UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

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DESARROLLO Y APLICACIÓN DE NUEVAS HERRAMIENTAS (BIO)ANALÍTICAS PARA EL CONTROL DE LA SEGURIDAD DE LOS PRODUCTOS COSMÉTICOS Y DE CUIDADO PERSONAL

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Memoria para optar al grado de Doctor en Química

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Santiago de Compostela, abril de 2015.

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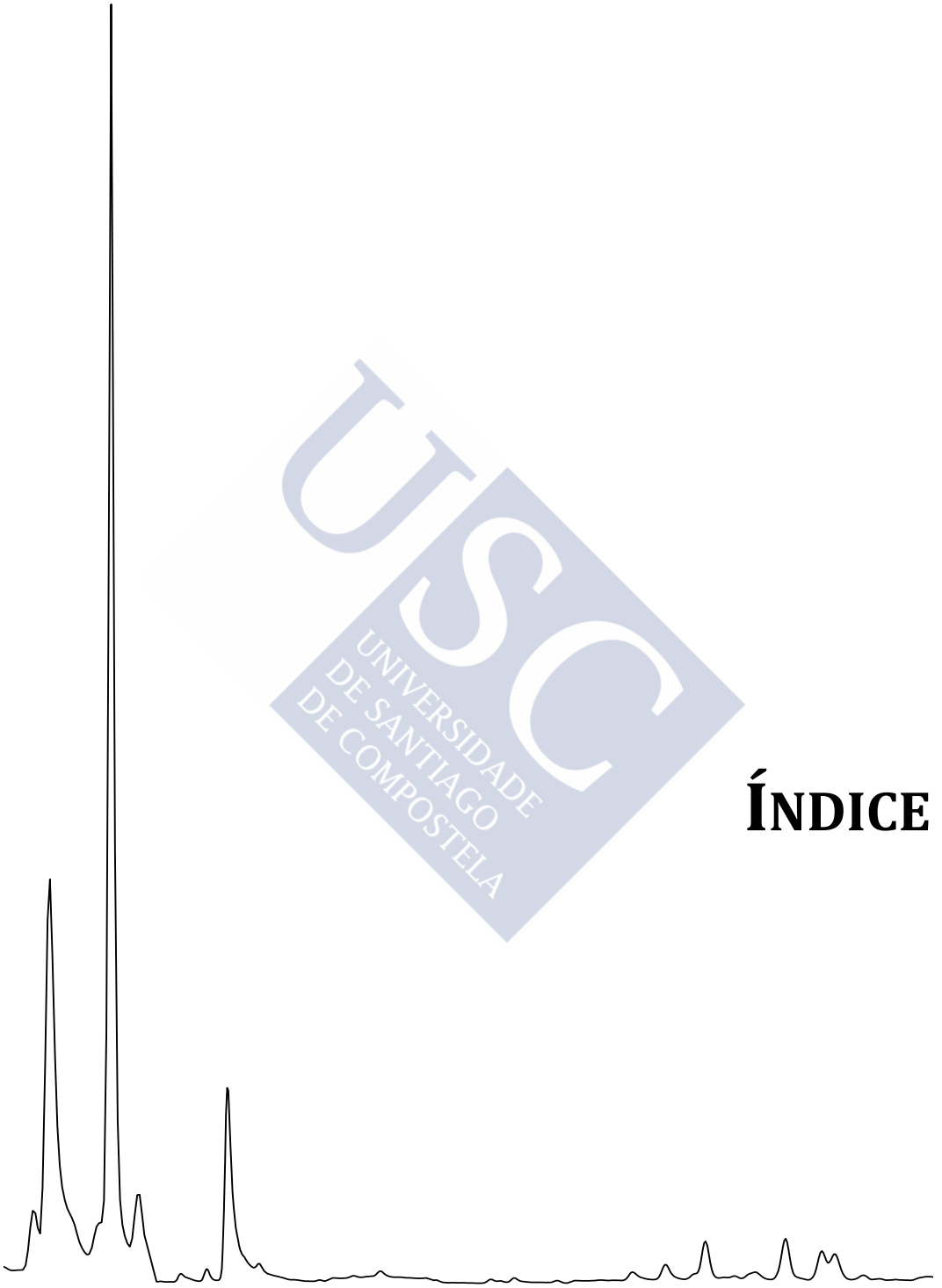

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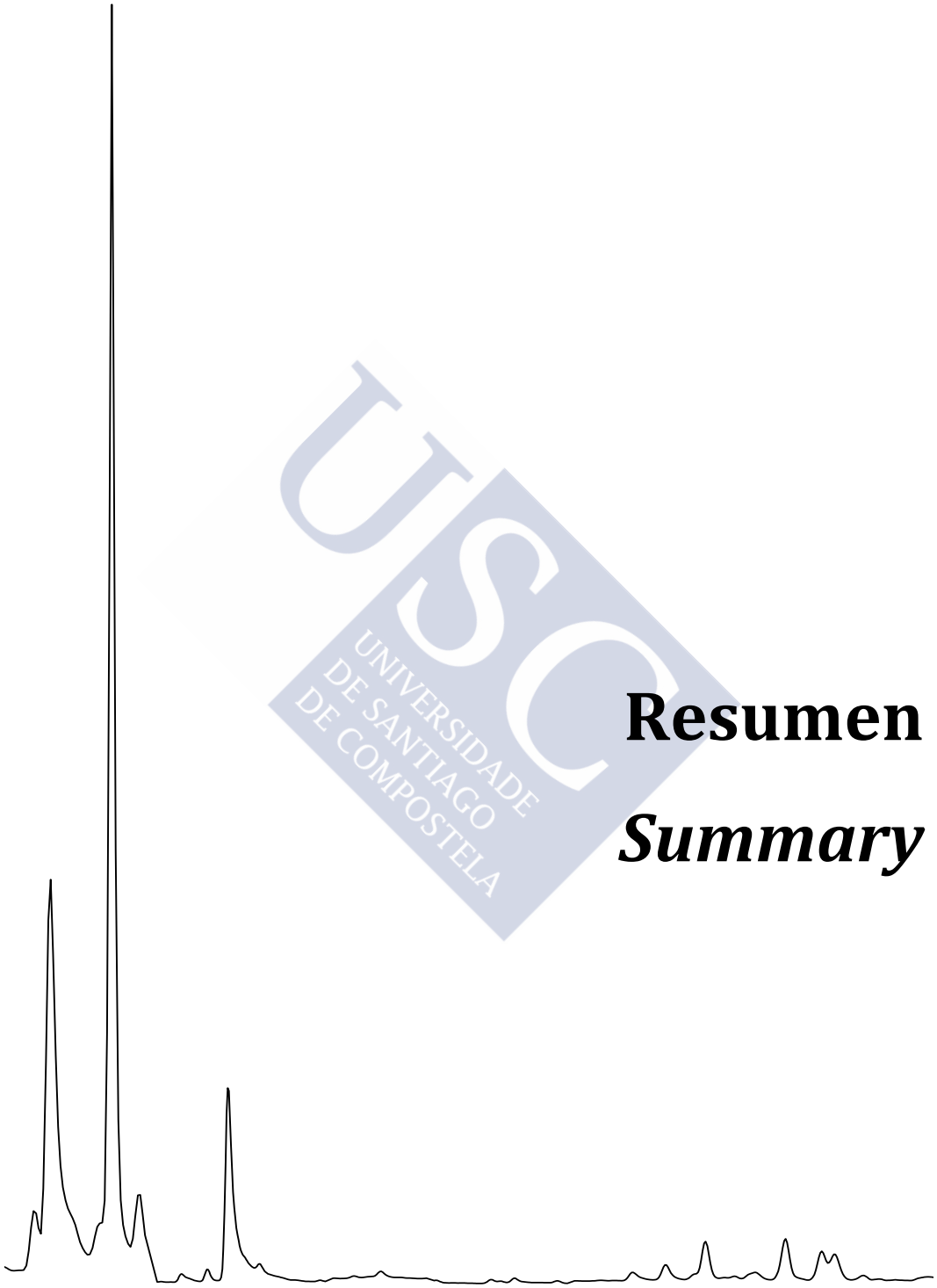

Resumen

Summary

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Resumen

En los últimos años, el uso ampliamente generalizado de los productos cosméticos y de cuidado personal ha suscitado una creciente preocupación social acerca de los efectos secundarios que ciertos ingredientes pueden causar en la salud humana. Productos de uso diario como jabones, geles de ducha, desodorantes, cremas hidratantes, protectores solares, pastas de dientes, etc., juegan un papel esencial en el mantenimiento de la higiene y del cuidado personal cotidiano. Entre los grupos de ingredientes de cosméticos más destacados se encuentran los conservantes y las fragancias alergénicas.

La adición de conservantes se considera necesaria debido a los riesgos para el consumidor asociados al desarrollo microbiano en el producto cosmético. Sin embargo, los conservantes están considerados entre los ingredientes que con más frecuencia causan alergias y dermatitis de contacto [1, 2]; y su presencia se ha relacionado además con efectos mucho más importantes, como actividad estrogénica [3], o la capacidad de inducir cáncer de mama en el ser humano [4].

Por su parte, las fragancias son constituyentes de los perfumes y se encuentran en un gran número de productos cosméticos de muy diversa índole. Su misión es provocar sensaciones agradables al consumidor, como sentirse limpio y aseado, enmascarando a su vez otros olores no deseados. Sin embargo, estas sustancias están relacionadas con ciertas alergias por contacto cuando se aplican sobre la piel [5-7]. Además, en este grupo hay sustancias de las que se sospechan efectos carcinogénicos [8], como el metileugenol, lo que hace pensar que el riesgo toxicológico de estas fragancias puede ser mayor del que se les atribuye hoy en día.

Las empresas fabricantes de cosméticos están obligadas a asegurar la inocuidad de sus productos comerciales, y de sus ingredientes, bajo condiciones razonables de uso, según la legislación vigente. Esta imposición legal acerca de la seguridad de un producto cosmético implica tres consideraciones importantes que han marcado el desarrollo de la presente tesis doctoral:

i Es necesario garantizar que la presencia de los ingredientes en la formulación cosmética cumple con las restricciones impuestas en la legislación, así como descartar la presencia de sustancias prohibidas o potencialmente tóxicas en productos cosméticos.

ii El fabricante ha de garantizar la perfecta conservación de su producto evitando la proliferación de microorganismos, tanto a lo largo del proceso de fabricación como en el producto final.

iii Es importante controlar la posible formación de subproductos que puedan originarse por la exposición de los ingredientes a condiciones ambientales oxidativas (ej. la radiación solar).

Sin embargo, el número de sustancias a controlar es enorme, su naturaleza química es muy variada y las matrices que pueden contenerlas son muy diversas, todo lo cual conlleva dificultades analíticas de alcance para los escasos y, en general, obsoletos métodos oficiales de análisis de ingredientes en cosméticos. Además, los métodos existentes al inicio de esta tesis doctoral empleaban técnicas de extracción clásicas o procedimientos multietapa, que generalmente implican extracción o dilución con disolventes, mezclado, sonicación, calentamiento, adición de ácidos o bases, centrifugación, y filtrado. Estos procedimientos suelen ser tediosos, consumen mucho tiempo, y habitualmente requieren el uso de disolventes peligrosos para la salud humana y el medioambiente. En esta línea, los métodos existentes para el control microbiológico también se basan en procedimientos clásicos como el conteo en placa. Estos métodos basados en el crecimiento, aunque no requieren una gran inversión en infraestructura y materiales, tienen limitaciones inherentes bien conocidas, tanto por la cantidad de tiempo requerido en el procedimiento, como por la laboriosidad de la recogida de datos. Por otra parte, los estudios de la estabilidad fotoquímica de conservantes así como su posible fototransformación en subproductos potencialmente tóxicos en productos cosméticos aplicados sobre la piel son prácticamente inexistentes.

La presente tesis doctoral surge con el objetivo de proporcionar herramientas analíticas que permitan solventar los inconvenientes debidos a la complejidad de la muestra, y abordar nuevas necesidades en el control de la seguridad de los productos cosméticos. Para ello se proponen nuevas metodologías más eficientes, sensibles y selectivas, basadas en técnicas avanzadas de extracción, como la dispersión de matriz en fase sólida (*Matrix Solid-Phase Dispersion MSPD*), la microextracción en fase sólida (*Solid-Phase Microextraction, SPME*), y la extracción con líquidos presurizados (*Pressurized Liquid Extraction, PLE*), que requieren bajo consumo de disolvente y minimizan la generacion de residuos. La combinación de dichas técnicas con métodos de cromatografía líquida de alta resolución (HPLC) o cromatografía de gases (GC), acoplados a detectores selectivos de espectrometría de masas (MS) o espectrometría de masas en tándem (MS/MS), ha permitido la determinación de especies de diversa naturaleza química, en una gran variedad de muestras cosméticas.

La memoria de esta tesis doctoral se compone de cuatro capítulos. En el primer capítulo (*I. Justificación y objetivos*) se contextualiza la problemática y se exponen los objetivos propuestos para la realización de este trabajo. En el segundo capítulo (*II. Introducción*) se ofrece una visión general acerca de los ingredientes objeto de estudio, así como una introducción al problema de la contaminación microbiana en productos cosméticos y a los estudios de fotodegradación. Además, se revisan las técnicas y procedimientos analíticos utilizados. En el capítulo *III (Experimental. Resultados y Discusión)* se exponen los desarrollos metodológicos, y los resultados discutidos que se han obtenido a los largo de la presente tesis doctoral. Finalmente las conclusiones generales se detallan en el capítulo *IV (Conclusiones generales).* Los trabajos publicados que constituyen esta tesis se han clasificado en los tres apartados siguientes, de acuerdo con los tres bloques temáticos abordados:

I. *Determinación de conservantes y fragancias alergénicas en productos cosméticos*

Para responder a la necesidad de actualizar los métodos existentes, los mayores esfuerzos se han centrado en el desarrollo de metodologías de extracción, capaces de aislar de forma eficaz los ingredientes objeto de estudio de una gran variedad de muestras cosméticas.

Consecuentemente, se han propuesto dos métodos basados en MSPD para la determinación multicomponente de conservantes y fragancias alergénicas por separado. En ambos casos se han obtenido condiciones óptimas de extracción similares, utilizando florisil como agente dispersante y una mezcla de hexano/acetona (1:1) como disolvente de elución. Para la determinación de conservantes se ha introducido una etapa de derivatización (acetilación) posterior a la extracción, con la finalidad de mejorar el análisis cromatográfico de los compuestos fenólicos. Los extractos obtenidos se analizaron mediante GC-MS. Las prestaciones de ambos métodos han resultado ser satisfactorias, lo que ha permitido su aplicación al análisis de distintas matrices cosméticas, como lociones corporales, cremas y antiarrugas, leches solares, maquillajes, etc.

Ambos métodos han ofrecido resultados comparables con metodologías previamente desarrolladas basadas en PLE. Dicha comparativa se ha realizado en el marco de un estudio de mercado sobre productos de cuidado e higiene para bebes y niños, en el que se han monitorizado un total de 38 ingredientes (25 fragancias alergénicas y 13 conservantes). Aunque se han cumplido las especificaciones legales en lo referente a las concentraciones máximas permitidas para conservantes y fragancias alergénicas en los productos analizados,

los resultados revelaron el alto contenido alergénico en algunos productos destinados a permanecer en contacto con la delicada piel del bebé.

Como alternativa a los métodos anteriores se ha propuesto una metodología simple y sensible para el análisis multiconservante, que combina la simplicidad de la SPME con la sensibilidad de GC-MS/MS. En el proceso de extracción mediante SPME se ha utilizado derivatización (acetilación) *in situ* y adición de modificador orgánico, con el doble objetivo de mejorar la respuesta cromatográfica de los conservantes fenólicos y compensar el efecto matriz. La metodología validada se aplicó exitosamente al análisis de formulaciones cosméticas de distinta naturaleza, demostrando ser un método fiable para el control de rutina, con posibilidad de automatización.

Debido al marcado carácter hidrofílico del grupo de conservantes de tipo isotiazolinona, se ha propuesto una alternativa analítica basada en HPLC-MS/MS. Empleado MSPD como procedimiento de extracción se han obtenido recuperaciones satisfactorias. Las condiciones óptimas de extracción se obtuvieron empleando florisil como agente dispersante y metanol como disolvente de elución. Los extractos se han podido analizar directamente debido a la compatibilidad con la fase móvil (metanol/agua), y no se ha observado efecto matriz en la fuente de ionización por electrospray (HESI). De la misma forma que en los casos anteriores, los buenos resultados de la validación han permitido la aplicación de este método al análisis de diversas muestras cosméticas y de productos del hogar.

II. *Detección de contaminación microbiana en productos cosméticos*

Con la finalidad de contribuir a la demanda de la industria cosmética de disponer de métodos rápidos para el control microbiológico, se ha explorado un enfoque novedoso para la detección de microbiota contaminante en productos cosméticos, basada en el rastreo de compuestos orgánicos volátiles de origen microbiano (MVOCs), mediante HSSPME-GC-MS. El muestreo en espacio de cabeza de muestras cosméticas intencionadamente contaminadas ha permitido la identificación de biomarcadores de actividad microbiana. Entre estos compuestos se han identificado metil cetonas y alcanoles de número impar de átomos de carbono (C_9–C_{15}) de origen microbiano. Los resultados sugieren que es claramente factible la identificación cualitativa de microorganismos viables presentes entre la microbiota contaminante en este tipo de productos, lo que supone un complemento de gran utilidad a los métodos rápidos existentes.

III. *Estudio del comportamiento fotoquímico de conservantes cosméticos*

La escasez de información sobre el comportamiento fotoquímico de conservantes, tanto en productos cosméticos como una vez liberados al medioambiente a través de las aguas residuales, ha motivado la realización de los estudios que se describen en este apartado.

Los experimentos de fotodegradación acuosa realizados con conservantes demostraron que la radiación UV promueve la formación de especies halogenados de parabenos y benzoatos en agua clorada. Se han identificado derivados bromados y bromoclorados, y se ha demostrado la transformación de benzoatos en parabenos, mediante procesos de hidroxilación fotoinducida.

El uso de fibras de SPME con distinta polaridad ha permitido la identificación de diversos fotoproductos de una gran variedad de estructuras químicas. Además, se han propuesto rutas de transformación entre subproductos estructuralmente relacionados, consistentes con su comportamiento cinético. Entre los productos de transformación de mayor interés se puede destacar la 2- y la 4-hidroxibenzofenona, y la 2,8-diclorodibenzo-p-dioxina (2,8-DCDD). Estos fotoproductos se han identificado por primera vez en matrices cosméticas.

Los últimos experimentos de fotodegradación de conservantes aplicados sobre un modelo de piel sintética han confirmado, no sólo la formación de las dos hidroxibenzofenonas y la 2,8-DCDD, si no también la generación de otros fotoproductos de tipo dioxina y derivados de BHT, de toxicidad desconocida. Estos resultados suponen una contribución importante al reciente llamamiento de instituciones públicas como la FDA (*Food and Drugs Administration*) sobre la necesidad de investigar la formación de subproductos de fotodegradación potencialmente tóxicos, como la 2,8-DCDD, sobre la piel humana.

Paralelamente al desarrollo de esta tesis, otros autores han realizado grandes esfuerzos en el desarrollo de metodologías, no sólo para la determinación de conservantes y fragancias, sino también para determinar otros ingredientes como fragancias policíclicas (musks sintéticas), ftalatos y otros plastificantes, que han suscitado cierta preocupación científica y social. En consecuencia, al final de esta memoria, en apartado *Artículo de Revisión*, se ha incluido una revisión publicada de métodos cromatográficos para el control de dichos

ingredientes. Estos métodos proporcionan herramientas poderosas para el necesario control de la seguridad de los productos cosméticos y de cuidado personal.

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Summary

In recent years, the widespread use of cosmetics and personal care products all over the world has raised a great social concern due to the unintended side effects that some ingredients may cause on consumers health. Daily used consumer products such as soaps, bath gels, deodorants, moisturizing creams, sunscreens, tooth pastes, etc., play an essential role on daily hygiene and personal care. Amongst the most relevant cosmetic ingredients are cosmetics and fragrance allergens.

The addition of preservatives is assumed to be necessary in order to prevent microbial growth. However, preservatives are highly allergenic, frequently causing contact dermatitis [1, 2]. Its presence has also been related to even more acute side effects such as estrogenic activity [3] or the capacity to induce breast cancer in humans [4].

As for allergenic fragrances, they are essential ingredients in perfumes, but they are also added to a wide variety of personal care products. The aim of fragrances is to evoke pleasant sensations to consumers, like feeling cleansed, thereby masking undesirable odors. Nevertheless, these substances are related to some contact allergies when they are applied on the skin [5-7]. In addition, it is worthy to notice that this group also includes some substances, such as methyleugenol, which are suspected to have genotoxic effects [8]. This fact led to think that the toxicological risk of fragrance allergens can be even higher than expected nowadays.

Cosmetic companies are urged to guaranty that the products placed on the market, and their ingredients, are safe under reasonable conditions of use, in accordance to the current legislation. This legal imposition about cosmetic products safety involves three considerations to be taken into account:

i It is necessary to guaranty that the presence of ingredients included in cosmetic formulations comply the restrictions imposed by the law, as well as to discard the occurrence of banned or potentially toxic substances in cosmetic products.

ii The manufacturer has to guaranty the perfect preservation of the product, avoiding microbiological growth both during the manufacturation process and in the final product.

iii Additionally, it is important to control the possible by-products formation, which may arise due to the exposition of the parent ingredients to oxidative conditions (e.g. solar radiation).

However, the huge number of substances to be controlled, their wide range of chemical structures, and the broad variety of matrices where they can be contained, pose a great challenge for the scarce and, in general, obsolete official methods for cosmetic ingredients analysis. Moreover, the existent methods before this PhD dissertation used to apply traditional extraction techniques or extraction processes, 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. In this line, the existing method intended for microbiological testing are also based on traditional plate count. These growth-based methods, although demanding no expensive infrastructure and being rather cheap in consumables, have well-known inherent limitations such as time-consuming procedures, both in operation and data collection, being laborious to perform. On the other hand, the photochemical stability studies of cosmetic products applied on the skin are almost nonexistent.

This PhD dissertation arise aiming to provide new analytical tools, in order to overcome the main drawbacks of the existent methods, and to develop novel methods to tackle the new requirements in the safety control of cosmetic products. For this purpose, new efficient, selective and environmentally friendly methodologies, based on advanced extraction techniques, such as solid-phase microextraction (SPME), matrix solid-phase dispersion (MSPD), and pressurized liquid extraction (PLE), have been proposed. These extraction procedures, in combination with high performance liquid chromatography (HPLC) or gas chromatography (GC), coupled to selective detectors based on mass spectrometry (MS), or tandem mass spectrometry (MS/MS), allowed the sensitive determination of a broad range of chemical species in a wide variety of complex cosmetic matrices.

The present PhD dissertation comprises four chapters or sections. The first chapter (*I. Justification and aims*) contextualize the motivation of this work, and presents the main aims to be tackled throughout this dissertation. In the second chapter (*II. Introduction*) a general overview about the target cosmetic ingredients is given, as well as an introduction to the microbial contamination in cosmetics, and photodegradation studies. Moreover the applied analytical techniques and procedures are also revised. In chapter *III (Experimental Results and Discussion)* the developed methodology and the results obtained throughout this PhD dissertation are exposed and further discussed. Finally, general conclusions are detailed in

chapter *IV (General Conclusions).* The published works that constitute this dissertation have been classified into three subsections according to the following three topics:

IV. *Determination of preservatives and fragrance allergens in cosmetic products.*

In order to face the need for updating the existent methods, the efforts have been mainly focused on the development of effective extraction methodologies, capable to isolate the target ingredients from a wide variety of cosmetic samples.

Consequently, two methods based on MSPD were proposed to the multianalyte determination of preservatives and fragrance allergens, separately. In both cases, similar optimal extraction conditions were obtained, using florisil as dispersing agent, and a mixture of hexane/acetone (1:1) as elution solvent. For preservatives determination, a derivatization (acetylation) step was included, after extraction, in order to improve the chromatographic performance of phenolic compounds. The obtained extracts were analyzed by GC-MS. The performances of both methods were proved to be satisfactory, which allowed their application to the analysis of different cosmetic matrices, such as body lotions, moisturizing and antiwrinkle creams, sun milks, make-ups, etc.

Comparable results were obtained between the abovementioned MSPD-based methods and a previously developed PLE-based methodology. Both approaches were applied to a market survey of hygiene and care products intended to be used by babies and children. In this study, a total of 38 target ingredients (25 fragrance allergens and 13 preservatives) were monitored. Although compliance with the current EU Cosmetic Regulation was fulfilled regarding the fragrances and preservatives limits, the results reveal the high allergenic content in some products intended to be in prolonged contact with the baby's skin.

As an alternative, a simple and sensitive methodology was developed for the multipreservative analysis, combining the simplicity of SPME with the outstanding sensitivity of GC-MS/MS. *In situ* acetylation and subsequent organic modifier addition have been successfully implemented in the SPME process as an effective extractive strategy for matrix effect compensation and chromatographic performance improvement. The validated methodology was successfully applied to the analysis of different types of cosmetic formulations, demonstrating to be a reliable multi-preservative methododology for routine control, with the possibility of automation.

Due to the strong hydrophylicity of isothiazolinone-type preservatives, an analytical alternative based on HPLC-MS/MS has been proposed. Using MSPD as extraction procedure,

satisfactory recoveries were obtained. Optimal extraction conditions were achieved using florisil as dispersing agent and methanol as elution solvent. Extracts were suitable to be readily analyzed due to their compatibility with the mobile phase (methanol/water). No matrix effect was observed in the ionization source by heated electrospray (HESI). As in the previous studies, the satisfactory validation results allowed the application of this method to the analysis of several cosmetic samples and household products.

V. *Detection of microbial contamination in cosmetic products.*

In order to contribute to the demand from the cosmetic industry for rapid methods intended for the microbiological control, a novel approach for the microbial contamination detection in cosmetic products, based on microbial volatile organic compounds (MVOCs) screening analysis by HSSPME-GC-MS, was explored. The head-space sampling above intentionally contaminated cosmetic samples has led to the identification of microbial activity biomarkers. The volatile fraction analysis revealed the presence of several odd-numbered carbon (C_9–C_{15}) methyl ketones and alkanols, amongst other compounds, which have been reported as characteristic volatiles of bacterial origin. These results suggest that it could be clearly feasible to qualitatively identify viable microorganisms in cosmetics, or even specific strains, by detecting their volatile biomarkers, which is a valuable complement for other rapid methods.

VI. *Study of the photochemical behavior of cosmetic preservatives.*

The scarcity of photochemical stability surveys about preservatives, either contained in the cosmetic product or after released into the environment from urban wastewaters, has motivated the development of the studies described in this section.

The aqueous photodegradation experiments of cosmetic preservatives demonstrated that UV-irradiation enhances the photo-induced formation of parabens and benzoate halogenated by-products in chlorine containing water. Brominated and bromochlorinated derivatives have been identified, and the transformation of benzoates into parabens through photo-induced hydroxylation processes was demonstrated.

The use of different SPME fiber coatings with diverse polarities has led to the identification of a broad variety of photoproducts with different chemical structures. Additionally, tentative transformation pathways of structurally related by-products, consistent with their photoformation–photodecay kinetics, were proposed. Amongst the

most outstanding photoproducts, it is worthy to highlight the identification of 2- and 4-hydroxybenzophenone, and 2,8-dichlorodibenzo-p-dioxin (2,8-DCDD) in cosmetic products for the first time.

The ultimate photodegradation experiments of preservatives applied onto an artificial skin model confirmed the formation of the aforementioned hydroxybenzophenones and 2,8-DCDD, as well as the generation of other dioxin-like photoproducts, and BHT derivatives with unknown toxicological properties. These results represent a rewarding contribution to the recent call of public institutions like the FDA (*Food and Drugs Administration*) for the need of new data regarding the potential for formation of triclosan photodegradation products, such as 2,8-DCDD, on human skin as a result of the consumers use.

In parallel with the development of this PhD dissertation, great efforts have been made by other authors on the development of methodologies, not only for the preservatives and fragrance allergens determinations, but also for other ingredients such as synthetic musks, phthalates, and other plasticizers, which have become subject of great social and scientific concern. Consequently, a published review of chromatographic methodologies for the control of these ingredients has been included at the end of this dissertation, in section *Artículo de revisión (Review article)*. The new methods provide powerful tools to successfully tackle the needed safety control of cosmetic and personal care products.

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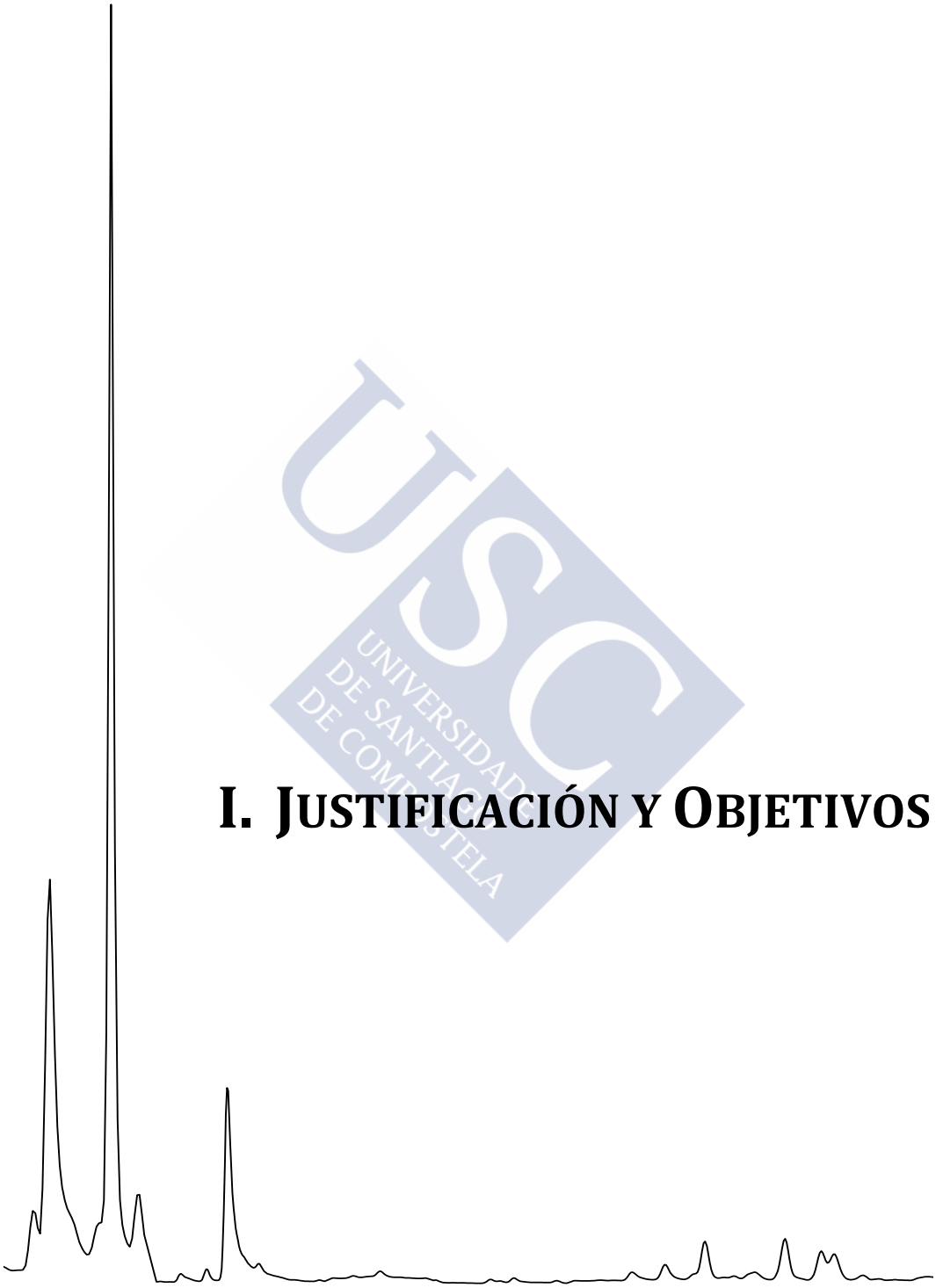
I. JUSTIFICACIÓN Y OBJETIVOS



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En la Unión Europea (UE), así como en los Estados Unidos (EEUU) y en la principales zonas del mercado global, la evaluación de la seguridad de los productos cosméticos y de sus ingredientes ha de tener en cuenta su principal premisa legal: los productos comercializados han de ser seguros; esto es, no pueden causar daño a la salud humana cuando se apliquen bajo condiciones normales o razonablemente previsibles de uso. Las regulaciones se consensuan (o se proponen) en el ámbito de la Unión, pero se ejecutan a nivel nacional, y cada país de la EU tiene un cuerpo competente propio, responsable de asegurar su cumplimiento. En última instancia, los fabricantes son los responsables de asegurar que los productos cosméticos cumplen con la normativa antes de su comercialización.

Sin embargo, la enorme cantidad de sustancias a controlar, la amplia variedad de estructuras químicas, y la complejidad de las matrices en las que pueden estar contenidas supone dificultades analíticas de alcance. Entre los grupos más destacados de ingredientes de cosméticos se encuentran las fragancias y los conservantes, objeto de estudio en esta tesis doctoral.

En el marco de la UE existen varios métodos oficiales propuestos para la determinación de algunos ingredientes de productos cosméticos, recogidos en distintas directivas de la UE. La última gran revisión general de los métodos analíticos publicados para cosméticos [1] puso de manifiesto que, aunque existen algunos métodos oficiales de análisis aprobados por las diferentes legislaciones, no son suficientes para llevar a cabo el necesario control de los mismos. Por su parte, *Cosmetics Europe – The Personal Care Association* (anteriormente COLIPA), en colaboración con otras instituciones, ha publicado una compilación de métodos [2] destinados al control de sustancias listadas en varios anexos del Reglamento Europeo de Productos Cosméticos, aunque estos métodos no son oficiales. Posteriormente, y durante el desarrollo de esta tesis doctoral, se han publicado normas estándar oficiales para el análisis de algunos ingredientes cosméticos, entre los que figura un método para el análisis de fragancias alergénicas [3], y otro para la determinación del conservante 3-iodo-2-propynyl butylcarbamate (IPBC) [4]; compuestos que han sido incluidos para su estudio en esta tesis. También se han publicado métodos estándar para el análisis de otros ingredientes, como filtros UV y ftalatos. Sin embargo, estos métodos cubren un número relativamente pequeño de ingredientes, teniendo en cuenta la gran cantidad de sustancias actualmente reguladas por el Reglamento Europeo.

En otras revisiones sobre control microbiológico en estos productos se advierte de la importancia para la industria de disponer de métodos más rápidos para la detección de microorganismos que el tradicional cultivo en placa [5, 6], de uso ampliamente generalizado en los actuales métodos estándar para el control microbiológico de productos cosméticos.

Por lo tanto, se hace necesario proporcionar a las autoridades competentes nuevas herramientas (bio)analíticas para la monitorización, tanto de los ingredientes, como del contenido microbiológico del producto cosmético. Además del control del contenido de los ingredientes regulados, la determinación de sustancias prohibidas o potencialmente tóxicas presentes en productos cosméticos; debidos tanto a su adición voluntaria como a su posible formación como subproducto, ha de ser objetivo de las nuevas metodologías desarrolladas.

En esta línea, la formación de subproductos de degradación o transformación de un determinado ingrediente es un hecho particularmente relevante, no solamente por la pérdida de actividad del propio ingrediente, sino por la posibilidad de que puedan originarse otras sustancias que entrañen un mayor riesgo para la salud. Aparte de la degradación por contaminación microbiológica, los procesos fotoinducidos son uno de los principales procesos que pueden alterar la composición de los productos cosméticos. En general, hay muy poca información sobre la estabilidad y las transformaciones fotoquímicas de los compuestos incluidos en este proyecto, y los estudios de fotodegradación en cosméticos son prácticamente inexistentes. La fotodescomposición no sólo inactiva el ingrediente en cuestión, sino que puede generar fotoproductos potencialmente dañinos, incluso de forma directa sobre la piel, especialmente en zonas expuestas a la radiación solar. Además, en el caso de los cosméticos, la interacción de los productos de fotodegradación con los excipientes o con componentes de la propia piel, como el sebo, puede conducir a la formación de nuevas moléculas de propiedades toxicológicas desconocidas; lo cual es una evidencia adicional de la necesidad de abordar este tipo de estudios.

En vista de las necesidades surgidas en torno a esta problemática, la presente tesis doctoral se ha desarrollado en base a los siguientes objetivos:

I. Desarrollo de métodos analíticos robustos, eficaces, rápidos y selectivos para la determinación multicomponente de conservantes y fragancias alergénicas en matrices cosméticas:

   - Conservantes: concretamente se han estudiado un total de 22 conservantes cosméticos, la mayor parte de ellos se encuentran listados en el anexo V del Reglamento Europeo.
   - Fragancias: se incluyen 25 fragancias alergénicas reguladas en el anexo III del Reglamento Europeo.

   Para ello se propone metodología basada en técnicas de extracción avanzada, como la microextracción en fase sólida (SPME), la dispersión de matriz en fase sólida (MSPD),

o la extracción con líquidos presurizados (PLE) más eficaces y rápidas, que reducen el uso de disolventes, amoldándose a los principios de la "química verde". Asimismo, se propone el acoplamiento de dichas técnicas extractivas a métodos cromatográficos de alta resolución con detectores selectivos como la espectrometría de masas (MS) o espectrometría de masas en tándem (MS/MS), que permiten la resolución de un gran número de especies de diversa naturaleza, evitando, a su vez, los problemas ocasionados por la matriz de la muestra.

II. Aplicación de la metodología desarrollada al análisis de productos cosméticos y de cuidado personal, tanto en productos que se aclaran como de permanencia, con la finalidad de evaluar la presencia de los ingredientes objeto de estudio en muestras reales, y el grado de cumplimiento de la legislación vigente.

III. Exploración de nuevos métodos de detección de microbiota contaminante de productos cosméticos, basados en el uso de técnicas de extracción en espacio de cabeza, para el rastreo de biomarcadores volátiles del crecimiento microbiano.

IV. Investigación de los procesos de transformación fotoquímicos de conservantes cosméticos seleccionados, e identificación de posibles subproductos de fototransformación. Dichos estudios se plantean tanto en matrices acuosas como cosméticas, así como en un modelo de piel sintética.

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II. INTRODUCCIÓN

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1. PRODUCTOS COSMÉTICOS Y DE CUIDADO PERSONAL: ASPECTOS GENERALES, SEGURIDAD Y CONTROL

1.1 CONSUMO Y MERCADO DE LOS PRODUCTOS COSMÉTICOS

Los productos cosméticos o productos de cuidado personal (PCPs) representan un grupo importante de productos de consumo que desempeña un papel esencial en la vida diaria de millones de personas en todo el mundo. Además de los cosméticos tradicionales, reconocidos en maquillajes o perfumes, los PCPs también incluyen productos fundamentales para el cuidado y mantenimiento de la higiene personal cotidiana como desodorantes, cremas, jabones, geles de ducha, o pastas dentífricas entre otros. A menudo, también se denomina PCPs a los ingredientes químicos, generalmente compuestos orgánicos de origen sintético, añadidos frecuentemente en las formulaciones de estos productos. El uso de productos cosméticos ha experimentado un crecimiento exponencial en los últimos años, no solo en adultos, sino también en bebés y en niños.

La cosmética constituye un sector industrial de carácter global y dinámico con sus principales mercados localizados en la Unión Europea (UE), Estados Unidos (EEUU), China y Japón. Estas cuatro zonas representan un valor de mercado de 69, 47, 29 y 18 mil millones de euros, respectivamente, de acuerdo con los datos compilados en el informe anual de actividad de Cosmetics Europe – The Personal Care Association (antes COLIPA) de 2013 [1]. La industria cosmética europea supone un tercio del mercado cosmético global, siendo también el mayor exportador mundial de productos cosméticos, y contribuyendo significativamente al crecimiento económico europeo. Por su parte, España está situada entre los cinco países con mayor volumen de mercado de productos cosméticos de la UE, solo superado por Italia, Reino Unido, Francia y Alemania. A pesar del último período de fuerte recesión económica, la demanda de productos cosméticos y de cuidado personal se ha mantenido constante [1]. Esto destaca la percepción pública de estos productos como componentes esenciales de uso cotidiano, y no como lujos, debido a los notables beneficios aportados por los PCPs a la calidad de vida de los consumidores; higiene corporal, cuidado dental o protección contra los efectos adversos de la radiación ultravioleta.

1.2 SEGURIDAD DE LOS PRODUCTOS COSMÉTICOS

Recientemente, el uso tan ampliamente generalizado de estos productos ha suscitado cierta preocupación social acerca de los efectos perjudiciales que algunos ingredientes comúnmente utilizados en formulaciones cosméticas pueden tener en la salud del

consumidor. Aunque el contacto humano externo con una determinada sustancia no implica necesariamente su penetración a través de la piel, el uso de PCPs puede provocar cierto grado de exposición local en zonas de la piel, la cavidad oral, la cara, los labios, los ojos o al entrar en contacto con las mucosas. Por ello, la exposición sistémica a ciertos ingredientes no puede descartarse completamente.

Durante las últimas décadas, la seguridad de los PCPs y sus ingredientes ha atraído la atención de los científicos, hasta alcanzar los estándares de seguridad de los que disfrutamos actualmente, pero no siempre ha sido así. Desde la época clásica hasta principios del siglo veinte era habitual el uso de tintes y maquillajes que contenían metales pesados altamente tóxicos como el plomo, el mercurio y los óxidos de cadmio. Se usaban preparados como sublimado de mercurio para eliminar manchas, tintes de cabello a base de sulfuro de plomo, y rojo de labios a base de sulfuro de mercurio. Más recientemente, en los años 30 se han detectado casos de intoxicación severa, y en algunos casos letal, provocada por un producto depilatorio que contenía talio [2]. Entre los años 1958-59 unos productos cosméticos comercializados en Reino Unido provocaron reacciones fotoalérgicas por su contenido en salicilanilida halogenada [3], y entre los años 50 y 60, ciertos desodorantes que contenían circonio provocaron un brote de alergias inflamatorias en la piel, detectadas en Europa y en EEUU [4, 5].

Hasta los años 60 se aceptaba de forma generalizada que los PCPs permanecían en la superficie del cuerpo humano, y por lo tanto la mayor preocupación residía en el efecto local que estos provocaban. Sin embargo, en las últimas décadas se reconoció que las sustancias de aplicación tópica pueden penetrar a través de la piel, pudiendo provocar una exposición sistémica en el ser humano. Así se establecieron los test de potencial de penetración percutánea para los ingredientes de PCPs, así como la evaluación de su toxicidad sistémica potencial. En los últimos años, y motivado por los cambios legislativos, se están desarrollando nuevos métodos para evaluar la toxicidad in vitro como substitución a los métodos basados en experimentación con animales [6].

Teniendo en cuenta la posibilidad de una exposición incontrolada del consumidor a los PCPs, la seguridad de estos productos ha de ser exhaustivamente evaluada y controlada antes de su salida al mercado [6]. Tanto en los países de la UE como en EEUU entre otros, la puesta en el mercado de un producto cosmético ha de cumplir con una premisa legal fundamental: tiene que ser seguro bajo condiciones normales de uso. Esta afirmación, aunque puede parecer bastante obvia, implica una profunda investigación previa acerca de las propiedades de todos los ingredientes incluidos en el producto cosmético final y los riesgos que estos pueden comportar para la salud del consumidor. Así, en términos de

evaluación del riesgo, el ámbito cosmético se distingue de otros sectores como el farmacéutico, el de aditivos alimentarios o el de biocidas, en algunas peculiaridades procedentes en su mayoría de la legislación cosmética europea. En este caso, el término "evaluación del riesgo" es reemplazado convenientemente por el de "evaluación de la seguridad", ya que un producto cosmético ha de ser seguro *a priori*. Además, la responsabilidad de que el producto sea seguro recae enteramente sobre el fabricante.

Sin embargo, en la actualidad no hay una armonización a nivel global sobre los tipos de test requeridos, como en el caso de la evaluación de la seguridad de los compuestos farmacéuticos. De hecho, la definición legal de cosmético o PCP no está del todo clara. En la UE, las cremas de protección solar son consideradas cosméticos, mientras que en los EEUU son clasificadas como medicamentos de venta libre (*Over–the-Counter drugs, OTC*) por la FDA; los tintes de pelo son cosméticos en la UE, pero *quasi*-medicamentos (*Quasi-Drugs, QDs*) en Japón, y por lo tanto, su seguridad estaría sujeta a dosieres de seguridad del tipo de los medicamentos [7].

Vista la complejidad de la legislación global en materia de productos cosméticos, a lo largo de la presente tesis doctoral se hará referencia a productos cosméticos o productos de cuidado personal (PCPs) indistintamente para designar el mismo tipo de productos de consumo.

1.3 LEGISLACIÓN EN MATERIA DE PRODUCTOS COSMÉTICOS

Para asegurar un alto nivel de protección, los cuatro principales mercados de productos cosméticos, Europa, EEUU, China y Japón, están regulados mediante sus respectivos modelos legislativos. Así, el Reglamento Europeo de Productos Cosméticos [8] aprobado por la Comisión Europea en 2009; la *Food, Drug and Cosmetic Act (FD&C Act)*, junto con la *Fair Packaging and Labeling Act (FPLA)* redactados por la *Food and Drug Administration (FDA)* [9] en EEUU; la *Hygienic Standard for Cosmetics* publicada en 2007 por el *Ministry of Health* en China; y finalmente la *Standards for Cosmetics* [10] adoptada en 2000 por el *Ministry of Health and Welfare* en Japón, constituyen los cuatro principales marcos legislativos en materia de productos cosméticos.

En la UE, el presente Reglamento (CE) No 1223/2009 [8] sustituye, desde el 11 de julio de 2013, a la anterior Directiva del Consejo de la UE 76/768/CEE [11] y sus sucesivas enmiendas y adaptaciones. De acuerdo con la nueva normativa se define producto cosmético como *"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". También incluye la lista de sustancias prohibidas que las formulaciones cosméticas no pueden contener, las restricciones aplicadas a otras sustancias y las llamadas listas positivas, que engloban los colorantes, conservantes y filtros UV, cuyo uso es legal en el marco de la UE; así como las reglas para el correcto etiquetado y otros asuntos relacionados con la seguridad, como la prohibición de los experimentos con animales.

Bajo dicha definición cabe incluir un amplísimo rango de productos cosméticos específicos y generales actualmente disponibles en el mercado para propósitos tales como:

i *Tratamiento facial (incluyendo labios, ojos y cabello) y cuidado del cuerpo*
Cremas, emulsiones, lociones, geles y aceites para la piel, mascarillas de belleza, maquillajes de fondo (líquidos, pastas, polvos), polvos de maquillaje, colorantes para el cabello, productos para la ondulación, alisado y fijación del cabello, productos para marcado del cabello, productos para la limpieza del cabello (lociones, polvos, champús), productos para el mantenimiento del cabello (lociones, cremas, aceites), productos para el peinado (lociones, lacas, brillantinas), productos antiarrugas, productos destinados a aplicarse en los labios, productos para el cuidado y maquillaje de las uñas.

ii *Productos de higiene personal*
Polvos para aplicar después del baño, polvos para la higiene corporal, jabones de tocador, jabones, perfumes, aguas de tocador y aguas de colonia, preparados para baño y ducha (sales, espumas, aceites, geles), depilatorios, desodorantes y antitranspirantes.

iii *Cremas solares y productos relacionados*
Productos para el sol, productos para el bronceado sin sol y productos para el blanqueo de la piel.

Los anexos a dicho Reglamento contienen las listas de sustancias cuyo uso en los productos cosméticos está permitido (anexos IV, V y VI), prohibido (anexo II) o restringido (anexo III). También se prohíben algunos colorantes (distintos a los que figuran en el anexo IV), conservantes (distintos a los que figuran en el anexo V) y filtros ultravioleta (distintos a los que figuran en el anexo VI).

Por otra parte, en el marco legislativo de los EEUU, la definición de cosmético no es tan amplia como en el marco europeo, y contiene pocas restricciones con respecto a los ingredientes que pueden usarse, mientras que los test de seguridad a realizar los determina el fabricante. En este modelo, no hay una lista positiva de ingredientes, aunque hay una lista corta de sustancias prohibidas o restringidas por la FDA para su uso en productos cosméticos. Las regulaciones en China y Japón se sitúan entre el modelo europeo y el americano. Aunque la legislación japonesa es más cercana al modelo de la UE, ya que cuenta con una lista positiva de ingredientes, las sustancias permitidas y los contenidos autorizados son bastante diferentes [10].

En los últimos año, el Reglamento Europeo se ha convertido en un modelo internacional, que resulta fácil de comprender y más o menos fácil de aplicar.

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USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

2. CONSERVANTES Y FRAGANCIAS ALERGÉNICAS EN PRODUCTOS COSMÉTICOS

2.1 CONSERVANTES COSMÉTICOS

2.1.1 Definición

Teniendo en cuenta el uso tan generalizado que hacemos de ellos, la composición y estabilidad de los productos cosméticos es de particular relevancia en nuestra vida diaria. En este sentido, los conservantes antimicrobianos juegan un papel crucial ya que previenen del crecimiento de microorganismos en el producto. Pero además, debe tenerse en cuenta que no solamente se puede estropear un producto cosmético por la acción de microorganismos, sino también debido a otros agentes externos, como el aire (si contiene ingredientes fácilmente oxidables) o la luz solar (si contiene ingredientes fotosensibles). Sin embargo, de acuerdo con la Regulación Europea [1], los conservantes se definen como: "(…) *sustancias que pueden añadirse a productos cosméticos con el objetivo principal de inhibir el desarrollo de microorganismos en tales productos*". Aunque los antioxidantes y sustancias absorbentes de luz solar no son considerados estrictamente como conservantes en el marco legislativo, sí se estudiarán como tales a lo largo de la presente tesis doctoral. A continuación se describen los compuestos más empleados de cada uno de los dos grupos de conservantes cosméticos.

2.1.2 Conservantes antimicrobianos

En este grupo se clasifican los conservantes añadidos a cosméticos con la finalidad de inhibir el crecimiento microbiano. La amplia variedad de conservantes antimicrobianos se puede clasificar de acuerdo con el grupo funcional predominante en la estructura molecular [2].

i. Ácidos orgánicos, sus ésteres y sus sales

Ejemplos de ácidos orgánicos usados como conservantes son: ácido dehidroacético, ácido sórbico, ácido salicílico, ácido propiónico y sus sales; también el ácido benzoico y sus sales, así como sus ésteres de alquilo.

Además, el ácido 4-hidroxibenzoico se usa ampliamente junto a sus ésteres (generalmente conocidos como parabenos) y sus sales. Los más comunes son el metil-, etil-, propil-, y el butil- éster. Los parabenos son los conservantes antimicrobianos más utilizados en productos cosméticos. La actividad antimicrobiana de estos compuestos incrementa con el aumento en el número de átomos de carbonos de la cadena alquílica, mientras que, por el contrario, su solubilidad en agua decrece. La actividad antimicrobiana de los ésteres

individuales es generalmente selectiva, así que su mezcla ofrece una poderosa actividad antimicrobiana contra un espectro extremadamente amplio de microorganismos. Esta es la razón por la que muchos cosméticos y productos de cuidado facial contienen parabenos como conservantes tanto en Europa como en Estados Unidos.

También se pueden considerar los derivados de carbamatos dentro de esta clase, como por ejemplo, el yodopropinil butilcarbamato (IPBC).

ii. Aldehídos y conservantes liberadores de formaldehído

El aldehído más comúnmente usado es el conocido como oximetileno o formalina. Es un conservante económico, más fácilmente soluble en agua que en aceite y grasa, usado en preparaciones acuosas como champús, acondicionadores, geles de ducha, líquidos lavamanos y baños de espumas.

A este mismo grupo también pertenecen aquellos compuestos que en disolventes polares actúan liberando formaldehído, tales como la imidazolidinil urea, diazolidinil urea, hidroximetilglicerato sódico y benzilhemiformal. Todos ellos están regulados en base a su contenido de formaldehído liberado.

También el 2-bromo-2-nitropropane-1,3- diol (Bronopol®, de aquí en adelante bronopol) y la DMDM hidantoína actúan liberando folmaldehído, y son los principales conservantes de un gran número de formulaciones comerciales y de productos de cuidado personal. A este mismo subgrupo también pertenece el Quaternium-15, así como el glutaraldehído.

iii. Aminas, amidas, piridinas y sales de benzalconio

Algunos ejemplos de de este grupo numeroso de conservantes son: Triclocarbán, hexamidina, dibromohexamidina, dibromopropamidina, clorohexidina, cloruro de benzalconio, metanamina, Quaternium-15, piritiona sódica, cloruro de cetilpiridinio, 3-cloro-N-metil acetamida.

iv. Fenoles y derivados

Los fenoles también son utilizados habitualmente como conservantes. Los más usados son: fenol, o-fenilfenol, p-cloro-m-cresol, o-cimen-5-ol, clorofeno y triclosán (TCS), entre otros.

v. Alcoholes y derivados

En esta clase se encuentran el fenoxietanol, fenoxiisopropanol, diclorobencil alcohol, bencil alcohol, clorobutanol, y 2-bromo-2-nitropropano-1,3-diol.

vi. Derivados del imidazol

Esta familia comprende compuestos tales como el climbazol, DMDM hidantoina, imidazolidinil urea y la diazolidinil urea, entre otros.

vii. Otros conservantes

Otro tipo de compuestos ampliamente distribuidos como conservantes en cosméticos, que se no encajan en las categorías mencionadas anteriormente son, por ejemplo: el bromo-5-nitro-1,3 dioxano (Bronidox®, de aquí en adelante bronidox), metildibromoglutaronitrilo, clorometilisotiazolinona (CMI) y metilisotiazolinona (MI).

Estos dos últimos ingredientes pertenecen a un grupo de conservantes muy efectivos clasificados y legislados también como biocidas, que presentan gran aplicación, no solo en productos cosméticos, sino también en productos domésticos e industriales con base acuosa [3-5].

2.1.3 Conservantes antioxidantes

Este tipo de compuestos son capaces de inhibir reacciones promovidas por el oxígeno, evitando así la oxidación y rancidez de productos usados habitualmente, como grasas, aceites, ceras, surfactantes y perfumes. Estos son habitualmente agentes reductores y captadores de radicales libres [2].

Entre los compuestos que han sido empleados como antioxidantes en cosméticos podemos citar el ácido cítrico, ácido gálico y sus ésteres, el ácido nordihidroguaiarético, ácido lipoico y sus derivados ácidos dihidrolipoicos, y el ácido glicólico entre otros muchos. Pero sin ninguna duda los más usados son el BHT (hidroxitolueno butilado) y el BHA (hidroxianisol butilado). El uso de mezclas de ambos es muy común debido al incremento sinérgico de su potencial antioxidante [2].

2.1.4 Estructura y propiedades físico-químicas

En la tabla 1 se recoge el listado de los conservantes cosméticos objeto de estudio en la presente tesis doctoral, junto a su estructura química, peso molecular, y algunas propiedades físico-químicas de interés analítico como la presión de vapor (Pv), el pK_a y el logaritmo decimal del coeficiente de partición (log K_{ow}). Todos los datos proceden de la base de datos SciFinder®.

Los parabenos son relativamente solubles en agua e hidrolíticamente estables en un amplio intervalo de pH. Presentan una acidez entre baja e intermedia y, al pH al que se encuentran la mayoría de las aguas (5.5-8), están en forma neutra. Su solubilidad en este medio aumenta al aumentar el pH (pasan a la forma aniónica) y desciende con el incremento de la longitud de la cadena hidrocarbonada del grupo éster.

El triclosán es un derivado clorado del hidroxifenil éter con una volatilidad y solubilidad bajas en agua. Este compuesto es un antimicrobiano de amplio espectro que posee gran capacidad biocida contra multitud de bacterias, hongos y levaduras [6]. Es un compuesto resistente a la hidrólisis, pero no a la degradación fotoquímica o térmica [7, 8]. Es menos soluble en agua que los parabenos y más lipofílico, por lo que presenta una mayor tendencia a adherirse a materiales sólidos. Su distribución en medios acuosos viene determinada por el pH: es ligeramente ácido y, en medios básicos, se encuentra en forma aniónica (más soluble).

Los conservantes bromados, bronopol y bronidox, son compuestos que poseen un alto poder bactericida, tanto contra bacterias Gram- como Gram+ [9]. El IPBC pertenece a la familia de los carbamatos. Es muy soluble en disolventes orgánicos, moderadamente soluble en agua y fácilmente hidrolizable en medio alcalino [10]. Se caracteriza por ser buen fungicida y antimicrobiano [11, 12].

Los conservantes fenólicos, BHA y BHT, son antioxidantes sintéticos que se usan para preservar el frescor, el aroma y el color, no sólo de productos cosméticos, sino también de productos alimentarios [13]. Se añaden principalmente en productos grasos, proporcionando estabilidad y duración contra los procesos de rancidez oxidativa de las grasas. Su uso está muy extendido debido a su estabilidad química, disponibilidad y bajo coste de producción [14].

Tabla 1. Estructura y propiedades físico-químicas de los conservantes objeto de estudio. Datos recogidos de la base de datos Scifinder®.

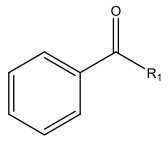
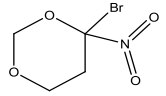
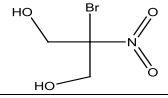
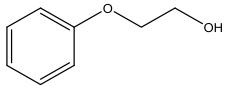
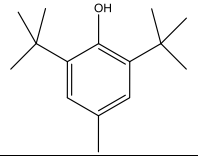
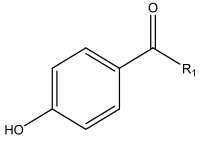
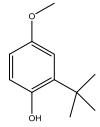
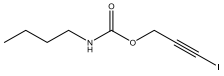
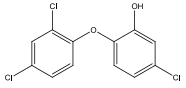
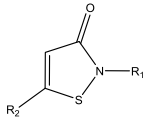
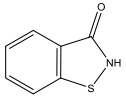
Estructura química	Abreviatura	Compuesto	R1	R2	Formula molecular	Peso molecular	pK_a	Log K_{oW}	Pv (mm Hg, 25 ºC)
	MeBz	Metil benzoato	CH_3	-	$C_8H_8O_2$	136,15	-	2,12	0,34
	EtBz	Etil benzoato	CH_2-CH_3	-	$C_9H_{10}O_2$	150,17	-	2,63	0,18
	BuBz	Butil benzoato	(-CH_2-)$_3$-CH_3	-	$C_{11}H_{14}O_2$	178,23	-	3,65	2,17E-02
	PhBz	Fenil benzoato	Ph	-	$C_{13}H_{10}O_2$	198,22	-	3,04	4,79E-04
	Brox	Bronidox	-	-	$C_4H_6BrNO_4$	212,00	-	0,75	3,71E-03
	Brol	Bronopol	-	-	$C_3H_6BrNO_4$	199,99	12,02	1,15	1,45E-06
	2-Phox	2-Fenoxietanol	-	-	$C_8H_{10}O_2$	138,17	14,36	1,25	0,0155
	BHT	Hidroxitolueno butilado	-	-	$C_{15}H_{24}O$	220,35	12,76	5,17	6,24E-03
	MeP	Metil parabén	CH_3	-	$C_8H_8O_3$	152,05	8,30	1,96	2,37 E-4
	EtP	Etil parabén	CH_2-CH_3	-	$C_9H_{10}O_3$	166,06	8,34	2,47	9,29 E-5
	iPrP	i-Propil parabén	CH-(CH_3)$_2$	-	$C_{10}H_{12}O_3$	180,08	8,40	2,91	1,16 E-3
	PrP	Propil parabén	(-CH_2-)$_2$-CH_3	-	$C_{10}H_{12}O_3$	180,08	8,23	3,04	5,55 E-4
	iBuP	i-Butil parabén	CH_2-CH-(CH_3)$_2$	-	$C_{11}H_{14}O_3$	194,09	8,17	3,4	3,81 E-4
	BuP	Butil parabén	(-CH_2-)$_3$-CH_3	-	$C_{11}H_{14}O_3$	194,09	8,47	3,57	1,86 E-4
	BzP	Bencil parabén	CH_2-Ph	-	$C_{14}H_{12}O_3$	228,08	8,18	3,56	3,37 E-6

Tabla 1 (continuación). Estructura y propiedades físico-químicas de los conservantes objeto de estudio. Datos recogidos de la base de datos Scifinder®.

Estructura química	Abreviatura	Compuesto	R1	R2	Formula molecular	Peso molecular	pK_a	Log K_{oW}	Pv (mm Hg, 25 ºC)
	BHA	Hidroxianisol butilado	-	-	$C_{11}H_{16}O_2$	180,24	11,83	3,09	4,46E-3
	IPBC	Iodopropinil butilcarbamato	-	-	$C_8H_{12}INO_2$	281,09	12,03	3,38	2,91E-4
	TCS	Triclosan	-	-	$C_{12}H_7Cl_3O_2$	287,95	7,80	4,76	6,45 E-7
	MI	Metil isotiazolinona	CH_3	-	C_4H_5NOS	115,15	-	−0,49	0,797
	CMI	Clorometil isotiazolinona	CH_3	Cl	C_4H_4ClNOS	149,60	-	0,4	0,328
	OI	Octil isotiazolinona	$(-CH_2-)_7-CH_3$	-	$C_{11}H_{19}NOS$	213,34	-	2,45	8,72E-4
	BzI	Bencil isotiazolinona	-	-	C_7H_5NOS	151,19	10,19	1,24	0,183

Las isotiazolinonas son derivados heterocíclicos de la 2H-isotiazolin-3-ona, que presenta una parte que contiene azufre, capaz de oxidar residuos tiólicos (presentes en los aminoácidos de tipo cisteína). Esta estructura ofrece una poderosa capacidad biocida contra un amplio espectro de bacterias y hongos [15]. Las isotiazolinonas como MI y CMI son los ingredientes activos de una mezcla (3:1 CMI/MI) vendida comercialmente con el nombre de Kathon®. La industria cosmética incluye esta mezcla con mucha frecuencia en una amplia variedad de formulaciones tanto de permanencia (*leave-on*) como de aclarado (*rinse-off*) debido a su alta efectividad a muy bajas concentraciones [16]. El uso de MI y CMI se ha incrementado considerablemente, ganando presencia junto con sus congéneres 1,2-benzisotiazolinone (BzI) and 2-octil-3-isotiazolinone (OI) en productos de limpieza, y otros productos industriales y domésticos, como pinturas, adhesivos, conservantes para madera, etc. [17, 18].

2.1.5 Toxicidad

A pesar de los innegables beneficios, en términos de preservación, que estos ingredientes aportan a los productos que los contienen, el uso de conservantes puede provocar efectos secundarios en la salud del consumidor. Estos efectos indeseables pueden aparecer justo después de la aplicación del producto o años más tarde, después de un uso continuado del mismo.

Aproximadamente el 6% de la población general presenta alergias relacionadas fundamentalmente con la presencia de conservantes y fragancias alergénicas [19]. Otros efectos que pueden llegar a producir estas sustancias abarcan desde irritación leve de la piel hasta actividad estrogénica, e incluso se ha planteado la posibilidad de que pudieran tener efectos neurotóxicos y genotóxicos [20, 21].

Algunos conservantes como el bronidox y el bronopol pueden descomponerse liberando agentes nitrosantes. Estos pueden reaccionar con aminas alifáticas, como la dietanolamina (DEA), trietanolamina (TEA) y monoetanolamina (MEA), añadidas comúnmente a champús y a otros productos de higiene personal, produciendo nitrosaminas carcinogénicas, que pueden ser absorbidas rápidamente por la piel [22].

El BHA y el BHT, también pueden causar serios efectos secundarios a largo plazo. Particularmente, el BHA puede actuar como modulador y disruptor del sistema endocrino [23]. Además, se le atribuye la capacidad de provocar daños en los tejidos del pulmón [24], e incluso deficiencias en el desarrollo del sistema reproductor [25]. En cuanto a BHT, aunque

el informe de evaluación de su seguridad indica que este compuesto no puede considerarse como genotóxico, tiene la capacidad de poder modificar la genotoxicidad de otros agentes [26].

Ensayos con animales han demostrado que TCS puede inducir desregulación de la función tiroidea, disminuyendo los niveles de tiroxina en el plasma. Además, también puede actuar como disruptor de hormonas sexuales presentando actividad antiestrogénica o antiandrogénica [27, 28]. Recientemente se ha visto que el TCS puede reaccionar con el cloro del agua de grifo y producir cloroformo gas [29]. En este caso merecen especial atención productos como las pastas de dientes, cuyo contenido de triclosan está permitido hasta una concentración máxima de 0.3 % (p/p). Por otro lado, el mayor potencial tóxico de este compuesto está en sus productos de descomposición o de transformación, ya que puede dar lugar a subproductos como clorofenoles, dioxinas y compuestos policlorados [7, 8, 30, 31].

El IPBC no presenta propiedades carcinogénicas conocidas para el ser humano, pero sí resulta tóxico para organismos del medio acuático [10]. Por otro lado, aunque sólo se han documentado casos de irritación ocular [32], es también tóxico por inhalación [32].

Los conservantes pertenecientes al grupo de los aldehídos liberan folmaldehído; un gas irritante, incoloro y de olor acre, que puede causar irritación en los ojos y garganta, nauseas, dificultad en la respiración, alergias y, a mayores concentraciones, puede ocasionar dolores de cabeza y ataques de asma [33, 34]. Estudios científicos recientes indican que el folmaldehído es sospechoso de producir cáncer [35], considerándose inseguros aquellos productos cosméticos con contenidos de folmaldehído superior al 0,2% [36].

Tanto MI como CMI han demostrado ser fuertes alérgenos y sensibilizadores al contacto con la piel [16, 37, 38]. Por ello, la legislación europea ha restringido su presencia en productos cosméticos a concentraciones máximas permitidas de 0,0015% (p/p) para mezclas 3:1 de CMI/MI, o 0,01% para MI [1]. Además, la *Cosmetic Toiletry and Fragrance Association (CTFA)* recomienda el uso de concentraciones no superiores a 0,00075% de la mezcla CMI/MI en productos de permanencia (*leave-on*) [39]. Por otro lado, el *Scientific Committee on Consumer Safety (SCCS)*, informó que no hay datos suficientes como para apoyar el uso seguro del la mezcla comercial Kathon en productos de permanencia [40], por lo que actualmente, sólo se permite su uso en productos de aclarado. Aunque la concentración del derivado no halogenado MI utilizado de forma individual, podría ser segura, siendo un sensibilizador más débil comparado con CMI, se han publicados nuevos casos de dermatitis por contacto que confirman su fuerte alergenicidad [37]. Investigaciones recientes también sugieren que MI puede ser un biocida neurotóxico, al observar su efecto

en ratones, después de una exposición prolongada a este ingrediente, incluso a bajas concentraciones [21]. También se han publicado varios informes sobre casos de dermatitis por contacto asociados a la exposición doméstica u ocupacional a productos que contienen BzI y OI [41, 42].

Por último, es importante destacar la existencia de evidencias científicas que indican que el uso inapropiado de productos biocidas puede contribuir al incremento de cepas bacterianas resistentes a antibióticos, tanto en los seres humanos como en el medioambiente [43].

2.1.6 Legislación

Los conservantes permitidos para su uso en productos cosméticos en el contexto de la UE se recogen en el anexo V del Reglamento Europeo [1], donde se establecen las limitaciones, requerimientos, normas de etiquetado, y las concentraciones máximas permitidas. Además, los conservantes incluidos en una formulación cosmética deberán aparecer listados en la etiqueta junto con otros ingredientes en orden descendente de concentración en el producto final.

Recientemente se han introducido nuevas enmiendas en el Reglamento Europeo que afecta al Anexo V, por las cuales se introducen cambios en las restricciones aplicables a los conservantes.

Así, según el Reglamento (UE) No 1003/2014 de la Comisión Europea [44], la mezcla de MI/CMI en la proporción de 3:1 sólo puede utilizase en productos de aclarado. Además, según el Reglamento delegado (UE) No 358/2014 de la Comisión Europea [45], el *Scientific Committee on Consumer Products (SCCP)*, consideró que el uso continuado de triclosán como conservante en la actual concentración máxima del 0,3 % en todos los productos cosméticos no era seguro para los consumidores, debido a la magnitud de la exposición agregada. No obstante, el SCCP consideró que su uso a una concentración máxima del 0,3 % es seguro en dentífricos, pastillas de jabón, jabones líquidos, geles de ducha, desodorantes, polvos faciales y cremas correctoras. Asimismo, el SCCS consideró seguro para el consumidor el uso de triclosán en productos para uñas, a una concentración máxima del 0,3 %, cuando el uso previsto sea limpiar las uñas de manos y pies antes de aplicar sistemas de uñas artificiales; y en colutorios, a concentraciones no superiores al 0,2 % [45].

Tabla 2. Nombre, número CAS y restricciones aplicadas en el anexo V del Reglamento Europeo de los conservantes cosméticos objeto de estudio.

CLAVE	Compuesto	Numero CAS	Restricciones legales		
			Tipo de producto, parte del cuerpo	Concentración máxima permitida	Otras restricciones
MeBz	Metil benzoato	93-58-3			
EtBz	Ethyl benzoato	93-89-0			
BuBz	Butil benzoato	136-60-7			
PhBz	Fenil benzoato	93-99-2			
	5-Bromo-5-nitro-1,3-dioxano	30007-47-7	A	0.1%	G
	2-Bromo-2-nitropropano-1,3-diol	52-51-7			
2Phox	2-Fenoxietanol	122-99-6			
BHA	2-tert-Butil-4-metoxifenol	121-00-6			
BHT	2,6-Bis(1,1-dimetiletil)-4-metilfenol	128-37-0			
MeP	Metil 4-hidroxibenzoato	99-76-3		0.4 % (como ácido)[a] 0.8 % (como ácido)[b]	
EtP	Etil 4-hidroxibenzoato	120-47-8			
PrP	Propil 4-hidroxibenzoato	94-13-3		0,14 % (como ácido)[c] 0,8 % (como ácido)[d]	H
BuP	Butil 4-hidroxibenzoato	94-26-8			
iPrP	Isopropil 4-hidroxibenzoato	4191-73-5		Prohibido	
iBuP	Isobutil 4-hidroxibenzoato	04247-02-3		Prohibido	
BzP	Benzil 4-hidroxibenzoato	94-18-8			
IPBC	Butil-3-yodo-2-propinil éster del ácido carbámico	55406-53-6	A	0.02 %	I, K
			B	0.01 %	I, J, K
			C	0.0075 %	I, K
TCS	2,4,4'-Tricloro-2'-hidroxidifenil éter	3380-34-5	D	0.3%	
			E	0.2%	
MI	2-Methyl-3-isothiazolinone	2682-20-4	F	0,0015 % (mezclas 3:1 de CMI:MI) 0.01% para MI solo	
CMI	5-Chloro-2-methyl-3-isothiazolinone	26172-55-4			
OI	2-Octyl-3-isothiazolinone	26530-20-1			
BzI	1,2-Benzisothiazolinone	2634-33-5			

[a] Para ésteres simples
[b] Para mezclas de ésteres
[c] Para la suma de las concentraciones individuales
[d] Para las mezclas de ésteres y sus sales, cuando la suma de las concentraciones de butylparaben y propylparaben y sus sales no exceda del 0,14 %

A: Productos que se aclaran
B: Productos que se no se aclaran
C: Desodorantes y antitranspirantes
D:Dentífricos, pastillas de jabón, jabones líquidos y geles de ducha, desodorantes (no en aerosol), polvos faciales y cremas correctoras, productos para limpiar las uñas de manos y pies antes de aplicar sistemas de uñas
E: colutorios
F:Productos que se aclaran en mezclas 3:1 de CMI:MI
G: Debería evitarse la formación de nitrosaminas.
H: No debe utilizarse en productos que no se aclaran concebidos para la zona del pañal de niños menores de tres años.
I: No utilizar en productos para niños menores de 3 años.
J: No utilizar en lociones y cremas corporales
K: No utilizar en productos bucales ni labiales

Por otra parte, el isopropilparabeno, el isobutilparabeno, el fenilparabeno, el bencilparabeno y el pentilparabeno quedaron prohibidos para su uso en productos cosméticos mediante el Reglamento (UE) No 1004/2014 Comisión [46], mientras que el resto de parabenos queda sujeto a nuevas restricciones.

En la tabla 2 se recoge el listado de conservantes objeto de estudio en esta tesis doctoral, identificados por su nombre y su número CAS, junto a su concentración máxima permitida y las restricciones actualizadas según la normativa europea.

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2.2 FRAGANCIAS ALERGÉNICAS

2.2.1 Introducción

Los perfumes están íntimamente relacionados con los productos cosméticos. Estos ingredientes tienen la capacidad de generar sensaciones agradables al consumidor, como sentirse limpio y aseado, o simplemente hacer que el usuario del cosmético se sienta cómodo y atractivo. Por otra parte, los perfumes también se añaden a los cosméticos con la intención de enmascarar olores indeseados provocados por otros ingredientes presentes en la formulación.

En cualquier caso, la mayoría de los consumidores disfruta el agradable efecto psicoactivo de las fragancias y, aparentemente, no sufre ningún tipo de efecto secundario. Sin embargo, a medida que el número y la cantidad de estos ingredientes se incrementa, los casos de efectos negativos en el usuario de productos perfumados también han ido aumentando [47].

Por fragancia se entiende una mezcla de compuestos químicos, que permiten diferenciar e identificar los distintos productos por el aroma que desprenden. Sin embargo, es habitual hablar de fragancia refiriéndose a cada uno de los componentes químicos que componen dicho aroma. Aunque existen distintos tipos de fragancias empleadas en productos cosméticos, en esta tesis doctoral se han incluido para su estudio las denominadas fragancias alergénicas (*Suspected Allergens*, SAs), reguladas como tales en la actual legislación en materia de productos cosméticos. Estos ingredientes son susceptibles de provocar irritaciones agudas en la piel, de acuerdo con el *SCCS* [48].

2.2.2 Estructura y propiedades físico-químicas

Las fragancias son ingredientes orgánicos de bajo peso molecular (<300 uma) con una presión de vapor por debajo de 2 mmHg [49]. El grupo de fragancias catalogadas inicialmente como potencialmente alergénicas (SAs) está formado por un total de 26 sustancias [47, 50], de las cuales 24 son compuestos químicos volátiles y de naturaleza muy diversa; terpenos, terpenoides, esteres, alcoholes, aldehídos, etc. Las dos restantes se corresponden con extractos naturales de líquenes, cuyos principales constituyentes son los compuestos alergénicos cloroatranol (en *Evernia prunastri* o musgo del roble, oakmoss) y atranol (en *Evernia furfuracea*, treemoss). Sin embargo, estos dos últimos extractos presentan una composición muy compleja, hecho que provoca que sean excluidas de la mayoría de los estudios [51].

En la tabla 3 se recoge el listado de las fragancias objeto de estudio en la presente tesis doctoral, junto a su estructura química, peso molecular nominal, y algunas propiedades físico-químicas de interés analítico. Se incluyen las 24 fragancias alergénicas susceptibles de ser analizadas por cromatografía de gases (GC), catalogadas inicialmente como SAs, y el metileugenol; un caso peculiar sujeto a cambios recientes en la legislación, tal y como se comenta en el siguiente apartado.

2.2.3 Toxicidad

Algunas de las fragancias más ampliamente usadas han resultado ser causantes de varios efectos secundarios como sensibilidad en la piel, dermatitis, sarpullido, tos, ataques de asma, migraña, etc. [52-55]. Se han documentado multitud de reacciones alérgicas derivadas del uso de distintos productos que contienen fragancias, la mayoría de ellas, reacciones dermatológicas (eczemas), debido a la aplicación directa sobre la piel de perfumes o cremas. [53, 56]. En Europa, alrededor de un 10% de la población con eczemas, presenta alergia o sensibilidad al contacto con este tipo de sustancias [56].

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 [57]. Otras afecciones derivadas del uso de fragancias se traducen en dolores de cabeza [58] o alteraciones de la mucosa nasal [59]. Como consecuencia, la población con patologías respiratorias, cuyo origen no sea el contacto con fragancias alergénicas, experimentará un mayor efecto irritante en presencia de estas sustancias [60].

Entre las fragancias que pueden comportan mayor riesgo para la salud cabe destacar el metileugenol. Esta sustancia había sido incluida por la UE en la lista de sustancias prohibidas en cosméticos en el año 2002 [61], dados los indicios de efectos genotóxicos y carcinogénicos [62]. Posteriormente, en la última revisión de la normativa europea de cosméticos [1], actualmente vigente, el metileugenol pasó a formar parte de la lista de ingredientes que se pueden incluir en dichos productos, aunque con limitaciones. En la actualidad siguen constatándose las propiedades genotóxicas de esta fragancia [63], lo que deja la puerta abierta a futuros cambios en la legislación en lo referente a su toxicidad.

Tabla 3. Identidad y propiedades físico-químicas de las fragancias alergénicas objeto de estudio. Datos recogidos de la base de datos Scifinder.

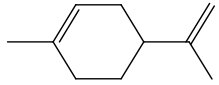
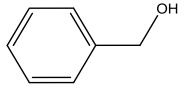
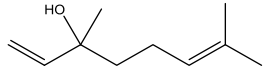
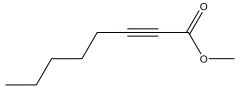
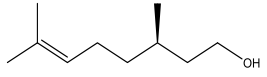
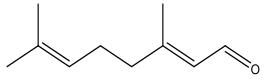
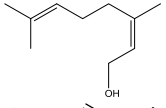
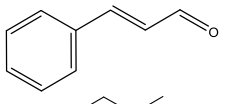
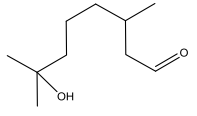
Compuesto	Nombre IUPAC	Número CAS	Estructura química	Fórmula molecular	Peso molecular	Tª de ebullición (°C)	Log K_{ow}
Limoneno	1-Metil-4-(1-metiletenil)-ciclohexeno	5989-27-5		$C_{10}H_{16}$	136	176	4,57
Alcohol bencílico	Fenilmetanol	100-51-6		C_7H_8O	108	205	1,05
Linalol	3,7-Dimetilocta-1,6-dien-3-ol	78-70-6		$C_{10}H_{18}O$	154	198	3,28
2-Octinoato de metilo	Metil-2-Octinoato	111-12-6		$C_9H_{14}O_2$	154	219	2,60
Citronelol	3,7-Dimetiloct-6-en-1-ol	106-22-9		$C_{10}H_{20}O$	156	225	3,38
Citral	3,7-Dimetilocta-2,6-dienal	5392-40-5		$C_{10}H_{16}O$	152	229	3,17
Geraniol	3,7-Dimetil-2,6-octadien-1-ol	106-24-1		$C_{10}H_{18}O$	154	229	3,28
Cinamaldehido	3-Phenyl-2-propenal	104-55-2		C_9H_8O	132	252	2,22
Hidroxicitronellal	7-Hydroxy-3,7-dimetiloctanal	107-75-5		$C_{10}H_{20}O_2$	172	240	1,54

Tabla 3 (continuación). Identidad y propiedades físico-químicas de las fragancias alergénicas objeto de estudio. Datos recogidos de la base de datos Scifinder.

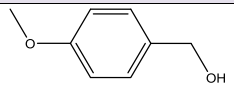
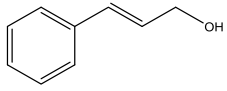
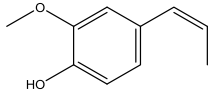
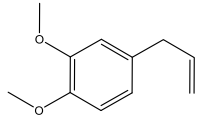
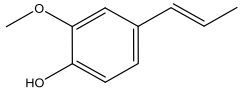
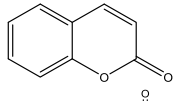
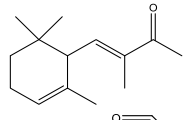
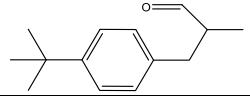
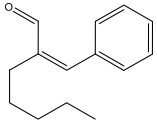
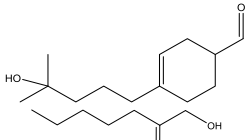
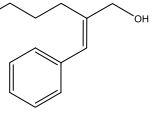
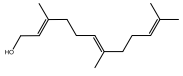
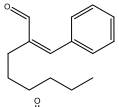
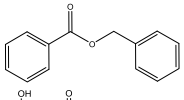
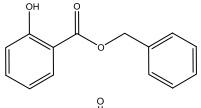
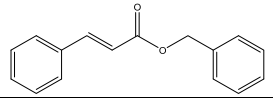
Compuesto	Nombre IUPAC	Número CAS	Estructura química	Fórmula molecular	Peso molecular	Tª de ebullición (°C)	Log K_{ow}
Anis alcohol	(4-Metoxifenil)metanol	105-13-5		$C_8H_{10}O_2$	138	259	1,10
Alcohol cinámico	3-Fenil-2-propen-1-ol,	104-54-1		$C_9H_{10}O$	134	250	1,93
Eugenol	2-Metoxi-4-prop-2-enil-fenol	97-53-0		$C_{10}H_{12}O_2$	164	256	2,20
Metileugenol	1,2-Dimethoxy-4-(2-propenyl)-benceno	93-15-2		$C_{11}H_{14}O_2$	178	248	2,9
Isoeugenol	2-metoxi-4-(1-propenil)-fenol	97-54-1		$C_{10}H_{12}O_2$	164	267	2,45
Cumarina	2H-1-Benzopiran-2-one	91-64-5		$C_9H_6O_2$	146	301	1,39
Alfa isometil ionona	4-(2,6,6-Trimetyl 2-ciclohexen-1-il)-3-metil-3-buten-2-one	127-51-5		$C_{14}H_{22}O$	206	266	4,41
Lilial®	3-(4-tert-Butilfenil)-2-metilpropanal	80-54-6		$C_{14}H_{20}O$	204	279	4,07

Tabla 3 (continuación). Identidad y propiedades físico-químicas de las fragancias alergénicas objeto de estudio. Datos recogidos de la base de datos Scifinder.

Compuesto	Nombre IUPAC	Número CAS	Estructura química	Fórmula molecular	Peso molecular	Tª de ebullición (°C)	Log K_{ow}
Amil cinamal	2-(Fenilmetilen)-1-Heptanal	122-40-7		$C_{14}H_{18}O$	202	248	4,80
Lyral®	4-(4-Hidroxi-4-metilpentil)-ciclohex-3-en-1-carbaldehido	31906-04-4		$C_{13}H_{22}O_2$	210	319	2,53
Amil cinamil alcohol	2-(Fenilmetilen)-1-Heptanol	101-85-9		$C_{14}H_{20}O$	204	>200	4,37
Farnesol	3,7,11-Trimetildodeca-2,6,10-trien-1-ol	4602-84-0		$C_{15}H_{26}O$	222	111	5,31
Hexilcinamaldehido	2-(Fenilmetilen)-octanal	101-86-0		$C_{15}H_{20}O$	216	174	4,82
Benzoato de bencilo	Benzoato de fenilmetilo	120-51-4		$C_{14}H_{12}O_2$	212	324	3,97
Salicilato de bencilo	2-Hidroxibenzoato de fenilmetilo	118-58-1		$C_{14}H_{12}O$	228	208	4,31
Cinamato de bencilo	3-Fenil-2-propenoato de fenilmetilo	103-41-3		$C_{16}H_{14}O_2$	238	198	3,65

La cumarina es otro ejemplo de fragancia con efectos carcinogénicos demostrados en experimentos con animales [64]. Este compuesto también forma parte de la lista de sustancias permitidas, aunque sujeta a restricciones en productos cosméticos. El citral junto con el geraniol, si bien no presentan efectos carcinogénicos, sí presentan actividad estrogénica, pudiendo provocar el agrandamiento de la próstata en los varones [65].

2.2.4 Legislación

Las legislaciones vigentes en los principales mercados de productos cosméticos a nivel global establecen que todos los ingredientes presentes en un cosmético han de ser declarados en la etiqueta del producto. Ésta podría ser una tarea extremadamente complicada, teniendo en cuenta que un perfume puede estar compuesto por cientos de productos químicos, que deberían ser mencionados en la etiqueta junto con el resto de ingredientes cosméticos. Así, de acuerdo con la anterior Directiva y el actual Reglamento Europeo [1], en el caso de perfumes y composiciones aromáticas, así como sus materias primas, todos ellos pueden ser etiquetados bajo los términos "perfume" o "aroma".

Por esta razón, el *Scientific Committee on Cosmetic and Non-Food-Products intended for Consumers (*SCCNFP) en su opinión SCCNFP/0017/98 de 1999 [50], identificó 26 fragancias alergénicas de las que debería informarse al consumidor acerca de su presencia en productos cosméticos. Esto se implementó en la anterior Directiva europea de productos cosméticos como "la regla de los 26 alérgenos". Estos 26 alérgenos se incluyen en el anexo III del actual Reglamento Europeo sujetos a las siguientes restricciones:

i. Sustancias que pueden ser incluidas hasta una concentración máxima permitida: 0,002 % (p/p) en cremas con fragancia, 0,001% en productos de aclarado, y 0,0002% en productos de permanencia. En el caso excepcional del bencil alcohol, la concentración máxima permitida es del 1%.

ii. Sustancias para las cuales su presencia tiene que declararse en la lista de ingredientes cuando su concentración exceda de 0,001 % (w/w) en productos de permanencia y 0,01% en productos de aclarado.

Desde que se identificaron las primeros 26 alérgenos, se han detectado otras fragancias que desencadenan reacciones alérgicas. Basándose en los informes disponibles y en varios casos de alergias presentados por dermatólogos, el SCCS identificó 82 sustancias (54 productos químicos y 28 extractos naturales) que pueden clasificarse como “alérgenos de contacto comprobados” en seres humanos, incluidos los 26 que ya estaban en la lista [48].

A partir de los resultados combinados de experimentos con animales y el análisis de su estructura química, otras 26 sustancias químicas individuales se clasificaron como "alérgenos de contacto probables". Además, 35 sustancias químicas individuales y 13 extractos naturales se clasificaron como "posibles alérgenos de contacto". Otras tres sustancias específicas fueron reconocidas como "alérgenos potentes" y no se consideran seguras en productos de consumo. El SCCS recomienda que estos ingredientes, así como los que podrían "activarse" y convertirse en sustancias alergénicas, deben añadirse a la lista de ingredientes alergénicos de cuya presencia en productos cosméticos se debe informar a los consumidores [66].

El actual Reglamento Europeo, en vigor desde 2013, incluye la transferencia de metileugenol desde el Anexo II (lista de sustancias prohibidas en productos cosméticos) al Anexo III (lista de sustancias que los productos cosméticos no pueden contener excepto las sujetas a las restricciones establecidas). Así las concentraciones máximas permitida para estas sustancias en distintos productos son las siguientes: 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%.

Recientemente, el SCCS ha propuesto transferir la fragancia lyral® desde su actual ubicación en el anexo III (lista de sustancias permitidas en cosméticos con restricciones) al anexo II (lista de sustancias prohibidas en productos cosméticos)[48]. Además, el mismo comité concluye que los alérgenos naturales cloroatranol y atranol no deben estar presentes en productos de consumo.

Los posibles efectos negativos en la salud de estas sustancias pueden conllevar, en un futuro cercano, a un descenso de las concentraciones máximas permitidas. Actualmente, ya se puede observar la inclusión del término "libre de fragancias" como una característica positiva en distinto productos cosméticos disponibles en el mercado.

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USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

3. CONTAMINACIÓN MICROBIANA Y CONSERVACIÓN DE LOS PRODUCTOS COSMÉTICOS

La mayoría de las formulaciones cosméticas, debido a que contienen un elevado porcentaje de agua y a que muchas de las sustancias utilizadas en su fabricación pueden ser degradadas biológicamente por microorganismos, son productos que se deterioran con el paso del tiempo. La presencia de microorganismos en los productos cosméticos puede producir cambios en el aspecto físico, color, olor y textura. En estas ocasiones, cuando el consumidor detecta signos visibles de alteración, reacciona rechazando el producto. Sin embargo, cuando la contaminación microbiológica no modifica el aspecto del cosmético, representa un importante riesgo para la salud del consumidor, ya que en estas condiciones los productos contaminados pueden causar irritaciones o infecciones, particularmente si el producto se aplica sobre piel dañada, en los ojos o en niños [1].

3.1 LA CONTAMINACIÓN MICROBIOLÓGICA EN LOS COSMÉTICOS

La contaminación microbiana de un producto cosmético puede tener distintos orígenes: materias primas, medio ambiente, equipo o personal de fabricación y envasado, y la utilización por el consumidor.

Para evitar la contaminación microbiológica de los cosméticos, el fabricante controla cada una de las posibles vías de entrada mediante el análisis microbiológico de materias primas, ambiente y equipos, a la vez que se sigue un plan de buenas prácticas de fabricación. Sin embargo, aún en estas condiciones "ideales" de fabricación y manipulación, el producto obtenido no es estéril, por lo que siempre existe la posibilidad de que algún microorganismo se incorpore al producto. Cuando esto ocurra, los microorganismos proliferarán si se dan las condiciones adecuadas, ya que los cosméticos proporcionan condiciones ideales de contenido en agua, oxígeno y nutrientes, necesarios para su adecuado desarrollo. Por otra parte, una vez el producto llega al consumidor, el fabricante desconoce si éste realizará un uso adecuado o no del cosmético (envase destapado, adición de agua, uso en deficientes condiciones higiénicas) y en qué condiciones será almacenado (elevadas temperaturas y humedad). Por todos estos motivos, se puede afirmar con toda seguridad que, en estas condiciones, el cosmético sufrirá una degradación química y microbiológica, viéndose comprometida su calidad y la seguridad de su uso [1].

Tabla 4. Tipos de microorganismos aislados de productos cosméticos contaminados.

Gram-Negativas		Gram-Positivas	Levaduras	Mohos
No fermentativas	Fermentativas			
Acinetobacter	*Citrobacter freundii*	*Bacillus*	*Candida*	*Absidia*
Alcaligenes	*Enterobacter cloacae*	*Staphylococcus aureus*	*Saccharomyces*	*Alternaria*
Burkholderia cepacia	*E. agglomerans*	*S. epidermis*	*Torula*	*Aspergillus*
Pseudomona putida	*E. aerogenes*	*Enterococcus*	*Zygosaccharomyces*	*Citromyces*
P. fluorescens	*E. gergoviae*	*Micrococcus*		*Cladosporium*
P. aeruginosa	*Klebsiella oxytoca*	*Streptococococcus*		*Dematium*
P. paucimobilis	*K. pneumoniae*	*Propionibacterium*		*Fusarium*
P. maltophilia	*Proteus*			*Helminthosporium*
	Serratia liquefaciens			*Hormodendrum*
	S. marescens			*Mucor*
	S. odorifera			*Geotrichum*
	S. rubidaea			*Paecilomyces*
				Penicillium
				Phoma
				Auerobasidium
				Rhizopus
				Thamnidium
				Trichothecium
				Verticillium

Los microorganismos más frecuentes en matrices cosméticas se han identificado en aquellos casos en los que su presencia ha derivado en daños en la salud del consumidor. Sin embargo, no es tan habitual que se documenten infecciones individuales como las infecciones debidas a brotes en hospitales. Así, en los últimos años se informó de brotes de infecciones de *Burkholderia* debido al uso de enjuague bucal contaminado en individuos hospitalizados [2-4]. Otro brote documentado de *Burkholderia cepacia* fue causado por leche hidratante contaminada en una unidad de cuidados intensivos [5]. Anteriormente, se había publicado un brote de *Pseudomonas aeruginosa* debido a infecciones causadas por cosméticos, también en individuos hospitalizados [6, 7].

Sin embargo, no sólo se han documentado episodios de contaminación microbiana en hospitales. Anelich y Korsten [8], y Wong et al. [9] investigaron la contaminación de diversos productos cosméticos. Los dos primeros encontraron varios géneros de microorganismos en 50 cremas distintas en Sudáfrica. El género más frecuentemente encontrado fue *Pseudomonas* (30%), seguido de *Enterobacter* (17%), *Aspergillus* (13%), y *Staphylococcus* (9%). Wong et al., por su parte, encontraron patógenos como *P. aeruginosa* and *B. cepacia* en 25 (45%) y 19 (33%) de 56 productos diferentes investigados en los EE.UU.

Desde 2005 hasta mayo de 2008, la UE advirtió sobre la contaminación microbiológica detectada en 24 productos cosméticos diferentes, y al menos el 42% de los productos destacados estaban contaminados por *P. aeruginosa* [10]. Por otra parte, un estudio realizado en EE.UU. sobre 3.000 muestras cosméticas de prueba a disposición del público, mostró que el 50% de los productos estaban contaminados con bacterias y el 10% contaminados con hongos [11, 12]. Estudios como los referidos anteriormente, han permitido aislar los diferentes tipos de microorganismos más habitualmente presentes en productos cosméticos contaminados, algunos de los cuales se recogen en la tabla 4 [13].

3.2 MECANISMOS DE ACTUACIÓN DE LOS CONSERVANTES

A diferencia de los antibióticos, de los que se conoce el modo de acción a nivel molecular frente a los microorganismos, de los conservantes únicamente se conocen de forma generalizada los puntos de actuación. Casi todos actúan desnaturalizando las proteínas o afectando a la permeabilidad de la membrana de los microorganismos y, por tanto, bloqueando el transporte y la generación de energía. Se pueden clasificar de acuerdo con su mecanismo de acción: agentes que dañan la membrana, agentes desnaturalizantes y agentes modificadores de grupos funcionales [1].

i. Agentes que dañan la membrana

Los disolventes orgánicos como los alcoholes y los tensioactivos catiónicos (p. ej., las sales de amonio cuaternarias) dañan la integridad estructural de la membrana, es decir, alteran la disposición ordenada de lípidos y proteínas, lo que origina interferencias con procesos de transporte y metabolismo energético de la célula.

Los ácidos débiles como *p*-hidroxibenzoico, benzoico o dehidroacético actúan alterando el potencial eléctrico de membrana y su permeabilidad, bloqueando la generación de energía y limitando su transporte.

ii. Agentes desnaturalizantes de proteínas

Alcoholes y donadores de formaldehído, entre otros, facilitan la agregación y precipitación de las proteínas del citoplasma y de las membranas de los microorganismos.

iii. Agentes modificadores de grupos funcionales

Los conservantes liberadores de formaldehído o las isotiazolinonas se caracterizan por actuar alterando los grupos funcionales de las proteínas de las membranas, centros activos de enzimas y ácidos nucleicos.

3.3 CONTROL DE LA EFICACIA DEL CONSERVANTE Y DEL CONTENIDO MICROBIOLÓGICO

Para la industria cosmética y la farmacéutica es de vital importancia disponer de metodología apropiada para testar la eficacia antimicrobiana de los conservantes elegidos en las formulaciones y, por otra parte, poder verificar que el producto es seguro desde un punto de vista microbiológico.

Con el fin de testar la eficacia antimicrobiana se han diseñado los denominados Test de Eficacia o "Challenge Tests", cuyo protocolo experimental se encuentra estandarizado en la Norma ISO 11930:2012 – *Evaluation of the antimicrobial protection of a cosmetic product* [14]. Esta norma internacional consta de dos partes: un ensayo de eficacia de la conservación y un procedimiento para evaluar la protección antimicrobiana global del producto cosmético. Ambas partes son de aplicación sólo a cosméticos que no estén considerados de bajo riesgo, de acuerdo con la ISO 29621:2010 –*Guidelines for the risk assessment and identification of microbiologically low-risk products* [15], que permite determinar aquellos productos que tienen riesgo de contaminación microbiana.

Este tipo de ensayo se realiza una vez finalizada la etapa de desarrollo de la fórmula y consiste en inocular una elevada carga de microorganismos específicos en un cosmético sin diluir. A tiempo cero horas, 7, 14, 21 y 28 días se examina el número de microorganismos supervivientes. Se considera que un sistema conservante es adecuado cuando en las condiciones de realización del ensayo se produce un descenso significativo del número de microorganismos inoculado[16].

Actualmente desde el punto de vista legislativo, no existe ninguna norma de obligado cumplimiento que regule los límites de contenido microbiológico en productos cosméticos. El único requisito que está estipulado en el Reglamento Europeo es que estos productos "no pueden causar daño en la salud humana cuando se apliquen bajo condiciones de uso normales o razonablemente previsibles". Debido a la falta de directrices oficiales para cosméticos en esta materia, se han publicado algunas recomendaciones por parte de gobiernos y asociaciones cosméticas. Así, se pueden encontrar recomendaciones en cuanto al límite de contaminación microbiana en productos cosméticos en las notas explicativas creadas por el SCCP [17]. Según estas notas, los cosméticos pueden dividirse en dos categorías:

i. Productos específicamente destinados a niños menores de 3 años, o para ser usados en el área ocular y en zonas mucosas. Para estos productos el contenido máximo de microorganismos viables totales no debe exceder de 100 unidades formadoras de

colonias (Colony Forming Units, CFU)/g en 0,5 g de producto, y además, se requiere la ausencia de los microorganismos patógenos *P. aeruginosa*, *S. aureus* y *C. albicans.*

ii. Para el resto de productos enmarcados en la segunda categoría, el contenido máximo de microorganismos viables totales no debe exceder de 1000 CFU/g en 0,1 g de producto, con ausencia de los patógenos mencionados del grupo anterior.

Sin embargo, los métodos de control microbiológico que estas asociaciones aplican a cosméticos se basan habitualmente en el tradicional conteo de cepas en placa Petri. Estos métodos basados en el crecimiento, aunque no requieren una infraestructura cara y emplean material bastante económico, tienen limitaciones inherentes bien conocidas, tales como el consumo excesivo de tiempo, tanto en la operación como en la recogida de datos, son laboriosos, conllevan el uso de grandes volúmenes de medios líquidos, medio sólido y reactivos, y conducen a resultados ambiguos asociados con el uso de la turbidez como método de determinación [18, 19]. Por ello, varios autores han destacado la necesidad de proporcionar métodos rápidos y fiables a los microbiólogos cosméticos para detectar contaminación microbiana [18-20].

En esta línea, a lo largo de la presente tesis doctoral se ha propuesto el desarrollo de un método bioanalítico para la detección rápida de contaminación microbiana, que permita el control microbiológico de productos cosméticos, tanto durante el proceso de fabricación como en el producto final. El desarrollo metodológico se describe en el apartado 2.1 del capítulo III (Resultados y Discusión) de esta tesis.

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4. FOTODEGRADACIÓN DE CONSERVANTES COSMÉTICOS

4.1 INTRODUCCIÓN A LOS ESTUDIOS DE FOTODEGRADACIÓN

La Unión de Química Pura y Aplicada (*International Union of Pure and Applied Chemistry, IUPAC*) define el fenómeno de la fotodegradación como la transformación fotoquímica de una molécula en fragmentos de menor peso molecular, normalmente en un proceso de oxidación. Además, se añade en la misma definición que este término es ampliamente usado en la destrucción de contaminantes mediante procesos que utilizan luz ultravioleta (UV). Por otra parte, se define reacción fotoquímica como una reacción química provocada por absorción de luz UV, visible (vis), o infrarroja (IR) [1].

Una transformación fotoquímica puede transcurrir a través de dos tipos de procesos diferentes:

i *Procesos fotoquímicos directos (fotodegradación directa)*: estos se refieren a la absorción de luz por un compuesto a las longitudes de onda que corresponden al espectro de absorción del mismo, con un flujo global de radiación de magnitud suficiente como para ocasionar una transformación química.

ii *Proceso fotoquímico sensibilizado (fotodegradación indirecta):* se refiere a la absorción de energía luminosa por parte de una molécula sensibilizadora intermedia. La energía luminosa absorbida se puede transferir a otros compuestos que no absorben luz o puede iniciar la producción de compuestos químicos oxidantes altamente reactivos, como el oxígeno singlete o radicales peroxi- e hidroxi-, los cuales pueden reaccionar con una molécula de sustrato originando una transformación química [2].

4.2 CINÉTICA DE LOS PROCESOS DE FOTODEGRADACIÓN

Los procesos de degradación suelen describirse típicamente en la bibliografía mediante ecuaciones de primer orden [3]. Las cinéticas de degradación de primer orden se expresan de la siguiente forma:

$$dC/dt = -kC \qquad \text{(ecuación 4.1)}$$

siendo *C* la concentración de compuesto degradado en el tiempo *t,* y *k* la constante de velocidad de primer orden.

Si se integra la ecuación 4.1 anterior, se obtiene la siguiente expresión:

$$C(t) = C_0\, e^{-kt} \qquad \text{(ecuación 4.2)}$$

donde C_0 es la concentración inicial del compuesto (t = 0 min).

Tal y como se deduce de la ecuación 4.2, la concentración de compuesto es una función exponencial del tiempo de degradación. Si se toman logaritmos en la ecuación 4.2, la ecuación resultante se puede expresar como:

$$\ln C(t) = \ln C_0 - kt \qquad \text{(ecuación 4.3)}$$

Así, en las cinéticas de fotodegradación de primer orden, el logaritmo neperiano de la concentración es una función lineal del tiempo de irradiación (ecuación 4.3).

El tiempo de vida media ($t_{1/2}$) se define como el tiempo en el que la concentración de un compuesto se reduce a la mitad de la concentración inicial. Se trata de un parámetro muy útil a la hora de comparar cinéticas de fotodegradación y, a partir de la ecuación 4.3, se obtiene lo siguiente:

$$C = C_0/2$$

$$\ln (C_0/2) = \ln C_0 - k\, t_{1/2}$$

$$\ln C_0 - \ln 2 = \ln C_0 - k\, t_{1/2}$$

$$t_{1/2} = \ln 2/k \qquad \text{(ecuación 4.4)}$$

4.3 ETAPAS EN EL ESTUDIO DE LA FOTODEGRADACIÓN DE COMPUESTOS ORGÁNICOS

El estudio de los procesos de fotodegradación se lleva a cabo generalmente con el interés de alcanzar los siguientes objetivos:

i. Estudio de la fotoestabilidad de los compuestos degradados mediante la evaluación de los aspectos cinéticos del proceso (tiempo de vida media, velocidad de degradación, fotólisis, fotocatálisis, etc.)

ii. Identificación de productos de transformación y seguimiento de sus perfiles de evolución cinética.

iii. Estudio de la estabilidad de los fotoproductos generados, lo que permite conocer su persistencia bajo las condiciones de degradación estudiadas.

iv. Establecimiento de rutas de fotodegradación que justifiquen la formación de los productos de transformación identificados.

v. Estudio de la toxicidad de los fotoproductos mediante la realización de bioensayos (*Vibrio fisheri*, *Daphnia magna*, etc.).

La realización de estos estudios conlleva una elevada complejidad, fundamentalmente debido a las dificultades analíticas que supone la extracción de los productos de transformación de la matriz objeto de estudio y su posterior identificación. Por ello, el procedimiento de preparación de muestra empleado previamente a la determinación es de vital importancia [3].

Así, los estudios de fotodegradación se llevan a cabo en tres etapas:

1) Irradiación de la muestra que contiene los analitos a estudiar, habitualmente con lámparas UV, simuladores solares, o luz solar natural.

2) Extracción de los analitos y de sus fotoproductos. Lo normal es que las concentraciones de los productos de degradación sean muy bajas, por lo que es necesario pre-concentrar la muestra o aislar los fotoproductos. En el caso de fotodegradación acuosa, las técnicas de enriquecimiento y concentración utilizadas normalmente son la extracción en fase solida (SPE) [4-6]. En algunos casos también se ha utilizado la microextracción en fase sólida (SPME) como procedimiento de extracción [7-9], como en la presente tesis doctoral (ver apartado 3.1 y 3.3, capítulo III).

3) Determinación y análisis del compuesto de partida y de los productos que se puedan haber formado. En la mayoría de los casos, la identificación se lleva a cabo mediante GC-MS o LC-MS.

El uso de técnicas como la LLE para el aislamiento de fotoproductos en los estudios clásicos de fotodegradación acuosa ha sido reemplazado por la SPE por su capacidad de concentración, posibilitando la identificación de los fotoproductos que se pueden generar a concentraciones muy bajas. Sin embargo, esta metodología también implica un procedimiento multietapa, lo que acarrea una serie de desventajas:

i. Se incrementan la duración y la posibilidad de cometer errores.

ii. Aumentan las posibilidades de perder algunos fotoproductos

iii. Frecuentemente se requiere el uso de disolventes orgánicos, dañinos para el medioambiente y para el operador.

Algunas de estas desventajas se pueden evitar utilizando la microextracción en fase solida (SPME) como técnica de extracción y preconcentración. Comparada con las técnicas de preparación de muestra tradicionales, la SPME se caracteriza por ser una alternativa rápida, sensible y económica. Además, la SPME es un procedimiento respetuoso con el medioambiente ya que no requiere el uso de disolventes orgánicos [10].

En cuanto a los estudios de fotodegradación en muestras reales complejas, la identificación de productos de transformación procedentes de un determinado precursor supone un importante reto analítico. La dificultad más destacada de estos estudios radica en la capacidad de recuperar los productos de degradación formados en la matriz, y la capacidad de detectarlos y dilucidar la identidad de los mismos mediante cromatografía líquida o de gases acoplada a espectrometría de masas. Una solución propuesta en la bibliografía se basa en el uso de especies precursoras marcadas con un isótopo radioactivo [11, 12]. Sin embargo esta propuesta, además de cara puede resultar muy complicada en ocasiones.

Otro enfoque alternativo consiste en realizar el estudio en dos etapas:

1. La primera se basa en la identificación de los productos de transformación en un soporte modelo (superficie de vidrio, películas de cera, etc.) fortificado con el compuesto objeto de estudio. El soporte ha de ser un material con propiedades fisicoquímicas que imite lo máximo posible las características de la matriz real, pero que a su vez permita recuperar fácilmente los productos de transformación generados y el compuesto precursor.
2. En una segunda etapa, se realiza un estudio dirigido a detectar los fotoproductos presentes en los extractos procedentes de la matriz real irradiada [13, 14] .

La técnica de extracción más habitual para extraer fotoproductos de muestras reales, como por ejemplo suelos agrícolas, consiste en sumergir el sólido en un disolvente, aplicando extracción sólido-líquido y añadiendo posteriormente una etapa de filtración [15].

Las fibras de SPME, con recubrimiento de polidimetilsiloxano (PDMS) han sido utilizadas como soporte alternativo en la investigación de las rutas de transformación fotoquímica de pesticidas y contaminantes emergentes [16-18]. En estos estudios, la SPME se aplicó con una doble función: como técnica de extracción, incorporando los compuestos

objeto de estudio en la fibra; y posteriormente como soporte o matriz de degradación, exponiendo los compuestos adsorbidos a radiación UV o luz solar natural y simulada.

Esta metodología, denominada *Photo-SPME* o fotodegradación *on-fibre*, se desarrolló en el seno del grupo de investigación en el que se realizó la presente tesis doctoral. Los fotoproductos se generan *in situ* en la propia fibra, y ésta es inmediatamente desorbida en el puerto de inyección del sistema cromatográfico para proceder a la determinación de los mismos. Mediante este sistema todos los fotoproductos se generan directamente en la fibra de SPME, por lo que no son necesarias etapas posteriores de extracción, facilitando su identificación debido a los elevados factores de concentración.

El uso de otras alternativas avanzadas de extracción, como la microextracción en fase sólida (*Matrix Solid-Phase Dispersion, MSPD*) o la extracción con líquidos presurizados (*Pressurized Liquid Extraction, PLE*), se ha aplicado en esta tesis doctoral, por primera vez, para el aislamiento de productos de transformación en estudios de fotodegradación en muestras cosméticas y en un sustrato de piel sintética (ver apartados 3.2 y 3.3, capítulo III).

4.4 FOTODEGRADACIÓN DE CONSERVANTES COSMÉTICOS EN AGUA

4.4.1 Degradación en el medio ambiente

Al igual que sucede con muchos otros ingredientes de productos de cuidado personal, los conservantes son continuamente liberados en aguas residuales urbanas, debido al ingente consumo diario de productos cosméticos en todo el mundo. Estos PCPs, procedentes de la industria farmacéutica, efluentes de hospitales, efluentes domésticos (productos cosméticos y del hogar), etc., [19] alcanzan niveles de concentración relativamente elevados [20] en influentes de plantas de tratamiento de aguas, contribuyendo a engrosar la lista de los denominados contaminantes emergentes.

Teniendo en cuenta que las plantas de tratamiento de agua residual (*Wastewater Treatment Plants, WWTPs*) se optimizan con el objetivo prioritario de eliminar fósforo inorgánico y especies nitrogenadas, sin tener en cuenta los denominados microcontaminantes, los PCPs pueden ser liberados finalmente al medioambiente a través de las descargas de los efluentes de las WWTPs y del uso de lodos digeridos como fertilizantes en agricultura [21] (Figura 1).

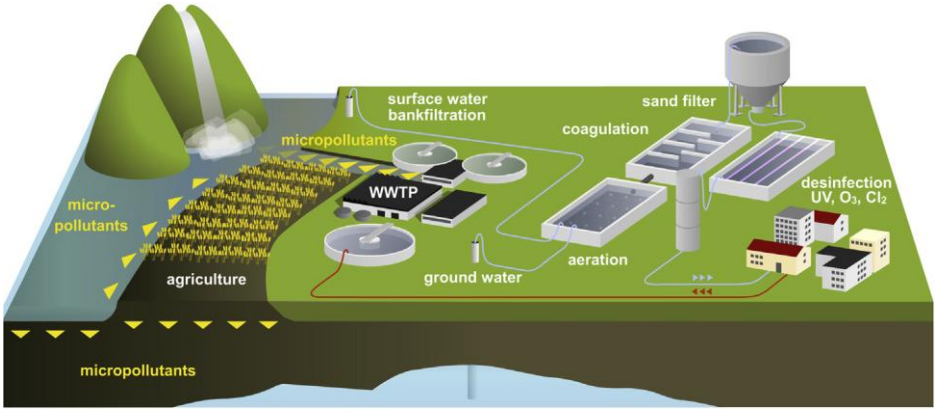

Figura 1. Esquema de las principales vías de liberación de PCPs al medio ambiente, según [22].

De hecho, los efluentes de las WWTPs se consideran la mayor fuente de contaminación medioambiental de productos farmacéuticos, dado que no están preparadas para la eliminación completa de estos microcontaminantes [23, 24]. Así, estos contaminantes pueden alcanzar el medio acuático en donde han sido detectados en concentraciones que abarcan desde 0,1 a 20 μg L^{-1}, en agua residual tratada, agua superficial, agua subterránea e incluso agua de bebida [25-27].

Muchos de estos compuestos no son fácilmente degradables y tienden a acumularse en organismos acuáticos o terrestres a través de la cadena alimentaria, provocando efectos medioambientales y ecológicos a largo plazo. La eliminación total de estos contaminantes procedentes de fuentes contaminantes es urgente, y para ello se requieren procedimientos terciarios/cuaternarios avanzados [28, 29]. Como contaminantes emergentes, los PCPs no están cubiertos por la legislación existente en el ámbito de la calidad del agua, y su impacto medioambiental no está suficientemente estudiado, aunque se piensa que son potencialmente dañinos para los ecosistemas medioambientales y para la salud humana. Sin embargo, uno de los efectos perjudiciales más conocidos de estos compuestos es su capacidad de disrupción endocrina en especies salvajes, afectando a su crecimiento, fisiología y reproducción [30]. Por ello, los PCPs requiere un mayor número de estudios tanto de su distribución en el medioambiente como de sus efectos [31].

Una vez que alcanzan el medio ambiente, el destino de los PCPs está controlado por procesos biológicos, físicos y químicos, siendo la biodegradación y la fotodegradación los principales procesos de degradación medioambientales. Un aspecto importante a tener en

cuenta es que la transformación medioambiental puede dar lugar a subproductos de mayor toxicidad y estabilidad que la de los compuestos de partida [32].

Los fenómenos de fotodegradación pueden tener lugar en la atmósfera o en la superficie del suelo o del agua. La luz del sol que alcanza la tierra tiene una longitud de onda en torno a 286 nm. La mayoría de los rayos UV son absorbidos en la capa superficial del agua (hasta una profundidad de 2 m). En las capas superficiales, la radiación solar promueve procesos fotoquímicos que pueden ser los responsables de la fotodegradación de los contaminantes orgánicos [33].

Los estudios sobre el comportamiento fotoquímico de conservantes cosméticos en el medioambiente no son muy numerosos en la bibliografía; probablemente por su condición de contaminantes emergentes. La fototransformación del antimicrobiano triclosan en agua, agua residual y aguas superficiales ha sido bien estudiada por el grupo de investigación en el que se desarrolla esta tesis doctoral, por su similitud estructural con contaminantes altamente tóxicos y persistentes, como las dioxinas. Está bien documentado que la degradación de triclosan mediante fotólisis directa genera 2,8-diclorodibenzo-p-dioxina (2,8-DCDD) y otros fotoproductos como clorofenoles [34-36]. Éste es, por tanto, un proceso de especial interés en el cual los intermedios de transformación son productos de mayor toxicidad que el compuesto de partida. Aunque la 2,8-DCDD no es el congénere mas tóxico de las dioxinas, su impacto ambiental es considerablemente más alto que el del triclosan [37].

4.4.2 Degradación en plantas de tratamiento de agua con luz UV

La eliminación de PCPs en plantas de tratamiento de aguas residuales convencionales es un reto para la producción de agua apta para la bebida [38]. Recientemente, ha habido un importante desarrollo en el campo de las tecnologías avanzadas para el tratamiento de agua, diseñadas para ser parte de la solución a dicho problema [29]. El uso de luz UV es un método establecido para la desinfección de agua de bebida y un método en desarrollo para la purificación de agua residual, que puede ser utilizado en combinación con fuertes oxidantes en los denominados Procesos de Oxidación Avanzada (*Advanced Oxidation Processes, AOPs*)[39], como substitutos del cloro, aplicándose en la última etapa del tratamiento.

Los AOPs combinan el uso de fuertes oxidantes, como por ejemplo O_3 o H_2O_2, con fuentes de alta energía, como la ultravioleta (UV), los ultrasonidos (US) o el haz de electrones (*Electron Beam, EB*); y catalizadores como Fe^{2+}, o fotocatalizadores como TiO_2. Además incorporan tecnologías de membrana (microfiltración, ultrafiltración, nanofiltración y

ósmosis reversa) y procesos de adsorción/intercambio iónico (resinas, carbón activado), cada una con sus ventajas y sus inconvenientes [40].

Se ha prestado especial atención a los AOPs en los que los radicales hidroxi (·OH) son los agentes responsables de la oxidación y mineralización de prácticamente cualquier molécula orgánica, produciendo CO_2 e iones inorgánicos como productos finales, debido a su fuerte poder oxidativo [41]. La fotocatálisis heterogénea con UV/TiO_2 se ha revelado como una técnica prometedora para la detoxificación de agua residual [40, 42]. Sin embargo, a pesar de que los AOPs están siendo ampliamente utilizados en el tratamiento de agua residual para la eliminación de contaminantes orgánicos, así como para incrementar la biodegradabilidad de los efluentes, la oxidación parcial de los contaminantes puede dar lugar a la formación de intermedios más tóxicos que los compuestos de partida [43]. Por este motivo se hace indispensable la identificación de los fotoproductos resultantes que permita el estudio de su toxicidad individual o del conjunto [44].

Existen algunos estudios en la bibliografía sobre la aplicación de AOPs a ingredientes cosméticos como parabenos y benzoatos. Así, la fotólisis directa del butilparaben resultó ser ineficiente debido al bajo rendimiento cuántico del proceso [39]. Aunque otros procesos oxidativos, como la ozonización, han resultado ser más efectivos que la fotólisis [45], la degradación de BuP, usando la acción combinada de la radiación UV y H_2O_2, tuvo lugar de forma más eficiente y rápida [46]. A este respecto, la fotooxidación con aditivos como H_2O_2 o TiO_2 se vio que mejoraba la degradación de metilparaben [47], mientras que la combinación de dos o más sistemas de oxidación simples incrementó la reactividad por efecto sinérgico [48]. La fotocatálisis heterogénea mediada por TiO_2 también ha sido utilizada para degradar bencilparaben [49] y acido benzoico [50]. En estos dos últimos estudios, se han identificado, además, varios productos de transformación, tales como benzoatos hidroxilados y fenoles, al igual que en otros estudios de degradación mediante ozonización de parabenos [38].

La degradación inducida por luz UV de los antioxidantes BHA y BHT también ha sido investigada aplicando fotólisis acuosa [51] y el enfoque alternativo de fotodegradación *on-fibre* [18]. En ambos casos se ha propuesto la identificación tentativa de los productos de transformación detectados. En cuanto al triclosán, como ya se ha mencionado en el apartado anterior, varios autores han estudiando su comportamiento fotoquímico, confirmando su degradación y su ciclación intramolecular fotoinducida para la formación de diclorodibenzo-p-dioxina en agua pura y en agua residual [34, 52].

La mayoría de los trabajos anteriormente revisados se llevaron a cabo usando lámparas de mercurio de baja presión con emisión de luz a 254 nm. Por ello, la evaluación de

los resultados de estos estudios podría extrapolarse, en cierta media, a condiciones medioambientales [13,18].

Por otra parte, es bien conocido que los compuestos que contienen grupos fenólicos en su estructura química, como los parabenos, pueden reaccionar fácilmente con cloro libre en agua clorada (a niveles de cloro entre 0,4 y 5 mg Cl_2 L^{-1}), dando lugar a subproductos de desinfección clorados [53, 54]. De hecho, se han detectado varios parabenos clorados en muestras de agua procedentes de piscinas públicas [55], así como en muestras de agua residual no tratada [54], que finalmente son liberados al medio acuático. Los estudios de toxicidad revelan que los subproductos clorados resultantes generan respuestas de toxicidad más agudas que los compuestos de partida en los tests *D. magna* y *V. fischeri* [56]. Además, se ha visto que los parabenos y los fenoles bromados son significativamente más citotóxicos y genotóxicos que sus análogos clorados [57-59]. Hoy en día, el uso de clorofenoles se ha restringido ampliamente, o prohibido definitivamente en muchos países, debido a sus posibles efectos adversos en la salud, incluso a concentraciones bajas [60].

Los estudios de fotodegradación de parabenos y benzoatos en agua con cloro, desarrollados en esta tesis doctoral, demostraron la formación fotoinducida de hidroxibenzoatos y fenoles halogenados bajo radiación UV (ver apartado 3.2, capítulo III) [9].

4.5 FOTODEGRADACIÓN DE CONSERVANTES SOBRE LA PIEL

4.5.1 Interacción de la luz solar con la piel

La luz solar se compone de un espectro continuo de radiación electromagnética, que se divide en tres regiones principales de longitudes de onda: ultravioleta (UV), visible, e infrarrojo. La radiación ultravioleta comprende longitudes de onda desde 200 nm a 400 nm, un rango un poco más corto que el de la luz visible (400–700 nm). La radiación ultravioleta se divide, a su vez, en tres secciones, cada una de las cuales provoca un efecto biológico distinto: UV-A (320–400 nm), UV-B (280–320 nm), y UV-C (200–280 nm). La radiación UV-C es bloqueada de forma muy efectiva por la capa de ozono en la estratosfera, lo que previene de sus efectos en la superficie de la tierra; aunque puede tener lugar cierta exposición accidental procedente de fuentes de radiación artificiales, como las lámparas germicidas. Tanto la radiación UV-A como UV-B alcanzan la superficie de la tierra con la intensidad suficiente como para tener importantes consecuencias biológicas en la piel y en los ojos. Las longitudes de onda en la región UV-B del espectro solar son absorbidas por la piel

produciendo eritema, quemaduras, y finalmente cáncer de piel. Aunque los rayos UV-A son el componente predominante de la radiación UV del sol a la que estamos expuestos, se supone que es un carcinógeno débil, causante del envejecimiento y arrugas en la piel [61].

La piel humana está constituida por tres capas: el estrato córneo, la epidermis y la dermis. En la dermis están presentes las células epiteliales incluyendo los melanocitos, encargados de generar el pigmento de melanina responsable de la coloración natural de la piel. La radiación UV en torno a 300 nm (UV-B) penetra a través del estrato córneo y de la epidermis. La radiación por encima de 350 nm (UV-A) penetra la tercera capa, la dermis, estimulando la formación de melanina y produciendo la tinción, que actúa en primera instancia como defensa para la protección de las quemaduras del sol [62].

Para que la energía de los fotones pueda tener algún efecto sobre la piel, es necesario que tengan lugar dos procesos esenciales. En primer lugar, la luz ha de alcanzar algún punto de la estructura de la piel y, una vez allí, los fotones han que ser absorbidos por un cromóforo. La subsiguiente relajación del cromóforo excitado al estado fundamental produce la liberación de la energía almacenada, provocando reacciones químicas, calor o emisión de luz [63].

4.5.2 Comportamiento fotoquímico de ingredientes cosméticos sobre la piel

Se ha demostrado claramente que no todos los compuestos aplicables sobre la piel con capacidad de absorber radiación UV-A y UV-B presentan suficiente fotoestabilidad. Bajo exposición UV, algunos de ellos pueden experimentar cambios espectrales o actuar como fotooxidantes, mediante la generación de radicales libres y especies de oxígeno reactivo, solos o en combinación con otras sustancias, cuando entran en contacto directo con la piel [64-66].

Por motivos prácticos, es imprescindible que un producto destinado a actuar como protector frente a la radiación solar, como en el caso de las pantallas solares, presente cierta estabilidad fotoquímica. Por esta razón, la mayor parte de las publicaciones sobre el comportamiento fotoquímico de ingredientes cosméticos, que se pueden encontrar en la bibliografía, se basan en estudios de la fotoestabilidad individual o combinada de filtros UV bajo radiación UV con diferentes aparatos de simulación solar, basados en lámparas de arco de xenón o lámparas fluorescentes [64-68]. Sin embargo, en espacios abiertos, las personas no están únicamente expuestas a la radiación UV-A y UV-B, sino a toda la radiación solar. Dado que ésta es policromática, sus mayores efectos no son solamente el resultado de la

acción de cada longitud de onda individualmente, sino también de las interacciones sinérgicas o antagonistas entre las diferentes longitudes de onda [69].

Los tests de laboratorio con fuentes de luz UV artificial que separan los efectos de la radiación UV-A y UV-B, no reproducen los niveles de inestabilidad de los ingredientes como bajo luz solar natural. Además, el espectro de la luz solar es cambiante [70], y la intensidad de la radiación que alcanza la superficie de la tierra depende fuertemente del ángulo de incidencia del sol debido a la latitud, de la estación, del momento del día, e incluso de la cantidad de ozono en la atmósfera [71, 72]. Los valores altos del factor de protección solar (*Sun Protection Factor, SPF*) implican fotoprotección duradera en pantallas solares. En cambio, esto solo está garantizado si los filtros UV permanecen estables durante todo el periodo de exposición a la luz solar, o si sus subproductos poseen un efecto protector comparable [73].

Estos intermedios reactivos procedentes de filtros UV o de cualquier otro ingrediente fotoinestable, al entrar en contacto directo con la piel, pueden actuar como fotooxidantes o también como promotores de fototoxicidad o dermatitis de contacto fotoalérgica. Ambas formas de fotosensibilización se describen a continuación.

4.5.3 Fotosensibilización: fototoxicidad y fotoalergia

Cuando una persona experimenta una reacción de fotosensibilidad, en realidad sufre una de las siguientes reacciones conocidas como reacciones fototóxicas o reacciones fotoalérgicas:

i. *Las reacciones fototóxicas* son el resultado de una liberación de energía por el agente fotosensibilizador, causando un daño a largo plazo en las zonas de la piel en torno a dicho agente. Le energía procedente de la radiación UV del sol es absorbida inicialmente por estas moléculas, e indirectamente la luz solar puede provocar la muerte de las células de la piel. Aunque el agente que causa la reacción puede detenerse rápidamente, la propia reacción puede, ocasionalmente, durar hasta 20 años después de que la sustancia se haya eliminado. Cualquier sustancia que puede tomarse por vía oral, por aplicación tópica (cremas aplicadas en la piel) o por inyección, puede provocar reacciones fototóxicas. Es una respuesta no inmunológica inducida por la luz (dermatitis) a un compuesto químico fotoactivo, y la respuesta de la piel se caracteriza por eritemas, y algunas veces, por edemas, vesiculación y pigmentación [74].

ii. *Las respuestas fotoalérgicas* suceden cuando los rayos UV causan transformación en la estructura de una molécula dando lugar a una nueva sustancia, provocando una respuesta en el sistema inmune para atacar a este antígeno foráneo. A medida que se producen los anticuerpos y atacan al sensibilizador, se desarrolla una erupción cutánea, que cursará durante unos días posteriormente a la aplicación de la sustancia. El brote no está limitado a las áreas expuestas al sol, sino que puede extenderse a otras partes del cuerpo [75]. Se conocen múltiples agentes capaces de causar reacciones fotoalérgicas entre los que se incluyen conservantes antimicrobianos de aplicación tópica, fragancias, ingredientes de pantallas solares tales como 6-metilcumarina, benzofenona, medicamentos antiinflamatorios no esteroideos (NSAIDs), prometzina, benzocaína o ácido para-aminobenzoico, entre otros.

Los fotoalérgenos son haptenos que requieren radiación UV para ser capaces de unirse a proteínas portadoras y provocar respuestas alérgicas [76]. El potencial fotoalergénico de los productos químicos se puede expresar por su capacidad de unirse a la *serum*-albúmina humana seguida de exposición UV [77]

Poco se conoce acerca de las transformaciones fotoquímicas que los ingredientes cosméticos pueden experimentar cuando son aplicados sobre la piel. La interacción de los productos de fotodegradación con los excipientes del propio cosmético que los contiene, o con componentes de la piel como el sebo, pueden dar lugar a nuevas moléculas con propiedades toxicológicas desconocidas [78, 79]. Consecuentemente, existe una creciente preocupación acerca de las propiedades fototóxicas o fotoalérgicas de los ingredientes cosméticos presentes en productos de aplicación tópica destinados a ser expuestos a la radiación solar [80] .

En esta tesis doctoral se han realizado los primeros estudios de fotodegradación de conservantes cosméticos sobre un sustrato de piel artificial, con la finalidad de identificar los posibles fotoproductos que pudieran formase en condiciones realistas de aplicación tópica y exposición UV (ver apartado 3.3, capítulo III).

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5. TÉCNICAS DE EXTRACCIÓN

La preparación de la muestra es un factor fundamental a tener en cuenta para el desarrollo exitoso de cualquier metodología analítica. Debido a la complejidad de las muestras cosméticas, los procedimientos utilizados para la extracción de sus ingredientes dependen del tipo de producto (emulsión, crema, champú, etc.), y de las características de las técnicas analíticas que se utilizarán para la determinación dichos ingredientes.

Para el análisis cromatográfico de muestras acuosas, basado tanto en técnicas de cromatografía liquida como de gases, generalmente es posible aplicar una dilución en un disolvente adecuado, o extraer los compuestos objeto de estudio con mezclas orgánicas o hidroorgánicas. Sin embargo, para la mayoría de las muestras cosméticas, no es posible realizar una simple dilución en un disolvente, previamente a su análisis, ya que muchas de ellas no se solubilizarían por completo y no se obtendría extractos homogéneos. Asimismo, la complejidad de las disoluciones obtenidas podría causar contaminación cromatográfica después de unos pocos análisis, así como la coelución de componentes de la matriz, haciendo realmente difícil la obtención de resultados analíticos satisfactorios. En la mayoría de los procedimientos clásicos, la preparación de muestra se lleva a cabo habitualmente en varias etapas que pueden incluir extracción con disolventes o simple dilución, mezcla, sonicación, calentamiento, adición de ácidos o bases, centrifugación y filtración. Estos procesos de preparación de muestra resultan muy tediosos y consumen mucho tiempo, además de utilizar grandes volúmenes de disolventes que pueden resultar perjudiciales para la salud [1].

Para solventar estos inconvenientes, en esta tesis doctoral se han empleado técnicas de extracción avanzadas como la microextracción en fase sólida (*Solid-Phase Microextraction, SPME*), la extracción con líquidos presurizados (*Pressurized Liquid Extraction, PLE*) o la dispersión de matriz en fase sólida (*Matrix Solid-Phase Dispersion, MSPD*). Estas técnicas han permitido aislar con gran eficacia los ingredientes objeto de estudio, así como sus productos de transformación, en una gran diversidad de matrices cosméticas. A continuación se describen las principales características de cada una de estas técnicas, sus procedimientos de extracción, y algunas aplicaciones en el análisis de PCPs.

5.1 MICROEXTRACCIÓN EN FASE SÓLIDA (SPME)

5.1.1 Introducción

La Microextracción en Fase Solida (*Solid-Phase MicroExtraction*, SPME) es una técnica de preparación de muestra desarrollada por Pawliszyn en 1989 [2], con el objetivo de satisfacer la necesidad de un método de muestreo rápido, libre de disolventes y compatible con los trabajos de campo. La SPME se basa en la utilización de una fibra de sílice fundida, químicamente inerte, estable a altas temperaturas y recubierta de una fase estacionaria ab/adsorbente de naturaleza polimérica. La fibra se expone a la muestra a analizar durante un tiempo determinado y, posteriormente, el analito extraído es desorbido de la fibra en el inyector de un cromatógrafo. [3]

La fibra utilizada en SPME se sitúa en el interior de una aguja de acero inoxidable. Esta aguja forma parte de una jeringa especialmente diseñada para alojar la fibra. En la Figura 2 se muestra el esquema del dispositivo mas empleado en SPME, introducido por Supelco en 1993.

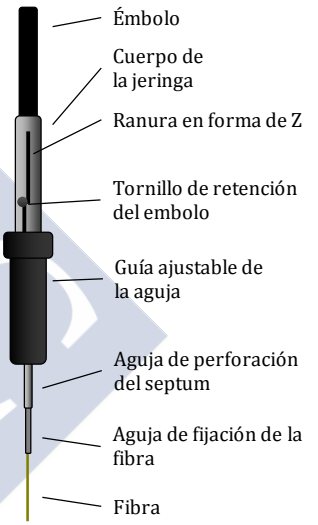

Figura 2. Esquema del dispositivo de SPME

Entre las principales ventajas de la SPME cabe destacar las siguientes:

i La extracción y concentración de los analitos tiene lugar en una sola etapa, evitando los inconvenientes de tediosas etapas de extracción, secado o concentración. Esto conlleva un ahorro considerable de tiempo.

ii Permite una extracción rápida y una transferencia directa y sencilla al equipo de separación y análisis.

iii No requiere el uso de disolventes. Se trata de una técnica respetuosa con el medio ambiente.

iv Permite obtener una elevada sensibilidad, puesto que lo analitos extraídos están concentrados en la fibra y son introducidos directamente, sin dilución, en el puerto de inyección del equipo de análisis.

Esta técnica presenta gran aplicación en el campo medioambiental, aunque cada vez son más numerosas las aplicaciones clínicas y alimentarias [4-6].

5.1.2 Principios de la SPME

La microextracción en fase sólida es una técnica de equilibrio en la que el analito se distribuye entre las distintas fases existentes en el sistema. Los equilibrios se producen entre el polímero que recubre la fibra y la muestra, el espacio de cabeza y la muestra, y entre el recubrimiento de la fibra y el espacio de cabeza. Se considera que la SPME se ha completado cuando la concentración del analito ha alcanzado el equilibrio de distribución entre la matriz de la muestra y el recubrimiento de la fibra. En la práctica esto significa que, una vez que se ha alcanzado el equilibrio, la cantidad extraída permanece constante dentro de los límites del error experimental, y es independiente de un incremento posterior del tiempo de extracción. Las condiciones de equilibrio se pueden describir mediante la siguiente ecuación:

$$n = \frac{K_{fs}\, V_f\, C_0}{K_{fs}\, V_f + V_s} \qquad \text{(ecuación 5.1)}$$

Donde n es la cantidad de analito extraída por el recubrimiento, K_{fs} es el coeficiente de distribución entre la fibra y la muestra, V_f es el volumen del recubrimiento de la fibra, V_s es el volumen de muestra, y C_0 es la concentración inicial del analito considerado en la muestra analizada.

Considerando que el volumen de la muestra (V_s) es mucho mayor que el volumen del recubrimiento de la fibra (V_f), puede cumplirse que $K_{fs}V_f << V_s$, en este caso:

$$n = K_{fs}\ V_f\ C_0 \qquad \text{(ecuación 5.2)}$$

Esto es, una vez alcanzado el equilibrio, la cantidad de analito extraído será independiente del volumen de muestra y directamente proporcional a su concentración inicial en la muestra, el volumen de fase contenida en la fibra de SPME y la constante de reparto entre la fibra y muestra. Como se observa en la deducción anterior, la constante de distribución Kfs indica que la eficacia de la extracción es dependiente del polímero que recubre la fibra de SPME, así como de su espesor.

La SPME es una técnica de equilibrio, y por eso no extrae exhaustivamente. No obstante, de la ecuación 5.2 se deduce que la concentración del analito en la muestra es directamente proporcional a la cantidad extraída, y ésta es la base de la cuantificación [2].

5.1.3 Procedimiento de extracción SPME

El proceso de microextracción en fase sólida se desarrolla en dos etapas básicas:

1. *Etapa de extracción*: Se expone la fibra a la muestra contenida en un vial sellado para que se produzca la migración de los analitos hacia la fibra durante un tiempo dado.
2. *Etapa de desorción de la fibra de SPME:* Se introduce la fibra en el inyector de un sistema analítico (cromatógrafo de gases o de líquidos) donde los analitos son desorbidos térmicamente o por disolución en la fase móvil [3].

El muestreo puede llevarse a cabo en dos modos diferentes, según las características de los analitos y de la muestra, tal y como se muestra en la Figura 3:

i. *Extracción directa o por inmersión (SPME)*: Se introduce la fibra directamente en la muestra líquida, produciéndose la migración directa de los analitos desde la matriz hasta la fibra. Se suele seleccionar cuando las muestras son relativamente sencillas y los analitos poco volátiles.

ii. *Extracción en espacio de cabeza (HSSPME):* La fibra se expone al espacio de cabeza existente sobre la muestra, de modo que los analitos difunden desde la muestra al espacio de cabeza, y de ahí al recubrimiento polimérico. Es adecuado para compuestos volátiles en matrices complejas, siendo además selectivo con respecto a aquellos analitos de elevado peso molecular [2].

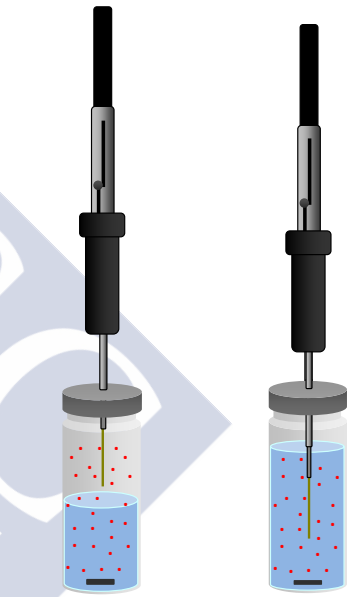

Figura 3. Modos de muestreo mediante SPME

5.1.4 Factores que afectan a la eficacia de la SPME

Existen una serie de factores sobre los que se puede actuar con el objetivo de desplazar el equilibro de reparto de los analitos entre la fibra y la muestra, incrementando la difusión de los analitos hacia la fibra.

i. Recubrimiento de la fibra

Existen diferentes tipos de fases poliméricas comerciales con distintas polaridades, que abarcan un amplio rango de afinidades por multitud de estructuras químicas. El espesor de la fase también es una variable a tener en cuenta ya que influye en el fenómeno de ab/adsorción de los analitos. En la Figura 4 se muestra un esquema de la distribución de las fibras de SPME más habitualmente utilizadas en función de sus propiedades.

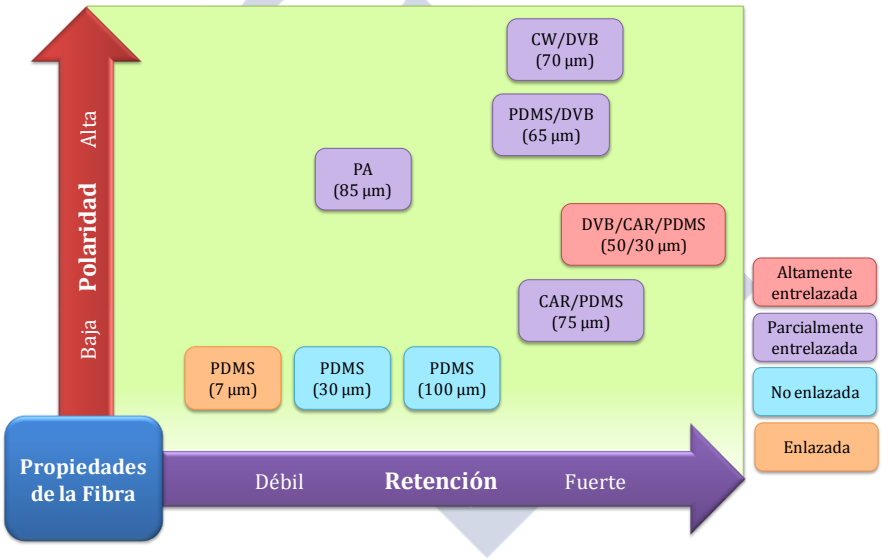

Figura 4. Propiedades de las principales fibras comercializadas de SPME [7].

ii. Tiempo de extracción

La máxima cantidad de analito se extraerá una vez transcurrido el tiempo necesario para alcanzar el equilibrio. Los compuestos con bajas constantes de distribución necesitan más tiempo para alcanzar el equilibrio. Por ello, en estos casos se suelen seleccionar tiempos más cortos que los de equilibrio. Cuando esto ocurre, el tiempo de exposición de la fibra requiere un control estricto para poder obtener suficiente reproducibilidad [7].

iii. Temperatura de extracción

En SPME, la temperatura de extracción tiene dos efectos contrapuestos. Por una parte, el incremento de temperatura durante la extracción favorece la difusión de los analitos hacia la fibra. Además, en HSSPME, la temperatura facilita la transferencia de los analitos hacia el espacio de cabeza. Por otro lado, puesto que la etapa de absorción es un proceso exotérmico, un incremento de temperatura reduce la constante de distribución fibra/muestra (K_{fs}) de los analitos [2].

iv. Efecto salino

La adición de sales (NaCl, KCl, etc) provoca un aumento de la fuerza iónica, variando las constantes de distribución de los analitos entre la muestra y la fibra, y con ello, la eficacia del proceso extractivo. Se produce, por tanto, una disminución de la solubilidad de los analitos en agua, forzando su paso a otras fases del sistema, como espacio de cabeza y fibra, originando un incremento de la sensibilidad. En general, la masa de analito extraída aumenta con la fuerza iónica, aunque cuando la concentración de sal es muy elevada puede provocar el efecto contrario para ciertos compuestos [3].

v. El pH de la muestra

El grado de disociación de compuestos con grupos ácidos o básicos influye decisivamente en su solubilidad. Para obtener la máxima eficacia de extracción, se ha de trabajar a un pH dos unidades por debajo del pKa en caso de compuestos ácidos, y dos unidades por encima en caso de los básicos.

vi. Volumen de la muestra

En general, el volumen de la muestra es mayor que el de la fibra, de modo que la cantidad de especie a extraer puede ser simplemente proporcional a la concentración en la muestra, el volumen de la fibra y el coeficiente de reparto K_{fs}. En ocasiones la cantidad de analito en la fibra aumenta con el volumen de muestra (V_s) hasta que se cumple que $V_s >> K_{fs}V_f$. A partir de ese momento, la eficacia de extracción no aumenta con el volumen de la muestra, denominándose *volumen infinito*. En todo caso, en SPME es muy poco habitual usar volúmenes de muestra por encima de 100 mL.

vii. Volumen de espacio de cabeza

Si el volumen del espacio de cabeza es muy grande, los compuestos volátiles se diluyen en él, disminuyendo su concentración en la fibra. Por otra parte, cuanto menor es el tamaño del espacio de cabeza, más rápida es la cinética del proceso de extracción.

viii. Agitación de la muestra

La agitación favorece la difusión de los analitos que se encuentran en matrices diluidas hasta la fibra, acelerando la cinética de extracción. La influencia de la agitación es especialmente significativa cuanto mayor sea el peso molecular de los compuestos y, sobre todo, cuando se opera en la modalidad de muestreo por inmersión.

ix. Adición de disolvente

La adición de disolventes orgánicos a muestras acuosas, normalmente, reduce la cantidad de analito extraída. Sin embargo, para muestras sólidas aumenta la eficacia de extracción, ya que favorece la difusión de los analitos desde la matriz a la fibra [2].

5.1.5 Aplicaciones de la SPME a la determinación de PCPs

Entre los ingredientes más frecuentemente utilizados en PCPs, los parabenos son, con diferencia, los compuestos más ampliamente estudiados, tanto en muestras medioambientales como cosméticas. Así, la SPME ha sido de gran utilidad en la determinación con gran sensibilidad de parabenos en un buen número de trabajos realizados en agua [8]. Canosa et al. [9] desarrollaron un método para la determinación de cinco parabenos en agua superficial y en agua residual. López-Darias et al. [10] expandieron la utilización de materiales poliméricos de líquidos iónicos en SPME, usando muestreo en modo inmersión directa para el análisis de muestras reales de agua. Regueiro et al. [11] desarrollaron un método basado en SPME con acetilación *in situ* para el análisis de parabenos, triclosan y fenoles relacionados en muestras de agua.

Aunque con menor presencia en la bibliografía, también se han publicado algunos trabajos para la determinación de parabenos y otros ingredientes en matrices cosméticas. El carácter no volátil de los parabenos implica que, en la mayoría de los casos, se necesita la extracción en modo directo [12, 13]. Aunque la extracción en espacio de cabeza puede ser ventajosa, ya que se evita el contacto con la muestra, los parabenos tienen que derivatizarse empleando este modo de muestreo [14]. Así, Yang et al. [15] propusieron un nuevo

acoplamiento entre SFE con derivatización *in situ,* y HSSPME *on-line* con GC-MS, para la determinación de parabenos y antioxidantes polifenólicos en cosméticos.

Las fibras comerciales de sílica, recubiertas con poliacrilato (PA) se han usado para la extracción de estos compuestos en su forma ionizada [13, 15]. En particular, Tsai et al. [13] evaluaron diferentes fibras comerciales con PA, PDMS, PDMS/DVB, CAR/PDMS y CW/DVB. En este estudio, el pretratamiento de la muestra consistió en dilución en metanol, para mejorar la dispersión y posterior dilución en agua. Las fibras de PA ofrecieron las mayores eficiencias de extracción para parabenos en cosméticos.

La SPME también se aplicó a la determinación de otros conservantes como bronidox en muestras cosméticas que se aclaran [16], así como a la determinación de fragancias alergénicas en agua de baño de bebé [17]. En la presente tesis doctoral se ha aplicado la SPME al desarrollado un nuevo método rápido y selectivo para de análisis multiconservante en productos cosméticos, empleando GC-MS/MS como técnica de determinación [18] (ver apartado 1.4, capítulo III)

5.1.6 Aplicaciones de la SPME a la determinación de MVOCs

Muchos compuestos orgánicos volátiles de naturaleza microbiana (*Microbial Volatile Organic Compouds, MVOCS*) son generados por microorganismos durante sus procesos metabólicos [19, 20]. Hasta la fecha existe un buen número de trabajos publicados en los que se ha intentado establecer una correlación entre la presencia de compuestos orgánicos volátiles de origen microbiano y cepas específicas, y consecuentemente, determinar si algunos MVOCs pueden servir como marcadores para la determinación de ciertas especies microbianas. Así, se han identificado algunos biomarcadores de cáncer procedentes de tejido estomacal canceroso, infectado con *Helicobacter pylori* [21, 22]. También ha sido posible identificar compuestos volátiles, característicos de bacterias, relacionadas con procesos de sinusitis en moco infectado [23]. Otros estudios se llevaron a cabo para la caracterización de MVOCs con actividad nematicida [24] y fungicida [25, 26], procedentes de un grupo de bacterias del suelo. El perfil volátil de rizobacterias [27] y bacterias aisladas de la flora de putrefacción del salmón ahumado [28] también han sido estudiados.

En estas investigaciones, el muestreo en modo de HSSPME es la técnica más utilizada. De hecho, el acoplamiento HSSPME con GC-MS se considera una herramienta de gran utilidad para el análisis de MVOCs [20], tal y como se demostró en uno de los trabajos desarrollados en la presente tesis doctoral (ver apartado 2, capítulo III)[29].

5.2 DISPERSIÓN DE LA MATRIZ EN FASE SÓLIDA (MSPD)

5.2.1 Introducción

Desde su introducción en 1989 por Barker et al. [30], la dispersión de matriz en fase sólida (M*atrix Solid-Phase Dispersion*, MSPD) ha demostrado ser una técnica eficiente, y a su vez genérica, aplicable a la extracción de un amplísimo rango de medicamentos, pesticidas, productos naturales, PCPs y otros compuestos, procedentes de una gran variedad de muestras complejas, tanto de animales como de plantas. La MSPD ha encontrado aplicación, particularmente, como procedimiento analítico para la preparación, extracción y fraccionamiento de muestras sólidas, semisólidas o altamente viscosas. Esta técnica se fundamenta en principios químicos y físicos basados en la aplicación de fuerzas, muestra mediante el mezclado mecánico, para producir la completa disrupción de la muestra y favorecer la interacción de la matriz de la muestra con el soporte sólido [31].

En su concepción original, la MSPD implicaba la mezcla de una muestra viscosa, sólida o semisólida con un soporte sólido de sílica (SiO_2), previamente derivatizado, para producir en su superficie una fase orgánica enlazada, como por ejemplo octadecilsilil (C_{18}). Este soporte sólido tiene la misma utilidad que la arena cuando ésta se utiliza como abrasivo: las fuerzas cortantes que surgen de la mezcla entre la muestra y el soporte sólido con un mortero y un pistilo (u otro dispositivo mecánico) perturban la arquitectura bruta de la muestra, rompiendo el material en pedazos de menor tamaño. Además, la presencia de una fase orgánica enlazada proporciona una dimensión adicional al proceso: los componentes de la muestra se disuelven y dispersan en la fase orgánica enlazada sobre la superficie de las partículas del soporte sólido, conduciendo a la disrupción completa de la muestra y a su dispersión sobre dicha superficie. Así, a través de las interacciones hidrofóbicas e hidrofílicas de los diferentes componentes de la muestra sobre la superficie de la fase enlazada, se produce otra fase con características únicas para llevar a cabo el aislamiento del analito de interés [32].

En los últimos años, muchas aplicaciones de la MSPD han supuesto la mezcla de las muestras con silicatos sin derivatizar (sílica gel, arena, etc.), u otros sólidos orgánicos (fibras grafitadas) o inorgánicos (Florisil, alúmina, etc.), que también provocan la disrupción de la muestra pero que no poseen, aparentemente, las mismas propiedades dispersivas que el C_{18} [31, 33, 34].

Entre sus principales ventajas con respecto a los métodos clásicos, cabe destacar las siguientes:

i. Permite realizar extracción y limpieza a la vez, lo que reduce las posibilidades de contaminación de la muestra durante el procedimiento [32, 35].
ii. Reduce considerablemente la manipulación de la muestra y, con ello, el tiempo total del análisis.
iii. Limita el consumo de muestra y de disolventes orgánicos.
iv. No requiere el empleo de instrumentación costosa y específica, ya que se utilizan condiciones de extracción moderadas (temperatura ambiente y presión atmosférica).

Los desarrollos y aplicaciones en MSPD se han ido compilando en varios artículos de revisión [31-33, 35, 36].

5.2.2 Factores que afectan a la eficacia de extracción

Entre los principales factores influyentes en el proceso de extracción mediante MSPD, cabe destacar los siguientes:

i. Agente dispersante

Las aplicaciones clásicas de MSPD emplean como agente dispersante un adsorbente con base de sílice. Estos materiales presentan la ventaja adicional de contener grupos silanoles libres, capaces de formar enlaces de hidrógeno con las moléculas de agua, actuando simultáneamente como agentes dispersantes y desecantes. Dentro de las sílices, las utilizadas con mayor frecuencia han sido las sílices enlazadas de fase reversa (particularmente C_{18} y C_8), que provocan la disolución de los componentes de la matriz sobre su superficie, favoreciendo la completa disrupción de la muestra. Para aislar analitos más polares se han empleado adsorbentes de fase normal, como sílices enlazadas a grupos polares (ej. aminas) u óxidos inorgánicos no enlazados (la propia sílice, la alúmina, el Florisil). Estos últimos interaccionan con los componentes de la matriz solamente por adsorción y, en consecuencia, no producen su disolución; no obstante, sus propiedades adsorbentes pueden modificarse dependiendo de su contenido en agua y de su carácter ácido o básico [32].

En los últimos años, la gran innovación en MSPD ha sido la utilización de nuevos materiales dispersante poco convencionales como los polímeros de impresión molecular de alta selectividad (*Highly Selective Molecularly-Imprinted Polymers, MIPs*), o los nanotubos de

carbono (*Multi-Walled Carbon Nanotubes, MWCNTs*). Más concretamente, los MWCNTs, particularmente después de una funcionalización adecuada, se han propuesto como adsorbentes emergentes para numerosas aplicaciones de extracción en fase solida (*Solid-Phase Extraction, SPE*) [33].

Aparte de la composición de la muestra, otro parámetro crítico es la relación muestra/dispersante. Generalmente, se dispersan 0,5 g de muestra con un soporte solido en relación 1:1 o 1:4 [36].

ii. Modificación de la matriz

A veces es necesario alterar el estado de ionización de los componentes de la muestra para asegurar que tienen lugar ciertas interacciones con la fase enlazada del soporte solido, y/o el disolvente de elución. Esto se puede llevar a cabo mediante la adición de ácidos, bases, sales, agentes quelatantes o desquelatantes, antioxidantes, etc., durante la etapa de mezclado de la muestra, y/o como aditivo en el disolvente de elución [32].

iii. Disolvente de elución

La naturaleza del disolvente de elución influye decisivamente en el proceso de MSPD, cuyo objetivo es el de desorber los analitos de forma selectiva, mientras que los componentes de la matriz quedan retenidos en el adsorbente de la columna. La mayoría de los adsorbentes se han probado en combinación con una gran variedad de disolventes, como alcanos, tolueno, doclorometano, alcoholes, y hasta agua a elevadas temperaturas [35].

iv. Presión y temperatura

Recientemente, varias aplicaciones han combinado la extracción con disolventes presurizados (*Pressurized Liquid Extraction, PLE*) con la MSPD, con el objetivo de conseguir extracciones más rápidas, mas automatizadas y más exhaustivas [34].

5.2.3 Procedimiento básico de la MSPD

El procedimiento básico de MSPD consta de las siguientes etapas (Figura 5):

1) La muestra se coloca en un mortero con el adsorbente, y se dispersa mecánicamente hasta obtener una mezcla de aspecto homogéneo. El mortero y el pistilo han de ser de vidrio o ágata, ya que se ha demostrado que la porcelana y otros materiales porosos pueden producir pérdidas de analito. La cantidad de muestra suele ser bastante pequeña (en torno a 0,5 g) y su relación con respecto a la masa de adsorbente suele

estar comprendida entre 1/4 y 1/1. En algunas aplicaciones se ha empleado, adicionalmente o como propio agente abrasivo, un agente desecante como la sílice o el sulfato sódico anhidro, obteniéndose una mezcla seca muy adecuada si la etapa de elución se lleva a cabo con disolventes apolares.

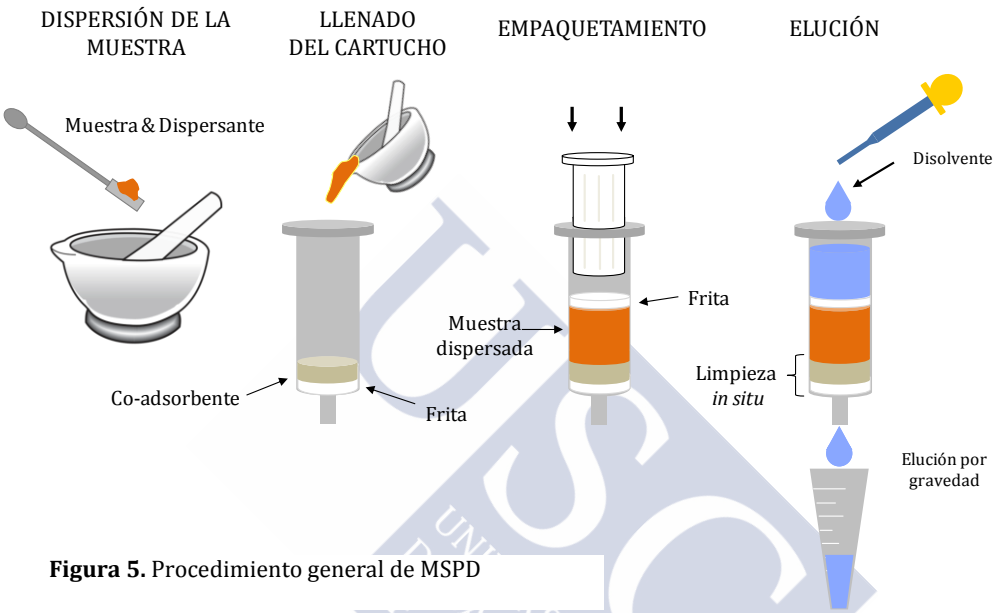

Figura 5. Procedimiento general de MSPD

2) Una vez que la muestra ha sido dispersada, el material resultante se transfiere a una columna o un cartucho-jeringa vacío de SPE, provisto de una frita de acero inoxidable o polipropileno, un filtro de celulosa o un tapón de lana de vidrio en su parte inferior. Generalmente se coloca una segunda frita en la parte superior de la muestra y, a continuación, se procede a su compresión utilizando el émbolo de una jeringa. En esta etapa son aplicables los principios de una buena cromatografía: evitar la formación de canales preferentes en la columna y no comprimir el material en exceso.

3) Finalmente, se lleva a cabo la elución de los analitos, que se puede realizar de dos formas distintas:

   i. los analitos se quedan retenidos en la columna y las interferencias se eluyen en una etapa de lavado y, a continuación, los analitos se eluyen con un disolvente diferente; o,

ii. las interferencias de la matriz se retienen selectivamente en la columna y los analitos se eluyen directamente.

Dado que toda la muestra está presente en la columna, es posible llevar a cabo una elución fraccionada de la misma, aislando diferentes compuestos o familias de compuestos mediante la utilización de disolventes diferentes. La mayoría de eluciones se lleva a cabo por gravedad, aunque es posible iniciar el flujo mediante la aplicación de presión positiva o negativa, o controlarlo durante todo el proceso mediante el empleo de un sistema de vacío.

4) Antes de la elución o de forma simultánea a ella, es posible introducir una etapa de limpieza para eliminar interferencias. El uso de una segunda columna (co-columna de limpieza), obtenida habitualmente como resultado de incorporar un material adsorbente en el fondo de la columna de MSPD (co-adsorbente), puede permitir obtener extractos más limpios, mayor purificación o un fraccionamiento extra del eluato [36].

5.2.4 Miniaturización de la MSPD

En ocasiones, la cantidad de muestra disponible para su análisis es limitada, particularmente en el caso de muestras biológicas, por lo que los errores en la etapa de preparación de muestra se hacen inadmisibles, y los protocolos que implican el uso de pequeñas cantidades de muestra pasan a ser muy valiosos. Así, algunos autores han optimizado procedimientos extractivos basados en micro-MSPD utilizando alícuotas de muestra de 100 mg o menos, y el volumen de disolvente de elución se ha reducido a 1 ml o unos pocos microlitros [37-39]. Entre estos desarrollos, cabe destacar las recientes aportaciones al análisis de productos cosméticos realizadas por el grupo de investigación en el que se ha desarrollado esta tesis. Uno de estos trabajos forma parte de la misma (ver apartado 3.2, capítulo III) [38].

5.2.5 Aplicaciones de la MSPD a la determinación de PCPs

En una reciente revisión de las aplicaciones de la MSPD a problemas analíticos concretos publicados desde el año 2009, se destaca que la mayor parte de estos artículos (63%) corresponde estudios de contaminantes en alimentación, en tejidos animales y vegetales, seguidos de compuestos naturales de origen animal y vegetal (17%), muestras

biológicas humanas (9%), y por último, sólo un 2% de los artículos están dedicados al análisis de productos cosméticos [33]

Los antimicrobianos triclosan y metil triclosan se determinaron en muestras de suelo, sedimento y lodo mediante MSPD por dos grupos de investigación distintos, utilizando GC-MS, pero con distintas condiciones de MSPD [40, 41]. Aunque la carga orgánica de los sedimentos y de los lodos es muy diferente, en ambos casos se obtuvieron recuperaciones muy similares, manteniendo las mismas condiciones de extracción. Por su parte, Sánchez-Brunete et al. [42] usaron el mismo método descrito anteriormente para triclosan y metil triclosan para el análisis de parabenos, octilfenol y nonilfenol en suelo, mediante GC-MS con derivatización. Sin embargo, las aplicaciones de la MSPD a muestras sólidas medioambientales (suelos, sedimentos secos o lodos) son relativamente pocas, en comparación con las técnicas basadas en ultrasonidos y *PLE*.

Recientemente, la MSPD ha demostrado ser una herramienta muy adecuada para la determinación de una amplia variedad de ingredientes cosméticos en productos de cuidado personal, como conservantes, fragancias alergénicas, plastificantes y fragancias policíclicas (*musks) [37, 43]*. En la presente tesis doctoral la MSPD se ha aplicado a la extracción de conservantes y fragancias alergénicas en PCPs, así como a la determinación de productos de fotodegradación de conservantes en matrices cosméticas (ver secciones 1.1 - 1.3, 1.5 y 3.2, apartado III) [38, 44-46].

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5.3 EXTRACCIÓN CON DISOLVENTES PRESURIZADOS (PLE)

5.3.1 Introducción a la PLE

La extracción con líquidos presurizados (*Pressurized Liquid Extraction*, PLE™, Fluid Management Systems, Inc., MA, USA), también conocida como extracción con fluidos presurizados (*Pressurized Fluid Extraction,* PFE), extracción con disolventes presurizados (*Pressurized Solvent Extraction, PSE*), o también por extracción acelerada por disolventes (Accelerated Solvent Extraction, ASE™, Dionex, Sunnyvale, CA, USA), se introdujo por vez primera en 1996 [47].

En los procedimientos de PLE convencional, la muestra, típicamente dispersada en un sorbente desecante o inerte, como sulfato de sodio o tierra de diatomeas, se empaqueta en una celda de acero inoxidable y, una vez insertada en un sistema de flujo cerrado, se extrae con un disolvente a temperaturas por encima del punto de su ebullición a presión atmosférica (hasta aproximadamente 200 °C). En PLE, el proceso de extracción se lleva a cabo a elevadas presiones, precisamente para poder trabajar con disolventes en fase líquida a temperaturas por encima de su punto de ebullición [48].

Los principales procesos que controlan el transporte de los analitos desde la matriz hasta el seno del disolvente durante la extracción [49] son:

1. Desorción desde una partícula solida
2. Difusión a través del disolvente localizado en el interior del poro de la partícula
3. Transferencia hacia el seno del flujo de disolvente (solubilización)

Entre sus principales ventajas cabe destacar:

i. Procesos de extracción más rápidos que los procedimientos clásicos (ej. Soxhlet), y alta eficiencia de extracción.

ii. Utiliza volúmenes inferiores de disolventes, disminuyendo la dilución de la muestra, y cumpliendo además con los requisitos de la química verde.

iii. Se trata de una técnica altamente automatizada, por lo que es especialmente útil a la hora de realizar análisis de rutina.

Por contra, su uso se ha visto parcialmente limitado debido a su elevado coste [49]. Sin embargo, la rápida aceptación de la PLE, le ha llevado a ser incluida en el método oficial

de la EPA (método 3545) para la extracción de compuestos orgánicos semivolátiles, insolubles o ligeramente solubles en agua, de suelos, arcillas, sedimentos, lodos y residuos sólidos [50]. Sus principales áreas de aplicación se centran en el análisis medioambiental y en la extracción de componentes nutricionales y bioactivos en alimentos [51]

5.3.2 Procedimiento de extracción de PLE

En la Figura 6 se muestra un esquema de un instrumento de PLE. La mayoría de las aplicaciones publicadas utilizan el equipo comercializado por Dionex (ASE 150) que permite trabajar a temperaturas de hasta 200 °C, y a presiones de hasta 21 MPa, en celdas de extracción de 1, 5, 11, 22 o 33 mL.

Antes de la extracción, es fundamental un adecuado pretratamiento de la muestra para conseguir aumentar la eficacia del proceso. A menudo es necesario someter la muestra a un proceso de homogeneización, molienda, tamizado y secado. La difusión de los analitos desde la muestra hacia el disolvente se puede incrementar considerablemente disminuyendo el tamaño de partícula. A continuación se suele mezclar la muestra con un material inerte como arena lavada con AEDT, alúmina básica, sulfato sódico, arena de cuarzo o tierra de diatomeas, con la finalidad de evitar la agregación de las partículas de la muestra que podría alterar la eficiencia de la extracción [51].

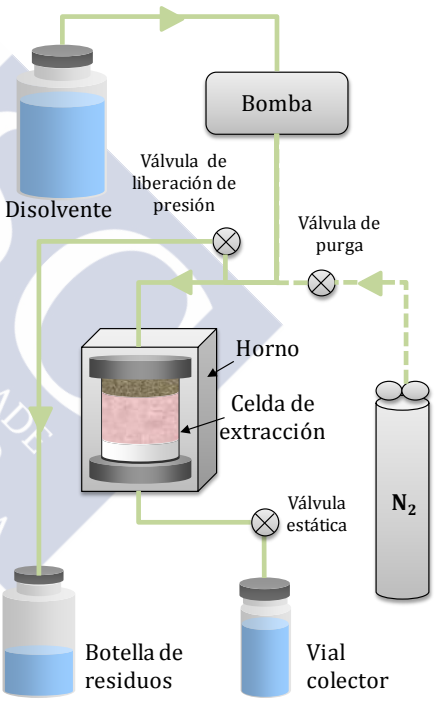

Figura 6. Esquema de un sistema PLE

El proceso de extracción puede realizarse en modo estático o en modo dinámico:

i. *Modo estático*: se comienza calentando la celda, con la muestra en su interior, hasta una temperatura apropiada durante un tiempo de equilibrio (aproximadamente 5 min), que viene seguido de un proceso de extracción estático. Este proceso se puede repetir varias veces si en una sola etapa se obtienen bajas recuperaciones.

ii. *Modo dinámico:* se puede asimilar a una elución por HPLC, llevada a cabo a temperaturas elevadas, en donde el empaquetamiento de la columna se reemplaza por la muestra. Aunque de esta manera se mejora la transferencia de materia, este tipo de extracción apenas se utiliza, principalmente debido al elevado consumo de disolvente en comparación con el modo estático.

Tras la extracción, el extracto se transfiere al vial colector, y la muestra y los tubos conectores se enjuagan con varias porciones de disolvente nuevo (*flush*). A continuación, todo el sistema se purga con el doble objetivo de eliminar, en la medida de lo posible, todos los residuos de la muestra para mejorar la recuperación del analito y, por otra parte, el de preparar al sistema para el siguiente proceso de extracción [49, 51].

5.3.3 Factores que afectan a la eficacia de extracción

Se pueden modificar distintos parámetros para conseguir optimizar la eficiencia de la extracción. Los más importantes son el disolvente de extracción, la temperatura, la presión, el tiempo de extracción estática y el número de ciclos [52].

i. Temperatura

Las altas temperaturas durante el proceso de extracción afectan a las propiedades del disolvente, incrementando su difusión y la capacidad de solubilizar el analito. Las interacciones entre los analitos y la matriz se debilitan, incrementándose la viscosidad y la tensión superficial. Por otra parte, las temperaturas demasiado altas pueden afectar a la estabilidad de compuestos termolábiles, sujetos a desintegración o degradación por hidrólisis.

ii. Presión

El motivo principal de la utilización de altas presiones en PLE es el de mantener el disolvente en estado líquido a elevadas temperaturas muy por encima de su punto de ebullición. Las altas presiones favorecen la penetración del disolvente en los poros de la muestra, con lo que se incrementa el poder de recuperación de los analitos. Cuanto mayor es la presión sobre el disolvente, mayor es su poder de solvatación, debido al incremento de la densidad. Sin embargo, el aumento de la densidad provoca, a su vez, un descenso de los coeficientes de difusión, que podría provocar la disminución de los valores de recuperación, debido a una lenta cinética del proceso de extracción [51].

iii. Disolvente

En general, a la hora de elegir un disolvente han de tenerse en cuenta, tanto sus propiedades fisicoquímicas como punto de ebullición, polaridad, densidad específica, así como su toxicidad. Disolventes como metanol, agua, tolueno, diclorometano, acetato de etilo y acetonitrilo se han utilizado en procedimientos PLE para la extracción de compuestos en alimentos y otras muestras complejas [51]. La polaridad del disolvente ha de parecerse a la de los analitos a extraer, aunque en general, las mezclas de disolventes polares y no polares ofrecen mayores recuperaciones [53, 54].

iv. Tiempo de extracción y número de ciclos

Los tiempos de extracción en PLE son muy cortos en comparación con los de las técnicas convencionales de extracción sólido-líquido. En ciertas matrices, los analitos pueden estar retenidos dentro de los poros u otras estructuras. En estos casos, el incremento del tiempo de extracción puede permitir la difusión de estos compuestos hacia el disolvente de extracción. El efecto del tiempo de extracción siempre se debe evaluar en conjunto con el número de ciclos, con el objetivo de conseguir una extracción completa de la forma más eficiente posible.

El uso de ciclos estáticos se desarrolló para introducir disolvente nuevo durante el proceso de extracción, lo cual ayuda a mantener un equilibrio de extracción favorable. Se ha comprobado que las extracciones de varios ciclos son especialmente útiles para muestras con una elevada concentración de los analitos o para matrices de difícil penetración [55].

5.3.4 Aplicaciones de la PLE a la determinación de PCPs

El mayor número de aplicaciones de PLE se centra en la extracción de contaminantes orgánicos presentes en matrices ambientales como suelos, sedimentos y residuos sólidos [52]. Además, también se ha aplicado a muestras biológicas y alimentarias [51].

La extracción con PLE se aplicó a la determinación de parabenos y otros conservantes antimicrobianos, como triclosan, en diferentes matrices de muestras sólidas, incluyendo polvo de espacios interiores [56], lodos residuales [57] y suelos [58]. En estos trabajos, se emplearon temperaturas de extracción entre 80 y 100 °C, aplicando presiones de entre 12 y 14 MPa. Los parabenos se extrajeron en dos o tres ciclos de extracción [14].

Recientemente se han desarrollado nuevas metodologías de análisis de productos cosméticos que incorporan la PLE como técnica de extracción para la determinación de

conservantes y fragancias alergénicas en cremas, lociones, productos de cuidado del cabello y otros productos de cuidado personal [53, 54]. En estos trabajos, el procedimiento de extracción tiene lugar durante 15 min a 120 °C y 10,3 MPa de presión, empleando una mezcla de hexano/acetona (1:1) como disolvente. La co-extracción de interferencias se minimizó incorporando florisil en el fondo de la celda de PLE. En el caso de los conservantes, se aplicó derivatización (acetilación) simultánea durante la extracción, incluyendo directamente los reactivos derivatizantes en la celda de extracción.

La PLE también se aplicó en un método multicomponente para la determinación de un gran número de ingredientes cosméticos, entre los que se incluyeron, conservantes, fregancias alergénicas, fragancias policíclicas y ftalatos, en toallitas de bebé y papel de baño húmedo para niños [59].

En la presente tesis doctoral, la PLE ha permitido extraer con gran eficacia los productos de transformación de conservantes cosméticos de piel sintética, empleada como sustrato modelo en los experimentos de fotodegradación (ver apartado 3.3, capítulo III)

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6. TÉCNICAS DE DETERMINACIÓN

Debido a la complejidad de las formulaciones cosméticas, constituidas muy a menudo por mezclas de sustancias de naturaleza muy diversa, el análisis multicomponente de sus ingredientes se convierte en una tarea muy complicada sin una buena etapa de separación previa. En este sentido, la cromatografía de gases y la cromatografía de líquidos, acopladas a distintos detectores, son las técnicas de mayor utilización en el análisis de ingredientes cosméticos.

La cromatografía de líquidos es la más comúnmente usada para el análisis de ingredientes como conservantes, filtros ultravioleta (UV) o colorantes, siendo la detección ultravioleta/visible (UV/vis) la técnica de detección con mayor presencia en la bibliografía.

Por otra parte, la cromatografía de gases ha sido utilizada principalmente para la determinación de fragancias y algunos conservantes, debido a las prestaciones de esta técnica al análisis de compuestos volátiles, ofreciendo una alta capacidad de separación de mezclas complejas. Los detectores de ionización de llama (*Flame Ionization Detector, FID*), captura electrónica (*Electron Capture Detector, ECD*) o espectrometría de masas (*Mass Spectrometry, MS*) son los más usados en acoplamiento con cromatografía de gases [1].

En la presente tesis doctoral se han utilizado ambas modalidades cromatográficas acopladas a MS y espectrometría de masas en tándem (MS/MS) como sistemas de detección. A continuación se describen las características más destacadas de ambas técnicas, a la vez que se recoge una breve revisión bibliográfica de sus aplicaciones a la determinación de ingredientes cosméticos.

6.1 CROMATOGRAFÍA DE GASES

La cromatografía gas-líquido, abreviada normalmente como cromatografía de gases (*Gas Chromatography*, GC), se basa en la distribución de los analitos entre una fase móvil gaseosa (el gas portador) y una fase estacionaria, generalmente líquida, inmovilizada sobre la superficie de un sólido (la columna cromatográfica). Es la técnica de elección para la separación de compuestos volátiles o semivolátiles térmicamente estables, ya que la muestra suele introducirse en la columna en fase gas y, para ello, las muestras líquidas deben experimentar una etapa previa de volatilización a elevadas temperaturas.

6.1.1 Derivatización

En el caso de compuestos polares y/o termosensibles, el análisis mediante GC está condicionado a un proceso previo de derivatización mediante el cual se modifica una determinada funcionalidad del analito, generando especies más apolares y suficientemente volátiles como para eluir a una temperatura razonable sin descomposición térmica o reorganización molecular. Este procedimiento mejora la resolución cromatografía e incrementa la respuesta de los analitos en el sistema de detección [2].

La acetilación es uno de los procedimientos más comunes para la derivatización de compuestos fenólicos. Consiste en la sustitución de un hidrógeno perteneciente a un grupo hidroxilo por un grupo acetilo, mediante la formación de un enlace carbono-oxígeno. Las ventajas radican en la alta eficacia obtenida durante el proceso usando reactivos de bajo precio, especialmente comparado con el coste de los reactivos empleados en otros procesos derivatizantes como la sililación. La acetilación con anhídrido acético como reactivo acetilante, y piridina como catalizador, se ha utilizado en esta tesis doctoral para la derivatización de conservantes fenólicos en medio orgánico [3], mientras que las reacciones de acetilación llevadas a cabo en medio acuoso se realizaron empleando hidrógeno fosfato de sodio como sustituto de la piridina [4] (ver apartados 1.1 y 1.5, capítulo III).

6.1.2 Acoplamiento a espectrometría de masas

La espectrometría de masas es una de las técnicas analíticas instrumentales de mayor utilidad que existen en la actualidad, al ser universal, sensible y extremadamente específica, ya que proporciona información acerca de la naturaleza, la composición y la estructura de las especies individuales detectadas. El acoplamiento GC-MS es, probablemente, la técnica híbrida de más amplia utilización, ya que combina el elevado poder de resolución de la cromatografía de gases con la alta sensibilidad y la información estructural aportadas por la espectrometría de masas.

Un espectrómetro de masas se compone de tres elementos fundamentales: la fuente de ionización (las especies neutras se transforman en iones que, adicionalmente, pueden experimentar algún tipo de fragmentación), el analizador de masas (los iones se filtran en función de su relación masa/carga, *m/z*) y el detector.

Entre las fuentes de ionización en fase gas, la fuente de ionización electrónica (*Electron Ionization*, EI) es la más común, y ha sido utilizada en la presente memoria en todos los análisis llevados a cabo por GC-MS. En ella, los electrones emitidos por un filamento

caliente de renio o wolframio son acelerados mediante un potencial de aproximadamente 70 eV, que se aplica entre el filamento y el ánodo. La trayectoria de estos electrones de elevada energía es perpendicular al camino de entrada de las moléculas en la fuente, de forma que, cuando ambos se acercan lo suficiente, cada molécula pierde un electrón por repulsión electrostática. Además, alcanza un estado rotacional o vibracional excitado desde el cual la subsecuente relajación se produce vía fragmentación en un gran número de iones positivos de diversas masas menores (y en ocasiones mayores) que la del ion molecular. Los complejos espectros de masas resultantes permiten la identificación de los compuestos detectados. Sin embargo, la extensa fragmentación puede ser también un inconveniente cuando da lugar a la desaparición del pico del ion molecular, perdiéndose la información más importante para establecer el peso molecular [5].

De entre los analizadores de masas disponibles actualmente en el mercado, los cuadrupolos (*Quadrupole, Q*) y las trampas de iones (*Ion Trap, IT*) son los filtros más comúnmente usados en acoplamiento con GC para el análisis de compuestos orgánicos de bajo peso molecular. A lo largo de la presente tesis doctoral se han utilizado dos configuraciones GC-MS basadas en analizadores de masas cuadrupolares

El analizador cuadrupolar consta de cuatro barras cilíndricas paralelas que actúan como electrodos, conectadas eléctricamente entre sí en pares opuestos. A dichos pares (polos) se les aplica una tensión de radiofrecuencia variable que sintoniza con un determinado ion. Cuando existe sintonía entre el ion que está pasando por ellas y la frecuencia aplicada, dicho ion continúa su camino, desviándose todos los demás no sintonizados fuera del cuadrupolo sin alcanzar el detector.

En la monitorización en modo SIM (S*elected Ion Monitoring*), estos potenciales se mantienen constantes y sólo los iones con un valor específico de *m/z* consiguen atravesar completamente el analizador. En el modo *scan* o de barrido, los potenciales se incrementan simultáneamente desde cero hasta un valor máximo, manteniéndose su relación ligeramente inferior a 6; el resultado es la filtración continua de iones con valores de *m/z* crecientes. Los espectrómetros de masas cuadrupolares convencionales resuelven fácilmente iones que difieren en una unidad de *m/z*, llegando hasta 3000 o 4000 *m/z*. Presentan una elevada velocidad de barrido que les permite obtener un espectro de masas completo en menos de 100 ms [5].

Otra configuración posible cuando se trabaja con este tipo de analizadores es la espectrometría de masas en tándem con triple cuadrupolo, que consiste en la disposición de tres cuádruplos en serie. El primero (Q1) y el tercer (Q3) cuadrupolos actúan filtrando los

iones de acuerdo con su *m/z*. El cuadrupolo situado en medio (Q2) se emplea como celda de colisión, en la que se induce una disociación por colisión de los iones precursores procedentes de Q1 empleando Ar, He o N_2. Los fragmentos o iones producto son filtrados en Q3.

La configuración de triple cuadrupolo permite operar en MS/MS en cuatro modos de trabajo diferentes:

i. *Product ion scan:* el primer cuadrupolo opera en modo SIM, seleccionando un ion de masa conocida que, posteriormente, se fragmenta en el segundo cuadrupolo. Dichos fragmentos se filtran en modo *scan* en Q_3, generando el espectro de fragmentación (*full-product ion spectrum*) del ion anterior. Esto permite obtener información estructural del ion original.

ii. *Precursor ion scan:* un determinado ion producto se selecciona en Q_3, y las masas precursoras se escanean en Q_1. Este es un modo de adquisición selectivo para iones que tienen un grupo funcional particular (ej. un grupo fenilo) liberado por la fragmentación en Q_2.

iii. *Neutral loss scan:* ambos cuadrupolos Q_1 y Q_3 operan en *full-scan*. Esto permite el reconocimiento selectivo de todos los iones que, mediante fragmentación en Q_2, sufren la pérdida de un determinado fragmento neutro (por ej. H_2O, NH_3). Este método es útil para la identificación selectiva de compuestos similares en una mezcla.

iv. *Selective reaction monitoring* (SRM): ambos cuadrupolos Q_1 y Q_3 trabajan en modo SIM, seleccionando un único ion precursor y, tras su fragmentación, un único ion producto. De esta forma se minimizan y, en algunos casos, se eliminan por completo las interferencias, reduciéndose el ruido químico de los cromatogramas, alcanzando excelente selectividad y sensibilidad.

En general, el análisis de PCPs mediante GC-MS se puede realizar en modo *scan* y/o SIM con propósitos identificativos y cuantitativos, respectivamente. Seleccionando iones de fragmentos característicos con alta intensidad, con un valor de *m/z* singular y con poco ruido procedente de interferencias, el análisis en modo SIM mediante GC-MS proporciona buena sensibilidad y selectividad para el análisis cuantitativo de PCPs. Sin embargo en el caso de matrices complejas, en las que hay problemas de coelución de interferencias de la matriz con los analitos de interés, el uso de GC-MS/MS se convierte en una herramienta indispensable para alcanzar niveles óptimos de selectividad y relación señal/ruido [6].

La espectrometría de masas en tándem contribuye a la supresión del ruido procedente de la matriz. Esto supone una ventaja muy importante en la cuantificación de niveles bajos de los analitos presentes en matrices altamente complejas como los productos cosméticos, con altos niveles de interferencias de fondo. Los iones precursores se seleccionan y las condiciones de MS/MS se optimizan para obtener un equilibrio entre máxima sensibilidad, mínimas interferencias espectrales y suficiente información para la identificación inequívoca [7].

6.1.3 Aplicación de GC-MS a la determinación de conservantes cosméticos y fragancias alergénicas.

La mayor parte de los esfuerzos para el análisis de conservantes se han centrado en la determinación de parabenos [8-21], mientras que los métodos para la determinación de otros conservantes en formulaciones cosméticas son más limitados. Debido a la naturaleza polar de la mayoría de los conservantes, la etapa de derivatización previa al análisis mediante GC es altamente recomendable para reducir la adsorción en el sistema cromatográfico, y mejorar la sensibilidad y la simetría de pico. Se han empleado procedimientos de derivatización empleando N-metil-N-(tert-butildimetilsilil) trifluoroacetamida, o una mezcla de N,O-bis(trimetilsilil)trifluoroacetamida y trimetilclorosilano para la determinación de parabenos en agua y en cosméticos [10, 19]. Sin embargo, la acetilación es uno de los procedimientos de derivatización más comunes para compuestos fenólicos [22, 23], aplicado a la determinación de parabenos y triclosan en agua [24], y recientemente empleado en el análisis de conservantes en muestras cosméticas [3, 4, 25]. Dos de estos últimos trabajos forman parte de la presente tesis (ver apartados 1.1 y 1.5, capítulo III).

Existe abundante bibliografía sobre la determinación de parabenos mediante GC acoplada a MS o MS/MS en muestras medioambientales [23, 26-29], y en menor medida en productos cosméticos. Otros trabajos utilizan métodos basados en el acoplamiento de la GC con detectores FID [20, 30]. Sin embargo, los sistemas FID carecen de la selectividad y especificidad que proporciona la MS [31].

Las fragancias se pueden encontrar en una gran variedad de muestras complejas, en un rango de concentración que puede fluctuar desde la baja parte por millón (μg/g) hasta los mg/g. A pesar de que la presencia de grupos cromóforos, en la mayoría de las fragancias, permite el uso de cromatografía líquida de alta resolución (HPLC) con detección UV/vis [32], el acoplamiento GC-MS puede considerarse como la principal técnica de elección para el análisis de este tipo de sustancias volátiles [33-36].

El uso de la monitorización en modo SIM para el análisis de fragancias ha demostrado ser más sensible y preciso [37] que el uso de iones extraídos de un análisis en modo *scan* [38]. Así, el modo SIM fue adoptado por la Asociación Internacional de Fragancias (*International Fragrance Association, IFRA*), que propuso un método para ser usado como referencia en los laboratorios de la industria de fragancias para la determinación de SAs [39]. No obstante, en caso de mezclas altamente complejas de fragancias, varios autores recomiendan acometer la determinación mediante GC-MS en modo *scan*, con el objetivo de evitar falsos positivos (fundamentalmente debido a la coelución de constituyentes del perfume que exhiben iones comunes con SAs) y falsos negativos (debido a la deriva que ciertos picos coeluyentes, y no relacionados, pueden promover en el tiempo de retención de los SAs) [33, 40, 41].

Una alternativa podría ser la elección de iones más selectivos en modo SIM, usando ionización química (*Chemical Ionization, CI*) más blanda. Esto daría lugar a una menor fragmentación que con el uso de EI, generando iones con masas altas más abundantes, incrementando la selectividad [42].

Para solucionar algunos inconvenientes causados por las interferencias de la matriz, también pueden aplicarse otros enfoques cromatográficos alternativos. La cromatografía de exclusión por tamaño (*Size-Exclusion Chromatography, SEC*) en combinación con GC-MS mostró mayores beneficios en comparación con otros métodos, debido a la flexibilidad de la limpieza con SEC, que permite la determinación de una gran variedad de compuestos en matrices difíciles con GC-MS [35].

La cromatografía de gases unidimensional (GC) y bidimensional (GC×GC) acoplada a espectrometría de masas [43], así como la cromatografía de gases multidimensional *(Multidimensional Gas Chromatography, MDGC)* con espectrometría de masas [44] es una herramienta poderosa de separación y análisis que ha sido aplicada a la determinación de SAs en productos cosméticos. La cromatografía bidimensional puede ser especialmente útil para confirmar la presencia de compuestos alergénicos detectados, cuando la deconvolución espectral no es posible. Por otra parte el procesado de los datos obtenidos con MDGC es más simple que utilizando en enfoque GC×GC [45].

Recientemente se ha publicado un método de análisis multicomponente de gran fiabilidad y alto rendimiento analítico, aplicable en laboratorios de control de perfumes, tanto en empresas como instituciones [46]. Permite la determinación de un total de 52 compuestos pertenecientes a cuatro clases diferentes de familias de ingredientes, en un único análisis mediante GC-MS: seis conservantes (parabenos y BHT), doce fragancias

sintéticas tipo *musk* (cinco nitromusks, seis policíclicas y una macrocíclica), veintiséis fragancias alergénicas y ocho ftalatos. Asimismo, también se han desarrollado métodos para la determinación de SAs en productos cosméticos y de cuidado personal; tanto de aclarado como de permanencia, así como en toallitas de bebé, mediante GC-MS [47-49]. El último de estos trabajos forma parte de la presente tesis (ver apartado 1.2, capítulo III).

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6.2 CROMATOGRAFÍA DE LÍQUIDOS

La cromatografía de líquidos (*Liquid Chromatography*, LC) se basa en la distribución de los analitos entre una fase estacionaria sólida y una fase móvil líquida, y es la técnica por excelencia para separar compuestos no volátiles, polares y/o térmicamente inestables.

6.2.1 Acoplamiento a espectrometría de masas

El acoplamiento LC-MS nace en la década de 1970, centrándose en los veinte años posteriores en resolver los dos problemas fundamentales derivados de la combinación de ambas técnicas: transformar las moléculas en disolución en iones en fase gaseosa sin que se produzca su degradación térmica, y eliminar la gran cantidad de gas y vapor procedente de la fase líquida antes de entrar en la región de alto vacío del espectrómetro de masas. Con este objetivo se han diseñado diferentes fuentes de ionización.

Las fuentes de ionización a presión atmosférica combinan los procesos de volatilización e ionización en una sola etapa. Suministran energía a la muestra sólida o líquida en condiciones de presión atmosférica, provocando la formación de iones gaseosos y dando lugar a espectros muy simplificados (técnicas blandas de ionización). Dentro de ellas, la ionización por electrospray (*Electrospray Ionisation*, ESI) se ha convertido en una de las más importantes y ha sido empleada en los análisis llevados a cabo por LC-MS en la presente tesis doctoral. En ESI, la disolución de la muestra es nebulizada a través de una aguja capilar que se mantiene a un potencial de varios kV con respecto a un electrodo cilíndrico circundante. Las microgotas cargadas resultantes se desolvatan por repulsiones coulómbicas y con la ayuda adicional de flujos de gas y calor, originando iones en fase gas con una o múltiples cargas. Este sistema permite controlar la fragmentación (presencia/ausencia) variando los potenciales de los electrodos y es compatible con MS/MS. Su principal inconveniente radica en que es muy sensible a los efectos de la matriz: los componentes presentes en ella pueden intervenir en reacciones ácido-base, disminuyendo o incrementando la ionización de los analitos. Además, pueden competir con ellos en su acceso a la superficie de las gotas del espray, dificultando su desolvatación, o variar (reduciendo o aumentando) la tensión superficial de las gotas [5].

En la presente tesis doctoral la cromatografía líquida se ha utilizado en combinación con la espectrometría de masas de triple cuadrupolo (QqQ) para la detección selectiva de conservantes cosméticos del tipo isotiazolinona (ver sección 1.5, apartado III Experimental, Resultados y Discusión).

6.2.2 Aplicación de LC a la determinación de conservantes cosméticos

Los métodos clásicos para el análisis simultáneo de más de un tipo de conservante en productos cosméticos se basan fundamentalmente en cromatografía líquida, tanto de fase normal como de fase reversa, con detección UV/vis [50-52]. La electroforesis capilar (C*apillary Electrophoresis, CE*) [53-55], la electroforesis capilar en zona (*Capillary Zone Electrophoresis, CZE*) [9, 56] y la cromatografía electrocinética micelar (*Micellar Electrokinetic Chromatography, MERKC*) [15, 57-59], también han sido ampliamente utilizadas. Estas técnicas han sido muy populares en el análisis de conservantes en PCPs, tanto para la determinación de compuestos cargados como hidrofóbicos. El análisis de inyección en flujo también se ha utilizado para mejorar el rendimiento [60].

Los métodos basados en LC para la determinación de parabenos suelen emplear columnas de C_8 y C_{18} con diferentes diámetros, longitud y tamaño de partícula. La combinación de distintos disolventes en la fase móvil ha sido la opción más extendida, mientras que la elución en isocrático es mucho menos popular. Se han utilizado detectores como UV, red de diodos (*Diode Array Detector, DAD*), y espectrometría de masas (*Mass Spectrometry Detector, MSD*), siendo el detector UV el mas empleado [61].

Recientemente, el detector de MS ha adquirido popularidad en su aplicación al análisis de PCPs, por su selectividad y gran capacidad de identificación. Sin embargo, las aplicaciones del acoplamiento LC-MS a la determinación de conservantes en productos cosméticos son bastante escasas [62]. Lee et al., emplearon LC-MS/MS para la determinación de cuatro parabenos (MeP, EtP, PrP, BuP) y cuatro antioxidantes (entre los que se incluyeron BHA y BHT) en productos cosméticos de diversa naturaleza [50]. En este método se utilizó un analizador de triple cuadrupolo e ionización ESI, tanto en modo positivo como negativo. Recientemente se han publicado normas estándar oficiales para el análisis de algunos ingredientes cosméticos, entre los que figura un método para el análisis del conservante 3-iodo-2-propynyl butylcarbamate (IPBC) mediante LC-MS [63]. Durante la realización de esta tesis se ha desarrollado un método basado en HPLC-MS/MS, empleando HESI en modo positivo, para la determinación de cuatro conservantes del tipo isotiazolinonas utilizados en productos cosméticos y del hogar [64] (ver apartado 1.5, capítulo III).

La aplicación de LC-MS para la determinación de conservantes cosméticos en muestras ambientales también es bastante limitada, aunque existen más trabajos publicados que en muestras cosméticas. Así, LC-MS/MS con triple cuadrupolo se ha empleado para la determinación de parabenos en suelos y sedimentos [65], así como en ríos y muestras de agua residual [66]. MS con trampa de iones se ha utilizado en la determinación de

descriptores endocrinos (incluyendo parabenos) en muestras de agua medioambiental [67]; en este trabajo se compararon distintos métodos de ionización con resultado similar.

La MS permite la identificación cualitativa de los analitos mediante la comparación de sus espectros de masas con los espectros teóricos de una librería espectral [14]. No obstante en LC-MS no es posible identificar compuestos comparando el perfil de su espectro de masas con el espectro de interés, mientras que con LC acoplado a espectrometría de masas en tándem (MS/MS) se obtienen resultados de iones de fragmentos específicos. Por otra parte la LC-MS está sujeta al efecto matriz, debido a las interacciones entre los analitos y algunos componentes de la muestra durante el proceso de ionización en la fuente, provocando un error proporcional en la respuesta del analito. Todo ello, sumado al alto precio de la instrumentación, hace de GC-MS la alternativa más habitual para el análisis de conservantes cosméticos, siempre que las características del analito lo permitan [61].

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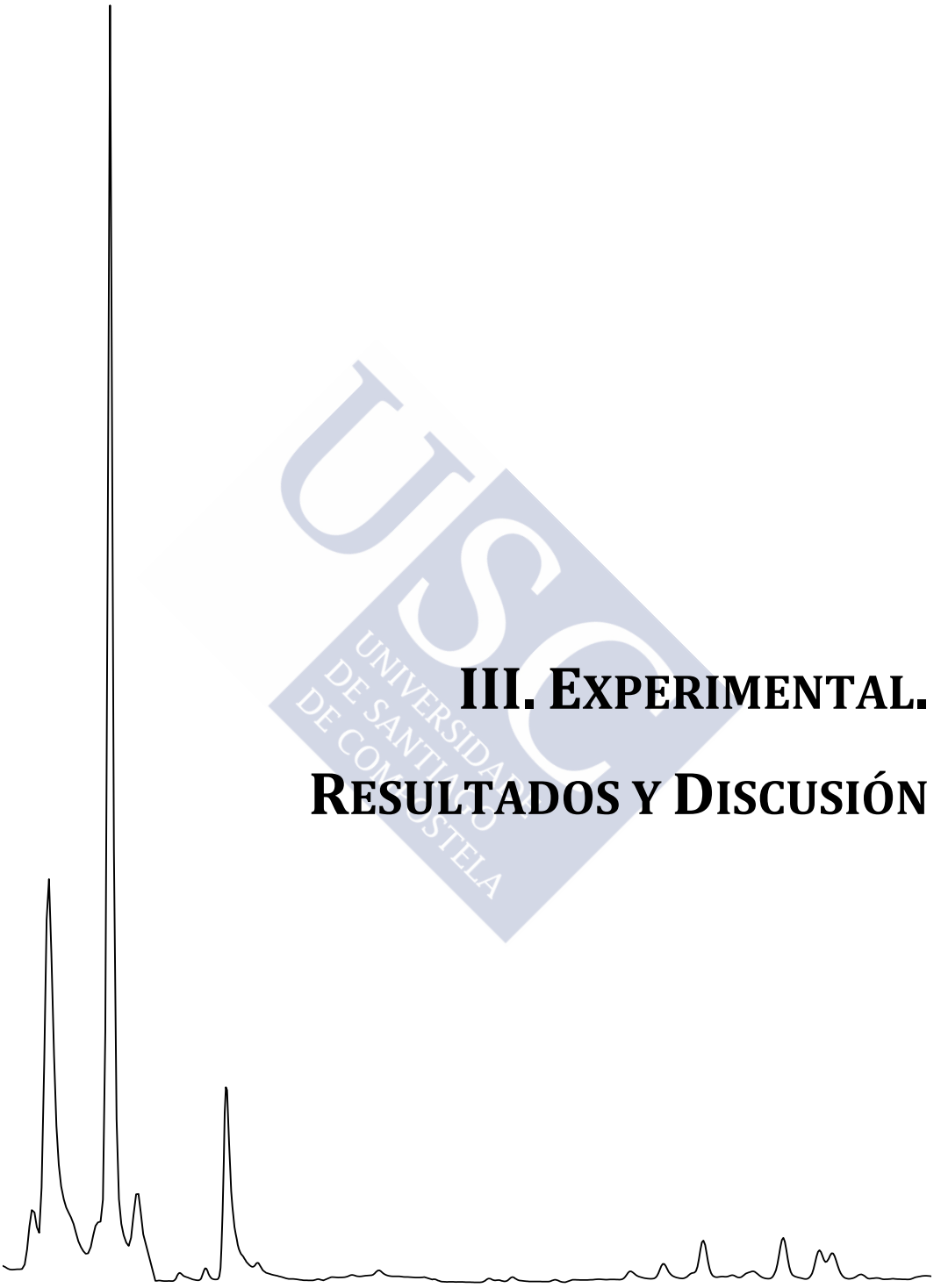
III. EXPERIMENTAL. RESULTADOS Y DISCUSIÓN



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III.1

DETERMINACIÓN DE CONSERVANTES Y FRAGANCIAS ALERGÉNICAS EN PODUCTOS COSMÉTICOS

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Los conservantes y las fragancias alergénicas constituyen dos grupos de ingredientes ampliamente distribuidos en los productos cosméticos y son los que con mayor frecuencia se asocian con casos de alergias y dermatitis de contacto. Estudios recientes sugieren que estos ingredientes no solo pueden provocar daños locales en la piel, si no que la exposición sistémica a algunos de estos ingredientes podría ocasionar efectos estrogénicos, carcinogénicos e incluso neurotóxicos a largo plazo para la salud humana (ver apartados 2.1.5 y 2.2.3, capítulo II).

Para asegurar un alto nivel de protección al consumidor, los conservantes y las fragancias alergénicas permitidos para su uso en productos cosméticos en el marco de la UE se recogen listados en los anexos V y III, del Reglamento (CE) No 1223/2009 de Productos Cosméticos, respectivamente. Además, en estos anexos se indican las limitaciones, requerimientos, advertencias para su etiquetado, así como las concentraciones máximas permitidas en el producto final. Sin embargo, los nuevos estudios de toxicidad de estos ingredientes generan nuevas opiniones del SCCS, que evidencian la necesidad de revisar los niveles de la seguridad de algunos de ellos; lo que conlleva la publicación de continuos cambios en las restricciones, a través de las sucesivas enmiendas y adaptaciones en la normativa vigente.

Las continuas modificaciones legislativas evidencian la necesidad de adaptación y desarrollo de nuevos métodos analíticos para garantizar el control adecuado de los conservantes y las fragancias alergénicas en productos cosméticos, y asegurar así el cumplimiento de las normativas actuales y futuras. En esta línea se ha centrado buena parte del trabajo de la presente tesis doctoral, cuyos resultados publicados se exponen en este apartado.

Al inicio de esta tesis, la mayor parte de los esfuerzos analíticos se habían centrado en la determinación de parabenos, mientras que los métodos para la determinación de otros conservantes en formulaciones cosméticas eran muy limitados o inexistentes. Por su parte, el desarrollo de métodos analíticos para la determinación de fragancias alergénicas en productos cosméticos resultaba tan complejo como necesario, teniendo en cuenta la escasez de metodología a este respecto. La mayoría de los métodos existentes, incluía procedimientos de preparación de muestra que implicaban múltiples etapas, lo que se traduce en procesos tediosos que implican mayor tiempo de análisis, y generalmente, un consumo de mayores volúmenes de disolventes.

En consecuencia, en este trabajo de tesis, los mayores esfuerzos se han centrado en el desarrollo de métodos analíticos de mayor simplicidad, incorporando procedimientos de

extracciones eficaces, capaces de romper las interacciones entre la matriz y la muestra, liberando los analitos para poder ser selectivamente extraídos *a posteriori*. Así, teniendo en cuenta las ventajas de la microextracción en fase sólida (MSPD) se han desarrollado dos métodos basados en MSPD para la determinación multicomponente de 13 conservantes y 25 fragancias alergénicas por separado. La incorporación de una etapa de limpieza *in situ*, permite reducir la posibilidad de contaminación de la muestra, reduciendo los tiempos de extracción y el uso de disolventes. Para la determinación de conservantes se ha introducido una etapa de derivatización (acetilación), posterior a la extracción, con la finalidad de mejorar el análisis cromatográfico de los compuestos fenólicos. Entre los conservantes estudiados, se han incluido siete parabenos, dos conservantes bromados (bronidox y bronopol), los antioxidantes BHA y BHT, el IPBC y el triclosan; todos ellos regulados para su uso en cosméticos a excepción de BHA y BHT. Entre las fragancias se han incluido los 24 alérgenos susceptibles de ser analizadas por GC, catalogadas inicialmente como SAs, y el metileugenol; un caso peculiar sujeto a cambios recientes en la legislación, tal y como se comenta en el apartado 2.2.3 de la Introducción.

La obtención de condiciones óptimas de extracción similares en ambos métodos, ha permitido realizar un estudio de mercado conjunto: se ha evaluado la presencia simultánea de ambos grupos de ingredientes en productos de cuidado personal destinados a ser utilizados por bebés y niños. En este mismo estudio también se han comparado las prestaciones de los nuevos métodos propuestos (MSPD-GC-MS) con la metodología desarrollada previamente en nuestro grupo de investigación, para la determinación del mismo grupo de ingredientes (PLE-GC-MS). Dicha comparativa ha puesto de manifiesto eficacias de extracción comparables para los grupos de ingredientes objeto de estudio, lo que presenta la MSPD como una alternativa más económica y de gran capacidad de extracción, aplicada por primera vez al análisis de productos cosméticos.

Como alternativa y complemento de los métodos anteriores, se ha desarrollado una metodología simple y sensible basada en SPME-GC-MS/MS para el análisis multiconservante de los siete parabenos, cuatro benzoatos, bronidox, 2-phenoxyetanol, BHA, BHT y triclosán. En el proceso de SPME se ha utilizado derivatización (acetilación) *in situ* y adición de modificador orgánico, con el doble objetivo de mejorar la respuesta cromatográfica de los conservantes fenólicos y compensar el efecto matriz. El resultado es una metodología atractiva, por la simplicidad y la gran capacidad de preconcentración de la SPME, que integra las etapas de muestreo, extracción y concentración en un único proceso ininterrumpido, a lo que hay que sumar la posibilidad de automatización.

Finalmente, se ha propuesto abordar el análisis de cuatro conservantes pertenecientes a un grupo de biocidas del tipo isotiazolinona, ampliamente presentes en productos cosméticos y del hogar, debido a su capacidad para provocar dermatitis por contacto, y a los indicios que sugieren actividad neurotóxica, incluso a niveles bajos, para alguno de estos ingredientes. Teniendo en cuenta el marcado carácter hidrofílico de este grupo de conservantes, se ha propuesto una alternativa analítica basada en HPLC-(HESI+)-MS/MS. Como técnica de extracción se ha propuesto, una vez más, la MSPD por su demostrada capacidad para aislar dichos compuestos de matrices cosméticas.

A continuación se exponen los resultados publicados resultado de la metodología desarrollada. Es importante destacar que desde el inicio de esta tesis, no sólo se ha producido el cambio desde la anterior Directiva del Consejo de la UE 76/768/CEE al nuevo Reglamento (CE) No 1223/2009 de Productos Cosméticos, sino que la nueva normativa también ha sufrido modificaciones que afectan a los ingredientes estudiados. Dichos cambios en han sido revisados en los apartados 2.1.6 y 2.2.4 de la Introducción de esta Tesis, que sirven de actualización a los apartados introductorios de cada una de los trabajos publicados.

Por otra parte, durante el desarrollo de esta tesis, se han publicado normas estándar para el análisis de algunos ingredientes cosméticos, como las fragancias alergénicas y el conservante 3-iodo-2-propynyl butylcarbamate (IPBC), incluidos para su estudio en los trabajos que se expondrán a continuación. En algunas de estas normas el método propuesto permite el análisis cromatográfico de muestras preparadas para inyectar; esto es, no se aplica ningún método de preparación de muestra, más allá de una simple dilución en un disolvente adecuado. Sin embargo, como se indica en el apartado 5 de la introducción de esta tesis, para la mayoría de las muestras cosméticas no es posible realizar una simple dilución en un disolvente, previamente a su análisis, ya que muchas de ellas no se solubilizarían por completo y no se obtendría extractos homogéneos. Asimismo, la complejidad de las disoluciones obtenidas podría causar contaminación cromatográfica después de unos pocos análisis, así como la coelución de componentes de la matriz, haciendo realmente difícil la obtención de resultados analíticos satisfactorios.

Los métodos que se describen a continuación permiten solventar los problemas ocasionados por la complejidad de las matrices cosméticas, así como la determinación multicomponente tanto de ingredientes sujetos a nuevas restricciones como de ingredientes recientemente prohibidos. Además la metodología propuesta ha sido rigurosamente validada, con límites de detección y cuantificación varios órdenes de magnitud por debajo de las concentraciones máximas establecidas en la legislación, lo que permite su aplicación con garantías al control de la seguridad de los productos cosméticos.

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Capítulo III.1.1

Analysis of multi-class preservatives in leave-on and rinse-off cosmetics by matrix solid-phase dispersion

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Analysis of multi-class preservatives in leave-on and rinse-off cosmetics by matrix solid-phase dispersion

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ABSTRACT

Matrix solid-phase extraction has been successfully applied for the determination of multi-class preservatives in a wide variety of cosmetic samples including rinseoff and leave-on products. After extraction, derivatization with acetic anhydride, and gas chromatography–mass spectrometry analysis were performed. Optimization studies were done on real non-spiked and spiked leave-on and rinse-off cosmetic samples. The selection of the most suitable extraction conditions was made using statistical tools such as ANOVA, as well as factorial experimental designs. The final optimized conditions were common for both groups of cosmetics and included the dispersion of the sample with Florisil (1:4), and the elution of the MSPD column with 5 mL of hexane/acetone (1:1). After derivatization, the extract was analyzed without any further cleanup or concentration step. Accuracy, precision, linearity and detection limits were evaluated to assess the performance of the proposed method. The recovery studies on leave-on and rinse-off cosmetics gave satisfactory values (>78% for all analytes in all the samples) with an average relative standard deviation value of 4.2%. The quantification limits were well below those set by the international cosmetic regulations, making this multi-component analytical method suitable for routine control. The analysis of a broad range of cosmetics including body milk, moisturizing creams, anti-stretch marks creams, hand creams, deodorant, shampoos, liquid soaps, makeup, sun milk, hand soaps, among others, demonstrated the high use of most of the target preservatives, especially butylated hydroxytoluene, methylparaben, propylparaben, and butylparaben.

Keywords: Matrix solid-phase dispersion; Cosmetics; Preservatives; Antioxidants; Personal-care products; Experimental design; Derivatization; GC-MS.

1. Introduction

Cosmetic products are complex formulations containing many additives and ingredients. To ensure protection of human health, cosmetic products are regulated and controlled worldwide. The European Union (EU) Cosmetic Products Regulation [1] constitutes the main regulatory system on cosmetic products in Europe. Among the different groups of cosmetic additives, preservatives (added to maintain the integrity of the product), especially phydroxybenzoic acid (parabens) and potentially nitrosating compounds, are a matter of scientific and social concern due to the negative side effects on human health.

The esters of parabens, iodopropynyl butylcarbamate (IPBC), 2,4,4′-trichloro-2′-hydroxydiphenyl ether (triclosan, TCS), and bromine-containing preservatives 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 [2]. 2-tert-Butyl-4-methoxyphenol (butylated hydroxyanisole, BHA) and 2,6-bis(1,1-dimethylethyl)-4-methylphenol (butylated hydroxytoluene, 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 [3].

The preservatives allowed in the EU context are listed in the Annex V of the EU Cosmetics Regulation [1] where limitations, requirements, label warnings, and the maxima permissible concentrations are indicated (see Table 1 last column for the target preservatives).

To guarantee 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. A great part of the analytical effort has been focused on parabens determination [4-7] while methods for the determination of other preservatives in cosmetic formulation are very limited or inexistent. Simultaneous analysis of more than one class of preservatives is scarce and mainly based on liquid chromatography [8-10] and capillary electrophoresis [11, 12]. Flow injection analysis has also been employed enhancing sample throughput [13].

For most cosmetic samples, it is not possible to simply dilute the sample in an adequate solvent prior analysis since several sample components would not be solubilized

and we would not obtain homogeneous extracts. In addition, the complexity of the obtained solutions would cause chromatographic contamination after few analyses, and coelution of matrix components, making really hard to obtain satisfactory analytical results for the target compounds. In most procedures, sample preparation is usually tedious and time consuming, and the use of hazardous solvents is frequently 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 [8, 14] or solid-phase extraction [4] have been recently applied for the determination of different additives in cosmetics. Recently, the authors have developed a pressurized solvent extraction procedure followed by gas chromatography–mass spectrometry (GC-MS) analysis for the determination of various groups of preservatives in leave-on cosmetics [15].

Matrix solid-phase dispersion (MSPD) was introduced by Barker et al. [16]. The possibility of performing extraction and clean-up at the same time is one of the main advantages of this technique, which reduces sample contamination during the procedure and decreases the amount of solvent required [17, 18]. MSPD developments and applications are compiled in several reviews [17-20]. This technique has been applied for the isolation of a wide variety of analytes, such as drugs, pesticides [21], polychlorinated biphenyls, antibiotics, surfactants, and naturally occurring compounds, in different matrices. It has also been applied to the analysis of some cosmetic ingredients including preservatives [21, 22] in environmental samples, but to our knowledge, this technique has not been applied to cosmetic samples.

The aim of this work is to develop a simple, efficient, and inexpensive MSPD method followed by derivatization GC-MS analysis to simultaneously determine different classes of preservatives such as bromine-containing preservatives, parabens, IPBC, TCS, and the antioxidants BHA and BHT, in a broad spectrum of cosmetic samples, including both rinse-off and leave-on products.

Table 1. IUPAC names, retention times, quantification, and identification ions of the target analytes.

Key	Compound	IUPAC name	CAS	Retention time (min)	Selected ions[a]	Maximum concentration[c]
	Bronidox	5-Bromo-5-nitro-1,3-dioxane	30007-47-7	7.09	85, 107, <u>135</u>, 137	0.1%[d]. The formation of nitrosamines should be avoided
	2-Phenoxyethanol	2-Phenoxy-1-ethanol	122-99-6	7.17	77, <u>94</u>, 138[b]	0.1%. The formation of nitrosamines should be avoided
	Bronopol	2-Bromo-2-nitropropane-1,3-diol	52-51-7	8.90	<u>115</u>,195[b]	
MeP	Methylparaben	Methyl 4-hydroxybenzoate	99-76-3	9.12	<u>121</u>, 152[b]	0.4 % (as acid) for single ester 0.8 % (as acid) for mixtures of esters
BHA	Butylated hydroxyanisole	2-tert-Butyl-4-methoxyphenol	121-00-6	9.18	<u>165</u>, 180[b]	
BHT	Butylated hydroxytoluene	2,6-Bis(1,1-dimethylethyl)-4-methylphenol	128-37-0	9.35	<u>205</u>, 220	
EtP	Ethylparaben	Ethyl 4-hydroxybenzoate	120-47-8	9.61	<u>121</u>, 138, 166[b]	0.4 % (as acid) for single ester
iPrP	Isopropylparaben	Isopropyl 4-hydroxybenzoate	4191-73-5	9.84	<u>121</u>, 138, 180[b]	0.8 % (as acid) for mixtures of esters
PrP	Propylparaben	Propyl 4-hydroxybenzoate	94-13-3	10.34	121, <u>138</u>, 166[b]	
IPBC		Carbamic acid, butyl-3-iodo-2-propynyl ester	55406-53-6	10.40	<u>165</u>, 182, 281	0.02 % (rinse-off)[e,f] 0.01 % (leave-on)[e,f,g] 0.0075 % (deodorants)[e,f]
iBuP	Isobutylparaben	Isobutyl 4-hydroxybenzoate	03/02/4247	10.82	93, 121, <u>138</u>[b]	0.4 % (as acid) for single ester
BuP	Butylparaben	Butyl 4-hydroxybenzoate	94-26-8	11.25	<u>121</u>, 138, 194[b]	0.8 % (as acid) for mixtures of esters
BzP	Benzylparaben	Benzyl 4-hydroxybenzoate	94-18-8	17.24	91, <u>121</u>, 228[b]	
TCS	Triclosan	2,4,4'-Trichloro-2'-hydroxydiphenyl ether	3380-34-5	17.37	218, <u>288</u>, 290[b]	0.3%

[a] Quantification ion is represented underlined

[b] Correspondent to the acetylated derivative

[c] Final concentration (%, w/w) in ready for use preparation according to Regulation (EC) No 1223/2009

[d] Only allowed in rinse-off products

[e] Not to be used in products for children under 3 years of age except in bath products/shower gel and shampoos

[f] Not to be used in oral and lip products

2. Experimental

2.1 Chemicals

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

Deuterated methyl-4-hydroxybenzoate-2,3,5,6-d_4 ($MePd_4$; 98 atom % D), used as surrogate standard, was obtained from C/D/N Isotopes (Quebec, Canada). The internal standard (IS) 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) was purchased from Supelco Analytical (Bellefonte, PA, USA). Neutral alumina, C_{18}, and sand (50–70 mesh) were achieved from Sigma-Aldrich (St. Louis, MO, USA). Silica gel 60 (230–240 mesh) was obtained from Merck KGaA (Darmstadt, Germany). Before being used, Florisil, alumina, and silica were activated at 130 °C for 12 h and then allowed to cool down 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 °C. All solvents and reagents were of analytical grade. Calibration standards were prepared covering a concentration range from 0.02 to 10 μg mL^{-1} in hexane/ acetone. Surrogate and the internal standards were added. The solutions were derivatized by adding 100 μL of acetic anhydride containing 2.5% of pyridine to 1 mL of the standard. The mixture was then maintained at 80 °C for 10 min, and then allowed to cool down before GC analysis.

2.2 Cosmetic samples

Different cosmetics from national and international brands were purchased from local sources. They included leave-on and rinse-off products such as body milk, moisturizing creams, anti-stretch marks creams, hand creams, makeup, sun milk, deodorant, shampoos

and liquid soaps, hand soaps, among others. Samples were kept in their original containers at room temperature until their analysis.

2.3 MSPD and derivatization procedures

A 0.5 g of cosmetic sample was exactly weighted into a 10 mL glass vial and spiked with 20 µL of $MePd_4$ surrogate solution (1,000 µg mL^{-1}). 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. After homogenization, the sample was gently blended with 1 g of a drying agent (anhydrous Na_2SO_4) and 2 g of dispersing sorbent into a glass mortar using a glass pestle until a homogeneous mixture was obtained (ca. 5 min). Then, the mixture was transferred into a column with a polypropylene frit at the bottom containing 0.5 g of Florisil (to obtain a further degree of fractionation and sample clean-up). A second frit was placed on top of the sample before compression with a syringe plunger. Elution was made by gravity flow with hexane/acetone (1:1, v/v), collecting 5 mL of extract into a graduated conical tube; 100 µL of PCB 30 solution (10 µg mL^{-1}) were finally added.

Acetylation was carried out by adding 100 µL of acetic anhydride containing 2.5% of pyridine to 1 mL of the standard or extract solutions. The mixture was then maintained at 80 °C for 10 min, and then allowed to cool down to room temperature. The derivatized MSPD extracts, diluted when necessary, were directly analyzed by GC-MS.

2.4 GC-MS analysis

The GC-MS analysis was performed using an Agilent 7890A (GC)-Agilent 5975C inert mass spectra detector (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 290, 150, and 230 °C, 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 80 °C (held 2 min) to 100 °C at 8 °C min^{-1}, to 150 °C at 20 °C min^{-1}, to 200 °C at 25 °C min−1 (held 5 min), to 220 °C at 8 °C min^{-1}, and a final ramp to 280 °C at 30 °C min^{-1}. Pulsed splitless mode was used for injection (25 psi, held 1.2 min). After 1 min, the split was opened at a flow of 75 mL min^{-1} and the injector temperature was kept at 220 °C. The injection volume was 1 µL. The MSD operated in selected ion

monitoring mode, monitoring at least two ions per compound (Table 1). The electron multiplier was set at a nominal value of 1,200 V.

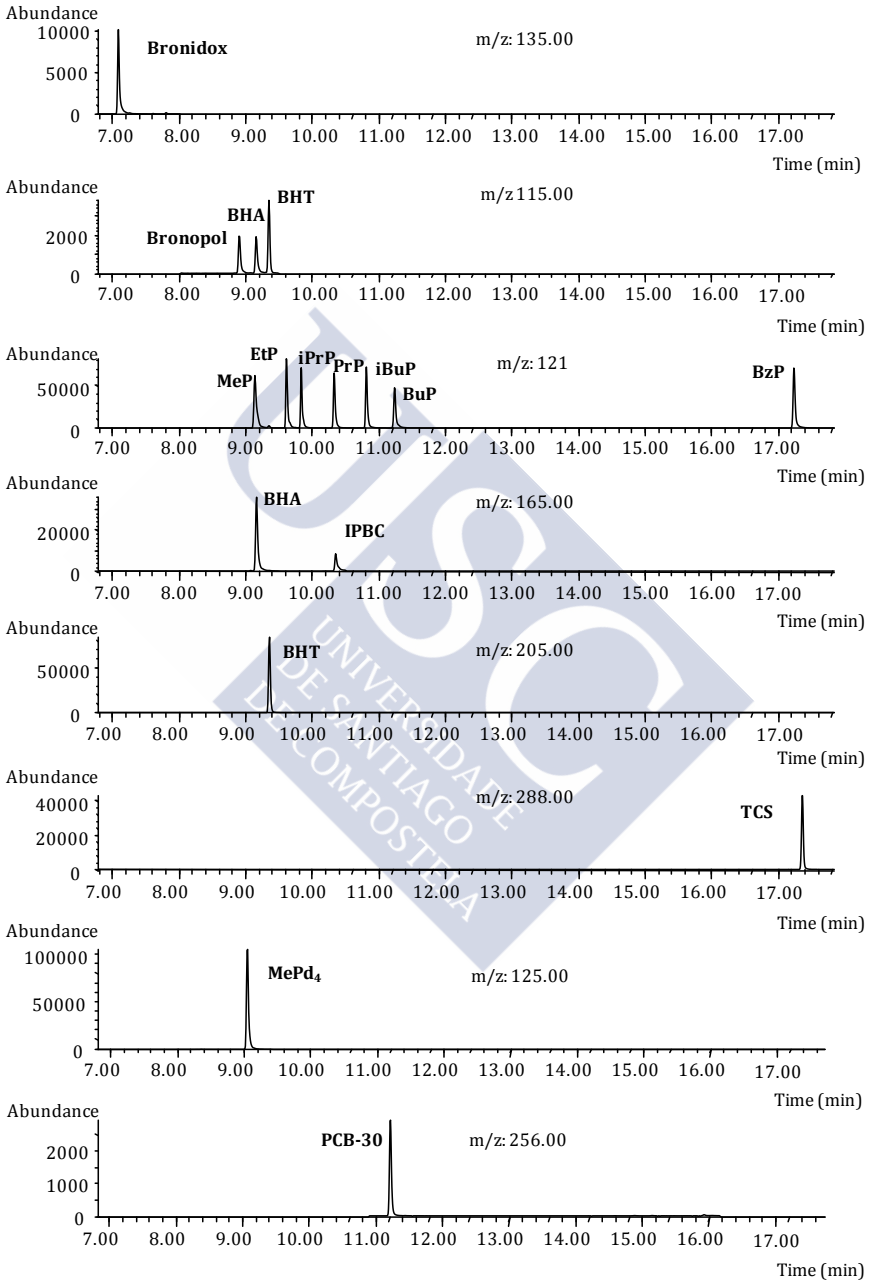

Fig. 1. Extracted ion chromatograms of a hexane/acetone standard solution containing 2 μg mL^{-1} of each preservative.

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 MSPD.

3. Results and discussion

3.1 Derivatization and GC-MS analysis

Optimization of the chromatographic conditions including a previous derivatization step to improve the chromatographic analysis was optimized elsewhere [15].

Different families of preservatives are studied in this work (see Table 1). Acetylation with acetic anhydride is one of the most simple and cheap derivatization procedures for phenolic compounds. Three of the compounds (bronidox, IPBC, and BHT) did not undergo derivatization. Bronidox and IPBC do not undergo derivatization since these compounds do not have chemical groups susceptible to acetylation. In the case of BHT, the acetylation could not be demonstrated since the retention time, peak shape, chromatographic response, and mass spectra were equivalent before and after the addition of the acetylation reagents. The highly hindered hydroxyl group with poor nucleophilicity may prevent the acetylation under the studied conditions. For the other compounds, reaction yield was quantitative, and satisfactory, improving significantly the chromatographic analysis of the target compounds.

No trace of the underivatized analytes was detected. The reaction was carried out with standard solutions in ethyl acetate and hexane/acetone (1:1, v/v) demonstrating the suitability of these solvents to accomplish derivatization. Derivatization time was studied in order to improve the throughput of the method; identical results were obtained when it was reduced from 30 [15] to 10 min and, therefore, this time was selected for the rest of the experiments. Figure 1 shows the extracted ion chromatograms of a derivatized standard solution obtained from the direct injection of a hexane/acetone solution containing 2 μg mL^{-1} of each preservative.

3.2 MSPD optimization

Efficiency of MSPD extraction depends on various experimental factors, being one of the most important the nature of the dispersing phase. Preliminary experiments were

conducted to study the influence of this parameter. Five different solid phases were tested: Florisil, neutral alumina, C_{18}, silica gel, and sand. The sample used consisted of a leave-on cosmetic (body milk) containing several of the target analytes. In these experiments we decided to work with the sample as it, without compound addition, to really evaluate the capability of MSPD to break analyte-matrix interactions in this kind of complex sample. In all cases, the MSPD was conducted applying the most usual sample/solid support material ratio (1 to 4), blending 2 g of solid support with 0.5 g of sample [20]. Since drying of the sample is essential for an efficient extraction, in all experiments 1 g of anhydrous sodium sulfate was added. In these initial trials, the MSPD column was eluted with two fractions of 5 mL of ethyl acetate, which were analyzed separately. All experiments were performed twice. The results obtained for the first fraction were analyzed by ANOVA, and they are given in Fig. 2. As can be seen, the responses obtained were statistically equivalent for the different phases.

Regarding the second fraction, the percentage of compound was in all cases lower than 0.5% compared with the first fraction, excluding alumina (1–2%), and so, only one fraction of 5 mL was considered for further experiments.

To investigate the simultaneous effect of the elution solvent and the dispersing phase, an experimental design was conducted. Considering the previous ANOVA study, only two sorbents (factor A) were selected: Florisil and sand. Sand was chosen due to its low cost, and Florisil was selected taken into account its dispersive and clean-up properties. For an efficient extraction, the solvent must solubilize the target compounds while leaving the sample matrix as intact as possible [20]. Considering the polar analyte nature, the derivatization and chromatographic performance, and our own experience [15], two

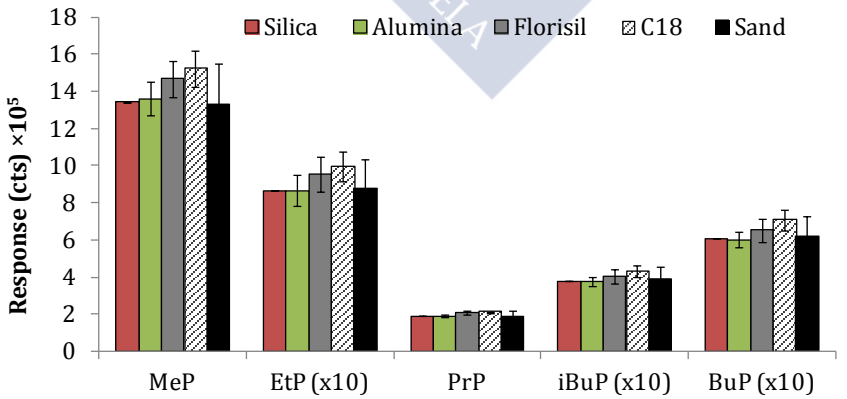

Fig. 2. Mean plots comparing the effect of the dispersing phase obtained in the one-way ANOVA study. The intervals displayed are based on Fisher's least significant difference (LSD).

solvents (factor B) were investigated: hexane/acetone (1:1, v/v) and ethyl acetate. Since we intended to develop a general method applicable to leave-on and rinse-off cosmetic samples, two different samples (factor C) were included in this study: a moisturizing lotion (leave-on) and a liquid hand soap (rinse-off). For this study, and with the aim of extending the research to all target compounds, the samples were fortified with all analytes (100 μg g^{-1}). The factorial experimental design 23 was carried out in duplicate, so the total number of experiments was sixteen. The extracts were acetylated at 80 °C for 10 min before GC-MS analysis (see experimental section). Data analysis was made with the statistical software package Statgraphics-Plus v5.1.

The global analysis of the results showed that the type of sample was statistically significant for most compounds. Therefore, we decided to analyze the design results in two blocks, the first one corresponding to the leave-on experiments and the second one to the rinse-off ones. Numerical analysis of the results obtained leads to the ANOVA table shown in Table 2. Regarding the rinse-off sample, main factors, dispersant and solvent, were not significant in all cases (excluding the dispersant BzP). However, the interaction factor was statistically significant for all compounds. The design results can be represented using several graphic tools. In Fig. 3 some representative graphics, showing the general behavior, are included. Figure 3a illustrates the Pareto charts. In these graphs, the length of each bar is

Table 2. *F* ratios and *p* values obtained in the analysis of variance.

	Rinse-off						Leave on					
	Dispersant (A)		Solvent (B)		Interaction (AB)		Dispersant (A)		Solvent (B)		Interaction (AB)	
	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	6.19		0.10		26.74	+	1.90		30.31	+	0.95	
Bronopol	2.46		0.39		20.99	+	0.32		3.14		2.94	
MeP	6.18		2.18		52.63	+	0.16		35.17	+	1.49	
BHA	0.30		6.56		12.65	+	2.72		46.49	+	5.83	
BHT	3.08		1.78		23.03	+	22.40	+	87.60	+	8.36	
EtP	5.57		0.30		44.32	+	0.38		38.69	+	2.26	
iPrP	5.37		0.09		53.74	+	1.05		41.39	+	2.79	
PrP	4.80		0.65		48.33	+	0.34		41.21	+	1.47	
IPBC	1.40		0.25		14.94	+	0.48		1.10		1.32	
iBuP	3.55		0.16		37.69	+	0.40		28.87	+	1.28	
BuP	3.20		0.37		37.57	+	0.09		31.19	+	2.49	
BzP	21.40	+	0.00		50.92	+	0.26		8.72		1.11	
TRC	5.72		0.03		29.96	+	0.76		11.89	+	1.03	

"+" denotes statistical significance (p<0.05)

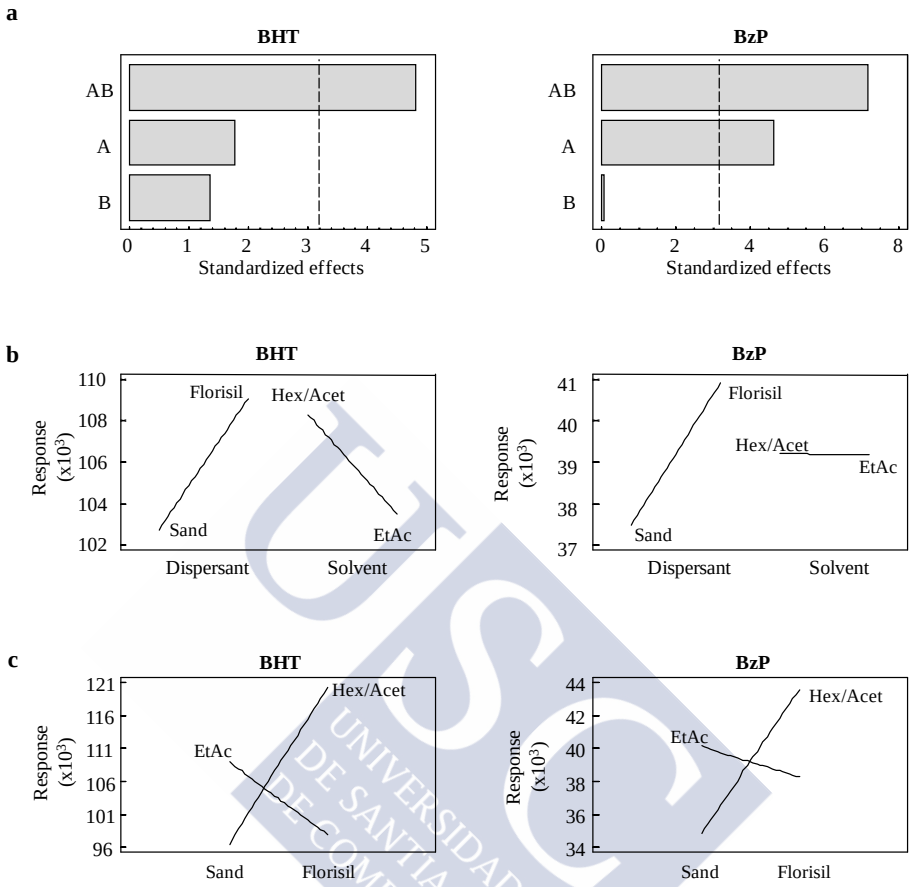

Fig. 3. Representative graphics showing the experimental design results for the rinse-off cosmetic: (a) Pareto charts, (b) main effects plots, and (c) interaction diagrams.

proportional to the absolute value of the standardized factor effect (vertical line represents the significance bound at the 95% confidence level). Figure 3b shows the main effects diagrams. 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. Finally, Fig. 3c shows the interaction plots, where the importance of the second order factor is clearly represented. Best extractions were obtained using Florisil as dispersing phase and hexane/acetone as eluting solvent.

Regarding the leave-on sample (see Table 2), the solvent employed was significant for most compounds, and the extraction was most efficient using hexane/acetone. The dispersant was non-significant with the exception of BHT (Florisil). The interaction between

both factors was also non-significant. Some illustrative graphics are included in Fig. 4, showing the most favorable conditions.

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In view of the results, the selected general conditions for the simultaneous extraction of the target preservatives and antioxidants both in leave-on and rinse-off cosmetics comprise the use of Florisil (2 g), as dispersant and the elution with hexane/acetone (5 mL).

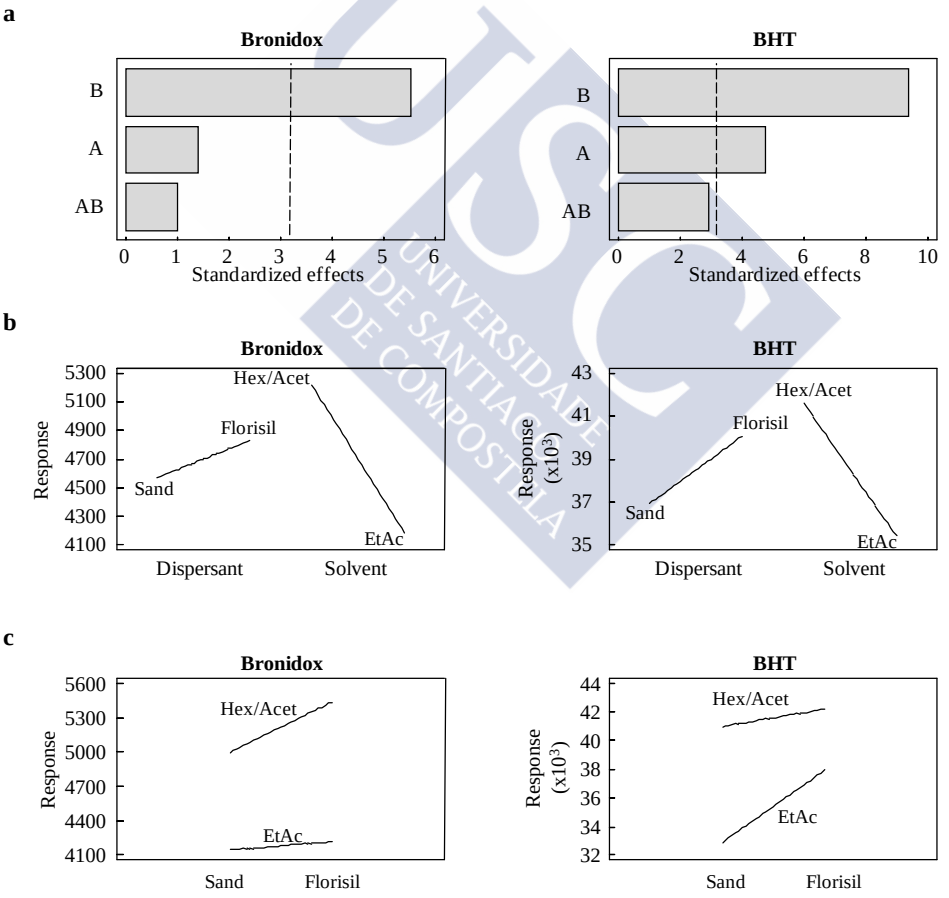

Fig. 4. Representative graphics showing the experimental design results for the leave-on cosmetic: (a) Pareto charts, (b) mean effects plots, and (c) interaction diagrams.

Table 3. Quality parameters of the method.

Compound	Correlation coefficient (R)	IDL (ng mL^{-1})	Recovery (RSD) (%)				LOD (%, w/w)	LOQ (%, w/w)
			100 µg g^{-1}			20 µg g^{-1}		
			HS2[a]	LS3	BM3[a]	HS2[a]		
Bronidox	0.9999	7.6	96.5 (2.7)	88.3	93.9 (6.0)	107 (6.1)	0.0000076	0.000025
Bronopol	0.9977	11	106 (11)	97.2	89.5 (11)	89.3 (12)	0.000012	0.000037
MeP	0.9989	0.35	99.4 (5.5)	n.c.	94.1 (6.4)	106 (4.1)	0.00000035	0.0000016
BHA	0.9990	0.54	104 (2.3)	94.5	100 (5.8)	91.2 (5.2)	0.00000034	0.0000011
BHT	0.9987	0.20	90.9 (3.6)	93.6	94.9 (10)	108 (5.9)	0.00000030	0.00000099
EtP	0.9990	0.15	95.6 (2.9)	88.9	93.9 (6.9)	110 (4.6)	0.00000022	0.00000073
iPrP	0.9989	0.88	91.0 (9.3)	93.6	98.9 (0.7)	107 (4.6)	0.00000038	0.0000013
PrP	0.9986	0.69	104 (0.9)	97.0	104 (1.6)	110 (3.5)	0.00000014	0.00000047
IPBC	0.9961	3.9	106 (1.3)	104	95.7 (8.9)	99.3 (4.1)	0.0000039	0.000013
iBuP	0.9990	0.23	96.6 (3.4)	104	95.7 (7.0)	101 (6.8)	0.00000025	0.00000085
BuP	0.9993	0.43	97.6 (2.4)	95.7	97.5 (7.0)	109 (4.8)	0.00000050	0.0000017
BzP	0.9997	0.20	95.8 (2.0)	110	101 (4.3)	96.0 (7.4)	0.00000021	0.00000071
TRC	0.9994	0.15	95.4 (0.8)	92.6	97.6 (5.1)	105 (4.2)	0.00000015	0.00000052

[a] (n=3).
n.c.: not calculated, the compounds were present in the sample at quite high concentration.

3.3 Method performance. Application to real samples

Method quality parameters were evaluated (Table 3). The instrumental linearity was proved at a concentration range between 0.02 and 10 μg mL^{-1} (including six concentration levels) using derivatized standard solutions prepared in hexane/acetone (see experimental section). Each concentration level was injected in duplicate or triplicate and the relative area (A/AIS) of each analyte was plotted versus its corresponding concentration. The response function was found to be linear with correlation coefficients (R) higher than 0.9961.

Instrumental detection limits were calculated as the concentration giving a signal-to-noise ratio of three (S/N= 3). Values ranged from 0.15 to 11 ng mL^{-1}, as can be seen in Table 3. Other validation parameters were calculated using real cosmetic samples. In this way, recovery studies were carried out by applying the optimized method to the extraction of three real samples spiked at 100 μg g^{-1}: a liquid soap (LS3), a body milk (BM3), and a hand soap (HS2). This last sample was also spiked at 20 μg g^{-1}. Previous analyses of the samples showed the presence of some of the target compounds (see Tables 4 and 5), and these initial concentrations were taken into account to calculate the recoveries. As can be seen in Table 3, recoveries were between 88% and 110% in all cases. Precision was also evaluated and relative standard deviation (RSD) values were lower than 10%, with an average value of 4.2%, excluding bronopol (11%).

The limits of detection and quantification of the overall method were calculated as the compound concentration giving a signal-to-noise ratio of three (S/N=3) and ten (S/N=10), respectively. These values are shown in Table 3, expressed as percentage (%, w/w) in order to be consequent with the units used in the European Cosmetics Regulation [1]. The obtained limits are much lower than the established restrictions, and it is important to emphasize that, if necessary, these limits could be reduced by concentrating the extract (5 mL).

Finally, the method was applied to the analysis of real cosmetic samples including rinse-off (hand soaps, hair conditioner, shampoo, and liquid soaps) as well as leave-on (lipstick and makeup, hand cream, sun milk, body milks, moisturizing creams, anti-stretch marks creams, deodorant, and face cream) products. Results are shown in Tables 4 and 5 for rinse-off and leave-on cosmetics, respectively. For all the samples, the recoveries of $MePd_4$ (surrogate standard) were satisfactory, with values ranging from 78.7 to 102 in rinse-off samples and ranged from 75.3 to 107% in leave-on samples (see last row, Tables 4 and 5). The maximum allowed concentration of parabens in ready for use preparations is 0.4% for single ester and 0.8% for mixtures of esters, expressed as acid [1]. For this reason, the total

content of parabens in the samples was determined and expressed as % (w/w) as acid, being included in both Tables.

In the case of rinse-off cosmetics (Table 4), BHT was the most frequently found target preservative, appearing in four out of seven samples. BHA was found in two samples, in one of them associated to 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 amount and concentration of parabens in the samples were quite low with an average value of less than two parabens per sample and an average total paraben concentration lower than 0.1% (w/w) expressed as acid. Triclosan was found in three samples. Bronidox was not detected in any sample while bronopol and IPBC were detected in just one sample each.

Regarding the leave-on samples, the general preservative content was clearly higher. Most samples presented five to six of the target analytes. BHT and the parabens MeP, PrP, and BuP were the most abundant preservatives found in more than ten out of 16 leave-on samples (Table 5). Triclosan was found in five samples, BHA in two, and Bronopol in one sample. Although in most of the samples the total paraben content can be considered quite high (>0.1% expressed as acid), the legal restrictions were fulfilled in all cases. The selected ion monitoring chromatogram corresponding to the sample BM2 is shown in Fig. 5.

Table 4. Analysis of rinse-off samples (%, w/w).

	HS1	HS2	HC	Sh	LS1	LS2	LS3
Bronidox							
Bronopol		0.004816					
MeP	0.057392		0.036310				0.116563
BHA		0.000371			0.000033		
BHT		0.009331	0.000061			0.000022	0.001011
EtP	0.013483		0.008605				
iPrP							
PrP	0.030030	0.000038	0.004851				
IPBC				0.000708			
iBuP			0.004222	<LOQ			
BuP		0.000056	0.008549				
BzP			<LOQ				
TCS	0.079422		0.000214			0.000006	
Total parabens[b]	*0.08634*	*0.000069*	*0.05293*				*0.10583*
Rec MePd₄ (%)[c]	*78.7*	*99.6*	*98.4*	*102*	*91.9*	*98.6*	*90.4*

[a]HS: hands soap, HC: hair conditioner, Sh: shampoo, LS: liquid soap
[b]Expressed as acid
[c] Recovery of the surrogate (%)
Blank spaces mean the values are below the LOD

Table 5. Analysis of leave-on samples (%, w/w).

	LS	MU1	MU2	HG	SM	BM1	BM2	BM3	BM4	MC	AS1	AS2	HC1	HC2	De	FC
Bronidox																
Bronopol					0.014183											
MeP		0.130004	0.169633		0.083229	0.163594	0.083761		0.000568		0.032191	0.079745	0.083707	0.071012		0.079370
BHA			0.026398						0.000591							
BHT	0.000034	0.000119	0.000251	0.000044	0.002509				0.072174		0.000017	0.006991	0.000021	0.000545	0.006067	
EtP		0.031379	0.003123			0.011557	0.028908		0.000114			0.019574				0.019427
iPrP																
PrP	0.136834	0.016105	0.084301		0.030361	0.048098	0.018393		0.000381	0.000012	0.014903	0.012391	0.057053	0.067021		0.011395
IPBC																
iBuP		0.014106		0.000010		0.005056	0.011769									0.008172
BuP	0.000448	0.030921	0.006489	<LOQ	0.000150	0.012831	0.029549		0.000141	<LOQ	0.000058	0.026357	0.000072	0.000188		0.019872
BzP															0.000020	
TCS				0.076628		0.000040						0.000031			0.075505	0.000059
Total parabens[b]	*0.10522*	*0.18849*	*0.22585*	*0.000017*	*0.09895*	*0.20773*	*0.143571*		*0.00100*	*0.00001*	*0.04070*	*0.11692*	*0.11979*	*0.11599*	*0.00001*	*0.11690*
Rec $MePd_4$ (%)[c]	*79.5*	*102*	*104*	*85.3*	*92.5*	*75.3*	*97.0*	*82.1*	*104*	*107*	*81.2*	*92.2*	*97.5*	*91.6*	*87.4*	*105*

[a]LS: lipstick, MU: makeup, HG: hands gel, SM: sun milk, BM: body milk, MC: moisturizing cream, AS: anti stretch marks, HS: hands cream, De: deodorant, FC: face cream
[b]Expressed as acid
[c] Recovery of the surrogate (%)
Blank spaces mean the values are below the LOD

According to the European Regulation [1], a list of ingredients should be included on the label of the cosmetic product. The most of the samples were properly labeled with some exceptions. Bronopol was included as ingredient of all the samples containing this compound. Parabens were, in general, included as ingredients on the cosmetic labels. Nevertheless, BHA was just included on the label of one of the samples (MU2), BHT was only included in the 25% of the labels of the positive samples, TCS was listed in three out of eight positive samples, and IPBC was not included as an ingredient of sample Sh.

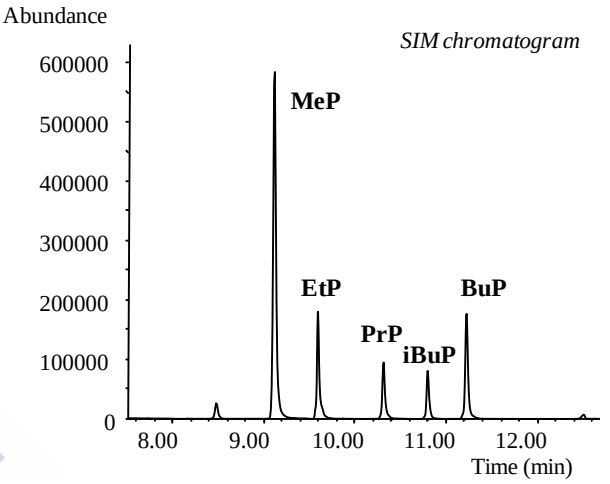

Fig. 5. Chromatogram of a real cosmetic sample (BM2, see concentrations in Table 5)

4. Conclusions

MSPD followed by derivatization and GC-MS analysis has been successfully applied to the determination of multiclass preservatives, including two bromine-containing preservatives, seven parabens, IPBC, TCS, and the antioxidant preservatives BHA and BHT, in leave-on and rinseoff cosmetics. To our knowledge, this study constitutes the first application of MSPD to cosmetics analysis. Optimization was carried out using real cosmetic samples and several statistical tools. Recovery studies were performed on leave-on and rinse-off samples, verifying the reliability of the optimized procedure.

The method was applied to a broad range of cosmetics demonstrating the high use of most of the target preservatives, especially BHT, and MeP, PrP, and BuP. Regarding paraben content, the concentrations found were below the legal limits in all cases.

With the developed method, some of the most relevant criteria required for an extraction procedure, such as low solvent consumption, and short process times are fulfilled. In addition, MSPD presents other clear advantages: it does not require special extraction equipment, it is cheap, and it can be easily implemented in any laboratory.

Acknowledgments

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Capítulo III.1.2

Determination of suspected fragrance allergens in cosmetics by matrix solid-phase dispersion gas chromatography-mass spectrometry analysis

Lucia Sánchez-Prado, J. Pablo Lamas, Gerardo Álvarez-Rivera, Marta Lores, Carmen García-Jares, and María Llompart

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Determination of suspected fragrance allergens in cosmetics by matrix solid-phase dispersion gas chromatography mass spectrometry analysis

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Abstract

An effective low cost sample preparation methodology and easily implementation in any laboratory, compared to that previously developed based on pressurized solvent extraction (PSE), for the determination of regulated fragrance allergens in leave-on and rinse-off cosmetics has been developed applying, for the first time, matrix solid-phase dispersion (MSPD) to this kind of analytes and samples.

The selection of the most suitable extraction conditions was made using statistical tools such as ANOVA, as well as a factorial multifactor experimental design. These studies were carried out using real cosmetic samples. In the final conditions, 0.5 of sample, previously mixed with 1 g of anhydrous Na_2SO_4, were blended with 2 g of dispersive sorbent (Florisil), and the MSPD column was eluted with 5 mL of hexane/acetone (1:1). The extract was then analyzed by GC-MS without any further clean-up or concentration step.

Accuracy, precision, linearity and detection limits (LODs) were evaluated to assess the performance of the proposed method. Quantitative recoveries (>75%) were obtained and RSD values were lower than 10% in all cases. The quantification limits were well below those set by the international cosmetic regulations, making this multi-component analytical method suitable for routine control. Finally, a wide variety of cosmetic products were analyzed. All the samples contained several of the target cosmetic ingredients, with and average number of seven. The total fragrance allergen content was in general quite high, even in baby care products, with values close to or up to 1%, for several samples.

Keywords: Matrix solid-phase dispersion; Cosmetics; Fragrance allergens; Fragrances; Experimental design; Personal care products; GC–MS.

1. Introduction

Some of the fragrance chemicals, widely used in every day products, have been shown to cause various side effects, like skin sensitivity, rashes, dermatitis, coughing, asthma attacks, migraine, etc. [1-4]. Legislations in the European Union [5], the United States (US) [6] and Japan [7], establish that all the components of cosmetics should be included on the label. Two different restrictions are applied to several suspected fragrance allergens in the Annex III of the EU Cosmetics Directive [5], 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% (w/w) in leave-on products and 0.01% (w/w) in rinse-off products (see in Table 1 the compounds considered in this study and their limitations). The confirmed and suspected negative effects on the health of such substances may drive in the future to lower these limits and even to establish maximum allowed concentration for many of these substances. In fact, it has been already observed the inclusion of the term "fragrance free" in several cosmetic products as a positive characteristic. These requirements imply reliable procedures to detect and quantify low levels of these ingredients in highly complex mixtures. These procedures must be versatile considering the wide variety of cosmetic products and the range of fragrance allergen concentrations [8]. Sample preparation is an essential step since the direct analysis of cosmetic samples, such as creams, lotions, etc. is quite problematic due to the difficulty to obtaining homogeneous solutions, the coelution of the matrix components, and the contamination of the chromatographic system [9]. Given the highly complex mixtures of fragrances and raw materials used in cosmetics, and to prevent false positives and false negatives, the GC-MS determination in full scan mode is recommended to accomplish the analysis of fragrance allergens in cosmetics [10-12]. Therefore, the development of analytical methods for the determination of fragrance allergens in leave-on as well as rinse-off cosmetics is as challenging as necessary; moreover, considering that the literature is somewhat scarce in this subject.

Recently, the authors have developed a pressurized solvent extraction (PSE) procedure followed by GC-MS analysis for the determination of regulated fragrance allergens in leave-on cosmetics [13]. The PSE method has proved to be an efficient and rapid technique for the extraction of this kind of targets from cosmetic samples. The obtained results were fully satisfactory in terms of LODs, recoveries, repeatability and reproducibility. Nevertheless, one of the main drawbacks of this methodology is the high cost of the instrumentation compared to the low cost of other techniques such as matrix solid-phase dispersion (MSPD), which does not require special instrumentation.

MSPD was introduced by Barker et al [14]. MSPD involves blending a viscous, solid or semisolid sample with a solid support. The shearing forces of blending with a mortar and pestle disrupt the gross architecture of the sample, breaking the material in smaller pieces. At the same time, sample components dissolve and disperse into the bound organic phase on the surface of the particle, leading to complete disruption of the sample and its dispersion over the surface. The possibility of performing extraction and clean-up at the same time is one of the main advantages of this technique, which reduces sample contamination during the procedure and decreases the amount of solvent required [15, 16]. MSPD developments and applications are compiled in several reviews [15-19]. This technique has been applied for the isolation of a wide variety of analyte classes, such as drugs, pesticides, polychlorinated biphenyls, antibiotics and antibacterial, surfactants and naturally occurring compounds, in very different matrices (food, biota, vegetables and environmental samples) [20-22].

The aim of this work is to develop a simple, rapid and low cost method based on MSPD followed by gas chromatography-mass spectrometry (GC-MS) to simultaneously identify and quantify 25 fragrances in multi-matrix cosmetic samples, including both products designed to remain on the skin and rinse-off products, which can be easily implemented in any laboratory. To our knowledge, MSPD 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 part

2.1 Reagents and materials

The 25 fragrance allergens considered in this study are listed in Table 1, where their common and chemical name, CAS number, purity and supplier are included.

Internal standard PCB-30 (2,4,6-trichlorobiphenyl) was purchased from Dr. Ehrenstorfer (Augsburg, Germany). Acetone, ethyl acetate, and *n*-hexane were provided by Merck (Darmstadt, Germany). Florisil (60-100 mesh) was purchased from Supelco Analytical (Bellefonte, PA, USA). Neutral alumina, C_{18}, and sand (50-70 mesh) were achieved from Sigma-Aldrich (St. Louis, MO, USA). Silica gel 60 (230-240 mesh) was obtained from Merck KGaA (Darmstadt, Germany). Before being used, Florisil, alumina and silica were activated at 130 °C for 12 h and then allowed to cool down in a desiccator. Sodium sulphate anhydrous (99%) was purchased by Panreac (Barcelona, Spain).

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

Common name	Chemical name	CAS number	Purity	Retention time (min)	Qualifiers and quantifiers
Limonene[a]	(4R)-1-Methyl-4-(1-methylethenyl)cyclohexene	5989-27-5	97%[d]	4.55	68,93,121,136
Benzyl alcohol[b]	Benzene methanol	100-51-6	99%[e]	4.66	77,79,107,108
Linalool[a]	3,7-Dimethyl-1,6-octadien-3-ol	78-70-6	97%[d]	5.67	71,93,121,136
Methyl-2-octynoate[a]	Methyl heptin carbonate	111-12-6	≥99%[f]	6.90	79,95,123,139
Citronellol[a]	(±)-3,7-Dimethyloct-6-en-1-ol	106-22-9/ 26489-01-0	95%[d]	7.16	69,81,95,123
Citral[a]	3,7-Dimethyl-2,6-octadienal	5392-40-5	95%[d]	7.32 7.59	69,84,94,109
Geraniol[a]	3,7-Dimethyl- (2E)-2,6-octadien-1-ol	106-24-1	≥96%[g]	7.43	69,93,111,123
Cinnamal[a]	3-Phenyl-2-propenal	104-55-2	≥93%[f]	7.63	77,103,131
Anise alcohol[a]	4-Methoxybenzyl alcohol	105-13-5	98%[d]	7.70	94,109,121,138
Hydroxycitronellal[a]	7-Hydroxy-3,7-dimethyloctanal	107-75-5	≥95%[f]	7.73	43,59,71
Cinnamyl alcohol[a]	3-Phenyl-2-propen-1-ol	104-54-1	98%[f]	7.91	92,105,115,134
Eugenol[a]	2-Methoxy-4-(2-propenyl)-phenol	97-53-0	99%[d]	8.31	131,149,164
Methyleugenol[c]	1,2-Dimethoxy-4-(2-propenyl)-benzene	93-15-2	99%[d]	8.61	147,163,178
Isoeugenol[a]	2-Methoxy-4-(1-propenyl)-phenol	97-54-1	98%[d]	8.67 8.98	131,149,164
Coumarin[a]	2H-1-Benzopyran-2-one	91-64-5	99%[d]	8.92	89,118,146
α-Isomethyl ionone[a]	3-Methyl-4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-3-buten-2-one	127-51-5	≥85%[g]	9.16	107,135,150
Lilial®[a]	2-(4-tert-Butylbenzyl) propionaldehyde	80-54-6	≥95%[g]	9.45	131,147,189
Amyl cinnamal[a]	2-Benzylideneheptanal	122-40-7	97%[e]	10.30	115,129,202
Lyral®[a]	Hydroxyhexyl-3-cyclohexene carboxaldehyde	31906-04-4	≥97%[g]	10.42	93,105,136,192
Amylcinnamyl alcohol[a]	2-Pentyl-3-phenylprop-2-en-1-ol	101-85-9	≥85%[g]	10.58	91,115,133,204
Farnesol[a]	3,7,11-Trimethyl-2,6,10-dodecatrien-1-ol	4602-84-0	95%[d]	10.63 10.84	69,81,93,133
Hexyl cinnamal[a]	2-Benzylideneoctanal	101-86-0	≥95%[f]	11.15	115,129,145,216
Benzyl benzoate[a]	Phenylmethyl benzoate	120-51-4	98%[e]	11.38	77,91,105,212
Benzyl salicylate[a]	Benzyl-2-hydroxybenzoate	118-58-1	≥99%[g]	12.63	65,91,228
Benzyl cinnamate[a]	3-Phenyl-2-propenoic acid phenylmethyl ester	103-41-3	99%[d]	16.20	91,103,131,192

[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 and 0.01% in rinse-off products.
[b] Maximum allowed concentration 1% (for use other than as a preservative).
[c] Maximum allowed concentration in fragrance cream: 0.002 %, rinse-off products: 0.001%; and other leave-on products: 0.0002 %.
Purchased from:
[d] Sigma-Aldrich Chemie GmbH (Germany); [e] Chem Service (West Chester, USA); [f] SAFC Supply Solutions (St. Louis, USA); [g] Fluka Chemie GmbH (Steimheim, Germany).

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 °C. 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 leave-on and rinse-off products such as moisturizing creams and lotions, anti-cellulite creams, hand creams, shampoos and gels, hair conditioners, and hand soaps. Samples were kept in their original containers at room temperature until their analysis.

2.3 MSPD procedure

0.5 grams of cosmetic sample were exactly weighted 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. The sample was gently blended with 1 g of a drying agent (anhydrous Na_2SO_4) and 2 g of the dispersive sorbent into a glass mortar using a glass pestle, until a homogeneous mixture was obtained (ca. 5 min). Then, the mixture was transferred into a column with a polypropylene frit at the bottom containing 0.5 g of Florisil (to obtain a further degree of fractionation and sample clean-up). A second frit was placed on top of the sample before compression with a syringe plunger. Elution was made by gravity flow with ethyl acetate or hexane/acetone (1:1, v/v), depending on the experiment. 5 mL of eluents were collected into a graduated conical tube and 50 µL of PCB 30 solution (200 µg mL^{-1}) were finally added. The MSPD extracts, diluted when necessary (dilution factors of 1:10 to 1:1000), were directly analyzed by GC-MS.

2.4 GC-MS analysis

Analyses were performed on 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 290, 150 and 230 °C, respectively. The system was operated by Agilent MSD ChemStation E.02.00.493 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 80 °C (held 2 min) to 100 °C at 8 °C min^{-1}; to 150 °C at 20 °C min^{-1}; to 200 °C (held 5 min) at 25 °C min^{-1}; to 220 °C at 8 °C min^{-1}; and a final ramp to 290 °C (held 6 min) at 30 °C min^{-1} (total analysis time = 25 min). Pulsed splitless mode was used for injection (30 psi, held 1.2 min). After 1 min the split was opened at a flow of 75 mL min^{-1} and the injector temperature was kept at 220 °C. The injection volume was 2 µL.

Table 2. F-ratios and p-values obtained in the analysis of variance study.

Compound	*F*-ratio	*p*-value
Limonene	86.75	**0.0001**
Benzyl alcohol	6.51	**0.0322**
Linalool	10.33	**0.0124**
Geraniol	6.06	**0.0371**
Coumarin	6.89	**0.0288**
α-Isomethyl- ionone	5.95	**0.0385**
Lilial	3.63	0.0950
Hexyl cinnamal	2.70	0.1522
Benzyl benzoate	3.69	0.0925
Benzyl salicylate	2.3	0.1933

p-values lower than 0.05 indicate statistical significance

The mass spectra detector (MSD) was operated in the scan mode and the mass range was varied from 40 to 300 m/z, starting at 4 minutes and ending at 25 min. The electron multiplier was set at a nominal value of 1300 V. The analytes were positively identified by comparison of their mass spectra and retention times to those of the standards. Table 1 summarizes the retention times as well as the qualification and quantification ions of the target analytes.

3. Results and discussion

The chromatographic method for the separation of the target fragrance allergens was optimized elsewhere [23, 24] and it is described in the Experimental section.

3.1 MSPD Optimization

One of the most important steps in the development of an efficient MSPD method is the selection of the dispersive phase. First experiments were carried out to study the influence of this parameter, using a real non-spiked cosmetic sample. Five sorbents were considered: alumina, Florisil, silica, sand and C_{18}.The sample consisted of a leave on cosmetic (a body milk), containing 10 of the target compounds. We chose a sample with a high

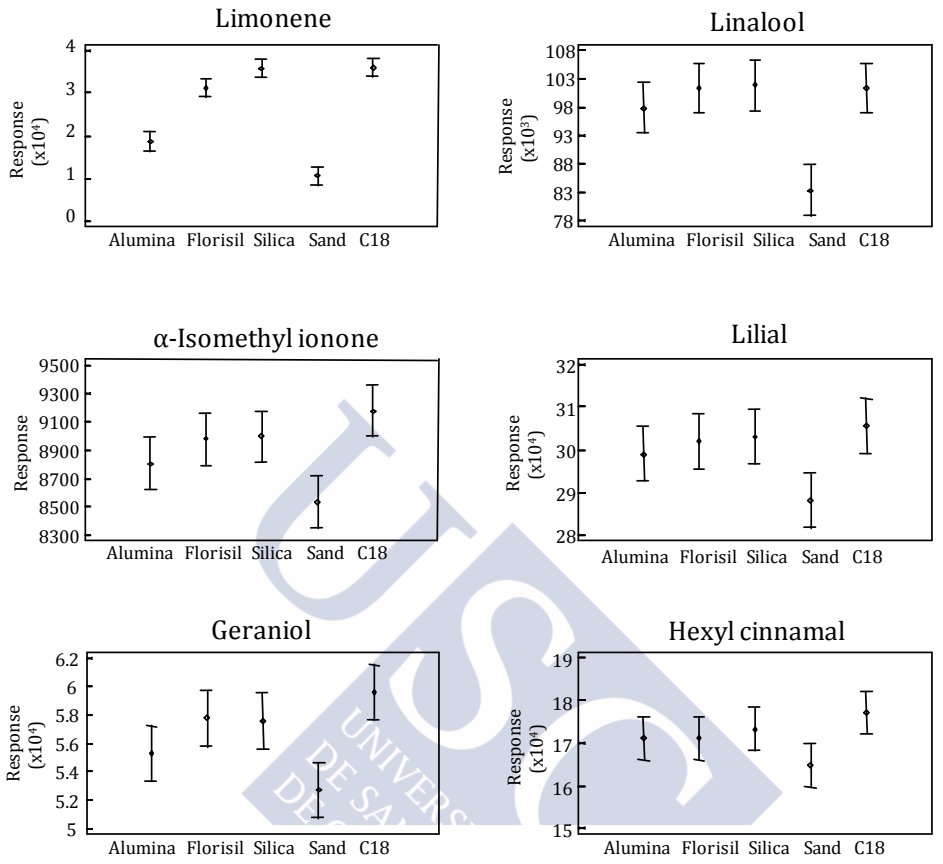

Fig. 1. Mean plots for several representative allergens obtained in the one-way ANOVA study.

number of fragrance allergens since we wanted to work with the sample as it (without spiking), to really evaluate the capability of MSPD to break the analyte-matrix interactions. MSPD was conducted applying the most usual sample/solid support material ratio (1 to 4), blending 2 g of solid support with 0.5 g of sample [17]. Since drying of the sample is essential for an efficient extraction, 1 g of anhydrous sodium sulphate was added in all experiments. The MSPD column was eluted with two fractions of 5 mL of hexane/acetone that were analyzed separately by GC-MS. All experiments were performed twice. The 10 fragrances included in the cosmetic label were extracted, and they were detected in the first fraction, independently of the sorbent used. Regarding the second fraction, only three of the compounds (lilial®, hexyl cinnamal and benzyl salicylate) were detected; in those cases, the

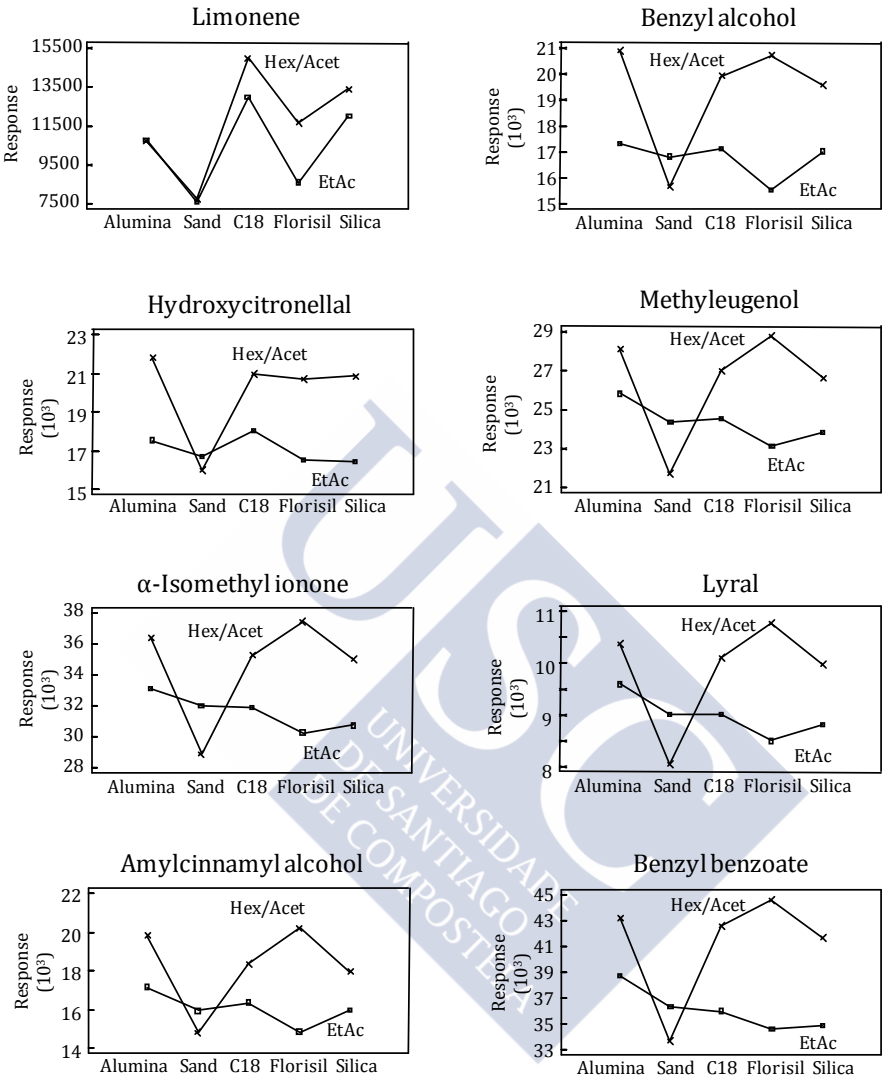

Fig. 2. Interaction plots for some representative fragrance allergens (Hex/Acet: hexane/acetone; EtAc: ethyl acetate).

chromatographic response was lower than 0.2% compared with the first fraction, which may indicate that a solvent volume of 5 mL is sufficient to elute the MSPD column.

The results obtained for the first fraction were analyzed by ANOVA. For most compounds, the sorbent used was statistically significant (Table 2). Analyzing the multiple

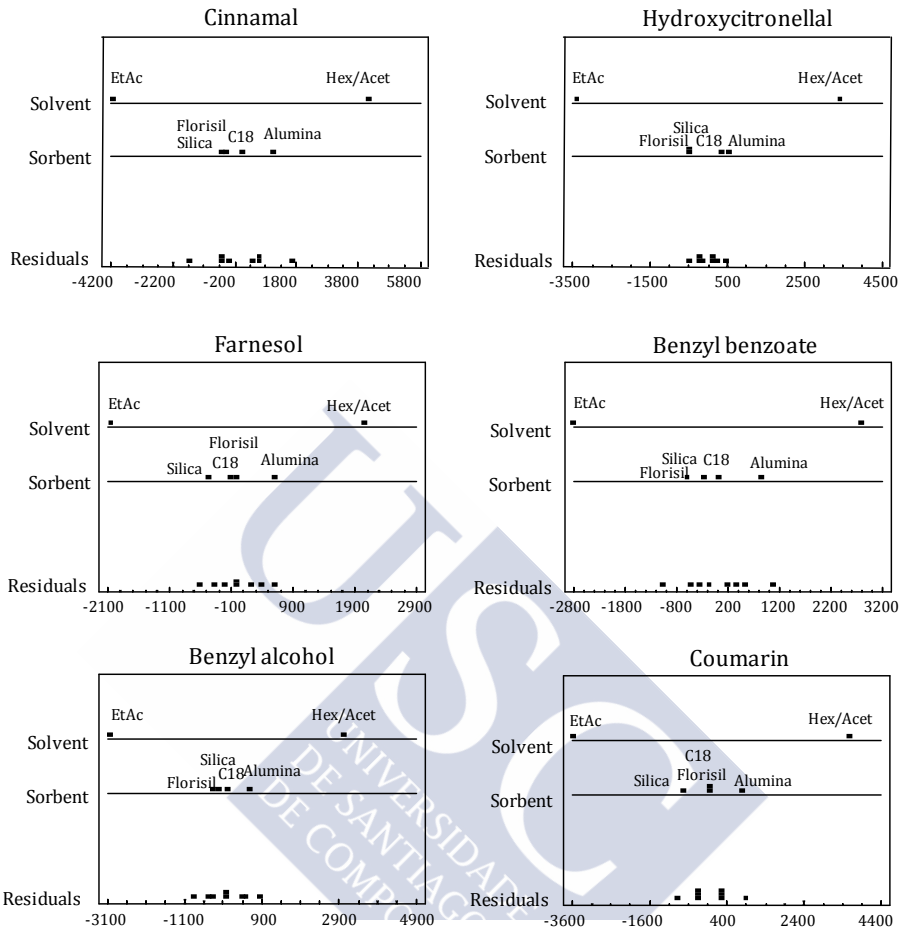

Fig. 3. ANOVA plots for several fragrance allergens.

range tests, we could realize that, in many cases, the results with the different sorbents were equivalent excluding sand, which gave lower general results (Figure 1).

To extent the study to all the 25 compounds considered (see Table 1), we perform an experimental design using, in this case, a moisturize lotion originally containing a low number (only 2) and low concentration of fragrance allergens. The sample was spiked with the target compounds (100 μg g^{-1}). Although considering the previous ANOVA the sand could be discarded, it would not significantly reduce the number of experiments, so we maintained all 5 sorbents (factor A). The second factor considered was the elution solvent (factor B), that it was studied at two levels: hexane/acetone (1:1, v/v), and ethyl acetate. Both solvents have

intermediate polarity, which should favour the simultaneous extraction of all the analytes. In both cases, the solvent volume was 5 mL. The study consisted of a multifactor categorical 5x2 design, involving 10 randomized experiments.

The results obtained are represented in the two factor plots in Figure 2. For simplicity, only 8 of the 25 compound graphic are included. As can be seen, the sand was once again a not a suitable sorbent, displaying the lowest extraction efficiency. On the other hand, we can observe that hexane/acetone is the most suitable solvent in all cases excluding sand, which gave higher response in combination with ethyl acetate. ANOVA analysis of the design results was performed excluding the sand experiments. The ANOVA results are graphically displayed in Figure 3, which includes some representative examples, since the general behaviour was equivalent for all analytes. This kind of plots shows the scaled effects of each factor so that the natural variance of the points in the diagram can be compared to that of the residuals, displayed at the bottom of the plot. By comparing the variability amongst the factors to that of the residuals, it is easy to identify those showing differences of a greater magnitude than could be solely accounted by the experimental error. It can be clearly observed that the solvent is a significant factor, and higher efficient is achieved using hexane/acetone (on the right of the graphics). On the other hand, the sorbent is not significant for all the compounds.

After optimization, the proposed MSPD conditions include the dispersion of the sample with Florisil (2 g), although other three sorbents (silica, C_{18}, and alumina) are also suitable, and the elution with 5 mL hexane/acetone (1:1, v/v).

3.2 Method performance

Method quality parameters were evaluated and they are included in Table 3. The instrumental linearity was proved at a concentration range between 0.05 and 10 μg mL^{-1} (including seven concentration levels) using standard solutions prepared in hexane/acetone (see Experimental section). Each concentration level was injected in triplicate or duplicate and the response function was found to be linear with correlation coefficients (R) higher than 0.9995.

Instrumental detection limits (IDL) were calculated as the concentration giving a signal-to-noise ratio of three (S/N=3). Values ranged from 1 to 12 ng mL^{-1} in most cases (Table 3). The other figures of merit were calculated using real cosmetic samples.

Table 3. Quality parameters of the method.

Compound	Correlation Coefficient (R)	IDL (ng mL^{-1})	Intra-day precision (%)[a]	Inter-day precision (%)[b]	Recovery (RSD) (%)[a]				LOD (%, w/w)	LOQ (%, w/w)
					"Leave-on"		"Rinse-off"			
					20 μg g^{-1}	100 μg g^{-1}	20 μg g^{-1}	100 μg g^{-1}		
Limonene	0.9997	5.5	0.19	5.9	85.4 (5.3)	98.3 (9.1)	79.3 (8.0)	75.3 (2.5)	0.000021	0.000070
Benzyl alcohol	1.0000	6.0	1.5	3.0	95.0 (3.3)	101 (3.7)	98.9 (1.0)	111 (6.1)	0.0000060	0.000020
Linalool	1.0000	5.4	2.0	5.5	98.2 (6.8)	92.8 (6.6)	105 (5.5)	105 (3.9)	0.0000081	0.000027
Methyl-2-octynoate	0.9999	11	0.8	4.1	88.0 (11)	107 (2.7)	100 (0.7)	101 (4.2)	0.000011	0.000037
Citronellol	1.0000	12	0.3	10	89.1 (2.3)	101 (6.2)	108 (1.3)	106 (3.4)	0.000042	0.00014
Citral	0.9999	12	3.9	4.6	98.5 (6.8)	99.3 (3.6)	83.5 (11)	87.3 (7.0)	0.000095	0.00032
Geraniol	0.9999	14	3.0	6.5	91.1 (8.2)	106 (6.7)	110 (2.8)	108 (4.7)	0.000071	0.00024
Cinnamal	0.9999	2.0	1.1	4.0	93.3 (12)	101 (0.9)	92.8 (9.8)	75.9 (9.9)	0.0000020	0.0000067
Anise alcohol	1.0000	11	3.1	5.8	83.5 (10)	95.8 (7.6)	104 (1.5)	103 (5.0)	0.000011	0.000037
Hydroxycitronellal	0.9999	3.0	4.1	5.3	93.5 (8.0)	103 (0.9)	92.1 (9.0)	77.2 (2.2)	0.000015	0.000050
Cinnamyl alcohol	0.9999	11	3.3	8.4	100 (13)	106 (3.9)	116 (3.1)	105 (9.2)	0.000011	0.000037
Eugenol	1.0000	2.0	0.84	2.0	112 (2.1)	99.7 (7.0)	93.9 (5.1)	104 (4.3)	0.0000020	0.0000067
Methyleugenol	0.9998	1.5	1.4	3.7	109 (3.8)	91.0 (6.2)	103 (8.5)	106 (4.1)	0.0000015	0.0000050
Isoeugenol	0.9995	12	1.9	8.9	108 (4.4)	110 (4.7)	115 (1.3)	102 (4.7)	0.000012	0.000040
Coumarin	0.9998	12	2.5	3.5	102 (3.0)	99.7 (2.2)	96.8 (9.3)	114 (8.1)	0.000012	0.000040
α-Isomethyl ionone	0.9998	3.8	0.80	4.7	112 (2.2)	94.2 (4.9)	109 (7.3)	102 (4.2)	0.0000038	0.000013
Lilial®	1.0000	12	3.3	4.0	103 (11)	115 (3.5)	n.c.	n.c.	0.000012	0.000040
Amyl cinnamal	0.9998	13	0.21	5.3	105 (3.7)	96.8 (1.2)	113 (7.2)	100 (5.3)	0.000013	0.000043
Lyral®	0.9999	11	0.52	3.5	94.8 (1.4)	112 (2.3)	97.9 (2.4)	77.6 (5.3)	0.000011	0.000037
Amylcinnamyl alcohol	0.9999	12	1.2	4.7	93.5 (6.3)	96.5 (4.0)	113 (4.0)	109 (1.9)	0.000012	0.000040
Farnesol	0.9999	60	1.5	8.3	94.5 (5.8)	90.4 (1.7)	99.9 (5.1)	99.0 (3.2)	0.00006	0.00020
Hexyl cinnamal	0.9997	7.9	0.94	4.8	106 (1.8)	101 (3.0)	106 (6.0)	100 (6.2)	0.0000079	0.000026
Benzyl benzoate	0.9996	4.2	1.5	4.4	95.2 (4.9)	101 (1.9)	102 (2.6)	104 (4.3)	0.0000042	0.000014
Benzyl salicylate	0.9999	11	3.1	3.4	102 (7.5)	118 (1.7)	109 (4.2)	88.7 (7.2)	0.000011	0.000037
Benzyl cinnamate	0.9999	11	1.6	3.6	112 (1.6)	98.1 (4.9)	115 (3.3)	108 (3.5)	0.000011	0.000037

n.c.: not calculated, lilial was present in the sample at high concentration.
[a] (n=3).
[b] (n=5)

Recovery studies were carried out by applying the optimized method to the extraction of three real samples, including both leave on and rinse off samples spiked at 20 μg g^{-1} and 100 μg g^{-1}. Previous analyses of the samples showed the presence of some of the target analytes and these initial concentrations were taken into account to calculate the recoveries. As can be seen in Table 3, recoveries were between 75 and 118% in all cases. Precision was also evaluated and RSD values were in most cases lower than 10% with an average value of 1.7% and 5.1% for intra- and inter-day precision, respectively.

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 three (S/N=3) and ten (S/N=10), respectively. These values are shown in Table 3, expressed as percentage (%, w/w) in order to be consequent with the units used in the European Cosmetics Regulation [5]. The obtained limits are much lower than the established restrictions, and it is important to emphasize that, if necessary, these limits could be reduced by concentrating the extract (5 mL).

3.3 Application to real samples

The method was applied to the analysis of real leave-on and rinse-off cosmetic samples. Leave-on cosmetics included: moisturizing creams (MC) and lotions (ML), an anti-cellulite cream (AC), moisturizing lotions for babies (MLB), and a hand cream (HC). As regards rinse-off products, three different shampoos (Sh), a hair conditioner (HC), gels (G) and hand soap (HS), and baby gels (BG) were analyzed.

Found concentrations in rinse-off products are included in Table 4 and they ranged from 0.000057 (eugenol in sample G) to 0.47% (w/w) (anise alcohol in sample Sh2), with an average value of 6 fragrance allergens per sample, and an average concentration of 0.025% (w/w). All the samples contained at least three different targets. 9 out of 25 fragrance allergens were not detected in any sample, whereas the most common ones were linalool (identified in 7 out of 8 samples) and limonene and benzyl alcohol (both found in 6 out of 8 samples). The most of the samples were properly labelled (the component was indicated on the label when its concentration exceeds the 0.01% (w/w)) with the exception of Sh1 and Sh2. For these samples, lilial® was present in the shampoo at higher concentrations than 0.01% (w/w) (Table 4) and their presence was not included on the label. It is noticeable the quite high total fragrance allergen content in a baby gel (BG1), 0.21% (w/w), the second highest value of the analyzed samples.

In the case of leave-on cosmetics (Table 5), found concentrations ranged from 0.000076 (benzyl alcohol in sample MC1) to 1.01% (w/w) (lilial® in sample ML), with an average value of 5 fragrance allergens per sample and an average concentration of 0.064% (w/w). The most frequently found was linalool, identified in all the analyzed samples, followed by limonene, present in 7 out of 9 samples. All samples were properly labelled, and the component was indicated on the label when its concentration exceeds the 0.001% (w/w). The highest total fragrance allergen contents were found in the moisturizing lotions, ML with the value of 1.42% (w/w) and two lotions for babies, MLB2 and MLB3 with contents of 0.530 and 0.820% (w/w), respectively.

Table 4. Analysis of real rinse-off cosmetic samples (Sh: shampoo; HC: hair conditioner; G: gel; HS: hands soap; BG: baby gel).

% (w/w)	Sh1	Sh2	Sh3	HC	G	HS	BG1	BG2
Limonene	0.000096	0.000545	0.051106		0.000629		0.034125	0.000229
Benzyl alcohol	0.001055	0.002264		0.008715	0.016589	0.001457		0.013072
Linalool	0.039104	0.012726	0.062486		0.011862	0.000212	0.056376	0.001119
Citronellol	0.036997							
Citral				0.009415				
Geraniol			0.010466		0.002922	<LOQ		0.000380
Anise alcohol		0.466929						
Eugenol					0.000057			
Methyl eugenol			0.000305					
Coumarin	0.001558		0.003668			0.008033		
α-Isomethyl ionone	0.019767	0.004758						
Lilial	0.017002	0.046826				0.082463	0.105540	0.002492
Lyral		0.009352						
Hexyl cinnamal		0.021230		0.000274	0.034138		0.010947	0.000513
Benzyl benzoate		0.000441						0.001751
Benzyl salicylate		0.007574	0.003752		0.000367			0.000185
Total fragrance allergen content	*0.116*	*0.573*	*0.132*	*0.0184*	*0.0666*	*0.0922*	*0.207*	*0.0197*

Blank cells mean values below LODs.

Table 5. Analysis of real leave-on cosmetic samples (MLB: moisturizing lotion for babies; MC: moisturizing cream; AC: anti-cellulite cream; ML: moisturizing lotion; HC: hands cream).

% (w/w)	MLB1	MLB2	MLB3	MC1	MC2	MC3	AC	ML	HC
Limonene		0.024311	0.101416	0.000932	0.004773		0.001352	0.011883	0.000422
Benzyl alcohol				0.000076		0.001015		0.000625	0.002337
Linalool	0.006570	0.031511	0.104257	0.013790	0.004191	0.000617	0.011808	0.070680	0.005137
Citronellol									0.001166
Geraniol		0.007229		0.000258					0.000798
Citral		0.010814	0.007833						
Hydroxycitronellal			0.000428						0.000737
Coumarin								0.020720	
α-Isomethyl ionone				0.002953				0.002470	0.000703
Lilial®		0.481264	0.678693	0.051238				1.010490	0.006109
Lyral®				0.006212					
Hexyl cinnamal			0.029158	0.007360				0.087747	0.003606
Benzyl benzoate	0.000259			0.000502				0.002845	
Benzyl salicylate					0.012318			0.228331	0.010465
Total fragrance allergen content	*0.0068*	*0.530*	*0.820*	*0.0824*	*0.0165*	*0.00163*	*0.0118*	*1.42*	*0.0311*

Blank cells mean values below LODs.

4. Conclusions

A fast, efficient, and cheap method based on MSPD followed by GC-MS for the simultaneous determination of fragrance allergens (including the 24 regulated in the EU Cosmetics Directive) in multi-matrix cosmetic samples has been developed. Optimization was carried out using real cosmetic samples and several statistical tools. Recovery studies were performed on leave-on and rinse-off samples, demonstrating the reliability of the optimized procedure.

The method was applied to a broad range of cosmetics. Target ingredients were present in all the analyzed samples and, in most cases, a quite high number of fragrance allergens were detected, although compliance with the actual European Regulation in terms of labelling was observed in most cases. To our knowledge, this is the first application of MSPD to the analysis of cosmetics, as well as to the analysis of fragrance allergens.

Acknowledgements

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Capítulo III.1.3

Content of suspected allergens and preservatives in marketed baby and child care products

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Content of suspected allergens and preservatives in marketed baby and child care products

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Abstract

Baby hygiene and care products are daily used cosmetics intended to cleanse and protect the delicate skin of the baby. However, the formulation of this kind of products may contain a highly complex mixture of chemicals such as fragrances and preservatives, some of which have been shown to act as strong sensitizers and allergens. As a result of the widespread use of these compounds in baby care products, there is an increasing social concern about the possible harmful consequences that the exposure to some of these ingredients could have on babies' health. To ensure a high level of protection, in the European framework, both preservatives and suspected fragrance allergens are subjected to restrictions according to the EU Cosmetic Directive. Due to the special sensitivity of infants, further restrictions and conditions are applied to these substances in cosmetics intended to be used in children under three. In this work a wide analytical screening of a great variety of rinse-off and leave-on products and personal care products (PCPs) targeted to infant care was accomplished. Matrix-solid phase dispersion (MSPD) and pressurized liquid extraction (PLE) procedures followed of gas chromatography-mass spectrometry (GC-MS) were successfully applied for the determination of 38 targets (25 suspected allergen fragrances and 13 preservatives). Although the compliance with the current EU Cosmetic Regulation was fulfilled regarding the fragrance and preservatives limits, the results reveal the high allergenic content in some products intended to be in prolonged contact with the baby's skin. The results further highlight that some of the target compounds have been omitted in the label.

Keywords: Matrix solid-phase dispersion; Pressurized liquid extraction; Cosmetics; baby and child care products; Preservatives; Fragrance allergens; GC-MS.

1. Introduction

Nowadays there is an exponential growing use of products devoted to the hygiene and personal care, not only by adults but also in babies and children. Baby hygiene or care products may contain a complex mix of chemicals, including artificial dyes and colors, fragrances, and preservatives, among others. Some of these substances may lead to unintended side effects on baby's health, which is an increasing matter of social concern and a challenge for baby-care products manufacturers.

Infants breathe more air and have more skin surface per pound of body weight than adults [1]. Different absorption and distribution factors due to the immaturity of physiological functions of young children may cause ineffective inactivation and elimination kinetics and thus higher internal exposure to the same external dose of certain chemicals in young children compared to adults [2-4]. Because major organ systems are still developing after birth, babies do not have fully functioning metabolic systems for getting rid of toxins as efficiently as adults and can be particularly susceptible to endocrine, immune or nervous system insults. Additionally, their blood-brain barrier, which prevents chemicals from travelling from the blood into the brain, is not fully developed [5, 6]. Infants also ingest products that are not designed for this purpose by putting the fingers and hands into their mouths. As regards cosmetic products, all this problematic is enhanced since many of such products contain "penetration enhancers" which further increase the absorption of chemicals through the skin.

The Regulation (EC) No 1223/2009 [7] establishes rules to be complied with by any cosmetic product available on the European market, in order to ensure a high level of protection of human health. This protects consumers by warranting that all cosmetic products on the European market are safe. To meet their obligations under the Directive, companies fulfil specific duties before placing a product on the market. These include a safety assessment, provision of product information and compliance with ingredient and labelling rules. The Directive includes the restrictions applied to some substances, and those prohibited ones which must not be included in the cosmetic formulations. PCPs intended for use on children below the age of three must undergo a specific safety assessment that takes account of their special needs. Annex III of the Cosmetics Directive [7] lists restrictions and conditions on specific substances and includes substances that must not be used in products for children under three.

Preservatives and suspected fragrance allergens (SAs) are included in the European Cosmetics Regulation, among other families of compounds. Fragrance ingredients are

frequent cause of allergic contact dermatitis and, in most cases, are caused by cosmetic products [8]. The occurrence of several volatile SAs needs to be determined since, in the EU market, their presence must be indicated on the label when its concentration exceeds the 0.001% in leave-on products and the 0.01% in rinse-off products.

Preservatives are used in PCPs to protect them against microbial growth, maintaining product integrity. Nevertheless, unintended possible side effects of these ingredients are a matter of concern. Parabens is the name given to a group of p-hydroxybenzoic acid esters used in over 22000 cosmetics as preservatives [9]. Their antimicrobial activity increases but water solubility decreases with increasing ester chain length. Because microbial replication takes place primarily in the water phase of a cosmetic product, preservative effectiveness is a combination of antimicrobial activity and water solubility. Parabens and butylated hydroxyanisole (BHA), an antioxidant frequently used to prevent oxidation, may modulate and disrupt the endocrine system [10]. Iodopropynyl butylcarbamate (IPBC), 2,4,4'-trichloro-2'-hydroxydiphenyl ether (TCS, triclosan) and bromine containing preservatives such as 5-bromo-5-nitro-1,3-dioxane (bronidox) and 2-bromo-2-nitropropane-1,3-diol (bronopol) are also included in a variety of PCPs. IPBC could cause acute inhalation toxicity [11]; TCS may disrupt the thyroid function [12], and can form toxic by-products in tap water and in the environment [13-16]; whereas bronidox and bronopol are known to be effective nitrosating agents; whenever these preservatives are coformulated with constituents having secondary amine structures, substantial carcinogenic nitrosamine formation is to be expected[17, 18]. Butylated hydroxytoluene (BHT) is a substituted toluene used in a wide range of cosmetic formulations as an antioxidant. According to the European Regulation on cosmetic products [7], the maximum allowed concentration of parabens is 0.4% (expressed as acid) for a single ester and 0.8% for mixtures of esters. IPBC can be added in a concentration lower than 0.02% in rinse-off products, 0.01% in leave-on products and 0.0075% in deodorants and antiperspirants. This preservative cannot be used in oral and lip products, in products for children under 3 years (except bath products, shower gels and shampoo), and in body lotions and creams. Bronidox is allowed only in rinse-off products being its maximum allowed concentration in ready for use preparation of 0.1%. The maximum concentration allowed for bronopol is also 0.1% but it can be used in rinse-off and leave-on products. Additionally, the manufacturer should also control that the formation of nitrosamines is avoided in products containing both brominated preservatives. The maximum allowed concentration in the finished product for triclosan is 0.3%. BHA and BHT are closely related synthetic antioxidants used as preservatives in lipsticks and moisturizers, among other cosmetics. 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%).

Although cosmetic products are regulated worldwide to ensure their safety and efficacy, the degree of restriction vary greatly between the main regulatory frameworks. This fact raises an intense public debate about the safety of some substances commonly found in personal care products. Thus, in recent days, some baby care products manufacturers and commercial distributors have been forced to remove some suspected products from the market and to modify the formulation therein. This is the case of those products tested positive for 1,4-dioxane and formaldehyde, in USA and China, which have been characterized as probable carcinogens by the Environmental Protection Agency. Furthermore, other PCPs have been advised to be removed from the market because of the risk of nitrosamines formation. Hence, the development and application of analytical methodology in exhaustive cosmetic controls has to be tackled as a matter of priority, in order to guarantee consumers health and ensure compliance to existing or future regulation.

Recently, the authors developed simple and fast analytical methods based on PLE [19, 20] and MSPD [21, 22] extraction techniques followed by gas chromatography-mass spectrometry (GC-MS) analysis, for the determination of preservatives and SAs in multi-matrix cosmetic samples including leave-on and rinse-off products. Both PLE and MSPD methodologies allowed the quantification of 25 SAs and 13 preservatives belonging to different chemical families at concentration levels below the limits established by the European legislation on cosmetics [7].

In the present work, MSPD and PLE developed methodologies are indistinctly applied to the analysis of a great variety of rinse-off and leave-on cosmetic and PCPs intended for baby and child care. A total of 47 samples were analyzed for determining the presence of the 38 targets (25 suspected allergen fragrances and 13 preservatives) (Table 1), allowing checking the compliance of the tested products with the European Regulation with regards to both the maximum allowed concentration of the target ingredients and their correct labelling.

Table 1. IUPAC names, retention times, quantification and identification ions of the target analytes.

Key	Compound	IUPAC name	CAS	Retention time (min)	Selected ions[a]
Suspected allergen substances					
SA1	Limonene	(4R)-1-Methyl-4-(1-methylethenyl)cyclohexene	5989-27-5	4.55	68, 93 (83), 121 (28), 136 (26)
SA2	Benzyl alcohol	Benzene methanol	100-51-6	4.66	77 (68), 79 (113), 107 (72), 108
SA3	Linalool	3,7-Dimethyl-1,6-octadien-3-ol	78-70-6	5.67	71 (123), 93,121 (32), 136 (11)
SA4	Methyl-2-octynoate	Methyl heptin carbonate	111-12-6	6.90	79 (85), 95 (131), 123, 139 (17)
SA5	Citronellol	(±)-3,7-Dimethyloct-6-en-1-ol	106-22-9/ 26489-01-0	7.16	69, 81 (59), 95 (52), 123 (28)
SA6	Citral	3,7-Dimethyl-2,6-octadienal	5392-40-5	7.32 7.59	69, 84 (29), 94 (35), 109 (32) 69, 84 (28), 94 (18), 109 (10)
SA7	Geraniol	3,7-Dimethyl- (2E)-2,6-octadien-1-ol	106-24-1	7.43	69, 93 (16), 111 (7), 123 (14)
SA8	Cinnamal	3-Phenyl-2-propenal	104-55-2	7.63	51 (20), 77 (33), 103 (48), 131
SA9	Anise alcohol	4-Methoxybenzyl alcohol	105-13-5	7.70	94 (38), 109 (75), 121 (55), 138
SA10	Hydroxycitronellal	7-Hydroxy-3,7-dimethyloctanal	107-75-5	7.73	43 (41), 59, 71 (44), 81 (14)
SA11	Cinnamyl alcohol	3-Phenyl-2-propen-1-ol	104-54-1	7.91	92, 105 (55), 115 (55), 134 (73)
SA12	Eugenol	2-Methoxy-4-(2-propenyl)-phenol	97-53-0	8.32	103 (28), 131 (28), 149 (33), 164
SA13	Methyleugenol	1,2-Dimethoxy-4-(2-propenyl)-benzene	93-15-2	8.61	103 (27), 147 (30), 163 (27), 178
SA14	Isoeugenol	2-Methoxy-4-(1-propenyl)-phenol	97-54-1	8.67 8.98	103 (27), 131 (22), 149 (31), 164 103 (22), 131 (22), 149 (32), 164
SA15	Coumarin	2H-1-Benzopyran-2-one	91-64-5	8.92	89 (42), 90 (41), 118, 146 (86)
SA16	α-Isomethyl ionone	3-Methyl-4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-3-buten-2-one	127-51-5	9.16	107 (55), 123 (18), 135, 150 (59)
SA17	Lilial®	2-(4-tert-Butylbenzyl) propionaldehyde	80-54-6	9.45	131 (28), 147 (39), 189, 204 (20)
SA18	Amyl cinnamal	2-Benzylideneheptanal	122-40-7	10.30	115 (91), 117 (89), 129, 202 (53)
SA19	Lyral®	Hydroxyhexyl-3-cyclohexene carboxaldehyde	31906-04-4	10.42	93 (69), 105 (33), 136, 192 (26)
SA20	Amylcinnamyl alcohol	2-Pentyl-3-phenylprop-2-en-1-ol	101-85-9	10.58	91 (77), 115 (59), 133, 204 (18)
SA21	Farnesol	3,7,11-Trimethyl-2,6,10-dodecatrien-1-ol	4602-84-0	10.63 10.84	69, 81 (58), 93 (26), 133 (26) 69, 81 (39), 93 (222), 133 (12)
SA22	Hexyl cinnamal	2-Benzylideneoctanal	101-86-0	11.15	115 (79), 129, 145 (51), 216 (42)
SA23	Benzyl benzoate	Phenylmethyl benzoate	120-51-4	11.38	77 (29), 91 (46), 105, 212 (27)
SA24	Benzyl salicylate	Benzyl-2-hydroxybenzoate	118-58-1	12.63	65 (11), 91, 228 (14)
SA25	Benzyl cinnamate	3-Phenyl-2-propenoic acid phenylmethyl ester	103-41-3	16.20	91 (97), 103 (43), 131, 192 (74)

[a] Quantification ion is represented underlined. The relative ion abundances (%) of the qualifier ions with respect to the quantifier ion are included between brackets

Table 1. (continued).

Key	Compound	IUPAC name	CAS	Retention time (min)	Selected ions[a]
Multiclass preservatives					
	Bronidox	5-Bromo-5-nitro-1,3-dioxane	30007-47-7	7.09	107 (48), 135, 137 (99)
	Bronopol	2-Bromo-2-nitropropane-1,3-diol	52-51-7	8.90	115, 195 (44)[b]
MeP	Methylparaben	Methyl 4-hydroxybenzoate	99-76-3	9.12	121, 152 (62)[b]
BHA	Butylated hydroxyanisole	2-tert-Butyl-4-methoxyphenol	121-00-6	9.18	165, 180 (57)[b]
BHT	Butylated hydroxytoluene	2,6-Bis(1,1-dimethylethyl)-4-methylphenol	128-37-0	9.35	205, 220 (26)
EtP	Ethylparaben	Ethyl 4-hydroxybenzoate	120-47-8	9.61	121, 138 (44), 166 (55)[b]
iPrP	Isopropylparaben	Isopropyl 4-hydroxybenzoate	4191-73-5	9.84	121, 138 (85), 180 (55)[b]
PrP	Propylparaben	Propyl 4-hydroxybenzoate	94-13-3	10.34	121 (74), 138, 180 (30)[b]
IPBC	Iodopropynyl butylcarbamate	Carbamic acid, butyl-3-iodo-2-propynyl ester	55406-53-6	10.40	165, 182 (53), 281 (3)
iBuP	Isobutylparaben	Isobutyl 4-hydroxybenzoate	4247-02-3	10.82	93 (8), 121 (80), 138[b]
BuP	Butylparaben	Butyl 4-hydroxybenzoate	94-26-8	11.25	121 (56), 138, 194 (16)[b]
BzP	Benzylparaben	Benzyl 4-hydroxybenzoate	94-18-8	17.24	91 (43), 121, 228 (30)[b]
TCS	Triclosan	2,4,4'-Trichloro-2'-hydroxydiphenyl ether	3380-34-5	17.37	218 (31), 288, 290 (99)[b]

[a] Quantification ion is represented underlined. The relative ion abundances (%) of the qualifier ions with respect to the quantifier ion are included between brackets
[b] Retention times as well as qualifiers and quantifiers correspond to the acetylated derivative

2. Experimental

2.1 Reagents and materials

Limonene (97%), linalool (97%), citronellol (95%), citral (95%), anise alcohol (98%), eugenol (99%), methyleugenol (99%), isoeugenol (98%), coumarin (99%), farnesol (95%), and benzyl cinnamate (99%) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Benzyl alcohol (99%), amyl cinnamal (97%), and benzyl benzoate (98%) were acquired from ChemService (West Chester, PA, USA). Methyl-2-octynoate (≥99%), cinnamal (≥93%), hydroxycitronellal (≥95%), cinnamyl alcohol (98%), and hexyl cinnamal (≥95%) were purchased from SAFC Supply Solutions (Saint Louis, MO, USA). Geraniol (≥96%), α-

isomethyl ionone (≥85%), lilial® (≥95%), lyral® (≥97%), amylcinnamyl alcohol (≥85%), benzyl salicylate (≥99%), and bronidox (≥99%) were obtained from Fluka Chemie GmbH (Steinheim, Germany). Bronopol (98%), methylparaben (99%), ethylparaben (99%), propylparaben (99%), butylparaben (99%), benzylparaben (99%), butylated hydroxyanisole (≥98.5%), butylated hydroxytoluene (99%), iodopropynyl butylcarbamate (97%), and triclosan (≥97.0%) were purchased from Aldrich (St. Louis, MO, USA). Isopropylparaben (≥99%) and isobutylparaben (≥97%) were purchased from TCI Europe (Zwijndrecht, Belgium).

Deuterated methyl-4-hydroxybenzoate-2,3,5,6-d_4 ($MePd_4$; 98 atom % D) and benzyl-d_7 alcohol (98 atom % D) used as surrogate standards, were obtained from C/D/N Isotopes (Quebec, Canada) and Aldrich (St. Louis, MO, USA), respectively.

Acetone and *n*-hexane were provided by Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Florisil (60–100 mesh) was purchased from Supelco Analytical (Bellefonte, PA, USA). Before being used, Florisil was activated at 130 °C for 12 h and then allowed to cool down in a desiccator. Sodium sulphate 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 and hexane/acetone (1:1, v/v). All solutions were stored in amber glass vials at −20 °C. All solvents and reagents were of analytical grade.

2.2 Cosmetic samples

Different cosmetics intended for baby and child care were purchased from local shops, supermarkets, department stores and pharmacies. They included leave-on and rinse-off products from national and international brands, covering a wide range of prices.

Twenty eight different leave-on samples were analysed including body care products: body lotion (BL), cream (BC), milk (BM), and moisturizing oil (MO); face creams (FC) to prevent the irritation of the baby face from cold and moisture; emulsions and creams to treat the irritation caused by diapers (DC); eau de toilette for kids (ET); louses lotion (LL); baby wipes (BW); sun care products (SP). Regarding rinse-off products, nineteen different samples were analysed and included shampoos (Sh), liquid soaps (LS), bubble baths (BB), shower gels (Ge), toothpaste for children (TP) and a washing powder for baby clothes (WP).

Samples were kept in their original containers at room temperature until their

analysis. Surrogates (20 μL of 1000 μg mL^{-1} $MePd_4$ solution, and 20 μL of 1000 μg mL^{-1} benzyl-d_7 alcohol solution) were added to each sample before the extraction procedure.

The samples were extracted using pressurized liquid extraction (PLE) [19, 20] and/or matrix solid-phase dispersion (MSPD) [21, 22]. In both cases, the obtained extracts were levelled to a final volume of 20 mL and divided in two aliquots. One of them was analysed as it by GC-MS full scan method to determine and quantify the SAs, whereas the other aliquot was derivatized and analysed by GC-MS selected ion monitoring (SIM) mode for the determination of the target preservatives and antioxidants. Due to the huge range of concentrations in which these compounds can be included in the samples, convenient dilutions were also injected in some cases. The general use of the targets in all classes of PCPs and cleaning products demanded special precautions during all analytical procedures to minimize contamination risk.

2.3 Pressurized liquid extraction (PLE)

Extractions were performed on an ASE 150 (Dionex, Co., Sunyvale, CA, USA), equipped with 10 mL stainless steel cells and 60 mL collection vials. Two cellulose filters (Dionex) were placed at each end of the PLE cell. 0.5 g of sample (after the addition of the surrogate solutions) mixed with the drying agent (1 g of Na_2SO_4) and the dispersing sorbent (2 g of Florisil), was introduced into the cell, where previously 1 g of clean sand (50-70 mesh particle size, Sigma-Aldrich) was placed. Finally, the dead volume of the cell was filled with sand. The cell was tightly closed and placed into the PLE 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) was employed as the extraction solvent. The extraction temperature and extraction time were 120 °C and 15 min, respectively. The extract was levelled in all cases to a final volume of 20 mL.

2.4 Matrix solid-phase dispersion (MSPD)

0.5 g of the cosmetic sample was exactly weighted into a 10-mL glass vial and spiked with the surrogate solutions. After homogenization, the sample was gently blended with 1 g of a drying agent (anhydrous Na_2SO_4) and 2 g of dispersing sorbent (Florisil) into a glass mortar using a glass pestle until a homogeneous mixture was obtained (ca. 5 min). Then, the mixture was transferred into a column with a polypropylene frit at the bottom containing 0.5 g of Florisil (to obtain a further degree of fractionation and sample clean-up). A second frit

was placed on top of the sample before compression with a syringe plunger. Elution was made by gravity flow with hexane/acetone (1:1, v/v), collecting 5 mL of extract into a graduated conical tube. The extract was finally levelled to a final volume of 20 mL.

2.5 Derivatization procedure

Acetylation was carried out by adding 100 µL of acetic anhydride containing 2.5% of pyridine to 1 mL of the standard or extract solutions. The mixture was then maintained at 80 °C for 10 min, and then allowed to cool down to room temperature. The derivatized PLE and MSPD extracts, diluted when necessary, were directly analyzed by GC-MS. Optimization of the derivatization conditions to improve the chromatographic analysis of phenolic preservatives was optimized elsewhere [20, 21]. Acetylation with acetic anhydride is one of simplest and cheapest derivatization procedures for phenolic compounds. Three of the compounds (bronidox, IPBC, and BHT) did not undergo derivatization given that bronidox and IPBC do not have chemical groups susceptible to acetylation. The acetylation of BHT could not be demonstrated as the highly hindered hydroxyl group with poor nucleophilicity may prevent the acetylation under the studied conditions. For the other preservatives and antioxidants, reaction yield was quantitative, and satisfactory, improving significantly the chromatographic behaviour of the target compounds.

2.6 GC-MS analysis

The GC-MS analysis was performed using an Agilent 7890A (GC)-Agilent 5975C inert mass spectra detector (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 290, 150, and 230 °C, 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 80 °C (held 2 min) to 100 °C at 8 °C min^{-1}, to 150 °C at 20 °C min^{-1}, to 200 °C at 25 °C min^{-1} (held 5 min), to 220 °C at 8 °C min^{-1}, and a final ramp to 280 °C at 30 °C min^{-1}. Pulsed splitless mode was used for injection (30 psi, held 1.2 min). After 1 min, the split was opened at a flow of 75 mL min^{-1} and the injector temperature was kept at 220 °C. The injection volume was 1 µL.

For the analysis of SAs the mass spectra detector (MSD) was operated in the scan mode and the mass range was varied from 40 to 300 m/z, starting at 4 min and ending at 30

min. The electron multiplier was set at a nominal value of 1300 V. The analytes were positively identified by comparison of their mass spectra and retention times to those of the standards. Table 1 summarizes the retention times as well as the qualification and quantification ions of the target analytes.

When preservatives and antioxidants were determined, the MSD was operated in selected ion monitoring (SIM) mode, monitoring at least two ions per compound (see also Table 1). The electron multiplier was set at a nominal value of 1,200 V.

3. Results and discussion

The samples were extracted using pressurized liquid extraction (PLE) [19, 20] and/or matrix solid-phase dispersion (MSPD) [21, 22]. PLE and MSPD optimized conditions were equivalent for both families of compounds (SAs and preservatives), excluding the extraction solvent. The most favourable solvent was hexane-acetone for SAs and ethyl acetate for preservatives. Taking into account that the solvent was not a significant factor for preservatives extraction, hexane-acetone was chosen. In any case, both PLE and MSPD methods were re-evaluated to confirm good performance. Quantitative recoveries were achieved, and precision was in all cases below 12% RSD. To indistinctly apply PLE or MSPD based methodologies to the samples, both methods were applied to a number of different real non-spiked leave-on and rinse-off cosmetic samples. Statistical hypothesis test (t-test) demonstrated no significant differences between the results obtained by both techniques at 95% significant level. Recoveries of the surrogates were quantitative: 79%- 117%, and 81%-118%, for benzyl-d_7 alcohol in leave-on and rinse-off samples, respectively; 70%-117%, and 74%-111% for $MePd_4$ in leave-on and rinse-off samples, respectively. Figures 1 and 2, and Tables S5 and S6, show that PLE and MSPD gave similar results in the broad variety of samples considered.

3.1 Analysis of real samples

Tables 2 and 3 summarize the average and the range of concentrations of the targets found in the baby and child PCPs analyzed. Individual data are shown in Supplementary information (Tables S1-S4).

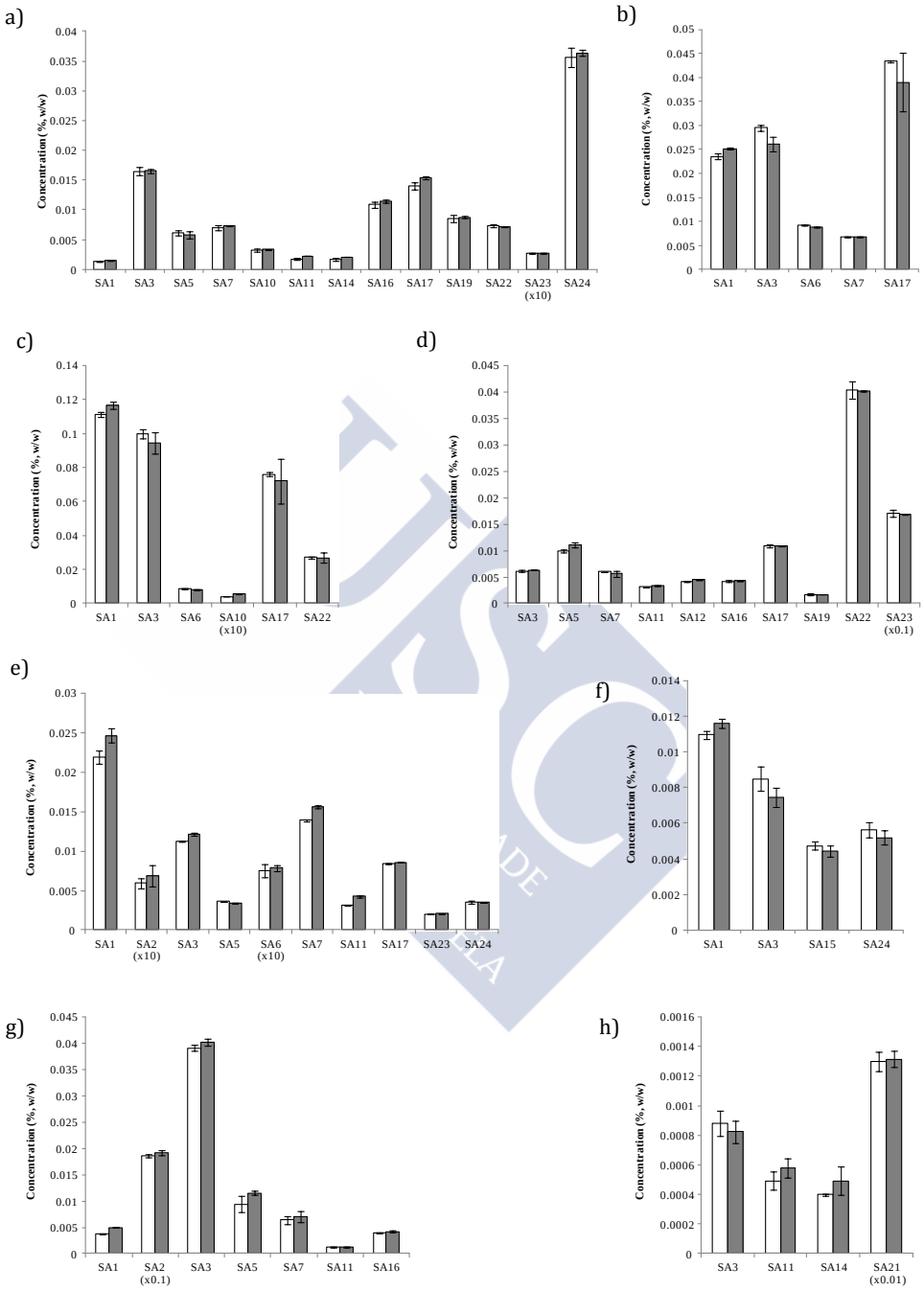

Fig. 1. Application of MSPD and PLE methods to different leave-on (a) body lotion BL2; (b) body milk BM2; (c) body milk BM3; (d) moisturizing oil MO1; (e) sun care product SP1, and rinse-off cosmetic samples (f) shampoo Sh1; (g) shampoo Sh3; (h) shower gel Ge1, for the determination of SAs (white bars: MSPD; grey bars: PLE; SA key in Table 1. Error bars show standard deviation values (n = 3–4)).

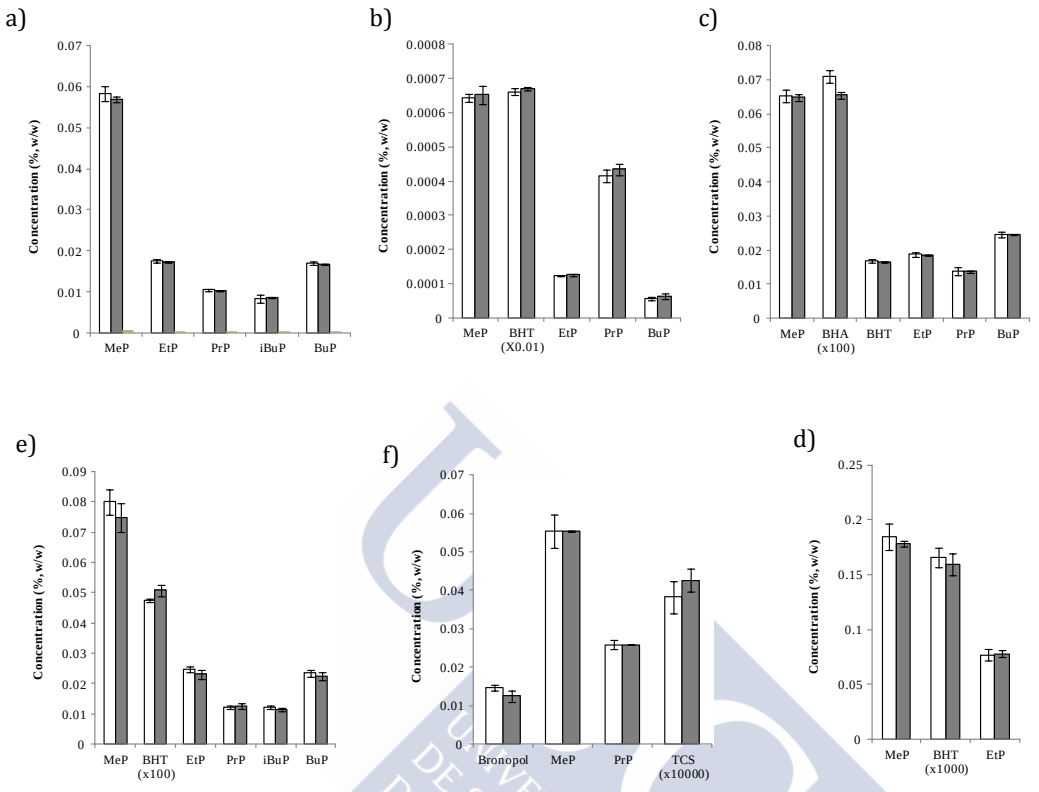

Fig. 2. Application of MSPD and PLE methods to different leave-on ((a): body lotion BL2; (b): body milk BM2; (c): body milk BM3; (d): sun protecting lotion SP1) and rinse-off cosmetic samples ((e): shampoo Sh3; (f): gel Ge1) for the determination of preservatives (white bars: MSPD; grey bars: PLE; error bars show standard deviation values (n = 3–4)).

3.2 Suspected fragrance allergens

The 25 studied SAs are allowed in PCPs formulations; nevertheless, its presence must be indicated on the label when concentration exceeds 0.001% in leave-on products and 0.01% in rinse-off products. Two SAs are more restricted, i.e. benzyl alcohol (maximum allowed concentration of 1.0%) and methyleugenol (maximum allowed concentration of 0.01% in fine fragrance, 0.004% in eau de toilette, 0.002% in fragrance cream and 0.0002% in other leave-on products and oral products). Since the LOQs obtained with both MSPD and PLE based methods are well below the limit set on the European Regulation, both methods are adequate to verify that marketed PCPs comply with currently in force EU legislation.

From a total of 47 samples analyzed, only 12 of them did not include any SA. The average number of SAs was five per sample, with limonene and linalool being the most frequently detected (included in 21 and 26 samples, respectively). SAs average concentrations ranged from 0.000067 % (cinnamal) to 0.137 % (benzyl alcohol) (Table 2).

3.2.1 Leave-on samples

The leave-on cosmetics contained between 1 and 13 SAs per sample, with an average of five SAs per sample, standing out a body lotion (BL2), moisturizing oil (MO1) and a sunscreen product (SP1) with 13, 10, and 10 SAs, respectively (Table S1).

Linalool, limonene and lilial were the most frequently detected SAs; linalool was found in 12 out of 18 samples, whereas limonene and lilial were detected in half of the positive samples each. It is relevant to point out the total SAs content (expressed as the sum of the all the SAs concentrations of each sample) of the samples. Figure 3a shows that 50% of the leave-on baby products contain SAs in a percentage ≥ 0.1%. Although there is no regulation on the maximum allowed for the total content of SAs in cosmetics, it seems that products designed to remain on the skin for child care should be formulated with lower SAs contents.

The highest SAs individual concentrations corresponded to benzyl alcohol (0.450% in BM1), limonene (0.212% in ET2), lilial (0.170% in ET2), and benzyl benzoate (0.169% in MO1) (Tables 2 and S1). It must be highlighted the concern about these high concentrations of SAs in baby care products, especially in the case of benzyl alcohol in a body milk (BM1), found so close to the regulated maximum.

Eight SAs were not detected in leave-on samples, i.e. methyl-2-octynoate, cinnamal, anise alcohol, methyl eugenol, amyl cinnamal, amyl cinnamyl alcohol, farnesol and benzyl cinnamate. Thirty five percent of the samples (10 out of 28) did not contain any of the analysed SAs. These samples were moisturizing oil for babies (MO2), three creams to prevent the irritation of the baby face from cold and moisture (FC2-4), all the diaper creams (DC), and one of the baby wipes analyzed (BW2). Figure 4 shows the extracted ion chromatograms obtained for the body moisturizing lotion BL2 (see concentrations in Table S1).

Table 2. Concentration (%, w/w) of SAs in baby and child care cosmetics.

Compound	Leave-on PCPs (28 samples)				Rinse-off PCPs (19 samples)				All products (47 samples)	
	N	Average	Minimum	Maximum	N	Average	Minimum	Maximum	N	Average
Limonene	9	0.0521	0.00148	0.212	12	0.0254	0.000330	0.143	21	0.0369
Benzyl alcohol	2	0.225	0.000688	0.450	3	0.0783	0.000144	0.192	5	0.137
Linalool	12	0.0365	0.00388	0.134	14	0.0228	0.000371	0.0655	26	0.0291
Citronellol	4	0.00778	0.00344	0.0116	4	0.00936	0.00372	0.0159	8	0.00856
Citral	7	0.0342	0.000786	0.138	2	0.00883	0.00736	0.0103	9	0.0286
Geraniol	6	0.00721	0.00333	0.0156	5	0.0110	0.000403	0.0279	11	0.00894
Cinnamal					1	0.0000671			1	0.0000671
Hydroxycitronellal	5	0.0206	0.0000673	0.0847	2	0.00568	0.00314	0.00821	7	0.0164
Cinnamyl alcohol	4	0.00264	0.000645	0.00431	2	0.000929	0.000492	0.00137	6	0.00207
Eugenol	3	0.00163	0.0000582	0.00444	4	0.00125	0.000212	0.00329	7	0.00141
Isoeugenol	1	0.00201	0.00201	0.00201	2	0.000245	0.000185	0.000299	3	0.000833
Coumarin	1	0.00223	0.00223	0.00223	2	0.00419	0.00396	0.00442	3	0.00354
α-Isomethyl ionone	4	0.00591	0.000448	0.0115	4	0.00432	0.0000666	0.0114	8	0.00511
Lilial®	9	0.0399	0.0000432	0.170	1	0.00946			10	0.0369
Lyral®	3	0.00952	0.00171	0.0181					3	0.00952
Farnesol					1	0.130			1	0.130
Hexyl cinnamal	7	0.0177	0.000439	0.0402	4	0.0213	0.0104	0.0504	11	0.0190
Benzyl benzoate	3	0.0570	0.0000465	0.169	4	0.000223	0.0000272	0.000704	7	0.0215
Benzyl salicylate	4	0.0153	0.000204	0.0363	4	0.0105	0.0000743	0.0363	8	0.0129
Total SA content	*18*	*0.142*	*0.000043*	*0.566*	*17*	*0.0753*	*0.000182*	*0.262*	*35*	*0.110*

Table3. Concentration (%, w/w) of preservatives in baby and child care cosmetics.

Compound	Leave-on PCPs (28 samples)				Rinse-off PCPs (19 samples)				All products (47 samples)	
	N	Average	Minimum	Maximum	N	Average	Minimum	Maximum	N	Average
MeP	19	0.126	0.000330	0.457	7	0.0593	0.00117	0.120	26	0.108
EtP	10	0.0285	0.000125	0.0915	4	0.0158	0.0000549	0.0237	14	0.0249
PrP	18	0.0457	0.0000153	0.147	6	0.0112	0.000471	0.0258	24	0.0371
iBuP	5	0.00428	0.0000999	0.0117	3	0.0120	0.0111	0.0131	8	0.00719
BuP	11	0.0164	0.0000598	0.0910	6	0.0103	0.0000137	0.0231	17	0.0143
Total paraben content	*24*	*0.159*	*0.000084*	*0.493*	*15*	*0.0688*	*0.0000137*	*0.124*	*39*	*0.112*
Bronopol					2	0.00762	0.000613	0.0146	2	0.00762
IPBC					3	0.00410	0.00316	0.00541	3	0.00410
TCS	1	0.0000114			3	0.00000957	0.00000282	0.0000219	4	0.0000100
BHA	4	0.000249	0.0000313	0.000656	1	0.000809			5	0.000361
BHT	12	0.0210	0.000166	0.0724	7	0.000601	0.000116	0.00132	19	0.0135

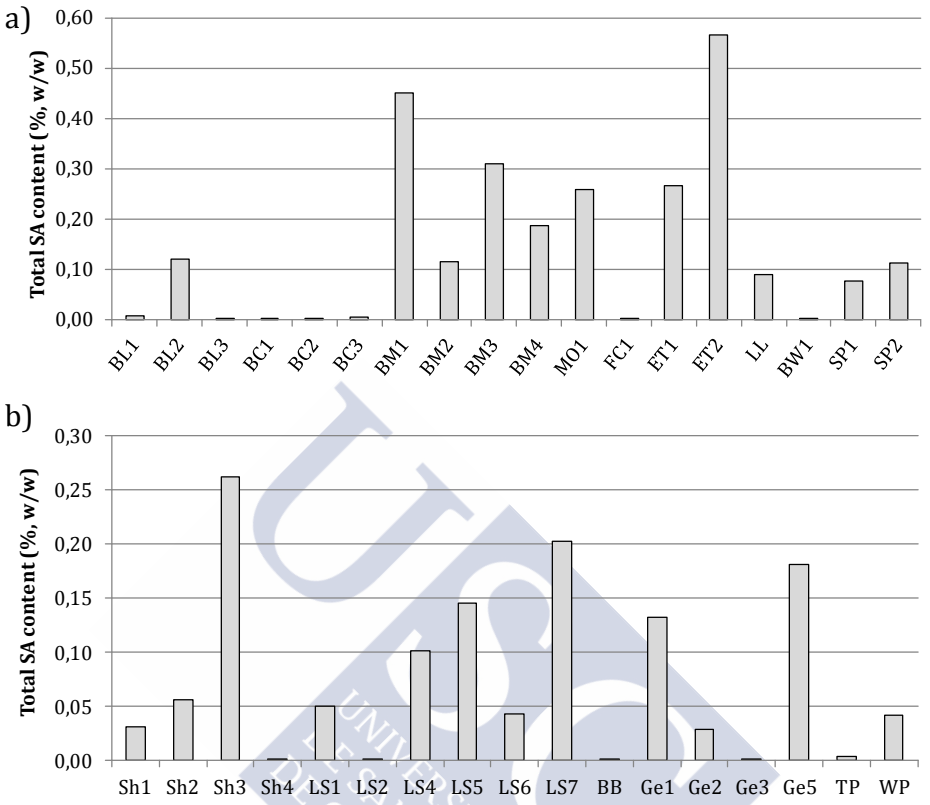

Fig. 3. Total SAs content (%, w/w) in (a) leave-on and (b) rinse-off samples. (BL: body lotion; BC: body cream; BM: body milk; MO: moisturizing oil; FC: face cream; ET: eau de toilette; LL: louses lotion; BW: baby wipes; SP: sun products; Sh: shampoo; LS: liquid soap; BB: bubble baths; Ge: gel; TP: toothpaste for children; WP: washing powder for baby clothes).

3.2.2 Rinse-off samples

Samples contained between 1 and 10 SAs, with an average of four SAs per sample. The sample with a higher number (10) of SAs was a liquid soap (LS7). The most frequently detected SAs were limonene and linalool, 12 and 14 out of 19 samples, respectively. The rest of the detected SAs appeared in less than five samples each one. Besides benzyl alcohol, the compounds present in highest concentrations were limonene (0.143% in LS7), linalool (0.0655% in LS5), hexyl cinnamal (0.0504% in Ge5), and benzyl salicylate (0.0363% in Ge5). Around 30% of the samples showed concentrations of SAs ≥ 0.1% (Figure 3b). The highest total SAs content was found in a shampoo (Sh3, 0.262%), a liquid soap (LS7, 0.202%), and a shower gel (Ge5, 0.181%). Liquid soaps LS4 and LS5 as well as gel Ge1 showed values

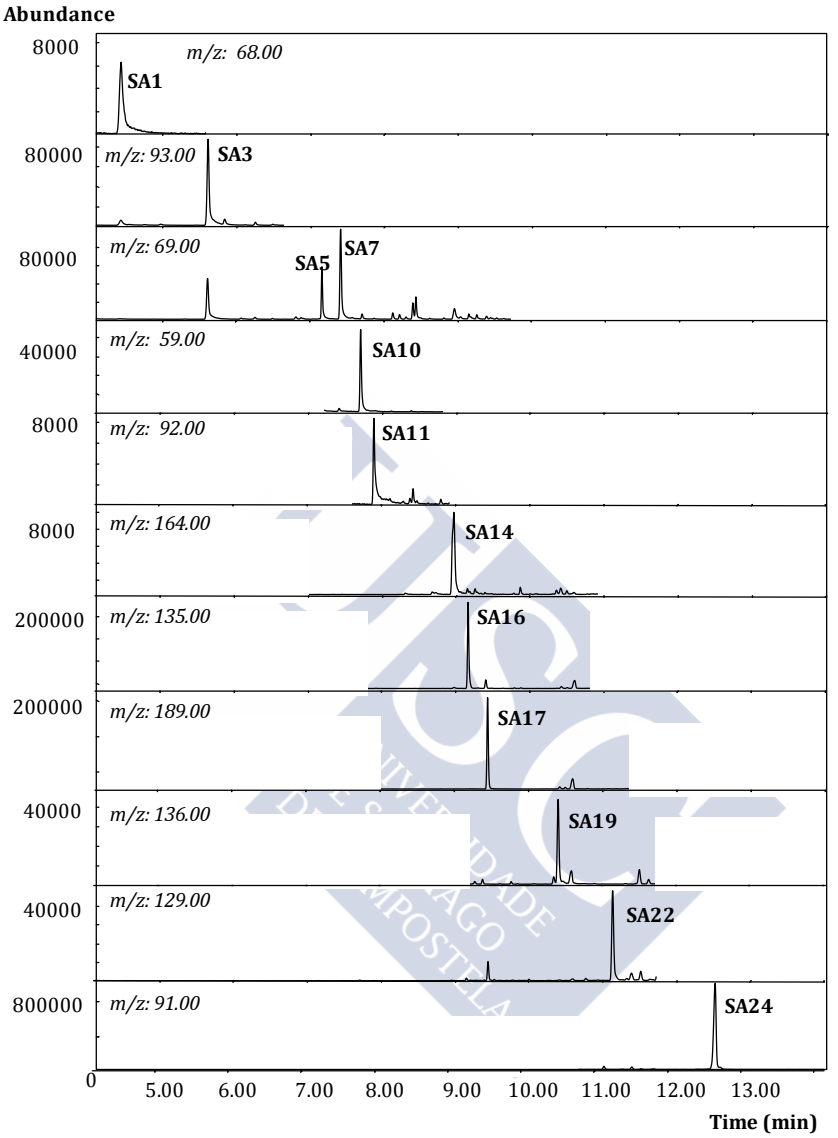

Fig. 4. Extracted ion chromatograms of the body lotion BL2 (see SA key in Table 1, and concentration values in Table S1).

around 0.1%, i.e. 0.101%, 0.146% and 0.132%, respectively. Only two samples did not contain any SA (a liquid soap and a shower gel), and only two samples included just one each, i.e. linalool, in Sh4, and benzyl alcohol in LS6.

3.3 Multiclass preservatives

Preservatives were evaluated in the 47 PCPs intended for child and baby care. Results are shown in Table 3 and Tables S3 and S4. Twenty-six samples contained at least one paraben. Bronopol, IPBC and TCS were found in two, three and four samples, respectively. BHT was included in the formulation of 40% of the samples, whereas BHA was only detected in five samples.

3.3.1 Leave-on samples

Eighty percent of the samples contained at least one of the studied preservatives and one- third of the samples presented five of the targets (Table 3 and Table S3). MeP and PrP were the most abundant preservatives, found in 19 and 18 out of 28 samples, respectively; BHT, BuP, and EtP were present in 12, 11, and 10 samples, respectively. BHA was found in four samples, in three of them associated to BHT, which increases the antioxidant power due to synergism. The use of both compounds is allowed in cosmetics for the time being, although there is some concern about their safety [10]. TCS was detected in just one sample (FC1) at low concentration (0.000011%).

Samples contained an average of three parabens per sample and an average total paraben concentration of 0.16% expressed as acid (see Tables 3 and S3). As can be seen in Figure 5, the highest total paraben content was found in body lotions, body creams, and face creams, reaching concentrations (about 0.5%) close to the legal limit. This should be a cause of concern for several reasons. Firstly, the highest concentrations were found in products aimed to be applied on the body, which means the application of higher amounts of the cosmetic product over a higher surface, and taking into account the higher body surface area to body mass ratio of babies compared to older children and adults, which finally can lead to a higher exposure per kg body weight to this body applied products. Secondly, face creams also presented a relatively high total parabens content. These cosmetics are aimed to be applied to irritated skin, usually around the mouth, due to the action of the wind, cold, dribbles, etc. According to an opinion of the Cosmetic Ingredients Review (CIR) Expert Panel (March 2012), irritated skin shows a higher dermal absorption. This Expert Panel underlined the risk, especially in the case of children below the age of 6 months. As regards the analyzed diaper creams, two of them contained around 0.1% of parabens. Figure 6 shows the selected ion chromatograms obtained for the diaper cream DC1 (see concentrations in Table S3). Regarding leave-on cosmetics designed for application on the nappy area, the presence of parabens especially in this irritated area is of particular concern due to their estrogen-

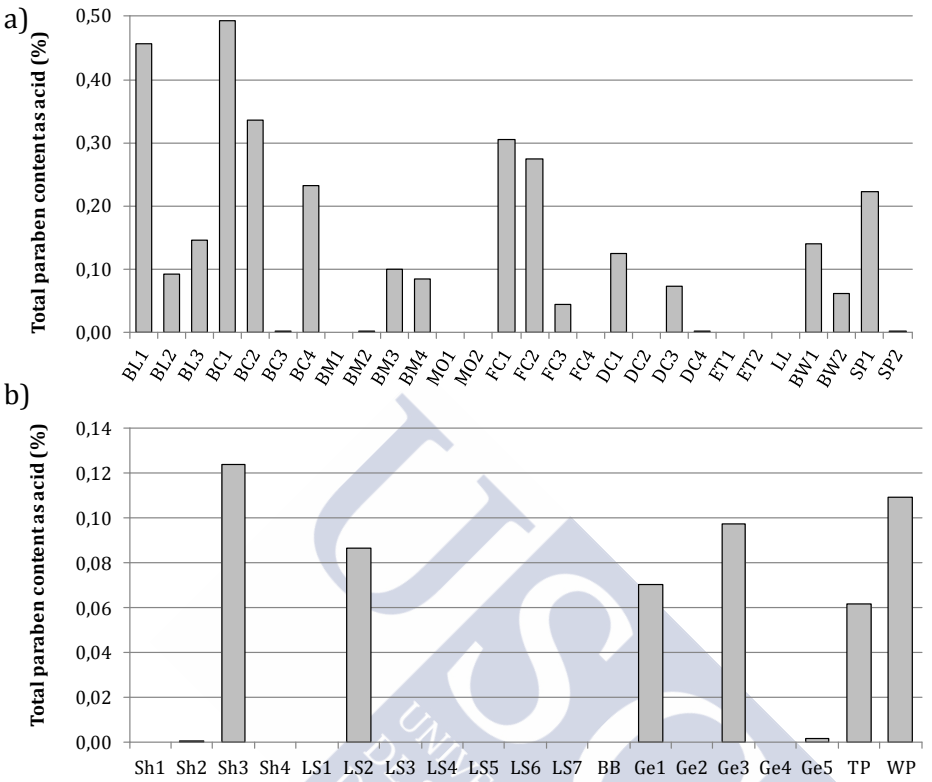

Fig. 5. Total paraben content expressed as acid (%, w/w) in (a) leave-on and (b) rinse-off samples. (BL: body lotion; BC: body cream; BM: body milk; MO: moisturizing oil; FC: face cream; DC: diaper cream; ET: eau de toilette; LL: louses lotion; BW: baby wipes; SP: sun products; Sh: shampoo; LS: liquid soap; BB: bubble baths; Ge: gel; TP: toothpaste for children; WP: washing powder for baby clothes).

mimicking endocrine disruption action. The highest paraben concentrations corresponded to MeP (average of 0.126%), and PrP with an average concentration of 0.0457%, although PrP was found in four samples close or higher than 0.1%. A single paraben was found in only one of the samples. This is in agreement with the general practice of using mixtures to get a powerful antimicrobial activity against an extremely broad spectrum of microorganisms since parabens posses a selective antimicrobial activity.

Five of the preservatives were not detected in any sample, i.e. bronidox (which is not allowed to be used in leave-on products [7], bronopol, IPBC and the parabens iPrP and BzP. Only 4 out of 28 samples did not contain any of the targets. These samples were: a body milk, two moisturizing oils, and a diaper cream. Only four of the samples did not contain parabens.

3.3.2 Rinse-off samples

Nineteen samples were analyzed and the most of them (14) contained at least one of the target preservatives (Table 3 and Table S4). Bronidox, iPrP and BzP were not detected in any sample. Seven samples contained BHT with an average concentration of 0.00060% and one of them also contained BHA. Two samples included bronopol in its composition, a liquid soap (LS6) and a gel (Ge1), this last showed a quite high concentration (0.015%). IPBC was detected in three samples: two liquid soaps (LS1 and LS6) and a gel (Ge5) being its average concentration of 0.0041%. TCS was also detected in three samples at low concentrations (average 0.000010%).

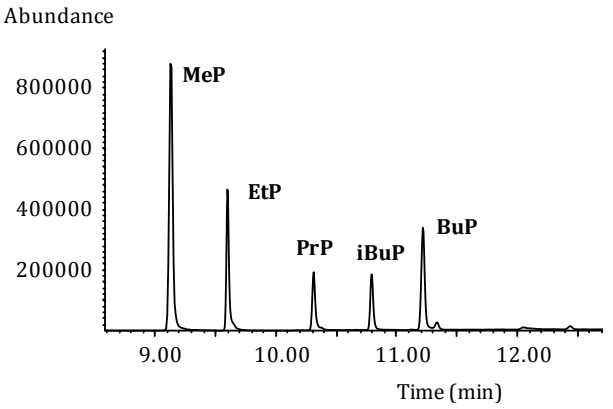

Fig. 6. SIM chromatogram (m/z = 121) of the diaper cream DC1 (see preservatives key in Table 1, and concentration values in Table S3).

The presence of parabens in the rinse-off cosmetics is less frequent than in leave-on cosmetics; eight samples contained at least one paraben, and among these samples the average content was three parabens per cosmetic product. MeP was detected in seven of the samples at an average concentration of 0.059%, followed by PrP and BuP both present in six samples with average concentrations of 0.011 and 0.010%, respectively. EtP and iBuP were detected in four and three samples, respectively. Their average concentrations were also around 0.01%. As can be seen in Figure 5 the total paraben content in rinse-off PCPs was lower than in leave-on cosmetics. The average of the total paraben content, taking into account the positive samples, was 0.069% less than the half of the average found in leave-on cosmetics.

3.4 Labelling

Proper labelling is an important aspect of putting a cosmetic product on the market. Labelling is used to inform consumers about the ingredients of a product and, in this way, to prevent the use of PCPs containing any specific chemical(s) that they cannot tolerate; and also by the dermatologists, as a guide to the compounds that maybe a cause of skin reactions or other adverse effects [23].

The degree of compliance of analyzed PCPs with the regulation on the labelling has been evaluated. The results showed that 24 out of 28 leave-on samples were properly labelled for all the SAs tested (about 85 % of the samples), which means that the SAs present in a concentration greater than 0.001 % were included on the label. Three samples were mislabelled with respect to one or two SAs: hexyl cinnamal in an eau de toilette (ET2), coumarin in the louses lotion (LL), benzyl benzoate in a moisturizing oil (MO1), linalool and geraniol in a body lotion (BL1). In some cases the labels of the cosmetics contain SAs although their concentrations were lower than the levels stipulated in the Regulation [7] . This is the case of the sun cream SP1 which included two SAs (benzyl alcohol and citral) at concentrations lower than 0.001%.

Eighteen out of 19 rinse-off PCPs were properly labelled, i.e. all the SAs present in a concentration higher than 0.01%, were listed on the label. The sample Sh3 was mislabelled with respect to a single SA since citronellol should be included on the label.

On the other hand, the label of the shampoo Sh2 included citral but this compound was not detected in the sample. Finally the list of ingredients of the liquid soaps LS1 and LS7 included limonene and citral, respectively, although their concentrations were lower than 0.01%.

EU Regulation [7] establishes that the label should contain a list of ingredients. MeP, EtP and PrP were listed as ingredients in more than 79, 90 and 83%, respectively, of the leave-on PCPs which contained them; whereas iBuP, BuP, and BHT were included on the label of half of them. BHA and TCS were not listed as ingredients in any of the products.

As regards rinse-off PCPs, EtP, iBuP, bronopol, and IPBC were included in the list of ingredients of all the products containing them. MeP, PrP, and BuP were labelled in more than 70% of the PCPs. BHA, BHT and TCS were not included in any of the labels corresponding to rinse-off products.

4. Conclusions

In the present paper, a wide-ranging market research on the content of suspected fragrance allergens and preservatives in a total of 47 different baby and child care products has been described. MSPD and PLE followed by gas chromatography-mass spectrometry (GC-MS) were successfully applied for the screening analysis of 38 targets (25 suspected allergen fragrances and 13 preservatives) in the samples. This study highlights their widespread presence in the broad range of market products intended for infant care, which

should be a matter of concern. The average number of fragrance allergens was 5 per sample, which is a high number if bearing in mind the possible synergic effects, and that some of these substances may behave as strong sensitizers and allergens, at total concentrations reaching 0.1%, and even 0.5% in cosmetics formulated to remain on baby skin. MeP and PrP were the most abundant preservatives followed by BHT, BuP, and EtP. Parabens were found in some products at concentrations close to the legal limit.

Labelling should be used as valuable information for consumers, in order to avoid the use of those PCPs containing any chemical(s) they cannot tolerate. With regards to SAs, five samples did not accomplish with the EU Cosmetic Regulation since they were mislabelled with respect to one or two SAs. As regards the preservatives, the Regulation does not specify their labelling. MeP, EtP and PrP were not listed in almost 20 % of the samples, whereas BHA and BHT were listed in about 50% of the products, and TCS was not listed in any product.

Some findings of this study reveal certain matters of concern, such as the high number of compounds present in some products, as well as the higher concentrations of compounds found in leave-on products intended to be applied on the body and even on irritated skin (e.g. diaper cream), staying in prolonged contact with the delicate and sensitive skin of the baby.

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Capítulo III.1.4

Development of a multi-preservative method based on solid–phase microextraction–gas chromatography–tandem mass spectrometry for cosmetic analysis

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Development of a multi-preservative method based on solid–phase microextraction–gas chromatography–tandem mass spectrometry for cosmetic analysis.

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Abstract

A simple methodology based on solid-phase microextraction (SPME) followed by gas chromatography-tandem mass spectrometry (GC-MS/MS) has been developed for the simultaneous analysis of different classes of preservatives including benzoates, bronidox, 2-phenoxyethanol, parabens, BHA, BHT and triclosan in cosmetic products. *In situ* acetylation and subsequent organic modifier addition have been successfully implemented in the SPME process as an effective extractive strategy for matrix effect compensation and chromatographic performance improvement. Main factors affecting SPME procedure such as fiber coating, sampling mode, extraction temperature and salt addition (NaCl) were evaluated by means of a $3 \times 2^{3-1}$ factorial experimental design. The optimal experimental conditions were established as follows: direct solid-phase microextraction (SPME) at 40 °C and addition of NaCl (20%, w/v), using a DVB/CAR/PDMS fiber coating. Due to the complexity of the studied matrices, method performance was evaluated in a representative variety of both rinse-off and leave-on samples, demonstrating to have a broad linear range ($R^2 > 0.9964$). In general, quantitative recoveries (>85% in most cases) and satisfactory precision (RSD < 13% for most of compounds) were obtained, with limits of detection (LODs) well below the maximum authorized concentrations established by the European legislation. One of the most important achievements of this work was the use of external calibration with cosmetic-matched standards to accurately quantify the target analytes. The validated methodology was successfully applied to the analysis of different types of cosmetic formulations including body milks, moisturizing creams, deodorants, sunscreen, bath gel, dental cream and make-up products amongst others, demonstrating to be a reliable multi-preservative methododology for routine control.

Keywords: Preservatives; Antioxidants; Cosmetics; Solid-phase microextraction; Personal care products; GC–MS/MS.

1. Introduction

Preservatives are essential ingredients widely added in daily used cosmetics and personal care products (PCPs) such as toothpastes, shampoos, creams, deodorants, etc., with the primary purpose of preventing spoilage, whether from microbial growth or undesirable oxidative processes. The esters of benzoic acid (benzoates) and *p*-hydroxybenzoic acid (parabens), 2,4,4'-trichloro-2'-hydroxydiphenyl ether (triclosan, TCS), 2-phenoxyethanol, bromine-containing preservatives as 5-bromo-5-nitro-1,3-dioxane (bronidox), as well as the antioxidants 2-tert-butyl-4-methoxyphenol (BHA) and 2,6-bis(1,1-dimethylethyl)-4-methylphenol (BHT) are frequently used preservatives in cosmetic formulations.

Despite their protective effect, the widespread use of these products has led to a social concern about the unintended harmful effects that some of these ingredients could have on consumer's health. Although benzoates were determined to be safe in the present practices of use in cosmetics [1], other ingredients such as parabens have been reported to have estrogenic/antiandrogenic-like properties [2, 3]. In addition, a potential relationship between breast cancer and prolonged dermal exposure to paraben-containing products is suggested [4]. BHA and TCS may also modulate and disrupt the endocrine system [5, 6]; whereas in the case of bronidox, the formation of carcinogenic nitrosamines is to be expected when co-formulated with products containing amines or amino derivatives [7].

In the EU context, these ingredients are subjected to several restrictions according to the EU Cosmetics Regulation [8], where limitations, requirements, label warnings, and the maxima permissible concentrations are indicated. As regards the above mentioned preservatives, the European legislation has established maxima allowed concentrations ranging from 0.1% (w/w), for bronidox, to 1% (w/w) for 2-phenoxyethanol. The maximum allowed limit for each individual paraben, as acid, has been set at 0.4% (w/w), and 0.8% (w/w) for mixtures of esters, while the maximum allowed level for TCS is 0.3% (w/w).

Therefore, to allow authorities to control the content of preservatives in the wide variety of marketed personal care products, effective and convenient methodologies are required. Methods for preservatives analysis in cosmetic samples are mainly focused on parabens determination, whereas analytical methods for the analysis of more than one class of preservatives are still a field under development. Liquid chromatography (LC) [9-11], capillary electrophoresis (CE) [12-14], capillary zone electrophoresis (CZE) [15, 16] and

micellar electrokinetic chromatography (MERKC) [17-20] have been widely used. Flow injection analysis (FIA) has also been employed enhancing sample throughput [21]. Although in less extent, gas chromatography (GC) coupled to mass spectrometry detectors has been applied [22]. Nevertheless, using GC approach, a derivatization step previous to phenolic preservatives analysis is highly recommendable to improve chromatographic performance [23, 24]. Acetylation is an advantageous derivatization procedure, offering a high efficient derivatization process using low-cost reagents, compared to silylation agents. This derivatization strategy, using acetic anhydride and pyridine has been firstly applied by the authors, to the determination of multi-class preservatives in cosmetics previous to GC–MS analysis [25, 26]. A variation of this reaction, employing acetic anhydride and sodium hydrogen phosphate, has also been performed in aqueous samples for the determination of phenolic preservatives such as parabens and triclosan in water [27].

However, the chromatographic analysis of cosmetic samples becomes a challenging task without a good sample pretreatment. Common sample preparation strategies involving several steps are frequently tedious and time-consuming, and the use of hazardous solvents is usually required. Moreover, the possible presence of interferences that could distort the results is not rejectable. In an attempt to overcome these problems, advanced extraction techniques such as supercritical fluid extraction (SFE) [24], solid-phase extraction (SPE) [28], pressurized liquid extraction (PLE) [26] and matrix solid-phase dispersion (MSPD) [25, 29], have been recently applied for the determination of preservatives and other different additives in cosmetics.

Solid-phase microextration (SPME) is one of the most attractive extraction techniques due to its simplicity and high extraction efficiency. SPME integrates sampling, extraction, concentration and sample introduction into a single uninterrupted process, which makes this procedure a valuable alternative analytical technique to more traditional procedures, reducing the laboratory generated waste and time for sample preparation [30]. This technique has been applied for the determination of parabens and some antioxidants in cosmetic samples [31, 32]. In these papers, the optimization of variables affecting the SPME procedure was carried out using the one-factor-at-a-time approach. By contrast with the factorial design method, this classical approach has the following drawbacks: (i) it requires more runs for the same precision in effect estimation; (ii) it cannot estimate interactions effects; (iii) the conclusions from its analysis are not general; (iv) it can miss optimal settings of factors [33]. Thus, using an experimental design approach, the optimization of a SPME procedure for the analysis of bronidox in rinse-off cosmetics was reported [34].

This work aims to optimize, validate and put into practise a simple methodology based on solid-phase microextraction followed by gas chromatography–tandem mass spectrometry (SPME–GC–MS/MS) for the simultaneous analysis of different classes of preservatives in a representative and wide variety of rinse-off and leave-on cosmetics. *In situ* acetylation was used for target compounds derivatization using acetic anhydride and sodium hydrogen phosphate. Major efforts were focused on avoiding matrix effects, an essential requirement to make feasible the analysis of cosmetic ingredients by external calibration, considering the broad diversity of cosmetic products. After that, factorial design was selected to simultaneously evaluate the main experimental factors affecting SPME. It is important to highlight that this is the first time MS/MS has been applied to the detection of target preservatives in personal care products (except for some parabens [22]), which is expected to increase the selectivity of the determinations in such complex matrices.

2. Experimental

2.1 Reagents and materials

Methylbenzoate (MeBz; 99%), ethylbenzoate (EtBz; 99%), butylbenzoate (BuBz) and phenylbenzoate (PhBz; 99%) were supplied by ChemService (West Chester, USA). 2-phenoxyethanol (Phox; 99%) was obtained from Fluka Chemie GmbH (Steinheim, Germany). Bronidox (≥99.0%) was acquired from Fluka (Sigma–Aldrich Chemie GmbH, Buchs, Switzerland). Methylparaben (MeP; 99%), ethylparaben (EtP; 99%), propylparaben (PrP; 99%), butylparaben (BuP; 99%), benzylparaben (BzP; 99%), butylated hydroxyanisole (BHA; ≥98.5%), butylated hydroxytoluene (BHT; 99%), and triclosan (TCS; ≥97.0%) were purchased from Aldrich (Milwaukee, WI, USA). Isopropylparaben (*i*-PrP; ≥99%) and isobutylparaben (*i*-BuP; ≥97%) were acquired from TCI Europe (Belgium).

As isotopically labeled internal standard (IS), deuterated methyl-4-hydroxybenzoate-2,3,5,6-d_4 (MeP-d_4; 98 atom% D), propyl 4-hydroxybenzoate-2,3,5,6-d_4 (PrP-d_4; 98 atom% D), and triclosan-d_3 (2,4-dichlorophenoxy-d_3) (TCS-d_3, 97 atom% D), was obtained from C/D/N Isotopes (Quebec, Canada), whereas 2,6-Di(tert-butyl-d_9)-4-methyl(phenol-3,5,O-d_3) (BHT-d_{21}, 98 atom% D) were purchased from Sigma–Aldrich.

Acetone, ethyl acetate, and acetic anhydride were provided by Merck (Darmstadt, Germany). Sodium chloride (99.7%) was supplied by Prolabo (VWR, Fontenay-sous-Bois, France). Sodium hydrogenphosphate heptahydrate was acquired from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). All solvents and reagents were of analytical grade.

Individual stock solutions of each compound were prepared in acetone. Further dilutions and mixtures in the same solvent were prepared by convenient dilution of the stock solution to spike cosmetic samples (when needed). Derivatized standards in ethyl acetate were prepared by adding 100 µL of acetic anhydride containing 2.5% of pyridine to 1 mL of the standard solution. The mixture was then maintained at 80 °C for 10 min, and then allowed to cool down before GC analysis [25]. Stock and working solutions were stored in a freezer at −20 °C protected from light. For daily evaluation of the GC equipment, a derivatized solution of 0.5 µg mL^{-1} of the target compound in ethyl acetate was also prepared to direct injection into the chromatograph.

Commercially available 100 µm polydimethylsiloxane (PDMS), 65 µm polydimethylsiloxane–divinylbenzene (PDMS/DVB), 85 µm polyacrylate (PA), 75 µm carboxen–polydimethylsiloxane (CAR/PDMS), and 50/30 µm divinylbenzene–carboxen–polydimethylsiloxane (DVB/CAR/PDMS) fibers housed in manual SPME holders were obtained from Supelco (Bellefonte, PA, USA). The fibers were conditioned as recommended by the manufacturer, inserting them in the injection port with carrier gas flow: PDMS and PDMS/DVB were kept at 250 °C for 0.5 h, PA at 300 °C for 2 h, CAR/PDMS at 220 °C for 1–2 h, and DVB/CAR/PDMS at 270 °C for 1 h.

2.2 Cosmetic samples

Different cosmetics and personal care products from national and international brands were purchased from local markets and drugstores. Leave-on products including two body milks (BM), and two baby body milks (BBM), a moisturizing cream (MC), two deodorants (De), a sun screen (SS), a baby after sun (BAS), a moisturizing lotion (ML), a make-up (Mu), and an eye make-up remover (EMuR) were used. As regards rinse-off samples, a liquid soap (LS), a child bath gel (CBG), tooth paste (TP), two shampoos (Sh), three hair conditioners (HC), a facial cleansing milk (FCM), and an after shave (ASh) were considered. Samples were kept in their original containers at room temperature until analysis. Many of these products were labeled as containing any of target preservatives.

A representative group of different types of cosmetic products (Sh: shampoo; ASh: after shave; De: deodorant; ML: moisturizing lotion; TP: tooth paste; MC: moisturizing cream; HC: hair conditioner) were used as blanks for method optimization and validation. Such samples were fortified at two levels, 0.1% and 0.002% (w/w), by adding 100 µL of acetone solution containing the target preservatives at 10,000 and 200 µg mL^{-1}, respectively, over accurately weighed aliquots of approximately 1 g. Subsequently, spiked samples were

homogenized by agitation in a vortex and then kept at 4 °C for more than 2 h to allow analyte–matrix interactions.

Due to the complexity of the analyzed samples, all SPME experiments were performed with cosmetic samples diluted in ultrapure water. Aliquots of approximately 1 g of the cosmetic samples were firstly homogenized with 9 mL of ultrapure water by shaking until the mixture was dispersed, and then diluted in ultrapure water to achieve final dilution ratios ranging from 1:100 to 1:10,000 (w/w). Dilutions were carried out in one or two steps in order to avoid handling large volumes, particularly for the final dilution ratio (1:10,000).

2.3 Solid-phase microextraction procedure

Aliquots of 8 mL of diluted cosmetic samples were placed in 10 or 22 mL vials with addition of 0.1 g sodium hydrogenphosphate heptahydrate, 100 μL acetic anhydride, and sodium chloride at 20% (w/v) in the required experiments. Vials were sealed with aluminum caps furnished with PTFE-faced septa and immersed in a water bath maintained at 40 °C during 15 min for derivatization. After that vials were cold down under tap water stream, 2 mL of acetone were added. Then, samples were immersed again in the water bath at the working temperature and the SPME fiber was exposed to the headspace over the sample (headspace solid-phase microextraction, HSSPME) or immersed into the sample (direct solid-phase microextraction, SPME) for 10–60 min, depending on the experiment. In all extractions, the samples were magnetically stirred during the sampling. After extraction, the fiber was retracted and transferred to the GC injection port and chromatographic analysis was carried out. Desorption time was set at 10 min and desorption temperature was 260 °C. In order to extend the lifetime of the fibers and to protect the chromatographic system, fibers were immersed for a few seconds (three times) in ultrapure water after SPME extractions with NaCl.

2.4 Gas chromatography–tandem mass spectrometry determination

The GC–MS/MS analysis was performed using a Thermo Scientific Trace 1310 gas chromatograph coupled to a triple quadrupole mass spectrometer (TSQ 8000) from Thermo Scientific (San Jose, CA, USA). The system was operated by Xcalibur 2.2 and Trace Finder TM 3.0.

Separation was carried out on a TG-5 SILMS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) obtained from Thermo Scientific (San Jose, 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 80 °C (held 3 min) to 280 °C at 14 °C min^{-1} (held 3 min). Splitless mode (held 2 min) was used for injection. The injector temperature was between 260 and 290 °C depending on the fiber used. Injector was operating in the splitless mode and programmed to return to the split mode after 2 min from the beginning of a run.

The mass spectrometer (MSD) was operated in the electron impact (EI) ionization positive mode (+70 eV). The temperatures of the transfer line, and the ion source were set at 290 and 350 °C, respectively. Selected reaction monitoring acquisition mode (SRM) was used. Quantitation was performed using the Trace Finder software.

Matrix match calibration was used for sample quantification. The calibration standards were obtained by serial dilution of an aqueous standard mixture of 1 μg mL^{-1} of each target in a 1:10,000 diluted sample. The linearity was assessed by preparing standard solutions from 0.5 to 1000 ng mL^{-1}. MeP-d_4, PrP-d_4, BHT-d_{21} and TCS-d_3 were selected as ISs to minimize instrumental variability in the performance experiments.

3. Results and discussion

3.1 GC–MS/MS analysis

Chromatographic conditions were optimized using a standard mixture solution of all target compounds in ethyl acetate. Based on previous works reported by the authors [25, 35], a derivatization step involving acetylation with acetic anhydride was introduced previous to GC analysis to improve chromatographic performance, avoiding the hydroxyl group interactions with the chromatographic system (see Section 2.1). All target phenolic preservatives underwent acetylation, with the exception of BHT, whose highly hindered hydroxyl group with poor nucleophilicity may prevent the acetylation under the studied conditions [26]. As expected, no differences on the chromatographic response were observed for benzoates and bronidox, since these compounds do not have chemical groups susceptible to acetylation. Noticeable improvement in the direct analysis of the acetylated derivatives was observed in comparison with the appreciable tailing shown before derivatization. Retention times of derivatized preservatives were shifted to higher values than those of the corresponding underivatized compounds. Both derivatized and underivatized forms of BHA, parabens and TCS showed similar mass spectra, although slightly different intensities of ion ratios were observed. Additionally, molecular ions of acetylated forms were not present or showed very low intensity, most probably, due to the

loss of the acetyl group upon ionization [36]. In the case of 2-phenoxyethanol, the most intense fragment ion for the acetylated derivative (*m*/*z* 87) corresponds to the ethyl acetate moiety.

MS/MS detection was chosen to improve sensitivity and selectivity in complex cosmetic matrices. Working conditions were optimized using the automated selected reaction monitoring (AutoSRM) tool implemented in the TSQ8000 GC–MS/MS software. In this automated process three different steps are involved: (i) precursor ion study; (ii) product ion study; and (iii) SRM optimization. Thus, working in electron ionization (EI) mode, the precursor ion for each compound was determined by full scan mode. In a second step, the mass spectrometer was set in product-ion-scan mode, and the most intense precursor ions were selected to subsequent fragmentation with different collision energies in Q2.

The most intense transitions in the MS/MS spectra of parabens lead to the product ions 121 *m*/*z*. Methyl- and benzyl- paraben main transitions corresponded to the loss of the $[CH_3O]$– and $[C_7H_7O]$–moieties, respectively, belonging to the ester bond. For the rest of parabens, the major transition revealed the loss of a hydroxyl group from the 4-hydroxybenzoic acid. In the case of benzoates, the fragmentation of the base peak in the MS spectra (105 *m*/*z*) showed the loss of a carbonyl group, leading to the product ion *m*/*z* 77 (phenyl ion) as the main transition. As regards TCS, the most intense product ion was observed at 218 *m*/*z*, which might resulted from the loss of an aromatic ring, whereas BHA and BHT showed the main transitions corresponding to the removal of a carbonyl and a methyl group (28 and 15 *m*/*z*) from the base peak of the MS spectra, respectively. In the MS/MS spectra of bronidox, two intense product ions appeared at 107 and 109 *m*/*z*, in a cluster of ions typical of bromine-containing compounds, which corresponds to the loss of a carbonyl group. On the other hand, the acetylated and non-acetylated forms of 2-phenoxyethanol underwent different fragmentation, showing the main product ions 94 (phenolic moiety) and 65 *m*/*z*, respectively, as the most intense transitions. Regarding the deuterated derivatives (MeP-d_4, PrP-d_3, BHT-d_{21} and TCS-d_3), the isotopic shift was observed in the corresponding aforementioned fragments, both in the parent ion and in the product ion.

Finally, the optimization of collision energy for each transition was conducted. Two or three transitions were selected for each compound according to ion abundance. The most intense transition was used for quantification purposes, whereas the second and the third ones were employed for identification/confirmation purposes (Table 1). Full scan and product ion mass spectra of acetylated preservatives can be seen in Figs. S1–S12.

Table 1. Experimental GC–MS/MS parameters of the target preservatives.

Compound	RT (min)	SRM_1 (Quantification)	CE_1[a]	SRM_2 (Confirmation)	CE_2[a]	SRM_3 (Confirmation)	CE_3[a]	IS[b]
MeBz	5.23	136 > 105	10	105 > 77	10			MeP-d_4
EtBz	6.09	122 > 105	10	105 > 77	10			MeP-d_4
Brox	6.55	135 > 107	5	137 > 109	5			MeP-d_4
Phox	6.64	138 > 94	10	94 > 66	10	94 > 65	15	MeP-d_4
BuBz	8.24	123 > 79	10	105 > 77	10			MeP-d_4
MeP-d_4	9.18	156 > 125	10	125 > 97	10	125 > 69	20	-
MeP	9.20	152 > 121	10	121 > 65	15	121 > 93	10	MeP-d_4
BHA	9.26	165 > 137	10	137 > 77	20	180 > 165	10	MeP-d_4
BHT-d_{21}	9.33	222 > 154	15	222 > 191	10	240 > 222	10	-
BHT	9.45	220 > 205	10	205 > 145	15	205 > 177	10	BHT-d_{21}
EtP	9.85	138 > 121	10	121 > 65	15	121 > 93	10	PrP-d_4
i-PrP	10.13	138 > 121	10	121 > 65	15	121 > 93	10	PrP-d_4
PrP-d_4	10.69	142 > 125	10	125 > 69	15	125 > 97	10	-
PrP	10.71	138 > 121	10	121 > 65	15	121 > 93	10	PrP-d_4
PhBz	10.85	165 > 127	35	105 > 77	10			MeP-d_4
i-BuP	11.18	138 > 121	10	121 > 65	15	121 > 93	10	MeP-d_4
BuP	11.55	138 > 121	10	121 > 65	15	121 > 93	10	MeP-d_4
BzP	14.54	228 > 121	10	121 > 93	10	121 > 65	20	MeP-d_4
TCS-d_3	14.57	291 > 221	20	291 > 149	20	292 > 151	20	-
TCS	14.58	288 > 218	15	218 > 127	30	218 > 155	20	TCS-d_3

[a] Collision energie (eV)
[b] Internal Standard

3.2 In situ derivatization SPME

In situ derivatization approach, not only can led to the improvement in chromatographic behavior and detection properties but also can be used to enhance extraction efficiency, since derivatives typically possess higher distribution constants towards the coating [37]. Acetylation procedure with acetic anhydride in the presence of hydrogenphosphate heptahydrate, instead of hydrogencarbonate or carbonate, has been shown as a valuable alternative for the derivatization of phenolic compounds such as parabens and triclosan in water [27]. This approach represents an advantageous and suitable procedure for *in situ* derivatization SPME for the analysis of phenolic preservatives, avoiding the possible drawbacks derived from overpressure inside the vial at high extraction temperatures caused by carbon dioxide bubbles from carbonate salts.

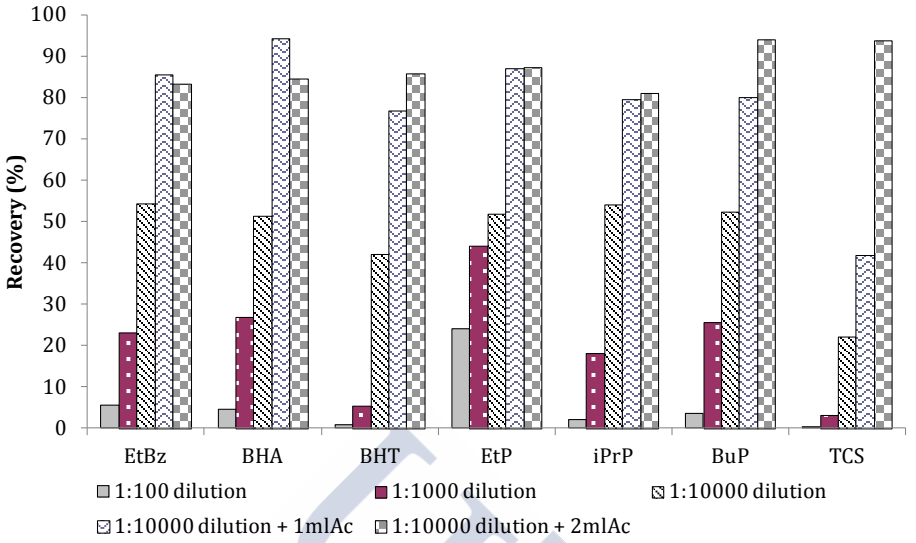

Fig. 1. Influence of sample dilution and acetone addition on matrix effect for some representative target preservatives.

In order to assess the feasibility of the *in situ* acetylation reaction for target preservatives, aliquots of 10 mL of water spiked with the targets at 20 ng mL^{-1} were initially used. As indicated by Regueiro et al. [27], 0.1 g sodium hydrogenphosphate, and 100 μL acetic anhydride as derivatization reagent were directly added to the SPME sampling vial. DVB/CAR/PDMS fiber was chosen for these initial trials, since it allows the extraction of analytes ranging different polarities. Extractions were performed in the direct sampling mode at room temperature for 15 min, after 15 min of thermostatization/derivatization at 40 °C, and using magnetic stirring to favor mass transfer. The SPME–GC–MS/MS analysis showed the presence of the corresponding acetyl derivatives of the target phenolic preservatives; whereas as expected, the derivatized form of BHT was not observed. In the case of 2-phenoxyethanol, the amount of acetylated derivative was very low (<10%). Anyway, the underivatized 2-phenoxyethanol, and BHT peak shape and chromatographic response were satisfactory, and they were considered as it, in subsequent experiments.

3.3 Sample pretreatment and matrix effect

An appropriate sample pretreatment is an essential step to minimize matrix effect, which becomes mandatory in order to apply external calibration. Otherwise, considering the

complexity and broad variety of cosmetic matrices, the use of standard addition calibration may result in a tedious quantification approach when analyzing a large number of different cosmetic products. Hence, a first approach based on sample dilution with Milli-Q water was applied with three basic aims: (I) minimize matrix effect, (II) improve mass transfer of targets between the sample and the fiber, and (III) allow working in direct sampling mode avoiding damages and contaminations in the fiber coating, as well as in the subsequent chromatographic analysis.

Dilution ratios of 1:100, 1:1000 and 1:10,000 were tested for matrix effect evaluation of MC1 sample with the target preservatives (20 ng mL^{-1}). Lower dilutions were discarded considering the low solubility of the samples at high concentrations, whereas no higher dilutions were considered since a significant reduction on method sensitivity was observed.

In a SPME–GC-MS process, the matrix effect is strictly related to the extraction yield of the sample process. In this case, following the validation criteria included in the ISO 12787 international standard [38] to the analysis of different cosmetics, matrix effect is given by the recoveries of the samples spiked with target analytes (R1) relative to standards in water at the same concentration (R2). Therefore, recovery values (%) of diluted samples, calculated as 100 × R1/R2, are depicted in Fig. 1 (three first bars). As expected, sample dilution lead to higher recoveries, increasing more than two or three times with respect to the 10-folds lower dilution. Thus, as dilution ratio decreases, a significant reduction on matrix effect for the target preservatives was observed, being particularly remarkable in the case of TCS and BHT. Although sometimes, operating in head-space mode may led to matrix effect reduction, similar results were obtained using both head-space (HSPME) or direct sampling mode (SPME) for the target compounds in the analyzed sample.

Since the aim of matrix effect reduction was not completely achieved (recoveries below 60%; see three first bars in Fig. 1), a second approach based on organic modifier addition was applied. Acetone was proposed as suitable organic solvent with intermediate polarity between water and high lipophilic cosmetic samples. Thus, different volumes of acetone were added (0.5, 1 and 2 mL) to the 1:10,000 diluted MC1 sample, spiked with the targets. As shown in Fig. 1 (two last bars), this time, satisfactory recoveries were obtained with the addition of 1 or 2 mL of acetone except for TCS, for which 2 mL are required. In order to check the generalized behavior in different matrices, four additional samples (TP, Sh1, De1, and HC1), were tested.Fig. 2 compares the SPME recoveries obtained for target preservatives spiked in diluted cosmetic samples, with (B) and without (A) acetone addition. As can be seen in Fig. 2A, the satisfactory recoveries obtained for some of the targets spiked in high water content samples (TP, Sh1 and De1), contrast with the poor extraction efficiency

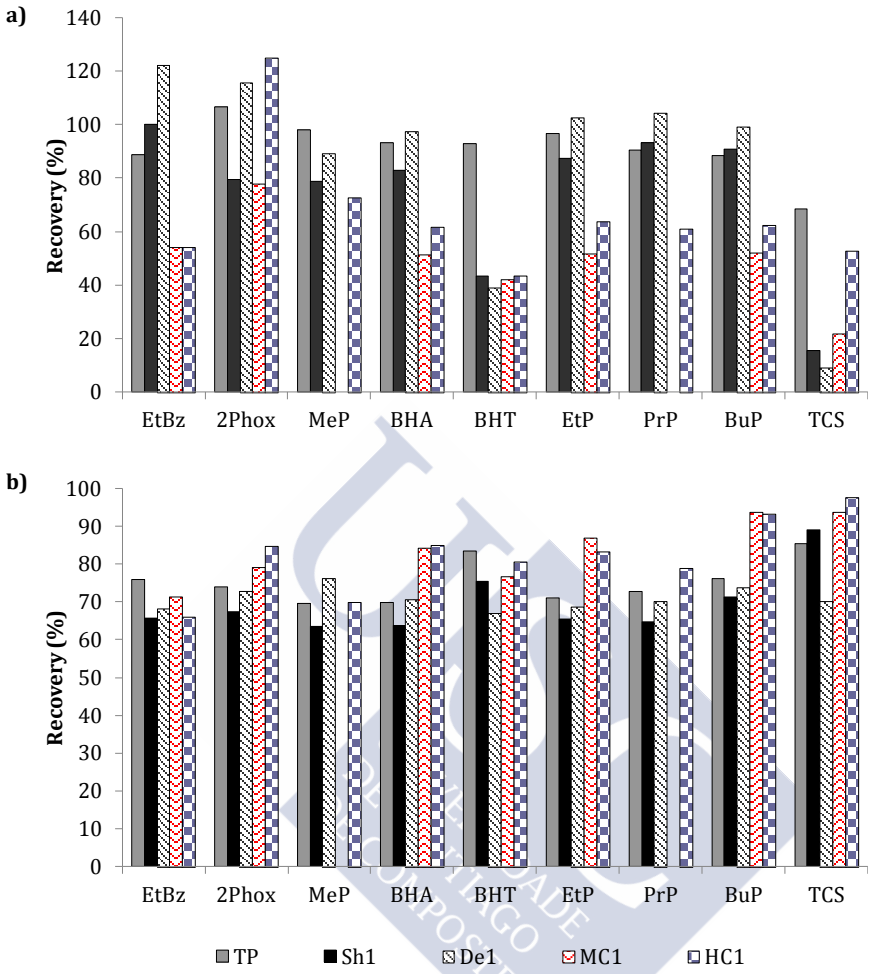

Fig. 2. Influence of sample dilution and acetone addition on matrix effect for some representative target preservatives.

observed for samples with apparent higher fatty content (FC and HC1). In addition, particularly low recoveries were found, in general, for BHA and TCS. On the contrary, a more homogeneous behavior on the extraction yield can be clearly observed after acetone addition (Fig. 2B). The presence of organic solvent has been proved to compensate matrix effect of different cosmetic matrices with different lipophilic nature.

The influence of sample dilution in the response was also evaluated. Dilution experiments with spiked BBM2 sample at 0.002% (w/w) were also conducted at the same three dilution ratios (1:100, 1:1000, 1:10,000). Unexpectedly, a sharp raise of response was

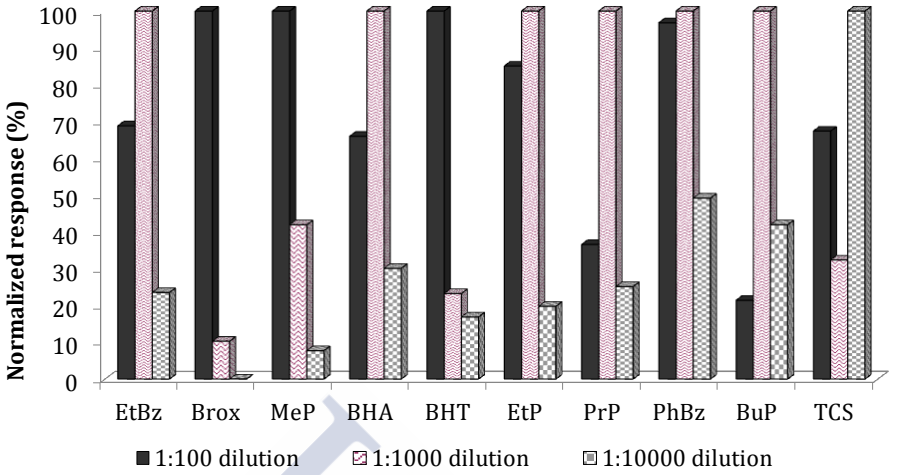

Fig. 3. Influence of sample dilution ratio on the analytical response of some representative targets. Responses are normalized to the highest signal for each compound.

observed for the most apolar compounds when applying 1:1000 sample dilution (see Fig. 3). In the case of 1:10,000 dilution, a generalized fall of reponse was noticed in comparison with the 10-folds lower dilution, however some preservatives such as *i*-BuP, BuP, BzP and TCS, showed even higher responses compared with the highest dilution. These results could be explained considering that, although sample dilution lead to lower (10 or 100 times below) analyte concentration, the matrix effect becomes weaker, favoring mass transfer. Hence, taking into account these results and considering that small dilution factors can decrease the life-time of the SPME fiber and increase background drawbacks in the chromatographic signal, the subsequent experiments were carried out using the 1:10,000 dilution ratios.

In further experiments, variable amounts of underivatized parabens and triclosan, ranging from 2% to 25%, were detected. Since acetone might led to low acetylation efficiency of target phenolic preservatives, some experiments were carried out to improve derivatization yield. Results indicated that when derivatization is conducted at 40 °C for 15 min before acetone addition, the amounts of underivatized compounds decrease below 5%.

Therefore, a SPME procedure based on sample dilution and *in situ* acetylation before acetone addition was proposed for the subsequent experiments, as an effective extractive strategy with matriz effect compensation.

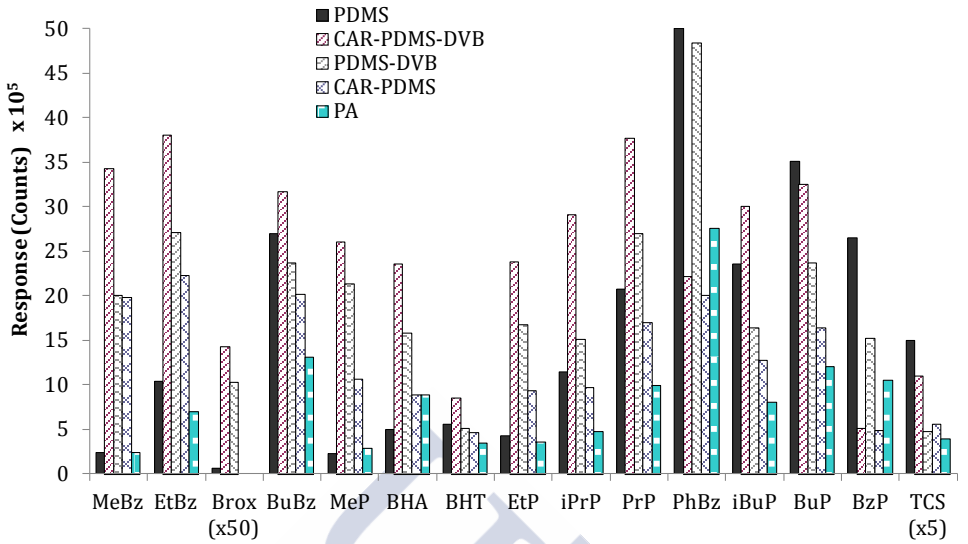

Fig. 4. Influence of the fiber coating on the extraction of the target preservatives.

3.4 SPME optimization

3.4.1 Influence of the fiber coating

Selection of an appropriate coating is one of the most important stages, and the first step to be considered when accomplishing a SPME optimization process. In this sense, preliminary experiments were performed in order to select the levels of this factor to be included in the subsequent experimental design. Five different fiber coatings (PDMS, DVB/CAR/PDMS, PDMS/DVB, CAR/PDMS, and PA) were compared to evaluate the capability of derivatized and non derivatized preservatives to be extracted by SPME. A 1:10,000 diluted cosmetic sample, spiked with the targets at 50 ng mL^{-1} was used to test the relative sorption affinity of each fiber for the target compounds in a real diluted matrix. As displayed in Fig. 4, the highest extraction efficiencies were achieved, in general, using DVB/CAR/PDMS fiber, with the exception of BzP and PhBz, which underwent a significant response improvement employing PDMS and PDMS/DVB. Close or comparable extraction yields were obtained using PDMS/DVB and DVB/CAR/PDMS for most volatile preservatives, whereas similar or higher responses were observed with PDMS fiber for less volatile compounds. On the contrary, the lowest extraction efficiency was observed, in general, using PA, due to its affinity for more polar compounds, whereas intermediate responses were obtained with CAR/PDMS, showing small differences with PDMS/DVB in some cases. Hence, PDMS, DVB/CAR/PDMS, and

PDMS/DVB were selected for the experimental design, since they were the most suitable coatings for the simultaneous analysis of the target compounds in cosmetic samples.

Similar results were obtained in reported works by Lokhnauth and Snow [39] and Regueiro et al. [27], where DVB/CAR/PDMS and PDMS/DVB were demonstrated to be the fiber coatings with better efficiency for the extraction of parabens in pharmaceutical formulations, and for parabens and triclosan extraction in water samples, respectively. However these results differ from those reported by Tsai and Lee [31], who proposed the use of PA fiber for the extraction of parabens and some antioxidants in cosmetic samples; although it is worthy to note that this study is referred to the extraction of underivatized compounds.

3.4.2 Experimental design

A factorial design was conducted to simultaneously evaluate the influence of the main factors affecting the solid-phase microextraction process, as well as to estimate their interaction effects. Taken into account the results obtained from *in situ* derivatization and sample pretreatment studies, some experimental conditions such as dilution factor, volume of acetone and the amount of derivatization reagent, were kept constant. On the other hand, four variables affecting SPME were considered as experimental factors to be included in the factorial design: fiber coating, extraction mode, temperature, and addition of sodium chloride. On the basis of the preliminary experiments, fiber coating was evaluated at three levels: PDMS, DVB/CAR/PDMS, and PDMS–DVB. The other three factors were studied at two levels: direct sampling (SPME) and headspace sampling (HSSPME) for extraction mode, 40 and 100 °C for the temperature, and 0% and 20% (w/w) for sodium chloride concentration. Table 2 summarizes the selected factor and their levels.

A $3 \times 2^{3-1}$ mixed-level fractional factorial design, involving a total of 12 randomized experiments, was proposed. Moreover, four additional experiments including central points (70 °C for the temperature, and 10% (w/w) for sodium chloride concentration) were

Table 2. Experimental factors and levels included in the experimental design.

Factor	Code	Low level (-)	Central level (0)	High level (+)	Continuous
Fiber coating	A	PDMS	DVB/CAR/PDMS	PDMS-DVB	No
Sampling mode	B	SPME		HS-SPME	No
Extraction T (°C)	C	40	70	100	Yes
NaCl (%, w/v)	D	0	10	20	Yes

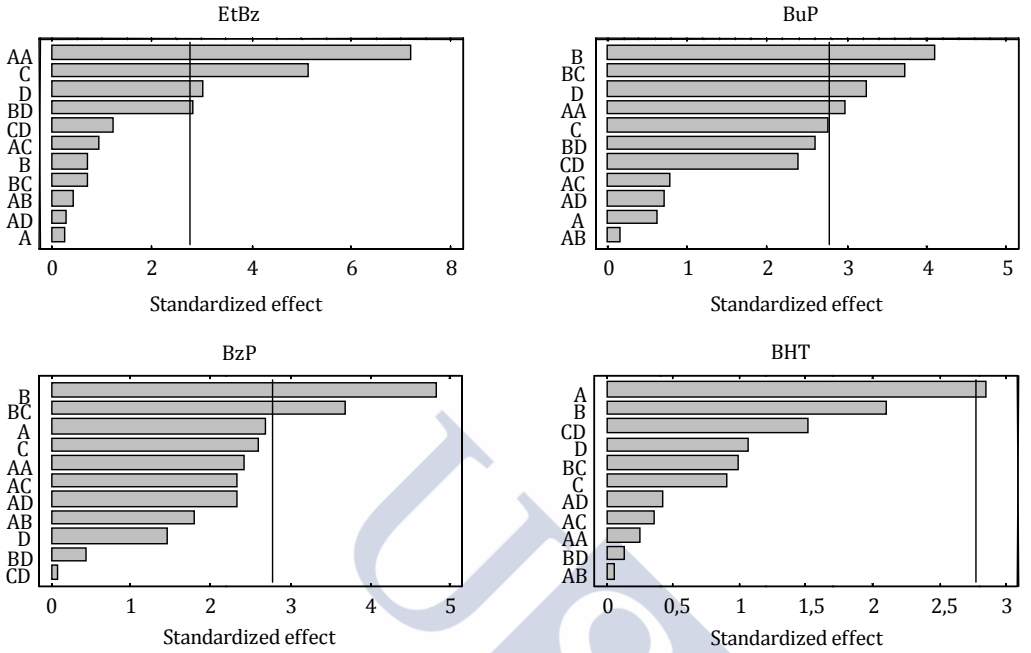

Fig. 5. Pareto charts for some representative target preservatives.

included, in order to gain sufficient degrees of freedom to estimate the experimental error. This sort of factorial design has resolution V, which means that main effects and all two-factor interactions can be estimated assuming that higher order interactions are not significant. Hence, it is possible to evaluate the influence on response of a total of four factor and their interactions with each other, with a small number of experiments (16 runs), compared with a full factorial design, which requires a total of 24 runs. Each experiment was carried out using a 1:10,000 dilution of MC1 sample, spiked at 0.002% (w/w) with each target preservative. After addition of 2 mL of acetone, a final volume of 10 mL was sampled in 22 mL vials, in order to make possible HSSPME experiments. Magnetic stirring was used, as it was widely demonstrated to favor mass transfer. Fifteen 15 min of extraction were initially set.

Data obtained from the factorial design can be easily interpreted with the statistical tools provided by the Statgraphics software. The graphical outcomes are depicted in Fig. 5 and Fig. 6, showing the representative behavior of the studied compounds. In the Pareto charts (Fig. 5), the length of each bar is proportional to the influence that each variable (factors) or second-order interaction (two-factors interaction) has on the response of the

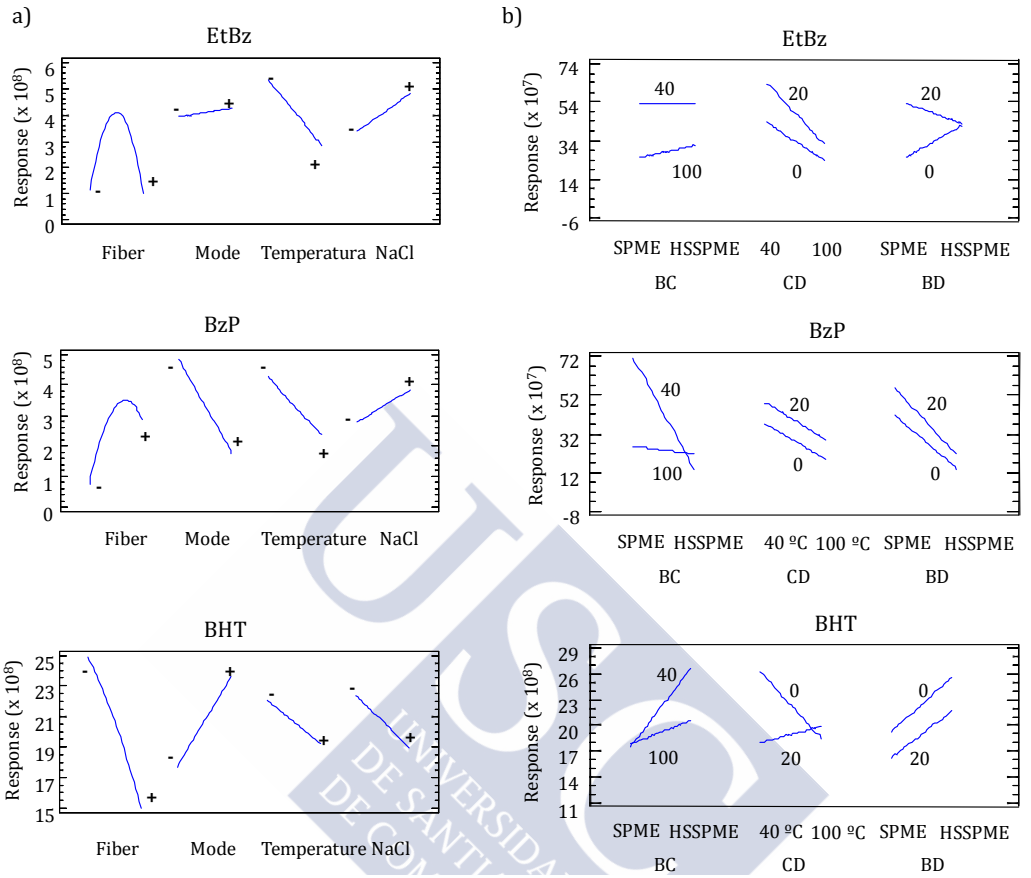

Fig. 6. Main effects plots for some selected preservatives (a), and interactions plots of relevant two-factors interaction (b).

target preservatives. In addition, those factors or interaction amongst these factors exceeding the vertical line limit are to be considered statistically significant at the 95% confidence level. Directly linked to the Pareto charts, the main effects plots (Fig. 6A) represent the response of the low and the high level of a factor connected by a line. The length of the line is proportional to the effect magnitude of the factor in the extraction process, whereas the sign of the slope indicates the level of the factor that produces the highest response.

As can be seen in Fig. 5, extraction mode (B) was one of the most influential variables, showing, in general, a sharp negative slope in the main effects plots (Fig. 6A). This result indicates that SPME was the most favorable sampling mode for the most targets, with the

exception of BHT, and two benzoates (see the former one in Fig. 6A). Nevertheless, for these compounds, sampling mode did not show a significant influence on its extraction.

The concentration of sodium chloride (D) is always a key factor to be considered in SPME, since the salt addition can improve mass transfer due to the so-called 'salting-out effect'. As expected, the addition of sodium chloride was a significant factor for the extraction of eight of the investigated preservatives (EtBz, BuBz, EtP, *i*-PrP, *i*-BuP, BuP, PrP and BHA). In general, according to the main effects chart, the 'salting-out' effect was shown to enhance the extraction for the studied compounds. In accordance with Tsai and Lee [31], the addition of salt did not improve the extraction of BHT from the cosmetic sample.

Extraction temperature is also one of the most relevant parameters to optimize in SPME, since it affects both thermodynamics and kinetics of the extraction [30]. For five of target preservatives (MeBz, EtBz, BuBz, BHA and EtP), temperature (°C) was a significant variable. For the rest of the compounds, temperature has been shown and important factor, showing the highest responses at the lowest level (40 °C). This behavior might be explained considering that high temperatures do not favor the adsorption kinetics of targets on fiber.

Finally, the fiber coating (A) was significant only for BHT. In this case, the best extraction yield was obtained using the PDMS coating.

In view of these plots for some representative compounds, it can be clearly appreciated that both extraction mode and temperature show a high influence on response. In general, higher extraction efficiency is obtained in the direct sampling mode at 40 °C. Moreover, the addition of salt led to higher extraction yields.

As far as second-order interactions are concerned, three of them were significant for any of the studied compounds: AA, BC, and BD. Fiber–fiber (AA) interaction has proven to have an essential influence on the extraction (Fig. 6B). The so-called 'squared factor' (AA), corresponding to the central level of the fiber coating variable: DVB/CAR/PDMS, was demonstrated to have a significant and positive effect on the SPME of most of the studied preservatives, with the already mentioned exception of BHT.

Regarding the interaction between extraction mode and temperature (BC), the higher responses are reached in the SPME mode when extraction at 40 °C is performed. This important interaction was significant for nine of the analytes. As indicated above, in the case of BHT, better responses were obtained using HSSPME mode at 40 °C. For the rest of the preservatives, the temperature effect was negligible operating in this mode. This behavior

contrast with the results reported in other studies, dealing with SPME of different groups of analytes, where the influence of the temperature is much higher for HSSPME than for SPME.

Interaction between extraction mode and NaCl addition (BD), was only significant for BHA and EtBz. In general, it is observed that better responses are obtained working in direct sampling mode at 20% (w/w) salt. Nevertheless, slight differences between the addition of 0% and 20% of salt were observed when working in HS mode.

Concerning the combined effect of the extraction temperature and sodium chloride (CD interaction), no significant differences are observed operating at 40 °C between 0% and 20% salt, except for BHA. In general, higher responses are obtained with 20% salt. On the contrary, BHT reached higher extraction efficiency in the absence of sodium chloride, although this effect is not significant. Working at 100 °C, no difference was observed at any NaCl concentration.

Attending the above discussion, some particular conditions might be considered to be applied in the case of individual analysis of some of the studied compounds such as BHT. For the extraction of this antioxidant, HSSPME at 40 °C, using PDMS coating without salt addition is recommended for an optimal SPME performance and for lower limits achievement. Moreover, it is important to notice that for several of the target preservatives PDMS/DVB and DVB/CAR/PDMS does not show significant differences on extraction efficiency, which may drive to the preferable use of the former coating, taken into account the lower carry over effect.

Considering that the aim of this work is the development of a multi-preservative method, the following general conditions are recommended for the simultaneous extraction of the 16 target preservatives in cosmetic samples: DVB/CAR/PDMS fiber coating, SPME sampling mode, 40 °C and addition of NaCl (20%, w/v). Since SPME was observed as the optimal sampling mode, the subsequent experiments were carried out employing 10 mL vials, in order to avoid the head-space volume.

Extraction time was finally evaluated analyzing spiked diluted samples after different times of fiber exposure in order to improve sample throughput. After 15 min of extraction, small differences were noticed in the extraction yield for most of target compounds. Therefore, 15 min of SPME after 15 min of derivatization was finally proposed.

Table 3. Linearity, and limits of detection of the proposed method.

Compound	Linearity		LOD[a] (%, w/w) × 10^4	LOQ[a] (%, w/w) × 10^4
	R^2	Range (%, w/w) × 10^4		
MeBz	0.9998	50 - 5000	0.92	3.1
EtBz	0.9998	10 - 5000	0.22	0.74
Bronidox	0.9995	200 - 5000	9.1	30
2Phox	0.9964	10 - 5000	0.78	2.6
BuBz	0.9999	10 - 1000	0.077	0.25
MeP	0.9998	5 - 5000	0.016	0.053
BHA	0.9999	5 - 5000	0.081	0.27
BHT	0.9999	10 - 2000	0.040	0.13
EtP	0.9991	5 - 5000	0.26	0.89
i-PrP	0.9997	5 - 5000	0.043	0.14
PrP	0.9995	5 - 5000	0.017	0.059
PhBz	0.9999	10 - 500	0.39	1.3
i-BuP	0.9994	5 - 1000	0.12	0.41
BuP	0.9992	5 - 1000	0.0075	0.025
BzP	0.9996	5 - 2000	0.018	0.060
TCS	0.9987	5 - 1000	0.0059	0.020

[a] equivalent to μg g^{-1}

3.5 Method performance

To assess the performance of the SPME–GC–MS/MS method, analytical quality parameters were evaluated (Table 3 and Table 4).

Considering the complexity and broad variety of studied matrices, the feasibility of the optimized microextraction procedure must be demonstrated in different cosmetic and personal care products. In this sense, a wide range of real samples (Sh1, ASh, De, ML, TP, MC) were selected for method accuracy evaluation. Since leave-on products (lipophilic samples) are expected to represent more complex matrices in aqueous solution in comparison with rinse-off products (high water content samples), greatest efforts were focused on leave-on cosmetics validation. These samples were fortified with known concentrations of each preservative at two levels: 0.1% (w/w) and 0.002% (w/w) (see details in Section 2.2). Recovery values were calculated as the ratio of the response obtained for spiked samples to the response measured for standards (at the same concentration level) prepared in a matrix matched solution (1:10,000 dilution of De1), and expressed as percentage (Table 4). In some particular cases, recovery values could not be estimated, since certain samples contained higher concentrations of some of targets than the corresponding to the spiked levels.

Table 4. Recoveries and repeatability obtained in representative leave-on and rinse-off cosmetic matrices.

Compound	Recovery (RSD) (%, n = 3)																	
	0.1 % (w/w)									0.002 % (w/w)								
	Sh1[a]	Ash		De1		ML		TP		MC1	MC2	Sh1		Ash		De1[a]		ML[a]		TP[a]		MC1
MeBz	101	90.1 (3.4)	*(7.4)*	89.3 (3.3)		94.4 (0.2)		97.0 (1.6)	*(5.3)*	87.8 (12)	92.1 (0.2)	102 (3.3)		88.9 (0.1)		104		101		92.0		115 (2.2)
EtBz	97.4	86.4 (2.0)	*(7.2)*	85.9 (7.0)		97.6 (2.6)		103 (4.5)	*(5.0)*	96.2 (9.2)	94.8 (6.6)	117 (3.8)		96.6 (3.6)		96.3		107		111		120 (2.1)
Brox	85.0	79.9 (0.3)	*(14)*	81.9 (1.2)		101 (6.0)		110 (15)	*(7.5)*	110 (8.0)	107 (16)	n.c.[b]		n.c.[b]		n.c.[b]		n.c.[b]		n.c.[b]		n.c.[b]
Phox	89.2	86.1 (2.0)	*(10)*	81.1 (0.4)		106 (6.7)		97.7 (3.9)	*(11)*	83.5 (9.1)	96.5 (0.1)	94.4 (7.2)		96.9 (3.3)		107		102		108		88.7 (8.5)
BuBz	86.7	86.6 (3.8)	*(6.8)*	80.1 (1.1)		103 (7.5)		97.9 (3.9)	*(11)*	95.9 (7.6)	92.3 (9.0)	104 (5.4)		94.7 (4.3)		111		98.6		108		95.9 (7.8)
MeP	99.7	101 (1.8)	*(1.5)*	97.4 (0.7)		109 (6.3)		103 (1.2)	*(1.6)*	108 (1.1)	105 (2.4)	104 (2.6)		n.c.[c]		111		n.c.[c]		69.8		n.c.[c]
BHA	112	103 (1.3)	*(8.0)*	84.9 (2.5)		107 (3.4)		96.7 (2.2)	*(6.8)*	79.9 (2.2)	97.7 (8.8)	114 (14)		110 (7.0)		95.0		120		130		113 (13)
BHT	104	104 (3.6)	*(11)*	96.9 (1.0)		102 (3.8)		103 (3.2)	*(10)*	130 (5.7)	111 (6.2)	100 (11)		103 (4.9)		126		90.9		118		91.5 (8.4)
EtP	97.1	93.2 (1.3)	*(7.2)*	97.8 (3.7)		96.9 (1.6)		100 (0.7)	*(10)*	94.5 (4.0)	95.3 (1.3)	99.9 (0.1)		92.1 (0.4)		93.8		106		94.9		101 (1.5)
i-PrP	103	93.7 (1.7)	*(13)*	103 (0.6)		91.8 (5.1)		97.0 (5.3)	*(15)*	86.5 (6.3)	96.6 (0.5)	101 (4.9)		103 (4.3)		98.2		103		103		106 (2.0)
PrP	107	99.3 (1.5)	*(2.9)*	99.3 (4.5)		83.9 (2.7)		102 (3.5)	*(4.1)*	99.9 (8.6)	103 (2.0)	104 (0.5)		95.4 (3.6)		98.5		n.c.[c]		96.7		112 (0.1)
PhBz	94.4	88.3 (7.0)	*(17)*	77.8 (6.3)		111 (9.6)		105 (7.9)	*(11)*	112 (2.8)	110 (1.8)	87.0 (9.3)		78.7 (3.3)		107		102		107		87.5 (4.0)
i-BuP	101	102 (7.3)	*(6.7)*	91.3 (2.6)		107 (1.7)		109 (7.6)	*(10)*	126 (6.9)	117 (8.7)	94.6 (2.1)		86.1 (3.9)		114		88.7		88.5		102 (0.2)
BuP	101	99.0 (8.2)	*(9.4)*	90.3 (2.3)		108 (0.9)		107 (7.7)	*(9.4)*	126 (7.3)	115 (6.8)	93.8 (3.8)		79.9 (2.5)		115		89.5		84.4		95.0 (0.7)
BzP	125	108 (2.3)	*(11)*	94.1 (5.6)		105 (3.8)		110 (6.7)	*(12)*	98.5 (5.7)	98.4 (4.3)	94.8 (8.6)		66.5 (5.3)		108		95.7		87.3		98.7 (6.8)
TCS	103	104 (1.8)	*(4.0)*	101 (2.3)		98.8 (3.1)		99.6 (4.0)	*(3.0)*	108 (2.3)	98.2 (0.7)	102 (2.0)		107 (2.9)		130		80.7		103		102 (2.4)

Sh: shampoo; ASh: after shave; De: deodorant; ML: moisturizing lotion; TP: tooth paste; MC: moisturizing cream

RSD values in italics: reproducibility

[a] (n=2)

[b] n.c., not calculated, since the spiked concentration was below LOQ.

[c] n.c., not calculated, since the concentration in sample is higher than the spiked concentration (see concentration values in Table 5).

In view of the results displayed in Table 4, satisfactory recoveries were achieved (above 85%) for most of targets and, therefore, no matrix effect was observed on tested samples. These results confirm the suitability of acetone addition as a simple strategy for avoiding matrix effect, allowing external calibration using matrix match solutions.

Method precision was evaluated at two fortified levels (0.1 and 0.002% w/w) as well as in non-spike samples containing the target analytes. The results were expressed in terms of relative standard deviation (RSD) in Table 4. In general, repeatability (intra-day) values were below 12%, reaching values no higher than 16% in some isolated cases, whereas slightly higher values were shown for reproducibility (inter-day) ranging from 4% to 15% for most of compounds. The variability was compensated by using four deuterated internal standards (MeP-d_4, PrP-d_4, BHT-d_{21}, TCS-d_3) (See Table 1).

Instrumental linearity was evaluated using the diluted De1 sample, as matrix matched solution free of target preservatives, spiked at different concentrations levels (between 6 and 10) ranging from 0.0005% to 0.5% (w/w), depending on the linear range of the studied compound. The calibration curves were obtained analyzing each concentration level in duplicate or triplicate. The response function was found to be linear for all compounds, with determination coefficients (R^2) between 0.9964 and 0.9999 (Table 3).

Regarding the limits of detection (LODs) and quantification (LOQs), they were calculated as the concentration giving a signal-to-noise ratio of three (S/N = 3) and 10 (S/N = 10), respectively, taking into account the dilution factors. These values were obtained for real samples spiked below the lowest level of the calibration curve, and expressed as a percentage (%, w/w) in accordance with the concentration units used in the European Cosmetics Regulation (Table 3). Procedural blanks showed the presence of some of targets such as BHT, which forced the LODs achieved for this compound to those corresponding to the average amount of analyte giving a response that is the blank signal plus three times the standard deviation. The source of BHT in blanks might be due to their ubiquitous presence at low levels in the ultrapure water. As can be seen in Table 3, LODs were below 0.000092% (w/w) for most of compounds, except for bronidox with LOD of 0.00091% (w/w).

The comparison with other reported methods in literature, based on MSPD [25] or PLE [26], shows that slightly higher LODs values were obtained with the proposed acetylation–SPME method, considering that high dilution levels are required. However, the proposed method provides a simple and less-time consuming alternative, allowing a higher throughput of analysis. Anyway, LODs described above were far below the maximum authorized concentration established by the EU legislation, between 0.1% and 1% (w/w), for

target compounds. Moreover, compared to other methods applying SPME [31], the developed methodology includes a wider range of preservatives to be analyzed in a single run using a cheap derivatization procedure and selective MS/MS detection.

3.6 Application to real samples

The developed SPME–GC–MS/MS methodology was applied to the analysis of a wide range of real cosmetic samples, which were labeled as containing any of the studied preservatives. Both rinse-off and leave-on samples were considered. ISs were added to each sample before the extraction in order to correct instrumental variability and slight variations that might arise due to the matrix effect. External calibration with matrix match was applied for accurate quantification. The results corresponding to the analyzed samples are shown in Table 5.

In general, parabens were the most frequently found preservatives in any of the analyzed samples. In particular, MeP and PrP, both associated with an increase in the preservative activity, were the most abundant preservatives followed by EtP, PrP, and BuP. These parabens were found in more than 10 samples, at concentrations no higher than 0.326% (w/w), quite close to the legal limit for single esters. In addition, the total content of parabens in the samples was also calculated and expressed as % (w/w) as acid according to the European Regulation. Although the legal restrictions were fulfilled in all cases, in half of samples, the total paraben content can be considered quite high (>0.1% expressed as acid). In some leave-on samples, the total paraben content attained even higher values than the highest levels detected in rinse-off samples. This can be particularly worrying, taking into account that leave-on products are intended to be in prolonged contact with skin. These findings confirm the results obtained in previous studies, which evidence the high content of parabens in marketed care products [40].

BHT was found in eight samples at concentrations ranging from 0.00306 to 0.19886% (w/w). Although BHT can be found associated to BHA as a synergic interaction, this last antioxidant was not found in any of the analyzed samples. Surprisingly, BHT reached the highest concentration values in leave-on products intended to be applied in the delicate and sensitive skin of the baby (BBM2 and BAS). Although there is some concern about the safety of both antioxidants, there are no restrictions about their use in the European Regulation.

Table 5. Concentration (%, w/w × 10^4) a of target preservatives in analyzed cosmetic samples.

	Leave-on														Rinse-off				
	FCM	EMuR	De2	BM1	BM2	BBM1	BBM2	MC1	MC2	SS	ASh	BAS	ML	Mu	CBG	Sh2	LS	HC2	HC3
Bronidox																			343
Phox		1210		309	260				74.5					48.7					
MeP	1290	16.2		3260	2330	1040	10.2	131		1980	1710	24.4	1760	1650	621	636	969	715	15.3
BHT	85.2	43.9	53.6				1830			14.1	30.6	1990		34.5					71.1
EtP	263	7.71		200	1370	202	1.69			213	7.13			940	260	230		118	8.19
PrP	144			870	9.42	493	3.21	63.1		902		10.3	727	3090		145	917	68.1	21.2
i-BuP	183			163						110				320		150		70.5	
BuP	328			282						272				625		235		136	
TCS			1250																
Total parabens[b]	*1870*	*27.5*		*4110*	*3260*	*1500*	*13.8*	*167*		*2940*	*1560*	*34.2*	*2490*	*5320*	*786*	*1220*	*1580*	*947*	*38.5*

Blank spaces: below LOQ
FCM: facial cleansing milk; EMuR: eye make-up remover; De: deodorant BM: body milk; BBM: baby body milk; MC: moisturizing cream; SS: sun screen; ASh: after shave; BAS: baby after sun; ML: moisturizing lotion; Mu: make-up; CBG: child bath gel; LS: liquid soap; HC: hair conditioner.
[a] equivalent to μg g^{-1}
[b] Expressed as acid

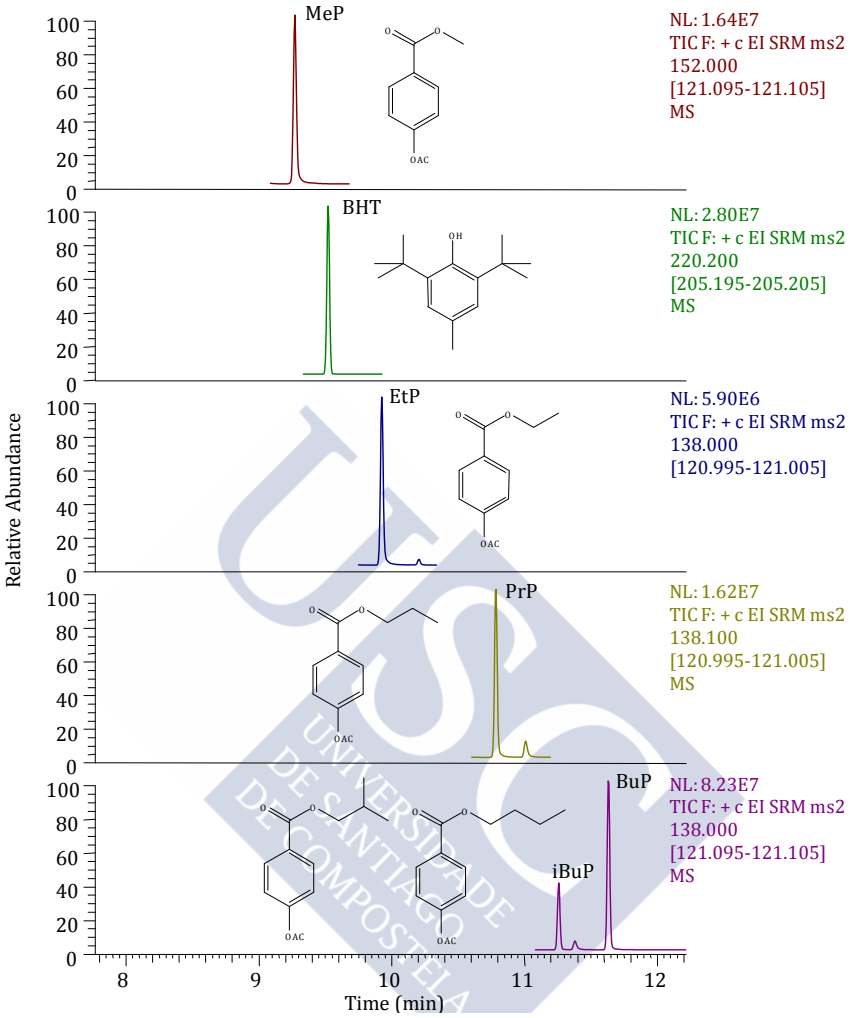

Fig.7. GC–MS/MS reconstructed chromatograms for a facial cleansing milk (FCM) containing six targets (see concentrations in Table 5).

2-Phenoxyethanol was included in six samples at concentrations between 0.00487 and 1.21% (w/w), exceeding the maxima authorized concentration (0.1%, w/w) in an eye make-up remover (EMuR). In the case of TCS, the highest concentration (0.125% w/w) was found in one of the analyzed deodorants (De2) with a value quite close to the legal limit 0.3% (w/w). It is reported that the presence of chemicals with proved endocrine disrupting and genotoxic properties (such as TCS) in cosmetic products applied to the underarm and breast area might be related to breast cancer [41].

Regarding product labeling, it is important to notice that eight of the analyzed samples were mislabeled with respect to one or more preservatives. 2-phenoxyethanol and BHT were not included on de label of two and three samples, respectively. As regards parabens, it is particularly noteworthy the absence of PrP, *i*-BuP and BuP in the label of a shampoo (Sh2). Fig. 7 shows the GC–MS/MS reconstructed chromatograms for a facial cleansing milk (FCM) containing six targets (see concentrations in Table 5).

4. Conclusions

SPME–GC–MS/MS was shown to be a simple, efficient and sensitive methodology for the rapid determination of multi-preservatives in cosmetic samples. In situ acetylation and organic modifier addition have been successfully implemented in the solid-phase microextraction process as an effective extractive strategy with matrix effect compensation, and improved chromatographic performance. A multivariate approach, based on a factorial experimental design, was applied for the optimization of the main variables affecting SPME. The validation of the optimized methodology was performed using a wide variety of rinse-off and leave-on samples, showing satisfactory recoveries and precision (RSD < 13%), demonstrating its reliability for routine control. In addition, short sample preparation time is needed (30 min), and low solvent consumption is required, which enables a high sample throughput and fulfils the most relevant criteria of a feasible extraction procedure.

The developed method was successfully applied to the analysis of a wide range of cosmetics and personal care products, revealing the high content of some of targets, although legal limits were fulfilled in general. Furthermore, the results evidence that some of the analyzed samples did not show compliance with the European Cosmetics Regulation with regards to labeling requirements, since several of target preservatives were not listed on the label. These findings demonstrate the suitability of the proposed method which is a valuable contribution to the required multi-preservative methodology for the quality control of cosmetic products.

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Capítulo III.1.5

Determination of isothiazolinone preservatives in cosmetics and household products by matrix solid-phase dispersion followed by high-performance liquid chromatography-tandem mass spectrometry

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Determination of isothiazolinone preservatives in cosmetics and household products by matrix solid-phase dispersion followed by high-performance liquid chromatography-tandem mass spectrometry.

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Abstract

In this work, the development of a new efficient methodology applying, for the first time, matrix solid phase dispersion (MSPD) for the determination of sensitizer isothiazolinone biocides in cosmetics and household products – 2-methyl-3-isothiazolinone (MI), 5-chloro-2-methyl-3-isothiazolinone (CMI), 1,2- benzisothiazolinone (BzI) and 2-octyl-3-isothiazolinone (OI) – is described. The main factors affecting the MSPD extraction procedure, the dispersive phase and the elution solvent, are assessed and optimized through a multicategorical experimental design, using a real cosmetic sample. The most suitable extraction conditions comprise the use of 2 g of florisil as dispersive phase and 5 mL of methanol as elution solvent. Subsequently, the extract is readily analyzed by HPLC–MS/MS without any further clean-up or concentration steps. Method performance was evaluated demonstrating to have a broad linear range ($R^2 > 0.9980$) and limits of detection (LOD) and quantification (LOQ) at the low nanogram per gram level, which are well below the required limits for UE regulation compliance. Satisfactory recoveries above 80%, except for MI (mean values close to 60%), were obtained. In all cases, the method precision (% RSD) was lower than 7%, making this low cost extraction method reliable for routine control. The validated methodology was finally applied to the analysis of a wide variety of cosmetics and household products. Most of the real samples analyzed have been shown to comply with the current European Cosmetic Regulation, although the results obtained for some rinse-off cosmetics (e.g. baby care products) revealed high isothiazolinone content.

Keywords: Isothiazolinones; Cosmetics; Matrix solid-phase dispersion; Biocides; Preservatives; Personal care products; LC–MS/MS.

1. Introduction

Isothiazolinone-type biocides are a group of effective preservatives widely used for the control of microbial organisms in a wide variety of aqueous-based industrial or domestic products and applications [1-3]. These compounds are heterocyclic derivatives of 2H-isothiazolin-3-one with an active sulfur moiety capable of oxidizing thiol-containing residues, thereby offering a powerful preservation activity against a broad spectrum of fungi and bacteria [4]. The most commonly used isothiazolinones which find manifold commercial applications are listed in Table 1. Methylisothiazolinones like 2-methyl-3-isothiazolinone (MI) and 5-chloro-2-methyl-3-isothiazolinone (CMI) are the active ingredients of a 3:1 CMI/MI mixture commercially sold under the name of Kathon. Cosmetic industry commonly includes Kathon CG (cosmetic grade) in a wide range of both rinse-off and leave-on formulations such as shampoos, gels, hair and skin care products, due to their high effectiveness at very low concentrations [5]. The use of MI and CMI has dramatically risen, and even gained presence at much higher concentrations together with 1,2-benzisothiazolinone (BzI) and 2-octyl-3-isothiazolinone (OI) in cleaning agents and other household and industrial products (water-based coatings, paints, adhesives, wood preservatives, etc.) [6, 7].

As a result of the widespread use of these compounds, there is an increased concern about the possible unintended side effects that these biocides could have on human health and environment. Both MI and CMI have been shown as strong skin sensitizers and allergens [5, 8, 9]. Therefore, EU legislation has restricted their presence in cosmetic products to maximum concentrations of 0.0015% for 3:1 CMI/MI mixtures or 0.01% for MI [10]. Besides, the Cosmetic Toiletry and Fragrance Association (CTFA) recommends using concentrations of no more than 0.00075% of the CMI/MI mixtures in leave-on products [11]. Recently, the Scientific Committee on Consumer Safety (SCCS) reported that no adequate data was given to support safe use of the Kathon mixture in leave-on cosmetics [12]. Although the concentration of the non-halogenated derivative MI alone might be safe, being a weaker sensitizer compared to CMI, new cases of contact dermatitis have been reported [8]. Current scientific experiments also suggest that MI may be a neurotoxic biocide after prolonged exposures even at low levels [13]. Other evidences of contact allergy have been deduced from accumulated case reports associated with occupational or domestic exposure to products containing BzI and OI [14, 15].

Thus, in order to guarantee consumers health and ensure compliance to existing or future regulation, reliable methodologies to identify and quantify isothiazolinone preser-

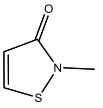
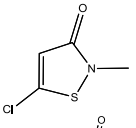
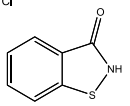
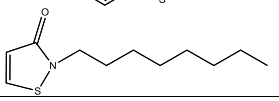
vatives are required. The case of cosmetics, toiletries and household products, to which people are most directly exposed, is of special relevancy considering the lack of updated methods in the literature for that purpose. These products include a broad variety of highly complex matrices which imply serious analytical difficulties.

Isothiazolinone analysis has been scarcely accomplished in matrices such as water, non-formalin adhesives, or food paper by liquid chromatography (LC or HPLC) [6, 16-19] and, in a lesser extent, by gas chromatography (GC) [2, 3]. The use of GC does not show, in general, good performance for MI and CMI analysis and, additionally, BzI requires derivatization to reduce adsorption in the chromatographic system and improve sensitivity. Recently, high performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) has been described for isothiazolinones analysis [6], providing high selectivity and sensitivity and avoiding an awkward derivatization step. Liquid–liquid extraction (LLE), ultrasonication or solid-phase extraction (SPE) are the procedures reported for isothiazolinones isolation. However, poor MI extraction yields have been obtained [2, 6] from high water-content matrices, due to the high polarity and water solubility of MI. Regarding isothiazolinone cosmetic analysis, the number of studies is really low and methods only include MI and CMI. They are usually based on solvent extraction followed by HPLC–UV detection, requiring extract purification [20, 21].

To overcome the drawbacks arising from the extraction of complex matrices, matrix solid-phase dispersion (MSPD) is presented as a valuable choice. The possibility of performing extraction and clean-up at the same time is one of the main advantages of this technique, which also reduces the amount of solvent required [22, 23]. MSPD has been applied for the isolation of a wide variety of analytes in matrices such as food, biota, vegetables, and environmental samples [22-25]. Recently, MSPD followed by GC–MS have been applied, for the first time, to cosmetic products analyzing fragrance allergens [26] and phenolic preservatives such as parabens [27]. Moreover, the analytical usefulness of applying chemometric tools (e.g. experimental design) for the optimization of MSPD experimental conditions has been demonstrated [28-30].

The aim of this study is the development, of an efficient method for the determination of isothiazolinone biocides in multi-matrix cosmetics and household products employing MSPD followed by HPLC–MS/MS. To the best of our knowledge, this is the first time that MSPD-HPLC–MS/MS is applied to the analysis of isothiazolinones in these complex matrices. It constitutes an important methodological improvement enabling the control of isothiazolinones content in these daily products.

Table 1. Characteristics and molecular properties of the target isothiazolinones.

Key	Compound	Chemical structure	CAS No.	MW (g/mol)	Water solubility (g/L)	$LogK_{ow}$
MI	2-Methyl-3-isothiazolinone[a]		2682-20-4	115.1	24	-0.49[c]
CMI	5-Chloro-2-methyl-3-isothiazolinone[a]		26172-55-4	149.6	10	0.40[c]
BzI	1,2-Benzisothiazolinone[b]		2634-33-5	151.2	0.56	1.24[d]
OI	2-Octyl-3-isothiazolinone[b]		26530-20-1	213.3	0.077	2.45[d]

[a] According to REGULATION (EC) No 1223/2009, the presence of this preservative substance must be indicated in the list of ingredients. Maximum allowed concentration: 0.0015 % (of a mixture in the ratio 3:1 CMI/MI) or 0.01% of MI in rinse-off and leave-on cosmetics.
[b] Not listed in Annex VI of REGULATION (EC) No 1223/2009, and therefore cannot be used as a preservative in cosmetic products.
[c] [17]. [d] [19].

2. Experimental

2.1 Chemicals and reagents

Chemical characteristics and CAS numbers of the studied compounds are presented in Table 1. As can be seen, the differences in chemical properties of the target isothiazolinones are evidenced by the wide range of hydrophilicity with log Kow values spanning more than three orders of magnitude.

A mixture of 2-methyl-3-isothiazolinone and 5-chloro-2-methyl-3-isothiazolinone (1:3) was acquired as Proclin reference standard (1.51% in water: 0.39% MI, 1.12% CMI) from Supelco (Bellefonte, PA, USA). 1,2-Benzisothiazolinone (BzI, ≥97.0% purity) and 2-octyl-3-isothiazolinone (OI, 99.9% purity) were purchased from Fluka (Sigma–Aldrich Chemie GmbH, Steinheim, Germany).

Acetone (analytical grade), n-hexane (analytical grade) and acetonitrile (HPLC-gradient grade) were supplied by Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Methanol (HPLC grade), water (HPLC grade) and formic acid (LC/MS grade) were supplied by J.T. Baker (Deventer, The Netherlands). Ammonium acetate (LC/MS grade) was obtained from Sigma–Aldrich (Steinheim, Germany). Argon BIP (99.995%) was purchased from

Carburos Metalicos (Aranjuez, Spain). Nitrogen, 99% was produced from a Mistral 4 generator (Schmidlin, Genève, Switzerland).

MSPD extraction procedure was conducted with four different sorbents tested for their ability to extract the target compounds from the studied samples. Florisil (60–100 mesh) was purchased from Supelco Analytical (Bellefonte, PA, USA). Neutral alumina and C_{18} (50–70 mesh) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Silica gel 60 (230–240 mesh) was obtained from Merck KGaA (Darmstadt, Germany). Before being used, florisil, alumina and silica were activated at 130 °C for 12 h and then allowed to cool down. Sodium sulphate anhydrous (99%) was purchased from Panreac (Barcelona, Spain). MSPD extraction was performed in commercially available 15 mL plastic cartridges. Polyethylene (PE) filter frits of 20 μm pore size from Supelco Analytical (Bellefonte, PA, USA) were used to compact clean-up phase and dispersed sample in the MSPD column. Acetonitrile, methanol and hexane/ acetone (1:1, v/v) were tested as extraction solvent.

Individual stock solutions of BzI (15,000 μg mL^{-1}) and OI (12,000 μg mL^{-1}) were prepared in acetone, whereas the commercial Proclin reference standard (15,100 μg mL^{-1}) was used as stock solution of MI (3900 μg mL^{-1}) and CMI (11,200 μg mL^{-1}). The use of Proclin as standard for MI and CMI is justified by the widespread use of this mixture in the market, which is the most common form in which these compounds are present in cosmetic formulations. Further dilutions and mixtures were prepared in methanol. Stock and working solutions were stored in amber glass vials at –20 °C.

2.2 Cosmetic samples

A wide variety of cosmetics and house-hold products were purchased from local markets and drugstores. As regards rinse-off cosmetics, six different shampoos (SH1-SH6), a face cleaning gel (FG), a dental cream (DC), a hair mask (HM), two baby liquid soaps (BS1, BS2), three baby bath gels (BG1-BG3) and a baby soft shampoo (BSH) were used. Regarding leave-on cosmetics, a hands cream (HC), two fluid make-up (MU1, MU2), a hair gel (HG), and two baby body milks (BBM1, BBM2) were also studied. On the other hand, three laundry detergents (LD1–LD3), a liquid dishwasher detergent (LDD), three different surface cleaners (SCC, WSC and LSC), and a laundry softener (LS) were also selected as house-hold products. Samples were kept in their original containers at room temperature until analysis. Many of these products were labeled as containing isothiazolinones.

Nine of the previously mentioned samples (SH1, SH2, BS1, BG1, HC, MU1, BBM1, LDD, and SCC) were used for method optimization and validation. In most of them, the presence of

isothiazolinones was not detected. The samples were spiked with 25 μL of standard solution, containing 20 μg mL^{-1} and 200 μg mL^{-1} of Proclin (1:3 MI/CMI), BzI and OI, to give a final concentration of 0.001% and 0.0001%, respectively. Subsequently, fortified samples were homogenized by vortex agitation and then kept for at least 1 h before extraction to allow analyte–matrix interactions.

2.3 MSPD procedure

0.5 g of cosmetic sample were exactly weighted into a 10 mL glass vial. The sample was gently blended with 1 g of a drying agent (anhydrous Na_2SO_4) and 2 g of dispersing sorbent into a glass mortar using a glass pestle until a homogeneous mixture was obtained. Then, the mixture was transferred into a column with a polypropylene frit at the bottom containing 0.5 g of florisil (to obtain a further degree of fractionation and sample clean-up). A second frit was placed on the top of the sample before compression with a syringe plunger. Elution was made by gravity flow with 5 mL of eluting solvent that were collected into a graduated conical tube.

The MSPD extracts were conveniently diluted to match the composition of mobile phase. Due to the high sensitivity of the HPLC–MS/MS method and the usual concentrations of the compounds found in the samples, a dilution 1:5 in mobile phase was generally applied. Fig. 1 shows the chromatogram corresponding to a standard solution of 200 ng mL^{-1} of Proclin (MI: 51.7 μg mL^{-1}, CMI: 148.3 μg mL^{-1}), BzI and OI prepared in methanol and in mobile phase 70% (A)/30% (B) (see Section 2.4). As can be seen, much better peak shape was obtained using this last option.

2.4 HPLC–MS/MS conditions

A Thermo Scientific (San Jose, CA, USA) instrument consisting of a Quantum Access triple quadrupole mass spectrometer equipped with a heated electrospray ionization (HESI) source was used. A Thermo Scientific Hypersil Gold aQ column, 3 μm, 100 mm × 2.1 mm, was used at a flow rate of 200 μL min^{-1} and at 25 °C with an Accela HPLC pump/autosampler.

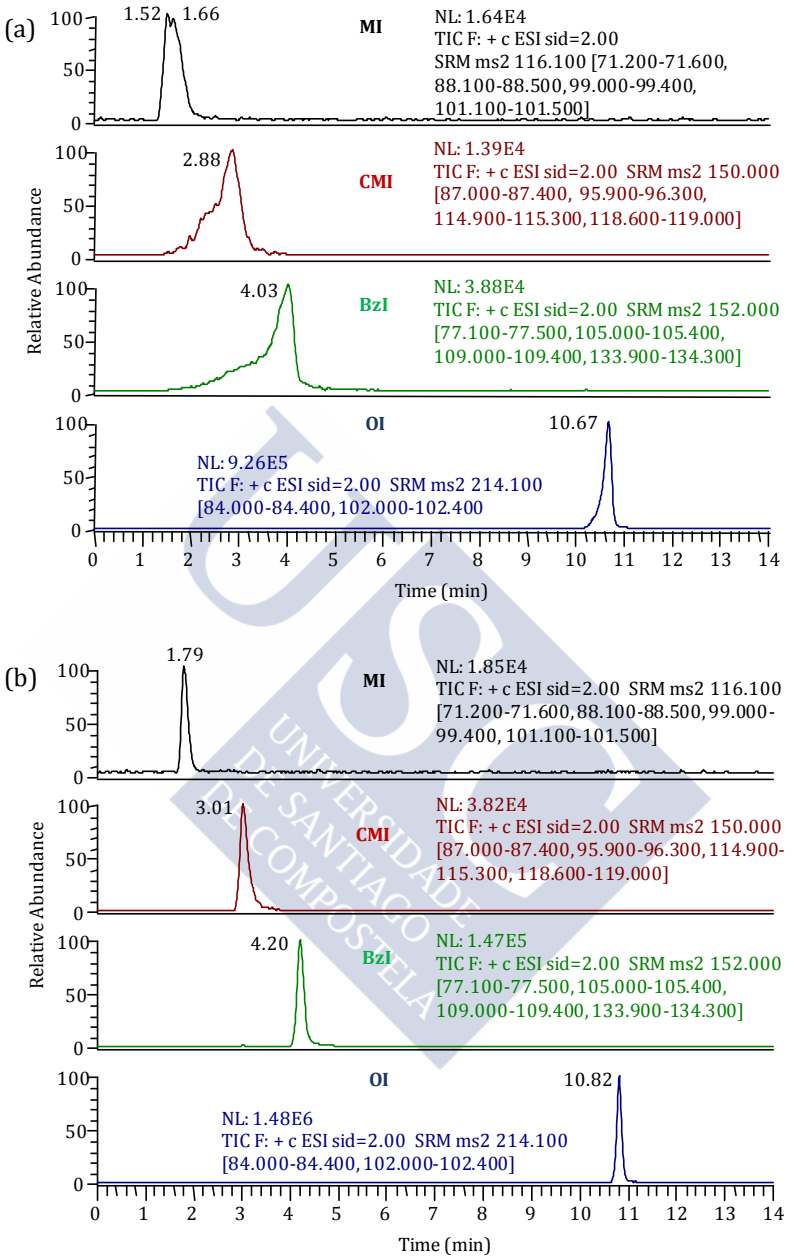


Fig. 1. HPLC-HESI (+)–MS/MS reconstructed chromatograms for standard solutions containing the target compounds at a concentration of 200 ng mL^{-1}: (a) in methanol and (b) in mobile phase 70% (A)/30% (B).

A gradient of the two following mobile phases starting at 70% (A)/30% (B) (maintained 1 min) and ending at 0% (A)/100% (B) (after 13 min) was used. The gradient turned to the starting conditions after 19 min. The mobile Phase A was constituted of water/formic acid (0.1%)/ ammonium acetate (5 mM) and the mobile phase B of methanol/formic acid (0.1%)/ ammonium acetate (5 mM). 10 µL of the standard solution or sample extract were injected into the HPLC column.

The mass spectrometer and the HESI source were working in positive mode with the following parameters: skimmer offset (2 V), sheath gas (38 au (arbitrary unit)), auxiliary gas (5 au), capillary temperature (350 °C), ion sweep cone gas (3 au), and vaporizer temperature (250 °C) and spray voltage (3000 V). The target biocides were directly introduced into the ion source and the collision energies of the selective reaction monitoring (SRM) transitions were optimized for each of them (Table 2). The first mass transition was used for quantification, and the second and third ones were used for identification/confirmation purposes.

The peak width was 0.7 Da both in Q1 and in Q3 and the argon pressure in the collision cell (Q2) was set to 0.002 mbar. For the LC–MS/MS analysis, all SRM transitions were acquired in only one segment with a scan time of 0.040 s. Biocide quantitation was performed using the LC Quan software.

External calibration was used for sample quantification. The calibration standards were obtained by serial dilution of a methanolic standard mixture of 200 µg mL^{-1} of Proclin (MI: 51.7 µg mL^{-1}, CMI: 148.3 µg mL^{-1}), BzI and OI in mobile phase. The linearity was assessed by preparing standard solutions from 1 to 1000 ng mL^{-1}.

2.5 Statistical analysis

Regression analysis, analysis of variance (ANOVA) and experimental design were accomplished using Statgraphics Centurion XV (Manugistics, Rockville, MD, USA), as software package. A multifactor categorical design was applied for the optimization of the MSPD extraction procedure to analyze the simultaneous effect of the sorbent and the elution solvent affecting MSPD. The chromatographic peak area of each one of the target analytes was used as the selected response.

Table 2. Retention times and MS/MS experimental parameters for the studied compounds.

Compound	Retention time (min)	Precursor ion (m/z)	Product ion for quantitation (m/z)	1st Product ion for identification (m/z)	2nd Product ion for identification (m/z)
MI	1.79	116.1	101.3 (24)[a]	99.2 (11)[a] (89.6 ±9.4%)[b]	71.4 (22)[a] (52.1 ±11.7%)[b]
CMI	3.01	150.0	87.2 (38)[a]	115.1 (20)[a] (51.8 ±10.7%)[b]	96.1 (22)[a] (22.7 ±9.1%)[b]
BzI	4.20	152.0	134.1 (22)[a]	109.2 (20)[a] (85.1 ±4.1%)[b]	77.3 (36)[a] (40.6 ±4.4%)[b]
OI	10.82	214.1	102.2 (15)[a]	84.2 (42)[a] (3.5 ±5.5%)[b]	

[a] Collision energy (V)
[b] relative ion intensities and +/- % rsd.

3. Results and discussion

3.1 Optimization of the MSPD extraction

MSPD selectivity strictly depends on both the nature of the sorbent materials and the elution solvent employed [25]. Therefore, the simultaneous effect evaluation of the dispersive phase (Factor A) and the extraction solvent (Factor B) on the MSPD procedure was performed by setting up an experimental design. Four sorbents were included in the study: alumina, florisil, silica and C_{18}, whereas factor B was studied at three levels: methanol, acetonitrile and hexane/acetone (1:1, v/v). These three solvents, with intermediate and upper-intermediate polarities are supposed to favor the simultaneous extraction of the four isothiazolinones, despite their different chemical structures and properties. Thus, a multifactor categorical 4×3 design, involving 12 randomized experiments, was proposed. Three of the assays were performed twice, gaining sufficient degrees of freedom to evaluate the statistical significance of the studied factors.

A real sample with high water content – a shampoo (SH2) – containing the two isothazolinones authorized in cosmetics, MI and CMI, was chosen to evaluate the capability of MSPD to break the interactions between the matrix and the analytes. In order to perform the simultaneous optimization of the four studied isothiazolinones, the sample was fortified with 0.001% (w/w) of Bz and OI. The most usual sample/solid support material ratio was used to accomplish MSPD, blending 2 g of sorbent material with 0.5 g of sample [23]. Due to the high water solubility of some target compounds, drying of the sample was essential. Hence, 1 g of anhydrous sodium sulphate was added in all MSPD experiments. After eluting the columns, 5 mL of solvent were collected.

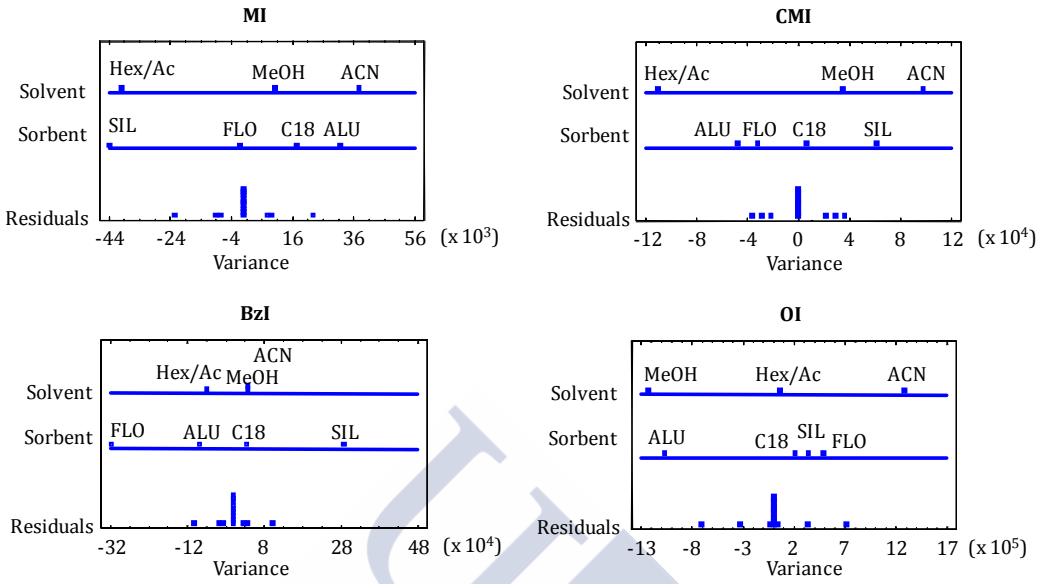


Fig. 2. ANOVA plots showing the statistical significance of the main factors (MeOH: methanol; Hex/Ac: hexane/acetone 1:1 (v/v); ACN: acetonitrile).

The results of the multifactor experimental design can be easily interpreted with the statistical tools provided by the Statgraphics software. The analysis of variance (ANOVA) describes the effect of the studied factors on the obtained response (Table 3). 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. In this table, the relevance of the solvent in the extraction process is evidenced. This factor was significant at the 95% confidence level (p value < 0.05) for all analytes, excluding Bzl. The sorbent was statistically significant for this last compound as well as the second order factor (AB). Besides, the interaction was very close to the significance level for MI (p = 0.052). ANOVA results for the main factors are graphically displayed in Fig. 2. This kind of plots shows scaled effects of each factor by comparing the natural variance of the

Table 3. ANOVA table showing the significance of main effects and second order interactions (F ratios and p values).

Compound	A: Sorbent		B: Solvent		Interaction AB	
	F ratio	*p* value	*F* ratio	*p* value	*F* ratio	*p* value
MI	9.09	0.051	*11.3*	*0.040*	8.69	0.052
CMI	4.74	0.117	*20.3*	*0.018*	3.31	0.177
BzI	*27.4*	*0.011*	1.58	0.340	*16.4*	*0.021*
OI	4.32	0.130	*11.6*	*0.039*	5.89	0.087

Values in italics denote statistical significance.

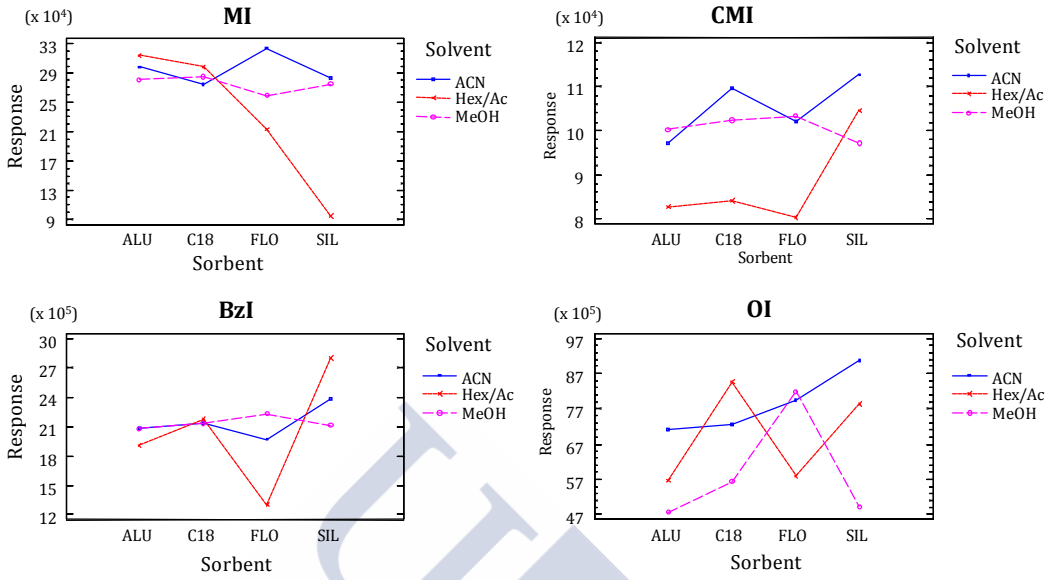


Fig. 3. Interaction plots for the studied isothiazolinones.

points in the diagram to that of the residuals, displayed at the bottom of the plot. Thus, it is easy to identify those factors showing differences of greater magnitude than which could be accounted solely by the experimental error. As indicated, the solvent was significant for MI, CMI, and OI, presenting the major influence on the variability of the response; on the other hand, the sorbent nature was only significant for BzI.

The two-factor plots display the least squares means at all combinations of two factors, which allows studying the effect of both factors simultaneously. Fig. 3 shows the sorbent–solvent plots for the target isothiazolinones. In general, higher extraction efficiencies and comparable responses were obtained using methanol or acetonitrile as elution solvent, especially for MI, CMI and BzI. Hexane/ acetone was in most cases unfavorable, especially for CMI using alumina, C_{18} and florisil, and for MI using silica; therefore, this solvent was discarded. Regarding OI, higher extraction efficiency was reached for florisil and silica employing acetonitrile, whereas only florisil offered comparable responses using methanol.

In view of these results, two different solvents can be use to achieve high extraction efficiency: methanol or acetonitrile. Considering the better compatibility of the solvent with the HPLC mobile phase employed, methanol was finally selected.

Table 4. Linearity, precision and LOQs of the proposed method.

Compound	Linearity			IDL (ng ml^{-1})	Intra-day (RSD, %)		Inter-day (RSD, %)			LOQ (%, w/w)
	R^2	LOF test								
		F-ratio	*P*-value		SH1[a]	BBM1[a]	SH1[b]	BBM1[b]	BS1[a]	
MI	0.9999	1.60	0.310	1.8	4.1	5.4	5.3	11	1.1	0.0000060
CMI	0.9991	0.31	0.893	1.5	0.4	1.2	2.9	7.1	5.6	0.0000049
BzI	0.9997	0.23	0.938	2.0	2.2	1.7	2.0	6.2	n.c.	0.0000060
OI	0.9980	0.23	0.870	0.2	3.4	4.9	2.9	6.9	3.7	0.00000066
MI+CMI[c]	0.9999	2.61	0.138	1.8	1.5	4.3	4.1	6.8	4.5	0.0000060

SH: shampoo. BBM: body body milk. BS: baby liquid soap
n.c: not calculated, since the sample was not spiked.
[a] (n=3) [b] (n=4)
[c] 3:1 CMI/MI mixture.

In brief, the selected experimental conditions for the MSPD extraction of the target compounds comprise the use of 2 g of florisil, as dispersive phase, and subsequent elution with 5 mL of methanol.

3.2 Method performance

The performance of the proposed analytical method was assessed in terms of linearity, accuracy, precision, and limits of detection and quantification. Taking into account the matrix complexity of the studied samples, this evaluation was made using real samples. The validation parameters are shown in Tables 4 and 5.

The instrumental linearity was tested using standards prepared in mobile phase and including seven concentrations between 10 ng mL^{-1} and 1000 ng mL^{-1} for Proclin (1:3 MI/CMI) and BzI, and between 1 ng mL^{-1} and 100 ng mL^{-1} for OI. This last compound exhibits a much better detection limit than the other isothiazolinones. The calibration curves were obtained injecting each concentration level in duplicate. The response function was found to be linear for the four compounds MI, CMI, BzI, and OI as well as for Proclin (MI + CMI peak area) with determination coefficients (R^2) between 0.9980–0.9999. 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. Results of the LOF test at a confidence level of 95% are also shown in Table 4. Since p values are greater than 0.05, the linear regression models are adequate to describe the experimental data.

Instrumental detection limits (IDL) were calculated as the concentration giving a signal to noise ration of three (S/N = 3). These limits were evaluated injecting standard

Table 5. Recoveries in different cosmetics and home care products.

Compound	Recovery (%)										Average recovery (%)
	0.001% (w/w)								0.0001% (w/w)		
	SH1[b]	SH2[b]	BG1[b]	BBM1[c]	HC[c]	MU1[c]	LDD[d]	SCC[d]	SH1[b]	BBM1[c]	
MI	45.6	60.6	45.5	57.1	n.c.	52.1	59.1	83.7	46.8	n.c.	56.3
CMI	92.0	86.4	94.3	95.3	94.1	96.3	86.3	94.1	100	70.4	90.9
BzI	91.2	74.0	87.3	97.7	90.2	99.2	82.5	n.c.	86.0	83.6	88.0
OI	86.7	82.2	77.8	88.5	89.4	92.2	79.7	91.8	82.4	78.5	84.9

[a] SH: shampoo, BBM: baby body milk, HC: hands cream, BG: bath Gel, MU: make up, SCC: surface cleaner cream , LDD: liquid dishwasher detergent
[b] Rinse-off cosmetics
[c] Leave-on cosmetics
[d] Cleaning products
n.c.: not calculated, since the concentration in sample is higher than the added concentration.

solutions of the lowest concentration levels prepared in mobile phase. Values ranging from 0.2 to 2 ng mL^{-1} were obtained.

Recovery studies were conducted in a high number of rinse-off and leave-on cosmetics (eight samples) including two shampoos (SH1, SH2), a bath gel (BG1), a hands cream (HC), a make-up (MU1) and a baby body milk (BBM1), as well as in a liquid dishwasher detergent (LDD), and a surface cleaner cream (SCC). These samples were fortified with known concentrations of each isothiazolinone at two levels: 0.001% (w/w) and 0.0001% (w/w) (see details in Section 2.2). Recovery values were obtained as the ratio of the found concentration, after subtracting the concentration of the non-spiked sample, to the spiked concentration, and expressed as percentage (Table 5). Since the previous analysis of three of the samples revealed the presence of higher concentrations of MI (for HC and BBM1) and of BzI (for SCC) than the spiked levels, recoveries could not be assessed in these isolated cases. Satisfactory recoveries, above 80%, were obtained for CMI, BzI and OI, both in rinse-off and leave-on cosmetic, as well as in the cleaning agents. The recovery values achieved for MI were somewhat lower, with an overall average recovery of 56%, probably due to the high water solubility and low log K_{ow} of this compound (Table 1). These results are substantially better than those found in the literature for MI extraction from different aqueous samples by LLE or SPE [2, 6]. In these studies, the best approaches resulted in extraction yields up to 25%, which prove the serious difficulties for MI isolation. However, using MSPD, recoveries close to 60% were reached, which are quite satisfactory taking into account the high water content of the studied samples.

The influence of water on isothiazolinone extraction was evaluated by performing MSPD in two different cosmetic samples, rinse-off (SH1) and leave-on (BBM1), using variable ratios of water/methanol as elution solvent. As can be seen in Fig. 4, the use of a 70:30 H2O/MeOH mixture showed the worst recoveries for all target compounds. On the contrary, the best isothiazolinone recoveries were generally obtained with 100% methanol, except for MI. The use of a 50:50 H2O/MeOH mixture increased significantly the extraction yield for MI above 70% in leave-on cosmetics. However, OI could be poorly recovered falling dramatically below 40% due to its high hydrophobicity. Hence, these trials confirm methanol as the most suitable elution solvent.

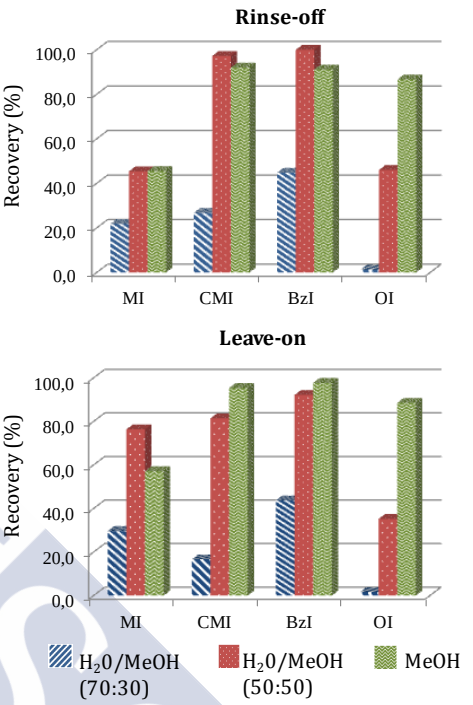


Fig. 4. Influence of water on isothiazolinone extraction from rinse-off and leave-on cosmetics.

Assuming the incomplete recovery of MI, the elution of the MSPD column with an additional volume of 5 mL of methanol was evaluated. The chromatographic responses for the target compounds in the second fraction showed an average value of 2% of the total spiked concentration, except for MI in rinse-off cosmetics (<10%). Therefore, only the first fraction of 5 mL was collected for further experiments.

Method precision was evaluated in spiked samples (0.001%, w/w) as well as in non-spike samples containing the target analytes. Relative standard deviation (RSD) within a day (n = 3) and among days (n = 3 and n = 4) were, in general, below 7%, reaching values lower than 11% (Table 4).

Since method accuracy and precision in real samples were satisfactory and no matrix effects were observed amongst the wide variety of cosmetics studied, quantification was performed using standard solutions in mobile phase, as previously described (see above in this section). The high selectivity of the method providing clean extracts, and the dilution (1:5) of the sample extracts prior to LC–MS/MS analysis, prevent the occurrence of matrix effects into the ion source.

Table 6. Analysis of cosmetic samples (%, w/w).

Compound	Rinse-off								
	SH2	SH3	SH4	SH5	SH6	FG	DC	HM	BS1
MI	0.000375	0.000038	0.000114	0.0000790	0.000475	0.0000354	0.0000590	0.00131	0.00258
CMI	0.000838	0.000112	0.000304	0.000270	0.000934	0.000107			0.00418
OI									0.00144
MI+CMI[b]	0.00121	0.000150	0.000418	0.000349	0.00141	0.000142			0.00676

Compound	Rinse-off				Leave-on			
	BS2	BG2	BG3	BSH	MU2	HG	BBM1	BBM2
MI	0.0111	0.00657	0.000205	0.000324	0.0000833	0.0000724	0.000112	0.00261
CMI	0.0000712		0.000335	0.000154	0.0000182	0.0000222		
OI								
MI+CMI[b]	0.0000953[c]		0.000540	0.000478	0.0000241[c]	0.0000301[c]		

[a] SH: Shampoo, FG: face gel, DC: dental cream, HM: hair mask, BS: baby liquid soap, BG: Bath Gel, BSH: baby shampoo, MU: make-up, HG: hair gel, BBM: baby body milk.
[b] 3:1 CMI/MI mixture
[c] Based on CMI content
[d] When MI is used alone
Blank cells mean values below LODs.

Regarding the limits of detection (LODs) and quantification (LOQs) of the overall method, they were calculated using the chromatograms obtained for real samples fortified at 0.0001% (w/w). LODs and LOQs were both far below the maximum authorized concentration established by the EU legislation in cosmetics. In addition it is important to notice that, if necessary, these limits could be reduced by concentrating the extract, since no losses of the target compounds have been shown during evaporation with nitrogen (data not shown).

3.3 Application to real samples

The validated MSPD-HPLC–MS/MS method was applied to analyze the target isothiazolinones in a total of 25 samples including cosmetics and household products. All analyzed samples were labeled as containing any of the studied compounds.

Because of the widespread use of MI and CMI in hair and skin care products, more than half of the samples corresponded to rinse-off cosmetics which included: five different shampoos (SH2-SH6), a face cleaning gel (FG), a dental cream (DC), a hair mask (HM), two baby liquid soaps (BS1, BS2), two baby bath gels (BG2, BG3) and a baby soft shampoo (BSH).

As can be seen in Table 6, most of these samples contained the CMI/MI mixture in the ratio 3:1, while three of them only contained MI. The results revealed concentrations of MI/CMI mixture ranging from 0.000095 to 0.0067% (w/w). Considering the maximum allowed concentrations set by the European Cosmetics Regulation (0.0015% for 3:1 CMI/MI mixture and 0.01% for MI) [10], the legal limits have been far exceeded, quite surprisingly, in a liquid baby soap (BS1). This sample also contained a significant concentration of OI, which is not included in the list of preservatives admitted for their use in cosmetics (UE Regulation, Annex V). The extracted ion chromatograms for BS1 sample are shown in Fig. 5. The remaining rinse-off samples were properly labeled according to the current regulation, containing isothiazolinone levels below the legal limits, although two shampoos (SH2 and SH6) almost attained the maximum concentration limit for the CMI/MI mixture. This threshold was even reached for MI in another baby liquid soft soap (BS2).

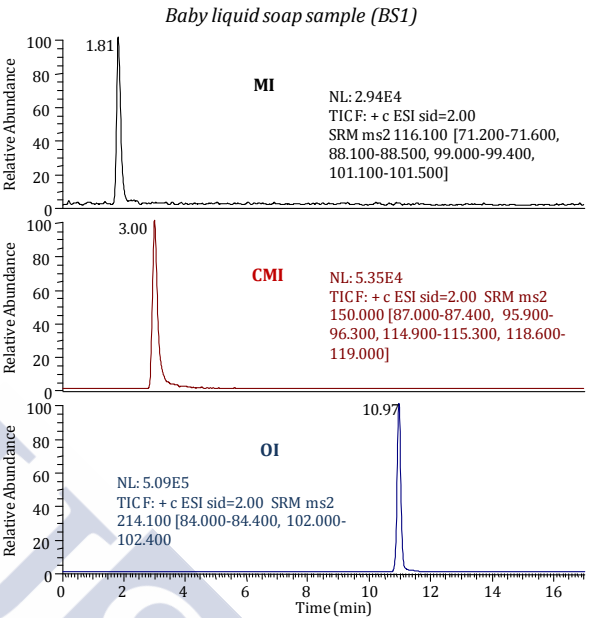


Fig. 5. HPLC-HESI (+)–MS/MS reconstructed chromatograms for a baby liquid soap sample (BS1) containing MI, CMI and OI (see concentrations in Table 6).

In case of leave-on cosmetics, the analyzed samples included: a fluid make-up (MU2), a hair gel (HG) and two baby body milks (BBM1, BBM2). MI and CMI contents were clearly lower, with concentrations ranging from 0.000018 to 0.00261% (Table 6). Unlike rinse-off cosmetics, MI content was shown to be higher than CMI in leave-on products, probably due to a preservative strategy based on increasing the concentration of the less sensitizing nonhalogenated derivative without reducing the preservative efficacy [8]. Although legal restrictions were fulfilled in this case, it is important to highlight that in the make-up, MI was not indicated as an ingredient in the product label.

Table 7. Analysis of home care products (%, w/w).

Compound	LDD	LD1	LD2	LD3	LS	SCC	WSC	LSC
MI	0.000179			0.000118	0.000271		0.000267	0.00290
CMI	0.000419			0.000502			0.000844	
BzI		0.0255				0.0116		0.00377
OI			0.000056	0.000035	0.000121			
MI+CMI[b]	0.000598			0.000620			0.00111	

[a] LDD: liquid dishwasher detergent, LD: laundry detergent, LS: laundry softener, SCC: surface cleaner cream, WSC: wood surface cleaner, LSC: liquid surface cleaner.
[b] 3:1 CMI/MI mixture
Blank cells mean values below LODs

Regarding the household products, three laundry detergents (LD1–LD3), a liquid dishwasher detergent (LDD), three different surface cleaners (SCC, WSC and LSC) and a laundry softener were analyzed. MI and CMI mixtures were found in LDD, LD3 and in WSC at concentrations between 0.0006 and 0.0011% (w/w) (Table 7). Unlike cosmetic samples, BzI was detected in three of the analyzed cleaning products, at quite high concentrations (average value of 0.015%). BzI, which is used as substitute of the chlorinated isothiazolinone, was found alone (LD1, SCC) or in combination with MI (LSC). On the other hand, OI was detected in three cleaning agent samples at lower levels than BzI, 0.00021% (w/w) as average, being found alone (LD2), only with MI (LS) or with MI and CMI (LD3).

4. Conclusions

In this work, a novel methodology based on MSPD followed by HPLC–MS/MS has been developed for the determination of isothiazolinone biocides (MI, CMI, BzI and OI) in multi-matrix cosmetics and household samples. MSPD procedure was optimized using real samples by means of an experimental design methodology. Performance evaluation in several rinse-off and leave-on cosmetics as well as in detergents and cleaning products revealed satisfactory recovery and precision (RSD < 10%). The developed method fulfills the most relevant criteria required for a suitable extraction procedure such as low cost, minimum solvent consumption and short process times, since high sample throughput is attained performing the extraction in about 20 min. The limits of detection (LODs) and quantitation (LOQs) at the nanogram per gram level were far below the established restrictions in the European Regulation, demonstrating the suitability of the proposed method for routine control.

The validated method has been successfully applied to the analysis of a wide range of real samples, showing for most of them, compliance with the current European Cosmetic Regulation. Nevertheless, some rinse-off cosmetics revealed high contents of the sensitizer compounds, quite close and even exceeding the maximum authorized limits. The results also suggested that some of the target compounds have been omitted in the label. These findings of particular concern highlight the reliability and suitability of the proposed methodology for controlling and ensuring compliance to existing or future restrictions in the regulatory framework of cosmetics and household products.

Acknowledgements

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III.2

DETECCIÓN DE CONTAMINACIÓN MICROBIANA EN PRODUCTOS COSMÉTICOS

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La contaminación microbiológica de los productos cosméticos es un problema de gran envergadura para la industria y supone una de las principales causas de pérdidas económicas. Por ello, el control microbiológico, tanto durante el proceso de fabricación como en el producto terminado, representa una prioridad en este sector. En consecuencia, los métodos rápidos de detección microbiológica resultan de gran importancia para la industria cosmética para facilitar la rápida salida de productos al mercado. Existen actualmente algunos métodos basados en bioluminiscencia, impedancia o citometría de flujo, espectrometría de masas, o en el análisis de ácidos nucleicos, que permiten detectar contaminación microbiana en 24h. Sin embargo, muchos de estos métodos no son capaces de alcanzar la especificidad de los enfoques microbiológicos clásicos, que siguen resultando indispensables para aislar e identificar microorganismos.

En el trabajo publicado que se expone a continuación, se ha explorado un enfoque novedoso para la detección de microbiota contaminante en productos cosméticos, basado en el rastreo de compuestos orgánicos volátiles de origen microbiano (MVOCs). El objetivo de este estudio, a modo de prueba de concepto, es el de demostrar la posibilidad de identificar metabolitos volátiles que sirvan de biomarcadores de contaminación microbiana en matrices cosméticas. Para ello, se ha empleado la capacidad de la SPME para aislar con gran eficacia y sensibilidad metabolitos volátiles en espacio de cabeza, en acoplamiento con GC-MS para la identificación estructural de los mismos. A diferencia de otros métodos rápidos, la metodología propuesta permitiría descartar la presencia de microorganismos no viables, ya que no son metabólicamente activos y no presentan ningún riesgo desde el punto de vista microbiológico. Por otra parte, podría detectar microorganismos estresados aunque no sean cultivables, ya que incluso en estos casos existe cierto grado de producción metabólica que se vería reflejado en la presencia de MVOCs característicos.

La publicación de este trabajo ha despertado el interés de un destacado miembro de la compañía Procter & Gamble y de la Sociedad Americana de Microbiología (ASM). Además, el trabajo ha sido seleccionado como ganador del *7º Premio Club de Usuarios de SPME*, patrocinado por la casa comercial Sigma-Aldrich, por su innovación en las aplicaciones de la SPME, y por su aplicabilidad en la industria.

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Capítulo III.2.1

A novel outlook on detecting microbial contamination in cosmetic products: analysis of biomarker volatile compounds by solid-phase microextraction gas chromatography-mass spectrometry

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A novel outlook on detecting microbial contamination in cosmetic products: analysis of biomarker volatile compounds by solid-phase microextraction gas chromatography-mass spectrometry

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Abstract

Cosmetic companies are required to control the optimal preservation of their commercial products, since microbial contamination in cosmetics represents an important risk for consumer health. Fast methods for cosmetic microbiological testing are of great industrial importance, facilitating the rapid release of products into the market. In this work, a novel approach based on microbial volatile organic compound (MVOC) analysis was proposed, for the first time, as an alternative method for the rapid detection of microbial contamination in cosmetics by SPME-GC-MS. Microbial volatiles from typical contaminants of cosmetic products were sampled above the headspace of standard cultures and from incubated samples. The volatile fraction analysis revealed, amongst other compounds, the presence of several odd-numbered carbon (C_9–C_{15}) methyl ketones and alkanols, which have been reported as characteristic compounds of bacterial origin. Some of them were found both in pure bacterial cultures and in the samples. However, other compounds not seen in cultures were seen in the cosmetics, suggesting that substrate is a very influential factor. These results also suggest that it could be clearly feasible to qualitatively identify viable microorganisms in cosmetics or even specific strains by detecting their volatile biomarkers, which would be a rewarding complement for other rapid methods. The results of the kinetic study performed on real samples further suggest the possibility of monitoring the contamination progress.

Keywords: Microbial contamination; Cosmetics; Personal care products; Solid-phase microextraction; Microbial volatile organic compounds; Biomarkers; GC–MS.

1. Introduction

Most of the cosmetic formulations, due to their composition and high water content, are products suitable for biological degradation by microorganisms [1]. Microbial contamination in cosmetics represents an important risk for consumer health, since it can lead to irritations or infection, especially when these products are applied on damaged skin, eyes or on babies [2]. Proof of that are the reported outbreaks of microbial infection from hospitalized individuals caused by contaminated mouthwash [3-5] and moisturizing milk [6]. Other studies about different contaminated cosmetics, recalled from the USA and EU, highlight *P. aeruginosa* as the main pathogen found in this kind of product [7, 8]. Besides, the genera *Klebsiella spp*. and *Serratia spp.* were also found amongst the most typical contaminants isolated from cosmetics, being the genus *Pseudomonas spp.* the most frequently responsible and the main problem for the cosmetics industry [9].

Nowadays, regulations regarding the microbiological content in cosmetic products do not exist; the only requirement for cosmetics in the UE Directive is that they "must not cause damage to human health when applied under normal or reasonably foreseeable conditions of use" [10]. Due to the lack of official cosmetic guidelines on this matter, some recommendations have been published by governments and cosmetic associations. Recommendations on limits of microbial contamination in cosmetic products can be found in the notes of guidance prepared by the EU's Scientific Committee of Consumer Products (SCCP) [11]. Similar challenge tests are described in the European Pharmacopoeia [12], the US Pharmacopoeia, the CTFA (Cosmetic, Toiletries and Fragrance Association) and the American Society for Testing and Materials (ASTM), as sources for guidelines covering testing of preservation efficacy in cosmetic and toiletry products [9].

Nevertheless, microbiological testing methods applied to cosmetics by these associations are usually based on traditional plate counts. These growth-based methods, although demanding no expensive infrastructure and being rather cheap in consumables, have well-known inherent limitations such as time-consuming procedures both in operation and data collection are laborious to perform, demand large volumes of liquid or solid media and reagents, and lead to ambiguous results associated with using turbidity as a detection endpoint [13, 14]. Thus, the need for providing fast and reliable methods to cosmetic microbiologists and manufacturers to detect microbial contamination has been highlighted by several authors [13-15].

Most recently, the development of fast microbiological methods such as bioluminescence, impedance or cytometry, based on the metabolic state of microorganisms, as well

as mass spectrometry and nucleic-acid based methods allow the detection of microbial contamination within 24 h, both in the finished product and in raw materials. Therefore, these methods are of great industrial importance, since they facilitate the rapid release of products into the market. Despite the advantages, fast methods have not been widely introduced for routine microbiological testing yet, mainly, due to the non-specificity in microbial detection and the lack of equivalence with the official methods [14, 16].

Many volatile organic compounds (MVOCs) are generated by microorganisms during metabolic processes [17, 18]. To date, many reports have attempted to establish correlations between MVOCs and specific specimens, and consequently to determine whether some MVOCs could serve as markers for the detection of certain microbial species. Thus, some cancer biomarkers were identified from cancer stomach tissues infected with *H. Pylori* [19, 20]. It was also possible to identify the volatile compounds characteristic of sinus-related bacteria from infected sinus mucus [21]. Other studies were carried out for characterizing MVOCs with nematicidal [22] and fungicidal [23, 24] activity from a group of soil bacteria, the volatile pro file of the rhizobacteria [25] and the bacteria isolated from the spoilage flora of cold-smoked salmon [26]. In these studies, headspace solid-phase microextraction (HSSPME) is the most used sampling technique, since SPME integrates extraction, concentration and introduction in one step, which results in reducing the sample preparation time and simultaneously increasing its sensitivity over other extraction techniques [27]. In fact, HSSPME coupled with gas chromatography-mass spectrometry (GC-MS) is considered as a valuable choice for the analysis of MVOCs [18]. In addition, GC-MS is a technique with great potential to identify the volatile characteristic that could be exploited for detection by sensor-based electronic recognition methods [21].

In order to characterize the MVOCs of the typical microbial contaminants in cosmetic products, the HSSPME was applied, in this work, to collect headspace volatiles above pure bacterial cultures as well as above inoculated real samples. The subsequent separation and identification of MVOCs was performed by GC-MS analysis of the collected volatile fraction.

In this “proof-of-concept” study, a MVOC-based method is proposed for the first time for the rapid detection of microbial contamination in cosmetics. The results suggest that it could be clearly feasible to qualitatively identify viable microorganisms in cosmetics or even specific strains by detecting their volatile biomarkers, which would be a rewarding complement for other rapid methods.

2. Experimental

2.1 Chemicals, materials and solutions

2-Tridecanone (>98% purity) was purchased from Alfa Aesar GmbH (Karlsruhe, Germany), 1-undecene (99.5% purity) was acquired from Dr Ehrenstorfer GmbH (Augsburg, Germany), while 2-undecanone (99% purity), tridecanal (90% purity) and 2-nonanone (99.9% purity) were supplied by Sigma-Aldrich Chemie GmbH (Steinheim, Germany). The alkane standard solution C_8–C_{20} (40 mg mL^{-1} each) was purchased from Fluka (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Tryptic soy broth (TSB) for bacterial growth was purchased from Cultimed (Barcelona, Spain). Individual stock solutions of each compound were prepared in acetone. Further dilutions and mixtures were prepared by convenient dilution of the stock solution in acetone to spike cosmetic samples when needed. Stock and working solutions were stored in amber glass vials at -20 °C. A solid-phase microextraction 50/30 μm divinylbenzene–carboxen–polydimethylsiloxane (DVB/CAR /PDMS) fiber housed in a manual SPME holder was obtained from Supelco (Bellefonte, PA, USA).

2.2 Cosmetic samples

Different cosmetics and personal care products including shampoo, moisturizing cream, hand cream, bath gel, make-up, make-up remover and revitalizing tonic were purchased from local markets and drugstores. Samples were kept in their original containers at room temperature until their inoculation, incubation and further analysis.

2.3 Culture of bacteria and cosmetic sample inoculation

Amongst the microorganisms that can be most commonly found in contaminated cosmetic samples, four representative bacterial species of different genera have been considered in this study: *Pseudomonas (P.) fuorescens, Serratia (S.) marcescens, Escherichia (E.) coli,* and *Klebsiella (K.) pneumoniae.* These specimens were grown in favourable media at the Department of Microbiology and Parasitology of the University of Santiago de Compostela, and further manipulated aseptically to inoculate cosmetic samples. Bacterial strains were grown in TSB culture medium and incubated at 27 or 37 °C, for 18 h in order to obtain optimal growth prior to cosmetic inoculation.

An aliquot of approximately 100 mg of the cosmetic was placed in a sterile 22 mL vial and intentionally contaminated with 50 µL of an overnight pure bacterial culture. Due to the complexity of this kind of samples, all inoculation experiments were performed with cosmetics diluted in 2 mL of ultrapure water, with the purpose of improving bacterial growth. The diluted samples were incubated up to a maximum period of 12 days. Those inoculated but non-incubated samples (t = 0 days) were used to discard further volatiles from the starter inocula. In addition, the non-inoculated samples were considered as blanks (BLANK 1A) as well as controls of unintended contamination during the incubation period (BLANK 1B).

Pure bacterial cultures were also grown in a 22 mL glass vial, in order to compare the microbial volatiles from standard cultures to those obtained from the contaminated cosmetic samples. Therefore, 2mL of fresh TSB medium were inoculated, similarly, with 50 µL of an overnight bacterial culture and then incubated for 48 h. In the same way as samples, the non-inoculated TSB was run as blank (BLANK 2A) and also as control of random contamination during the incubation period (BLANK 2B, incubation for 48 h).

In any of these cases, vials were sealed with a headspace aluminium cap furnished with a PTFE-faced septum and incubated at 27 or 37 ºC, depending on the strain, for a maximum period of 12 days. The incubated sample vials were stored in the freezer at -20 °C until the sampling of the volatiles was performed.

2.4 SPME procedure

Vials, taken from the freezer, were immersed in a water bath maintained at the sampling temperature (60 °C). Samples were let to equilibrate for 5 min and the SPME fiber was then introduced into the glass vial through the septum and exposed to the headspace over the sample for 30 min. During all the sampling processes, magnetic stirring was performed using a stir bar previously introduced into the sample vial in order to improve mass transfer of the studied volatile metabolite to the headspace. After extraction, the fiber was retracted into the needle of the holder syringe and readily inserted into the GC injector. The fiber was desorbed for 5 min at 250 °C and subsequently conditioned in another GC injector for 5 min at 270 °C to avoid any carryover-effect.

2.5 Gas chromatography/mass spectrometry (GC-MS) analysis

Analyses were performed on an Agilent 7890A (GC)-Agilent 5975C inert MSD with a 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 °C, respectively. The system was operated by Agilent MSD ChemStation E.02.00.493 software.

Separation was carried out on a HP5 capillary column (30 m × 0.25 mm i.d., 0.25 mm film thickness) from Agilent Technologies (Palo Alto, CA, USA). Helium (purity 99.999%) was employed as the carrier gas at a constant column flow rate of 1.0 mL min^{-1}. The GC oven temperature was programmed from 50 °C held for 3 min, then ramped 4 °C min^{-1} to 200 °C and held for 2 min, next ramped 20 °C min^{-1} to 290 °C and finally held for 3 min (total analysis time = 50 min). Pulsed splitless mode was used for injection (30 psi, held 1.2 min). After 1 min the split was opened at a flow rate of 75 mL min^{-1} and the injector temperature was kept at 250 °C.

The mass spectrometric detector (MSD) was operated in the scan mode and the mass range was varied from 40 to 300 m/z, starting at 4 minutes and ending at 50 min. The electron multiplier was set at a nominal value of 1200 V.

Positive identification of microbial volatile compounds was performed by comparing their retention time with that of commercial standards, when available, and on the basis of the mass spectrum and Kovats retention indices in agreement with corresponding data in the literature (NIST MS Search 2.0 database). Otherwise, tentative identification of target compounds was proposed taking into account the mass spectrum and Kovats retention indices. Table 1 summarizes the MVOCs identified both in pure bacterial cultures and in the bacteria incubated samples. None of these compounds were found in their respective blanks.

Table 1. MVOCs identified above the headspace of pure bacterial cultures in TSB and bacteria-incubated cosmetic samplesa

Compound	CAS No.	Ret. time (min)	Kovats Ret. Index[c]	*Pseudomonas fluorescens*									*S. marcescens*			*K. pneumoniae*			*E. coli*		Reliability of identif.[b]	Qualifier and quantifier Ions
				TSB	HC1	Sh	MC	HC2	BG	MU	MUR	RT	TSB	HC2	RT	TSB	HC2	RT	TSB	RT		
3-methyl-1-butanol	123-51-3	3,50	706	X												X					B-C	42, 55, 70
2-Heptanone	110-43-0	6,25	871		X		X			X			X	X	X	X		X		X	B-C	43, 58, 71
2-Nonanone	821-55-6	13,25	1088	X				X		X	X		X	X	X	X	X	X	X	X	A-B-C	43, 58, 71
1-Undecene	821-95-4	13,50	1091		X		X					X									A-B-C	43, 55,70, 83,97
2-Phenylethanol	60-12-8	13,90	1105	X												X			X		B-C	91, 122
1-Nonanol	143-08-8	16,10	1149			X						X									B-C	41, 55, 70, 83,97
1-Decanol	112-30-1	19,60	1267	X								X	X						X		B-C	43,55,70,83
2-Undecanone	112-12-9	20,40	1274	X			X	X		X			X	X			X	X		X	A-B-C	43, 58, 71
Indole	120-72-9	20,50	1293													X			X	X	B-C	90, 117
2-Undecanol	1653-30-1	20,80	1302	X									X		X						B-C	45
Undecanal	112-44-7	20,85	1304			X			X			X									B-C	41, 57, 68, 82, 96
1-Undecanol	112-42-5	23,00	1370			X						X			X				X		B-C	55,69,83,97,11
3-Dodecanone	1534-27-6	23,80	1394			X			X												B-C	57, 72, 85, 155
1-Dodecanol	112-53-8	25,95	1464	X									X			X			X		B-C	43,55,69,83,97, 111
2-Tridecanone	593-08-8	26,80	1480					X		X			X	X		X	X	X		X	A-B-C	43, 58, 71
2-Tridecanol	1653-31-2	26,85	1490	X									X		X	X			X		B-C	45
Tridecanal	10486-19-8	27,40	1511		X	X			X			X									A-B-C	43, 57, 68, 82, 96
1-Tridecanol	112-70-9	29,10	1569	X	X				X										X		B-C	55, 69, 83, 97, 111
Tetradecanal	124-25-4	30,40	1614			X			X			X									B-C	57, 68, 82, 96
1-Tetradecanol	112-72-1	32,00	1672													X			X		B-C	55, 69, 83, 97, 111
2-Pentadecanone	2345-28-0	32,35	1686										X		X	X		X	X	X	B-C	43,58,71
1-Pentadecanol	629-76-5	33,60	1731	X					X			X	X						X		B-C	55,69,83,97,111

[a] Sh: shampoo, MC: moisturizing cream, HC: hands cream, BG: bath gel, MU: make-up, MUR: make-up remover, RT: revitalizing tonic, TSB: tryptic soy broth.
[b] The reliability of the identification is indicated as follows: A, retention time in agreement with reference standards; B, mass spectrum and C, Kovats indices in agreement with corresponding data in the literature
[c] Kovats indices were calculated for the DB5 stationary phase of a capillary column.

3. Results and discussion

Preliminary consideration in the MVOCs analysis MVOC analysis is usually accomplished using the GC-MS approach. Mass spectral libraries such as the NIST library, which is used in this work, are of immense utility as identification tools.

However, in some cases, related compounds such as geometrical and positional isomers of long-chain aliphatic compounds and methyl-branched compounds near the end of an alkyl chain can generate similar mass spectra [17]. Under these circumstances, compound identification is not possible exclusively by considering only the mass spectra. Thereby, additional data are necessary in order to perform a reliable structure elucidation. Despite the fact that the best option is always the direct comparison with a reference standard, the Kovats retention indices are very useful tools, additionally employed in this work, which allow a quite accurate identification of the studied bacterial volatiles, especially when the standards are not available.

Apart from the reliability of the identified compounds, special attention has been paid to running blank analyses of culture media (BLANK 2A) as well as of cosmetic samples (BLANK 1A). Besides, each inoculated sample was incubated, at the same time, together with its blank (BLANK 1B) in order to discard non-intended contamination. As can be seen in Fig. 1, both culture media and cosmetic samples blanks are themselves abundant sources of volatiles. Bearing in mind such complex profiles, high challenge is posed in distinguishing the volatiles of the blank from those of the bacteria. This apparent drawback could be readily overcome due to the selectivity of MS by operating in the selected-ion monitoring (SIM) mode.

3.1 Selection of typical bacterial contaminants of cosmetics

Given the serious problems that *Pseudomonas* can cause in the cosmetics industry, this genus was considered in this study. *P. aeruginosa* is the most virulent opportunistic pathogen of this genus, since it can produce life-threatening infections (especially in immunocompromised people), being very difficult to eradicate from manufacturing plants [28]. Thus, in order to perform the analysis of the volatile compounds produced by this kind of bacteria in cosmetics, a non-pathogenic representative member of this genus, *P. fluorescens*, was selected. On the other hand, *S. marcescens* and *K. pneumoniae*, which are also amongst the typical contaminants found in cosmetics [9], were also considered as representative specimens of their genera. Finally, E. coli was further included due to its ubiquitous presence in the human life.

3.2 MVOCs from bacterial cultures

Volatile metabolites from bacterial growth in standard culture media were firstly analyzed with the purpose of searching for similarities with those of the cosmetic samples. Previously, headspace analysis of glass vials containing only culture media was performed. The results obtained from the volatile fraction of TSB medium BLANK 2A (Fig. 1A) revealed the presence of several compounds in a background of siloxanes, most likely originated from the media culture during the autoclaving process, plastic, septum and SPME fiber degradation products, in agreement with Preti et al. [21].

The selected bacteria were grown on TSB medium and the produced volatile compounds were collected by SPME and analysed by GC-MS as described in the

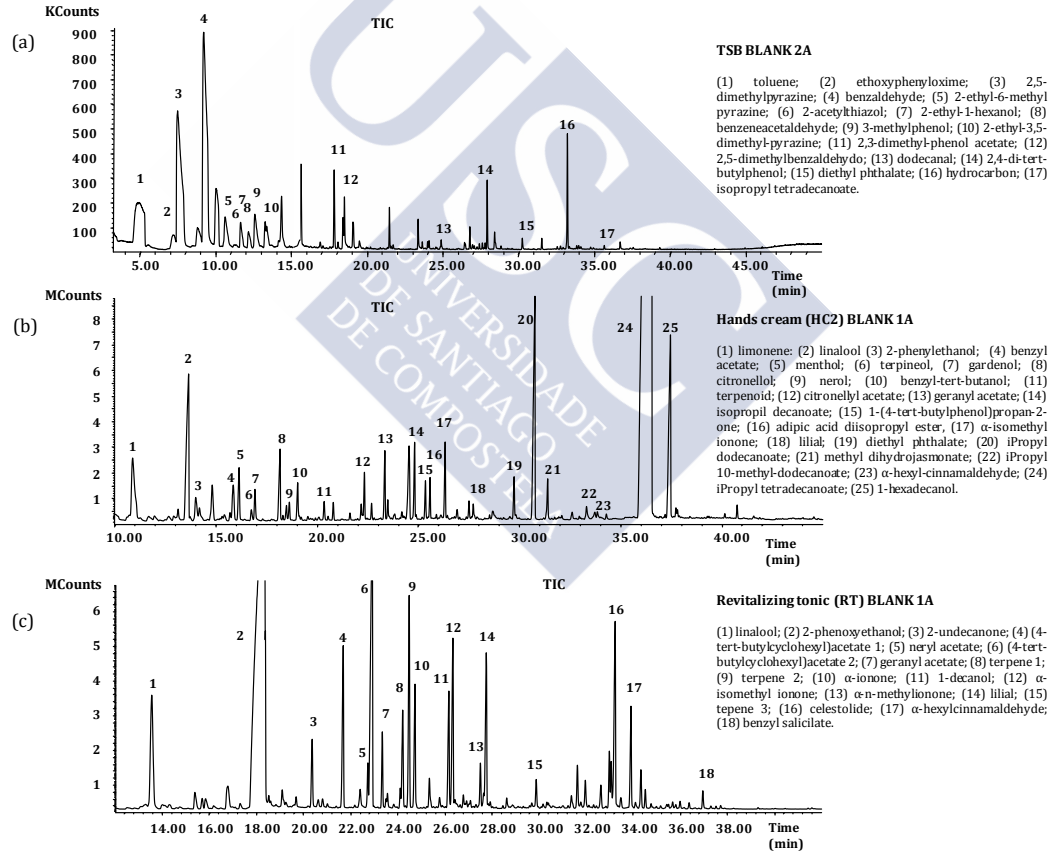

Fig. 1. Total ion chromatograms of volatiles collected above the headspace of: (A) the TSB culture medium (BLANK 2A) used to grow the target bacteria; (B) hand cream (HC2) blank (BLANK 1A); and (C) revitalizing tonic (RT) blank (BLANK 1A). Non-labeled peaks: mainly siloxanes.

Experimental section. Several MVOCs such as 2-nonanone, 2-phenylethanol, 1-decanol, 2-undecanone, 1-dodecanol, 2-tridecanone, 2-tridecanol, 2-pentadecanone or 1-pentadecanol were seen to be common, at least, for three of the studied bacteria, although each specimen showed a particularly distinctive profile. The overlapped total ion chromatograms (TICs) of the TSB culture medium (BLANK 2B) and the incubated TSB culture medium with *P. fluorescens* are shown in Fig. 2. About ten compounds identified in the bacterial culture, most probably related to bacterial growth, can be clearly distinguished from the blank. These MVOCs are listed in Table 1.

3.3 MVOCs from inoculated cosmetic samples

A total of eight real cosmetic samples were studied: a shampoo (Sh), a moisturizing cream (MC), two hand creams (HC), a bath gel (BG), a make-up (MU), a make-up remover (MUR) and a revitalizing tonic (RT). Before the inoculation assays, an exhaustive analysis of the cosmetic blanks (BLANK 1A) was accomplished. The compounds most frequently and abundantly found were terpenic fragrances and their derivatives, acetates, long-chain esters and alcohols as well as some musks and phthalates amongst others. The chromatograms corresponding to the blank analysis of the samples HC2 and RT, as representative leave-on and rinse-off cosmetics, are shown in Fig. 1B and C respectively.

SPME-GC-MS analysis of volatiles from cosmetic samples inoculated with *P. fluorescens* was performed on the eight considered personal care products. Two of them (HC2 and RT) were inoculated with *S. marcescens* and *K. pneumoniae* separately, whereas *E. coli* was used to inoculate RT only (see Table 1).

A number of MVOCs ranging from 3 to 10 per sample were identified. The microbial volatiles most frequently found were 2-heptanone, 2-nonanone, 1-undecene, 2-undecanone, 2-tridecanone, tridecanal, 2-pentadecanone and 1-pentadecanol amongst others (Table 1). These odd-numbered carbon compounds have been reported as characteristic volatiles of bacterial origin, most likely formed by modification of products from the fatty acid biosynthesis pathway where decarboxylation steps very often yield compounds such as 1-alkenes [29] or methyl ketones [30]. In addition, the reduction of the carboxy groups to aldehydes and 1-alkanols is another possible alternative [17].

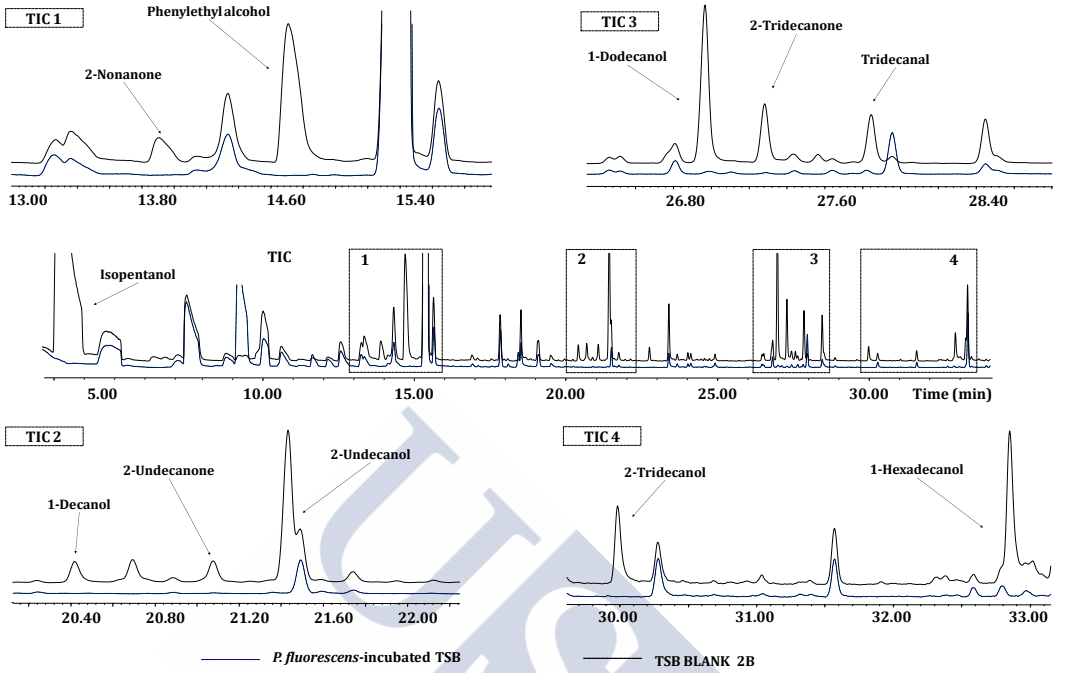

Fig. 2. Overlapped total ion chromatograms of the TSB culture medium (BLANK 2B) and incubated TSB culture medium with *P. fluorescens*.

C_9-, C_{11}- and C_{13}-methyl ketones were found in several cosmetic samples inoculated with the tested bacteria. These three compounds have been found in the cultures of several strains of *Pseudomonas*, such as *P. fluorescens* and *P. aeruginosa* [31]. Besides, the production of 2-nonanone and 2-undecanone was also reported for *Serratia* strains [32], and in this work, the production of 2-nonanone by *E. coli* and *K. pneumoniae* bacterial cultures could be confirmed. Fig. 3 shows the volatile profile of a hand cream incubated with *P. fluorescens*. The contaminated sample shows a distinctive profile displayed, in the SIM chromatogram (m/z 58), which clearly differs fromthe BLANK 1B, allowing us to detect the presence of *P. fluorescens* in the cosmetic sample.

Other homologue methyl ketones like 2-heptanone were found in some samples and culture media inoculated with target bacteria, whereas 2-pentadecanone, which can occur in many bacteria [33-35], was also identified in RT samples incubated with *S. marcescens*, *K. pneumoniae* or *E. coli*, being also detected in their respective TSB cultures. The formal reduction of the methyl ketones produces methyl carbinols such as 2-undecanol [36] and 2-tridecanol [37]. Both compounds appeared in the TSB cultures of *P. fluorescens* and *S.*

marcescens, as well as in the RT sample incubated with the *Serratia* strain. Besides, C12-carbinol was also shown in *K. pneumoniae* and E. coli cultures.

1-Nonanol and its homologues up to C15 were present, although to a lesser extent, in some of the investigated contaminated samples. Most of these alkanols have been reported in different combinations in several bacterial cultures [36, 38, 39]. Analogues C_{11}-, C_{13}-, C_{14}-aldehydes were found in the samples inoculated with *P. fluorescens*. Although C_{11}- and C_{12}-aldehydes occur in some bacteria [38], unbranched aldehydes are relatively rare, most probably because of their high reactivity [17]. Regarding the alkenes, 1-undecene, which often occurs in *Pseudomonas* [40, 41] was the only alkene identified in three of the analyzed samples (HC1, MC and RT) incubated with *P. fluorescens*.

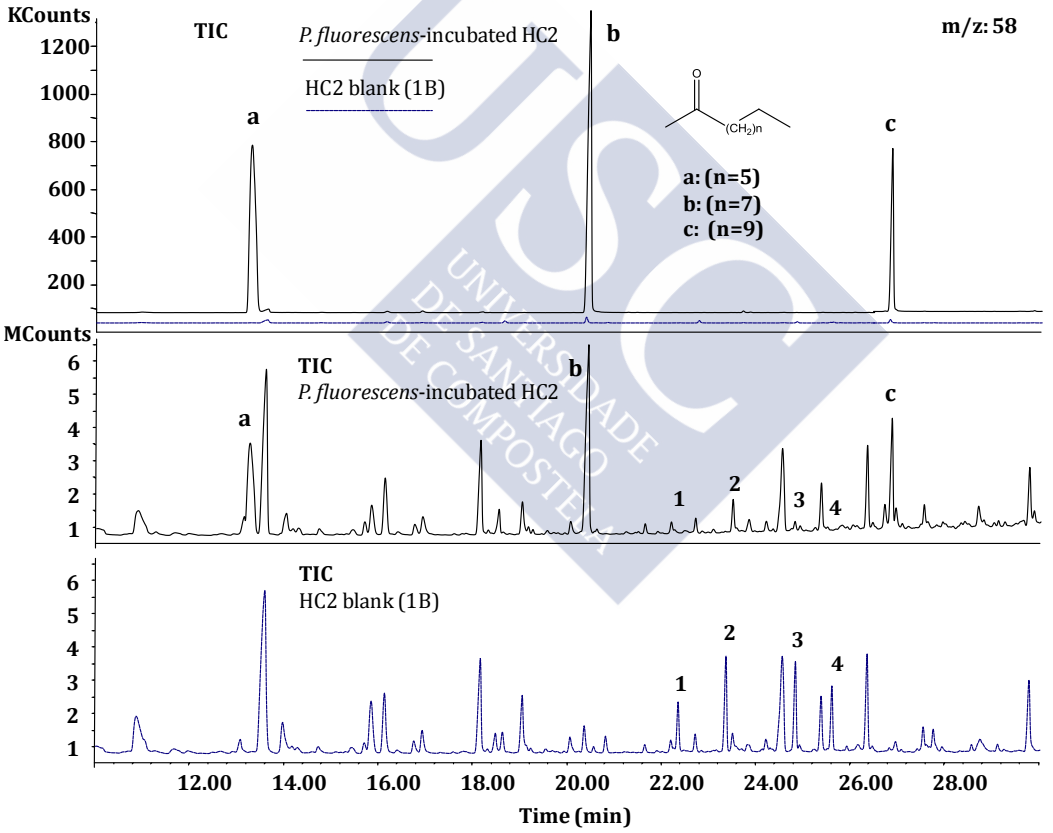

Fig. 3. TIC and extracted ion chromatograms for the collected volatiles from a hand cream (HC2) incubated with *P. fluorescens*. MVOCs identified in the bacteriumincubated sample: (a) 2-nonanone; (b) 2-undecanone; and (c) 2-tridecanone. Volatile compounds with a different profile in the incubated sample and in the BLANK 1B: (1) citronellyl acetate; (2) geranyl acetate; (3) isopropyl decanoate; and (4) adipic acid diisopropyl ester.

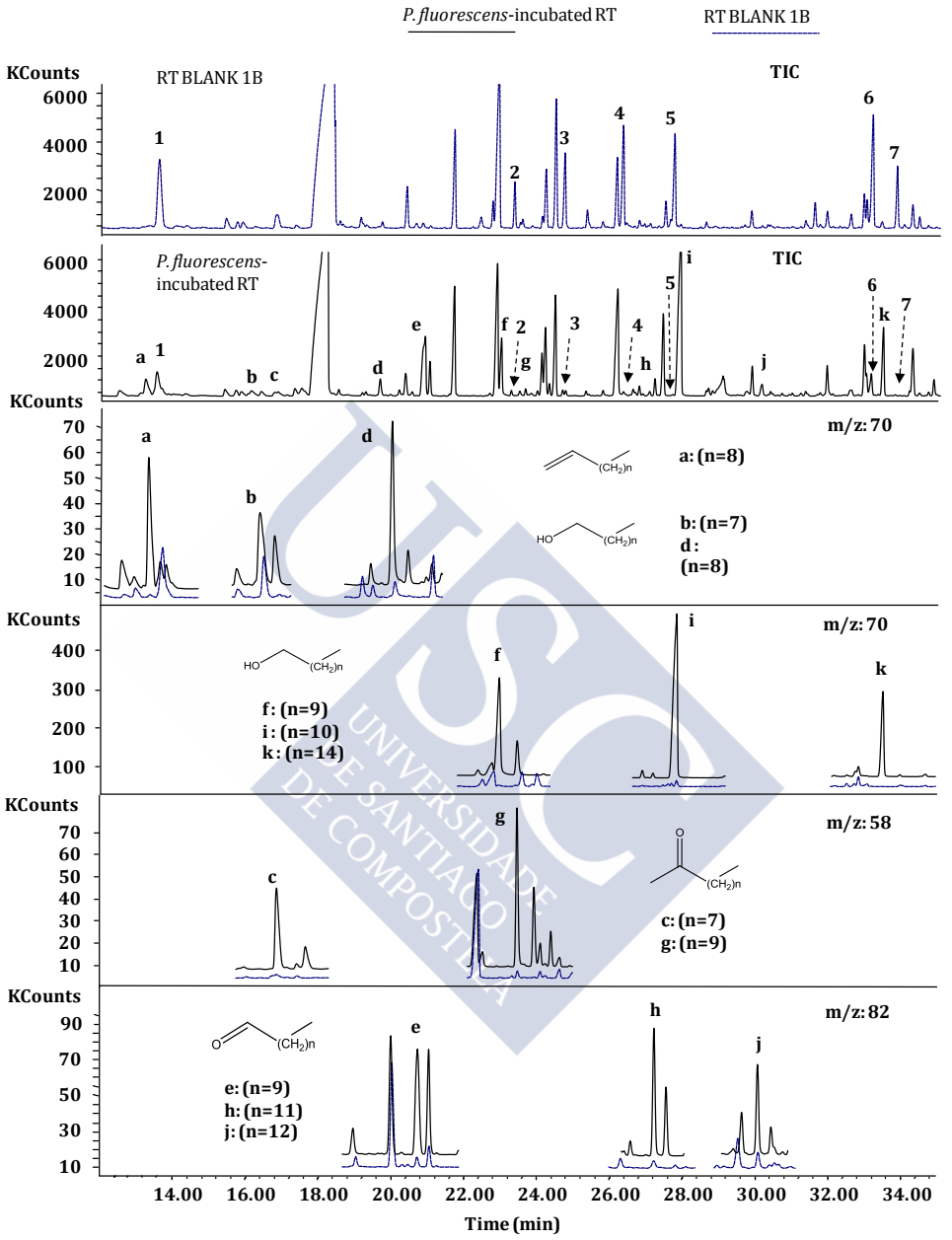

Fig. 4. TIC and extracted ion chromatograms for the collected volatiles from a revitalizing tonic (RT) incubated with *P. fluorescens*. MVOCs identified in the bacteriumincubated sample: (a) 1-undecene; (b) 1-nonanol; (c) 2-decanone; (d) 1-decanol; (e) undecanal; (f) 1-undecanol; (g) 2-dodecanone; (h) tridecanal; (i) 1-dodecanol; (j) tetradecanal; and (k) 1-hexadecanol. Volatile compounds with a different profile in the incubated sample and in the BLANK 1B: (1) linalool; (2) geranyl acetate; (3) aionone; (4) α-isomethyl ionone; (5) lilial; (6) celestolide; and (7) α -hexylcinnamaldehyde.

Amongst the very minority volatile compounds found in the headspace of analyzed cosmetic samples, indole and 3-dodecane were identified. The heteroatomic compound indole, derived from L-tryptophan, can be found in *Klebsiella* [42] and *E. coli* strains [17, 40, 43]. The presence of this volatile compound was confirmed in *K. pneumoniae* and E. coli TSB culture medium and even in the RT sample for this last bacterium. On the other hand, 3-dodecanone, produced by *P. fluorescens* in SH and MU samples could be produced as a result of the use of acetate or propionate instead of isovalerate as a starter unit in the biosynthesis pathway of fatty acid derivatives [17].

Finally, 3-methyl-1-butanol and 2-phenyl-ethanol were the only two analyzed volatiles that could not be found in any of the studied samples, being solely present in the TSB cultures of *P. fluorescens* and *K. pneumoniae*. In the case of E. coli only 2-phenylethanol was found. This last compound is one of the most widespread aromatic volatiles reported to be produced by the genera *Escherichia* and *Klebsiella* [17, 32, 44, 45] as is confirmed in this work. Fig. 4 shows the TIC and extracted ion chromatograms corresponding to the SPME-GC-MS analysis of a revitalizing tonic sample inoculated with *P. fluorescens*. The letters labelled peaks correspond to the volatile compounds clearly identified by MS in the SIM mode. The presence of such compounds in the sample, which are missing in the blank, can be solely attributable to the presence of the bacteria, most probably produced during its growth in the sample. With respect to the overall analyzed cultures and

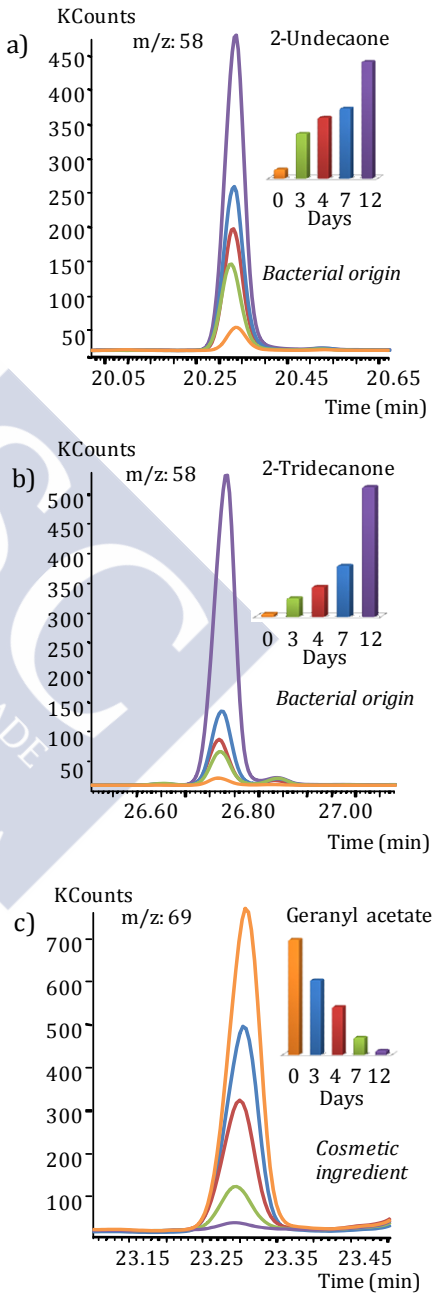

Fig. 5. Kinetic profiles showing the production of (a) 2-undecanone and (b) 2-tridecanone, and the degradation of (c) geranyl acetate in a hand cream (HC2) incubated with *P. fluorescens*.

samples, several common MVOCs were found in both pure bacterial cultures and in the cosmetic samples. However, other compounds not seen in the cultures were seen in the cosmetics and vice versa. Bearing in mind that each sample or culture medium represents a different substrate to each microorganism, the metabolites produced during the bacterial growth therein will differ from each other. These results are in agreement with Preti et al. [21] suggesting an important role for the growth substrate, as is demonstrated in this work. A summary of the overall identified compounds, both in culture medium and in the cosmetic samples is shown in Table 1.

3.4 Kinetic behavior of MVOCs

The kinetic profile for some volatiles identified as potential biomarkers of microbial contamination was studied. A hand cream (HC2) inoculated with *P. fluorescens* was incubated at 27 °C for different periods of time up to 12 days, in order to evaluate the kinetic behavior of the MVOCs found in this sample. As can be seen in Fig. 5a and b, the response obtained for 2-undecanone and 2-tridecanone showed a meaningful growth since the beginning of the incubation period. Especially sharp was the increase for 2-tridecanone from the seventh to the twelfth day. These results suggest quite similar kinetic profiles for the volatiles to those of the bacterial growth, with an exponential trend. Thus, it could be hypothesized that such MVOCs might be related to microbial growth in the contaminated cosmetic products. In strict terms, only feeding experiments with labelled precursors can conclusively prove whether a certain compound is actually produced by a bacterium [17].

However, bearing in mind the confirmed data in the literature and the kinetic evidence, it can be affirmed that the identified volatile compounds are biomarkers of bacterial presence.

Proof of the bacterial metabolic activity in cosmetic substrates is the consumption kinetics of some cosmetic ingredients. Thus, comparative analyses show that some of the volatiles in the blank were missing or found at lower concentration in the bacteria-incubated sample. This can be clearly observed in Fig. 3 and 4, where these “consumed compounds” are number-labeled in both blank and bacterium-incubated chromatograms. Fig. 5c shows the decreasing profile obtained for geranyl acetate (cosmetic ingredient frequently used for fragrance) in a *P. fluorescens*-incubated hand cream. These results evidence the capability of some bacteria to survive and grow in cosmetic substrates by metabolizing their ingredients.

4. Conclusions

A novel methodology based on MVOC analysis was proposed for the rapid determination of microbial contamination in cosmetic products. SPME-GC-MS was used for collecting and analyzing the volatiles above the headspace of cultures and samples incubated with typical bacterial contaminants. A total amount of twenty-two MVOCs were identified in the experiments performed. Many of them were present both in the samples and in the cultures. However some other volatiles were only found in the samples, depending on the cosmetics, demonstrating the influential role of the substrate in the bacterial growth. Data presented here suggest that it is clearly feasible to detect microbiological contamination in cosmetic matrices by analyzing the emitted microbial volatile compounds. Moreover, the identification of bacteria-specific volatiles from a contaminated sample might be possible. However, this research work should be continued using a large number of different cosmetics and bacteria, in order to look for specific biomarkers.

This promising method might be easily implemented as a rapid screening methodology in the cosmetics industry for detecting microbial contamination both in the finished product and in raw materials, facilitating the rapid release of products into the market.

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III.3

ESTUDIO DEL COMPORTAMIENTO FOTOQUÍMICO DE CONSERVANTES COSMÉTICOS

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Aunque los estudios de estabilidad fotoquímica de ingredientes cosméticos no son muy abundantes en la bibliografía, se ha demostrado claramente que ciertos conservantes son capaces de absorber radiación ultravioleta, e incluso experimentar fenómenos de fotodegradación o transformación fotoquímicas, mediante fotólisis o procesos de fotocatálisis con fuertes oxidantes en los denominados Procesos de Oxidación Avanzada. Estos procesos pueden tener lugar bajo radiación solar natural o durante el tratamiento de aguas residuales, en las que los PCPs son liberados diariamente procedentes de la industria farmacéutica, efluentes de hospitales, efluentes domésticos (ej. productos cosméticos y del hogar), etc. Sin embargo, un tratamiento incompleto de estos compuestos puede dar lugar a la formación de subproductos de fotodegradación cuya liberación al medioambiente puede implicar consideraciones ecotoxicológicas.

En esta línea, se ha decidido abordar el estudio de la fotodegradación acuosa de algunos de los conservantes más frecuentemente empleados en formulaciones cosméticas, como benzoatos, parabenos, BHA, BHT y triclosan; con el objetivo de evaluar las transformaciones fotoquímicas que pueden sufrir estos ingredientes bajo tratamiento con luz ultravioleta. La SPME se empleó en estos estudios como técnica extractiva, dada su demostrada eficacia para la extracción de los conservantes estudiados, así como por su capacidad de incrementar la detectabilidad de posibles fotoproductos generados a concentraciones bajas. La identificación de los productos de transformación ha sido posible gracias al acoplamiento con GC-MS.

Los primeros resultados de estos estudios demostraron, por primera vez, que la radiación UV promueve la formación de especies halogenados de parabenos y benzoatos en agua clorada. Se han identificado derivados bromados y bromoclorados, así como la transformación de benzoatos en parabenos, mediante procesos de hidroxilación fotoinducida. La importancia de estos resultados radica en la formación de subproductos halogenados de mayor toxicidad que los conservantes de partida.

En una aproximación, el uso de fibras de SPME con distinta polaridad ha permitido la detección de fotoproductos con una gran variedad de estructuras químicas, algunos de ellos no había sido previamente identificados. Los derivados estructuralmente similares se han podido relacionar a través de rutas de transformación, que han permitido explicar las transformaciones ocurridas.

Dada la demostrada capacidad de los conservantes seleccionados de generar una gran cantidad de productos de transformación, algunos de ellos con propiedades toxicológicas desconocidas, es razonable pensar que dichas transformaciones fotoquímicas también

pudieran tener lugar en formulaciones tópicas aplicadas en zonas de la piel expuestas directamente a la radiación solar. Para demostrar esta hipótesis se han planteado dos aproximaciones. Por una parte se han realizado experimentos de fotodegradación en productos cosméticos fortificados sobre una superficie inerte (vidrio), y por otra parte se ha reproducido una situación más realista al irradiar un cosmético fortificado aplicado sobre un modelo de piel sintética.

Para conseguir aislar los fotoproductos de las muestras cosméticas irradiadas se ha propuesto una versión miniaturizada de MSPD, por el reducido tamaño de muestra utilizado. La extracción en los experimentos con piel sintética se realizó mediante PLE, una técnica de gran eficacia y más adecuada para un sustrato sintético difícilmente dispersable (piel artificial). El análisis de los extractos obtenidos se realizó mediante GC-MS, lo que permitió la utilización de la librería de espectros para la identificación inequívoca de fotoproductos.

Estos estudios han confirmado la formación fotoinducida de hidroxibenzofenonas, 2,8-diclorodibenzo-p-dioxina, así como otros fotoproductos de tipo dioxina y derivados de BHT. Los resultados obtenidos constituyen las primeras evidencias de la transformación fotoquímica de conservantes tanto en formulaciones cosméticas como en un modelo de piel artificial.

A continuación se presentan los trabajos publicados o en vía de ser publicados, derivados de los estudios de fotodegradación desarrollados durante la última etapa de esta tesis doctoral.

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Capítulo III.3.1

Identification of halogenated photoproducts generated after ultraviolet-irradiation of parabens and benzoates in water containing chlorine by solid-phase microextraction and gas chromatography-mass spectrometry

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Identification of halogenated photoproducts generated after ultraviolet-irradiation of parabens and benzoates in water containing chlorine by solid-phase microextraction and gas chromatography-mass spectrometry

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Abstract

This work presents a new solid-phase microextraction (SPME)-based approach to investigate the formation of halogenated by-products generated by the UV-induced photodegradation of parabens and their congener benzoates in water containing chlorine. Degradation of parent species, and further identification of their transformation by-products were monitored by gas chromatography coupled to mass spectrometry (GC-MS). In order to improve detectability, SPME was applied as a preconcentration step after UV-irradiation of target preservatives. Experiments performed with dechlorinated water, ultrapure water, and tap water showed that under UV-light, the presence of even low levels of free chlorine increases the photodegradation rate of target preservatives, enhancing the formation of halogenated photoproducts. Monobrominated, dibrominated and bromochlorinated hydroxybenzoates were identified, and the transformation of benzoates into halogenated parabens was also confirmed. Bromination is expected to occur when free chlorine is present, due to the presence of traces of bromide in water samples. Five halogenated phenols (mainly brominated) were detected as breakdown photoproducts from both families of target preservatives. On the basis of the appearance of the aforementioned by-products, a tentative transformation pathway, consistent with the photoformation–photodecay kinetics of the by-products, is proposed herein for the first time.

Keywords: Personal care products; Photodegradation; Halogenated by-products; Bromophenols; Solid-phase microextraction; GC–MS.

1. Introduction

Benzotes and parabens, alkyl esters of benzoic acid and para-hydroxybenzoic acid, respectively, are structurally related chemicals that are widely used as preservatives in cosmetics and personal care products (PCPs), as well as in canned food and pharmaceuticals [1-3]. Although no harmful effects on consumer health have been attributed to the use of benzoates or their salts in cosmetics [4], some studies indicate that most benzoic acid derivatives may exhibit toxicity and cause serious public health and environmental problems[2]. In fact, sodium and potassium benzoic acid salts have been shown to be clastogenic, mutagenic and cytotoxic to human lymphocytes in vitro [5]. Parabens are suspected endocrine disrupting compounds with oestrogenic- and antiandrogenic-like properties [6, 7]. Moreover, recent studies suggest a potential relationship between the application of paraben-containing products and some types of allergies [8] and even breast cancer [9]. The continuous release of PCPs into the environment has raised scientific concern about the effects that these ubiquitous compounds and their transformation products might have on human health and on aquatic organisms. The efficient removal of these compounds in conventional wastewater treatment plants is a challenge for drinking water production[10]. UV light is an established method of drinking water disinfection and a developing method for wastewater purification, which can be used in combination with strong antioxidants in the so-called Advanced Oxidation Processes (AOPs) [11].

Although literature about photodegradation of parabens and benzoates is rather scarce, some studies dealing with photolytic or photocatalytic processes applying AOPs have been reported. Thus, direct photolysis of butylparaben (BuP) [11] was found to be inefficient due to low quantum yield. Although other oxidative processes such as ozonation are reported to be more effective than photolysis [12], degradation of BuP using combined action of UV radiation and H_2O_2 occurred rapidly and effectively [13]. In this respect, photooxidation with additives such as H_2O_2 or TiO_2 was found to enhance methylparaben degradation [14], whereas the combination of two or more simple oxidation systems increased the reactivity by a synergic effect [15]. Heterogeneous TiO_2-mediated photocatalysis has also been used to degrade benzylparaben [16] and benzoic acid [17]. In the latter two studies, several photoproducts were identified, such as hydroxylated benzoates and phenols, as in other ozonization studies about parabens [10].

On the other hand, it is well known that compounds containing phenolic hydroxyl groups, such as parabens, can react readily with free chlorine in chlorinated water (0.4–

5 mg Cl_2 L^{-1}) yielding chlorinated by-products [18, 19]. In fact, several chlorinated parabens, which are eventually released into aquatic environments, have been detected in water samples from public swimming pools [20] and from raw sewage water samples [19]. Toxicity studies reveal that the resulting chlorinated by-products generate more acute toxicity responses than the parent compounds in the *Daphnia magna* and *Vibrio fischeri* tests [21]. Moreover, evidence has shown that brominated parabens and phenols are generally significantly more cytotoxic and genotoxic than their chlorinated analogs [22-24]. Nowadays, the use of chlorophenols has been restricted or widely banned in many countries due to possible adverse health effects, even at low concentrations [25].

To our knowledge, the photochemical behavior of parabens and their congener benzoates under UV light in water containing chlorine has not yet been investigated. Photohalogenated derivatives and halogenated breakdown photochemical by-products are suspected to be generated. Thus, in this work, a reliable methodology based on solid-phase microextraction coupled to gas chromatography mass-spectrometry (SPME-GC-MS) was developed and applied in order to tackle the following aims: (i) to investigate the photodegradation of parabens and benzoates in water samples containing chlorine, (ii) to identify their potential degradation and transformation by-products on the basis of their mass spectra, information reported in the literature and the NIST Mass Spectral Database, (iii) to monitor their photoformation–photodegadation kinetics, and (iv) to propose the transformation pathways for the preservatives under study. We suggest herein the implementation of SPME-GC-MS as highly valuable tool, which allow, for the first time, the monitorization of photohalogenated by-products from the target emerging pollutants.

2. Experimental

2.1 Reagents and materials

Methylbenzoate (MeBz; 99%), ethylbenzoate (EtBz; 99%), and butylbenzoate (BuBz) were supplied by ChemService (West Chester, USA). Methylparaben (MeP; 99%), ethylparaben (EtP; 99%), propylparaben (PrP; 99%), butylparaben (BuP; 99%), isopropylparaben (iPrP; ≥99%) and isobutylparaben (iBuP; ≥97%) were acquired from TCI Europe (Belgium). The retention times and the selected quantification and identification ions of the target preservatives are included in Table 1. For confirmation purposes, 3,5-dibromomethylparaben (3,5-DBrMeP) was obtained form Sigma–Aldrich.

Acetone (99.9%) and sodium thiosulphate (>98%) were provided by Merck (Darmstadt, Germany) and sodium chloride (99.7%) was supplied by Prolabo (VWR, Fontenay-sous-Bois, France). All solvents and reagents were of chromatographic grade.

Individual stock solutions of each compound were prepared in acetone. Further dilutions and mixtures in the same solvent were prepared by convenient dilution of the stock solution to spike water samples. Stock and working solutions were stored in a freezer at −20 °C protected from light.

Commercially available fibers housed in manual SPME holders were obtained from Supelco (Bellefonte, PA, USA): 100 μm polydimethylsiloxane (PDMS), 65 μm polydimethylsiloxane–divinylbenzene (PDMS/DVB), 75 μm carboxen–polydimethylsiloxane (CAR/PDMS). The fibers were conditioned as recommended by the manufacturer.

2.2 Water samples

Three different water samples containing different amounts of free chlorine were tested for photodegradation studies with the target preservatives: dechlorinated ultrapure water (TH-W), ultrapure water (MQ-W), and tap water (TP-W). Use of the *N*,*N*-diethyl-*p*-phenylenediamine procedure with photometric detection [26] confirmed the presence of free chlorine in tap water (0.6 mg L^{-1}) and also in ultrapure water. In the case of MQ-W, free chlorine was not possible to quantify, since the concentration was below the LOQ (0.1 mg L^{-1}). The pH of the samples was similar, ranging from 6.4 to 7.1. Dechlorinated water was obtained by removing the available free chlorine from ultrapure water with sodium thiosulphate (1 mM).

2.3 Photodegradation and solid-phase microextraction procedure

Two aliquots of water (3 ml each) previously spiked with the target preservatives (at 50 ng mL^{-1}) were placed in two quartz cuvettes. Irradiation experiments were conducted in a laboratory-made photoreactor equipped with two low-pressure mercury lamps (8 and 10 W, 254 nm). Quartz cuvettes were placed inside the photoreactor box at a distance of 9 cm from the lamps, and irradiated with UV light (254 nm) at 8 or 18W-UV (depending on the experiment) for the designated time (2–60 min). In the control experiment (0 min of UV irradiation), the water samples were immediately extracted by SPME.

Irradiated solutions (6 ml of water) were transferred to 10 ml vials with addition of sodium chloride at 20% (w/v). Vials were sealed with aluminum caps furnished with PTFE-faced septa and immersed in a water bath maintained at the working temperature (25 or 100 °C). Samples were let to equilibrate for 5 min before placing the SPME device. Afterwards, the fiber was exposed to the headspace over the sample (HSSPME) or immersed into the sample (SPME) for 15 min. Three different fiber coatings (PDMS, PDMS/DVB, and CAR/PDMS) were compared to evaluate the capability of SPME to extract the parent compounds and photochemical by-products. After extraction, the fibers were thermally desorbed at 270 °C in the GC injector port for 5 min, for GC–MS analysis. Under the final optimized conditions, target compounds were extracted at 25 °C for 15 min in direct sampling mode, using a CAR/PDMS fiber over the stirred samples, with addition of 20% (w/v) of sodium chloride.

Dark test were performed by placing the cuvettes in the photoreactor box, maintaining the lamps switched off for a certain time (0–60 min), and covering the whole device with aluminum foil. The SPME procedure was then applied.

2.4 Gas chromatography–mass spectrometry analysis

The GC–MS analysis was performed with a Thermo Scientific Trace 1310-GC gas chromatograph coupled to a triple quadrupole mass spectrometer (TSQ 8000; Thermo Scientific; San Jose, CA, USA). The system was operated by Xcalibur 2.2 and Trace Finder TM 3.0.

Separation was carried out on a TG-5 SILMS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) obtained from Thermo Scientific (San Jose, CA, USA). Helium (purity 99.999%) was used as carrier gas, at a constant column flow of 1.0 mL min^{-1}. The GC oven temperature was programmed from 80 °C (held 2 min) to 230 °C at 8 °C min^{-1}, and to 270 °C at 15 °C min^{-1} (held 5 min). Splitless mode (held 2 min) was used for injection. The injector temperature was between 260 and 290 °C depending on the fiber used.

The mass spectrometry detector (MSD) was operated in the electron impact (EI) ionization positive mode (+70 eV). All determinations were carried out in the full scan acquisition mode, in a mass range of m/z 40–450. The transfer line and the ion source temperatures were set at 290 and 350 °C, respectively.

Table 1. Retention times (RTs), quantification and identification ions, and reverse match (R. Match) of target parabens, benzoates and the tentatively identified photoproducts (see general structures in Fig. 7).

Label	Compound	RT(min)	Qualifier and quantifier ions[a]	Identification based on[b]	R. Match
Benzoates					
MeBz	Methyl benzoate	5.86	136 (39), *105*, 77 (62)	-	-
EtBz	Ethyl benzoate	7.15	150 (22), 122 (36), *105*, 77 (44)	-	-
BuBz	Butyl benzoate	10.65	123 (65), *105*, 77 (45)	-	-
Parabens					
MeP	Methylparaben	11.85	152 (38), *121*, 93 (24)	-	-
EtP	Ethylparaben	12.93	*121*, 138 (20), 166 (23)	-	-
iPrP	Isopropylparaben	13.40	*121*, 138 (37), 180 (11)	-	-
PrP	Propylparaben	14.43	*121*, 138 (57), 180 (66)	-	-
iBuP	Isobutylparaben	15.28	93 (11), *121*, 138 (57)	-	-
BuP	Butylparaben	15.90	*121*, 138 (84), 194 (68)	-	-
Paraben derivatives					
3-BrMeP	3-Bromomethylparaben	12.76	230 (26), *199*, 171 (14)	A	807
3,5-DBrMeP	3,5-Dibromomethylparaben	16.88	310 (40), *279*, 251 (14), 170 (16)	A	815
3-Br-5-ClMeP	3-Bromo-5-chloromethylparaben	15.57	266, 235, 179, 126	B	-
3-BrEtP	3-Bromoethylparaben	13.85	244(22), 216 (37), *199*, 171 (15), 143 (13)	C	-
3,5-DBrEtP	3,5-Dibromoethylparaben	17.82	324 (25), 296 (54), *279*, 251 (16), 170 (22)	D	-
3-Br-5-ClEtP	3-Bromo-5-chloroethylparaben	16.56	280, 252, 235, 179, 126	B	-
3-Br-iPrP	3-Bromoethylparaben	14.31	258 (12), 216 (57), *199*, 171 (12), 143 (11)	C	-
3,5-DBr-iPrP	3,5-Dibromoethylparaben	18.18	338 (22), 296 (81), *279*, 251 (11), 170 (24)	D	-
3-Br-5-Cl-iPrP	3-Bromo-5-chloroethylparaben	16.96	294, 252, 235, 179, 126	B	-
3-BrPrP	3-Bromopropylparaben	15.33	258 (8), 216 (80), *199*, 171 (14), 143 (13)	C	-

[a] Quantification ions are set in italics. The relative ion abundances (%) of the qualifier ions with respect to the quantifier ion are included in brackets

[b] A: theoretical mass spectrum, B: experimental ion justification (see Fig 4), C: theoretical mass spectrum of 3-bromo-4-hydroxybenzoic acid, D: theoretical mass spectrum of 3,5-dibromo-4-hydroxybenzoic acid, E: theoretical mass spectrum of 3-bromosalicilic acid, F: theoretical mass spectrum of 3-

Table 1. (Continued)

Label	Compound	RT(min)	Qualifier and quantifier ions[a]	Identification based on[b]	R. Match
3,5-DBrPrP	3,5-Dibromopropylparaben	19.10	338 (14), *296*, 279 (98), 251 (12), 172 (24)	D	-
3-Br-5-ClPrP	3-Bromo-5-chloropropylparaben	17.88	294, 252, 235, 179, 126	B	-
3-Br-iPrP	3-Bromoisobutylparaben	16.12	216 (96), *199*, 171 (14), 143 (13)	C	-
3,5-DBr-iPrP	3,5-Dibromoisobutylparaben	19.75	*296*, 279 (83), 251 (10), 172 (22)	D	-
3-Br-5-Cl-iPrP	3-Bromo-5-chloroisobutylparaben	18.57	*252*, 235, 179, 126	B	-
3-BrBuP	3-Bromobutylparaben	16.80	258 (8), 216 (80), *199*, 171 (14), 143 (13)	C	-
3,5-DiBrBuP	3,5-Dibromobutylparaben	20.39	*296*, 279 (67), 251 (10), 172 (21)	D	-
3-Br-5-ClBuP	3-Bromo-5-chlorobutylparaben	19.21	*252*, 235, 179, 126	B	-
Benzoate derivatives					
MeS	Methyl 2-hydroxybenzoate (Methyl salicylate)	7.57	152 (49), *120*, 92 (54)	A	922
5-BrMeS	5-Bromomethylsalicylate	11.85	230 (25), *198*, 170 (50)	E	-
3,5-DBrMeS	3,5-Dibromomethylsalicylate	16.26	310 (33), *278*, 250 (22), 197 (37), 143 (42)	A	785
3-OHMeBz	Methyl 3-hydroxybenzoate	11.38	*152 (35), 121*, 93 (27)	F	-
3-BrMeP	3-Bromomethylparaben	12.76	230 (26), *199*, 171 (14)	A	807
3,5-DBrMeP	3,5-Dibromomethylparaben	16.88	310 (40), *279,* 251 (14), 170 (16)	A	-
EtS	Ethyl 2-hydroxybenzoate (Ethyl salicylate)	8.88	166 (30), *120*, 121 (30), 92 (44)	A	943
5-BrEtS	5-Bromoethylsalicylate	12.97	244 (23), 218 (4), *198*, 170 (41), 143 (12)	E	-
3,5-DBrEtS	3,5-Dibromoethylsalicylate	17.19	324 (21), *278*, 250 (10), 197 (21), 143 (35)	G	-
3-OHEtBz	Ethyl 3-hydroxybenzoate	12.48	166 (33), 138 (34), *121*, 93 (29)	A	835
3,5-DBrEtP	3,5-Dibromoethylparaben	17.82	324 (25), 296 (54), *279,* 251 (16), 170 (22)	D	-

[a] Quantification ions are set in italics. The relative ion abundances (%) of the qualifier ions with respect to the quantifier ion are included in brackets
[b] A: theoretical mass spectrum, B: experimental ion justification (see Fig 4), C: theoretical mass spectrum of 3-bromo-4-hydroxybenzoic acid, D: theoretical mass spectrum of 3,5-dibromo-4-hydroxybenzoic acid, E: theoretical mass spectrum of 3-bromosalicilic acid, F: theoretical mass spectrum of 3-hydroxybenzoic , acid, G: theoretical mass spectrum of 3,5-dibromosalicilic acid.

Table 1. (Continued)

Label	Compound	RT(min)	Qualifier and quantifier ions[a]	Identification based on[b]	R. Match
3-Br-5-ClEtP	3-Bromo-5-chloroethylparaben	16.56	233, 235, 252, 254	B	-
BuS	Butyl 2-hydroxybenzoate (Butyl salicylate)	12.21	194 (21), 138 (20), *120*, 121 (26), 92 (21)	A	952
5-BrBuS	5-Bromobutylsalicylate	15.83	272 (12), 216 (32), *198*, 170 (24), 143 (22)	E	-
3,5-DBrBuS	3,5-Dibromobutylsalicylate	19.72	352 (12), 296 (28), *278*, 197 (10), 143 (34)	G	-
3-OHBuBz	Butyl 3-hydroxybenzoate	15.42	194 (11), 138 (95), *121*, 93 (27)	F	-
3-BrBuP	3-Bromobutylparaben	16.80	258 (8), 216 (80), *199*, 171 (14), 143 (13)	C	-
3,5-DiBrBuP	3,5-Dibromobutylparaben	20.39	*296*, 279 (67), 251 (10), 172 (21)	D	-
Halogenated phenol					
2-BrPh	2-Bromophenol	5.44	174 (98), *172*, 93 (20), 65 (30)	A	851
4-BrPh	4-Bromophenol	9.07	174 (99), *172*, 93 (41), 65 (66)	A	854
2,6-DBrPh	2,6-Dibromophenol	10.17	254 (49), *252*, 250 (52), 143 (18)	A	942
2,4,6-TClPh	2,4,6-Trichlorophenol	10.27	200 (30), 198 (94), *196,* 160 (19), 132 (53)	A	946
4-Br-3,5-DClPh	4-Bromo-3,5-dichlorophenol	11.75	*242,* 240 (64), 244 (46), 178 (17), 133 (24)	A	702
2,4,6-TBrPh	2,4,6-Tribromophenol	14.71	328 (34), *330,* 332 (98), 334 (30), 250 (26)	A	920

[a] Quantification ions are set in italics. The relative ion abundances (%) of the qualifier ions with respect to the quantifier ion are included in brackets

[b] A: theoretical mass spectrum, B: experimental ion justification (see Fig 4), C: theoretical mass spectrum of 3-bromo-4-hydroxybenzoic acid, D: theoretical mass spectrum of 3,5-dibromo-4-hydroxybenzoic acid, E: theoretical mass spectrum of 3-bromosalicilic acid, F: theoretical mass spectrum of 3-hydroxybenzoic , acid, G: theoretical mass spectrum of 3,5-dibromosalicilic acid.

Identification was accomplished using the NIST Mass Spectral Database (NIST MS Search 2.0) and by comparing retention times with those of commercial standards, when available. Quantification was performed using Trace Finder software.

3. Results and discussion

3.1 SPME-GC-MS analysis.

SPME was used to investigate the photochemical behavior of parabens and benzoates in water containing chlorine. Under the conditions specified in Section 2, the highest extraction efficiencies were achieved with CAR/PDMS fiber, and performing the extraction in direct sampling mode at room temperature (25 °C). The addition of NaCl (20%, w/v) improved extraction yields. Magnetic stirring was also used, as it is known to favor mass transfer. Since parabens showed satisfactory responses at the studied concentration, no derivatization step was applied for not to introduce additional complexity in the photoproducts identification process. These results are in good agreement with those obtained by Regueiro et al. [27] for the extraction of acetylated parabens from water samples, although the addition of NaCl improved extraction efficiency for non-derivatized parabens as well as for benzoates. GC–MS analysis of the extracted compounds was then performed. Under the optimized conditions, the method precision was evaluated (n = 4) in terms of relative standard deviations (RSDs), and satisfactory results (RSD < 12%) were obtained for the target compounds.

3.2 Aqueous photodegradation and photohalogenation

3.2.1 Influence of irradiation power

Following the photodegradation procedure described in the experimental section, preliminary experiments were performed to test the photostability of the target preservatives in ultrapure water. The photodegradation kinetics of parabens and benzoates were monitored by studying the influence of irradiation time (0–20 min) on the analytical response. The results of experiments carried out at 8W and 18W-UV irradiation were compared. The kinetic curves obtained for the photochemical behavior of BuP and BuBz, as representative compounds of parabens and benzoates, are shown in Fig. 1 (results are expressed as relative responses regarding the non-degraded compound). As expected, degradation was faster observed at the highest power. As can be seen in Fig. 1A, after 20 min irradiation at 8W-UV, less than 20% of the parabens remained undegraded, yielding a kinetic

curve with a slow decrease in the signal, whereas benzoates did not undergo photodegradation even after 20 min.

Working at 18W-UV, parabens exhibited pseudo-first order kinetics, which was demonstrated by plotting the natural logarithm of the percentage of undegraded compound against irradiation time (Fig. 1B). On the other hand, the benzoates showed slower degradation, and pseudo-first order behavior can be observed from 5 min of irradiation. For practical reasons, the stability experiments of benzoates were carried out at 18W-UV. Slower degradation, achieved by irradiation at 8W-UV, was performed for a better understanding of the photodegradation/photoformation kinetics of some by-products.

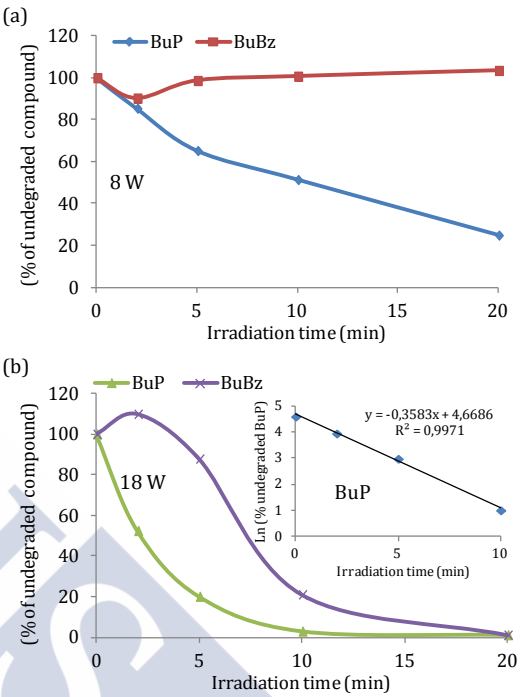

Fig. 1. Photodegradation kinetic curves obtained for BuP and BuBz as representativetargets: (a) 8W-UV irradiation; (b) 18W-UV irradiation. Demonstration of first-orderkinetic behavior of parabens (BuP) is also included.

The results of the dark test in MQ-W show that after 20 min of 18W-UV irradiation, a reduction in the peak areas (>95%) was clearly observed for both parabens and benzoates, whereas no significant decrease in the analytical response was observed in the experiments performed in darkness (see Fig. 2A for PrP in MQ-W). These results demonstrate that the decrease in analytical responses after irradiation of aqueous solutions was only due to the action of photons, and loss of the target analytes does not occur through other processes, such as e.g. volatilization.

3.2.2 Influence of chlorine content

Several halogenated derivatives (mainly brominated and bromochlorinated by-products) were identified during aqueous photodegradation studies of both parabens and benzoates (see Section 3.2.3) performed in different types of ultrapure water (Milli-Q water and LC–MS grade water). In view of these results, chlorine and bromine at trace levels were suspected to affect the photochemical behavior of target preservatives. As reported in

literature, in chlorinated water, compounds containing phenolic hydroxyl groups, such as parabens, exhibit favorable chlorination kinetics [21]. Moreover, free chlorine can oxidize bromide rendering bromine, which can react with aromatic compounds through electrophilic substitution reactions. Thus, chlorination and bromination are expected to compete to react with parabens through substitution reactions, being bromination a more favorable reaction than chlorination [19].

Dechlorinated water (TH-W), ultrapure water (MQ-W) and tap water (TP-W) were tested to determine the extent to which the presence of free chlorine may affect parabens and benzoates photodegradation. The results of the photochemical degradation of PrP, as a representative compound, in the aforementioned water samples is illustrated in Fig. 2.

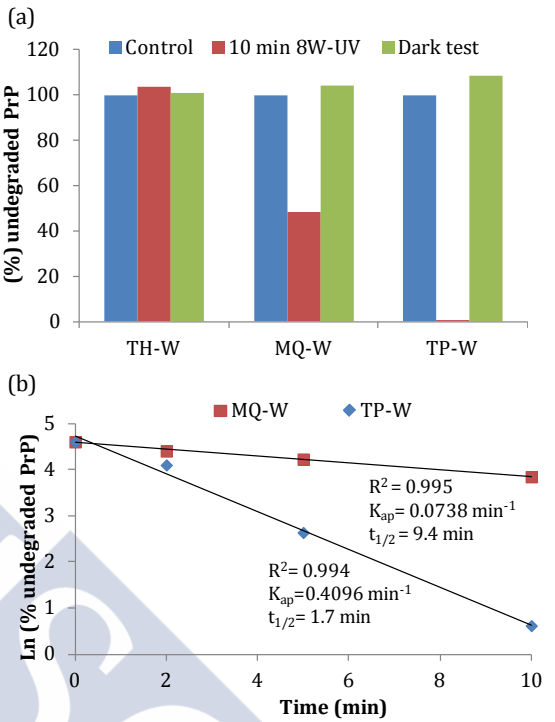

Fig. 2. Influence of chlorine in aqueous photodegradation of PrP at 8W-UV. (a) Dark test and aqueous photodegradation in comparison with the control (non irradiated) experiment in TH-W, MQ-W and TP-W water samples. (b) Pseudo-first-order kinetic behavior and kinetic parameters in MQ-W and TP-W.

After 10 min of irradiation at 8W-UV, the target compounds diluted in ultrapure water and tap water underwent a significant reduction in the peak area of 50% and 98%, respectively, whereas no decrease in the analytical response was observed in dechlorinated water (Fig. 2A). Further experiments indicate that irradiation for 60 min at 18W-UV is required to achieve 80% degradation of parabens in TH-W. These results demonstrate that, in the absence of free chlorine, photodegradation yield decrease significantly.

In view of the results of the dark test carried out in the three types of water (Fig. 2A), spontaneous chemical halogenation can be discarded, and the decrease in analytical responses is considered to be due only to the photohalogenation induced by irradiation of aqueous solutions. Hence, it can be assumed that, the presence of even low levels of free

chlorine (as in ultrapure water), increases the degradation rate of the target preservatives during UV-irradiation.

The photochemical kinetic behavior for target preservatives in MQ-W and TP-W water samples was monitored. The natural logarithm of the percentage of undegraded PrP, in both water samples, was plotted against irradiation time (Fig. 2B). In both cases, experimental data showed satisfactory linear fit, indicating that aqueous photodegradation of PrP in the presence of bromine (brominated parabens were the most favorable photoproducts; see Section 3.2.3) follows a pseudo-first-order kinetic model. The rate constants and the half-life times were also calculated for the target compounds. Apparent first-order rate constants (k_{ap}) were determined from the slopes of the straight-line equations obtained from the least-squares regression: $\ln C$ versus time plot, $\ln C = -k_{ap}t + \ln C_0$, where C_0 and C represent the percentage of undegraded compound at times zero and t, respectively. Half-life times were calculated using the expression: $t_{1/2} = \ln 2/k_{ap}$.

Table 2. Half-lives times ($t_{1/2}$) for parabens and benzoates in ultrapure and tap water.

Compounds	MQ-W [a]		TP-W [b]	
	$t_{1/2}$ (min)	R^2	$t_{1/2}$ (min)	R^2
	18W-UV			
MeBz	7.3	0.999	7.4	0.999
EtBz	7.3	0.999	7.2	0.999
BuBz	6.7	0.989	7.1	0.998
	8W-UV			
MeP	9.3	0.995	3.0	0.999
EtP	9.9	0.989	3.4	0.991
iPrP	8.8	0.986	1.7	0.994
PrP	9.6	0.987	1.7	0.992
iBuP	8.3	0.987	1.6	0.979
BuP	8.5	0.991	1.3	0.958

[a] Chlorine concentration (< 0.1 mg L^{-1})
[b] Chlorine concentration (0.6 mg L^{-1})

Thus, the kinetic parameters show that the photodegradation rate increases with the concentration of free chlorine in water for the target parabens, whereas no significant differences were observed for benzoates in MQ-W and TP-W (Table 2). The half-life values for both families of target preservatives were obtained at different irradiation powers, since degradation kinetics of benzoates was shown to be much slower at 8W-UV, as can be deduced from Fig. 1.

3.2.3 Halogenated photoproducts

As a result of UV-irradiation of target preservatives in the studied water samples, several halogenated by-products were detected. The identified photoproducts as well as the retention times, identification and quantification ions of the MS spectra, the identification

approach for each photoproduct, and the reverse match (R. Match) values given by NIST Mass Spectral Database are summarized in Table 1. Three halogenated species were identified for each paraben, whereas four halogenated and two hydroxylated by-products were obtained for each benzoate. Furthermore, six halogenated phenols were detected as photodegradation by-products derived from both parabens and benzoates under photochemical degradation/halogenation. Individual studies have revealed the origin of each particular photoproduct.

The formation of the photohalogenated parabens can be clearly observed in Fig. 3, where the overlapped total ion chromatograms (Fig. 3a), as well as the extracted ion chromatograms obtained after 0, 2, 5, and 10 min (Fig. 3b–e) of irradiation at 18W-UV are shown. Each chromatogram illustrates the response of the halogenated parabens reaching the highest response at each time, as well as the experimental mass spectrum of the corresponding halogenated 4-hydroxybenzoic acid moiety. Although different intensities of ion ratios were observed in the mass spectra, both ester and acid forms share similar fragmentation patterns. These are mainly characterized by two major clusters of ions, typical of bromine- and chlorine-containing compounds: 199 and 216 m/z clusters for mono-brominated, 277 and 294 m/z clusters for di-brominated, and 233 and 250 m/z clusters for bromochlorinated parabens. This pattern is consistent with $[M\text{-}OC_nH_2CH_3]^+$ and $[M\text{-}C_{n+1}H_2CH_3]^+$, respectively (with the exception of methyl esters), attributed to the loss of the alkoxy group and the alkyl chain from the parent compound (see experimental ion justification for PrP photohalogenated by-products in Fig. 4a–c). Moreover, the molecular ions and the most intense peaks of mono- and di-brominated species were shifted 78 and 156 m/z units, respectively, with respect to those observed in the mass spectra of the corresponding paraben. This behavior indicates the substitution of one or two atoms of hydrogen per bromine in the benzenic ring of the parent compound. In the case of the bromochlorinated derivatives, the observed shift was 112 m/z (78 + 34 m/z) units, corresponding to the substitution of two atoms of hydrogen per bromine and chlorine. Due to the influence of the ester moiety (an electron-withdrawing group or meta-director), meta-substitution is expected to be the most favorable option for halogenated parabens. Additionally, this position is also favored due to the presence of the phenolic group (ortho/para director). This assumption was confirmed by comparing the retention times of the dibrominated photoproduct of MeP with that of the corresponding commercial standard 3,5-DBrMeP.

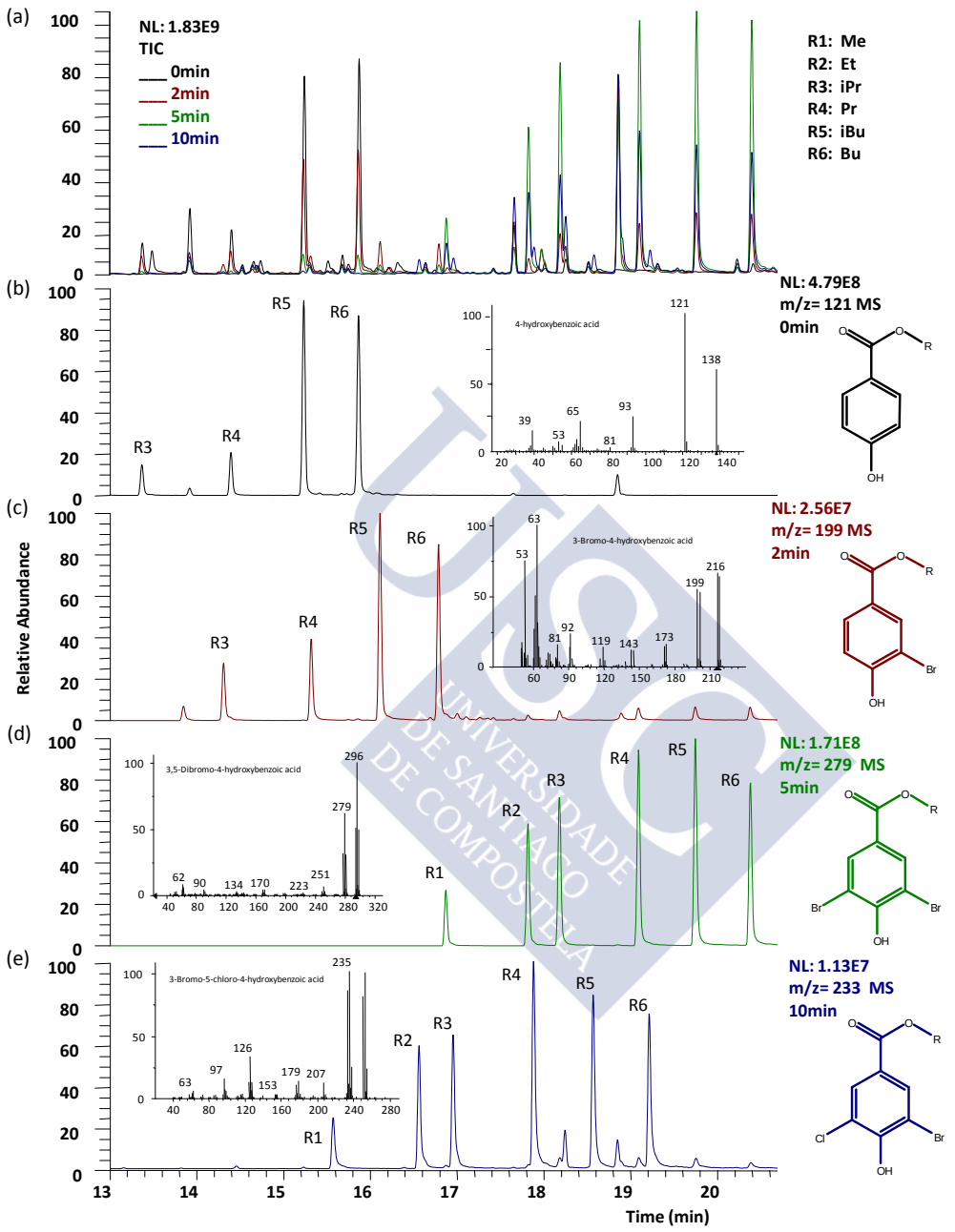

Fig. 3. Overlapped total ion chromatograms (a), and extracted ion chromatograms for (b) parabens; (c) monobrominated parabens; (d) dibrominated parabens, and (e) bromo chlorinated parabens, obtained for 0, 2, 5, and 10 min of UV irradiation. The mass spectrum of the corresponding halogenated 4-hydroxybenzoic acid moiety is also included.

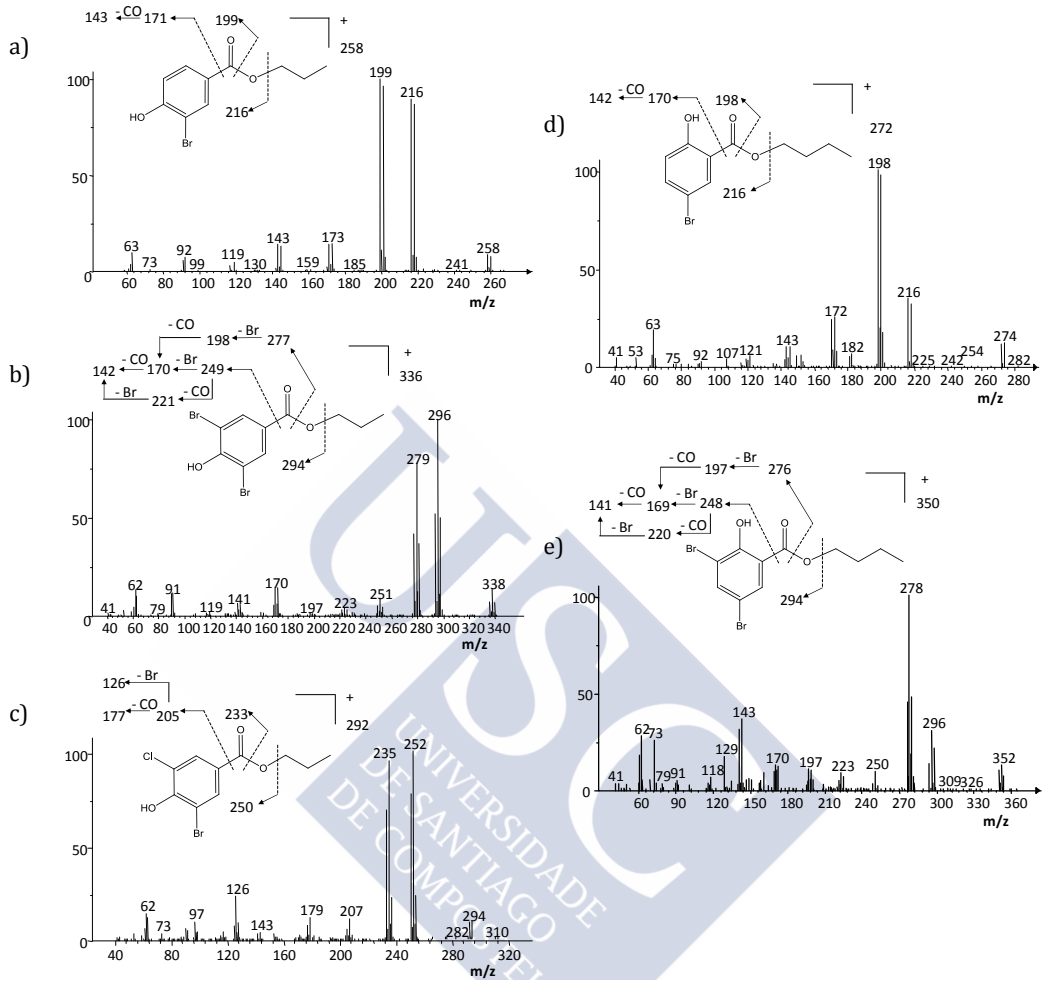

Fig. 4. Experimental ion justification of representative halogenated hydroxybenzoic acid alkyl esters: (a) 3-bromopropylparaben; (b) 3,5-dibromopropylparaben; (c) 3-bromo,5-chloropropylparaben; (d) 5-bromobutylsalicylate; and (e) 3,5-dibromobutylsalicylate.

Halogenated parabens are susceptible to further photodegradation, as can be easily deduced from Fig. 5a, which shows PrP photodegradation and photohalogenated by-products photoformation–photodecay kinetic curves. After reaching their maximum concentrations (between 2 and 10 min at 18W-UV) the three halogenated by-products began to degrade. Nevertheless, kinetic curves obtained at 8W-UV (Fig. S1), show that dibrominated and bromochlorinated parabens are significantly more resistant to degradation, indicating that these photoproducts might be more persistent if formed in the

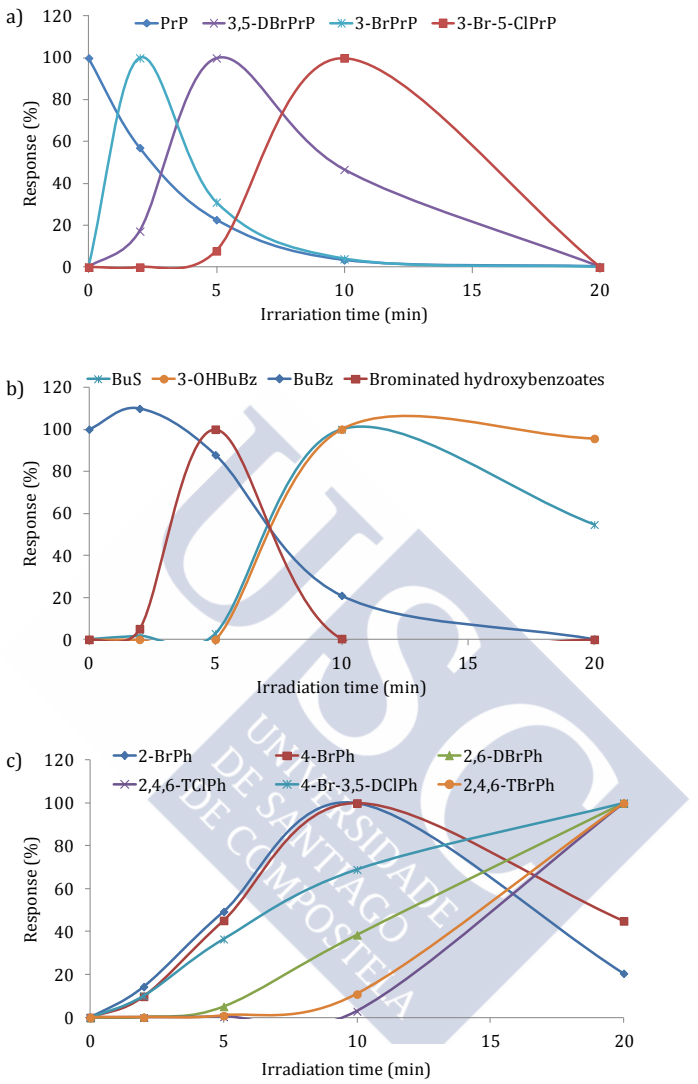

Fig. 5. Photodegradation–photoformation kinetic curves under UV irradiation of: (a) PrP and photohalogenated by-products at 18W-UV; (b) BuBz and photohydroxylatedand halogenated by-products at 18W-UV; (c) photohalogenated phenols at 8W-UV. Responses are normalized to the maximum response obtained for each product.

aquatic environment under natural sunlight or after incomplete disinfection water treatment based on UV-light.

Photodegradation experiments with benzoates revealed the formation of four brominated by-products for each alkyl ester, identified as mono- and di-brominated forms of

both ortho- and para-hydroxybenzoates. Moreover, meta-hydroxybenzoic acid alkyl esters were also detected. The ortho-hydroxylated derivatives, also known as salicylates, were clearly identified in the NIST library. These compounds showed slightly different fragmentation patterns than the corresponding meta- and para-derivatives, with the base peak appearing at m/z 120 rather than at m/z 121. Apart from the molecular ion $[M]^+$, the mass spectra of ortho-hydroxylated derivatives are mainly characterized by the ions m/z 138 (except for methyl esters), m/z 120 and m/z 92, consistent with $[M\text{-}C_{n+1}H_2CH_3]^+$, $[m/z\ 138\text{-}H_2O]^+$, and $[m/z\ 120\text{-}CO]^+$. On the other hand, the meta- and para-derivatives show m/z 121, and m/z 93 as distinctive ions, corresponding to the fragments $[m/z\ 138\text{-}OH]^+$, and $[m/z\ 121\text{-}CO]^+$. The slightly different fragmentation pattern exhibited by these regioisomers is due to the so-called *ortho* effect. This effect promotes the loss of a molecule of water instead of a molecule of hydroxyl in ortho-substituted benzoates, as the phenolic hydroxyl is located on a carbon atom adjacent to a carbon atom with an acid moiety [28].

Bromination of salicylates was assumed to take place in meta- position to the ester group, which was confirmed by mass spectral library for 3,5-DBrMeS. The most intense peaks for mono-brominated salicylates were obtained for the cluster of ions of m/z 170 and 172, whereas dibrominated salicylates revealed ions of m/z 276, 278 and 280 as the most abundant cluster. These peaks represent the loss of the alkoxy group from mono- and di-chloro hydroxybenzoate in the mass spectrum (see experimental ion justification for BuBz photohalogenated by-products in Fig. 4d and e). The presence of these fragment ions confirms the bromination and hydroxylation of the aromatic ring of the benzoates. The photoformation/photodegradation kinetic profile of BuBz and the halogenated by-products, as well as the corresponding hydroxylated derivatives are illustrated in Fig. 5b.

As a result of the photolytic process, several brominated and bromochlorinated phenols were obtained, probably as breakdown photoproducts or by successive halogenation of the phenolic moiety of the abovementioned halogenated hydroxybenzoates. Fig. 6 shows the extracted ion chromatograms obtained for 0, 2, 5, 10, and 20 min of UV irradiation, illustrating the formation and degradation of halogenated phenols, as well as the experimental and theoretical mass spectra. The fragmentation pattern of these photoproducts shows the molecular ions as the most intense peaks in a cluster of ions typical of halogenated compounds. Two monobrominated compounds were tentatively identified as ortho- and para-bromophenols. Di- and tri-bromo phenol were also generated, showing a shift of 156 and 234 m/z units, corresponding to the addition of two and three atoms of bromine, respectively. Moreover, in the mass spectra of two further phenolic compounds the

shift observed was 102 m/z and 146 units. These were identified as trichloro and bromodichloro phenols. Ortho- and para-derivatives are expected to be the most favorable halogenated phenolic by-products. The photoformation–photodecay kinetic curves for these compounds are shown in Fig. 5c.

It is usually accepted that the results of photochemical experiments performed using radiation below 300 nm, to some extent, can be extrapolated to environmental condition [13, 29]. Consequently, under natural solar irradiation, brominated species are expected to be the major photochemical by-products of parabens and benzoates released to disinfected (chlorinated) water containing natural traces of bromide. The presence of the target halogenated photoproducts may be particularly important in outdoor recreational waters exposed to intense solar irradiation, very often crowded with bathers using sunscreens containing several of the target preservatives. Furthermore, the release of this brominated species into the environment after incomplete UV wastewater treatment cannot be discarded. Although the degradation by-products were not quantified in the present study, it is important to bear in mind that the signal of the identified photohalogenated by-products is not at all negligible. In fact, some photoproducts such as dibrominated parabens yielded even higher response than the parent compounds in the TIC chromatogram (Fig. 3). In coastal cities, where water sources can be affected by inputs of salt water, exposure to brominated by-products can be favored [30].

3.2.4 Photohalogenation and photodegradation pathways

A tentative transformation pathway is proposed in Fig. 7. Halogenation was found to be a significant reaction underwent by hydroxylated benzoates at the aromatic ring, whereas no halogenated by-products were identified for the non-hydroxylated forms. Thus, three hydroxylated intermediates obtained from alkyl benzoates (P, Q and S) are proposed. They are presumably generated via photo-hydroxylation reactions with hydroxyl radicals (·OH) arising from the photolytic decomposition of water under UV irradiation. Ortho- and para-hydroxybenzoates (S and P, respectively) can further react with bromine and chlorine, rendering stable meta-substituted halogenated photoproducts. However no halogenated species were observed for the meta-hydroxylated forms of benzoates (Q).

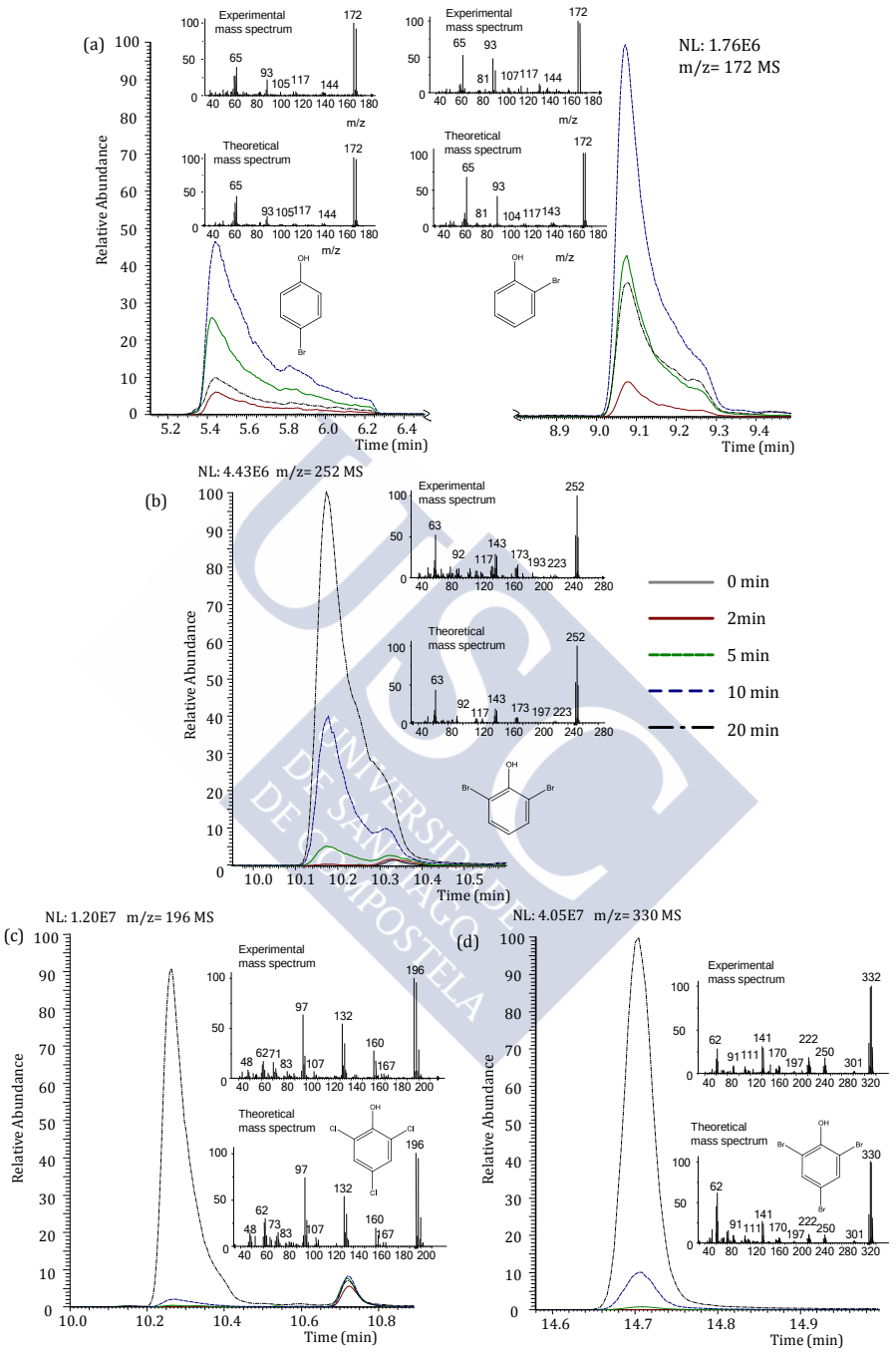

Fig. 6. Extracted ion chromatograms, and mass spectra showing the formation-degradation of photohalogenated phenols: (a) monobromophenols; (b) dibromophenol; (c) trichlorophenol, and (d) tribromophenol, obtained for 0, 2, 5, 10, and 20 min of irradiation at 8W-UV.

Pathways I and II describe the successive halogenation of salicylates and parabens respectively. The proposed sequence of halogenation can be sustained by the corresponding kinetic profiles shown in Fig. 5a and b. It can be easily deduced from Fig. 5a that, in the presence of UV light, parabens react with free bromine rendering the mono-brominated by-product, which in turn undergo further bromine substitution reaction yielding the di-brominated paraben. Formation of the bromochlorinated paraben subsequently takes place, evidencing that both chlorine and bromine compete to react with parabens through photo-induced substitution reactions, with bromination favored over chlorination. These results are consistent with those reported by Canosa et al. [19], who studied the chemical halogenation of parabens in chlorinated water.

Regarding halogenated salicylates, both mono- and di-brominated forms showed the same kinetic profile in benzoates photodegradation at 18W-UV, and they are depicted under the same kinetic curve in Fig. 5b. It can also be observed that once the brominated forms reached maximal concentrations (after irradiation for 5 min) a sharp decrease in response was observed, whereas hydroxylated species undergo a significant increase. This kinetic behavior can be explained considering that as soon as hydroxybenzaotes are formed, they readily undergo photochemical bromination. Subsequent degradation of this brominated species, after 5 min of intense UV-irradiation (18W), led to increasing concentrations of debrominated hydroxybenzoates by means of a reductive photochemical debromination mechanism. This behavior was also observed by Sanchez-Prado et al. [31] in photochemical studies concerning polybrominated diphenyl ethers. Para-hydroxybenzoates were not detected after debromination. However, meta-hydroxybenzoates were identified, suggesting photoisomerization toward this last form.

As regards the formation of halogenated phenols, proposal of detailed photodegradation/photohalogenation pathways is complicated as there are different possibilities to explain the generation of the identified photoproducts by means of radical mechanisms. Logical photochemical mechanisms are proposed in pathways IV–VII. For example, it seems feasible that mono and dibrominated phenols will appear as a result of the photolytic breakdown of C-C bond between the phenol moiety and the carboxylic group of brominated hydroxybenzoates. Thus, tentatively identified para- and ortho-monobromophenols (X1 and X2) may be generated through radicals R1 and R2, after loss of the carboxylic moiety from photoproducts S1 and P1 respectively. Since these by-products were identified in individual photodegradation studies of both parabens and benzoates, photo-isomerization of both forms is also expected to occur. On the other hand, dibrominated phenol (X3) may be directly generated from photoproduct P2 through radical R3, or by photosubstitution of an hydrogen

atom by bromine in photoproduct X2. In turn, X4 might undergo further photobromination yielding tribrominated phenol (X4). Alternatively, the reaction of R4 with another radical of bromine, or the successive bromination of X1/X2, may also led to the formation of X4. Finally, bromodichlorophenol (X5) is expected to be generated by subsequent meta-substitution of two atoms of hydrogen per chlorine in X1. Furthermore, X5 could also undergo isomerization and subsequently exchange the bromine in the para position for chlorine, rendering trichlorophenol (X6). This proposal is consistent with the formation–photodegradation curves of the abovementioned photoproducts (see Fig. 5c), which support the belated generation of X4 and X6 as secondary photoproducts.

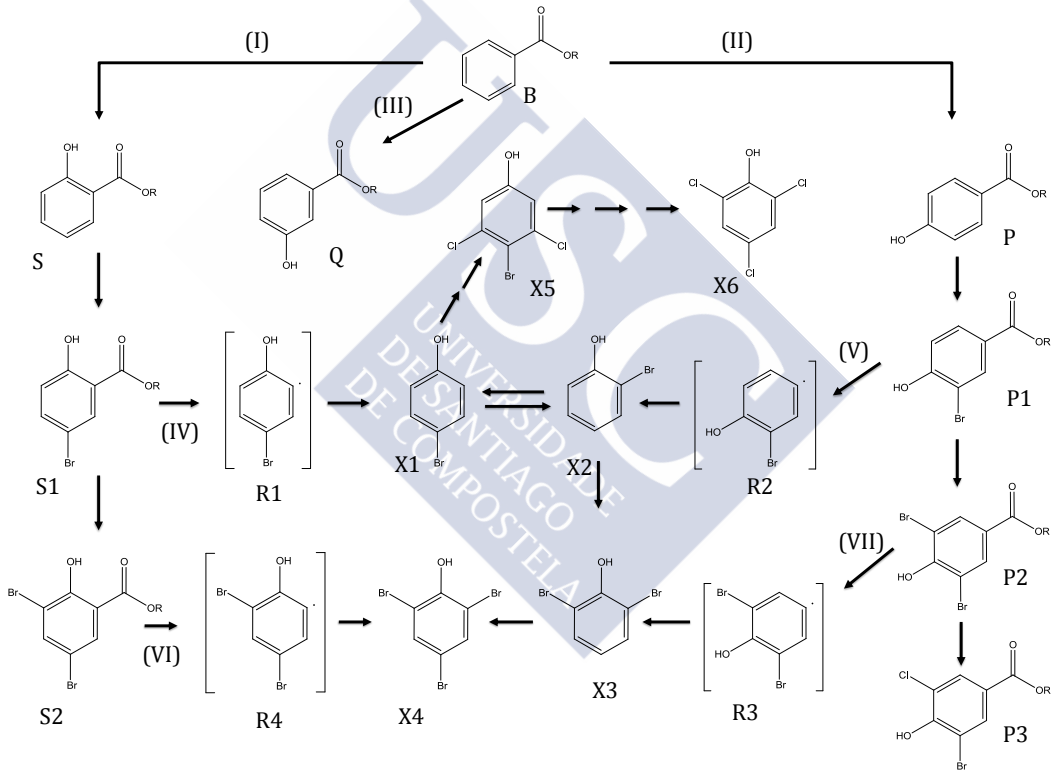

Fig. 7. Proposed tentative photohalogenation pathways of hydroxybenzoates and their phenolic photo-degradation by-products.

4. Conclusions

SPME-GC-MS has been successfully applied to the study of the photochemical behavior of parabens and benzoates in chlorine-containing water. The capacity of UV light to induce the formation of halogenated by-products has been demonstrated. Even at very low levels of chlorine, if trace amounts of bromide are present, hydroxylated benzoates can react with these to yield halogenated by-products (mainly brominated species), and breakdown photoproducts such as halogenated phenols. The proposed photohalogenation-photodegradation pathway evidences the transformation of benzoates into halogenated parabens.

In this work, the efforts were focused on detection and identification of photo-induced halogenation by-products using low-pressure mercury lamps (254 nm). Nevertheless, further photodegradation studies should be carried out in order to: (i) evaluate photohalogenation of target preservatives using a solar simulator or under natural sunlight irradiation, (ii) monitor the fate of target brominated species under environmetal conditions, and (iii) quantify extent to which these are formed after incomplete UV-light wastewater treatment.

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Capítulo III.3.2

Identification of unwanted photoproducts of cosmetic preservatives in personal care products under uv-light using solid-phase microextraction and micro-matrix solid-phase dispersion

Gerardo Álvarez-Rivera, María Llompart, Carmen García-Jares, Marta Lores

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Identification of unwanted photoproducts of cosmetic preservatives in personal care products under UV-light using solid-phase microextraction and micro-matrix solid-phase dispersion.

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Abstract

The photochemical transformation of widely used cosmetic preservatives including benzoates, parabens, BHA, BHT and triclosan has been investigated in this work applying an innovative double-approach strategy: identification of transformation products in aqueous photodegradation experiments (UV-light, 254 nm), followed by targeted screening analysis of such photoproducts in UV-irradiated cosmetic samples. Solid-phase microextraction (SPME) was applied, using different fiber coatings, in order to widen the range of detectable photoproducts in water, whereas UV-irradiated personal care products (PCPs) containing the target preservatives were extracted by micro-matrix solid-phase dispersion (micro-MSPD). Both SPME and micro-MSPD-based methodologies were successfully optimized and validated. Degradation kinetics of parent species, and photoformation of their transformation by-products were monitored by gas chromatography coupled to mass spectrometry (GC–MS). Thirty nine photoproducts were detected in aqueous photodegradation experiments, being tentatively identified based on their mass spectra. Transformation pathways between structurally related by-products, consistent with their kinetic behavior were postulated. The photoformation of unexpected photoproducts such as 2- and 4-hydroxybenzophenones, and 2,8-dichlorodibenzo-p-dioxin in PCPs are reported in this work for the first time.

Keywords: Cosmetics; Preservatives; Personal care products; Solid-phase microextraction; GC–MS; Photodegradation; Micro-matrix solid-phase dispersion; 2,8-Dichlorodibenzo-p-dioxina.

1. Introduction

The use of preservatives in cosmetic and personal care products (PCPs) is an essential requirement to maintain the integrity of the product. Among the most frequently found preservatives in cosmetic formulations, the esters of benzoic acid (benzoates) and p-hydroxybenzoic acid (parabens), and 2,4,4'-trichloro-2'-hydroxydiphenyl ether (triclosan, TCS) are added for the primary purpose of inhibiting the development of microorganisms (antimicrobial function). Other preservatives such as the antioxidants 2-tert-butyl-4-methoxyphenol (BHA) and 2,6-bis(1,1-dimethylethyl)-4-methylphenol (BHT) are commonly employed to protect cosmetics against damage and degradation caused by the exposure to oxygen (antioxidant function). The widespread use of these ingredients has become a matter of concern due to their potential side effects on consumer's health. In fact, some of these preservatives have been reported to have estrogenic/antiandrogenic-like properties [1]. In addition, a potential relationship between breast cancer and prolonged dermal exposure to paraben-containing products is suggested [2]. BHA and TCS may also modulate and disrupt the endocrine system [3, 4].

Upon direct exposure to UV-light, the photodegradation of the aforementioned preservatives may occur. Some photochemical studies under different oxidative conditions in water have been reported for some of the target ingredients. Thus, the degradation of butylparaben using the combined action of UV-irradiation and H_2O_2 [5] was shown to be more effective than photolysis [6]. Likewise, photooxidation with additives such as H_2O_2 or TiO_2 was found to enhance methylparaben degradation [7]. Additionally, the degradation of benzylparaben [8] and benzoic acid [9] by heterogeneous TiO_2-mediated photocatalysis was also investigated and several photoproducts, such as hydroxylated benzoates and phenols were identified. UV-light induced degradation of antioxidants BHA and BHT has also been investigated applying aqueous photolysis [10] and the *on-fiber* photodegradation [11] approach. In both cases, tentatively identified transformation by-products were proposed. Regarding TCS, several authors have studied the photochemical behavior of this preservative, confirming its photochemical degradation and the photoinduced ring closure of triclosan to the photoformation of dichlorodibenzo-p-dioxin in pure water and waste water samples [12, 13].

In the case of cosmetic products, particularly those intended to be in prolonged contact with the skin, exposure to artificial UV-light or solar radiation not only may cause the inactivation of the preservatives, but also may produce potentially hazardous photoproducts. Besides, the complexity of the cosmetic matrix (mainly oil-in-water emulsions) may affect

the photostability of the preservatives and their phototransformation, due to the interaction of target compounds with other ingredients. Hence, the study of the photochemical behavior of these ingredients in cosmetics is an issue of special relevancy, considering the lack of literature on this matter. However, due to the complexity of the cosmetic extracts, chromatographic analysis pose a great challenge to the identification of minor transformation by-products, as coelution of matrix components can led to misinterpretation of mass spectra. Accordingly, a first approach based on previous photodegradation and by-products identification in pure water, can be a valuable reference for the subsequent study of the photochemical transformations of preservatives in complex cosmetics matrices.

The application of solid-phase microextraction (SPME) to water samples is an attractive alternative to other extraction techniques frequently used for this purpose like solid-phase extraction (SPE), avoiding equilibration, loading, washing and elution steps. SPME integrates sampling, extraction, concentration and sample introduction into a single uninterrupted process, reducing the laboratory generated waste and time for sample preparation [14]. This technique has been applied for the determination of target preservatives both in water [15] and in cosmetic samples [16], as well as in the aqueous photodegradation study of triclosan [12, 17]. On the other hand, matrix solid-phase dispersion (MSPD) has been reported as a valuable choice for the extraction of complex solid and semi-solid or viscous samples. The possibility of performing extraction and clean-up at the same time is one of the main advantages of this technique [18], which can even reduce the amount of solvent and sample portion required using the micro-MSPD approach [19]. Recently, MSPD and micro-MSPD has been applied to cosmetic products analyzing preservatives, fragrance allergens [19-21], and isothiazolinone-type biocides [22].

In this work, SPME and micro-MSPD coupled to gas chromatography-mass spectrometry (GC–MS) are proposed to elucidate the possible phototransformations of cosmetic preservatives. Validation and application of this methodology to the identification of transformation by-products were the major aims of this study. A double approach strategy, from the simple (aqueous photodegradation) to the complex (photodegradation in cosmetic matrix), was applied in order to improve by-products detectability through targeted screening analysis of irradiated cosmetic samples. The use of micro-MSPD-GC-MS is applied herein, for the first time, for the extraction and identification of transformation photoproducts in cosmetic matrices.

2. Experimental

2.1 Reagents and material

Acetone, n-hexane and ethyl acetate for trace analysis were supplied by Merck (Darmstadt, Germany), and sodium chloride (99.7%) was obtained by Prolabo (VWR, Fontenay-sous-Bois, France). Methyl benzoate (MeBz; 99%), ethyl benzoate (EtBz; 99%), butyl benzoate (BuBz) and phenyl benzoate (PhBz; 99%) were supplied by Chem Service (West Chester, USA). Methylparaben (MeP; 99%), ethylparaben (EtP; 99%), propylparaben (PrP; 99%), butylparaben (BuP; 99%), benzylparaben (BzP; 99%), butylated hydroxyanisole (BHA; ≥98.5%), butylated hydroxytoluene (BHT; 99%), and triclosan (TCS; ≥97.0%) were purchased from Aldrich (Milwaukee, WI, USA). Individual stock solutions of each compound were prepared in acetone. Further dilutions and mixtures in the same solvent were prepared by convenient dilution of the stock solution to spike water or cosmetic samples. Stock and working solutions were stored at −20 °C protected from light.

Commercially available fibers housed in manual SPME holders were obtained from Supelco (Bellefonte, PA, USA): 100 μm polydimethylsiloxane (PDMS), 65 μm polydimethyl-siloxane-divinylbenzene (PDMS/DVB), 75 μm carboxen-polydimethylsiloxane (CAR/PDMS). The fibers were conditioned as recommended by the manufacturer.

Micro-MSPD extraction was performed in commercially available 2.5 mL plastic cartridges. Glass wool for laboratory, from Sigma-Aldrich, was used to compact clean-up phase and disperse the sample in the MSPD column. Florisil (60–100 mesh) was used as sorbent and purchased from Supelco Analytical (Bellefonte, PA, USA). It was activated at 130 °C for 12 h and then allowed to cool down. Sodium sulphate anhydrous (99%) was purchased from Panreac (Barcelona, Spain).

2.2 Aqueous photodegradation and SPME procedure

Photodegradation experiments were conducted in a laboratory-made photoreactor equipped with two low-pressure mercury lamps (8 and 10 W), exhibiting equivalent spectrum of radiation (200–280 nm), mainly emitting at 254 nm, and differing in the nominal power.

Two quartz cuvettes containing 6 mL of ultrapure water, spiked with target preservatives at 100 ng mL^{-1}, were placed inside the photoreactor box at a distance of 9 cm from the lamps, and irradiated with UV light (254 nm) at 8 or 18 W-UV (depending on the experiment) for the designated time (0–20 min). Irradiated solutions were transferred to

10 ml vials with addition of sodium chloride at 20% (w/v). Vials were sealed with aluminum caps furnished with PTFE-faced septa and immersed in a water bath maintained at the working temperature 25 °C. Samples were let to equilibrate for 5 min, and the fiber was then immersed into the sample for 20 min. Three different fiber coatings (PDMS, PDMS/DVB, and CAR/PDMS) were compared to evaluate the capability of SPME to extract the parent compounds and the phototransformation products. After extraction, the fibers were thermally desorbed at 270 °C in the GC injector port for 5 min, for GC–MS analysis.

Dark tests were performed by placing the cuvettes in the photoreactor box, maintaining the lamps switched off for a certain time (0–20 min), and covering the whole device with aluminum foil. The SPME procedure was then applied.

2.3 Photodegradation in PCPs and micro-MSPD procedure

A moisturizing cream (MC) and a hair conditioner (HC), purchased from local markets, were used to perform photodegradation experiments in cosmetic matrices. Both PCPs were fortified with the target preservatives at a concentration level frequently found in real cosmetic samples (100 μg g^{-1}). Thus, 1 g of sample was mixed with 1 mL of a standard solution containing the target preservatives at 100 μg mL^{-1} in acetone. Portions of the resulting liquid mixture (ca. 100 mg) were transferred onto the bottom of a beaker (surface area, 16 cm^2) and uniformly spread by circular movements. After acetone evaporation, a cosmetic layer of ca. 3.5 mg/cm^2 was obtained. This experimental procedure was a modified and adapted version of the Sun Protection Factor (SPF) Test Method from COLIPA [23]. Samples were exposed to the 10 W-UV lamp in the photoreactor for a certain time (0–60 min).

After UV-irradiation, the samples were blended with 0.4 g of a drying agent (anhydrous Na_2SO_4) and 0.4 g of a dispersing agent (Florisil). The mixture was transferred into a glass mortar and gently homogenized using a glass pestle (ca. 5 min). Then, the mixture was transferred into a syringe cartridge (2.5 mL), with a small amount of glass wool at the bottom, containing 0.2 g of Florisil (to obtain a further degree of fractionation and sample clean-up). Another small amount of glass wool was placed on top of the sample before compression. Elution was made by gravity flow with hexane/acetone (1:1), collecting 1 mL of extract in a volumetric flask. The micro-MSPD extracts, conveniently diluted when necessary, were directly analyzed by GC–MS.

2.4 GC–MS analysis

A Thermo Scientific Trace 1310-GC gas chromatograph coupled to a triple quadrupole mass spectrometer (TSQ 8000; Thermo Scientific; San Jose, CA, USA) was used. The system was operated by Xcalibur 2.2 and Trace Finder TM 3.0.

Separation was carried out on a TG-5 SILMS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) obtained from Thermo Scientific (San Jose, CA, USA). Helium (purity 99.999%) was used as carrier gas, at a constant column flow of 1.0 mL min^{-1}. The GC oven temperature was programmed from 80 °C (held 2 min) to 230 °C at 8 °Cmin^{-1}, and to 270 °C at 15 °C min^{-1} (held 5 min). Splitless mode (held 2 min) was used for injection. The injector temperature was 270 °C.

The mass spectrometry detector (MSD) was operated in the electron impact (EI) ionization positive mode (+70 eV). MSD operated in full scan acquisition mode, in a mass range of m/z 40–450. The transfer line and the ion source temperatures were set at 290 and 350 °C, respectively.

Identification was accomplished using the NIST Mass Spectral Database (NIST MS Search 2.0) and by comparing retention times with those of commercial standards, when available. Quantification was performed using Trace Finder software.

3. Results and discussion

3.1 SPME and micro-MSPD-GC–MS performance

As some transformation products can be generated at low concentration levels, SPME was selected in aqueous photodegradation experiments as extraction and concentration technique at the same time. Different types of commercial fibers were employed. Suitable coatings for polar compounds such as PDMS/DVB and CAR/PDMS were tested, together with the non-polar PDMS, in order to increase the possibility of extracting a wider range of photoproducts with different structures and polarities. Extraction conditions (temperature and sampling mode) were optimized, and the highest extraction efficiencies for the target preservatives, considered as parent species, were achieved using direct sampling mode at room temperature (25 °C), with addition of NaCl (20%, w/v) in line with previous reported works for some of targets in water [15, 24].

Table 1. Retention times, quantification and identification m/z ratios, and validation parameters of the proposed SPME and micro-MSPD methods.

Compound	Retention time (min)	Qualifier and quantifier ions (m/z)[a]	Aqueous Photodegradation (SPME)		In-cosmetic photodegradation (micro-MSPD)		
			Precision (RSD, %)[b]	LOD[c] (ng ml-1)	Precision (RSD, %)[b]	Recovery (%)	LOD[d] (% w/w) ×10⁴
MeBz	5.85	136 (39), *105*, 77 (62)	3.3	0.21	8.0	53.8	0.043
EtBz	7.14	150 (22), 122 (36), *105*	4.3	0.17	7.3	69.3	0.044
BuBz	10.62	123 (65), *105*, 77 (45)	12	0.033	6.6	92.7	0.036
MeP	11.84	152 (38), *121*, 93 (24)	10	0.22	6.8	104	0.11
BHA	12.38	180 (45), *165*, 137 (73)	7.9	0.031	3.2	91.6	0.067
BHT	12.68	220 (23), *205*, 177 (18)	12	0.11	0.4	102	0.031
EtP	12.92	*121*, 138 (20), 166 (23)	5.8	0.55	0.7	106	0.093
PhBz	15.00	198 (5), *105*, 77 (30)	12	0.016	1.7	111	0.075
BuP	15.88	*121*, 138 (84), 194 (68)	3.0	0.43	3.7	102	0.11
TCS	20.69	*288*, 218 (86), 146 (62)	7.1	0.28	4.1	111	0.12
BzP	21.04	228 (18), 121, 91 (53)	11	0.17	5.2	102	0.17

[a]Quantificaion ions are set in italics. The relative ion abundances (%) of the qualifier ions with respect to the quantifier ion are included in bracket.
[b] n=3
[c] Estimated for the most sensitive fiber coating (PDMS/DVB)
[d] Equivalent to μg g^{-1}.

Irradiated cosmetic samples were extracted using an optimized micro-MSPD version described in Section2.3. Florisil (0.4 g) was used as dispersing sorbent, as it was demonstrated to be suitable for the isolation of preservative compounds from cosmetic matrices [21]. Although similar recovery values were obtained for the parent species using 1 mL of ethyl acetate compared to hexane/acetone (1:1), slightly higher extraction yields were achieved employing the latter solvent, allowing the quantitative recovery of the phenolic species. Thus, satisfactory recoveries above 92%, except for the most volatile benzaotes MeBz and EtBz (mean values around 50% and 70%, respectively), were obtained for the analyzed samples using the proposed micro-MSPD methodology.

After extraction, both SPME fibers and micro-MSPD extracts were readily analyzed by GC–MS without any derivatization step for not to introduce additional complexity in the identification process of photoproducts. Method precision, expressed in terms of relative standard deviation (%RSD), was lower than 12% and 8% for SPME and micro-MSPD, respectively. Aqueous photodegradation experiments showed limits of detection (LODs) below the ng L^{-1} level, whereas LOD values between 0.031and 0.17 μg g^{-1} were obtained for the parent preservatives in the studied cosmetic samples. It is important to notice that

micro-MSPD limits have been reduced five times compared to the conventional MSPD procedure, which is crucial for photoproducts identification purposes, considering the small sample portion (50 mg) used in these experiments. Compared to other reported methods in literature these limits were shown to be slightly higher, considering that no derivatization step was introduced in this work. Anyhow, these limits are sufficiently low to permit the photochemical studies of the selected compounds and tentatively identify their transformation products. Retention times, quantification m/z ratios and validation parameters of the proposed methods are summarized in Table 1.

3.2 Aqueous photodegradation of cosmetic preservatives

3.2.1 Influence of irradiation power and time

Two aqueous photodegradation experiments were carried out at two different irradiation powers; 8 W-UV and 18 W-UV, in order to test the feasibility of the photochemical studies in a reasonable range of time. As can be seen in Fig. 1, after 10 min of UV irradiation, a degradation up to 50% was observed for parabens at 8 W-UV, whereas 18 W-UV are required to achieve a significant signal reduction around 80% for alkyl benzoates. On the other hand, BHA, BHT, TCS, BzP and PhBz showed degradation levels close to 100% at both irradiation powers, which evidences their higher photolability. For practical reasons, subsequent photodegradation experiments of these latter compounds were carried out at 8 W-UV, whereas alkyl benzoates and parabens were irradiated with the 18 W-UV system. Dark test was also accomplished as described in Section 2.2, to check the photolytic origin of the observed degradation. As can be clearly seen in Fig. 1 (fourth bar) no analyte losses are observed during 20 min of exposure to darkness. These results demonstrate that the

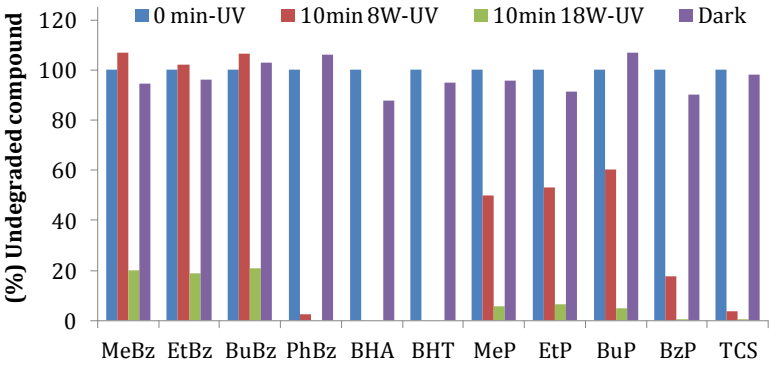

Fig. 1. Influence of irradiation power (8 and 18 W-UV) on target preservatives photodegradation in water after 10 min-UV, compared to the control (no irradiated) and dark test. Responses are normalized to the control experiment.

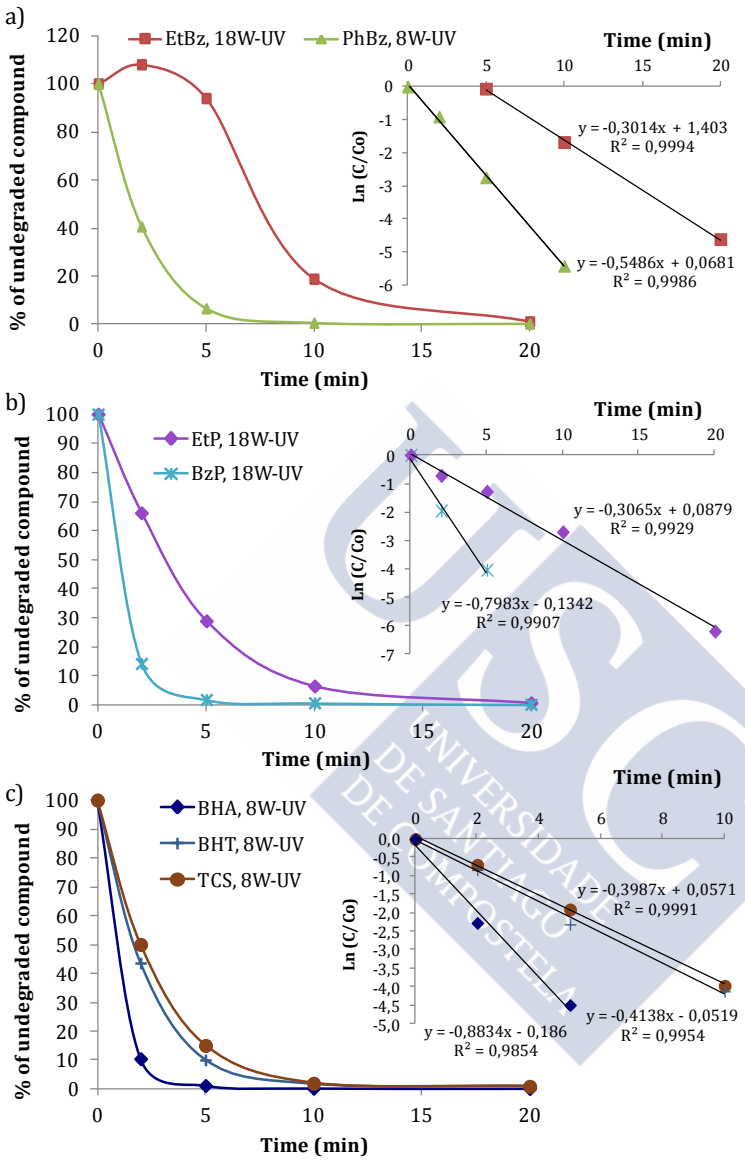

Fig. 2. Influence of irradiation time on aqueous photodegradation for representative target preservatives. Linear fit of logarithmic values vs. irradiation time, demonstrating pseudo-first order kinetic behavior. C_0 and C represent the percentage of undegraded compound at times zero and t, respectively. (a) EtBz and PhBz at 18 W-UV; (b) EtP and BzP at 18 W-UV; (c) BHA, BHT and TCS at 8 W-UV.

decrease of signal observed during the irradiation experiments of target preservatives is only due to the effect of UV-light, and no other processes such as volatilization occur.

The kinetic behavior of the target preservatives under UV-light (254 nm) was evaluated, following the experimental procedure described in Section 2.2. The photodegradation kinetics were monitored by studying the influence of irradiation time (0–20 min) on the analytical response and the results were compared. Fig. 2 illustrates the kinetic curves

obtained for representative target compounds photodegraded in water. After 20 min of UV-light exposure, most of compounds were nearly completely degraded (<1%).

Despite being structurally related compounds, alkyl parabens (p-hydroxybenzoates) showed faster degradation than alkyl benzoates, with less than 30% of compound remaining in water at 5 min of 18 W-UV (Fig. 2a). In contrast, at the same time and irradiation power negligible reduction of signal was observed for alkyl benzoates in water (Fig. 2b). In the case of BHA, BHT and TCS, the photodecay curves show almost complete disappearance after short irradiation times (5 min) at 8 W-UV. Therefore their degradation study was performed at 8 W, originating kinetic curves with a slower drop of the signal as illustrated in Fig. 2c.

Table 2. Calculated kinetic parameters for the target preservatives in water.

Compound	Linear range (min)	R^2	K_{ap} (min^{-1})	$t_{1/2}$ (min)
		18W-UV		
MeBz	5-20	0.999	0.31	7.27
EtBz	5-20	0.999	0.30	7.30
BuBz	5-20	0.999	0.30	7.32
MeP	0-20	0.996	0.31	2.22
EtP	0-20	0.993	0.26	2.70
PrP	0-20	0.997	0.30	2.34
BuP	0-20	0.993	0.31	2.26
		8W-UV		
BHA	0-5	0.985	0.88	0.78
BHT	0-10	0.995	0.52	1.34
PhBz	0-10	0.999	0.55	1.25
BzP	0-20	0.993	0.18	3.87
TCS	0-10	0.999	0.40	1.73

Experimental data were adjusted to a pseudo-first-order kinetic model by plotting the natural logarithm of the percentage of undegraded compound against irradiation time. Time ranges were tested until the point from which the kinetic curves start to tend asymptotically towards zero. Satisfactory linear correlation was obtained between both variables, with correlation coefficients above 0.985. The kinetic parameters for the studied preservatives are summarized in Table 2.

3.2.2 Identification of photoproducts

Upon UV-irradiation of target preservatives, a large number of transformation products were detected in aqueous photodegradation experiments. Several of them were tentatively identified on the basis of their mass spectra, reported data in literature, and using the NIST Mass Spectral Database. Retention times, identification and quantification ions, the identification approach of these photoproducts, as well as the reverse match (R. Match) values given by NIST library are listed in Table 3 and Table 4. Additionally, the positively identified transformation products using different fibers coatings are indicated with a "X" sign and a number.

Table 3. Retention times, quantification and identification ions, and reverse match of tentatively identified photoproducts of benzoates and parabens in aqueous photodegradation experiments using different SPME fiber coatings.

Key	Photoproduct	Retention time (min)	SPME fiber coating[c]			Qualifier and quantifier ions(m/z)[a]	Reverse match	Identification based on[b]
			PDMS	PDMS/DVB	CAR/PDMS			
BP1	Benzaldehyde	3.90	X	2X	3X	*105*, 106, 77	952	A
BP2	Benzyl alcohol	4.88	X	ND	8X	*108*, 107, 78, 77	876	A
MeBz	Methylbenzoate	5.85	X	3X	7X	136, *105*, 77	914	A
EtBz	Ethylbenzoate	7.15	X	X	4X	150, 122, *105*	947	A
BP3-a	Methyl 2-hydroxybenzoate	7.57	X	5X	11X	152, *120*,92	931	A
BP3-b	Ethyl 2-hydroxybenzoate	8.87	X	X	3X	166, *120*, 92	920	A
BuBz	Butylbenzoate	10.65	X	X	3X	178, 123, *105*, 77	923	A
BP4-a	Methyl 3-hydroxybenzoate	11.38	X	20X	10X	152, *121*, 93	_	D
MeP	Methylparaben	11.84	X	21X	12X	152, *121*, 93	920	A
BP5-a	Methyl 2-hydroxy-4-methoxybenzoate	12.10	X	5X	7X	*150*, 122, 107	_	B
BP3-c	Butyl 2-hydroxybenzoate	12.21	3X	X	4X	*120*, *138*, 194, 92	944	A
BP4-b	Ethyl 3-hydroxybenzoate	12.48	X	X	4X	166, 138, *121*, 93	_	D
EtP	Ethylparaben	12.90	X	X	4X	166, 138, *121*, 93	934	A
BP6-a	Methyl 4-methoxybenzoate	12.84	X	5X	2X	135, *152*, 107,	_	C
BP5-b	Ethyl 2-hydroxy-4-methoxybenzoate	13.20	X	X	5X	*150*, 122, 107	_	B
BP6-b	Ethyl 4-methoxybenzoate	13.92	X	X	3X	135, *152*, 107,	_	C
BP5-c	Butyl 2-hydroxy-4-methoxybenzoate	15.53	2X	X	4X	224, *150*, 122, 107	_	B
BP4-c	Butyl 3-hydroxybenzoate	15.42	X	X	3X	194, 138, *121*, 93	_	D
BuP	Butylparaben	15.89	X	X	3X	194, 138, *121*, 93	947	A
BP7	2-Hydroxybenzophenone	15.98	X	X	2X	*197*, 121, 105,77	927	A
BP8	4-Hydroxybenzylphenol	16.24	X	5X	5X	*184*, 165, 107, 91	858	A
BP6-c	Butyl 4-methoxybenzoate	16.88	X	X	2X	135, *152*, 107, 208	808	C
BP9	4-hydroxybenzophenone	19.58	X	30X	8X	*121*, 198, 105, 77	932	A

[a]Quantification ions are set in italics.

[b] (A) Theoretical mass spectrum, (B) theoretical mass spectrum of methyl 2-hydroxy-4-methoxylbenzoate, (C) theoretical mass spectrum of 4-methoxylbenzoic acid, (D) theoretical mass spectrum of 3-hydroxybenzoic acid

[c] The "X"sign represents the positive detection of the photoproduct. The number represents the relative analytical response obtained with respect to the other fibers for the same photoproduct. ND: not detected

Table 4. Retention times, quantification and identification ions, and reverse match of tentatively identified photoproducts of BHA, BHT and TCS in aqueous photodegradation experiments using different SPME fiber coatings.

Key	Photoproduct	Retention time (min)	SPME fiber coating[c]			Qualifier and quantifier ions (m/z)[a]	Reverse match	Identification based on[b]
			PDMS	PDMS/DVB	CAR/PDMS			
BH1	tert-Butyl-1,4-benzoquinone	7, 67	X	ND	4X	164, *149*, 136, 121	798	A
BH2	2,6-Ditert-butyl-4-methoxy-4-methyl-2,5-cyclohexadien-1-one	11.03	2X	ND	X	250, 235, 194, *179*	916	A
BH3	3,5-di-tert-Butyl-4-hydroxyanisole/ 3,5-di-tert-Butyl-4-hydroxybenzyl alcohol	11.50	X	6X	5X	236, *221*, 193	656/550	A
BH4	3-tert-Butyl-2,4,5-trihydroxyanisole	11.65	X	2X	2X	197, *165*, 137, 109	_	B
BH5	BHA isomer 1	11.97	3X	X	2X	221, 180, *165*, 137	_	B
BH6	2,6-di-tert-Butyl-1,4-benzoquinone	12.05	10X	X	2X	220, *177*, 149, 135	796	A
BH7	5-tert-Butyl-1,2,3-benzenetriol	12.28	ND	X	X	182, *167*, 139, 121	708	A
BH8	BHA isomer 2	13,09	X	X	X	180, *165*, 137	_	B
BH9	BHA isomer 3	13,27	X	X	3X	180, *165*, 137	_	B
BH10	tert-Butylhydroquinone	13.33	X	3X	4X	166, *151*, 123	761	A
BH11	4,6-di-t-butyl-m-cresol (or isomers)	13,54	X	ND	ND	220, *205*, 177, 129	_	C
BH12	1,2,4-Trimethoxybenzene	13.93	2X	X	2X	*168*, 153, 125	730	A
BH13	5-tert-Butyl-3,4-dihydroxyanisole	15,20	X	2X	3X	196, *181*, 153	_	B
BH14	3,5-di-t-butyl-4-hydroxybenzaldehyde	16.36	4X	X	2X	234, *219*, 191, 175	828	A
T1	Dichlorobenzene	4.47	X	ND	X	146, *149*, 111, 75	848	A
T2	2,8-Dichlorodibenzo-p-dioxin	19.24	X	ND	ND	*252*, 189, 126	960	A

[a]Quantification ions are set in italics.
[b] (A) Theoretical mass spectrum, (B) experimental ion justification, (C) theoretical mass spectrum of BHT.
[c] The sign X represent the positive detection of the photoproduct. The number represents the relative analytical response obtained with respect to the other fibers for the same photoproduct. ND: not detected

Individual studies have revealed the origin of each particular photoproduct. Since some of target preservatives are structurally related compounds, i.e. benzoates-parabens, and BHA-BHT, they share some common transformation products and photodegradation pathways. Therefore, those photoproducts arising from benzoates and/or parabens are labeled with letters BP (Table 3). Photoproducts obtained from BHA and/or BHT photodegradation are designated with letters BH, whereas triclosan by-products are labeled with letter T (Table 4). It is noteworthy that, although BP photoproducts were, to a greater or a lesser extent, identified using any of the considered fiber coatings, several BH and T transformation products were selectively extracted by only one or two fibers.

3.2.2.1. Parabens and benzoates photoproducts. Hydroxylation in the benzenic ring of benzoates has been previously reported by the authors [24] as an important phototransformation pathway of alkyl benzoates in water. In this line, three different hydroxylation by-products, with similar fragmentation pattern of their mass spectra, were obtained for each alkyl ester. These photoproducts were identified as ortho-, meta- and para-hydroxybenzoates, on the basis of their positive match on the NIST library or by comparing with the reference mass spectrum of its corresponding hydroxybenzoic acid moiety. Ortho-hydroxylated benzoates (BP3), also known as salicylates, are characterized by the ions m/z138 $[M\text{-}C_{n+1}H_2CH_3]^+$, m/z 120 $[m/z\ 138\text{-}H_2O]^+$ and m/z 92 $[m/z\ 120\text{-}CO]^+$, whereas meta- and para-derivatives (BP4 and parabens, respectively), show m/z 138, m/z121 $[m/z\ 138\text{-}OH]^+$, and m/z 93 $[m/z\ 120\text{-}CO]^+$ as the most representative peaks in the mass spectra. This slightly different fragmentation pattern can be explained considering that during fragmentation, ortho-substituted benzoates lose a molecule of water instead of a hydroxyl group, as the phenolic hydroxyl is located on a carbon atom adjacent to a carbon atom with an acid moiety [25].

Individual experiments of parabens revealed that these compounds can lose the hydroxyl group through photolysis, yielding benzoates, which in turns may produce new hydroxylated by-products. These findings evidence that under UV-irradiation, parabens and benzoates are interconvertible structures, and the photodegradation by-products from both parent species can be explained within the same phototransformation pathway, as illustrated in Fig. 3.

As a result of the photodegradation process, benzaldehyde (BP1) was shown to be the major breakdown photoproduct generated during photolysis of parabens and benzoates. BP1 was clearly identified by comparing its mass spectrum with the NIST spectral database. This photoproduct might be generated after photolytic ester cleavage of benzoates or

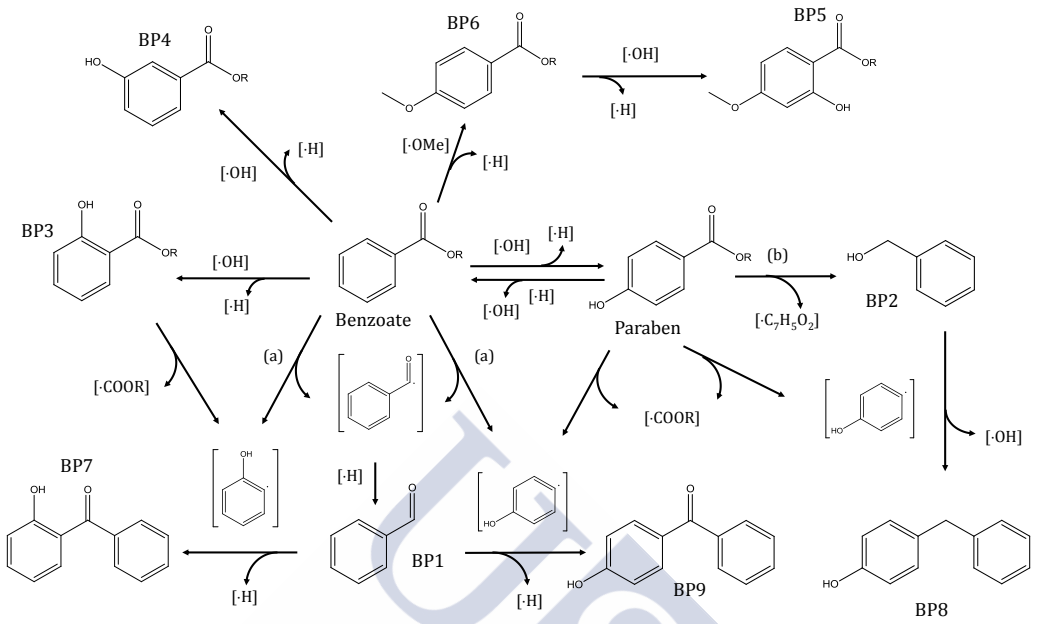

Fig. 3. Postulated phototransformation pathway for parabens and benzoates. (a) R = Phenyl; (b) R = Benzyl.

dehydroxylated parabens. Although the formation of phenol from hydroxybenzoates might be quite obvious, free phenol was not found under the studied experimental conditions. This fact might be explained considering the high reactivity of phenolic radicals, which play an essential role in the phototransformation pathway of parabens and benzoates.

Phenolic radicals may react with benzaldehyde to form the photoproducts BP7 and BP9, which were identified as hydroxybenzophenones on the basis of their theoretical mass spectra found in the NIST library. Both structures exhibit similar fragmentation patterns consistent with *m/z* 198 $[M]^+$, *m/z* 121 $[M\text{-}C_6H_5]^+$, *m/z* 105 $[M\text{-}C_6H_6O]^+$ and *m/z* 77 $[C_6H_5]^+$. In the case of BP6, the presence of the ion *m/z* 197 as the base peak in the mass spectrum reveal the easy loss of a proton $[M\text{-}H]^+$, most probably from the hydroxyl group in ortho- to the ketone group. As ortho- and para-derivatives are expected to be the most favorable hydroxylated by-products, BP7 and BP9 were identified as 2-hydroxy and 4-hydroxybenzophenones, respectively.

Photoproduct BP8 could be either 2-hydroxy- or 4-hydroxybenzylphenol according to the NIST spectral database. In both cases, the most intense peaks in the mass spectrum correspond to the ions *m/z* 184, *m/z*165, and *m/z* 107. However, the slightly different

intensity of ion ratios makes the 4-hydroxylated derivative the most likely transformation product. BP8 might arise from the reaction of phenolic radicals with benzyl alcohol (BP2), which was identified as breakdown photoproduct when BzP is present. Although BP2 has been previously reported in literature as BzP photoproduct [8], the formation of the hydroxybenzophenones (BP7 and BP9) and the hydroxybenzylphenol (BP8) from parabens and benzoates is detected herein for the first time. Generation and subsequent photodegradation of both benzophenones can be clearly observed in Fig. 4a and b.

Methoxylated benzoates (BP6) could be identified on the basis of the reference mass spectra of some methoxybenzoic acid esters. The mass spectra of these photoproducts are mainly characterize by the ions m/z 152 $[M\text{-}C_{n+1}H_2CH_3]^+$, m/z 135 $[m/z$ 152-OH$]^+$, m/z 107 $[m/z$ 135-CO$]^+$, being the para-methoxylated derivative the most likely structure, according to the relative intensity of ions. Similarly, fragmentation pattern of photoproducts BP5 is consistent with m/z 150 $[M\text{-}OHC_{n+1}H_2CH_3]^+$, m/z 122$[m/z$ 150-CO$]^+$, and m/z 107$[m/z$ 122-$CH_3]^+$, which reveal the addition of a hydroxyl group to the benzenic ring of their precursors methoxylated benzoates. As para-position is occupied by the methoxyl group, ortho-hydroxylation seems to be the most favorable option, being 2-hydroxy-4-methoxybenzoates the assigned structures. This proposal is plausible from the similarity with the mass spectrum of methyl 4-methoxy-2-hydroxybenzoate. Methoxyl groups can be released by photolytic ester cleavage of parent compounds.

The postulated phototransformation pathway, illustrated in Fig. 3, is consistent with the corresponding kinetic profiles of the abovementioned photoproducts (Fig. 5a and b). As can be deduced form Fig. 5a, benzoates can be generated from parabens photodegradation, and subsequently undergo hydroxylation and methoxylation reactions. Moreover, Fig. 5b supports the formation of the hydroxylated and methoxylated benzoates as primary photoproducts of benzoates, and confirms the belated generation of BP5, which might be attributed to its character of secondary photoproduct.

3.2.2.2. BHA and BHT photoproducts. Despite the structural similarity, the relation between BHA and BHT through photochemical processed is not evident from data reported in literature. Therefore, the transformation of both antioxidants under UV-light are comparatively studied below. The proposed photodegradation pathways are shown in Fig. 6.

Four transformation products were detected in BHA photodegradation experiments (BH1, BH4, BH10 and BH13, see Table 4). The loss of a methyl group from the methoxyl substituent in the aromatic ring of BHA can led to the formation of BH10, which in turn may

undergo subsequent oxidation rendering 2-tert-butyl-1,4-benzoquinone (BH1). They were identified by positive match on the NIST library. The formation of these transformation products have been previously reported in oxidation experiments during water chlorination of BHA [26]. Although the theoretical mass spectra of BH13 and BH4 could not be found in the mass spectral database, their experimental fragmentation pattern showed some similarities with BHA and its isomers. Assuming m/z 196 and m/z 212 as the molecular ions of the hypothetical photoproducts BH13 and BH4, these peaks were 16 and 32 mass units higher than the molecular ion of BHA (m/z 180), suggesting the addition of one and two oxygen atoms to the parent compound. Thus, both species could be tentatively identified, as monohydroxylated and dihydroxylated derivatives of BHA (Figs. S1 and S2 in Supplementary information). These results are in agreement with previous works that report the occurrence of hydroxylated derivatives upon UV-irradiation of BHA [10].

The photodegradation of BHT originated a wider range of structures, eight of which were tentatively identified (Table 4). During the photolytic process, the aromatic system of BHT can be attacked through hydroxylation in para-position to the hydroxyl group, and subsequently undergo o-methylation, yielding 2,6-ditert-butyl-4-methoxy-4-methyl-2,5-cyclohexadien-1-one (BH2). Although the hydroxylated intermediate was not detected, its formation is supported by previous studies about phototransformation of phenolic antioxidants like BHT [27]. The formation of BH2 is depicted in Fig. 4c, which could be readily identified for the first time as BHT photoproduct according to the corresponding theoretical mass spectra in the NIST library.

In turn, BH2 can lose the methyl group in position 4 of the aromatic ring, rendering 3,5-di-t-Butyl-4-hydroxyanisole (BH3), which might suffer o-demethylation to generate2,6-di-t-Butyl-quinone (BH6) via the semiquinone radical as an intermediate. Besides, the photolytic process can led to bond splitting by breaking one of the t-butyl chain of BH3 yielding three different photoproducts (BH5, BH8 and BH9) with very similar mass spectra (Fig. S3). While photoproducts BH3 and BH6 were identified by searching in the NIST library, the structures of BH5, BH8, and BH9 could not be confidently assigned to a single structure. However, the high level of similarity between the fragmentation patterns observed for these latter photoproducts and the mass spectra of BHA, suggest that these by-products might be most likely BHA isomers, showing a fragmentation pattern consistent with m/z 180 $[C_{11}H_{16}O_2]^+$, m/z 165 $[180\text{-}CH_3]^+$, m/z137 $[180\text{-}C(CH_3)_3]^+$. These findings evidence, for the first time, that BHA and BHT photodegradation pathways are clearly linked, as evidenced in Fig. 6.

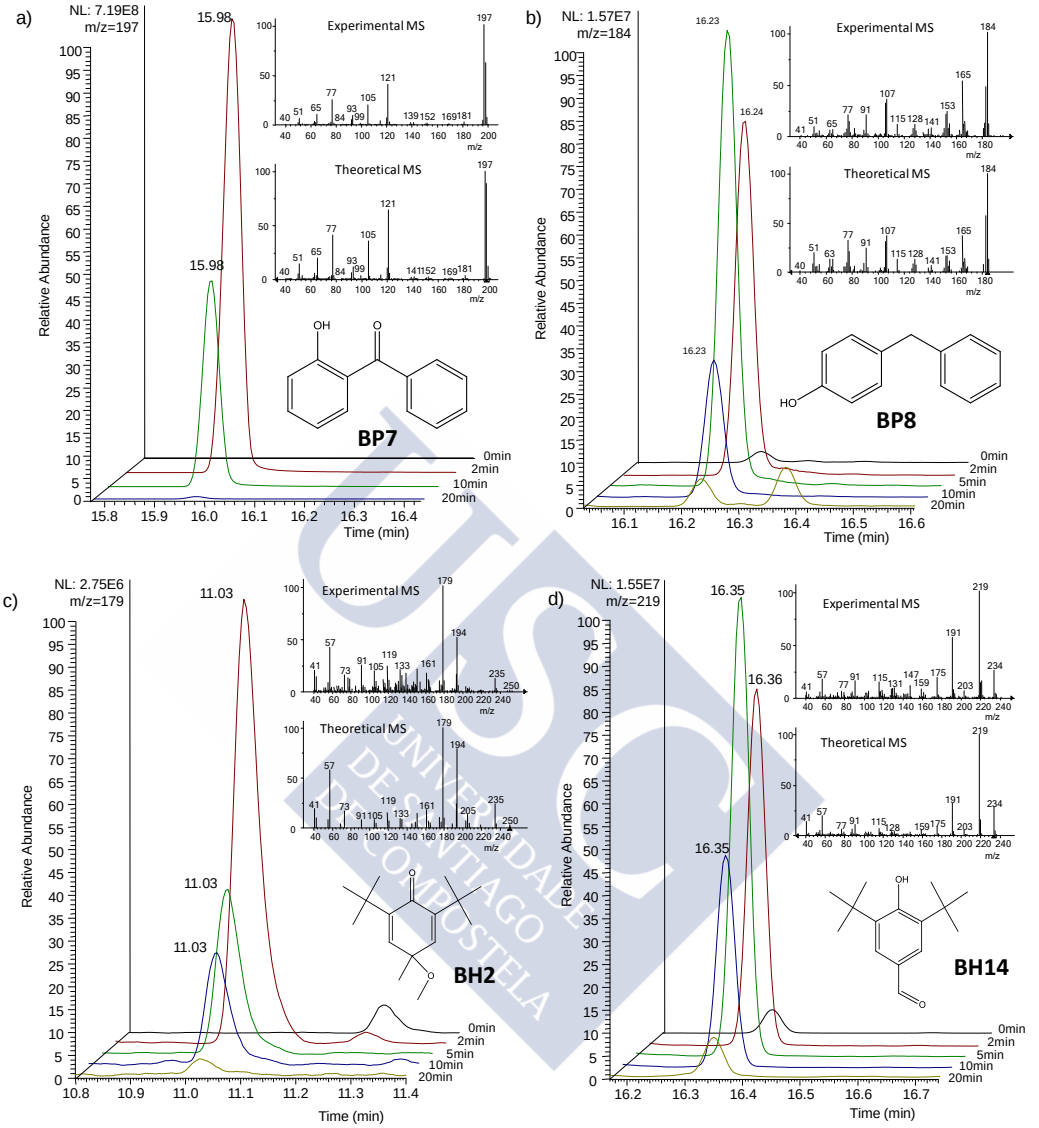

Fig. 4. SPME-GC–MS extracted ion chromatogram and mass spectra showing the formation-degradation of photoproducts: (a) BP7; (b) BP8; (c) BH2; (d) BH14, obtained atdifferent UV-irradiation times (0–20 min) in aqueous photodegradation experiments.

Alternatively, oxidation of methyl group in position 4 of the benzenic ring after H-subtraction can led to the formation of BH14. This compound, previously detected as BHT photoproduct [11], was identified in the mass spectral data base as 3,5-di-t-butyl-4-hydroxybenzaldehyde. Its photoformation is illustrated in Fig. 4d. Additionally, BHT can

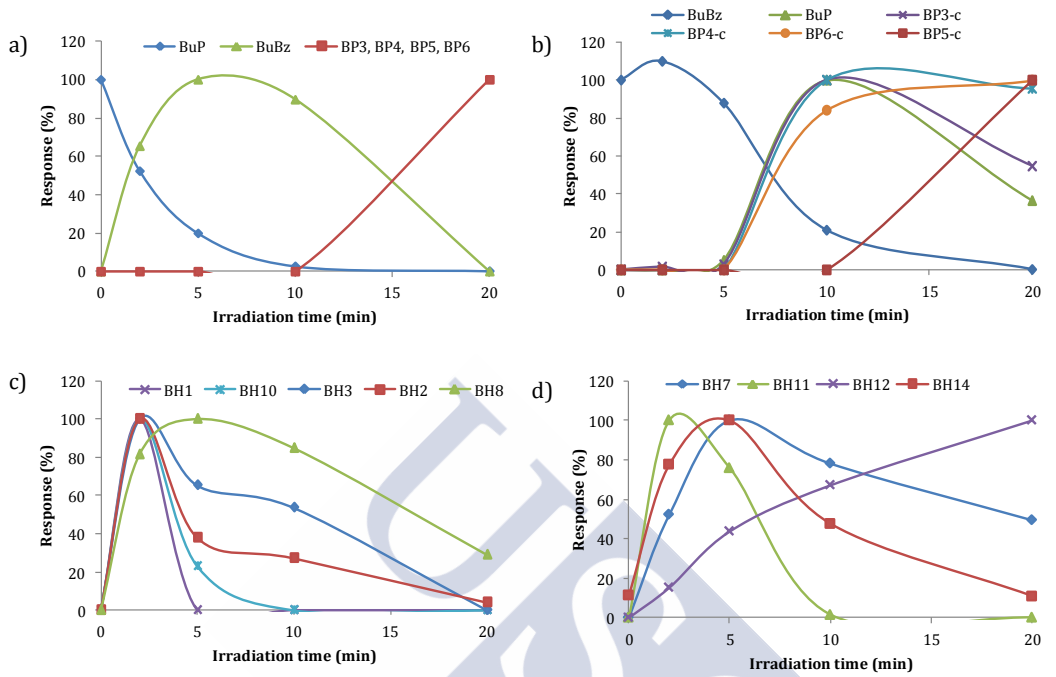

Fig. 5. Kinetic profile for representative parents and transformation products in aqueous photodegradation experiments: (a) BuP, BuBz, and photohydroxylated/methoxylatedby-products at 18 W-UV; (b) BuBz and photohydroxylated/methoxylated by-products at 18 W-UV; (c) and (d) BHA and BHT photoproducts at 8 W-UV.

undergo isomerization, leading to photoproducts such as BH11, which exhibit a fragmentation with a good match with the mass spectrum of the parent compound. The remaining two by-products (BH7 and BH12) might be generated through the loss of a tertbutyl and methyl group of BHT, which could activate the aromatic system and induce further hydroxylation in the benzenic ring. BH7 and BH12 were tentatively proposed as 5-tert-Butyl-1,2,3-benzenetriol and 1,2,4-trimethoxybenzene, respectively, on the basis of the best match with the corresponding theoretical mass spectra found in the NIST library.

In general, BHA and BHT photoproducts are generated very quickly, as can be deduced from their kinetic profiles depicted in Fig. 5b and c. Once they reach their maximum concentration after short UV exposure times (2–5 min), rapid degradation can be clearly observed for BH1, BH10 and BH11, whereas other by-products such as BH12 or BH7, are shown to be more persistent.

3.2.2.3. Triclosan and dioxin formation. Due to the structural similarity with highly toxic and persistent contaminants such as dioxins, triclosan and its phototransformation processes have been extensively studied in literature. The conversion of triclosan into 2,8-DCDD, has been previously reported by Latchet al. [13], and further confirmed by Mezcua et al. [28] and Sánchez-Prado et al. [17] in water and waste water samples. This by-product shows a mass spectrum characterized by the ions *m/z* 152, *m/z* 189, and *m/z* 126, and has been confirmed in this work as the major photoproduct of triclosan. As can be seen in Table 4, the identification of 2,8-DCDD was only possible using PDMS fiber coating, whereas no analytical response was obtained employing the other two fiber. This behavior can be explained considering the high log K_{ow} (5.7) value for this photoproduct [29], which is expected to have high affinity for hydrophobic phases such as polydimethylsiloxane. In agreement with data reported in literature [30], the formation of 2,8-DCDD was demonstrated to occur at the pH conditions proposed in these aqueous photodegradation studies (pH 5.5–7), which mimics quite well the real pH conditions of a pH-balanced personal care product.

Although photolysis of triclosan can led to the formation of chlorinated phenols, they were not detected under the employed irradiation conditions. Instead, diclorobenzene (T1) was tentatively identified as an alternative breakdown photoproduct by positive match on the NIST library. The exact substitution of chlorine atoms could not be confirmed. However 1,2- or 1,4-dichlorobenzene seems to be the most feasible structures.

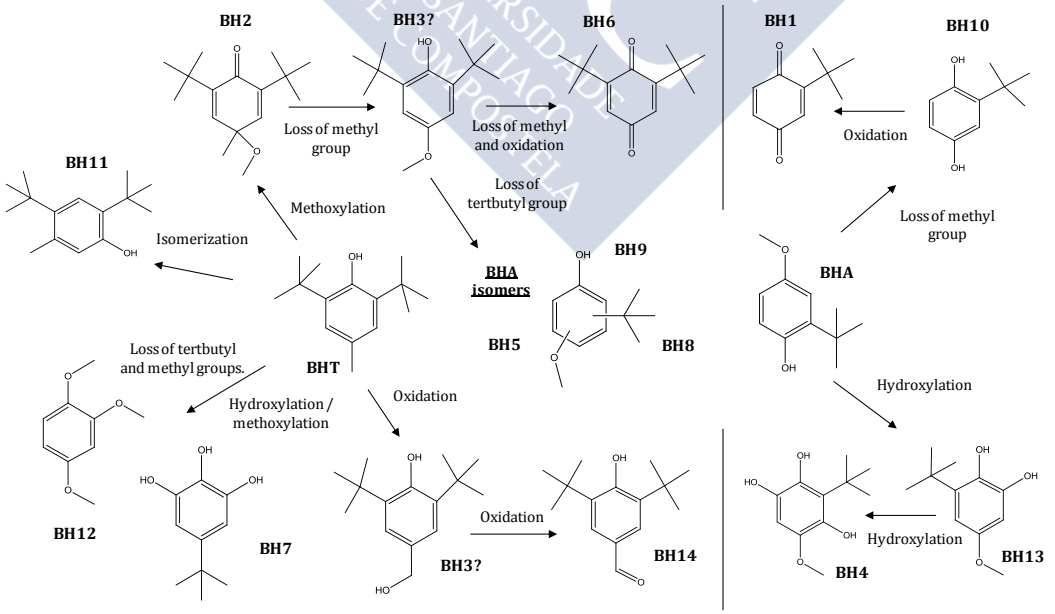

Fig. 6. Proposed phototransformation pathway for the antioxidants BHA and BHT.

3.3 Photochemical behavior of cosmetic preservatives and photoproducts identification in PCPs

From a toxicological point of view, it is relevant to know if the above discussed phototransformations can also occur when commercial cosmetic products are exposed to UV-light. Under these conditions, the interaction with other ingredients or with other components in the cosmetic matrix may affect the photodegradation of the studied preservatives. Therefore, in order to evaluate the photochemical behavior of cosmetic preservatives in a more realistic scenario, two leave-on PCPs were considered in this study: a moisturizing cream (MC) and a hair conditioner (HC). These PCPs were fortified at concentration level frequently found in real cosmetic samples (100 μg g^{-1}), with a standard solution containing a mixture of the target preservatives in acetone. Previous analyses of the non-spiked samples did not showed the presence of any of the target compounds. The photodegradation experiments and subsequent extraction by micro-MSPD were performed according to the experimental procedure described in Section 2.3.

Spiked samples were irradiated using the 10 W-UV lamp during different periods of time, from 0 to 60 min, and the photodecay of target preservatives was monitored. After 20 min of UV-irradiation, a slightly lower degradation level was observed for parabens in MC (30–40% undegraded), compared to aqueous degradation (20% undegraded). Besides, BzP, which was shown to be the most photolabile paraben in water, showed similar kinetic decay with respect to its congeners alkyparabens, exhibiting higher photostability in the cream (Fig. S 4a).

As expected from the experiments in water, both antioxidants BHA and BHT underwent very fast degradation in MC, reaching degradation yields higher than 95% after 2 min of UV-irradiation (Fig. S 4b). Comparable photodegradation patterns were obtained in both sets of experiments (in water and in the cosmetics) for TCS, with a half-life time around 2.5 min under the considered irradiation conditions.

On the other hand, wide differences on the photodegradation behavior were observed for benzoates depending on the ester moiety (Fig. S 4c). Although, PhBz was shown to be again the most easily degradable benzoate, the photostability of alkyl derivatives in the cream increases with the length of the alkyl chain. Thus, after 20 min of UV-light exposure, the order of photostability of benzoates in MC was BuBz (64% of undegraded compound) > EtBz (39%) > MeBz (20%) > PhBz (2%). The lower photostability of PhBz was expected from aqueous photodegradation experiments.

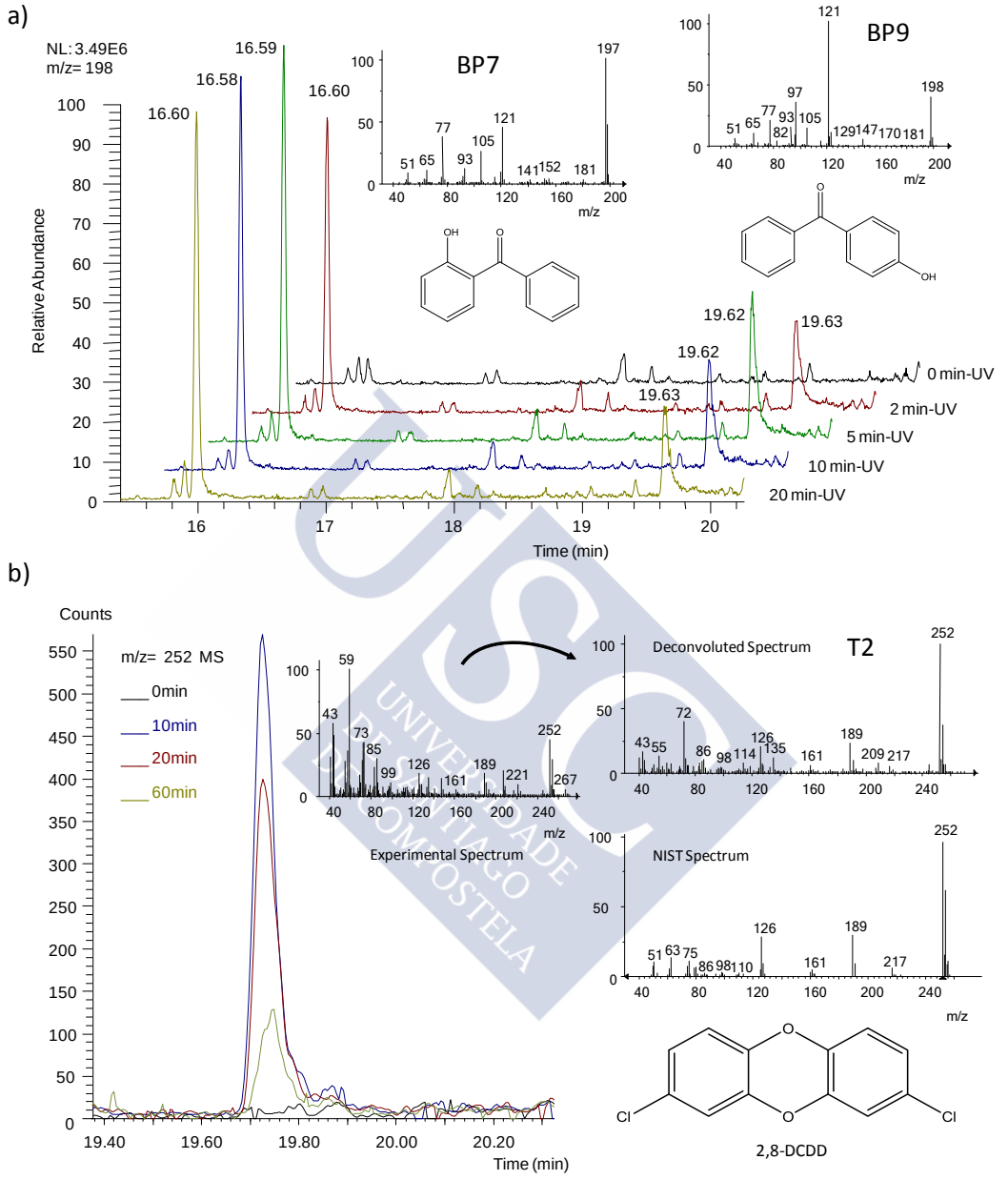

Fig. 7. Micro-MSPD-GC–MS extracted ion chromatograms of (a) a moisturizing cream (MC), and (b) a hair conditioner (HC), showing the formation of the photoproductsBH7, BP9 and T2. The mass spectra of the identified compounds are also included.

Nevertheless, the relative photostability of the alkyl benzoates is not in agreement with kinetic data obtained in water. This unexpected behavior might be explained considering the interactions between the oil phase of the emulsion, and the target alkyl benzoates. Cosmetic ingredients with higher log K_{ow} (high lipophilicity) are expected to exhibit stronger interactions with the oil (non-polar) phase, which might led to a reduction on the absorption of UV-light by the preservative. Therefore, it seems reasonable to think that more lypophilic preservatives show higher photostability. According to this hypothesis, the relative increasing photostability of target alkyl benzoates, at the working conditions, is consistent with the increasing log K_{ow}values; MeBz (1.99), EtBz (2.64) and BuBz (3.84). On the other hand, log K_{ow} values of alkyl parabens; MeP (1.66), EtP (2.19) and BuP (3.24), are comparatively lower than their congeners alkyl benzoates, which can explain a more similar kinetic behavior to that obtained in aqueous photodegradation.

Screening analysis of irradiated samples revealed the formation of three relevant photoproducts described in Section 3.2: BP7, BP9 and T2. Fig. 7a and b shows the overlapped extracted ion chromatograms obtained at different irradiation times of samples MC and HC, respectively, illustrating the formation of the detected benzophenones and dioxin, as well as their experimental mass spectra. BP4 (2-hydroxybenzophenone) was shown to be the major photoproduct detected in both samples and it could be identified by the most intense ions of its mass spectrum (m/z 197, 121). However, the identification of other transformation products with low phototransformation yield or less amenable to the proposed analytical procedure proved to be more challenging. Thus, the experimental mass spectra of BP7 (4-hydroxybenzophenone) and T2 (2,7/8-DCDD) in the obtained micro-MSPD extracts evidence this complexity. Only after an additional on-batch clean-up of the extracts with florisil, T2 could be clearly identified in the NIST library applying spectral deconvolution (See Fig. 7b).

After the first 5 min of irradiation at 10 W-UV, the formation of BP7 and BP9 accounted for more than 90% of its quantity in the cosmetic, whereas T2 exhibit its maximum concentration at 10 min. These by-products were rather resistant to undergo further degradation, being more stable than in aqueous degradation experiments, demonstrating the high stability of these photoproducts in cosmetic matrices. These findings suggest that if they are generated in a real life situation, their persistence in the cosmetic product appears to be feasible. Both benzophenones belong to a family of UV filters, widely used in sunscreens, which might explain their high stability.

4. Conclusions

In this work, SPME and micro-MSPD-based methodologies were successfully developed and applied to the study of the photochemical transformation of cosmetic preservatives under UV-light in water and PCPs.

The use of different SPME fiber coatings in aqueous photodegradation experiments allowed the identification of a wide range of photoproducts with different structures and polarities, some of which were detected herein for the first time. A total of 23 compounds were identified as parabens and benzaote by-products, whereas 14 compounds were proposed as BHA and BHT transformation products. Moreover, 2,8-DCDD was confirmed as triclosan photoproduct. Photoformation and photodecay of detected by-products were monitored by GC–MS, and phototransformation pathways, consistent with the kinetic information, were postulated.

Targeted screening analysis of micro-MSPD extracts, obtained from UV-irradiated cosmetic samples, revealed the rapid formation of two unexpected hydroxylated benzophenones, and 2,8-DCDD. The observed stability of these photoproducts in PCPs suitable for topical application might be a matter of concern due to their potential toxicity. However, further experiments are necessary to assess the extent to which the observed photoproducts may occur in daily life on human skin and under natural sunlight conditions.

The results obtained in this work demonstrate the suitability of the proposed methodology to track the phototransformation of regulated cosmetic preservatives, and detect their potentially hazardous by-products in highly complex matrices like cosmetic.

Acknowledgements

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Capítulo III.3.3

Photoinduced generation of 2,8-dichlorodibenzo-p-dioxin and other cosmetic preservative photoproducts in topical cream applied onto artificial skin.

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Photoinduced generation of 2,8-dichlorodibenzo-p-dioxin and other cosmetic preservative photoproducts in topical cream applied onto artificial skin.

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Abstract

The antioxidant BHT, as well as the antimicrobials triclosan (TCS) and phenyl benzoate (PhBz) are frequently used cosmetic preservatives, which have been reported to undergo photochemical degradation, rendering several well identified photoproducts. However, the photochemical stability and further phototransformations of these preservatives in topical applications of cosmetics exposed to UV-light are largely unknown. In this study the photochemical transformation of BHT, TCS and PhBz in artificial skin model was investigated through two sets of photodegradation experiments: (i) UV-Irradiation (8W, 254 nm) of artificial skin directly spiked with the target preservatives, (ii) UV-irradiation of artificial skin after the application of a cosmetic cream fortified with the target compounds. Subsequently, pressurized liquid extraction (PLE) was used to isolate the target preservatives and their transformation products. The follow-up of the photodegradation kinetics of the parent preservatives, the identification of the arising by-products (TPs), and the monitorization of their kinetic profile was performed by gas chromatography-mass spectrometry (GC-MS). The photochemical transformation of triclosan into 2,8-dichlorodibenzo-p-dioxin (2,8-DCDD) and other dioxin-like photoproducts has been confirmed in this work. Furthermore, seven BHT photoproducts and three benzophenones as PhBz by-products, most of them with unknown toxicological properties, have also been identified. These findings reveal the first evidences of cosmetic ingredient phototransformation into unwanted transformation products on an artificial skin model, which represents a valuable contribution to the call of public institutions like the Federal Food and Drug Administration (FDA) for the need of new data regarding the potential for formation of photodegradation products on human skin.

Keywords: Cosmetics; Preservatives; Personal care products; Photodegradation; Pressurized liquid extraction; Artificial skin; 2,8-Dichlorodibenzo-p-dioxina; GC–MS.

1. Introduction

Personal care products (PCPs) such as moisturizing creams, body lotions or sunscreens, amongst others, are daily used topical products intended to be in prolonged contact with the skin. Some of the most frequently found cosmetic ingredients (e.g. the antioxidant BHT, or the antimicrobials triclosan and phenyl benzoate) exhibit chemical structures capable of absorbing UV-light, thereby undergoing photochemical reactions [1-3]. Hence, it is reasonable to think that photochemical transformations of topical formulation of these ingredients when applied onto skin areas directly exposed to intense solar radiation cannot be discarded.

It has been clearly demonstrated that not all UVA and UV absorbers are sufficiently photostable [4]. Upon UV exposure of topically applied PCPs, cosmetics ingredients can act as photooxidants via generation of free radicals and reactive oxygen species alone or in combination with other substances when coming into direct contact with the skin [5-7]. Moreover, the reactive intermediates of photounstable ingredients may behave as photo-oxidants or may also promote phototoxic or photoallergic contact dermatitis. The interaction of photodegradation products with sunscreen excipients or skin components like sebum may lead to the formation of new molecules with unknown toxicological properties, or even more toxic than the parent compound [8, 9]. Consequently, there is an increasing concern about the potential phototoxicity and photoallergy of cosmetic ingredients included in topical application products, intended to be exposed to the solar radiation [10] .

Due to their practical applications, there is a considerable amount of literature dealing with studies on the UV-filters photostability. The behavior of the individual filters or the final sunscreen products upon UV-irradiation were extensively studied using various solar simulators based on xenon arc lamps or fluorescent lamps [5, 11-14]. Nevertheless, the photostability of other ingredients most frequently found in PCPs, such as preservatives, has not been sufficiently studied so far.

In previous works, the authors studied the phototransformation of several cosmetic preservatives in both UV-irradiated water and cosmetic samples, revealing the formation of some relevant photoproducts [3]. Thus, the phenolic antioxidant BHT was shown to easily photodegrade, rendering several tranformation products whose toxicity has been little

studied so far. The unexpected formation of 2-hydroxy and 4-hydroxybenzophenones, mainly from phenyl benzoate after photolytic ester cleavage and subsequent intramolecular rearrangement, was demonstrated in UV-irradiated personal care products. Furthermore, as expected from previous photodegradation experiments in water [15-17], outstandingly, the photochemical transformation of triclosan into 2,8-dichlorodibenzo-p-dioxin (2,8-DCDD) was detected in a real cosmetic matrix under UV-light at 254 nm.

Although it is well documented that triclosan in aqueous solution can be degraded into 2,8-DCDD and other degradation products [3, 15-19], some discrepancies on dioxin formation can be found in literature regarding the experimental conditions for its generation (mainly pH) [15, 20]. However the aforementioned photochemical experiments in cosmetics confirmed that triclosan ring closure reaction can occur under the conditions of a pH-balanced personal care product, that is, the natural pH of the skin. In this regard, a recent official document reported by the FDA about over-the-counter healthcare antiseptic drugs [21], states that, although the data support photodegradation in aqueous solution, there is no data regarding whether photodegradation of triclosan can occur on human skin after topical application of triclosan-containing products. Moreover, this document calls for the need of new data regarding the potential for formation of triclosan photodegradation products on human skin as a result of the consumers use.

However, the identification of transformation products (TPs) contained in a biological substrate like human skin smeared with a cosmetic matrix is certainly a very challenging issue. Moreover, the monitorization of the photochemical behavior of TPs, generally occurring at low concentration levels, becomes even a more complicated task, since coelution of matrix components during chromatographic analysis can led to misinterpretation of mass spectra. Therefore, the use of model substrates (e.g. artificial skin), which mimics the surface properties of human skin (topography, pH, critical surface tension and ionic strength) from where the parent compounds and potential TPs can be easily recovered, is a simpler alternative approach.

Pressurized liquid extraction (PLE) has been recently applied for the analysis of a broad range of cosmetic ingredients such as preservatives, fragrance allergens, phthalates and musks in cosmetic matrices, such as leave-on and rinse-off products, as well as in baby wipes [22-25].

In this work, a PLE coupled to gas chromatography-mass spectrometry (GC-MS) based methodology was developed and applied in order to demonstrate the photochemical transformation of three cosmetic preservatives in artificial skin. Photostability of parent

preservatives in the skin matrix model was evaluated by following their degradation kinetic behavior. Moreover, the photoformation-photodegradation kinetics of the identified transformation products were monitored. The identification, for the first time, of 2,8-DCDD and other photoproducts of unknown toxicological properties, after UV-induced photodegradation of cosmetic preservatives in artificial skin is a relevant matter of concern.

2. Experimental

2.1. Reagents and material

Butylated hydroxytoluene (BHT; 99%), and triclosan (TCS; ≥97.0%) were purchased from Aldrich (Milwaukee, WI, USA), whereas phenyl benzoate (PhBz; 99%) was supplied by ChemService (West Chester, USA). Sodium sulfate anhydrous (99%) was purchased by Panreac (Barcelona, Spain) and sand (200–300 μm mesh particle size) was provided by Scharlau (Barcelona, Spain). Acetone, n-hexane and ethyl acetate for trace analysis were supplied by Merck (Darmstadt, Germany), and sodium chloride (99.7%) was obtained by Prolabo (VWR, Fontenay-sous-Bois, France).

Individual stock solutions of each compound were prepared in acetone. Further dilutions and mixtures in the same solvent were prepared by convenient dilution of the stock solution to spike water or cosmetic samples. Stock and working solutions were stored at −20 °C protected from light.

Artificial skin (VITRO-SKIN®) was purchased from IMS testing group (ME, USA, www.ims-usa.com) as photodegradation model substrate. It is an advanced testing substrate that effectively mimics the surface properties of human skin. It has been formulated to have topography, pH, critical surface tension and ionic strength that are similar to human skin.

2.2. Cosmetic sample content

A preservatives-free and fragrances-free basic cream, acquired from VWR International®, was used in photodegradation experiments as model topical cream, containing the following main ingredients: aqua (water), paraffinum liquidum, glycerin, glyceryl stearate, bisdiglyceryl polyacyladipate-2, stearic acid, cetearyl alcohol, urea, potassium stearate, creatine, 1,2-hexenediol, caprylyl glycol, and tropolone.

2.3. Photodegradation in artificial skin samples

Two different sets of photodegradation experiments were performed using artificial skin: (i) VITRO-SKIN® was directly spiked with the target preservatives (ii) target compounds were previously added to a cosmetic cream and subsequently applied to VITRO-SKIN®.

The sheet of artificial skin was cut into 3 cm × 3 cm pieces and homogeneously spiked with 20 µL of the target preservatives at 1000 µg ml^{-1} in acetone by dotting it at approximately equally spaced points across the substrate with the "skin topography" side up. After homogeneous absorption acetone was left to evaporate, leding to a realistic preservative dose of 2.2 µg cm^{-2}.

For the second set of experiments a moisturizing cram model was fortified with the target preservatives at a concentration level frequently found in real cosmetic samples (500 µg g^{-1}). Thus, 0.5 g of sample was mixed with 0.5 mL of a standard solution containing the target preservatives at 500 µg mL^{-1} in acetone. Portions of the resulting liquid mixture (ca. 40 mg) were pipetted onto artificial skin, by dotting it at approximately equally spaced points across the substrate with the "skin topography" side up. Immediately after the cream application, the product was rubbed into the film with a gloved finger and uniformly spread by circular movements. After acetone evaporation, a cosmetic layer of ca. 2.4 mg/cm^2 was obtained.

In both experiments, the artificial skin was laid over a glass Petri dish with the "skin topography" side up and then placed inside the photoreactor box at a distance of 9 cm from the lamps. The substrate was irradiated with 8W-UV light (254 nm) for the designated time (0–60 min).

2.4. PLE procedure

Extractions were performed on an ASE 150 (Dionex, Co., Sun-nyvale, CA, USA), equipped with 10 mL stainless steel cells and 60 mL collection vials. Two cellulose filters (Dionex, 27 mm) were placed at each end of the PLE cell. Individual sheets of artificial skin were directly introduced into the cell, where previously 5g of clean sand were placed. 1 g of sodium sulfate anhydrous as drying agent was also added. Finally, the dead volume was immediately filled with sand to avoid losses of the most volatiles analytes. The cell was tightly closed and placed into the PLE system. The extraction pressure was 1500 psi, the flush volume was 60%, the purge time 60 s, and the temperature 110 °C. Ethyl acetate, and

the mixture hexane/acetone (1:1, v/v) were tested as extraction solvents. In all cases, the extracts were leveled to a final volume of 20 mL with the extraction solvent. Then, PLE extracts were filtered through a 0.20 μm PTFE filters, and analyzed by GC-MS.

2.5. GC-MS analysis

The GC–MS analysis was performed using an Agilent 7890A(GC)-Agilent 5975 C inert MSD with triple axis detector and an Agilent 7693 autosampler from Agilent Technologies (Palo Alto, CA,USA). The system was operated by Agilent MSD ChemStation E.02.00.493 software.

Separation was carried out on a SLBTM-5ms capillary column (30m × 0.25 mm i.d., 0.25 μm film thickness) obtained from Supelco Analytical (Bellefonte, PA, 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 80 °C (held 2 min) to 230 °C at 8 °C min^{-1}, and to 270 °C at 15 °C min^{-1} (held 5 min). Splitless mode (held 2min) was used for injection. The injector temperature was 270 °C.

The mass spectrometry detector (MSD) was operated in the electron impact (EI) ionization positive mode (+70 eV). MSD operated in full scan acquisition mode, in a mass range of *m/z* 40–450. The transfer line and the ion source temperatures were set at 290 and 350 °C, respectively.

Identification was accomplished using the NIST Mass Spectral Database (NIST MS Search 2.0) or by experimental mass spectrum interpretation (experimental ion justification).

3. Results and discussion

3.1. Method validation

For the extraction of target cosmetic preservatives and their transformation products from artificial skin PLE was the technique of choice, due to its proved high extraction efficiency. Extraction conditions were optimized to maximize the recoveries for the target preservatives considered as the parent species in this work.

Extraction temperature and time were set at 120 °C and 15 min, as it has been previously shown to be suitable conditions for the extraction of target preservatives from leave-on cosmetic samples [22, 25]. Sand was selected as extraction sorbent, as it was

proved to provide clean extracts in the determination of cosmetic preservatives in baby wipes, as reported by Celeiro et al. [24]. Two different solvents; ethyl acetate and hexane/acetone (1:1) were tested due to their intermediate polarity, which makes them suitable for the extraction of target preservatives and their foreseeable phototransformation by-products. Higher recovery values were obtained using ethyl acetate as extraction solvent, compared to hexane/acetone (1:1), for the extraction of the target compounds from the artificial skin substrate. Therefore, ethyl acetate was the selected extraction solvent. Thus, using the optimal experimental conditions described in the experimental section, satisfactory validation parameters were obtained (Table 1).

As can be seen in Table 1, although almost quantitative recoveries (≥ 92%) were achieved when target ingredients are directly spiked in Vitro-Skin®, lower recoveries were obtained in the experiments where Vitro-Skin® is rubbed with the studied moisturizing cream (Vitro-Skin® & MC). However, the accuracy was shown to be satisfactory in order to tackle the proposed photodegradation experiments, considering that precision was below 9% in terms of RSD. When necessary, the extracts were concentrated by nitrogen stream evaporation and subsequent reconstitution in ethyl acetate, in order to increase minor by-products detectability.

Table 1. Retention time, quantification and identification m/z ratios, accuracy and LODs of the proposed PLE-GC-MS methodology

Compound	RT (min)	Qualifier and quantifier ions (m/z)[a]	Recovery (± RSD, %)[b]	
			Vitro-Skin®	Vitro-Skin® & MC
BHT	12,68	220, *205,* 177	95 ± 3	72 ± 7
PhBz	15,00	198, *105*, 77	92 ± 3	66 ± 8
TCS	20,69	*288,* 218, 146	94 ± 1	102 ± 9

[a] Quantification ions are set in italics.

[b] n = 3

3.2. Photostability of cosmetic preservatives in artificial skin

First, experiments were carried out in order to test the photostability of the target preservatives when submitted to photodegradation in Vitro-Skin® (VS). Additionally, dark tests were performed to demonstrate the photochemical origin of such degradation.

As illustrated in Fig 1, significant losses (from 75 to 30 %) of the target analytes were observed during the UV-irradiation of the studied cosmetic preservatives in VS. On the other hand, the obtained results during the photodegradation experiments in Vitro-Skin® with moisturizing cream (VS&MC) indicated that the decrease of the signal only took place after UV-irradiation, confirming that no analyte losses through other processes, such as volatilization, occurred in this set of experiments. These results reveal that the presence of the cosmetic matrix contributes to retain the target preservatives on the skin. However, despite the losses, a significant photodegradation was observed in both VS and VS&MC experiments after 40 min of UV-irradiation.

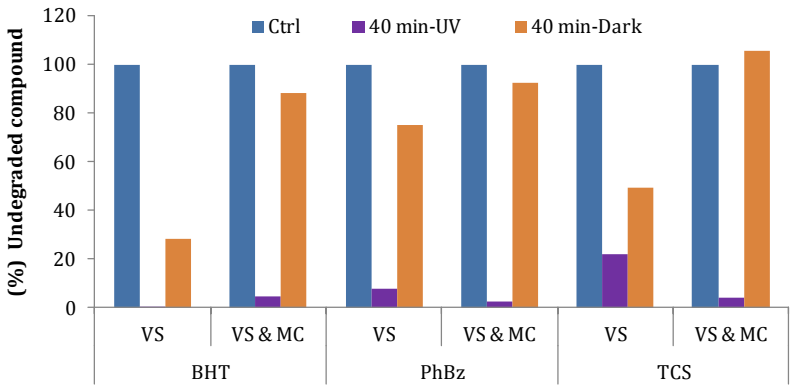

Fig. 1. Influence of irradiation time (40 min 8W-UV) on the target preservatives photodegradation, compared to the control experiment (0 min) and the dark test. Comparison of the relative responses obtained in VS (VitroSkin®) and VS&MC (Vitro-Skin® with moisturizing cream) photodegradation experiments.

The photodegradation kinetics of the target compounds were monitored by studying the influence of the irradiation time on their analytical response. Fig. 2 illustrates the photodecay curves obtained for each compound during VS&MC experiments. As can be seen, after 20 min, less than 10% of the target preservatives remained undegraded in the artificial skin.

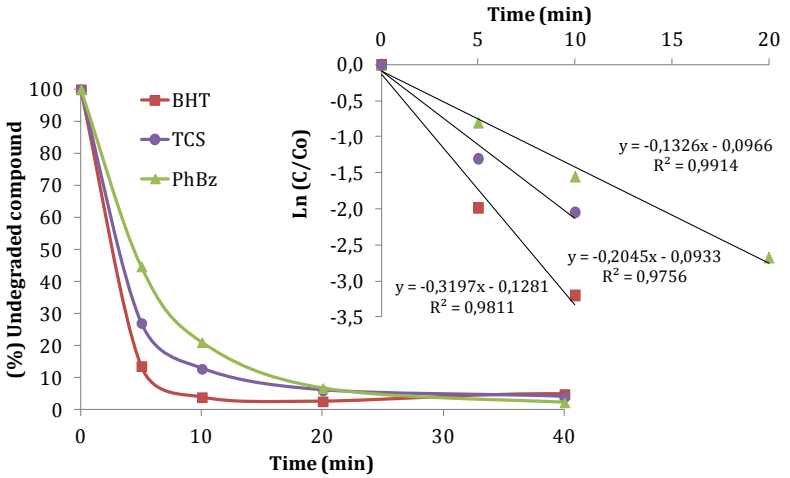

Fig. 2. Photodegradation kinetics of the target preservatives. Linear fit of logarithmic values vs. irradiation time, demonstrating pseudo-first order kinetic behavior. C_0 and C represent the percentage of undegraded compound at times *zero* and *t*, respectively.

The pseudo-first-order kinetic behavior was demonstrated for all the photodegradation reactions by plotting the natural logarithm of the ratio C/C_0 (percentage of undegraded compound at times *t* and *zero*, respectively), against irradiation time. Correlation coefficients, obtained for the corresponding least-squares regressions, were in the range of 0.98-0.99 (Fig. 2).

3.3. Photoproducts identification

A thorough screening analysis of the irradiation experiments revealed the formation of 14 transformation products. These photodegradation products were tentatively identified on the basis of their mass spectra, using the NIST Mass Spectral Database and/or reported information in the literature. The identified photoproducts with their corresponding retention times and identification and quantification ions are listed in Table 2. The last two columns of this table also show the identification approach of these photoproducts, as well as the reverse match (R. Match) values given by NIST library as an index of identification reliability.

Table 2. Retention times, quantification and identification ions, and reverse matches of the tentatively identified photoproducts in VS (VitroSkin®) and VS&MC (Vitro-Skin® with moisturizing cream) photodegradation experiments.

Key	Photoproduct	VS	VS & MC	RT (min)	Qualifier and quantifier ions (m/z)a	Reverse match	Identif [b]
Ph	Phenol	X	nd	4,65	94, 66	780	A
BH1	4,6-Di-t-butyl-m-cresol	X	nd	14,67	205, 220, 189, 149	690	A
BH2	2,4-Di-t-butyl-6-methylphenol	X	X	15,54	205, 220, 189, 149	769	A
BH3	2,6-Di-tert-butyl-4-methoxymethylphenol	nd	X	16,23	235, 250, 219	768	A
BP1	2-Hydroxybenzophenone	X	X	16,76	197, 121, 105	927	A
BP2	4-methyl-benzophenone	nd	X	17,16	196, 119, 105	940	A
BH4	3,5-di-t-butyl-4-hydroxy-benzaldehyde	X	X	17,25	219, 234,191	880	A
BH5	3,5-di-t-Butyl-4-hydroxybenzyl alcohol	X	X	17,30	221, 236, 161	808	A
TC1	2,8-Dichlorodibenzo-p-dioxin (2,8-DCDD)	X	X	19,70	252, 189, 126	850	A
TC2	Dichloro-hydroxydiphenyl ether	X	X	19,79	254, 256, 112	-	B
BP3	4-Hydroxybenzophenone	X	X	20,36	121, 198, 105, 93	839	A
BH6	2,5-Di-t-butyl-p-benzoquinone	X	nd	20,41	205, 220, 177, 149	693	A
BH7	4,4'-Ethylenebis(2,6-di-tert-butylphenol)	X	nd	21,72	219, 438, 204	829	A
TC3	Dichloro-hydroxy-dibenzofuran/ DCDD	nd	X	22,00	252, 189, 126	-	C

[a] Quantification ions are set in italics.

[b] Identification based on: (A) Theoretical mass spectrum, (B) theoretical mass spectrum of methyl 2-hydroxy-4-methoxylbenzoate, (C) theoretical mass spectrum of 4-methoxylbenzoic acid, (D) theoretical mass spectrum of 3-hydroxybenzoic acid.

[c] The "X" sign represents the positive detection of the photoproduct; nd: not detected.

3.3.1 BHT photoproducts

Seven transformation products were identified in the photodegradation experiments of BHT on artificial skin. They are designated with the prefix BH in Table 2.

BH1 and BH2 exhibit very similar fragmentation pattern to the mass spectra of BHT. As photoisomerization reactions of BHT are well reported in literature, both photoproducts

are expected to be BHT isomers. Thus, 4,6-di-t-butyl-m-cresol and 2,4-di-t-butyl-6-methylphenol showed the best matches on the NIST library for BH1 and BH2, respectively.

Oxidation of the methyl group in position 4 of the benzenic ring after H-subtraction can lead to the formation of the alcohol derivative BH5; which, in turn, can subsequently undergo further oxidation, rendering the aldehyde derivative BH4. These photoproducts were identified as 2,6-di-tert-butyl-4-methoxymethylphenol (BH3), 3,5-di-t-butyl-4-hydroxy-benzaldehyde (BH4) and 3,5-di-t-Butyl-4-hydroxybenzyl alcohol (BH5) and on the basis of the best match with the NIST library. Although BH3 has been identified in this work form the first time, BH4 and BH5 have been previously detected as oxidation photoproducts of BHT in water [26] and in *on-fiber* photodegradation studies[1].

The formation of BH6, tentatively identified as 2,5-di-t-butyl-p-benzoquinone, was also expected from previous works as BHT metabolite [27] and photooxidation by-product [3]. Di-t-butyl-p-benzoquinones can be generated from di-t-butyl-4-hydroxyanisole intermediates via the semiquinone radical [3]. Although these intermediates were not detected, its formation is supported by previous studies about the phototransformation of phenolic antioxidants like BHT.

The last BHT photoproduct (BH7) was identified as the BHT dimer 4,4'-ethylenebis(2,6-di-tert-butylphenol). This assumption is plausible according with the fragmentation patters exhibited by this transformation product, which show m/z 438 $[M]^+$ and m/z 219 $[(m/z\ 438)/2]^+$ as the most representative ions. These compound had been previously identified by Fries et al. as BHT metabolite in water [28]. Nevertheless, BH7 was detected herein for the first time as BHT phototransformation product.

3.3.2 Phenyl benzoate photoproducts

Photoproducts BP1 and BP3 exhibit similar fragmentation patterns, consistent with m/z 198 $[M]^+$, m/z 121 $[M\text{-}C_6H_5]^+$, m/z105 $[M\text{-}C_6H_6O]^+$and m/z 77 $[C_6H_5]^+$. They were readily identified as hydroxybenzophenones on the basis of their NIST theoretical mass spectra . In the case of BP1, the presence of the base peak at m/z 197 reveals the easy loss of a proton $[M\text{-}H]^+$, most probably from the hydroxyl group in the ortho- position to the ketone group. As ortho- and para-derivatives are expected to be the most favorable hydroxylated by-products, BP1 and BP3 were identified as 2-hydroxy and 4-hydroxybenzophenones, respectively. Hydroxybenzophenones might be generated through intramolecular rearrangement after photolytic cleavage of the ester bond of the phenyl benzoate. Thus, the resulting phenolic and benzaldehyde radicals can react with each other, as reported in

previous studies. The formation of BP1 and BP3 in Vitro-Skin® with moisturizing cream experiments is illustrated in Fig 3.

The unambiguous identification of BP2 as 4-methyl-benzophenone in VS&MC photodegradation experiments reveals the effect of the cosmetic matrix during phenyl benzoate phototransformation. The presence of the methyl substituent in the benzophenone structure is most probably due to the interaction with cosmetic excipients.

3.3.3 Triclosan photoproducts

As a result of the photochemical degradation of triclosan during UV-irradiation of the cosmetic applied on artificial skin, two dioxin-like transformation products (TC1 and TC3) and a dichlorohydroxydiphenyl ether (TC2) were detected. The generation of the triclosan photoproducts, as well as their experimental mass spectra, is clearly illustrated in Fig. 4.

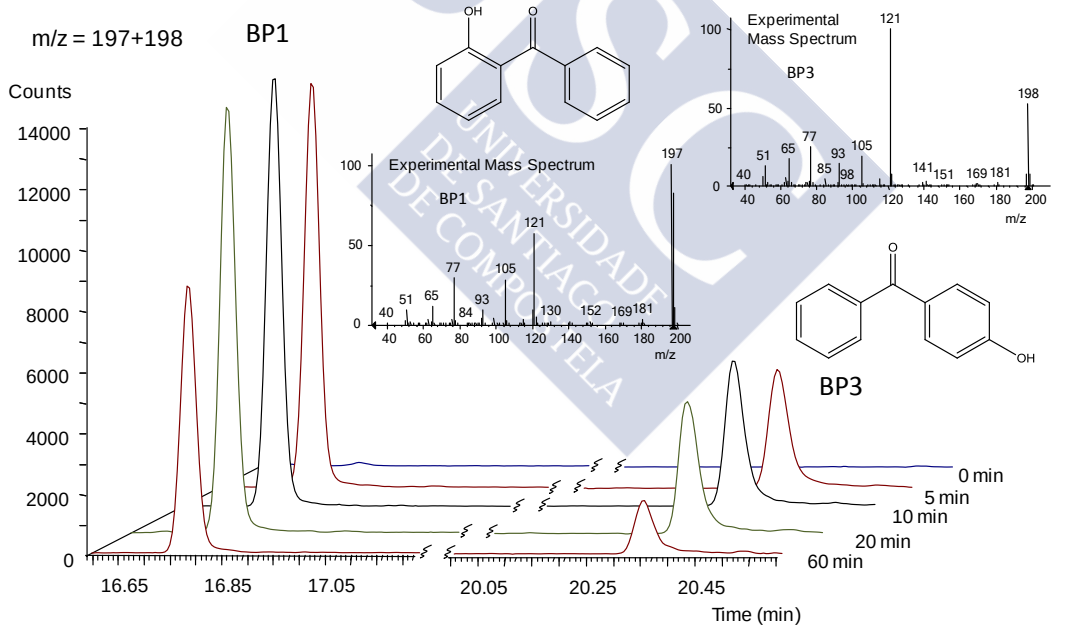

Fig. 3. PLE-GC–MS extracted ion chromatograms showing the formation-degradation of phenyl benzoate photoproducts BP1 and BP3, obtained at different UV-irradiation times (0–60 min) in Vitro-Skin® with moisturizing cream experiments. Experimental mass spectra are also displayed.

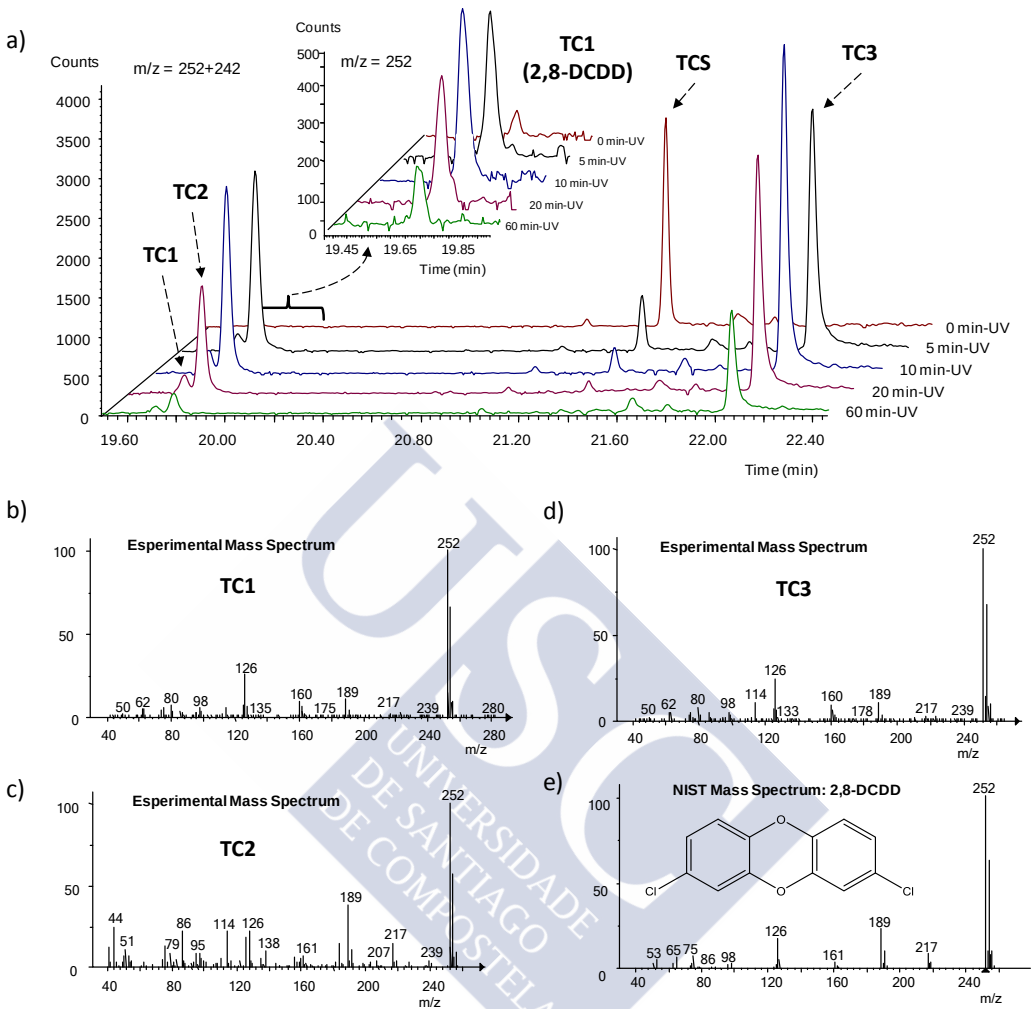

Fig. 4. PLE-GC–MS extracted ion chromatograms showing the formation-degradation of triclosan photoproducts TC1, TC2 and TC3, obtained at different UV-irradiation times (0–60 min) in Vitro-Skin® with moisturizing cream experiments. Experimental mass spectra and theoretical mass spectrum of 2,8-DCDD are comparatively displayed.

TC1 was identified as 2,8-Dichlorodibenzo-p-dioxin (2,8-DCDD), showing positive match in the NIST library (see theoretical spectrum in Fig. 4e). The spectrum of this photoproduct (Fig. 4b) is mainly characterized by the peaks m/z 252 and m/z 254, corresponding to the molecular ions $[M]^+$ and $[M+2]^+$, respectively. A detailed view on the extracted ion chromatogram m/z 252 (Fig. 4a) shows the isolated signal of 2,8-DCDD. The

possibility of the chlorine atoms being in 2,7- or 2,8-positions (2,7-DCDD, 2,8-DCDD) has been suggested by different authors; however, the most likely product of triclosan ring closure is considered to be 2,8-DCDD [18]. Although a simple photoisomerization could lead to the 2,7-DCDD, the relative position of TC1 in the chromatogram with respect to triclosan, in terms of retention time, is consistent with the position of 2,8-DCDD in previous works [17].

Photoproduct T2 was tentatively identified as dichlorohydroxydiphenyl ether. The mass spectrum is mainly characterized by the ions m/z=254 and m/z=256, corresponding to the ions $[M]^+$ and $[M+2]^+$, respectively. The base peak ion is m/z=184, formed by loss of two chlorine atoms from the molecular ion. T2 is generated by reductive dechlorination (loss of a chlorine atom and its replacement by a hydrogen atom). Its formation in aqueous media has been previously reported in literature [2, 29, 30].

Followed by T1 and T2, the photoproduct T3 was shown to be the major photoproduct of triclosan, in terms of abundance (peak area). The base peak ions are the cluster m/z=252 and m/z=254, corresponding to the ions $[M]^+$ and $[M+2]^+$, respectively. The ion m/z=189 is formed by loss of a chlorine atom and an ethyl group. On the basis of the mass spectrum similarity with that of 2,8-DCDD, T1 and T2 could be isomers, therefore, another DCDD might has been photogenerated. The formation of other DCDD isomers by the photodegradation of triclosan has not previously been reported. Nevertheless, the formation of a dichlorohydroxydibenzofuran during UV irradiation studies of Irgasan DP300 (triclosan) was reported by Kanetoshi et al.[31]. In addition, similarities between the mass spectra of T3 and that of dichlorohydroxydibenzofuran reported in literature are evident [2]. Thus, T3 can be either a dioxin or a furan; anyhow, both benzofurans and dioxins are considered to be highly toxic chemicals.

3.4. Kinetic profile of the photoproducts

The kinetic behavior of the identified photoproducts was also evaluated in order to demonstrate the generation and evolution of these compounds in artificial skin. As depicted in Fig 6, the obtained photoproducts are generated very quickly, reaching their maxima concentrations between 5 and 10 min at 8W-UV. After 10 min of UV irradiation, the transformation products began to degrade in a greater or lesser extent.

The fast degradation of photoproducts BH2, BH3 and BH5 contrast with the higher photostability of phenyl benzoate and triclosan photoproducts. In fact, BP1 did not show a

visible decrease in their signal in the time interval of the experiment, whereas between 60 and 40 % of its congeners BP2 and BP3, respectively, remain undegraded after 60 min of UV irradiation. The high photostability of these photoproducts can be explained considering the structural characteristics of benzophenones, widely used as UV filters in sunscreens.

The parallel kinetic behavior of TC1 and TC3 is consistent with their tentatively proposed structural similarity, deduced from their highly similar fragmentation pattern. As can be seen in Fig. 5, although both by-products exhibit slightly higher photolability that benzophenones, their slow photodecay evidence their significant persistence, even after 60 min of UV irradiation. This fact should be taken into account, because of the potential toxicity of these dioxin and dioxin-like photoproducts.

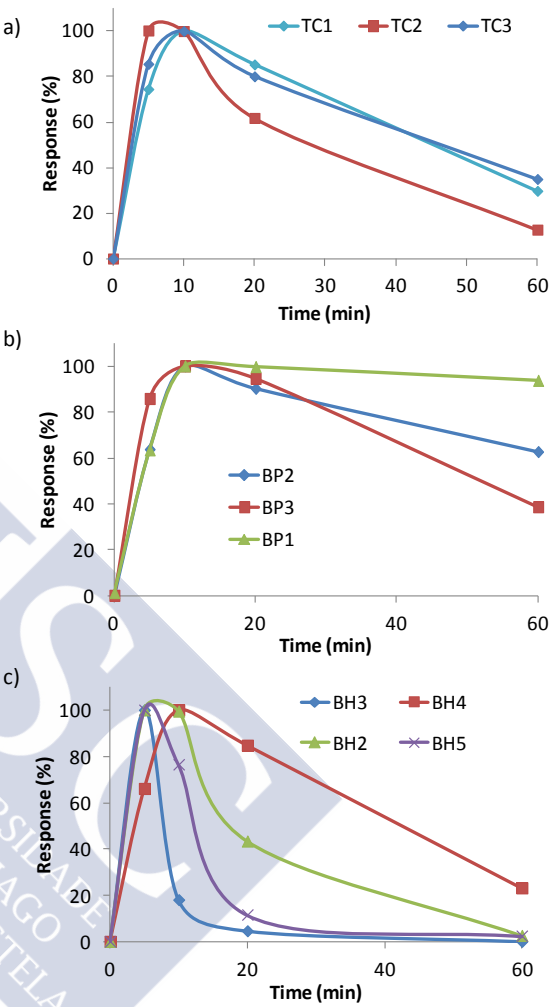

Fig. 5. Kinetic profile for representative transformation products in aqueous photodegradation experiments: (a) triclosan photopro-ducts; (b) Phenyl benzoate photoproducts (c) Representative BHT photoproducts, at 8 W-UV.

4. Conclusions

The photochemical transformation of the cosmetic preservatives BHT, TCS and PhBz on an artificial skin model under UV light (254 nm) has been investigated. PLE was successfully implemented as a high throughput extraction technique for the isolation of the target cosmetics ingredientes and their transformation byproducts, from the artificial skin substrate. BHT resulted to be the most photolabile compound, whereas PhBz exhibited more stability. A faster kinetic degradation was observed when the cosmetic matrix was applied on the skin. Targeted screening analysis by GC-MS revealed the

generation of 2,8-DCDD and other dioxin-like or furan-like photoproducts, which are considered to be more toxic that their precursor triclosan. Moreover, seven BHT photoproducts and three benzophenones as PhBz by-products, most of them with unknown toxicological properties, were also identified. These findings reveal the first evidences of cosmetic ingredient phototransformation into unwanted transformation products on an artificial skin model, which is particularly relevant, considering the widespread presence of this ingredient in daily life products.

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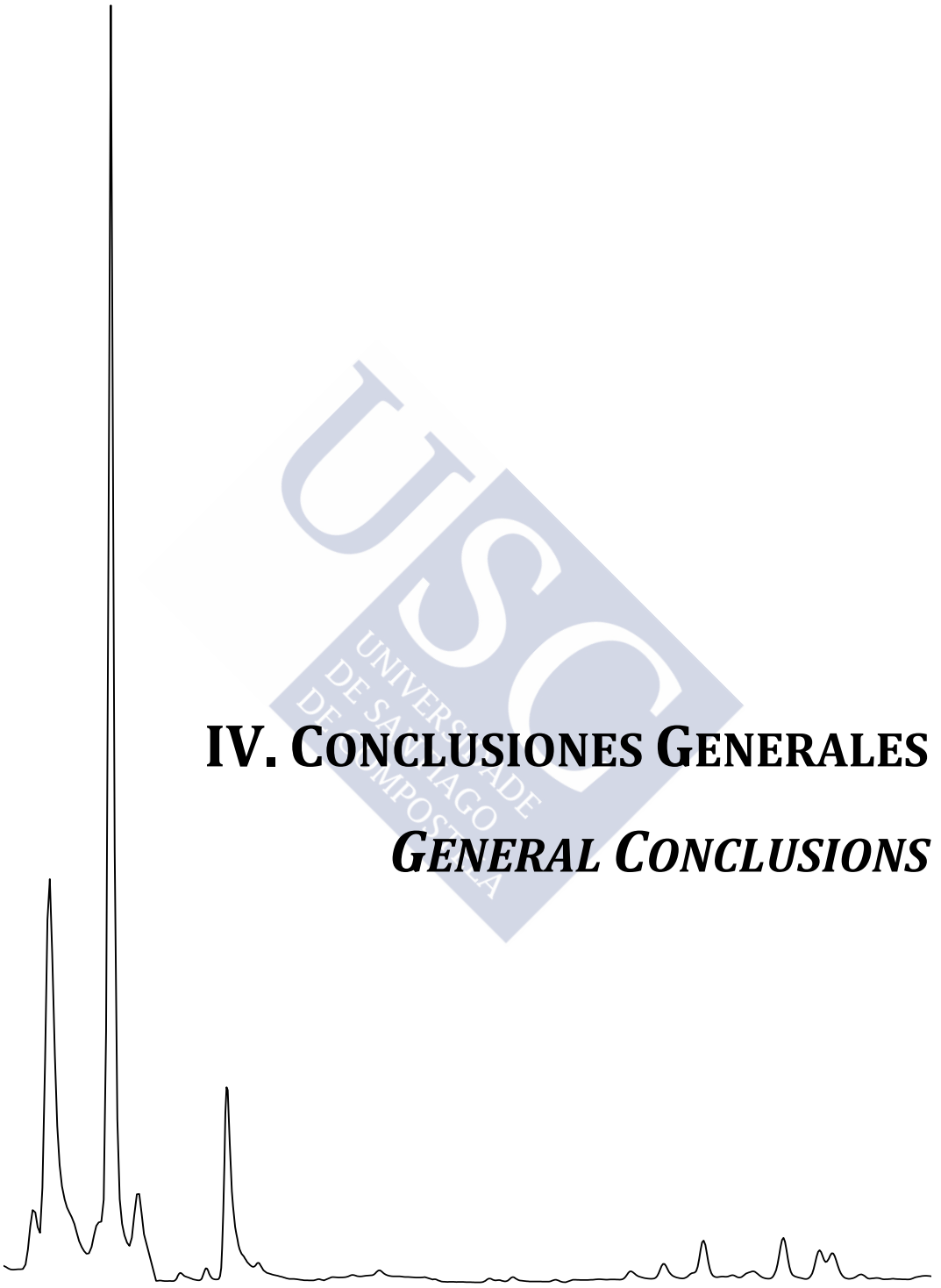
IV. CONCLUSIONES GENERALES

GENERAL CONCLUSIONS



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Los procedimiento analíticos desarrollados esta tesis doctoral para la determinación de conservantes y fragancias alergénicas, así como para la detección de contaminación microbiana, representan una propuesta metodológica basada en la simplicidad procedimental, la rapidez y la robustez del análisis, pudiendo implementarse fácilmente en cualquier laboratorio de control. Además, estos métodos emplean técnicas como la MSPD y la SPME, que pueden calificarse como respetuosos con el medio ambiente, debido a la baja generación de residuos, y al mínimo consumo de disolventes utilizados.

Los procedimientos de extracción se han optimizado empleando herramientas quimiométricas. Así, se han utilizado diseños experimentales para evaluar la influencia de los principales factores en el proceso de extracción, así como las interacciones entre estos, empleando el número mínimo de experimentos necesarios.

Asimismo, la evaluación de las prestaciones analíticas de los métodos desarrollados; en términos de linealidad, exactitud y precisión, han generado resultados satisfactorios, obteniéndose limites de detección y cuantificación muy por debajo de las concentraciones máximas permitidas en el Reglamento Europeo para los ingredientes objeto de estudio. La aplicabilidad de dichos métodos se ha demostrado mediante análisis de muestras reales y estudios de mercado.

Por otra parte, se ha estudiado el comportamiento fotoquímico de los conservantes cosméticos en agua, en la propia matriz cosmética y en un modelo de piel sintética. Para acometer estos estudios se han aplicado técnicas de extracción como SPME, micro-MSPD y PLE, lo que ha permitido aislar los productos de transformación de los conservantes considerados. Estos estudios suponen una aportación novedosa, teniendo en cuenta que, hasta la fecha, no existe información publicada acerca de los procesos de transformación fotoquímica de conservantes en productos cosméticos.

En definitiva, la metodología y los estudios presentados en esta tesis suponen un avance en el desarrollo de procedimientos analíticos para el control de la seguridad de los productos cosméticos en tres campos destacables, cuyas conclusiones generales se exponen a continuación:

I. *Determinación de conservantes y fragancias alergénicas en productos cosméticos*

i. La aplicación, por primera vez, de la MSPD en matrices cosméticas ha resultado ser de gran utilidad para la determinación de conservantes y fragancias alergénicas en una gran variedad de muestras cosméticas. La capacidad de disrupción de la muestra que posee esta técnica ha permitido aislar con gran eficacia los ingredientes objeto de

estudio en matrices de gran complejidad como cremas, jabones, barras de labios, o pastas dentífricas, entre otras.

ii. La posibilidad de añadir co-adsorbentes en el fondo de la columna de MSPD, a modo de etapa de limpieza *in situ*, ha permitido obtener extractos limpios utilizando una pequeña cantidad de disolvente y sin necesidad de introducir etapas adicionales.

iii. La eficacia de los procesos de extracción mediante MSPD y PLE ha resultado ser comparable para la mayoría de los conservantes y fragancias estudiados, lo que presenta a la MSPD como una alternativa económica para el análisis de estos ingredientes en productos cosméticos.

iv. El uso de GC-MS, empleando monitorización en modo SIM ha permitido la determinación selectiva y sensible de los ingredientes estudiados en los extractos obtenidos mediante MSPD.

v. La aplicación de los métodos desarrollados a muestras reales ha permitido confirmar la presencia generalizada de metilparabén, propilparabén y butilparabén, además del antioxidante BHT, a concentraciones relativamente elevadas.

vi. Los resultados del estudio de mercado realizado en productos de cuidado personal para su uso en bebés y niños han puesto de manifiesto el elevado número de fragancias alergénicas por muestra, así como el alto contenido alergénico en alguna de las muestras de permanencia analizadas, destinadas a permanecer en contacto con la delicada piel del bebé. Además, se ha identificado un número significativo de muestras que han incumplido el Reglamento Europeo por no incluir algunos conservantes, como BHT o algunos parabenos, en la etiqueta.

vii. Se ha demostrado la utilidad de la SPME acoplada a GC-MS/MS para la determinación de distintos conservantes cosméticos. A pesar del alto grado de dilución aplicado a las muestras cosméticas para reducir el efecto matriz, la gran capacidad de concentración de la SPME en combinación con la alta selectividad de la monitorización en modo SRM han dado como resultado una metodología de gran sensibilidad y simplicidad, con posibilidad de automatización.

viii. Debido a su marcado carácter hidrofílico, se ha propuesto una metodología alternativa, basada en HPLC-MS/MS con analizador de triple cuadrupolo, para el análisis de conservantes del tipo isotiazolinona. La MSPD se ha aplicado, por primera vez, a la extracción de estos conservantes en productos cosméticos y del hogar. Utilizando florisil como fase dispersante y como co-adsorbente se han obtenido

extractos limpios en metanol, directamente compatibles con la fase móvil ($MeOH/H_2O$), sin efecto matriz durante la ionización de los analitos en la interfase de electrospray (HESI)

II. *Detección de contaminación microbiana en productos cosméticos*

i. Por primera vez, se ha explorado un nuevo enfoque bioanalítico para la detección rápida de microbiota contaminante en muestras cosméticas, basado en la rastreo de biomarcadores volátiles de bacterias, hongos o levaduras.

ii. Se ha demostrado que es claramente factible la identificación de microorganismos viables (o incluso cepas específicas) en matrices cosméticas, mediante el análisis de compuestos orgánicos volátiles de origen microbiano, como metil cetonas y alcanoles, de número impar de átomos de carbono, empleando la SPME en espacio de cabeza como técnica de extracción acoplada a GC-MS.

III. *Estudio del comportamiento fotoquímico de conservantes cosméticos*

i. La aplicación de la SPME en los experimentos de fotodegradación acuosa ha demostrado ser una alternativa rápida y simple frente a otra técnica de extracción ampliamente utilizada para el mismo fin, como la LLE o la SPE. La capacidad de extracción y preconcentración de esta técnica ha permitido incrementar la detectabilidad de los fotoproductos generados a concentraciones muy bajas.

ii. El uso de fibras de SPME con diferentes fases adsorbentes ha permitido la extracción sencilla de un amplio rango de fotoproductos con estructuras químicas muy diversas, algunos de ellos identificados por primera vez en esta tesis doctoral.

iii. Se ha aplicado por primera vez una versión miniaturizada de la MSPD para aislar subproductos de transformación fotoquímica en muestras cosméticas. El uso de la micro-MSPD permitió trabajar con pequeñas cantidades de muestra (50 mg), sin afectar a la detectabilidad de los fotoproductos.

iv. Se ha demostrado la capacidad de la radiación ultravioleta de inducir la formación de subproductos halogenados de parabenos y benzoatos en agua clorada, incluso a niveles bajos de cloro. La presencia de trazas de bromuro, naturalmente presentes en el agua, dio lugar a la formación preferente de especies bromadas, que presentan mayor toxicidad que sus análogos clorados.

v. Por primera vez, se han realizado experimentos de fotodegradación de ingredientes cosméticos sobre un modelo de piel artificial. Los resultados obtenidos han confirmado la transformación fotoinducida del conservante antimicrobiano triclosan en 2,8-DCDD y otros subproductos de tipo dioxina; así como la transformación de fenil benzoato y BHT en photoproductos de toxicidad desconocida. Estos resultados aportan los primeros datos relativos al comportamiento fotoquímico de ingredientes cosméticos aplicados directamente sobre un modelo de piel sintética, lo que supone una contribución a los requerimientos de instituciones públicas como la FDA sobre este tema.

vi. Finalmente, se ha demostrado la utilidad del acoplamiento de las técnicas de extracción empleadas en esta tesis doctoral con la técnica híbrida GC-MS para el aislamiento y determinación de productos de transformación. Las condiciones estandarizadas de fragmentación utilizadas en estos estudios (EI a 70 eV) han permitido el uso de librerías de espectros, lo que ha facilitado enormemente la difícil tarea de la identificación de los productos de transformación.

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The analytical procedures developed throughout the present PhD dissertation for the determination of preservatives and fragrance allergens, as well as for microbial contamination detection, represent a methodological proposal based on procedural simplicity, rapidity and robustness of the analysis, which can be easily implemented in any control laboratory. Moreover, these methods, applying MSPD and SPME, can be described as environmentally friendly, due to the low generation of residues, and minimal solvents consumption.

The extraction procedures have been optimized applying chemometrical tools. Thus, experimental designs were used in order to evaluate the influence of the main factors on the extraction process, as well as the interactions between factors, employing the minimum required number of experiments.

Furthermore, the evaluation of the methods performance; in terms of linearity, accuracy and precision, were shown to be satisfactory, and the limits of detection and quantification were far below the legal limits established in the European Regulation for the target ingredients. The applicability of such methods has been demonstrated by analyzing real samples and performing an extensive market research on baby and child care products.

On the other hand, the photochemical behavior of selected cosmetic preservatives in water, in the cosmetic matrix itself, and in a synthetic skin model has been studied. In order to accomplish such studies, extraction techniques such as SPME, micro-MSPD and PLE have been applied, which allowed the isolation of several transformation products. The obtained results represent a rewarding contribution, considering that no information about the photochemical transformation of preservatives in cosmetic products had been reported up to now.

In short, the methodology and the studies included in this dissertation represent a significant advance in the development of analytical procedures for the safety control of cosmetic products in three remarkable fields, which general conclusions are drawn below:

I. *Determination of preservatives and fragrance allergens in cosmetic products.*

i. The application, for the first time, of MSPD to cosmetic matrices has proven to be particularly useful for cosmetic preservatives and fragrance allergens determination in a broad variety of cosmetic samples. The capacity of this technique for sample disruption allowed the efficient isolation of target ingredients in highly complex matrices such as creams, soaps, lipsticks, or tooth pastes, among others.

ii. The possibility of co-adsorbents addition at the bottom of the MSPD column, as *in situ* clean-up step, allowed obtaining clean extracts by using small solvent volumes, without the need of additional steps.

iii. The extraction efficiency of MSPD and PLE procedures has been proved to be comparable for most of the studied preservatives and fragrance allergens, which presents the MSPD-based approach as a low-cost alternative for the analysis of these ingredients in cosmetic products.

iv. The use of GC-MS employing monitorization in SIM mode allowed the selective and sensitive determination of the studied cosmetic ingredients in the extracts obtained by MSPD.

v. The application of the developed methods to the analysis of real samples has confirmed the widespread presence of methylparaben, propylparaben and butylparaben, as well as the antioxidant BHT, at relatively high concentrations.

vi. The results of the market survey on baby and child care products revealed the high number of allergenic fragrances per sample, and the high allergenic content of some analyzed leave-on samples, which are intended to be in prolonged contact with the delicate baby's skin. In addition, a relatively high number of these samples did not comply with the European Regulation, as some preservatives, such as parabens or BHT, were not included in the label.

vii. It has been demonstrated the usefulness of SPME coupled to GC-MS/MS for the multipreservatives analysis in cosmetic products. Despite the high dilution rate applied to the samples in order to reduce matrix effect, the preconcentration capacity of SPME in combination with the high selectivity or the SRM mode has resulted in a methodology of great simplicity and sensitivity, which can be easily automated.

viii. An alternative methodology, based on HPLC-MS/MS with triple quadrupole, has been successfully developed for the analysis of the strong hydrophilic isothiazolinone-type preservatives. MSPD has been applied, for the first time, to the isolation of the target isotiazolinones from cosmetics and household products. The use of florisil as dispersing sorbent and co-adsorbent led to clean methanolic extracts, directly compatible with the mobile phase (MeOH/H2O), which did not show matrix effect during the analytes ionization in the HESI source.

II. *Detection of microbial contamination in cosmetic products*

i. A novel bioanalytical approach has been explored for the rapid detection of microbial contamination in cosmetic samples, based on the identification of volatile biomarkers of bacteria, fungi or yeasts.

ii. It has been demonstrated that the identification of viable microorganisms (or even specific strains) growing in cosmetic matrices is clearly feasible, through the targeted screening analysis of microbial volatile organic compounds (MVOCs), such as odd-numbered carbon methyl ketones and alkanols, by employing SPME in head-space sampling mode as extraction technique, coupled to GC-MS.

III. *Study of the photochemical behavior of cosmetic preservatives*

i. The application of SPME in aqueous photodegradation experiments has demonstrated to be a rapid and simple alternative to other extraction techniques commonly used in this type of experiments (eg. LLE or SPE). Furthermore, the capacity of extraction and preconcentration of SPME allowed increasing minor photoproducts detectability.

ii. The use of different SPME fibers coatings allowed the simple extraction of a broad range of photoproducts with different chemical structures. The photoformation of some of them had not been previously reported in literature.

iii. For the first time, a miniaturized version of MSPD has been applied to the isolation of photochemical transformation products from cosmetic samples. The micro-MSPD approach allowed working with small portions of sample (50 mg), without diminishing by-products detectability.

iv. The capacity of UV-irradiation to induce the formation of halogenated by-products of parabens and benzoates in water-containing chlorine has been demonstrated, even at very low levels of chlorine. The presence of naturally occurring trace levels of bromine in water led to the formation of brominated by products, which are expected to be more toxic than the parent compounds and their chlorinated analogues.

v. The results of the photodegradation experiments of selected cosmetic preservatives on an artificial skin model confirmed the photoinduced transformation of the antimicrobial triclosan into 2,8-DCDD and other dioxin like photoproducts; as well as the phototransformation of phenyl benzoate and BHT into photoproducts with unknown toxicological properties. These results provide the first data regarding the

photochemical behavior of cosmetic ingredients directly applied on the skin, which represent a contribution to the requirements of public institutions like FDA in this issue.

vi. Finally, it has been demonstrated the usefulness of the coupling of the employed extraction techniques with the hyphenated technique GC-MS for the isolation and determination of transformation products. The standardized fragmentation conditions employed (EI at 70 eV) allowed the use of spectra libraries, which greatly facilitates the hard task of transformation products identification.

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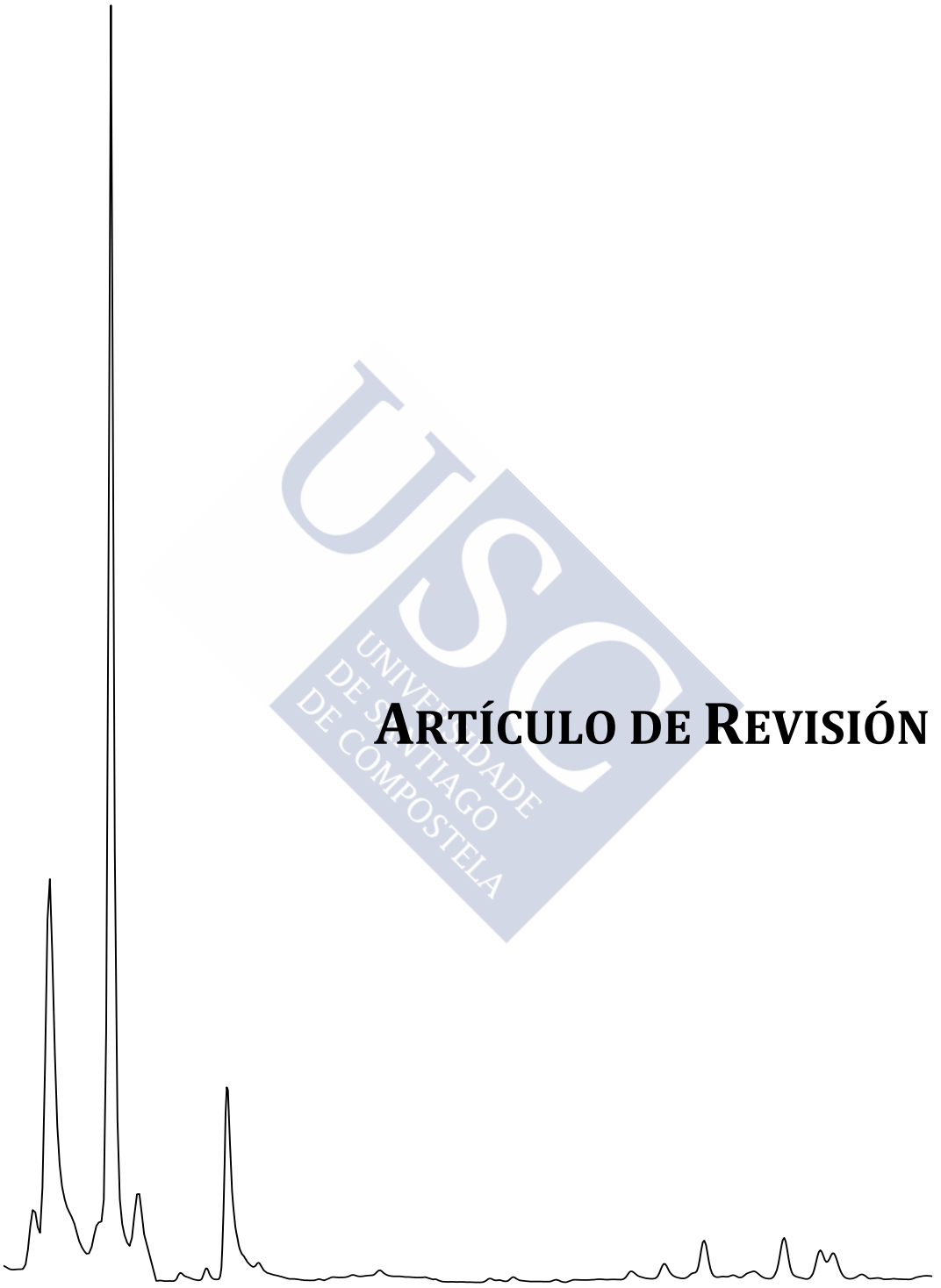

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Cosmetics and Toiletries: Chromatography

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Cosmetics and Toiletries: Chromatography

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Abstract

In recent years, great efforts have been focused on the development of new analytical methodologies to determine the main groups of cosmetic ingredients, among which preservatives, fragrance allergens, synthetic musks, phthalates, and other plasticizers have become subject of great social and scientific concern. Due to the complexity of cosmetic matrices, advanced extraction techniques, in combination with gas and liquid chromatography coupled to mass spectrometry or tandem-mass spectrometry, have been proved as powerful tools to successfully tackle cosmetic analysis. Both cosmetic ingredients safety issues, and recent chromatographic applications for its quality control are dealt with in this article.

1. COSMETIC INGREDIENTS AND COSMETIC SAFETY: GENERAL OVERVIEW.
   - *1.1 Introduction*
   - *1.2 Preservatives*
   - *1.3 Fragrances*
     - *1.3.1 Suspected fragrance allergens*
     - *1.3.2 Synthetic musks*
   - *1.4 Phthalates and Other Plasticizers*
   - *1.5 Microbial Contamination in Cosmetics*

2. CHROMATOGRAPHIC APPLICATIONS FOR COSMETIC QUALITY CONTROL.
   - *2.1 Preservatives*
   - *2.2 Suspected Fragrance Allergens (SAs)*
   - *2.3 Plasticizers and Synthetic Musks*
   - *2.4 Case Study: Multicomponent Analysis in Perfumes*
   - *2.5 Chromatographic Approach for Microbial Contamination Detection*
   - *2.6 Application of the ISO 12787 International Standard*

3. CONCLUSION AND FUTURE TRENDS
4. REFERENCES

Keywords: Chromatography; Cosmetics; Derivatization; Experimental design; Fragrance allergens; Matrix solid-phase dispersion (MSPD); Perfumes; Personal care products (PCPs); Phthalates; Plasticizers;Preservatives; Pressurized liquid extraction (PLE); Solid-phase icroextraction (SPME); Synthetic musks; Tandem-mass spectrometry (MS/MS).

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1. Cosmetic Ingredients and Cosmetic Safety: General Overview

1.1 Introduction

Cosmetics are important consumer products with an essential role in everyone's life: apart from traditional cosmetic products, such as make-up and perfumes, they also include products for hygiene and personal care, for example tooth-care products, shampoos, and soaps. Cosmetic industry is a dynamic and global industry sector with their main markets found in the European Union (EU), the United States (US), and Japan with a value of 72, 59, and 30 billion euros, respectively, according to data compiled in Cosmetics Europe Activity Report 2012.

In recent years, the use of cosmetics and personal care products (PCPs) have grown exponentially, not only for adults but also for babies and children, providing solutions for needs which could not even be envisaged a few years back. Concurrently, the widespread use of these products has led to a social concern about the unintended harmful effects that some cosmetic ingredients could have on consumers health.

To ensure a high level of protection, cosmetic products are regulated and controlled worldwide. The EU Cosmetic Products Regulation, the federal Food, Drug and Cosmetic Act, the Fair Packaging and Labeling Act drawn up by the Food and Drug Administration (FDA) in US, and finally, the Pharmaceutical Affairs Law adopted in Japan, constitute the three main regulatory systems on cosmetic products.

In the EU, the legislative framework is constituted by the new EU Cosmetic Products Regulation (EC) No 1223/209 [1], where a cosmetic is defined as "any substance or preparation intended to be placed in contact with the various external parts of the human body (epidermis, hair system, nails, lips, and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance and/or correcting body odors and/or protecting them or keeping them in good condition." It also includes the list of the prohibited substances which cosmetic formulations must not contain, the restrictions applied to other substances, as well as the rules for proper labeling and other affairs related to consumers safety.

On the other hand, in the US framework, a narrow definition of cosmetics is given, with few restrictions on the ingredients that can be used and the type of safety testing to be undertaken is determined by manufacturers. In this model, 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 [2]. Regulations in Japan is somewhat between the EU and the US models. Japanese legislation is closer to the EU model, with a positive list of ingredients, but the allowed substances and authorized contents are quite different [3]. In recent years the EU Regulation has become an international model, which is easy to understand and more or less easy to apply.

The EU requires that cosmetic products placed on the European market be safe; that is, they 'must not cause damage to human health when applied under normal or reasonably foreseeable conditions of use.' Manufacturers are responsible for ensuring that cosmetic products comply with the law before they are marketed. Regulations are enforced at the national level, and each country in the EU has an authoritative body that is responsible for ensuring compliance. However the huge number of substances to be controlled, their wide range of chemical structures, and the variety of complex matrices in which they can be contained, pose a great challenge for the analytical methodology to be developed.

Within the EU framework there are several official analytical methods proposed to determine some ingredients in cosmetic products, which are described in different EU directives and grouped in a book edited by the European Commission (1999). However, a recent general review of published methodology for cosmetic analysis [4] highlights that, although there exist official analytical methods approved by different regulations, they are not sufficient to carry out their necessary control [5]. These methods only cover a relatively small number of ingredients considering the large amount of substances which are currently regulated by the EU Cosmetics Regulation.

Therefore, the number of official methods of analysis can be considered scarce, thus making it difficult for authorities to control the presence and/or contents of regulated ingredients and the presence of banned substances in cosmetics either through voluntary addition or as a by-product. It should be noted that many ingredients banned in cosmetics, concern pharmaceutical substances that, in the context of the European Pharmacopeia, are covered by specifications and analysis methods used to verify conformity to these specifications. Additionally, COLIPA (the new Cosmetics Europe – The Personal Care Association), in collaboration with other institutions has published a compilation of analytical methods targeted to the control of substances listed in several annexes of the EU Cosmetics Directive, however these methods are 'not official' [6].

In recent years, due to the increasing social and scientific awareness, great efforts have been focused on the development of new analytical methodology, in order to determine the main groups of cosmetic ingredients among which preservatives, fragrance allergens,

synthetic musks, phthalates, and other plasticizers have been tackled. These new contributions lead to the update of some obsolete methods, as well as the improvement of the existing official ones. Both cosmetic ingredients safety and recent developed methodology for its quality control are dealt with in this article.

1.2 Preservatives

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 preservatives allowed in the EU context are listed in the Annex VI of the EU Cosmetics Regulation [1] where limitations, requirements, label warnings, and the maxima permissible concentrations are indicated. Some of the preservatives with most widespread use in cosmetic formulations are described below.

The esters of p-hydroxybenzoic acid (PHBA, parabens), iodopropynyl butylcarbamate (IPBC), 2,4,4'-trichloro-2'- hydroxydiphenyl ether (triclosan, TCS), and bromine-containing preservatives 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 [2].

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 [5].

Isothiazolinone-type biocides are a group of effective preservatives used in a wide variety of aqueous-based industrial or domestic products and applications [7-9]. These compounds are heterocyclic derivatives of 2H-isothiazolin-3-one with an active sulfur moiety capable of oxidizing thiol-containing residues, thereby offering a powerful preservation activity against a broad spectrum of fungi and bacteria [10]. Isothiazolinones like 2-methyl-3-isothiazolinone (MI), and 5-chloro-2-methyl-3-isothiazolinone (CMI) are the active ingredients of a 3:1 CMI/MI mixture commercially sold under the name of Kathon®.

Cosmetic industry commonly includes Kathon® cosmetic grade in a wide range of both rinse-off and leave-on formulations such as shampoos, gels, hair, and skin care products, due to their high effectiveness at very low concentrations [11]. The use of MI and CMI has dramatically risen, and even gained presence at much higher concentrations together with 1,2-benzisothiazolinone (BzI) and 2-octyl-3-isothiazolinone (OI) in cleaning agents and other household and industrial products (water-based coatings, paints, adhesives, wood preservatives, etc.) [12, 13].

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. Parabens and butylated hydroxyanisole (BHA), may modulate and disrupt the endocrine system [14]. IPBC could cause acute inhalation toxicity [15]; TCS may disrupt thyroid function [16], and can form toxic by-products in tap water and in the environment [17-20]; whereas bronidox and bronopol are known to be effective nitrosating agents; whenever these preservatives are coformulated with constituents having secondary amine structures, substantial carcinogenic nitrosamine formation is to be expected [21]. According to the European Regulation on cosmetic products [1] the maximum allowed concentration of parabens is 0.4% (expressed as acid) for a single ester and 0.8% for mixtures of esters. IPBC can be added in a concentration lower than 0.02% in rinse-off products, 0.01% in leave-on products and 0.0075% in deodorants and antiperspirants. This preservative cannot be used in oral and lip products, in products for children under 3 years (except bath products, shower gels, and shampoos), and in body lotions and creams. Bronidox is allowed only in rinse-off products with its maximum allowed concentration in ready-for-use preparations being 0.1%. The maximum concentration allowed for bronopol is also 0.1% but it can be used in rinse-off and leave-on products. Additionally, the manufacturer should also ensure that formation of nitrosamines is avoided in products containing both brominated preservatives. The maximum allowed concentration in the finished product for TCS is 0.3%. 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 [22]; however the report concludes that BHT is safe at the low concentration currently used in cosmetic formulations (from 0.0002% to 0.5%).

Both MI and CMI have been shown as strong skin sensitizers and allergens [11, 23, 24]. Therefore, EU legislation has restricted their presence in cosmetic products to maximum concentrations of 0.0015% for 3:1 CMI/MI mixtures or 0.01% for MI [1]. Besides, the Cosmetic, Toiletry and Fragrance Association (CTFA) recommends using concentrations of

no more than 0.00075% of the CMI/MI mixtures in leave-on products [25]. Recently, the Scientific Committee on Consumer Safety reported that no adequate data was given to support safe use of the Kathon® mixture in leave-on cosmetics [26]. Although the concentration of the nonhalogenated derivative MI alone might be safe, being a weaker sensitizer compared to CMI, new cases of contact dermatitis have been reported [23]. Current scientific experiments also suggest that MI may be a neurotoxic biocide after prolonged exposures even at low levels [27]. Other evidences of contact allergy have been deduced from accumulated case reports associated with occupational or domestic exposure to products containing BzI and OI [28, 29].

There is also current scientific evidence which 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.

1.3 Fragrances

The safety of fragrance ingredients is a top priority for the cosmetics industry in general and in particular, for perfume 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.

Because of the nature of the use of perfumes (leave-on cosmetics), there is a high potential for human exposure. So, it is important that ingredient labeling 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 or other adverse effects [30].

However, not just perfumes contain fragrances as they are also contained in the majority of personal care, household, and laundry products in the market. The term ‘fragrance’ or ‘perfume’ in a cosmetic ingredients list usually represents a complex mixture of dozens of chemicals, which are dominated by synthetic compounds.

1.3.1 Suspected fragrance allergens

Some of the fragrance chemicals have been shown to cause various side effects, like skin sensitivity, rashes, dermatitis, coughing, asthma attacks, migraine, etc. [31-33]. Legislations in force in the three principal markets regarding cosmetic products, that is, in the EU [1], the US [2], and Japan [3], establish that all the ingredients for cosmetics should be included on the label. According to the EU Cosmetic Products Regulation, 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 (SAs) are included in this annex. Two different restrictions are applied to them, that is, 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. The possible negative effects on health by such substances may drive to at least a decrease of these values. In fact, it has been already observed that the inclusion of the term 'fragrance free' in several cosmetic products, is a positive characteristic.

European legislation requires monitoring 27 compounds used in perfumery as they might elicit skin sensitization, the so-called potentially allergen substances 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 [34]. 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, so its control has also been proposed in some surveys included in this article.

1.3.2 Synthetic musks

Synthetic musks are used as an alternative for the natural musk, a class of aromatic substance obtained from the glandular secretion of animals such as the musk deer, or from numerous plants emitting similar fragrances. These substances [35] exhibit a strong, warm, sensual, and long-lasting odor, which makes them essential in modern perfumery and form

the base note foundations of most perfume formulas. Nitromusks dominated the market for many years but declined significantly in the 1990s [36]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 [37]. Nevertheless, these compounds are still largely used in PCPs, sanitation products, and fragrances [38]. 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 consequently replaced it gradually by a third group of fragrances, consisting of partially artificial and partially nature identical members, the macrocyclic musks [39].

Some fragrance ingredients are not perfuming agents themselves but enhance the performance of perfuming agents. For example, diethyl phthalate (DEP) is widely used in cosmetic fragrances to make the scent linger. However, the European Commission on Endocrine Disruption has listed DEP as a Category 1 priority substance, based on evidence that it interferes with hormone function [40]. Despite the potential health risks, phthalates are still choice ingredients in cosmetics because they are cheap and versatile. This group of ingredients is dealt with below.

1.4 Phthalates and Other Plasticizers

Phthalates (esters of phthalic acid) are a group of chemicals with a wide variety of industrial applications [41, 42]. Regarding cosmetics, they are used as plasticizers in products such as nail polishes and hair sprays, and as solvents and perfume fixatives in many other products. Phthalates are commonly found in perfumes mainly as carriers or solvents for synthetic musks. Among the principal phthalates used in cosmetics, dimethyl phthalate and 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; so their analytical control has also been considered in some recently developed methods. 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 nonnegligible amounts of DEP [43]. Other phthalates such as: di-n-butyl phthalate

(DBP), bis(2-ethylhexyl) phthalate (DEHP), bis(2-methoxyethyl) phthalate (DMEP), and di-n-pentyl phthalate (DPP), are forbidden by the EU [1].

This class of chemicals has been linked to hormone disruption, which can affect development and fertility[44]. Due to their potential risks for human health and environment, several of them have been included in the priority list of pollutants of different organizations. Although some phthalates such as DBP or DEHP are being phased out of cosmetics, others such as DEP are still used without restrictions in many products including fragrances, as mentioned before.

1.5 Microbial Contamination in Cosmetics

Most of the cosmetic formulations, due to their composition and high water content, are products suitable for biological degradation by microorganisms [45]. Microbial contamination in cosmetics represents an important risk for consumer health, since it can lead to irritations or infection, especially when these products are applied on damaged skin, eyes, or on babies [46]. Proof of that are the reported outbreaks of microbial infection from hospitalized individuals caused by contaminated mouthwash [47-49] and moisturizing milk [50]. Other studies about different contaminated cosmetics, recalled from the US and EU, highlight *Pseudomonas aeruginosa* as the main pathogen found in this kind of product [51, 52]. Besides, the genera *Klebsiella spp.* and *Serratia spp*. were also found amongst the most typical contaminants isolated from cosmetics, being the genus *Pseudomonas spp*., the most frequently responsible and the main problem for the cosmetics industry [53].

Nowadays, regulations regarding the microbiological content in cosmetic products do not exist; the only requirement for cosmetics in the EU Directive is that they must not cause damage to human health when applied under normal or reasonably foreseeable conditions of use [1]. Due to the lack of official cosmetic guidelines on this matter, some recommendations have been published by governments and cosmetic associations.

Recommendations on limits of microbial contamination in cosmetic products can be found in the notes of guidance prepared by the EU's Scientific Committee of Consumer Products [54]. Similar challenging tests are described in the European Pharmacopeia [55], the US Pharmacopeia, the Cosmetic, Toiletries and Fragrance Association and the American Society for Testing and Materials, as sources for guidelines covering, testing of preservation efficacy in cosmetic and toiletry products [53].

The microbiological testing methods applied to cosmetics by these associations are usually based on traditional plate counts. These growth-based methods, although demanding no expensive infrastructure and being rather cheap in consumables, have well known inherent limitations such as time-consuming procedures both in operation and data collection, are laborious to perform, demand large volumes of liquid or solid media and reagents, and lead to ambiguous results associated with using turbidity as a detection end point [56, 57]. Thus, the need for providing fast and reliable methods to cosmetic microbiologists and manufacturers to detect microbial contamination has been highlighted by several authors [56-58]. In this way, a novel chromatographic approach to microbiological analysis of contaminated cosmetic products is described in Section 'Chromatographic Approach for Microbial Contamination Detection' of this article.

2. Chromatographic Applications for Cosmetic Quality Control

Since cosmetic formulations very often contain complex mixtures of different classes of ingredients, the required multicomponent analysis becomes a challenging task without a previous separation step. In this sense, liquid chromatography (LC) and gas chromatography (GC) coupled to different detectors are the most commonly used techniques for the analysis of cosmetic ingredients.

LC is the most commonly used technique for the analysis of ingredients such as preservatives, UV filters or dyes, being UV/vis the most widespread used detection technique in literature. Traditionally, thin layer chromatography (TLC) has been widely used for qualitative separation in method development for identification purposes. On the other hand, GC has been mainly used for determination of fragrance ingredients and some preservatives as this technique is used to analyze volatile compounds offering a high separation capability of complex mixtures. Flame-ionization detector (FID), electron capture detector or mass spectrometry (MS) detector were most commonly used coupled to the GC instrument. In terms of selectivity, MS and tandem MS/MS represent, at present, powerful detection tools for both chromatographic techniques.

To achieve a good chromatographic performance, sample preparation is a vital factor to be considered to succeed in the development of a new methodology. Due to the complexity of cosmetics samples, the procedures used to extract ingredients depend on the nature of the products (emulsion, cream, shampoos, etc.) and also the characteristics of the analytical

techniques to be employed to determine the active substances. For chromatographic analyses, based on liquid or gas chromatographic techniques, it is generally possible to dilute aqueous samples in a suitable solvent or extract target compounds with organic or hydroorganic mixtures [5].

However, sample preparation techniques in these procedures are frequently tedious and time-consuming, and the use of hazardous solvents is usually required. For most cosmetic samples, it is not possible to simply dilute the sample in an adequate solvent prior to analysis since several sample components would not be solubilized and we would not obtain homogeneous extracts. In addition, the complexity of the obtained solutions would cause chromatographic contamination after few analyses, and coelution of matrix components, making really hard to obtain satisfactory analytical results for the target compounds. 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. Moreover, the possible presence of interferences that could distort the results is not rejectable.

To overcome some of these drawbacks, advanced extraction techniques such as supercritical fluid extraction (SFE), solid-phase extraction (SPE), and solid-phase microextraction (SPME), pressurized liquid extraction (PLE) and matrix solid-phase dispersion (MSPD), have been recently applied for the determination of different additives in cosmetics. These extraction procedures in combination with GC–MS, GC–MS/MS or high performance LC (HPLC)–MS/MS have arisen as powerful methods to successfully tackle cosmetic analysis.

The most recently developed analytical methods for the determination of the main groups of cosmetic ingredients which have arisen as social concern; preservatives, fragrance allergens, synthetic musks, phthalates, and other plasticizers are reviewed below. These new chromatographic applications represent an updated analytical alternative for testing cosmetic products.

2.1 Preservatives

Simultaneous analysis of more than one class of preservatives are mainly based on LC, in particular both ion-pair and reversed-phase LC with UV/vis detection [59-61]. Capillary electrophoresis [62-64], capillary zone electrophoresis [65, 66] and micellar electrokinetic chromatography (MERKC) [67-70] have also been widely used. These techniques have become popular separation tools in preservative analysis, used to determine both charged and hydrophobic compounds after addition to the running buffer of a surfactant or a

modifier[5]. Flow injection analysis has also been employed, enhancing sample throughput [71].

Most of the sample treatment and extraction procedures reported using the aforementioned analytical techniques are mainly based on simple sample dilution in a suitable solvent, vortex extraction or ultrasound-assisted extraction with organic or hydroorganic mixtures. However, sometimes, these extraction processes also needs a cleanup step. In this sense, some authors have recently adopted advanced extraction procedures, for preservatives analysis, such as SFE [59, 66, 72], SPE [73], SPME [74, 75], PLE [76, 77], or MSPD [78, 79], which do not require great amount of solvent since they are preconcentration techniques, avoiding further cleanup.

As far as preservatives are concerned, a great part of the analytical effort has been focused on parabens determination [66, 67, 72, 73, 80-89], while methods for the determination of other preservatives in cosmetic formulation are more limited. Due to the polar nature of most preservatives such as parabens, a derivatization step previous to GC analysis is highly recommended to reduce adsorption in the chromatographic system, and improve sensitivity, peak separation, and peak symmetry [72, 80]. Acetylation is one of the most common derivatization procedures for phenolic compounds [90, 91], applied for the determination of parabens and TCS in water [92] and recently employed in the analysis of such preservatives in cosmetic samples [77, 79]. The advantages of acetylation are the high efficiency obtained using low-cost reagents, especially compared with silylation agents.

Before GC analysis, an effective one-step sample preparation methodology applying PLE has been developed for the analysis of multiclass preservatives in leave-on cosmetics [76]. This technique is quick, 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. This is the first time PLE is applied to this kind of matrices using a simple, cheap, and quick derivatization procedure: acetylation with acetic anhydride and pyridine was used (Figure 1). 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. One of the main advantages of this procedure is 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.

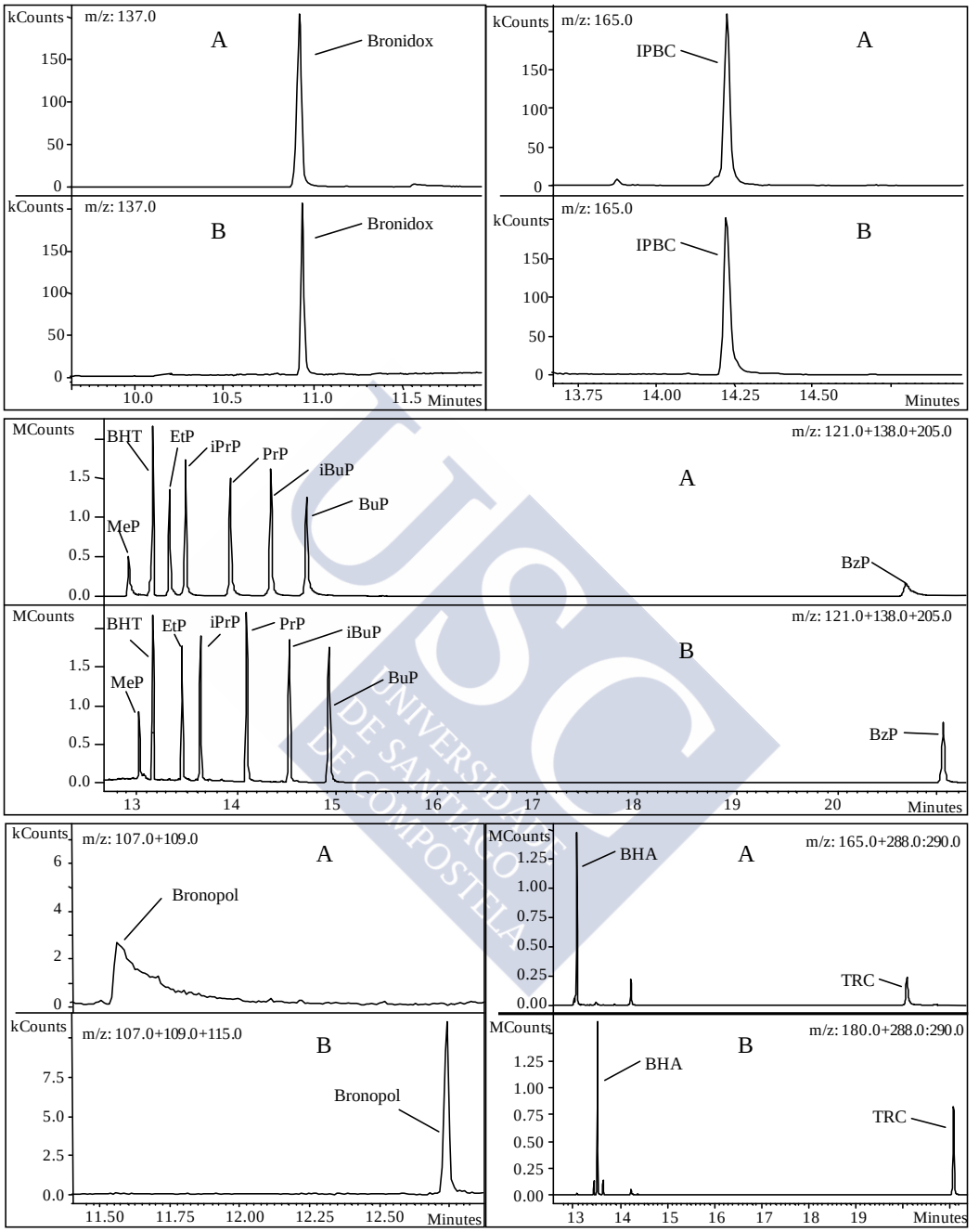

Fig. 1. Extracted ion chromatograms corresponding to a 10 mg ml^{-1} solution of the target analytes before (A) and after (B) derivatization.

MSPD, introduced by Barker et al. [93] in 1989, has also been shown as a suitable extraction procedure for cosmetic ingredients. MSPD involves blending a viscous, solid, or semisolid sample with a solid support. The shearing forces of blending with a mortar and pestle disrupt the gross architecture of the sample, breaking the material into smaller pieces. At the same time, sample components dissolve and disperse into the bound organic phase on the surface of the particle, leading to complete disruption of the sample and its dispersion over the surface. The possibility of performing extraction and cleanup at the same time is one of the main advantages of this technique, which reduces sample contamination during the procedure and decreases the amount of solvent required [94, 95]. MSPD developments and applications are compiled in several reviews [94-97].

MSPD has been successfully applied for the determination of multiclass preservatives including two bromine-containing preservatives, seven parabens, IPBC, TCS, and the antioxidant preservatives BHA and BHT in a wide variety of cosmetic samples including rinse-off and leave-on products [79]. After extraction, derivatization with acetic anhydride, and GC–MS analysis were performed. Optimization studies were done on real non-spiked and spiked leave-on and rinse-off cosmetic samples. The selection of the most suitable extraction conditions was made using statistical tools such as ANOVA, as well as factorial experimental designs (Figures 2 and 3). The final optimized conditions were common for both groups of cosmetics and included the dispersion of the sample with florisil (1:4), and the elution of the MSPD column with 5 ml of hexane/acetone (1:1). After derivatization, the extract was analyzed without any further cleanup or concentration step. Recovery studies were performed on leave-on and rinse-off samples, verifying the reliability of the optimized procedure. The quantification limits were well below those set by the international cosmetic regulations, making this multicomponent analytical method suitable for routine control.

Both PLE-GC-MS and MSPD-GC-MS methods were further compared and indistinctly applied to the analysis of a great variety of rinse-off and leave-on cosmetic and PCPs intended for baby and child care [98]. In a total of 47 different baby and child care products, methyl paraben (MeP) and propyl paraben (PrP) were the most abundant preservatives followed by BHT, butyl paraben (BuP), and ethyl paraben (EtP). Parabens were found in some products at concentrations close to the legal limit.

New liquid-liquid extraction (LLE) approaches, such as Dispersive liquid–liquid microextraction (DLLME) [83, 88] has been shown as an efficient method for the extraction of several parabens in cosmetic products, on the basis of a safe organic solvent, octanol.

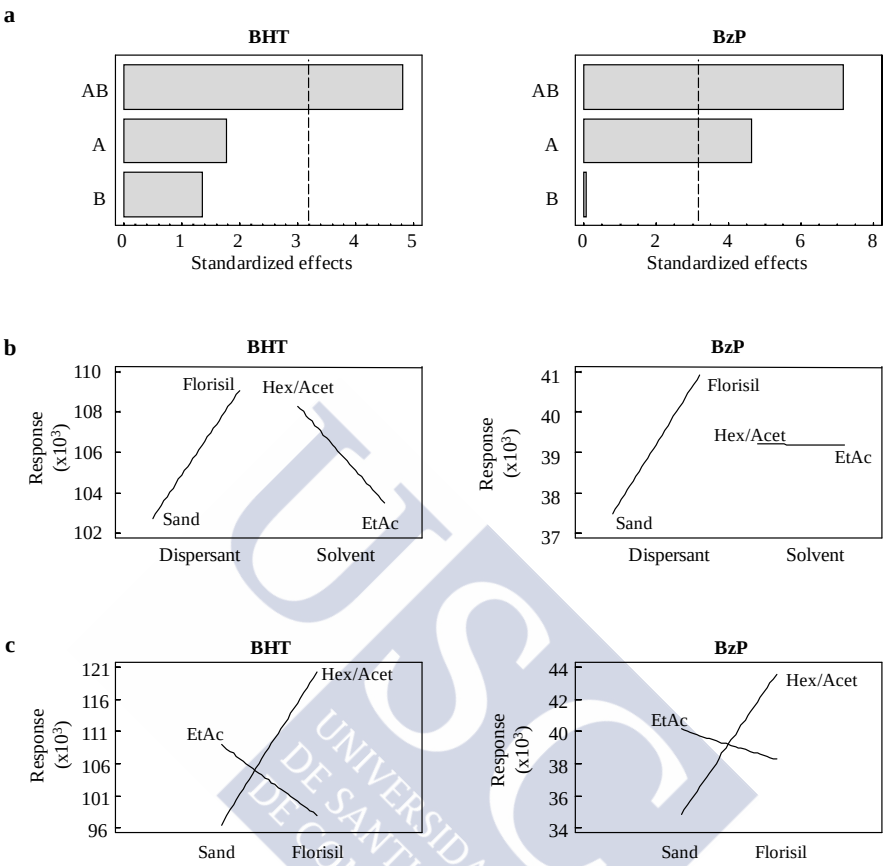

Fig. 2. Representative graphics showing the experimental design results for the rinse-off cosmetic: (a) Pareto charts; (b) main effects plots; and (c) interaction diagrams. Factor A, dispersant; factor B: solvent.

The analytical use of ultrasound-generated emulsions has found a growing interest to improve efficiency in LLE since they increase the speed of the mass transfer between the two immiscible phases implied. Thus, dispersed droplets can act as efficient liquid–liquid microextractors in the continuous phase, and later they can be readily separated by centrifugation [99]. A method based on ultrasound-assisted emulsification microextraction (USAEME) was developed for the determination of sodium benzoate (SB), PHBA, methyl-paraben, ethyl-paraben, and propyl-paraben in different samples [100].

GC coupled to tandem MS (GC–MS/MS) with dynamic selected reaction monitoring was very recently applied to parabens determination [82] in PCPs. A rapid and sensitive

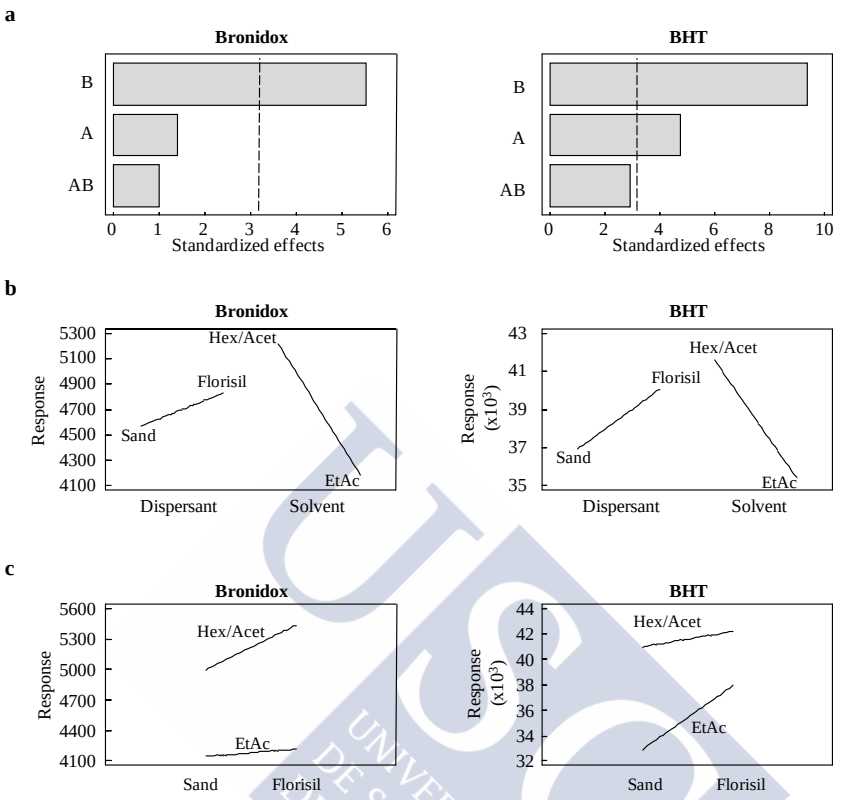

Fig. 3. Representative graphics showing the experimental design results for the leave-on cosmetic: (a) pareto charts; (b) main effects plots; and (c) interaction diagrams. Factor A, dispersant; factor B, solvent.

analytical method was developed for the analysis of methyl-, ethyl-, propyl-, and butyl parabens, using the corresponding isotopically labeled parabens as internal standards to correct for recovery and matrix effects.

Regarding isothiazolinone analysis in cosmetic products, the number of studies is really low and methods only include MI and CMI. They are mainly based on solvent extraction followed by HPLC–UV detection, requiring extract purification [60, 101]. Isothiazolinone-type preservatives analysis has been accomplished in other matrices such as water, nonformalin adhesives, or food paper by LC or HPLC [12, 102-105] and, in a lesser extent, by GC [8, 9]. The use of GC does not show, in general, good performance for MI and CMI analysis and, additionally, BzI requires derivatization to reduce adsorption in the chromatographic system and improve sensitivity. LLE, ultrasonication or SPE are the procedures reported for isothiazolinones isolation. However, poor MI extraction yields have

been obtained [8, 12] from high water-content matrices, due to the high polarity and water solubility of MI.

MSPD coupled to HPLC–tandem MS (MSPD-HPLC–MS/MS) has been described for isothiazolinones analysis in cosmetics, providing high selectivity and sensitivity and avoiding an awkward derivatization step [106]. The main factors affecting the MSPD extraction procedure, the dispersive phase and the elution solvent, were assessed and optimized through a multicategorical experimental design, using a real cosmetic sample. The most suitable extraction conditions comprise the use of 2 g of florisil as dispersive phase and 5 ml of methanol as elution solvent. Subsequently, the extract is readily analyzed by HPLC–MS/MS without any further cleanup or concentration steps. This kind of methodologies fulfills the most relevant criteria required for a suitable extraction procedure such as low cost, minimum solvent consumption, and short process times, since high-sample throughput is attained performing the extraction in about 20 min.

Several analytical procedures for the determination of bronidox in cosmetic samples have been reported, most of them based on TLC [107] and LC [30, 108-111]. GC-based methods have been also developed but all of them came out in low international repercussion and, consequently, hard-to-reach journals.

A procedure based on SPME combined with GC-mECD for the determination of preservative bronidox in rinse-off cosmetics has been reported. SPME integrates sampling, extraction, concentration, and sample introduction into a single uninterrupted process, resulting in high-sample throughput [74]. This advanced extraction technique has been also applied to the analysis of other kind of preservatives such as parabens in cosmetic samples [75]. The implementation of SPME to bronidox analysis is considered as a valuable alternative analytical technique to more traditional procedures, reducing the laboratory generated waste and time for sample preparation. Negative-matrix effects due to the complexity of the studied samples were reduced by dilution with ultrapure water. A factorial design was selected to study and optimize main experimental factors affecting SPME. The optimized method was validated and applied to the determination of the target compound in different cosmetic samples. SPME-based methodologies have the advantages of short duration, simplicity, and absence of organic solvents.

Other preservatives such as TCS were individually determined by an efficient in-tube based ultrasound-assisted salt-induced liquid–liquid microextraction (IT-USA-SI-LLME) technique [112]. IT-USA-SI-LLME method is a variation of USAEME, based on the rapid phase separation of water-miscible organic solvent from the aqueous phase in the presence of high

concentration of salt (salting-out phenomena) under ultrasonication. The resulting extract was further analyzed by LC.

2.2 Suspected Fragrance Allergens

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. Despite the presence of chromophoric groups, most of these fragrances allow the use of HPLC with ultraviolet detection [113]. However, GC–MS can be considered the technique of choice for the analysis of this kind of volatile substances [114-117] .

The use of ions extracted from an analysis in scan mode [118], has been shown to be less sensitive and accurate than selected ion monitoring (SIM) approach [119], where only a few previously selected ions are monitored[118]. Therefore, the higher the sensitivity obtained, the lower the sample amount to be injected is required, avoiding the column overload and improving peak resolution. In fact, SIM mode was adopted by the International Fragrance Association (IFRA), which proposed a method that can be used as a reference in the laboratories of the fragrance industry to determine SAs in fragrance concentrates [34]. However, in the case of highly complex mixtures of fragrances, the GC–MS determination in full scan mode is recommended to accomplish, by several authors in order to prevent false positives (mainly due to coelutions of perfume constituents exhibiting isobaric ions in common with SAs) and false negatives (due to the shift that a large nonrelated eluting peak in front of the SIM window can promote on the SAs retention time) [114, 120, 121].

An alternative is to choose more selective ions in the SIM mode, using softer chemical ionization, which gives rise to less fragmentation than electronic impact (EI), yielding more abundant ions at higher masses and increasing selectivity [122].

To overcome some drawbacks arising from the matrix interferences, alternative chromatographic approaches can be applied. Size-exclusion chromatography (SEC) combined with GC–MS shows bigger benefits compared to other methods, since the flexible cleanup with SEC allows the determination of a large range of compounds in difficult matrices with GC–MS [116].

One-dimensional/two-dimensional GC–MS (GC×GC) [123] and target multidimensional GC (MDGC)–MS [124] have been applied for the determination of SAs in cosmetic products. A combination of full evaporation-dynamic headspace (FEDHS) with selectable

one-dimensional/two-dimensional GC–MS can be used. The FEDHS approach allows the nondiscriminating extraction and injection of both apolar and polar fragrance compounds, without contamination of the analytical system by high molecular weight nonvolatile matrix compounds. The two-dimensional mode is especially useful to confirm the presence of detected allergen compounds when mass spectral deconvolution is not possible. On the other hand, the MDGC approach has the advantage of simpler data processing compared to GC×GC approach.

Although GC is considered as the best option for SAs analysis, LC–MS/MS was proposed for quantitative determination of atranol and chloratranol [125], being further applied to investigate the presence of both chemicals in perfumes and similar products on the European market [126].

While liquid samples such as perfumes or perfumed oils, can be directly analyzed usually after sample dilution [121, 127, 128], the direct analysis of other cosmetic samples, such as creams and lotions, is quite problematic. The main reasons are the contamination of the chromatographic inlet and column, which occurs after a few analyses [115], 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, several extractive approaches have been developed for SAs analysis in cosmetics.

In sorptive extraction methods such as SPME and stir-bar sorptive extraction (SBSE), the extraction phase can be exposed either directly to the sample or to the headspace of the sample being further desorbed in the injection system. The simplicity of these techniques makes them suitable for sampling automation. However, to avoid matrix effect, dilution of an aliquot of cosmetic sample in a large amount of water is required. The sorptive extraction can still be improved using a cold fiber approach, which can be even fully automated and miniaturized [129].

By using packed liners or cartridges with polydimethylsiloxane (PDMS) particles, the on-line thermal fractionation of a sample can be easily achieved with a thermo desorber of headspace cartridges [130], or in the injector itself [115]. In this technique, cosmetic matrix does not interfere with quantification, as volatile analytes are isolated from the rest of the sample [131].

PLE has been recently applied for SAs extraction. PLE is fast, increases automatation, 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. In this way, solid-phase dispersion-PLE followed by GC–MS has been applied

for the determination of 26 SAs (all that regulated in the EU Cosmetics Directive amenable by GC, as well as pinene and methyleugenol) in cosmetic samples [76]. The effects of the temperature, extraction time and solvent, and dispersing sorbent, affecting the procedure, have been evaluated using a multifactor strategy. The optimal conditions after the analysis of main and second order effects entailed the extraction at 12 °C for 15 min, using hexane/acetone as solvent, and florisil as dispersing sorbent. The method's performance has been studied, as showing good linearity and precision. Detection limits (S/N = 3) ranged from 0.000001% to 0.0002% (w/w), values far below the established restrictions as regard labeling 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 ten samples (several moisturizing and antiwrinkle 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.

The PLE method has proved to be an efficient and rapid technique for the extraction of this kind of targets from cosmetic samples. Nevertheless, one of the main drawbacks of this methodology is the high cost of the instrumentation compared to the low cost of other techniques such as MSPD, which does not require special equipment. The possibility of performing extraction and cleanup at the same time is one of the main advantages of this technique, which reduces sample contamination during the procedure and decreases the amount of solvent required [94, 95].

An effective low-cost sample preparation methodology for the determination of regulated fragrance allergens in leave-on and rinse-off cosmetics has been developed applying, for the first time, MSPD to this kind of analytes and samples [78]. The selection of the most suitable extraction conditions was made using statistical tools such as ANOVA, as well as a factorial multifactor experimental design (Figure 4). These studies were carried out using real cosmetic samples. In the final conditions, 0.5 of sample, previously mixed with 1 g of anhydrous Na_2SO_4, were blended with 2 g of dispersive sorbent (florisil), and the MSPD column was eluted with 5 ml of hexane/acetone (1:1). The extract was then directly analyzed by GC–MS. Quantitative recoveries (>75%) were obtained and RSD values were lower than 10% in all cases. The quantification limits were well below those set by the international cosmetic regulations, making this multicomponent analytical method suitable for routine control. In addition, the MSPD method can be implemented in any laboratory at low cost since it does not require special equipment. A wide variety of cosmetic products were

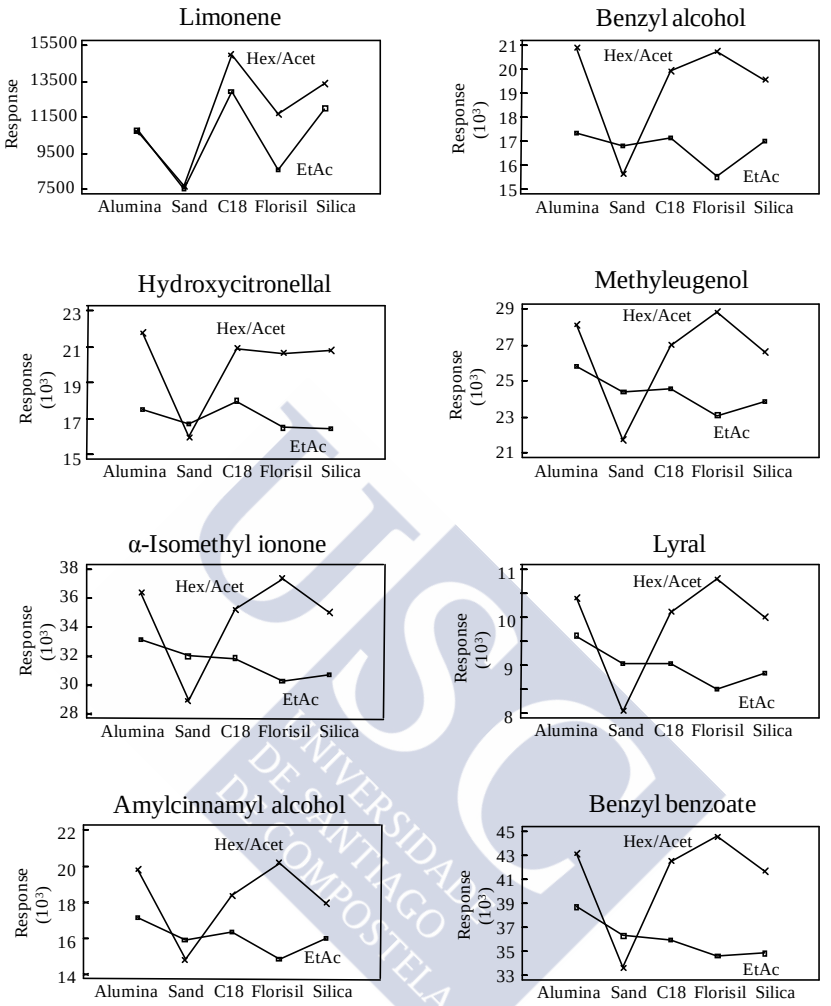

Fig. 4. Interaction plots for some representative fragrance allergens (Hex/Acet, hexane/acetone; EtAc, ethyl acetate).

analyzed. All the samples contained several of the target cosmetic ingredients, with an average number of seven. The total fragrance allergen content was in general quite high, even in baby care products, with values close to or up to 1%, for several samples, although the actual European Cosmetic Regulation was fulfilled.

2.3 Plasticizers and Synthetic Musks

A look at the scientific literature evidences the lack of studies devoted to the development of methodology for the determination of musks in cosmetics, as well as the scarcity of studies about phthalates analysis in cosmetic products.

Currently, no official method exists for the determination of phthalates and musks in cosmetic, and only few methods can be found in the literature [132-140]. HPLC/UV is the most commonly described method for the determination of phthalates in cosmetic products [133-135, 137-139], with EtOH, MeOH, or acetonitrile (ACN) being the most widely used elution solvents. Most of these works are focused on the identification and quantification of the phthalates most commonly found in cosmetics (DEP, DBP, DEHP, and butyl benzyl phthalate (BBP)), whereas some others also deal with less used phthalates such as di-n-heptyl phthalate (DnHP) [135] or di-allyl phthalate [139]. In addition, in most of these studies the number of analytes considered is generally low.

Although to a lesser extent, GC coupled to FID and MS has also been applied for the quantification and the identification of phthalates in cosmetic products [134, 136]. In the case of MS detection, EI as ionization mode, and SIM approach (m/z = 149) were used.

Sample preparation procedure for phthalates extraction, usually consists of solvent extraction by mechanical shaking or by sonication [134-137], followed by centrifugation or filtration. In some cases, a SPE cleanup or dilution step is included. Other methods, based on the extraction of phthalates from several cosmetic samples with hexane on a celite column have been reported [132, 133].

Modern techniques such as PMMEs (polymer monolith microextraction) [138] and a new LLE approach with USAEME SFO (USAEME with droplet solidification of floating organic) [139] have also been proposed for phthalates extraction from cosmetics. The USAEME technique proposed by Regueiro et al. in 2008 [99] has recently been applied to the analysis of phthalate esters in cosmetics and environmental water samples [139]. Other studies regarding the analysis of phthalates, musks, and other ingredients in cosmetics have been published, dealing with the analysis of perfumes, by direct analysis or after sample dilution by GC–MS [140, 141].

A new sorptive extraction approach based on ethylene glycol-silicone coated stir bars, which have recently become commercially available, was developed for the simultaneous determination of bisphenol A, bisphenol F, and bisphenol Z by SBSE in combination with thermal desorption-GC–MS (TD-GC–MS) [142].

MSPD, which has been recently proposed for the analysis of preservatives and fragrance allergens in cosmetic samples, providing efficient and low cost extractions, and meeting the requirements of the 'green chemistry' [78, 79, 106], has also been proved to be a suitable analytical tool for the extraction or the determination of 18 plasticizers and 12 musk fragrances in leave-on and rinse-off cosmetics [143]. Multivariate optimization was carried out using real cosmetic samples and method's quality parameters were also evaluated on real cosmetic samples. The MSPD method was miniaturized and customized to avoid or minimize risks of phthalate contamination and to reduce residues and costs. It does not require special equipment since the extraction is performed in glass Pasteur pipettes with glass wool plugs and, thus, it can be easily implemented in any laboratory at negligible costs. Method accuracy and precision were satisfactory, showing mean recovery values from 85% to 105%, and RSD was generally below 8%. The method was also applied to a broad range of cosmetics demonstrating the suitability of the optimized procedure. Twenty-five out of thirty targets were detected in the samples. The presence of EU regulation prohibited phthalates (mainly DBP and DEHP, at concentrations up to 141 mg g^{-1}) and nitromusk was confirmed in a high number of cosmetics. None of the phthalates, nor the musks were included in the list of ingredients of the products.

Concurrently, other analytical methods were proposed for the separation and quantification of 12 phthalates by GC-MS including the eight phthalates regulated by the European Regulation (DBP, DEHP, BBP, DMEP, DnPP, diisopentyl phthalate (DiPP), DPP, and diisobutyl phthalate (DiBP)) [144]. This work applies the approach for result validation described in the ISO 12787 international standard 'Cosmetics–Analytical methods–Validation criteria for analytical results using chromatographic techniques,' which is reviewed in Section 'Application of the ISO 12787 International Standard' of this article.

A chromatographic alternative applying nonaqueous MERKC can also be considered for the analysis of polycyclic musks in perfumes [145]. This optimized methodology can be successfully applied to the analysis of commercial perfume samples for the determination of Tonalide®, Galaxolide®, and Traseolide® polycyclic musks.

2.4 Case Study: Multicomponent Analysis in Perfumes

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 [146]. Nevertheless, there is an increasingly pressing demand to know perfume's composition in-

depth, in particular due to the possible biological activities of some ingredients [147], like many of the compounds whose control has been dealt with in this article. 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 [77, 148-150] or combinations of preservatives (parabens) and phthalates [134] or preservatives including antioxidants [59, 71, 75, 151], but in no case focused on the analysis of perfumes. Otherwise, in the case of fragrance allergens, the developed methodology has been based mainly on GC–MS [34, 128, 131, 152] or comprehensive two-dimensional gas chromatograph with MS detection [153, 154] or, more recently, with FID detection combined with chemometrical tools [155]. 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 [39].

A reliable multicomponent analytical method applicable in control laboratories, from companies and institutions, with a high-throughput level was reported [140]. 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), 12 synthetic musks (five nitromusks, six polycyclic, and one macrocyclic), 26 fragrance allergens, and eight phthalates. Instrument and analytical conditions have been optimized; no pretreatment 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. The obtained LODs are far below the established restrictions as regards labeling in the European Cosmetics Regulation.

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 maximum allowed concentrations of the ingredients and/or ingredient labeling. All samples contained some of the target ingredients. Several samples do not comply with the regulations concerning the presence of phthalates (Figure 5). Musks data confirmed the trend about the replacement of nitromusks by polycyclic musks; as well as the

noticeable introduction of macrocyclic musks in the perfume composition. The prohibited musk moskene has been detected in one sample in an appreciable concentration. The average number of fragrance allergens is 12 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. Preservative 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 labeling has been evaluated in a subset of samples, and only about 38% of the perfumes were properly labeled for the allergens tested.

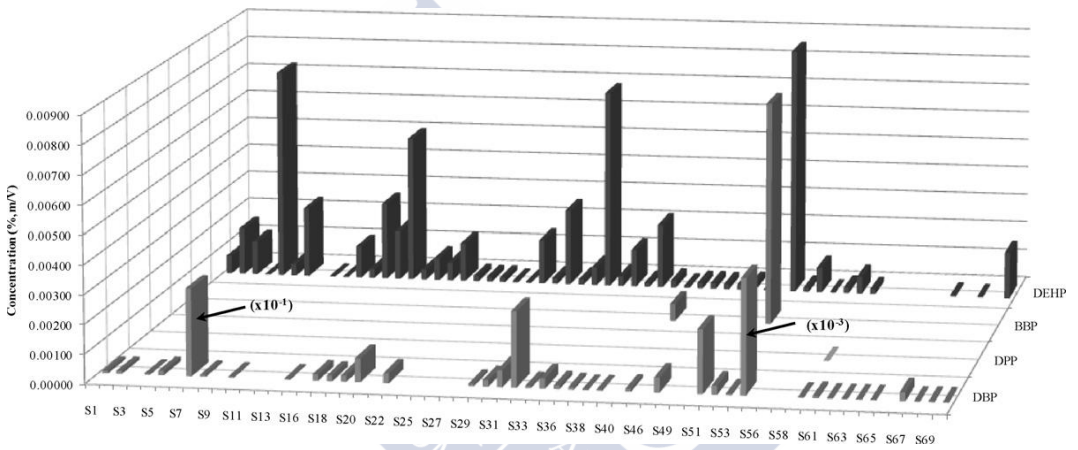

Fig. 5. Partial distribution of the phthalates in the samples. The plot shows the irregular samples containing phthalates banned in the EU.

2.5 Chromatographic Approach for Microbial Contamination Detection

As an alternative to more traditional microbiological testing methods applied to cosmetics, the development of quick methods such as bioluminescence, impedance or cytometry, based on the metabolic state of microorganisms, as well as MS and nucleic-acid based methods, allow the detection of microbial contamination within 24 h, both in the finished product and in raw materials. Therefore, these methods are of great industrial importance, since they facilitate the rapid release of products into the market. Despite the advantages, quick methods have not been widely introduced for routine microbiological testing yet, mainly, due to the nonspecificity in microbial detection and the lack of equivalence with the official methods [57, 156].

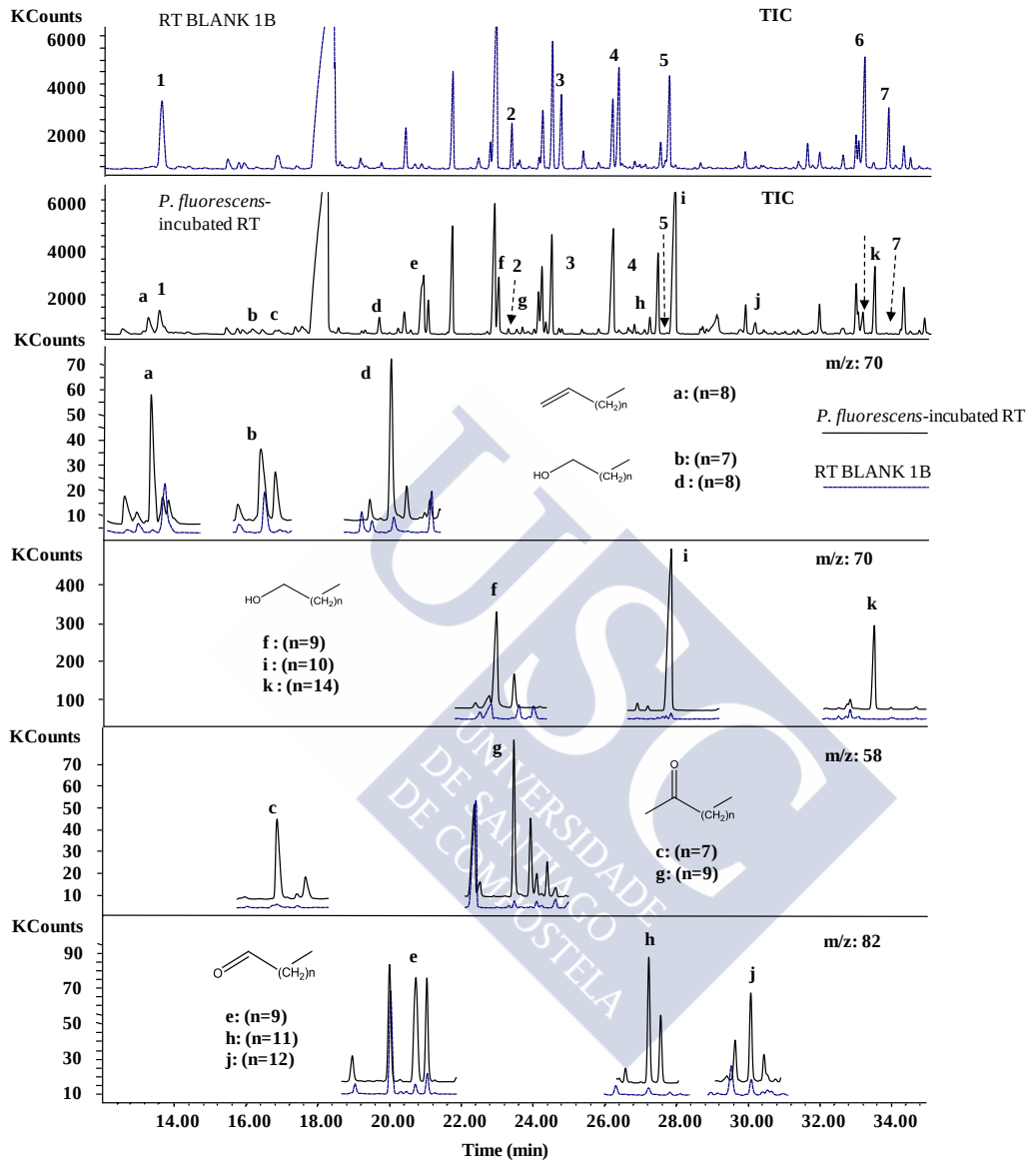

Fig. 6. Total ion chromatogram (TIC) and extracted ion chromatograms for the collected volatiles from a revitalizing tonic (RT) incubated with *P. fluorescens*. MVOCs identified in the bacterium-incubated sample: (a) 1-undecene; (b) 1-nonanol; (c) 2-decanone; (d) 1-decanol; (e) undecanal; (f) 1-undecanol; (g) 2-dodecanone; (h) tridecanal; (i) 1-dodecanol; (j) tetradecanal; and (k) 1-hexadecanol. Volatile compounds with a different profile in the incubated sample and in the BLANK 1B: (1) linalool; (2) geranyl acetate; (3) α-ionone; (4) α-isomethyl ionone; (5) lilial; (6) celestolide; and (7) α-hexylcinnamaldehyde.

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Many volatile organic compounds (MVOCs) are generated by microorganisms during metabolic processes [157, 158]. To date, many reports have attempted to establish correlations between MVOCs and specific specimens, and consequently to determine whether some MVOCs could serve as markers for the detection of certain microbial species. Thus, some cancer biomarkers were identified from cancer stomach tissues infected with *Helicobacter pylori* [159, 160]. It was also possible to identify the volatile compounds characteristic of sinus-related bacteria from infected sinus mucus. Other studies were carried out for characterizing MVOCs with nematicidal and fungicidal activity from a group of soil bacteria [161, 162], the volatile profile of the rhizobacteria [163] and the bacteria isolated from the spoilage flora of cold-smoked salmon [164]. In these studies, headspace SPME (HSSPME) is the most used sampling technique, since SPME integrates extraction, concentration and introduction in one-step, which results in reducing the sample preparation time and simultaneously increasing its sensitivity over other extraction techniques [165]. In fact, HSSPME coupled with GC-MS is considered as a valuable choice for the analysis of MVOCs [158]. In addition, GC-MS is a technique with great potential to identify volatiles characteristic of microbial metabolism that could be exploited for detection by sensor-based electronic recognition methods [166].

A novel approach based on MVOC analysis was proposed, for the first time, as an alternative method for the rapid detection of microbial contamination in cosmetics by SPME-GC-MS [167]. In order to characterize the MVOCs of the typical microbial contaminants in cosmetic products, HSSPME was applied, to collect headspace volatiles above pure bacterial cultures as well as above inoculated real samples (Figure 6). The subsequent separation and identification of MVOCs was performed by GC-MS analysis of the collected volatile fraction. The volatile fraction analysis revealed, amongst other compounds, the presence of several odd-numbered carbon (C9–C15) methyl ketones and alkanols, which have been reported as characteristic compounds of bacterial origin. Some of them were found both in pure bacterial cultures and in the samples. However, other compounds not seen in cultures were seen in the cosmetics, suggesting that substrate is a very influential factor.

In this 'proof-of-concept' study, a MVOC-based method is proposed for the first time, for the rapid detection of microbial contamination in cosmetics. The results suggest that it could be clearly feasible to qualitatively identify viable microorganisms in cosmetics or even specific strains by detecting their volatile biomarkers, which would be a rewarding complement for other rapid methods. This promising method might be easily implemented as a rapid screening methodology in the cosmetics industry for detecting microbial

contamination both in the finished products and in raw materials, facilitating the rapid release of products into the market.

2.6 Application of the ISO 12787 International Standard

In view of above mentioned, some general analytical methods exist, or are to be developed to assess the quality of cosmetics in accordance with the European Union Cosmetics Products Regulation [1]. Although the existing or under development general methods might not be strictly certifiable, they are intended to be widely usable, comprehensible and transferable. Therefore a specific validation approach to ensure the reliability of the results is required in order to apply these methods to cosmetic analysis.

In this context, the ISO 12787 international standard 'Cosmetics–Analytical methods–Validation criteria for analytical results using chromatographic techniques' *[168]* defines validation criteria with which analytical results obtained from the analysis of cosmetic products should comply in order to give confidence in performance, reliability, and quality of the final results. It proposes an analytical approach that can be used by a single laboratory in order to perform chromatographic analysis on a given sample, or samples.

The validation criteria defined in this International Standard include specificity, selectivity, recovery, confidence interval, limit of detection, limit of quantification (LoQ), accuracy and linearity, which are supposed to be specific to the sample matrix. Therefore, this criterion shall be determined for each sample matrix. If similar matrices are used, validation results obtained for each criteria can be extended to all other analyzed samples in the same concentration range.

This standard comprises three different steps to be performed:

• First step – minimum validation criteria on standard solutions.

The first part consists of determining, using standard solutions, the main characteristics of the analytical method used before performing tests on samples. The validation criteria on standard solutions checked during this first step are: the analyte LOD and/or LoQ; the conformity of the chromatographic analysis (Rs: resolution, As: asymmetry); the linear range of the analyte signal and the standard accuracy. This first step is carried out once at the beginning of the analytical program and should be performed again or at least checked if any analytical parameter of the method is changed (calibration solvent, injection volume, chromatographic column type, separation conditions, calibration range, etc.).

• Second step – sample screening.

The aim of this step is to evaluate the quantity of analyte in the sample by injecting a calibration curve in the linearity range determined in the previous step, a control standard and the sample(s) with and without internal standards.

• Third step – assays.

The purpose of this step is to evaluate the presence of the analyte in the sample using two different protocols.

The first one is intended to ensure that the measured signal is not influenced by an interference compound or analytical problem (e.g., a bad extraction yield). If the analyte is not detected or detected at concentrations less than the LOQ, assays are performed using spiked recoveries.

The aim of the second protocol is to determine the analyte concentration in the sample as well as several validation parameters, like the matrix effect, the extraction yield, the accuracy, and the confidence interval. These parameters are determined by performing statistical analyses on six preparations of the same sample: three unspiked preparations, two PrEMS (samples spiked with the analyte of interest at the beginning of the analytical procedure) and one PoEMS (sample taken through the entire extraction procedure and spiked with the analyte of interest at the end of the extraction immediately before, or very close to, detection).

Thus, the validation criteria of the evaluated methodology are obtained from the PoEMS and/or PrEMS recoveries:

• Matrix effect can be determined depending on the PoEMS recovery relative to calibration standards.

• The extraction yield of the analytical process is obtained from the difference between the PoEMS and the PrEMS recoveries, relative to calibration standards.

• The accuracy of the method is estimated by the recovery obtained for the PrEMS relative to the PoEMS.

• The RSD of the essay is obtained by a statistical analysis of the replicates.

3. Conclusion and Future Trends

The complexity and variety of cosmetic products are mainly due to the diversity and complexity of their formulations. Among the wide variety of cosmetic ingredients, preservatives, fragrance allergens, synthetic musks, phthalates, and other plasticizers have currently raised social and scientific awareness, due to the unintended side effects that these compounds may cause on consumers health.

In this article, the most recently developed analytical methods to the determination of the main groups of cosmetic ingredients are reviewed. In some of these methods, advanced extraction techniques such as SFE, SPE, SPME, PLE, and MSPD, have been recently applied in combination with GC–MS, GC–MS/MS, or HPLC–MS/MS, which have arisen as powerful tools to successfully tackle cosmetic analysis.

The development and validation of further multicomponent methods with suitable analytical features, covering wider ranges of different families of ingredients, is still a field under development. These new methodologies are expected to use modern and efficient techniques with selective detectors, as well as rapid and simple sample preparation procedures with high level of automatation, preferably green analytical methods avoiding or reducing the use of toxic reagents in analytical procedures. The challenge is, therefore, focused on the development of robust and versatile methodologies ranging a broad spectrum of cosmetic products and ingredients.

At last but not least, it is important to notice that both the existing methodologies cited in literature and the coming ones, should comply with the validation criteria defined in the ISO 12787 international standard in order to give confidence in performance, reliability, and quality of the final results.

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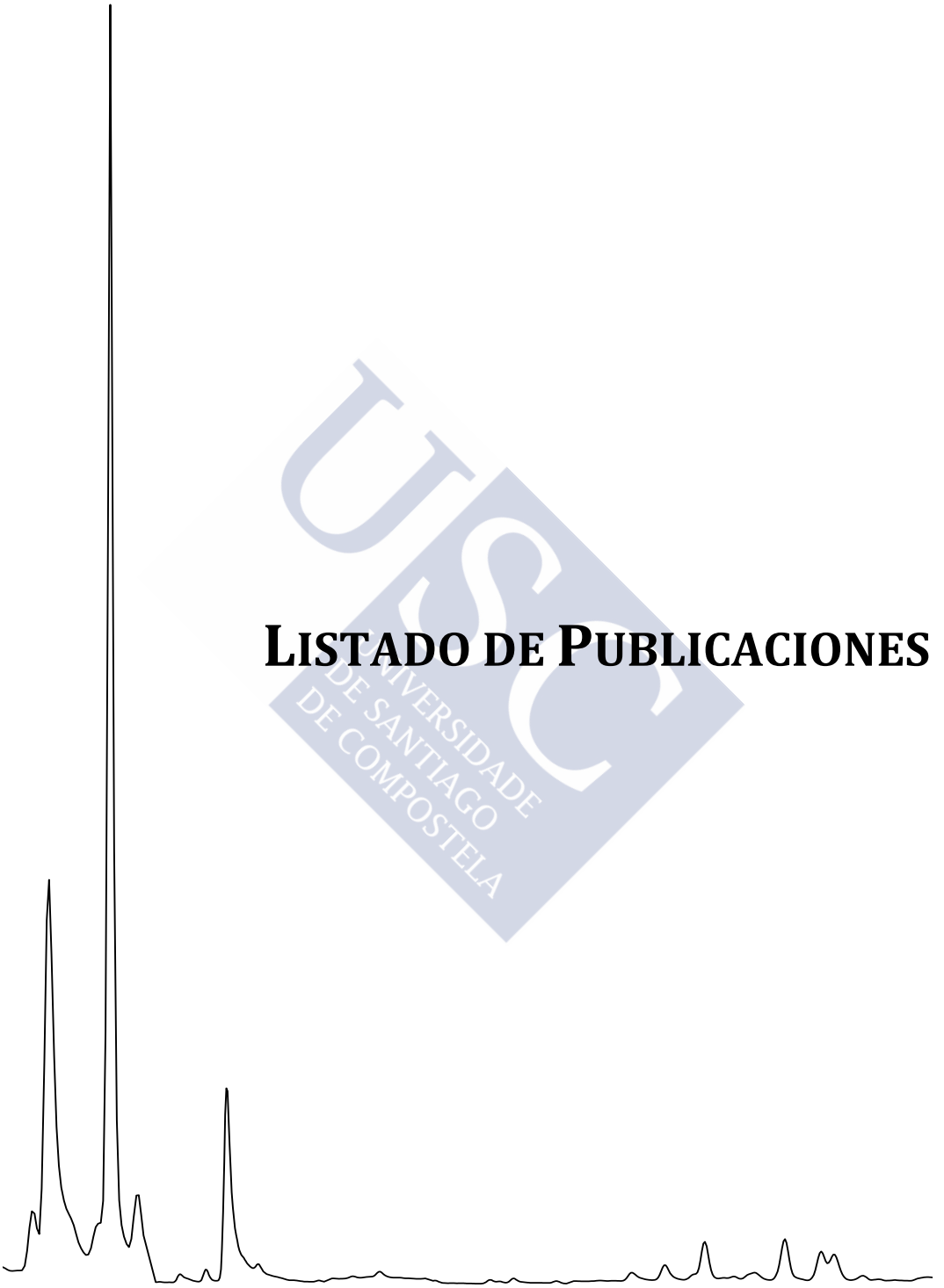

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Durante el desarrollo de la presente tesis doctoral han sido publicados o están pendientes de publicación en revistas científicas los siguientes trabajos:

- ✓ Sanchez-Prado, L.; Alvarez-Rivera, G.; Lamas, J. P.; Lores, M.; Garcia-Jares, C.; Llompart, M., Analysis of multi-class preservatives in leave-on and rinse-off cosmetics by matrix solid-phase dispersion. *Analytical and Bioanalytical Chemistry* 401 (2011) 3293-3304.

- ✓ Sanchez-Prado, L.; Lamas, J. P.; Alvarez-Rivera, G.; Lores, M.; Garcia-Jares, C.; Llompart, M., 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.

- ✓ Alvarez-Rivera, G.; Dagnac, T.; Lores, M.; Garcia-Jares, C.; Sanchez-Prado, L.; Lamas, J. P.; Llompart, M., Determination of isothiazolinone preservatives in cosmetics and household products by matrix solid-phase dispersion followed by high-performance liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A* 1270 (2012) 41-50.

- ✓ Sanchez-Prado, L.; Alvarez-Rivera, G.; Lamas, J. P.; Llompart, M.; Lores, M.; Garcia-Jares, C., Content of suspected allergens and preservatives in marketed baby and child care products. *Analytical Methods* 5 (2013) 416-427.

- ✓ Alvarez-Rivera, G.; Lores, M.; Llompart, M.; Garcia-Jares, C., Cosmetics and Toiletries: Chromatography. *Reference Module in Chemistry, Molecular Sciences and Chemical Engineering*, Elsevier: 2013. doi:10.1016/B978-0-12-409547-2.05839-X.

- ✓ Alvarez-Rivera, G.; De Miguel, T.; Llompart, M.; Garcia-Jares, C.; Villa, T. G.; Lores, M., A novel outlook on detecting microbial contamination in cosmetic products: analysis of biomarker volatile compounds by solid-phase microextraction gas chromatography-mass spectrometry. *Analytical Methods* 5 (2013) 384-393.

- ✓ Alvarez-Rivera, G.; Vila, M.; Lores, M.; Garcia-Jares, C.; Llompart, M., Development of a multi-preservative method based on solid-phase microextraction-gas chromatography-tandem mass spectrometry for cosmetic analysis. *Journal of Chromatography A* 1339 (2014) 13-25.

- ✓ Alvarez-Rivera, G.; Llompart, M.; Garcia-Jares, C.; Lores, M., Identification of halogenated photoproducts generated after ultraviolet-irradiation of parabens and benzoates in water containing chlorine by solid-phase microextraction and gas chromatography-mass spectrometry. *Journal of Chromatography A* 1349 (2014) 105-15.

- ✓ Alvarez-Rivera, G.; Llompart, M.; Garcia-Jares, C.; Lores, M., Identification of unwanted photoproducts of cosmetic preservatives in personal care products under ultraviolet-light using solid-phase microextraction and micro-matrix solid-phase dispersion. *Journal of Chromatography A* 1390 (2015) 1-12.

- ✓ Alvarez-Rivera, G.; Llompart, M.; Garcia-Jares, C.; Lores, M., Photoinduced generation of 2,8-dichlorodibenzo-p-dioxin and other cosmetic preservative photoproducts in topical cream applied onto artificial skin. *To be submitted*.

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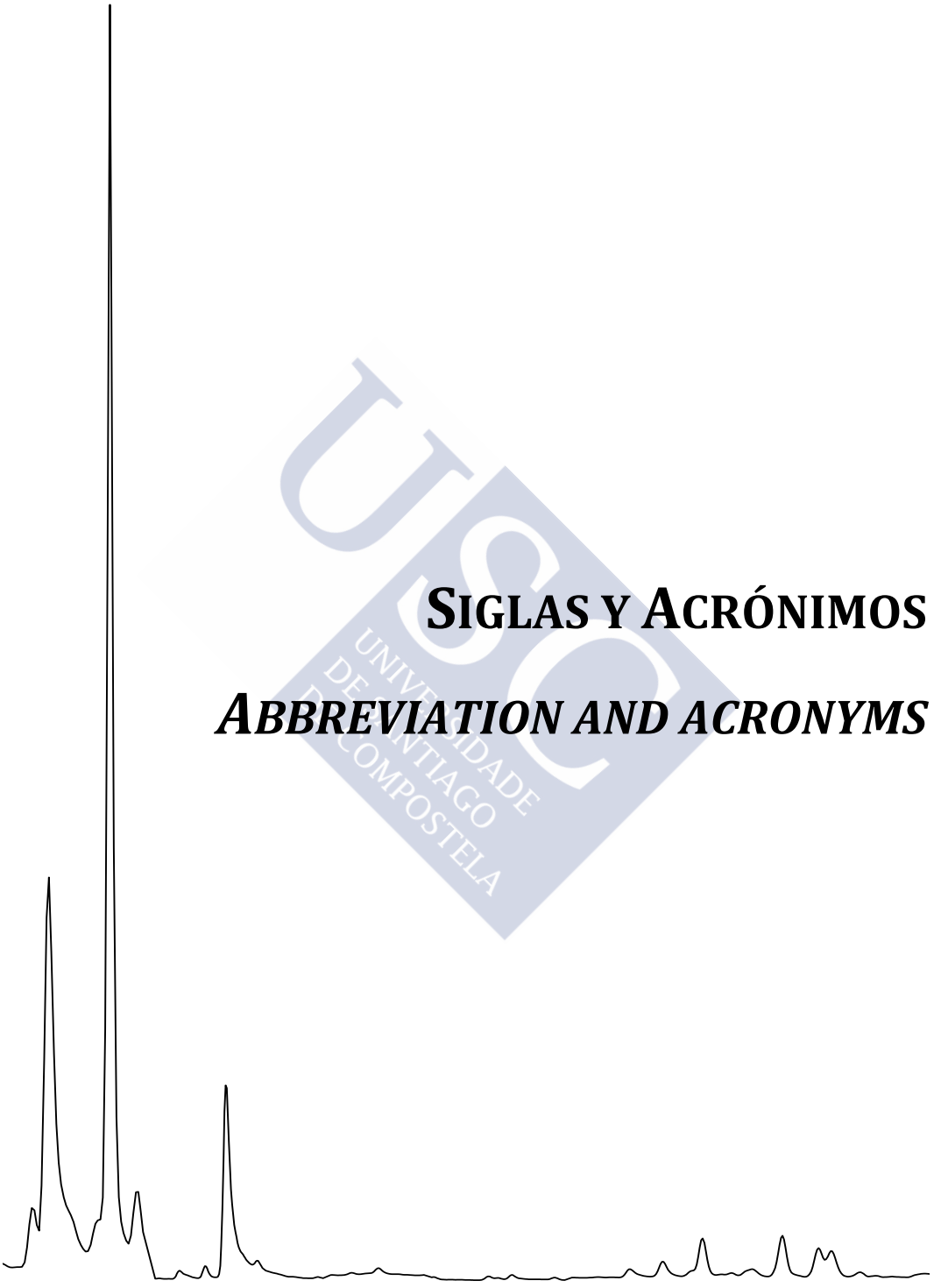
SIGLAS Y ACRÓNIMOS

ABBREVIATION AND ACRONYMS



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ANOVA	Analysis of variance	Análisis de varianza
AOP	Advanced oxidation process	Proceso de oxidación avanzado
ASE	Accelerated solvent extraction	Extracción con disolventes acelerados
CAR	Carboxen	Carboxen
CAS	Chemical Abstracts Service	-
CE	European Commision	Comisión Europea
CE	Capillary electrophoresis	Electroforesis capilar
CFU	Colony forming unit	Unidad formadora de colonia
CI	Chemical ionization	Ionización química
COLIPA	The European Cosmetics Association	Asociación europea de cosméticos
CTFA	Cosmetic Toiletry and Fragrance Association	-
CW	Carbowax	Carbowax
CZE	Capillary zone electrophoresis	Electroforesis capilar en zona
DAD	Diode array detector	Detector de red de diodos
DVB	Divinylbenzene	Divinilbenceno
EB	Electron beam	Haz de electrones
EC	European Commision	Comisión Europea
ECD	Electron capture detector	Detector de captura electrónica
EEUU	United States of America	Estados Unidos de América
EI	Electronic impact	Impacto electrónico
EPA	Environmental protection agency	Agencia de protección medioambiental
ESI	Electrospray ionization	Ionización por electrospray
EU	European Union	Unión europea
FDA	Food and Drug Administration	-
FD&C Act	Federal Food, Drug and Cosmetic Act	-
FIA	Flow injection analysis	Análisis por inyección en flujo

FID	Flame ionization detector	Detector de ionización de llama
FPLA	Fair Packaging and Labeling Act	-
GC	Gas chromatography	Cromatografía de gases
HESI	Heated electrospray ionization	Ionización por electrospray calentado
HPLC	High performance liquid chromatography	Cromatografía líquida de alta resolución
HSSPME	Headspace-solid phase microextraction	Microextracción en fase sólida en espacio de cabeza
IDL	Instrumental detection limit	Límite de detección instrumental
IFRA	International Fragrance Association	-
IR	Infrared	Infrarrojo
IT	Ion trap	Trampa de iones
IUPAC	International Union of Pure and Applied Chemistry	Unión internacional de química pura y aplicada
K_{ow}	Partition constant octanol-water	Constante de partición octanol-agua
LC	Liquid chromatography	Cromatografía líquida
LLE	Liquid-liquid extraction	Extracción líquido-líquido
LOD	Limit of detection	Límite de detección
LOF	Lack of fit	Falta de ajuste
LOQ	Limit of quantification	Límite de cuantificación
MeOH	Methanol	Metanol
min	Minutes	Minutos
MDGC	Multidimensional gas chromatography	Cromatografía de gases multidimensional
MERKC	Micellar Electrokinetic Chromatography	Cromatografía electrocinética micelar

MIPs	Highly selective molecularly-imprinted polymers	Polímeros de impresión molecular de alta selectividad.
MS	Mass spectrometry	Espectrometría de masas
MS/MS	Tandem mass spectrometry	Espectrometría de masas en tándem
MSPD	Matrix solid-phase dispersion	Dispersión de matriz en fase sólida
MVOCs	Microbial volatile organic compounds	Compuestos orgánicos volátiles de origen microbiano
MWCNTs	Multi-walled carbon nanotubes	-
PA	Polyacrylate	Poliacrilato
PAL	Pharmaceutical Affairs Law	-
PCP	Personal care product	Producto de cuidado personal
PDMS	Polydimethylsiloxane	Polidimetilsiloxano
PFE	Pressurized fluids extraction	Extracción con fluidos presurizados
PLE	Pressurized liquid extraction	Extracción con líquidos presurizados
PSE	Pressurized solvent extraction	Extracción con disolventes presurizados
Pv	Vapor pressure	Presión de vapor
Q	Quadrupole	Cuadrupolo
QqQ	Triple quadrupole	Triple cuadrupolo
RSD	Relative standard deviation	Desviación estándar relativa
SAs	Suspected allergens	Sustancias alergénicas
SCCNFP	Scientific committee on cosmetic products and non-food products	Comité científico para productos cosméticos y no alimentarios
SCCP	Scientific committee on consumer Products	Comité científico para productos de consumo
SCCS	Scientific committee on Consumer Safety	Comité científico para la seguridad del consumidor
SEC	Size-exclusion chromatography	Cromatografía de exclusión por tamaño
SIM	Selected Ion monitoring	Monitorización de iones seleccionados
SPE	Solid-phase extraction	Extracción en fase sólida

SPF	Solar protection factor	Factor de protección solar
SPME	Solid-phase microextraction	Microextracción en fase sólida
UE	European Union	Unión Europea
US	Ultrasounds	Ultrasonidos
UV	Ultraviolet	Ultravioleta
vis	Visible	Visible
WWTP	Wastewater treatment plants	Planta de tratamiento de agua residual

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INFORMACIÓN SUPLEMENTARIA

SUPPLEMENTARY INFORMATION

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Supplementary Information of Section III.1.3

Table S1 SAs concentrations (%, w/w) in real leave-on samples of baby and child care products. (BL: body moisturizing lotion, BC: body moisturizing cream, BM: body milk, MO: moisturizing oil, FC: face cream, DC: diaper cream, ET: eau de toilette, LL: louses lotion, BW: baby wipes, SP: sunscreen products; see compound codes in Table 1)

Table S2 SAs concentrations in real rinse-off samples of baby and child care products (%, w/w) (Sh: shampoo, LS: liquid soap, BB: bubble bath, Ge: gel, WP: washing powder for baby clothes, TP: toothpaste for kids; see compound codes in Table 1)

Table S3 Preservatives concentrations (%, w/w) in real leave-on samples of baby and child care products (BL: body moisturizing lotion, BC: body moisturizing cream, BM: body milk, MO: moisturizing oil, FC: face cream, DC: diaper cream, ET: eau de toilette, LL: louses lotion, BW: baby wipes, SP: sunscreen products)

Table S4 Preservatives concentrations in real rinse-off samples of baby and child care products (%, w/w) (Sh: shampoo, LS: liquid soap, BB: bubble bath, Ge: gel, WP: washing powder for baby clothes, TP: toothpaste for kids)

Table S5 SAs average concentrations and standard deviations (%, w/w) comparing MSPD and PLE methods in different leave-on (body lotion BL2; body milk BM2; body milk BM3; moisturizing oil MO1; sun care product SP1) and rinse-off baby care products (shampoo Sh1; shampoo Sh3; shower gel Ge1)

Table S6 Preservatives average concentrations and standard deviations (%, w/w) comparing MSPD and PLE methods in different leave-on (body lotion BL2; body milk BM2; body milk BM3; sun protecting lotion SP1) and rinse-off baby care products (shampoo Sh3; gel Ge1)

Table S1. SAs concentrations (%, w/w) in real leave-on samples of baby and child care products. (BL: body moisturizing lotion, BC: body moisturizing cream, BM: body milk, MO: moisturizing oil, FC: face cream, DC: diaper cream, ET: eau de toilette, LL: louses lotion, BW: baby wipes, SP: sunscreen products; see compound codes in Table 1)

Samples[a]	SA1	SA2	SA3	SA5	SA6	SA7	SA10	SA11	SA12	SA14	SA15	SA16	SA17	SA19	SA22	SA23	SA24
BL1			0.00388			0.00333											
BL2	0.00148		0.0165	0.00575		0.00731	0.00334	0.00220		0.00201		0.0115	0.0164	0.00870	0.00718	0.000275	0.0363
BL3													0.0000432				
BC1								0.000645									
BC2													0.0000474				
BC3			0.00430						0.000378								
BM1		0.44989															
BM2	0.0249		0.0279		0.00866	0.00681							0.0460				
BM3	0.121		0.0908		0.00807		0.00058						0.0646		0.0252		
BM4	0.0654		0.0569		0.00579								0.0426		0.0159		
MO1			0.00631	0.0116		0.00563		0.00340	0.00444			0.00426	0.0109	0.00171	0.0402	0.169	
FC1															0.000439	0.000046	0.000204
ET1	0.00204		0.0372		0.138	0.00459	0.0847		0.000058								
ET2	0.212		0.134		0.0138		0.0146					0.000448	0.170	0.0181	0.00280		
LL	0.00877		0.0138		0.0648						0.00223						
BW1							0.0000673										
SP1	0.0247	0.00069	0.0121	0.00344	0.000786	0.0156		0.00431					0.00858			0.00207	0.00349
SP2	0.00890		0.0333	0.0103								0.00749			0.0319		0.0210

[a] Samples MO2, FC2-FC4, DC1-DC5, and BW2 did not contain any of the analyzed SAs (values below LOD).
Blank spaces means values below LOD.

Table S2. SAs concentrations in real rinse-off samples of baby and child care products (%, w/w) (Sh: shampoo, LS: liquid soap, BB: bubble bath, Ge: gel, WP: washing powder for baby clothes, TP: toothpaste for kids; see compound codes in Table 1)

Samples[a]	SA1	SA2	SA3	SA5	SA6	SA7	SA8	SA10	SA11	SA12	SA14	SA15	SA16	SA17	SA21	SA22	SA23	SA24
Sh1	0.0138		0.00746									0.00442						0.00520
Sh2	0.0167		0.0290			0.00603						0.00396						
Sh3	0.00513	0.192	0.0402	0.0114		0.00769			0.00137				0.00407					
Sh4			0.00154															
LS1	0.00408		0.00755		0.0103	0.0279				0.000749								
LS2	0.000831		0.000371															
LS4	0.0328		0.0485											0.00946		0.0104		
LS5	0.0739		0.0655					0.00314		0.00329								
LS6		0.0430																
LS7	0.143		0.0262	0.00372	0.00736			0.00821			0.000185		0.00177			0.0111	0.000120	0.000074
BB																	0.000704	0.000337
Ge1			0.000881						0.000492		0.000299				0.130			
Ge2	0.00114		0.00766	0.00640			0.000067			0.000212						0.0133		
Ge3		0.000144															0.000038	
Ge5	0.01267		0.0540	0.0159									0.0114			0.0504		0.0363
WP	0.000330		0.0277			0.0131							0.000067				0.000027	
TP	0.000791		0.00215							0.000744								

To simplify, methyl-2-octynoate, anise alcohol, methyl eugenol, amyl cinnamal, lyral®, amyl cinnamyl alcohol, and benzyl cinnamate are not included in the table since they were not detected in any sample. Blank spaces means values below LOD.

[a] Samples LS3 and Ge4 did not contain any of the analyzed SAs (values below LOD).

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Table S3. Preservatives concentrations (%, w/w) in real leave-on samples of baby and child care products (BL: body moisturizing lotion, BC: body moisturizing cream, BM: body milk, MO: moisturizing oil, FC: face cream, DC: diaper cream, ET: eau de toilette, LL: louses lotion, BW: baby wipes, SP: sunscreen products)

Samples[a]	MeP	BHA	BHT	EtP	PrP	iBuP	BuP	TCS
BL1	0.224			0.0915	0.147		0.0910	
BL2	0.0569			0.0172	0.0102	0.00860	0.0167	
BL3	0.160							
BC1	0.457		0.0173		0.101	0.000479	0.000995	
BC2	0.312	0.000136	0.00827		0.0664		0.000524	
BC3					0.000110			
BC4	0.135				0.142			
BM2	0.000648		0.0701	0.000125	0.000425		0.0000598	
BM3	0.0630	0.000656	0.0163	0.0182	0.0128		0.0240	
BM4	0.0537		0.0138	0.0154	0.0105		0.0203	
FC1	0.284				0.0613	0.000477	0.00119	0.0000114
FC2	0.244				0.0683			
FC3	0.000330	0.000171	0.0417		0.0575			
FC4			0.00145					
DC1	0.0668			0.0315	0.0142	0.0117	0.0252	
DC3	0.00244		0.00152		0.0933			
DC4	0.000645			0.000282	0.000155	0.0000999	0.000234	
ET1			0.00837					
ET2			0.000173					
LL		0.0000313						
BW1	0.0910			0.0353	0.0359			
BW2	0.0687				0.0000153			
SP1	0.176		0.000166	0.0756				
SP2	0.00101		0.0724	0.000187	0.000456		0.0000918	

To simplify, bronidox, bronopol, iPrP, IPBC and BzP are not included in the table since they were not detected in any sample. Blank spaces means values below LODs.

[a] Samples BM1, MO1, MO2, and DC2 did not contain any of the analyzed preservatives (values below LOD).

Table S4 Preservatives concentrations in real rinse-off samples of baby and child care products (%, w/w) (Sh: shampoo, LS: liquid soap, BB: bubble bath, Ge: gel, WP: washing powder for baby clothes, TP: toothpaste for kids)

Samples[a]	Bronopol	MeP	BHA	BHT	EtP	PrP	IPBC	iBuP	BuP	TCS
Sh1				0.000116						
Sh2				0.000367					0.0000137	0.00000282
Sh3		0.0763		0.000526	0.0237	0.0132		0.0118	0.0231	
LS1							0.00541			
LS2		0.0445			0.0183	0.0132		0.0111	0.0180	
LS4			0.000809	0.00132						
LS6	0.000613						0.00316			
LS7				0.000806						
Ge1	0.0146	0.0554				0.0258				0.00000397
Ge2				0.000231						
Ge3		0.0503			0.0210	0.0134		0.0131	0.0207	
Ge4										
Ge5		0.00117		0.000844	0.0000549	0.000471	0.00373		0.0000485	
WP		0.120				0.00107			0.0000172	
TP		0.0677								0.0000219

To simplify, bronidox, iPrP and BzP are not included in the table since they were not detected in any sample. Blank spaces means values below LODs.

[a] Samples Sh4, LS3, LS5, BB and Ge4 did not contain any of the analyzed preservatives (values below LOD).

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Table S5 SAs average concentrations and standard deviations (%, w/w) comparing MSPD and PLE methods in different leave-on (body lotion BL2; body milk BM2; body milk BM3; moisturizing oil MO1; sun care product SP1) and rinse-off baby care products (shampoo Sh1; shampoo Sh3; shower gel Ge1)

Compoud	BL2				BM2				BM3				MO1			
	MSPD		PLE		MSPD		PLE		MSPD		PLE		MSPD		PLE	
	Average	Desvest	Average	Desvest	Average	Desvest	Average	Desvest	Average	Desvest	Average	Desvest	Average	Desvest	Average	Desvest
SA1	0,001307	0,000119	0,001485	0,000028	0,023550	0,000535	0,025155	0,000258	0,111300	0,001508	0,116704	0,002078				
SA3	0,016477	0,000676	0,016497	0,000385	0,029525	0,000566	0,026114	0,001620	0,099697	0,002611	0,094318	0,006186	0,006162	0,000226	0,006315	0,000002
SA5	0,006098	0,000467	0,005755	0,000553									0,009935	0,000321	0,011036	0,000480
SA6					0,009216	0,000110	0,008723	0,000088	0,008743	0,000179	0,008257	0,000318				
SA7	0,007003	0,000484	0,007310	0,000135	0,006796	0,000111	0,006801	0,000092					0,006022	0,000004	0,005629	0,000596
SA10	0,003211	0,000264	0,003337	0,000034					0,000411	0,000018	0,000589	0,000028				
SA11	0,001699	0,000147	0,002198	0,000000									0,003085	0,000052	0,003395	0,000034
SA12													0,004104	0,000082	0,004442	0,000042
SA14	0,001612	0,000232	0,002014	0,000004												
SA16	0,010905	0,000514	0,011455	0,000196									0,004232	0,000135	0,004260	0,000028
SA17	0,013933	0,000592	0,015437	0,000282	0,043318	0,000161	0,038976	0,006123	0,076101	0,001306	0,072210	0,013235	0,010933	0,000283	0,010890	0,000040
SA19	0,008510	0,000535	0,008704	0,000171									0,001714	0,000157	0,001708	0,000014
SA22	0,007281	0,000241	0,007180	0,000053					0,027065	0,000560	0,026926	0,002922	0,040430	0,001627	0,040211	0,000167
SA23	0,000275	0,000010	0,000266	0,000005									0,170729	0,007132	0,168878	0,000496
SA24	0,035650	0,001628	0,036316	0,000503												

Blank spaces means values below LODs

Table S5 (continued)

Compound	SP1				Sh1				Sh3				Ge1			
	MSPD		PLE		MSPD		PLE		MSPD		PLE		MSPD		PLE	
	Average	Desvest	Average	Desvest	Average	Desvest	Average	Desvest	Average	Desvest	Average	Desvest	Average	Desvest	Average	Desvest
SA1	0,021912	0,000865	0,024683	0,000951	0,010976	0,000231	0,011596	0,000253	0,003766	0,000059	0,004947	0,000026				
SA2	0,000591	0,000065	0,000688	0,000131					0,185489	0,002559	0,191771	0,004642				
SA3	0,011197	0,000075	0,012117	0,000198	0,008492	0,000686	0,007459	0,000559	0,039088	0,000544	0,040166	0,000638	0,000881	0,000086	0,000824	0,000076
SA5	0,003605	0,000028	0,003444	0,000063					0,009428	0,001556	0,011551	0,000367				
SA6	0,000756	0,000082	0,000786	0,000037												
SA7	0,013888	0,000082	0,015599	0,000196					0,006371	0,000701	0,007078	0,001080				
SA11	0,003128	0,000049	0,004239	0,000086					0,001186	0,000129	0,001223	0,000159	0,000492	0,000061	0,000579	0,000066
SA14													0,000399	0,000008	0,000493	0,000097
SA15					0,004737	0,000225	0,004423	0,000320								
SA16									0,003864	0,000096	0,004180	0,000215				
SA17	0,008449	0,000047	0,008581	0,000010												
SA21													0,129948	0,006764	0,131500	0,005577
SA23	0,002048	0,000029	0,002071	0,000035												
SA24	0,003533	0,000189	0,003490	0,000004	0,005632	0,000420	0,005200	0,000384								

Blank spaces means values below LODs

Table S6 Preservatives average concentrations and standard deviations (%, w/w) comparing MSPD and PLE methods in different leave-on (body lotion BL2; body milk BM2; body milk BM3; sun protecting lotion SP1) and rinse-off baby care products (shampoo

Compound	BL2				BM2				BM3			
	MSPD		PLE		MSPD		PLE		MSPD		PLE	
	Average	Desvest	Average	Desvest	Average	Desvest	Average	Desvest	Average	Desvest	Average	Desvest
MeP	0,058362	0,001793	0,056901	0,000680	0,000644	0,000012	0,000652	0,000026	0,065292	0,001907	0,064777	0,001116
BHA									0,000711	0,000019	0,000655	0,000010
BHT					0,066191	0,000882	0,067007	0,000536	0,016923	0,000549	0,016423	0,000105
EtP	0,017575	0,000499	0,017187	0,000219	0,000124	0,000001	0,000126	0,000003	0,018752	0,000619	0,018550	0,000191
PrP	0,010426	0,000380	0,010157	0,000115	0,000414	0,000019	0,000434	0,000017	0,013816	0,001020	0,013672	0,000260
iBuP	0,008340	0,001069	0,008598	0,000161								
BuP	0,017039	0,000479	0,016700	0,000181	0,000056	0,000005	0,000062	0,000008	0,024648	0,000807	0,024502	0,000193

Compound	SP1				Sh3				Ge1			
	MSPD		PLE		MSPD		PLE		MSPD		PLE	
	Average	Desvest	Average	Desvest	Average	Desvest	Average	Desvest	Average	Desvest	Average	Desvest
Bronopol									0,014634	0,000743	0,012467	0,001532
MeP	0,184588	0,011903	0,178170	0,002929	0,080013	0,004091	0,074810	0,004814	0,055425	0,004279	0,055359	0,000047
BHT	0,000166	0,000009	0,000159	0,000010	0,000475	0,000007	0,000509	0,000019				
EtP	0,076959	0,004890	0,077763	0,003130	0,024767	0,001067	0,023112	0,001519				
PrP					0,012093	0,000514	0,012535	0,000821	0,025805	0,001201	0,025779	0,000018
iBuP					0,012163	0,000468	0,011395	0,000661				
BuP					0,023406	0,001031	0,022386	0,001332				
TCS									0,000004	0,000000	0,000004	0,000000

Blank spaces means values below LODs.

Supplementary Information of Section III.1.4

Figure S1. Full scan (a) and product ion (b-d) mass spectra of acetylated MeP.

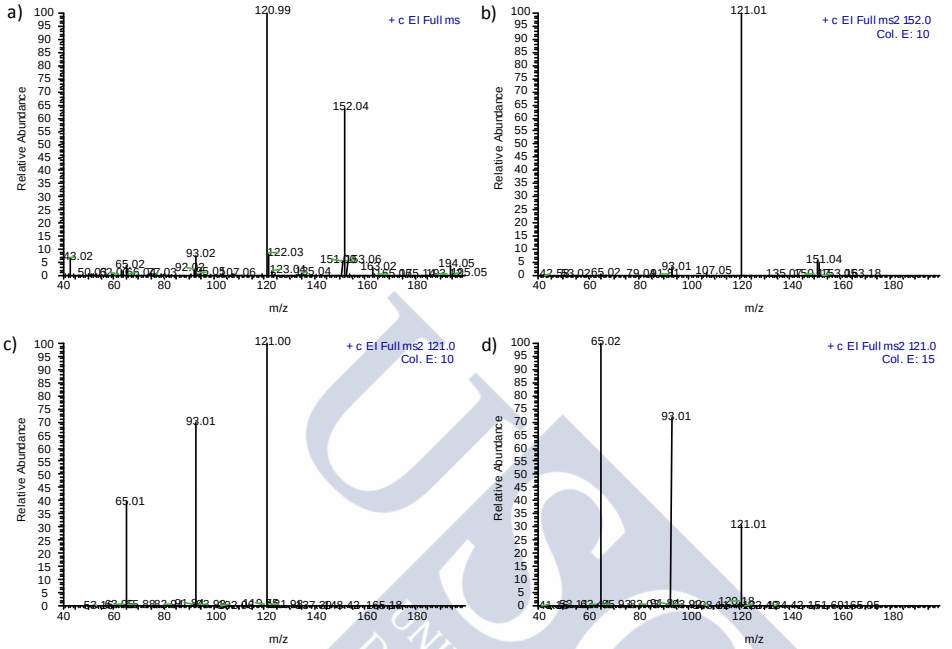


Figure S2. Full scan (a) and product ion (b-d) mass spectra of acetylated MeP-d_4.

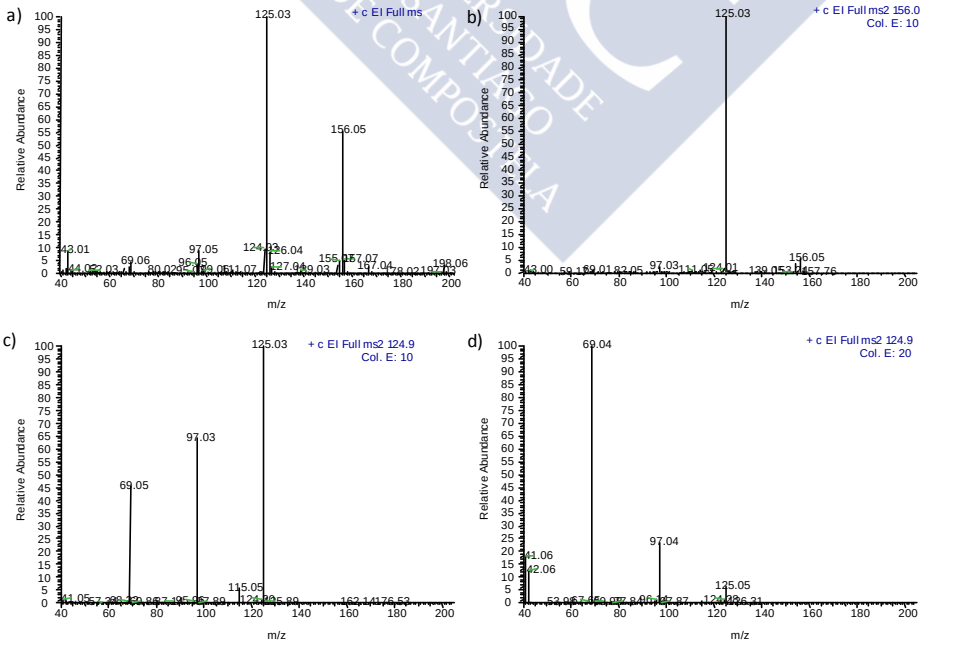


Figure S3. Full scan (a) and product ion (b-d) mass spectra of acetylated BHA.

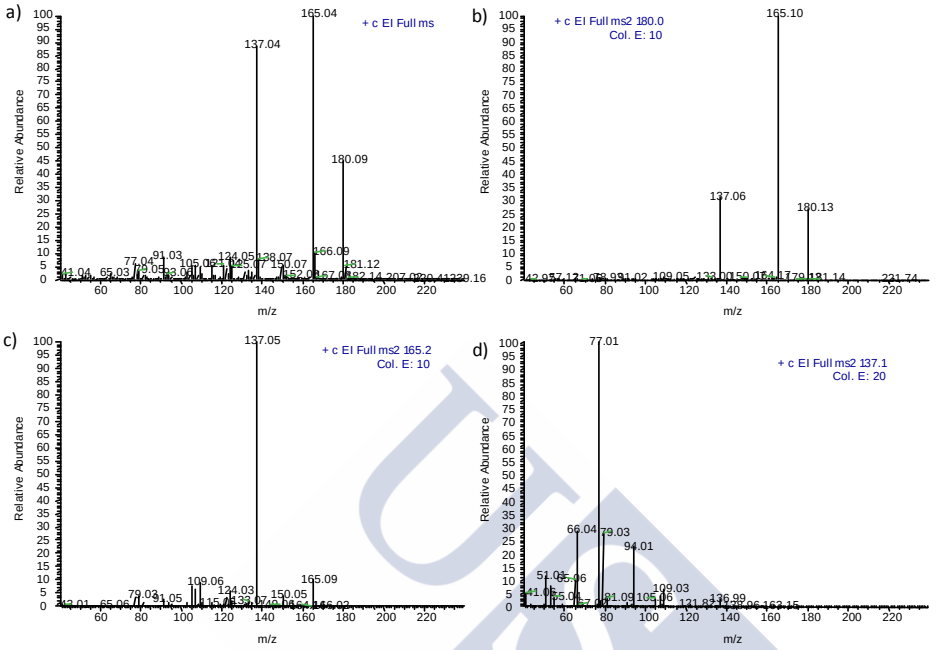


Figure S4. Full scan (a) and product ion (b-d) mass spectra of acetylated EtP.

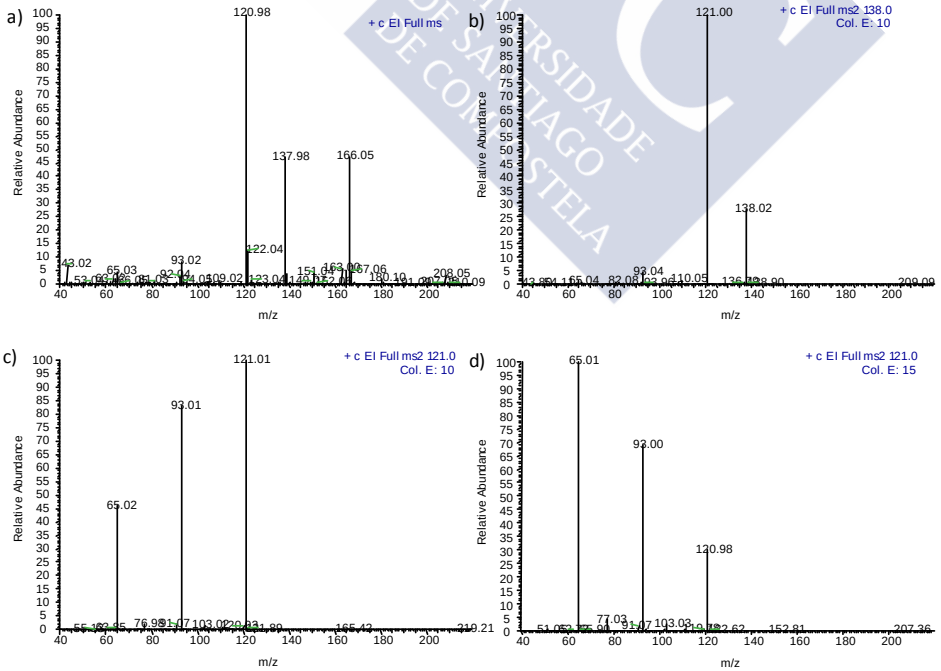


Figure S5. Full scan (a) and product ion (b-d) mass spectra of acetylated *i*-PrP

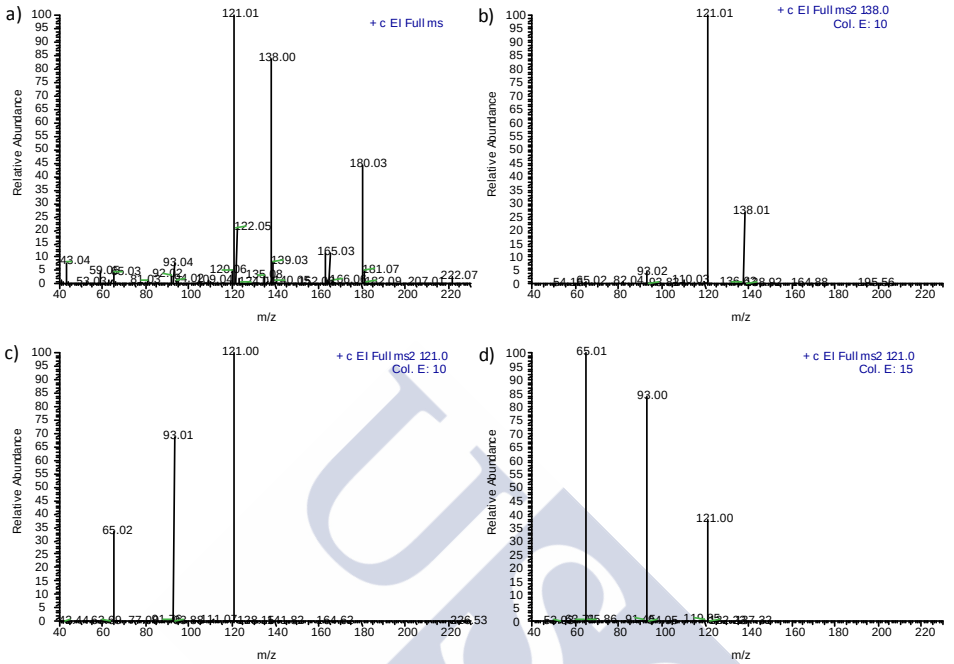


Figure S6. Full scan (a) and product ion (b-d) mass spectra of acetylated PrP

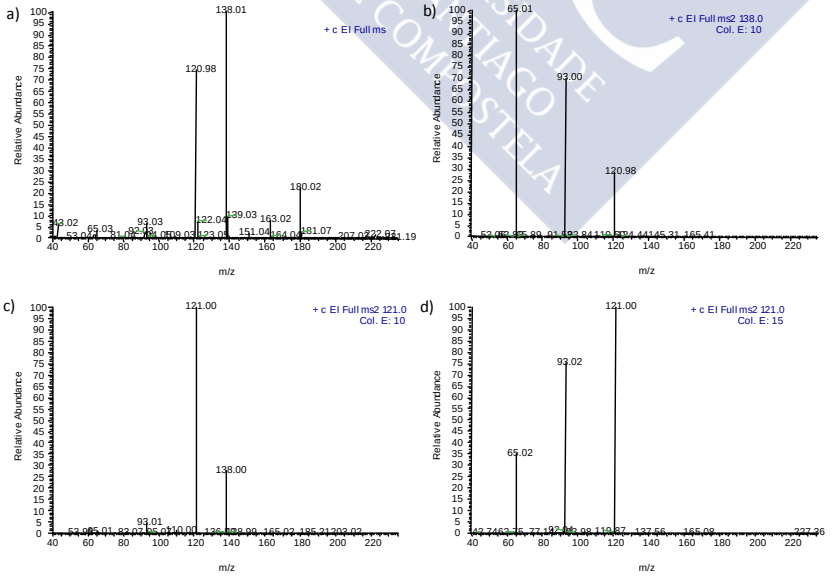


Figure S7. Full scan (a) and product ion (b-d) mass spectra of acetylated PrP-d_4

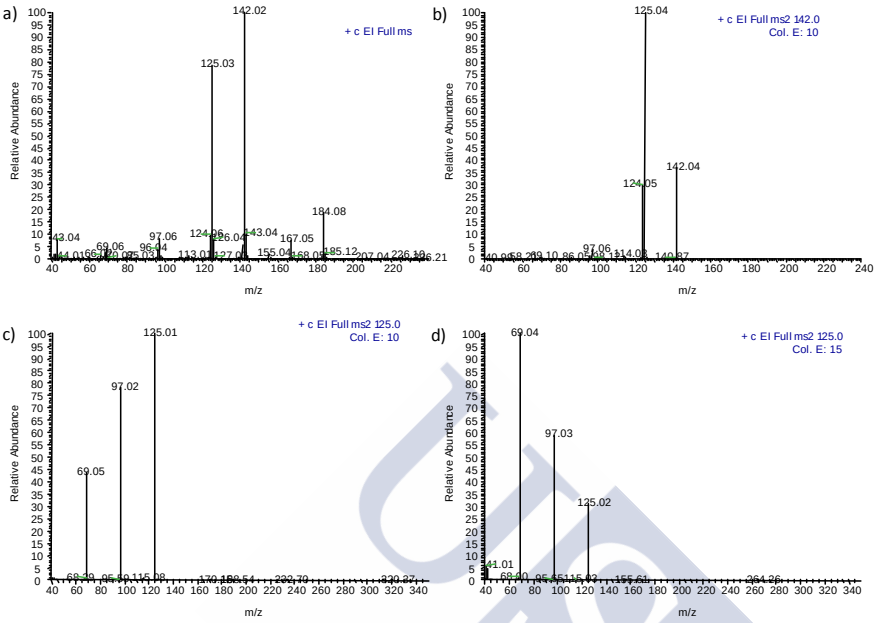


Figure S8. Full scan (a) and product ion (b-d) mass spectra of acetylated *i*-BuP

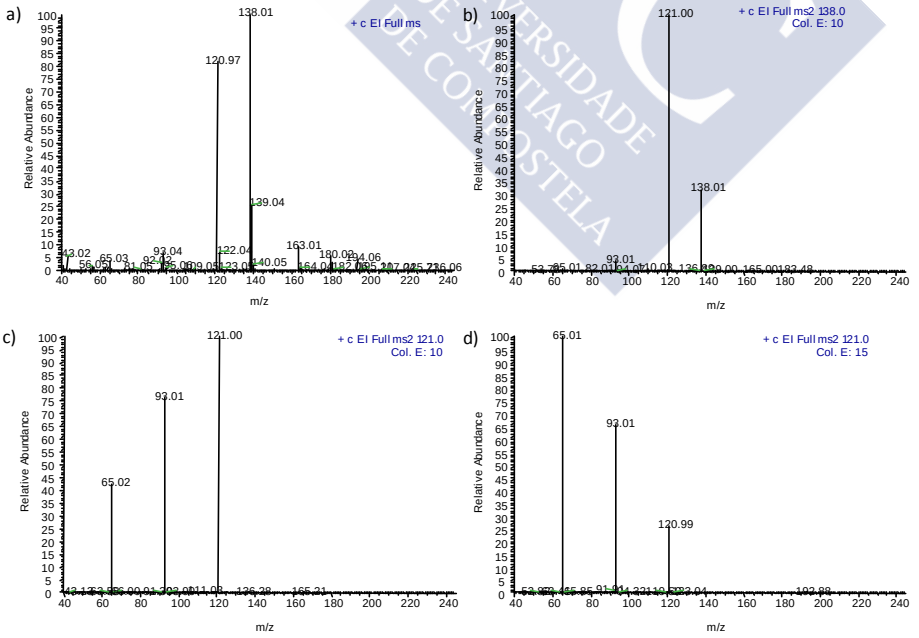


Figure S9. Full scan (a) and product ion (b-d) mass spectra of acetylated BuP

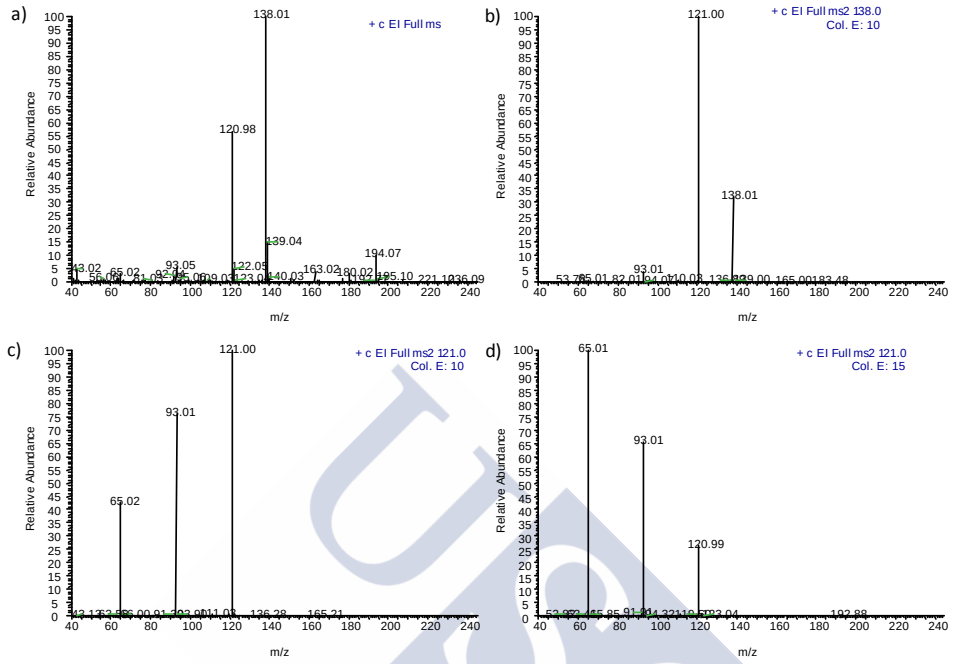


Figure S10. Full scan (a) and product ion (b-d) mass spectra of acetylated BzP

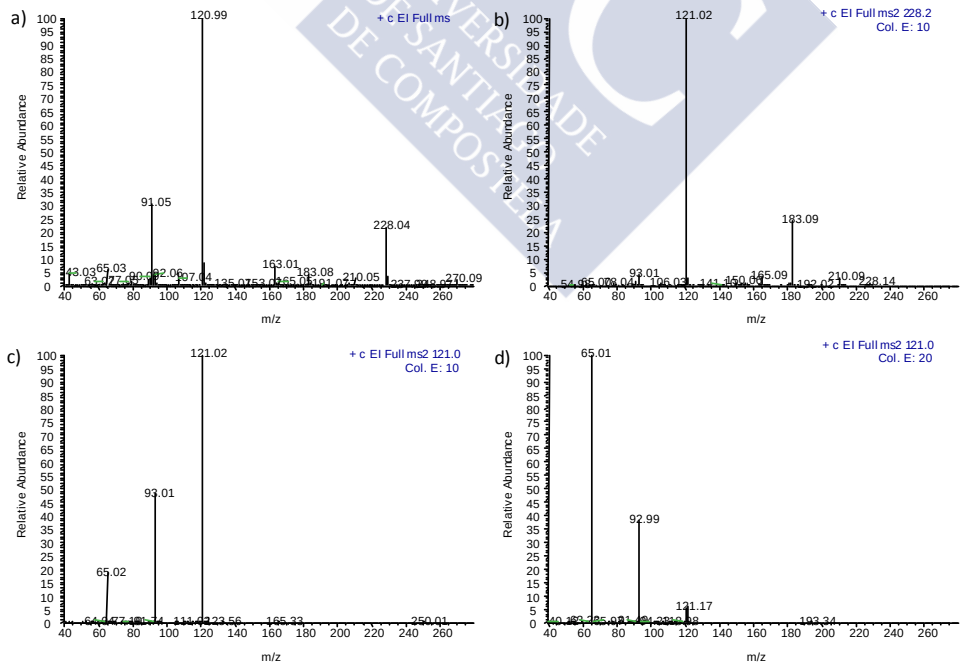


Figure S11. Full scan (a) and product ion (b-d) mass spectra of acetylated TCS

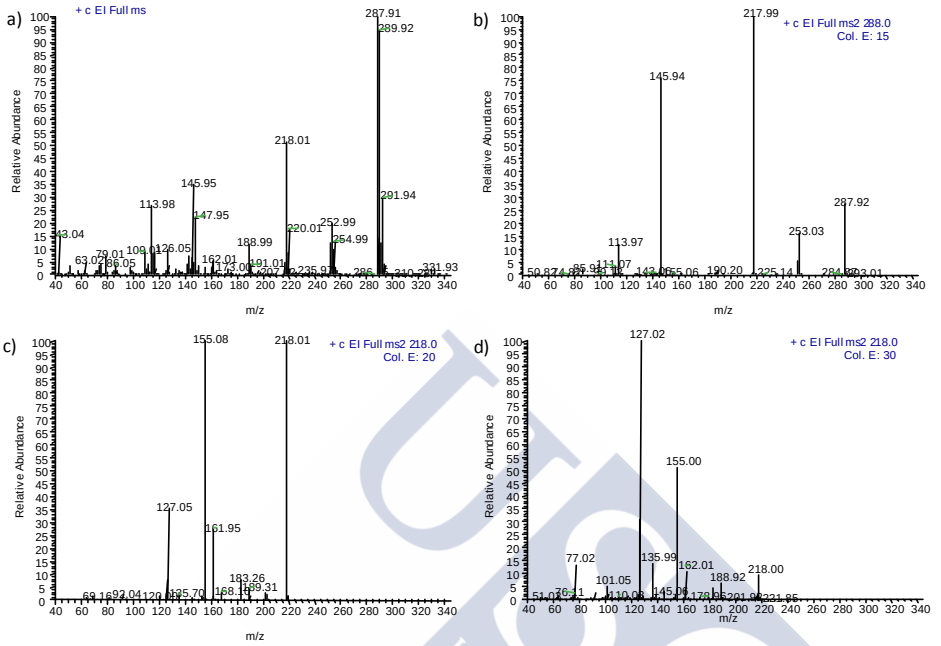
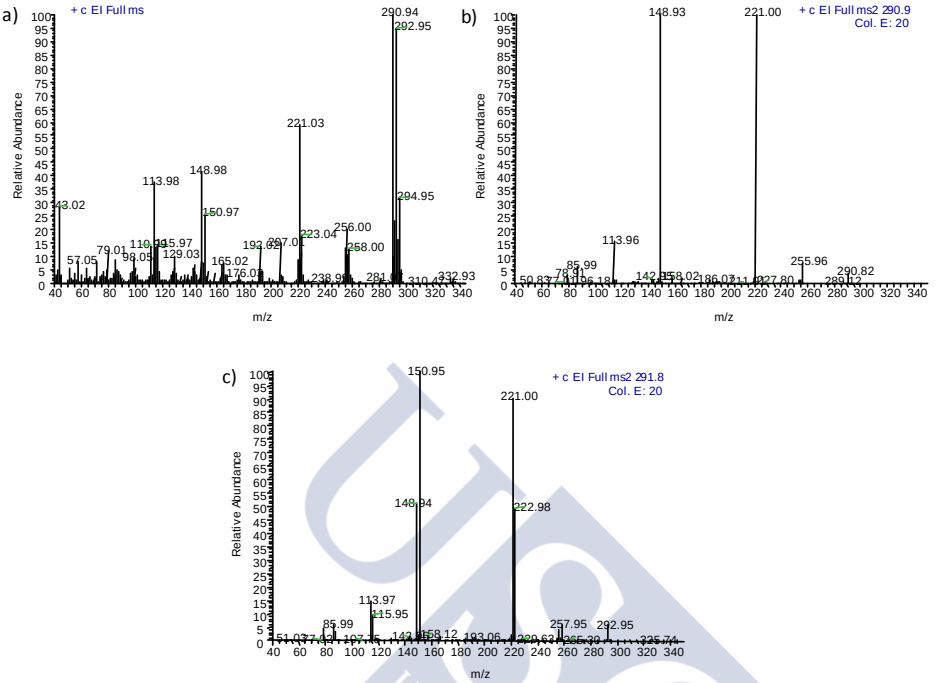
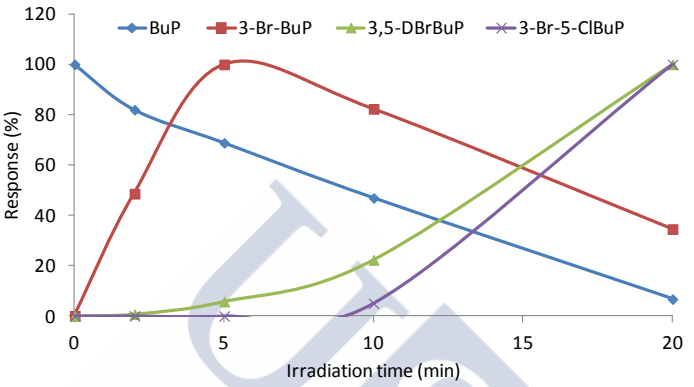


Figure S12. Full scan (a) and product ion (b-d) mass spectra of acetylated TCS-d_3



Supplementary Information of Section III.3.1

Figure S1. Photodegradation–photoformation kinetic curves under UV irradiation of: (a) BuP and photohalogenated by-products at 8W-UV.



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Supplementary Information of Section III.3.2

Figure S1. Mass spectrum and experimental ion justification of photoproduct BH4.

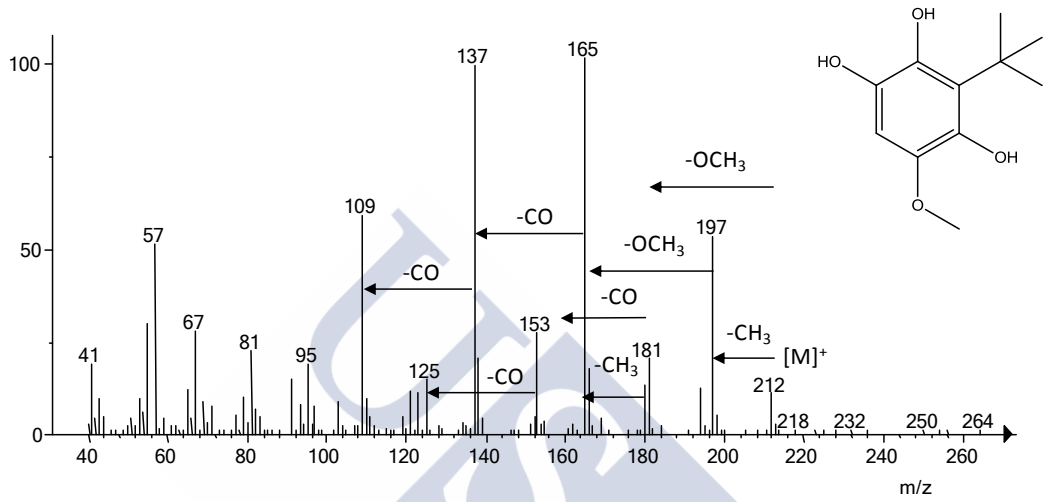


Figure S2. Mass spectrum and experimental ion justification of photoproduct BH13.

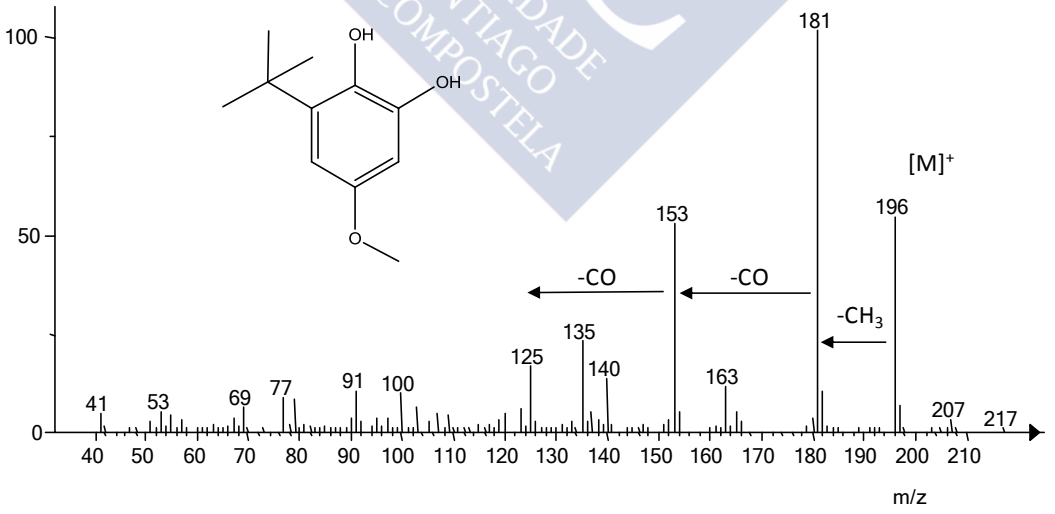


Figure S3. Mass spectra of (a) BHA, and BH photoproducts identified as (b) BHA isomer 1, (c) BHA isomer 2, and (d) BHA isomer 3.

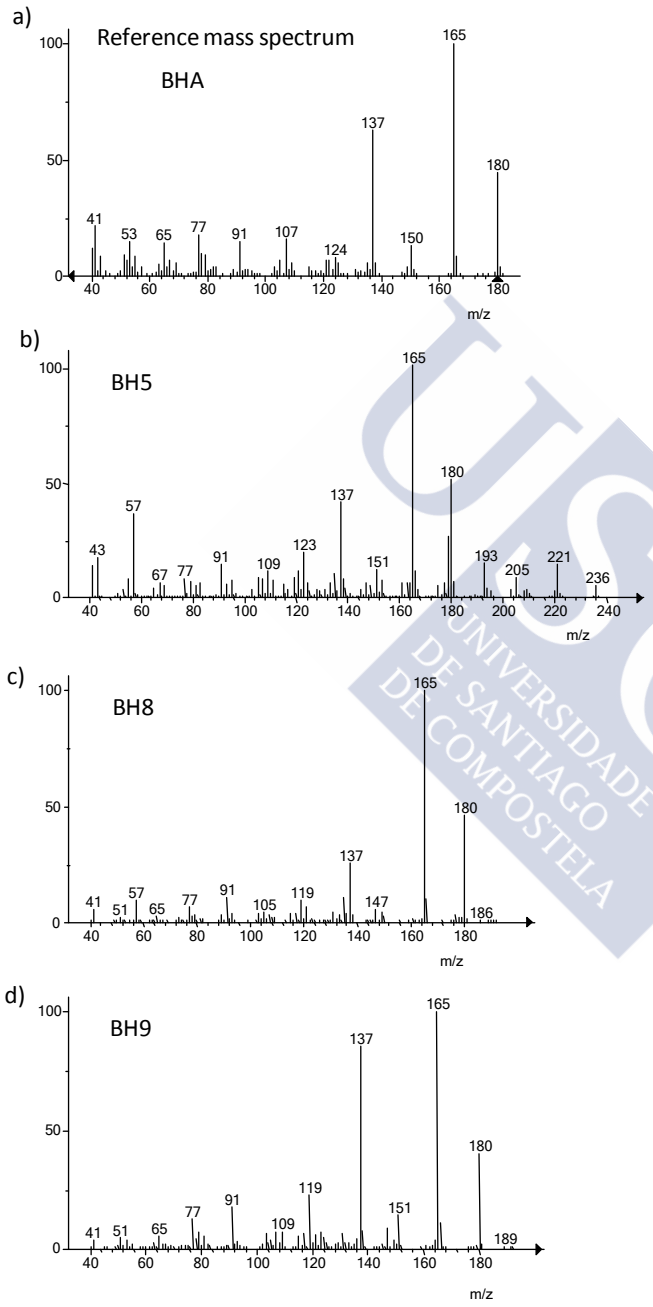
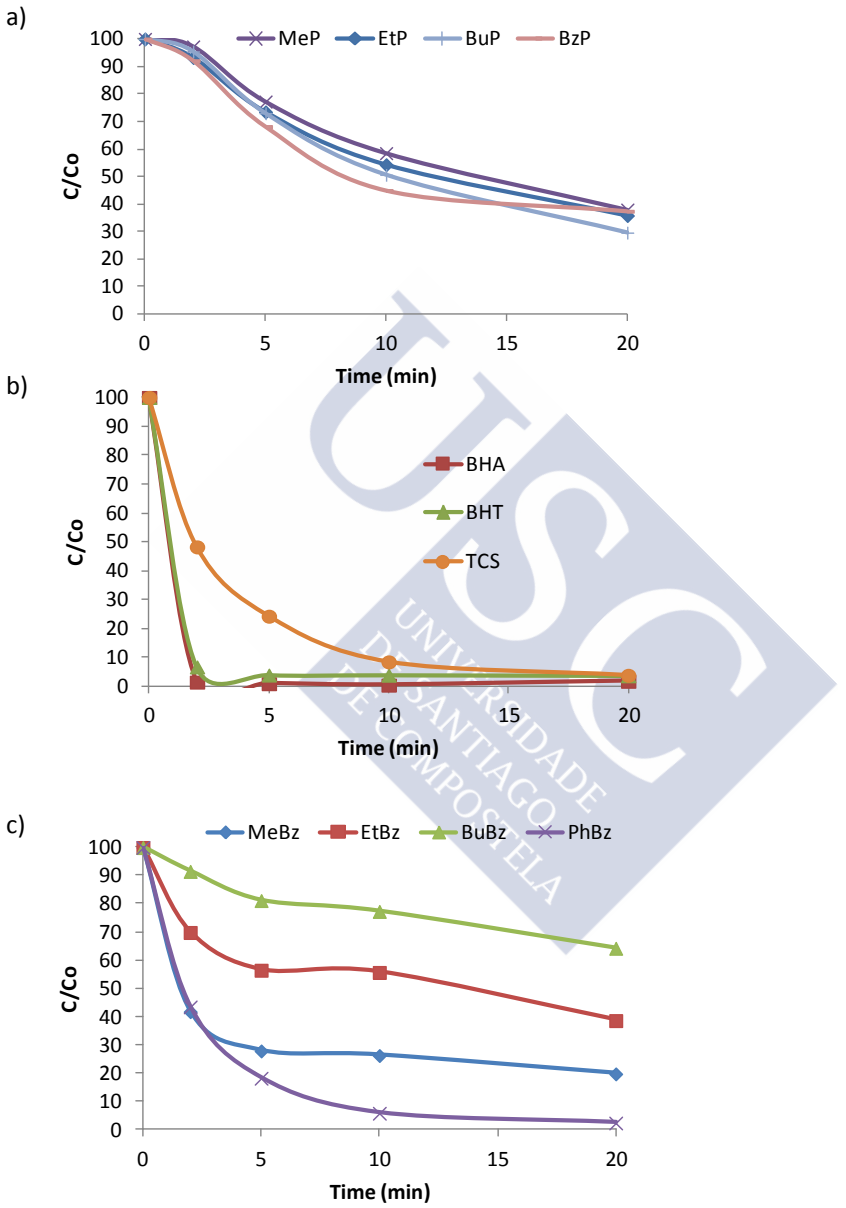


Figure S4. Influence of irradiation time on photodegradation of (a) target parabens; (b) BHA, BHT and TCS; and (c) target benzoates, in a moisturizing cream (MC) irradiated at 10W-UV.



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