034
	MAA	0.40	0.034
	EDMA	2.00	0.395
	AIBN	0.12	0.020
	MNPs	-	0.025
mMIP-0.5%	MAA	0.40	0.034
	EDMA	2.00	0.395
	AIBN	0.12	0.020
	MNPs	-	0.025
mMIP-2%	Cocaine	0.10	0.034
	MAA	0.40	0.034
	EDMA	2.00	0.395
	AIBN	0.12	0.020
	MNPs	-	0.100
mNIP-2 %	MAA	0.40	0.034
	EDMA	2.00	0.395
	AIBN	0.12	0.020
	MNPs	-	0.100

SEM characterization



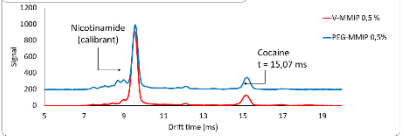
mMIP extraction conditions



Loading capacity (µg g^{-1})

V-mMIP-0,5 %	102 ± 17
PEG-mMIP-0,5 %	124,2 ± 1,3
V-mMIP-2%	1,6 ± 0,2
PEG-mMIP-2%	1,14 ± 0,08

IMS plasmagram



Analytical features

LOD = 11 µg L^{-1} LOQ = 36 µg L^{-1} RSD (100 µg L^{-1}) = 2,5%

[Cocaine] (µg L^{-1})	Water recovery (% ± s)			
	V-mMIP-2 %	PEG-mMIP-2%	V-mMIP-0,5 %	PEG-mMIP-0,5 %
70	< LOQ	<LOQ	110 ± 20	73 ± 11
230	17 ± 3	16,2 ± 0,8	119 ± 10	78 ± 3
460	-	-	76 ± 9	68 ± 11

[Cocaine] (µg L^{-1})	Saliva recovery (% ± s)	
	V-MMIP 0,5 %	PEG-MMIP 0,5 %
80	93 ± 12	99 ± 10
270	95 ± 10	80 ± 4
560	88 ± 7	80 ± 5

Analysis of field samples

Sample	Detected compounds	[Cocaine] (µg L^{-1} ± s)		Bias (%)
	LF-IA	mMIP-IMS	mMIP-UHPLC-MS-MS	
1	Cocaine	350 ± 40	329 ± 5	7
2	Cocaine	367 ± 15	354 ± 5	4
3	Cocaine Benzodiazepines	298 ± 8	383 ± 6	-22
4	Cocaine Cannabis	350 ± 50	361 ± 5	-4
5	Cocaine Cannabis	315 ± 16	358 ± 5	-12
6	Cocaine Cannabis	760 ± 20	615 ± 9	23
7	Cocaine Cannabis	547 ± 13	528 ± 8	3

Lateral flow immunoassay (LF-IA)

UHPLC-MS-MS

Conclusions

The developed procedure provided a limit of detection of 11 µg L^{-1} in saliva samples with cocaine recoveries ranging from 80 to 99 % using mMIPs while those values decreased to less than 5 % using magnetic non-imprinted polymers (mNIPs) for saliva samples spiked with cocaine at 80-560 µg L^{-1}. The qualitative performance of the method was compared with a reference procedure based on lateral-flow immunoassay and quantitative results obtained from the analysis of saliva samples of cocaine users by mMIP-IMS procedure were statistically comparable to those obtained by a reference procedure based on liquid chromatography-mass spectrometry. In summary, the specificity of mMIPs extraction coupled to the high sensitivity of IMS measurements makes this combination useful for quick, selective, and sensitive determination of cocaine residues in saliva samples.

Acknowledgements: Authors acknowledge the financial support the Ministerio de Economia y Competitividad-Feder (CTQ 2014-52841-P and CTQ 2014-52765-R) and Generalitat Valenciana (project **PROMETEO-II 2014-077**).

X CIE - XXVI RNE SPECTROSCOPY

SELECTIVITY EVALUATION OF METHAMPHETAMINE BASED MOLECULARLY IMPRINTED POLYMER

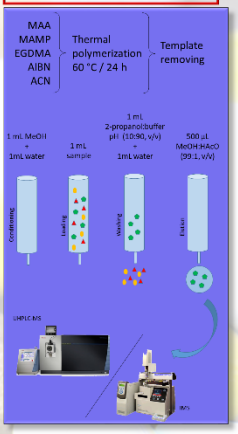
Sorribes-Soriano A., Armenta S., Esteve-Turrillas F.A., Herrero-Martínez J.M.
Department of Analytical Chemistry, University of Valencia, 50th Dr. Moliner St., 46100 Burjassot, Spain
*aitor.sorribes@uv.es

M. de la Guardia
A. Pastor
S. Garrigues
M.L. Cervera
A.E. Morales
S. Armenta
SOLINQUIANA

SUMMARY

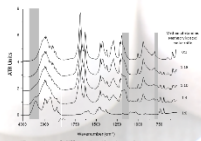
Nowadays, the illicit drug market has evolved by the rising of New Psychoactive Substances (NPS) carrying important healthy risks all around the world. Analytical methods for the analysis of NPS in biological fluids typically requires a preliminary sample treatment step. The most widely employed treatment is solid phase extraction (SPE) with generic solid sorbents, which leads to co-extraction of endogenous compounds and other interferents. Molecularly imprinted polymers (MIPs) are synthetic materials prepared using a template (usually the target molecule), a monomer and a cross-linker. After polymerization and a washing step, the polymer has recognition sites able to selectively extract template and molecules with similar structure. MIPs have demonstrated that provide cleaner extracts with less matrix effects, improving limits of detection (LOD), trueness and precision. This study is focused on the development of a methamphetamine (MAMP) based-MIP, for the extraction of amphetamine related NPS from oral fluid. MIP characterization has been done by infrared spectroscopy (IR), nitrogen adsorption-desorption, scanning electron microscopy (SEM). The procedure was applied for the analysis of oral fluid samples by Ion Mobility Spectrometry (IMS) and Ultra-High Performance Liquid Chromatography – tandem mass spectrometry (UHPLC-MS).

EXPERIMENTAL

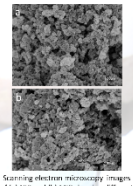


RESULTS

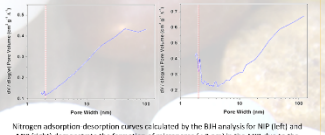
MIP characterization



IR spectra of different ratios template-monomer (MAMP:MAA). It shows the bands located at 3500, 1175 and 780 cm^{-1} were displaced due to the formation of hydrogen bonds between template and monomer.

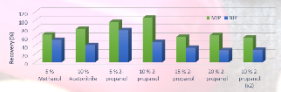


Scanning electron microscopy images of (a) NIP and (b) MIP showing different sizes of the globules.

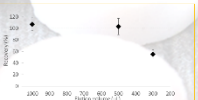


Nitrogen adsorption desorption curves calculated by the BJH analysis for NIP (left) and MIP (right) demonstrate the formation of micropores (< 2 nm) in the MIP, due to the presence of template in the polymerization mixture.

Study of SPE conditions

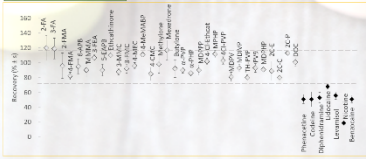


The best washing solvent was phosphate buffer (pH 9; 0.1 M) prepared in 10 % (v/v) 2-propanol, providing quantitative amphetamine recoveries and a 2.2 MIP/NIP imprinting factor.



At least 500 µL 1 % (v/v) acetic acid in metanol was required to elute quantitatively amphetamine from the MIP.

Selectivity study



A 80 % recovery and a RSD below 15 % was obtained for the SPE of 32 NPS with similar structure to MAMP using the proposed MIP. However, recoveries lower than 56 % were obtained for the analysis of 7 conventional drugs with molecular structures non-related to MAMP.

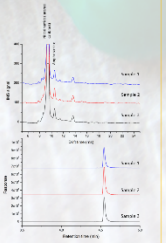
Real sample analysis

Analytical features of some representative NPS from different families involved in this study.

Analyte	IMS		UHPLC-MS	
	k_d [cm^2 V^{-1} s^{-1}]	LOD [µg L^{-1}]	RT [min]	LOD [µg L^{-1}]
Amphetamine	1.668	15	4.55	0.5
5-PA	1.605	21	2.47	0.2
5-APB	1.419	30	2.40	0.8
6-APB	1.436	43	2.28	0.06
α-PVP	1.306	26	2.73	0.4
Ethcathinone	1.519	15	2.11	0.3
4-MEC	1.447	23	3.19	0.07
2C-E	1.326	17	2.68	0.7

Recoveries obtained for field samples spiked with amphetamine by IMS and UHPLC-MS after consumption of different interferents.

Sample	Recovery ± s (%)		Interferents
	IMS	UHPLC-MS	
1	99 ± 6	86 ± 4	Fluticasone
2	122 ± 2	112 ± 6	Caffeine
3	104 ± 3	84 ± 4	Cetirizine



IMS plasmagrams (a) and UHPLC-MS cromatograms (b) obtained for the analysis of field saliva samples analyzed by the proposed procedure.

CONCLUSIONS

- IR spectra show interactions between the template and the monomer that prove the formation of prepolimerization complex.
- SEM images shows slight differences in the NIP/MIP globules size.
- The nitrogen adsorption-desorption study allows to distinguish the presence of mesopores in both polymers, although pore sizes of less than 2 nm were observed in MIP. This fact suggests that the template molecule create (micro)pores with appropriate size to recognize molecules with similar size.
- The selectivity study shows that MAMP-like NPS tested were able to be specifically recognized by the MIP.
- Field saliva samples analyzed by the proposed methodology show the effectiveness of the sample treatment, providing LODs from 15 to 43 µg L^{-1} for IMS and from 0,5 to 15 µg L^{-1} for UHPLC-MS.
- The IMS can be proposed as a a vanguard approach for the fast determination of NPS in saliva while UHPLC-MS can be considered as a rearguard technique for confirmatory analysis.

Acknowledgements
Authors gratefully acknowledge the financial support of the Ministerio de Economía y Competitividad (CTQ 2014-52841) and Generalitat Valenciana (project PROMETEO-II 2014-077 and PROMETEO/2016/145). A.Sorribes-Soriano acknowledge the financial support of Generalitat Valenciana (ACIF-2017/386).

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MOLECULARLY-IMPRINTED PIPETTE-TIP EXTRACTION OF AMPHETAMINE-TYPE SUBSTANCES FROM ORAL FLUIDS

Alejandro Valencia, Aitor Sorribes-Soriano, José Manuel Herrero-Martínez, Sergio Armenta*, Francesc A. Esteve-Turrillas

Department of Analytical Chemistry, University of Valencia, 50th Dr. Moliner St., 46100 Burjassot, Spain

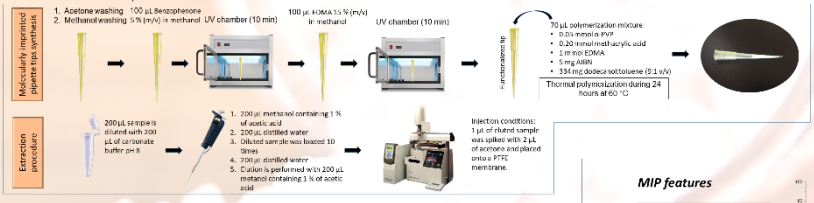
*sergio.armenta@uv.es, Tel: +34-963-544-004, Fax: +34-963-544-838

INTRODUCTION

Molecularly imprinted polymers (MIP) are synthetic materials prepared in presence of target molecule (or template) which create a pre-polymerization complex with a monomer. After the polymerization and removing the template, MIPs provides cavities complementary in terms of shape, size and chemical properties able to bind selectively target molecule or similar molecular structures. Molecular imprinting have been applied widely in several formats such as Solid Phase Extraction (SPE), disks, Stir-Bar Sorptive Extraction (SBSE), capillary columns, dispersive SPE, magnetic SPE or Solid Phase Microextraction (SPME) among others. Recently new formats of molecular imprinting are being developed such micropipette tips-MIPs in order to ease the extraction procedure.

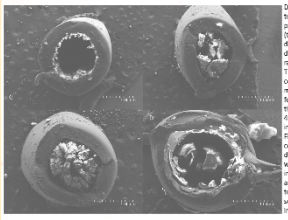
In this study the development of molecularly imprinted pipette tips is proposed for amphetamine-type drugs analysis in oral fluid samples. For our proposal first is necessary to modify the walls of polypropylene (PP) tips by the use of benzophenone and ethylene glycol dimethacrylate (EDMA) via UV radiation to allow binding of MIP to the tip. After the modification, polymerization mixture prepared using α-PVP as a template, methacrylic acid as monomer and EDMA as cross-linker is introduced into the tip and carried by thermal or UV activation. In the research wide number of polymerization mixtures were tested in order to find the best physical properties, so surface morphology of obtained MIPs was observed by Scanning Electron Microscopy (SEM) characterizing the material and allowing to chose the best polymerization mixture. Methanol containing 1 % of acetic acid was used as activation and elution solvent, the simplicity of the matrix allow to use water as washing solvent to remove the possible interferes, for sample loading the pH used was 8 to ease the formation of hydrogen bonds between the drug and the MIP. MIP capabilities such as loading capacity, reproducibility between different tips and reusability were studied. Analytical technique used in this study have been Ion mobility spectrometry (IMS) which provides short analysis time, simplicity and appropriate sensibility. Finally water sampled and field saliva samples were spiked with different volumes of α-PVP in order to compare if the complexity of oral fluid versus water and validate the method.

EXPERIMENTAL

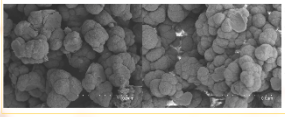


RESULTS

Characterization



Different polymerization mixtures were studied in order to obtain the monolithic tips with suitable physical properties. Molar ratios of 1:4:20 and 1:6:30 (template/monomer/crosslinker) were studied using different porogenic solvents including chloroform and dodecanol containing 10 % of toluene with percentages ranging from 60 to 91 % of porogen in the mixture. Thermal and UV chamber polymerization were also compared. Most of the obtained tips showed breakable monoliths or impermeable so they were discarded for future studies. The four best monoliths are shown in the figure. Figure A shows a 1:4:20 polymer containing 60 % of porogen polymerized by UV activation. SEM image proves a lack of anchorage of the later to the tip. Figure B and C were taken from 1:4:20 polymer containing 70 % (B) and 60 % (C) of porogen using dodecanol-toluene mixture where permeable material was obtained. Finally, in figure D can be seen the SEM image of 1:4:20 polymer containing 60 % of chloroform as porogen were lack of porosity is observed. Attending to the SEM images and the capability to flow different solvents and physical robustness the material obtained in figure C was selected for future studies.



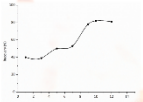
SEM images show the morphology of NIP (left) and MIP (right) where it can be seen smaller globules in the MIP. The presence of template during the synthesis creates more cavities in the polymer providing then, a higher porosity. This facts where proven when the samples where flowed through the tips being easier to flow oral fluid samples through the MIP than NIP where the absorption was slower.

Analytical features of IMS

Parameter	Value
Linear range	25 – 500 µg L^{-1}
Linearity (R^2)	0.9958
LOD	8 µg L^{-1}
LOQ	25 µg L^{-1}
Repetibility (n=4)	
• Intra-day	4 %
• Inter-day	6 %
Analysis duration	30 s

Analytical features show good linearity and a wide linear range for the analysis of α-PVP, low LOD and LOQ and good reprintibility being a perfect analytical technique for the analysis of α-PVP

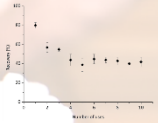
Extraction procedure



The number of cycles that the sample have to be percolated was studied in order to obtain the minumum loadings necessary to reach the equilibrium

MIP features

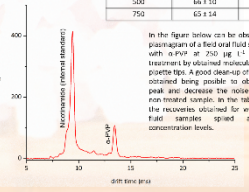
Feature	Value
Loading capacity	5.3 mg g^{-1}
Tips repetibility (n=3)	
• Water samples	14 %
• Oral fluid	11 %
Reusability	1



Analytical features show good linearity and a wide linear range for the analysis of α-PVP in oral fluid samples where the concentration is usually between 300 and 1000 µg L^{-1}. Low LOD and LOQ were obtained and good reprintibility being a perfect analytical technique for the analysis of α-PVP. As can be seen in the figure after one use the retention of the α-PVP decrease considerably, so the pipette tips are not reusable.

Sample analysis

Concentration α-PVP (µg L^{-1})	Recovery ± s (%)	
	Water	Oral Fluid
100	101 ± 10	149 ± 10
250	105 ± 5	103 ± 13
500	66 ± 10	72 ± 7
750	65 ± 14	59 ±8



In the figure below can be observed an IMS plasmagram of a fiedl oral fluid sample spiked with α-PVP at 250 µg L^{-1} after sample treatment by obtained molecularly imprinted pipette tips. A good clean-up of the sample is obtained being posible to obtain a single peak and decrease the noise provided by non treated sample. In the table is showed the recoveries obtained for water and oral fluid samples spiked at different concentration levels.

CONCLUSIONS

- Different polymers have been obtained and compared in terms of physical properties obtaining as the best monolith when a mixture of 1:4:20 (α-PVP/methacrylic acid/EDMA) solved using 60 % of dodecanol containing 10 % of toluene as a porogenic solvent and polymerizing by thermal initiation.
- The proposed extraction procedure have been tested and optimized with regards to the number of loadings necessary to reach the equilibrium.
- The analytical features of IMS have been proved for the analysis of those substance providing acceptable values in terms of linearity, limit of detection, repetibility and time consuming.
- The obtained MIP have been studied and the main parameters of its quality have been tested such as loading capacity, repetibility and reusability
- Finally the studied procedure have been applied to the analysis of field saliva samples spiked with α-PVP providing an excellent clean-up of the sample and suitable recoveries.

- As a future prospectives the studied procedure will be applied to the analysis of more amphetamine-type substances and liquid chromatography tandem mass spectrometry (LC-MS) will be used as a reguard technique comparing the obtained results with IMS (vanguard technique)

Acknowledgements

Authors gratefully acknowledge the financial support of the Ministerio de Economía y Competitividad (CTQ 2014-52841) and Generalitat Valenciana (project PROMETEO-II 2014-077 and PROMETEO/2016/145). A.Sorribes-Soriano acknowledge the financial support of Generalitat Valenciana (ACIF-2017/386).

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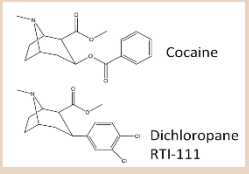
Ion mobility spectrometry determination of dichloropane in oral fluid by microextraction by packed sorbent

Amparo Monedero, Aitor Sorribes-Soriano, Francesc A. Esteve-Turrillas, Sergio Armenta*
Department of Analytical Chemistry, University of Valencia, 50th Dr. Moliner St., 46100 Burjassot, Spain
**e-mail address of corresponding author:* *sergio.armenta@uv.es

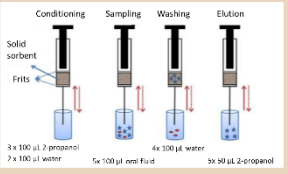
Introduction

New psychoactive substances (NPS) are defined by the United Nations Office on Drugs and Crime (UNODC) as substances of abuse which have similar effects to drugs under international control conventions, but not controlled yet. The NPS market is very dynamic and NPS belonging to diverse chemical groups are continuously emerging at an unprecedented rate. Dichloropane, also known as RTI-111, is a synthetic stimulant substance of the tropane chemical class, structurally related to cocaine with stimulant and anorectic properties and unknown pharmacology, metabolism, and toxicity in humans. Abuse drug consumption analysis is carried out in plasma and serum. However, the use of oral fluid as alternative matrix due to its simplicity and non-invasive collection is gradually increasing. Moreover, non-metabolized drugs are typically found in oral fluid analysis. The usefulness of ion mobility spectrometry (IMS) for the analysis of NPS in biological fluids has been previously demonstrated after different sample treatments, such as liquid-liquid microextraction and solid phase microextraction. Microextraction by packed sorbent (MEPS) was introduced in 2004 as a simple, fast, on-line sample-preparation technique, with advantages like low sample and solvent consumption and high possibilities for automation. Two different MEPS sorbents like octyl silica (C8) and octadecyl (C18) silica were evaluated for the extraction of dichloropane from oral fluids and its analysis by IMS, using a gas chromatography-mass spectrometry (GC-MS) procedure as reference method.

Experimental



C_8 and C_{18} MEPS sorbents

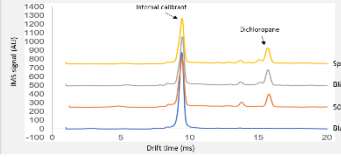


GC-MS
Capillary column HP-5: 30m x 0.25 mm x 0.25 µm
Injection volume: 1 µL
Temperature program: 1 min at 150 °C, 10 min temperature increase at 10 °C min^{-1} up to 250 °C, 250°C during 20 min.
Internal standard: Cocaine-D_3

IMS
IONSCAN-LS from Smiths Detection
Ionization source: ^{63}Ni foil - positive mode
Desorption, inlet and drift tube temperatures were adjusted to 260, 270 and 232 °C

Results

Analytical signal

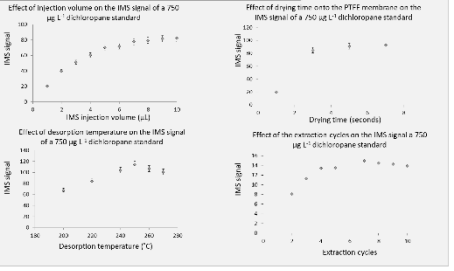


Analytical features

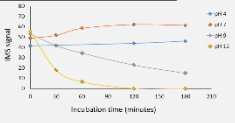
Analytical parameters obtained for the determination of dichloropane by microextraction by packed sorbent using C_8 and C_{18} sorbents and ion mobility spectrometry determination.

Parameter	C8	C18
Slope (L $µg^{-1}$)	0.102 ± 0.002	0.123± 0.005
Intercept	-0.1 ± 0.9	4 ± 3
R^2	0.999	0.990
LOD (µg L^{-1})	30	50
LOQ (µg L^{-1})	90	150
RSD (%)	6	11

Experimental and instrumental conditions



Effect of sample pH



Higher recoveries of dichloropane can be obtained at higher pH, however, substantial basic hydrolysis can be observed increasing incubation time. Consequently, a neutral pH was selected for further experiments.

Recovery of samples spiked with dichloropane

Recoveries obtained for the analysis of water, synthetic saliva and field saliva samples spiked at different levels dichloropane by using C_8 MEPS followed by IMS.

Sample	[Spiked] (µg L^{-1} ± s)	[Found] (µg L^{-1} ± s)	Recovery (% ± s)
Water	250	220 ± 30	87 ± 14
	500	460 ± 20	92 ± 4
	750	741 ± 6	99 ± 1
Synthetic saliva	250	260 ± 30	105 ± 12
	500	550 ± 15	110 ± 3
	750	675 ± 15	90 ± 2
Field saliva	250	240 ± 18	96 ± 7
	500	535 ± 10	107 ± 2
	750	640 ± 30	85 ± 4

Analysis of blind spiked saliva samples

Blind spiked saliva samples were analyzed by the proposed C_8 MEPS-IMS procedure and a GC-MS reference procedure.

Sample	GC-MS (µg L^{-1} ± s)	IMS (µg L^{-1} ± s)
OF-1	255 ± 21	228 ± 35
OF-2	483 ± 16	460 ± 20
OF-3	681 ± 20	734 ± 6

Conclusions

An analytical procedure based on the combined use of MEPS and IMS has been developed and validated for dichloropane determination in oral fluid samples. MEPS allows preconcentration of dichloropane from oral fluid samples removing potential interferences from the matrix. The extraction procedure was optimized in terms of pH of the loading step, the number of extraction cycles and the nature of solid sorbent. Additionally, the IMS instrumental conditions were also evaluated in terms of injection volume, desorption temperature and drying time. The proposed procedure provided a RSD values of 6 and 11 %, for C8 and C18 solid sorbents, and LOD values of 30 and 50 µg L^{-1}, for C8 and C18 solid sorbents, being adequate for dichloropane analysis in oral fluids. Finally, C8 sorbent was selected for sample analysis due to its superior characteristics. Recoveries for spiked samples were in the range of 85-110 % for analyte concentration ranging from 250 to 750 µg L^{-1}, being the results obtained for blind spiked samples comparable with those provided by a reference GC-MS procedure. In summary, the proposed methodology is fast, sensitive and precise, very appropriate for screening of dichloropane abuse.

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Molecularly-imprinted polymer-stir cake sorptive extraction of ecgonine methyl ester from water and biological samples for the evaluation of cocaine abuse

Rocío Arráez, Aitor Sorribes-Soriano, José Manuel Herrero-Martínez, Sergio Armenta, Francesc A. Esteve-Turrillas*

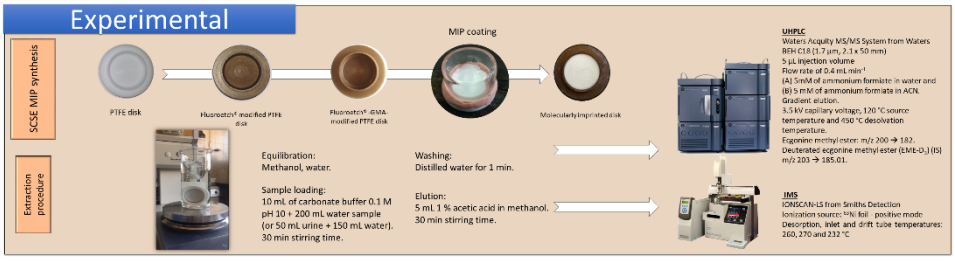
Department of Analytical Chemistry, University of Valencia, 50th Dr. Moliner St., 46100 Burjassot, Spain

**e-mail address of corresponding author: *francesc.a.esteve@uv.es*

Introduction

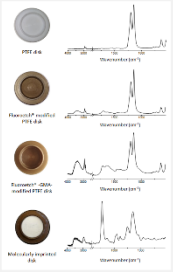
The use of drugs of abuse is increasing worldwide and causes not only a well-known serious social problem but also concern as environmental emerging contaminants. Cocaine is one of the most widely consumed drug worldwide, according to the United Nation Office of Drug and Crime (UNODC) World Drug Report, 2017. Following consumption, cocaine is metabolized in their metabolites benzoylecgonine and ecgonine methyl ester and excreted in urine and feces, being released to the aquatic environment. The presence of these substances in sewage systems and other environmental compartments is a topic of growing concern for ecological health and to estimate levels of community consumption. Different studies on surface water and wastewater from European cities have been published being observed that cocaine is one of the most consumed drugs. Analytical methods for the analysis of abuse drugs are based on liquid chromatography–mass spectrometry (LC-MS). Due to the concentration of the analytes and the complexity of the sample, most of these methods require a preliminary sample treatment step typically based on solid-phase extraction (SPE). In this study, we propose the development of a molecularly-imprinted polymer (MIP) for the ecgonine methyl ester extraction from wastewater and urine for the estimation of cocaine abuse. The main advantage of specific solid sorbents is that based on the reduction of the interfering compounds co-extraction which decreases the limits of detection. Additionally, the stir cake sorptive extraction (SCSE) mode integrates extraction, enrichment and clean-up into one step, being its main advantages related to simple operation, high cost-efficiency, and high extraction capacity.

Experimental

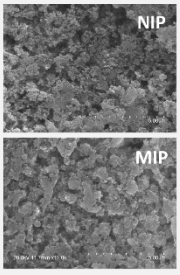


Results

Characterization of antibody columns

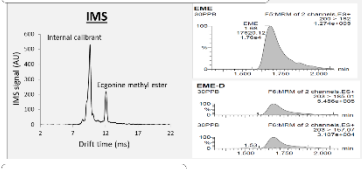


Sodium naphthalene was used to activate disk surface for polymer anchoring. C-F bounds (1700 – 1000 cm-1) are replaced by C-H, C-OH, and –COOH (3500 cm-1). The introduction of –OH groups allows GMA bounding to the PTFE through the epoxy group leaving the vinyl group free for covalent binding to polymer. In the third spectra can be seen an increasing of the signal provided by O-H vibration because the introduction of GMA provides a higher number of C-OH bounds. Finally after polymerization, the bands obtained are completely attributed to the MIP structure and the surface of the disk can not be observed.



MIP and NIP were characterized by SEM in order to study the differences between both polymers. It can be seen that the surface of the MIP have bigger globules than NIP were the absence of the template during the synthesis provides the formation of smaller globules.

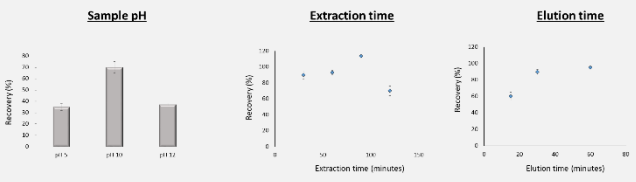
Analytical signals



Analytical features

	UHPLC-MS/MS	IMS
Linear range (µg L-1)	2-500	14-200
LOD (µg L-1) water/urine	0.006/0.02	0.1/0.4
LOQ (µg L-1) water/urine	0.02/0.08	0.3/1.2
RSD (%)	5	7
Time (min)	6	1
R2	0.9998	0.9992

Study of extraction conditions



Analysis of water samples

Sample	Concentration ± s (ng L-1)	
	IMS	UHPLC-MS/MS
Port of Valencia	< LOD	143 ± 10
Pond (Almenara)	< LOD	48 ± 3
Lagoon (Marjal dels moros)	< LOD	67 ± 5
Irrigation ditch (Puçol)	187*	98 ± 8
Irrifation ditch (Lliria)	< LOD	37 ± 3
Urban wastewater	250*	195 ± 13

* Below the LOQ of the procedure

Analysis of spiked urine samples

Sample	Concentration ± s (µg L-1)	
	Spiked	UHPLC-MS/MS
U-1	2.00	2.03 ± 0.06

Conclusions

A MIP-SCSE has been synthetized for ecgonine methyl ester extraction from water samples. The MIP allow preconcentration of ecgonine methyl ester present in water and remove interferences from the matrix. The extraction procedure was optimized in terms of extraction time and pH for loading step. The main features of the MIP were studied such as selectivity, obtaining that all benzoylecgonine was removed while 56 % of cocaine was retained by the MIP. This approach of molecular imprinting provides a high IF (26) due to a decreasing of non-specific interactions between substances and polymer. The binding capacity obtained (17.7 µg g-1) makes suitable the use of MIP-SCSE for surface or wastewater analysis. The comparison of the evaluated analytical techniques shows better parameters by UHPLC-MS/MS providing a reliable method for ecgonine methyl ester analysis, being IMS an appropriate screening method. Surface water and a wastewater were analyzed by both methodologies obtaining detectable EME. Finally for urine samples the proposed method provided recovery values of 84 %.

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DETERMINATION OF METHYLONE IN ORAL FLUIDS BY MICROEXTRACTION BY PACKED SORBENT AND ION MOBILITY SPECTROMETRY

S. Sánchez-Martínez, A. Sorribes-Soriano, S. Armenta, F.A. Esteve-Turrillas

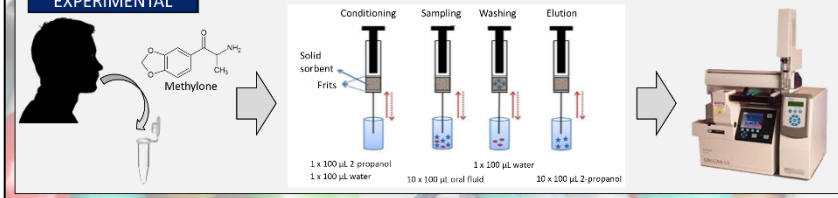
Department of Analytical Chemistry, University of Valencia, 50th Dr. Moliner St., 46100, Burjassot, Spain.

e-mail address of corresponding author: francesc.a.esteve@uv.es

INTRODUCTION AND OBJECTIVE

Methylone (3,4-methylenedioxy-N-methylcathinone) is a synthetic cathinone with euphoric and stimulants effects, but other effects like hyperthermia, seizures and kidney damage may also appear. Drug analysis usually has been carried out in plasma, serum or urine but these methods usually mean invasive methods and very complex matrices. In this study, the analysis of methylone in oral fluid is proposed using microextraction by packed sorbent (MEPS) followed by ion mobility spectrometry (IMS) detection. MEPS consist in a solid phase extraction where a small amount of sorbent(4 mg) is packed at the end of a needle. There are different possible commercial sorbents available but in these study the sorbent chosen was octadecyl silica (C_{18}) because their good features against low polar compounds. Besides, MEPS provides some benefits such as their low sample/solvent consumption, simplicity of the extraction procedure and possibility on-line of coupling. On the other hand, IMS is proposed as detection technique that in positive mode shows good sensitivity against molecules which present amine groups in addition to its quickness, simplicity and low cost. Thus, methylone could be determined by a fast, miniaturized and automatized method.

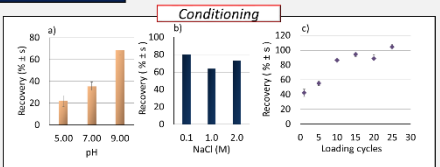
EXPERIMENTAL



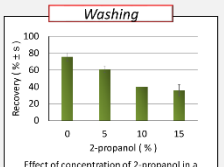
IMS
IONSCAN-LS from Smiths Detection
Ionization source: ^{63}Ni foil - positive mode
Desorption, inlet and drift tube temperatures were adjusted to 260, 270 and 232 °C

RESULTS

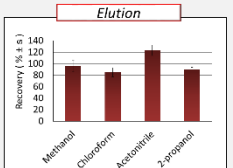
EXTRACTION CONDITIONS



Effect of the (a) pH (b) concentration of NaCl and (c) loading cycles during the loading step for a 250 µg L^{-1} of methylone standard prepared in deionized water.

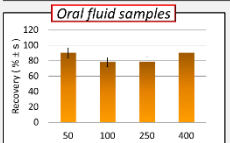


Effect of concentration of 2-propanol in a phosphate buffer (pH 9.0) for washing solvent in the extraction of 250 µg L^{-1} of methylone standard prepared in deionized water.

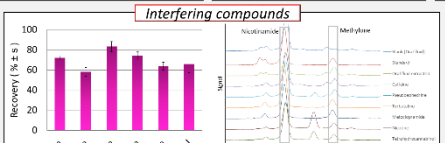


Effect of the nature of elution solvent for the extraction of 250 µg L^{-1} of methylone standard prepared in deionized water.

FIELD SAMPLES



Extraction of methylone from different field oral fluid samples spiked at different concentration levels.



Recoveries and plasmagrams obtained from field oral fluid samples spiked with metyhone at 250 µg L^{-1} containing several interfering compounds.

ANALYTICAL FEATURES

Feature	Value
LOD	4 µg L^{-1}
LOQ	14 µg L^{-1}
Linear range	14-500 µg L^{-1}
Linearity (R^2)	0,994
RSD	< 10 %

CONCLUSIONS

In this study, a method for methylone determination in saliva has been developed using MEPS followed by IMS determination. Firstly, the most appropriate extraction conditions were studied including the pH, ionic strength and the number of required aspiration cycles for the loading step. The best washing solvent found was a pH 9.0 phosphate buffer because the addition of 2-propanol elutes the analyte. The use of different elution solvents demonstrated that its nature does not affect significantly the elution, thus the 2-propanol was chosen due to its high compatibility to IMS analysis. The proposed procedure was used for the analysis of field oral fluid samples spiked at different concentration of methylone and containing some potential interfering compounds showing in all cases good recoveries. Finally, the analytical features of the method such as LOD (4 µg L^{-1}) or RSD (< 10 %) demonstrated the opportunities of MEPS-IMS to methylone determination.

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