TESIS DOCTORAL

Título: *Aplicación de la cromatografía líquida acoplada a la espectrometría de masas para la evaluación de la exposición interna a contaminantes alimentarios*

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Nombre: **Pablo Dualde Marín**

DIRECTORES:

Dr. Vicent Yusà Pelechá

Dr. Agustín Pastor García

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Vicent Yusà Pelechá, Doctor en Ciencias Químicas e investigador de FISABIO, y Agustín Pastor García, Doctor en Ciencias Químicas y Catedrático de Universidad

CERTIFICAN que:

Pablo Dualde Marín, Licenciado en Farmacia por la Universidad de Valencia, ha estado trabajando bajo nuestra dirección para la elaboración de la tesis "Aplicación de la cromatografía líquida acoplada a la espectrometría de masas para la evaluación de la exposición interna a contaminantes alimentarios", y que presenta para optar al Título de Doctor por la Universitat de València.

Y para que así conste a los efectos oportunos, firmamos el presente documento en Valencia a 12 de mayo de 2020.

YUSÀ I PELECHÁ, VICENT (AUTENTICACIÓN)
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Fecha: 2020.07.03 10:08:04 +02'00'

AGUSTIN IGNACIO |PASTOR| GARCIA
Firmado digitalmente por AGUSTIN IGNACIO|PASTOR|GARCIA
Fecha: 2020.07.03 11:00:51 +02'00'

Vicent Yusà Pelechá

Agustín Pastor García

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ÍNDICE DE CONTENIDO

ÍNDICE DE TABLAS

ÍNDICE DE FIGURAS

i) LISTADO DE TRABAJOS ORIGINALES

Capítulo	Artículo científico	Revista	Cuartil
1	Retrospective analysis of pesticide metabolites in urine using liquid chromatography coupled to high-resolution mass spectrometry	Talanta	1
2	Optimization of Resolving Power, Fragmentation and Mass Calibration in an Orbitrap Spectrometer for Analysis of 24 Pesticide Metabolites in Urine	International Journal of Analytical Chemistry	3
3	Determination of four parabens and bisphenols A, F and S in human breast milk using QuEChERS and liquid chromatography coupled to mass spectrometry	Journal of Chromatography B	2
4	Biomonitoring of bisphenols A, F, S in human milk and probabilistic risk assessment for breastfed infants	Science of Total Environment	1
5	Biomonitoring of parabens in human milk and estimated daily intake for breastfed infants	Chemosphere	1
6	Risk assessment of exposure to phthalates in breastfeeding women using human biomonitoring	Chemosphere	1

ii) ABREVIATURAS

2,4,5-T (Ácido 2,4,5-triclorofenoxiacético)
2,4-D (Ácido 2,4-diclorofenoxiacético)
2cx-MMHP (Mono[2-carboximetilhexil] ftalato)
3-KC ('3-ketocarbofuran')
4,6-DMP ('4,6-dimethoxy-2-pyrimidinamine')
AAVal ('N-2-carbamoylethylvaline')
ADI (Ingesta diaria admisible)
AIC (criterio de información de Akaike)
ALAM (Alachlor mercapturate)
AM (Media aritmética)
AP (Acefato)
APCI ('Atmospheric presure chemical ionization')
APPI ('Atmospheric pressure photoionization')
ATSDR ('Agency for Toxic Substances and Disease Registry')
ATZM (Atrazine mercapturate)
BE ('Biomonitoring Equivalent')
BFR (Retardante de llama bromado)
BH (Biomonitorización humana)
BIC (Criterio de información bayesiano)
BP (Butil paraben)
BPA (Bisfenol A)
BPF (Bisfenol F)
BPS (Bisfenol S)
BRIICS (Brasil, Rusia, India, Indonesia, China y Sudáfrica)
BzBP (Benzilbutil ftalato)
CDC ('Centers for Disease Control and Prevention')
CE ('Capillary Electrophoresis')
CID ('Collision Induced Dissociation')
cis-DCCA ('cis-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid')
CMHC ('3-Chloro-7-hydroxy-4-methylcoumarin')
COPHES ('Consortium to Perform Human Biomonitoring on a European Scale')
DALYs (Años de vida ajustados por discapacidad)
DAP (Dialquilfosfato)
DBCA ('cis-(2,2-Dibromovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid')
DBP (Di-n-butil ftalato)
DCP (Diciclohexil ftalato)
DEAMPY ('2-Diethylamino-6-methyl-6-hydroxypyrimidine')
DEHP (Di-2-etilhexil ftalato)
DEMOCOPHES ('Demonstration of a Study to Coordinate and Perform Human Biomonitoring on a European Scale')
DEP (Diethyl phosphate)
DEPHT (Dietil ftalato)

DETP (‘Diethyl thiophosphate’)
DF (Frecuencia de detección)
DiBP (Diisobutil ftalato)
DIMET (Dimetoato)
DiNP (Diisonoil ftalato)
DMDTP (‘Dimethyl dithiophosphate’)
DME (‘Diethylmaleate’)
DMP (Dimetil ftalato)
DMTP (‘Dimethyl thiophosphate’)
DMT (‘Diethyl malate’)
DLLME (Microextracción líquido-líquido dispersiva)
DOP (Di-n-octil ftalato)
d-SPE (Extracción en fase sólida dispersiva)
EC (Calibración externa)
EDI (Ingesta diaria estimada)
EFSA (Autoridad Europea de Seguridad Alimentaria)
EI (Impacto electrónico)
ENNS (‘Étude nationale nutrition santé’ - ‘French National Nutrition and Health Survey’)
ELFE (‘French longitudinal study of children’)
EP (Etil paraben)
EPA (Agencia de protección ambiental de EEUU)
ESI (Electronebulización)
Esteban (‘Environment, Health,Biomonitoring, physical Activity, Nutrition’)
FD (‘Forward dosimetry’)
FFT (Transformada de Fourier Rápida)
FLD (‘Fluorescence Detection’)
FLEHS (‘Flemish Environment and Health Study’)
FPBA (‘4-Fluoro-3-phenoxybenzoic acid’)
FT-ICR MS (Espectrómetro de resonancia iónica ciclotrónica por transformada de Fourier)
FWHM (Anchura a media altura)
GAVal (‘N-2-carbamoyl-2-hydroxyethylvaline’)
GC (Cromatografía gaseosa)
GerESs (German Environmental Surveys)
GM (Media geométrica)
HBM (Valor guía para biomonitorización del ‘German Human Biomonitoring’)
HCD (‘Higher Energy Collision Induced Dissociation’)
HPLC (Cromatografía líquida de alta resolución)
HQ (‘Hazard quotient’)
HRMS (Espectrometría de masas de alta resolución)
IC (Intervalo de confianza)
IMPY (‘2-Isopropyl-4-methyl-6-hydroxypyrimidine’)
INMA (proyecto Infancia y Medioambiente)
InVS (‘French Institute for Public Health Surveillance’)
LC (Cromatografía líquida)

LDR (Rango dinámico lineal)
LLE (Extracción líquido-líquido)
LoQ (Límite de cuantificación)
MBzP (Mono-benzil ftalato)
MCHP (Mono-ciclohexil ftalato)
MCPP (Mono-3-carboxipropil ftalato)
MECPP (Mono-2-etil-5-carboxipentil ftalato)
MEHHP (Mono-2-etil-5-hidroxihexil ftalato)
MEHP (Mono-2-etilhexil ftalato)
MEOHP (Mono-2-etil-5-oxohexil ftalato)
MEP (Mono-etil ftalato)
METM ('Metolachlor mercapturate')
MiBP (Mono-isobutil ftalato)
MiNP (Mono-isononil ftalato)
MIP-SPE ('Molecularly imprinted polymer for microdisc solid-phase extraction')
MLE ('Máximum likelihood estimation method')
MMC ('Matrix-matched calibration')
MMP (Mono-metil ftalato)
MnBP(Mono-n-butil ftalato)
MNP ('3-Methyl-4-nitrophenol')
MOP (Mono-n-octil ftalato)
MP (Metil paraben)
MS (Espectrometría de masas)
MS/MS (Espectrometría de masas en tándem)
MRL ('Maximum residue limit')
MRM ('Multiple reaction monitoring')
NICI ('Negative ion chemical ionization')
NEMHA ('N-(2-Ethyl-6-methylphenyl)-2-hydroxyacetamide')
NHANES ('National Health and Nutrition Examination Survey')
NOAEL ('non-observed adverse effect level')
NOEL ('non-observed effect level')
OCPs (Plaguicidas organoclorados)
OECD (Organización para la Cooperación y el Desarrollo Económicos)
OMET (Ometoato)
OLS (Mínimos cuadrados ordinarios)
PAHs (Hidrocarburos aromáticos policíclicos)
PBA ('3-Phenoxybenzoic acid')
PBDEs (Polibromodifenil éteres)
PCA (Análisis de componentes principales)
PCBs (Policlorobifenilos)
PFCs (Perfluorados)
PNP (p-nitrophenol)
PP (Propil paraben)
POA ('Propachloroxanilic acid')

POPs (Contaminantes orgánicos persistentes)
PSA (‘Primary and secondary amine’)
PVC (Policloruro de vinilo)
QqQ (Espectrómetro de masas de triple cuadrupolo)
QuEChERS (‘Quick, Easy, Cheap, Effective, Rugged, and Safe’)
RfD (Dosis de referencia)
RD (‘Reverse dosimetry’)
RRM (Modelo de regresión robusta)
RSD (Coeficiente de variación)
RT (Tiempo de retención)
SBSE (‘stir-bar sorptive extraction’)
SM-SLLME (‘stir-membrane solid–liquid–liquid microextraction’)
SPE (Extracción en fase sólida)
SPME (‘in-tube solid phase microextraction’)
SRM (‘Selected reaction monitoring’)
TCPy (‘3,5,6-Thrichloro-2-pyridinol’)
TDI (Ingesta diaria tolerable)
TOF (Especrómetro de masas de tiempo de vuelo)
trans-DCCA (‘trans-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid’)
UAE (extracción asistida por ultrasonidos)
UA-MSPDE (extración en fase sólida magnética dispersiva asistida por ultrasonidos)
UF (Factor de incertidumbre)
UE (Unión Europea)
UHPLC (‘ultrahigh performance liquid cromatography’)
VOCs (compuestos orgánicos volátiles y semivolátiles)
XIC (‘extracted ion chromatogram’)

1. RESUMEN

1. RESUMEN

La contaminación química es actualmente un grave problema de salud pública. La producción de sustancias químicas aumenta a un ritmo vertiginoso, y aunque los países desarrollados (OECD) producen la mayoría de las mismas, el crecimiento en países como Brasil, India, Indonesia, China o Sudáfrica (BRIICS) se produce con mayor velocidad.

Un porcentaje elevado de estas sustancias son potencialmente peligrosas para la salud, y el ser humano está expuesto a las mismas principalmente mediante ingestión, inhalación o por vía dérmica. Consecuentemente, es importante para la protección de la salud estudiar el grado de exposición de la población a las distintas sustancias químicas, ya sea mediante la monitorización ambiental, estudiando la presencia de contaminantes en los diferentes compartimentos ambientales (agua, alimentos, aire, suelo, etc…), o mediante la biomonitorización humana, determinando los niveles de biomarcadores de exposición en matrices biológicas (sangre, orina, leche materna, etc…). La biomonitorización humana nos proporciona datos sobre la exposición interna por lo que integra todas las vías de exposición. La presente tesis doctoral está centrada en el estudio de la biomoniotorización humana de contaminantes alimentarios mediante el desarrollo de metodologías analíticas basadas en cromatografía líquida acoplada a espectrometría de masas y la aplicación de las mismas en estudios poblacionales para conocer la exposición de la población y llevar a cabo la evaluación del riesgo.

La tesis está conformada por seis capítulos, que se corresponden con los artículos científicos que la sustentan. En general, se pueden agrupar en artículos de desarrollo de metodología analítica (capítulos 1, 2 y 3) y en artículos de niveles poblacionales (exposición) y de evaluación del riesgo (capítulos 4, 5 y 6).

Los capítulos 1 y 2 se centran en el análisis de muestras de orina para la determinación de plaguicidas mediante cromatografía líquida acoplada a espectrometría de masas de alta resolución. El primero está más enfocado a la identificación de nuevos metabolitos en orina, mientras que el segundo se centra en la optimización de un método analítico de cuantificación.

En el capítulo 3 se describe el desarrollo de una metodología analítica multiresiduo para la determinación de bisfenoles y parabenos en leche materna mediante cromatografía líquida acoplada a espectrometría de masas con analizador de triple cuadrupolo. La metodología desarrollada se empleó posteriormente en los capítulos 4 y 5 para determinar los niveles poblacionales de bisfenoles y parabenos, respectivamente, en madres lactantes y llevar a cabo la evaluación del riesgo para sus hijos.

Por último, en el capítulo 6 se determinan metabolitos de ftalatos en orina en madres lactantes y se lleva a cabo la correspondiente evaluación del riesgo.

Con respecto a los resultados, en el capítulo 1 se llevó a cabo con éxito un análisis retrospectivo mediante diferentes enfoques: análisis de sospechosos, análisis de desconocidos y análisis multivariante en diferentes poblaciones. El estudio permitió la

identificación y confirmación de seis metabolitos de plaguicidas mediante búsqueda de sospechosos, la identificación de un metabolito mediante búsqueda de desconocidos, así como la diferenciación mediante análisis de componentes principales de las diferentes poblaciones estudiadas.

En el capítulo 2 se optimizaron los parametros espectroméricos de un método de cromatografía líquida acoplada a espectrometría de masas de alta resolución tipo Orbitrap. Como resultado, el poder de resolución (25.000 FWHM), el tipo de fragmentación (CID 40 eV) y el tipo de calibración de masas (cafeína como calibrante interno para ESI positivo), demostraron mejorar las prestaciones del análisis en términos de sensibilidad y exactitud.

En el capítulo 3, la utilización de la extracción mediante QuEChERS combinada con la determinación mediante espectrometría de masas en tándem y aplicando ionización en modo negativo, permitió el desarrollo de un método optimizado y validado para la determinación de tres bisfenoles y cuatro parabenos en leche materna con límites de cuantificación (LoQ) de entre 0,1 y 0,25 ng/mL.

En los capítulos 4 y 5 el bisfenol A y los parabenos se detectaron en más del 60% de las muestras analizadas, mientras que los bisfenoles F y S presentaron frecuencias de detección reducidas (<25%). Las concentraciones de bisfenoles y parabenos en leche se encontraron en un rango de entre <LoQ y 49 ng/mL. El estudio de evaluación del riesgo para bisfenol A y parabenos en recién nacidos lactantes concluyó que las ingestas diarias estimadas de estos compuestos a través de la lactancia no se consideran un riesgo para la salud de la población estudiada.

En el capítulo 6, nueve de los metabolitos de ftalatos estudiados se detectaron en más de un 80% de las muestras, mientras que los niveles de metabolitos oscilaron entre <LoQ y 1291 ng/mL. Los estudios de evaluación del riesgo determinaron que la exposición a ftalatos en la población de madres estudiada no superaba los valores límite y, por tanto, no se considera que hubiera un riesgo para su salud.

La cromatografía líquida acoplada a espectrometría de masas permite la determinación de biomarcadores de contaminantes alimentarios en los niveles presentes en las muestras biológicas. La combinación del uso de técnicas de cuantificación ‘target’ así como otros enfoques como la búsqueda de sospechosos o de desconocidos permite conocer el grado de exposición de la población a contaminantes y llevar a cabo estudios de evaluación del riesgo. En general, se observa que la presencia de contaminantes alimentarios fue elevada en las muestras biológicas analizadas, sin embargo, los niveles detectados no se consideran peligrosos para las poblaciones estudiadas.

1. RESUM

La contaminació química és actualment un greu problema de salut pública. La producció de substàncies químiques augmenta a un ritme molt elevat, i, tot i que els països desenvolupats (OECD) produeixen gran part de les mateixes, el creiximent en països com Brasil, India, Indonesia, Xina o Sudàfrica (BRIICS) es produeix a major velocitat.

Un percentatge elevat d'aquestes substàncies són potencialment perilloses per a la salut i l'ésser humà està exposat principalment mitjançant ingestió, inhalació o per via dèrmica. Consequentment, és important per a la protecció de la salut estudiar el grau d'exposició de la població a les distintes substàncies químiques, ja siga mitjançant la monitorització ambiental, estudiant la presència de contaminants en els diferents compartiments ambientals (aigua, aliments, aire, sòl, etc…), o mitjançant la biomonitorització humana, determinant els nivells de biomarcadors d'exposició en matrius biològiques (sang, orina, llet materna, etc…). La biomonitorització humana integra totes les vies d'exposició i ens proporciona dades sobre l'exposició interna. La present tesi doctoral està centrada en l'estudi de la biomonitorització humana de contaminants alimentaris mitjançant el desenvolupament de metodologies analítiques basades en cromatografia líquida acoblada a espectrometria de masses i l'aplicació de les mateixes en estudis poblacionals per coneixer l'exposició de la població i dur a terme l'avaluació del risc.

La tesi està formada per sis capítols, que es corresponen amb els articles científics que componen la tesi. En general, es poden agrupar en artícles de desenvolupament de metodologia analítica (capítols 1, 2 i 3) i en articles de nivells poblacionals (exposició) i d'avaluació del risc (capítols 4, 5 i 6).

Els capítols 1 i 2 es centren en l'anàlisi de mostres d'orina per a la determinació de plaguicides mitjançant cromatografia líquida acoblada a espectrometria de masses d'alta resolució. El primer està més enfocat a la identificació de nous metabòlits en orina, i el segon es centra en l'optimització d'un mètode analític de quantificació.

Al capítol 3 es descriu el desenvolupament d'una metodologia analítica multiresidu per a la determinació de bisfenols i parabens en llet materna mitjançant cromatografia líquida acoblada a espectrometria de masses amb analitzador de triple quadrupol. La metodologia desenvolupada es va utilitzar posteriorment als capítols 4 i 5 per a determinar els nivells poblacionals de bisfenols i parabens, respectivament, en mares lactants i dur a terme l'avaluació del risc per als seus fills.

Per últim, al capítol 6 es determinen metabòlits de ftalats en orina en mares lactants i es du a terme la corresponent avaluació del risc.

Respecte als resultats, al capítol 1 es va dur a terme en èxit un anàlisi retrospectiu mitjançant diferents enfocaments: anàlisi de sospitosos, anàlisi de desconeguts i anàlisi multivariant de diferents poblacions. L'estudi va permetre la identificació i confirmació de sis metabòlits de plaguicides mitjançant búsqueda de sospitosos i anàlisi multivariant de diferents poblacions. L'estudi va permetre la identificació i confirmació de sis

metabòlits de plaguicides mitjançant búsqueda de sospitosos, la identificació d'un metabolit mitjançant búsqueda de desconeguts i la diferenciació mitjançant anàlisi de components principals de les diferents poblacions estudiades.

Al capítol 2 s'optimitzaren els paràmetres espectromètrics d'un mètode de cromatografia líquida acoblada a espectrometria de masses d'alta resolució tipus Orbitrap. Com a resultat, el poder de resolució (25.000 FWHM), el tipus de fragmentació (CID 40 eV) i el tipus de calibració de masses (cafeïna com a calibrant intern per a ESI positiu), mostraren les millors prestacions en quant a sensibilitat i exactitud.

Al capítol 3, la utilització de l'extracció mitjaçant QuEChERS combinada amb la determinació mitjançant espectrometria de masses en tàndem i aplicant ionització en mode negatiu, van permetre el desenvolupament d'un mètode optimizat i validat per a la determinació de tres bisfenols i quatre parabens en llet materna amb límits de quantificació (LoQ) entre 0,1 i 0,25 ng/mL.

Als capítols 4 i 5 el bisfenol A i els parabens es detectaren en més del 60% de les mostres analitzades, mentre que els bisfenols F i S presentaren freqüències de detecció reduïdes (<25%). Les concentracions de bisfenols i parabens en llet es trobaren en un interval de entre <LoQ i 49 ng/mL. L'estudi d'avaluació del risc per a bisfenol A i parabens en nadons lactants va concloure que les ingestes diaries estimades d'aquestos compostos a través de la lactància no eren perilloses per a la salut de la població estudiada.

Al capítol 6, nou dels metabòlits de ftalats estudiats es detectaren en més de un 80% de les mostres, mentre que els nivells de metabòlits oscil·laren entre <LoQ i 1291 ng/mL. Els estudis d'avaluació del risc determinaren que l'exposició a ftalats en la població de mares estudiada no superava els valors límit i, per tant, no es considera que hagués un risc per a la seua salut.

La cromatografia líquida acoblada a espectrometria de masses permet la determinació de biomarcadors de contaminants alimentaris als nivells presents en les mostres biològiques. La combinació de l'ús de tècniques de quantificació 'target' i altres metodologies amb un enfocament de búsqueda de sospitosos o desconeguts permet coneixer el grau d'exposició de la població a contaminants i dur a terme estudis d'avaluació del risc. En general, s'observa que la presència de contaminants alimentaris va ser elevada en les mostres biològiques analitzades, tanmateix, els nivells detectats no es consideren perillosos per a les poblacions estudiades.

1. ABSTRACT

Chemical pollution is currently a paramount public health concern. Chemical production has spread rapidly and despite its production is mainly focused in OECD nations, in countries as Brazil, India, Indonesia, China or South Africa (BRIICS) the production is growing rapidly. A high ratio of these compounds are considered potentially life-threatening and human beings are exposed to them through ingestion, inhalation and dermal exposure mainly. Consequently, in order to ensure health safety, the exposure of population to chemicals must be assessed through environmental monitoring, studying the presence of contaminants in environmental compartments (water, food, air, soil, etc…), or through human biomonitoring, assessing the levels of exposure biomarkers in biological matrices (blood, urine, human milk, etc…), which informs about the internal exposure and integrates all the routes of exposure. The present doctoral thesis is focused in the study of human biomonitoring of food contaminants through the development of analytical methodologies fundamented on liquid chromatography coupled to mass spectrometry in order to assesss the exposure of the populantion and implement the risk assessment.

The thesis is composed of six chapters, which correspond with six scientific papers. In general they can be grouped as analitcal method development papers (chapters 1, 2 and 3) and papers focused on exposure and risk assessment (chapters 4, 5 and 6).

Chapters 1 and 2 are focused on the determination of pesticide biomarkers in urine samples through liquid chromatography coupled to high resolution mass spectrometry. In the first chapter the objective is the identification of new metaoblites in urine, while the second is focused in the optimization of a quantification analytical method.

In chapter 3 the development of a multiresidue analytical methodology for the determination of bisphenols and phthalates in human milk is described. The method is based on liquid chromatography coupled to triple quadrupole mass spectrometry. This method is applied in chapters 4 and 5 for the determination of levels of bisphenols and parabens, respectively, in lactating mothers and the implementation of a risk assessment study in breastfed newborns.

Finally, in chapter 6 urinary phthalate metabolites are determined in urine of lactating mothers and a risk asssessment study is implemented.

Regarding results, in chapter 1 a retrospective analysis was implemented through different approaches: suspect screening, unkonwn analysis and multivariant analysis of different populations. The study allowed the identification and confirmation of six pesticide metabolites through suspect screening, the identification of one metabolite through unknonwn analysis, and the separation of the different studied populations through principal component analysis.

In chapter 2, the spectrometric parameters of a method of liquid chromatography coupled to an Orpitrap high resolution mass spectrometer were optimized. As a result, the

resolving power (25,000 FWHM), the fragmentation (CID 40 eV) and the mass calibration in ESI positive with caffeine as lock mass were selected in order to improve the analytical performance in terms of sensitivity and accuracy.

In chapter 3, QhEChERS extraction combined with the determination using tandem mass spectrometry and negative ionization allowed the development of an analytical method for the determination of three bisphenols and four parabens in human milk with limits of quantification (LoQ) ranging from 0.1 to 0.25 ng/mL.

In chapter 4 and 5, bisphenol A and parabens were detected in more than 60% of the samples, while the bisphenols F and S showed reduced detection frequencies (<25%). The concentrations of bisphenols and parabens in human milk ranged from <LoQ and 49 ng/mL. The risk assessment study for bisphenol A and parabens concluded that the estimated daily intakes of these compounds through breasfeeding in newborns were not considered dangerous for the studied population.

In chapter 6, nine phthalate metabolites were detected in more than 80% of samples and the concentrations ranged from <LoQ to 1291 ng/mL. The risk assessment studies determined that the phthalate exposure of the mother population studied did not exceeded the threshold values and, therefore, a health risk derived from the exposure to phthalates was discarded.

Liquid chromatography coupled to mass spectrometry allows the determination of food contaminant biomarkers at concentrations usually present in biological matrices. The use of quantification approaches (target) combined with other approaches such as suspect screening or unkonwn analysis allows to study the exposure of the population to contaminants and to implement risk assessment studies. In general, the presence of food contaminants was elevated in the samples analyzed, however, the levels detected were not considered dangerous for the populations studied.

2. INTRODUCCIÓN

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Las sustancias químicas forman parte de la vida cotidiana de la sociedad actual, juegan un papel muy importante en todos los sectores económicos y facilitan la vida de millones de personas. La producción de sustancias químicas continúa creciendo en todo el mundo, especialmente en los países fuera de la OECD (**Figura 1**). En la Unión Europea (UE) se produjeron en 2016 cerca de 350 millones de toneladas de sustancias químicas **(EUROSTAT, 2020)**, las cuales tienen una gran variedad de usos como en productos de limpieza, materiales de construcción, productos textiles, plásticos, aditivos alimentarios, baterías, pinturas, artículos del hogar, etc. Sin embargo, gran parte de estas sustancias pueden representar un riesgo para la salud **(Li & Suh, 2019)**. Se estima que aproximadamente un 62% de las sustancias químicas consumidas en la UE en 2016 eran potencialmente peligrosas para la salud **(UNEP, 2019)**. Junto a las sustancias fabricadas con alguna finalidad tecnológica, algunos compuestos químicos peligrosos se generan de forma involuntaria como productos secundarios o subproductos en procesos industriales, o se emiten como consecuencia de actividades antropogénicas (p. ej. el tráfico) o causas naturales como los incendios o erupciones volcánicas. A este último grupo pertenecen muchos de los grupos de los contaminantes altamente tóxicos como las dioxinas y los hidrocarburos aromáticos policíclicos **(Dioxins book, 2003; Abdel-Shafy & Mansour, 2016)**

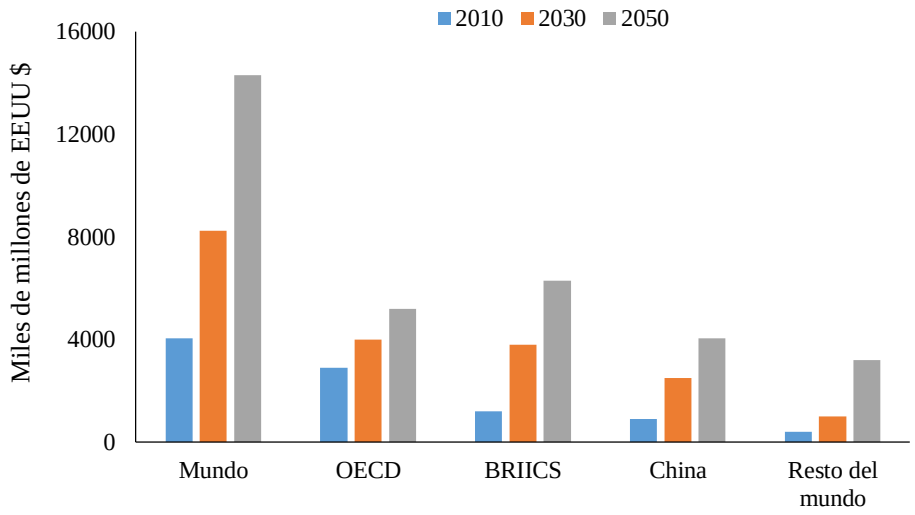


Figura 1. Proyección de producción de compuestos químicos por región (en ventas) entre 2010 y 2050 **(**adaptado de **OECD, 2012)**.

La contaminación química es uno de los principales factores de morbilidad y mortalidad. Los principales efectos sobre la salud por exposición a contaminantes son las alteraciones en el neurodesarrollo y las interacciones con el sistema endocrino **(Landrigan et al. 2018)**. Las muertes atribuidas a la contaminación química en el mundo en 2016 ascendieron a 1,6 millones de personas, y cerca de 45 millones de años de vida ajustados por discapacidad (DALYs) **(WHO, 2018)**. En la **Figura 2** se detallan las distintas patologías y causas que generaron estas muertes. Además, actualmente ha crecido la exposición a contaminantes químicos emergentes que están comenzando a ser considerados como potenciales amenazas para la salud. Esta situación ha llevado a las

Naciones Unidas a establecer entre los objetivos de la Agenda 2030 la reducción del número de muertes y enfermedades debidas a exposición a sustancias químicas y a la contaminación del aire, agua y suelo **(UN, 2019)**.

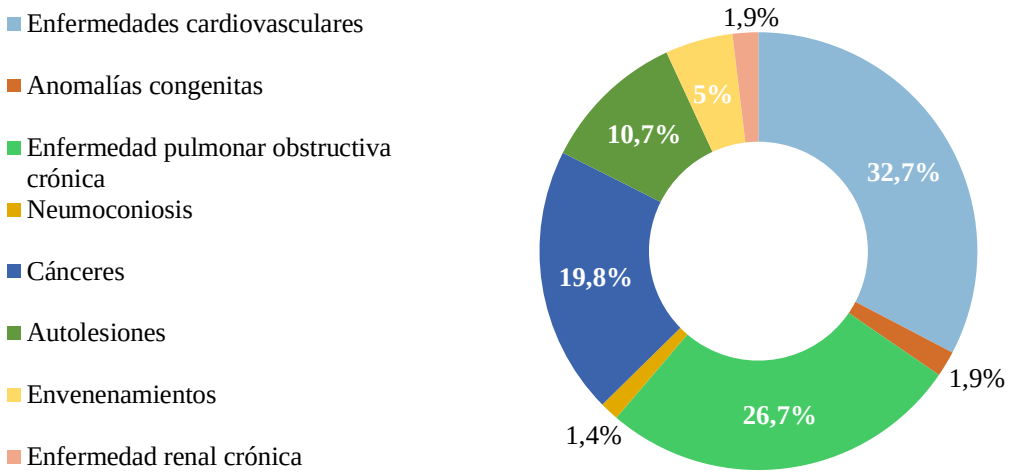


Figura 2. Distribución de las causas de muerte (1,6 millones) relacionadas con la exposición a sustancias químicas **(**adaptado de **UNEP, 2019)**.

Las contaminantes se distribuyen desde las fuentes al medioambiente y entran en el cuerpo humano por inhalación, absorción dérmica o ingestión (ver **Figuras 3** y **4**).

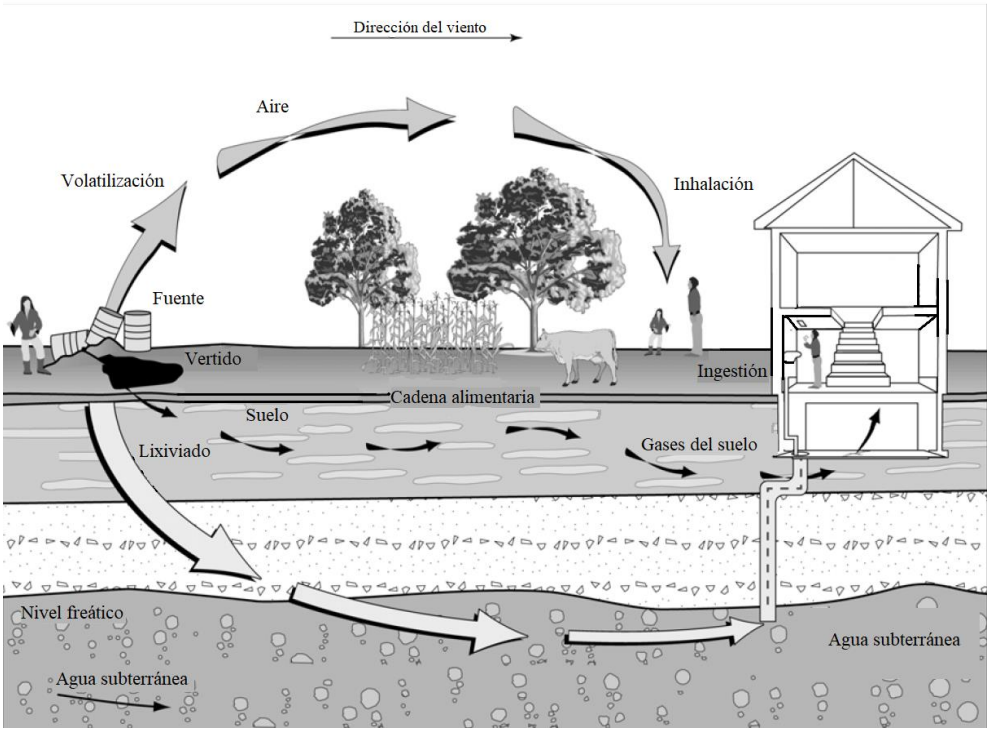


Figura 3. Esquema de las fuentes y rutas de exposición a contaminantes **(**adaptado de **ATSDR, 2005)**.

La población está expuesta por vía inhalatoria a los contaminantes presentes tanto en el aire ambiente como en el aire interior y la absorción de los mismos se produce en los pulmones. Los contaminantes más estudiados en aire son los NO_x, el SO_2, el O_3, el CO, las partículas en suspensión, los compuestos orgánicos volátiles y semivolatiles (VOCs), y el radón **(Leung, 2015)**. Otros contaminantes químicos presentes en aire son los PAHs **(Kim et al., 2013)** y las dioxinas **(Lohmann & Jones, 1998)**, así como contaminantes emergentes como retardantes de llama, sustancias perfluoroalquiladas, plastificantes (ftalatos y bisfenoles), conservantes (parabenos), fármacos y plaguicidas **(Barroso et al., 2019)**.

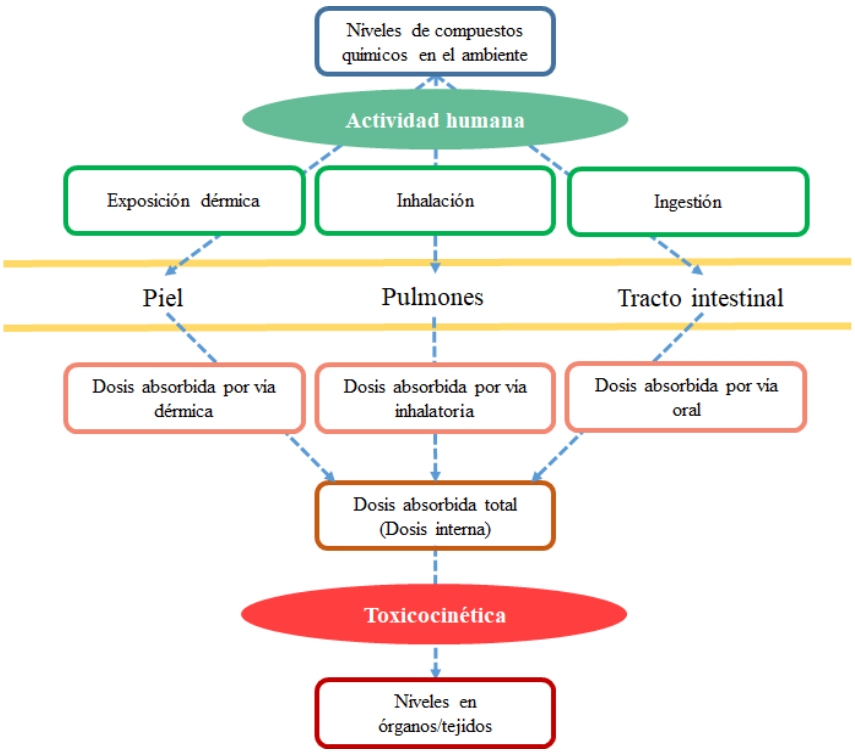


Figura 4. Esquema de los mecanismos de absorción de contaminantes químicos en el cuerpo humano **(**adaptado de **Rice et al., 2008)**.

La absorción dérmica ocurre principalmente a través de la piel aunque también a través de otros órganos como los ojos. El contacto dérmico se produce en diferentes situaciones: contacto con agua durante el baño, lavado de manos o natación; contacto con el suelo en actividades como la jardinería, la construcción o juegos en el exterior; contacto con productos comerciales como cosméticos; en el desarrollo de ciertas actividades profesionales como la aplicación de plaguicidas; o contacto con polvo o residuos químicos presentes en alfombras, suelos o ropa (**EPA, 2011**). Entre los contaminantes a los que la población está expuesta por contacto dérmico están: los retardantes de llama; algunos metales como níquel, cromo o cobalto; algunos plaguicidas como piretroides, organofosforados y carbamatos; filtros solares como benzofenonas; conservantes como parabenos; y ftalatos presentes en plásticos o productos cosméticos **(IPCS, 2014)**.

La exposición mediante ingestión se produce principalmente a través de la dieta. La contaminación química de los alimentos es un problema global de seguridad alimentaria. Las causas de la contaminación de los alimentos son variadas: ésta puede ser debida a la presencia de contaminantes en el medioambiente como en el caso del mercurio o las dioxinas; a actividades humanas como la aplicación de plaguicidas u otros productos químicos agrícolas; a la generación de contaminantes durante la elaboración o procesado de alimentos; o también se puede producir por migración de los contaminantes desde el material de envasado a los alimentos **(Thompson & Darwish, 2019)**. Además, la población está expuesta también a través de la ingesta de agua. La contaminación del agua puede producirse en la fuente de suministro (agua subterránea o agua superficial); durante el tratamiento de las aguas con compuestos químicos; o después del tratamiento, mediante el lixiviado de contaminantes desde las vías de conducción de agua o la migración desde los envases donde se almacena **(EPA, 2011)**. En el caso de los recién nacidos, la exposición mediante ingestión se debe principalmente a la leche materna. La leche materna es la principal fuente de nutrición para muchos recién nacidos durante los primeros meses de vida y presenta numerosos beneficios ya que los protege de infecciones y favorece su desarrollo cognitivo. Sin embargo, algunos contaminantes pueden encontrarse en la leche materna debido a la exposición de las madres a los mismos **(EPA, 2011)**. Aunque los beneficios de la lactancia son superiores a los riesgos por exposición que implica, se deben llevar a cabo estudios para controlar y tratar de reducir la contaminación **(Picone & Paolillo, 2013)**.

Los contaminantes a los que la población está expuesta a través de la dieta son de grupos muy variados como metales, contaminantes orgánicos persistentes, plaguicidas, plastificantes, conservantes o productos formados durante el procesado de los alimentos como los PAHs y la acrilamida. Algunos de los metales presentes en la dieta que presentan un mayor riesgo para la población son: el mercurio, presente principalmente en peces de gran tamaño **(Carocci et al., 2016)**; el plomo en cereales **(EFSA, 2010a)**; el cadmio en cereales y vegetales **(EFSA, 2012)**; y el arsénico en arroz **(Upadhyay et al., 2019)**. Entre los contaminantes orgánicos persistentes están las dioxinas, furanos, policlorobifenilos (PCBs) y plaguicidas organoclorados (OCPs), que se encuentran en alimentos muy variados como huevos, aceite, lácteos, pescados, productos cárnicos, frutas y verduras **(Guo et al., 2019)**. Durante las últimas décadas se han diseñado plaguicidas no persistentes como organofosforados, carbamatos y piretroides. Estos compuestos no se bioacumulan pero pueden presentar efectos nocivos sobre la salud y se encuentran principalmente en vegetales **(Carvalho, 2017)**. Por otro lado, los alimentos en contacto con materiales plásticos pueden contener bisfenoles o ftalatos que migran desde los envases y se consideran disruptores endocrinos **(Almeida et al., 2018; Serrano et al., 2014)**. Algunos aditivos alimentarios como los conservantes pueden tener efectos nocivos para la salud a grandes dosis. Por ejemplo, los parabenos, que se utilizan principalmente en productos cosméticos y fármacos aunque también en la industria alimentaria **(Bledzka et al 2014)**. También existen contaminantes que se forman durante el procesado y/o cocción de los alimentos, como los PAHs o la acrilamida **(Zelinkova & Wenzl, 2015; Capuano & Fogliano, 2011)**.

Debido a la variedad de fuentes y rutas de exposición y al ingente número de contaminantes que existen, se han desarrollado diferentes metodologías para evaluar el grado de exposición de la población a los compuestos químicos y facilitar el estudio del exposoma.

2.1. Exposoma

El exposoma es un concepto relativamente reciente acuñado por Wild **(2005)** definido inicialmente como 'el conjunto de la exposición a factores ambientales a lo largo de la vida del individuo'. Con este término se pretende enfatizar el papel de la exposición ambiental en el desarrollo de enfermedades crónicas y de este modo complementar la relevancia del genoma. Posteriormente, Miller & Jones **(2014)** redefinieron el concepto del exposoma como 'la medida acumulada de factores ambientales y sus respuestas biológicas asociadas a lo largo de la vida incluyendo las exposiciones derivadas del ambiente, la dieta, el comportamiento y los procesos endógenos'. Es importante resaltar que en esta segunda definición del exposoma se incluye el papel de los 'procesos endógenos', es decir, los cambios producidos en el organismo derivados de la exposición a factores ambientales (ej: mutaciones en el ADN, formación de aductos, alteración de proteínas…) y las modificaciones que produce el organismo en los agentes ambientales (ej: metabolismo de contaminantes químicos). Por otro lado, se debe destacar que, a diferencia del genoma, el objetivo del estudio del exposoma está principalmente enfocado a la toma de decisiones en el ámbito de la salud pública **(Wild, 2012)**.

Debido a la complejidad y amplitud del concepto de exposoma, es necesario definir y clasificar los agentes y factores que lo componen. Wild **(2012)** clasifica el exposoma en tres categorías: exposición interna, exposición externa específica y exposición externa general **(Figura 5)**. La exposición interna incluye los procesos internos del organismo tales como el metabolismo, las hormonas, la morfología del cuerpo, la actividad física, los procesos de inflamación, el estrés oxidativo, el envejecimiento o la microflora intestinal. La exposición externa específica engloba a los contaminantes químicos, la radiación, los agentes infecciosos, la dieta, el estilo de vida (consumo de alcohol, tabaco…). Por último, la exposición externa general incluye los factores sociales, económicos y psicológicos, como la educación, el estatus financiero, el estrés psicológico y mental o el clima. Hay que señalar que la frontera que divide a estas tres categorías en algunas ocasiones es difusa y algunos tipos de exposición pueden ser englobados en más de una categoría como por ejemplo la actividad física que puede formar parte tanto de la exposición interna como de la exposición externa específica. Además, algunas exposiciones están interconectadas entre sí, en especial, las exposiciones internas pueden ser consecuencia directa o indirecta de una exposición externa (p. ej. una exposición interna como la inflamación puede derivar de una exposición externa específica como la contaminación del aire).

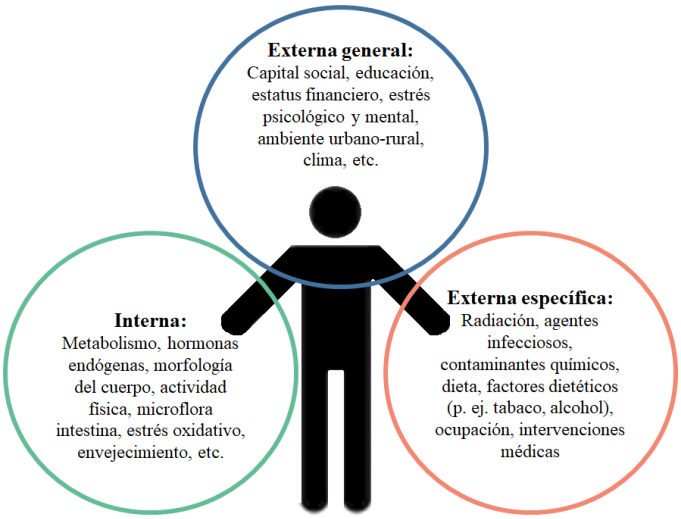


Figura 5. Exposoma: clasificación de sus componentes en función del tipo de exposición **(**adaptado de **Wild, 2012)**.

Existen diferentes estrategias para llevar a cabo el estudio del exposoma. En primer lugar, debe tenerse en cuenta que el concepto del exposoma implica una variación a lo largo del tiempo ya que incluye todas las exposiciones acumuladas desde la concepción hasta la muerte del individuo. Esta variación temporal incrementa la complejidad del estudio del exposoma ya que evaluar la exposición de forma continua implica una serie de retos analíticos y económicos que son inabordables en la mayoría de los casos. Como alternativa, se puede evaluar el exposoma mediante la medición puntual en etapas críticas de la vida, en especial en aquellas en las que se lleva a cabo el desarrollo del individuo. Así, se pueden realizar 'capturas' del exposoma en cada una de las etapas de la vida: gestación, primera infancia, infancia, adolescencia y madurez **(Rappaport & Smith, 2010; Wild, 2012)**.

Por otro lado, también existen diferentes enfoques para el estudio del exposoma en función de si la medición se lleva a cabo en la fuente de exposición (estrategia descendente o 'bottom-up') o en el medio interno (estrategia ascendente o 'top-down') **(Rappaport & Smith, 2010)**. En el caso de la exposición a compuestos químicos o exposoma químico, la estrategia descendente corresponde a la monitorización ambiental y la estrategia ascendente a la biomonitorización en humanos (BH) (**Figura 6**). La monitorización ambiental se centra en la determinación de los contaminantes en matrices ambientales como aire, agua y alimentos a través de las cuales la población está expuesta **(EEA, 2008)**. Este enfoque nos permite conocer cuáles son las fuentes de exposición a contaminantes, así como llevar a cabo una evaluación del riesgo basada en los niveles de los contaminantes en las fuentes de exposición **(Eskola et al., 2019)**. En contraste, la BH se centra en la determinación de los contaminantes o sus metabolitos en muestras biológicas, de manera que mide de manera integrada la contaminación proveniente de todas las fuentes y vías de contaminación y por lo tanto permite llevar a cabo una

evaluación del riesgo global, por lo que es útil para evaluar de manera conjunta la eficacia de las medidas de prevención del riesgo **(Aylward, 2018; Roca & Yusà, 2013)**.

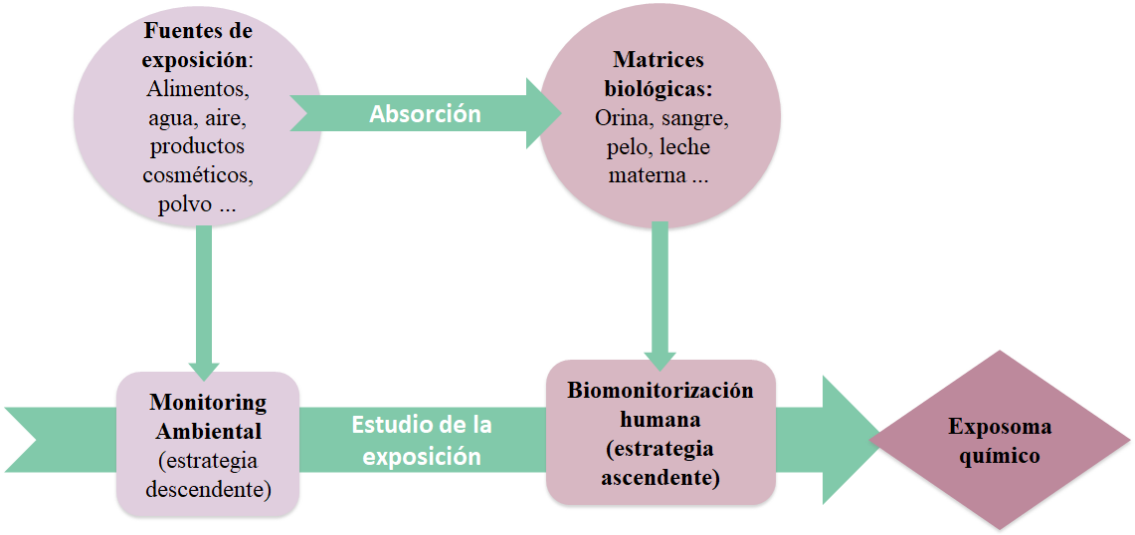


Figura 6. Estrategias de estudio de la exposición a contaminantes químicos.

2.2. Biomonitorización humana

Inicialmente, la BH se utilizó para estudiar la exposición ocupacional a contaminantes. Sin embargo, durante la segunda mitad del siglo XX se empezó a implantar en estudios ambientales en la población general, siendo uno de los ejemplos más conocidos la detección del descenso de niveles de plomo en sangre de la población estadounidense durante la segunda mitad del siglo XX como consecuencia de la restricción del uso de plomo en la gasolina, pinturas y otros artículos **(Creager, 2018)**. Durante las dos últimas décadas el número de estudios de BH se ha incrementado significativamente **(Bocato et al., 2019)**. En la **Figura 7** se muestra el número de programas de BH implantados en el mundo en los que se estudian diferentes tipos de contaminantes alimentarios **(Choi et al., 2015a)**. Los contaminantes alimentarios más estudiados en BH son los metales **(Castaño et al., 2015; Berglund et al., 2015)**; seguidos por los contaminantes orgánicos bioacumulables como PCBs, OCPs, perfluorados (PFCs), polibromodifenil éteres (PBDEs), dioxinas y furanos **(Haines et al., 2017; Weldon & LaKind, 2016);** y los contaminantes orgánicos no bioacumulables como fenoles, ftalatos, PAHs, acrilamida, clorofenoles y algunas familias de plaguicidas (organofosforados, piretroides y carbamatos) **(Khoury et al., 2018; Timchalk, 2011)**. Las matrices más utilizadas son la sangre y la orina **(Choi et al., 2015a)**.

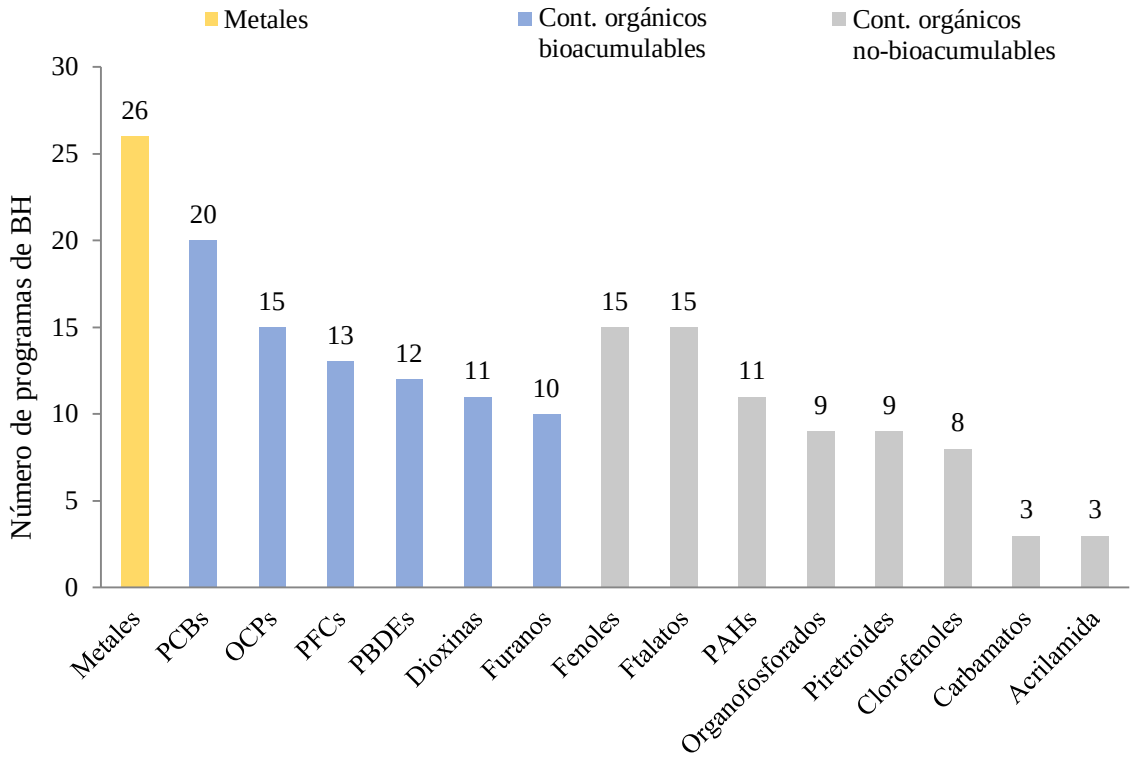


Figura 7. Número de programas de BH en el mundo con los distintos tipos de contaminantes alimentarios incluidos en los mismos **(**adaptado de **Choi et al., 2015a)**.

La mayoría de los estudios utilizan lo que se podría denominar BH tradicional o análisis 'target', es decir, análisis de muestras biológicas para cuantificar un reducido o no muy amplio número de biomarcadores ya conocidos. Sin embargo, las mejorías de los equipos analíticos y la implantación del concepto de exposoma han motivado el desarrollo de métodos de 'screening' que permiten la identificación de un elevado número de sustancias potencialmente presentes en las muestras a analizar ('suspect screening' o análisis de sospechosos) o de métodos orientados a la búsqueda de sustancias no conocidas ('unknown analysis' o análisis de desconocidos) **(Bocato et al., 2019)**.

2.2.1. Matrices

Las matrices más utilizadas para la biomonitorización de la población general son la sangre y la orina. Sin embargo, la BH también se puede llevar a cabo en otras matrices como el pelo **(Castaño et al., 2015)**, las uñas **(Li et al., 2013)**, los dientes **(Andra et al., 2015)**, la saliva **(Michalke et al., 2015a)**, el sudor **(Omokhodion & Howard, 1991)** o las heces **(Sahlstrom et al., 2015)**. También se pueden estudiar otros tipos de matrices biológicas características de algunos grupos de población o de etapas de la vida concretas como la gestación (placenta, cordón umbilical, líquido amniótico y meconio) **(Cooke, 2014)**, las madres lactantes (leche materna) **(Björklund et al., 2012)**, los hombres adultos

(semen) **(Song et al., 2018)** o los pacientes de ciertas patologías (cálculos renales) **(Kuta et al., 2016)**. Por último, también se han llevado a cabo estudios en otras matrices más invasivas y difíciles de recolectar como el líquido cefalorraquídeo **(Michalke et al., 2015b)** o el hígado **(Cooke, 2014)**. Se deben tener en cuenta los procesos toxicocinéticos de absorción, distribución, metabolismo y excreción **(Gupta, 2016)** para seleccionar la matriz más adecuada para estudiar los contaminantes de interés. Además, en función del tipo de matriz y del analito a estudiar, la información que proporciona el análisis puede corresponder a una exposición reciente o acumulada tal y como se indica en la **Figura 8 (Needham & Sexton, 2000)**.

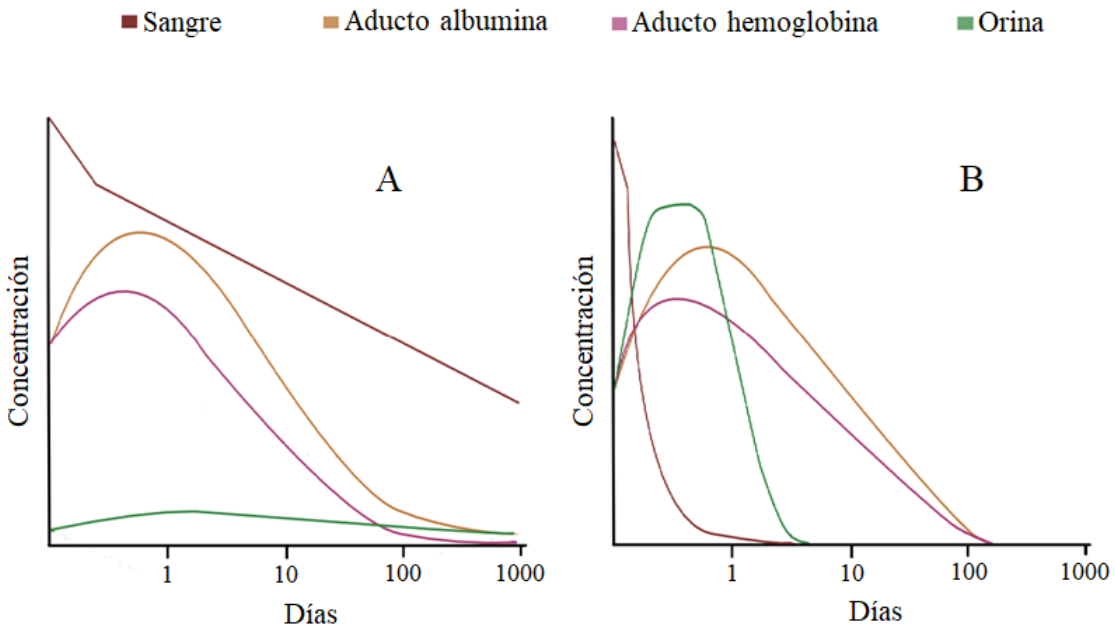


Figura 8. Variación temporal, tras exposición, de las concentraciones de biomarcadores de contaminantes bioacumulables (A) y de excreción rápida (B) libres en sangre, formando aductos en sangre con albumina o hemoglobina, y en orina **(**adaptado de **Needham & Sexton, 2000)**.

La **Tabla 1** describe algunas de las características de interés de las principales matrices utilizadas en BH (sangre, orina, leche materna y pelo).

La sangre es una de las matrices más utilizadas en la BH debido a que permite la detección de un gran número de contaminantes y además irriga todos los órganos y tejidos del cuerpo, por lo que el nivel de un contaminante en sangre es buen indicador del grado de exposición al mismo **(Angerer et al., 2007)**. Es especialmente útil en el caso de contaminantes persistentes ya que los niveles de estos contaminantes en

Tabla 1. Características de las principales matrices biológicas utilizadas en los estudios de BH.

	Sangre (Sangre total, suero o plasma)	**Orina (orina 24h o 'spot urine')**	**Leche materna**	**Pelo**
Contaminantes estudiados	Metales como Pb, Hg total, metilmercurio, Cd, o As total **(Saravanabhavan et al., 2017)**; contaminantes orgánicos persistentes como OCPs, PBDEs, PCBs, dioxinas y furanos **(Pumarega et al., 2016)**; y, en menor medida, algunos contaminantes orgánicos no persistentes como acrilamida formando aductos con hemoglobina **(Hays & Aylward, 2008)**	Principalmente metales como Sb, As total, especies de As, Hg inorgánico, Pb o Cd **(Saravanabhavan et al., 2017);** y biomarcadores de contaminantes orgánicos no persistentes como ftalatos, PAHs, plaguicidas, bisfenoles **(Khoury et al., 2018),** acrilamida **(Hays & Aylward, 2008)**; o parabenos **(Honda et al., 2018)**.	Metales como Pb, Hg o Cd **(Yurdakök, 2015)**; contaminantes orgánicos persistentes como OCPs, PBDEs, PCBs, dioxinas y furanos **(Mannetje et al., 2012);** y no persistentes como bisfenoles **(Mendonca et al., 2014)**, parabenos o metabolitos de ftalatos **(Schlumpf et al., 2010)**.	Metales como Hg o Pb **(Esplugas et al., 2019)**; contaminantes orgánicos persistentes como OCPs, PBDEs, PCBs, dioxinas, furanos y PFCs **(Król et al., 2013)**; y algunos contaminantes orgánicos no persistentes como parabenos, bisfenoles o metabolitos de ftalatos **(Martín et al., 2019; Katsikantami et al., 2020)**.
Biomarcadores /Ventana exposición	Para biomarcadores de contaminantes persistentes los niveles en sangre reflejan exposición acumulada (meses o años). En el caso de contaminantes persistentes y no persistentes que forman aductos con proteinas o ADN la ventana de exposición es de días o pocos meses **(Needham & Sexton, 2000)**.	Los niveles de biomarcadores de contaminantes no persistentes en esta matriz reflejan exposición reciente (horas o días) **(Needham & Sexton, 2000)**.	En contaminantes persistentes indica exposición acumulada pero se deben tener en cuenta los procesos de depuración a lo largo de la lactancia **(National Research, 2006; LaKind et al., 2004)**.	Permite conocer exposición a largo plazo y exposición relativamente reciente. En función de la distancia entre el segmento de pelo a analizar y el extremo del pelo en contacto con el cuero cabelludo se puede determinar el periodo de tiempo en el que se produjo la exposición **(Smolders et al., 2009)**. Sin embargo, tan solo está validado el uso de esta matriz para estudiar los niveles de Hg.
Ajuste niveles	-	En el caso de 'spot urine' se usan los niveles de creatinina, la osmolalidad o la gravedad específica (**Middleton et al., 2016)**.	En el caso de compuestos persistentes se usa la masa de grasa en leche **(LaKind et al., 2004)**.	-

Tabla 1. (Continuación)

	Sangre (Sangre total, suero o plasma)	**Orina (orina 24h o 'spot urine')**	**Leche materna**	**Pelo**
Ventajas	Permite conocer la exposición en la población general. Irriga todos los órganos y tejidos del cuerpo por lo que es un buen indicador del grado de exposición **(Angerer et al., 2007)**.	Permite conocer la exposición de la población general. Facilidad de toma de muestra en el caso de 'spot urine'. No existe limitación de volumen salvo en recién nacidos o niños pequeños (<.4 años) **(National Research, 2006)**.	Permite conocer la exposición tanto de las madres como de los recién nacidos lactantes **(Schlumpf et al., 2010)**.	Permite conocer la exposición de la población general. Permite conocer el periodo de tiempo en el que se produjo la exposición. Facilidad de toma de muestra y conservación **(Esteban & Castaño, 2009)**.
Limitaciones	Se trata de una matriz invasiva, existe limitación de volumen y requiere de personal especializado para la toma de muestra. Dificultad de transporte y conservación de la muestra **(Rockett et al., 2004; Tuck et al., 2009; National Research, 2006)**.	En el caso de orina 24h la toma de muestra puede implicar ciertas dificultades. En el caso de 'spot urine' los niveles deben ajustarse **(Esteban & Castaño, 2009)**.	Solo disponible en un grupo poblacional reducido (madres lactantes). Dificultad para tomar muestras durante los primeros días/semanas tras el parto, especialmente en madres primiparas **(National Research, 2006)**.	Posibilidad de contaminación externa **(Scharmm, 2008)**. Excepto en el caso del Hg, no existen biomarcadores de exposición validados.

sangre están en equilibrio con los niveles en los depósitos grasos del organismo. Tras la absorción, los niveles de contaminante en sangre decrecen rápidamente hasta que se alcanza un equilibrio entre los niveles en los depósitos y la sangre, y, a partir de este momento, el descenso de los niveles se produce paulatinamente. Por lo tanto, la sangre es válida para evaluar la exposición interna a estos contaminantes a largo plazo **(National Research, 2006)**. La sangre también se utiliza para la biomonitorización de contaminantes no persistentes que forman aductos con proteinas o ADN y, por tanto, aumentan el periodo de tiempo en el que están presentes en sangre tras su exposición **(Needham & Sexton, 2000)**, como la acrilamida que forma aductos con hemoglobina **(Hays & Aylward, 2008) (**ver **Tabla 1)**. En función del tipo de biomarcador a determinar, se puede analizar la sangre total, el plasma y/o el suero sanguíneo **(Harvey et al., 2016)**. Por otro lado, la sangre presenta algunos inconvenientes principalmente relacionados con la dificultad de la toma de muestra mediante venopunción ya que se trata de un muestreo invasivo, precisa de personal cualificado y conlleva un riesgo de contagio para el personal que toma la muestra **(Rockett et al., 2004)**. Además, en el caso de necesitar grandes volúmenes para llevar a cabo el análisis, puede no ser adecuada en algunas poblaciones como niños o mujeres embarazadas **(National Research, 2006)**. Otros inconvenientes son la dificultad de transporte y la conservación de las muestras y, en caso de llevar a cabo el análisis en plasma y/o suero, se debe llevar a cabo un pretratamiento de muestra **(Rockett et al., 2004; Tuck et al., 2009)**. Existen alternativas a la toma de muestra por venopunción como el 'dried blood spot' o gota de sangre seca **(Poothong et al., 2019; Li & Tse, 2010)** que tan solo precisan de pequeños volúmenes de sangre y facilitan el proceso de muestreo y conservación de la muestra, reduciendo los costes e inconvenientes del muestreo tradicional.

Junto con la sangre, la orina es la matriz más utilizada en estudios de BH **(Angerer et al., 2007)**. Las personas generan alrededor de 1,4 L de orina al día con un residuo sólido seco de 59 g/día. Las variaciones en el volumen excretado de orina se deben principalmente a diferencias en edad, actividad física, condiciones ambientales y toma de agua, sal y proteínas. Su pH es de alrededor de 6,2 y algunos de sus componentes principales son el agua, el potasio, el calcio, el magnesio, la urea, la creatina, la creatinina y el ácido úrico **(Rose et al., 2015; National Research, 2006)**. A diferencia de la sangre, la toma de muestra de orina no es invasiva **(Alves et al., 2014)** y permite la recolección de grandes volúmenes de muestra por lo que facilita la detección de contaminantes presentes a bajas concentraciones **(Angerer et al., 2007)**. En el caso de recién nacidos el volumen de muestra sí que puede ser limitante debido a que es muy reducido y debe recogerse de los pañales. También debe tenerse en cuenta que en niños de 2 a 4 años el volumen de muestra también es más reducido que en la población general **(National Research, 2006)**. En orina, los contaminantes pueden encontrarse inalterados, como metabolitos de fase I y/o como metabolitos de fase II conjugados **(Moos et al., 2016; Frederiksen et al., 2007)**. Cuando se estudian contaminantes que dan lugar a metabolitos conjugados con grupos glucuronido y/o sulfato, generalmente se lleva a cabo un tratamiento enzimático con β-glucuronidasas y/o sulfatasas con objeto de hidrolizarlos previamente a su cuantificación **(Dwivedi et al., 2018)**. A la hora de llevar a cabo el muestreo existen varias opciones:

recolectar el total de orina excretada durante un día (‘24h urine’), la recolección de muestras de orina puntuales (‘spot urine’) o recolección de la primera orina de la mañana. La recolección de las dos últimas es menos costosa pero tiene como inconveniente que no permite diferenciar el grado de dilución de las orinas por lo que es recomendable ajustar las concentraciones de los biomarcadores detectados con los niveles de creatinina, la osmolalidad, la gravedad específica u otros métodos **(Esteban & Castaño, 2009; Middleton et al., 2016)**. La concentración de creatinina en orina oscila entre 0,3 y 2,1 g/L. Se debe tener en cuenta que la concentración varía en base a la dilución de la orina pero también está correlacionada con el peso corporal, la masa muscular y el consumo de proteínas, siendo mayor en hombres que en mujeres **(Rose et al., 2015; Middleton et al., 2016)**. Tal y como se observa en la **Tabla 1**, la biomonitorización en orina está especialmente indicada para el estudio de metales y contaminantes orgánicos o metabolitos hidrosolubes con semividas cortas, por lo que es indicador de exposición reciente o de una exposición continuada **(Esteban & Castaño, 2009; National Research, 2006)**. Aunque existen algunos estudios de BH de contaminantes persistentes en orina en general se recomiendan otras matrices para evaluar la exposición a este tipo de compuestos **(Esteban & Castaño, 2009; Alves et al., 2014)**.

La BH en leche materna es de enorme utilidad ya que proporciona información tanto de la exposición interna de las madres lactantes como de la exposición externa de los recién nacidos a través de la ingesta. Esta última tiene una gran relevancia en el contexto de la evaluación del riesgo ya que los neonatos presentan una mayor vulnerabilidad a los contaminantes debido a que su organismo se encuentra en fase de desarrollo **(Mallozzi et al., 2016)**. Sin embargo, la toma de muestra de esta matriz presenta limitaciones ya que se restringe a un grupo poblacional muy concreto **(Esteban & Castaño, 2009)** y la toma de muestra durante los primeros días o semanas tras el parto puede ser difícil, especialmente para madres primiparas **(National Research, 2006)**. La leche se forma en las células epiteliales de las glándulas mamarias a través de 5 rutas simultáneas, de manera que transforman los precursores presentes en la sangre o el líquido intersticial en los componentes de la leche. Estas rutas son: i) exocitosis (en especial de los compuestos hidrofílicos); ii) síntesis y secreción de grasa (a partir de ácidos grasos presentes en el plasma o en las células epiteliales de las glándulas mamarias); iii) transporte a través de la membrana apical (en el caso de moléculas pequeñas como sodio, potasio, algunos monosacáridos o agua); iv) transcitosis (permite el paso de algunas proteínas desde el plasma a la leche); v) ruta paracelular (paso de células del sistema inmunitario y otros componentes) **(Lovelady et al., 2002)**. La leche está compuesta principalmente por agua, en la que el resto de compuestos están disueltos o en suspensión. El resto de compuestos mayoritarios de la leche son lípidos (30-50 g/L), carbohidratos, proteínas (1% de la leche), aminoácidos, minerales, vitaminas, enzimas, inmunoglobulinas, ácidos nucleicos, hormonas, factores del crecimiento y células. La composición de la leche varía a lo largo de la lactancia de manera que provee de los nutrientes necesarios al niño a lo largo de su desarrollo. Tras el parto, en primer lugar se produce el calostro y a partir de la primera o la segunda semana empieza a producirse la leche madura. La composición de la leche continua variando con el tiempo ya que a lo largo de la lactancia los niveles de lípidos

aumentan y los niveles de otros compuestos como el zinc o el cobre disminuyen **(LaKind et al., 2004)**. Debido a que se trata de una matriz con alto contenido lipídico, la biomonitorización en leche materna permite el estudio de contaminantes persistentes lipofílicos. Los contaminantes lipofílicos se acumulan en los depósitos grasos del cuerpo y pasan a la leche materna para su excreción. Por lo tanto, en la madre lactante existe una reducción de los niveles de contaminantes lipofílicos a lo largo de la lactancia denominada depuración. Para una correcta cuantificación de los contaminantes lipofílicos, su concentración debe corregirse respecto al contenido total de lípidos en leche **(Esteban & Castaño, 2009)**. La leche materna también se ha utilizado para determinación de niveles de metales **(Björklund et al., 2012)** y contaminantes orgánicos no persistentes y sus metabolitos (ver **Tabla 1**). Al igual que en la orina, los contaminantes que presentan metabolitos conjugados de fase II deben hidrolizarse mediante un tratamiento enzimático previamente a su cuantificación **(Cao et al., 2015)**.

El pelo es una matriz no invasiva, fácil de muestrear, transportar y conservar. Permite obtener información tanto de exposición a largo plazo como de exposición relativamente reciente. Es la matriz más utilizada para evaluar la exposición a Hg. Teniendo en cuenta que el pelo crece alrededor de 1 cm cada mes, el análisis de los diferentes segmentos del cabello permite conocer las variaciones en la exposición a lo largo del tiempo **(Esteban & Castaño, 2009; Smolders et al., 2009)**. De manera general, las muestras se toman en la parte occipital del cráneo, tan cerca de la raíz del pelo como sea posible. Los segmentos más cercanos al cuero cabelludo hacen referencia a una exposición más reciente. El mayor inconveniente de la biomonitorización en pelo es la dificultad de distinguir entre la presencia de contaminantes debida a la exposición interna y la contaminación externa de la muestra por deposición atmosférica sobre el pelo, por lo que se deben aplicar procedimientos de purificación previos al análisis del pelo para evitar la posible contaminación externa. Otras dificultades para una adecuada interpretación de los niveles de contaminantes en pelo están relacionados con la variabilidad en el crecimiento o la influencia del sexo, la edad, el color o los tratamientos capilares **(Scharmm, 2008)**. Tal y como se indica en la **Tabla 1**, la BH en pelo se ha utilizado para la determinación de metales **(Castaño et al., 2015)**, contaminantes orgánicos persistentes **(Appenzeller & Tsatsakis, 2012; Schramm, 2008)** y contaminantes orgánicos no persistentes y sus metabolitos **(Katsikantami et al., 2020; Martín et al., 2019)**.

2.2.2. Biomarcadores

Para estudiar la exposición interna a contaminantes se utilizan diferentes tipos de indicadores biológicos o biomarcadores: biomarcadores de exposición, biomarcadores de efecto y biomarcadores de susceptibilidad (ver **Figura 9**). Los biomarcadores deben ser específicos, sensibles, accesibles y se debe conocer su toxicocinética. Además, se debe disponer de metodologías que permitan analizarlos adecuadamente.

- Los biomarcadores de exposición son el propio contaminante, sus metabolitos y/o el producto de la interacción entre el contaminante y una molécula o célula diana que puedan ser medidos en algún compartimiento del organismo.
- Los biomarcadores de efecto son alteraciones en el organismo susceptibles de ser medidas, es decir, cambios bioquímicos, fisiológicos o de comportamiento que en algunos casos pueden estar asociados con alguna enfermedad o alteración de la salud.
- Los biomarcadores de susceptibilidad son indicadores de la capacidad del organismo para responder frente a la exposición a un contaminante.

No siempre es fácil definir la frontera entre los diferentes tipos de biomarcadores y en algunos casos un biomarcador puede englobarse dentro de varios grupos **(National Research, 2006)**.

En la mayoría de los casos, los estudios de BH estudian la exposición interna utilizando biomarcadores de exposición y, en concreto, se centran en la detección de los contaminantes y sus metabolitos. Por ello, es importante conocer los mecanismos metabólicos específicos de cada contaminante con objeto de seleccionar el biomarcador adecuado para evaluar la exposición interna.

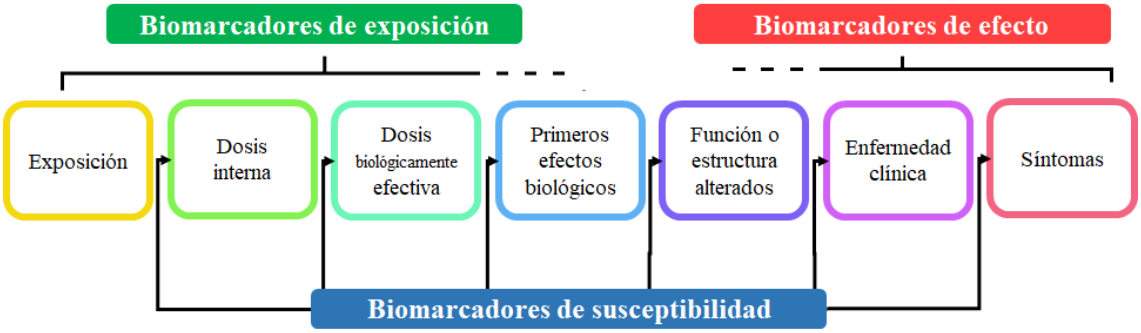


Figura 9.Tipos de biomarcadores **(**adaptado de **National Research, 2006)**.

2.2.2.1. Metabolismo de los xenobióticos

La absorción de los contaminantes se puede dar mediante ingestión (en el tracto grastrointestinal), inhalación (en los pulmones) o por vía dérmica (en la piel). Tras su absorción los contaminantes pasan al torrente circulatorio donde se distribuyen por todo el organismo. El organismo posee mecanismos metabólicos mediados por enzimas que facilitan la eliminación de los contaminantes y otros xenobióticos como los fármacos. El metabolismo de xenobióticos se produce principalmente en el hígado, sin embargo, otros órganos también poseen la capacidad de metabolizar, como el tracto gastrointestinal, los pulmones, la piel o los riñones **(Caldwell et al., 1995)**. Generalmente, el metabolismo de los xenobióticos favorece su detoxificación, sin embargo, en algunos casos los

metabolitos son más tóxicos que los compuestos precursores **(Macherey & Dansette, 2015)**. La principal vía de eliminación de los xenobióticos y sus metabolitos es la orina aunque también se pueden excretar por las heces, el sudor, la saliva, la leche materna o el aire expirado **(Boussery et al., 2008)**.

Generalmente, se distinguen dos tipos de metabolismo de xenobióticos: reacciones de fase I y de fase II. Las reacciones de fase I son reacciones de funcionalización que introducen o revelan un grupo funcional en el xenobiótico. Las reacciones de fase I se pueden englobar en reacciones de oxidación, reducción e hidrólisis. Las reacciones de fase II son reacciones de conjugación catalizadas por transferasas que facilitan la unión covalente entre el grupo funcional de un xenobiótico o un metabolito de fase I y una molécula endógena polar que aumenta la hidrosolubilidad del metabolito formado y por tanto facilita su excreción. Las reacciones de conjugación se agrupan en reacciones de metilación, sulfonación, glucuronidación, acetilación y conjugación con glutation. Se debe tener en cuenta que no todos los xenobióticos sufren procesos metabólicos, algunos xenobióticos son eliminados o almacenados sin ser metabolizados, además, algunos metabolitos de fase I pueden ser excretados sin necesidad de formar conjugados **(Testa & Clement, 2015; Caldwell et al., 1995; Klaassen, 2013)**.

2.2.3. Contaminantes alimentarios

La dieta es una de las principales vías de exposición a contaminantes **(Figura 4)**, y distintos contaminantes están asociados a diferentes grupos de alimentos (p. ej. el mercurio en peces de gran tamaño **(Carocci et al., 2016)**; el arsénico en arroz **(Upadhyay et al., 2019)**, o los plaguicidas en vegetales **(Sang et al., 2020)**). Al igual que en los estudios de monitorización ambiental (p. ej. estudios de dieta total **(Marín et al., 2017)**), existen numerosas familias de contaminantes alimentarios cuya exposición ha sido estudiada mediante biomonitorización. Se pueden dividir en 3 grupos: metales, contaminantes orgánicos persistentes y contaminantes orgánicos no-persistentes tal y como se describe en la **Tabla 2**.

2.2.3.1. Metales

Los metales son uno de los grupos de compuestos más estudiados mediante biomonitorización **(Choi et al., 2015a)**. Se han llevado a cabo estudios en sangre **(Cusick et al., 2018)**, orina **(Saravanabhavan et al., 2017)**, leche materna **(Björklund et al., 2012)**, pelo **(Liang et al., 2017)**, meconio **(Yang et al., 2013)** y otras matrices **(Kim & Kim, 2011; Arruda-Neto et al., 2010)**. Se debe tener en cuenta que algunos de los metales como el zinc, el hierro, el cobre o el selenio son considerados micronutrientes debido a que son esenciales para la salud humana, sin embargo, pueden presentar efectos tóxicos si las exposiciones son elevadas **(Nieder et al., 2018)**. Aunque se ha evaluado la exposición a decenas de metales mediante estudios de biomonitorización **(Björklund et**

al., 2012), los más estudiados son los elementos tóxicos como el mercurio, el plomo o el cadmio **(Choi et al., 2015a).**

2.2.3.2. Contaminantes orgánicos persistentes

Los contaminantes orgánicos persistentes (POPs), son compuestos orgánicos que poseen semividas de eliminación de años o décadas en suelos y sedimentos y de varios días en la atmósfera. Se trata de sustancias tóxicas con elevada lipofilia que se acumulan y biomagnifican a través de la cadena alimentaria y, en general, son resistentes al metabolismo **(Jones & de Voogt., 1999)**. Existen diversos tipos de clasificación de los POPs, pero de manera general se pueden agrupar en los POPs que se producen intencionadamente y los POPs que se generan inintencionadamente. Dentro del primer grupo se encuentran los plaguicidas organoclorados (OCPs) y otros productos químicos industriales como los policlorobifenilos (PCBs) y algunos retardantes de llama bromados (BFRs), aunque estos últimos no siempre son considerados como POPs. Dentro del segundo grupo se encuentran las dioxinas y los furanos **(Ashraf, 2017; Haines et al., 2017)**. La exposición interna a POPs ha sido extensamente estudiada en diferentes estudios de biomonitorización. Al tratarse de compuestos lipofílicos, en general se utilizan matrices que permiten la determinación de estas sustancias, como el suero y plasma sanguíneos **(Tan et al., 2008; Haines et al., 2017)**, el pelo **(Król et al., 2013)**, la leche materna **(Fang et al., 2015)**, el tejido adiposo **(Lee et al., 2017)** o el meconio **(Jeong et al., 2016)**.

2.2.3.3. Contaminantes orgánicos no persistentes

Existe un amplio abanico de compuestos orgánicos no persistentes usados en distintas aplicaciones como aditivos alimentarios **(Bledzka et al., 2014)**, plastificantes **(Wittassek et al., 2011)** o plaguicidas **(Yusa et al., 2015)**; y otros que pueden generarse involuntariamente durante el procesado y/o cocción de los alimentos como la acrilamida **(Exon, 2006)** o los PAHs **(Abdel-Shafy & Mansour, 2016)**. La mayoría tiene semividas cortas en el organismo (de horas o días) y en las últimas décadas han sido ampliamente estudiados mediante biomonitorización. Entre los compuestos más estudiados mediante BH están los parabenos, bisfenoles, ftalatos, plaguicidas polares, acrilamida y PAHs. En la mayoría de los casos se han estudiado en orina, pero también se han estudiado en otras matrices como sangre, leche materna o pelo (ver **Tabla 2**).

Tabla 2. Contaminantes alimentarios estudiados en BH

Tipo de contaminante	Contaminante	Biomarcadores	Matrices	Referencia
Metales	Mercurio elemental y mercurio divalente	Hg	Orina (refleja exposición a largo plazo).	**(Boerleider et al., 2017)**
	Metilmercurio	Hg	Pelo y sangre (reflejan exposición a medio-largo plazo)	**(Boerleider et al., 2017)**
	Cd	Cd	Sangre (refleja exposición reciente en la población general y exposición a largo plazo en no fumadores), orina (refleja exposición acumulada).	**(Tiesjema & Mengelers, 2016)**
	Pb	Pb	Sangre (semivida: 1 mes), orina y saliva.	**(Klotz & Goen, 2017)**
		Biomarcadores de efecto	Eritrocitos, orina y/o plasma	**(Klotz & Goen, 2017)**
Contaminantes orgánicos persistentes	OCPs (Aldrin, clordano, dicloro difenil tricloroetano (DDT), hexaclorobenceno y hexaclorociclohexano, entre otros).	Plaguicidas inalterados o metabolizados en el ambiente o en el organismo	Suero y plasma sanguíneos, leche materna, meconio, pelo y tejido adiposo.	**(Tan et al., 2008; Haines et al., 2017; Aerts et al., 2019; Jeong et al., 2016; Król et al., 2013)**
	PCBs	PCBs inalterados	Suero y plasma sanguíneos, leche materna, meconio, pelo y tejido adiposo.	**(Tan et al., 2008; Haines et al., 2017; Fang et al., 2015; Jeong et al., 2016; Król et al., 2013).**

Tabla 2 (Continuación)

Tipo de contaminante	**Contaminante**	**Biomarcadores**	**Matrices**	**Referencia**
Contaminantes orgánicos persistentes (continuación)	BFRs [polibromodifenil éteres (PBDEs), tetrabromobisphenol-A (TBBPA), hexabromociclododecano (HBCDD) y polibromobifenilos (PBBs)]	BFRs inalterados	Sangre, plasma y suero sanguíneos, leche materna, pelo, tejido adiposo y meconio.	**(Aerts et al., 2019; Tan et al., 2008; Król et al., 2013; Jeong et al., 2016; Haines et al., 2017; Fang et al., 2015)**
	Policlorodibenzodioxinas y policlorodibenzofuranos (PCDD/Fs)	PCDD/Fs inalterados	Sangre, plasma y suero sanguíneos, tejido adiposo, pelo y leche materna.	**(Tan et al., 2008; Król et al., 2013; Fang et al., 2015)**
Contaminantes orgánicos no-persistentes	Parabenos	Parabenos inalterados o metabolitos oxidados y/o conjugados	Orina, leche materna, sangre, plasma sanguíneo y tejido adiposo	**(Ye et al., 2006a; Azzouz et al., 2016a; Mulla et al., 2015; kolatorova et al., 2018; Artacho-Cordon et al., 2018; Moos et al., 2016)**
	Bisfenoles	Bisfenoles inalterados o metabolitos conjugados	Orina, leche materna, suero sanguíneo y sudor.	**(Völkel et al. 2002; Cao et al., 2015; Genuis et al., 2012)**
	Ftalatos	Metabolitos (monoesteres, oxidados y/o conjugados) o ftalatos inalterados	Orina**,** leche materna, suero sanguíneo, saliva y semen.	**(Wittassek et al., 2011; Fromme et al., 2011; Wang et al., 2019)**
	Plaguicidas polares (organofosforados, piretroides, neonicotinoides, carbamatos y algunos herbicidas)	Generalmente metabolitos de plaguicidas	Orina, sangre, plasma, suero, leche materna y pelo	**(Yusa et al., 2015; Roca et al., 2014a; Tsatsakis et al., 2010; Feo et al., 2012)**

Tabla 2 (Continuación)

Tipo de contaminante	**Contaminante**	**Biomarcadores**	**Matrices**	**Referencia**
Contaminantes orgánicos no-persistentes (continuación)	Acrilamida	N-acetyl-S-(2-carbamoylethyl)-cysteine, N-acetyl-S-(2-carbamoylethyl)-l-cysteine-sulfoxide, N-acetyl-S-(2-carbamoyl-2-hydroxyethyl)-cysteine y aductos con hemoglobina como N-2-carbamoylethylvaline (AAVal) y N-2-carbamoyl-2-hydroxyethylvaline (GAVal)	Orina y sangre	**(Wang et al., 2017; Urban et al., 2006)**
	PAHs	Metabolitos hidroxilados y conjugados o PAHs inalterados.	Orina, sangre y leche materna.	**(Jacob & Seidel, 2002; Thai et al., 2015; Singh et al., 2008; Wang et al., 2018)**

2.2.4. Programas de biomonitorización

El estudio de la exposición interna mediante biomonitorización ha sido implantado en diferentes países y organismos internacionales durante las últimas décadas. Además han surgido proyectos de investigación internacionales que tienen como objetivos principales evaluar la exposición de la población a contaminantes mediante biomonitorización humana y armonizar los procedimientos de muestreo y analíticos con objeto de obtener resultados comparables **(Choi et al., 2015b; Louro et al., 2019)**.

En Norteamerica destacan dos grandes programas de BH, uno en Estados Unidos y otro en Canadá **(NHANES, 2009; CHMS, 2017)**. En Estados Unidos, los 'Centers for Disease Control and Prevention' (CDC) estudian la exposición interna de la población estadounidense desde 1999 mediante estudios de BH. Los resultados son publicados en el 'National Report on Human Exposure to Environmental Chemicals', desde el 2009 y se actualiza cada dos años. Las matrices utilizadas para estudiar la exposición interna son la orina, la sangre y el suero sanguíneo en alrededor de 2500 participantes del 'National Health and Nutrition Examination Survey' (NHANES). Se trata de uno de los estudios más extensos de BH y evalúa la exposición a más de 200 contaminantes y/o metabolitos incluyendo metales, contaminantes orgánicos persistentes y no-persistentes **(NHANES, 2009; NHANES, 2019)**.

En Canadá, el 'Canadian Health Measures Survey' estudia la exposición de la población canadiense en alrededor de 6000 participantes desde 2007. Hasta la actualidad han hecho públicos informes de 4 ciclos de BH. Las matrices utilizadas para llevar a cabo los estudios de BH son la sangre y la orina y desde el inicio del programa se han determinado más de 150 contaminantes y/o metabolitos **(CHMS, 2017)**.

En Europa existen varios programas de BH a nivel nacional además de estudios en el ámbito europeo como el COPHES/DEMOCOPHES y el HBM4EU. El estudio COPHES/DEMOCOPHES fue impulsado por el COPHES (Consortium to Perform Human Biomonitoring on a European Scale) y se llevó a cabo mediante el proyecto DEMOCOPHES ('Demonstration of a Study to Coordinate and Perform Human Biomonitoring on a European Scale') desarrollado entre 2010 y 2012. En el estudio participaron 3688 voluntarios (madres y niños) de 17 países de la Unión Europea y se determinaron metales, bisfenol A y biomarcadores de exposición a tabaco en pelo y orina **(DEMOCOPHES, 2013)**.

El HBM4EU es un proyecto de investigación a nivel europeo que se está llevando a cabo actualmente (2017-2021) en el que participan más de veinte países. El estudio se centra en la biomonitorización de muestras humanas y, entre otros, tiene como objetivos armonizar los protocolos de los estudios de biomonitorización entre los países participantes, establecer valores guía de biomonitorización y establecer nexos entre la exposición humana a contaminantes químicos y el desarrollo de enfermedades **(Louro et al., 2019)**.

En Alemania, los 'German Environmental Surveys' (GerESs) son estudios de BH a nivel nacional que se comenzaron a implementar en 1985. Hasta la actualidad se han llevado a cabo 5 estudios. Los dos primeros (GerES I y GerES II) se centraron en el estudio de metales pesados, en el primero se tomaron muestras de 2700 adultos y en el segundo muestras de 4000 adultos y de más de 700 de niños. A partir del tercero se incluyó el análisis de contaminantes orgánicos. El GerES III se llevó a cabo en adultos con un total de 4800 participantes, mientras que el GerES IV (n=1790) y el GerES V (n=2500) se han centrado en el estudio de niveles en niños y adolescentes. Entre las matrices analizadas se incluyen la orina y la sangre **(GerES-V, 2015; WHO, 2015)**.

En España, el estudio nacional BIOAMBIENT.ES se llevó a cabo para evaluar la exposición interna a metales, POPs, PAHs y otros contaminantes químicos en la población española. Entre 2009 y 2010 se recogieron muestras de sangre, orina y pelo de casi 2000 participantes mayores de 16 años **(Pérez-Gómez et al., 2013)**. Por otro lado, el proyecto INMA (Infancia y Medioambiente) también se lleva a cabo en España y está centrado en el estudio de la exposición a contaminantes durante la gestación y la infancia. El proyecto se inició en 2003 y está conformado por varias cohortes en Menorca, Granada, Ribera d'Ebre, Valencia, Sabadell, Asturias y Guipúzcoa y ha recogido muestras de más de 3500 madres y sus hijos. INMA ha estudiado la exposición a metales, POPs, ftalatos, bisfenoles y otros contaminantes orgánicos en muestras de sangre, placenta, orina, saliva y pelo, además de recoger información de cuestionarios, parámetros clínicos y medidas ambientales **(Gascon et al., 2017)**. Además, el programa BIOVAL se estableció en la Comunitat Valenciana con objeto de conocer la exposición interna de la población infantil a contaminantes alimentarios mediante el análisis de muestras de orina y pelo. En total, 666 niños de 6 a 11 años participaron en el programa, cuyas muestras fueron recogidas a lo largo del año 2016 **(Perez et al., 2017)**.

En Francia, el estudio 'French National Nutrition and Health Survey' (ENNS – Étude nationale nutrition santé) evaluó la exposición interna de la población francesa a metales, plaguicidas y PCBs mediante BH. Para ello, durante 2006 y 2007, se tomaron muestras de sangre, orina y pelo de aproximadamente 3.100 adultos de entre 18 y 74 años y 1.700 niños de entre 3 y 17 años en la Francia continental **(Fréry et al., 2010; Fréry et al., 2012)**. Este estudio sirvió como referencia para establecer una estrategia nacional de BH coordinada por el 'French Institute for Public Health Surveillance' (InVS). Este estrategia integra muestras de dos estudios diferentes: uno que abarca a la población general: 'Environment, Health, Biomonitoring, physical Activity, Nutrition' (Esteban); y otro que se centra en el periodo perinatal: 'French longitudinal study of children' (ELFE) **(Fréry et al., 2012; Dereumeaux et al; 2017)**. En el contexto de Esteban se recogieron muestras de orina, sangre y pelo de residentes en la Francia continental entre los años 2014 y 2016 que tuviesen entre 6 y 74 años, con objeto de conocer la exposición de la población y establecer valores de referencia. Además, el estudio incluye la recogida de datos de salud y nutrición. Alrededor de 3000 adultos (18-74 años) y 1300 niños (6-17 años) participaron en el estudio. La lista de compuestos priorizados para ser analizados incluye bisfenoles, ftalatos, parabenos, plaguicidas polares, OCPs, dioxinas, furanos, PCBs, BFRs, PFCs y

metales, entre otros. **(Dereumeaux et al; 2017)**. Por otro lado, para el estudio de BH durante el periodo perinatal, se tomaron muestras de alrededor de 4100 madres embarazadas de entre las 18000 participantes del estudio ELFE. Las madres fueron incluidas en el estudio durante 2011 y se evaluó la exposición a bisfenol A (BPA), ftalatos, plaguicidas polares, OCPs, dioxinas, furanos, PCBs, BFRs, PFCs y metales mediante el análisis de muestras de orina, suero sanguíneo, pelo y sangre del cordón umbilical que fueron tomadas en los momentos previos, durante y/o en los días posteriores al parto **(Fréry et al., 2012; Dereumeaux et al; 2017)**.

En Flandes (Bélgica), se desarrolla el estudio de BH 'Flemish Environment and Health Study' (FLEHS). De 2002 a 2014 se han llevado tres ciclos de este estudio que ha evaluado la exposición interna a metales, PCBs, OCPs, ftalatos y PAHs entre otros contaminantes en alrededor de 5800 participantes mediante el análisis del cordón umbilical de recién nacidos y sangre y orina de adolescentes (14-15 años) y adultos (20-40 y 50-65 años) **(Schoeters et al., 2017)**.

Además, existen estudios a nivel nacional de BH en otros países europeos como Noruega, Dinamarca, Rusia, Italia, la República Checa y en algunos países asiáticos como Corea del Sur **(WHO, 2015; Choi et al., 2015b; Magnus, 2017; Cerná et al., 2017)**.

2.3. Métodos analíticos

Los biomarcadores de contaminantes orgánicos están presentes en las muestras biológicas a niveles traza, y además, las matrices biológicas presentan un alto grado de complejidad con un elevado número de compuestos interferentes **(National Research, 2006)**. Por lo tanto, es necesario desarrollar metodologías analíticas con tratamientos de muestra y equipos analíticos que posean la suficiente sensibilidad y especificidad para poder identificar y cuantificar los biomarcadores a los niveles a los que está expuesta la población **(Bader et al., 2010; Boogaard et al., 2011)**.

El elevado número de muestras en estudios de BH **(Choi et al., 2015a)** y la dificultad en la recolección de volúmenes de muestra elevados de algunas matrices, como la leche materna **(National Research, 2006)**, hacen necesario el desarrollo de metodologías multi-residuo que permitan determinar varios analitos en el mismo análisis y/o el desarrollo de metodologías que utilicen volúmenes de muestra reducidos.

A continuación, se describen las diferentes etapas de los métodos analíticos de BH, centrándose principalmente en los analitos (bisfenoles, parabenos, ftalatos y plaguicidas) y matrices (orina y leche materna) estudiados en la presente tesis. En la **Figura 10** se muestra el esquema general de las metodologías analíticas.

2.3.1. Tratamiento de muestra

El tratamiento de muestra viene determinado por el tipo de matriz y la naturaleza y niveles de los analitos a determinar **(Fernández-Peralbo & Luque de Castro, 2012)**. Además, se debe de tener en cuenta el tipo de estrategia analítica que se va a llevar a cabo. Si se va a llevar a cabo análisis 'target', en el que se busca identificar y cuantificar un analito o grupo de analitos con unas características similares, se utilizan tratamientos de muestra más selectivos, que disminuyan la presencia de interferentes antes de analizar la muestra. En el caso de enfoques tipo análisis de sospechosos o análisis de desconocidos, el tratamiento de muestra debe permitir identificar un amplio número de analitos con propiedades fisico-químicas muy diferentes entre sí **(Plassmann et al., 2015)**.

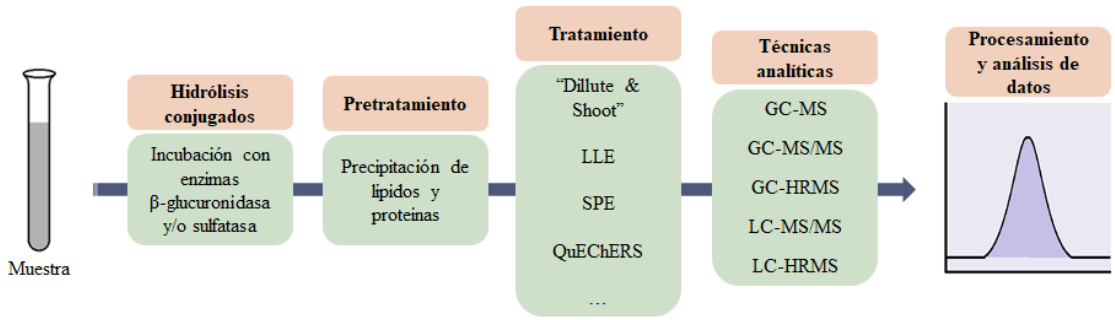


Figura 10. Esquema general de las metodologías analíticas en BH.
LLE: Extracción líquido-líquido; SPE: extracción en fase sólida; GC: cromatografía de gases; LC: cromatografía de líquidos; MS: espectrometría de masas; MS/MS: espectrometría de masas en tandem; HRMS: espectrometría de masas de alta resolución.

2.3.1.1. Hidrólisis enzimática

La presencia de metabolitos de fase II o conjugados de contaminantes orgánicos en muestras biológicas como la orina o la leche materna, hace necesaria la hidrólisis de los mismos previamente a su análisis **(Vela-Soria, 2016; Roca et al., 2014a)**. Al igual que otros xenobióticos, muchos contaminantes orgánicos y sus metabolitos de fase I se conjugan con grupos glucurónido y/o sulfato para aumentar su hidrosolubilidad y facilitar su eliminación mediante la orina **(Testa & Clement, 2015)**. El análisis de estos conjugados incrementaría el coste y la complejidad de los análisis mediante biomonitorización por lo que se recomienda la hidrólisis de estos conjugados previamente al análisis mediante la adición de enzimas hidrolíticas **(Grignon et al., 2017)**.

El proceso de hidrólisis se basa en la adición de la enzima junto con un tampón a un pH adecuado para su actividad y se incuba a una temperatura y tiempo determinados con

objeto de garantizar la hidrólisis completa de los conjugados con glucurónido y/o sulfato en la muestra biológica. Las condiciones adecuadas dependen del tipo de analito a determinar, del tipo de muestra y de la enzima utilizada. Existen diferentes tipos de enzimas con actividad β-glucuronidasa y/o sulfatasa. En algunos casos es imprescindible seleccionar adecuadamente la enzima ya que algunas de ellas tienen una actividad lipasa no específica y pueden hidrolizar enlaces ester de algunos biomarcadores. Éste es el caso de los ftalatos, en los que la cadena de los monoesteres podría ser hidrolizada, por lo que el análisis daría lugar a una subestimación de los niveles. Dwivedi et al. recomendaron el uso de la enzyma β-glucuronidasa de *E. coli* K-12 para el análisis de metabolitos de ftalatos, ya que evita la hidrólisis de los monoésteres **(Dwivedi et al., 2018)**.

2.3.1.2. Pretratamiento

La elevada presencia de proteínas en matrices biológicas como la leche o la sangre, o de lípidos en leche materna hacen que sea recomendable el pretratamiento de la muestra para precipitar o eliminar estos interferentes **(Yusa et al., 2015; Rodríguez-Gómez et al., 2014a)** Existen diferentes opciones para eliminar esos compuestos. En la determinación de bisfenoles y parabenos en leche materna se añade o bien una solución ácida de sales de zinc y tungsteno para precipitar lípidos y proteínas **(Rodríguez-Gómez et al., 2014b)** o bien disolventes como la acetona para precipitar las proteínas **(Deceuninck et al., 2015)**.

2.3.1.3. Extracción y/o purificación

Para la determinación de bisfenoles y parabenos en leche materna, los procesos de extracción y/o purificación más usados son las técnicas tradicionales como la extracción líquido-líquido (LLE) **(Fisher et al., 2017)**, la extracción en fase sólida (SPE) o la combinación de ambas **(Ye et al., 2008)**. Sin embargo, también se han aplicado técnicas de extracción más recientemente desarrolladas. Modificaciones de la SPE como la 'in-tubo solid phase microextraction' (SPME) **(Souza et al., 2016)** o la 'molecularly imprinted polymer for microdisc solid-phase extraction' (MIP-SPE) **(Melo et al., 2013)** han sido utilizadas con resultados satisfactorios. Asimismo, se ha aplicado técnicas que incluyen la aplicación de la sonicación para facilitar el proceso de extracción, como la extracción asistida por ultrasonidos (UAE) **(Rodríguez-Gómez et al., 2015)** o la combinación del uso de ultrasonidos con técnicas magnéticas como la extración en fase sólida magnética dispersiva asistida por ultrasonidos (UA-MSPDE) **(Filippou et al., 2017)**. También se han aplicado otras metodologías de extracción como la 'stir-bar sorptive extraction' (SBSE) **(Rodríguez-Gómez et al., 2014a)**, la 'stir-membrane solid–liquid–liquid microextraction' (SM-SLLME) **(Rodríguez-Gómez et al., 2014c)**, o la microextracción líquido-líquido dispersiva (DLLME) **(Alshana et al, 2015)**. Algunos de esos métodos incluyen procedimientos muy laboriosos que pueden ser evitados mediante el uso de QuEChERS ('Quick, Easy, Cheap, Effective, Rugged, and Safe'), un tipo de

tratamiento de muestra que consiste en una extracción asistida por la adición de sales que provocan un efecto 'salting out', favoreciendo el paso de los contaminantes de la muestra a la fase orgánica, y un proceso de purificación mediante la adición de diferentes combinaciones de C_{18}, 'primary secondary amine' PSA y/o otros sorbentes en fase dispersiva **(González-Curbelo et al., 2015)**. El uso de QuEChERS ha sido aplicado satisfactoriamente en la determinación de bisfenoles y/o parabenos en matrices humanas como la orina **(Correia-Sa et al., 2018)** o la leche materna **(Vela-Soria et al., 2018)**.

En la determinación de metabolitos de ftalatos en orina, el proceso de extracción se lleva a cabo generalmente mediante SPE **(Sabaredzovic et al., 2015; Feng et al., 2015)**. Sin embargo, el uso del 'dilute & shoot' también ha sido utilizado con éxito **(Lien et al., 2018)**, facilitando y reduciendo el tiempo del tratamiento de muestra.

Con respecto a la determinación de biomarcadores de plaguicidas en orina, la mayoría de las técnicas de extracción y/o purificación utilizadas son de LLE o SPE **(Yusà et al., 2015)**. Sin embargo, también se han desarrollado métodos de extracción con QuEChERS **(Roca et al., 2014b)** que simplifican el proceso de tratamiento de muestra y amplían el espectro de analitos que pueden ser determinados.

En estudios de análisis de sospechosos o de desconocidos en orina, Díaz et al. utilizaron el 'dilute & shoot' para la detección de medicamentos y sustancias ilícitas **(Díaz et al., 2012)**. Por otro lado, Plassmann et al. compararon el uso de QuEChERS y la inyección directa para el análisis de sospechosos de biomarcadores de contaminantes en orina. La inyección directa evitó la pérdida de compuestos previamente al análisis pero disminuía la sensibilidad de la técnica por el aumento de la supresión de la señal, mientras que los QuEChERS mejoraron la sensibilidad de la técnica pero impedían la determinación de los compuestos más polares. Por consiguiente, se recomendó el uso combinado de ambas técnicas para llevar a cabo este tipo de estudios **(Plassmann et al., 2015)**.

2.3.2. Técnicas analíticas

Debido a que los biomarcadores de los contaminantes se encuentran a niveles traza en las muestras biológicas, y a la complejidad de las mismas **(National Research, 2006)**, en los estudios de HB las metodologías analíticas más utilizadas son la cromatografía líquida (LC) y la gaseosa (GC) acopladas a espectrometría de masas (MS) **(Choi et al., 2015a)**. La GC es utilizada principalmente para la determinación de compuestos volatiles como los POPs y compuestos muy polares previa derivatización de los biomarcadores, mientras que la LC es utilizada para determinar compuestos polares. En concreto, la LC acoplada a espectrometría de masas en tandem (MS/MS) es una de las técnicas analíticas más utilizadas para la determinación de estos compuestos mediante biomonitorización **(Yusà et al., 2015; Mercogliano & Santonicola, 2018)**. El desarrollo de la 'ultrahigh performance liquid cromatography' (UHPLC) permite la reducción de la anchura de pico e incrementa la resolución y la sensibilidad siempre que se disponga de detectores capaces de realizar barridos o 'scans' en intervalos cortos de tiempo **(Andra et al., 2017)**. En la

fuente de ionización, en la mayoría de los casos estos analitos se ionizan en modo negativo, debido a que los biomarcadores estudiados (bisfenoles, metabolitos de ftalatos, parabenos, y la mayoría de metabolitos de plaguicidas) presentan grupos funcionales que pueden reducirse y liberar grupos H^+ con facilidad. Respecto al modo de ionización, la electronebulización (ESI) es la más técnica más utilizada **(Roca et al., 2014b; 28, Rodríguez-Gómez., et al 2014b; 36, Rodríguez-Gómez., et al 2015; Sabaredzovic et al., 2015**). Sin embargo, la 'atmospheric presure chemical ionization' (APCI) también ha sido utilizada con éxito para la determinación de bisfenoles **(Ye et al., 2006b)**.

La determinación se realiza fundamentalmente en modo masas en tandem (MS/MS) mediante el uso de la espectrometría de masas de triple cuadrupolo (QqQ). La principal ventaja de esta técnica es su selectividad y sensibilidad. En la literatura se pueden encontrar numerosos ejemplos de su uso en la determinación de diferentes biomarcadores de plaguicidas, ftalatos, bisfenoles y parabenos en orina y/o leche materna **(Rodríguez-Gómez et al., 2014b; Jeong et al., 2011; Roca et al., 2014a)**.

Sin embargo, en los últimos años el campo de la espectrometría de masas de alta resolución (HRMS) ha experimentado un gran desarrollo. La principal ventaja que ofrece la HRMS es la capacidad de adquirir en modo 'full scan' con una elevada resolución y exactitud de masa **(Hernández et al., 2012)**. Estos aspectos operacionales han permitido que la HRMS sea una herramienta muy potente capaz de almacenar la información relativa a gran parte de los analitos ionizables de una muestra en un solo análisis y posibilitar el análisis retrospectivo de analitos no buscados en el análisis 'target' inicial. Esta estrategia ha sido utilizada en diferentes aplicaciones como la detección de medicamentos humanos y/o veterinarios en aguas **(Hernández et al., 2011)** y en piensos **(León et al., 2016)**; o la detección de plaguicidas en aire **(López et al., 2016)** y en orina **(Roca et al., 2014b)**. Hay diversos tipos de espectrómetros de masas de alta resolución, entre los que destacan los espectrómetros de tiempo de vuelo (TOF), los espectrómetros de resonancia iónica ciclotrónica por transformada de Fourier (FT-ICR MS) y el espectrómetro de masas tipo Orbitrap **(Marshall & Hendrickson, 2008; Andra et al., 2017; Makarov et al., 2009)**.

Existen varios ejemplos del uso de LC-HRMS en la determinación de contaminantes oránicos (y/o sus metabolitos) en muestras biológicas **(Roca et al., 2014b; Cortejade et al., 2016).** Además, recientemente se han desarrollado equipos híbridos en los que además del analizador/detector se incorpora un cuadrupolo (Q-Orbitrap o Q-TOF) y ya se están aplicando en el análisis de contaminantes ambientales **(Blanco-Zubiaguirre et al., 2020)**. En la presente tesis se ha empleado en algunos trabajos un equipo de UHPLC-HRMS tipo Orbitrap.

2.3.2.1. Espectrómetro de masas de alta resolución Orbitrap

En la **Figura 11** se presenta un esquema de un espectrómetro de masas tipo Orbitrap (Exactive^{TM}). El funcionamiento general del equipo se describe a continuación: Tras una

separación de los analitos mediante LC, se forman los iones en la fuente de ionización y, mediante una serie de lentes y multipolos, los iones entran en la C-trap (trampa de iones curvada) donde son almacenados hasta que entran en grupos de iones al Orbitrap. Dentro del Orbitrap, los iones giran orbitalmente alrededor del electrodo central del analizador de masas. En función de la frecuencia de la oscilación armónica axial que presentan y mediante una transformada de Fourier, se determina su valor m/z. Opcionalmente, los iones pueden ser fragmentados en la fuente de ionización ('Collision Induced Dissociation', CID) o pasar por una celda de colisión 'Higher Energy Collision Induced Dissociation' (HCD), donde son fragmentados debido a la aplicación de un voltaje y dando lugar a fragmentos que podrán ser analizados posteriormente en el Orbitrap **(Exactive, 2010).**

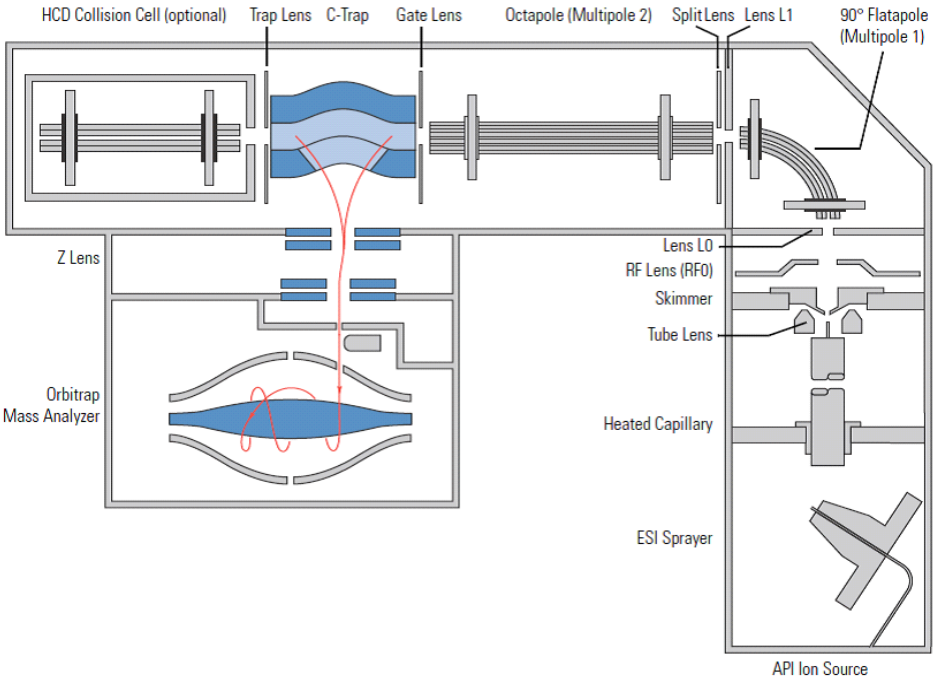


Figura 11. Esquema Orbitrap Exactive™ **(Exactive, 2010)**.

En la **Figura 12** se representa el analizador de masas tipo Orbitrap. Como se aprecia en la figura, está formado por dos electrodos: un electrodo central, denominado 'spindle' (eje) alrededor del cual los iones se mueven en espiral; y otro electrodo externo que está dividido por la mitad por un anillo cerámico aislante (**Exactive, 2010)**.

Desde la C-trap, los paquetes o grupos de iones de cada rango de m/z, viajan tangencialmente hacia el Orbitrap. Para mantener a los iones en su interior, el Orbitrap no usa radiofrecuencias o un campo magnético sino que los iones que se mueven alrededor del Orbitrap son atrapados en un campo electrostático creado por el 'spindle' y por los electrodos externos. La atracción electrostática hacia el electrodo central es

compensada por la fuerza centrífuga que se origina por la velocidad tangencial inicial de los iones. Axialmente, los iones son dirigidos desde las zonas externas del Orbitrap más estrechas hacia la zona interna más ancha, lo cual provoca oscilaciones axiales sin necesidad de una excitación adicional, es por esta razón que el Orbitrap tiene esa forma característica. La frecuencia de rotación de cada valor de m/z depende de parámetros como la energía, la posición y el ángulo de los iones (**Perry et al., 2008; Exactive, 2010)**.

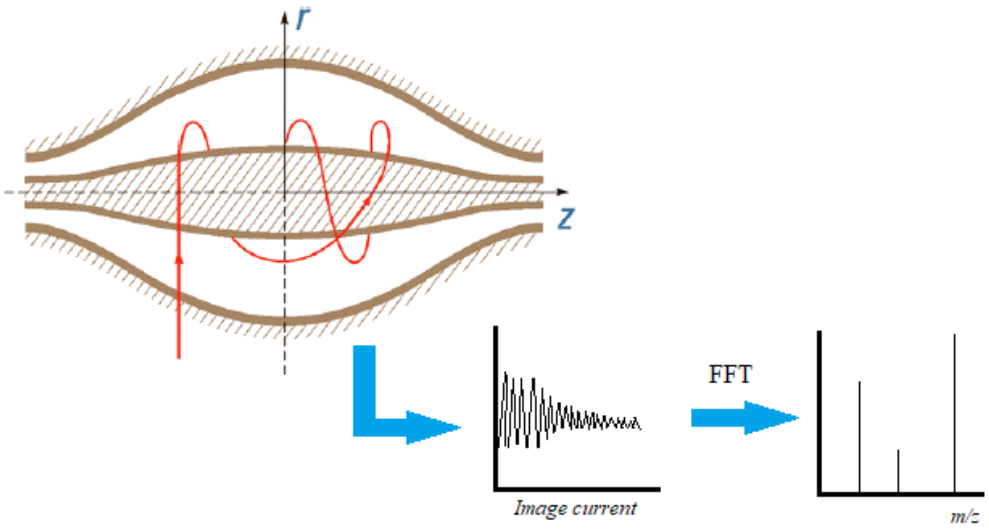


Figura 12. Visión esquemática de un analizador Orbitrap **(**adaptado de **Exactive, 2010)**

Como se puede observar en la **Figura 12**, las trayectorias de un ion combinan la ya mencionada rotación alrededor del electrodo central con oscilaciones armónicas a lo largo de él dependientes del componente axial. La frecuencia ω de estas oscilaciones armónicas a lo largo del eje z es independiente de la energía, del ángulo y de la posición inicial del ión, por lo que depende únicamente del cociente masa carga (m/q ó m/z) del ion y de la constante instrumental k. Esta frecuencia se transforma mediante una Transformada de Fourier Rápida (FFT) en su valor de m/z correspondiente, con gran precisión y alta resolución, tal y como describe la siguiente ecuación (**Perry et al., 2008; Exactive, 2010; Makarov et al., 2009)**

$$\omega_z = \sqrt{\frac{k}{m/q}}$$

En el Orbitrap, la resolución es directamente proporcional al tiempo de adquisición, así a mayor tiempo de adquisición, mayor es la resolución. Se debe llegar a un compromiso entre el tiempo de adquisición necesario y la resolución deseada **(Makarov & Scigelova, 2010)**. Además, la resolución decrece conforme aumenta la masa.

2.3.2.2. Estrategias analíticas en la biomonitorización humana

Debido a la compleja naturaleza del exposoma, se requieren diferentes estrategias o enfoques para conseguir desentrañar el conjunto del espectro completo de exposiciones de interés a lo largo de la vida de un individuo. Dentro del estudio del exposoma químico mediante BH, las técnicas analíticas están basadas en la espectrometría de masas, tal y como hemos visto anteriormente, y se pueden utilizar tres estrategias analíticas: análisis 'target', análisis de sospechosos y análisis de desconocidos **(Xue et al., 2019)**.

El análisis 'target' es la estrategia de análisis clásica en el que se dispone de información de los analitos diana y de los patrones de los mismos **(Andra et al., 2017)**. Este tipo de enfoque es el más utilizado en los programas actuales de BH, y es útil para conocer la exposición de la población a los contaminantes más comunes y conocidos. Sin embargo, por sí solo no permite conocer la totalidad del exposoma químico ya que generalmente son técnicas enfocadas a la determinación de una familia de compuestos químicos en concreto **(Dennis et al., 2017)**. Esta estrategia puede ser llevada a cabo tanto en equipos de baja resolución, como el QqQ, como en equipos de HRMS. El análisis mediante equipos de alta resolución presenta algunas ventajas en el caso de analitos difíciles de fragmentar, ya que permiten la identificación del mismo gracias a la información de la masa monoisotópica y del perfil isotópico **(Xue et al., 2019; Andra et al., 2017)**. Además, la posibilidad de adquirir en 'full scan' permite realizar un análisis retrospectivo reprocesando los datos **(Roca et al., 2014b)**. Los equipos híbridos (MS/MS-HRMS) aumentan las capacidades y posibilidades del análisis **(Xue et al., 2019).**

El análisis de sospechosos se usa cuando se conoce información de los compuestos a determinar (formula molecular, estructura química, propiedades fisicoquímicas, fragmentos, etc…) pero no se dispone de patrones. En estos casos, el uso de equipos de HRMS es imprescindible ya que permiten adquirir en 'full scan' y medir la masa monoisotópica y el perfil isotópico con elevada exactitud (**Xue et al., 2019)** Este enfoque permite aumentar el número de analitos identificados utilizando largas bases de datos de biomarcadores propias o disponibles en la bibliografía, sin embargo, no permite la cuantificación exacta de los analitos detectados ni la identificación inequívoca de compuestos sin la adquisición de patrones **(Bocato et al., 2019; Roca et al., 2014b; Plassmann et al., 2015).**

El análisis de desconocidos se aplica cuando la búsqueda se lleva a cabo sin información previa acerca de los compuestos que se van a detectar. El análisis de desconocidos hipotéticamente permite la medición de un número ilimitado de compuestos en una muestra mediante la adquisición con equipos de HRMS, convirtiéndolo en una técnica prometedora para el estudio del exposoma **(Xue et al., 2019)**. Se debe que tener en cuenta que tanto en el análisis de sospechosos como en el análisis de desconocidos, los resultados están condicionados por el tipo de tratamiento de muestra utilizado, el tipo de cromatografía y el tipo de ionización. No existe una metodología capaz de detectar la totalidad de los contaminantes de una muestra **(Andra et al., 2017)**. Sin embargo, el desarrollo de herramientas bioinformáticas ligadas a la mejora en las prestaciones de los

equipos analíticos permitirá avanzar en el estudio del exposoma mediante correlaciones entre los datos analíticos obtenidos e información acerca del desarrollo de enfermedades u otras características de los participantes **(Dennis et al., 2017)**.

2.4. Evaluación del riesgo mediante biomonitorización humana

La evaluación del riesgo tiene como objetivo estimar el riesgo derivado de la exposición a un contaminante o grupo de contaminantes en una población. La evaluación del riesgo forma parte de la metodología del análisis del riesgo. Se trata de un proceso que comprende varios pasos entre los que se encuentran la evaluación de la exposición y la caracterización del riesgo **(IPCS, 2004)**. En la **Figura 13** se muestra un esquema de la evaluación del riesgo en seguridad alimentaria en el que se detallan los dos posibles enfoques, exposición externa y exposición interna.

La evaluación de la exposición en seguridad alimentaria se ha llevado a cabo tradicionalmente a través de la exposición externa, combinando los datos de niveles de los contaminantes en los alimentos y los datos de frecuencia de consumo de los mismos para conocer la exposición diaria a los contaminantes. Estos datos son comparados con valores guía en alimentos como la ingesta diaria admisible (ADI), la ingesta diaria tolerable (TDI) y la dosis de referencia (RfD) y tienen definiciones similares. La ADI se define como la estimación de la cantidad máxima de un agente, expresada en base a la masa del cuerpo a la que los miembros de una población o subpoblación pueden estar expuestos diariamente de por vida sin que conlleve un riesgo para la salud aparente. La ADI se refiere a agentes que se añaden intencionadamente a los alimentos. La TDI es un valor muy similar a la ADI pero para contaminantes y la RfD se define como una estimación de la dosis de exposición diaria que aparentemente no tiene efectos perjudiciales aunque la exposición se mantenga a lo largo de toda la vida **(Marín, 2014; IPCS, 2004)**.

La información de la exposición interna a contaminantes que proporciona la BH permite llevar a cabo la evaluación del riesgo a contaminantes de manera integrada, aunando la exposición proveniente de las diferentes rutas y fuentes de exposición. Sin embargo, para llevar a cabo una adecuada evaluación del riesgo mediante BH es necesario conocer correctamente la toxicocinética de los contaminantes a estudiar y seleccionar los biomarcadores y matrices más adecuados para llevar a cabo la evaluación del riesgo **(Angerer et al., 2011)**.

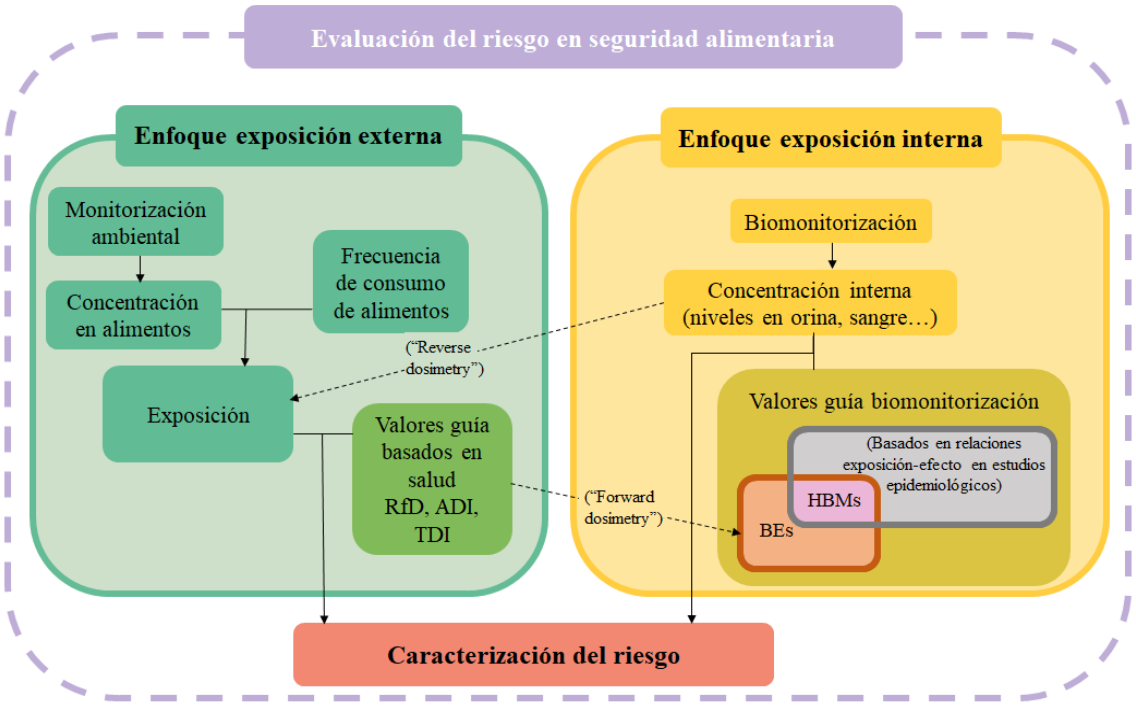


Figura 13. Evaluación del riesgo en seguridad alimentaria.

Como se muestra en la **Figura 13**, la evaluación del riesgo mediante BH puede realizarse comparando los niveles internos con valores guía para biomonitorizión o estimando la ingesta a partir de los niveles internos mediante 'reverse dosimetry' (RD) **(Zidek et al., 2017)**. A su vez, los valores guía para BH se pueden establecer de dos maneras: se pueden estimar en base a relaciones exposición efecto en estudios epidemiológicos como en el caso de algunos valores guía del 'German Human Biomonitoring' (HBM-I y HBM-II), o se pueden estimar a partir de valores guía de ingesta mediante 'forward dosimetry' (FD). Para estimar valores guía en BH mediante FD, se parte de un valor guía de ingesta basado en salud, como las ADI o TDI, y teniendo en cuenta los procesos toxicocinéticos del compuesto en el organismo, se estiman los niveles del biomarcador en una matriz compuesta que equivalen al valor guía de ingesta del que se partía. Este procedimiento es el que se utiliza para establecer los 'Biomonitoring Equivalents' (BEs) y algunos valores HBM-I y HBM-II **(Angerer et al., 2011; Zidek et al., 2017)**.

Los BEs se definen como la concentración de un compuesto químico o metabolito en un medio biológico que equivale a un valor de referencia de exposición existente, como la TDI o la RfD. Los BEs pueden ser utilizados como valores guía para evaluar los datos derivados de la BH e identificar compuestos químicos que se encuentran por debajo, cerca o por encima de estos valores **(Hays & Aylward, 2009)**. Se han establecido BEs para alrededor de 50 contaminantes y metabolitos. La mayoría están establecidos en sangre y orina. Sin embargo, para algunos analitos también existen BEs en otras matrices biológicas como el suero y el plasma sanguíneos o la leche materna **(Steckling et al., 2018)**. Es importante resaltar que los BEs no tienen un valor diagnóstico, no deben usarse para interpretar datos a nivel individual y no marcan un límite definido entre seguridad y

riesgo. Sin embargo, sí que sirven para priorizar las sustancias sobre las que se debe llevar a cabo una evaluación del riesgo más exhaustiva **(Hays & Aylward, 2009)**. En la **Figura 14** se indica como utilizar los BEs en la toma de decisiones. Si los niveles del biomarcador en estudio no superan el BE, se considera que no son sustancias prioritarias para la salud pública. Sin embargo, si superan el BE, estas sustancias deben considerarse prioritarias para organismos de salud pública y se deben llevar a cabo más estudios para evaluar el riesgo **(Hays & Aylward, 2012)**.

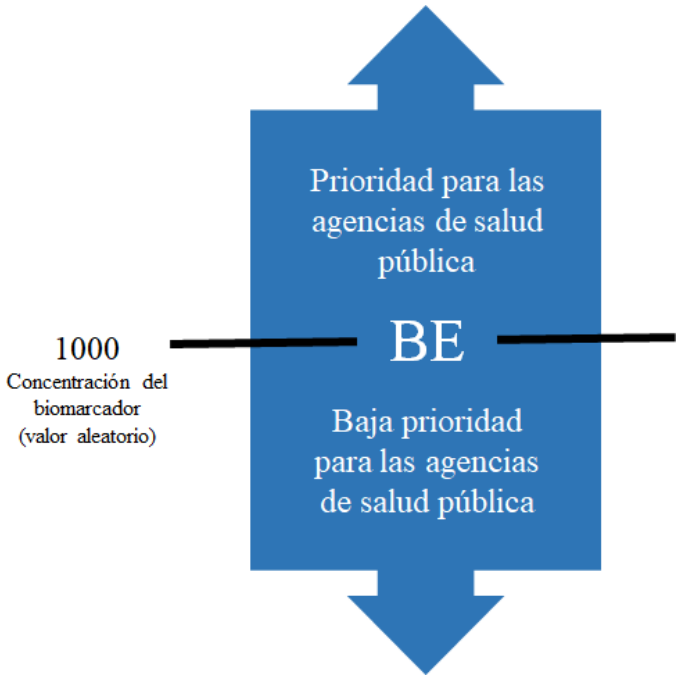


Figura 14. Empleo de los BEs para la toma de decisiones en salud pública **(**adaptado de **Hays & Aylward, 2012)**.

Los valores guía HBM-I y HBM-II son valores establecidos por la 'German Human Biomonitoring Comission' y se determinan en base a estudios epidemiológicos o se derivan de valores guía como la ADI o la TDI **(Angerer et al., 2011)**. Los valores HBM se establecen para toda la población o para subgrupos en el caso de que haya recomendaciones para grupos vulnerables. El HBM-I representa la concentración de una sustancia en una matriz biológica humana a la cual y por debajo de la cual no hay riesgo de que haya efectos sobre la salud y por lo tanto no es necesario actuar. Los valores HBM-II describen la concentración de una sustancia en una matriz biológica a la cual y por encima de la cual la aparición de efectos adversos sobre la salud es posible y por lo tanto es necesario reducir la exposición. Para niveles entre HBM-I y HBM-II no se pueden descartar efectos adversos sobre la salud y se debe llevar a cabo un seguimiento para determinar si existe una exposición elevada. Si al repetir las medidas se repiten los niveles, se recomienda buscar la posible fuente de exposición al contaminante y reducir o eliminar la exposición **(Apel et al., 2017)**. Los valores HBM-II solo se determinan cuando se calculan en base a estudios epidemiológicos **(Angerer et al., 2011)**. Existen alrededor de 20 compuestos y/o metabolitos para los cuales se ha establecido al menos un valor HBM-I en orina, sangre, suero y/o plasma **(Apel et al., 2017)**.

En el caso de compuestos para los que no existen valores guía de BH, se puede estudiar la caracterización del riesgo aplicando la RD, de manera que la concentración interna de un biomarcador se puede utilizar para estimar la ingesta del contaminate y, por tanto, compararla con valores guía de ingesta como ADI o TDI. Para llevar a cabo esta estrategia es necesario conocer los datos toxicocinéticos del biomarcador **(Zidek et al., 2017; Katsikantami et al., 2019)**.

Para muchos compuestos no existen valores guía HBM o BEs en leche materna **(Steckling et al., 2018)**, ni existen datos toxicocinéticos de distribución en esta matriz, por lo que es difícil llevar a cabo una evaluación del riesgo para las madres en base a la concentración de algunos biomarcadores en leche. Sin embargo, con los datos la ingesta diaria de leche materna por parte de los recién nacidos se puede estimar la ingesta diaria de contaminantes a través de la lactancia y, por tanto, se puede comparar con valores guía como el ADI o el TDI y llevar a cabo una evaluación del riesgo en los recién nacidos **(Schlumpf et al., 2010)**.

2.5. Contaminantes estudiados en la presente tesis

En la presente tesis se estudiaron los niveles de biomarcadores de ftalatos y plaguicidas polares en orina y de bisfenoles y parabenos en leche materna.

2.5.1. Ftalatos

Los ftalatos son compuestos químicos producidos en grandes cantidades a nivel mundial que se usan como plastificantes en un gran número de aplicaciones **(ECHA, 2019)**. Sus propiedades y usos dependen de sus cadenas alquílicas. Los ftalatos de cadena larga como el di-2-etilhexil ftalato (DEHP) y el diisonoil ftalato (DiNP) son principalmente usados en materiales de policloruro de vinilo (PVC) como envases en contacto con alimentos, recubrimientos del suelo, ropa y juguetes. Por otro lado, los ftalatos de cadena corta como el dimetil ftalato (DMP), el dietil ftalato (DEPHT), el benzilbutil ftalato (BzBP) y el diisobutil ftalato (DiBP) también son usados en productos cosméticos, pinturas o el recubrimiento de cápsulas **(Wittassek et al., 2011)**. En la Unión Europea, el uso de ftalatos está regulado en materiales en contacto con alimentos **(Comission Directive EC, 2007)**, juguetes y artículos destinados a niños que puedan ser introducidos en la boca **(Regulation EC, 2006)**, cosméticos **(Regulation EC, 2009)**, medicinas **(EMA, 2014)** y en productos sanitarios **(Directive EC, 2007)**.

Como los ftalatos no están unidos covalentemente al material, pasan con facilidad desde los productos que lo contienen al aire, alimentos, agua y polvo. Como resultado, la población general está continuamente expuesta a ftalatos a través de la ingestión, inhalación y/o la exposición dérmica **(Weiss et al., 2018)**. Después de entrar en el organismo, los ftalatos se metabolizan. Durante el primer paso metabólico, se hidroliza una de las cadenas de los diésteres formando monoesteres. Además, son frecuentes otros

tipos de transformaciones como la oxidación o la hidroxilación de los monoésteres de ftalatos de cadena larga como el DEHP. Durante el metabolismo de fase II, los metabolitos de ftalatos se conjugan con grupos glucurónido. Un gran porcentaje de la dosis absorbida de ftalatos se excreta durante las primeras 24h ya sea como metabolitos libres o conjugados **(Frederiksen et al., 2007; Wittassek et al., 2011)**. Algunos de los metabolitos urinarios de ftalatos más comunes se muestran en la **Tabla 3**.

Existen diferentes estudios que han evaluado la toxicidad de los ftalatos y han concluido que tienen efectos tóxicos sobre la reproducción y el desarrollo en animales. Además, son considerados como posibles disruptores endocrinos en humanos **(Heudorf et al., 2007)**. Se considera que a los niveles a los que está expuesta la población general, el riesgo de provocar alteraciones elevadas en el sistema reproductivo masculino es débil. Sin embargo, la evidencia de que puede afectar a la calidad del semen es moderada **(Kay et al., 2014)**. Con respecto al neurodesarrollo, la exposición prenatal a ftalatos se asocia negativamente con el comportamiento y el desarrollo cognitivo de los niños (p. ej. menor cociente intelectual, problemas de la atención, hiperactividad y peor comunicación social) **(Ejaredar et al., 2015)**.

La BH es la mejor manera de evaluar la exposición global a ftalatos. Estos han sido determinados en varias matrices biológicas (orina, sangre, leche materna…). Sin embargo, en la mayoría de los casos la orina ha sido la matriz seleccionada para estudiar la exposición interna a ftalatos **(Wittassek et al, 2011)**. Los niveles de metabolitos de ftalatos en orina reflejan una exposición reciente y en general se detectan a niveles traza. Como ejemplo, los últimos resultados publicados por el NHANES en la población estadounidense entre los años 2013 y 2014 muestran medias geométricas de los metabolitos de ftalatos más comúnes en un rango de entre 1,36 ng/mL (MEHP) y 35,7 ng/mL (MEP) **(NHANES, 2019)**.

Tabla 3. Ftalatos y sus metabolitos **(NHANES, 2009; Frederiksen et al., 2007)**

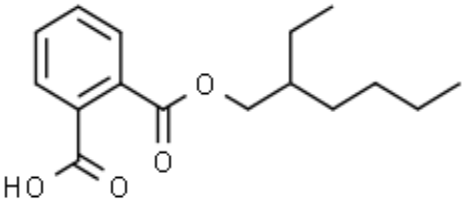
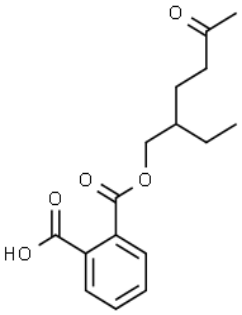
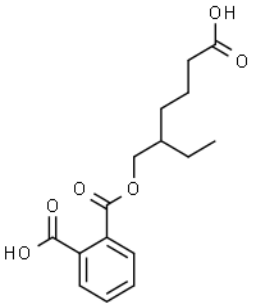
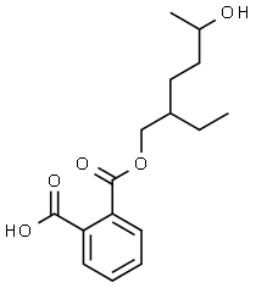
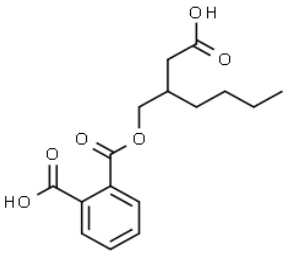
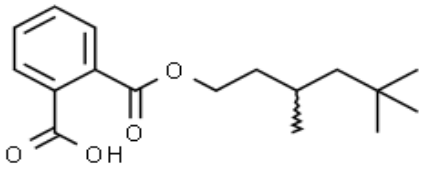
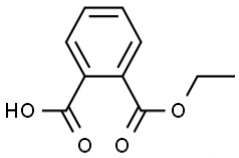
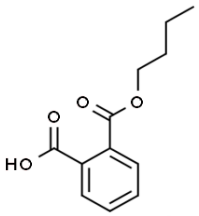
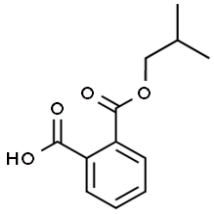
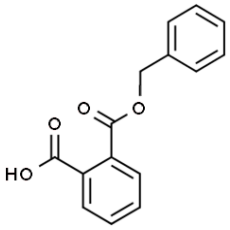
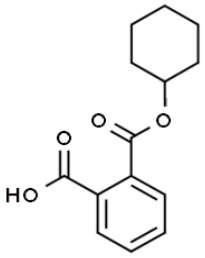
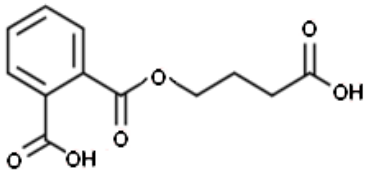
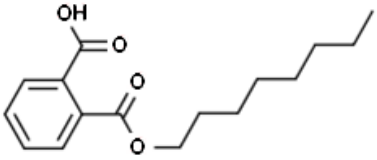
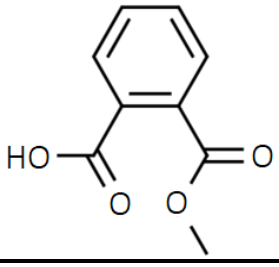
Ftalato (Acrónimo)	**Metabolito (Acrónimo)**	**Estructura**
Di-2-etilhexil ftalato (DEHP)	Mono-2-etilhexil ftalato (MEHP)	
	Mono-(2-etil-5-oxohexil) ftalato (MEOHP)	
	Mono-(2-etil-5-carboxipentil) ftalato (MECPP)	
	Mono-(2-etil-5-hidroxihexil) ftalato (MEHHP)	
	Mono[2-(carboximetil)hexil] ftalato (2cx-MMHP)	
Di-isononil ftalato (DiNP)	Mono-isononil ftalato (MiNP)	

Tabla 3. (Continuación)

Ftalato (Acrónimo)	Metabolito (Acrónimo)	Estructura
Dietil ftalato (DEPHT)	Mono-etil ftalato (MEP)	
Di-n-butil ftalato (DBP)	Mono-n-butil ftalato (MnBP)	
Di-isobutil ftalato (DiBP)	Mono-isobutil ftalato (MiBP)	
Benzilbutil ftalato (BzBP)	Mono-benzil ftalato (MBzP)	
Diciclohexil ftalato (DCP)	Mono-ciclohexil ftalato (MCHP)	
Di-(n-octil) ftalato (DOP)	Mono-(3-carboxipropil) ftalato (MCPP)	
	Mono-n-octil ftalato (MOP)	
Dimetil ftalato (DMP)	Mono-metil ftalato (MMP)	

En España, se han estudiado niveles de metabolitos de ftalatos en mujeres embarazadas, mujeres y niños, cuyas muestras se han recogido entre 2004 y 2012. Para la mayoría de los metabolitos estudiados la frecuencia de detección (DF) fue mayor del 80% y el metabolito que se encontró a mayores niveles fue el MEP con medias geométricas que oscilaban entre 34.9 y 336 ng/mL en función del estudio **(Casas et al 2016; Cutanda et al., 2015; Casas et al 2011)**.

Existen valores guía para BH (tanto valores BE como HBM) que permiten llevar a cabo una estimación de la evaluación del riesgo a partir de los niveles de metabolitos de ftalatos en orina (ver **Tabla 4**).

Tabla 4. Valores guía para BH (BE y HBM-I) para ftalatos en orina

Ftalato	BE					HBM I	Referencia
	Derivado del TDI de Health Canada	Derivado de la RfD de US EPA	Derivado del MRL crónico de ATSDR	Derivado del MRL intermedio de ATSDR	Derivado de la TDI de EFSA		
DEHP (como la suma de cinco metabolitos: MEHP, MEHHP, MEOHP, 5cx-MEPP, y 2cx-MMHP) ng/mL (µg/g creatinina)	1000 (1300)	430 (550)	1300 (1700)	3200 (4100)	1100 (1400)	-	**(Aylward et al., 2009ª)**
DEHP (como la suma de dos metabolitos: MEHHP yMEOHP) ng/mL	-	-	-	-	-	300*	**(Schulz et al., 2012)**
DEPHT (como MEP) ng/mL (µg/g creatinina)	-	18000 (23000)	-	-	-	-	**(Aylward et al., 2009b)**
BzBP (como MBzP) ng/mL (µg/g creatinina)	31000 (40000)	3800 (4900)	-	-	12000 (15000)	-	**(Aylward et al., 2009b)**
DnBP (como MnBP) ng/mL (µg/g creatinina)	1400 (1800)	2700 (3500)	-	-	200 (280)	-	**(Aylward et al., 2009b)**
DiNP (como MiNP) ng/mL	-	-	-	-	0,5**	-	**(Hays et al., 2011)**

*Para mujeres en edad reproductiva

**Para mujeres >16 años

2.5.2. Plaguicidas polares

El uso de plaguicidas ha mejorado la productividad de la agricultura y ha reducido la propagación de enfermedades transmitidas por insectos alrededor del mundo. Tras la prohibición o restricción de los plaguicidas persistentes, estos han sido sustituidos por plaguicidas más polares como los organofosforados, los carbamatos o los piretroides. Sin embargo, el uso intensivo de esos compuestos puede tener un impacto sobre la salud pública **(González-Alzaga, 2015)**. Existen varias familias de plaguicidas polares utilizadas como insecticidas, herbicidas o fungicidas en la agricultura y/o en los hogares **(Yusa et al., 2015)**. En la **Tabla 5** se muestran algunos de los biomarcadores de plaguicidas polares más estudiados en orina humana.

Hay evidencias de efectos carcinogénicos, neurológicos, reproductivos, inmunológicos y genotóxicos asociados a la exposición con plaguicidas no persistentes en adultos **(Koureas et al., 2012)**. Además, varios estudios han establecido que la exposición a plaguicidas puede provocar efectos adversos sobre la reproducción **(Rauch et al., 2012)** y problemas en el neurodesarrollo en niños **(Marks et al., 2010; 5, Rauh et al., 2012)**. La exposición a estos compuestos es principalmente debida a la ingesta de agua y alimentos como frutas y vegetales contaminados, así como por inhalación en zonas cercanas a la aplicación de plaguicidas **(Sang et al., 2020; López et al., 2016)**.

Para estudiar la exposición interna a plaguicidas no-persistentes con semividas cortas en el organismo, la orina es la matriz más adecuada. Los niveles de metabolitos de plaguicidas en orina proporciona información acerca de una exposición reciente **(Roca et al., 2014a**). Sin embargo, también se han realizado estudios en sangre, plasma, suero, pelo y otras matrices. En la mayoría de los casos los biomarcadores de exposición son los metabolitos de los compuestos originales **(Yusa et al., 2015)**.

Tabla 5. Biomarcadores de plaguicidas polares en orina humana **(Roca et al., 2014b)**.

Familia	Plaguicida	Biomarcador	Acrónimo
Organofosforados	Chlorpirifós, chlorpirifós-metil	3,5,6-Thrichloro-2-pyridinol	TCPy
	Paratión, metil paratión	p-nitrophenol	PNP
	Pirimifos-metil	2-Diethylamino-6-methyl-6-hydroxypyrimidine	DEAMPY
	Diazinon	2-Isopropyl-4-methyl-6-hydroxypyrimidine	IMPY
	Coumafós	3-Chloro-7-hydroxy-4-methylcoumarin	CMHC
	Fenitrotión	3-Methyl-4-nitrophenol	MNP
	Dimetoato	Dimetoato	DIMET
	Ometoato	Ometoato	OMET
	Acefato	Acefato	AP
	Metamidofós	Metamidofós	Metamidofós
	Cloretoxifós, clorpirifós, coumafós, diazinón, disulfotón, etión, paratión, forato, fosalón, sulfotep, terbufós, azinfos-metil, diclorvós, dicrotofós, dimetoato, fenitrotión, fentión, malatión, metil paratión, triclorfón, clorpirifos-metil, metidatión, mevinphos, oxidemetón-metil, fosmet, pirimifos-metil, temefós, tetraclorvinfós, naled.	Diethyl phosphate	DEP
		Diethyl thiophosphate	DETP
		Dimethyl thiophosphate	DMTP
		Dimethyl dithiophosphate	DMDTP
Herbicidas tipo fenoxi	Ácido 2,4-diclorofenoxiacético	Ácido 2,4-diclorofenoxiacético	2,4-D
	Ácido 2,4,5-triclorofenoxiacético	Ácido 2,4,5-triclorofenoxiacético	2,4,5-T
Herbicidas tipo cloroacetanilida	Atrazina	Atrazine mercapturate	ATZM
	Alachlor	Alachlor mercapturate	ALAM
	Metolaclor	Metolachlor mercapturate	METM
Piretroides	Piretroides comerciales	3-Phenoxybenzoic acid	PBA
	Ciflutrina	4-Fluoro-3-phenoxybenzoic acid	FPBA
	Permetrina, cipermetrina, Ciflutrina	cis-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid	cis-DCCA
		trans-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid	trans-DCCA
	Deltametrina	cis-(2,2-Dibromovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid	DBCA

2.5.3. Bisfenoles

El BPA es un compuesto químico producido a gran escala y ampliamente usado en materiales en contacto con alimentos y bebidas hechos de plásticos tipo policarbonato, en resinas epoxi usadas en el recubrimiento interno de las latas de conserva, así como en papeles térmicos **(Mercogliano & Santonicola, 2018)**.

Se considera que la dieta es la principal fuente de exposición al BPA para la población general, aunque también existen otras fuentes que pueden contribuir a la exposición global como el papel térmico o el contacto con juguetes **(Geens et al., 2012)**. Según la EFSA, la comida enlatada (50%) y los productos cárnicos no enlatados (20%) fueron las principales fuentes de exposición a BPA en la población general en Europa. En el caso de los niños, las fórmulas infantiles constituyeron entre el 25 y el 37% de la exposición total **(EFSA, 2015)**. Una normativa europea reciente **(EU, 2018)** ha intensificido las restricciones en el uso de BPA en materiales en contacto con alimentos. Esta normativa establece que la migración no deben superar el límite de 0,05 mg de BPA por kg de alimento, prohíbe el uso de BPA en biberones y establece que no se permite la migración de BPA desde los recubrimientos aplicados a materiales en contacto con alimentos destinados para niños menores de 3 años.

En una reciente opinión científica de EFSA **(EFSA, 2015)**: a) se identifica la exposición a BPA con efectos adversos sobre el riñón y el hígado; b) establece que no hay suficiente evidencia científica para correlacionar la exposición a BPA a bajas dosis con alteraciones en el sistema reproductivo; c) indica que la exposición prenatal a BPA puede estar asociada con efectos sobre el neurodesarrollo, sin embargo, señala que no hay suficiente evidencia científica como para confirmarlo.

Así mismo, varios estudios han concluido que el BPA puede interactuar con el sistema endocrino (ej: receptores de estrógenos), pero no se puede asegurar que los efectos observados puedan ocurrir a las concentraciones a las que la población está expuesta ni si esta exposición puede dar lugar a efectos adversos sobre la salud **(WHO, 2010)**. En base a los datos toxicológicos, se ha establecido una TDI temporal (t-TDI) de 4µg /Kg peso corporal (pc) día para la exposición oral a BPA **(EFSA, 2015)**.

Las restricciones en el uso de BPA han estimulado a los fabricantes a emplear compuestos alternativos para sustituir al BPA. Entre ellos, el BPF y el BPS son los más usados **(Chen et al., 2016; Liao et al., 2021a)**. Sin embargo, existen estudios recientes que muestran que el BPF y el BPS presentan efectos sobre el sistema endocrino similares a los del BPA **(Rochester & Bolden, 2015)**. Además, el proyecto europeo HBM4EU ha incluido al BPF y al BPS, junto con el BPA, en el listado de sustancias prioritarias para ser analizadas en estudios de biomonitorización **(HBM4EU, 2017)**. Las estructuras del BPA, BPF y BPS se muestran en la **Figura 15**.

Tras la ingesta oral, el BPA es metabolizado en el hígado para formar el conjugado con glucurónido. En humanos, el BPA tiene una semivida de menos de 6h y se excreta en orina principalmente en su forma conjugada **(Völkel et al., 2002)**. Sin embargo, se ha

encontrado BPA libre en varias muestras biológicas, indicando que la población está expuesta internamente a formas estrogénicamente activas de BPA **(Vandenberg et al., 2010)**.

En las madres lactantes, la porción absorbida de BPA se transfiere rápidamente a la leche materna, por lo que el BPA en leche se considera un biomarcador de exposición reciente **(Migeot, 2013)**. Se han observado altas concentraciones de BPA en la leche horas después de su consumo **(Tateoka et al., 2015)**. Sin embargo, los niveles de BPA en calostro reflejan la exposición acumulada durante la segunda mitad de la gestación. **(Migeot, 2013)**.

El BPA se encuentra en leche materna tanto libre como conjugado **(Cao et al., 2015)**. Sin embargo, el BPA conjugado no posee ninguna actividad estrogénica **(Migeot, 2013)**; en consecuencia, los niveles de BPA libres en leche son más adecuados para llevar a cabo la evaluación del riesgo de los niños lactantes. Un comité de expertos de FAO/WHO **(WHO, 2010)** estimó que la ingesta de BPA de los recién nacidos que se alimentaban exclusivamente mediante lactancia (0-6 meses de edad) era de 0.3 y 1.3 µg/kg peso corporal por día (media y percentil 95, respectivamente).

Mercogliano & Santonicola **(2018)** llevaron a cabo una revisión de niveles de BPA en leche materna desde 2003. Sin embargo, la mayoría de los estudios solo analizaron un número reducido de muestras ($n \leq 30$).

El metabolismo y la distribución del BPF y el BPS en humanos no ha sido tan estudiado como el del BPA, sin embargo, algunos experimentos sugieren que el metabolismo del BPF y el BPS es similar al del BPA **(Rochester & Bolden 2015)**. Se han llevado a cabo varios estudios para determinar los niveles en orina de BPF y BPS **(Liao et al. 2012b; Yang et al. 2014; Zhou et al. 2014)**. No obstante, muy pocos han estudiado la presencia de estos bisfenoles en leche materna y, además, analizaron un número reducido de muestras ($n \leq 30$) **(Deceunink et al., 2015; Niu et al., 2017)**.

Debido a que los bisfenoles, en especial el BPA, son contaminantes ubicuos, se debe tener especial cuidado durante el muestreo, tratamiento de muestra y análisis para la determinación de estas sustancias en estudios de BH **(Ye et al., 2013)**. Los sacaleches y recipientes de conservación de la muestra pueden ser una fuente de contaminación a BPA **(Arbuckle et al., 2015; Mendonca et al., 2014)**. Además, se recomienda verificar que el material de polipropileno usado durante la preparación y el análisis de las muestras está libre de BPA **(Arbuckle et al. 2015)**.

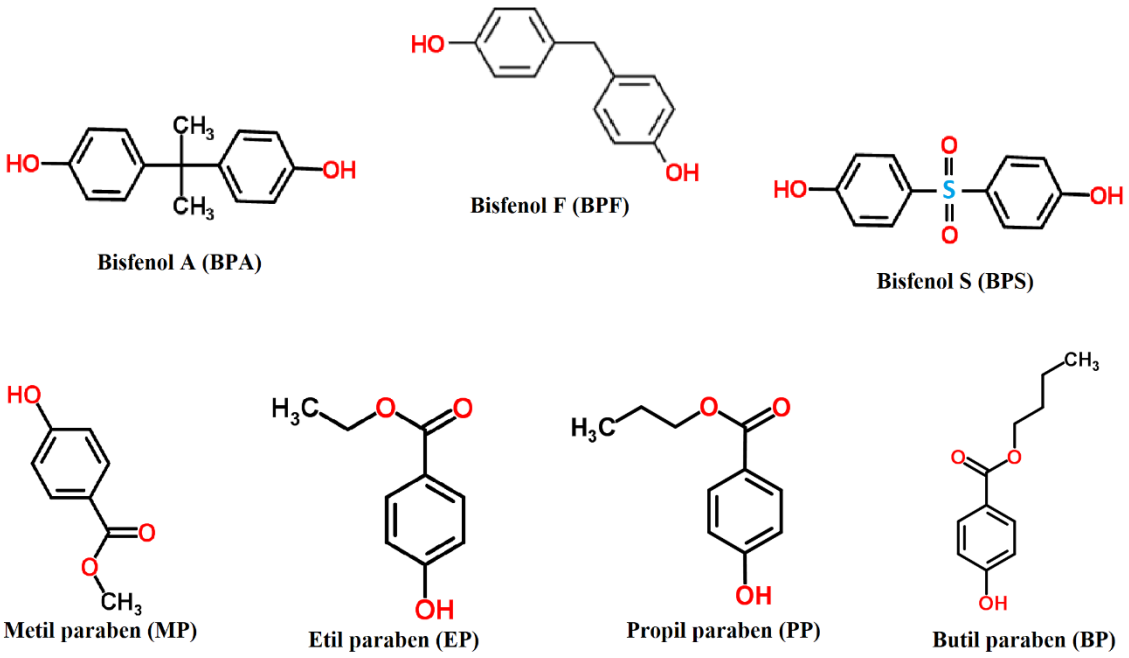


Figura 15. Estructuras moleculares de los bisfenoles y parabenos estudiados en la presente tesis.

2.5.4. Parabenos

Los parabenos, o ésteres del ácido p-hidroxibenzoico, son un grupo de compuestos químicos muy utilizados como conservantes debido a sus propiedades antimicrobianas **(Bledzka et al., 2014)**. Existen diferentes tipos de parabenos diferenciados por la conformación de su cadena, que también define sus propiedades fisicoquímicas. El MP, el EP, el PP y el BP son los parabenos más comúnmente usados (ver **Figura 15**) **(Nowak et al., 2018)**.

La UE permite y regula el uso de parabenos en alimentos **(EU Directive, 2006)**, productos farmacéuticos **(SANTE 2017a; SANTE, 2017b)** y cosméticos **(EC, 2009; EC, 2014a; EC 2014b)**. Debido al elevado uso de estos compuestos, los investigadores han detectado niveles de parabenos en muestras de agua **(Carmona et al., 2014)**; alimentos **(Liao et al., 2013a; Liao et al., 2013b)**, polvo **(Wang et al., 2012)** y otras matrices como vegetales, suelo, pescado o aire **(Bledka et al., 2014)**.

En humanos, las principales vías de exposición son la absorción dérmica al utilizar productos de cuidado personal, con una exposición estimada máxima de 2400 µg/kg pc-día, y la ingestión de productos farmacéuticos y alimentos, con ingestas diarias estimadas (EDIs) máximas de 417 µg/kg pc-día y de 13 µg/kg pc-día, respectivamente. Algunos estudios con animales han correlacionado la exposición a parabenos con efectos sobre la salud, principalmente con alteraciones en la actividad estrogénica durante la gestación y la primera infancia **(Kang et al., 2002; Ahn et al., 2012; Guerra et al., 2017)**. Los niveles de parabenos podrían sobrepasar la acción endógena del estradiol y se debe tener

especial cuidado con el PP ya que su margen de seguridad es más bajo. Además, se necesitan estudios toxicológicos que combinen la exposición a parabenos con la exposicón a otros contaminantes con efectos estrogénicos **(Boberg et al., 2010)**. A pesar de estos estudios, en general se considera que los niveles a los que la población está expuesto son seguros, aunque son necesarios más estudios toxicológicos **(Bledzka et al., 2014)**.

Tras la absorción dérmica y gastrointestinal, los parabenos son principalmente metabolizados a ácido p-hidroxibenzoico. Sin embargo, este metabolito es un biomarcador inespecífico de la exposición a parabenos y la toxicidad puede variar entre los diferentes parabenos **(Ye et al., 2008)**. Además, los parabenos también pueden formar metabolitos oxidados que se excretan en orina junto con los parabenos inalterados. Los parabenos son excretados rápidamente mediante la orina por lo que se consideran biomarcadores de exposición reciente **(Moos et al., 2016)**.

Los estudios de BH de parabenos se han centrado en los niveles en orina **(Ye et al., 2006a)**. Sin embargo, los parabenos también han sido estudiados en otras matrices como la sangre **(Mulla et al., 2015)**, el plasma **(Kolatrova et al., 2018)** o el tejido adiposo **(Artacho-Cordon et al., 2018)**. Los parabenos se han detectado en leche materna tanto libres como conjugados **(Azzouz et al., 2016a)**, sin embargo, muchos de los estudios en leche materna fueron enfocados en el desarrollo de una metodología analítica y analizaron un número limitado de muestras (n<20) **(Souza et al., 2016)**. Tan sólo Fisher et al. y Schlumpf et al. analizaron un mayor número de muestras y detectaron niveles de parabenos en un rango entre <LoQ y 16 ng/mL con frecuencias de detección (DF) en un rango del 0 al 82% **(Fisher et al., 2017; Schlumpf et al., 2010)**.

La EFSA ha establecido el ADI para la suma de MP y EP en 0-10 mg/kg pc por día en base a estudios que establecieron ‘non-observed-adverse-effects-levels’ (NOAELs) de 1000 mg/kg pc por día para ambos parabenos. Sin embargo, no se pudo definir un ADI para el PP debido a estudios que mostraron alteraciones sobre las hormonas sexuales y los órganos reproductores masculinos en ratas **(EFSA, 2004)**. Tan solo Schlumpf et al. han estudiado la exposición a parabenos de los recién nacidos a través de la lactancia **(Schlumpf et al., 2010)**.

3. OBJETIVOS

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Los objetivos generales de la presente tesis son el desarrollo de metodologías analíticas para la determinación de contaminantes alimentarios y sus metabolitos en muestras biológicas humanas, y aplicar dichas metodologías en estudios poblacionales para llevar a cabo una evaluación de la exposición y del riesgo.

Los objetivos principales se concretan en seis objetivos específicos:

1) Aplicación de la LC-HRMS para la determinación de biomarcadores de plaguicidas en orina mediante un análisis retrospectivo de sospechosos, de desconocidos y multivariante.

2) Optimización de los parámetros instrumentales de un HRMS Orbitrap para la determinación de biomarcadores de exposición de plaguicidas en orina.

3) Desarrollo de una metodo multiresiduo para la determinación de bisfenoles y parabenos en leche materna mediante LC-MS/MS.

4) Estudio de las concentraciones de bisfenoles en leche materna y evaluación del riesgo en recién nacidos lactantes.

5) Estudio de las concentraciones de parabenos en leche materna y evaluación del riesgo en recién nacidos lactantes.

6) Estudio de las concentraciones de metabolitos de ftalatos en orina y evaluación del riesgo en madres lactantes.

4. MATERIALES Y MÉTODOS

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En esta sección se describen los materiales, reactivos, patrones y equipos utilizados en la presente Tesis Doctoral. Además, se describen las poblaciones estudiadas, como se ha llevado a cabo la toma de muestra, las técnicas de preparación de muestra y las metodologías analíticas mediante LC-MS utilizadas.

También se señalan los criterios de identificación y confirmación que se han utilizado para la detección y cuantificación de los biomarcadores, la metodología empleada para el estudio de los determinantes de la exposición y la metodología utilizada para llevar a cabo la evaluación del riesgo en la población estudiada.

4.1. Materiales, reactivos, patrones, equipos y software.

A) Material empleado:

-Sobres de extracción QuEChERS EN con 4 g $MgSO_4$, 1 g NaCl, 1 g citrato sódico anhidro, 0.5 g de monohidrogencitrato disódico sesquihidrato de Agilent Technologies (Madrid, España).
-Sobres de extracción QuEChERS (Original) con 4 g $MgSO_4$, 1 g NaCl de Agilent Technologies (Madrid, España).
-Kit de extracción en fase sólida dispersiva AOAC en tubos de 15mL de polipropileno con 400 mg de PSA, 200 mg de C_{18} y 12000 mg de $MgSO_4$, de Agilent Technologies (Madrid, España).
-Piezas de cerámica para la homogenización de Agilent Technologies (Madrid, España).

B) Reactivos utilizados:

-Acetonitrilo para análisis de residuos de plaguicidas de Scharlab (Barcelona, España).
-Acetonitrilo grado LC-MS de VWR Prolabo (Barcelona, España).
-Metanol grado LC-MS de VWR Prolabo (Barcelona, España).
-Metanol para análisis de residuos de plaguicidas de Scharlab (Barcelona, España).
-Ácido acético (pureza 98-100%) de Merck KGaA (Darmstadt, Alemania).
-Acetato de sodio anhidro de Merck KGaA (Darmstadt, Alemania).
-Agua ultrapura producida con un sistema Milli-Q de Millipore (Bedford, EEUU).
-Enzima β-glucuronidasa/sulfatasa de *Helix pomatia* tipo H1 de Sigma Aldrich (St. Louis, MO, EEUU).
-Enzima β-glucuronidasa arilsulfatasa de *Helix pomatia* de Merck KGaA (Darmstadt, Alemania)
-Enzima β-glucuronidasa (*E. coli* K12) de Roche (Mannheim, Alemania).
-Orina sintética SURINE de Sigma-Aldrich (Saint Louis, MO, EEUU).

C) Patrones:

-2-Diethylamino-6-methyl-6-hydroxypyrimidine de Dr. Ehrenstorfer (Ausburg, Alemania).
-2-Isopropyl-4-methyl-6-hydroxypyrimidine de Dr. Ehrenstorfer (Ausburg, Alemania).
-3-Chloro-7-hydroxy-4-methylcoumarin de Santa Cruz Biotechnology, Inc (Heidelberg, Alemania). (Ausburg, Alemania).
-3-Ketocarbofuran de Sigma Aldrich (Barcelona, España).
-3-Methyl-4-nitrophenol de Dr. Ehrenstorfer (Augsburg, Alemania).
-3-Phenoxybenzoic acid de Dr. Ehrenstorfer (Ausburg, Alemania).
-3,5,6-Trichloro-2-pyridinol de Sigma Aldrich (Barcelona, España).
-4,6-dimethoxy-2-pyridinamine de Dr. Ehrenstorfer (Ausburg, Alemania).
-4-Fluro-3-phenoxybenzoic acid de Dr. Ehrenstorfer (Ausburg, Alemania).
-Acefato de Analytical Standard Solutions (Saint Jean d'Illac, Francia).
-Ácido 2,4-diclorofenoxiacético de Dr. Ehrenstorfer (Ausburg, Alemania).
-Ácido 2,4,5-triclorofenoxiacético de Dr. Ehrenstorfer (Ausburg, Alemania).
-Alachlor mercapturate de Dr. Ehrenstorfer (Ausburg, Alemania).
-Atrazine mercapturate de Santa Cruz Biotechnology, Inc (Heidelberg, Alemania).
-cis-(2,2-dibromovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid de Dr. Ehrenstorfer (Ausburg, Alemania).
-cis,trans-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid de Dr. Ehrenstorfer (Ausburg, Alemania).
-Diethyl dithiophosphate de Sigma-Aldrich (Barcelona, España).
-Diethyl malate de Sigma Aldrich (Barcelona, España).
-Diethyl maleate de Sigma Aldrich (Barcelona, España).
-Diethyl phosphate de Chromlab (Barcelona, España).
-Diethyl thiophosphate de Cerilliant-Certificated Reference Materials (Texas, EEUU).
-Di-n-butylphosphate de Dr. Ehrenstorfer (Ausburg, Alemania).
-Dimethyl dithiophosphate de Cerilliant-Certificated Reference Materials (Texas, EEUU).
-Dimethyl thiophosphate de Chiron AS (Trondheim, Noruega).
-Dimetoato de Dr. Ehrenstorfer (Ausburg, Alemania).
-Malathion dicarboxylic acid de Dr. Ehrenstorfer (Ausburg, Alemania).
-Methyl-N-(3-hydroxyphenyl)-carbamate de Dr. Ehrenstorfer (Ausburg, Alemania).
-Methyl-N-phenylcarbamate de Sigma Aldrich (Barcelona, España).
-Metamidofós de Dr. Ehrenstorfer (Ausburg, Alemania).
-Metolachlor mercapturate de Dr. Ehrenstorfer (Ausburg, Alemania).
-N-(2-Ethyl-6-methylphenyl)-2-hydroxyacetamide de Sigma Aldrich (Barcelona, España).
-Ometoato de Dr. Ehrenstorfer (Ausburg, Alemania).
-p-nitrophenol de Analytical Standard Solutions (Saint Jean d'Illac, Francia).
-Propachloroxanilic acid de Sigma Aldrich (Barcelona, España).
-4-fluoro-3-phenoxybenzoic acid ($^{13}C_6$, 99%) de Cambridge Isotope Laboratories (Massachusetts, EEUU).

-Ácido 2,4-diclorofenoxiacético-d_3 de Dr. Ehrenstorfer (Ausburg, Alemania).
-Atrazine mercapturate (ring-$^{13}C_3$, 99%) de Cambridge Isotope Laboratories (Massachusetts, EEUU).
-Dimetoato d_6 de Dr. Ehrenstorfer (Ausburg, Alemania).
-Metamidofós-d_6 de CDN Isotopes (Quebec, Canadá).
-p-nitrophenol-2,3,5,6-d_4 de Dr. Ehrenstorfer (Ausburg, Alemania).
-trans-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid-$^{13}C_2$, d_1 de Cambridge Isotope Laboratories (Massachusetts, EEUU).
-Bifenol A de Dr. Ehrenstorfer (Ausburg, Alemania).
-Bifenol F de Dr. Ehrenstorfer (Ausburg, Alemania).
-Bifenol S de Dr. Ehrenstorfer (Ausburg, Alemania).
-Bifenol A -d_{14} de Dr. Ehrenstorfer (Ausburg, Alemania).
-Bifenol F-d_{10} de Toronto Research Chemicals (Toronto, Canadá).
-Bifenol S-d_8 de CDN Isotopes (Quebec, Canadá).
-Metil paraben de Sigma-Aldrich (Barcelona, España).
-Etil paraben de Sigma-Aldrich (Barcelona, España).
-Propil paraben de Sigma-Aldrich (Barcelona, España).
-Butil paraben de Sigma-Aldrich (Barcelona, España).
-$^{13}C_6$-Butil paraben de Cambridge Isotope Laboratories (Massachusetts, EEUU).
-$^{13}C_6$-Metil paraben de Cambridge Isotope Laboratories (Massachusetts, EEUU).
-Etil paraben-d_5 de Santa Cruz Biotechnology (Heidelberg, Alemania).
-Mono-(2-etil-5-carboxipentil) ftalato (MECPP) de Cambridge Isotope Laboratories (Andover, MA, EEUU).
-Mono-(2-etil-5-hidroxihexil) ftalato (MEHHP) de Cambridge Isotope Laboratories (Andover, MA, EEUU).
-Mono-(2-etil-5-oxohexil) ftalato (MEOHP) de Cambridge Isotope Laboratories (Andover, MA, EEUU).
-Mono-(3-carboxipropil) ftalato (MCPP) de Cambridge Isotope Laboratories (Andover, MA, EEUU).
-Mono[2-(carboximetil)hexil] ftalato (2cx-MMHP) de Cambridge Isotope Laboratories (Andover, MA, EEUU).
-Monoisobutil ftalato (MiBP) de Cambridge Isotope Labs. (Andover, MA, EEUU).
-Monoisononil ftalato (MiNP) de Cambridge Isotope Labs. (Andover, MA, EEUU).
-Monobenzil ftalato (MBzP) de AccuStandard, Inc. (New Haven, CT, EEUU).
-Monoetil ftalato (MEP) de AccuStandard, Inc. (New Haven, CT, EEUU).
-Mono-n-octil ftalato (MOP) de AccuStandard, Inc. (New Haven, CT, EEUU).
-Monociclohexil ftalato (MCHP) de Fluka, Sigma-Aldrich (Saint Louis, MO, EEUU).
-Monomethyl ftalato (MMP) de Fluka, Sigma-Aldrich (Saint Louis, MO, EEUU).
-Mono-2-etilhexil ftalato (MEHP) de Dr. Ehrenstorfer (Ausburg, Alemania).
-Mono-n-butil ftalato (MnBP) de Dr. Ehrenstorfer (Ausburg, Alemania).
-$^{13}C_4$-Mono-(2-etil-5-oxohexil) ftalato (MEOHP-$^{13}C_4$) de Cambridge Isotope Laboratories (Andover, MA, EEUU).
-$^{13}C_2$-Mono-n-butil ftalato (MnBP-$^{13}C_2$) de Cambridge Isotope Laboratories (Andover, MA, EEUU).

D) Equipos:

-Columna cromatográfica Hypersil Gold aQ (100 mm x 2.1 mm, 1.9 µm) de Thermo Fisher Scientific (Bremen, Alemania).
-Columna cromatográfica Hypersil Gold (100 mm x 2.1 mm, 1.9 µm) de Thermo Fisher Scientific (Bremen, Alemania).
-Columna cromatográfica Symmetry C_{18} (150 mm x 2.1mm, 5 µm) de Waters (Milford, Massachusetts, EEUU).
-Columna C_{18} Luna Omega 50 x 2.1 mm (1.6 µm) de Phenomenex Inc. (Torrance, CA, EEUU).
-Cromatógrafo UHPLC Accela™ de Thermo Fisher Scientific (Bremen, Alemania).
-Cromatógrafo HPLC Finnigan Surveyor™ de Thermo Fisher Scientific (Bremen, Alemania)
-Cromatógrafo, Dionex™ UltiMate™ 3000 de Thermo Scientific™ (Bremen, Alemania).
-Espectrómetro de masas de alta resolución tipo Orbitrap, Exactive™ con fuente de ionización mediante electronebulización (HESI-II) de Thermo Scientific (Bremen, Alemania).
-Espectrómetro de masas de triple cuadrupolo, Finnigan TSQ Quantum Ultra Detector Analyser equipado con una fuente de ionización APCI (San José, CA, EEUU).
-Espectrómetro de masas de triple cuadrupolo, TSQ Quantiva con fuente de ionización tipo ESI de Thermo Scientific™ (Bremen, Alemania).
-MIRIS HMA™ de MIRIS AB (Uppsala, Suecia).

E) Software:

-Mass Frontier 7.0.2014 from Thermo Scientific en capítulo 1.
-MINITAB, Release 14 de Minitab Inc. (Birmingham, Reino Unido) en capítulos 3-5.
-MzMine 2.10 (software libre) **(Pluskal et al., 2010)** en capítulo 1.
-R, versión 3.3.1. (software libre) en capítulos 4-6.
-SIMCA v13.0 de Umetrics (Umeå, Suecia) en capítulo 1.
-SPSS, versión 17.0 de IBM en capítulo 4-6.
-TraceFinder™ 3.1 de Thermo Scientific (Bremen, Alemania) en capítulos 1 y 2.
-TraceFinder™ 3.2 de Thermo Scientific (Bremen, Alemania) en capítulos 3-6.
-Xcalibur™ 2.2 de Thermo Scientific (Bremen, Alemania) en todos los capítulos.

4.2. Población estudiada

El capítulo 1 se estudiaron diferentes enfoques analíticos para la determinación e identificación de biomarcadores de plaguicidas en orina humana. La metodología analítica fue aplicada en muestras de orina de 49 madres embarazadas cedidas por el proyecto FP7-ENV-DENAMIC ‘Developmental Neurotoxicity Assessment of Mixtures

in Children' (cod 282957). Las madres donantes residían en Sabadell (España) (n=30), Valencia (España) (n=10) y en diferentes regiones de Eslovaquia (n=9).

En los capítulos 4-6 de la presente tesis se han estudiado las concentraciones de parabenos, bisfenoles y metabolitos de ftalatos en matrices biológicas (leche y orina) de las participantes en el proyecto Bettermilk. Las participantes fueron madres lactantes que había dado a luz entre junio y noviembre de 2015 en el Hospital Universitario y Politécnico 'La Fe' (Valencia, España). Los criterios de selección para unirse al proyecto fueron: i) haber tenido un embarazo y parto normales; ii) la madre había vivido en la Comunidad Valenciana durante al menos 10 años; y iii) la madre había decidido amamantar a su hijo/a. Un total de 120 madres de entre 20 y 45 años participaron en el estudio. El estudio fue aprobado por el Comité Ético de la Dirección General de Salud Pública y Centro Superior de Investigación en Salud Pública y por el Comité Ético de Investigación Biomédica del Hospital Universitario y Politécnico la Fe y todas las madres firmaron el consentimiento informado. El Biobanco IBSP-CV (PT13/0010/0064) fue el responsable de la conservación de las muestras y de la información de las participantes.

4.2.1. Toma de muestra.

Para el estudio de bisfenoles y parabenos se analizó una muestra de leche de cada madre lactante tomada a las dos semanas después del parto. Además, algunas madres (n=56) dieron dos muestras extra tomadas a las cinco y ocho semanas después del parto con objeto de estudiar la evolución de los niveles de contaminantes en leche a lo largo de la lactancia. Las muestras se recogieron tras lavar el pecho con abundante agua y utilizando un sacaleches libre de BPA. Las muestras se guardaron en un recipiente de vidrio y fueron conservadas en el congelador de las casas a (-20) ºC hasta su traslado al Biobanco IBSP-CV, donde fueron alicuotadas y conservadas a (-80) ºC.

Para el estudio de metabolitos de ftalatos se analizó una muestra de orina de cada madre lactante tomada entre las dos y las ocho semanas después del parto. Las muestras se tomaron a primera hora de la mañana y se conservaron en la nevera del domicilio de las madres hasta su traslado por parte del personal investigador ese mismo día al Biobanco IBSP-CV, donde fueron alicuotadas y conservadas en recipientes de vidrio a (-80) ºC.

4.2.2. Cuestionarios

Las participantes del estudio Bettermilk cumplimentaron una serie de cuestionarios que proporcionaron información sociodemográfica, dietética y de hábitos de vida de las madres lactantes. Los resultados de los cuestionarios se muestran en las **Tablas 6-9**.

4.3. Preparación de las muestras.

En todos los casos, previamente al tratamiento de muestra se llevó a cabo una hidrólisis enzimática para la desconjugación de los biomarcadores con grupos glucurónido y/o sulfato (metabolitos de fase II). En general se utilizó una enzima β-glucuronidasa-aryl-sulfatasa de Helix pomatia, excepto en el caso de la determinación de metabolitos de ftalatos en la que se utilizó una enzima β-glucuronidasa de *E. coli* K-12 para evitar la hidrólisis de los monoésteres por parte de la enzima **(Dwivedi et al., 2018)**. En general, el procedimiento de la hidrólisis consistió en añadir a la muestra la enzima junto con una solución tampón, e incubar a 37ºC. Para la determinación de bisfenoles y parabenos en leche, las muestras fueron tratadas tanto con enzima como sin ella con objeto de conocer la proporción de analitos libres y conjugados en muestra.

Con respecto al tratamiento de muestra se siguieron de manera general dos tipos de preparación de muestra: QuEChERS y 'Dilute & Shoot'. En el caso de la determinación de biomarcadores de plaguicidas en orina y de bisfenoles y parabenos en leche materna, la extracción se llevó a cabo utilizando QuEChERS, mientras que para la determinación de metabolitos de ftalatos en orina se ha utilizado 'Dilute & Shoot'.

Con respecto a la extracción de muestra con QuEChERS, se llevaron a cabo dos procedimientos diferentes, en el caso de la determinación de plaguicidas en orina, tan solo se utilizó la primera fase de extracción facilitada por 'salting-out'. Sin embargo, para la determinación de bisfenoles y parabenos en leche materna, el procedimiento incluía tanto la fase de 'salting out', como la purificación posterior.

Tabla 6. Características generales de las madres lactantes y los recién nacidos (n=120)

Características		**n (%)**
Madre		
Número de hijos		
	1	69 (57.5)
	2	40 (33.3)
	≥ 3	11 (9.2)
Edad (años)		33 (20 - 45)[a]
	Datos faltantes	1 (0.8)
Peso antes del embarazo (kg)		60 (42 - 92)[a]
	Datos faltantes	2 (1.7)
Altura (cm)		164 (150 - 184)[a]
	Datos faltantes	2 (1.7)
Índice de masa corporal antes del embarazo (kg/m^2)		21.9 (16.6 - 35.4)[a]
	Datos faltantes	2 (1.7)
Dieta específica durante el embarazo		
	Sí	17 (14.2)
	No	101 (84.2)
	Datos faltantes	2 (1.7)

Tabla 6. (Continuación)

Características	**n (%)**
País de nacimiento	
España	104 (86.7)
Otro	14 (11.7)
Datos faltantes	2 (1.7)
Lugar de residencia	
Urbano	85 (70.8)
Rural	21 (17.5)
Datos faltantes	14 (11.7)
Nivel educacional	
Solo educación primaria	13 (10.8)
Hasta educación secundaria	24 (20.0)
Estudios universitarios	83 (69.2)
Situación laboral	
Empleada	100 (83.3)
Desempleada	18 (15.0)
Datos faltantes	2 (1.7)
Tiempo trabajado fuera de casa (años)	10 (0 - 28)[a]
Datos faltantes	2 (1.7)
La madre fue amamantada	
Sí	81 (67.5)
No	34 (28.3)
Datos faltantes	5 (4.2)
Ejercicio físico	
3 o más días a la semana	19 (15.8)
1 o 2 días a la semana	18 (15.0)
Ocasionalmente	50 (41.7)
Nunca	30 (25.0)
Datos faltantes	3 (2.5)
Fumadora	
Sí	9 (7.5)
Ex-fumadora	48 (40.0)
Nunca	63 (52.5)
Recién nacido	
Edad gestacional (semanas)	40 (35 - 41)[a]
Datos faltantes	28 (23.3)
Sexo	
Niño	47 (39.2)
Niña	70 (58.3)
Datos faltantes	3 (2.5)
Peso al nacer (g)	3360 (2160 - 4350)[a]
Datos faltantes	3 (2.5)
Altura al nacer (cm)	51 (46 - 55)[a]
Datos faltantes	43 (35.8)
Perímetro craneal al nacer (cm)	34 (32.5 - 37)[a]
Datos faltantes	68 (56.7)

[a]Valores expresados como mediana (mínimo- máximo).

Tabla 7. Madres lactantes: consumo de alimentos por grupos (gramos/mes), Índice MED-DQI y consumo de productos envasados en las 72h previas (n=120).

Consumo de alimentos por grupos (g/mes) **Datos faltantes: 6 (5.0)*[c]	**Mediana (mínimo - máximo)**
Huevos	700 (30 - 1800)
Lácteos	13000 (1400 - 40000)
Productos cárnicos	6000 (700 - 14000)
Productos de la pesca	4000 (1000 - 16000)
Vegetales	13000 (1200 - 40000)
Frutas	16000 (4000 - 80000)
Legumbres y cereales	5000 (2000 - 30000)
Aceites y grasas	800 (150 - 1500)
Bollería y pastelería	1300 (170 - 7000)
Miscelánea	1400 (300 - 30000)
Bebidas[a]	44000 (1700 - 80000)
Índice MED-DQI[b]	5 (0 - 8)
Bueno	56 (46.7)[c]
Medio-bueno	63 (52.5)[c]
Medio-malo	1 (0.8)[c]
Malo	0 (0)[c]
Nº raciones productos envasados (72h previas)	14 (1 - 39)

[a]Valores expresados en mL/mes.
[b]Valores expresados en unidades arbitrarias (a.u.).
[c]Valores expresados como frecuencia absoluta (porcentaje).

Tabla 8. Frecuencia de uso de productos cosméticos en madres lactantes (n=120).

Frecuencia de uso de productos cosméticos	**n (%)**
Productos para el cuidado de la piel	
Frecuencia: Nunca o antes del embarazo	25 (20.8)
Frecuencia: Diariamente	68 (56.7)
Frecuencia: Varias veces a la semana	20 (16.7)
Frecuencia: Varias veces al mes	4 (3.3)
Datos faltantes	3 (2.5)
Perfumes	
Frecuencia: Nunca o antes del embarazo	55 (45.8)
Frecuencia: Diariamente	34 (28.3)
Frecuencia: Varias veces a la semana	22 (18.3)
Frecuencia: Varias veces al mes	4 (3.3)
Datos faltantes	5 (4.2)
Desodorantes	
Frecuencia: Nunca o antes del embarazo	9 (7.5)
Frecuencia: Diariamente	96 (80.0)
Frecuencia: Varias veces a la semana	6 (5.0)
Datos faltantes	9 (7.5)
Protectores solares	
Frecuencia: Nunca o antes del embarazo	74 (61.7)
Frecuencia: Diariamente	17 (14.2)
Frecuencia: Varias veces a la semana	10 (8.3)
Frecuencia: Varias veces al mes	3 (2.5)
Frecuencia: Ocasionalmente	10 (8.3)
Datos faltantes	6 (5.0)
Tintes para el cabello	
Veces al año	2 (0 - 26)[a]
Datos faltantes	12 (10.0)

Tabla 8. (Continuación).

Frecuencia de uso de productos cosméticos	**n (%)**
Tinte para el cabello	
Última aplicación: ≤1 semana	9 (7.5)
Última aplicación: < 1 mes	10 (8.3)
Última aplicación: ≥ 1 mes, < 3 meses	34 (28.3)
Última aplicación: ≥ 3 meses	12 (10.0)
Última aplicación: Nunca o antes del embarazo	51 (42.5)
Datos faltantes	4 (3.3)
Pintalabios	
Frecuencia: Nunca o antes del embarazo	87 (72.5)
Frecuencia: Diariamente	11 (9.2)
Frecuencia: Varias veces a la semana	8 (6.7)
Frecuencia: Varias veces al mes	13 (10.8)
Datos faltantes	1 (0.8)
Maquillaje	
Frecuencia: Nunca o antes del embarazo	65 (54.2)
Frecuencia: Diariamente	16 (13.3)
Frecuencia: Varias veces a la semana	16 (13.3)
Frecuencia: Varias veces al mes	20 (16.7)
Datos faltantes	3 (2.5)

[a]Valores expresados como mediana (mínimo - máximo).

Tabla 9. Número de raciones de alimentos ingeridas durante las 72h previas por las madres lactantes (n=120).

Nº raciones consumidas 72 h previas	Mediana (Mínimo - Máximo)
Zumos envasados	0 (0 - 12)
Huevos	1 (0 - 6)
Productos cárnicos	4.3 (0 - 10)
Productos de la pesca	1.3 (0 - 7)
Lácteos	6 (0 - 17)
Vegetales	3 (0 - 12)
Frutas	6 (0 - 15)
Legumbres y cereales	6.3 (2 – 20)
Aceites y grasas	6 (0 - 21)

Posteriormente, los extractos orgánicos se evaporaron aplicando corriente de nitrógeno en un baño de agua a 37ºC y se reconstituyeron. Tras la reconstitución, se filtraron las muestras previamente a su inyección en un equipo de LC-MS.

Con respecto al tratamiento de muestra mediante 'Dilute & Shoot', 500 µL de muestra se diluyeron con tampón acetato amónico y enzima (requeridos para la hidrólisis enzimática) y agua, disolución de patrón interno (y disolución de patrón en el caso de curvas de calibrado y controles de calidad) hasta alcanzar un volumen final de 900 µL. Las muestras diluidas fueron centrifugadas y el sobrenadante fue filtrado para su posterior inyección en un equipo de LC-MS.

En la **Tabla 10** se detallan las preparaciones de muestra utilizadas en la presente tesis.

4.4. Etapa de análisis.

En la presente tesis, la etapa de análisis se ha llevado a cabo utilizando equipos de LC acoplada a MS. Para la separación cromatográfica se han empleado diferentes columnas y fases móviles y para la detección se han utilizado equipos de MS/MS tipo QqQ y de HRMS tipo Orbitrap. En la **Tabla 10** se describen los diferentes métodos cromatográficos y de detección utilizados.

Tabla 10. Preparaciones de muestra y técnicas LC-MS utilizadas en la presente tesis.

Parámetros	Capítulo 1 Capítulo 2	Capítulos 3, 4 y 5	Capítulo 6
Analitos (matriz)	Plaguicidas y sus metabolitos (orina)	Bisfenoles y parabenos (leche)	Metabolitos de ftalatos (orina)
Volumen muestra	5 mL	10 mL	0.5 mL
Uso patrón interno	Sí (sólo se usó para el análisis 'target') Sí	Sí	Sí
Tratamiento enzimático	Adición de 20 μL de enzima β-glucuronidasa aril sulfatasa (de *Helix pomatia*) y 1 mL de tampón acetato 0,2 M (incubación a 37ºC durante la noche)	Adición de 1 mL de solución (enzima β-glucuronidasa aril sulfatasa de *Helix pomatia* en tampón acetato de amonio 1M) (incubación a 37ºC durante 17 h). Las muestras fueron analizadas con hidrólisis enzimática y sin hidrólisis.	Adición de 10 μL de enzima β-glucuronidasa (*E. Coli* K12) y 200 μL de tampón de acetato de amonio 1M (incubación a 37ºC durante 90 min).
Tratamiento de muestra	**1.** Adición 10 mL ACN y un sobre QuEChERS (EN), agitación y centrifugación (3.500 rpm, 10 min). **2.** Trasvase fase orgánica y evaporación (corriente N_2, 37ºC) **3.** Reconstitución con 200 µL de MeOH:H_2O (10:90,v/v) con 0.1% de ácido acético **4.** Ultracentrifugación y filtración en eppendorf con filtro 0.2 µm (11.000 rpm, 3 min, 10° C) **5.** Trasvase del filtrado a un vial de inyección	**1.** Adición 10 mL ACN y un sobre QuEChERS (Original), agitación y centrifugación (8.000 rpm, 10 min). **2.** Trasvase fase orgánica y congelación (-20ºC, durante la noche) **3.** Agitación, adición sobre QuEChERS (AOAC), agitación y centrifugación (4.500 rpm, 15 min) **4.** Trasvase fase orgánica y evaporación (corriente N_2, 37ºC) **5.** Reconstitución con 250 µL of MeOH:H_2O (20:80,v/v) **6.** Ultracentrifugación y filtración en eppendorf con filtro 0.2 µm (13.000 rpm, 1 min, 5° C) **7.** Trasvase del filtrado a un vial de inyección	**1.** Dilución con enzima y tampón acetato de amonio (ver tratamiento enzimático) y agua y disoluciónes patrón hasta un volumen final de 900 μL. **2.** Centrifugación (4500 rpm, 10 min). **3.** Trasvase de 400 μL de sobrenadante y ultracentrifugación (13.000 rpm, 10 min, 4ºC). **4.** Trasvase de 200 μL de sobrenadante a vial inyección.

Tabla 10. (Continuación).

Parámetros	Capítulo 1	Capítulo 2	Capítulos 3, 4 y 5	Capítulo 6
Cromatografía	UHPLC		HPLC	UHPLC
Columna cromatográfica	Columna Hypersil Gold aQ (100 mm x 2,1 mm, 1,9 μm)	Columna Hypersil Gold (100 mm x 2,1 mm, 1,9 μm)	Columna Symmetry C_{18} (2,1×150 mm, 5 µm)	Columna C_{18} Luna Omega 50 x 2,1 mm (1,6 µm)
Fases móviles (LC)	Fase móvil A: H_2O (ácido acético 0,1%). Fase móvil B: MeOH (ácido acético 0,1%).		Fase móvil A: H_2O Fase móvil B: MeOH	Fase móvil A: H_2O (ácido acético 0,1%). Fase móvil B: MeOH:ACN (90:10, v/v) (ácido acético 0,1%)
Flujo (LC)	400 (µL/min)		300 (µL/min)	400 (µL/min)
Modo de ionización	ESI en modo positivo y negativo		APCI en modo negativo	ESI en modo negativo
Detector	HRMS tipo Orbitrap		MS/MS tipo QqQ	MS/MS tipo QqQ
Modo adquisición	‘Full scan’ ‘Full scan’, HCD 20eV	‘Full scan’, CID 40eV	‘Selected reaction monitoring’ (SRM)	SRM

4.5. Criterios de identificación y confirmación.

Los criterios de identificación y confirmación de los analitos estudiados en la presente tesis doctoral están basados en la guía SANTE/11813/2017 y en el Capítulo 1 en la guía SANCO/12571/2013 **(SANTE, 2017c; SANCO 2013)**. A continuación se describen los diferentes criterios utilizados:

A) Criterios de identificación y confirmación para el estudio 'target' mediante LC-HRMS (Capítulos 1 y 2):

i) Exactitud de masa del ion molecular < 5 ppm
ii) Exactitud de masa del fragmento (si existe) < 5 ppm
iii) Exactitud de masa de los iones del perfil isotópico (A+1, A+2 y/o A+4) < 5 ppm.
iv) La diferencia en el tiempo de retención entre la muestra y el patrón debe ser ≤ 0.1 min (en el caso del Capítulo 1, cuyas muestras fueron analizadas y procesadas previamente, se utilizó el criterio de la SANCO/12571/2013: La diferencia en el tiempo de retención entre la muestra y el patrón debe ser ≤ 0.2 min).

B) Criterios de identificación y confirmación para el análisis retrospectivo de sospechosos mediante LC-HRMS (Capítulo 1).

Estos criterios se basaron en los parámetros de identificación que ofrece el software TraceFinder™ 3.1:

Para los iones moleculares:

i) Área mínima: 10,000
ii) Relación señal-ruido > 5
iii) Exctitud de masa < 5 ppm

Para los fragmentos:

iv) Intensidad mínima: 5,000
v) Exctitud de masa < 5 ppm

Con respecto al perfil isotópico:

vi) Acoplamiento mínimo con respecto al teórico: 90%
vii) Máxima desviación de la intensidad relativa: 30%
viii) Exctitud de masa < 5 ppm

C) Criterios de identificación y confirmación para el análisis retrospectivo de desconocidos mediante LC-HRMS (Capítulo 1):

Para el análisis de desconocidos, la búsqueda de metabolitos de plaguicidas se llevó a cabo usando el enfoque 'relación fragmentación-degradación' **(García-Reyes et al., 2007)**. Esta metodología se basa en el hecho de que los compuestos de bajo peso molecular generalmente tienen rutas de degradación y fragmentación paralelas. Por lo tanto, si el fragmento de una molécula 'A' aparece tanto al tiempo de retención de la molécula 'A', como a otro tiempo de retención, podría indicar la presencia de una molécula 'B' con una estructura similar a la molécula 'A' (un posible metabolito). Mediante la revisión de los 'extracted ion chromatogram' (XIC) de los fragmentos, se puede comprobar la presencia de picos a tiempos de retención que no corresponden con los de la molécula padre y, por tanto, permiten la posible detección de nuevos metabolitos. Para una confirmación de los nuevos analitos se requiere de patrones de referencia certificados (si están disponibles) mediante la comparación de tiempos de retención.

D) Criterios de procesado e identificación para el análisis multivariante mediante LC-HRMS (Capítulo 1):

Previamente al análisis multivariante, se procesaron de los 'raw data' adquiridos en 'full scan' sin fragmentación y 'full scan' con fragmentación mediante el programa MzMine 2.10 con objeto de convertir datos tridimensionales (m/z, tiempo de retención e intensidad de señal ('ion current') en picos cromatrograficos (m/z, tiempo de retención) con sus áreas asociadas. El procesado de los datos incluyó 6 pasos con los criterios de identificación y procesado de señales del MzMine que se describen a continuación:

i) Detección de masas aplicando el algoritmo 'masa exacta'.
ii) 'Construcción del cromatograma': altura mínima (10^4); 'período de tiempo mínimo' (0,09 min); y 'tolerancia m/z' (5 ppm, tolerancia relativa).
iii) 'Chromatogram deconvolution': Aplicación del algoritmo 'baseline cut-off'.
iv) 'Agrupamiento isotópico: 'tolerancia m/z' (5 ppm, tolerancia relativa); 'toleracia RT' (0,1 min, absoluta); 'monotonic shape' (Sí); 'carga máxima' (1); 'isótopo representativo' (más intenso).
v) Para corregir las diferencias en RT y de m/z entre muestras, los picos se alinearon utilizando el algoritmo 'Join aligner' con los siguientes parámetros: 'tolerancia m/z' (10 ppm); 'peso de la m/z (90); 'peso del RT' (10); 'Tolerancia del RT' (0,25 min); 'requiere el mismo estado de carga' (Sí).

E) Criterios de identificación y confirmación para LC-MS/MS (Capítulos 3-6)

i) Detección de dos transiciones SRM por analito (en el caso de patrones internos solo se usa 1 transición).
ii) Relación entre las áreas de las dos transiciones SRM en las muestras debía ser ≤ 30% a la relación en los patrones
iii) Relación señal/ruido > 3
iv) La diferencia en el tiempo de retención entre la muestra y el patrón ≤ 0,1min

4.6. Análisis estadístico.

El análisis estadístico se llevó a cabo utilizando los programas SPSS (versión 17.0.) para el análisis estadístico descriptivo, R (versiones 3.3.1 y 3.3.2.) para el análisis de determinantes y la evaluación del riesgo, y SIMCA (v13.0) para el análisis multivariante.

4.6.1. Análisis estadístico descriptivo

Se llevó a cabo un análisis estadístico descriptivo de los niveles de bisfenoles y parabenos en leche y de metabolitos de ftalatos en orina descritos en los Capítulos 4, 5 y 6 que presentaban una DF superior al 40%. Los valores inferiores al límite de cuantificación fueron estimados utilizando el 'máximum likelihood estimation method' (MLE) descrito en EFSA, **(2010b)**. Este método asume que los datos se distribuyen de acuerdo a una distribución paramétrica determinada. Se asumió una distribución log-normal para las concentraciones de biomarcadores. Para describir los niveles de biomarcadores, se calculó la concentración mínima y máxima, los percentiles 25, 50, 75 y 95 (P25, P50, P75 y P95, respectivamente) y la media aritmética (AM) y geométrica (GM) de cada biomarcador.

4.6.2. Análisis de determinantes

En los Capítulos 4, 5 y 6 se estudiaron posibles determinantes de los niveles de bisfenoles y parabenos en leche materna y de ftalatos en orina. Para ello, se construyeron modelos de regresión robusta (RRMs) tanto simples como múltiples para identificar asociaciones entre los niveles de biomarcadores y las variables independientes (sociodemográficas, dietéticas, de uso de cosméticos y de niveles de macronutrientes). Debido a la presencia de valores atípicos entre los niveles de biomarcadores, se decidió utilizar la regresión robusta como una alternativa a los métodos de estimación de mínimos cuadrados ordinarios (OLS). Para conseguir una distribución normal en la variable respuesta se utilizó la transformación logarítmica de las concentraciones de biomarcadores.

En primer lugar se llevaron a cabo RRMs simples entre las variables dependientes (concentraciones de contaminantes) y todas las variables independientes. Posteriormente,

se llevaron a cabo RRMs multiples, introduciendo para cada contaminante las variables independientes que habían mostrado un p-valor < (0,05 - 0,2) en los RRMs simples. Los RRMs mútliples se construyeron siguiendo un procedimiento de selección de variables hacia atrás basado en diferentes criterios: criterio de información bayesiano (BIC), criterio de información de Akaike (AIC) y p-valor. Finalmente, se seleccionaron aquellos modelos que mostraron un mejor ajuste de los datos. En el caso de los parabenos y de ftalatos se seleccionaron los modelos construidos en base al BIC y en el caso de los bisfenoles los modelos construidos en base a la significación del p-valor (<0,05). El modelo general de RRM múltiple se describe a continuación:

$$log(y_i) = \beta_0 + \beta_1 \cdot x_{1i} + \cdots + \beta_p \cdot x_{pi} + \varepsilon_i, \quad i = 1, \dots, n.$$

Donde y_i es la concentración de biomarcador en matriz de cada participante, (β_0, β_1, … , β_p) son los parámetros del método, (x_{1i}, …,x_{pi}) son los valores de las variables independientes de cada participante, ε_i es el error y n es el número de participantes. La estimación de los parámetros del modelo se realizó considerando el estimador M con ponderación bicuadrada permitiendo que observaciones con residuos elevados tengan un menor peso en la estimación. En los RRMs múltiples se consideraron significativas aquellas variables cuyo p-valor fue <0,05.

En el caso de los estudios en orina, las concentraciones de biomarcadores no fueron ajustadas en base a la concentración de creatinina para los RRMs, sino que se introdujeron las concentraciones de creatinina como una variable indenpendiente en los RRMs múltiples tal y como recomendaron Barr et al., **(2005)**.

Con objeto de estimar la correlación entre los niveles de los diferentes biomarcadores estudiados en matrices biológicas, se estudió el coeficiente de correlación de Spearman para comparar sus concentraciones. En el caso de biomarcadores en orina, se utilizaron las concentraciones ajustadas por los niveles de creatinina.

4.6.3. Análisis multivariante

En el Capítulo 1, se llevó a cabo un análisis de datos multivariante para identificar diferencias entre los grupos de muestras analizadas en base a la procedencia de las mismas. Previamente al análisis multivariante se aplicó un ‘Pareto scaling’ a todas las variables analizadas con objeto de reducir el ruido y los datos y, por lo tanto, mejorar la calidad de la información. Los datos fueron analizados utilizando análisis de componentes principales (PCA). Los picos bidimensionales (RT y m/z) fueron utilizados como variable independiente. La validez y robustez del modelo PCA se evaluó mediante $R^2(X)$ y $Q^2(X)$. El PCA se aplicó a las muestras adquiridas en modo ESI positivo.

4.7. Evaluación del riesgo.

En la presente tesis se llevó a cabo una evaluación del riesgo de la población estudiada en los capítulos 4-6, es decir, los recién nacidos lactantes y las madres. En los recién nacidos lactantes se llevó a cabo una evaluación del riesgo basada en la exposición externa a través de la ingesta de leche materna (Capítulos 4 y 5). En el caso de las madres se llevó a cabo una evaluación del riesgo basada en la exposición interna utilizando los niveles en orina (Capítulo 6).

4.7.1. Evaluación del riesgo en recién nacidos lactantes.

Para estimar la exposición de los bebés lactantes a contaminantes a través de la leche materna, se tuvo en cuenta la concentración de los contaminantes en leche y la ingesta diaria de leche, que se estimó en base a las recomendaciones para la lactancia de la agencia de protección ambiental de EEUU (EPA). La EPA **(2011**), indicó valores recomendados de ingesta diaria de leche materna para bebés de 1 a 3 meses. Siendo la media 140 mL/kg-pc/día y el percentil superior (media más dos desviaciones estándar) 190 mL/kg-pc/día.

La estimación de la ingesta de contaminantes a través de la leche materna se llevó a cabo mediante dos enfoques: i) determinista and ii) probabilístico.

En el enfoque determinista, la ingesta diaria estimada (EDI) se calculó de acuerdo a la ecuación:

EDI = C x M

Donde ‘C’ es la concentración media del contaminante en leche y ‘M’ es la media de ingesta diaria de leche (140 mL/kg -pc/día). Asimismo, se calculó la EDI utilizando el percentil superior de ingesta diaria de leche (190 mL/kg-pc/día).

En el enfoque probabilístico, la distribución de la ingesta diaria de leche por los recién nacidos fue estimada asumiendo que los datos de ingesta se distribuían de acuerdo con una distribución log-normal. Los parámetros estimados se obtuvieron indicando como valor medio de la distribución 140 mL/kg-pc/día e indicando que la probabilidad de que la ingesta sea de menor de 190 mL/kg-pc/día es del 95%.

$$e^{\mu+\sigma^2/2} = 140;\ F(190) = 0.95$$

donde μ y σ son los parámetros de la distribución log-normal y F representa la función de distribución. Además, se consideró una ingesta mínima de leche de 120 mL/kg-pc/día. Tras la estimación de la distribución de la ingesta, se estimaron valores de ingesta que se ajustaran a esta distribución (uno por cada participante). La ingesta de contaminantes fue calculada multiplicando los valores de ingesta de leche estimados para cada participante por la concentración de contaminante en leche.

Tras su cálculo, la EDI se comparó con valores límite de ingesta de referencia como ADI o TDI para los contaminantes estudiados.

4.7.2. Evaluación del riesgo en madres lactantes.

Para llevar a cabo la evaluación del riesgo, las concentraciones de biomarcadores en orina fueron comparados con los valores guía de BH de la literatura (BEs y HBMs). El 'hazard quotient' (HQ) se calculó comparando el P95 de la concentración del biomarcador estudiado con sus BEs y/o HBMs correspondientes.

HQ=P95/(BE ó HBM)

Los HQs calculados se utilizaron para caracterizar el riesgo de la población. Si el HQ es <1, es poco probable que exista un riesgo para la salud. Sin embargo, si el HQ>1, es posible que exista un riesgo para la salud.

5. RESULTADOS

5.1. Capítulo 1: Análisis retrospectivo de metabolitos de plaguicidas en orina mediante UHPLC-HRMS

En el presente capítulo se desarrolló una estrategia analítica para la identificación de metabolitos de plaguicidas en orina que combina un enfoque 'target' previamente desarrollado **(Roca et al., 2014b)**, un análisis de retrospectivo de sospechosos, un análisis de desconocidos utilizando un enfoque 'relación fragmentación-degradación' y un análisis multivariante mediante PCA de los datos adquiridos en HRMS. Para el análisis de sospechosos de plaguicidas se usó una base de datos con 263 compuestos (**Tabla SI-1. Cap. 1**), la cual incluye metabolitos de organofosforados (63), cloracetamidas (36), carbamatos (20), triazinas (15), triazoles (15), sulfonilureas (11) y otras clases de plaguicidas. El método analítico está basado en una extracción genérica utilizando QuEChERS y UHPLC-HRMS. La metodología analítica fue aplicada en muestras de orina de 49 madres embarazadas que residían en Sabadell (España) (n=30), Valencia (España) (n=10) y en diferentes regiones de Eslovaquia (n=9).

5.1.1. Resultados y discusión

5.1.1.1. Análisis 'target'

Los metabolitos de plaguicidas estudiados mediante un enfoque 'target' se muestran en la **Tabla SI-2. Cap. 1**. De manera global, 9 metabolitos de plaguicidas se detectaron en las 49 muestras de orina analizadas: 4 dialquilfosfatos (DAPs) (DEP, DETP, DMTP y DMDTP); 4 metabolitos específicos de plaguicidas organofosforados (DEAMPY, IMPY, PNP y TCPy) y el herbicida 2,4-D. Entre los 9 metabolitos detectados, las concentraciones oscilaron entre 0.9 y 98.9 ng/mL, con DFs de entre 3 y 71%.

5.1.1.2. Análisis retrospectivo de sospechosos

Se construyó una base de datos teórica de 263 metabolitos de plaguicidas. Para cada sustancia, la base de datos incluía información de la fórmula molecular, la masa molecular exacta del ión molecular y de los fragmentos teóricos. Para construir la base de datos se utilizó información de bases de datos **(PDCL, 2020; PPDB, 2020a; Chemspider, 2020; PubChem, 2020),** en el software Mass Frontier 7.0.2014 y en la literatura previa **(Mol et al., 2012; Muñoz et al., 2014; Muñoz et al., 2012; Gómez-Pérez et al., 2014; Muñoz et al., 2011a; Muñoz et al., 2011b)**. (Ver **Tabla SI-3. Cap. 1).** Utilizando la base de datos teórica se aplicó un análisis retrospectivo en el que 24 metabolitos de plaguicidas fueron identificados provisionalmente (Ver **Tablas 11 y 12)**. Utilizando 6 soluciones de patrones de referencia disponibles se confirmó la detección de los metabolitos: 3-ketocarbofuran (3-KC); 4,6-dimethoxy-2-pyrimidinamine (4,6-DMP); diethyl malate (DMT); diethyl maleate (DME); N-(2-Ethyl-6-methylphenyl)-2-hydroxyacetamide

(NEMHA); y propachloroxanilic acid (POA) (ver **Tabla 12**). El resto de metabolitos identificados provisionalmente no pudieron ser confirmados debido a la falta de patrones de referencia disponibles. En la **Figura 16** se muestra como ejemplo el XIC del ion molecular y del fragmento, así como el perfil isotópico del NEMHA.

Los compuestos identificados son metabolitos de cinco plaguicidas (**Tabla 12**), de los cuales, el carbofuran **(EFSA, 2009)**, el propaclor **(EFSA, 2011)** y el metolaclor **(PPDB, 2020b)** estaban prohibidos en la UE en el momento de análisis.

Tabla 11. Metabolitos de plaguicidas detectados pero no confirmados en el análisis de sospechosos (n=49).

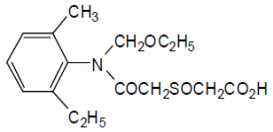
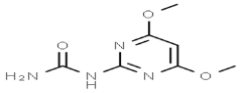
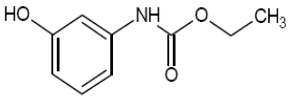
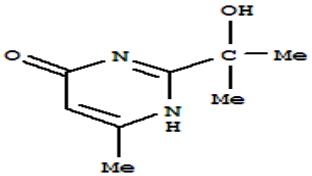
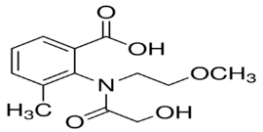
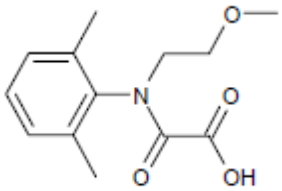
Metabolito	nºCAS	Estructura	Plaguicida precursor	Masa monitorizada* $[M+H]^+$	Δm (ppm) ($[M+H]^+$)	Δm (ppm) (FRAG. 1)	Nª
Acetic acid, {2-[(ethoxymethyl)(2-ethyl-6-methylphenyl)-amino]-2-oxoethyl} sulfinyl	618113-86-3		Acetoclor	342,13697	-1,98-(-1,41)	-0,87-(-0,48)	2
(4,6-dimethoxypyrimidin-2-yl)urea	151331-81-6		Bensulfuron-metil	199,08256	-1,33-0,65	-0,43-0,98	18
EHPC	7159-96-8		Desmedifam	182,081169	-0,93-0,43	-0,27-1,11	10
2-(1-hydroxy-methyl)-ethyl-4-methyl-6-hydroxypirimidine	28175-97-5		Diazinon	169,09715	-2,56-1,87	-1,28-1,83	4
2-[(2-hydroxyacetyl)-(2-methoxy-ethyl)-amino]3-methyl-benzoic acid	1138220-18-4		Dimetaclor	268,11794	-2,16- (-0,68)	-0,69-3,44	2
N-(2,6-dimethylphenyl)-N-(2-methoxyethyl)oxalamic acid			Dimetaclor	208,09682	-1,43-0,87	-1,12-1,38	25

Tabla 11. (Continuación).

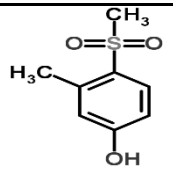
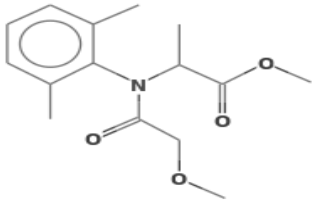
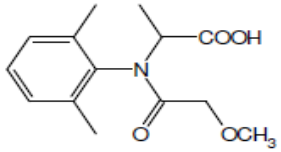
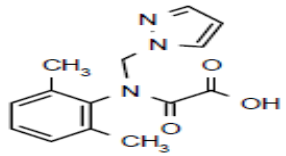
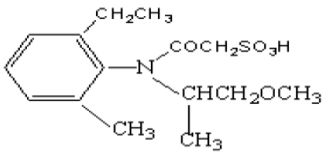
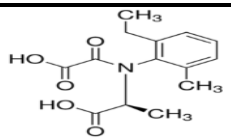
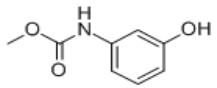
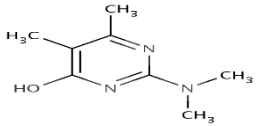
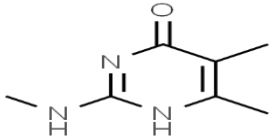
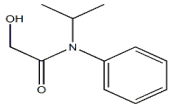
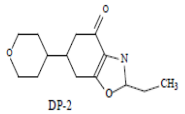
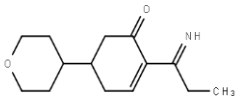
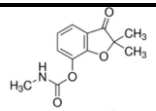
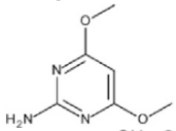
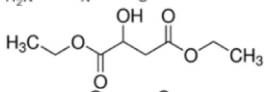
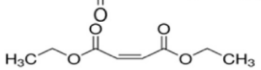
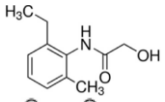
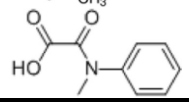
Metabolito	nºCAS	Estructura	Plaguicida precursor	Masa monitorizada* $[M+H]^+$	Δm (ppm) ($[M+H]^+$)	Δm (ppm) (FRAG. 1)	Nª
Fenamiphos sulphone phenol	14270-40-7		Fenamifós	187,04234	0,57	1,22	1
N-(2,6-dimethylphenyl)-N-(methoxyacetyl)-alanine	467430-42-8		Metalaxil	281,13868	-0,96-0,78	-0,55-0,68	8
R-2-[2,6-dimethylphenyl)-meyhoxyacetyl-amino]-propionic acid	75596-99-5		Metalaxil	266,13868	-1,78-1,04	-0,25-0,66	20
N-(2,6-dimethylphenyl)-N-(1H-pyrazol-1-ylmethyl)oxalamide	1231244-60-2		Metazaclor	274,11861	-0,43-0,71	-0,53-0,21	8
Metolachlor ethane sulfonic acid	171118-09-5		Metolaclor	330,13697	-2,24	2,28	1

Tabla 11. (Continuación).

Metabolito	nºCAS	Estructura	Plaguicida precursor	Masa monitorizada* $[M+H]^+$	Δm (ppm) ($[M+H]^+$)	Δm (ppm) (FRAG. 1)	Nª
(S)-2-[(Oxalyl)(2-ethyl-6-methylphenyl)amino] propionic acid	1217465-10-5		Metolaclor	280,11794	-2,22- 1,14	-0,27-1,85	13
Methyl-N-hydrophenyl-carbamate	13683-89-1		Fenmedifam	168,06551	-0,57-1,46	-1,27-0,86	24
2-dimethylamino-5,6-dimethylpyrimidin-4-ol	40778-16-3		Pirimicarb	168,11313	-0,25	0,61	1
5,6-dimethyl-2-(methylamino)pyrimindin-4-ol	78195-30-9		Pirimicarb	154,09748	0,09	-0,61	1
Propachlor alcohol	42404-06-8		Propaclor	194,11756	-0,43	-0,25	1
(RS)-2-ethyl-6,7-dihidro-6-perhydropyran-4yl-benzoxazol-4-(5H)-one			Tepraloxidim	250,14377	-2,50-1,87	-3,63-2,43	10
3-hydroxy-2-(1-iminopropyl)-5-perhydropyran-4-ylcyclohex-2-en-1-one			Tepraloxidim	252,15942	-1,53-2,01	-0,87-1,23	8

*Acoplamiento perfil isotópico> 90%; Nª: número de muestras en los que se ha detectado

Tabla 12. Metabolitos de plaguicidas identificados y confirmados con patrones en el análisis de sospechosos (n=49).

Metabolito	nº CAS	Estructura	Plaguicida precursor	Masa monitorizada $[M+H]^+$	Δm (ppm) $([M+H]^+)$	Δm (ppm) (Frag. 1)	RT patrón (min)	Rango RT muestras (min)	Número de muestras detectadas	Concentración estimada (ng/mL) Media / Rango
3-KC	16709-30-1		Carbofuran	236,09173	-0,87 - 0,56	-1,15 – 3,43	2,87	2,85 -2,91	4	8,34 / 4,0 -14,4
4,6-DMP	36315-01-2		Bensulfuron-metil	156,07675	-0,63 - 0,57	-0,23 – 0,53	2,45	2,38 -2,51	6	14,67 /1,86-31,28
DMT	7554-12-3		Malation	191,09140	0,26 - 2,22	2,12 – 2,54	2,67	2,62 -2,66	2	1,24 /1,06 -1,42
DME	141-05-9		Malation	173,08083	-0,78 - 2,48	-0,63 – 2,19	3,06	3,02 - 3,11	9	14,58/2,01-45,99
NEMHA	97055-05-5		Metolaclor	194,11755	-0,76 - 2,25	-0,93 – 1,43	2,97	2,91 - 3,02	7	2,47/0,79-9,70
POA	70628-36-3		Propaclor	208,09682	-1,45 - 1,33	-2,12 – 0,86	2,83	2,77 - 2,88	8	67,95/ 17,02-141,0

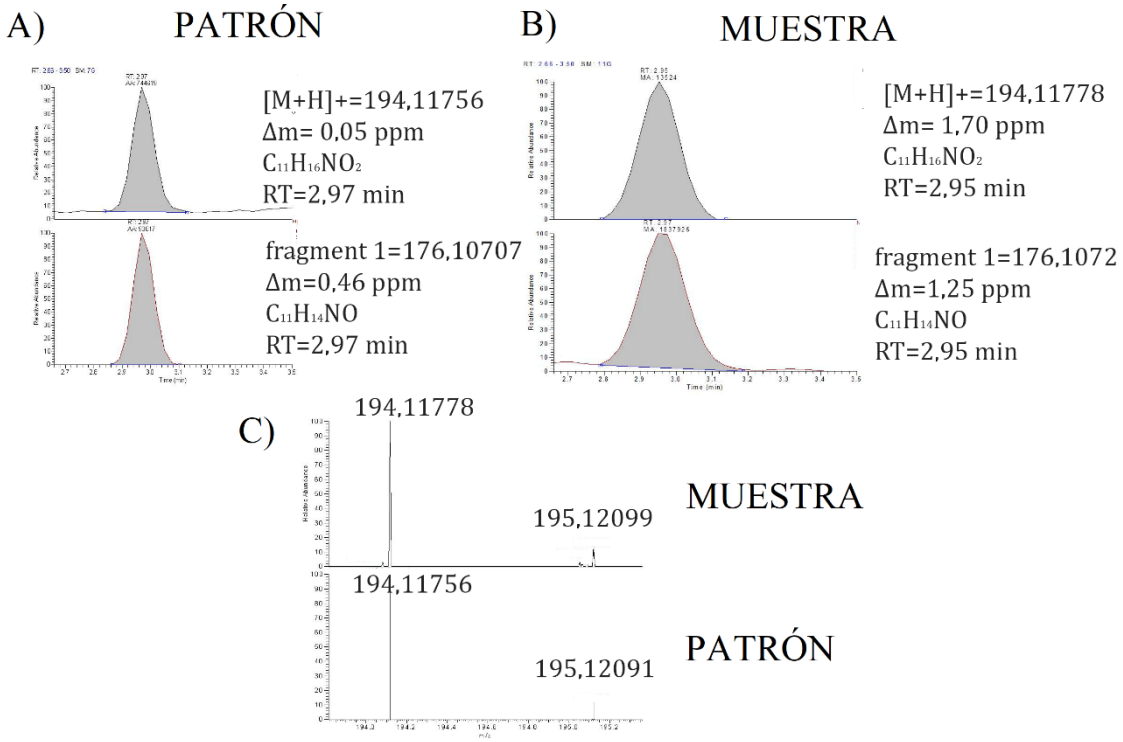


Figura 16. XIC del ion molecular y del fragmento, en un patrón (A) y en una muestra (B) así como el perfil isotópico del NEMHA en una muestra y el patrón (C).

5.1.1.3. Análisis retrospectivo de desconocidos

El tercer paso de la estrategia analítica llevada a cabo, fue un análisis para búsqueda de desconocidos mediante el enfoque 'relación fragmentación-degradación'. Mediante este enfoque se identificó el methyl-N-phenyl carbamate, un producto de transformación del methyl-N-3-hydroxyphenyl carbamate.

Como se observa en la **Figura 17a**, el methyl-N-3-hydroxyphenyl fue identificado a RT de 2,86 min. Gracias al estudio de los XIC de los fragmentos del methyl-N-3-hydroxyphenyl (m/z 151,06276 y 85,05685 Da), se identificaron dos picos a RT 1,90 min en 6 muestras que correspondían a otro compuesto posiblemente relacionado con el methyl-N-3-hydroxyphenyl. Posteriormente (**Figura 17b**), se estudió el XIC del mayor de los fragmentos (m/z 151,06276) sin aplicar fragmentación y se observó un pico a 1,90 min con área superior a cuando se aplicaba la fragmentación. Por lo tanto, el Methyl-N-phenylcarbamate (C8H9NO2), que se identificó y confirmó en 6 muestras, gracias a la presencia del ion molecular $[M+H]^+$ (151,06276 Da) y de un fragmento iónico (m/z (85.05685 Da) y a una similitud entre el perfil isotópico en muestra y el teórico del 98%. En consecuencia, el ion con m/z 151,06276 (C8H9NO2) podría ser a la vez un fragmento y un producto de transformación del metil-N-3-hydroxyphenol-carbamate.

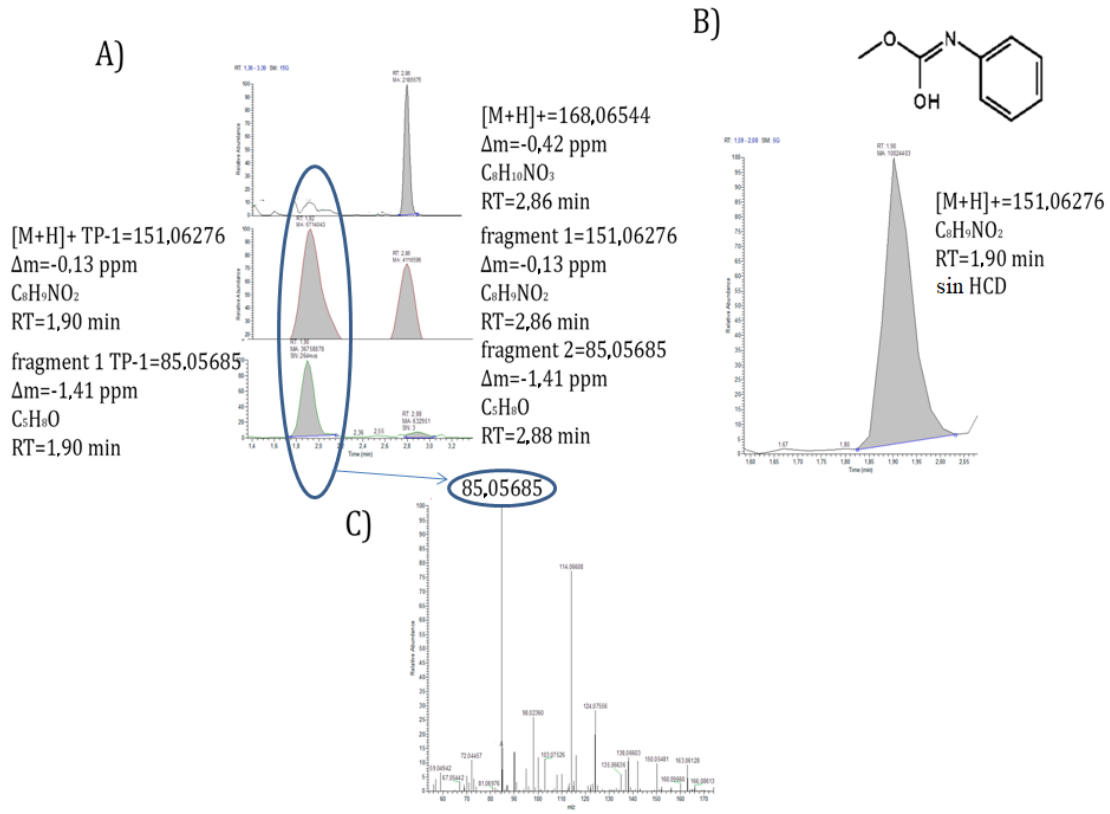


Figura 17. (A) XICs aplicando HCD (20 eV) a m/z 168,06544 (m/z del $[M+H]^+$ del methyl-N-3-hydroxyphenyl carbamate), a m/z 151,06276 (m/z del $[M+H]^+$ del methyl-N-phenylcarbamate) y a m/z 85,05685; XIC sin aplicar HCD a m/z 151,06276 (m/z del $[M+H]^+$ del methyl-N-phenylcarbamate); C) Espectro de masas del 'full scan' aplicando HCD (20 eV) al RT 1,90 min.

5.1.1.4. 'Metabolic profiling' y análisis multivariante

Se llevó a cabo un 'metabolic profiling' gracias a los cromatogramas en 'full scan' de las muestras de orina adquiridas en UHPLC-HRMS. El listado de picos (mz,RT), obtenido con el programa MzMine tal y como se describe en la sección 4.5.D, permitió llevar a cabo un PCA para explorar las relaciones entre las muestras y resaltar un pequeño número de componentes latentes que resumían el conjunto de datos adquiridos sin que se produjese una gran pérdida de información.

El modelo PCA, construido en base al procedimiento descrito en la sección 4.6.3, mostró claramente la separación entre las orinas de España (Valencia y Sabadell) y Eslovaquia (ver **Figura 18**). La validez y la robustez del modelo PCA fueron evaluadas de la siguiente manera. Se utilizó un modelo PCA de 5 componentes en el que el parámetro descriptivo ($R^2(X)$)=0,782 y el parámetro predictivo ($Q^2(X)$) = 0,351. $R^2(X)$ y $Q^2(X)$ varían entre 0 y 1, donde 1 significa que el modelo se adecúa perfectamente y 0 que no se adecúa en absoluto. En consecuencia, se obtuvo un modelo con una capacidad descriptiva aceptable ($R^2(X)$=0,782).

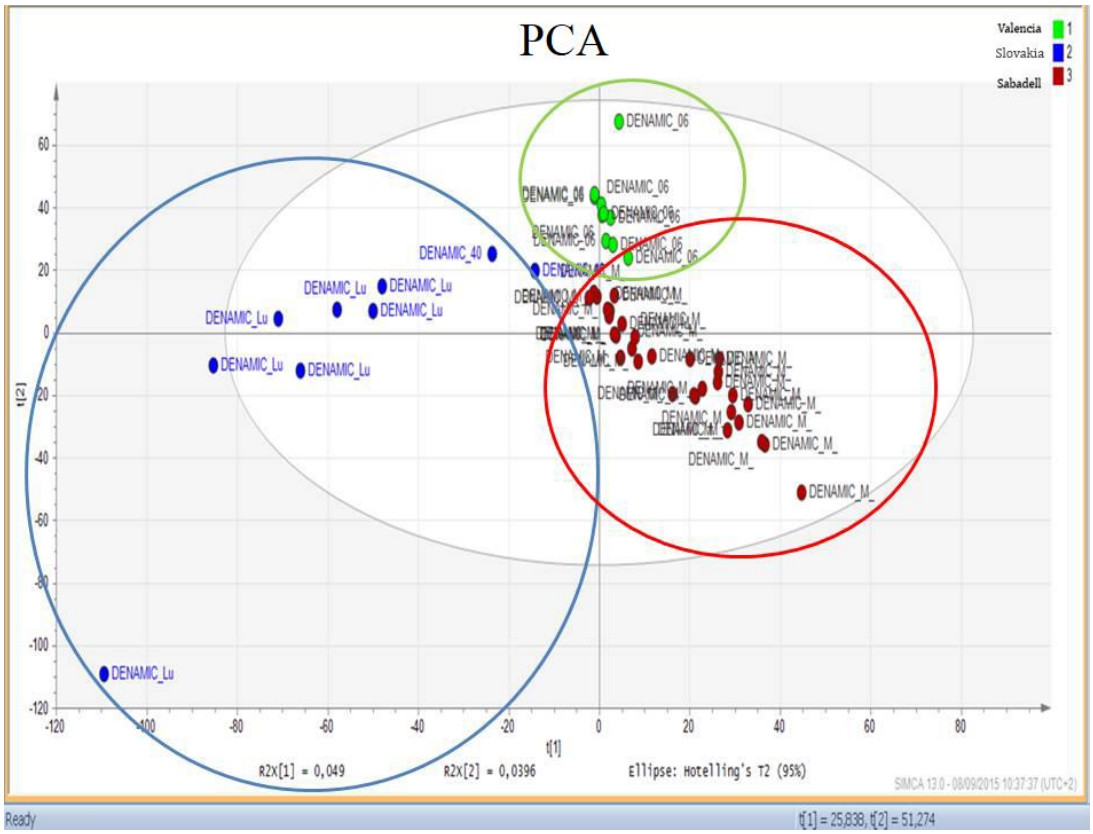


Figura 18. 'Score plot' bidimensional de un PCA de las muestras de Sabadell (n=30), Valencia (n=10) y Eslovaquia (n=9).

5.1.2. Conclusiones

-Se desarrolló una estrategia para el análisis de sospechosos de metabolitos de plaguicidas en orina, usando UHPLC-HRMS que ha demostrado ser útil para la identificación de sustancias que no están incluidas en los análisis 'target'.

-La mayoría de los 24 metabolitos identificados provisionalmente no habían sido detectados en orina con anterioridad, y 6 de ellos fueron confirmados gracias a la inyección de patrones de referencia (3-KC, 4,6-DMP, DMT, DME, NEMHA y POA).

-El análisis de desconocidos mediante el enfoque 'relación fragmentación-degradación' permitió identificar, y posteriormente confirmar usando patrones de referencia, un producto de transformación desconcido, el methyl-N-phenylcarbamate. Aunque esta metodología permite la identificación de analitos desconocidos, es necesario un mayor desarrollo de los procedimientos computacionales o utilizar equipos de análisis con más prestaciones, como los equipos híbridos MS/MS-HRMS, que permitan aumentar la eficiencia del análisis de desconocidos.

-Por otro lado, el enfoque metabolómico profundiza en el estudio de señales analíticas que permiten identificar relaciones entre muestras y posiblemente dar pié a la identificación de nuevos biomarcadores.

5.1.3. Artículo 1: Retrospective analysis of pesticide metabolites in urine using liquid chromatography coupled to high-resolution mass spectrometry

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Retrospective analysis of pesticide metabolites in urine using liquid chromatography coupled to high-resolution mass spectrometry

Antonio López [a,b,c], Pablo Dualde [a,b,c], Vicent Yusà [a,b,c,*], Clara Coscollà [a,c]

[a] Foundation for the Promotion of Health and Biomedical Research in the Valencian Region, FISABIO-Public Health, 21, Avenida Catalunya, Valencia, 46020 Spain
[b] Analytical Chemistry Department, University of Valencia,, Edifici Jeroni Muñoz, Dr. Moliner 50, 46100 Spain
[c] Public Health Laboratory of Valencia, 21, Avenida Catalunya, Valencia, 46020 Spain

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ABSTRACT

A comprehensive retrospective analysis of pesticide metabolites in urine was developed, using liquid chromatography coupled to Orbitrap high-resolution mass spectrometry (UHPLC-HRMS) that includes both post-run target (suspect screening) and non-target screening. An accurate-mass database comprising 263 pesticide metabolites was built and used for the post-run screening analysis. For non-target analysis, a "fragmentation-degradation" relationship strategy was selected. The proposed methodology was applied to 49 real urine samples from pregnant women. In the post-target analysis 26 pesticide metabolites were tentatively identified, 8 of which (2-diethylamino-6-methyl-pyrimidinol; 3-keto-carbofuran; 4,6-dimethoxy-2-pyrimidinamine; 4-hydroxy-2-isporopyl-6-methylpyrimidine; diethyl malate; diethyl maleate; N-(2-Ethyl-6-methylphenyl)-2-hydroxyacetamide and propachlor oxanilic acid) were confirmed using analytical standards. Likewise, one unknown degradation product, methyl-N-phenylcarbamate was elucidated in the non-target screening. Finally, the real urine samples were grouped according to their origin applying a metabolomic approach.

1. Introduction

The use of pesticides has increased crop yield and reduced insect-borne diseases around the world. After banning or restricting the use of environmental persistent pesticides, non-persistent pesticides, such as organophosphates (OPs), N-methyl carbamates and pyrethroids, have been increasingly used. Nevertheless, the intensive use of these compounds may impact public health, especially of vulnerable populations such as pregnant women and children living in agricultural communities [1]. There is evidence of carcinogenic, neurological, reproductive, immunological and genotoxic effects associated with the exposure to non-persistent pesticides in adults [2]. In addition, several studies have found a greater risk of adverse reproductive effects [3] and delayed or deranged neurobehavioral development in children [4,5]. Biomonitoring is the preferred approach to assess human internal exposure to environmental pollutants. However, the number of studies in pesticide exposure is limited and most of them do not include a wide range of biomarkers. The European Union (EU) is currently promoting HBM (human biomonitoring) across Europe [6]. Their aim is to integrate biomonitoring studies and environmental and health monitoring programmes, to assess human exposure to chemicals in different population groups.

For assessing internal exposure to non-persistent pesticides that have a short-life in the human body, urine is the matrix of choice. Pesticide metabolites in urine are representative of recent exposure because non-persistent pesticides are rapidly metabolized and eliminated in a few days [7].

Conventional targeted analysis of pesticide metabolites in urine using LC-MS/MS is based on establishing a method to determine a list of known analytes, which requires the use of reference standards and a purposeful chromatographic method development [8,9]. As an alternative, LC-HRMS offers the possibility of detecting hundreds of polar contaminants in a quantitative target approach due to its sensitivity and selectivity in full-scan analysis, combined with high-resolving power (> 50,000 FWHM) and accurate mass measurement (1–5 ppm) [10]. Furthermore, it allows the post-run detection of compounds suspected of being present in environmental samples (suspect screening) without reference standards, and the detection of unknown compounds [11,12].

Despite these advantages, to our knowledge only few works

* Corresponding author at: Foundation for the Promotion of Health and Biomedical Research in the Valencian Region, FISABIO-Public Health, 21, Avenida Catalunya, 46020 Valencia, Spain.
E-mail address: yusa_vic@gva.es (V. Yusà).

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have been published using UHPLC-HRMS for biomonitoring of pesticide metabolites. Cortéjade et al., 2016 [13] developed an analytical method for the targeted screening and multi-residue quantification of 38 environmental contaminants in urine, including 12 pesticides, a single pesticide metabolite (tributyl phosphate) and other compounds of different families. Roca et al., 2014 [14] developed an analytical strategy for biomonitoring of pesticides in urine that included a target analysis of 29 metabolites of pesticides (OPs, pyrethroids, herbicides and carbamates) with LOQs ranging from 0.8 to 3.2 $\mu g\,L^{-1}$, and a reduced post-target screening of different types of metabolites (60 compounds) such as pesticides (12), PAHs (12), phenols (14) and other environmental pollutants. A more in-depth study of the post-target screening for pesticide metabolites is necessary, which should combine target and post-target LC-HRMS strategies. This methodology could be used as a good analytical tool for HBM studies.

In the present study we have developed a comprehensive analytical strategy for pesticide metabolites in urine that combines a previously developed quantitative target analysis, with a massive retrospective screening of pesticide metabolites, using a database containing 263 compounds (Table SI-1) (suspect screening), including metabolites of organophosphates (63), chloroacetamides (36), carbamates (20), triazines (15), triazoles (15), sulfonylureas (11) and other chemical pesticide classes. The method uses a generic extraction based on QuEChERS and UHPLC-HRMS. The analytical methodology was applied to urine samples from pregnant women participating in a study to describe prenatal exposure to pesticides. To our knowledge, there has been no work reported previously on the analysis of pesticide metabolites using a comprehensive database for pesticide metabolites in post-target analysis, and on the use of the non-target analysis.

2. Experimental

2.1. Reagents and chemicals

All solvents used were specific for pesticide residue analysis and of analytical grade. Acetonitrile and methanol were supplied by Scharlab (Barcelona, Spain). Acetic acid (purity 98–100%), β-glucoronidase aryl sulfatase enzyme, and anyhydrous sodium acetate were obtained from Merck (KGaA, Darmstadt, Germany). Deionized water was organically and biologically purified by using a Mili-Q Ultrapure System (Milipore, Darmstadt, Germany). QuEChERS EN extraction kits, containing 4 g $MgSO_4$; 1 g NaCl, 1 g NaCitrate; 0.5 g disodium citrate sesquihydrate, were obtained from Agilent Technologies (Madrid, Spain).

2.2. Standard and stock solution

Certified commercial standards were of high purity (ranging between 75–100%) and purchased from Dr Ehrenstorfer (Augsburg, Germany), Sigma-Aldrich (Barcelona, Spain), Cerilliant-Certificated Reference Materials (Texas, USA), and Cambridge Isotope Laboratories (Massachussetts, USA). Table SI-2 shows the pesticide metabolites and internal standards (IS) used in the target analysis. Stock standard solutions of individual compounds (with concentrations between 20 and 500 $mg\,L^{-1}$) were prepared in acetonitrile by weighing powder solutions and stored at −20 °C. Multi-analyte intermediate standard solutions were prepared by diluting the individual stock solutions with acetonitrile and used for preparing working mixed-standards solutions in acetonitrile: water (10:90 v/v). The concentration of the analytes in working solutions ranged from 1000 to 5000 $ng\,mL^{-1}$ depending on the compound. A working solution of 1000 $ng\,mL^{-1}$ was also prepared containing internal standards.

2.3. Study population

The proposed analytical strategy was applied to 49 real urine samples from pregnant women from two regions of Spain: Sabadell (n=30) and Valencia (n=10), and various regions of Slovakia (n=9). Study subjects were participants in the DENAMIC project, "Developmental Neurotoxicity Assessment of Mixtures in Children" (http://www.denamic-project.eu/). Women participating in the study signed an informed consent form in each phase and the research protocol was approved by the Ethics Committee involved in the study.

2.4. Sample preparation

The sample preparation was performed following a previously developed method [14]. In short, after the homogenization of the whole sample, 5 mL of urine were mixed into a 15 mL tube with 1 mL of 0.2 M acetate buffer (3.1 mL of glacial acetic acid and 9.7 g of sodium acetate diluted in 1 L of deionized water), 20 μL of β-glucuronidase aryl sulfatase enzyme (to hydrolyze possible glucoronide-or sulfatase-conjugated metabolites), and the internal standard solution. The samples were incubated overnight at 37 °C (10–17 h).

After the enzymatic hydrolysis, a simplified QuEChERS procedure was employed to extract metabolites from urine samples. Briefly, 10 mL of acetonitrile, and a pouch of QuEChERS extraction salt packet were added to the hydrolysed sample in a 50 mL polypropylene tube. The mixture was strongly shaken and centrifuged during 10 min at 3500 rpm. The acetonitrile layer obtained was immediately transferred into a 15 mL tube and evaporated to dryness in a water bath at 37 °C under a stream of nitrogen. The dry residue was then dissolved in 200 μL of methanol: water (10/90, v/v) containing 0.1% of acetic acid, placed into a Milipore 0.2 μm Eppendorf and ultra-centrifuged (11,000 rpm, 3 min and 10° C). The final extract was transferred into an injection vial and analysed with the UHPLC-HRMS system.

2.5. UHPLC-HRMS Orbitrap analysis

Chromatographic separation was performed on an Accela liquid chromatography UHPLC system equipped with a Hypersil Gold aQ column (100 mm × 2.1 mm, 1.9 μm) both from ThermoFisher Scientific (Bremen, Germany). The flow rate used was 400 $\mu L\,min^{-1}$ and the injection volume was 10 μL. Separations were performed using a binary gradient. The analysis started with 95% H_2O with 0.1% acetic acid (solvent A). After 1 min, this percentage was linearly decreased down to 45% within 5 min. After that, solvent A was decreased quickly to 0% in 0.5 min and maintained for 1.5 min. The composition was increased to initial conditions in 0.5 min, followed by a re-equilibration time of 12.5 min. The total run time was 20 min

Data acquisition was performed on the Orbitrap mass spectrometer Exactive™ analyzer (Thermo Scientific, Bremen, Germany). The system was equipped with a heat electrospray ionization interface (HESI-II). The ion source parameters were as follow: 3.5 kV (positive mode) and 2.5 kV (negative mode); sheath gas flow-rate: 55; auxiliary gas flow-rate: 10; skimmer voltage: 23 V; heater temperature: 300 °C; capillary temperature: 150 °C; capillary voltage: 45 V and tube lens voltage: 120 V. In this study two independent runs were carried out using electrospray ionization in positive (ESI +) and negative (ESI -) mode with HCD (Higher collision-induced dissociation cell) switching for the fragmentation of ions in a non-selective manner operating with N_2 (>95%) and employing a collision energy of 20 eV.

The system operating in full-scan mode (50–800 *m/z*) at a resolving power of 25,000 FWHM (scan time=250 ms). No-specific

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lock mass was used for internal mass axis correction. The automatic gain control (AGC) was set to 1×10^6. Data were processed with the Trace Finder 3.1 software (Thermo Scientific, Bremen, Germany).

2.6. Identification and confirmation criteria for pesticide metabolites

For compound identification in the target analysis, the following criteria were established [15]: (i) Mass accuracy of the molecular ion < 5 ppm; (ii) mass accuracy of the fragment ion < 5 ppm; (iii) isotopic pattern similar to the theoretical isotopic pattern (the relative intensity of the A+1 and/or A+2 isotope peaks in the real sample shall correspond to the theoretical relative intensities). As we use reference standard solutions for target analysis, the confirmation criteria included: (iv) retention time (t_R) similar to that of the reference standard ± 0.20 min

In post-run target screening, searching for metabolite compounds was carried out by using automated software tools. The identification and confirmation settings in the Trace Finder programme included a threshold override of 10,000, with S/N of 5, and a mass tolerance of 5 ppm for the molecular ion; an intensity threshold of 5,000 and a mass tolerance of 5 ppm for fragments. For the isotopic pattern a fit threshold of 90%, an allowed relative intensity (RI) deviation of 30%, and a mass deviation of 5 ppm were selected in the Trace Finder software. The resulting one-point calibration (50 ng mL^{-1}) on suspected peaks yielded semi-quantitative concentrations. The semiquantitative approach gives only approximate concentrations, because the purpose of a post-target study is basically to identify and, if possible, to confirm pesticide metabolites but it is not an accurate quantification method.

In the non-target approach, the search of pesticide metabolites was undertaken using the "fragmentation-degradation relationship" approach [16]. This methodology relies on the fact that low-molecular weight compounds often display parallel degradation and fragmentation pathways. Therefore, should a fragment ion appear not only at the t_R corresponding to the parent molecule, but also at a different t_R, it may indicate the presence of a molecule with similar structure, i.e., a possible metabolite. Checking the extracted ion chromatogram (XIC) of fragment ions and their mass spectra at retention times for peaks not corresponding to the parent molecule allows the straightforward detection of novel metabolites. A final confirmation was performed comparing retention times to those obtained for reference standards (when available).

2.7. Quality control procedure

The limit of quantification (LOQ) for each compound was established at the lowest validated spiked level to meet the performance acceptability criteria of the method defined for pesticide residue (mean recoveries ranged between 60% and 120% with RSD < 30%). In each analytical batch, various quality control samples (QC) were used to check the extraction procedure efficiency and to ensure a good quantification of real samples. The QC were prepared spiking blank urine samples using the working standard solutions and were subjected to the same extraction and analysis procedures of real samples and calibration curve points. Blank urine samples were introduced in each analytical batch in order to estimate possible contamination. Samples with signals for pesticide metabolites lower than 30% of LOQ were considered not contaminated.

2.8. Metabolic profiling and data handling

Data handling tasks in metabolomics can be roughly divided into two steps: data processing and data analysis. The data processing step consists of low-level processing of raw data with signal processing methods and combining data between measurements. These tasks transform the raw data into format that is easy to use in the subsequent data analysis step. The data analysis stage includes tasks for analysis and interpretation of processed data. This typically includes multivariate analyses such as clustering of metabolic profiles or discovering important differences between groups of samples. [17].

2.8.1. Data processing

The analytical platform in metabolomic experiments generates large amounts of data from a single sample which must be preprocessed before multivariate analysis. Following the acquisition by a given analytical tool, metabolic fingerprints need to be deconvoluted to allow conversion of the three dimensional raw data (*m/z*, retention time, ion current) to time- and mass-aligned chromatographic peaks with associated peak areas.

In this study, data were processed by the open-source MzMine 2.10 software and the following steps were carried out: 1. Mass detection: applying the exact mass algorithm, the noise level was set at 10^6; 2. Chromatogram builder: minimum height = 10^4, minimum time span = 0.09 min and *m/z* tolerance = 5 ppm (relative tolerance); 3. Chromatogram deconvolution: applying the baseline cut-off algorithm, minimum peak height = 40000, peak duration range = 0.05–0.5 min and baseline level = 60000; 4. Isotope grouping: *m/z* tolerance = 5 ppm (relative tolerance), RT tolerance = 0.1 min (absolute), monotonic shape = OK, maximum charge = 1, representative isotope = most intense; 5. Alignment: to correct differences in time and masses between samples, the peaks were aligned using the Join aligner algorithm and the following parameters: *m/z* tolerance = 10 ppm, weight for *m/z* = 90, weight for RT = 10, retention time tolerance = 0.25 min, require same charge state = OK.

2.8.2. Data analysis

SIMCA (v13.0, Umetrics, Umeå, Sweden) was used to perform multivariate data analysis. Before multivariate statistical analysis, Pareto scaling was applied to all variables in order to reduce the noise in the data and thereby enhance the information content and quality. The data were analysed by Principal Component Analysis (PCA). The various mass peaks constituting the mass fingerprints (pairs of chromatographic retention time and *m/z* ratio) were considered as independent variables. The validity and robustness of the PCA model was evaluated by $R^2(X)$ and $Q^2(X)$.

The PCA model was applied in ESI+ mode to 49 analysed samples of urine from different regions of Spain and Slovakia.

3. Results and discussion

3.1. Target analysis

The first step of the developed analytical strategy was the target analysis of samples following a previously developed method [14]. Overall, nine pesticide metabolites were detected in the 49 urine samples analysed: 4 generic dialkyl phosphate metabolites such as diethyl phosphate (DEP), diethyl thiophosphate (DETP), dimethyl thiophosphate (DMTP) and dimethyl dithiophosphate (DMDTP); 4 specific metabolites of organophosphate pesticides such as 2-diethylamino-6-methyl-4-pyrimidol (DEAMPY), 2-isopropyl-6-methyl-4-pyrimidinol (IMPY), 4-nitrophenol (PNP), 3,5,6-trichloro-2-pyridinol (TCPY) and one specific phenoxy herbicide (2,4-dichlorophenoxyacetic acid: 2,4-D). Table 1 shows the concentration of these metabolites in the real samples. The concentrations ranged from 0.9 to 98.9 ng mL^{-1}, with frequencies of

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Table 1
Average concentrations and frequency of detection of target pesticide metabolites in urine (ng mL^{-1}) (N=49).

Station		Sabadell (N=30)			Valencia (N=10)			Slovakia (N=9)		
Metabolite	**Acronym**	**Frequency of detection (%)[a]**	**Range (ng mL^{-1})**	**Average (ng mL^{-1})[b]**	**Frequency of detection (%)[a]**	**Range (ng mL^{-1})**	**Average (ng mL^{-1})[b]**	**Frequency of detection (%)[a]**	**Range (ng mL^{-1})**	**Average (ng mL^{-1})[b]**
Diethyl phosphate	DEP	13.3	18.1–98.9	61.6	–	–	–			
Diethyl thiophosphate	DETP	3.3	–	13.3	–	–	–			
Dimethyl thiophosphate	DMTP	43.3	2.9–26.9	12.0	10	–	5.1			
2-diethylamino-6-methyl-4-pyrimidinol	DEAMPY	26.7	1.7–3.9	2.6	–	–	–			
4-nitrophenol	PNP	3.3	–	2.0	–	–	–			
3,5,6-trichloro-2-pyridinol	TCPY	16.7	1.1–23.2	5.9	30	2.1–5.5	4.3	71.4	0.9–7.8	3.8
2,4-dichlorophenoxyacetic acid	2,4-D	3.3	–	1.5	–	–	–			
Dimethyl dithiophosphate	DMDTP	–	–	–	30	2.81–15.5	9.4			
2-isopropyl-6-methyl-4-pyrimidinol	IMPY	–	–	–	20	1.8–4.2	3.0			

N= total samples
[a] Frequency of detection was calculated for samples with concentrations above the limit of detection (LOD)
[b] The average was calculated form the arithmetic mean with concentrations above LOQ

detection from 3 to 71%.

As an example, Figure SI-1 shows the accurate mass extracted-ion chromatograms of the molecular ion (with HCD) for 2,4-D, with a mass window of 10 ppm (5 ppm mass error) of a urine sample collected in Sabadell. The compound fulfilled the identification criteria for target analysis.

3.2. Post-run target screening of real samples

A customised theoretical database was built containing 263 pesticide metabolites. For each substance, the screening database included the elemental composition (molecular formula) and the theoretical accurate mass of the monitored ion. Existing data from available databases [18–21] and the previous literature [22] were used. This is a theoretical database where no standards were analysed to get characteristic fragments. Information about fragments was included when available in the literature, mainly from HRMS (exact mass) and QqQ (nominal mass) studies [18,19,23–28] (see Table SI-3).

The second step of the strategy included a post-run target screening using the built database and the full-scan signals from the analysis. Twenty six pesticide metabolites were tentatively identified (Table SI-4) in the retrospective analysis of the chromatograms. All these substances met the identification criteria described previously. Using the only 8 commercially available reference standard solutions, we confirmed the detection of the following metabolites: 2-diethylamino-6-methyl-pyrimidinol (2-DMP); 3-ketocarbofuran (3-KC); 4,6-dimethoxy-2-pyrimidinamine (4,6-DMP); 4-hydroxy-2-isopropyl-6-methylpyrimidine (HIMPY); diethyl malate (DMT); diethyl maleate (DME); N-(2-Ethyl-6-methylphenyl)-2-hydroxyacetamide (NEMHA) and propachlor oxanilic acid (POA)) (Table 2). The rest of metabolites could not be confirmed because no standards were commercially available. All confirmed metabolites presented low frequencies of detection ranging from 4% (diethyl malate and 2-diethylamino-6-methyl-4-pyrimidinol) to 18% (diethyl maleate). Regarding the estimated concentrations (semiquantitative), the highest levels were observed for propachlor oxanilic acid (maximum concentration of 141.04 ng mL^{-1}). The other metabolites contained low average concentration levels ranging from 1.24 ng mL^{-1} (diethyl malate) to 14.67 ng mL^{-1} (4,6-Dimethoxy-2-pyrimidinamine).

As an example of the pesticide metabolites detected in the post-run target screening, Fig. 1 shows the accurate mass extracted-ion chromatograms (XIC) of the molecular ion, characteristic fragments and isotopic pattern for the 3-keto-carbofuran metabolite. Fig. SI 2-8 shows the XIC of the other confirmed metabolites.

3-ketocarbofuran (C12H13NO4) is a metabolite of carbofuran [29], which is currently banned in the EU and was identified in 4 samples (Fig. 1). Confirmation was accomplished by accurate mass measurements ($[M+H]^+$=236.09180, $C_{12}H_{14}NO_4$), isotope pattern matching and the presence of a diagnostic fragment ion (m/z 151.07505, $C_9H_{11}O_2$), fulfilling the identification criteria. The theoretical and experimental isotopic patterns for 3-ketocarbofuran (M+1, ^{13}C) are also shown, with estimated average concentrations around 8 ng mL^{-1}.

Propachlor oxanilic acid ($C_{11}H_{13}NO_3$) is a metabolite of propachlor, a herbicide banned in EU since 2008 [30]. It was identified in 8 samples (Fig. SI-2). Confirmation was accomplished by accurate mass measurements ($[M+H]^+$=208.09680, $C_{11}H_{14}NO_3$), isotope pattern matching and the presence of a diagnostic fragment ion (m/z 150.05487, $C_8H_8NO_2$). This pesticide metabolite fulfilled the identification criteria. The theoretical and experimental isotopic patterns for propachlor oxanilic acid (M+1, ^{13}C) are also shown.

2-diethylamino-6-methyl-pyrimidinol ($C_9H_{15}N_3O$) is a metabolite of pyrimiphos-methyl, an organophosphate used to control insects and miles [31] and was identified in only 2 samples (Fig. SI-3). Confirmation was accomplished by accurate mass measurements ($[M+H]^+$=182.12879, $C_9H_{16}N_3O$), isotope pattern matching and the presence of a diagnostic fragment ion (m/z 154.09784, $C_7H_{12}N_3O$). This pesticide metabolite fulfilled the identification criteria. The theoretical and experimental isotopic patterns for 2-diethylamino-6-methyl-pyrimidinol (M+1, ^{13}C) are also shown, with estimated average concentrations around 7 ng mL^{-1}.

4,6-dimethoxy-2-pyrimidinamine ($C_6H_9N_3O_2$) is a metabolite of bensulfuron-methyl, a herbicide used in cereals such as rice [32] and was identified in 12% of the samples (Fig. SI-4). Confirmation was accomplished by accurate mass measurements ($[M+H]^+$=156.07675, $C_6H_9N_3O_2$), isotope pattern matching and the presence of one diagnostic fragment ion (m/z 139.05021, $C_6H_7N_2O_2$). This pesticide metabolite fulfilled the identification criteria. The theoretical and experimental isotopic patterns for 4,6-dimethoxy-2-pyrimidinamine (M+1, ^{13}C) are also shown, with estimated average concentrations around 15 ng mL^{-1}.

4-hydroxy-2-isopropyl-6-methylpyrimidine ($C_8H_{12}N_2O$) is a metabolite of diazinon, an organophosphate banned in EU [33] and was identified in 16% of the samples (Fig. SI-5). Confirmation was accomplished by accurate mass measurements ([M+H]

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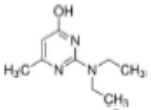
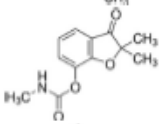
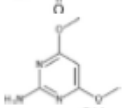
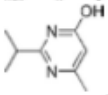
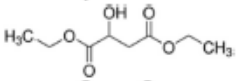
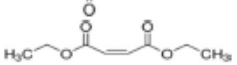
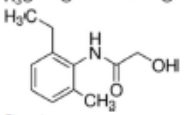
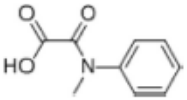
Table 2
Pesticide metabolites identified and confirmed with standards in the post-run target screening (suspected screening) (n=49).

Metabolite	CAS number	Structure	Parent	Monitored Mass*[M+H]+	Δm (ppm) ([M+H]+)	Δm (ppm) (FRAG. 1)	Retention time of standard (min)	Range of Retention time of samples (min)	Number of detected samples	Average/ Estimated range level (ng mL−1)a
2-diethylamino-6-methyl-4-pyrimidinol	42487–72-9		Pyrimiphos-methyl	182.12879	−0.12–0.99	−1.52–3.37	2.41	2.35–2.40	2	6.91 / 5.89–7.93
3-ketocarbofuran	16709–30–1		Carbofuran	236.09173	−0.87–0.56	1.15–3.43	2.87	2.85–2.91	4	8.34/ 4.0–14.40
4,6-Dimethoxy-2-pyrimidinamine	36315–01-2		Bensulfuron-methyl	156.07675	−0.63–0.57	−0.23–0.53	2.45	2.38–2.51	6	14.67/ 1.86–31.28
4-hydroxy-2-isopropyl-6-methylpyrimidine	2814–20–2		Diazinon	153.10224	−1.80– 0.59	−0.93–0.86	2.36	2.30–2.43	8	4.71 / 1.63–9.62
Diethyl malate	7554–12-3		Malathion	191.09140	0.26–2.22	2.12–2.54	2.67	2.62–2.66	2	1.24/ 1.06–1.42
Diethylmaleate	141–05–9		Malathion	173.08083	−0.78–2.48	−0.63–2.19	3.06	3.02–3.11	9	14.58/ 2.01–45.99
N-(2-Ethyl-6-methylphenyl)– 2-hydroxyacetamide	97055–05-5		Metolachlor	194.11755	−0.76–2.25	−0.93–1.43	2.97	2.91–3.02	7	2.47 /0.79–9.70
Metabolite	**CAS number**	**Structure**	**Parent**	**Monitored Mass*[M+H]+**	**Δm (ppm) ([M+H]+)**	**Δm (ppm) (FRAG. 1)**	**Retention time of standard (min)**	**Range of Retention time of samples (min)**	**Number of detected samples**	**Average/ Estimated range level (ng mL−1)a**
Propachlor oxanilic acid	70628–36-3		Propachlor	208.09682	−1.45–1.33	−2.12-0-86	2.83	2.77–2.88	8	67.95/ 17.02–141.04

* All isotopic pattern >90%.
a Semiquantitative concentrations.

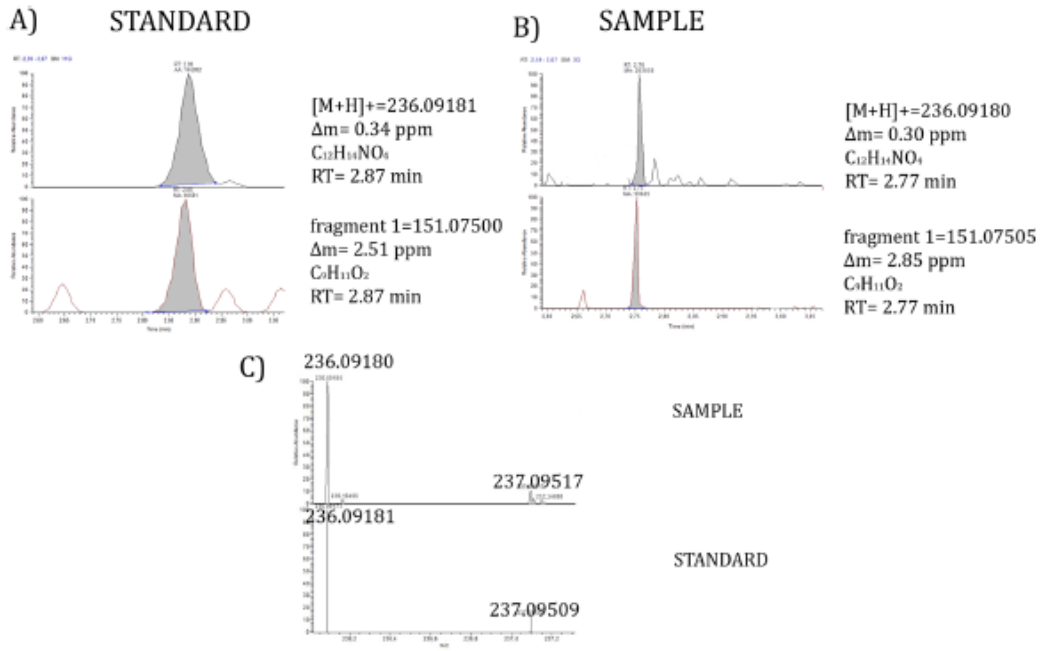


Fig. 1. (A) Accurate mass extracted ion chromatograms (XIC) of the molecular ion and a characteristic fragment for 3-ketocarbofuran in a standard; (B) Accurate mass extracted ion chromatograms (XIC) of the molecular ion and a characteristic fragment for 3-ketocarbofuran in a real sample; (C) Isotopic patterns of the molecular ion of the sample and the standard.

$^{+}$ = 153.10224, $C_{11}H_{14}NO_3$), isotope pattern matching and the presence of one diagnostic fragment ion (*m/z* 137.07094, $C_8H_8NO_2$), fulfilling the identification criteria. The theoretical and experimental isotopic patterns for 4-hydroxy-2-isopropyl-6-methylpyrimidine (M+1, ^{13}C) are also shown, with estimated average concentrations around 5 ng mL^{-1}.

Diethyl malate ($C_8H_{14}O_5$) and diethylmaleate ($C_8H_{12}O_4$) are metabolites of malathion, an insecticide used in vegetables [34]. Diethyl malate was identified in only 2 samples (Fig. SI-6) and diethylmaleate was identified in 9 samples (Fig. SI-7). Diethyl malate was confirmed by accurate mass measurements ([M+H]$^{+}$ = 191.09140, $C_8H_{15}O_5$), isotope pattern matching and the presence of one diagnostic fragment ion (*m/z* 150.05487, $C_6H_9O_4$). Diethylmaleate was confirmed by accurate mass measurements ([M+H]+ = 173.08083, $C_8H_{13}O_4$), isotope pattern matching and the presence of one diagnostic fragment ion (*m/z* 127.05487, $C_6H_7O_3$). Both pesticide metabolites fulfilled the identification criteria. The theoretical and experimental isotopic patterns for both metabolites (M+1, ^{13}C) are also shown, with estimated average concentrations around 2 ng mL^{-1} and 15 ng mL^{-1} respectively.

N-(2-Ethyl-6-methylphenyl)−2-hydroxyacetamide ($C_{11}H_{15}NO_2$) is a metabolite of metolachlor, a herbicide banned in EU and was identified in 7 samples (Fig. SI-8). Confirmation was accomplished by accurate mass measurements ([M+H]$^{+}$ = 194.11755, $C_{11}H_{16}NO_2$), isotope pattern matching and the presence of one diagnostic fragment ion (*m/z* 176.10699, $C_{11}H_{14}NO$), fulfilling the identification criteria. The theoretical and experimental isotopic patterns for N-(2-Ethyl-6-methylphenyl)−2-hydroxyacetamide (M+1, ^{13}C) are also shown, with estimated average concentrations around 2.5 ng mL^{-1}.

3.3. Non-target and metabolomic analysis

3.3.1. Non-target analysis

The third step of the developed analytical strategy was a non-target analysis following the 'fragmentation-degradation' relationship approach [16]. In this approach, accurate mass measurements of ions of interest are used to establish relationships between fragmentation of parent pesticides in the instrument (HCD-fragmentation) and possible degradation products in urine. One non-target metabolite was identified (methyl-N-phenylcarbamate, $C_8H_9NO_2$), a transformation product of methyl-N-3-hydroxyphenyl-carbamate.

Methyl-N-phenylcarbamate, (C8H9NO2) was identified and confirmed in 6 samples, using the presence of the [M+H]+ and one fragment (RT=1.90 min). Methyl-N-phenylcarbamate was confirmed using the presence of [M+H]$^{+}$=151.06276, the presence of one diagnostic fragment ion (*m/z* 85.05685) and the isotopic pattern matching (98%) (Fig. 2). Following the screening procedure, the extracted ion chromatogram (EIC) of the fragment ion was checked. An additional peak on the EIC was found at RT=1.90 min (Fig. 2). Furthermore, the ion of 151.06276 was present in the recorded XIC without HCD (no fragmentation) at higher response. The mass spectrum at 1.90 min revealed that 85.05685 was the most abundant ion (C_5H_8O, a fragment ion of methyl-N-phenylcarbamate) applying HCD=20 eV. Consequently, the ion with *m/z* 151.06276 ($C_8H_9NO_2$) could be at the same time a fragment and a transformation product of methyl-N-3-hydroxyphenyl-carbamate.

3.3.2. Metabolomic profiling and multivariate analysis

The full-scan chromatograms of urine samples acquired using LC-HRMS provided a metabolic profiling of samples. Following a series of steps routinely used in metabolomics such as signal

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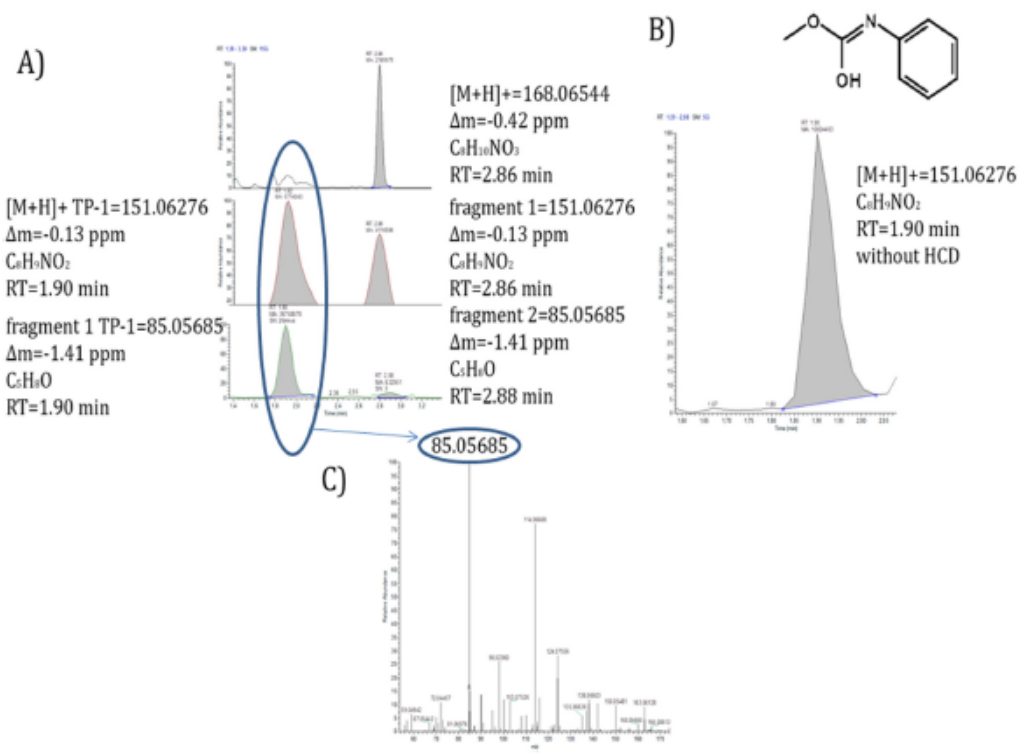

Fig. 2. (A) Extracted ion chromatograms (XIC) of methyl-N-3-hydrophenyl-carbamate ($m/z=168.06544$); its fragment ($m/z=151.06276$) and a transformation product of methyl-N-3-hydrophenyl-carbamate (RT=1.90 min); (B) extracted ion chromatogram at m/z 151.06276 without HCD. Accurate mass extracted ion chromatograms (XIC) of the molecular ion and a characteristic fragment for methyl-N-3-hydrophenyl-carbamate; (C) Accurate mass spectrum at 1.90 min, which corresponds to transformation product-1.

processing and multivariate data analysis (see the Experimental section), we performed a principal component analysis (PCA) of the samples analysed. PCA is the most commonly used method to explore relationships between samples in metabolic profiling studies. It aims to extract a small number of latent components that summarize the measured data with minimal information being lost by taking advantage of the correlation structure of peak intensities.

The PCA model built clearly shows the separation between urine samples from the two different locations in Spain (Valencia and Sabadell) and Slovakia (see Fig. 3), which indicates different metabolic profiles for each group of samples.

The validity and the robustness of this PCA model were checked as follows. A five component PCA model was selected, with the descriptive parameter ($R^2(X)=0.782$) and the predictive parameter ($Q^2(X)=0.351$). $R^2(X)$ and $Q^2(X)$ vary between 0 and 1, where 1 means a perfectly fitting model and 0 no fit at all. Consequently, acceptable descriptive capability was obtained ($R^2(X)=0.782$).

A further study will allow us to identify biomarkers and determine which compounds have the strongest discriminatory power between sample groups.

4. Conclusions

We have developed a comprehensive strategy for retrospective analysis of pesticide metabolites in urine, using UHPLC-HRMS, that has proved to be useful for the identification of new substances not included in the scope of the target analysis. Twenty-six metabolites, the majority of which had not previously been detected in urine, were identified, with eight of them having been fully confirmed using analytical standards (2-DMP; 3-KC; 4,6-DMP; HIMPY; DMT; DME; NEMHA and POA).

One unknown transformation product, using the 'fragmentation-degradation' relationship was identified and finally confirmed using analytical standards, methyl-N-phenylcarbamate, and its transformation product of methyl-N-(3-hydroxyphenyl) carbamate. Although this methodology provided, in some cases, good results and unknown metabolites could be elucidated, more computational efforts are necessary for the identification of unknown metabolites.

Complementarily to the application of the developed analytical strategy, it is possible to use a metabolomics approach to deepen the study of the analytical signals in order to identify relationships between samples, and possibly to find some new biomarkers.

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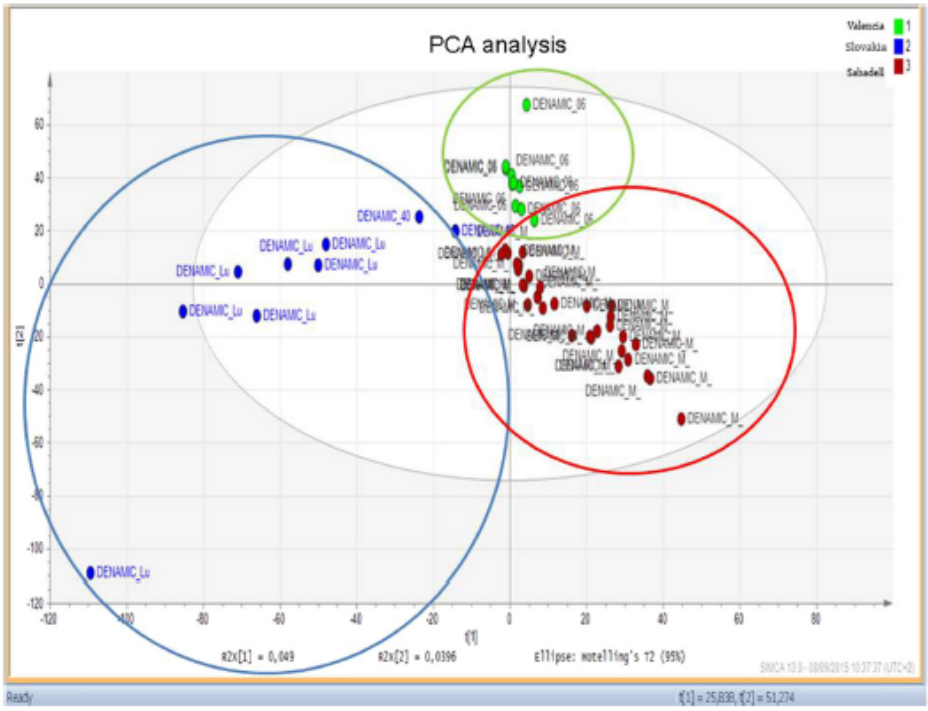

Fig. 3. Two dimensional PCA score plot of human urine samples for Sabadell (N=30), Valencia (N=10) and Slovakia (N=9).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.talanta.2016.07.065.

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5.2. Capítulo 2: Optimización del poder de resolución, fragmentación y calibración de masas en un Orbitrap MS para el análisis de 24 metabolitos de plaguicidas en orina

En el presente capítulo se optimizaron distintos parámetros del espectrómetro de masas de alta resolución Orbitrap de un método HPLC-HRMS previamene desarrollado para la determinación de metabolitos de plaguicidas en orina humana **(Roca et al., 2014b)**. La adquisición se llevó a cabo en 'full scan' (m/z 50-800) y, en concreto, se optimizaron los siguientes parámetros espectrométricos: Poder de resolución, tipo y energía de fragmentación y tipo de calibración de masas.
Para la optimización del poder de resolución, se compararon tres poderes de resolución diferentes (10.000, 25.000 y 50.000 FWHM). En el caso de la fragmentación se comparó la fragmentación CID a diferentes energías con la fragmentación HCD 20eV utilizada en el método original. Por último, se comparó el uso de calibración externa con la calibración externa e interna y se evaluó en base a la exactitud de masa obtenida.

5.2.1. Resultados y discusión

5.2.1.1. Optimización del poder de resolución

Para la optimización del poder de resolución se utilizarón blancos de orina (n=6) fortificados con patrones de los 24 plaguicidas de la **Tabla 5** a 50 ng/mL. La evaluación del poder de resolución se llevo a cabo en base a la intensidad de señal (área) y exactitud de masa (Δm) en los iones moleculares y en los fragmentos. La fragmentación utilizada fue HCD 20 eV, se adquirió en positivo y negativo en inyecciones separadas y se usó calibración de masas externa.

Con respecto a los iones moleculares, algunos metabolitos como DEAMPY, IMPY, PNP, TCPy, MNP, DEP, DETP o DIMET, presentaron respuestas (áreas) similares a los tres poderes de resolución evaluados. Sin embargo, para DMDTP, Methamidophos, OMET, cisDCCA, DBCA, ATZM, ALAM, METM y 2,4,5-T, las mayores áreas se obtuvieron a una resolución de 25,000 FWHM. Con respecto a los 7 compuestos restantes, el poder de resolución de 25,000 FWHM presentó respuestas similares a las obtenidas en el mejor de los casos para cada compuesto.
En cuanto a los fragmentos, 17 de los 20 compuestos en los que se evaluaron los fragmentos iónicos, presentaron las mayores respuestas con un poder de resolución de 50,000 FWHM.
En cuando a la exactitud de masa, tal y como se observa en la **Tabla 13**, todos los poderes de resolución permitieron exactitudes de masa inferiores a 5 ppm, excepto en dos iones a 10,000 FWHM.
En resumen, tanto el poder de resolución a 25,000 como a 50,000 FWHM ofrecieron resultados similares. Sin embargo, teniendo en cuenta que en el Orbitrap la velocidad de

scan está relacionada con el poder de resolución y que, por tanto, a mayor resolución, menor número de puntos por pico cromatográfico, se decidió seleccionar la resolución de 25,000 FWHM como la más óptima.

Tabla 13. Recuento de iones (moleculares y de fragmentos) agrupados en base a la exactitud de masa Δm en ppm a 10,000, 25,000 and 50,000 FWHM (n=6).

Poder de resolución	**Δm(ppm)**					
	≤ 1	**]1 – 2]**	**]2 – 3]**	**]3 – 4]**	**]4 – 5]**	**> 5**
10.000	2	12	19	9	0	2
25.000	1	22	19	2	0	0
50.000	0	30	11	3	0	0

Condiciones de adquisición: ESI+/ESI- in inyecciónes separadas, Fragmentación a 20 eV y calibración de masas externa

5.2.1.2. Optimización del modo de fragmentación

Para la optimización de la fragmentación en primer lugar se estudió el modo de fragmentación CID a cuatro energías de fragmentación diferentes (10, 20, 30 y 40 eV) utilizando cinco blancos de orina fortificados (50 ng/mL) utilizando el poder de resolución previamente optimizado (25,000 FWHM) y calibración de masas externa. Las mayores respuestas (áreas) en la mayoría de los iones fragmento fueron obtenidas con CID (40eV). A modo de ejemplo, la **Figura 19** muestra el área del fragmento de PBA a diferentes energías de fragmentación.

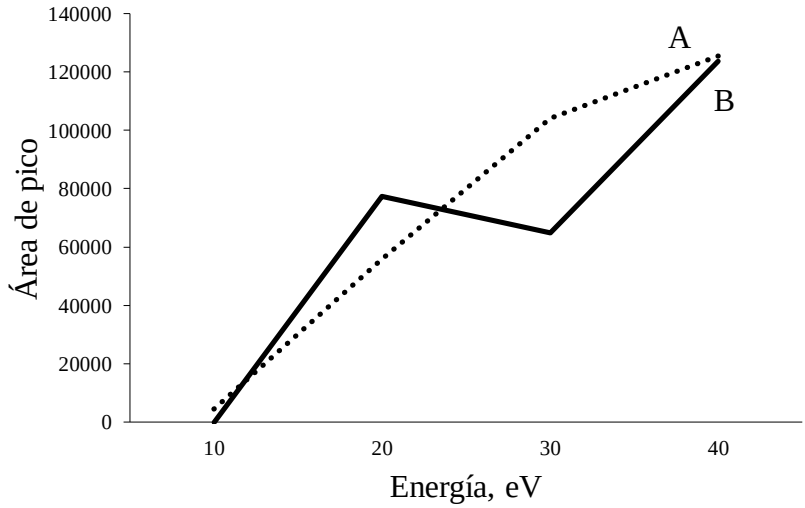

Figura 19. Área del fragmento de PBA a diferentes energías de fragmentación en modo CID. A) En una inyección adquirida solo en positivo; B) En una inyección adquirida en positivo y negativo a la vez. Se utilizó un poder de resolución de 25.000 FWHM y calibración de masas externa en todos los experimentos (n=5).

Posteriormente, se compararon las áreas de fragmentos obtenidas utilizando CID (40 eV) y HCD (20 eV) utilizando 6 blancos de orina fortificados. Al mismo tiempo, se compararon los resultados de áreas de fragmentos obtenidas realizando la adquisición en positivo y negativo en una inyección o en inyecciones separadas. Los resultados se muestran en la **Figura 20**.

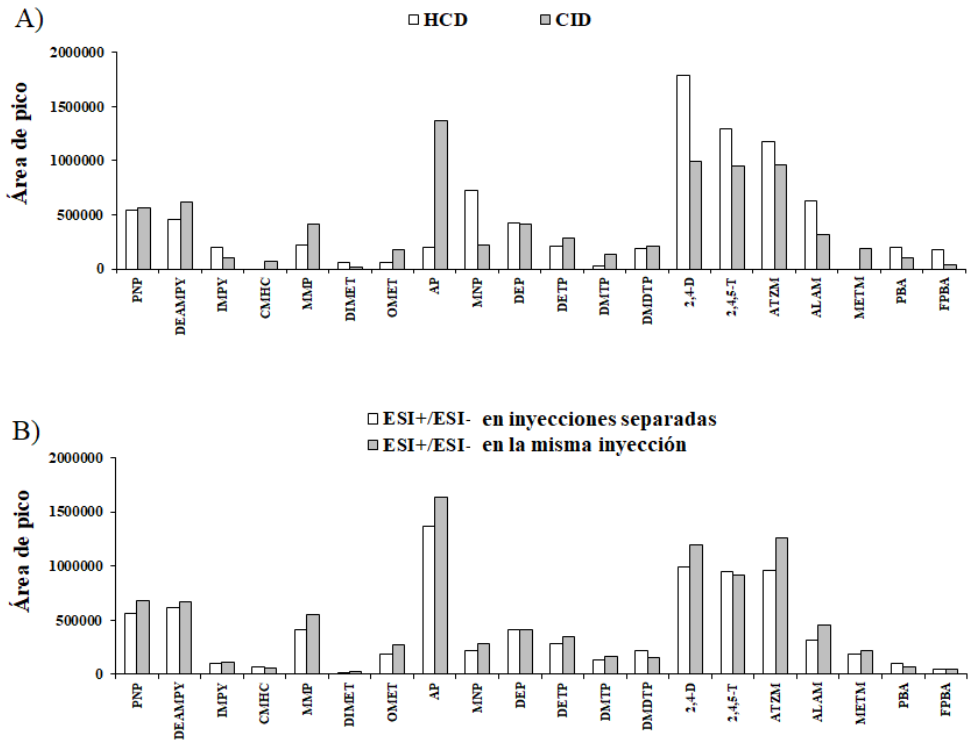

Figura 20. A) Comparación entre las áreas de fragmentos utilizando CID (40 eV) y HCD (20 eV) B) Comparación entre las áreas de fragmentos utilizando CID (40 eV) adquiriendo en positivo y negativo en inyecciones separadas o en la misma inyección. En todos los experimentos se usó un poder de resolución de 25,000 FWHM y calibración de masas externa.

En cuanto a la comparación entre CID y HCD, tal y como se observa en la **Figura 20 A**, se obtuvieron áreas similares. Sin embargo, se seleccionó CID (40 eV) como el método óptimo debido a las áreas bajas que presentaban algunos fragmentos (CMHC y METM) en HCD (20 eV). Por último, como se observa en la **Figura 20 B** se obtuvieron áreas de fragmentos similares o mejores al adquirir en positivo y negativo en la misma inyección, por lo tanto se seleccionó esta opción debido a que la adquisición simultánea en positivo y negativo reduce el tiempo de análisis.

5.2.1.3. Optimización del método de calibración de masas.

Para la optimización del modo de calibración de masas, se utilizó el poder de resolución (25,000 FWHM) y modo de fragmentación (CID 40eV) previamente optimizados, además se adquirió en positivo y negativo en la misma inyección. El uso de tan solo calibración de masas externa fue comparado con el uso de calibración de masas externa

e interna para los analitos adquiridos en modo positivo. Para ello se utilizó cafeína como calibrante interno ([M+H]+ m/z = 195, 08765 Da). La idoneaidad del modo de calibración se estableció calculando la exactitud de masa (Δm en ppm) en cinco blancos de orina fortificados.

Tal y como se observa en la **Tabla 14**, el uso de cafeína como calibrante interno mejora la exactitud de masa tanto en los iones moleculares como en los fragmentos. Por lo que se seleccionó el uso de cafeína como calibrante interno en positivo.

Tabla 14. Exactituades de masa medias (Δm) (ppm) y desviaciones estándar usando calibración externa + calibración interna o usando solo calibración externa para iones moleculares y fragmentos en ESI positivo (n=5).

Biomarcador	**Ión molecular**				**Fragmento**			
	Calibración interna + externa		**Calibración externa**		**Calibración interna + externa**		**Calibración externa**	
	Δm(ppm)	**Desv. est.**	**Δm(ppm)**	**Desv. est.**	**Δm(ppm)**	**Desv. est.**	**Δm(ppm)**	**Desv. est.**
DEAMPY	0,21	0,13	0,47	0,26	0,17	0,08	1,15	0,19
IMPY	0,08	0,06	0,62	0,26	3,55	0,08	2,64	0,23
DIMET	0,27	0,07	0,76	0,29	0,44	0,05	0,50	0,12
OMET	0,14	0,10	0,53	0,39	0,12	0,11	0,90	0,33
AP	0,12	0,10	0,41	0,21	0,15	0,08	0,99	0,27
ATZM	0,49	0,15	0,99	0,41	0,19	0,04	0,91	0,36
ALAM	0,24	0,27	1,14	0,55	0,17	0,14	0,59	0,17
METM	1,52	1,38	2,23	1,07	0,77	0,45	1,96	0,39

Condiciones de adquisición: ESI+/ESI- en la misma inyección, poder de resolución (25.000 FWHM) y fragmentación CID (40 eV).

5.2.2. Conclusiones

-Se ha llevado a cabo la optimización de parámetros espectrométricos como el poder de resolución, el modo de fragmentación y el tipo de calibración de masas para la determinación de 24 metabolitos de plaguicidas en orina.

-Se seleccionó un poder de resolución de 25,000 FWHM, el uso de fragmentación CID y el uso de calibración de masas interna para mejorar la intensidad de la señal (área de pico cromatográfico) y exactitud de masa de los iones molecular y fragmentos

-El poder de resolución seleccionado ofrece suficiente resolución para evitar interferencias isotópicas y además permite adquirir en modo negativo y positivo en la misma inyección, reduciendo el tiempo de análisis.

-El uso de fragmentación CID (40eV) fue seleccionado, sin embargo, no se estudiaron energías de fragmentación en modo CID superiores.

5.2.3. Artículo 2: Optimization of Resolving Power, Fragmentation and Mass Calibration in an Orbitrap Spectrometer for Analysis of 24 Pesticide Metabolites in Urine

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Research Article

Optimization of Resolving Power, Fragmentation, and Mass Calibration in an Orbitrap Spectrometer for Analysis of 24 Pesticide Metabolites in Urine

Pablo Dualde,[1,2] **Clara Coscollà**,[1,3] **Agustin Pastor**,[2] **and Vicent Yusà**[1,2,3]

[1] *Foundation for the Promotion of Health and Biomedical Research in the Valencian Region, FISABIO-Public Health, 21 Avenida Catalunya, 46020 Valencia, Spain*
[2] *Analytical Chemistry Department, University of Valencia, Edifici Jeroni Muñoz, Dr. Moliner 50, 46100 Burjassot, Spain*
[3] *Public Health Laboratory of Valencia, 21 Avenida Catalunya, 46020 Valencia, Spain*

Correspondence should be addressed to Vicent Yusà; yusa_vic@gva.es

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Mass spectrometer parameters such as Resolving Power, type of fragmentation, and mass calibration mode were optimized in the analysis of 24 pesticide metabolites in human urine using Ultra-High Pressure Liquid Chromatography coupled to Orbitrap High-Resolution Mass Spectrometer (UHPLC-HRMS). The best results were achieved with a Resolving Power of 25,000 FWHM and by applying Collision Induced Dissociation fragmentation mode (40 eV).

1. Introduction

The ever growing number of chemicals being used, such as pesticides, care products, UV filters, parabens, and so on, has an impact on the environment and therefore on humans, especially in vulnerable populations [1]. To protect consumers from such contaminants, an evaluation of the exposure to these chemicals and the subsequent risk assessment is necessary. The National Report on Human Exposure to Environmental Chemicals in the United States [2] and the Human Biomonitoring Report of Environmental Chemicals in Canada [3] are both examples of biomonitoring programs that assess the exposition of a population to environmental chemicals over time. The European Environment and Health Strategy also encourages the adoption of human biomonitoring studies across Europe [4].

Some pesticide metabolites are biomarkers of pesticide exposure. These metabolites are present in urine at concentrations of few ng mL^{-1} [2], and consequently metabolite determination requires sensitive and selective analytical methods. The usual analytical technique for polar metabolites in human biomonitoring studies is the LC-MS/MS [5]. However, the introduction of the high-resolution (>10,000 FWHM) mass spectrometers, which allow to obtain mass accuracies lower than 5 ppm, such as Q-TOF or Orbitrap, has allowed the implementation of combined quantitative target and postrun target analytical strategies for comprehensive determination of pesticides and other emerging contaminant metabolites [6].

Over the last few years, liquid chromatography coupled to Orbitrap high-resolution mass spectrometry (LC-HRMS) has been applied in human biomonitoring studies [6]. Recently, Cortejade *et al.* (2016) developed an analytical method for the targeted screening and multiresidue quantification of 38 contaminant metabolites in urine, including 12 pesticides, a pesticide metabolite (tributyl phosphate), and other compounds of different families [7]. Likewise, Roca *et al.* (2014) developed an analytical method that combined the quantitative target analysis of urinary metabolites of pesticides with a retrospective analysis using liquid chromatography coupled to HRMS. In this study, the main factors governing the ion-source ionization were optimized [6]. In addition, *López et al.* (2016) developed a retrospective analytical methodology for the analysis of pesticide metabolites in urine by LC-HRMS [8].

In the existing literature on application of HRMS to food and feed contaminants [9–14] and in the field of human biomonitoring [6, 15–21], the emphasis so far has mainly been the detectability of the analytes based on retention time and the exact mass of the most abundant analyte ion and on quantitative determination. However, Resolving Power (R) and mass calibration should be further investigated in order to avoid interferences and improve mass accuracy. Likewise, the selection of the fragmentation mode (High-energy Collision Dissociation, HCD, or Collision Induced Dissociation, CID) and their optimization could improve the sensitivity of the confirming ions (fragments).

How much Resolving Power is necessary to apply to a specific problem should be a frequent analytical question [22]. Orbitrap mass spectrometers allow a wide range of Resolving Powers. Theoretically, a higher Resolving Power provides a better resolution of analytes from isobaric interferences present in complex matrices such as urine. However, in Orbitrap, greater Resolving Power requires longer measurement time. A higher Resolving Power leads to the monitoring of fewer data points per time unit [23]. Therefore, for quantitative analysis, the Resolving Power for each matrix-analyte combination should be selected taking into account the isobaric interferences and the number of data points required for provide good peak shape. The Resolving Power has been studied in the literature for veterinary drugs in food and animal samples [22] as well as for other residues and contaminants in food and feed [24].

In order to obtain a suitable mass accuracy (< 5 ppm) in an Orbitrap spectrometer, a proper mass calibration must be employed. External mass calibration is performed previously to the analysis by direct infusion of a mix of compounds with known masses; the experimental m/z values obtained are then corrected with the theoretical m/z values in order to fit the accuracy of the analyzer. Until now, external mass calibration in Orbitrap has been widely employed in biological samples, food, and feed [6, 7, 11, 12, 18, 20]. However, as Leendet *et al.* (2015) have pointed out "improper external mass calibration can lead to large systematic errors in mass measurements" and "external mass calibration range must include the mass range of interest" [25]. Another strategy is the use of internal mass calibration, which is achieved by introducing a compound with a known theoretical m/z value (lock mass) during the analysis. The comparison between the theoretical and experimental errors is used to normalize the m/z values of the rest of peaks [26]. Thus, Strano-Rossi *et al.* (2015) successfully determined stimulants and drugs in food supplements using internal mass calibration in an Orbitrap system [27]. Internal calibration is recommended to avoid drifts of the external calibration over time [25].

Unlike conventional quadrupole (QqQ) instruments, Orbitrap Exactive™ users do not implement a compound-specific fragmentation optimization. In Exactive™, two all ion fragmentation (AIF) modes are allowed: HCD and CID [28]. In HCD, the ions are fragmented in a collision cell using N_2 as a collision gas, while CID allows the ions dissociation through interaction with neutral target species. Consequently, some authors have studied the fragmentation, optimizing the HCD cell for different substances in biological samples, food and feed [6, 11, 12, 18, 20] in an Orbitrap detector. Optimization of HRMS (Orbitrap) fragmentation mode has also been employed in areas such as proteomics [28] or for the identification of oligosaccharides [29]. Optimization of the different types of fragmentation modes (HCD and CID) will probably improve the sensitivity of these methods.

In a previous work [6], we studied the influence of the HCD collision energy on the fragmentation of various pesticide metabolites in urine, working at 50,000 FWHM. In the present paper, we want to increase the speed of the analysis using the polarity switching function (ESI^+ and ESI^- in the same injection) and to study more in depth other factors that can have a decisive influence on the sensitivity, accuracy and speed of the analysis of pesticide metabolites in urine. Consequently, the aims of the present work are to (i) study the influence of Resolving Power (R) on the signal intensity and mass accuracy of the different ions; (ii) compare the two all ions fragmentations (AIF) modes (HCD; CID); (iii) evaluate the two options for mass calibration (internal and external) to improve mass accuracy for this specific application.

2. Materials and Methods

This study has been developed in the framework of the DENAMIC project, which included all the required ethical approvals.

2.1. Reagents and Chemicals. Solvents were specific for pesticide residue analysis and of analytical grade. Acetonitrile and methanol were supplied by Scharlab (Barcelona, Spain). Acetic acid (purity 98-100%), β-glucuronidase arylsulfatase enzyme, and anhydrous sodium acetate were obtained from Merck (KGaA, Darmstadt, Germany). Deionized water was organically and biologically purified by using a Milli-Q Ultrapure System (Millipore, Darmstadt, Germany). QuEChERS EN extraction kits, containing 4 g $MgSO_4$; 1 g NaCl; 1 g Sodium Citrate; 0.5g Sodium Hydrogencitrate Sesquihydrate, were obtained from Agilent Technologies (Madrid, Spain).

Standards of pesticide metabolites (Table 1) were achieved. All commercial standards were of high purity and were obtained from Dr. Ehrenstorfer (Augsburg, Germany), Sigma-Aldrich (Barcelona, Spain), Cerilliant-Certificated Reference Materials (Texas, USA), Cambridge Isotope Laboratories (Massachusetts, USA), Santa Cruz Biotechnology (Heidelberg, Germany), and AccuStandard (New Haven, USA). Stock standard solutions containing 20–500 mg L^{-1} of the individual compounds were prepared by weighing each compound and dissolving it in acetonitrile. Solutions were stored at −20°C. Multianalyte intermediate standard solutions were prepared by diluting the individual stock solutions in acetonitrile and used for preparing working mixed-standard solutions in acetonitrile: water (10:90, v/v). The concentration of the analytes in working solutions ranged from 1000 to 5000 ng·mL^{-1} depending on the compound.

TABLE 1: Pesticide metabolites: diagnostic and fragment ions used for analysis.

Class	Compound	Metabolite	Acronym	Elemental composition	Diagnostic ion	Exact mass m/z diagnostic ion (Da)	Fragment elemental composition	m/z fragment ion (Da)
Organophosphate insecticides	Chlorpyrifos, chlorpyrifos-methyl	3,5,6-Trichloro-2-pyridinol	TCPy	$C_5H_2NOCl_3$	$[M-H]^-$	195.91292	-	-
	Parathion, methyl parathion	p-nitrophenol	PNP	$C_6H_5NO_3$	$[M-H]^-$	138.01966	$C_6H_4O_2$	108.02167
	Pirimiphos-methyl	2-Diethylamino-6-methyl-6-hydroxypyrimidine	DEAMPY	$C_9H_{15}N_3O$	$[M+H]^+$	182.12879	$C_7H_{12}N_3O$	154.09748
	Diazinon	2-Isopropyl-4-methyl-6-hydroxypyrimidine	IMPY	$C_8H_{12}N_2O$	$[M+H]^+$	153.10224	C_4H_6NO	84.04439
	Coumaphos	3-Chloro-7-hydroxy-4-methylcoumarin	CMHC	$C_{10}H_7ClO_3$	$[M-H]^-$	209.00109	$C_9H_5O_2$	145.02841
	Fenitrothion	3-Methyl-4-nitrophenol	MNP	$C_7H_7NO_3$	$[M-H]^-$	152.0353l	$C_7H_6O_2$	122.03733
	Dimethoate	Dimethoate	DIMET	$C_5H_{12}NO_3PS_2$	$[M+H]^+$	230.00690	$C_2H_6O_3PS$	124.98206
	Omethoate	Omethoate	OMET	$C_5H_{12}NO_4PS$	$[M+H]^+$	214.02974	$C_4H_8O_4PS$	182.98754
	Acephate	Acephate	AP	$C_4H_{10}NO_3PS$	$[M+H]^+$	184.01917	$C_2H_8O_3PS$	142.99262
	Methamidophos	Methamidophos	MMP	$C_2H_8NO_2PS$	$[M+H]^+$	142.00861	-	112.01577
	Chlorethoxyphos, chlorpyrifos coumaphos, diazinon, disulfoton, ethion, parathion, phorate, phosalone, sulfotep, terbufos, azinphos-methyl, dichlorvos, dicrotophos, dimethoate, fenitrothion, fenthion, malathion, methyl parathion, trichlorfon, chlorpyrifos-methyl, methidathion, mevinphos, oxydemeton-methyl, phosmet, pirimiphos-methyl, temephos, tetrachlorvinphos, isazofos-methyl, naled	Diethyl phosphate	DEP	$C_4H_{11}O_4P$	$[M-H]^-$	153.03221	$C_2H_6O_4P$	125.00092
		Diethyl thiophosphate	DETP	$C_4H_{11}O_3PS$	$[M-H]^-$	169.00937	$C_2H_6O_3PS$	140.97807
		Dimethyl thiophosphate	DMTP	$C_2H_7O_3PS$	$[M-H]^-$	140.97807	CH_3O_3PS	125.95460
		Dimethyl dithiophosphate	DMDTP	$C_2H_7O_2PS_2$	$[M-H]^-$	156.95523	$CH_3O_2PS_2$	141.93174
Phenoxy herbicides	2,4-Dichlorophenoxyacetic acid	2,4-Dichlorophenoxyacetic acid	2,4-D	$C_8H_6O_3Cl_2$	$[M-H]^-$	218.96212	$C_6H_3OCl_2$	160.95664
	2,4,5-Trichlorophenoxyacetic acid	2,4,5-Trichlorophenoxyacetic acid	2,4,5-T	$C_8H_5O_3Cl_3$	$[M-H]^-$	252.92315	$C_6H_2OCl_3$	194.91767
Chloroacetanilide herbicides	Atrazine	Atrazine mercapturate	ATZM	$C_{13}H_{22}N_6O_3S$	$[M+H]^+$	343.15468	$C_8H_{16}N_5S$	214.11209
	Alachlor	Alachlor mercapturate	AIAM	$C_{19}H_{28}N_2O_5S$	$[M+H]^+$	397.17916	$C_5H_8N_2O_3$	130.04987
	Metolachlor	Metolachlor mercapturate	METM	$C_{20}H_{30}N_2O_5S$	$[M+H]^+$	411.19481	$C_{15}H_{24}NO_2S$	282.15223

Table 1: Continued.

Class	Compound	Metabolite	Acronym	Elemental composition	Diagnostic ion	Exact mass m/z diagnostic ion (Da)	Fragment elemental composition	m/z fragment ion (Da)
	Commercial Pyrethroids	3-Phenoxybenzoic acid	PBA	$C_{13}H_{10}O_3$	$[M-H]^-$	213.05571	$C_{12}H_9O$	169.06589
	Cyfluthrin	4-Fluoro-3-phenoxybenzoic acid	FPBA	$C_{13}H_9FO_3$	$[M-H]^-$	231.04629	$C_{12}H_8OF$	187.05647
Pyrethroid Insecticides	Permethrin, cypermethrin, cyfluthrin	cis (2,2-Dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid	cis-DCCA	$C_8H_{10}O_2Cl_2$	$[M-H]^-$	206.99850	-	-
		trans-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid	trans-DCCA	$C_8H_{10}O_2Cl_2$	$[M-H]^-$	206.99850	-	-
	Deltamethrin	cis-(2,2-Dibromovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid	DBCA	$C_8H_{10}O_2Br_2$	$[M-H]^-$	294.89747	-	-
Internal Standards		PNP-D4		$C_6HD_4NO_3$	$[M-H]^-$	142.04477	-	-
		FPBA-13C6		$^{13}C_6C_7H_9FO_3$	$[M-H]^-$	237.06642	-	-
		DCCA-13C2		$^{13}C_2C_8H_9DCl_2O_2$	$[M-H]^-$	210.01149	-	-
		ATZM-13C3		$_{13}C_3C_{10}H_{22}N_6O_3S$	$[M+H]^+$	346.16475	-	-
		24-D-D3		$C_8H_3Cl_2O_3D_3$	$[M-H]^-$	221.98095	-	-
		MMP-D6		$C_7H_2NO_2PSD_6$	$[M+H]^+$	148.04627	-	-
		DIMET-D6		$C_5H_6NO_3PS_2D_6$	$[M+H]^+$	236.04455	-	-
		DBP		$C_8H_{19}O_4P$	$[M-H]^-$	209.09481	-	-

2.2. Sample Preparation and UHPLC-HRMS. A previously developed sample preparation was used [6]. Briefly, in order to hydrolyze the possible glucuronide- or sulfate-conjugated metabolites, 5 mL of urine was mixed with 1 mL of 0.2 M acetate buffer 20 μL of β-glucuronidase aryl sulfatase enzyme and the internal standard solution mix in the analysis of real samples. The samples were maintained at 37°C overnight for hydrolysis.

Metabolites were extracted from the urine samples employing the dispersive solid phase extraction QuEChERS kits. In a 50 mL polypropylene tube, the urine was mixed with 10 mL of acetonitrile, a QuEChERS pouch, and 2 ceramic pieces. After centrifugation, the acetonitrile phase was transferred and evaporated to dryness in a water bath at 37°C under a nitrogen stream. Subsequently, 200 μL of methanol: water (10:90, v/v) containing 0.1% of acetic acid was added and the solution was transferred into a Millipore 0.2 μm Eppendorf and ultra-centrifuged. The final extract was transferred into an injection vial and analyzed in the UHPLC-HRMS system.

Chromatographic separation was performed on an ultra-high performance liquid chromatography (UHPLC) system Accela™ equipped with a Hypersil Gold column (100 mm x 2.1 mm, 1.9 μ) from ThermoFisher Scientific (Bremen, Germany). The chromatographic separation was optimized in a previous study [6]. Briefly, the flow rate used was 400 μL min^{-1} and the injection volume was 10 μL. A binary gradient was used: acetic acid 0.1% (v/v) in water was used as mobile phase A, while acetic acid 0.1% (v/v) in methanol was used as mobile phase B. The analysis started with 95% mobile phase A. After 1 min, this percentage was linearly decreased down to 45% within 5 min. After that, solvent A was decreased quickly to 0% in 0.5 min and maintained for 1.5 min. The total run time was 20 min.

Mass analysis was performed on the Orbitrap mass spectrometer Exactive™ analyzer (Thermo Scientific, Bremen, Germany). The system was equipped with a heat electrospray ionization interface (HESI-II). The ion-source parameters were previously optimized as follows: spray voltage: 3.5 kV (positive mode) and 2.5 kV (negative mode); sheath gas flow rate: 55; auxiliary gas flow rate: 10; skimmer voltage: 23 V; heater temperature: 300°C; capillary temperature: 150°C; capillary voltage: 45 V and tube lens voltage: 120 V. For more details of the HRMS analysis see Roca *et al.* (2014) [6].

2.3. Compound Identification Criteria. The criteria for target compound identification were established following the SANTE/11813/2017 guideline [30]: (i) mass accuracy of the molecular ion< 5 ppm; (ii) mass accuracy of the fragment ion < 5 ppm; (iii) isotopic pattern similar to the theoretical isotopic pattern (the relative intensity of the A+1 and/or A+2 isotope peaks in the real sample shall correspond to the theoretical relative intensities). For confirmation we used the reference standard solutions of those compounds available in the market. In this case the confirmation criteria were included: (iv) retention time (t_R) similar to that of the reference standard ± 0.1 min.

2.4. HRMS Orbitrap Parameters Optimization

2.4.1. Resolving Power Optimization. To optimize the Resolving Power (R), the system operating in full-scan mode (50-800 m/z) was tested at the R of 10,000; 25,000; and 50,000 FWHM. 6 blank matrix urine aliquotes spiked with a mixed-standard solution of 24 target pesticide metabolites (see Table 1) (50 ng·mL^{-1}) were analyzed.

The Resolving Power was evaluated measuring the peak area (signal intensity) and the mass accuracy (Δm) for the diagnostic and fragment ions of each metabolite. A scheme of the Resolving Power optimization study is detailed in Table SI.1. Resolving Power optimization data were acquired with ESI+/- in separated injections, using HCD fragmentation 20 eV and external mass calibration.

2.4.2. Fragmentation Optimization. After the selection of the most suitable R, HCD and CID fragmentations were evaluated. Five spiked urine samples were injected with CID energies of 10, 20, 30, and 40 eV. We previously set the energy for HCD fragmentation to 20 eV. Once the CID energy was optimized, five different methods, in six spiked samples (50 ng·mL^{-1}), were studied using or not HCD and CID fragmentations and using ESI+ and ESI- in the same or in different injections. The response was evaluated measuring the peak area of the fragment ions in (i) ESI^+ with and without HCD (HCD= 20 eV); (ii) ESI^- with and without HCD (HCD= 20 eV); (iii) ESI^+ with and without CID; (iv) ESI^- with and without CID; (v) ESI^+ and ESI^- in the same injection with and without CID. Fragmentation optimization data were acquired using the previously optimized R and external mass calibration.

2.4.3. Internal and External Mass Calibration Study. With respect to mass calibration, both external and internal mass calibrations were evaluated. External calibration was performed using the mixtures ProteoMass™ LTQ/FT-Hybrid ESI Cal Mix in Pos an Neg Mode (Supelco, Bellefonte, PA, USA). Internal mass calibration was achieved introducing caffeine ($M+H^+$ m/z = 195.08765 Da) in the mobile phase as a lock mass for positive ionization (ESI^+).

In total, five spiked aliquotes were analyzed using internal and external mass calibration separately. The analytical response was evaluated measuring peak areas and mass accuracies (Δm) for the diagnostic and fragment ions. All mass calibration study data were acquired using the previously optimized R and fragmentation settings.

Data were processed using the TraceFinder™ 3.1 (Thermo Scientific, Bremen, Germany) and Xcalibur™ 2.2 (Thermo Scientific, Bremen, Germany) software.

3. Results and Discussion

3.1. Resolving Power Optimization. In order to select the most appropriate R for the determination of pesticide metabolites in urine, the influence of this parameter on the signal and mass accuracy of the 24 compounds was investigated. Tables 2 and 3 show the signal intensity (peak area) and the mass

Table 2: Average peak area and coefficient of variation (CV, %) obtained at 3 different Resolving Powers (R) for pesticide metabolites diagnostic and fragment ions (n = 6).

Metabolite	Peak area	Diagnostic ion			Fragment ion		
		R=10,000	R=25,000	R=50,000	R=10,000	R=25,000	R=50,000
CMHC	Average	908337	839333	570456	12927	26726	39492
	CV (%)	1.51	2.91	7.49	24.38	21.36	52.87
DEAMPY	Average	994268	880944	834834	63190	161187	175626
	CV (%)	17.15	28.51	10.49	22.31	16.20	40.94
IMPY	Average	1447089	1425080	1663104	Not found	47097	116409
	CV (%)	0.36	7.92	13.22	Not found	1.23	41.28
PNP	Average	6388647	6280026	6007343	3938342	3858484	4005830
	CV (%)	1.70	2.06	1.92	7.36	4.40	6.67
TCPy	Average	1090750	1148221	943686	-	-	-
	CV (%)	1.73	7.04	8.08	-	-	-
MNP	Average	3938892	3813784	3650660	503016	474896	634023
	CV (%)	3.10	3.85	4.18	11.40	13.27	19.46
DMTP	Average	76340	68339	88833	2644	6361	9973
	CV (%)	39.41	5.74	16.55	57.74	37.34	57.74
DMDTP	Average	111502	321392	247711	2679	16962	53675
	CV (%)	36.39	10.13	30.71	57.74	110.51	11.56
DEP	Average	1719737	1870997	1556112	196269	272546	272666
	CV (%)	4.09	4.33	5.83	21.00	12.49	6.12
DETP	Average	2224008	2154624	1819644	50934	141703	169339
	CV (%)	0.08	6.97	6.56	63.42	24.47	24.00
AP	Average	58729	74307	90733	157684	157729	206069
	CV (%)	8.34	33.62	17.63	7.21	4.59	13.43
MMP	Average	69818	178190	112470	19869	169131	69306
	CV (%)	7.38	2.89	4.58	25.93	3.05	7.43
OMET	Average	45484	226016	149173	13032	25505	32670
	CV (%)	69.04	23.35	17.68	35.04	22.09	32.95
DIMET	Average	380660	399855	329466	60934	83874	87121
	CV (%)	8.48	11.44	14.44	6.51	10.55	31.27
PBA	Average	648211	571829	363693	121191	138365	166373
	CV (%)	8.82	15.34	16.11	11.30	20.44	23.68
FPBA	Average	612579	519635	405204	73743	82755	112916
	CV (%)	13.94	23.07	11.99	16.68	13.95	31.57
cis DCCA	Average	171464	273263	194626	-	-	-
	CV (%)	28.60	16.39	13.55	-	-	-
trans DCCA	Average	453810	436816	314120	-	-	-
	CV (%)	6.12	6.49	13.04	-	-	-
DBCA	Average	6904	32503	32185	-	-	-
	CV (%)	31.56	40.91	16.74	-	-	-
ATZM	Average	273078	1179224	756085	1314830[a]	1421843[a]	1252716[a]
	CV (%)	71.21	3.96	2.01	3.57	4.46	9.05
ALAM	Average	67659	687712	511658	831200[a]	1255816[a]	1242225[a]
	CV (%)	12.30	15.92	7.77	7.02	2.35	5.40
METM	Average	268530	644807	446473	2762	5381	6657
	CV (%)	8.80	4.93	9.49	57.74	57.74	57.74
2,4-D	Average	1234998	1225331	907979	1130961	1117200	1224757
	CV (%)	6.27	4.39	5.67	3.85	8.45	3.68
2,4,5-T	Average	731224	786035	477627	677796	718415	777837
	CV (%)	10.79	10.86	10.65	7.95	12.15	13.05

Acquisition conditions: ESI+/ESI- in separated injections, HCD fragmentation 20 eV, and external mass calibration.
-: no fragment ions monitored.
[a]Irregular peak shape caused by isobaric interferences. The measured areas could be affected by the interferences.

Table 3: Average and range of mass accuracies (Δm) (ppm) obtained at 3 different Resolving Powers (R) for pesticide metabolites diagnostic and fragment ions (n=6).

Metabolite	Δm (ppm)	Diagnostic ion			Fragment ion		
		R=10,000	R=25,000	R=50,000	R=10,000	R=25,000	R=50,000
CMHC	Average	2.27	1.38	1.24	3.11	2.52	3.36
	Range	1.60 – 2.75	0.07 – 2.32	0.93 – 1.65	1.61 – 4.16	1.10 – 3.81	3.33 – 3.40
DEAMPY	Average	1.09	2.68	1.30	3.19	1.70	1.44
	Range	0.05 – 2.58	1.65 – 3.66	0.73 – 1.90	1.70 – 4.53	0.69 – 3.37	0.49 – 2.38
IMPY	Average	1.81	1.56	1.33	> 5	1.61	1.99
	Range	1.25 – 2.14	1.36 – 1.76	1.26 – 1.36	> 5	1.35 – 1.90	1.90 – 2.07
PNP	Average	2.69	1.94	1.63	3.64	2.18	1.96
	Range	2.31 – 3.08	0.77 – 2.87	1.20 – 1.86	2.92 – 4.99	1.09 – 3.13	1.48 – 2.28
TCPy	Average	2.23	2.01	1.83	-	-	-
	Range	0.28 – 3.01	0.42 – 3.12	1.05 – 3.38	-	-	-
MNP	Average	2.59	1.32	1.59	2.36	1.18	1.89
	Range	2.56 – 2.66	1.05 – 1.56	1.15 – 2.06	1.70 – 2.93	0.88 – 1.70	1.62 – 2.11
DMTP	Average	0.79	0.66	2.43	2.41	1.02	1.28
	Range	0.11 – 1.40	0.34 – 1.09	1.96 – 2.82	1.51 – 4.52	0.75 – 1.19	0.08 – 2.78
DMDTP	Average	2.47	1.49	2.14	1.96	2.67	1.31
	Range	1.26 – 3.21	0.87 – 2.52	1.65 – 2.43	0.25 – 4.03	1.67 – 4.26	0.49 – 2.10
DEP	Average	2.66	2.02	1.84	1.88	2.05	2.35
	Range	1.43 – 3.26	1.16 – 2.84	1.44 – 2.14	0.89 – 2.81	1.03 – 3.11	1.98 – 2.71
DETP	Average	3.46	2.02	1.96	2.26	3.13	2.10
	Range	3.28 – 4.99	1.27 – 2.63	1.30 – 2.29	0.61 – 4.22	2.30 – 4.22	1.31 – 3.04
AP	Average	1.66	1.41	1.39	1.91	2.26	1.79
	Range	0.42 – 4.80	0.60 – 2.10	0.48 – 2.57	0.06 – 4.00	1.30 – 3.16	1.13 – 2.11
MMP	Average	2.21	1.67	1.69	3.06	1.67	3.45
	Range	0.28 – 4.14	1.24 – 2.43	0.92 – 2.69	2.62 – 3.49	1.24 – 2.43	3.22 – 3.63
OMET	Average	0.86	1.92	2.09	1.55	3.36	1.90
	Range	0.03 – 1.39	1.11 – 2.61	1.86 – 2.38	0.47 – 2.39	2.17 – 4.98	1.19 – 2.99
DIMET	Average	2.79	2.55	2.13	2.38	2.34	2.67
	Range	1.18 – 4.62	2.18 – 2.92	1.14 – 3.10	0.38 – 4.06	1.10 – 3.90	2.20 – 3.11
PBA	Average	1.79	1.33	1.12	2.25	1.55	1.77
	Range	1.25 – 2.11	0.18 – 2.85	0.68 – 1.97	1.65 – 3.25	0.28 – 3.53	1.18 – 2.26
FPBA	Average	1.17	2.09	1.38	3.10	1.85	1.41
	Range	0.15 – 1.80	0.80 – 3.49	0.66 – 2.41	2.09 – 4.20	0.30 – 3.46	0.10 – 1.92
cisDCCA	Average	1.84	1.42	1.76	-	-	-
	Range	1.20 – 2.31	0.31 – 2.18	0.61 – 2.67	-	-	-
transDCCA	Average	2.37	1.82	1.41	-	-	-
	Range	1.05 – 3.47	0.23 – 3.04	0.31 – 2.45	-	-	-
DBCA	Average	2.92	2.07	1.78	-	-	-
	Range	1.80 – 4.10	1.08 – 2.74	0.36 – 3.16	-	-	-
ATZM	Average	3.61	2.29	2.98	1.18	1.84	1.85
	Range	2.29 – 4.97	1.43 – 2.87	2.17 – 4.12	0.83 – 1.63	1.42 – 2.32	1.56 – 2.27
ALAM	Average	3.41	2.57	3.29	2.30	2.14	1.96
	Range	1.24 – 4.76	1.69 – 2.85	2.85 – 4.76	1.38 – 3.25	1.61 – 3.38	1.61 – 2.43
METM	Average	2.37	2.68	2.77	> 5	1.52	2.13
	Range	0.90 – 4.38	1.70 – 3.87	1.19 – 4.31	1.09 – >5	0.42 – 2.35	1.82 – 3.13
2,4-D	Average	2.65	1.92	1.72	3.21	2.19	1.89
	Range	1.98 – 3.62	0.97 – 2.56	0.74 – 2.77	2.82 – 3.87	1.08 – 3.00	1.76 – 2.14
2,4,5-T	Average	1.65	1.30	2.28	2.89	2.15	1.71
	Range	0.46 – 2.26	0.22 – 1.85	1.30 – 4.14	2.16 – 3.65	1.42 – 2.78	1.29 – 2.23

Acquisition conditions: ESI+/ESI- in separated injections, HCD fragmentation 20 eV, and external mass calibration.
-: no fragment ions monitored.

Table 4: Number of ions (diagnostic and fragment ions) into the six mass accuracy ranges considered at 10,000, 25,000, and 50,000 FWHM.

Resolving Power	Δm (ppm)					
	≤ 1	]1 – 2]	]2 – 3]	]3 – 4]	]4 – 5]	> 5
10,000	2	12	19	9	0	2
25,000	1	22	19	2	0	0
50,000	0	30	11	3	0	0

Acquisition conditions: ESI+/ESI- in separated injections, HCD fragmentation 20 eV, and external mass calibration.

accuracy (Δm), respectively, of the diagnostic and fragment ions.

Regarding diagnostic ions, some specific metabolites such as DEAMPY, IMPY, PNP, TCPy, MNP, DEP, DETP, or DIMET presented similar areas at the three R checked (Table 2). However, R = 25,000 FWHM provided the largest areas for DMDTP, MMP, OMET, cis DCCA, DBCA, ATZM, ALAM, METM, and 2,4,5-T. For the remaining 7 compounds a R= 25,000 FWHM presented areas close to that provided by the best R for each compound (10,000 FWHM or 50,000 FWHM).

For fragments, 17 out of 20 compounds presented higher intensities with R = 50,000 FWHM (Table 2). In some cases, as for METM, fragment ions areas are very low in comparison with the diagnostic ion response. It is relatively frequent that AIF produces fragment ions with low abundances, partly because the optimal fragmentation energy is not equal for all diagnostic ions. It could affect the analysis of real samples, because some diagnostic ions may not be confirmed because their fragment ions signal is below the noise. To sum up, R = 25,000 FWHM seems more suitable for diagnostic ions and 50,000 FWHM for fragment ions. However, taking into account the total ions (diagnostic plus fragments) and the variability of the response, a R = 25,000 FWHM was selected because it presented more number of ions with high area (see Figure SI.1). We have not found a clear explanation why some ions present higher responses when acquired at a R (e.g., 25,000 FWHM) while other ions present higher responses when acquired at another R (e.g., 50,000 FWHM).

In addition, the R of 25,000 FWHM presented more data points (scans) per peak than R = 50,000 FWHM because in an Orbitrap analyser the scan speed decrease when R increase (i.e., 2 Hz at R = 50,000 FWHM; 4 Hz at R = 25,000 FWHM). The number of data points is important for peak shape and quantification.

Table 3 shows the average and range of mass accuracy (Δm, ppm) of pesticide metabolites diagnostic and fragment ions obtained at the three R tested. Table 4 summarizes the results showing the number of ions in each of the six ranges of mass accuracy considered. As can be observed (Table 4), good mass accuracies (<3 ppm) in most of the ions were obtained applying R of 25,000 and 50,000 FWHM.

Taking into account these results, a R = 25,000 FWHM was chosen because (i) it gave the highest signal (peak area) for more analytes; (ii) it presented good mass accuracy; and (iii) the scan time gave a suitable number of data points. An added advantage of using this intermediate R is that the instrument presents a sufficient speed to be able to use the detection of positive (ESI^+) and negative (ESI^-) ions

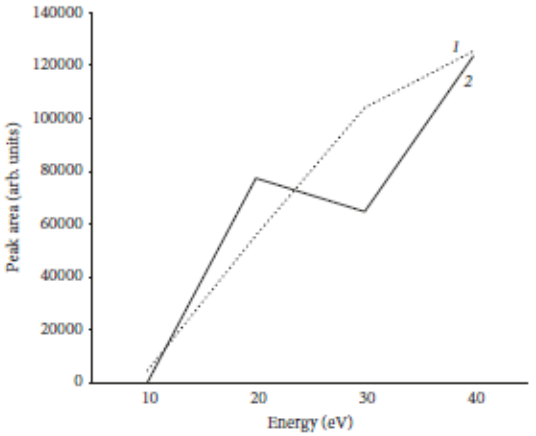

Figure 1: CID fragmentation of PBA (m/z = 169.06589). Variation of the fragment ion response (peak area) with the applied energy (eV). (1) Applying only ESI^+; (2) using the polarity switching (ESI^+, ESI^- in the same injection). Acquisition conditions: R= 25,000 FWHM and external mass calibration.

in the same injection, increasing the speed of analysis and the throughput of the method. Martínez-Dominguez *et al.* (2016) also applied R = 25,000 FWHM for the analysis of organic contaminants in food using a UHPLC-Orbitrap detector [12]. QTOF systems also employed similar Resolving Powers (18,000-22,500 FWHM) for the analysis of pesticides, organophosphate flame retardants, and chemical agents in urine and drinking water [15, 16, 31]. However, in Orbitrap analyzer a higher Resolving Power (50,000 FWHM) was used for the analysis of pesticides and drugs in urine, food, and feed [6, 11, 27] and for the analysis of mycotoxins in food (70,000 FWHM) [14]. In contrast, a lower Resolving Power was employed in other studies ranging from 7,000 to 17,500 FWHM in biological and food samples [7, 12, 18]. Kaufmann *et al.* (2010) compared liquid chromatography selectivity in LC-MS/MS and LC-HRMS, applying different Resolving Powers (10,000-100,000 FWHM). They indicated that a Resolving Power of 50,000 FWHM was the most suitable for the analysis of veterinary drugs in food using LC-Orbitrap detector [22].

3.2. Fragmentation Optimization. Regarding the optimization of the CID energy, the highest fragment areas were obtained using a fragmentation energy of 40 eV. As an

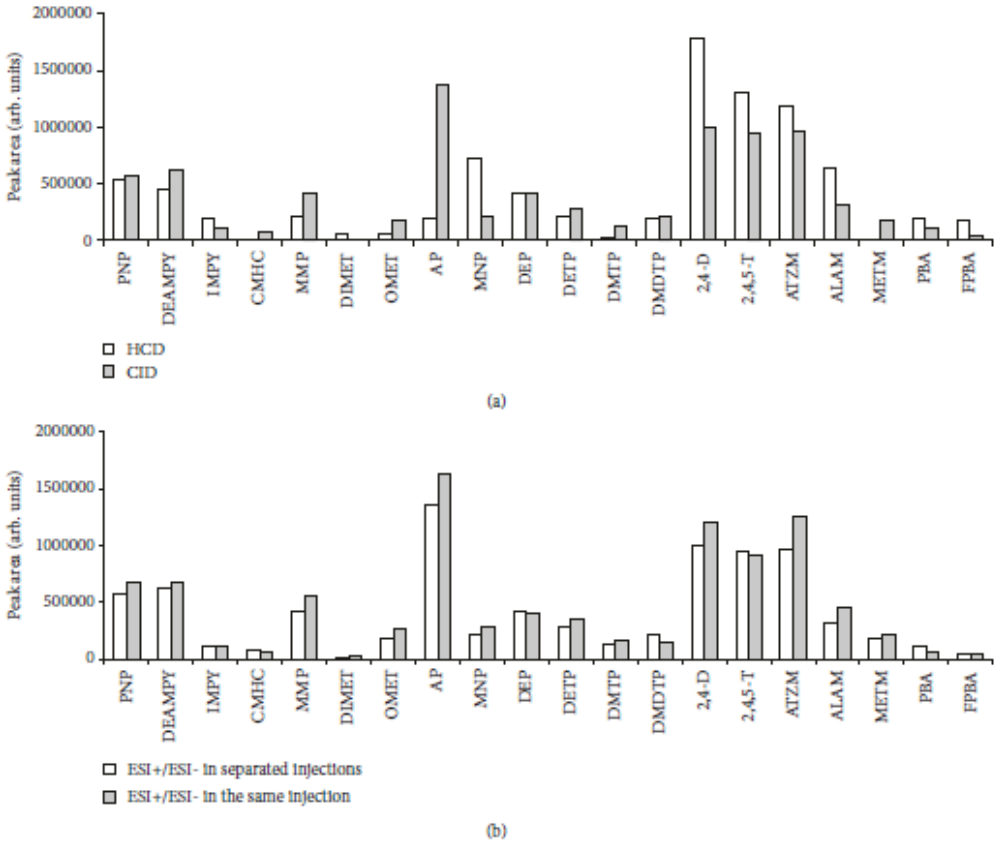

Figure 2: (a) Comparison between responses (peak area) of fragments obtained using CID (40 eV) and HCD (20 eV). (b) Comparison between CID fragmentation (40 eV) using the polarity switching function (ESI^+ and ESI^-) in the same injection and in two separate injections. Acquisition conditions: R= 25,000 FWHM and external mass calibration.

example, Figure 1 shows the fragment ion areas obtained using CID at different energies for PBA.

Comparing the results obtained using CID and HCD, Figure 2(a) shows similar areas for fragment ions obtained with CID and HCD modes. However, some compounds such as CMHC and METM were only fragmented applying CID. Consequently, CID energy (40 eV) was selected for all compounds. Similar results were obtained using ESI^+ and ESI^- in the same injection than in two separate injections (see Figure 2(b)). Consequently, a method with ionization mode in ESI^+ and ESI^- in the same injection and CID fragmentation was selected. Strano-Rossi *et al.* (2015) also selected CID energy (40 eV) in LC-Orbitrap for the determination of stimulants and drugs in food supplements [27]. Tak *et al.* (2014) employed higher CID energy (150 eV) in an LC-QTOF detector for the determination of chemical warfare agents in drinking water [31]. However, HCD is the most widely fragmentation mode used in HRMS Orbitrap detectors. HCD energies from 20 to 30 eV have been applied to fragment compounds such as pesticides, biotoxins, mycotoxins, veterinary drugs, and other toxins in biological, food, and feed samples [6, 11, 12, 18, 20]. Optimization of the mode of fragmentation in Orbitrap detectors is also carried out in other fields as proteomics. Jedrychowski *et al.* (2011) have evaluated CID/HCD fragmentation for murine phosphoproteomics [28].

3.3. Internal and External Mass Calibration Study. In general, the use of lock mass (internal calibration) improves mass accuracy. In addition, in the present method some fragments are below 138.06619 Da, which is the low mass in the ESI^+ external calibration solution; consequently a continuous correction of the acquired masses (internal calibration) could avoid an excessive mass drift.

In order to check whether this general rule is applicable to this particular application, we studied the influence of the calibration mode on the mass accuracy of eight substances analyzed in ESI^+ mode, using caffeine as internal

Table 5: Average mass accuracies (Δm) (ppm) and standard deviations (Std dev) using internal and external calibration for diagnostic and fragment ions in ESI positive mode (n=5).

Metabolite	Diagnostic ion				Fragment ion			
	Internal calibration		External calibration		Internal calibration		External calibration	
	Δm (ppm)	Std dev	Δm (ppm)	Std dev	Δm (ppm)	Std dev	Δm (ppm)	Std dev
DEAMPY	0.21	0.13	0.47	0.26	0.17	0.08	1.15	0.19
IMPY	0.08	0.06	0.62	0.26	3.55	0.08	2.64	0.23
DIMET	0.27	0.07	0.76	0.29	0.44	0.05	0.50	0.12
OMET	0.14	0.10	0.53	0.39	0.12	0.11	0.90	0.33
AP	0.12	0.10	0.41	0.21	0.15	0.08	0.99	0.27
ATZM	0.49	0.15	0.99	0.41	0.19	0.04	0.91	0.36
ALAM	0.24	0.27	1.14	0.55	0.17	0.14	0.59	0.17
METM	1.52	1.38	2.23	1.07	0.77	0.45	1.96	0.39

Acquisition conditions: ESI+/ESI- in the same injection, R = 25,000 FWHM, and CID fragmentation 40 eV.

standard. Table 5 shows the mass accuracies (Δm, ppm) for the diagnostic and fragment ions obtained with internal and external mass calibration. As it can be observed, a better Δm was obtained when working with internal mass calibration. Hence, the use of caffeine as lock mass was selected.

In general, external mass calibration is used in the literature for HRMS Orbitrap detector and internal mass calibration for HRMS QTOF detectors. External mass calibration of Orbitrap in urine, plasma, food, and feed has been widely performed for pesticides, parabens, veterinary drugs, biotoxins, mycotoxins, and other substances [6, 8, 11, 12, 18, 20]. However, internal mass calibration has also been used, as in the method described by Strano-Rossi *et al.* (2015), for the analysis of various stimulants in food supplements. In this case the authors used diisooctyl phthalate ionic species, m/z 391.2843 Da, in order to compensate any possible mass axis drifts, obtaining Δm < 1 ppm for all ionic species [27]. In contrast, QTOF detectors normally calibrate with negative internal mass calibration (leucine enkephalin) [15, 16, 19] or positive internal mass calibration (purine and hexakis-(1H,1H,3H-tetrafluoropentoxy)phosphazene) [31].

4. Conclusions

Mass spectrometry parameters such as Resolving Power, fragmentation mode, and type of mass calibration have been optimized for the analysis of 24 pesticide metabolites in urine. A Resolving Power of 25,000 FWHM, internal calibration, and CID fragmentation were selected as the best options to improve the signal intensity and the mass accuracy of diagnostic and fragment ions. This Resolving Power provides enough resolution to avoid isotopic interferences and allows the use of the polarity switching function (ESI^+ and ESI^- in the same injection), hence reducing the analysis time.

The optimized HRMS parameters allow the determination of pesticide metabolites in urine samples; however a further validation of the method is required to determine the LOD and other performance parameters.

5. Study Limitation

We have not tested CID energies higher than 40 eV.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Supplementary Materials

Table SI.1: scheme of the Resolving Power optimization study. Figure SI.1: number of ions with high ([100-80]%) and low (<80%) peak areas at R=10,000, R=25,000, and R=50,000 (FWHM), for total, diagnostic, and fragment ions. *(Supplementary Materials)*

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5.3. Capítulo 3: Determinación de cuatro parabenos y bisfenol A, F y S en leche materna utlizando QuEChERS y HPLC-MS/MS

En el presente capítulo se desarrolló una metodolo analítico basado en la extracción y purificación con QuEChERS y en la detección mediante HPLC-APCI-MS/MS(QqQ) para la determinación de bisfenoles (BPA, BPF, BPS) y parabenos (MP, EP, PP y BP) en leche materna. Además, el método permite opcionalmente la hidrólisis enzimática de los analitos conjugados. Se llevó a cabo un estudio del efecto matriz y el método fue validado.

En primer lugar se llevó a cabo una búsqueda bibliográfica de métodos analíticos para la determinación de bisfenoles y/o parabenos en leche materna. El resultado de la búsqueda se muestra en la **Tabla 15**. Ninguno de los métodos previamente desarrollado había permitido la determinación conjunta de BPA, F y S y parabenos. En la mayoría de los casos se habían incluido pretratamientos para la precipitación de grasa y proteínas y con respecto a la extracción y purificación y muchos métodos habían empleado LLE, SPE, metodologías más laboriosas o el uso de QuEChERS. La detección se había llevado a cabo principalmente mediante LC-MS/MS, aunque el uso de otras técnicas, como GC, también se habían utilizado en la determinación de bisfenoles y parabenos. Respecto al modo de ionización en LC-MS, generalmente se había usado ESI. El modo APCI no había sido previamente utilizado para la determinación de parabenos, BPF o BPS en leche materna.

5.3.1. Resultados y discusión

5.3.1.1. Optimización de la cromatografía

Se utilizó una columa C18 (Symmetry, 2.1 x 150 mm, 5µ) para llevar a cabo la separación cromatográfica. Varios modificadores (ácidos, bases y tampones) fueron probados en la fase móvil acuosa y en la fase móvil orgánica (metanol). Sin embargo, el uso de fases móviles sin modificadores permitió obtener las mejores formas y áreas de pico, por lo que se seleccionó agua como fase móvil A y metanol como fase móvil B.

Los parámetros cromatográficos tras la optimización se describen a continuación. La temperatura de la columna fue de 35ºC y el flujo usado de 0,3 mL/min. Asimismo, el volumen de inyección fue de 20 µL. El gradiente utilizado se inicia con 98% de fase móvil A y, a continuación, A decrece al 0% en un periodo de 6 min y se mantiene durante 1 min. Posteriormente se recuperan las condiciones iniciales en 0,5 min, seguidas de un tiempo de reequilibrado de 4,5 min.

Tabla 15. Métodos analíticos previamente descritos para el análisis de bisfenoles y/o parabenos en leche materna.

Referencia	Biomarcadores analizados	Volumen muestra (mL)	Preparación de muestra	Técnica analítica	Tiempo análisis (min)	Fuente de ionización	Curva de calibración	Analitos*	Precisión (RSD (%))	Recuperación (%)	R^2 curva de calibrado	LoQ (ng/mL)	LDR (ng/mL)
(Ye et al., 2008)	Libres + Totales	0,1	Hidrólisis enzimática (opcional) + LLE (MeOH) + columna 'on-line' automatizada SPE (RP-18).	HPLC-MS/MS	15 (incluyendo SPE)	APPI (-)	EC	MP	3,5 - 8,3 (Intra e interdía)	90-119 (4 niveles)	-	0,1 (LoD)	-
								EP			-	0,1 (LoD)	-
								PP			-	0,1 (LoD)	-
								BP			-	0,1 (LoD)	-
								BPA			-	0,3 (LoD)	-
(Rodríguez-Gómez et al., 2014a)	Libres	9,9	Para LC-MS: Precipitación de lípidos y proteínas (Sales de zinc (Zn) y tungsteno (W) en solución ácida) + 'Stir-bar sorptive extraction' (SBSE) / Para análisis GC-MS: Precipitación de lípidos y proteínas (Sales de Zn y W en solución ácida) + SBSE + Derivatización	GC-MS/MS y UHPLC-MS/MS	26 (GC-MS) / 10 (LC-MS)	EI (GC-MS) / ESI (-) (LC-MS)	MMC	MP	1,7 - 8,4 (GC-MS) / 2,8 - 15 (LC-MS) (Intra e interdía)	97-114 (GC-MS) / 92-111 (LC-MS) (3 niveles)	0,991 - 0,999	0,4 (GC-MS) / 0,2 (LC-MS)	0,4 - 100 (GC) / 0,2 - 100 (LC-MS)
								EP				0,4 (GC-MS) / 0,3 (LC-MS)	0,4 - 100 (GC-MS) / 0,3 - 100 (LC-MS)
								PP				0,3 (GC-MS) / 0,7 (LC-MS)	0,3 - 100 (GC-MS) / 0,7 - 100 (LC-MS)
								BP				0,3 (GC-MS) / 0,2 (LC-MS)	0,3 - 100 (GC-MS) / 0,2 - 100 (LC-MS)
								BPA				0,5 (GC-MS) / 0,3 (LC-MS)	0,5 - 100 (GC-MS) / 0,3 - 100 (LC-MS)
(Rodríguez-Gómez et al., 2014b)	Libres	9,9	Precipitación de lípidos y proteínas (Sales de Zn y W en solución ácida) + Extracción en fase sólida dispersiva (d-SPE) (150 mg C_{18} y 100 mg $MgSO_4$)	UHPLC - MS/MS	13	ESI (-)	MMC	MP	2,0 - 12,4 (Intra e interdía)	91,0 - 119,8 (3 niveles)	0,998 - 0,999	0,09	0,09 - 25,0
								EP				0,09	0,09 - 25,0
								PP				0,09	0,09 - 25,0
								BP				0,1	0,10 - 25,0
								BPA				0,15	0,15 - 25,0
(Rodríguez-Gómez et al., 2015)	Libres	9,9	Extracción asistida por ultrasonidos (UAE) + d-SPE (500 mg $MgSO_4$ y 300 mg C_{18})	UHPLC - MS/MS	10	ESI (-)	MMC	MP	1,3 - 11,1 (Intra e interdía)	93,8 - 112,2	0,996 - 0,998	0,5	0,5 - 50
								EP				0,5	0,5 - 50
								PP				0,4	0,4 - 50
								BP				0,7	0,7 - 50
								BPA				0,5	0,5 - 50
(Azzouz et al., 2016b)	Libres	1	LLE(ACN) + SPE(LiChrolut ENcolumn) + Derivatización	GC-MS	24.5	-	MMC	MP	4,9 - 7 (Intra e interdía)	87-104 (3 niveles)	> 0,995	0,026	0,026 - 50
								EP				0,029	0,029 - 50
								PP				0,028	0,028 - 50
								BP				0,028	0,028 - 50
								BPA				0,0034	0,0034 - 50
(Azzouz et al., 2016a)	Libres + Totales	0,5	Hidrólisis enzimática (opcional) + LLE (etil acetato) + clean up con un sistema continuo SPE (LiChrolut EN) + Derivatización	GC-MS	25	-	MMC	MP	5,2 - 7,2 (Intra e interdía)	92 - 99 (2 niveles)	> 0,994	0,055	0,055 - 75
								EP		98 - 101 (2 niveles)		0,055	0,055 - 75
								PP		100 - 103 (2 niveles)		0,05	0,050 - 75
								BP		88 - 100 (2 niveles)		0,055	0,055 - 75
								BPA		99 - 102 (2 niveles)		0,007	0,007 - 75
(Vela-Soria et al., 2016)	Total	0,25	Hidrólisis enzimática + Desnaturación protéica (acetona) + Microextracción líquido-líquido dispersiva (DLLME)	LC-MS/MS	8.5	-	MMC	MP	5,1 - 14,6 (Intra e interdía)	90,2 - 111,8 (3 niveles)	0,994 - 0,996	0,5	0,5 - 80
								EP				0,5	0,5 - 80
								PP				0,5	0,5 - 80
								BP				0,5	0,5 - 80
								BPA				0,5	0,5 - 80
(Deceuninck et al., 2015)	Libres + Totales	3g	Hidrólisis enzimática (optional) + Precipitación de proteinas (acetona) + HRX(poliestireno-divinilbenceno)-SPE + 'molecularly imprinted polymers'-SPE + Derivatización	GC-MS/MS	19	-	EC	BPA	13 - 20 (2 niveles)	94 - 105 (3 niveles)	0,9987	< 0,010 µg/kg	< 0,010 - 5 µg/kg
								BPF	-	103 – 109 (3 niveles)	0,9985	0,018 µg/kg	0,018 – 5 µg/kg
								BPS	-	93-100 (3 niveles)	0,9993	0,003 µg/kg	0,003 – 5 µg/kg
(Filippou et al., 2107)	Libres	1g	Precipitación protéica(solvente apropiado) + Extración en fase sólida magnética dispersiva asistida por ultrasonidos (UA-MSPDE)	LC-UV	-	-	MMC	BPA	0,5 - 3,7 (Intra e interdía)	89,1 - 99,4 (Intra e interdía)	0,9997	2,5 µg/kg	2,5 - 5000 µg/kg

Tabla 15. (Continuación).

Referencia	Biomarcadores analizados	Volumen muestra (mL)	Preparación de muestra	Técnica analítica	Tiempo análisis (min)	Fuente de ionización	Curva de calibración	Analitos*	Precisión (RSD (%))	Recuperación (%)	R^2 curva de calibrado	LoQ (ng/mL)	LDR (ng/mL)
(Arbuckle et al., 2015)	Libres + Totales	0,1	Hidrólisis enzimática(opcional)+ Precipitación protéica(ACN, NaCl) + LLE(clorobutano) + Derivatización + LLE(H_2O + diclorometano:hexano (8:92, v:v))	GC-MS/MS	-	NICI	-	BPA	-	-	-	0,3 (LoD)	-
(Ye et al., 2006b)	Libres + Totales	0,1	Hidrólisis enzimática (opcional) + LLE (2-propanol) + SPE on-line	HPLC-MS/MS	24(SPE incluido)	APCI (-)	EC	BPA	8,2 - 11,4 (2 niveles)	97 - 106 (4 niveles)	> 0,99	0,28 (LoD)	-
(Alshana et al., 2015)	Libres	>1	Extraction 'Salting out' (Ác. fosfórico + ACN + NaCl) + DLLME (Ác. fosfórico y H_2O)	CE	-	-	EC	MP	0,7 - 1,2 (n = 3, Intra e interdía)	90 - 100 (n = 3, 2 niveles))	0,9996	0,3	0,3 - 6,0
								EP	1,5 - 2,2 (n = 3, Intra e interdía)	93,3 - 101,7 (n = 3, 2 niveles)	0,9993	0,3	0,3 - 6,0
								PP	0,5 - 0,8 (n = 3, Intra e interdía)	93,3 - 100 (n = 3, 2 niveles))	0,9998	0,3	0,3 - 6,0
								BP	0,3 - 0,4 (n = 3, Intra e interdía)	96,7 - 100 (n = 3, 2 niveles))	0,9999	0,3	0,3 - 6,0
(Fotouhi et al., 2017)	Libres	0,2	'Magnetically assisted matrix solid phase dispersion' (SorbentE: Poly(indole-thiophene) coated magnetic graphene oxide (MGO@PIT); Sal secante and dispersante de la matriz: Na_2SO_4) + DLLME (1-octanol)	LC-UV y LC-MS/MS	15 (LC-UV) / 28 (LC-MS)	ESI (-) (LC-MS)	MMC	MP	5,3 - 11,3 (Intra e interdía) (sólo datos UV)	87 (sólo datos UV)	0,999 (sólo datos UV)	50 (sólo datos UV)	50 - 4000 (sólo datos UV)
								EP	5,7-10,5 (Intra e interdía) (sólo datos UV)	92 (sólo datos UV)	0,998 (sólo datos UV)	50 (sólo datos UV)	50 - 4000 (sólo datos UV)
								PP	5,1-10,4 (Intra e interdía) (sólo datos UV)	83 (sólo datos UV)	0,999 (sólo datos UV)	50 (sólo datos UV)	50 - 4000 (sólo datos UV)
(Rodríguez-Gómez et al., 2014c)	Libres	1	Liofilización + 'stir-membrane solid–liquid–liquid microextraction' (SM-SLLME)	LC-MS/MS	10	ESI (-)	MMC	MP	1,1 - 7,7 (n = 18, 3 niveles, Intra e interdía)	91 - 103 (n = 18, 3 niveles, Intra e interdía)	0,998	0,5	0,5 - 100
								EP	3,6 - 8,0 (n = 18, 3 niveles, Intra e interdía)	93 - 106 (n = 18, 3 niveles, Intra e interdía)	0,997	0,5	0,5 - 100
								PP	3,9 - 7,5 (n = 18, 3 niveles, Intra e interdía)	95 - 103 (n = 18, 3 niveles, Intra e interdía)	0,997	0,4	0,4 - 100
								BP	1,8 - 6,0 (n = 18, 3 niveles, Intra e interdía)	96 - 104 (n = 18, 3 niveles, Intra e interdía)	0,997	0,5	0,5 - 100
(Melo et al., 2013)	Libres	-	Precipitación protéica (MeCN) + 'Molecularly imprinted polymer for microdisc solid-phase extraction' (MIP-SPE)	LC-UV	-	-	-	MP EP PP	< 13 (Interdía)	86 - 117	> 0,992	10 - 20	10 - 150
(Souza et al., 2016)	Libres	0,2	?In-tube solid phase microextraction? (SPME) (Sorbente: 'Molecularly imprinted polymer modified with a hydrophilic external layer' (RAM-MIP))	UHPLC-MS/MS	-	ESI (-)	MMC	MP	3,5 - 18,7 (n = 5, 6 niveles)	-	0,9989	10	10 - 400
								EP	7,0 - 13,7 (n = 5, 6 niveles)	-	0,9953	10	10 - 400
								PP	2,7 - 14,4 (n = 5, 6 niveles)	-	0,9992	10	10 - 400
								BP	1,0 - 16,0 (n = 5, 6 niveles)	-	0,9999	3	3 - 400
(Fisher et al., 2017)	Totales	-	Hidrólisis enzimática + LLE (1-clorobutano)	LC-MS/MS	-	ESI (-)	-	MP	7,2	69	-	0,1(LoD)	-
								EP	8,3	75	-	0,1(LoD)	-
								PP	7	79	-	0,1(LoD)	-
								BP	3,8	80	-	0,1(LoD)	-
(Niu et al., 2017)	Totales	0,2	Hidrólisis enzimática + LLE (ACN) +'Bond Elut Enhanced Matrix Removal-Lipid purification'+ Derivatización	LC-MS/MS	10.1	ESI(+)	MMC	BPA	3,23 – 6,36 (n=6, 3 niveles)	97,36 – 110,86 (n=6, 3 niveles)	>0,99	0,05	0,05 – 50
								BPF	6,15 – 12,9 (n=6, 3 niveles)	89,42 – 110,00 (n=6, 3 niveles)	>0,99	0,005	0,005 – 5
								BPS	2,06 – 16,6 (n=6, 3 niveles)	99,33 – 109,80 (n=6, 3 niveles)	>0,99	0,01	0,01 – 10

Tabla 15. (Continuación).

Referencia	Biomarcadores analizados	Volumen muestra (mL)	Preparación de muestra	Técnica analítica	Tiempo análisis (min)	Fuente de ionización	Curva de calibración	Analitos*	Precisión (RSD (%))	Recuperación (%)	R^2 curva de calibrado	LoQ (ng/mL)	LDR (ng/mL)
(Vela-Soria et al., 2018)	Totales	1	Hidrólisis enzimática + QuEChERS (LLE con ACN y 150 mg NaCl y150 mg $MgSO_4$) + QuEChERS (d-SPE con 250 mg PSA, 50 mg C_{18}, y 25 mg $MgSO_4$)	UHPLC-MS/MS	12,5	(-)	MMC	MP	6 – 13 (n=18, 3 niveles)	98 – 109 (n=18, 3 niveles)	0,993	0,4	0,4-80
								EP	6 – 8 (n=18, 3 niveles)	101-102 (n=18, 3 niveles)	0,995	0,3	0,3-80
								PP	5 – 12 (n=18, 3 niveles)	99- 103 (n=18, 3 niveles)	0,992	0,4	0,4-80
								BP	9 – 11 (n=18, 3 niveles)	97-103 (n=18, 3 niveles)	0,992	0,5	0,5-80
(Xiong et al., 2018)	Libres	5g	QuEChERS (LLE con ACN + ácido fórmico + 1g $MgSO_4$ + 2 g NaCl	HPLC-FLD	28	-	MMC	BPA	6,3 -11,2 (n= 18, 3 niveles, Intra e interdía)	76 – 88 (n= 18, 3 niveles, Intra e interdía)	0,9992	9,8 µg/kg	10 – 100 µg/kg

*Sólo se han descrito los datos para BPA, BPF, BPS, MP, EP, PP y BP

'Fluorescence Detection' (FLD); 'Capillary Electrophoresis' (CE); 'Atmospheric pressure photoionization' (APPI); Impacto electrónico (EI); 'Negative ion chemical ionization' (NICI); Calibración externa (EC); Matrix-matched calibration (MMC); Límite de cuantificación (LoQ); Rango dinámico lineal (LDR); Coeficiente de variación (RSD).

5.3.1.2. Optimización de la ionización y parámetros del detector

Se comparó el uso de la fuente de ionización en modo ESI y APCI, ambas en polaridad negativa, siendo el modo APCI el que dio lugar a mayores respuestas analíticas para todos los analitos tal y como se observa en la **Tabla SI-1. Cap. 3**.

Las condiciones de la fuente de ionización en APCI neg (temperatura de vaporización, temperatura del capilar, 'discharge current', 'seath gas pressure' y 'auxiliary gas pressure') fueron optimizadas con un diseño de experimentos. En primer lugar se utilizó un diseño Placket Burman para estimar la influencia relativa de estos parámetros en la respuesta analítica y posteriormente se llevó a cabo un diseño central compuesto (ver **SI-1. Cap. 3**, **Tablas SI-2-4 Cap. 3** y **Figura 21**). Los valores de los parámetros finalmente seleccionados se describen a continuación: i) temperatura de vaporización (400 ºC) ii) temperatura del capilar (250ºC) iii) 'discharge current' (4 µA); iv) 'sheath gas pressure' (43 psi); y v) 'auxiliary gas flow rate': 4 unidades arbitrarias.

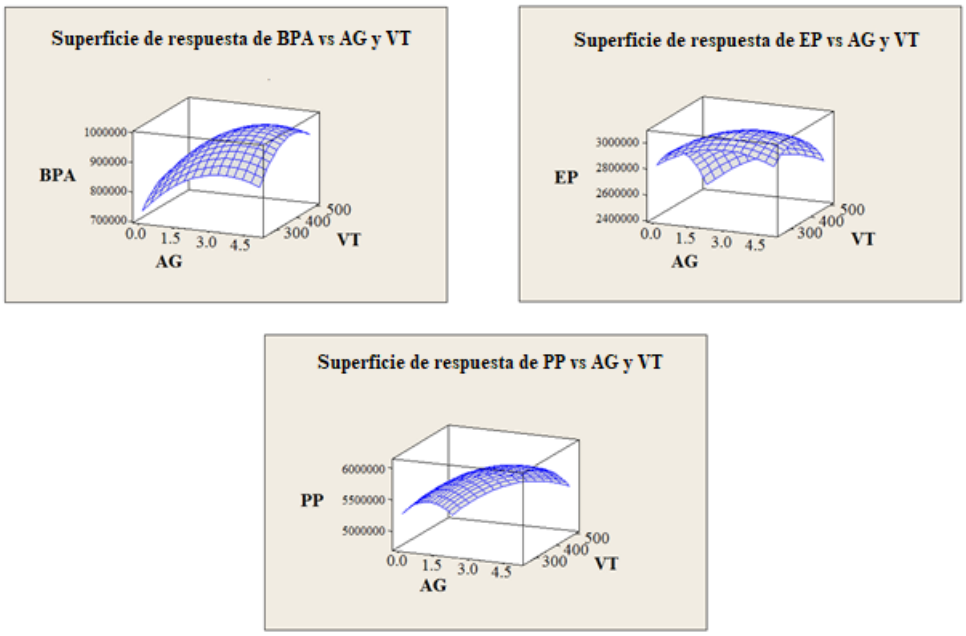

Figura 21. Optimización de los parámetros APCI. Superficies de respuesta obtenidos para BPA, EP y PP en el diseño central compuesto.
VT: Temperatura de vaporización; AG: 'Auxiliary gas flow'

Respecto a la adquisición, se trabajó en modo SRM y se utilizaron dos transiciones por cada analito, de manera que se estudió un ión de cuantificación y otro cualitativo (excepto en patrones internos que solo se utilizó una transición). La **Tabla 16** muestra los parámetros espectrométricos utilizados.

Tabla 16. Parámetros espectrométricos utilizados para la determinación en el QqQ.

Analito	Fórmula química	Transiciones (m/z)[a]	CE (eV)	'Tube lens offset voltage '(V)
Bisfenol A (BPA)	$C_{15}H_{16}O_2$	227,1 → 133,0[b]	-25	-78
		227,1 → 211,9[c]	-31	-78
Bisfenol F (BPF)	$C_{13}H_{12}O_2$	199,1 → 105,0[b]	-23	-90
		199,1 → 93,1[c]	-23	-90
Bisfenol S (BPS)	$C_{12}H_{10}O_4S$	249,1 → 108,0[b]	-20	-90
		249,1 → 156,0[c]	-20	-90
Metil paraben (MP)	$C_8H_8O_3$	151,0 → 92,1[b]	-22	-72
		151,0 → 136,1[c]	-19	-72
Etil paraben (EP)	$C_9H_{10}O_3$	165,1 → 92,0[b]	-27	-51
		165,1 → 136,0[c]	-16	-51
Propil paraben (PP)	$C_{10}H_{12}O_3$	179,1 → 92,0[b]	-22	-77
		179,1 → 136,1[c]	-19	-77
Butil paraben (BP)	$C_{11}H_{14}O_3$	193,1 → 92,0[b]	-28	-78
		193,1 → 136,1[c]	-20	-78
BPA-d_{14}[d]	$C_{15}H_2D_{14}O_2$	241,2 → 141,9[b]	-31	-78
BPF-d_{10}[d]	$C_{13}H_2D_{10}O_2$	209,0 → 97,0[b]	-23	-90
BPS-d_8[d]	$C_{12}H_2D_8O_4S$	257,0 → 112,0[b]	-20	-90
$^{13}C_6$-MP[d]	$^{13}C_6C_2H_8O_3$	157,0 → 98,0[b]	-22	-72
EP-d_5[e]	$C_9H_5D_5O_3$	170,1 → 92,1[b]	-27	-51
$^{13}C_6$-BP [d]	$C_5\,^{13}C_6H_{14}O_3$	199,1 → 98,0[b]	-28	-78

[a] Transiciones SRM
[b] Transición usada para la cuantificación
c Transición usada para la cuantificación
[d] Patrón interno
[e] Patrón interno usado para corregir las concentraciones de EP y PP

5.3.1.3. Optimización del tratamiento de muestra

La extracción y purificación mediante QuEChERS fueron optimizadas probando diferentes proporciones de las sales y reactivos. En el paso de extracción facilitada por 'salting-out', se compararon dos combinaciones (Combinación A: 4g $MgSO_4$ y 1g NaCl; Combinación B: 4g $MgSO_4$, 1g NaCl, 1g de citrato sódico anhidro y 0,5g de monohidrogencitrato disódico sesquihidrato). Debido a que la combinación A presentaba mejores recuperaciones y formas de pico, así como una eliminación de la grasa excelente, se seleccionaron los QuEChERS con 4g $MgSO_4$ y 1g NaCl. Con respecto al paso de purificación se estudiaron tres combinaciones (Combinación A: 150 mg C_{18}, 1200 mg $MgSO_4$; Combinación B: 400 mg C_{18} y 1200 mg $MgSO_4$; y Combinación C: 400 mg C_{18}, 400 mg PSA and 1200 mg $MgSO_4$). Se seleccionó la Combinación C debido a que permitió la obtenición de señales óptimas debidas a la reducción del ruido, además de permitir detectar niveles a concentraciones más bajas de BP y BPA.

Tras la optimización de los procesos de extracción y purificación, se seleccionó el tratamiento de muestra definitivo (ver **Figura 22** y **Tabla 10**).

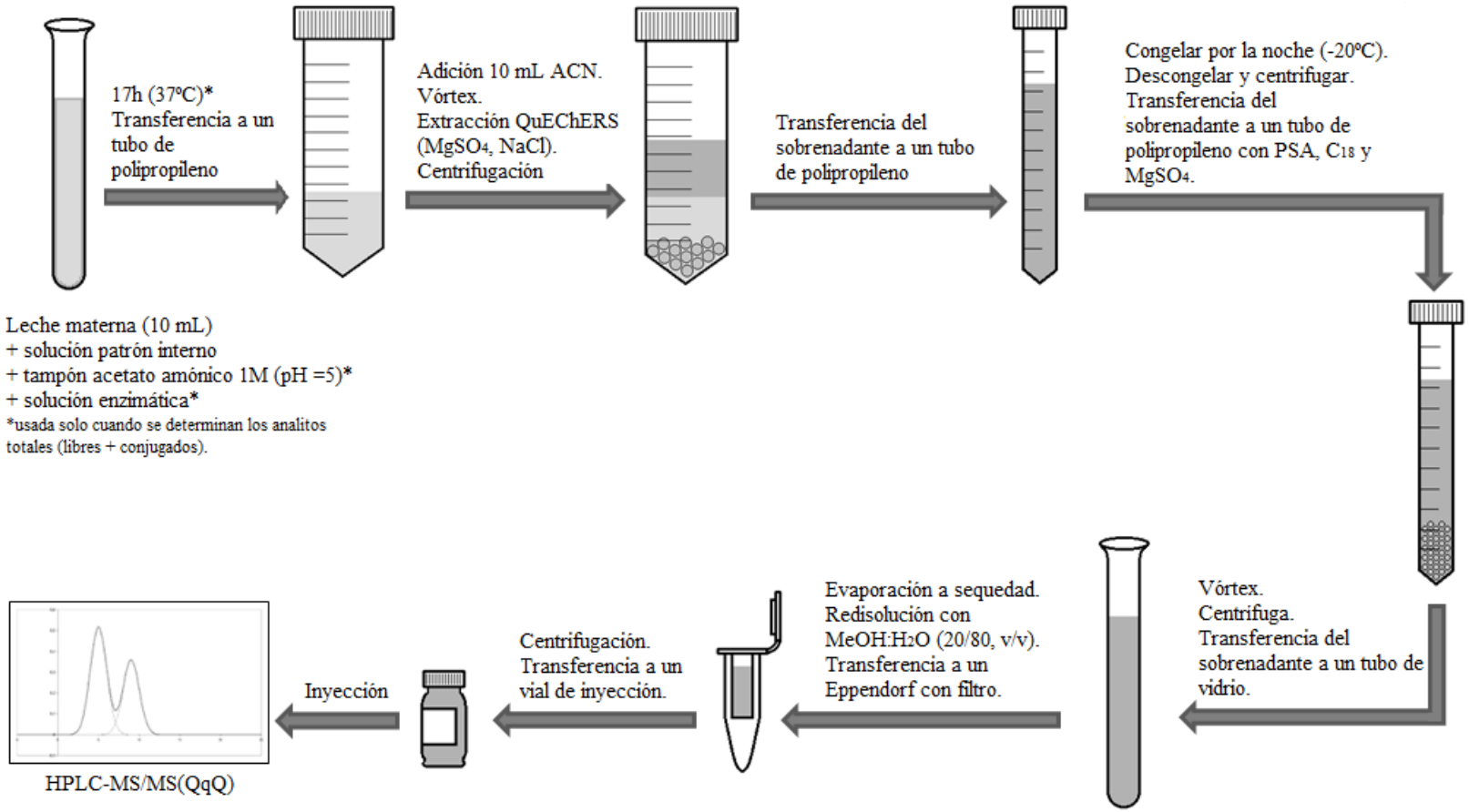

Figura 22. Tratamiento de muestra para la determinación de bisfenoles y parabenos en leche materna.

5.3.1.4. Estudio del efecto matriz

Para el estudio del efecto matriz se prepararon dos sets. El set A estaba formado por 5 replicas de MeOH:H2O (20:80,v/v) fortificada a 5 ng/mL con patrones de los analitos. El set B estaba formado por 5 replicas de blanco de leche fortificada tras la preparación de muestra, para obtener una concentración en vial de inyección de 5 ng/mL.

Para cada analito, el efecto matriz se calculó de la siguiente manera:

Efecto matriz (%) = (B/A)*100

Donde, A y B eran las áreas medias de los analitos detectados en los sets A y B, respectivamente.

El estudio demostró que el MP y BPA presentaban un efecto matriz moderado (75% y 127%, respectivamente), mientras que en el resto de analitos estudiados (EP, PP y BP) el efecto matriz no era significativo ya que estaba en un rango de entre el 86 y el 92%. Con objeto de corregir el efecto matriz en MP y BPA, se utilizaron patrones internos y MMC. El estudio del efecto matriz no se llevó a cabo sobre el BPF ni el BPS ya que se introdujeron posteriormente en el método.

5.3.1.5. Validación del método

Los resultados de la validación del método se muestran en la **Tabla 17**. Se obtuvo una buena linealidad para todos los compuestos (R^2>0.99). El rango lineal de las curvas MMC se estableció entre 0.1 y 50 ng/mL excepto para el BPF y S (0.125-50 y 0.25-50 ng/mL, respectivamente). Los LoQ obtenidos fueron adecuados y consistentes con los niveles encontrados en la literatura. Por último, se obtuvieron recuperaciones en un rango entre el 83 y el 115 % y precisiones con coeficientes de variación (RSD) menores al 16%. El método fue aplicado a muestras reales (ver Capítulos 4 y 5). En la **Figura 23** se muestran cromatogramas de un blanco de muestra fortificado y de una muestra real.

Tabla 17. Parámetros de validación del método.

Analito	**Linealidad (R^2)**	**LoQ (ng/mL)**	**LDR (ng/mL)**	**Exactitud (Recuperación (%)**			**Precisión (RSD (%))**		
				Bajo (n=5)	**Medio (n=5)**	**Alto (n=5)**	**Bajo (n=5)**	**Medio (n=5)**	**Alto (n=5)**
MP [a]	0,996 – 0,999	0,1	0,1 – 50	104	93	95	11	6	1
EP [a]	0,991 – 0,999	0,1	0,1 – 50	105	105	93	12	6	5
PP [a]	0,991 – 0,998	0,1	0,1 – 50	92	83	95	16	6	16
BP [a]	0,995 – 0,999	0,1	0,1 – 50	107	96	92	14	5	6
BPA [a]	0,992 – 0,999	0,1	0,1 – 50	100	101	97	12	7	13
BPF [b]	0,993 – 0,998	0,125	0,125 – 50	115	98	90	6	10	11
BPS [c]	0,991 – 0,999	0,250	0,250 – 50	108	97	99	12	8	11

LoQ: Límite de cuantrificación; LDR: Rango dinámico lineal

[a] Bajo: Concentración 0.1 ng/mL; Medio: Concentración 5 ng/mL; Alto: Concentración 50 ng/mL

[b] Bajo: Concentración 0.125 ng/mL; Medio: Concentración 5 ng/mL; Alto: Concentración 50 ng/mL

[c] Bajo: Concentración 0.250 ng/mL; Medio: Concentración 5 ng/mL; Alto: Concentración 50 ng/mL

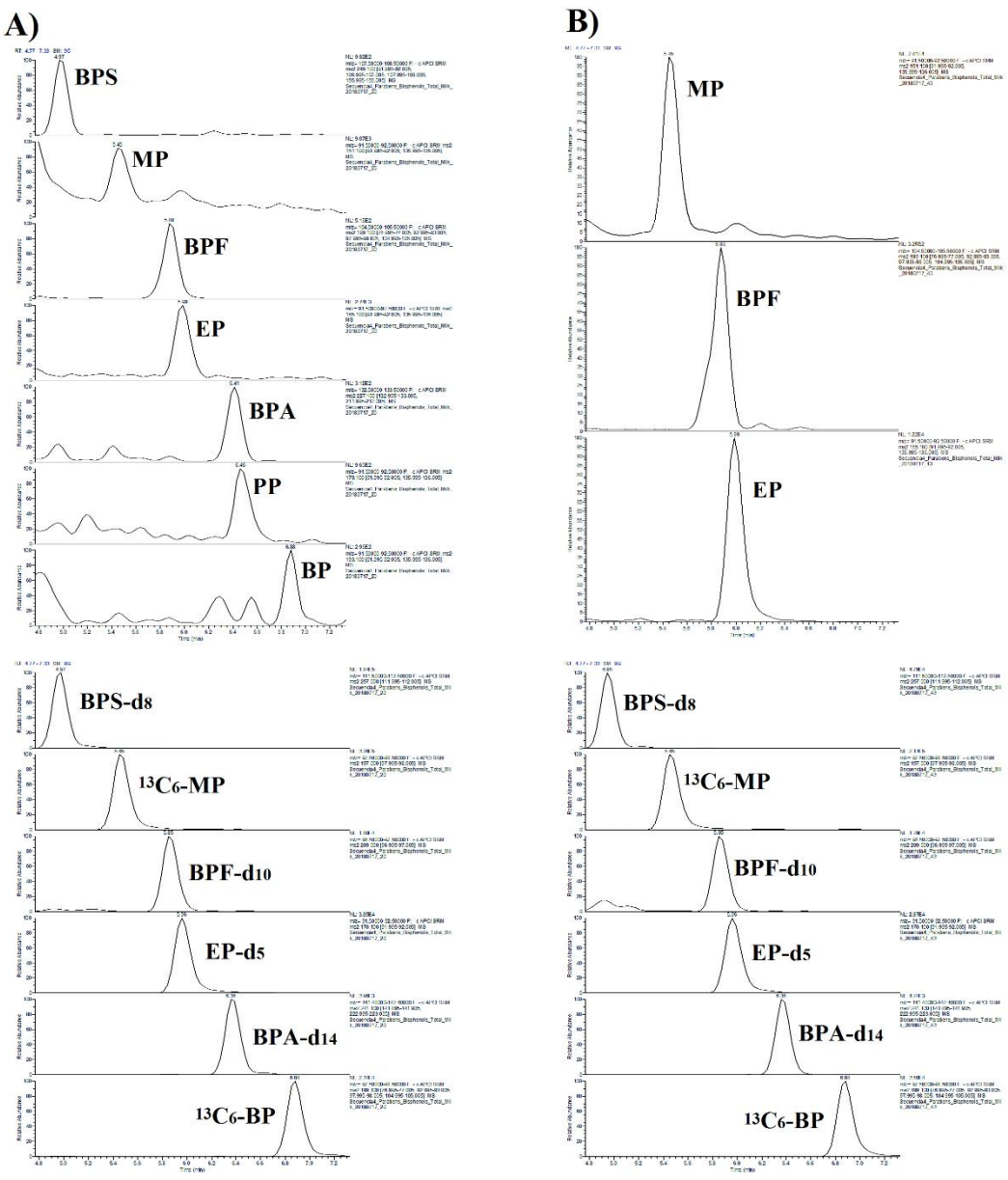

Figura 23. A) Cromatogramas de un blanco de muestra fortificado al LoQ. B) Cromatogramas de una muestra. Los analitos detectados en la muestra en concentraciones superiores al LoQ fueron: MP (1,54 ng/mL), BPF (0,13 ng/mL) and EP (1,46 ng/mL).

5.3.2. Conclusiones

-Se ha desarrollado un método multiresiduo para la determinación de bisfenoles y parabenos en leche materna sensible, exacto y preciso.

-Por primera vez, un solo métododo analítico tiene la capacidad de determinar BPA, BPF, BPS y parabenos en leche materna.

-Además, el uso de APCI como modo de ionización ha sido usado por primera vez para la determinación de BPF, S y parabenos en leche materna.

-La combinación del uso de QuEChERS como tratamiento de muestra y el análisis LC-(APCI)-MS/MS ha permitido alcanzar LoQs de 0,1 ng/mL para la mayoría de los analitos estudiados, lo cual asegura su detección en leche humana en los niveles encontrados previamente en la literatura.

-El método ha sido aplicado satisfactoriamente en la determinación de bisfenoles y parabenos en leche materna, tal y como se muestra en los Capítulos 4 y 5.

5.3.3. Artículo 3: Determination of four parabens and bisphenols A, F and S in human breast milk using QuEChERS and liquid chromatography coupled to mass spectrometry

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Determination of four parabens and bisphenols A, F and S in human breast milk using QuEChERS and liquid chromatography coupled to mass spectrometry

Pablo Dualde[a,b], Olga Pardo[c], Sandra F. Fernández[a], Agustín Pastor[b], Vicent Yusà[a,b,c,*]

[a] Public Health Laboratory of Valencia, Av. Catalunya, 21, 46020, Valencia, Spain
[b] Department of Analytical Chemistry, University of Valencia, Doctor Moliner 50, 46100 Burjassot, Spain
[c] Foundation for the Promotion of Health and Biomedical Research in the Valencia Region, FISABIO-Public Health, Av. Catalunya 21, 46020, Valencia, Spain

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ABSTRACT

For the first time, a multiresidue, sensitive and high throughput method for determination of bisphenol A, F and S and 4 parabens (methyl paraben, ethyl paraben, propyl paraben and butyl paraben) in human breast milk was developed. The proposed method includes an extraction and clean-up procedure based on QuEChERS methodology followed by liquid chromatography coupled to triple quadrupole mass spectrometry determination. Negative atmospheric pressure chemical ionization in the selected reaction monitoring mode was used for mass detection. During the method validation the recoveries varied between 83 and 115% with a precision lower than 20% for all analytes using spiked levels from 0.1 to 50 ng mL^{-1}. The LOQ was 0.10 ng mL^{-1} for most of the analytes. The proposed method was successfully applied for the determination of these compounds in 10 breast milk samples from volunteer lactating mothers from the Valencian region (Spain). Among parabens, methyl paraben presented the highest detection frequency (80%) with a concentration range of 0.11–7.00 ng mL^{-1}, while bisphenol A was detected more frequently than BPF and BPS (80% of detection frequency) with concentrations ranging from 0.13 to 1.62 ng mL^{-1}.

1. Introduction

Bisphenols and parabens are some of the most widely used chemicals worldwide [1,2]. The plastic monomer and plasticizer bisphenol A (BPA) (see Table 1), is used by the manufacturers as an intermediate in the production of polycarbonate and epoxy resins, flame retardants, and other products. Final products include adhesives, protective coatings, polycarbonate flasks and a host of other items frequently used in food packaging [1,3,4]. The main source of exposure of the population to BPA is the ingestion of packaged food or water. However, in infants the predominant routes of exposure are breastfeeding and/or use of polycarbonate bottles [3]. Although BPA can be used in food contact materials in the European Union (EU) [5], it is included in the REACH "Candidate List of substances of very high concern for Authorisation" for its endocrine disrupting properties [6,7] and for its toxicity on reproduction [8].

The recent limitations in the use of BPA have favoured the use of analogue compounds as bisphenol F (BPF) and S (BPS) [9]. However, some studies suggest that BPF and BPS show similar endocrine-disrupting effects to BPA [10] and they have been included in the list of prioritized substances in the Human Biomonitoring for Europe project (HBM4EU) [11].

The alkyl esters of p-hydroxybenzoic acid (parabens) (Table 1) are a group of chemicals used as preservatives in cosmetic products, pharmaceuticals and food [12,13]. Consequently, the main route of exposure to parabens is dermal absorption. After being absorbed by the skin, parabens are metabolized by esterases and/or conjugated and excreted in urine and bile [2]. Some studies found a possible relationship between exposure to parabens and oestrogenic and antiandrogenic activities [2,12].

The European Environmental and Health Strategy and its corresponding Action Plan 2004–2010 encouraged the adoption of human biomonitoring studies in Europe [14], as an effective and necessary tool for assessing human exposure to chemicals in different population groups, mainly in children. These biomonitoring programs require appropriate analytical methods [1,13,15,16].

The selection of the appropriate matrix is capital in biomonitoring studies and breast milk samples provide information about the exposure

* Corresponding author at: Av. Catalunya 21, 46021, Valencia, Spain.
E-mail address: yusa_vic@gva.es (V. Yusà).

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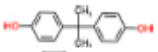
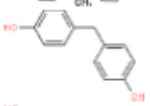
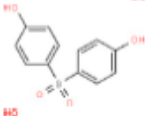
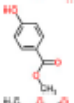
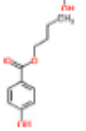
Table 1
Elemental composition, selected transitions, collision energy (CE) and tube lens offset voltage employed for each parent ion-product ions transitions.

Analyte	Stucture	Elemental composition	Transitions (m/z)[a]	CE (eV)	Tube lens offset voltage (V)
Bisphenol A (BPA)		$C_{15}H_{16}O_2$	227.1 → 133.0[b] 227.1 → 211.9[c]	-25 -31	-78 -78
Bisphenol F (BPF)		$C_{13}H_{12}O_2$	199.1 → 105.0[b] 199.1 → 93.1[c]	-23 -23	-90 -90
Bisphenol S (BPS)		$C_{12}H_{10}O_4S$	249.1 → 108.0[b] 249.1 → 156.0[c]	-20 -20	-90 -90
Methyl paraben (MP)		$C_8H_8O_3$	151.0 → 92.1[b] 151.0 → 136.1[c]	-22 -19	-72 -72
Ethyl paraben (EP)		$C_9H_{10}O_3$	165.1 → 92.0[b] 165.1 → 136.0[c]	-27 -16	-51 -51
Propyl paraben (PP)		$C_{10}H_{12}O_3$	179.1 → 92.0[b] 179.1 → 136.1[c]	-22 -19	-77 -77
Butyl paraben (BP)		$C_{11}H_{14}O_3$	193.1 → 92.0[b] 193.1 → 136.1[c]	-28 -20	-78 -78
BPA-d_{14}[d]	-	$C_{15}H_2D_{14}O_2$	241.2 → 141.9[b]	-31	-78
BPF-d_{10}[d]	-	$C_{13}H_2D_{10}O_2$	209.0 → 97.0[b]	-23	-90
BPS-d_8[d]	-	$C_{12}H_2D_8O_4S$	257.0 → 112.0[b]	-20	-90
$^{13}C_6$-MP[d]	-	$^{13}C_6C_2H_8O_3$	157.0 → 98.0[b]	-22	-72
EP-d_5[e]	-	$C_9H_5D_5O_3$	170.1 → 92.1[b]	-27	-51
$^{13}C_6$-BP[d]	-	$C_5\,^{13}C_6H_{14}O_3$	199.1 → 98.0[b]	-28	-78

[a] Ion ratio measures for confirmation (see Section 2.8.) For each compund the average ion ratio were the following: BPA (0.25); BPF (0.54); BPS (0.63); MP (0.62); EP (0.31); PP (0.43); BP (0.43). This ion ratio presents variability between sequences.
[b] SRM transition used for quantification.
[c] SRM transition used for confirmation.
[d] Internal standard.
[e] Internal standard used to correct matrix effects of EP and PP.

of both the mother and her child [17]. However, since only lactating women can donate breast milk, few biomonitoring studies and analytical procedures have been developed using this matrix in comparison with other matrices such as urine [18–21]. Diverse studies have proved the presence of bisphenols (range of 0.01 to 30 ng·mL^{-1} approximately) [22–27] and parabens (ranges from 0.1 to 1000 ng mL^{-1} approximately) [25,28–32] in breast milk samples. Their presence in breast milk at trace levels and the complexity of the matrix require the use of sensitive and selective analytical methodologies. Furthermore, the large number of samples usually required in biomonitoring programs, and the complexity of breast milk sampling makes it necessary to develop multi-residue methods for the determination of various contaminants in the same analysis.

Both unconjugated and conjugated forms of bisphenols and parabens can be present in breast milk as they can conjugate with glucuronide and sulfate groups, thus reducing their toxicity and increasing their excretion [24,28]. For the determination of total (conjugated and unconjugated) species, the hydrolysis of the conjugates must be achieved. Generally a β-glucuronidase/sulfatase enzyme is used for the hydrolysis [33]. Regarding BPA, the ratio unconjugated/total BPA in breast milk presented median values around 0.6 in most of the studies [26,34]. To our knowledge, no previous studies have compared the ratio unconjugated/total BPF or BPS. For parabens ratios unconjugated/total BPA ranged from 0.2 to 1 [33,35].

Table 2 summarizes some of the analytical procedures previously developed for the determination of parabens and/or bisphenols in breast milk. None of the methods previously described allowed the determination of parabens and BPA, BPF and BPS in a single run. Sample preparation is a critical step in complex matrices such as breast milk owing to the presence of lipids and proteins that hinder the determination of the analytes. Sample pre-treatment techniques for fat and protein precipitation are commonly used. Regarding the extraction and clean-up steps, previously developed analytical methods usually employed traditional techniques such as liquid-liquid extraction (LLE) and solid phase extraction (SPE). However, more recently-developed extraction techniques have also been applied. Some of these methods involve laborious steps which can be avoided with the use of QuEChERS, a simple sample treatment methodology consisting in a salting-out extraction step followed by a clean-up step using d-SPE, which has been successfully used in the determination of bisphenols and parabens in matrices such as human urine [48] and breast milk (Table 2([46,47])).

Regarding instrumental techniques, both gas and liquid chromatography (GC and LC) have been employed (Table 2). However, liquid chromatography–tandem mass spectrometry (LC–MS/MS) has become the most frequently used analytical technique for the biomonitoring of

Table 2
Analytical procedures previously described for the determination of parabens and/or bisphenols in breast milk.

Reference	Scope	Sample volume (mL)	Sample preparation	Analytical Determination	Run time (min)	Ionization source
[33]	Unconjugated + Total	0.1	Enzymatic hydrolysis (optional) + LLE (MeOH) + Automated on-line column SPE (RP-18)	HPLC-MS/MS	15 (including SPE)	APPI (−)
[30]	Unconjugated	9.9	For LC-MS analysis: Fat and protein precipitation (Zinc (Zn) and tungsten (W) salts in acidic solution) + Stir-bar sorptive extraction (SBSE)/For GG-MS analysis: Fat and protein precipitation (Zn and W salts in acidic solution) + SBSE + Derivatization	GC–MS/MS and UHPLC-MS/MS	26 (GC–MS) / 10 (LC-MS)	EI (GC–MS)/ESI (−) (LC-MS)
[28]	Unconjugated	9.9	Fat and protein precipitation (Zn and W salts in acidic solution) + Dispersive solid phase extraction (d-SPE) clean-up (150 mg C18 and 100 mg $MgSO_4$)	UHPLC -MS/MS	13	ESI (−)
[36]	Unconjugated	9.9	Ultrasound-assisted extraction (UAE) + d-SPE clean-up (500 mg $MgSO_4$ and 300 mg C18)	UHPLC -MS/MS	10	ESI (−)
[37]	Unconjugated	1	LLE(ACN) + Continuous SPE(LiChrolut Encolumn) + Derivatization	GC–MS	24.5	–
[35]	Unconjugated + Total	0.5	Enzymatic hydrolysis (optional) + LLE (ethyl acetate) + clean up by continuous SPE system (LiChrolut EN) + Derivatization	GC–MS	25	–
[38]	Total	0.25	Enzymatic hydrolysis + Protein denaturation (acetone) + Dispersive Liquid Liquid Microextraction (DLLME)	LC-MS/MS	8.5	–
[24]	Unconjugated + Total	3 g	Enzymatic hydrolysis (optional) + Protein precipitation(acetone) + HRX(polystyrene-divinylbenzene)-SPE + MIMP(molecularly imprinted polymers)-SPE + Derivatization	GC–MS/MS	19	–
[39]	Unconjugated	1 g	Protein precipitation(appropriate solvent) + Ultrasonic Assisted Magnetic Solid Phase Dispersive Extraction (UA-MSPDE)	LC-UV	–	–
[40]	Unconjugated + Total	0.1	Enzymatic hydrolysis (optional) + Protein precipitation(ACN,NaCl) + LLE (chlorobutane) + Derivatization + LLE(water + dichloromethane:hexane (8:92, v:v))	GC–MS/MS	–	NICI
[41]	Unconjugated + Total	0.1	Enzymatic hydrolysis (optional) + LLE (2-propanol) + on-line SPE	HPLC-MS/MS	24 (including SPE)	APCI (−)
[42]	Unconjugated	> 1	Salting out extraction (SoE) (Phosphoric acid + ACN + NaCl) + DLLME (Phosphoric acid and water)	CE	–	–
[32]	Unconjugated	0.2	Magnetically assisted matrix solid phase dispersion (MA-MSPD) (Sorbent: Poly(indole-thiophene) coated magnetic graphene oxide (MGO@PIT); Drying salt and matrix dispersing agent: Na_2SO_4) + DLLME (1-octanol)	LC-UV and LC-MS/MS	15 (LC-UV)/28 (LC-MS)	ESI (−) (LC-MS)
[43]	Unconjugated	1	Liophilization + stir-membrane solid–liquid–liquid microextraction (SM-SLLME)	LC-MS/MS	10	ESI (−)
[44]	Unconjugated	–	Protein precipitation (MeCN) + Molecularly imprinted polymer for microdisc solid-phase extraction (MIP-SPE)	LC-UV	–	–
[45]	Unconjugated	0.2	In-tube solid phase microextraction (SPME) (Sorbent: Molecularly imprinted polymer modified with a hydrophilic external layer (RAM-MIP))	UHPLC-MS/MS	–	ESI (−)
[31]	Total	–	Enzymatic hydrolysis + LLE (1-chlorobutane)	LC-MS/MS	–	ESI (−)

(continued on next page)

Table 2 (*continued*)

Reference	Scope	Sample volume (mL)	Sample preparation	Analytical Determination	Run time (min)	Ionization source
[27]	Total	0.2	Enzymatic hydrolysis + LLE (ACN) + Bond Elut Enhanced Matrix Removal-Lipid purification+ Derivatization	LC-MS/MS	10.1	ESI(+)
[46]	Total	1	Enzymatic hydrolysis + QuEChERS (LLE with ACN and 150 mg NaCl and 150 mg $MgSO_4$) + QuEChERS (dSPE with 250 mg PSA, 50 mg C_{18} and 25 mg $MgSO_4$)	UHPLC-MS/MS	12.5	(−)
[47]	Unconjugated	5g	QuEChERS (LLE with ACN + formic acid + 1 g $MgSO_4$ + 2 g NaAc)	HPLC-FLD	28	–

Reference	Calibration curve	Analytes[a]	Precision Coefficients of variation (RSD (%))	Spiked recoveries (%)	Calibration curve R^2	LoQ (ng·mL^{-1})	LDR (ng·mL^{-1})
[33]	EC	MP	3.5–8.3 (Intra and interday)	90–119 (4 levels)	–	0.1 (LoD)	–
		EP			–	0.1 (LoD)	–
		PP			–	0.1 (LoD)	–
		BP			–	0.1 (LoD)	–
		BPA			–	0.3 (LoD)	–
[30]	MMC	MP	1.7–8.4 (GC-MS)/2.8–15 (LC-MS) (Intra and interday)	97–114 (GC-MS)/ 92–111 (LC-MS) (3 levels)	0.991–0.999	0.4 (GC-MS)/0.2 (LC-MS)	0.4–100 (GC)/0.2–100 (LC-MS)
		EP				0.4 (GC-MS)/0.3 (LC-MS)	0.4–100 (GC-MS)/0.3–100 (LC-MS)
		PP				0.3 (GC-MS)/0.7 (LC-MS)	0.3–100 (GC-MS)/0.7–100 (LC-MS)
		BP				0.3 (GC-MS)/0.2 (LC-MS)	0.3–100 (GC-MS)/0.2–100 (LC-MS)
		BPA				0.5 (GC-MS)/0.3 (LC-MS)	0.5–100 (GC-MS)/0.3–100 (LC-MS)
[28]	MMC	MP	2.0–12.4 (Intra and interday)	91.0–119.8 (3 levels)	0.998–0.999	0.09	0.09–25.0
		EP				0.09	0.09–25.0
		PP				0.09	0.09–25.0
		BP				0.1	0.10–25.0
		BPA				0.15	0.15–25.0
[36]	MMC	MP	1.3–11.1 (Intra and interday)	93.8–112.2	0.996–0.998	0.5	0.5–50
		EP				0.5	0.5–60
		PP				0.4	0.4–60
		BP				0.7	0.7–50
		BPA				0.5	0.5–60
[37]	MMC	MP	4.9–7 (Intra and interday)	87–104 (3 levels)	> 0.995	0.026	0.026–90
		EP				0.029	0.029–50
		PP				0.028	0.028–90
		BP				0.028	0.028–50
		BPA				0.0034	0.0034–50
[35]	MMC	MP	5.2–7.2 (Intra and interday)	92–99 (2 levels)	> 0.994	0.055	0.055–75
		EP		98–101 (2 levels)		0.055	0.055–75
		PP		106–103 (2 levels)		0.05	0.050–75
		BP		88–100 (2 levels)		0.055	0.055–75
		BPA		99–102 (2 levels)		0.007	0.007–75
[38]	MMC	MP	5.1–14.6 (Intra and interday)	90.2–111.8 (3 levels)	0.994–0.996	0.5	0.5–80
		EP				0.5	0.5–80
		PP				0.5	0.5–80
		BP				0.5	0.5–80
		BPA				0.5	0.5–80

(*continued on next page*)

Table 2 (*continued*)

Reference	Calibration curve	Analytes[a]	Precision Coefficients of variation (RSD (%))	Spiked recoveries (%)	Calibration curve R^2	LoQ (ng·mL^{-1})	LDR (ng·mL^{-1})
[24]	EC	BPA	1.3–20 (2 levels)	94–105 (3 levels)	0.9987	< 0.010 μg kg^{-1}	< 0.010–5 μg kg^{-1}
		BPF	–	103–109 (3 levels)	0.9985	0.018 μg kg^{-1}	0.018–5 μg kg^{-1}
		BPS	–	93–100 (3 levels)	0.9993	0.003 μg kg^{-1}	0.003–5 μg kg^{-1}
[39]	MMC	BPA	0.5–3.7 (intra and interday)	89.1–99.4 (intra and interday)	0.9997	2.5 μg kg^{-1}	2.5–5000 μg kg^{-1}
[40]	–	BPA	–	–	–	0.3 (LoD)	–
[41]	External calibration	BPA	8.2–11.4 (2 levels)	97–106 (4 levels)	> 0.99	0.28 (LoD)	–
[42]	EC	MP	0.7–1.2 (n = 3, intra and interday)	90–100 (n = 3, 2 levels)	0.9996	0.3	0.3–6.0
		EP	1.5–2.2 (n = 3, intra and interday)	93.3–101.7 (n = 3, 2 levels)	0.9993	0.3	0.3–6.0
		PP	0.5–0.8 (n = 3, intra and interday)	93.3–100 (n = 3, 2 levels)	0.9998	0.3	0.3–6.0
		BP	0.3–0.4 (n = 3, intra and interday)	96.7–100 (n = 3, 2 levels)	0.9999	0.3	0.3–6.0
[32]	MMC	MP	5.3–11.3 (intra and interday) (UV data only)	87 (UV data only)	0.999 (UV data only)	50 (UV data only)	50–4000 (UV data only)
		EP	5.7–10.5 (Intra and interday) (UV data only)	92 (UV data only)	0.998 (UV data only)	50 (UV data only)	50–4000 (UV data only)
		PP	5.1–10.4 (Intra and interday) (UV data only)	83 (UV data only)	0.999 (UV data only)	50 (UV data only)	50–4000 (UV data only)
[43]	MMC	MP	1.1–7.7 (n = 18, 3 levels, intra and interday)	91–103 (n = 18, 3 levels, intra and interday)	0.998	0.5	0.5–100
		EP	3.6–8.0 (n = 18, 3 levels, intra and interday)	92–106 (n = 18, 3 levels, intra and interday)	0.997	0.5	0.5–100
		PP	3.9–7.5 (n = 18, 3 levels, intra and interday)	95–103 (n = 18, 3 levels, intra and interday)	0.997	0.4	0.4–100
		BP	1.8–6.0 (n = 18, 3 levels, intra and interday)	96–104 (n = 18, 3 levels, intra and interday)	0.997	0.5	0.5–100
[44]	–	MP	< 13 (Interday)	86–117	> 0.992	10–20	10–150
		EP					
		PP					
[45]	MMC	MP	3.5–18.7 (n = 5, 6 levels)	–	0.9989	10	10–400
		EP	7.0–13.7 (n = 5, 6 levels)	–	0.9953	10	10–400
		PP	2.7–14.4 (n = 5, 6 levels)	–	0.9992	10	10–400
		BP	1.0–16.0 (n = 5, 6 levels)	–	0.9999	3	3–400
[31]	–	MP	7.2	69	–	0.1(LoD)	–
		EP	8.3	75	–	0.1(LoD)	–
		PP	7	79	–	0.1(LoD)	–
		BP	3.8	80	–	0.1(LoD)	–
[27]	MMC	BPA	3.23–6.36 (n = 6, 3 levels)	97.36–110.86 (n = 6, 3 levels)	> 0.99	0.05	0.05–50
		BPF	6.15–12.9 (n = 6, 3 levels)	89.42–110.00 (n = 6, 3 levels)	> 0.99	0.005	0.005–5
		BPS	2.06–16.6 (n = 6, 3 levels)	99.33–109.80 (n = 6, 3 levels)	> 0.99	0.01	0.01–10
[46]	MMC	MP	6–13 (n = 18, 3 levels)	98–109 (n = 18, 3 levels)	0.993	0.4	0.4–80
		EP	6–8 (n = 18, 3 levels)	101–102 (n = 18, 3 levels)	0.995	0.3	0.3–60
		PP	5–12 (n = 18, 3 levels)	99–103 (n = 18, 3 levels)	0.992	0.4	0.4–80
		BP	9–11 (n = 18, 3 levels)	97–103 (n = 18, 3 levels)	0.992	0.5	0.5–60

(continued on next page)

Table 2 (*continued*)

Reference	Calibration curve	Analytes[a]	Precision Coefficients of variation (RSD (%))	Spiked recoveries (%)	Calibration curve R^2	LoQ ($ng mL^{-1}$)	LDR ($ng mL^{-1}$)
[47]	MMC	BPA	6.3–11.2 (n = 18, 3 levels, intra and interday)	76–88 (n = 18, 3 levels, intra and interday)	0.9992	9.8 $\mu g kg^{-1}$	10–100 $\mu g kg^{-1}$

Fluorescence Detection (FLD); Capillary Electrophoresis (CE); Atmospheric pressure photoionization (APPI); Electron impact (EI); Negative ion chemical ionization (NICI); External Calibration (EC); Matrix-matched calibration (MMC).

[a] Only BPA, BPF, BPS, MP, EP, PP and BP have been described.

bisphenols and parabens, allowing the detection of these compounds at trace levels in biological matrices. Despite using advanced analytical instrumentation (LC-MS/MS), LoQs below 0.1 ng mL^{-1} have only been achieved for bisphenols by Niu et al., 2017 (Table 2 ([27])). As far as we know, the use of atmospheric pressure chemical ionization (APCI) has not been employed for determining parabens, BPF or BPS in breast milk, being, instead, electrospray (ESI) the most frequently employed LC-MS ionization technique (Table 2([28,30,36,38])). APCI is useful for analysing low weight molecules with non-polar or relatively low polar properties [49] and it has previously been used successfully in the determination of BPA in breast milk (Table 2([41])). Although APCI has been clearly less investigated than the ESI source, it has been generally reported to be less susceptible to matrix effects because ionization takes place in the gas phase [50].

Due to the ubiquitous presence of bisphenols and parabens, mainly BPA, in the environment, some authors have indicated that special care during collection, handling and analysis of samples for the biomonitoring of these substances should be taken [51]. It has been stated that the use of containers and breast pumps during sampling can be a source of contamination of BPA [34,40,52]. Also, the preparative steps involved in the sample analysis could be a cause of BPA contamination due to the use of non-properly cleaned glassware and contaminated reagents or polypropylene material [40,53]. Furthermore, BPA can be found in experimental apparatus plastic devices, or even in the dust [51].

In the present study we have developed a new analytical strategy based on QuEChERS for the extraction and purification steps and LC-APCI-MS/MS for a sensitive determination of total bisphenols (BPA, BPF, BPS) and parabens (MP, EP, PP and BP) in human breast-milk samples. In a first step we developed the method for the determination of parabens and BPA only. However, due to the recent EU interest in the human biomonitoring of BPF and BPS, including them in the list of priority substances of HBM4EU [11], we added, later to the method optimization, both BPF and BPS. Consequently, BPF and BPS were not included in the optimization steps, but the seven analytes were included in the method validation and field samples analysis. The developed method presents two main advantages: i) is the first reported method which can determine four parabens and bisphenols A, F and S in breast milk in a single run; ii) the LoQs obtained allow the determination of these compounds at the levels usually found in breast milk samples. The proposed method was satisfactorily validated and successfully applied in 10 breast milk samples from volunteer lactating mothers from the Valencian region (Spain).

2. Materials and methods

2.1. Reagents and chemicals

Acetonitrile and methanol were of LC-MS grade and supplied by VWR Prolabo (Barcelona, Spain). Ultra-pure water produced with a Milli-Q Gradient system (Millipore, Bedford, USA) was used throughout the study.

QuEChERS Extract Pouches-EN method (salt packet containing 4 g $MgSO_4$, 1 g NaCl), ceramic homogenizers for QuEChERS extraction and QuEChERS fatty dispersive-SPE AOAC kit, 15 mL polypropylene tube containing 400 mg PSA, 200 mg C_{18} and 1200 mg $MgSO_4$ were obtained from Agilent Technologies (Madrid, Spain).

β-Glucuronidase/sulfatase from *Helix pomatia* type H1 was obtained from Sigma Aldrich (St. Louis, MO, USA). The enzymatic solution was prepared weekly disolving the enzyme purified powder in ammonium acetate 1 M (pH = 5) to obtain a solution of 3500 U/mL.

2.2. Standards and solutions

Certified commercial standards were of high purity. BPA, BPF and BPS and BPA-d14 were obtained from Dr. Ehrenstorfer (Ausburg,

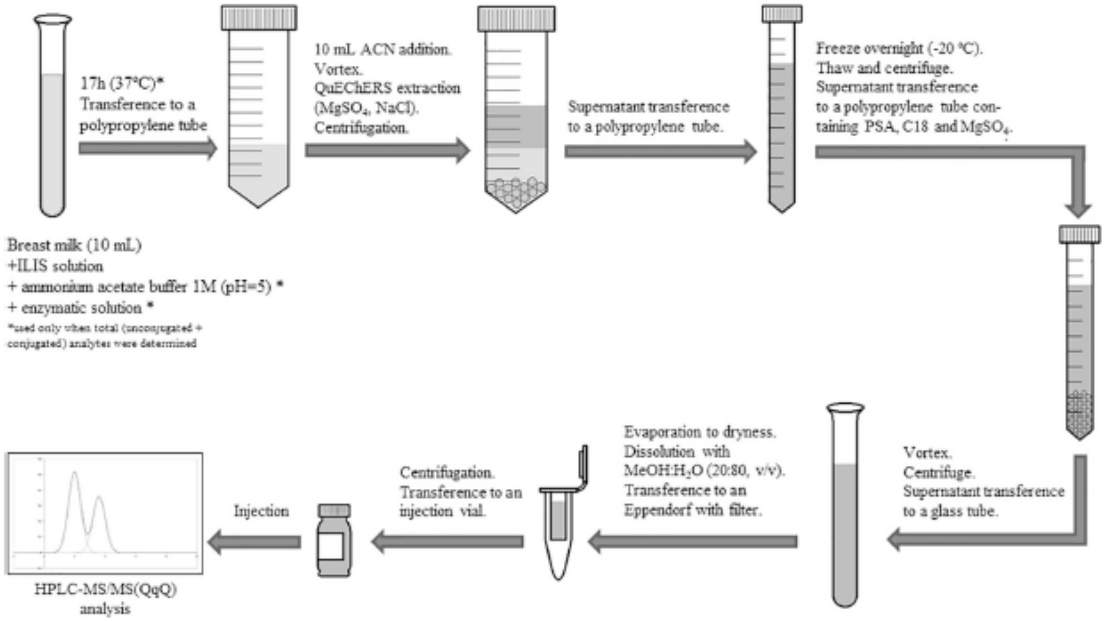


Fig. 1. Diagram of the extraction procedure.

Germany). BPF-d10 and BPS-d8 were obtained from Toronto Research Chemicals (Toronto, Canada) and CDN Isotopes (Quebec, Canada), respectively. MP, EP, PP and BP were supplied by Sigma-Aldrich (Barcelona, Spain), $^{13}C_6$-BP and $^{13}C_6$-MP were supplied by Cambridge Isotope Laboratories (Massachusetts, USA) and EPB-d_5 was purchased from Santacruz Biotechnology (Heidelberg, Germany). Table 1 shows all the compounds considered for the present study. Each commercial standard was weighed and/or dissolved in acetonitrile to obtain a stock solution of approximately 200,000 ng mL^{-1}. The stock solutions were stored in the dark at −20 °C for < 6 months. Multi-analyte intermediate standard solutions containing 100, 1000 and 20,000 ng mL^{-1} were prepared by diluting the individual stock solutions with methanol:water (20:80, *v*/v) (similar composition to mobile phase) and were used for the matrix-match calibration curve and the spiked samples for recovery calculations. The intermediate solutions were stored in the dark at 4 °C for < 3 months.

A mix solution containing the isotopically-labelled internal standards (ILIS) (Table 1) 1000 ng·mL^{-1} was prepared by diluting individual standard solutions in methanol:water (20:80, v/v). This solution was stored in the dark at 4 °C for < 3 months.

2.3. Sample collection and storage

The 10 samples of human breast milk analyzed in this study are part of a research biomonitoring project. The study and sampling protocol were approved by the Scientific Ethics Committee of the Valencian Research Centre for Public Health (FISABIO) of the Valencian Government (Dirección General de Salud Pública, DGSP) and the Biomedical Scientific Ethics Committee of the University and Polytechnic Hospital La Fe. All participants signed an Informed Consent approved by the Ethics Committee. Samples were obtained during 2015 from 10 Spanish women (28–40 years old) living in the Valencian Region (Spain) that provided breast milk 2 weeks after delivery, as part of a collaboration with the perinatology group of Health Research Institute La Fe in Valencia. In order to avoid bisphenols and parabens contamination (see point 2.4. Control of contamination), the breast pumps used for sample collection were BPA free. Furthermore the mothers avoided the use of personal care products in their nipples and cleaned them with abundant water before sampling. Samples were stored at −80 °C in the IBSP-CV BioBank (PT13/0010/0064), integrated in the Spanish National Biobanks Network and in the Valencian Biobanking Network.

2.4. Control of contamination

In order to avoid and check the contamination, mainly of BPA, during sample collection and sample treatment some special measures were taken. As parabens are present in cosmetics, mothers avoided the use of personal care products in their nipples and cleaned them with abundant water before sampling. The breast pumps and containers used for sampling were BPA free (specified by the supplier). Additionally a migration study was applied to them in order to confirm the absence of contamination. The migration study was based on [54] with some modifications; the simulant used was ethanol 50% (*v*/v) (simulant D1) and the conditions of the migration study were 2 h at 70 °C following the European Union Reglament 11/2011 [5]. The analysis was performed in the same LC-MS/MS equipment used for the determination of bisphenols and parabens in human milk.

Regarding sample treatment, special care was taken in order to avoid dust or air contamination, working in safety cabinets. Also, the polypropylene material used during sample treatment was also BPA free.

As indicated in section 2.7 "Validation and Quality control procedure", each batch included a Reagent blank, and a Milk blank which suffered the same procedures as the study samples in order to control the contamination during sample treatment.

In addition, various mobile phases which consisted of methanol:water (20:80, *v*/v) were injected throughout the batches in order to check the absence of BPA migration from the analytical equipment.

2.5. Sample preparation

A scheme of sample preparation is detailed in Fig. 1. For the analysis of total analytes, a deconjugation step was implemented after the defreezing and homogenization of the sample. Briefly, in a glass tube, 10 mL of the sample, 125 µL of the ILIS solution and 1 mL of the enzymatic solution were added. The mix was homogenized and incubated during 17 h at 37 °C. After the deconjugation step, the sample was transferred into a 50 mL polypropylene centrifuge tube. A simplified QuEChERS procedure was employed to extract compounds from human breast milk. Briefly, 10 mL of acetonitrile was added and the mixture was shaken for 1 min with a Vortex and introduced in an ice bath. Consecutively, a pouch of QuEChERS salt packet containing 4 g anhydrous $MgSO_4$, 1 g NaCl and 2 homogenizer ceramics were added. The content was shaken for 1 min and the tube was introduced in the ice bath again. Then, the mixture was centrifuged at 8000 rpm and 20 °C during 10 min. The total acetonitrile layer was taken and transferred into a 10 mL polypropylene tube. The mixture was stored overnight at −20 °C.

After 24 h, the extract was transferred into a 15 mL QuEChERS fatty dispersive-SPE (d-SPE) AOAC kit (400 mg PSA, 200 mg C_{18} and 1200 mg $MgSO_4$) polypropylene tube. The mixture was shaken for 1 min and centrifuged for 15 min at 4500 rpm and 15 °C. The cleaned supernatant was transferred into a 15 mL glass tube and evaporated under N_2 stream at 37 °C to dryness. The dry residue was then dissolved in 250 µL of methanol:water (20:80, v/v) and placed into a Millipore 0.2 µm Eppendorf and ultra-centrifuged (13,000 rpm, 1 min and 5 °C). The final extract was transferred into an injection vial and analyzed on the HPLC–MS/MS system.

2.6. HPLC-MS/MS analysis

2.6.1. Chromatographic conditions

Chromatographic separation was carried out with the high performance liquid chromatography (HPLC) system TSQ Quantum from Thermo Fisher Scientific (Bremen, Germany) equipped with a Symmetry C18 column (2.1 × 150 mm, 5 µm) from Waters (Milford, Massachusetts, USA). The column temperature was 35 °C, the flow rate used was 300 $\mu L \cdot min^{-1}$ and the injection volume was 20 µL. The mobile phase consisted of (A) water and (B) methanol. The analysis started with 98% mobile phase A. Then, solvent A decreased to 0% in 6 min and was maintained for 1 min. The initial conditions were restored in 0.5 min, followed by a re-equilibration time of 4.5 min. The total run time was 12 min. Data acquisition was performed by the Thermo Scientific Trace Finder™ 3.2 software.

2.6.2. Mass spectrometry detector settings

The influence of the ionization source on the analytical responses was studied by injecting a standard solution using ESI(−) and APCI (−). Although ESI is nowadays the most commonly used technique because of its wide range of applicability, APCI is suitable for thermally stable polar and nonpolar compounds. In order to compare the analytical responses obtained with ESI(−) and APCI(−), a 100 $ng\,mL^{-1}$ standard mix solution was injected in triplicate using both sources.

Mass analysis was performed on the Finnigan TSQ Quantum Ultra Detector analyser (San José, CA, USA) equipped with an APCI source. Collision gas pressure was 1.5 mTorr. Tube lens offset voltages were optimized for each compound using the automated optimization procedure in the syringe infusion mode provided by the manufacturer. The ion source settings were optimized by design of experiments (DOE): vaporization temperature (VT), 400 °C; capillary temperature (CT), 250 °C; discharge current (DC), 4 µA; sheath gas pressure (SG), 43 psi; and auxiliary gas flow rate (AG), 4 arbitrary units (a.u.). Statistical data manipulation and numerical analysis of data resulting from experimental design were carried out by means of the statistical package MINITAB for Windows, Release 14 (Minitab Inc., Birmingham, UK). A Plackett-Burman design (B-M) was chosen as a screening method to estimate the relative influence of the five factors on the analytical response. Subsequently, in order to obtain a more accurate optimization of the factors with a higher influence, a central composite design (CCD) was used (see Supplementary Material SI-1 and Table S-1.

APCI and selected reaction monitoring (SRM) in negative mode were used for all the analytes. The use of two transitions for each compound allowed simultaneous quantification and identification in one run. Elemental composition, time of retention (t_R), selected transitions, collision energy (CE) and tube lens offset voltage employed for each parent ion-product ion transition are shown in Table 1.

2.6.3. Matrix effect study

The matrix effect can be calculated a) comparing the slope of a calibration curve reagent and a matrix-matched calibration curve [41] b) and it can also be studied according to the procedure described in Leon et al., and Roca et al., [55,56]. We have selected the second option. Briefly, two analytical sets A and B were prepared. The Matrix effect (ME,%) of the method was evaluated by comparing the absolute peak areas of the two sets. The procedure for each set is described as follows:

Set A: Consisted of five replicates of a standard solution containing the 5 analytes at a concentration of 5 $ng \cdot mL^{-1}$ in mobile phase.

Set B: Consisted of five replicates of a pool blank of breast milk spiked, after sample preparation, with 250 µL of the standard solution used on set A.

The matrix effect was calculated via the formula:

$$ME\ (\%) = (B/A) * 100$$

where, A and B are the mean peak areas of the set A and B, respectively. The matrix effect was classified into three different categories considering the calculated values. There was no matrix effect when the ME factor was between 80% and 120%. A medium matrix effect was considered when the values ranged between 40% and 80% or 120% and 150%. A percentage below 40% or above 150% was classified as a high matrix effect [55].

2.7. Validation and quality control procedure

The method validation was based on the recommendations established in the "Bioanalytical Method Validation, FDA Guidance for Industry" [57]. Validation experiments and quality controls were performed using a pool of blank breast milk samples. The blank milk pool was obtained mixing milk from lactating mothers participating in the project Bettermilk ($n = 120$) which presented signals clearly lower than the LoQs of bisphenols and parabens.

The linearity of the method was evaluated using eight-point matrix-matched calibration curves, spiking the pool of blank breast milk at various concentration levels (from 0.1 to 50.0 $ng\,mL^{-1}$ (except for BPF and BPS wich levels ranged from 0.125 to 50 $ng\,mL^{-1}$ and 0.250 to 50 $ng\,mL^{-1}$ respectively). Analysis of Variance (ANOVA) Mandel's fitting test and $R^2 > 0.99$ were used for assessing the validity of the linear model. The accuracy of the method was calculated in terms of relative recovery in each batch by spiking the pool of blank breast milk at three different concentration levels: LoQ level and the intermediate and the highest level of the calibration curve. Inter- and intra-day precision was also evaluated by measuring replicates of each concentration daily and during five different days, respectively.

The limit of quantification (LoQ) for each compound was established at the lowest validated spiked level to meet the performance acceptability criteria (mean recoveries ranging between 80 and 120% with RSDs < 20%). Furthermore, a minimal signal-to-noise ratio of 10 was required and the confirmation criteria defined in section 2.8. were achieved.

In each analytical batch, various quality control samples (QC) were used to check the extraction procedure efficiency and ensure a good

quantification of real samples. The QC were prepared by spiking the pool of breast milk blanks at LoQ level and the intermediate and the highest level of the calibration curve and were subjected to the same extraction and analysis procedures as real samples and calibration curve points. A Reagent blank and a Milk blank were also included in each batch in order to control contamination and interferences.

2.8. Quantification and confirmation criteria

For quantitation, procedural matrix-matched calibration curves were used. MP, EP, PP, BP, BPA, BPF and BPS in samples were quantified using a linear calibration function. The quantification was performed with the area corresponding to the most abundant fragment ion of the analyte obtained in SRM mode. To correct possible matrix effects and losses occurred during extraction, analogous ILIS were used (Table 1).

Compound identification and confirmation was based on SANTE/11813/2017 [58]:

a) 2 product ions, one used for quantification and another used for confirmation (except in IS, where only the quantification ion was required)
b) a tolerance of ± 0.1 min for Relative Retention Time (RRT) is allowed between the suspicious analyte in the sample and the analyte in the quality control samples;
c) the ratio between the fragment ions obtained in the sample should not differ > 30% with respect to the average ratio of the analyte in the calibration standards from the same sequence.

3. Results and discussion

3.1. Control of contamination

The breast pump migration analysis confirmed the absence of contamination. In absence of clearly defined criteria for the contamination control in biomonitoring analytical methods, we followed the criteria described in the SANTE/11813/2017 (regularly used in our laboratory), which establishes that the signal in the blanks should not be higher than the 30% of the reporting limit signal (in our case the LoQ) [58]. During breast milk analysis, Milk blank and Reagent blank analytes areas were < 30% than the LoQ area in all batches involved in validation and field samples analysis, and Milk blank and Reagent blank calculated concentrations were lower than 0.03 ng mL^{-1} (LoQ = 0.1 ng mL^{-1}). Also, the absence of signal at the retention time and *m/z* value of BPA in the Mobile phase (methanol:water (20:80, *v/v*)) injections denies the existence of BPA migration from the analytical equipment. Fig. S2 shows, as an example, the SRM chromatograms of BPA of LoQ, Milk blank, Reagent blank and Mobile phase in one batch. Consequently, we considered the analytes external contamination negligible in comparison with LoQ levels.

3.2. Chromatographic separation

A C18 column (Symmetry, 2.1 × 150 mm, 5 µm) was selected to achieve chromatographic separation. Several modifiers (acids, bases and buffers) were tested in water (mobile phase A), and in methanol (mobile phase B) since modifiers are frequently used in multiresidue LC-MS/MS methods for the determination of BPA and parabens in breast milk [28,30,36,38]. However, the use of acids, bases and/or buffers can reduce the lifetime of the column [59,60]. Despite everything, mobile phases with no added modifiers provided the best peak shapes and the highest responses. Therefore, water was selected as mobile phase A and methanol as mobile phase B.

3.3. Optimization of the ionization modes and detector settings

The results of comparison between ESI(−) and APCI(−) can be seen in Table S-2. Using APCI(−) higher analytical responses for all analytes were obtained. In addition, APCI do not require the addition of acid or basic compounds in the mobile phase and, as stated before, the presence of these modifiers can contribute to decrease the life of the LC column [59,60]. Hence, APCI(−) was selected as the ionization source. As can be seen in Table 2, most of the methodologies based on LC-MS employed ESI (−). Only Ye et al., [41] used APCI for the analysis of BPA, and to our knowledge, APCI had never been employed previously for the determination of parabens, BPF or BPS in breast milk.

The full-scan spectra were monitorized through infusion (50 µL min^{-1}) of individual standard solutions of 1 µg mL^{-1}. Then, the fragmentation of the precursor ion was optimized.

The ion source settings optimization is fully described in the Supplementary material (SI-1. Ion source settings optimization, Table S-3, Table S-4, Fig. S-1). Briefly, five factors (k = 5) were selected as potentially affecting de APCI efficiency: SG, AG, CT, VT and DC. After a Placket Burman screening, AG and VT were selected for optimization with a CCD. The optimized factor settings were: SG, 43 psi; AG, 4 a.u.; CT, 250 °C; VT, 400 °C; and DC, 4 µA.

3.4. Sample treatment procedure

Both extraction and clean-up steps were achieved using QuEChERS. Diverse proportions of salts and reagents were studied to optimize the sample treatment. The main objective was the quantitative extraction of all the compounds studied as well as the removal of lipids and other matrix interferences that can compromise the selective and sensitive detection.

The first step of the sample treatment was based on the QuEChERS salting out extraction. Acetonitrile was selected as the extraction solvent and various QuEChERS salts combinations (Combination A: 4 g $MgSO_4$ and1 g NaCl; Combination B: 4 g $MgSO_4$ and 1 g NaCl, 1 g sodium citrate and 0.5 g sodium hydrogencitrate sesquihydrate) were tested to select the most appropriate extraction method for this study. Finally, QuEChERS extraction tubes containing 1 g NaCl and 4 g $MgSO_4$ were selected since this combination presented higher recoveries with good peak shapes and an excellent removal of fat content. This extraction procedure is simple in comparison with other methodologies as that described by Rodrígez-Gómez et al., 2015 (Table 2 ([36]) which needed 3 sonication steps to achieve the extraction. Alshana et al., 2015 also applied a salting-out extraction using phosphoric acid, ACN and a saturated NaCl solution for the determination of parabens in breast milk (Table 2([42])).

To remove co-extracted interfering substances from breast milk a d-SPE based on the QuEChERS procedure clean-up was designed. Various amounts and combinations of sorbents (150 mg end-capped C_{18} with 1200 mg $MgSO_4$; 400 mg end-capped C_{18} with 1200 mg $MgSO_4$ and finally 400 mg end-capped C_{18} with 400 mg PSA and 1200 mg $MgSO_4$) were tested in order to select the combination that maximized the analytical signal while removing most of the matrix interferences.

In the present study it was observed that the combination of 400 mg end-capped C_{18} with 400 mg PSA and 1200 mg $MgSO_4$ provided the optimum signals in terms of reducing background noise and allowing to reach a low LoQ value for BP and BPA, the two analytes that presented the lowest signal intensity. Therefore, the 400 mg end-capped C_{18}, 400 mg PSA and 1200 mg $MgSO_4$ combination was selected for the clean-up step. Similar results were reported by Vela-Soria et al. (Table 2 [46]), which highlighted the significant positive effect of PSA in the clean-up procedure. However, Rodríguez et al. (Table 2 [36]) indicated that although PSA removed matrix interferences, they observed lower areas when PSA was added than when only C_{18} and $MgSO_4$ were used during the clean-up step. The use of d-SPE as clean-up proved to be a good alternative to the SPE used in most of the methods described for

Table 3
Method validation parameters.

Analyte	Linearity (R^2)	LoQ (ng mL^{-1})	LDR (ng mL^{-1})	Accuracy (Recovery (%)			Precision (RSD (%)		
				Low (n = 5)	Medium (n = 5)	High (n = 5)	Low (n = 5)	Medium (n = 5)	High (n = 5)
MP[a]	0.996–0.999	0.1	0.1–50	104	93	95	11	6	1
EP[a]	0.991–0.999	0.1	0.1–50	105	105	93	12	6	5
PP[a]	0.991–0.998	0.1	0.1–50	92	83	95	16	6	16
BP[a]	0.995–0.999	0.1	0.1–50	107	96	92	14	5	6
BPA[a]	0.992–0.999	0.1	0.1–50	100	101	97	12	7	13
BPF[b]	0.993–0.998	0.125	0.125–50	115	98	90	6	10	11
BPS[c]	0.991–0.999	0.250	0.250–50	108	97	99	12	8	11

LDR: linear dynamic range.
[a] Low: Concentration 0.1 ng mL^{-1}; Medium: Concentration 5 ng mL^{-1}; High: Concentration 50 ng mL^{-1}.
[b] Low: Concentration 0.125 ng mL^{-1}; Medium: Concentration 5 ng mL^{-1}; High: Concentration 50 ng mL^{-1}.
[c] Low: Concentration 0.250 ng mL^{-1}; Medium: Concentration 5 ng mL^{-1}; High: Concentration 50 ng mL^{-1}.

the determination of BPA and parabens in breast milk [24,33,35,37,41] or other SPE-derived techniques also used in these determinations as SPME [45] MIP-SPE [44] or UA-MSPDE [39].

3.5. Matrix effect study

When using LC-MS for the analysis of complex matrices such as breast milk, matrix effect must be studied during method development to acknowledge the effect of the matrix over the ion suppression or the signal enhancement [28,30,36,38]. APCI is clearly less investigated than the ESI source and, although it is generally reported that the former is less susceptible to a matrix effect because ionization takes place in the gas phase, the matrix effect was evaluated before studying its performance characteristics [61].

Results showed that MP and BPA presented medium matrix effect (MP 75% indicating ion suppression and BPA 127% reflecting ion enhancement). For EP, PP and BP, the calculated ME factor was 92%, 86% and 88%, respectively, indicating the absence of matrix effect.

Although some authors have proposed that matrix effects in bioanalytical HPLC-MS/MS assays can be minimized by employing multistep sample clean-up procedures [50], additional clean-up steps are time-consuming and involve more sample manipulation, which can increase the analytical errors associated to sample preparation. Sample dilution might also be a good option to avoid matrix effect although it would reduce method sensitivity, so this possibility was directly discarded. Consequently, in order to correct the matrix effect observed for BPA and MP we used both ILIS and matrix matched calibration. The use of ILIS also ensures a better quantification. As can be seen in Table 2 most of the multi-residue methods for the determination of both BPA and parabens in breast milk also employed the matrix-matched calibration to correct matrix effects.

3.6. Validation

The performance parameters of the method are shown in Table 3. A good linearity for all compounds fulfilling the linearity criteria was obtained, since in all cases $R^2 > 0.99$.

The Linear Dynamic Range (LDR) was set at 0.1–50 ng mL^{-1} except for BPF (0.125–50 ng mL^{-1}) and BPS (0.250–50 ng mL^{-1}). This range covers the majority of compound levels found in real samples described in the literature (0.1–43.5 ng mL^{-1}) [22,23,26,28,30–37,41,52,62]. To our knowledge, only Fotouhi et al., with concentrations higher than 50 ng mL^{-1} and Niu et al., with concentrations lower than 0.1 ng mL^{-1} quantified analyte levels in breast-milk samples out of the LDR ranges presented here [31,36]. Moreover, the present method has one of the lowest LoQ (0.1 ng mL^{-1} for most of the compunds) in comparison with previously-developed methods (Table 2). Therefore, the present analytical method is suitable for the quantification of bisphenols and parabens in breast milk as it has a low LoQ in comparison with previously-developed methods, especially considering that several of them do not study the recovery and precision at the LoQ, but at higher levels [28,35], and a LDR covering the previously described levels of bisphenols and parabens in breast milk samples.

Appropriate recoveries, for the three levels validated (including the LoQ) were obtained for the target compounds ranging from 83% (PP, at the medium level) to 115% (BPF, at the lowest level) similar to the recoveries obtained in methods that had been previously developed (Table 2). The intra- and inter-day precision of the method was assured by the calculated RSDs at the three levels, which were ≤ 19% for all compounds. As can be seen in Table 2, most of the methods that had been previously developed obtained also intra- and inter-day precisions with RSDs < 20% in all cases. A chromatogram of the compounds from MB spiked at the limit of quantification is presented in Fig. 2.A.

3.7. Analysis of field samples

The proposed analytical strategy was applied to 10 real breast milk samples. The analysis allowed the identification and quantification of bisphenols and parabens, as can be seen in Table 4. The quantified levels were lower than in previous studies: BPA was quantified in 80% of samples with a geometric mean (GM) of 0.37 ng mL^{-1} (calculated only with positive samples) while other studies presented DF from 26 to 90% and GMs from 0.67 to 0.8 ng mL^{-1} [34,52,63]; BPF was found only in 3 samples with concentrations ranging from 0.13 to 0.32 ng mL^{-1}, while higher DF were found in Niu et al., (60%) [27]. BPS was only quantified in 1 sample with a concentration of 0.37 ng mL^{-1}, similarly to Niu et al., wich only quantified BPS in one sample (DF 5%) with a concentration of 0.68 ng mL^{-1} [27]; MP presented a DF of 80% in a range between 0.11 and 7.00 ng mL^{-1}, which are lower values than found in the literature, where DF as well as concentrations were found in a range between 67 and 100% and 0.5–21 ng mL^{-1} respectively [25,38,46]; EP DF was 40% and the concentrations ranged from 0.49 to 4.05 ng mL^{-1} while higher DF and concentrations were found in other studies (0–86% and 0.6–22 ng mL^{-1}, respectively) [33,35,46]; PP was quantified in 60% of samples in a range between 0.13 and 0.76 ng mL^{-1}, which are lower than in previous studies where DF and concentration range from 13 to 100% and 0.1–12 ng mL^{-1}, respectively [25,26,38] and BP presented a DF of 20% with a concentration range from 0.17 to 0.34 ng mL^{-1} while in the majority of studies did not detect levels of BP [25,31], only Azzouz et al., found BP with a DF of 29% and

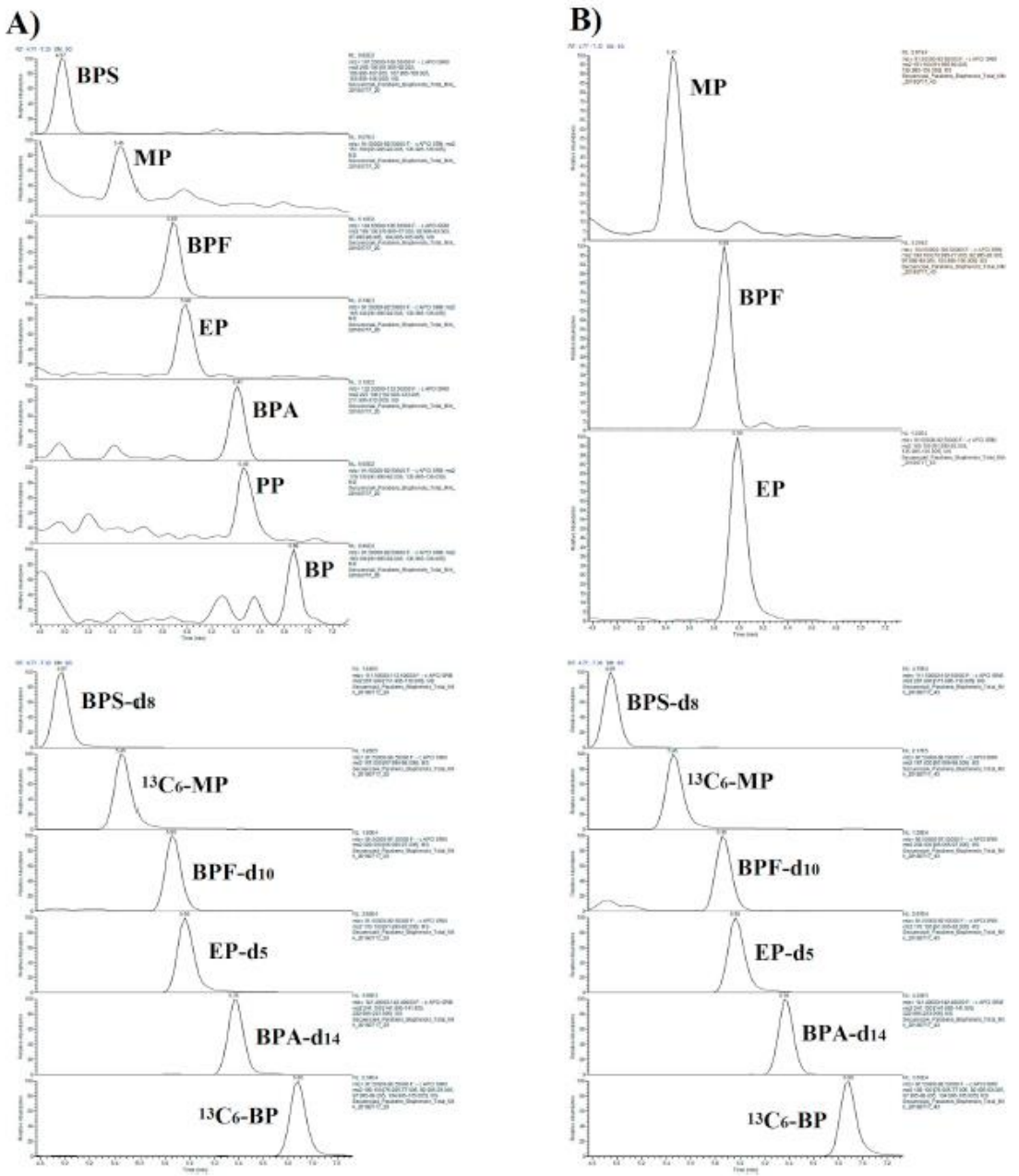


Fig. 2. A) SRM chromatograms of a spiked blank at the LoQ. B) SRM chromatograms of a field sample. The analytes detected with a concentration higher than the LoQ in the field sample were: MP (1.54 ng mL^{-1}), BPF (0.13 ng mL^{-1}) and EP (1.46 ng mL^{-1}).

concentrations ranging from 0.81 and 1.10 ng mL^{-1} [35]. It should be stressed that this is not a monitoring study, as few samples have been analyzed ($n = 10$). However, the detection and quantification of the analytes proves the validity of the method for the analysis of BPA, BPF, BPS, MP, EP, PP and BP in breast milk for future studies.

Fig. 2. shows the SRM chromatograms for all analytes and their ILIS in a field sample (Fig. 2.B.) and in a MB spiked with standards at LoQ. (Fig. 2.A.) The identification criteria for quantification analysis were achieved.

Table 4
Determination of parabens and bisphenols in 10 field breast milk samples. Results expressed in ng mL^{-1}.

Sample	BPA	BPF	BPS	MP	EP	PP	BP
1	0.22	0.32	< LoQ	1.09	4.05	0.76	< LoQ
2	1.62	< LoQ	0.37	7.00	< LoQ	0.40	< LoQ
3	0.66	0.15	< LoQ	1.39	0.49	0.17	0.34
4	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
5	0.14	< LoQ	< LoQ	2.21	< LoQ	0.34	0.17
6	0.13	< LoQ	< LoQ	0.11	< LoQ	< LoQ	< LoQ
7	< LoQ	0.13	< LoQ	1.54	1.46	< LoQ	< LoQ
8	0.72	< LoQ	< LoQ	0.44	< LoQ	0.17	< LoQ
9	0.64	0.21	< LoQ	< LoQ	< LoQ	0.13	< LoQ
10	0.17	< LoQ	< LoQ	2.12	0.71	< LoQ	< LoQ

4. Conclusions

A sensitive, accurate and precise multi-residue method for the determination of bisphenols and parabens in breast milk has been developed. For the first time a single multiresidue method allows the determination of the bisphenols A, F and S and parabens. These multiresidue approaches are essential in biomonitoring exposure assessment studies, since the toxicological effect of contaminants (as endocrine disrupting chemicals) must be studied as a whole. Moreover, the use of APCI as the ion source in the LC-MS/MS analysis, which increased the sensitivity of the method in comparison with ESI, has been applied for the first time in the determination of BPF, BPS and parabens in breast milk. The combination of the QuEChERS as sample preparation and the LC-(APCI)-MS/MS analysis allowed achieving an LoQ of 0.1 ng mL^{-1} for most of the analytes which ensures their detection in breast milk at the levels previously found in literature. The performance parameters of the method were studied and the procedure was validated. The analytical method was successfully applied for the analysis of 10 breast milk samples proving that it is useful for future human biomonitoring studies in breast milk.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2019.03.004.

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5.4. Capítulo 4: Biomonitorización de BPA, F y S en leche materna y evaluación del riesgo en lactantes

En el presente capítulo se estudiaron los niveles de bisfenoles en leche materna de 120 madres del proyecto Bettermilk. El análisis se realizó tanto con hidrólisis enzimática (n=100) como sin hidrólisis (n=120) para comparar los niveles de bisfenoles totales (libres + conjugados) y libres en leche materna recogida a las 2 semanas del parto. Además, se estudiaron los niveles de BPA libres a las 5 y a las 8 semanas después del parto para conocer la evolución de la exposición a lo largo de la lactancia (n=49). Para llevar a cabo los análisis se utilizó la metodología analítica desarrollada en el Capítulo 3. Posteriormente se estudiaron los determinantes de las concentraciones de BPA en leche y se efectuó la evaluación del riesgo en lactantes por ingesta de BPA a través de la leche materna.

5.4.1. Resultados y discusión

5.4.1.1. Concentraciones de bisfenoles en leche materna

En la **Tabla 18** se muestran las concentraciones de bisfenoles en leche materna a las 2 semanas después del parto. Respecto a los niveles de bisfenoles totales, los niveles de BPA presentaron una DF (83%) muy superior a la del BPF (22%) y el BPS, que solo fue cuantificado en una muestra. Por otro lado, si comparamos los niveles de BPA libre y BPA total, presentan una DF similar. Las GM del BPA-libre y del BPA-total fueron 0,15 y 0,29 ng/mL, respectivamente. La GM del ratio [BPA-libre]/[BPA-total] (calculado solo para las muestras con ambos valores >LoQ) fue de 0,58, indicando la presencia de metabolitos conjugados en leche materna. Los histogramas del logaritmo de las concentraciones de BPA-libre y total se muestran en la **Figura 24**.

Tabla 18. Niveles de BPA, BPF and BPS en leche materna de madres de Valencia (España).

	BPA-libre (ng/mL)	**BPA-total (ng/mL)**	**BPF-total (ng/mL)**
Número de muestras (n)	120	100	91
> LoQ	77,4 %	83,0%	22,0 %
Mínimo	<LoQ	<LoQ	<LoQ
P25	0,10	0,10	<LoQ
Mediana	0,10	0,26	<LoQ
GM	0,15	0,29	-
AM	1,1	1,6	-
P75	0,18	0,57	<LoQ
P95	2,8	6,4	0,21
Máxima	41	42	0,46
Desviación estándar	4,8	5,8	0,12

LoQ: Límite de cuantificación; BPS-total (n=91) sólo se cuantificó (> LoQ) en una muestra (0.37 ng/mL)

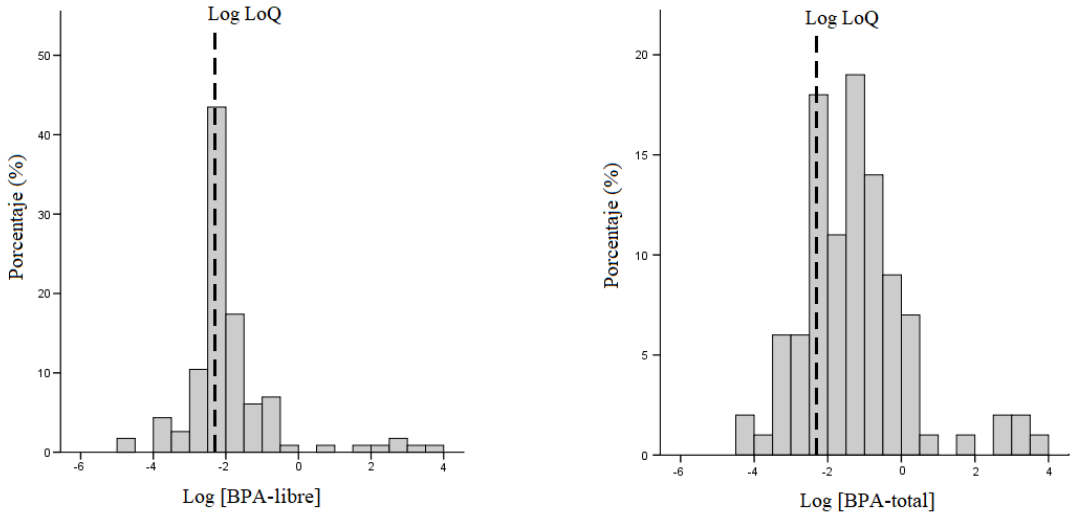

Figura 24. Histogramas del logaritmo de las concentraciones de BPA libre (n=120) y total (n=100) en leche materna a las 2 semanas después del parto.

Las concentraciones de BPA en leche materna descritas en la literatura presentan medianas de 0,10-7,6 ng/mL y 0,11-10,4 para BPA-libre y total, respectivamente **(Cao et al., 2015; Yi et al., 2010)**. Con respecto al BPF y BPS tan solo han sido estudiados previamente en tres estudios, que contaban con un número reducido de muestras (n≤30). En la mayoría de los estudios se detectaron pocas muestras positivas para ambos analitos, excepto Deceuninck et al., que reportaron un DF para BPF del 60% (**Deceuninck et al., 2015; Niu et al., 2017; Tuzimski et al., 2018)**.

Con respecto a la evolución de los niveles de BPA libre a lo largo de la lactancia, la GM de las concentraciones a las 2, 5 y 8 semanas en las participantes que dieron tres muestras de leche (n=49) fueron 0,12, 0,17 y 0,14 ng/mL, respectivamente. Sobre se estos resultados se aplicó el test de Friedman que indicó que no había diferencias significativas en los niveles de BPA-libre en leche entre las 2 y las 8 semanas (p-valor = 0,329).

5.4.1.2. Determinantes de niveles de BPA

Se llevaron a cabo RRMs simples y multiple para estudiar los determinantes de los niveles de BPA en leche materna tomada a las 2 semanas. Las variables independientes estudiadas fueron datos socioeconómicos, dietéticos y de uso de cosméticos de las madres. En la **Tabla 19** se muestran los resultados del RRM múltiple para BPA-total. Como se observa las variables independientes 'lugar de residencia durante los últimos 10 año's y 'frecuencia de uso de productos para el cuidado de la piel' se correlacionaron con los niveles de BPA-total. Las madres que residían en un medio rural tenían niveles significativamente menores que las que vivían en un medio urbano. Asimismo, las madres que usaban productos para el cuidado de la piel tenían niveles significativamente más elevados de BPA en compración con las madres que no los usaban.

Algunos estudios previos han encontrado correlaciones entre el lugar de residencia y los niveles urinarios de BPA en diferentes poblaciones, sin embargo, en estos estudios, los niveles de BPA en orina eran mayores en poblaciones que vivían en áreas rurales **(Cutanda et al., 2015; Tratnik et al., 2019)**. No se ha encontrado un motivo claro que explique la relación entre el lugar de residencia y los niveles de BPA en leche materna en nuestro estudio. Por otro lado, la presencia de niveles de BPA en productos de cuidado personal como lociones para la piel **(Liao & Kannan, 2014)**, podría explicar la correlación positiva entre la frecuencia de uso de productos para el cuidado de la piel y los niveles de BPA en leche materna que se ha encontrado en el presente estudio.

Tabla 19. Resultados del RRM multiple de los niveles BPA-total en leche materna.

Variable	Coeficientes estimados (95% IC)	Error estándar	p-valor
'Intercept'	-1,780 (-2,211 - -1,350)	0,220	<0,001*
Lugar de residencia durante los últimos 10 años: Rural	-0,875 (-1,402 - -0,348)	0,269	0,002*
Frecuencia de uso de productos para el cuidado de la piel: Diariamente	0,71 (0,197 - 1,223)	0,262	0,008*
Frecuencia de uso de productos para el cuidado de la piel: Varias veces a la semana	0,472 (-0,217 - 1,16)	0,351	0,183
Frecuencia de uso de productos para el cuidado de la piel: Mensualmente	0,559 (-0,654 - 1,773)	0,619	0,369

*p-valor <0,05; IC (Intervalo de confianza)

5.4.1.3. Evaluación del riesgo en lactantes

Se utilizó un enfoque determinista y uno probabilístico para la estimación de la ingesta de BPA en lactantes tal y como se describe en la sección 4.7.1. Utilizando un enfoque determinista, las EDI de BPA-libre y BPA-total, estimando una que la ingesta media de leche de 140 mL/kg-pc/día **(EPA, 2011)**, fueron de 0,02 y 0,04 µg/kg-pc/día, respectivamente. Si se se calculan las EDIs en base a la ingesta de leche en el percentil superior (190 mL/kg-pc/día) **(EPA, 2011)**, las EDIs para BPA-libre y total fueron 0,03 y 0,06 µg/kg-pc/día, respectivamente.

Utilizando un enfoque probabilístico, la distribución log-normal de ingesta de leche se muestra en la **Figura 25**. La **Tabla 20** muestra la EDI de BPA-libre y total para lactantes utilizando el enfoque probabilístico. Como se puede observar, se estima que el 95% de los lactantes tienen una EDI de BPA-total menor a 1 µg/kg-pc/día.

Según EFSA **(2015)**, la exposición estimada media a BPA a través de la dieta es de 0,165 µg/kg-pc/día para neonatos de entre 6 días y 3 meses de edad. Esta exposición es mucho mayor que las exposición medias estimadas en el presente estudio (0,03-0,04 µg/kg-pc/día para BPA-total). En cualquier caso, en ninguno de los enfoques del estudio actual (ni determinista ni probabilístico), se estimó que la población de lactantes estudiada tuvo una EDI de BPA superior a la TDI temporal de BPA de 4 µg/kg-pc/día establecida por la EFSA **(EFSA, 2015)**. Por otro lado, WHO **(2010)** estimó que cuando los recién nacidos eran alimentados con fórmulas infantiles en polvo, la exposición a BPA a través de la dieta era de 2,0 y 2,7 µg/kg-pc/día para la media y el percentil 95, respectivamente, valores superiores a los estimados en lactancia en este estudio.

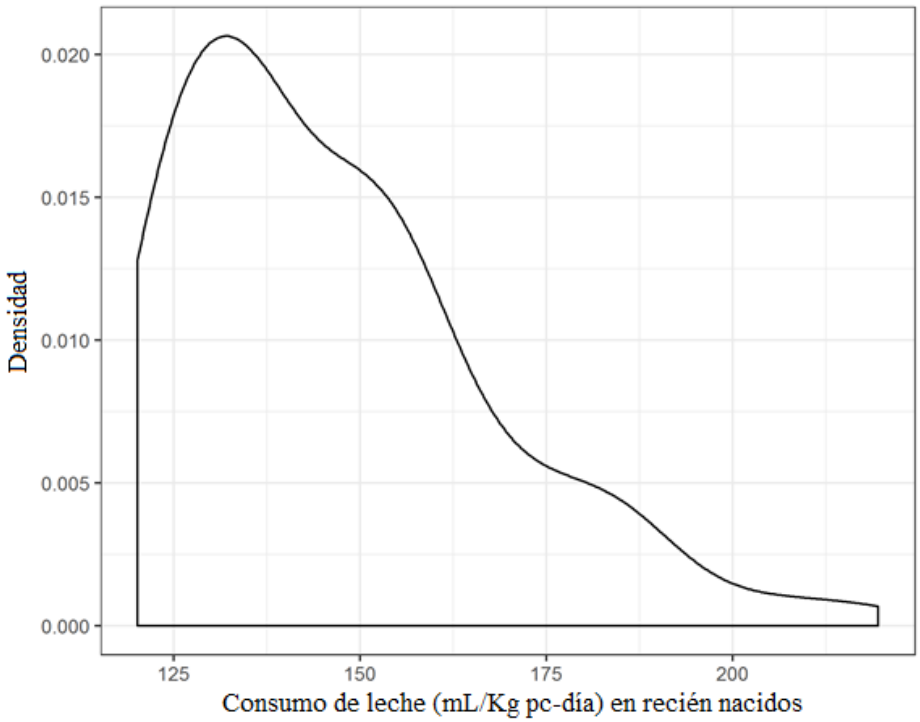

Figura 25. Distribución logarítimico normal truncada del consumo de leche

Tabla 20. EDI en µg/Kg pc día de BPA para lacatantes utilizando el enfoque probabilístico.

	Exposición BPA-libre (µg/Kg pc día)	**Exposición BPA total (µg/Kg pc día)**
Mínimo	0,001	0,001
P25	0,01	0,02
Mediana	0,02	0,04
GM	0,03	0,04
AM	0,20	0,30
P75	0,03	0,10
P95	0,60	1,0
Máximo	5	7
Desv. estándar	0,6	0,9

5.4.2. Conclusiones

-La exposición a BPA por parte de la población lactante estudiada es menor a la TDI temporal establecida por la EFSA de 4 µg/kg pc día.

-Los niveles de BPA libre no variaron significativamente durante la lactancia, y ni la edad de las madres ni el número de hijos se asociaron con los niveles de BPA en leche, lo cual hace pensar que los niveles de BPA en leche hacen referencia a una exposición reciente.

-Aunque el BPA está siendo reemplazado por sus análogos F y S, estos presentaron niveles y DF muy bajos en la población estudiada.

5.4.4. Artículo 4: Biomonitoring of bisphenols A, F, S in human milk and probabilistic risk assessment for breastfed infants

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Biomonitoring of bisphenols A, F, S in human milk and probabilistic risk assessment for breastfed infants

Pablo Dualde [a,b,c], Olga Pardo [a], Francisca Corpas-Burgos [a], Julia Kuligowski [d], María Gormaz [e], Máximo Vento [e], Agustín Pastor [b], Vicent Yusà [a,b,c,*]

[a] Foundation for the Promotion of Health and Biomedical Research in the Valencian Region, FISABIO-Public Health, 21, Avenida Catalunya, 46020 Valencia, Spain
[b] Analytical Chemistry Department, University of Valencia, Edifici Jeroni Muñoz, Dr. Moliner 50, 46100 Burjassot, Spain
[c] Public Health Laboratory of Valencia, 21, Avenida Catalunya, 46020 Valencia, Spain
[d] Neonatal Research Unit, Health Research Institute La Fe, Avenida Fernando Abril Martorell 106, 46026 Valencia, Spain
[e] Neonatal Division, University and Polytechnic Hospital La Fe, Avenida Fernando Abril Martorell, 106, 46026 Valencia, Spain

HIGHLIGHTS

- BPA, BPS and BPF levels were determined in human milk samples from Spanish mothers.
- DF of total and u-BPA were 83 and 77%, and a GM of 0.29 and 0.15 ng/mL, respectively.
- BPF and BPS present low frequencies of detection on the studied population.
- Levels of u-BPA do not change during lactation.
- Studied infants BPA estimated daily intake was below EFSA t-TDI of 4 μg/kg bw.

GRAPHICAL ABSTRACT

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ABSTRACT

The present study addresses the presence of bisphenols A (BPA) and its analogs bisphenol F (BPF) and S (BPS) in milk of 120 mothers living in Valencia (Spain) and participating in the BEITERMILK project (year 2015). We also studied the factors that could influence the BPA levels and estimated the exposure and the risk for breast fed infants. The frequency of detection of total (conjugated + unconjugated) and unconjugated-BPA were 83% and 77%, with a geometric mean of 0.29 ng/mL and 0.15 ng/mL, respectively. The frequency of detection was much lower for total-BPF (22%) and total-BPS (1.1%).
The place of residence of the mother and the use of personal care products showed significant association with BPA concentrations. The estimated daily intake of total-BPA for breastfed infants amounted to a geometric mean of 0.04 μg/kg bw and a 95th percentile of 1.0 μg/kg bw, below the tolerable daily intake of 4 μg/kg bw-day established by EFSA. To our knowledge, this is the largest biomonitoring study of bisphenols in human milk in Europe.

* Corresponding author at: Foundation for the Promotion of Health and Biomedical Research in the Valencian Region, FISABIO-Public Health, 21, Avenida Catalunya, 46020 Valencia, Spain.
E-mail address: yusa_vic@gva.es (V. Yusà).

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1. Introduction

Bisphenol A (BPA) is a high production volume chemical, widely used in food and liquid packages made of polycarbonate plastics, epoxy resins (used as internal coating in cans), and also in thermal papers (Mercogliano and Santonicola, 2018).

Diet is considered main source of BPA exposure of the general population, although non-dietary sources such as thermal paper or toys can contribute to the total exposure (Geens et al., 2012). According to EFSA (2015), in the European population canned food (50%), non-canned meat products (20%) were the main sources of total exposure for all studied population, and infant formula (25–37%) for infants.

A new European regulation (EU, 2018) has tightened the restrictions on the use of BPA in food contact materials. This regulation has lowered the migration limits (0.05 mg of BPA per kg of food), expands the ban of use of BPA in baby bottles and prohibits the migration of BPA from varnishes or coatings applied to materials in contact with food for infants and children 0–3 years old.

Hazard identification of BPA from a recent EFSA scientific opinion (EFSA, 2015) include: a) the changes in the kidney and liver are critical endpoints in BPA general toxicity; b) there is not enough evidence to infer a causal link between BPA exposure and reproductive effects in humans at low dose levels; c) prenatal BPA exposure may be associated with neurodevelopmental effects, although there is not sufficient evidence to infer a causal link.

Likewise, several studies have concluded that BPA could interact with the endocrine system (i.e. estrogen receptors), but is uncertain if the observed effects can occur at concentrations relevant to human exposure or this can lead to adverse health effects (WHO, 2010). Taking into account the "general toxicity" a temporary tolerable daily intake (t-TDI) of 4 μg kg bw^{-1} day^{-1} for oral exposure to BPA has been derived (EFSA, 2015).

The restrictions in the use of BPA have stimulated manufacturers to use alternative substances to replace BPA. Between them, bisphenol analogues, bisphenol F (BPF) and S (BPS) are the most used (Chen et al., 2016; Liao et al., 2012a). However, recent studies have shown that BPF and BPS present similar endocrine-disrupting effects to BPA (Rochester and Bolden, 2015). Consequently, the research program promoted by EU (HBM4EU) has included both BPF and BPS, next to BPA, in the list of prioritised substances to be determined in biomonitoring studies (HBM4EU, 2017).

After oral uptake, BPA undergoes substantial detoxification metabolism in the liver to BPA-glucuronide. Humans excrete conjugate forms of BPA into urine with a half-life of <6 h (Völkel et al., 2002). However, unconjugated BPA (aglycone BPA) has been detected in a variety of human samples indicating that humans are exposed internally to estrogenically active BPA (Vandenberg et al., 2010).

In mothers producing milk, the absorbed portion of BPA is quickly transferred into the breast, resulting in the observed high concentration of BPA in the human milk quickly (hours) after BPA consumption (Tateoka, 2015). BPA, as somewhat lipophilic chemical (K_{ow}: 2.2–3.4), incorporates to human milk at levels comparable to the concentrations in fatty tissues in the body (Migeot et al., 2013; Zimmers et al., 2014).

BPA is present in human milk as unconjugated or in the conjugated form (Cao et al., 2015). However, conjugated BPA does not display any estrogenic activity (Migeot et al., 2013); consequently, the measurement of unconjugated-BPA (u-BPA) is more suitable in risk assessment of BPA in breastfeeding infants. BPA in mature milk reflects the recent exposure of the mother. This is different from BPA content in colostrum, that reflect accumulated mother exposure during the second half of the pregnancy (Migeot et al., 2013).

Human milk is the major or exclusive food source of nourishment for infants. If mothers are exposed to environmental pollutants, these pollutants may contaminate human milk and consequently breast fed infants are exposed to BPA. A report of the Joint FAO/WHO Expert Meeting (WHO, 2010) estimates an intake of 0.3 and 1.3 μg/kg bw per day (mean and 95th percentile) for breast fed infants 0–6 months of age. (EU, 2011a). In a relevant review, Mercogliano and Santonicola (2018) reported studies of BPA levels in human milk since 2003. However, most of the studies analysed a small number of samples ($n \leq 30$). Given the significant interest in protecting the health of neonates and infants, assessing exposure to BPA from human milk through biomonitoring studies with a significant number of participants is of great importance.

The metabolism and distribution of BPF and BPS in humans have not been as well studied as in case of BPA, nevertheless, some experiments suggest that BPF and BPS metabolism is similar to BPA metabolism (Rochester and Bolden, 2015). Several large biomonitoring studies have been implemented in order to determine urinary levels of BPF and BPS in urine (Liao et al., 2012b; Yang et al., 2014; Zhou et al., 2014). However, to our knowledge, only Deceuninck et al. (2015), Niu et al. (2017) and Tuzimski et al. (2018) have studied human milk levels of BPF and/or BPS in small populations ($n = 20–30$ samples).

Since bisphenols, mainly BPA, are ubiquitously present in the environment, some studies have noted that special care during sampling, and biomonitoring of these substances should be taken (Ye et al., 2013). It has been indicated that the containers and breast pumps can be a source of contamination of BPA (Arbuckle et al., 2015; Mendonca et al., 2014). Furthermore, during sample preparation, the use of non-cleaned glassware, contaminated reagents or polypropylene material can be a source of BPA contamination (Arbuckle et al., 2015; Deceuninck et al., 2014). Also, BPA can be found in plastic devices that form the analytical equipment, or in the dust (Ye et al., 2013). In the present study we have taken into account these requirements and recommendations to avoid contamination.

The aim of this study was i) to determine the occurrence of BPA, BPF and BPS in human milk; ii) study the factors influencing the BPA levels; iii) evaluate the variations BPA during lactation and iv) estimate the exposure and the risk assessment for breast feed infants to BPA.

2. Materials and methods

2.1. Standards and reagents

Certified standards of BPA, BPF and BPS and BPA-d_{16} were purchased from Dr. Ehrenstorfer (Ausburg, Germany). BPF-d_{10} and BPS-d_8 were purchased from Toronto Research Chemicals (Toronto, Canada) and CDN Isotopes (Quebec, Canada), respectively. β-glucuronidase/sulfatase from *Helix pomatia* type H1 was obtained from Sigma Aldrich (St. Louis, MO, USA). The enzymatic solution was prepared weekly by disolving the β-glucuronidase/sulfatase purified powder in ammonium acetate 1 M ($pH = 5$) to obtain a solution of 3500 U/mL.

2.2. Control of contamination

Some procedures were applied in order to avoid and check the contamination, mainly of BPA, during sampling and sample analysis. The breast pumps and containers used for sample collection were BPA free (specified by the supplier), also a migration study was developed in order to dismiss the BPA contamination of human milk during sampling. The migration study was based on Santillana et al. (2011); the simulant used was ethanol 50% (v/v) (simulant D1) and the conditions of the study were 2 h at 70 °C following the European Union reglament 10/2011 (EU, 2011b). The analysis was performed in the same LC-MS/MS equipment used for the determination of bisphenols in human milk. The BPA LoQs of the migration study was 0.0025 ng/g.

Sample analysis was implemented in safety cabinets in order to avoid dust or air contamination. Furthermore, the polypropylene material used for sample analysis and reagents (including QuEChERS) were checked previously for BPA contamination. During sample analysis, each batch included a reagent blank and a milk blank which suffered

the same treatment as the field samples to check the contamination during the treatment steps.

Also, several mobile phases which consisted of methanol:water (20:80, v/v) were injected throughout the sequences in order to control the possible leaching from the analytical equipment.

2.3. Study design, population and sample collection

2.3.1. Study area and population

Regarding the study area and population, 120 breastfeeding women were recruited in Valencia (2015) as a part of the project BETTERMILK. The details of the study population were described in previous papers (Yusa et al., 2017).

2.3.2. Samples and data collection

All the participants provided a milk sample 2 weeks after birth, and some of them (n = 49) provided two extra samples (collected 5 and 8 weeks after birth) in order to evaluate the BPA variation during lactation. After cleaning their breasts with abundant clean water, samples were collected by the mothers in a glass container using a BPA free breast pump (Philips Avent SCF330). Samples remained in the household freezer (−20 °C) until they were transferred to the IBSP-CV BioBank where they were aliquoted and stored at (−80)°C.

As explained in detail in Yusa et al. (2017) sociodemographic and diet questionnaires were filled out by the mothers. Also, in the present study, a cosmetic use questionnaire was used. Table 1, SD1 and SD2 show the results of the questionnaires.

The IBSP-CV BioBank (PT13/0010/0064) was the responsible of managing the samples and the participants information and the study was approved by the ethical and scientific committees (see Yusa et al., 2017).

2.3.3. Mediterranean Diet Quality Index (Med-DQI) and food intake calculation

Calculation of Med-DQI was based on Gerber (2006). Briefly, % saturated fatty acids (SFA) energy, mg cholesterol · day $^{-1}$, meat g · day $^{-1}$, olive oil mL · day $^{-1}$, fish g · day $^{-1}$, cereals and legumes g · day $^{-1}$ and vegetables and fruits g · day $^{-1}$ were calculated. Following the rules described in Table SD3, diet was classified in: Good (1–4 arbitrary units "a.u."), Half-good (5–7 a.u.), Half-poor (8–10 a.u.) and Poor (11–14 a.u.).

Fook intake calculation was described in Yusa et al. (2017).

2.4. Chemical analysis

2.4.1. Sample preparation

Concerning the analysis of unconjugated analytes, after the thawing and shaking the sample (10 mL), it was transferred into a polypropylene centrifuge tube (free of BPA) where was spiked with an isotopically labelled internal standard (ILIS) solution in order to reach, after homogenization, an ILIS concentration in sample of 12.5 ng/mL. Next, 10 mL of acetonitrile, two ceramic pieces and a QuEChERS salt packet (4 g anhydrous $MgSO_4$, 1 g NaCl) were added. After shaking and centrifugation (8000 rpm, 20 °C, 10 min), the total acetonitrile layer was isolated in a polypropylene tube and stored overnight at −20 °C.

After 24 h, the solid residue was discarded and the extract was transferred into a 15 mL QuEChERS fatty dispersive-solid phase extraction (d-SPE) AOAC kit (400 mg primary secondary amine (PSA), 400 mg C_{18} and 1200 mg $MgSO_4$) polypropylene tube. The mixture was shaken and centrifuged for 15 min at 4500 rpm and 15 °C. The acetonitrile supernatant was transferred to a glass tube and evaporated using a N_2 stream at 37 °C. 250 μL of methanol: water (20:80, v/v) were added to the dry residue and after homogenization, the mixture was transferred into a Millipore 0.2 μm Eppendorf. A final ultra-centrifugation (13,000 rpm, 1 min and 5 °C) was applied in order to filter the extract and the obtained volume was injected on the HPLC–MS/MS equipment.

Table 1
Characteristics of the studied population.

Characteristics		n (%) (N = 120)
Mother		
Number of children		
	1	69 (57.5)
	2	40 (33.3)
	≥3	11 (9.2)
Age (years)		33 (20–45)[a]
Weight before pregnancy (kg)		60 (42–92)[a]
Height (cm)		164 (150–184)[a]
BMI before pregnancy (kg/m^2)		21.9 (16.6–35.4)[a]
Special diet during pregnancy		
	Yes	17 (14.4)
	No	101 (85.6)
	Missing data	2
Country of birth		
	Spain	104 (88.1)
	Foreign	14 (11.9)
	Missing data	2
Place of residence		
	Urban	85 (80.2)
	Rural	21 (19.8)
	Missing data	14
Education level		
	Only primary school	13 (10.8)
	Secondary school	24 (20.0)
	University	83 (69.2)
Occupational status		
	Employed	100 (84.6)
	Unemployed	18 (15.3)
	Missing data	2
Time worked outside the home (years)		10 (0–28)[a]
Use of cosmetics at work		
	Yes	12 (10)
	No	108 (90)
Mother was breastfed		
	Yes	81 (70.4)
	No	34 (29.6)
	Missing data	5
Physical exercise		
	3 or more days/week	19 (16.2)
	1 or 2 days/week	18 (15.4)
	Occasionally	50 (42.7)
	Never	30 (25.6)
	Missing data	3
Smoker		
	Yes	9 (7.5)
	Ex-smoker	48 (40.0)
	Never	63 (52.5)
Child		
Gestational age (weeks)		40 (35–41)[a]
Sex		
	Boy	47 (40.2)
	Girl	70 (59.8)
	Missing data	3
Weight (g)		3360 (2160–4350)[a]
Height (cm)		51 (46–55)[a]
Cranial perimeter (cm)		34 (32.5–37)[a]

[a] Values expressed as median (minimum - maximum).

For the analysis of total (conjugated + unconjugated) analytes, prior to the extraction with acetonitrile in a polypropylene tube, a deconjugation step was implemented in a glass tube by adding 10 mL of sample, the ILIS solution and 1 mL of the enzymatic solution. The mix was homogenized and incubated for 17 h at 37 °C.

In this study we have analyzed total-BPA, total-BPF, total-BPS and u-BPA.

2.4.2. HPLC-MS/MS analysis

The chromatographic separation was carried out with the HPLC system TSQ Quantum from Thermo Fisher Scientific (Bremen, Germany) equipped with a Symmetry C18 column (2.1 × 150 mm, 5 μm) from Waters (Milford, Massachusetts, USA). Chromatographic conditions are

described in Table SD-4. Data acquisition was performed by the Thermo Scientific Trace Finder ™ 3.2 software.

Regarding mass analysis, the Finnigan TSQ Quantum Ultra Detector analyser (San José, CA, USA) equipped with an atmospheric pressure chemical ionization (APCI) source was employed. Negative APCI mode and selected reaction monitoring (SRM) mode were used for all the analytes. Ion source settings are described in Table SD-5. The use of two transitions for each compound allowed their quantification and identification. For ILIS only the quantification transition was used (Table SD-6).

2.4.3. Quality assurance/Quality control (QA/QC) and validation

The laboratory works under the quality assurance system established by ISO/IEC/EN 17025. For quantification, procedural matrix-matched calibration curves were used. The quantification was performed with the area corresponding to the SRM quantification transition. To correct possible matrix effects and losses occurred during extraction, analogous ILIS were used (Table SD-6).

Compound identification and confirmation were based on SANTE/11813/2017 (SANTE, 2017):

a) 2 Product ions, one used for quantification and another used for confirmation (except for ILIS, where only the quantification ion was required)

b) The ratio between the product ions in a sample should not differ >30% with respect to the average ratio of the analyte in the calibration standards from the same sequence.

c) A tolerance of ±0.1 min for relative retention time (RRT) is allowed between the suspicious analyte in the sample and the analyte in the spiked samples;

Each set of samples was analysed under QA protocols, including a matrix-matched calibration curve, procedural blanks and fortified samples. The method validation parameters are shown in Table SD-7.

The method linearity was studied using eight-point matrix-matched calibration curves ranging from limits of quantification (LoQ) to 50 ng/mL for all analytes. In order to assess the validity of linearity, analysis of variance (ANOVA) Mandel's fitting test and a $R^2 > 0.99$ was required. The accuracy of the method was calculated in terms of relative recovery in each batch by spiking the pool of blank human milk at three different concentration levels: LoQ, the intermediate and the highest level of the calibration curves. Inter- and intra-day precision were also studied. The acceptance criteria were recovery (80–120%) and precision (RSD ≤ 20%).

The LoQ for each compound was established at the lowest validated spiked level to meet the performance acceptability criteria (recovery, precision and linearity) and confirmation criteria previously described. Furthermore, a minimal signal-to-noise ratio of 10 was required.

Likewise, different blanks were used to confirm the lack of external contamination (see section 2.2.)

2.4.4. Human milk macronutrients determination

Human milk macronutrients were determined using a MIRIS HMA™ (MIRIS AB, Uppsala Sweden), based on mid-infrared transmission spectroscopy. MIRIS HMA™ quantifies the levels of fat, carbohydrate, protein, total solids (TS) and energy in human milk. The determinations, QCs and calibrations performed followed the standard operation procedure given by the manufacturer. In order to distinguish between real proteins and other nitrogen (N) compounds (as oligossacarides, urea ...), real protein concentration was calculated multiplying crude protein concentration by 0.8 since non-protein N represents the 20% of total N compounds (Miris HMA™, 2017).

2.5. Statistical analysis

BPA levels below the quantification limit (left-censored results) were estimated following the maximum likelihood estimation method (MLE) described in (EFSA, 2010). This method is based on the assumption the data are distributed according to a certain parametric distribution and estimates the parameters of this distribution so that the probability of obtaining the observed sample is maximized. A log-normal distribution was assumed for BPA levels in human milk.

The statistical description of all the variables (Tables 1, SD-1, SD-2, SD-8) and the bisphenols levels was carried out as detailed in Yusa et al. (2017). The Friedman test was used to compare the u-BPA levels at 2, 5 and 8 weeks after delivery within each mother.

Simple and multiple robust linear regression models (RRMs) were built to assess the relationship between BPA levels and sociodemographic, dietary (including packaged food consumption) and use of cosmetics variables. Robust regression was considered as an alternative to ordinary least squares estimation methods (OLS) given the presence of some outlier values in BPA levels. Both u-BPA and total-BPA levels were studied separately. The logarithmic transformation of u-BPA and total-BPA levels was considered to get the normality of the response variable. Multiple RRMs were built following a backward variable selection procedure based on *p*-value criterion and considering a significance level of 0.05. RRMs have been done only for BPA because BPF and BPS presented too low frequencies of detection.

The general multiple linear regression model is described here:

$$\log(y_i) = \beta_0 + \beta_1 \cdot x_{1i} + \ldots + \beta_p \cdot x_{pi} + \varepsilon_i;\ i = 1, \ldots, n.$$

where y_i is the BPA milk level of each donor, $(\beta_0, \beta_1, \ldots, \beta_p)$ are the method parameters, $(x_{1i}, \ldots, x_{pi})$ are the values of the independent variables for each donor, ε_i is the error and n is the number of donors. The estimation of the parameters of the model was carried out considering the M-estimator with bisquare weighting allowing observations with higher residuals (outliers) will have a lower weight in the estimation.

The previously described statistical analysis was carried out using the version 3.3.1. of the "R" software.

2.6. Exposure assessment

In order to obtain an estimate of BPA exposure of breastfed children in our study, we combined information on milk consumption and information on u-BPA and total-BPA levels in the milk of the mothers of our study. We estimated the exposure of breastfed infants to BPA following two approaches: i) deterministic and ii) probabilistic.

In the deterministic approach, the estimated daily intake (EDI) was calculated according the following equation:

$$\text{EDI}\left(\text{mg kg}^{-1}\text{ body weight day}^{-1}\right) = \text{C x M};$$

where C is the average BPA level in the study population and M is the mean milk intake rate (quantity of human milk consumed per day) over six months of breast-feeding. We have used a human milk intake of 140 mL/kg bw-day (EPA, 2011). We also calculated the BPA exposure using the upper percentile (mean plus 2 standard deviation) of the human milk intake recommended by EPA (2011) for 1–3 months of age (190 mL/kg-day).

In the probabilistic approach, the distribution of milk consumption of infants was estimated assuming that the consumption data are distributed according to a log-normal distribution. The estimation of the parameters of this distribution will be obtained by imposing that the mean of the distribution is 140 mL/kg bw-day and the probability that the milk consumption is <190 mL/kg bw-day is from 95%, i.e.

$e^{\mu+\sigma^2/2} = 140$; $F(190) = 0.95$. Where μ and σ are the parameters of the distribution log-normal and F represent the distribution function. In addition, a minimum milk intake of 120 mL/kg bw-day was also considered. Once the milk consumption distribution of the breastfed children was estimated, 120 values of this distribution are generated that correspond to each one of the children of the mothers of our study. The intake

of BPA in children was estimated by multiplying the consumption values generated by the BPA levels in milk samples analyzed.

3. Results

3.1. Control of contamination

The migration study evidenced the absence of BPA contamination in the breast pumps or in the polypropylene material, all the chromatograms showed BPA areas <LoQ (LoQ BPA = 0.0025 ng/g). Fig. SD-1 shows as an example SRM chromatogram of the quantitative ions of BPA in a simulant spiked at LoQ and in two breast pumps in the migration study. As a contamination control protocol for biomonitoring analytical methods has not been clearly defined, we followed the measures described in the SANTE/11813/2017 (regularly used in our laboratory), which determines that the signal in the blanks must be lower than the 30% of the reporting limit signal (in our case the LoQ) (SANTE, 2017). The milk blank and reagent blank analytes areas were < 30% than the LoQ areas in all the sequences involved in the human milk samples analysis. Also, the mobile phase (methanol:water (20:80, v/v)) BPA areas were several orders of magnitude lower than the LoQ areas. Fig. SD-2 shows, as an example, the SRM chromatograms of BPA in a milk blank spiked at the LoQ, a milk blank, a reagent blank and a mobile phase (methanol:water (20:80, v/v)) in one sequence. Consequently, it was considered that the analytes external contamination (blank milk contamination, reagents contamination, mobile phase contamination) did not alter significantly the quantification of analytes in field samples at concentrations equal or higher than the LoQ.

3.2. Bisphenols concentrations

The concentrations of main macronutrients in human milk samples are described in Table SD-8.

The bisphenols levels quantified are shown in Table 2, examples of chromatograms of the bisphenols quantification ions (SRM) in milk blank and milk sample are shown in Fig. SD-3. Total-BPA showed the highest frequency of detection (DF) (83%) and the highest GM (0.29 ng/mL). As expected, concentrations and DF of total-BPA were higher than u-BPA levels (see Table 2). The histograms of u-BPA and total-BPA concentrations are depicted in Fig. 1. The ratio [u-BPA]/[total-BPA] (calculated only for samples with both u-BPA and total-BPA concentrations > LOQ) presented a GM of 0.54, a median of 0.58 and a range from 0.16 to 1.0. Regarding total-BPF, it was detected in the 22% of the samples ranging from <0.13–0.46 ng/mL. Total-BPS was only quantified in one sample.

In order to evaluate the possible variation of the levels of u-BPA over time, three samples of milk for 49 participants were collected during 2, 5 and 8 weeks after delivery. Table SD-9 shows the evolution of u-BPA levels in human milk, the GM detected were 0.12, 0.17 and 0.14 ng/mL, respectively. The Friedman test presented a p-value = 0.329 (>0.05) which shows no significant differences of the individual levels of u-BPA between 2 and 8 weeks.

Table 2
Levels of BPA, BPF and BPS in human milk of mothers in Valencian Region.

	u-BPA (ng/mL)	total-BPA (ng/mL)	total-BPF (ng/mL)
Number of samples	n = 120	n = 100	n = 91
> LOQ	77.4%	83.0%	22.0%
Minimum	< LOQ	<LOQ	<LOQ
25th percentile	0.10	0.10	<LOQ
Median	0.10	0.26	<LOQ
Geometric mean	0.15	0.29	
Arithmetic mean	1.1	1.6	
75th percentile	0.18	0.57	<LOQ
95th percentile	2.8	6.4	0.21
Maximum	41	42	0.46
Standard deviation	4.8	5.8	0.12

LOQ: Limit of quantification.
Total-BPS (n = 91) quantified > LoQ only in one sample (0.37 ng/mL).

3.3. Factors of influence on BPA levels

Simple and multiple RRMs were employed to analyze the confounders (e.g. age, canned food) and covariates (other determinants) that may be related with the BPA levels. Levels of total-BPA in human milk were not found to be related with the anthropometric variables of the participants or their children (height, weight, gestational age…). Regarding the mothers sociodemographic variables, place of residence during the last 10 years showed correlation with the total-BPA levels (p-value <0.05). (see Table SD-10). We also investigated the possible correlation between food consumption, packaged food consumption and cosmetic usage and the levels of total-BPA in milk. Only skin care products and makeup frequency of use was significantly correlated with total-BPA levels (p-value <0.05) (see Table SD-11).

Furthermore, we analyzed the macronutrients (fat, proteins, carbohydrates…) for all milk samples. Human milk carbohydrates (g/100 mL) and TS (g/100 mL) were significantly correlated with total-BPA levels (p-value <0.05) (Table SD-12).

We built a multiple RRM with the five mentioned independent variables (place of residence, frequency of use of skin care products and make up and levels of carbohydrates and TS in milk) and total-BPA levels as the dependent variable. Only place of residence and frequency of use of skin care products presented significant association with total-BPA levels (see Table 3). The mothers living in rural areas presented lower concentrations of total-BPA than mothers living in urban areas and mothers who used skin care products daily showed significantly higher total-BPA concentrations than the mothers who never used skin care products.

The same study was applied to u-BPA levels. The results of the simple RRMs between the independent variables with the levels of u-BPA in human milk are shown in Tables SD-13, SD-14 and SD-15. The parameters which showed the lowest p-values (<0.05) using the simple RRM were studied using a multiple RRM for u-BPA levels in human milk. As observed in Table SD-16, the participants living in rural areas presented lower concentrations of u-BPA as happened with total-BPA. Furthermore, mothers who had dyed their hair during the week previous to the sampling had significantly higher u-BPA levels than mothers who had dyed their hair between one week and one month previous to the sampling. Finally a significant association was found between fruit consumption and BPA levels. However, since the estimated coefficient was close to 0, the influence of fruit consumption over u-BPA levels should not be relevant.

3.4. Exposure and risk assessment for breastfed infants

In the deterministic approach, the EDI was calculated following the formula (see Section 2.6):

$$\text{EDI}\left(\text{mg kg}^{-1}\text{ body weight day}^{-1}\right) = \text{C x M}.$$

C was the average of u-BPA or total-BPA levels in the studied population (GM = 0.15 ng/mL and 0.29 ng/mL respectively), and M the mean milk intake rate (quantity of human milk consumed per day) over six months of breast-feeding (140 mL/kg bw-day). Consequently, the EDI amounted to 0.02 and 0.04 μg/kg bw-day for u-BPA and total-BPA, respectively. We also calculated the u-BPA exposure using the upper percentile of the human milk intake (190 mL/kg-day). Taking this intake, the exposure was of 0.03 and 0.06 μg/kg bw-day for u-BPA and total-BPA, respectively. In all scenarios the estimated intake is two orders of magnitude lower than the temporary t-TDI of 4 μg/kg bw established by EFSA (2015).

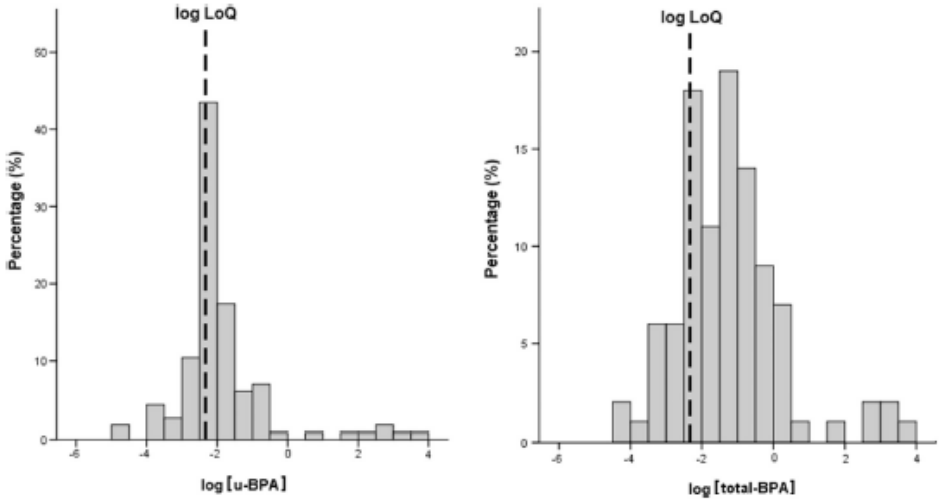

Fig. 1. Histograms of log transformed u-BPA and log transformed total-BPA levels.

In the probabilistic approach, the estimated log-normal milk consumption distribution is shown in Fig. SD-4. Table 4 shows the estimated exposure to u-BPA and total-BPA for breastfed infants.

As can be seen in Table 4, 50% of breastfed infants have an estimated intake of u-BPA and total-BPA lower than 0.02 and 0.04 μg/kg bw-day respectively. These values are similar to the calculated using the deterministic approach. Likewise, the 95% of children have total-BPA estimated intakes lower than 1.0 μg/kg bw-day, which does not exceed the t-TDI of 4 μg/kg bw.

We have not estimated the risk assessment of BPF/S owing to their low frequencies of detection. In any case their exposure and risk will be much lower than for BPA.

4. Discussion

To our knowledge, in the scientific literature covering the period from 2006 until now, eighteen studies have described concentrations of BPA in mature human milk (See Table 5). However, most of them were focused on the analytical methodology development: twelve of the studies analyzed <25 samples and only three studied BPA levels in >100 samples. From European mothers, Deceuninck et al. (2015) analysed 30 human milk samples. Total BPA was quantified in 90% of the samples, with a median of 0.11 μg/kg, and a maximum of 1.16 μg/kg.

Among the studies carried out in North America, Cao et al. (2015) developed the more relevant in terms of number of populations, with 278 human milk samples from Canada. u-BPA was detected in 16% of the samples, with concentrations ranging from <0.036 to 2.3 ng/g with a median of 0.10 ng/g, a maximum of 2.3 ng/g. The authors have also analyzed the total-BPA, that present concentration ranging from <0.036 to 2.5 ng/g, with a detection frequency of 26%. In this study the GM and median ratios of [u-BPA]/[total-BPA] were 0.57 and 0.70 respectively. This ratios are similar to the ratios obtained in the present study and in other studies (see Table SD-17).

The levels detected in our study, with a median concentration of 0.10 ng/mL and 0.26 ng/mL for u-BPA and total-BPA, are similar or higher than levels reported in the literature (Ye et al. (2006), Cao et al. (2015), Niu et al. (2017), Deceuninck et al. (2015)). However other studies presented higher levels than in the present study (see Table 5).

As far as we know, only three studies have previously described levels of BPF and/or BPS in human milk. Deceuninck et al. (2015) studied levels of both bisphenols in 30 milk samples from French mothers, total-BPF (LOQ = 0.018 μg/kg) was not detected in any sample and total-BPS (LOQ = 0.003 μg/kg) was only detected in one sample (0.23 μg/kg). In China, Niu et al. (2017) detected levels of total-BPF and total-BPS in human milk samples (n = 20), total-BPF (LOQ = 0.005 μg/L) was detected in the 60% of the samples with concentrations ranging from 0.010 to 0.166 μg/L, total-BPS (LOQ = 0.010 μg/L) was detected in one sample with a concentration of 0.683 μg/L. Tuzimski et al. (2018) analyzed 20 human milk samples from Lublin (Poland) in order to detect BPS. Four of the samples presented levels higher than the LoQ (0.20 ng/mL) with concentrations ranging from 0.20 to 0.33 ng/mL. Higher levels have been detected in human urine. In a study carried out by Philips et al. (2018), the urinary median concentrations of BPF and BPS were 0.57 and 0.36, respectively, with detection frequencies higher than 40% in pregnant woman from Netherlands recruited in years 2004 and 2005. Ashrap et al. (2018) found urinary geometric mean concentrations of 0.3 and 3.2 ng/mL for BPF and BPS, respectively, with a BPS detection frequency of 90% in a Northern Puerto Rico population.

Table 3
Results of the multiple robust regression model for total-BPA levels in human milk.

Variable	Estimated coefficients (95% CI)	Standard error	p-value
Intercept	−1.780 (−2.211 – −1.350)	0.220	<0.001*
Place of residence last 10 years: Rural	−0.875 (−1.402 – −0.348)	0.269	0.002*
Frequency skin care products: Daily	0.71 (0.197–1.223)	0.262	0.008*
Frequency skin care products: Several times a week	0.472 (−0.217–1.16)	0.351	0.183
Frequency skin care products: Monthly	0.559 (−0.654–1.773)	0.619	0.369

* p-values <0.05.

Table 4
Estimated BPA exposure in μg kg^{-1} bw day^{-1} for breastfeed infants using the probabilistic approach.

	Exposure u-BPA (μg/kg bw-day)	Exposure total-BPA (μg/kg bw-day)
Minimum	0.001	0.001
25th percentile	0.01	0.02
Median	0.02	0.04
Geometric mean	0.03	0.04
Arithmetic mean	0.20	0.30
75th percentile	0.03	0.10
95th percentile	0.60	1.0
Maximum	5	7
Standard deviation	0.6	0.9

In order to achieve a better estimation of the intake of BPA by breast fed infants, we studied the variation of levels of u-BPA over the course of lactation (from 2 to 8 weeks). No individual significant changes in the concentrations were found. For different chemicals, mainly those that are persistent, bioaccumulative and toxic (PBT) (e.g. Dioxins, PCBs) a decline in chemical concentration over the duration of breastfeeding have been described (depuration rates) (LaKind et al., 2001). The depuration rates of PBTs could be influenced, among other factors, by initial chemical concentrations, age of the mother, parity, however this depletion is poorly understood (LaKind et al., 2004). BPA in mature milk reflects recent exposure, consequently the possible variations during lactation are mainly linked to the exposure (via mother diet) between feedings, and probably their levels are not linked to the depuration process such as those PBTs stored in adipose tissues of the mother.

As mentioned in the results section, the place of residence of the mother showed significant statistical association with the levels of BPA in human milk, the mothers who lived in a rural area presented lower levels than the ones who lived in an urban area. Previous studies have found different correlations between residence location and urine BPA levels. Cutanda et al. (2015) found significantly higher levels of BPA in urine of children who lived in rural areas in comparison with children who lived in urban areas in Spain. Snoj Tratnik et al. (2019) also found higher levels of urinary BPA in mothers who lived in rural areas in Slovenia. However, they found significantly higher levels of urinary BPA in urban children than in rural children. Other studies of BPA levels in urine and adipose tissue did not found significant differences between urban and rural areas (Zhang et al., 2016; Artacho-Cordon et al., 2018). No clear explanation could be found to explain the relation between place of residence and human milk total-BPA levels in our study. Furthermore, in the present study, BPA levels in human milk were positively correlated with the use of personal care products. The presence of BPA in personal care products, including skin lotions and body washes, can explain this association (Liao and Kannan, 2014). To our knowledge no other studies have analyzed previously the influence of potential determinants on the levels of BPA in human milk. It has been observed for more hydrophobic environmental chemicals, mainly those that accumulate in the lipids of humans (e.g. PCDD/F, PCBs, α-HCH), that age presented a positive relationship with their levels in milk (Rawn et al., 2017; Haraguchi et al., 2009). The number of children of the mother also has been associated (negatively) with the levels of persistent organic pollutants (PCBs, OC insecticides) in milk, although in the literature this association is not totally consistent (Tanabe and Kunisue, 2007). The fact that no association between age and levels of BPA has been found is consistent with the fact that BPA is not accumulated in the body and reflects recent exposure. Probably, the lack of association between levels of BPA in human milk and consumption of canned foods is due to the fact that the questionnaire did not accurately reflect the differences in consumption of these products.

According to EFSA (2015), the estimated average dietary exposure was 0.165 μg/kg bw per day for infants from 6 days up to 3 months.

Table 5
Human milk BPA levels in other studies.

Country (City/Region), Year sampling	Age babies	Type	n° participants	LoD (LoQ)	DF (%)	AM	GM	Median	P95	Range	Reference
–	–	Unconjugated	20	0.28	60	1.3	–	0.4	–	<LoD - 6.3	Ye et al., 2006
–	–	Total	20	0.28	90	1.9	–	1.1	–	<LoD - 7.3	Ye et al., 2006
–	–	Unconjugated	4	0.3	100	0.8	0.69	0.62	1.43	0.41–1.54	Ye et al., 2008
–	–	Total	4	0.3	100	1.02	0.96	0.86	1.51	0.73–1.62	Ye et al., 2008
Korea	2 weeks	Unconjugated	100	0.39 (1.3)	100	–	–	6.6	–	0.65–29.9	Yi et al., 2010
Korea	2 weeks	Total	100	0.39 (1.3)	100	–	–	10.4	–	0.65–42.6	Yi et al., 2010
France	Few days	Unconjugated	3	0.09 (0.40)	100	2.39	2.01	3.07	3.27	0.8–3.3	Cariot et al., 2012
USA	–	Unconjugated	30	0.3	29	–	–	<LoD	0.6 (P75)	–	Duty et al., 2013
USA	–	Total	30	0.3	100	–	–	1.3	2.5 (P75)	–	Duty et al., 2013
USA, 2006–2008	3–18 months	Unconjugated	23	0.3	22	1.7	0.4	<LoD	18.8	<LoD - 23.6	Mendonca et al., 2014
USA, 2006–2008	3–18 months	Total	23	0.3	75	2.1	0.8	0.8	19.4	<LoD - 22.6	Mendonca et al., 2014
USA	–	Unconjugated	21	0.22	62	3.13	1	0.68	10.7	<LoD-108	Zimmers et al., 2014
Spain (Granada)	–	Unconjugated	10	0.1 (0.3)	60	–	–	–	–	<LoD-11.5	Rodriguez-Gomez et al., 2014a
Spain (Granada)	–	Unconjugated	10	0.05 (0.15)	60	–	–	–	–	<LoD-13.8	Rodriguez-Gomez et al., 2014b
Canada, 2009–2011	2–3 months	Unconjugated	56	0.3	4	<LoD	<LoD	<LoD	<LoD	<LoD-1.6	Arbuckle et al., 2015
Canada, 2009–2011	2–3 months	Total	56	0.3	5	<LoD	<LoD	<LoD	0.4	<LoD-1.9	Arbuckle et al., 2015
Canada, 2009–2011	2–10 week	Unconjugated	278	0.21 *	17	–	0.11 *	0.10 *	0.39 *	<LoD - 2.3*	Cao et al., 2015
Canada, 2009–2011	2–10 week	Total	278	0.21*	26	–	0.13 *	0.11 *	0.65 *	<LoD-2.5*	Cao et al., 2015
France	–	Total	30	<0.003*(<0.01 *)	87	0.23*	–	0.11*	–	<LoQ-1.16*	Deceuninck et al., 2015
Japan	–	–	19	–	–	–	–	–	–	1.4–380**	Nakao et al., 2015
Spain (Granada)	–	Unconjugated	10	0.2 (0.5)	40	–	–	–	–	<LoD-2.1	Rodriguez-Gomez et al., 2015
Spain (Jaen)	–	Unconjugated	6	0.001	33	–	–	–	–	<LoD-2.9	Azzouz et al., 2016a
Spain (Andalusia)	–	Unconjugated	7	0.002 (0.007)	0	–	–	–	–	–	Azzouz et al., 2016b
Spain (Andalusia)	–	Total	7	0.002 (0.007)	57	–	–	–	–	<LoQ- 9.3	Azzouz et al., 2016b
China, 2014	–	Total	20	0.017 (0.05)	85	0.14***	0.10***	0.07***	0.44***	<LoD-0.55	Niu et al., 2017
Korea, 2011–2012	1 month	Total	127	0.30	80	–	0.85	0.74	7.74	<LoD-43.20	Lee et al., 2017
Spain (Valencia), 2015	2 weeks	Unconjugated	120	(0.10)	77	1.1	0.15	0.10	2.8	<LoQ-41	Present study
Spain (Valencia), 2015	2 weeks	Total	100	(0.10)	83	1.6	0.29	0.26	6.4	<LoQ-42	Present study

LoD (limit of detection); LoQ (limit of quantification); DF (detection frequency); AM (arithmetic mean); GM (geometric mean); P95 (95th percentile); P75 (75th percentile).
* ng/g.
** ng/g lipid.
*** Data derived from Niu et al., 2017 (values <LoQ expressed as LoQ/√2).

This exposure is several times higher than that found in our study (0.03 and 0.04 μg/kg bw.day). Likewise, as noted before, all the approaches considered (deterministic or probabilistic approaches, in both, average or 95th percentile (P95), scenarios) showed an exposure much lower than the health-based reference value (t-TDI). However, in the EFSA study a very conservative decision scenario was used, considering the total-BPA in mature milk as entirely deconjugated. A WHO Expert Meeting (2010) concluded that breastfed infants, between 0 and 6 months, of age were exposed to 0.3 and 1.3 μg/kg body weight of BPA per day (mean and 95th percentile). When infants were fed with powdered formula (prepared as consumed), the estimate exposure to BPA was 2.0 and 2.7 μg/kg body weight per day at the mean and 95th percentile, respectively. (WHO, 2010).

5. Conclusions

Breastfeeding can potentially cause exposures of chemical pollutants to nursing infants. The present study (sample collection year: 2015) shows that Valencian breastfed infants present very low exposure to BPA, far below the health-based reference value established by EFSA of 4 μg/kg bw-day.

Likewise, the results show that the individual levels of u-BPA do not change during lactation, and neither the age of the mothers nor the parity are associated with the levels of u-BPA in human milk, which represent a recent exposure.

Although BPA is being replaced by its analogues F and S, these present low frequencies of detection on the studied population

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.03.024.

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5.5. Capítulo 5: Biomonitorización de parabenos en leche materna e ingesta diaria admisible para lactantes

En el presente capítulo, se estudiaron los niveles de parabenos en leche materna de 120 madres del proyecto Bettermilk. El análisis se realizó tanto con hidrólisis enzimática (n=102) como sin hidrólisis (n=120) para comparar los niveles de parabenos totales (libres + conjugados) y libres en leche materna recogida a las 2 semanas del parto. Además, se estudiaron los niveles de parabenos libres a las 5 y a las 8 semanas después del parto para conocer la evolución de la exposición a lo largo de la lactancia (n=51). Para llevar a cabo los análisis se utilizó la metodología analítica desarrollada en el Capítulo 3. Posteriormente se estudiaron los determinantes de las concentraciones de parabenos totales en leche y se estimó la evaluación del riesgo en lactantes por ingesta de parabenos a través de la leche materna

5.5.1. Resultados y discusión

5.5.1.1. Concentraciones de parabenos en leche materna

En la **Tabla 21** se muestran las concentraciones de parabenos en leche materna a las 2 semanas después del parto. En todos los casos las DF para parabenos totales fueron > 60% y el MP presentó la mayor DF (89%). Con respecto a las concentraciones, los niveles de parabenos totales oscilaron entre <LoQ y 49 ng/mL, y sus GMs se encontraban en un rango entre 0,1 ng/mL (BP) y 0,36 ng/mL (MP). El ratio [parabeno-libre]/[parabeno-total] se calculó solo con las muestras cuyas concontraciones de parabeno libre y total fueron >LoQ. Las GMs de estos ratios fueron de 0,39 (MP), 0,42 (EP), 0,50 (PP) y 0,76 (BP), por lo tanto, se confirmó la presencia de metabolitos conjugados en leche materna. En la **Figura 26** se muestran los histogramas de los logaritmos de las concentraciones de parabenos totales en leche materna.

Las **Tablas SI-7. Cap. 5 y SI-8. Cap. 5** muestran el coeficiente de correlación de Spearman entre los niveles de parabenos en leche materna tomada dos semanas despúes del parto. Los niveles de todos los parabenos estaban correlacionados significativamente entre ellos (p-valores<0,005), lo cual sugiere que se emplean conjuntamente como conservantes.

Para evaluar los posibles cambios en los niveles de parabenos durante la lactancia, se compararon los niveles de parabenos libres en leche a las 2, 5 y 8 semanas después del parto (ver **Tabla 22**). Mediante un test de Friedman, se determinó que las diferencias en las concentraciones de MP, EP y BP a lo largo de la lactancia eran significativas (p-valores <0.05). La estadística descriptiva (**Tabla 22**) muestra que en general los niveles de parabenos eran mayores en la semana 2 que en la semana 8. Por lo tanto, los niveles de MP, EP y BP se redujeron significativamente durante la lactancia.

Tabla 21. Concentraciones de parabenos en muestras de leche de madres de Valencia (España) tomadas 2 semanas después del parto.

	MP (ng/mL)		EP (ng/mL)		PP (ng/mL)		BP (ng/mL)	
	Libre	**Total**	**Libre**	**Total**	**Libre**	**Total**	**Libre**	**Total**
Número de muestras	n=120	n=91	n=120	n=102	n=120	n=102	n=120	n=102
LoQ	0,10	0,10	0,10	0,10	0,10	0,10	0,10	0,10
> LoQ	60%	89%	41%	70%	47%	72%	53 %	61%
Mínimo	< LoQ	<LoQ	< LoQ	<LoQ	< LoQ	<LoQ	< LoQ	<LoQ
P25	< LoQ	0,10	< LoQ	<LoQ	< LoQ	<LoQ	< LoQ	<LoQ
Mediana	0,10	0,19	< LoQ	0,10	< LoQ	0,10	0,10	0,10
GM	0,13	0,36	< LoQ	0,13	< LoQ	0,14	< LoQ	0,10
AM	0,95	2,7	0,17	0,44	0,25	0,52	0,11	0,13
P75	0,36	1,0	0,10	0,23	0,10	0,18	0,10	0,10
P95	3,8	19	0,30	1,5	1,00	3,5	0,27	0,36
Máximo	31	49	5,2	9,0	6,5	8,0	1,1	1,3
Desv. estándar	3,5	7,7	0,62	1,2	0,76	1,3	0,12	0,16

LoQ: Límite de cuantificación (0,10 ng/mL para todos los compuestos)

Tabla 22. Niveles de parabenos libres en leche humana en las semanas 2, 5 y 8 después del parto (n=51).

	MP (ng/mL)			EP (ng/mL)			PP (ng/mL)			BP (ng/mL)		
Semanas después del nacimiento	**2**	**5**	**8**	**2**	**5**	**8**	**2**	**5**	**8**	**2**	**5**	**8**
> LoQ	71%	71%	57%	43%	29%	20%	41%	45%	47%	61%	25%	43%
Mínimo	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ
P25	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ
Mediana	0,10	0,10	0,10	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	0.10	<LoQ	<LoQ
GM	0,23	0,17	0,15	<LoQ	<LoQ	<LoQ	0,10	0,10	0,10	<LoQ	<LoQ	<LoQ
AM	1,3	0,63	0,63	0,18	0,13	0.10	0,36	0,28	0,23	0,12	0,16	<LoQ
P75	0,85	0,32	0,41	0,10	0,10	<LoQ	0,14	0,10	0,10	0,10	0,10	0,10
P95	5,6	4,4	2,8	0,28	0,75	0,43	2,2	1,9	1,6	0,31	1,2	0,32
Máximo	31	6,1	15	4,7	1,2	1,5	6,5	4,2	1,8	1,1	2,2	0,36
Desv. estándar	4,5	1,4	2,1	0,60	0,23	0,21	1,0	0,71	0,43	0,15	0,37	0,07
'Mean rank'	2,18	2,07	1,75	2,19	2,05	1,76	2,05	2,00	1,95	2,24	1,81	1,95
Friedman test χ^2		6,12			9,6			0,40			7,6	
Friedman test p-valor		0,047*			0,008*			0,817			0,023*	

*p-valor < 0,05; LoQ (Límite de cuantificación: 0,10 ng/mL)

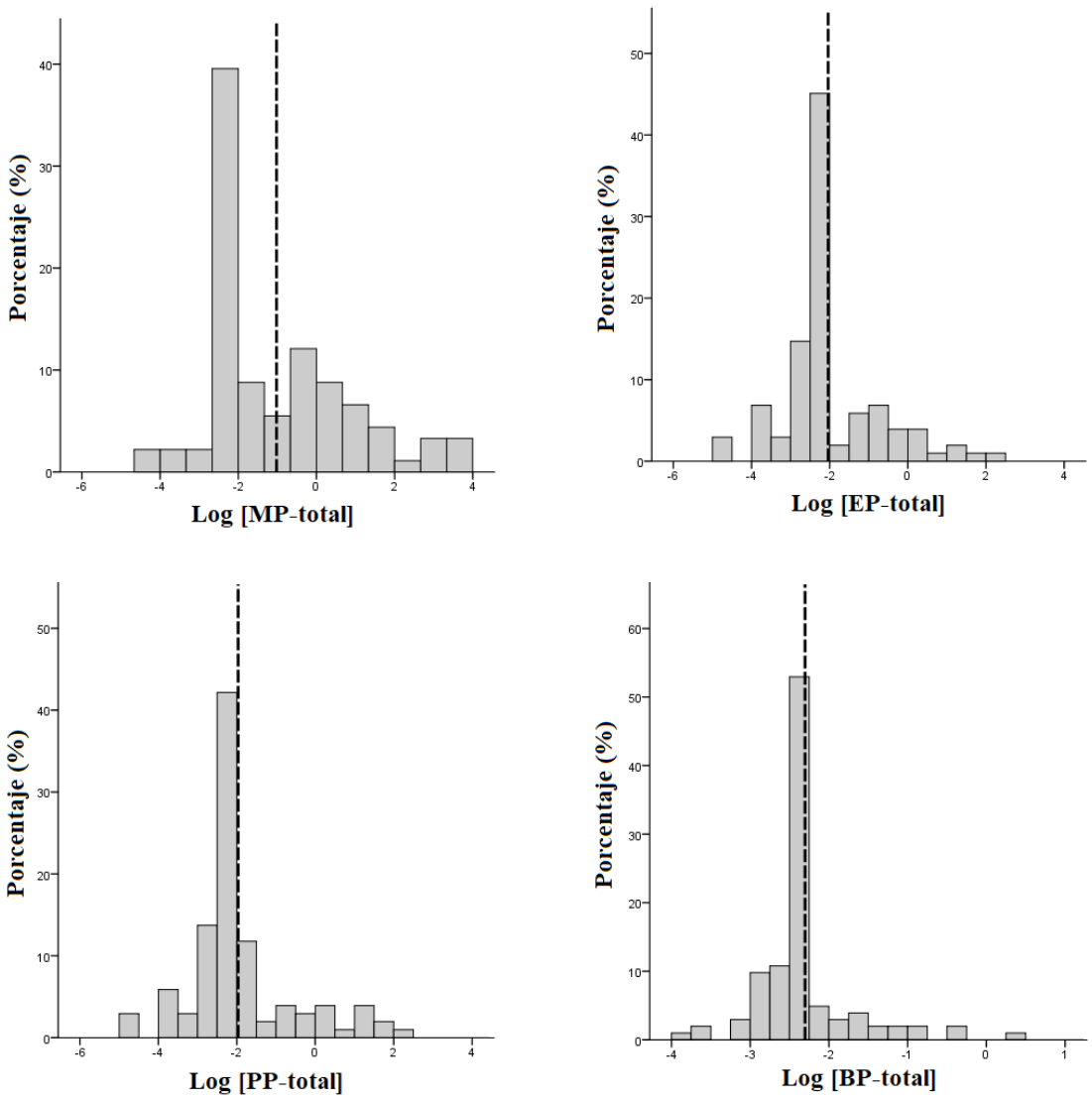

Figura 26. Histogramas de los logaritmos de las concentraciones de parabenos totales en leche materna a las 2 semanas después del parto. Las líneas verticales muestran el log de las GM.

Este es el mayor estudio de BH de parabenos en leche materna. Schlumpf et al. determinaron parabenos libres en leche de madres suizas y detectaron niveles entre 1,26 ng/mL (EP) y 2,18 ng/mL, mientras que el BP no fue detectado **(Schlumpf et al., 2010)**. En otro estudio, Fisher et al. determinaron parabenos totales en leche de madres canadienses y reportaron GMs por debajo de su LoD (0,1 ng/mL) y AMs en un rango entre 0,12 ng/mL (EP) y 0,99 ng/mL (MP). El BP no fue detectado **(Fisher et al., 2017)**.

5.5.1.2. Determinantes de parabenos en leche materna

Se llevaron a cabo RRMs simples y multiples para estudiar los determinantes de los niveles de parabenos en leche materna tomada a las 2 semanas. Las variables independientes estudiadas fueron datos sociodemográficos, dietéticos, uso de cosméticos

de las madres y niveles de macronutrientes en leche materna. En la **Tabla 23** se muestran los resultados de los RRM múltiple para parabenos-total.

La frecuencia de uso de algunos cosméticos fue positivamente correlacionada con los niveles de EP y PP ya que las madres que usaban diariamente algunos de estos cosméticos (perfumes y cremas solares) presentaron mayores niveles de estos parabenos en leche que las madres que no los usaban nunca. Además, los niveles de proteínas en leche fueron correlacionados positivamente con los niveles de MP y PP.

Tan solo dos estudios han analizado previamente la influencia de potenciales determinantes de los niveles de parabenos en leche materna. Schlumpf et al. **(2010)** observaron que las concentraciones de EP in leche materna crecían significativamente al aumentar edad de la madre. No se encontraron correlaciones de niveles de parabenos en leche con la dieta. Fisher et al. **(2017)** detectó un aumento significativo de los niveles de PP en leche de madres que habían usado recientemente maquillaje o limpiadores de piel. Sin embargo, no encontraron diferencias significativas con los niveles de MP y no se estudiaron los determinantes de los niveles de BP ni EP en leche. La correlación entre la frecuencia de uso de algunos cosméticos y los niveles de EP y PP en el presente estudio puede ser debido al uso de parabenos como conservantes en los cosméticos **(Bledzka et al., 2014)**.

5.5.1.3. EDI de parabenos en lactantes

Se utilizó un enfoque determinista y uno probabilístico para la estimación de la ingesta de parabenos en lactantes tal y como se describe en la sección 4.7.1. Las EDIs de parabenos calculados utilizando un enfoque determinista se muestran en la **Tabla SI-15. Cap. 5**. Con respecto al enfque probabilistico, la **Figura 25** muestra la distribución log-normal de la ingesta de leche y la **Tabla 24** muestra la EDI de parabenos libres y totales en lactantes. En cualquier caso, ninguna de las EDIs calculadas presenta valores mayores o similares a la ADI establecida por la EFSA para la suma de MP y EP de 0-10,000 µg/kg pc/día **(EFSA, 2004)**, por lo que no se puede considerar que haya un riesgo para los lactantes. Con respecto a PP y BP, la EFSA no ha establecido un ADI debido a que no se ha determinado un valor NOAEL. Sin embargo, sí que existen 'non-observed effect levels' (NOELs) para PP y BP (6500 µg/kg pc/día and 700 µg/kg pc/día, respectivamente **(Boberg et al., 2010)**. Los EDIs de PP y BP en el presente estudio **(Tabla SI-15. Cap. 5, Tabla 24)** fueron de varios órdenes de magnitud inferiores que el cociente NOELs/ factor de incertidumbre (UF) (el UF utilizado fue de 100 para garantizar las posibles variabilidades debidas a factores interespecies e interindividuales). Schlumpf et al. **(2010)** también calcularon los EDIs de u-MP, u-EP y u-PP, (0,42, 0,22 y 0,28 µg/kg pc/día, respectivamente). Al igual que en el presente estudio, no se encontraron EDIs superiores a los ADIs.

Tabla 23. Resultados de RRM multiples de niveles de parabenos-totales en leche materna.

Biomarcador	Variable	Parámetros estimados (95% IC)	Error estándar	p-valor
MP-total	'Intercept'	-2,874 (-5,166 – -0,582)	1,169	0,016*
	Huevos (g/mes)	$-9\cdot10^{-4}$ ($-0,002 – 2\cdot10^{-4}$)	$6\cdot10^{-4}$	0,122
	Frecuencia uso productos cuidado piel: Semanalmente o mensualmente	-0,923 (-1,868 – 0,023)	0,482	0,060
	Frecuencia uso productos cuidado piel: Nunca	-0,8758 (-1,778 – 0,026)	0,640	0,061
	Frecuencia uso perfumes: Semanalmente o mensualmente	-0,406 (-1,461 – 0,650)	0,538	0,454
	Frecuencia uso perfumes: Nunca	-0,816 (-1,738 – 0,107)	0,471	0,088
	Proteínas en leche (g/100mL)	2,729 (0,933 – 4,525)	0,916	0,004*
EP-total	'Intercept'	-4,441 (-5,665 - -3,218)	0,624	<0,001*
	Consumo tabaco: Ex-fumadora	1,125 (0,405 - 1,845)	0,367	0,003*
	Consumo tabaco: No-fumadora	1,562 (0,866 - 2,259)	0,355	<0,001*
	Vegetales (g/mes)	$1,7\cdot10^{-5}$ ($-7,7\cdot10^{-6} – 4,1\cdot10^{-5}$)	$1,2\cdot10^{-5}$	0,182
	Bollería y pastelería (g/mes)	$2\cdot10^{-4}$ ($1\cdot10^{-4} – 4\cdot10^{-4}$)	$1\cdot10^{-4}$	0,006*
	Frecuencia uso protector solar: Semanalmente	-0,207 (-0,954 - 0,540)	0,381	0,588
	Frecuencia uso protector solar: Mensualmente u ocasionalmente	-0,221 (-0,989 - 0,548)	0,392	0,575
	Frecuencia uso protector solar: Nunca	-0,617 (-1,138 - -0,096)	0,266	0,023*
	Proteínas en leche (g/100mL)	0,636 (-0,218 - 1,489)	0,436	0,149
PP-total	'Intercept'	-2,383 (-4,401 - -0,364)	1,030	0,023*
	Índice de masa corporal antes del embarazo (kg/m^2)	-0,072 (-0,135 - -0,010)	0,032	0,025*
	Dieta especial: No	0,459 (-0,252 - 1,170)	0,363	0,209
	Frecuencia uso productos cuidado piel: Semanalmente o mensualmente	-0,263 (-0,855 - 0,330)	0,302	0,387
	Frecuencia uso productos cuidado piel: Nunca	-0,367 (-0,919 - 0,185)	0,282	0,196
	Frecuencia uso perfumes: Semanalmente o mensualmente	-0,418 (-1,058 - 0,222)	0,327	0,204
	Frecuencia uso perfumes:Nunca	-1,037 (-1,587 - -0,487)	0,281	<0,001*
	Proteínas en leche (g/100mL)	2,009 (0,988 - 3,030)	0,521	<0,001*
BP-total	'Intercept'	-2,237 (-2,405 - -2,069)	0,086	<0,001*
	Frutas (g/mes)	$-5,2\cdot10^{-6}$ ($-9,9\cdot10^{-6}$ - $-5,4\cdot10^{-7}$)	$2,4\cdot10^{-6}$	0,031*
	Frecuencia uso protector solar: Semanalmente	-0,162 (-0,387 - 0,064)	0,115	0,164
	Frecuencia uso protector solar: Mensualmente u ocasionalmente	-0,119 (-0,336 - 0,099)	0,111	0,288
	Frecuencia uso protector solar: Nunca	-0,041 (-0,196 - 0,114)	0,079	0,604

* p-valor < 0,05.

Tabla 24. EDIs (en µg/Kg pc-día) de parabenos en lactantes calculadas utilizando un enfoque probabilístico.

	EDI (µg/Kg pc-día)							
	MP-libre	**MP-total**	**EP-libre**	**EP-total**	**PP-libre**	**PP-total**	**BP-libre**	**BP-total**
Mínimo	0,0001	0,0008	0,0006	0,0010	0,0007	0,0010	0,003	0,004
P25	0,006	0,014	0,005	0,011	0,004	0,012	0,009	0,012
Mediana	0,015	0,030	0,011	0,014	0,012	0,015	0,013	0,014
GM	0,02	0,11	0,011	0,03	0,012	0,04	0,015	0,03
AM	0,15	0,4	0,03	0,07	0,04	0,08	0,017	0,02
P75	0,06	0,14	0,015	0,03	0,02	0,03	0,016	0,016
P95	0,6	3,0	0,05	0,2	0,15	0,6	0,04	0,06
Máximo	4,8	6,7	1,0	1,2	1,0	1,4	0,19	0,2
Desv. estándar	0,6	1,2	0,10	0,18	0,12	0,2	0,02	0,03

5.5.2. Conclusiones

-En el presente capítulo se demuestra la viabilidad de usar la BH para evaluar la exposición a parabenos en niños lactantes

-Los EDIs de MP y EP fueron muy inferiores a la ADI de 10.000 µg/kg-pc/día establecida por la EFSA para la suma de MP y EP.

-Los bajos ratios [parabeno-libre]/[paraben-total] indican la presencia de parabenos conjugados en leche materna.

-El estudio mostró una correlación entre niveles de parabenos en leche materna y la frecuencia de consumo de algunos cosméticos, así como con los niveles de proteínas en leche materna.

5.5.3. Artículo 5: Biomonitoring of parabens in human milk and estimated daily intake for breastfed infants

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Biomonitoring of parabens in human milk and estimated daily intake for breastfed infants

Pablo Dualde [a, b], Olga Pardo [a], Francisca Corpas-Burgos [a], Julia Kuligowski [c], María Gormaz [d], Máximo Vento [d], Agustín Pastor [b], Vicent Yusà [a, b, e, *]

[a] Foundation for the Promotion of Health and Biomedical Research in the Valencian Region, FISABIO-Public Health, 21, Avenida Catalunya, 46020, Valencia, Spain
[b] Analytical Chemistry Department, University of Valencia, Edifici Jeroni Muñoz, Dr. Moliner 50, 46100, Burjassot, Spain
[c] Neonatal Research Unit, Health Research Institute La Fe, Avenida Fernando Abril Martorell 106, 46026, Valencia, Spain
[d] Neonatal Division at the University and Polytechnic Hospital La Fe, Avenida Fernando Abril Martorell, 106, 46026, Valencia, Spain
[e] Public Health Laboratory of Valencia, 21, Avenida Catalunya, 46020, Valencia, Spain

HIGHLIGHTS

- Paraben levels were determined in human milk samples from 120 Spanish mothers.
- Detection of parabens ranged from 61 to 89% with GMs from 0.10 to 0.36 ng/mL.
- Both conjugated and unconjugated parabens were present in human milk.
- Paraben levels were correlated with the frequency of use of cosmetics.
- Breastfed infants intake of parabens was far below the ADI.

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ABSTRACT

In this study, we assessed the presence of four parabens in human milk of 120 mothers from Valencia (Spain) which took part in a human biomonitoring project (BETTERMILK). The detection frequency ranges of parabens were 41–60% and 61–89% for unconjugated- and total (unconjugated + conjugated)-parabens, respectively. The concentrations ranged from <LoQ to 31 ng/mL and from <LoQ to 49 ng/mL for unconjugated- and total-parabens, respectively. The frequency of use of some cosmetic products and human milk protein levels were the main predictors of parabens in milk. The study evidences the presence of both conjugated and unconjugated paraben forms in human milk. The newborns estimated daily intake of parabens through human milk was several orders of magnitude lower than the 0–10 mg/kg bw-day acceptable daily intake for the sum of methyl and ethyl paraben established by EFSA. To our knowledge, this is currently the largest biomonitoring study of parabens in human milk.

1. Introduction

The esters of p-hydroxybenzoic acid (parabens) are a group of synthetically produced chemicals used as preservatives due to their antimicrobial properties (Bledzka et al., 2014). There are different types of substituents, being the alkyl esters methyl paraben (MP), ethyl paraben (EP), propyl paraben (PP) and butyl paraben (BP) the most commonly used. The length of the alkyl chain defines their physicochemical properties (Nowak et al., 2018).

The EU allows and regulates their use in food (EU Directive, 2006), pharmaceuticals (SANTE, 2017a; SANTE, 2017b) and cosmetic products (EC, 2009; EC, 2014a; EC, 2014b). Owing to the widespread use of parabens and their environmental pathways, researchers have detected levels of parabens in different

* Corresponding author. Foundation for the Promotion of Health and Biomedical Research in the Valencian Region, FISABIO-Public Health, 21, Avenida Catalunya, 46020, Valencia, Spain.
E-mail address: yusa_vic@gva.es (V. Yusà).

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environmental compartments such as water (Carmona et al., 2014), foodstuff (Liao et al., 2013a, 2013b) and indoor dust (Wang et al., 2012) or others, including plants, fish, soil, or indoor air (Bledzka et al., 2014).

In humans, the main routes of exposure to parabens are the dermal absorption of personal care products, with maximum estimated levels of 2400 μg/kg bw-day, and the ingestion of pharmaceuticals and foodstuff, with maximum estimated daily intakes (EDIs) of 417 μg/kg bw-day and 13 μg/kg bw-day, respectively. Some studies with animals have linked paraben exposure to health risks, mainly by relating paraben exposure to altered estrogenic activity during fetal and neonatal stages (Kang et al., 2002; Ahn et al., 2012; Guerra et al., 2017). Paraben levels may exceed the action of endogenous estradiol in children and special care with propylparaben should be taken since its safety margin is very low. Furthermore, studies of the combined exposure to parabens and other chemicals with estrogenic effects are needed (Boberg et al., 2010).

After dermal and oral absorption, parabens are mainly metabolized to p-hydroxybenzoic acid. Nevertheless, this metabolite is a non-specific biomarker for paraben exposure, while the estrogenic activity can vary between different parabens. Therefore, in order to study the internal exposure to parabens in humans, the unchanged parabens and their conjugates should be used as biomarkers (Ye et al., 2008). Parabens are rapidly excreted in urine after consumption, hence, they must be considered as biomarkers of recent exposure (Moos et al., 2016).

Biomonitoring studies of parabens have been focused on urinary levels (Ye et al., 2006). However, parabens have also been studied in other matrices such as blood (Mulla et al., 2015), plasma (Kolatorova et al., 2018) or adipose tissue (Artacho-Cordon et al., 2018). Parabens have been detected in human milk in both unconjugated and conjugated forms (Azzouz et al., 2016a), however, most of these studies were focused in the development of an analytical methodology and analyzed a limited number of milk samples ($n < 20$) (Souza et al., 2016). Only Fisher et al. (2017) and Schlumpf et al. (2010) analyzed a higher number of samples and detected parabens levels ranging from < LoQ to 16 ng/mL with detection frequencies (DF) ranging from 0 to 82%.

The use of human milk for biomonitoring studies is convenient, since it provides information on the exposure of both, the mother and the breastfed infant, in a period of high vulnerability to endocrine disruptors (Mallozzi et al., 2016). The European Food Safety Authority (EFSA) established an acceptable daily intake (ADI) of 0–10 mg/kg (body weight) bw-day for the sum of MP, EP acid esters and their sodium salts based on studies which showed non-observed-adverse-effects-levels (NOAELs) of 1000 mg/kg bw-day for both parabens. Nevertheless, an ADI for PP could not be defined due to studies which showed PP effects on sex hormones and male reproductive organs in juvenile rats (EFSA, 2004). Only Schlumpf et al. (2010) studied the newborns exposure to parabens through lactation.

In this context, the major knowledge gaps that the present study intends to cover are: i) the low number of biomonitoring studies of parabens in human milk; ii) the low number of risk assessment studies which assess the breastfed infants exposure to parabens through lactation; iii) the lack of biomonitoring studies assessing the range unconjugated/conjugated paraben levels in human milk. In order to address these knowledge gaps, the objectives of the present study were: i) determine the concentration of unconjugated (u-parabens) and total parabens in human milk; ii) study the changes of u-paraben levels during breastfeeding iii) study the correlation of total-paraben levels with lifestyle, diet and other variables iv) estimate the exposure of breastfed newborns to u-parabens and total-parabens.

2. Materials and methods

2.1. Standards and reagents

MP, EP, PP and BP certified standards were acquired from Sigma Aldrich (Steinheim, Germany). The isotopically labeled internal standards (ILIS) $^{13}C_6$-MP and $^{13}C_6$-BP were acquired from Cambridge Isotope Laboratories (Tewksbury, Massachusetts, USA) and EP-d_5 was obtained from Santa Cruz Biotechnology (Dallas, Texas, USA). The enzyme β-Glucuronidase sulfatase from Helix pomatia type H1 was purchased from Sigma Aldrich (St. Louis, MO, USA). The β-Glucuronidase purified powder was dissolved in ammonium acetate 1 M (pH = 5) to obtain a 3500 U/mL enzymatic solution that was weekly prepared.

2.2. Study design and sample collection

The recruitment of volunteers has been previously described elsewhere (Yusa et al., 2017). Briefly, 120 lactating mothers aged between 20 and 45 years were recruited as donors in the University and Polytechnic Hospital la Fe (Valencia, Spain, 2015) in the framework of the BETTERMILK project.

Human milk was sampled 2 weeks after birth. Furthermore, in order to study the change of paraben levels during breastfeeding, 51 volunteers provided two extra samples collected 5 and 8 weeks after birth. Samples were collected after cleaning their breasts with abundant clean water. Milk samples were sampled in a glass container using breast pumps (Philips Avent SCF330). Samples were stored in the participants freezer at −20 °C inmediatly until their transfer to the IBSP-CV BioBank where they were conserved at −80 °C. Other sampling details are described in Dualde et al. (2019a).

Volunteers filled in questionnaires providing information on sociodemographic characteristics, lifestyle, use of cosmetics and diet. Table 1, Table SD1 and SD2 show the variables studied. The questionnaires are described in detail in Yusa et al., 2017).

As explained in Yusa et al. (2017), samples and data from volunteers included in this study, approved by the ethical and scientific committees, were administered by the IBSP-CV BioBank (PT13/0010/0064).

2.3. Chemical analysis

2.3.1. Parabens determination

The analytical method has been previously described (see Dualde et al., 2019b). In short, 10 mL of sample were spiked and mixed with an ILIS solution. Subsequently, analytes were extracted adding 10 mL of acetonitrile and a QuEChERS salt pouch (4 g anhydrous $MgSO_4$, 1 g NaCl). After mixing and centrifugation (8000 rpm, 10 min), the organic layer was transferred to a polypropylene tube. A QuEChERS fatty dispersive-solid phase extraction (d-SPE) AOAC kit (400 mg primary secondary amine (PSA), 400 mg C18 and 1200 mg $MgSO_4$) was added. After shaking and centrifugation (4500 rpm, 15 min), the acetonitrile layer was transferred to a glass tube and the solvent was evaporated under a N_2 stream (37 °C). Finally 250 μL of methanol:water (20:80, v/v) were added to the residue. The extract was homogenized and filtered previously to the injection into the high performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) system. For the analysis of total parabens, an enzymatic deconjugation step was implemented previously to the extraction. A TSQ Quantum (HPLC) system from Thermo Fisher Scientific (Bremen, Germany) equipped with a Symmetry C18 column (2.1 × 150 mm, 5 μm) from Waters (Milford, Massachusetts, USA) was used for chromatographic separation. Mass analysis was performed on the

Table 1
Characteristics of the studied population.

Characteristics	n (%) (N = 120)
Mother	
Number of children	
1	69 (57.5)
2	40 (33.3)
≥3	11 (9.2)
Age (years)	33 (20–45)[a]
Weight before pregnancy (kg)	60 (42–92)[a]
Height (cm)	164 (150–184)[a]
BMI before pregnancy (kg/m^2)	21.9 (16.6–35.4)[a]
Special diet during pregnancy	
Yes	17 (14.4)
No	101 (85.6)
Missing data	2
Country of birth	
Spain	104 (88.1)
Foreign	14 (11.9)
Missing data	2
Place of residence	
Urban	85 (80.2)
Rural	21 (19.8)
Missing data	14
Education level	
Only primary school	13 (10.8)
Secondary school	24 (20.0)
University	83 (69.2)
Occupational status	
Employed	100 (84.8)
Unemployed	18 (15.2)
Missing data	2
Time worked outside the home (years)	10 (0–28)[a]
Use of cosmetics at work	
Yes	12 (10)
No	108 (90)
Mother was breastfed	
Yes	81 (70.4)
No	34 (29.6)
Missing data	3
Physical exercise	
3 or more days/week	19 (16.2)
1 or 2 days/week	18 (15.4)
Occasionally	50 (42.7)
Never	30 (25.6)
Missing data	3
Smoker	
Yes	9 (7.5)
Ex-smoker	48 (40.0)
Never	63 (52.5)
Child	
Gestational age (weeks)	40 (35–41)[a]
Sex	
Boy	47 (40.2)
Girl	70 (59.8)
Missing data	3
Weight (g)	3360 (2160–4350)[a]
Height (cm)	51 (46–55)[a]
Cranial perimeter (cm)	34 (32–37)[a]

[a] Values expressed as median (minimum – maximum).

Finnigan TSQ Quantum Ultra Detector analyzer (San José, CA, USA). Negative APCI mode and selected reaction monitoring (SRM) mode were used for the determination of parabens. Data acquisition was performed by the Thermo Scientific Trace Finder TM 3.2 software. Details of the HPLC-MS/MS method are described in Tables SD3–SD5. HPLC-MS/MS chromatograms of a milk blank before and after spiking with the parabens at the LoQ level are shown in Fig. SD-1.

2.3.2. Quality assurance/quality control (QA/QC)

The laboratory works under the quality assurance system established by ISO/IEC/EN 17025. The analytical validation has been described in Dualde et al. (2019b) and the method validation parameters are shown in Table SD6. Each set of samples was analyzed following QA/QC protocols, including a matrix-matched calibration curve, procedural blanks (reagent blank and milk blank) and fortified blank samples at three levels: 0.10 ng/mL (LoQ); 5.0 ng/mL (Intermediate level) and 50 ng/mL (Higher level), in order to monitor the analytical sequence and confirm the lack of external contamination.

The acceptance criteria for QA/QC were: Recovery of spiked blank samples, 80–120%; precision, RSD ≤20%; reagent blank and milk blank areas ≤30% LoQ area.

The LoQ was established at the lowest validated spiked level to meet the performance acceptability criteria for QA/QC previously described. The LoQ for all analytes was 0.10 ng/mL.

The liquid chromatography coupled to tandem mass spectrometry quantification and confirmation criteria described in Dualde et al. (2019b) were applied.

2.3.3. Human milk macronutrients determination

Human milk fat, carbohydrate, protein, total solids and energy were determined using a MIRIS HMA™ (MIRIS AB, Uppsala Sweden), based on mid-infrared transmission spectroscopy. The determinations, QCs and calibrations performed followed the standard operation procedure given by the manufacturer (MIRIS HMA™, 2017). Other details have been described in Dualde et al. (2019a).

2.4. Statistical analysis

Statistical analysis were performed using the R software (version 3.3.1) and IBM SPSS (version 17.0). For the estimation of paraben levels below the LoQ the maximum likelihood estimation method (MLE) described in EFSA (2010) was employed assuming a log-normal distribution (see Dualde et al., 2019a for more details).

The descriptive analysis of the variables studied was described in Dualde et al. (2019a). The Friedman test was used to compare the u-paraben levels 2, 5, and 8 weeks after delivery.

In order to study the correlation between the levels of the different types of parabens in milk the Spearman correlation test was used.

To study the relationship between total-paraben levels and sociodemographic, dietary, use of cosmetics and macronutrients levels variables, simple and multiple robust linear regression models (RRMs) were employed. RRM was considered an alternative to ordinary least squares estimation methods (OLS) given the presence of some outlier values in paraben levels. In order to achieve the normality of the response variable, paraben levels were logarithmically transformed. Multiple RRMs were built following a backward variable selection procedure based on the Bayesian Information Criterion (BIC) (Konishi and Kitagawa, 2008) and considering as explanatory variables those with a p-value below 0.10 in the simple RRMs.

The correlation between paraben levels in milk and some variables of infants (gestational age, weight at birth, height at birth and cranial perimeter) was also studied using the Spearman correlation test.

2.5. Exposure assessment

In order to estimate the exposure of breastfed newborns to parabens, we estimated the breastfed infants daily intake (EDI) using two approaches (deterministic and probabilistic) as detailed in Dualde et al. (2019a).

In the deterministic approach, the EDI was calculated following the equation:

$$EDI\left(\frac{\mu g}{kg\ bw \times day}\right) = C\left(\frac{\mu g}{L}\right) \times M\left(\frac{L}{kg\ bw \times day}\right)$$

where C is the average paraben level in the studied population and M is the mean milk intake rate (quantity of human milk consumed per day) over six months of breast-feeding (140 mL/kg bw-day). We also calculated the paraben exposure using the 95th percentile of human milk intake during the first 3 months of age (190 mL/kg-day) (see Dualde et al., 2019a).

In the probabilistic approach, the infants EDI was calculated assuming that the consumption data were distributed according to a log-normal distribution, with a mean of 140 mL/kg bw-day and a probability that the milk consumption is < 190 mL/kg bw-day of 95%:

$$e^{\mu+\sigma^2/2} = 140; F(190) = 0.95$$

where μ and σ are the parameters of the log-normal distribution and F represents the distribution function. In addition, a minimum milk intake of 120 mL/kg bw-day was also considered. Once the milk consumption distribution of the breastfed infants was estimated, 120 values of this distribution were generated corresponding to each one of the infants recruited in this study. The infant's paraben intake was estimated by multiplying the generated consumption values generated by the paraben levels found in milk samples (see Dualde et al., 2019a).

3. Results

3.1. Paraben levels

The descriptive statistics of parabens in human milk two weeks after delivery are shown in Table 2. In all cases the detection frequency (DF) for total-parabens was higher than 60% and MP presented the highest DF (89%). Regarding concentrations, total-paraben levels ranged from <LoQ to 49 ng/mL and their geometric means (GMs) ranged from 0.10 ng/mL (BP) to 0.36 ng/mL (MP).

The ratio [u-paraben]/[total-paraben], calculated only in samples with both unconjugated and total paraben concentrations > LoQ, presented GMs of 0.39 (MP), 0.42 (EP), 0.50 (PP) and 0.76 (BP) and ranges from 0.05 to 1.00 (MP), 0.08–1.00 (EP), 0.05–1.00 (PP) and 0.40–1.00 (BP). Most of them showed low ratios (<0.50). Therefore, in general levels of conjugated parabens in human milk were higher than levels of the unconjugated forms, except for BP.

Tables SD7 and SD8 show the Spearman correlation between the levels of parabens in human milk two weeks after delivery. All levels of parabens are significantly correlated with each other (p-values <0.005).

In order to measure the possible changes of paraben levels during lactation, 51 participants donated three milk samples collected 2, 5 and 8 weeks after delivery. Table 3 shows the evolution of u-parabens human milk levels after delivery. Since total-paraben levels were not determined in the 5th and 8th week samples (see Section 5.1), the evolution of total-paraben levels throughout lactation was not studied. When comparing the 2, 5 and 8 weeks paraben levels using the Friedman test, the p-values obtained were <0.05 for MP, EP and BP. The descriptive statistics (Table 3) show that, in general, paraben levels were higher in the 2nd week than in the 8th week. Therefore, MP, EP and BP levels decreased significantly during lactation.

Levels of the main human milk macronutrients are presented in Table SD9.

3.2. Determinants of total paraben levels

The relation of total-paraben levels (two weeks after delivery) with socio-demographic characteristics, diet habits, use of cosmetics and other factors was studied. In Tables SD10–13 simple RRMs of log normal levels of total-parabens with the independent variables are presented. Fig. 1 shows histograms of log transformed total-paraben levels. Subsequently, variables with p-values <0.10 were included in the multiple RRMs. The results of the multiple RRMs are shown in Table 4.

The frequency of use of some cosmetic products was positively associated with levels of EP and PP, the daily use of some cosmetic products was associated with higher levels of human milk paraben levels than in participants which never used those cosmetic products. Regarding diet, consumption of fruits and pastries was significantly correlated with levels of total-parabens. However, as the estimated parameters calculated were close to 0, the influence of those variables over paraben levels is negligible. The concentration of proteins in milk was also positively associated with the levels of MP and PP. Likewise, mother with low BMI presented significantly higher levels of PP. Finally, the levels of EP were significantly lower in smokers. However, since the number of smokers was low (n = 9) this association could be inconsistent.

Some questionnaire variables related to the children infants were considered as dependent variables and they were not included in the RRMs. The correlation of these variables (gestational age, weight at birth, height at birth and cranial perimeter at birth) with paraben levels was studied using a Spearman

Table 2
Levels of parabens in human milk of mothers in Valencian Region (2 weeks after delivery).

	MP (ng/mL)		EP (ng/mL)		PP (ng/mL)		BP (ng/mL)	
	Unconjugated	Total	Unconjugated	Total	Unconjugated	Total	Unconjugated	Total
Number of samples	n = 120	n = 91	n = 120	n = 102	n = 120	n = 102	n = 120	n = 102
LoQ	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
> LoQ	60%	89%	41%	70%	47%	72%	53%	61%
Minimum	< LoQ	<LoQ	< LoQ	<LoQ	< LoQ	<LoQ	< LoQ	<LoQ
25th percentile	< LoQ	0.10	< LoQ	<LoQ	< LoQ	<LoQ	< LoQ	<LoQ
Median	0.10	0.19	< LoQ	0.10	< LoQ	0.10	0.10	0.10
Geometric mean	0.13	0.36	< LoQ	0.13	< LoQ	0.14	< LoQ	0.10
Arithmetic mean	0.95	2.7	0.17	0.44	0.25	0.52	0.11	0.13
75th percentile	0.36	1.0	0.10	0.23	0.10	0.18	0.10	0.10
95th percentile	3.8	19	0.30	1.5	1.00	3.5	0.27	0.36
Maximum	31	49	5.2	9.0	6.5	8.0	1.1	1.3
Standard deviation	3.5	7.7	0.62	1.2	0.76	1.3	0.12	0.16

LoQ: Limit of quantification (0.10 ng/mL for all compounds).

Table 3
Levels of unconjugated parabens in human milk in weeks 2, 5 and 8 after birth (n=51).

	MP (ng/mL)			EP (ng/mL)			PP (ng/mL)			BP (ng/mL)		
Weeks after birth	2	5	8	2	5	8	2	5	8	2	5	8
>LoQ	71%	71%	57%	43%	29%	20%	41%	45%	47%	61%	25%	43%
Minimum	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ
25th percentile	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ
Median	0.10	0.10	0.10	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	0.10	<LoQ	<LoQ
Geometric mean	0.23	0.17	0.15	<LoQ	<LoQ	<LoQ	0.10	0.10	0.10	<LoQ	<LoQ	<LoQ
Arithmetic mean	1.3	0.63	0.63	0.18	0.13	0.10	0.36	0.28	0.23	0.12	0.16	<LoQ
75th percentile	0.85	0.32	0.41	0.10	0.10	<LoQ	0.14	0.10	0.10	0.10	0.10	0.10
95th percentile	5.6	4.4	2.8	0.28	0.75	0.43	2.2	1.9	1.6	0.31	1.2	0.32
Maximum	31	6.1	15	4.7	1.2	1.5	6.5	4.2	1.8	1.1	2.2	0.36
Standard deviation	4.5	1.4	2.1	0.60	0.23	0.21	1.0	0.71	0.43	0.15	0.37	0.07
Mean rank	2.18	2.07	1.75	2.19	2.05	1.76	2.05	2.00	1.95	2.24	1.81	1.95
Friedman test χ^2	6.12			9.6			0.40			7.6		
Friedman test p-value	0.047*			0.008*			0.817			0.023*		

*p-value < 0.05.
LoQ (Limit of quantification: 0.1 ng/mL).

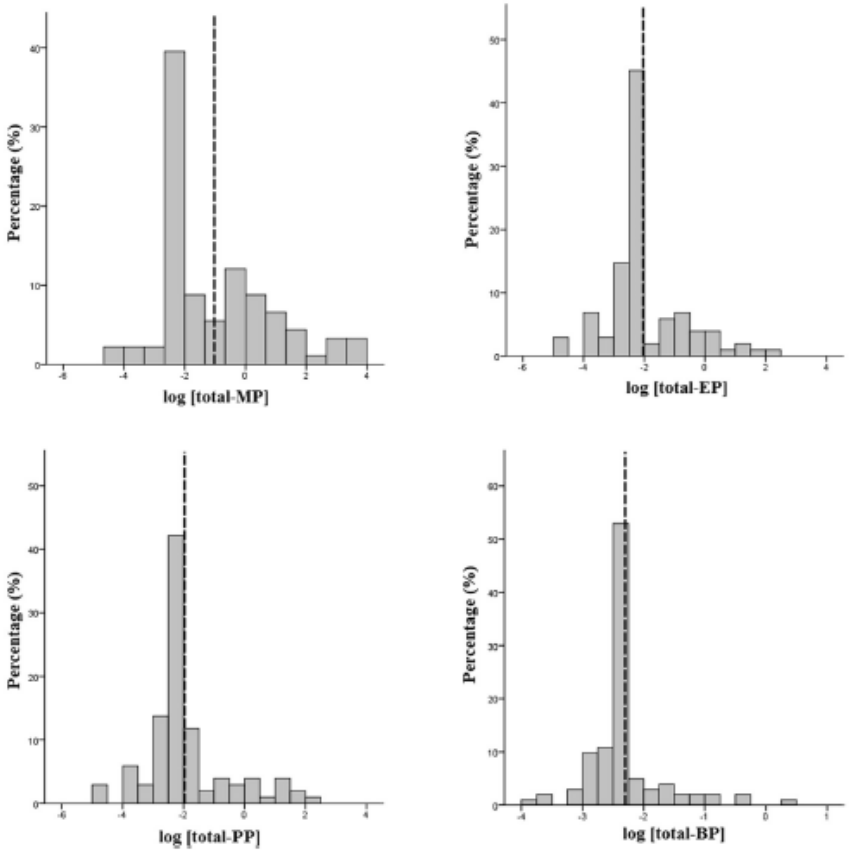

Fig. 1. Histograms of log transformed total-paraben levels. The vertical lines show the log transformed geometric means.

correlation test (see Table SD14). In general, paraben levels were negatively correlated with those variables.

3.3. *Infants exposure to parabens*

The EDIs obtained using the deterministic approach are shown

Table 4
Results of the multiple linear robust regression models for total-parabes levels in human millk.

Analyte	Variable	Estimated parameters (95% CI)	Standard error	P-value
Total-MP	**Intercept**	−2.874 (−5.166–−0.582)	1.169	0.016*
	Eggs (g/month)	$-9\cdot10^{-4}$ ($-0.002-2\cdot10^{-4}$)	$6\cdot10^{-4}$	0.122
	Skin care frequency: Weekly or monthly	−0.923 (−1.868–0.023)	0.482	0.060
	Skin care frequency: Never	−0.8758 (−1.778–0.026)	0.640	0.061
	Perfume frequency: Weekly or monthly	−0.406 (−1.461–0.650)	0.538	0.454
	Perfume frequency: Never	−0.816 (−1.738–0.107)	0.471	0.088
	Protein (g/100 mL)	2.729 (0.933–4.525)	0.916	0.004*
Total-EP	Intercept	−4.441 (−5.665–−3.218)	0.624	<0.001*
	Smoker status: Ex-smoker	1.125 (0.405–1.845)	0.367	0.003*
	Smoker status: Non -smoker	1.562 (0.866–2.259)	0.355	<0.001*
	Vegetables (g/month)	$1.7\cdot10^{-5}$ ($-7.7\cdot10^{-6}-4.1\cdot10^{-5}$)	$1.2\cdot10^{-5}$	0.182
	Pastries (g/month)	$2\cdot10^{-4}$ ($1\cdot10^{-4}-4\cdot10^{-4}$)	$1\cdot10^{-4}$	0.006*
	Sunscreen frequency: Weekly	−0.207 (−0.954–0.540)	0.381	0.588
	Sunscreen frequency: Occasionally	−0.221 (−0.989–0.548)	0.392	0.575
	Sunscreen frequency: Never	−0.617 (−1.138–−0.096)	0.266	0.023*
	Protein (g/100 mL)	0.636 (−0.218–1.489)	0.436	0.149
Total -PP	Intercept	−2.383 (−4.401–−0.364)	1.030	0.023*
	Mother BMI before pregnancy (kg/m^2)	−0.072 (−0.135–−0.010)	0.032	0.025*
	Special diet: No	0.459 (−0.252–1.170)	0.363	0.209
	Skincare frequency: Weekly or monthly	−0.263 (−0.855–0.330)	0.302	0.387
	Skincare frequency: Never	−0.367 (−0.919–0.185)	0.282	0.196
	Perfume frequency: Weekly or monthly	−0.418 (−1.058–0.222)	0.327	0.204
	Perfume frequency: Never	−1.037 (−1.587–−0.487)	0.281	<0.001*
	Protein (g/100 mL)	2.009 (0.988–3.030)	0.521	<0.001*
Total-BP	Intercept	−2.237 (−2.405–−2.069)	0.086	<0.001*
	Fruits (g/month)	$-5.2\cdot10^{-6}$ ($-9.9\cdot10^{-6}$ - $-5.4\cdot10^{-7}$)	$2.4\cdot10^{-6}$	0.031*
	Sunscreen frequency: Weekly	−0.162 (−0.387–0.064)	0.115	0.164
	Sunscreen frequency: Occasionally	−0.119 (−0.336–0.099)	0.111	0.288
	Sunscreen frequency: Never	−0.041 (−0.196–0.114)	0.079	0.604

* P-value < 0.05.

in Table SD15. In both GM and 95th estimations, the intake was several orders of magnitude lower than the ADI of 0–10,000 μg/kg bw-day for the sum of MP and EP (EFSA, 2004).

Regarding the probabilistic approach, Fig. SD2 shows the estimated log-normal milk consumption distribution and Table 5 shows the estimated exposure to u- and total-parabens for breastfed newborns.

As can be seen in Table 5, the GMs and P95 EDIs obtained were higher than those obtained by the deterministic approach. Nevertheless, using both approaches the estimated values were several orders of magnitude lower than the ADI of 0–10,000 μg/kg bw-day for the sum of MP and EP (EFSA, 2004). Regarding PP and BP, the EFSA has not estimated an ADI.

4. Discussion

This is the largest human biomonitoring study reporting on concentrations of parabens in human milk. To our knowledge, since 2008, thirteen studies have determined parabens in this matrix (Table 6). Nevertheless, eleven of them were focused on the development of analytical methods and analyzed only a low number of samples ($n < 20$). The study with the highest number of samples recruited 56 donors (Fisher et al., 2017). From the thirteen studies, seven studied only u-parabens, four studied only total parabens and only two studied both unconjugated and total parabens. In the literature (Table 6), MP presented the highest DFs (34–100%) and BP the lowest DFs (0–80%). Similar results were obtained in the present study, where MP and BP presented also the highest and the lowest DFs, respectively. In previous studies, paraben levels ranged from 0.1 to 87.5 ng/mL. Focusing on the studies with the largest number of samples ($n > 20$), Schlumpf et al. (2010) determined u-parabens in defatted milk of Swiss mothers and detected levels from 1.26 ng/mL (u-EP) to 2.18 ng/mL (u-MP) while BP was not detected. However, these levels have been questioned since the use of defatted milk could lead to an underestimation of the levels of parabens, especially in the case of BP, which is the most lipophilic paraben (Bledzka et al., 2014). In another study, Fisher et al. (2017) determined total-parabens in human milk from

Table 5
Estimated exposure parabens in μg/Kg bw-day for breastfed infants using the probabilistic approach.

	Estimated daily intake (μg/Kg bw-day)							
	u-MP	total-MP	u-EP	total-EP	u-PP	total-PP	u-BP	total-BP
Minimun	0.0001	0.0008	0.0006	0.0010	0.0007	0.0010	0.003	0.004
25th percentile	0.006	0.014	0.005	0.011	0.004	0.012	0.009	0.012
Median	0.015	0.030	0.011	0.014	0.012	0.015	0.013	0.014
Geometric mean	0.02	0.11	0.011	0.03	0.012	0.04	0.015	0.03
Arithmetic mean	0.15	0.4	0.03	0.07	0.04	0.08	0.017	0.02
75th percentile	0.06	0.14	0.015	0.03	0.02	0.03	0.016	0.016
95th percentile	0.6	3.0	0.05	0.2	0.15	0.6	0.04	0.06
Maximun	4.8	6.7	1.0	1.2	1.0	1.4	0.19	0.2
Standard deviation	0.6	1.2	0.10	0.18	0.12	0.2	0.02	0.03

Table 6
Human milk parabens levels in other studies.

Country (City/Region), year sampling	Age newborns	Biomarkers	N° samples	MP (ng/mL)					EP (ng/mL)					PP (ng/mL)					BP (ng/mL)					Reference
				DF (%)	LoQ	AM	GM	Range	DF (%)	LoQ	AM	GM	Range	DF (%)	LoQ	AM	GM	Range	DF (%)	LoQ	AM	GM	Range	
Spain (Valencia), 2015	2 weeks	u	120	60	0.1	0.95	0.13	<LoQ-31	41	0.1	0.17	<LoQ	<LoQ-5.2	47	0.1	0.25	<LoQ	<LoQ-6.5	53	0.1	0.11	<LoQ	<LoQ-1.1	Present study
Spain (Valencia), 2015	2 weeks	Total	91–102	89	0.1	2.74	0.36	<LoQ-49	70	0.1	0.44	0.13	<LoQ-9.0	72	0.1	0.52	0.14	<LoQ-8.0	61	0.1	0.13	0.10	<LoQ-1.3	Present study
USA, 2007	–	u	4	50	0.1 (LoD)	–	–	0.32[a]-3.04	0	0.1 (LoD)	–	–	–	25	0.1 (LoD)	–	–	0.32–0.32	0	0.1 (LoD)	–	–	–	Ye et al. (2008)
USA, 2007	–	Total	4	100	0.1 (LoD)	–	–	0.53[a]-3.00	0	0.1 (LoD)	–	–	–	25	0.1 (LoD)	–	–	0.33–0.33	0	0.1 (LoD)	–	–	–	Ye et al. (2008)
Switzerland (Basel), 2005–2006	4 to >30 days	u	41	34	–	2.18[a]	–	1[a]-8	20	–	1.26[a]	–	1[a]-1.5	15	–	1.42[a]	–	1[a]-2	0	–	–	–	–	Schlumpf et al., 2010[b]
Spain (Granada)	–	u	10	50	0.2	–	–	0.7–7.8	70	0.3	–	–	0.7[a]-15	80	0.7	–	–	0.7[a]-43.5	40	0.2	–	–	0.6[a]-14.5	Rodriguez-Gómez et al. (2014a)
Spain (Granada)	–	u	10	90	0.09	–	–	0.4–3.5	90	0.09	–	–	0.2–3.4	90	0.09	–	–	0.1–7.5	80	0.1	–	–	0.2[a]-1.3	Rodriguez-Gómez et al. (2014b)
Spain (Granada)	–	u	10	80	0.5	–	–	1.26[a]-16.3	60	0.5	–	–	0.97[a]-18.1	70	0.4	–	–	1.02[a]-12.6	50	0.7	–	–	1.06[a]-12.1	Rodriguez-Gómez et al. (2015)
USA (North Carolina), 2004–2005	2 weeks- 4 months	Total	8	100	0.1 (LoD)	–	–	0.5–2.3	50	0.1 (LoD)	–	–	–	100	0.1 (LoD)	–	–	0.1–0.6	0	0.1 (LoD)	–	–	–	Hines et al. (2015)
Spain (Jaen)	–	u	6	50	0.026	–	–	1.2[a]-8.1	66	0.029 (LoD)	–	–	1.3[a]-5.1	0	0.027	–	–	–	17	0.029	–	–	0.36[a]-0.36	Azzouz et al. (2016b)
Spain (Andalusia), 2015	–	u	7	57	0.016 (LoD)	–	–	0.2[a]-0.73	71	0.016 (LoD)	–	–	2.7[a]-7.4	14	0.015 (LoD)	–	–	0.96[a]-0.96	0	0.016 (LoD)	–	–	–	Azzouz et al. (2016a)
Spain (Andalusia), 2015	–	Total	7	86	0.016 (LoD)	–	–	0.98[a]-3	86	0.016 (LoD)	–	–	5.7[a]-22	29	0.015 (LoD)	–	–	2.3[a]-2.5	29	0.016 (LoD)	–	–	0.81[a]-1.10	Azzouz et al. (2016a)
Spain (Granada)	–	Total	15	67	0.5	–	–	1.8[a]-18.6	13	0.5	–	–	5.3[a]-6.7	13	0.5	–	–	0.7[a]-3.8	0	0.5	–	–	–	Vela-Soria et al. (2016)
Brazil (São Paulo)	–	u	16	94	10	–	–	10.8[a]-39.8	50	10	–	–	11.5[a]-29.6	0	10	–	–	–	19	3	–	–	3[a]-8.1	Souza et al. (2016)
Canada (Ottawa), 2009–2010	2–3 months	Total	56	82	0.1 (LoD)	0.991	0.0672	<LoD-16.325	57	0.1 (LoD)	0.123	0.0023	<LoD-2.183	66	0.1 (LoD)	0.334	0.0277	<LoD-4.588	0	0.1 (LoD)	–	–	–	Fisher et al. (2017)
Spain (Granada)	–	Total	15	100	0.4	–	–	0.9[a]-21	7	0.3	–	–	0.6[a]-0.6	40	0.4	–	–	0.4[a]-12	0	0.5	–	–	–	Vela-Soria et al. (2018)
Brazil (Sao Paulo)	–	u	16	100	5	–	–	7.5–87.5	100	5	–	–	7.6–18.1	6	–	–	–	<LoQ–8.2	0	5	–	–	–	Grecco et al. (2019)

u (unconjugated parabens).
NA (not analyzed).
[a] Data calculated only with samples > LoQ.
[b] Concentrations extracted from defatted samples.

Canadian mothers sampled 2–3 months after delivery. GMs of paraben levels were below the LoD (0.1 ng/mL) and the AMs ranged from 0.12 (total-EP) to 0.99 (total-MP) ng/mL, BP was not detected. In the present study, both AMs and GMs of total-parabens were higher than those detected by Fisher et al. (2017). As far as we know, the present research is the first large biomonitoring study that quantifies levels of BP in human milk.

The main form (unconjugated or conjugated) in which parabens are present in human milk has not been previously studied in a large biomonitoring study. As mentioned before, only two studies with a low number of samples ($n \leq 7$) determined both unconjugated and total parabens in the same samples (Table 6). Results from Ye et al. (2008) showed that parabens in human milk were mainly present in their unconjugated forms. However, Azzouz et al. (2016a), observed higher DF when determining total-parabens (Table 6) and the ratios [u-paraben]/[total-paraben] were lower than 0.5 (GMs: 0.24, 0.22 and 0.38 for MP, EP and PP, respectively). In the present study we observed ratios [u-paraben]/[total-paraben] with GMs ranging from 0.31 to 0.50, except for BP (GM ratio = 0.76). Therefore, the present study supports the data showed by Azzouz et al. (2016a) indicating that conjugated metabolites were the main form of parabens in human milk except for BP.

Likewise, we studied the relationship between the levels of the 4 parabens included in the present study (Tables SD7 and SD8). The high correlation found between all 4 parabens suggests the combined use of parabens as preservatives.

In the literature (Table 6), parabens were determined in human milk sampled between 4 days and 4 months after delivery. However, most of the studies did not indicate the time after delivery sampling period. To our knowledge, the variation of paraben levelsin human milk during lactation has not been studied previously. In the present study, we assessed changes in paraben human milk levels comparing samples collected 2, 5 and 8 weeks after delivery and we found significant reductions of unconjugated MP, EP and BP levels over time (Table 3). For persistent chemicals such as dioxins or PCBs, a decrease in analyte concentrations over the duration of lactation have been described (depuration rates) (LaKind et al., 2001). The depuration rates could be influenced, among other factors, by initial chemical concentrations, age of the mother, and parity; however this depletion is poorly understood (LaKind et al., 2004). Nevertheless, despite the presence of parabens in adipose tissue (Artacho-Cordon et al., 2018), parabens are considered as non-persistent chemicals that show rapid urinary excretion after consumption (Moos et al., 2016). Therefore, variations in paraben levels could be explained by a lower paraben exposure throughout lactation (e.g. use of less cosmetics).

As far as we know, only two studies have previously analyzed the influence of potential determinants on the levels of parabens in human milk. Schlumpf et al. (2010), observed that concentration of EP in human milk increased significantly with the mother's age. However, they did not observe this correlation for MP or PP. No correlations of paraben levels with diet were observed. Fisher et al. (2017) detected a significant increase on human milk PP levels in mothers who had recently used eye makeup, makeup and skin cleaners. Nevertheless, for MP no significant differences were found and BP and EP determinants were not studied owing to their low detection frequencies. In the present study (Table 4), total levels of some parabens were significantly higher in mothers which used some cosmetic products, such as sunscreen and perfume, on a daily basis, than in mothers which never used those cosmetics. Paraben levels in milk are positively correlated with use of cosmetics since they are used as preservatives in personal care products (Bledzka et al., 2014).

In this study, other predictors such as smoking status, BMI and protein levels in human milk were correlated with paraben levels in milk. The negative association between EP levels and smoker status has not been stated previously. However, the number of participants who indicated that were smokers was low ($n = 9$), and therefore, this association could be inconsistent. The inverse association of paraben levels with BMI has not been shown in previous works, however, Bethea et al. (2019) found urinary methyl paraben levels inversely correlated with BMI in black women from Detroit (USA) recruited from 2010 to 2012. Regarding protein levels in milk, a significantly positive correlation between paraben levels (MP and PP) and protein levels was found in the present study. To our knowledge, this association has not been previously stated. Paraben's binding to proteins could explain this association. However, we have not found evidence of this fact in the literature.

In this study (Table SD14) higher levels of parabens in breast milk were associated with lower height and weight at birth and with a minor gestational age. To our knowledge, there are no previous studies in the literature that focus on the relationship between paraben milk levels and gestational age and newborns anthropometric parameters. Geer et al. (2017) studied relationships between paraben levels in maternal biofluids (urine and cord blood plasma) collected during pregnancy and anthropometric measures and gestational age of newborns from New York. Significantly negative associations between BP levels and gestational age and birth weight were found and BP levels were positively correlated with preterm birth. Regarding PP, this compound was negatively associated with body length at birth. Similar associations were found in the present study. However, paraben levels during pregnancy were not studied here, therefore, we cannot conclude that maternal paraben exposure has effects on the newborn's anthropometric measures or gestational age.

According to EFSA (2004), the ADI for the sum of MP and EP is 0–10,000 μg/kg bw-day. The newborn's paraben EDIs in the present study were several orders of magnitude lower than the ADI (see Table 5). A similar low risk was described by Schlumpf et al. (2010), who calculated the EDI AM (using only samples > LoQ) for u-MP, u-EP and u-PP (0.42, 0.22 and 0.28 μg/kg bw-day, respectively). Regarding PP and BP, the EFSA has not established an ADI since a NOAEL value has not been determined. PP and BP Non observed effect levels (NOELs) are 6500 μg/kg bw-day and 700 μg/kg bw-day (Boberg et al., 2010), respectively. The EDI values for PP and BP obtained in the present study (see Table 5) were several orders of magnitude lower than the NOELs/UF (an "uncertainty factor, UF" of 100 and is applied to guarantee a conservative outcome due to interspecies and intraspecies variability).

5. Conclusions

In the present study we showed the feasibility of using human biomonitoring to evaluate the paraben exposure of breastfed infants. The concentrations of parabens were in the same range as those detected in preceding studies; although most of the previous works studied very few human milk samples.

The levels of parabens indicate that the exposure of newborns to MP and EP through human milk was several orders of magnitude lower than the ADI of 10,000 μg/kg bw-day established by the EFSA for the sum of both compounds.

The low ratios [u-paraben]/[total-paraben] evidence the high presence of conjugated parabens in human milk. Additionally, the study showed an association between paraben levels in human milk and the frequency of use of some cosmetic products and protein levels in human milk mainly.

5.1. Study limitations

Owing to the limited milk volume, only unconjugated parabens were determined in the samples collected in weeks 5 and 8 after delivery. Consequently, the evolution of paraben content throughout lactation could not be estimated using total paraben levels. Furthermore, the mothers who provided samples were recruited in just one hospital and the educational level of the present study participants was skewed toward university studies. Therefore, the conclusions cannot be extrapolated to the whole Valencian population.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2019.124829.

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5.6. Capítulo 6: Evaluación del riesgo de la exposición a ftalatos en mujeres lactantes usando biomonitorización humana

En el presente capítulo, se estudiaron los niveles de 14 metabolitos de ftalatos en orina de 104 madres del proyecto Bettermilk. Las orinas fueron recogidas entre las semana 2 y la 8 después del parto. Para llevar a cabo los análisis se utilizó la metodología analítica descrita en la **Tabla 10**. Las condiciones de ionización, los parámetros espectrométricos, los LoQs y los resultados de la validación de la metodología se muestan en las **Tablas SI-4-6. Cap. 6**. Posteriormente se estudiaron los determinantes de las concentraciones de ftalatos en orina y se estimó la evaluación del riesgo en madres por exposición interna a ftalatos.

5.6.1. Resultados y discusión

5.6.1.1. Concentraciones de metabolitos de ftalatos en orina

En la **Tabla 25** se muestran las concentraciones de los metabolitos de ftalatos estudiados en orina. De los 14 metabolitos estudiados, 9 presentaron DFs > 80%, siendo el MEP y el MECPP los metabolitos que presentaron las mayores DFs (100%). MCPP se detectó tan solo en 5 muestras y MiNP, MCHP, MMP y MOP no se detectaron en ninguna. Los niveles de metabolitos oscilaron entre <LoQ y 1291 ng/mL, siendo el MEP el metabolito que presentó mayores niveles (GM = 34,9 ng/mL).

En cuanto a los diésteres, el DEHP fue calculado como la suma de cinco (MEHP, MECPP, MEHHP, MEOHP, and 2cx-MMHP) o dos (MEHHP and MEOHP) metabolitos. Considerando el DEHP como la suma de cinco metabolitos, el DEHP estuvo presente en todas las muestras con niveles que oscilaban entre 5,67 y 1978,9 ng/mL. La **Figura 27** muestra los histogramas del logaritmo de las concentraciones de metabolitos de ftalatos en orina.

En estudios previos se han estudiado los niveles de metabolitos de ftalatos en orinas de mujeres lactantes en Carolina del Norte (EEUU) **(Hines et al., 2009)**, Lund (Suecia) **(Högberg et al., 2008)** y Shanghai (China) **(Dong et al., 2019)**. De manera similar a los resultados del presente capítulo, las DF de la mayoría de los metabolitos de ftalatos estudiados fueron mayores del 80%. Con respecto a las concentraciones, los niveles de los metabolitos de DEHP en estudios anteriores (medianas 3-27ng/mL) fueron similares o superiores al presente estudio (medianas 3-14 ng/mL). En cuanto a los metabolitos de ftalatos de cadena corta, los niveles de MEP fueron también mayores en previos estudios (medianas 37-74 ng/mL) que en el presente estudio (mediana de 33 ng/mL).

En estudios de niveles de ftalatos en otras poblaciones españolas (mujeres lactantes, madres y niños) (ver **Tabla 26**), las DFs para la mayoría de los metabolitos de ftalatos

fueron también superiores al 80% en todos los estudios y las concentraciones de metabolitos de ftalatos fueron superiores que en el estudio actual.

La **Tabla SI-9. Cap. 6** muestra el coeficiente de correlación de Spearman entre los niveles de ftalatos en orina. Los niveles de DBP (como MnBP) fueron significativamente correlacionados con todos los ftalatos, y DEHP fue significativamente correlacionado con DBP, DiBP y BzBP.

5.6.1.2. Determinantes de metabolitos de ftalatos

La exposición a ftalatos se estudió usando RRMs. Los RRMs multiple se muestran en la **Tabla 27**. El consumo de zumos envasados en las 72h previas al muestreo fue asociado positivamente con los niveles de DiBP y DEPHT en orina. Además, los nivels de DEPHT se asociaron positivamente con la frecuencia de uso de desodorante y negativamente con la frecuencia con la que realizaban actividad física. Las participantes que practicaban actividad física regularmente (3 o más días a la semana) tenían menores niveles de MEP que las que solo practicaban actividad física ocasionalmente o nunca. Sin embargo, esta diferencia solo resultó significativa (p-valor <0.05) entre las mujeres que practicaban actividad física regularmente y aquellas que lo hacían ocasionalmente.

La migración de ftalatos desde los materiales de envasado a zumos ha sido estudiada previamente. Rastkari et al. **(2018)** estudiaron el efecto de diferentes condiciones de almacenamiento (tiempo, luz solar, temperatura) sobre la migración de ftalatos desde el material al zumo, demostrando la presencia de DEPHT, DEHP y DnBP en dicha matriz.

Por otro lado, la 'Regulation (EC) No 1223/2009 of the European Parliament on cosmetic products' **(Regulation EC, 2009)** prohibió el uso de algunos ftalatos (DnBP, DEHP, y BzBP) en cosméticos. Sin embargo, permite el uso de DEPHT. Además, la 'Food and Drug Administration' detectó niveles de DEPHT en productos de belleza, incluyendo desodorantes **(Hubinger, 2010)**. Por lo tanto, la asociación entre niveles en orina de metabolitos de DEPHT y la frecuencia de uso de desodorantes se puede deber a la presencia de DEPHT en algunos desodorantes.

Tabla 25. Niveles de metabolitos de ftalatos en muestras de orina (n=104).

	MEHP ng/mL (µg/g creatinina)	**MEOHP ng/mL (µg/g creatinina)**	**MECPP ng/mL (µg/g creatinina)**	**MEHHP ng/mL (µg/g creatinina)**	**2cx-MMHP ng/mL (µg/g creatinina)**	**MEP ng/mL (µg/g creatinina)**	**MnBP ng/mL (µg/g creatinina)**	**MiBP ng/mL (µg/g creatinina)**	**MBzP ng/mL (µg/g creatinina)**	**DEHP como la suma de 5 metabolitos [a] (µg/g creatinina)**	**DEHP como la suma de 2 metabolitos [b] (µg/g creatinina)**
Número de muestras	104	104	104	104	104	104	104	104	104	104	104
LoQ (ng/mL)	2	0,5	1	2	2	2	0,5	2	1	-	-
DF(%)	91,3	99,0	100	96,2	80,8	100	99,0	99,0	85,6	100	99,0
Mínimo	<LoQ	<LoQ	1,91 (4,06)	<LoQ	<LoQ	2,44 (2,86)	<LoQ	<LoQ	<LoQ	5,67 (10,06)	2,29 (3,49)
P25	<LoQ	3,52 (4,01)	7,96 (9,57)	4,80 (5,67)	<LoQ	15,35 (16,29)	5,56 (6,91)	7,32 (8,23)	1,20 (1,39)	21,16 (25,49)	8,69 (9,62)
Mediana	3,08 (3,05)	6,13 (6,41)	14,40 (14,27)	8,50 (8,40)	3,39 (3,56)	32,60 (35,65)	12,21 (11,61)	12,49 (12,08)	2,20 (2,27)	35,64 (35,66)	15,33 (15,13)
GM	3,13 (3,18)	6,61 (6,71)	15,52 (15,76)	8,86 (9,00)	3,82 (3,88)	34,90 (35,44)	11,34 (11,52)	13,78 (14,00)	2,19 (2,23)	39,15 (39,76)	15,58 (15,82)
AM	6,54 (5,82)	13,03 (11,51)	30,73 (26,69)	17,00 (15,66)	8,56 (7,57)	68,08 (61,68)	17,47 (15,70)	21,28 (19,42)	3,20 (3,05)	75,86 (67,26)	30,03 (27,17)
P75	6,28 (5,52)	10,75 (9,35)	25,95 (21,63)	13,53 (12,01)	6,30 (5,15)	80,29 (69,14)	20,16 (17,27)	23,26 (22,14)	4,26 (3,32)	61,52 (53,30)	23,60 (20,90)
P95	23,34 (20,06)	36,53 (27,49)	93,82 (69,99)	42,96 (35,50)	17,83 (19,84)	170,06 (195,68)	48,66 (44,40)	80,64 (60,47)	10,25 (7,86)	209,90 (196,85)	76,82 (66,84)
Máximo	82,90 (62,80)	340,60 (258,03)	860,60 (651,97)	469,40 (355,61)	225,40 (170,76)	1291,00 (978,03)	148,80 (113,59)	137,04 (144,25)	16,70 (28,81)	1978,90 (1499,17)	810,00 (613,64)
Desv. estándar	11,64 (9,23)	34,45 (26,89)	86,08 (65,92)	47,23 (39,51)	24,36 (20,27)	137,00 (105,58)	20,87 (15,65)	24,46 (20,62)	3,13 (3,38)	200,74 (158,50)	81,46 (66,10)

LoQs (ng/mL) de otros metabolitos: MCPP (2); MiNP (0,5); MCHP (0,5); MMP (1); MOP (0,5).

MiNP, MCHP, MMP y MOP no se detectaron en ninguna muestra. MCPP La DF (%) de MCPP fue de 4.8 ; Rango de concentraciones de MCPP cuantificadas (ng/mL): 2,2 – 4,9.

[a] Suma de MEHP, MEOHP, MECPP, MEHHP y 2cx-MMHP.

[b] Suma de MEOHP y MEHHP.

Tabla 26. Concentraciones de metabolitos de ftalatos en orina en poblaciones españolas

Biomarcador	Ciudad o región	Periodo de muestreo	Población	nº muestras	LoQ (ng/mL)	DF%	AM ng/mL(µg /g creat)	GM ng/mL (µg/g creat)	Mediana (P50) ng/mL (µg/g creat)	P95 ng/mL (µg/g creat)	Rango ng/mL (µg/g creat)	Referencia
MEHP	Valencia	2015	Madres lactantes (2-8 semanas después del parto)	104	2	91,3	6,54 (5,82)	3,13 (3,18)	3,08 (3,05)	23,34 (20,06)	<LoQ - 82,90 (62,80)	Estudio actual
	Sabadell	2004-2006	Mujeres embarazadas (1er-3er trimestre embarazo)	390	1*	99,5-99,2	-	9,6 (11,3)	-	-	1,3 (1,8)– 202,0 (266,9)	Casas et al., 2016
	Madrid y Toledo	Octubre 2011-Enero 2012	Niños 6-11 años (urbano)	59	0,5	>96	-	5,70 (6,24)	-	19,50 (13,75)	-	Cutanda et al., 2015
			Niños 6-11 años (rural)	60			-	6,86 (7,50)	-	21,95 (19,36)	-	
			Madres (urbano)	59			-	6,94 (6,35)	-	28,30 (15,77)	-	
			Madres (rural)	59			-	7,27 (6,93)	-	23,41 (23,41)	-	
	Asturias, Gipuzkoa, Sabadell y Valencia	2004–2008	Mujeres embarazadas (3er trimestre embarazo)	118	1,2*	84,9	-	-	4,4	-	-	Casas et al., 2011
	Granada	2005-2006	Niños (género masculino) (4 años)	19		100	-	-	6,2	-	-	

Tabla 26. (Continuación).

Biomarcador	Ciudad o región	Periodo de muestreo	Población	nº muestras	LoQ (ng/mL)	DF%	AM ng/mL(µg /g creat)	GM ng/mL (µg/g creat)	Mediana (P50) ng/mL (µg/g creat)	P95 ng/mL (µg/g creat)	Rango ng/mL (µg/g creat)	Referencia
MEOHP	Valencia	2015	Madres lactantes (2-8 semanas después del parto)	104	0,5	99	13,03 (11,51)	6,61 (6,71)	6,13 (6,41)	36,53 (27,49)	<LoQ - 340,60 (258,03)	Estudio actual
	Sabadell	2004-2006	Mujeres embarazadas (1er-3er trimestre embarazo)	390	0,5*	100	-	19,0 (21,7)	-	-	2,3 (4,1) - 342,8 (378,3)	Casas et al., 2016
	Madrid y Toledo	Octubre 2011-Enero 2012	Niños 6-11 años (urbano)	59	0,2	>96	-	19,40 (21,23)	-	47,60 (49,36)	-	Cutanda et al., 2015
			Niños 6-11 años (rural)	60			-	25,34 (27,69)	-	101,30 (75,66)	-	
			Madres (urbano)	59			-	12,95 (11,90)	-	48,40 (24,50)	-	
			Madres (rural)	59			-	14,80 (14,10)	-	65,40 (49,45)	-	
	Asturias, Gipuzkoa, Sabadell y Valencia	2004–2008	Mujeres embarazadas (3er trimestre embarazo)	118	0,7*	100	-	-	15,7	-	-	Casas et al., 2011
	Granada	2005-2006	Niños (género masculino) (4 años)	19		100	-	-	44,6	-	-	
MECPP	Valencia	2015	Madres lactantes (2-8 semanas después del parto)	104	1	100	30,73 (26,69)	15,52 (15,76)	14,40 (14,27)	93,82 (69,99)	1,91 (4,06) - 860,60 (651,97)	Estudio actual
	Sabadell	2004-2006	Mujeres embarazadas (1er-3er trimestre embarazo)	390	1*	100-99,7	-	36,2 (41,4)	-	-	4,6 (7,7)– 476,1 (718,9)	Casas et al 2016
	Asturias, Gipuzkoa, Sabadell y Valencia	2004–2008	Mujeres embarazadas (3er trimestre embarazo)	118	0,6*	100	-	-	32,2	-	-	Casas et al., 2011
	Granada	2005-2006	Niños (género masculino) (4 años)	19		100	-	-	115,0	-	-	

Tabla 26. (Continuación).

Biomarcador	Ciudad o región	Periodo de muestreo	Población	nº muestras	LoQ (ng/mL)	DF%	AM ng/mL(µg /g creat)	GM ng/mL (µg/g creat)	Mediana (P50) ng/mL (µg/g creat)	P95 ng/mL (µg/g creat)	Rango ng/mL (µg/g creat)	Referencia
MEHHP	Valencia	2015	Madres lactantes (2-8 semanas después del parto)	104	2	96,2	17,00 (15,66)	8,86 (9,00)	8,50 (8,40)	42,96 (35,50)	<LoQ - 469,40 (355,61)	Estudio actual
	Sabadell	2004-2006	Mujeres embarazadas (1er-3er trimestre embarazo)	390	0,5*	100	-	25,5 (29,0)	-	-	3,2 (5,3)– 536,0 (503,4)	Casas et al., 2016
	Madrid y Toledo	Octubre 2011-Enero 2012	Niños 6-11 años (urbano)	59	0,2	>96	-	31,80 (34,80)	-	101,90 (94,88)	-	Cutanda et al., 2015
			Niños 6-11 años (rural)	60			-	38,62 (42,21)	-	143,20 (110,94)	-	
			Madres (urbano)	59			-	21,19 (19,48)	-	73,60 (46,01)	-	
			Madres (rural)	59			-	21,70 (20,68)	-	83,70 (60,71)	-	
	Asturias, Gipuzkoa, Sabadell y Valencia	2004–2008	Mujeres embarazadas (3er trimestre embarazo)	118	0,7*	100	-	-	17,3	-	-	Casas et al., 2011
	Granada	2005-2006	Niños (genero masculino) (4 años)	19		100	-	-	57,4	-	-	
2cx-MMHP	Valencia	2015	Madres lactantes (2-8 semanas después del parto)	104	2	80,8	8,56 (7,57)	3,82 (3,88)	3,39 (3,56)	17,83 (19,84)	<LoQ - 225,40 (170,76)	Estudio actual
	Sabadell	2004-2006	Mujeres embarazadas (3er trimestre embarazo)	288	2*	100	(58,3)	(45,9)	-	-	(14,4) - (1086,5)	Agay-Shay et al., 2015

Tabla 26. (Continuación).

Biomarcador	Ciudad o región	Periodo de muestreo	Población	nº muestras	LoQ (ng/mL)	DF%	AM ng/mL(µg /g creat)	GM ng/mL (µg/g creat)	Mediana (P50) ng/mL (µg/g creat)	P95 ng/mL (µg/g creat)	Rango ng/mL (µg/g creat)	Referencia
MEP	Valencia	2015	Madres lactantes (2-8 semanas después del parto)	104	2	100	68,08 (61,68)	34,90 (35,44)	32,60 (35,65)	170,06 (195,68)	2,44 (2,86) - 1291,00 (978,03)	Estudio actual
	Sabadell	2004-2006	Mujeres embarazadas (1er-3er trimestre embarazo)	390	1*	100-99,7	-	335,6 (389,1)	-	-	21,9 (34,0)– 5115,1 (9379,8)	Casas et al., 2016
	Madrid y Toledo	Octubre 2011-Enero 2012	Niños 6-11 años (urbano)	59	0,25	>96	-	138,5 (151,6)	-	727,1 (1705,0)	-	Cutanda et al., 2015
			Niños 6-11 años (rural)	60			-	237,7 (259,8)	-	1127,5 (440,9)	-	
			Madres (urbano)	59			-	143,4 (131,9)	-	1376,1 (851,3)	-	
			Madres (rural)	59			-	181,0 (172,6)	-	1675,2 (2239,6)	-	
	Asturias, Gipuzkoa, Sabadell y Valencia	2004–2008	Mujeres embarazadas (3er trimestre embarazo)	118	0,8*	100	-	-	324		-	Casas et al., 2011
	Granada	2005-2006	Niños (género masculino) (4 años)	19		100	-	-	755,0		-	

Tabla 26. (Continuación).

Biomarcador	Ciudad o región	Periodo de muestreo	Población	nº muestras	LoQ (ng/mL)	DF%	AM ng/mL(µg /g creat)	GM ng/mL (µg/g creat)	Mediana (P50) ng/mL (µg/g creat)	P95 ng/mL (µg/g creat)	Rango ng/mL (µg/g creat)	Referencia
MnBP	Valencia	2015	Madres lactantes (2-8 semanas después del parto)	104	0,5	99	17,47 (15,70)	11,34 (11,52)	12,21 (11,61)	48,66 (44,40)	<LoQ - 148,80 (113,59)	Estudio actual
	Sabadell	2004-2006	Mujeres embarazadas (1er-3er trimestre embarazo)	390	1*	99,2	-	29,0 (32,7)	-	-	3,4 (5,8) – 402,6 (835,7)	Casas et al., 2016
	Madrid y Toledo	Octubre 2011-Enero 2012	Niños 6-11 años (urbano)	59	1	>96	-	42,88 (46,92)	-	162,4 (150,9)	-	Cutanda et al., 2015
			Niños 6-11 años (rural)	60			-	50,55 (55,25)	-	190,3 (215,7)	-	
			Madres (urbano)	59			-	33,76 (31,03)	-	161,5 (78,82)	-	
			Madres (rural)	59			-	31,63 (30,16)	-	178,1 (107,5)	-	
	Asturias, Gipuzkoa, Sabadell y Valencia	2004–2008	Mujeres embarazadas (3er trimestre embarazo)	118	0,6*	100	-	-	27,5	-	-	Casas et al., 2011
	Granada	2005-2006	Niños (género masculino) (4 años)	19		100	-	-	30,2	-	-	

Tabla 26. (Continuación).

Biomarcador	Ciudad o región	Periodo de muestreo	Población	nº muestras	LoQ (ng/mL)	DF%	AM ng/mL(µg /g creat)	GM ng/mL (µg/g creat)	Mediana (P50) ng/mL (µg/g creat)	P95 ng/mL (µg/g creat)	Rango ng/mL (µg/g creat)	Referencia
MiBP	Valencia	2015	Madres lactantes (2-8 semanas después del parto)	104	2	99	21,28 (19,42)	13,78 (14,00)	12,49 (12,08)	80,64 (60,47)	<LoQ - 137,04 (144,25)	Estudio actual
	Sabadell	2004-2006	Mujeres embarazadas (1er-3er trimestre embarazo)	390	0,5*	100	-	28,8 (33,0)	-	-	4,0 (5,1)– 367,6 (334,2)	Casas et al., 2016
	Madrid y Toledo	Octubre 2011-Enero 2012	Niños 6-11 años (urbano)	59	1	>96	-	60,73 (60,73)	-	172,80 (172,8)	-	Cutanda et al., 2015
			Niños 6-11 años (rural)	60			-	62,03 (62,03)	-	289,50 (289,5)	-	
			Madres (urbano)	59			-	40,52 (37,25)	-	149,30 (96,57)	-	
			Madres (rural)	59			-	34,56 (32,94)	-	106,20 (64,28)	-	
	Asturias, Gipuzkoa, Sabadell y Valencia	2004–2008	Mujeres embarazadas (3er trimestre embarazo)	118	0,3*	100	-	-	29,9	-	-	Casas et al., 2011
	Granada	2005-2006	Niños (género masculino) (4 años)	19		100	-	-	41,9	-	-	

Tabla 26. (Continuación).

Biomarcador	Ciudad o región	Periodo de muestreo	Población	nº muestras	LoQ (ng/mL)	DF%	AM ng/mL(µg /g creat)	GM ng/mL (µg/g creat)	Mediana (P50) ng/mL (µg/g creat)	P95 ng/mL (µg/g creat)	Rango ng/mL (µg/g creat)	Referencia
MBzP	Valencia	2015	Madres lactantes (2-8 semanas después del parto)	104	1	85,6	3,20 (3,05)	2,19 (2,23)	2,20 (2,27)	10,25 (7,86)	<LoQ - 16,70 (28,81)	Estudio actual
	Sabadell	2004-2006	Mujeres embarazadas (1er-3er trimestre embarazo)	390	0,5*	99,2	-	11,1 (12,6)	-	-	0,8 (1,5)– 336,4 (405,1)	Casas et al., 2016
	Madrid y Toledo	Octubre 2011-Enero 2012	Niños 6-11 años (urbano)	59	0,2	>96	-	12,99 (14,21)	-	56,50 (47,08)	-	Cutanda et al., 2015
			Niños 6-11 años (rural)	60			-	12,45 (13,60)	-	65,05 (52,46)	-	
			Madres (urbano)	59			-	9,51 (8,75)	-	48,10 (31,21)	-	
			Madres (rural)	59			-	7,66 (7,30)	-	39,40 (29,76)	-	
	Asturias, Gipuzkoa, Sabadell y Valencia	2004–2008	Mujeres embarazadas (3er trimestre embarazo)	118	0,3*	99,2	-	-	10,5	-	-	Casas et al., 2011
	Granada	2005-2006	Niños (género masculino) (4 años)	19		100	-	-	33,0	-	-	
MCPP	Valencia	2015	Madres lactantes (2-8 semanas después del parto)	104	2	4,7	-	-	-	-	<LoQ-4,9 (5,08)	Estudio actual
	Asturias, Gipuzkoa, Sabadell y Valencia	2004–2008	Mujeres embarazadas (3er trimestre embarazo)	118	0,2*	97,5	-	-	1,5	-	-	Casas et al., 2011
	Granada	2005-2006	Niños (género masculino) (4 años)	19		100	-	-	6,1	-	-	

* LoD

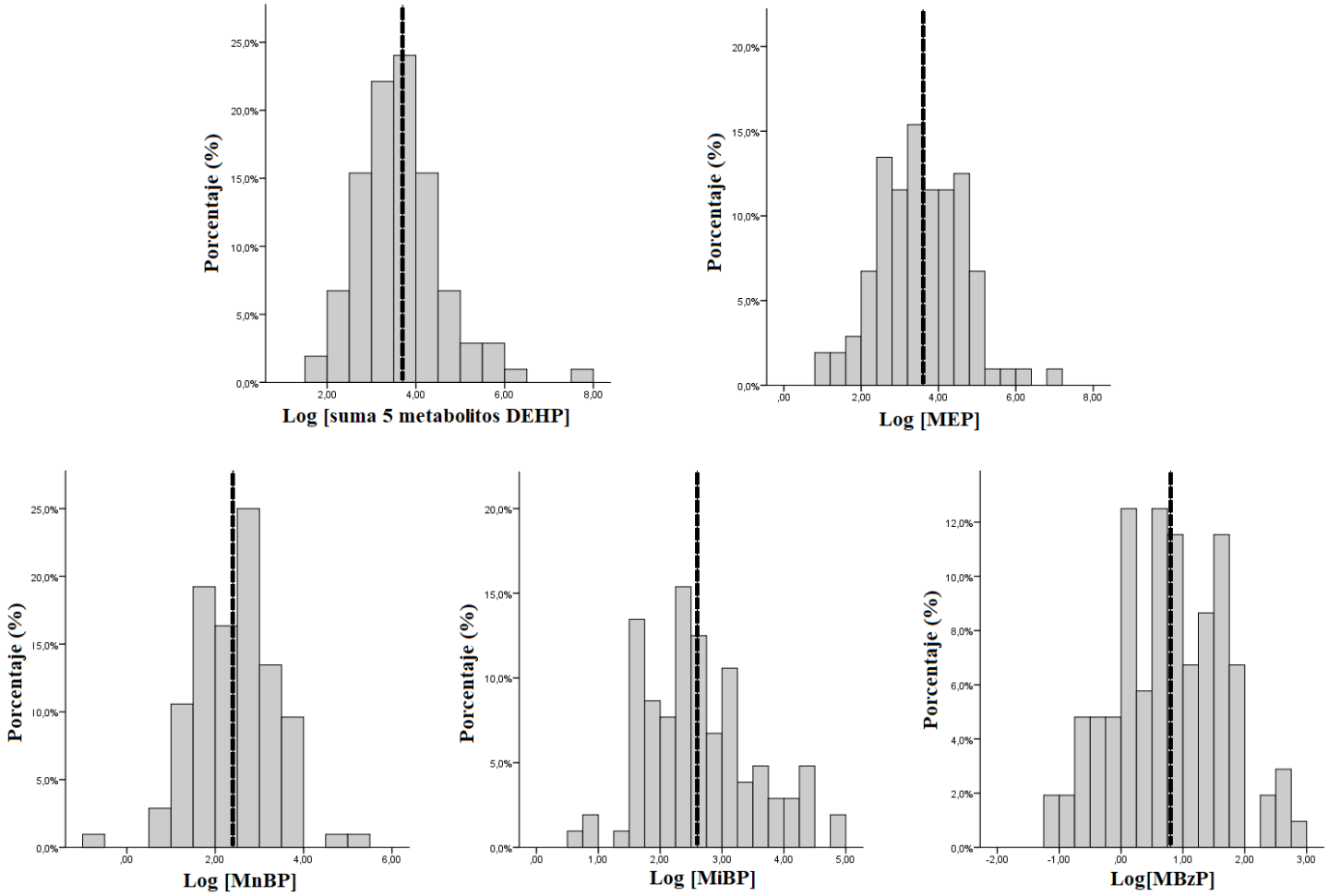

Figura 27. Histogramas del logaritmo de la concentración de metabolitos de ftalatos en orina. Las líneas verticales muestran el log de las GM.

Tabla 27. Resultados de los RRMs múltiples para niveles de ftalatos en orina.

Ftalato (Biomarcador)	Variable	Parámetros estimados (95% IC)	Error estándar	p-valor
BzBP (como MBzP)	'Intercept'	-0,381 (-0,755 - -0,007)	0,191	0,049*
	Creatinina	0,9578 (0,651 - 1,264)	0,156	<0,001*
	Lugar de residencia últimos 10 años: Rural	0,2924 (-0,112 - 0,696)	0,206	0,159
	Desempleada	0,4672 (0,031 - 0,904)	0,223	0,039*
DiBP (como MiBP)	'Intercept'	1,4183 (1,088 - 1,749)	0,169	<0,001*
	Creatinina	0,8829 (0,603 - 1,163)	0,143	<0,001*
	País de nacimiento: Otros	0,2672 (-0,130 - 0,665)	0,203	0,190
	Zumo envasado (nº raciones 72h previas)	0,118 (0,045 - 0,191)	0,037	0,002*
DnBP (como MnBP)	'Intercept'	1,0886 (0,681 – 1,496)	0,208	<0,001*
	Creatinina	1,0546 (0,742 – 1,368)	0,160	<0,001*
	Frecuencia uso perfumes: Diariamente	0,2387 (-0,114 – 0,592)	0,180	0,188
	Frecuencia uso perfumes: Weekly or monthly	0,3394 (-0,025 – 0,704)	0,186	0,071
DEPHT (como MEP)	'Intercept'	1,5407 (0,663 - 2,418)	0,448	<0,001*
	Creatinina	0,7314 (0,301 - 1,162)	0,219	0,001*
	Frecuencia ejercicio físico: 1 or 2 days/week	0,4193 (-0,262 - 1,1006)	0,348	0,231
	Frecuencia ejercicio físico: Ocasionalmente	0,6504 (0,085 - 1,216)	0,289	0,027*
	Frecuencia ejercicio físico: Nunca	0,6288 (-0,056 - 1,314)	0,350	0,076
	Frecuencia uso desodorantes: Diariamente	0,6151 (0,011 - 1,219)	0,308	0,049*
	Zumo envasado (nº raciones 72h previas)	0,1334 (0,026 - 0,241)	0,055	0,017*
DEHP (como la suma de 5 metabolitos)	'Intercept'	2,3032 (1,446 - 3,161)	0,438	<0,001*
	Creatinina	1,1204 (0,875 - 1,366)	0,126	<0,001*
	Índice de masa corporal antes del embarazo (kg/m2)	-0,0004 (-0,034 - 0,033)	0,017	0,980

* P-value < 0.05.

Las RRMs se aplicaron solo a los ftalatos que presentaban una DF(%) >40 en orina.

5.6.1.3. Evaluación del riesgo en madres lactantes

Los resultados de la evaluación del riesgo se muestran en la **Figura 28**. Los HQs se calcularon comparando los niveles de ftalatos en el P95 de la población con el valor guía en BH (BEs o HBM-I). Todos los HQs fueron menores que 1, por lo tanto, no se puede considerar que hubiese un riesgo por exposición a ftalatos en la población estudiada. El DEHP (como la suma de 5 metabolitos) fue el ftalato que presentó un mayor HQ (0,49).

Con respecto al DiNP (medido como MiNP), el BE (derivado del TDI de la EFSA) es de 0,5 ng/mL. Teniendo en cuenta que en nuestro estudio el LoQ del MiNP era de 0,5 ng/mL y que MiNP no fue detectado, se puede concluir que la exposición a DiNP era menor al BE. Sin embargo, para mejorar la evaluación del riesgo del DiNP, se debería reducir el LoQ del MiNP o estudiar otros metabolitos oxidados del DiNP que suelen presentar concentraciones mayores en orina **(Hays et al., 2011)**.

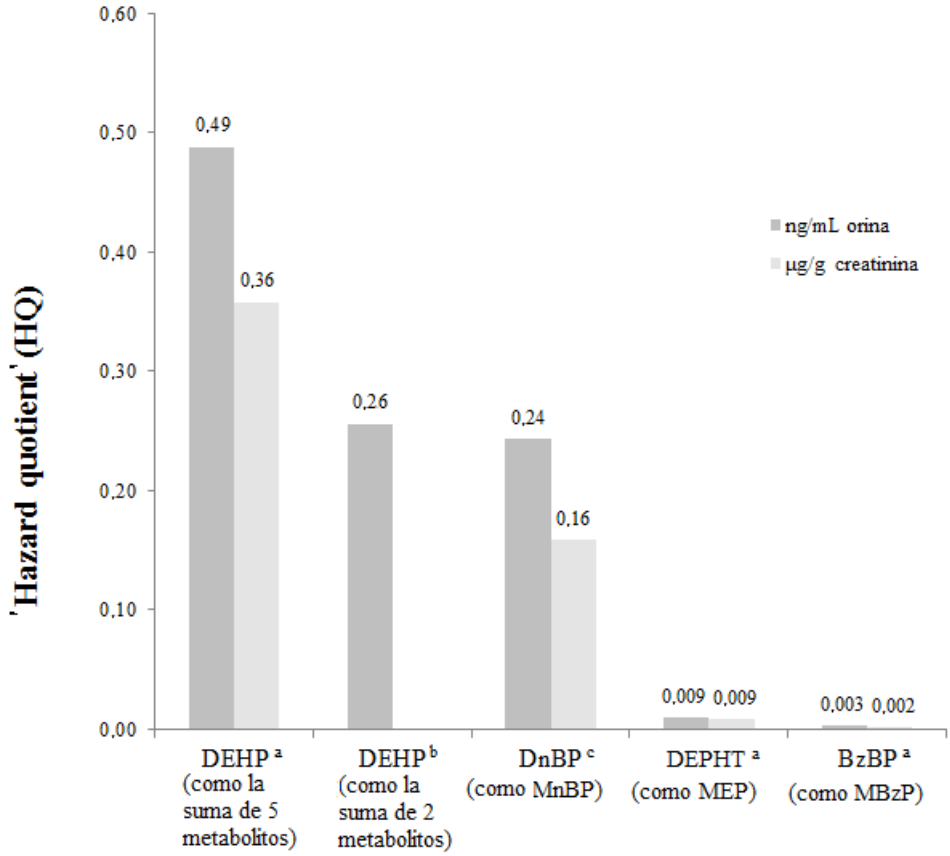

Figura 28. HQs de los niveles de ftalatos en orina. [a]BE derivado de la US EPA, [b]HBM I, [c]BE derivado de EFSA TDI.

5.6.2. Conclusiones

-La exposición a ftalatos en la población en estudio fue menor que en otras poblacionales descritas de la literatura.

-Además, los niveles detectados se encontraban por debajo de los valores guía de BH para ftalatos. Por lo tanto, se considera que no existió un riesgo asociado a la exposción a ftalatos en la población estudiada.

-Para mejorar la evaluación del riesgo a DINP, se recomienda estudiar metabolitos oxidados del MINP.

5.6.3. Artículo 6: Risk assessment of exposure to phthalates in breastfeeding women using human biomonitoring

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Risk assessment of exposure to phthalates in breastfeeding women using human biomonitoring

Pablo Dualde [a, b, c], Nuria Leon [a], Olga Pardo [b], Clara Coscollà [b], Máximo Vento [d], Agustín Pastor [c], Vicent Yusà [a, b, c, *], on behalf of BETTERMILK project[1]

[a] Public Health Laboratory of Valencia, 21, Avenida Catalunya, 46020, Valencia, Spain
[b] Foundation for the Promotion of Health and Biomedical Research in the Valencian Region, FISABIO-Public Health, 21, Avenida Catalunya, 46020, Valencia, Spain
[c] Analytical Chemistry Department, University of Valencia, Edifici Jeroni Muñoz, Dr. Moliner 50, 46100, Burjassot, Spain
[d] Neonatal Division at the University and Polytechnic Hospital La Fe, Avenida Fernando Abril Martorell, 106, 46026, Valencia, Spain

HIGHLIGHTS

- Phthalate metabolites levels were determined in urine of 104 Spanish mothers.
- metabolites showed detection frequencies >80%.
- Concentrations ranged from <LoQ to 1291 ng/mL.
- Some phthalates were associated with packaged juice consumption and use of deodorants.
- Risk associated to the phthalate exposure in the present study seems not a concern.

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ABSTRACT

In this study, we assessed the presence of 14 phthalate metabolites in the urine of 104 lactating mothers from Valencia (Spain) who took part in the human biomonitoring project BETTERMILK. Nine of the metabolites studied showed detection frequencies >80%, whereas the rest of the metabolites presented low detection frequencies (<5%). The concentrations ranged from <LoQ to 1291 ng/mL with monoethyl phthalate showing the highest concentration, with a geometric mean of 34.90 ng/mL. In general, the phthalate metabolite levels quantified in the present study were lower than the urinary levels found in previous studies that involved lactating mothers. The consumption of packaged juices and the frequency of deodorant usage were predictors of some phthalate metabolite levels in urine. In order to put the biomonitoring data in a risk assessment context, guide values for the different phthalates were used and the respective hazard quotients were calculated, which ranged from 0.0036 (benzylbutyl phthalate) to 0.49 (di-2-ethylhexyl phthalate) at the 95th percentile level. Consequently, no risk was appreciated in the studied population.

1. Introduction

Phthalates are high production volume chemicals used as plasticizers in a wide range of applications (ECHA, 2019). Their properties and uses depend on their alkyl chains. Long-branched phthalates like di-2-ethylhexyl phthalate (DEHP) and di-isononyl phthalate (DiNP) are used mainly in polyvinyl chloride (PVC) applications such as food contact materials, flooring, clothing, or toys. On the other hand, short-chain phthalates such as dimethyl phthalate (DMP), diethyl phthalate (DEP), benzylbutyl phthalate (BzBP), di-n-butyl phthalate (DnBP) and di-isobutyl phthalate (DiBP) are also employed in personal care products, paints or enteric-coated tablets (Wittassek et al., 2011). In the European Union, the use of phthalates is regulated in materials and articles in contact with food (Commission Directive, 2007), toys and childcare

* Corresponding author. Foundation for the Promotion of Health and Biomedical Research in the Valencian Region, FISABIO-Public Health, 21, Avenida Catalunya, 46020, Valencia, Spain.
E-mail address: yusa_vic@gva.es (V. Yusà).
[1] BETTERMILK: Gormaz M.; Kuligowski J.; Cernada, M.; Sánchez A.; Torres E.; Correcher P.; Núñez A.; Parra A. and Ramón A.

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articles which can be placed in the mouth (Regulation EC, 2006), personal care products (Regulation EC, 2009), medicines (EMA, 2015), and medical devices (Directive, 2007).

Since phthalates are not covalently bound, they are frequently released into the environment by leaching and migration from products into air, food, water, and dust. As a result, the general population is continuously exposed to phthalates through ingestion, inhalation, or dermal exposure (Weiss et al., 2018). After entering the human body, phthalates undergo metabolism. During the first metabolic step, phthalate monoesters are formed by hydrolysis. Moreover, phase I biotransformations such as oxidation and hydroxylation of the formed monoesters are common, primarily in long-branched phthalates such as DEHP. During phase II biotransformation, the metabolites can be conjugated with glucuronide. A high percentage of the absorbed dose is excreted in urine during the first 24h as free or conjugated metabolites (Frederiksen et al., 2007; Wittassek et al., 2011). Some of the most common urinary phthalate metabolites are shown in Table 1. Different studies have evaluated phthalate toxicity and have concluded that they have toxic effects on reproduction and development in studies with animals, and they are suspected endocrine disruptors in humans (Heudorf et al., 2007).

Table 1
Most common used phthalates and their metabolites (CDC, 2009; Frederiksen et al., 2007).

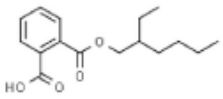
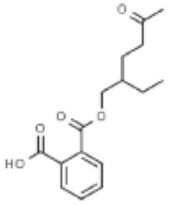
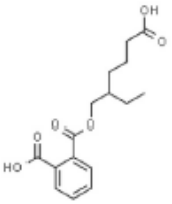
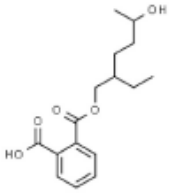
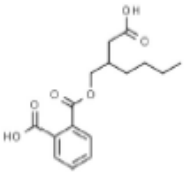
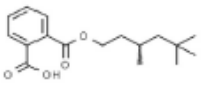
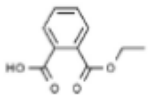
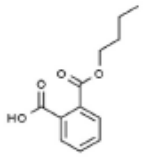
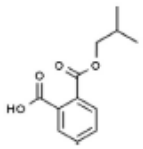
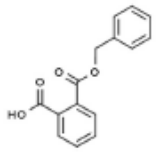
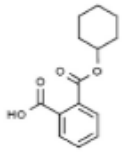
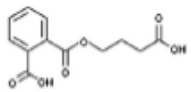
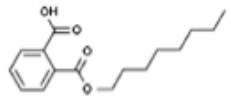
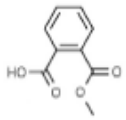
Phthalate (Acronym)	Metabolite (Acronym)	Structure	LoQ present study (ng/mL)
Di-2-ethylhexyl Phthalate (DEHP)	Mono-2-ethylhexyl phthalate (MEHP)		2
	Mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP)		0.5
	Mono-(2-ethyl-5-carboxypentyl) phthalate (MECPP)		1
	Mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP)		2
	Mono[2-(carboxymethyl)hexyl] phthalate (2cx-MMHP)		2
Di-isononyl Phthalate (DiNP)	Mono-isononyl phthalate (MiNP)		0.5

Table 1 (*continued*)

Phthalate (Acronym)	Metabolite (Acronym)	Structure	LoQ present study (ng/mL)
Diethyl Phthalate (DEP)	Mono-ethyl phthalate (MEP)		2
Di-n-butyl phthalate (DBP)	Mono-n-butyl phthalate (MnBP)		0.5
Di-isobutyl phthalate (DiBP)	Mono-isobutyl phthalate (MiBP)		2
Benzylbutyl Phthalate (BzBP)	Mono-benzyl phthalate (MBzP)		1
Dicyclohexyl Phthalate (DCP)	Mono-cyclohexyl phthalate (MCHP)		0.5
Di-(n-octyl) Phthalate(DOP)	Mono-(3-carboxypropyl) phthalate (MCPP)		2
	Mono-n-octyl phthalate (MOP)		0.5
Dimethyl Phthalate (DMP)	Mono-methyl phthalate (MMP)		1

In order to assess the global exposure of the population to phthalates, human biomonitoring (HBM) is the most appropriate approach. Phthalates can be determined in various biological fluids (urine, blood, human milk, etc.). However, in several studies, urine was the matrix selected to study phthalate exposure (Wittassek et al., 2011). Urine possesses some advantages in comparison with other biomonitoring matrices since it is non-invasive and can be easily collected. In general, phthalate metabolites in urine reflect recent exposure and are detected at trace levels. As an illustration, the most common phthalate metabolites had geometric mean concentrations ranging from 1.36 ng/mL (MEHP) to 35.7 ng/mL (MEP) in the United States (2013–2014), as described in the Fourth National Report (CDC, 2019).

Different approaches have been used to collect urine samples in biomonitoring studies for short biological half-life chemicals (24-h, spot and first-morning urine samples). While in some studies 24-h urine samples were preferred for biomonitoring of chemicals with short half-life (Husoy et al., 2019), the most relevant international surveys such as the Canadian Health Measurement Survey (Health Canada, 2010), the National Health and Nutrition Examination Survey (NHANES) (CDC, 2009), the German Environmental Survey of Children and Adolescents (GerES) (Schwedler et al., 2020) or the

DEMOCOPHES Human Biomonitoring Study (Cullen et al., 2017; Schwedler et al., 2017) collects spot or first-morning urine samples Frederiksen et al. (2013) compared the temporal variability in phthalate metabolite excretion on spot, first morning and 24-urine samples, concluding that the slightly better variability obtained with 24-h urine does not justify the logistic efforts needed for using it. Likewise, Barr et al. (2005), indicated that 24-h sample collection can cause incomplete or improper collection. Therefore, in the present study, first-morning urine samples were collected to assess the exposure to phthalates.

For using HBM in a risk assessment context, several guidance values such as the German HBM-I and HBM-II (**Schulz et al., 2012**), or the biomonitoring equivalents (BE) (Aylward et al., 2009a, 2009b; Hays et al., 2011) have been proposed. This guidance values can be useful as screening values for comparison with internal exposure data for risk assessment or risk management but are not meaningful for medical diagnostic (Angerer et al., 2011).

The objectives of the present paper are: i) to determine the urinary concentrations of phthalate metabolites in a study group of lactating mothers from Valencia (Spain), ii) to study the socio-deomographic, dietetic and other factors which influence urinary phthalate concentrations, and iii) to estimate the risk assessment of the study population to phthalates.

2. Materials and methods

2.1. Study design, population, and sample collection

2.1.1. Study area and population

During 2015, lactating mothers (n = 120) from Valencia (Spain) were recruited into the BETTERMILK project. This project has been previously described in detail in Yusa et al. (2017).

2.1.2. Samples and data collection

Between June and November 2015, the mothers provided a first-morning urine sample collected 2–8 weeks post-partum, and 107 urine samples were analyzed for phthalate metabolites. Samples were aliquoted and preserved at −80 °C in a glass container in the IBSP-CV (Investigación Biomédica y en Salud Pública de la Comunitat Valenciana) biobank.

As described in Yusa et al. (2017), sociodemographic and diet information of the participants was obtained. Furthermore, a questionnaire regarding personal care product use was employed. Table 2, **SD1** and **SD2** show the results of the questionnaires mentioned.

The IBSP-CV BioBank (PT13/0010/0064) was responsible for managing the samples and the participants' information. The study was approved by the ethical and scientific committees as detailed in Yusa et al. (2017). The study and sampling protocol were approved by the Biomedical Scientific Ethics Committee of the University and Polytechnic Hospital La Fe (Valencia) and the Scientific Ethics Committee of the Valencian Research Centre for Public Health (FISABIO) of the Valencian Government (Dirección General de Salud Pública, DGSP). All participants signed an Informed Consent approved by the Ethic Committees.

2.1.3. Mediterranean diet quality index (Med-DQI)

To study the correlation between the adherence to the Mediterranean diet of the mothers and the exposure to phthalates, the Med-DQI was calculated to classify the mothers according to their adherence to this diet. Subsequently, Med-DQI was compared with the levels of phthalate metabolites in urine through regression models as described in Section 2.3. Calculation of Med-DQI was based on Gerber (2006). Briefly, the percentage of saturated fatty acids (SFA) energy, cholesterol mg/day, meat g/day, olive oil mL/day, fish g/day, cereals and legumes g/day and fruits and vegetables g/day were calculated. Following the rules described in Table SD3, diet was classified in: Good (1–4 arbitrary units, "a. u."), Half-good (5–7 a.u.), Half-poor (8–10 a.u.) and Poor (11–14 a.u.).

Table 2
Characteristics of the studied population.

Characteristics	n (%) (N = 104)
Mother	
Number of children	
First time mother	62 (59.6)
2 or more	42 (40.4)
Age (years)	34 (20–45)[a]
Weight before pregnancy (kg)	60 (42–92)[a]
Height (cm)	164 (150–184)[a]
BMI before pregnancy (kg/m^2)	21.8 (17.0–35.0)[a]
Special diet during pregnancy	
Yes	13 (12.7)
No	89 (87.3)
Missing data	2
Country of birth	
Spain	88 (86.3)
Foreign	14 (13.7)
Missing data	2
Place of residence	
Urban	77 (82.8)
Rural	16 (17.2)
Missing data	11
Education level	
Only primary school	11 (10.6)
Secondary school	22 (21.2)
University	71 (68.3)
Occupational status	
Employed	90 (86.5)
Unemployed	14 (13.5)
Time worked outside the home (years)	10 (0–28)[a]
Physical exercise	
3 or more days/week	16 (15.8)
1 or 2 days/week	17 (16.8)
Occasionally	46 (45.5)
Never	22 (21.8)
Missing data	3
Smoker	
Yes	6 (5.8)
Ex-smoker	44 (42.3)
Never	54 (51.9)
Child	
Gestational age (weeks)	40 (35–41)[a]
Sex	
Boy	37 (36.6)
Girl	64 (63.4)
Missing data	3
Weight (g)	3360 (2160–4350)[a]
Height (cm)	51 (46–55)[a]
Cranial perimeter (cm)	34 (33 - 37)[a]

[a] Values expressed as median (minimum – maximum).

2.2. Chemical analysis

2.2.1. Sample treatment

Sample treatment was based on previous studies (Calafat, 2013; Sabaredzovic et al., 2015; Servaes et al., 2013). First, 10 μL of the isotope-labeled internal standard (ILIS) mix solution were mixed with 500 μL of urine in an amber glass tube (15 mL). Subsequently, 160 μL of ultrapure water and 200 μL of $AcNH_4$ buffer 1 M (pH 6.5) were added. In order to hydrolyze the conjugates, enzyme β-glucuronidasefrom *E. coli* K12 was selected in order to avoid the hydrolysis of the phthalate monoesters chain (Dwivedi et al., 2018). 10 μL of enzyme were added, and the samples were incubated for 90 min at 37 °C. Then, the tubes were centrifuged at 4500 rpm for

10 min, and 400 μL of the supernatant were transferred to an Eppendorf tube where the sample was ultracentrifuged (13000 rpm, 10 min, 4 °C). Finally, 200 μL of the supernatant were transferred to the injection vial, and 10 μL were injected in the liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) system. Furthermore, creatinine levels were measured using the Jaffé's reaction to adjust the levels of metabolite phthalates in urine.

2.2.2. UHPLC-MS/MS analysis

Analyte separation was performed using a chromatograph, Dionex™ UltiMate™ 3000 from Thermo Scientific™ (Bremen, Germany) and a C18 column Luna Omega 50 × 2.1 mm (1.6 μm) from Phenomenex Inc. (Torrance, CA, USA). The chromatographic separation details are described in Table SD4. After chromatographic separation, analytes were detected in the TSQ Quantiva triple quadrupole mass spectrometer from Thermo Scientific™ (Bremen, Germany). The ionization conditions and mass transitions are detailed in Tables SD5 and SD6, respectively. Trace Finder™ 3.2. software from Thermo Scientific™ was used for data acquisition and processing.

2.2.3. Validation and quality assurance/quality control

The laboratory follows the ISO/IEC/EN 17025 quality assurance system. In Table SD7, the method validation parameters are shown. For quantification, eight-point matrix-matched calibration curves and ILIS were used (Table SD6), using synthetic urine as a matrix blank.

Furthermore, the linearity of the method was evaluated by calculating the deviation (RSD < 20%) for each point of the calibration curve and the coefficient of determination ($R^2 > 0.98$) of the linear regression equations of matrix-matched calibration curves The accuracy of the method was calculated in terms of the relative recovery in each batch by spiking the pool of blank human milk at three different concentration levels: the limit of quantification (LoQ), and the intermediate and the highest levels of the calibration curves with an acceptance criteria of 75–120%. Regarding precision, the acceptance criteria was RSD ≤25%.

The identification and confirmation requirements followed the SANTE/11813/2017 guidelines (SANTE/11813/, 2017):

a) Detection of two product ions using selected reaction monitoring (SRM) acquisition, one used for quantification and the other for confirmation (for the ILIS, only the quantification ion detection was required)
b) Ion ratio from sample extracts should be within ±30% (relative) of average of calibration standards from same sequence
c) The maximum allowed retention time (RT) difference between the suspicious analyte in the sample and the analyte in the spiked blanks was ±0.1 min.

The LoQ for each compound was established at the lowest validated spiked level to accomplish the performance acceptability criteria in terms of recovery, precision, linearity, and the confirmation criteria described above. The LoQs for the phthalate metabolites are depicted in Tables 1 and 3.

Each set of samples was analyzed according to quality assurance protocols: a matrix-matched calibration curve, procedural blanks, and spiked samples. In Table SD7, the method validation parameters are shown.

Owing to the widespread use of phthalates, samples are prone to external contamination with phthalate diesters during storage, treatment, and analysis. In biomonitoring studies, the determination of phthalate metabolites instead of phthalate diesters can reduce the presence of external contamination. In particular, the oxidized metabolites formed in the human body are not prone to external contamination (Wittassek et al., 2011). However, several procedures were followed to avoid and check the external contamination. For example, urine samples were aliquoted and stored in glass tubes to avoid contact with PVC. Furthermore, the

Table 3
Levels of phthalate metabolites in urine samples (n = 104).

	MEHP ng/mL (μg/g creatinine)	MEOHP ng/mL (μg/g creatinine)	MECPP ng/mL (μg/g creatinine)	MEHHP ng/mL (μg/g creatinine)	2cx-MMHP ng/mL (μg/g creatinine)	MEP ng/mL (μg/g creatinine)	MnBP ng/mL (μg/g creatinine)	MiBP ng/mL (μg/g creatinine)	MBzP ng/mL (μg/g creatinine)	DEHP as the sum of five metabolites [a] (μg/g creatinine)	DEHP as the sum of two metabolites [b] (μg/g creatinine)
Number of samples	104	104	104	104	104	104	104	104	104	104	104
LoQ (ng/mL)	2	0.5	1	2	2	2	0.5	2	1	–	–
DF(%)	91.3	99.0	100	96.2	80.8	100	99.0	99.0	85.6	100	99.0
Minimum	<LoQ	<LoQ	1.91 (4.06)	<LoQ	<LoQ	2.44 (2.86)	<LoQ	<LoQ	<LoQ	5.67 (10.06)	2.29 (3.49)
25th percentile	<LoQ	3.52 (4.01)	7.96 (9.57)	4.80 (5.67)	<LoQ	15.35 (16.29)	5.56 (6.91)	7.32 (8.23)	1.20 (1.39)	21.16 (25.49)	8.69 (9.62)
Median	3.08 (3.05)	6.13 (6.41)	14.40 (14.27)	8.50 (8.40)	3.39 (3.56)	32.60 (35.65)	12.21 (11.61)	12.49 (12.08)	2.20 (2.27)	35.64 (35.66)	15.33 (15.13)
Geometric mean	3.13 (3.18)	6.61 (6.71)	15.52 (15.76)	8.86 (9.00)	3.82 (3.88)	34.90 (35.44)	11.34 (11.52)	13.78 (14.00)	2.19 (2.23)	39.15 (39.76)	15.58 (15.82)
Arithmetic mean	6.54 (5.82)	13.03 (11.51)	30.73 (26.69)	17.00 (15.66)	8.56 (7.57)	68.08 (61.68)	17.47 (15.70)	21.28 (19.42)	3.20 (3.05)	75.86 (67.26)	30.03 (27.17)
75th percentile	6.28 (5.52)	10.75 (9.35)	25.95 (21.63)	13.53 (12.01)	6.30 (5.15)	80.29 (69.14)	20.16 (17.27)	23.26 (22.14)	4.26 (3.32)	61.52 (53.30)	23.60 (20.90)
95th percentile	23.34 (20.06)	36.53 (27.49)	93.82 (69.99)	42.96 (35.50)	17.83 (19.84)	170.06 (195.68)	48.66 (44.40)	80.64 (60.47)	10.25 (7.86)	209.90 (196.85)	76.82 (66.84)
Maximum	82.90 (62.80)	340.60 (258.03)	860.60 (651.97)	469.40 (355.61)	225.40 (170.76)	1291.00 (978.03)	148.80 (113.59)	137.04 (144.25)	16.70 (28.81)	1978.90 (1499.17)	810.00 (613.64)
Standard deviation	11.64 (9.23)	34.45 (26.89)	86.08 (65.92)	47.23 (39.51)	24.36 (20.27)	137.00 (105.58)	20.87 (15.65)	24.46 (20.62)	3.13 (3.38)	200.74 (158.50)	81.46 (66.10)

Other metabolite LoQs (ng/mL): MCPP (2); MiNP (0.5); MCHP (0.5); MMP (1); MOP (0.5).
MiNP, MCHP, MMP and MOP were not detected in any sample. MCPP DF (%) = 4.8; MCPP level range (ng/mL): 2.2–4.9.
[a] Sum of MEHP, MEOHP, MECPP, MEHHP and 2cx-MMHP.
[b] Sum of MEOHP and MEHHP.

external contamination during sample treatment and analysis was assessed by adding a matrix blank and a reagent blank that underwent the same procedures than the samples to each batch. If phthalate metabolites were detected in the blanks, their quantitative ion areas needed to be <30% of the area at the LoQ.

In addition, the laboratory participated in the G-EQUAS intercomparison program, round 62, in 2018, for the available urinary phthalate metabolites (MEHP, MECPP, MEHHP, MEOHP, MBzP, MnBP, and MiBP) at two concentration levels, and in the ICI-EQUAS intercomparison program, round 03, in 2019, for the available urinary phthalate metabolites (MEP, MEHP, MECPP, MEHHP, MEOHP, MBzP, MnBP, MiBP, MCHP, and MOP) at two concentration levels, to guarantee the reliability of the results. The laboratory successfully fulfilled the requirements for all the phthalate metabolites evaluated in both programs.

2.3. Statistical analysis

The statistical analysis was performed using R (version 3.3.1) and IBM SPSS (version 17.0). The statistical analysis of the phthalate metabolite levels was performed for those analytes that presented a detection frequency (DF) > 40%. Assuming a log-normal distribution of the phthalate metabolite levels in urine, left-censored values (concentrations < LoQ) were estimated employing the maximum likelihood estimation (MLE) method described by the European Food Safety Authority (EFSA, 2010) (see Dualde et al., 2019a for more details).

The phthalate metabolite levels were summarized by calculating the minimum, 25th (P25), 50th (P50), 75th (P75) and 95th (P95) percentiles, arithmetic and geometric means, maximum and standard deviation.

The Spearman correlation test was used to assess the correlation between the urinary concentrations of the different phthalate metabolites adjusted for creatinine.

To study the relationship between phthalate metabolite levels and sociodemographic, dietary, and the use of personal care products characteristics, simple and multiple robust linear regression models (RRMs) were employed as described in Dualde et al. (2019b). To achieve the normality of the response variable, phthalate metabolite levels were logarithmically transformed. The RRM was used as an alternative to the ordinary least squares estimation method (OLS) owing to the presence of some outlier values in the phthalate metabolite levels. Multiple RRMs were built following a backward variable selection procedure based on the Bayesian information criterion (BIC) (Konishi and Kitagawa, 2008) and by introducing as dependent variables, those with a p-value <0.20 in the simple RRMs. For the RRMs, phthalate metabolite levels were not creatinine adjusted. As recommended by Barr et al. (2005), creatinine levels were introduced as a separate independent variable in the multiple regression analyses.

2.4. Risk assessment

To perform the risk assessment, the levels of phthalates in urine were compared with the guidance values established in the literature (Table SD8). HBM I is a health-related biological exposure limit. If the concentration of a substance in a biological matrix is below the HBM I threshold, a health risk does not exist. A BE is a toxicological guidance value that can be defined as the concentration of a biomarker of exposure (a chemical or its metabolites) in a human fluid that is equivalent to an exposure guidance value of the chemical, e.g. a reference dose (RfD) (Steckling et al., 2018). The definition of HBM value in the case of phthalates (DEHP) is similar to the definition of BE, and uses TDI as exposure guidance value (Angerer et al., 2011).

DEHP levels, as the sum of two metabolites (MEHHP and MEOHP), were compared with the health-based guidance value HBM I. Furthermore, levels DEHP as the sum of five metabolites (MEHP, MEHHP, MEOHP, MECPP, and 2cx-MMHP), and DEP, BzBP, and DnBP studied as MEP, MBzP, and MnBP, respectively, were compared with BE guidance values. If several BEs for each phthalate existed, the lowest BE was selected as the reference value for each phthalate (conservative scenario): the BEs for DEHP, DEP, and BzBP derived from the RfD from the U.S Environmental Protection Agency (EPA) were selected, and the BE for DnBP derived from the TDI established by EFSA was chosen. The hazard quotient (HQ) was calculated comparing the P95 of the phthalate biomarker levels in the present study with the above-mentioned guidance values:

$$HQ = \frac{P95}{BE \text{ or } HBM\ I}$$

The HQs were used to evaluate the health risks of the population. If the HQ was below 1, a health risk was not expected. However, a health risk derived from the exposure to phthalates could not be discarded if the HQ was above 1.

3. Results

3.1. Urinary levels

As can be seen in Table SD9, three of the 107 urine samples presented creatinine levels not within the normal range of 0.3–3 g/L (Barr et al., 2005) and were excluded from the study. Therefore, phthalate metabolite levels of 104 urine samples were studied (see Table 3). Nine of the 14 phthalate metabolites presented DFs >80%, MEP and MECPP presented the highest DFs (100%). MCPP was quantified in only five samples, and MiNP, MCHP, MMP, and MOP were not detected in any sample. Levels of phthalate metabolites ranged from <LoQ to 1291 ng/mL, MEP showed the highest levels [geometric mean (GM) = 34.9 ng/mL]. Focusing on parent phthalates, DEHP was calculated as the sum of five (MEHP, MECPP, MEHHP, MEOHP, and 2cx-MMHP) or two (MEHHP and MEOHP) metabolites. In the first case, DEHP was present in all samples with levels ranging from 5.67 to 1978.90 ng/mL. Fig. 1 shows the histograms of the phthalate metabolite levels in urine. Likewise, Table SD10 shows the Spearman correlation coefficients between levels of phthalates in urine. DnBP (as MnBP) was significantly correlated with all the phthalates, and DEHP was significantly correlated with DnBP, DiBP, and BzBP.

3.2. Phthalate determinants

Phthalate exposure predictors were studied using simple RRMs (see Tables SD11-SD15) and multiple RRMs (see Table 4). The consumption of packaged juices within 72h previous to sample collection was positively associated with the levels of DiBP (as MiBP) and DEP (as MEP) in urine. DEP levels were also positively associated with the frequency of deodorant usage and negatively associated with the frequency of physical activity. Women who engaged in physical activity regularly (3 or more days a week) presented lower levels of phthalate metabolites than those who only did physical activity occasionally or never. However, this difference was only significant (p-value <0.05) among women who did physical activity regularly and those who did it occasionally. BzBP (as MBzP) levels were significantly higher in unemployed women.

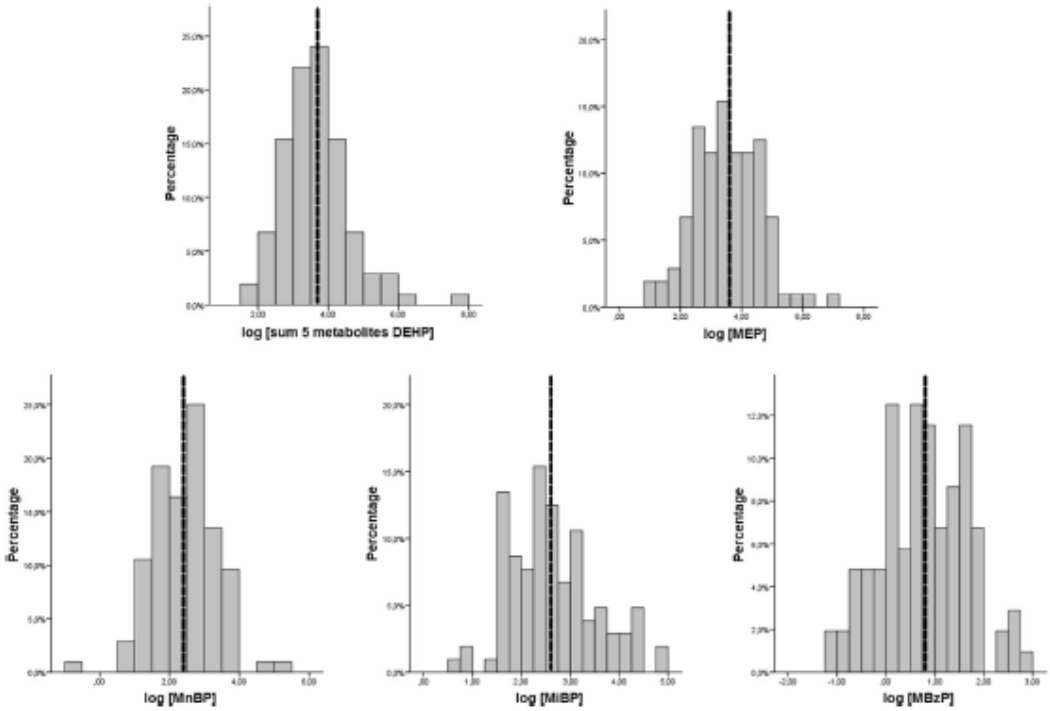


Fig. 1. Histograms of log transformed phthalate metabolites levels. The vertical lines show the log transformed geometric means.

Table 4
Results of the multiple linear robust regression models for phthalate levels in urine.

Analyte	Variable	Estimated parameters (95% CI)	Standar error	P-value
BzBP (as MBzP)	**Intercept**	−0.381 (−0.755–−0.007)	0.191	0.049*
	Creatinine	0.9578 (0.651–1.264)	0.156	<0.001*
	Place of residence last 10 years: Rural	0.2924 (−0.112 - 0.696)	0.206	0.159
	Occupational status: unemployed	0.4672 (0.031–0.904)	0.223	0.039*
DiBP (as MiBP)	**Intercept**	1.4183 (1.088–1.749)	0.169	<0.001*
	Creatinine	0.8829 (0.603–1.163)	0.143	<0.001*
	Country of birth: Other	0.2672 (−0.130 - 0.665)	0.203	0.190
	Packaged juice (nº rations 72h)	0.118 (0.045–0.191)	0.037	0.002*
DnBP (as MnBP)	**Intercept**	1.0886 (0.681–1.496)	0.208	<0.001*
	Creatinine	1.0546 (0.742–1.368)	0.160	<0.001*
	Perfume frequency: Daily	0.2387 (−0.114 – 0.592)	0.180	0.188
	Perfume frequency: Weekly or monthly	0.3394 (−0.025 – 0.704)	0.186	0.071
DEP (as MEP)	**Intercept**	1.5407 (0.663–2.418)	0.448	<0.001*
	Creatinine	0.7314 (0.301–1.162)	0.219	0.001*
	Physical exercise: 1 or 2 days/week	0.4193 (−0.262 - 1.1006)	0.348	0.231
	Physical exercise: Occasionally	0.6504 (0.085–1.216)	0.289	0.027*
	Physical exercise: Never	0.6288 (−0.056 - 1.314)	0.350	0.076
	Deodorants frequency: Daily	0.6151 (0.011–1.219)	0.308	0.049*
	Packaged juice (nº rations 72h)	0.1334 (0.026–0.241)	0.055	0.017*
DEHP (as sum of five metabolites)	**Intercept**	2.3032 (1.446–3.161)	0.438	<0.001*
	Creatinine	1.1204 (0.875–1.366)	0.126	<0.001*
	BMI before pregnancy (kg/m2)	-4e-04 (−0.034 - 0.033)	0.017	0.980

* P-value < 0.05.
Regression models were only applied to phthalate metabolites which presented DF(%) >40 in urine.

3.3. Risk assessment

The results of the risk assessment are shown in Fig. 2. All HQs were lower than 1, i.e. none of the phthalate concentrations at the P95 were higher than the health-based guidance values (BE or HBM). DEHP, as the sum of five metabolites, presented the highest HQ (0.49). The rest of the phthalates were far below their respective guidance values.

4. Discussion

Other studies of phthalate metabolite levels in urine of lactating mothers have been conducted in North Carolina (USA), Lund (Sweden), and Shanghai (China) (see Table 5). Similar to our results,

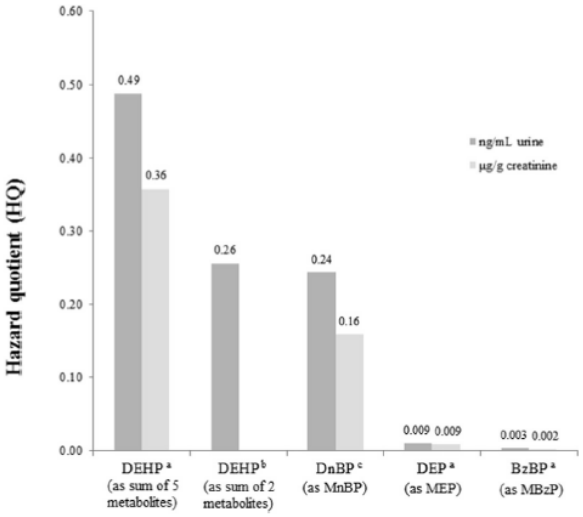


Fig. 2. Phthalate hazard quotients. [a]BE derived from US EPA, [b]HBM I, [c]BE derived from EFSA TDI.

most of the metabolites in these studies presented DF > 80%, showing the widespread exposure to phthalates worldwide. MCCP and MMP presented low DFs in the studies found in the literature and especially in our study (DF < 5%). MiNP, MCHP, and MOP urinary levels were not assessed in previous studies involving lactating women. Regarding phthalate metabolite levels, DEHP metabolites in previous studies showed higher or similar concentrations than in our study (medians ranging 3–27 ng/mL and 3–14 ng/mL, respectively). Focusing on short-chain phthalate metabolites, MEP levels were also higher in previous studies (medians ranging from 35 to 74 ng/mL) than in the present study (median of 33 ng/mL). Although MiBP, MnBP, and MBzP levels were lower in our study in comparison with most of the other studies, MiBP and MnBP showed similar or lower levels in the North Carolina study (medians ranging from 2.1 to 14 ng/mL) than in our study (median of 12 ng/mL). In addition, MBzP showed lower levels in the Shanghai study than in the present study (median of 1.28 and 2.23 µg/g creatinine, respectively).

In other Spanish populations including pregnant women, mothers and children (sampling periods 2004–2012) (Agay-Shay et al., 2015; Casas et al., 2011, 2016; Cutanda et al., 2015) (see Table SD16), the DFs for most of the phthalate metabolites were >80% in all studies (MiNP, MCHP, MMP, and MOP levels were not assessed in previous studies in Spain). Furthermore, the phthalate metabolite levels in the present study were lower than in previous Spanish studies. This could indicate a reduction of the exposure owing to the restrictions in the use of phthalates and the substitution of phthalates by other plasticizers, such as 1,2-cyclohexane dicarboxylic acid diisononyl ester (DINCH) (Silva et al., 2013); however, more data are necessary.

Regarding phthalate level determinants, the consumption of packaged juice was positively associated with levels of DEP (as MEP) and DiBP (as MiNP) in urine. The migration of phthalates from packaging materials to juice has been studied previously. Rastkari et al. (2018) studied the effect of different storage conditions (time, sunlight, and temperature) on the migration of phthalates from high density polyethylene (HDPE) and polyethylene terephthalate (PET) packages to juices. As a result, levels of DEP, DEHP, and DBP were found in juices (DiBP was not studied). Furthermore, Guo et al. (2010) studied the migration of phthalates from PVC bottles to orange juice, detecting levels of DEP and DEHP in the beverage (DiBP was not studied). In the present study, DEP (as MEP) levels were also positively associated with the frequency of deodorant usage. Even though the use of some phthalates (DBP, DEHP, and BzBP) in cosmetic products are prohibited, Regulation (EC) No 1223/2009 of the European Parliament on cosmetic products (Regulation EC, 2009) does not include DEP in the list of prohibited substances. Furthermore, the U.S. Food and Drug Administration detected DEP levels in beauty products, including deodorants (Hubinger, 2010). Previous studies have assessed the influence of personal care product use on the levels of phthalates in urine. Parlett et al. (2013) found an association between deodorant usage and MEP urinary levels in U.S. women. Likewise, Nassan et al. (2017) found a positive association between MEP urinary levels and deodorant usage in U.S. men. Larsson et al. (2014) found an association between the use of sunscreen and MEP urinary levels in Swedish women, however they did not found this association with the use of deodorants.

Furthermore, in our results, levels of DEP (as MEP) were negatively associated with the frequency of physical activity. In contrast, Reeves et al. (2019) found that postmenopausal women from the United States who did moderate physical activity presented lower levels of some phthalate metabolites (MCPP and monocarboxynonyl phthalate). As mentioned by Reeves et al. (2019), the nexus between physical activity and phthalate exposure and/or metabolism is not clear and requires further investigation.

In the present study, BzBP (as MBzP) levels were significantly higher in unemployed women. However, we have not found any explanation for this association, and since only 14 unemployed women were included in the present study, this association could be inconsistent.

Regarding the risk assessment, none of the phthalate metabolites at the P95 levels exceeded their respective guidance values (Fig. 2), consequently there is no concern regarding the risk derived from the exposure to phthalates in the studied population.

In the present study, DEHP and DnBP showed the highest HQs. Therefore, the HQs of these analytes were also calculated (as described in Section 2.5.) based on the data from previous studies of lactating women using the P95 or the P75 (Table 5). In most of the cases, a health risk derived from phthalate exposure should not be expected (HQ < 1). However, in the North Carolina study, DEHP (as the sum of two metabolites) showed a HQ of 1.87 during the first sampling visit (2–7 weeks after delivery). On the contrary, during the second visit (3–4 months after delivery), the HQ was <1 (0.87). Furthermore, DnBP showed a HQ > 1 (1.07) in the Shanghai study using the P75 (creatinine-corrected levels).

DiNP has been associated with anemia, liver, kidney and testicular effects and with anti-androgenic activity in toxicology studies with rats and mice. The reference value (BE) derived from EFSA's TDI is 0.5 ng/mL of DiNP (as MiNP) (Hays et al., 2011). Considering that in our study the LoQ for MiNP was 0.5 ng/mL, and MiNP was not detected, we can conclude that the exposure to DiNP was lower than the BE. However, in order to improve the risk assessment of DiNP, a lower LoQ for MiNP should be achieved. In addition, other DiNP metabolites such as mono(hydroxyl-isononyl) phthalate (OH-MINP), mono(oxoisononyl) phthalate (oxo-MINP), and mono(carboxy-isooctyl) phthalate (carboxy-MINP) usually show higher levels in urine (Hays et al., 2011). Therefore, the study of these metabolites could improve the risk assessment of DiNP.

Further work should also include the biomonitoring of emerging plastizicers as Hexamoll® DINCH (di-(iso-nonyl)-cyclohexane-1,2-dicarboxylate) or DPHP (di-(2-propylheptyl) phthalate which production has increased since they were introduced in the 2000s as substitutes for high molecular weight phthalates (Schwedler et al.,

Table 5
Urinary phthalate levels in populations of lactating mothers.

Analyte	Country (City or region)	Year sampling	Sampling time (after delivery)	Sample size	LoQ (ng/mL)	DF%	AM ng/mL (μg/g creat)	GM ng/mL (μg/g creat)	Median ng/mL (μg/g creat))	P95 ng/mL (μg/g creat)	Range ng/mL (μg/g creat)	Reference
MEHP	Spain (Valencia)	2015	2–8 weeks	104	2	91,3	6.54 (5.82)	3.13 (3.18)	3.08 (3.05)	23.34 (20.06)	<LoQ - 82.90 (62.80)	Present study
	USA (North Carolina)	December 2004–July 2005	(1st visit) 2–7 weeks	33	0,25*	91	–	–	3.0 (7.6) **	86.2 (46.9)**	–	Hines et al., 2009***
			(2nd visit) 3–4 months	30		50	–	–	<LoD (3.6)**	21.1 (58.0)**	–	
	Sweden	2001	2–3 weeks	38	0.98*	100	13 (18)	–	9 (15)	17 (24)****	2.9 (4.6) - 57 (74)	Hogberg et al., 2008
	China (Shangai)	March–May 2014	1–9 months	138	0.6*	100	–	(23.67)	(22.45)	(48.46)****	–	Dong et al. (2019)
MEOHP	Spain (Valencia)	2015	2–8 weeks	104	0,5	99	13.03 (11.51)	6.61 (6.71)	6.13 (6.41)	36.53 (27.49)	<LoQ - 340.60 (258.03)	Present study
	USA (North Carolina)	December 2004–July 2005	(1st visit) 2–7 weeks	33	0,16*	100	–	–	12.0 (17.9)**	224.2 (122.1)**	–	Hines et al., 2009***
			(2nd visit) 3–4 months	30		93	–	–	8.4 (13.5)**	77.8 (45.2)**	–	
	Sweden	2001	2–3 weeks	38	1.1*	97	19 (18)	–	11 (15)	24 (24)****	0.54 (3) - 83 (57)	Hogberg et al., 2008
	China (Shangai)	March–May 2014	1–9 months	138	0.1*	100	–	(10.75)	(9.75)	(18.97)****	–	Dong et al. (2019)
MECPP	Spain (Valencia)	2015	2–8 weeks	104	1	100	30.73 (26.69)	15.52 (15.76)	14.40 (14.27)	93.82 (69.99)	1.91 (4.06) - 860.60 (651.97)	Present study
	USA (North Carolina)	December 2004–July 2005	(1st visit) 2–7 weeks	33	0,4*	100	–	–	27.3 (36.8)**	364.4 (247.9)**	–	Hines et al., 2009***
			(2nd visit) 3–4 months	30		100	–	–	24.8 (32.5)**	236.9 (134.8)**	–	
	China (Shangai)	March–May 2014	1–9 months	138	0.2*	100	–	(17.13)	(15.94)	(28.31)****	–	Dong et al. (2019)
MEHHP	Spain (Valencia)	2015	2–8 weeks	104	2	96,2	17.00 (15.66)	8.86 (9.00)	8.50 (8.40)	42.96 (35.50)	<LoQ - 469.40 (355.61)	Present study
	USA (North Carolina)	December 2004–July 2005	(1st visit) 2–7 weeks	33	0,11*	100	–	–	18.6 (24.5)**	336.2 (183.0)**	–	Hines et al., 2009***
			(2nd visit) 3–4 months	30		93	–	–	10.9 (17.0)**	181.9 (80.7)**	–	
	Sweden	2001	2–3 weeks	38	0.95*	100	25 (25)	–	15 (24)	29 (33)****	1.4 (5.1) - 126 (86)	Hogberg et al., 2008
	China (Shangai)	March–May 2014	1–9 months	138	0.03*	100	–	(21.78)	(19.98)	(38.35)****	–	Dong et al. (2019)
2cx-MMHP	Spain (Valencia)	2015	2–8 weeks	104	2	80,8	8.56 (7.57)	3.82 (3.88)	3.39 (3.56)	17.83 (19.84)	<LoQ - 225.40 (170.76)	Present study
	China (Shangai)	March–May 2014	1–9 months	138	0.5*	100	–	(5.83)	(5.46)	(8.91)****	–	Dong et al. (2019)
MEP	Spain (Valencia)	2015	2–8 weeks	104	2	100	68.08 (61.68)	34.90 (35.44)	32.60 (35.65)	170.06 (195.68)	2.44 (2.86) - 1291.00 (978.03)	Present study
	USA (North Carolina)	December 2004–July 2005	(1st visit) 2–7 weeks	33	0,26*	100	–	–	73.1 (145.0)**	6891.8 (3155.6)**	–	Hines et al., 2009***
			(2nd visit) 3–4 months	30		100	–	–	74.3 (113.0)**	1662.2 (2444.4)**	–	
	Sweden	2001	2–3 weeks	38	1*	97	84 (101)	–	35 (39)	80 (119)****	0.5 (5.5) - 761 (862)	Hogberg et al., 2008
	China (Shangai)	March–May 2014	1–9 months	138	0.2*	100	–	(52.45)	(40.68)	(139.01)****	–	Dong et al. (2019)
MnBP	Spain (Valencia)	2015	2–8 weeks	104	0,5	99	17.47 (15.70)	11.34 (11.52)	12.21 (11.61)	48.66 (44.40)	<LoQ - 148.80 (113.59)	Present study
	USA (North Carolina)	December 2004–July 2005	(1st visit) 2–7 weeks	33	0,45*	97	–	–	14(18.3)**	53.5 (40.8)**	–	Hines et al., 2009***
			(2nd visit) 3–4 months	30		80	–	–	6.6 (18.2)**	155.9 (133.0)**	–	
	Sweden	2001	2–3 weeks	38	1.1*	100	53 (56)	–	46 (50)	68 (68)****	5.1 (18) - 198 (191)	Hogberg et al., 2008
	China (Shangai)	March–May 2014	1–9 months	138	0.04*	100	–	(115.08)	(102.52)	(299.36)****	–	Dong et al. (2019)
MiBP	Spain (Valencia)	2015	2–8 weeks	104	2	99	21.28 (19.42)	13.78 (14.00)	12.49 (12.08)	80.64 (60.47)	<LoQ - 137.04 (144.25)	Present study
	USA (North Carolina)	December 2004–July 2005	(1st visit) 2–7 weeks	33	0,9*	97	–	–	3.8 (5.2)**	12.1 (13.9)**	–	Hines et al., 2009***
			(2nd visit) 3–4 months	30		67	–	–	2.1 (3.4)**	19.0 (12.2)**	–	
	Sweden	2001	2–3 weeks	38	1*	89	21 (21)	–	16 (15)	30 (23)****	0.52 (1.1) - 130 (110)	Hogberg et al., 2008

(continued on next page)

Table 5 (*continued*)

Analyte	Country (City or region)	Year sampling	Sampling time (after delivery)	Sample size	LoQ (ng/mL)	DF%	AM ng/mL (μg/g creat)	GM ng/mL (μg/g creat)	Median ng/mL (μg/g creat))	P95 ng/mL (μg/g creat)	Range ng/mL (μg/g creat)	Reference
	China (Shangai)	March–May 2014	1–9 months	138	0.04*	100	–	(127.49)	(123.77)	(299.01)****	–	Dong et al. (2019)
MBzP	Spain (Valencia)	2015	2–8 weeks	104	1	85,6	3.20 (3.05)	2.19 (2.23)	2.20 (2.27)	10.25 (7.86)	<LoQ - 16.70 (28.81)	Present study
	USA (North Carolina)	December 2004–July	(1st visit) 2–7 weeks	33	0,4*	100	–	–	9.6 (14.4)**	75.6 (70.2)**	–	Hines et al., 2009***
		2005	(2nd visit) 3–4 months	30		100	–	–	11.0 (13.4)**	141.1 (62.6)**	–	
	Sweden	2001	2–3 weeks	38	1*	100	16 (20)	–	13 (17)	20 (27)****	2.2 (4.5) - 38 (63)	Hogberg et al., 2008
	China (Shangai)	March–May 2014	1–9 months	138	0.2*	64,5	–	(1.28)	(0.88)	(3.18)****	–	Dong et al. (2019)
MCPP	Spain (Valencia)	2015	2–8 weeks	104	2	4,7	–	–	–	–	<LoQ-4.9 (5.08)	Present study
	USA (North Carolina)	December 2004–July	(1st visit) 2–7 weeks	33	0,32*	100	–	–	3.2 (3.5)**	11.4 (17.9)**	–	Hines et al., 2009***
		2005	(2nd visit) 3–4 months	30		93	–	–	3.4 (3.7)**	18.2 (10.2)**	–	
	Sweden	2001	2–3 weeks	38	1*	66	1.9 (2.4)	–	1.5 (1.6)	2.5 (2.7)****	0.5 (0.5) - 9.1 (16)	Hogberg et al., 2008
MMP	Spain (Valencia)	2015	2–8 weeks	104	1	0	–	–	–	–	–	Present study
	USA (North Carolina)	December 2004–July	(1st visit) 2–7 weeks	33	1*	18	–	–	<LoD (4.1)**	4.5 (97.1)**	–	Hines et al., 2009***
		2005	(2nd visit) 3–4 months	30		7	–	–	<LoD (4.9)**	4.8 (6.4)**	–	
	Sweden	2001	2–3 weeks	38	1*	53	2.3 (2.5)	–	1.2 (1.9)	2.8 (2.8)****	0.5 (0.3)- 15 (12)	Hogberg et al., 2008
	China (Shangai)	March–May 2014	1–9 months	138	0.02*	100	–	(29.98)	(25.01)	(77.1)****	–	Dong et al. (2019)

Limit of quantification (LoQ); Detection frequency (DF); Arithmetic mean (AM); Geometric mean (GM); 95th percentile (P95).
*Limit of detection (LoD).
** Calculated using data > LoD.
*** Mothers fasted 1.5 h before sampling.
**** 75th percentile.

2019).

5. Conclusions

The exposure to phthalates in lactating mothers from Valencia (Spain) was lower than those found in other comparable populations documented in the literature. Furthermore, the levels detected were below the guidance values, consequently the health risk associated to phthalate exposure does not appear to be a concern.

Additionally, the study showed an association between some phthalate levels in urine and the consumption of packaged fruit juice and the frequency of deodorant usage.

6. Study limitations

The mothers who provided samples were recruited in only one hospital, and the educational level of the study participants was skewed toward university studies. Therefore, the conclusions cannot be extrapolated to the entire population of the Valencian region (Spain). Likewise, the sample size (n = 104), although greater than other recently published studies (Table 5), do not permit extrapolate their findings to the general Spanish population of lactating women.

Declaration of competing interest

None.

CRediT authorship contribution statement

Pablo Dualde: Formal analysis, Validation, Investigation, Data curation, Writing - original draft. **Nuria Leon:** Formal analysis, Validation. **Olga Pardo:** Writing - review & editing. **Clara Coscollà:** Writing - review & editing. **Máximo Vento:** Resources, Supervision, Project administration. **Agustín Pastor:** Supervision. **Vicent Yusà:** Conceptualization, Methodology, Resources, Writing - review & editing, Project administration.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at

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6. CONCLUSIONES

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La biomonitorización permite conocer la exposición de la población a un amplio número de contaminantes procedentes de distintas rutas y fuentes de contaminación de manera integrada, por lo que es útil para conocer la exposición global. Sin embargo, implica algunas limitaciones ya que en muchas ocasiones no se conocen adecuadamente los procesos toxicocinéticos de los contaminantes en el organismo y, además, requiere de métodologías analíticas lo suficientemente sensibles para detectarlos en matrices biológicas a niveles traza.

A lo largo de la presente tesis se han desarrollado diversas metodologías analíticas que permiten conocer la exposición interna de la población desde diferentes enfoques. Mediante el uso de la espectrometría de masas de alta resolución, el análisis retrospectivo de sospechosos y de desconocidos permite ampliar el conocimiento cualitativo de la exposición, es decir, permite identificar nuevos biomarcadores que actualmente no se incluyen en los análisis a larga escala. Sin embargo, estas metodologías son tediosas en lo que se refiere al procesado de los datos, por lo que se precisa de técnicas computacionales más avanzadas y de equipos analíticos con más prestaciones, como los híbridos MS/MS-HRMS, de manera que se creen metodos de procesado más eficientes.

Por otro lado, el desarrollo de técnicas ‘target’ para el análisis multiresiduo mediante LC-MS/MS, permite el estudio de biomarcadores conocidos en profundidad, de manera que se puedan conocer los niveles a los que está expuesto la población. Estos datos pueden ser usados para compararlos con valores guía y llevar a cabo una evaluación del riesgo. En la presente tesis se llevó a cabo una evaluación del riesgo mediante datos de exposición interna para madres lactantes (ftalatos en orina) y datos de exposición externa para los recién nacidos lactantes (bisfenoles y parabenos en orina). En general, se puede concluir que los contaminantes estudiados se detectaron en la mayoría de las muestras analizadas, sin embargo, los niveles cuantificados no suponían un peligro para la población estudiada.

Por último, si se combina la información de niveles de contaminantes en matrices biológicas con encuestas sociodemográficas o de consumo, la biomonitorización permite conocer o al menos dar pistas acerca de cuales son las fuentes y rutas de exposición a contaminantes.

7. BIBLIOGRAFÍA

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ANEXOS

ANEXO CAPÍTULO 1

Información suplementaria de 'Retrospective analysis of pesticide metabolites in urine using liquid chromatography coupled to high-resolution mass spectrometry'

Tabla de información suplementaria

Tabla SI-1. Capítulo 1. Chemical class of pesticide metabolites.

Nº	Compound	CAS number	Elemental composition	Chemical class
1	2,4-dichlorophenoxyacetic acid	94-75-7	C8H6O3Cl2	Phenoxy herbicide
2	2,4,5-Trichlorophenoxyacetic acid	93-76-5	C8H5O3Cl3	Phenoxy herbicide
3	Abamectin metabolite (5-O-demethyl-Avermectin A1a)	113665-89-7	C48H72O14	Insecticide, Acaricide, Nematicide
4	Abamectin metabolite (5-O-demethyl-25-de(1-methylpropyl)-25-(1-methylethyl)-Avermectin A1a)	65195-56-4	C47H70O14	Insecticide, Acaricide, Nematicide
5	Acetamiprid metabolite[N-methyl-(6-chloro-3-pyridyl)methylamine]	120739-62-0	C7H9ClN2	Insecticide, Neonicotinoid
6	Acephate	30560-19-1	C4H10NO3PS	Organophosphate insecticide
7	Acetochlor metabolite [N-(ethoxymethyl)-N-(2-ethyl-6-methylphenyl) acetamide]	34256-82-1	C14H21NO2	Herbicide, chloroacetamide
8	Acetochlor metabolite [Acetic acid, [(ethoxymethyl)(2-ethyl-6-methylphenyl)-amino]oxo]		C14H19NO4	Herbicide, chloroacetamide
9	Acetochlor metabolite [Acetic acid, {2-[(ethoxymethyl)(2-ethyl-6-methylphenyl)-amino]-2-oxoethyl}sulfinyl]		C16H23NO5S	Herbicide, chloroacetamide
10	Acetochlor metabolite [2-[(2-ethyl-6-methylphenyl)amino]-2-oxoethanesulfonic acid; [(2-Ethyl-6-methylphenyl)-carbamoyl]-methanesulfonic acid]		C11H15NO4S	Herbicide, chloroacetamide
11	Acetochlor metabolite [Ethanesulfonic acid, 2-[(2-ethyl-6-methylphenyl)(ethoxymethyl)amino]-2-oxo]		C14H21NO5S	Herbicide, chloroacetamide
12	Alachlor metabolite (Alachlor mercapturate)	116482-92-9	C19H28N2O5S	Herbicide, chloroacetamide
13	Ametryn metabolite[Deethyl ametrin]	4147-57-3	C9H17N5S	Herbicide, triazine
14	Amitraz metabolite(N-2 4-Dimethylphenyl-N'methylformamidine)	33089-74-6	C10H14N2	Herbicide, triazine
15	Atrazine metabolite (6-deisopropyl atrazine)	1007-28-9	C5H8ClN5	Herbicide, triazine
16	Atrazine metabolite (Deethyl atrazine)	6190-65-4	C6H10ClN5	Herbicide, triazine
17	Atrazine metabolite (Atrazine mercapturate)	138722-96-0	C13H22N6O3S	Herbicide, triazine
18	Azinphos-methyl metabolite (phosphorodithioic O,O,S-trimethyl ester)	2953-29-9	C3H9O2PS2	Insecticide, acarecide, organophosphate
19	Azinphos-methyl metabolite (phosphorothioic O,O,S-trimethyl ester)	152-20-5	C3H9O3PS	Insecticide, acarecide, organophosphate
20	Azinphos-methyl metabolite (Benzamide)	55-21-0	C7H7NO	Insecticide, acarecide, organophosphate
21	Azinphos-methyl metabolite (Pyrido[3,4-d] pyrimidin-4-ol)	19178-25-7	C7H5N3O	Insecticide, acarecide, organophosphate
22	Azinphos-methyl metabolite (3-methyl-benzotriazin-4-one)	22305-44-8	C8H7N3O	Insecticide, acarecide, organophosphate
23	Azoxystrobin metabolite [4-(2-cyanophenoxy)-6-hydroxypyrimidine]		C11H7N3O2	Fungicide, strobilurin

Tabla SI-1. Capítulo 1. (Continuación).

Nº	Compound	CAS number	Elemental composition	Chemical class
24	Azoxystrobin metabolite [2-[6-(2-cyanophenoxy)pyrimidin-4yloxy]benzoic acid]		C18H11N3O4	Fungicide, strobilurin
25	Bensulfuron methyl metabolite[methyl 2-({[(4-hydroxy-6-methoxypyrimidin-2yl)carbamoyl]sulfamoyl}methyl)benzoate]		C15H16N4O7S	Herbicide, sulfonyurea
26	Bensulfuron methyl metabolite (4,6-Dimethoxy-2-pyrimidinamine)	36315-01-2	C6H9N3O2	Herbicide, sulfonyurea
27	Bensulfuron methyl metabolite [methyl 2-(sulfamoylmethyl)benzoate]	112941-26-1	C9H11NO4S	Herbicide, sulfonyurea
28	Bensulfuron methyl metabolite [(4,6-dimethoxypyrimidin-2-yl)urea]		C7H10N4O3	Herbicide, sulfonyurea
29	Bensulfuron methyl metabolite [{[(4,6-dimethoxypyrimidin-2-yl)carbamoyl]sulfamoyl}acetic acid]		C9H12N4O7S	Herbicide, sulfonyurea
30	Bensulfuron methyl metabolite [2-(sulfamoylmethyl)benzoic acid]		C8H9NO4S	Herbicide, sulfonyurea
31	Bifenox metabolite (Bifenox acid)	59024-05-4	C13H7Cl2NO5	Herbicide, diphenylether
32	Bifenox metabolite[2-Amino-5-(2,4-dichlorophenoxy)-benzoic acid]	59216-76-1	C13H9Cl2NO3	Herbicide, diphenylether
33	Bitertanol metabolite (1,2,4-triazole)	288-88-0	C2H3N3	Fungicide, triazole
34	Bitertanol metabolite (1H-1,2,4-triazol-1-ylacetic acid)	28711-29-7	C4H5N3O2	Fungicide, triazole
35	Bromopropylate metabolite(4,4-dibromobenzilic acid)	30738-49-9	C14H10Br2O3	Acaricide, propilate
36	Cadusafos metabolite (Methyl-2-butyl sulfone)		C8H18O2S	Insecticide, Nematicide, organophosphate
37	Captan metabolite (THPAM)	4795-29-3	C5H11NO	Fungicide, bactericide, phtalimide
38	Captan metabolite (THPI)	1469-48-3	C8H9NO2	Fungicide, bactericide, phtalimide
39	Carbaryl metabolite (1-naphtol)	90-15-3	C10H8O	Insecticide, plant growth regulator, carbamate
40	Carbaryl metabolite (Methylamine)	200-820-0	CH5N	Insecticide, plant growth regulator, carbamate
41	Carbendazim metabolite(2-aminobenzimidazole)	934-32-7	C7H7N3	Fungicide, benzoimidazole
42	Carbofuran metabolite (3-Keto-Carbofuran)	16709-30-1	C12H13NO4	Insecticide, nematicide, acaricide, carbamate
43	Carbofuran metabolite (Carbofuran-7-phenol)	1563-38-8	C10H12O2	Insecticide, nematicide, acaricide, carbamate
44	Carboxin metabolite (Carboxin sulfoxide)	17757-70-9	C12H13NO3S	Fungicide, oxathiin
45	Chloridazon metabolite(Desphenyl Chloridazon)	6339-19-1	C4H4ClN3O	Herbicide, pyridazinone
46	Chloridazon metabolite (Methyl-desphenyl-chloridazon)	17254-80-7	C5H6ClN3O	Herbicide, pyridazinone
47	Chlorpropham metabolite(HSA-4)		C10H12ClNO6S	Herbicide, plant growth regulator, carbamate
48	Chlorthalonil metabolite [2-amido-3,5,6-trichlo-4-cyanobenzenesulphonic acid]		C8H3O4Cl3N2S	Fungicide, chloronitrile
49	Chlorthalonil metabolite [2,4-bis-amido-3,5,6-trichloro benzenesulfonic acid]		C8H5Cl3N2O5S	Fungicide, chloronitrile

Tabla SI-1. Capítulo 1. (Continuación).

Nº	Compound	CAS number	Elemental composition	Chemical class
50	Chlorthalonil metabolite [4-amido-2,5-dichloro-6-cyano benzene-1,3-disulfonic acid]		C8H4Cl2N2O7S2	Fungicide, chloronitrile
51	Chlorthalonil metabolite [2,5-dichloro-4,6-dicyano-benzene-1,3-disulfonic acid]		C8H2Cl2N2O6S2	Fungicide, chloronitrile
52	Chlorthalonil (3-carbamyl-2,4,5-trichlorobenzoic acid)	142733-37-7	C8H4Cl3NO3	Fungicide, chloronitrile
53	Chlorsulfuron metabolite (2-chlorobenzenesulfonamide)	6961-82-6	C6H6ClNO2S	Herbicide, sulfonyurea
54	Chlorsulfuron metabolite [2-amino-4-methoxy-6-methyl-1,3,5-triazine]	1668-54-8	C5H8N4O	Herbicide, sulfonyurea
55	Chlorsulfuron metabolite [N-[(N-carbamoylcarbamimidoyl)carbamoyl]-2-chlorobenzenesulfonamide]		C5H8N4O	Herbicide, sulfonyurea
56	Chlorotoluron metabolite [3-(3-chloro-p-tolyl)-1-methylurea]	22175-22-0	C10H13ClN2O	Herbicide, urea
57	Chlorotoluron metabolite [2-Chloro-4-(3,3-dimethyl-ureido)-benzoic acid; 1-(3-Chlor-4-carboxyphenyl)-3,3-dimethylharnstoff]	59587-01-8	C10H11ClN2O3	Herbicide, urea
58	Chlorotoluron metabolite [Desmethylchlortoluron]	22175-22-0	C9H11ClN2O	Herbicide, urea
59	Chlorpyrifos metabolite (3,5,6-trichloro-2-pyridinol)	6515-38-4	C5H2Cl3NO	Insecticide, organophosphate
60	Chlorpyrifos, chlorpyrifos-metyol metabolite (3,5,6-trichloro-2-pyridinol)	6515-38-4	C5H2NOCl3	Insecticide, organophosphate
61	Chlorpyrifos metabolite (dimethyl 3,5,6-trichloro-2-pyridylphosphate)	5598-52-7	C7H7NO4Cl3P	Insecticide, organophosphate
62	Chlorpyrifos metabolite (Chlorpyrifos oxon)	5598-15-2	C9H11Cl3NO4P	Insecticide, organophosphate
63	Chlorpyrifos-methyl metabolite(Dimethyl 3,5,6-trichloro-2-pyridinyl phosphate)		C7H7Cl3NO4P	Insecticide, acaricide, organophosphate
64	Chlorpyrifos-methyl metabolite(3,5,6-Trichloro-2(1H)-pyridinone)	6515-38-4	C5H2NOCl3	Insecticide, acaricide, organophosphate
65	Chlorpyrifos-methyl metabolite(Desmethyl chlorpyrifos)		C6H5NO4Cl3P	Insecticide, acaricide, organophosphate
66	Coumaphos metabolite [3-Chloro-7-hydroxy-4-methylcoumarin]	6174-86-3	C10H7ClO3	Antiparasitic, insecticide, acaricide, anthelmintic, ectoparasitide, organophosphate
67	Cyfluthrin metabolite (4-fluoro-3-phenoxy benzoic acid)	77279-89-1	C13H9FO3	Insecticide, pyrethroids
68	Cymoxanil metabolite [3-ethyl-4-(methoxyamino)-2,5-dioxoimidazolidine-4-carbonitrile]	644972-55-4	C7H10N4O3	Fungicide, cyanoacetamide oxime
69	Cymoxanil metabolite[3-ethyl-4-(methoxyamino)-2,5-dioxoimidazolidine-4-carboxamide]	644972-61-2	C7H12N4O4	Fungicide, cyanoacetamide oxime
70	Cyromazine metabolite (Ammelide)	645-93-2	C3H4N4O2	Insecticide, veterinary substance, triazine
71	Cyromazine metabolite (Ammeline)	645-92-1	C3H5N5O	Insecticide, veterinary substance, triazine
72	Cyromazine metabolite (Melamine)	108-78-1	C3H6N6	Insecticide, veterinary substance, triazine

Tabla SI-1. Capítulo 1. (Continuación).

Nº	Compound	CAS number	Elemental composition	Chemical class
73	Cyproconazole metabolite (1,2,4-triazole)	288-88-0	C2H3N3	Fungicide, triazole
74	Cyproconazole metabolite(1H-1,2,4-triazol-1-ylacetic acid)	28711-29-7	C4H5N3O2	Fungicide, triazole
75	Deltamethrin metabolite (Decamethrinic acid)	53179-78-5	C8H10Br2O2	Insecticide, veterinary substance, pyrethroid
76	Deltamethrin metabolite (3-phenoxybenzoic acid)	3739-38-6	C13H10O3	Insecticide, veterinary substance, pyrethroid
77	Deltamethrin metabolite (cis-(2,2-dicbromovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid)	63597-73-9	C8H10O2Br2	Insecticide, veterinary substance, pyrethroid
78	Desmedipham metabolite (EHPC)	7159-96-8	C9H11NO3	Herbicide, carbamate
79	Diazinon metabolite (Pyrimidol)	557-01-7	C4H4N2O	Insecticide, acarecide, repellent, veterinary substance, organophosphate
80	Diazinon metabolite (4-hydroxy-2-isopropyl-6-methylpyrimidine)	2814-20-2	C8H12N2O	Insecticide, acarecide, repellent, veterinary substance, organophosphate
81	Diazinon metabolite(Diethylphosphate)	598-02-7	C4H11O4P	Insecticide, acarecide, repellent, veterinary substance, organophosphate
82	Diazinon metabolite (2-Isopropyl-6-methyl-pyrimidinol)	2814-20-2	C8H12N2O	Insecticide, acarecide, repellent, veterinary substance, organophosphate
83	Diazinon metabolite [2-(1-hydroxy-1-methyl)-ethyl-4-methyl-6-hydroxpyrimidine]	28175-97-5	C8H12N2O2	Insecticide, acarecide, repellent, veterinary substance, organophosphate
84	Dicamba metabolite (3,6-dichloro-2-hydroxy benzoic acid)	3401-80-7	C7H4Cl2O3	Herbicide, benzoic acid
85	Dichlobenil metabolite (2,6-Dichlorobenzamide)	2008-58-4	C7H5Cl2NO	Herbicide, benzonitrile
86	Dichlofluanid metabolite [N,N-Dimethylaminosulfanilide]	4710-17-2	C8H12N2O2S	Fungicide, sulphamide
87	Dichlorvos metabolite (2,2-Dichloroacetic acid)	79-43-6	C2H2Cl2O2	Insecticide, acarecide, organophosphate
88	Dichlorvos metabolite (2,2 dichloro-acetaldehyde)	79-02-7	C2H2Cl2O	Insecticide, acarecide, organophosphate
89	Diclofop-methyl metabolite (diclofop-phenol)	40843-73-0	C12H8Cl2O2	Herbicide, Aryloxyphenoxypropionate
90	Dimethachlor metabolite[N-(2,6-dimethylphenyl)-N-(2-methoxyethyl)oxalamic acid]	1086384-49-7	C13H17NO4	Herbicide, chloroacetmide
91	Dimethachlor metabolite [(2,6-Dimethylphenyl)-(2-methoxyethyl)-carbamoyl]-methanesulfonic acid]		C13H19NO5S	Herbicide, chloroacetmide
92	Dimethachlor metabolite [(2,6-Dimethylphenylcarbamoyl)-methanesulfonic acid]		C10H13NO4S	Herbicide, chloroacetmide

Tabla SI-1. Capítulo 1. (Continuación).

Nº	Compound	CAS number	Elemental composition	Chemical class
93	Dimethachlor metabolite [(2,6-Dimethyl-phenyl)-(2-sulfo-acetyl)-amino]-acetic acid]		C12H15NO6S	Herbicide, chloroacetmide
94	Dimethachlor metabolite [2-[(2-hydroxy-acetyl)-(2-methoxy-ethyl)-amino]3-methyl-benzoic acid]		C13H17NO5	Herbicide, chloroacetmide
95	Dimethachlor metabolite [3-{2-[(2,6-dimethyl-phenyl)-(2-hydroxyacetyl)-amino]-ethylsulfanyl}-2-hydroxy-propionic acid]		C15H21NO5S	Herbicide, chloroacetmide
96	Dimethachlor metabolite [(2,6-dimethylphenyl)-2-methoxyethyl)carbamoyl]methanesulfonic acid sodium salt]		C13H18O5NaNS	Herbicide, chloroacetmide
97	Dimethachlor metabolite [(2,6-dimethylphenylcarbamoyl)-methanesulfonic acid sodium salt]		C10H12NNaO4S	Herbicide, chloroacetmide
98	Dimethenamid metabolite [sodium[(2,4-dimethyl-thiophen-3-yl)-(2-methoxy-1-methyl-ethyl)-carbamoyl]methanesulfonate]		C12H19NO5S2	Herbicide, chloroacetmide
99	Dimethenamid metabolite [N-(2,4-dimethyl-thiophen-3-yl)-N-(2-methoxy-1-methyl-ethyl)-oxalamic acid]	380412-59-9	C12H17NO4S	Herbicide, chloroacetmide
100	Dimethoate		C5H12NO3PS2	Insecticide, acarecide, organophosphate
101	Dimethoate Metabolite(Omethoate)	1113-02-6	C5H12NO4PS	Insecticide, acarecide, organophosphate
102	Dimethomorph metabolite (4-chlorophenyl)(3,4-dimethoxyphenyl)methanone)	116412-84-1	C15H13O3Cl	Fungicide, morpholine
103	Dimethomorph metabolite (4-formylmorpholine)	113009-82-8	C5H9O2N	Fungicide, morpholine
104	Dimoxystrobin metabolite [(E)-0-[(2-hydroxycarbonyl-5-methyl)phenoxymethyl]-2-methoxyimino-Nmethylphenylacetamide]		C19H20N2O5	Fungicide, strobilorium
105	Diuron metabolite [1-(3,4-dichlorophenyl)-3-methylurea]	3567-62-2	C8H8Cl2N2O	Herbicide, phenylurea
106	Diuron metabolite (3,4-dichlorophenyl urea)	2327-02-8	C7H6Cl2N2O	Herbicide, phenylurea
107	Diuron metabolite(3,4-dichloroaniline)	95-76-1	C6H5Cl2N	Herbicide, phenylurea
108	Ethiofencarb metabolite (Ethiofencarb-sulfoxide)	53380-22-6	C11H15NO34S	Insecticide, carbamate
109	Fenamiphos metabolite (fenamiphos-sulfone-phenol)		C8H10O3S	Nematicide, organophosphate
110	Fenhexamid metabolite(Deschlorofenhexamid)	1335041-78-5	C14H19NO2	Fungicide, hydroxyanilide
111	Fenhexamid metabolite(Biphenyl-fenhexamid)		C28H32Cl4N2O4	Fungicide, hydroxyanilide
112	Fenitrothion metabolite(3-methyl-4-nitrophenol)	2581-34-2	C7H7NO3	Insecticide, organophosphate
113	Fenitrothion metabolite (3-methyl-4-nitrophenol)		C7H7NO3	Insecticide, organophosphate
114	Fenitrothion metabolite(Dimethyl-3-methyl-4-nitrophenyl phosphate)	2255-17-6	C8H10NO6P	Insecticide, organophosphate
115	Fenitrothion metabolite(Methyl 3-methyl-4-nitrophenyl hydrogen phosphate)	15930-84-4	C9H12NO6P	Insecticide, organophosphate
116	Fipronil metabolite(Fipronil amide)		C12H6Cl2F6N4O2S	Insecticide, veterinary substance, phenylpyrazole
117	Fipronil metabolite(Fipronil sulphide)	120067-83-6	C12H4Cl2F6N4S	Insecticide, veterinary substance, phenylpyrazole
118	Fipronil metabolite (Fipronil sulfone)	120068-36-2	C12H4Cl2F6N4O2S	Insecticide, veterinary substance, phenylpyrazole

Tabla SI-1. Capítulo 1. (Continuación).

Nº	Compound	CAS number	Elemental composition	Chemical class
119	Fludioxonil meyabolite [3-Carbamoyl-2-cyano-3-(2,2-difluorobenzo[1,3]-dioxo-4-yl)-oxirane-2-carboxylic acid]		C12H6F2N2O6	Fungicide, phenylpyrrole
120	Fludioxonil meyabolite [2,2-difluorobenzo[1,3]dioxole-4-carboxylic acid]	126120-85-2	C8H4F2O4	Fungicide, phenylpyrrole
121	Fludioxonil meyabolite [4-(2,2-difluoro-benzo[1,3]dioxol-4-yl)-2,5-dioxo-2,5-dihydro-1H-pyrrole-3-carbonitrile]		C12H6F2N2O4	Fungicide, phenylpyrrole
122	Flufenacet metabolite [[(4-Fluorophenyl)isopropylcarbamoyl]-methanesulfonic acid]		C11H14FNO4S	Herbicide, oxyacetamide
123	Flufenacet metabolite [1,3,4,-Thiadiazol-2(3h)-one, 5-(trifluoromethyl)-]	84352-75-0	C3HF3N2OS	Herbicide, oxyacetamide
124	Fluopicolide metabolite (3-Sulfo-5-trifluoromethylpyridine-2-carboxylic acid)		C7H4F3NO5S	Fungicide, Benzamide
125	Fluopicolide metabolite (dichlorbenzamide)	4659-54-4	C7H5Cl2NO	Fungicide, Benzamide
126	Fluopicolide metabolite (3-Chloro-5-trifluoromethylpyridine-2-carboxylic acid)	80194-18-9	C7H5ClF3NO2	Fungicide, Benzamide
127	Flupyrsulfuron metabolite (Flupyrsulfuron-methyl)	144740-53-4	C15H14F3N5O7S	Herbicide, Pyrimidinylsulfonylurea
128	Fluquinconazole metabolite [3-(2,4-dichlorophenyl)-6-fluoro-quinazolin-2,4(3H)-dione]	168900-02-5	C14H7Cl2FN2O2	Fungicide, triazole
129	Fluquinconazole metabolite (1,2,4-triazole)	288-88-0	C2H3N3	Fungicide, triazole
130	Flurtamone metabolite (2,2,2-Trifluoroacetic acid)	76-05-1	C2HF3O2	Herbicide, pyridanizone
131	Flusilazole metabolite [(bis(4-fluorophenyl)methyl silanol]	156162-13-9	C13H12SiOF2	Fungicide, triazole
132	Glufosinate metabolite (3-methylphosphinico-propionic acid)	15090-23-0	C4H9O4P	Herbicide, organophosphate
133	Glyphosate metabolite (Aminomethylphosphonic acid)	1066-51-9	CH6NO3P	Herbicide, Phosphonoglycine
134	Haloxyfop metabolite (DE-535 pyridinol)	76041-71-9	C6H3ClNOF3	Herbicide, Aryloxyphenoxypropionate
135	Imazalil metabolite[1-(2,4-dichlorophenyl)2-imidazol-1-ylethanol]	24155-42-8	C11H10Cl2N2O	Fungicide, imidazole
136	Imazalil metabolite (Methyl isothiocyanate)	556-61-6	C2H3NS	Fungicide, imidazole
137	Imazosulfuron (2-Chloroimidazo[1,2-a]pyridin-3-sulfonamid)		C7H6ClN3O2S	Herbicide, sulfonyurea
138	Imidaclorprid metabolite [1-[(6-chloro-3-pyridinyl)methyl]N-nitro-1H-imidazol-2-amine]	115086-54-9	C9H8ClN5O2	Insecticide, neonicotinoid
139	Imidaclorprid metabolite (6-chloronicotinic acid)	5326-23-8	C6H4ClNO2	Insecticide, neonicotinoid
140	Iprodione metabolite [N-(3,5-dichlorophenyl)3-isopropyl-2,4-dioxoimidazoline-1-carboxamide]	63637-89-8	C13H13Cl2N3O3	Fungicide, dicarboximide
141	Iprovalicarb metabolite(p-methyl-phenethylamine)	3261-62-9	C9H13N	Fungicide, carbamate
142	Isoproturon metabolite (p-methyl-phenethylamine)	3261-62-9	C9H13N	Herbicide, urea
143	Isoproturon metabolite (Desmethylisoproturon)	34123-57-4	C11H16N2O	Herbicide, urea
144	Kresoxim methyl metabolite [(E)-methoxyamino(alpha-(o-tolyloxy)-o-tolyl)acetic acid]		C17H17NO4	Fungicide, bactericide, strobilurin
145	Linuron metabolite (3,4-dichloroaniline)	95-76-1	C6H5Cl2N	Herbicide, urea

Tabla SI-1. Capítulo 1. (Continuación).

Nº	Compound	CAS number	Elemental composition	Chemical class
146	Malathion metabolite(ethyl ester-2-hydroxyl-3-thionyl 2-butenoic acid)		C8O5SH12	Insecticide, acarecide, organophosphate
147	Malathion metabolite(Diethyl malate)	7554-12-3	C8H14O5	Insecticide, acarecide, organophosphate
148	Malathion metabolite[(7S,8R,9S)-7,8,9-Trihydroxy-6-oxaspiro[4.5]dec-7-yl]methyl dihydrogen phosphate)		C10H19O8P	Insecticide, acarecide, organophosphate
149	Malathion metabolite(Diethylsuccinate)	123-25-1	C8H14O4	Insecticide, acarecide, organophosphate
150	Malathion metabolite(Dimethyl 2,3-dihydroxy-2,3-dimethylsuccinate)	15309-47-4	C8H14O6	Insecticide, acarecide, organophosphate
151	Malathion metabolite (diethylmaleate)	141-05-9	C8H12O4	Insecticide, acarecide, organophosphate
152	Malathion metabolite (Malathion dicarboxylic acid)	1190-28-9	C6H11O6PS2	Insecticide, acarecide, organophosphate
153	Malathion metabolite (Malathion dicarboxilid acid)		C6H11O6PS2	Insecticide, acarecide, organophosphate
154	Malathion metabolite (Malathion monocarboxylic acid)	35884-76-5	C8H15O6PS2	Insecticide, acarecide, organophosphate
155	Malathion metabolite (Isomalathion)	3344-12-5	C10H19O6PS2	Insecticide, acarecide, organophosphate
156	Malathion metabolite [diethyl (dimethoxy-phosphoryl) succinate]		C10H19O8P	Insecticide, acarecide, organophosphate
157	Malathion metabolite [diethyl (methyl sulphonyl) succinate]		C9O6H16S	Insecticide, acarecide, organophosphate
158	Malathion metabolite [2-hydroxyl-3-thionyl-2-butene-diethylester]		C8H12O5S	Insecticide, acarecide, organophosphate
159	Malathion metabolite (malaoxon)	1634-78-2	C10H19O7PS	Insecticide, acarecide, organophosphate
160	Mancozeb metabolite (Ethylenethiourea)	13966-32-0	C3H6N2S	Fungicide, carbamate
161	Mancozeb metabolite (Ethyleneurea)	120-93-4	C3H6N2O	Fungicide, carbamate
162	Metalaxyl metabolite[N-(2,6-dimethylphenyl)-N-(methoxyacetyl)alanine]	467430-42-8	C14H19NO4	Fungicide, phenylamide
163	Metalaxyl metabolite [(R)-2-[(2,6-dimethyl-phenyl)-methoxyacetyl-amino]-propionic acid]	75596-99-5	C14H19NO4	Fungicide, phenylamide
164	Metalaxyl metabolite [2-[(1-carboxyethyl)-methoxyacetyl-amino]-3-methyl-benzoic acid]	104390-56-9	C14H17NO6	Fungicide, phenylamide
165	Metamitron metabolite [4,5-dihydro-3-methyl-6-phenyl-1,2,4-triazin-5-one]	36993-94-9	C10H9N3O	Herbicide, triazinone
166	Metazachlor metabolite [N-(2,6-dimethylphenyl)-N-(1H-pyrazol-1-ylmethyl)aminocarbonylmethylsulfonic acid]	172960-62-2	C14H17N3O4S	Herbicide, chloroacetamide
167	Metazachlor metabolite [methyl N-(2,6-dimethylphenyl)-N-(1Hpyrazol-1-ylmethyl)aminocarbonylmethylsulfoxide]		C15H19N3O2S	Herbicide, chloroacetamide
168	Metazachlor metabolite [N-[(2-hydroxycarbonyl-6-methyl)phenyl]-N-(1-H-pyrazol-1-ylmethyl)oxalamide]		C14H13N3O5	Herbicide, chloroacetamide
169	Metazachlor metabolite [N-(2,6-dimethylphenyl)-N-(1H-pyrazol-1-ylmethyl)aminocarbonylmethylsulfinyl acid]		C16H19N3O4S	Herbicide, chloroacetamide

Tabla SI-1. Capítulo 1. (Continuación).

Nº	Compound	CAS number	Elemental composition	Chemical class
170	Metazachlor metabolite [N-(2,6-dimethylphenyl)-N-(1H-pyrazol-1-ylmethyl)oxalamide]		C14H15N3O3	Herbicide, chloroacetamide
171	Methamidophos	10265-92-6	C2H8NO2PS	Organophosphate insecticide
172	Methiocarb Metabolite(Methiocarb-sulfone)	2179-25-1	C11H15NO4S	Insecticide, Molluscicide, Bird repellent, Carbamate
173	Methiocarb Metabolite(Methiocarb-sulfoxide)	2635-10-1	C11H15NO3S	Insecticide, Molluscicide, Bird repellent, Carbamate
174	Methyl-Thiophanate metabolite (Carbendazim)	10605-21-7	C9H9N3O2	Fungicide, benzimidazole
175	Metolachlor metabolite(Metolachlor ethane sulfonic acid)	171118-09-5	C15H23NO5S	Herbicide, chloroacetamide
176	Metolachlor metabolite(Metolachlor oxanilic acid)	152019-73-3	C15H21NO4	Herbicide, chloroacetamide
177	Metolachlor metabolite [(S)-2-[(Oxalyl)(2-ethyl-6-methylphenyl)amino]propionic acid]		C14H17NO5	Herbicide, chloroacetamide
178	Metolachlor metabolite [(2-Ethyl-6-methylphenyl)-carbamoyl]-methanesulfonic acid]		C11H15NO4S	Herbicide, chloroacetamide
179	Metolachlor metabolite [N-(2-Ethyl-6-methylphenyl)-2-hydroxyacetamide]	97055-05-5	C11H15NO2	Herbicide, chloroacetamide
180	Metolachlor metabolite [2-[((S)-1-Carboxyethyl)(2-ethyl-6-methylphenyl)amino]2-oxo-ethanesulfonic acid]		C14H19NO6S	Herbicide, chloroacetamide
181	Metolachlor metabolite [N-(2-Ethyl-6-methylphenyl)-L-alanine	82508-03-0	C12H17NO2	Herbicide, chloroacetamide
182	Metolachlor metabolite [N-(2-Ethyl-6-methylphenyl)-oxalamic acid]	152019-74-4	C11H13NO3	Herbicide, chloroacetamide
183	Metolachlor metabolite [2-[((S)-1-Carboxyethyl)(2-ethyl-6-methylphenyl)amino]-2-oxo-ethanesulfonic acid disodium salt]	1418095-19-8	C14H17NNa2O6S	Herbicide, chloroacetamide
184	Metolachlor metabolite (Metolachlor mercapturate)	159956-64-6	C20H30N2O5S	Herbicide, chloroacetamide
185	Myclobutanil metabolite(1,2,4-triazole	288-88-0	C2H3N3	Fungicide, triazole
186	Myclobutanil metabolite (1H-1,2,4-triazol-1-ylacetic acid)	28711-29-7	C4H5N3O2	Fungicide, triazole
187	Molinate metabolite (Molinate sulfoxide)	52236-29-0	C9H17NO2S	Herbicide, thiocarbamate
188	Napropamide metabolite [α-naphthol-2-methyl-naphthol(1,2-b)-2Hfuran-3-one]		C13H10O2	Herbicide, alkanamide
189	Napropamide metabolite [(N,N,N',N',Tetraethyl -4.4'-dihydroxyalpha,alpha'-2'-dimethyl[1,1-binaphthalene]-3,3'-diacetamide]		C34H40N2O4	Herbicide, alkanamide
190	Napropamide metabolite [N,N-diethyl-4-hydroxy-α-methyl-2-naphthaleneacetamide]		C17H21NO2	Herbicide, alkanamide
191	Napropamide metabolite [N,N-diethyl-4-hydroxy-α-methyl-1-naphthaleneacetamide]		C17H21NO2	Herbicide, alkanamide
192	Organophosphate metabolite (Dimethyl thiophosphate)	1112-38-5	C2H6O3PS	Organophosphate
193	Organophosphate metabolite (Dimethyl dithiophosphate)	756-80-9	C2H5O2PS2	Organophosphate
194	Organophosphate metabolite (Diethyl phosphate)	598-02-7	C4H11O4P	Organophosphate
195	Organophosphate metabolite (Diethyl thiophosphate)	298-06-6	C4H11O3PS	Organophosphate

Tabla SI-1. Capítulo 1. (Continuación).

Nº	Compound	CAS number	Elemental composition	Chemical class
196	Organophosphate metabolite (Diethyl dithiophosphate)		C4H11O2PS2	Organophosphate
197	Parathion, methyl parathion metabolite (4-nitrophenol)	100-02-7	C6H5NO3	Insecticide, acarecide, organophosphate
198	Paclobutrazol metabolite [4H-1,2,4-triazol-3-ol]	122442-66-4	C2H3N3O	Plant growth regulator; Fungicide, Triazole
199	Paclobutrazol metabolite [(2RS)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1H-1,2,4 triazol-1-yl) pentan-3-one]	63190-87-4	C15H18ClN3O	Plant growth regulator; Fungicide, Triazole
200	Permethrin, cypermethrin, cyfluthrin metabolite [cis-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid]	55701-05-8	C8H10O2Cl2	Pyrethroid insecticide
201	Permethrin, cypermethrin, cyfluthrin metabolite [trans-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid]	55701-05-8	C8H10O2Cl2	Pyrethroid insecticide
202	Pethoxamid metabolite [N-(2-Ethoxyethyl)-N-(2-methyl-1-phenylpropenyl)-2-sulfoacetamide]		C16H23NO5S	Herbicide, chloroacetamide
203	Phenmedipham metabolite [Methyl-N-(3-hydroxyphenyl)-carbamate]	13683-89-1	C8H9NO3	Herbicide, carbamate
204	Picolinafen metabolite [6-[(3-trifluoromethylphenoxy)picolinic acid]	137640-84-7	C13H8F3NO3	Herbicide, pyridine compounds
205	Pinoxaden metabolite [8-(2,6-diethyl-4-methyl-phenyl)-tetrahydropyrazolo[1,2-d][1,4,5]oxadiazepine-7,9-dione]	314020-44-5	C18H24N2O4	Herbicide, unclassified
206	Pirmicarb metabolite [2-amino-5,6-dimethylpyrimidin-4-yldimethylcarbamate]		C9H14N4O2	Insecticide, carbamate
207	Pirmicarb metabolite [5,6-dimethyl-2-(methylamino)pyrimidin-4-ol]	78195-30-9	C7H11N3O	Insecticide, carbamate
208	Pirmicarb metabolite [2-dimethylamino-5,6-dimethylpyrimidin-4-ol]	40778-16-3	C8H13N3O	Insecticide, carbamate
209	Propachlor metabolite (propachlor sulphonic acid)		C11H15NO4S	Herbicide, chloroacetamide
210	Propachlor metabolite (propachlor sulphinylacetic acid)		C13H17NO4S	Herbicide, chloroacetamide
211	Propachlor metabolite (propachlor oxanilic acid)	70628-36-3	C11H13NO3	Herbicide, chloroacetamide
212	Propachlor metabolite (propachlor alcohol)	42404-06-8	C11H15NO2	Herbicide, chloroacetamide
213	Propaquizafop metabolite (Quizalofop)	76578-12-6	C17H13ClN2O4	Herbicide, Aryloxyphenoxypropionate
214	Propazine Metabolite(hydroxypropazine)	7374-53-0	C9H17N5O	Herbicide, triazine
215	Propineb metabolite (4-Methyl-imidazolidin-2-thione)	2122-19-2	C4H8N2S	Fungicide, carbamate
216	Propyzamide metabolite (2-(3,5-dichlorophenyl)-4,4-dimethyl-5-methylene-2-oxazoline)		C12H11Cl2NO	Herbicide, benzamide
217	Propyzamide metabolite [3,5-dichloro-N-(1,1-dimethyl)-2-oxo-npropyl)benzamide]	29939-97-7	C12H13Cl2NO2	Herbicide, benzamide
218	Prothioconazole metabolite (1H-1,2,4-Triazole-1-ethanol, alpha-(1-chlorocyclopropyl)-alpha-(2-chlorophenyl)methyl)		C14H15Cl2N3O	Fungicide, Triazolinthione

Tabla SI-1. Capítulo 1. (Continuación).

Nº	Compound	CAS number	Elemental composition	Chemical class
219	Pyraflufen-ethyl metabolite [2-chloro-5-(4-chloro-5-difluoromethoxy-1-methylpyrazol-3-yl)-4-fluorophenoxyacetate]	129630-17-7	C13H9Cl2F3N2O4	Herbicide, Dessicant, phenylpyrazole
220	Pyraflufen-ethyl metabolite [2-chloro-5-(4-chloro-5-difluoromethoxy-1-methylpyrazole-3yl)-4-fluorophenol]		C11H7Cl2F3N2O2	Herbicide, Dessicant, phenylpyrazole
221	Pyrethroid metabolite (3-Phenoxybenzoic acid)	3739-38-6	C13H10O3	Pyrethroid insecticide
222	Pyridaben metabolite [2-tert-butyl-4-(4-tertbutylbenzoyl)pyridazin-3(2H)-one-5-sulfonic acid]		C19H24N2O5S	Insecticide, Acaricide, Pyridazinone
223	Pyridaben metabolite [2-tert-butyl-5-(4-tert-butylbenzylsulfinyl)-4-chloropyridazin-3(2H)-one]		C19H25ClN2O2S	Insecticide, Acaricide, Pyridazinone
224	Pyridaben metabolite [2-tert-butyl-5-[4-(1-carboxy-1-methylethyl)benzylthio]-4-chloropyridazin-3(2H)-one]		C19H23ClN2O3S	Insecticide, Acaricide, Pyridazinone
225	Pyridate metabolite (6-chloro-3-phenylpyridazin-4-ol)	40020-01-7	C10H7ClN2O	Herbicide, Phenylpyridazine
226	Pyrimiphos methyl metabolite [Phosphoric acid, 2-(diethylamino)-5-methyl-4-pyrimidinyl dimethyl ester]		C11H20N3O4P	Insecticide, Acaricide, Organophosphate
227	Pyrimiphos methyl metabolite[2-diethylamino-6-methyl-4-pyrimidinol]	42487-72-9	C9H15N3O	Insecticide, Acaricide, Organophosphate
228	Pyrimiphos methyl metabolite (4-[4-(Diethoxyphosphino)-1H-1,2,3-triazol-1-yl]butanoic acid)		C10H18N3O4P	Insecticide, Acaricide, Organophosphate
229	Pyroxsulam metabolite (Aminotriazole)	61-82-5	C9H9F3N6O3S	Herbicide, Graminicide, Triazolopyrimidine
230	Pyroxsulam metabolite (5,7-Dihydroxy-Pyroxsulam)		C12H9F3N6O5S	Herbicide, Graminicide, Triazolopyrimidine
231	Pyroxsulam metabolite (7-Hydroxy-Pyroxsulam)		C13H11F3N6O5S	Herbicide, Graminicide, Triazolopyrimidine
232	Quinmerac metabolite (7-chloro-2-hydroxy-3-methylquinoline-8-carboxylic acid)		C11H8ClNO3	Herbicide, quinoline
233	Quinmerac metabolite (7-chloro-3,8-quinoline-dicarboxylic acid)	90717-07-0	C11H6ClNO4	Herbicide, quinoline
234	Simazine metabolite (6-deisopropyl atrazine)	1007-28-9	C5H8ClN5	Herbicide, triazine
235	Simazine metabolite (2-hydroxy-4,6-bis(ethlyamino)-triazine)	2599-11-3	C7H13N5O	Herbicide, triazine
236	Spirodiclofen metabolite (3-(2,4-dichlorophenyl)-4-hydroxy-1-oxaspiro[4,5]dec-3-en-2-one)	148476-22-6	C15H14Cl2O3	Acaricide, Insecticide, tetronic acid
237	Sulcotrione metabolite (2-chloro-4-(methylsulfonyl)-benzoic acid)	53250-83-2	C8H7ClO4S	Herbicide, triketone
238	Tebuconazole metabolite (1,2,4-triazole)	288-88-0	C2H3N3	Fungicide, Plant growth regulator, Triazole
239	Tebufenozide metabolite[4-(N'-(3,5-dimethylbenzoyl-N-(1,1-dimethylethyl)hydrazinocarbonyl)phenyl acetic acid]		C22H26N2O4	Insecticide, Diacylhydrazine
240	Tebufenozide metabolite [N-(1,1-dimethyethyl)-N-(4-acetylebenzoyl)-3,5-dimethylbenzohydrazine]		C22H26N2O3	Insecticide, Diacylhydrazine

Tabla SI-1. Capítulo 1. (Continuación).

Nº	Compound	CAS number	Elemental composition	Chemical class
241	Tepraloxydim metabolite [(RS)-2-ethyl-6,7-dihydro-6-perhydropyran-4-ylbenzoxazol-4-(5H)-one]		C14H21NO3	Herbicide, cyclohexadione
242	Tepraloxydim metabolite [3-hydroxy-2-(1-iminopropyl)-5-perhydropyran-4-ylcyclohex-2-en-1-one]		C14H21NO3	Herbicide, cyclohexadione
243	Terbuthylazine Metabolite(Terbuthylazine-2-hydroxy)	66753-07-9	C9H17N5O	Herbicide, Microbiocide, Algicide, triazine
244	Terbuthylazine Metabolite(Desethyl-terbuthylazine)	30125-63-4	C7H12ClN5	Herbicide, Microbiocide, Algicide, triazine
245	Tetraconazole metabolite [2-(2,4-dichlorophenyl)-3-(1H-1,2,4-triazol-1-yl)propan-1-ol]		C13H12Cl2N6O	Fungicide, triazole
246	Tetraconazole metabolite [2-(2,4-dichlorophenyl)-3-(1H-1,2,4-triazol-1-yl)propanoic acid]		C11H9Cl2N3O2	Fungicide, triazole
247	Tetraconazole metabolite [1H-1,2,4-triazol-1-ylacetic acid]	28711-29-7	C4H5N3O2	Fungicide, triazole
248	Thiacloprid metabolite (Thiacloprid sulfonic acid)		C10H13CIN4SO5	Insecticide, Molluscicide, neonicotinoid
249	Thiacloprid metabolite (Thiacloprid-amide)	676228-91-4	C10H11ClN4OS	Insecticide, Molluscicide, neonicotinoid
250	Thiamethoxam metabolite (Clothianidim)	210880-92-5	C6H8ClN5O2S	Insecticide, neonicotinoid
251	Tolclofos-methyl metabolite (2,6-Dichloro-4-methylphenyl dimethyl phosphate)		C9H11Cl2O4P	Fungicide, chlorophenyl
252	Tolclofos-methyl metabolite (1,4-dichloro-3-methylphenol)	17788-00-0	C7H6Cl2O	Fungicide, chlorophenyl
253	Tolclofos-methyl metabolite (Desmethyl tolclofos)		C8H9Cl2O4P	Fungicide, chlorophenyl
254	Tolyfluanid metabolite(DMST)	66840-71-9	C9H14N2O2S	Fungicide, sulfamide
255	Tolyfluanid metabolite (N,N-Dimethylsulfamide)	4315-09-7	C2H8N2O2S	Fungicide, sulfamide
256	Triclorfom Metabolite(Dichlorvos)	62-73-7	C4H7Cl2O4P	Insecticide, organophosphate
257	Triclorfom Metabolite(Desmethyl dichlorvos)	17650-82-7	C2H3Cl2O4P	Insecticide, organophosphate
258	Triclorfom metabolite (Dichlorivinylphosphate)		C2HCl2O4P	Insecticide, organophosphate
259	Triclorfom metabolite (Dichloroethanol)		C2H4Cl2O	Insecticide, organophosphate
260	Trifluralin metabolite [3-nitro-N2,N2-dipropyl-5-(trifluoromethyl)benzene-1,2-diamine]	2078-04-8	C13H18F3N3O2	Herbicide, dinitroaniline
261	Tritosulfuron metabolite (2-trifluoromethyl-benzenesulfonamide)	1869-24-5	C7H6F3NO2S	Herbicide, sulfonyurea
262	Vinclozolin metabolite (3,5-dichlorophenylcarbamic acid-(1-carboxyl-1-methyl)-2-propenyl-ester)		C12H11Cl2NO4	Fungicide, oxazole
263	Vinclozolin metabolite (N-(3,5-dichlorophenyl)-2-hydroxy-2-methyl-3-butenic acid-amide)		C11H11Cl2NO2	Fungicide, oxazole

Tabla SI-2. Capítulo 1. Target compounds, including classification, possible precursor, elemental composition, accurate mass and fragments data

Class	Posible precursor compound	Metabolite	Acronym	Purity degree (%)	Elemental composition	Diagnostic ion	Exact mass m/z diagnositic ion (Da)	Fragment	
								Elemental composition	m/z ion (Da)
Organophosphate insecticides	Coumpahos	3-Chloro-7-hydroxy-4-methylcoumarin (3-Chloro-4-methylumbelliferone)	**CMHC**	99.2	$C_{10}H_7ClO_3$	[M-H]	209.00109	$C_9H_5O_2$	145.02841
	Pirimiphos-methyl	2-diethylamino-6-methyl-4-pyrimidinol	**DEAMPY**	99	$C_9H_{15}N_3O$	[M+H]	154.09742	$C_7H_{12}ON_3$	154.09748
	Diazinon	2-isopropyl-6-methyl-4-pyrimidinol	**IMPY**	99.9	$C_8H_{12}N_2O$	[M+H]	153.10146	C_4H_6ON	84.04465
	Parathion, methyl parathion	4-nitrophenol	**PNP**	99	$C_6H_5NO_3$	[M-H]	138.01966	$C_6H_4O_2$	108.02156
	Chlorpyrifos, chlorpyrifos-methyl	3,5,6-trichloro-2-pyridinol	**TCPY**	99.8	$C_5H_2NOCl_3$	[M-H]	195.91292	-	-
	Fenitrothion	3-methyl-4-nitrophenol	**MNP**	99	$C_7H_7NO_3$	[M-H]	152.03531	$C_7H_6O_2$	122.03733
	Malathion	Malathion dicarboxilic acid	**MDA**	99	$C_6H_{11}O_6PS_2$	[M-H]	272.96619	-	-
	Chlorethoxyphos, chlorpyrifos coumaphos, diazinon, disulfoton, ethion, parathion, phorate, phosalone, sulfootep, terbufos	Dimethyl thiophosphate	**DMTP**	98	$C_2H_6O_3PS$	[M-H]	140.97807	CH_3O_3PS	125.95462
		Dimethyl dithiophosphate	**DMDTP**	98	$C_2H_5O_2PS_2$	[M-H]	156.95523	$CH_3O_2PS_2$	141.93174
		Diethyl phosphate	**DEP**	75	$C_4H_{11}O_4P$	[M-H]	153.03221	$C_2H_6O_4P$	125.00102
		Diethyl thiophosphate	**DETP**	99	$C_4H_{11}O_3PS$	[M-H]	169.00937	$C_2H_6O_3PS$	140.97815
		Diethyl dithiophosphate	**DEDTP**	90	$C_4H_{11}O_2PS_2$	[M-H]	184.98653	$C_2H_6O_2PS_2$	156.95523
	Acephate	Acephate	**AP**	96	$C_4H_{10}NO_3PS$	[M+H]	184.01917	$C_2H_8O_3PS$	142.99256
	Methamidophos	Methamidophos	**MMP**	98	$C_2H_8NO_2PS$	[M+H]	142.00861	CH_5O_2NP	94.00534
	Omethoate	Omethoate	**OMET**	98	$C_5H_{12}NO_4PS$	[M+H]	214.02923	$C_4H_8O_4PS$	182.98754
	Dimethoate	Dimethoate	**DIMET**	98.5	$C_5H_{12}NO_3PS_2$	[M+H]	230.0069	$C_2H_6O_2PS$	124.98206

Tabla SI-2. Capítulo 1. (Continuación).

Class	Posible precursor compound	Metabolite	Acronym	Purity degree (%)	Elemental composition	Diagnostic ion	Exact mass m/z diagnositic ion (Da)	Fragment	
								Elemental composition	m/z ion (Da)
Pyrethroid insecticides	Commercial pyrethroids	3-Phenoxybenzoic acid	**PBA**	99	$C_{13}H_{10}O_3$	[M-H]	213.05571	$C_{12}H_9O$	169.06572
	Cyfluthrin	4-fluoro-3-phenoxy benzoic acid	**FPBA**	95.5	$C_{13}H_9FO_3$	[M-H]	231.04629	$C_{12}H_8OF$	187.0563
	Permethrin, cypermethrin, cyfluthrin	cis-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid	**cis-DCCA**	99	$C_8H_{10}O_2Cl_2$	[M-H]	206.9985	-	-
		trans-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid	**trans-DCCA**	99	$C_8H_{10}O_2Cl_2$	[M-H]	206.9985	-	-
	Deltamethrin	cis-(2,2-dibromovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid	**DBCA**	99.3	$C_8H_{10}O_2Br_2$	[M-H]	406.07197	-	-
Chloroacetanilide herbicides	Atrazine	Atrazine mercapturate	**ATZM**	96	$C_{13}H_{22}N_6O_3S$	[M+H]	343.15468	$C_8H_{16}N_5S$	214.1119
	Alachlor	Alachlor mercapturate	**ALAM**	98	$C_{19}H_{28}N_2O_5S$	[M+H]	397.17916	$C_5H_7O_3N$	130.04974
	Metolachlor	Metolachlor mercapturate	**METM**	95	$C_{20}H_{30}N_2O_5S$	[M+H]	411.19481	$C_{15}H_{24}O_2NS$	282.15088
Phenoxy herbicides	2,4-dichlorophenoxyacetic acid	2,4-dichlorophenoxyacetic acid	**2.4-D**	99	$C_8H_6O_3Cl_2$	[M-H]	218.96212	$C_6H_3OCl_2$	160.95645
	2,4,5-Trichlorophenoxyacetic acid	2,4,5-Trichlorophenoxyacetic acid	**2.4.5-T**	99	$C_8H_5O_3Cl_3$	[M-H]	252.92315	$C_6H_2OCl_3$	194.91749
Internal standards		4-nitrophenol D_4	**PNP-D_4**	99	$C_6HD_4NO_3$	[M-H]	142.04477	-	-
		4-fluoro-3-phenoxybenzoic acid ($^{13}C_6$)	**FPBA-$^{13}C_6$**	98.3	$^{13}C_6C_7H_9FO_3$	[M-H]	237.06642	-	-
		DCCA $^{13}C_2$. D_1	**DCCA-$^{13}C_2$. D_1**	100	$C_6{}^{13}C_2H_9DCl_2O_2$	[M-H]	210.01149	-	-

Tabla SI-2. Capítulo 1. (Continuación).

Class	Posible precursor compound	Metabolite	Acronym	Purity degree (%)	Elemental composition	Diagnostic ion	Exact mass m/z diagnositic ion (Da)	Fragment	
								Elemental composition	m/z ion (Da)
Internal standards		Atrazine mercapturate (Ring-$^{13}C_3$)	**ATZM-$^{13}C_3$**	98	$^{13}C3C_{10}H_{22}N_6O_3S$	[M+H]	346.16475	-	-
		2,4-D D_3	**2.4-D-D_3**	97.6	$C_8H_3Cl_2O_3D_3$	[M-H]	221.98095	-	-
		Methamidophos-D_6	**METHA-D6**	98.7	$C_2H_2NO_2PSD_6$	[M+H]	148.04627	-	-
		Dimethoate-D_6	**DIMET-D6**	98	$C_5H_6NO_3PS_2D_6$	[M+H]	236.04455	-	-
		Dibuthyl phosphate	**DBP**	98	$C_8H_{19}O_4P$	[M-H]	209.09481	-	-

Tabla SI-3. Capítulo 1. Compounds searched in the database for post-run target screening, including elemental composition of the compound, theoretical accurate mass and fragments

Nº	Compound	CAS number	Elemental composition	Monitored ion	Monitored Mass	Frag. 1	Structure	Frag. 2	Structure	Frag.3	Structure	Matrix(*)	Ref
1	2,4-dichlorophenoxyacetic acid	94-75-7	C8H6O3Cl2	M-H	218.96212	160.95645	C6H4OCl2					S	[12]
2	2,4,5-Trichlorophenoxyacetic acid	93-76-5	C8H5O3Cl3	M-H	252.92315	194.91749	C6H3OCl3					-	[12]
3	Abamectin metabolite (5-O-demethyl-Avermectin A1a)	113665-89-7	C48H72O14	M+H	873.49948	831.452533	C45H67O14	641.36841	C37H53O9	295.17513	C13H27O7	W	[1],[2], [3]
4	Abamectin metabolite (5-O-demethyl-25-de(1-methylpropyl)-25-(1-methylethyl)-Avermectin A1a)	65195-56-4	C47H70O14	M+H	859.48383	583.326544	C34H47O8	333.19078	C16H29O7	175.096485	C8H15O4	W	[1],[2], [3]
5	Acetamiprid metabolite[N-methyl-(6-chloro-3-pyridyl)methylamine]	120739-62-0	C7H9ClN2	M+H	157.0527	126.010503	C6H5ClN	121.076025	C7H9N2	111.994853	C5H3NCl	S	[2],[3]
6	Acephate	30560-19-1	C4H10NO3PS	M+H	184.01917	142.99256	C2H7O3PS					-	[12]
7	Acetochlor metabolite [N-(ethoxymethyl)-N-(2-ethyl-6-methylphenyl) acetamide]	34256-82-1	C14H21NO2	M+H	236.16450	177.114815	C11H15NO	136.112076	C9H14N	121.101177	C9H13	W	[1],[2], [3]
8	Acetochlor metabolite [Acetic acid, [(ethoxymethyl)(2-ethyl-6-methylphenyl)-amino]oxo]		C14H19NO4	M+H	266.13868	236.091734	C12H14NO4	119.085527	C9H11	192.138291	C12H18NO	W	[1],[2], [3]
9	Acetochlor metabolite [Acetic acid, {2-[(ethoxymethyl)(2-ethyl-6-methylphenyl)-amino]-2-oxoethyl}sulfinyl]		C16H23NO5S	M+H	342.13697	313.097844	C14H19NO5S	296.095105	C14H18NO4S	190.122641	C12H16NO	W	[1],[2], [3]
10	Acetochlor metabolite [2-[(2-ethyl-6-methylphenyl)amino]-2-oxoethanesulfonic acid; [(2-Ethyl-6-methylphenyl)-carbamoyl]-methanesulfonic acid]		C11H15NO4S	M+H	258.07945	148.112076	C10H14N	140.001205	C2H6NO4S	226.053241	C10H12NO3S	W	[1],[2], [3]
11	Acetochlor metabolite [Ethanesulfonic acid, 2-[(2-ethyl-6-methylphenyl)(ethoxymethyl)amino]-2-oxo]		C14H21NO5S	M+H	316.12132	270.079455	C12H16NO4S	240.068891	C11H14NO3S	190.122641	C12H16NO	W	[1],[2], [3]
12	Alachlor metabolite (Alachlor mercapturate)	116482-92-9	C19H28N2O5S	M+H	397.17916	130.04974	C5H7O3N						[12]
13	Ametryn metabolite[Deethyl ametrin	4147-57-3	C9H17N5S	M+H	228.12774	212.096442	C8H14N5S	170.049493	C5H8N5S	136.061772	C5H6N5	S	[2],[3]
14	Amitraz metabolite(N-2 4-Dimethylphenyl-N'methylformamidine)	33089-74-6	C10H14N2	M+H	163.12297	132.080775	C9H10N	134.096426	C9H12N	147.091675	C9H11N2	W	[4]
15	Atrazine metabolite (6-deisopropyl atrazine)	1007-28-9	C5H8ClN5	M+H	174.0541	157.027550	C5H6ClN4	146.022799	C3H5ClN5	111.053947	C3H5N5	S	[2],[3]
16	Atrazine metabolite (Deethyl atrazine)	6190-65-4	C6H10ClN5	M+H	188.06975	146.0228	C3H5N5Cl	136.061772	C5H6N5	128.99625	C3H2ClN4	S	[5]
17	Atrazine metabolite (Atrazine mercapturate)	138722-96-0	C13H22N6O3S	M+H	343.15468	214.1119	C8H15N5S					-	[12]
18	Azinphos-methyl metabolite (phosphorodithioic O,O,S-trimethyl ester)	2953-29-9	C3H9O2PS2	M+H	172.98543	140.959219	C2H6OPS2	124.982063	C2H6O2PS	93.0099925	C2H6O2P	W	[6]
19	Azinphos-methyl metabolite (phosphorothioic O,O,S-trimethyl ester)	152-20-5	C3H9O3PS	M+H	157.00827	124.982063	C2H6O2PS	109.004907	C2H6O3P			W	[6]
20	Azinphos-methyl metabolite (Benzamide)	55-21-0	C7H7NO	M+H	122.06004	105.033491	C7H5O	77.0385768	C6H5			W	[6]

Tabla SI-3. Capítulo 1. (Continuación).

Nº	Compound	CAS number	Elemental composition	Monitored ion	Monitored Mass	Frag. 1	Structure	Frag. 2	Structure	Frag.3	Structure	Matrix(*)	Ref
21	Azinphos-methyl metabolite (Pyrido[3,4-d] pyrimidin-4-ol)	19178-25-7	C7H5N3O	M+H	148.05053	130.039973	C7H4N3					W	[6]
22	Azinphos-methyl metabolite (3-methyl-benzotriazin-4-one)	22305-44-8	C8H7N3O	M+H	162.06618	134.071273	C7H8N3	146.071274	C8H8N3			W	[6]
23	Azoxystrobin metabolite [4-(2-cyanophenoxy)-6-hydroxypyrimidine]		C11H7N3O2	M+H	214.0611	196.050538	C11H6N3O	187.050204	C10H7N2O2	118.02874	C7H4NO	S,W	[2],[3]
24	Azoxystrobin metabolite [2-[6-(2-cyanophenoxy)pyrimidin-4yloxy]benzoic acid]		C18H11N3O4	M+H	334.08223	318.087317	C18H12N3O3	307.071333	C17H11N2O4	171.055289	C10H7N2O	S,W	[2],[3]
25	Bensulfuron methyl metabolite[methyl 2-({[(4-hydroxy-6-methoxypyrimidin-2yl)carbamoyl]sulfamoyl}methyl)benzoate]		C15H16N4O7S	M+H	397.08124	367.070681	C14H15N4O6S	339.075767	C13H15N4O5S	185.066917	C6H9N4O3	W	[1],[2],[3]
26	Bensulfuron methyl metabolite (4,6-Dimethoxy-2-pyrimidinamine)	36315-01-2	C6H9N3O2	M+H	156.07675	124.050538	C5H6N3O	139.050204	C6H7N2O2			W	[1],[2],[3]
27	Bensulfuron methyl metabolite [methyl 2-(sulfamoylmethyl)benzoate]	112941-26-1	C9H11NO4S	M+H	230.04815	199.005955	C8H7O4S	170.027026	C7H8NO2S	135.044056	C8H7O2	W	[1],[2],[3]
28	Bensulfuron methyl metabolite [(4,6-dimethoxypyrimidin-2-yl)urea]		C7H10N4O3	M+H	199.08256	156.076753	C6H10N3O2	124.050538	C5H6N3O	139.050204	C6H7N2O2	W	[1],[2],[3]
29	Bensulfuron methyl metabolite [{[(4,6-dimethoxypyrimidin-2-yl)carbamoyl]sulfamoyl}acetic acid]		C9H12N4O7S	M+H	321.04995	289.023731	C8H9N4O6S	182.056018	C7H8N3O3	156.076753	C6H10N3O2	W	[1],[2],[3]
30	Bensulfuron methyl metabolite [2-(sulfamoylmethyl)benzoic acid]		C8H9NO4S	M+H	216.03250	180.995391	C8H5O3S	170.027026	C7H8NO2S	121.028406	C7H5O2	W	[1],[2],[3]
31	Bifenox metabolite (Bifenox acid)	59024-05-4	C13H7Cl2NO5	M+H	327.97740	292.000726	C13H7ClNO5	236.986847	C12H7Cl2O	166.013484	C7H4NO4	W	[1],[2],[3]
32	Bifenox metabolite[2-Amino-5-(2,4-dichlorophenoxy)-benzoic acid]	59216-76-1	C13H9Cl2NO3	M+H	298.00322	280.976676	C13H7Cl2O3	251.997746	C12H8Cl2NO	236.986847	C12H7Cl2O	W	[1],[2],[3]
33	Bitertanol metabolite (1,2,4-triazole)	288-88-0	C2H3N3	M+H	70.039970							S, GW, Pl, An	[2],[3]
34	Bitertanol metabolite (1H-1,2,4-triazol-1-ylacetic acid)	28711-29-7	C4H5N3O2	M+H	128.04545	98.0348883	C3H4N3O	82.0399737	C3H4N3	112.050538	C4H6N3O	Soil, Pl, An	[2],[3]
35	Bromopropylate metabolite(4,4-dibromobenzilic acid)	30738-49-9	C14H10Br2O3	M+H	384.90695	338.901466	C13H9Br2O	156.964739	C6H6Br	184.959654	C7H6BrO	S	[2],[3]
36	Cadusafos metabolite (Methyl-2-butyl sulfone)		C8H18O2S	M+H	179.11003	109.031776	C3H9O2S	91.0212121	C3H7OS			S,W	[2],[3]
37	Captan metabolite (THPAM)	4795-29-3	C5H11NO	M+H	102.09134	85.0647915	C5H9O	71.0491414	C4H7O			W	[1],[2],[3]
38	Captan metabolite (THPI)	1469-48-3	C8H9NO2	M+H	152.07060	131.000179	C7H1ONO	109.064792	C7H9O			W	[1],[2],[3]
39	Carbaryl metabolite (1-naphtol)	90-15-3	C10H8O	M+H	145.06479	127.0752	C10H7					S,Hu	[2],[3]
40	Carbaryl metabolite (Methylamine)	200-820-0	CH5N	M+H	32.04948							S	[2],[3]
41	Carbendazim metabolite(2-aminobenzimidazole)	934-32-7	C7H7N3	M+H	134.07127	133.0634	C7H7N3	105.0578	C7H7N	117.044724	C7H5N2	S	[2],[3]
42	Carbofuran metabolite (3-Keto-Carbofuran)	16709-30-1	C12H13NO4	M+H	236.09173	208.060434	C10H10NO4	186.990009	C9H1ONO3	151.07536	C9H11O2	W	[4]
43	Carbofuran metabolite (Carbofuran-7-phenol)	1563-38-8	C10H12O2	M+H	165.09100	135.044056	C8H7O2	107.049141	C7H7O			W	[1],[2],[3]

Tabla SI-3. Capítulo 1. (Continuación).

Nº	Compound	CAS number	Elemental composition	Monitored ion	Monitored Mass	Frag. 1	Structure	Frag. 2	Structure	Frag.3	Structure	Matrix(*)	Ref
44	Carboxin metabolite (Carboxin sulfoxide)	17757-70-9	C12H13NO3S	M+H	252.06889	220.079061	C12H14NOS	196.042676	C9H10NO2S	120.04439	C7H6NO	W	[1],[2],[3]
45	Chloridazon metabolite(Desphenyl Chloridazon)	6339-19-1	C4H4ClN3O	M+H	146.01156	128.985017	C4H2ClN2O	110.034888	C4H4N3O	130.016651	C4H5ClN3	W	[1],[2],[3]
46	Chloridazon metabolite (Methyl-desphenyl-chloridazon)	17254-80-7	C5H6ClN3O	M+H	160.02721	143.000667	C5H4ClN2O	124.050538	C5H6N3O			W	[1],[2],[3]
47	Chlorpropham metabolite(HSA-4)		C10H12ClNO6S	M+H	310.01466	292.004097	C10H11ClNO5S	274.037984	C10H12NO6S	251.972797	C7H7ClNO5S	W	[4]
48	Chlorthalonil metabolite [2-amido-3,5,6-trichlo-4-cyanobenzenesulphonic acid]		C8H3O4Cl3N2S	M+H	328.89518	311.868637	C8HCl3NO4S	301.884288	C7H3Cl3NO4S	292.918509	C8H3Cl2N2O4S	W	[1],[2],[3]
49	Chlorthalonil metabolite [2,4-bis-amido-3,5,6-trichloro benzenesulfonic acid]		C8H5Cl3N2O5S	M+H	346.90575							W	[1],[2],[3]
50	Chlorthalonil metabolite [4-amido-2,5-dichloro-6-cyano benzene-1,3-disulfonic acid]		C8H4Cl2N2O7S2	M+H	374.89097							W	[1],[2],[3]
51	Chlorthalonil metabolite [2,5-dichloro-4,6-dicyano-benzene-1,3-disulfonic acid]		C8H2Cl2N2O6S2	M+H	356.88040							W	[1],[2],[3]
52	Chlorthalonil (3-carbamyl-2,4,5-trichlorobenzoic acid)	142733-37-7	C8H4Cl3NO3	M+H	267.93295							W	[1],[2],[3]
53	Chlorsulfuron metabolite (2-chlorobenzenesulfonamide)	6961-82-6	C6H6ClNO2S	M+H	191.98805	156.011375	C6H6NO2S	142.971675	C6H4ClS	110.999604	C6H4Cl	W	[1],[2],[3]
54	Chlorsulfuron metabolite [2-amino-4-methoxy-6-methyl-1,3,5-triazine]	1668-54-8	C5H8N4O	M+H	141.07708	124.050538	C5H6N3O	109.050873	C4H5N4			W	[1],[2],[3]
55	Chlorsulfuron metabolite [N-[(N-carbamoylcarbamimidoyl)carbamoyl]-2-chlorobenzenesulfonamide]		C5H8N4O	M+H	141.07708	124.050538	C5H6N3O	109.050873	C4H5N4			W	[1],[2],[3]
56	Chlorotoluron metabolite [3-(3-chloro-p-tolyl)-1-methylurea]	22175-22-0	C10H13ClN2O	M+H	213.07892	168.021068	C8H7ClNO	163.08659	C9H11N2O	161.07094	C9H9N2O	S,W	[2],[3]
57	Chlorotoluron metabolite [2-Chloro-4-(3,3-dimethyl-ureido)-benzoic acid; 1-(3-Chlor-4-carboxyphenyl)-3,3-dimethylharnstoff]	59587-01-8	C10H11ClN2O3	M+H	243.05309	225.042531	C10H10ClN2O2	207.076419	C10H11N2O3	193.060769	C9H9N2O3	W	[1],[2],[3]
58	Chlorotoluron metabolite [Desmethylchlortoluron]	22175-22-0	C9H11ClN2O	M+H	199.06326	168.021068	C8H7ClNO	163.08659	C9H11N2O	142.041804	C7H9ClN	W	[1],[2],[3]
59	Chlorpyrifos metabolite (3,5,6-trichloro-2-pyridinol)	6515-38-4	C5H2Cl3NO	M+H	197.92747	179.916908	C5HCl3N	161.950796	C5H2Cl2NO	145.955881	C5H2Cl2N	S	[2],[3]
60	Chlorpyrifos, chlorpyrifos-metyol metabolite (3,5,6-trichloro-2-pyridinol)	6515-38-4	C5H2NOCl3	M-H	195.91292	-	-					S	[12]
61	Chlorpyrifos metabolite (dimethyl 3,5,6-trichloro-2-pyridylphosphate)	5598-52-7	C7H7NO4Cl3P	M+H	304.91728	273.89889	C6H4O3NCl3P	269.94843	C7H7O4NPCl2	179.91691	C5HNCl3	A	[3],[7]
62	Chlorpyrifos metabolite (Chlorpyrifos oxon)	5598-15-2	C9H11Cl3NO4P	M+H	333.95640	179.916908	C5HCl3N	297.979726	C9H11Cl2NO4P	241.917126	C5H3Cl2NO4P	W	[3],[8]
63	Chlorpyrifos-methyl metabolite(Dimethyl 3,5,6-trichloro-2-pyridinyl phosphate)		C7H7Cl3NO4P	M+H	305.92104	273.898889	C6H4Cl3NO3P	269.948426	C7H7Cl2NO4P	179.916909	C5HCl3N	A	[3],[7]
64	Chlorpyrifos-methyl metabolite(3,5,6-Trichloro-2(1H)-pyridinone)	6515-38-4	C5H2NOCl3	M+H	197.92744	179.916908	C5HCl3N	161.950796	C5H2Cl2NO	145.955881	C5H2Cl2N	A	[3],[7]
65	Chlorpyrifos-methyl metabolite(Desmethyl chlorpyrifos)		C6H5NO4Cl3P	M+H	291.90945	259.883239	C5H2Cl3NO3P	255.932776	C6H5Cl2NO4P	179.916909	C5HCl3N	A	[3],[7]
66	Coumaphos metabolite [3-Chloro-7-hydroxy-4-methylcoumarin]	6174-86-3	C10H7ClO3	M-H	209.00109	145.02841	C9H6O2					-	[12]

Tabla SI-3. Capítulo 1. (Continuación).

Nº	Compound	CAS number	Elemental composition	Monitored ion	Monitored Mass	Frag. 1	Structure	Frag. 2	Structure	Frag.3	Structure	Matrix(*)	Ref
67	Cyfluthrin metabolite (4-fluoro-3-phenoxy benzoic acid)	77279-89-1	C13H9FO3	M-H	231.04629	187.0563	C12H9OF					-	[12]
68	Cymoxanil metabolite [3-ethyl-4-(methoxyamino)-2,5-dioxoimidazolidine-4-carbonitrile]	644972-55-4	C7H10N4O3	M+H	199.08257	172.071667	C6H10N3O3	153.040702	C5H5N4O2	152.045453	C6H6N3O2	S,W	[2],[3]
69	Cymoxanil metabolite[3-ethyl-4-(methoxyamino)-2,5-dioxoimidazolidine-4-carboxamide]	644972-61-2	C7H12N4O4	M+H	217.09313	200.066582	C7H10N3O4	170.056018	C6H8N3O3	127.050204	C5H7N2O2	S,W ,Sed	[2],[3]
70	Cyromazine metabolite (Ammelide)	645-93-2	C3H4N4O2	M+H	129.04070	112.014152	C3H2N3O2	111.030137	C3H3N4O	112.014152	C3H2N3O2	W	[1],[2],[3]
71	Cyromazine metabolite (Ammeline)	645-92-1	C3H5N5O	M+H	128.05668	111.030137	C3H3N4O	110.046122	C3H4N5	111.030137	C3H3N4O	W	[1],[2],[3]
72	Cyromazine metabolite (Melamine)	108-78-1	C3H6N6	M+H	127.07267	110.046121	C3H4N5	68.0243237	C2H2N3	110.046121	C3H4N5	W	[1],[2],[3]
73	Cyproconazole metabolite (1,2,4-triazole)	288-88-0	C2H3N3	M+H	70.039970							S, GW, Pl, An	[2],[3]
74	Cyproconazole metabolite(1H-1,2,4-triazol-1-ylacetic acid)	28711-29-7	C4H5N3O2	M+H	128.0455	110.034888	C4H4N3O	98.0348883	C3H4N3O	82.0399737	C3H4N3	S, Pl, An	[2],[3]
75	Deltamethrin metabolite (Decamethrinic acid)	53179-78-5	C8H10Br2O2	M+H	296.91203	250.906552	C7H9Br2	216.985869	C8H10BrO2	113.059706	C6H9O2	S	[2],[3]
76	Deltamethrin metabolite (3-phenoxybenzoic acid)	3739-38-6	C13H10O3	M+H	215.07027	105.033491	C7H5O	169.064792	C12H9O	121.028406	C7H5O2	S,W	[2],[3]
77	Deltamethrin metabolite (cis-(2,2-dicbromovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid)	63597-73-9	C8H10O2Br2	M-H	406.07197	-	-					-	[12]
78	Desmedipham metabolite (EHPC)	7159-96-8	C9H11NO3	M+H	182.08116	170.995094	C9H1ONO2	136.039305	C7H6NO2	93.0334914	C6H5O	W	[1],[2],[3]
79	Diazinon metabolite (Pyrimidol)	557-01-7	C4H4N2O	M+H	97.03964	79.0290747	C4H3N2					W	[2],[3]
80	Diazinon metabolite (4-hydroxy-2-isopropyl-6-methylpyrimidine)	2814-20-2	C8H12N2O	M+H	153.10224	137.070939	C7H9N2O	135.091675	C8H11N2	119.060375	C7H7N2	An	[2],[3]
81	Diazinon metabolite(Diethylphosphate)	598-02-7	C4H11O4P	M+H	154.03895	143.960696	C4H1OO3P	110.984172	CH4O4P	98.9841716	H4O4P	A	[3],[9]
82	Diazinon metabolite (2-Isopropyl-6-methyl-pyrimidinol)	2814-20-2	C8H12N2O	M+H	153.10224	137.070939	C7H9N2O	121.039639	C6H5N2O	84.04465	C4H5ON	W	[1],[2],[3]
83	Diazinon metabolite [2-(1-hydroxy-1-methyl)-ethyl-4-methyl-6-hydroxpyrimidine]	28175-97-5	C8H12N2O2	M+H	169.09715	151.086589	C8H11N2O	109.039639	C5H5N2O			W	[1],[2],[3]
84	Dicamba metabolite (3,6-dichloro-2-hydroxy benzoic acid)	3401-80-7	C7H4Cl2O3	M+H	206.96102	188.950461	C7H3Cl2O2	170.984348	C7H4ClO3	160.955547	C6H3Cl2O	W	[1],[2],[3]
85	Dichlobenil metabolite (2,6-Dichlorobenzamide)	2008-58-4	C7H5Cl2NO	M+H	189.98209	154.005418	C7H5ClNO	144.960632	C6H3Cl2	110.999604	C6H4Cl	W	[1],[2],[3]
86	Dichlofluanid metabolite [N,N-Dimethylaminosulfanilide]	4710-17-2	C8H12N2O2S	M+H	201.06922	156.011375	C6H6NO2S	169.04301	C7H9N2OS			W	[1],[2],[3]
87	Dichlorvos metabolite (2,2-Dichloroacetic acid)	79-43-6	C2H2Cl2O2	M+H	128.95046	110.939896	C2HCl2O	90.981943	C2H2ClNO			W	[1],[2],[3]
88	Dichlorvos metabolite (2,2 dichloro-acetaldehyde)	79-02-7	C2H2Cl2O	M+H	112.95554	110.939896	C2HCl2O					W	[1],[2],[3]
89	Diclofop-methyl metabolite (diclofop-phenol)	40843-73-0	C12H8Cl2O2	M+H	254.99741	236.986846	C12H7Cl2O	219.020734	C12H8ClO2	160.955547	C6H3Cl2O	W	[1],[2],[3]

Tabla SI-3. Capítulo 1. (Continuación).

Nº	Compound	CAS number	Elemental composition	Monitored ion	Monitored Mass	Frag. 1	Structure	Frag. 2	Structure	Frag.3	Structure	Matrix(*)	Ref
90	Dimethachlor metabolite[N-(2,6-dimethylphenyl)-N-(2-methoxyethyl)oxalamic acid]	1086384-49-7	C13H17NO4	M+H	252.12303	234.112469	C13H16NO3	204.101905	C12H14NO2	105.069877	C8H9	W	[1],[2],[3]
91	Dimethachlor metabolite [(2,6-Dimethylphenyl)-(2-methoxyethyl)-carbamoyl]-methanesulfonic acid]		C13H19NO5S	M+H	302.10567	240.068890	C11H14NO3S	220.133205	C13H18NO2	192.138291	C12H18NO	W	[1],[2],[3]
92	Dimethachlor metabolite [(2,6-Dimethylphenylcarbamoyl)-methanesulfonic acid]		C10H13NO4S	M+H	244.06380	162.091340	C10H12NO	134.096426	C9H12N	105.069877	C8H9	W	[1],[2],[3]
93	Dimethachlor metabolite [(2,6-Dimethyl-phenyl)-(2-sulfo-acetyl)-amino]-acetic acid]		C12H15NO6S	M+H	302.06928	284.058719	C12H14NO5S	240.068891	C11H14NO3S	220.09682	C12H14NO3	W	[1],[2],[3]
94	Dimethachlor metabolite [2-[(2-hydroxy-acetyl)-(2-methoxy-ethyl)-amino]3-methyl-benzoic acid]		C13H17NO5	M+H	268.11794	250.107384	C13H16NO4	192.06552	C10H10NO3	135.044056	C8H7O2	W	[1],[2],[3]
95	Dimethachlor metabolite [3-{2-[(2,6-dimethyl-phenyl)-(2-hydroxyacetyl)-amino]-ethylsulfanyl}-2-hydroxy-propionic acid]		C15H21NO5S	M+H	328.12132	306.079455	C15H16NO4S	134.096426	C9H12N	280.100191	C14H18NO3S	W	[1],[2],[3]
96	Dimethachlor metabolite [(2,6-dimethylphenyl)-2-methoxyethyl)carbamoyl]methanesulfonic acid sodium salt]		C13H18O5NaNS	M+H	284.05631	284.095105	C13H18NO4S	268.100191	C13H18NO3S	192.138291	C12H18NO	W	[1],[2],[3]
97	Dimethachlor metabolite [(2,6-dimethylphenylcarbamoyl)-methanesulfonic acid sodium salt]		C10H12NNaO4S	M+H	266.04574	226.053240	C10H12NO3S	210.058326	C10H12NO2S	134.096426	C9H12N	W	[1],[2],[3]
98	Dimethenamid metabolite [sodium[(2,4-dimethyl-thiophen-3-yl)-(2-methoxy-1-methyl-ethyl)-carbamoyl]methanesulfonate]		C12H19NO5S2	M+H	322.07774							W	[1],[2],[3]
99	Dimethenamid metabolite [N-(2,4-dimethyl-thiophen-3-yl)-N-(2-methoxy-1-methyl-ethyl)-oxalamic acid]	380412-59-9	C12H17NO4S	M+H	272.09510							W	[1],[2],[3]
100	Dimethoate		C5H12NO3PS2	M+H	230.0069	124.98206	C2H5O2PS					-	[12]
101	Dimethoate Metabolite(Omethoate)	1113-02-6	C5H12NO4PS	M+H	214.02974	124.9821	C2H6O2PS	154.9926	C3H8O3PS	182.98754	C4H7O4PS	S	[4]
102	Dimethomorph metabolite (4-chlorophenyl)(3,4-dimethoxyphenyl)methanone)	116412-84-1	C15H13O3Cl	M+H	277.06259	245.036383	C14H10ClO2	227.070271	C14H11O3	215.025819	C13H8ClO	W	[1],[2],[3]
103	Dimethomorph metabolite (4-formylmorpholine)	113009-82-8	C5H9O2N	M+H	116.07060	98.0600404	C5H8NO	71.0491414	C4H7O			W	[1],[2],[3]
104	Dimoxystrobin metabolite [(E)-0-[(2-hydroxycarbonyl-5-methyl)phenoxymethyl]-2-methoxyimino-Nmethylphenylacetamide]		C19H20N2O5	M+H	357.14449							W	[1],[2],[3]
105	Diuron metabolite [1-(3,4-dichlorophenyl)-3-methylurea]	3567-62-2	C8H8Cl2N2O	M+H	219.0086	204.992994	C7H7Cl2N2O	187.966446	C7H4Cl2NO	159.97272		S	[2],[3]

Tabla SI-3. Capítulo 1. (Continuación).

Nº	Compound	CAS number	Elemental composition	Monitored ion	Monitored Mass	Frag. 1	Structure	Frag. 2	Structure	Frag.3	Structure	Matrix(*)	Ref
107	Diuron metabolite(3,4-dichloroaniline)	95-76-1	C6H5Cl2N	M+H	161.98718	144.960632	C6H3Cl2	126.010504	C6H5ClN	91.04165	C6H5N	S,An	[2],[3]
108	Ethiofencarb metabolite (Ethiofencarb-sulfoxide)	53380-22-6	C11H15NO34S	M+H	242.08454	107.0491	C7H7O	185.0631	C9H13O2S			S	[4]
109	Fenamiphos metabolite (fenamiphos-sulfone-phenol)		C8H10O3S	M+H	187.04234	169.031776	C8H9O2S	155.016127	C7H7O2S	107.049141	C7H7O	S	[1],[2],[3]
110	Fenhexamid metabolite(Deschlorofenhexamid)	1335041-78-5	C14H19NO2	M+H	234.14886	216.138290	C14H18NO	142.122641	C8H16NO	125.096092	C8H13O	S,W	[2],[3]
111	Fenhexamid metabolite(Biphenyl-fenhexamid)		C28H32Cl4N2O4	M+H	601.11889	565.142217	C28H32Cl3N2O4	317.904151	C12H4Cl4NO	175.966446	C6H4Cl2NO	S	[2],[3]
112	Fenitrothion metabolite(3-methyl-4-nitrophenol)	2581-34-2	C7H7NO3	M+H	154.04987	136.039305	C7H6NO2	124.051881	C7H8O2	107.049141	C7H7O	S	[2],[3]
113	Fenitrothion metabolite (3-methyl-4-nitrophenol)		C7H7NO3	M-H	152.03531	122.03733	C7H7O2					S	[12]
114	Fenitrothion metabolite(Dimethyl-3-methyl-4-nitrophenyl phosphate)	2255-17-6	C8H10NO6P	M+H	262.04750	215.046771	C9H12O4P	201.031122	C8H10O4P	138.01857	C6H4NO3	A	[3],[10]
115	Fenitrothion metabolite(Methyl 3-methyl-4-nitrophenyl hydrogen phosphate)	15930-84-4	C9H12NO6P	M+H	248.03185	152.034219	C7H6NO3	230.021285	C8H9NO5P	201.031122	C8H10O4P	A	[3],[10]
116	Fipronil metabolite(Fipronil amide)		C12H6Cl2F6N4O2S	M+H	454.95655	418.979869	C12H6ClF6N4O2S	368.983063	C11H6ClF4N4O2S	336.986527	C11H6Cl2F3N4O	S	[2],[3]
117	Fipronil metabolite(Fipronil sulphide)	120067-83-6	C12H4Cl2F6N4S	M+H	420.95107	403.924519	C12H2Cl2F6N3S	393.940169	C11H4Cl2F6N3S	318.975963	C11H4Cl2F3N4	S	[2],[3]
118	Fipronil metabolite (Fipronil sulfone)	120068-36-2	C12H4Cl2F6N4O2S	M+H	452.94090	435.914348	C12H2Cl2F6N3O2S	425.929998	C11H4Cl2F6N3O2S	212.948017	C7H2Cl2F3	S	[4]
119	Fludioxonil meyabolite [3-Carbamoyl-2-cyano-3-(2,2-difluorobenzo[1,3]-dioxo-4-yl)-oxirane-2-carboxylic acid]		C12H6F2N2O6	M+H	313.02666	296.000119	C12H4F2NO6	286.01577	C11H6F2NO6	268.005205	C11H4F2NO5	W	[1],[2],[3]
120	Fludioxonil meyabolite [2,2-difluorobenzo[1,3]dioxole-4-carboxylic acid]	126120-85-2	C8H4F2O4	M+H	203.01504	185.004476	C8H3F2O3	157.009562	C7H3F2O2	137.023321	C7H5O3	W	[1],[2],[3]
121	Fludioxonil meyabolite [4-(2,2-difluoro-benzo[1,3]dioxol-4-yl)-2,5-dioxo-2,5-dihydro-1H-pyrrole-3-carbonitrile]		C12H6F2N2O4	M+H	279.02119	254.025940	C11H6F2NO4	238.031026	C11H6F2NO3	215.045119	C11H7N2O3	W	[1],[2],[3]
122	Flufenacet metabolite [[(4-Fluorophenyl)isopropylcarbamoyl]-methanesulfonic acid]		C11H14FNO4S	M+H	276.07003	242.064554	C11H14NO4S	124.055704	C7H7FN			W	[1],[2],[3]
123	Flufenacet metabolite [1,3,4,-Thiadiazol-2(3h)-one, 5-(trifluoromethyl)-]	84352-75-0	C3HF3N2OS	M+H	170.98344	142.98853	C2H2F3N2S	112.966732	C2F3S			W	[1],[2],[3]
124	Fluopicolide metabolite (3-Sulfo-5-trifluoromethylpyridine-2-carboxylic acid)		C7H4F3NO5S	M+H	271.98350	201.980469	C7H3F3NO4S	190.01104	C6H4NO5S			W	[1],[2],[3]
125	Fluopicolide metabolite (dichlorbenzamide)	4659-54-4	C7H5Cl2NO	M+H	189.98209	162.971197	C7H3Cl2O	154.005418	C6H5Cl2O			W	[1],[2],[3]
126	Fluopicolide metabolite (3-Chloro-5-trifluoromethylpyridine-2-carboxylic acid)	80194-18-9	C7H5ClF3NO2	M+H	225.98771	209.992802	C7H4ClF3NO	192.02669	C7H5F3NO2	155.984683	C6H3ClNO2	W	[1],[2],[3]
127	Flupyrsulfuron metabolite (Flupyrsulfuron-methyl)	144740-53-4	C15H14F3N5O7S	M+H	466.06387	434.037664	C14H11F3N5O6S	406.04275	C13H11F3N5O5S	267.98859	C8H5F3N04S	W	[1],[2],[3]

Tabla SI-3. Capítulo 1. (Continuación).

Nº	Compound	CAS number	Elemental composition	Monitored ion	Monitored Mass	Frag. 1	Structure	Frag. 2	Structure	Frag.3	Structure	Matrix(*)	Ref
128	Fluquinconazole metabolite [3-(2,4-dichlorophenyl)-6-fluoro-quinazolin-2,4(3H)-dione]	168900-02-5	C14H7Cl2FN2O2	M+H	324.99414	289.017459	C14H7ClFN2O2	281.988324	C13H7Cl2FNO	144.960632	C6H3Cl2	S	[2],[3]
129	Fluquinconazole metabolite (1,2,4-triazole)	288-88-0	C2H3N3	M+H	70.03997							S,GW,Pl, An	[2],[3]
130	Flurtamone metabolite (2,2,2-Trifluoroacetic acid)	76-05-1	C2HF3O2	M+H	115.00014	96.9895758	C2F3O	94.9939122	C2HF2O2			W	[1],[2], [3]
131	Flusilazole metabolite [(bis(4-fluorophenyl)methyl silanol]	156162-13-9	C13H12SiOF2	M+H	251.06982	233.059259	C13H11F2Si	231.063596	C13H12OFSi	113.03972	C6H6FO	S,An	[2],[3]
132	Glufosinate metabolite (3-methylphosphinico-propionic acid)	15090-23-0	C4H9O4P	M+H	153.03112	135.020557	C4H8O3P	107.025643	C3H8O2P	93.0099925	C2H6O2P	W	[1],[2], [3]
133	Glyphosphate metabolite (Aminomethylphosphonic acid)	1066-51-9	CH6NO3P	M+H	112.01580	94.989257	CH4O3P	94.0052415	CH5NO2P			W	[1],[2], [3]
134	Haloxyfop metabolite (DE-535 pyridinol)	76041-71-9	C6H3ClNOF3	M+H	197.99280	179.982238	C6H2ClF3N	162.016125	C6H3F3NO			W	[1],[2], [3]
135	Imazalil metabolite[1-(2,4-dichlorophenyl)2-imidazol-1-ylethanol]	24155-42-8	C11H10Cl2N2O	M+H	257.02429	239.013730	C11H9Cl2N2	227.972107	C11H1OClN2O	188.986847	C8H7Cl2O	S,W	[2],[3]
136	Imazalil metabolite (Methyl isothiocyanate)	556-61-6	C2H3NS	M+H	74.0059							S	[2],[3]
137	Imazosulfuron (2-Chloroimidazo[1,2-a]pyridin-3-sulfonamid)		C7H6ClN3O2S	M+H	231.99420	214.967652	C7H4ClN2O2S	196.017524	C7H6N3O2S	151.005752	C7H4ClN2	W	[1],[2], [3]
138	Imidaclorprid metabolite [1-[(6-chloro-3-pyridinyl)methyl]N-nitro-1H-imidazol-2-amine]	115086-54-9	C9H8ClN5O2	M+H	254.04393	218.067251	C9H8N5O2	192.032302	C9H7ClN3	126.010504	C6H5ClN	S,An , Pl	[2],[3]
139	Imidaclorprid metabolite (6-chloronicotinic acid)	5326-23-8	C6H4ClNO2	M+H	158.00033	139.989768	C6H3ClNO	122.023655	C6H4NO2	111.994853	C5H3ClN	W	[2],[3]
140	Iprodione metabolite [N-(3,5-dichlorophenyl)3-isopropyl-2,4-dioxoimidazoline-1-carboxamide]	63637-89-8	C13H13Cl2N3O3	M+H	330.04067	294.063995	C13H13ClN3O3	283.998808	C11H8Cl2N3O2	270.967174	C10H5Cl2N2O3	W	[1],[2], [3]
141	Iprovalicarb metabolite(p-methyl-phenethylamine)	3261-62-9	C9H13N	M+H	136.11208	91.05393	C7H7					S	[2],[3]
142	Isoproturon metabolite (p-methyl-phenethylamine)	3261-62-9	C9H13N	M+H	136.11208	119.085526	C9H11	118.065126	C8H8N	105.069877	C8H9	S	[2],[3]
143	Isoproturon metabolite (Desmethylisoproturon)	34123-57-4	C11H16N2O	M+H	193.13354	119.085527	C9H11	179.11789	C10H15N2O	151.08650	C8H11N2O	W	[5]
144	Kresoxim methyl metabolite [(E)-methoxyamino(alpha-(o-tolyloxy)-o-tolyl)acetic acid]		C17H17NO4	M+H	300.12303							S,W	[2],[3]
145	Linuron metabolite (3,4-dichloroaniline)	95-76-1	C6H5Cl2N	M+H	161.9872	144.960632	C6H3Cl2	126.010504	C6H5ClN	91.0416508	C6H5N	S,An	[2],[3]
146	Malathion metabolite(ethyl ester-2-hydroxyl-3-thionyl 2-butenoic acid)		C8O5SH12	M+H	221.04782	187.060099	C8H11O5	161.026691	C6H9O3S	151.005956	C4H7O4S	W	[3],[6]
147	Malathion metabolite(Diethyl malate)	7554-12-3	C8H14O5	M+H	191.0914	161.044449	C6H9O5	157.085921	C8H13O3	145.049535	C6H9O4	W	[3],[6]
148	Malathion metabolite[(7S,8R,9S)-7,8,9-Trihydroxy-6-oxaspiro[4.5]dec-7-yl]methyl dihydrogen phosphate)		C10H19O8P	M+H	299.08903	267.062815	C9H16O7P	253.047166	C8H14O7P	209.020951	C6H10O6P	W	[3],[6]
149	Malathion metabolite(Diethylsuccinate)	123-25-1	C8H14O4	M+H	151.09648	145.049535	C6H9O4	121.085921	C5H13O3			W	[3],[6]
150	Malathion metabolite(Dimethyl 2,3-dihydroxy-2,3-dimethylsuccinate)	15309-47-4	C8H14O6	M+H	207.08631	177.039364	C6H9O6	157.085921	C8H13O3	145.049535	C6H9O4	W	[3],[6]

Tabla SI-3. Capítulo 1. (Continuación).

Nº	Compound	CAS number	Elemental composition	Monitored ion	Monitored Mass	Frag. 1	Structure	Frag. 2	Structure	Frag.3	Structure	Matrix(*)	Ref
151	Malathion metabolite (diethylmaleate)	141-05-9	C8H12O4	M+H	173.08083	143.033885	C6H7O4	129.054621	C6H9O3	127.038971	C6H7O3	W	[3],[6]
152	Malathion metabolite (Malathion dicarboxylic acid)	1190-28-9	C6H11O6PS2	M+H	274.98074	256.970177	C6H10O5PS2	226.959613	C5H8O4PS2	200.980349	C4H10O3PS2	S	[2],[3]
153	Malathion metabolite (Malathion dicarboxilid acid)		C6H11O6PS2	M-H	272.96619	-	-					S	[12]
154	Malathion metabolite (Malathion monocarboxylic acid)	35884-76-5	C8H15O6PS2	M+H	303.01204	285.001477	C8H14O5PS2	270.985828	C7H12O5PS2	257.006563	C7H14O4PS2	S	[2],[3]
155	Malathion metabolite (Isomalathion)	3344-12-5	C10H19O6PS2	M+H	331.04334	124.982063	C2H6O2PS	173.080835	C8H13O4	236.998107	C7H10O5SP	PChI	[2],[3]
156	Malathion metabolite [diethyl (dimethoxy-phosphoryl) succinate]		C10H19O8P	M+H	331.07886	267.062815	C9H16O7P	253.047166	C8H14O7P	209.020951	C6H10O6P	W	[3],[6]
157	Malathion metabolite [diethyl (methyl sulphonyl) succinate]		C9O6H16S	M+H	252.06621	223.027085	C7H11O6S	219.032171	C8H11O5S	203.037256	C8H11O4S	W	[3],[6]
158	Malathion metabolite [2-hydroxyl-3-thionyl-2-butene-diethylester]		C8H12O5S	M+H	221.04782	203.037256	C8H11O4S	187.0601	C8H11O5	179.037256	C6H11O4S	W	[3],[6]
159	Malathion metabolite (malaoxon)	1634-78-2	C10H19O7PS	M+H	315.06618	129.054621	C6H9O3	142.992628	C2H8O3PS	127.01542	C2H8O4P	A	[3],[6]
160	Mancozeb metabolite (Ethylenethiourea)	13966-32-0	C3H6N2S	M+H	103.03244	77.0167955	CH5N2S					W	[1],[2],[3]
161	Mancozeb metabolite (Ethyleneurea)	120-93-4	C3H6N2O	M+H	87.055289	61.0396394	CH5N2O					W	[1],[2],[3]
162	Metalaxyl metabolite[N-(2,6-dimethylphenyl)-N-(methoxyacetyl)alanine]	467430-42-8	C14H19NO4	M+H	266.13868	180.1015	C10H12O3	95.08516	C7H11	213.15	C14H19NO4	S	[2],[3]
163	Metalaxyl metabolite [(R)-2-[(2,6-dimethyl-phenyl)-methoxyacetyl-amino]-propionic acid]	75596-99-5	C14H19NO4	M+H	266.13868	248.128119	C14H18NO3	206.117555	C12H16NO2	164.106991	C10H14NO	W	[1],[2],[3]
164	Metalaxyl metabolite [2-[(1-carboxyethyl)-methoxyacetyl-amino]-3-methyl-benzoic acid]	104390-56-9	C14H17NO6	M+H	296.11286	278.102299	C14H16NO5	252.123035	C13H18NO4			W	[1],[2],[3]
165	Metamitron metabolite [4,5-dihydro-3-methyl-6-phenyl-1,2,4-triazin-5-one]	36993-94-9	C10H9N3O	M+H	188.08183	162.066188	C8H8N3O	145.076025	C9H9N2			W	[1],[2],[3]
166	Metazachlor metabolite [N-(2,6-dimethylphenyl)-N-(1H-pyrazol-1-ylmethyl)aminocarbonylmethylsulfonic acid]	172960-62-2	C14H17N3O4S	M+H	324.10125	290.095774	C14H16N3O2S	256.063805	C11H14NO4S			W	[1],[2],[3]
167	Metazachlor metabolite [methyl N-(2,6-dimethylphenyl)-N-(1Hpyrazol-1-ylmethyl)aminocarbonylmethylsulfoxide]		C15H19N3O2S	M+H	306.12707	242.128788	C14H16N3O	238.089626	C12H16NO2S			W	[1],[2],[3]
168	Metazachlor metabolite [N-[(2-hydroxycarbonyl-6-methyl)phenyl]-N-(1-H-pyrazol-1-ylmethyl)oxalamide]		C14H13N3O5	M+H	304.09279	286.082232	C14H12N3O4	244.071668	C12H10N3O3			W	[1],[2],[3]
169	Metazachlor metabolite [N-(2,6-dimethylphenyl)-N-(1H-pyrazol-1-ylmethyl)aminocarbonylmethylsulfinyl acid]		C16H19N3O4S	M+H	350.11690	322.121988	C15H20N3O3S	304.111424	C15H18N3O2S			W	[1],[2],[3]
170	Metazachlor metabolite [N-(2,6-dimethylphenyl)-N-(1H-pyrazol-1-ylmethyl)oxalamide]		C14H15N3O3	M+H	274.11861	256.108053	C14H14N3O2	230.128789	C13H16N3O			W	[1],[2],[3]
171	Methamidophos	10265-92-6	C2H8NO2PS	M+H	142.00861	94.00534	CH4O2NP					S	[12]

Tabla SI-3. Capítulo 1. (Continuación).

Nº	Compound	CAS number	Elemental composition	Monitored ion	Monitored Mass	Frag. 1	Structure	Frag. 2	Structure	Frag.3	Structure	Matrix(*)	Ref
172	Methiocarb Metabolite(Methiocarb-sulfone)	2179-25-1	C11H15NO4S	M+H	258.07945	201.058	C9H13O3S	122.0726	C8H10O			S	[4]
173	Methiocarb Metabolite(Methiocarb-sulfoxide)	2635-10-1	C11H15NO3S	M+H	242.08454	185.0631	C9H13O2S	170.0396	C8H10O2S			S	[4]
174	Methyl-Thiophanate metabolite (Carbendazim)	10605-21-7	C9H9N3O2	M+H	192.07675	160.05035	C8H6N3O					S	[2],[4]
175	Metolachlor metabolite(Metolachlor ethane sulfonic acid)	171118-09-5	C15H23NO5S	M+H	330.13697	316.12132	C14H22NO5S	310.110755	C15H20NO4S	268.100191	C13H18NO3S	S	[2],[3]
176	Metolachlor metabolite(Metolachlor oxanilic acid)	152019-73-3	C15H21NO4	M+H	280.15433	262.14377	C15H20NO3	246.11247	C14H16NO3	194.08117	C10H12NO3	S,W	[2],[3]
177	Metolachlor metabolite [(S)-2-[(Oxalyl)(2-ethyl-6-methylphenyl)amino]propionic acid]		C14H17NO5	M+H	280.11794	266.102299	C13H16NO5	244.09682	C14H14NO3	218.117555	C13H16NO2	W	[1],[2],[3]
178	Metolachlor metabolite [(2-Ethyl-6-methylphenyl)-carbamoyl]-methanesulfonic acid]		C11H15NO4S	M+H	258.07945	244.063805	C10H14NO4S	224.073976	C11H14NO2S	176.106991	C11H14NO	W	[1],[2],[3]
179	Metolachlor metabolite [N-(2-Ethyl-6-methylphenyl)-2-hydroxyacetamide]	97055-05-5	C11H15NO2	M+H	194.11755	176.106990	C11H14NO	148.112076	C10H14N	136.112076	C9H14N	W	[1],[2],[3]
180	Metolachlor metabolite [2-[((S)-1-Carboxyethyl)(2-ethyl-6-methylphenyl)amino]2-oxo-ethanesulfonic acid]		C14H19NO6S	M+H	330.10058	316.084934	C13H18NO6S	312.09002	C14H18NO5S	232.133205	C14H18NO2	W	[1],[2],[3]
181	Metolachlor metabolite [N-(2-Ethyl-6-methylphenyl)-L-alanine	82508-03-0	C12H17NO2	M+H	208.13320	194.117555	C11H16NO2	148.112076	C10H14N	135.10420	C9H13N	W	[1],[2],[3]
182	Metolachlor metabolite [N-(2-Ethyl-6-methylphenyl)-oxalamic acid]	152019-74-4	C11H13NO3	M+H	208.09682	190.086255	C11H12NO2	164.106991	C10H14NO	136.112076	C9H14N	W	[1],[2],[3]
183	Metolachlor metabolite [2-[((S)-1-Carboxyethyl)(2-ethyl-6-methylphenyl)amino]-2-oxo-ethanesulfonic acid disodium salt]	1418095-19-8	C14H17NNa2O6S	M+H	374.06447	356.053909	C14H16NNa2O5S	346.033174	C12H14NNa2O6S	280.0614	C11H15NNaO4S	W	[1],[2],[3]
184	Metolachlor metabolite (Metolachlor mercapturate)	159956-64-6	C20H30N2O5S	M+H	411.19481	282.15088	C15H23O2NS					-	[12]
185	Myclobutanil metabolite(1,2,4-triazole	288-88-0	C2H3N3	M+H	70.03997							S, GW, Pl, An	[2]
186	Myclobutanil metabolite (1H-1,2,4-triazol-1-ylacetic acid)	28711-29-7	C4H5N3O2	M+H	128.04545	98.0348883	C3H4N3O	82.0399737	C3H4N3	112.050538	C4H6N3O	S,An ,Pl	[2],[3]
187	Molinate metabolite (Molinate sulfoxide)	52236-29-0	C9H17NO2S	M+H	204.10528	174.058325	C7H12NO2S	147.034851	C5H9NO2S	126.091341	C7H12NO	S	[2],[3]
188	Napropamide metabolite [α-naphthol-2-methyl-naphthol(1,2-b)-2Hfuran-3-one]		C13H10O2	M+H	199.07535	161.059706	C10H9O2	143.049141	C10H7O	127.054227	C10H7	W	[1],[2],[3]
189	Napropamide metabolite [(N,N,N',N',Tetraethyl -4.4'-dihydroxyalpha,alpha'-2'-dimethyl[1,1-binaphthalene]-3,3'-diacetamide]		C34H40N2O4	M+H	541.30608	495.264219	C32H35N2O3	468.216935	C30H30NO4	414.20637	C27H28NO3	W	[1],[2],[3]
190	Napropamide metabolite [N,N-diethyl-4-hydroxy-α-methyl-2-naphthaleneacetamide]		C17H21NO2	M+H	272.16450	256.133205	C16H18NO2	254.153941	C17H20NO	199.075356	C13H11O2	W	[1],[2],[3]
191	Napropamide metabolite [N,N-diethyl-4-hydroxy-α-methyl-1-naphthaleneacetamide]		C17H21NO2	M+H	272.16450	254.15394	C17H20NO	216.10191	C13H14NO2	199.07536	C13H11O2	W	[1],[2],[3]

Tabla SI-3. Capítulo 1. (Continuación).

Nº	Compound	CAS number	Elemental composition	Monitored ion	Monitored Mass	Frag. 1	Structure	Frag. 2	Structure	Frag.3	Structure	Matrix(*)	Ref
192	Organophosphate metabolite (Dimethyl thiophosphate)	1112-38-5	C2H6O3PS	M-H	140.97807	125.95462	CH4O3PS					-	[12]
193	Organophosphate metabolite (Dimethyl dithiophosphate)	756-80-9	C2H5O2PS2	M-H	156.95523	141.93174	CH4O2PS2					-	[12]
194	Organophosphate metabolite (Diethyl phosphate)	598-02-7	C4H11O4P	M-H	153.03221	125.00102	C2H7O4P					-	[12]
195	Organophosphate metabolite (Diethyl thiophosphate)	298-06-6	C4H11O3PS	M-H	169.00937	140.97815	C2H7O3PS					-	[12]
196	Organophosphate metabolite (Diethyl dithiophosphate)		C4H11O2PS2	M-H	184.98653	156.95523	C2H7O2PS2					-	[12]
197	Parathion, methyl parathion metabolite (4-nitrophenol)	100-02-7	C6H5NO3	M-H	138.01966	108.02156	C6H5O2					-	[12]
198	Paclobutrazol metabolite [4H-1,2,4-triazol-3-ol]	122442-66-4	C2H3N3O	M+H	86.034888	68.0243237	C2H2N3					W	[1],[2], [3]
199	Paclobutrazol metabolite [(2RS)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1H-1,2,4 triazol-1-yl) pentan-3-one]	63190-87-4	C15H18ClN3O	M+H	292.12111	262.074166	C13H13ClN3O	256.144439	C15H18N3O	223.088419	C13H16ClO	W	[1],[2], [3]
200	Permethrin, cypermethrin, cyfluthrin metabolite [cis-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid]	55701-05-8	C8H10O2Cl2	M-H	206.9985	-	-						[12]
201	Permethrin, cypermethrin, cyfluthrin metabolite [trans-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid]	55701-05-8	C8H10O2Cl2	M-H	206.9985	-	-						[12]
202	Pethoxamid metabolite [N-(2-Ethoxyethyl)-N-(2-methyl-1-phenylpropenyl)-2-sulfoacetamide]		C16H23NO5S	M+H	342.13697	308.131490	C16H22NO3S	300.09002	C13H18NO5S	270.079455	C12H16NO4S	W	[1],[2], [3]
203	Phenmedipham metabolite [Methyl-N-(3-hydroxyphenyl)-carbamate]	13683-89-1	C8H9NO3	M+H	168.06551	151.06278	C8H9NO2	110.06004	C6H8NO	135.03148	C7H5NO2	W	[1],[2], [3]
204	Picolinafen metabolite [6-[(3-trifluoromethylphenoxy)picolinic acid]	137640-84-7	C13H8F3NO3	M+H	284.05290	237.0396	C12H6F3NO	214.04987	C12H8NO3	198.054955	C12H8NO2	W	[1],[2], [3]
205	Pinoxaden metabolite [8-(2,6-diethyl-4-methyl-phenyl)-tetrahydropyrazolo[1,2-d][1,4,5]oxadiazepine-7,9-dione]	314020-44-5	C18H24N2O4	M+H	333.180883	315.170319	C18H23N2O3	275.139019	C15H19N2O3	177.127392	C12H17O	W	[1],[2], [3]
206	Pirmicarb metabolite [2-amino-5,6-dimethylpyrimidin-4-yldimethylcarbamate]		C9H14N4O2	M+H	211.11895	195.087652	C8H11N4O2	182.092403	C8H12N3O2	124.050538	C5H6N3O	W	[1],[2], [3]
207	Pirmicarb metabolite [5,6-dimethyl-2-(methylamino)pyrimidin-4-ol]	78195-30-9	C7H11N3O	M+H	154.09748	152.081838	C7H10N3O	123.055289	C6H7N2O	135.079099	C7H9N3	W	[1],[2], [3]
208	Pirmicarb metabolite [2-dimethylamino-5,6-dimethylpyrimidin-4-ol]	40778-16-3	C8H13N3O	M+H	168.11313	150.102574	C8H12N3	136.050538	C6H6N3O	124.063114	C6H8N2O	W	[1],[2], [3]
209	Propachlor metabolite (propachlor sulphonic acid)		C11H15NO4S	M+H	258.07945	224.073975	C11H14NO2S	176.106991	C11H14NO	160.112076	C11H14N	W	[1],[2], [3]
210	Propachlor metabolite (propachlor sulphinylacetic acid)		C13H17NO4S	M+H	284.09510							W	[1],[2], [3]
211	Propachlor metabolite (propachlor oxanilic acid)	70628-36-3	C11H13NO3	M+H	208.09682	190.086255	C11H12NO2	150.054955	C8H8NO2	132.06552	C5H10NO3	W	[1],[2], [3]
212	Propachlor metabolite (propachlor alcohol)	42404-06-8	C11H15NO2	M+H	194.11756	152.070605	C8H10NO2	163.06278	C9H9NO2	132.080776	C9H1ON	W	[1],[2], [3]
213	Propaquizafop metabolite (Quizalofop)	76578-12-6	C17H13ClN2O4	M+H	345.06366	327.053096	C17H12ClN2O3	309.086983	C17H13N2O4	165.054621	C9H9O3	W	[4]
214	Propazine Metabolite(hydroxypropazine)	7374-53-0	C9H17N5O	M+H	212.15059	196.00		112.00				S	[2],[3]

Tabla SI-3. Capítulo 1. (Continuación).

Nº	Compound	CAS number	Elemental composition	Monitored ion	Monitored Mass	Frag. 1	Structure	Frag. 2	Structure	Frag.3	Structure	Matrix(*)	Ref
215	Propineb metabolite (4-Methyl-imidazolidin-2-thione)	2122-19-2	C4H8N2S	M+H	117.04809	101.016795	C3H5N2S	82.0525498	C4H6N2			W	[1],[2], [3]
216	Propyzamide metabolite (2-(3,5-dichlorophenyl)-4,4-dimethyl-5-methylene-2-oxazoline)		C12H11Cl2NO	M+H	256.02904	220.052368	C12H11ClNO	201.982096	C8H6Cl2NO	162.971197	C6H5Cl2O	W	[1],[2], [3]
217	Propyzamide metabolite [3,5-dichloro-N-(1,1-dimethyl)-2-oxo-npropyl)benzamide]	29939-97-7	C12H13Cl2NO2	M+H	274.03961	252.066006	C12H13ClN2O2	230.013396	C10H10Cl2NO	203.997746	C8H8Cl2NO	W	[1],[2], [3]
218	Prothioconazole metabolite (1H-1,2,4-Triazole-1-ethanol, alpha-(1-chlorocyclopropyl)-alpha-(2-chlorophenyl)methyl)		C14H15Cl2N3O	M+H	312.06649	294.055929	C14H14Cl2N3	284.035194	C12H12Cl2N3O	211.007582	C11H9Cl2	W	[1],[2], [3]
219	Pyraflufen-ethyl metabolite [2-chloro-5-(4-chloro-5-difluoromethoxy-1-methylpyrazol-3-yl)-4-fluorophenoxyacetate]	129630-17-7	C13H9Cl2F3N2O4	M+H	384.99642	366.985858	C13H8Cl2F3N2O3	349.019746	C13H9ClF3N2O4	316.989052	C12H8Cl2FN2O3	W	[1],[2], [3]
220	Pyraflufen-ethyl metabolite [2-chloro-5-(4-chloro-5-difluoromethoxy-1-methylpyrazole-3yl)-4-fluorophenol]		C11H7Cl2F3N2O2	M+H	326.99094							W	[1],[2], [3]
221	Pyrethroid metabolite (3-Phenoxybenzoic acid)	3739-38-6	C13H10O3	M-H	213.05571	169.06572	C12H10O						[12]
222	Pyridaben metabolite [2-tert-butyl-4-(4-tertbutylbenzoyl)pyridazin-3(2H)-one-5-sulfonic acid]		C19H24N2O5S	M+H	393.14786							W	[1],[2], [3]
223	Pyridaben metabolite [2-tert-butyl-5-(4-tert-butylbenzylsulfinyl)-4-chloropyridazin-3(2H)-one]		C19H25ClN2O2S	M+H	381.13980	307.066638	C15H16ClN2OS	247.030253	C9H12ClN2O2S	233.014603	C8H10ClN2O2S	W	[1],[2], [3]
224	Pyridaben metabolite [2-tert-butyl-5-[4-(1-carboxy-1-methylethyl)benzylthio]-4-chloropyridazin-3(2H)-one]		C19H23ClN2O3S	M+H	395.11906							W	[1],[2], [3]
225	Pyridate metabolite (6-chloro-3-phenylpyridazin-4-ol)	40020-01-7	C10H7ClN2O	M+H	207.031967	104.0495	C7H6N	189.021402	C10H6ClN2	171.055289	C10H7N2O	W	[3], [10]
226	Pyrimiphos methyl metabolite [Phosphoric acid, 2-(diethylamino)-5-methyl-4-pyrimidinyl dimethyl ester]		C11H20N3O4P	M+H	290.12641	274.095119	C10H17N3O4P	258.100204	C10H17N3O3P	219.05292	C7H12N2O4P	A	[3],[7]
227	Pyrimiphos methyl metabolite[2-diethylamino-6-methyl-4-pyrimidinol]	42487-72-9	C9H15N3O	M+H	182.12879	166.097488	C8H12N3O	154.09748	C7H12ON3	150.102574	C8H12N3	A	[3],[7]
228	Pyrimiphos methyl metabolite (4-[4-(Diethoxyphosphino)-1H-1,2,3-triazol-1-yl]butanoic acid)		C10H18N3O4P	M+H	276.11077	244.084554	C9H15N3O3P	228.053254	C8H11N3O3P	215.045429	C7H10N3O3P	A	[3],[7]
229	Pyroxsulam metabolite (Aminotriazole)	61-82-5	C9H9F3N6O3S	M+H	338.04034	319.041941	C9H9F2N6O3S	292.047442	C9H9F3N5OS	172.017524	C5H6N3O2S	W	[1],[2], [3]
230	Pyroxsulam metabolite (5,7-Dihydroxy-Pyroxsulam)		C12H9F3N6O5S	M+H	406.03017	375.011784	C11H6F3N6O4S	337.034965	C11H9N6O5S	225.978025	C6H3F3NO3S	W	[1],[2], [3]
231	Pyroxsulam metabolite (7-Hydroxy-Pyroxsulam)		C13H11F3N6O5S	M+H	420.04582	389.027434	C12H8F3N6O4S	351.050615	C12H11N6O5S	208.975285	C6H2F3NO2S	W	[1],[2], [3]
232	Quinmerac metabolite (7-chloro-2-hydroxy-3-methylquinoline-8-carboxylic acid)		C11H8ClNO3	M+H	238.02654	220.015982	C11H7ClNO2	202.04987	C11H8NO3	192.021068	C10H7ClNO	W	[1],[2], [3]

Tabla SI-3. Capítulo 1. (Continuación).

Nº	Compound	CAS number	Elemental composition	Monitored ion	Monitored Mass	Frag. 1	Structure	Frag. 2	Structure	Frag.3	Structure	Matrix(*)	Ref
233	Quinmerac metabolite (7-chloro-3,8-quinoline-dicarboxylic acid)	90717-07-0	C11H6ClNO4	M+H	252.00581	233.995247	C11H5ClNO3	216.029134	C11H6NO4	154.989434	C7H4ClO2	W	[1],[2],[3]
234	Simazine metabolite (6-deisopropyl atrazine)	1007-28-9	C5H8ClN5	M+H	174.05410	157.027550	C5H6ClN4	146.022799	C3H5ClN5	111.053947	C3H5N5	S	[2],[3]
235	Simazine metabolite (2-hydroxy-4,6-bis(ethlyamino)-triazine)	2599-11-3	C7H13N5O	M+H	184.11929	166.108722	C7H12N5	139.061437	C5H7N4O	111.030137	C3H3N4O	S	[2],[3]
236	Spirodiclofen metabolite (3-(2,4-dichlorophenyl)-4-hydroxy-1-oxaspiro[4,5]dec-3-en-2-one)	148476-22-6	C15H14Cl2O3	M+H	313.03927	277.062598	C15H14ClO3	242.961026	C10H5Cl2O3	158.976282	C7H5Cl2	W	[1],[2],[3]
237	Sulcotrione metabolite (2-chloro-4-(methylsulfonyl)-benzoic acid)	53250-83-2	C8H7ClO4S	M+H	234.98263	199.005955	C8H7O4S	188.977154	C7H6ClO2S	171.011041	C7H7O3S	W	[1],[2],[3]
238	Tebuconazole metabolite (1,2,4-triazole)	288-88-0	C2H3N3	M+H	70.03997							S, GW, Pl, An	[2],[3]
239	Tebufenozide metabolite[4-(N'-(3,5-dimethylbenzoyl-N-(1,1-dimethylethyl)hydrazinocarbonyl)phenyl acetic acid]		C22H26N2O4	M+H	383.19653	323.175404	C20H23N2O2	281.128454	C17H17N2O2	165.054621	C9H9O3	S,W, Pl	[2],[3]
240	Tebufenozide metabolite [N-(1,1-dimethyethyl)-N-(4-acetylebenzoyl)-3,5-dimethylbenzohydrazine]		C22H26N2O3	M+H	367.20162	323.175404	C20H23N2O2	309.123369	C18H17N2O3	149.059706	C9H9O2	S, GW, Pl, An	[2],[3]
241	Tepraloxydim metabolite [(RS)-2-ethyl-6,7-dihydro-6-perhydropyran-4-ylbenzoxazol-4-(5H)-one]		C14H21NO3	M+H	250.14377	209.153606	C13H21O2	197.117221	C11H17O3	179.106656	C11H15O2	W	[1],[2],[3]
242	Tepraloxydim metabolite [3-hydroxy-2-(1-iminopropyl)-5-perhydropyran-4-ylcyclohex-2-en-1-one]		C14H21NO3	M+H	252.15942	235.132871	C14H19O3	224.12812	C12H18NO3	179.106656	C11H15O2	W	[1],[2],[3]
243	Terbuthylazine Metabolite(Terbuthylazine-2-hydroxy)	66753-07-9	C9H17N5O	M+H	212.15059	156.08802	C5H10N5O	114.06604	C4H8N3O	86.03456	C2H4ON3	S,Sed,W	[2],[4]
244	Terbuthylazine Metabolite(Desethyl-terbuthylazine)	30125-63-4	C7H12ClN5	M+H	202.0854	185.058850	C7H10ClN4	166.108722	C7H12N5	128.99625	C3H2ClN4	S,Sed,W	[2],[3]
245	Tetraconazole metabolite [2-(2,4-dichlorophenyl)-3-(1H-1,2,4-triazol-1-yl)propan-1-ol]		C13H12Cl2N6O	M+H	339.05224	254.024629	C11H1OCl2N3	236.058516	C11H11ClN3O	172.991932	C8H7Cl2	S,An	[2],[3]
246	Tetraconazole metabolite [2-(2,4-dichlorophenyl)-3-(1H-1,2,4-triazol-1-yl)propanoic acid]		C11H9Cl2N3O2	M+H	286.01446	268.003893	C11H8Cl2N3O	250.037781	C11H9ClN3O2	216.981761	C9H7Cl2O2	S,An	[2],[3]
247	Tetraconazole metabolite [1H-1,2,4-triazol-1-ylacetic acid]	28711-29-7	C4H5N3O2	M+H	128.04545	98.0348883	C3H4N3O	82.0399737	C3H4N3	112.050538	C4H6N3O	S,An, Pl	[2],[3]
248	Thiacloprid metabolite (Thiacloprid sulfonic acid)		C10H13ClN4SO5	M+H	337.03679	303.031315	C10H12ClN4O3S	301.060117	C10H13N4SO5	229.04868	C8H10ClN4O2	S	[2],[3]
249	Thiacloprid metabolite (Thiacloprid-amide)	676228-91-4	C10H11ClN4OS	M+H	271.04149	235.064808	C10H11N4OS	167.037053	C8H8ClN2	158.038259	C5H8N3OS	S	[2],[3]
250	Thiamethoxam metabolite (Clothianidim)	210880-92-5	C6H8ClN5O2S	M+H	250.01600	131.96660	C4H3NClS	217.9897847	C5H5ClN5OS	214.039322	C6H8N5O2S	S	[4]
251	Tolclofos-methyl metabolite (2,6-Dichloro-4-methylphenyl dimethyl phosphate)		C9H11Cl2O4P	M+H	284.98447	252.958262	C8H8Cl2O3P	249.0078	C9H11ClO4P	160.955547	C6H3Cl2O	A	[3], [11]
252	Tolclofos-methyl metabolite (1,4-dichloro-3-methylphenol)	17788-00-0	C7H6Cl2O	M+H	176.98685	158.976282	C7H5Cl2	126.994519	C6H4ClO	141.010169	C7H6ClO	A	[3], [11]

Tabla SI-3. Capítulo 1. (Continuación).

Nº	Compound	CAS number	Elemental composition	Monitored ion	Monitored Mass	Frag. 1	Structure	Frag. 2	Structure	Frag.3	Structure	Matrix(*)	Ref
254	Tolyfluanid metabolite(DMST)	66840-71-9	C9H14N2O2S	M+H	215.08487	170.027025	C7H8NO2S	183.05866	C8H11N2OS	91.0542268	C7H7	W	[4]
255	Tolyfluanid metabolite (N,N-Dimethylsulfamide)	4315-09-7	C2H8N2O2S	M+H	125.03792	108.011375	C2H6NO2S	93.9957256	CH4NO2S			W	[1],[2], [3]
256	Triclorfom Metabolite(Dichlorvos)	62-73-7	C4H7Cl2O4P	M+H	220.95318	127.0155	C2H8O4P	109.0049	C2H6O3P	188.926962	C3H4Cl2O3P	S	[4]
257	Triclorfom Metabolite(Desmethyl dichlorvos)	17650-82-7	C2H3Cl2O4P	M+H	192.92188	174.911312	C2H2Cl2O3P	138.934635	C2HClO3P	80.973607	H2O3P	S,W	[2],[3]
258	Triclorfom metabolite (Dichlorivinylphosphate)		C2HCl2O4P	M+H	190.90623							S	[2],[3]
259	Triclorfom metabolite (Dichloroethanol)		C2H4Cl2O	M+H	114.97120	61.9917794	C2H3Cl	78.994519	C2H4ClO			S	[2],[3]
260	Trifluralin metabolite [3-nitro-N2,N2-dipropyl-5-(trifluoromethyl)benzene-1,2-diamine]	2078-04-8	C13H18F3N3O2	M+H	306.14239	289.115838	C13H16F3N2O2	259.14166	C13H18F3N2	205.021939	C7H4F3N2O2	S,W, Sed	[2],[3]
261	Tritosulfuron metabolite (2-trifluoromethyl-benzenesulfonamide)	1869-24-5	C7H6F3NO2S	M+H	226.01441	208.987861	C7H4F3O2S	156.011376	C6H6NO2S	145.025961	C7H4F3	W	[1],[2], [3]
262	Vinclozolin metabolite (3,5-dichlorophenylcarbamic acid-(1-carboxyl-1-methyl)-2-propenyl-ester)		C12H11Cl2NO4	M+H	304.01378	268.037112	C12H11ClNO4	254.021462	C11H9ClNO4	205.97701	C7H6Cl2NO2	W	[1],[2], [3]
263	Vinclozolin metabolite (N-(3,5-dichlorophenyl)-2-hydroxy-2-methyl-3-butenic acid-amide)		C11H11Cl2NO2	M+H	260.02396	230.013395	C10H10Cl2NO	224.047283	C11H11ClNO2	154.005418	C7H5ClNO	W	[1],[2], [3]

(*) Matrix where the metabolite was found in the literature
A= Air; W=Water; S=Soil; Pl=Plants; An=Animals; Hu=Humans; GW=Groundwater

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[7] A. Muñoz, T. Vera, H. Sidebottom, M. Ródenas, E. Borrás, M. Vázquez, M. Raro, W. Mellouki. Studies on the atmospheric degradation of chlorpyrifos-methyl. Environ.Sci.Technol. 45(5) (2011) 1880-1886.
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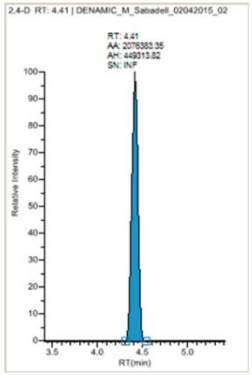
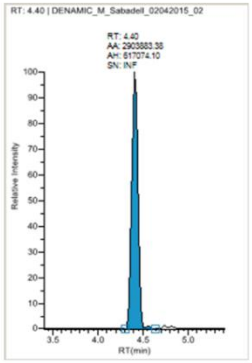
[11] A. Muñoz, T. Vera, H. Sidebottom, M. Ródenas, E. Borrás, M. Vázquez, M. Raro, W. Mellouki. Studies on the atmospheric fate of propachlor (2-chloro-N-isopropylacetanilide) in the gase phase. Atmos. Environ. 49 (2012) 33-40.
[12] M. Roca, N. León, A. Pastor, V. Yusà. Comprehensive analytical strategy for biomonitoring of pesticidas in urine by liquid chromatography-orbitrap high resolution mass spectrometry. J. Chromatogr. A 1374 (2014) 66-76.

A) STANDARD

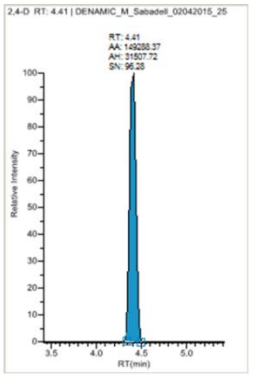


[M-H]-=218.96204
Δm= -0.38 ppm
$C_8H_6O_3Cl_2$
RT= 4.41 min

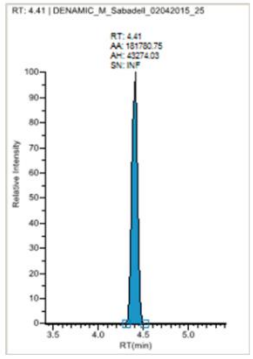


fragment 1=160.95604
Δm= -2.54 ppm
$C_6H_4OCl_2$
RT= 4.40 min

B) SAMPLE



[M-H]-=218.96202
Δm= -0.45 ppm
$C_8H_6O_3Cl_2$
RT= 4.41 min



fragment 1=160.95630
Δm= -0.93 ppm
$C_6H_4OCl_2$
RT= 4.41 min

Figura SI-1. Capítulo 1. A) Accurate mass extracted ion chromatograms (XIC) of the molecular ion [M-H] and a characteristic fragment for 2,4-D in a standard; B) Accurate mass extracted ion chromatogram (XIC) of the molecular ion [M-H] and a characteristic fragment for 2,4-D in a real sample.

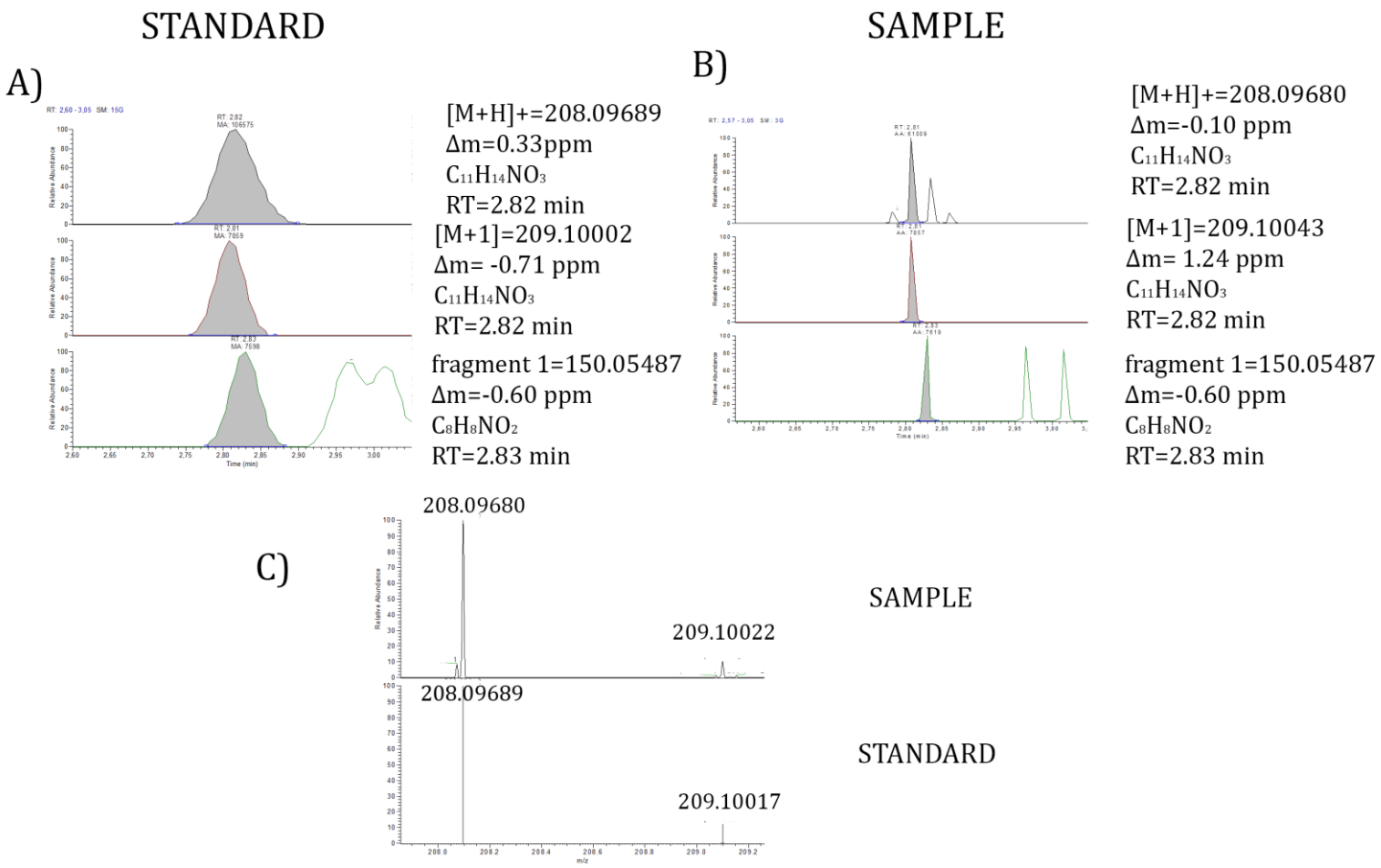


Figura SI-2. Capítulo 1. A) Accurate mass extracted ion chromatograms (XIC) of the molecular ion and a characteristic fragment for propachlor oxanilic acid in a standard; B) Accurate mass extracted ion chromatograms (XIC) of the molecular ion and a characteristic fragment for propachlor oxanilic acid in a real sample; C) Isotopic patterns of the molecular ion of the sample and the standard.

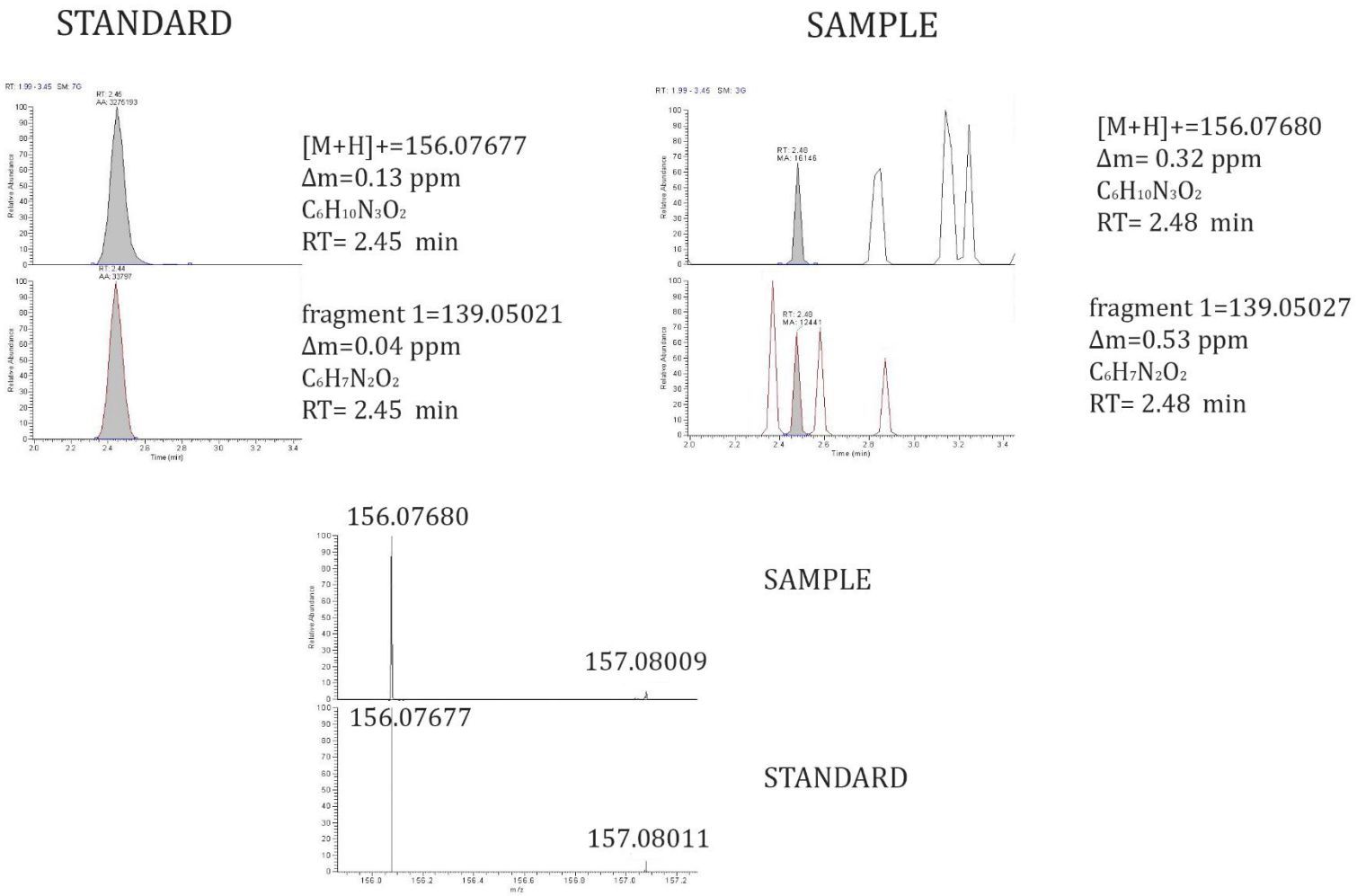


Figura SI-3. Capítulo 1. Accurate mass extracted ion chromatograms of the molecular ion and a characteristic fragment of 4,6-Dimethoxy-2-pyrimidinamine: a) standard; b) real sample; c) Isotopic patterns of the molecular ion of the sample and standard.

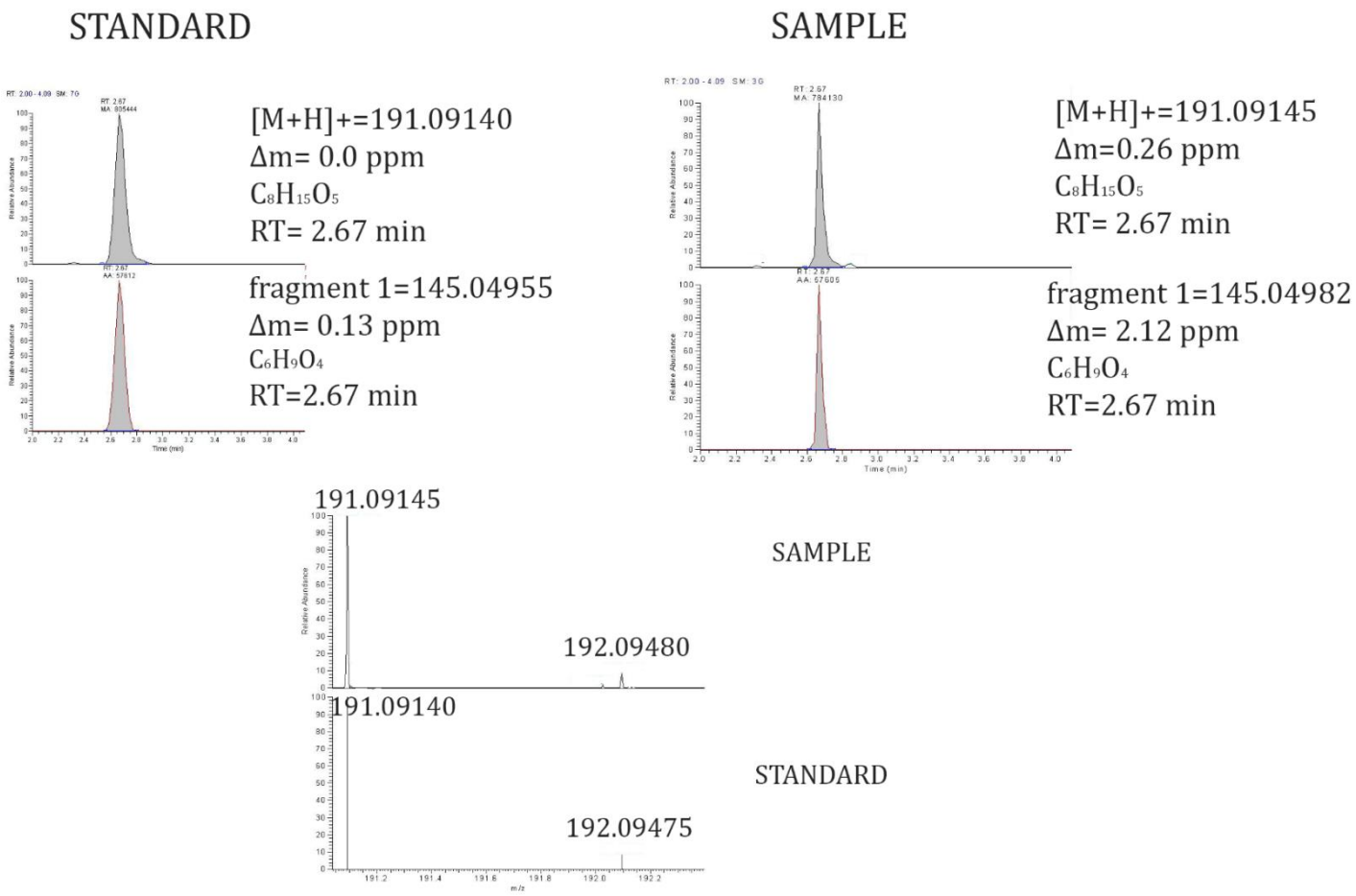


Figura SI-4. Capítulo 1. Accurate mass extracted ion chromatograms of the molecular ion and a characteristic fragment of diethyl malate: a) standard; b) real sample; c) Isotopic patterns of the molecular ion of the sample and standard.

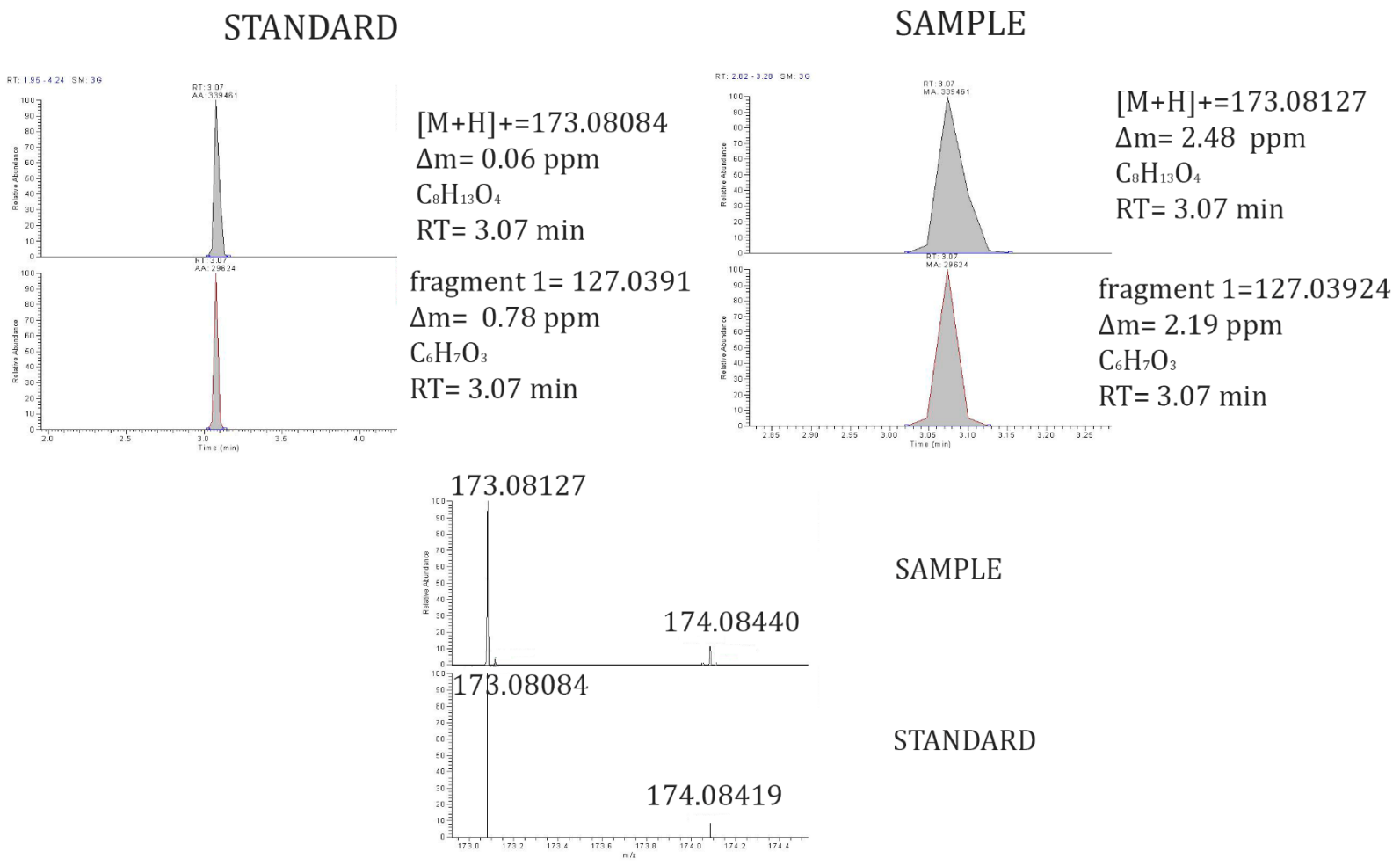


Figura SI-5. Capítulo 1. Accurate mass extracted ion chromatograms of the molecular ion and a characteristic fragment of diethylmaleate: a) standard; b) real sample; c) Isotopic patterns of the molecular ion of the sample and standard.

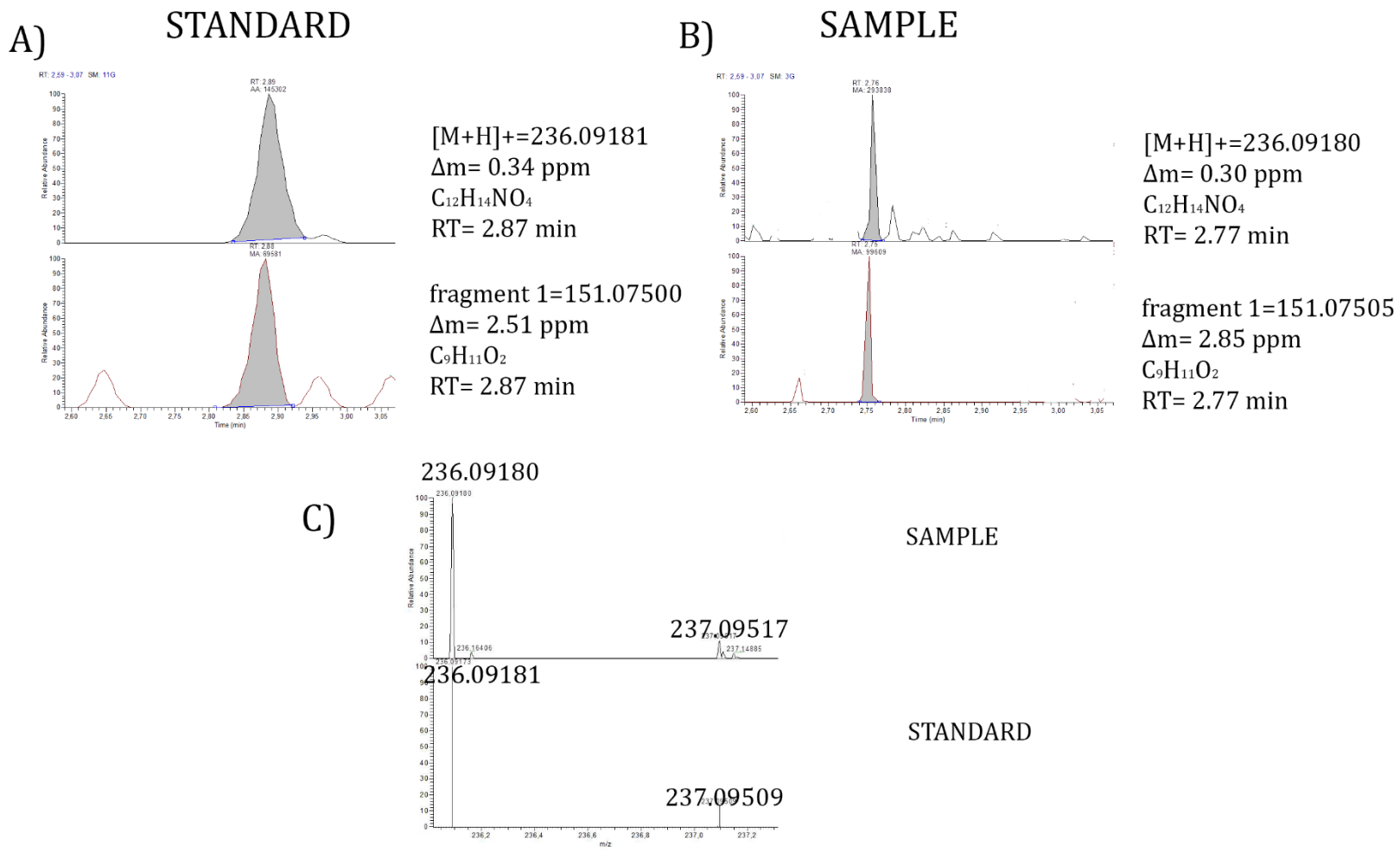


Figura SI-6. Capítulo 1. Accurate mass extracted ion chromatograms of the molecular ion and a characteristic fragment of 3-ketocarbofuran: a) standard; b) real sample; c) Isotopic patterns of the molecular ion of the sample and standard.

ANEXO CAPÍTULO 2

Información suplementaria de 'Optimization of Resolving Power, fragmentation and mass calibration in an Orbitrap Spectrometer for analysis of 24 pesticide metabolites in urine'

Tabla de información suplementaria

Tabla SI-1. Capítulo 2. Scheme of the Resolving power optimization study

Ionization mode	**ESI NEG**			**ESI POS**		
Resolving power (FWHM)	**10,000**	**25,000**	**50,000**	**10,000**	**25,000**	**50,000**
Nº aliquotes (spiked urine) injected[a]	6	6	6	6	6	6
Nº acquisitions non-applying fragmentation[a] (diagnostic ions)	6	6	6	6	6	6
Nº acquisitions applying fragmentation (HCD 20 eV)[a] (fragment ions)	6	6	6	6	6	6

[a]Each injection allowed both 'applying fragmentation' and 'non-applying fragmentation' acquisitions quasi-simultaneously

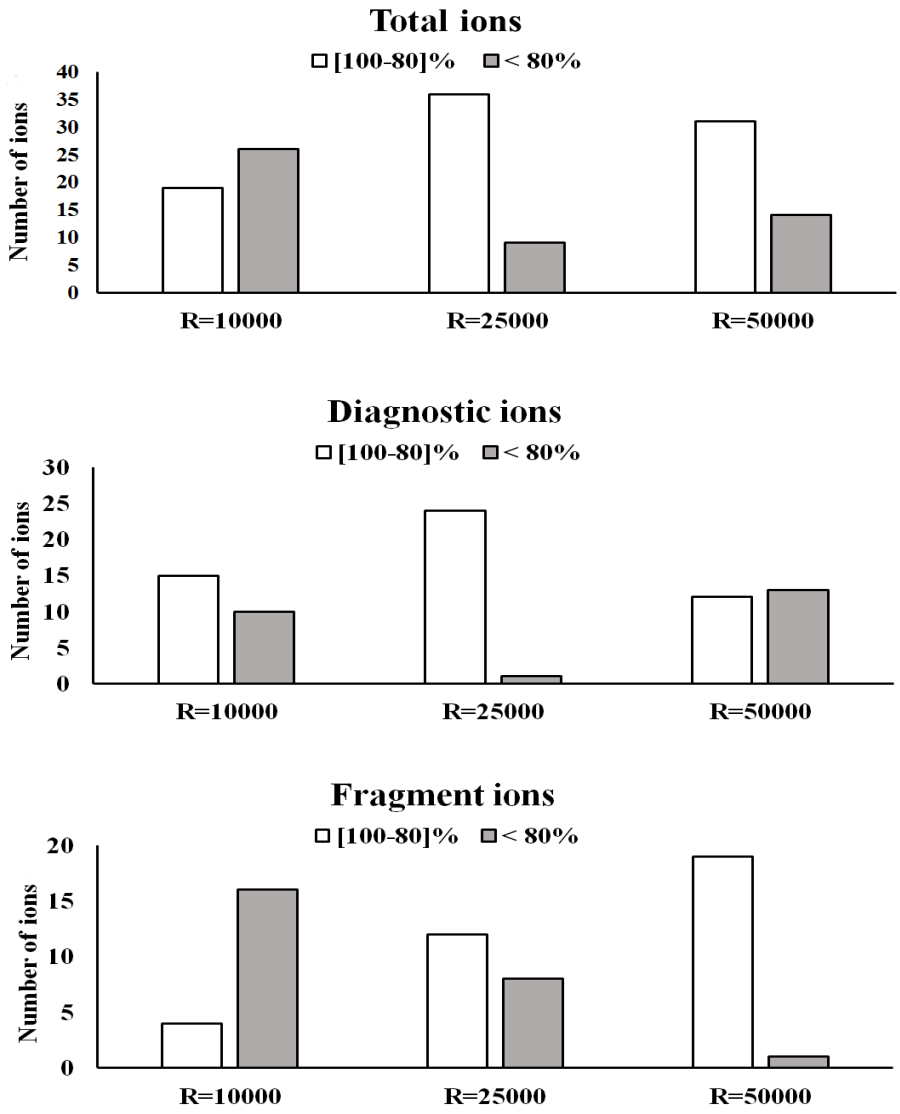


Figura SI-1. Capítulo 2. Number of ions with high ([100-80]%) and low (<80%) peak areas at R=10,000, R=25,000 and R=50,000 (FWHM), for total, diagnostic and fragment ions. The percentage (%) is calculated dividing the area of an ion by the highest area obtained for that ion and multiplying by 100.

ANEXO CAPÍTULO 3

Información suplementaria de 'Determination of four parabens and bisphenols A, F and S in human breast milk using QuEChERS and liquid chromatography coupled to mass spectrometry'

Tabla de información suplementaria

SI-1. Capítulo 3. Ion source settings optimization

SI-1.1. Capítulo 3. Screening design

Considering the literature and previous studies carried out in our laboratory, five factors (k=5) were selected as potentially affecting de APCI efficiency: SG, AG, CT, VT and DC.

A Plackett-Burman design (B-M) was chosen as a screening method to estimate the relative influence of the five factors on the analytical response. With this screening design the main effects were calculated with a reduced number of experiments (12 runs, 1 block), and the experiment run order was randomized to provide protection against the effect of hidden variables. The ranges investigated in this screening were: SG (25 – 60 psi), AG (0 – 5 a.u.), CT (150 – 300ºC), VT (250 – 500ºC) and DC (3 – 5 µA).

The values corresponding to every factor in each experiment are shown in **Tabla SI-2. Capítulo 3**. The estimated effect of the five factors over the analyte responses and their statistical significance at 95% confidence level are shown in **Tabla SI-3. Capítulo 3**. As can be seen, AG and VT presented the lowest p-values on the analytical response and the highest effects for most of the compounds studied, especially BPA, which showed the lowest peak areas and focused the ion source setting optimization efforts. In consequence, these factors were selected for further optimization despite VT not presenting a p-value < 0.05 for any compound. For the next stage in the optimization process, SG, CT and DC were fixed at 43 psi (medium value since it presented a low effect for BPA), 250ºC (high value since it presented a high positive effect for BPA) and 4 µA (medium value since it presented a low effect for BPA), respectively.

SI-1.2. Capítulo 3. Central composite design

In order to obtain a more accurate optimization of AG and VT, a central composite design (CCD) was chosen. This type of experimental design allows to build a response surface and to select the factor settings or operating conditions that maximize the analyte responses. The CCD model selected included 8 cube points, 8 axial points and 10 central points in cube, with an Alpha value of 1.41421, involving 26 chromatographic injections (13 runs in duplicate). The values corresponding to every factor in each experiment are shown in **Tabla SI-4. Capítulo 4**.

The responses were fitted by a multiple regression equation, including second order and interaction terms. Three-dimensional response surfaces displayed the effect of the two independent variables on the analytical response (arbitrary units of peak area).

The following step was to select the factor settings that simultaneously optimize the responses of the five compounds. This was done by using the 'response optimizer' from the response-surface design in the MINITAB program. As there were multiple responses (one for each analyte), and as the response surfaces were different for each compound, it was necessary to find a factor setting that simultaneously maximized the desirability for each response. It must be noticed that the desirability is 0.0 for the lowest values obtained in the CCD; and it increases as response values increase; being 1.0 for the highest response obtained in the experiments. For this reason, we maximized a composite desirability that combined the individual desirability of all the response variables into a single measure taking into account that all the response variables had the same importance. The optimized responses had a global desirability of 0.66 and the factor settings established were: SG, 43 psi; AG, 4 a.u.; CT, 250ºC; VT, 400ºC; and DC, 4 µA. **Figura 21** shows, as an example, some response surfaces obtained by using the aforementioned model for BPA, EP and PP.

Tabla SI-1. Capítulo 3. Comparison of the quantitative ions peak areas using APCI and ESI for the analysis of a standard mix solution (100 ng/mL). (BPF and BPS not included in the optimization)

Analytes	**Peak area arithmetic mean ± standard deviation (n = 3)**	
	APCI (-)	**ESI (-)**
MP	2353001 ± 120451	644310 ± 109096
EP	2009881 ± 113363	290360 ± 49921
PP	3078318 ± 123962	450723 ± 82121
BP	2603080 ± 87183	611216 ± 115315
BPA	121735 ± 14218	155 ± 190

Tabla SI-2. Capítulo 3. Experimental conditions of Placket-Burman design used for optimization of detector settings for parabens and BPA (BPF and BPS not included in the optimization)

Run	Sheath Gas (psi)	Auxilliary Gas (a.u.)	Capillary Temperature (ºC)	Vaporization Temperature (ºC)	Discharge Current (µA)
1	60	0	300	250	3
2	25	0	150	500	5
3	60	5	300	250	5
4	43	3	225	375	4
5	43	3	225	375	4
6	60	0	150	250	5
7	60	5	150	500	3
8	60	0	300	500	3
9	25	0	300	500	5
10	25	0	150	250	3
11	25	5	150	250	3
12	43	3	225	375	4
13	25	5	300	500	3
14	60	5	150	500	5
15	25	5	300	250	5

Tabla SI-3. Capítulo 3. Plackett- Burman screening. Estimated effects (α=0.05) and p-values (within parenthesis) for the compounds for the five selected parameters. (BPF and BPS not included in the optimization)

Compound	Sheath gas	Auxilliary gas	Capillary Temperature	Vaporization Temperature	Discharge Current
MP	-123031 (0.531)	988630 (0.001)[a]	197485 (0.324)	-128876 (0.512)	295142 (0.155)
EP	436775 (0.148)	823659 (0.017)[a]	263120 (0.363)	-124820 (0.659)	-192425 (0.501)
PP	-180440 (0.611)	616307 (0.109)	-24394 (0.945)	-30714 (0.931)	-8758 (0.980)
BP	-348679 (0.272)	382813 (0.232)	85430 (0.780)	-37497 (0.902)	-71016 (0.816)
BPA	17734 (0.883)	247627 (0.067)	147820 (0.242)	180125 (0.162)	-41803 (0.730)

[a]p-value < 0.05

Tabla SI-4. Capítulo 3. Experimental conditions of the CCD design used for optimization of detector settings for parabens and BPA (BPF and BPS not included in the optimization)

Run	Auxilliary Gas (a.u.)	Vaporization Temperature (ºC)
1	1	287
2	3	500
3	3	375
4	4	287
5	0	375
6	3	375
7	3	500
8	4	287
9	1	463
10	3	375
11	3	375
12	0	375
13	3	375
14	1	287
15	4	463
16	5	375
17	1	463
18	5	375
19	3	375
20	3	375
21	3	375
22	3	375
23	3	250
24	3	250
25	3	375
26	4	463

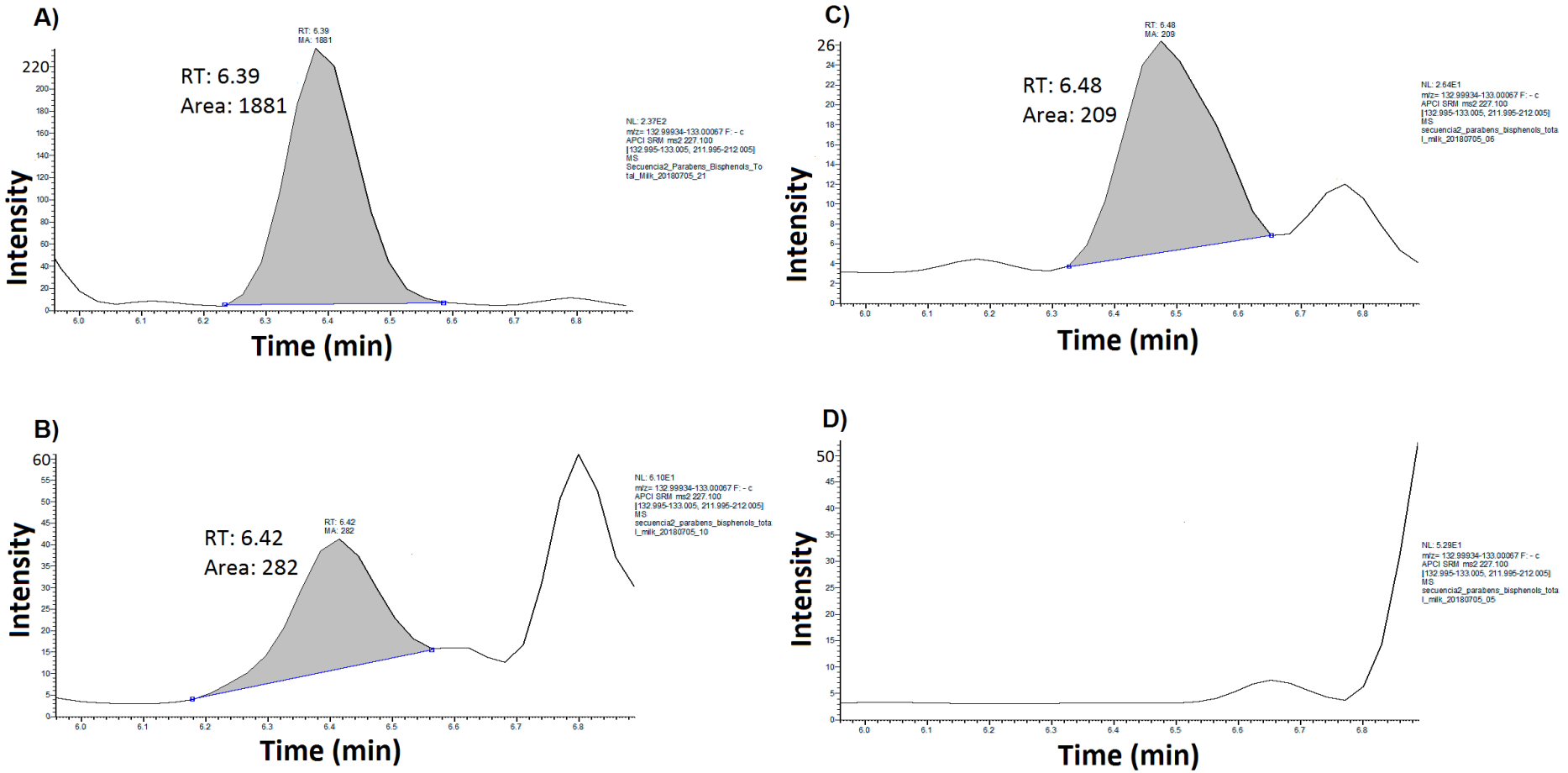


Figura SI-1. Capítulo 3. BPA SRM chromatograms in: A) Milk blank spiked at the LoQ; B) Milk blank; C) Reagent blank; and D) mobile phase (methanol:water (20:80, v/v))

ANEXO CAPÍTULO 4

Información suplementaria de 'Biomonitoring of bisphenols A, F, S in human milk and probabilistic risk assessment for breastfed infants'

Tabla de información suplementaria

Tabla de contenidos (Continuación)

Tabla SI-1. Capítulo 4. Food consumption by groups in the population (grams/month), index MED-DQI and recent packaged products consumption

Food consumption by groups (g/month)	**Median (minimum - maximum)**
Eggs	700 (30 - 1800)
Dairy products	13000 (1400- 40000)
Meat products	6000 (700- 14000)
Fishing products	4000 (1000 - 16000)
Vegetables	13000 (1000 - 40000)
Fruits	16000 (4000 - 80000)
Legumes and cereals	5000 (2000 - 30000)
Oils and fats	800 (150 - 1500)
Pastries	1000 (170 - 7000)
Miscellany	1400 (300 - 30000)
Drinks[a]	40000 (1700 - 80000)
Index MED-DQI[b]	5 (0 - 8)
Good	56 (46.7)[c]
Medium-good	63 (52.5)[c]
Half-poor	1 (0.8)[c]
Packaged products nº rations (72h)	14 (1 - 39)

[a]Values expressed as mL/month
[b]Values expressed in arbitray units (a.u.)
[c]Values expressed as absolute frequency (percentage).

Tabla SI-2. Capítulo 4. Frequency of use of cosmetics products.

Cosmetics products	**n (%)**
Skin care	
Frequency	
Never or before pregnancy	25 (21.4)
Daily	68 (58.1)
Several times a week	20 (17.1)
Sometimes in the month	4 (3.4)
Missing data	3
Parfums	
Frequency	
Never or before pregnancy	55 (47.8)
Daily	34 (29.6)
Several times a week	22 (19.1)
Monthly	4 (3.5)
Missing data	5
Deodorants	
Frequency	
Never or before pregnancy	9 (8.1)
Daily	96 (86.5)
Several times a week	6 (5.4)
Missing data	9
Sun screen	
Frequency	
Never or before pregnancy	74 (64.9)
Daily	17 (14.9)
Several times a week	10 (8.8)
Monthly	3 (2.6)
Ocasionally	10 (8.8)
Missing data	6
Hair colour	
Times/year	2 (0 - 26)[a]
Last application	
≤1 week	9 (7.8)
< 1 month	10 (8.6)
≥ 1 month, < 3 months	34 (29.3)
≥ 3 months	12 (10.3)
Never or before pregnancy	51 (44.0)
Missing data	4
Lipstick	
Frecuency	
Never or before pregnancy	87 (73.1)
Daily	11 (9.2)
Several times a week	8 (6.7)
Monthly	13 (10.9)
Missing data	1

Tabla SI-2. Capítulo 4. (Continuación)

Cosmetics products	**n (%)**
Makeup	
Frecuency	
Never or before pregnancy	65 (55.6)
Daily	16 (13.7)
Several times a week	16 (13.7)
Monthly	20 (17.1)
Missing data	3

[a]Values expressed as median (minimum - maximum).

Tabla SI-3. Capítulo 4. Mediterranean Diet Quality Index (Med-DQI) calculation

Score	SFA(Saturated fatty acids) (%energy)	Cholesterol (mg/day)	Meat (g/day)	Olive oil (mL/day)	Fish (g/day)	Legumes and cereals (g/day)	Fruits and vegetables (g/day)
0	<10	<300	<25	>15	>60	>300	>700
1	10 - 13	300 - 400	25 - 125	5 - 15	30 - 60	100 - 300	400 - 700
2	>13	>400	>125	>5	>30	>100	>400

Tabla SI-4. Capítulo 4. Chromatographic conditions

Time (min)	Mobile phase A (water) (%)	Mobile phase B (methanol) (%)
0	98	2
6	0	100
7	0	100
7.5	98	2
12	98	2

Injection volume: 20 µL
Flow rate: 300 µL/min

Tabla SI-5. **Capítulo 4.** Ion source settings

Vaporization temperature	Capillary temperature	Discharge current	Sheath gas pressure	Auxiliary gas flow rate
400°C	250°C	4 µA	43 psi	4 arbitrary units

Tabla SI-6. Capítulo 4. Elemental composition of BPA, BPF, BPS and the ILIS, selected transitions, collision energy (CE) and tube lens offset voltage employed for each parent ion-product ions transitions

Analyte	Elemental composition	Transitions (m/z)	CE (eV)	Tube lens offset voltage (V)
BPA	$C_{15}H_{16}O_2$	227.1 → 133.0[a]	-25	-78
		227.1 → 211.9[b]	-31	-78
BPF	$C_{13}H_{12}O_2$	199.1 → 105.0[a]	-23	-90
		199.1 → 93.1[b]	-23	-90
BPS	$C_{12}H_{10}O_4S$	249.1 → 108.0[a]	-20	-90
		249.1 → 156.0[b]	-20	-90
BPA-d_{14}[c]	$C_{15}H_2D_{14}O_2$	241.2 → 141.9[a]	-31	-78
BPF-d_{10}[c]	$C_{13}H_2D_{10}O_2$	209.0 → 97.0[a]	-23	-90
BPS-d_8[c]	$C_{12}H_2D_8O_4S$	257.0 → 112.0[a]	-20	-90

[a]SRM transition used for quantification
[b]SRM transition used for confirmation
[c]ILIS

Tabla SI-7. Capítulo 4. Method validation parameters

Analyte	Linearity (R^2)	LoQ (ng/mL)	LDR (ng/mL)	Accuracy (recovery (%))			Precision (RSD (%))		
				Low (n=5)	Medium (n=5)	High (n=5)	Low (n=5)	Medium (n=5)	High (n=5)
u-BPA [a]	0.996 – 0.999	0.10	0.10 – 50	105	96	86	19	16	14
BPA total [a]	0.992 – 0.999	0.10	0.10– 50	100	101	97	12	7	13
BPF total [b]	0.993 – 0.998	0.13	0.13– 50	115	98	90	6	10	11
BPS total [c]	0.991 – 0.999	0.25	0.25 – 50	108	97	99	12	8	11

LDR: Linear Dynamic Range;
[a] Low: Concentration 0.10 ng/mL; Medium: Concentration 5.0 ng/mL; High: Concentration 50 ng/mL
[b] Low: Concentration 0.12 ng/mL; Medium: Concentration 5.0 ng/mL; High: Concentration 50 ng/mL
[c] Low: Concentration 0.25 ng/mL; Medium: Concentration 5.0 ng/mL; High: Concentration 50 ng/mL

Tabla SI-8. Capítulo 4. Levels of macronutrients in milk of mothers in the Valencian Region.

Macronutrients	**Median (minimum - maximum)**
Fat (g/100 ml)	3.16 (0.82 - 6.85)
Crude Protein (g/100 ml)	1.49 (0.75 - 2.19)
Real protein (g/100 ml)	1.18 (0.61 - 1.77)
Carbohydrate (g/100 ml)	7.21 (3.85 - 7.58)
TS (g/100 ml)	12.10 (7.82 - 15.49)
Energy (kcal/100 ml)	64.72 (42.32 - 97.84)

TS (Total Solids)

Tabla SI-9. Capítulo 4. Levels of unconjugated-BPA in human milk in weeks 2, 5 and 8 after birth (n=49)

unconjugated-BPA concentration (ng/mL)			
Weeks after birth	**2**	**5**	**8**
Minimum	<LoQ	<LoQ	<LoQ
25th percentile	<LoQ	<LoQ	<LoQ
Median	0.10	0.13	0.11
Geometric mean	0.12	0.17	0.14
Arithmetic mean	0.68	0.64	1.3
75th percentile	0.15	0.30	0.19
95th percentile	2.8	4.8	8.8
Maximum	22	11	40
Standard deviation	3.2	1.9	5.8

Tabla SI-10. Capítulo 4. Relationship between total-BPA levels in human milk and the characteristics and habits of the study population using simple robust regression

Variable	Total-BPA levels Median (minimum-maximum)	Estimated parameters (Simple robust regression)	CI 95%	p-value
Mother				
Nº children				
1	0.24 (0.01 – 22)	-	-	-
2	0.25 (0.01 – 42)	-0.003	(-0.516 - 0.510)	0.992
3 or more	0.50 (0.07 – 1.4)	0.682	(-0.220 - 1.584)	0.142
Age (years)	-	0.043	(-0.010 - 0.096)	0.118
Weight before pregnancy (kg)	-	0.003	(-0.021 - 0.027)	0.797
Height (cm)	-	-0.003	(-0.044 - 0.038)	0.892
BMI before pregnancy (kg/m^2)	-	0.005	(-0.061 - 0.070)	0.884
Special diet during pregnancy				
Yes	0.27 (0.05 – 28)	-	-	-
No	0.26 (0.01 – 42)	0.001	(-0.710 - 0.712)	0.998
Country of birth				
Spain	0.28 (0.01 – 42)	-	-	-
Other	0.19 (0.05 – 28)	-0.048	(-0.779 - 0.684)	0.899
Place of residence last 10 years				
Urban	0.28 (0.01 – 28)	-	-	-
Rural	0.10 (0.03 – 42)	-0.817	(-1.388 - -0.246)	0.006*
Education level				
Only Primary School	0.18 (0.05 – 1.5)	-	-	-
Secondary School	0.16 (0.01 – 28)	-0.261	(-1.142 - 0.621)	0.563
University	0.28 (0.01 – 42)	-0.009	(-0.772 - 0.755)	0.983
Occupational status				
Employed	0.25 (0.01 – 42)	-	-	-
Unemployed	0.29 (0.03 – 2.09)	0.125	(-0.584 - 0.835)	0.730

Tabla SI-10. Capítulo 4. (Continuación)

Variable	Total-BPA levels Median (minimum-maximum)	Estimated parameters (Simple robust regression)	CI 95%	p-value
Period working outside the home (years)	-	0.020	(-0.027 - 0.067)	0.397
Use of cosmetics at work				
Yes	0.22 (0.04 – 28)	-	-	-
No	0.28 (0.01 – 42)	0.433	(-0.374 - 1.241)	0.296
Breastfed				
Yes	0.24 (0.01 – 42)	-	-	-
No	0.35 (0.05 – 22)	0.309	(-0.247 - 0.866)	0.278
Physical exercise				
3 or more days/week	0.18 (0.05 – 1.6)	-	-	-
1 or 2 days/week	0.25 (0.01 – 42)	-0.018	(-0.935 - 0.899)	0.970
Occasionally	0.30 (0.01 – 28)	0.146	(-0.618 - 0.91)	0.709
Never	0.20 (0.03 – 15)	-0.264	(-1.072 - 0.543)	0.523
Smoker				
Yes	0.29 (0.04 – 1.5)	-	-	-
Ex-smoker	0.25 (0.01 – 16)	-0.108	(-1.061 - 0.846)	0.825
Never	0.25 (0.03 – 42)	-0.130	(-1.074 - 0.815)	0.788
Child				
Gestational age (weeks)	-	-0.038	(-0.264 - 0.188)	0.741
Gender				
Boy	0.29 (0.01 – 2.1)	-	-	-
Girl	0.25 (0.01 – 42)	-0.096	(-0.599 - 0.406)	0.707
Weight (g)	-	$-4 \cdot 10^{-4}$	($-0.001 – 9 \cdot 10^{-5}$)	0.115
Height (cm)	-	-0.084	(-0.240 - 0.072)	0.295
Cranial perimeter (cm)	-	-0.037	(-0.359 - 0.286)	0.825

*p-values <0.05

Tabla SI-11. Capítulo 4. Relationship between total-BPA levels in human milk with food consumption and cosmetic use of the study population using simple robust regression

Variable	Total-BPA levels Median (minimum-maximum)	Estimated parameters (Simple robust regression)	CI 95%	p-value
Food consumption				
Frequency (g/month)				
Eggs	-	$5 \cdot 10^{-4}$	(-3·10-4 - 0.001)	0.224
Dairy products	-	$4.93 \cdot 10^{-6}$	($-2.88 \cdot 10^{-5}$ – $3.86 \cdot 10^{-5}$)	0.775
Meat products	-	$9.70 \cdot 10^{-5}$	($-1.88 \cdot 10^{-5}$ – $2.13 \cdot 10^{-4}$)	0.104
Fishery products	-	$3.22 \cdot 10^{-5}$	($-6.02 \cdot 10^{-5}$ – $1.24 \cdot 10^{-4}$)	0.496
Vegetables	-	$1.30 \cdot 10^{-6}$	($-3.31 \cdot 10^{-5}$ – $3.57 \cdot 10^{-5}$)	0.941
Fruits	-	$2.96 \cdot 10^{-6}$	($-1.81 \cdot 10^{-5}$ – $2.39 \cdot 10^{-5}$)	0.783
Legumes and cereals	-	$-8.59 \cdot 10^{-5}$	(-1.7·10-4 – $1.25 \cdot 10^{-6}$)	0.056
Oils and fats	-	$3.73 \cdot 10^{-5}$	(-0.001 - 0.001)	0.925
Pastries	-	$8.72 \cdot 10^{-5}$	($-1 \cdot 10^{-4}$ – $2.8 \cdot 10^{-4}$)	0.378
Miscellany	-	$-2.26 \cdot 10^{-5}$	($-1.5 \cdot 10^{-4}$ – $1 \cdot 10^{-4}$)	0.728
Drinks (mL/month)	-	$2.43 \cdot 10^{-6}$	($-2.13 \cdot 10^{-5}$ – $2.62 \cdot 10^{-5}$)	0.842
Index-MED-DQI	-	-0.071	(-0.233 - 0.092)	0.397
Good	0.28 (0.03 – 42)	-	-	-
Medium-good	0.25 (0.01 – 28)	-0.072	(-0.562 - 0.418)	0.774
Half-poor	1.1 (1.1 – 1.1)	1.519	(-0.933 - 3.97)	0.228
Packaged products nº rations /72h	-	0.005	(-0.028 - 0.038)	0.762
Personal care products				
Skin care				
Frequency				
Never or before pregnancy	0.10 (0.04 – 15)	-	-	-
Daily	0.34 (0.01 – 42)	0.788	(0.254 - 1.322)	0.005*
Several times a week	0.29 (0.05 – 5.92)	0.734	(0.027 - 1.441)	0.045*
Monthly	0.25 (0.10 – 0.43)	0.490	(-0.859 - 1.839)	0.478

Tabla SI-11. Capítulo 4. (Continuación).

Variable	Total-BPA levels Median (minimum-maximum)	Estimated parameters (Simple robust regression)	CI 95%	p-value
Parfums				
Frequency				
Never or before pregnancy	0.19 (0.01 – 22)	-	-	-
Daily	0.33 (0.04 – 42)	0.374	(-0.208 - 0.957)	0.211
Several times a week	0.28 (0.01 – 1.6)	0.377	(-0.278 - 1.033)	0.262
Monthly	0.25 (0.10 – 0.43)	0.138	(-1.298 - 1.574)	0.851
Deodorants				
Frequency				
Never or before pregnancy	0.16 (0.03 – 1.1)	-	-	-
Daily	0.29 (0.01 – 42)	0.329	(-0.591 - 1.25)	0.485
Several times a week	0.17 (0.01 – 0.34)	-0.599	(-2.17 - 0.973)	0.457
Sunscreen				
Frequency				
Never or before pregnancy	0.25 (0.04 – 28)	-	-	-
Daily	0.33 (0.03 – 22)	0.238	(-0.486 - 0.963)	0.521
Several times a week	0.42 (0.10 – 42)	0.453	(-0.420 - 1.327)	0.312
Monthly	0.12 (0.09 – 0.14)	-0.747	(-2.504 - 1.010)	0.407
Occasionally	0.25 (0.01 – 15)	0.050	(-0.823 - 0.923)	0.910
Hair colour				
Times/year	-	0.004	(-0.052 - 0.059)	0.897
Last application				
≤ 1 week	0.42 (0.01– 22)	-	-	-
< 1 month	0.17 (0.04 – 2.09)	-0.722	(-1.876 - 0.431)	0.223
≥ 1 month, < 3 months	0.21 (0.03 – 16)	-0.781	(-1.741 - 0.178)	0.114
≥ 3 months	0.31 (0.03 – 1.6)	-0.559	(-1.643 - 0.524)	0.314
Never or before pregnancy	0.27 (0.04 – 28)	-0.518	(-1.435 - 0.4)	0.272

Tabla SI-11. Capítulo 4. (Continuación).

Variable	Total-BPA levels Median (minimum-maximum)	Estimated parameters (Simple robust regression)	CI 95%	p-value
Lipstick				
Frequency				
Never or before pregnancy	0.25 (0.01 – 42)	-	-	-
Daily	0.14 (0.01 – 1.5)	-0.232	(-1.036 - 0.572)	0.572
Several times a week	0.50 (0.24 – 1.2)	0.744	(-0.269 - 1.756)	0.153
Monthly	0.25 (0.04 – 16)	-0.194	(-0.998 - 0.610)	0.637
Makeup				
Frequency				
Never or before pregnancy	0.23 (0.01 – 22)	-	-	-
Daily	0.34 (0.01 – 16)	0.168	(-0.575 - 0.912)	0.658
Several times a week	0.56 (0.08 – 28)	0.788	(0.090 - 1.486)	0.029*
Monthly	0.17 (0.03 – 42)	-0.244	(-0.923 - 0.435)	0.483

*p-values <0.05

Tabla SI-12. Capítulo 4. Relationship between total-BPA concentration in human milk with levels of macronutrients using simple robust regression

Macronutrients	Estimated parameters (Simple robust regression)	CI 95%	p-value
Fat (g/100 mL)	0.112	(-0.106 - 0.330)	0.318
Real Protein (g/100 mL)	-0.051	(-1.198 - 1.096)	0.931
Carbohydrate (g/100 mL)	0.518	(0.039 - 0.997)	0.037*
TS (g/100 mL)	0.199	(0.006 - 0.392)	0.046*
Energy (kcal/100 mL)	0.018	(-0.005 - 0.041)	0.131

*p-values <0.05
TS (Total Solids)

Tabla SI-13. Capítulo 4. Relationship between u-BPA levels in human milk and the characteristics and habits of the study population using simple robust regression

Characteristics	u-BPA levels Median (minimum - maximum)	Estimated parameters (Simple robust regression)	CI (95 %)	p-value
Mother				
Number of children				
1	0.10 (0.02 - 22)	-	-	-
2	0.10 (0.01 - 41)	-0.080	(-0.341 - 0.182)	0.552
3 or more	0.14 (0.02 - 0.87)	0.427	(-0.012 - 0.867)	0.059
Age (years)	-	0.010	(-0.015 - 0.036)	0.430
Weight before pregnancy (kg)	-	0.001	(-0.010 - 0.013)	0.844
Height (cm)	-	0.011	(-0.008 - 0.030)	0.259
BMI before pregnancy (kg/m^2)	-	-0.008	(-0.038 - 0.022)	0.600
Special diet during pregnancy				
Yes	0.10 (0.06 - 15)	-	-	-
No	0.10 (0.01 - 41)	-0.028	(-0.343 - 0.287)	0.862
Country of birth				
Spain	0.10 (0.01 - 41)	-	-	-
Other	0.12 (0.03 - 15)	0.165	(-0.182 - 0.512)	0.354
Place of residence last 10 years				
Urban	0.12 (0.02 - 22)	-	-	-
Rural	0.10 (0.02 - 41)	-0.308	(-0.570 - -0.046)	0.023*
Education level				
Only primary school	0.10 (0.02 - 0.87)	-	-	-
Secondary school	0.10 (0.02 - 15)	-0.185	(-0.612 - 0.242)	0.398
University	0.11 (0.01 - 41)	0.083	(-0.282 - 0.448)	0.657
Occupational status				
Employed	0.10 (0.01 - 41)	-	-	-
Unemployed	0.10 (0.02 - 0.87)	0.033	(-0.292 - 0.359)	0.842
Period woriking outside the home (years)	-	-0.017	(-0.040 - 0.006)	0.157

Tabla SI-13. Capítulo 4. (Continuación)

Characteristics	u-BPA levels Median (minimum - maximum)	Estimated parameters (Simple robust regression)	CI (95 %)	p-value
Use of cosmetics at work				
Yes	0.09 (0.02 - 15)	-	-	-
No	0.10 (0.01 - 41)	0.564	(0.164 - 0.964)	0.007*
Breastfed				
Yes	0.10 (0.01 - 41)	-	-	-
No	0.12 (0.02 - 22)	0.073	(-0.180 - 0.325)	0.575
Physical exercise				
3 or more days/week	0.11 (0.03 - 0.47)	-	-	-
1 or 2 days/week	0.14 (0.05 - 41)	0.089	(-0.345 - 0.522)	0.689
Occasionally	0.10 (0.02 - 22)	0.026	(-0.336 - 0.387)	0.889
Never	0.10 (0.01 - 14)	-0.142	(-0.529 - 0.246)	0.475
Smoker				
Yes	0.10 (0.02 - 0.22)	-	-	-
Ex-smoker	0.10 (0.01 - 14)	0.008	(-0.457 - 0.474)	0.972
Never	0.11 (0.02 - 41)	0.043	(-0.415 - 0.500)	0.855
Child				
Gestational age (weeks)	-	0.026	(-0.076 - 0.128)	0.621
Gender				
Boy	0.10 (0.01 - 0.47)	-	-	-
Girl	0.10 (0.02 - 41)	-0.094	(-0.325 - 0.138)	0.430
Weight (g)	-	$-2.04 \cdot 10^{-5}$	$(-2.69 \cdot 10^{-4} – 2.28 \cdot 10^{-4})$	0.873
Height (cm)	-	-0.040	(-0.105 - 0.024)	0.224
Cranial perimeter (cm)	-	-0.026	(-0.173 - 0.121)	0.733

Tabla SI-14. Capítulo 4. Relationship between u-BPA levels in human milk and food consumption and cosmetic use of the study population using simple robust regression

Variable	u-BPA levels Median (minimum-maximum)	Estimated parameters (Simple robust regression)	CI (95%)	p-value
Food consumption				
Frequency (g/month)				
Eggs	-	$4.07 \cdot 10^{-4}$	($3.80 \cdot 10^{-5}$ - 0.001)	0.033*
Dairy products	-	$4 \cdot 10^{-6}$	($-1.20 \cdot 10^{-5}$ – $2 \cdot 10^{-5}$)	0.620
Meat products	-	$2.6 \cdot 10^{-5}$	($-3.1 \cdot 10^{-5}$ – $8.3 \cdot 10^{-5}$)	0.372
Fishery products	-	$2.1 \cdot 10^{-5}$	($-2.2 \cdot 10^{-5}$ – $6.5 \cdot 10^{-5}$)	0.342
Vegetables	-	$7 \cdot 10^{-6}$	($-9 \cdot 10^{-6}$ – $2.3 \cdot 10^{-5}$)	0.405
Fruits	-	$1.1 \cdot 10^{-5}$	($1 \cdot 10^{-6}$ – $2.1 \cdot 10^{-5}$)	0.037*
Legumes and cereals	-	$-1.8 \cdot 10^{-5}$	($-5.9 \cdot 10^{-5}$ – $2.4 \cdot 10^{-5}$)	0.401
Oils and fats	-	$3.3 \cdot 10^{-4}$	($-3.7 \cdot 10^{-5}$ - 0.001)	0.080
Pastries	-	$1.9 \cdot 10^{-5}$	($-7.3 \cdot 10^{-5}$ – $1.1 \cdot 10^{-4}$)	0.692
Miscellany	-	$9 \cdot 10^{-6}$	($-2.6 \cdot 10^{-5}$ – $4.3 \cdot 10^{-5}$)	0.618
Drinks (mL/month)	-	$6 \cdot \cdot 10^{-6}$	($-4 \cdot 10^{-6}$ – $1.6 \cdot 10^{-5}$)	0.246
Index MED-DQI	-	-0.084	(-0.165 - -0.003)	0.044*
Good	0.10 (0.01 - 41)	-	-	-
Medium-good	0.10 (0.02 – 15)	-0.082	(-0.310 - 0.145)	0.479
Half-poor	0.87 (0.87 – 0.87)	1.974	(0.753 - 3.196)	0.002*
Packaged products nº rations / 72h	-	0.001	(-0.014 - 0.016)	0.902
Personal care products				
Skin care				
Frequency				
Never or before pregnancy	0.10 (0.01 - 14)	-	-	-
Daily	0.10 (0.02 - 41)	0.214	(-0.100 - 0.529)	0.185
Several times a week	0.13 (0.03 - 4.8)	0.406	(0 - 0.812)	0.053
Monthly	0.12 (0.10 - 0.17)	0.262	(-0.457 - 0.98)	0.477

Tabla SI-14. Capítulo 4. (Continuación).

Variable	u-BPA levels Median (minimum-maximum)	Estimated parameters (Simple robust regression)	CI (95%)	p-value
Parfums				
Frequency				
Never or before pregnancy	0.10 (0.02 - 22)	-	-	-
Daily	0.10 (0.02 - 41)	-0.120	(-0.400 - 0.159)	0.401
Several times a week	0.10 (0.01 - 1.9)	-0.004	(-0.326 - 0.318)	0.980
Monthly	0.10 (0.08 - 0.12)	-0.201	(-0.849 - 0.447)	0.544
Deodorants				
Frequency				
Never or before pregnancy	0.10 (0.07 - 0.57)	-	-	-
Daily	0.10 (0.01 - 41)	0.142	(-0.259 - 0.543)	0.489
Monthly	0.10 (0.02 - 0.15)	0.120	(-0.520 - 0.760)	0.713
Sun screen				
Frequency				
Never or before pregnancy	0.12 (0.01 - 15)	-	-	-
Daily	0.10 (0.02 - 22)	-0.333	(-0.692 - 0.026)	0.072
Several times a week	0.10 (0.04 - 41)	-0.245	(-0.704 - 0.214)	0.299
Monthly	0.09 (0.08 - 0.13)	-0.288	(-1.054 - 0.477)	0.462
Occasionally	0.17 (0.10 - 14)	0.110	(-0.350 - 0.569)	0.641
Hair colour				
Times/year	-	-0.008	(-0.033 - 0.016)	0.521
Last application				
≤1 week	0.19 (0.10 - 22)	-	-	-
< 1 month	0.10 (0.04 - 0.10)	-0.757	(-1.319 - -0.195)	0.010*
≥ 1 month, < 3 months	0.10 (0.02 - 11)	-0.370	(-0.820 - 0.079)	0.109
≥ 3 months	0.10 (0.01 - 0.87)	-0.400	(-0.926 - 0.126)	0.139
Never or before pregnancy	0.12 (0.02 - 15)	-0.189	(-0.621 - 0.244)	0.394

Tabla SI-14. Capítulo 4. (Continuación).

Variable	u-BPA levels Median (minimum-maximum)	Estimated parameters (Simple robust regression)	CI (95%)	p-value
Lipstick				
Frequency				
Never or before pregnancy	0.10 (0.01 - 41)	-	-	-
Daily	0.11 (0.03 - 0.38)	0.227	(-0.202 - 0.655)	0.302
Several times a week	0.20 (0.10 - 1.94)	0.384	(-0.09 - 0.858)	0.115
Monthly	0.10 (0.04 - 11)	-0.039	(-0.450 - 0.372)	0.853
Makeup				
Frequency				
Never or before pregnancy	0.10 (0.01 - 22)	-	-	-
Daily	0.11 (0.03 - 11)	0.023	(-0.356 - 0.403)	0.904
Several times a week	0.13 (0.04 - 15)	0.105	(-0.255 - 0.465)	0.568
Monthly	0.10 (0.04 - 41)	-0.051	(-0.395 - 0.292)	0.771

Tabla SI-15. Capítulo 4. Relationship between u-BPA concentration in human milk and levels of macronutrients using simple robust regression

Macronutrients	Estimated parameters (Simple robust regression)	CI (95%)	p-value
Fat (g/100 ml)	0.100	(-0.006 - 0.205)	0.066
Real protein (g/100 ml)	-0.199	(-0.716 - 0.318)	0.452
Carbohydrate (g/100 ml)	0.105	(-0.149 - 0.358)	0.420
TS (g/100 ml)	0.101	(0.002 - 0.201)	0.049*
Energy (kcal/100 ml)	0.011	(0 - 0.023)	0.052

*p-value < 0.05.

Tabla SI-16. Capítulo 4. Results of the multiple robust regression model for u-BPA levels in human milk.

Variable	Estimated coefficients (95% CI)	Standard error	P-value
Intercept	-1.948 (-2.393 - -1.503)	0.227	<0.001*
Place of residence last 10 years: Rural	-0.309 (-0.594 - -0.024)	0.146	0.037*
Frequency (g/month): Fruits	$1.05 \cdot 10^{-5}$ ($1.50 \cdot 10^{-6}$ – $1.94 \cdot 10^{-5}$)	$4.6 \cdot 10^{-6}$	0.025*
Hair colour last application: < 1 month	-0.665 (-1.23 - -0.101)	0.288	0.023*
Hair colour last application: ≥ 1 month, < 3 months	-0.35 (-0.806 - 0.105)	0.232	0.135
Hair colour last application: ≥ 3 months	-0.325 (-0.891 - 0.242)	0.289	0.265
Hair colour last application: Never or before pregnancy	-0.263 (-0.707 - 0.18)	0.226	0.247

Tabla SI-17. Capítulo 4. Ratios [unconjugated-BPA]/[total-BPA] in different studies

Study	GM	Median	Range
Present	0.54	0.58	0.16 – 1.00
Cao et al., 2015	0.57	0.70	0.08 – 1.00
Ye et al., 2008	0.71*	0.76*	0.49 – 0.99*
Mendonca et al., 2014	0.50**		
Yi et al., 2010		0.63***	

*Derived from individual samples levels
**Derived from GM levels: (GM unconjugated-BPA) / (GM total-BPA)
***Derived from median levels: (median unconjugated-BPA) / (median total-BPA)
GM (geometric Mean)

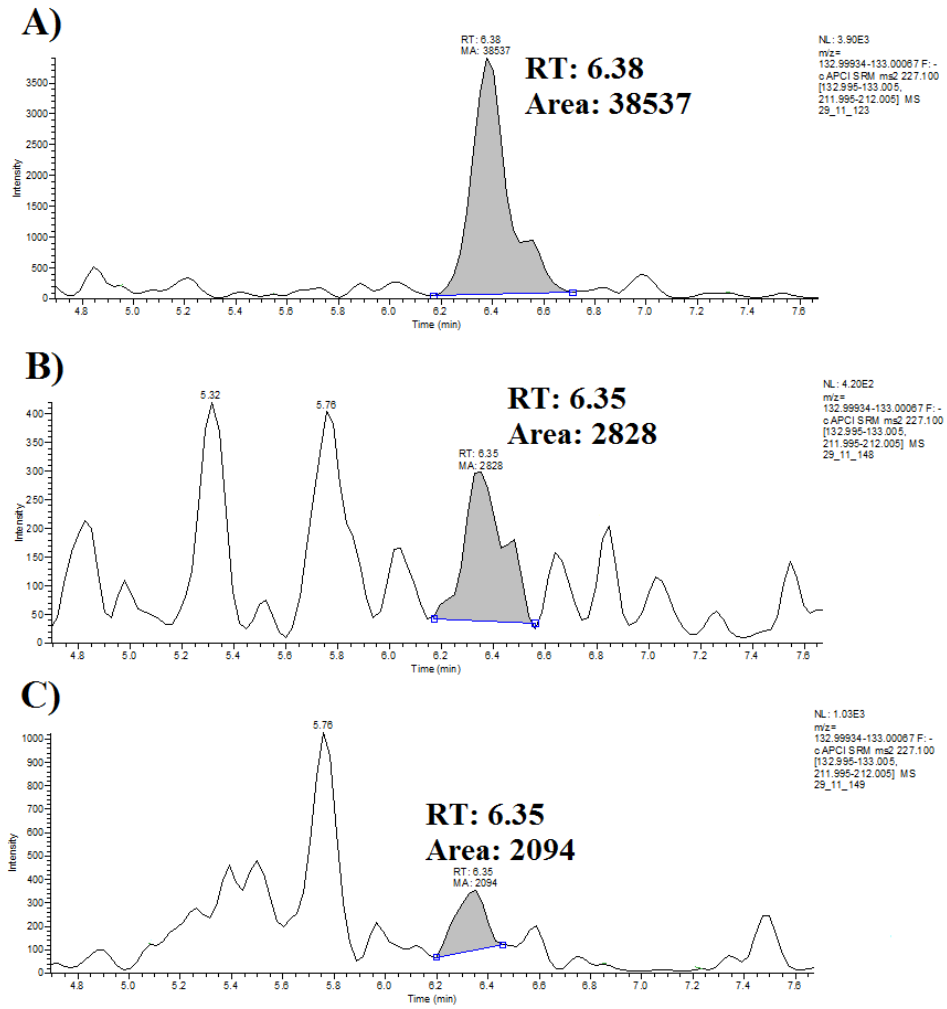


Figura SI-1. Capítulo 4. BPA SRM chromatograms in the migration study: A)simulant spiked at LoQ; B) and C) breast pumps.

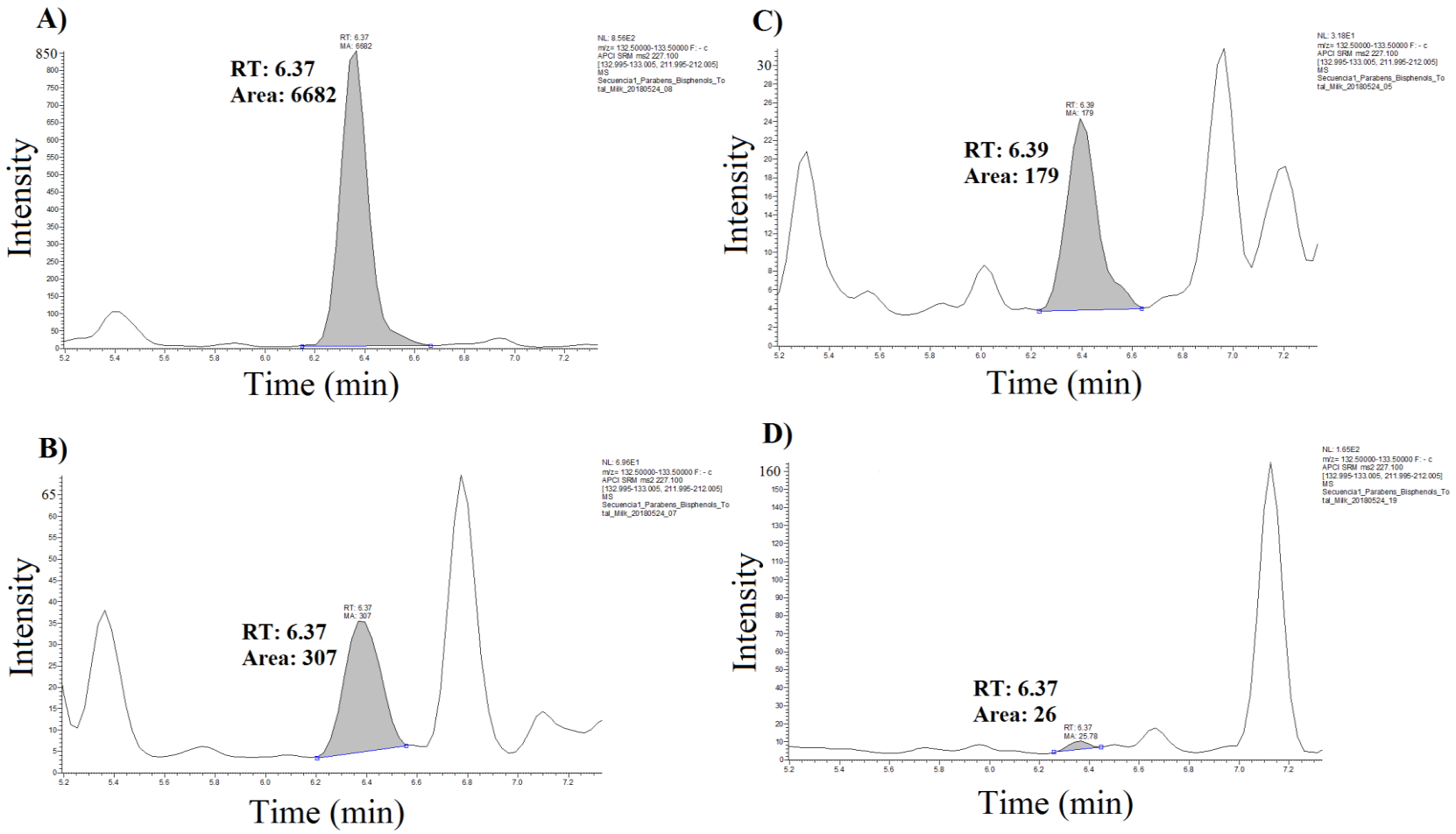


Figura SI-2. Capítulo 4. BPA SRM chromatograms in: A) Milk blank spiked at the LoQ; B) Milk blank; C) Reagent blank; and D) mobile phase (methanol:water (20:80, v/v))

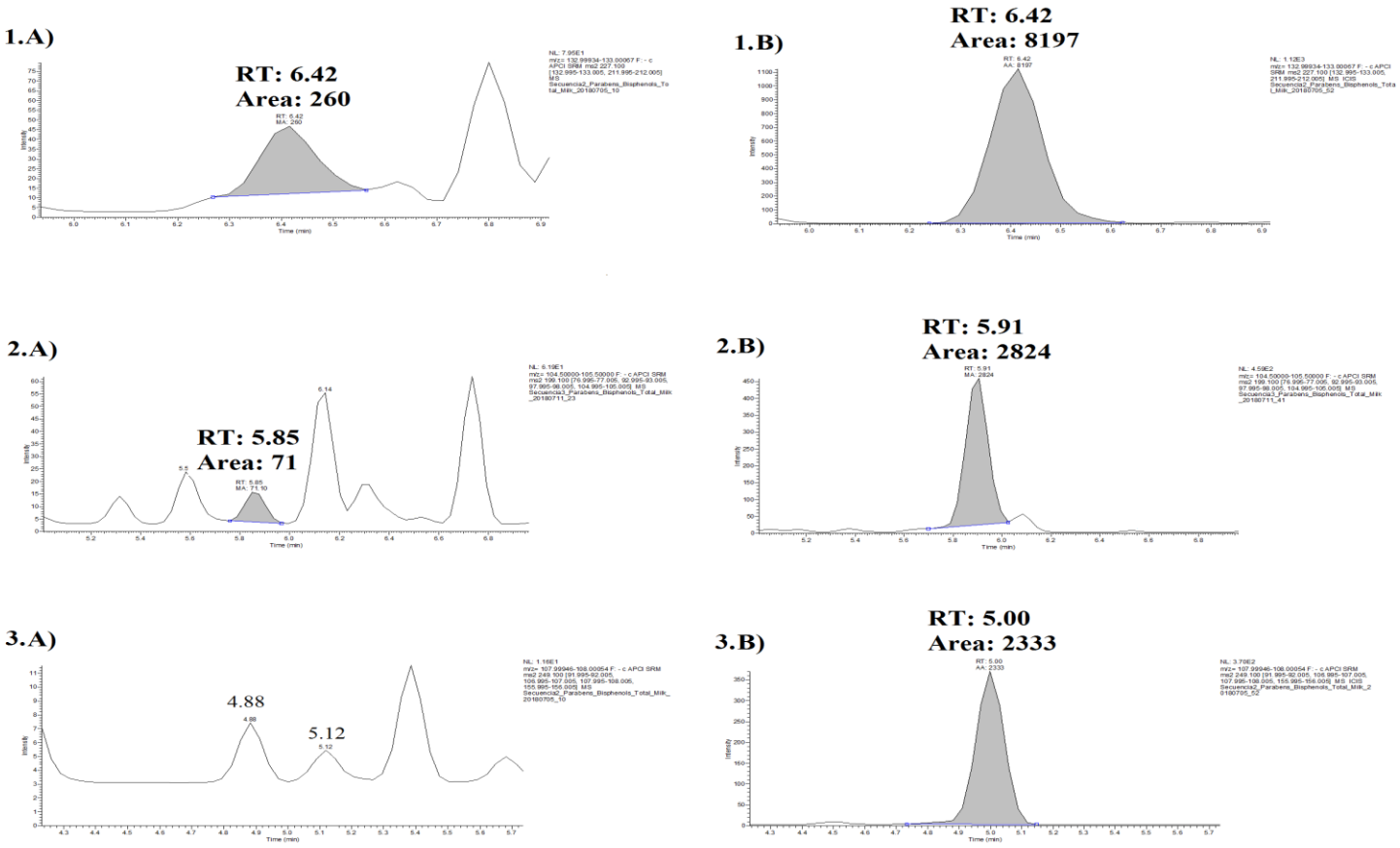


Figura SI-3. Capítulo 4. SRM chromatograms of the quantification ions of BPA(1), BPF(2) and BPS(3) in milk blank(A) and milk sample(B).

ANEXO CAPÍTULO 5

Información suplementaria de 'Biomonitoring of parabens in human milk and estimated daily intake for breastfed infants'

Tabla de información suplementaria

Tabla SI-1. Capítulo 5. Food consumption by groups in the population (grams/month), Index MED-DQI and recent packaged products consumption.

Food consumption by groups (g/month)	**Median (minimum - maximum)**
Eggs	720 (30 - 1800)
Dairy products	13000 (14000- 43000)
Meat products	5600 (740 - 14000)
Fishing products	4200 (1000 - 16000)
Vegetables	13000 (1200 - 3900)
Fruits	16000 (3700 - 84000)
Legumes and cereals	5000 (2000 - 27000)
Oils and fats	800 (150 - 1500)
Pastries	1300 (170 - 7300)
Miscellany	1400 (340 - 30000)
Drinks[a]	44000 (1700 - 77000)
Index MED-DQI[b]	5 (0 - 8)
Good	56 (46.7)[c]
Medium (Half-good or Half-poor)	64 (53.3)[c]

[a]Values expressed as mL/month
[b]Values expressed in arbitray units (a.u.)
[c]Values expressed as absolute frequency (percentage).

Tabla SI-2. Capítulo 5. Frequency of use of cosmetics products.

Cosmetic products	**n (%)**
Skin care	
Frequency	
Never or before pregnancy	25 (21.4)
Daily	68 (58.1)
Weekly or monthly	24 (20.5)
Missing data	3
Perfume	
Frequency	
Never or before pregnancy	55 (47.8)
Daily	34 (29.6)
Weekly or monthly	26 (22.6)
Missing data	5
Deodorants	
Frequency	
Never or before pregnancy	9 (8.1)
Daily or almost daily	102 (91.9)
Missing data	9
Sunscreen	
Frequency	
Never or before pregnancy	74 (64.9)
Daily	17 (14.9)
Several times a week	10 (8.8)
Monthly or ocasionally	13 (11.4)
Missing data	6
Hair colour	
Times/year	2 (0 - 26)[a]
Last application	
≤1 week	9 (7.8)
< 1 month	10 (8.6)
≥ 1 month, < 3 months	34 (29.3)
≥ 3 months	12 (10.3)
Never or before pregnancy	51 (44.0)
Missing data	4

Tabla SI-2. Capítulo 5. (Continuación).

Cosmetic products	**n (%)**
Lipstick	
Frequency	
Never or before pregnancy	87 (73.1)
Daily	11 (9.2)
Weekly or monthly	21 (17.6)
Missing data	1
Makeup	
Frequency	
Never or before pregnancy	65 (55.6)
Daily	16 (13.7)
Several times a week	16 (13.7)
Monthly	20 (17.1)
Missing data	3

[a]Values expressed as median (minimum - maximum)

Tabla SI-3. Capítulo 5. Liquid chromatography settings

Time (min)	**Mobile phase A: H_2O (%)**	**Mobile phase B: MeOH (%)**	**Flow ($mL \cdot min^{-1}$)**
0	98	2	300
6	0	10	300
7	0	10	300
7.5	98	2	300
12	98	2	300

Injection volume: 20µL

Tabla SI-4. Capítulo 5. Ion source settings

Ion source settings	
Ionization mode	APCI $[M-H]^-$
Seath gas pressure (psi)	43
Auxiliary gas flow rate (a.u.)	4
Capillary temperature (°C)	250
Vaporization temperature (°C)	400
Discharge current (µA)	4
Collision gas pressure (mTorr)	1.5

Tabla SI-5. Capítulo 5. Elemental composition, selected transitions, collision energy (CE) and tube lens offset voltage employed for each parent ion-product ions transitions

Analyte	Elemental composition	Transitions (m/z)	CE (eV)	Tube lens offset voltage (V)
Methyl paraben (MP)	$C_8H_8O_3$	151.0 → 92.1[a]	-22	-72
		151.0 → 136.1[b]	-19	-72
Ethyl paraben (EP)	$C_9H_{10}O_3$	165.1 → 92.0[a]	-27	-51
		165.1 → 136.0[b]	-16	-51
Propyl paraben (PP)	$C_{10}H_{12}O_3$	179.1 → 92.0[a]	-22	-77
		179.1 → 136.1[b]	-19	-77
Butyl paraben (BP)	$C_{11}H_{14}O_3$	193.1 → 92.0[a]	-28	-78
		193.1 → 136.1[b]	-20	-78
$^{13}C_6$-MP[c]	$^{13}C_6C_2H_8O_3$	157.0 → 98.0[a]	-22	-72
EP-d_5[d]	$C_9H_5D_5O_3$	170.1 → 92.1[a]	-27	-51
$^{13}C_6$-BP [c]	$C_5\ ^{13}C_6H_{14}O_3$	199.1 → 98.0[a]	-28	-78

[a]SRM transition used for quantification
[b]SRM transition used for confirmation
[c]Internal standard
[d]Internal standard used to correct matrix effects of EP and PP

Tabla SI-6. Capítulo 5. LC-MS/MS method validation parameters

Analyte	Calibration equation[a]	Linearity (R^2)	LoQ (ng/mL)	LDR (ng/mL)	Accuracy (Recovery (%)			Precision (RSD (%)		
					Low (n=5)	Medium (n=5)	High (n=5)	Low (n=5)	Medium (n=5)	High (n=5)
MP	y=0.0663x + 0.0124	0.996 – 0.999	0.1	0.1 – 50	104	93	95	11	6	1
EP	y=0.284x + 0.0114	0.991 – 0.999	0.1	0.1 – 50	105	105	93	12	6	5
PP	y=0.156x + 0.0029	0.991 – 0.998	0.1	0.1 – 50	92	83	95	16	6	16
BP	y=0.074x + 0.0035	0.995 – 0.999	0.1	0.1 – 50	107	96	92	14	5	6

LDR: Linear Dynamic Range;
Low: Concentration 0.1 ng/mL; Medium: Concentration 5 ng/mL; High: Concentration 50 ng/mL
[a] Example of one calibration equation; y (Area ratio); x (concentration (ng/mL))

Tabla SI-7. Capítulo 5. Spearman's correlation coefficients between unconjugated parabens levels in human milk (2 weeks after delivery)

Paraben	u-MP (ng/mL)	u-EP (ng/mL)	u-PP (ng/mL)	u-BP (ng/mL)
u-MP (ng/mL)	-	0.404**	0.440**	0.295*
u-EP (ng/mL)	-	-	0.347**	0.430**
u-PP (ng/mL)	-	-	-	0.289*
u-BP (ng/mL)	-	-	-	-

*p-value < 0.005
**p-value < 0.001

Tabla SI-8. Capítulo 5. Spearman's correlation coefficients between total parabens levels in human milk (2 weeks after delivery)

Paraben	Total-MP (ng/mL)	Total-EP (ng/mL)	Total-PP (ng/mL)	Total-BP (ng/mL)
Total-MP (ng/mL)	-	0.561**	0.514**	0.317**
Total-EP (ng/mL)	-	-	0.348**	0.375**
Total-PP (ng/mL)	-	-	-	0.367**
Total-BP (ng/mL)	-	-	-	-

*p-value < 0.005
**p-value < 0.001

Tabla SI-9. Capítulo 5. Levels of macronutrients in human milk of mothers in the Valencian Region.

Macronutrients	Median (minimum - maximum)
Fat (g/100 ml)	3.2 (0.82 - 6.9)
Protein (g/100 ml)	1.2 (0.61 – 1.8)
Carbohydrate (g/100 ml)	7.2 (3.9 - 7.6)
Total Solids (g/100 ml)	12 (7.8 - 15.5)
Energy (kcal/100 ml)	65 (42 - 98)

Tabla SI-10. Capítulo 5. Relationship between total-MP levels in human milk with the characteristics and habits of the study population using simple linear robust regression

Variable	**Total-MP levels Median (minimum-maximum)**	**Estimated parameters (Simple linear regression)**	**CI 95%**	**p-value**
Mother				
Nº children				
1	0.10 (0.01 – 49)	-	-	-
2	0.27 (0.01 – 34)	0.152	(-0.679 – 0.983)	0.717
3 or more	0.24 (0.10 – 1.4)	-0.017	(-1.516 – 1.482)	0.982
Age (years)	-	0.004	(-0.083 – 0.090)	0.935
Weight before pregnancy (kg)	-	-0.023	(-0.060 – 0.014)	0.221
Height (cm)	-	0.008	(-0.063 – 0.079)	0.825
BMI before pregnancy (kg/m^2)	-	-0.074	(-0.175 – 0.028)	0.153

Tabla SI-10. Capítulo 5. (Continuación).

Variable	Total-MP levels Median (minimum-maximum)	Estimated parameters (Simple linear regression)	CI 95%	p-value
Special diet during pregnancy				
Yes	0.10 (0.01 – 5.1)	-	-	-
No	0.24 (0.01 – 49)	0.905	(-0.274 – 2.084)	0.131
Country of birth				
Spain	0.25 (0.01 – 49)	-	-	-
Other	0.11 (0.01 – 5.1)	-0.650	(-1.755 – 0.454)	0.245
Place of residence last 10 years				
Urban	0.13 (0.01 – 28)	-	-	-
Rural	0.14 (0.02 – 49)	0.653	(-0.338 – 1.644)	0.193
Education level				
Only Primary School	0.15 (0.08 – 4.4)	-	-	-
Secondary School	0.10 (0.01 – 28)	-0.058	(-1.491 – 1.375)	0.936
University	0.25 (0.01 – 49)	0.391	(-0.871 – 1.653)	0.540
Occupational status				
Employed	0.17 (0.01 – 49)	-	-	-
Unemployed	0.30 (0.10 – 16)	0.039	(-1.118 – 1.196)	0.946
Period working outside the home (years)	-	-0.015	(-0.086 – 0.055)	0.664
Use of cosmetics at work				
Yes	0.10 (0.08 – 28)	-	-	-
No	0.24 (0.01 – 49)	0.145	(-1.225 – 1.514)	0.834
Breastfed				
Yes	0.24 (0.01 – 34)	-	-	-
No	0.14 (0.04 – 49)	-0.191	(-1.101 – 0.718)	0.677

Tabla SI-10. Capítulo 5. (Continuación).

Variable	Total-MP levels Median (minimum-maximum)	Estimated parameters (Simple linear regression)	CI 95%	p-value
Physical exercise				
3 or more days/week	0.27 (0.01 – 12)	-	-	-
1 or 2 days/week	0.61 (0.02 – 49)	0.904	(-0.494 – 2.302)	0.202
Occasionally	0.11 (0.04 – 34)	-0.124	(-1.318 – 1.070)	0.837
Never	0.10 (0.10 – 2.7)	-0.325	(-1.586 – 0.937)	0.610
Smoker				
Yes	0.10 (0.01 – 4.4)	-	-	-
Ex-smoker	0.36 (0.08 – 49)	0.966	(-0.538 – 2.470)	0.205
Never	0.15 (0.01 – 34)	0.463	(-1.030 – 1.956)	0.539
Child				
Gender				
Boy	0.44 (0.02 – 34)	-	-	-
Girl	0.14 (0.01 – 49)	-0.040	(-0.848 – 0.768)	0.921
Food consumption				
Frequency (g/month)				
Eggs	-	-0.001	$(-0.002 - 4.53\cdot10^{-5})$	0.059
Dairy products	-	$1.36\cdot10^{-5}$	$(-6.62\cdot10^{-5} - 3.89\cdot10^{-5})$	0.607
Meat products	-	$-5.66\cdot10^{-5}$	$(-2.45\cdot10^{-4} - 1.32\cdot10^{-4})$	0.551
Fishery products	-	$2.01\cdot10^{-5}$	$(-1.39\cdot10^{-4} - 1.80\cdot10^{-4})$	0.802
Vegetables	-	$1.22\cdot10^{-5}$	$(-4.16\cdot10^{-5} - 6.59\cdot10^{-5})$	0.654
Fruits	-	$-7.17\cdot10^{-6}$	$(-4.01\cdot10^{-5} - 2.57\cdot10^{-5})$	0.666
Legumes and cereals	-	$4.87\cdot10^{-5}$	$(-8.89\cdot10^{-5} - 1.86\cdot10^{-4})$	0.484
Oils and fats	-	$-7.93\cdot10^{-4}$	$(-0.002 - 4.21\cdot10^{-4})$	0.197
Pastries	-	$4.01\cdot10^{-5}$	$(-2.79\cdot10^{-4} - 3.59\cdot10^{-4})$	0.803
Miscellany	-	$3.36\cdot10^{-5}$	$(-1.78\cdot10^{-4} - 2.45\cdot10^{-4})$	0.753
Drinks (mL/month)	-	$-3.16\cdot10^{-5}$	$(-6.92\cdot10^{-5} - 5.96\cdot10^{-6})$	0.098

Tabla SI-10. Capítulo 5. (Continuación).

Variable	Total-MP levels Median (minimum-maximum)	Estimated parameters (Simple linear regression)	CI 95%	p-value
Index-MED-DQI				
Good	0.21 (0.02 – 49)	-	-	-
Medium	0.14 (0.01 – 34)	-0.042	(-0.820 – 0.736)	0.915
Packaged products nº rations /72h	-	0.010	(-0.045 – 0.066)	0.710
Personal care products				
Skin care				
Frequency				
Never or before pregnancy	0.10 (0.01 – 12)	-	-	-
Daily	0.51 (0.04 – 49)	0.891	(-0.043 – 1.824)	0.061
Weekly or monthly	0.10 (0.01 – 23)	-0.324	(-1.477 – 0.829)	0.577
Perfume				
Frequency				
Never or before pregnancy	0.10 (0.01 – 12)	-	-	-
Daily	0.61 (0.10 – 49)	1.124	(0.193 – 2.055)	0.019*
Weekly or monthly	0.16 (0.02 – 34)	0.293	(-0.652 – 1.237)	0.540
Deodorants				
Frequency				
Never or before pregnancy	0.44 (0.10 – 2.2)	-	-	-
Daily or almost daily	0.23 (0.01 – 49)	0.118	(-1.375 – 1.610)	0.876
Sunscreen				
Frequency				
Never or before pregnancy	0.12 (0.01 – 49)	-	-	-
Daily	0.60 (0.05 – 34)	0.714	(-0.512 – 1.940)	0.250
Several times a week	0.55 (0.03 – 28)	0.742	(-0.663 – 2.148)	0.296
Monthly or occasionally	0.10 (0.10 – 1.1)	-0.629	(-1.855 – 0.598)	0.311

Tabla SI-10. Capítulo 5. (Continuación).

Variable	Total-MP levels Median (minimum-maximum)	Estimated parameters (Simple linear regression)	CI 95%	p-value
Hair colour				
Times/year	-	0.019	(-0.072 – 0.109)	0.682
Last application				
≤ 1 week	0.45 (0.11 – 49)	-	-	-
< 1 month	0.38 (0.10 – 2.3)	-0.612	(-2.512 – 1.298)	0.526
≥ 1 month, < 3 months	0.25 (0.03 – 34)	-0.384	(-1.977 – 1.208)	0.632
≥ 3 months	0.10 (0.02 – 7.0)	-1.332	(-3.150 – 0.486)	0.149
Never or before pregnancy	0.12 (0.01 – 23)	-1.002	(-2.516 – 0.512)	0.192
Lipstick				
Frequency				
Never or before pregnancy	0.17 (0.01 – 49)	-	-	-
Daily	0.34 (0.10 – 23)	0.191	(-1.072 – 1.454)	0.765
Weekly or monthly	0.10 (0.01 – 28)	-0.360	(-1.425 – 0.706)	0.504
Makeup				
Frequency				
Never or before pregnancy	0.12 (0.01 – 49)	-	-	-
Daily	0.19 (0.10 – 34)	0.516	(-0.630 – 1.662)	0.373
Several times a week	0.44 (0.05 – 7.0)	0.284	(-0.863 – 1.430)	0.624
Monthly	0.55 (0.10 – 28)	1.114	(-0.032 – 2.260)	0.057
Macronutrients				
Fat (g/100 mL)	-	0.211	(-0.125 – 0.548)	0.215
Protein (g/100 mL)	-	2.405	(0.636 – 4.174)	0.008*
Carbohydrate (g/100 mL)	-	-0.348	(-1.092 – 0.395)	0.354
TS (g/100 mL)	-	0.200	(-0.110 – 0.510)	0.203
Energy (kcal/100 mL)	-	0.024	(-0.012 – 0.060)	0.187

*p-values <0.05
TS (Total Solids)

Tabla SI-11. Capítulo 5. Relationship between total-EP levels in human milk and the characteristics and habits of the study population using simple linear robust regression

Variable	Total-EP levels Median (minimum-maximum)	Estimated parameters (Simple linear regression)	CI 95%	p-value
Mother				
Nº children				
1	0.10 (0.01 – 9.0)	-	-	-
2	0.10 (0.01 – 1.5)	-0.115	(-0.662 – 0.431)	0.677
3 or more	0.24 (0.04 – 2.7)	0.658	(-0.262 – 1.579)	0.159
Age (years)	-	-0.002	(-0.059 – 0.056)	0.949
Weight before pregnancy (kg)	-	-0.010	(-0.035 – 0.015)	0.435
Height (cm)	-	0.007	(-0.035 – 0.049)	0.728
BMI before pregnancy (kg/m^2)	-	-0.033	(-0.100 – 0.034)	0.335
Special diet during pregnancy				
Yes	0.10 (0.02 – 1.3)	-	-	-
No	0.10 (0.01 – 9.0)	0.146	(-0.586 – 0.879)	0.692
Country of birth				
Spain	0.10 (0.01 – 9.0)	-	-	-
Other	0.10 (0.05 – 2.9)	0.142	(-0.613 – 0.898)	0.710
Place of residence last 10 years				
Urban	0.10 (0.01 – 6.6)	-	-	-
Rural	0.10 (0.01 – 9.0)	0.037	(-0.639 – 0.712)	0.914
Education level				
Only Primary School	0.09 (0.02 – 2.7)	-	-	-
Secondary School	0.10 (0.01 – 9.0)	-0.015	(-0.951 – 0.920)	0.974
University	0.10 (0.01 – 6.6)	0.299	(-0.509 – 1.107)	0.465
Occupational status				
Employed	0.10 (0.01 – 9.0)	-	-	-
Unemployed	0.10 (0.02 – 2.7)	-0.077	(-0.816 – 0.663)	0.838

Tabla SI-11. Capítulo 5. (Continuación).

Variable	Total-EP levels Median (minimum-maximum)	Estimated parameters (Simple linear regression)	CI 95%	p-value
Period working outside the home (years)	-	0.007	(-0.042 – 0.056)	0.781
Use of cosmetics at work				
Yes	0.10 (0.04 – 0.38)	-	-	-
No	0.10 (0.01 – 9.0)	0.351	(-0.508 – 1.210)	0.419
Breastfed				
Yes	0.10 (0.01 – 9.0)	-	-	-
No	0.10 (0.02 – 6.6)	-0.248	(-0.823 – 0.327)	0.394
Physical exercise				
3 or more days/week	0.10 (0.01 – 2.9)	-	-	-
1 or 2 days/week	0.10 (0.05 – 1.5)	0.123	(-0.858 – 1.104)	0.804
Occasionally	0.10 (0.02 – 9.0)	0.060	(-0.755 – 0.875)	0.884
Never	0.10 (0.01 – 6.6)	0.025	(-0.835 – 0.884)	0.955
Smoker				
Yes	0.03 (0.01 – 0.07)	-	-	-
Ex-smoker	0.10 (0.01 – 9.0)	1.480	(0.536 – 2.424)	0.002*
Never	0.10 (0.03 – 6.6)	1.653	(0.721 – 2.585)	0.001*
Child				
Gender				
Boy	0.10 (0.01 – 6.6)	-	-	-
Girl	0.10 (0.01 – 9.0)	-0.299	(-0.821 – 0.224)	0.259

Tabla SI-11. Capítulo 5. (Continuación).

Variable	Total-EP levels Median (minimum-maximum)	Estimated parameters (Simple linear regression)	CI 95%	p-value
Food consumption				
Frequency (g/month)				
Eggs	-	$-1.96 \cdot 10^{-4}$	$(-0.001 – 0.001)$	0.668
Dairy products	-	$-2.25 \cdot 10^{-5}$	$(-5.80 \cdot 10^{-5} - 1.30 \cdot 10^{-5})$	0.211
Meat products	-	$-2.14 \cdot 10^{-5}$	$(-1.51 \cdot 10^{-4} - 1.09 \cdot 10^{-4})$	0.745
Fishery products	-	$5.31 \cdot 10^{-5}$	$(-4.66 \cdot 10^{-5} - 1.53 \cdot 10^{-4})$	0.293
Vegetables	-	$4.57 \cdot 10^{-5}$	$(9.98 \cdot 10^{-6} - 8.14 \cdot 10^{-5})$	0.013*
Fruits	-	$1.49 \cdot 10^{-5}$	$(-7.61 \cdot 10^{-6} - 3.73 \cdot 10^{-5})$	0.192
Legumes and cereals	-	$2.23 \cdot 10^{-5}$	$(-7.56 \cdot 10^{-5} - 1.20 \cdot 10^{-4})$	0.653
Oils and fats	-	$3.40 \cdot 10^{-5}$	$(-8.02 \cdot 10^{-4} – 0.001)$	0.934
Pastries	-	$3.00 \cdot 10^{-4}$	$(9.78 \cdot 10^{-5} – 0.001)$	0.004*
Miscellany	-	$1.30 \cdot 10^{-4}$	$(-3.41 \cdot 10^{-6} - 2.64 \cdot 10^{-4})$	0.056
Drinks (mL/month)	-	$2.32 \cdot 10^{-5}$	$(-4.83 \cdot 10^{-5} - 1.88 \cdot 10^{-6})$	0.069
Index-MED-DQI				
Good	0.10 (0.02 – 9.0)	-	-	-
Medium	0.10 (0.01 – 6.6)	-0.245	(-0.758 – 0.269)	0.347
Packaged products nº rations /72h	-	-0.012	(-0.046 – 0.021)	0.467

Tabla SI-11. Capítulo 5. (Continuación).

Variable	Total-EP levels Median (minimum-maximum)	Estimated parameters (Simple linear regression)	CI 95%	p-value
Personal care products				
Skin care				
Frequency				
Never or before pregnancy	0.10 (0.01 – 9.0)	-	-	-
Daily	0.10 (0.02 – 6.6)	472	(-0.147 – 1.092)	0.134
Weekly or monthly	0.10 (0.02 – 1.5)	-0.026	(-0.796 – 0.745)	0.947
Perfume				
Frequency				
Never or before pregnancy	0.10 (0.01 – 9.0)	-	-	-
Daily	0.10 (0.02 – 6.6)	0.057	(-0.576 – 0.689)	0.859
Weekly or monthly	0.10 (0.02 – 4.1)	0.163	(-0.504 – 0.830)	0.628
Deodorants				
Frequency				
Never or before pregnancy	0.10 (0.10 – 6.6)	-	-	-
Daily or almost daily	0.10 (0.01 – 9.0)	-0.563	(-1.468 – 0.342)	0.220
Sunscreen				
Frequency				
Never or before pregnancy	0.10 (0.01 – 9.0)	-	-	-
Daily	0.13 (0.02 – 6.63)	0.604	(-0.116 – 1.324)	0.099
Several times a week	0.10 (0.05 – 0.34)	-0.002	(-0.894 – 0.891)	0.997
Monthly or occasionally	0.10 (0.02 – 0.70)	-0.146	(-0.964 – 0.672)	0.724

Tabla SI-11. Capítulo 5. (Continuación).

Variable	Total-EP levels Median (minimum-maximum)	Estimated parameters (Simple linear regression)	CI 95%	p-value
Hair colour				
Times/year	-	-0.006	(-0.065 – 0.052)	0.829
Last application				
≤ 1 week	0.10 (0.09 – 0.27)	-	-	-
< 1 month	0.10 (0.01 – 1.2)	0.074	(-1.190 – 1.337)	0.908
≥ 1 month, < 3 months	0.10 (0.01 – 6.6)	0.253	(-0.793 – 1.299)	0.632
≥ 3 months	0.10 (0.04 – 2.7)	0.207	(-0.980 – 1.393)	0.730
Never or before pregnancy	0.10 (0.01 – 9.0)	-0.035	(-1.038 – 0.968)	0.945
Lipstick				
Frequency				
Never or before pregnancy	0.10 (0.01 – 9.0)	-	-	-
Daily	0.10 (0.10 – 0.27)	-0.120	(-0.993 – 0.752)	0.785
Weekly or monthly	0.10 (0.02 – 1.2)	-0.304	(-1.017 – 0.410)	0.400
Makeup				
Frequency				
Never or before pregnancy	0.10 (0.01 – 6.6)	-	-	-
Daily	0.10 (0.02 – 0.89)	-0.011	(-0.792 – 0.770)	0.978
Several times a week	0.10 (0.01 – 1.23)	-0.206	(-0.964 – 0.552)	0.591
Monthly	0.10 (0.02 – 9.0)	0.441	(-0.297 – 1.178)	0.238
Macronutrients				
Fat (g/100 mL)	-	0.002	(-0.230 – 0.234)	0.987
Protein (g/100 mL)	-	1.469	(0.322 – 2.616)	0.013*
Carbohydrate (g/100 mL)	-	-0.312	(-0.825 – 0.200)	0.230
TS (g/100 mL)	-	0.006	(-0.209 – 0.221)	0.957
Energy (kcal/100 mL)	-	0.001	(-0.024 – 0.026)	0.952

*p-values <0.05
TS (Total Solids)

Tabla SI-12. Capítulo 5. Relationship between total-PP levels in human milk and the characteristics and habits of the study population using simple linear robust regression

Variable	Total-PP levels Median (minimum-maximum)	Estimated parameters (Simple linear regression)	CI 95%	p-value
Mother				
Nº children				
1	0.10 (0.01 – 6.1)	-	-	-
2	0.11 (0.01 – 8.0)	0.202	(-0.376 – 0.781)	0.489
3 or more	0.10 (0.01 – 0.75)	-0.166	(-1.140 – 0.809)	0.737
Age (years)	-	0.001	(-0.059 – 0.061)	0.979
Weight before pregnancy (kg)	-	-0.022	(-0.048 – 0.004)	0.099
Height (cm)	-	0.011	(-0.033 – 0.056)	0.617
BMI before pregnancy (kg/m^2)	-	-0.070	(-0.141 – 0.001)	0.052
Special diet during pregnancy				
Yes	0.09 (0.01 – 0.69)	-	-	-
No	0.10 (0.01 – 8.0)	0.758	(-0.008 – 1.525)	0.053
Country of birth				
Spain	0.10 (0.01 – 8.0)	-	-	-
Other	0.10 (0.01 – 0.69)	-0.416	(-1.218 – 0.386)	0.306
Place of residence last 10 years				
Urban	0.10 (0.01 – 6.1)	-	-	-
Rural	0.10 (0.01 – 3.8)	0.165	(-0.510 – 0.840)	0.629
Education level				
Only Primary School	0.10 (0.02 – 0.75)	-	-	-
Secondary School	0.10 (0.01 – 4.0)	0.287	(-0.693 – 1.266)	0.563
University	0.10 (0.01 – 8.0)	0.481	(-0.365 – 1.328)	0.262
Occupational status				
Employed	0.10 (0.01 – 8.0)	-	-	-
Unemployed	0.10 (0.02 – 3.5)	0.060	(-0.727 – 0.846)	0.881

Tabla SI-12. Capítulo 5. (Continuación).

Variable	Total-PP levels Median (minimum-maximum)	Estimated parameters (Simple linear regression)	CI 95%	p-value
Period working outside the home (years)	-	0.026	(-0.024 – 0.076)	0.308
Use of cosmetics at work				
Yes	0.10 (0.05 – 4.0)	-	-	-
No	0.10 (0.01 – 8.0)	-0.144	(-1.047 – 0.759)	0.753
Breastfed				
Yes	0.10 (0.01 – 8.0)	-	-	-
No	0.10 (0.01 – 6.1)	0.163	(-0.447 – 0.773)	0.597
Physical exercise				
3 or more days/week	0.11 (0.01 – 0.47)	-	-	-
1 or 2 days/week	0.10 (0.02 – 5.0)	1.179	(0.190 – 2.167)	0.020*
Occasionally	0.10 (0.01 – 8.0)	0.232	(-0.589 – 1.053)	0.576
Never	0.10 (0.04 – 6.1)	0.327	(-0.538 – 1.193)	0.455
Smoker				
Yes	0.08 (0.02 – 0.22)	-	-	-
Ex-smoker	0.10 (0.01 – 5.0)	0.803	(-0.229 – 1.835)	0.126
Never	0.10 (0.01 – 8.0)	0.961	(-0.058 – 1.980)	0.064
Child				
Gender				
Boy	0.10 (0.03 – 8.0)	-	-	-
Girl	0.10 (0.01 – 5.0)	-0.077	(-0.637 – 0.484)	0.787

Tabla SI-12. Capítulo 5. (Continuación).

Variable	**Total-PP levels Median (minimum-maximum)**	**Estimated parameters (Simple linear regression)**	**CI 95%**	**p-value**
Food consumption				
Frequency (g/month)				
Eggs	-	$-1.36\cdot10^{-4}$	(-0.001 – 0.001)	0.759
Dairy products	-	$2.51\cdot10^{-5}$	($-9.22\cdot10^{-6}$ - $5.93\cdot10^{-5}$)	0.150
Meat products	-	$5.65\cdot10^{-5}$	($-6.89\cdot10^{-5}$ - $1.82\cdot10^{-4}$)	0.373
Fishery products	-	$5.06\cdot10^{-6}$	($-9.20\cdot10^{-5}$ - $1.02\cdot10^{-4}$)	0.918
Vegetables	-	$-1.07\cdot10^{-5}$	($-4.64\cdot10^{-5}$ - $2.49\cdot10^{-5}$)	0.553
Fruits	-	$-1.09\cdot10^{-5}$	($-3.27\cdot10^{-5}$ - $1.09\cdot10^{-5}$)	0.324
Legumes and cereals	-	$-5.69\cdot10^{-5}$	($-1.51\cdot10^{-4}$ - $3.72\cdot10^{-5}$)	0.233
Oils and fats	-	$-4.18\cdot10^{-4}$	(-0.001 - $3.86\cdot10^{-4}$)	0.305
Pastries	-	$1.86\cdot10^{-5}$	($-1.86\cdot10^{-4}$ - $2.23\cdot10^{-4}$)	0.857
Miscellany	-	$-2.28\cdot10^{-5}$	($-1.55\cdot10^{-4}$ - $1.09\cdot10^{-4}$)	0.733
Drinks (mL/month)	-	$-1.01\cdot10^{-5}$	($-3.47\cdot10^{-5}$ - $1.46\cdot10^{-5}$)	0.419
Index-MED-DQI				
Good	0.10 (0.01 – 3.8)	-	-	-
Medium	0.10 (0.01 – 8.0)	0.083	(-0.458 – 0.624)	0.761
Packaged products nº rations /72h	-	-0.008	(-0.043 – 0.027)	0.653

Tabla SI-12. Capítulo 5. (Continuación).

Variable	Total-PP levels Median (minimum-maximum)	Estimated parameters (Simple linear regression)	CI 95%	p-value
Personal care products				
Skin care				
Frequency				
Never or before pregnancy	0.10 (0.02 – 3.5)	-	-	-
Daily	0.10 (0.01 – 8.0)	0.616	(-0.030 – 1.261)	0.061
Weekly or monthly	0.10 (0.01 – 3.2)	-0.070	(-0.873 – 0.733)	0.863
Perfume				
Frequency				
Never or before pregnancy	0.10 (0.01 – 3.5)	-	-	-
Daily	0.12 (0.05 – 6.1)	1.097	(0.480 – 1.714)	0.001*
Weekly or monthly	0.10 (0.01 – 8.0)	0.416	(-0.235 – 1.066)	0.207
Deodorants				
Frequency				
Never or before pregnancy	0.17 (0.02 – 6.1)	-	-	-
Daily	0.10 (0.01 – 8.0)	-0.657	(-1.625 – 0.310)	0.310
Sunscreen				
Frequency				
Never or before pregnancy	0.10 (0.01 – 5.0)	-	-	-
Daily	0.10 (0.02 – 8.0)	0.119	(-0.670 – 0.909)	0.765
Several times a week	0.10 (0.03 – 4.0)	0.106	(-0.873 – 1.085)	0.830
Monthly or occasionally	0.10 (0.01 – 0.12)	-0.775	(-1.673 – 0.123)	0.123

Tabla SI-12. Capítulo 5. (Continuación).

Variable	Total-PP levels Median (minimum-maximum)	Estimated parameters (Simple linear regression)	CI 95%	p-value
Hair colour				
Times/year	-	0.008	(-0.055 – 0.071)	0.801
Last application				
≤ 1 week	0.14 (0.01 – 3.8)	-	-	-
< 1 month	0.10 (0.04 – 1.0)	-0.192	(-1.504 – 1.120)	0.772
≥ 1 month, < 3 months	0.10 (0.03 – 8.0)	0.155	(-0.932 – 1.242)	0.778
≥ 3 months	0.10 (0.04 – 0.75)	-0.334	(-1.567 – 0.899)	0.592
Never or before pregnancy	0.10 (0.01 – 5.0)	-0.339	(-1.381 – 0.702)	0.519
Lipstick				
Frequency				
Never or before pregnancy	0.10 (0.01 – 8.0)	-	-	-
Daily	0.12 (0.01 – 5.0)	0.004	(-0.917 – 0.926)	0.993
Several times a week	0.10 (0.01 – 4.0)	-0.208	(-0.962 – 0.545)	0.584
Makeup				
Frequency				
Never or before pregnancy	0.10 (0.01 – 6.1)	-	-	-
Daily	0.13 (0.08 – 8.0)	1.012	(0.197 – 1.827)	0.015*
Several times a week	0.11 (0.04 – 0.7)	0.242	(-0.549 – 1.033)	0.545
Monthly	0.10 (0.04 – 4.0)	0.653	(-0.117 – 1.422)	0.096
Macronutrients				
Fat (g/100 mL)	-	0.098	(-0.145 – 0.342)	0.424
Protein (g/100 mL)	-	1.434	(0.222 – 2.645)	0.021*
Carbohydrate (g/100 mL)	-	-0.145	(-0.688 – 0.387)	0.596
TS (g/100 mL)	-	0.116	(-0.110 – 0.341)	0.310
Energy (kcal/100 mL)	-	0.013	(-0.014 – 0.039)	0.344

*p-values <0.05
TS (Total Solids)

Tabla SI-13. Capítulo 5. Relationship between total-BP levels in human milk and the characteristics and habits of the study population using simple linear robust regression.

Variable	Total-BP levels Median (minimum-maximum)	Estimated parameters (Simple linear regression)	CI 95%	p-value
Mother				
Nº children				
1	0.10 (0.04 – 0.70)	-	-	-
2	0.10 (0.02 – 1.3)	-0.075	(-0.335 – 0.185)	0.567
3 or more	0.10 (0.06 – 0.45)	0.250	(-0.189 – 0.688)	0.261
Age (years)	-	-0.022	(-0.049 – 0.005)	0.111
Weight before pregnancy (kg)	-	-0.007	(-0.018 – 0.005)	0.274
Height (cm)	-	-0.006	(-0.026 – 0.014)	0.547
BMI before pregnancy (kg/m^2)	-	-0.015	(-0.047 – 0.017)	0.355
Special diet during pregnancy				
Yes	0.10 (0.05 – 0.64)	-	-	-
No	0.10 (0.02 – 1.3)	-0.106	(-0.452 – 0.240)	0.546
Country of birth				
Spain	0.10 (0.03 – 1.3)	-	-	-
Other	0.10 (0.02 – 0.12)	-0.225	(-0.580 – 0.130)	0.212
Place of residence last 10 years				
Urban	0.10 (0.02 – 0.70)	-	-	-
Rural	0.10 (0.04 – 0.21)	0.079	(-0.205 – 0.364)	0.580
Education level				
Only Primary School	0.10 (0.04 – 0.45)	-	-	-
Secondary School	0.09 (0.03 – 0.20)	-0.092	(-0.534 – 0.351)	0.682
University	0.10 (0.02 – 1.3)	0.116	(-0.266 – 0.499)	0.547
Occupational status				
Employed	0.10 (0.02 – 1.3)	-	-	-
Unemployed	0.10 (0.06 – 0.45)	0.019	(-0.335 – 0.374)	0.914

Tabla SI-13. Capítulo 5. (Continuación)

Variable	Total-BP levels Median (minimum-maximum)	Estimated parameters (Simple linear regression)	CI 95%	p-value
Period working outside the home (years)	-	-0.009	(-0.033 – 0.014)	0.426
Use of cosmetics at work				
Yes	0.10 (0.06 – 0.12)	-	-	-
No	0.10 (0.02 – 1.3)	0.087	(-0.321 – 0.496)	0.673
Breastfed				
Yes	0.10 (0.02 – 1.3)	-	-	-
No	0.10 (0.04 – 0.70)	0.119	(-0.160 – 0.398)	0.398
Physical exercise				
3 or more days/week	0.10 (0.04 – 0.64)	-	-	-
1 or 2 days/week	0.10 (0.03 – 0.21)	-0.065	(-0.513 – 0.383)	0.773
Occasionally	0.10 (0.04 – 1.3)	0.052	(-0.320 – 0.424)	0.783
Never	0.10 (0.03 – 0.70)	0.049	(-0.343 – 0.442)	0.804
Smoker				
Yes	0.08 (0.04 – 0.11)	-	-	-
Ex-smoker	0.10 (0.03 – 0.45)	0.223	(-0.241 – 0.687)	0.343
Never	0.10 (0.02 – 1.3)	0.428	(-0.031 – 0.886)	0.067
Child				
Gender				
Boy	0.10 (0.02 – 1.3)	-	-	-
Girl	0.10 (0.03 – 0.64)	-0.069	(-0.317 – 0.180)	0.585

Tabla SI-13. Capítulo 5. (Continuación).

Variable	Total-BP levels Median (minimum-maximum)	Estimated parameters (Simple linear regression)	CI 95%	p-value
Food consumption				
Frequency (g/month)				
Eggs	-	$-2.89 \cdot 10^{-4}$	$(-7.15 \cdot 10^{-4} - 1.38 \cdot 10^{-4})$	0.182
Dairy products	-	$-5.64 \cdot 10^{-6}$	$(-2.26 \cdot 10^{-5} - 1.13 \cdot 10^{-5})$	0.511
Meat products	-	$7.71 \cdot 10^{-6}$	$(-5.41 \cdot 10^{-5} - 6.95 \cdot 10^{-5})$	0.805
Fishery products	-	$-2.48 \cdot 10^{-5}$	$(-7.22 \cdot 10^{-5} - 2.26 \cdot 10^{-5})$	0.302
Vegetables	-	$1.08 \cdot 10^{-5}$	$(-6.62 \cdot 10^{-6} - 2.82 \cdot 10^{-5})$	0.222
Fruits	-	$1.26 \cdot 10^{-5}$	$(-2.31 \cdot 10^{-5} - -2.16 \cdot 10^{-6})$	0.019*
Legumes and cereals	-	$-9.18 \cdot 10^{-7}$	$(-4.75 \cdot 10^{-5} - 4.57 \cdot 10^{-5})$	0.969
Oils and fats	-	$-1.97 \cdot 10^{-6}$	$(-4.00 \cdot 10^{-4} - 3.96 \cdot 10^{-4})$	0.992
Pastries	-	$6.03 \cdot 10^{-5}$	$(-3.96 \cdot 10^{-5} - 1.60 \cdot 10^{-4})$	0.234
Miscellany	-	$2.93 \cdot 10^{-5}$	$(-3.53 \cdot 10^{-5} - 9.38 \cdot 10^{-5})$	0.370
Drinks (mL/month)	-	$4.47 \cdot 10^{-6}$	$(-7.63 \cdot 10^{-6} - 1.66 \cdot 10^{-5})$	0.465
Index-MED-DQI				
Good	0.10 (0.03 – 0.44)	-	-	-
Medium	0.10 (0.02 – 1.3)	-0.066	(-0.310 – 0.179)	0.594
Packaged products nº rations /72h	-	$-8.44 \cdot 10^{-4}$	(-0.017 – 0.015)	0.917

Tabla SI-13. Capítulo 5. (Continuación).

Variable	Total-BP levels Median (minimum-maximum)	Estimated parameters (Simple linear regression)	CI 95%	p-value
Personal care products				
Skin care				
Frequency				
Never or before pregnancy	0.09 (0.03 – 0.64)	-	-	-
Daily	0.10 (0.02 – 1.3)	0.116	(-0.176 – 0.409)	0.431
Weekly or monthly	0.10 (0.04 – 0.36)	-0.081	(-0.444 – 0.283)	0.660
Perfume				
Frequency				
Never or before pregnancy	0.10 (0.03 – 0.45)	-	-	-
Daily	0.10 (0.04 – 0.70)	0.181	(-0.117 – 0.479)	0.230
Weekly or monthly	0.10 (0.02 – 1.3)	0.008	(-0.306 – 0.322)	0.961
Deodorants				
Frequency				
Never or before pregnancy	0.10 (0.02 – 1.3)	-	-	-
Daily or almost daily	0.10 (0.03 – 0.70)	-0.041	(-0.492 – 0.410)	0.858
Sunscreen				
Frequency				
Never or before pregnancy	0.10 (0.02 – 0.45)	-	-	-
Daily	0.10 (0.06 – 1.3)	0.497	(0.168 – 0.827)	0.004*
Several times a week	0.09 (0.07 – 0.21)	-0.053	(-0.462 – 0.356)	0.796
Monthly or occasionally	0.10 (0.03 – 0.10)	-0.248	(-0.623 – 0.127)	0.192
Hair colour				
Times/year	-	-0.007	(-0.034 – 0.021)	0.633
Last application				
≤ 1 week	0.09 (0.04 – 0.44)	-	-	-
< 1 month	0.10 (0.07 – 0.15)	0.067	(-0.504 – 0.639)	0.815
≥ 1 month, < 3 months	0.10 (0.04 – 1.3)	0.305	(-0.168 – 0.778)	0.203
≥ 3 months	0.10 (0.06 – 0.45)	0.150	(-0.387 – 0.686)	0.581
Never or before pregnancy	0.10 (0.02 – 0.36)	-0.043	(-0.497 – 0.410)	0.850

Tabla SI-13. Capítulo 5. (Continuación).

Variable	Total-BP levels Median (minimum-maximum)	Estimated parameters (Simple linear regression)	CI 95%	p-value
Lipstick				
Frequency				
Never or before pregnancy	0.10 (0.03 – 1.3)	-	-	-
Daily	0.09 (0.02 – 0.15)	-0.409	(-0.808 – -0.009)	0.045*
Weekly or monthly	0.10 (0.05 – 0.16)	-0.208	(-0.535 – 0.118)	0.208
Makeup				
Frequency				
Never or before pregnancy	0.10 (0.03 – 0.70)	-	-	-
Daily	0.10 (0.02 – 1.3)	0.028	(-0.336 – 0.392)	0.878
Several times a week	0.10 (0.05 – 0.25)	-0.064	(-0.417 – 0.289)	0.720
Monthly	0.10 (0.06 – 0.64)	0.218	(-0.126 – 0.562)	0.211
Macronutrients				
Fat (g/100 mL)	-	0.017	(-0.094 – 0.127)	0.764
Protein (g/100 mL)	-	0.257	(-0.306 – 0.820)	0.368
Carbohydrate (g/100 mL)	-	0.022	(-0.224 – 0.269)	0.858
TS (g/100 mL)	-	0.028	(-0.074 – 0.131)	0.587
Energy (kcal/100 mL)	-	0.003	(-0.009 – 0.015)	0.668

*p-values <0.05
TS (Total Solids)

Tabla SI-14. Capítulo 5. Spearman correlation between determinant variables and parabens levels 2 weeks after pregnancy (p-value)

	u-MP (ng/mL)	**Total-MP (ng/mL)**	**u-EP (ng/mL)**	**Total-EP (ng/mL)**	**u-PP (ng/mL)**	**Total-PP (ng/mL)**	**u-BP (ng/mL)**	**Total-BP (ng/mL)**
Gestational age (weeks)	-0.187 (0.079)	-0.118 (0.316)	-0.016 (0.884)	-0.186 (0.103)	-0.121 (0.259)	0.039 (0.734)	-0.265 (0.012)*	-0.154 (0.179)
Children weight at birth (g)	-0.130 (0.171)	0.026 (0.810)	-0.103 (0.279)	-0.017 (0.870)	0.011 (0.906)	0.013 (0.897)	-0.106 (0.264)	-0.214 (0.034)*
Children height at birth (cm)	-0.303 (0.009)*	-0.094 (0.477)	-0.144 (0.221)	-0.041 (0.748)	-0.123 (0.297)	-0.108 (0.398)	-0.181 (0.122)	-0.122 (0.340)
Children cranial perimeter (cm)	-0.005 (0.971)	-0.021 (0.895)	-0.171 (0.234)	-0.078 (0.616)	-0.011 (0.939)	-0.064 (0.681)	-0.145 (0.315)	-0.186 (0.227)

*p-value <0.05

Tabla SI-15. Capítulo 5. EDI using the deterministic approach

Parabens (µg/Kg bw-day)	u-MP	Total-MP	u-EP	Total-EP	u-PP	Total-PP	u-BP	Total-BP
Milk intake rate GM (140 mL/kg bw-day)	0.018	0.05	0.008	0.018	0.010	0.02	0.011	0.014
Milk intake rate 95th percentile (190 mL/kg bw-day)	0.02	0.07	0.011	0.02	0.013	0.03	0.02	0.02

GM (geometric mean)

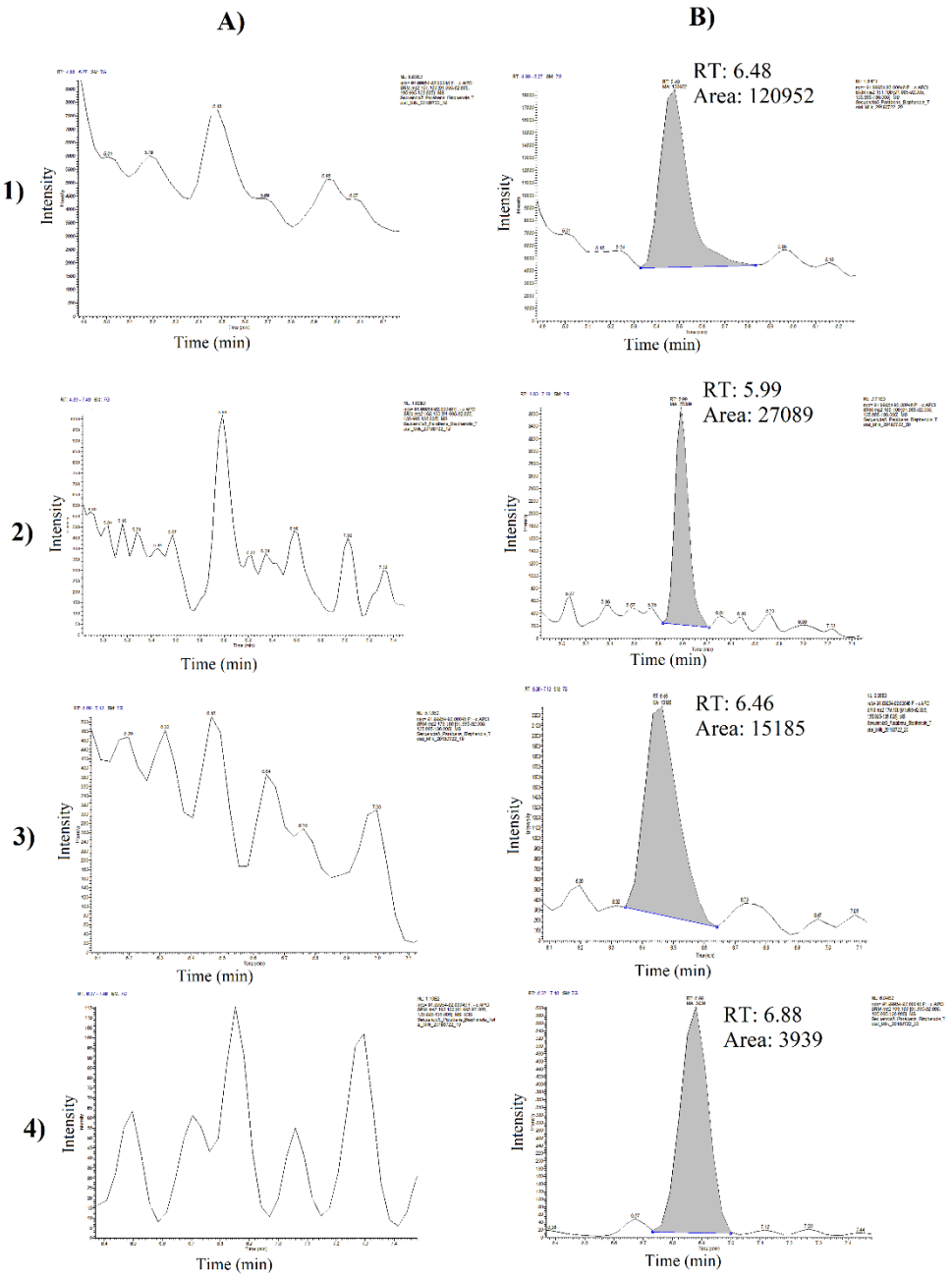


Figura SI-1. Capítulo 5. HPLC-MS/MS chromatograms of milk blank before and after spiking with parabens at the LoQ level. A) before spiking; B) after spiking; 1) MP SRM chromatograms; 2) EP SRM chromatograms; 3) PP SRM chromatograms; 4) BP SRM chromatograms. For all analytes the blank milk area (A) was <30% of the area of the milk spiked at the LoQ (B).

ANEXO CAPÍTULO 6

Información suplementaria de 'Risk assessment of exposure to phthalates in breastfeeding women using human biomonitoring'

Tabla de información suplementaria

Tabla SI-1. Capítulo 6. Food consumption by groups in the population (nº rations/last 72h) and Index MED-DQI

Food consumption by groups (g/month)	**Median (minimum - maximum)**
Eggs	1 (0 - 6)
Meat products group	5 (0 – 10)
Fishery products group	2 (0 – 7)
Dairy products group	6 (0 – 17)
Vegetables group	3 (0 – 12)
Fruits group	6 (0 – 15)
Legumes and cereals group	6 (2 – 20)
Oils and fats group	6 (0 – 21)
Packaged juice	0 (0 – 12)
Index MED-DQI[a]	5 (0 - 8)
Good	50 (48.1)[b]
Medium (Half-good or Half-poor)	54 (51.9)[b]

[a]Values expressed in arbitray units (a.u.)
[b]Values expressed as absolute frequency (percentage).

Tabla SI-2. Capítulo 6. Frequency of use of personal care products.

Personal care products	**n (%)**
Skin care	
Frequency	
Never or before pregnancy	23 (22.3)
Daily	58 (56.3)
Weekly or monthly	22 (21.4)
Missing data	1
Perfume	
Frequency	
Never or before pregnancy	49 (49)
Daily	27 (27)
Weekly or monthly	24 (24)
Missing data	4
Deodorants	
Frequency	
Not daily	13 (13.5)
Daily	83 (86.5)
Missing data	8
Sunscreen	
Frequency	
Never or before pregnancy	64 (64)
Daily	14 (14)
Sometimes (Weekly, monthly or occasionaly)	22 (22)
Missing data	4
Hair colour last application	
≤1 month	16 (15.8)
>1 month	39 (38.6)
Never or before pregnancy	46 (45.5)
Missing data	3
Lipstick	
Frequency	
Never or before pregnancy	76 (73.1)
Daily or sometimes	28 (26.9)
Makeup	
Frequency	
Never or before pregnancy	58 (56.9)
Daily	13 (12.7)
Sometimes (Weekly, monthly or occasionaly)	31 (30.4)
Missing data	2

Tabla SI-3. Capítulo 6. Chromatographic conditions

Time (min)	**Mobile phase A (%)** ***H_2O (HAc 0.1%)***	**Mobile phase B (%)** ***MeOH:ACN (90:10, v/v) (HAc 0.1%)***	**Flow (mL/min)**	**Temperature (ºC)**
0.0	70	30	0.4	50
2.0	70	34	0.4	50
4.1	60	40	0.4	50
6.1	45	55	0.4	50
8.1	20	80	0.4	50
8.5	0	100	0.4	50
10.0	70	30	0.4	50
12.0	70	30	0.4	50

Tabla SI-4. Capítulo 6. Mass spectrometer ionization settings

Ionization source	H-ESI
Ionization mode	Negative
Spray voltage (V)	2500
Sheath gas (a.u.)	45
Aux gas (a.u.)	13
Sweep Gas (a.u.)	1
Ion transfer tube temperature (ºC)	342
Vaporizer temperature (ºC)	358

H-ESI (Heated electrospray ionization)
a.u. (Arbitrary units)

Tabla SI-5. Capítulo 6. SRM transitions, fragmentation conditions, retention time for the analytes studied and the isotopically labelled internal standards (ILIS) used.

Metabolite	RT (min)	ILIS	Precursor (m/z)	Fragments (m/z)	CE (V)	RF Lens (V)
MECPP	7.22	External	307.18	159.17 (Q)	13.5	49
				113.17 (q)	27.4	49
				121.11 (q)	24.3	49
MEHHP	7.52	MEOHP-$^{13}C_4$	293.18	145.17 (Q)	14.5	68
				121.11 (q)	18.4	68
MEOHP	7.01	MEOHP-$^{13}C_4$	291.18	143.15 (Q)	14.4	68
				121.11 (q)	18.5	68
MCPP	1.29	External	251.12	103.11 (Q)	10.2	37
				164.99 (q)	11.8	37
				121.13 (q)	19.6	37
2cx-MMHP	7.73	MEOHP-$^{13}C_4$	307.18	159.17 (Q)	13.5	49
				141.11 (q)	23.4	49
				113.17 (q)	27.4	49
MBzP	6.92	MBP-$^{13}C_2$	255.15	183.11 (Q)	10.2	56
				105.11 (q)	15.5	56
MCHP	7.90	MEOHP-$^{13}C_4$	247.15	77.11 (Q)	19.3	55
				147.05 (q)	16.4	55
MEP	2.22	MEOHP-$^{13}C_4$	193.15	77.17 (Q)	17.2	50
				121.11 (q)	10.3	50
MiBP	6.53	MBP-$^{13}C_2$	221.12	134.11 (Q)	13.6	55
				77.1 (q)	18.0	55
MiNP	9.75	MBP-$^{13}C_2$	291.18	141.22 (Q)	19.2	68
				121.11 (q)	18.5	68
MMP	1.17	MEOHP-$^{13}C_4$	179.17	77.11 (Q)	17.8	45
MEHP	9.58	MEOHP-$^{13}C_4$	277.18	127.15 (Q)	16.9	63
				121.11 (q)	16.4	63
MOP	9.71	MBP-$^{13}C_2$	277.18	127.15 (Q)	16.9	63
				125.17 (q)	15.0	63
				121.11 (q)	16.4	63
MnBP	6.67	MBP-$^{13}C_2$	221.12	77.11 (Q)	18.0	55
				149.18 (q)	10.8	55
				177.18 (q)	10.2	55
MEOHP-$^{13}C_4$	7.01	-	295.18	143.18 (Q)	14.5	65
MBP-$^{13}C_2$	6.67	-	225.15	79.17 (Q)	17.9	55

CE (Collision energy)
RF (Radio frequency)
Q (Quantification ion)
q (Confirmation ion)

Tabla SI-6. Capítulo 6. Method validation parameters

Compound	Linearity (R^2)	LoQ (ng/mL)	LDR (ng/mL)	Accuracy (Recovery (%))			Precision (RSD (%))			Uncertainty /%)		
				Low	Medium	High	Low	Medium	High	Low	Medium	High
MECPP	0.998 - 1.000	1.0	1.0 - 100	98[a]	92[a]	92[a]	12[a]	6[a]	5[a]	28[a]	14[a]	12[a]
MEHHP	0.999 - 1.000	2.0	2.0 - 200	97[b]	93[b]	92[b]	10[b]	5[b]	5[b]	24[b]	11[b]	11[b]
MEOHP	0.996 - 1.000	0.5	0.5 - 50	101[c]	96[c]	93[c]	12[c]	10[c]	12[c]	28[c]	23[c]	27[c]
MCPP	0.993 - 0.999	2.0	2.0 - 200	89[b]	94[b]	100[b]	19[b]	10[b]	9[b]	44[b]	23[b]	21[b]
2cx-MMHP	0.999 - 1.000	2.0	2.0 - 200	101[b]	92[b]	92[b]	13[b]	7[b]	5[b]	31[b]	15[b]	13[b]
MBzP	0.997 - 1.000	1.0	1.0 - 100	104[a]	94[a]	92[a]	14[a]	5[a]	5[a]	32[a]	11[a]	13[a]
MCHP	1.000 - 1.000	0.5	0.5 - 50	98[c]	93[c]	93[c]	10[c]	5[c]	5[c]	24[c]	11[c]	12[c]
MEP	0.986 - 1.000	2.0	2.0 - 200	98[b]	94[b]	93[b]	11[b]	13[b]	8[b]	25[b]	30[b]	18[b]
MiBP	0.998 - 0.999	2.0	2.0 - 200	98[b]	95[b]	91[b]	11[b]	6[b]	7[b]	26[b]	15[b]	17[b]
MiNP	0.998 - 1.000	0.5	0.5 - 50	105[c]	92[c]	93[c]	10[c]	7[c]	7[c]	23[c]	16[c]	17[c]
MMP	0.997 - 1.000	1.0	1.0 - 100	109[a]	97[a]	102[a]	21[a]	9[a]	12[a]	50[a]	22[a]	27[a]
MEHP	0.999 - 1.000	1.0	1.0 - 100	78[a]	92[a]	92[a]	12[a]	6[a]	5[a]	40[a]	17[a]	11[a]
MOP	0.999 - 1.000	0.5	0.5 - 50	99[c]	92[c]	94[c]	17[c]	7[c]	5[c]	28[c]	13[c]	12[c]
MnBP	0.999 - 1.000	0.5	0.5 - 50	96[c]	91[c]	90[c]	20	5[c]	8[c]	46[c]	11[c]	18[c]

LoQ (Limit of quantification); Linear dynamic range (LDR); Relative standard deviation (RSD)
[a]Low (1.0 ng/mL); Medium (10 ng/mL); High (100 ng/mL)
[b]Low (2.0 ng/mL); Medium (20 ng/mL); High (200 ng/mL)
[c]Low (0.5 ng/mL); Medium (5 ng/mL); High (50 ng/mL)

Tabla SI-7. Capítulo 6. Biomonitoring equivalents (BE) and HBM I guidance values for phthalates in urine

Phthalate	BE					HBM I	Reference
	Derived from Health Canada TDI	Derived from US EPA RfD	Derived from ATSDR chronic MRL	Derived from ATSDR Intermediate MRL	Derived from EFSA TDI		
DEHP (as the sum of five metabolites: MEHP, MEHHP, MEOHP, 5cx-MEPP, and 2cx-MMHP) ng/mL (µg/g creatinine)	1000 (1300)	430 (550)	1300 (1700)	3200 (4100)	1100 (1400)	-	Aylward et al 2009a
DEHP (as the sum of two metabolites: MEHHP and MEOHP) ng/mL	-	-	-	-	-	300*	Schulz et al., 2012
DEP (as MEP) ng/mL (µg/g creatinine)	-	18000 (23000)	-	-	-	-	Aylward et al 2009b
BzBP (as MBzP) ng/mL (µg/g creatinine)	31000 (40000)	3800 (4900)	-	-	12000 (15000)	-	Aylward et al 2009b
DnBP (as MnBP) ng/mL (µg/g creatinine)	1400 (1800)	2700 (3500)	-	-	200 (280)	-	Aylward et al 2009b
DiNP (as MiNP) ng/mL	-	-	-	-	0.5**	-	(Hays et al., 2011)

*For women of reproductive age
**For women >16 years old

Tabla SI-8. Capítulo 6. Levels of urinary creatinine of mothers in the Valencian Region.

Parameter	Median (minimum - maximum)
Creatinine (g/L)	1.04 (0.32 – 2.41)*

*n=104. Three samples were out of the range 0.3 – 3 g/L and were removed from the study

Tabla SI-9. Capítulo 6. Spearman's correlation coefficients between levels of phthalate metabolites in urine

Analyte	BzBP (as MBzP) (µg/g creat)	DiBP (as MiBP) (µg/g creat)	DnBP (as MnBP) (µg/g creat)	DEP (as MEP) (µg/g creat)	DEHP (as sum of five metabolites) (µg/g creat)
BzBP (as MBzP) (µg/g creat)	-	0.171	0.279*	0.152	0.378**
DiBP (as MiBP) (µg/g creat)	-	-	0.622**	0.176	0.421**
DnBP (as MnBP) (µg/g creat)	-	-	-	0.307*	0.416**
DEP (as MEP) (µg/g creat)	-	-	-	-	0.074
DEHP (as sum of five metabolites) (µg/g creat)	-	-	-	-	-

*p-value < 0.01
**p-value <0.001

Tabla SI-10. Capítulo 6. Relationship between MBzP levels in human milk with the characteristics and habits of the study population using simple linear robust regression

Variable	MBzP levels Median (minimum-maximum)	Estimated parameters (Simple linear robust regression)	Standard error	p-value
Mother				
Nº children				
Primipara mother	2.20 (0.39 – 16.70)	-	-	-
2 or more children	1.96 (0.36 – 10.80)	-0.151	0.186	0.420
Age (years)	-	-0.023	0.021	0.263
Weight before pregnancy (kg)	-	-0.002	0.010	0.263
Height (cm)	-	-0.004	0.014	0.759
BMI before pregnancy (kg/m^2)	-	0.002	0.026	0.942
Special diet during pregnancy				
Yes	1.74 (0.63 – 4.80)	-	-	-
No	2.20 (0.36 – 16.70)	-0.057	0.275	0.835
Country of birth				
Spain	2.05 (0.39 – 16.70)	-	-	-
Other	2.50 (0.36 – 5.70)	-0.089	0.266	0.740
Place of residence last 10 years				
Urban	1.83 (0.36 – 16.70)	-	-	-
Rural	3.00 (1.00 – 14.27)	0.540	0.254	0.036**
Education level				
Only Primary School	2.00 (0.55 – 3.60)	-	-	-
Secondary School	2.10 (0.36 – 6.27)	0.120	0.348	0.731
University	2.13 (0.43 – 16.70)	0.113	0.305	0.713
Occupational status				
Employed	1.91 (0.36 – 14.27)	-	-	-
Unemployed	4.18 (1.07 – 16.70)	0.496	0.264	0.063*
Period working outside the home (years)	-	-0.012	0.017	0.484

Tabla SI-10. Capítulo 6. (Continuación)

Variable	MBzP levels Median (minimum-maximum)	Estimated parameters (Simple linear robust regression)	Standard error	p-value
Physical exercise				
3 or more days/week	1.50 (0.43 – 5.70)	-		-
1 or 2 days/week	1.70 (0.36 – 14.27)	0.010	0.327	0.975
Occasionally	2.80 (0.39 – 16.70)	0.184	0.272	0.678
Never	2.00 (0.70 – 7.14)	0.018	0.308	0.954
Smoker				
Yes	2.66 (0.39 – 5.70)	-		-
Ex-smoker	1.87 (0.43 – 13.54)	0.009	0.406	0.981
Never	2.32 (0.36 – 16.70)	-0.084	0.402	0.835
Food consumption				
nº rations previous 72h				
Eggs	-	-0.084	0.097	0.389
Packaged juice	-	0.040	0.049	0.411
Meat products group	-	-0.016	0.050	0.755
Fishery products group	-	-0.025	0.061	0.688
Dairy products group	-	-0.032	0.025	0.212
Vegetables group	-	0.048	0.044	0.278
Fruits group	-	0.010	0.028	0.734
Legumes and cereals group	-	-0.012	0.028	0.673
Oils and fats group	-	0.011	0.029	0.694
Index-MED-DQI				
Good	2.32 (0.39 – 16.70)	-		-
Medium	1.99 (0.36 – 13.54)	-0.099	0.184	0.593

Tabla SI-10. Capítulo 6. (Continuación)

Variable	MBzP levels Median (minimum-maximum)	Estimated parameters (Simple linear robust regression)	Standard error	p-value
Personal care products				
Skin care				
Frequency				
Never or before pregnancy	1.61 (0.43 – 14.27)	-	-	-
Daily	2.20 (0.36 – 16.70)	0.140	0.228	0.542
Weekly or monthly	2.05 (0.39 – 10.80)	0.109	0.276	0.693
Perfume				
Frequency				
Never or before pregnancy	2.80 (0.43 – 16.70)	-		-
Daily	1.83 (0.39 – 10.80)	-0.322	0.224	0.153*
Weekly or monthly	2.19 (0.36 – 14.27)	-0.220	0.233	0.348
Deodorants				
Frequency				
Not daily	2.50 (0.72 – 4.10)	-		-
Daily	2.00 (0.36 – 16.70)	-0.181	0.278	0.516
Sunscreen				
Frequency				
Never or before pregnancy	2.59 (0.36 – 14.27)	-		-
Daily	2.29 (0.55 – 10.80)	0.092	0.274	0.739
Sometimes	1.44 (0.58 – 16.70)	-0.208	0.230	0.368
Hair colour				
Last application				
≤ 1 month	1.44 (0.79 – 4.07)	-		-
> 1 month	1.99 (0.39 – 16.70)	-0.105	0.280	0.711
Never or before pregnancy	2.47 (0.36 – 10.80)	-0.046	0.275	0.867
Lipstick				
Frequency				
Never or before pregnancy	2.20 (0.39 – 14.27)	-		-
Daily or sometimes	1.83 (0.36 – 16.70)	0.025	0.207	0.903

Tabla SI-10. Capítulo 6. (Continuación)

Variable	MBzP levels Median (minimum-maximum)	Estimated parameters (Simple linear robust regression)	Standard error	p-value
Makeup				
Frequency				
Never or before pregnancy	2.12 (0.36 – 14.27)	-		-
Daily	1.19 (0.55 – 4.70)	-0.230	0.232	0.416
Sometimes	2.5 (0.68 – 16.70)	0.163	0.205	0.428
Urinary levels				
Creatinine (g/L)	-	1.024	0.147	<0.001**

*p-values <0.20
**p-values <0.05

Tabla SI-11. Capítulo 6. Relationship between MiBP levels in human milk with the characteristics and habits of the study population using simple linear robust regression.

Variable	MiBP levels Median (minimum-maximum)	Estimated parameters (Simple linear robust regression)	Standard error	p-value
Mother				
Nº children				
Primipara mother	10.84 (4.50 – 88.40)	-	-	-
2 or more children	13.25 (1.70 – 89.40)	0.283	0.181	0.120*
Age (years)	-	-0.024	0.020	0.221
Weight before pregnancy (kg)	-	-0.009	0.009	0.317
Height (cm)	-	0.001	0.014	0.929
BMI before pregnancy (kg/m^2)	-	-0.031	0.025	0.204
Special diet during pregnancy				
Yes	11.04 (4.50 – 60.20)	-	-	-
No	11.51 (1.70 – 89.40)	-0.071	0.267	0.790

Tabla SI-11. Capítulo 6. (Continuación).

Variable	MiBP levels Median (minimum-maximum)	Estimated parameters (Simple linear robust regression)	Standard error	p-value
Country of birth				
Spain	10.47 (1.70 – 89.40)	-	-	-
Other	16.90 (6.41– 60.20)	0.442	0.254	0.085*
Place of residence last 10 years				
Urban	11.30 (1.70 – 89.40)	-	-	-
Rural	20.30 (4.70 – 78.60)	0.312	0.248	0.211
Education level				
Only Primary School	7.89 (5.03 – 12.80)	-	-	-
Secondary School	13.14 (4.58 – 60.20)	0.316	0.334	0.346
University	12.04 (1.70 – 89.40)	0.015	0.293	0.958
Occupational status				
Employed	10.82 (1.70 – 78.60)	-	-	-
Unemployed	16.91 (4.61 – 89.40)	0.244	0.262	0.355
Period working outside home (years)	-	-0.004	0.017	0.823
Physical exercise				
3 or more days/week	8.99 (1.70 – 60.20)	-		-
1 or 2 days/week	12.62 (4.70– 30.37)	0.485	0.310	0.121*
Occasionally	11.30 (3.90 – 89.40)	0.347	0.258	0.182*
Never	13.12 (2.70 – 61.80)	0.390	0.293	0.186*
Smoker				
Yes	7.66 (4.61– 22.70)	-		-
Ex-smoker	12.04 (2.70 – 88.40)	0.297	0.398	0.458
Never	11.95 (1.70 – 89.40)	0.295	0.394	0.456

Tabla SI-11. Capítulo 6. (Continuación).

Variable	MiBP levels Median (minimum-maximum)	Estimated parameters (Simple linear robust regression)	Standard error	p-value
Food consumption				
nº rations previous 72h				
Eggs	-	-0.084	0.094	0.369
Packaged juice	-	0.164	0.044	<0.001**
Meat products group	-	0.049	0.049	0.315
Fishery products group	-	-0.063	0.060	0.295
Dairy products group	-	-0.035	0.025	0.167*
Vegetables group	-	0.055	0.043	0.201
Fruits group	-	-0.013	0.028	0.647
Legumes and cereals group	-	-0.018	0.027	0.512
Oils and fats group	-	-0.009	0.028	0.754
Index-MED-DQI				
Good	10.83 (1.70 – 78.60)	-		-
Medium	12.96 (2.70– 89.40)	-0.115	0.180	0.523
Personal care products				
Skin care				
Frequency				
Never or before pregnancy	12.60 (2.70 – 43.40)	-		-
Daily	11.84 (1.70 – 89.40)	-0.118	0.225	0.600
Weekly or monthly	10.47 (3.90 – 60.20)	-0.222	0.273	0.417
Perfume				
Frequency				
Never or before pregnancy	11.30 (2.70 – 88.40)	-		-
Daily	12.80 (1.70 – 89.40)	0.122	0.221	0.584
Weekly or monthly	11.20 (5.03– 78.60)	0.169	0.230	0.465

Tabla SI-11. Capítulo 6. (Continuación).

Variable	MiBP levels Median (minimum-maximum)	Estimated parameters (Simple linear robust regression)	Standard error	p-value
Deodorants				
Frequency				
Not daily	14.70 (4.58 – 43.40)	-		-
Daily	11.20 (1.70 – 89.40)	-0.049	0.267	0.854
Sunscreen				
Frequency				
Never or before pregnancy	12.70 (1.70 – 88.40)	-		-
Daily	13.95 (4.50 – 89.40)	0.121	0.271	0.658
Sometimes	10.83 (2.70– 53.70)	-0.284	0.227	0.215
Hair colour				
Last application				
≤ 1 month	10.38 (1.70 – 21.33)	-		-
> 1 month	11.19 (3.90 – 88.40)	0.010	0.266	0.969
Never or before pregnancy	12.89 (2.70 – 89.40)	0.048	0.260	0.855
Lipstick				
Frequency				
Never or before pregnancy	12.16 (2.70 – 89.40)	-		-
Daily or sometimes	10.88 (1.70 – 60.20)	0.054	0.203	0.790
Makeup				
Frequency				
Never or before pregnancy	12.61 (2.70 – 89.40)	-		-
Daily	9.54(1.70 – 88.40)	-0.175	0.276	0.526
Sometimes	10.00 (4.50– 61.80)	-0.217	0.200	0.280
Urinary levels				
Creatinine (g/L)	-	0.972	0.152	<0.001**

*p-values <0.20
**p-values <0.05

Tabla SI-12. Capítulo 6. Relationship between MnBP levels in human milk with the characteristics and habits of the study population using simple linear robust regression

Variable	MnBP levels Median (minimum-maximum)	Estimated parameters (Simple linear robust regression)	Standard error	p-value
Mother				
Nº children				
Primipara mother	10.56 (2.88 – 35.62)	-	-	-
2 or more children	12.60 (0.43 – 148.80)	0.062	0.186	0.741
Age (years)	-	-0.032	0.021	0.119*
Weight before pregnancy (kg)	-	-0.011	0.010	0.277
Height (cm)	-	-0.017	0.015	0.250
BMI before pregnancy (kg/m^2)	-	-0.016	0.026	0.541
Special diet during pregnancy				
Yes	6.95 (4.09 – 50.30)	-	-	-
No	12.35 (0.43– 148.80)	-0.013	0.279	0.963
Country of birth				
Spain	11.50 (0.43 – 148.80)	-	-	-
Other	12.40 (3.35 – 53.80)	0.301	0.270	0.266
Place of residence last 10 years				
Urban	11.95 (0.43 – 52.80)	-	-	-
Rural	12.70 (3.50 – 148.80)	0.207	0.260	0.428
Education level				
Only Primary School	6.28 (3.35 – 12.50)	-	-	-
Secondary School	12.21 (2.88 – 52.80)	0.449	0.340	0.191*
University	12.55 (0.43 – 148.80)	0.230	0.299	0.443
Occupational status				
Employed	10.60 (0.43 – 148.80)	-	-	-
Unemployed	13.62 (3.60 – 50.00)	0.159	0.268	0.554

Tabla SI-12. Capítulo 6. (Continuación).

Variable	MnBP levels Median (minimum-maximum)	Estimated parameters (Simple linear robust regression)	Standard error	p-value
Period working outside home (years)	-	-0.024	0.017	0.153*
Physical exercise				
3 or more days/week	9.19 (0.43 – 50.30)	-		-
1 or 2 days/week	15.00 (2.88 – 52.80)	0.408	0.315	0.199*
Occasionally	12.11 (3.35 – 148.80)	0.308	0.263	0.244
Never	12.70 (1.79 – 50.00)	0.072	0.297	0.809
Smoker				
Yes	8.81 (3.60 – 27.10)	-		-
Ex-smoker	12.26 (1.79 – 50.00)	-0.157	0.406	0.699
Never	12.20 (0.43 – 148.80)	-0.271	0.401	0.501
Food consumption				
nº rations previous 72h				
Eggs	-	-0.011	0.095	0.912
Packaged juice	-	0.088	0.044	0.070*
Meat products group	-	0.016	0.050	0.743
Fishery products group	-	-0.010	0.061	0.875
Dairy products group	-	-0.033	0.025	0.195*
Vegetables group	-	0.021	0.044	0.633
Fruits group	-	0.002	0.028	0.938
Legumes and cereals group	-	-0.043	0.027	0.115*
Oils and fats group	-	-0.002	0.029	0.944

Tabla SI-12. Capítulo 6. (Continuación).

Variable	MnBP levels Median (minimum-maximum)	Estimated parameters (Simple linear robust regression)	Standard error	p-value
Index-MED-DQI				
Good	10.60 (0.43 – 148.80)	-		-
Medium	12.68 (1.79 – 52.80)	-0.003	0.183	0.989
Personal care products				
Skin care				
Frequency				
Never or before pregnancy	12.98 (1.79 – 50.00)	-		-
Daily	12.03(0.43 – 148.80)	0.029	0.232	0.903
Weekly or monthly	12.10 (3.35– 50.30)	-0.030	0.281	0.915
Perfume				
Frequency				
Never or before pregnancy	11.95 (1.79 – 50.30)	-		-
Daily	11.81 (0.43 – 50.00)	0.087	0.223	0.698
Weekly or monthly	12.30 (3.35 – 148.80)	0.309	0.232	0.185*
Deodorants				
Frequency				
Not daily	14.10 (2.70 – 21.52)	-		-
Daily	12.10 (0.43 – 148.80)	0.155	0.276	0.575
Sunscreen				
Frequency				
Never or before pregnancy	12.21 (0.43 – 148.80)	-		-
Daily	12.78 (3.35 – 42.70)	0.164	0.278	0.557
Sometimes	10.82 (1.79 – 50.00)	-0.056	0.233	0.810
Hair colour				
Last application				
≤ 1 month	10.02 (0.43 – 16.51)	-		-
> 1 month	11.80 (2.88 – 35.62)	0.108	0.278	0.699
Never or before pregnancy	12.21 (1.79 – 148.80)	0.126	0.271	0.643

Tabla SI-12. Capítulo 6. (Continuación).

Variable	MnBP levels Median (minimum-maximum)	Estimated parameters (Simple linear robust regression)	Standard error	p-value
Lipstick				
Frequency				
Never or before pregnancy	12.25 (1.79 – 148.80)	-		-
Daily or sometimes	12.03 (0.43 – 52.80)	0.230	0.204	0.262
Makeup				
Frequency				
Never or before pregnancy	12.45 (1.79 – 148.80)	-		-
Daily	7.09 (0.43 – 30.60)	-0.299	0.288	0.301
Sometimes	12.21 (3.50 – 27.10)	-0.181	0.209	0.389
Urinary levels				
Creatinine (g/L)	-	1.047	0.151	<0.001**

*p-values <0.20
**p-values <0.05

Tabla SI-13. Capítulo 6. Relationship between MEP levels in human milk with the characteristics and habits of the study population using simple linear robust regression

Variable	MEP levels Median (minimum-maximum)	Estimated parameters (Simple linear robust regression)	Standard error	p-value
Mother				
Nº children				
Primipara mother	31.65 (3.00 – 1291.00)	-	-	-
2 or more children	34.88 (2.44 – 196.57)	0.158	0.225	0.485
Age (years)	-	-0.024	0.025	0.347
Weight before pregnancy (kg)	-	0.002	0.012	0.883
Height (cm)	-	-0.015	0.018	0.398

Tabla SI-13. Capítulo 6. (Continuación).

Variable	MEP levels Median (minimum-maximum)	Estimated parameters (Simple linear robust regression)	Standard error	p-value
BMI before pregnancy (kg/m^2)	-	0.023	0.032	0.471
Special diet during pregnancy				
Yes	30.80 (2.44 – 170.70)	-	-	-
No	32.97 (3.00 – 1291.00)	0.100	0.337	0.767
Country of birth				
Spain	31.60 (2.44 – 1291.00)	-	-	-
Other	43.20 (10.90 – 170.70)	0.357	0.323	0.272
Place of residence last 10 years				
Urban	33.09 (2.44 – 1291.00)	-	-	-
Rural	25.10 (13.60 – 145.00)	-0.032	0.309	0.918
Education level				
Only Primary School	26.20 (5.91 – 120.80)	-	-	-
Secondary School	46.35 (2.44 – 170.70)	-0.187	0.414	0.652
University	31.72 (3 – 1291.00)	-0.371	0.364	0.310
Occupational status				
Employed	31.72 (2.44 – 446.50)	-	-	-
Unemployed	72.39 (12.31 – 1291.00)	0.310	0.323	0.340
Period working outside home (years)	-	-0.015	0.021	0.477
Physical exercise				
3 or more days/week	32.62 (2.44 – 170.70)	-		-
1 or 2 days/week	30.42 (10.99 – 446.50)	0.176	0.379	0.643
Occasionally	51.47 (3.00 – 1291.00)	0.530	0.316	0.097
Never	27.28 (3.34 – 133.90)	0.091	0.358	0.800
Smoker				
Yes	69.55 (12.31 – 80.20)	-		-
Ex-smoker	27.55 (2.44 – 1291.00)	-0.172	0.489	0.726
Never	32.97 (9.58 – 170.70)	-0.255	0.483	0.598

Tabla SI-13. Capítulo 6. (Continuación).

Variable	MEP levels Median (minimum-maximum)	Estimated parameters (Simple linear robust regression)	Standard error	p-value
Food consumption				
nº rations previous 72h				
Eggs	-	0.160	0.112	0.158
Packaged juice	-	0.163	0.055	0.004**
Meat products group	-	0.023	0.060	0.697
Fishery products group	-	-0.040	0.073	0.588
Dairy products group	-	-0.008	0.031	0.790
Vegetables group	-	-0.016	0.053	0.769
Fruits group	-	-0.012	0.034	0.730
Legumes and cereals group	-	-0.050	0.033	0.132*
Oils and fats group	-	-0.002	0.034	0.964
Index-MED-DQI				
Good	32.18 (3.00 – 196.57)	-		-
Medium	34.75 (2.44 – 1291.00)	0.040	0.221	0.856
Personal care products				
Skin care				
Frequency				
Never or before pregnancy	25.00 (3.00– 120.80)	-		-
Daily	40.01 (5.91 – 1291.00)	0.434	0.274	0.126*
Weekly or monthly	39.46 (2.44 – 170.70)	0.471	0.332	0.160*
Perfume				
Frequency				
Never or before pregnancy	27.28 (2.44 – 1291.00)	-		-
Daily	34.64 (9.58 – 446.50)	0.157	0.267	0.557
Weekly or monthly	43.35 (5.91 – 196.57)	0.410	0.277	0.142*

Tabla SI-13. Capítulo 6. (Continuación).

Variable	MEP levels Median (minimum-maximum)	Estimated parameters (Simple linear robust regression)	Standard error	p-value
Deodorants				
Frequency				
Not daily	15.63 (2.44 – 196.57)	-		-
Daily	39.46 (5.91 – 1291.00)	0.467	0.332	0.163*
Sunscreen				
Frequency				
Never or before pregnancy	28.85 (3.00 – 1291.00)	-		-
Daily	56.65 (17.40 – 133.90)	0.217	0.338	0.523
Sometimes	36.15 (2.44 – 120.80)	0.035	0.283	0.903
Hair colour				
Last application				
≤ 1 month	20.41 (3.00 – 196.57)	-		-
> 1 month	43.28 (9.24 – 1291.00)	0.160	0.335	0.634
Never or before pregnancy	32.41 (2.44 – 446.50)	-0.248	0.327	0.451
Lipstick				
Frequency				
Never or before pregnancy	31.65 (2.44 – 1291.00)	-		-
Daily or sometimes	43.28 (9.58 – 446.50)	0.564	0.245	0.023**
Makeup				
Frequency				
Never or before pregnancy	32.27 (3.00 – 170.70)	-		-
Daily	28.25 (9.58 – 1291.00)	0.437	0.352	0.217
Sometimes	34.64 (2.44 – 196.57)	0.208	0.255	0.416
Urinary levels				
Creatinine (g/L)	-	0.868	0.209	<0.001**

*p-values <0.20
**p-values <0.05

Tabla SI-14. Capítulo 6. Relationship between DEHP (as sum of five metabolites) levels in human milk with the characteristics and habits of the study population using simple linear robust regression

Variable	DEHP levels Median (minimum-maximum)	Estimated parameters (Simple linear robust regression)	Standard error	p-value
Mother				
Nº children				
Primipara mother	32.56 (7.51 – 1978.90)	-	-	-
2 or more children	38.35 (5.67 – 454.20)	0.162	0.176	0.358
Age (years)	-	-0.018	0.020	0.366
Weight before pregnancy (kg)	-	-0.014	0.009	0.139*
Height (cm)	-	0.002	0.014	0.902
BMI before pregnancy (kg/m^2)	-	-0.039	0.024	0.116*
Special diet during pregnancy				
Yes	36.56 (12.77 – 294.85)	-	-	-
No	34.66 (5.67 – 1978.90)	-0.272	0.623	0.302
Country of birth				
Spain	33.03 (5.67 – 1978.90)	-	-	-
Other	57.90 (21.42 – 137.40)	0.384	0.249	0.126*
Place of residence last 10 years				
Urban	35.00 (5.67 – 1978.90)	-	-	-
Rural	33.71 (13.75 – 86.16)	0.180	0.250	0.718
Education level				
Only Primary School	36.79 (21.42 – 51.00)	-	-	-
Secondary School	36.10 (10.31 – 137.40)	-0.039	0.319	0.904
University	34.01 (5.67 – 1978.90)	-0.288	0.230	0.306
Occupational status				
Employed	33.37 (5.67 – 1978.90)	-	-	-
Unemployed	72.31 (10.31 – 454.20)	0.500	0.253	0.051*
Period working outside home (years)	-	$4.67 \cdot 10^{-5}$	0.016	0.998

Tabla SI-14. Capítulo 6. (Continuación).

Variable	DEHP levels Median (minimum-maximum)	Estimated parameters (Simple linear robust regression)	Standard error	p-value
Physical exercise				
3 or more days/week	41.91 (5.67 – 85.50)	-		-
1 or 2 days/week	26.60 (7.51 – 121.17)	-0.231	0.311	0.459
Occasionally	39.40 (10.31 – 1978.90)	0.136	0.259	0.601
Never	36.28 (6.79– 330.87)	0.084	0.293	0.774
Smoker				
Yes	34.35 (11.22– 124.50)	-		-
Ex-smoker	34.36 (6.79 – 330.87)	-0.060	0.388	0.878
Never	35.80 (5.67 – 1978.90)	-0.095	0.384	0.806
Food consumption				
nº rations previous 72h				
Eggs	-	0.091	0.208	0.835
Packaged juice	-	0.048	0.046	0.297
Meat products group	-	0.025	0.047	0.603
Fishery products group	-	-0.056	0.058	0.337
Dairy products group	-	-0.044	0.024	0.073*
Vegetables group	-	0.065	0.041	0.123*
Fruits group	-	-0.004	0.027	0.878
Legumes and cereals group	-	0.010	0.026	0.691
Oils and fats group	-	-0.005	0.027	0.842
Index-MED-DQI				
Good	33.37 (5.67 – 1978.90)	-		-
Medium	36.79 (6.79 – 454.20)	0.018	0.174	0.917

Tabla SI-14. . Capítulo 6. (Continuación).

Variable	DEHP levels Median (minimum-maximum)	Estimated parameters (Simple linear robust regression)	Standard error	p-value
Personal care products				
Skin care				
Frequency				
Never or before pregnancy	46.70 (6.79 – 330.87)	-		-
Daily	29.32 (5.67 – 454.20)	-0.279	0.216	0.198*
Weekly or monthly	38.30 (16.22 – 1978.90)	-0.338	0.261	0.197
Perfume				
Frequency				
Never or before pregnancy	34.31 (6.79 – 1978.90)	-		-
Daily	30.76 (5.67 – 454.20)	-0.205	0.214	0.341
Weekly or monthly	38.30 (11.64 – 85.12)	-0.110	0.223	0.624
Deodorants				
Frequency				
Not daily	46.70 (13.75– 330.87)	-		-
Daily	33.71 (5.67 – 1978.90)	-0.310	0.253	0.222
Sunscreen				
Frequency				
Never or before pregnancy	35.64 (5.67 – 294.85)	-		-
Daily	37.07 (17.99 – 1978.90)	0.077	0.263	0.769
Sometimes	29.50 (6.79 – 330.87)	-0.203	0.220	0.360
Hair colour				
Last application				
≤ 1 month	33.85 (5.67 – 63.08)	-		-
> 1 month	34.01 (7.51 – 294.85)	0.019	0.258	0.941
Never or before pregnancy	35.64 (6.79 – 1978.90)	-0.171	0.252	0.500
Lipstick				
Frequency				
Never or before pregnancy	37.29 (6.79 – 1978.90)	-		-
Daily or sometimes	28.68 (5.67 – 294.85)	-0.048	0.196	0.807

Tabla SI-14. . Capítulo 6. (Continuación).

Variable	DEHP levels Median (minimum-maximum)	Estimated parameters (Simple linear robust regression)	Standard error	p-value
Makeup				
Frequency				
Never or before pregnancy	38.85 (6.79 – 1978.90)	-		-
Daily	21.60 (5.67 – 137.40)	-0.332	0.272	0.226
Sometimes	35.16 (7.51 – 294.85)	-0.171	0.197	0.389
Urinary levels				
Creatinine (g/L)	-	1.106	0.126	<0.001**

*p-values <0.20
**p-values <0.05