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DESARROLLO DE MÉTODOS ANALÍTICOS PARA LA DETERMINACIÓN DE TRAZAS DE COMPUESTOS DE INTERÉS EN PRODUCTOS COSMÉTICOS

Memoria presentada por Lorenza Schettino
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ÍNDICE GENERAL

RESUMEN

RESUMEN

La actual demanda social de productos cosméticos en los países desarrollados, por un lado, y las mayores exigencias en materia de seguridad que establece la normativa europea para este tipo de productos (Reglamento (CE) Nº 1223/2009 del Parlamento Europeo y del Consejo de 30 de noviembre de 2009 sobre los productos cosméticos), por otro, requiere el continuo desarrollo de métodos analíticos que permitan a las empresas y los organismos oficiales llevar a cabo el correcto control de calidad de los productos cosméticos, con la clara finalidad de garantizar la seguridad de los usuarios.

En la presente Tesis Doctoral se han desarrollado y validado diversos métodos analíticos para la determinación de sustancias de interés en productos cosméticos, tanto ingredientes autorizados como sustancias prohibidas, con el fin de ofrecer unas herramientas útiles para el control de calidad de este tipo de productos, y así contribuir a la evaluación de la seguridad de los mismos en cumplimiento con lo regulado según la normativa vigente.

La Tesis Doctoral se ha estructurado en seis capítulos agrupados en dos secciones:

La **Sección I** corresponde a la **Introducción** y consta de dos capítulos:

- En el ***Capítulo 1*** se describe la legislación vigente en Europa en materia de productos cosméticos. Se introducen las sustancias objeto de estudio y se describen los antecedentes bibliográficos más relevantes sobre su determinación.

- Dado que en la presente Tesis Doctoral se lleva a cabo la determinación de compuestos de interés a nivel de trazas, haciendo uso en la mayoría de los casos de técnicas de microextracción, en el ***Capítulo 2*** se hace una discusión general sobre las técnicas de microextracción que han sido empleadas en el control analítico de los productos cosméticos.

La **Sección II**, que consta de los Capítulos 3 a 6, recoge un resumen del trabajo experimental y los resultados obtenidos en el desarrollo de la presente Tesis Doctoral. Así,

- En el ***Capítulo 3*** se describe un método analítico para la determinación de cannabidiol, un activo de gran interés en la actualidad por su creciente empleo en productos cosméticos, del que se reivindican propiedades beneficiosas para la piel, como la antioxidante y sebostática. El analito se determina a nivel de trazas después de lixiviarse de la matriz cosmética con etanol, sin necesidad de una etapa de preconcentración.

- En el ***Capítulo 4*** se describe un método analítico para la determinación de acrilamida, una sustancia prohibida en productos cosméticos por ser mutagénica y cancerígena. El método se basa en una derivatización previa del analito seguida de la microextracción líquido-líquido dispersiva como técnica de preconcentración.

- En el ***Capítulo 5*** se describe un segundo método analítico para la determinación de acrilamida sin necesidad de derivatización, basado en la microextracción líquido-líquido dispersiva en fase reversa asistida por vórtex.

- En el ***Capítulo 6*** se describe un método analítico para la determinación de diversas nitrosaminas, sustancias cancerígenas prohibidas en productos cosméticos, basado también en la microextracción líquido-líquido dispersiva asistida por vórtex.

Por último, se presentan las **Conclusiones Generales** de la presente Tesis Doctoral y, como **Anexo,** se incluyen las publicaciones científicas a las que ha dado lugar.

SECCIÓN I

Introducción

Capítulo 1

Productos cosméticos: legislación y análisis

1.1. Normativa vigente sobre productos cosméticos

En la Unión Europea (UE), el instrumento jurídico que regula los productos cosméticos en todos los Estados miembros es el Reglamento (CE) Nº 1223/2009 del Parlamento Europeo y del Consejo de 30 de noviembre de 2009 [1] (en adelante Reglamento Europeo), que derogó la anterior Directiva 76/768/CEE de 1976 en materia de productos cosméticos [2].

El Reglamento Europeo se publicó en 2009 con el fin de armonizar la normativa entre los Estados de la UE, y así generar un libre mercado interno con normas definidas y procedimientos simplificados, y tiene pleno efecto en toda su extensión desde el 11 de julio de 2013.

En el Reglamento Europeo, se define producto cosmético como *"toda aquella sustancia o mezcla destinada a ser puesta en contacto con las partes superficiales del cuerpo humano (epidermis, sistema piloso y capilar, uñas, labios y órganos genitales externos) o con los dientes y las mucosas bucales, con el fin exclusivo o principal de limpiarlos, perfumarlos, modificar su aspecto, protegerlos, mantenerlos en buen estado o corregir los olores corporales"* [1].

Atendiendo a esta definición, algunos ejemplos de productos cosméticos serían:

- cremas, emulsiones, lociones, geles y aceites para la piel
- mascarillas de belleza, jabones, perfumes, aguas de tocador y aguas de colonia, preparados para baño y ducha
- depilatorios
- desodorantes y antitranspirantes
- colorantes para el cabello, productos para la ondulación, alisado, fijación, limpieza, mantenimiento y peinado del cabello
- productos para el afeitado
- maquillaje y productos para desmaquillar
- productos para el cuidado bucal y dental
- productos para el cuidado y maquillaje de las uñas
- productos de higiene íntima externa

- productos para la protección solar y productos para el bronceado sin sol
- productos para el blanqueamiento de la piel

1.2. Seguridad de los productos cosméticos

El Reglamento Europeo tiene como principal objetivo garantizar la seguridad de los usuarios. En este sentido, remarca que *"los productos cosméticos deben ser seguros en condiciones de utilización normales o razonablemente previsibles"*.

Por esta razón, y según el Reglamento Europeo, los fabricantes de aquellos productos cosméticos que se vayan a introducir en el mercado europeo tienen el deber de:

- elaborar los productos conforme a las buenas prácticas de fabricación (UNE-EN ISO 22716) [3];
- cumplir con las prohibiciones y restricciones aplicables a las sustancias que figuran en los anexos del Reglamento Europeo, y llevar a cabo, cuando sea necesario, el control de calidad, tanto de los productos cosméticos acabados como de las materias primas utilizadas como ingredientes, para así garantizar dicho cumplimiento;
- disponer de un expediente de información sobre cada producto fabricado, que conste de un informe sobre la evaluación de la seguridad del producto cosmético llevada a cabo por una persona cualificada.

Además, las autoridades competentes tienen el deber de velar por la seguridad de los usuarios a través de controles en el mercado y de inspecciones/auditorías a las empresas para comprobar que cumplen con el Reglamento Europeo.

1.3. Los ingredientes de los productos cosméticos

Según el Reglamento Europeo, se considera ingrediente cosmético "*cualquier sustancia o mezcla que se utiliza intencionalmente en el producto cosmético durante el proceso de fabricación*" [1].

En los productos cosméticos, cada ingrediente desempeña una función concreta, y por esto se clasifican en distintas categorías según la función específica que ejercen en la fórmula. Dentro de estas categorías se clasifican generalmente por sus características químicas.

Una primera clasificación de los ingredientes podría establecerse en base a la función que desempeñan en la formulación cosmética:

- **Ingredientes activos.** Son las sustancias que atribuyen al producto cosmético la función para la que ha sido diseñado, proporcionando un beneficio para el usuario. Son, por tanto, los responsables de la eficacia del producto. Pueden ser de origen sintético, mineral, vegetal o animal.

- **Excipientes.** Son los ingredientes que constituyen el vehículo de la fórmula. En un producto cosmético puede haber uno o más excipientes, cuya elección está condicionada por la naturaleza químico-física de los ingredientes activos a incorporar, sin excluir otros aspectos como el tipo de producto, la zona de aplicación, el público al que va dirigido, etc.

- **Compuestos de adición**. Pueden considerarse como tales las sustancias que se añaden al producto: para modificar sus propiedades organolépticas (color, olor) haciéndolo más atractivo para el usuario, para evitar la proliferación de microorganismos (bacterias, hongos) o para proteger la formulación de la oxidación u otras posibles alteraciones causadas por el medio (luz, calor, humedad).

La tendencia en la industria cosmética, por lo que se refiere a la selección de los ingredientes, se está enfocando actualmente hacia

"*cosmética natural*", que prioriza precisamente el uso de materias primas de origen natural que hayan sido sometidas a un procesamiento exiguo, minimizando las transformaciones químicas o la adición de aditivos. El reto de las industrias cosméticas, hoy en día, es elaborar productos seguros y de calidad, cuyas fórmulas sean acordes a las necesidades de usuarios concienciados, que identifican el concepto de cosmética natural con valores como el bienestar, la salud y el compromiso con el medio ambiente.

1.4. Restricciones para determinadas sustancias en los productos cosméticos

En el Artículo 14 del Reglamento Europeo se establecen las restricciones para las sustancias enumeradas en una serie de listados (en forma de anexos), y se afirma que los productos cosméticos no podrán contener:

- → las ***sustancias prohibidas*** enumeradas en el Anexo II de dicho Reglamento;

- → las ***sustancias sujetas a restricción*** enumeradas en el Anexo III si no se utilizan con arreglo a las restricciones establecidas en dicho anexo, atendiendo al tipo de producto, partes del cuerpo donde se aplica, concentración máxima en el producto acabado, así como advertencias que deben figurar en el etiquetado del producto;

- → los ***colorantes*** que no estén enumerados en el Anexo IV o que no se usen con arreglo a las condiciones establecidas en dicho anexo;

- → Los ***conservantes*** que no estén enumerados en el Anexo V o que no se usen con arreglo a las condiciones establecidas en dicho anexo;

→ los ***filtros ultravioleta*** que no estén enumerados en el Anexo VI o que no se usen con arreglo a las condiciones establecidas en dicho anexo,

En resumen, el Anexo II y el Anexo III son, respectivamente, listados de sustancias prohibidas y restringidas en productos cosméticos, mientras que los Anexos IV, V y VI son listados positivos, referidos a las sustancias que pueden utilizarse como colorantes, conservantes y filtros ultravioleta, respectivamente, atendiendo a las restricciones allí establecidas.

Toda aquella sustancia no incluida en estos anexos puede utilizarse como ingrediente en la fabricación de un producto cosmético sin ningún tipo de restricción, siempre que la persona responsable pueda proporcionar la información necesaria y suficiente para demostrar la seguridad del producto elaborado con dicho ingrediente, conforme al Reglamento Europeo.

Cabe indicar que los anexos se actualizan constantemente por la Comisión Europea al estar sujetos a continuas incorporaciones de nuevas sustancias o modificaciones de lo definido para sustancias ya presentes, según las opiniones emitidas por el *Comité Científico de Seguridad de los Consumidores* (SCCS, del inglés *Scientific Committee on Consumer Safety*), una institución de la Comisión Europea que evalúa los riesgos para la salud y la seguridad de los productos de consumo.

1.4.1. Sustancias prohibidas en los productos cosméticos

Según el Reglamento Europeo, todas aquellas sustancias, o grupos de sustancias, enumeradas en su Anexo II se consideran prohibidas en los productos cosméticos y, por esta razón, no deben ser añadidas voluntariamente a los productos cosméticos durante su formulación y posterior fabricación [1]. Dada la seriedad y el compromiso social de la industria cosmética nacional, y europea, no es de esperar que los productos cosméticos contengan compuestos prohibidos en una concentración más o menos elevada como consecuencia de su

adición intencionada. No obstante, existe la posibilidad de que alguna sustancia prohibida pueda encontrarse en los productos cosméticos a nivel de trazas por causas involuntarias, como sería el caso de aquellas impurezas procedentes de las materias primas, tanto naturales como sintéticas, o aquellas procedentes del proceso de fabricación, o de la degradación de algún ingrediente de la fórmula, o que se forme como subproducto por reacción entre ingredientes, o bien del almacenamiento o de la migración desde el envase.

En estos casos, la presencia fortuita de pequeñas cantidades de sustancias prohibidas, cuando sea técnicamente inevitable bajo las buenas prácticas de fabricación, se permitirá si los productos cosméticos que se comercialicen son seguros para la salud humana, siempre que sean utilizados en las condiciones normales o razonablemente previsibles de uso [1]. Por ello, es obligación del fabricante, en el caso de presencia de trazas de una sustancia prohibida en un producto cosmético, probar su inevitabilidad técnica, asegurar la inocuidad del producto, y determinar la concentración de la sustancia prohibida para verificar que cumple con los límites de seguridad establecidos por el Reglamento Europeo para aquella sustancia.

Por esta razón, es de gran interés desarrollar métodos analíticos para el análisis de sustancias prohibidas en productos cosméticos, tratando de:

- consultar periódicamente la información sobre las posibles nuevas prohibiciones de sustancias que se puedan declarar en la Comisión Europea.

- desarrollar y validar métodos analíticos con la sensibilidad suficiente para el análisis de trazas de las sustancias prohibidas, así como con la selectividad necesaria para evitar interferencias en el análisis de muestras complejas.

Además, teniendo en cuenta las características de interés en el control de calidad en el caso de los productos cosméticos, es necesario tratar de que los métodos desarrollados sean rápidos, sencillos y asequibles (por su coste y por la instrumentación

requerida) para su implementación en las empresas del sector cosmético y/o en los laboratorios autorizados.

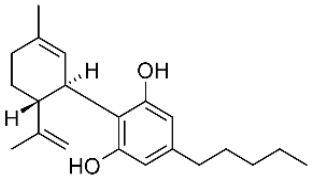
1.5. Analitos objeto de estudio

A continuación, se describen los analitos objeto de estudio en la presente Tesis Doctoral y los principales antecedentes bibliográficos sobre su determinación.

1.5.1. El cannabidiol

En relación al objetivo de la presente Tesis Doctoral, en el **Capítulo 3** se presenta un método analítico para la determinación de ***cannabidiol*** (2-[(1*R*,6*R*)-6-isopropenil-3-metilciclohex-2-en-1-il]-5-pentilbenzen-1,3-diol), un ingrediente activo de origen natural procedente de extractos vegetales, cuya información más relevante se muestra en la *Tabla 1.1*.

Tabla 1.1. Información sobre el cannabidiol estudiado en el *Capítulo 3.*

Nombre	Número CAS	Estructura química	M_r (g mol^{-1})	Log $K_{o/w}$
Cannabidiol	13956-29-1		314.5	6.5

El cannabidiol, o CBD, es un fitocannabinoide presente en la planta del cáñamo o cannabis (*Cannabis sativa L.*) [4] que ha despertado un creciente interés por parte de la comunidad científica en el ámbito farmacológico, debido a sus efectos como antiinflamatorio, anticonvulsivo, antiemético, ansiolítico, y antipsicótico [5]. En el ámbito cosmético, según la Base de Datos de Ingredientes Cosméticos

(CosIng) [6], se atribuyen al CBD propiedades como antiseborreico, antioxidante, acondicionador de la piel y protector de la piel [7].

Más concretamente, a nivel de la piel, el CBD actúa sobre las glándulas sebáceas a través del sistema endocannabinoide, suprimiendo la proliferación de los sebocitos e inhibiendo la producción de sebo. De hecho, gracias también a su acción antiinflamatoria, contrarresta los principales factores patogénicos del acné (sobreproducción de sebo, sobreproliferación de sebocitos e inflamación), convirtiéndose en un agente terapéutico prometedor para el tratamiento del acné vulgar [7]. Además, algunos estudios indican que el CBD contrarresta el daño celular oxidativo generado por los radicales libres, ayudando a disminuir los signos visibles del envejecimiento de la piel [8].

El CBD no presenta propiedades psicoactivas, a diferencia de su isómero, el tetrahidrocannabinol (THC), que es un conocido estupefaciente [4]. Por ello, para evitar la presencia de THC en los productos cosméticos, la legislación indica que solo puede utilizarse CBD si proviene de plantas obtenidas a partir de semillas certificadas de cáñamo con un contenido de THC inferior al 0.2 %, aptas para cultivos con fines industriales [9], o si se ha producido sintéticamente [6].

Por lo que se refiere a su determinación, en la bibliografía analítica existen diversos artículos relacionados con la determinación de CBD y otros cannabinoides basados en el uso de diferentes estrategias de preparación de muestra [10] y técnicas cromatográficas [11]. Estas sustancias se han determinado en distintos tipos de matrices, analizándose directamente en la planta [12], en aceites [13–15], en productos alimenticios [16], o en cabello [17,18] y fluidos biológicos, como orina [19,20], sangre [20], saliva [21,22] y sudor [22], para el control en abuso de drogas, o para el estudio de individuos tratados con preparados farmacéuticos de cannabis medicinal. En este sentido, se han utilizado diferentes técnicas, como la cromatografía de gases acoplada a espectrometría de masas (GC-MS, del inglés *gas chromatography-mass spectrometry*) para el análisis de cabello [17,23–26], saliva [27,28] y alimentos conteniendo ingredientes derivados del cáñamo [16]; la cromatografía de gases acoplada a espectrometría de masas en tándem (GC-MS/MS) para el análisis de

cabello [29–32]; la cromatografía de líquidos con detección ultravioleta (LC-UV, del inglés *liquid chromatography-ultraviolet*) para el análisis de orina [19,20] y cerebro y sangre de ratones [20]; y la cromatografía de líquidos acoplada a espectrometría de masas en tándem (LC-MS/MS, del inglés *liquid chromatography-tandem mass spectrometry*) para el análisis de cabello [18], saliva [21,22], parches de sudor [22] y suero y orina [22,33].

Por otra parte, las publicaciones relacionadas con la determinación de CBD y otros cannabinoides en productos cosméticos son escasas y recientes, y en este contexto se describen brevemente a continuación.

Meng et al. [34] analizaron muestras comerciales de cremas por LC-MS/MS después de efectuar una extracción asistida por ultrasonidos. El procedimiento consistía en la dilución de las muestras con metanol conteniendo un 0.005% de ácido fórmico y un 5% de agua, seguida de sonicación y centrifugación.

Un enfoque parecido realizaron Ocaña-Rios et al. [35], analizando diferentes geles y cremas mediante GC-MS, después de su dilución en metanol y extracción asistida por ultrasonidos.

Nemeskalová et al. [36] analizaron diversos productos cosméticos de naturaleza liposoluble por LC-UV y LC-MS/MS mediante la dispersión de la muestra con una mezcla de acetato de etilo:isopropanol (1:1), agitando con vórtex y calentando. Sigue la separación del sobrenadante después de enfriamiento a -20 ºC, la dilución con una mezcla agua:acetonitrilo 40:60 (v/v) y la filtración previa al análisis.

Huber et al. [37] determinaron el CBD en cremas mediante LC-UV tras microextracción asistida por ultrasonidos a 40 ºC, empleando metanol y un líquido iónico (-butoxy-3-ethoxy-2-ethylimidazolium bis(trifluoromethane)sulfonimide), evaporación del sobrenadante obtenido y reconstitución del líquido iónico restante con una disolución de acetonitrilo.

Debido a que la mayoría de los antecedentes bibliográficos revisados requieren de distintas etapas de preparación de muestra, en el **Capítulo 3** de la presente Tesis Doctoral se propone un método analítico en el que se ha prestado especial atención a la simplificación

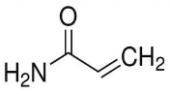
del procedimiento experimental y la reducción de los tiempos de análisis.

En el contexto actual que tiende hacia una cosmética natural, el interés de la determinación del CBD surge de la gran popularidad que esta sustancia ha alcanzado entre los extractos vegetales y los compuestos procedentes de plantas que se pueden emplear como ingredientes cosméticos. De hecho, en los últimos años la presencia en el mercado de productos cosméticos que contienen tanto CBD como otros derivados de la planta del cannabis ha aumentado exponencialmente, traduciéndose en la necesidad de las empresas fabricantes de disponer de métodos analíticos eficaces para llevar a cabo el control de calidad de sus productos.

1.5.2. La acrilamida

En relación con el objetivo de la presente Tesis Doctoral, en los **Capítulos 4 y 5** se estudia la ***acrilamida*** (2-propenamida), cuya información más relevante se muestra en la *Tabla 1.2*.

Tabla 1.2. Información sobre la acrilamida estudiada en los *Capítulos 4 y 5*.

Nombre	Número CAS	Estructura química	M_r (g mol^{-1})	Log $K_{o/w}$
Acrilamida	79-06-1		71.08	-0.56

Según el Anexo II, entrada 681, del Reglamento Europeo [1], la acrilamida es una sustancia prohibida en los productos cosméticos. Se trata de una sustancia con una elevada toxicidad, ya que se considera potencialmente cancerígena, al demostrarse su implicación en una mayor incidencia de tumores en diversos órganos. Por esta razón, está clasificada como sustancia cancerígena perteneciente al grupo 2A por

el Centro Internacional de Investigaciones sobre el Cáncer (IARC, del inglés *International Agency for Research on Cancer*) [38].

La acrilamida es también mutagénica, ya que efectúa una acción genotóxica induciendo mutaciones *in vivo* tanto en células somáticas como en células germinales y, en el caso de estas últimas, se ha demostrado que induce mutaciones hereditarias [38,39]. Además, se ha demostrado que la acrilamida es tóxica para la reproducción, sobre todo para el aparato reproductivo masculino, ya que causa una alteración de la fertilidad asociada con efectos sobre el recuento de espermatozoides y sus parámetros de motilidad [39].

La toxicidad sistémica y el daño biológico causados por la acrilamida se deben a su carácter nucleofílico, en su capacidad de formar aductos covalentes con grupos químicos de moléculas biológicas, como grupos azufrados en los aminoácidos de las proteínas, y grupos amino o cetónicos en las bases del ADN, formando una unión covalente con estas macromoléculas que impide su correcto funcionamiento [40,41].

Aunque el uso de la acrilamida como ingrediente cosmético está prohibido, muchos polímeros sintetizados a partir de ella, conocidos con el nombre genérico de poliacrilamidas, se utilizan como ingredientes cosméticos debido a sus múltiples y variadas funciones, como estabilizantes, antiestáticos, formadores de espuma, aglutinantes, filmógenos, fijadores, espesantes o modificadores reológicos [42,43].

El polímero de acrilamida más sencillo es la poliacrilamida, formado a partir de la polimerización lineal de monómeros de acrilamida por adición de un radical aniónico iniciador [44], tal y como se muestra en la *Figura 1.1*.

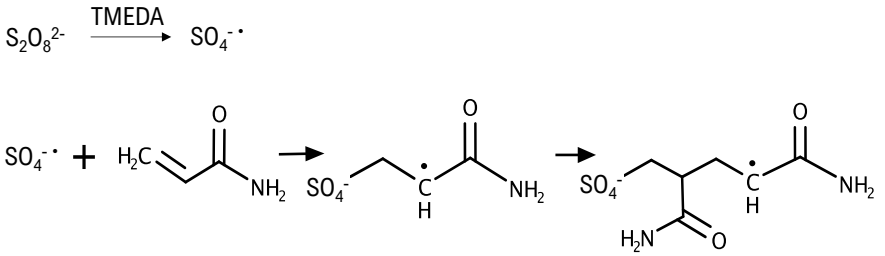


Figura 1.1. *Polimerización de la acrilamida en presencia de persulfato y tetrametiletilendiamina (TMEDA). El TMEDA cataliza la descomposición del persulfato, que se convierte en el radical aniónico iniciador de la reacción de polimerización.*

Según la Base de Datos de Ingredientes Cosméticos (CosIng) [45] se consideran pertenecientes al grupo de las poliacrilamidas todos los polímeros listados a continuación:

- Acrylamide/ammonium acrylate copolymer
- Acrylamide/ethalkonium chloride acrylate copolymer
- Acrylamide/ethyltrimonium chloride acrylate/ ethalkonium chloride acrylate copolymer
- Acrylamide/sodium acrylate copolymer
- Acrylamide/sodium acryloyldimethyltaurate copolymer
- Acrylamide/sodium acryloyldimethyltaurate/acrylic acid copolymer
- Acrylamides copolymer
- Acrylamides/DMAPA acrylates/methoxy PEG methacrylate copolymer
- Acrylamidopropyltrimonium chloride/acrylamide copolymer
- Acrylates/acrylamide copolymer
- Aminoethylpropanediol-acrylates/acrylamide copolymer
- Corn starch/acrylamide/sodium acrylate copolymer
- Gelatin/lysine/polyacrylamide
- Hydroxypropyltrimonium chloride
- Polyacrylate-10
- Polyacrylate-11
- Polyacrylate-13
- Polyacrylate-2
- Polyacrylate-7
- Polyquaternium-15

- → Polyquaternium-32
- → Polyquaternium-33
- → Polyquaternium-39
- → Polyquaternium-43
- → Polyquaternium-5
- → Polyquaternium-53
- → Polyquaternium-63
- → Polyquaternium-7
- → Polyquaternium-94
- → Poly[oxymethylene melamine acrylates/acrylamide]
- → Potassium acrylates/acrylamide copolymer
- → Sodium acrylate/sodium acryloyldimethyl taurate/acrylamide copolymer
- → Sodium acryloyldimethyl taurate/acrylamide/VP copolymer
- → Starch/acrylates/acrylamide copolymer
- → Styrene/acrylamide copolymer

Estos polímeros no son tóxicos por sí mismos, sin embargo, pueden arrastrar consigo impurezas de acrilamida, ya que las largas cadenas poliméricas que los constituyen se forman por reacción entre monómeros de acrilamida, por lo que existe la posibilidad de que pequeñas cantidades de estos monómeros, que no hayan reaccionado durante la polimerización, acaben en los productos cosméticos y, en consecuencia, provoquen un riesgo para el usuario [42,46].

Por todo lo descrito, el Reglamento Europeo prohíbe el uso de acrilamida en los productos cosméticos y restringe el empleo de poliacrilamidas para minimizar el contenido máximo residual de acrilamida al límite considerado seguro según la Opinión del Comité Científico de Productos Cosméticos y Productos No Alimentarios (SCCNFP del inglés *Scientific Committee on Cosmetic and Non-Food Products Intended for Consumers*) (actualmente, SCCS) [1,42]. Así pues, según el Anexo III, entrada 66, este límite está establecido en 0.1 mg/kg en productos corporales sin aclarado y 0.5 mg/kg en el resto de los productos [1,42].

No obstante, debido al uso masivo de poliacrilamidas en la formulación de productos cosméticos, es de gran importancia el desarrollo de

métodos analíticos para la determinación de acrilamida residual en estos tipos de productos. Cabe indicar que no existen métodos analíticos oficiales ni artículos publicados en la bibliografía donde se lleve a cabo la determinación de acrilamida en productos cosméticos. Es por ello por lo que, en el contexto de la presente Tesis Doctoral, se consideró de gran interés desarrollar un método analítico para la determinación de acrilamida en productos cosméticos que pudiera suplir la falta de métodos oficiales y publicaciones científicas para la determinación de esta sustancia en matrices cosméticas.

Por otra parte, la acrilamida se ha determinado ampliamente en otras matrices como los productos alimenticios, en los cuales se ha descubierto que se forma fácilmente durante la cocción diaria a altas temperaturas (en procesos como hornear, freír y asar) y también durante el procesamiento industrial (a temperaturas superiores a 120 °C y con poca humedad), a través de un proceso químico conocido como reacción de Maillard, que implica a azúcares y aminoácidos (principalmente asparagina) presentes de forma natural en muchos alimentos [41,47].

Debido al elevado riesgo para la salud que supone la exposición constante a esta sustancia a través de la dieta, existen numerosos métodos analíticos publicados para la determinación de esta sustancia en matrices de productos alimenticios, que utilizan principalmente LC-MS [48,49], LC-MS/MS [50–54], LC-UV [55–58] o LC con detección por fluorescencia [59], y también GC-MS [60–64], GC con detector de ionización en llama (GC-FID, del inglés GC-*flame ionization detector*) [65], o electroforesis capilar de zona (CZE) con detección UV [66].

Además, se han empleado diferentes procedimientos de derivatización en el desarrollo de métodos analíticos para la determinación de acrilamida en matrices de productos alimenticios, como es el caso de la sililación [67], la bromación [68], y el uso de ácido trifluoroacético [69], de tioles [56,59] o de ácido mercaptobenzóico [58].

En cuanto al tratamiento de las muestras de alimentos, debido a las complejas y variadas matrices que también presentan este tipo de productos, existen diferentes artículos publicados que hacen uso de técnicas de microextracción, como la microextracción líquido-líquido

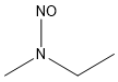
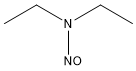
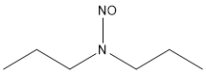
dispersiva (DLLME, del inglés *dispersive liquid-liquid microextraction*) [54,56,62,70], la microextracción en gota (SDME, del inglés *single drop microextraction*) [71] y la microextracción en fase sólida (SPME, del inglés *solid-phase microextraction*) [61,65,67].

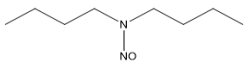
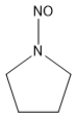
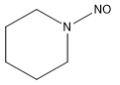
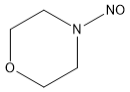
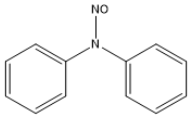
Ante la inexistencia de un método oficial, así como de publicaciones científicas basadas en la determinación de acrilamida en las complejas matrices de los productos cosméticos, en la presente Tesis Doctoral se han desarrollado dos métodos, que se describen en los **Capítulos 4 y 5**, siendo los primeros trabajos presentados con este fin.

1.5.3. Las nitrosaminas

En relación con el objetivo de la presente Tesis Doctoral, en el **Capítulo 6** se centra la atención en las ***N-nitrosaminas***, cuya información más relevante se muestra en la *Tabla 1.3*.

Tabla 1.3. Información sobre las nitrosaminas estudiadas en el *Capítulo 6*.

Nombre	Número CAS	Estructura química	M_r (g mol^{-1})	Log $K_{o/w}$
N-nitrosodimetilamina (NDMA)	62-75-9		74.08	-0.50
N-nitrosoetilmetilamina (NMEA)	10595-95-6		88.11	0.01
N-nitrosodietilamina (NDEA)	55-18-5		102.14	0.52
N-nitrosodipropilamina (NDPA)	621-64-7		130.19	1.54

N-nitrosodibutilamina (NDBA)	924-16-3		158.24	2.56
N-nitrosopiperidina (NPIP)	100-75-4		114.15	0.44
N-nitrosopirrolidina (NPYR)	930-55-2		100.12	-0.09
N-nitrosomorfolina (NMOR)	59-89-2		116.12	-0.59
N-nitrosodifenilamina (NDPhA)	86-30-6		198.22	3.13

Las N-nitrosaminas son derivados alifáticos o aromáticos de aminas secundarias, con un grupo nitroso (-NO) unido al nitrógeno. Son una familia de sustancias prohibidas en los productos cosméticos según el Anexo II, entrada 410, del Reglamento Europeo [1], por presentar efectos cancerígenos, mutagénicos y teratogénicos [72,73]. Aunque su prohibición ya se recogía en la anterior normativa, y data de 1992 [74], la presencia de estas sustancias en los productos cosméticos sigue siendo un problema persistente.

De hecho, es posible encontrar trazas de nitrosaminas en estos productos, ya que pueden formarse con relativa facilidad por reacción entre ingredientes, constituyendo un riesgo para la salud del usuario.

La reacción que da lugar a la formación de las nitrosaminas (*Figura 1.2*), ocurre entre aminas secundarias o terciarias [75] y un agente nitrosante, como nitritos u óxidos de nitrógeno [73,76,77].

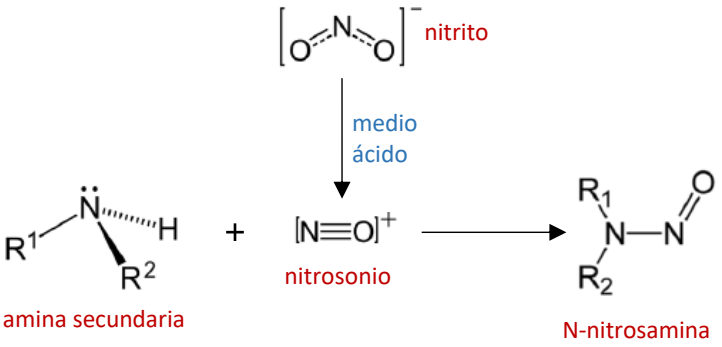


Figura 1.2. *Formación de nitrosaminas a partir de aminas secundarias.*

Por este motivo, con el objetivo de reducir la formación de estos compuestos en los productos cosméticos y, por tanto, el riesgo para la salud de los usuarios, los fabricantes de productos cosméticos deben exigir a los proveedores de materias primas un listado de las impurezas que pudieran contener y, además, evitar cualquier incompatibilidad entre ingredientes que pudiera causar reacciones de nitrosación.

Con este fin, se limita el uso de ingredientes que contengan o liberen iones nitrito, de modo que si son empleados deberán de acompañarse de sistemas de inhibición de la reacción de nitrosación a través de compuestos antioxidantes (α-tocoferol, ácido ascórbico, etc) [76]. Además de las nitrosaminas, se prohíben también las alquil- y alcanolaminas secundarias y sus sales (II/411), y se restringe el uso de las dialquilamidas y dialcanolamidas de ácidos grasos y sus sales (III/60), las monoalquilaminas, monoalcanolaminas y sus sales (III/61), y las trialquilaminas, trialcanolaminas y sus sales (III/62) [1,76].

Además, en 2012, el SCCS, al evaluar el riesgo para la salud asociado a la presencia de nitrosaminas en productos cosméticos, estableció un límite máximo de 50 µg/kg para trazas de nitrosaminas, tanto en las materias primas como en los productos cosméticos terminados [72,76].

En la bibliografía científica se pueden encontrar algunos métodos analíticos para la determinación de estas sustancias en productos

cosméticos. La mayoría de ellos se basan únicamente en la determinación de N-nitrosodietanolamina (NDELA), una nitrosamina hidrofílica para cuya determinación en productos cosméticos existen dos normas internacionales: la ISO 10130:2009 [78] y la ISO 15819:2014 [79].

El hecho de que la mayoría de las publicaciones científicas sobre este tema se centren exclusivamente en la determinación de esta nitrosamina se debe a que la NDELA es la nitrosamina que más frecuentemente se detecta en los productos cosméticos [72,75].

En cuanto a los métodos publicados para su determinación, se emplean distintas técnicas analíticas, principalmente, LC con analizador térmico de energía (TEA, del inglés thermal energy analizer) [80–82], LC-UV [83–86], LC-MS/MS [87–89], LC con polarografía [90]; GC-TEA [91], GC-MS [83,92], GC con detector de conductividad electrolítica [93] y captura electrónica [94]; y electrocromatografía capilar [95]. En cuanto al tratamiento de la muestra, destacan principalmente las técnicas de (micro)extracción en fase sólida, como la extracción en fase sólida (SPE, del inglés *solid-phase extraction*) [87,88] y la SPME [92]; mientras que es más reducido el empleo de técnicas de microextracción en fase líquida [84,86].

En lo referente a métodos analíticos para la determinación de varias N-nitrosaminas de forma simultánea en productos cosméticos, no existen métodos oficiales y hay pocos artículos científicos al respecto en la bibliografía, y esto se debe, entre otras cosas, al diferente rango de polaridad que presentan, lo que dificulta su extracción simultánea durante el tratamiento de la muestra. Sin embargo, se han publicado algunos estudios enfocados a algunos grupos de nitrosaminas, basados en técnicas cromatográficas como GC-TEA [96,97], LC-TEA [98], LC-UV [99], GC-MS/MS [100], GC-MS [101,102], LC-MS [103] y LC-MS/MS [104].

En cuanto al pretratamiento de las muestras cosméticas, en la bibliografía se pueden encontrar varios artículos en los que se emplean técnicas de extracción tanto en fase sólida como en fase líquida para la preconcentración de las N-nitrosaminas. De las técnicas en fase líquida, se utiliza la DLLME en fase reversa asistida por vórtex

(VA-RP-DLLME, del inglés *vortex-assisted* (VA) *reversed-phase* (RP)) [103], mientras que, entre las técnicas en fase sólida, destacan la SPE [100], la extracción en fase sólida dispersiva (DSPE del inglés *dispersive solid-phase extraction*) [105], la SPME en espacio de cabeza (HS-SPME, del inglés *headspace*) [102] y la microextracción dispersiva por sorción sobre barra agitadora (SBSDME, del inglés *stir bar sorptive dispersive microextraction*) [104].

El método propuesto en el **Capítulo 6** pretende ofrecer una respuesta a la escasez de métodos analíticos que permitan la determinación simultánea de un grupo de nitrosaminas en matrices cosméticas, con la sensibilidad adecuada para determinar los bajos niveles de concentraciones contemplados por la legislación.

Capítulo 2

Uso de técnicas de microextracción para el control analítico de productos cosméticos

El contenido de este capítulo ha sido publicado en el artículo:
A comprehensive review on the use of microextraction techniques in the analysis of cosmetic products
L. Schettino, G. Peris-Pastor, J.L. Benedé, A. Chisvert, **Advances in Sample Preparation** 3 (2022) 100024

2.1. Introducción a las técnicas de microextracción

La preparación de la muestra es un punto clave en el desarrollo de un método analítico ya que implica el acondicionamiento de la muestra a los requerimientos de la instrumentación utilizada, la separación de los analitos de una matriz compleja, y/o la preconcentración de los analitos cuando se encuentran a bajos niveles de concentración, mejorando así los límites de detección y cuantificación del método.

En las últimas décadas ha habido un interés creciente por desarrollar metodologías sostenibles que cumplan en la medida de lo posible con los principios de la denominada Química Analítica Verde (*Green Analytical Chemistry*) [106] y, más concretamente, con los principios de la recientemente propuesta Preparación de Muestra Verde (*Green Sample Preparation*) [107].

Las técnicas de microextracción presentan distintas ventajas que casan con estos principios, y surgieron como claras alternativas a las técnicas de extracción tradicionales (esto es, la extracción líquido-líquido (LLE, del inglés *liquid-liquid extraction*) y la extracción en fase sólida (SPE). De hecho, en comparación con estas técnicas tradicionales, en las técnicas de microextracción se emplean cantidades muy pequeñas de disolvente de extracción o sorbente y volúmenes de muestra reducidos, lo que hace que se generen menos cantidad de residuos (lo que las convierte en enfoques más ecológicos). Además, presentan tiempos de análisis más cortos, así como una automatización y acoplamiento con los instrumentos analíticos más viable. Todo ello, en clara concordancia con los principios anteriormente mencionados, ha contribuido a incrementar su uso y a primar su elección en las últimas décadas [108].

Las técnicas de microextracción se clasifican en dos grandes grupos en función de la naturaleza de la fase de extracción:

- las llamadas en **fase sólida**, esto es, basadas en sorbentes
- las llamadas en **fase líquida**, esto es, basadas en disolventes

Las técnicas de microextracción se han aplicado con éxito a la determinación de una amplia gama de analitos en diferentes matrices, como alimentos [109], muestras ambientales [110], productos farmacéuticos [111] o fluidos biológicos [112], entre otros.

El análisis de los productos cosméticos no ha sido ajeno a esta tendencia, ya que las técnicas de microextracción pueden ser de gran utilidad para la determinación de sustancias en muy bajas concentraciones, como las sustancias prohibidas por el Reglamento Europeo, siendo necesario preconcentrarlas para alcanzar los límites de detección/cuantificación requeridos. Además, teniendo en cuenta que los productos cosméticos son matrices complejas formadas por un elevado número de sustancias [113], las técnicas de microextracción son útiles para separar los analitos de interés de la matriz antes de su medida instrumental con fines de limpieza.

El primer precedente de microextracción en productos cosméticos data de 1997, es decir, hace veinticinco años. La *Figura 2.1* muestra un histograma de todos los artículos en este ámbito, según el año de publicación, desde entonces hasta este año 2022, reflejando el alto potencial y la gran atención que la comunidad científica ha prestado a las técnicas de microextracción para el análisis de productos cosméticos en los últimos veinticinco años. Como se puede observar, la evolución temporal muestra que ambas categorías de técnicas de microextracción han alcanzado y mantenido un nivel de aplicación que no ha disminuido con el tiempo, estableciéndose entre los procedimientos más utilizados en el análisis de productos cosméticos cuando se requiere realizar una extracción por los motivos anteriormente mencionados.

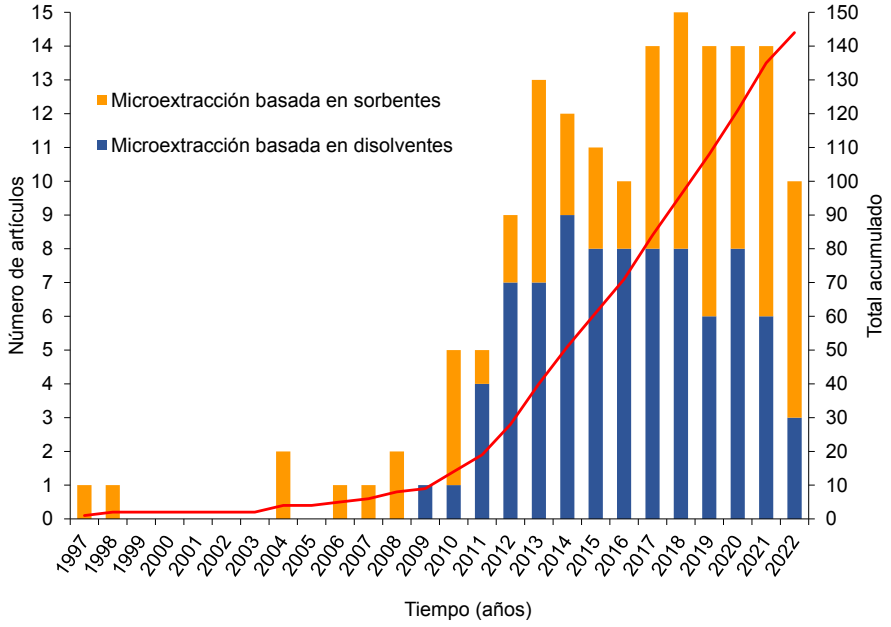


Figura 2.1. *Evolución temporal del número de artículos publicados sobre análisis de productos cosméticos en los que se emplean técnicas de microextracción. La línea roja indica el total acumulado.*

Cabe destacar que, en la mayoría de los casos, aplicar una técnica de microextracción directamente a un producto cosmético no es posible a causa de la naturaleza y complejidad de las matrices de este tipo de productos (emulsiones, geles, sólidos, soluciones con alto contenido alcohólico, etc.). Por ello, en la mayoría de los casos es necesario un tratamiento preliminar de la muestra, como la dilución en un disolvente adecuado [114], o la lixiviación de los analitos de la matriz cosmética a un disolvente apropiado mediante ultrasonidos [115] o vórtex [116], o un paso previo de limpieza, como LLE [117,118], SPE [119], extracción con líquidos presurizados (PLE, del inglés *pressurized liquid extraction*) [120], DSPE [121,122] o dispersión de matriz en fase sólida (MSPD, del inglés *matrix solid-phase dispersion*) [123], entre otros, para eliminar gran parte de los componentes de la matriz que, de lo contrario, podrían afectar negativamente a la aplicación de la técnica de microextracción.

2.2. Técnicas de microextracción basadas en sorbentes

Las técnicas de microextracción basadas en sorbentes hacen uso de materiales sólidos para separar los analitos de interés.

Aunque el procedimiento experimental es diferente según la técnica concreta, en todas ellas se inmoviliza el material sorbente sobre la superficie de un soporte inerte que se expone a la fase dadora para extraer los analitos de interés. Posteriormente, estos son desorbidos al poner en contacto el sorbente con un disolvente apropiado (desorción líquida), o desorbiendo los analitos térmicamente por acoplamiento directo con el instrumento analítico (desorción térmica).

El uso de sorbentes adecuados es un factor crítico para lograr una extracción selectiva de los analitos, por lo que se deben tener en consideración la naturaleza y las propiedades fisicoquímicas del material sorbente para su correcta selección [124].

A continuación, se describen las técnicas de microextracción basadas en sorbentes que se han utilizado en el control analítico de productos cosméticos, y se comentan con más detalle algunos ejemplos de su aplicación.

2.2.1. Microextracción en fase sólida

La SPME fue introducida por primera vez en 1990 [125], y rápidamente se convirtió en una de las técnicas de microextracción más utilizadas hasta la fecha.

Esta técnica se basa en la transferencia de los analitos de la matriz de la muestra a una fibra adsorbente alojada en el interior de una aguja metálica que protege la fibra y que se encuentra fijada en un soporte similar a una jeringa. Para proceder a la extracción, la fibra se saca de la aguja y se sumerge directamente en la disolución a extraer, o se puede exponer en el espacio de cabeza para el caso de analitos (semi)volátiles [126]. Para acelerar la cinética de la extracción, la disolución a extraer se somete a agitación con ayuda de un imán y un

agitador magnético. Después de la extracción, la fibra se retrae en la aguja para posteriormente realizar la desorción de los analitos. La *Figura 2.2* muestra el esquema de la SPME.

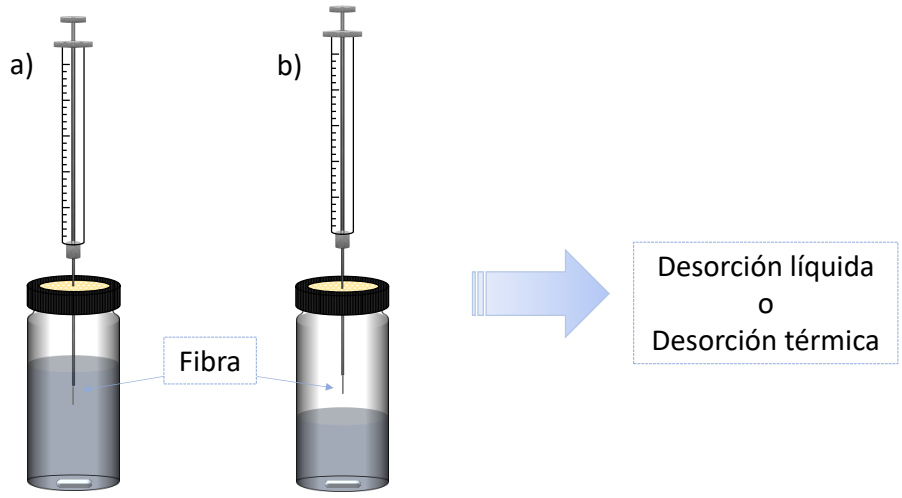


Figura 2.2. *Esquema de la SPME: (a) modo de inmersión directa y (b) modo de espacio de cabeza*

La SPME fue la primera técnica de microextracción utilizada para el análisis de productos cosméticos, cuando Struppe et al. [127], en 1997, la utilizaron para determinar nitro-almizcles en cremas. Después de este primer trabajo, muchos investigadores han utilizado la SPME como técnica de microextracción para el análisis de productos cosméticos y, hasta el día de hoy, sigue siendo la técnica de microextracción basada en sorbentes más utilizada en este campo. El material sorbente más empleado es el polidimetilsiloxano (PDMS), ya sea solo [127–129] o en combinación con otros materiales como el divinilbenceno (DVB) [130–135] o el carboxeno (CAR) [102]. Las combinaciones PDMS-DVB y PDMS-CAR, o incluso PDMS-DVB-CAR [136], permiten la extracción simultánea de analitos tanto polares como apolares, como formaldehído [132,133], alérgenos de fragancias [135], nitrosaminas [102] y ftalatos [130]. Otro material que ha sido frecuentemente utilizado es el poliacrilato, para la determinación de

antioxidantes y conservantes [137], y diferentes aditivos [138]. Además de estos materiales comerciales, también se han diseñado en el laboratorio materiales a medida, en función de las características fisicoquímicas de los analitos, para mejorar la selectividad de la extracción, como el diacrilato de polietilenglicol para determinar parabenos [139], o un compuesto basado en un líquido iónico polimérico aromático para la determinación de cinco hidrocarburos aromáticos policíclicos (PAHs, del inglés *Polycyclic Aromatic Hydrocarbons*) [140], o materiales derivados del óxido de grafeno (GO, del inglés *graphene oxide*) para la determinación de PAHs [141], derivados del benceno [142], parabenos [143] y alérgenos de fragancias [144], entre otros.

2.2.2. Extracción por sorción sobre barra agitadora

En la extracción por sorción sobre barra agitadora (SBSE) [145], el sorbente recubre una barra agitadora sumergida en la fase dadora líquida, que se agita magnéticamente para promover el contacto entre analitos y sorbente, y así facilitar la extracción de los compuestos de interés. Una vez realizada la extracción, la barra agitadora se retira y se seca con papel antes de proceder a la desorción de los analitos. La Figura 2.3 muestra el esquema de la SBSE.

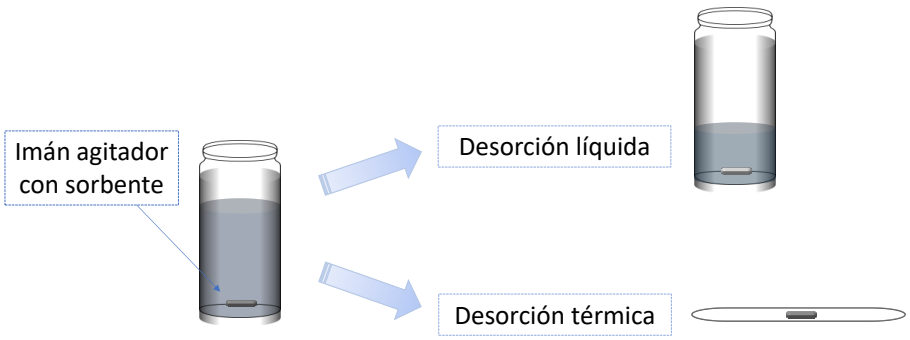


Figura 2.3. *Esquema de la SBSE*

Las primeras aplicaciones de SBSE en el análisis de productos cosméticos utilizaron PDMS como sorbente para la extracción de parabenos [146] y triclosán [147]. Al igual que con la SPME, se emplearon también recubrimientos a medida para aumentar la selectividad y la sensibilidad a la hora de extraer. Por citar algún ejemplo, se empleó una estructura metal-orgánica inmovilizada sobre la superficie de la barra agitadora para extraer parabenos [148], o una espuma compuesta de GO, polietilenglicol (PEG) y látex natural, atravesada por un alambre de acero inoxidable que permitía su agitación de forma similar a SBSE, para la extracción de butilhidroxitolueno (BHT), butilhidroxianisol (BHA) y otros antioxidantes [149].

2.2.3. Extracción en fase sólida dispersiva

En la DSPE, el sorbente se dispersa en la fase dadora para aumentar la superficie de contacto entre el sorbente y los analitos [150,151], y así mejorar la cinética de extracción. La dispersión puede lograrse por diferentes vías, como agitación manual, magnética, asistida por vórtex, asistida por ultrasonidos o por efervescencia [152], y una vez transcurrido el tiempo de extracción, el sorbente se recupera por centrifugación, y en menor medida por filtración, para someterlo a la etapa de desorción posterior. La Figura 2.4 muestra el esquema de la DSPE.

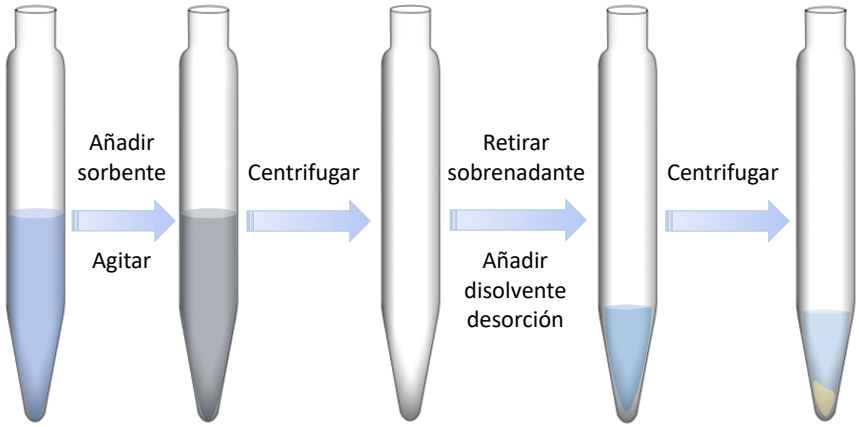


Figura 2.4. *Esquema de la DSPE*

Esta técnica se hizo mucho más popular cuando se empezaron a emplear sorbentes magnéticos, ya que facilitan su separación de la fase dadora mediante la simple aplicación de un campo magnético externo proporcionado por un imán, evitando así la etapa de centrifugación o de filtración de la DSPE convencional [153]. Debido a estas ventajas, se han utilizado materiales magnéticos para la determinación de diferentes analitos como, por ejemplo, parabenos, utilizando MIL-101(Cr) en combinación con nanopartículas magnéticas de Fe_3O_4 y nanotubos de carbono de paredes múltiples [154], nanopartículas magnéticas de Fe_3O_4 con GO magnético cargado con curcumina [155] o el líquido iónico 1-butyl-3-methylimidazolium chloride (BMIM-Cl) cargado en la superficie de un polímero de β-ciclodextrina injertado con nanopartículas magnéticas de Fe_3O_4 [156]; glucocorticoides utilizando polímeros de impresión molecular magnéticos (MIPs, del inglés *molecularly imprinted polymers*) [157,158]; o el colorante rodamina B con nanopartículas magnéticas de Fe_3O_4 recubiertas de poli(anilina-naftilamina) (Fe_3O_4@PANI-NA) para su determinación en sombras de ojos, champús y jabón de manos [159].

Un nuevo enfoque de esta técnica, desarrollada por el grupo de investigación en el que se ha desarrollado la presente Tesis Doctoral, es la microextracción dispersiva por sorción sobre barra agitadora

(SBSDME) [160], En esta técnica, que combina los principios de la SBSE y de la DSPE, un sorbente magnético que recubre una barra de agitación magnética se dispersa en la fase dadora al agitar a velocidad elevada. Cuando se detiene la agitación, el sorbente magnético que contiene los analitos se recupera nuevamente por la barra agitadora magnética sin necesidad de un campo magnético externo adicional [161]. La Figura 2.5 muestra el esquema de la SBSDME.

Figura 2.5. *Esquema de la SBSDME*

Esta técnica se ha utilizado con éxito para la determinación de ocho nitrosaminas en un gel de ducha y una crema corporal, mediante el uso de nanopartículas magnéticas de $CoFe_2O_4$ incrustadas en la estructura metal-orgánica MIL-101(Fe) ($CoFe_2O_4$/MIL-101(Fe)) como fase extractante [104]. Posteriormente, se aplicó la misma técnica para determinar diez PAHs empleando un material compuesto de $CoFe_2O_4$ y óxido de grafeno reducido (rGO) ($CoFe_2O_4$/rGO)[162].

2.2.4. Otros enfoques de microextracción basados en sorbentes

Además de las técnicas de microextracción descritas anteriormente, se han publicado otros enfoques basados en sorbentes para el análisis

de productos cosméticos. Se describen algunos ejemplos a continuación.

Cabe mencionar la microdispersión de matriz en fase sólida (μ-MSPD), que es una versión miniaturizada de la clásica MSPD, en la que la muestra se mezcla con un agente desecante y un sorbente dispersante. En esta versión miniaturizada, en la que todas las cantidades son reducidas respecto a la MSPD, la mezcla se carga en una pipeta Pasteur de vidrio o en una punta de pipeta con una pequeña cantidad de lana de vidrio para evitar pérdidas de material, y se utiliza una cantidad reducida de disolvente para eluir los compuestos [163]. Esta técnica se ha empleado para la determinación de una amplia variedad de analitos como plastificantes, almizcles, colorantes, conservantes, alérgenos de fragancias, filtros UV, PAHs y glicoles, entre otros [163–170].

En la microextracción capilar (CME, del inglés *capillary microextraction*), la fase dadora pasa a través de un capilar que contiene el sorbente empaquetado en su interior o recubriendo la pared interna. En la modalidad más utilizada, conocida como microextracción en fase sólida en tubo (IT-SPME, del inglés *in-tube SPME*), se pasa un disolvente adecuado a través del capilar para desorber los analitos, que generalmente se acopla directamente al sistema cromatográfico, lo que permite que los analitos sean transferidos en línea [171]. En este contexto, por ejemplo, se ha utilizado un monolito de poli(ácido metacrílico-co-etilenglicol dimetacrilato) como sorbente para la determinación de ftalatos en tónicos faciales [172] y hormonas en quitaesmaltes y perfumes [173].

La microextracción por sorbente empaquetado (MEPS, del inglés *microextraction by packed sorbent*) es otra variante de microextracción basada en sorbentes en la que el sorbente se coloca en una jeringa y los analitos se retienen en ella cuando se aspira la fase dadora a través de la jeringa y pasa a través del sorbente [174]. Esta técnica es un procedimiento miniaturizado alternativo a la SPE, que reduce drásticamente el uso de disolventes orgánicos y permite, a través de la jeringa, la desorción líquida de los analitos directamente en el instrumento analítico. Con esta técnica solo se ha empleado C18 como sorbente en el análisis de productos cosméticos, aplicado a la

determinación de parabenos [175], benzofenonas [176] y ftalatos [177].

En la técnica de microextracción por adsorción en barra (BAμE, del inglés *bar adsorptive microextraction*) [178], se utiliza una barra sobre la cual se fija el sorbente mediante un adhesivo de doble cara, que luego se introduce en la fase dadora mientras se agita, operando bajo flotación. Posteriormente, se toma la barra de extracción y se lleva a otro vial con el correspondiente disolvente para eluir los analitos. Esta técnica se ha utilizado para la determinación de benzofenonas [179] y parabenos [180].

La extracción por sorción sobre tejido (FPSE, del inglés *fabric-phase sorptive extraction*) es otro enfoque de microextracción basado en sorbentes en el que una pequeña pieza de tejido plana recubierta con el sorbente se sumerge en una disolución de la muestra agitada magnéticamente para la extracción de los analitos [181]. Posteriormente, la desorción se lleva a cabo simplemente sumergiendo el tejido en el disolvente apropiado. Esta técnica se ha empleado para determinar parabenos usando PEG como recubrimiento [182,183].

La llamada extracción en fase sólida protegida por membrana (MP-SPE, del inglés *membrane-protected solid-phase extraction*) también se ha utilizado en el análisis de productos cosméticos. En esta técnica, el sorbente se coloca dentro de una fibra hueca porosa, que se sumerge en la fase dadora líquida y se agita durante un período definido. Esta técnica se ha utilizado para la detección de parabenos [184] y aldehídos alifáticos [185] en muestras cosméticas.

2.3. Técnicas de microextracción basadas en disolventes

Las técnicas de microextracción basadas en disolventes (comúnmente conocidas como técnicas de microextracción en fase líquida (LPME, del inglés *liquid-phase microextraction*)), tienen lugar entre una fase dadora líquida, que contiene los analitos a extraer, y un volumen muy

pequeño (unos pocos microlitros) de una fase aceptora también líquida inmiscible en la fase dadora.

Debido a la creciente necesidad de reducir el uso de disolventes orgánicos convencionales en favor de disolventes más respetuosos con el medio ambiente, en los últimos años se han popularizado mucho disolventes alternativos como los líquidos iónicos (IL, del inglés *ionic liquid*) [186], los disolventes supramoleculares (SUPRAs) [187] y los disolventes eutécticos profundos (DES, del inglés *deep eutectic solvent*) [188], para ser empleados como fases aceptoras en estas técnicas de microextracción.

El desafío de aplicar técnicas de microextracción basadas en disolventes en el análisis de productos cosméticos se manifiesta al tratar con la complejidad de las matrices cosméticas, y por esta razón se han utilizado muchas variaciones de estas técnicas para superar este obstáculo.

A continuación, se describen las técnicas de microextracción basadas en disolventes que se han empleado en el análisis de productos cosméticos, y se detallan algunos ejemplos de su aplicación.

2.3.1. Microextracción en gota

La microextracción en gota (SDME) fue la primera modalidad de LPME que se empleó en el análisis de productos cosméticos, en 2009 [189].

La técnica se basa en extraer los analitos mediante una microgota de disolvente orgánico que cuelga de la punta de la aguja de una jeringa, directamente sumergida en la fase dadora acuosa, o en su espacio de cabeza para el caso de analitos (semi)volátiles. Después de la extracción, la microgota se retrae en la jeringa y el extracto se introduce en el instrumento analítico. La Figura 2.6 muestra el esquema de la SDME.

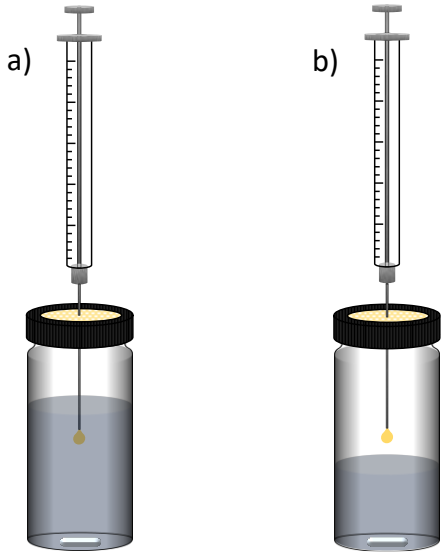


Figura 2.6. *Esquema de la SDME: (a) modo de inmersión directa y (b) modo de espacio de cabeza*

La primera aplicación de esta técnica en matrices cosméticas permitió determinar parabenos en geles limpiadores y capilares utilizando acetato de hexilo como solvente extractante [189]. La modalidad de espacio de cabeza se aplicó para determinar residuos de disolventes volátiles y semivolátiles en muestras cosméticas, como la acetona [190], el etanol [191] y el 1,4-dioxano [192–194], este último prohibido.

Recientemente, se ha empleado el IL 1-butyl-3-methylimidazolium hexafluorophosphate para determinar cadmio, arsénico, plomo y níquel en maquillaje de ojos [195].

2.3.2. Microextracción en fase líquida con fibra hueca

La microextracción en fase líquida con fibra hueca (HF-LPME, del inglés *hollow fiber*) [196,197], es una técnica de LPME en la cual la gota extractante de disolvente orgánico está alojada dentro de una fibra hueca porosa tubular, quedando así protegida y minimizando el

riesgo de que se desprenda de la punta de la aguja como puede ocurrir fácilmente en la SDME. La Figura 2.7 muestra el esquema de la HF-LPME.

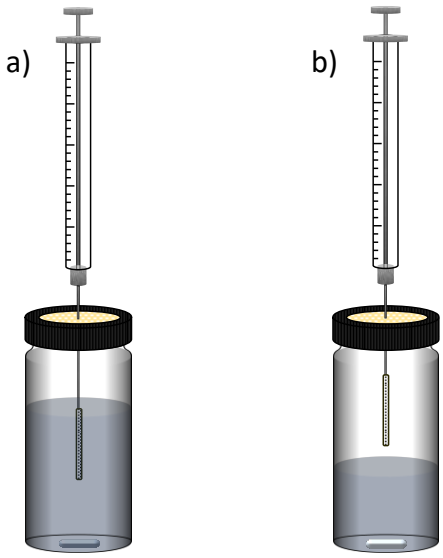


Figura 2.7. *Esquema de la HF-LPME: (a) modo de inmersión directa y (b) modo de espacio de cabeza*

En el control analítico de productos cosméticos, se usó esta técnica con 1-octanol como disolvente de extracción para determinar rodamina 6G en barras de labios [198] y parabenos en cremas y champús [199]; con tolueno para la extracción de filtros UV en cremas, lociones y bases de maquillaje [200]; y con el IL 1-octyl-3-methylimidazolium hexafluorophosphate para la extracción de diferentes hormonas sexuales en cremas y lociones [201].

2.3.3. Microextracción líquido-líquido dispersiva

La microextracción líquido-líquido dispersiva (DLLME) se introdujo por primera vez en 2006 [202], y es una técnica que destaca por su bajo coste, su fácil aplicación y su rapidez.

La DLLME es la técnica de LPME más empleada en el control analítico de productos cosméticos, y a la que se ha recurrido en los métodos presentados en los **Capítulos 4, 5, y 6** de la presente Tesis Doctoral.

En su modo convencional se basa en un sistema ternario de disolventes:

- la fase dadora de naturaleza acuosa que contiene el/los analito/s a extraer,
- el disolvente de extracción (normalmente un disolvente halogenado debido a su mayor densidad respecto al agua, lo que facilita su recolección tras la separación de las fases), y
- el disolvente dispersante (miscible tanto en la fase dadora acuosa como en el disolvente de extracción).

Para llevar a cabo la DLLME, se inyecta rápidamente una mezcla apropiada de los disolventes dispersante y de extracción en la fase dadora acuosa, situada en un tubo de punta cónica, formando una microemulsión más o menos estable, donde el disolvente dispersante provoca la dispersión del disolvente de extracción en forma de múltiples microgotas. De este modo, el área superficial de contacto entre el disolvente de extracción y la muestra es extremadamente grande, acelerando la trasferencia del analito desde la fase dadora acuosa al disolvente de extracción. La extracción es prácticamente instantánea y, a continuación, se separan las fases por centrifugación, de modo que el extracto se deposita en el fondo del tubo de punta cónica, lo que facilita su recolección. Es por ello por lo que se emplean disolventes de extracción más densos que el agua. El extracto sedimentado se recolecta y se transfiere al instrumento seleccionado para su análisis [203]. Un esquema general de la DLLME se muestra en la *Figura 2.8*.

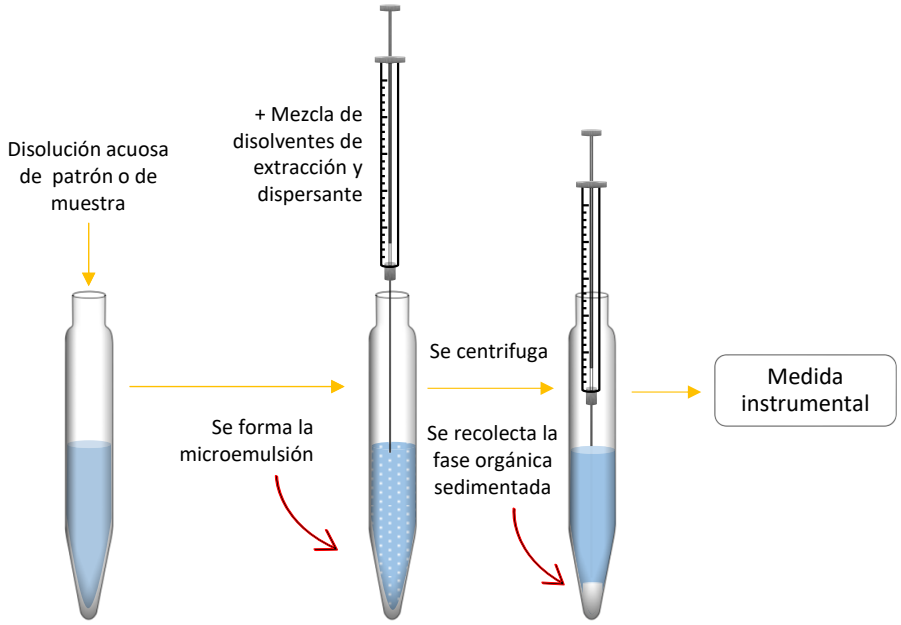


Fig. 2.8 *Representación gráfica del proceso de la DLLME*

En el análisis de productos cosméticos, se han publicado diversos artículos que emplean la DLLME convencional con disolventes de extracción halogenados, como cloroformo o tetracloruro de carbono, para la determinación de níquel [204], bismuto [205], parabenos [206] y otros conservantes [207], atranol y cloroatranol [208], ftalatos [209] y vitamina E [120].

Para evitar el uso de disolventes halogenados, se han propuesto disolventes de extracción alternativos menos dañinos para el investigador y el medio ambiente, como ILs que se han empleado para la extracción de especies de mercurio [210], filtros UV tipo benzofenona [211], hexaclorofeno [121], nanopartículas de plata [212], parabenos [122,213], compuestos fenólicos [214], colorante FCF azul brillante [215], y bergapten y bergamottin [216], o SUPRAs para extraer parabenos [217].

La DLLME convencional se ha empleado como técnica de microextracción en el método descrito en el **Capítulo 4** de la presente

Tesis Doctoral, en el cual, para determinar trazas de acrilamida en cremas y geles, se realiza una etapa de limpieza (*clean-up*) con hexano mediante una LLE, una derivatización de la acrilamida con 2-naftalentiol para, entre otras cosas, conferirle características apolares para ser extraída, mediante una etapa de DLLME con cloroformo y provocar una preconcentración y limpieza previa al análisis por LC-UV.

Por otra parte, en su modalidad de fase reversa (RP-DLLME) [218] se aprovechan las ventajas de la técnica convencional invirtiendo las fases para poder extraer compuestos altamente polares, que con la DLLME convencional no se podrían extraer debido a la naturaleza no polar de los disolventes de extracción utilizados. De hecho, en la RP-DLLME, es un pequeño volumen de agua el que actúa como disolvente de extracción para extraer compuestos polares, dispersándose en una fase dadora orgánica que actúa de soporte.

Esta técnica se ha aplicado para la extracción de sustancias prohibidas en productos cosméticos como NDELA [86] (la nitrosamina más habitual en productos cosméticos) y formaldehído [116].

En esta modalidad, una variable fundamental a considerar es la naturaleza del disolvente que actúa de soporte y por tanto de fase dadora en el proceso de extracción, prefiriendo disolventes menos densos que el agua, para así poder recoger fácilmente el extracto acuoso en la punta cónica del tubo utilizado para la extracción. Un esquema general de la RP-DLLME se muestra en *la Figura 2.9.*

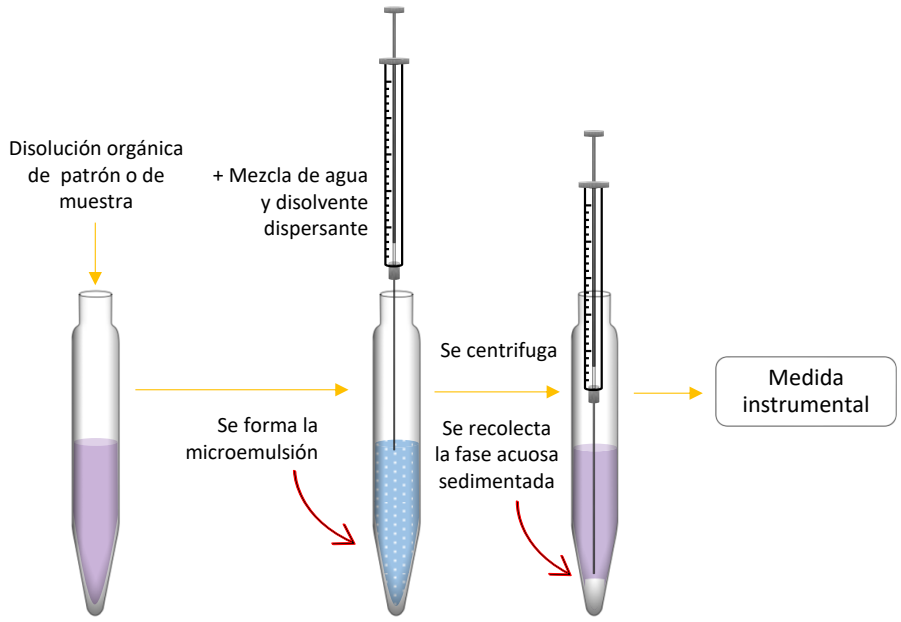


Fig. 2.9 *Representación gráfica del proceso de la RP-DLLME*

En algunos casos, tanto en la DLLME convencional como en la RP-DLLME, el disolvente dispersante puede actuar disminuyendo el coeficiente de reparto de los analitos entre el disolvente de extracción y la fase dadora, reduciendo así la eficiencia de la extracción. Para evitar este efecto no deseado, es posible optar por emplear un procedimiento alternativo para generar la microemulsión, a través de diferentes mecanismos de dispersión, tales como la agitación con ultrasonidos, la agitación por vórtex, la agitación magnética o través de un flujo de aire. En estos casos, el uso de un disolvente dispersante no es necesario. Así:

- La DLLME asistida por ultrasonidos (USA-DLLME, del inglés *ultrasound-assisted*) utiliza ultrasonidos para formar la dispersión del solvente de extracción en la fase acuosa, presentando la ventaja de promover rápidamente la homogeneización y la transferencia de masa entre fases inmiscibles. La primera aplicación publicada de USA-DLLME en el análisis de productos cosméticos fue la determinación de triclosán en diferentes

muestras de jabón [219]. También se ha utilizado para extraer alcanolaminas y alquilaminas en cremas, protectores solares, lociones, champús y polvos [220]; formaldehído en champús, acondicionadores y geles de ducha [221]; conservantes en cremas, pastas dentales, desmaquillantes, champús y productos de protección solar [222–224], entre otras aplicaciones.

- La DLLME asistida por vórtex (VA-DLLME) utiliza el vórtex como instrumento de agitación para permitir la rápida formación de la microemulsión. Algunos ejemplos de esta modalidad aplicada al control analítico de productos cosméticos son la determinación de cloramina T [225], parabenos [226] y ftalatos [114]; el empleo de un DES de DL-mentol/PEG para determinar parabenos en cremas, y de otro de choline cloruro/etilenglicol para extraer parabenos en aceites cosméticos [227,228]; el uso de los ILs 1-hexyl-3-methylimidazolium tetrafluoroborate para la extracción de hormonas [229], Octylguanidinium chloride para extraer parabenos [230] y 1-butoxy-3-ethoxy-2-ethyl-imidazolium bis(trifluoromethane)sulfonamide para extraer cannabidiol [37].

  Esta modalidad se ha empleado en el trabajo descrito en el **Capítulo 6** de la presente Tesis Doctoral, para la determinación simultánea de niveles traza de nueve N-nitrosaminas prohibidas en productos cosméticos, que se basa en la VA-DLLME, como técnica de limpieza y preconcentración, seguida del análisis instrumental por GC-MS.

  También hay ejemplos del uso de esta técnica en fase reversa (VA-RP-DLLME), utilizando agua como disolvente de extracción [103], y en el **Capítulo 5** de la presente Tesis Doctoral se propone un método en el que este enfoque ha permitido determinar de manera rápida y sencilla trazas de acrilamida en diversos productos cosméticos. En el método descrito se propone la VA-RP-DLLME como técnica de limpieza y preconcentración previa al análisis de los extractos mediante LC-MS/MS, sin la necesidad de llevar a cabo una etapa de derivatización previa como fue necesario en el **Capítulo 4**.

- La DLLME asistida por agitación magnética (MSA-DLLME, del inglés *magnetic stirring-assisted*), utiliza un agitador magnético y un imán introducido en la disolución de muestra para lograr la dispersión. Una ventaja de la MSA-DLLME es que la utilización de placas calefactoras con agitación magnética puede permitir mantener la temperatura controlada mientras se realiza la extracción. Ejemplos de aplicación de esta modalidad en el análisis de productos cosméticos son la determinación de rodamina B y rodamina 6G en barras de labios [231], y de parabenos en cremas [123].

- La DLLME asistida por aire (AA-DLLME, del inglés *air-assisted*) logra la dispersión del disolvente de extracción en la disolución acuosa de muestra a través de ciclos repetidos de aspiración/propulsión con ayuda de una jeringa [232]. Esta simple acción conduce a una dispersión eficiente del disolvente de extracción en la solución de muestra, y se estudia el número de ciclos de aspiración-expulsión necesarios para lograr el mejor rendimiento de extracción. Ejemplos de aplicación de esta técnica en el análisis de productos cosméticos son la determinación de parabenos en distintas matrices cosméticas [115,233].

Finalmente, la última modalidad de DLLME que se ha empleado en el análisis de muestras cosméticas es la llamada microextracción por solidificación de gota orgánica flotante (DLLME-SFOD, del inglés *solidification of floating organic droplet*), en la que se utiliza un disolvente de extracción menos denso que el agua, de modo que después de la extracción la gota de extracto se queda en la superficie de la fase dadora acuosa formando una capa difícil de recoger. Para facilitar su recolección, se congela sumergiendo el vial de extracción en un baño de hielo para que solidifique en forma de gota y se pueda recoger fácilmente con ayuda de una espátula. Esta técnica se empleó, por ejemplo, para extraer parabenos usando SUPRAs [234] o parabenos y plomo usando 1-undecanol [119,235]. En esta técnica también se implementó la sonicación (USA-DLLME-SFOD), utilizando alcoholes grasos como disolventes de extracción para determinar

ftalatos [236] y sustancias potencialmente alergénicas [237] en diferentes matrices cosméticas.

2.3.4. Extracción en punto de nube

La extracción en punto de nube (CPE, del inglés *cloud point extraction*) consiste en la separación de los analitos de la fase acuosa mediante pseudo-fases de micelas de tensioactivo no iónico. Al alterar las propiedades de la disolución, como la temperatura, la presión o el contenido de sal, las micelas se separan en dos fases isotrópicas: una fase compuesta casi en su totalidad por el tensioactivo conteniendo el analito, y una fase acuosa en la que la concentración del tensioactivo es cercana a la concentración micelar crítica, que es la concentración mínima de tensioactivo a partir de la cual se forman micelas espontáneamente en una disolución. Posteriormente, es necesaria una retro-extracción del analito en un disolvente orgánico para reducir las interferencias del tensioactivo en el instrumento analítico. La Figura 2.10 muestra el esquema de la CPE.

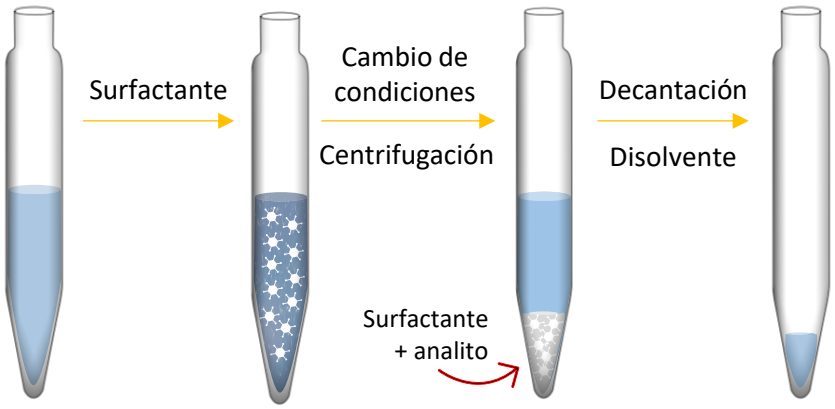


Fig. 2.10 *Esquema de la CPE*

Esta técnica ha sido empleada, por ejemplo, para la extracción de cadmio utilizando PEG tert-octilfenil éter (Triton X-114) en champú, lápiz labial y esmalte de uñas [238], o para la extracción de antioxidantes, como BHA, BHT, tert-butilhidroquinona (TBHQ) y galato de propilo, utilizando dodecilpolioxietilen éter (AEO 9) como disolvente de extracción [239].

2.4. Tendencias en la aplicación de técnicas de microextracción al análisis de productos cosméticos

El trabajo de revisión sobre el uso de técnicas de microextracción en el análisis de productos cosméticos, que se incluye en el Anexo de la presente Tesis Doctoral, presenta una recopilación exhaustiva de los trabajos publicados en este campo, desde el primer precedente en 1997 hasta el presente. De este trabajo de revisión, que compila y comenta un total de 144 artículos centrados en la etapa de microextracción, se observan ciertas tendencias en cuanto a aplicación, que se comentan a continuación.

Así, se concluye que, entre las técnicas de microextracción basadas en sorbentes (*Figura 2.11*), la SPME (42%), y más concretamente la modalidad en espacio de cabeza, es sin duda la más utilizada. La ventaja de esta modalidad radica en que no necesita de un disolvente en la etapa de desorción al acoplarla directamente al instrumento de medida (es decir, GC), haciendo la técnica más versátil y rápida. Otras técnicas, como la DSPE (17%) y la SBSE (10%), también se han empleado, aunque en menor medida. La principal ventaja de la DSPE es la posibilidad de diseñar y utilizar una amplia variedad de sorbentes, con diferentes características dependiendo de los analitos a extraer, que dispersándose en la fase dadora reducen drásticamente el tiempo de extracción en comparación con los enfoques estáticos (es decir, SPME y SBSE).

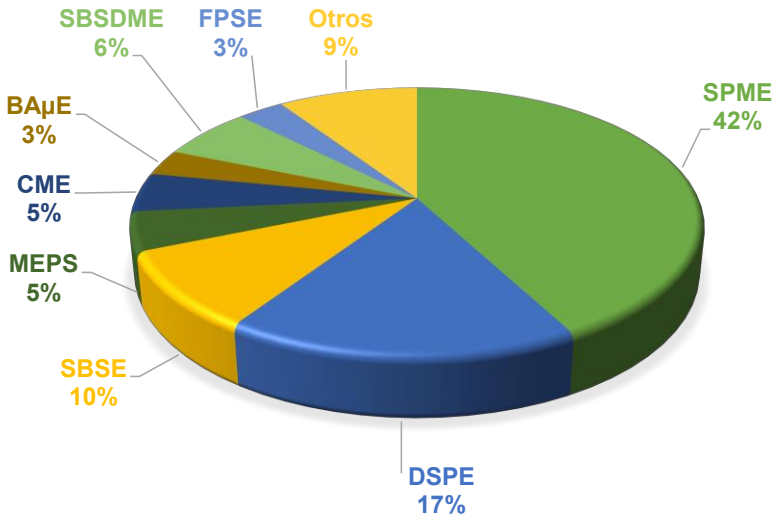


Figura 2.11 *Frecuencia de uso de las técnicas de microextracción basadas en sorbentes aplicadas al análisis de productos cosméticos*

Por otro lado, entre las LPME, la modalidad que presenta mayor relevancia en este campo es la DLLME (*Figura 2.12*), ya que en concreto el 79% de los métodos revisados basados en LPME hacen uso de esta técnica. La rapidez, la sencillez y el bajo coste son seguramente las principales ventajas sobre los otros enfoques, en los que normalmente no se alcanza el estado de equilibrio. Dentro de esta técnica se han presentado diferentes modalidades, siendo la convencional (32%), y las asistidas por vórtex (25%) y ultrasonidos (14%) las más utilizadas.

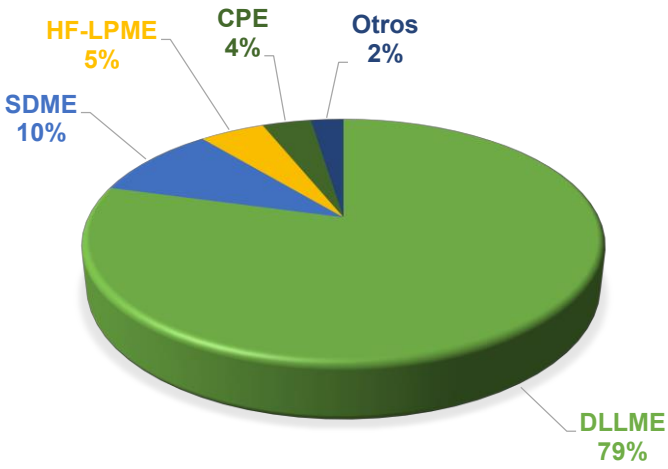


Figura 2.12 *Frecuencia de uso de las técnicas de microextracción basadas en disolventes aplicadas al análisis de productos cosméticos.*

2.5. Analitos de interés en el desarrollo de técnicas de microextracción aplicadas al análisis de productos cosméticos

Los analitos que se investigan en los productos cosméticos son de gran variedad, y pueden ser:

- ingredientes activos empleados en bajas concentración que, frente a la posible degradación o inestabilidad en la fórmula, deben ser cuantificados para asegurar su concentración y así confirmar la eficacia del producto (por ejemplo, vitaminas y cannabidiol);

- excipientes y aditivos que deben ser cuantificados para asegurar que su concentración cumpla con los límites de seguridad definidos de acuerdo con la normativa vigente sobre productos cosméticos;

- compuestos prohibidos por la legislación vigente por su toxicidad demostrada, cuya presencia debe ser controlada en caso de que exista la posibilidad de que estén presentes en la formulación por causas no intencionadas.

En la *Figura 2.13* se muestra la tendencia sobre categorías de sustancias que se han estudiado en productos cosméticos con técnicas de microextracción, haciendo referencia a los artículos examinados en el trabajo de revisión incluido en el Anexo.

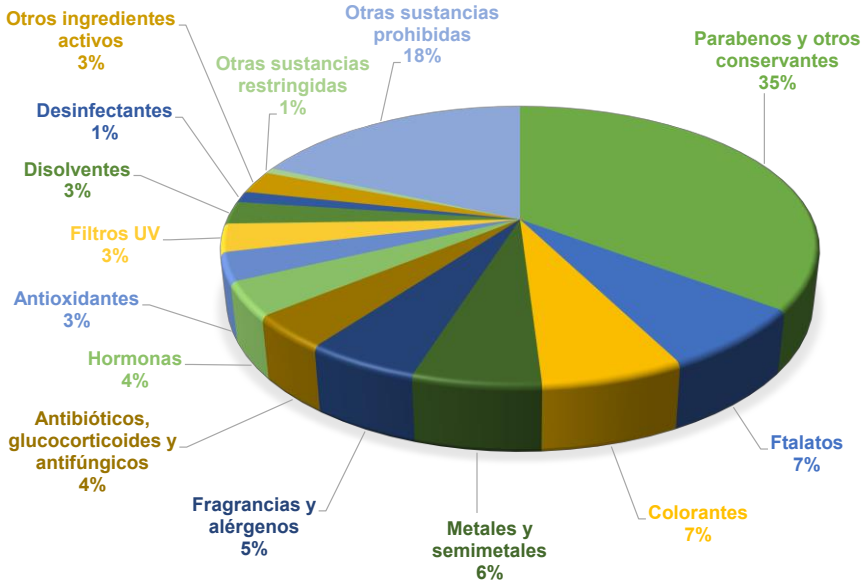


Figura 2.13 *Analitos de interés determinados en productos cosméticos aplicando técnicas de microextracción.*

Los *conservantes* han sido la categoría de sustancias más investigada (35%) con técnicas de microextracción, y entre ellos destacan los parabenos, probablemente porque, por un lado, su nivel de concentración tiene que respetar los límites definidos por el Reglamento Europeo como conservantes, y por otro lado porque, debido a que algunos de ellos están actualmente prohibidos (por ejemplo, bencilparabeno, isobutilparabeno, isopropilparabeno, fenilparabeno), en los últimos años se ha difundido información engañosa generalizada a todos los parabenos sobre la toxicidad y el peligro de exposición a estos compuestos. Sin embargo, no todos los parabenos están prohibidos en el marco de la UE, y los que sí están permitidos se consideran seguros para la salud humana si son utilizados en los productos cosméticos dentro de los límites establecidos por la normativa vigente. Otros conservantes, cuyos niveles en los productos cosméticos se han investigado, son el triclosán, bronopol, 3-iodo-2-propinil butilcarbamato (IPBC), fenoxietanol, ácido benzoico, ácido salicílico, ácido sórbico y benzoato de sodio, entre otros.

Los *ftalatos*, que se utilizan comúnmente como plastificantes en la producción de esmaltes de uñas o lacas para el cabello, como disolventes, o como fijadores en los perfumes, son otra categoría de ingredientes que destaca entre las más analizadas (7%). Como se ha demostrado que algunos ftalatos son tóxicos y se han prohibido en los productos cosméticos, el interés hacía las técnicas de microextracción para determinar estos analitos se debe a la necesidad de comprobar su ausencia en el producto final, ya que podrían estar presentes a causa de impurezas en las materias primas o, sobre todo, a causa de migración desde los envases de plástico utilizados.

También se han determinado con frecuencia, utilizando técnicas de microextracción, *colorantes* y *filtros UV* (7% y 3%, respectivamente), ya que también son sustancias sujetas a restricciones de concentración según el tipo de producto y la población a la que se destinan, especialmente los filtros UV, cuya concentración debe comprobarse para asegurar el cumplimiento con los límites permitidos y la concordancia con la información declarada en la etiqueta, para garantizar el factor de protección solar declarado.

Otras sustancias determinadas a través de estas técnicas son *metales* y *semimetales* (6%), *antioxidantes* (3%), y *fragancias* y *alérgenos* (5%). Este último grupo incluye muchas moléculas, algunas de las cuales están prohibidas (por ejemplo, lilial), mientras que otras (por ejemplo, linalool, citral, geraniol, bencil alcohol), debido a su acción alergénica, deben declararse en etiqueta si superan una determinada concentración según el tipo de producto.

También se han publicado artículos en los que se aplican técnicas de microextracción a la determinación de *ingredientes activos*, como vitaminas, cannabidiol, antraquinonas, y disolventes como el alcohol, con el interés de cuantificar su concentración en el producto final para garantizar una cierta eficacia o reclamar su presencia en la etiqueta.

Finalmente, se han publicado también artículos en los que se aplican técnicas de microextracción a la determinación de *sustancias prohibidas* en los productos cosméticos que deben investigarse para asegurar su ausencia antes de su comercialización, o para analizar un producto ya comercializado en una inspección de mercado. Estas sustancias incluyen, además de algunos parabenos y ftalatos mencionados anteriormente, hormonas, antibióticos y antifúngicos, que no están permitidos como ingredientes cosméticos pero que en ocasiones se usan ilegalmente para conferir ciertas propiedades al producto; disolventes tóxicos, como el 1,4-dioxano; y sustancias cancerígenas como nitrosaminas y formaldehído, que pueden encontrarse en el producto acabado a causa de reacciones secundarias o por liberarse a partir de otros ingredientes.

SECCIÓN II

Resumen de los resultados experimentales

Capítulo 3

Desarrollo de un método rápido y sensible para la determinación de cannabidiol en productos cosméticos mediante cromatografía de líquidos acoplada a espectrometría de masas en tándem

El contenido de este capítulo ha sido publicado en el artículo:
A rapid and sensitive method for the determination of cannabidiol in cosmetic products by liquid chromatography–tandem mass spectrometry
L. Schettino, M. Prieto, J.L. Benedé, A. Chisvert, A. Salvador, **Cosmetics** 8 (2021) 30

INTRODUCCIÓN

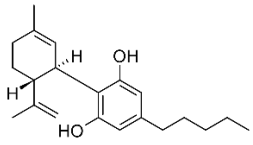
Objetivo

Desarrollar y validar un método analítico para la determinación de cannabidiol (CBD) en diferentes tipos de productos cosméticos y materias primas mediante cromatografía de líquidos acoplada a espectrometría de masas en tándem (LC-MS/MS).

Compuesto estudiado

- Cannabidiol (CBD)

Tabla 3.1. Información de interés sobre el compuesto estudiado

Nombre	Número CAS	Estructura química	M_r (g mol^{-1})	Log $K_{o/w}$
Cannabidiol	13956-29-1		314.5	6.5

Resumen

Se ha desarrollado y validado un método analítico para la determinación de CBD, un ingrediente activo con actividad seboestática y antioxidante, mediante LC-MS/MS tras lixiviar el analito de la matriz cosmética con etanol. Los bajos límites de detección (0.22 ng mL^{-1}) y cuantificación (0.74 ng mL^{-1}) alcanzados permiten la determinación de CBD a nivel de trazas sin necesidad de una etapa de preconcentración. A su vez, el amplio intervalo dinámico lineal del método también permite la determinación de CBD en concentración altas sin necesidad de una elevada dilución. El método se aplicó con

éxito al análisis de seis productos cosméticos y una materia prima empleada en la fabricación de productos cosméticos con CBD.

PROCEDIMIENTO EXPERIMENTAL

Instrumentación

- → Cromatógrafo de líquidos 1100 Series de Agilent Technologies, equipado con un desgasificador, una bomba cuaternaria, un inyector automático y un horno para la columna, acoplado a un detector Agilent 6410B Triple Quad MS/MS
- → Agitador vórtex ZX3 de VELP Scientifica
- → Generador de nitrógeno NiGen LCMS 40-1 de Claind

Reactivos

- → CBD (1.0 mg mL^{-1} en metanol) como patrón
- → CBD-d3 (100 µg mL^{-1} en metanol) como patrón interno
- → Etanol y agua ultrapura como disolventes en la preparación de muestras y patrones
- → Ácido fórmico, metanol grado-MS y agua grado-MS como disolventes para la preparación de la fase móvil
- → Nitrógeno empleado como nebulizador y gas de cortina en la fuente de iones del MS/MS

Muestras

Se analizaron cuatro cremas hidratantes (muestras A-D), un gel de ducha (muestra E) y una mascarilla capilar (muestra F) como muestras de productos cosméticos, y un aceite de semillas de *Cannabis sativa L.* estandarizado al 1.3% en CBD como materia prima (muestra G).

Según las etiquetas de los productos seleccionados, las cuatro cremas y la materia prima contenían CBD entre los ingredientes, mientras que el gel de ducha y la mascarilla capilar indicaban el aceite de semillas de *Cannabis sativa L.* entre los ingredientes, sin mencionar la presencia de CBD.

Método propuesto

Preparación de patrones

Mediante diluciones sucesivas de la disolución patrón madre de CBD de 1.0 mg mL^{-1}, se preparó una disolución de CBD de 100 ng mL^{-1} en etanol. A partir de esta, se prepararon disoluciones patrón de trabajo de diferentes concentraciones de CBD (de 1 a 30 ng mL^{-1}), diluyendo con los volúmenes apropiados de etanol y agua para obtener una disolución final etanol:agua 1:1 (v/v). Las disoluciones patrón de trabajo también contenían el volumen apropiado de una disolución de CBD-d3 de 200 ng mL^{-1} en etanol para obtener una concentración de patrón interno de 8 ng mL^{-1}. Las disoluciones obtenidas se trasvasaron a viales de inyección para el análisis cromatográfico.

Preparación de muestras

Se pesó 1 g de muestra, bien de producto cosmético o de materia prima, en un matraz aforado, se llevó a un volumen final de 10 mL con etanol, y se facilitó la lixiviación del CBD mediante un agitador vórtex. Las disoluciones de muestra obtenidas se filtraron a través de filtros de nylon con un tamaño de poro de 0.45 µm. A continuación, se tomó 1 mL de esta disolución inicial, o un volumen menor si la concentración de CBD en la muestra era relativamente alta, y se transvasó a un matraz aforado de 10 mL, donde se enrasó con los volúmenes apropiados de etanol y agua para obtener una disolución final etanol:agua 1:1 (v/v). Al igual que en las disoluciones patrón, las disoluciones de muestra contenían el volumen apropiado de una disolución de CBD-d3 de 200 ng mL^{-1} en etanol para conseguir una

concentración de patrón interno de 8 ng mL^{-1}. Las disoluciones de muestra obtenidas se trasvasaron a viales de inyección para el análisis cromatográfico.

Análisis cromatográfico

El análisis se llevó a cabo por LC-MS/MS, y las variables instrumentales del método propuesto se resumen en la *Tabla 3.2*.
La medida de los analitos se llevó a cabo mediante ionización por electronebulización (ESI, del inglés *electrospray ionization*) en modo positivo y por monitorización de reacciones múltiples (MRM, del inglés *multiple reaction monitoring*).

Tabla 3.2. Variables instrumentales del equipo LC-MS/MS

LC	Volumen de inyección	10 µL					
	Columna	Agilent Zorbax SB-C18 (50 mm x 2.1 mm; 1.8 µm)					
	Temperatura del horno	35 ºC					
	Caudal	0.2 mL min^{-1}					
	Modo	Isocrático					
	Fase móvil	Metanol (0.1% ácido fórmico):agua (0.1% ácido fórmico) 80:20 (v/v)					
MS/MS	Ion precursor	**315 (CBD)**			**318 (CBD-d3)**		
	m/z	193 [a]	41	123	196 [a]	41	123
	Fragmentor	132 V	132 V	132 V	114 V	114 V	114 V
	Energía de colisión	18 V	70 V	34 V	18 V	70 V	34 V
	Voltaje del capilar (ESI+)	3 kV					
	Temperatura del gas	310 °C					
	Caudal del gas	12 L min^{-1}					
	Presión del nebulizador	50 psi					

[a] Fragmento de cuantificación

RESULTADOS

Estudio de las variables instrumentales en la espectrometría de masas

En primer lugar, se realizó la optimización y selección de las transiciones de los iones precursor→producto y de la fuente de ionización mediante el software *MassHunter Optimizer* y *Source Optimizer*, respectivamente.

Las variables relacionadas con el detector se estudiaron inyectando una disolución patrón de CBD y una disolución de CBD-d3, ambas de 1 μg mL^{-1} y en proporción 1:1 etanol:agua. Se estudiaron las transiciones de los iones precursor→producto generadas a partir de los dos iones precursores, y luego se optimizaron los valores del fragmentor y la energía de colisión para cada uno de los iones producto.

Los valores optimizados para cada variable se han mostrado en la *Tabla 3.2*.

Estudio de la etapa de preparación de muestra

La preparación de la muestra no requiere de ningún paso previo de preconcentración del analito, ya que se observó que era suficiente con lixiviar el analito de la matriz en etanol, favoreciendo el proceso mediante agitación con vórtex. A continuación, se separó la fracción insoluble por filtración a través de filtros de nylon de 0.45 μm de tamaño de poro, para evitar que posibles partículas sólidas llegasen a la columna. Las disoluciones filtradas se diluyeron con agua para obtener disoluciones de etanol:agua 1:1 (v/v), ya que de lo contrario se provocaba desdoblamiento de los picos cromatográficos al presentar el disolvente de inyección (etanol) una fuerza de elución muy superior a la fase móvil.

Parámetros analíticos del método propuesto

Linealidad

Se estudió la linealidad con disoluciones patrón que contenían concentraciones de CBD entre 0.75 y 200 ng mL^{-1} y una concentración fija de CBD-d3 de 8 ng mL^{-1}. Se empleó el cociente de áreas entre el pico del analito y el pico del patrón interno como señal analítica, y se estimó la linealidad mediante el coeficiente de determinación (R^2). Se obtuvo un valor de 0.9992, lo que demostró un intervalo dinámico lineal amplio y por tanto adecuado para determinar concentraciones a niveles relativamente bajos y altos.

Límites de detección y cuantificación

Los límites de detección y cuantificación instrumentales se calcularon como tres y diez veces, respectivamente, la relación señal/ruido de un patrón, mientras que los límites de detección y cuantificación del método se calcularon teniendo en cuenta el procedimiento de preparación de la muestra.

Los valores obtenidos se resumen en la *Tabla 3.3*, y demuestran que con el método propuesto es posible determinar el CBD en productos cosméticos a nivel de trazas sin necesidad de pasos previos de preconcentración.

Tabla 3.3. Límites de detección y cuantificación

LOD[a] (ng mL^{-1})	LOQ[a] (ng mL^{-1})	MLOD[b] (ng g^{-1})	MLOQ[b] (ng g^{-1})
0.22	0.74	22	74

[a] LOD: límite de detección instrumental; LOQ: límite de cuantificación instrumental

[b] MLOD: límite de detección del método; MLOQ: límite de cuantificación del método

Repetibilidad

La repetibilidad de las medidas, expresada como desviación estándar relativa (RSD, del inglés *relative standard deviation*), se evaluó aplicando el método propuesto a cinco réplicas de dos disoluciones patrón que contenían diferentes niveles de concentración de CBD (2 y 4 ng mL^{-1}) en una misma sesión de trabajo (repetibilidad intra-día) y en cinco sesiones de trabajo distintas (repetibilidad inter-día).

Los resultados obtenidos, que se muestran en la *Tabla 3.4*, indican que se logró una buena repetibilidad (RSD < 8.5%).

Tabla 3.4. Estudio de la repetibilidad

RSD (%) [a]			
Intra-día (N^b = 5)		**Inter-día (N^b = 5)**	
2 ng mL^{-1}	**4 ng mL^{-1}**	**2 ng mL^{-1}**	**4 ng mL^{-1}**
4.6	5.1	5.9	8.5

[a] desviación estándar relativa
[b] N: número de réplicas

Análisis de muestras

El método propuesto se aplicó al análisis de seis productos cosméticos disponibles comercialmente (que contenían CBD o aceite de semillas de *Cannabis sativa L.* entre sus ingredientes) y una materia prima (aceite de semillas de *Cannabis sativa L.* con una cantidad conocida de CBD). Los resultados, que se muestran en la *Tabla 3.5*, revelan que se cuantificó CBD en la materia prima y en las muestras cosméticas en las que se indicaba CBD como ingrediente en la etiqueta, mientras que no se detectó CBD en aquellas muestras que contenían aceite de semillas de *Cannabis sativa L.* como ingrediente.

Para evaluar la exactitud del método, se fortificaron las seis muestras cosméticas analizadas a dos niveles de concentración distintos (2 y 4 ng mL^{-1}) en las disoluciones de medida. Se calculó el coeficiente de recuperación relativa para las seis muestras, y los valores obtenidos, que también se muestran en la *Tabla 3.5*, oscilaron entre 100 y 114%, lo que prueba que el efecto matriz era despreciable.

Tabla 3.5. Aplicación del método al análisis de muestras comerciales

Muestra[a]	**Cantidad fortificada**[b] **(µg g^{-1})**	**Cantidad encontrada**[b] **(µg g^{-1})**	**R (%)**[b]
A	-	140 ± 8	-
	19 ± 1	159 ± 2	101 ± 4
	40 ± 2	186 ± 4	114 ± 16
B	-	316 ± 8	-
	34 ± 3	352 ± 3	105.6 ± 1.2
	72 ± 9	395 ± 14	109.0 ± 1.1
C	-	2060 ± 50	-
	189 ± 16	2250 ± 16	100 ± 5
	354 ± 14	2416 ± 40	100 ± 7
D	-	341 ± 17	-
	61 ± 18	409 ± 14	113 ± 8
	110 ± 30	480 ± 50	109 ± 11
E	-	< LOD	-
	0.044 ± 0.001	0.045 ± 0.010	100 ± 2
	0.087 ± 0.001	0.088 ± 0.004	100 ± 5
F	-	< LOD	-

	120 ± 40	130 ± 30	110 ± 11
	410 ± 150	510 ± 110	101 ± 13
G	-	1304 ± 14	-

[a] A, B, C y D: Cremas hidratantes conteniendo CBD como ingrediente; E: Gel de ducha conteniendo aceite de semillas de Cannabis sativa L. como ingrediente; F: Mascarilla capilar conteniendo aceite de semillas de Cannabis sativa L. como ingrediente; G: Aceite de semillas de Cannabis sativa L. estandarizado conteniendo 1300 μg g^{-1} de CBD

[b] coeficiente de recuperación relativa, expresado como media ± desviación estándar de tres réplicas

A modo de ejemplo, en la *Figura 3.1* y en la *Figura 3.2* se muestran los cromatogramas obtenidos aplicando el método propuesto a una muestra de crema (muestra C) y a la materia prima (muestra G), respectivamente.

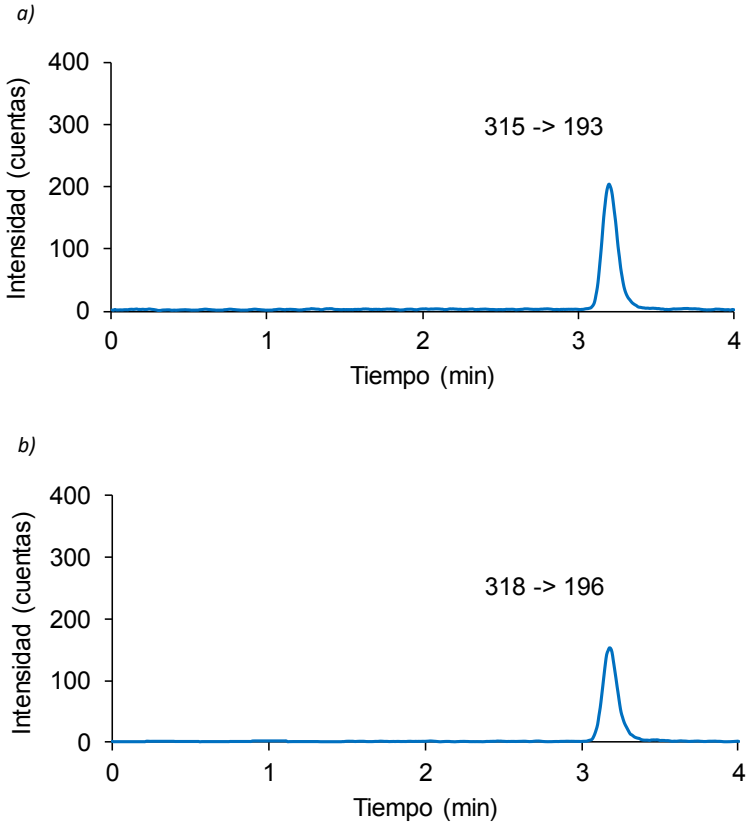


Figura 3.1. *Cromatogramas obtenidos aplicando el método propuesto a una crema cosmética (muestra C) ((a) CBD, (b) CBD-d3).*

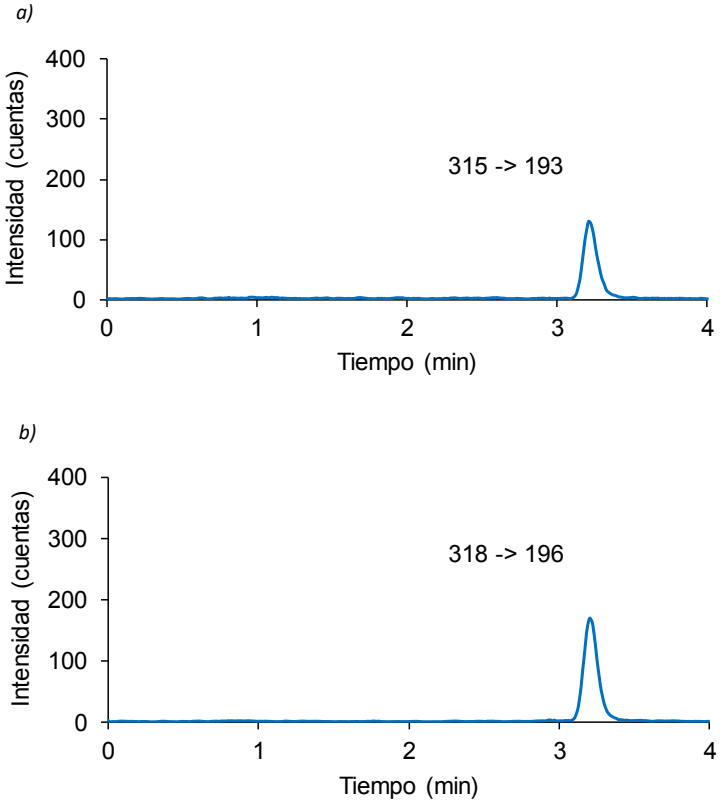


***Figura 3.2**. Cromatogramas obtenidos aplicando el método propuesto a la materia prima (muestra G) ((a) CBD, (b) CBD-d3).*

CONCLUSIONES

En este capítulo:

Se ha desarrollado y validado un nuevo método analítico para la determinación de CBD en productos cosméticos y materias primas.

- → El método propuesto se basa en la determinación directa del analito mediante LC-MS/MS
- → El método analítico propuesto es de simple ejecución y presenta una elevada sensibilidad, ya que permite la determinación de CBD a niveles de trazas. Además, el amplio rango de linealidad del método permite la determinación de CBD también en muestras con concentraciones relativamente elevadas
- → El método propuesto se ha aplicado al análisis de productos cosméticos acabados, con matrices hidrofílicas y lipofílicas, y a una materia prima
- → Las características analíticas del método y los resultados obtenidos demuestran su idoneidad para el control de calidad de los productos cosméticos que contengan CBD, asegurando así que su concentración en el producto final sea la deseada
- → El método propuesto es inocuo para el operador y el medio ambiente, de acuerdo con los principios de la Química Analítica Verde, ya que no se utilizan disolventes orgánicos tóxicos durante la preparación de las muestras

Capítulo 4

Desarrollo de un método analítico para la determinación de trazas de acrilamida en productos cosméticos basado en microextracción líquido-líquido dispersiva seguida de cromatografía de líquidos con detección espectrofotométrica

El contenido de este capítulo ha sido publicado en el artículo:
Development of a sensitive method for determining traces of prohibited acrylamide in cosmetic products based on dispersive liquid-liquid microextraction followed by liquid chromatography-ultraviolet detection.
L. Schettino, J.L. Benedé, A. Chisvert, A. Salvador, **Microchemical Journal** 159 (2020) 105402

Y presentado como cartel en la XXII Reunión de la Sociedad Española de Química Analítica, Valladolid, 2019

INTRODUCCIÓN

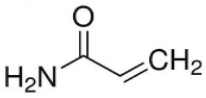
Objetivo

Desarrollar, por primera vez, un método analítico para la determinación de acrilamida en productos cosméticos. El método se basa en una microextracción líquido-líquido dispersiva (DLLME) como etapa de preconcentración seguida de la determinación por cromatografía de líquidos con detección espectrofotométrica (LC-UV).

Compuesto estudiado

- Acrilamida

Tabla 4.1. Información de interés sobre el compuesto estudiado

Nombre	Número CAS	Estructura química	M_r (g mol^{-1})	Log $K_{o/w}$
Acrilamida	79-06-1		71.08	-0.56

Resumen

En este trabajo se presenta por primera vez un método analítico para la determinación de trazas de acrilamida en productos cosméticos. El método se basa en una etapa de preconcentración por DLLME, seguida del análisis por LC-UV. Previa a la microextracción, se realizó una extracción líquido-líquido (LLE) con hexano para eliminar los componentes lipofílicos de la matriz. A continuación, se realizó una derivatización de la acrilamida con 2-naftalentiol para obtener un compuesto más lipofílico, y por tanto extraíble con el disolvente apolar

empleado en DLLME, a la vez que se añadió un grupo cromóforo que permitió su medida mediante LC-UV a longitudes de onda mayores, evitando así las posibles interferencias que podrían darse a bajas longitudes de onda. Se estudiaron las principales variables involucradas tanto en la etapa de derivatización como en la de extracción para proporcionar los mejores factores de preconcentración.

El método fue validado con éxito mostrando una buena linealidad (de 10 ng mL^{-1} a 20 µg mL^{-1}), un factor de preconcentración de 103 ± 2, límites de detección y cuantificación de 3.0 y 9.8 ng mL^{-1}, que corresponden respectivamente a 0.7 y 2.4 µg kg^{-1} en la muestra, y una buena repetibilidad (RSD < 14%).

Finalmente, el método analítico propuesto se aplicó a muestras de productos cosméticos comerciales de diferente naturaleza, obteniéndose coeficientes de recuperación cuantitativos (85 – 112%), lo que demuestra un efecto matriz insignificante.

Reacción de derivatización

La reacción de derivatización propuesta y optimizada en el presente trabajo es la adición tiol-Michael catalizada por una base. Se empleó 2-naftalentiol [240] como reactivo derivatizante, que se muestra en la *Figura 4.1*.

En esta reacción, la presencia de una base facilita la adición de un grupo tiol a un compuesto carbonílico α,β-insaturado para formar un producto de adición de tipo tioéter (nuevo enlace C-S). Para ello, la base toma el protón del grupo tiol del 2-naftalentiol, generando un anión tiolato que, al ser un nucleófilo fuerte, ataca el carbono beta de la acrilamida, deficiente en electrones. Se genera así un intermedio aniónico que, al ser una base fuerte, toma de nuevo el protón, formando el tioéter (3-(2-naftiltio)propanamida) como producto final y la base regenerada.

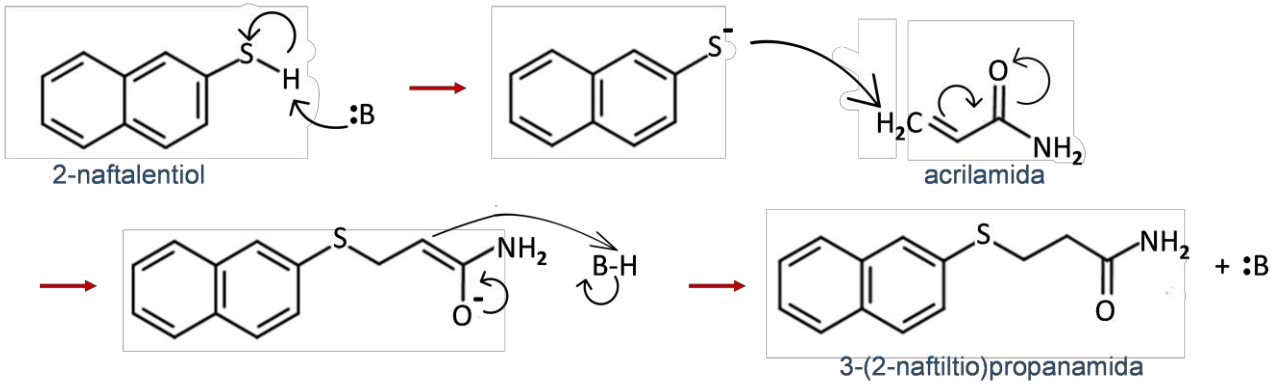


Figura 4.1. *Reacción de derivatización llevada a cabo en el método propuesto: adición tiol-Michael catalizada por una base entre acrilamida y 2-naftalentiol*

PROCEDIMIENTO EXPERIMENTAL

Instrumentación

- → Cromatógrafo de líquidos 1220 Infinity de Agilent Technologies, compuesto por un desgasificador, una bomba programable, un inyector automático, un horno de columna termostatizado y un detector UV
- → Horno de microondas MicroSYNTH de Milestone con reactores de PTFE de 70 mL
- → Concentrador de muestras Stuart SBHCONC/1 de Cole-Parmer
- → Agitador vórtex ZX3 de VELP Scientifica S.r.l.
- → Centrífuga EBA 21 Hettich®
- → Medidor de pH Basic 20 de Crison Instruments S.A.
- → Generador de nitrógeno NiGen LCMS 40-1 de Claind S.r.l.

Reactivos

- → Acrilamida ≥ 99% como patrón
- → Agua ultrapura como disolvente en la preparación de muestras y patrones
- → Cloruro de sodio (NaCl) grado reactivo 99.5% para ajustar la fuerza iónica de las disoluciones
- → Hexano grado-LC 96% como fase orgánica en la LLE
- → 2-naftalentiol ≥ 99% como agente derivatizante
- → Tetraborato de sodio para crear el medio básico en la reacción de derivatización
- → Acetona ultrapura como disolvente dispersante y cloroformo grado-reactivo como disolvente de extracción en la DLLME
- → Nitrógeno 99% para la evaporación de los extractos
- → Etanol grado-LC y agua ultrapura como disolventes para la preparación de la fase móvil

Muestras

Se analizaron dos geles hidrofílicos, uno exfoliante corporal (muestra A) y uno para piernas cansadas (muestra B), y una crema fotoprotectora lipofílica (muestra C). Todos ellos contenían derivados de la poliacrilamida en su listado de ingredientes.

Método propuesto

Preparación de patrones y muestras

Se preparó una disolución madre de 500 µg mL^{-1} de acrilamida en agua. A continuación, una alícuota de esta disolución se diluyó con agua para preparar una disolución patrón intermedia de 20 µg mL^{-1} que se usó para preparar las disoluciones acuosas de trabajo (10–200 ng mL^{-1}), que se ajustaron al 2% de NaCl (m/v).

En cuanto a la preparación de la muestra, se pesaron entre 0.15 y 0.5 g en matraces aforados de 10 mL, enrasando con agua. Para favorecer la dispersión de la muestra, se agitó con un agitador vórtex durante 5 min, formando una emulsión que sucesivamente se rompió añadiendo 0.2 g de NaCl.

A continuación, se colocaron 6.5 mL de la disolución acuosa, ya fuera patrón o muestra, en un tubo de vidrio con 3.5 mL de hexano. De este modo se llevó a cabo la LLE, mezclando la fase acuosa y la fase orgánica con vórtex durante 30 s, y luego separándolas por centrifugación a 6000 rpm durante 10 min. Finalmente, 5 mL de la fase acuosa inferior se transvasaron a un reactor de microondas de PTFE para llevar a cabo la reacción de derivatización.

Etapa de derivatización

Para llevar a cabo la reacción de derivatización, se adicionaron 250 µL de una disolución etanólica de 2-naftalentiol de 0.5 mg mL^{-1} (como agente derivatizante) y 250 µL de una disolución acuosa de tetraborato

de sodio de 4 mg mL^{-1} (como base) a todos los reactores de PTFE que contenían 5 mL de disolución patrón o muestra. Además, en el caso de las muestras, se preparó una disolución a la que, en lugar de 2-naftalentiol, se le añadieron 250 µL de agua desionizada para, de esa manera, no producir la reacción de derivatización (en adelante se hará referencia a esta réplica como la disolución de muestra no derivatizada).

A continuación, se colocaron los reactores de PTFE en el horno de microondas a una potencia de 500 W y se aplicó el siguiente programa de temperatura: de temperatura ambiente a 70 °C en 1 min, temperatura constante a 70 ºC durante 10 min. A continuación, se dejó enfriar durante 10 min a temperatura ambiente y la mezcla de reacción de cada reactor de PTFE se recolectó y transvasó a un tubo de vidrio de 15 mL para realizar la etapa de DLLME.

Etapa de DLLME

Se prepararon mezclas de 250 µL de acetona (como disolvente dispersante) y 80 µL de cloroformo (como disolvente de extracción) y se inyectaron con una jeringa en cada una de las disoluciones a extraer, formando instantáneamente las correspondientes microemulsiones. A continuación, los tubos de vidrio se centrifugaron a 6000 rpm durante 5 min, y las fases sedimentadas (aproximadamente 50 µL) se recolectaron usando una jeringa Hamilton 1705 RNR de 100 µL. Los extractos se trasvasaron a insertos de 200 µL situados dentro de viales de inyección de 1.5 mL, y se evaporaron hasta sequedad a temperatura ambiente bajo corriente de nitrógeno. Finalmente, se reconstituyeron en 30 µL de una mezcla de etanol:agua 50:50 (v/v) y se analizaron mediante LC-UV.
La *Figura 4.2* muestra un esquema de todo el procedimiento experimental.

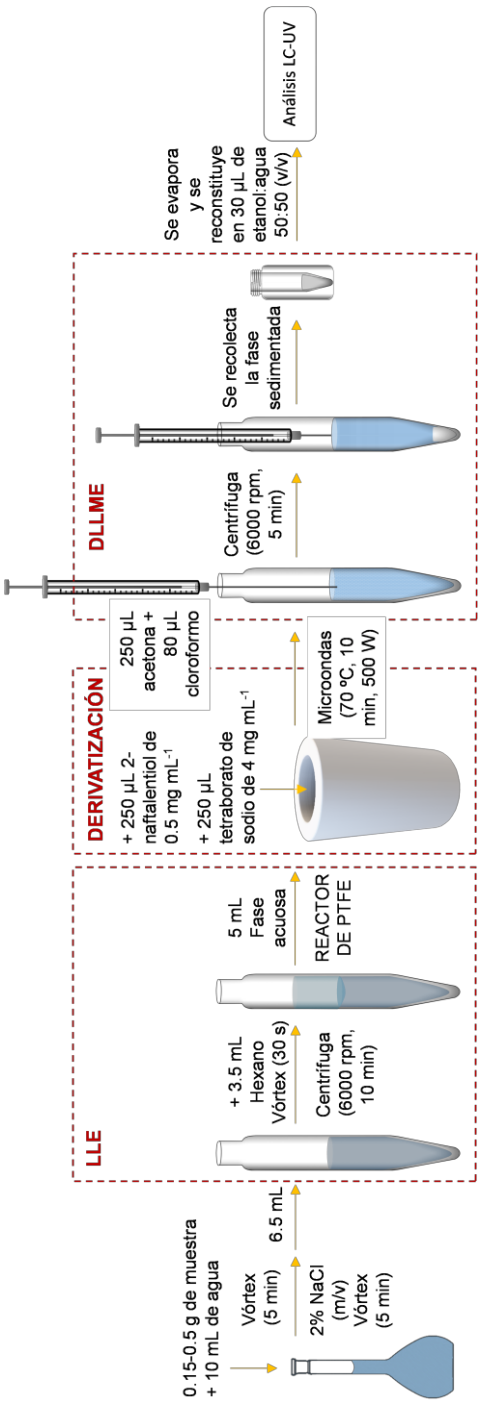


***Figura 4.2.** Esquema del método propuesto*

Análisis cromatográfico

En la Tabla 4.3 se describen los valores para las variables instrumentales del equipo de LC-UV.

Tabla 4.3. Variables instrumentales del equipo LC-UV

LC	Volumen de inyección	20 µL		
	Columna	Purospher® RP-18 (125 mm x 4 mm; 5 µm)		
	Temperatura del horno	25 ºC		
	Caudal	1 mL min^{-1}		
	Modo	Gradiente		
	Fase móvil	**t (min)**	**Etanol (%)**	**Agua (%)**
		0	50	50
		3	50	50
		4	100	0
		5	100	0
		5.5	50	50
		8	50	50
UV	Longitud de onda	254 nm		

RESULTADOS

Estudio de las variables experimentales involucradas en la etapa de derivatización

Existen diferentes variables que pueden afectar a la reacción de derivatización, como la temperatura, el tiempo, la concentración del reactivo derivatizante y el pH del medio. Con el fin de mejorar la sensibilidad del método, estas variables fueron estudiadas con una estrategia univariante, empleando 5 mL de una disolución patrón de acrilamida de 20 μg mL^{-1}.

Cada variable se evaluó por triplicado, añadiendo 250 μL de una disolución etanólica de 2-naftalentiol, como agente derivatizante, y 250 μL de una disolución acuosa de tetraborato de sodio (4 mg mL^{-1}), como regulador del pH.

Después de cada experimento de derivatización, la mezcla de reacción se sometió a la etapa de DLLME utilizando 80 μL de cloroformo como disolvente de extracción y 500 μL de acetona como disolvente dispersante. Posteriormente, el extracto orgánico se trasvasó a un inserto de vidrio ubicado en un vial de inyección, que fue evaporado hasta sequedad bajo una corriente de nitrógeno.

Finalmente, cada extracto se reconstituyó con 30 μL de una mezcla de etanol:agua 50:50 (v/v), y se analizó mediante LC-UV, donde el área del pico cromatográfico de la acrilamida derivatizada ($A_{derivatizada}$) fue la función respuesta.

Temperatura de derivatización

Se estudió la influencia de la temperatura en la reacción de derivatización realizando diferentes pruebas a través del programa de temperatura del horno de microondas.

Se estudió la temperatura de reacción a 50, 60, 70 y 80 °C empleando una rampa de 1 min y manteniéndola constante durante 20 min. No se probaron temperaturas más altas debido al riesgo de polimerización de la acrilamida por encima de los 80 °C [241]. Para llevar a cabo la reacción, se utilizó una concentración de 2-naftalentiol de 2 mg mL^{-1} y no se efectuó ningún ajuste del pH del medio más allá del proporcionado por la base (tetraborato), que venía a ser aproximadamente 9.5.

Como se muestra en la *Figura 4.3*, la señal aumentó con la temperatura hasta 70 °C. Más allá de este valor, la señal disminuyó probablemente a causa de la polimerización de la acrilamida [241]. Por lo tanto, se seleccionó 70 °C como temperatura de derivatización para los siguientes experimentos ya que, en igualdad de condiciones, la reacción de derivatización tiene lugar en mayor extensión.

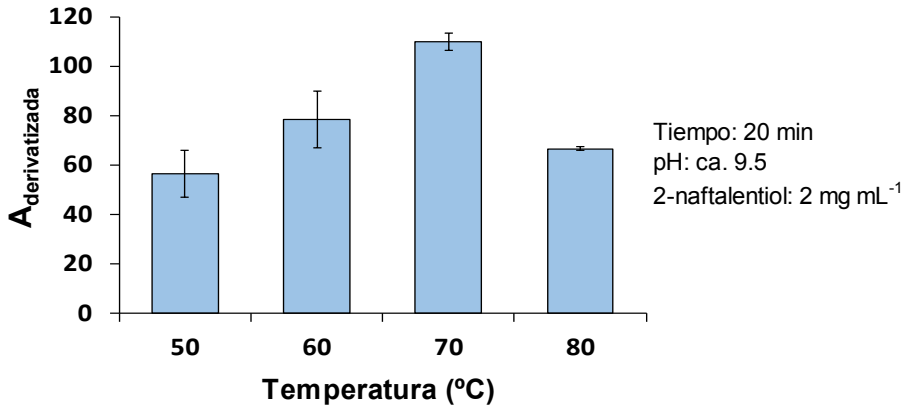


Figura 4.3. *Optimización de la temperatura de derivatización*

Tiempo de derivatización

De manera similar se estudió la influencia del tiempo de reacción, utilizando siempre una concentración de 2-naftalentiol de 2 mg mL^{-1} y

ningún ajuste del pH del medio más allá del proporcionado por la base (tetraborato), que venía a ser aproximadamente 9.5.

Se realizaron diferentes pruebas aumentando la temperatura a 70 °C en 1 min y luengo manteniéndola constante durante 5, 10, 20, 30 y 40 min.

Los resultados obtenidos, que se muestran en la *Figura 4.4,* ponen de manifiesto que la señal aumentaba hasta un tiempo de derivatización de 20 min, pero disminuía a valores más altos, quizás debido a la polimerización parcial de la acrilamida. Debido a que no se observó una diferencia significativa en la señal entre 10 y 20 min, se decidió seleccionar el valor de 10 min como tiempo de reacción para los siguientes experimentos, ya que se conseguía el mismo efecto, pero en menor tiempo.

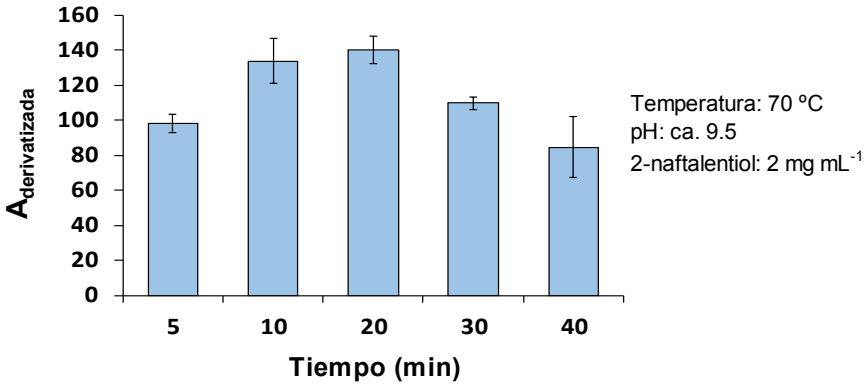


Figura 4.4. *Optimización del tiempo de derivatización*

pH del medio

Como se ha mencionado anteriormente, la reacción de derivatización es una adición de tiol-Michael catalizada por una base, lo que significa que requiere de un pH básico para llevarse a cabo.

La influencia del pH del medio se estudió con disoluciones acuosas de tetraborato de sodio de 4 mg mL^{-1}, ajustadas a los valores de pH: 8, 8.5, 9, 9.5 y 10.

Como se puede observar en la *Figura 4.5*, no hay una diferencia significativa entre los valores de pH de 9 a 10. Considerando que la solución acuosa de tetraborato de sodio de 4 mg mL^{-1} proporciona un valor de pH de aproximadamente 9.5 a la mezcla de reacción, se decidió continuar sin ajustar el pH para experimentos posteriores y trabajar al pH que proporcionaba la propia disolución de tetraborato.

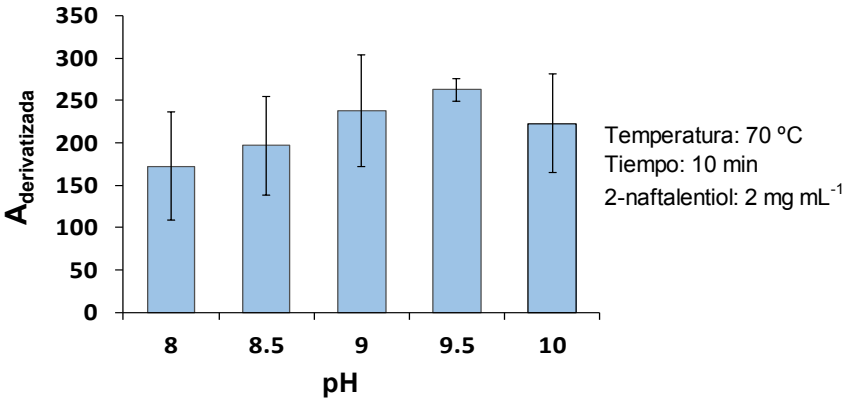


Figura 4.5. *Optimización del pH del medio en la reacción de derivatización*

Concentración del agente derivatizante

Finalmente, se estudió el efecto de la concentración del agente derivatizante (es decir, el 2-naftalentiol) sobre la señal analítica. En los experimentos anteriores se había utilizado una concentración de 2 mg mL^{-1}, pero el exceso de derivatizante en la mezcla de reacción provocaba una ligera turbidez que dificultaba el proceso DLLME. Por esta razón, se disminuyó esta concentración a valores inferiores (0.25, 0.5, 1 y 2 mg mL^{-1}).

Considerando los resultados obtenidos, que se muestran en la *Figura 4.6*, se seleccionó la concentración de 0.5 mg mL^{-1}, ya que permitía mantener una señal analítica suficientemente elevada sin aparición de turbidez, permitiendo seguir con las otras etapas del método sin dificultades.

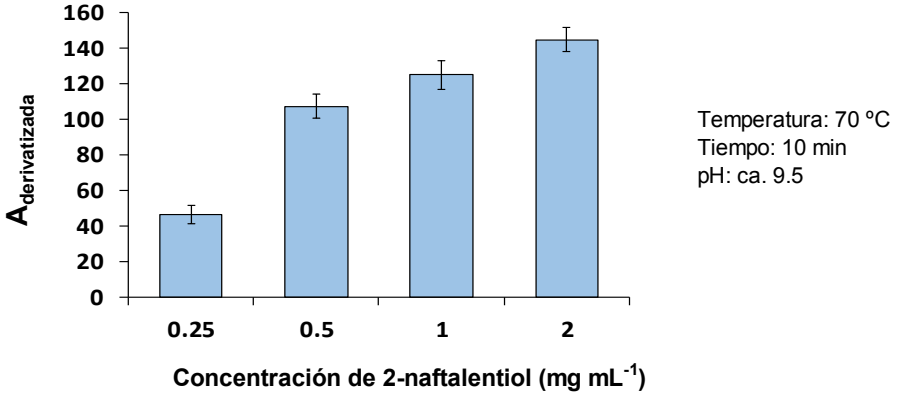


Figura 4.6. *Optimización de la concentración de agente derivatizante*

Estudio de las variables experimentales involucradas en la etapa de DLLME

Existen diferentes variables que pueden afectar al proceso de DLLME, como la naturaleza y el volumen de los disolventes de extracción y dispersante, y la fuerza iónica de la fase dadora. Para el estudio de estas variables, cada condición experimental se evaluó por triplicado utilizando 5 mL de una disolución patrón de acrilamida de 20 µg mL^{-1} sometida al proceso de derivatización antes optimizado.

Se evaluó la influencia de cada variable en base al factor de preconcentración (FP) alcanzado, definido como FP = C_{ext}/ C_0 (donde C_{ext} es la concentración de la acrilamida derivatizada en el extracto

final y C_0 es la concentración inicial de este compuesto en la fase acuosa antes de la extracción).

No se consideró como variable de estudio el tiempo de extracción ya que se define como el tiempo que transcurre entre la inyección de la mezcla de disolventes y el inicio de la centrifugación. Después de la formación de la microemulsión, el área superficial entre el disolvente de extracción y la fase dadora acuosa es tan elevada que el estado de equilibrio se logra rápidamente y, por lo tanto, el tiempo de extracción es tan corto que se puede considerar despreciable [242].

En el caso de compuestos potencialmente ionizables, también es importante optimizar el pH de la fase dadora, pero no es el caso de la acrilamida derivatizada, que no se ve influenciada por el pH, por lo que esta variable tampoco se consideró en el estudio.

Naturaleza de los disolventes de extracción y dispersante

Las propiedades recomendadas para el disolvente de extracción son que sea inmiscible en agua y que tenga mayor densidad que esta, para que cuando se separen las fases después de la centrifugación sedimente en la punta cónica del tubo para ser recogido con más facilidad. En el caso del disolvente dispersante, debe ser miscible en ambas fases para lograr una buena dispersión del disolvente de extracción en la fase dadora. Por tanto, es muy importante encontrar la combinación adecuada de disolvente de extracción y dispersante para llevar a cabo el proceso DLLME.

En este sentido, se ensayaron diferentes combinaciones de diclorometano y cloroformo (como disolventes de extracción) y etanol y acetona (como disolventes dispersantes). No se logró la separación de fases con la combinación cloroformo-etanol, mientras que no se observó la formación de la dispersión cuando se usó el diclorometano. Por tanto, se seleccionó la combinación cloroformo-acetona para los siguientes experimentos.

Volumen de los disolventes de extracción y dispersante

En primer lugar, se estudió la influencia del volumen del disolvente de extracción, realizando la DLLME con mezclas de diferentes volúmenes de cloroformo, comprendidos entre 60 y 90 µL, y 500 µL de acetona.

De acuerdo con la *Figura 4.7*, la respuesta aumentó con el volumen de cloroformo hasta 80 µL y luego disminuyó al usar un volumen mayor, ya que la dispersión no se formó correctamente, sino que se formaron gotas grandes del disolvente de extracción, las cuales sedimentaron rápidamente. Por lo tanto, se seleccionó un volumen de 80 µL para el disolvente de extracción.

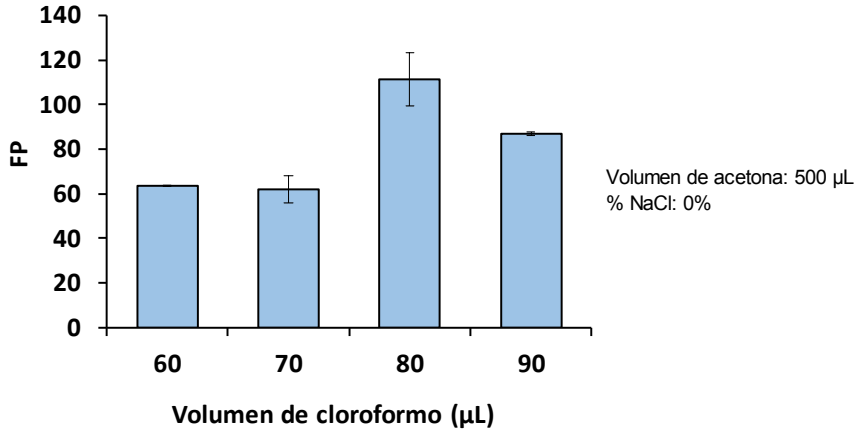


Figura 4.7. *Optimización del volumen de disolvente de extracción*

A continuación, se estudió la influencia del volumen del disolvente dispersante, realizando la DLLME con mezclas de 80 µL de cloroformo con diferentes volúmenes de acetona, comprendidos entre 125 y 750 µL.

De acuerdo con la *Figura 4.8*, la señal más alta se obtuvo para 250 µL, mientras que a volúmenes más altos de disolvente dispersante

disminuyó el FP, probablemente a causa de la disminución del coeficiente de partición del analito en el disolvente de extracción [242].

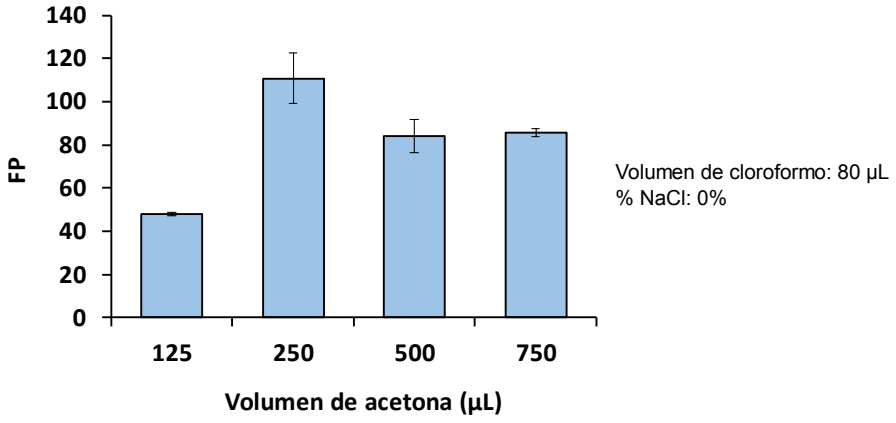


Figura 4.8. *Optimización del volumen de disolvente dispersante*

Fuerza iónica de la fase dadora

Generalmente, el aumento de la fuerza iónica conduce a una disminución de la solubilidad tanto del analito no polar como del disolvente orgánico de extracción en la fase acuosa debido al efecto salino. La influencia de esta variable se estudió añadiendo diferentes concentraciones de NaCl a la fase dadora, comprendidas entre 0 y 5% (m/v), antes de realizar la DLLME.

Como se puede ver en la *Figura 4.9*, el FP aumentó ligeramente con NaCl al 1%, pero la adición de cantidades más altas de sal tuvo un efecto negativo en el FP de la acrilamida derivatizada. Este efecto negativo podría deberse al aumento de la viscosidad y, por tanto, a una mayor dificultad en la transferencia de masa del analito desde la fase dadora a la fase de extracción [243]. Además, también aumenta

el volumen de la fase sedimentada, produciendo así una dilución de los analitos.

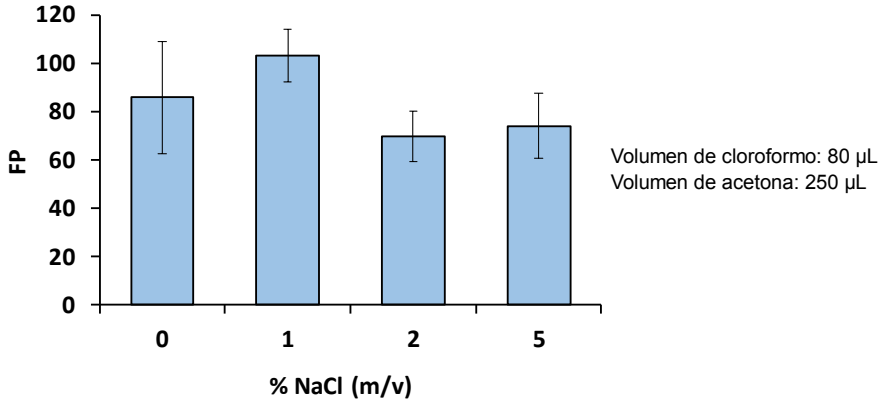


Figura 4.9. *Optimización de la fuerza iónica de la fase dadora*

Estudio del pretratamiento de la muestra

Una vez optimizada la etapa de extracción, se analizaron muestras con diferente composición cosmética.

Teniendo en cuenta la alta solubilidad en agua de la acrilamida, y la matriz grasa que se encuentra muy frecuentemente en algunas muestras cosméticas, se consideró oportuno lixiviar la acrilamida de la matriz con agua. Se valoró la posibilidad de asistir la lixiviación por ultrasonidos, pero, mediante unas pruebas previas con una disolución patrón del analito de 20 µg mL^{-1}, se observó que la señal analítica disminuía a medida que aumentaba el tiempo de agitación, probablemente debido a la polimerización de la acrilamida. Por este motivo, se probó una alternativa mediante agitación con vórtex y, dado

que la señal no disminuía, se seleccionó este modo de agitación en lugar de la agitación con ultrasonidos para realizar la lixiviación.

Sin embargo, la agitación con vórtex provocaba que se formara una emulsión como consecuencia de la agitación vigorosa que promovía este agitador. Para resolver este problema, se añadió NaCl (hasta 2% m/v) para romper la emulsión formada, perturbando las capas de iones alrededor de las micelas y reduciendo el potencial zeta. Como consecuencia, se reajustó el % de NaCl en el método general de tal forma que, aunque disminuyese levemente el FP como se ha comprobado anteriormente, era posible realizar el procedimiento con las muestras.

Posteriormente, se agregó hexano para extraer los componentes lipofílicos que pudieran haberse co-lixiviado con la acrilamida, y se realizó así un paso de limpieza a través de una LLE. De este modo, se redujo la posibilidad de que otros compuestos lipofílicos presentes en la matriz pudieran ser arrastrados durante el procedimiento y perturbar la DLLME.

Parámetros analíticos del método propuesto

Linealidad

Se estudió la linealidad con disoluciones patrón de acrilamida sometidas al procedimiento de microextracción. Se obtuvo un alto nivel de linealidad, que alcanzó, al menos, hasta 20 µg mL^{-1}, aunque, teniendo en cuenta los bajos niveles de acrilamida esperados en las muestras, el rango de trabajo se fijó entre 10 y 200 ng mL^{-1}, obteniéndose un valor de coeficiente de determinación (R^2) de 0.9990.

Factor de preconcentración

El factor de preconcentración (FP) obtenido fue de 103 ± 2.

Límites de detección y cuantificación

Los límites de detección y cuantificación se calcularon como la concentración correspondiente a tres y diez veces, respectivamente, el valor de la relación señal/ruido de un patrón sometido al procedimiento de derivatización/extracción, mientras que los límites de detección y cuantificación del método se calcularon teniendo en cuenta además el procedimiento de preparación de la muestra.

Los valores obtenidos, que se indican en la *Tabla 4.2*, demuestran que el método propuesto permite detectar y cuantificar satisfactoriamente los valores umbral establecidos por el Reglamento Europeo de productos cosméticos para los residuos de acrilamida (0.1 mg kg^{-1} (100 µg kg^{-1}) en los productos cosméticos sin aclarado y 0.5 mg kg^{-1} (500 µg kg^{-1}) en el resto de los productos).

Tabla 4.2. Límites de detección y cuantificación

LOD[a] (ng mL^{-1})	LOQ[a] (ng mL^{-1})	MLOD[b] (µg kg^{-1})	MLOQ[b] (µg kg^{-1})
3.0	9.8	0.7	2.4

[a] LOD: límite de detección; LOQ: límite de cuantificación
[b] MLOD: límite de detección del método; MLOQ: límite de cuantificación del método

Repetibilidad

La repetibilidad, expresada como desviación estándar relativa (RSD), se evaluó aplicando el método propuesto a cinco réplicas de dos disoluciones patrón que contenía acrilamida a dos niveles de concentración (40 y 80 ng mL^{-1}), el mismo día (intra-día) y durante cinco días consecutivos (inter-día).

Los resultados obtenidos, que se muestran en la *Tabla 4.3*, indican que se logró una buena repetibilidad (RSD < 14%).

Tabla 4.3. Estudio de la repetibilidad

RSD (%)[a]			
Intra-día (N[b] = 5)		Inter-día (N[b] = 5)	
40 ng mL^{-1}	**80 ng mL^{-1}**	**40 ng mL^{-1}**	**80 ng mL^{-1}**
8.9	3.7	13.5	8.1

[a] desviación estándar relativa

[b] N: número de réplicas

Análisis de muestras

Se analizaron, utilizando el método propuesto, tres muestras cosméticas distintas que contenían poliacrilamidas en su formulación: un gel exfoliante corporal, un gel para piernas cansadas y una crema fotoprotectora).

Mediante el análisis de las disoluciones de muestras no derivatizadas mencionadas anteriormente, se verificó que algunas de las muestras mostraban un pico nativo en el tiempo de retención de la acrilamida derivatizada. En el caso de aparecer un pico nativo al mismo tiempo de retención de la acrilamida derivatizada, ha sido necesario substraer su señal a la señal de acrilamida derivatizada para no comprometer los resultados obtenidos.

Los resultados, que se muestran en la *Tabla 4.4*, revelaron que los contenidos de acrilamida encontrados fueron relativamente altos, considerando que esta sustancia está prohibida en los productos cosméticos, y que están incluso por encima de los valores umbral de seguridad.

Para realizar los estudios de recuperación y, por tanto, evaluar el efecto matriz, se fortificaron las tres muestras cosméticas a dos niveles de concentración de acrilamida. Los valores del coeficiente de

recuperación relativa obtenidos, que también se muestran en la *Tabla 4.4*, oscilaron entre 85 y 112%, demostrando que el efecto matriz era despreciable y, por lo tanto, se podía utilizar la calibración externa como se describe en el método propuesto.

Tabla 4.4. Aplicación del método propuesto al análisis de muestras comerciales

Muestra[a]	Cantidad fortificada[b] (mg kg^{-1})	Cantidad encontrada[b] (mg kg^{-1})	Recuperación relativa (%)[b]
	-	0.7 ± 0.1	-
A	1.7 ± 0.2	2.1 ± 0.2	85 ± 2
	3.3 ± 0.5	4.5 ± 0.9	109 ± 9
	-	1.5 ± 0.6	-
B	1.3 ± 0.1	3.0 ± 0.1	109 ± 8
	2.5 ± 0.1	4.3 ± 0.3	109 ± 7
	-	0.3 ± 0.1	-
C	1.8 ± 0.1	1.9 ± 0.1	98 ± 6
	3.2 ± 0.3	3.8 ± 0.6	112 ± 10

[a] A: gel exfoliante corporal; B: gel para piernas cansadas; C: crema fotoprotectora
[b] expresado como media ± desviación estándar de tres réplicas

A modo de ejemplo, la *Figura 4.10* muestra los cromatogramas de una muestra de gel sometida al método propuesto, tanto sin fortificar como fortificada con 2.5 mg kg^{-1}.

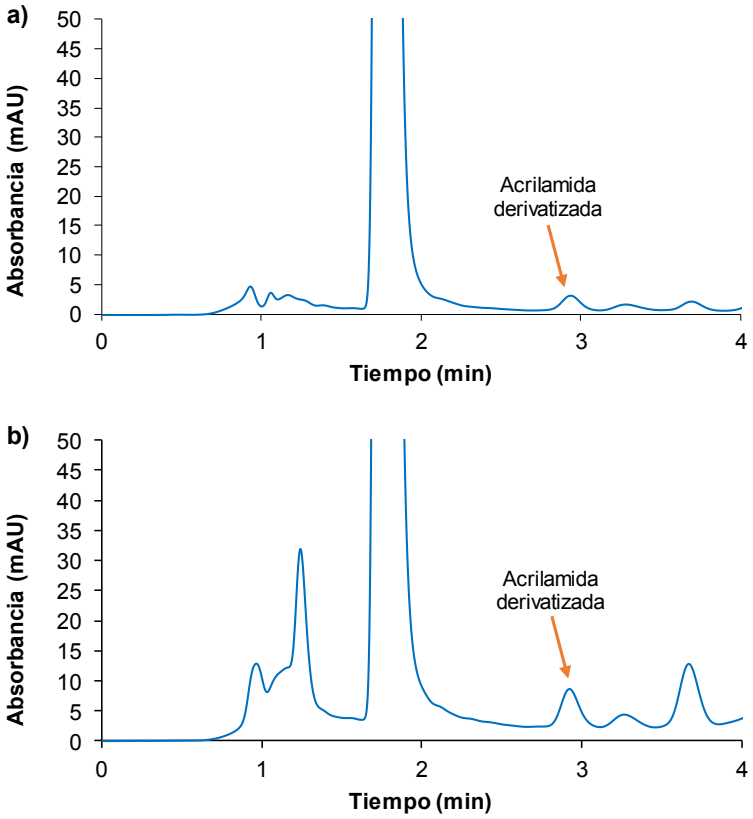


Figura 4.10. *Cromatogramas obtenidos aplicando el método DLLME-LC-UV propuesto a una muestra de gel: (a) sin fortificar y (b) fortificado a 2.5 mg* kg^{-1}

Comparación con otros métodos publicados anteriormente sobre la determinación de acrilamida

Cabe destacar que el método descrito en este capítulo es el primer método enfocado a la determinación de acrilamida en productos cosméticos. En este sentido, se compararon las características más relevantes del método propuesto con las de otros métodos publicados para la determinación de acrilamida en matrices distintas de las

cosméticas. Para llevar a cabo una comparación más objetiva, los métodos a comparar con el método propuesto se seleccionaron en base a las similitudes en el tratamiento de la muestra (es decir, etapa de derivatización y/o extracción por DLLME) y/o en la técnica instrumental (es decir, LC-UV).

Cabe decir que la combinación propuesta en este método, la derivatización con 2-naftalentiol con la DLLME, permite alcanzar un MLOD en el rango bajo de μg kg^{-1} a pesar de utilizar instrumentación de menor sensibilidad como LC-UV, siendo este un valor similar a los obtenidos usando instrumentos más sofisticados y costosos como LC-MS [53,54] o GC-MS [62].

A partir de los datos que se muestran en la *Tabla 4.5*, se concluye que la DLLME permite la extracción más rápida de la acrilamida (en su forma derivatizada) en comparación con otros métodos que utilizan otras técnicas de (micro)extracción. Aunque es cierto que los métodos que llevan a cabo una etapa de derivatización requieren de un tiempo de análisis adicional, con el método propuesto se logra una reducción considerable del tiempo de derivatización. Eso se debe al uso de microondas, que hace que el tiempo de reacción en el presente método sea más breve en comparación con la misma reacción de derivatización desarrollada en otros trabajos sin usar microondas [56,59]. En cualquier caso, los métodos analíticos reportados permiten la determinación de acrilamida a nivel de trazas, con coeficientes de recuperación relativa cuantitativos, a pesar de que algunos de ellos requieren calibración por adición de patrón para lograrlo [54,59]

Tabla 4.5. Comparación del método propuesto con otros métodos que determinan acrilamida en otro tipo de muestras

Muestra	Técnica de extracción[a]	Tiempo de extracción (min)	Técnica instrumental[b]	Agente derivatizante (tiempo de reacción)	MLOD[c]	Recuperación relativa (%)	Referencia
Productos cosméticos	DLLME	Instantáneo	LC-UV	2-naftalentiol (10 min)	0.7 μg kg^{-1}	85 - 112	Trabajo propuesto
Pan y galletas	DLLME	Instantáneo	LC-UV	2-naftalentiol (45 min)	n.r.[d]	90 - 96	[56]
Patatas fritas	DLLME	Instantáneo	GC-MS	Xantidrolo (40 min)	0.6 μg kg^{-1}	97	[62]
Café	DLLME	Instantáneo	LC-MS/MS	-	0.9 μg L^{-1}	97 - 106	[54]
Café	IL-DLLME	10	GC-MS	-	n.r.	n.r.	[70]
Patatas fritas	LLE	-	LC-FLD	2-naftalentiol (40 min)	129.2 μg kg^{-1}	105 - 108	[59]
Alimentos	LLE	10	LC-MS/MS	D-cisteina (50 min)	0.04 μg kg^{-1}	92 - 104	[53]
Espárragos	SLE	10	LC-UV	Ácido mercaptobenzoico (100 min)	25 μg kg^{-1}	107	[58]
Alimentos	SDME	5	GC-ECD	Ácido bromhídrico (15 min)	n.r.	97 - 104	[71]
Galletas	SPME	20	LC-UV	-	1.3 μg kg^{-1}	86 - 98	[57]
Comida china	SLE+SPE	30	LC-UV	-	8.0 μg kg^{-1}	89 - 103	[55]

[a] DLLME: microextracción líquido-líquido dispersiva; IL: líquido iónico; LLE: extracción líquido-líquido; SLE: extracción sólido-líquido; SDME: microextracción en gota; SPME: microextracción en fase sólida; SPE: extracción en fase sólida

[b] ECD: detector de captura de electrones; FLD: detección por fluorescencia; GC: cromatografía de gases; LC: cromatografía de líquidos; MS: espectrometría de masas; MS/MS: espectrometría de masas en tándem; UV: detección ultravioleta

[c] MLOD: Límite de detección del método; [d] n.r.: no reportado

CONCLUSIONES

En este capítulo:

Se ha propuesto el primer método analítico para la determinación de acrilamida en productos cosméticos.

- → El método propuesto se basa en una reacción de derivatización con 2-naftalentiol asistida por microondas, seguida por DLLME y análisis mediante LC-UV
- → El método propuesto se ha aplicado de manera eficiente al análisis de productos cosméticos acabados de distinta naturaleza química
- → El método propuesto presenta buenas características analíticas y, gracias a su elevada sensibilidad, permite detectar la acrilamida a nivel de trazas, en el rango bajo de μg kg^{-1}, a pesar de utilizar instrumentación de menor sensibilidad pero de bajo coste como LC-UV
- → El método es idóneo para el control de calidad de los productos cosméticos que contienen ingredientes que son potenciales liberadores de acrilamida, con el fin de garantizar la seguridad del usuario y el cumplimiento de la legislación vigente

Capítulo 5

Desarrollo de un método analítico para la determinación de trazas de acrilamida en productos cosméticos mediante microextracción líquido-líquido dispersiva en fase reversa asistida por vórtex y cromatografía de líquidos acoplada a espectrometría de masas en tándem

El contenido de este capítulo ha sido publicado en el artículo:
Trace determination of prohibited acrylamide in cosmetic products by vortex-assisted reversed-phase dispersive liquid-liquid microextraction and liquid chromatography-tandem mass spectrometry.
L. Schettino, A. García-Juan, L. Fernández-Lozano, J.L. Benedé, A. Chisvert, **Journal of Chromatography A** 1687 (2023) 463651

Y presentado como cartel en la XXI Reunión de la Sociedad Española de Cromatografía y Técnicas Afines, Almería, 2022

INTRODUCCIÓN

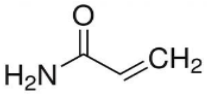
Objetivo

Desarrollar un método analítico para la determinación de trazas de acrilamida en productos cosméticos sin necesidad de etapas de derivatización como en el método presentado en el capítulo anterior.

Compuesto estudiado

- Acrilamida

Tabla 5.1. Información de interés sobre el compuesto estudiado

Nombre	Número CAS	Estructura química	M_r (g mol^{-1})	Log $K_{o/w}$
Acrilamida	79-06-1		71.08	-0.56

Resumen

Se propone un nuevo método analítico para la determinación de residuos de acrilamida en aquellos productos cosméticos que contienen ingredientes potencialmente liberadores de acrilamida. El método se basa en una etapa de VA-RP-DLLME, seguida de LC-MS/MS para su determinación.

Dada la elevada polaridad de la acrilamida, se optó por emplear DLLME en su modalidad en fase inversa (esto es, utilizando agua como fase de extracción), lo que permite extraer el analito evitando el paso de derivatización efectuado en el método presentado en el Capítulo 4. Finalmente, el análisis se realizó directamente por LC-

MS/MS, por lo que no es necesaria la introducción de un grupo cromóforo, lo que evita también la necesidad de la derivatización realizada en el capítulo anterior.

El método analítico propuesto se ha aplicado a muestras de productos cosméticos comerciales. El empleo de acrilamida-d3 como patrón interno corrige el efecto matriz, obteniéndose coeficientes de recuperación relativa cuantitativos (88 – 108%).

PROCEDIMIENTO EXPERIMENTAL

Instrumentación

- Cromatógrafo de líquidos 1100 Series de Agilent Technologies, equipado con un desgasificador, una bomba cuaternaria, un inyector automático y un horno para la columna acoplado a un detector Agilent 6410B Triple Quad MS/MS
- Agitador vórtex ZX3 de VELP Scientifica S.r.l.
- Centrífuga EBA 21 Hettich®
- Generador de nitrógeno NiGen LCMS 40-1 de Claind S.r.l.

Reactivos

- Acrilamida ≥ 99% como patrón
- Acrilamida-d3 (500 µg mL^{-1} en acetonitrilo) como patrón interno
- Acetonitrilo grado-HPLC para la preparación de las disoluciones madre de patrón
- Tolueno grado-reactivo para la preparación de las disoluciones patrón de trabajo de acrilamida y acrilamida-d3, y como disolvente de soporte en la etapa de VA-RP-DLLME
- Metanol grado-LC-MS para la preparación de las disoluciones de trabajo de acrilamida y acrilamida-d3 en el análisis de muestras
- Agua grado-LC-MS como fase aceptora
- Agua grado-LC-MS y metanol grado-LC-MS como disolventes y fluoruro de amonio (NH_4F) como tampón para la preparación de la fase móvil
- Nitrógeno empleado como nebulizador y gas de cortina en la fuente de iones del MS/MS

Muestras

Se analizaron cinco productos cosméticos, un gel revitalizante (muestra A), una leche desmaquillante (muestra B), un jabón líquido de manos (muestra C), un crema fotoprotectora (muestra D), y un gel de baño para bebés (muestra E). Estas muestras se seleccionaron porque contenían poliacrilamidas en su listado de ingredientes. Solo la muestra de gel de baño para bebés no mencionaba ningún polímero a base de acrilamida en su etiqueta.

Método propuesto

Preparación de patrones y muestras

Se preparó una disolución madre de 500 µg mL^{-1} de acrilamida en acetonitrilo. A partir de esta se preparó una disolución patrón intermedia de 5 µg mL^{-1} en tolueno y, a partir de esta última, se preparó una disolución patrón de trabajo de 50 ng mL^{-1} en este mismo disolvente.

En cuanto a la acrilamida-d3, se preparó una disolución patrón intermedia de 50 µg mL^{-1} en tolueno diluyendo la disolución comercial de 500 µg mL^{-1} y, a partir de esta, se preparó una disolución de trabajo de 100 ng mL^{-1} en tolueno. Adicionalmente, se preparó una disolución madre de acrilamida-d3 de 50 µg mL^{-1} en metanol y, a partir de esta, una disolución de trabajo de 100 ng mL^{-1} también en metanol.

A partir de las disoluciones de trabajo anteriores, se prepararon nueve disoluciones patrón en 5 mL de tolueno. Para ello se utilizaron tubos de vidrio de punta cónica de 15 mL y se adicionaron alícuotas de volumen creciente de la disolución de acrilamida, para obtener un rango de concentración de 0.005 a 5 ng mL^{-1}, y una alícuota constante de la disolución de patrón interno en tolueno, para obtener una concentración de 0.5 ng mL^{-1}. Finalmente se añadió tolueno hasta 5 mL.

En cuanto a la preparación de la muestra, se pesaron 0.01 g en un tubo de polipropileno de punta cónica de 15 mL, y se agregaron 25 µL de metanol y 25 µL de la disolución de acrilamida-d3 de 100 ng mL^{-1} en metanol (para tener un volumen total de 50 µL de metanol), para de este manera simular de forma más realista la integración del patrón interno en la matriz de la muestra. La muestra se agitó mediante vórtex durante 1 min hasta observar la formación de una dispersión homogénea. Se agregaron 5 mL de tolueno y la muestra se agitó nuevamente durante 1 min. A continuación, se centrifugó a 6000 rpm durante 5 min y, finalmente, el sobrenadante se decantó en un tubo de vidrio de punta cónica de 15 mL para realizar la etapa de VA-RP-DLLME.

Etapa de VA-RP-DLLME

Para llevar a cabo la microextracción, se agregaron 50 µL de agua como fase extractante a cada disolución patrón o de muestra. A continuación, se agitó con vórtex durante 1 min para favorecer la formación de la microemulsión, y luego se centrifugó a 6000 rpm durante 5 min. La fase sedimentada se recogió con una jeringa Hamilton 1705 RNR de 100 µL y se trasvasó a un inserto de vidrio de 200 µL situado dentro de un vial de inyección de 1.5 mL para efectuar el análisis mediante LC-MS/MS.

La *Figura 5.1* muestra un esquema del procedimiento experimental.

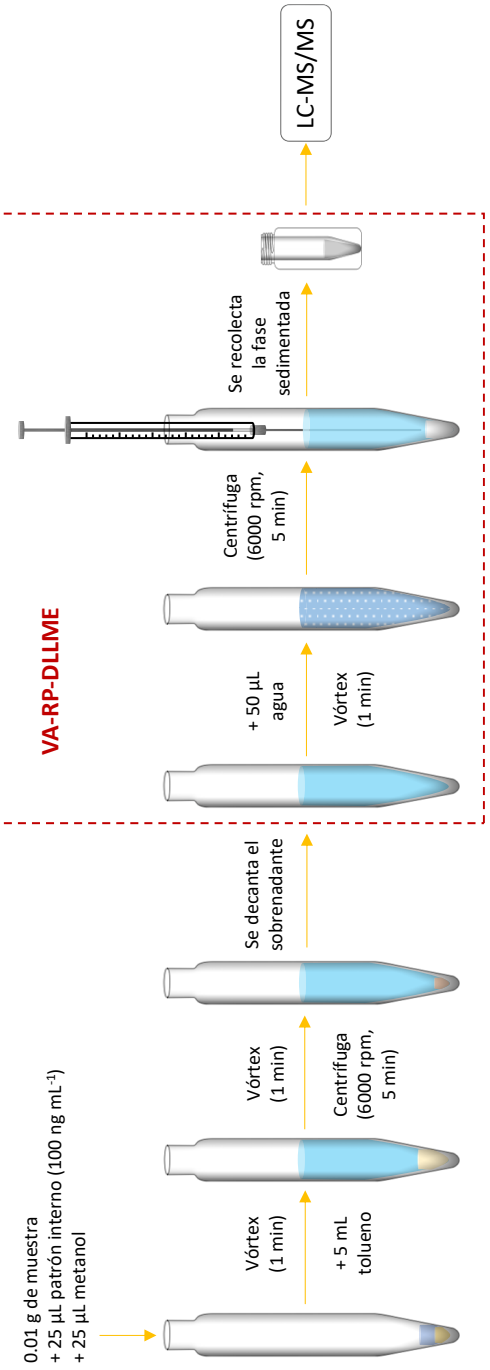


Figura 5.1. *Esquema del método propuesto*

Análisis cromatográfico

El análisis se llevó a cabo por LC-MS/MS, y las variables instrumentales del método propuesto se resumen en la *Tabla 5.2*.

La detección de los analitos por espectrometría de masas se llevó a cabo mediante ionización por electronebulización (ESI) en modo positivo y por monitorización de reacciones múltiples (MRM).

A modo de ejemplo, la *Figura 5.2* muestra el cromatograma de una disolución patrón de acrilamida y de acrilamida-d3, ambas en una concentración de 0.5 ng mL^{-1}, sometida al método analítico propuesto.

Tabla 5.2. Variables instrumentales del LC-MS/-MS

LC	Volumen de inyección	5 µL					
	Columna	Agilent Zorbax SB-C18 (50 mm x 2.1 mm; 1.8 µm)					
	Temperatura del horno	40 ºC					
	Caudal	0.2 mL min^{-1}					
	Modo	Isocrático					
	Fase móvil	Metanol:Agua (0.5 mM de NH_4F) 40:60 (v/v)					
MS/MS	Ion precursor	**72 (Acrilamida)**			**75 (Acrilamida-d3)**		
	m/z	55[a]	44	27	58[a]	44	30
	Fragmentor	40 V	40 V	40 V	45 V	45 V	45 V
	Energía de colisión	10 V	26 V	18 V	10 V	22 V	34 V
	Voltaje del capilar (ESI+)	5 kV					
	Temperatura del gas	340 °C					
	Caudal del gas	13 L min^{-1}					
	Presión del nebulizador	40 psi					

[a] Fragmento de cuantificación

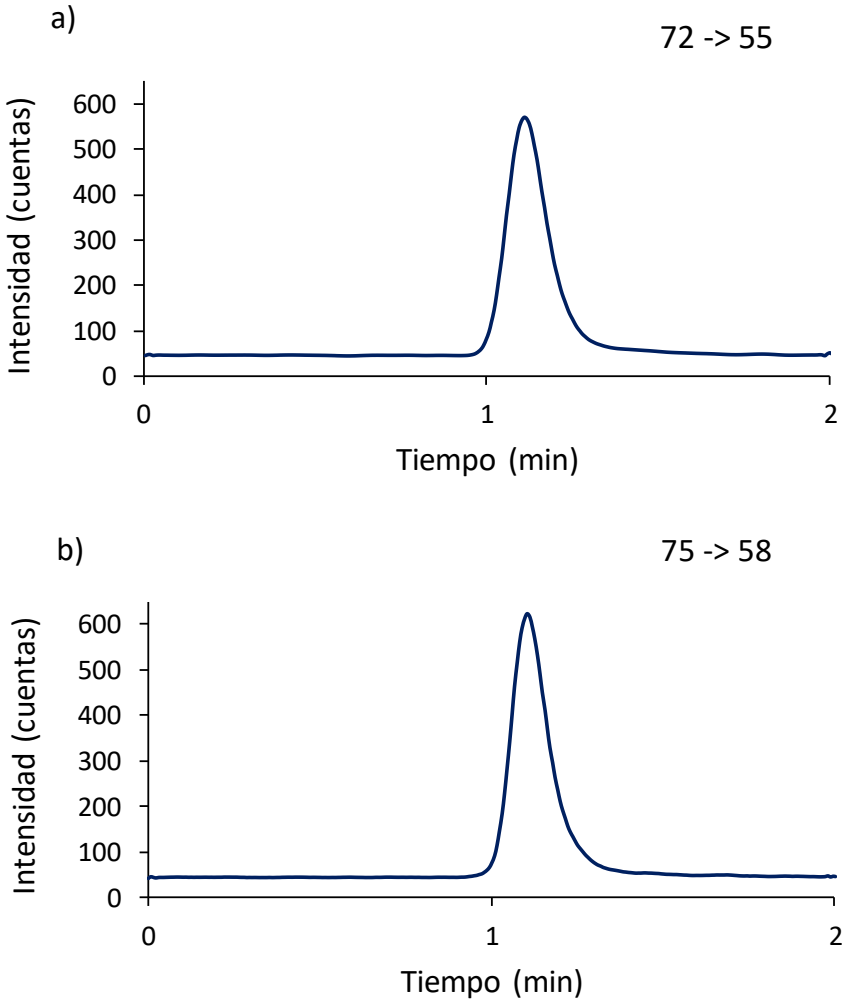


Figura 5.2. *Cromatograma obtenido aplicando el método propuesto a una disolución patrón conteniendo (a) 0.5 ng mL^{-1} de acrilamida y (b) 0.5 ng mL^{-1} de acrilamida-d3*

RESULTADOS

Estudio de las variables experimentales involucradas en el análisis por LC-MS/MS

La optimización de las transiciones ión precursor → ión producto y sus valores de energía de colisión y fragmentor se realizó mediante el software *MassHunter Optimizer*, mientras que la optimización de las variables de la fuente de ionización se realizó mediante el software *Source Optimizer*. Durante los estudios de optimización, se operaron el modo de ionización positiva por electronebulización (ESI+) y por monitorización de reacciones múltiples (MRM).
Para la optimización de las transiciones ión precursor → ión producto de ambos analitos, se inyectó una solución de acrilamida y otra de acrilamida-d3, ambas de 1 μg mL^{-1} en agua. El ion precursor de cada compuesto estaba formado por el ion pseudomolecular por transferencia de protón ($[M+H]^+$). A continuación, se obtuvieron los tres iones producto que presentaban mayor abundancia, así como sus valores óptimos de energía de colisión y fragmentor. En cuanto a la optimización de las variables de la fuente de ionización, se inyectó una disolución que contenía 1 μg mL^{-1} de acrilamida y acrilamida-d3 en agua.
Los valores obtenidos con estos estudios de optimización se han mostrado en la *Tabla 5.2*.

Estudio de las variables experimentales involucradas en la etapa de VA-RP-DLLME

En el procedimiento de VA-RP-DLLME, existen diferentes variables que pueden afectar al rendimiento de extracción. En este trabajo, las variables que se estudiaron fueron la naturaleza del disolvente de soporte, que actúa como fase dadora, y del disolvente dispersante, el

volumen del disolvente de extracción y el tiempo de agitación con vórtex.

Para el estudio de estas variables, cada condición experimental se evaluó por triplicado utilizando 5 mL de una disolución patrón de acrilamida de 20 ng mL^{-1}. La influencia de cada variable se evaluó utilizando como función respuesta el área del pico correspondiente a la acrilamida.

Naturaleza del disolvente de soporte que actúa como fase dadora

En RP-DLLME, la fase dadora debe ser un disolvente orgánico inmiscible con agua y preferiblemente con una densidad menor que esta para facilitar la sedimentación de la gota de agua, que actúa como extractante, en la punta cónica del tubo de vidrio y así facilitar su recolección. En este sentido, se estudiaron el tolueno y el hexano como posibles disolventes de soporte. Para ello, se tomaron 5 mL de una disolución patrón de 20 ng mL^{-1} de acrilamida, preparada en tolueno o hexano, y se introdujeron en tubos de vidrio de punta cónica. A continuación, se añadieron 100 µL de agua a cada tubo, se agitó con vórtex durante 0.5 min y se centrifugó a 6000 rpm durante 5 min. Las gotas sedimentadas se recogieron con una jeringa Hamilton 1705 RNR de 100 µL y se introdujeron en insertos de vidrio de 200 µL situados en viales de inyección para su análisis mediante LC-MS/MS.

De acuerdo con la *Figura 5.3*, la extracción empleando hexano proporcionó resultados muy poco satisfactorios en comparación a los obtenidos con tolueno, por lo que se seleccionó este último como disolvente de soporte.

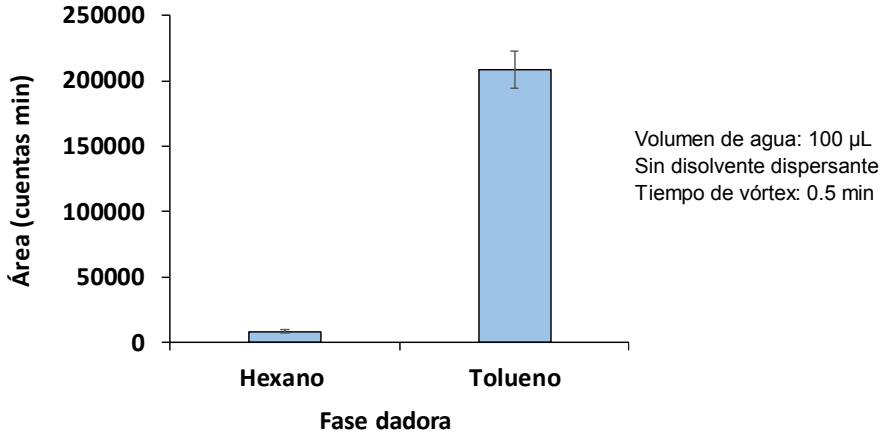


Figura 5.3. *Selección del disolvente de soporte que actúa como fase dadora*

Naturaleza del disolvente dispersante

Una vez seleccionado el disolvente de soporte, se evaluó la posibilidad de emplear etanol, acetona o acetonitrilo como posibles disolventes dispersantes. Se prepararon mezclas de 100 µL de agua y 250 µL de disolvente dispersante en microtubos de centrífuga para cada disolvente considerado. A continuación, estas mezclas se inyectaron rápidamente con una jeringa en las disoluciones patrón de 20 ng mL^{-1} de acrilamida en tolueno, formando de este modo la microemulsión de manera instantánea. Finalmente, los tubos se centrifugaron a 6000 rpm durante 5 min y cada gota de extracto se recolectó y midió mediante LC-MS/MS.

Cuando se utilizó etanol como disolvente dispersante, se obtuvieron extractos ligeramente turbios debido a la formación de una emulsión, por lo que se descartó este disolvente para estudios posteriores.

Adicionalmente, también se consideró la posibilidad de no utilizar disolvente dispersante. En este caso, solo se introdujeron 100 µL de agua en los tubos de vidrio, de modo que la microextracción se asistió por vórtex durante 0.5 min para favorecer la formación de la

microemulsión ya que, en ausencia de un disolvente dispersante, no se genera espontáneamente.

Como se puede observar en la *Figura 5.4*, se observaron resultados similares tanto con los disolventes dispersantes estudiados, como en ausencia de ellos. Por este motivo, dado el similar rendimiento de extracción, y con el fin de reducir el consumo de disolventes orgánicos, se decidió evitar el uso del disolvente dispersante y emplear agitación con vórtex.

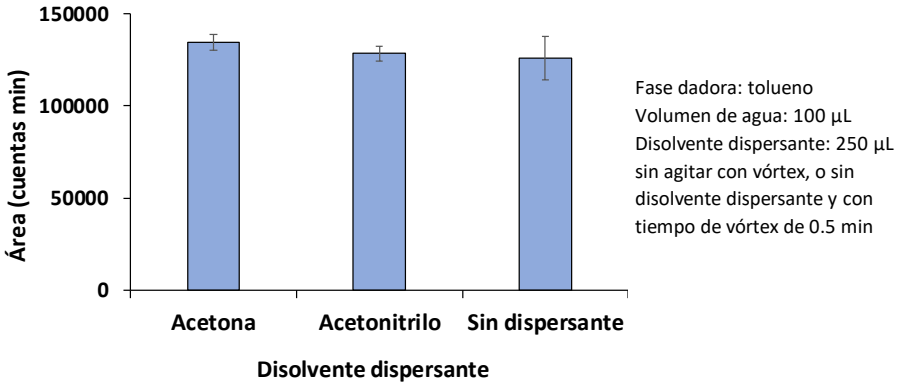


Figura 5.4. *Optimización del disolvente dispersante*

Volumen del disolvente de extracción

A continuación, se optimizó el volumen de agua utilizada como disolvente de extracción. Para ello se evaluaron por triplicado 50, 75, 100 y 125 µL de agua. Una vez añadida el agua, el tiempo de agitación con vórtex fue de 0.5 min.

La *Figura 5.5* muestra que al utilizar 50 µL de agua se logró una mayor preconcentración del analito en comparación con el resto de los volúmenes considerados. Aunque la tendencia de esta variable indica que un volumen inferior a 50 µL podría resultar en una mayor

preconcentración del analito, no se han considerado volúmenes más pequeños para el estudio porque las gotas de extracto a recoger eran demasiado pequeñas para ser manipuladas. Por lo tanto, se decidió utilizar un volumen de 50 µL para el disolvente de extracción.

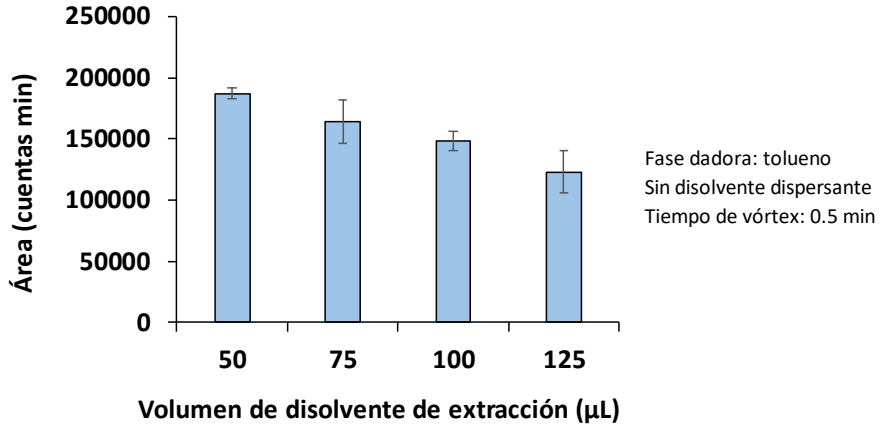


Figura 5.5. *Optimización del volumen de disolvente de extracción*

Cabe indicar que se realizó un ensayo de Análisis de Varianza (ANOVA) de los resultados obtenidos durante el estudio de esta variable, obteniendo un valor de significación de 0.0636. Dado que este valor es mayor que 0.05, se puede concluir que no existe una diferencia estadísticamente significativa entre los valores promedio obtenidos para los diferentes volúmenes de extracción ensayados a un nivel de confianza del 95%.

Tiempo de agitación con vórtex

Finalmente se optimizó el tiempo de agitación con vórtex. Para cada réplica, se agregaron 50 µL de agua como disolvente de extracción a 5 mL de una disolución de acrilamida de 20 ng mL^{-1} en tolueno. A continuación, se agitó con vórtex durante 0, 0.5, 1 y 1.5 min, cada valor por triplicado.

La *Figura 5.6* muestra que, en ausencia de un disolvente dispersante, se requiere asistir la microextracción con vórtex para favorecer la transferencia del analito de la fase dadora a la fase extractante. De hecho, un mayor tiempo de vórtex proporciona una mayor extracción del analito, aunque tiempos de agitación superiores a 1 min no proporcionaron una mejoría. Por esta razón, se seleccionó 1 min como el tiempo óptimo de agitación con vórtex para el proceso de microextracción.

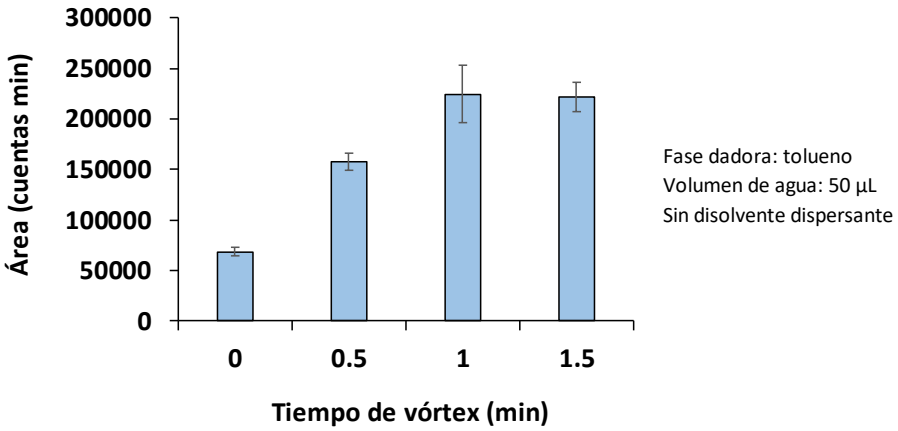


Figura 5.6. *Optimización del tiempo de agitación con vórtex*

Se realizó un ensayo ANOVA para los resultados obtenidos durante el estudio de esta variable, obteniendo un valor de significación de 8,47385E-06. Dado que este valor es inferior a 0.05, se puede concluir que existe una diferencia estadísticamente significativa entre los valores promedio obtenidos para los diferentes tiempos de extracción ensayados a un nivel de confianza del 95.0 %.

Estudio del pretratamiento de muestra

Durante estudios preliminares del método propuesto con muestras reales, se comprobó que no era posible disolver o dispersar la muestra

cosmética directamente en tolueno debido a la formación de grumos, lo que dificultaba la realización de la VA-RP-DLLME.

En este sentido, se optó por dispersar la muestra en la mínima cantidad de un disolvente orgánico polar miscible en tolueno que permitiera romper la estructura de la matriz cosmética. Para ello se ensayaron acetonitrilo y metanol, observándose que, a igualdad de volumen, se obtenía una dispersión completa y homogénea de la muestra con metanol, mientras que no se conseguía una dispersión homogénea con acetonitrilo. Así, una vez pesada la muestra, se añadieron 50 µL de metanol, obteniéndose una dispersión homogénea agitando con vórtex durante 1 min. Acto seguido se adicionaron 5 mL de tolueno para llevar a cabo la microextracción.

Para verificar que esta cantidad de metanol no afectaba los resultados de la microextracción actuando como un disolvente dispersante, se realizó un estudio comparativo aplicando el método propuesto, por triplicado, a una disolución acuosa de acrilamida de 2.5 ng mL^{-1} con y sin la adición de 50 µL de metanol. Se aplicó un test de t de Student que arrojó un valor de significación de 0.45. Dado que este valor es mayor que 0.05, se puede concluir que no hay diferencias significativas a un nivel de confianza del 95% al añadir metanol.

Estudio del efecto matriz

Para estudiar el efecto matriz en el proceso de extracción, se prepararó un calibrado externo y un calibrado por adición de patrón (ambos de 0 a 5 ng mL^{-1} de acrilamida) empleando, a modo de ejemplo, la muestra de leche desmaquillante, y se sometieron al procedimiento de microextracción.

La elección de una muestra cosmética constituida por una emulsión para efectuar estos estudios se debió a que, a diferencia de otros cosméticos con una formulación más minimalista, este tipo de muestra representa el "peor caso" a superar para la microextracción propuesta, ya que esta matriz presenta un elevado número de ingredientes, tanto

hidrofílicos como lipofílicos, y una considerable carga de tensioactivos que podrían afectar negativamente al procedimiento VA-RP-DLLME.

El efecto matriz se calculó como la relación entre la pendiente de la recta de calibrado por adición de patrón y la de la calibración externa. Se obtuvo un valor de 0.74, indicando la presencia de efecto matriz.

Al haberse demostrado la existencia de efecto matriz, se propuso utilizar un patrón interno para corregirlo.

Por esta razón, se repitieron ambas calibraciones, pero conteniendo en este caso 3 ng mL^{-1} de acrilamida-d3 como patrón interno que, en el caso de la preparación del calibrado por adición de patrón, se añadió directamente sobre la muestra antes de su dilución. Se eligió la acrilamida-d3 como patrón interno por varias razones:

- → al ser un compuesto deuterado, no está presente en las muestras cosméticas
- → su estructura química es igual a la del analito y, por lo tanto, sus comportamientos son idénticos
- → a pesar de eluir al mismo tiempo de retención que el analito, al tener distinta masa, la transición precursor → producto es diferente, lo que no interfiere en su determinación

En este caso, representando gráficamente A_i/A_{sur} frente a la concentración (donde A_i es el área del pico de la acrilamida y A_{sur} es el área de la acrilamida-d3), la relación entre la pendiente de la recta de calibrado por adición de patrón y la de la calibración externa dio un valor próximo a 1.00, lo que demostró que la adición de acrilamida-d3 como patrón interno corrigió, como se esperaba, el efecto matriz.

Parámetros analíticos del método propuesto

Linealidad

Se estudió la linealidad con disoluciones patrón de acrilamida sometidas al procedimiento entero. Se obtuvo un alto nivel de

linealidad, que alcanzó, al menos, hasta 20 ng mL^{-1}. Teniendo en cuenta los bajos niveles de acrilamida esperados en las muestras, el rango de trabajo se fijó entre 0.005 y 5 ng mL^{-1}, obteniéndose un valor de coeficiente de determinación (R^2) de 0.998.

Factor de preconcentración

El factor de preconcentración (FP) obtenido, calculado como FP = C_{ext}/C_0), donde C_{ext} es la concentración del analito en el extracto y C_0 es la concentración del analito en la fase dadora, fue de 52.

Límites de detección y cuantificación

Los límites de detección y cuantificación se calcularon como la concentración correspondiente a tres y diez veces, respectivamente, el valor de la relación señal/ruido para una disolución patrón de acrilamida de 0.005 ng mL^{-1} sometida al procedimiento de microextracción propuesto, mientras que los límites de detección y cuantificación del método se calcularon teniendo en cuenta además el procedimiento de preparación de la muestra.

Los valores obtenidos, que se indican en la *Tabla 5.3*, están muy por debajo de los valores umbral establecidos por el Reglamento Europeo sobre productos cosméticos (0.1 mg kg^{-1} (100 µg kg^{-1}) para productos sin aclarado, y 0.5 mg kg^{-1} (500 µg kg^{-1}) en el resto), lo que muestra que el método propuesto es adecuado para cumplir con el propósito para el que fue desarrollado.

Tabla 5.3. Límites de detección y cuantificación

LOD[a] (ng mL^{-1})	LOQ[a] (ng mL^{-1})	MLOD[b] (µg kg^{-1})	MLOQ[b] (µg kg^{-1})
0.001	0.003	0.51	1.69

[a] LOD: límite de detección; LOQ: límite de cuantificación
[b] MLOD: límite de detección del método; MLOQ: límite de cuantificación del método

Repetibilidad

La repetibilidad de la extracción, expresada como desviación estándar relativa (RSD), se evaluó aplicando el método propuesto a cinco réplicas de dos disoluciones patrón en tolueno que contenían acrilamida a dos niveles de concentración (0.5 y 1 ng mL^{-1}), el mismo día (intra-día) y durante cinco días consecutivos (inter-día).

Los resultados obtenidos, que se muestran en la *Tabla 5.4*, indican que se logró una buena repetibilidad (RSD < 4.1%).

Tabla 5.4. Estudio de la repetibilidad

RSD (%) [a]			
Intra-día (N^b = 5)		Inter-día (N^b = 5)	
0.5 ng mL^{-1}	**1 ng mL^{-1}**	**0.5 ng mL^{-1}**	**1 ng mL^{-1}**
2.5	2.6	4.0	4.1

[a] desviación estándar relativa

[b] N: número de réplicas

Análisis de muestras

Se analizaron, mediante el método propuesto, cinco muestras de productos cosméticos disponibles comercialmente (un gel revitalizante, una leche desmaquillante, jabón líquido de manos, una crema fotoprotectora y un gel de baño para bebés).

Como puede verse en los resultados que se muestran en la *Tabla 5.5*, se detectó y determinó acrilamida en cuatro de las cinco muestras analizadas. Además, cabe señalar que el contenido de acrilamida en

una de las muestras se encontraba por encima de 0.1 mg kg^{-1} y, por lo tanto, dicho producto no cumplía con el Reglamento Europeo [1].

Por otro lado, para verificar que el uso del patrón interno corregía el efecto matriz en las muestras, se aplicó el método propuesto a las cinco muestras analizadas, fortificándolas para evaluar el coeficiente de recuperación. Para ello, las muestras se fortificaron durante la etapa de pretratamiento de la muestra con alícuotas de 5 y 10 µL de la disolución patrón de acrilamida de 500 ng mL^{-1} en metanol, se agregaron 25 µL de la disolución de trabajo de 100 ng mL^{-1} de acrilamida-d3 en metanol y se añadió la diferencia en metanol para llegar a los 50 µL necesarios para dispersar la muestra como se ha descrito anteriormente. Se obtuvieron de este modo dos niveles de fortificación, y como se puede observar en la *Tabla 5.5*, los valores del coeficiente de recuperación relativa obtenidos oscilaron entre 88 y 108%, lo que demuestra que, utilizando patrón interno, se corrigió el efecto matriz.

Tabla 5.5. Aplicación del método propuesto al análisis de muestras comerciales

Muestra[a]	Cantidad fortificada[b] (mg kg^{-1})	Cantidad encontrada[b] (mg kg^{-1})	R (%)[b]
A	-	0.38 ± 0.04	-
	0.23 ± 0.04	0.62 ± 0.05	101 ± 3
	0.55 ± 0.19	0.97 ± 0.16	108 ± 8
B	-	< LOD	-
	0.26 ± 0.02	0.23 ± 0.03	88 ± 4
	0.48 ± 0.10	0.47 ± 0.06	94 ± 9
C	-	0.020 ± 0.003	-
	0.19 ± 0.05	0.19 ± 0.05	90 ± 1

	0.41 ± 0.12	0.45 ± 0.11	106 ± 6
	-	0.002 ± 0.001	-
D	0.20 ± 0.05	0.20 ± 0.05	98 ± 6
	0.44 ± 0.10	0.43 ± 0.09	98 ± 6
	-	0.0031 ± 0.0003	-
E*	0.22 ± 0.03	0.22 ± 0.01	100 ± 9
	0.40 ± 0.02	0.39 ± 0.03	99 ± 5

[a] A: gel revitalizante; B: leche desmaquillante; C: jabón líquido de manos; D: crema fotoprotectora; E: gel de baño para bebés
[b] coeficiente de recuperación relativa, expresado como media ± desviación estándar de tres réplicas
* La muestra no presenta poliacrilamidas en el listado de ingredientes

CONCLUSIONES

En este capítulo:

Se ha propuesto un segundo método analítico para la determinación de acrilamida en productos cosméticos.

- → El método analítico desarrollado es muy sensible y permite determinar residuos de acrilamida a nivel de trazas en productos cosméticos
- → El método se basa en un pretratamiento de la muestra, en el que se realiza una etapa de VA-RP-DLLME seguida de LC-MS/MS
- → Empleando el método propuesto no es necesario un paso previo de derivatización como en el método descrito en el Capítulo 4, evitando así una mayor manipulación de muestra
- → El método analítico propuesto permite la determinación de acrilamida en diferentes tipos de matrices cosméticas muy por debajo de los valores umbral establecidos por el Reglamento Europeo sobre productos cosméticos
- → Las buenas características analíticas, la sencillez y el bajo coste del procedimiento lo convierten en un método adecuado para las empresas en el control de calidad para así garantizar la seguridad de los usuarios y el cumplimiento de la normativa europea sobre productos cosméticos

Capítulo 6

Desarrollo de un método analítico para la determinación de N-nitrosaminas prohibidas en productos cosméticos mediante microextracción líquido-líquido dispersiva asistida por vórtex y cromatografía de gases acoplada a espectrometría de masas

El contenido de este capítulo ha sido publicado en el artículo:

Determination of nine prohibited N-nitrosamines in cosmetic products by vortex-assisted dispersive liquid-liquid microextraction prior to gas chromatography-mass spectrometry. L. Schettino, J.L. Benedé, A. Chisvert, **RSC Advances** 13 (2023) 2963

Y presentado como cartel en la XXI Reunión de la Sociedad Española de Cromatografía y Técnicas Afines, Almería, 2022

INTRODUCCIÓN

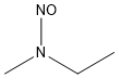
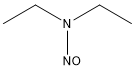
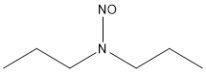
Objetivo

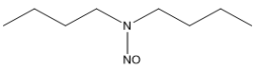
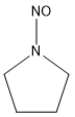
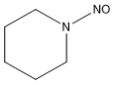
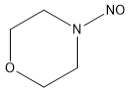
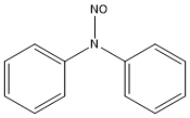
Desarrollar un nuevo método analítico para la determinación simultánea de nueve N-nitrosaminas prohibidas en productos cosméticos. El método se basa en una etapa de microextracción líquido-líquido dispersiva asistida por vórtex (VA-DLLME), seguida de análisis por cromatografía de gases-espectrometría de masas (GC-MS).

Compuestos estudiados

Los analitos objeto de estudio se indican en la *Tabla 6.1*.

Tabla 6.1. Información de interés sobre los compuestos estudiados

Nombre	Número CAS	Estructura química	M_r (g mol^{-1})	Log $K_{o/w}$
N-nitrosodimetilamina (NDMA)	62-75-9		74.08	-0.50
N-nitrosoetilmetilamina (NMEA)	10595-95-6		88.11	0.01
N-nitrosodietilamina (NDEA)	55-18-5		102.14	0.52
N-nitrosodipropilamina (NDPA)	621-64-7		130.19	1.54

N-nitrosodibutilamina (NDBA)	924-16-3		158.24	2.56
N-nitrosopiperidina (NPIP)	100-75-4		114.15	0.44
N-nitrosopirrolidina (NPYR)	930-55-2		100.12	-0.09
N-nitrosomorfolina (NMOR)	59-89-2		116.12	-0.59
N-nitrosodifenilamina (NDPhA)	86-30-6		198.22	3.13

Resumen

El método propuesto en este capítulo permite la determinación de nueve N-nitrosaminas a nivel de trazas, y se basa en una VA-DLLME para extraer y preconcentrar los analitos seguida de GC-MS para su determinación.

Las variables involucradas en el proceso de microextracción se optimizaron utilizando un diseño de Box-Behnken. Además, se estudiaron diferentes enfoques para el pretratamiento de la muestra, como LLE, SPE, filtración y lixiviación, con el fin de realizar un paso de limpieza y eliminar interferentes que pudiesen afectar negativamente la aplicación de la técnica de microextracción.

El método fue validado con éxito mostrando una buena linealidad hasta, al menos, 20 ng mL^{-1}, factores de preconcentración de 2 a 100 dependiendo del analito, límites de detección y cuantificación a niveles bajos de µg kg^{-1} y buenos valores de repetibilidad (< 13 %).

Finalmente, el método analítico desarrollado se aplicó a muestras de productos cosméticos comerciales de diferente naturaleza, corrigiendo el efecto matriz mediante calibración por adición de patrón. En las muestras se encontraron cantidades considerables de algunas de las N-nitrosaminas, incluso superando el límite de seguridad establecido en 50 µg kg^{-1} [76].

El método propuesto resulta ser rápido, simple y asequible, además no necesita de equipos sofisticados, cualidades que lo convierten en un excelente recurso para llevar a cabo el control de calidad de los productos cosméticos en la mayoría de los laboratorios.

PROCEDIMIENTO EXPERIMENTAL

Instrumentación

- → Cromatógrafo de gases 8860 acoplado a un espectrómetro de masas de cuadrupolo simple 5977B, ambos de Agilent Technologies, y un automuestreador PAL LSI 85 de CTC Analytics.
- → Agitador vórtex ZX3 de VELP Scientifica S.r.l.
- → Centrífuga EBA 21 Hettich®

Reactivos

- → Disolución comercial EPA 8270 Appendix IX Nitrosamine Mix, 2000 µg mL^{-1} de cada N-nitrosamina en metanol, como patrón
- → Metanol grado LC-MS para la preparación de las disoluciones patrón madre e intermedias
- → Agua ultrapura para la preparación de las disoluciones patrón de trabajo
- → Cloroformo grado-reactivo como fase aceptora en la etapa de VA-DLLME

→ Hexano grado LC al 96 %, cartuchos de SPE (Discovery® DSC-Diol, Strata-X^{TM} y Strata SDB-L) para el estudio del pretratamiento de la muestra

Muestras

Se analizaron un gel calmante para después del sol (muestra A), una crema hidratante (muestra B) y una crema reafirmante (muestra C), para ensayar y validar el método propuesto. Todas ellas contenían en su fórmula ingredientes que podían causar la formación de nitrosaminas.

Método propuesto

Preparación de muestras y patrones

Se preparó una disolución madre de los analitos de 500 µg mL^{-1} en metanol y, a partir de esta, se preparó una disolución intermedia de 50 µg mL^{-1} en este mismo disolvente.

Tomando una alícuota de la disolución intermedia y diluyéndola con agua ultrapura, se obtuvo una disolución de trabajo de 100 ng mL^{-1}.

Con respecto a la preparación de la muestra, las disoluciones de muestra se prepararon mediante calibración por adición de patrón. De este modo, se pesaron 0.05 g de muestra en matraces aforados de 5 mL, y se le añadieron diferentes alícuotas de la disolución patrón de trabajo de 100 ng mL^{-1}, obteniéndose un rango de concentración de los analitos de 0.01 a 20 ng mL^{-1} una vez enrasado con agua. Finalmente, las disoluciones de muestra se agitaron con vórtex durante 1 min aprox. hasta obtener una dispersión homogénea, y se transvasaron a tubos de polipropileno de 15 mL de punta cónica para realizar la etapa de VA-DLLME.

Etapa de VA-DLLME

Para realizar la VA-DLLME, se añadieron 120 µL de cloroformo, como disolvente de extracción, a los 5 mL de las disoluciones de calibración descritas con anterioridad. A continuación, cada disolución se agitó con vórtex durante 1 min para favorecer la formación de la microemulsión, y se centrifugó a 6000 rpm durante 5 min. La fase extractante, sedimentada en la punta cónica del tubo, se recogió con una jeringa Hamilton 1705 RNR de 100 µL y se transvasó a un inserto de vidrio de 200 µL situado dentro del vial de inyección para efectuar el análisis mediante GC-MS.

La *Figura 6.1* muestra un esquema del procedimiento experimental.

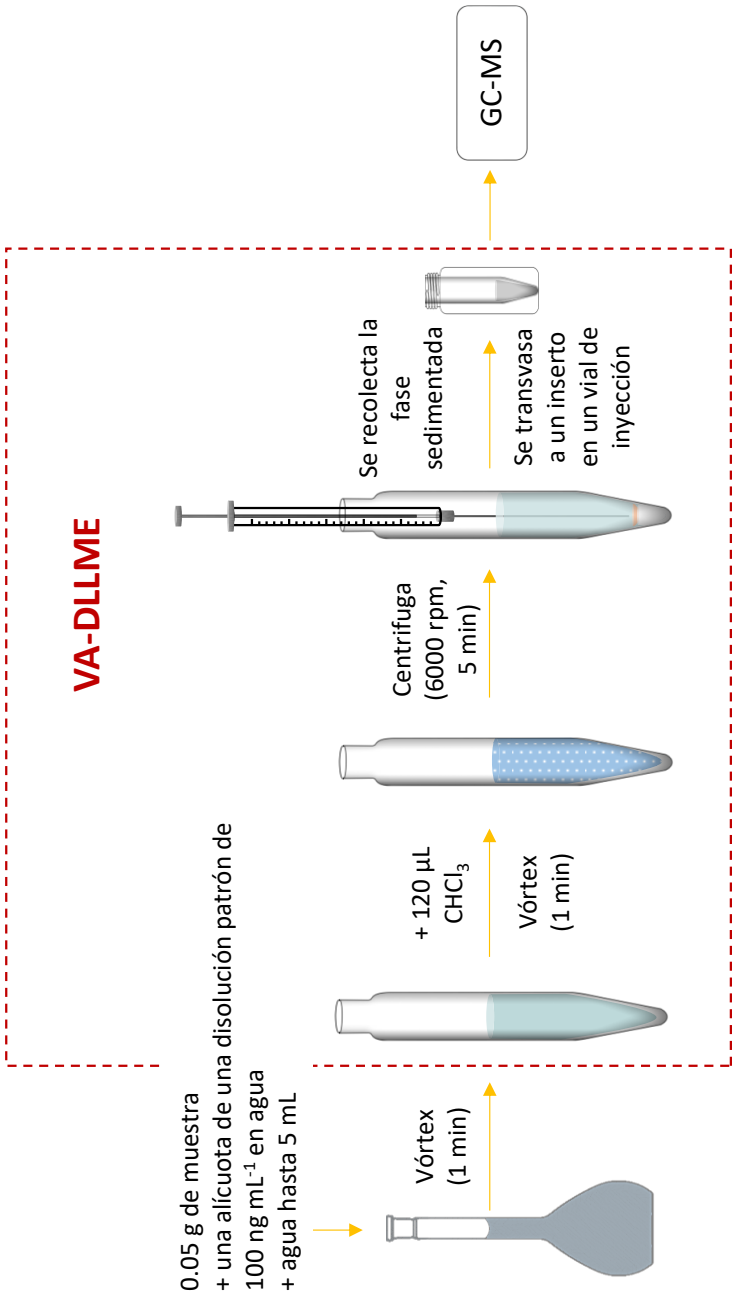


Figura 6.1. *Esquema del método propuesto*

Análisis cromatográfico

El análisis se lleva a cabo por GC-MS y las variables instrumentales del método propuesto se resumen en la *Tabla 6.2*.

Tabla 6.2. Variables instrumentales del GC-MS

GC	Volumen de inyección	2 µL
	Modo de inyección	Sin división de flujo
	Temperatura de inyección	230 ºC
	Gas portador	Helio
	Caudal del gas portador	1 mL min^{-1}
	Columna	VF-WAXms (30 m x 0.25 mm; 0.25 µm)
	Temperatura del horno	60 °C (2min) → 10 °C/min → 160 °C → 40 °C/min → 240 °C (4min)
	Temperatura de la interfase	230 ºC
MS	Modo de ionización	Ionización electrónica (EI)
	Rango de masas	de 40 a 200 uma
	Energía de electrones	70 eV
	Temperatura de la fuente	230 °C
	Temperatura del cuadropolo	150 ºC

La adquisición se llevó a cabo en el modo de monitorización de ión seleccionado (SIM, del inglés *selected ion monitoring*). Para mejorar la sensibilidad, se establecieron seis ventanas de adquisición según sus tiempos de retención. Estas se muestran en la *Tabla 6.3* junto a los iones de monitorización (m/z).

Tabla 6.3. Tiempo de retención e iones de monitorización de los analitos estudiados

Analito	Ventanas de adquisición (min)	Tiempo de retención (min)	Ion de cuantificación (m/z)	Iones de identificación (m/z)	
NDMA	6.00-6.90	6.68	74	43	42
NMEA	6.90-8.50	7.33	88	56	42
NDEA		7.73	102	56	42
NDPA	8.50-11.00	9.52	130	70	43
NDBA	11.00-12.15	11.72	116	84	57
NPIP		12.01	114	55	42
NPYR	12.15-14.00	12.36	100	68	41
NMOR		12.81	116	86	56
NDPhA	15.00-18.00	16.66	169	168	167

Bajo las condiciones descritas, el tiempo requerido para el análisis cromatográfico de todos los analitos fue de 18 min.

La *Figura 6.2* muestra un cromatograma de una disolución patrón de los analitos de 10 ng mL^{-1} sin extraer y después de haberla sometida al método propuesto.

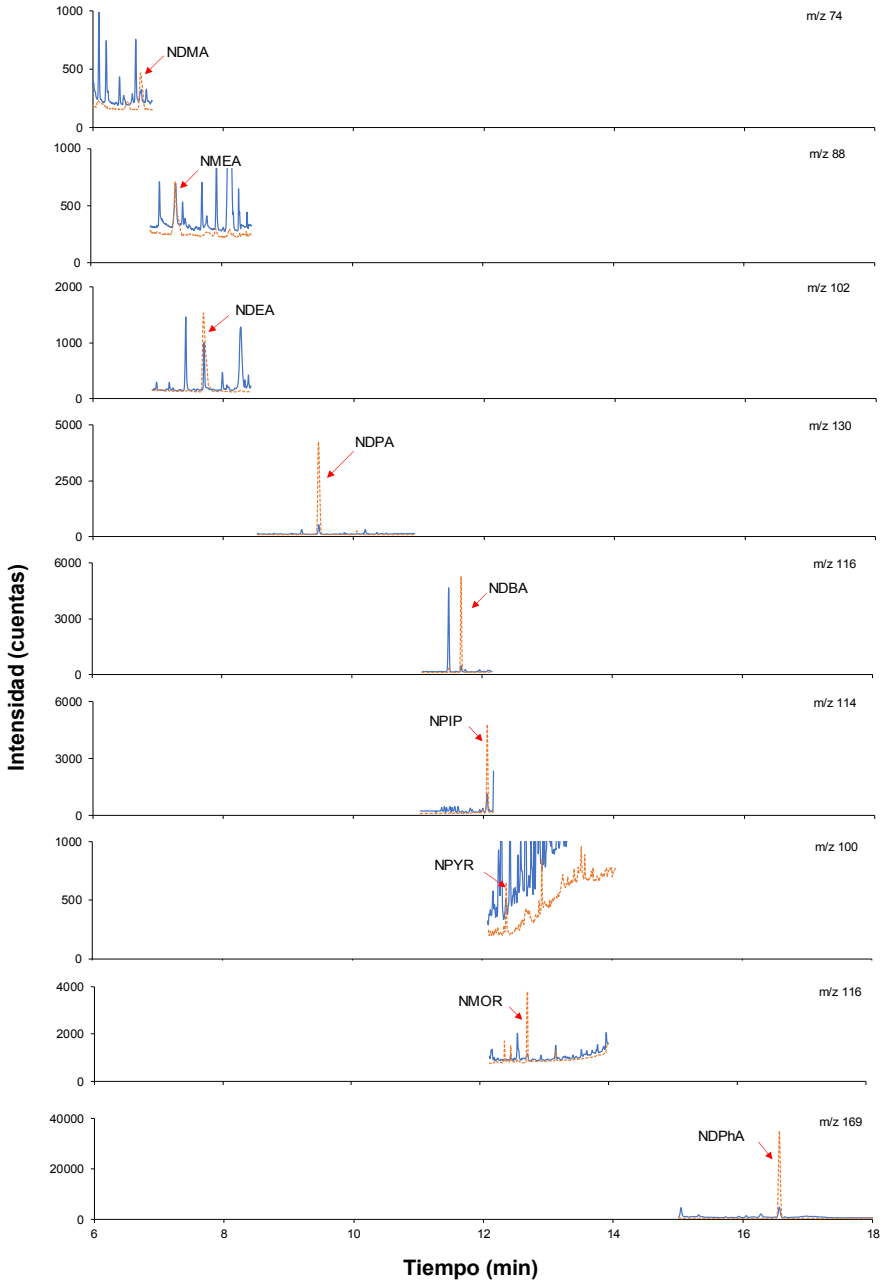


***Fig. 6.2**. Cromatograma de una disolución patrón de los analitos de 10 ng mL^{-1} sin extraer (azul) y después de haber sido sometida al método propuesto (naranja).*

RESULTADOS

Optimización multivariante de las variables experimentales involucradas en la etapa de VA-DLLME

Como paso previo a la optimización de las variables experimentales involucradas en la etapa de microextracción, se realizaron estudios preliminares para evaluar la naturaleza del disolvente de extracción, que es aconsejable que sea más denso que el agua para permanecer en el fondo del tubo de extracción tras la centrifugación. Además, desde el principio se evaluó la posibilidad de evitar el uso del disolvente dispersante utilizando en su lugar agitación vórtex para favorecer la formación de la microemulsión. Por esta razón, se seleccionaron el cloroformo y el diclorometano como posibles disolventes de extracción y se llevó a cabo el procedimiento VA-DLLME, extrayendo 5 mL de una disolución patrón de 5 ng mL^{-1} de los analitos utilizando 100 µL de disolvente extractante y agitando con vórtex durante 60 s.

Al utilizar diclorometano no se obtuvo la formación de la microemulsión, por lo que se seleccionó cloroformo como fase extractante.

A continuación, se optimizaron las variables involucradas en el procedimiento VA-DLLME mediante una metodología de superficie de respuesta (RSM, del inglés *response surface methodology*), mostrando las interacciones entre ellas. En concreto, se llevó a cabo un diseño Box-Behnken para evaluar las variables de extracción. El análisis estadístico de los resultados se realizó utilizando el software StatGraphics Centurion XVI de StatGraphics Technologies.

Las variables estudiadas (y los rangos) fueron el volumen del disolvente de extracción (60-150 µL), la fuerza iónica de la fase dadora (0-10%, NaCl (m/v)) y el tiempo de agitación con vórtex (30-90 s).

Todos los experimentos se realizaron en la misma sesión de trabajo utilizando 5 mL de una disolución patrón de los analitos de 5 ng mL^{-1} en agua como fase dadora.

Diseño Box-Benhken

Los diseños de Box-Benhken son una clase de diseños de segundo orden giratorios o casi giratorios que se utilizan para generar una respuesta de orden elevado.

El número total de experimentos necesarios para el desarrollo del diseño de Box-Benhken (N) se define mediante la siguiente ecuación:

$N = 2k(k-1) + Cp$ (1)

donde k es el número de factores (o variables) a evaluar (en este caso 3) y Cp es el número de réplicas del punto central. De acuerdo con la Ecuación (1), se calcularon 15 experimentos, seleccionando 3 réplicas del punto central. Cada factor se ensayó en un rango definido por tres niveles codificados (bajo (-1), medio (0) y alto (+1)). En la la *Tabla 6.4* se muestran los valores codificados y su correspondencia con los valores reales ensayados de cada uno de los experimentos realizados.

Table 6.4. Diseño Box-Behnken para la optimización multivariante de las variables

Experimento	Volumen de $CHCl_3$ (µL)		Tiempo de vórtex (s)		Fuerza iónica (%NaCl, m/v)	
	Sin codificar	Codificado	Sin codificar	Codificado	Sin codificar	Codificado
1	60	-1	60	0	0	-1
2	150	1	60	0	0	-1
3	60	-1	60	0	10	1
4	150	1	60	0	10	1
5	60	-1	30	-1	5	0

6	150	1	30	-1	5	0
7	60	-1	90	1	5	0
8	150	1	90	1	5	0
9	105	0	30	-1	0	-1
10	105	0	30	-1	10	1
11	105	0	90	1	0	-1
12	105	0	90	1	10	1
13	105	0	60	0	5	0
14	105	0	60	0	5	0
15	105	0	60	0	5	0

Los resultados obtenidos se ajustaron a una superficie de respuesta empleando la siguiente ecuación cuadrática:

$$Y = \beta_o + \Sigma \beta_i X_i + \Sigma \beta_{ii} X_i^2 + \Sigma \beta_{ij} X_i X_j + \varepsilon \quad (2)$$

donde Y es la respuesta analítica; β_0 es el término constante; β_i, β_{ii} y β_{ij} representan los coeficientes de regresión del diseño; χ_i y χ_j son las diferentes variables; y ε es el error residual.

Posteriormente, se aplicó la deseabilidad global del experimento (D) para estudiar las diferentes respuestas y lograr las condiciones de extracción óptimas para potenciar las respuestas analíticas de los analitos:

$$D = (d_1(Y_1) \cdot d_2(Y_2) \cdots d_n(Y_n))^{1/n} \quad (3)$$

donde n es el número de respuestas en el proceso de optimización y $d_i(Y_i)$ es la deseabilidad individual de cada respuesta en el experimento. La deseabilidad individual se calcula como:

$$di = Y_i - Y_{min} / Y_{max} - Y_{min} \quad (4)$$

Las respuestas se clasifican entre 0 y 1, para definir, respectivamente, las respuestas indeseables y las respuestas completamente deseables.

Para evaluar la adecuación del modelo se consideró el coeficiente de determinación (R^2), el cual para todas las nitrosaminas dio un valor > 0.87, indicando que el modelo diseñado fue eficiente para predecir respuestas.

La *Figura 6.3* muestra los gráficos de las superficies de respuesta en términos de deseabilidad para las tres variables estudiadas.

La *Figura 6.3 (a)* muestra que no hubo grandes diferencias en el tiempo de vórtex, pero se obtuvieron mejores respuestas utilizando 50-70 s, consiguiendo la dispersión adecuada del disolvente de extracción en la fase dadora. Por esta razón, se seleccionó el valor medio de 60 s para experimentos posteriores.

Por otro lado, como se muestra en la *Figura 6.3 (a) y (b)*, la mejor respuesta para el volumen de disolvente de extracción se obtuvo utilizando 60 µL de cloroformo. Sin embargo, con este volumen se generaba una fase sedimentada demasiado pequeña (aprox. 15 µL) que dificultaba su manipulación e inyección en el sistema GC-MS, por lo que se decidió seleccionar un volumen mayor (80 µL).

Finalmente, como se observa en la *Figura 6.3 (c)*, la mejor respuesta para la fuerza iónica de la fase dadora se mostró tanto para el valor de 0 % como para el de 10 % NaCl (m/v). Esto ocurre probablemente debido a dos efectos diferentes. Por un lado, la presencia de sales disminuye la solubilidad del disolvente de extracción, por lo que aumenta el volumen del extracto, provocando la dilución de los analitos. Por el contrario, cuando se utiliza una mayor concentración de sal, la solubilidad de los analitos en la fase acuosa también disminuye y se potencia su paso a la fase orgánica (efecto salino), obteniendo una mayor extracción de los analitos. En base a estos resultados, y para reducir el consumo de reactivos, se seleccionó el valor de NaCl al 0 % (m/v) para los siguientes experimentos.

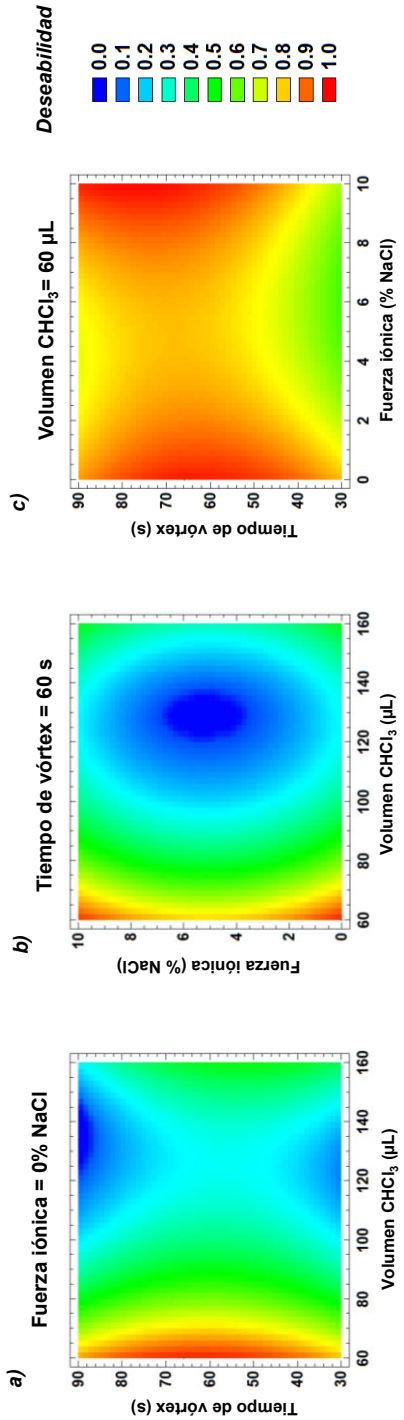


Figura 6.3. *Superficie de respuesta de la función de deseabilidad que representa la relación entre las diferentes variables que afectan la extracción: (a) tiempo de agitación con vórtex vs volumen de $CHCl_3$, (b) fuerza iónica vs volumen de $CHCl_3$ y (c) tiempo de agitación con vórtex vs fuerza iónica.*

Estudio del pretratamiento de muestra

Una vez optimizada la etapa de extracción, se llevaron a cabo unas pruebas realizando la VA-DLLME directamente sobre una disolución de muestra obtenida a partir de una crema cosmética libre de nitrosaminas. La elección de una crema como muestra cosmética para estos estudios preliminares se debió a que, a diferencia de otros cosméticos con una formulación más minimalista, una crema representa el "peor caso" a superar para la microextracción propuesta. Esto se debe a que esta matriz compleja presenta un elevado número de componentes lipofílicos y tensioactivos que podrían afectar negativamente al procedimiento de VA-DLLME.

Se observó que, en efecto, al realizar la microextracción en las condiciones definidas por la optimización anteriormente descrita, los compuestos lipofílicos contenidos en la matriz también pasaban al cloroformo, precipitando y apareciendo en el fondo del tubo tras la centrifugación junto con la fase sedimentada, provocando la formación de una gota turbia nada aconsejable de inyectar en el sistema GC-MS.

Para solventar este inconveniente, se realizaron diferentes planteamientos para el pretratamiento de la muestra, con el fin de realizar un paso previo de limpieza para eliminar estas impurezas que afectaban negativamente la aplicación de la técnica de microextracción. Para llevar a cabo estos estudios, se preparó una disolución de muestra de una crema cosmética libre de nitrosaminas pesando 0.5 g de muestra en un matraz de 50 mL y enrasándolo con agua, después de fortificar a una concentración de 1 ng mL^{-1} con las nitrosaminas estudiadas.

En un primer intento, antes de realizar la etapa de VA-DLLME, se realizó un paso de limpieza por medio de una LLE con 1 mL de hexano sobre 5 mL de disolución de muestra. En un segundo intento, se percolaron 5 mL de la disolución de muestra a través de diferentes cartuchos SPE (Discovery® DSC-Diol, Strata-X^{TM} y Strata-SDB-L), después de activar cada cartucho de acuerdo con las especificaciones del fabricante.

Como se puede observar en la *Figura 6.4*, el mejor resultado se logró realizando la LLE con 1 mL de hexano. Entre los cartuchos SPE, los mejores resultados se obtuvieron con el cartucho Discovery® DSC-Diol, cuyo rendimiento fue el más cercano al alcanzado mediante LLE, e incluso se obtuvieron resultados ligeramente mejores para las nitrosaminas más lipofílicas (es decir, NDPA, NDBA y NDPhA), que era más probable que se perdieran con el hexano y, por lo tanto, dieran señales más bajas.

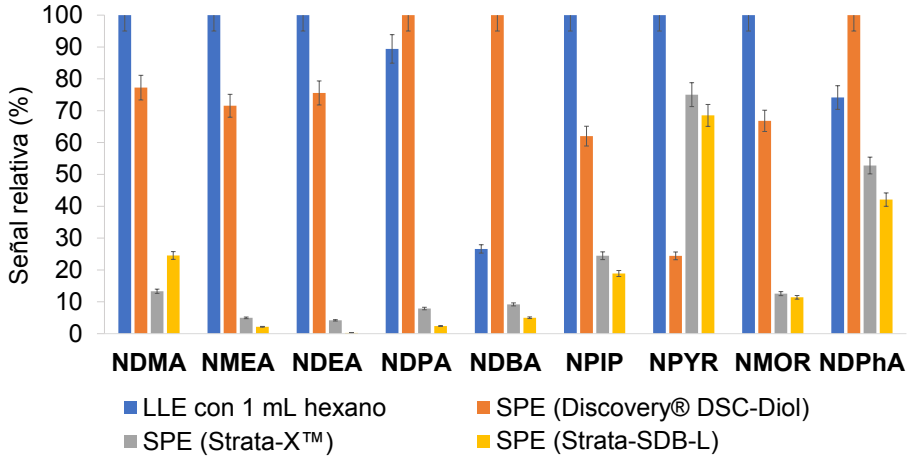


Figura 6.4. *Estudio de pretratamiento de muestra: comparación entre la limpieza mediante LLE con 1 mL de hexano y SPE con diferentes cartuchos (Discovery® DSC-Diol, Strata-X™ y Strata-SDB-L)*

Una vez seleccionada la LLE con hexano como pretratamiento, se estudió la posibilidad de reducir el volumen de hexano de 1 mL a 200 µL (cantidad mínima de disolvente que permite recolectarlo fácilmente). Asimismo, también se evaluó la opción de no realizar una LLE, y se plantearon dos nuevos enfoques: por un lado, filtrar la disolución de muestra a través de un filtro de nylon de tamaño de poro de 0.45 µm para eliminar la parte no soluble de la matriz antes de la microextracción, y por otro lado, no realizar ninguna etapa de limpieza y simplemente lixiviar los analitos de la muestra en agua.

Esta última opción se planteó porque se observó, en experimentos previos, que la gota turbia mencionada antes, que se formaba cuando la microextracción se realizaba directamente sobre la disolución de muestra con 80 µL de cloroformo, se convertía en una fina capa entre la fase dadora acuosa y la fase extractante si se aumentaba el volumen de cloroformo. De este modo se conseguía generar un pequeña gota transparente de extracto en la punta del tubo de microextracción, fácil de recoger e inyectar en el sistema GC-MS. Sin embargo, en este caso, fue necesario aumentar el volumen de cloroformo hasta 120 µL para efectuar la VA-DLLME.

La *Figura 6.5* compara estos enfoques y muestra que, en general, los mejores resultados se lograron aumentando el volumen de cloroformo sin realizar un paso previo de limpieza, a pesar de no utilizar el valor considerado como óptimo para el volumen de disolvente de extracción.

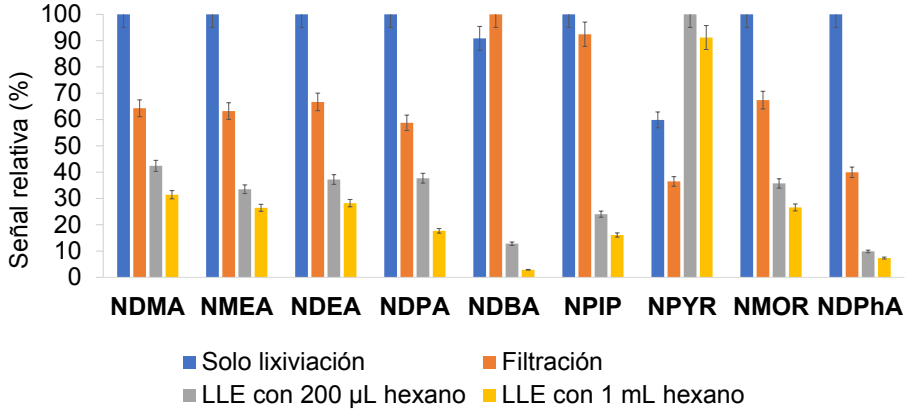


Figura 6.5. *Estudio de pretratamiento de muestra: comparación entre la limpieza por medio de LLE con 1 mL y 200 µL de hexano, filtración y ningún paso de limpieza con el aumento del volumen del disolvente de extracción.*

Los resultados obtenidos con la metodología seleccionada fueron muy positivos, ya que casi no se perdieron analitos durante el proceso en comparación con los otros enfoques considerados, en los cuales se manifiesta una perdida generalizada de los analitos. Además, el

enfoque seleccionado permite evitar un paso de pretratamiento previo a la microextracción, reduciendo así el tiempo de análisis, y minimiza la cantidad de residuos producidos, que de lo contrario hubiera sido mayor.

Estudio del efecto matriz

Para evaluar el efecto matriz causado por las matrices cosméticas durante el procedimiento analítico, se realizó una calibración externa con disoluciones patrón de los analitos en agua de 0.01 a 10 ng mL^{-1} que se sometieron al proceso VA-DLLME optimizado. Asimismo, se prepararon y sometieron a la etapa de microextracción disoluciones de muestra sin fortificar y fortificadas a dos niveles de concentración de los analitos (0.5 y 1 ng mL^{-1}).

Los valores de los coeficientes de recuperación relativa obtenidos con este estudio oscilaron entre 11 y 431%, demostrando que el proceso analítico se vio afectado por un efecto matriz considerable. Por lo tanto, se propuso la calibración por adición de patrón para corregir el efecto matriz observado.

Parámetros analíticos del método propuesto

Linealidad

Se estudió la linealidad con disoluciones patrón de las N-nitrosaminas estudiadas sometidas al procedimiento propuesto. Se obtuvo un alto grado de linealidad, que alcanzó, al menos, hasta 20 ng mL^{-1}, obteniéndose valores de coeficiente de determinación (R^2) superiores a 0.990.

Factor de preconcentración

Los factores de preconcentración (FP) obtenidos, calculados como FP = C_{ext}/C_0), donde C_{ext} es la concentración del analito en el extracto y C_0 es la concentración del analito en la fase dadora, variaron entre 2 y 100, debido a las diferentes polaridades de las N-nitrosaminas en estudio, que afectan a la eficiencia de extracción de cada una de ellas.

Límites de detección y cuantificación

Los límites de detección y cuantificación se calcularon como tres y diez veces, respectivamente, la relación señal/ruido de un patrón sometido al procedimiento de extracción, mientras que los límites de detección y cuantificación del método se calcularon teniendo en cuenta además el procedimiento de preparación de la muestra.

Los valores obtenidos, que se indican en la *Tabla 6.5*, están muy por debajo del valor umbral de 50 μg kg^{-1} para trazas de N-nitrosaminas en productos cosméticos establecido por el Reglamento Europeo [1,76], lo que confirma que el método propuesto es adecuado para la determinación de estos compuestos en este tipo de matrices.

Tabla 6.5. Límites de detección y cuantificación

Analito	LOD[a] (ng L^{-1})	LOQ[a] (ng L^{-1})	MLOD[b] (μg kg^{-1})	MLOQ[b] (μg kg^{-1})
NDMA	74.3	247.5	7.4	24.8
NMEA	91.5	304.9	9.2	30.5
NDEA	19.1	63.6	1.9	6.4
NDPA	4.4	14.7	0.4	1.5
NDBA	0.2	0.6	0.02	0.06
NPIP	18.6	61.9	1.9	6.2
NPYR	33.3	110.9	3.3	11.1

NMOR	83.8	279.3	8.4	27.9
NDPhA	35.0	116.8	3.5	11.7

[a] LOD: límite de detección; LOQ: límite de cuantificación
[b] MLOD: límite de detección del método; MLOQ: límite de cuantificación del método

Repetibilidad

La repetibilidad de la extracción, expresada como desviación estándar relativa (RSD), se evaluó aplicando el método propuesto a cinco réplicas de tres disoluciones patrón en agua que contenían las N-nitrosaminas a tres diferentes niveles de concentración (0.1, 0.5 y 5 ng mL^{-1}), el mismo día (intra-día) y durante cinco días consecutivos (inter-día).

Los resultados obtenidos, que se muestran en la *Tabla 6.6*, indican que se logró una buena repetibilidad (RSD% < 13%)

Tabla 6.6. Estudio de la repetibilidad

Analito	**RSD (%) [a]**					
	Intra-día (N^b = 5)			**Inter-día (N^b = 5)**		
	0.1 ng mL^{-1}	**0.5 ng mL^{-1}**	**5 ng mL^{-1}**	**0.1 ng mL^{-1}**	**0.5 ng mL^{-1}**	**5 ng mL^{-1}**
NDMA	n.a.[b]	6.7	3.3	n.a.	10.9	4.3
NMEA	n.a.	4.6	2.1	n.a.	7.2	9.1
NDEA	3.0	3.4	3.3	7.3	4.6	4.6
NDPA	4.6	1.1	4.5	6.0	7.6	12.4
NDBA	6.0	4.4	3.7	6.6	8.4	12.5
NPIP	5.3	4.2	4.8	9.7	12.5	7.6

NPYR	7.4	2.2	3.8	6.1	11.1	5.2
NMOR	n.a.	2.0	2.7	n.a.	8.4	5.5
NDPhA	4.3	2.4	6.9	9.9	6.7	7.7

[a] desviación estándar relativa
[b] N: número de réplicas
[c] n.a.: no aplicable, ya que la concentración está por debajo del LOQ

Análisis de muestras

Se analizaron, mediante el método propuesto, tres muestras cosméticas disponibles comercialmente (un gel calmante para después del sol, una crema hidratante y una crema reafirmante).

Como se puede observar en la *Tabla 6.7*, los resultados obtenidos en el análisis de las tres muestras cosméticas muestran que en todas ellas se detectaron y determinaron algunas de las N-nitrosaminas.

En el gel calmante se determinaron seis N-nitrosaminas, tres de las cuales superaban el límite de seguridad de 50 μg kg^{-1} definido por el Reglamento Europeo (770 ± 90 μg kg^{-1} de NMEA, 50.6 ± 0.3 μg kg^{-1} de NPIP y 114 ± 5 μg kg^{-1} de NPYR).

En cuanto a las cremas, en la primera crema solo se cuantificó NMOR (870 ± 60 μg kg^{-1}), que superaba ampliamente el límite de seguridad, mientras que en la segunda crema solo se cuantificó NMEA (560 ± 20 μg kg^{-1}), que también se encontró por encima del límite de seguridad.

Cabe señalar que las etiquetas de estas tres muestras analizadas incluían dos ingredientes que, si bien son ingredientes permitidos (bronopol como conservante y trietanolamina como regulador de pH), pueden reaccionar bajo ciertas condiciones y llegar a formar involuntariamente N-nitrosaminas.

Tabla 6.7. Aplicación del método propuesto al análisis de muestras

Analito	Cantidad encontrada[a] (µg kg^{-1})		
	Gel calmante para después del sol	Crema hidratante	Crema reafirmante
NDMA	< LOD	< LOD	< LOD
NMEA	770 ± 90	< LOD	560 ± 20
NDEA	< LOD	< LOD	< LOD
NDPA	1.19 ± 0.01	< LOD	< LOD
NDBA	16.5 ± 0.1	< LOD	< LOD
NPIP	50.6 ± 0.3	< LOD	< LOD
NPYR	114 ± 5	< LOD	< LOD
NMOR	< LOD	870 ± 60	< LOD
NDPhA	10.57 ± 0.03	< LOD	< LOD

[a] expresado como media ± desviación estándar de tres réplicas

Comparación del método propuesto con publicaciones anteriores

La *Tabla 6.8* muestra la comparación del método propuesto con otros métodos publicados anteriormente con el mismo propósito, tanto basados en extracción en fase sólida como en fase líquida. Como puede verse, los MLOD son comparables o incluso mejores que cuando se utilizan instrumentos analíticos más sensibles y caros (por ejemplo, detección MS/MS). En cuanto al tiempo de extracción, se evidencia que las técnicas de extracción basadas en disolventes, como el método propuesto, son mucho más rápidas ya que la extracción se logra prácticamente de manera instantánea (en menos de 1 min). Además, no se necesitan equipos sofisticados ni materiales comerciales (como sorbentes, cartuchos o fibras) para realizar la

extracción, lo que hace que el método sea asequible para la mayoría de los laboratorios. Por otro lado, el principal inconveniente del método propuesto es el uso de un disolvente organoclorado como fase de extracción, lo que va en contra de las tendencias actuales en Química Analítica, aunque solo se utilizan unos pocos microlitros por extracción.

Con respecto a este último aspecto, la ecosostenibilidad de la preparación de muestra de cada uno de los métodos comparados se evaluó utilizando la nueva herramienta métrica denominada AGREEprep [244]. Esta herramienta métrica considera diversos factores de impacto ambiental y de salud como el uso de reactivos, la energía consumida, los posibles riesgos laborales y los residuos generados, entre otros. Esta herramienta puntúa la etapa de preparación de la muestra en una escala de 0 a 1, donde 0 es la peor puntuación y 1 la máxima puntuación. Como se puede observar en la *Tabla 6.8*, la puntuación obtenida por el método propuesto es positivamente comparable a los otros métodos, ya que el pretratamiento de la muestra es sencillo (es suficiente la lixiviación de los analitos en agua), a diferencia de aquellos métodos donde se requieren mayores cantidades de muestra o de disolventes, como metanol, diclorometano y/o hexano.

Table 6.8. Comparación del método propuesto con otros métodos basados en técnicas de extracción para la determinación de N-nitrosaminas en cosméticos

N-nitrosaminas	Pretratamiento de muestra[a]	Técnica de extracción[b]	Tiempo[c]	Solventes orgánicos[a]	Técnica instrumental[d]	MLOD (μg g^{-1})	AGREEprep score	Ref.
10	1 g + 10 mL MeOH:DCM; Sonicación 10 min; Centrifugación 15 min	SPE	n.r.	6 mL MeOH (Acondicionamiento); 3 mL MeOH (30%) (Lavado); 8 mL MeOH (Desorption); Evaporación + 1 mL MeOH	GC-MS/MS	700 - 3000	0.16	[100]
13	0.2 g + 4 mL H_20	LLE	Vórtex 1 min (Extracción) Centrifugación 3 + 2 min	3 mL ACN x 2 (Extracción) Evaporación + 1 mL EtAc	GC-MS	3 - 15	0.50	[101]
7	5 g + 5 mL DCM:MeOH; Sonicación 30 min	HS-SPME	30 min (Extracción)	-	GC-MS	0.46 - 36.54	0.24	[102]
11	1 g + 7 mL ACN; Sonicación 10 min; Centrifugación 3 min	DSPE	Vórtex 20 min (Extracción) Centrifugación 3 min	Evaporación + 1 mL MeOH	LC-MS/MS	7 - 250	0.25	[105]
7	0.1 g + 5 mL HEX; Vórtex; Centrifugación 5 min	VA-RP-DLLME	Vórtex 0.5 min (Extracción) Centrifugación 5 min	-	LC-MS	1.8 - 50	0.48	[103]

8	0.5 g + 25 mL H_20; Vórtex; 1 mL HEX; Centrifugación 15 min	SBSDME	30 min (Extracción) 1 min (Desorción)	1 mL ACE (Desorción)	LC-MS/MS	3 - 13	0.37	[104]
10	0.1 g + 0.2 Na_2SO_4 + 0.4 g Florisil	µ-MSPD	n.r.	0.1 Florisil 1+10 mL EtAC (Desorción)	GC-MS	12 - 150	0.49	[169]
9	0.05 g + 5 mL H_20	VA-DLLME	Vórtex 1 min (Extracción) Centrifugación 5 min	120 µL CH_3Cl (Extracción)	GC-MS	0.06 - 30.5	0.59	Trabajo propuesto

[a] ACE: acetona; ACN: acetonitrilo; EtAc: etil acetato; DCM: diclorometano; HEX: hexano; MeOH; metanol

[b] DSPE: extracción dispersiva en fase sólida; HS-SPME: microextracción en fase sólida en espacio de cabeza; LLE: extracción líquido-líquido; µ-MSPD: microdispersión de matriz en fase sólida; SBSDME: microextracción dispersiva por sorción sobre barra agitadora; SPE: extracción en fase sólida; VA-RP-DLLME: microextracción líquido-líquido dispersiva en fase reversa assistida por vórtex

[c] n.r.: no reportado

[d] GC: cromatografía de gases; LC: cromatografía de líquidos; MS: espectometría de masas; MS/MS: espectometría de masas en tándem

CONCLUSIONES

En este capítulo:

Se ha propuesto un método analítico para la determinación de nueve N-nitrosaminas prohibidas en productos cosméticos.

- → El método propuesto se basa en un pretratamiento de la muestra relativamente simple, consistente en la lixiviación de los analitos en agua, seguido de VA-DLLME y análisis mediante GC-MS
- → Se han optimizado las variables que intervienen en la etapa de microextracción y se han realizado estudios comparativos sobre el pretratamiento de muestra para encontrar la mejor estrategia que permitiese determinar el mayor número de nitrosaminas de forma simultánea con la sensibilidad requerida, favoreciendo su extracción de las matrices cosméticas complejas sin perder analitos durante el procedimiento
- → El método propuesto presenta buenas características analíticas y un procedimiento sencillo y asequible, características que lo convierten en un método adecuado para el control de calidad de los cosméticos con el fin de garantizar la seguridad de los usuarios y el cumplimiento del Reglamento Europeo sobre productos cosméticos

CONCLUSIONES GENERALES

En la presente Tesis Doctoral se han desarrollado y validado diferentes métodos analíticos para la determinación de sustancias de interés en productos cosméticos.

Durante el desarrollo de los métodos analíticos propuestos se han considerado las pautas establecidas por el Reglamento Europeo para las sustancias en examen (en el caso de las sustancias prohibidas), garantizando la sensibilidad requerida, además de demostrar la aplicabilidad del método a diferentes tipologías de matrices, considerando la elevada variabilidad que existe en las formulaciones de los productos cosméticos.

Además, se ha prestado especial atención a las directrices de la Química Analítica Verde y los principios de la Preparación de Muestra Verde proponiendo, en la medida de lo posible, métodos que fueran inocuos para el operador y respetuosos con el medio ambiente. En este sentido, en las estrategias propuestas se ha intentado reducir el volumen de disolventes orgánicos utilizados, reducir la cantidad de muestra empleada, utilizar equipos con menor consumo energético y generar la cantidad mínima de residuos.

Los analitos objeto de estudio han sido ingredientes activos (cannabidiol) y sustancias prohibidas (acrilamida y N- nitrosaminas).

En relación con la determinación de cannabidiol:

→ Se ha desarrollado y validado un método analítico para su determinación en diferentes tipos de productos cosméticos y una materia prima (**Capítulo 3**).

El método contempla una preparación de la muestra rápida y sencilla, ya que consta solo de una etapa de lixiviación del analito en etanol y del acondicionamiento de la disolución de medida, previo al análisis mediante LC-MS/MS. Con esta estrategia se obtiene una amplia linealidad que permite determinar el cannabidiol tanto a nivel de trazas, sin necesidad de una etapa de preconcentración, como a concentraciones elevadas, sin necesidad de una elevada dilución.

En relación con la determinación de acrilamida, una sustancia mutagénica y potencialmente cancerígena prohibida en los productos cosméticos, se presentan dos métodos analíticos, y cabe destacar que el primero de ellos ha dado lugar a la primera publicación científica sobre este tema:

- → El primero de los métodos propuestos consta de distintas etapas cuya finalidad es obtener una buenas selectividad y sensibilidad utilizando un equipo asequible para la mayoría de los laboratorios (LC-UV). El método se basa en una etapa previa de limpieza con hexano, seguida por la derivatización de la acrilamida con 2-naftalentiol y el empleo de DLLME convencional como técnica de microextracción para preconcentrar el analito antes de su determinación (**Capítulo 4**).

- → El segundo método propuesto se desarrolla ofreciendo un enfoque distinto, con el fin de evitar reacciones de derivatización y hacer más práctico el método. Por esta razón, se desarrolla y valida un procedimiento basado en VA-RP-DLLME, como técnica de microextracción, seguida de análisis mediante LC-MS/MS (**Capítulo 5**).

Finalmente, en relación con la determinación de N-nitrosaminas:

- → Se ha desarrollado y validado un método analítico para la determinación de nueve N-nitrosaminas prohibidas en productos cosméticos, basado en VA-DLLME, como técnica de microextracción, seguida de análisis mediante GC-MS. Las variables involucradas en el proceso de microextracción se optimizaron mediante un diseño de Box-Behnken, se estudiaron diferentes enfoques para el tratamiento de muestras y, además, se comparó el método propuesto con otros publicados anteriormente, evaluando la ecosostenibilidad de la preparación de muestra utilizando la nueva herramienta métrica AGREEprep (**Capítulo 6**).

Todos los métodos desarrollados en el marco de la presente Tesis Doctoral permiten la determinación de los compuestos de interés de forma rápida y sencilla. Además, se alcanzan sobradamente los límites de seguridad establecidos por los organismos reguladores, si es el caso. Todo ello hace de estos métodos una herramientas extremadamente útiles para el control de calidad en la industria cosmética atendiendo a la demanda de las propias empresas y organismos reguladores.

LISTA DE SIGLAS Y ACRÓNIMOS

μ-MSPD

Microdispersión de matriz en fase sólida

AA

Air-assisted

ANOVA

Análisis de Varianza

BAμE

Microextracción por adsorción en barra

BHA

Butilhidroxianisol

BHT

Butilhidroxitolueno

CAR

Carboxeno

CAS

Chemical Abstracts Service

CBD

Cannabidiol

CE

Comisión Europea

CME

Microextracción capilar

CosIng

Cosmetic Ingredient database

CPE

Extracción en punto de nube

CZE

Electroforesis capilar de zona

DES

Disolvente eutéctico profundo

DLLME

Microextracción líquido-líquido

dispersiva

DSPE

Extracción en fase sólida dispersiva

DVB

Divinilbenceno

EN

Norma Europea

ESI

Ionización por electronebulización

FID

Detector de ionización en llama

FP

Factor de preconcentración

FPSE

Extracción por sorción sobre tejido

GC

Cromatografía de gases

GO

Óxido de grafeno

HF

Fibra hueca

HPLC

Cromatografía de líquidos de alto rendimiento

HS

Espacio de cabeza

IARC

International Agency for Research on Cancer

IL

Líquido iónico

ISO

Organización Internacional de Normalización

IT

In tube

LC

Cromatografía de líquidos

LLE

Extracción líquido-líquido

LOD

Límite de detección

LOQ

Límite de cuantificación

LPME

Microextracción en fase líquida

MEPS

Microextracción por sorbente empaquetado

MIPs

Polímeros de impresión molecular

MLOD

Límite de detección del método

MLOQ

Límite de cuantificación del método

MP-SPE

Extracción en fase sólida protegida por membrana

MRM

Monitorización de reacciones múltiples

MS

Espectrometría de masas

MS/MS

Espectrometría de masas en tándem

MSA

Asistida por agitación magnética

MSPD

Dispersión de matriz en fase sólida

NDBA

N-nitrosodibutilamina

NDEA

N-nitrosodietilamina

NDELA

N-nitrosodietanolamina

NDMA

N-nitrosodimetilamina

NDPA

N-nitrosodipropilamina

NDPhA

N-nitrosodifenilamina

NMEA

N-nitrosoetilmetilamina

NMOR

N-nitrosomorfolina

NPIP

N-nitrosopiperidina

NPYR

N-nitrosopirrolidina

PAHs

Hidrocarburos aromáticos policíclicos

PDMS

Polidimetilsiloxano

PEG

Polietilenglicol

PLE

Extracción con líquidos presurizados

PTFE

Politetrafluoroetileno (teflón)

RP

Fase reversa

RSD

Desviación estándar relativa

RSM

Metodología de superficie de respuesta

SBSE

Extracción por sorción sobre barra agitadora

SBSDME

Microextracción dispersiva por sorción sobre barra agitadora

SCCNFP

Scientific Committee on Cosmetic and Non-Food Products Intended for Consumers

SCCS

Scientific Committee on Consumer Safety

SDME

Microextracción en gota

SFOD

Solidificación de gota orgánica flotante

SIM

Monitorización de ion seleccionado

SPE

Extracción en fase sólida

SPME

Microextracción en fase sólida

SUPRAs

Disolventes supramoleculares

TBHQ

Tert-butilhidroquinona

TEA

Analizador térmico de energía

THC

Tetrahidrocannabinol

TIC

Total ion chromatogram

TMEDA

Tetrametiletilendiamina

UE

Unión Europea

UNE

Asociación Española de Normalización (Una Norma Española)

USA

Asistido por ultrasonidos

UV

Ultravioleta

VA

Asistido por vórtex

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ANEXO

PUBLICACIONES DE LA TESIS DOCTORAL

A comprehensive review on the use of microextraction techniques in the analysis of cosmetic products

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A comprehensive review on the use of microextraction techniques in the analysis of cosmetic products

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ABSTRACT

Microextraction techniques have gained special relevance since their appearance in the 90s, and the analysis of cosmetic products has not been immune to this trend. The first application of microextraction techniques in this field was published 25 years ago and, since then, these techniques have become the most widely used methodologies in the analysis of these everyday products, demonstrating that the advantages they provide in sample preparation are in line with the needs and principles currently pursued. This review presents a comprehensive description of those contributions in the analysis of cosmetic products based on microextraction techniques, from the first precedent to the present. The trends within the topic have been properly discussed, highlighting the most recent extraction phases and the benefits they present over the traditional ones.

1. Introduction

Sample preparation has become one of the strengths in the Analytical Chemistry field since it involves sample conditioning to the requirements for its analysis. This stage is especially relevant when it is used to isolate the target analytes from a complex matrix, and/or to preconcentrate them when they are at low concentration levels.

For the last decades, there has been an increasing interest to develop methodologies with good analytical parameters, especially those related to sensitivity and selectivity, in accordance with the principles of Green Analytical Chemistry [1] and, most specifically, with those of the recently proposed Green Sample Preparation [2]. For this purpose, the so-called microextraction techniques provide an excellent opportunity to achieve both purposes at the same time. In these techniques, that emerged as clear alternatives to traditional extraction techniques (i.e., liquid-liquid extraction (LLE) and solid-phase extraction (SPE)), very small amounts of extraction solvent or sorbent are used in comparison to those required in LLE or SPE, respectively. These are not only the unique advantages but also they produce less wastes, which make them eco-friendly approaches; the analysis time is reduced; the automation and coupling with analytical instruments is more feasible; sensitivity and enrichment factors are improved; and the required sample volumes are lower. All that, which is in clear agreement with the aforementioned principles, has contributed to increase their use in the last decades [3].

Microextraction techniques can be classified in two main groups depending on the nature of the extraction phase: those based on solid extraction phases and those based on solvents as extraction phases. It should be noted that, despite their appearance about three decades ago, microextraction techniques are in continuous development in order to improve operational aspects, such as reducing the manual intervention and speeding up the analysis time. Indeed, they have evolved towards the design, synthesis, and application of greener and sustainable sorbents (in the case of sorbent-based microextraction techniques) and solvents (in the case of solvent-based microextraction techniques), all of them with interesting and effective extraction properties [4]. Thus, they have become one of the axes on which researchers across the globe have turned in recent decades.

As evidence of it, microextraction techniques have been successfully applied for the determination of a wide range of analytes in different matrixes [5] such as food [6], environment [7], pharmaceuticals [8], or biological fluids [9], among others. The analysis of cosmetic products has not been immune to this trend, and different contributions on this topic can be found in the literature [10].

It should be said that cosmetic products are regulated worldwide to ensure the functioning of the internal market and a high level of protection of human health. In Europe, the European Regulation on Cosmetic Products (Regulation (EC) N° 1223/2009) [11] is the current regulatory framework for cosmetic products. In this regulation, there is a list of substances that are prohibited to add to cosmetic products (Annex II) including more than 1600 chemical compounds. However, despite the goodwill of the manufacturers, some of these harmful substances to the user's health may be found in the finished cosmetic product due to un-

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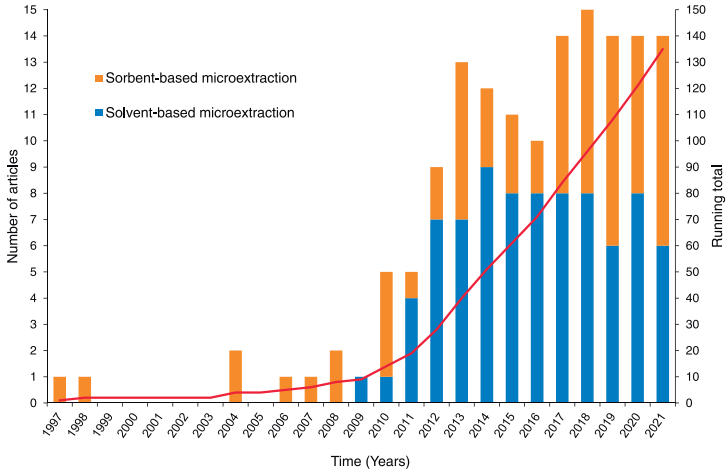

Fig. 1. Time trend of microextraction techniques applied to cosmetic products. The red line indicates the cumulative total.

intentional causes, such as deficiencies in the purification of raw materials, degradation of some ingredients, formation of by-products during the production or storage, and/or contamination from the container. Thus, due to their low concentration levels expected, microextraction techniques can be very useful for the determination of these prohibited substances, needing to preconcentrate them to reach the required limits of detection/quantification.

Additionally, EU Regulation also includes lists of substances subjected to restrictions in terms of maximum allowed concentration, type of product, target user, etc. (Annexes III-VI). Therefore, it is important the development of reliable analytical methods to improve and facilitate the quality control in the cosmetic industry and ensure that these conditions are met. As these restricted compounds can be freely employed not beyond the conditions there laid down, they are not expected to be at trace level, and thus microextraction techniques would not be necessary. However, it should be take into account that cosmetic products are complex matrices formed by a high number of substances variable depending on the cosmetic product [12]. In this way, it may be necessary to separate the target analytes from the matrix prior to instrumental analysis in order to avoid matrix effects. Therefore, again microextraction techniques might be useful, in this case for clean-up purposes. The same should be applied to those allowed ingredients that are not subjected to any restriction, and thus not listed in the EU Regulation.

This review presents a comprehensive compilation of those published papers on the application of microextraction techniques in the analysis of cosmetic products, spanning from the first precedent in 1997, that means, 25 years ago, to the present. A total of 139 articles are compiled and briefly discussed focusing on the microextraction stage, and an exhaustive consensus of the names and acronyms of all the developed techniques was made in order to avoid duplication of acronyms for the same techniques. Fig. 1 shows a histogram of all these articles according to the year of publication, which reflects the high potential and the great attention that scientific community has paid to microextraction techniques for the analysis of cosmetic products in the last twenty-five years.

As can be seen, the time trend shows that both types of microextraction techniques have reached and maintained a level of application that has not diminished over time, ranking among the most used procedures in the analysis of cosmetic products as they are cutting-edge techniques that easily adapt to new research trends, and to environmental and operational needs. The application of sorbent-based microextraction techniques to the analysis of cosmetic products began long before the application of solvent-based microextraction techniques. However, there was a clear trend towards greater use of the latter per year (more pronounced in the period 2011–2016), likely due to their greater simplicity, rapidity and low cost, mainly caused by use of solvents as extraction phase. From 2016 to present, the number of publications of both types of techniques has remained almost similar, most probably due to the awareness of researchers to design new sorbent materials with better extraction capabilities (i.e., selectivity) in a simpler way.

It should be emphasized that applying a microextraction technique directly to a cosmetic product is not possible in most of the cases due to the nature and complexity of the matrixes (emulsions, gels, solids, solutions with high alcohol content, etc.). Then, a preliminary sample pretreament, such as dilution in a suitable solvent [13], leaching the analytes from the cosmetic matrix to an appropriate solvent by ultrasounds [14] or vortex [15], is often required. In other cases, it might be even necessary to perform a previous clean-up step, either by LLE [16,17], SPE [18], pressurized liquid extraction (PLE) [19], dispersive solid-phase extraction (DSPE) [20,21] or by matrix solid-phase dispersion (MSPD) [22], among others, thus eliminating the matrix that might negatively affect the application of the subsequent microextraction technique. This review is focused on the microextraction step, but not on the entire sample treatment, and thus an exhaustive description of these sample pretreatment methodologies is not included.

2. Sorbent-based microextraction techniques

Sorbent-based microextraction techniques make use of sorbents to isolate the target analytes. Usually, this sorbent is placed on the surface of an inert support exposed to the donor phase, dispersed into the donor phase itself, or packed into a device. Later, the analytes need to be eluted from the sorbent, what is also a crucial step to their determination. For this purpose, an appropriate solvent is directly contacted with the sorbent to elute the analytes (liquid desorption), or the sorbent is thermally desorbed by direct coupling with the analytical instrument.

The use of suitable sorbents is a critical factor to get selective, precise, and accurate extraction of the analytes, so the nature and physicochemical properties of the sorbent material are very important [23].

Those papers describing analytical methods for the analysis of cosmetic products based on sorbent-based microextraction techniques are discussed below. The employed sorbent, analytes, type of samples, analytical technique, and some relevant analytical performances are summarized in Table 1 .

2.1. Solid-phase microextraction

Since the introduction of solid-phase microextraction (SPME) in 1990 [24], it quickly became one of the most widely used microextraction techniques to date, if not the most. This technique is based on the transfer of the analytes from the sample matrix to an adsorbent fiber held inside a needle holder by exposing it either directly immersed to the sample (DI-SPME) or to its headspace (HS-SPME) [25], the latter broadly employed for (semi)volatile analytes. To enhance the extraction performance by promoting the contact between analytes and the adsorbent fiber, the sample matrix is stirred by a magnet. After extraction, the fiber is retracted into the needle to later perform the desorption of the analytes.

This technique was the first microextraction technique used for the analysis of cosmetic products, when Struppe et al. in 1997 [26] determined nitro musks in creams. By this way, they developed a selective and sensitive method that simplified the cosmetic sample preparation procedure. After this work, many researchers used SPME as microextraction technique for the analysis of cosmetic products and, to this day, it is still the most widely used sorbent-based microextraction technique in this field.

The non-polar polydimethylsiloxane (PDMS) is the most commonly used material as fiber coating, either alone [26–28] or in combination with other materials such as divinylbenzene (DVB) [29–34] or carboxen (CAR) [35]. PDMS-DVB and PDMS-CAR, or even PDMS-DVB-CAR [36], which present bipolar character, allow the simultaneous extraction of both polar and non-polar analytes, such as formaldehyde, fragrance allergens, nitrosamines and phthalates as it is shown in Table 1. It should be remarked that the widely use of these materials is due to the fact that they are commercially available. Other commercially available material that has been employed for the determination of antioxidants and preservatives [37], and different additives [38] is polyacrylate (PA).

In addition, to improve the selectivity of the extraction, different home-made materials have been used depending on the physicochemical characteristics of the analytes. For this purpose, a polyethylene glycol diacrylate (PEGDA) fiber was employed to determine parabens due to the high surface area and good extraction efficiency [39]. A composite based on an aromatic polymeric ionic liquid (PIL) was employed for the determination of five polycyclic aromatic hydrocarbons (PAHs) due to its hydrophobic and aromatic nature [40]. Graphene oxide (GO)-derived composite materials were also exploited for the determination of different compounds such as PAHs [41], benzenes [42], parabens [43] and fragance allergens [44].

For the extraction of the nitrosamine N-nitrosodiethanolamine (NDELA) from different shampoo samples, an aluminium hydroxide grafted fused silica fiber that acts as a Lewis acid as well as an oxophile was employed [45]. A porous aromatic framework (PAF) was synthesized by employing melamine and 4,4'biphenyldiboronic acid, according to a feasible Chan-Lam reaction, and a home-made SPME device was prepared for the determination of antioxidants and parabens [46]. Recently, even a 3D printed device has been employed using silver-polyaniline (Ag/PANI) coated pencil lead as extractant phase for the determination of phthalates in different cosmetic matrixes [47].

The preferred instrumental technique was gas chromatography (GC) coupled either with flame ionization detector (FID) or with mass spectrometry (MS), due to the possibility of inserting the fiber into the GC inlet without the need for liquid desorption. Liquid chromatography (LC) was employed in only one method, where parabens were determined [39].

2.2. Stir bar sorptive extraction

Stir bar sorptive extraction (SBSE) [48], although in a lesser extent than SPME, has also been employed in the analysis of cosmetic products. In this technique, the sorbent coats a stir bar usually submerged in the liquid donor phase, which is magnetically stirred to facilitate the extraction of the target compounds. Once the extraction is accomplished, the stir bar is taken out and dried with a tissue prior the desorption of the analytes.

The first applications of SBSE in the analysis of cosmetic products used PDMS as sorbent phase, due to its commercial availability, and it was applied for the extraction of parabens [49] and triclosan [50]. As in the SPME, home-made coatings were later employed to increase the selectivity and sensitivity. In this sense, parabens were also extracted by a metal-organic framework (MOF) (i.e., MIL-68) immobilized onto the stir bar surface [51], and by an amino and hydroxyl groups enriched bifunctional microporous organic network (NH_2/OH B-MON) [52].

Other analytes were also extracted by SBSE-based procedures. Thus, Siritham et al. used a composite foam made of GO, polyethylene glycol and natural latex (GO/PEG/NL), crossed by a stainless steel wire that allowed its stirring similarly to SBSE, for the extraction of butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and other antioxidants [53]; and Zang et al. extracted different chlorophenols using a hollow tube filled with a magnetic composite made of Fe_3O_4 magnetic nanoparticles (MNPs), reduced graphene oxide (rGO) and graphitic carbon nitride (g-C_3N_4) (Fe_3O_4/rGO/g-C_3N_4) agitated by itself under a magnetic field [54].

2.3. Dispersive solid-phase extraction

In dispersive solid-phase extraction (DSPE), the extractant sorbent is dispersed in the donor phase [55,56], increasing the contact area between the sorbent and the analytes, thus improving the extraction kinetics and, in this way, increasing the extraction performance considerably. The dispersion can be achieved by different ways, such as shaking, stirring, vortex assisted, ultrasound assisted or by effervescence [57]. Once the extraction time is completed, the sorbent is usually recovered by decantation and/or centrifugation for desorption stage.

MOF HKUST-1 [58] and a molybdenum disulfide-graphene oxide (MoS_2/GO) [59] have been employed for the determination of parabens. Moreover, the MOF MIL-101(Cr) was employed for the first time for the determination of hormones [60].

It should be noted that DSPE become more widespread due to the employment of magnetic sorbents since they allow their effective separation from the donor phase by applying an external magnetic field simply provided by a magnet, and thus no separation methods such as centrifugation or filtration, typically used in conventional DSPE, are required [61]. Due to these advantages, magnetic materials have been used for the determination of different analytes, such as parabens [62–64]. They have also been applied for the determination of glucocorticoids using magnetic molecularly imprinted polymers (MIPs) [65,66] with higher selectivity than non-imprinted polymers (NIPs) due to the complementary shape, size, and molecular interactions of imprinting sites of MIPs cavities to the analytes formed during their synthesis. MIPs can be synthesized for just one analyte or can be prepared with multiple templates to recognize different analytes. Glucocorticoids have also been determined by a magnetically functionalized g-C_3N_4 bonded to the MOF MIL-101(Cr) [67]. Dye rhodamine B was extracted with Fe_3O_4 MNPs coated with poly(aniline-naphthylamine) (Fe_3O_4@PANI-NA) for its determination in eye shadows, shampoos and hand washing products [68].

An effervescence-assisted DSPE procedure, where a tablet made of a carbon dioxide source (sodium carbonate), a proton donor (citric acid)

Table 1
Published papers on the use of sorbent-based microextraction techniques in the analysis of cosmetic products[a].

Extraction phase[b]	Analytes[c]	Matrix	Instrumental technique[d]	R (%)	LOD (ng mL^{-1})	RSD (%)	Ref.
Solid-phase microextraction (SPME)							
PDMS	Nitro musks	Bath cream	GC-AED	n.r.	500-1000[e]	<9.0	[26]
PDMS	1,4-dioxane	Shampoo and liquid soap	GC-MS	96.0-98.8	60-510	<3.2	[27]
PDMS	Perfume compounds	Shampoo	GC-FID	n.r.	0.02-0.20	<5.5	[28]
PDMS-DVB	Phthalates	Glycerin and liquid paraffin	GC-MS	78-119	0.05-86.40[e]	<13.2	[29]
PDMS-DVB	Fragrance allergens	Baby oil, lip balm, olive cream, deodorant, antiperspirant, face mask, face lotion, shampoo and toothpaste	GC-FID	>80	7-2700	<7.6	[30]
PDMS-DVB	Formaldehyde	Shampoo, conditioner, face wash and face mask	GC-MS	80	n.r.	n.r.	[31]
PDMS-DVB	5-bromo-5-nitro-1,3-dioxane	Shampoo, body cleansers and facial exfoliants	GC-μECD	n.r.	66[e]	<9.0	[32]
PDMS-DVB	Formaldehyde	Nail polish, shower gel and make-up	GC-FID	89-98	40	<9.5	[33]
PDMS-DVB	Formaldehyde	Hair gel	GC-FID	n.r.	4.6	12	[34]
CAR-PDMS	Nitrosamines	Cream	GC-MS	54-98	0.46-36.54[e]	<19.0	[35]
DVB-CAR-PDMS	Preservatives	Body milk, moisturizing cream, deodorant, sunscreen, baby after sun, moisturizing lotion, make-up, eye make-up remover, liquid soap, child bath gel, tooth paste, shampoo, hair conditioner, facial cleansing milk and after shave	GC-MS/MS	79.9-130.0	5.9-910[e]	<15	[36]
PA	Antioxidants, preservatives	Cream	GC-MS	83-98	0.4-8.5[e]	<15.2	[37]
PA	Additives	Emulsion, lotion and body cream	GC-MS	n.r.	0.5-8.3[e]	<7.8	[38]
PEGDA	Parabens	Sun block, hand lotion, cream and body lotion	LC-UV/Vis	90.2-97.7	120-150	<5.4	[39]
[C_8VIM][NS]	PAHs	Hair spray and nail polish	GC-FID	n.r.	0.005-0.010	<11.7	[40]
g-C_3N_4/rGO	PAHs	n.r.	GC-MS	70-118	0.001-0.002	<8.9	[41]
P(BT-3-MeT)/rGO	Benzenes	Nail polish, hair dye and correction fluid	GC-FID	85.8-106.2	0.00525-0.0125	<5.4	[42]
Ppy/MnO_2-rGO/[AVIM][NTf_2]	Parabens	Perfume	GC-FID	82.8-116.8	0.0028-0.0064	<10.2	[43]
β-CD/GO	Fragrance allergens	Shampoo and skin care water	GC-FID	70.1-94.1	0.15-0.05	<11.4	[44]
Al(OH)$_3$ grafted	NDELA	Hair shampoo, body shampoo and hand washing liquid	GC-MS	95-99	1[e]	<7.2	[45]
PAF	BHA, BHT, parabens	n.r.	GC-FID	81.5-110.2	0.12-0.30	<7.2	[46]
Ag/PANI	Phthalates	Rubbing alcohol, contact lens cleaner, saline solution, eye cleaner and antibacterial disinfectant liquid	GC-FID	81.09-92.92	3.65-4.91	<5.5	[47]
Stir bar sorptive extraction (SBSE)							
PDMS	Parabens	Body cream, antiperspirant cream and sunscreen	LC-UV/Vis	90-99	n.r.	<5.3	[49]
PDMS	TCS	Toothpaste	LC-UV/Vis	78.5	0.1	3.6	[50]
MIL-68	Parabens	Sunscreen cream	LC-MS/MS	88.48-101.82	0.001-0.002	<8.5	[51]
NH_2/OH B-MON	Parabens	Lipstick	LC-UV/Vis	81.2-109.6	0.010-0.035	<7.3	[52]
GO/PEG/NL	MI, BHT, BHA	Mouthwash, hair shampoo and conditioner	GC-MS	84-107	0.5-5	<3	[53]
Fe_3O_4/rGO/g-C_3N_4	Chlorophenols	Toner	LC-UV/Vis	85-104	0.2-0.3[e]	<12	[54]
Dispersive solid-phase extraction (DSPE)							
HKUST-1	Parabens	Cream	LC-UV/Vis	63.7-121.0	100-600	<12	[58]
MoS_2/GO	Parabens	Toner	LC-UV/Vis	91.3-124.0	0.4-2.3	<9.9	[59]
MIL-101(Cr)	Hormones	n.r.	LC-UV/Vis	93-102	0.36-0.91	<6.1	[60]
Fe_3O_4/MWCNTs@MIL-101(Cr)	Parabens and phthalates	Skin cream, sunblock cream and foot cream	LC-UV/Vis	74.0-97.2	0.03-0.15	<9.1	[62]
Fe_3O_4/GO/curcumine	Parabens	Tooth paste and mouthwash	LC-UV/Vis	71.6-120.3	0.4-1.0	<7.7	[63]
Fe_3O_4@β-CD-TDI@[C_4MIM][Cl]	Parabens	Cream	LC-UV/Vis	80.3-117.3	0.02-0.09	<14.9	[64]
Dexamethasone-MIP	Dexamethasone	Skincare cosmetics	LC-UV/Vis	93.8-97.6	50	<2.7	[65]
Fe_3O_4@dtMIP	Glucocorticoids	Lotion, toner and mask	LC-UV/Vis	87-102	15	<2.6	[66]

(*continued on next page*)

Table 1 (*continued*)

Extraction phase[b]	Analytes[c]	Matrix	Instrumental technique[d]	R (%)	LOD (ng mL^{-1})	RSD (%)	Ref.
Fe_3O_4/g-C_3N_4/MIL-101(Cr)	Glucocorticoids	Facial mask and toner	LC-MS/MS	77-113	0.002	<5.5	[67]
Fe_3O_4@PANI-NA	Rhodamine B	Shampoo, eye shadow and hand washing liquid	FL	94-99	0.1	<8.2	[68]
Fe_3O_4/Zn-Al-LDH/PANI/SDBS	Heavy metals	Lipstick and brightening cream	FAAS	88.3-95.3	0.9-2.1	<6.1	[69]
Stir bar sorptive dispersive microextraction (SBSDME)							
$CoFe_2O_4$/MIL-101(Fe)	Nitrosamines	Shower gel and body cream	LC-MS/MS	96-109	3-13[e]	<17.0	[72]
$CoFe_2O_4$/rGO	PAHs	Face cream, cleansing milk, body milk and body cream	GC-MS	n.r.	0.02-2.50	<10.0	[73]
Micro matrix solid-phase dispersion (*μ*-MSPD)							
Florisil	Plasticizers and musks	Shampoos, soaps, body milks, sunscreens, creams, aftershave lotions and deodorants	GC-MS	84-105	1.4-300[e]	<10	[74]
Florisil	Dyes	Decorative makeup, lipsticks, lip gloss, toothpastes, regenerating creams, shampoos and eye shadows	LC-MS/MS	70-120	0.01-0.62	<15	[75]
Florisil	Fragrance allergens and preservatives	Shampoos, body milk, moisturizing milk, toothpaste, hand creams, gloss lipstick, sunblock, deodorants and liquid soaps	GC-MS/MS	83-115	0.4-37[e]	<15	[76]
Florisil	Preservatives	Toner, spray and lotion	GC-FID	80-124	53-180	<12	[77]
Florisil	Fragrance allergens, plasticizers, synthetic musks, preservatives and UV filters	Sunscreen, hair products, creams, make-up, lip balms, make-up and lipsticks	GC-MS/MS	97-111	n.r.	<10	[78]
Florisil	Fragrance allergens, preservatives, plasticizers and musks	Gels, shampoos, soaps, sunscreen, lotions, body milks, creams and deodorants	GC-MS	80-110	3-700[e]	<15	[79]
Florisil	PAHs, pesticides, phthalates, nitrosamines, dyes and fragrances	Hand creams and shower gels	GC-MS	72-116	0.09-1.3[e]	<15	[80]
Florisil	Glycols, Glycol ethers and their acetates	Moisturizing hand cream and shower gel	GC-MS	91-108	30-440[e]	<18	[81]
C18	Dyes	Hand soap, face painting, eye shadow, toothpaste, coloured hairspray and shampoo	LC-MS/MS	69-121	14.2-476[e]	<15	[82]
Sand	Colouring agents	Eye shadow, toothpaste and lipstick	LC-MS/MS	83-115	0.4-37[e]	<15	[83]
Silica	Preservatives	Creams	LC-UV/Vis	63-83	30-40[e]	<7.9	[84]
Capillary microextraction (CME)							
MAA-EGDMA	Phthalates	Lacquer remover and perfume	LC-UV/Vis	81.2-108.8	0.7-3.7	<7.7	[86]
MAA-EGDMA	Hormones	Toner	LC-UV/Vis	83-119	2.3-4.6	<7.7	[87]
BMA-EDMA/GN	Glucocorticoids	n.r.	LC-MS	83.7-103.8	0.12-1.93	<14.5	[88]
Microextraction by packed sorbent (MEPS)							
C18	Parabens	Shampoo	LC-UV/Vis	89-105	2-5	<12.0	[90]
C18	Benzophenones	Shampoo and hair mask	GC-MS	44.0-69.7	1.8-3.2	<15.5	[91]
C18	Phthalates	Cream and perfume	GC-MS	88.23-184.40	0.003-0.015	<4.7	[92]
Bar adsorptive microextraction (BAμE)							
AC	Benzophenones	After shave and sun protection cream	LC-UV/Vis	91.1-103.5	0.3-0.5	<13.0	[94]
AC	Parabens	Body lotion and hand cream	LC-UV/Vis	77.4-103.1	0.1	<12.9	[95]
Fabric phase sorptive extraction (FPSE)							
PEG	Parabens	Rose water, deodorant, hair serum and cream	LC-UV/Vis	88-122	0.252-0.580	<5	[97]
PEG	Parabens	Hair cream, hair foam, hand-face cream, blusher, headlight, lipstick and eye pen	LC-UV/Vis	87.3-105.2	2.75-2.98	<3.8	[98]
Membrane protected solid-phase extraction (MP-SPE)							
SBA-15/PANI-p-TSA	Parabens	Sunscreens, antiperspirants, creams and lotions	LC-UV/Vis	82-108	0.1-0.4	<7	[99]

(*continued on next page*)

Table 1 (*continued*)

Extraction phase[b]	Analytes[c]	Matrix	Instrumental technique[d]	R (%)	LOD (ng mL^{-1})	RSD (%)	Ref.
NH_2-β-CD-(St-DVB-MAA)	Aliphatic aldehydes	Toner and moisturized cream	LC-UV/Vis	81.7-114.9	0.024-2.5	<7.7	[100]
MAA-EGDMA	Glucocorticoids	n.r.	LC-UV/Vis	69.0-113.3	1.5-15.0	<10.6	[101]
Multi-stir-rod solid-phase extraction							
MWCNTs-COOH	Parabens	Mouthwashes, foam, hand washes and toner	LC-UV/Vis	83-103	0.63-0.8	<5.8	[102]
Pipette-tip SPE (PT-SPE)							
Cr-based MOF	Parabens	Shampoo	UV/Vis	95.4-101.9	0.24-0.25	<5.8	[103]
Spin-column SPE (SC-SPE)							
PAN/Fe-Mg-MIL-88B	Phthalate esters	Lipstick and cream	GC-FID	86.4-102.8	0.15-0.30	<7.9	[104]

[a] n.r.: not reported

[b] **AC**: Activated carbon; **[AVIM] [NTf$_2$]**: 1-ally-3-vinylimidazolium bis(trifluoromethylsulfonyl)imide; **β-CD**: β-cyclodextrin; **BMA**: Butyl methacrylate; **B-MON**: Bifunctional microporous organic network; **[C$_4$MIM] [Cl]**: Butylimidazolium chloride; **[C$_8$VIM] [NS]**: octylvinylimidazolium naphthalene sulfonate; **CAR**: Carboxen; **dtMIP**: Dual-template molecularly imprinting polymer; **DVB**: Divinylbenzene; **EDMA**: Ethylene dimethacrylate; **EGDMA**: Ethylene glycol dimethacrylate; **g-C$_3$N$_4$**: Graphitic carbon nitride; **GN**: Graphene nanosheets; **GO**: Graphene oxide; **LDH**: Layered double hydroxide; **MAA**: Methacrylic acid; **MIP**: Molecularly imprinting polymer; **MOF:** metal-organic framework; **MWCNTs**: Multiwalled carbon nanotubes; **NA**: naphthylamine; **NL**: Natural latex; **PA**: Polyacrylate; **PAF**: Porous aromatic framework; **PAN**: Polyacrylonitrile; **PANI**: Polyaniline; **P(BT-3-MeT)**: Poly(2,2-bithiophene-co-3-methylthiophene); **PDMS**: Polydimethylsiloxane; **PEG**: Poly(ethylene glycol); **PEGDA**: Poly(ethylene glycol) diacrylate; **Ppy**: Polypyrrole; **p-TSA**: para-toluenesulfonic acid; **rGO**: Reduced graphene oxide; **SDBS**: Sodium dodecylbenzenesulfonate; **St**: Styrene; **TDI**: Toluene-2,4-diisocyanate

[c] **BHA**: Butylated hydroxyanisole; **BHT**: Butylated hydroxytoluene; **MI**: 2-methyl-3-isothiazolinone; **NDELA**: N-nitrosodiethanolamine; **PAHs**: Polycyclic aromatic hydrocarbons; **TCS**: Triclosan

[d] **AED**: Atomic emission detector; **μECD**: Micro electron capture detector; **FAAS**: Flame atomic absorption spectrometry; **FID**: Flame ionization detector; **FL**: Fluorimetry; **GC**: Gas chromatography; **LC**: Liquid chromatography; **MS**: Mass spectrometry; **MS/MS**: Tandem mass spectrometry; **UV/Vis**: Ultraviolet/visible spectrometry

[e] LOD expressed as ng g^{-1}

and the sorbent (i.e., a zinc-aluminium layered double hydroxide (Zn-Al-LDH) modified with PANI and sodium dodecylbenzenesulfonate (SDBS) (Fe_3O_4/Zn-Al-LDH/PANI/SDBS)) was used to extract heavy metals [69]. In this modality, no external devices are required for the dispersion of the sorbent.

At this point, it should be cited the stir bar sorptive dispersive microextraction (SBSDME) approach, which combines the principles of SBSE and DSPE [70], in such a way a magnetic sorbent coating a magnetic stir bar is dispersed in the donor phase by high stirring rate. When the stirring is stopped, the magnetic sorbent containing the analytes is retrieved again by the magnetic stir bar without requiring an additional external magnetic field [71]. This technique has been successfully used for the determination of eight nitrosamines in shower gel and body cream, by using $CoFe_2O_4$ MNPs embedded in MOF MIL-101(Fe) (i.e., $CoFe_2O_4$/MIL-101(Fe)) as extractant phase due to its high porosity and presence of oxygen groups on its surface, resulting in a high surface area and moderated polarity, and thus making it suitable for the effective interaction with polar organic analytes [72]. Later, it was applied to determine ten PAHs employing a $CoFe_2O_4$/rGO composite due to its strong affinity for carbon-based ring structures [73].

2.4. Other sorbent-based microextraction approaches

Besides the microextraction techniques described above, other extraction approaches for the analysis of cosmetic products have been published.

It is worthy to mention micro matrix solid-phase dispersion (μ-MSPD), which is a miniaturized version of the classical MSPD. As in classical MSPD, the sample is blended with a drying agent and a dispersing sorbent, but the amounts are reduced. Then, the mixture is loaded into a glass Pasteur pipette or into a pipette tip with a small amount of glass wool and dispersing sorbent at the bottom, rather than in a larger cartridge like in conventional MSPD. Finally, a reduced amount of elution solvent is used to elute the target compounds [74]. In this approach, Florisil is the most commonly used disperser sorbent. It has been employed for the determination of a wide variety of analytes such as plasticizers, musks, dyes, preservatives, fragrance allergens, UV filters, PAHs and glycols, among others [74–81]. Other disperser sorbents, such as C18 [82], sand [83] or silica [84], have also been employed for the determination of dyes, coloring agents and preservatives, respectively.

In capillary microextraction (CME), the donor phase is passed through a capillary containing the extraction phase, which can be packed or coating the inner wall. In the most used modality, known as in-tube solid-phase microextraction (IT-SPME), a suitable solvent is then passed through the capillary to desorb the analytes, which is usually coupled to a chromatographic system thus allowing the analytes be transferred on-line [85]. The main advantages of this technique are the elimination of toxic solvents, and the integration of extraction and injection to the chromatographic system in a single device.

In this context, poly(methacrylic acid-co-ethylene glycol dimethacrylate) (MAA-EGDMA) monolith was used as sorbent for the determination of phthalates in toner samples [86], and hormones in lacquer remover and perfume [87]. This material showed good reusability during a 3-month trial, which is a key factor for this technique. Poly(butyl metacrylate-ethylene dimethacrylate-graphene nanosheets) (BMA-EDMA/GN) was employed to determine glucocorticoids [88]. GN were employed instead other carbon nanomaterials because they have excellent properties, such as high loading capacity, easy modification and π-electron system. Furthermore, reduced GN still contains a small amount of hydroxyl groups and carboxyl groups from the protection step, which can enhance the water wettability of GN, improving the adsorption of weak polar analytes due to the specific interaction, such as dipole-dipole and hydrogen bonding interaction. On the other hand, BMA possesses hydrophobic groups at the surface of porous polymer skeleton, so it was expected to show a good extraction capability for weak polar and non-polar compounds.

Microextraction by packed sorbent (MEPS) is a technique where the sorbent phase is placed into a syringe and the analytes are retained on it when the donor phase is passed through the sorbent [89]. This tech-

nique is a fast and simple miniaturized alternative procedure to SPE, reducing drastically the use of organic solvents. Furthermore, the syringe allows the liquid desorption of the analytes directly into the analytical instrument. Although different materials, either commercial or lab-made, have been reported in this technique [89], only commercial C18 has been used as extraction phase in the analysis of cosmetic products, where parabens [90], benzophenones [91], and phthalates [92] were successfully determined.

In bar adsorptive microextraction (BAμE) [93], a bar is used but in a different way as in SBSE. In this technique, the sorbent is fixed to the bar by using double-sided adhesive, and then it is inserted in the donor phase while it is stirred, operating under the floating sampling technology. After that, the extraction bar is taken out and transferred to other vial to elute the analytes in a proper solvent. This technique has been used for the determination of four benzophenone-type UV comparing the performance obtained employing activated carbon (AC) and a modified pyrrolidone polymer, showing both sorbent phases similar analytical parameters [94]. Later, AC was also employed for the determination of parabens [95].

Fabric-phase sorptive extraction (FPSE) is another sorbent-based microextraction approach in which a small piece of fabric coated with a thin sol-gel is immersed in a magnetically stirred sample solution for extraction of the analytes [96]. Later, the desorption procedure is carried out by just immersing the fabric phase into the appropriate solvent. In this technique, the sorbent is chemically bonded to the fabric substrate, allowing a high and readily accessible active extraction surface for fast and high-efficiency extraction of analytes. In the field of cosmetic analysis, it has been applied to determine parabens by using PEG as coating [97,98].

The so-called membrane protected solid-phase extraction (MP-SPE) has been also used in the analysis of cosmetic products. In this technique, the sorbent is put inside a porous hollow fiber, which is immersed into the liquid donor phase and stirred for a defined period. Ara et al. [99] used an ordered mesoporous silica material (i.e., SBA-15) coated with p-toluenesulfonic acid-doped PANI (SBA-15/PANI-p-TSA) for the extraction of parabens, exploiting the high surface area of SBA-15 and the hydrophobic, π–π and electrostatic interactions between PANI-p-TSA and parabens. Trace aliphatic aldehydes were also determined by this approach. In this case, a one-step MP-SPE, using an amino-modified β-cyclodextrin anchored to a styrene-divinilbenzene-methacrylic acid polymeric network (i.e., NH_2-β-CD-(St-DVB-MAA)), and derivatization with 2,4-dinitrophenylhydrazine strategy was successfully employed [100]. The authors concluded that the high selectivity was because of the size and interactions provided by NH_2-β-CD. Finally, a MAA-EGDMA polymer was in situ polymerized inside a hollow fiber and then it was used for the extraction of glucocorticoids in toner samples [101].

Makkliang et al. [102] proposed a microextractor device where used monolith rod-shape multiwalled carbon nanotubes functionalized with carboxylic groups (MWCNTs-COOH) by polymerizing MAA-EGDMA (i.e., MWCNTs-COOH/MAA-EGDMA). This device consisted of different small DC motors immersed in the sample solution in a similar way than SPME. Given the number of microextractors, the device could extract up to six samples simultaneously. It was applied to the determination of parabens in different cosmetic samples.

Recently, a chromium-based MOF sorbent for pipette-tip SPE (PT-SPE) was used for the determination of parabens in shampoo samples [103]. In this approach, the sorbent was packed in a pipette-tip, and cotton was placed at both ends of the tip to avoid sorbent loss. The sample solution was aspirated/dispensed through the tip seven times to extract the analytes.

In this line, a spin-column SPE (SC-SPE) approach has been proposed for the determination of phthalates in creams and lipsticks [104]. In this case, the sorbent was an electrospun composite made of polyacrylonitrile (PAN) and a Fe-Mg bimetallic MOF (i.e., PAN/Fe-Mg-MIL-88B) and it was packed inside a spin column. After the sample loading, it was centrifuged to pass the solution through the packed sorbent, and it was repeated twice.

3. Solvent-based microextraction techniques

In solvent-based microextraction techniques (commonly known as liquid-phase microextraction techniques (LPME)), the extraction takes place between a liquid donor phase, which contains the analytes to be extracted, and a very small volume (a few microliters) of an acceptor phase immiscible in the donor phase, which allows reaching high enrichment factors.

Due to the growing need to reduce the use of conventional organic solvents as acceptor phases, more environmentally friendly alternative solvents have become popular, such as ionic liquids (ILs) [105], supramolecular solvents (SUPRAs) [106] and deep eutectic solvents (DES) [107].

The challenge of applying solvent-based microextraction techniques in the analysis of cosmetic products is manifested in dealing with the complexity of cosmetic matrices, and for this reason many variations of these techniques have been used to overcome this obstacle.

As previously done for sorbent-based microextraction techniques, Table 2 summarizes the methods for the analysis of cosmetic products based on solvent-based microextraction techniques. The papers therein are briefly discussed below.

3.1. Single drop microextraction

Single drop microextraction (SDME) was the first solvent-based microextraction technique modality presented in 1996 [108,109], but it was not until 2009 when it was used in the analysis of cosmetics, being the first LPME technique used for this purpose. The technique is based on the extraction of the analytes by means of a microdrop of only a few microliters of organic solvent hanging from a syringe needle tip, directly immersed in the aqueous donor phase (DI), or in its headspace (HS), which allows the extraction of volatile or semi-volatile analytes if the sample is heated at moderate temperatures. After extraction, the microdroplet is retracted into the syringe and the extract is introduced into the analytical system.

The first application of this technique to cosmetic matrices, based on the DI mode, was for the extraction of parabens in cleanser and hair gels using hexyl acetate as extraction phase [110]. Since then, the HS mode was applied to the extraction of volatile and semi-volatile solvents from cosmetic samples, such as residual acetone using an aqueous ethanolic drop as extraction solvent, which contained the fluorophores 7-hydroxy-4-methylcoumarin and 7-diethylamino-4-methylcoumarin, in such a way the extracted acetone induced proportional changes in their fluorescence [111]. Similarly, the same authors proposed a fluorescence probe for the determination of ethanol, based on its extraction with an aqueous drop containing the enzyme alcohol dehydrogenase and the cofactor nicotinamide adenine dinucleotide (NAD) [112], in such a way the alcohol was oxidized to acetaldehyde and NAD reduced to NADH increasing the fluorescence proportionally. Prohibited 1,4-dioxane was also extracted by SDME using either methyl benzoate [113], or a mixture of hexane and dichloromethane [114] or 1-octanol [115] as extraction solvents, and in all cases employing gas chromatography-flame ionization detector (GC-FID). The use of an aqueous droplet was also used to preconcentrate parabens employing an aqueous solution of sodium hydroxide [16]. In this case, a previous clean-up step was performed with a mixture of isooctane and 1-octanol in a sodium hydroxide solution, and then, after acidification, the analytes were first extracted with a mixture of isooctane and 1-octanol, and then back-extracted in a droplet of aqueous sodium hydroxide solution.

Recently, with the intention of using greener solvents, a new modality of SDME based on the use of ILs (e.g., $[C_4MIM][PF_6]$) as extraction phase rather than a conventional organic solvent has been used to extract cadmium, arsenic, lead and nickel from eye makeup [116]. The

Table 2
Published papers on the use of solvent-based microextraction techniques in the analysis of cosmetic products[a]

Extraction phase[b]	Analytes[c]	Matrix	Instrumental technique[d]	R (%)	LOD(ng mL^{-1})	RSD (%)	Ref.
Single drop microextraction (SDME)							
Hexyl acetate	Parabens	Makeup removing gel and hair gel	GC-MS	92.4-104.5	0.001-0.015	<12.1	[110]
EtOH/water	Residual acetone	Cream, baby cream and deodorant	FL	89-109	260[e]	<5.3	[111]
Water	Ethanol	Hand cream, moisturizing cream, deodorants, facial cleansing gel, nappy cream, hair gel and body gel	FL	93-108	40[e]	<5.3	[112]
Methyl benzoate	1,4-Dioxane	Shampoo and hand soap	GC-FID	>84	400[e]	<14.2	[113]
n-hexane/dichloromethane	1,4-Dioxane	Shampoos	GC-FID	88-99	0.52[e]	<6.9	[114]
1-octanol	1,4-Dioxane	Shampoos, toothpaste, lotion, and liquid soap	GC-FID	89-99	0.97[e]	<4.7	[115]
Water	Parabens	Creams, foot cream, face cream, lotion, sunscreen lotion and body milk	LC-UV/Vis	94.5-105.3	1.7-4.3[e]	<9.6	[16]
[C_4MIM][PF_6]	Arsenic, cadmium, lead, nickel	Eye makeup	ETAAS	95.1-98.8	0.049-0.262	<4.2	[116]
Hollow fiber liquid-phase microextraction (HF-LPME)							
1-octanol	Rhodamine 6G	Lipstick	LC-UV/Vis	90-96.5	0,9	<3.5	[119]
1-octanol	Parabens	Cream and shampoo	LC-UV/Vis	85.6-103	0.2-0.5	<6.3	[120]
Toluene	UV filters	Cream, lotion, lipstick, and foundation	LC-UV/Vis	n.r.	0.001-0.1	<5.2	[121]
[C_8MIM][PF_6]	Hormones	n.r.	LC-UV/Vis	91.3-106.2	0.91-1.01	<5.47	[122]
Dispersive liquid-liquid microextraction (DLLME)							
Conventional							
Chloroform	Acrylamide	Creams and gels	LC-UV/Vis	85-112	0.7[e]	<14	[17]
Chloroform	Nickel	Hand cream	FAAS	97	1	<1.2	[125]
Chloroform	Bismuth	Hair dye	FAAS	95-101	4	3	[126]
Chloroform	Parabens	Moisturisers, hair conditioners and shaving creams	GC-FID	86.4-101.4	0.029-0.102	<6.86	[127]
Chloroform	Preservatives	Creams and lotions	CE-UV/Vis	71-113	200-375[e]	<5	[128]
Chloroform	Atranol and chloroatranol	Perfumes	GC-MS	79-110	2.4-5[e]	<9	[129]
Carbon tetrachloride	Phthalates	Shampoos, gels, creams, deodorants, and make-up	LC-MS/MS	84-124	0.042-0.12	<1.10	[130]
Carbon tetrachloride	Vitamin E	Creams, make-up, and shampoos	LC-UV/Vis	87-115	0.13-0.46	<1.8	[131]
[C_6MIM][PF_6]	Mercury species	Hand cream and skin refreshener	LC-ICP-MS	88.2-101.2	0.0013-0.0072	<7.4	[133]
[C_6MIM][PF_6]	Benzophenone-type UV filters	Sunscreens	MEKC	80.2-117.7	3.9-6.7	.2.77	[134]
[C_6MIM][PF_6]	Hexachlorophene	Moisturizer, toner and body lotion	LC-UV/Vis	74.5–97.7	140	<6.7	[20]
[C_6MIM][PF_6]	Silver nanoparticles (AgNPs)	Face creams	UV/Vis	73.2-96.6	150	<10.2	[135]
[C_6MIM][PF_6]	Parabens	Creams	UV/Vis	83.3-90	4.2-4.8	<4.3	[136]
[C_8MIM][PF_6]	Phenolic compounds	Toner, lotion, make-up remover and perfume	CE-UV/Vis	82-119	5-100	<12.8	[137]
[C_{10}MIM][BF_4]	Brilliant blue FCF	Eau de toilette and shampoos	LC-UV/Vis	99-103	0.34	<1	[138]
[PrEtIMO] [Tf_2N]	Bergapten and bergamottin	Hydroalcoholic cosmetic formulations	LC-UV/Vis-FL	75-99	11-22	<11.7	[139]
$C_{14}C_2C_{14}$ (Met) SUPRA	Parabens	n.r.	LC-UV/Vis	92-108	0.5-0.7	<11.9	[140]
[C_8MIM][PF_6]	Parabens	n.r.	LC-UV/Vis	84.2-98.4	0.064-0.135	<5.6	[141]
Water	NDELA	Creams and shower gels	LC-UV/Vis	87-117	1.1	<8	[143]
Water	Formaldehyde	Gels, masks, creams, and shampoos	LC-UV/Vis	91-113	0.7	<9	[144]
Ultrasound-assisted (USA-DLLME)							
Isopropanol	Triclosan	Body soap, hand soap and face soap	LC-UV/Vis	90.4-98.5	0.09	<5.3	[145]
Cyclohexane	Alkanolamines and alkylamines	Creams, sunscreens, lotions, shampoos, and powders	IC-NSCD	87-109	72-120	<6	[146]
Dichloromethane	Formaldehyde	Conditioners, shampoo, baby shampoo and shower gel	UV/Vis	n.r.	20[e]	5,87	[147]
1-octanol	Preservatives	Sunscreen, shampoos and toothpastes	LC-UV/Vis	n.r.	0.3.8.3	<10	[148]
1-octanol	Parabens	Shampoo	LC-UV/Vis	90.5–98.7	0.05-0.2	<5.6	[149]

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Table 2 (*continued*)

Extraction phase[b]	Analytes[c]	Matrix	Instrumental technique[d]	R (%)	LOD(ng mL^{-1})	RSD (%)	Ref.
1-octanol	Parabens, Benzoic acid	Skin cleanser, whitening cream, toothpaste, and moisturizer cream	LC-UV/Vis	70-138.1	0.03-10	<7	[150]
1-octanol	Parabens	Face masks, creams, and hair conditioners	GC-FID	82-109	2000-9500[e]	<5	[151]
THF-decanoic acid SUPRA	Rhodamine B	Lipstick, rouge, and nail polish	UV/Vis	99-104	0.49	<5.8	[152]
THF-decanoic acid SUPRA	Phthalates	Hair gel and perfumes	LC-UV/Vis	91.0-108.5	0.10-0.70	<11.7	[153]
Tetra-n-butylammonium bromide/decanoic acid DES	Ponceau 4R	Detergent and cologne	UV/Vis	98-107	5.97	<5	[154]
Tetra-n-butylammonium chloride/decanoic acid DES	Rhodamine B	Cologne, lipsticks, and nail polish cleaner	UV/Vis	97-110	2.2	<2.3	[155]
Sorbitol/glycerol DES	Niacinamide	Creams	UV/Vis	97.3-98.9	0.33	<2.8	[156]
Zinc chloride/acetamide DES	Lead and cadmium	Lipsticks and eye shadows	FAAS	96.6-98.5	8.75-10.5	<2.3	[157]
$[C_6MIM][PF_6]$	Anthraquinones	n.r.	LC-UV/Vis	81.7-110.9	0.01-0.09	<9.8	[158]
$[C_6MIM][PF_6]$	Triclosan	Handwash gel and moisturizing cream	UV/Vis	n.r.	18[e]	2,6	[159]
$[C_8MIM][PF_6]$	Parabens	Toning lotion	CE-UV/Vis	71.9-119.2	0.45-0.72	<8.7	[160]
Vortex-assisted (VA-DLLME)							
1-hexanol	Chloramine-T	Facial cleanser, face cream and face emulsion	LC-UV/Vis	90.8-100.9	8.2[e]	<6.5	[162]
Chloroform	Parabens	Moisturizer, toner, lotion, and soap	UV/Vis	82-97	476	<6	[163]
Chlorobenzene	Phthalates	Perfumes	GC-MS	85.6-95.8	0.003-0.070	<4.2	[13]
DL-menthol/PEG DES	Parabens	Creams and lotions	LC-UV/Vis	66-97	0.3-2	<5	[164]
Choline chloride/EG DES	Parabens	Cosmetic-oil products	LC-UV/Vis	82.0-102.5	49-61	<2.91	[165]
$[C_6MIM][BF_4]$	Hormones	Liquid-like and gel-like cosmetics	LC-UV/Vis	93.2-114.2	0.03-0.24	<5.2	[166]
$[C_8Gu][Cl]$	Parabens	Facial tonics	LC-UV/Vis	82-114	0.5-1.4	<16	[167]
[BuEtEtIMO][Tf_2N]	Cannabidiol	Creams	LC-UV/Vis	80	10000[e]	<2.5	[168]
$[P_{6,6,6,14}]_2[CoCl_4]$	Estrogens	Lotion	LC-UV/Vis	96-111	5-15	<10.5	[169]
Anisole	UV filters	Sunscreens	LC-UV/Vis	88-105	15	<4.9	[170]
Water	Nitrosamines	Creams and shower gels	LC-MS	80-113	1.8-50[e]	<9.8	[171]
Hexane	Preservatives	Creams, and gels	LC-UV/Vis	84-118	20-60	<10	[172]
Water	Bronopol	Creams, gels and shampoos	LC-UV/Vis	91-104	900	<10.9	[173]
Magnetic stirring-assisted (MSA-DLLME)							
1-octanol	Rhodamine B, Rhodamine 6G	Lipsticks	LC-UV/Vis	90-95	1.15-1.23	<2.53	[174]
THF/1-decanol SUPRA	Parabens	Creams	LC-UV/Vis	86-102	30-40[e]	<8	[175]
Air-assisted (AA-DLLME)							
Carbon tetrachloride	Parabens	Cream and lipstick	GC-FID	98-109	0.5-1	<9.4	[177]
p-xylene	Parabens	Shampoo, toothpaste, body lotion and moisturizing cream	GC-FID	74-86	0.9-2.7	<9	[178]
Methanol/acetonitrile	Antibiotics, glucorticoids and antifungal drugs	n.r.	MS	81.4-110.1	15-45[e]	<8.7	[179]
$[C_6MIM][PF_6]$	Dyes	Lipstick	LC-UV/Vis	88.8-99.3	3.9-84.8	<6.9	[180]
Fe_3O_4@oleic acid DL-menthol/decanoic acid ferrofluid	Parabens	Lipstick, eyeliner, blusher, eye shadow, and hand cream	LC-UV/Vis	85.9-99.0	0.8-1.3	<5.52	[181]
Thymol/enanthic acid DES	Parabens	Gel and tonic	LC-UV/Vis	95.8-105.2	0.2-0.3	<11.2	[182]
1-decanol	Parabens	Gel and tonic	GC-FID	96-103.2	1.0-1.7	<3.89	[183]
DLLME-solidification of floating organic drop (DLLME-SFOD)							
Tetrabutyl ammonium hydroxide/decanoic acid SUPRA	Parabens	Sunscreen, cream and aftershave	LC-UV/Vis	92.2-108.7	0.2-0.5	<11.9	[184]
1-undecanol	Parabens	Shampoos	LC-UV/Vis	82.8-108.4	0.3-1.7	<3.1	[18]
1-undecanol	Lead	Lipstick and hair dye	GFAAS	90-109.7	0.1[e]	<8.3	[185]
THF	Dyes	Lipsticks and eye shadows	LC-UV/Vis	90-106	250-3200[e]	<3.3	[186]
1-undecanol	Phthalates	Shampoos, after shave gels and hair sprays	LC-UV/Vis	n.r.	0.005-0.01	<12.6	[187]
2-dodecanol	Fragance allergens	Eau de toilettes, colognes, and perfumes	LC-UV/Vis	90-138	1-154	<12	[188]
1-dodecanol	Phthalates	Emulsions	LC-UV/Vis	n.r.	20-170	<4	[189]
Cloud point extraction (CPE)							
Triton X-114 5.0 % (v/v)	Cadmium	Shampoo, nail polish and lipstick	UV/Vis	97.2-103	2.5	<5.1	[190]

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Table 2 (*continued*)

Extraction phase[b]	Analytes[c]	Matrix	Instrumental technique[d]	R (%)	LOD(ng mL^{-1})	RSD (%)	Ref.
AEO_9	Antioxidants	Facial cleanser, skin milk and toner	LC-UV/Vis	90-110	1.4-8.5	<4.8	[191]
SDS	Estrogens	Toner	LC-UV/Vis	77.3-104.1	0.2-0.7	<10.4	[192]
Gas diffusion-microextracion (GDME)							
Acetylacetone	Formaldehyde	n.r.	LC-MS/MS	n.r.	1980[e]	<5.2	[193]
Acetylacetone	Formaldehyde	Fixative gels, body moisturizer, hand cream, hair conditioner, shampoo, and shower gel	Smartphone reader	n.r.	200[e]	<19	[194]

[a] n.r.: not reported

[b] **AEO_9**: Dodecylpolyoxyethylene ether; **[BuEtEtIMO] [Tf_2N]**:1-butoxy-3-ethoxy-2-ethyl-imidazolium bis(trifluoromethane)sulfonamide; **[C_4MIM] [PF_6]**: 1-butyl-3-methylimidazolium hexafluorophosphate; **[C_6MIM] [BF_4]**: 1-hexyl-3-methylimidazolium tetrafluoroborate; **[C_6MIM] [PF_6]**: 1-hexyl-3-methylimidazolium hexafluorophosphate; **[C_8Gu] [Cl]**: Octylguanidinium chloride; **[C_8MIM] [PF_6]**: 1-octyl-3-methylimidazolium hexafluorophosphate; **[C_{10}MIM] [BF_4]**: 1-decyl-3-methylimidazolium tetrafluoroborate; **DES**: Deep eutectic solvent; **EG**: ethylene glycol; **EtOH**: ethanol; **[$P_{6,6,6,14}$]$_2$[[$CoCl_4$]**: Trihexyl(tetradecyl)phosphonium tetrachlorocobaltate(II); **PEG**: polyethylene glycol; **[PrEtIMO] [Tf_2N]**: 1-propyloxy-3-ethoxyimidazolium bis(trifluoromethane)sulfonamide; **SDS**: Sodium dodecylsulfate; **SUPRA**: Supramolecular solvent; **THF**: tetrahydrofuran; **Triton X-114**: Polyethylene glycol tert-octylphenyl ether.

[c] **NDELA**: N-nitrosodiethanolamine; **UV-filters**: ultraviolet filters.

[d] **CE**: Capillary electrophoresis; **ETAAS**: Electrothermal atomic absorption spectrometry; **FAAS**: Flame atomic absorption spectrometry; **FID**: Flame ionization detector; **FL**: Fluorimetry; **GFAAS**: Graphite furnace atomic absorption spectrometry; **GC**: Gas chromatography; **IC**: Ion chromatography; **ICP**: inductively coupled plasma; **LC**: Liquid chromatography; **MEKC**: Micellar electrokinetic chromatography; **MS**: Mass spectrometry; **NSCD**: nonsuppressed conductivity detection; **UV/Vis**: Ultraviolet/visible spectrometry.

[e] LOD expressed as ng g^{-1}

extraction was carried out in a long and narrow column that contains the cosmetic sample and where the IL was added, forming very fine droplets that go down the column extracting the analytes and then become a single drop after reaching the lower part of the column where it was collected. The authors termed this approach as single drop ionic liquid based non-dispersive microextraction.

3.2. Hollow fiber liquid-phase microextraction

In 1999, hollow fiber liquid-phase microextraction (HF-LPME) [117,118] was proposed, in which a tubular porous hollow fiber lodges a drop of organic solvent, thus being protected and minimizing the risk of detaching from the needle tip as in SDME can easily occur.

1-octanol was used as extraction solvent for the extraction of rhodamine 6G from lipsticks [119] and parabens from creams and shampoos [120]; and toluene for the extraction of UV filters from creams, lotions and foundations [121].

On the other hand, the ionic liquid [C_8MIM]PF_6] was used for the extraction of different sex hormones [122] from creams and lotions. In this latter case, the hollow fiber contained a stir bar inside, which was immersed in the donor phase and magnetically stirred.

3.3. Dispersive liquid-liquid microextraction

Dispersive liquid-liquid microextraction (DLLME) was introduced in 2006 [123], and it is originally based on a ternary solvents system: an aqueous sample containing the analytes to extract, an extraction solvent (typically halogenated solvents due to their higher density than water that facilitates their collection after phase separation) and a disperser solvent (miscible with both the aqueous sample and extraction solvent). An appropriate mixture of the disperser and extraction solvents is rapidly injected into the aqueous donor phase forming a cloudy solution, so that the contact surface area between the extraction solvent and the sample is infinitely large, favouring the analyte transfer from the aqueous donor phase to the extraction solvent. Then, the phase separation is achieved by centrifugation and the extracting phase is collected and transferred to the analytical instrument [124].

The DLLME technique allows to achieve high enrichment factors, and it is cheap, simple, and very quick, since the extraction is obtained practically instantaneously.

In the last decade, the use of DLLME in the analysis of all type of samples has increased considerably, including in cosmetics where it has become the most widely used LPME applied to these matrices.

DLLME was reported in its most classic modality with high-density extraction solvents, such as chloroform for the determination of acrylamide [17], nickel [125], bismuth [126], parabens [127] and other preservatives [128], and atranol and chloroatranol [129]; and carbon tetrachloride for the extraction of phthalates [130] and vitamin E [131].

In order to avoid the use of halogenated solvents as extraction solvents, other greener alternatives have been proposed in order to be less harmful to the researcher and the environment [132]. In this sense, different ILs has been applied to the extraction of mercury species [133], benzophenone-type UV filters [134], hexachlorophene [20], silver nanoparticles [135] parabens [136], phenolic compounds [137], Brilliant blue FCF dye [138], and bergapten and bergamottin [139]. Additionally, in 2017, a SUPRAs was used to extract parabens from several cosmetics [140].

DLLME has also been applied in combination with sorbent-based microextraction techniques such as DSPE for the analysis of parabens [141]. Thus, ILs-DLLME was used to concentrate the target analytes in the extraction solvent, and then, to avoid time-consuming centrifugation, magnetic graphene oxide (i.e., Fe_3O_4@GO) was added to retrieve the IL containing the target analytes by hydrophobic interactions.

Reversed-phase-DLLME [142] is a modality of DLLME in which a small volume of water acts as extraction solvent by being dispersed in an organic donor phase to extract highly polar compounds that the original DLLME could not be able to extract due to the typically non-polar nature of the solvents used. This technique has been applied for the extraction of prohibited substances in cosmetic products such as the nitrosamine NDELA [143] and formaldehyde [144]. In this modality, a fundamental variable is the nature of the donor phase, which is studied by comparing the extraction efficiency when using different organic solvents. In the aforementioned studies, toluene was used as the donor phase, which is less dense than water, and thus it allows the easy collection of the small volume of aqueous extract in the conical tip of the tube used for the extraction.

It should be added that in some cases, both in the conventional DLLME and in the RP-DLLME, the disperser solvent usually decreases the partition coefficient of the analytes in the extraction solvent. In order to avoid this undesired effect, the formation of the microemulsion through different dispersion mechanisms, such as vortex or magnetic

stirring, ultrasonication or a gas flow, among others, is preferred. These dispersion modalities are presented separately below.

3.3.1. Ultrasound-assisted DLLME

Ultrasound-assisted DLLME (USA-DLLME) uses ultrasounds to allows the dispersion of the extraction solvent in the aqueous phase and has the advantage of rapidly promoting homogenization and mass transfer between immiscible phases.

The first reported USA-DLLME application in the analysis of cosmetic products describes the development of a salt-induced liquid–liquid microextraction based on the rapid phase separation of isopropanol (water-miscible organic solvent) from the aqueous phase in the presence of high concentration of salt (salting-out effect) for the determination of triclosan in different soap samples [145].

USA-DLLME has been used with cyclohexane to extract alkanolamines and alkylamines from creams, sunscreens, lotions, shampoos, and powders [146]; with dichloromethane to extract formaldehyde from shampoos, conditioners, and shower gels [147]; and with 1-octanol to extract preservatives from creams, toothpastes, cleansers, shampoos and sunscreen products [148–150]. Wei et al. [151] presented a different way to achieve the dispersion based on the use of ultrasounds. In this work, an ultrasonic nebulizer produces an 'ultrasonic fountain' that disperses the cosmetic sample and the extraction solvent (i.e., 1-octanol), obtaining an aerosol that is sprayed in the extraction vessel with the aqueous solution. It was applied to the determination of six parabens in cosmetic products.

Along the line of using greener solvents, SUPRAs were used for rhodamine B determination [152], and to extract phthalates from hair gels and perfumes [153]; different types of DES have been used in USA-DLLME for the determination of Ponceau 4R [154], rhodamine B [155], niacinamide [156], and lead and cadmium [157]; while ILs were used to extract anthraquinones [158], triclosan [159], and parabens [160];

3.3.2. Vortex-assisted DLLME

The vortex-assisted DLLME technique uses vortex as a stirring instrument to allow the rapid formation of the microemulsion (cloudy solution), generally without the need to use a disperser solvent [161].

With this modality, the use of 1-hexanol was reported for the extraction of chloramine T [162], and chloroform and chlorobenzene were reported to extract parabens [163] and phthalates [13], respectively.

The use of DESs was also reported to extract parabens from creams and cosmetic oils [164,165].

ILs were also used as extraction solvents with this modality, for the extraction of hormones [166], parabens [167] and cannabidiol [168]. Moreover, one study used a $[CoCl_4^{2-}]$-based magnetic IL for the extraction of estrogens in lotions [169].

Anisole has recently been used as extraction solvent in a combination of ultrasounds and vortex-assisted-DLLME (i.e., USA-VA-DLLME) technique as a green alternative to classical organic solvents for the extraction of UV filters from sunscreen products [170].

Water was used as extraction solvent as well, in a reversed-phase approach (VA-RP-DLLME) in which seven nitrosamines were extracted from an n-hexane donor phase [171].

Finally, a procedure named vortex-assisted liquid-liquid semi-microextraction (VALLsME) was developed by Miralles et al. [172] for the determination of five alternative preservatives such as phenethyl alcohol, methylpropanediol, phenylpropanol, caprylyl glycol, and ethylhexylglycerin in creams and gels, using 1 mL of n-hexane as extraction solvent and vortex stirring to form the microemulsion. The target analytes were extracted after in situ derivatization with benzoyl chloride. This procedure was termed 'semi'-microextraction due to the amount of extraction solvent used, which although it is not just a few microliters, it is a small amount compared to that used in non-miniaturized techniques. A reversed-phase modality of this technique was also presented, extracting the restricted preservative bronopol with 0.5 mL of water from a sample solution of n-hexane and ethanol [173].

3.3.3. Magnetic stirring-assisted DLLME

The dispersion has been also achieved by means of a magnetic stirrer in the analysis of rhodamine B and rhodamine 6G in lipsticks [174]. In this operational mode (MSA-DLLME), unlike other methods of agitation, the device allows the integration of a bath to maintain the temperature controlled while the extraction is carried out. The extraction of parabens from creams has been also conducted by mean of this modality, but combining with matrix-solid phase dispersion (MSPD), in such a way the magnetic stirring allows the formation of a SUPRA and extraction at the same time [175].

3.3.4. Air-assisted DLLME

In this modality, the dispersion of the extraction solvent in the aqueous solution is achieved through a gas flow of air. In the most common methodology, known as air assisted in syringe-DLLME, a syringe is used to aspirate and dispense the mixture of extraction solvent and aqueous solution multiple times allowing the formation of the microemulsion [176]. This simple action leads to efficient dispersion of the extraction solvent in the sample solution. This technique was applied to the simultaneous derivatization and extraction of parabens from lipsticks and creams using carbon tetrachloride [177]. In this work, it was possible to separate the phases without the need of centrifugation by filtering just after extraction with a nylon membrane filter applied to the same syringe. In another work, parabens were extracted with the same technique but using p-xylene as extraction solvent, and since it is lighter than water, a specific device was designed to collect the extract, also avoiding the centrifugation step [178].

An "all-in-one" device system that combines air-assisted in syringe microextraction, clean up, and needle spray ionization with a specially designed syringe assembly was developed to determine antibiotics, glucocorticoids and antifungal drugs in cosmetic samples, extracting them with a mixture of methanol and acetonitrile [179].

Sonication was also implemented in this technique (USA-air-assisted in-syringe-DLLME), using the ionic liquid $[C_6MIM][PF_6]$ as extraction solvent to determine illegal azo dyes in lipsticks [180].

A simple and green variation of this technique was applied to preconcentrate parabens in make-up and hand cream samples: two syringes were connected to each other through a metallic connection, one containing the sample with a DES-based ferrofluid whereas the other one was empty, and the content was injected going from one to another and vice versa eight times. Subsequently, the DES-based ferrofluid was isolated using an external magnet and the analytes were desorbed from the DES with methanol [181].

In 2021, Azizi et al. presented two works with a new version of this technique to perform the determination of parabens in personal care products, one extracting with a DES [182] and one extracting with 1-decanol [183]. To perform the method, they created a home-made extraction device in which a stream of nitrogen bubbles dispersed the extraction solvent into the aqueous donor phase.

3.3.5. DLLME-solidification of floating organic drop

Another modality of DLLME is the microextraction by solidification of floating organic drop (DLLME-SFOD), in which an extraction solvent less dense than water is used. In this case, the drop of the extraction solvent is located on the surface of the donor aqueous phase, and it is frozen by immersing the extraction vial in an ice bath, thus the extractant solidifies facilitating its collection.

This technique was used to extract parabens using SUPRAs [184], or 1-undecanol [18] as extraction solvents. This last solvent was also used to extract lead [185], with microwave radiation to cause rapid and homogeneous heating of some cosmetic matrices such as lipstick, eye shadow or hair dye. Microwave assistance was also used to extract dyes from lipstick and eye shadows, by using tetrahydrofuran as extraction solvent [186].

Sonication was also implemented in this technique (USA-DLLME-SFOD), using fatty alcohols as extraction solvents. After dispersion, they

were solidified by means of cold baths to be collected and analyzed with chromatography. With this modality, five phthalates [187] and eighteen potentially allergenic substances [188] have been determined in different cosmetic matrices.

Finally, a work compare DLLME with DLLME-SFOD for the determination of phthalates, and the authors suggested that out of a total of ten phthalates, the best extraction method was DLLME-SFOD, with the exception of di-n-pentyl phthalate (DPP) and dicyclohexyl phthalate (DCHP) for which DLLME was more suitable [189].

3.4. Cloud point extraction

Cloud point extraction (CPE) consists of the separation of the analytes from the aqueous phase by pseudo-phases of non-ionic surfactant micelles. When altering properties of the solution, such as temperature, pressure or salt content, the micelles separate into two isotropic phases: a phase composed almost entirely of surfactant and the analyte, and an aqueous phase in which the concentration of the surfactant is close to the critical micelle concentration. A back-extraction of the analyte in an organic solvent is necessary to reduce the interferences of the surfactant in the analytical instrument.

This technique has been reported for the extraction of cadmium using PEG tert-octylphenyl ether (i.e., Triton X-114) in selected cosmetic products such as shampoo, lipstick and nail polish [190]; and for the extraction of antioxidants, such as BHA, BHT, tert-butylhydroquinone (TBHQ), and propyl gallate (PG), using dodecylpolyoxyethylene ether (i.e., AEO_9) as an environmentally-friendly extraction solvent [191].

Furthermore, CPE was used in combination with the co-precipitation of estrogens with aluminium hydroxide to determine these analytes effectively in facial toners using sodium dodecylsulfate (SDS) as extraction solvent [192].

3.5. Other solvent-based microextraction techniques

In 2017, Brandão et al. [193] reported the application of an extraction technique known as gas diffusion-microextraction (GDME) for the simultaneous derivatization, extraction, and preconcentration of formaldehyde from cosmetic samples. This technique is based on a two-part sealed module: the GDME module with a porous membrane impregnated with acetylacetone as reagent, placed vertically over a flask containing the sample. When the sample is heated, the vapours rise towards the membrane and formaldehyde reacts with the reagent in the presence of ammonium forming 3,5-diacetyl-1,4-dihydrolutidine (DDL), which is then collected and diluted in water before analyze it by chromatography [193], or through a smartphone reader, as it was proposed a year later [194].

4. Discussion and trends

As can be seen from the reviewed articles, and as already highlighted above, the common objectives in sample preparation in the last decades, and specifically in microextraction techniques, have been to reduce the sample and solvent consumption, the execution costs, and the analysis time, all this to prioritize the development of environmentally friendly methodologies, according to the principles of green sample preparation (GSP) [2].

A priority factor to consider in the selection of a microextraction technique is to identify the most suitable extraction solvent or sorbent, depending on the case, to obtain good selectivity for the analyte(s) of interest. Depending on the nature and chemical structure of the analytes, and thus the interactions they may generate, it is possible to choose from a wide range of extractants.

Regarding sorbent-based microextraction techniques, the design of tailor-made sorbents (e.g., lab-made fibers for SPME, or functionalized MNPs for DSPE) has been one of the focusses of attention to increase

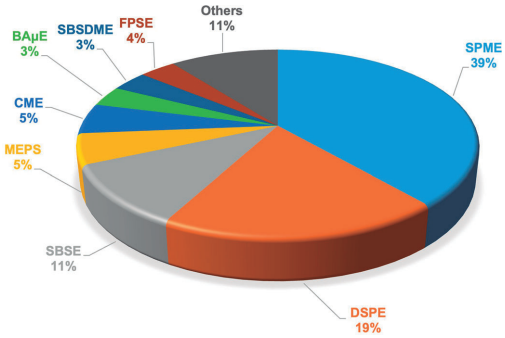

Fig. 2. Pie chart on SBME techniques applied to cosmetic products.

the selectivity regarding the target analytes, with respect to conventional and/or commercially available ones (e.g., PDMS-based devices). Additionally, magnetic sorbents occupy an important place, mainly due to their easy retrieval after extraction by means of an external magnetic field, reducing sample pre-treatment time and simplifying the whole procedure.

As for solvent-based microextraction techniques, the research has gone towards the employment of less toxic and selective solvents (i.e., ILs, DES, SUPRAs) than the traditional ones (i.e., conventional organic solvents). In the latter, the degree of selectivity is lower, since the extraction is done mainly by affinity in terms of polarity, so that other compounds of the matrix with similar characteristics to the analytes can be also extracted.

Regardless the selected extraction phase, another important input to take into account is the extraction kinetics. In general terms, the extraction goes faster in those techniques where the extraction phase is dispersed within the donor phase (e.g., DSPE, DLLME), since the contact area between both phases is increased.

Finally, the volatility of the analytes could tip the balance to choose a microextraction approach or another. In this sense, if the analytes are volatile, a microextraction technique that allows the analytes to be directly desorbed into the analytical instrument (e.g., SPME or SBSE) may be a good option.

4.1. Trends in the application of microextraction techniques to the analysis of cosmetic products

Among the sorbent-based microextraction techniques (Fig. 2), the well-stablished SPME (41%), and more specifically HS-SPME, is undoubtedly the most used, although other later techniques, such as DSPE (21%) and SBSE (10%), have also been used but in a lesser extent. HS-SPME represents an advantage because it does not use solvents in the desorption stage, since the extraction phase containing the analytes is directly coupled to the analytical instrument (i.e., GC). On the other hand, the main advantages of DSPE are the possibility of designing and using a wide variety of tailor-made sorbents with different properties depending on the analytes, and their dispersion into the donor phase, in such a way the extraction time required is drastically reduced compared with static approaches (i.e., SPME and SBSE).

On the other hand, the application of LPME in the analysis of cosmetic products started with SDME in 2009. Later, other LPME techniques began to be used and, as has been shown, the approach that obtained special relevance in this field is DLLME (Fig. 3), since specifically 66% of the reviewed LPME-based methods make use of this technique. The rapidness and simplicity are the main advantages over the other approaches, where equilibrium state is not usually achieved. Within this

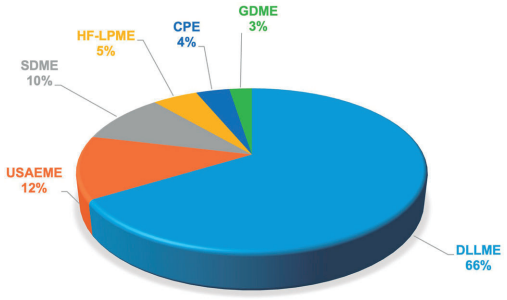

Fig. 3. Pie chart on LPME techniques applied to cosmetic products.

technique, different modalities have been presented, being the conventional (34%), and those assisted by vortex (19%) and ultrasound (15%) the most used since they required common laboratory equipment, in addition to those assisted by magnetic stirring, by air, and the DLLME with floating organic droplet solidification.

4.2. Analytes of interest in the development of microextraction techniques applied to cosmetic products

The analytes investigated in cosmetic products are of great variety: active ingredients that must be quantified to ensure their concentration and consequently confirm the efficacy of the product (e.g., vitamins, cannabidiol); excipients and additives that must be quantified to ensure that their concentration complies with the safety limits defined according to the international regulations on cosmetic products; and prohibited compounds by current legislations due to their demonstrated toxicity, whose presence must be controlled in case there is a possibility to be present in the formulation due to the unintentional causes mentioned above.

In Fig. 4, it is shown the research trends among cosmetic ingredients and the categories of substances that have been mainly studied.

Preservatives have been the most investigated substances (37%), and parabens stand out among them. This is mainly due to two reasons: on the one hand, because as preservatives, their concentration level has to respect very precise defined limits depending on the type of product and the category to which it is destined, and on the other hand because, due to the fact that some of them are prohibited according to the EU Regulation (e.g., benzylparaben, isobutylparaben, isopropylparaben, phenylparaben), in recent years they have been in the spotlight since information on the toxicity and danger of exposure of all parabens was disseminated. However, not all parabens are prohibited in the EU framework, and if used in a cosmetic product within the limits allowed by the EU Regulation, they are considered safe for humans. Other preservatives that have been investigated include triclosan, bronopol, IPBC, phenoxyethanol, benzoic acid, salicylic acid, sorbic acid, and sodium benzoate, among others.

Phthalates, which have been commonly used as plasticizers in the production of cosmetics such as nail polish or hairspray, as solvents, or as plasticizers in production of polyvinyl chloride (PVC) plastics, are another category of ingredients that stands out as one of the most analyzed (8%). As some phthalates have been shown to be toxic, they have been banned in cosmetics, such as dibutyl phthalate, benzyl butyl phthalate, and bis(2-ethylhexyl) phthalate, among others. The interest in microextraction techniques for these analytes is due to the need to verify their absence in the final product, although they could be present due to impurities in the raw materials or migration from the plastic containers used.

Dyes and UV filters are also frequently determined (6% and 4%, respectively), since they are substances subject to concentration restrictions depending on the type of product and the population they are intended for, especially UV filters, whose concentration must be checked to ensure compliance with the permitted limits and concordance with the information declared on the label to guarantee the sun protection factor claimed.

Other substances typically determined are metals and semi-metals (6%), antioxidants (4%), and fragrance and allergens (5%). This latter

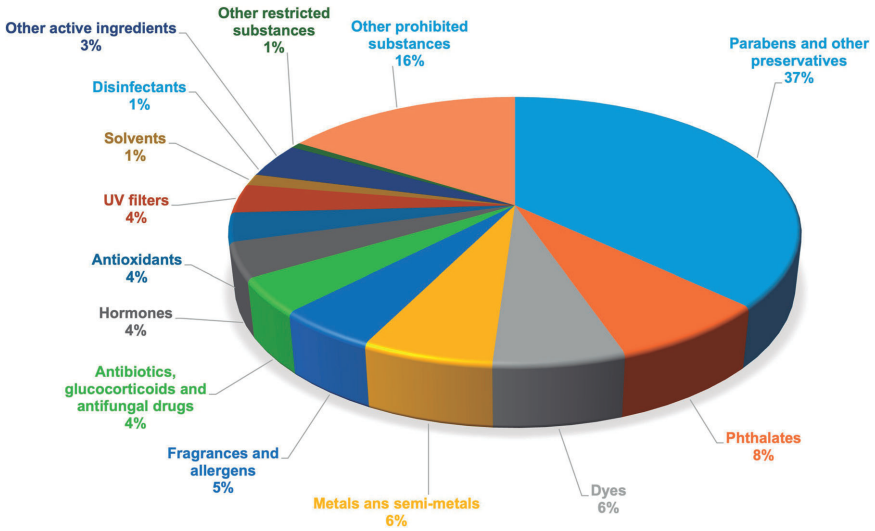

Fig. 4. Analytes of interest in microextraction techniques applied to cosmetic products.

group includes many molecules, some of which are prohibited (e.g., lilial), while others must be declared on product labels if surpass a nominal concentration due to their higher allergenic properties (e.g., citral, geraniol, or benzyl alcohol).

The application of these microextraction techniques to the analysis of active ingredients such as vitamins, cannabidiol, anthraquinones, perfuming substances or solvents such as ethanol was also reported, with the interest of quantified their concentration in the final product to guarantee a certain efficacy or claim their presence on the label.

Finally, special attention is paid to prohibited substances in cosmetic products, which can be investigated to ensure their absence in a cosmetic before it is marketed, or to analyze a product already marketed against possible alerts. These substances include, in addition to some of the parabens and phthalates already mentioned, hormones, antibiotics, and antifungals that are not allowed as cosmetic ingredients but are sometimes used illegally to confer certain properties to the product; toxic solvents such as 1,4-dioxane; and carcinogenic substances such as nitrosamines, formaldehyde, and acrylamide, which can derive from secondary reactions or be released from other ingredients.

5. Conclusions and future trends

All the different approaches and modalities of microextraction techniques that have been reported in this review, both sorbent-based and solvent-based, were presented fundamentally to satisfy the need to combine the demands of the cosmetic industry with technological advances in the field of analytical chemistry. In this way, it was possible to develop control measures that ensure the quality and safety of cosmetic products, quickly adapting to new advances and legislative changes.

Since the first precedent twenty-five years ago, the increased number of published scientific articles related to the use of microextraction techniques in the analysis of cosmetic products reflects the high potential that these miniaturized techniques offer to sample preparation in this field.

Numerous advances have already been addressed throughout all these years in terms of the microextraction modalities developed and the extraction phases used, in order to have a greater selectivity towards the analytes and facilitate the procedure. Special mention deserve the magnetic phases, either functionalized magnetic nanoparticles or composite materials, that allow to be easily handled and retrieved from the donor solution. Future trends should be shifted to not just the development of new materials, both sorbents and solvents, to further increase this selectivity even more, but also to diminish their toxicity and environmental impact and thus comply with the established principles of Green Analytical Chemistry and Green Sample Preparation. This fact should be emphasized even more when the purpose of the use of microextraction techniques is to reduce the detrimental impact of analytical methodologies, since otherwise, the environment impact would be collaterally increased, and the green nature of the method diminishes.

On the other hand, many of the analytes determined in cosmetic samples by means of microextraction techniques are at a very low concentration since they are prohibited compounds and therefore their presence is involuntary. Thus, it is necessary to use microextraction techniques that improve the limits of detection, although it is true that this depends not only on the preparation technique used but also on the instrumental analysis technique. Moreover, when possible, suitable methods have been designed to apply directly in industries to carry out the 'in process' quality control.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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A rapid and sensitive method for the determination of cannabidiol in cosmetic products by liquid chromatography–tandem mass spectrometry

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A Rapid and Sensitive Method for the Determination of Cannabidiol in Cosmetic Products by Liquid Chromatography–Tandem Mass Spectrometry

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Abstract: Cannabidiol is a phytocannabinoid with proven pharmacological properties that is also used in the cosmetic industry for its sebostatic and antioxidant activities, being considered a new anti-aging ally. An analytical method is proposed for the determination of CBD in cosmetic products by liquid chromatography with tandem mass spectrometry, after leaching the CBD from the cosmetic matrix with ethanol. Low instrumental limits of detection (0.22 ng mL^{-1}) and quantification (0.74 ng mL^{-1}) allow the determination of CBD at trace levels without needing preconcentration, whereas the wide linearity of the method allows the determination of CBD in more concentrated samples without high dilution. The method was successfully applied to the analysis of six cosmetic products and a raw material. The proposed method is suitable for the quality control of cosmetic products containing CBD, being able to quickly and easily determine this compound, ensuring that its concentration in the finished product is the desired one.

Keywords: cannabidiol; cosmetic products; liquid chromatography; mass spectrometry

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1. Introduction

Cannabidiol (CBD) is a phytocannabinoid found in the *Cannabis* plant that does not have psychoactive activity, unlike tetrahydrocannabinol (THC) also present in *Cannabis*.

CBD has aroused a lot of interest in recent years due to its various pharmacological properties, which include analgesic, anti-inflammatory, antineoplastic, and chemopreventive activity. In addition, it has recently started to be used in the cosmetic industry for its antioxidant, skin conditioning, and sebostatic properties. CBD acts on the function of sebaceous glands, behaving like a highly effective sebostatic agent, inhibiting the proliferation of sebocytes and the production of sebum. CBD also exerts anti-inflammatory actions that, combined with its lipostatic and antiproliferative effects, make it a promising therapeutic agent for the treatment of acne vulgaris, since it counteracts the multiple pathogenic factors of acne: sebum overproduction, sebocyte overproliferation, and inflammation [1]. Moreover, CBD is a powerful antioxidant that helps counteract oxidative cell damage generated by free radicals by helping to decrease the visible signs of skin aging [2], and it is mainly for this reason that it is becoming one of the star ingredients of the cosmetic sector in recent years [3,4].

Current European legislation does not prohibit the use of synthetically obtained CBD in cosmetic products. *Cannabis* seeds and leaves (without the upper part of the plant, flowers, or fruits) obtained from varieties of hemp with a low THC content (less than 0.2%) can be also used, both after proper treatment (such as to obtain oils, e.g., *Cannabis sativa* seed oil) or after a process to obtain and purify CBD for direct use as an ingredient [5–7].

Many articles related to CBD and other cannabinoids determination in plants [8], oils [9], or other matrices, such as biological fluids, hair, or food products can be found in the analytical literature based on the use of different sample preparation strategies [10] and chromatographic techniques [11]. Gas chromatography coupled to simple mass spectrometry (GC-MS) was used for the analysis of hair [12–16], oral fluid [17,18], and hemp food [19] samples. Gas chromatography with tandem mass spectrometry (GC-MS/MS) was also used for hair sample analysis [20–23]. Liquid chromatography with ultraviolet detection (LC-UV) was used for the analysis of urine [24,25] and brain and blood mice [25]. Liquid chromatography with tandem mass spectrometry (LC-MS/MS) was used for the analysis of hair [26] and oral fluid samples [27], and ultra-high performance liquid chromatography with tandem mass spectrometry (UHPLC-MS/MS) was used for serum and urine [28,29] and oral fluid and sweat patches [28] analysis.

Only a few recent publications are related to the determination of CBD and other cannabinoids in cosmetic products. In this context, Meng et al. [30] analyzed commercial creams by LC-MS/MS after dilution of samples with methanol (MeOH) containing 0.005% of formic acid and 5% of water, sonication, and centrifugation, thus obtaining a limit of detection (LOD) of 0.048 ng mL^{-1} of CBD in the measurement solutions. Nemeskalová et al. [31] analyzed hydrophobic cosmetics by LC-UV and LC-MS/MS by treatment with ethyl acetate:isopropanol 1:1 by vortex, followed by heating to get a good dispersion and separation of the supernatant after cooling to −20 °C and finally dilution with a 60% aqueous acetonitrile solution, shaking by vortex, and filtering before analysis; a LOD of 0.2 μg g^{-1} of CBD in samples was obtained. Huber et al. [32] determined CBD in creams by LC-UV by microextraction using MeOH and an ionic liquid and sonication at 40 °C, followed by centrifugation, evaporation of the supernatant, and dilution of the remaining ionic liquid with an acetonitrile solution; a LOD of 0.01 mg g^{-1} of CBD in samples was obtained.

The aim of this work is to develop and validate an analytical method for the determination of CBD in different types of cosmetic products, with good analytical features for use in the quality control of the cosmetic industry, such as replacement or reduction of toxic organic reagents, quickness, and simplicity, especially in the sample preparation stage. Moreover, the proposed method allows the determination of CBD both at trace level and in higher concentrations. Recommended performance parameters in single-laboratory validation of analytical methods for cosmetics given by the Joint Research Centre Guidelines [33] have been studied.

2. Materials and Methods

2.1. Apparatus

An Agilent Technologies 1100 Series liquid chromatography system, equipped with a degasser, a quaternary pump, an automatic injector, and an oven, was used. The chromatography system was coupled to an Agilent 6410B Triple Quad LC-MS/MS detector. Reverse-phase chromatographic separations were carried out using an Agilent Zorbax SB-C18 column (50 mm in length, 21 mm in internal diameter, and 1.8 μm in particle size). The acquisition and processing of data were carried out using a computer equipped with the Agilent Technologies MassHunter Workstation LC/QQQ software (Version B.08.02).

A ZX3 vortex mixer from VELP Scientifica (Usmate Velate, Italy) was used to facilitate the lixiviation of the analyte from the sample.

2.2. Reagents and Samples

A solution of CBD in MeOH with a concentration of 1.0 mg mL^{-1} from Sigma-Aldrich (Saint Louis, MO, USA) (purity 99.3%) was used as a stock standard solution.

A solution of Cannabidiol-D3 (CBD-D3) in MeOH with a concentration of 100 μg mL^{-1} from Sigma-Aldrich (Saint Louis, MO, USA) (purity 99.7%) was used as a stock internal standard solution.

Ethanol (EtOH) HPLC grade from Panreac (Barcelona, Spain) and deionized water obtained through a Connect water purification system from Adrona (Riga, Latvia) were used for sample and standards preparation.

A chromatographic mobile phase composed of LC-MS grade MeOH and LC-MS grade water from Panreac (Barcelona, Spain) was used, both phases containing 0.1% of formic acid prepared from formic acid provided by VWR chemicals (Fontenay-sous-Bois, France).

Six commercial cosmetic products (i.e., four creams, a shower gel, and a hair mask) and a raw material (*Cannabis sativa* oil standardized in 1.3% CBD) were analyzed, and their brands are not mentioned for confidentiality reasons. According to the labels, the four creams and the raw material contained CBD among the labeling ingredients, while the labels on the shower gel and the hair mask indicated that they contained the ingredient *Cannabis sativa* oil.

2.3. Proposed Method

2.3.1. Standards Preparation

An ethanolic CBD solution of 100 ng mL^{-1} was prepared by successive dilutions of the 1.0 mg mL^{-1} CBD stock standard solution with EtOH. Working standard solutions containing different concentrations of CBD (1 to 30 ng mL^{-1}) were prepared by dilution of the 100 ng mL^{-1} ethanolic standard solution with the appropriate volumes of EtOH and water to get 1:1 EtOH:water solutions. These solutions also contained the appropriate volume of a 200 ng mL^{-1} ethanolic CBD-D3 solution to get a fixed internal standard concentration of 8 ng mL^{-1}. The obtained standard solutions were transferred to injection vials for chromatographic analysis.

2.3.2. Samples Preparation

First, 1 g of sample was weighed into a volumetric flask and brought to a final volume of 10 mL with EtOH. To facilitate CBD leaching, the sample was shaken with a vortex mixer. Then, 1 mL of this initial solution was taken and transferred to a 10 mL volumetric flask and the appropriate volumes of EtOH and water were added to get a 1:1 EtOH:water solution. A lower mass of sample or a lower volume of the initial sample solution was taken if CBD concentration was relatively high. As in the standards, sample solutions contained the appropriate volume of the 200 ng mL^{-1} ethanolic CBD-D3 solution to get a fixed internal standard concentration of 8 ng mL^{-1}. The obtained solutions were filtered through nylon filters with a pore size of 0.45 μm and transferred to injection vials for LC-MS/MS.

2.3.3. LC-MS/MS Analysis

Ten microliters of sample or standard solutions were injected into the chromatographic system and the ratio of the analyte (CBD) peak area to the internal standard (CBD-D3) peak area was used as an analytical signal to carry out the determination of CBD. The mobile phase consisted of MeOH:water (both with 0.1% of formic acid) by isocratic elution at a mixing ratio of 80:20% (*v/v*). The flow rate was 0.2 mL min^{-1}, and the column temperature was maintained constant at 35 °C. The run time was below 4 min.

The triple quadrupole MS operated in positive electrospray ionization mode (ESI+, capillary voltage at 3 kV) by multiple reaction monitoring (MRM). The other conditions were gas temperature at 310 °C, nebulizer gas flow rate at 12 L min^{-1}, and nebulizer gas at 50 psi.

The collision energy and the m/z precursor→product ion transitions for quantification are indicated in Table 1, together with the summary of the instrumental variables.

Table 1. Instrumental variables of LC-MS/MS analysis.

	Instrumental Variable						
LC	Injection volume	10 µL					
	Column temperature	35 °C					
	Flow rate	0.2 mL min^{-1}					
	Mode	Isocratic					
	Mobile phase	80% MeOH: 20% H_2O (both with 0.1% of formic acid)					
		Precursor ion: 315 (CBD)			**Precursor ion: 318 (CBD-D3)**		
		m/z = 193 [a]	**m/z = 41**	**m/z = 123**	**m/z = 196 [a]**	**m/z = 41**	**m/z = 123**
MS/MS	Fragmentor	132 V	132 V	132 V	114 V	114 V	114 V
	Collision energy	18 V	70 V	34 V	18 V	70 V	34 V
	Capillary voltage (ESI^+)	3 kV					
	Gas temperature	310 °C					
	Gas flow	12 L min^{-1}					
	Nebulizer	50 psi					

[a] Used as quantification transitions.

3. Results and Discussion

3.1. Chromatographic Conditions

The standards and samples were prepared as EtOH:water solutions (see Section 2.3), which allowed the sample preparation step to be as environmentally friendly as possible. However, commercialized EtOH does not reach the degree of purity necessary to be used as a mobile phase in LC-MS/MS, so MeOH:water was used in the chromatographic step.

The optimization and selection of MS/MS transitions were performed using Agilent MassHunter Optimizer software in multiple reaction monitoring (MRM) mode in positive polarity. The variables related to the detector were studied by injecting a CBD standard solution of 1 µg mL^{-1} and a CBD-D3 solution of 1 µg mL^{-1}, both 1:1 EtOH:water. The product ions generated from the two precursor ions were studied, and then the fragmentor and the collision energy were optimized for each of the product ions. The optimized parameters are shown in Table 1. It was also determined that the quantification ion, the one that provides a higher signal, is the one that corresponds to the transition 315 > 193 for CBD and the one that corresponds to the transition 318 > 196 for CBD-D3. Finally, the capillary voltage was optimized for the product ions, being +3 kV in all of them.

Figure 1 shows the chromatogram of a standard solution containing 4 ng mL^{-1} of CBD and 8 ng mL^{-1} of CBD-D3 obtained under these conditions. The CBD peak is observed at a retention time of approximately 3.2 min with a good resolution.

3.2. Standards Preparation

EtOH is proposed as a solvent for the stock standard solutions, since it is a harmless and relatively inexpensive solvent compared to other organic solvents in which CBD is also soluble. However, elution force differences between MeOH (used in the mobile phase) and EtOH (used as solvent) caused a split of the peak if only EtOH was used as the solvent. Then, before injection to the LC-MS/MS system, the ethanolic standard solutions were diluted with water to obtain EtOH:water 1:1 solutions that prevented splitting.

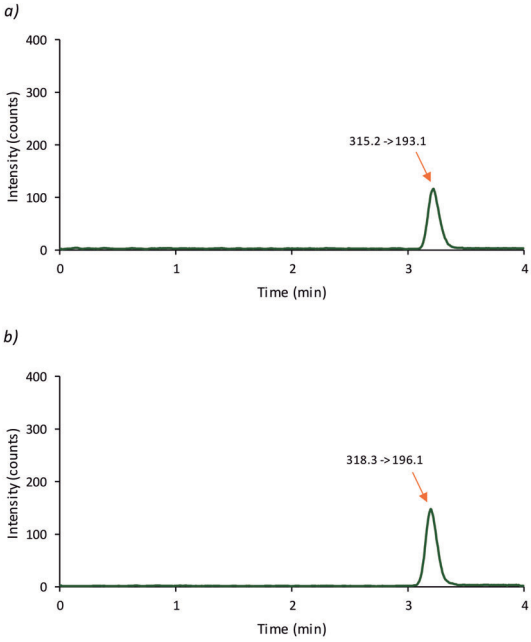

Figure 1. Chromatogram obtained applying the proposed LC-MS/MS method to a standard solution containing 4 ng mL^{-1} of analyte and 8 ng mL^{-1} of internal standard ((**a**) CBD; (**b**) CBD-D3).

3.3. Samples Preparation

The method was tested by applying it to six commercial cosmetic products and a raw material with different types of matrix.

The sample preparation process does not require any prior preconcentration steps, only brief shaking of the samples with EtOH to achieve leaching of CBD from the cosmetic matrix. Samples were filtered through a 0.45 µm pore size nylon filter, preventing solid particles from reaching the injector or column. As for standard solutions, filtered samples were diluted with water to obtain EtOH:water 1:1 solutions.

3.4. Analytical Figures of Merit of the Proposed Method

Quality parameters such as linearity, limit of detection (LOD), limit of quantification (LOQ), repeatability, and recovery were evaluated to validate the proposed method. The results are summarized in Table 2.

Table 2. Main analytical parameters of the proposed LC-MS/MS method.

LOD (ng mL^{-1})	LOQ (ng mL^{-1})	MLOD (ng g^{-1})	MLOQ (ng g^{-1})	Repeatability (%RSD)			
				Intra-Day (N = 5)		Inter-Day (N = 5)	
				2 ng mL^{-1}	4 ng mL^{-1}	2 ng mL^{-1}	4 ng mL^{-1}
0.22	0.74	22	74	4.6	5.1	5.9	8.5

N: Number of replicates.

The linearity can be defined in this case as the ability of the LC-MS/MS proposed method to induce an analytical signal whose intensity is directly proportional to CBD concentration. Analytical signals (analyte and internal standard peaks area ratio) of six standards containing CBD concentrations between 0.75 and 200 ng mL^{-1} and a fixed concentration of CBD-D3 (8 ng mL^{-1}) were calculated, and linearity was estimated by the coefficient of determination (R^2). An R^2 value of 0.9992 was obtained, showing that it is possible to determine CBD in samples with a relatively high concentration without needing to add more dilution steps than those recommended in the proposed method.

The instrumental LOD and LOQ, i.e., the smallest CBD concentration that can be detected or quantified with acceptable accuracy, were calculated as three and 10 times, respectively, the signal-to-noise ratio. Methods LOD and LOQ were calculated as the corresponding values in the sample, according to the preparation procedure. The LOD and LOQ were 0.22 and 0.74 ng mL^{-1} in the measurement solutions (i.e., 22 and 74 ng g^{-1} in the sample), respectively. These values allow the determination of CBD at trace levels in cosmetic formulations without needing pre-concentration steps.

The repeatability of the measurements is expressed as relative standard deviation (RSD) of several replicates of the same solution. It was evaluated by applying the proposed method to five replicates of two standard solutions containing different concentration levels of CBD (2 and 4 ng mL^{-1}) on the same day (intra-day) and for five consecutive days (inter-day). The results revealed that good repeatability was achieved (RSD < 8.5%).

The proposed method was applied to the analysis of six commercially available cosmetics (containing cannabidiol or *Cannabis sativa* seed oil) and a raw material (containing *Cannabis sativa* oil and a known amount of CBD).

The results are shown in Table 3. It revealed that CBD was detected in the raw material and in the cosmetic samples in where CBD was indicated on the label as an ingredient, while CBD was not detected in those samples in which *Cannabis sativa* seed oil was indicated as an ingredient.

Table 3. Application of the method to the analysis of commercial samples.

Sample [a]	Found Amount [b] (μg g^{-1})	Repeatability of Results RSD (%)	Relative Recovery [b] (for 2 ng mL^{-1}) (%)	Relative Recovery [b] (for 4 ng mL^{-1}) (%)
A	140 ± 8	5.7	101 ± 4	114 ± 16
B	316 ± 8	2.5	105.6 ± 1.2	109.0 ± 1.1
C	2060 ± 50	2.4	100 ± 5	100 ± 7
D	341 ± 17	5.0	113 ± 8	109 ± 11
E	N.D.	-	100 ± 2	100 ± 5
F	N.D.	-	110 ± 11	101 ± 13
G	1304 ± 14	1.1	-	-

[a] A, B, C, and D: Commercial cosmetic creams containing CBD as an ingredient; E: Shower gel containing *Cannabis sativa* seed oil as an ingredient; F: Hair mask containing *Cannabis sativa* seed oil as an ingredient; G (raw material): Labeled as *Cannabis sativa* oil standardized in 1300 μg g^{-1} CBD; [b] expressed as mean ± standard deviation of three replicates.

In order to evaluate the recovery of the method, six cosmetic samples were spiked with 1 mL of a CBD standard solution of 200 or 400 ng in the first step of the procedure (before treatment with EtOH), which results in 2 and 4 ng mL^{-1} in the measurement solutions. The recovery of CBD was calculated as the percentage of the CBD obtained by using the proposed method. As can be seen, the relative recovery values obtained ranged between 100% and 114%, which shows negligible matrix effects.

Figure 2 shows the chromatogram of the raw material and Figure 3 corresponds to a cream sample.

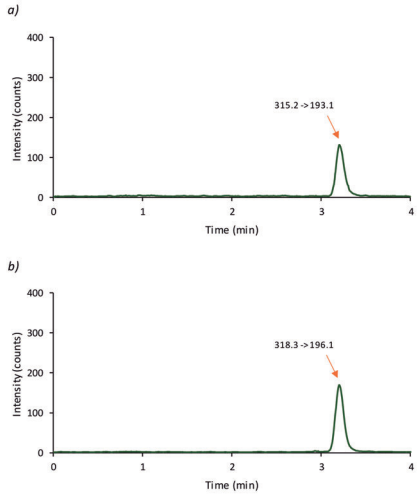

Figure 2. Chromatogram obtained applying the proposed LC-MS/MS method to *Cannabis sativa* oil raw material (sample G) ((**a**) CBD; (**b**) CBD-D3).

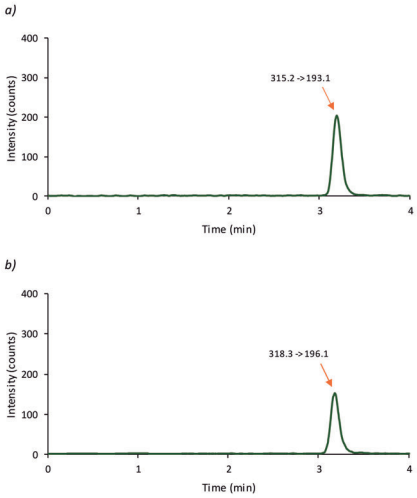

Figure 3. Chromatogram obtained applying the proposed LC-MS/MS method to a cosmetic cream (sample C) ((**a**) CBD; (**b**) CBD-D3).

4. Conclusions

A sensitive analytical method is proposed to determine CBD in cosmetic products using LC-MS/MS.

The proposed analytical method is simple and highly sensitive, since it allows the determination of CBD at trace levels. Likewise, the wide linearity range of the method allows the determination of CBD in samples with relatively high concentrations.

The characteristics of the method and the results obtained show its usefulness in carrying out this determination simply and quickly. The compound has been efficiently

determined in cosmetic samples of different natures with good analytical features. For this reason, the proposed method is suitable for quality control of cosmetic products that contain this ingredient, thus assuring that its concentration in the finished product is the desired one.

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Development of a sensitive method for determining traces of prohibited acrylamide in cosmetic products based on dispersive liquid-liquid microextraction followed by liquid chromatography-ultraviolet detection.

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Development of a sensitive method for determining traces of prohibited acrylamide in cosmetic products based on dispersive liquid-liquid microextraction followed by liquid chromatography-ultraviolet detection

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ABSTRACT

According to the European Regulation on cosmetic products, the presence of acrylamide in these consumer products is not allowed due to its mutagenic and potentially carcinogenic effects. Despite this ban, acrylamide might be present in those cosmetic products containing acrylamide-based polymers. However, there is no analytical method for its determination in this type of matrices. Based on this, the development of analytical methods for the determination of acrylamide in cosmetic products is required to guarantee consumer safety. In this work, an analytical method for determining traces of prohibited acrylamide in cosmetic products is presented for the first time. The method is based on dispersive liquid-liquid microextraction (DLLME), followed by liquid chromatography–ultraviolet (LC-UV) detection. A previous derivatization of acrylamide with 2-naphthalenethiol was performed to obtain a more lipophilic compound, and therefore, more extractable in the DLLME step, in addition to the bathochromic displacement of the measurement wavelength. The main variables involved in both derivatization and DLLME step were studied to provide the best enrichment factors. Under the optimized conditions, 6.5 mL of aqueous sample solution was cleaned-up from lipophilic matrix components by liquid-liquid extraction (LLE) using 3.5 mL of hexane. Then, 5 mL were taken and subjected to microwave derivatization with a mixture of 250 μL of 2-naphthalenethiol (0.5 mg mL^{-1}) and 250 μL of di-sodium tetraborate (4 mg mL^{-1}). Afterwards, it was subjected to DLLME by injecting a mixture of 250 μL of acetone (as disperser solvent) and 80 μL of chloroform (as extraction solvent). The extracts were evaporated to dryness, reconstituted in mobile phase and injected into the LC-UV system. The method was successfully validated showing good linearity (from 10 ng mL^{-1} to 20 μg mL^{-1}), an enrichment factor of 103 ± 2, instrumental limits of detection and quantification of 3.0 and 9.8 ng mL^{-1}, which, according to sample treatment, correspond to 0.7 and 2.4 μg kg^{-1} in the sample, respectively, and a good repeatability (RSD < 14%). Finally, the proposed analytical method was applied to the determination of acrylamide in commercial cosmetic samples of different nature with good relative recovery values (85 – 112%), which shows that the matrix effect is negligible. This method could help the cosmetic industry to control the cosmetic products containing potential acrylamide-releasing ingredients.

1. Introduction

Cosmetic industries must guarantee that the products they manufacture are safe for users, and therefore, cosmetic products are not expected to contain substances prohibited by the European Regulation [1]. Despite that, the potential presence of prohibited substances must be controlled since some of them might be present due to unintentional causes, such as degradation of some ingredient in the formulation, formation of by-products by reaction between ingredients, deficiencies during the purification of raw materials, or migration of substances from the package.

An example of these prohibited substances that might be present in cosmetics is acrylamide, which is a mutagenic and potentially carcinogenic compound according to the International Agency for Research on Cancer (IARC) [2,3]. The toxic effects of acrylamide are caused by itself and glycidamide, its reactive epoxide metabolite, because these compounds can react with the chemical moieties of biological molecules, especially the thiol moiety of cysteine residues in proteins and the amino or ketone moieties of DNA bases, forming a covalent bond with these macromolecules which prevents its correct functioning [4,5].

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Acrylamide might be present in those cosmetic products containing acrylamide-based polymers, such as polyacrylamide, acrylamide/sodium acrylate copolymer, acrylamides/acrylates/DMPA/methoxy PEG methacrylate copolymer, acrylates/acrylamide copolymer, acrylates/diacetoneacrylamide copolymer and others [6], which are synthetized through the polymerization of acrylamide monomers by the addition of an anionic radical initiator. These polymers are widely used in cosmetic creams and gels as stabilizing agents, antistatic agents, binders, film-forming agents, fixatives, thickeners, or rheology modifiers due to their properties [7,8], and small amounts of unreacted acrylamide monomers could accompany them. For this purpose, the European Regulation not only forbids the use of acrylamide as ingredient in cosmetic products, but it also restricts the use of polyacrylamide so that the maximum residual acrylamide content is 0.1 mg kg^{-1} for leave-on cosmetic products and 0.5 mg kg^{-1} for the rest [1,7,9].

To the best of our knowledge, it should be noted that there are neither official nor other published analytical methods for the determination of acrylamide in cosmetic products. Therefore, the development of analytical methods to determine it in these consumer products is of great interest in order to guarantee the consumer safety.

Acrylamide has been determined in other matrices, such as food, by liquid chromatography (LC) coupled to mass spectrometry (MS) [10–15], or with ultraviolet–visible (UV/Vis) [16–19] or fluorescence [20] detection, and also by gas chromatography (GC) coupled to MS [21–25] or electron capture detection [25,26], or capillary zone electrophoresis (CZE) with UV/Vis detection [27]. Regarding the treatment of the food samples, different procedures taking into account the advantages offered by microextraction techniques, such as dispersive liquid-liquid microextraction (DLLME) [15,17,24,25], single-drop microextraction (SDME) [26] and solid-phase microextraction (SPME) [23], have been published.

The aim of this work is to develop, for the first time, an analytical method to determine acrylamide in cosmetic products, exploiting the high potential of DLLME as enrichment step before its determination by LC. To improve the selectivity and sensitivity of the analysis, a previous clean-up with hexane was performed to separate those lipophilic ingredients that could be present in the cosmetic formulation, followed by a derivatization step to convert acrylamide to a more lipophilic compound to be extracted by DLLME. In addition, the derivatization reaction introduced a chromophore moiety that produced the bathochromic displacement of the measurement wavelength so that it can be measured by LC-UV without the potential interferences that might be encountered at low wavelengths. Different derivatization procedures to determine acrylamide in other matrices by LC-UV have been described in the analytical literature, based on the use of d-cysteine [14], mercaptobenzoic acid [19], xanthydrol [24], hydrobromic acid [26], or on the thiol-Michael addition reaction with 2-naphthalenethiol [17,20]. In this work, a base catalyzed thiol-Michael addition reaction [28] was carried out, using 2-naphthalenethiol at basic pH as derivatizing agent. Under basic pH, the addition of the thiol group to acrylamide is catalyzed forming a thioether type addition product (hereinafter derivatized-acrylamide). Furthermore, for the first time this derivatization reaction was microwave assisted, which accelerated the derivatization reaction by reducing the reaction times.

The proposed method allows to establish if the final cosmetic product meets the requirements stipulated in the European Regulation to ensure the consumer safety.

2. Experimental

2.1. Apparatus

An Agilent 1220 Infinity LC system, comprised of a degasser, a programmable pump, an autosampler, a thermostated column oven and a UV/Vis detector, was employed throughout the study. A Purospher® RP-18 (125 mm × 4 mm i.d., 5 μm particle size) column from Merck (Darmstadt, Germany) was used.

A MicroSYNTH microwave oven from Milestone (Sorisole, Italy) with 70-mL PTFE reactors was used for the derivatization reaction.

A Stuart SBHCONC/1 sample concentrator from Cole-Parmer (Staffordshire, United Kingdom) was used to evaporate the extracts after the extraction process.

A ZX3 vortex mixer from VELP Scientifica (Usmate Velate, Italy) was used to facilitate the leaching of the analyte from the sample, an EBA 21 centrifuge Hettich® (Tuttlingem, Germany), was used for phase separation, and a Basic 20 pHmeter from Crison (Alella, Spain) was used for the pH adjustments.

2.2. Reagents and samples

Acrylamide ≥ 99% used as standard and 2-naphthalenethiol 99% used as derivatization agent were purchased from Sigma-Aldrich (Steinheim, Germany).

LC-grade ethanol (EtOH), LC-grade hexane 96%, ultrapure acetone and analytical reagent-grade chloroform were all purchased from Scharlau Chemie (Barcelona, Spain). Deionized water was obtained from a Connect water purification system provided by Adrona (Riga, Latvia).

Analytical reagent grade sodium chloride 99.5% from Scharlau (Barcelona, Spain) was used to adjust the ionic strength, and di-sodium tetraborate from Merck Eurolab (Briare Le canal, France) was used to adjust the pH needed in the derivatization step.

Nitrogen 99.9%, used for the evaporation of the extracts in the sample concentrator, was obtained by means of a NiGen LCMS 40-1 nitrogen generator from Claind (Lenno, Italy).

Three commercial cosmetic products, i.e. a lipophilic cream and two hydrophilic gels, were analysed. They were selected from different brands and the names are not shown for confidentiality reasons.

2.3. Proposed method

2.3.1. Standards and sample preparation

A stock solution containing 500 μg mL^{-1} of acrylamide was prepared in deionized water. Then, an aliquot of this solution was diluted with deionized water to prepare a standard aqueous solution (20 μg mL^{-1}) used to prepare the working standard aqueous solutions (10–200 ng mL^{-1}), which were adjusted to 2% NaCl (w/v).

Regarding sample preparation (either cream or gel), 0.15–0.5 g were weighted in triplicate into 10 mL volumetric flasks, which were filled to the line with deionized water. The sample dispersion was shaken with a vortex mixer (5 min) forming an emulsion. Then, 0.2 g of NaCl was added to break the emulsion. A fourth replicate was prepared in the same way but it was not subjected to the derivatization step (see Section 2.3.2).

Next, 6.5 mL of the standard or the sample aqueous solutions were placed in a glass tube with 3.5 mL of hexane, and both phases were mixed with a vortex mixer (30 s), and then centrifuged (6000 rpm, 10 min). Finally, 5 mL of the lower aqueous phase was transferred to a microwave PTFE reactor.

2.3.2. Derivatization step

For this step, 250 μL of an ethanolic 2-naphthalenethiol solution (0.5 mg mL^{-1}) and 250 μL of an aqueous di-sodium tetraborate solution (4 mg mL^{-1}) were added to all PTFE reactors containing 5 mL of the sample or the standard solution described above, except for the reactor containing the sample that is not going to be derivatized (hereinafter, the non-derivatized sample solution), to which 250 μL of deionized water was added instead of the 2-naphthalenethiol solution.

Then, the PTFE reactors were inserted in the microwave oven (500 W) and the following temperature program was applied: from room temperature to 70 °C in 1 min and kept constant for 10 min. Once cooled, the reaction mixture of each PTFE reactor was collected and transferred to a 15-mL glass tube to perform the DLLME step.

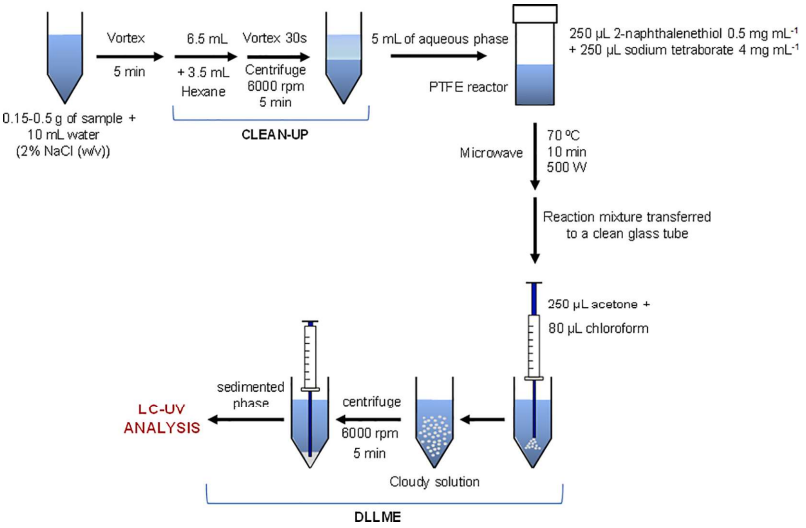

Fig. 1. Schematic diagram of the proposed method.

2.3.3. Dispersive liquid-liquid microextraction (DLLME)

A mixture of 250 µL of acetone (as disperser solvent) and 80 µL of chloroform (as extraction solvent) was quickly injected with a syringe into each solution forming a microemulsion. Then, the glass tube was centrifuged (6000 rpm, 5 min), and the sedimented phase (ca. 50 µL) was collected using a 100 µL Hamilton 1705 RNR syringe (Bonaduz, Switzerland) and transferred into a 200 µL insert placed inside a 1.5-mL injection vial.

The extracts were evaporated to dryness at room temperature under a nitrogen stream, and they were finally reconstituted in 30 µL of a mixture of EtOH and deionized water (50:50) for LC-UV analysis.

Fig. 1 shows a schematic diagram of the whole experimental procedure.

2.3.4. LC-UV analysis

At this point, 20 µL of each reconstituted extract were injected into the LC system described before (see Section 2.1). The mobile phase consisted of water (solvent A) and EtOH (solvent B). The pumps supplied the following gradient at 1 mL min^{-1} and at room temperature: 0–3 min, 50% solvent B; 3–4 min linear gradient to 100% solvent B, held for 1 min; 5–5.5 min return to 50% solvent B and held for 2.5 min. Peak area monitoring was performed at 254 nm. Fig. S1 shows the chromatogram obtained for a standard solution of 80 ng mL^{-1} subjected to the whole proposed method.

Calibration curves were constructed by plotting the peak area of the target analyte versus concentration.

Note: if there was a native peak in the sample, observed in the chromatogram of the non-derivatized sample solution, this signal should be subtracted from the sample solutions subjected to the derivatization reaction.

3. Results and discussion

3.1. Study of the experimental variables involved in the derivatization step

A previous selection of the experimental derivatization variables was essential in order to obtain the highest signals and, therefore, the lowest limits of detection. Different variables can affect the derivatization reaction, such as the temperature at which the reaction takes place, the derivatization time, the concentration of the derivatization reagent and the pH. These variables were studied with the one-variable-at-a-time approach, where each condition was performed in triplicate using 5 mL of a standard aqueous solution of acrylamide at 20 µg mL^{-1} to which 250 µL of an ethanolic 2-naphthalenethiol solution, as derivatization reagent, and 250 µL of an aqueous di-sodium tetraborate solution (4 mg mL^{-1}), as pH regulator, were added. After each derivatization experiment, the reaction mixture was subjected to DLLME using volumes of extraction and disperser solvents not yet optimized at that time (respectively 80 µL of chloroform and 500 µL of acetone). After that, the organic phase was transferred to a glass insert located in a 1.5 mL injection vial and dried using a stream of nitrogen. Finally, the extract was reconstituted with 30 µL of an ethanol and deionized water mixture (50:50) for LC-UV/Vis analysis, where the chromatographic peak area of the derivatized-acrylamide was the response function.

3.1.1. Derivatization temperature

The effect of the microwave temperature program on the response function was studied. Different tests were carried out increasing the temperature to 50, 60, 70 and 80 °C in 1 min and keeping it constant for 20 min. No higher temperatures were tested due to the risk of acrylamide polymerization above 80 °C [29]. As shown in Fig. 2a, the signal increased with temperature up to 70 °C. Beyond this value the signal decreased most probably due to the polymerization of acrylamide [29]. Therefore, 70 °C was selected as derivatization temperature for further experiments.

3.1.2. Derivatization time

Similarly, the effect of reaction time was studied. Different tests were carried out increasing the temperature to 70 °C in 1 min and keeping it constant for 5, 10, 20, 30 and 40 min. The results (Fig. 2b) showed that the response function increased with a derivatization time of 10–20 min, but decreased with higher values, maybe due to the partial polymerization of acrylamide. Therefore, a reaction time of 10 min was set for further experiments.

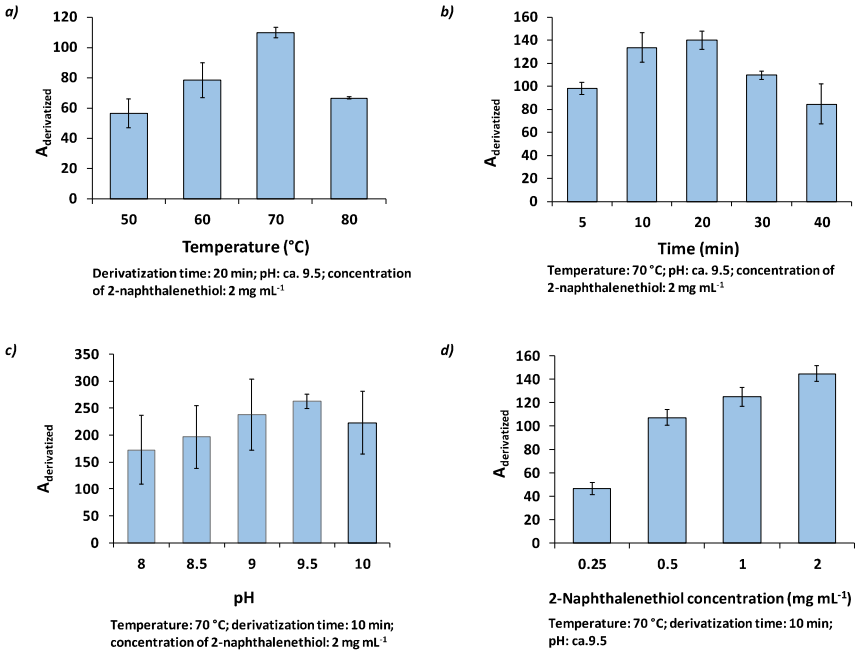

Fig. 2. Optimization of derivatization step conditions: (a) derivatization temperature, (b) derivatization time, (c) pH and (d) concentration of derivatization agent. Extraction conditions: 80 μL of chloroform; 500 μL of acetone; 0% NaCl. Error bars show the standard deviation for N = 3.

3.1.3. pH

As said before, the derivatization reaction is a base catalyzed thiol-Michael addition, which requires a basic pH to be carried out [28]. The influence of this variable was studied with aqueous di-sodium tetraborate solutions of 4 mg mL^{-1}, adjusted to different pH values ranging from 8 to 10. As shown in Fig. 2c, there were no significant differences between the pH values tested and, considering that the original prepared solution of sodium tetraborate had a pH value ca. 9.5 by itself, it was decided to not adjust the pH for further experiments.

3.1.4. Derivatization agent concentration

Finally, the effect of derivatization agent concentration (i.e., 2-naphthalenethiol) on the response function was also studied. The excess of this agent in the reaction mixture caused a slight turbidity that could hinder the DLLME process. Based on the results of Fig. 2d, a concentration of 0.5 mg mL^{-1} was selected, since it allowed maintaining a sufficiently high analytical signal without the appearance of turbidity.

3.2. Study of the experimental variables involved in the DLLME procedure

During this study, the enrichment factor (EF), defined as $EF = C_{ext}/C_0$ (where C_{ext} is the concentration of derivatized-acrylamide in the final extract and C_0 is the initial concentration of this compound in the aqueous phase before the extraction) was used as response function.

Different variables may affect the DLLME process, such as the type and the volume of extraction and disperser solvents, and the ionic strength of the donor phase. For the study of these variables, each experimental condition was evaluated in triplicate using 5 mL of a standard aqueous solution of acrylamide at 20 μg mL^{-1} subjected to the derivatization process as described before.

The extraction time was not studied, since it is defined as the time between the injection of the mixture of solvents and the start of the centrifugation, and it has been shown that it has a negligible effect on DLLME [30].

In the case of potentially ionisable compounds, it is also important to optimize the pH of the donor phase, but it is not the case of derivatized-acrylamide, which is not influenced by pH, so this variable was not considered in this study.

3.2.1. Type of extraction and disperser solvent

In order to obtain a suitable combination of both extraction and disperser solvent to carry out the DLLME process, the recommended properties of the extraction solvent are low water solubility and higher density than water, while the disperser solvent must be miscible in both extraction solvent and water to achieve a good dispersion. It should be noted that, during the DLLME step, the disperser solvent causes the dispersion of the extraction solvent in form of multiple small-size droplets, so that the surface area between the extraction solvent and the donor solution was infinitely large, favoring the transfer of the analyte from the aqueous donor phase to the extraction solvent.

In this sense, different combinations of dichloromethane and chloroform (as extraction solvents) and ethanol and acetone (as disperser solvents) were tested. No phase separation was achieved with the chloroform-ethanol combination, whereas no dispersion was observed when dichloromethane was used. Similar results were obtained in previous studies conducted in our research group [31,32]. Thus, the combination of chloroform-acetone was selected for further experiments.

3.2.2. Volume of the extraction and disperser solvents

To study the effect of the volume of the extraction solvent, mixtures of 500 μL of acetone and different volumes of chloroform, ranging from

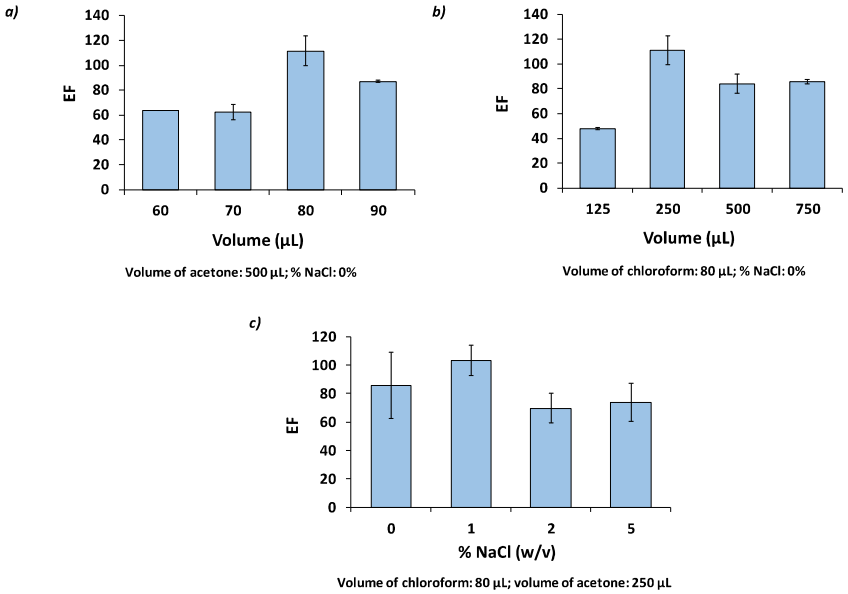

Fig. 3. Optimization of DLLME conditions: (a) extraction solvent volume, (b) disperser solvent volume and (c) ionic strength of the donor phase. Derivatization conditions: temperature: 70 °C; time: 10 min; pH: ca. 9.5; concentration of 2-naphthalenethiol: 0.5 mg mL^{-1}. Error bars show the standard deviation for N = 3.

60 to 90 µL, were tested. According to Fig. 3a, the response increased with the volume of chloroform up to 80 µL. However, the response decreased using a higher volume, since the dispersion was not properly formed, but rather large droplets of the extraction solvent were formed, which were quickly sedimented. Therefore, a volume of 80 µL was selected for the extraction solvent.

Next, mixtures of 80 µL of chloroform with different volumes of acetone ranging from 125 to 750 µL were tested. According to Fig. 3b, the highest signal was obtained for 250 µL, whereas higher volumes of disperser solvent decreased the EF, probably because the partition coefficient of the analyte into the extraction solvent decreased [30].

3.2.3. Ionic strength of donor phase

In general terms, the increase of ionic strength leads to a decrease in the solubility of both the non-polar analyte and the organic extraction solvent in the aqueous phase, due to the salting-out effect. The influence of this variable was studied by adding different concentrations of sodium chloride to the donor phase, ranging from 0 to 5% (w/v), before performing the DLLME step.

As can be seen in Fig. 3c, the EF increases slightly with 1% sodium chloride, but the addition of higher amounts of salt has a significant negative effect on the EFs of derivatized-acrylamide. This negative effect could be due to the increase of viscosity and hence, to the difficulty of the mass transfer of the target analyte [33].

3.3. Study of the pretreatment of the sample

Once the whole method was optimized, samples with different cosmetic composition were analyzed.

At the beginning there was a problem with the insoluble matrix, which made the DLLME step difficult. Considering the high water-solubility of acrylamide and the very frequent fatty matrix encountered in some cosmetic samples, acrylamide was leached from the sample by the addition of water. Leaching was initially assisted by ultrasonic agitation, but the signal decreased as the agitation time increased, probably due to acrylamide polymerization. For this reason, an alternative was tested by shaking with the vortex mixer and, since the signal did not decrease, the vortex mixer was selected instead of the ultrasonic agitation to carry out leaching.

However, another shortcoming arises, since an emulsion was formed as a consequence of the vigorous agitation with the vortex mixer. In this sense, NaCl (up to 2% w/v) was added to break the formed emulsion by disturbing the ion layers around the micelles and reducing the zeta potential, making the emulsion unstable. As consequence, the NaCl was readjusted in the overall method, in such a way the EF was slightly decreased (see Section 3.2.2) but it allowed carrying out the DLLME step in the samples.

Later, hexane was added to extract the lipophilic components that might be co-leached with the acrylamide, and in this way to perform a cleaning step to reduce the possibility that other lipophilic compounds present in the matrix could drag during the procedure and disturb the DLLME step.

It is important to consider that some of the analyzed samples showed a native peak in the retention time of derivatized-acrylamide when these samples were not derivatized. This could jeopardizes the results and, therefore, it is mandatory to know if each sample has a native peak where derivatized-acrylamide is eluted. In this sense, the sample solutions must be injected without and with derivatization, to subtract the signal of the native peak from the derivatized-acrylamide signal.

3.4. Analytical figures of merit of the proposed method

Quality parameters such as linearity, enrichment factor (EF), limit of detection (LOD), limit of quantification (LOQ) and repeatability were evaluated to validate the proposed method. The results are summarized in Table 1.

A high level of linearity, that reached at least 20 µg mL^{-1}, was

Table 1
Main analytical parameters of the proposed DLLME-LC-UV method.

EF[a] ± s	LOD[b] (ng mL^{-1})	LOQ[b] (ng mL^{-1})	MLOD[c] (μg kg^{-1})	MLOQ[c] (μg kg^{-1})	Repeatability (%RSD) (N = 5) [d]			
					Intra-day		Inter-day	
					40 ng mL^{-1}	80 ng mL^{-1}	40 ng mL^{-1}	80 ng mL^{-1}
103 ± 2	3.0	9.8	0.7	2.4	8.9	3.7	13.5	8.1

[a] EF: Enrichment factor, as the mean of three replicates, s: standard deviation
[b] LOD: Limit of detection; LOQ: Limit of quantification, calculated as 3 and 10 times, respectively, the signal-to-noise ratio
[c] MLOD: Method limit of detection; MLOQ: Method limit of quantification, according to the sample pretreatment
[d] RSD: Relative standard deviation; N: number of replicates

obtained. Nevertheless, taking into account the low acrylamide levels expected in the samples, the working range was set from 10 to 200 ng mL^{-1} and the equation obtained from the calibration curve was:

$$A_{254} = (0.49 \pm 0.01)\cdot C_{acrylamide} (-2 \pm 2); \quad R^2 = 0.9990; \quad N = 5$$

where A_{254} is the peak area obtained for derivatized-acrylamide at 254 nm; $C_{acrylamide}$ is the concentration of acrylamide in ng mL^{-1}; R^2 is the coefficient of determination and N is the number of calibration points.

The achieved EF = C_{ext}/C_0 was 103 ± 2.

The LOD and LOQ, calculated as the concentration value corresponding to a signal-to-noise value of 3 and 10 respectively, were 3.0 and 9.8 ng mL^{-1} (i.e., 0.7 and 2.4 μg kg^{-1} in the sample), respectively. These values are more than sufficient to detect and to determine the threshold values established by the European Regulation of cosmetic products (i.e., 0.1 mg kg^{-1} in leave-on cosmetic products and 0.5 mg kg^{-1} in the rest) [1].

The repeatability, expressed as relative standard deviation (RSD), was evaluated by applying the proposed method to five replicates of standard aqueous solution containing the target analyte at two different levels of concentration (40 and 80 ng mL^{-1}) on the same day (intra-day) and for five consecutive days (inter-day). Results revealed that good repeatability was achieved (RSD < 14%).

3.5. Application to the analysis of commercial cosmetic products

Three different commercially available cosmetic samples (one lipophilic cream and two hydrophilic gels) containing acrylamide-based polymers were analyzed using the proposed DLLME-LC-UV method.

The results, shown in Table 2, revealed that the acrylamide contents found were relatively high considering that this substance is prohibited in cosmetic products, and that they are even above the threshold values (i.e., 0.1 mg kg^{-1} in leave-on cosmetic products and 0.5 mg kg^{-1} in the rest) [1].

Table 2
Acrylamide contents found in three cosmetic samples and their relative recovery values obtained by applying the developed DLLME-LC-UV method.

Sample [a]	Fortified amount (mg kg^{-1}) [b]	Found amount (mg kg^{-1}) [b]	Relative recovery (%) [b]
A	–	0.7 ± 0.1	–
	1.7 ± 0.2	2.1 ± 0.2	85 ± 2
	3.3 ± 0.5	4.5 ± 0.9	109 ± 9
B	–	1.5 ± 0.6	–
	1.3 ± 0.1	3.0 ± 0.1	109 ± 8
	2.5 ± 0.1	4.3 ± 0.3	109 ± 7
C	–	0.3 ± 0.1	–
	1.8 ± 0.1	1.9 ± 0.1	98 ± 6
	3.2 ± 0.3	3.8 ± 0.6	112 ± 10

[a] A and B: hydrophilic gel; C: lipophilic cream
[b] expressed as mean ± standard deviation of three replicates

To perform recovery studies, and thus evaluate matrix effect, the three cosmetic samples were fortified with the target analyte at two concentration levels. The concentrations of derivatized-acrylamide were used to calculate the recovery as follows:

$$Recovery\,(\%) = \frac{Concentration_{fortified\ sample} - Concentration_{unfortified\ sample}}{Concentration_{added}} \times 100$$

As can be seen in Table 2, the relative recovery values obtained ranged between 85 and 112%, which demonstrates that the three types of cosmetic matrices of different nature have negligible effects on the results and, therefore, external calibration can be used as described in the proposed method. Typical chromatograms of an unfortified and fortified gel sample at 2.6 mg kg^{-1} are shown in Fig. S2.

3.6. Comparison with other previously published methods on acrylamide determination

It should be emphasized that the proposed method is the first one focused on the determination of acrylamide in cosmetic products. In this sense, the most relevant characteristics of the proposed method were compared with those achieved by other previously published methods for the determination of acrylamide but in other matrices instead of cosmetics. In order to carry out a more objective comparison, the methods to compare with the proposed method were selected on the basis of having similarities on sample pretreatment (i.e., derivatization step and/or extraction by DLLME) and/or on the analytical technique (i.e., LC-UV). It should be said that combining derivatization with 2-naphthalenethiol and DLLME allows to achieve a MLOD in the low μg kg^{-1} range despite using low-cost instrumentation like LC-UV, being similar to those obtained by using more sophisticated and expensive instruments such as LC–MS [10–15] or GC–MS [21–25]. From the data shown in Table 3, it is concluded that DLLME allows a faster extraction of acrylamide (or its derivative) when compared with other methods using other (micro)extraction techniques. It is true that those methods, including the proposed one, carrying out a derivatization step require an additional time. Nevertheless, a considerable reduction in the derivatization time is achieved in the proposed method due to microwave assistance when compared to the same derivatization reaction without using microwaves [17,20]. In any case, the reported analytical methods allow the determination of acrylamide at trace levels, as it was the objective. Finally, as it is mandatory, the reported methods allow the determination of acrylamide with quantitative recoveries, despite some of them require standard addition calibration to achieve it [15,20].

4. Conclusions

A sensitive analytical method to determine acrylamide in cosmetic products is proposed here for the first time. It is based on a microwave-assisted derivatization reaction with 2-naphthalenethiol followed by dispersive liquid-liquid microextraction and liquid chromatography with UV detection. With this new approach, the compound has been efficiently determined in cosmetic samples of different nature, besides

Table 3
Comparison of the proposed DLLME-LC-UV approach with other methods for the determination of acrylamide in different samples.

Sample	Sample pretreatment[a]	Extraction time (min)	Instrumental technique[b]	Derivatization agent (time)	MLOD[c]	Relative recovery (%)	Ref.
Cosmetic products	DLLME	Instantaneous	LC-UV	2-naphthalenethiol (10 min)	0.7 μg kg^{-1}	85–112	This work
Bread and biscuits	DLLME	Instantaneous	LC-UV	2-naphthalenethiol (45 min)	n.r. [d]	90–96	[17]
Potato chips	DLLME	Instantaneous	GC–MS	xanthydrol (40 min)	0.6 μg kg^{-1}	97	[24]
Brewed coffee	DLLME	Instantaneous	LC–MS/MS	–	0.9 μg L^{-1}	97–106	[15]
Brewed coffe	IL-based *in situ* DLLME	10	GC–MS	–	n.r.	n.r.	[25]
Potato crisps	LLE	–	LC-FLD	2-naphthalenethiol (40 min)	129.2 μg kg^{-1}	105–108	[20]
Foods	LLE	10	LC-MS/MS	d-cysteine (50 min)	0.04 μg kg^{-1}	92–104	[14]
Radix asparagi	SLE	10	LC-UV	2-mercaptobenzoic acid (100 min)	25 μg kg^{-1}	107	[19]
Foods	SDME	5	GC-ECD	hydrobromic acid (15 min)	n.r.	97–104	[26]
Biscuits	MSPE	20	LC-UV	–	1.3 μg kg^{-1}	86–98	[18]
Chinese food	SLE + SPE	30	LC-UV	–	8.0 μg kg^{-1}	89–103	[16]

[a] DLLME: Dispersive liquid-liquid microextraction; IL: ionic liquid; LLE: Liquid-liquid extraction; MSPE: Magnetic solid-phase extraction; SDME: Single-drop microextraction; SLE: Solid-liquid extraction; SPE: Solid-phase extraction
[b] ECD: Electron capture detection; FLD: Fluorescence detection; GC: Gas chromatography; LC: Liquid chromatography; MS: Mass spectrometry; MS/MS: Tandem mass spectrometry; UV: Ultraviolet detection
[c] MLOD: method limit of detection
[d] n.r.: not reported

providing good analytical characteristics. The proposed analytical method is simple and highly sensitive, since the enrichment step allows to determine the analyte at trace levels below the threshold values established by the European Regulation of cosmetic products. All these features make the proposed method useful for the quality control of cosmetic products containing acrylamide-based ingredients, in order to ensure user safety and compliance with current legislation.

CRediT authorship contribution statement

Lorenza Schettino: Validation, Investigation, Data curation, Writing - original draft. **Juan L. Benedé:** Conceptualization, Methodology, Writing - review & editing. **Alberto Chisvert:** Conceptualization, Methodology, Writing - review & editing, Supervision, Project administration. **Amparo Salvador:** Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2020.105402.

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SUPPORTING MATERIAL

Development of a sensitive method for determining traces of prohibited acrylamide in cosmetic products based on dispersive liquid-liquid microextraction followed by liquid chromatography-ultraviolet detection

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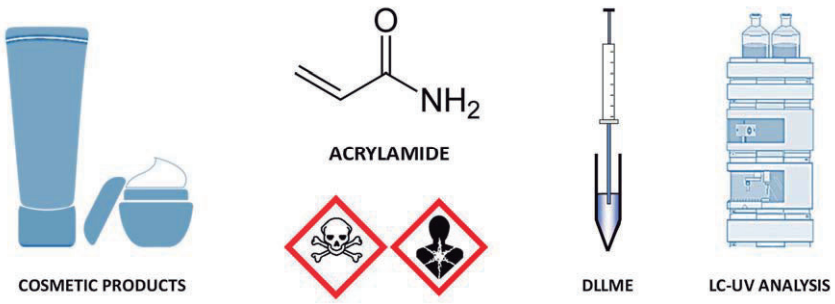
Graphical abstract



SUPPORTING MATERIAL

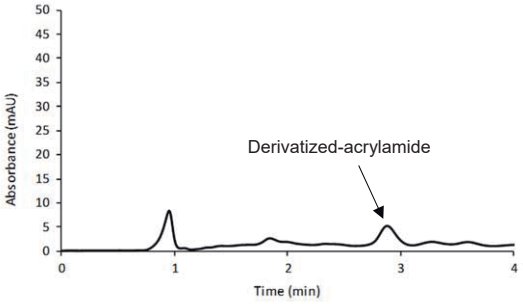


Fig. S1. Chromatogram obtained applying the proposed DLLME-LC-UV method to a standard solution containing acrylamide at 80 ng mL^{-1}

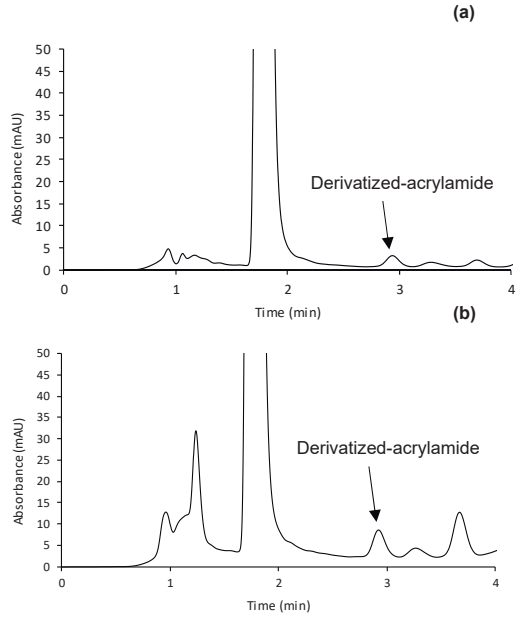


Fig. S2. Chromatograms obtained applying the proposed DLLME-LC-UV method to a gel sample: (a) unfortified and (b) fortified at 2.6 mg kg^{-1}

Trace determination of prohibited acrylamide in cosmetic products by vortex-assisted reversed-phase dispersive liquid-liquid microextraction and liquid chromatography-tandem mass spectrometry.

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Trace determination of prohibited acrylamide in cosmetic products by vortex-assisted reversed-phase dispersive liquid-liquid microextraction and liquid chromatography-tandem mass spectrometry

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ABSTRACT

An analytical method for the determination of residual acrylamide in cosmetic products containing potential acrylamide-releasing ingredients is presented. The method is based on vortex-assisted reversed-phase dispersive liquid-liquid microextraction (VA-RP-DLLME) to extract and preconcentrate acrylamide by using water as extraction solvent taking advantage the highly polar behavior of this analyte, followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) for its determination. Under optimized conditions (5 mL toluene as supporting solvent, 50 µL of water as extraction solvent, 1 min for vortex extraction time) the method was properly validated obtaining good analytical features (linearity up to 20 ng mL^{-1}, method limits of detection and quantification of 0.51 and 1.69 ng g^{-1}, respectively, enrichment factor of 52, and good repeatability (RSD < 4.1%)). The proposed analytical method was applied to the determination of acrylamide in commercial samples that were weighed and dispersed in the minimum quantity of methanol (50 µL) by vortex stirring before applying the VA-RP-DLLME procedure. Through the pretreatment of the sample and the use of acrylamide-d_3 as surrogate, the matrix effect was overcome, obtaining good relative recovery values (88–108%). The proposed method has shown efficacy, simplicity, and speed, and it allows the determination of acrylamide at trace levels easily, which could make it very useful for companies in the quality control of cosmetic products containing potential acrylamide-releasing ingredients to fulfill the safety limits imposed by European Regulation.

1. Introduction

The Annex II of the European Regulation 1223/2009 on cosmetic products [1] includes a list of prohibited substances in cosmetic products. These compounds cannot be present in this type of household products and their residual presence is just accepted if they are technologically inevitable with correct manufacturing procedures and provided that the finished product is safe. Therefore, these substances must be controlled in cosmetic products, since some of them could be present unintentionally due to, for example, the formation of by-products resulting from reaction between ingredients, or deficiencies in the purification of the raw materials, or degradation of some ingredients or migration of components from the packaging.

One of these prohibited substances in cosmetic products is acrylamide, which presents mutagenic and potentially carcinogenic effects. It belongs to the group of compounds 2A, defined as probably carcinogenic to humans, according to the classification of the International Agency for Research on Cancer (IARC) [2], and it has high systemic toxicity since it can bind covalently with macromolecules such as proteins and DNA, blocking its proper functioning [3,4].

Although the use of acrylamide as an ingredient in cosmetics is prohibited, many polymers synthesized from acrylamide are recurrently used as ingredients in cosmetic formulations due to their multiple and varied functions, such as stabilisers, antistatic agents, foam builders, binders, film-formers, fixatives, thickeners, or rheology modifiers, becoming widely used in the cosmetic industry [5,6]. These ingredients are known as the category of polyacrylamides, which includes a long list of various acrylamide copolymers and crosspolymers, such as the well-known polyacrylates and polyquaterniums, or others such as acrylamide/ammonium

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acrylate copolymer, acrylamide/sodium acrylate copolymer, or acrylamide/isopropylacrylamide crosspolymer [5,7].

However, it is important to highlight that the use of these polymers is correlated to the presence of traces of acrylamide in cosmetic products. Long polymeric chains are made-up by reaction between acrylamide monomers, so there is the possibility that small amounts of unreacted acrylamide monomers accompany them, ending up in the finished product and, consequently, exposing the consumer to a risk [5].

To this end, the European Regulation not only prohibits the use of acrylamide as an ingredient in cosmetic products, but also restricts the use of acrylamide-based polymers to ensure that the maximum content of residual acrylamide is reduced to less than 0.1 mg kg^{-1} in leave-on cosmetics, and less than 0.5 mg kg^{-1} in all other types of cosmetics [1,5,8]. Therefore, it is of great interest to develop new methods to determine that the concentration of acrylamide in cosmetic products is below the safety limits dictated by the European Regulation.

Although acrylamide was extensively determined in other matrices such as food [9–30], to the best of our knowledge, there is only one published analytical method for its determination in cosmetic products [31]. In this antecedent, proposed by our research group, a clean-up with hexane was performed through a liquid-liquid extraction, followed by a microwave-assisted derivatization of acrylamide with 2-naphthalenethiol, and finally the analyte was preconcentrated by means of dispersive liquid-liquid microextraction (DLLME) using chloroform as extraction solvent. The extract was dried and reconstituted in an ethanol:water solution to be finally analyzed by liquid chromatography-ultraviolet detection (LC-UV). The derivatization step was necessary to convert the acrylamide into a more lipophilic compound in order to be extracted by DLLME and, at the same time, to introduce a chromophore moiety that would allow its detection by UV spectrometry.

Herein, a new analytical method for the determination of acrylamide in cosmetic products is proposed. This new method consists of a preconcentration and cleaning step through vortex-assisted reserved-phase DLLME (VA-RP-DLLME) prior to liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analysis. Working in reversed phase (i.e., water as extraction phase) is due to the high polarity and water-solubility of acrylamide and therefore its affinity for the extracting aqueous phase, which allows it to be extracted avoiding the derivatization step. Finally, the analysis is carried out directly by LC-MS/MS, for which the introduction of a chromophore group is not necessary, obtaining excellent selectivity and sensitivity. This work improves the most inconvenient laborious and time-consuming stages of the methodology proposed in the past, thus proposing a faster and more affordable method that allows stablishing if the finished cosmetic product complies with the requirements dictated by the European Regulation to guarantee the safety of consumers.

2. Experimental

2.1. Apparatus

An Agilent Technologies 1100 Series liquid chromatography system equipped with a degasser, quaternary pump, autosampler, and thermostatic column oven, coupled to an Agilent 6410B Triple Quad MS/MS detector was employed for chromatographic analysis. Chromatographic separation was carried out using an Agilent Zorbax SB-C18 column (50 mm x 2.1 mm, 1.8 µm particle size) purchased to Agilent Technologies (Waldbronn, Germany). Data acquisition and processing was carried out using a computer equipped with the "Agilent MassHunter Workstation Data Acquisition" software.

During the pretreatment of the sample and the VA-RP-DLLME, a ZX3 vortex mixer from VELP Scientifica (Usmate Velate, Italy) and an EBA 21 centrifuge from Hettich® (Tuttlingem, Germany) were employed.

2.2. Reagents and samples

Acrylamide ≥99% and a deuterated acrylamide solution (acrylamide-d3 standard solution) of 500 μg mL^{-1} in acetonitrile, both purchased from Sigma Aldrich (Steinheim, Germany), were used as analytical standard and surrogate, respectively.

HPLC-grade acetonitrile from Panreac (Barcelona, Spain) was used in the preparation of the acrylamide stock solutions. Reagent grade toluene from Scharlau (Barcelona, Spain) was used in the preparation of the analyte and surrogate working solutions for the standards preparation, and as supporting solvent in the VA-RP-DLLME stage, while LC-MS grade methanol from VWR Chemicals (Fontenay-sous-Bois, France) was used in the preparation of the analyte and surrogate working solutions for the sample preparation. LC-MS grade water from Panreac was used as acceptor phase.

For the mobile phase employed in the chromatographic separation, LC-MS grade methanol from VWR Chemicals, LC-MS grade water from Panreac and ammonium fluoride (NH_4F) from Acros Organics (Geel, Belgium) were used.

Nitrogen employed as nebulizer and curtain gas in the MS/MS ion source was obtained using a NiGen LCMS 40 nitrogen generator from Claind S.r.l. (Lenno, Italy). The extra-pure nitrogen (> 99.999%) used as collision gas in the MS/MS collision cell was provided by Praxair (Madrid, Spain).

Five commercially-available cosmetic products were analysed, three of them with hydrophilic-type matrix (i.e., a revitalizing gel for legs, a liquid hand soap, and a baby bath gel), and the other two with lipophilic-type matrix (i.e., a make-up remover milk and a sunscreen cream). These samples were chosen because they contained acrylamide-based polymers as ingredients, except the baby bath gel sample which did not mention any acrylamide-based polymer in its label. For reasons of confidentiality, the brands of the cosmetic products used as samples in this work are not indicated.

2.3. Proposed analytical method

2.3.1. Standards and sample preparation

A stock solution containing 500 μg mL^{-1} of acrylamide was prepared in acetonitrile. Then, an aliquot of this solution was diluted to prepare a standard intermediate solution (5 μg mL^{-1}) in toluene, and, from this one, a working standard solution (50 ng mL^{-1}) was also prepared in toluene.

Regarding acrylamide-d3, an intermediate solution of 50 μg mL^{-1} in toluene was prepared by diluting the 500 μg mL^{-1} commercial solution in acetonitrile, and, from this one, a 100 ng mL^{-1} working solution was also prepared in toluene. Additionally, for the sample preparation step, a 50 μg mL^{-1} acrylamide-d3 stock solution was prepared in methanol and, from this one, a 100 ng mL^{-1} working solution was prepared also in methanol.

From the previous working standard solutions, nine standard calibration solutions were prepared in 5 mL toluene using 15 mL glass tubes with conical bottom, by adding aliquots of increasing volumes of the acrylamide solution to obtain a concentration range from 0.005 to 5 ng mL^{-1}, and a constant aliquot of the surrogate solution to get a concentration of 0.5 ng mL^{-1}.

Regarding sample preparation, 0.01 g were weighed into a 15 mL polypropylene tube with a conical bottom, and 25 μL of methanol and 25 μL of the 100 ng mL^{-1} acrylamide-d3 working solution in methanol were added (for a total of 50 µL of methanol). The sample was vortexed until the formation of a homogeneous

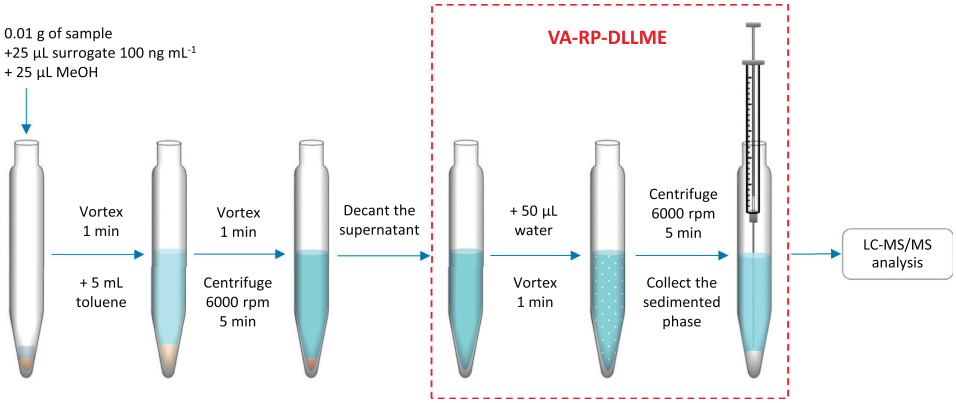

Fig. 1. Schematic diagram of the proposed method.

dispersion was observed. 5 mL of toluene were added, and the sample was vortexed again for 1 min and then centrifuged at 6000 rpm for 5 min. The supernatant was decanted into 15 mL glass tube with conical bottom to perform the VA-RP-DLLME step.

2.3.2. VA-RP-DLLME

To carry out the microextraction, 50 μL of water were added as extractant phase to each standard or sample solution (as above prepared). Next, the solutions were stirred with vortex for 1 min to favor the formation of the cloudy solution, and then they were centrifuged at 6000 rpm for 5 min. The sedimented phases were collected using a 100 µL Hamilton 1705 RNR syringe (Bonaduz, Switzerland) and transferred into a 200-µL glass inserts placed inside injection vials for further LC-MS/MS analysis.

Fig. 1 shows a schematic diagram of the whole experimental procedure.

2.3.3. LC-MS/MS analysis

At this point, 5 µL of each extract, from standard or sample solutions, were injected into the LC system described before (see Section 2.1). The chromatographic method was carried out with a mobile phase consisted of solvent A (methanol) and solvent B (water, 0.5 mM NH_4F), by isocratic elution at a mixing ratio of 40:60 (v/v); the flow rate was set at 0.2 mL min^{-1}, and the column temperature was kept constant at 40 °C. The run time was 2 min.

The MS triple quadrupole detector operated in positive electrospray ionization mode (ESI+), with capillary voltage at 5 kV, by multiple reaction monitoring (MRM). Gas temperature was set at 340 °C, nebulizer gas flow rate at 13 L min^{-1}, and nebulizer gas pressure at 40 psi.

The precursor → product *m/z* transitions for identification and quantification, collision energies and fragmentor values, both for the analyte and the surrogate, are shown in Table 1.

3. Results and discussion

3.1. Study of the variables involved in the MS/MS detection

The optimization of the precursor→product *m/z* transitions and their values of collision energy and fragmentor were carried out using the Agilent MassHunter Optimizer software, whereas the optimization of the ionization source variables was carried out using the Agilent Source Optimizer software, in both cases operated in positive electrospray ionization mode (ESI^+) and multiple reaction monitoring (MRM).

Table 1
Instrumental variables of MS/MS detection.

Instrumental variable	Acrylamide		Acrylamide-d3			
Precursor ion (m/z)	72		75			
Product ions (m/z)	55[a]	44	27	58 [a]	44	30
Fragmentor	40 V	40 V	40 V	45 V	45 V	45 V
Collision energy	10 V	26 V	18 V	10 V	22 V	34 V
Capillary voltage (ESI^+)	5 kV					
Gas temperature	340 °C					
Gas flow	13 L min^{-1}					
Nebulizer	40 psi					

[a] Used as quantification transitions.

For the optimization of the precursor → product *m/z* transitions of both analytes, individual solutions of acrylamide and acrylamide-d3, both of 1 μg mL^{-1} in water, were injected. The protonated molecule (i.e., $[M + H]^+$) was the selected precursor ion for each compound since it provided the highest sensitivity. Next, the three product ions with the highest abundance were selected, as well as their optimal collision energy and fragmentor values. The results obtained are shown in Table 1.

Regarding the optimization of the ionization source variables, a solution containing 1 μg mL^{-1} of acrylamide and acrylamide-d3 in water was injected. The optimized values for these variables are also shown in Table 1.

3.2. Study of the experimental variables involved in the VA-RP-DLLME procedure

In the VA-RP-DLLME procedure, different variables may affect the extraction performance. In this work, the variables that have been studied are the nature of both the supporting solvent acting as donor phase and the disperser solvent, the volume of the extraction solvent, and the vortex time. The influence of each variable has been evaluated using the peak area corresponding to acrylamide as response function.

3.2.1. Nature of the supporting solvent acting as donor phase

In RP-DLLME, the donor phase must be an organic solvent immiscible with water and preferably with a lower density than water to facilitate the sedimentation of the aqueous extractant droplet

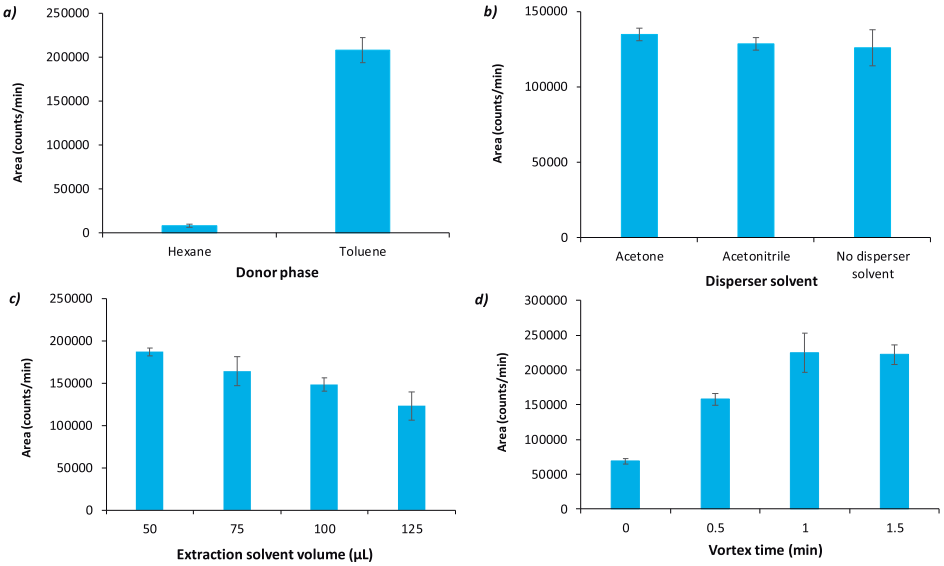

Fig. 2. Optimization of VA-RP-DLLME conditions: (a) nature of the donor phase, (b) nature of the disperser solvent, (c) extraction solvent volume, and (d) vortex time.

in the conical bottom of the glass tube and thus facilitate its collection at the end of the process. To this regard, toluene and hexane were studied as supporting solvents acting as donor phase. For this purpose, 5 mL of standard solution of 20 ng mL^{-1} of acrylamide, prepared in toluene and hexane, respectively, were taken and introduced into glass tubes with conical bottom. Next, 100 μL of water were added, vortexed for 30 s and centrifuged at 6000 rpm for 5 min. The sedimented droplets were collected with a microsyringe and injected into LC-MS/MS. The study was performed in triplicate for each solvent. According to Fig. 2a, extraction was barely achieved by using hexane, whereas toluene presented excellent results and, therefore, the latter was selected to continue the experiments.

3.2.2. Nature of the disperser solvent

Once the supporting solvent was selected, ethanol, acetone and acetonitrile were evaluated as disperser solvents. For each replicate, 5 mL of a 20 ng mL^{-1} acrylamide solution in toluene were introduced into glass tubes. Mixtures of 100 μL of water and 250 μL of disperser solvent were prepared in triplicate in centrifuge microtubes for each solvent considered. Then, these mixtures were rapidly injected by syringe into the solutions, forming the microemulsion. Then, the tubes were centrifuged for 5 min at 6000 rpm and each droplet of extract was collected and injected into LC-MS/MS.

In the case of using ethanol as disperser solvent, slightly cloudy droplets were obtained due to the formation of an emulsion, so it was not considered for further studies.

Additionally, the possibility of not using disperser solvent was also considered. In this case, only 100 μL of water were introduced into the glass tubes, and the RP-DLLME was assisted by vortex for 0.5 min to favor the formation of the microemulsion that, in the absence of a disperser solvent, was not spontaneously generated.

As can be seen in Fig. 2b, similar results were observed between the studied disperser solvents, and in absence of them. For this reason, given a similar extraction performance, it was decided to avoid the disperser solvent.

3.2.3. Volume of the extraction solvent

The next variable to optimize was the volume of water used as extraction solvent. For this purpose, 50, 75, 100 and 125 μL were evaluated in triplicate. The donor phase was a solution of 20 ng mL^{-1} of acrylamide in toluene and, once the water was added, the vortex time was 0.5 min. Fig. 2c shows the obtained results, which were compared by an ANOVA test. A p-value of 0.0636 was obtained (i.e., > 0.05), so there were not statistically significant differences between the obtained values for a 95% confidence level, despite the observed trend shows higher signal values for lower extraction volumes. Smaller volumes were not considered because the droplets to be collected would have been too small to handle. Based on this, an extraction volume of 50 μL was selected for further experiments.

3.2.4. Vortex time

The last variable to optimize was the vortex time. For each replicate, 50 μL of water as extraction solvent was added to 5 mL of 20-ng mL^{-1} acrylamide solution in toluene. Then, it was shaken with vortex for 0, 0.5, 1 and 1.5 min, each value in triplicate. Fig. 2d shows the results, which were compared by ANOVA test. A p-value of 8.47×10^{-6} was obtained (i.e., < 0.05), so there were statistically significant differences between the obtained values for a 95% confidence level. This confirms that, in the absence of a disperser solvent, vortex agitation favored the transference of the analyte from the donor phase to the extractant phase. Vortex times greater than 1 min did not ensure higher performance. For this reason, a vortex time of 1 min was selected for the microextraction process.

3.3. Study of the pretreatment of the sample

In an analytical method for trace determination, the sample preparation stage usually consists of a process of extraction and preconcentration of the analyte, generally by means of (micro)extraction techniques. Cosmetic products are very complex matrices and highly varied, so often the (micro)extraction technique cannot be applied directly to them. In these cases, a pretreatment of the sample is necessary so that it does not negatively affect the application of the (micro)extraction technique [32].

Initially, during preliminary studies of the proposed method with real samples, it was shown that it was not possible to dissolve or disperse the cosmetic sample directly in toluene due to the insolubility of the matrices, which made the RP-DLLME step difficult to carry out. For experimental reasons, it was necessary to introduce a previous stage to disperse the sample in the minimum amount of an organic solvent miscible in toluene that would allow breaking the structure of the cosmetic matrix. For this purpose, acetonitrile and methanol were tested, showing that, at equal volume, a complete and homogeneous dispersion of the sample was obtained with methanol, while with acetonitrile it was not possible to obtain a homogeneous dispersion. For this reason, once the sample was weighed, 50 μL of methanol were added and the sample was easily dispersed by vortexing for 1 min.

To verify that this amount of methanol did not affect the extraction performance by acting as a disperser solvent, the proposed method was applied (in triplicate) to a 2.5 ng mL^{-1} acrylamide aqueous standard solution with and without the addition of 50 μL of methanol. A Student's t-test was applied to compare both signals and a p-value of 0.45 was obtained (i.e., $>$ 0.05), thus showing no significant differences when methanol was added.

3.4. Study of the matrix effect

To study the matrix effect in the extraction process, an external calibration and a standard addition calibration with the make-up remover milk sample (both from 0 to 5 ng mL^{-1} of acrylamide) were prepared and subjected to the optimized VA-RP-DLLME. This study was performed with the make-up remover milk because, unlike other cosmetics with a more minimalist formulation, this type of sample represents the "worst case" to overcome for the proposed microextraction. This complex matrix is an emulsion containing a high number of ingredients, both hydrophilic and lipophilic, including surfactants, which could negatively affect the VA-RP-DLLME procedure.

Matrix effects were calculated as the ratio between the slope of the standard addition to that of the external calibration. A value of 0.74 was obtained, suggesting a negative matrix effect.

With the aim of avoiding this matrix effect, it was proposed to use a surrogate. To this regard, both calibrations were repeated but containing 3 ng mL^{-1} of acrylamide-d3 as surrogate. Acrylamide-d3 was chosen as surrogate for various reasons: (1) it is a deuterated compound so that it is not present in cosmetic samples, (2) its chemical structures is equal to the target analyte, and thus their behaviours are identical, and (3) despite eluting at the same retention time as the analyte, it does not interfere when using an MS detector due to mass, and therefore the transition precursor $\rightarrow$ product m/z, is different. In this case, when plotting A_i/A_{sur}, the ratio between the slope of the standard addition (0.2708 mL ng^{-1}) to that of the external calibration (0.2707 mL ng^{-1}) was 1.00. Thus, the addition of acrylamide-d3 as a surrogate corrected, as expected, the matrix effect.

It should be emphasized that the addition of the surrogate was considered more appropriate in the dispersion step of the sample in methanol, from a working solution in methanol, rather than later when sample is diluted with toluene. In this way, the integration of the surrogate in the sample matrix is simulated in a more realistic way.

3.5. Analytical figures of merit of the proposed method

Analytical parameters such as linearity, enrichment factor (EF), instrumental and method limits of detection (LOD and MLOD, respectively) and quantification (LOQ and MLOQ, respectively), and repeatability were evaluated to validate the proposed method.

A high level of linearity was obtained by applying the proposed method under optimized conditions, reaching at least 20 ng mL^{-1}. However, due to the very low concentration of acrylamide expected in the samples, the working range was set from 0.005 ng mL^{-1} to 5 ng mL^{-1}, with a determination coefficient (R^2) of 0.998.

The EF, defined as EF $=C_{ext}/C_0$, where C_{ext} is the concentration of the analyte in the extract and C_0 is the initial concentration of the analyte in the donor phase before the extraction, was calculated using an acrylamide solution of 2 ng mL^{-1} as initial concentration. The obtained EF was 52.

LOD and LOQ, calculated as 3 and 10 times, respectively, the signal-to-noise ratio of a standard solution at 0.005 ng mL^{-1} subjected to the VA-RP-DLLME procedure, were 0.001 ng mL $^{-1}$ and 0.003 ng mL $^{-1}$, respectively. The MLOD and MLOQ values were obtained considering sample weight and dilution. Hence, values of 0.51 µg kg^{-1}and 1.69 µg kg^{-1} were obtained, respectively. These values are well below the threshold values established by the European Regulation on cosmetic products (i.e., 0.1 mg kg^{-1} (100 µg kg^{-1}) for leave-on products and 0.5 mg kg^{-1} (500 µg kg^{-1}) in the rest), which confirms that the method is suitable for the purpose for which it was developed.

The repeatability, expressed as relative standard deviation (RSD), was evaluated by applying the proposed VA-RP-DLLME method to five independent replicates of acrylamide standard solution in toluene at two different concentration, 0.5 and 1 ng mL^{-1}, on the same day (intra-day), obtaining RSD values of 2.5 and 2.6%, respectively, and for five consecutive days (inter-day), obtaining RSD values of 4.0 and 4.1%, respectively

3.6. Application to the analysis of commercial cosmetic products

In order to evaluate the analytical utility of the proposed method, five different commercially available cosmetic samples (i.e., a revitalizing gel for legs, a make-up remover milk, a liquid hand soap, a sunscreen cream and a baby bath gel) were analyzed by the proposed VA-RP-DLLME method.

As can be seen in the results shown in Table 2, the acrylamide concentration was quantitatively determined in four of the five samples analyzed. It should be noted that, in one of the samples, the acrylamide content was above 0.1 mg kg^{-1}, the maximum concentration for leave-on body products that contain polyacrylamides as an ingredient. Therefore, this product does not comply with European Regulation [1].

Additionally, to verify that the use of the deuterated surrogate corrected the matrix effect in the samples, the proposed method was applied to the five analyzed samples and recovery studies were performed. The samples were spiked during the sample treatment stage (see Section 2.3.1.), with aliquots of 5 and 10 μL of the 500-ng mL^{-1} acrylamide standard solution in methanol plus the difference in methanol to arrive at 50 μL, and thus obtain two levels of fortification. As can be seen in Table 2, the obtained relative recoveries values ranged between 88 and 108%, which demonstrated that, using the proposed method with addition of surrogate, the matrix effect was corrected.

Fig. 3 shows chromatograms of a sample solution (baby bath gel) (unspiked (a) and spiked with 0.5 ng mL^{-1} acrylamide (b) both containing acrylamide-d3 (surrogate) at 0.5 ng mL^{-1}).

Table 2
Acrylamide contents found in five cosmetic samples and their relative recovery values obtained by applying the developed method.

Sample[a]	Spiked amount (μg g^{-1})	Found amount (μg g^{-1})[b]	Relative recovery (%)[b]
A	-	0.38 ± 0.04	-
	0.23 ± 0.04	0.62 ± 0.05	101 ± 3
	0.55 ± 0.19	0.97 ± 0.16	108 ± 8
B	-	< LOD	-
	0.26 ± 0.02	0.23 ± 0.03	88 ± 4
	0.48 ± 0.10	0.47 ± 0.06	94 ± 9
C	-	0.020 ± 0.003	-
	0.19 ± 0.05	0.19 ± 0.05	90 ± 1
	0.41 ± 0.12	0.45 ± 0.11	106 ± 6
D	-	0.002 ± 0.001	-
	0.20 ± 0.05	0.20 ± 0.05	98 ± 6
	0.44 ± 0.10	0.43 ± 0.09	98 ± 6
E*	-	0.0031 ± 0.0003	-
	0.22 ± 0.03	0.22 ± 0.01	100 ± 9
	0.40 ± 0.02	0.39 ± 0.03	99 ± 5

[a] A: revitalizing gel for legs; B: make-up remover milk; C: hand soap; D: sunscreen cream; E: baby bath gel.
[b] expressed as mean ± standard deviation of three replicates.
* The sample does not present acrylamide-based polymers in its label information.

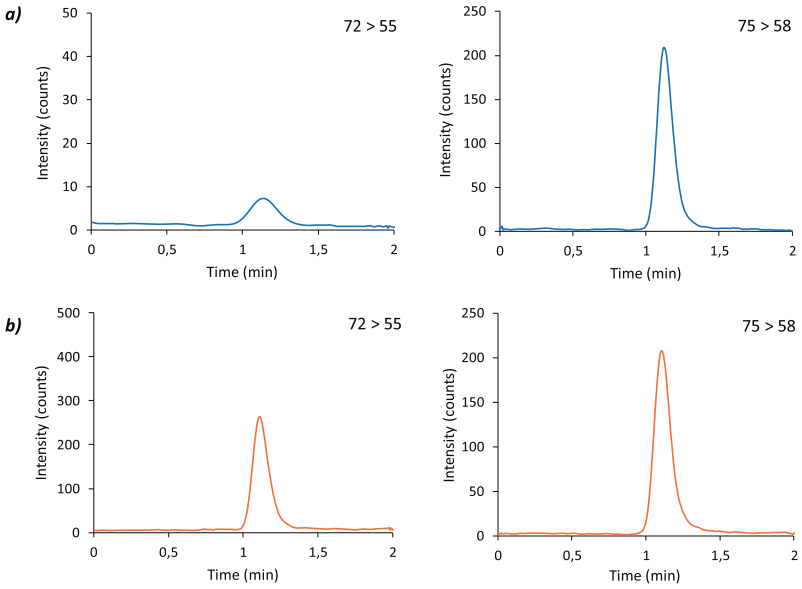

Fig. 3. Chromatograms of a sample solution (baby bath gel) (unspiked (a) and spiked with 0.5 ng mL^{-1} acrylamide (b) both containing acrylamide-d3 (surrogate) at 0.5 ng mL^{-1}) subjected to the proposed analytical method.

4. Conclusions

A sensitive analytical method to determine residual acrylamide at trace level in cosmetic products has been successfully developed and validated. The proposed method is based on an appropriate sample pre-treatment, in which vortex-assisted reversed-phase dispersive liquid-liquid microextraction (VA-RP-DLLME) was followed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The variables involved in both the microextraction and the detection steps have been optimized.

The proposed analytical method is fast, simple, and highly sensitive allowing the determination of acrylamide in different kind of cosmetic matrices well below the threshold values established by the European Regulation on cosmetic products. It should be emphasized that by employing VA-RP-DLLME, a prior derivatization step is not necessary, thus overcoming the laborious stages of our previous work in which acrylamide was determined in cosmetics.

The good analytical characteristics, simplicity and affordable procedure make it a suitable method to guarantee the safety of users and compliance with European Regulation on cosmetic products.

Against, it should be noted that the main disadvantage of the proposed methodology is the consumption of 5 mL of toluene as

donor phase. However, this volume could be reduced to the detriment of the enrichment factor.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Lorenza Schettino: Methodology, Validation, Investigation, Data curation, Writing – original draft. **Alejandro García-Juan:** Validation, Investigation, Data curation. **Laura Fernández-Lozano:** Validation, Investigation, Data curation. **Juan L. Benedé:** Methodology, Writing – review & editing, Supervision. **Alberto Chisvert:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Funding acquisition.

Data Availability

Data will be made available on request.

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Determination of nine prohibited *N*-nitrosamines in cosmetic products by vortex-assisted dispersive liquid–liquid microextraction prior to gas chromatography-mass spectrometry†

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An analytical method for the simultaneous determination of nine prohibited *N*-nitrosamines in cosmetic products is presented. *N*-nitrosamines are banned compounds in cosmetic products due to their harmful effects. Therefore, these compounds are not intentionally added to these products but, however, small amounts of them may be present due to unintentional causes, and thus sensitive methods for their analytical control are required. The proposed method is based on vortex-assisted dispersive liquid–liquid microextraction (VA-DLLME) to extract and preconcentrate the analytes, followed by gas chromatography-mass spectrometry (GC-MS) for their determination. The variables involved in the VA-DLLME process were optimized by using a Box–Behnken design and, due to the different polarity of the *N*-nitrosamines studied, several approaches for sample treatment were compared to achieve the best results. The method was successfully validated, showing a good linearity at least up to 20 ng mL^{-1}, enrichment factors from 2 to 100 depending on the target analyte, limits of detection and quantification at the low μg kg^{-1} level, and good repeatability values (<13%). Finally, the proposed analytical method was applied to the determination of *N*-nitrosamines in commercial cosmetic samples of different nature, avoiding the matrix effect by means of standard addition calibration. Significant amounts of some of the *N*-nitrosamines, even exceeding the established regulatory limit, were found in the samples. The resulting method is fast, simple, and affordable to carry out the quality control of cosmetic products to ensure consumer safety for most laboratories.

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Introduction

N-Nitrosamines are *N*-nitroso derivatives of secondary amines with mutagenic, carcinogenic, and teratogenic effects,[1] which can be found in cosmetic products without having been intentionally added during the manufacturing process, thus constituting a risk to consumer health. *N*-nitrosamines are easily formed when secondary or tertiary amines react with nitrosating agents, such as nitrites or nitrogen oxides.[2,3] This implies that ingredients containing or releasing nitrite ions should not be used, but if they are employed, nitrosation reaction inhibition systems should be used (such as the use of α-tocopherol, ascorbic acid and other substances).[3]

For this reason, with the aim of reducing the formation of these compounds in cosmetics, and therefore the health risk of consumers, European legislation has prohibited not only *N*-nitrosamines in cosmetic products,[4] but also secondary alkyl- and alkanolamines. Moreover, the use of fatty acid dialkylamides and dialkanolamides, monoalkylamines, monoalkanolamines, trialkylamines, trialkanolamines, and their salts present restrictions in these products.[3,5] Furthermore, in 2012, the European Scientific Committee on Consumer Safety (SCCS) established a maximum content limit of 50 μg kg^{-1} for traces of *N*-nitrosamines, both in raw materials used as ingredients in cosmetics and in finished cosmetic products.[3] In this regard, according to this scientific opinion, cosmetic industries are required to perform quality control analysis for raw materials and for those products whose constituents may unintentionally cause the formation of nitrosamines, and to avoid impurities and incompatibilities between ingredients to prevent nitrosation reactions.

Different analytical methods for *N*-nitrosamines determination in cosmetic products can be found in the scientific literature. Most of them are based on the determination of only *N*-nitrosodiethanolamine (NDELA),[6–16] a hydrophilic nitrosamine for whose determination in cosmetic products two official analytical methods have been approved (*i.e.*, ISO 10130[17] and ISO 15819[18]).

Beyond the NDELA, there are few methods in the literature that simultaneously determine several *N*-nitrosamines in

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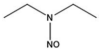
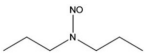
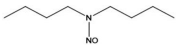
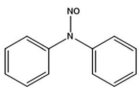
cosmetic products. This is mainly due to the different polar character that exists between them, which hinder to extract them simultaneously in the treatment of the sample. In this context, some studies based on chromatographic techniques have been published by using gas chromatography (GC) coupled to thermal energy analyzer (TEA),[19,20] single mass spectrometry (MS)[21–23] or in tandem (MS/MS);[24] or liquid chromatography (LC) with TEA,[25] ultraviolet (UV),[26] or MS[27] and MS/MS[28] detection.

Regarding the pretreatment of the cosmetic samples, both solid- and liquid-phase (micro) extraction techniques have been used for the enrichment of *N*-nitrosamines. Among the solid-based techniques, solid-phase extraction (SPE),[24] dispersive solid-phase extraction (DSPE),[29] headspace solid-phase microextraction (HS-SPME),[22] stir bar sorptive-dispersive microextraction (SBSDME)[28] and micro-matrix solid-phase dispersion (μMSPD)[23] have been employed. On the other hand, among liquid-based techniques, liquid–liquid extraction (LLE)[21] and vortex-assisted reversed-phase dispersive liquid–liquid microextraction (VA-RP-DLLME)[27] are the only applications, to the best of our knowledge. However, the limits of detection of some of these methods are higher than the regulatory limit (*i.e.*, 50 μg kg^{-1}), they consume large amounts of organic solvents and/or they are time-consuming procedures that hinder the sample throughput. Additionally, some of them use unaffordable instruments (*e.g.*, TEA and MS/MS detectors) for the most quality control laboratories of cosmetic manufacturers.

These reasons motivated us to develop an analytical method for the simultaneous determination of nine prohibited *N*-nitrosamines (see Table 1) at trace level in cosmetic products. The method is based on vortex-assisted dispersive liquid–liquid microextraction (VA-DLLME), followed by gas chromatography-mass spectrometry (GC-MS) analysis. Unlike the conventional DLLME, in which a polar organic solvent is used as disperser solvent, in the VA-DLLME it is the vortex agitation that helps the formation of the cloudy solution, and thus reducing the consumption of additional organic solvents beyond the extraction solvent. Moreover, due to the different polarity of the target nitrosamines (log $K_{o/w}$ from −0.59 to 3.13), different approaches for the sample treatment, such as LLE, SPE, filtration and leaching, were compared to achieve the best results during the procedure.

Table 1 Chemical structure and relevant data of the target *N*-nitrosamines

Analyte[a]	CAS number	Chemical structure	Molecular weight (g mol^{-1})	Log $K_{o/w}$
NDMA	62-75-9		74.08	−0.50
NMEA	10 595-95-6		88.11	0.01
NDEA	55-18-5		102.14	0.52
NDPA	621-64-7		130.19	1.54
NDBA	924-16-3		158.24	2.56
NPIP	100-75-4		114.15	0.44
NPYR	930-55-2		100.12	−0.09
NMOR	59-89-2		116.12	−0.59
NDPhA	86-30-6		198.22	3.13

[a] NDMA, *N*-nitrosodimethylamine; NMEA, *N*-nitrosoethylmethylamine; NDEA, *N*-nitrosodiethylamine; NDPA, *N*-nitrosodipropylamine; NDBA, *N*-nitrosodibutylamine; NPIP, *N*-nitrosopiperidine; NPYR, *N*-nitrosopyrrolidine; NMOR, *N*-nitrosomorpholine; NDPhA, *N*-nitrosodiphenylamine.

Experimental

Apparatus

An 8860-gas chromatography system coupled to a simple quadrupole 5977B mass spectrometer, both from Agilent Technologies (Palo Alto, CA, USA), and a PAL LSI 85 autosampler from CTC Analytics (Zwingen, Switzerland) was used.

During the VA-DLLME, a ZX3 vortex mixer from VELP Scientifica (Usmate Velate, Italy) and an EBA 21 centrifuge from Hettich® (Tuttlingem, Germany) were also employed.

Reagents and samples

EPA 8270 Appendix IX Nitrosamine Mix (2000 μg mL^{-1} of each component in methanol) from Sigma–Aldrich (Steinheim, Germany) was used as standard.

LC-MS grade methanol from VWR Chemicals (Fontenay-sous-Bois, France) was used in the preparation of the standard stock and intermediate solutions.

Deionized water (resistivity ≥18 MΩ cm), obtained from a Connect water purification system provided by Adrona (Riga, Latvia), was used in the preparation of working solutions, and analytical reagent-grade chloroform purchased from Scharlau Chemie (Barcelona, Spain) was used as acceptor phase during the VA-DLLME stage.

LC-grade hexane 96% purchased from Scharlau Chemie (Barcelona, Spain) was used for the study of the sample pretreatment.

SPE cartridges (*i.e.*, Discovery® DSC-Diol (50 μm particle size) from Supelco (Bellefonte, PA), and Strata-X™(25 μm particle size) and Strata SDB-L (100 μm particle size), both from Phenomenex (Torrance, CA)) were also used for the study of the sample pretreatment.

Three sample of commercial cosmetic products (*i.e.*, an aftersun gel, and two different body creams) were analysed, and for reasons of confidentiality the brands of these commercial samples are not shown.

Proposed method

Standards and sample preparation. A stock solution containing 50 μg mL^{-1} of the analytes in methanol was prepared by diluting the commercial standard solution described before. Taking an aliquot from this stock solution and diluting it with water, a working solution of 100 ng mL^{-1} was obtained.

Regarding sample preparation, working sample solutions were prepared by standard addition calibration approach as follows: 0.05 g of cosmetic sample were weighed into 5 mL volumetric flasks, then they were spiked with different aliquots (*i.e.*, from 0.5 μL to 1 mL) of the 100 ng mL^{-1} aqueous standard working solution reaching spiked concentrations of the target analytes from 0.01 to 20 ng mL^{-1}, and filled up to the line with water. Then, they were mixed by vortex stirring (*ca.* 1 min) until a homogeneous dispersion was obtained.

After that, the sample solutions were transferred to 15 mL polypropylene tube with a conical bottom to perform the VA-DLLME procedure.

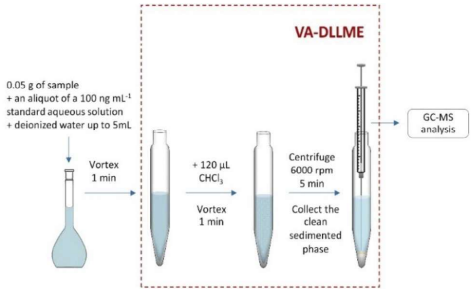


Fig. 1 Schematic diagram of the proposed method.

VA-DLLME. To perform the VA-DLLME, 120 μL of chloroform as extraction solvent were added to 5 mL of each standard addition calibration solution prepared as described in the previous section. The solution was then vortexed for 1 min to favor microemulsion formation, and then centrifuged at 6000 rpm for 5 min. The settled extractant phase was collected with a 100 μL Hamilton 1705 RNR syringe and transferred to a 200 μL glass insert placed inside the injection vial for subsequent GC-MS analysis.

Fig. 1 shows a schematic diagram of the whole experimental procedure.

GC-MS analysis. GC-MS analysis was performed with the equipment mentioned above (see Section apparatus). Chromatographic separation was achieved using a VF-WAXms (polyethylene glycol) column of 30 m length, 0.25 mm diameter and 0.25 μm film thickness from Agilent Technologies (Palo Alto, CA, USA).

The GC oven temperature was maintained at 60 °C for 2 min, and then ramped to 160 °C at 10 °C min^{-1}, and to 240 °C at a rate of 40 °C min^{-1}, holding this temperature for 4 min. The carrier gas was helium with a constant flow of 1 mL min^{-1}. The injection was performed at 230 °C in splitless mode, and the

Table 2 Summary of retention times (min) and monitoring ions (*m*/*z*) of target *N*-nitrosamines

Analyte[a]	Acquisition time windows (min)	Retention time (min)	Quantification ion (*m*/*z*)	Qualifier ions (*m*/*z*)	
NDMA	6.00–6.90	6.68	74	43	42
NMEA	6.90–8.50	7.33	88	56	42
NDEA		7.73	102	56	42
NDPA	8.50–11.00	9.52	130	70	43
NDBA	11.00–12.15	11.72	116	84	57
NPIP		12.01	114	55	42
NPYR	12.15–14.00	12.36	100	68	41
NMOR		12.81	116	86	56
NDPhA	15.00–18.00	16.66	169	168	167

[a] NDMA, *N*-nitrosodimethylamine; NMEA, *N*-nitrosoethylmethylamine; NDEA, *N*-nitrosodiethylamine; NDPA, *N*-nitrosodipropylamine; NDBA, *N*-nitrosodibutylamine; NPIP, *N*-nitrosopiperidine; NPYR, *N*-nitrosopyrrolidine; NMOR, *N*-nitrosomorpholine; NDPhA, *N*-nitrosodiphenylamine.

injected volume was 2 μL. Ion source operated by electronic ionization at 70 eV at 230 °C, and transfer line and quadrupole temperatures were set at 230 °C and 150 °C, respectively. Acquisition was carried out in both full scan and Selected Ion Monitoring (SIM) mode. To improve the sensitivity, selected ions were acquired in six-time windows depending on their retention times, as shown in Table 2.

Under the described conditions, the required time for the chromatographic analysis of all analytes was 18 min.

Results and discussion

Optimization of the VA-DLLME variables

Before the optimization for the extraction step conditions (see Section VA-DLLME), preliminary considerations were made to assess the nature of the extraction solvent. It must be denser than water to remain, after centrifugation, at the bottom of the extraction tube. In addition, from the outset the possibility of avoiding the use of disperser solvent by using instead vortex stirring to provide the cloudy solution formation was evaluated. For this reason, chloroform and dichloromethane were considered as possible extraction solvents, and VA-DLLME procedure was performed by extracting 5 mL of standard solution at 5 ng mL^{-1} of the analytes. When using dichloromethane, neither the formation of the microemulsion nor the phase separation was obtained, so chloroform was selected as the extractant phase, since a fine microemulsion was formed by vortexing, demonstrating the possibility of assisting the DLLME procedure with vortex and thus avoiding the use of a disperser solvent.

Next, the variables involved in the VA-DLLME procedure were optimized through a response surface methodology (RSM), showing the interactions between them. In this work, the studied variables were the volume of the extraction solvent, the ionic strength of the donor phase and the vortex time. The statistical analysis of the results was performed using StatGraphics Centurion XVI software from StatGraphics Technologies, Inc. (The Plains, VA, USA). A Box–Behnken design was performed to assess the three significant extraction variables, performing 15 experimental runs with three levels for each factor (see ESI†). The independent factors (and the ranges) studied were the volume of extraction solvent (60–150 μL), the ionic strength of the donor phase (0–10% NaCl (w/v)), and the vortex time (30–90 s). All experiments were carried out on the same day using 5 mL of a standard solution of the analytes at 5 ng mL^{-1} in water as donor phase and they are summarized in Table S1.†

To evaluate the suitability of the model, the coefficient of determination (R^2) was considered, which a value >0.87 was obtained for all target analytes, indicating that the designed model was efficient for predicting responses. Fig. 2 shows the response surface plots in terms of desirability (estimated as described in ESI†) for the three factors studied.

Fig. 2a shows that there were no great differences in the vortex time, but the best responses were obtained using 50–70 s, getting the proper dispersion of the extraction solvent in the donor phase. For that reason, 60 s was established for further experiments as mean value. On the other side, as it is shown in Fig. 2a and b, the best response for the volume of extraction solvent was obtained using 60 μL of chloroform. However, a very small droplet (*ca.* 15 μL) was obtained, which was considered too low to be handled and injected into the GC-MS system, and therefore it was decided to continue selecting the volume of 80 μL for further experiments.

Finally, as can be seen in Fig. 3c, the best response for the ionic strength of the donor phase was showed from 0 to 10% NaCl (w/v). This occurs due to two different effects. On the one hand, the presence of salts decreases the solubility of the extraction solvent (*i.e.*, chloroform), thereby the volume of the extract increases, causing the dilution of the analytes. On the other hand, when a higher salt concentration is used, the solubility of the analytes in the aqueous phase decreases and enhance their transfer to the organic phase (salting-out effect), obtaining a greater extraction of the analytes, with good signals despite the effect of dilution. Based on these results, and to reduce reagent consumption, 0% NaCl (w/v) was selected in further experiments.

In summary, the optimized method consisted of 80 μL of chloroform, 60 s of vortex stirring time and no NaCl adjustment. Fig. S1† shows a chromatogram of a standard solution containing the analytes at 10 ng mL^{-1} subjected to the optimized VA-DLLME procedure, compared to the chromatogram obtained for an unextracted standard solution of same concentration.

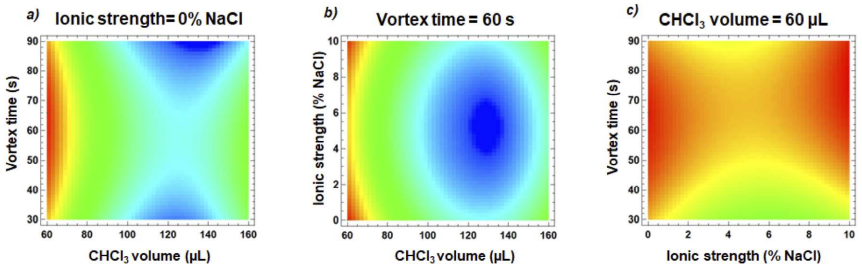


Fig. 2 Response surface plots of the desirability function representing the relation between the different variables affecting the extraction: (a) vortex time *vs.* $CHCl_3$ volume, (b) ionic strength *vs.* $CHCl_3$ volume, and (c) vortex time *vs.* ionic strength.

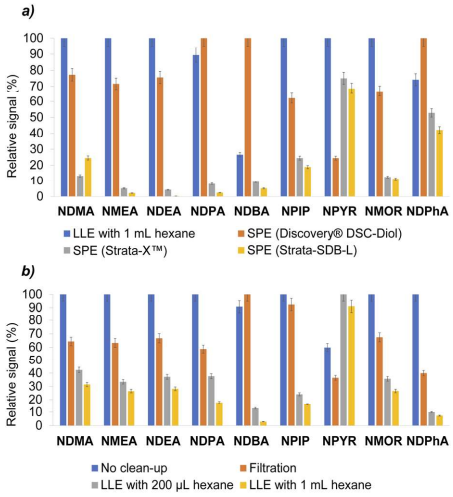


Fig. 3 Sample pretreatment studies carried out with a *N*-nitrosamine free sample solution spiked to 1 ng mL^{-1} with target analytes: (a) comparison between clean-up through LLE with 1 mL hexane and SPE with different cartridges; (b) comparison between clean-up through LLE with 1 mL and 200 µL of hexane, filtration, and no clean-up step with increasing of the extraction solvent volume (*i.e.*, 120 µL of chloroform).

Study of the pretreatment of the sample

Once the entire extraction stage was optimized, studies were carried out by performing the VA-DLLME directly on a sample solution obtained from a *N*-nitrosamines-free cosmetic cream. This type of complex matrices has a high number of lipophilic components and surfactants that could negatively affect the VA-DLLME procedure. Indeed, it was observed that when performing the microextraction, the lipophilic compounds contained in the matrix also passed to the chloroform, precipitating at the bottom of the centrifuge tube, and thus causing the formation of a cloudy drop that was not possible to inject into the GC-MS system. For this reason, different strategies were carried out for the pretreatment of the sample to perform a clean-up step, prior to the VA-DLLME procedure, with the aim of eliminating these impurities. For these studies, a sample solution of a *N*-nitrosamines-free cosmetic cream was prepared by weighing 0.5 g of sample into a 50 mL volumetric flask and filling to the line with water, after spiking it to 1 ng mL^{-1} with target analytes.

In a first attempt, LLE clean-up was carried out with 1 mL of hexane over 5 mL of the aqueous sample solution. In a second attempt, 5 mL of the aqueous sample solution were percolated throughout different SPE cartridges (*i.e.*, Discovery® DSC-Diol, Strata-X™, and Strata-SDB-L) subjected to vacuum.

As can be seen in Fig. 3a, the best performance was achieved by means of LLE with 1 mL of hexane. Among the SPE cartridges, the best results were obtained with the Discovery® DSC-Diol cartridge, whose performance was the closest to LLE with hexane, obtaining slightly better results only for the more lipophilic nitrosamines (*i.e.*, NDPA, NDBA and NDPhA), which were more likely to be removed with the hexane and therefore lower signal were obtained in these three cases.

Thus, it was considered to continue testing the LLE with hexane, and thus to discard the percolation of the sample solution through the SPE cartridges because the analytes interacted partially with the different sorbents, preventing their immediate elution and causing their loss.

Then, the possibility of reducing the volume of hexane from 1 mL to 200 µL (minimum amount of solvent to make it easy to collect) was studied. Likewise, the option of not doing an LLE was also evaluated through two other approaches: (1) filtering the sample solution through a 0.45 µm particle size nylon filter to remove impurities from the matrix prior to microextraction, and (2) do not perform any clean-up step and just leach the analytes from the sample into water. The latter approach was also considered because the aforementioned cloudy droplet that formed when VA-DLLME was performed directly on the sample solution with 80 µL of chloroform became a layer between the aqueous donor phase and the extractant phase if the volume of chloroform was increased to 120 µL. In this way, a small clean drop of extractant phase, easy to be collected and injected into the GC-MS system, was obtained at the bottom of the microextraction tube.

Fig. 3b compares these approaches and shows that, in general terms, the best results were achieved by increasing the volume of chloroform without performing a clean-up step, despite not using the previous optimized value.

The results obtained with the selected methodology were very positive compared to all the approaches considered in the sample pretreatment studies described above, not only because no analytes were lost during the procedure and a pretreatment step prior to microextraction was avoided reducing the time of analysis, but also because the amount of waste produced is minimized. Otherwise, the waste would be higher if filters, cartridges, or hexane were used in a clean-up step.

Study of the matrix effect

To evaluate the matrix effect caused by cosmetic matrices during the analytical procedure, an external calibration was performed with aqueous standard solutions from 0.01 to 10 ng mL^{-1} and subjected to the optimized VA-DLLME process. Likewise, non-spiked samples and spiked with 0.5 and 1 ng mL^{-1} sample solutions were prepared and subjected to microextraction. The relative recovery (RR) was evaluated as $RR\% = 100 \times (C_{M+S} - C_M)/C_{S\ added}$, being C_M the concentration of the measurement solution in the original sample, C_{M+S} the concentration of the measurement solution in the fortified sample, and $C_{S\ added}$ the standard concentration added.

The RR values obtained with this study ranged between 11 and 431%, demonstrating that the analytical process was affected by a consistent matrix effect, either positive or negative depending on the analyte and on the sample. Hence, standard addition calibration was employed to correct the observed matrix effects.[28]

Analytical performance of the proposed method

Different analytical parameters were evaluated to validate the proposed method. These results are summarized in Table 3.

It should be noticed that, as standard addition calibration is used to correct the matrix effects, and these are different for each analyte in each tested sample, thus affecting the signal in different extension, those parameters depending on the signal, such as linearity, limits of detection (LOD) and quantification (LOQ), and enrichment factor (EF), are matrix-dependent. In this sense, in order to obtain values just to know the magnitude order and to compare with other methods, they were obtained by using aqueous solutions.

The method achieved a good linearity at least up to 20 ng mL^{-1}, with determination coefficients (R^2) > 0.990.

LODs and LOQs, calculated as 3 and 10 times the signal-to-noise ratio of a standard solution subjected to the proposed method, ranged from 0.2 to 91.5 ng L^{-1} and from 0.6 to 304.9 ng L^{-1}, respectively. Therefore, further considering sample dilution, method LODs (MLODs) ranged from 0.02 to 9.2 μg kg^{-1}, and the method LOQs (MLOQs) ranged from 0.06 to 30.5 μg kg^{-1}, in the cosmetic samples. These values are well below the threshold value of 50 μg kg^{-1} for traces of *N*-nitrosamines in cosmetic products established by the European Regulation,[3,5] which confirms that the method is suitable for the determination of these compounds in this kind of matrices.

The achieved EFs, defined as $EF = C_{ext}/C_0$, where C_{ext} is the concentration of the analyte in the extract and C_0 is the initial concentration of the analyte in the donor phase before the extraction, was calculated using a standard solution at 1 ng mL^{-1} of the analytes as initial concentration, and ranged from 2 to 100, due to the different polarities of the *N*-nitrosamines studied that respectively affect the extraction efficiency of each of them.

The repeatability, expressed as relative standard deviation (RSD), was evaluated by applying the proposed VA-DLLME method to five replicates of aqueous standard solution at three different concentrations (*i.e.*, 0.1, 0.5 and 5 ng mL^{-1}) on the same day (intra-day) and for five consecutive days (inter-day). The intra-day repeatability values ranged from 3.0 to 7.4% at 0.1 ng mL^{-1}, from 1.1 to 6.7% at 0.5 ng mL^{-1}, and from 2.1 to 6.9 at 5 ng mL^{-1}. The inter-day repeatability values ranged from 6.0 to 9.9% at 0.1 ng mL^{-1}, from 4.6 to 12.5% at 0.5 ng mL^{-1}, and from 4.3 to 12.5 at 5 ng mL^{-1}. Results show that good repeatability values have been achieved with the proposed method.

Table 4 shows the comparison of the new developed method with those previously published with the same purpose, both solid- and liquid-phase extraction-based methods.

As can be seen, the MLODs are of the same magnitude order, even that in those methods based on more expensive analytical instruments (*e.g.*, MS/MS detection). Regarding the extraction time, liquid-based extraction techniques, as the proposed method, are much faster since the extraction is achieved practically instantaneously (less than 1 min), which is beneficial to get a high-throughput method. Moreover, no sophisticated equipment or commercial materials (*e.g.*, sorbents, cartridges, or fibers) are needed to perform the extraction, making the method affordable for most laboratories. On the other hand, the main drawback of the proposed method is the use of an organochloride solvent as extraction phase, which is against current trends in Analytical Chemistry, but just a low volume is used.

Regarding this last matter, the greenness of the sample preparation of each work was evaluated by using the new metric tool termed AGREEprep.[30] This metric tool considers various environmental and health impact factors such as the use of reagents, the consumed energy, possible occupational hazards, and generated wastes, among others. This tool scores the sample preparation stage on a scale from 0 to 1, where 0 is the worst score and 1 is the maximum score. As can be seen in Table 4, the score obtained by the proposed method is positively comparable to the other methods since the sample pretreatment is simple (just lixiviation of the analytes in water), in contrast to those methods where higher amounts of sample and solvents such as methanol, dichloromethane and/or hexane are employed.

Table 3 Main analytical parameters of the proposed method

						Repeatability[d] (%RSD)					
						Intra-day ($N = 5$)			Inter-day ($N = 5$)		
Analytes	LOD[a] (ng L^{-1})	LOQ[a] (ng L^{-1})	MLOD[b] (μg kg^{-1})	MLOQ[b] (μg kg^{-1})	EF[c]	0.1 ng mL^{-1}	0.5 ng mL^{-1}	5 ng mL^{-1}	0.1 ng mL^{-1}	0.5 ng mL^{-1}	5 ng mL^{-1}
NDMA	74.3	247.5	7.4	24.8	2	n.a.[e]	6.7	3.3	n.a.	10.9	4.3
NMEA	91.5	304.9	9.2	30.5	18	n.a.	4.6	2.1	n.a.	7.2	9.1
NDEA	19.1	63.6	1.9	6.4	38	3.0	3.4	3.3	7.3	4.6	4.6
NDPA	4.4	14.7	0.4	1.5	100	4.6	1.1	4.5	6.0	7.6	12.4
NDBA	0.2	0.6	0.02	0.06	73	6.0	4.4	3.7	6.6	8.4	12.5
NPIP	18.6	61.9	1.9	6.2	91	5.3	4.2	4.8	9.7	12.5	7.6
NPYR	33.3	110.9	3.3	11.1	25	7.4	2.2	3.8	6.1	11.1	5.2
NMOR	83.8	279.3	8.4	27.9	10	n.a.	2.0	2.7	n.a.	8.4	5.5
NDPhA	35.0	116.8	3.5	11.7	66	4.3	2.4	6.9	9.9	6.7	7.7

[a] LOD: limit of detection; LOQ: limit of quantification, calculated as 3 and 10 times, respectively, the signal-to-noise ratio. [b] MLOD: method limit of detection; MLOQ: method limit of quantification, according to the sample pretreatment. [c] EF: enrichment factor. [d] RSD: relative standard deviation. [e] n.a.: not applicable, since the concentration is below the LOQ.

Table 4 An overview on reported extraction-based methods for the determination of the target analytes in cosmetic products (chronological order)

Target *N*-nitrosamines	Sample pretreatment[a]	Extraction technique[b]	Time[c]	Organic solvent [a]	Instrumental technique[d]	MLOD ($\mu g\ g^{-1}$)	AGREEprep score	Ref.
10	1 g + 10 mL MeOH : DCM; sonicated 10 min; centrifuged 15 min	SPE	n.r.	6 mL MeOH (conditioning); 3 mL MeOH (30%) (washing); 8 mL MeOH (desorption); evaporation + 1 mL MeOH	GC-MS/MS	700–3000	0.16	24
13	0.2 g + 4 mL H_2O	LLE	Vortex 1 min (extraction) centrifugation 3 + 2 min	3 mL ACN × 2 (extraction) evaporation + 1 mL EtAc	GC-MS	3–15	0.50	21
7	5 g + 5 mL DCM : MeOH; sonicated 30 min	HS-SPME	30 min (extraction)	—	GC-MS	0.46–36.54	0.24	22
11	1 g + 7 mL ACN; sonicated 10 min; centrifuged 3 min	DSPE	Vortex 20 min (extraction) centrifugation 3 min	Evaporation + 1 mL MeOH	LC-MS/MS	7–250	0.25	29
7	g + 5 mL HEX; vortex; centrifuged 5 min	VA-RP-DLLME	Vortex 0.5 min (extraction) centrifugation 5 min	—	LC-MS	1.8–50	0.48	27
8	0.5 g + 25 mL H_2O; vortex; 1 mL HEX; centrifuged 15 min	SBSDME	30 min (extraction) 1 min (desorption)	1 mL ACE (desorption)	LC-MS/MS	3–13	0.37	28
10	0.1 g + 0.2 Na_2SO_4 + 0.4 g Florisil®	μ-MSPD	n.r.	0.1 g Florisil® 1 + 10 mL EtAC (desorption)	GC-MS	12–150	0.49	23
9	0.05 g + 5 mL H_2O	VA-DLLME	Vortex 1 min (extraction) centrifugation 5 min	120 μL CH_3Cl (extraction)	GC-MS	0.06–30.5	0.59	This work

[a] ACE: acetone; ACN: acetonitrile; EtAc: ethyl acetate; DCM: dichloromethane; HEX: hexane; MeOH; methanol. [b] DSPE: dispersive solid-phase extraction; HS-SPME: headspace-solid phase microextraction; LLE: liquid–liquid extraction; μ-MSPD: micro matrix solid-phase dispersion; SBSDME: stir bar sorptive dispersive microextraction; SPE: solid-phase extraction; VA-RP-DLLME: vortex-assisted reversed-phase dispersive liquid–liquid microextraction. [c] n.r.: no reported. [d] GC: gas chromatography; LC: liquid chromatography; MS: mass spectrometry; MS/MS: tandem mass spectrometry.

Application to the analysis of commercial cosmetic products

Three commercially available cosmetic samples, an aftersun gel and two different body creams, were analyzed using the proposed VA-DLLME method. As can be seen in Table 5, the results obtained in the analysis of the three cosmetic samples show that *N*-nitrosamines have been quantitatively determined in all of them.

Table 5 *N*-nitrosamines contents found in three cosmetic samples obtained by applying the developed method

	Found amount ($\mu g\ kg^{-1}$)[a]		
Analytes	Aftersun gel	Body cream 1	Body cream 2
NDMA	< LOD	< LOD	< LOD
NMEA	770 ± 90	< LOD	560 ± 20
NDEA	< LOD	< LOD	< LOD
NDPA	1.19 ± 0.01	< LOD	< LOD
NDBA	16.5 ± 0.1	< LOD	< LOD
NPIP	50.6 ± 0.3	< LOD	< LOD
NPYR	114 ± 5	< LOD	< LOD
NMOR	< LOD	870 ± 60	< LOD
NDPhA	10.57 ± 0.03	< LOD	< LOD

[a] expressed as mean ± standard deviation of three replicates.

The results obtained in the aftersun gel revealed that six *N*-nitrosamines were determined, three of which exceed the safety limit of 50 $\mu g\ kg^{-1}$ defined by the European Regulation (*i.e.*, NMEA 770 ± 90 $\mu g\ kg^{-1}$, NPIP 50.6 ± 0.3 $\mu g\ kg^{-1}$, and NPYR 114 ± 5 $\mu g\ kg^{-1}$).

Regarding the creams, only NMOR has been quantified in the first cream, which widely exceeds the safety limit, being found at a concentration of 870 ± 60 $\mu g\ kg^{-1}$, while in the second cream only NMEA was quantified, which was also found to be above the safety limit, at a concentration of 560 ± 20 $\mu g\ kg^{-1}$. Chromatograms of the sample solutions subjected to the proposed VA-DLLME method are shown in Fig. S2.†

It should be noted that, as declared on the labels, these three samples analyzed included two ingredients that, even though they are permitted ingredients as they are a preservative (*i.e.*, bronopol) and a pH regulator (*i.e.*, triethanolamine), their reaction can unintentionally cause the formation of nitrosamines.

Conclusions

A sensitive analytical method for determining trace levels of nine banned *N*-nitrosamines in cosmetic products has been successfully developed and validated. The proposed method is based on vortex-assisted dispersive liquid–liquid microextraction (VA-DLLME) followed by gas chromatography-mass spectrometry (GC-MS). The variables involved in the microextraction stage have been optimized, and comparative studies of sample pretreatment have been carried out to find the best methodology that allows the analysis of the greatest number of nitrosamines at the same time with the required sensitivity, favoring their extraction from the complex cosmetic matrices without losing analytes during the procedure.

The proposed method has good analytical characteristics that, in addition to being a simple and affordable procedure, make it suitable for quality control of cosmetics in order to guarantee the safety of users and compliance with the European Regulation on cosmetic products. It should not be forgotten that one of the requirements of routine analysis methods is that they allow a high sample throughput, *i.e.*, analyze several samples in a short period of time, and this is achieved by DLLME-based methods.

Author contributions

L. Schettino: methodology, validation, investigation, data curation, writing – original draft; J. L. Benedé: methodology, writing – reviewing and editing, supervision; A. Chisvert: conceptualization, methodology, resources, writing – reviewing and editing, supervision, funding acquisition.

Conflicts of interest

The authors declare that there are no conflicts to declare, they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Electronic Supplementary Material (ESI) for RSC Advances.

SUPPLEMENTARY INFORMATION

Determination of nine prohibited N-nitrosamines in cosmetic products by vortex-assisted dispersive liquid–liquid microextraction prior to gas chromatography-mass spectrometry

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Table S1. Box-Behnken design for multivariate optimization of the critical variables.

Step	$CHCl_3$ volume (µL)		Vortex time (s)		Ionic strength (%NaCl, w/v)	
	Uncoded	Coded	Uncoded	Coded	Uncoded	Coded
1	60	-1	60	0	0	-1
2	150	1	60	0	0	-1
3	60	-1	60	0	10	1
4	150	1	60	0	10	1
5	60	-1	30	-1	5	0
6	150	1	30	-1	5	0
7	60	-1	90	1	5	0
8	150	1	90	1	5	0
9	105	0	30	-1	0	-1
10	105	0	30	-1	10	1
11	105	0	90	1	0	-1
12	105	0	90	1	10	1
13	105	0	60	0	5	0
14	105	0	60	0	5	0
15	105	0	60	0	5	0

Box-Benhken design

Box-Benhken designs are a class of rotatable or nearly rotatable second-order designs used to generate high order response.

The total number of experiments required for the development of the Box-Benhken design (N) is defined by the following equation:

$$N = 2k(k-1) + Cp \quad (1)$$

where k is the number of factors (i.e., 3), and Cp is the number of replicates of the central point. In this sense, performing 3 replicates of the central point and according to Equation (1), 15 experiments were required. As shown in Table 3, the range of each factor was defined between a high and low value.

The following quadratic polynomial equation was applied to evaluate the multiple linear regression for each response:

$$Y = \beta_o + \Sigma \beta_i X_i + \Sigma \beta_{ii} X_i^2 + \Sigma \beta_{ij} X_i X_j + \varepsilon \quad (2)$$

where Y is the analytical response; β_0 is the constant term; β_i, β_{ii} and β_{ij} represent the regression coefficients of the design; χ_i and χ_j are the different variables; and ε is the residual error.

Afterwards, the global desirability of the experiment (D) was applied to study the different responses and achieve the optimal extraction conditions to enhance the analytical responses of the target analytes:

$$D = (d_1(Y_1) \cdot d_2(Y_2) \cdots d_n(Y_n))^{1/n} \quad (3)$$

where n is the number of responses in the optimization process, and $d_i(Y_i)$ is the individual desirability of each response in the experiment. The individual desirability is calculated as:

$$di = Y_i - Y_{min} / Y_{max} - Y_{min} \quad (4)$$

Responses are ranked between 0 and 1, respectively, to define undesirable responses and fully desirable responses.

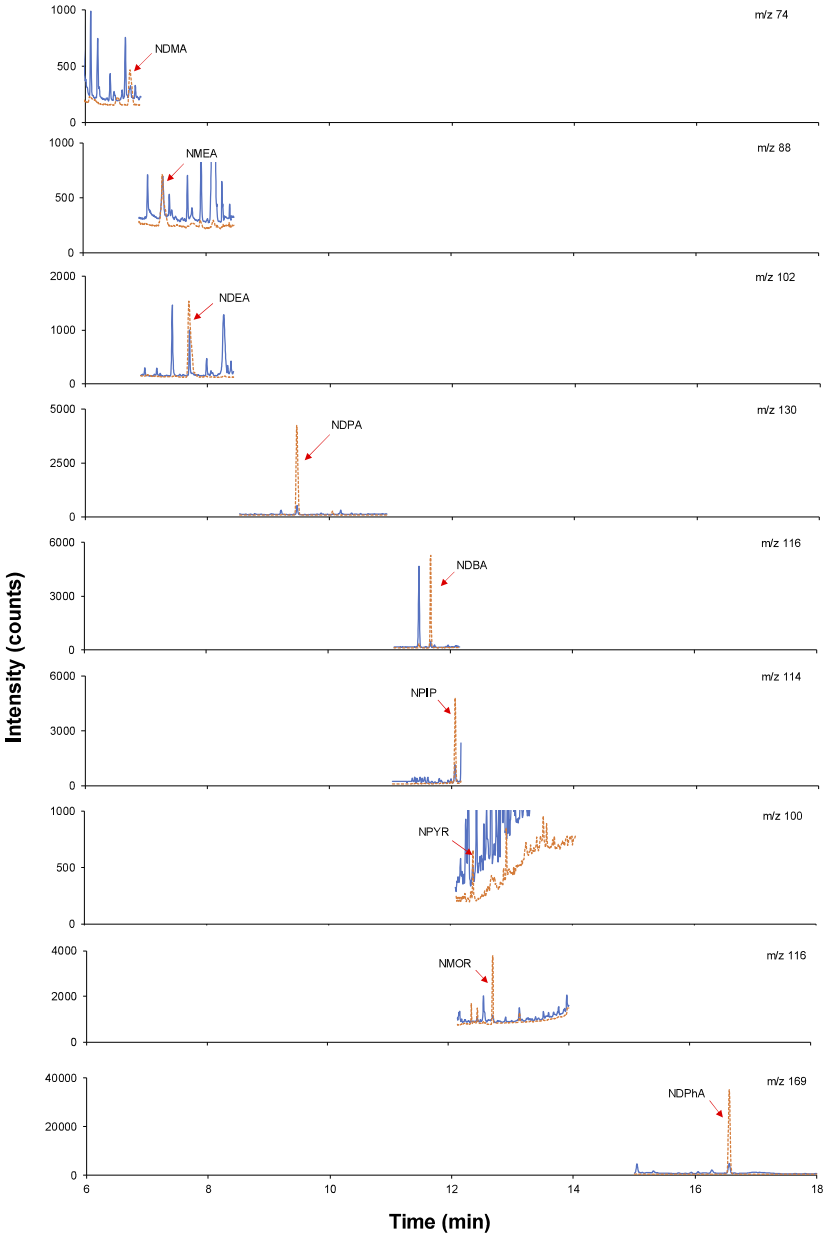

Figure S1. Chromatogram of a standard solution containing the analytes at 10 ng mL^{-1} subjected to the VA-DLLME procedure (dotted orange line), and (b) unextracted (continuous blue line).

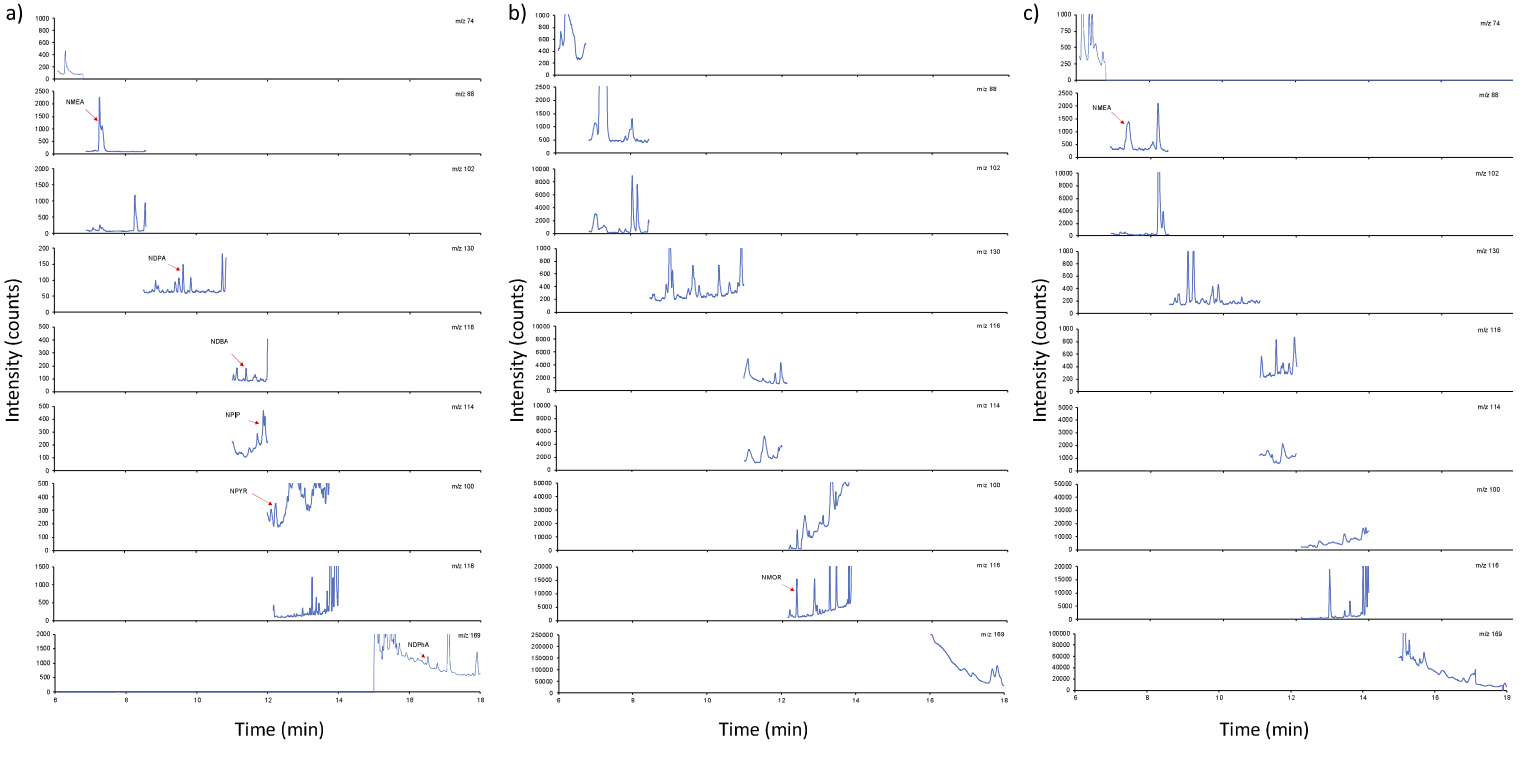

Figure S2. Chromatograms of sample solutions subjected to the proposed VA-DLLME method: (a) aftersun gel, (b) body cream 1, and (c) body cream 2.