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DETERMINACIÓN DE FRAGANCIAS, CONSERVANTES Y OTROS ADITIVOS REGULADOS EN PRODUCTOS DE USO COTIDIANO Y ATMÓSFERAS INTERIORES

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Al Licenciado Juan Pablo Lamas Castro a la presentación del trabajo titulado "DETERMINACIÓN DE FRAGANCIAS, CONSERVANTES Y OTROS ADITIVOS REGULADOS EN PRODUCTOS DE USO COTIDIANO Y ATMÓSFERAS INTERIORES" que ha realizado bajo su dirección, para optar al grado de Doctor en Química.

Y para que así conste, firman la presente autorización en Santiago de Compostela, a día 22 de Septiembre de 2011.

María Llompart Vizoso **Carmen García Jares** **Lucía Sánchez Prado**

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RESUMEN

Las políticas de control promovidas por la EU han comenzado a interesarse por la fabricación y utilización industrial de algunos ingredientes de productos de uso muy frecuente en el ámbito doméstico y del cuidado personal. Estos ingredientes incluyen los componentes de las fragancias y los conservantes, algunos de los cuales están reconocidos como alérgenos potenciales y probables disruptores endocrinos, entre otros muchos aditivos de naturaleza diversa. Para muchos de los compuestos considerados en este trabajo de Tesis doctoral, no se disponía de metodología analítica o las existentes eran claramente inadecuadas.

En este trabajo se han desarrollado métodos analíticos para la determinación de varias familias de ingredientes y aditivos en productos de uso cotidiano, principalmente en productos de higiene y cuidado personal.

Las metodologías propuestas se basan en procedimientos rápidos de extracción/dilución, incluyendo la microextracción en fase sólida, la extracción asistida por ultrasonidos y la extracción con disolventes presurizados; para el análisis se utiliza la cromatografía de gases junto con la espectrometría de masas como técnica de detección.

De manera general a lo largo del trabajo de Tesis se ha tratado de cumplir con los principios de la Química Analítica verde, diseñando procedimientos con nulo o mínimo consumo de reactivos y disolventes.

Por otra parte, el desarrollo metodológico se llevó a cabo mediante la aplicación de estrategias de diseño experimental. La validación se realizó utilizando herramientas estadísticas, demostrándose la fiabilidad de los procedimientos propuestos en términos de exactitud, precisión. Los límites de detección y cuantificación obtenidos estuvieron en todos los casos muy por debajo de los requerimientos establecidos en las normativas de regulación.

En todos los casos, dichos procedimientos fueron aplicados a un abanico variado de muestras, prestando especial atención a los productos dirigidos a la población infantil, por tratarse del sector más vulnerable a la acción de los tóxicos.

Como objetivo adicional, y teniendo en cuenta la alta volatilidad de algunas de estas sustancias, lo que convierte a la inhalación en una de las principales vías de

entrada en el organismo, se desarrollaron métodos para el control de la presencia de fragancias alergénicas en atmósferas interiores. Para ello se utilizaron aproximaciones analíticas novedosas, basadas en la extracción en fase sólida, la microextracción en fase sólida y la extracción asistida por ultrasonidos. Los métodos, una vez validados, se aplicaron a muestras de aire interior de distinto origen, poniendo de manifiesto el elevado grado de exposición a estos tóxicos a través de esta vía.

SUMMARY

The politics of control promoted by the EU became interested in the manufacture and industrial utilisation of some ingredients of products of very frequent use in the domestic field and for personal care. These ingredients include the components of the fragrances and the preservatives, some of which are recognised like potential alergens and likely endocrine disruptors, amongst a lot of other additives of diverse nature. For many of the compounds considered in this doctoral Thesis, analytical methodology was inexistent or the existent ones were clearly unsuitable.

Therefore, in this Thesis analytical methods for the determination of several families of ingredients and additives in products of daily use, mainly in products of hygiene and personal care, have been developed. The proposed methodologies are based on fast procedures of extraction/dilution, and high resolution chromatographic analysis, currently using mass spectrometry as detection technique. Along the work of this Thesis, it was treated to fulfil with the principles of the green Analytical Chemistry, designing procedures with none or minimum consumption of reagents and dissolvents. On the other hand, the methodological development was supported on the analysis of the results obtained by application of experimental design strategies. The validation was performed using statistical tools, showing the reliability of the procedures proposed in terms of accuracy, precision, and with limits of detection and quantification very underneath of the requests established by regulations.

In all the cases, the proposed procedures were applied to a high variety of samples, accounting for special attention to the products headed to the childish population, as it constitutes the most vulnerable sector to the action of the toxics.

As an additional objective, and taking into account the high volatility of some of these substances, which converts inhalation in one of the main ways of entrance in the organism, methods for the control of the presence of allergenic fragrances in inner atmospheres have been developed. For this purpose, new analytical approximations based on solid phase extraction, solid phase microextraction, and ultrasounds-assited extraction have been developed. The methods, once validated, were applied to samples of indoor air of distinct origin, showing the high degree of exposition to these toxics through inhalation.

I. JUSTIFICACIÓN Y OBJETIVOS

Actualmente, en los países desarrollados existe un importante aumento de problemas en la salud de la población relacionados con el uso creciente de materiales y productos de consumo de origen muy diverso. Este hecho crea la necesidad de realizar un control riguroso de los productos existentes en el mercado, velando siempre por un cumplimiento estricto de la normativa aplicable, en constante modificación y renovación, aportando de esta forma garantías de seguridad al consumidor. Para realizar dicho control es preciso disponer de metodología analítica adecuada, sin embargo, en muchos casos dicha metodología es poco eficaz, está obsoleta o simplemente no existe.

Es por ello que en esta Tesis se plantea el desarrollo de nueva metodología analítica para la determinación de sustancias potencialmente tóxicas presentes en distintos productos de consumo.

En primer lugar se evalúa la exposición indirecta de la población infantil a sustancias potencialmente alergénicas presentes en productos de cuidado e higiene personal diseñados específicamente para este sector de la población. Este estudio se centra en la determinación de estas sustancias en aguas de baño de bebés. Para el desarrollo de este trabajo se utiliza la microextracción en fase sólida (SPME), junto con una técnica cromatográfica selectiva de determinación, como es el caso de la cromatografía de gases acoplada a espectrometría de masas (GC/MS).

Continuando con esta línea se aborda la determinación de distintas familias de componentes de productos cosméticos. Para ello se proponen desarrollos analíticos en los que se hace uso de técnicas afines a los principios de "química verde". La extracción con disolventes presurizados (PSE) encaja en esta definición y junto con la GC/MS, se van a aplicar a la determinación de fragancias alergénicas y de un grupo amplio de conservantes pertenecientes a familias químicas muy diversas. El estudio de estos tipos de aditivos es de vital importancia, ya que se extiende a la práctica totalidad de los productos cosméticos. Las metodologías desarrolladas deberán ser sencillas y robustas, aunque al mismo tiempo deban demostrar su eficacia de aplicación a un elevado rango de muestras representativas del mercado actual de productos de cuidado personal.

En una segunda parte de la Tesis se pretende desarrollar metodología para determinar la presencia de sustancias potencialmente alergénicas en ambientes

interiores, resultado de la aplicación y uso de numerosos productos para el hogar y de productos de cuidado e higiene personal. Las técnicas de determinación en las condiciones descritas, aparte de ser sencillas y respetuosas con el entorno, tienen que desarrollarse de forma que se minimice la manipulación de las muestras. Con este fin se propone aplicar la combinación de la extracción en fase sólida (SPE) para la toma de muestra, con técnicas de extracción de analitos como la SPME y la extracción asistida por ultrasonidos (US).

Los últimos estudios de esta Tesis son consecuencia de un problema de intoxicación concreto, el caso del "síndrome del sofá tóxico" también denominado como "dermatitis del sofá chino" y "dermatitis por calzado". En la investigación de estos casos documentados por primera vez en el año 2008, se determinó que la sustancia que provocaba estas reacciones en la piel de los consumidores era el dimetilfumarato (DMF), compuesto biocida con conocidos efectos perjudiciales para la salud humana. El origen de esta sustancia se encontró en las pequeñas bolsas con agentes desecantes distribuidas con distintos productos de consumo. Sin embargo, la metodología analítica existente por aquel entonces para la determinación del DMF, era prácticamente inexistente o inadecuada. Con este fin, se propuso el uso de una técnica de extracción sencilla, US, combinada con cromatografía de gases. La eficacia del método se demostró con su aplicación a un elevado número de muestras representativas de una gran variedad de agentes desecantes presentes en los productos de consumo, principalmente calzado.

Colateralmente, se observó la presencia de otras sustancias potencialmente nocivas y alergénicas en estos materiales desecantes. Tales sustancias son el benzotiazol (BT) y el p-tertbutilfenol (TBP), para los que también se desarrolló metodología analítica para su determinación en la matriz citada.

Todas las metodologías desarrolladas cumplen con las premisas de ser sencillas, rápidas, robustas y de fácil adopción e implementación en cualquier laboratorio analítico. Por otro lado, permiten cumplir las exigencias legales vigentes.

II. INTRODUCCIÓN

1. ADITIVOS POTENCIALMENTE TÓXICOS Y ALERGÉNICOS EN PRODUCTOS DE CONSUMO Y CUIDADO PERSONAL

Los productos de consumo y cuidado personal se fabrican y comercializan a nivel mundial y van dirigidos a individuos de cualquier nivel socio-económico. Millones de personas de cualquier edad o sexo utilizan o están en contacto diariamente con estos productos.

La población, normalmente, hace un uso masivo de los distintos productos cosméticos y del hogar que existen en el mercado, sin reparar en los posibles efectos adversos que puedan tener en su salud. Muchos de estos productos (jabones, cremas, colonias, friegasuelos, ambientadores, etc.) incluyen en su formulación distintas sustancias que pueden llegar a ser perjudiciales para la salud humana.

Por otro lado, hay productos que, careciendo de peligrosidad para la salud humana en sí mismos, implican un riesgo importante para la misma debido a las sustancias que se utilizan para conservarlos durante el almacenamiento y transporte: muebles de madera, sofás, zapatos, distintos artículos de piel, etc.

En los últimos años se ha desatado una creciente alarma social acerca de los posibles efectos adversos que la composición y formulación de los distintos productos que se encuentran en el mercado pueden tener sobre la salud. De esta forma, se pone de manifiesto la necesidad de controlar la composición de dichos artículos dando así respuesta a la demanda social existente.

Para ejercer este control y garantizar el uso y consumo de la gran variedad de productos que ofrece el mercado, se crean y renuevan las distintas regulaciones o normas que rigen su fabricación y distribución [1,2].

ADITIVOS TÓXICOS EN PRODUCTOS COSMÉTICOS

Respecto a los cosméticos, existen evidencias del uso de estos productos dedicados exclusivamente al cuidado personal que se remontan unos 9000 años. Egipcios, griegos y romanos usaban habitualmente distintos productos para cuidar y aromatizar los cuerpos [3].

Desde la aparición y uso de estos productos por las antiguas civilizaciones, el mundo de los productos cosméticos o de cuidado personal ha ido creciendo de forma exponencial siempre de la mano del deseo de mantener el cuerpo joven y saludable. Existen productos cosméticos adaptados a cualquier parte del cuerpo, champús para el cabello, cremas faciales, pintura de ojos o labios, cremas para las manos, etc. Todos estos productos tienen como objetivo garantizar la higiene y mejorar la imagen de los individuos, aportando juventud, resaltando virtudes, ocultando defectos, etc.

Los cosméticos han originado un mercado con un importante peso en la economía mundial. En Europa, la facturación de productos cosméticos a finales del año 2010 ascendía a 67 billones de euros, seguido de los EEUU y Japón con 38 y 29 billones de euros respectivamente [3]. Estos son los tres grandes escenarios del mercado de productos cosméticos. En cada uno de ellos existe normativa que regula y controla la fabricación y uso de la multitud de productos que está al alcance del consumidor, con el único objetivo de garantizar un elevado nivel de protección para la salud de los individuos.

En Europa, la primera regulación de los productos cosméticos se realiza a través de la Directiva 76/768/CEE del 27 de Julio de 1976 [4], siendo reemplazada por el Reglamento 1223/2009 actualmente vigente [2]. Esta es la normativa que se debe cumplir respecto la fabricación, composición, etiquetado y uso de los distintos productos cosméticos.

Esta regulación define como producto cosmético "toda 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". Bajo esta definición se incluyen multitud de productos de consumo como: cremas, emulsiones, lociones, geles y aceites para la piel, mascarillas de belleza, maquillajes, polvos para higiene personal, jabones, perfumes, aguas de colonia, sales de baño, productos para el afeitado, productos para la depilación, productos para el cuidado del cabello, productos para el bronceado, etc.[2].

Por otro lado, los fabricantes de los distintos productos cosméticos están obligados a asegurar la inocuidad de los mismos así como de sus componentes. Esta garantía abarca un uso de los productos según las instrucciones del fabricante y para los fines que han sido fabricados.

Esta Tesis se ha enfocado hacia el desarrollo de nuevas metodologías analíticas para el análisis de diferentes grupos de sustancias presentes en productos de consumo y cuidado personal: fragancias, conservantes, ftalatos y otros compuestos alergénicos.

1.1 FTALATOS

1.1.1. Definición

Los ftalatos son una familia de compuestos químicos ampliamente utilizados en la industria como sustancias plastificantes para aportar flexibilidad en productos tales como adhesivos, recubrimientos, acetatos de polivinilo y especialmente en la fabricación de cloruro de polivinilo (PVC) [5-9].

Además, estos compuestos se encuentran en productos de cuidado personal (perfumes, lacas para el pelo, esmalte de uñas, etc.), calzado, material médico etc.[5,9,10]. Debido a su liberación durante la fabricación y uso de los productos industriales o de consumo su presencia es ubicua en múltiples compartimentos ambientales [5].

La gran cantidad de usos y aplicaciones de los ftalatos ha desencadenado que su producción mundial ascienda a varios millones de toneladas por año [8,11]. En Europa se consumen sobre 1 millón de toneladas anuales, de las cuales el 93% se utiliza en la fabricación de PVC [9].

La estructura general de los ftalatos, figura 1, se corresponde a ésteres dialquílicos o diarílicos del ácido 1,2-bencenocarboxílico (ácido ftálico).

Figura 1. Estructura general de los ésteres de ácido ftálico: ftalatos

Usando como base la estructura del ácido ftálico, se pueden obtener numerosos ftalatos con un amplio rango de pesos moleculares (dependiendo del número de átomos de carbono de los sustituyentes R y R’) por reacción con numerosos alcoholes. Como resultado de esta modificación del ácido ftálico se obtienen, generalmente, compuestos estables que permanecen en estado líquido a temperatura ambiente. De todas las combinaciones propuestas de ésteres del ácido ftálico (~ 10000), solamente son comerciales entre 50 y 100 [9].

De entre los ftalatos de bajo peso molecular destacan, por los niveles de producción, el dietilhexilftalato (DEHP), dibutilftalato (DBP), diisobutilftalato (DIBP) y bencilbutilftalato (BBP). Éstos representan un 15% de los ftalatos comercializados en Europa, siendo el DEHP el de mayor volumen de producción con un 10% del total de ftalatos [9]. Durante la última década se ha observado una importante disminución de la producción de este grupo de ftalatos, y sobre todo del DEHP, a favor de los ftalatos de mayor peso molecular.

En este último grupo de ftalatos (de elevado peso molecular) destacan el diisononilftalato (DINP), diisodecilftalato (DIDP), dipropilheptilftalato (DPHP), diisoundecilftalato (DIUP) y ditridecilftalato (DTDP), entre otros, representando el 80% de la producción de dicha familia de compuestos en Europa [9].

En esta Tesis se consideran los ftalatos detallados en la tabla 1.

Tabla 1. *Número CAS, peso molecular, coeficiente de partición octanol-agua (log K_{ow}) y estructura de los ftalatos estudiados.*

Compuesto	Número CAS	PM	Log K_{ow}	Estructura
Dimetilftalato DMP	131-11-3	194.2	1.61	
Dietilftalato DEP	84-66-2	222.2	2.54	
Dibutilftalato DBP	84-74-2	278.3	4.27	
Dimetoxietilftalato DMEP	117-82-8	282.3	2.9	
Dipentilftalato DPP	131-18-0	306.4		
Bencilbutilftalato BBP	85-68-7	312.4	4.70	
Dietilhexilftalato DEHP	117-81-7	390.6	7.73	
Di-n-octilftalato DnOP	117-84-0	390.6	7.73	

1.1.2. Aplicaciones

Anteriormente se ha mencionado como principal aplicación de los ésteres del ácido ftálico la producción de PVC, aunque los ftalatos pueden estar presentes en

cualquier material polimérico en calidad de plastificante, aportando flexibilidad y resistencia [5,12]. Existen numerosas aplicaciones de los ftalatos en multitud de productos de consumo: embalajes de alimentos, juguetes, piel artificial, productos de plástico con nitrocelulosa, acetato de polivinilo, material médico, material deportivo, adhesivos, insecticidas, gomas sintéticas, calzado, cosméticos, etc.[6,7,9,10].

1.1.3. Distribución y toxicidad

Debido a que los ftalatos no están unidos químicamente a los distintos materiales en que se emplean, pueden ocurrir procesos de migración o volatilización al medio ambiente, ya sea durante la manufacturación de los distintos productos o durante la manipulación del producto elaborado y preparado para su consumo [8,10].

La aplicación directa sobre la piel de productos cosméticos de cuidado personal que contengan estas sustancias es una de las formas más rápida y sencilla de entrar en contacto con los ftalatos. Este tipo de aplicaciones, junto con el uso de ftalatos como el DEHP en material médico, o el uso del DBP en el recubrimiento de medicamentos [8], ponen de manifiesto una exposición fácil y directa de la población a este tipo de compuestos.

Por lo tanto, la exposición de la población a los ftalatos puede tener lugar por ingestión, inhalación y exposición dérmica, y este contacto tiene lugar a lo largo de toda la vida de los individuos [8,10,12].

Si bien los ftalatos presentan baja toxicidad aguda, algunos de ellos han mostrado toxicidad crónica, como es el caso del DBP, BBP y DEHP, los cuales presentan genotoxicidad en linfocitos humanos y células mucosas [13]. Actualmente, la Unión Europea los ha clasificado como sustancias tóxicas para la reproducción, prohibiendo su uso y comercialización a partir del año 2015 [14].

En general, los ftalatos se consideran disruptores endocrinos ya que muestran toxicidad para la reproducción y el desarrollo en animales, produciendo un descenso de los niveles de testosterona y malformaciones del sistema reproductor [8,15-17].

1.1.4. Los Ftalatos en los productos cosméticos

Los ftalatos, en la industria cosmética se utilizan como disolventes y diluyentes sin olor (en lacas de uñas, quitaesmaltes, en lacas para el pelo), y como fijadores de perfumes (en aguas de colonia, entre otros). Algunos de estos compuestos (DBP, BBP y DEHP) están considerados como posibles agentes mutágenos, carcinógenos y tóxicos para la reproducción humana a través del Reglamento (UE) 143/2011 [14], incluyéndolos de esta forma en el anexo XIV del Reglamento (CE) No 1907/2006 [18].

En lo que concierne a la industria cosmética, la UE ya prohibió en el año 2009 [2] el uso de estos tres compuestos junto con el ftalato de bis (2-metoxietilo), los dipentil ésteres ramificados y lineales (n-pentilisopentil, diisopentil, di-n-pentil) y los alquil ésteres de di-C7 a C11 ramificados y lineales, en la fabricación de cualquier producto cosmético.

Referente a legislación fuera de la UE, la EPA ha propuesto el control de seis ftalatos: DMP, DEP, DBP, BBP, DEHP y di-n-octilftalato (DnOP) y en la actualidad ya no incluye al DEP y DMP en la lista de sustancias permitidas en la elaboración de diversos productos [19].

Esta reciente aparición de nueva normativa en la que se prohíbe o limitan los ftalatos, refleja una clara tendencia a la eliminación de estos compuestos de los distintos productos de consumo.

Los ftalatos que más se utilizan en cosmética son el DBP, DMP, DEP y DEHP. De estos cuatro compuestos, solamente están prohibidos en la UE el DBP y el DEHP, mientras que sobre DMP y DEP existe cierta controversia en cuanto a su potencial toxicidad [20], ya que se los relaciona con efectos perjudiciales sobre el ADN del esperma humano, entre otros [21].

1.2. FRAGANCIAS

Por fragancia se entiende una mezcla de compuestos químicos, que permiten diferenciar e identificar los distintos productos por el aroma que desprenden [22]. Sin embargo, es habitual hablar de fragancia, refiriéndose a un único compuesto de esta familia.

En la actualidad, en la fabricación de distintas fragancias se emplean más de 2000 ingredientes y la mayoría de estos han sido utilizados desde hace décadas. A pesar de la larga historia de estas sustancias, se requiere una vigilancia continua que garantice la seguridad de los consumidores [23].

Hay dos grandes grupos de fragancias que habitualmente son objeto de estudio por sus posibles efectos perjudiciales sobre la salud humana o el medioambiente: Fragancias sintéticas y Fragancias alergénicas.

1.2.1. Fragancias sintéticas

1.2.1.1. Definición

Las fragancias sintéticas (almizcles sintéticos) se usan como alternativa a las fragancias naturales (almizcles naturales) [22].

Según su estructura química, las fragancias sintéticas se pueden dividir en tres grandes grupos: fragancias o almizcles policíclicos, fragancias o almizcles nitrogenados y fragancias o almizcles macrocíclicos. Estas últimas presentan estructura y propiedades similares a los compuestos aromáticos naturales [5,12,22,24].

Habitualmente se usa el término en inglés "musk" para referirse a este tipo de almizcles, por lo tanto, se usará indistintamente junto con los vocablos en español.

Las fragancias sintéticas nitrogenadas aparecieron por primera vez a finales del siglo XIX como sustituto económico de las fragancias naturales.

Este grupo de sustancias, denominado frecuentemente como "nitromusks", está formado por cinco compuestos derivados del di- y tri- nitrobenceno: musk xylene® (MX), musk ketone® (MK), musk ambrette® (MA), musk moskene® (MM) y musk tibetene® (MT) [5,24,25].

Las fragancias sintéticas policíclicas (musks policíclicas), se desarrollaron a mediados del siglo XX y durante los últimos años han ido sustituyendo o reemplazando a las nitromusk.

La familia de las musks policíclicas está formada por sustancias cuya estructura obedece básicamente a sustituciones por grupos metilo de dos moléculas principales: indano y tetralina. Cinco de ellas se pueden clasificar como derivadas del indano: galaxolide® (HHCB), celestolide® (ADBI), phantolide® (AHMI), cashmeran® (DPMI), traseolide® (ATII). Por lado, la tonalide® (AHTN) y versalide® (ATTN), se clasifican como derivadas de la tetralina [5,24]. De todas ellas, cabe destacar por sus volúmenes de producción la tonalide® y galaxolide® [5].

Desde el punto de vista de sus propiedades, las musks sintéticas (nitromusks y musks policíclicas) se pueden definir como sustancias muy solubles en disolventes orgánicos, lipofílicas y persistentes en tejidos grasos [24,26-28]; además, presentan una alta estabilidad química y una baja biodegradabilidad y alto potencial de bioacumulación.

El grupo de las musks macrocíclicas está compuesto por una mezcla de compuestos sintéticos y naturales. El origen de este grupo de sustancias se encuentra en las musk muscone, que es el almizcle natural. Básicamente son cetonas macrocíclicas (origen animal), lactonas y bis-lactonas (origen vegetal). En esta categoría se engloban: ambrettolide®, exaltolide®, musk R1®, musk T® (etilenbrasilato), civetone® y muscone (almizcle natural). Este grupo de compuestos se caracteriza por su elevado coste de producción [12,22,25,29]. Teniendo en cuenta la estructura de las musks macrocíclicas, se puede producir una fácil descomposición microbiana de las mismas, convirtiéndolas en compuestos de estabilidad química reducida y mayor biodegradabilidad que las nitromusks y musks policíclicas [5].

Estas características aportan a las fragancias macrocíclicas una gran ventaja frente al resto de musks sintéticas, puesto que las convierte en menos perjudiciales para la salud y medioambiente. Este hecho ha provocado un descenso importante en la producción de las nitromusks y musks policíclicas a favor de las macrocíclicas [5].

En el presente trabajo de tesis se estudiarán las fragancias sintéticas detalladas en la tabla 2.

1.2.1.2. Aplicaciones

Las fragancias naturales se usan en Asia desde hace más de 5000 años para la elaboración de medicinas y perfumes. En Europa, el uso de este tipo de fragancias comenzó a reducirse al principio de la década de los 90 debido al alto coste que tenía el introducirlas en los perfumes. Actualmente el uso de almizcles naturales se reduce a un mercado exclusivo y de alto poder adquisitivo [25,29].

Anteriormente se ha mencionado que la aparición de las primeras musks sintéticas (nitromusks) se remonta a finales del siglo XIX. El objetivo de su desarrollo se centraba únicamente en la sustitución industrial de las musks naturales, ya que el uso de éstas últimas resultaba muy costoso [12,25].

La mayor producción de fragancias se centra en el sector cosmético, destacando los perfumes. En menor medida las podemos encontrar en multitud de productos de consumo como champús, detergentes, suavizantes, lociones, jabones, productos de limpieza, ambientadores, productos perfumados del hogar, aditivos en tabaco, en herbicidas, explosivos, cebos de pesca, et.[5,24-27,30].

De todas las fragancias presentes en el mercado, las más importantes por volumen de producción (miles de toneladas anuales) son las musks galaxolide® y tonalide®, repartiéndose el 95% de la producción mundial de musks policíclicas [25,29,30].

Tabla 2. *Número CAS, peso molecular, coeficiente de partición octanol-agua (log K_{ow}) y estructura de las fragancias sintéticas estudiadas.*

Compuesto	Número CAS	PM	Log K_{ow}	Estructura
Cashmeran® DPMI	33704-61-9	206.3	4.9	
Celestolide® ADBI	13171-00-1	244.4	6.6	
Phantolide® AHMI	15323-35-0	244.4	6.7	
Traseolide® ATII	68140-48-7	258.4	6.3	
Galaxolide® HHCB	1222-05-5	258.4	5.9	
Tonalide® AHTN	21145-77-7	258.4	5.7	
Musk Ambrette® MA	83-66-9	268.3	3.71	
Musk xylene® MX	81-15-2	297.3	4.8	
Musk moskene® MM	116-66-5	278.3	5.8	
Musk tibetene® MT	145-39-1	266.3	5.0	
Musk ketone® MK	81-14-1	294.3	4.3	
Ambrettolide®	28645-51-4	252.4	5.4	

1.2.1.3. Distribución y toxicidad

Las primeras musks que hicieron saltar la alarma sobre su potencial toxicidad, fueron las nitromusks. Se detectaron por primera vez en seres humanos en los años 90, demostrándose en estudios posteriores su alto potencial de bioacumulación [26,31].

Las musks ambrette®, tibetene® y moskene® (nitromusks) cuyo auge de producción y consumo fue en los años 80, fueron las primeras musks prohibidas y retiradas del mercado a finales de los años 90 (1995-1998) debido, principalmente, a su neurotoxicidad y genotoxicidad [26,27]. Recientemente, la Unión Europea (UE) ha prohibido el uso y comercialización de la musk xylene® a partir del 21 de Julio de 2014, catalogándola como sustancia muy persistente y bioacumulable [14]. En los Estados Unidos (EEUU), la Environmental Protection Agency (EPA) también empieza a poner restricciones a la musk xylene® [19].

Dentro del grupo de las nitromusks, las de mayor producción y uso son las musks xylene® y ketone®. Las prohibiciones sobre musk xylene® en la UE y EEUU ya se han comentado, mientras que la musk ketone® está permitida bajo ciertas restricciones, por generar sospechas de inducir al cáncer o facilitar e incrementar el efecto carcinogénico de otros compuestos en animales y en humanos [24,32,33]. Únicamente se ha prohibido su uso en productos de higiene bucal [2].

La probada toxicidad y alto potencial de bioacumulación de las nitromusks, características que implican efectos perjudiciales tanto para la salud humana como para el medioambiente, han provocado una drástica caída de su producción a lo largo de los años a favor de las musks policíclicas, sobre todo de la tonalide® y galaxolide® [26,30]. Estas últimas tienen una menor toxicidad que las nitromusks, pero el carácter lipofílico y lenta degradación que presentan, provoca que estos compuestos sean fácilmente bioacumulables, y por lo tanto, también son potencialmente perjudiciales para la salud humana y medioambiente. Adicionalmente las musks xylene, ketone y tonalide producen actividad estrogénica en humanos [26,33].

La principal vía de contacto de la población humana con las musk es a través de la absorción dérmica [26] debido al uso de gran cantidad de productos cosméticos por parte de la población.

Este hecho, junto a las propiedades ya mencionadas de bioacumulación y lipofilia, provoca que las musks se acumulen con facilidad en diversas partes del cuerpo humano: tejidos, leche, sangre, etc. [14,24,26].

1.2.1.4. Las fragancias sintéticas en los productos cosméticos

Las prohibiciones de uso que afectan a las musks ambrette®, tibetene®, moskene® y xylene, han provocado que del grupo de las fragancias sintéticas nitrogenadas, sólo esté permitida en productos cosméticos la musk ketone®. Este compuesto está regulado a través del Reglamento 1223/2009 [2] en el cual se autoriza el uso de dicha sustancia en todos los productos cosméticos excepto en los de higiene bucal. De todas formas, el contenido en musk ketone® en los distintos productos cosméticos está limitado por dicho reglamento a un máximo de 0.042%, valor que puede llegar a 0.56% en las aguas de colonia y a 1.4% en los perfumes. Este mismo tipo de limitación, es la que presentaba el Reglamento de cosméticos para la musk xylene®, cuyo uso está prohibido en cualquier producto a partir del año 2014 [14].

Las fragancias sintéticas policíclicas (celestolide®, phantolide®, cashmeran®, traseolide®, galaxolide® y tonalide®) han ido sustituyendo a las fragancias nitrogenadas a lo largo de estos años. Si bien no se ha demostrado toxicidad en el uso de estas fragancias, conviene remarcar que se parecen mucho químicamente a la versalide, fragancia prohibida a finales de los años 70 debido a su neurotoxicidad [34].

Por otra parte, el Comité Científico sobre Productos Cosméticos y No Alimentarios destinados al consumidor (SCCNFP, hoy SCCP) indicó que el uso de la tonalide® debe restringirse en la composición de fragancias para aguas de perfume y perfumes [35], y la OSPAR (Oslo and Paris Commission) incluyó a la galaxolide® y a la tonalide® en la lista de sustancias químicas de acción prioritaria [36]. Así, la UE ha establecido una concentración máxima autorizada en el cosmético acabado del 0,1% en tonalide® para productos de permanencia (excepto productos hidroalcohólicos 1%, fragancia fina 2,5% y fragancia en crema 0,5%) y del 0,2% en productos de aclarado. Para la phantolide® se establece el límite del 2% sólo en productos de permanencia [37].

1.2.2. Fragancias alergénicas

1.2.2.1. Definición

Típicamente los ingredientes de las fragancias consisten en moléculas orgánicas de bajo peso molecular (<300uma) con una presión de vapor por debajo de 2 mm Hg [38].

El grupo de fragancias potencialmente alergénicas (SAs, suspected allergenics) está formado por un total de 27 sustancias [2,39], y son constituyentes principales de perfumes y de multitud de productos de consumo.

Su principal función es la de proporcionar olores agradables y atrayentes para el cuerpo humano, consiguiendo enmascarar olores indeseados (función similar a las musks). Sin embargo, se las relaciona con distintas alergias cuando entran en contacto con el cuerpo humano, tanto en su aplicación directa sobre la piel, como por inhalación.

De las 27 sustancias pertenecientes a este grupo, 25 se definen químicamente como volátiles. Las dos restantes se corresponden con extractos naturales de líquenes de composición muy compleja, hecho que provoca que sean excluidas de la mayoría de los estudios [40], ya que no pueden ser analizadas por cromatografía de gases.

Este grupo de 25 fragancias alergénicas volátiles, está formado por compuestos de naturaleza química muy diversa (tabla 3): terpenos, terpenoides, esteres, alcoholes, aldehídos, etc.

Tabla 3. *Número CAS, peso molecular, coeficiente de partición octanol-agua (log K_{ow}) y estructura de las fragancias alergénicas estudiadas.*

Compuesto	Número CAS	PM	Log K_{ow}	Estructura
Pineno	80-56-8	136	4.37	
Limoneno	5989-27-5	136	4.57	
Alcohol bencílico	100-51-6	108	1.05	HO
Linalol	78-70-6	154	3.28	HO
Octanoato de 2 metilo	111-12-6	154	2.60	O O
Citronelol	106-22-9	156	3.38	OH
Citral	5392-40-5	152	3.17	O
Geraniol	106-24-1	154	3.28	OH
Cinamaldehido	104-55-2	132	2.22	O
Hidroxicitronelal	107-75-5	172	1.54	HO O
Anis alcohol	105-13-5	138	1.10	O OH
Alcohol cinamílico	104-54-1	134	1.93	OH
Eugenol	97-53-0	164	2.20	OH O
Metileugenol	93-15-2	178	2.9	O O
Isoeugenol	97-54-1	164	2.45	OH O

Tabla 3.Continuación

Compuesto	Número CAS	PM	Log K_{ow}	Estructura
Cumarina	91-64-5	146	1.39	
Alfa isometil ionona	127-51-5	206	4.41	
Lilial®	80-54-6	204	4.07	
Amil cinamal	122-40-7	202	4.80	
Lyral®	31906-04-4	210	2.53	
Amil cinamil alcohol	101-85-9	204	4.37	
Farnesol	4602-84-0	222	5.31	
Hexilcinamaldehido	101-86-0	216	4.82	
Benzoato de bencilo	120-51-4	212	3.97	
Salicilato de bencilo	118-58-1	228	4.31	
Cinamato de bencilo	103-41-3	238	3.65	

1.2.2.2. Aplicaciones

Actualmente existen distintas restricciones en cuanto a la introducción de fragancias alergénicas en los distintos productos de consumo. Estas restricciones varían en función del ámbito geográfico de producción y consumo de las mismas,

pero en esencia son muy similares. En Europa, el organismo que se encarga de velar por la seguridad de los productos de consumo es el Comité Científico de Seguridad de los Consumidores (CCSC o SCCS, respondiendo a los términos en inglés) según la decisión 2008/721/CE de la Comisión Europea [41]. Pero es la propia Comisión Europea la encargada de elaborar la legislación necesaria. En los Estados Unidos los organismos encargados de controlar el mercado de las fragancias son la "Food and Drug Administration" (FDA) y "Consumer Products Safety Commision" (CPSC) [17]. El objetivo principal de todos estos organismos, es el de velar por la seguridad de los consumidores.

El uso de fragancias en los productos de consumo facilita que el consumidor pueda identificar por el olor, generalmente agradable, los distintos productos que ofrece el mercado.

En la actualidad podemos encontrar fragancias en más de 5000 productos. Estos datos, aportados por el "Research Institute for Fragrance Materials (RIFM), 2011" [42], ponen de manifiesto el potencial de aplicación de las fragancias alergénicas por parte de la industria.

Las fragancias se pueden encontrar en productos como: alimentos, bebidas, artículos de tocador-cosméticos, ambientadores, productos para el hogar, pesticidas, etc.… [17,19,43,44]. De entre todas las familias de productos donde se pueden encontrar estas sustancias, hay que destacar los cosméticos. En este tipo de productos es en donde se encuentra la mayor aplicación de las fragancias potencialmente alergénicas.

1.2.2.3. Distribución y toxicidad

Existen gran cantidad de productos de consumo que tienen en su formulación fragancias alergénicas, lo que pone de manifiesto la facilidad con la que el consumidor puede entrar en contacto con estas sustancias [23].

Se han documentado multitud de reacciones derivadas del uso de los distintos productos que contienen fragancias, la mayoría de ellas, reacciones dermatológicas (eczemas) por la aplicación directa sobre la piel de perfumes, cremas, etc. siendo las manos, axilas y la cara las partes del cuerpo más expuestas

a este tipo de contacto [39,45,46]. En Europa, alrededor de un 10% de la población con eczemas, presenta alergia o sensibilidad al contacto con este tipo de sustancias [39].

Por otro lado, estos compuestos pueden inducir o empeorar distintos problemas respiratorios, como el asma, debido a las propiedades irritantes que poseen muchos de ellos [47,48]. Otras afecciones derivadas de uso de fragancias se traducen en dolores de cabeza [49], alteraciones de la mucosa nasal [50], y otros tipos de alergias [39].

Como consecuencia, la población que padezca diversas enfermedades tipo asma, sinusitis, rinitis, etc., cuyo origen no sea el contacto con fragancias alergénicas, experimentará un mayor efecto irritante en presencia de estas sustancias. Este efecto sinérgico se puede producir incluso cuando la exposición se produce con valores inferiores de la concentración necesaria para provocar la misma reacción en la población sana [17].

De los 25 alérgenos clasificados como volátiles, cabe resaltar al metil eugenol. Este compuesto había sido incluido por la Unión Europea, en la lista de sustancias prohibidas en cosméticos en el año 2002 [51], puesto que se sospechaba de sus efectos genotóxicos y cancerígenos [43,44]. Posteriormente en la última revisión de la normativa europea de cosméticos [2], actualmente vigente, esta sustancia pasa a formar parte de la lista de ingredientes que se pueden incluir en dichos productos, pero con limitaciones. Actualmente, siguen constatándose las propiedades genotóxicas de esta fragancia [52], lo que deja la puerta abierta a futuros cambios en la legislación en lo referente la toxicidad de esta fragancia.

Otro ejemplo de fragancia con efectos carcinogénicos en animales es la cumarina [53]; pese a ello, esta sustancia también forma parte de la lista de sustancias permitidas, aunque restringidas en productos cosméticos.

El citral junto con el geraniol, si bien no presentan efectos carcinogénicos, si presentan actividad estrogénica y provocan el agrandamiento de la próstata en los varones [54].

Debido a la problemática derivada del uso de productos de consumo que contengan en su formulación fragancias alergénicas, es necesario que se proporcione al consumidor la información necesaria sobre su presencia en los mismos. Esta

información se materializa en forma de etiquetas o en su defecto, documentación complementaria y detallada de la composición del producto [2,19,39].

1.2.2.4 Las fragancias alergénicas en los productos cosméticos

La presencia de estas fragancias es muy habitual en los productos cosméticos, sobre todo en perfumes.

Debido a los posibles efectos negativos para la salud humana de estas sustancias, la UE regula a través del Reglamento 1223/2009 [2] el uso de estos compuestos en los distintos productos cosméticos. En este sentido, el Reglamento exige la declaración en la etiqueta del producto de la presencia de estos compuestos alergénicos cuando sus niveles de concentración se encuentren por encima de niveles de 0.01 o 0.001% según se trate de un producto cosmético de aclarado (por ej. gel de ducha) o de permanencia en la piel (por ej. loción hidratante), respectivamente. La única excepción a esta regla, es para el Metileugenol. Este compuesto estaba incluido inicialmente entre las sustancias totalmente prohibidas en los productos cosméticos (Anexo II) [4], pero en la última revisión del correspondiente al Reglamente 1223/2009 [2] se ha incluido en el Anexo III con las siguientes restricciones de concentración máxima permitida en los productos: perfumes 0.01%, agua de colonia 0.004%, crema de fragancia 0.002%, otros productos de permanencia y productos bucales 0.0002% y, finalmente, en productos de aclarado 0.001%.

El reglamento 1223/2009 de la UE incluye a este grupo de 25 sustancias volátiles en el anexo III: lista de sustancias que no puede haber en los productos cosméticos salvo que cumplan con las restricciones establecidas. Por otro lado se propone el control del monoterpeno alfa-pineno cuyo control no está recogido en la Regulación Europea de productos cosméticos [2], pero está reconocido como alérgeno en otras normativas y estudios [55,56].

1.3 CONSERVANTES

1.3.1 Definición

Los conservantes son sustancias cuya principal función es evitar el desarrollo de microorganismos (antimicrobianos) [2,57], aunque también se emplean para evitar la degradación por exposición al oxígeno de los distintos productos (antioxidantes).

Estos compuestos forman parte de la composición de numerosos productos de consumo: jabones, cosméticos, alimentos, fármacos, pasta de dientes, entre otros.

Dentro de la definición de conservantes se incluyen distintas familias de compuestos, siempre que posean alguna de las propiedades anteriormente mencionadas. Entre los conservantes estudiados (tabla 4) se encuentran: los derivados del ácido p-hidroxibenzoico (parabenes), el butilcarbamato de iododopropinilo (IPBC), el 5-cloro-2-(2,4-diclorofenoxi)fenol (triclosán, TCS), los conservantes bromados (5-Bromo-5-nitro-1,3-dioxano (bronidox) y 2-bromo-2-nitropropano-1,3-diol (bronopol), el butilhidroxianisol (BHA) y el butilhidroxitolueno (BHT).

Tabla 4. *Número CAS, peso molecular, coeficiente de partición octanol-agua (log K_{ow}) y estructura de los conservantes estudiados.*

Compuesto	Número CAS	PM	Log K_{ow}	Estructura
Metilparaben (MeP)	99-76-3	152.2	1.91	HO, O, O
Etilparaben (EtP)	120-47-8	166.2	2.34	HO, O, O
Isopropilparaben (iPrP)	4191-73-5	180.2	2.91	HO, O, O
Propilparaben (PrP)	94-13-3	180.2	2.94	HO, O, O

Tabla 4. Continuación.

Compuesto	Número CAS	PM	Log K_{ow}	Estructura
Isobutilparaben (iBuP)	4247-02-3	194.2	3.40	
Butilparaben (BuP)	94-26-8	194.2	3.50	
Benzilparaben (BzP)	94-18-8	228.2	3.56	
Triclosán (TCS)	3380-34-5	289.5	4.8	
Butilcarbamato de iododopropinilo (IPBC)	55406-53-6	281.1	2.45	
Bronidox	30007-47-7	212.0	0.25	
Bronopol	52-51-7	199.99	-0.64	
Butilhidroxianisol (BHA)	121-00-6	180.2	3.50	
Butilhidroxitolueno (BHT)	128-37-0	220.3	5.10	

En el grupo de los parabenes, destacan por volumen de producción y uso el metilparaben, etilparaben y propilparaben. Los otros parabenes considerados, son menos usados: isopropilparaben, isobutilparaben y bencilparaben junto con sus sales sódicas.

En general los parabenes son sólidos, estables al aire y resistentes a la hidrólisis en agua. Su solubilidad en agua disminuye a medida que aumenta el tamaño de la cadena hidrocarbonada [58].

El IPBC pertenece a la familia de los carbamatos. Es sólido de color blanco, muy soluble en disolventes orgánicos, moderadamente soluble en agua y fácilmente hidrolizable en medio alcalino [59]. Se caracteriza por ser buen fungicida y antimicrobiano [59-62].

El triclosán es un derivado clorado del hidroxifenil éter con una volatilidad y solubilidad en agua bajas. Este compuesto es un antimicrobiano de amplio espectro con una producción mundial de más de 1500 toneladas por año [63], posee gran capacidad biocida contra multitud de bacterias, numerosos hongos y levaduras [63].

Los conservantes bromados, bronopol y bronidox, son compuestos que poseen un alto poder bactericida, sobre todo contra las bacterias Gram- y Gram+ [64]. La producción mundial de estos compuestos bromados es de más de 5000 toneladas anuales, dato indicativo de la distribución de estas sustancias en los distintos productos de consumo.

Los conservantes fenólicos, BHA y BHT, se caracterizan por su poder antioxidante. Su uso también está muy extendido debido a su estabilidad química, disponibilidad y bajo coste de producción [65], con una producción mundial que supera las 60000 toneladas anuales [66]. Estos compuestos normalmente se usan de forma combinada para potenciar el efecto antioxidante [67].

1.3.2 Aplicaciones

El uso de conservantes antimicrobianos está muy extendido en multitud de productos de consumo: productos cosméticos, productos para el hogar, productos de cuidado personal (PCPs) y productos farmacéuticos.

La versatilidad de uso de parabenes por parte de la industria es muy amplia, pudiéndose encontrar también en productos edulcorantes, congelados, bebidas, salsas, etc.

La capacidad antimicrobiana de estos compuestos aumenta al aumentar la cadena hidrocarbonada. Ésta es muy selectiva, así que se suelen emplear mezclas de

los mismos o con otros conservantes (BHA, BHT, triclosán,..), para potenciar y ampliar su actividad [68].

Las propiedades antimicrobianas del IPBC han ampliado mucho su espectro de uso por parte de la industria, pudiéndose utilizar como antimicrobiano en la madera, fungicida industrial, desinfectante de agua, etc.[60]. Además numerosos productos de consumo lo contienen en su formulación como es el caso de cosméticos, pegamentos, pinturas, tejidos, etc.[59,61,62].

Otro conservante importante, por las cifras de producción, es el triclosán. La elevada estabilidad térmica y gran potencial como antimicrobiano, convierte al triclosán en un componente idóneo para su uso en campos bien diferenciados del sector productivo como la fabricación de plásticos y tejidos o en la industria cosmética [69].

En cuanto a los conservantes bromados, bronopol y bronidox, se aplicaron inicialmente como conservantes en fármacos y desinfectantes del agua. Con el tiempo, sus usos se han extendido a multitud de productos de consumo incluyendo cosméticos, y productos del hogar.

Los conservantes fenólicos (BHA, BHT) se emplean como antioxidantes en un amplio abanico de productos de consumo (cosméticos, comida, productos farmacéuticos, caucho, pinturas, plásticos, aceites, etc.) [66,70].

1.3.3 Distribución y toxicidad

La presencia de conservantes en los distintos productos de consumo es necesaria para evitar riesgos al consumidor derivados del crecimiento microbiano en dichos productos. Sin embargo, su presencia puede provocar irritaciones cutáneas y otras afecciones en la salud humana. Los parabenes y el BHA se consideran disruptores endocrinos [71], el IPBC se considera tóxico por inhalación [72], los parabenes son sospechosos de provocar cáncer de mama [73]. Además, conservantes como el BHA, productos de transformación del triclosán [74], bronidox y bronopol, son sospechosos de inducir distintos tipos de cáncer [75,76].

Actualmente, el CCSC no considera que existan datos suficientes para afirmar que conservantes como los parabenes sean cancerígenos, pero si acepta su

actividad estrogénica. Por ello recomienda disminuir los valores de concentración de parabenes, sobre todo los de cadena más larga [77].

El IPBC no presenta propiedades cancerígenas conocidas, pero sí resulta tóxico para organismos del medio acuático [59,61]. Por otro lado, respecto a las reacciones de este compuesto en contacto con el cuerpo humano sólo se pueden documentar fuertes irritaciones oculares [62,72].

Hasta no hace muchos años, no se asociaba el triclosán con propiedades tóxicas, cancerígenas o teratogénicas. Estudios recientes prueban que este compuesto puede provocar tumores en animales [78]. Respecto a la distribución del triclosán en el cuerpo humano, se ha encontrado en muestras de suero sanguíneo [79], orina [80] y en leche materna [81]. Por otro lado, el mayor potencial tóxico de este compuesto está en sus productos de descomposición o de reacción, puesto que puede dar lugar a compuestos perjudiciales como clorofenoles, dioxinas y compuestos policlorados [74,82,83].

Respecto al bronidox y bronopol, su uso en Europa está permitido, pero con restricciones. El fabricante debe de garantizar que no existen posibilidades de formación de nitrosaminas dentro de los productos [2]. En otras legislaciones que suelen ser más permisivas, como es el caso de la legislación japonesa, el uso de estos conservantes bromados está totalmente prohibido [84]. En sí mismos, estos compuestos no suponen ningún riesgo, pero sus productos de descomposición pueden reaccionar con otras sustancias presentes generando nitrosaminas, compuestos considerados como altamente cancerígenos [75].

Los conservantes fenólicos BHA y BHT se consideran potencialmente nocivos para la salud humana al provocar la pérdida de nutrientes y distintos efectos tóxicos en el cuerpo humano [70]. Su acumulación en el cuerpo se centra en el tejido adiposo, hígado y riñones [85]. El efecto cancerígeno de estos compuestos fenólicos no está totalmente claro. En la actualidad existen distintos estudios en los que consideran al BHT y al BHA como sustancias potencialmente cancerígenas [85,86]. Sin embargo, otros autores concluyen que estos compuestos no tienen propiedades cancerígenas [87], o incluso los clasifican como sustancias con propiedades anticancerígenas [88].

Por otro lado, el uso masivo o mal uso de todos estos biocidas puede provocar una mayor resistencia bacteriológica a los antibióticos tanto en el medioambiente como en humanos [89].

1.3.4 Los conservantes en los productos cosméticos

La UE regula el uso de los conservantes en productos cosméticos a través de Anexo V del Reglamento 1223/2009 [2] que hace referencia a las sustancias admitidas en este tipo de productos. Entre los conservantes más utilizados en la industria cosmética se pueden destacar los ésteres de ácido p-hidroxibenzoico y sus sales (parabenes), el triclosán (TCS), el butilcarbamato de yodopropinilo (IPBC), los conservantes bromados (bronopol y bronidox) y los conservantes fenólicos (BHA y BHT). Todas estas sustancias (excepto BHA y BHT) están incluidas en el anexo V del Reglamento 1223/2009 de la UE, donde se establecen las concentraciones máximas permitidas de cada conservante en los productos cosméticos.

Dentro del grupo de los ésteres del ácido p-hidroxibenzoico (parabenes), la concentración máxima permitida es de 0.4% para un sólo éster y del 0.8% para mezclas de ésteres, expresadas como ácido. Actualmente el CCSC ha propuesto reducir la concentración individual o conjunta del propilparaben y butilparaben a 0.19%, puesto que se han encontrado claras evidencias de su actividad estrogénica [77].

Para el triclosán, la concentración máxima permitida en los productos cosméticos es del 0.3%, aunque es una sustancia que está continuamente vigilada. El CCSC ya ha adoptado la propuesta de la European Cosmetics Association (COLIPA) del 22 de Marzo del 2011 sobre el triclosán, por lo que la concentración de 0.3% no se considera segura para el consumidor debido, sobre todo, a la magnitud de la exposición acumulativa derivada del uso de distintos productos de permanencia en piel como lociones hidratantes o aerosoles [90]. Por otro lado, se propone como concentración límite segura de entre 0.15 o 0.2% para los enjuagues bucales puesto que el actual límite se considera peligroso desde una perspectiva toxicológica.

En lo referente a IPBC y a sus restricciones en los distintos productos cosméticos de la UE, el Reglamento 1223/2009 establece distintos valores en función del uso del cosmético. Así, en los productos de aclarado, la concentración máxima

permitida es de 0.02%, en productos de permanencia prolongada en la piel es del 0.01% y en desodorantes y antitranspirantes es del 0.0075%. Por otro lado, según este Reglamento esta sustancia no puede estar presente en productos bucales, labiales, ni en lociones y cremas corporales (productos destinados a ser aplicados en grandes extensiones corporales). Respecto a la seguridad infantil, el IPBC no puede estar presente en productos destinados a niños menores de 3 años, excepto en productos de baño, gel de ducha y champú (0.02%) [2].

Para los conservantes bromados, bronopol y bronidox, la concentración máxima permitida en productos cosméticos es de 0.1% en la UE (en el caso del bronidox solamente se autoriza en productos de aclarado), pero siempre que el fabricante garantice que no es posible la formación de nitrosaminas al entrar en contacto estos compuestos con las distintas sustancias presentes en los productos.

1.4. Otros compuestos alergénicos: dimetilfumarato, benzotiazol y 4-terbutilfenol

1.4.1 Definición

Es muy común encontrar en los hogares distintos productos que aparentemente son inocuos, pero que provocan serios daños en la salud de los consumidores.

Esto se debe a que muchos productos están acompañados de distintas sustancias que son potencialmente peligrosas para la salud del consumidor. Un buen ejemplo de este tipo de sustancias son el dimetilfumarato (DMF), benzotiazol (BT) y el 4-tert-butilfenol (TBP) (tabla 5). La presencia de estas sustancias no suele estar indicada en los distintos productos ya que no forman parte de la formulación de los mismos.

El DMF (éster insaturado del ácido fumárico) es un sólido estable de color blanco cristalino, muy volátil y lipofílico.

El BT es un compuesto organosulfurado de la familia de los tiazoles, líquido y de color amarillo.

El TBP, con estructura de alquilfenol, es un sólido estable de color blanco.

Estas tres sustancias tienen en común su uso como fungicidas o biocidas, que pueden provocar alta irritabilidad de la piel, los ojos y el tracto respiratorio [91-94].

Tabla 4. *Número CAS, peso molecular, coeficiente de partición octanol-agua (log Kow) y estructura los compuestos estudiados.*

Compuesto	Número CAS	PM	Log K_{ow}	Estructura
Dimetilfumarato DMF	624-49-7	144.13	0.62	
Benzotiazol BT	95-16-9	135.19	2.01	
p-Tert-butilfenol TBP	98-54-4	150.22	3.29	

1.4.2 Aplicaciones

El DMF es un poderoso fungicida usado habitualmente como protector contra en moho que puede aparecer en el calzado, prendas de piel, muebles y otros muchos productos, generalmente durante el transporte o almacenamiento en los climas cálidos y húmedos. Esta protección se consigue habitualmente introduciendo dentro de los productos o de sus envoltorios pequeñas bolsas con la sustancia.

La producción del BT en Europa supera las 30000 toneladas anuales, por lo que se considera un compuesto de producción elevada (HPV, por las siglas en inglés). La mayor parte de la producción de BT se usa industrialmente como acelerante en procesos de vulcanización de caucho, actuando también, como inhibidor de los procesos de corrosión [94,95].

Por otro lado, el BT también se emplea como biocida en la fabricación de papel y de productos de piel [92,93]. Debido a las propiedades de este compuesto como inhibidor de la corrosión, también se puede encontrar en la fabricación de líquidos refrigerantes o anticongelantes [96].

La producción mundial del p-tert-butilfenol, TBP, es de miles de toneladas anuales [86,97], por ello se considera también como un compuesto de HPV.

El TBP es buen estabilizador UV e inhibidor de la corrosión [91]. Por estas características el TBP se emplea en la fabricación de caucho, insecticidas, diversos materiales de construcción, pegamentos impermeables (para piel, zapatos, muebles), madera contrachapada, fibra de vidrio, etc.[86,91]

1.4.3 Distribución y toxicidad

Anteriormente se comentó la presencia del DMF sobre todo en pequeñas bolsas que acompañan a muchos productos. En la Unión Europea, la presencia del DMF en cualquier producto, se considera como un riesgo grave para la salud [1].

Esta sustancia penetra a través de la ropa hasta la piel [99] causando una dolorosa dermatitis de contacto acompañada de picores, irritación, quemaduras, e incluso problemas respiratorios agudos y riesgo de lesiones oculares graves [1,99].

La dermatitis causada por el DMF es muy difícil de tratar, lo que provocó que la UE, a través de la Directiva 98/8/EC [100], prohibiese todos aquellos productos que en su fabricación estuviese presente el DMF. Lo que no impedía esta Directiva, es la entrada de este tipo de productos desde países que no perteneciesen a la UE. Ante la alarma causada por el "síndrome del sofá tóxico", provocado por la importación de productos fabricados en Asia y conservados con DMF, la UE prohibió en Marzo del 2009 [1] la importación de cualquier producto tratado o conservado con DMF. De esta forma, se pretendía evitar la posible entrada de productos con esta sustancia en los países de la UE y, consecuentemente, proteger a los consumidores europeos. Esta Decisión del 17 de Marzo de 2009 implicó la retirada del mercado de todos los productos con DMF y también la exigencia de la recuperación de todos los productos estuviesen en manos de los consumidores [1].

Actualmente está prohibición sigue vigente hasta Marzo de 2012 a través de la Decisión del 1 de Marzo de 2011 [101].

En lo referente al BT, sus efectos sobre la salud no son tan nocivos como los del DMF, pero también es muy tóxico por inhalación, pudiendo provocar fuertes irritaciones en la piel, ojos y aparato respiratorio. Es poco biodegradable y potencialmente tóxico para el medio acuático [94].

La principal vía de exposición al TBP es la inhalación del aerosol o contacto a través de la piel. Es irritante para la piel, ojos, tracto respiratorio y también es responsable de fuertes decoloraciones de la piel [86]. Se considera disruptor endocrino [102] y se ha probado que provoca cáncer de estómago en animales [86]. Esta última observación, parece estar de acuerdo con las propiedades claramente cancerígenas del 2(3)-tert-butil-metoxifenol, sustancia con una estructura muy similar al TBP.

Como se puede desprender de todo lo expuesto anteriormente parece evidente la necesidad de desarrollar medidas de control, incluyendo procedimientos de análisis fiables y sensibles, que permitan garantizar la seguridad del consumidor.

2. TÉCNICAS DE PREPARACIÓN DE MUESTRA

El objetivo de la etapa de tratamiento de muestra en el proceso de análisis, es separar de la matriz los distintos compuestos presentes en una forma química adecuada para su posterior análisis.

En realidad esta etapa suele ser un paso crítico y fundamental a la hora de diseñar el proceso analítico. De la decisión de cómo tratar la muestra, dependerá todo el desarrollo de la metodología analítica que se quiera emplear para la determinación de los compuestos objetivo.

En primer lugar, es necesaria una etapa de extracción de los analitos desde la matriz. En este paso se puede utilizar disolventes o instrumentación compatibles con los sistemas de separación y determinación, orientando de esta forma la etapa de tratamiento de la matriz hacia las siguientes fases del método analítico. Se puede aprovechar esta etapa de tratamiento, para la eliminación de especies o sustancias interferentes, obteniendo de esta forma un extracto "limpio" y compatible con el método de determinación propuesto.

Habitualmente para conseguir un nivel medible instrumentalmente, son necesarios pasos intermedios de concentración o transformación química de los analitos en especies compatibles con los sistemas de determinación empleados: derivatización, modificación del pH, etc.…

En la actualidad existe una tendencia creciente a diseñar o desarrollar técnicas de preparación de muestra sencillas y compatibles con la automatización, que permitan sustituir a las técnicas clásicas consiguiendo reducir tiempo y coste de los análisis, minimizando el uso de disolventes orgánicos y de residuos (en línea con los principios de la Química Verde).

En los trabajos desarrollados en la presente Tesis se han seleccionado técnicas como la microextracción en fase sólida (SPME), extracción en fase sólida (SPE), extracción con disolventes presurizados (PSE) y extracción asistida por ultrasonidos (US).

2.1 MICROEXTRACCIÓN EN FASE SÓLIDA (SPME)

La SPME (Solid-Phase Microextraction) es una técnica de tratamiento de muestra rápida y que no requiere el uso de disolventes. La base de esta técnica consiste en la extracción desde la muestra de los analitos objetivo mediante una fibra de sílice fundida recubierta de una fase estacionaria polimérica ad/absorbente. Una vez extraídos los analitos, se procede a la desorción térmica de los mismos en el puerto de inyección de un cromatógrafo. Esta desorción también se puede realizar en un disolvente orgánico que posteriormente se introduce en un sistema cromatográfico [103].

Se trata de una técnica sencilla y rápida con aplicación a muestras sólidas, líquidas y gaseosas, empleada principalmente en el campo medioambiental, aunque actualmente se está abriendo paso en otros campos como el de análisis clínico.

El soporte donde va alojada la fibra de sílice es muy similar a una jeringuilla manual, básicamente se trata de un émbolo que extrae o retrae la fibra. En la figura 2 se muestra el detalle del soporte comercializado por Supelco.

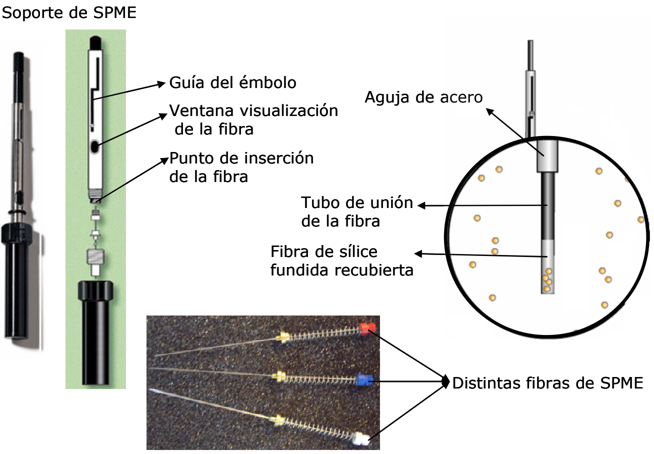


Figura 2. Dispositivo de SPME (Supelco).

La fibra de sílice fundida es químicamente inerte y estable a altas temperaturas. Está recubierta de un polímero con características ab/adsorbentes dando lugar a una geometría cilíndrica que se ubicada dentro de una aguja protectora de acero inoxidable.

Esta técnica de extracción presenta importantes ventajas frente a otras técnicas clásicas. Destaca principalmente por la ausencia de disolventes orgánicos, aunque alguna aplicación puede requerir pequeñas cantidades de disolvente (del orden de µL). Se trata de una técnica fácilmente automatizable, muy sensible y necesita poco volumen de muestra.

Con esta técnica las etapas de muestreo, extracción y concentración se reducen a un único paso, convirtiéndola en una técnica rápida, sencilla y de bajo coste.

La extracción mediante SPME, no es una extracción completa de los analitos como ocurre con las técnicas clásicas, sino que se extrae una pequeña cantidad de analito hasta alcanzar el equilibrio de distribución entre las distintas fases implicadas [103].

Existen dos formas básicas de realizar el muestreo mediante SPME [103]:

- Extracción por inmersión o directa (DSPME): en este modo de muestreo la fibra se introduce directamente dentro de la muestra ocurriendo una migración de los analitos desde la muestra a la fase extractante de la fibra. Este modo de extracción se recomienda para matrices acuosas no muy complejas. En esta modalidad de muestreo, la fibra puede deteriorarse fácilmente con la muestra, por lo que se puede realizar la extracción utilizando una membrana de protección de la fibra.
- Extracción en espacio de cabeza (HSSPME): la fibra se expone al espacio de cabeza sobre la muestra, de modo que ocurre la extracción de los analitos que han migrado desde la muestra a la fase gaseosa del espacio de cabeza. Este modo de muestreo está indicado para la determinación de los compuestos más volátiles que puede tener una muestra o para aquellas muestras que debido a su complejidad, necesiten modificadores de pH, digestiones, o cualquier otro tipo de tratamiento. Por otro lado, la aplicación y ventajas de la técnica en el análisis de diversas familias de compuestos semivolátiles han sido a su vez claramente demostradas [11,104,105].

La extracción por SPME es un proceso basado en el equilibrio que se puede favorecer optimizando distintas variables.

- Por un lado debemos de elegir el **recubrimiento polimérico** de la fibra que tenga una mayor afinidad por los analitos. Actualmente existen varios recubrimientos comercializados por Supelco con distintos espesores de fase: polidimetilsiloxano (PDMS), poliacrilato (PA), polidimentilsiloxano/divinilbenceno, (PDMS/DVB), carbowax/polietilenglicol (CW/PEG), polidimetilsiloxano/carboxen (PDMS/CAR) y divinilbenceno/carboxen/polidimetilsiloxano (DVB/CAR/PDMS). Estos recubrimientos cubren un amplio rango de compuestos de distintas polaridades.

- La SPME es un proceso de equilibrio. Para alcanzar dicho equilibrio se necesita un **tiempo de extracción,** que dependerá de los compuestos a estudiar. La utilización de tiempos de extracción inferiores al equilibrio es posible siempre que se consiga la sensibilidad suficiente para la aplicación deseada. En este caso es necesario un control exhaustivo del tiempo de extracción [106].

- La **temperatura de extracción** habitualmente es una de las variables más importantes a la hora de optimizar el método de extracción, puesto que es un factor con doble efecto. Por un lado, el incremento de la temperatura favorece la migración de los analitos hacia la fibra, reduciendo el tiempo necesario para alcanzar el equilibrio. Por otro lado como la etapa de absorción es un proceso exotérmico, un aumento de temperatura en condiciones de equilibrio, implica una disminución de la cantidad de analito extraída.

- Usando modificadores del medio o de la matriz se puede favorecer el proceso de extracción de compuestos [103].

  Se puede modificar la **fuerza iónica** adicionando distintas sales, produciendo una disminución de la solubilidad de los analitos neutros en l agua, favoreciendo así su extracción.

  Con la **modificación del pH** se mejora la extracción de aquellos analitos que tienden a estar disociados en medio acuoso. Así, trabajando siempre

a pH por debajo (en especies ácidas) o por encima (en especies básicas) del pKa, se extraerán los analitos en la forma más favorable: sin disociar.

La **adición de agua o disolventes orgánicos** a muestras sólidas, facilita la liberación de analitos desde la matriz y mejora la difusión hacia el recubrimiento de la fibra.

-La **agitación** de la muestra incrementa normalmente la difusión de los analitos desde la muestra al espacio de cabeza o a la fibra, disminuyendo el tiempo de extracción.

-Otras variables que intervienen en la extracción son el **volumen de muestra** o volumen del **espacio de cabeza**. Los volúmenes de muestra elevados favorecen la extracción de compuestos con coeficientes de distribución elevados y los volúmenes de espacio de cabeza pequeños provocan una concentración de los analitos en el mismo con el consecuente aumento de sensibilidad.

En esta Tesis, la SPME se ha combinado con la SPE para realizar el muestreo y determinación de fragancias alergénicas en aire interior. Esta aproximación había sido desarrollada previamente por nuestro grupo de investigación, demostrándose su utilidad para la determinación de diversas familias de contaminantes orgánicos [105,107,108]. Es este caso la SPE se emplea para la toma de muestra de aire (figura 3), quedando los analitos retenidos en el adsorbente del dispositivo.

Una vez retenidos los distintos compuestos en el adsorbente (cartucho de SPE), se procede a su extracción por SPME para su posterior análisis.

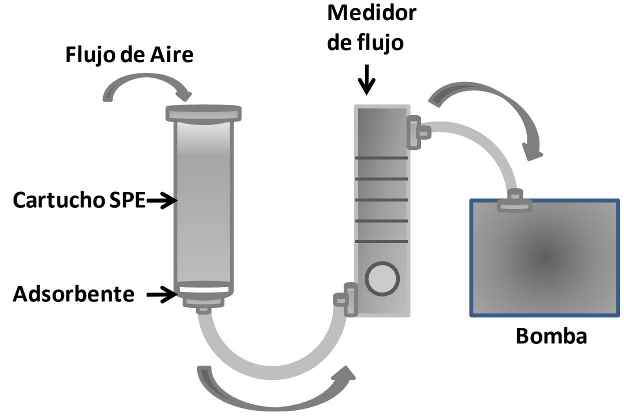


Figura 3. Descripción de un montaje de muestreo de aire

2.2 EXTRACCIÓN CON DISOLVENTES PRESURIZADOS (PSE)

La extracción con disolventes presurizados (pressurized solvent extraction, PSE) se conoce también como extracción con fluidos presurizados (PFE) o por los nombres comerciales de extracción acelerada por disolventes (ASE™, Dionex, Sunnyvale, CA, USA) o extracción con líquidos presurizados (PLE™, Fluid Management Systems, Inc., MA, USA).

La extracción mediante esta técnica se realiza a temperaturas y presiones elevadas; de esta forma es posible trabajar con disolventes en fase líquida a temperaturas superiores a su punto de ebullición. Con este modo de trabajo se consiguen tiempos de extracción muy inferiores a los clásicos (Soxhlet por ejemplo) y se reduce el volumen de disolvente empleado en la extracción con la consecuente disminución del factor de dilución de la muestra. Es esta última característica la que convierte la PSE en una técnica de extracción "verde" debido al bajo volumen de disolvente orgánico empleado en la extracción [109].

El objetivo que se persigue al trabajar a temperaturas elevadas es incrementar la capacidad de los disolventes para solubilizar los distintos analitos y aumentar las velocidades de difusión de los mismos. Además, las temperaturas elevadas favorecen la ruptura de las fuertes interacciones soluto-matriz y

disminuyen la viscosidad del disolvente, facilitando de esta forma la penetración del disolvente en seno de la matriz.

Para que las extracciones a temperaturas elevadas sean eficaces, es necesario que el disolvente permanezca en estado líquido. Esto se consigue elevando la presión dentro del sistema. Con este aumento de presión también se favorece la extracción de los analitos desde los poros de la matriz y la solubilización de las burbujas de aire que pueden impedir el contacto de los analitos con el disolvente.

En la figura 4 se muestra un esquema de un equipo de extracción de PSE de la marca DIONEX (ASE 200). Básicamente consta de una bomba para impulsar disolvente (similar a las de HPLC) a través de una celda donde se encuentra la muestra. Esta celda se puede presurizar hasta 3000 psi y se encuentra ubicada dentro de un horno que permite termostatizarla hasta 200 ºC. Finalmente, el extracto se recoge en un vial colector [110] .

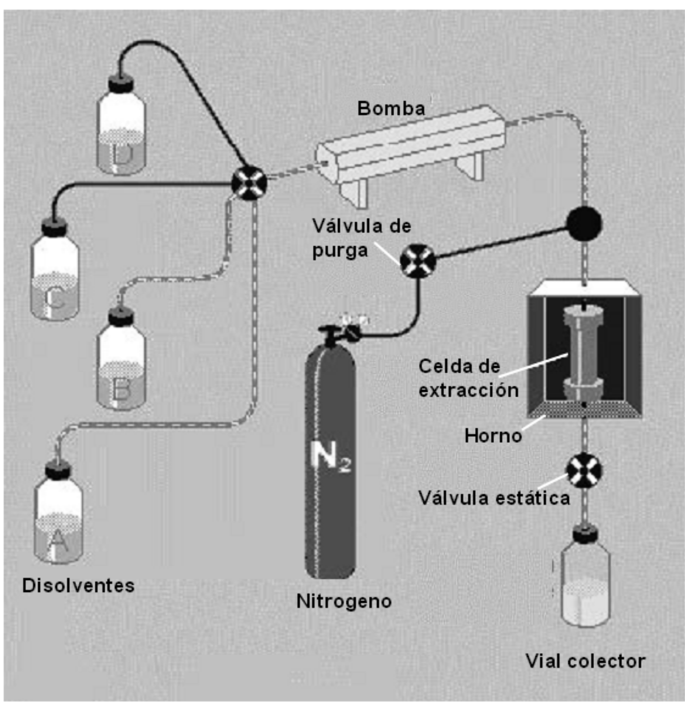


Figura 4. Esquema equipo de extracción PSE (ASE) [111].

Los pasos a seguir para llevar a cabo una extracción PSE son los siguientes:

1. Preparación de la muestra para su introducción en la celda. En este paso la muestra debe ser homogenizada en el tamaño de partícula y debe estar seca.
2. La muestra se introduce en la celda de extracción. En función de la cantidad de muestra a analizar se puede variar el tamaño de la celda (1, 5, 11, 22 o 33 mL). Este tamaño de celda está correlacionado con el volumen de extracto obtenido. Por otro lado, dentro de la celda se pueden combinar distintos adsorbentes cuya función es la de limpieza *in situ* del extracto (arena, alúmina, florisil, sílica, etc.). Es importante llenar completamente la celda para obtener un menor volumen de extracto y que éste sea reproducible. Para ello se suele utilizar arena o tierra de diatomeas.
3. La celda se coloca en el sistema y comienza el calentamiento y llenado con disolvente. Los disolventes más comunes son: acetato de etilo, metanol, acetonitrilo, hexano, acetona, etc.
4. El siguiente paso es una etapa, normalmente estática, en la que el disolvente está un tiempo determinado en contacto con la muestra a la temperatura y presión escogida.
5. Finalmente, se lleva a cabo la extracción recogiendo todo el extracto en un vial colector.

El extracto obtenido puede ser directamente analizado, aunque dependiendo de la técnica de determinación empleada, pueden ser necesarios tratamientos posteriores sencillos como la filtración, derivatización, concentración, etc.

2.3 EXTRACCIÓN ASISTIDA POR ULTRASONIDOS (US)

Los ultrasonidos (US) son ondas acústicas cuya frecuencia es inaudible para el oído humano. Esta técnica de extracción se caracteriza por ser sencilla, rápida y de bajo coste, con buenos resultados en la extracción de muestras sólidas y líquidas. Existen numerosas ventajas y aplicaciones de dicha técnica que se han recogido y analizado exhaustivamente en la bibliografía [6,112-120].

Esta técnica se basa en el uso de un disolvente orgánico al que se le aplica energía de ultrasonidos, para llevar a cabo la extracción de muestras sólidas o líquidas. La aplicación de ultrasonidos provoca una agitación continua de la muestra en el disolvente, facilitando de este modo los procesos de transferencia de masa entre ambas fases al haber una mayor penetración del disolvente en las distintas matrices. Como resultado se obtienen extracciones muy eficaces en periodos muy cortos de tiempo [6,121].

Los ultrasonidos viajan a través del disolvente en forma de onda. Lo hacen de forma alternada provocando contracciones y expansiones de dicho medio. Durante los ciclos de expansión se produce un aumento negativo de la presión formándose burbujas o cavidades (fenómeno que se conoce como cavitación), que terminan explotando y liberando energía [122]. Esta energía liberada provoca un aumento de la temperatura que facilita la solubilidad de los analitos. Este aumento de temperatura, junto con las elevadas presiones alcanzadas, provocan una mayor penetración del disolvente en la muestra [123].

El instrumental más común para realizar la extracción por ultrasonidos consta de un baño de agua sobre el que se aplica siempre la misma frecuencia de US (en torno a unos 40 KHz, dependiendo del fabricante), con control del tiempo y también puede estar dotado de control de temperatura (figura 5).

Como alternativa al baño de ultrasonidos está la sonda de ultrasonidos. Ésta permite regular la frecuencia de la energía de ultrasonidos y se puede introducir directamente dentro de la muestra; de esta forma se pueden realizar extracciones más energéticas en aquellas aplicaciones que así lo requieran.

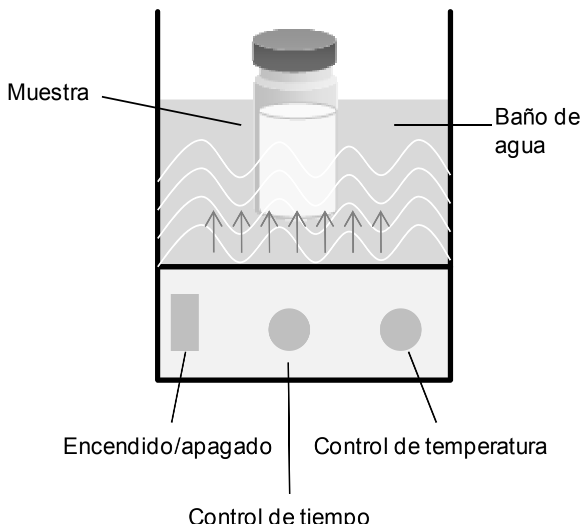


Figura 5. Esquema de un baño de ultrasonidos convencional

3. TÉCNICAS DE DETERMINACIÓN

La cromatografía de gases es una técnica analítica que actualmente se usa de forma rutinaria en cualquier tipo de laboratorio de control y o de investigación y desarrollo.

El fundamento de esta técnica cromatográfica es la separación de los distintos componentes de una mezcla debido a la distribución de los mismos entre una fase estacionaria, que puede ser líquida o sólida, y una fase móvil o gas portador. De las dos modalidades la más usada y extendida es en la que interviene la fase estacionaria líquida. El resultado es una técnica con una alta resolución, sensibilidad y selectividad, características ampliamente reconocidas [124].

Esta técnica es la adecuada para la separación y determinación de compuestos volátiles o semivolátiles, térmicamente estables y no susceptibles a la adsorción o descomposición en el soporte sólido de la columna. Por otro lado, la aplicación de esta técnica se puede extender a compuestos que no cumplan con alguno de los requisitos anteriores, mediante reacciones de derivatización [124].

En esta Tesis se ha llevado a cabo la separación de los distintos analitos en columnas cromatográficas con tres fases estacionarias distintas: DB-WAX (100% polietilen glicol), VF-1701 (14% cianopropil fenil – 86% polidimetilsiloxano), y HP5 (5% fenil – 95% polidimetilsiloxano). Como sistemas de detección se ha usado el detector de captura de electrones (ECD) y el detector de espectrometría de masas (MS).

3.1 Cromatografía de gases con detección de captura electrónica (GC/ECD).

Este tipo de detector es muy selectivo y especialmente sensible a moléculas que contienen grupos halogenados, carbonilos conjugados, metilo, nitro y compuestos organometálicos [124].

El sistema consta de una fuente radiactiva de ^{63}Ni que emite radiación β que colisiona contra las moléculas de gas portador, generalmente nitrógeno, ionizándolas. De este modo se genera una corriente de electrones entre dos electrodos que da lugar a la señal constante del detector. Cuando llegan al detector

moléculas capaces de captar electrones, se produce una disminución del flujo de electrones, lo que se traduce en una señal analítica [125].

El detector de captura electrónica empleado en el presente trabajo de Tesis (μECD) se caracteriza por tener una celda de detección 10 veces inferior a las celdas tradicionales. Esto implica volúmenes inferiores de gas dentro de la celda, que se traduce en una menor dilución de los analitos dentro de la misma lo que conlleva un aumento de la sensibilidad.

3.2 Cromatografía de gases con detección de espectrometría de masas (GC/MS).

La asociación de la cromatografía de gases con los detectores de espectrometría de masas (GC/MS) proporciona una herramienta muy potente para la identificación de compuestos en muestras complejas [124].

Esta combinación se ha convertido en la actualidad en una de las herramientas analíticas más potentes. Se caracteriza por ser universal, específica y muy rápida para la determinación de multitud de analitos. Por otro lado, aporta fiabilidad a los resultados obtenidos al proporcionar espectros de masas de cada componente dando lugar a su correcta identificación. Estos espectros también aportan información estructural de las distintas sustancias desconocidas presentes en las muestras [126].

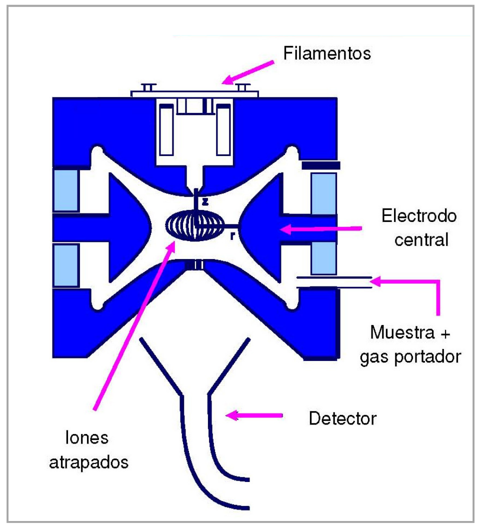
Actualmente es muy frecuente encontrar en laboratorios de rutina equipos cromatográficos acoplados con los principales detectores de espectrometría de masas: la trampa de iones y el cuadrupolo. El funcionamiento de ambos detectores está basado en el mismo principio: modifican la trayectoria de los iones variando la radiofrecuencia aplicada. En las figuras 6 y 7 se muestra un esquema de una trampa de iones y de un cuadrupolo, respectivamente.

La trampa de iones es un detector que funciona en el tiempo, funcionamiento pulsado. Este proceso se realiza en cuatro fases que se desarrollan dentro de la trampa:

1. Ionización: mediante electrones energéticos.
2. Almacenamiento de iones: hasta alcanzar la capacidad óptima de la trampa. Calculado por el software.
3. Barrido de masas: incrementando el voltaje del electrodo central se expulsan los iones desde m/z bajas a altas.
4. Detección: en el electromultiplicador. Amplifica los iones estabilizados en la trampa.

El cuadrupolo es un detector que funciona en el espacio, funcionamiento continuo. El proceso se lleva a cabo de forma secuencial, las distintas partes están separadas y diferenciadas.

1. Ionización: se produce en una fuente externa.
2. Enfoque de los iones (lentes): los iones son inmediatamente extraídos de la fuente, enfocados y acelerados hacia el campo cuadrupolar.
3. Analizador cuadrupolar: “Filtra” las masas para una transmisión selectiva. La selección de diferentes combinaciones de potenciales de radiofrecuencia (RF) permiten la transmisión estable de un rango de m/z. Los iones con movimientos inestables se pierden, chocando contra los cuadrupolos negativos ó contra las paredes del analizador.
4. Detección: en el electromultiplicador. Amplifica los iones que pasan a través del analizador.



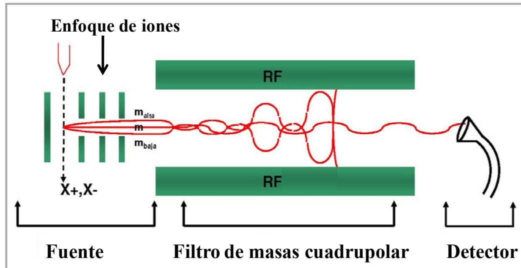


Figura 6. Esquema de una trampa de iones

Figura 7. Esquema correspondiente a un cuadrupolo

4. BIBLIOGRAFÍA

1. Commission Decision of 17 March 2009 requiring Member States to ensure that products containing the biocide dimethylfumarate are not placed or made available on the market (notified under document number C(2009) 1723). Official Journal of the European Union.

2. Reglamento (CE) no 1223/2009 del Parlamento Europeo y del Consejo de 30 de noviembre de 2009 sobre los productos cosméticos (versión refundida). Diario Oficial de la Unión Europea L342 (2009) 59.

3. The European Cosmetics Association, COLIPA. Activity Report 2010, Science, Beauty and Care Innovating for a Sustainable Future,.

4. Directiva del Consejo (76/768/CEE) de 27 de julio de 1976 relativa a la aproximación de las legislaciones de los Estados miembros en materia de productos cosméticos. Diario Oficial n° L 262 de 27/09/1976 p. 0169.

5. R.J.B. Peters, Phthalates and artificial musks in perfumes. TNO-report R&I-A R 2005/011 (2005).

6. J. Regueiro, M. Llompart, C. Garcia-Jares, J.C. Garcia-Monteagudo, R. Cela, J. Chromatogr. A 1190 (2008) 27.

7. R. Stringer, I. Labunska, D. Santillo, P. Johnston, J. Siddorn, A. Stephenson, Environ. Sci. Pollut. R. 7 (2000) 27.

8. U. Heudorf, V. Mersch-Sundermann, J. Angerer, Int. J. Hyg. Envir. Heal. 210 (2007) 623.

9. European council for plasticizers and intermediates, 2011. Plasticizers. http://www.ecpi.org (accesed July 03, 2011).

10. R. Benson, Regul. Toxicol. Pharm. 53 (2009) 90.

11. M. Polo, M. Llompart, C. Garcia-Jares, R. Cela, J. Chromatogr. A 1072 (2005) 63.

12. C. Garcia-Jares, J. Regueiro, R. Barro, T. Dagnac, M. Llompart, J. Chromatogr. A 1216 (2009) 567.

13. N.H. Kleinsasser, B.C. Wallner, E.R. Kastenbauer, H. Weissacher, U.A. Harréus, Teratogen. Carcin. Mut. 21 (2001) 189.

14. Reglamento (UE) nº 143/2011 de la Comisión de 17 de febrero de 2011 por el que se modifica el anexo XIV del Reglamento (CE) nº 1907/2006 del Parlamento Europeo y del Consejo, relativo al registro, la evaluación, la autorización y la restricción de las sustancias y preparados químicos (REACH). Diario Oficial de la Unión Europea. L 44/2 (2011).

15. J. Naarala, A. Korpi, Toxicol. Lett. 188 (2009) 157.

16. J. Borch, M. Axelstad, A.M. Vinggaard, M. Dalgaard, Toxicol. Lett. 163 (2006) 183.

17. B. Bridges, Flavour. Frag. J. 17 (2002) 361.

18. Reglamento (CE) Nº 1907/2006 del Parlamento Europeo y del Consejo de 18 de diciembre de 2006 relativo al registro, la evaluación, la autorización y la restricción de las sustancias y preparados químicos (REACH), por el que se crea la Agencia Europea de Sustancias y Preparados Químicos. Diario Oficial de la Unión Europea (2006). Diario Oficial de la Unión Europea L 136/3.

19. Environmental Protection Agency, EPA, Fragrance Ingredient List. (2011). http://www.epa.gov/opprd001/inerts/fmaingredient.pdf. (accesed July 19, 2011).

20. M.A. Kamrin, J. Toxicol. Env. Health. Part B 12 (2009) 157.

21. S.M. Duty, A.M. Calafat, M.J. Silva, L. Ryan, R. Hauser, Hum. Reprod. 20 (2005) 604.

22. C. Sommer, The role of musk and musk compounds in the fragrance industry. The Handbook of Environmental Chemistry Part X, vol. 3 (2004) 1.

23. R.A. Ford, B. Domeyer, O. Easterday, K. Maier, J. Middleton, Regul. Toxico. Pharm. 31 (2000) 166.

24. G.G. Rimkus, M. Wolf, Chemosphere 33 (1996) 2033.

25. T. Heberer, Acta. Hydroch. Hydrob. 30 (2002) 227.

26. H.P. Hutter, P. Wallner, W. Hartl, M. Uhl, G. Lorbeer, R. Gminski, V. Mersch-Sundermann, M. Kundi, Int. J. Hyg. Envir. Heal. 213 (2010) 124.

27. R. Gatermann, H. Hühnerfuss, G. Rimkus, A. Attar, A. Kettrup, Chemosphere 36 (1998) 2535.

28. G.G. Rimkus, R. Gatermann, H. Hühnerfuss, Toxicol. Lett. 111 (1999) 5.

29. G.G. Rimkus, Toxicol. Lett. 111 (1999) 37.

30. A.M. Peck, K.C. Hornbuckle, Environ. Sci. Technol. 38 (2004) 367.

31. G. Rimkus, B. Rimkus, M. Wolf, Chemosphere 28 (1994) 421.

32. S. Apostolidis, T. Chandra, I.C.J. Demirhan, H.W. Doerr, A. Chandra, Anticancer Res. 22 (2002) 2657.

33. N. Bitsch, C. Dudas, W. Körner, K. Failing, S. Biselli, G. Rimkus, H. Brunn, Arch. Environ. Con. Tox. 43 (2002) 0257.

34. D. Opdyke, Food Cosmet. Toxicol. 17 (1979) 357.

35. Opinion of the SCCNFP intended for Consumers concerning 6-acetyl-1,1,2,4,4,7-hexamethyl tetraline (AHTN). SCCNFP/0609/02, final (2002).

36. Oslo and Paris Commission (OSPAR). Background Document on musk xylene and other musks. Series Point and Diffuse Sources no. 101. (2004).

37. Commission Directive 2008/42/EC of 3 April 2008 amending Council Directive 76/768/EEC, concerning cosmetic products, for the purpose of adapting

Annexes II and III thereto to technical progress. Official Journal of the European Union L 93/13 (2008).

38. D. Salvito, R. Senna, C. McIntosh, Fragrance materials and the environment - is there a risk?. SETAC Globe (Learned Discourses section) (2006) 42.

39. Opinion of the SCCNFP intended for fragrance allergy in consumers. A review of the problem. Analysis of the need for appropriate consumer information and identification of consumer allergens. SCCNFP/0017/98 Final (1999).

40. Opinion of the SCCNFP intended for Consumers concerning atranol and chloroatranol present in natural extracts (e.g. Oak moss and Tree Moss extracts), SCCP/0847/04 adopted by the SCCP on 7 December 2004. EU Commission (2004).

41. Decisión de la Comisión (2008/721/CE) de 5 de agosto de 2008 por la que se crea una estructura consultiva de Comités científicos y expertos en el ámbito de la seguridad de los consumidores, la salud pública y el medio ambiente y se deroga la Decisión 2004/210/CE. Diario Oficial de la Unión Europea L 241/21 (2008).

42. Research Institute for Fragance Materials, RIFM. The International Scientific Authority for the Safe Use of Fragrance Materials. http://www.rifm.org/about.php (accesed July 11, 2011).

43. J.D. Johnson, M.J. Ryan, J.D. Toft, S.W. Graves, M.R. Hejtmancik, M.L. Cunningham, R. Herbert, K.M. Abdo, J. Agric. Food Chem. 48 (2000) 3620.

44. Opinion of the Scientific Committee on Food on Methyleugenol (4-Allyl-1,2-dimethoxybenzene). European Commission Health & Consumer Protection Directorate-General Scientific Committee on Food (SCF) (2001).

45. A.C. De Groot, P.J. Frosch, Contact Dermatitis 36 (1997) 57.

46. J.D. Johansen, Am. J. Clin. Dermatol. 4 (2003) 789.

47. K. Rumchev, J. Spickett, M. Bulsara, M. Phillips, S. Stick, Thorax 59 (2004) 746.

48. C. Shim, M.H. Williams Jr, Am. J. Med. 80 (1986) 18.

49. A. Farrow, H. Taylor, K. Northstone, J. Golding, Arch. Environ. Health. 58 (2003) 633.

50. J. Elberling, A. Linneberg, A. Dirksen, J.D. Johansen, L. Frølund, F. Madsen, N.H. Nielsen, H. Mosbech, Clinical & Experimental Allergy 35 (2005) 75.

51. Vigesimosexta Directiva 2002/34/CE de la Comisión de 15 de abril de 2002 por la que se adaptan al progreso técnico los anexos II, III y VII de la Directiva 76/768/CEE del Consejo relativa a la aproximación de las legislaciones de los Estados miembros en materia de productos cosméticos. Diario Oficial de las Comunidades Europeas L 102/19 (2002).

52. W. Ding, D.D. Levy, M.E. Bishop, L.E. Lyn-Cook, R. Kulkarni, C.-W. Chang, A. Aidoo, M.G. Manjanatha, Toxicol. Sci. 123(1) (2011) 103.

53. National Toxicology Program, TR-422.Toxicology and carcinogenesis studies of coumarin (CAS No. 91-64-5) in F344/N rats and B6C3F1 mice (gavage studies) http://ntp.niehs.nih.gov/ntp/htdocs/LT_rpts/tr422.pdf, U.S Departament of Health and Human Services, National Institute of Health (NIH U.S). (1993).

54. A.A. Geldof, C. Engel, B.R. Rao, Urol. Res. 20 (1992) 139.

55. K. Koistinen, D. Kotzias, S. Kephalopoulos, C. Schlitt, P. Carrer, M. Jantunen, S. Kirchner, J. McLaughlin, L. Mølhave, E.O. Fernandes, B. Seifert, Allergy 63 (2008) 810.

56. New Jersey Department of Health and Senior Services, DHSS, list of hazardous substance fact sheets. http://www.state.nj.us/health/eoh/rtkweb/documents/factsheetlist.pdf. (accesed July 19, 2011).

57. C.G. Daughton, T.A. Ternes, Environ. Health. Persp.107 Suppl 6 (1999) 907.

58. F. Giordano, R. Bettini, C. Donini, A. Gazzaniga, M.R. Caira, G.G.Z. Zhang, D.J.W. Grant, J. Pharm. Sci. 88 (1999) 1210.

59. L. Juergensen, J. Busnarda, P.-Y. Caux, R. Kent, Environ. Toxicol. 15 (2000) 201.

60. R. Smith, K. Gaglani, Antifungal compositions of 3-iodpropynyl-N-n-butylcarbamate and 2-n-N-butyl-1,2-benzisothiazolin-3-one and methods for use in industrial applications. From U.S. Pat. Appl. Publ. (2011), US 20110077278 A1 20110331.

61. A.P. Farrell, E. Stockner, C.J. Kennedy, Arch. Environ. Con. Tox. 35 (1998) 472.

62. Environmental Protection Agency, EPA, Registration Eligibility Decision (RED) 3-Iodo-2-propynylbutylcarbamate (IPBC), EPA 738-R-97-003 (1997). http://www.epa.gov/oppsrrd1/REDs/2725red.pdf (accessed July 13, 2011)

63. H. Singer, S. Müller, C. Tixier, L. Pillonel, Environ. Sci. Technol. 36 (2002) 4998.

64. M. Ghannoum, M. Thomson, W. Bowman, S. Al-Khalil, Folia Microbiol. 31 (1986) 19.

65. M.-H. Yang, H.-J. Lin, Y.-M. Choong, Food. Res. Int. 35 (2002) 627.

66. Organisation for Economic Cooperation and Development (OECD), Screening Information Data Set (SIDS). Initial Assessment Report: 2,6-DI-TERT-BUTYL-P-CRESOL (BHT). SIDS Initial Assessment Meeting (SIAM) 14, Paris, France, 26-28 March 2002.

67. S. Polati, F. Gosetti, M.C. Gennaro, S. Amparo, C. Alberto, C. Amparo Salvador, T. Alan, in Analysis of Cosmetic Products, Elsevier, Amsterdam, 2007, p. 211.

68. C. Charnock, T. Finsrud, J. Clin. Pharm. Ther.32 (2007) 567.

69. B.D. Andrea, A. Hontela, J. Appl. Toxicol. 31 (2011) 285.

70. K.H.G. Freitas, O. Fatibello-Filho, Talanta 81 (2010) 1102.

71. DHI Water and Environment. Study on Enhancing the Endocrine Disrupter Priority List with a Focus on Low Production Volume Chemicals. Revised Report to DG Environment. Hersholm, Denmark: DHI, 2007. (2007).

72. R.S. Lanigan, Int. J. Toxicol. 17 (S5) (1998) 1.

73. P.D. Darbre, A. Aljarrah, W.R. Miller, N.G. Coldham, M.J. Sauer, G.S. Pope, J. Appl. Toxicol. 24 (2004) 5.

74. M. Lores, M. Llompart, L. Sanchez-Prado, C. Garcia-Jares, R. Cela, Anal. Bioanal. Chem. 381 (2005) 1294.

75. M.T. Matyska, J.J. Pesek, L. Yang, J. Chromatogr. A 887 (2000) 497.

76. Cosmetic Ingredient Review Safety of Triclosan as a Preservative in Cosmetics, http://www.cir-safety.org/newreports.shtml (accessed July, 2010).

77. The European Cosmetics Association, COLIPA, OPINION on Parabens, n° P82 SCCS/1348/10, Revision 22 March. European Union (2011).

78. J.V. Rodricks, J.A. Swenberg, J.F. Borzelleca, R.R. Maronpot, A.M. Shipp, Cr. Rev. Toxicol.40 (2010) 422.

79. M. Allmyr, F. Harden, L.-M.L. Toms, J.F. Mueller, M.S. McLachlan, M. Adolfsson-Erici, G. Sandborgh-Englund, Sci. Total. Environ. 393 (2008) 162.

80. C. Queckenberg, J. Meins, B.l Wachall, O. Doroshyenko, D. Tomalik-Scharte, B. Bastian, M. Abdel-Tawab, a.U. Fuhr, Antimicrob. Agents. Chemother. 54 (2010) 570.

81. M. Allmyr, M. Adolfsson-Erici, M.S. McLachlan, G. Sandborgh-Englund, Sci. Total. Environ. 372 (2006) 87.

82. K. Hell, E.R. Altwicker, L. Stieglitz, R. Addink, Chemosphere 40 (2000) 995.

83. C. Tixier, H.P. Singer, S. Canonica, S.R. Müller, Environ. Sci. Technol. 36 (2002) 3482.

84. K. Lintner, Global Regulatory Issues for the Cosmetics Industry. William Andrew Inc (2009) 31.

85. G. Hocman, Int. J. Biochem. 20 (1988) 639.

86. Organisation for Economic Cooperation and Development (OECD), Screening Information Data Set (SIDS): P-TERT-BUTYL PHENOL. SIDS Initial Assessment Meeting (SIAM) 10th (2000).

87. A.A.M. Botterweck, H. Verhagen, R.A. Goldbohm, J. Kleinjans, P.A. van den Brandt, Food Chem. Toxicol. 38 (2000) 599.

88. G.M. Williams, M.J. Iatropoulos, Cancer Lett. 104 (1996) 49.

89. Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) Assessment of the Antibiotic Resistance Effects of Biocides. European Commission (2009).

90. Scientific Committee on Consumer Safety, SCCS, OPINION on Triclosan, The European Cosmetics Association, COLIPA n° P32, ADDENDUM to the SCCP Opinion on Triclosan (SCCP/1192/08) from January 2009 (2011).

91. L. Badoil, D. Benanou, Anal. Bioanal. Chem. 393 (2009) 1043.

92. B.G. Brownlee, J.H. Carey, G.A. MacInnis, I.T. Pellizzari, Environ. Toxicol. Chem. 11 (1992) 1153.

93. A. Orlita, Int. Biodeter. Biodegr. 53 (2004) 157.

94. T. Reemtsma, O. Fiehn, G. Kalnowski, M. Jekel, Environ. Sci. Technol. 29 (1995) 478.

95. A. Kloepfer, M. Jekel, T. Reemtsma, Environ. Sci. Technol. 39 (2005) 3792.

96. C.M. Reddy, J.G. Quinn, Environ. Sci. Technol. 31 (1997) 2847.

97. A.V. Barse, T. Chakrabarti, T.K. Ghosh, A.K. Pal, S.B. Jadhao, Pestic. Biochem. Phys. 86 (2006) 172.

98. J.D.L. Williams, I.H. Coulson, P. Susitaival, S.M. Winhoven, Br. J. Dermatol. 159 (2008) 233.

99. Resolución de 22 de diciembre de 2008, del Instituto Nacional del Consumo, por la que se acuerda la publicación del acuerdo de iniciación de procedimiento para prohibir la comercialización de calzado, sillones y cualquier artículo que por su uso va a estar en contacto con la piel que contenga dimetilfumarato. Publicado en BOE, 21 Enero . Boletín Oficial de Estado Núm. 18 Sec. V-B. (2009) 5474.

100. Directiva 98/8/CE del Parlamento Europeo y del Consejo de 16 de febrero de 1998 relativa a la comercialización de biocidas. Diario Oficial de las Comunidades Europeas L 123/1 (1998).

101. Commission Decision of 1 March 2011 extending the validity of Decision 2009/251/EC requiring Member States to ensure that products containing the biocide dimethylfumarate are not placed or made available on the market (notified under document C(2011) 1174) (Text with EEA relevance) (2011/135/EU). Official Journal of the European Union. (2011).

102. T.E. Haavisto, N.A. Adamsson, S.A. Myllymäki, J. Toppari, J. Paranko, Reprod. Toxicol. 17 (2003) 593.

103. Pawliszyn J., Wiley-VCH, New York (1997).

104. M. Fernandez-Alvarez, M. Llompart, J.P. Lamas, M. Lores, C. Garcia-Jares, R. Cela, T. Dagnac, J. Chromatogr. A 1188 (2008) 154.

105. J. Regueiro, C. Garcia-Jares, M. Llompart, J.P. Lamas, R. Cela, J. Chromatogr. A 1216 (2009) 2805.

106. H. Kataoka, H.L. Lord, J. Pawliszyn, J. Chromatogr. A 880 (2000) 35.

107. R. Barro, S. Ares, C. Garcia-Jares, M. Llompart, R. Cela, J. Chromatogr. A 1072 (2005) 99.

108. R. Barro, Optimización de nuevos métodos para la determinación de contaminantes orgánicos en aire. Tesis doctoral. Universidad de Santiago de Compostela (2006).

109. J.A. Mendiola, M. Herrero, A. Cifuentes, E. Ibañez, J. Chromatogr. A 1152 (2007) 234.

110. ASE® 200 Accelerated solvent extractor operator’s manual. Dionex Corporation Document No. 031149, Revision 04 (1999).

111. AutoASE® Software User’s Guide. Dionex Corporation. Document No. 031259, Revision 03 (2002).

112. I. Pumure, J.J. Renton, R.B. Smart, Chemosphere 78 (2010) 1295.

113. M.D. Luque de Castro, F. Priego-Capote, Anal. Chim. Acta 583 (2007) 2.

114. E. Becerril-Bravo, J. Pablo Lamas, L. Sanchez-Prado, M. Lores, C. Garcia-Jares, B. Jimenez, M. Llompart, Chemosphere 81 (2010) 1378.

115. J.-L. Capelo-Martínez, Anal. Bioanal. Chem. 395 (2009) 543.

116. M.D. Luque de Castro, F. Priego-Capote, Talanta 72 (2007) 321.

117. J. Regueiro, M. Llompart, E. Psillakis, J.C. Garcia-Monteagudo, C. Garcia-Jares, Talanta 79 (2009) 1387.

118. J.P. Bossio, J. Harry, C.A. Kinney, Chemosphere 70 (2008) 858.

119. J.L. Capelo, A.M. Mota, Curr. Anal. Chem.1 (2005) 193.

120. Environmental Protection Agency, EPA, method 3550C ultrasonic extraction (2007). http://www.epa.gov/osw/hazard/testmethods/sw846/pdfs/3550c.pdf. (accesed 19 July, 2011).

121. J.A. Pérez-Serradilla, F. Priego-Capote, M.D. Luque de Castro, Anal. Chem. 79 (2007) 6767.

122. W. Lauterborn, C.-D. Ohl, Ultrason. Sonochem. 4 (1997) 65.

123. M.D. Luque de Castro, F. Priego-Capote, Analytical Applications of Ultrasound . Elsevier, Amsterdam, The Netherlands. (2007).

124. L. Hernandez, C. González, Introducción al análisis instrumental. Capítulo 11, cromatografía de gases. Editorial Ariel, S.A. (2002).

125. D.A. Skoog, F.J. Holler, T. Nieman, Principios de análisis instrumental, Mc Graw Hill, 5th Ed, 2000.

126. C.F. Poole, J. Chem. Educ. 80 (2003) 883.

III. PARTE EXPERIMENTAL. RESULTADOS Y DISCUSIÓN

1. DETERMINACIÓN DE ADITIVOS TÓXICOS Y ALERGÉNICOS EN PRODUCTOS COSMÉTICOS

La seguridad de ciertos grupos de ingredientes usados en productos de cuidado personal ha despertado gran interés en los últimos años, ya que muchos de ellos pueden ser perjudiciales para la salud humana al presentar propiedades alergénicas, estrogénicas o incluso carcinogénicas. Este tipo de sustancias están controladas en la UE mediante la regulación de Productos Cosméticos (CE) nº 1223/2009 del Parlamento Europeo y del Consejo del 30 de Noviembre de 2009 (refundición de la Directiva 76/768 de la UE) [1].

Con el fin de garantizar el cumplimiento de esta normativa, es necesario desarrollar metodologías analíticas que nos permitan la determinación de estas sustancias en los productos cosméticos.

La metodología existente al inicio de la presente investigación para la monitorización y control de estos ingredientes, estaba obsoleta o no existía. En ella se hace uso de técnicas clásicas, laboriosas y muy alejadas de los principios que contempla la "química verde".

Por ello, se planteó el desarrollo de nueva metodología para la determinación de varios grupos de ingredientes de gran interés actual que incluye a fragancias (almizcles y sustancias potencialmente alergénicas (SAs)), ftalatos y conservantes.

En una primera aproximación se decidió iniciar la investigación con el grupo de fragancias alergénicas. Para simplificar este primer estudio se trabajo indirectamente con los cosméticos, analizando aguas de baño de bebés. Este enfoque nos pareció muy interesante, ya que las aguas de baño suponen un claro contacto de estos compuestos alergénicos con la población infantil.

Este sector de la población es más sensible y vulnerable al contacto con sustancias químicas. Esto se debe principalmente, a los bebés poseen un sistema inmune inmaduro, hecho que agrava los efectos de la posible entrada de sustancias tóxicas en su organismo. Por otro lado, en los países desarrollados es una práctica común el baño diario de bebés y niños, añadiendo con frecuencia varios productos cosméticos de forma simultánea. Estos productos contienen detergentes que pueden romper la barrera natural de la piel, facilitando de esta forma la entrada de sustancias irritantes, alergénicas o tóxicas a su organismo. Este proceso está favorecido por el menor espesor de la piel y la mayor superficie de contacto que presentan los niños en comparación con su peso. El contacto con sustancias

peligrosas durante el baño se produce también por ingestión directa del agua de baño o por inhalación, ya que muchas de las sustancias presentes son volátiles o semivolátiles.

Hasta ese momento no existía ninguna metodología que determinase las fragancias alergénicas en agua. En este trabajo se propone la aplicación de la Microextracción en Fase Sólida (SPME) como técnica de extracción de los analitos en dichas muestras, ya que es una técnica rápida, sencilla y adecuada para compuestos volátiles. Para la determinación y cuantificación de las fragancias se utiliza la cromatografía de gases a acoplada con espectrometría de masas (GC-MS), técnica que aporta fiabilidad y robustez al análisis.

En este estudio se analizaron muestras reales de agua de baño de bebés en las cuales se utilizaron productos de cuidado diario dirigidos a esta parte de la población: champús, baños de burbujas, lociones, aceites, etc. Como el objetivo era determinar a qué sustancias pueden estar expuestos los bebés durante su baño diario, no se llevó ningún control sobre la cantidad de agua en la bañera, temperatura o cantidad de producto añadido. La intención era enfrentarse a la realidad de que no todas las bañeras llevan la misma cantidad de agua y que no hay ningún control sobre la cantidad de producto que se añade (las etiquetas de los productos no ponen ninguna instrucción en este sentido).

Los resultados fueron bastante sorprendentes al encontrarse valores elevados para los 15 compuestos considerados. Este estudio despertó gran interés en la comunidad científica siendo destacado como "paper in forefront". Al mismo tiempo el SINC (Servicio de Información y Noticias Científicas) resaltaba los resultados obtenidos en este trabajo. Además, el trabajo resultó ganador de la "4º Edición del premio al mejor trabajo de SPME del año 2010" patrocinado por la casa comercial Sigma-Aldrich y entregado por el creador de la técnica el Dr. Janusz Pawliszyn.

Teniendo en cuenta los resultados obtenidos, se decidió abordar la temática desde el principio, es decir, determinar y cuantificar la presencia de dichos compuestos directamente en los productos cosméticos. Curiosamente, por entonces era muy difícil encontrar en los envases o etiquetas de los distintos productos cosméticos los términos: "fragrance free", "phthalate free" o "non-paraben

products", refiriéndose a la ausencia en los distintos productos de fragancias, ftalatos y parabenes. Hoy en día, sin embargo, empieza a no ser infrecuente, otorgándole valor añadido al producto que está exento de dichas sustancias.

Para llevar a cabo los análisis se propone la extracción con fluidos presurizados, comúnmente denominada por sus siglas en inglés PLE, como técnica de extracción de los distintos compuestos en productos cosméticos. Hasta la fecha no se había encontrado ningún trabajo que aplicase PLE a muestras de cosméticos, posiblemente debido a la complejidad que presenta la matriz.

La justificación de la elección de PLE como técnica de extracción se basa principalmente en su sencillez y eficacia, además de ser una técnica oficialmente aceptada para la determinación de compuestos semivolátiles en muestras sólidas medioambientales [2].

Siguiendo esta línea de trabajo se ha conseguido la puesta a punto de dos metodologías robustas para la determinación de 26 fragancias alergénicas, por un lado, y la determinación de un número destacado de conservantes, pertenecientes a distintas familias, incluyendo los populares parabenes.

El último estudio de este bloque consistió en el desarrollo de un método multicomponente para el control analítico de perfumes en laboratorios de rutina, bien de la industria cosmética o de distintos organismos y agencias de control. Este trabajo se ideó y desarrolló a raíz de la necesidad por parte de una empresa multinacional del sector, que demandaba metodología analítica para el análisis y el control de perfumes.

La metodología desarrollada permite llevar a cabo el análisis simultáneo de 52 ingredientes de cosméticos pertenecientes a distintas familias (conservantes, fragancias nitrogenadas, fragancias policíclicas, fragancias macrocíclicas, fragancias alergénicas y ftalatos) en perfumes.

Es importante destacar que el método de análisis no requiere realizar ningún tratamiento complejo de la muestra; tan sólo es necesario realizar una o varias diluciones de la misma en un disolvente adecuado y su determinación por GC-MS.

Por tanto en esta primera parte del trabajo de Tesis, que constituye el núcleo central de la misma, se presenta, el desarrollo de nuevas metodologías para la

determinación de distintos aditivos tóxicos y alergénicos en una variada gama de muestras de productos cosméticos. Estos nuevos desarrollos analíticos han dado lugar a los siguientes estudios (discutidos en el siguiente capítulo):

- "Solid-phase microextraction gas chromatography-mass spectrometry determination of fragrance allergens in baby bathwater".
- "Development of a solid phase dispersion-pressurized liquid extraction method for the analysis of suspected fragrance allergens in leave-on cosmetics".
- "Simultaneous in-cell derivatization pressurized liquid extraction for the determination of multiclass preservatives in leave-on cosmetics".
- "Multicomponent analytical methodology to control phthalates, synthetic musks, fragrance allergens and preservatives in perfumes".

Bibliografía:

[1] Reglamento (CE) No 1223/2009 del parlamento europeo y del consejo de 30 de noviembre de 2009 sobre los productos cosméticos (versión refundida). Diario Oficial de la Unión Europea L342 (2009) 59.

[2] EPA Method 3545, Pressurised fluid extraction, in: Test Methods for Evaluating Solid Waste, Physical/Chemical Methods, 3rd ed., U.S. GPO, Washington, DC, 2008, Final Update IV; EPA SW-846.

1.1 SOLID-PHASE MICROEXTRACTION GAS CHROMATOGRAPHY-MASS SPECTROMETRY DETERMINATION OF FRAGRANCE ALLERGENS IN BABY BATHWATER

SOLID-PHASE MICROEXTRACTION GAS CHROMATOGRAPHY-MASS SPECTROMETRY DETERMINATION OF FRAGRANCE ALLERGENS IN BABY BATHWATER

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Abstract

A method based on solid-phase microextraction (SPME) and gas chromatography-mass spectrometry (GCMS) has been optimized for the determination of fragrance allergens in water samples. This is the first study devoted to this family of cosmetic ingredients performed by SPME. The influence of parameters such as fibre coating, extraction and desorption temperatures, salting-out effect and sampling mode on the extraction efficiency has been studied by means of a mixed-level factorial design, which allowed the study of the main effects as well as two-factor interactions. Excluding desorption temperature, the other parameters were, in general, very important for the achievement of high response. The final procedure was based on headspace sampling at 100 ºC, using polydimethylsiloxane / divinylbenzene fibres. The method showed good linearity and precision for all compounds, with detection limits ranging from 0.001 to 0.3 ng mL^{-1}. Reliability was demonstrated through the evaluation of the recoveries in different real water samples, including baby bathwater and swimming pool water. The absence of matrix effects allowed the use of external standard calibration to quantify the target compounds in the samples. The proposed procedure was applied to the determination of allergens in several real samples. All the target compounds were found in the samples, and, in some cases, at quite high concentrations. The presence and the levels of these chemicals in baby bathwater should be a matter of concern.

Keywords: Fragrance allergens, Allergens, Cosmetics, Personal care products, Solid-phase microextraction, Water analysis, Multifactor optimization, Factorial design.

Introduction

Personal care products play an intimate role in our daily routines [1, 2]. They are complex formulations that contain a whole host of chemicals. Baby products are not different from this. The average baby hygiene product and especially baby bath products and bubble baths contain a complex mix of chemicals, including artificial dyes and colours, fragrances, phthalates, preservatives, harsh chemicals and sodium lauryl sulphate (used to promote lathering).

While children have never been better protected in some ways, mounting evidence suggests they have never been more vulnerable to things we have always considered safe: air, water and consumer products such as bubble bath. Children inhale more air than adults do, their skin is about five times thinner and is significantly more permeable: what goes on it, in general, goes into the child's body in some form. And once in there, it may have a surprisingly strong effect [3]. According to one briefing on baby toiletries from the Women's Environmental Network [4], infants lack a blood-brain barrier to prevent blood-borne toxins entering the brain until they are 6 months old and, in consequence, low-level exposures that would have little or no effect on an adult brain can sabotage a fetal one.

Most of us assume that the chemicals we put on our babies and children daily have been thoroughly tested and regulated, and are safe. This is technically correct: the ingredients within any toiletry and cosmetic product are subject to EU regulations and testing [5, 6]. But some toxicologists, and many environmental groups, believe such tests are inadequate. Even when the product is correctly labelled indicating the presence of those regulated ingredients, there will be no guaranties it has been tested for safety by an independent regulatory panel. In addition, of chemicals that have been tested, few have been examined for their effects on children's health [3, 7].

In the opinion of some experts, the escalating use of unnecessary products in the name of hygiene could certainly be doing our children's skin more harm than good [8]. Our consumption of products such as bubble baths, lotions, oils, talcs, wipes and even baby perfumes has shot up in recent decades. The number of children affected nowadays by eczema and asthma has increased significantly.

This problem has transcended the scientific world and is a subject of social concern [9]. The most used baby products are those intended for use in the bath, such as shampoos, bubble baths, shower gels and soaps. These products also contain some detergents that can break down the skin's natural barrier, and so it allows other irritants and allergens to penetrate it. In developed countries, the daily baby bath is a common practice. Some babies and children spend a long time playing in the bath. The exposure to these chemicals is not only through dermal absorption, but is also by inhalation and ingestion (it is quite common to swallow bathwater either intentionally or unintentionally). In this sense, some experts recommend considering bathing babies a little less often and using soap only when it is truly required.

A group of these hazardous chemicals included in most personal care products are the allergen fragrances. The Scientific Committee on Cosmetic Products and Non-Food Products (SCCNFP) has identified 26 of these ingredients as likely to cause contact allergies [5, 10], and the EU Cosmetic Directive [5] requires an indication of the presence of potential fragrance allergens in cosmetic products if a limit of 0.01% for rinse-off and 0.001% for leave-on products is exceeded [5].

It is important to underline that some of these substances not only pose the risk of causing allergies, but there is also evidence of brain and nervous system effects at low doses, as well as immunotoxicity and broad systemic effects in animal studies [11, 12]. In fact, one of these ingredients, methyleugenol, was included in 2002 in the list of forbidden substances in the EU Cosmetic Directive [5],owing to the potential risk of inducing cancer [13].

Analytical methods for the determination of this group of substances are mainly based on gas chromatography-mass spectrometry (GC-MS) [14–17]. Most of these methods are focused on the determination of these compounds in cosmetics. Owing to the difficulty of obtaining a good compound resolution as well as with other matrix components, advanced methods based on multidimensional chromatography have been proposed [18, 19].

Table 1. Chemical Abstract Service (CAS) number, IUPAC names, molecular structures and formulas, and main properties of the allergens studied

Compound	CAS number	IUPAC name	Molecular structure	Molecular formula	MW (g mol^{-1})	Log K_{OW}	Boiling Point (ºC)	Solubility (mg L^{-1})
Pinene	80-56-8	2,6,6-trimethylbicyclo[3.1.1]hept-2-ene		$C_{10}H_{16}$	136	4.37	155	18
Limonene	95327-98-3	1-methyl-4-prop-1-en-2-yl-cyclohexene		$C_{10}H_{16}$	136	4.57	176	13.8
Linalol	78-70-6	3,7-dimethylocta-1,6-dien-3-ol	HO	$C_{10}H_{18}O$	154	3.28	198	1589
Citronellol	106-22-9	3,7-Dimethyloct-6-en-1-ol	OH	$C_{10}H_{20}O$	156	3.38	225	322
Citral	5392-40-5	3,7-dimethylocta-2,6-dienal	O	$C_{10}H_{16}O$	152	3.17	229	590
Geraniol	106-24-1	3,7-dimetil-2,6-octadien-1-ol	OH	$C_{10}H_{18}O$	154	3.28	229	531
Hydroxy-citronellal	107-75-5	7-hydroxy-3,7-dimethyloctanal	HO, O	$C_{10}H_{20}O_2$	172	1.54	240	23800
Eugenol	97-53-0	2-methoxy-4-prop-2-enyl-phenol	OH, O	$C_{10}H_{12}O_2$	164	2.2	256	<1000
Coumarin	91-64-5	2H-1-Benzopyran-2-one	O, O	$C_9H_6O_2$	146	1.39	301	2500
Isoeugenol	97-54-1	Phenol, 2-methoxy-4-(1-propenyl)	OH, O	$C_{10}H_{12}O_2$	164	2.45	267	984
Lilial®	80-54-6	3-(4-tert-Butylphenyl)-2-methylpropanal	O	$C_{14}H_{20}O$	204	4.07	279	33
Farnesol	4602-84-0	3,7,11-trimethyldodeca-2,6,10-trien-1-ol	OH	$C_{15}H_{26}$	222	5.31	111	267
Hexylcinnmal-dehyde	101-86-0	Octanal, 2-(phenylmethylene)	O	$C_{15}H_{20}O$	216	4.82	174	2.8
Benzyl benzoate	120-51-4	Benzyl benzenecarboxylate	O, O	$C_{14}H_{12}O_2$	212	3.97	323.5	19.8
Benzyl salicylate	118-58-1	phenylmethyl 2-hydroxybenzoate	O, O, HO	$C_{14}H_{12}O$	228	4.31	208	<1000

In the present study, we developed a method for the identification and quantification of 15 of the most common fragrance allergens [5, 10] in baby bathwaters (see Table 1). We have not found other studies on the determination of these fragrances in water. The analytical procedure is based on solid-phase microextraction (SPME) followed by GCMS analysis. This is also the first application of SPME specifically devoted to the determination of fragrance allergens. The

proposed method was finally applied to a variety of baby bathwaters collected at the end of the bath. All the target compounds were found in the samples, and, in some cases, at quite high concentrations.

Experimental

Reagents and materials

3,7-Dimethyl-1,6-octadien-3-ol (linalol; 97%), 3,7-dimethyloct-6-en-1-ol (β-citronellol; 95%), 2-methoxy-4-prop-2-enylphenol (eugenol; 99%), 2H-1-benzopyran-2-one (coumarin; 99%); 3,7,11-trimethyldodeca-2,6,10-trien-1-ol (farnesol, mixture of isomers; 95%), 3,7-dimethylocta-2,6-dienal (citral, cis/trans; 95%), 1-methyl-4-prop-1-en-2-yl-cyclohexene [(R)-(+)-limonene; 97%) and 2-methoxy-4-(1-propenyl) phenol (isoeugenol, cis/trans; 98%) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). 2,6,6- Trimethylbicyclo[3.1.1]hept-2-ene [(−)-α-pinene; ≥99%], 3,7-dimethyl-2,6-octadien-1-ol (geraniol; ≥96%), 2-hydroxyphenylmethyl ester benzoic acid (benzyl salicylate; ≥99%) and 3-(4-tert-butylphenyl)-2-methylpropanal (lilial®; ≥95%) were purchased from Fluka Chemie (Steinheim, Germany). 7-Hydroxy-3,7-dimethyloctanal (hydroxycitronellal; ≥95%) and 2-(phenylmethylene)octanal (hexylcinnamaldehyde; ≥95%), were purchased from SAFC Supply Solutions (St. Louis, USA). Phenylmethyl benzoate (benzyl benzoate; 98.5%) was purchased from Chem Service (West Chester, USA). Table 1 shows the Chemical Abstract Service registry numbers, the physicochemical properties and the chemical structures of the target compounds.

Methanol, ethyl acetate and acetone were provided by Merck (Darmstadt, Germany). Individual stock solutions of each compound (2,000 μg mL^{-1}) were prepared in methanol. Further dilutions and mixtures were prepared inethyl acetate and acetone. The latter were employed for spiking water samples. Working Milli-Q water solutions with concentrations from 0.1 to 50 ng mL^{-1} were made by appropriate dilution and were then stored in amber glass vials at −20 ºC.

NaCl was provided by VWR Prolabo (Fontenay-sous-Bois, France). All solvents and reagents were of analytical grade. Ultrapure water was obtained from a Milli-Q water purification system (Millipore, Billerica, MA, USA).

The SPME manual holders and fibres were supplied by Supelco (Bellefonte, PA, USA). Five different commercial fibre coatings were used throughout the present work: 85 µm polyacrylate (PA), 100 µm polydimethylsiloxane (PDMS), 65 µm polydimethylsiloxane / divinylbenzene (PDMS/DVB), 75 µm Carboxen / polydimethylsiloxane (CAR/PDMS) and 50/30 µm divinylbenzene / Carboxen / polydimethylsiloxane (DVB/CAR/PDMS). Prior to first use, fibres were conditioned as recommended by the manufacturer.

Different real baby bathwater samples as well as an indoor swimming pool water sample were collected in amber glass containers. The products for daily baby care, including shampoos and bubble baths, were among the most frequently used in Spain. The excess of free chlorine in the water samples was removed by addition of sodium thiosulphate (0.1 mg mL^{-1}). Samples were stored in the dark at 4 ºC until analysis.

Gas chromatography-mass spectrometry

The GC-MS analysis was performed using a Varian 3800-GC gas chromatograph (Varian Chromatography Systems, Walnut Creek, CA, USA) coupled to a Varian Saturn 2000 ion trap mass spectrometer (Varian Chromatography Systems) equipped with a 1079 split / splitless injector. The system was operated by Saturn GC-MS workstation version 5.4 software.

Separation was carried out on a J&W DB-WAX capillary column (50 m × 0.20-mm inner diameter, 0.20- µm film thickness) from Agilent Technologies (Palo Alto, CA, USA). Helium (purity 99.999%) was employed as the carrier gas at a constant column flow of 1.0 mL min^{-1}. The gas chromatograph oven temperature was programmed from 45 ºC (held for 2 min) to 230 ºC at 15 ºC min^{-1} (held for 16 min) (total analysis time 30.33 min).

Splitless mode (maintained for 2 min) was used for injection; the split flow was set at 50 mL min^{-1} and the injector temperature was kept at 260 ºC.

The ion trap mass spectrometer was operated in the electron impact ionization mode (+70 eV). Manifold, ion trap and transfer line temperatures were maintained at 110, 200 and 220 ºC, respectively.

In the full-scan mode the mass range was varied from 39 to 250 *m/z* at 0.7 s per scan, starting at 4 min and ending at 15 min. From 15 min until the end of the analysis (30.33 min), the mass range of the full-scan mode was varied from 50 to 250 *m/z* at 0.7 s per scan. The filament emission current was 10 μA. The analytes were positively identified by comparison of their mass spectra and retention times with those of standards. The identification and quantification ions and retention times for each target compound are listed in Table 2.

Table 2 Retention times, quantification and identification ions of the target compounds.

Retention time (min)	**Compound**	**Quantification ions**	**Identification ions**
7.96	Pinene	93,77	93,77,41,121
8.41	Limonene	67,93	67,93,39,136
11.61	Linalol	93,71,43	93,71,43,55
12.84 13.33	Citral	39,41,69	39,41,69,84
13.41	Citronellol	67,69,41	67,69,41,81
14.05	Geraniol	41,69	41,69,123,93
14.79	Hydroxy-citronellal	43,59,71	43,59,71,41
15.79	Lilial®	189	189,147,131
16.79	Eugenol	164	164,131,103
18.10 18.22 18.57	Farnesol	69,41	69,41,81,121
18.74	Isoeugenol	164	164,77,103
19.00	Hexylcinna-maldehyde	129,216	129,216,117
20.81	Coumarin	146,118	146,118,89
23.93	Benzyl benzoate	105,194	105,194,91,77
28.28	Benzyl salicylate	91	91,65,39

Solid-phase microextraction

Aliquots of 10 mL water sample were placed in 22-mL headspace vials, into which 0, 1 or 2 g NaCl, as necessary for the experiment, had previously been added. Then, the vials were sealed with aluminium caps furnished with Teflon-faced septa and immersed in a water bath maintained at the selected extraction temperature (50 , 75 or 100 ºC). Samples were allowed to equilibrate for 5 min before placement of the SPME device. Afterwards, the fibre was exposed to the headspace over the sample (headspace SPME, HS-SPME) or immersed into the sample (SPME) for 20 min, depending on the experiment. A stir bar was introduced into the sample vial and magnetic stirring was performed during all the extraction process. Once the exposure period had finished, the fibre was retracted into the needle of the holder syringe and immediately inserted into the gas chromatograph injector. Desorption was carried out at 220 or 260 ºC depending on the experiment.

Under the final optimized conditions, the target compounds were extracted at 100 ºC for 20 min in the headspace mode using a PDMS/DVB fibre over the stirred samples with addition of 2 g NaCl. Fibre desorption was performed at 220 ºC. Possible carryover was checked and, under the selected conditions, it was not observed. Blanks were periodically run during the analysis to confirm the absence of contamination.

Results and discussion

Multivariate optimization of the SPME process

First, experiments were conducted to optimize the chromatographic separation of the target analytes as well as to select the quantification ions to obtain the maximum signal-to- noise ratio. The chromatographic conditions were summarized in “Experimental” and the identification and quantification ions are included in Table 2. Figure 1 shows the ion chromatograms of an ethyl acetate standard mixture of allergen fragrances at a concentration of 50 μg mL^{-1}.

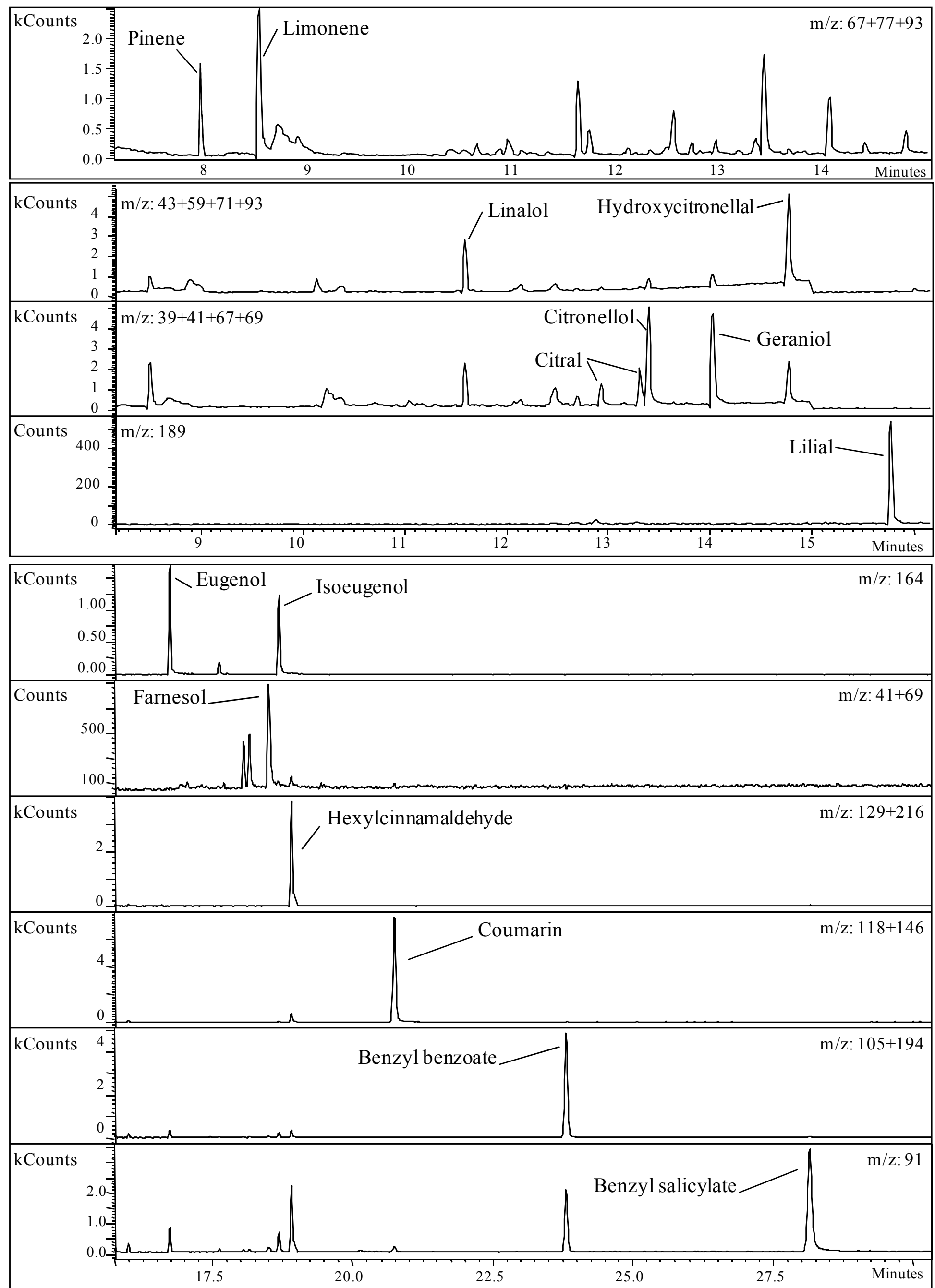


Fig. 1 Gas chromatography-mass spectrometry (GC-MS) ion chromatograms obtained from the direct injection of an ethyl acetate solution containing 50 µg mL^{-1} of each fragrance allergen

The analytes included in this study comprise alcohols, aldehydes, esters, ethers and one lactone, compounds with very different physicochemical properties, so an appropriate optimization is essential for the development of a suitable method for the simultaneous extraction of all analytes (see Table 1). It is important to study not only the individual effects of the factors affecting the SPME process, but also the possible interaction effects between the main factors. Thus, the optimization was carried out with the use of an experimental design strategy.

Initial SPME experiments were carried out with the purpose of selecting the factors as well as the factor levels to be considered in the multivariate experimental approach. In these experiments, five fibres were tested (PDMS, PDMS/DVB, DVB/CAR/PDMS, CAR/PDMS, PA) with sampling in the headspace mode (HS-SPME) at 50 ºC for 20 min. The results obtained for some of the target analytes are represented in Figure. 2. Three of the fibres were not satisfactory, giving, in general, low responses. The most appropriate fibres were PDMS/DVB and DVB/CAR/ PDMS; nevertheless, the latter fibre was not adequate for the extraction of limonene and coumarin. Therefore, PDMS/DVB was selected as the most suitable fibre for the simultaneous determination of all analytes.

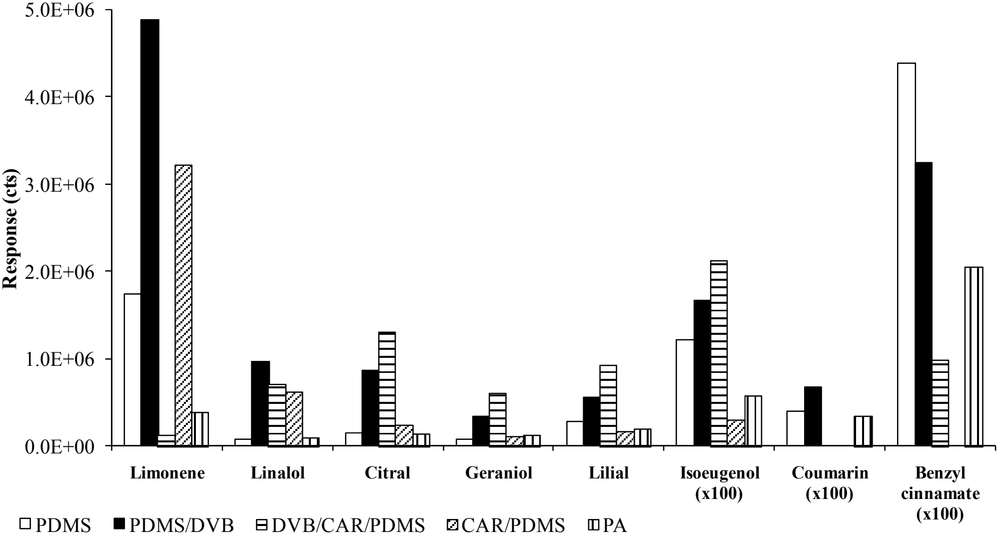


Fig. 2 Influence of the fibre coating on the headspace solid-phase microextraction (HS-SPME) of some selected allergens. PDMS polydimethylsiloxane, DVB divinylbenzene, CAR Carboxen, PA polyacrylate.

Some previous tests were also performed at different extraction temperatures in both direct (SPME) and head-space (HS-SPME) sampling modes, demonstrating that both factors, extraction temperature and sampling mode, were very important and interrelated, and that both deserved their inclusion in the factorial design optimization. Room temperature was inappropriate for HS-SPME, giving lower results than 50 ºC for SPME; therefore, this temperature value was discarded for further optimization.

A multivariate strategy of optimization was carried out to assess the influence of the main factors on the microextraction process, to select the optimal working conditions. The factors included in the factorial design were as follows: extraction temperature (A), at three levels (50, 75 and 100 ºC); sampling mode (B), at two levels (SPME and HS-SPME,); salt addition (NaCl) (C), at two levels (0 and 20%), and desorption temperature (D), at two levels (220 and 260 ºC). The factors and levels considered, as well as the corresponding codes, are summarized in Table 3.

A $3\times2^{3-1}$ mixed-level multifactor design, which involved 12 runs and two central points, was selected, allowing two degrees of freedom to estimate the experimental error. This design has resolution V, which means that it is capable of evaluating all main effects and all two-factor interactions. Numerical analysis of data resulting from the experimental design was performed with the statistical software package Statgraphics Centurion XV (Manugistics, Rockville, MD,USA). The experiments were carried out with 10-mL aliquots of Milli-Q water spiked at 25 ng mL^{-1} with the target analytes. Samples were magnetically stirred and the sampling time was set at 20 min to allow maximum throughput.

The selected design allows us to interpret the results using statistical tests and graphic tools to determine which factors and interactions have a statistically significant effect.

Table 3. Factors and levels considered in the experimental design.

Factor	Code	Low level (-)	High level (+)	Continuous
Extraction temperature (ºC)	A	50	100	Yes
Sampling mode	B	SPME	HS-SPME	No
NaCl (%, w/v)	C	0.0	20	Yes
Desorption temperature (ºC)	D	220	260	Yes

HS headspace, SPME solid-phase microextraction

Pareto charts of the standardized effects for the main factors and two-factor interactions are shown in Figure. 3. The length of each bar is proportional to the absolute value of its associated standardized effect. The standardized effect is obtained by dividing the estimated effect of each factor or interaction by its standard error. The vertical dotted line in the graph represents the statistically significant bound at the 95% confidence level. As can be seen in the figure, desorption temperature (D) was not statistically significant for any of the target compounds, excluding benzyl benzoate; in contrast, the other three main factors were, in general, quite important, although their effects varied depending on the compound. The interactions AB and BC (see the factor codes in Table 3) were also significant in many cases. The quadratic term AA was also significant for some analytes, which means that the factor extraction temperature presents a minimum or a maximum in the domain studied.

Figure 4 shows the main effect plot for various selected allergens. This kind of plot shows the main effects with a line drawn between the low and the high level of the corresponding factors. The length of the lines is proportional to the magnitude of the effect of each factor, and the sign of the slope indicates the level of the factor that produces the highest response. The influence of the extraction temperature, as well as the quadratic term of this factor (AA) in some cases, is clearly appreciated in the figure. The optimal temperature varies depending on the compound. For the most volatile analytes, such as pinene and limonene, the most favourable temperature was 50 ºC (see the last one in the figure). Most of the other compounds

presented optimal response at intermediate temperatures, which is represented in the graphic by a maximum (factor AA statistically significant; see also Figure. 3). Finally, some compounds, such as lilial®, showed better extraction efficiency at the highest level of this factor, i.e. at 100 ºC.

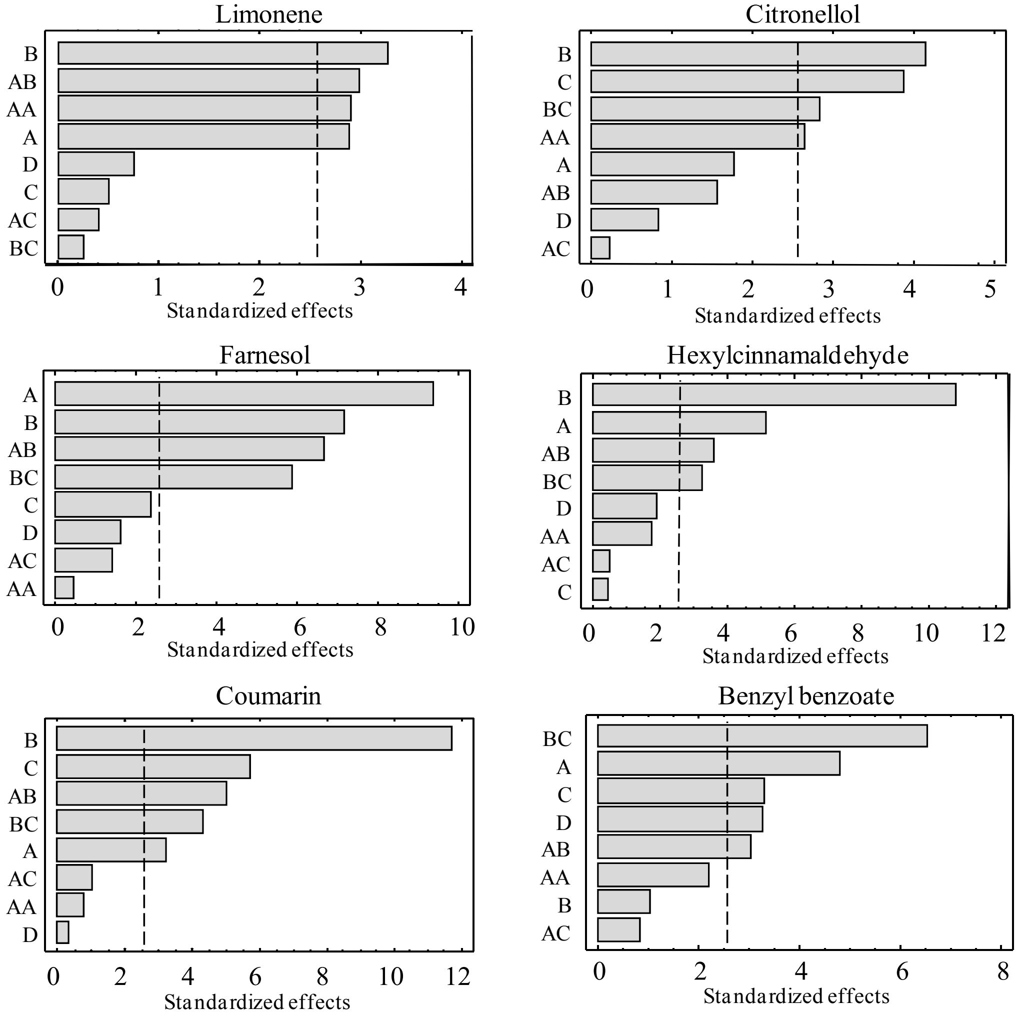


Fig. 3. Pareto charts for some selected allergens
(see the factor codes in Table 3)

Regarding the sampling mode, the analyte behavior was also quite different. Nine of the compounds presented better responses in the headspace mode, whereas five of them preferred direct extraction (see some examples in Figure. 4). Finally, the influence of the addition of salt was positive for most of the compounds.

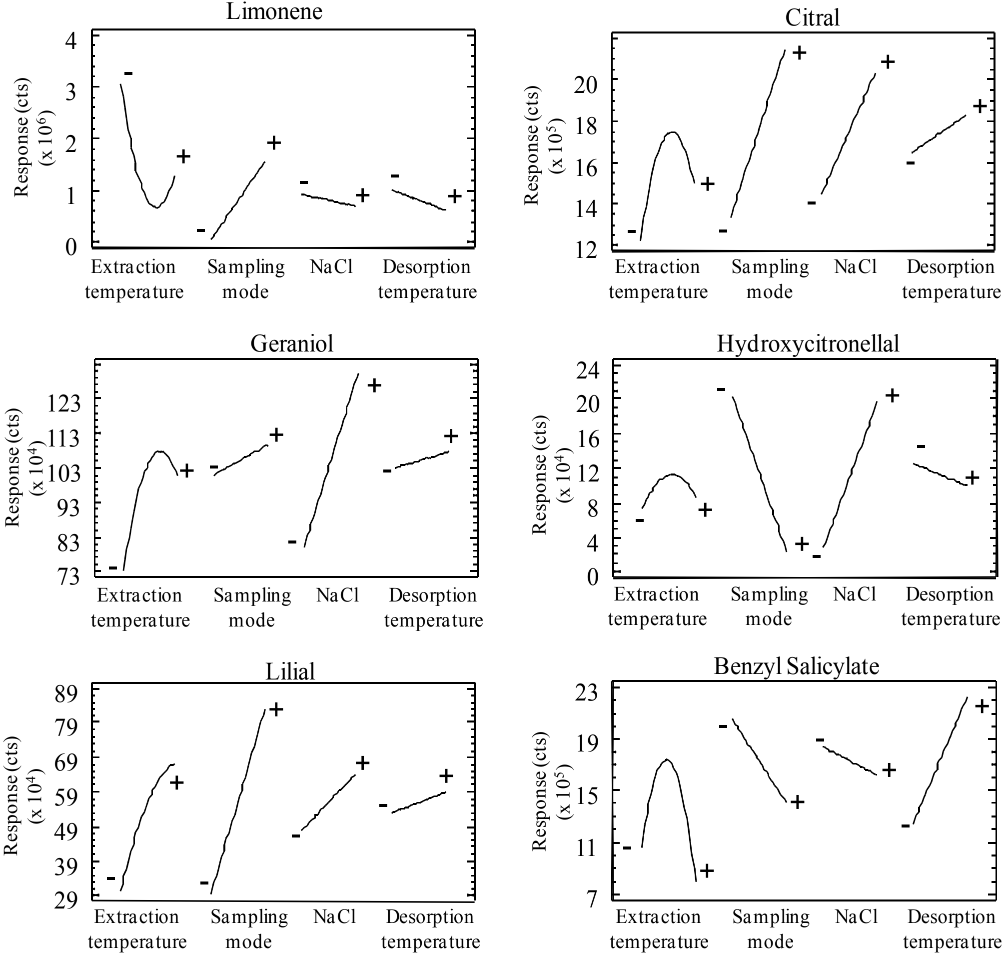


Fig. 4. Main effects plots for some selected allergens (see the factor codes and levels in Table 3)

As mentioned above, interaction factors such as BC, and especially AB, were also significant for many compounds and, consequently, they must be analysed. Interaction graphs for the relevant two-factor interactions of several representative

allergens are depicted in Figure. 5. Especially interesting is the AB interaction effect, which can be clearly observed in the figure. As previously discussed, many of the compounds showed the most favourable extraction at intermediate temperature (see Figure. 4). Nevertheless, analysing the AB factor, we can observe that the maximum appears shifted towards the high level (100 ºC) when sampling was performed in the headspace mode. In fact, for most analytes HS-SPME gives much higher responses at 100 ºC or very close temperatures. On the other hand, the direct sampling mode SPME does not show such an important extraction efficiency variation with temperature although, usually, the optimal extraction temperature was around 75 ºC (e.g. citral and geraniol, Figure. 5). It is also important to note that for most of the compounds that showed more favourable extraction by SPME (e.g. eugenol, Figure. 5), analysing the second-order factor AB, we can clearly see that the response achieved by SPME is much higher than that obtained by HS-SPME at low temperature values, but it is also very close to the HSSPME responses at 100 ºC. Regarding the BC interaction, we can also observe in the graphs that the addition of salt clearly benefits HS-SPME, whereas its effect on SPME is not so important, and even in some cases is negative.

Therefore, after analysing the interaction effects, we can conclude that the most favourable general conditions for the simultaneous extraction of the target analytes are HS-SPME at 100 ºC, after the addition of salt. As mentioned above, the desorption temperature was not significant, so we decided to set this parameter to 220 ºC, with the aim of achieving more selectivity in the analysis of real samples, since other compounds requiring higher desorption temperatures will not be introduced into the gas chromatography system. These general conditions are only unfavourable for four compounds: limonene and pinene, which will be betterextracted at lower temperature, and coumarin and hydroxycitronellal, which would give higher responses by SPME.

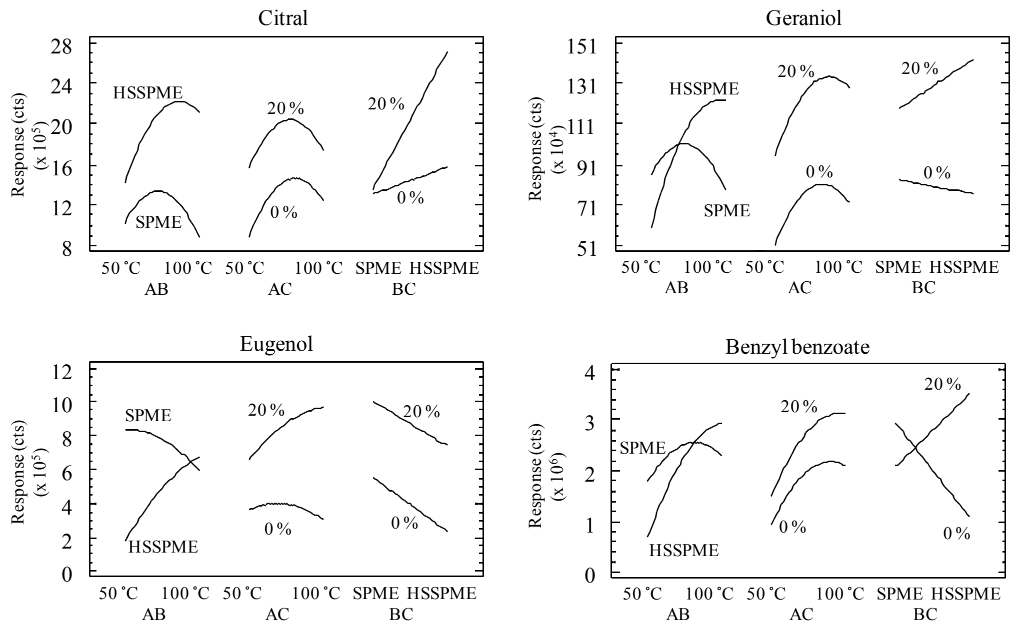
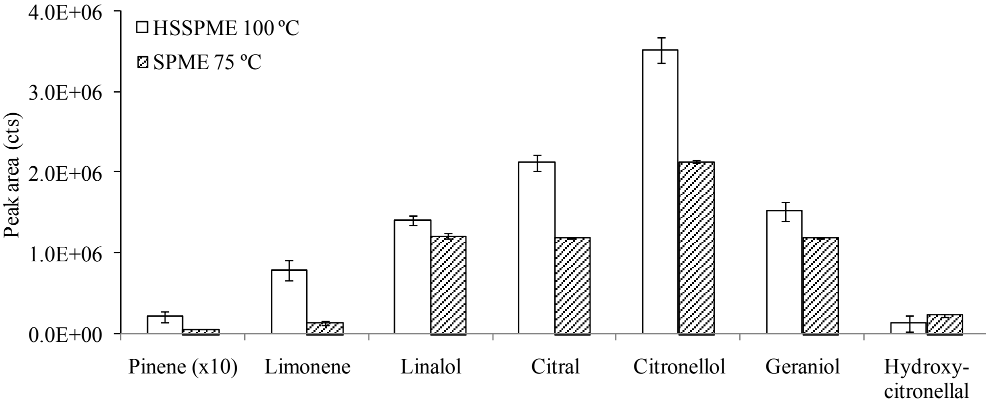


Fig. 5. Interactions plots for some selected allergens
(see the factor codes in Table 3)

In Figure. 6, the responses achieved by the selected conditions (HS-SPME, 100 ºC, 20% NaCl) and by direct SPME at 75 ºC (the general most favourable extraction temperature for direct sampling) and 0% NaCl are compared. The improvement achieved by HS-SPME is very clear, excluding the two exceptions mentioned above: coumarin and hidroxycitronellal. In both cases (HS-SPME and SPME), method repeatability was satisfactory.



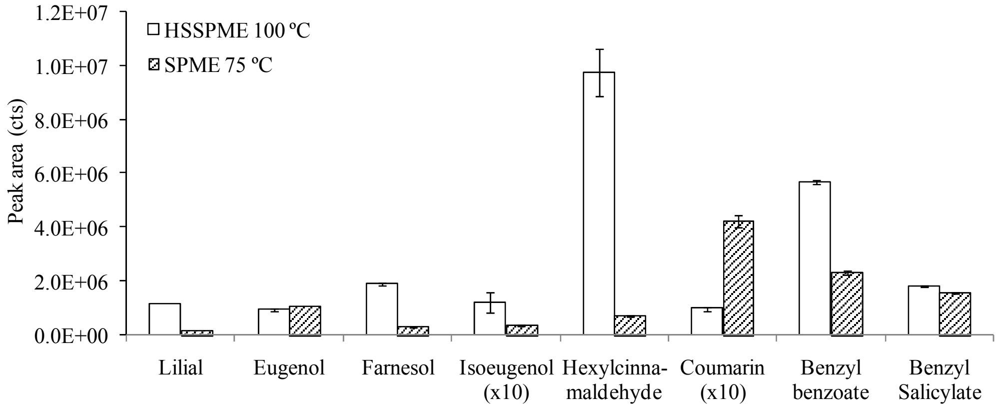


Fig. 6. Comparison between HS-SPME (100 ºC) and SPME (75 ºC) (concentration 20 ng mL^{-1})

Performance study of the HS-SPME method

To evaluate method linearity, a calibration study was performed by selecting the optimal conditions indicated above. The calibration range was from 0.1 to 50 ng mL^{-1} and the number of calibration levels was six. The calibration solutions were prepared in Milli-Q water. The method exhibited a directly proportional relationship between the extracted amount of each fragrance allergen and its initial concentration in the sample, with correlation coefficients (R) ranging from 0.993 to 1.000 (Table 4).

Table 4. Precision (relative standard deviation, %), linearity, limit of detection (LOD) and limit of quantification (LOQ) of the proposed method

Compound	Intraday precision[a]			Interday precision[b]	Linearity[c] (*R*)	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
	1 ng mL^{-1}	5 ng mL^{-1}	20 ng mL^{-1}	20 ng mL^{-1}			
Pinene	16	3.8	13	10	0.994	0.188	0.625
Limonene	17	12	1.3	10	0.996	0.097	0.323
Linalol	5.1	3.0	5.7	7.7	0.993	0.115	0.385
Citral	3.9	9.7	4.9	8.9	0.997	0.273	0.909
Citronellol	4.0	3.3	2.9	2.8	0.995	0.176	0.588
Geraniol	9.7	10	2.5	5.2	0.998	0.250	0.833
Hydroxycitronellal	0.8	5.8	13	10	0.993	0.273	0.909
Lilial®	4.8	4.4	1.0	8.2	0.995	0.012	0.041
Eugenol	3.6	7.9	0.9	5.0	0.996	0.021	0.070
Farnesol	5.0	14	4.4	21	0.999	0.055	0.182
Isoeugenol	17	9.7	8.6	12	0.996	0.300	1.000
Coumarin	8.7	5.6	4.8	8.7	0.998	0.136	0.455
Hexylcinnamaldehyde	5.6	9.2	2.2	2.0	0.999	0.001	0.004
Benzyl benzoate	5.6	1.4	0.4	5.6	1.000	0.002	0.008
Benzyl salicylate	16	3.9	5.5	5.7	0.999	0.013	0.042

[a] n=3
[b] n=5
[c] Concentration range 0.1-50 ng mL^{-1}

The precision of the experimental procedure was assessed at three concentrations: 1, 5 and 20 ng mL^{-1}. The results showed good intraday and interday precision, with relative standard deviation, in general, lower than 10% (see Table 4).

Limits of detection (signal-to-noise ratio of 3) and limits of quantification (signal-to-noise ratio of 10) are also presented in Table 4. These limits are at the subnanogram per millilitre level, and, therefore, the sensitivity of the proposed method can be considered satisfactory.

Application to real baby bathwater samples

The reliability of the proposed method was demonstrated by analysing real baby bathwater samples (BBW1-BBW9) and also an indoor swimming pool water sample (SPW). Two of the samples, one baby bathwater and the swimming pool water, were selected for matrix effect and recovery studies. These samples were spiked at 20 and 5 ng mL^{-1}, respectively.

Table 5. Recovery of the target analytes from two different samples spiked at 5 ng mL^{-1} (swimming pool water) and 20 ng mL^{-1} (baby bathwater)

Compound	Swimming pool water	Bathwater	Swimming pool water with adition of sodium tiosulphate	Bathwater with adition of sodium tiosulphate
Pinene	36	116	100	109
Limonene	44	35	85	76
Linalol	72	29	75	75
Citronellol	81	60	74	79
Citral	85	70	90	85
Geraniol	87	48	75	80
Hydroxy-citronellal	100	102	90	94
Eugenol	20	68	107	86
Coumarin	102	105	111	116
Isoeugenol	37	9	85	96
Lilial®	104	92	80	90
Farnesol	85	94	105	124
Hexylcinna-maldehyde	99	87	91	81
Benzyl benzoate	109	117	92	103
Benzyl salicylate	129	146	105	99

Recoveries were calculated as the ratio of the measured concentration, after subtracting the initial concentration in the non-spiked sample, to the spiked concentration, and expressed as a percentage. Concentrations were calculated by external calibration using ultrapure water standards. Recoveries were satisfactory for eight of the target allergens, but they were low for six compounds: pinene, limonene, linalool, geraniol, eugenol and isoeugenol. On the other hand, recoveries were too high for benzylsalicylate (see Table 5). The samples were then treated with sodium thiosulphate (0.1 mg mL^{-1}), to remove the excess of free chlorine, and, in this way preventing analyte oxidation, and the recovery studies were performed again. All the results were satisfactory and the values were in most cases over 80% (see Table 5, last two columns). These recoveries can be considered quantitative and, in consequence, after the addition of sodium thiosulphate no matrix effects were observed. Therefore, quantification by external calibration using ultrapure water standard solutions is suitable.

The method was finally applied to determine the levels of the target analytes in baby bathwater samples (BBW1-BBW9). Additionally, the levels found in the

swimming pool water sample (SPW) were also calculated. In the case of the bathwater samples, the products used included shampoos, bubble baths and moisturizing soaps intended for babies. Sodium thiosulphate was added to all the samples after the sampling step. The results are shown in Table 6. Figure 7 shows the HSSPME-GC-MS ion chromatograms obtained for a real baby bathwater sample containing ten of the target analytes (BBW1, see concentrations in Table 6). As can be seen, all the target compounds were detected in at least two of the samples. It is interesting to underline the high concentrations found for some compounds in several samples, reaching values even greater than 100 ng mL^{-1} in some cases. Taking into account that it is not infrequent for babies to spend 15 min or even longer times in the bath every day, and that the exposure pathway to these and other ingredients is not only dermal absorption but also inhalation, and frequently ingestion, the presence and the levels of these chemicals in bathwater should be a matter of special concern.

Table 6. Levels found (ng mL^{-1}) in real samples

Compound	BBW1	BBW2	BBW3	BBW4	BBW5	BBW6	BBW7	BBW8	BBW9	SPW
Pinene	39.0		8.6		354		5.5			
Limonene	5.4		56.3	0.4	568	0.3	1.7		3.0	
Linalol	11.9		47.3	2.1	108	0.6	26.2	2.3	19.3	
Citral	1.1			11.7		2.3	38.2	6.8	1.8	
Citronellol	4.3		34.0	1.7	14.8	3.3	5.7	6.5	13.8	
Geraniol	9.6		25.3	2.6	40.5		11.3		10.8	
Hydroxycitronellal							103		88.5	
Lilial®	0.4	0.7		0.5	0.8	0.6	3.0	0.7		7.9
Eugenol	2.6	0.1	31.9		40.7					
Farnesol		2.9	27.4		62.5	2.2	17.7	1.6	2.7	
Isoeugenol										
Hexylcinnamaldehyde		0.2	0.21		0.2	0.01	3.1			0.1
Coumarin	2.9	10.0	157	2.7						1.4
Benzyl benzoate		2.3	12.1	0.03	0.3	0.03		0.03		0.3
Benzyl salicylate	0.4	3.5	4.1	3.3	1.6	0.4			0.06	0.1

Empty cells indicate concentrations lower than LODs.

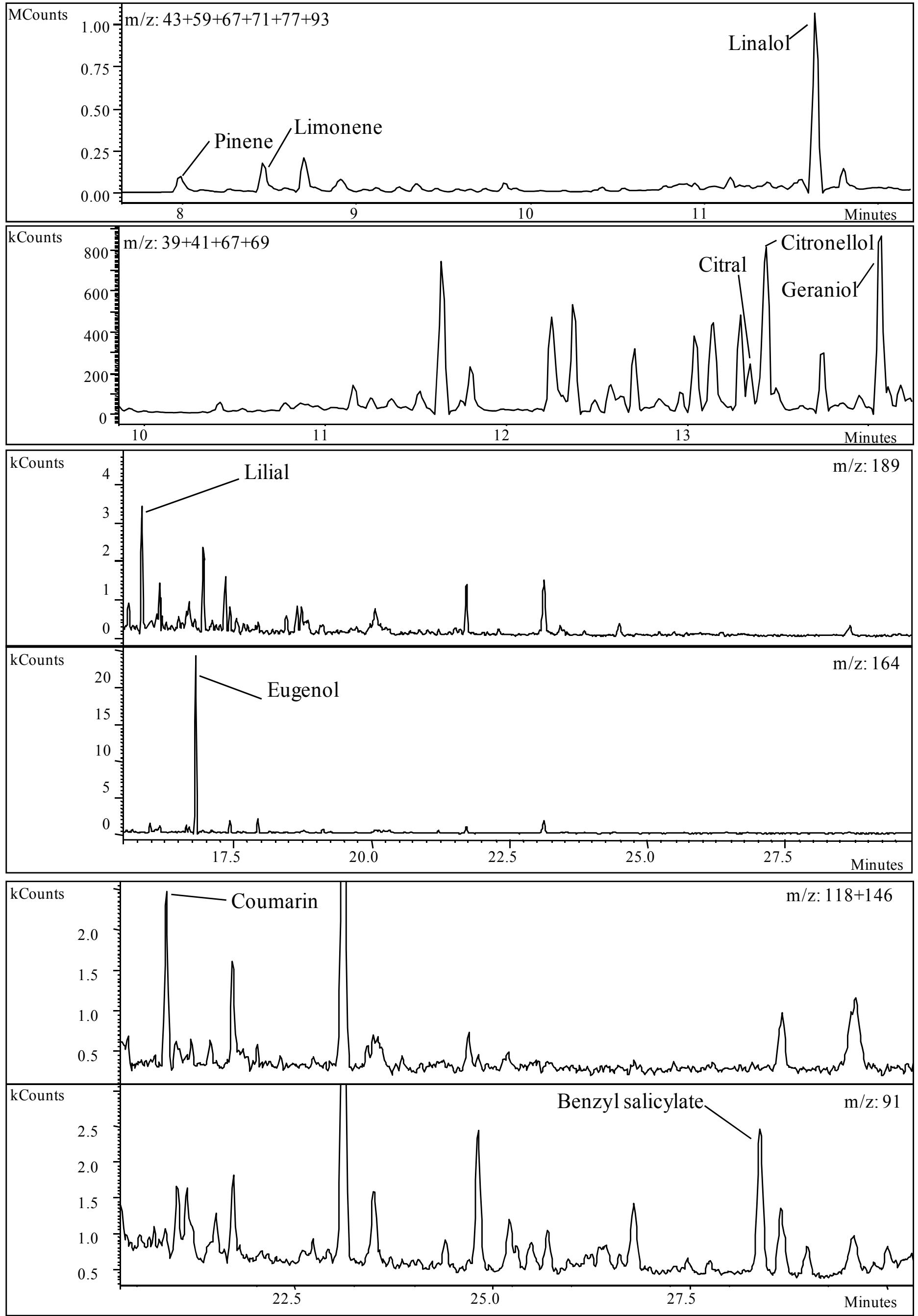


Fig. 7. HS-SPME-GC-MS ion chromatograms obtained for a real non-spiked baby bathwater sample (BBW1).

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References

1. CG. Daughton, TA. Ternes, Environ Health Perspect 107 (S6) (1999) 907.
2. CG. Daughton, TL. Jones-Lepp (eds) (2001) Pharmaceuticals and personal care products in the environment. ACS symposium series 791. Oxford University Press, Washington.
3. P. Grandjean, D. Bellinger, A. Bergman, S. Cordier, G. Davey-Smith, B. Eskenazi, D. Gee, K. Gray, M. Hanson, P. Van den Hazel, JJ. Heindel, B. Heinzow, I. Hertz-Picciotto, H. Hu, TT-K. Huang, TK. Jensen, PJ. Landrigan, IC. McMillen, K. Murata, B. Ritz, G. Schoeters, NE. Skakkebaek, S. Skerfving, P. Weihe, Basic Clin. Pharmacol. Toxicol. 102 (2) (2008) 73.
4. Women's Environmental Network (2009) WEN. http://www.wen.org.uk. Accessed 14 Feb 2009.
5. European Commission (2008) Council Directive of 27 July 1976 on the approximation of the laws of the Member States relating to cosmetic products (76/768/EEC) (OJ L 262, 27.9.1976, p. 169) consolidated version of 24/04/2008. European Commission, Brussels.
6. L. Gagliardi, S. Dorato, In: Analysis of cosmetic products. A. Salvador, A. Chisvert (eds) Elsevier, Amsterdam (2007), chap 1.
7. P. Grandjean, PJ. Landrigan, Lancet 368 (2006) 2167.
8. MJ. Cork, DA. Robinson, Y. Vasilopoulos, A. Ferguson, M. Moustafa, A. MacGowan, GW. Duff, SJ. Ward, R. Tazi-Ahnini, J. Allergy Clin. Immunol. 118 (1) (2006) 3.

9. The Guardian (2004) Throw out the bath water? http://www.guardian.co.uk/chemicalworld/story/0,14534,1210214,00.html. Accessed 21 Feb 2009.
10. Scientific Committee on Cosmetic Products and Non-Food Products (1999) Fragrance allergy in consumers, SCCNFP/0017/98 final, December 1999. European Commission, Brussels.
11. Environmental Working Group (2009) Eugenol. http://www.cosmeticsdatabase.com/ingredient.php?ingred06=702373&refurl=/product.php?prod_id=171315&¬hanks=1. Accessed 1 Mar 2009.
12. T. Uehara, N. Kiyosawa, T. Shimizu, K. Omura, M. Hirode, T. Imazawa, Y. Mizukawa, A. Ono, T. Miyagishima, T. Nagao, T. Urushidani, Hum. Exp. Toxicol. 27 (1) (2008) 23.
13. JD. Johnson, MJ. Ryan, JD. II. Toft, SW. Graves, MR. Hejtmancik, ML. Cunningham, R. Herbert, KM. Abdo, J. Agric. Food Chem. 48 (2000) 3620.
14. SC. Rastogi, JD. Johansen, T. Menne, P. Frosch, M. Bruze, KE. Andersen, JP. Lepoittevin, S. Wakelin, IR. White, Contact Dermatitis 41 (2) (1999) 84.
15. H. Leijs, J. Broekhans, L. van Pelt, C. Mussinan, J. Agric. Food Chem. 53(14) (2005) 5487.
16. M. Bassereau, A. Chaintreau, S. Duperrex, D. Joulain, H. Leijs, G. Loesing, N. Owen, A. Sherlock, C. Schippa, P-J. Thorel, M. Vey, J. Agric. Food Chem. 55(1) (2007) 25.
17. M. Niederer, R. Bollhalder, C. Hohl, J. Chromatogr. A 1132 (1–2) (2006) 109.
18. MS. Dunn, N. Vulic, RA. Shellie, S. Whitehead, P. Morrison, PJ. Marriott, J. Chromatogr. A 1130(1) (2006) 122.
19. L. Mondello, A. Casilli, PQ. Tranchida, D. Sciarrone, P. Dugo, G. Dugo, LC-GC Eur. 21(3) (2008) 130.

1.2 DEVELOPMENT OF A SOLID PHASE DISPERSION-PRESSURIZED LIQUID EXTRACTION METHOD FOR THE ANALYSIS OF SUSPECTED FRAGRANCE ALLERGENS IN LEAVE-ON COSMETICS

DEVELOPMENT OF A SOLID PHASE DISPERSION-PRESSURIZED LIQUID EXTRACTION METHOD FOR THE ANALYSIS OF SUSPECTED FRAGRANCE ALLERGENS IN LEAVE-ON COSMETICS

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Abstract

A new method based on solid phase dispersion-pressurized liquid extraction (PLE) followed by gas chromatography-mass spectrometry (GC-MS) has been developed for the determination of 26 suspected fragrance allergens (all the regulated in the EU Cosmetics Directive amenable by GC, as well as pinene and methyleugenol) in cosmetic samples. The effects of the temperature, extraction time and solvent, and dispersing sorbent, affecting the whole proposed procedure, have been evaluated using a multifactor strategy. The optima conditions after the analysis of main and second order effects entailed the extraction at 120 ºC for 15 min, using hexane / acetone as solvent, and florisil as dispersing sorbent. The method performance has been studied, showing good linearity ($R \geq 0.996$) as well as good precision (RSD≤10%).Detection limits (S/N = 3) ranged from 0.000001 to 0.0002% (w/w), values far below the established restrictions as regard labelling in the European Cosmetics Regulation. Reliability was demonstrated through the quantitative recoveries of all the studied compounds. The absence of matrix effects allowed quantification of the compounds by calibration with standard solutions. The analysis of 10 samples (several moisturizing and anti-wrinkle creams and lotions, hand creams, and sunscreen and after-sun creams), covering very different matrices, showed the presence of suspected allergens in all the analyzed samples; in fact, half of the samples contained an elevated number of them. Although the ubiquity of these compounds was demonstrated, labelling was in all cases in consonance with the European Cosmetics Regulation.

Keywords: Pressurized liquid extraction; Fragrance allergens; Cosmetics; Experimental design; Personal care products.

1. Introduction

The majority of personal care, household and laundry products on the market contain fragrances. Some of the fragrance chemicals have been shown to cause various side effects, like skin sensitivity, rashes, dermatitis, coughing, asthma attacks, migraine, etc. [1–3]. Legislations in force in the three principal markets regarding cosmetic products, i.e., in the European Union [4], the United States (US) [5] and Japan [6], establish that all the ingredients for cosmetics should be included on the label. According to the EU Cosmetics Directive [4], in the case of perfume and aromatic compositions and their raw materials, all together can be referred to under the word "perfume" or "aroma"; nevertheless, its Annex III consists of a list of restricted substances used as ingredients of cosmetic products. Several suspected fragrance allergens are included in this Annex. Two different restrictions are applied to them, i.e., substances that can be included up to a maximum allowed concentration, and substances for which their presence must be indicated in the list of ingredients when their concentrations exceed the 0.001% in leave-on products and 0.01% in rinse-off products (see in Table 1 the compounds considered in this study and their limitations). The possible negative effects on the health of such substances may drive to at least a decrease of these values. In fact, it has been already observed the inclusion of the term "fragrance free" in several cosmetic products as a positive characteristic.

Hence routine analytical methods are required to ensure that regulations are observed by producers and importers. The variety of matrices in which fragrance compounds have to be analyzed is very broad and includes very complex matrices. In addition, the concentration range of the fragrance compounds in these matrices may fluctuate from low micrograms per gram to milligrams per gram. While liquid samples such as perfumes or perfumed oils, can be directly analyzed usually after simple dilution [7–9], the direct analysis of other cosmetic samples, such as creams and lotions, is quite problematic since the contamination of the chromatographic inlet and column occurs after a few analyses [10], the difficulty of achieving accurate determinations due to the complexity of obtaining homogeneous solutions of the samples, and the coelution of the matrix components.

Therefore, the development of analytical methods for the determination of fragrance allergens in leave-on cosmetics is as challenging as necessary; even though, up to our knowledge, the literature is somewhat scarce in this subject.

Despite the presence of chromophoric groups in the most of these fragrances allows the use of high performance liquid chromatography (HPLC) with ultraviolet detection [11], gas chromatography-mass spectrometry (GC-MS) can be considered the technique of choice for the analysis of this kind of volatile substances [10,12,13].

Pressurized liquid extraction (PLE) has been applied for the analysis of other cosmetic ingredients such as UV filters [14–16], musks [16], preservatives and antimicrobials [15,16] in environmental matrices such as sediments [14] and sewage sludge [15,16]. This technique is fast, increases automation, decreases the amount of organic solvents, and offers the possibility of controlling the selectivity of the extraction by loading different sorbents instead of inert materials into the extraction cell.

The aim of this work is to develop a method based on PLE followed by gas chromatography-mass spectrometry (GC-MS) to simultaneously identify and quantify 26 fragrances in multi-matrix cosmetic samples. To our knowledge, PLE is applied for the first time to the analysis of cosmetics and it is also the first time that it is applied to the analysis of suspected fragrance allergens.

2. Experimental

2.1. Reagents and materials

The 26 studied fragrance allergens, their chemical names and the purity of the standards are summarized in Table 1. The internal standard PCB-30 (2,4,6-trichlorobiphenyl) was purchased from Dr. Ehrenstorfer (Augsburg, Germany).

Table 1. Target fragrance allergens, their CAS numbers and purity, chromatographic retention times, as well as their qualification and quantification ions.

Common name	Chemical name	CAS number	Purity	Boiling point (°C)	Retention time (min)	Qualifier and quantifier ions
Pinene	2,6,6-Trimethylbicyclo[3.1.1]-hept-2-ene	80-56-8	≥99%[c]	155	4.75	77,91,93
Limonene[a]	(4R)-1-Methyl-4-(1-methylethenyl)cyclohexene	5989-27-5	97%[d]	176	6.62	67,93,121
Benzyl alcohol[a]	Benzene methanol	100-51-6	99%[e]	205	6.87	77,79,108
Linalool[a]	3,7-Dimethyl-1,6-octadien-3-ol	78-70-6	97%[d]	198	8.05	71,93,121
Methyl-2-octynoate[a]	Methyl heptin carbonate	111-12-6	≥99%[f]	219	9.74	67,95,123
Citronellol[a]	(±)-3,7-Dimethyloct-6-en-1-ol	106-22-9/ 26489-01-0	95%[d]	225	10.06	67,69,81,95
Citral[a]	3,7-Dimethyl-2,6-octadienal	5392-40-5	95%[d]	229	10.20 10.50	67,69,109
Geraniol[a]	3,7-Dimethyl- (2E)-2,6-octadien-1-ol	106-24-1	≥96%[c]	229	10.35	67,69,111,123
Cinnamal[a]	3-Phenyl-2-propenal	104-55-2	≥93%[f]	252	10.52	77,103,131
Anise alcohol[a]	4-Methoxybenzyl alcohol	105-13-5	98%[d]	259	10.66	77,109,121,138
Hydroxycitronellal[a]	7-Hydroxy-3,7-dimethyloctanal	107-75-5	≥95%[f]	241	10.68	59,81,95
Cinnamyl alcohol[a]	3-Phenyl-2-propen-1-ol	104-54-1	98%[e]	250	10.88	91,92,115,134
Eugenol[a]	2-Methoxy-4-(2-propenyl)-phenol	97-53-0	99%[d]	256	11.34	131,149,164
Methyl-eugenol[b]	1,2-Dimethoxy-4-(2-propenyl)-benzene	93-15-2	99%[d]	248	11.71	147,163,178
Isoeugenol[a]	2-Methoxy-4-(1-propenyl)-phenol	97-54-1	98%[d]	267	11.75 12.04	131,149,164
Coumarin[a]	2H-1-Benzopyran-2-one	91-64-5	99%[d]	298	11.98	90,118,146
α-Isomethyl ionone[a]	3-Methyl-4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-3-buten-2-one	127-51-5	≥85%[c]	266	12.24	107,135,150
Lilial®[a]	2-(4-tert-Butylbenzyl) propionaldehyde	80-54-6	≥95%[c]	279	12.54	131,147,189
Amyl cinnamal[a]	2-Benzylideneheptanal	122-40-7	97%[d]	289	13.21	91,115,203
Lyral®[a]	Hydroxyhexyl-3-cyclohexene carboxaldehyde	31906-04-4	≥97%[c]	319	13.29	77,79,136
Amylcinnamyl alcohol[a]	2-Pentyl-3-phenylprop-2-en-1-ol	101-85-9	≥85%[c]	>200	13.40	91,115,133
Farnesol[a]	3,7,11-Trimethyl-2,6,10-dodecatrien-1-ol	4602-84-0	95%[d]	283	13.44 13.56	69,81,93
Hexyl cinnamal[a]	2-Benzylideneoctanal	101-86-0	≥95%[f]	308	13.73 13.88	91,115,216
Benzyl benzoate[a]	Phenylmethyl benzoate	120-51-4	98%[e]	324	13.85	91,105,194
Benzyl salicylate[a]	Benzyl-2-hydroxybenzoate	118-58-1	≥99%[c]	320	14.56	65,91,228
Benzyl cinnamate[a]	3-Phenyl-2-propenoic acid phenylmethyl ester	103-41-3	99%[d]	371	16.86	91,131,192,193

[a] According to Regulation (EC) No 1223/2009, the presence of the substance must be indicated in the list of ingredients when its concentration exceeds 0.001% in leave-on products.
[b] Maximum allowed concentration in fragrance cream: 0.002%, and other leave-on products: 0.0002%.
[c] Purchased from: Fluka Chemie GmbH (Steimheim, Germany).
[d] Sigma–Aldrich Chemie GmbH (Germany).
[e] Chem Service (West Chester, USA).
[f] SAFC Supply Solutions (St. Louis, USA).

Acetone, ethyl acetate, and *n*-hexane were provided by Merck (Darmstadt, Germany). Florisil (60–100 mesh) and C18 (70–230 mesh) were achieved from Aldrich (Milwaukee, WI, USA). Before being used, florisil was activated at 130 ºC for 12 h and then allowed to cool down in a desiccator. Anhydrous sodium sulphate (99%) was purchased by Panreac (Barcelona, Spain).

Individual stock solutions of each compound were prepared in acetone. Further dilutions and mixtures were prepared in acetone, hexane / acetone (1:1, v/v), and ethyl acetate. All solutions were stored in amber glass vials at −20 ºC. All solvents and reagents were of analytical grade.

2.2. Cosmetic samples

Different cosmetics from national and international brands were purchased from local sources. They included moisturizing and antiwrinkle creams and lotions, hand creams, sunscreen and after-sun creams. Samples were kept in their original containers at room temperature until their analysis.

1 g of the cosmetic sample was weighted exactly into a 10-mL glass vial. When it was necessary, the sample was spiked with 50 μL of the corresponding acetone solution of the target compounds to get the desired final concentration in the cosmetic sample. The sample was then thoroughly mixed with 2 g of drying agent (anhydrous sodium sulphate, Na_2SO_4) and 2 g of dispersing sorbent (C18 or florisil).

2.3. PLE procedure

Extractions were performed on an ASE 200 system (Dionex, Co.,Sunyvale, CA, USA) equipped with a 24-sample carousel, 11-mL stainless steel cells and 40-mL collection vials. Two cellulose filters (Dionex) were placed at each end of the PLE cell. The sample, mixed with the drying agent and the dispersing sorbent, was introduced into the cell, where previously 1 g of clean sand was placed. Finally, the dead volume of the cell was filled up with sand. The cell was tightly closed and placed into the carousel of the ASE system. Extractions were performed by preheating the cell before filling with solvent (preheat method). The extraction

pressure was set to 1500 psi, the flush volume was 60% and the purge time was set to 60 s. Hexane / acetone (1:1, v/v) or ethyl acetate were employed as extraction solvents, depending on the experiment. The extraction temperature and extraction time varied during the optimization of the method. After extraction, 20 μL of PCB 30 (100 μg mL^{-1}) were added to the final extract (~15 mL) to correct possible variations of the extract volume. Then, PLE extracts were directly analyzed by GC-MS, without a pre-concentration step, since the detection limits achieved are low enough considering the current cosmetic regulations.

2.4. GC-MS analysis

Analyses were performed on a Varian CP 3900 gas chromatograph (Varian Chromatography Systems, Walnut Creek, CA, USA) equipped with a 1177 split / splitless injector and an ion trap spectrometer Varian Saturn 2100 (Varian Chromatography Systems).Separation was carried out on a HP5 capillary column (30 m ×0 .25 mm i.d., 0.25 μm film thickness) from Agilent Technologies (Palo Alto, CA, USA). Injection volume was 2 μL. Helium (purity 99.999%) was employed as carrier gas at a constant column flow of 1.0 mL min^{-1}. The GC oven temperature was programmed from 45 ºC (held 2min) to 100 ºC at 8 ºC min^{-1}; to 150 ºC at 20 ºC min⁻1; to 200 ºC at 25 ºC min^{-1} (held 5min); and a final ramp to 280 ºC (held 4min) at 35 ºC min^{-1} (total analysis time = 25min). The splitless mode (held 2min) was used for injection, after that the split flow was set at 20 mL min^{-1}. The injector temperature was kept at 220 ºC. Trap, manifold and transfer-line temperatures were 220 ºC, 120 ºC and 280 ºC, respectively.

The GC-MS system was operated by Saturn GC-MS workstation v5.52 software. In the full scan mode the mass range was varied from 50 to 320 *m/z* at 0.6 s $scan^{-1}$, starting at 4 min and ending at 22.5 min. The filament emission current was 15 μA. The analytes were positively identified by comparison of their mass spectra and retention times to those of the standards.

2.5. Statistical analysis

Basic and descriptive statistics, as well as experimental design analysis were performed using Statgraphics-Plus v5.1 (Manugistics, Rockville, MD, USA) as software package. The experimental design was applied in the optimization of the extraction method, to analyze the simultaneous effect of the main parameters affecting PLE.

3. Results and discussion

3.1. Optimization of the dispersive pressurized liquid extraction process.

The chromatographic method for the separation of the target allergens was optimized elsewhere [17,18] and it is described in Section 2. Table 1 summarizes the retention times as well as the qualification and quantification ions of the target analytes.

Different parameters affecting the pressurized liquid extraction (PLE) can be optimized in order to achieve fast and efficient extraction. In the usual working range for this technique, pressure generally has a negligible effect on the extraction yield [19], and so, we decided to conduct the experiments at 1500 psi, which is the standard operating pressure in PLE extractions [20]. Flush volume and purge time were set at 60% and 60 s, respectively. The influence of the remaining variables was studied using a multifactor strategy. The studied factors were: extraction temperature (factor A), extraction time (factor B), solvent (factor C) and dispersing sorbent (factor D) (see Table 2). Extraction temperature was studied at three levels from 80 to 120 ºC, whereas the other factors were studied at two levels. The second factor considered was the static extraction time that it was assessed at 5 and 15 min. The extraction solvent is one of the most important parameters to optimize in PLE. Two solvents were investigated, hexane / acetone (1:1, v/v), recommended in the 3545 EPA method [21], and ethyl acetate; both solvents with intermediate polarity that should be suitable for the varied range of polarities of the target analytes. The inclusion of an in situ clean-up step by adding certain sorbents to the PLE cells favours to the obtaining of clean extracts. In this way, lipids and other co-extractable materials are prevented from coming out to the extract. In addition,

these materials can act as dispersing phase, contributing to the consecution of a more efficient extraction. Thus, 2 g of dispersing sorbent (C18 or florisil) were mixed with the sample and packed in the cell.

Table 2. Factors and levels considered in the experimental design

Factor	Key	Levels		
		Lower (-)	Intermediate	Upper (+)
Temperature (°C)	A	80	100	120
Time (min)	B	5		15
Solvent	C	Hexane/Acetone		Ethyl
Dispersing sorbent	D	C18		Florisil

The study consisted of a $3\times2^{3-1}$ mixed level fraction factorial design, involving 12 randomized experiments. Experiments were performed using 1 g of a real moisturizing cream sample containing some of the target analytes (pinene, limonene, linalool, citronellol, geraniol, coumarin, ionone, lilial®, hexyl cinnamal, and benzyl salicylate) and fortified with all compounds at 100 μg g^{-1}. Since drying of the sample is essential for an efficient PLE, in all experiments 2 g of anhydrous sodium sulphate were added. Sand was employed to avoid dead volume.

Numerical analysis of the results leads to the ANOVA results shown in Table 3. As it can be seen, temperature (factor A) and time (factor B) were significant for several analytes. In the cases that temperature was significant and the time was also significant. The extraction solvent (factor C) was significant for fewer compounds; and the last factor, the type of dispersing sorbent (factor D), was only significant for two of the most volatile compounds, pinene and limonene. However, the most important factor, which was significant for 25 out of 26 compounds, was a second order factor, the interaction time and extraction solvent (BC). This factor was also the most influential one (see *F*-values) for most of the analytes. Another interaction effect that must be considered is temperature and extraction solvent (AC), which was significant for 10 compounds. Finally, other interactions were less important and only significant in few cases.

Table 3. *F* ratios and *p* values obtained in the analysis of variance

	Main effects								Interactions													
	A: Temperature		B: Time		C: Solvent		D: Sorbent		AA		AB		AC		AD		BC		BD		CD	
	F ratio	*p* value	*F* ratio	*p* value	*F* ratio	*p* value	*F* ratio	*p* value	*F* ratio	*p* value	*F* ratio	*p* value	*F* ratio	*p* value	*F* ratio	*p* value	*F* ratio	*p* value	*F* ratio	*p* value	*F* ratio	*p* value
Pinene	1		37		4		447	+			114		257	+	111		782	+	42		1	
Limonene	281	+	439	+	284	+	640	+			860	+	2414	+	97		4167	+	60		14	
Benzyl alcohol	53		2		420	+	8		48		212	+	721	+			1322	+	87		41	
Linalool	1		1		9		4				8		14		5		87	+			3	
Methyl-2-octynoate	0.1		4		0.1		0.03		3		11		14				64	+	6			
Citronellol	100	+	24	+	8		1		4		60	+	22	+					16		36	+
Citral	12		14		15		1		27		51		83		3		397	+	18			
Geraniol	1		17		558	+	43		268	+	68		497	+	97		1128	+			172	+
Cinnamal	0.2		4		6		0.5				11		16		2		79	+	3			
Anise alcohol	1		17		9		3				24		53		13		300	+	9		14	
Hydroxycitronellal	0.01		5		4		1		2		3		7		1		26	+				
Cinnamyl alcohol	0.02		4		2		0.3		1		2		4		1		20	+				
Eugenol	0.3		7		1		0.5				5		11		3		53	+	2			
Methyleugenol	3		11		0.2		1				8		23	+	5		107	+	2			
Isoeugenol	0.1		28	+	0.4		4		3		14		18				90	+	19	+		
Coumarin	17		41	+	22	+	6		16						2		75	+	21	+	13	
α-Isomethyl ionone	0.4		8		1		0.1				4		16		3		45	+	2			
Lilial®	110		90		0.4		32		10		2		68				888	+	12		3	
Amyl cinnamal	405	+	598	+	6		33				53		688	+	322	+	3138	+	2		57	
Lyral®	49		237	+	29		36				36		50		47		305	+	7		37	
Amylcinnamyl alcohol	187	+	44		319	+	120		64		19		136		37		597	+			97	
Farnesol	24	+	29	+	10		2				15		15		2		42	+	5			
Hexyl cinnamal	17		51	+	0.4		5				10		35	+	13		182	+	1			
Benzyl benzoate	22	+	61	+	5		0.1				9		26	+	3		109	+	6			
Benzyl salicylate	2835	+	6720	+	409	+	76		202	+			6110	+	2694	+	13608	+	295	+	1252	+
Benzyl cinnamate	15		137		17		0.4		39		152		94				399	+	185	+	10	

+ cell, *p*-value<0.05; empty cell, *p*-value>0.05.

The information included in the ANOVA can be graphically plotted by means of the Pareto charts. In Fig. 1, some examples are showed. In these graphics the length of each bar is proportional to the absolute value of its associated standardized effect. The standardized effect is obtained by dividing the estimated effect of each factor or interaction by the standard error. Vertical line in the graphs represents the statistically significant bound at the 95% confidence level. We can clearly appreciate the notable influence of BC in all cases. Other significant factors were the interaction AC, and the main factors temperature (factor A) and extraction time (factor B).

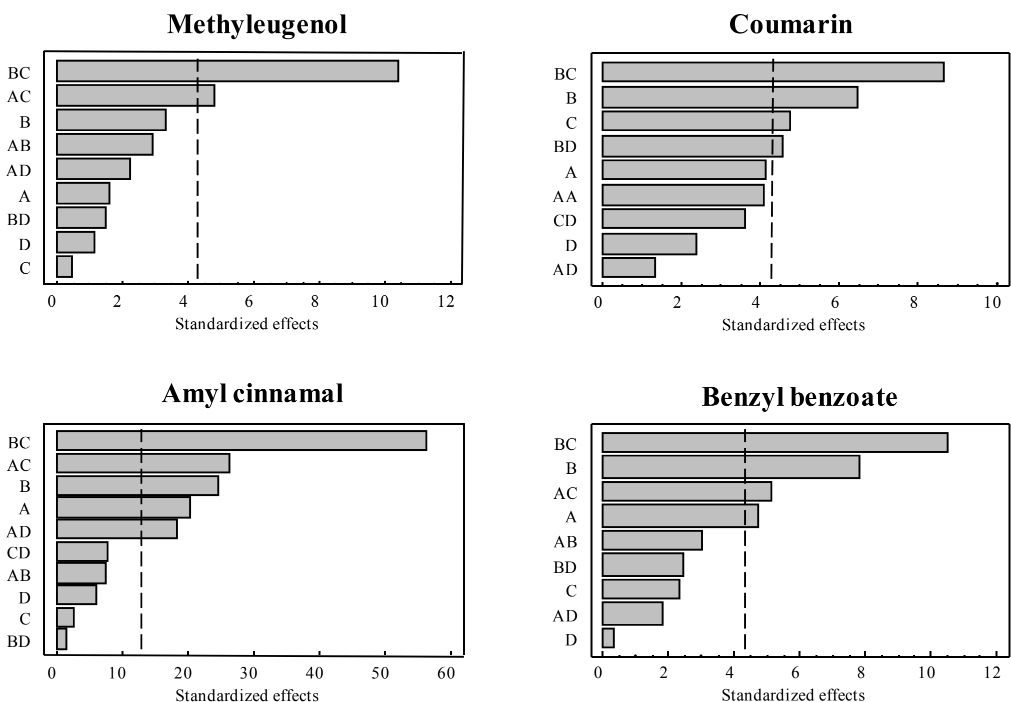

Fig. 1. Pareto charts showing the significant factors (95%) for some selected analytes (see factor codes in Table 2).

A very useful graphic option provided by the statistic software is the main effects plot. Fig. 2 shows the main effects diagrams for several representative compounds since the general behaviour was common in most cases. This kind of plots shows the main effects with a line drawn between the low and the high level of the corresponding factors. The length of the line is proportional to the effect

magnitude of each factor in the extraction process, and the sign of the slope indicates the level of the factor which produces the highest response. Regarding factors temperature and time, best extractions were generally obtained at the high level of the factors, which means at 120 ºC and 15 min. The solvent was only significant for six compounds, being for some of the analytes more favourable the use of hexane / acetone (see as example benzyl alcohol plot in Fig. 2), and for other compounds ethyl acetate (see coumarin plot). Nevertheless, this factor must be carefully analyzed since it is involved in the most important second order effects: its interaction with the temperature and the extraction time (AC and BC, respectively). Dispersing sorbent was non-significant and, therefore, characterized by a horizontal line, excluding the two most volatile compounds, for which C18 is more suitable than florisil.

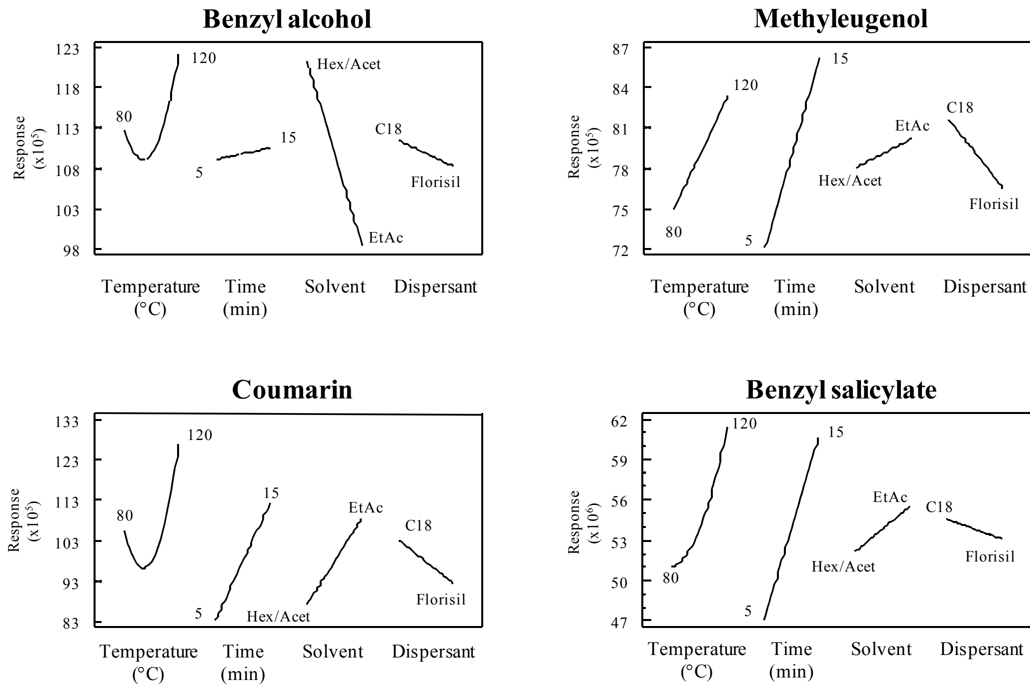

Fig. 2. Main effects plots for some representative fragrance allergens (Hex/Acet: hexane/acetone; EtAc: ethyl acetate).

As previously commented, the interaction effects must be considered before proposing a general method for the simultaneous extraction of the 26 fragrance allergens, and especially, time–solvent (BC) which was significant for 25 among 26 compounds. The most important second order effects are shown in Fig. 3 for some analytes, as example, since the trends were, in general, the same. Analyzing BC interaction, the most favourable extraction conditions implies the extraction with hexane / acetone for 15 min. Even for those compounds, such as coumarin or benzyl salicylate, for which ethyl acetate seemed more favourable after the analysis of only main factors (see Fig. 2), the analysis of second order factors, especially BC, shows the convenience of using hexane / acetone. Regarding AC interaction, the most favourable conditions consist on hexane/acetone extraction at 120 ºC. The other interaction effects were not very important with the exception of AD for benzyl salicylate, for which the most favourable conditions are the extraction at 120 ºC employing florisil as dispersing phase. In summary, the general conditions selected after the analysis of main and second order effects, involved the extraction at 120 ºC for 15 min, using hexane / acetone as solvent, and florisil as dispersing sorbent. Although the use of C18 would be also suitable, florisil was selected since this last dispersant, once it is mixed with the samples, was easier to manipulate as well as the lower prize compared to C18.

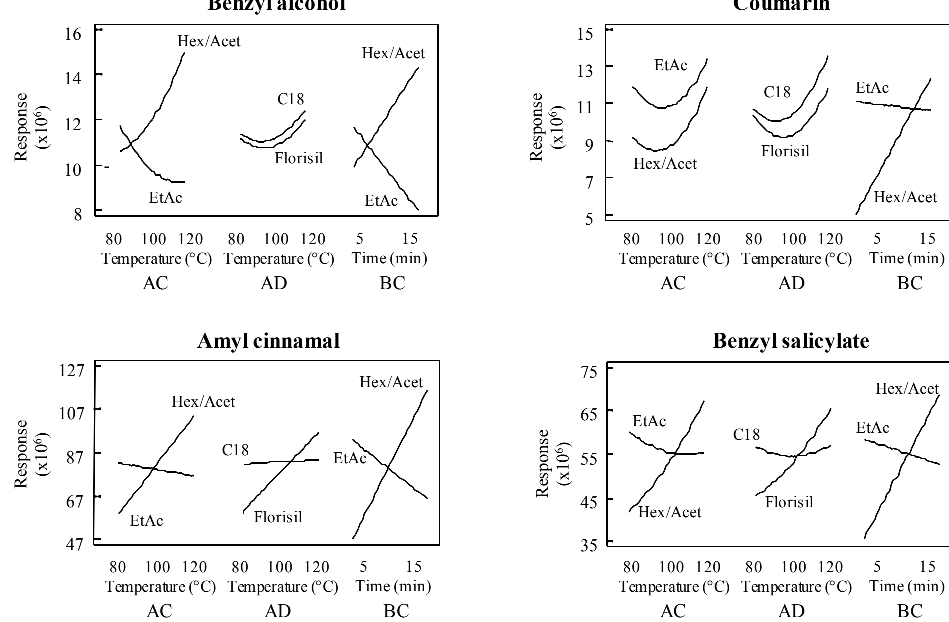

Fig. 3. Interaction effects plots: AC (temperature–solvent); AD (temperature–sorbent); and BC (time–solvent).

3.2. Method validation

Method quality parameters were estimated (Table 4). The instrumental linearity was evaluated at a concentration range between 0.02 and 10 μg mL^{-1} (including seven concentration levels). Each concentration level was injected in triplicate and the response function was found to be linear with correlation coefficients (*R*) higher than 0.996. Instrumental limits of detection (IDL) were calculated as the concentration giving a signal-to noise ratio of three (S/N = 3). Values ranged from 0.83 ng mL^{-1} (methyleugenol) to 25 ng mL^{-1} (citral) (see Table 4).

The other figures of merit were calculated using real cosmetic samples.

Recovery studies were carried out by applying the optimized PLE method to the extraction of cream samples spiked at two different levels, 15 and 75 μg g^{-1}. Previous analyses of this sample showed the presence of some of the target analytes, and these initial concentrations were taken into account to calculate the

recoveries. Recoveries were between 85 and 114% (see Table 4). Precision was also evaluated and RSD values were in all cases lower than 10% with an average value of 4.2%.

Table 4. Quality parameters of the method.

Compound	Correlation coefficient (*R*)	IDL (ng mL^{-1})	Recovery[a] (RSD) %		LOD (%, w/w)	LOQ (%, w/w)
			15 μg g^{-1}	75 μg g^{-1}		
Pinene	0.998	4.9	86.1 (4.4)	89.2 (5.0)	0.000052	0.00017
Limonene	0.998	6.0	105 (6.9)	105 (6.1)	0.0000083	0.000028
Benzyl alcohol	0.998	7.5	95.1 (7.4)	95.8 (1.0)	0.000012	0.000040
Linalool	0.999	6.2	98.7 (1.5)	110 (9.7)	0.0000085	0.000028
Methyl-2-octynoate	0.999	5.9	114 (9.2)	86.4 (3.1)	0.000014	0.000046
Citronellol	0.997	7.4	87.4 (9.2)	90.8 (7.2)	0.000043	0.00014
Citral	0.997	25	96.1 (2.5)	92.7 (4.4)	0.000026	0.000086
Geraniol	0.998	11	110 (9.5)	114 (4.2)	0.000021	0.000071
Cinnamal	0.999	6.0	90.4 (2.1)	85.1 (0.3)	0.000018	0.000061
Hydroxycitronellal	0.999	3.8	88.0 (1.4)	98.5 (0.2)	0.000011	0.000021
Anise alcohol	0.998	8.6	111 (0.3)	110 (6.8)	0.000017	0.000055
Cinnamyl alcohol	0.996	17	107 (3.0)	101 (2.2)	0.000021	0.000068
Eugenol	0.999	3.7	91.5 (6.8)	109 (0.6)	0.0000019	0.0000062
Methyleugenol	0.998	0.83	96.0 (4.0)	95.6 (8.0)	0.0000012	0.0000040
Isoeugenol	0.998	5.6	101 (0.8)	99.9 (2.8)	0.0000075	0.000025
Coumarin	0.998	1.5	112 (0.5)	92.0 (6.7)	0.0000036	0.000012
α-Isomethyl ionone	0.998	1.3	87.7 (1.6)	99.0 (4.4)	0.0000032	0.000011
Lilial®	0.999	4.7	97.2 (0.2)	106 (7.2)	0.0000076	0.000025
Amyl cinnamal	0.997	2.6	108 (0.8)	114 (0.5)	0.0000042	0.000014
Lyral®	0.997	5.6	113 (6.9)	91.3 (1.3)	0.000029	0.000097
Amylcinnamyl alcohol	0.998	3.9	91.0 (6.8)	94.0 (6.1)	0.000012	0.000039
Farnesol	0.998	22	85.9 (8.8)	88.3 (4.3)	0.00018	0.00060
Hexyl cinnamal	0.998	2.5	109 (2.6)	112 (1.1)	0.0000063	0.000021
Benzyl benzoate	0.999	1.7	100 (4.0)	85.6 (1.7)	0.0000073	0.000024
Benzyl salicylate	0.998	3.8	n.c.	102 (0.6)	0.0000095	0.000032
Benzyl cinnamate	0.999	6.0	90.4 (5.5)	86.3 (8.9)	0.000012	0.000039

n.c.: not calculated
[a] (n=3).

The limits of detection (LODs) and quantification (LOQs) corresponding to the overall method were calculated as the concentration giving a signal-to-noise ratio of three (S/N = 3) and ten (S/N = 10), respectively. These values are also summarized in Table 4, expressed as percentage (%, w/w) in order to be consequent with the units used in the European Cosmetics Regulation [4]. As it can be seen, the obtained LODs and LOQs are several orders of magnitude lower than the established restrictions (see Table 1); and it is important to emphasize that, if necessary, these limits can be easily reduced (at least one order of magnitude) by concentrating the PLE extract (~15 mL).

3.3. Application to real samples

The method was finally applied to the analysis of several real cosmetic samples including moisturizing creams and lotions, sun-screen and after-sun creams, anti-wrinkle, and hand creams. The PSE extracts were directly analyzed without any further concentration step. In some cases, the extract was properly diluted due to the high concentration of some of the analytes in several samples. Results are shown in Table 5. Fig. 4 shows the extracted ion chromatograms obtained for a moisturizing cream (MC1). Found concentrations ranged from 0.00006% (methyleugenol in sample HC1) to 0.23% (hexyl cinnamal in MC2). Half of the samples contained an elevated number of the studied compounds; in fact, four of the samples included more than eight fragrance allergens. Three compounds were detected in two samples (limonene and benzyl alcohol in HC2, and citral in SC) labelled as "fragrance free", although the calculated concentrations were below the limits established in the European Cosmetics Regulation [4]. Only six of the target fragrances (methyl-2-octynoate, cinnamal, anise alcohol, amyl cinnamal, amylcinnamyl alcohol, and benzyl cinnamate) were not detected in any sample. Limonene was present in seven out often samples, in some cases at quite high concentrations (see concentration values for MC1, MC2, and ML in Table 5). Total fragrance allergen content in the samples almost reached the 1% (0.73%) in some case, with an average value of 0.15%.

Table 5. Analysis of real cosmetic samples (MC: moisturizing cream; ML: moisturizing lotion; AW: anti-wrinkle cream; HC: hands cream; SC: sunscreen cream; AS: after-sun cream).

	Concentration (%, w/w)									
	MC1	MC2	MC3	ML	AW1	AW2	HC1	HC2	SC	AS
Pinene	0.00073			0.00030	0.00121					
Limonene[a]	0.02052	0.07904	0.00638	0.01990	0.00050		0.00358	0.00019		
Benzyl alcohol[a]			0.00023	0.00032			0.00433	0.00014		
Linalool[a]	0.06590	0.20321	0.00883	0.01118						
Citronellol[a]	0.00450			0.00101			0.00196			
Citral[a]		0.00114	0.00036	0.00581			0.00192		0.00049	
Geraniol[a]	0.01516						0.00128			
Hydroxy-citronellal[a]				0.00216						
Cinnamyl alcohol[a]				0.00074		0.00101				
Eugenol[a]				0.00379	0.00023		0.00027			
Methyleugenol[b]							0.00006			
Isoeugenol[a]					0.00029		0.00012			
Coumarin[a]	0.00211		0.00030	0.00134						
α-Isomethyl ionone[a]	0.00673			0.01511		0.00099	0.00175			
Lilial®[a]	0.19343	0.19835					0.06534			
Lyral®[a]		0.00314								
Farnesol[a]										0.00684
Hexyl cinnamal[a]	0.00369	0.23213		0.02100			0.00859			
Benzyl benzoate[a]		0.01248					0.00486			
Benzyl salicylate[a]	0.12932	0.00019	0.13440							
Total content	0.44210	0.72967	0.15050	0.08265	0.00223	0.00200	0.09406	0.00033	0.00049	0.00684

[a] According to Regulation (EC) No 1223/2009, the presence of the substance must be indicated in the list of ingredients when its concentration exceeds 0.001% in leave-on products.

[b] Maximum allowed concentration in fragrance cream: 0.002%, and other leave-on products: 0.0002%. Blank cells mean values below LODs.

As it was commented in Section 1, the presence of these ingredients must be included in the cosmetic label when its concentration exceeds 0.001% (w/w) in ready for use preparation, in the case of leave-on products. The labelling in the samples containing some of these compounds was in consonance with the actual European Cosmetics Regulation.

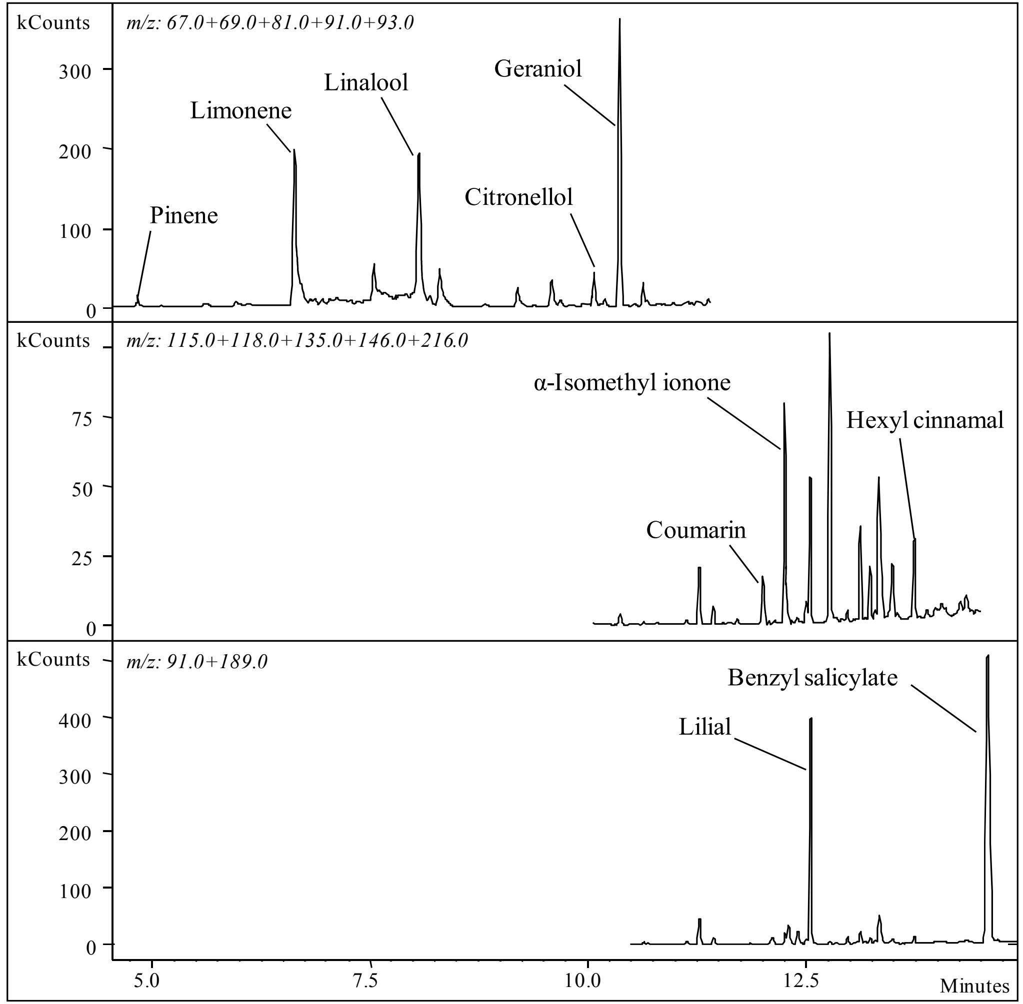

Fig. 4. Extracted ion chromatograms of sample MC1 (see concentrations in Table 5).

4. Conclusions

PLE followed by gas chromatography-mass spectrometry (GC-MS) is applied for the simultaneous determination of 26 fragrance allergens in multi-matrix cosmetic samples. This is the first application of PLE to the analysis of cosmetics as well as to the analysis of fragrance allergens. The direct GC-MS analysis without any further step was possible since the obtained extracts were homogeneous and clear, and matrix interferences were not observed in any case. The absence of matrix

effect allowed the use of calibration with standard solutions avoiding, in this way, the need of standard addition based quantification procedures. The obtained LODs are far below the established restrictions in Cosmetic Regulations, making this analytical method suitable for routine control. The reliability of the method was demonstrated through a broad range of leave-on cosmetics. The ubiquity of these compounds was demonstrated since they were present in all the analyzed samples and, in most cases, a quite high number of fragrance allergens per sample were detected.

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References

1. A.C. De Groot, P.J. Frosch, Contact Dermatitis 36 (1997) 57.
2. J.D. Johansen, Am. J. Clin. Dermatol. 4 (2003) 789.
3. D.R. Bickers, P. Calow, H.A. Greim, J.M. Hanifin, A.E. Rogers, J.H. Saurat, I.G. Sipes, R.L. Smith, H. Tagami, Regul. Toxicol. Pharm. 37 (2003) 218.
4. Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products (recast), Off. J. Eur. Union L342/59 (2009).
5. Ingredients Prohibited & Restricted by FDA Regulations, http://www.fda.gov/Cosmetics/ProductandIngredientSafety/SelectedCosmeticIngredients/ucm127406.htm , accessed July 2010.
6. Standards for Cosmetics, Ministry of Health and Welfare, http://www.mhlw.go.jp/english/topics/cosmetics/index.html , accessed July 2010.
7. R. Shellie, P. Marriott, A. Chaintreau, Flavour Frag. J. 19 (2004) 91.

8. H. Leijs, J. Broekhans, L. van Pelt, C. Mussinan, J. Agric. Food Chem. 53 (2005) 5487.
9. L. Mondello, D. Sciarrone, A. Casilli, P.Q. Tranchida, P. Dugo, G. Dugo, J. Sep. Sci. 30 (2007) 1905.
10. F. David, C. Devos, D. Joulain, A. Chaintreau, P. Sandra, J. Sep. Sci. 29 (2006) 1587.
11. C. Villa, R. Gambaro, E. Mariani, S. Dorato, J. Pharmaceut. Biomed. 44 (2007) 755.
12. M. Niederer, R. Bollhalder, C. Hohl, J. Chromatogr. A 1132 (2006) 109.
13. M. del Nogal Sánchez, J. Pérez-Pavón, B. Moreno Cordero, Anal. Bioanal. Chem. 397 (2010) 2579.
14. R. Rodil, M. Moeder, Anal. Chim. Acta 612 (2008) 152.
15. A. Nieto, F. Borrull, R.M. Marcé, E. Pocurull, J. Chromatogr. A 1216 (2009) 5619.
16. A. Nieto, F. Borrull, E. Pocurull, R.M. Marcé, TrAC Trends Anal. Chem. 29 (2010) 752.
17. J.P. Lamas, L. Sanchez-Prado, C. Garcia-Jares, M. Llompart, J. Chromatogr. A 1217 (2010) 1882.
18. J.P. Lamas, L. Sanchez-Prado, M. Lores, C. Garcia-Jares, M. Llompart, J. Chromatogr. A 1217 (2010) 5307.
19. R. Carabias-Martínez, E. Rodríguez-Gonzalo, P. Revilla-Ruiz, J. Hernández-Méndez, J. Chromatogr. A 1089 (2005) 1.
20. Methods Optimization in Accelerated Solvent Extraction (ASE®), Technical Note 208, Dionex Corporation, Sunnyvale, CA (2004).
21. EPA Method 3545, Pressurised fluid extraction, in: Test Methods for Evaluating Solid Waste, Physical/Chemical Methods, 3rd ed., U.S. GPO, Washington, DC, 2008, Final Update IV; EPA SW-846.

1.3 SIMULTANEOUS IN-CELL DERIVATIZATION PRESSURIZED LIQUID EXTRACTION FOR THE DETERMINATION OF MULTICLASS PRESERVATIVES IN LEAVE-ON COSMETICS

SIMULTANEOUS IN-CELL DERIVATIZATION PRESSURIZED LIQUID EXTRACTION FOR THE DETERMINATION OF MULTICLASS PRESERVATIVES IN LEAVE-ON COSMETICS

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Abstract

An effective one-step sample preparation methodology for the determination of multiclass preservatives in cosmetics has been developed, applying, for the first time to this kind of matrix, pressurized liquid extraction (PLE) and a very simple, cheap, and fast derivatization procedure: acetylation with acetic anhydride and pyridine. A multifactorial experimental design has been used to evaluate and optimize the main experimental parameters potentially affecting the extraction process. In the final conditions the sample was mixed with Florisil as the dispersing sorbent and extracted with ethyl acetate for 15 min at 120 ºC. One of the main goals of this work was to demonstrate the possibility of carrying out direct cosmetic preservative acetylation by simply adding the derivatization reagents into the PLE cell. The extract was then analyzed by GC / MS without any further cleanup or concentration step. The accuracy, precision, linearity, and detection limits (LODs) were evaluated to assess the performance of the proposed method. Quantitative recoveries were obtained, and relative standard deviation values were lower than 10% in all cases. The obtained LODs ranged from 0.000004% to 0.0001% (w/w), values far below the established restrictions in the European Cosmetics Regulation, making this multicomponent analytical method suitable for routine control. Finally, several cosmetic products such as moisturizing and antiwrinkle creams and lotions, hand creams, sunscreen and after-sun creams, baby lotions, and hair care products were analyzed. All the samples contained several of the target cosmetic ingredients, in some cases at quite high concentrations, although the actual European Cosmetics Regulation was fulfilled in all cases.

INTRODUCTION

Preservatives are substances added to cosmetics for the primary purpose of inhibiting the development of microorganisms (antimicrobial function), but may also be added to protect such products against damage and degradation caused by the exposure to oxygen (antioxidant function).

The esters of *p*-hydroxybenzoic acid (parabens), iodopropynyl butylcarbamate (IPBC), 2,4,4′-trichloro-2′-hydroxydiphenyl ether (triclosan, TCS), and bromine-containing preservatives such as 5-bromo-5-nitro-1,3-dioxane (Bronidox) and 2-bromo-2-nitropropane- 1,3-diol (Bronopol) are included in a wide variety of cosmetics and personal-care products to prevent or retard bacterial growth. Parabens are the most widely used antimicrobial preservatives in cosmetic products. Their antimicrobial activity is generally selective, so their mixtures or mixtures with other classes of preservatives offer powerful antimicrobial activity against an extremely broad spectrum of microorganisms.[1]

2-*tert*-Butyl-4-methoxyphenol (BHA) and 2,6-bis(1,1-dimethylethyl)-4-methylphenol (BHT) are antioxidant preservatives frequently used to prevent oxidation in foods and cosmetics. The use of mixtures of both of them is very common since there is a synergic increase of their antioxidant power.[2]

Together with the positive protective effects of cosmetic preservatives, unintended possible side effects of these ingredients are a matter of concern, because exposure to some of these compounds could have harmful effects on human health. Some of these ingredients, such as parabens and BHA, may modulate and disrupt the endocrine system,[3] IPBC could cause acute inhalation toxicity,[4] and some compounds such as BHA or some transformation products of triclosan, Bronidox, and Bronopol are even suspected carcinogenics.[5-7] There is also current scientific evidence that indicates that the use or misuse of biocidal products may contribute to the increased occurrence of antibiotic-resistant bacteria, both in humans and in the environment.[8]

To ensure a high level of protection of human health, cosmetic products are regulated and controlled worldwide. The new European Union (EU) Cosmetic Products Regulation[9] (which is, to a great extent, a recast of the previous Cosmetics Directive[10] and its successive amendments and adaptations), the federal Food, Drug

and Cosmetic Act (FD&C Act) and the Fair Packaging and Labeling Act (FPLA) drawn up by the Food and Drug Administration (FDA) in the United States, and, finally, the Pharmaceutical Affairs Law (PAL) adopted in Japan constitute the three main regulatory systems on cosmetic products. The preservatives allowed in the EU context are listed in Annex V of the EU Cosmetics Regulation,[9] where limitations, requirements, label warnings, and the maximum permissible concentrations are indicated (see Table S-1 (Supporting Information) for the target preservatives of this study). In Japanese legislation there is also a positive list of preservatives, but the allowed substances and authorized contents are quite different.[11] In the U.S. framework there is not a positive list of preservatives, although there is a short list of substances, published by the FDA, banned or restricted in cosmetics, including different compounds formerly used as preservatives.[1]

Thus, to protect consumer health and ensure compliance to existing government regulations, there is a need for the development of effective and convenient methodologies to identify and determine preservatives in cosmetics both accurately and sensitively.

A great part of the analytical effort has been focused on paraben determination,[12-15] while methods for the determination of other preservatives in cosmetic formulations are very limited or inexistent. However, multicomponent analytical methods are required since cosmetic products very often contain mixtures of preservatives belonging to different chemical classes. Simultaneous analysis of more than one class of preservatives is scarce and mainly based on liquid chromatography (LC)[16-18] and capillary electrophoresis (CE).[19,20] Flow injection analysis (FIA) has also been employed, enhancing sample throughput.[21]

In most of these procedures, sample preparation is usually performed through several steps which can include solvent extraction or dilution, mixing, sonication, heating, addition of acids or bases, centrifugation, and filtration. These procedures are frequently tedious and time-consuming, and the use of hazardous solvents is usually required. In addition, the possible presence of interferences that could distort the results is not rejectable. To overcome some of these drawbacks, supercritical fluid extraction (SFE),[16,22] solid-phase extraction (SPE),[12] and solid-phase microextraction (SPME)[23] have been recently applied for the determination of different additives in cosmetics. Pressurized liquid extraction (PLE) has been applied

for the analysis of cosmetic ingredients (parabens and TCS, among them) in environmental matrixes, such as sewage sludge.[24,25] PLE is fast, increases automation, decreases the amount of organic solvents, and offers the possibility of controlling the selectivity of the extraction by loading different sorbents instead of inert materials into the extraction cell.

Due to the polar nature of most preservatives, a derivatization step previous to gas chromatography (GC) analysis is highly recommended to reduce adsorption in the chromatographic system and improve sensitivity, peak separation, and peak symmetry.[14,22] Acetylation is one of the most common derivatization procedures for phenolic compounds,[26,27] and it has been applied for the determination of parabens and triclosan in water,[27,28] but to our knowledge, this derivatization procedure has never before been employed for cosmetic samples. The advantages of acetylation are the high efficiency obtained using low-cost reagents, especially compared with silylation agents. The aim of this work is to develop a method based on PLE with acetylation followed by gas chromatography/mass spectrometry (GC / MS) for the simultaneous determination of different classes of preservatives including two bromine-containing preservatives, seven parabens, IPBC, TCS, and the antioxidant preservatives BHA and BHT in multimatrix cosmetic samples. The possibility of performing simultaneous derivatization and extraction by adding the acetylation reagents into the PLE cell will be evaluated. To our knowledge, both acetylation and PLE are applied for the first time to the analysis of cosmetics.

MATERIALS AND METHODS

Chemicals.

Bronidox (≥99.0%) was acquired from Fluka (Buchs, Switzerland). Bronopol (98%), methylparaben (99%, MeP), ethylparaben (99%, EtP), propylparaben (99%, PrP), butylparaben (99%, BuP), benzylparaben (99%, BzP), butylated hydroxyanisole (≥98.5%, BHA), butylated hydroxytoluene (99%, BHT), IPBC (97%), and triclosan (≥97.0%, TCS) were purchased from Aldrich (Milwaukee, WI). Isopropylparaben (≥99%, iPrP) and isobutylparaben (≥97%, iBuP) were purchased from TCI Europe (Belgium). Table S-1 (Supporting Information) shows the IUPAC names and chemical structures of the studied compounds.

Deuterated methyl 4-hydroxybenzoate-2,3,5,6-*d4* ($MePd_4$, 98.3 atom % D) was obtained from C/D/N Isotopes (Quebec, Canada). The internal standard PCB-30 (2,4,6-trichlorobiphenyl) was purchased from Dr. Ehrenstorfer (Augsburg, Germany). Acetone, ethyl acetate, *n*-hexane, pyridine, and acetic anhydride (Ac_2O) were provided by Merck (Darmstadt, Germany). Florisil (60-100 mesh) and C18 (70-230 mesh) were obtained from Aldrich (Milwaukee, WI). Before being used, Florisil was activated at 130 ºC for 12 h and then allowed to cool in a desiccator. Sodium sulfate anhydrous (99%) was purchased by Panreac (Barcelona, Spain). Individual stock solutions of each compound were prepared in acetone. Further dilutions and mixtures were prepared in acetone, hexane, hexane/acetone (1:1, v/v), and ethyl acetate. All solutions were stored in amber glass vials at -20 ºC. All solvents and reagents were of analytical grade.

Acetylation was carried out by adding 100 *μL* of acetic anhydride containing 2.5% pyridine to 1 mL of the standard or extract solutions. The mixture was then maintained at 80 ºC for 30 min and then allowed to cool to room temperature.

Cosmetic Samples.

Different cosmetics from national and global companies were purchased from local stores. They included moisturizing and antiwrinkle creams and lotions, hand creams, sunscreen and after-sun creams, and baby lotions. Two products for hair care were also considered. Samples were kept in their original containers at room temperature until their analysis.

A 0.5 g portion of cosmetic sample was weighed exactly into a 10 mL glass vial. When it was necessary, the sample was spiked with 50 *μ*L of the corresponding acetone solution of the target compounds to get the desired final concentration in the cosmetic sample. The sample was then mixed with 1 g of a drying agent (anhydrous Na_2SO_4) and 1 g of dispersing sorbent (C18 or Florisil).

PLE and Derivatization Procedures.

Extractions were performed on an ASE 200 system (Dionex Co., Sunnyvale, CA) equipped with a 24-sample carousel, 11 mL stainless steel cells, and 40 mL collection vials. Two cellulose filters (Dionex) were placed at each end of the PLE cell. The sample, mixed with the drying agent and the dispersing sorbent, was introduced into the cell, where previously 1 g of clean sand (50-70 mesh particle size, Sigma-Aldrich) was placed. In all experiments, 20 μL of $MePd_4$ surrogate solution (2500 μg mL^{-1}) was added to each sample before extraction. Finally, the dead volume of the cell was filled with sand. The cell was tightly closed and placed into the carousel of the ASE system. Extractions were performed by preheating the cell before filling with solvent (preheat method). The extraction pressure was set to 1500 psi, the flush volume was 60%, and the purge time was set to 60 s. Hexane/acetone (1:1, v/v) or ethyl acetate was employed as the extraction solvent, depending on the experiment. The extraction temperature and extraction time varied during the optimization of the method. After extraction, 20 μL of PCB 30 (100 μg mL^{-1}) was added to the final extract (~15 mL) to correct possible variations of the extract volume. Then PLE extracts were derivatized and analyzed by GC/MS.

In the simultaneous derivatization-extraction experiments, 100 μL of acetic anhydride containing 2.5% pyridine was added to the cosmetic sample before the addition of the drying agent and the dispersing sorbent. Then the PLE procedure previously described was carried out. Finally, the extracts were directly analyzed since in-cell derivatization was accomplished during extraction. GC / MS Analysis. Analyses were performed on a Varian CP 3900 gas chromatograph (Varian Chromatography Systems, Walnut Creek, CA) equipped with a 1177 split/splitless injector and an ion trap spectrometer, Varian Saturn 2100 (Varian Chromatography Systems). Separation was carried out on an HP5 capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) from Agilent Technologies (Palo Alto, CA). Helium (purity 99.999%) was employed as the carrier gas at a constant column flow of 0.8 mL min^{-1}. Two different GC oven temperature programs were tested. The first was used for the derivatization studies, and it consisted of the following: 45 ºC (held 2 min) to 100 ºC at 8 ºC min^{-1}, to 150 ºC at 20 ºC min^{-1}, to 200 ºC at 25 ºC min^{-1} (held 5 min), to 220 ºC (held 1 min) at 8 ºC min^{-1}, and a final ramp to 260 ºC (held 7 min) at 30 ºC min^{-1}. The second program was optimized to keep good resolution of the target compounds, increasing the sample throughput: 60 ºC (held 2 min) to 200

ºC at 30 ºC min^{-1} and a final ramp to 260 ºC (held 4 min) at 40 ºC min^{-1} (total analysis time 15 min). The injector was programmed to return to the split mode after 2 min from the beginning of a run. The split flow was set at 20 mL min^{-1}. The injector temperature was held constant at 260 ºC. The trap, manifold, and transfer-line temperatures were 220, 120, and 280 ºC, respectively. The GC/MS system was operated by Saturn GC / MS Workstation v5.52 software. In the full scan mode the mass range was varied from 50 to 320 *m/z* at 0.6 s $scan^{-1}$, starting at 4 min and ending at 15 min. The filament emission current was 15 μA. The analytes were positively identified by comparison of their mass spectra and retention times to those of the standards.

Statistical Analysis.

Basic and descriptive statistics and experimental design analysis were performed using Statgraphics- Plus v5.1 (Manugistics, Rockville, MD) as the software package. The experimental design was applied in the optimization of the extraction method to analyze the simultaneous effect of the main parameters affecting PLE.

RESULTS AND DISCUSSION

Derivatization and GC/MS Analysis.

Optimization of the chromatographic conditions was accomplished using a standard mixture solution of all target compounds in *n*-hexane. Direct analysis produced peaks with appreciable tailing for most compounds due to the interaction of hydroxyl groups with the chromatographic system. Therefore, a derivatization step was introduced prior to GC determination to improve the chromatographic analysis. Acetylation with acetic anhydride is one of the most simple and cheap derivatization procedures for phenolic compounds. The procedure to obtain standard solutions of the corresponding acetylated compounds was based on a previous work dealing with the acetylation of other phenolic species[26] and a recent study including some of the target compounds,[28] and it is described in the Materials and Methods.

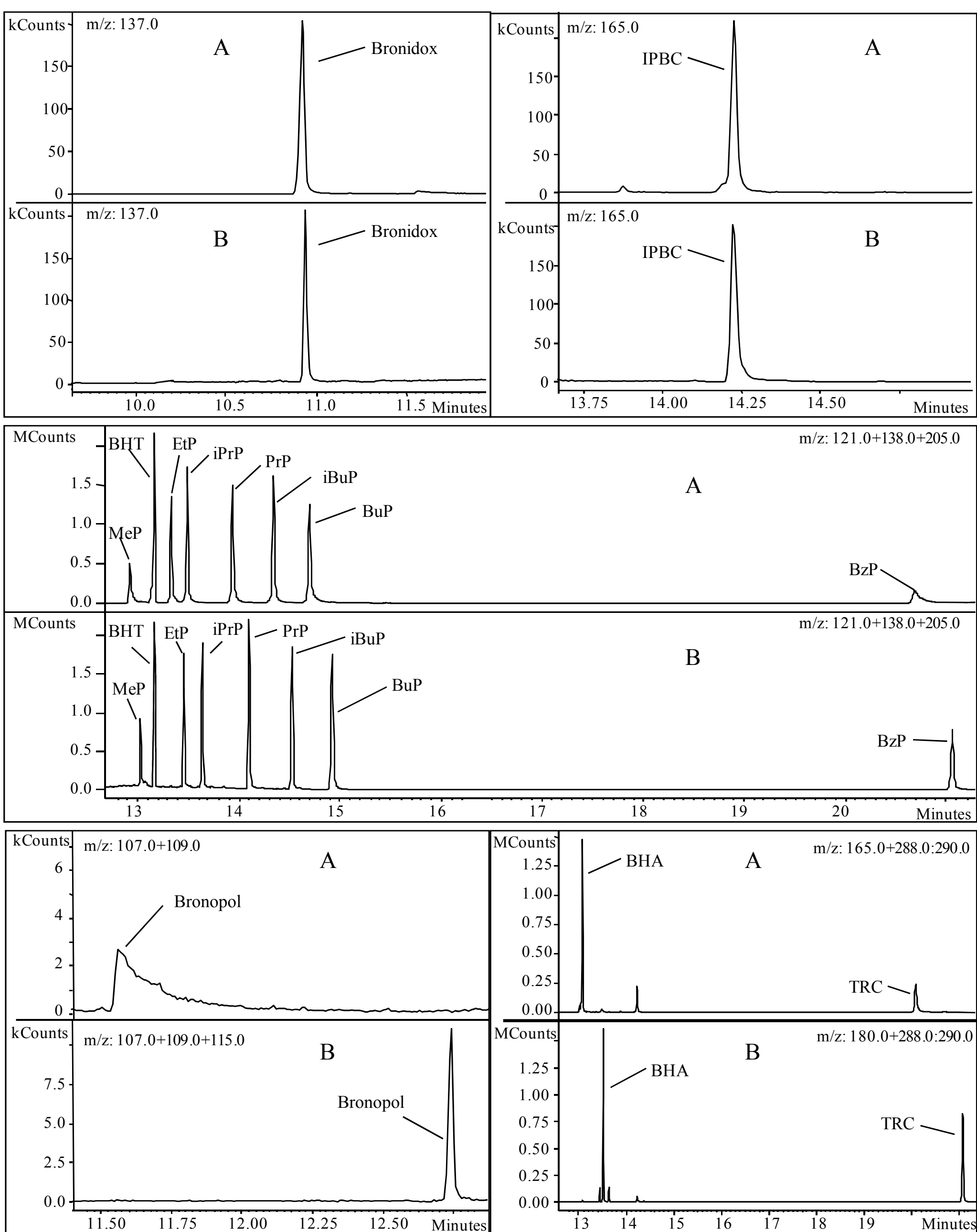

Figure 1. Extracted ion chromatograms corresponding to a 10 μg mL^{-1} solution of the target analytes before (A) and after (B) derivatization.

Different families of preservatives are studied in this work (Table S-1, Supporting Information), and for some of these compounds no previous studies on their acetylation reaction were found (e.g., Bronopol). It is necessary to ensure which compounds undergo derivatization and to demonstrate the chromatographic benefits of this reaction. Figure 1 shows the extracted ion chromatograms before (A) and after (B) acetylation, and Figure S-1 (Supporting Information) compares the chromatographic responses obtained. The retention times and the quantification and identification ions for the nonderivatized and derivatized analytes are included in Table S-2 (Supporting Information).

Bronidox and IPBC do not undergo derivatization since these compounds do not have chemical groups susceptible to acetylation; the retention times are not modified, and neither are their chromatographic responses (Figures 1 and S-1, Supporting Information).

On the contrary, parabens and triclosan are acetylated under selected conditions. This fact is confirmed because of the displacement of the retention times (see Figure 1), as well as the improvement in the peak shapes, since the tailing observed in the nonderivatized species disappears and peaks completely symmetric are obtained. This improvement is especially noticeable for MeP, BzP, and TCS; for these compounds, responses are also significantly higher (Figure S-1, Supporting Information). Additionally, small differences can also be observed in the obtained mass spectra. The ratio of ion intensities is slightly modified when the derivatization takes place (see as an example MeP in Figure S-2, Supporting Information). The molecular ions corresponding to the acetylated derivatives were not present in the mass spectra in most cases. This absence has been previously reported as a result of the loss of the acetyl group upon ionization.[28,29] Complete acetylation can be assured since nonderivatized species were not detected.

Regarding Bronopol, the effect of the derivatization on the peak shape and chromatographic response is much more evident as can be seen in Figures 1 and S-1 (Supporting Information). The retention time was also considerably modified (more than 1 min). In addition, the mass spectrum of the acetylated derivative differs significantly from the spectrum of Bronopol (see both spectra in Figure S-2). In this compound two hydroxyl groups are present (Table S-1), which means that the acetylation can take place in two reaction centers. In fact, in this case, the molecular

ion corresponding to the doubly acetylated compound was identified in the mass spectrum (*m/z* 283), confirming the above hypothesis. In addition, a cluster of ions, typical of bromine-containing compounds, around *m/z* 195 and 197 corresponding to the loss of two CH_3CO groups is present. The base peak (*m/z* 115) was formed by the subsequent loss of the bromine atom.

BHA also undergoes derivatization (see Figure 1). The most intense fragment ions for the acetylated derivative (see Figure S-2, Supporting Information) were also formed by the loss of CH_3CO, in such a way that the mass spectrum of the derivative was similar to that of the nonderivatized compound, with the exception of the ratio of ion intensities and the presence of the acetylated BHA molecular ion at *m/z* 222.

In the case of BHT, the acetylation could not be demonstrated since the retention time, peak shape, chromatographic response (Figure 1), and mass spectra were equivalent before and after the addition of the acetylation reagents. The highly hindered hydroxyl group with poor nucleophilicity (see the structure in Table S-1, Supporting Information) may prevent the acetylation under the studied conditions. This is in agreement with the study of Monsef-Mirzai,[30] who demonstrated that very hindered phenols, such as BHT, remain unacetylated. Anyway, the underivatized BHT peak shape and chromatographic response are both satisfactory.

In summary, three of the compounds (Bronidox, IPBC, and BHT) did not undergo derivatization. For the other compounds, the reaction yield was quantitative, since we could not find any trace of the underivatized analytes, and satisfactory, improving significantly the chromatographic analysis of the target compounds both qualitatively and quantitatively. The reaction was also carried out with standard solutions in ethyl acetate and hexane/acetone (1:1, v/v), demonstrating the suitability of these solvents to accomplish derivatization. The acetylated derivatives were stable for at least several weeks.

PLE Optimization.

The influence of the main variables potentially affecting the PLE process must be evaluated to obtain an efficient extraction. In the usual working range for this technique, the pressure generally has a negligible effect on the extraction yield,[31,32] so we decided to conduct the experiments at 1500 psi, which is the standard

operating pressure in PLE extractions.[33] The flush volume and purge time were set at 60% and 60 s, respectively. The influence of the remaining variables was studied using a multifactor strategy. The study consisted of a complete factorial 2^4 design, involving 16 randomized experiments and allowing 5 degrees of freedom to estimate the experimental error. This design has resolution V, which means that it is capable of evaluating all main effects and all two-factor interactions. Numerical analysis of data resulting from the experimental design was made with the statistical software package Statgraphics-Plus v5.1. The experiments were performed using 0.5 g of a real moisturizing cream sample initially labeled as containing some of the target compounds (Bronopol, MeP, BHT, and PrP) and fortified with all compounds at 100 μg g^{-1}. Since drying of the sample is essential for an efficient PLE, in all experiments 1 g of anhydrous sodium sulfate was added. Sand was employed to avoid dead volume. The studied factors were the extraction temperature (A), extraction solvent (B), dispersing sorbent (C), and extraction time (D).

The temperature factor (A) was studied at 80 and 120 ºC. The choice of an appropriate solvent is another essential aspect in the development of extraction methods. For an efficient extraction, the solvent must solubilize the target compounds while leaving the sample matrix as intact as possible.[33] Two solvents (factor B) were investigated: hexane/acetone (1:1, v/v) and ethyl acetate.

The inclusion of an in situ cleanup step by adding certain sorbents to the PLE cell contributes to obtaining clean extracts. In this way, lipids and other coextractable matrix materials are prevented from coming out to the extract. In addition, these sorbents can act as a dispersing phase, contributing to the execution of a more efficient extraction. Thus, 1 g of dispersing sorbent (factor C), C18 or Florisil, was mixed with the sample and packed in the PLE cell. The last factor considered was the extraction time (factor D), and it was assessed at 5 and 15 min.

The 16 experiments were carried out; after extraction, the extracts were acetylated at 80 ºC for 30 min before GC/MS analysis (see the Materials and Methods). Numerical analysis of the results obtained leads to the analysis of variance (ANOVA) results shown in Table 1. As can be seen, the most important factor, with statistical significance for most of the target compounds, is the extraction solvent. The extraction time was also significant for many analytes, whereas the temperature and the dispersing sorbent were each significant for five compounds. Some second-

order effects are also important, especially interactions AB (temperature-solvent) and BD (solvent-time).

Table 1. *F* Ratios and *p* Valuesa Obtained in the Analysis of Variance.

	Main effects								Interactions									
	A: Temperature		B: Solvent		C: Dispersant		D: Time		AB		AC		AD		BC		BD	
	F ratio	*p* value	*F* ratio	*p* value	*F* ratio	*p* value	*F* ratio	*p* value	*F* ratio	*p* value	*F* ratio	*p* value	*F* ratio	*p* value	*F* ratio	*p* value	*F* ratio	*p* value
Bronidox	20	+	11	+	8	+	1		18	+	8	+	22	+	3		21	+
Bronopol	5		15	+	0.3		4		6		5		1		0		6	
MeP	5		19	+	0.1		14	+	5		4		2		2		5	
BHA	16	+	28	+	2		40	+	10	+	9	+	0.01		3		13	+
BHT	1		1		0		2		5		0.2		1		1		1	
EtP	12	+	19	+	2		28	+	13	+	3		6		0.01		9	+
iPrP	7	+	10	+	1		17	+	7	+	2		3		0.01		8	+
PrP	0		31	+	4		6		1		0.2		1		0.1		1	
IPBC	8	+	40	+	37	+	19	+	12	+	4		5		0.01		10	+
iBuP	5		54	+	7	+	35	+	11	+	3		0.1		0.1		5	
BuP	6		36	+	9	+	41	+	12	+	6		2		0.3		7	+
BzP	1		0.5		7	+	11	+	1		0.3		0.2		0.2		11	+
TRC	1		1		6		3		0		1		1		0.4		12	+

+ cell, *P* value<0.05; empty cell, *P* value>0.05.

The information included in the ANOVA can be graphically plotted by means of the Pareto charts. In Figure S-3 (Supporting Information), some representative graphics are shown. The length of each bar is proportional to the absolute value of its associated standardized effect. The vertical line in the graphs represents the statistically significant bound at the 95% confidence level.

Figure 2 shows the main effects diagrams for several representative compounds. This kind of plot shows the main effects with a line drawn between the low and the high levels of the corresponding factors. The length of the lines is proportional to the effect magnitude of each factor in the extraction process, and the sign of the slope indicates the level of the factor that produces the highest response. Regarding the significant factors B and D (see the ANOVA in Table 1), the best extractions were obtained at the high level of the factors for all compounds, which means ethyl acetate and 15 min. The other two main factors A and C were significant for less compounds (Table 1) but, in those cases, were also characterized by a positive slope, so better extractions were also achieved at the high level of the factors, 120 ºC and Florisil.

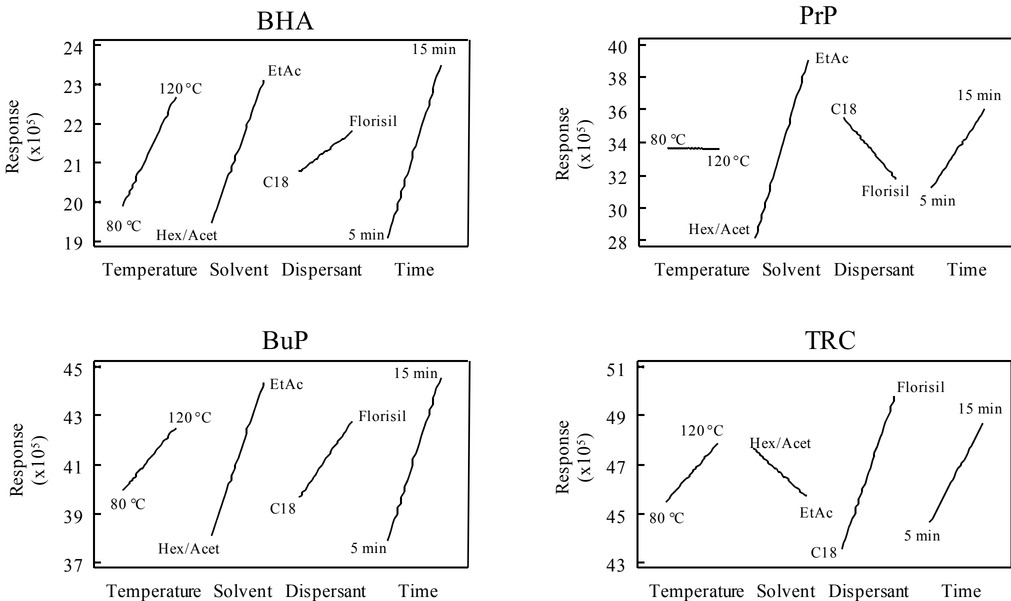

Figure 2. Main effects plots for some representative compounds (EtAc) ethyl acetate; (Hex/Acet) hexane/acetone.

Before a general method for the simultaneous extraction of the 13 target compounds is proposed, it is necessary to examine the interaction effects, since some of them, especially AB and BD (see Table 1), were significant for several analytes. These second-order effects are shown in Figure 3 for some analytes, as an example, since the trends were equivalent in all cases. Although the slopes of the lines are quite different, the lines do not intercept, so the general conditions established after analysis of the main effects do not change. Interaction AB shows again as the most favorable conditions the extraction at 120 ºC using ethyl acetate. Regarding the BD effect, the most favorable conditions were ethyl acetate and 15 min, although it is interesting to notice that in general the time is only significant when hexane/acetone is used. An exception to this behavior was BzP and TCS (see the BzP plot in Figure 3). For these two compounds, the most favorable conditions would involve the extraction with hexane/acetone for 15 min.

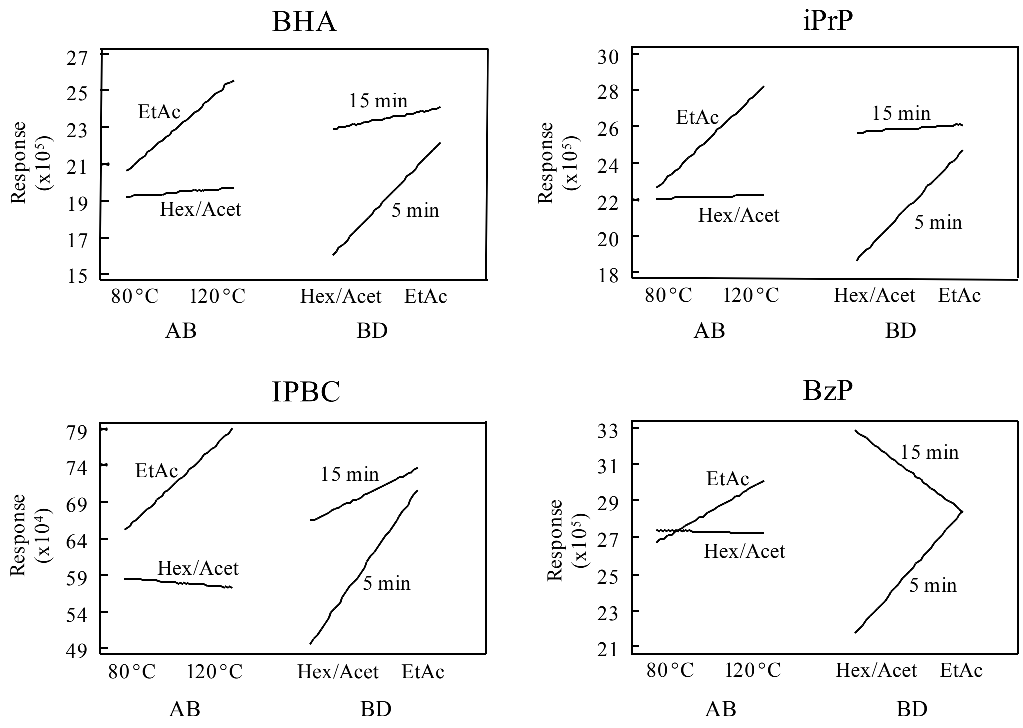

Figure 3. Interaction effects plots: AB (temperature-solvent) and BD (solvent-time).

In view of the results of the experimental design, the selected general conditions for the simultaneous extraction of the target preservatives and antioxidants were established as follows: extraction temperature of 120 ºC, ethyl acetate as solvent, Florisil as dispersing sorbent, and extraction time of 15 min.

Experiments were also run with the objective of studying the possibility of performing in-cell derivatization of the target compounds in the PLE cell. In the simultaneous derivatization-extraction experiments, 100 μL of acetic anhydride containing 2.5% pyridine was added to the cosmetic sample and the PLE procedure was carried out in the selected conditions indicated above. The initial results were fully satisfactory, obtaining equivalent extracts, and as a consequence, both processes, PLE followed by derivatization, as well as the simultaneous pressurized liquid derivatization- extraction, were considered for method validation.

Method Performance. Application to Real Samples.

Method quality parameters were evaluated (Table 2). The instrumental linearity was proved at a concentration range between 0.05 and 10 μg mL^{-1} (including six concentration levels) using derivatized standard solutions prepared in ethyl acetate (see the Materials and Methods). Each concentration level was injected in triplicate, and the response function was found to be linear with determination coefficients (R^2) higher than 0.9946.

Instrumental detection limits (IDLs) were calculated as the concentration giving a signal-to-noise ratio of 3 (S/N = 3). Values ranged from 0.41 to 18 ng mL^{-1}, as can be seen in Table 2.

The other figures of merit were calculated using real cosmetic samples.

Recovery studies were carried out by applying the optimized PLE method to the extraction of a real cream sample spiked at two different concentrations, 20 and 100 μg g^{-1}. Previous analyses of this sample showed the presence of some of the target compounds (see sample MC1 in Table 3), and these initial concentrations were taken into account to calculate the recoveries. As can be seen in Table 2, the recoveries were between 74% and 110% in all cases. The precision was also evaluated, and the relative standard deviation (RSD) values were lower than 10% with an average value of 4.2%.

As was commented, the possibility of performing simultaneous derivatization-extraction by adding the acetylation reagents in the PLE cell was also evaluated. Recoveries are also given in Table 2. As can be seen, the recoveries were satisfactory, with values ranging from 84% to 111%. The precision of the method expressed as the RSD was between 1% and 9%. Performing the combined derivatization-extraction process, the method quality parameters are equivalent and the method is even more simple and time saving.

Table 2. Quality Parameters of the Proposed Method[a]

	instrumental parameters		recovery[b] (RSD) % (n=3) derivatization after extraction		in-cell derivatization			
Compd	R^2	IDL (ng mL^{-1})	20 µg g^{-1}	100 µg g^{-1}	20 µg g^{-1}	100 µg g^{-1}	LODs (%, w/w)	LOQs (%, w/w)
Bronidox	0.9971	5.6	73.7 (1.5)	98.3 (2.7)	97.9 (1.5)	85.7 (9.0)	0.000094	0.00031
Bronopol	1.0000	18	n.c.	98.2 (7.3)	83.5 (3.7)	88.4 (1.9)	0.00015	0.00051
MeP	0.9991	1.0	n.c.	94.8 (9.5)	n.c.	113 (4.1)	0.0000053	0.000018
BHA	0.9996	1.2	110 (0.6)	93.0 (2.3)	87.9 (8.0)	90.1 (4.1)	0.0000081	0.000027
BHT	0.9994	0.41	91.0 (5.8)	98.1 (0.5)	107 (4.0)	105 (0.3)	0.0000041	0.000013
EtP	1.0000	1.4	95.5 (2.2)	101 (0.4)	109 (8.7)	111 (0.9)	0.0000080	0.000027
iPrP	0.9992	1.7	100 (7.0)	101 (0.8)	95.4 (8.1)	104 (1.8)	0.0000098	0.000033
PrP	0.9965	1.0	99.3 (8.4)	107 (0.1)	n.c.	89.7 (6.8)	0.0000058	0.000019
IPBC	0.9946	2.3	94.5 (5.3)	90.9 (5.2)	90.6 (7.9)	100 (2.7)	0.000085	0.00028
iBuP	0.9971	0.86	104 (6.0)	96.5 (1.6)	102 (6.4)	97.0 (4.8)	0.0000065	0.000022
BuP	0.9988	0.64	101 (4.2)	97.2 (2.3)	108 (1.1)	106 (5.1)	0.0000060	0.000020
BzP	0.9998	2.0	95.5 (7.2)	99.1 (9.1)	88.9 (4.1)	104 (1.3)	0.000068	0.00023
TRC	0.9977	0.73	109 (4.5)	110 (5.3)	93.6 (8.4)	111 (2.9)	0.000040	0.00013

[a] *n.c.*: no calculated since the concentrations in the sample were much higher than the added concentration. [b] Real sample MC1 (Table 3) was employed in the recovery studies.

The limits of detection (LODs) and quantification (LOQs) of the overall method were calculated as the compound concentration giving a signal-to-noise ratio of 3 (S/N = 3) and 10 (S/N = 10), respectively. These values are shown in Table 2, expressed as a percentage (w/w) to be equivalent with the units used in the European Cosmetics Regulation.9 The obtained limits are much lower than the established restrictions (see Table S-1, Supporting Information), and it is important to emphasize that, if necessary, these limits could be easily reduced (by at least 1 order of magnitude) by concentrating the PLE extract (~15 mL).

Finally, the method was applied to the analysis of real cosmetic samples including moisturizing creams (MCs) and lotions (MLs), antiwrinkle (AW) creams, hand creams (HCs), sunscreen creams (SCs), after-sun (AS) creams, baby lotions (BLs), and hair conditioning (CO) and shampoo (SH) products. The results are shown in Table 3. The extracted ion chromatogram of sample HC1 is shown in Figure S-4 (Supporting Information). For all the samples, the recoveries of $MePd_4$ (surrogate standard) were satisfactory, with values ranging from 83.7 to 115 (see the first row, Table 3). As commented in the first section of this paper, the presence of these

ingredients must be included in the cosmetic label, and these levels cannot exceed the regulated limit in each case. Regarding parabens, the compounds mainly found were MeP and PrP; both compounds are usually associated with an increase in the preservative activity. EtP, iBuP, and BuP were also found in the samples but much less frequently. The maximum allowed concentration of parabens in ready for use preparations is 0.4% for a single ester and 0.8% for mixtures of esters, expressed as acid (see the European Cosmetics Regulation limits in Table S-1). For this reason, the total content of parabens in the samples was determined and expressed as a percentage (w/w) of acid, being included in the last row of Table 3. All samples presented paraben concentrations below the legal limits, although one of the samples, a baby moisturizing lotion (BL2), was close to the total paraben maximum concentration limit. Most of the samples were correctly labeled with the exception of EtP, iBuP, and BuP in HC2, PrP in AW1 and MC5, and iPrP in CO, which were not included in the label. The antioxidant BHT was found in most of the samples, whereas BHA was found in four samples, in two of them associated with BHT, which increases the antioxidant power due to the synergism. Although there is some concern about the safety of both compounds, there are no restrictions about their use in cosmetic formulations. The presence of BHT and BHA was not indicated in the label with the exception of HC2 and BL1. IPBC was found in one rinse-off product (SH), and it was included in the product label. Finally, Bronopol was detected in one leave-on cosmetic (MC1), and in this case, it was also listed as an ingredient.

Table 3. Analysis of Real Cosmetic Samples (%, w/w)[a]

	MC1	MC2	MC3	MC4	MC5	ML1	ML2	AW1	AW2	HC1	HC2	SC	AS	BL1	BL2	CO	SH
$MePd_4$[b]	103	92	100	115	91.5	106	94	109	88.9	96.8	107	83.7	109	97.7	105	95.1	94.5
Bronidox																	
Bronopol	0.00703					0.00093											
MeP	0.05468	0.05038	0.0641	0.17033	0.00134	0.06291	0.30297		0.00441	0.04224	0.10207	0.16808		0.00488	0.25291	0.23893	
BHA					0.00031				0.00152		0.00015		0.00047				
BHT	2.7E-05	1.2E-05	1.5E-05				0.00001		0.00268	0.03542	0.00091	0.00011		0.03471	0.00007	0.00032	
EtP		0.01519	0.01825		0.00062					0.02445	0.00034	0.06925			0.1044		
iPrP																0.00195	
PrP	0.00884	0.00638	0.00824	0.02211	0.00112	0.00747	0.13025	0.00011		0.00786	0.01148			0.1583	0.24074	0.1229	
IPBC																	0.0015
iBuP		0.00603	0.00665							0.00811	7.3E-05					0.00021	
BuP		0.00949	0.01148	0.02938						0.00763	0.00023			0.00019	0.01293	0.00022	
BzP			<LOQ														
TRC																	
Total parabens as acid	0.05642	0.07429	0.09258	0.19249	0.00208	0.06284	0.37492	8.5E-05	0.00401	0.0759	0.10197	0.21017		0.12593	0.51017	0.31295	

[a] MC = moisturizing cream, ML = moisturizing lotion, AW = antiwrinkle cream, HC = hands cream, SC = sunscreen cream, AS = after-sun cream, BL = babies moisturizing lotion, CO = hair conditioning lotion, SH = shampoo). [b] Recovery of the surrogate (%) is given in the first row. Blank spaces mean the values are below the LOD.

CONCLUSIONS

A method based on acetylation PLE followed by GC/MS for the simultaneous determination of different classes of preservatives, including two bromine-containing preservatives, seven parabens, IPBC, TCS, and the antioxidant preservatives BHA and BHT, in multimatrix cosmetic samples has been developed. To our knowledge, both acetylation and PLE are applied for the first time to the analysis of cosmetics. We have demonstrated the possibility of performing simultaneous in situ derivatization by adding the acetylation reagents directly on the cosmetic sample into the PLE cell, making possible the GC/MS analysis of the extract without any further step. The obtained LODs are far below the established restrictions in the European Cosmetics Regulation, making this multicomponent analytical method suitable for routine control. The reliability of the method was demonstrated through a broad range of cosmetic products showing compliance with the actual European Cosmetics Regulation.

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References

1. Ingredients Prohibited & Restricted by FDA Regulations. http://www.fda.gov/Cosmetics/ProductandIngredientSafety/SelectedCosmeticIngredients/ucm127406.htm (accessed July 2010).
2. S. Polati, F. Gosetti, M. C. Gennaro, In Analysis of Cosmetic Products; A. Salvador, A. Chisvert, Eds.; Elsevier: New York, 2007, p 211.
3. DHI Water and Environment. Study on Enhancing the Endocrine Disrupter Priority List with a Focus on Low Production Volume Chemicals; Revised Report to DG Environment; DHI: Hersholm, Denmark, 2007. http://ec.europa.eu/environment/endocrine/documents/final_report_2007.pdf.

4. R. S. Lanigan, Int. J. Toxicol. 17 (Suppl. 5) (1998) 1.
5. M. T. Matyska, J. J. Pesek, L. Yang, J. Chromatogr. A 887 (2000) 497.
6. International Agency for Research on Cancer. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans; World Health Organization: Geneva, Switzerland, 1996; Vol. 40. http://monographs.iarc.fr/ENG/Monographs/vol40/volume40.pdf#12.
7. Cosmetic Ingredient Review. Safety of Triclosan as a Preservative in Cosmetics. http://www.cir-safety.org/newreports.shtml (accessed July 2010).
8. Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR). Assessment of the Antibiotic Resistance Effects of Biocides; European Commission: Brussels, Belgium, 2009.
9. Off. J. Eur. Union 2009, L342, 59-209.
10. Council Directive 76/768/EEC of 27 July 1976 on the Approximation of the Laws of the Member States Relating to Cosmetic Products; European Union: Brussels, Belgium.
11. Ministry of Health and Welfare. Standards for Cosmetics. http://www.mhlw.go.jp/english/topics/cosmetics/index.html (accessed July 2010).
12. Márquez-Sillero, I.; Aguilera-Herrador, E.; Cárdenas, S.; Valcárcel, M. J. Chromatogr., A 2010, 1217, 1–6.
13. S.-P. Wang, C.-L. Chang, Anal. Chim. Acta 377 (1998) 85.
14. M. Saraji, S. Mirmahdieh, J. Sep. Sci. 32 (2009) 988.
15. L. P. Melo, M. E. C. Queiroz, J. Sep. Sci. 33 (2010) 1849.
16. M.-R. Lee, C.-Y. Lin, Z.-G. Li, T.-F. Tsai, J. Chromatogr. A 1120 (2006) 244.
17. T. Wu, C. Wang, X. Wang, Q. Ma, Int. J. Cosmet. Sci. 30 (2008) 367.
18. L. Gagliardi, G. Cavazzutti, L. Turchetto, F. Manna, D. Tonelli, J. Chromatogr., A 508 (1990) 252.
19. J. Wang, D. Zhang, Q. Chu, J. Ye, Chin. J. Chem. 28 (2010) 313.

20. H.-Y. Huang, Y.-C. Lai, C.-W. Chiu, J.-M. Yeh, J. Chromatogr. A 993 (2003) 153.
21. J. F. García-Jiménez, M. C. Valencia, L. F. Capitán-Vallvey, Anal. Chim. Acta 594 (2007) 226.
22. T.-J. Yang, F.-J. Tsai, C.-Y. Chen, T. C.-C. Yang, M.-R. Lee, Anal. Chim. Acta 668 (2010) 188.
23. T.-F. Tsai, M.-R. Lee, Chromatographia 67 (2008) 425.
24. Nieto, F. Borrull, R. M. Marcé, E. Pocurull, J. Chromatogr. A 1216 (2009) 5619.
25. Nieto, F. Borrull, E. Pocurull, R. M. Marcé, TrAC Trends Anal. Chem. 29 (2010) 752.
26. M. P. Llompart, R. A. Lorenzo, R. Cela, J. R. J. Paré , J. M. R. Bélanger, K. Li, J. Chromatogr. A 757 (1997) 153.
27. J. Regueiro, E. Becerril, C. Garcia-Jares, M. Llompart, J. Chromatogr. A 1216 (2009) 4693.
28. J. Regueiro, M. Llompart, E. Psillakis, J. C. Garcia-Monteagudo, C. Garcia- Jares, Talanta 79 (2009) 1387.
29. T. R. Croley, B. C. Lynn, Jr. Rapid Commun. Mass Spectrom. 12 (1998) 171.
30. P. Monsef-Mirzai, Fuel 75 (1996) 1684.
31. R. Carabias-Martínez, E. Rodríguez-Gonzalo, P. Revilla-Ruiz, J. Hernández-Méndez, J. Chromatogr. A 1089 (2005) 1.
32. EPA Method 3545, Pressurised Fluid Extraction. Test Methods for Evaluating Solid Waste, Physical/Chemical Methods, 3rd ed.; Final Update IV; EPA SW-846; U.S. Government Printing Office: Washington, DC, 2008.
33. Methods Optimization in Accelerated Solvent Extraction (ASE®); Technical Note 208; Dionex Corp.: Sunnyvale, CA, 2004.

SUPPORTING INFORMATION AVAILABLE

Additional information as noted in the text.

Table S-1. Chemical structures and regulation limits of the target preservatives.

Key	Compound	IUPAC name	CAS	Chemical structure	Maximum concentration (EU)[a]
	Bronidox	5-Bromo-5-nitro-1,3-dioxane	30007-47-7		0.1 %[b]. The formation of nitrosamines should be avoided
	Bronopol	2-Bromo-2-nitropropane-1,3-diol	52-51-7		0.1 %. The formation of nitrosamines should be avoided
BHA	Butylated hydroxy-anisole	2-tert-Butyl-4-methoxyphenol	121-00-6		
BHT	Butylated hydroxy-toluene	2,6-Bis(1,1-dimethylethyl)-4-methylphenol	128-37-0		
IPBC		Carbamic acid, butyl-3-iodo-2-propynyl ester	55406-53-6		0.02 % (rinse-off)[c,e] 0.01 % (leave-on)[c,d,e] 0.0075 % (deodorants)[c,e]
MeP	Methyl-paraben	Methyl 4-hydroxybenzoate	99-76-3		
EtP	Ethyl-paraben	Ethyl 4-hydroxybenzoate	120-47-8		
iPrP	Isopropyl-paraben	Isopropyl 4-hydroxybenzoate	4191-73-5		0.4 % (as acid) for single ester 0.8 % (as acid) for mixtures of esters
PrP	Propyl-paraben	Propyl 4-hydroxybenzoate	94-13-3		
iBuP	Isobutyl-paraben	Isobutyl 4-hydroxybenzoate	4247-02-3		
BuP	Butyl-paraben	Butyl 4-hydroxybenzoate	94-26-8		
BzP	Benzyl-paraben	Benzyl 4-hydroxybenzoate	94-18-8		

Table S-2. Retention times, quantification and identification ions of the target analytes before and after acetylation.

	Non-derivatized		Acetylated derivatives	
	Retention time (min)	Quantifier and qualifier ions	Retention time (min)	Quantifier and qualifier ions
Bronidox	10.92	107,109,137		
Bronopol	11.57	107,109,125	12.75	115,195,197
MeP	12.92	93,121,152	13.02	93,121,152
BHT	13.16	177,205,220		
EtP	13.32	121,138,166	13.45	121,138,166
BHA	13.08	137,165,180	13.51	137,165,180
iPrP	13.48	121,138,165	13.64	121,138,180
PrP	13.93	65,121,138	14.10	121,138,139
IPBC	14.23	164,165,181		
iBuP	14.43	121,138,139	14.52	121,138,139
BuP	14.70	121,138,139	14.92	121,138,139
BzP	20.69	91,121,228	21.06	91,121,228
TRC	20.11	218,288,290	21.09	218,288,290

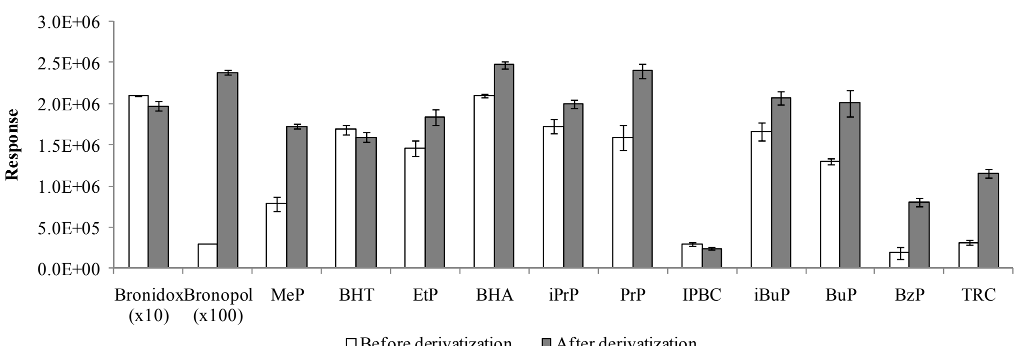

Figure S-1. Comparison of the chromatographic response before and after derivatization.

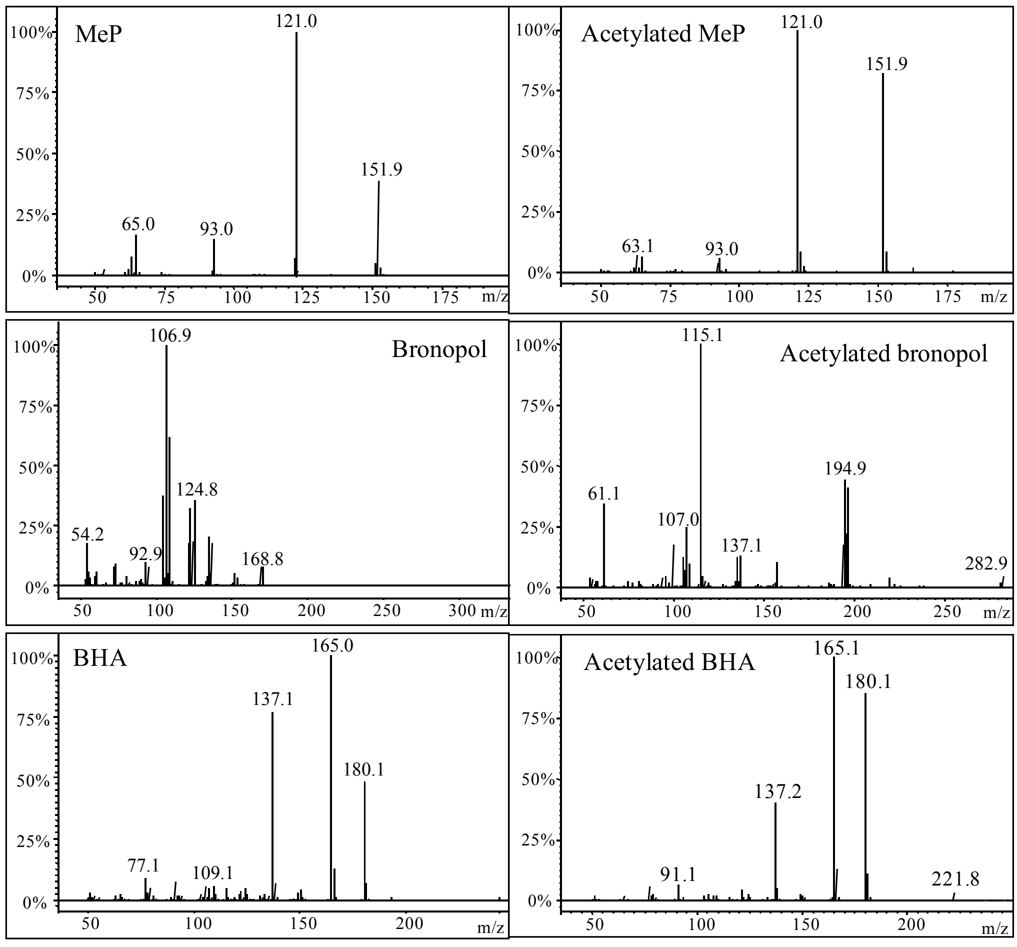

Figure S-2. Mass spectra corresponding to some selected preservatives and their acetylated derivatives.

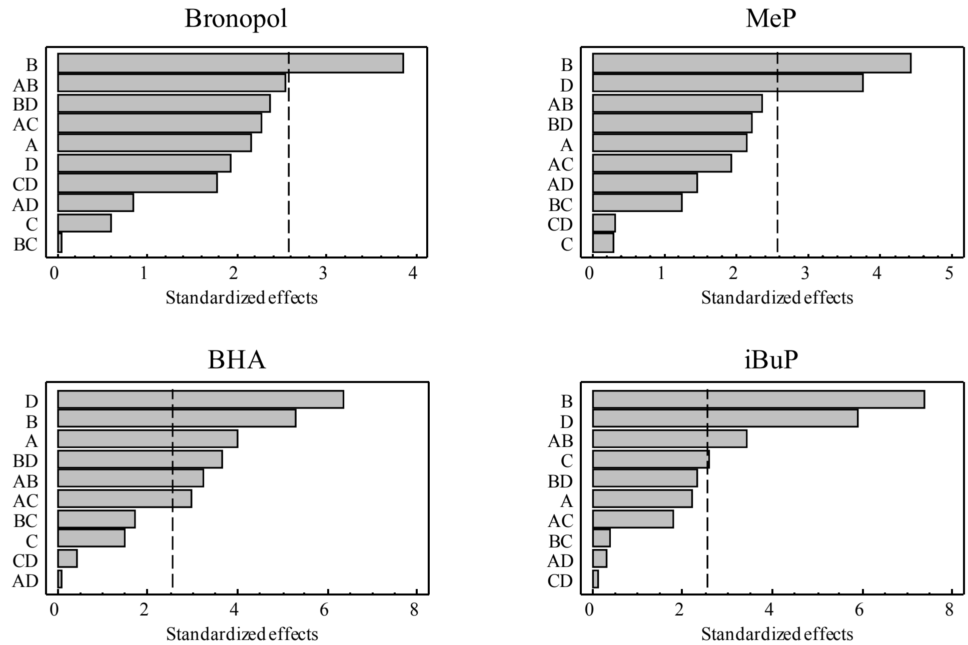

Figure S-3. Pareto charts showing the significant factors (95%) for some selected analytes.

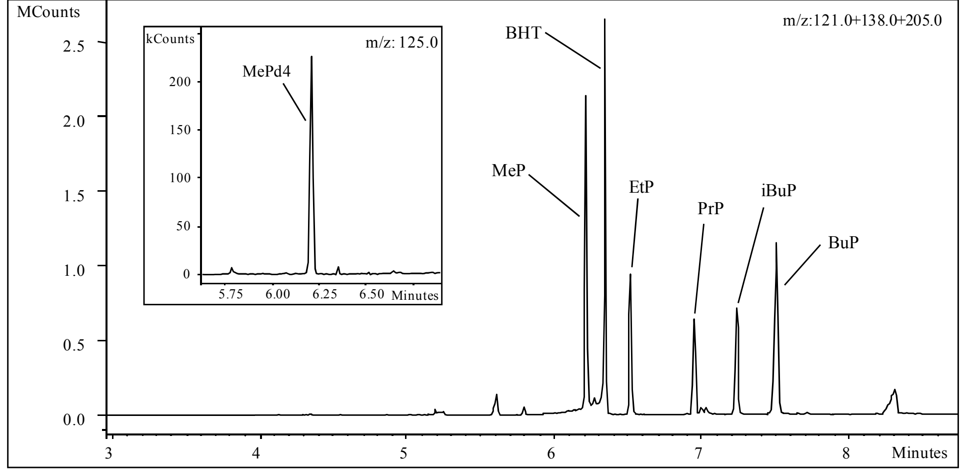

Figure S-4. Chromatogram of a real sample (HC1, see concentrations in Table 3).

1.4 MULTICOMPONENT ANALYTICAL METHODOLOGY TO CONTROL PHTHALATES, SYNTHETIC MUSKS, FRAGRANCE ALLERGENS AND PRESERVATIVES IN PERFUMES

MULTICOMPONENT ANALYTICAL METHODOLOGY TO CONTROL PHTHALATES, SYNTHETIC MUSKS, FRAGRANCE ALLERGENS AND PRESERVATIVES IN PERFUMES

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Abstract

A simple, fast, robust and reliable multicomponent analytical method applicable in control laboratories with a high throughput level has been developed to analyze commercial brands of perfumes. Contents of 52 cosmetic ingredients belonging to different chemical families can be determined in a single run. Instrumental linearity, precision of the method and recovery studies in real samples showed excellent results, so that quantification by external calibration can be effectively applied. Relevant limits of detection and quantification were obtained for all the targets considered, far below the legal requirements and amply adequate for its accurate analytical control.

A survey of 70 commercial perfumes and colognes has been performed, in order to verify whether these products complied with the recent changes in European legislation: regarding the maxima allowed concentrations of the ingredients and/or ingredient labelling. All samples contained some of the target ingredients. Several samples do not comply with the regulations concerning the presence of phthalates. Musks data confirmed the trend about the replacement of nitromusks by polycyclic musks; as well as the noticeable introduction of macrocyclic musks in the perfumes composition. The prohibited musk moskene has been detected in one sample in an appreciable concentration. The average number of fragrance allergens is twelve per sample; their presence must be indicated in the list of ingredients when its concentration exceeds the 0.001%, but values higher than 1% have been found in some samples. Preservatives data show that parabens, although ubiquitous in other cosmetic products, are not widely used in perfumery. In contrast, the presence of BHT is indeed widespread. The degree of compliance with the European Regulation on the labelling has been evaluated in a subset of samples, and only about the 38% of the perfumes were properly labelled for the allergens tested.

Keywords: Perfumes; Phthalates; Synthetic Musks; Fragrance Allergens; Preservatives; Cosmetics Analysis

1. Introduction

The safety of fragrance ingredients is a top priority for the cosmetics industry in general and, particularly, for perfumes manufacturers. New scientific data are constantly evaluated to ensure that the highest standards are applied to the creation of a fragrance. But a perfume may contain hundreds of substances, and any analytical method designed to analyze as many ingredients as possible with minimal effort would be welcome for both the industry and the control bodies worldwide.

The free circulation of cosmetic products in the market and the safety of cosmetics placed on it, which obviously include perfumes, have to be ensured and guaranteed by the respective governments. Recently, on 30 November 2009, in the European Union context, a new Cosmetic Products Regulation [1] (which is, to a great extent, a recast of the previous Cosmetics Directive [2] and its successive amendments and adaptations) has been adopted in order to strengthen certain elements of the regulatory framework for cosmetics, such as in-market control, with a view to ensuring a high level of protection of human health. This Regulation is already into force and it will apply with some exceptions from 11th July 2013. Among the new features of the Regulation, Article 19 from Ch. VI ("consumer information") is dedicated specifically to "labelling" and directly affects the groups of ingredients selected in this study. Previous (but currently applied) regulatory aspects for fragrance ingredients in cosmetics have been well reviewed and discussed [3,4].

Because of the nature of the use of perfumes (leave-on cosmetics), there is a high potential of human exposure. So, it is important that the ingredient labelling is correct, because this can be used by consumers to avoid the use of the products containing specific chemical(s) that they cannot tolerate; and by dermatologists, as a guide to the compounds that may be the cause of skin reactions [5] or other adverse effects. Among the different groups of chemicals used as additives in perfumes, the following four have been selected to be controlled in this study: phthalates, synthetic musks, fragrance allergens and preservatives.

Phthalates are commonly found in perfumes mainly as carriers or solvents for synthetic musks. Among the principal phthalates used in cosmetics, dimethyl phthalate (DMP) and diethyl phthalate (DEP) are not banned in Europe, but they are defined as contaminants to be controlled by the U.S. Environmental Protection

Agency (EPA), together with the di-n-octyl phthalate (DNOP); so their analytical control is also considered here. Backing up this decision, recent findings suggest that long-term exposure to DEP, one of the widely used phthalate esters, can lead to serious health problems, and most perfumes contain non-negligible amounts of DEP [6]. The rest of phthalates measured in this study are forbidden by the European Union [1, 2]: di-n-butyl phthalate (DBP), bis(2-ethylhexyl phthalate (DEHP), bis(2-methoxyethyl) phthalate (DMEP) and di-n-pentyl phthalate (DPP).

Synthetic musks are used as an alternative for the natural musk, an intensely smelling secretion of the male musk deer. Nitromusks dominated the market for many years but declined significantly in the 90 s [7] due to their bioaccumulative properties and health adverse reactions, which eventually led to the prohibition of musk tibetene, musk moskene and musk ambrette; while musk ketone and musk xylene can still be used in cosmetics but with restrictions. There was a parallel increase in the use of polycyclic musks, a second group of synthetic musks which comprises several high volume use products, such as tonalide® (AHTN) and galaxolide® (HHCB). However, reports on the presence of polycyclic musks in water, fish and human samples damped enthusiasm and caused production levels to decrease [8]. Nevertheless, these compounds are still largely used in personal care products, sanitation products and fragrances [9]. Although polycyclic musks have been tested in the past and showed no toxicological and dermatological effects, their high levels of use, chemical stability and low biodegradability have the consequence that have been gradually replaced by a third group of fragrances, consisting in partially artificial and partially nature-identical members, the macrocyclic musks [10].

European legislation requires monitoring 27 volatile compounds used in perfumery as they might elicit skin sensitization, the so-called potentially allergen substances (PAS) or fragrance allergens. Of these 27 substances, 25 are chemically defined volatile compounds whereas the other two are natural moss extracts and do not correspond to defined chemicals. Without presuming whether their possible sensitizing properties will be confirmed or invalidated, their occurrence in fragrance concentrates needs to be determined [11]. Recent changes in EU regulations include the transfer of methyleugenol from the Annex II (List of substances prohibited in cosmetic products) to the Annex III (List of substances which cosmetic products

must not contain except subject to the restrictions laid down) and thus this compound should also be considered for controlling. Besides, pinene is a monoterpene that may be allergenic (although it is not regulated as such) and its presence is very common in perfumes -as it will be shown below-, so its control is also included in this study; making a total of 26 compounds monitored (25 regulated and 1 proposed).

Preservatives are used in cosmetics to protect them against microbial growth, both to care for consumers and to maintain product integrity. The esters of *p*-hydroxybenzoic acid or parabens are the most widely used preservatives in cosmetic products, especially the mixtures of methyl-, propyl-, ethyl-, butyl- and/or benzylparaben. This ubiquity led us to select them for control in perfumes. Butylated hydroxytoluene (BHT) is a synthetic antioxidant widespread used as preservative. The Final Report on the safety assessment of BHT indicates that the compound itself is not generally considered genotoxic, although it can modify the genotoxicity of other agents [12]; however the report concludes that BHT is safe at the low concentration currently used in cosmetic formulations (from 0.0002% to 0.5%).

Analytical aspects related to perfumes involve, overall, the characterization and the quality control of the extracts obtained by perfume manufacturers, of new extracts obtained from different sources or with different methods, or of perfumery raw materials [3]. Nevertheless, there is an increasingly pressing demand to know perfumes composition in depth, in particular due to the possible biological activities of some ingredients [13], like many of the compounds whose control is proposed in this study. Due to the numerous interfering compounds, the analysis of fragrances composition remains a very challenging task for perfume and cosmetic manufacturers. In this sense, most of the advances in multicomponent analysis of cosmetics have been made in the determination of different groups of preservatives [14–17] or combinations of preservatives (parabens) and phthalates [18] or preservatives including antioxidants [19–22], but in no case focused on the analysis of perfumes. Otherwise, in the case of fragrances allergens, the developed methodology has been based mainly on GC-MS [11, 23–25] or comprehensive two-dimensional gas chromatography with MS detection [26,27] or, more recently, with FID detection combined with chemometrical tools [28]. In all the cited cases, the methodologies have been applied to very few perfume samples. In addition, in none

of the previous work, the analytical effort solves more than a group of ingredients focusing on perfumes and neither focused on the analysis of a large number of perfume samples to survey the levels of such ingredients in commercial products, as it is the case in the present study, with the exception of a recent survey by an international NGO, in which phthalates and synthetic musks have been determined in a random selection of 36 perfume brands [10].

The objective of the present work is to put forward a reliable multicomponent analytical method applicable in control laboratories, from companies and institutions, with a high throughput level. A total of 52 target compounds belonging to four different types of ingredients have been selected to be determined in a single GC-MS run: six preservatives (parabens and BHT), twelve synthetic musks (five nitromusks, six polycyclic and one macrocyclic), twenty-six fragrance allergens, and eight phthalates. Instrument and analytical conditions have been optimized; no pre-treatment of samples other than dilution is needed. The ruggedness of the proposed methodology is demonstrated by its application using two different instrumental set-ups, involving external and internal ion trap configurations as well as two different GC stationary phase polarities. To assess the performance of the proposed method, accuracy, precision, linearity and detection limits (LODs) have been evaluated obtaining quantitative recoveries and low RSD values. The obtained LODs are far below the established restrictions as regards labelling in the European Cosmetics Regulation [1]. A survey of a variety of perfumes and colognes for the target ingredients was conducted. Seventy samples, fragrances for men, women, children and babies, have been analyzed with the proposed methodology to check whether these products complied with the new European Regulation with respect to both the maximum allowed concentration (MAC) of the ingredients present and the correct labelling.

Table 1. Retention times, quantification and identification ions of the targets.

N	Compound	Chemical name	CAS number	Purity (%)	GC-MS detection	
					Retention time (min)	Quantifier and qualifiers
1	Pinene	2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene	80-56-8	≥99[a]	6.75	77,91,93
2	Limonene	(4R)-1-Methyl-4-(1-methylethenyl)cyclohexene	5989-27-5	97[b]	8.68	67,93,121
3	Benzyl alcohol	Benzene methanol	100-51-6	99[c]	8.94	77,79,108
4	Linalool	3,7-Dimethyl-1,6-octadien-3-ol	78-70-6	97[b]	9.84	43,93,121
5	Methyl-2-octynoate	Methyl heptin carbonate	111-12-6	≥99[d]	11.09	67,79,95
6	Citronellol	(±)-3,7-Dimethyloct-6-en-1-ol	106-22-9/ 26489-01-0	95[b]	11.36	69,81,95
7	Citral	3,7-Dimethyl-2,6-octadienal	5392-40-5	95[b]	11.51 11.78	39,69,109
8	Geraniol	3,7-Dimethyl-(2E)-2,6-octadien-1-ol	106-24-1	≥96[a]	11.60	41,69,111
9	Cinnamal	3-Phenyl-2-propenal	104-55-2	≥93[d]	11.90	77,103,131
10	Hydroxycitronellal	7-Hydroxy-3,7-dimethyloctanal	107-75-5	≥95[d]	11.94	43,59,81
11	Anise alcohol	4-Methoxybenzyl alcohol	105-13-5	98[b]	11.99	109,121,138
12	Cinnamyl alcohol	3-Phenyl-2-propen-1-ol	104-54-1	98[c]	12.18	77,92,134
13	Eugenol	2-Methoxy-4-(2-propenyl) phenol	97-53-0	99[b]	12.49	103,131,164
14	Methyleugenol	1,2-Dimethoxy-4-(2-propenyl)-benzene	93-15-2	99[b]	12.78	147,163,178
15	Isoeugenol	2-Methoxy-4-(1-propenyl) phenol	97-54-1	98[b]	12.86 13.15	103,131,164
16	DMP	Dimethyl phthalate	131-11-3	98[a]	13.14	77,163,194
17	Coumarin	2H-1-benzopyran-2-one	91-64-5	99[b]	13.18	89,118,146
18	MeP	Methyl 4-hydroxybenzoate	99-76-3	99[a]	13.21	65,121,152
19	α-Isomethyl ionone	3-Methyl-4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-3-buten-2-one	127-51-5	≥85[a]	13.29	107,135,150
20	BHT	Butylated hydroxytoluene	128-37-0	99[b]	13.45	57,205,220
21	Cashmeran	1,1,2,3,3-Pentamethyl-2,5,6,7-tetrahydroinden-4-one	33704-61-9	>95[e]	13.50	135,191,163
22	EtP	Ethyl 4-hydroxybenzoate	120-47-8	99[a]	13.61	65,121,138
23	Lilial®	2-(4-*tert*-Butylbenzyl) propionaldehyde	80-54-6	≥95[a]	13.63	131,147,189
24	DEP	Diethyl phthalate	84-66-2	98[a]	14.01	149,176,177
25	PrP	Propyl 4-hydroxybenzoate	94-13-3	99[a]	14.28	65,121,138
26	Amyl cinnamal	2-Benzylideneheptanal	122-40-7	97[b]	14.47	115,129,202
27	Lyral®	Hydroxyhexyl-3-cyclohexene carboxaldehyde	31906-04-4	≥97[a]	14.65	79,91,136
28	Amylcinnamyl alcohol	2-Pentyl-3-phenylprop-2-en-1-ol	101-85-9	≥85[a]	14.77	91,115,133
29	Celestolide	4-Acetyl-6-tert-butyl-1,1-dimethylindane	13171-00-1	>98[e]	14.97	173,229,244
30	Farnesol	3,7,11-Trimethyldodeca-2,6,10-trien-1-ol	4602-84-0	95[b]	14.99	41,69,81
31	BuP	Butyl 4-hydroxybenzoate	94-26-8	99[a]	15.14	65,121,138
32	Hexylcinnamal	2-Benzylideneoctanal	101-86-0	≥95[d]	15.34 15.60	115,129,216
33	Phantolide	6-Acetyl-1,1,2,3,3,5-hexamethylindan	15323-35-0	>98[f]	15.38	145,187,229
34	Benzyl benzoate	Phenylmethyl benzoate	120-51-4	98.5[c]	15.66	105,194,212
35	Musk ambrette (MA)	6-*tert*-Butyl-3-methyl-2,4-dinitroanisole	83-66-9	99[g]	16.21	91,251,253
36	Traseolide	5-Acetyl-3-isopropyl-1,1,2,6-tetramethylindane	68140-48-7	Tech. [f]	16.39	131,173,215
37	Galaxolide®	1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta(g)-2-benzopyran	1222-05-5	75[f]	16.50	171,213,243
38	Musk xylene (MX)[h]	1-*tert*-Butyl-3,5-dimethyl-2,4,6-trinitrobenzene	81-15-2		16.56	43,282,297
39	Tonalide®	6-Acetyl-1,1,2,4,4,7-hexamethyltetralin	1506-02-1	98[f]	16.58	159,187,243
40	Musk moskene (MM)[h]	1,1,3,3,5-Pentamethyl-4,6-dinitro-2H-indene	116-66-5		16.92	263,264,278
41	Benzyl salicylate	Benzyl-2-hydroxybenzoate	118-58-1	≥ 99[a]	16.98	65,91,228
42	Musk tibetene (MT)[g]	1-*tert*-Butyl-3,4,5-trimethyl-2,6-dinitrobenzene	145-39-1		17.70	43,251,266
43	Ambrettolide	17-Oxacycloheptadec-6-en-1-one	7779-50-2	>97[e]	17.87	67,81,95
44	DBP	Dibutyl phthalate	84-74-2	99[b]	18.10	149,150,223
45	Musk ketone (MK)[a]	4-*tert*-Butyl-3,5-dinitro-2,6-dimethyl acetophenone	81-14-1		18.45	279,280,294
46	DMEP	Bis(2-methoxyethyl) phthalate	117-82-8	94[g]	18.79	58,59,149
47	Benzyl cinnamate	3-Phenyl-2-propenoic acid phenylmethyl ester	103-41-3	99[b]	20.80	91,131,192
48	DPP	Dipentyl phthalate	131-18-0	99.2[g]	21.24	149,150,237
49	BzP	Benzyl hydroxybenzoate	94-18-8	99[a]	21.34	65,91,121
50	BBP	Benzyl butyl phthalate	85-68-7	98[b]	22.83	91,149,206
51	DEHP	Bis(2-ethylhexyl) phthalate	117-81-7	99.5[a]	23.89	149,150,167
52	DNOP	Di-n-octyl phthalate	117-84-0	≥98[a]	25.31	41,149,279

Obtained from: [a]Fluka Chemie GmbH, Germany (MK: 100 ng μL^{-1} in acetonitrile); [b]Sigma-Aldrich Chemie GmbH, Germany; [c]ChemService, West Chester, USA; [d]SAFC Supply Solutions, St. Louis, USA; [e]Ventos, Barcelona, Spain; [f]LGC Standards GmbH, Germany; [g]Dr. Ehrenstorfer, Germany (MT: 10 ng μL^{-1} in cyclohexane); [h]100 ng μL^{-1} in acetonitrile, Riedel de Haën, Germany.

2. Material and Methods

2.1. Reagents and materials

The studied compounds, their chemical names and the purity of the standards are summarized in Table 1. Ethyl acetate and acetone (analytical grade) were provided by Merck (Darmstadt, Germany). Individual stock solutions of each compound were prepared in acetone. Further dilutions and mixtures were prepared in ethyl acetate and then stored in amber glass vials at -20 ºC.

2.2. Gas chromatography-mass spectrometry

The samples were analyzed in two GC-MS equipments. The first one was a Varian 450-GC gas chromatograph (Varian Chromatography Systems, Walnut Creek, CA, USA) coupled to an ion trap mass spectrometer Varian 240-MS (Varian Chromatography Systems) with a waveboard for multiple MS (MS^n) analysis; and a sampler model CP-8400. The system was managed by Varian MS workstation v6.9.1 software. Separation was carried out on a HP5 capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness) from Agilent Technologies (Palo Alto, CA, USA). The ion trap mass spectrometer was operated in the electron impact (EI) ionization mode (+70 eV) using an external ionization configuration. Manifold, ion trap, ion source and transfer line temperatures, were maintained at 40, 150, 200 and 280 ºC, respectively. The filament emission current was 25 µA. The acquisition mass range was from 39 to 400 *m/z* at 3 µscans, starting at 5 minutes and ending at 30 min.

The second equipment used was a Varian 3800-GC gas chromatograph (Varian Chromatography Systems, Walnut Creek, CA, USA) coupled to a Varian Saturn 2000 ion trap mass spectrometer (Varian Chromatography Systems) equipped with a 1079 split/splitless injector. The system was operated by Saturn GC-MS workstation version 5.4 software, and separation was carried out on a VF-1701ms capillary column (30 m × 0.25 mm i.d. × 0.39 mm o.d., 0.25 µm film thickness) from Varian, Inc. (Lake Forest, CA, USA). The ion trap mass spectrometer was operated in the electron impact (EI) ionization mode (+70 eV). Manifold, ion trap, and transfer line temperatures, were maintained at 110, 200, and 280 ºC, respectively. The filament emission current was 10 µA. The acquisition mass range was the same described for the other equipment.

Common GC conditions to both systems were as follows. Helium (purity 99.999%) was employed as carrier gas at a constant column flow of 1.0 mL min^{-1}. The GC oven temperature was programmed from 45 ºC (held 2min) to 100 ºC at 8 ºC min^{-1}, to 130 ºC at 20 ºC min^{-1} (held 3min) and, to 200 ºC at 25 ºC min^{-1}; (total analysis time = 25min). Splitless mode (held 2min) was used for injection, the split flow was set at 20 mL min^{-1} and the injector temperature was kept at 220 ºC. The injection volume was 2 µL.

The analytes were positively identified by comparison of their mass spectra and retention times to those of standards. The identification and quantification ions and retention times for each target compound are listed in Table 1.

2.3. Sampling and sample pre-treatment

Seventy commercial perfumes and eau de toilettes (for men, women, children and babies) were purchased in perfumeries, supermarkets, and convenience stores from Galicia (Northwest Spain). The samples included international and national brands, covering a wide range of prices. Samples were stored at room temperature until analysis. All samples were clear liquids and no special pre-treatment was applied apart from homogenization and dilution 1:10 with ethyl acetate. Due to the huge range of concentrations in which these compounds can be included in the samples (from ng per mL to mg per mL), dilutions 1:100 and 1:1000 with ethyl acetate were also injected in some cases.

The general use of the targets in all classes of personal-care products and cleaning products, as well as the well-known ubiquitous presence of phthalates, demand special precautions during al analytical procedures to minimize contamination risk.

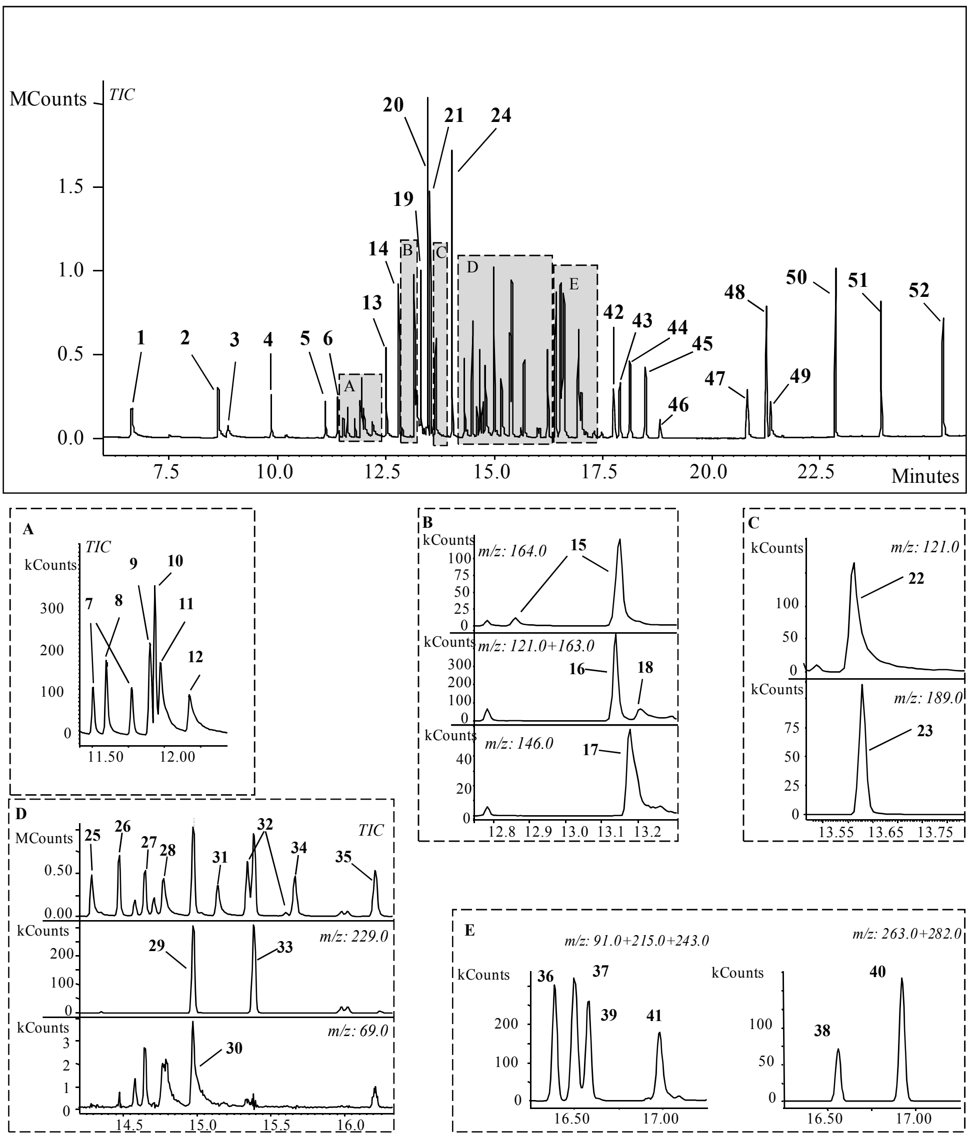
3. Results and Discussion

In this study we develop a chromatographic method useful to separate and identify 52 analytes with very different chemical and physical properties, and belonging to different cosmetic additive families including fragrance allergens (26), phthalates (8), synthetic musks (12), and preservatives (6). The objective is to

make possible the application of the chromatographic method to the analysis of these important groups of regulated cosmetic ingredients in cosmetic analysis in a single run. As previously commented, most of the studies found in the literature deals with only one group of these ingredients, and, in addition, the number of compounds considered is low. Some studies about the determination of regulated suspected allergens [29], one of the families included in our study, show the difficulties to achieve an effective separation of these compounds. Previously, we had developed a GC-MS method that makes possible the quantification of 24 of the fragrance allergens [30, 31]. This was the starting point to develop a chromatographic method including the other four analyte families. Different chromatographic columns and different temperature programs were tested to achieve the best possible separation conditions. The final selected chromatographic conditions are summarized in the material and methods section. Taking into account the high number of analytes considered, the global method resolution can be considered quite good; although some analytes were not (completely) resolved, the extracted ion chromatograms, permitted the proper quantification of all compounds. The selection of the quantification ions was based on the attainment of the most favourable resolution and the maximum signal-to-noise ratio. Fig. 1 shows a chromatogram composition obtained for a 5 μg mL^{-1} standard mixture including all target compounds. The 52 cosmetic ingredients could be analyzed in only 25 min.

3.1. Method Validation

It is well known that the most important problem concerning phthalate analysis is the risk of contamination, resulting in false positive results and over-estimated concentrations [32]. The sources of contamination can be present in any step of the analytical procedure. Special care was taken to avoid the contact of reagents and solutions with plastic materials. Laboratory glassware was washed prior to use with ultrapure water and dried at 300 ºC. This material was stored in aluminium foil to avoid adsorption of phthalates from the air. Besides, due to the occurrence of musk fragrances as ingredients of all kind of cleansing products and cosmetics, the risk of sample contamination when they are manipulated in the laboratory is not negligible, so it is advisable to extreme precautions to avoid sources of interference in the laboratory environment.



1: Pinene; 2: Limonene; 3: Benzyl alcohol; 4: Linalool; 5: Methyl-2-octynoate; 6: Citronellol; 7: Citral; 8: Geraniol; 9 Cinnamal; 10: Hydroxycitronellal; 11: Anise alcohol; 12: Cinnamyl alcohol; 13: Eugenol; 14: Methyleugenol; 15: Isoeugenol; 16: DMP; 17: Coumarin; 18: MeP; 19: α-Isomethyl ionone; 20: BHT; 21: Cashmeran; 22: EtP; 23: Lilial; 24: DEP; 25: PrP; 26: Amyl cinnamal; 27: Lyral®; 28: Amylcinnamyl alcohol; 29: Celestolide; 30: Farnesol; 31: BuP; 32: Hexylcinnamal; 33: Phantolide; 34: Benzyl benzoate; 35: MA; 36: Traseolide; 37: Galaxolide; 38: MX; 39: Tonalide; 40: MM; 41: Benzyl salicylate; 42: MT; 43: Ambrettolide; 44: DBP; 45: MK; 46: DMEP; 47: Benzyl cinnamate; 48: DPP; 49: BzP; 50: BBP; 51: DEHP; 52: DNOP

Fig. 1. Total and extracted ion chromatograms for a standard mixture (5 μg mL^{-1}).

Blank runs of the chromatographic system and direct injections of the solvent selected, ethyl acetate, must be daily done. In spite of all the precautions adopted, one of the phthalates, DEP, was found in all solvent blank runs and, in consequence, detection limit was calculated considering the average blank signal.

To verify that the developed GC-MS method was suitable for the quantitative determination of the selected groups of ingredients in perfumery commercial products, method quality parameters were estimated and summarized for each family of compounds in Table 2. Regarding to instrumental linearity, the method exhibited a direct proportional relationship between the amount of each analyte and the chromatographic response with correlation coefficients $R \geq 0.999$ for phthalates and musks fragrances, R≥0.996 for fragrance allergens and $R \geq 0.998$ for preservatives (Table 2). The sensitivity of the method, expressed as the slope of the calibration curve, is also included in Table 2.

Method precision was studied within a day and among days at several concentration levels between 0.01 and 10 μg mL^{-1}. The results for the levels 0.1 and 1.0 μg mL^{-1} are also included in Table 2. For phthalates, RSD values ranged from 0.81 to 5.9% (intraday precision, average 3.00%), and between 2.1 and 5.8% (inter-day precision, average 3.99%). Precision for musk fragrances was also satisfactory with RSD values ranging from 0.52 to 6.7% for intra-day and 1.5 and 5.7% for inter-day studies (the averages for intra-day and inter-day precision were 2.81 and 3.90%, respectively). For the suspected fragrance allergens, RSDs for the intra-day precision ranged from 0.38 to 7.7% with an average value of 4.04%, while the RSDs for the inter-day precision ranged from 1.1 to 7.5% with an average value of 4.61%. Finally, preservatives RSD values ranged from 1.6 and 5.7% (intra-day precision, average 3.34%), and between 2.3 and 6.5% (inter-day precision, average 5.00%).

Instrumental detection limits (IDLs) were calculated as the concentration giving a signal-to-noise ratio of three (S/N = 3) in all cases with the exception of DEP, which appeared in all the blanks. For this reason its IDL was calculated as the blank signal plus three times the standard deviation. IDLs are also shown in Table 2. The calculated values were, in general, in the low ng per mL. Derivatization, and, particularly, acetylation of parabens could improve the peak shape of these quite polar compounds and, therefore, their IDLs [17].

Table 2. Performance of the method for the target compounds.

	Sensitivity	Linearity *R*	Precision (% RSD)				IDLs (ng mL^{-1})	Recovery[f] (RSD) (%)	LOD[g] (%, w/v)
			Intra-day (n=3)		Inter-day (n=6)				
			0.1[e]	1.0[e]	0.1[e]	1.0[e]			
Phthalates[a]									
DMP	178	1.000	0.96	4.7	2.1	5.8	1.0	105 (2.7)	0.0000010
DEP	189	1.000	5.9	4.3	4.7	5.4	15	n.c.	0.000015
DBP	271	1.000	4.3	3.8	3.6	4.8	1.0	97.3 (4.4)	0.0000010
DMEP	10	1.000	0.90	1.5	2.9	4.0	6.4	105 (1.0)	0.0000064
DPP	187	1.000	1.7	1.1	3.7	3.9	0.65	106 (5.3)	0.00000065
BBP	113	1.000	2.9	3.7	2.1	3.8	5.0	97.9 (8.1)	0.0000050
DEHP	192	0.999	0.81	3.7	3.5	3.9	0.81	88.6 (5.4)	0.00000081
DNOP	297	0.999	4.8	3.0	4.7	5.0	0.65	95.1 (5.7)	0.00000065
Musk Fragrances[a]									
Cashmeran	78	1.000	1.9	6.7	1.5	4.9	0.48	107 (2.9)	0.00000048
Celestolide	157	1.000	4.2	2.0	3.6	5.5	0.20	92.8 (5.1)	0.00000020
Phantolide	179	1.000	4.4	3.7	3.6	4.4	0.19	101 (2.8)	0.00000019
Musk ambrette	23	0.999	0.52	1.7	1.9	3.9	1.0	99.2 (3.5)	0.0000010
Traseolide	197	0.999	0.83	3.2	3.3	3.5	0.25	96.8 (4.8)	0.00000025
Galaxolide®	319	1.000	2.6	4.4	2.5	5.5	0.21	n.c	0.00000021
Musk xylene	56	0.999	2.3	3.7	2.7	5.1	1.1	100 (2.1)	0.0000011
Tonalide®	183	1.000	3.6	3.5	2.8	5.6	0.27	94.1 (3.6)	0.00000027
Musk moskene	151	1.000	1.7	4.5	2.9	5.7	0.23	102 (2.3)	0.00000023
Musk tibetene	50	0.999	1.5	2.7	2.9	4.9	1.1	107 (6.1)	0.0000011
Ambrettolide	66	1.000	2.0	0.54	3.0	4.0	0.83	110 (8.0)	0.00000083
Musk ketone	83	1.000	1.4	3.9	4.7	5.1	0.88	99.6 (7.0)	0.00000088
Fragrance allergens									
Pinene[b]	56	1.000	6.2	1.0	5.0	6.0	0.98	94.9 (8.9)	0.0000010
Limonene[b]	58	0.999	6.6	3.2	6.6	3.5	0.26	n.c.	0.00000026
Benzyl alcohol[c]	50	0.999	n.c.	7.2	n.c.	7.5	25	106 (2.0)	0.0000033
Linalool[b]	25	0.999	7.7	2.4	5.8	2.4	1.7	n.c.	0.0000017
Methyl-2-octynoate[c]	18	0.996	n.c.	2.1	n.c.	6.5	15	105 (6.3)	0.000015
Citronellol[d]	38	0.998	7.4	7.3	6.7	2.9	15	104 (5.8)	0.000012
Citral[c]	4	0.998	n.c.	6.4	n.c.	5.0	29	105 (5.0)	0.000029
Geraniol[c]	5	0.997	n.c.	0.81	n.c.	5.9	27	97.7 (2.7)	0.000027
Cinnamal[c]	65	0.998	n.c.	5.8	n.c.	5.4	6.0	105 (8.0)	0.0000060
Hydroxycitronellal[b]	31	0.999	4.0	1.2	6.2	1.4	1.5	96.0 (5.2)	0.0000033
Anise alcohol[c]	23	0.999	n.c.	5.3	n.c.	4.1	21	106 (3.0)	0.000021
Cinnamyl alcohol[c]	17	1.000	n.c.	2.1	n.c.	6.5	26	103 (3.8)	0.000026
Eugenol[b]	56	0.999	3.6	3.6	7.1	2.9	4.5	93.9 (8.7)	0.0000015
Methyleugenol[b]	77	0.999	3.6	1.4	5.7	1.1	0.42	101 (2.5)	0.00000042
Isoeugenol[c]	65	0.999	n.c.	2.8	n.c.	3.5	6.3	106 (3.7)	0.0000063
Coumarin[d]	50	0.998	6.6	4.1	5.9	5.5	22	95.5 (6.6)	0.00000072
α-Isomethyl ionone[b]	77	0.999	4.9	3.5	6.7	3.8	0.75	101 (7.1)	0.00000075
Lilial®[b]	37	0.998	4.8	2.3	4.4	1.7	0.30	n.c.	0.00000030
Amyl cinnamal[b]	32	0.998	3.8	1.3	5.4	2.6	2.4	100 (5.7)	0.0000024
Lyral®[b]	33	0.998	2.5	4.1	2.5	3.8	3.0	n.c.	0.0000030
Amylcinnamyl alcohol[d]	24	0.999	3.3	4.4	6.9	4.2	18	89.4 (2.5)	0.000018
Farnesol[c]	3	0.999	n.c.	5.7	n.c.	4.6	29	n.c.	0.000069
Hexylcinnamal[b]	53	0.999	5.5	2.2	5.8	2.9	0.73	103 (1.3)	0.00000073
Benzyl benzoate[b]	76	0.999	6.0	4.9	4.9	3.8	2.4	90.7 (0.5)	0.0000024
Benzyl salicylate[b]	160	0.999	0.60	4.4	3.2	3.8	2.3	98.6 (5.1)	0.0000023
Benzyl cinnamate[d]	35	0.999	6.8	0.38	4.4	3.7	7.5	102 (2.2)	0.0000075
Preservatives									
MeP[d]	31	0.999	2.2	3.3	6.0	3.8	15	108 (4.5)	0.000015
BHT[a]	174	0.998	3.6	1.6	3.2	4.8	0.16	100 (4.8)	0.0000016
EtP[d]	106	1.000	4.6	4.8	6.5	6.2	15	98.3 (6.3)	0.000015
PrP[d]	118	1.000	1.8	2.4	5.7	3.9	9.0	105 (5.6)	0.0000090
BuP[d]	94	1.000	5.7	4.5	5.4	5.8	15	105 (2.6)	0.000015
BzP[d]	87	0.998	2.7	2.9	2.3	6.4	50	97.5 (5.9)	0.000050

Linearity ranged from: [a] 0.005 to 10 μg mL^{-1}, [b] 0.010 to 20 μg mL^{-1}, [c] 0.200 to 20 μg mL^{-1}, [d] 0.050 to 20 μg mL^{-1}, [e] Concentration levels (μg mL^{-1}).

[f] Spiked amounts on real samples (n=3) ranged from 0.00070 to 0.0028 % (w/V) for phthalates; from 0.00010 to 0.0027 % (w/v) for musks; from 0.0011 to 0.0083 % (w/v) for allergens; and from 0.0024 to 0.013 % (w/v) for preservatives.

[g] Calculated on real samples for a sample dilution factor of 1:10. n.c. not calculated.

The inconvenient of this process, which is carried out in a basic medium, is that can alter other targets containing for example alcohol groups. Since the aim of this work is to establish a reliable multicomponent analytical method with a high throughput level and the obtained IDLs without derivatization are discarded.

One of the most important difficulties regarding cosmetic analytical control is the wide range of concentrations at which the regulated ingredients can be found (between the parts per million and the high percentage). This problem becomes a challenge when the objective is the control of different compound families, enlarging significantly the number of analytes to determine. Due to the high concentration of some of the target ingredients, all real perfume samples analyzed were diluted by a factor of 10 to 1000. Frequently, and due to the broad range of concentrations found, several dilutions must be analyzed to achieve proper quantification of all compounds.

Recovery studies were carried out with real samples and they were quite challenging for several reasons. For these studies, three different real samples were used (S41, S42 and S57). The selection of the samples was difficult, since all the samples contained several of the targets. When the original target concentration of the sample is quite high, it was not possible to calculate the recovery; in the rest of the cases, the initial concentration was taken into account to calculate the recoveries. Another difficulty was the selection of the spiked amount, since the addition of the lowest possible percentage of solvent is preferable to avoid changes in the matrix and, in some cases, enough concentrated standards are not available. The spiked concentrations ranged from 0.00070 to 0.0028% (w/v) for the phthalates, from 0.00010 to 0.0027% (w/v) for the musk fragrances, from 0.0011 to 0.0083% (w/v) for the fragrance allergens, and 0.0024 to 0.013% (w/v) for the preservatives. The recovery of DEP, galaxolide®, limonene, linalool, lilial® and lyral® could not be evaluated since they were already present in the samples in a high concentration. On the other hand, the recovery of farnesol was not evaluated, since a high concentrated standard solution was not available.

Recoveries are shown in Table 2, and were satisfactory in all cases, with a minimum of 88.6% and a maximum of 110%. Therefore, quantification by external calibration can be effectively employed. Method detection limits were also evaluated in real samples and they are referred to a sample dilution factor of 1:10. As can be

seen in the Table 2, these limits for most analytes correspond to ten times the IDLs, excluding few exceptions for which the chromatographic background was higher than in the corresponding standard solution chromatogram, increasing in this way the LODs of the method.

3.2. Application to Real Samples

The validated method was applied to the analytical control of the 52 target ingredients in 70 different commercial products, designed to be used by men, women, children or babies. Individual data on the presence and found concentrations (%, w/v) of each of the ingredients in the real samples (named S1 to S70) can be found in Tables I to IV of the supplementary material. As a graphical example, Fig. 2 shows the analysis of the sample S11, in which a total of 22 ingredients have been detected in a single run: DEP and DEHP as phthalates; galaxolide®, tonalide® and MK as musks; 15 of the 26 allergenic fragrances and the preservatives MeP and BHT. A statistical summary of the data has been performed to facilitate the discussion of results, considering just the analytes detected in more than 10% of the consumer products.

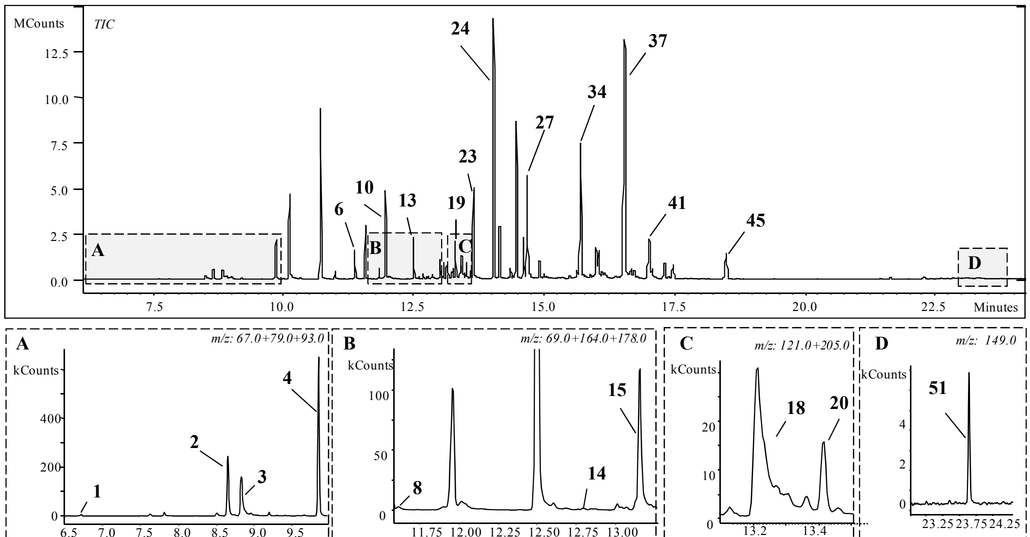


Fig. 2. Total and extracted ion chromatograms obtained for sample S11 (key numbers as in Fig. 1).

3.2.1. Phthalates

The degree of prohibition of phthalates in cosmetics is not homogeneous. Some of them can not be part of the composition of cosmetic products as DBP, DMEP, DPP, BBP and DEHP. Besides, some of the principal phthalates used in cosmetics, DMP and DEP are permitted in Europe, but are considered pollutants in USA -together with DNOP-, and thus their analytical control is also preventively considered in this study. A practical approach to control those banned phthalates can be that the LOQs of the selected method should be less than or equal to 0.0001%. Thus, the average LOD obtained for phthalates with the proposed methodology was 0.0000038%; with minima and maxima in the 10^{-6} % and 10^{-5} % order for LODs. In a previous USA Food and Drug Administration (FDA) study of consumer cosmetic products for phthalate esters [33], levels lower than 10 μg mL^{-1} (0.001%) were reported as not detected.

Table 3 shows a statistical distribution of the phthalates in the evaluated samples. The most common phthalates were DEP, DBP and DEHP present in 81.4%, 72.8% and 55.7%, of the perfumes tested, respectively; as it has been just commented, DBP and DEHP are banned by the EU. The presence of DMP, DPP and BBP was very limited, but again three of the samples did not comply with the regulations in this regard; and finally, DNOP and DMEP were not detected in any sample. Six samples are completely free of phthalates (S23, S43, S45, S48, S55 and S59). Table I (see supplementary material) shows the complete distribution of the phthalates in the samples. Fig. 3 is a partial extract showing the distribution of the banned phthalates in the irregular samples; mainly emphasizing the sample whose DBP concentration reaches 4% (S54). The number of irregular samples may seem high, but it should be noted that these compounds can come from many different sources (e.g. packaging of raw materials or perfumes themselves) and not necessarily from the manufacturing process.

Table 3. Concentration of the target compounds in real samples. Maximum concentration and statistic parameters (%, w/v).

Compound	Maximum concentration	25th percentile	50th percentile Median	75th percentile	90th percentile	N out of 70
Phthalates						
DMP	0.00259	0.00007	0.00017	0.00039	0.00089	9
DEP	3.158	0.2987	0.4686	0.7569	1.327	57
DBP	3.928	0.00004	0.00009	0.0003	0.00108	39
DMEP	<LOD	-	-	-	-	0
DPP	0.00002	-	-	-	-	1
BBP	0.00772	-	-	-	-	2
DEHP	0.00861	0.00016	0.00032	0.00133	0.00265	51
DNOP	<LOD	-	-	-	-	0
Musk Fragrances						
Cashmeran	0.1712	0.00093	0.0074	0.0328	0.068	27
Celestolide	0.0582	0.0008	0.00376	0.0105	0.0308	13
Phantolide	0.00482	0.00023	0.00055	0.00091	0.00205	14
Musk ambrette	<LOQ	-	-	-	-	0
Traseolide	0.00683	0.00289	0.00366	0.00452	0.00591	4
Galaxolide®	1.6	0.00096	0.1225	0.4846	0.9491	63
Musk xylene	0.00065	-	-	-	-	1
Tonalide®	1.268	0.00138	0.0703	0.3051	0.5799	34
Musk moskene	0.1164	-	-	-	-	1
Musk tibetene	<LOQ	-	-	-	-	0
Ambrettolide	0.457	0.00325	0.0157	0.0348	0.0862	39
Musk ketone	0.3761	0.00099	0.0172	0.1436	0.2385	15
Fragrance Allergens						
Pinene	0.1182	0.00137	0.0066	0.0162	0.0347	68
Limonene	1.123	0.0229	0.1353	0.3779	0.5735	69
Benzyl alcohol	0.3159	0.00936	0.0253	0.0413	0.1156	32
Linalool	1.085	0.1078	0.2137	0.3953	0.7327	70
Methyl-2-octynoate	<LOQ					0
Citronellol	2.325	0.0101	0.0485	0.1185	0.272	63
Citral	0.159	0.00739	0.0197	0.08	0.2459	34
Geraniol	1.648	0.00453	0.0126	0.0318	0.0436	51
Cinnamal	0.00735	0.00046	0.00095	0.00139	0.00293	10
Hydroxycitronellal	1.024	0.00417	0.0232	0.1138	0.2241	45
Anise alcohol	0.00871	-	-	-	-	2
Cinnamyl alcohol	0.0484	0.0284	0.0343	0.0405	0.0446	6
Eugenol	0.5676	0.00086	0.00298	0.0366	0.1185	43
Methyleugenol	0.0301	0.0003	0.00044	0.0012	0.00541	22
Isoeugenol	0.0568	0.00154	0.00227	0.0119	0.0474	9
Coumarin	0.8971	0.00705	0.0269	0.0808	0.1942	39
α-Isomethyl ionone	1.115	0.0181	0.0625	0.201	0.3176	56
Lilial®	5.812	0.0261	0.1641	0.3383	1.069	59
Amyl cinnamal	0.0312	-	-	-	-	4
Lyral®	2.252	0.0373	0.0961	0.1791	0.4995	28
Amylcinnamyl alcohol	0.00432	-	-	-	-	3
Farnesol	0.0733	-	-	-	-	1
Hexylcinnamal	1.462	0.0104	0.1366	0.2819	0.7379	35
Benzyl benzoate	0.7233	0.005	0.0373	0.1143	0.2992	44
Benzyl salicylate	2.318	0.00271	0.0326	0.1925	0.4676	59
Benzyl cinnamate	0.1452	0.00089	0.00107	0.0659	0.1275	9
Preservatives						
MeP	0.21481	-	-	-	-	2
BHT	0.24812	0.00098	0.00494	0.0191	0.0546	48
EtP	<LOQ	-	-	-	-	0
PrP	<LOQ	-	-	-	-	0
BuP	<LOQ	-	-	-	-	0
BzP	<LOQ	-	-	-	-	0

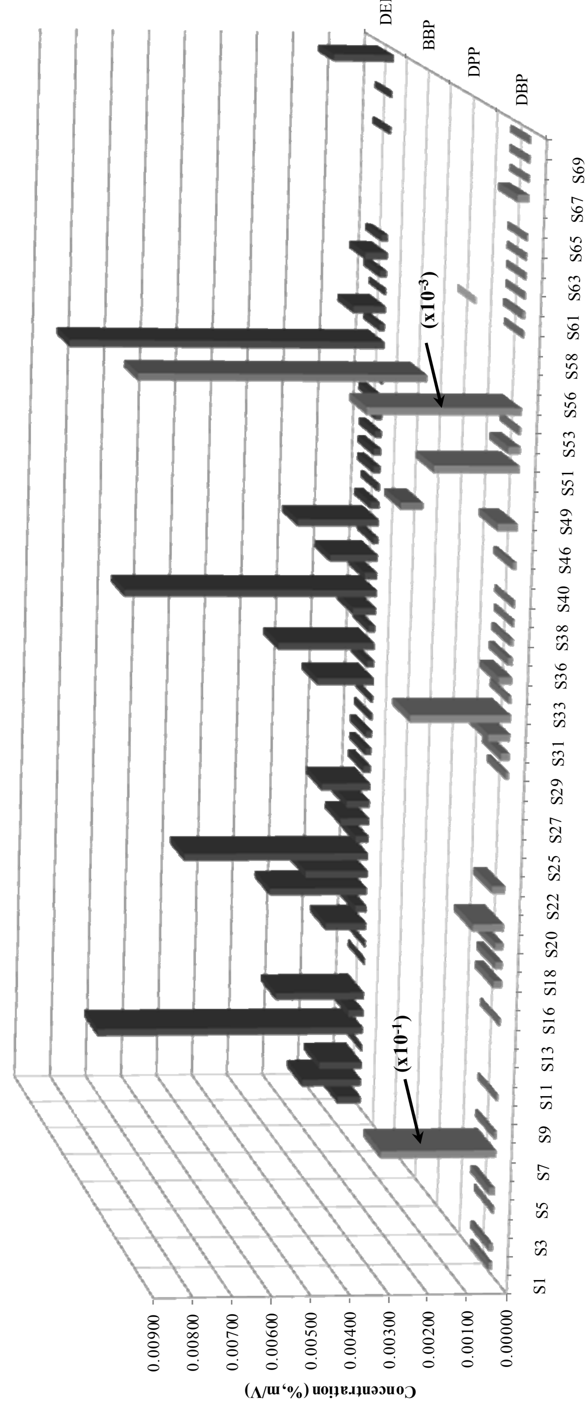


Fig. 3. Partial distribution of the phthalates in the samples. The plot shows the irregular samples containing phthalates banned in the EU.

3.2.2. Musk Fragrances.

There are three nitromusks that are not allowed in cosmetic formulations: ambrette, moskene and tibetene, and a LOQ lower or equal to 0.0001% can also be applied to their control. Other two nitromusks may be part of the composition of cosmetic products with some constraints, being the maximum permitted concentrations for musk xylene and musk ketone 1.0% and 1.4% in fine fragrance, 0.4% and 0.56% in eau de toilette, and 0.03% and 0.042% in other products, respectively. Although right now the polycyclic musks are considered safer than the nitromusks, it is likely that, given its vast global production and the amount and variety of potential sources, they will be in the spotlight sooner or later; thereby implementing their analytical control in cosmetics is to go one step ahead. An appropriate LOD average value was also obtained for all the twelve musks considered in this study: 0.00000056%; with minima and maxima values between 10^{-7} and 10^{-6} %. Musks real data (Tables 3 and II) confirm the commented trend about the replacement of nitromusks (present in the 21.4% of the perfume samples) by polycyclic musks (present in the 97.1%) with a clear prevalence of galaxolide® and tonalide®; as well as the noticeable introduction of macrocyclic musks such as ambrettolide, determined in a 55.7% of the perfumery products (Fig. 4). Two samples were completely free of musk fragrances (S10 and S59) and one else virtually free of them (S12). It is pertinent to project out here that the prohibited musk moskene has been detected in onesample (S16) in an appreciable concentration (0.12%).

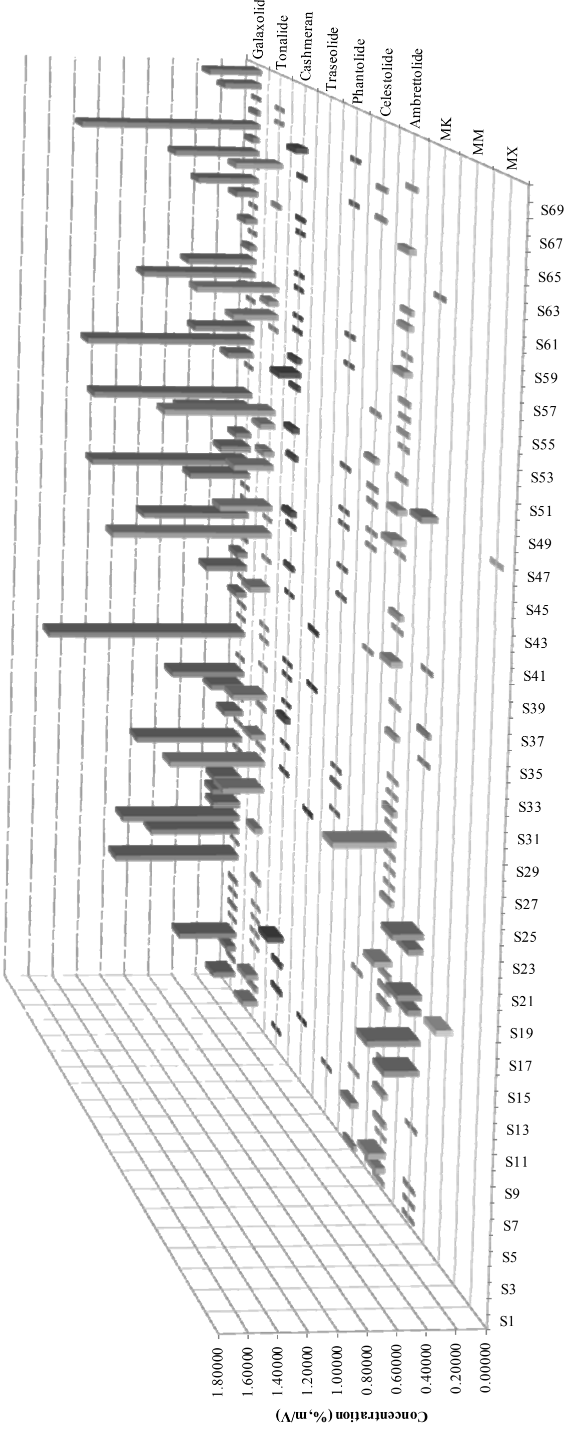


Fig. 4. Distribution of the musk compounds in the samples. The prohibited musk moskene has been detected in sample S16.

3.2.3. Fragrance allergens.

The presence of any potentially allergenic fragrance must be declared in the product label when present in concentrations greater than 0.001% for leave-on cosmetics and 0.01% in rinse-off products. The former limit has to be considered here, since perfumes are designed to remain on the skin. Thus, suitable LOD data were obtained for this group of substances, with an average value of 0.000010%, far below the legal requirements; being the minima and maxima in the 10^{-7}% and 10^{-5}% order, respectively.

The information concerning the allergenic fragrances is summarized in Tables 3 and III. The average amount of fragrance allergens is 12 per sample. Four fragrance allergens appeared in more than 90% of the samples, i.e. pinene in 68 samples, limonene in 69, linalool in all the samples, and citronellol in 63. The highest found concentrations corresponded to lilial® (5.81239%), citronellol (2.32510%), benzyl salicylate (2.31787%), and lyral® (2.25157%). Values higher than 1% were also obtained for six compounds more.

The degree of compliance with the Regulation on the labeling has been evaluated in a subset of 29 samples (41.4% of all perfumes tested). The in depth analysis of the results (see Table III of supplementary material) showed that only eleven of the twenty-nine samples were properly labelled for all the allergens tested (about the38%of the subset). Seven samples were mislabelled with respect to a single allergen, since the compound should be included as an ingredient but it is not: methyleugenol in S1, amyl-cinnamyl alcohol in S4 and S48, citronellol in S57; lilial® in S65, cinnamal in S66, and α-isomethyl ionone in S67. The remaining 11 samples showed labelling errors of varying degrees, highlighting S6 (with 14 missing names in the label of the 17 allergens determined), S42 (13 of 17), S49 (9 of 11) and S61 (12 of 14). Finally, in a different subgroup of another 11 samples one or more compounds were labeled as ingredients but were not found in the perfume: 1 in S10, S51, S60, S64 and S68; 2 in S24 and S67; 3 in S59, 4 in S66, 5 in S63, 9 in S70, and 12 in S69. The latter situation may be due to a strategy of some manufacturers of perfumes, which use a label with all allergens controlled for all consumer products that might contain them, regardless of the identity and actual amount of each allergen compound in each of the products. This is not a good practice; it does not

help the consumer to choice informed, not to dermatologists who are looking for the possible source of a specific allergy.

3.2.4. Preservatives.

Regarding to parabens, the MAC is 0.4% (expressed as acid) for one ester and 0.8% for mixtures of esters, so an average LOD of 0.000018% properly allows the application of the method for the analytical control of parabens in perfumery products. The CIR (Cosmetic Ingredient Review)[1] found BHT safe as used [12]; nevertheless, BHT is also in the crosshairs of some opinion groups that opt for the so-called "safe cosmetics". Given that the CIR indicates that its presence is safe at concentrations up to 0.5%, LOD values in the 10^{-5}% order are amply adequate for its accurate analytical control.

Preservatives data are shown in Tables 3 and IV. The only ester of p-hydroxybenzoic acid detected was methylparaben (in just 2 of the 70 samples). In contrast, BHT appeared in the 68.5% of the perfumes tested, confirming that its presence is indeed widespread.

4. Conclusions

The proposed multicomponent analytical methodology has proven to be useful for the control of 52 target ingredients (8 phthalates, 12 synthetic musks, 26 fragrance allergens and 6 preservatives) in 70 commercial perfumes from different brands, designed to be used by men, women, children or babies. The robustness of the methodology has been demonstrated using two different instrumental set-ups (external and internal ion traps with different GC stationary phase polarities). The good performance of the GC-MS method has been demonstrated in terms of linearity, accuracy, precision, IDLs, quantitative recoveries (> 88%) and LODs far below the European Cosmetics Regulation established limits of either control or restriction.

Only 10 samples accomplished with the Regulation on phthalates. Two of the three phthalates found as the most common in the analyzed samples are banned by the EU: 39 perfumes contained DBP and 51 perfumes, DEHP. Besides, three samples

did not comply with the regulations regarding the presence of DMP, DPP and BBP, although their levels were very limited.

Musks real data confirm the trend about the replacement of nitromusks by polycyclic musks and the perceptible introduction of macrocyclic musks. The prohibited musk moskene has been detected in one sample in an appreciable concentration.

The average number of fragrance allergens was 12 per sample. Four fragrance allergens appeared in more than 90% of the samples (pinene, limonene, linalool and citronellol). The degree of compliance with the Regulation on the labelling has been evaluated in a subset of samples (about 41% of the total), resulting that only 11 perfumes were properly labelled for all the allergens tested.

The generic use of BHT in perfumes has been confirmed, as well as the practical absence of parabens.

From a positive point of view, all perfumes met the Regulation either relating to the labelling or to the established control or restriction limits regarding to preservatives and all but one fulfill the Regulation regarding to musks.

Acknowledgements

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References

1. Official Journal of European Union, L342 (2009) 59.
2. Official Journal of European Union, 76/768/EEC (1976) L262.
3. A. Chisvert, A. Salvador, 6.1. Perfumes in cosmetics. Regulatory aspects and analytical methods for fragrance ingredients and other related chemicals in

cosmetics, in: A. Salvador, A. Chisvert (Eds.), Analysis of Cosmetic Products, Elsevier, Amsterdam, 2007, p. 249.

4. C. Bertram, 26 "allergens" in cosmetics: a challenge for all skateholders, in: K. Lintner (Ed.), Global regulatory issues for the cosmetics industry, William Andrew, New York, 2009.
5. S.C. Rastogi, Contact Dermatitis 43 (2000) 339.
6. K. Chingin, H. Chen, G. Gamez, L. Zhu, R. Zenobi, Anal. Chem. 81 (2008) 123.
7. G.G. Rimkus, Toxicol. Lett. 111 (1999) 37.
8. L. Roosens, A. Covaci, H. Neels, Chemosphere 69 (2007) 1540.
9. J.L. Reiner, K. Kannan, Chemosphere 62 (2006) 867.
10. R.J.B. Peters, TNO-report R&I-A R 2005/011, TNO, Environment and Geosciences, 2005.
11. A. Chaintreau, D. Joulain, C. Marin, C.-O. Schmidt, M. Vey, J. Agric. Food Chem. 51 (2003) 6398.
12. R.S. Lanigan, T.A. Yamarik, Int. J. Toxicol. 21 (2002) 19.
13. C. Bicchi, P. Rubiolo, C. Cordero, Anal. Bioanal. Chem. 384 (2006) 53.
14. K. Heinig, C. Vogt, Electrophoresis 20 (1999) 3311.
15. E. Marengo, M. Gennaro, V. Gianotti, J. Chromatogr. Sci. 39 (2001) 339.
16. E. Marengo, V. Gianotti, S. Angioi, M.C. Gennaro, J. Chromatogr. A 1029 (2004) 57.
17. L. Sanchez-Prado, J.P. Lamas, M. Lores, C. Garcia-Jares, M. Llompart, Anal. Chem. 82 (2010) 9384.
18. H.-Y. Shen, H.-L. Jiang, H.-L. Mao, G. Pan, L. Zhou, Y.-F. Cao, J. Sep. Sci. 30 (2007) 48.
19. L. Gagliardi, D. De Orsi, L. Manna, D. Tonelli, J. Liq, Chromatogr. R. T. 20 (1997) 1797.
20. M.-R. Lee, C.-Y. Lin, Z.-G. Li, T.-F. Tsai, J. Chromatogr. A 1120 (2006) 244.

21. J.F. García-Jiménez, M.C. Valencia, L.F. Capitán-Vallvey, Anal. Chim. Acta 594 (2007) 226.
22. T.-F. Tsai, M.-R. Lee, Chromatographia 67 (2008) 425.
23. A. van Asten, TrAC Trend Anal. Chem. 21 (2002) 698.
24. A. Chaintreau, 6.2 Analytical methods to determine potentially allergenic fragrance-related substances in cosmetics, in: A. Salvador, A. Chisvert (Eds.), Analysis of Cosmetic Products, Elsevier, Amsterdam, 2007, p. 257.
25. L. Mondello, D. Sciarrone, A. Casilli, P.Q. Tranchida, P. Dugo, G. Dugo, J. Sep. Sci. 30 (2007) 1905.
26. L. Mondello, A. Casilli, P.Q. Tranchida, G. Dugo, P. Dugo, J. Chromatogr. A 1067 (2005) 235.
27. G. Purcaro, P.Q. Tranchida, C. Ragonese, L. Conte, P. Dugo, G. Dugo, L. Mondello, Anal. Chem. 82 (2010) 8583.
28. F. Augusto, R.J. Poppi, M. Pozzobon Pedroso, L.A. Fonseca de Godoy, L. Wang Hantao, LCGC Europe (2010) 430–438.
29. M.S. Dunn, N. Vulic, R.A. Shellie, S. Whitehead, P. Morrison, P.J. Marriott, J.

Chromatogr. A 1130 (2006) 122.

30. J.P. Lamas, L. Sanchez-Prado, C. Garcia-Jares, M. Llompart, J. Chromatogr. A 1217 (2010) 1882.
31. J.P. Lamas, L. Sanchez-Prado, M. Lores, C. Garcia-Jares, M. Llompart, J. Chromatogr. A 1217 (2010) 5307.
32. M. Llompart, C. Garcia-Jares, P. Landin, Phthalate esters, in: L.M.L. Nollet (Ed.), Chromatographic Analysis of the Environment, CRC Press, Taylor and Francis Group, Boca Raton, 2006, pp. 1103–1153.
33. J.C. Hubinger, D.C. Havery, J. Cosmet. Sci. 57 (2006) 127.

Supplementary data

Table I. Found concentrations (%, w/v) of phthalates in real samples.

	DMP	DEP	DBP	DPP	BBP	DEHP
S1		0.00145	0.00009			0.00064
S2		0.33394	0.00011			0.00165
S3		1.25937				0.00119
S4		0.16490	0.00004			0.00003
S5		0.51411	0.00015			0.00737
S6		0.00427				0.00041
S7		0.30095	0.02975			0.00245
S8		0.68368	0.00008			
S9	0.00039	0.56822				0.00003
S10			0.00004			0.00003
S11		0.86790				0.00112
S12						0.00032
S13		1.20877				0.00270
S14		0.77721				
S15		0.28730	0.00005			0.00172
S16		0.56914				0.00510
S17		0.3661	0.00022			0.00036
S18		1.695	0.00019			0.00079
S19		0.8729	0.00021			0.00063
S20	0.00259	0.8272	0.00080			0.00135
S21	0.00046	0.5196				0.00020
S22		0.2987	0.00033			0.00016
S23						
S24		1.776				0.00017
S25		0.7717				0.00006
S26						0.00004
S27						0.00156
S28		1.574				0.00020
S29		0.7025	0.00008			0.00265
S30		0.3427	0.00022			0.00018
S31		0.3833	0.00056			0.00064
S32		1.767	0.00257			0.00693
S33		0.7513	0.00007			0.00034
S34		0.2954				
S35		0.00497	0.00035			0.00131
S36			0.00013			0.00014
S37	0.00015	0.6021	0.00008			0.00224
S38		0.2418	0.00004			0.00025
S39		0.3052	0.00004			0.00008
S40		0.3539			0.00060	0.00020
S41		0.4412				
S42	0.00019	0.4686	0.00010			0.00020
S43						
S44		0.00264				
S45						
S46		1.429				0.00018
S47		0.0217	0.00051			0.00017
S48						
S49		0.00247				0.00023
S50		0.7569				0.00004
S51		0.5421	0.00217			
S52	0.00017	0.5136	0.00030		0.00772	0.00861
S53		0.3507	0.00006			0.00015
S54	0.00004	0.5073	3.928			0.00089
S55						
S56						0.00003
S57		0.3670				0.00019
S58		0.0300				0.00061
S59						
S60	0.00004	0.00352	0.00003	0.00002		0.00017
S61		0.4006	0.00007			
S62		0.7630	0.00003			
S63		0.00181	0.00003			
S64		0.4654	0.00003			
S65		0.0358	0.00003			
S66						0.00006
S67		3.148	0.00030			
S68		0.3274	0.00003			0.00004
S69	0.00007	0.5891	0.00004			
S70		0.6940	0.00004			0.00163

Table II. Found concentrations (%, w/v) of musk fragrances in real samples.

	Cashmeran	Celestolide	Phantolide	Traseolide®	Galaxolide®	MX	Tonalide	MM	Ambrettolide	MK
S1		0.0264							0.00893	0.00083
S2	0.00134				0.17855		0.12069		0.0687	0.00114
S3					0.00636		0.01966		0.1263	0.00217
S4		0.0582	0.00009		0.07035		0.09939		0.00012	
S5	0.0160			0.00085	0.46693				0.0202	
S6		0.00075			0.00007		0.00003			
S7	0.0175				0.00613		0.00208		0.0312	0.00023
S8					0.00162		0.00006			
S9	0.1286				0.00755		0.00032		0.00785	
S10										
S11					1.012					0.2495
S12					0.00005					
S13		0.00149			0.7236				0.0221	0.3761
S14					0.9598				0.0157	
S15					0.2200		0.0629		0.0181	0.0951
S16					0.2277			0.1164	0.1304	0.1728
S17					0.2217					
S18							0.3320			
S19					0.00009					0.1060
S20			0.00072	0.00375	0.8605		0.7593		0.0249	0.2220
S21	0.00268				0.00009		0.00290		0.00012	
S22			0.00038		0.1495		0.1132		0.00010	
S23	0.00049				0.00036				0.00287	
S24			0.00482		0.2648		0.0101		0.4569	
S25	0.0416		0.00021		0.5884		0.2716		0.00363	
S26	0.00053				0.00025				0.0285	
S27							0.00010		0.00131	
S28	0.00055				1.600				0.00119	
S29	0.00077			0.00357	0.00068		0.00053			
S30					0.00097		0.00116			
S31					0.0837				0.0277	0.00177
S32					0.00015					
S33				0.00683	0.3311		0.1581		0.00448	0.0172
S34	0.00054				0.0832					
S35		0.00042			0.00028		0.00004			
S36	0.0176								0.0825	
S37			0.00212		0.8573		1.268			0.00049
S38					0.00023		0.00022		0.00151	
S39	0.00941		0.00039		0.00050		0.4166		0.0284	
S40	0.0477				0.4854					
S41					1.284					
S42		0.00014	0.00013		0.2396		0.3162			
S43		0.00031	0.00016		0.1225		0.0818		0.00061	
S44	0.0267								0.1010	
S45		0.00376			0.5781		0.1134			
S46	0.0506	0.00676	0.00189		1.284		0.8822		0.0688	
S47						0.00065				0.1143
S48		0.0319			0.00062				0.0108	
S49	0.0222				0.2029					
S50	0.1712				1.346				0.00048	
S51	0.0389	0.00124			0.4794				0.0125	
S52					0.00509		0.00281		0.0055	
S53	0.00354		0.00031		0.00094		0.3546		0.0108	
S54	0.00100				0.0780		0.0778			
S55			0.00093		0.9062		0.6499		0.0621	
S56	0.00090				0.5491				0.00042	
S57	0.00097				0.0555					
S58					0.00024				0.0383	
S59					0.0938				0.0209	
S60	0.00074				0.00036					
S61	0.00740				0.1713		0.0112			0.00050
S62					0.4838					
S63					0.00057				0.0506	
S64	0.00588	0.0105	0.00073		0.6747		0.3675			
S65					0.0537					
S66	0.0940	0.0105			1.435					
S67			0.00084		0.0256		0.00204		0.00803	
S68					0.00380		0.00023			
S69					0.2921					
S70					0.4171					

Table III. Found concentrations (%, w/v) of allergenic fragrances in real samples.

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
Pinene	*0.00065*	*0.00033*	*0.00786*	*0.00668*	*0.0497*	*0.0248*	*0.0107*	*0.00125*	*0.017*	*0.00045*
Limonene	0.00239	0.0102	0.2891	0.2724	1.066	**1.123**	0.421	0.0229	0.5192	0.00268
Benzyl alcohol	0.0452	0.062	0.0173				0.0153	0.0173	0.0411	
Linalool	0.0962	0.2161	0.1903	0.1635	0.7773	**0.2776**	0.1025	1.007	0.7289	0.0409
Citronellol	0.128	0.1254	0.2934		0.0643	**0.00588**	0.0166	0.0862	0.0951	
Citral		0.00328	0.0327	0.0165	0.0393	**0.0263**	0.0033	0.0335		0.159
Geraniol	0.0676	0.081	0.392		<LOQ	**0.0129**		0.1293	0.0224	
Cinnamal										
Hydroxycitronellal	0.207	0.00333	0.1934	0.0129	0.00777	**0.1138**	0.0664	0.1452	0.0102	0.0825
Anise alcohol										
Cinnamylalcohol	0.0397								0.0484	
Eugenol	0.0426		*0.00084*		*0.00048*	*0.00032*	0.038	0.0394		
Methyl eugenol	**0.0301**								*0.00057*	
Isoeugenol	0.00154						0.0075	0.00227		
Coumarin	0.00342				0.0269	**0.00803**		0.0814	0.8971	
α–Isomethyl ionone	0.0737	0.00512	0.3094	*0.00041*	0.0617	**0.0317**	0.0635	0.3258	0.7607	
Lilial®	0.0981	0.2411	1.875	0.2962	0.2007	**0.0111**	0.4194	0.003		
Amylcinnamal						**0.00998**				
Lyral®	0.0872	0.0841		0.0179		**0.00272**	0.1229		0.0832	
Amylcinnamyl alcohol				**0.00432**						
Farnesol					0.0733					
Hexyl-cinnamal	0.8412					**0.8667**		0.0848	0.4797	
Benzyl benzoate	0.1405	0.0481	0.0148	*0.00033*	0.3077	**0.00179**	0.0215	0.0329	0.2792	
Benzyl salicylate	0.3542	0.0393	0.612	*0.00053*	0.4186	*0.00019*	0.206	0.0118	0.0541	
Benzyl cinnamate	0.1231					**0.00113**		*0.00098*		

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Table III. Found concentrations (%, w/v) of allergenic fragrances in real samples (continued).

Pinene	**S11**	**S12**	**S13**	**S14**	**S15**	**S16**	**S17**	**S18**	**S19**	**S20**
Limonene	*0.00192*	*0.0206*	*0.00075*	*0.0271*	*0.00693*	*0.0501*	*0.0186*	*0.0159*	*0.00127*	*0.00677*
Benzyl alcohol	0.0598	0.8788	0.015	0.3779	0.2186	0.4254	0.7017	0.487	0.1263	0.2961
Linalool	0.1408		0.3159	0.00693		0.0394			0.0286	0.00468
Citronellol	0.3373	0.2471	0.4012	0.2684	0.5249	0.2936	0.4824	0.3525	0.0921	0.453
Citral	0.2508	0.00372	0.0592	0.0871	0.1512	0.4969	0.117	0.00597	0.0518	0.7066
Geraniol		0.00891				0.1214	0.0519	0.0336		0.0073
Cinnamal	0.0195	0.0124	0.2459	0.0368	0.00452	0.351	0.0088	0.00358	0.04	0.2766
Hydroxycitronellal										
Anise alcohol	1.024	0.00235	0.0201	0.0255		0.2264	0.001		0.2464	0.00417
Cinnamylalcohol										
Eugenol									0.0135	
Methyl eugenol	0.1728		0.1335			0.0248	*0.0009*		0.00124	0.0276
Isoeugenol	*0.00047*			0.00348		0.00562			*0.00029*	0.00921
Coumarin	0.0568									
α–Isomethyl ionone			0.0198	0.00194		0.4945		0.2015	0.0989	0.0215
Lilial®	0.1067	*0.00008*	0.0251	0.0962	0.0633	0.1858	0.1671	*0.00043*	0.044	0.2489
Amylcinnamal	0.6908	*0.00029*	0.1677	0.0872	1.142	0.2529	0.3931	0.2028		0.00319
Lyral®									0.00376	
Amylcinnamyl alcohol	0.6136			0.2382			0.1396	0.1589		
Farnesol										
Hexyl-cinnamal										
Benzyl benzoate				0.1031		0.2823		0.5831		0.00928
Benzyl salicylate	0.7233	*0.0003*	0.2513	0.0376		0.228	0.0075	0.0308	0.0419	0.6639
Benzyl cinnamate	0.3813		0.00646	0.0326		0.0445	0.0029	*0.00021*	0.045	0.0699

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Table III. Found concentrations (%, w/v) of allergenic fragrances in real samples (continued).

	S21	**S22**	**S23**	**S24**	**S25**	**S26**	**S27**	**S28**	**S29**	**S30**
Pinene		*0.0117*	*0.0119*	*0.0047*	*0.00686*	*0.00105*	*0.0001*	*0.0197*	*0.0106*	*0.00485*
Limonene	0.0032	0.5414	0.0844	0.0735	0.2194	0.0348	0.0048	0.2631	0.0557	0.0984
Benzyl alcohol	0.00208	**0.0102**				0.00364	0.0319	0.0032		0.0115
Linalool	0.1072	0.0871	0.2125	1.03	0.4493	0.9961	0.1709	0.2156	0.1431	0.1499
Citronellol	0.0097	0.00621	0.0163	2.325	0.0245	0.2773	0.0578		0.0186	0.3072
Citral		0.0232			0.00765			0.00494		
Geraniol	0.0126	0.00868	0.00484	1.648			0.0377		0.0192	0.102
Cinnamal					0.00244				*0.00089*	
Hydroxycitronellal	0.0348			**0.5204**	0.001	0.055		0.1329		0.0624
Anise alcohol								<LOQ		0.00871
Cinnamylalcohol										
Eugenol				0.408	0.0379	0.0353	0.003		0.00149	*0.00095*
Methyl eugenol		*0.00034*					*0.0002*			
Isoeugenol										
Coumarin		0.0175	0.00193				0.0497		0.0801	0.00922
α–Isomethyl ionone	*0.0003*	0.0402	*0.0001*	1.115	0.22	0.089		0.3414		0.382
Lilial®	*0.00099*	**0.1641**	*0.00043*	5.812		0.3033	0.5259	0.2283	1.051	0.2612
Amylcinnamal										
Lyral®	0.0148	**0.0189**		2.252	0.2107		0.1878			0.1762
Amylcinnamyl alcohol										
Farnesol										
Hexyl-cinnamal		0.1189		1.462	0.00757	0.2815			0.00665	
Benzyl benzoate	0.00167			**0.6888**		0.7159	0.0371	0.00591		0.038
Benzyl salicylate	0.1704	0.1282	*0.00023*	2.318		0.7352	0.9816	0.695	0.00803	0.0124
Benzyl cinnamate								*0.00065*		

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Table III. Found concentrations (%, w/v) of allergenic fragrances in real samples (continued).

	S31	S32	S33	S34	S35	S36	S37	S38	S39	S40
Pinene	*0.00326*	*0.00294*	*0.0127*	*0.0014*	*0.00562*	*0.00754*	*0.0011*	0.00441	*0.1182*	*0.035*
Limonene	0.00101	0.0582	0.2858	0.0405	0.0235	0.1832	0.0012	0.1224	**0.4092**	**0.9925**
Benzyl alcohol	0.0457	0.0419		0.0246	0.0018	0.0223		0.00489		
Linalool	0.0136	0.9058	0.4142	0.0513	0.1571	0.1497	0.0817	0.1446	**0.2952**	**1.085**
Citronellol	0.1398	0.0417	0.0195		0.00556	0.0485	0.0106	0.0665	**0.0226**	**0.0961**
Citral		0.0455			0.0169		0.0292			**0.0167**
Geraniol	0.1882	0.0638	0.0197		0.00383	0.0485		0.0119		**0.0057**
Cinnamal			*0.00031*				0.0014			
Hydroxycitronellal	*0.0006*		0.0203	0.0111	0.0835	0.123	**0.001**	0.0293		
Anise alcohol										
Cinnamylalcohol										
Eugenol	0.00454	0.1452	0.00054		*0.00023*		***2E-04***			
Methyl eugenol	0.00226	*0.00047*							*0.00008*	
Isoeugenol						0.00188				
Coumarin	0.00156	0.0443	0.0432		0.00137			0.192	**0.0163**	**0.048**
α–Isomethyl ionone	0.1304	0.0336	0.0308	0.051	0.0207	0.3867	**0.005**	0.2485	**0.2068**	
Lilial®		0.00186	0.147	0.1332	*0.00023*	0.5032	**0.035**		**0.2242**	**0.1131**
Amylcinnamal					*0.00025*					
Lyral®	0.0944			0.00743						
Amylcinnamyl alcohol										
Farnesol										
Hexyl-cinnamal	0.0233	0.00544	0.1865	0.1869	0.5547			0.2389	**0.1151**	
Benzyl benzoate	0.0862	*0.00082*	0.0213		*0.00066*					
Benzyl salicylate	0.086	*0.00086*	0.0499	0.2468	*0.00035*	0.3774	**0.011**	0.2697	*0.00081*	
Benzyl cinnamate										

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Grey square: the compound appears as an ingredient but was not found

Table III. Found concentrations (%, w/v) of allergenic fragrances in real samples (continued).

	S41	S42	S43	S44	S45	S46	S47	S48	S49	S50
Pinene	*0.00652*	*0.0198*	*0.00062*	*0.0104*	*0.00226*	*0.0373*	*0.005*	*0.00123*	*0.00125*	*0.00086*
Limonene	0.2953	**0.2564**	0.0207	0.1548	0.1353	0.4451	0.0112	0.00717	**0.1074**	0.0186
Benzyl alcohol	0.1311						0.0395	0.029		
Linalool	0.5531	**0.1612**	0.0311	0.3027	0.4347	0.6504	0.2925	0.1134	**0.1102**	0.00898
Citronellol	0.1304	**0.018**		0.1629	0.00285			0.1336	**0.0064**	0.00745
Citral	0.0245	**0.00557**	0.00233		0.00283		0.0016		**0.00439**	
Geraniol	0.0934	**0.0159**		0.0791		0.152	0.0023	0.0764		0.0047
Cinnamal		*0.00072*								
Hydroxycitronellal		*0.001*	*0.00034*	0.0664				0.2208		0.00525
Anise alcohol										
Cinnamylalcohol	0.0289							0.0407		
Eugenol	0.00115	**0.0188**		0.00141	0.00219	*0.00027*	0.0018	0.0448	**0.00475**	
Methyl eugenol	0.00139	*0.00033*				*0.00041*	*0.0004*			
Isoeugenol								*0.00053*		
Coumarin	0.062	**0.0664**				0.0819	0.0338	0.00171	**0.00407**	
α–Isomethyl ionone		**0.066**		0.2992			0.199	0.0843	**0.0536**	0.00617
Lilial®	0.2787	**0.0396**		0.8885		0.3732		0.1164	*0.00061*	3.283
Amylcinnamal										
Lyral®	0.8893	**0.0174**	0.1044				0.097	0.0953		
Amylcinnamyl alcohol								**0.00391**		
Farnesol										
Hexyl-cinnamal	0.1761	**0.00548**	*0.00054*	0.1716			0.004	0.9677	**0.0115**	0.0292
Benzyl benzoate	0.0649	**0.0663**	0.1056	0.0374	0.1454	0.027	0.0372	0.1469		
Benzyl salicylate	0.00757	**0.00276**		0.179			0.0654	0.4315	**0.00461**	0.0167
Benzyl cinnamate	*0.00089*			*0.00034*				0.1452		

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Black numbers: properly labelled
Bold numbers: the compound should be included as an ingredient but it is not
Italic numbers: the compound is not labelled but doesn't need to be included on the label because its concentration is lower than 0.001% or because it is not regulated
Grey square: the compound appears as an ingredient but was not found

Table III. Found concentrations (%, w/v) of allergenic fragrances in real samples (continued).

	S51	S52	S53	S54	S55	S56	S57	S58	S59	S60
Pinene	*0.0101*	*0.00495*	*0.0345*	*0.00069*	*0.00168*	*0.0118*	*0.0483*	*0.0205*	*0.00178*	*0.00113*
Limonene	0.0285	0.1775	0.2602	0.0306	0.00626	0.3884	0.4842	0.7984	0.1604	
Benzyl alcohol								0.1215		
Linalool	0.3378	0.2895	0.215	0.068	0.1023	0.6112	0.1855	0.7671	0.1309	*0.00053*
Citronellol	0.0339	0.0244	0.014	0.00583	0.0255	*0.00064*	**0.004**	0.5952	0.0636	0.0117
Citral	0.0376	0.00415		0.00417		0.0155	0.0067			
Geraniol	0.00677	0.0212	0.008	0.0106			0.0026	0.6732		
Cinnamal			0.00129				*0.0004*	0.00734		
Hydroxycitronellal				0.0119		0.0025		0.00242	0.017	
Anise alcohol										
Cinnamylalcohol										
Eugenol	0.0135	*0.00032*					0.0587	0.5676	*0.00022*	
Methyl eugenol		*0.00009*	*0.00025*				*0.0006*			
Isoeugenol							*0.0002*	0.045		
Coumarin		0.00128	0.00606		0.1923	0.017		0.5291	0.00843	
α–Isomethyl ionone	0.00885	*0.00056*		0.0755			*0.0006*	0.2432	0.0101	
Lilial®	0.0726	0.00211	0.0862			*0.00019*	0.0026	0.00325	0.0227	0.0712
Amylcinnamal										
Lyral®							0.1535	0.4506		
Amylcinnamyl alcohol										*0.00055*
Farnesol										
Hexyl-cinnamal								0.00112		0.3452
Benzyl benzoate					0.00134			0.00228		
Benzyl salicylate		*0.00099*	*0.00056*	0.00122		0.9371	0.0015	*0.00023*	0.0937	*0.00019*
Benzyl cinnamate										

Grey numbers: no considered in the labelling control subset
Black numbers: properly labelled
Bold numbers: the compound should be included as an ingredient but it is not
Italic numbers: the compound is not labelled but doesn't need to be included on the label because its concentration is lower than 0.001% or because it is not regulated
Grey square: the compound appears as an ingredient but was not found

Table III. Found concentrations (%, w/v) of allergenic fragrances in real samples (continued).

	S61	S62	S63	S64	S65	S66	S67	S68	S69	S70
Pinene	*0.00293*	*0.0358*	*0.00164*	*0.00214*	*0.00797*	*0.00973*	*0.0004*	*0.0214*		*0.00056*
Limonene	**0.0357**	**0.4988**	0.032	0.0217	0.3609	0.2359	0.0152	0.7069	0.00125	0.0147
Benzyl alcohol	**0.0017**		0.0289				0.026			0.0147
Linalool	**0.1073**	**0.296**	0.0157	0.2562	0.1327	0.1094	0.0326	0.3775	0.0135	0.1877
Citronellol	**0.00474**	**0.12**	0.0722	0.00375	0.00272	0.098	0.0507	0.00744	0.0127	0.0494
Citral		**0.00326**		0.00881	0.00959					
Geraniol		**0.014**		0.00135		0.1283	0.0063	0.0251	0.00468	0.0436
Cinnamal						**0.00101**		*0.00037*		
Hydroxycitronellal	*0.00065*		0.2859			0.0102	0.0232		0.0608	
Anise alcohol										
Cinnamylalcohol						0.0282				
Eugenol	**0.0155**			0.0197		0.00211	*0.0005*	*0.00024*	0.00557	0.00114
Methyl eugenol		*0.0006*		*0.00012*		*0.00039*				
Isoeugenol				0.0119						
Coumarin	**0.00224**	**0.04**				0.0907				0.0168
α–Isomethyl ionone	**0.0584**	**0.0536**	0.1205	0.0394	*0.00031*		**0.04**	**0.00115**	0.259	
Lilial®	**0.0296**	**0.2294**	1.143	0.096	**0.5676**	2.546	0.2103	0.0048	0.042	0.1688
Amylcinnamal										0.0312
Lyral®	**0.0415**						0.0244			0.0849
Amylcinnamyl alcohol										
Farnesol										
Hexyl-cinnamal	**0.1366**						0.0972	**0.00156**	0.2518	0.256
Benzyl benzoate	**0.0181**		0.0012		*0.00024*	0.0651	*0.0009*			0.0561
Benzyl salicylate	**0.0312**		0.1305	0.2753	*0.00021*	0.00635	0.0908	**0.00266**	0.0304	0.00413
Benzyl cinnamate			0.0659							

Grey numbers: no considered in the labelling control subset
Black numbers: properly labelled
Bold numbers: the compound should be included as an ingredient but it is not
Italic numbers: the compound is not labelled but doesn't need to be included on the label because its concentration is lower than 0.001% or because it is not regulated
Grey square: the compound appears as an ingredient but was not found

Table IV. Found concentrations (%, w/v) of preservatives in real samples.

	MEP	BHT		MEP	BHT
S1			**S36**		0.0154
S2		0.00481	**S37**		0.0101
S3		0.00868	**S38**		
S4		0.00058	**S39**		0.00051
S5		0.00109	**S40**		0.00305
S6		0.00278	**S41**		0.0622
S7		0.00016	**S42**		0.00030
S8			**S43**	0.2148	0.00003
S9		0.00385	**S44**		0.0140
S10		0.00806	**S45**		0.00507
S11	0.1258	0.00160	**S46**		0.0218
S12			**S47**		
S13		0.00264	**S48**		
S14			**S49**		0.00022
S15		0.0445	**S50**		0.0254
S16			**S51**		0.00102
S17		0.00761	**S52**		
S18		0.00056	**S53**		
S19			**S54**		0.00314
S20			**S55**		
S21			**S56**		0.00884
S22			**S57**		
S23			**S58**		0.00260
S24		0.00186	**S59**		0.0103
S25			**S60**		0.00006
S26			**S61**		0.1911
S27			**S62**		0.00022
S28		0.0407	**S63**		0.0306
S29		0.0514	**S64**		0.0182
S30		0.00086	**S65**		0.0103
S31			**S66**		0.00006
S32		0.00021	**S67**		0.3188
S33		0.0276	**S68**		0.00259
S34			**S69**		0.2481
S35		0.00783	**S70**		0.2346

2. DETERMINACIÓN DE SUSTANCIAS ALERGÉNICAS EN AIRE INTERIOR

El ritmo de vida presente en la sociedad moderna provoca que la mayor parte de la población desarrolle su vida cotidiana en ambientes interiores. Una persona puede llegar a pasar más del 90% de su tiempo en dichos ambientes: en el hogar, coche, metro, oficina, etc. Ésta es la razón principal de la creciente preocupación de la sociedad por la calidad del aire en este tipo de ambientes. Por ello es muy frecuente encontrarse con multitud de productos en el mercado con la única función de "purificar" el aire en ambientes interiores. Este tipo de ambientes, además de los contaminantes clásicos, pueden presentar una concentración elevada de contaminantes emergentes, y en concreto, de PCPs. El uso cotidiano de productos de higiene y cuidado personal como perfumes, jabones, maquillajes, etc., los convierten en el principal foco de entrada de estas sustancias en el organismo humano. Si añadimos factores como una ventilación inadecuada, valores de temperatura y humedad elevados en estos lugares cerrados, se obtiene un ambiente contaminado y tóxico para la salud.

Trasladando a este escenario la profusa utilización de productos con fragancias alergénicas, obtenemos un ambiente muchas veces enrarecido, irritante, molesto y alergénico por la presencia de estas sustancias sensibilizantes.

Hasta la publicación de los estudios que se recogen en esta Tesis, no se había publicado ningún método analítico capaz de determinar la presencia de las 26 fragancias alergénicas en aire interior.

El objetivo perseguido fue la puesta a punto de un método analítico con el que se pudiera determinar la presencia de estas sustancias en los ambientes interiores siempre con la premisa de que dicha metodología fuese sencilla, rápida y fácilmente trasladable a la rutina de cualquier laboratorio analítico.

Para ello se desarrollaron dos metodologías basadas en trabajos anteriores de nuestro grupo de investigación en los que se realizaba la determinación de musks sintéticas, pesticidas y otros compuestos en ambientes interiores [1,2]. Las metodologías propuestas combinan un muestreo activo mediante extracción en fase sólida (SPE) seguido por la extracción por ultrasonidos (US) o por la extracción y preconcentración mediante microextracción en fase sólida (SPME), de la fase utilizada durante el muestreo.

Tras la optimización, tanto de la etapa de muestreo como de las etapas de extracción propuestas, se aplicaron los métodos desarrollados al análisis de muestras procedentes de distintos hogares y otros ambientes interiores.

Tras el análisis de los resultados obtenidos, se observa que el uso de productos perfumados aporta al aire interior niveles elevados de estas fragancias alergénicas con el consiguiente riesgo para la salud.

En resumen, este apartado de Tesis se ha centrado en el desarrollo de metodología analítica para la determinación de 26 fragancias alergénicas en aire interior, originando los siguientes estudios, que se discuten en los apartados posteriores:

- "Determination of fragrance allergens in indoor air by active sampling followed by ultrasound-assisted solvent extraction and gas chromatography–mass spectrometry".
- "Sorbent trapping solid-phase microextraction of fragrance allergens in indoor air".

Bibliografía:

[1] R. Barro, C. Garcia-Jares, M. Llompart, M. Herminia Bollain, R. Cela, J. Chromatogr. A. 1111 (2006) 1.

[2] J. Regueiro, C. Garcia-Jares, M. Llompart, J.P. Lamas, R. Cela, J. Chromatogr. A 1216 (2009) 2805.

2.1 DETERMINATION OF FRAGRANCE ALLERGENS IN INDOOR AIR BY ACTIVE SAMPLING FOLLOWED BY ULTRASOUND-ASSISTED SOLVENT EXTRACTION AND GAS CHROMATOGRAPHY–MASS SPECTROMETRY

DETERMINATION OF FRAGRANCE ALLERGENS IN INDOOR AIR BY ACTIVE SAMPLING FOLLOWED BY ULTRASOUND-ASSISTED SOLVENT EXTRACTION AND GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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Abstract

Fragrances are ubiquitous pollutants in the environment, present in the most of household products, air fresheners, insecticides and cosmetics. Commercial perfumes may contain hundreds of individual fragrance chemicals. In addition to the widespread use and exposure to fragranced products, many of the raw fragrance materials have limited available health and safety data. Because of their nature as artificial fragrances, inhalation should be considered as an important exposure pathway, especially in indoor environments. In this work, a very simple, fast, and sensitive methodology for the analysis of 24 fragrance allergens in indoor air is presented. Considered compounds include those regulated by the EU Directive, excluding limonene; methyl eugenol was also included due to its toxicity. The proposed methodology is based on the use of a very low amount of adsorbent to retain the target compounds, and the rapid ultrasound-assisted solvent extraction (UAE) using a very low volume of solvent which avoids further extract concentration. Quantification was performed by gas chromatography coupled to mass spectrometry (GC–MS). The influence of main factors involved in the UAE step (type of adsorbent and solvent, solvent volume and extraction time) was studied using an experimental design approach to account for possible factor interactions. Using the optimized procedure, 0.2 m^{-3} air are sampled, analytes are retained on 25 mg Florisil, from which they are extracted by UAE (5 min) with 2 mL ethyl acetate. Linearity was demonstrated in a wide concentration range. Efficiency of the total sampling-extraction process was studied at several concentration levels (1, 5 and 125 μg m^{-3}), obtaining quantitative recoveries, and good precision (RSD < 10%). Method detection limits were ≤0.6 μg m^{-3}. Finally, the proposed method was applied to real samples collected in indoor environments in which several of the target compounds were determined.

Keywords: Fragrance allergens; Ultrasound-assisted solvent extraction; Indoor air; Air analysis; Factorial design optimization; GC–MS

1. Introduction

Indoor air quality has become an important global community concern due to the increased amount of personal time spent in indoor environments. Taking into account that people in developed countries spend up to 90% of their time indoors [1, 2], inhalation of indoor air is potentially the most important exposure pathway to many pollutants [2]. The high comfort achieved in developed countries increased the demand and the widespread consumption of fragranced household products, fresheners and cosmetics. Inadequate ventilation, high temperatures and humidity coupled with the slow indoor degradation processes may increase indoor levels of many components of these consumer products [3]. The primary purposes of fragrances are to impart a scent to a product, mask the odor of other materials in the product or, in some cases, alter mood. More than 2600 ingredients have been documented for use in fragrances [4] but many of the raw fragrance materials have little available health and safety data. The potential for exposure to these materials in our society is, therefore, very high. With increased usage and exposure there are increased anecdotal and clinical accounts of fragranced products causing, triggering and exacerbating health conditions. In addition to known dermatological problems [5, 6], fragrances can induce or worsen respiratory problems due to their irritant effect. They are thought to trigger asthma, asthmatic exacerbations, and other respiratory conditions [7, 8]; headaches [9]; and mucosal symptoms [10]. Those with asthma, allergies, sinus problems, rhinitis and other such conditions are more susceptible to the effects of irritants, often at levels that are many times lower than what would cause problems in the general population [11]. The long-term impact due to the possible bioaccumulation in human tissues is also cause of concern. In addition, there are environmental concerns, as fragranced products add to both air and water pollution.

The Scientific Committee on Cosmetic Products and Non-Food Products (SCCNFP) has identified 26 of these ingredients as likely to cause contact allergies [12]. They have been designated by the European Union (EU) as requiring labeling on cosmetic and detergent products [13, 14]. The presence of these fragrances must be indicated in the list of ingredients when its concentration exceeds the 0.001% in leave-on products and 0.01% in rinse-off products. The use of some of the 26 fragrance compounds is already more restricted, i.e. the finished cosmetic product

must not contain more than 0.01% of methyl-2-octynoate, 0.02% of isoeugenol and 1.0% hydroxycitronellal. Methyleugenol must not be part of the composition of cosmetic products, although there are some exceptions. The most of these substances are also restricted by the International Fragrance Association (IFRA) [15], the official representative body of the fragrance industry worldwide, with the main purpose of ensuring the safety of fragrance materials. Analytical methods for the determination of this group of substances are mainly based on gas chromatography–mass spectrometry (GC–MS) [16–19]. Most of these methods are focused on the determination of these compounds in cosmetics. Owing to the difficulty of obtaining a good compound resolution as well as with other matrix components, advanced methods based on multidimensional chromatography have been proposed [20–22]. Recently, a method for the quantification of 15 fragrance allergens in baby bathwaters has been published [23]. The analytical procedure is based on solid phase microextraction (SPME) and GC–MS analysis.

To our knowledge, there are no studies developing analytical methodology for the analysis and quantification of these fragrance allergens in indoor air. Few studies have reported the analysis of synthetic musk compounds in indoor air and suspended particulate matter. In all of them, musks have been collected by active sampling and, in general, reduced flow rates, using polyurethane foam as adsorbent [24–26]. The extraction of musk compounds from this adsorbent is carried out by Soxhlet using different solvent mixtures [25, 26] and pressurized solvent extraction (PSE) [24]. To overcome the drawbacks of these methods related to time consuming steps and large volumes of organic solvents required, Regueiro et al. [27] proposed the use of SPME as an alternative to solvent extraction. In this way, musk compounds are adsorbed onto a small amount of Tenax and analytes are transferred to a SPME fiber in the headspace mode. As an alternative to SPME fiber as the acceptor phase in the desorption of the analytes from the adsorbent, Barro et al. [28, 29] proposed a simple method based on the rapid desorption of the analytes adsorbed on Tenax to a small volume of *n*-hexane for the determination of polychlorinated biphenyls [28] and pyrethroid insecticides [29] in indoor air.

The aim of the present study was to develop a fast, simple and inexpensive method for the determination of 24 fragrance allergens in indoor air based on the use of a very low amount of adsorbent to retain the compounds, which allowed their

rapid desorption by UAE in a very low volume of solvent, avoiding further sample manipulation. The optimization of the methodological parameters was carried out using an experimental design approach to study the main factors as well as possible factor interactions. The performance of the method was studied in terms of linearity, precision, accuracy and limits of detection. The application to real samples collected in home and car environments allowed the determination of several of the target compounds at concentrations ranging from <1 to > 100 μg m^{-3}.

2. Experimental

2.1. Reagents and materials

3,7-Dimethyl-1,6-octadien-3-ol, 97% (linalool, CAS number 78-70-6); 3,7-dimethyloct-6-en-1-ol, 95% (citronellol, 106-22-9); 2-methoxy-4-prop-2-enyl phenol, 99% (eugenol, 97-53-0); 1,2-dimethoxy-4-(2-propenyl)-benzene, 99% (methyleugenol, 93-15-2); 2H-1-benzopyran-2-one, 99% (coumarin, 91-64-5); 3,7,11-trimethyldodeca-2,6,10-trien-1-ol, 95% (farnesol, mixture of isomers, 4602-84-0); 3,7-dimethylocta-2,6-dienal, 95% (citral, cis/trans, 5392-40-5); 1-methyl-4-prop-1-en-2-yl-cyclohexene 97% (limonene, 5989-27-5); 4-methoxybenzene methanol, 98% (anisyl alcohol, 105-13-5); 2-methoxy-4-(1-propenyl) phenol, 98% (isoeugenol, cis/trans, 97-54-1); 3-phenyl phenylmethyl ester-2-propenoic acid, 99% (benzyl cinnamate, 103-41-3); and 2-(phenylmethylene)-heptanal, 97% (amyl cinnamal, 122-40-7) were purchased from Aldrich (Sigma–Aldrich Chemie GmbH, Steinheim, Germany).

3-Methyl-4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-3-buten-2-one, ≥85% (ionone, 127-51-5); 3,7-dimetil-2,6-octadien-1-ol, ≥96% (geraniol, 106-24-1); 2-(phenylmethylene)-1-heptanol, ≥85% (amyl cinnamyl alcohol, 101,85-9); 3-(4-tertbutylphenyl)-2-methylpropanal, ≥95% (lilial®, 80-54-6); 4-(4-hydroxy-4-methylpentyl)cyclohex-3-ene-1-carbaldehyde, ≥97% (lyral®, 31906-04-4); and 2-hydroxy-phenylmethyl ester benzoic acid, ≥99% (benzyl salicylate, 118-58-1) were purchased from Fluka (Fluka Chemie GmbH, Steinheim, Germany). 2-Octynoic acid, methyl ester, ≥ 99% (methyl 2-octynoate, 111-12-6); 7-hydroxy-3,7-dimethyloctanal, ≥95% (hydroxycitronellal, 107-75-5); 3-phenyl-2-propenal, ≥93% (cinnamaldehyde, 104-55-2); 2-(phenylmethylene) octanal, ≥95% (hexyl

cinnamaldehyde, 101-86-0), were purchased from SAFC Supply Solutions (St. Louis, USA).

Benzene methanol, 99% (benzyl alcohol, 100-51-6); 3-phenyl-2-propen-1-ol, 98% (cinnamyl alcohol, 104-54-1); phenylmethyl benzoate, 98.5% (benzyl benzoate, 120-51-4) was purchased from Chem Service (West Chester, USA).

n-Hexane, ethyl acetate, and acetone were provided by Merck (Darmstadt, Germany). Individual stock solutions of each compound were prepared in acetone. Further dilutions and mixtures were prepared in acetone and then stored in amber glass vials at −20 ºC.

2.2. Gas chromatography–mass spectrometry

The GC–MS analysis was performed using an Agilent 7890A (GC)-Agilent 5975C inert MSD with triple axis detector and an Agilent 7693 autosampler from Agilent Technologies (Palo Alto, CA, USA). The temperatures of the transfer line, the quadrupole and the ion source were set at 280, 150 and 230 ºC, respectively. The system was operated by Agilent MSD ChemStation E.02.00.493 software.

Separation was carried out on a HP5-MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness). Helium (purity 99.999%) was employed as carrier gas at a constant column flow of 1.0 mL min^{-1}. The GC oven temperature was programmed from 45 ºC (held 2min) to 100 ºC at 8 ºC min^{-1}, to 150 ºC at 20 ºC min^{-1}, to 200 ºC at 25 ºC min^{-1} (held 5min) and a final ramp to 225 ºC at

8 ºC min^{-1}.

Splitless mode (held 2min) was used for injection, the split flow was set at 20 mL min^{-1} and the injector temperature was kept at 260 ºC.

In the full scan mode the mass range was varied from 39 to 300 *m/z*, starting at 5 min. The analytes were positively identified by comparison of their mass spectra and retention times to those of standards. The quantification ions for each target compound are listed in Table 1.

Table 1. Quantification ions and performance of the GC–MS method.

Key	Compound	MS detection	Linearity	Precision (% RSD)				
		Quantification Ions	Correlation coefficient (R)	Intra-day (n=4)			Inter-day (n=7)	
				0.05a	0.5[a]	10[a]	0.5a	10[a]
1	Limonene	93	1.0000	2.0	1.5	1.9	2.1	2.1
2	Benzyl alcohol	108	1.0000	17	3.9	1.6	10	2.9
3	Linalool	93	0.9997	2.3	2.2	1.7	0.8	1.7
4	Methyl-2-octynoate	95	0.9992	5.4	3.3	1.7	10	1.8
5	Citronellol	69	0.9971	-	3.2	2.0	5.4	1.8
6	Geraniol	69	0.9995	-	4.1	1.4	3.6	1.1
7	Citral	69	0.9999	4.6	2.3	1.8	3.2	1.8
8	Cinnamaldehyde	131	0.9991	3.8	4.1	0.98	3.5	1.7
9	Anisyl alcohol	138	0.9994	6.7	3.4	1.2	5.0	2.0
10	Hydroxycitronellal	59	0.9992	3.0	2.2	1.7	3.3	1.8
11	Cinnamyl alcohol	134	0.9996	-	5.5	2.2	8.3	2.4
12	Eugenol	164	1.0000	4.7	2.1	0.83	1.3	1.4
13	Methyleugenol	178	0.9997	2.8	0.7	1.1	5.7	1.4
14	Coumarin	146	0.9998	1.2	2.8	1.3	10	2.7
15	Isoeugenol	164	1.0000	5.4	2.4	1.1	3.4	1.7
16	Ionone	135	0.9990	1.2	1.4	1.3	1.2	1.1
17	Lilial®	189	0.9993	2.3	1.0	1.1	1.1	1.5
18	Amyl cinnamal	129	0.9991	2.3	1.7	1.1	14	1.6
19	Lyral®	136	0.9970	8.5	3.9	3.9	10	1.7
20	Amyl cinnamic alcohol	133	0.9988	-	5.5	0.95	12	2.9
21	Farnesol	69	0.9976	-	4.7	4.2	9.6	1.5
22	Hexyl cinnamaldehyde	129	0.9995	5.9	2.9	1.1	6.4	1.6
23	Benzyl benzoate	105	0.9989	4.4	1.1	1.7	4.6	1.8
24	Benzyl salicylate	91	0.9997	2.8	4.5	4.8	9.1	1.6
25	Benzyl cinnamate	131	0.9995	6.3	2.5	1.1	11	2.0

[a] Concentration levels (μg mL^{-1}).

2.3. Ultrasound-assisted extraction

To optimize the UAE of target compounds, a volume of 100 μL of standard mixtures of the analytes in acetone were directly spiked on 25 mg of the adsorbent: activated Florisil of 60–100 mesh (Aldrich, Steinheim, Germany) or Tenax TA of mesh size 60–80 (Supelco). Florisil was activated overnight in an oven at 130 ºC. The spike was left 2 h at room temperature allowing the evaporation of the solvent, and then the selected volume (1 or 2mL depending on the experiment) of the extractant organic solvent (ethyl acetate or *n*-hexane) was added to the glass vial, and sealed with a headspace aluminum cap furnished with PTFE-faced septum. The analytes were extracted from the samples to the organic solvent using an ultrasound bath (Ultrasons Med-II, J.P. Selecta, Barcelona, Spain) at 40 kHz of ultrasound frequency and 200W power at 25 ± 3 ºC or 45 ± 3 ºC for 5 or 10min, depending on the experiment. Afterwards, the extract was filtered through a 0.22 μm Millex®-GV

filter (13 mm diameter) (Millipore, Bedford, USA), and injected in the chromatographic system.

In the final optimized conditions, 25 mg of Florisil were sonicated with 2mL ethyl acetate for 5min at 25 ± 3 ºC. Blanks were periodically run during the analysis to confirm the absence of contamination.

2.4. Air sampling

To collect the target compounds from air, a known volume of air was pumped through a glass tube containing 25 mg of activated Florisil adsorbent by using a S-8 vacuum pump (Telstar, Tarrasa, Spain). Only PTFE tubing was used for all connections to minimize contaminations. Different volumes of air (0.05 – 1 m^3) were pumped at 0.010 $m^3\ min^{-1}$ through the microfiltration glass funnels containing 25 mg Florisil. The adsorbent with the retained compounds was then simply transferred from the glass funnel into a 10-mL headspace glass vial and the UAE was carried out under the optimized conditions.

For method validation experiments, the sampler was placed in a clean room provided of a laminar flow system in order to avoid external contamination.

To detect possible breakthrough of the adsorbent, some experiments required the coupling on-line of a second and a third glass tube filled with 25 mg of non-spiked Florisil to the first spiked one. Each portion of adsorbent was individually extracted.

2.5. Statistical analysis

Basic and descriptive statistics and experimental design analysis were performed using Statgraphics XV Centurion (Rockville, MD) as software package. The experimental design was applied in the optimization of the UAE method, to analyze the simultaneous effect of the main parameters.

3. Results and discussion

Difficulties described in literature dealing with the effective separation of the regulated suspected allergens [20] led to test different oven temperature programs in order to obtain a suitable chromatography of the compounds. First experiments also allowed the selection of the quantification ions to attain the maximum signal-to-noise ratio. In the GC–MS conditions summarized in the experimental section, all compounds could be determined in less than 21 min. Fig. 1 shows the chromatogram of a standard mixture of 25 allergen fragrances at a concentration of 5 μg mL^{-1}, in which the good separation of the compounds can be noticed.

Linearity of the GC–MS method was evaluated in the concentrations range from 0.025 to 20 μg mL^{-1} (9 levels). The correlation coefficients were higher than 0.997 for all compounds (see Table 1). Intra-day and inter-day precision were evaluated at several concentration levels and both were satisfactory (<5% in most cases).

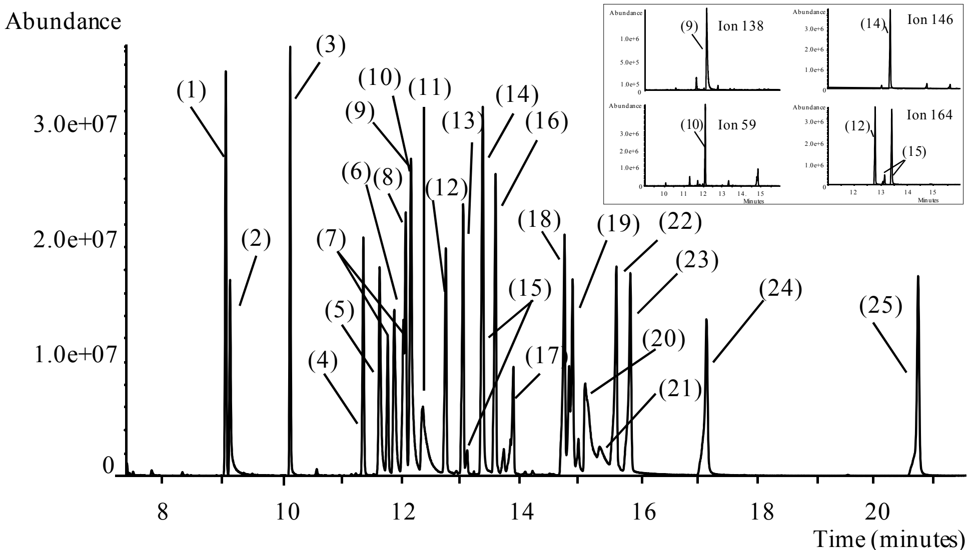

Fig. 1. GC–MS full scan chromatogram of a standard mixture of the fragrance allergens at 5 μg mL^{-1} in ethyl acetate (see number code equivalence in Table 1).

3.1. Optimization of the ultrasound-assisted solvent extraction

Desorption step determines the efficiency of the final method and then, experimental work was initially focused on the optimization of the UAE process using an experimental design approach. Five main factors were selected for this study: type of adsorbent, type and volume of extracting solvent, extraction temperature and ultrasounds application time. Tenax TA and Florisil were the choice for the two levels of factor type of adsorbent. The efficiency of Tenax TA and Florisil in the retention of some organic pollutants in air, even at such little amounts as 25 mg, was previously reported [30–32] and thus, both adsorbents were considered in the present study. Selection of the two solvents was related to the type of adsorbents we intended to check; on one hand, a very low polarity solvent such as n-hexane, and on the other, a medium polarity solvent such as ethyl acetate. This last factor was studied at three levels whereas all the other factors were studied at two levels. The factors selected and their levels are presented in Table 2.

Table 2. Factors and levels considered in the experimental design.

Factor	Code	Low level (-)	High level (+)	Continuous
Solvent	A	*n*-Hexane	Ethyl acetate	Yes
Temperature	B	25 ºC	45 ºC	Yes
Extraction time	C	5 min	10 min	Yes
Solvent volume	D	0.5 mL	2 mL	Yes
Adsorbent	E	Tenax	Florisil	No

A $3\times2^{(4-1)}$ mixed level fraction design was proposed (Statgraphics XV Centurion). The resolution of the design is V, enabling an estimation of all main effects and all two-factor interactions. Two center points were added to increase the degrees of freedom to evaluate the experimental error; thus, 26 experiments were run.

The outcomes of the experimental design can be simply interpreted by visualizing several intuitive software tools provided by Statgraphics. For practical reasons, only some representative examples are illustrated in Figs. 2–4. In the Pareto charts (Fig. 2), the standardized effects are plotted in decreasing order of

absolute magnitude, thus making easier to see which are the most important factors and interactions. In addition, the line drawn on the chart indicates if an effect is statistically significant at a specified significance level (in this case, 95%).

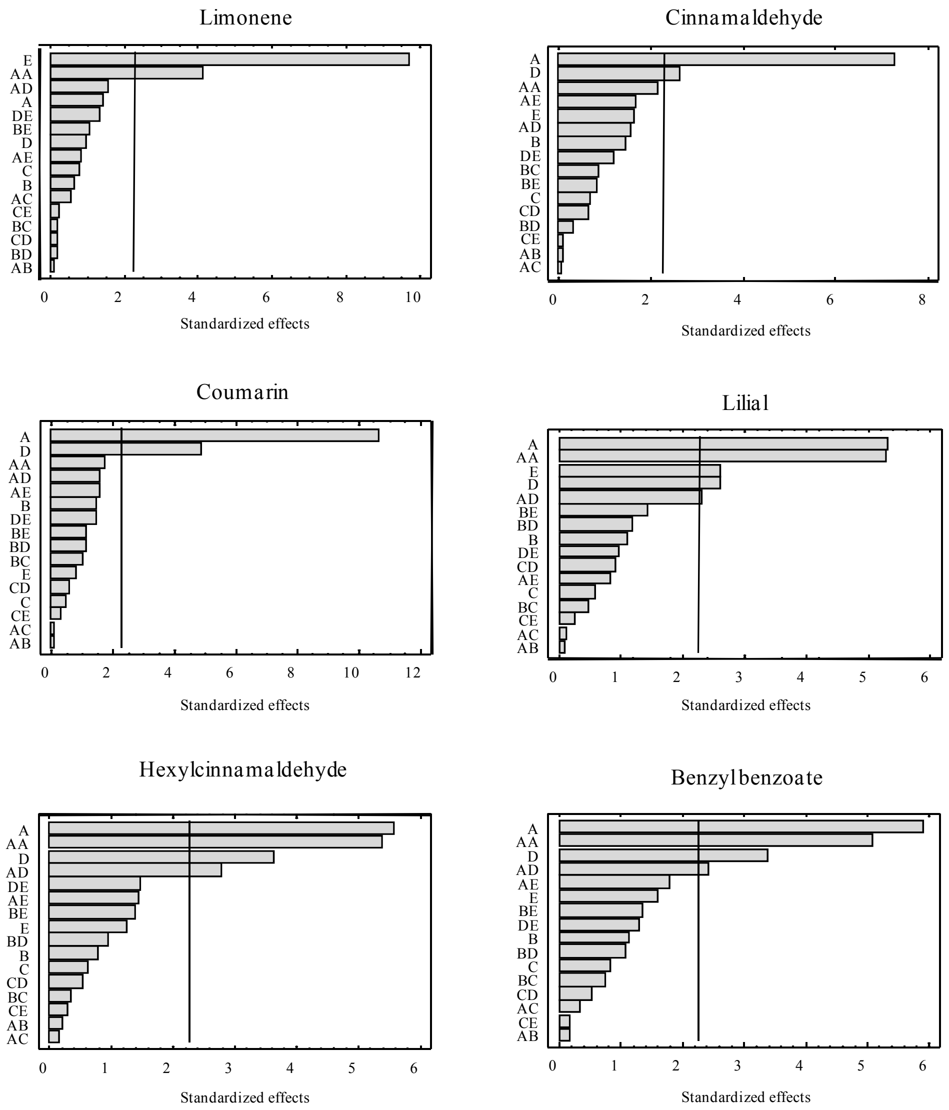

Fig. 2. Pareto charts showing the significant factors (95%) for some selected fragrance allergens (see factor codes in Table 2).

Analyzing the Pareto charts (Fig. 2), it was observed that type of solvent (A), and solvent volume (D) were the most important parameters for the extraction efficiency. Factor A was significant for all analytes excluding limonene, and factor D was significant for half of the target compounds. The type of adsorbent used (factor E) was only significant for limonene and lilial®. The standardized effect of the other 2 factors, B and C (temperature and extraction time, respectively) did not reach the significance border line. Fig. 3 shows the main effects plots for some representative compounds. These graphics show how the response varies when each factor is changed from its low level to its high level, while all other factors held at the center of the experimental domain. As can be seen, all analytes were more efficiently extracted from the adsorbent using 100% of ethyl acetate (the high level of this factor). The use of *n*-hexane provided lower responses than ethyl acetate and for some analytes the lowest results were obtained when a mix of both solvents was used (represented by a central minimum, e.g. linalool and benzyl benzoate, see Fig. 3). This last effect is also showed in the Pareto chart diagram (Fig. 2) with a significant effect for the quadratic term of this factor (AA) (for example, see lilial® and hexyl cinnamaldehyde in the figure). Regarding solvent volume, all analytes were better extracted at the high level of this factor, 2 mL. For the other 3 main factors, the differences between the analytical response obtained for the low and the high level of the factor were not important, and so, these factors are represented by a short and almost horizontal line, excluding factor E for limonene and lilial® as previously indicated, being the extraction more favorable from Tenax for limonene and from Florisil for lilial® (see Fig. 3). Concerning interaction effects, only AD interaction was significant for some analytes such as hexyl cinnamaldehyde and benzyl benzoate (Fig. 2), and this effect is shown in Fig. 4 for some representative compounds. In these plots, the predicted response for each combination of the low and high levels of two factors is displayed at the end of each line segment. As it can be observed, the extraction efficiency using n-hexane is considerably lower than using ethyl acetate, as it was already concluded from the main effects plots (Fig. 3). Using the first solvent, the responses obtained were in general quite similar for 0.5 and 2 mL. Nevertheless, when ethyl acetate was used, higher response and, in consequence, better extraction efficiency was achieved with 2 mL of solvent.

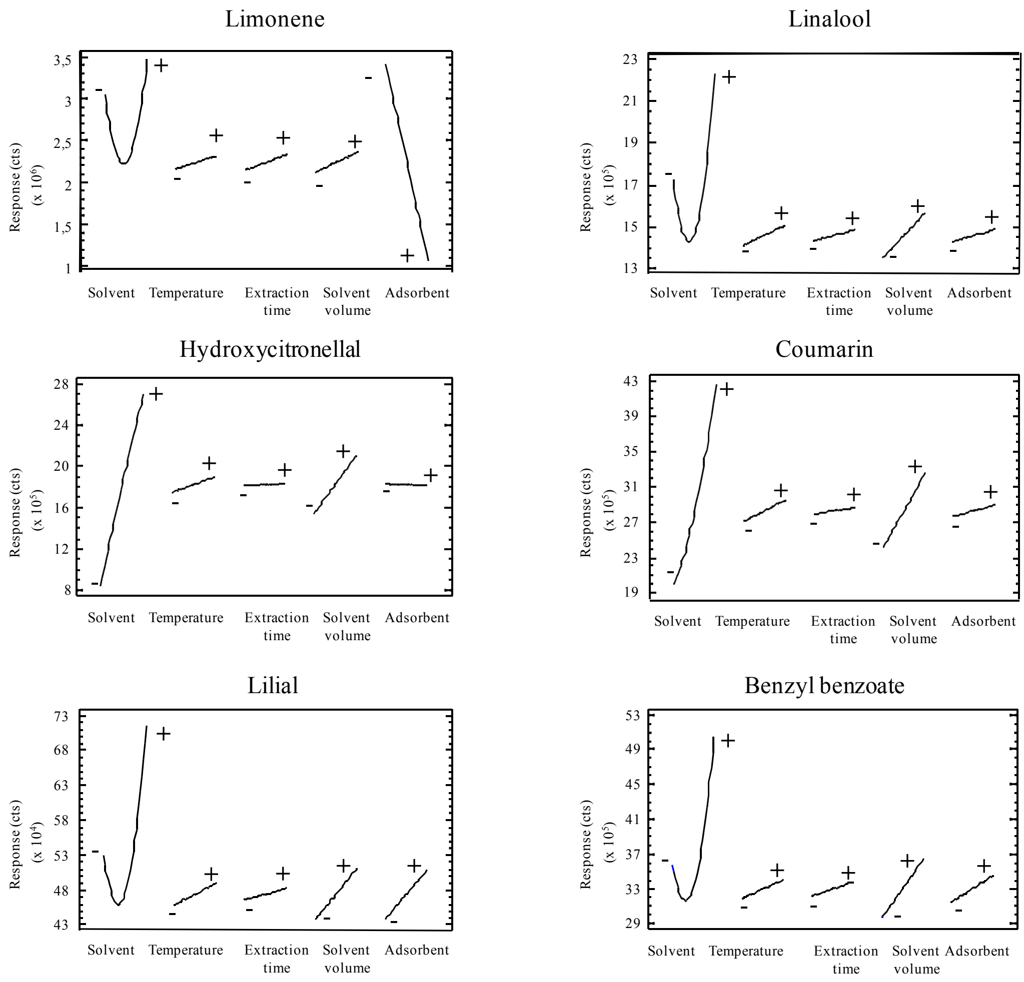

Fig. 3. Main effects plots for some selected fragrance allergens (see levels in Table 2).

After optimization of the investigated factors, the recommended procedure for the simultaneous UAE of the target analytes was established as follows: temperature 25 ºC, 2 mL of ethyl acetate, and 5min of extraction time using Florisil or Tenax as adsorbent.

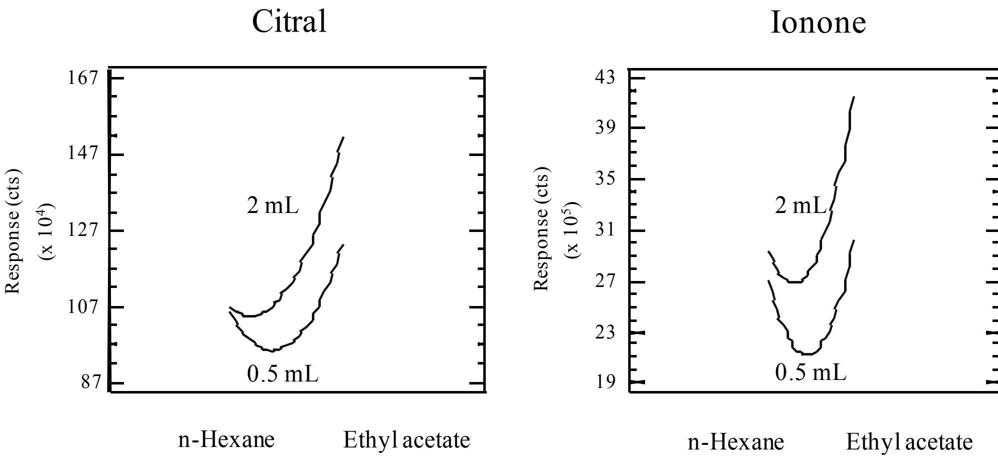

Fig. 4. Combined effect of factors type of solvent (A) and solvent volume (D) for two selected fragrance allergens: citral and ionone.

Under the experimental conditions selected, extraction efficiency was calculated using Florisil spiked at three levels (0.2, 2, and 25 μg of each compound) and, as can be seen in Table 3, average recoveries were satisfactory for most analytes (>80%), excluding benzyl salicylate for which recovery was about 50%. Anyway, the recovery for this last compound was consistent and equivalent at different concentration levels. The precision was also satisfactory with RSD in general lower than 10% (see Table 3); therefore, the extraction method can be considered suitable for all the target analytes.

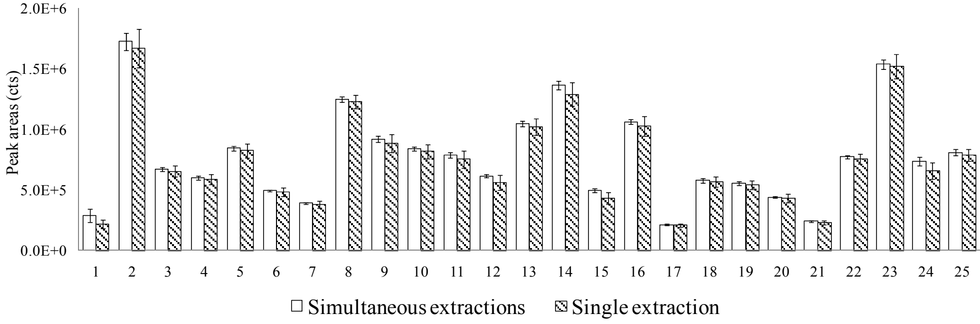

Fig. 5. Comparison of the responses obtained for simultaneous and single extractions (see number code equivalence in Table 1).

Table 3. Extraction efficiency (%) from Florisil at three spiked concentration levels.

Compound	0.2 µg (n=4)		1 µg (n= 4)		25 µg (n=4)	
	Recovery	RSD	Recovery	RSD	Recovery	RSD
Limonene	96.0	3.0	110	8.6	102	7.7
Benzyl alcohol	84.4	5.6	102	2.0	109	7.6
Linalool	98.9	6.0	105	4.2	105	7.2
Methyl-2-octynoate	88.5	5.6	106	3.1	108	8.3
Citronellol	111	13	105	8.5	108	8.4
Geraniol	98.8	6.5	110	3.2	106	8.2
Citral	90.3	2.7	108	2.8	108	8.1
Cinnamaldehyde	94.4	3.4	106	3.9	112	7.5
Anisyl alcohol	97.2	8.6	109	8.2	112	6.7
Hydroxycitronellal	93.9	5.8	104	4.0	112	6.8
Cinnamyl alcohol	79.2	7.8	101	6.7	114	6.6
Eugenol	90.0	5.5	81.1	5.2	93.4	10
Methyleugenol	89.0	6.5	107	2.8	110	7.7
Coumarin	93.4	5.0	103	4.0	113	3.6
Isoeugenol	104	11	94.2	8.6	86.6	11
Ionone	100	7.5	101	1.9	107	6.6
Lilial®	101	2.8	105	3.0	105	8.2
Amyl Cinnamal	100	7.1	105	1.5	114	7.8
Lyral®	90.4	7.3	111	2.4	104	7.2
Amyl cinnamyl alcohol	100	6.8	102	4.6	114	7.7
Farnesol	104	8.9	111	5.0	118	7.3
Hexyl cinnamaldehyde	99.5	3.5	100	2.1	114	7.5
Benzyl benzoate	94.2	5.9	110	2.1	115	5.1
Benzyl salicylate	44.9	9.6	43.6	8.1	59.8	10
Benzyl cinnamate	110	3.3	120	0.6	96.9	3.6

The possibility of performing simultaneous extractions was also evaluated and the results obtained (Fig. 5) were equivalent for single and multiple extractions (n = 6), allowing in this way to improve the throughput of this method step.

3.2. Optimization of the sampling step

Once optimized the extraction process and confirmed that the allergens could be recovered from the adsorbent, the sampling step was studied. Initial experiments using Tenax and Florisil demonstrated the inefficiency of the Tenax to effectively retain the analytes.

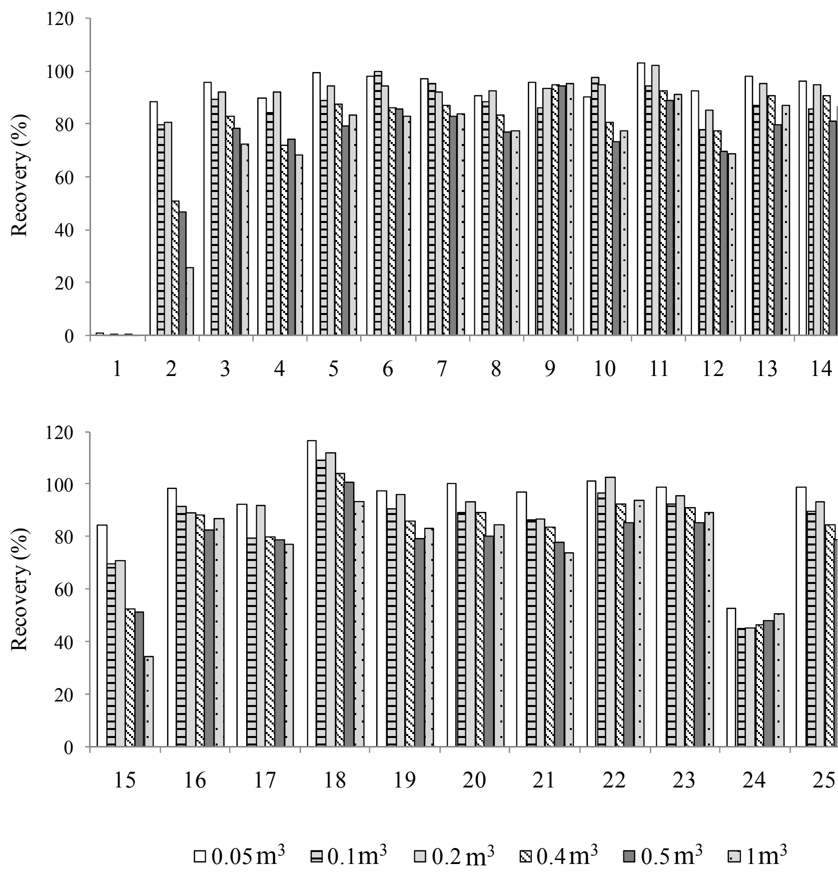

Fig. 6. Variation of the chromatographic response with the volume of air sampled (see number code equivalence in Table 1).

To evaluate the possible breakthrough, portions of 25 mg Florisil were spiked in duplicate with 10 µg of the analytes and then, different volumes of air ranging from 0.05 to 1 m^3 were sampled. The portions of adsorbent were individually

extracted under the optimized extraction conditions. Fig. 6 shows the results obtained. As it is clearly appreciated, limonene is almost completely lost in all experiments, even for a sample volume as low as 0.05 m^3. Benzyl alcohol and isoeugenol showed significant breakthrough in the sample range tested and analyte losses are evident above 0.2 m^3 air. Other compounds showed slightly lower responses for higher sample volumes, whereas for some compounds, in general the less volatile ones, no breakthrough was observed in the entire interval.

Some experiments were also run using larger amounts of adsorbent (up to 200 mg) and the results obtained were not improved. With the objective of mainly studying limonene losses and evaluating the possibility of recovering this compound satisfactorily, a series of experiments were carried out with 3 devices, each one containing 25 mg of Florisil, connected in series, and sampling only 0.05 m^3 of air. Limonene was detected and quantified in the three devices (at 1.6, 11 and 18% respectively), but the total recovery was only 31%. The other compounds were efficiently retained in the first device and only no significant amounts of some of the most volatile analytes were detected in the additional two Florisil portions.

Some additional UAE experiments performed leaving the spiked Florisil in an open vial for 10 min gave satisfactory recovery valuesfor all analytes but again a very low recovery of limonene (24%), demonstrating the easiness of this compound to be lost either by volatilization, transformation (e.g. oxidation), or both mechanisms [33].

Due to the need of exhaustive cleaning of the glass microfilter samplers to avoid memory effects, and also for other practical reasons (e.g. easiness in transport), the use of disposable SPE cartridges instead of the glass adsorbent supports, was evaluated. No significant differences were found in the obtained results (data not shown) and thus, both materials could be used in the same way.

In view of the results obtained and with the aim of establishing a general method for the analysis of all target allergens in air excluding limonene, a sampling volume of 0.2 m^3 was selected. If more sensitivity were required larger sample sizes (up to 1 m^3) could be collected assuming important losses only for two analytes (benzyl alcohol and isoeugenol) and slight losses (about 10–20%) for some other analytes.

3.3. Performance of the method

In all validation experiments, results obtained are referred to the sampling of 0.2 m^3 air. With the aim to assure blank samples, air blanks as well as adsorbent blanks were obtained in a clean room provided with a laminar flow system and analyzed before every set of experiments.

Efficiency of the total sampling-extraction process was evaluated at three concentration levels (1, 5, 125 μg m^{-3}). Recovery was satisfactory with values >80% in most cases (see Table 4). Recovery values for benzyl salicylate were corrected according to the extraction efficiency for this compound (see Section 3.1). Precision of the method can be considered good with RSD values generally <10%.

Limits of detection (LOD, S/N = 3) of the proposed method are also included in Table 4, showing values ≤0.6 μg m^{-3}, with the exception of farnesol (2.2 μg m^{-3}). LOD values at the low ng m^{-3} were obtained for several compounds (linalool, hydroxycitronellal, eugenol, methyleugenol, coumarin, ionone, lilial®, benzyl benzoate, and benzyl salicylate).

3.4. Application to real indoor air samples

Finally, the proposed method was applied to real samples collected in different home rooms (0.2 m^3, 0.010 m^3 min^{-1}) that had been treated with aerosols, electrical diffusion units, as well as different common cleaning products of general domestic use in Spain. The application of the products was made following the recommendations of the manufacturers regarding the appropriate amounts to be used, when available, and depended on the use of the sampled room and always respected the generalized habits people have in using this kind of products. The air inside of a car was also sampled (sample S9). Concentrations of the compounds are summarized in Table 5. As can be seen, several of the target analytes were present in the indoor air and could be determined. Linalool and lilial® were found in all the analyzed samples, whereas citronella and ionone were present in seven of the nine air samples. The highest found concentrations corresponded to lilial® (194 μg m^{-3}) and linalool (136 μg m^{-3}).

Table 4. Recovery (%), repeatability (%), and limits of detection of the total sampling-extraction process.

Compound	1 µg m^{-3} (n=4)		5 µg m^{-3} (n=4)		125 µg m^{-3} (n=4)		LOD µg m^{-3}
	Recovery	RSD	Recovery	RSD	Recovery	RSD	
Benzyl alcohol	98.7	5.8	86.4	5.5	76.5	0.7	0.19
Linalool	77.6	4.1	87.4	3.4	88.7	0.5	0.015
Methyl-2-octynoate	104	9.8	92.1	5.0	92.6	2.4	0.13
Citronellol	116	8.4	89.9	4.4	92.4	0.2	0.36
Geraniol	92.4	7.4	99.4	3.2	93.2	13	0.29
Citral	90.6	3.3	108	4.8	91.3	2.1	0.16
Cinnamaldehyde	94.4	8.2	97.6	6.0	87.6	2.3	0.12
Anisyl alcohol	98.6	9.5	107	11	86.7	1.4	0.23
Hydroxycitronellal	92.4	8.6	97.2	4.5	98.8	0.9	0.038
Cinnamyl alcohol	90.5	6.6	109	5.1	97.9	0.7	0.55
Eugenol	67.2	4.7	76.5	2.7	85.0	1.6	0.041
Methyleugenol	95.9	6.6	97.4	6.9	88.0	3.3	0.027
Coumarin	108	7.5	99.9	6.3	88.4	0.7	0.069
Isoeugenol	90.4	9.1	83.4	4.8	70.6	5.2	0.37
Ionone	91.6	6.4	94.7	6.8	83.6	10	0.017
Lilial®	102	7.2	100	3.6	112	1.2	0.019
Amyl Cinnamal	105	5.2	105	2.5	112	10	0.17
Lyral®	99.3	6.3	111	3.5	96.0	5.9	0.16
Amyl cinnamyl alcohol	102	8.4	106	5.8	85.4	1.0	0.18
Farnesol	101	7.9	114	6.6	85.2	6.4	2.2
Hexyl cinnamaldehyde	87.4	5.5	99.7	4.6	100	4.0	0.15
Benzyl benzoate	95.3	6.2	108	3.3	87.8	1.2	0.037
Benzyl salicylate[a]	111	10	97.4	8.4	90.4	1.5	0.036
Benzyl cinnamate	105	2.6	114	5.8	90.3	0.1	0.15

[a]Recovery values were corrected taking into account the average extraction efficiency for this compound.

Table 5. Compounds found (μg m^{-3}) in indoor air samples[a].

Compound	S1	S2	S3	S4	S5	S6	S7	S8	S9
Benzyl alcohol	<LOQ		<LOQ			<LOQ		3.9	
Linalool	14	<LOQ	104	136	43	3.08	100	38	<LOQ
Citronellol	<LOQ	1.1	11	3.8	5.7		9.1	9.5	35
Citral		2.9	4.5	10.1	24.3	0.23	0.91	6.1	2.2
Hydroxycitronellal									61
Eugenol			0.42	0.41			3.6	0.34	3.1
Coumarin				0.96					
Ionone	0.54	1.1	0.21	0.76	1.7	<LOQ	5.1	1.8	
Lilial®	1.2	1.4	3.1	1.9	15.1	0.33	64	60	194
Lyral®									4.6
Hexyl cinnamaldehyde	<LOQ	0.89	<LOQ		0.54		4.9	0.72	
Benzyl benzoate		<LOQ	<LOQ		<LOQ			<LOQ	0.53
Benzyl salicylate		0.16	<LOQ		<LOQ			0.17	

[a]Blank spaces mean values below LOD.

4. Conclusions

A very simple and sensitive method to analyze fragrance allergens in indoor air was developed. The active retention of the target compounds on a very small amount of Florisil and the subsequent desorption by application of ultrasounds using only 2 mL ethyl acetate, avoided for the requirements of extract concentration prior to chromatographic analysis. After optimization of the extraction step, the study of the retention efficiency from air demonstrated that for most compounds no breakthrough occurred up to 0.2 m^3. Only limonene was not efficiently retained even sampling very low air volumes. For all the other analytes a general methodology was satisfactorily developed and proposed. The study of method performance demonstrated its linearity, quantitative recoveries, and good sensitivity, with LODs ≤ 0.6 μg m^{-3}. In addition, the method allowed high sample throughput since the total sampling-extraction-analysis process is completed within one hour. The analysis of several air samples demonstrated the validity of the proposed method for the analysis of the target compounds in indoor environments.

Acknowledgements

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References

1. L. Molhave, G. Clausen, B. Berglund, J. De Ceaurriz, A. Kettrup, T. Lindvall, M. Maroni, A.C. Pickering, U. Risse, H. Rothweiler, B. Seifert, M. Younes, Indoor Air 7 (1997) 225.
2. S.K. Brown, M.R. Sim, M.J. Abramson, C.N. Gray, Indoor Air 4 (1994) 123.
3. D.R. Bickers, P. Calow, H.A. Greim, J.M. Hanifin, A.E. Rogers, J.H. Saurat, I.G. Sipes, R.L. Smith, H. Tagami, Regul. Toxicol. Pharm. 37 (2003) 218.
4. R.A. Ford, B. Domeyer, O. Easterday, K. Maier, J. Middleton, Regul. Toxicol. Pharm. 31 (2000) 166.
5. A.C. De Groot, P.J. Frosch, Contact Dermatitis 36 (1997) 57.
6. J.D. Johansen, Am. J. Clin. Dermatol. 4 (2003) 789.
7. K. Rumchev, J. Spickett, M. Bulsara, M. Phillips, S. Stick, Thorax 59 (2004) 746.
8. C. Shim, M.H. Williams Jr., Am. J. Med. 80 (1986) 18.
9. A. Farrow, H. Taylor, K. Northstone, J. Golding, Arch. Environ. Health 58 (2003) 633.
10. J. Elberling, A. Linneberg, A. Dirksen, J.D. Johansen, L. Frolund, F. Madsen, N.H. Nielsen, H. Mosbech, Clin. Exp. Allergy 35 (2005) 75.
11. B. Bridges, Flavour Frag. J. 17 (2002) 361.
12. SCCNFP/0017/98 Final Fragrance Allergy in Consumers, December 1999.
13. Directive 2003/15/EC of 27 February 2003 amending Council Directive 76/768/EEC on the approximation of the laws of the Member States relating to cosmetic products, Off. J. Eur. Union L 066 (2003) 26.

14. Council Directive of 27 July 1976 on the Approximation of the Laws of the Member States Relating to Cosmetic Products 76/768/EEC.
15. IFRA International Fragrance Association, http://www.ifraorg.org/Home/page.aspx/3.
16. S.C. Rastogi, J. High Resolut. Chromatogr. 18 (1995) 653.
17. H. Leijs, J. Broekhans, L. van Pelt, C. Mussinan, J. Agric. Food Chem. 53 (2005) 5487.
18. M.Bassereau, A. Chaintreau, S. Duperrex, D. Joulain, H. Leijs, G. Loesing, N.Owen, A. Sherlock, C. Schippa, P.J. Thorel, M. Vey, J. Agric. Food Chem. 55 (2007) 25.
19. M. Niederer, R. Bollhalder, C. Hohl, J. Chromatogr. A 1132 (2006) 109.
20. M.S. Dunn, N. Vulic, R.A. Shellie, S. Whitehead, P. Morrison, P.J. Marriott, J.Chromatogr. A 1130 (2006) 122.
21. L. Mondello, A. Casilli, P.Q. Tranchida, G. Dugo, P. Dugo, J. Chromatogr. A 1067 (2005) 235.
22. L. Mondello, A. Casilli, P.Q. Tranchida, D. Sciarrone, P. Dugo, G. Dugo, LC–GC Eur. 21 (2008) 130.
23. J.P. Lamas, L. Sanchez-Prado, C. Garcia-Jares, M. Llompart, Anal. Bioanal. Chem. 394 (2009) 1399.
24. H. Fromme, T. Lahrz, M. Piloty, H. Gebhart, A. Oddoy, H. Ruden, Indoor Air 14 (2004) 188.
25. R. Kallenborn, R. Gatermann, S. Planting, G.G. Rimkus, M. Lund, M. Schlabach, I.C. Burkow, J. Chromatogr. A 846 (1999) 295.
26. D. Chen, X. Zeng, Y. Sheng, X. Bi, H. Gui, G. Sheng, J. Fu, Chemosphere 66 (2007) 252.
27. J. Regueiro, C. Garcia-Jares, M. Llompart, J.P. Lamas, R. Cela, J. Chromatogr. A 1216 (2009) 2805.
28. R. Barro, S. Ares, C. Garcia-Jares, M. Llompart, R. Cela, Anal. Bioanal. Chem. 381 (2005) 255.

29. R. Barro, C. Garcia-Jares, M. Llompart, M. Herminia Bollain, R. Cela, J. Chromatogr. A 1111 (2006) 1.

30. R. Barro, S. Ares, C. Garcia-Jares, M. Llompart, R. Cela, J. Chromatogr. A 1045 (2004) 189.

31. R. Barro, S. Ares, C. Garcia-Jares, M. Llompart, R. Cela, J. Chromatogr. A 1072 (2005) 99.

32. R. Barro, C. Garcia-Jares, M. Llompart, R. Cela, J. Chromatogr. Sci. 44 (2006) 430.

33. A.-T. Karlberg, K. Magnusson, U. Nilsson, Contact Dermatitis 26 (1992) 332.

2.2 SORBENT TRAPPING SOLID-PHASE MICROEXTRACTION OF FRAGRANCE ALLERGENS IN INDOOR AIR

SORBENT TRAPPING SOLID-PHASE MICROEXTRACTION OF FRAGRANCE ALLERGENS IN INDOOR AIR

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Abstract

Exposure to fragrance substances is exponentially increasing in our daily life due to the enhanced use of scented products. Some fragrances are known to be important sensitizers, inhalation being an important exposure pathway in indoor environments. A simple and sensitive method based on solid-phase enrichment and solid-phase microextraction (SPME) followed by gas chromatography–mass spectrometry (GC–MS) has been developed for the analysis of 24 volatile fragrance allergens in indoor air. Suspected allergens present in the air (0.2m^3) were adsorbed onto a very small quantity of florisil (25 mg) and then transferred to a SPME fiber in the headspace mode (HS). To the best of our knowledge, this paper describes the first application of SPME for the determination of these compounds in air samples. The experimental parameters affecting the microextraction process have been optimized using a multifactor experimental design strategy. Accuracy, linearity, precision and detection limits (LODs) were evaluated to assess the performance of the proposed method. External calibration, using spiked sorbent standards, and not requiring the complete sampling process (only the SPME step), demonstrated to be suitable for the quantification of all suspected allergens. Recovery studies were performed at three concentration levels (0.04,1.00 and 50 μg m^{-3}), obtaining quantitative recoveries (≥85%) in most cases. LOD values at the low ng m^{-3} level were achieved for all the target compounds. The application of the method to daily home air samples demonstrated the ubiquity of this kind of fragrance ingredients in quotidian indoor environments, finding 18 of the 24 considered compounds in concentrations ranging from 0.01 to 56 μg m^{-3}. Benzyl alcohol, linalool, citronellol, ionone and lilial® were found in most analyzed samples.

Keywords: Fragrance allergens, Indoor air, Solid-phase microextraction, Gas chromatography–mass spectrometry, Experimental design optimization.

1. Introduction

Fragrance containing products are part of our daily life. The majority of personal-care products, household and laundry products on the market contain fragrances and, consequently, the exposure to these substances in our society is very high due to our lifestyle [1,2]. In addition to known dermatological problems [3,4], fragrances can induce or worsen respiratory problems due to their irritant effect. These product ingredients are thought to trigger asthma, asthmatic exacerbations, and other respiratory illness; headaches; and mucosal symptoms [5–9]. Indoor air quality is an increasingly important problem worldwide and home exposure to allergens and pollutants represents one of the major public health issues of the moment [10].

The Scientific Committee on Cosmetic Products and Non-Food Products (SCCNFP) has identified 24 fragrance compounds as likely to cause contact allergies [11]. The most of these substances are also restricted by the International Fragrance Association (IFRA) [12], the official representative body of the fragrance industry world-wide, with the main purpose of ensuring the safety of fragrance materials since many of the raw fragrance materials have little available health and safety data [13].

According to the European Union (EU) regulation on cosmetic products [14], the presence of these fragrances must be indicated in the list of ingredients when its concentration exceeds the 0.001% in leave-on products and 0.01% in rinse-off products. For benzyl alcohol the maximum concentration in ready for use preparation should not exceed the 1.0%; and for methyleugenol the maximum allowed concentration is 0.01% in fine fragrance, 0.004% in eau de toilette, 0.002% in fragrance cream, 0.0002% in other leave-on products and oral products, and 0.001% in rinse-off products.

In the last years, several analytical methods for determining musk compounds in air have been developed. Samples have typically been collected by active sampling of high volumes using different sorbents [15–17]. The samples were then solvent extracted and, usually, a clean-up step is needed, which entails time-consuming and tedious methods. Regueiro et al. [18] proposed a very simple method for the analysis of synthetic musks in indoor air, in which musk compounds

are adsorbed onto a small amount of Tenax and, then, analytes are transferred to a solid-phase microextraction (SPME) fiber. Ramirez et al. [19] developed a rapid and straightforward method for determining synthetic musks, parabens, and insect repellents in air samples, based on thermal desorption-gas chromatography–mass spectrometry (TDGC– MS).

Very recently we presented a simple methodology for the analysis of 24 fragrance allergens in indoor air [20]. The procedure is based on the use of a very low amount of sorbent to retain the target compounds, and the rapid ultrasound-assisted solvent extraction (UAE) with only 2mL of solvent, avoiding further concentration. As far as we know, this is the only work related to the determination of fragrance allergens in indoor air.

Regarding solid-phase microextraction (SPME) of fragrance allergens, Lamas et al. have developed a method for the quantification of 15 of these cosmetic ingredients in baby bathwaters [21], and Masuck et al. [22] proposed a sensitive method to determine and quantify the emission of 24 allergens from scented toys.

In the present study, we developed a simple and highly sensitive method based on solid-phase extraction followed by solid-phase microextraction (SPE-SPME) for the analysis of 24 volatile fragrance allergens in indoor air. To the best of our knowledge, this paper describes the first application of SPME for the determination of these compounds in air samples. To study and optimize main experimental parameters affecting the microextraction process, a multifactor experimental design is employed. Accuracy, linearity, precision and detection limits (LODs) are evaluated to assess the performance of the proposed method. Finally, several indoor air samples are analyzed in order to demonstrate the applicability of the method, and the high exposure levels to fragrance allergens in quotidian home indoor environments.

2. Experimental

2.1. Reagents and materials

3,7-Dimethyl-1,6-octadien-3-ol, 97% (linalool); 3,7-dimethyloct-6-en-1-ol, 95% (citronellol); 2-methoxy-4-prop-2-enyl phenol, 99% (eugenol); 1,2-dimethoxy-4-(2-propenyl)-benzene, 99% (methyleugenol); 2H-1-benzopyran-2-one, 99%(coumarin); 3,7,11-trimethyldodeca-2,6,10-trien-1-ol, 95% (farnesol, mixture of isomers); 3,7-dimethylocta-2,6-dienal, 95% (citral, cis/trans); 1-methyl-4-prop-1-en-2-yl-cyclohexene 97% (limonene); 4-methoxybenzene methanol, 98% (anisyl alcohol); 2-methoxy-4-(1-propenyl) phenol, 98% (isoeugenol, cis/trans); 3-phenyl phenylmethyl ester-2-propenoic acid, 99% (benzyl cinnamate); and 2-(phenylmethylene)-heptanal, 97% (amyl cinnamal were purchased from Aldrich (Sigma–Aldrich Chemie GmbH, Steinheim, Germany).

3-Methyl-4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-3-buten-2-one, ≥85% (ionone); 3,7-dimetil-2,6-octadien-1-ol, ≥96%(geraniol); 2-(phenylmethylene)-1-heptanol, ≥85% (amyl cinnamyl alcohol); 3-(4-tert-butylphenyl)-2-methylpropanal,≥95% (lilial®); 4-(4-hydroxy-4-methylpentyl)cyclohex-3-ene-1-carbaldehyde, ≥97% (lyral®); and 2-hydroxy-phenylmethyl ester benzoic acid, ≥99% (benzyl salicylate) were purchased from Fluka (Fluka Chemie GmbH, Steinheim, Germany). 2-Octynoic acid, methyl ester, ≥99% (methyl 2-octynoate); 7-hydroxy-3,7-dimethyloctanal, ≥95% (hydroxycitronellal); 3-phenyl-2-propenal, ≥93% (cinnamaldehyde); 2-(phenylmethylene) octanal,≥95% (hexyl cinnamaldehyde) were purchased from SAFC Supply Solutions (St. Louis, USA).

Benzene methanol, 99% (benzyl alcohol); 3-phenyl-2-propen-1-ol, 98% (cinnamyl alcohol); phenylmethyl benzoate, 98.5% (benzyl benzoate) were purchased from Chem Service (West Chester, USA).

Ethyl acetate and acetone were analytical grade and were provided by Merck (Darmstadt, Germany). Individual stock solutions of each compound and a mixture of them were prepared in acetone. Working solutions were made by appropriate dilution in ethyl acetate and acetone and then stored in amber glass vials at −20 ºC. The latter were employed for spiking samples. Water (50–100 µL, Milli-Q grade) was used to favour sorbent desorption.

SPME manual holders and fibers were supplied by Supelco (Bellefonte, PA, USA). Two different commercial fiber coatings were used throughout the present work: 65 μm polydimethylsiloxane / divinylbenzene (PDMS/DVB) and 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS). Prior to first use, fibers were conditioned according to the manufacturer's recommendations.

2.2. Air sampling: solid-phase extraction

Air samples were collected in several indoor environments using an active sampling procedure. A known volume of air was pumped (0.010 m^3 min^{-1}) through a microfiltration glass funnel containing 25mg of sorbent by using a S-8 vacuum pump (Telstar, Tarrasa, Spain). Florisil of 60–100 μm mesh (Aldrich, Steinheim, Germany) and Tenax TA of mesh size 60–80 (Supelco) were the sorbents of choice since they were successfully used in several studies employing similar setup. Florisil was activated overnight in an oven at 130 ºC. Only PTFE tubing was used for all connections to minimize contaminations. The sorbent with the retained compounds was then simply transferred from the glass funnel into a 10-mL headspace glass vial and sealed with an aluminum cap furnished with PTFE-faced septum. Finally, SPME was carried out under the optimized conditions.

For method validation experiments, the sampler was placed in a clean room provided of a laminar flow system in order to avoid external contamination.

2.3. Solid-phase microextraction

The headspace vial containing the enriched sorbent was placed into a water bath thermostated at the temperature of the experiment (50 or 100 ºC). Then, the compounds retained by the sorbent were extracted by exposing the SPME fiber to the headspace of the vial (HS-SPME) for 20 min. To favour desorption of the analytes from the sorbent to the fiber, the effect of adding a small volume (50–100 μL) of solvent to the sorbent before thermostatization was tested. Once SPME was completed, the fiber was inserted into the GC injection port and desorbed for 5min.

For method optimization, as well as in validation studies, the sorbent was spiked with the analytes at different concentrations and subjected to the SPME step or to the whole SPE-SPME process.

2.4. Gas chromatography–mass spectrometry

The GC–MS analysis was performed using a Varian 450-GC gas chromatograph (Varian Chromatography Systems, Walnut Creek, CA, USA) coupled to an ion trap mass spectrometer Varian 240-MS (Varian Chromatography Systems) with a waveboard for multiple MS (MS^n) analysis, equipped with an automatic injector CP-8400. The system was operated by Varian MS workstation v6.9.1 software.

Separation was carried out on a HP5 capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) from Agilent Technologies (Palo Alto, CA, USA). Helium (purity 99.999%) was employed as carrier gas at a constant column flow of 1.0 mL min^{-1}. The GC oven temperature was programmed from 45 ºC (held 2min) to 100 ºC at 8 ºC min^{-1}, to 150 ºC at 20 ºC min^{-1}, to 200 ºC at 25 ºC min^{-1} (held 5min) and a final ramp to 225 ºC (held 1 min) at 8 ºC min^{-1}.

The injector was operated in the splitless mode and programmed to return to the split mode after 2min from the beginning of a run. Split flow was set at 20 mL min^{-1} and the injector temperature was kept at 260 ºC.

The ion trap mass spectrometer was operated in the electron impact (EI) ionization mode (+70 eV) using an external ionization configuration. Manifold, ion trap, ion source and transfer line temperatures were maintained at 40, 150, 200 and 280 ºC, respectively.

In the full scan mode the mass range varied from 39 to 400 *m/z* at 3 μscans, starting at 5min and ending at 22.50 min. The filament emission current was 25 μA. The analytes were positively identified by comparison of their mass spectra and retention times to those of standards. The identification and quantification ions and retention times for each target compound are listed in Table 1, as well as their main properties.

Table 1. Main properties and quantification ions of the studied allergens.

Key	Retention time (min)	Compound	CAS number	IUPAC name	Molecular weight	log K_{OW}	Boiling point (ºC)	Solubility[a] (mg L^{-1})	Quantifier and qualifier (*m/z*)
1	9.29	Limonene	5989-27-5	1-Methyl-4-prop-1-en-2-yl-cyclohexene	136	4.57	176	13.8	67,93,121
2	9.46	Benzyl alcohol	100-51-6	Benzene methanol	108	1.05	205	40000	77,79,108
3	10.36	Linalool	78-70-6	3,7-Dimethylocta-1,6-dien-3-ol	154	3.28	198	1589	43,93,121
4	11.53	Methyl-2-octynoate	111-12-6	2-Octynoic acid, methyl ester	154	2.60	219	-	67,79,95
5	11.80	Citronellol	106-22-9	3,7-Dimethyloct-6-en-1-ol	156	3.38	225	322	69,81,95
6	11.93 12.19	Citral	5392-40-5	3,7-Dimethylocta-2,6-dienal	152	3.17	229	590	39,69,109
7	12.02	Geraniol	106-24-1	3,7-Dimetil-2,6-octadien-1-ol	154	3.28	229	531	41,69,111
8	12.31	Cinnamaldehyde	104-55-2	2-Propenal, 3-phenyl-	132	2.22	252	1420	77,103,131
9	12.31	Hydroxycitronellal	107-75-5	7-Hydroxy-3,7-dimethyloctanal	172	1.54	240	23800	43,59,81
10	12.38	Anisyl alcohol	105-13-5	Benzene methanol, 4-methoxy-	138	1.10	259	2070	77,109,138
11	12.57	Cinnamyl alcohol	104-54-1	2-Propen-1-ol, 3-phenyl-	134	1.93	250	1800	77,92,134
12	12.89	Eugenol	97-53-0	2-Methoxy-4-prop-2-enyl-phenol	164	2.20	256	<1000	103,131,164
13	13.17	Methyleugenol	93-15-2	Benzene, 1,2-dimethoxy-4-(2-propenyl)-	178	2.9	248	500	147,163,178
14	13.25 13.54	Isoeugenol	97-54-1	Phenol, 2-methoxy-4-(1-propenyl)-	164	2.45	267	984	103,131,164
15	13.57	Coumarin	91-64-5	2H-1-Benzopyran-2-one	146	1.39	301	2500	89,118,146
16	13.70	Ionone	127-51-5	4-(2,6,6-Trimethyl 2-cyclohexen-1-yl)-3-methyl-3-buten-2-one	206	4.41	266	16	107,135,150
17	14.08	Lilial®	80-54-6	3-(4-tert-Butylphenyl)-2-methylpropanal	204	4.07	279	33	131,147,189
18	15.03	Amyl cinnamal	122-40-7	Heptanal, 2-(phenylmethylene)-	202	4.80	289	8.5	115,129,202
19	15.24	Lyral®	31906-04-4	4-(4-Hydroxy-4-methylpentyl)cyclohex-3-ene-1-carbaldehyde	210	2.53	319	185-1045	79,91,136
20	15.38	Amyl cinnamyl alcohol	101-85-9	1-Heptanol, 2-(phenylmethylene)-	204	4.37	>200	26	91,115,133
21	15.61	Farnesol	4602-84-0	3,7,11-Trimethyldodeca-2,6,10-trien-1-ol	222	5.31	111	267	41,69,81
22	16.05 16.35	Hexylcinnamaldehyde	101-86-0	Octanal, 2-(phenylmethylene)-	216	4.82	174	2.8	115,129,216
23	16.43	Benzyl benzoate	120-51-4	Phenylmethyl benzoate	212	3.97	324	19.8	105,194,212
24	17.99	Benzyl salicylate	118-58-1	Benzoic acid, 2-hydroxy-, phenylmethyl ester	228	4.31	208	<1000	65,91,228
25	21.57	Benzyl cinnamate	103-41-3	2-Propenoic acid, 3-phenyl-, phenylmethyl ester	238	3.65	198	9	91,131,192

[a] Water, 25 ◦C.

The chromatographic method was evaluated by direct injection of solutions in ethyl acetate of different concentrations of the target analytes. Linearity was good in the studied concentration range (0.025 – 25 μg mL^{-1}, 10 points) and linear regression coefficients were ranged from 0.990 to 0.999. Repeatability (n = 5) in terms of relative standard deviation (RSD) was lower than 7.9%.

3. Results and discussion

3.1. Method development

The most relevant step for the application of the SPE-SPME procedure is the transfer of the analytes from the sorbent to the headspace of the vial and then to the SPME fiber. In this way, the optimization of the SPME process is clearly necessary to improve the amount of compound adsorbed by the fiber and hence the limits of detection and quantification of the method. A multivariate optimization that allows the simultaneous study of main variables potentially affecting SPME was performed.

In order to define the experimental domain, previous experiments were carried out. These experiments demonstrated the importance of the use of high temperature as well as the addition of a small volume of solvent to favour, or make even possible, the extraction of the analytes through the fiber coating (data not shown). Therefore, these two factors were included in the experimental design: the temperature at 50 and 100 ºC, and the addition of solvent at 50 and 100 μL. Other three factors were also included in this study: sorbent type, fiber coating, and type of solvent. Florisil and Tenax TA were the sorbents selected for the study due to their suitability to combine with SPME as it has been previously demonstrated in the analysis of several classes of pollutants in indoor air [18,23,24]. The addition of a small volume of solvent to the sorbent previously to the SPME has demonstrated an improvement of the extraction of some kinds of compounds. Regarding the fiber, DVB/CAR/PDMS and PDMS/DVB coatings were selected. This last fiber has recently been employed for the extraction of suspected allergens from baby bathwater, leading to excellent results [21]. On the other hand, the presence of CAR in the fiber DVB/CAR/PDMS and the double length of this fiber could improve the results of the PDMS/DVB for some analytes, and it was considered for study.

Table 2. Factors and levels considered in the screening experimental design.

Factor	Key	Low level (-)	High level (+)
Solvent volume	A	50 µL	100 µL
Extraction temperature	B	50 ºC	100 ºC
Fiber	C	PDMS/DVB	DVB/CAR/PDMS
Sorbent	D	Tenax	Florisil
Solvent	E	Water	Acetone

All these factors were then included in a screening study by means of a 2^{5-1} half fraction factorial design, involving a total of 16 randomized runs, with each parameter evaluated at two levels. Table 2 summarizes the factors and levels considered. All experiments were performed using a small amount of sorbent (25 mg) spiked with the target analytes at a level of 4 ng per milligram. The design has resolution V, which means that it is capable of evaluating all main effects and all two-factor interactions. Numerical analysis of data was made with the statistical software package Statgraphics Plus 5.1 (Manugistics, Rockville, MD, USA). The selected design allows studying the results using various statistical tests and graphic tools. Table 3 summarizes the analysis of variance (ANOVA) obtained. For simplicity, only *F*-ratios and *p*-values are given. The *F*-ratio measures the contribution of each factor or interaction on the variance of the response. The *p*-value tests the statistical significance of each factor and interaction. When *p*-value is less than 0.05, the factor has a statistically significant effect at the 95% confidence level. As can be seen, three of the main factors, type of sorbent (D), type of solvent added (E), and extraction temperature (B), as well as the interaction effect DE, were significant for most analytes. The other main factors were nonsignificant for almost all analytes but some other interaction effects such as AC, BD and BE were important in some cases. This information can clearly be visualized by means of the Pareto charts.

Table 3. *F*-ratios and *p*-values obtained in the analysis of variance.

	Main effects										Interactions							
	A: Volume		B: Temperature		C: Fiber		D: Sorbent		E: Solvent		AC		BD		BE		DE	
	F ratio	*p* value	*F* ratio	*p* value	*F* ratio	*p* value	*F* ratio	*p* value	*F* ratio	*p* value	*F* ratio	*p* value	*F* ratio	*p* value	*F* ratio	*p* value	*F* ratio	*p* value
Limonene	0.2		3		3		9	+	9	+	4		4		4		9	+
Benzyl alcohol	8	+	4		3		5		2		2		0.0		0.0		9	+
Linalool	0.3		7		2		14	+	14	+	8	+	10	+	9	+	14	+
Methyl-2-octynoate	10		98	+	9		364	+	364	+	138	+	180	+	132	+	370	+
Citronellol	0.1		18	+	4		56	+	61	+	27	+	39	+	30	+	65	+
Citral	0.2		20	+	4		62	+	67	+	24	+	35	+	24	+	70	+
Geraniol	0.1		13	+	6		70	+	75	+	23	+	39	+	26	+	81	+
Cinnamaldehyde	7		5		5		10		12	+	2		0.0		1		17	+
Hydroxycitronellal	0.4		0.2		2		1		1		0.2		1		1		2	
Anisyl alcohol	24	+	138	+	43	+	4		1		42	+	0.2		1		43	+
Cinnamyl alcohol	2		4		2		3		3		2		0.3		11		5	
Eugenol	1		0.1		3		20	+	25	+	0.1		2		0.4		29	+
Methyleugenol	4		41	+	41	+	361	+	246	+	8		1		1		331	+
Isoeugenol	2		6		3		14	+	16	+	3		0.1		1		24	+
Coumarin	16		1892	+	265	+	241	+	9		120		47		29		215	+
Ionone	1		15	+	9	+	339	+	203	+	2		3		3		330	+
Lilial®	1		9		4		493	+	449	+	8		0.2		1		534	+
Amyl cinnamal																		
Lyral®	0.3		51		4		35		4		33		4		1		49	
Amyl cinnamyl alcohol	1		289	+	3		322	+	219	+	217	+	214	+	156	+	288	+
Farnesol	3		84	+	2		80	+	77	+	84	+	67	+	67	+	93	+
Hexylcinnamaldehyde	9		178	+	2		359	+	204	+	89	+	117	+	51	+	277	+
Benzyl benzoate	6		895	+	17		1544	+	1202	+	650	+	778	+	568	+	1330	+
Benzyl salicylate	2		87	+	4		131	+	123	+	81	+	76	+	71	+	136	+
Benzyl cinnamate	1		3		2		5		3		1		3		1		4	

+ cell, p value<0.05; empty cell, p value>0.05.

Fig. 1 shows the Pareto charts including the main factors and two-factor interactions for several selected compounds. The length of each bar is proportional to the absolute value of its associated standardized effect. The standardized effect is obtained by dividing the estimated effect of each factor or interaction by its standard error. Vertical line in the graphs represents the statistically significant bound at the 95% confidence level. Pareto charts clearly show that the most relevant factors are the sorbent (D) and the type of solvent (E). The extraction temperature (B) was also significant in many cases, although the associated effect is low (see the corresponding bars in the figure). In addition, the fiber coating (C) and the solvent volume (A) were non-significant for most analytes. Second order factors were in general very important and in some cases more relevant than main factors. DE (sorbent-type of solvent) was significant for most of compounds, while other factor interactions such as BD (extraction temperature-sorbent), BE (extraction temperature-type of solvent), and AC (solvent volume-type of fiber) were also significant for some of the analytes.

Fig. 2 shows the main effects plots for several compounds of different chemical structures, representing general behaviours. This kind of plots shows the main effects with a line drawn between the low and the high level of the corresponding factors. The length of the lines is proportional to the effect magnitude of each factor in the extraction process, and the sign of the slope indicates the level of the factor that produces the highest response. These graphs again show the high influence on response of the sorbent and the type of solvent added. In all cases, higher responses were obtained selecting florisil and water. Regarding temperature, 100 ºC favours, in general, SPME extraction, excluding some of the most volatile analytes that prefer 50 ºC (e.g. citronellol). In the few cases in which the fiber type was significant (only for four compounds, see Table 3), higher responses were obtained using the DVB/CAR/PDMS coating (e.g. methyleugenol, Fig. 2).

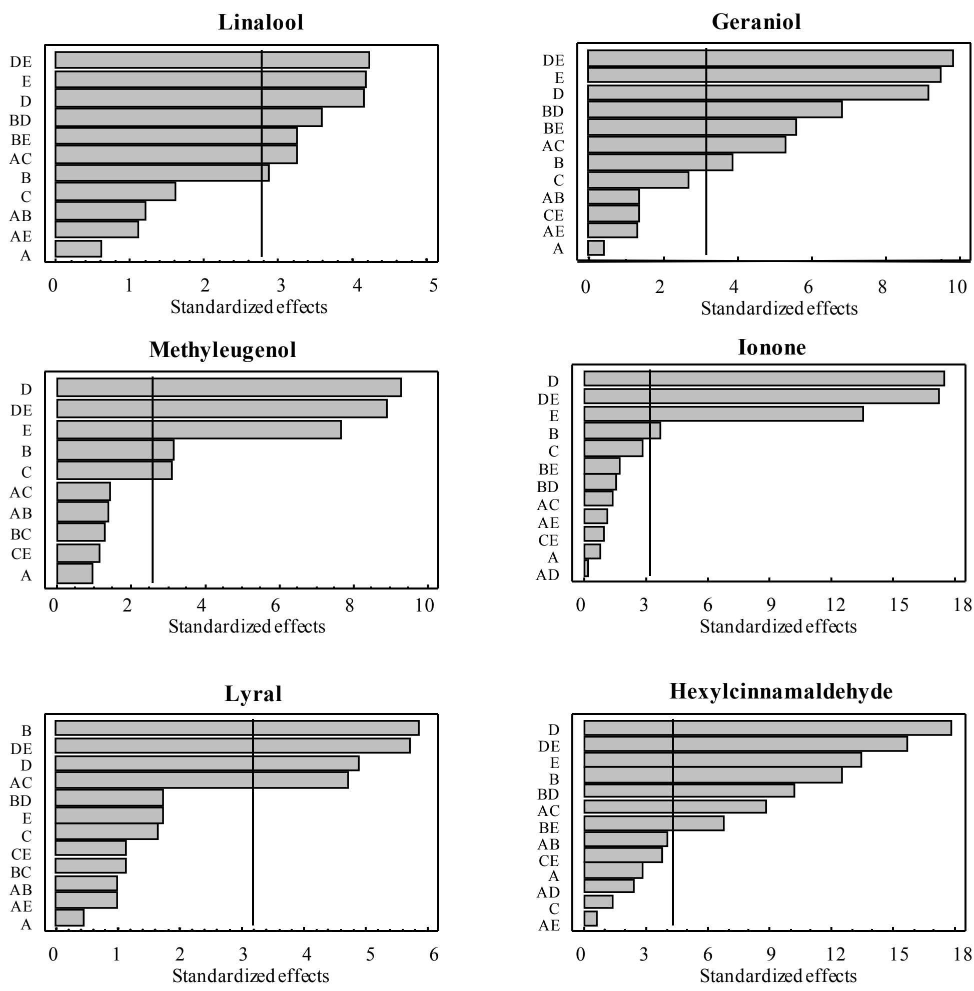

Fig. 1. Pareto charts showing the significant factors (95%) for some selected fragrance allergens (see factor codes in Table 2).

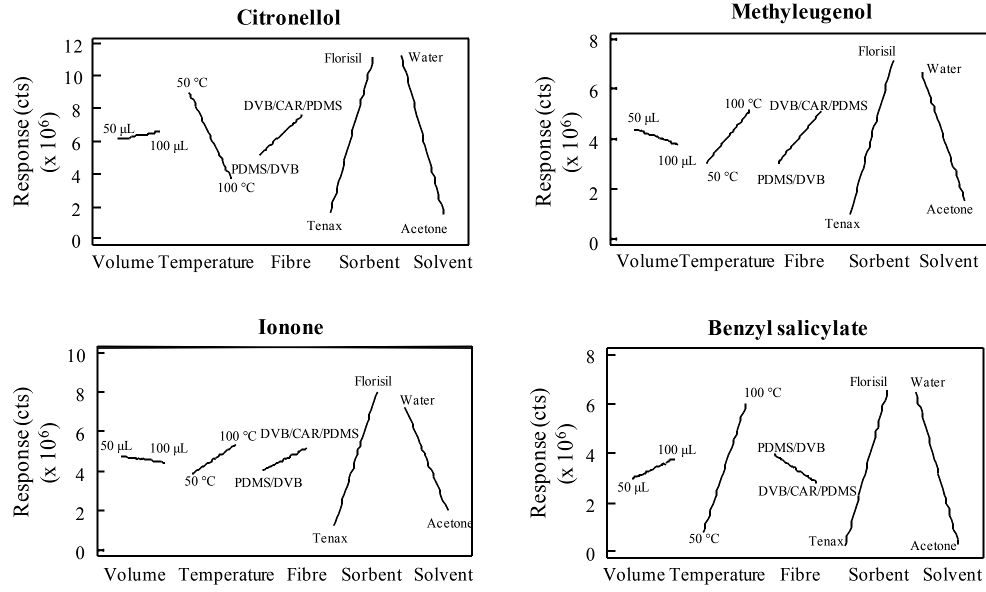

Fig. 2. Main effect plots for some selected fragrance allergens.

The interaction graphics (Fig. 3) are very useful and help to visualize optimal conditions. Only the most relevant second order effects (see Table 3) and those showing general behaviours will be commented here. The most strong interaction effect is the sorbent-type of solvent (DE) one. This effect was significant for almost all compounds (see Table 3). We can clearly appreciate in Fig. 3a that the response obtained using florisil is considerably improved when water instead of acetone is added, whereas the solvent has little effect in the case of Tenax, although the responses with this sorbent are very low. Other important interaction is BE (extraction temperature-type of solvent) (see Fig. 3b). For the most volatile compounds, the most favourable conditions are 50 ºC and water; nevertheless, for the rest of compounds, the responses at 50 ºC are quite low and similar independently of the solvent employed, but, at 100 ºC, extraction efficiency is considerably improved using water. BD (extraction temperature-sorbent) effect also shows the improvement in the response obtained using florisil at 100 ºC for the less volatile compounds (see Fig. 3c). Finally, and although the main factors A and C were mostly not significant, the interaction effect AC was significant for half of the compounds (see Table 3), showing as most favourable conditions the use of

DVB/CAR/PDMS and 100 μL of solvent for the most volatile compounds and PDMS/DVB and 100 μL of solvent for the less volatile ones (see Fig. 3d).

In summary, after optimization, the experimental conditions selected for the SPME of allergenic compounds involve the use of florisil as sampling sorbent, the addition of 100 μL water to the sorbent, and the extraction at 100 ºC using a DVB/CAR/PDMS fiber, although PDMS/DVB coating would also be suitable.

Regarding SPE air enrichment, breakthrough volume was studied in a previous work [20], demonstrating that air volumes of 0.2 m^3 could be sampled without significant losses of any compound, excluding limonene (almost completely lost even with lower sampling volumes), and isoeugenol (with recoveries about 70%). In the present study, this same volume was selected for method validation.

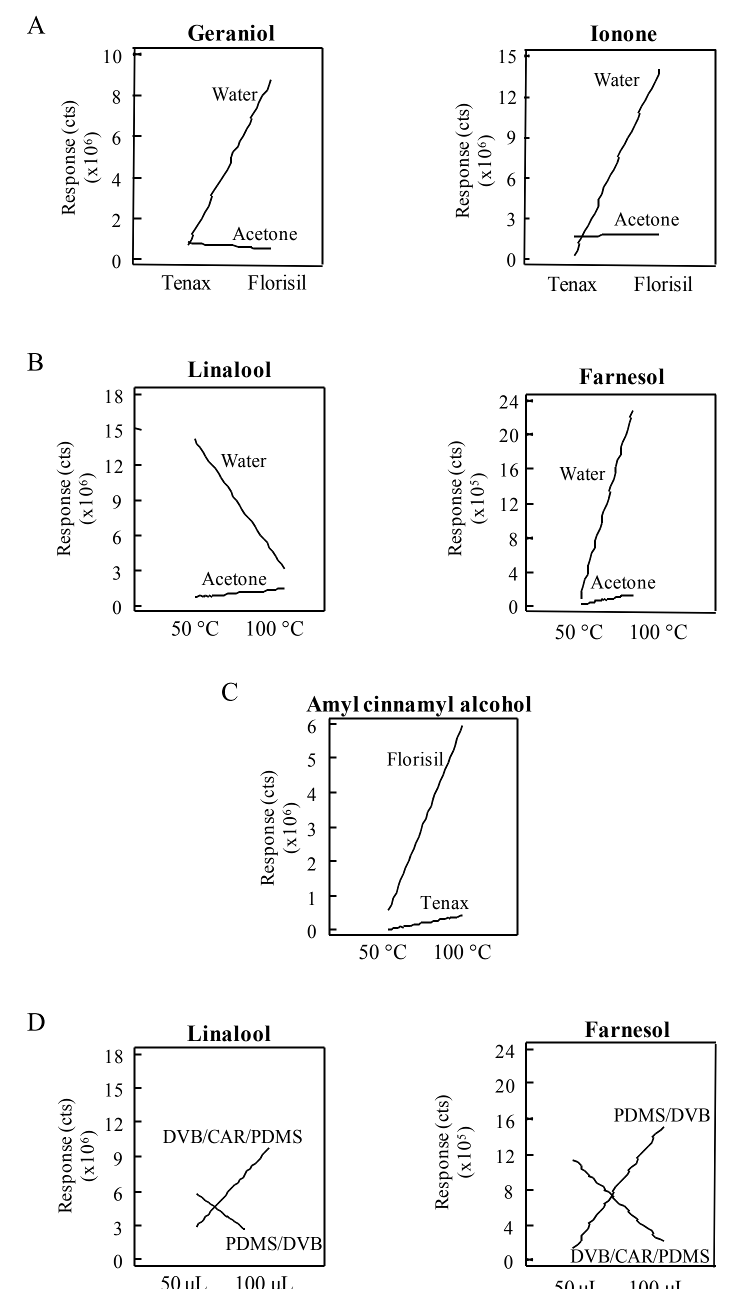

Fig. 3. Combined effect of factors for some selected fragrance allergens: A, type of sorbent and type of solvent; B, extraction temperature and type of solvent; C, extraction temperature and type of sorbent; D, solvent volume and type of fiber.

3.2. Method performance

In order to assess the performance of the proposed method, main analytical quality parameters were evaluated. Linearity was tested using sorbent portions spiked in the range from 0.08 to 40 ng mg^{-1} at 9 levels of concentration, each one analyzed in duplicate or triplicate. Determination coefficients (R^2) for the calibration curves are shown in Table 4. All compounds showed good correlation with R^2 values higher than 0.9947. To validate the regression data, an analysis of variance (ANOVA) was performed. The lack-of-fit (LOF) test is designed to determine whether the selected model is adequate to describe the experimental data. The test compares the variability of the proposed model residuals to the variability between observations (area counts) at replicate values of the independent variable. Results of the LOF test for the calibration range considered at a confidence level of 95% are also shown in Table 4. Since *p*-values for LOF test are greater than 0.05 for all compounds, the linear regression models appear to be adequate for the experimental data. For highly contaminated atmospheres, calibration in split mode might be necessary, and linearity in a higher range (20–800 ng mg^{-1} at 6 levels) was also proven.

Due to the general use of these fragrance compounds in all classes of personal-care products and cleaning products, trace analysis demands special precautions during all analytical procedures to minimize contamination risk, and blank samples must be daily run. To estimate the detection limits (LODs) of the method, blank air samples were collected in a clean room provided of a laminar flow system. None of the compounds with the exception of lilial® was detected in blank air samples. LODs were calculated as the average amount of analyte giving a response that is the blank signal plus three times the standard deviation (LOD = blank signal + 3SD). Thus, the estimated LODs considering a sample volume of 0.2 m^3 are shown in Table 4. Values ranged from 0.05 to 12 ng m^{-3} and are 1–2 orders of magnitude lower than the ones achieved by the previously proposed SPE-UAE method [20].

Recoveries using the proposed SPE-SPME procedure were estimated sampling 0.2 m^3 air at three different concentration levels: 0.04, 1.00 and 50 μg m^{-3}. Recoveries were calculated as the ratio of the measured concentration to the spiked concentration and expressed as a percentage. Table 4 shows that recoveries were satisfactory, between 80 and 115% in most cases. Therefore, quantification by

external calibration using directly spiked sorbent can be employed. Only one compound, isoeugenol, gave low recoveries (about 50%), and the concentration estimate for this compound in real samples can be only considered as semi-quantitative.

The precision of the method was evaluated at the three same concentration levels and results are also shown in Table 4. Repeatability can be considered good with RSD values below 10% in many cases.

Table 4. Linearity, limits of detection, recovery and precision of the method (20 min, 10 L min^{-1}).

	Linearity		LOD (ng m^{-3})	Recovery (RSD) % (n=3)		
Compound	R^2	LOF test (*p*)		0.04 µg m^{-3}	1.00 µg m^{-3}	50 µg m^{-3}
Benzyl alcohol	0.9997	0.216	2.5	90.3 (5.7)	110 (9.8)	99.9 (8.0)
Linalool	0.9984	0.406	3.1	85.9 (15)	98.9 (5.5)	95.2 (5.4)
Methyl-2-octynoate	0.9992	0.180	2.5	103 (6.4)	128 (7.3)	109 (8.3)
Citronellol	0.9972	0.164	2.3	89.4 (11)	106 (8.7)	105 (4.1)
Citral	0.9947	0.738	1.9	102 (3.5)	115 (3.0)	93.0 (11)
Geraniol	0.9992	0.143	2.5	89.6 (5.2)	110 (5.0)	117 (11)
Cinnamaldehyde	0.9988	0.748	3.6	72.5 (6.9)	98.7 (5.3)	107 (3.6)
Hydroxycitronellal	0.9984	0.459	12	110 (5.1)	127 (2.0)	99.0 (1.0)
Anisyl alcohol	0.9995	0.967	1.6	105 (12)	112 (9.6)	88.2 (14)
Cinnamyl alcohol	0.9995	0.881	1.8	80.6 (4.6)	110 (8.7)	99.0 (3.7)
Eugenol	0.9999	0.840	0.72	70.0 (2.6)	80.4 (1.9)	104 (10)
Methyleugenol	0.9977	0.072	0.047	72.3 (2.7)	107 (3.1)	109 (15)
Isoeugenol	0.9954	0.523	2.0	46.0 (4.2)	34.0 (11)	65.0 (10)
Coumarin	0.9974	0.431	1.5	73.1 (1.8)	79.0 (4.2)	92.0 (3.9)
Ionone	0.9959	0.168	0.50	116 (2.9)	105 (0.7)	111 (9.0)
Lilial®	0.9996	0.262	10	112 (14)	105 (2.3)	107 (6.8)
Amyl cinnamal	0.9994	0.839	2.5	83.8 (5.4)	73.3 (4.0)	108 (2.7)
Lyral®	0.9999	0.727	1.3	95.0 (9.4)	76.9 (4.9)	103 (1.1)
Amyl cinnamyl alcohol	0.9999	0.934	1.9	85.7 (5.4)	99.2 (7.6)	96.1 (11)
Farnesol	0.9948	0.741	12	102 (4.6)	92.4 (7.1)	110 (13)
Hexylcinnamaldehyde	0.9999	0.759	2.5	91.5 (11)	78.0 (4.0)	112 (3.6)
Benzyl benzoate	0.9997	0.839	1.1	96.0 (1.8)	103 (6.1)	120 (7.9)
Benzyl salicylate	0.9996	0.828	2.5	88.6 (5.4)	76.7 (6.5)	115 (10)
Benzyl cinnamate	0.9976	0.184	2.7	108 (0.5)	75.9 (10)	124 (8.0)

3.3. Method application: determination of fragrance allergens in indoor air

The SPE-SPME method was applied to the analysis of indoor air samples collected in different home places (0.2 m^3, 0.010 m^3 min^{-1} including kitchen (code K in Table 5), living room (L), washroom (WR), corridors (C), bedrooms (BR), storage room (SR) and laundry room (LR) samples, at normal daily conditions (Table 5). Other air room samples and a car sample (CAR) were taken after the application of different aerosol products such as insecticides and freshener aerosols or diffusers (Table 6). All samples were collected in duplicate and initially analyzed by GC–MS in the splitless mode; if analyte concentration exceeds the linear calibration range, the sample is also analyzed in the split mode. As can be seen in Table 5, 14 of 24 target analytes were present in the daily indoor air samples, at concentrations reaching µg m^{-3} in some cases. Four of them, benzyl alcohol, linalool, ionone, and lilial®, were found in almost all environments. This fact should attract the attention regarding the ubiquitous presence of this kind of compounds in quotidian indoor environments.

Table 5. Compounds found (µg m^{-3}) in indoor air samples[a] under normal daily conditions (WR, washroom; LR, laundry room; C, corridor; L, living room; K, kitchen; BR, bedroom; SR, storage room).

Compound	WR1	WR2	LR1	LR2	C1	C2	C3	L1	K1	BR1	SR
Benzyl alcohol	0.583	2.67	0.170	0.392	0.238	0.043	0.312	1.37	1.02	0.049	0.343
Linalool	2.11	6.11	0.153	2.43	0.629	0.185	0.863		2.85	0.231	0.980
Citronellol	0.192	0.342	0.171	0.144				0.476	0.926	0.017	0.307
Citral								0.642	0.447		
Geraniol		0.491						0.394			0.208
Cinnamaldehyde	0.020										0.631
Hydroxycitronellal	0.793		0.329			0.094			0.991		
Eugenol	0.036								0.018		0.028
Ionone	0.187	0.193	0.055	0.113	0.031	0.017	0.043	0.765	0.478	0.015	0.032
Lilial®	1.09	0.898	0.224	0.116	0.343		0.364	0.310	0.714		0.254
Farnesol		0.543			0.052		0.122	0.294			
Hexylcinnamaldehyde	0.020	0.035	0.081		0.024		0.011	0.189	0.057		
Benzyl benzoate	0.012	0.013						0.015	0.013		0.017
Benzyl salicylate								0.014	0.014		0.018

[a]Blank spaces mean values below LOD.

Table 6. Compounds found μg m^{-3}) in indoor air samples[a] after the application of different aerosol products (CAR, car; WR, washroom; C, corridor; L, living room; K, kitchen; BR, bedroom).

Compound	CAR	WR3	WR4	C4	L2	K2	K3	BR2	L3	K4	BR3
Benzyl alcohol	6.71	3.30	6.21	2.04	6.18	6.18	2.36	3.06	1.38		1.80
Linalool	30.8	53.6	23.7	15.9	31.6	30.8	26.2	20.9	7.54		6.84
Methyl-2-octynoate		7.33									
Citronellol	9.97	20.8	6.25	5.75	10.6	12.2	7.52	7.18	0.483		
Citral				3.19	1.50		1.12				
Geraniol	4.75	15.8	1.83		7.65	5.97	7.38	3.76			
Cinnamaldehyde		0.472		0.918				0.103			
Hydroxycitronellal					4.33	2.38	4.28		0.384		
Cinnamyl alcohol		4.32									
Eugenol		3.48	0.916	4.08	2.32	1.78	2.24	0.172			
Isoeugenol[b]			0.943	1.90	1.80	1.78	1.28				
Coumarin							0.692			0.204	
Ionone	1.54	7.57	0.577	1.19	0.274		0.523	1.69	0.144	0.008	0.423
Lilial®	7.71	13.8	2.70	6.92	6.99	5.35	7.41	9.78	1.30		0.169
Amyl cinnamal								0.088	3.87	0.051	
Farnesol											
Hexylcinnamaldehyde	1.07	14.7	1.44	0.111	0.911	0.136	0.271	3.84	0.216		0.213
Benzyl benzoate	0.511	0.599	0.202	0.976	0.521	0.184	0.329		1.23	0.235	
Benzyl salicylate	0.251	55.8	0.793	0.389	0.983	1.52		3.58	0.600	0.033	0.099

[a] Blank spaces mean values below LOD.
[b] Values were corrected taking into account the average extraction efficiency for this compound.

When different air fresheners (10 commercial brands were tested) were applied following the recommendations of the manufacturers (when available) into the home places (see eight first columns in Table 6), the measured concentrations of the studied allergenic fragrances were markedly high. Under these conditions, 18 of the 24 allergens were found in some samples, and 10 of them in almost all the samples. The highest concentrations were due to linalool (from 6.8 to 53.6 μg m^{-3}) but high concentrations above 10 μg m^{-3} were also found for citronellol, geraniol, lilial® and benzyl salicylate in some cases. L3, K4 and BR3 (Table 6) are samples taken into three different rooms after the application of different insecticides. Higher concentrations and higher number of allergen fragrances were found in L3 and BR3. The insecticides applied into these two rooms were aerosols labelled as "perfumed", whereas K4 corresponds to an insecticide for surface application.

The extracted ion chromatograms for one of the indoor air samples (L2) are shown in Fig. 4.

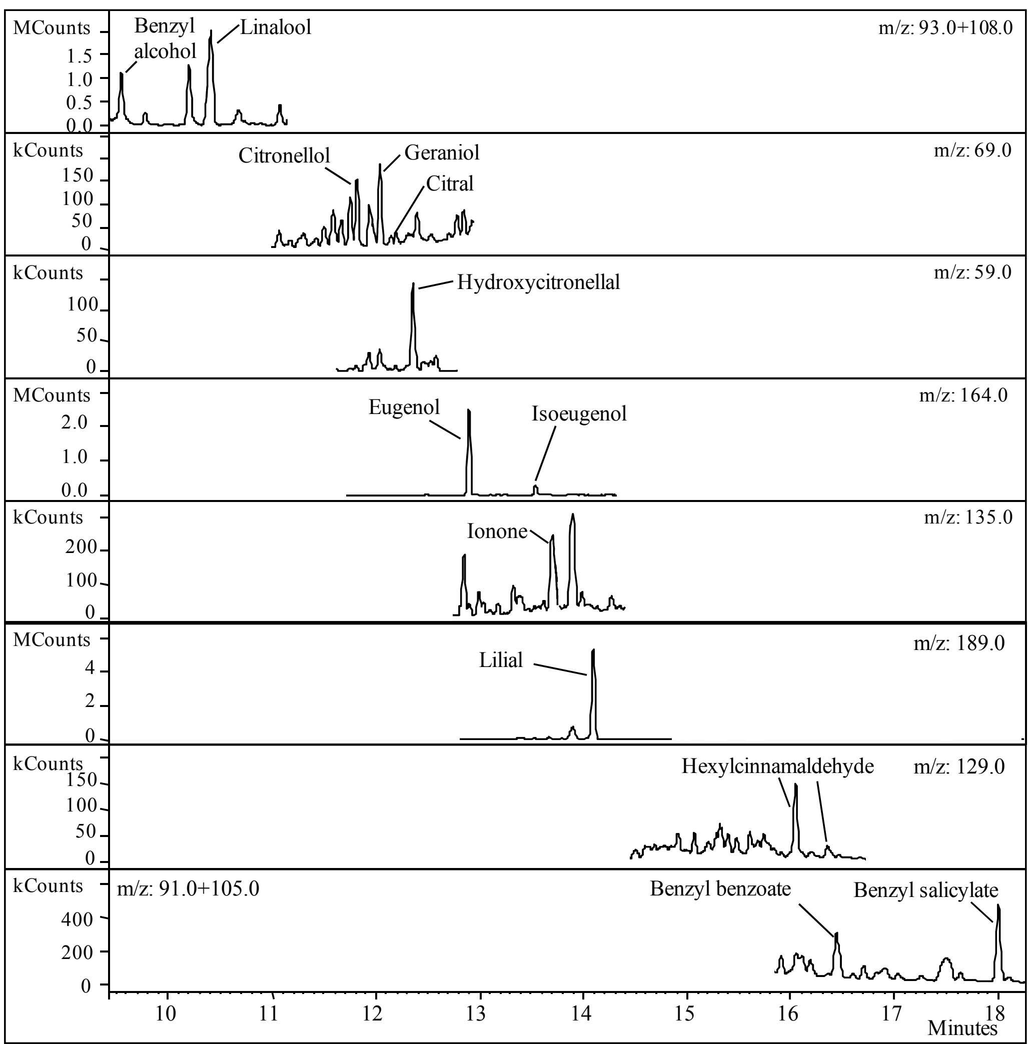

Fig. 4. Extracted ion chromatograms of sample L2 (see concentrations in Table 6).

4. Conclusions

A method based on sorbent trapping combined with SPME followed by GC–MS was optimized for the rapid analysis of 24 suspected allergens in indoors air samples. To the best of our knowledge, this is the first method based on SPME for the analysis of these fragrance compounds in air. Compounds in the pumped air could be retained on a very small amount of florisil (25 mg) and then extracted in the headspace mode by SPME. The SPME step was optimized by means of a factorial design demonstrating the importance of the interaction effects. The best conditions involve the use of florisil and the extraction at 100 ºC, after the addition of 100 µL of water to promote desorption, using DVB/CAR/PDMS or PDMS/DVB as fiber coating. One of the best attainments of the proposed method is the external calibration, which can be performed by direct spike of the sorbent with the target compounds. The performance of the method has been demonstrated in terms of linearity, accuracy (recoveries > 85% in most cases) and precision (RSD < 15%). The sensitivity of the method is very high; limits of detection below 4 ng m^{-3} were obtained for the majority of the target compounds. In addition, the proposed SPE-HS-SPME method is simple, fast, cheap, and easy to implement in non-specialized laboratories. The application of the method to daily home air samples demonstrates the ubiquity of this kind of fragrance ingredients in quotidian indoor environments, which were found in all the samples, and frequently at quite high levels.

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References

1. T.A.E. Platts-Mills, D. Vervloet, W.R. Thomas, R.C. Aalberse, M.D. Chapman, J. Allergy Clin. Immun. 100 (1997) S2.

2. A. Schnuch, E. Oppel, T. Oppel, H. Römmelt, M. Kramer, E. Riu, U. Darsow, B. Przybilla, D. Nowak, R.A. Jörres, Br. J. Dermatol. 162 (2010) 598.
3. A.C. De Groot, P.J. Frosch, Contact Dermatitis 36 (1997) 57.
4. J.D. Johansen, Am. J. Clin. Dermatol. 4 (2003) 789.
5. K. Rumchev, J. Spickett, M. Bulsara, M. Phillips, S. Stick, Thorax 59 (2004) 746.
6. C. Shim, M.H. Williams Jr., Am. J. Med. 80 (1986) 18.
7. A. Farrow, H. Taylor, K. Northstone, J. Golding, Arch. Environ. Health 58 (2003) 633.
8. J. Elberling, A. Linneberg, A. Dirksen, J.D. Johansen, L. Frolund, F. Madsen, N.H. Nielsen, H. Mosbech, Clin. Exp. Allergy 35 (2005) 75.
9. B. Bridges, Flavour Frag. J. 17 (2002) 361.
10. L.F. Gaunt, K.L. Jerrim, J.F. Hughes, Powder Technol. 135–136 (2003) 354.
11. SCCNFP/0017/98 Final Fragrance Allergy in Consumers (December 1999).
12. IFRA International Fragrance Association, http://www.ifraorg.org/Home/page.aspx/3.
13. R.A. Ford, B. Domeyer, O. Easterday, K. Maier, J. Middleton, Regul. Toxicol. Pharm. 31 (2000) 166.
14. Regulation (EC) No. 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products (recast). Official Journal of European Union, L342/59 (2009).
15. R. Kallenborn, R. Gatermann, S. Planting, G.G. Rimkus, M. Lund, M. Schlabach, I.C. Burkow, J. Chromatogr. A 846 (1999) 295.
16. H. Fromme, T. Lahrz, M. Piloty, H. Gebhart, A. Oddoy, H. Rüden, Indoor Air 14 (2004) 188.
17. A.M. Peck, K.C. Hornbuckle, Atmos. Environ. 40 (2006) 6101.
18. J. Regueiro, C. Garcia-Jares, M. Llompart, J.P. Lamas, R. Cela, J. Chromatogr. A 1216 (2009) 2805.
19. N. Ramirez, R.M. Marce, F. Borrull, J. Chromatogr. A (in press).

20. J.P. Lamas, L. Sanchez-Prado, C. Garcia-Jares, M. Llompart, J. Chromatogr. A 1217 (2010) 1882.

21. J.P. Lamas, L. Sanchez-Prado, C. Garcia-Jares, M. Llompart, Anal. Bioanal. Chem. 394 (2009) 1399.

22. I. Masuck, C. Hutzler, A. Luch, J. Chromatogr. A 1217 (2010) 3136.

23. R. Barro, S. Ares, C. Garcia-Jares, M. Llompart, R. Cela, J. Chromatogr. A 1072 (2005) 99.

24. R. Barro, C. Garcia-Jares, M. Llompart, R. Cela, J. Chromatogr. Sci. 44 (2006) 430.

3. DETERMINACIÓN DE DIMETILFUMARATO Y OTROS ALÉRGENOS POTENCIALES

Muchas veces los consumidores se encuentran con que productos supuestamente inocuos para su salud provocan graves lesiones en la misma. Este tipo de circunstancia ha ido adquiriendo mayor repercusión en los últimos años, llegando en algunos casos a la prensa diaria. Un claro ejemplo se encuentra en los múltiples casos de problemas para la salud de distintos individuos, derivado del uso de productos que contenían dimetilfumarato (DMF).

Las severas dermatitis vinculadas al uso de sofás o calzado, causaron gran alarma y preocupación en la sociedad provocando una rápida reacción de los servicios de alerta de la UE. En la investigación de estos casos documentados por primera vez en el año 2008 [1], se determinó que la sustancia que provocaba estas reacciones en la piel de los consumidores era el DMF, compuesto biocida con conocidos efectos perjudiciales para la salud humana.

Paralelamente, y con la presión de la industria de saber por qué su producto estaba provocando estas reacciones alérgicas en los consumidores, investigamos la procedencia de esta sustancia irritante, puesto que no se incluía en el proceso de fabricación de los distintos productos.

Esta búsqueda nos condujo a estudiar las pequeñas bolsas desecantes con sílica gel presentes en numerosos paquetes o cajas que contienen productos como calzado. El enfoque hacia el análisis de estas bolsas desecantes supuestamente inocuas, se debió a que junto con el cartón de las cajas, eran materiales que no estaban controlados por el fabricante en cuestión.

La primera sorpresa tras recibir dichas muestras para análisis, fue la aparición de una fuerte irritación y dolor de cabeza producidos durante la clasificación y almacenamiento de las muestras. Estos hechos, hicieron indispensables la manipulación de la muestra con extracción forzada y protección respiratoria y ocular.

Tras las primeras pruebas, se determinó la presencia de DMF en valores muy elevados.

Según la búsqueda bibliográfica realizada en ese momento, no existía ninguna metodología aplicada a la determinación de alguna sustancia en las "bolsitas

de sílica gel". Surgió entonces la necesidad de desarrollo de metodología analítica que permitiese la determinación de la composición de dichas muestras.

La apuesta por el desarrollo de metodologías analíticas sencillas y fácilmente trasladables a cualquier laboratorio de rutina, nos llevó a utilizar la extracción asistida por ultrasonidos (US) para el tratamiento de las muestras y la determinación por cromatografía de gases con detector de captura electrónica.

Mediante esta metodología se analizaron numerosas muestras de "bolsitas de sílica gel" de diversos orígenes, obteniendo unos resultados verdaderamente sorprendentes y alarmantes. A la vista de estos resultados, se decidió la divulgación inmediata de los mismos.

El siguiente paso con este tipo de muestra, fue la búsqueda de otras sustancias que también pudiesen estar presentes en las bolsas de "sílica gel". Tras una labor de "screenig", se encontraron otras dos sustancias potencialmente peligrosas para la salud y que no deberían estar presentes en dicho material. Estos compuestos son el benzotiazol (BT) y el 4-tert-butil phenol (TBP). El origen de estas dos sustancias en dichas muestras no está muy claro, pueden haber sido adicionadas directamente a la sílica gel, como el caso del DMF, o pueden aparecer en estas muestras como resultado de procesos de migración al entrar en contacto con productos que sí los incluyan en su composición.

Tras el desarrollo y optimización de la metodología analítica, basada también en la extracción asistida por US, pero usando la cromatografía de gases-espectrometría de masas (GC/MS) para la determinación, se procesó un número importante de muestras de distinto origen.

Lo realmente interesante de este estudio, son los niveles elevados de BT y TBP en las "bolsitas de sílica gel". Estos valores indican una posible concentración de estas sustancias la sílica gel, siempre y cuando no se hubiesen adiccionado directamente al desecante.

En este estudio también se encontraron valores de DMF realmente elevados, sobresaliendo una muestra entre todas las demás, ya que los valores cuantificados del compuesto reflejan que la muestra es 100% DMF.

Debe destacarse que, simultáneamente o pocos meses después de la realización de estos estudios, se prohibió el uso y comercialización de este compuesto en la UE [2,3]. Esta legislación incluye la obligación de la recuperación de todos los productos que contengan el DMF y estén en manos de los consumidores.

A raíz de toda esta problemática, en la actualidad es frecuente ver en los sacos con agentes desecantes textos en los que se informa de la ausencia del compuesto, "DMF free".

En resumen, este apartado de la Tesis se ha centrado en el desarrollo de metodología analítica para la determinación de varias sustancias tóxicas presentes en productos de consumo, originando los siguientes estudios discutidos a continuación:

- "Determination of dimethyl fumarate in desiccant and mouldproof agents using ultrasound-assisted extraction gas chromatography with electron-capture detection".
- "Determination of dimethyl fumarate and other potential allergens in desiccant and antimould sachets".

Bibliografía:

[1] T. Rantanen, British Journal of Dermatology. 159 (2008) 218.

[2] Commission Decision of 17 March 2009 requiring Member States to ensure that products containing the biocide dimethylfumarate are not placed or made available on the market (notified under document number C(2009) 1723) (1). Official Journal of the European Union.

[3] Commission Decision of 1 March 2011 extending the validity of Decision 2009/251/EC requiring Member States to ensure that products containing the biocide dimethylfumarate are not placed or made available on the market (notified under document C(2011) 1174) (Text with EEA relevance) (2011/135/EU). Official Journal of the European Union.

3.1 DETERMINATION OF DIMETHYL FUMARATE IN DESICCANT AND MOULDPROOF AGENTS USING ULTRASOUND-ASSISTED EXTRACTION GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

DETERMINATION OF DIMETHYL FUMARATE IN DESICCANT AND MOULDPROOF AGENTS USING ULTRASOUND-ASSISTED EXTRACTION GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

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Abstract

A fast, simple, low cost, and high throughput method has been developed for the determination of dimethyl fumarate (DMF) in desiccant and anti-mould agents employed for protecting clothes, footwear and accessories from humidity and mould. The procedure is based on ultrasound-assisted extraction followed by GC-µECD analysis. The method was conveniently optimized, and the analyte was efficiently extracted from the samples in only 5 min using such a low volume of solvent (ethyl acetate) as 1 mL. Simultaneous extractions could be carried out in also 5 min without loosing efficiency. The method has been validated showing good linearity and selectivity. Precision was satisfactory with RSD of 4–5%. Recovery was evaluated in two real samples at different DMF concentration levels and was >90% in all cases. Limit of detection and quantification were at the ng g^{-1} level. The proposed procedure was applied to the determination of DMF in several desiccant and anti-mould samples. Although most of them were labelled as "silica gel" all the samples tested with the exception of three presented important amounts of DMF, many of them at the high µg g^{-1} level. The presence and the high concentrations of this allergenic fungicide in desiccant and anti-mould sachets employed in many consumer products, such as clothes and footwear, should be a matter of concern.

Keywords: Dimethyl fumarate; Ultrasound-assisted extraction; GC-µECD analysis; Desiccant; Anti-mould agent; Allergy.

1. Introduction

Dimethyl fumarate (DMF) is a powerful fungicide, which has been found to act as an extreme sensitizer, causing severe acute reactions when come into contact with the skin of healthy individuals. Exposition to DMF has produced very difficult to treat allergic eczemas in patients from several European countries. First cases arose in 2006 from the use of sofas and chairs manufactured in China, and rapidly new cases related to the use of other consumer goods principally footwear were reported [1].

DMF is mainly used to avoid deterioration of leather, furniture, and other products by mould during the storage and transport in warm and humid climates. Sachets containing DMF are placed in origin inside clothes and couches, as well as inside shoeboxes, from which it spreads and impregnates the consumer product. In addition, there is recent evidence that DMF could be present in certain Chinese food such as high-fat cakes [2]. The severity of the damage together with the broad use of goods manufactured in China lead to a huge uncertainty among consumers, wondering which goods are safe and which are not.

From 1998, DMF is banned in biocide formulations for products manufactured in the EU (Directive 98/8/EC), but noncommunitarian producers can use it and thus enter the EU market. The Decision adopted by the EU in March 2009 tries to protect EU consumer from the risk of DMF by banning the importation of contaminated products, and recalling and withdrawing those products already on the market [3]. According with this Decision, the presence of DMF in products should be determined against the maximum limit of 0.1 mg kg^{-1} of product or part of the product, and the analytical methodology should be able to reliably quantify DMF at this concentration level. This amount is considered to be sufficiently below the concentration of 1 mg kg^{-1}, which showed a strong reaction in the patch test mentioned above.

However, the public concern about DMF and the non-negligible risk of finding contaminated desiccant sachets and consumer products in the EU market for still long time, makes imperative the availability of rapid, simple and efficient analytical methods for DMF which could be immediately implemented in control laboratories at low cost.

Up to now, and to the best of our knowledge, there is a lack of validated analytical methods for DMF in the international scientific literature. The use of HPLC for the analysis of DMF and monomethyl fumarate in keratinocytes related to the treatment of psoriasis with fumarates has been reported [4]. Rantanen [1] described the use of a headspace technique together with gas chromatography and mass spectrometry (GC/MS) to semiquantitatively determine DMF in pieces of chair seats and backrests, which allowed establishing the first relationship between DMF and the severe contact dermatitis cases appeared in Finland.

Thus, the aim of this work is to develop a method for the determination of DMF in desiccant and anti-mould sachets based on ultrasound-assisted extraction. The method was validated in terms of linearity, precision, sensitivity and selectivity demonstrating its reliability. Several samples obtained from foot wear, footwear boxes, clothes and accessories were analyzed. Almost all the samples presented high concentrations of this powerful fungicide.

2. Experimental

2.1. Reagents and materials

2-Butenedioic acid (E)-, dimethyl ester (Dimethyl fumarate, DMF, CAS number: 624-49-7) 97% purity was purchased from Aldrich (Sigma–Aldrich Chemie GmbH, Steinheim, Germany).

Methanol and ethyl acetate of analytical grade were provided by Merck (Darmstadt,Germany).A stock solution of 3000 μg mL^{-1} was prepared in methanol. Further dilutions were prepared in methanol (for spiking experiments) and ethyl acetate (for external calibration). All solutions were stored in amber glass vials at −20 ºC.

Real samples of desiccant and anti-mould sachets were collected from different kinds of clothes and accessories, as well as footwear and footwear boxes acquired in several shops and markets of Spain. The content of each sachet was placed in a mortar in order to grind and homogenize the sample. The obtained powder was placed in an amber glass vial and sealed till the extraction and analysis is carried out.

2.2. Chromatographic analysis

GC-μECD was performed using an Agilent Technologies 6890N Network gas chromatograph System equipped with 63Ni μ-ECD system, equipped with 7683B Series automatic injector. The system was operated by GC ChemStation Rev A.10.02 software.

Separation was carried out on a HP5 capillary column (30 m×0.32mm i.d., 0.25 μm film thickness) from Agilent Technologies (Palo Alto, CA, USA). Helium (purity 99.999%) was employed as carrier gas at a constant column flow of 2.0 mL min^{-1}, and nitrogen was employed as make-up gas at a constant flow of 30.0 mL min^{-1}. The GC oven temperature was programmed from 60 ºC (held 2min) to 260 ºC at 30 ºC min^{-1} (total analysis time = 8.67min). Detector temperature was set at 300 ºC.

Splitless mode (held 2min) was used for injection, the split flow was set at 50 mL min^{-1} and the injector temperature was kept at 260 ºC. The injection volume was 2μL. The analyte was positively identified by comparison of its retention time to that of a standard solution.

2.3. Ultrasound-assisted extraction

The selected volume (1 or 2 mL depending on the experiment) of the organic solvent (ethyl acetate or methanol) was added to the glass vial containing 50, 200 or 500 mg) of sample powder, and sealed with a headspace aluminium cap furnished with PTFE-faced septum. DMF was extracted from the samples to the organic solvent using an ultrasound bath (Ultrasons Med-II, J.P. Selecta, Barcelona, Spain) at 40 kHz of ultrasound frequency and 200W power at 25 ± 3 ºC or 50 ± 3 ºC for 5 or 10 min depending on the experiment. Afterwards, the extract was filtered through a 0.22 μm Millex®- GV filter (13mm diameter) (Millipore, Bedford, USA). When it was convenient, extracts were diluted previously to the injection in the chromatographic system.

In the final optimized conditions, 50-500 mg of sample were sonicated with 1 mL ethyl acetate for 5 min at 25 ± 3 ºC. Blanks were periodically run during the analysis to confirm the absence of contamination.

3. Results and discussion

Initial chromatographic experiments were carried out of DMF. DMF standard solutions of different concentrations were prepared in ethyl acetate. The chromatographic conditions were optimized (see experimental conditions in Section 2), and we could prove the suitability of the μECD to achieve a favourable and selective DMF response in a very short time (t_R = 4.75 min). Initial extraction experiments using real DMF contaminated samples demonstrated as well the selectivity of the method. Figure 1 shows the chromatograms corresponding to three real samples where we can easy appreciate the clean background obtained and, thus, the absence of chromatographic interferences.

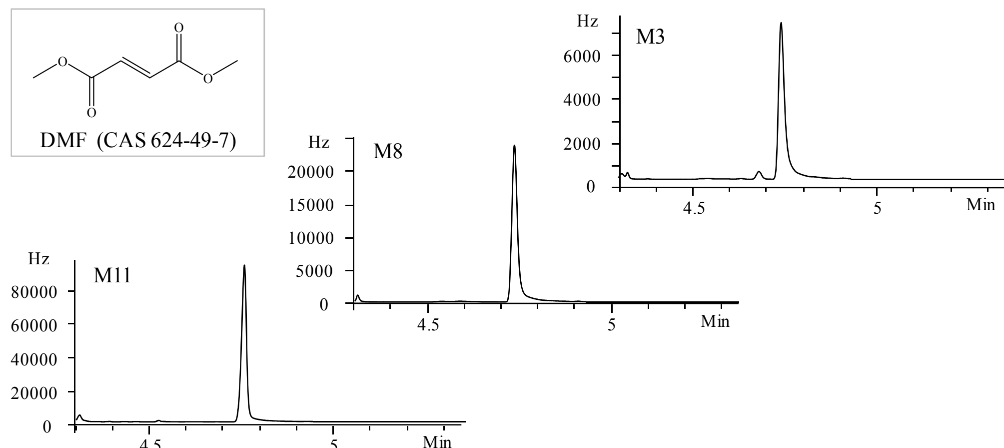

Figure. 1. GC-_ECD chromatograms of three real samples containing DMF (see sample concentrations in Table 4).

The chromatographic method was validated in terms of precision, linearity, and detection and quantification limits. To evaluate method linearity, a calibration study was performed using DMF standards prepared in ethyl acetate. The calibration range was from 0.1 to 50 μg mL^{-1}, and the number of calibration levels was seven. The method exhibited a direct proportional relationship between the amount of DMF and the chromatographic response with a determination coefficient (R^2) of 0.9992 (Table 1). To validate the regression data, an analysis of variance (ANOVA) was performed. The lack-of-fit (LOF) test is designed to determine whether the selected

model is adequate to describe the experimental data. The test compares the variability of the proposed model residuals to the variability between observations at replicate values of the independent variable. Results of the LOF test for the calibration range considered, at a confidence level of 95% are also shown in Table 1. Since *p*-value is greater than 0.05, the linear regression model is adequate for the experimental data. The precision of the method was evaluated by calculating the relative standard deviation (RSD) at three concentration levels, and results are shown in Table 1. Values were in general very homogeneous for the three levels tested, about 2.2% and 4.7% for the intra-day and inter-day determinations, respectively.

Table 1. Performance of the GC-μECD method

Linearity			Precision (RSD,%)		LOD (ng mL-1)	LOQ (ng mL-1)
R^2	LOF test		Intra-day (n=5)	Inter-day (n=6)		
	F-ratio	*p*-value				
0.9992	0.22	0.9227	2.0^a – 2.7^b– 1.8^c	5.9^a – 4.6^b – 3.6^c	7.8	26

Concentration level= (a) 0.5 μg mL^{-1}, (b) 5 μg mL^{-1}, (c) 50 μg mL^{-1}

Limits of detection (LOD) and quantification (LOQ), defined for a signal-to-noise ratio of 3 (*S/N* = 3) and 10 (*S/N* = 10), respectively, were estimated (see Table 1). Both limits are at the low ng mL^{-1} level.

In the development of the ultrasound-assisted extraction method for DMF, we have studied the effect of four factors that might affect extraction efficiency: type of solvent, MeOH and ethyl acetate; solvent volume, 1 and 2 mL; extraction temperature, 25 ºC and 50 ºC; and extraction time, 5 and 10 min. For this optimization step, a real DMF contaminated sample was employed. For all experiments, the sample size was set at 50 mg. In order to select the optimal conditions, a 2^4 complete factorial design was employed. This design allows studying of the influence of main factors as well as the two-factor interactions. The total number of experiments was 16, allowing 5 degrees of freedom to estimate the experimental error. None of the factors neither the interactions showed statistical

significance, as can be observed in the ANOVA analysis of Table 2. As can be seen in the table, all the factors and interactions showed *p*-values higher than 0.05 and, so, they were not statistically significant. Therefore, extraction efficiency is equivalent in all experimental conditions tested. Thus, we selected the most suitable conditions considering other criteria. Ethyl acetate was selected since it is a more suitable chromatographic solvent than MeOH; the low solvent volume (1 mL) was preferred since it provides more concentrated extracts and lower solvent consumption; extraction temperature was set at 25 ºC avoiding possible solvent concentration, saving energy and unfavouring interfering compounds coextraction; finally, an extraction time of 5 min was selected to achieve maximum throughput.

Table 2. ANOVA corresponding to the 2^4 factorial design

	Factors				Factor interactions					
	Solvent (A)	Volume (B)	Temperature (C)	Time (D)	AB	AC	AD	BC	BD	CD
F-ratio	0.05	0.01	0.13	0.09	0.21	0.76	0.65	0.38	0.51	0.01
p-value	0.82	0.91	0.74	0.78	0.67	0.42	0.46	0.56	0.51	0.91

We also studied the possibility of performing simultaneous extractions (n = 6). The responses obtained as well as the RSD were identical to the one obtained for single extractions (see Figure 2a). So, the possibility of performing up to six simultaneous extractions and in only 5 min was demonstrated.

Some experiments were as well carried out employing a higher amount of sample. In this case, aliquots of 200 mg and 500 mg of sample were extracted under the selected conditions. The responses were 4 and 10 times higher, respectively, than the ones obtained with 50 mg, demonstrating method efficiency for extracting larger amounts of solid sample (Figure 2b).

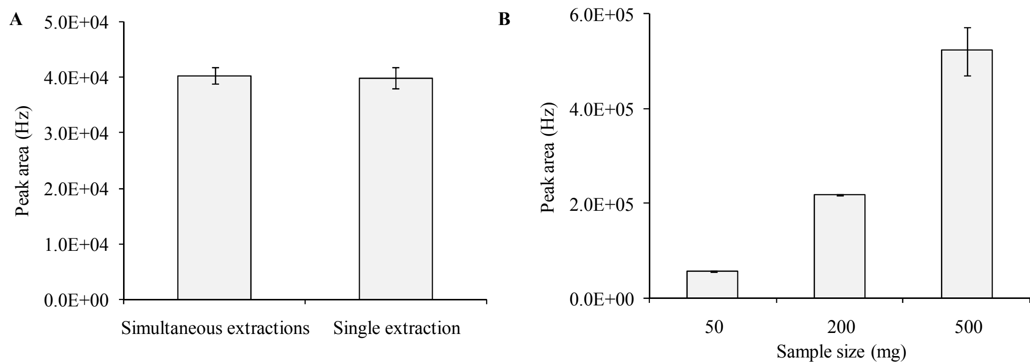

Figure 2. Comparison of the responses obtained for (a) single extractions and simultaneous extractions, (b) 50 mg, 200 mg and 500 mg of sample.

Precision of the ultrasound-assisted extraction GC-μECD experimental procedure was assessed using real samples containing different DMF concentrations. Results showed low intra-day and inter-day variation, with relative standard deviation (RSD) about 5% in all cases (see Table 3), and, thus, equivalent to the ones obtained considering only the chromatographic analysis (see Table 1).

LOD and LOQ, for the full ultrasound-assisted extraction GC- μECD method (sample size of 500 mg) are also presented in Table 3. These limits are below the maximum limit of 0.1 mg kg^{-1} imposed by the European Union [3]. Therefore, the proposed method is suitable to determine DMF content in real samples.

Table 3. Performance of the ultrasound-assisted extraction GC-μECD proposed method.

Precision (RSD %, n=5)		Recovery (%)		LOD (ng g^{-1})	LOQ (ng g^{-1})
Intra-day	Inter-day	Sample M3	Sample M1		
4.5[a] - 5.4[b]	4.5[a] - 4.6[b]	91[c] - 98[d]	94[d]	14	46

Concentration level: (a) 70.2 μg g^{-1}, (b) 2.0 μg g^{-1}
Addition level: (c) 614 μg g^{-1}, (d) 61.4 μg g^{-1}.

Recoveries were calculated as the ratio of the measured concentration, after subtracting the initial concentration in the non-spiked sample, to the spiked concentration, and expressed as percentage. Concentrations were calculated by

external calibration. Recoveries were satisfactory for the two tested samples (see Table 3), being in all cases quantitative (>90%). The method was finally applied to determine the levels of DMF in sachets labelled as “mouldproof agent”, “biochemical dry-desiccant”, “antimoulds-active mineral”, “desiccant-silica gel”, “silica gel” or “mouldproof agent” in the envelope. The samples presented different aspects (little balls, sand, powder) and colours (white, grey, yellow). For the final determinations, some of the extracts were conveniently diluted. Results are summarized in Table 4. All tested samples excluding three, showed detectable concentrations of DMF. Many of them had quite high concentrations even reaching the parts per thousand levels. It can be concluded that the presence and levels of this allergenic chemical in consumer goods should be a matter of concern.

Table 4. Concentration of DMF in several desiccant and anti-mould samples

Sample	Dimethyl fumarate (μg g^{-1})
M1	2.0
M2	105
M3	70.2
M4	139
M6	1429
M7	2639
M8	200
M9	511
M10	205
M11	683
M12	98
M14	n.d
M15	6.6
M16	n.d
M17	624
M18	n.d
M19	9.8
M20	102
M21	32
M22	132
M23	61.5
M24	513
M25	176

n.d: not detected

Acknowledgements

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References

1. T. Rantanen, British Journal of Dermatology 159 (2008) 218.
2. L.-Z. Jin, H.-J. Hu, Zhongguo Weisheng Jianyan Zazhi 16 (2006) 1209.
3. Commission Decision of 17 March 2009 requiring Member States to ensure that products containing the biocide dimethyl fumarate are not placed or made available on the market (notified under document number C(2009) 1723) (1), Official Journal of the European Union, L74 (17 March 2009) 32.
4. D. Jirovsky, W.Wiegrebe, Monatshefte für Chemie 135 (2004) 1563.

3.2 DETERMINATION OF DIMETHYL FUMARATE AND OTHER POTENTIAL ALLERGENS IN DESICCANT AND ANTIMOULD SACHETS

DETERMINATION OF DIMETHYL FUMARATE AND OTHER POTENTIAL ALLERGENS IN DESICCANT AND ANTIMOULD SACHETS

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Abstract

A method for the determination of dimethyl fumarate (DMF), benzothiazole (BT) and tert-butylphenol (TBP) in desiccant and antimould agents employed for protecting consumer products from humidity and mould has been developed. The method is based on ultrasoundassisted extraction (UAE) followed by GC-MS analysis. Parameters that could affect the extraction of the compounds have been optimised using a multivariate approach. In the final conditions, the extraction is performed using only 0.5 or 1 mL ethyl acetate and applying ultrasound energy for 5 min. Simultaneous extractions could also be carried out in 5 min without losing efficiency. The method was validated showing good linearity (R^2 >0.995). Both intra- and inter-day precisions were studied at several concentration levels, being satisfactory in all cases (RSD <10%). Recovery was evaluated in four real desiccant samples at different compound concentrations, ranging between 87% and 109%. Limits of detection and quantification were in the low nanogramme per gramme level, thus allowing the determination of DMF at concentrations well below the limit established by the recent EU Directive (0.1 µg g^{-1}). The proposed procedure was applied to the determination of the target compounds in several desiccant and antimould samples. Although most of them were simply labelled as "silica gel", more than 70% of the tested samples contained high amounts of DMF, many of them at the high microgram per gramme level. Many samples also showed the presence of the other two potential allergens. These results demonstrate that the content of the "desiccant" sachets and tablets in consumer products does not usually belong with the label of the desiccant, and hence, the high risk of exposition to the powerful allergen DMF and other potentially harmful chemicals through consumer goods should be a matter of concern.

Keywords: Dimethyl fumarate . Benzothiazol . tert-Butylphenol . Ultrasound-assisted extraction . GC-MS . Desiccant . Antimould agent . Allergy

Introduction

Consumers have become increasingly familiar with the presence of desiccant sachets in packaged goods such as footwear, leather clothes, handbags, electronics and also in pharmaceuticals and foods. Sachets of desiccants constitute a valuable help for the industry and for consumers since they control the humidity and avoid the damage caused in the materials by moisture and mould, thus preserving the quality, and in this way, extending the self-life of the consumer products. Most desiccant sachets usually contain either clay, minerals or silica gel. However, sachets may also contain substances used to provide or to reinforce the antimould action of the desiccant [1]. In this way, it is worthy of notice that sachets are simply labelled as desiccant, antimould, or even only as "do not eat".

Two most commonly used desiccants are silica gel and calcium oxide. Silica gel/silica dioxide is a natural mineral which changes to molecular or bead form after purification and processing. Since it is chemically and biologically inert, no harmful health effects will be expected even if it is accidentally ingested in small amounts. Calcium oxide is a white powder which can control humidity effectively by keeping the relative humidity below 10%. It is mostly used for packaging dehydrated foods or foods (e.g., biscuits or pastry food) that need to be stored in low-humidity environments. In contact with moisture, calcium oxide becomes caustic and may cause burning sensation, stomach cramps, and diarrhoea if swallowed accidentally. Other desiccants such as activated carbon, clay and calcium chloride, are less commonly used due to their higher cost.

Concern about desiccant sachets started when the first cases of allergic eczemas arose in 2006 related to the use of sofas and chairs manufactured in China. The relationship with the presence of the fungicide dimethyl fumarate (DMF) in desiccant sachets placed under the seats was established by Rantanen [2]. Strong reactions were observed in the most severe case down to 1 mg kg^{-1}. Rapidly, new cases of allergy associated to the use of other consumer goods principally footwear, were reported in several European countries, increasing the public concern up to date.

According to clinical tests, the health damage was caused by DMF, which is a powerful fungicide preventing moulds that may deteriorate leather furniture or

footwear during storage or transport in a humid climate. DMF, an extreme sensitizer, penetrated through the clothes onto consumers`skin [3] where it caused painful skin contact dermatitis, including itching, irritation, redness, and burns; in some cases, acute respiratory troubles were reported. The presence of DMF is thus a serious risk. From 1998, DMF is banned in biocide formulations for products manufactured in the EU (Directive 98/8/EC); thus, biocidal products containing DMF are not legally available in the community for the treatment of products against moulds, and thus no product manufactured in the EU can legally contain DMF. However, non communitarian producers can use it and thus enter the EU market. The decision adopted by the EU in March 2009 [4] tries to protect EU consumers from the risk of DMF by banning the importation of contaminated products, and recalling and withdrawing those products already on the market. According to this decision, the presence of DMF in products should be determined against the maximum limit of 0.1 mg per kg of product or part of the product, and the analytical methodology should be able to reliably quantify DMF at this concentration level. This amount is considered to be sufficiently below the concentration of 1 mg kg^{-1}, which showed a strong reaction in the patch test mentioned above.

Validated analytical methods for DMF have not been reported in the international scientific literature. HPLC was used for the analysis of DMF and monomethyl fumarate in keratinocytes related to the treatment of psoriasis with fumarates [5]. Rantanen [2] semiquantitatively determined DMF in pieces of chair seats and backrests by a headspace technique and GC-MS, which allowed the first relationship between DMF and severe contact dermatitis cases appeared in Europe.

In addition to DMF, desiccant sachets can contain, adsorb and, hence, spread to the consumer goods other potentially harmful substances. Benzothiazoles are high-production- volume chemicals widely used as biocides in paper and leather manufacturing, as corrosion inhibitors [6, 7], and as vulcanization accelerators in rubber production. Benzothiazole (BT) is known to be present in rubber components, since it is used in vulcanisation processes, and serve as a biocorrosion inhibitor [8]; it can also be a derivative of thiocyanate methylthiobenzothiazole (TMBT), used as a fungicide in leather processing. 4-*tert*-Butyl phenol (TBP) has a wide use as UV light stabiliser, rubber chemical, a corrosion inhibitor, and in insecticides [9], among others. TBP is considered as an endocrine disruptor [10], irritant to the skin, eyes,

and respiratory tract. A carcinogenicity study indicated that this chemical has promoted activity of fore stomach carcinogenesis in rats treated with N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) [11]. Besides, since the structurally related chemical 2(3)-tert-butyl-methoxylphenol is a clear carcinogen, the carcinogenic potential of this chemical should not be ruled out.

As a consequence of its widespread use, derivatives of benzothiazoles have been found in the environment, and they should be considered as emerging pollutants. BT has been detected in environmental [12–16] and drinking waters [16], wastewaters [15–18], and sediments [19]. For the determination of this compound, methods combining liquid–liquid extraction (LLE) [12] and solid phase extraction (SPE) [14–17, 19], with GC-MS [12, 14, 19], GC×GCTOF- MS [15], and LC-MS [16, 17] analysis, have been described.

TBP has been determined in river water after LLE followed by GC-MS and laser-induced fluorescence analysis [20], in seawater and water from an irrigation canal by SPE and HPLC-(ESI)MS) [21], in wastewater by continuous LLE-GC-MS [22], and SPE-HPLC-(ESI)MS [23]; in waste landfill leachates by stir bar sorptive extraction-GCMS after derivatization [9]. TBP has also been detected in outdoor and indoor air [24, 25] and in household dust samples [25].

In this work, a method based on ultrasound-assisted extraction is developed for the determination of DMF, BT and TBP, in desiccant and antimould sachets. To our knowledge, this is the first study about the presence of these harmful compounds in the usual desiccant sachets that can be found in hundreds of consumer goods and are plentiful in our homes. The method was validated in terms of linearity, precision, sensitivity and selectivity demonstrating its reliability. Obtained LOQ for DMF was widely below the established value in the recently published commission decision [4]. Several desiccant samples obtained from footwear, footwear boxes, clothes, furniture, and accessories were analysed. Almost all the samples presented high concentrations of the target analytes, and more than 70% of the samples presented DMF at high concentration levels, far exceeding the legal limit.

Experimental

Reagents and materials

(E)-2-Butenedioic acid, dimethyl ester (dimethyl fumarate, DMF) 97% purity, and benzothiazole (BT) 96 % purity, were purchased from Aldrich (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). 4-*tert*-Butylphenol (TBP) was purchased from Fluka (Fluka Chemie GmbH, Steinheim, Germany). Table 1 shows the physico-chemical properties of the target compounds.

Methanol and ethyl acetate of analytical grade were provided by Merck (Darmstadt, Germany). Stock solutions of each compound were prepared containing concentrations ranged between 2,000–4,000 μg mL^{-1} in methanol. Further mixture dilutions were prepared in methanol (for spiking experiments) and ethyl acetate (for external calibration). All solutions were stored in amber glass vials at −20 °C.

Table 1. Main physico-chemical properties of the target compounds

Compound	CAS number	Density (g cm^{-3})	Molecular weight (g mol-1)	Boiling point (°C)	Vapour pressure (Pa)	Aqueous Solubility (g L-1)	Partition coefficient (pKow)
DMF	624-49-7	1.12	144.13	193	63.3	22	0.62
BT	95-16-9	1.27	135.19	227	1.9	3	2.01
TBP	98-54-4	0.971	150.22	233.7	4.8	9	3.29

Real samples of desiccant and antimould sachets and tablets were collected inside of different kinds of clothes, accessories, and footwear, as well as furniture, acquired in several shops and markets of Spain. The content of each sachet was placed in a mortar in order to grind and homogenise the sample. The obtained powder was placed in an amber glass vial and sealed until the extraction and analysis is carried out.

Gas chromatography–mass spectrometry

The GC-MS analysis was performed using a Varian 450-GC gas chromatograph (Varian Chromatography Systems, Walnut Creek, CA, USA) coupled to an ion-trap mass spectrometer Varian 240-MS (Varian Chromatography Systems)

with a waveboard for multiple MS (MS^n) analysis; and equipped with an automatic injector CP-8400. The system was operated by Varian MS workstation v6.9.1 software.

Separation was carried out on a HP5 capillary column (30 m×0.25 mm i.d., 0.25 μm film thickness) from Agilent Technologies (Palo Alto, CA, USA). Helium (purity 99.999%) was employed as carrier gas at a constant column flow of 1.5 mL min^{-1}. The GC oven temperature was programmed from 45 ºC (held 2 min) to 150 ºC at 10 ºC min^{-1} (total analysis time=12.5 min). Splitless mode (held 2 min) was used for injection, the split flow was set at 20 mL min^{-1} and the injector temperature was kept at 260 ºC.

The ion-trap mass spectrometer was operated in the electron impact ionisation mode (+70 eV) using an external ionisation configuration. Manifold, ion trap, ion source and transfer line temperatures, were maintained at 40, 150, 200 and 280 ºC, respectively. In the full-scan mode the mass range was varied from 50 to 300 m/z at three microscans average, starting at 4 min and ending at 12.5 min. The filament emission current was 25 μA. The analytes were positively identified by comparison of their mass spectra and retention times to those of standards. The identification and quantification ions and retention times for each target compound are listed in Table 2.

Table 2. Retention time and ions used for identification and quantification of the target compounds

Compound	Retention time (min)	Quantification ions	Identification ions
DMF	7.23	85,113	85,113,114
BT	10.48	108,135	69,108,135
TBP	11.34	107,135	95,107,135

Ultrasound-assisted extraction

The selected volume (0.5, 1 or 2 mL, depending on the experiment) of the organic solvent (ethyl acetate or methanol) was added to the glass vial containing 50–500 mg of sample powder, and sealed with a headspace aluminium cap furnished with PTFE-faced septum. The analytes were extracted from the samples to the organic solvent using an ultrasound bath (Ultrasons Med-II, J.P. Selecta, Barcelona, Spain) at 40 kHz of ultrasound frequency and 200 W power at 25 ± 3 ºC or 50 ± 3 ºC for 5 or 10 min depending on the experiment. Afterwards, the extract was filtered through a 0.22 µm Millex®-GV filter (13 mm diameter; Millipore, Bedford, USA). When it was convenient, extracts were diluted previously to the injection in the chromatographic system.

In the final optimised conditions, samples (50 – 500 mg) were sonicated with ethyl acetate (0.5 – 2 mL) for 5 min at 25 ± 3 ºC. Blanks were periodically run during the analysis to confirm the absence of contamination.

Results and discussion

First experiments were conducted to optimise the chromatographic separation of the target analytes as well as to select the quantification ions to obtain maximum signal-to-noise ratio. The chromatographic conditions are summarised in the experimental section, and the identification and quantification ions are presented in Table 2. The GC-MS method was validated in terms of precision, linearity, and detection and quantification limits.

To evaluate method linearity, a calibration study was performed using standards prepared in ethyl acetate. The calibration range was from 10 ng mL^{-1} to 50 µg mL^{-1}, with seven calibration levels and two to three replicates by level. The method exhibited a direct proportional relationship between the amount of each analyte and the chromatographic response with a determination coefficient (R^2)>0.9986 (Table 3). To validate the regression data, an analysis of variance was performed. The lack-of-fit (LOF) test is designed to determine whether the selected model is adequate to describe the experimental data. The test compares the variability of the proposed model residuals to the variability between observations at replicate values of the independent variable. Results of the LOF test for the

calibration range considered, at a confidence level of 95%, are also shown in Table 3. Since *p* values are greater than 0.05, the linear regression models are adequate for the experimental data.

Table 3. Performance of the GC-MS method

Compound	Linearity			Precision (RSD,%)		LOD (ng mL^{-1})	LOQ (ng mL^{-1})
	R^2	LOF test					
		F-ratio	*P*-value	Intra-day[a] (n=3)	Inter-day[b] (n=6)		
DMF	0.9986	0.52	0.8171	1.3-7	1.0-3.1	2.2	7.4
BT	0.9949	0.22	0.1311	3.4-8	2.5-5.6	2.4	7.9
TBP	0.9953	0.26	0.9648	0.2-10	0.9-3.5	1.9	6.2

[a]Concentration level= 0.05 - 50 µg mL^{-1}
[b]Concentration level= 5 - 50 µg mL^{-1}

The precision of the method was evaluated by calculating the relative standard deviation (RSD) at several concentration levels of the compounds, and results are included in Table 3. RSD values were in general very homogeneous, lower than 10% for the intra-day precision (concentrations at 0.05, 0.10, 5, and 50 µg mL^{-1}), and lower than 5.6% for the inter-day precision (considering compound concentrations of 5 and 50 µg mL^{-1}).

Limits of detection (LOD) and quantification (LOQ), defined for a signal-to-noise ratio of 3 (S/N=3) and 10 (S/N=10), respectively, were estimated at the low nanogramme per millilitre for all the analytes (see Table 3).

In the development of the UAE method, a multivariate strategy of optimization was carried out with the aim of selecting the optimal working conditions. We studied the effect of four factors (each one at two levels) that might affect extraction efficiency: type of solvent, solvent volume, extraction temperature, and extraction time. The factors and levels considered, as well as the corresponding codes, are summarised in Table 4. For the optimization step, a real desiccant sample contaminated with DMF was employed. Since BT and TBP were not present in this sample, they were added to give a final concentration of 50 µg g^{-1}. In all experiments, the sample size was set at 50 mg (see sample preparation in the "Experimental" section). A 2^4 complete factorial design involving 16 experiments was

employed. This design has resolution V, which provides the advantage of being able to study main effects as well as two-factor interactions leaving five degrees of freedom to estimate experimental error. The experimental design data analysis was performed with the statistical software package Statgraphics Centurion XV (Manugistics, Rockville, MD, USA). The selected design allows to interpret the results using statistical tests and graphic tools in order to determine which factors and interactions have a statistically significant effect.

Table 4. Factors and levels considered in the experimental design

Factor	Code	Low level (-)	High level (+)	Continuous
Solvent	A	Methanol	Ethyl acetate	No
Volume (mL)	B	1.0	2.0	Yes
Temperature (°C)	C	25	50	Yes
Time (min)	D	5	10	Yes

The analysis of the results obtained after running the 16 experiments produced the standardised Pareto charts showed in Fig. 1. The length of each bar in the graphs is proportional to the absolute value of its associated standardised effect. The standardised effect is obtained by dividing the estimated effect of each factor or interaction by its standard error. The effects are displayed in decreasing order of importance, which allows easy identification of the most important factors. The vertical dotted line in the graph represents the statistically significant value bound at the 95% confidence level. As can be seen in the figure, the most important factor for the extraction of BT and TBP was the type of solvent. Temperature was also significant for one compound, TBP, and was very close to the significance for the other species. Extraction time and solvent volume were not significant factors for the extraction of any of the target compounds. Interactions between factors were not significant in any case.

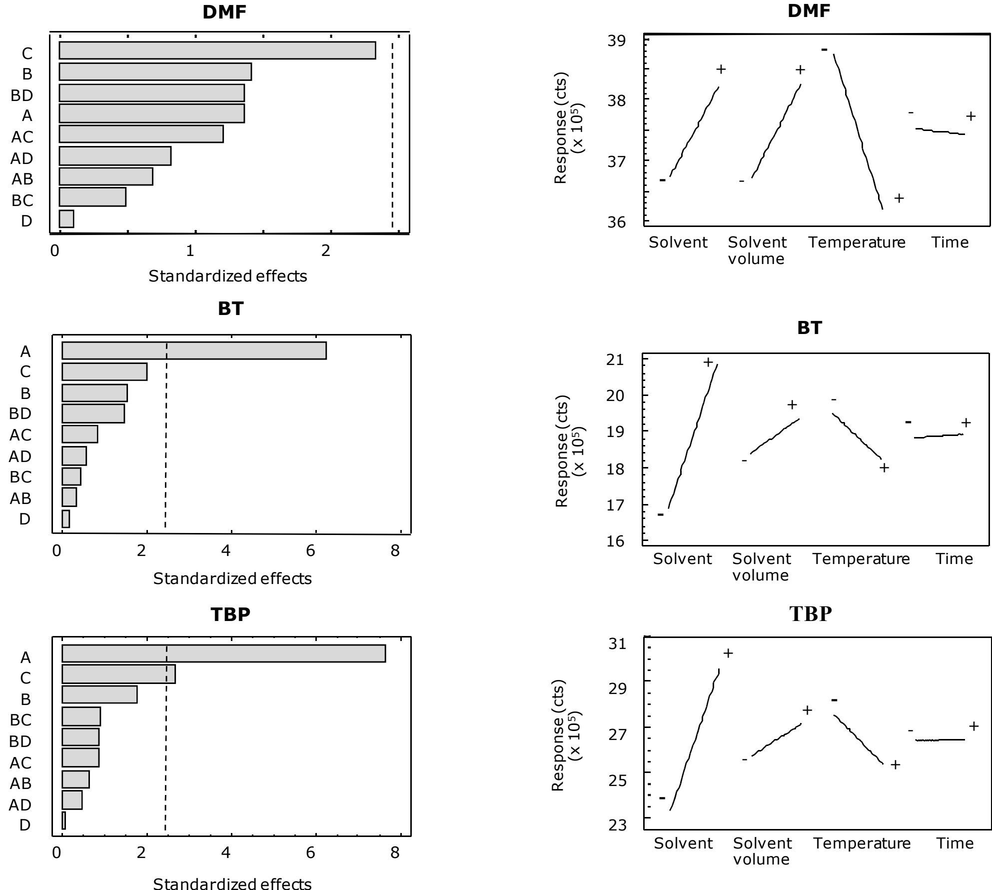


Fig. 1. Pareto charts for the target allergens (see factor keys and levels in Table 4)

Fig. 2. Main effects plots for the target allergens (see factor keys and levels in Table 4)

Figure 2 shows the main effect plots. This kind of plots shows the main effects with a line drawn between the low and the high level of the corresponding factors. The length of the lines is proportional to the effect magnitude of each factor, and the sign of the slope indicates the level of the factor that produces the highest response. As can be seen in the plots, the general tendencies were equivalent for all compounds. Regarding the significant factors, the most suitable solvent was ethyl acetate (the high level of this factor), and the most favourable extraction temperature was 25 ºC (the low level of this factor). Solvent volume was not significant and thus, we selected 1 mL since it provides more concentrated extracts

and lower solvent consumption. We decided to set the time at its lower level, 5 min, to achieve maximum sample throughput. In summary, the optimal UAE procedure involves the extraction of the samples at 25 ºC with 1 mL ethyl acetate for 5 min.

Sample throughput would also improve if simultaneous extractions could be performed. Thus, we studied this possibility by performing six (n=6) simultaneous extractions in the selected conditions. The responses obtained as well as the RSD were identical to those obtained for single extractions (see Fig. 3). So, the feasibility of simultaneous extract up to six samples in only 5 min was demonstrated.

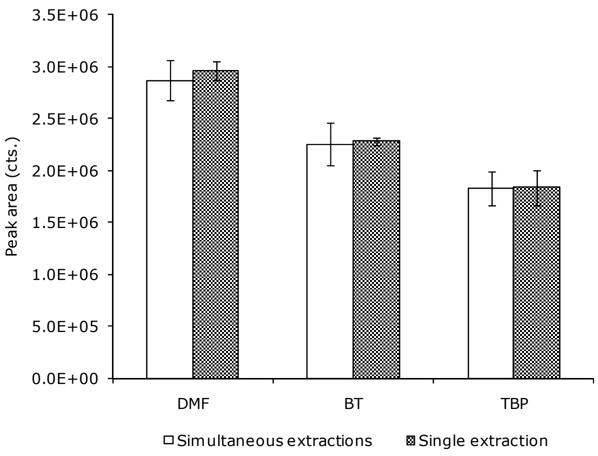
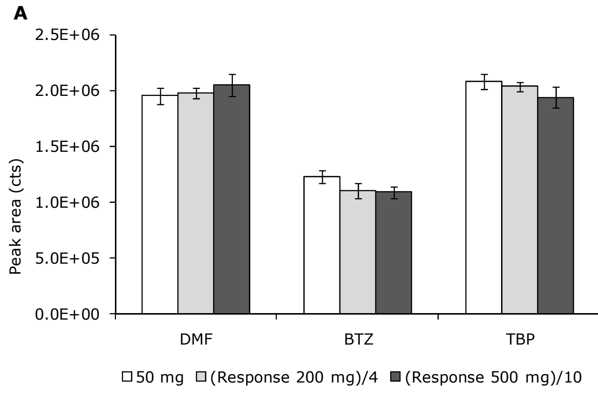


Fig. 3. Comparison of responses between single and simultaneous extractions (n=6)

Some experiments were well carried out employing higher amounts of sample and lower extraction solvent volume. In the first case, aliquots of 200 and 500 mg of sample spiked at 100 µg g^{-1} with the target compounds were extracted under the selected conditions, and the obtained responses were compared to those obtained using 50 mg sample. Results are depicted in Fig. 4a, in which, to facilitate the comparison, the responses obtained for 200 and 500 mg samples were divided by the expected increase factors (4 and 10, respectively) regarding the response for 50 mg. As can be seen, the analytes followed a similar behaviour, i.e. responses obtained using 200 and 500 mg sample were four- and tenfold higher than the ones obtained with 50 mg, respectively, demonstrating the method efficiency for extracting larger amounts of solid sample.

Additionally, the extraction with a lower volume of solvent (0.5 mL) was studied for sample amounts of 50 and 500 mg using a real sample containing the three target compounds at the low microgram per gramme level. Results showed that the obtained responses were twofold higher than those obtained using 1 mL (see Fig. 4b), thus indicating that the reduction in the extraction solvent volume could be useful if more sensitivity was eventually required.



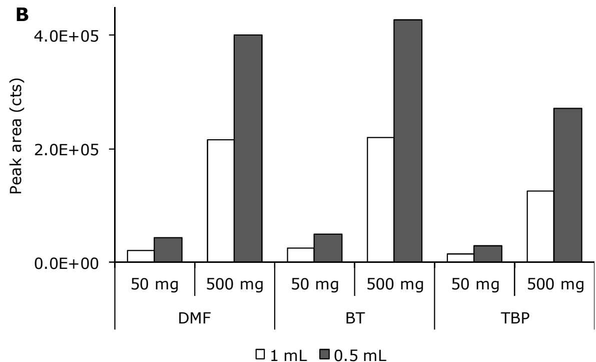


Fig. 4. A) Comparison of responses using different amounts of sample. B) Comparison of responses using different solvent volumes.

Performance study and application of the method

Precision of the UAE-GC-MS experimental procedure was assessed using real samples spiked at four concentration levels (1, 10, 100 and 500 μg g^{-1}). Results showed low intra- and inter-day variations, with relative standard deviation (RSD) values about 5% and below 10% in all cases (see Table 5), thus equivalent to the ones obtained considering only the chromatographic analysis (see Table 3).

Table 5. Performance of the proposed UAE-GC-MS method

Compound	Intra-day RSD (%, n=5)				Inter-day RSD (%, n=5)	Recovery (%)				LOD (ng g^{-1})	LOQ (ng g^{-1})
	Addition level (μg g^{-1})				Addition level (μg g^{-1})	Addition level (μg g^{-1})					
	1	10	100	500	100	1	10	100	500		
DMF	5.3	2.2	3.8	4.1	3.7(8.0 [a])	89	103	109	108	5.0	17
BT	2.1	4.6	3.8	5.2	5.1	103	95	92	89	11	36
TBP	9.9	6.7	3.1	3.7	3.8	98	94	87	99	12	39

[a] Addition level= 500 μg g^{-1}

Limits of detection (LOD, *S/N*=3) and limits of quantification (LOQ, *S/N*=10), for the full UAE-GC-MS method were estimated using a non-spiked real desiccant sample (500 mg) containing the three compounds at relatively low concentrations (3.70, 1.80, and 0.590 μg g^{-1} of DMF, BT and TBP, respectively). LOD and LOQ values are also presented in Table 5, and were found at the low nanogramme per gramme level. Therefore, the sensitivity of the proposed method can be considered high, and make it suitable to determine DMF content in real samples [4].

Recovery studies were performed by applying the optimised method to the extraction of four desiccant samples spiked with the target analytes at different concentrations (1, 10, 100 and 500 μg g^{-1}). Previous analysis of these samples showed the presence of some of the compounds, and these initial concentrations were taken into account for calculating the recoveries. Results are depicted in Table 5, showing that recoveries were satisfactory for the three compounds at all concentration levels considered in the tested samples.

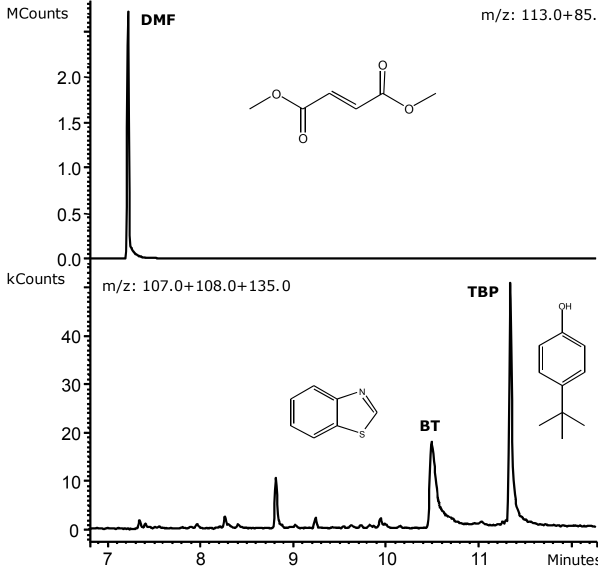


Fig. 5. UAE-GC-MS extracted ion chromatograms obtained for a real desiccant sachet sample (S7)

The method was finally applied to determine the levels of DMF, BT and TBP in sachets and tablets labelled as "mould proof agent", "biochemical dry—desiccant", "antimoulds—active mineral", "desiccant— silica gel", "silica gel" or just simply "do not eat" in the envelope. The samples presented different aspects (little balls, sand, and powder) and colours (colourless, white, grey, yellow). For the final determinations, some of the extracts were conveniently diluted. Results are summarised in Table 6. From the 37 samples tested, 27 contained DMF in concentrations ranging from 0.239 to 2640 μg g^{-1}, of which more than a half contained more than 100 μg g^{-1} of DMF, and one of the samples, a "mould proof agent" tablet, was constituted by about 100% DMF. These results demonstrate that the content of the commonly found "desiccant" sachets and tablets in consumer products does not belong with the label of the desiccant, and hence, the high risk of exposition to this powerful allergen chemical through consumer goods. Only two samples were free of any of the target compounds, whereas all samples except five, contained BT in concentrations ranging from 0.243 to 71.8 μg g^{-1}; and more than half contained TBP at concentrations of 0.0528–12.6 μg g^{-1}. Figure 5 shows the

extracted ion chromatograms of a sample containing the three target compounds (S7, see Table 6 for concentrations).

Table 6. Concentration (μg g^{-1}) of the compounds in desiccant samples

Sample	DMF	BT	TBP
S1	n.d.	12.2	4.07
S2	120	6.63	4.97
S3	71.9	4.12	0.531
S4	136	1.29	n.d.
S5	1460	16.3	n.d.
S6	2640	2.64	0.713
S7	219	6.95	6.54
S8	530	0.243	0.501
S9	209	n.d.	n.d.
S10	710	4.42	3.61
S11	115	5.38	2.61
S12	n.d.	n.d.	n.d.
S13	3.57	n.d.	n.d.
S14	n.d.	n.d.	n.d.
S15	684	2.15	0.511
S16	3.70	1.80	0.592
S17	9.39	2.25	0.341
S18	100	2.69	0.346
S19	28.5	n.d.	0.147
S20	150	4.23	1.38
S21	151	n.d.	n.d.
S22	45.4	3.54	2.40
S23	62.4	1.93	0.119
S24	586	1.42	0.177
S25	223	1.11	0.187
S26	0.947	4.51	n.d.
S27	0.527	1.43	n.d.
S28	0.323	2.04	n.d.
S29	n.d.	11.1	0.739
S30	n.d.	32.4	n.d.
S31	n.d.	43.2	0.0528
S32	0.239	24.7	12.6
S33	n.d.	31.9	1.88
S34	3.52	71.8	0.602
S35	n.d.	11.1	n.d.
S36	n.d.	5.45	n.d.
S37	1.06×10^{6}	n.d.	n.d.

n.d. not detected (<LOD)

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References

1. Rice J (1994), Food Processing, http://www.allbusiness.com/manufacturing/food-manufacturing/464544-1.html. Accessed date: 25 March 2009.
2. T. Rantanen, British J Dermatol 159 (2008) 218.
3. J.D.L. Williams, I.H. Coulson, P. Susitaival, S.M. Winhoven, British J. Dermatol. 159 (2008) 233.
4. Official Journal of the European Union, L74 (2009) 32-34.
5. D. Jirovsky, W. Wiegrebe, Monatshefte für Chemie /Chemical Monthly 135 (2004) 1563.
6. B.G. Brownlee, J.H. Carey, G.A. MacInnis, I.T. Pellizzari, Environ. Toxicol. Chem. 11 (1992) 1153.
7. A. Orlita, Int. Biodeterior. Biodegrad. 53 (2004) 157.
8. T. Reemtsma, O. Fiehn, G. Kalnowski, M. Jekel, Environ. Sci. Technol. 29 (1995) 478.
9. L. Badoil, D. Benanou, Anal. Bioanal. Chem. 393 (2009) 1043.
10. T.E. Haavisto, N.A. Adamsson, S.A. Myllymäki, J. Toppari, J. Paranko, Reprod.Toxicol. 17 (2003) 593.
11. SIDS Initial Assessment Report for 10th SIAM. P-TERT-BUTYL PHENOL, UNEP Publications (2000).

12. K. Bester, H. Hühnerfuss, W. Lange, N. Theobald, Sci. Total. Environ. 207 (1997) 111.
13. S. Lesage, Fresenius J. Anal. Chem. 339 (1991) 516.
14. H-G. Ni, F-H. Lu, X-L. Luo, H-Y. Tian, EY. Zeng, Environ. Sci. Technol. 42 (2008) 1892.
15. E. Jover, V. Matamoros, J.M. Bayona, J. Chromatogr. A. 1216 (2009) 4013.
16. T. van Leerdam, A. Hogenboom, M. van der Kooi, P. de Voogt, Int. J. Mass. Spectrom. 282 (2009) 99.
17. A. Kloepfer, M. Jekel, T. Reemtsma, J. Chromatogr. A. 1058 (2004) 81.
18. T. Reemtsma, S. Weiss, J. Mueller, M. Petrovic, S. Gonzalez, D. Barcelo, F. Ventura, T.P. Knepper, Environ. Sci. Technol. 40 (2006) 5451.
19. J. Slobodník, S. Ramalho, B.L.M. van Baar, A.J.H. Louter, U.A.T. Brinkman, Chemosphere 41 (2000) 1469.
20. E-J. Ko, K-W. Kim, S-Y. Kang, S-D. Kim, S-B. Bang, S-Y. Hamm, D-W. Kim, Talanta 73 (2007) 674.
21. L. Brossa, R.M. Marce, F. Borrull, E. Pocurull, Environ. Toxicol. Chem. 24 (2005) 261.
22. K.E. Conn, L.B. Barber, G.K. Brown, R.L. Siegrist, Environ. Sci. Technol. 40 (2006) 7358.
23. L. Brossa, E. Pocurull, F. Borrull, R.M. Marcé, Chromatographia 59 (2004) 419.
24. I. Saito, A. Onuki, H. Seto, Indoor Air 14 (2004) 325.
25. R.A. Rudel, D.E. Camann, J.D. Spengler, L.R. Korn, J.G. Brody, Environ. Sci. Technol. 37 (2003) 4543.

IV. CONCLUSIONES

Tras la exposición de los resultados obtenidos en esta Tesis, es necesario realizar una síntesis de los aspectos más significativos.

1. Aditivos potencialmente tóxicos y alergénicos en productos de consumo y cuidado personal.

En primer lugar analizaremos el trabajo que hace referencia a la **determinación de fragancias alergénicas en agua**.

Constituye el primer estudio documentado de este grupo de compuestos en medio acuoso, y los resultados obtenidos produjeron un gran impacto científico y social, debido a que se constató que la población infantil estaba en contacto directo y de forma continuada con estas sustancias alergénicas a niveles, en ocasiones, muy elevados.

Se demuestra que la SPME es una técnica muy adecuada para la determinación de fragancias alergénicas en medio acuoso.

Las condiciones optimizadas para la extracción de las fragancias alergénicas estudiadas en muestras de agua implican el uso de la fibra PDMS/DVB, a una temperatura de 100 ºC, en modo HS-SPME, con agitación magnética y en medio salino (20% de NaCl).

La oxidación de alguno de los compuestos presentes en las muestras acuosas cloradas se evitó adicionando tiosulfato sódico (0.1 mg mL^{1}).

El método propuesto presentó recuperaciones cuantitativas (>80%), junto con una precisión satisfactoria (<10%), y límites de detección (LODs) inferiores al ngmL^{-1} para la mayoría de los compuestos estudiados.

El método se aplicó al análisis de un número elevado de muestras de agua de baño de bebés, determinándose valores de concentración muy elevados en alguna de ellas, hasta 500 ng mL^{-1}.

Durante el estudio de los aditivos presentes en productos cosméticos, se desarrollaron metodologías analíticas que permiten, por un lado, **la determinación de sustancias alergénicas**, y por otro lado, la **determinación** de una gran variedad de **sustancias conservantes**, en los **cosméticos**.

Hasta la publicación de estos trabajos, los métodos disponibles para el análisis de fragancias alergénicas o conservantes en productos cosméticos se caracterizaban por ser laboriosos o poco automatizables. Con estos desarrollos se presentan las primeras aplicaciones de la extracción con disolventes presurizados, PSE o PLE, en productos cosméticos, consiguiendo la determinación de fragancias potencialmente alergénicas, y la determinación de sustancias conservantes mediante derivatización simultánea en celda.

La extracción de los compuestos mediante PSE se optimizó con diseños experimentales.

Para las fragancias alergénicas la mejor extracción se consigue a temperatura de 120ºC, con un tiempo de extracción estático de 15 minutos, una mezcla hexano/acetona (1:1) y el uso de florisil como dispersante.

En estas condiciones, los estudios de recuperación dieron como resultado valores superiores al 85% para la totalidad de los analitos estudiados.

Para los conservantes los valores óptimos para la extracción por PSE son: temperatura de 120ºC, acetato de etilo, florisil y un tiempo extracción de 15 minutos en la etapa estática.

La posibilidad de la derivatización de los conservantes con anhídrido acético en la propia celda de extracción, se presentó por primera vez en este trabajo. Esta reacción es una manera económica y rápida de derivatizar los compuestos fenólicos, y así poder determinarlos por cromatografía de gases.

La combinación de la acetilación (antes o después de la extracción) junto con las condiciones de extracción optimizadas, conducen a recuperaciones superiores al 90% prácticamente para la totalidad de los compuestos estudiados.

Los extractos obtenidos mediante ambas metodologías fueron limpios y homogéneos, y no mostraron efecto matriz.

Por otro lado, los LODs y LOQs, permiten la aplicación de estos métodos a la determinación de fragancias alergénicas y de conservantes en productos cosméticos, cumpliendo con las restricciones establecidas en la normativa vigente.

Los métodos descritos se aplicaron con éxito a un elevado número de muestras de productos cosméticos, demostrándose de esta forma su eficacia y utilidad para el análisis y control de dichas sustancias en productos cosméticos.

Dos de las muestras analizadas anunciaban en el envase de una forma muy llamativa la expresión "Fragrance Free", haciendo alusión a la ausencia de fragancias en el producto. En los análisis y contra-análisis realizados a dichas muestras, se determinaron compuestos como limoneno, alcohol bencílico o citral. Aunque, las concentraciones encontradas estaban por debajo de los límites establecidos en la legislación europea, referente a la información que debe aparecer en la etiqueta del producto, no se puede considerar correcto el uso de dicha expresión.

Centrándonos en los conservantes, una de las muestras analizadas presentó una concentración total de parabenes próxima al límite estipulado legalmente. Por otro lado, haciendo referencia a las etiquetas de los productos, tres de las muestras analizadas incumplen dicha normativa al no indicar la presencia de parabenes en el producto.

El resto de las muestras analizadas cumplen con la normativa europea de composición y etiquetado de productos cosméticos.

En el trabajo de **análisis multicomponente en muestras de perfumes** se consiguió la determinación simultánea de 52 compuestos en perfumes, entre los que se incluyen fragancias potencialmente alergénicas, ftalatos, *musks* sintéticas y conservantes. Hasta el momento de su publicación, en la literatura científica se describían metodologías de análisis sólo para grupos reducidos de ingredientes de perfumes.

El éxito de la separación cromatográfica, optimizada para dos fases de columnas cromatográficas distintas, junto con la rapidez y sencillez del método, favorecen la posible implantación del mismo en un laboratorio de análisis de rutina o control.

El tratamiento de la muestra se resume en una o varias diluciones, siempre en acetato de etilo, que dependerán de la concentración de los compuestos presentes en las muestras, pudiendo variar en varios órdenes de magnitud.

Tanto las recuperaciones como los LODs y LOQs obtenidos son satisfactorios para la totalidad de los compuestos estudiados, permitiendo cumplir con las exigencias legales actuales.

El método propuesto se aplicó a un total de 70 perfumes, detectándose un elevado número de muestras que no cumplen los límites o restricciones exigidos por la normativa europea vigente.

2. Determinación de sustancias alergénicas en aire interior

Otra parte de esta Tesis se centra en la **determinación de sustancias alergénicas en atmósferas o ambientes interiores**.

Se desarrollaron las primeras metodologías analíticas que permiten el estudio de fragancias alergénicas en ambientes interiores. La presencia de estas sustancias en estos ambientes es consecuencia, en parte, del extendido uso de productos para el mantenimiento del hogar y productos para el cuidado personal. En estos espacios potencialmente nocivos para la salud, los individuos desarrollan la mayor parte de la vida cotidiana. La peligrosidad para la salud de los ambientes interiores no sólo se debe a la presencia de sustancias alergénicas aquí estudiadas, sino que también a la presencia de contaminantes ambientales clásicos y otros emergentes (PPCPs) muy documentados en la literatura científica.

La aplicación del muestreo activo reteniendo los analitos en un cartucho de SPE, junto con la extracción asistida por ultrasonidos o con SPME, y la determinación por GC/MS, dio lugar a metodologías sencillas, robustas y de bajo coste para la determinación de sustancias alergénicas en aire interior.

La optimización del proceso de extracción asistida por US, así como de la SPME, se realizó mediante sendos diseños experimentales. Los factores objeto de estudio en cada diseño difieren dependiendo de la técnica empleada.

- Del diseño para la extracción mediante US se concluye que los mejores resultados se obtienen a temperatura ambiente, usando florisil como

adsorbente, un volumen 2 mL de acetato de etilo y un tiempo de 5 minutos de extracción.

- Para la SPME la extracción óptima se consigue a temperatura elevada (100ºC), con adición de 100µL de agua a 25 mg de florisil y empleando como fibra DVB/CAR/PDMS o PDMS/DVB.

La etapa de muestreo, que se combina con cualquiera de los dos procedimientos de extracción desarrollados, requiere solamente de 25 mg de florisil en el interior de un cartucho de SPE a través del cual se hacen pasar 0.2 m^3 de aire.

En ambos casos se obtuvieron recuperaciones superiores al 80% para la todos los analitos (excepto para el limoneno e isoeugenol).

Tanto la precisión, como los LODs y LOQs, fueron satisfactorios y adecuados para la determinación de compuestos en ambientes interiores.

Los procedimientos se aplicaron al muestreo y determinación de sustancias alergénicas en distintos ambientes interiores, encontrándose en todas las muestras analizadas varios de los analitos estudiados.

Varios de los compuestos como lilial, linalol, citronelol y ionona, están presentes en muchas de las muestras en concentraciones muy elevadas, llegando a superar valores de 100 µg m^{-3}.

3. Determinación de dimetilfumarato y otros alérgenos potenciales en productos de consumo.

Con los trabajos sobre la presencia de **sustancias peligrosas en muestras de agentes desecantes** se dio, por primera vez, respuesta a la demanda de metodología analítica para el análisis de sustancias contenidas en agentes desecantes, presentes en multitud de productos de consumo, a raíz de los problemas de toxicidad debidos al DMF.

La aplicación de energía de US para la extracción del DMF, BT y TBP, de muestras de agentes desecantes, combinada con GC/µECD y con GC/MS, dio lugar a metodologías analíticas rápidas, sencillas y de bajo.

La extracción por US del DMF, se optimizó mediante la aplicación de un diseño experimental en el que ninguno de los factores estudiados, ni sus interacciones, resultaron estadísticamente significativos. Las condiciones de extracción se propusieron en base a exigencias cromatográficas, mínimo consumo de disolvente y mayor rapidez de la extracción, es decir, 1mL acetato de etilo, a temperatura ambiente y un tiempo de extracción de 5 minutos.

Por otro lado, puesto que los compuestos BT y TBP no presentan señal analítica en el detector de captura electrónica, se optó por el uso de la espectrometría de masas (MS) para llevar a cabo la determinación de estos compuestos junto con el DMF.

La optimización de los parámetros de extracción por US del BT, TBP y DMF, se realizó mediante otro diseño experimental en el cual se observó un comportamiento homogéneo de los distintos compuestos frente a los factores estudiados, deduciéndose fácilmente las condiciones óptimas de extracción: 2 mL de acetato de etilo, a temperatura ambiente y durante 5 minutos.

Las validaciones de los métodos analíticos mostraron recuperaciones cuantitativas y una buena precisión para todos los analitos. Por otro lado, los LODs obtenidos son del orden del bajo ng g^{-1} cumpliendo ampliamente con las restricciones de la legislación vigente.

Los métodos propuestos se aplicaron a más de 60 muestras de agentes desecantes, o similares, que acompañaban a distintos productos de consumo. Casi todas las muestras analizadas contienen concentraciones muy elevadas de estos compuestos tóxicos para la salud humana.

En una de las muestras analizadas se determinó una concentración de DMF del 100%, este hecho pone de manifiesto un claro incumplimiento de la normativa europea.

Conclusiones finales:

En esta Tesis se desarrollaron por primera vez metodologías analíticas para:

- Determinación de fragancias potencialmente alergénicas en muestras de agua, productos cosméticos y en atmósferas interiores.
- Determinación de conservantes en productos cosméticos.
- Determinación de DMF, BT y p-TBP en muestras de agentes desecantes o similares presentes en distintos productos de consumo.

Todos los métodos propuestos son respetuosos con el medio ambiente y encajan perfectamente en la definición de "Química Verde".

Son métodos sencillos, robustos y constituyen una referencia para el análisis de fragancias alergénicas, conservantes, ftalatos y musks sintéticas.

La estrategia seguida para la optimización de las metodologías propuestas ha sido la aplicación de criterios de diseño experimental, minimizando de esta forma los experimentos a realizar y favoreciendo la interpretación de los resultados obtenidos.

Se han desarrollado métodos de análisis en distintas matrices relacionadas con productos de consumo de uso cotidiano: agua de baño de bebés, productos cosméticos y de cuidado personal, atmósferas interiores y agentes desecantes.

AXEXO I: ABREVIATURAS Y ACRÓNIMOS

A

Ac_2O		Anhídrido acético
ADBI	**A**cetyl-**d**imethyl-**b**utyl **i**ndan	Acetil-dimetil-butil indano
AHMI	**A**cetyl-**h**exa**m**ethyl **i**ndan	Acetil-hexametil indano
AHTN	**A**cetyl-**h**examethyl-**t**etrahydro **n**aphthalene	Acetil-hexametil-tetrahidro naftaleno
ANOVA	**An**alysis **o**f **va**riance	Análisis de varianza
AS	**A**fter-**s**un cream	
ASE	**A**ccelerated **S**olvent **E**xtraction	Extracción con disolventes acelerados
ATII	**A**cetyl-**t**etramethyl-**i**sopropyl **i**ndan	Acetil-tetrametil-isopropil indano
ATTN	**A**cetyl-ethyl-**t**etramethyl-**t**etrahydro-**n**aphthalene	Acetil-etil-tetrametil-tetrahidro naftaleno
AW	**A**nti-**w**rinkle cream	

B

BBP	**B**enzyl **b**utyl **p**hthalate	Ftalato de butil bencilo
BHA	**B**utylated **h**idroxy**a**nisole	Hidroxibutilanisol
BHT	**B**utylated **H**ydroxytoluene	Butilhidroxido tolueno
BL	**B**aby **l**otions	
BP	**B**oiling **p**oint	Punto de ebullición
BT	**B**enzo**t**hiazole	Benzotiazol
BuP	**B**utyl **p**araben	Butilparaben
BzP	**B**enzyl **p**araben	bencilparaben

C

CAS	**C**hemical **A**bstracts **S**ervice	
CCSC		**C**omité **C**ientífico de la **S**eguridad de los **C**onsumidores
CE		**C**omisión **E**uropea
CE	**C**apillary **e**lectrophoresis	
CIR	**C**osmetic **I**ngredient **R**eview	
CO	Hair Conditioning	
COLIPA	The European Cosmetics Association	
CPSC	**C**onsumer **P**roducts **S**afety **C**ommision	

CW/PEG	**C**arbo**w**ax/**P**oli**e**thylen**g**lycol	Carbowax/Polietilenglicol
D		
DBP	**Di**b**u**tyl **p**hthalate	Ftalato de dibutilo
DEHP	**D**i-2-**e**thyl**h**exyl **p**hthalate	Ftalato de di-2-etilhexilo
DEP	**Di**ethyl **p**hthalate	Ftalato de dietilo
DiBP	**Dii**so**b**utyl **p**hthalate	Ftalato de diisobutilo
DIDP	**Dii**so**d**ecyl **p**hthalate	Ftalato de diisodecilo
DINP	**Dii**so**n**onyl **p**hthalate	Ftalato de diisononilo
DIUP	**Dii**so**u**ndecyl **p**hthalate	Ftalato de isoundecilo
DMEP	**Dim**ethoxy**e**thyl **p**hthalate	Ftalato de dimetoxietilo
DMF	**Dim**ethyl **f**umarate	Dimetil fumarato
DMP	**Dim**ethyl **p**hthalate	Ftalato de dimetilo
DnOP	**D**i-n-**o**ctyl **p**hthalate	Ftalato de di-n-octilo
DPHP	**Dip**ropyl**h**eptyl **p**hthalate	Ftalato de dipropilheptilo
DPMI	Dihydro-**p**enta**m**ethyl **i**ndanone	Dihidro-pentametil indanona
DPP	**Dip**entyl **p**hthalate	Ftalato de dipentilo
DSPME	**D**irect-**S**olid-**p**hase **m**icro**e**xtraction	Microextracción en fase sólida directa
DTDP	**Ditrid**ecyl **p**hthalate	Ftalato de ditredecilo
DVB/CAR/PDMS	**Div**inyl**b**enzene/**Car**boxen/**P**oli**d**i**m**ethyl**s**iloxane	Divinilbenceno/Carboxen/polidimetilsiloxano
E		
EC	**E**uropean **C**ommunity	
ECD	**E**lectron **c**apture **d**etector	Detector de captura electrónica
EEUU		**E**stados **U**nidos
EI	**E**lectronic **i**mpact	Impacto electrónico
EPA	**E**nvironmental **P**rotection **A**gency	Agencia de protección medioambiental
EtP	**E**thyl **p**araben	Etilparaben
EU	**E**uropean **U**nion	
F		
FD&C Act	federal **F**ood,**D**rug and **C**osmetic **Act**	
FDA	**F**ood and **D**rug **A**dministration	

FIA	**F**low **i**njection **a**nalysis	
FPLA	**F**air **P**ackaging and **L**abeling **A**ct	

G

GC	**G**as **c**hromatography	Cromatografía de gases
GC/ECD	**G**as **c**hromatography/**E**lectron **c**apture **d**etector	Cromatografía de gases/Detector de captura electrónica
GC/MS	**G**as **c**hromatography/**M**ass **s**pectrometry	Cromatografía de gases/Espectrometría de masas

H

HC	**H**ands **c**ream	
HCA	**H**exyl**c**innam**a**ldehyde	Hexilcinamaldehido
HHCB	**H**exahydro-**h**examethyl-**c**yclopenta-**b**enzopyran	Hexahidro-hexametil-ciclopenta-benzopirano
HPLC	**H**igh **p**erformance **l**iquid **c**hromatography	Cromatografía líquida de alta resolución
HPV	**H**igh **p**roduction **v**olume	Compuestos de alto volumen de producción
HS	**h**ead**s**pace	Espacio de cabeza
HS-SPME	**H**ead**s**pace**-S**olid **p**hase **m**icroextraction	Microextracción en fase sólida en espacio de cabeza

I

iBuP	**i**so**B**utyl **p**araben	isobutilparaben
ID	**I**nternal **d**iameter	Diámetro interno
IDL	**I**nstrumental **d**etection **l**imit	Límite instrumental de detección
IFRA	**I**nternational **F**ragrance **A**ssociation	
IPBC	**I**odo**p**ropyl**b**utyl **c**arbamate	Butilcarbamato de iodopropinilo
iPrP	**i**so**P**ropyl **p**araben	Propilparaben
IT	**I**on **t**rap	Trampa de iones
IUPAC	**I**nternational **U**nion of **P**ure and **A**pplied **C**hemistry	Unión internacional de Química pura y aplicada

K

K_{ow}	Partition constant octanol-Water	Constante de partición octanol-agua
L		
LC	**L**iquid **c**hromatography	Cromatografía líquida
LC-MS	**L**iquid **c**hromatography-**M**ass **s**pectrometry	Cromatografía líquida-Espectrometría de masas
LLE	**L**iquid–**l**iquid **e**xtraction	Extracción líquido–líquido
LOD	**L**imit **of** **d**etection	Límite de detección
LOF	**L**ack **o**f **f**it	Falta de ajuste
LOQ	**L**imit **of** **q**uantification	Límite de cuantificación
M		
MA	**M**usk **a**mbrette	Almizcle de ambrette
MC	**M**oisturizing **c**ream	
MeOH	Methanol	Metanol
MeP	**M**ethyl **p**araben	Metilparaben
min		minutos
MK	**M**usk **ke**tone	Almizcle de cetona
ML	**M**oisturizing **L**otion	
MM	**M**usk **m**oskene	Almizcle de mosqueno
MS	**M**ass **s**pectrometry	Espectrometría de masas
MSPD	**M**atrix **s**olid-**p**hase **d**ispersion	Dispersion de matriz en fase sólida
MT	**M**usk **t**ibetene	Almizcle de tibeteno
MW	**M**olecular **w**eight	Peso molecular
MX	**M**usk **x**ylene	Almizcle de xileno
N		
NaCl	Sodiun Chloride	Cloruro sódico
O		
OSPAR	**Os**lo and **Par**is Commission	Comisión de Oslo y París
P		
PA	**P**oly**a**crylate	Poliacrilato
PAL	**P**harmaceutical **A**ffairs **L**aw	

PAS	**P**otentially **a**llergen substances	
PCB	**P**oly**c**hlorinated **b**iphenyl	Bifenilo policlorado
PCP	**P**ersonal **c**are **p**roduct	Producto de cuidado personal
PDMS	**P**oly**d**i**m**ethyl**s**iloxane	Polidimetilsiloxano
PDMS/CAR	**P**oly**d**i**m**ethyl**s**iloxane/**Car**boxen	Polidimetilsiloxano/Carboxen
PDMS/DVB	**P**oly**d**i**m**ethyl**s**iloxane/**D**i**v**inyl**b**enzene	Polidimetilsiloxano/Divinilbenceno
PFE	**P**ressurized **f**luids **e**xtraction	Extracción con fluidos presurizados
PLE	**P**ressurized **l**iquid **e**xtraction	Extracción con líquidos presurizados
PM		**P**eso **M**olecular
PPCP	**P**harmaceutical and **p**ersonal **c**are **p**roduct	Producto farmacéutico y de cuidado personal
PrP	**P**ropyl **p**araben	Propilparaben
PSE	**P**ressurized **s**olvent **e**xtraction	Extracción con disolventes presurizados
PTFE	**P**oly**t**etra**f**luoro**e**thylene	
PVC	**P**oly**v**inyl **c**hloride	Cloruro de polivinilo

R

R^2		Coeficiente de determinación
REACH	**R**egistration, **E**valuation, **A**uthorisation and **R**estriction of **Ch**emicals	Registro, evaluación, autorización y restricción de compuestos químicos
RIFM	**R**esearch **I**nstitute for **F**ragrance **M**aterials	
RSD	**R**elative **s**tandard **d**eviation	Desviación estándar relativa

S

S/N	**S**ignal-to-**n**oise ratio	Relación señal/ruido
SAs		**S**ustancias **a**lergénica**s**
SC	**S**unscreen **c**ream	
SCCNFP	**S**cientific **C**ommittee on **C**osmetic **P**roducts and **N**on-**F**ood **P**roducts	Comité Científico sobre Productos Cosméticos y No Alimentarios
SCCP	**S**cientific **C**ommittee on **C**onsumer **P**roducts	Comité Científico sobre Productos de consumo
SCCS	**S**cientific **C**ommittee on **C**onsumer **S**afety	

SH	Shampoo	
SINC		**S**ervicio de **I**nformación y **N**oticias **C**ientíficas
SPE	**S**olid-**p**hase **e**xtraction	Extracción en fase sólida
SPME	**S**olid-**p**hase **m**icro**e**xtraction	Microextracción en fase sólida

T

TBP	p-**t**ert-**B**utyl**p**henol	p-tert-butilfenol
TCS	**T**ri**c**lo**s**an	Triclosán
TDGC-MS	**T**hermal **d**eseorption-**g**as **c**hromatography- **m**ass **s**pectrometry	
Temp		Temperatura
TMBT	**M**ethyl**t**hio**b**enzo**t**hiazole	Metiltiobenzotiazol

U

UAE	**U**ltrasound-**a**ssisted **e**xtraction	
UE	European Union (EU)	**U**nión **E**uropea
US	**U**ltra**s**ounds	Ultrasonidos
US	**U**nited **S**tates of America	
USA	**U**nited **S**tate of **A**merica	Estados Unidos de America
UV	**U**ltra**v**iolet	Ultravioleta

AXEXO II: LISTADO DE PUBLICACIONES

A lo largo de la etapa investigadora, en la cual se integra esta Tesis Doctoral, se han publicado los siguientes trabajos:

- **J. Pablo Lamas, Carmen Salgado-Petinal, Carmen García-Jares,María Llompart, Rafael Cela, Mariano Gómez**, Solid-phase microextraction–gas chromatography–mass spectrometryfor the analysis of selective serotonin reuptake inhibitors in environmental wáter, Journal of Chromatography A, 1046 (2004) 241–247. doi:10.1016/j.chroma.2004.06.099
- **Carmen Salgado-Petinal, J. Pablo Lamas,Carmen Garcia-Jares, Maria Llompart, Rafael Cela**, Rapid screening of selective serotonin re-uptake inhibitors in urine samples using solid-phase microextraction gas chromatography–mass spectrometry, Analytical and Bioanalytical Chemistry 382 (2005) 1351–1359. doi:10.1007/s00216-005-3284-3
- **Maria Fernandez-Alvarez, Maria Llompart, J. Pablo Lamas, Marta Lores, Carmen Garcia-Jares, Rafael Cela, Thierry Dagnac,** Development of a solid-phase microextraction gas chromatography with microelectron-capture detection method for a multiresidue analysis of pesticides in bovine milk, Analytica Chimica Acta 617 (2008) 37–50. doi:10.1016/j.aca.2008.01.021
- **Maria Fernandez-Alvarez, Maria Llompart, J. Pablo Lamas, Marta Lores, Carmen Garcia-Jares, Rafael Cela, Thierry Dagnac,** Simultaneous determination of traces of pyrethroids, organochlorines and other main plant protection agents in agricultural soils by headspace solid-phase microextraction–gas chromatography, Journal of Chromatography A, 1188 (2008) 154–163. doi:10.1016/j.chroma.2008.02.080
- **J. Pablo Lamas, Lucia Sanchez-Prado, Carmen Garcia-Jares, Maria Llompart,** Determination of dimethyl fumarate in desiccant and mouldproof agents using ultrasound-assisted extraction gas chromatography with electron-capture detection, Journal of Chromatography A, 1216 (2009) 5755–5758. doi:10.1016/j.chroma.2009.06.028
- **J. Pablo Lamas, Lucia Sanchez-Prado, Jorge Regueiro, Maria Llompart, Carmen Garcia-Jares**, Determination of dimethyl fumarate and other potential

allergens in desiccant and antimould sachets, Analytical and Bioanalytical Chemistry (2009) 394:2231–2239. doi:10.1007/s00216-009-2923-5

- **J. Pablo Lamas, Lucia Sanchez-Prado, Carmen Garcia-Jares, Maria Llompart,** Solid-phase microextraction gas chromatography-mass spectrometry determination of fragrance allergens in baby bathwater, Analytical and Bioanalytical Chemistry (2009) 394:1399–1411. Doi:10.1007/s00216-009-2829-2
- **Jorge Regueiro, Carmen Garcia-Jares, Maria Llompart, J. Pablo Lamas, Rafael Cela,** Development of a method based on sorbent trapping followed by solid-phase microextraction for the determination of synthetic musks in indoor air, Journal of Chromatography A, 1216 (2009) 2805–2815. doi:10.1016/j.chroma.2008.09.062
- **P. Canosa, R. Montes, J.P. Lamas, M. García-López, I. Orriols, I. Rodríguez,** Pressurized liquid extraction followed by gas chromatography with atomic emission detection for the determination of fenbutatin oxide in soil samples, Talanta 79 (2009) 598–602. doi:10.1016/j.talanta.2009.04.033
- **R. Montes, P. Canosa, J. Pablo Lamas, A. Piñeiro, I. Orriols, R. Cela, I. Rodríguez**, Matrix solid-phase dispersion and solid-phase microextraction applied to study the distribution of fenbutatin oxide in grapes and white wine, Analytical and Bioanalytical Chemistry (2009) 395:2601–2610. Doi:10.1007/s00216-009-3175-0
- **Maria Fernandez-Alvarez, Maria Llompart, J. Pablo Lamas, Marta Lores, Carmen Garcia-Jares, Maria Garcia-Chao, and Thierry Dagnac,** Simultaneous Extraction and Cleanup Method Based on Pressurized Solvent Extraction for Multiresidue Analysis of Pesticides in Complex Feed Samples, Journal of Agricultural and Food Chemistry (2009) 57, 3963–3973. doi:10.1021/jf9004252
- **Maria Fernandez-Alvarez, Maria Llompart, J. Pablo Lamas, Marta Lores, Carmen Garcia-Jares, Rafael Cela, Thierry Dagnac,** Development of a matrix solid-phase dispersion method for the simultaneous determination of pyrethroid and organochlorinated pesticides in cattle feed, Journal of Chromatography A, 1216 (2009) 2832–2842. doi:10.1016/j.chroma.2008.10.026

- **Lucia Sanchez-Prado, J. Pablo Lamas, Marta Lores, Carmen Garcia-Jares, and Maria Llompart,** Simultaneous In-Cell Derivatization Pressurized Liquid Extraction for the Determination of Multiclass Preservatives in Leave-On Cosmetics, Analytical Chemistry (2010) 82, 9384–9392. Doi: 10.1021/ac101985h
- **J. Pablo Lamas, Lucia Sanchez-Prado, Carmen Garcia-Jares, Marta Lores, Maria Llompart,** Development of a solid phase dispersion-pressurized liquid extraction method for the analysis of suspected fragrance allergens in leave-on cosmetics, Journal of Chromatography A, 1217 (2010) 8087–8094. doi:10.1016/j.chroma.2010.10.120
- **J. Pablo Lamas, Lucia Sanchez-Prado, Marta Lores, Carmen Garcia-Jares, Maria Llompart**, Sorbent trapping solid-phase microextraction of fragrance allergens in indoor air, Journal of Chromatography A, 1217 (2010) 5307–5316. doi:10.1016/j.chroma.2010.06.036
- **J. Pablo Lamas, Lucia Sanchez-Prado, Carmen Garcia-Jares, Maria Llompart,** Determination of fragrance allergens in indoor air by active sampling followed by ultrasound-assisted solvent extraction and gas chromatography–mass spectrometry, Journal of Chromatography A, 1217 (2010) 1882–1890. doi:10.1016/j.chroma.2010.01.055
- **Maria Fernandez-Alvarez, J. Pablo Lamas, Lucia Sanchez-Prado, Maria Llompart,Carmen Garcia-Jares, Marta Lores,** Development of a solid-phase microextraction gas chromatography with microelectron-capture detection method for the determination of 5-bromo-5-nitro-1,3-dioxane in rinse-off cosmetics, Journal of Chromatography A, 1217 (2010) 6634–6639. doi:10.1016/j.chroma.2010.04.027
- **Elias Becerril, J. Pablo Lamas, Lucia Sanchez-Prado, Maria Llompart, Marta Lores, Carmen Garcia-Jares**, Analysis of regulated suspected allergens in waters, Talanta 83 (2010) 464–474. doi:10.1016/j.talanta.2010.09.038
- **Maria Fernandez-Alvarez, J. Pablo Lamas, Maria Garcia-Chao, Carmen Garcia-Jares, Maria Llompart, Marta Loresa and Thierry Dagnac,** Monitoring of pesticide residues in dairy cattle farms from NW Spain, Journal of Environmental Monitoring (2010) 12, 1864–1875. doi:10.1039/c0em00054j

- **Elias Becerril-Bravo, J. Pablo Lamas, Lucia Sanchez-Prado, Marta Lores, Carmen Garcia-Jares, Blanca Jimenez, Maria Llompart,** Ultrasound-assisted emulsification–microextraction of fragrance allergens in water, Chemosphere 81 (2010) 1378–1385. doi:10.1016/j.chemosphere.2010.09.028
- **Lucia Sanchez-Prado, Maria Llompart, J. Pablo Lamas, Carmen Garcia-Jares, Marta Lores,** Multicomponent analytical methodology to control phthalates, synthetic musks, fragrance allergens and preservatives in perfumes, Talanta 85 (2011) 370–379. doi:10.1016/j.talanta.2011.03.079
- **Lucia Sanchez-Prado, J. Pablo Lamas, Gerardo Alvarez-Rivera, Marta Lores, Carmen Garcia-Jares, Maria Llompart,** Determination of suspected fragrance allergens in cosmetics by matrix solid-phase dispersion gas chromatography–mass spectrometry analysis, Journal of Chromatography A, 1218 (2011) 5055– 5062. doi:10.1016/j.chroma.2011.06.013
- **Lucia Sanchez-Prado, Gerardo Alvarez-Rivera, J. Pablo Lamas, Marta Lores, Carmen Garcia-Jares, Maria Llompart,** Analysis of multi-class preservatives in leave-on and rinse-off cosmetics by matrix solid-phase dispersion, Analytical and Bioanalytical Chemistry, *in press,* 2011. doi:10.1007/s00216-011-5412-6