UNIVERSITAT DE VALÈNCIA
FACULTAD DE QUÍMICA
DEPARTAMENTO DE QUÍMICA ANALÍTICA

ANALYTICAL METHODS FOR THE DETERMINATION OF PERFUME-RELATED SUBSTANCES

Memoria presentada por Marina López Nogueroles
para la obtención del título de Doctor

Programa de Doctorado: 560 310E
Técnicas Experimentales en Química

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Dña Amparo Salvador Carreño, Catedrática de Universidad y D. Alberto Chisvert Sanía, Profesor Titular de Universidad, ambos del Departamento de Química Analítica de la Universitat de València, en calidad de Directores de la Tesis Doctoral (modalidad de Doctorado Europeo) presentada por Marina López Nogueroles, con el título "***Analytical methods for the determination of perfume-related substances***"

CERTIFICAN,

> Que la citada Tesis Doctoral se ha realizado en los laboratorios del Departamento de Química Analítica de la Facultad de Química de la Universitat de València y en los *Laboratoires de Sciences Analytiques, Bioanalytiques et Miniaturisation* de la *École Supérieure de Physique et de Chimie Industrielles de la ville de Paris* (París, Francia) y que han dirigido y supervisado los distintos aspectos del presente trabajo así como su redacción

Y para que así conste a los efectos oportunos, firman el presente certificado en Valencia, a 26 de Mayo de 2014.

Prof. Dra. Amparo Salvador　　　　Prof. Dr. Alberto Chisvert

Les grandes personnes aiment les chiffres. Quand vous leur parlez d'un nouvel ami, elles ne vous questionnent jamais sur l'essentiel. Elles ne vous disent jamais : « Quel est le son de sa voix? Quels sont les jeux qu'il préfère? Est-ce qu'il collectionne les papillons? » Elles vous demandent : « Quel âge a-t-il? Combien a-t-il de frères? Combien pèse-t-il? Combien gagne son père? » Alors seulement elles croient le connaître.

Antoine de Saint-Exupéry (Le Petit Prince)

A Amparo

Llegado el momento de escribir los agradecimientos de la tesis, podría parecer que todo el trabajo ya está hecho. Pero no es así. Queda una parte importante, la de agradecer a todas aquellas personas que me han ayudado o me han acompañado durante estos años y sin las cuales yo no habría llegado hasta aquí. Además, no hay que olvidar que esta es la única parte que seguro leerá todo el mundo por lo que también hay que esforzarse.

Sobre todo, y porque se lo merece, la tesis va enteramente dedicada a Amparo. Por siempre estar ahí, en lo laboral y lo personal, por apoyarme, por guiarme y por quererme tanto. Amparo, muchas gracias.

Por supuesto, tengo muchas cosas que agradecer a Alberto. Gracias por haberme ayudado tanto estos años y por siempre tener tiempo para mí, por liado que vayas. Por tus visitas y risas en el laboratorio. Espero no haberte dado muchos disgustos. ¡Ya verás cómo dentro de unos años solo te acuerdas de lo bueno!

No puedo olvidarme de mis compis del laboratorio 11: Zaca, Isuha, Isabel, Iván y JuanLu. Chicos, sin vosotros esto no habría sido igual. Por las risas, cotilleos, amores y desamores... Por esto y por todas las otras cosas que he tenido que omitir, os quiero y no os olvidaré. A mi P.E. favorita, por el soporte técnico. También agradecer el apoyo al resto de compañeros que han pasado por el laboratorio, sobre todo a Sonia, Elena y Aitor, que me ayudaron en algunos trabajos.

Gracias también al grupo de 'comida', a los que son y a los que en su momento fueron: Javi, Sergio, Noemí, Pablo, Dani, David, Manuela, Luis, Julia, Zaca, Isuha e Isabel (*last but not least)*. Por hacer de la comida uno de los mejores y más divertidos momentos del día. A los demás becarios del departamento, especialmente a Enrique, por ser tan majos y estar siempre dispuestos a ayudar.

Muchísimas gracias al Departamento de Química Analítica por haberme permitido realizar el trabajo. Al hombre más buscado del departamento, Javier, porque sabe de todo y siempre te ayuda. A las dos Marías por siempre ser tan eficientes, ir tan estupendas y ser tan sonrientes. Mostrar mi gratitud también a todos los profesores del departamento que me han ayudado todos estos años. Gracias también a Joan por ayudarme con algún examen durante la carrera, por recibirme y tratarme tan bien en su casa, por los patrones de ftalatos y, fundamentalmente, por ayudarme cuando lo he necesitado.

Gracias también al Ministerio de Ciencia e Innovación por la beca predoctoral que me concedieron y la ayuda extra en la estancia.

Tengo un muy buen recuerdo de la estancia, así que quiero agradecer a Valérie Pichon aceptarme en su grupo de investigación, *Laboratoires Sciences*

Analytiques, Bioanalytiques et Miniaturisation de *la École supérieure de physique et de chimie industrielles de la ville de Paris*. Siempre tuvo un momento y una sonrisa para mí. A Sonia Lordel por dedicarme tanto tiempo y enseñarme todo lo que yo sé de MIPs. Igualmente, a los compañeros del laboratorio de allí que me acogieron e hicieron sentir bien desde el primer día.

Gracias a Aitor y a Marina por enseñarme a usar el Photoshop, que tan útil es cuando eres doctorando. Gracias Aitor, por todas esas figuras en negro del Power Point.

Gracias a mis padres por ser como son y ayudarme en todo. Por enseñarme lo que es importante. A ellos, mi hermana, mi abuela, y mi demás familia los quiero nombrar porque son importantes en mi vida, están siempre ahí y, por supuesto, sin ellos tampoco estaría aquí.

Y por último, gracias a todas esas personas que, aunque no he nombrado aquí, me han ayudado y acompañado. Que no salgáis aquí no quiere decir que no me acuerde de vosotros.

SECTION I. INTRODUCTION

SECTION II. SUMMARY OF EXPERIMENTAL RESULTS AND DISCUSSION

Essential oils: analytical methods to control the quality of perfumes

A rapid and sensitive gas chromatography-mass spectrometry method for the quality control of perfumes: simultaneous determination of phthalates

Determination of atranol and chloroatranol in perfumes using simultaneous derivatization and dispersive liquid-liquid microextraction followed by gas chromatography-mass spectrometry

A chromatochemometric approach for evaluating and selecting the perfume maceration time

Dispersive liquid-liquid microextraction followed by gas chromatography-mass spectrometry for the determination of nitro musks in surface water and wastewater samples

Development of a selective solid phase extraction method for nitro musk compounds in environmental waters using molecular imprinted sorbents

Abstract

Fragrances are chemical compounds with a pleasant odour. They are used in many consumer products, providing them with their characteristic aroma. In this way, they can be found in all kinds of cosmetic products (such as perfumes, shampoos, deodorants or soaps) and household products (such as detergents, air fresheners or candles).

The use of cosmetic products has progressively increased over time and is now considered an indicator of the progress and prosperity of a country. The recent European Union (EU) Cosmetic Regulation harmonises the guidelines in order to achieve free movement of products and ensure a high level of protection of human health. This Regulation controls important aspects such as labelling and composition, indicating the prohibited substances and the maximum permitted quantity of certain cosmetic ingredients.

Concerning perfumes, these products require careful preparation to ensure that, in addition to the compliance of the Regulation, the finished product has the originally designed olfactory characteristics. Suitable analytical methods, that enable the appropriate quality controls, are required.

Furthermore, the EU Cosmetic Regulation prohibits or restricts the concentration of over 50 fragrances, due to possible side effects. Additionally, other substances, which are not fragrances themselves, but could appear in the finished product due to the manufacturing process or the product packaging, are also prohibited or restricted due to their side effects. Therefore, new analytical methods that enable the detection of prohibited or restricted ingredients in the raw materials and the finished products are required.

Moreover, all these fragrance-related substances could indirectly reach the environment if wastewater treatment plants are not sufficiently effective. In addition, because of their presence in cosmetics, they can also reach the environment due to activities such as bathing in seas or rivers. Therefore, it is also necessary to develop new analytical methods to estimate the potential for bioaccumulation in these ecosystems.

Some fundamental concepts about perfumes and legislation, the background of the topic, and the methodologies used in this PhD thesis are described in Section I.

The **aim** of this PhD thesis is the development of analytical methods for the detection and determination of fragrance compounds and perfume-related compounds that enable the adequate quality control of perfumes and the environmental impact assessment of fragrance substances.

Based on this aim, various analytical methods have been developed:

- a method for the determination of phthalates in perfumes. These analytes may be present in the product intentionally (used as fixatives, solvents of some fragrances, denaturing of the alcohols used in manufacturing...) or unintentionally (due to migration from plastic packaging to the product). The phthalates banned in the EU Cosmetic Regulation are determined using gas chromatography coupled to mass spectrometry (GC-MS) as analytical technique.
- a method to determine atranol and chloroatranol in perfumes. These two substances are found in natural plant extracts usually used as fragrances. However, they have an important allergenic potential and are in the process of inclusion in the list of prohibited substances of the EU Cosmetic Regulation. The method is based on GC-MS as analytical technique. As sample treatment liquid-liquid extraction (LLE) followed by dispersive liquid-liquid microextraction (DLLME) and simultaneous derivatization of the compounds is performed.
- a method to study and select the maceration time involved in the preparation of perfumes. The method is based on GC-FID (flame ionization detector) as analytical technique. The data are further processed by linear discriminant analysis (LDA).
- a method for the determination of nitro musks in environmental water and wastewater based on DLLME as sample treatment step followed by GC-MS as analytical technique. These compounds have been widely used as fragrances due to its pleasant odour. However, due to its harmful effects the use of musk ambrette, musk *tibetene* and musk moskene is prohibited in cosmetics and the use of musk xylene and musk ketone is restricted to a maximum concentration by the EU Cosmetic Regulation. Furthermore, these compounds are considered persistent pollutants with a strong tendency to bioaccumulate.
- a method for the determination of nitro musks in environmental water and wastewater based on solid phase extraction (SPE) using molecularly imprinted sorbents as sample treatment followed by GC-MS as analytical technique.

The **work methodology** followed to carry out the work of this doctoral thesis is:

1. *Development and optimization of the detection/determination step using standards of the substances studied.*

Due to the volatility of the analytes gas chromatography has been used. In general, a mass spectrometer detector has also been used, with which low limits of detection are obtained, suitable for trace analysis.

2. *Development and optimization strategies for cleaning and concentrating the analytes*

To carry out the determination of compounds at trace levels it is essential to include a step prior to the analysis, involving a concentration of the analytes. Besides, this stage should also remove interfering substances that may contain the sample matrix. In this sense, new methods that include DLLME or SPE using molecularly imprinted sorbents as sample treatment steps have been developed.

3. *Validation of the proposed methods.*

The developed methods have been evaluated based on their analytical characteristics, such as limits of detection and quantification, accuracy, precision or enrichment factors. The proposed methods have been applied to the analysis of real samples and the influence of the matrix has been evaluated by recovery studies.

4. *Conclusions and diffusion of results*

The results and conclusions have been diffused through the usual scientific means (presentation at conferences, published articles, etc.). The work performed in this PhD thesis has led to the six publications included in the Annex.

The detailed **conclusions** of each of the works are shown in the summaries written in Section II. Finally, the general conclusions of the doctoral thesis are shown.

Resumen

Las fragancias son productos químicos que desprenden un olor agradable y que se utilizan en multitud de productos de consumo a los que confieren su característico aroma. Así, se pueden encontrar en productos cosméticos de todo tipo (tales como perfumes, champús, desodorantes o jabones) y también en productos para el hogar (como detergentes, ambientadores o velas).

El uso de productos cosméticos se ha incrementado progresivamente con el paso del tiempo, considerándose un indicador del progreso y bienestar de un país. El reciente Reglamento Europeo sobre productos cosméticos armoniza íntegramente las normas comunitarias a fin de lograr la libre circulación de los productos y garantizar un elevado nivel de protección de la salud humana. Este Reglamento regula aspectos importantes, como el etiquetado y la composición, indicando las sustancias prohibidas, así como la cantidad máxima permitida de algunos ingredientes cosméticos y su aplicación.

En particular, los perfumes requieren una cuidadosa elaboración que asegure que, además de que se cumple el Reglamento, el producto acabado posee las características olfativas que se pretendía al diseñar su formulación. Para realizar los controles de calidad correspondientes se requieren métodos analíticos adecuados.

Además, el Reglamento Europeo sobre productos cosméticos prohíbe o restringe la concentración en el producto acabado de más de 50 fragancias, debido a sus posibles efectos secundarios para la salud del usuario. Además, otras sustancias, que no son fragancias propiamente dichas, pero que pueden aparecer en el producto acabado a través del proceso de fabricación o envasado también están prohibidas o restringidas debido a sus efectos secundarios. Por tanto, se requieren métodos analíticos capaces de detectar la posible presencia de trazas de ingredientes prohibidos y restringidos, en las materias primas y en los productos acabados.

Por otra parte, todas estas sustancias relacionadas con las fragancias pueden llegar indirectamente al medio ambiente si su eliminación en las plantas de tratamiento de aguas no es suficientemente efectiva y, también adicionalmente, debido a su presencia en productos cosméticos, pueden alcanzar directamente el medio ambiente debido a actividades como el baño en mares o ríos. Por lo tanto, es también necesario desarrollar métodos analíticos para estimar su potencial de bioacumulación en estos ecosistemas.

En la Sección I se describen algunos conceptos fundamentales sobre los perfumes y su legislación, los antecedentes del tema y las metodologías analíticas utilizadas en la presente Tesis Doctoral.

El **objetivo** de la presente Tesis Doctoral es desarrollar métodos analíticos para la detección y determinación de fragancias y sustancias relacionadas con los perfumes que permitan un adecuado control de calidad de los perfumes y una evaluación del impacto medioambiental de las fragancias.

En base a este objetivo, se han desarrollado diversos métodos analíticos:

- un método para la determinación de ftalatos en perfumes. Estos analitos pueden presentarse en el producto de forma intencionada (utilizados como fijadores, disolventes de algunas fragancias, desnaturalizantes de los alcoholes usados en su fabricación…) o de forma no intencionada (debido a su migración desde los envases de plástico al producto). Se han determinado los ftalatos prohibidos en el Reglamento Europeo utilizando cromatografía de gases acoplada a espectrometría de masas (GC-MS) como técnica analítica.
- un método para la determinación, en perfumes, de dos sustancias procedentes de extractos vegetales naturales utilizados como perfumantes, denominados *atranol* y *chloroatranol,* de conocido potencial alérgeno. Estas sustancias están en vía de inclusión en el listado de sustancias prohibidas del Reglamento Europeo. El método está basado en GC-MS como técnica analítica. Como etapa de tratamiento de la muestra se utiliza la extracción liquido-líquido (LLE) seguida de extracción liquido-líquido dispersiva (DLLME) y derivatización simultanea de los compuestos.
- un método que permite estudiar y seleccionar el tiempo de maceración implicado en la elaboración de los perfumes. El método está basado en GC-FID (ionización de llama) como técnica analítica. Los datos se tratan posteriormente mediante análisis discriminante lineal (LDA).
- un método para la determinación de nitro almizcles en aguas medioambientales y en aguas provenientes de plantas de tratamiento basado en DLLME como etapa de tratamiento de la muestra seguido de CG-MS como técnica analítica. Estos compuestos han sido ampliamente utilizados como fragancias debido a su agradable olor. Sin embargo, debido a sus efectos nocivos el uso de m*usk ambrette, musk tibetene* y *musk moskene* está prohibido en productos cosméticos y el de *musk xylene* y m*usk ketone* está restringido a unas concentraciones máximas por el Reglamento Europeo. Además, estos compuestos están considerados contaminantes persistentes con una fuerte tendencia a la bioacumulación.

- un método para la determinación de nitro almizcles en aguas medioambientales y en aguas provenientes de plantas de tratamiento basado en extracción en fase sólida (SPE) utilizando sorbentes molecularmente impresos como tratamiento de la muestra seguido de CG-MS como técnica analítica.

La **metodología de trabajo** utilizada en la realización de los trabajos que forman parte de esta Tesis Doctoral ha sido:

1. *Desarrollo y optimización de la etapa de detección/determinación empleando patrones de las sustancias a estudiar.*

Debido a la volatilidad de los analitos a estudiar se ha empleado la cromatografía de gases. En general, se ha utilizado la espectrometría de masas como detector, con el que se obtienen bajos límites de detección, aptos para el análisis de trazas.

2. *Desarrollo y optimización de estrategias de limpieza y concentración de los analitos.*

Para llevar a cabo la determinación de compuestos a nivel de trazas es de vital importancia incluir un paso previo al análisis, que implique una concentración de los analitos. Además esta etapa también debería eliminar las sustancias interferentes que pueda contener la matriz de la muestra. En este sentido, se han desarrollado nuevos métodos que utilizan como etapa de tratamiento de la muestra la DLLME o la SPE utilizando sorbentes molecularmente impresos.

3. *Validación de los métodos propuestos.*

Se han evaluado los métodos desarrollados en base a sus características analíticas, tales como límites de detección y cuantificación, exactitud, precisión o factor de enriquecimiento. Los métodos propuestos se han aplicado al análisis de muestras reales y se ha evaluado la influencia de la matriz mediante estudios de recuperación.

4. *Elaboración de conclusiones y difusión de los resultados*

Los resultados y conclusiones se han difundido mediante los medios científicos habituales (presentación en congresos, redacción de artículos, etc.) Los trabajos realizados en la presente Tesis Doctoral han dado lugar a 6 publicaciones que se incluyen en el Anexo presentado.

Las **conclusiones** detalladas de cada uno de los trabajos realizados, se muestran en los resúmenes elaborados en la Sección II. Finalmente, se presentan las conclusiones generales de la Tesis Doctoral presentada.

SECTION I.

INTRODUCTION

Chapter 1.

General view of fragrance-related compounds. Regulation and bibliographic record

Based on:

A. Chisvert, M. Lopez-Nogueroles, A. Salvador,
Essential Oils: Analytical Methods to Control the Quality of Perfumes,
in: M.J. Ramawat (Ed.), **Handbook of Natural Products**,
Springer-Verlag Berlin Heidelberg, 2013, pp. 3287-3310.

1.1. Fragrances and perfumes

"*Every fragrance is a unique combination of science and artistry.*" This phrase can be read in the web page of one the most important associations related to perfumes, the *International Fragrance Association* (IFRA). Indeed, fragrance companies make large investments in product innovation and this industry employs many scientists in research and development.

The sense of scent has a powerful influence in our lives. Scent stimulates emotions, such as attraction, stress or pain. It even triggers memories and alters moods. Moreover, it is a sense we use all the time. People, food, plants, everything has a particular natural odour, and we even add aromatic substances to many other things.

Fragrances are not usually sold directly. They are purchased to manufacturers of consumer products and embedded into different articles. Cosmetic products, such as shampoos, deodorants, soaps and perfumes, or household products such as laundry detergents, bleaches, air fresheners or candles contain fragrances that are responsible for their characteristic odour.

Among these aromatic chemicals, namely **fragrance chemicals**, or just simply **fragrances** it is very common to find compounds with the characteristic five-carbon isoprene unit, giving them the names of terpenes, such as terpene aldehydes, monoterpene alcohols, sesquiterpene alcohols, terpene ketones, terpene esters, monoterpene hydrocarbons or sesquiterpene hydrocarbons. In addition, other compounds, such as aldehydes, alcohols, ketones, esters, phenols or lactones are also very common. [1]

Fine fragrances, commonly called **perfumes**, are an alcoholic solution of hundreds of these chemicals. The word perfume is derived from the Latin word, "*per fumus*", meaning "through smoke", based on its incense origins.

The art of making perfumes started thousands of years ago by the Egyptian civilization. They associated them with the gods and recognised their positive effect on health and well-being. **Figure 1** shows a wall painting from ancient Egypt with Egyptian women smelling cones of scented unguent. The heat would melt the cones and they would apply it on their bodies.

After the Egyptians, perfumes were further developed by the Roman civilization, where they become popular among the wealthier class population. With the decline of the Roman Empire, its use decreased in Europe and perfumery became an oriental art, developed by the Persians and the Arabs. It was not until the 13th century that all this knowledge arrived to Europe due to Arabic influences. In the 14th and 15th century, France quickly became the European centre of perfume and cosmetic manufacture. A major industry grew in the south of France with the cultivation of flowers for perfume essences. Louis XV, in the 18th century, used it so much that his court was called "*le cour parfumee*" (the perfumed court). Nowadays, France is still the heart of the European's perfume industry.

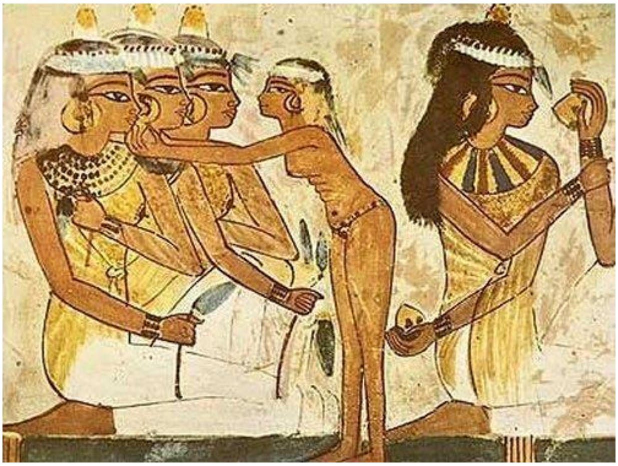


Figure 1. A wall painting from ancient Egypt

Since then, the perfume market has become very important. According to IFRA, it is estimated that fragrance dependent output throughout the fragrance value chain in Europe supported a total gross value (GVA) of over 51 billion of euros. Direct economic impacts within the value chain account for 30 billion. The remainder are the result of indirect multiplier impacts through purchases from European-based suppliers (13 billion), and induced multipliers due to additional consumption spending (8 billion). This information is shown in **Figure 2.**

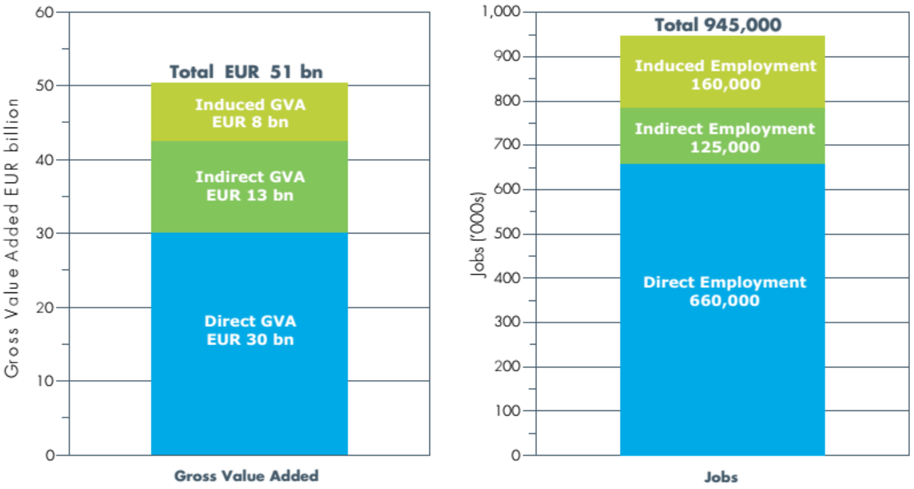


Figure 2. Total gross value added and jobs dependent on fragrance technologies in Europe in 2012. (Extracted from IFRA's web page)

1.2. Classification of perfumes

The classification of perfumes can be done according to different criteria. Most common classifications are carried out according to the origin of its fragrances, the type of odour of these fragrances or their final content in the product.

1.2.1 Nature of the perfume

Depending of its origin perfumes can be classified as **natural** or **synthetic** [2]. When the perfume raw material is of natural origin, it is called **extract** or **essential oil**. If not, **concentrate** is a more general term. All of these words are used to designate the concentrated hydrophobic liquid containing the volatile fragrances.

Natural perfumes can be obtained from plants (e.g. lavender, geranium) or from some of its parts, like flowers (e.g. jasmine, rose, gardenia), fruits (e.g. lemon, orange, vanilla), roots (e.g. vetiver, cistus, angelica), leaves (e.g. violet, patchouli, peppermint), wood (e.g. vetiver, sandalwood, cedarwood), bark (e.g. cinnamon, nutmeg), resin (e.g. benjui, tolu, galbanum) and seeds (e.g. angelica, celery, anis). They are also obtained from animal glands and organs. For example, natural musk fragrance are obtained from the testicles of the musk deer, civet is a secretion from glands of the civet cat, ambergris is obtained from a secretion from the intestine of the sperm whale and castoreum is obtained from glands near the reproductive organs of the beaver. **Figure 3** shows two photos of the process of natural perfume manufacture.

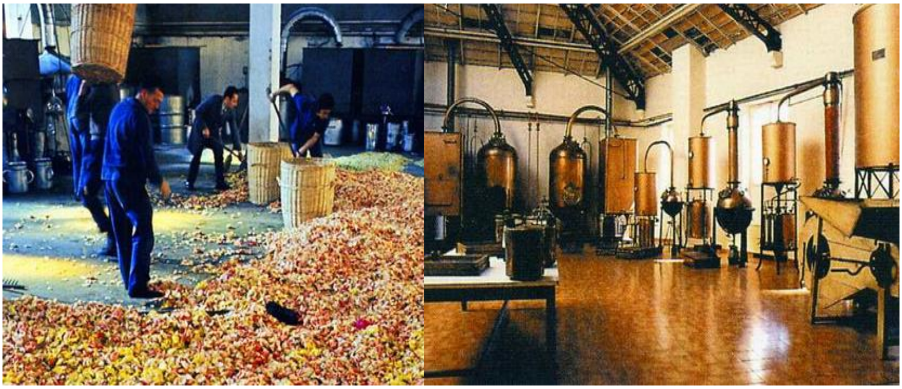


Figure 3. Workers collecting petals to make fragrances and an old perfume distillation factory (Extracted from http://blogs.elcorreo.com/miperfumeria/2012/07/16/creacion-perfumes/)

There are different extraction methods for natural perfumes or essential oils depending on the raw material and the chemical fragrances to extract:

▪ **Steam distillation** consists in passing water vapour through the raw plant material. This way the fragrances are extracted. Once the steam condenses the oil floats on the top of the water, so it is easily separated.

▪ In **hydrodistillation**, the raw material is immersed and boiled in water. The volatile essential oil is also obtained from the water vapour by condensation.

▪ In the case of **solvent extraction** raw materials are submerged in organic solvents, such as hexane, ethanol or petroleum ether and the fragrances extracted. Then, the solvent is eliminated by distillation resulting in the extract known as "concrete". The concrete can be further purified by a second extraction with ethanol resulting in a very pure essential oil known as "absolute".

▪ When the extraction solvent is pig or cow fat the process is known as **enfleurage**. This is a very time-consuming and expensive way of extraction.

■ **Maceration** is similar to enfleurage, but in this case the raw material is immersed in warmed oil.

■ In **rectification** the raw materials are directly heated without a solvent. This method is used when a toasted scent is wanted.

■ Another way of extraction is named **expression** and consists in manually or mechanically pressing the plants. This is very suitable for thermo labile compounds.

■ Finally, a more modern way of extraction is **supercritical fluid extraction**. In this process, carbon dioxide in supercritical conditions is used to extract the hydrophobic aromatic compounds from the raw material. It does not alter the nature of the aromatic compounds as it takes place at a low temperature and pressure. Removal of the carbon dioxide is easy as it vaporizes after depressurization. However, it is an expensive process and needs special instrumentation.

In contrast, **synthetic perfumes** are a mixture of fragrance chemicals synthetized in a laboratory with the objective of creating an odour similar to a natural fragrance or sometimes in search of something new and original. Unfortunately, a natural essential oil contains hundreds of different compounds and all, even those at trace levels, contribute to its characteristic odours. Thus, it is very difficult to exactly reproduce a natural perfume by just mixing different synthetic fragrances. Another difficulty encountered is sometimes two isomeric forms of a compound smell different and chiral synthesis is needed. Nevertheless, its lower cost compared to natural perfumes makes them increasingly common. Its price is not the only advantage. The quantity and quality of natural source supplies are in many ways unpredictable due to their dependence on crop quality or weather. Moreover, ethic concerns raise with the use of animal products.

1.2.2 Odour of the perfume

The fragrance expert Michael Edwards designed his own scheme of fragrance classification in 1983, taking into account the type of odour that fragrance provided. This classification is schemed in the **fragrance wheel**, shown in **Figure 4**. The fragrance wheel is a fragrance classification chart created in order to simplify fragrance classification and naming scheme. The wheel differences between four main groups:

■ **Floral** fragrances are those fragrances that reminds one of flowers such as jasmine, rose, heliotrope, etc.

■ **Fresh** fragrances include citrus odour fragrances, with aromas reminiscent of lemon, orange, lime, grapefruit, etc. The fruity ones are based on non-citrus fruity odours like peach, apple, banana, etc. Green fragrances are those that create the sensation of smelling grass or leaves while water main fragrances include marine and aquatic aromas.

■ **Woody** fragrances are those reminiscent of dry wood and trees.

■ Finally, **oriental** fragrances refer to those sweet strong fragrances reminiscent of vanilla, ambergris, etc

Figure 4. The Fragrance Wheel (Extracted from www.thefragranceshop.com)

Another important aspect is the persistence of the fragrance odour on the body. This characteristic gives rise to perfumery **notes**, which are related to the type of fragrance in the fragrance wheel. Notes in perfumery are descriptors of scents and are separated, according to volatility, into three classes. This classification is shown in **Figure 5**.

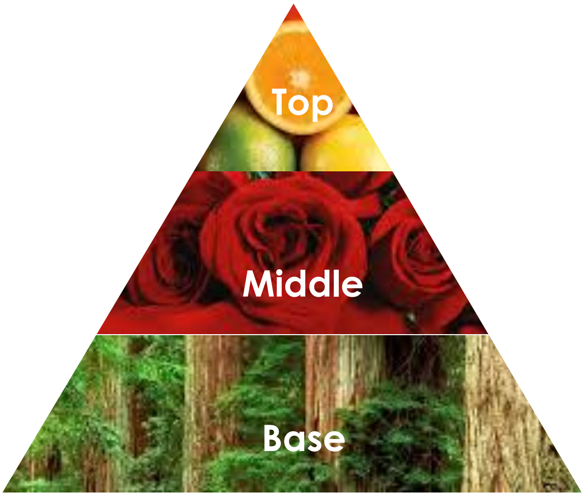


Figure 5. The fragrance notes pyramid.

■ The **top or head notes** are the most volatile and are responsible for the first impression. Their aroma lasts from a couple of minutes to an hour approximately and are usually those classified as fresh in the fragrance wheel.

■ After this, there is the **middle or heart notes**, composed by the medium-volatility fragrance chemicals, typically the floral essences. These are the most important as they are the identity of the perfume.

■ Finally, the **base notes** are those low volatility compounds that remain more than six hours after the application. Woody aromas tend to be this kind of compounds.

1.2.3 Fragrance content of the perfume

When the classification of perfumes depends on the fragrance raw materials (essential oil or concentrate) content, different types can be found:

- ***Eau de Cologne***
- ***Eau Fraiche,***
- ***Eau de Toilet,***
- ***Eau de Parfum***
- ***Parfum*.**

Figure 6 shows its typical concentrations. Almost all other cosmetic products contain fragrances, but obviously in a much lower content than that of fine fragrances. Moisturizing creams and body milks (0.1-0.5%), after-shave (0.5-2 %), toothpastes (0.5-1 %) and hair care products (0.01-0.1 %) are just some examples. Regarding cleaning household products, such as, laundry, dishwashing, bathroom or furniture products, nearly all contain perfume in variable amounts, to make them smell clean and mask unpleasant odours from other components of the product. [3]

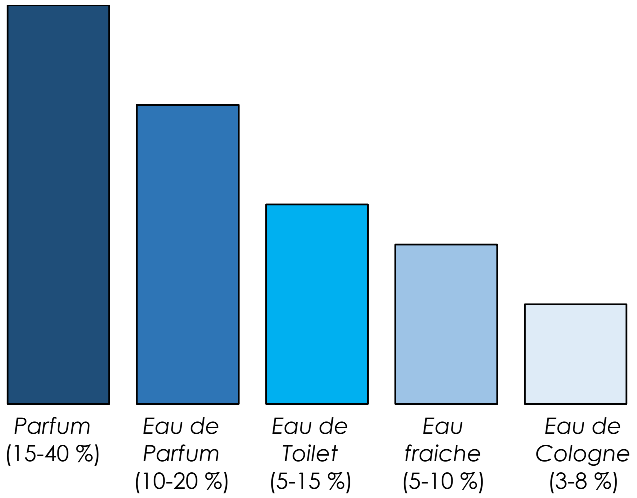


Figure 6. Approximate concentration of fragrance raw material in different types of fine fragrances

1.3. The manufacture of a perfume

The manufacture of a perfume involves several steps: [4]

■ The first of all the steps is the **obtaining of the perfume raw material** which, as already said, is named as extract or essential oil, in case it is of natural origin or as concentrate, in general terms.

This step includes the synthesis of aromatic chemicals in the laboratory by perfume chemists, in case of synthetic origin perfumes. In the case of natural origin perfumes, this step will consist in the obtaining of the animal products or the harvest, picking and transportation of plants. After this, the extraction of the natural fragrances from the raw materials is performed.

In order to obtain the concentrate, the different fragrances are mixed together according to the composition of the perfume. This perfume formula may contain hundreds or even thousands of different compounds.

■ Once the concentrate of the specific perfume is obtained, it is **blended** in different proportions with ethanol, depending on the type of fine fragrance desired. Other components are sometimes added in a much lower proportion, such as water, antioxidants, UV filters and colouring agents.

■ After the mixture is elaborated, it must be left some time in a process known as **maceration**, a process of maturing or aging of the perfume inside vats. The concentration of some components may change due to the transference of insoluble substances from the concentrate to the solution or due to the precipitation of cerate components. These changes occur until a steady state is achieved, in which the organoleptic characteristics of the wished perfume are obtained. It should be pointed out that the described perfume maceration process should not be confused with a procedure, already explained, which is an extraction method to obtain essential oils, also named maceration but referred to the raw material and not to the final product.

Usually, perfume maceration times last several weeks, even months, in order to assure that the process has been completed. This period frequently exceeds the time that would be necessary to obtain the organoleptic characteristics of the wished perfume, since the steady-state is generally reached before. This implicates important losses of time and its resulting economic cost as the perfumes remain in the vats of maceration more time than the necessary before being packed and ready to put on the market.

In this way, the aim of Chapter 5 of the present thesis is the development of an analytical method to detect any significant change in the process of perfume maceration, and thus to establish the perfume maceration time. This work, therefore, responds to a real problem of perfume companies. In fact, it was a result of an important company request to reduce costs of production in their perfume manufacture. Although this is an important topic in the field, no papers about the study and/or the establishment of the perfumes maceration time were found in bibliographic databases. In the preliminary steps of this work it was quickly assumed that to study the perfume maceration process from a chemical point of view, not only analytical sensitive and selective techniques were needed but multivariant treatment of the data was compulsory, provided that more than a compound might be altered during the maceration process.

■ Once the perfume maceration has concluded, the mixture is cooled and any sediment formed is removed by filtration. Finally, the perfume can be **bottled** and **commercialized**.

Quality control is an important issue in perfume manufacture and can be executed at any time of the whole process. The regulation in force in the country where the perfume is willing to be sold has to be complied. From an analytical chemistry standpoint, quality control focuses on the determination of compounds of interest, such as fragrance allergens or other prohibited or restricted compounds.

In this sense, Chapter 3 and 4 of the present thesis respond to this last need. The aim of Chapter 3 is the development of a method to determine in perfumes the family of phthalates banned by the EU Cosmetic Regulation on cosmetics. Besides, the aim of Chapter 4 is to develop a method to determine atranol and chloroatranol, fragrance allergens, in perfumes. Quality control can also assure that the composition of perfume is the one that was initially formulated. In addition, characterizing an essential oil is also very interesting.

1.4. Perfumes and the environment

Unfortunately, some of the ingredients used in cosmetic products, in general, and in perfumes, in particular, are harmful to the environment. The use of cosmetics has widespread so much that many of them have reached and are being accumulated in the environment, mainly in aquatic systems. In fact, some of them, such as sunscreens or musks, are already considered as **emerging contaminants** and are included in the prestigious annual reviews of Richardson dealing with water analysis [5]. Moreover, in the case of those compounds with lipophilic characteristics, these compounds have been bioaccumulated in different species. In the case of perfume related substances, it has to be taken into account that its presence in the environment may be due not only to cosmetic products but also to household products that also contain them.

All of these compounds have reached the aquatic environment by direct and indirect sources. Direct inputs can come from recreational activities (e.g., swimming and sunbathing) or industrial wastewater discharges, whereas indirect inputs can come via wastewater-treatment plants from different activities as showering, washing clothes, industrial wastewater discharges, etc. As a result, it is important to evaluate the potential for bioaccumulation on aquatic ecosystems of all these compounds that appear in the aquatic environment.

In this sense, the aim of Chapter 6 and 7 of this thesis is the development of analytical methods to determine nitro musks in wastewater and surface waters. These fragrance compounds are considered persistent pollutants due to their strong tendency to bioaccumulate [6] and are related to dermatitis, carcinogenic effects and endocrine dysfunction [7-11].

1.5. Regulation for fragrance chemicals

The European Union (EU) is a single market, which means that is a free trade area with common policies on product regulation. Concerning regulation on cosmetic products, Council Directive 76/768/EEC was adopted on the 27th of July of 1976. Since then, many amendments and adaptations have been made. Last recast of all these changes as one single text is Regulation 1223/2009 [12]. This single law addresses important aspects, as cosmetic composition or labelling, in order to protect public health. It controls and regulates the substances banned in cosmetics, as well as the maximum allowable amount of certain restricted substances according to their intended use. Moreover, the so-called European Inventory of Cosmetic Ingredients [13] lists the substances usually employed in cosmetic products. This list is not restrictive as any other substance may be used as long as it is not banned. Section two of this inventory deals with those ingredients used in perfume and aromatic raw materials.

Other important markets in the cosmetic global industry are the Unites States (US or USA) and Japan. In the USA, the Food and Drug Administration (FDA) has a much more permissive [14] regulation. Regarding Japan, the Pharmaceutical and Food Safety Bureau [15] is the regulatory body in charge of cosmetics. This regulation is more similar to the European and collects, as this one, lists of banned and restricted cosmetic ingredients.

A cosmetic product is defined by the EU Cosmetic Regulation as "*any substance or mixture intended to be placed in contact with the 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, protecting them, keeping them in good condition or correcting body odours*". Besides, the FDA defines a cosmetic product as "*an article intended to be applied to the human body for cleaning, beautifying, promoting attractiveness, or altering the appearance without affecting the body's structure or functions*". Not only fragrances and perfumes are a cosmetic product itself but are many times an ingredient added to make another cosmetic product smell nice or mask any undesirable odour of the product.

Regulation of cosmetic ingredients is imperative, as they are daily use products used by the population of all ages. Its use makes them vulnerable to be inhaled, ingested or absorbed through the skin into the bloodstream and transported throughout the body. Therefore, their dermatological effects or their effects on other organs of the body should be taken into account.

EU Cosmetic Regulation specifies that all the ingredients of cosmetic products must be indicated on their label. This is problematic in the case of perfumes as its essential oil or concentrates usually contain too many different compounds and are many times considered trade secrets. Therefore, perfume components are an exception and do not necessarily have to be specified but they can be grouped and labelled under the word "perfume" or "aroma" [2].

However, skin irritations or allergic reactions due to fragrance chemicals are relatively common. Different effects have been reported, such as skin sensitivity, rashes, dermatitis, coughing or asthma attacks. In order to improve the diagnosis of contact allergies among consumers and to facilitate these users to protect themselves from these compounds the 2003/15/EC Directive [16] listed 26 fragrances classified as **potentially allergenic substances** (PASs). Since then, any cosmetic product containing any of these 26 substances have to declare its presence on the label when present at a higher concentration than 0.001 % in those products to remain on the skin or 0.01 % in those intended to be rinsed off.

In February of 2014, the European Commission proposed a change to a tighter regulation [17]. The proposal is to add an extra list of allergens to the previous list of 26 allergens which presence has to be mentioned on the label and to ban some others. A 3 months public consultation was started in which any interested parties, including authorities of the Member States, manufacturers of cosmetic products, producers of the substances concerned, relevant industry and consumers associations can send any comment. After this period, the proposal, with some changes if necessary, will become a measure in force. In this new proposal there are three compounds planned to be banned in cosmetic products, hydroxyisohexyl 3-cyclohexene carboxaldehyde, **atranol** and **chloroatranol.** In this sense, the determination of these two last compounds in perfumes is the aim of Chapter 4 of this thesis.

At this point, it is worth mentioning that the term ‘fragrance free’ has no legal definition. In fact, even though this term is claimed on the label the cosmetic product will most probably still contain fragrance chemicals, just in a lower concentration. Similarly, the term ‘hypoallergenic’ has no legal definition and does not guarantee immunity to any allergic reaction. Not only fragrances produce allergies but also some preservatives, dyes, surfactants, solvents, etc. [3]

Besides the compliance of the legislation in force in each country, the fragrance industry is in some way self-regulated by some independent organizations. The *Research Institute for Fragrance Materials* (RIFM) evaluates and distributes scientific data on the safety assessment of fragrance substances found in cosmetics and other products. In fact, the IFRA establishes usage guidelines for fragrance ingredients based on RIFM evaluation results and recommends avoiding many ingredients. [18]

Some fragrances are related to other health problems, not related to allergies. The EU Cosmetic Regulation prohibits and restricts the amount used of more than 50 fragrance substances, like some extracts or synthetic musks, in cosmetic products. Other substances related to fragrances, such as **phthalate** esters which have been used as solvents and vehicles for fragrance ingredients, are also restricted because of their undesirable side effects. In this sense, a method for the determination in perfumes of those phthalates banned in cosmetic products is presented in Chapter 3 of this work.

Besides, in the case of household products, Regulation 648/2004 [19] regulates detergents in the EU. These products have similar labelling requirement for perfume content than cosmetic products. In this sense, a perfume or an essential oil shall be considered a single ingredient and will be labelled as “parfum” listing none of the substances that it contains. Additionally, as happens in the case of cosmetic products, there is an exception for those allergenic fragrance substances that appear on Annex III of the cosmetic regulation. These ingredients must also be labelled separately on the household product if their concentration exceeds 0.01 % in weight.

The European Directive on environmental quality standards in the field of water policy presents a list of priority substances. In it, **musk xylene** is listed as a substance subjected to review for possible identification as priority substance or priority hazardous substance [20]. According to legislation, the causes of pollution should be identified and emissions should be dealt and rectified for all those substances considered as priority in this Regulation. In addition, Member States should improve the knowledge and data available on the sources of these priority substances.

Accordingly, and as already mentioned, two methods to determine musk xylene, and other nitro musks, in surface waters is presented in Chapter 6 and 7 of this thesis.

1.6. Analytical aspects of fragrance-related compounds.

As already mentioned, perfume manufacturers perform quality control of commercial perfumes and raw materials to assure that they fulfil the desired quality. To do so, the instrumentation available in cosmetic industries is sometimes scarce. However, there are many easy and non-expensive quality control techniques that are performed to get information about the quality of the product, such as measurements of physical (density, colour, optical rotation, etc.) and chemical properties (acidity or carbonyl indexes). Spectrometric techniques, such as ultraviolet/visible (UV/VIS) or infrared spectrometry can also provide overall qualitative, or even quantitative, useful information for the quality control of a perfume by comparing the obtained spectra with those previously recorded and kept in the databases [1]. However, these techniques provide overall information. To obtain information about each component separately, and taking into account that perfume ingredients are volatile or semi-volatile compounds, gas chromatography (GC) is by far, the technique of choice [21], usually using a flame ionization detector (FID) or a mass spectrometer (MS).

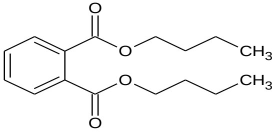
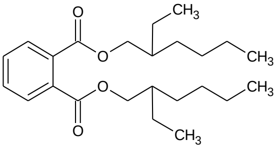
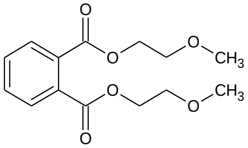
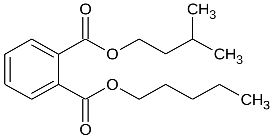
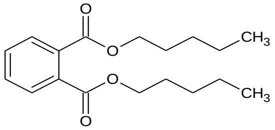
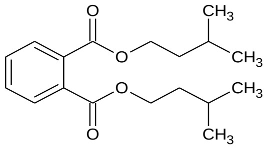
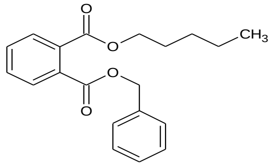
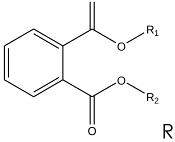
Electronic noses is a common technology in the food and beverage industry, for which many applications are found in the databases [22]. Medical and environmental applications are also found. This technology is also useful in the cosmetic and fragrance industry. This device mimics human olfactory system and consists of a sample system in headspace, a sensor array as detection system and a computer for data treatment. It is not a separative mechanism, it just gives signal patterns for a particular odour [23]. In a way, it measures smells. Electronic noses need a database of reference to treat the results. In this sense, the usual way to work in this technique is to start analysing a set of known samples and then treating this data statistically, for example with pattern recognition methods, such as principal component analysis (PCA), artificial neural network (ANN) or clusters. After that, new unknown samples can be recognized by comparing them with the database. Different works using an electronic nose in fragrance analysis can be found. For instance, Carrasco et al. [24] use an electronic nose to discriminate between different families of Yves Saint Laurent perfumes. Branca et al. [25] developed an electronic nose device combined with PCA and ANN to detect the presence of a perfume note called mangone in fragrances. Cano et al. [26] developed a PCA method to detect between original and counterfeit perfumes. Fernandez et al. [27] showed the applicability of an electronic nose for the discrimination of origin, qualities and harvesting year of a natural raw material called benzoin gum. Ye et al. [28] also used an electronic nose combined with PCA to discriminate between natural musk and adulterated.

1.6.1. Phthalates

The term phthalate is used to designate the dialkyl or alkyl aryl esters of phthalic acid. These compounds are used in the cosmetic industry mainly as denaturants of the alcohols used in their manufacture or as fixatives or solvents for some fragrances. Thus, it is usual to find them in nail polish, hairsprays and perfumes [29]. Moreover, these substances are mainly used in the manufacture of plastics to increase their flexibility. Therefore, they can be found in different manufactured products, not only as part of its formulation but as a result of migration during manufacture or storage [2].

Phthalates have been proven to be harmful to living organisms [30-32]. These lipophilic compounds are rapidly metabolized by humans and biotransformed in their respective monoesters and other oxidative products, which are excreted in urine and faeces [33]. Studies show that some phthalates and their metabolites are potentially toxic in animals due to endocrine-disruptive effects, which can cause adverse health effects, particularly for fertility and reproduction [34-36]. Moreover, some of them cause cancer in rats and mice [37]. Therefore, the presence of some phthalates is forbidden by the European legislation in some vulnerable products, such as toys [38] or personal care products [12]. Specifically, current EU Cosmetic Regulation forbids the presence in cosmetic products of the phthalates shown in **Table 1**

Table 1: Forbidden phthalates in cosmetic products according the European Union Regulation

Compound	Used acronym	CAS Nº	Molecular structure
Dibutyl phthalate	DBP	84-74-2	
bis(2-Ethylhexyl) phthalate	DEHP	117-81-7	
bis(2-Methoxyethyl) phthalate	DMEP	117-82-8	
n-pentyl isopentyl phthalate	NPIPP	84777-06-0	
di-n-Pentyl phthalate	DNPP	131-18-0	
Diisopentylphthalate	DIPP	605-50-5	
Benzyl butyl phthalate	BBP	85-68-7	
di-C7-11(linear and branched)-alkyl phthalates	DHNUP	68515-42-4	 R=C_7H_{15}, C_8H_{17}, C_9H_{19}, $C_{10}H_{21}$ or $C_{11}H_{23}$

Other phthalates like diethyl phthalate (DEP), dimethyl phthalate (DMP) or di-n-octyl-phthalate (DNOP) are allowed in cosmetic products in the EU without any restriction, even though they are defined as pollutants by the U.S. Environmental Protection Agency [39] and are also related to adverse health effects [40,41].

Concerning the phthalate determination the number of publications dealing with these compounds shows their great interest and their widespread presence in many consumer products and the environment. Specifically, in the case of the determination of phthalates in cosmetic products, several analytical methods for the identification and determination at the level of µg mL^{-1} and ng mL^{-1} have been published. The most commonly used techniques are liquid chromatography (LC) combined with UV/VIS and GC with FID or coupled to MS. Most of the publications deal with the determination of dibutyl phthalate (DBP), bis(2-ethylhexyl) phthalate (DEHP), benzyl butyl phthalate (BBP) and di-n-pentyl-phthalate (DNPP) within other non-prohibited phthalates as DEP or DNOP. Less common are studies that include bis(2-methoxyethyl) phthalate (DMEP), the most polar prohibited phthalate. Besides, no analytical methods for the determination of the mix of C_{7-11} branched and linear alkyl esters (DHNUP) have been found.

The determination of phthalates in cosmetic samples started many years ago, as shown in a work dated in 1973 where different phthalates were determined by GC-FID [42]. Many years later, Markovic et al. [43] published a work where the phthalate DMP and N,N-diethyl-m-toluamide, both used as insect repellent compounds, were determined in cosmetic samples by thin-layer chromatography (TLC) and UV scanning densitometry. Koo and Lee [44] estimated the median daily human exposure to phthalates by determining the presence of the phthalates DEHP, DEP, DBP and BBP by LC in a hundred different cosmetic samples, like perfumes, deodorants or nail polishes. Samples were weighted, dissolved in methanol, vortexed and centrifuged before injection. As expected, DEP, the non-banned phthalate, was found at high concentrations. Nevertheless, the other phthalates were also present in rather high concentrations in many samples.

Chen et al. [45] developed a method for the determination of six phthalate esters, DMP, DEP, DBP, BBP, DEHP and DNOP, in cosmetics by GC-FID. MS was also used but just to qualitatively confirm the presence of the target compounds. Sample pretreatment, similar to Koo and Lee's work, consisted in weighting the samples, dissolving them in methanol, sonicating and centrifuging. The upper clean layer was used previous elimination of water with anhydrous sodium sulphate. Compared to the previous works this method showed better sensitivity, with lower limits of detection (LOD). De Orsi et al. [46] continued with LC coupled to UV detection, reducing the run time of the previous work. DMP, DEP, dipropyl phthalate (DPP), diisobutyl phthalate (DIBP), BBP, DBP and DEHP were determined in nail cosmetics. Shen et al. [47] studied the determination of seven phthalates and four parabens, used as preservatives, in different kinds of cosmetic samples, using both LC-UV and GC-MS in SIM (single ion monitoring) mode. A clean-up step using C18 SPE (solid phase extraction) was carried out, which improved the LOD. However, the method is time-consuming and uses high volumes of methanol, so it could not be considered a green method suitable for routine analysis. The IFRA has a phthalate quantification procedure, available in its website [48]. The determination is performed in a GC-MS in SIM mode, with both a quantifier and a qualifier ion per compound.

Traditional extraction techniques that involve the use of high amounts of toxic solvents should be avoided. In these sense, Chingin et al. [49] presented a novel procedure based on extractive electrospray ionization mass spectrometry (EESI-MS) for the detection of DEP in perfumes with no need of sample pretreatment. Su et al. [50] determined DMP, DBP, dicyclohexyl phthalate (DCHP) and DNOP in perfumes and lacquer removers based on polymer monolith microextraction and LC-UV. The conditions for extraction were optimized leading to a sensitive method.

Later, Koniecki et al. [51] determined the phthalate levels in 252 cosmetic products from the Canadian market by GC-MS to estimate the dermal exposure. Sanchez-Prado et al. [52] also developed a method that allows the simultaneous determination of many compounds, including phthalates, in perfumes. The method consists in diluting the sample in ethyl acetate and injecting it directly into a GC-MS system. Kamerai et al. [53] used ultrasound-assisted emulsification microextraction with solidification of floating organic droplet as sample preparation technique followed by LC for the analysis of DBP and other non-prohibited phthalates in cosmetic products and water samples. Gimeno et al. [54] published a method to determine the eight prohibited phthalates by the EU Cosmetic Regulation, where sample preparation depends on the matrix. Thus, some cosmetic samples are just diluted and more complicated are assayed after a LLE (liquid-liquid extraction) with tert-methyl butyl ether. Analysis are then carried out by GC-MS. Validation criteria were obtained using the ISO 12787 [55] and the work proposed as a standard for the assay of phthalates in cosmetic samples at the European Comitee for Standardization. Later, Moradi et al. [56] presented an ultrasound-assisted emulsification microextraction (USAEME) followed by LC with UV detection for the determination of three non-prohibited phthalates. This method was successfully applied to hair gel, perfume and water samples. Finally, Llompart et al [57] published a matrix solid-phase dispersion (MSPD) and GC-MS method for the determination of the prohibited phthalates among other fragrance related substances in cosmetic products.

All studies with real perfume samples show the common presence of some of the prohibited phthalates in Europe, like DBP, BBP and DEHP, even in several hundred parts per million. In this sense, the FDA conducted studies of consumer cosmetic products for phthalate esters [58,59] where many of them were found. A Greenpeace study [29] of different commercial perfumes by GC-MS also found prohibited phthalates in the mg L^{-1} range. Cosmetic industry should try to avoid this issue using free phthalate plastics and solvents.

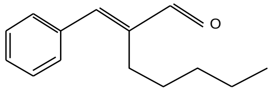
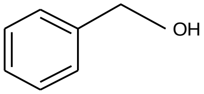
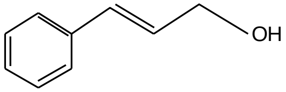
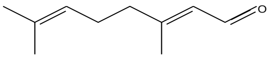
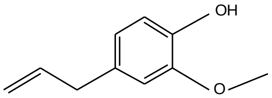
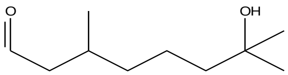
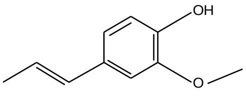
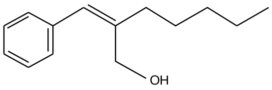
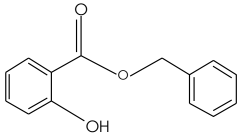
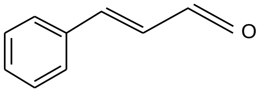
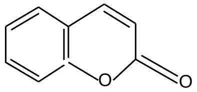
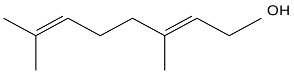
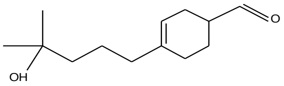
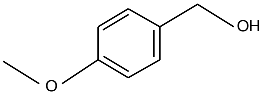
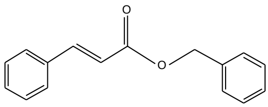
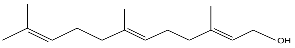
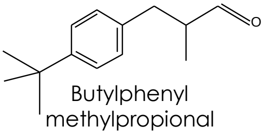
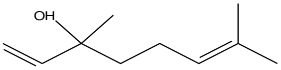
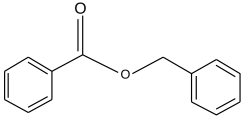
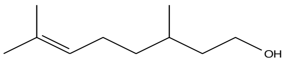
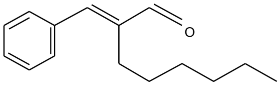
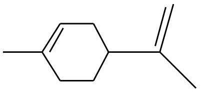
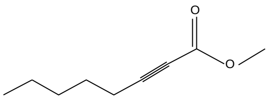
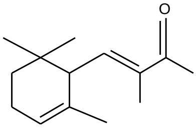
In order to contribute to the rigorous quality control of perfumes, Chapter 3 summarizes a GC-MS method for the simultaneous determination of phthalates in perfumes. The method uses ethanol as solvent and no dilution of the samples is performed. It was assayed in the determination of all the currently banned phthalates with the only exception of DHNUP, as it is a mixture of several compounds.

1.6.2. Potentially allergenic fragrance substances

The 26 substances listed in **Table 2** are currently declared as potentially allergenic substances (PASs) by the EU Cosmetic Regulation[16]. 24 of these 26 substances are chemically defined volatile compounds whereas the other two are natural moss extracts, thus, not defined chemicals, but a natural mixture of many of them. Within the 24 chemically defined allergens, it is important to note that they are very different classes of compounds, such as alcohols, carbonyl compounds, esters or phenols.

After this Regulation came into force, many efforts have been made for the development of new analytical methods for the determination of these skin-sensitizing agents. Most of the published works focus on the determination of the 24 chemically defined PASs, since the natural conditions of the two natural moss extracts, without a standardized industrial processing, makes the composition of these extracts variable and their accurate determination complicated.

Table 2. Chemical structure of those compounds declared as potentially allergenic substances by the EU Cosmetic Regulation.

 Amyl cinnamal	 Benzyl alcohol	 Cinnamyl alcohol
 Citral	 Eugenol	 Hydroxycitronellal
 Isoeugenol	 Amylcinnamyl alcohol	 Benzyl salicylate
 Cinnamal	 Coumarin	 Geraniol
 Hydroxyisohexyl 3-cyclohexene carboxaldehyde	 Anise alcohol	 Benzyl cinnamate
 Farnesol	 Butylphenyl methylpropional	 Linalool
 Benzyl benzoote	 Citronellol	 Hexyl cinnamal
 Limonene	 Methyl 2-octynoate	 Alpha-isomethyl ionona
Oakmoss extract (*Evernia prunastri* extract)	Treemoss extract (Evernia furfuracea extract)	

Their volatile characteristics makes GC the better technique of analysis, specially using FID or MS. This last selective detector is very useful as one of the biggest difficulties to face in their determination is the coelution of the target compounds with other of the many components of the perfume. Coelution leads to false positives and negatives, which published works try to solve operating either in SIM or extracted ion chromatogram (EIC) mode. Even working in SIM, these problems are not completely eliminated. Better ways are the use of comprehensive GC or clean up steps before the determination. [60]

In this sense, Rastogi proposed for the first time, even before the approval of the regulation, a SPE followed by GC method for the determination of 11 of the 26 substances declared as PASs [61]. GC-MS was used for previous identification while GC-FID was chosen for quantification. Some years later the same author published another work [62] using the same GC-MS method, with some modifications, to survey the content of the 24 PASs in cleaning products and other consumer products such as cosmetics or toys. In this method, the sample treatment depended on the type of sample, but in summary, samples were shaken with methanol, and the lixiviated fraction was purified with a silica gel column before its injection into the GC port. Detection was performed in full scan (FS) and quantification in EIC mode. In the same way, Ellendt et al. [63] did also use GC-MS, FS mode for identification and SIM mode for quantification, for the determination of the 24 PASs in deodorants. However, coelution problems persisted in complex fragrance mixtures.

Later, Chaintreau et al. [64] made a significant progress presenting a method to determine PASs in perfume concentrates, with direct injection of the samples. This work explores in the coelution problem between the target compounds and other components of the perfume. Four different columns were used to try to solve this problem. SIM mode of three different ions per compound (i.e., one ion for quantification and the others two as qualifiers), were also used and their relative abundances taken into account for correct identification, thus minimizing false positives and false negatives, by comparing the relative abundances with respect to those of the reference compound. Even though all this helped, false positives, due to coelution, still occurred. This work highlighted the need for clean-up techniques as the non-volatile parts of the cosmetic products remain in the injector disturbing future injections. This work was accepted as the official IFRA method for the quantification of potential fragrance allergens in fragrance compounds [65]. Some extra recommendations were added in a paper published some years later [66], which concluded that coelution problems could still occur and recommended to inject each sample in two different polarity columns as well as to monitor three ions per compounds to assure about the positive identification/quantification. A ring-test was conducted using this strategy, and the results published years later [67], in which the investigation of an automated data treatment procedure to aid the analyst during the interpretation of the analytical results was also proposed.

Leijs et al. [68] followed a similar strategy for the analysis of these 24 compounds in fragrance raw materials and perfume oils. These authors used a chromatograph with two injectors connected each one to a different polarity column. Both columns were connected to a MS interface using a dual-hole ferrule, thus carrying out a sequential dual-column analysis for each sample. However, they preferred operating in FS mode and quantifying in EIC mode, to avoid additional acquisition in case of coelutions, and to prevent false negatives as a result of retention time shift. Obviously, this mode of acquisition significantly increases the limit of quantification (LOQ).

Another work, from Mondello et al. [69] determined the PASs using fast GC-MS, by employing shorter and narrower columns besides high-speed MS detector. The MS operated in FS mode and quantification was performed in EIC mode. The main novelty of this work consisted in a very short run-time (less than 5 minutes). However, the same above-mentioned problems regarding coelutions are expected to occur.

As Chaintreau and co-workers pointed out, a clean-up step that separates the target compound from the matrix to avoid dirtiness of the system is advisable. Some works can be found in the literature describing clean-up steps before the determination of the 24 restricted allergens. For example, as mentioned before, Rastogi [62] used a silica gel column for purifying the extracts. Niederer et al. [70] proposed a clean-up step using size-exclusion chromatography prior to GC-MS for the determination of the 24 PAS in different cosmetic products, such as creams, body lotions and oils. David et al. [71,72] classified samples into four different groups based on matrix characteristics. Simple samples, with no presence of non-volatile material, were diluted and injected. More complex samples were also injected directly but in a two dimensional GC. This is not possible with dirtier matrices, where a programmed temperature vapourizing (PTV) inlet with an automated liner exchange (ALEX) was proposed. Thus, fractioning of the target compounds from this non-volatile matrix took place in the liner. When handling aqueous samples with the target compounds at very low concentrations a stir bar sorptive extraction (SBSE) step was added before the PTV approach. Regarding clean-up steps Chen et al. [73] used solid-phase microextraction (SPME) followed by GC-FID for the quantitative analysis of geraniol, a restricted allergen, and other flavour and perfume compounds in shampoo. Lamas et al. [74] proposed the use of solid-phase dispersion-pressurized liquid extraction (SPD-PLE) followed by GC-MS in EIC mode for the determination of the 24 regulated fragrance allergens and two other fragrance compounds in cosmetic products (creams and lotions). The same authors have published several works concerning fragrance allergens. They presented a method based on MSPD as concentration and clean-up step followed by GC-MS in SIM mode for PASs determination in cosmetic products [75] and a MSPD and PLE procedures followed by GC-MS to determine suspected allergens in marketed baby and child care products [76]. A recent work [77] from these authors develops an analytical method based on micro matrix solid phase dispersion for the analysis of fragrance allergens and preservatives in personal care products. The analysis is performed using a triple quadrupole-mass spectrometer (QqQ). Same authors also published a GC-MS method [52] with no pre-treatment of samples other than dilution for their determination among other fragrance related components in perfumes.

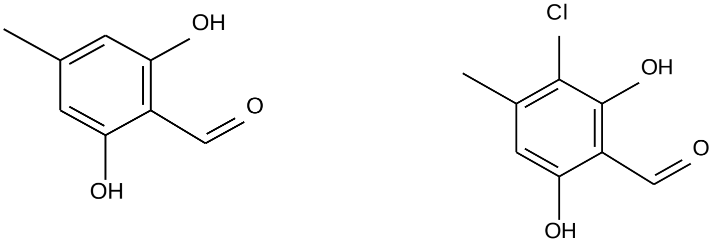
Development in the field of comprehensive GC (GCxGC) also seems a good way to handle the coelution problem. In this sense, Chaintreau and co-workers [78] presented a GCxGC-FID method for the determination of the 24 regulated substances plus two other compounds. The method could not completely eliminate component overlap, leading to similar results than using 1D-GC coupled to MS. Later, the same authors [79] presented the first work dealing with the quantification of the PASs based on comprehensive GC coupled to a MS (GCxGC-MS). As obviously expected, selectivity is improved compared to GC-MS and GCxGC-FID. Comprehensive GC can overcome coelution problems but is very time consuming during the interpretation of the analytical results. The same authors published another paper [80] presenting a software prototype to reprocess the data which shortens this step. Leco [81] presented a method for allergens in perfumes using GCxGC-(TOF)MS (time-of-flight mass spectrometer). Identification was better achieved than using a simple quadrupole but the equipment is too expensive to be used in routine analysis. Dunn et al. [82] compared two approaches for this analysis, GCxGC and multidimensional gas chromatography (MDGC), in which selected portions of the primary separation, rather than the whole sample, are transferred to a secondary column. MDGC seems to be a better option as data processing for the output is almost identical to a single column analysis while the data of a GCxGC experiment is still relatively labour intense. Cordero et al. [83] published a work where their determination by GCxGC-MS and GCxGC-FID is accomplished. Finally, Devos et al. [84] presented a method for fragrance allergens in cosmetic products using a combination of full evaporation-dynamic headspace (FEDHS) with selectable one-dimensional/two-dimensional GC–MS. The full evaporation dynamic headspace avoids contamination of the analytical system by high molecular weight non-volatile matrix compounds.

Works dealing with the determination of PASs with a different technique than GC are also found in the literature. Villa et al. [85] propose the first work where LC-UV is used for the determination of the 24 suspected allergens in different cosmetic products. A C18 column and a gradient acetonitrile/water were used. Samples are weighted, diluted, sonicated and filtered before injection. This can be a useful method for routine analysis in cosmetic industries where more expensive equipment is not available. However, coelution problems are present, not only between compounds but also for matrix components. In this sense, Furlanetto et al. [86] published a method for 18 of the 24 allergens by microemulsion electrokinetic chromatography (MEEKC) and UV detection. The method was applied to two rinse-off cosmetics, a shampoo and a bath gel. Rudback et al. developed for the first time a LC-MSxMS for the determination of three allergens in essential oils. Lately, Lopez-Gazpio et al. [87] also presented a micellar electrokinetic chromatography method (MEKC) for the determination of 15 suspected fragrance allergens and some preservatives in personal care products.

Concerning the determination of the two natural moss extracts restricted as potentially allergenic – treemoss and oakmoss –, the number of publications is more uncommon. The industrial processing varies considerably but basically harvested lichens, from oak trees in the case of oakmoss and pine and cedar trees in the case of treemoss, are extracted, usually with hexane or more polar solvents. Then they are further diluted in ethanol and usually submitted to physical treatments intended to remove their original colour. Taking into account their natural conditions and that their industrial processing is not standardised it is not surprising to find variability in the chemical composition of these extracts. Therefore, the quantification of some of their individual components is not indicative of their total amount. Joulain et al. published two comprehensive reviews on the composition of these extracts. 170 constituents are identified in oakmoss extracts and 90 in the case of treemoss [88,89].

From all the different compounds present in oakmoss and treemoss extracts, atranol and chloroatranol are very potent allergens [90-93]. **Figure 7** shows the chemical structure of these compounds. They are degradation products, formed after transesterification and decarboxylation of the lichen depsides atranorin and chloroatranorin, and are formed in the moss absolute production [94]. As already specified, although these compounds are not listed in the EU Cosmetic Regulation, the European Commission proposed in February of 2014 a change to a tighter regulation regarding fragrance allergens [17]. The proposal includes the prohibition of atranol and chloroatranol in cosmetic products, due to its high allergenic features. Therefore, these compounds will, most surely, be banned or at least restricted in the near future. Therefore, works dealing with their determination will be useful, specially taking into account the scarce number of analytical methods available for this purpose.

In this sense, the determination of these two last compounds in perfumes is the aim of Chapter 4 of this thesis.



Atranol Chloroatranol

Figure 7. Chemical structure of atranol and chloroatranol

Regarding their determination Bernard et al. [93] searched for allergenic or sensitizing molecules in oakmoss using GC-MS after chemical fractionation of the extract by gel permeation chromatography. David et al. [71] semiquantitatively determined chloroatranol in moss extracts by GC-MS using a programmed temperature vaporizing inlet with an automated liner exchange. The liner was packed with polydimethylsiloxane that retained non-volatile material. Hiserodt et al. [94] developed a qualitative method using liquid chromatography-tandem mass spectrometry (LC-MS/MS) to identify atranorin and some related potential allergens, including atranol and chloroatranol, in oakmoss absolute. Bossi et al. [95] further developed this method, with direct injection of the sample, to quantify atranol and chloroatranol in perfumes. The recovery of chloroatranol from spiked perfumes was 89±10%. Low recoveries (49±6%) were observed for atranol in spiked perfumes, indicating ion suppression caused by matrix components. Rastogi et al. [96,97] also quantified the presence of these two allergenic compounds in different commercial perfumes based on this method.

As many times aforementioned, clean-up step that separates the target compound from the matrix to avoid dirtiness of the system is advisable to obtain a good accuracy. Thus, accordingly, the work summarized in Chapter 4 proposes a LLE step to clean up the sample solution from lipophilic substances. After this, a simultaneous derivatization and dispersive liquid-liquid microextraction (DLLME) step that enables to change the solvent (for further GC-MS analysis) and to concentrate the target compounds is performed. The derivatization step is proposed since atranol and chloroatranol are relatively polar compounds, and without it, a poor extraction efficiency would be achieved in the DLLME process. Moreover, the derivatization allows volatility and thus increases sensitivity in GC-MS. The chosen approach was an in situ derivatization via acetylation of the hydroxyl groups under basic aqueous conditions, using acetic anhydride as acetylating agent. The derivatization is performed at the same time as DLLME, allowing the simplification of the procedure and a decrease in the time of analysis. This one step derivatization and DLLME was first introduced by Fattahi et al. [98] to determine clorophenols in waters and has been later used by other authors to determine phenols in a similar approach although also performing other types of derivatization reactions such as silylation [99,100] and O-alkoxycarbonylation [101,102].

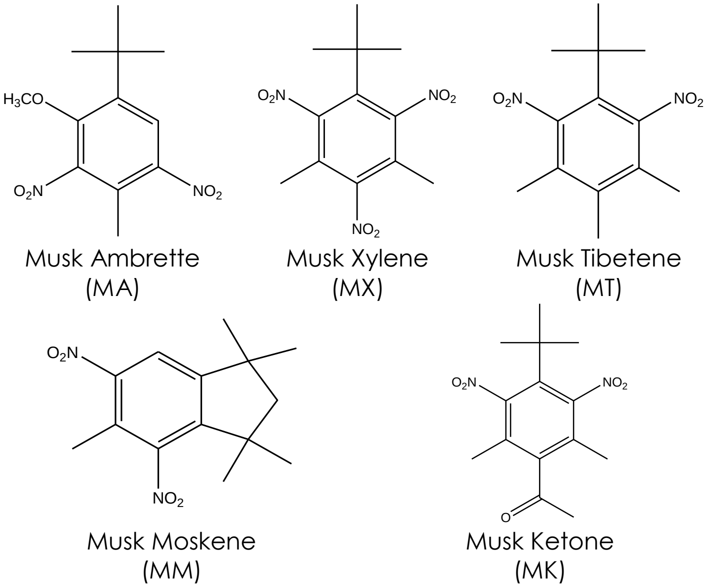
1.6.3. Musk compounds

Musk compounds have been widely used as fragrance chemicals in many consumer products such as cosmetics, detergents, food additives or household products [7,103]. They are valuable compounds not only for their unique odour but also for their fixative properties.

Natural musk, itself, was already used in ancient times and was obtained as a secretion produced by a gland of the musk deer. Traditionally, musk deer are killed to remove the musk gland, although musk can be extracted through the external orifice of the musk gland without the need of killing the animal. Nevertheless, its difficult availability make this natural product very expensive [104].

In addition, the term musk also refers to other compounds, with totally different chemical structure but possessing musk-like odour properties. These are commonly named synthetic musks and appeared as a response to economical and ethical motives. Artificial musks have been generally divided in three subgroups: **nitro musks, polycyclic musks and macrocyclic musks**.

The **nitro musks**, which are characterized by a nitro-aromatic moiety, were accidentally discovered by Baur in 1888 [105]. This group is basically formed by five compounds i.e., musk ambrette (MA), musk xylene (MX), musk moskene (MM), musk tibetene (MT) and musk ketone (MK). **Figure 8** shows the chemical structure of these compounds.



Musk Ambrette (MA)

Musk Xylene (MX)

Musk Tibetene (MT)

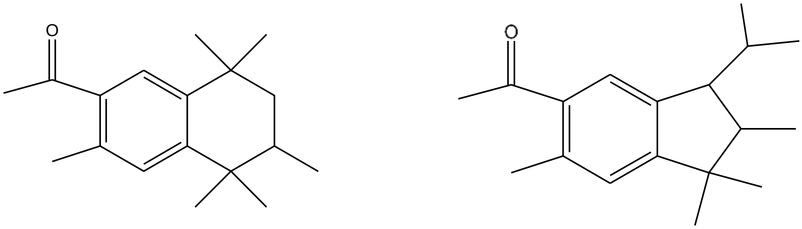
Musk Moskene (MM)

Musk Ketone (MK)

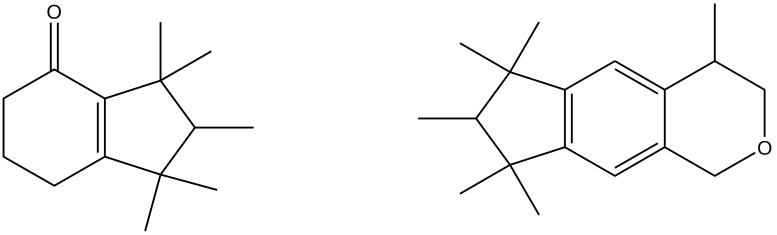
Figure 8. Chemical structure of nitro musk compounds

Despite their pleasant aroma, nitro musks are believed to be persistent pollutants due to their strong tendency to bioaccumulate [6,106]. Many papers have been written about their health risks, showing they are related with different types of dermatitis, carcinogenic effects and endocrine disruption [6-10,107]. In fact, the use in cosmetic products of MA, MT and MM is banned in the EU while the use of MX and MK is restricted. Nevertheless, its use is permitted in North America.

Regarding the **polycyclic musks**, these are composed of several cycles and do not have nitro substituents. **Figure 9** shows the structure of some of the most commonly used polycyclic musks, such as 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta(g)-2-benzopyran (HHCB) commonly known as galaxolide®, 6-acetyl-1,1,2,4,4,7-hexamethyltetralin (AHTN) commonly known as tonalide®, 5-acetyl-3-isopropyl-1,1,2,6-tetramethylindane (AITI) commonly known as traseolide®, and 1,1,2,3,3-pentamethyl-2,5,6,7-tetrahydroinden-4-one (DPMI) commonly known as cashmeran.



Tonalide® (AHTN) Traseolide® (AITI)

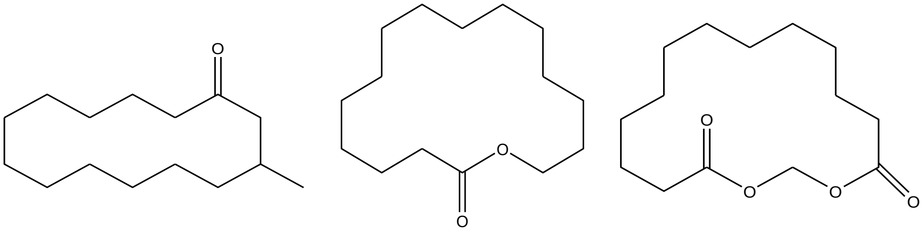


Cashmeran (DPMI) Galaxolide® (HHCB)

Figure 9. Chemical structure of polycyclic musk compounds

These musks were developed in 1950 and have slowly replaced the nitro musk compounds in Europe, since they are believed to have less harmful and toxic effects. However, in the later years, many studies show their bioaccumulation in the aquatic system and living organisms [108-112]. None polycyclic musk is banned in the EU and only some of them are restricted. The rest can be freely used in cosmetic products. Thus, polycyclic musks are more used in the EU while nitro musks are the common ones in North America [113].

Finally, **macrocyclic musks**, which are much larger compounds than the other two groups, have been developed in the recent years. Even though they have synthetic nature they are synthesized trying to imitate the natural musk compounds. As these compounds are not restricted by law and seem not to have biodegradability problems, their use have increased considerably and are replacing the polycyclic musk compounds [104]. Some of the most important macrocyclic musks are shown in **Figure 10**.



Muscone Exaltolide Ethylene brassylate

Figure 10. Chemical structure of macrocyclic musk compounds

Regarding the literature dealing with the determination of musks in cosmetic the preferred technique is GC, with MS or electron capture detector (ECD) in the case of nitro musks. Sommer [114] presented a method for quantification of nitro musks in cosmetics and detergents using GC with both, ECD and MS. Wineski et al. [115] determined MA in fragrance products with an internal standard addition technique by GC-ECD. The same author published another paper [116] some years later with a similar method to determine MA, MX and MK. Struppe et al. [117] proposed a headspace solid-phase microextraction (HS-SPME) and GC with atomic-emission detection method for the determination of MX, MK and MA in cosmetic samples. Four different commercially available fiber coatings were tested and a 100 µm polydimethylsiloxane gave the best results. To the best of our knowledge, Eymann et al. [118] published the first paper where the determination of polycyclic musks in cosmetics was achieved, together with nitro musks. GC was used, with ECD in the case of nitro musks and MS for polycyclic musks. Some years later, Sommer and Juhl [119] proposed a GC with FID and MS detection to determine macrocyclic musks in alcohol-containing cosmetics.

More recently several papers have been published on the determination of musks in cosmetic products. Roosens et al. [120] determined AHTN, HHCB, MX and MK in 82 personal care products, 19 of them perfumes, with GC-MS and combined these results with the average usage to estimate exposure profiles through dermal application concluding that exposure to the polycyclic musks is much higher than to the nitro musk compounds. Ma et al. [121] proposed an SPE followed by GC-MS/MS method for the determination of MX in cosmetics. Martinez-Girón [122] achieved the enantiomeric separation of four chiral polycyclic musks using capillary electrophoresis (CE). The method was applied to determine these enantiomers in perfumes. As already mentioned before, Sanchez-Prado et al. [52,57] also developed a method that allows the simultaneous determination of many compounds, including nitro musks and polycyclic musks, in perfumes. Lopez-Gazpio et al. [123] optimized and validated a nonaqueous micellar electrokinetic chromatography method with no sample pretreatment for the determination of three polycyclic musks in perfumes. Homem et al. [124] published a new method based on QuEChERS (quick, easy, cheap, effective, rugged, and safe) extraction followed by GC-MS for the analysis of musks, five nitro, five polycyclic and one macrocyclic musk, in different personal care products. Finally, Dong et al. [125] lately established a new method for the simultaneous determination of the five nitro musks and two polycyclic musks in creams by means of SPE followed by GC-MS/MS.

The extended use of these compounds has aid to their appearance in the environment. The removal in municipal sewage treatment plants has been estimated to be between 60 and 80% for nitro musks and between 40 and 60% for polycyclic musks [126]. Thus, nitro musks indirectly reach the aquatic environment via wastewater treatments plants. Additionally, owing to their presence in consumer products, they can directly reach the aquatic environment from, for example, swimming activities in seas, rivers and lakes. Unfortunately, these compounds are considered persistent pollutants [5] and with a moderate to high potential for bioaccumulation and bioconcentration. Therefore, it is important to develop new analytical methods to evaluate its potential for bioaccumulation on the environment.

It is worth mentioning that musk xylene was classified as a substance of high concern with a very persistent and very bioaccumulative designation under the authority of the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) regulation in 2008. All nitro musk compounds were said not to degrade easily, causing them to be highly stable and ubiquitous in the environment. [106,127]

Owing to all of these facts, many articles about the determination of artificial musks, in matrixes different from cosmetic products, can be found in the literature. The most abundant are environmental samples such as air, water and sediments [108,109,128]. Other works in biological fluids, such as blood [110,111,129] and human milk [105,130,131] or aquatic species [126,132,133] are also available. GC with mass detection is by far the most common choice. This is the aim of the two works summarized in Chapters 6 and 7 of this thesis, where two methods for the determination of the nitro musk compounds in environmental waters are presented.

From all the data found in the literature, it can be concluded that nitro musks have been largely replaced by polycyclic musks, especially in Europe, due to the banning of these compounds. However, nitro musks are still being produced in China and India and sometimes used in United States [106]. Moreover, due to their environmental persistence and concerns for the long-term effects the number of published scientific works on this subject have not decreased.

As nitro musks are found at trace level in the environment, extraction techniques to clean up and concentrate are very useful for their determination. Specifically in environmental waters, Gatermann et al. [134] published a work, where MX and MK were quantified for the first time in this type of matrix using a LLE with hexane followed by evaporation on a rotary evaporator. The analysis was then performed by GC, both with MS and FID detection. Same authors then published a work [135] where the monoamine metabolites of MX and MK and their parent compounds were analyzed in river waters, sewage samples, sediments and biota. Heberer et al. [136] determined artificial musks in the aquatic system in Berlin applying a SPME followed by GC-MS. Osemwengie et al. [137] determined both, nitro and polycyclic musks, in municipal sewage effluent water by an on-site SPE method developed by the US Environmental Protection Agency. Analysis was performed using a GC-MS operating in full scan mode. Same author [138] also published another work relating the concentration in waters and the associated biota. After that, Mitjans et al. [139,140] determined musks and other fragrance compounds using CLSA (closed loop stripping analysis) and GC-MS detection. The method was applied to different environmental waters in Europe.

Peck et al. [141] analysed MX and MK, among other polycyclic musks in Lake Michigan using GC-MS. Both of them were detected. This author published two years later an interesting review [109] on the determination of persistent ingredients of personal care products in environmental matrices. This work is a good summary of the work done for the musk analysis at that time. Three years later, Bester [108] wrote another review article, specifically about the analysis of musk fragrances in environmental samples. Yang and Metcalfe [142] analysed samples from a domestic wastewater treatment plant in Canada using LLE and GC-MS. Although polycyclic musks were found in higher concentrations, nitro musks were also detected. Polo et al. [143] also detected MK and MX by SPME and GC with micro electron capture detection.

The high development of more modern extraction techniques in the last years can be observed in the works concerning the determination of nitro musks in environmental waters. In this sense, Regueiro et al. published a work [144] using USAEME. Some nitro musks, among other emergent contaminants and pesticides, were determined in environmental waters. Ramirez et al. [145] developed a SBSE and thermal desorption GC-MS method for determining synthetic musks in water samples. Main advantage is the limited manipulation of the sample. As many times stated polycyclic musks were found in higher amounts but nitromusks (in this case MM and MK) were also detected. Shortly after Arbulu et al. [146] published a work using the same technique for natural and wastewaters. Vallecillos et al. [147] developed an interesting method based on automated ionic liquid headspace single drop miroextraction coupled to GC-MS/MS. Nitro (not detected) and polycyclic musks were determined in environmental water samples. Posada-Ureta et al. [148] published a membrane assisted solvent extraction coupled to large volume injection-GC-MS for analysis of synthetic musks in environmental water samples. A year later, some of the same authors [149] published another work on the subject but this time by means of microextraction by packed sorbents coupled to large volume injection-GC-MS. Very recently, Caballero-Diaz [150] et al. developed a method based on microextraction by packed sorbents combined with surface-enhanced Raman spectroscopy. This method was applied for the determination of MK in river water samples.

In this sense, Chapter 6 and 7 summarize two different methods for the analysis of the whole family of nitro musk compounds in environmental water samples. Chapter 6 is based on DLLME and Chapter 7 in SPE using molecular imprinted sorbents.

Chapter 2.

Methodologies used in this thesis

2.1. Linear discriminant analysis

As noted above, Chapter 5 focuses on the study of the process of maceration of a perfume. For this, the so-called linear discriminant analysis (LDA), a pattern recognition method, was used. The aim of this method is to describe, based on a mathematical model, the behavior of a categorical dependent variable through the information provided by a set of independent quantitative variables. It detects patterns of behavior and thus, establishes groups or categories. LDA is classified as a supervised pattern recognition method, since, unlike the unsupervised ones, observations (also called variables or measurements) from known classes are considered. It is with this set of samples, called training set, that the criteria to classify future observations are fixed and the model is constructed. Then, an unknown sample is assigned to one of the groups based on the values of its quantitative variables. [151]

In this work, LDA was used to assess whether the area peaks of the chromatograms of the macerated and non-macerated perfume samples have different patterns. First, based on this difference two distinct groups were established. Then, the minimum time required for the perfume maceration was set studying intermediate time samples and seeing if they were classified as macerated or as non-macerated samples.

Since the number of variables is often large, the starting point in LDA is to obtain the linear discriminant equation or function (LDF), which is a linear combination of the predictor variables. The form of the function is:

$$D = a_1 X_1 + a_2 X_2 + a_3 X_3 + \dots + a_i X_i$$

Where: D is the discriminant function

X is the predictor variable

a is the discriminant coefficient (or weight) for that variable

i is the total number of predictor variables

In the same way, each point on the discriminant function, called score, can be defined as:

$d = a_1 x_1 + a_2 x_2 + a_3 x_3 + \ldots + a_i x_i$

Where: d is the score of that observation

x is the corresponding value of the predictor variable

a is the discriminant coefficient (or weight) for that variable

i is the total number of predictor variables

It should be noted that among several independent variables, not all have the same discriminatory power. Moreover, some of them may have little or none discriminatory power and therefore, they are discarded from the function. Standardizing the variables ensures that scale differences between the variables are eliminated. When all variables are standardized, the absolute value of its weights (its coefficient in the discriminant function) ranks the variables in terms of their discriminating power. This way, good predictor variables will have large weights and will contribute mostly to differentiating the groups [152]. To discriminate between q groups or categories with the use of i predictor variables the lowest of q-1 or i is the maximum number of discriminant functions needed.

Among the infinite possible discriminant function, the one chosen is that that provides the maximum dispersion among the different groups and the minimum between the observations of the same group. This information can be studied with the so-called λ_{wilks} [153]. This parameter is calculated as the sum of squares of the distances between points belonging to the same category divided by the total sum of squares:

$$\lambda_{Wilks} = \frac{SS_{group}}{SS_{total}}$$

Being,

$$SS_{total} = \sum_{i=1}^{N} (x_i - \bar{x}_{global})^2 \qquad \text{and} \qquad SS_{group} = \sum_{j=1}^{k} \sum_{i=1}^{n} (x_{i,j} - \bar{x}_j)^2$$

SS_{total} is the sum of squares of distances of each observation to the mean value of all the objects, and SS_{group} the sum of squares of distances between the observations belonging to the same group and the mean value of that group. The values of λ_{wilks} range between zero and one. Values near one indicate that the categories are overlapped (i.e. no separation between groups), while values close to zero indicate that the groups are well resolved and therefore the model is able to distinguish groups.

In this work, the software SPSS (Statistical Package for the Social Sciences) was used. Thus, the SPSS stepwise algorithm established the predictors of the LDA models. This algorithm selects a variable predictor when the reduction of λ_{wilks} produced after its inclusion in the model exceeds F_{in}, the entrance threshold of a test of comparison of variances or F-test. As the inclusion of a new predictor changes the significance of other variables already present in the model, a rejection threshold, F_{out}, is used to remove, if necessary, any predictor from the model. This process finishes when no more predictors are entering or leaving the model. The probability values of F_{in} and F_{out}, 0.05 and 0.10, respectively, were adopted.

The model also provides the cut-off values, which are the boundaries to delimit each of the groups. For two-groups, when group sizes are equal, the cut-off is the mean of the two centroids.

In order to present a graphic explanation of the technique **Figure 11** shows a simple example of the training sets, with two groups (represented as red and blue) and two quantitative variables (axis x_1 and x_2). The new axis, D, is the discriminant function, a linear combination of the two variables, x_1 and x_2. The discrimination between the two groups using only one of the two variables (projecting the points onto each one of the axis) would not be possible. However, it is possible to construct this new axis, taking into account both variables, so that there is no group overlapping.

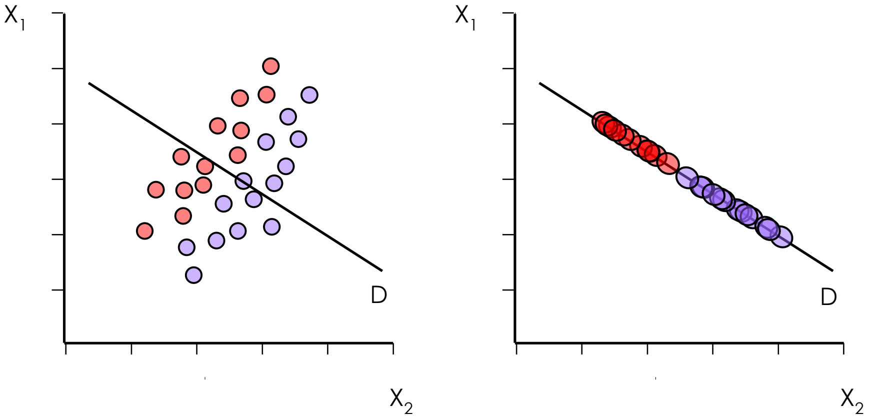


Figure 11. Graphical example of two training sets and its discriminant function.

Once the model has been established and before classifying unknown samples, it is interesting to calculate the percentage of correctly classified objects. To do so, a group of observations, different from the training set but with a known group, are represented on the discriminant function based on their scores to see if they are assigned to the appropriate group. However, it is common to have scarce experimental data and in this case, leave-one-out-cross-validation can be useful. In this sense, the model is constructed without one of the observations of the training set and then, it is studied if this one is correctly classified. The process is repeated for each observation and after that, it is easy to determine the percentage of objects that are correctly classified.

Finally, the model is applied to problem observations with an unknown group. In a two group situation, membership is calculated generating a score of the observation in the discriminant function. Depending on which side of the cut-off value is this score the observation is classified as one of the two groups.

LDA has been used in many fields of analytical chemistry as a classificatory tool, especially in food analysis, in order to discriminate between samples where the qualitative variable is the geographical origin, genetic variety, cultivar origin, botanical origin, genuine or adulterated, etc. [154]. Regarding perfume analysis assisted with LDA, only two articles have been found in the literature. The first one studies the relationship between the structure and fragrance properties of different chemicals, which belong to three categories (apple, pineapple and rose) [155]. In the second one, five terpenes are discriminated based on the use of different serum albumins acting as low-selectively receptors [156]. Neither applications of LDA nor other chemometric approaches for the study of the perfumes maceration process have been found in the literature. However, LDA has been applied to study some other time-dependant procedures. For example, LDA has been used to classify cheeses of different ripening ages (fresh, medium and/or old) based on water extract profiles obtained by LC [157], different proteolysis parameters [158], ethanol-water protein fraction profile obtained by capillary zone electrophoresis [159], or fat, protein, dry matter and different metals contents [160]. LDA has also been used to classify Spanish hams of different curing times (6, 8 and 12 months) based on the peptide profiles obtained by CZE [161]. Also, to establish the different age of wines based on their concentrations of some inorganic anions [162], or to study the influence of the storage time of young white wines on the phenolic composition and colour [163].

2.2. Molecular imprinted sorbents

Solid phase extraction (SPE) is a well-known technique, broadly used in analytical chemistry to clean-up and concentrate. The solutes dissolved in a liquid are retained in a solid (the sorbent) previously packed in a syringe-shaped cartridge. After that, the different compounds of the sample are eluted according to the affinity of the sample compounds for this sorbent. A clean-up step is performed as either, the desired analytes of interest or the impurities of the sample are retained in the sorbent. However, this technique presents some disadvantages as the co-extraction of interfering compounds, which interfere in the subsequent determination of the compounds of interest. Indeed, the classical SPE sorbents retain analytes by nonselective interactions as hydrophobic or polar interactions. An option, which has shown to improve selectivity in SPE, is the use of molecularly imprinted sorbents [164-169].

The specific biological interaction antigen–antibody inspired the concept of molecular imprinting, which has the principles of these bio-specific interactions but applied to a purely chemical aim. Molecular imprinting consists in the synthesis of a material that contains specific cavities of a molecule footprint, called template. To do so, a monomer that develops strong interactions with the template is needed. For this, the template should present multiple chemical functionalities that could interact with the monomer. The synthesis of the sorbent is carried out in the presence of high amounts of cross-linking molecules. Once the imprinted material are synthesized the links between the template and the monomers must be broken. This step is accomplished washing the polymeric network with the appropriate solvents in order to extract the template and leave the specific cavities. The cavities formed are complementary to the imprinted molecule in shape, size and arrangement of functional groups. As in the antigen-antibody interactions, the retention of the target molecule is based on a mechanism of molecular recognition. **Figure 12** shows the principles of molecular imprinting.

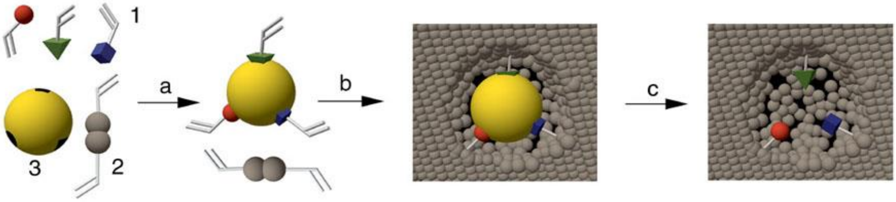


Figure 12. Principals of molecular imprinting**.** (Extracted from [170])

(1) Functional monomers, (2) cross-linker, and (3) template molecule.
(a) The functional monomers form a complex with the template molecule.
(b) The functional monomers copolymerize with the cross-linker.
(c) Removing the template liberates complementary binding sites that can reaccommodate the template in a highly selective manner.

Unfortunately, a complete removal of the template is not usually achieved. Therefore, residual leaking of the template molecule during the elution is common when using molecular imprinted sorbent for SPE. This fact leads to a lack of accuracy in the determination, especially in trace analysis. The simplest and most common way of avoiding this problem is not to use, in the synthesis of the material, the exact analyte of interest as template molecule but a structural analog, known as dummy molecule. Residual leaking of the template is still present but is no more a problem as it is a different molecule with different chromatographic behavior and thus, different retention times. This procedure is also a good option when the use of the target compound as template is not feasible due to its high price or that the conditions used in polymerization may cause degradation in undesired compounds. [168]

Once the imprinted sorbent is synthesized, and before the template removal, the resultant monolith must be ground and sieved to obtain particles, usually between 25 and 36 µm. Both, the grinding and the sieving, can be done by hand or automatically. Next, those particles under the desired size and still present after the sieving are eliminated by sedimentation of the desired particles. This step consists in mixing the particles with a hydroorganic mixture, shacking and leaving to sedimentation. Then, the supernatant, with its suspended fine particles, is discarded. The step is repeated several times until the supernatant becomes clear. Those particles settled at the bottom have the desired size to form the sorbent. They are finally left in the open air overnight to be dried and then a given amount can be weighted and introduced in a cartridge between two frits. It is important to note that the sieving and sedimentation steps present important losses and a yield below 50% is often obtained. After all of these steps is when the template molecule is removed. To do this, a solvent can be percolated through the extraction cartridge to try to break the monomer-template interactions and make available the binding sites. [171]

Molecular imprinted polymers

Those imprinted materials synthesized by radical polymerization are called molecularly imprinted polymers (MIPs). This is the most common imprinted material due to a rather straightforward synthesis and to the huge choice of available monomers. As already explained, the monomer must form interactions with the template, usually non-covalent interactions, such as hydrogen bonds, electrostatic, hydrophobic or π-π interactions. As some common examples, methacrylic acid can be used as monomer if the template has basic properties whereas vinylpyridine is preferred if it is acidic. In contrast, when the template molecule does not have acid-basic sites, styrene can be used to form hydrophobic or π-π interactions. Besides, acrylamide is an option to form hydrogen bonding. Regarding cross-linkers, most commonly used contain vinyl or acrylic groups.

Moreover, the synthesis porogen solvent has a great influence in the synthesis and needs to be chosen. Obviously, it must solubilize the organic reagents. Besides, its selection is very important, as it will affect the interactions between the template and the monomer and therefore, the selectivity of the sorbent. Moderately polar and aprotic solvents, such as toluene, dichloromethane, chloroform and acetonitrile are usually used. These organic media favor some interactions that will not necessarily occur in water. This explains the difficulty in applying MIP to aqueous samples as the target molecules interact with the MIP in an unspecific way. [172]

As important as the choice of the nature of the monomer, cross-linker and solvent is the relative proportion between all reagents. The crosslinking agent must be in molar excess to obtain a rigid structure with well-defined cavities, but not in too large excess. The proportion of functional monomers is determined by the amount of template and is usually introduced in a higher amount. The molar ratio most commonly used for MIP synthesis is 1/4/20 (template/monomer/crosslinking agent). This proportion appears to be most favorable and enable the formation of a strong monomer-template interaction. [171]

Molecular imprinted silicas

Another via of synthesis of molecular imprinted sorbents is the sol-gel approach to form the so-called molecularly imprinted silicas (MIS), based on organosilanes. In this case the monomer is composed of tetraalkyl orthosilane ($Si(OR)_4$). This methodology is an interesting alternative in the case of molecules that are difficult to imprint by radical polymerization. MISs are very rigid materials that provide a large surface area and a large porosity. Moreover, they are very stable materials that preserve the structure of the cavities and the spatial distribution of the functional groups. As already explained, the use of organic monomers implies the use of an organic solvent to solubilize them and consequently, the MIP is not directly selective in aqueous media. Thus, compared to the organic MIPs, most important property of MIS is they are synthesizable in water. In consequence, their cavities present a high selectivity and improved bond sites when working with aqueous samples.

The sol-gel polymerization consists of two steps. First, the hydrolysis of the alkoxide groups and then the condensation of the hydrolyzed products. In the hydrolysis step the alkoxide groups (OR) react in the presence of water to give hydroxyl groups (OH) and obtain the silanols. The use of a catalyst allows a more rapid and complete reaction.

$Si(OR)_4 + H_2O \rightarrow HO\text{-}Si(OR)_3 + R\text{-}OH$

Depending on the amount of water and catalyst used, the hydrolysis is continued:

$Si(OR)_4 + 4\ H_2O \rightarrow Si(OH)_4 + 4\ R\text{-}OH$

The polymer is obtained in the condensation step. The silanol groups formed during the hydrolysis step react with each other releasing a molecule of water or alcohol. This stage begins before the hydrolysis step is completed, so it occurs between molecules partially or completely hydrolyzed and molecules that may not yet have been hydrolyzed. During this process, the viscosity of the reaction medium increases.

$HO\text{-}Si(OR)_3 + HO\text{-}Si(OR)_3 \rightarrow (OR)_3Si\text{-}O\text{-}Si(OR)_3 + H_2O$

or

$Si(OR)_4 + HO\text{-}Si(OR)_3 \rightarrow (OR)_3Si\text{-}O\text{-}Si(OR)_3 + ROH$

To synthesize a MIS, a monomer with a hydrolyzable function (to generate the silicate network) and an organic function (to generate the "skeleton" and to form non-covalent interactions with the template) is needed. The crosslinking agents are generally tetraethoxysilane (TEOS) or tetramethoxysilane (TMOS), with four hydrolysable functions to promote the formation of a multidimensional network. [171]

Selective extraction procedure in molecular imprinted sorbents

It is important to keep in mind that having a molecular imprinted sorbent with the correct size cavities is not enough to have a selective extraction procedure. An adequate extraction procedure must still be optimized. This procedure must enable the specific interaction of the target compound in a real sample (with other compounds present) with the cavities and eliminate those non-specific interactions with the monomer residues in the surface of the polymer. To optimize the extraction procedure and evaluate the specific and non-specific interactions a non-imprinted polymer (NIP) or silica (NIS) is used. This non-imprinted sorbent is synthesized simultaneously and exactly in the same way than the molecular imprinted sorbent with the exception of not introducing the template molecule. Thus, the sorbent will not present cavities and its extraction of the compounds in the sample will work with the principles of the common SPE sorbents (i.e., non-specific interactions). The extraction procedure with the imprinted sorbent is tested in parallel with the non-imprinted sorbent and compared to study if the analyte retention is selective (due to the cavities) or not. [168]

Nitro musk selective extraction

The aim of the work presented in Chapter 7 was to find a molecularly imprinted SPE sorbent for the simultaneous selective extraction of the five compounds from the nitro musk family in wastewater and surface water samples.

MIPs, synthesized by radical polymerization of organic monomers, are the most commonly used imprinted sorbents. However, as already mentioned this type of sorbents are synthesized in organic solvents and consequently are many times not selective in aqueous media. Moreover, even in organic solvents the crucial interactions between the template and the monomer are difficult to achieve in the case of templates with a nitro group [173,174]. A strong retention by hydrogen bonds is unlikely and taking advantage of hydrophobic interactions by using the styrene as monomer is neither an option since nitro aromatic compounds inhibit the radical polymerization of the styrene. Thus, a different approach was taken and MIS sorbents, synthesized by sol–gel polymerization, were used.

In fact, a MIS synthesized using the same procedure as the one described by some authors of the work using 2,4-dinitrotoluene (2,4-DNT) as template was used because of its structural analogy with the target nitro musks [174].

Continuing with the aforementioned, a selective procedure that allowed the selective extraction of the nitro musks using this MIS was optimized. In this sense, the cavities were sufficiently large to accept the nitro musks but when applying specific conditions of extraction. The extraction procedure developed in the previous work for the selective extraction of nitro explosives could not be used for these compounds. Indeed, nitro musks were lost before the elution fraction. Moreover, the other way round also happened. It was observed that the use of the extraction procedure used for nitro musks lead to the loss of the nitro explosives in the washing step. This highlights the necessity to adapt the nature and the volume of the solvent constituting the washing fraction to the nature of the studied compounds.

2.3. Dispersive liquid-liquid microextraction

The use of the traditional liquid-liquid extraction technique (LLE), based on the transfer of target compounds from one solvent to another immiscible in the donor phase, has decreased over the years in the field of analytical chemistry. The main drawback of this technique is it consumes large amounts of organic solvents, often toxic and expensive. Moreover, LLE requires several successive extractions to achieve high yields and solvent evaporation and reconstitution, which often causes imprecision in the results due to loss of analytes. Since the mid-to-late 90's, new liquid phase microextraction techniques have been developed to replace traditional LLE. These new methods try to improve the technique searching for better yields, faster and more reproducible methodologies and the use of smaller volumes of solvents and sample. This last aim has led us to a miniaturization of the traditional LLE, leading to the so-called liquid phase microextraction techniques (LLME).

The most important types of liquid phase microextraction techniques are single drop microextraction (SDME), hollow fiber liquid phase microextraction (HF-LPME) and dispersive liquid-liquid microextraction (DLLME). SDME consists in suspending a drop of a few microlitres of extractant solvent at the end of the tip of a syringe needle. The droplet can be immersed in the donor phase or suspended in the headspace. The droplet is then left some time, collected in the syringe and transferred to chromatographic instrument. On one hand, its main advantage are its low cost and minimal solvent consumption. On the other hand, its main problems are that it is time-consuming and that the drop presents a high instability. SDME is not a robust technique and it requires working very carefully. A possibility to solve this problem is to protect the extracting solvent inside a tubular porous hollow fiber. This way the drop will not be so instable and it has the advantage that vigorous agitation to accelerate the kinetics of the extraction can be performed with no danger of detachment of the drop. The solvent soaks the pores of the fiber forming the so-called supported liquid membrane. Moreover, three phases systems were developed. In this methodology compounds are extracted from an aqueous solution through an organic solvent forming the supported liquid membrane and then further back-extracted into another aqueous solution, pH adjusted, situated in the lumen of the hollow fiber. [175]

Assadi and co-workers developed DLLME in 2006 [176], which has become, since then, in a very popular technique [177-179]. This technique was used in this thesis in the works summarized in Chapters 4 and 6.

DLLME has several advantages, including simplicity of the procedure, its speed and high enrichment factors. It consists in a ternary solvent extraction in which a mixture of a small volume of a dispersion solvent and a few microliters of an extraction solvent is introduced with the use of a syringe in an aqueous liquid sample. The extraction solvent must be denser than water and the disperser solvent must be miscible in both, the extraction solvent and the aqueous donor phase. As dispersion solvent generally methanol, acetonitrile or acetone are selected. As extraction solvent carbon disulfide, carbon tetrachloride, tetrachloroethylene, chloroform or dichloromethane are generally chosen. Once the mixture disperser-extractant solvent is quickly injected in the aqueous sample, a cloudy solution is formed. This cloudy solution consists in multiple droplets of the extraction solvent of very small size. The big surface of contact between the extracting solvent and the sample leads to an instantaneous equilibrium state, what makes this technique rather quick. After extraction, phase separation is performed by centrifugation. The sedimented phase containing the analytes is collected with a microsyringe, and can be transferred into the analytical instrument (e.g., injected directly in the chromatographic system). **Figure 13** shows a scheme of DLLME.

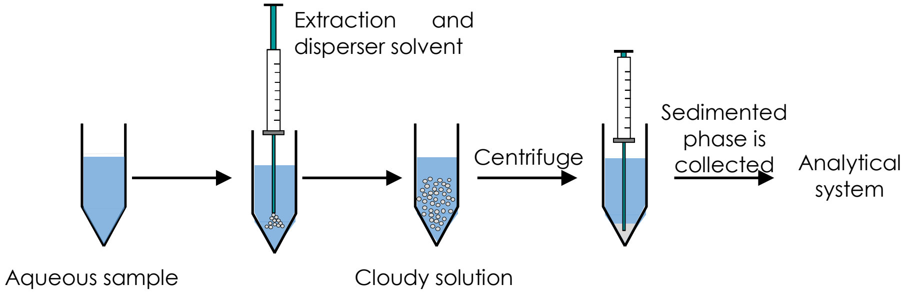


Figure 13. Scheme of dispersive liquid-liquid microextraction

After the development of DLLME some variations of this technique were presented. For example, ultrasound-assisted dispersive liquid-liquid microextraction (USA-DLLME) uses ultrasounds after the DLLME to improve the extraction efficiency. Instead of ultrasounds, vortex agitation has also been proposed in the so-called vortex-assisted liquid-liquid microextraction (VA-DLLME). [180]

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Section II.

SUMMARY OF EXPERIMENTAL RESULTS AND DISCUSSION

Chapter 3.

Determination of phthalates in perfumes using gas chromatography-mass spectrometry

Based on:

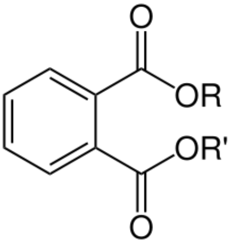
M. Lopez-Nogueroles, J.L. Benede, A. Chisvert, A. Salvador,
A rapid and sensitive gas chromatography-mass spectrometry method for the quality control of perfumes: simultaneous determination of phthalates,
Analytical Methods 5 (2013) 409-415.

AIM OF THE WORK

Aim	Development of a rapid and sensitive method to simultaneously determine in perfumes the phthalates banned by the EU Cosmetic Regulation.
Proposed method	Gas chromatography–mass spectrometry (GC-MS).
Interest	Current legislation on cosmetic products forbids the presence of several phthalates. Although it is a need in quality control of perfumes, no study for the simultaneous determination of all prohibited phthalates was found in the literature before the publication of this work. No dilution of the samples is performed.

TARGET COMPOUNDS

General structure of an phthalate:



Compound	Acronym	CAS Nº
Dibutyl phthalate	DBP	84-74-2
bis(2-Ethylhexyl) phthalate	DEHP	117-81-7
bis(2-Methoxyethyl) phthalate	DMEP	117-82-8
n-pentyl-isopentyl phtalate	NPIPP	84777-06-0
di-n-Pentyl phthalate	DNPP	131-18-0
Diisopentylphthalate	DIPP	605-50-5
Benzyl butyl phthalate	BBP	85-68-7

CALIBRATION MODE

Selected calibration mode

The use of a surrogate was considered in order to reduce the variability of the measurements.

The slopes of internal standard calibration with and without standard addition calibration were compared:

- At a 95% confidence interval most of the variance were comparable by Fisher's *F*-test.
- At a 95% confidence interval the slope values for many target compounds were not comparable by Student's t-test.

(See details in Table 3 of the article)

↓

Matrix effects

↓

Standard addition calibration combined with internal standard is recommended.

Analytical signal

$y = A_i/A_{sur}$

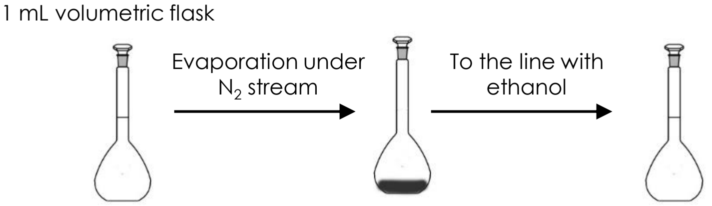
A_i = the peak area of the target analyte
A_{sur} = the peak area of the surrogate

Selected surrogate

Hexachlorobenzene (HClB)

- Suitable for GC-MS.
- High volatility.
- High sensitivity.

PROPOSED METHOD



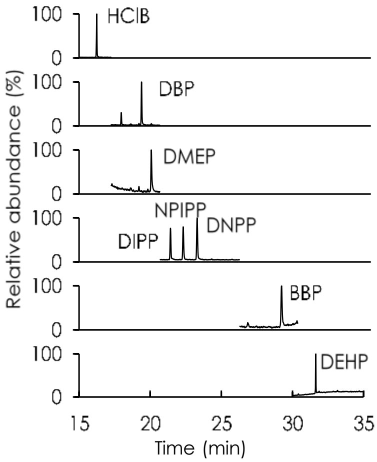
- 0.8 mL commercial perfume sample
- Surrogate (20 µL of 50 µg mL^{-1} HCIB)
- Increasing amounts of the standard solution
- To the line with ethanol

GC-MS Conditions	
Injection volume	1 µL (Splitless time 1 min)
Inlet temperature	280 °C
Transfer line temperature	280 °C
Ion source temperature	250 °C
Helium flow rate	1 mL/min
Column	HP-5MS Ultra Inert (30m, 0.25mm, 0.25µm)
Oven temperature	50 °C (1 min) 10 °C/min to 205 °C 1 °C/min to 217 °C 20 °C/min to 280 °C (10min)

Acquisition intervals

Compound	Identification and quantification ions[1]	Time (min)
HCIB (surrogate)	**284**	15.0 - 18.0
DBP	**149**, 150, 223	18.0 - 21.4
DMEP	58, **59**, 149	18.0 - 21.4
DIPP NPIPP DNPP	**149**, 150, 237	21.4 – 27.0
BBP	91, **149**, 206	27.0 - 31.0
DEHP	**149**, 167, 279	31.0 - 33.0

[1] The ion in bold was the one used to perform quantification



ANALYTICAL FIGURES OF MERIT

Working range

Selected for each analyte according to LODs and content in the samples

DBP: 0, 400, 600, 800 ng mL^{-1}
DEHP and DMEP: 0, 1000, 1500, 2000 ng mL^{-1}
NPIPP, DNPP, DIPP and BBP: 0, 50, 75, 100 ng mL^{-1}

Limits of detection and quantification of the method

Calculated with the standard addition calibration (with the six perfume sample as matrix).

Estimated as:

LOD: 3 $S_{y/x}$/b

LOQ: 10 $S_{y/x}$/b

$S_{y/x}$: Residual standard deviation of the calibration curve

b: Slope of the curve

(6 samples)

Analyte	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
DBP	75	250
DEHP	*	*
DMEP	75 - 294	250 - 980
NPIPP	6 - 34	20 - 113
DNPP	4 - 15	13 - 50
DIPP	6 - 17	20 - 57
BBP	3 - 14	10 - 47

* Not calculated because the analyte was present in the sample at high concentration.

The signal to noise ratio is not at all the same for standard solutions and for standard addition solutions which results in higher LODs but more realistic.

The LOD of DMEP is specially high due to its relatively low mass (m/z 59) quantification ion.

The obtained values are similar to those found in previous published articles.

Repeatability

Evaluated applying the proposed method to five replicates of a standard aqueous solution that contained 100 ng mL^{-1} of the target compounds

Analyte	RSD (%)
DBP	6
DEHP	7
DMEP	5
NPIPP	6
DNPP	5
DIPP	6
BBP	6

Analysis of real samples

Determination in 6 commercial perfumes from different brands (names are not shown for confidentiality reasons).

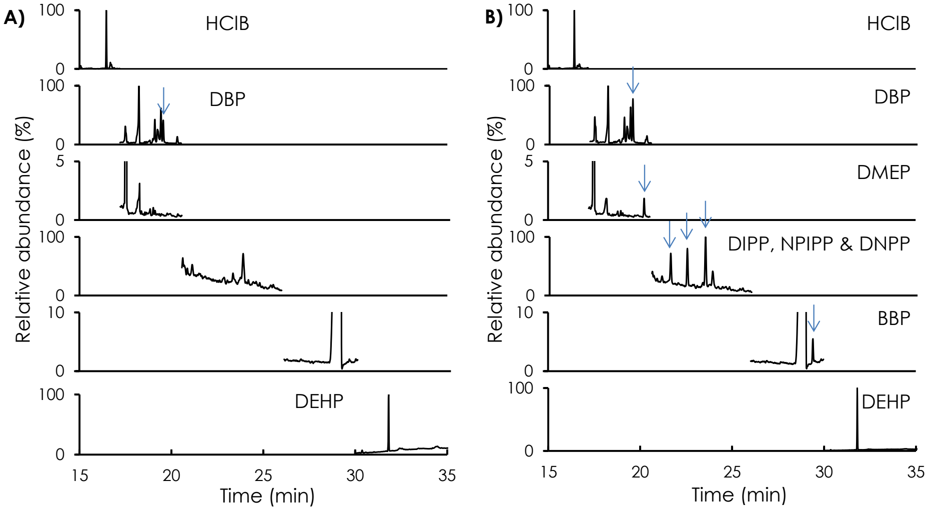
Sample	DBP	DEHP	DMEP	NPIPP	DNPP	DIPP	BBP
1	<LOD	1400±200	<LOD	<LOD	<LOD	<LOD	<LOD
2	760±50	1200±100	<LOD	<LOD	<LOD	<LOD	<LOD
3	150±20	660±60	<LOD	<LOD	<LOD	<LOD	<LOD
4	660±50	520±40	<LOD	<LOD	<LOD	<LOD	<LOD
5	<LOD	800±100	<LOD	<LOD	<LOD	<LOD	<LOD
6	100±10	800±100	<LOD	<LOD	<LOD	<LOD	<LOD

DBP was present in four samples (2, 3, 4 and 6) with values ranging between 100-760 ng mL^{-1}.

DEHP was present in all the analyzed samples with values ranging between 520 to 1400 ng mL^{-1}.

DMEP, NPIPP, DNPP, DIPP and BBP were below the limits of detection.

Chromatogram, as an example, of perfume 4 (A), and of the same sample spiked with the seven analytes (B):



Recovery studies

Samples were spiked with known amounts of those analytes not present in the sample and the proposed method was applied

Analyte	Sample	Concentration (ng mL^{-1})		Recovery (%)
		Spiked	Obtained	
DBP	1	400	401	100
	5	400	416	104
DMEP	1	1000	885	88
	2	1000	980	98
	3	1000	978	98
	4	1000	916	92
	5	1000	1027	103
	6	1000	974	97
NPIPP	1	50	51	102
	2	50	47	93
	3	50	55	109
	4	50	47	93
	5	50	53	106
	6	50	64	128
DNPP	1	50	47	95
	2	50	48	97
	3	50	51	101
	4	50	49	99
	5	50	51	102
	6	50	53	106
DIPP	1	50	46	92
	2	50	54	108
	3	50	51	101
	4	50	47	94
	5	50	52	103
	6	50	46	92
BBP	1	50	50	100
	2	50	49	99
	3	50	48	96
	4	50	45	91
	5	50	50	100
	6	50	49	99

DEHP's recovery was not assayed in any perfume because it was present in all of them.

DBP was only assayed in perfumes A and E, due to its presence in the other four samples.

The obtained recoveries are proximate to 100 %.

These results demonstrate the accuracy of the method.

CONCLUSIONS

■ The GC-MS proposed method enables the determination of the banned phthalates DBP, DEHP, DMEP, NPIPP, DNPP, DIPP and BBP, at trace level with accuracy and precision.

■ To our knowledge, no other study for the determination of all these prohibited phthalates in perfumes or other cosmetic products had been previously published.

■ It is worth mentioning the need to study matrix effects in perfume analysis, especially when low LODs are required and no dilution of sample is desired, as in phthalates determination.

■ The proposed method is accurate, rapid, sensitive and does not require other organic solvent than ethanol, being useful for perfume analysis with quality and safety purposes.

Chapter 4.

Determination of atranol and chloroatranol in perfumes using simultaneous derivatization and dispersive liquid liquid microextraction followed by gas chromatography-mass spectrometry

Based on:

M. López-Nogueroles, A. Chisvert, A. Salvador, *Determination of atranol and chloroatranol in perfumes using simultaneous derivatization and dispersive liquid–liquid microextraction followed by gas chromatography–mass spectrometry*,
Analytica Chimica Acta 826 (2014) 28-34.

AIM OF THE WORK

Aim

Development of a method to determine atranol and chloroatranol in perfumes with a good accuracy.

Proposed method

Liquid-liquid extraction (LLE) followed by a simultaneous dispersive liquid–liquid microextraction (DLLME) and derivatization. Finally gas chromatography–mass spectrometry (GC-MS).

Interest

They are present in oakmoss and treemoss extracts, used in perfume manufacture, and are very potent allergens.

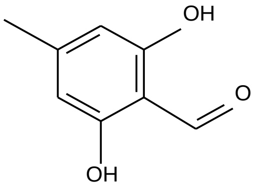
The number of publications regarding the determination of these compounds is scarce and they do not achieve accurate results.

The EU Scientific Committee on Consumer Products stated that these compounds are such potent allergens they should not be present at all in cosmetic products.

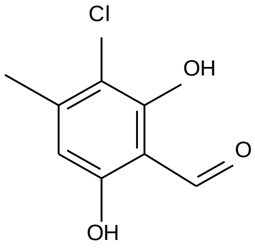
It is expected they will be banned or restricted in the coming years.

An effective sample preparation, that enables a significant clean-up, is crucial to obtain a good accuracy.

TARGET COMPOUNDS



Atranol



Chloroatranol

LLE STEP

Preliminary aspects

Perfumes contain many non-polar compounds. A good clean–up is important to avoid:

- Dirtiness in the injector port, the column head and the ion source.
- Jeopardized results.

DLLME needs an aqueous donor phase but perfumes are over 80 % ethanol. Direct dilution with water is not possible as it provides an opaque dispersion.

The target compounds have a relatively high polarity (log P of 1.99 and 2.99 for atranol and chloroatranol respectively) compared to most of the other primary constituents of an essential oil of a perfume.

↓

Conventional LLE is performed before DLLME to remove matrix compounds

Experimental procedure

1.5 mL of water and 1.5 mL of hexane are added to 1 mL of perfume and a conventional LLE process is performed.

Results

Atranol and chloroatranol remain in the hydro-alcoholic phase, whereas most of the matrix are transferred to the organic phase.

The injection of the rejected phases (hexane) in the GC-MS show that an important clean-up is performed.

Three extractions with aliquots of 1.5 mL of hexane are observed to be enough to eliminate most of the fat-soluble interferences of the perfume that made the solution used for DLLME not clear.

Extraction losses after the three extractions resulted in 18.9±0.4 % for atranol and 31.3±0.8 % for chloroatranol. This loss is worthy compared to the clean-up performed.

SIMULTANEOUS DERIVATIZATION AND DLLME STEP

Preliminary aspects

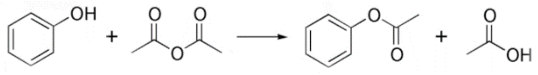
DLLME enables a change to a suitable GC-MS solvent and a concentration of the target compounds.

Due to the fact that atranol and chloroatranol are relatively polar compounds a poor extraction efficiency will be achieved in the DLLME process if they are not derivatized.

Moreover, derivatization rises volatility and thus increases sensitivity in GC-MS.

Chosen approach

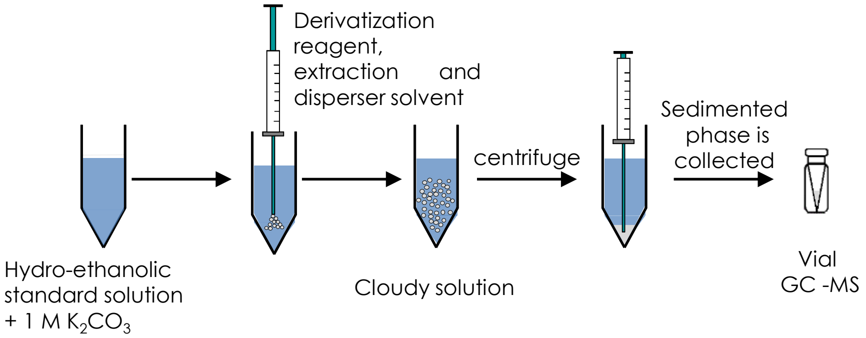
In situ derivatization via acetylation of the hydroxyl groups under basic aqueous conditions, using acetic anhydride as acetylating agent. Example:



The derivatization is performed at the same time as DLLME, allowing the simplification of the procedure and a decrease in the time of analysis.

GENERAL PROCEDURE FOR THE SIMULTANEOUS DERIVATIZATION AND DLLME

The extraction, the disperser solvents and the derivatization reagent are added to the basified hydro-ethanolic solution (sample or standard). After extraction, phase separation is achieved by centrifugation. the sediment phase is collected and analysed by GC-MS.



SELECTION OF THE SIMULTANEOUS DERIVATIZATION AND DLLME CONDITIONS

DLLME variables to be studied	A) Nature of extraction and disperser solvents B) Volume of extraction solvent C) Volume of disperser solvent D) Potassium carbonate amount E) Anhydride acetic volume F) Ionic strength of the aqueous donor phase G) Extraction and derivatization time
Response function	GC-MS peak area of each derivatized target analyte.
Standard hydro-ethanolic solution	1 mL of a 50 ng mL^{-1} ethanolic solution of the two target analytes, to which 9 mL of water are previously added.

A) SELECTION OF EXTRACTION AND DISPERSER SOLVENT

Required features for the extraction solvent	Immiscibility in the aqueous phase. Higher density than the aqueous donor phase. Good extraction of the target compounds. Good behaviour as GC solvent.
Assayed extraction solvents	Dichloromethane Chloroform
Required features for the disperser solvent	Formation of the cloudy solution. Miscibility in the aqueous donor phase and the extraction solvent.
Assayed disperser solvents	Acetone Acetonitrile Ethanol
Experimental procedure	1000 µL of each disperser solvent previously mixed with 100 µL of each extraction solvent and 100 µL of anhydride acetic were rapidly injected into 10 mL of the hydro-ethanolic standard solutions containing 1 mL of a 1M solution of K_2CO_3.
Results	Dichloromethane did not form cloudy solution in none of the three combinations tested. The volume of the sedimented phase was too low to handle when ethanol was used as disperser solvent. Slightly better analytical signals were obtained when using acetone than acetonitrile as disperser solvents.
Selected value	Extraction solvent: chloroform Disperser solvent: acetone

B) SELECTION OF THE VOLUME OF EXTRACTION SOLVENT

Starting hypothesis

Higher volumes will increase the extracted amount but it will also dilute it.

Assayed extraction volumes

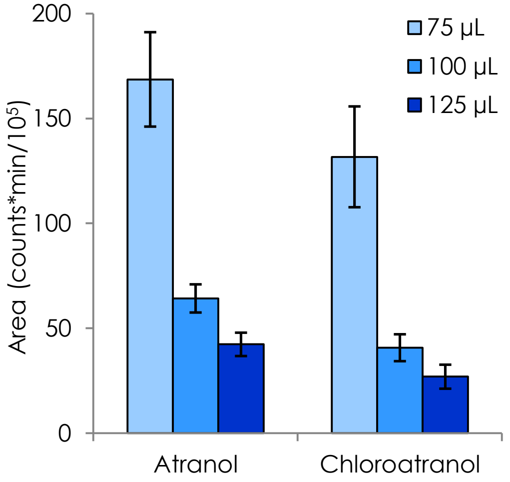
50 to 125 µL

Experimental procedure

Different volumes of extraction solvent previously mixed with 1000 µL of disperser solvent and 100 µL of anhydride acetic were rapidly injected into 10 mL of the standard hydro-ethanolic solution containing 1 mL of a 1M solution of K_2CO_3.

Results

Results are the average of 3 replicates, and the error bars show the standard deviation.



50 µL was discarded because the obtained sedimented phase volume was not enough to inject into the GC.

75 µL was discarded because the obtained sedimented phase volume was not enough to handle easily.

The signal decreases with the increase of the extraction solvent volume.

Selected value

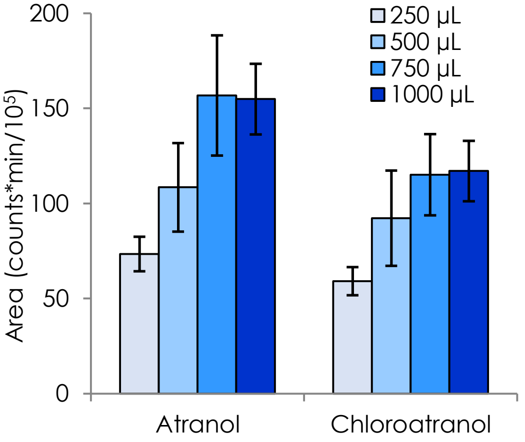
100 µL of extraction solvent.

C) SELECTION OF THE VOLUME OF DISPERSER SOLVENT

Starting hypothesis	Low volumes of disperser solvent will not produce a well-formed cloudy solution.
Assayed disperser volumes	250 to 1000 µL
Experimental procedure	Different volumes of disperser solvent previously mixed with 100 µL of extraction solvent and 100 µL of anhydride acetic were rapidly injected into 10 mL of the standard hydro-ethanolic solution containing 1 mL of a 1M solution of K_2CO_3.

Results

Results are the average of 3 replicates, and the error bars show the standard deviation.



The signal increases with the disperser solvent volume and tends to stabilize after 750 µL.

Selected value

750 µL of disperser solvent.

D) SELECTION OF THE POTASSIUM CARBONATE AMOUNT

Starting hypothesis

On one hand, an alkaline pH is needed to perform the derivatization reaction in a reasonable yield. On the other hand, potassium carbonate is a salt and it increases the ionic strength of the solution, what could affect the results.

Assayed K_2CO_3 amounts

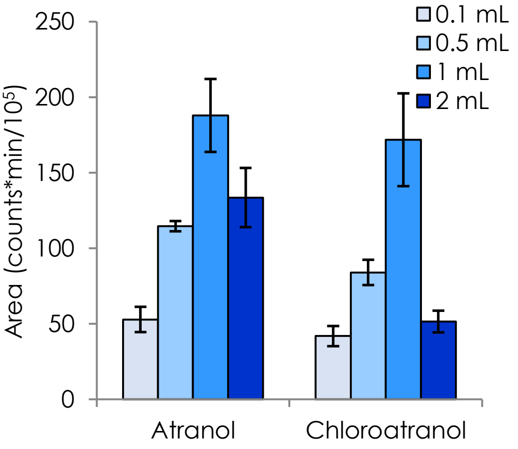
0.1 to 2 mL of a 1M solution of K_2CO_3

Experimental procedure

750 µL of disperser solvent previously mixed with 100 µL of extraction solvent and 100 µL of anhydride acetic were rapidly injected into 10 mL of the standard hydro-ethanolic solution containing different amounts of a 1M solution of $K_2CO_{3.}$

Results

Results are the average of 3 replicates, and the error bars show the standard deviation.



The best results were obtained when 1 mL of the solution was added.

The resulting pH is of 11.5 ± 0.2. As an alkaline pH in needed in order to perform the derivatization reaction, this pH value was kept for further experiments.

Selected value

Add 1 mL of a 1M solution of K_2CO_3 to the standard hydro-ethanolic solution before performing the simultanoeus DLLME and derivatization reaction.

E) SELECTION OF THE ACETIC ANHYDRIDE VOLUME

Starting hypothesis

Signal is expected to rise as the volume of anhydride acetic increases and it would be desirable that it was in excess. However, a high volume could decrease the signal due to a raise in the acidity.

Assayed acetic anhydride volumes

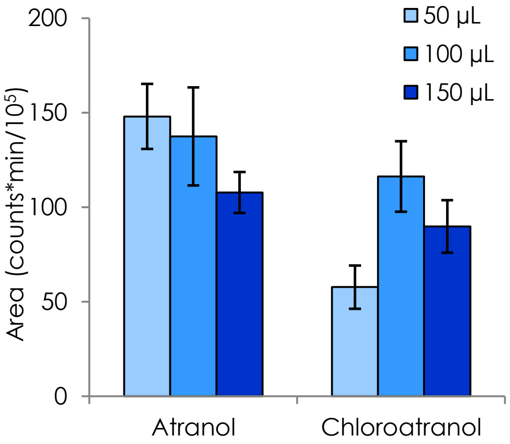
50 to 150 µL

Experimental procedure

Different volumes of anhydride acetic previously mixed with 750 µL of disperser solvent and 100 µL of extraction solvent were rapidly injected into 10 mL of the standard hydro-ethanolic solution containing 1 mL of a 1M solution of K_2CO_3.

Results

Results are the average of 3 replicates, and the error bars show the standard deviation.



When comparing 50 and 100 µL, similar results were obtained for atranol.

Best results were obtained when 100 µL were used in the case of chloroatranol.

Selected value

100 µL of acetic anhydride.

F) SELECTION OF THE IONIC STREGTH OF THE AQUEOUS DONOR PHASE

Starting hypothesis

The increase of the ionic strength will improve the extraction but will also increase the sedimented phase volume and dilute the analytes.

Assayed % NaCl

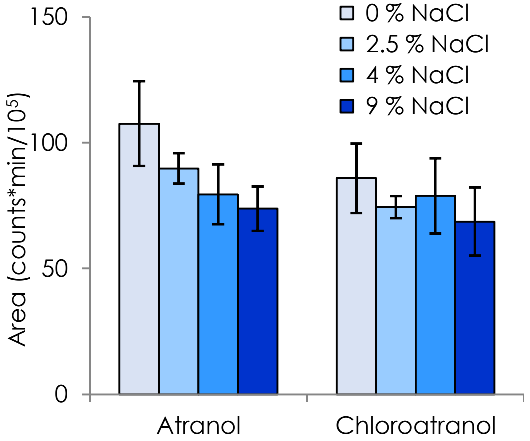
0 to 9 %

Experimental procedure

750 µL of disperser solvent previously mixed with 100 µL of extraction solvent and 100 µL of anhydride acetic were rapidly injected into 10 mL of the standard hydro-ethanolic solution containing 1 mL of a 1M solution of K_2CO_3 and different amounts of NaCl.

Results

Results are the average of 3 replicates, and the error bars show the standard deviation.



1 mL of a 1M potassium carbonate solution was previously added. So, there were already other salts that already contributed to the ionic strength of the donor phase.

The addition of salt reduces the signal of atranol, probably due to the fact that the volume of sedimented phase increases considerably with the ionic strength.

No significant differences were obtained in the case of chloroatranol.

Selected value

No addition of salt to the aqueous donor phase.

G) SELECTION OF THE EXTRACTION AND DERIVATIZATION TIME

Starting hypothesis

The equilibrium state is instantaneously achieved as the cloudy solution generates a very large surface area between the sample, the extraction solvent and the derivatization reagent.

Assayed time

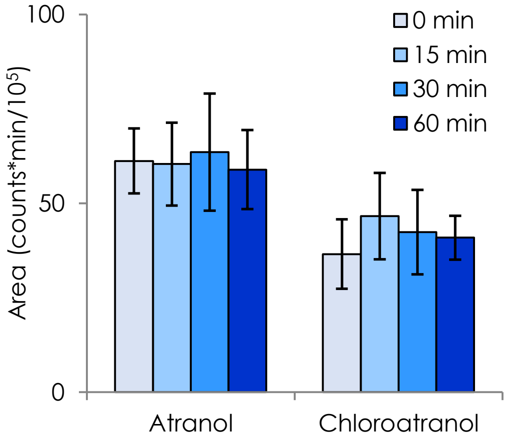
0 to 60 minutes

Experimental procedure

750 µL of disperser solvent previously mixed with 100 µL of extraction solvent and 100 µL of anhydride acetic were rapidly injected to 10 mL of the standard hydro-ethanolic solution containing 1 mL of a 1M solution of K_2CO_3. Then different times between this step and the centrifugation were assayed.

Results

Results are the average of 3 replicates, and the error bars show the standard deviation.



Results confirmed, as expected, that extraction and derivatization time does not affect the results obtained in the DLLME.

Selected value

The centrifugation step can be performed immediately after the DLLME process.

CALIBRATION MODE

Selected calibration mode

The use of a surrogate was considered in order to reduce the variability of the measurements.

Standard addition calibration using internal standard was performed for each sample and their slopes compared to that of conventional internal standard calibration

Sample	Calibration slopes ratio (%)[a]	
	Atranol	Chloroatranol
Perfume 1	116 ± 5	72 ± 6
Perfume 2	112 ± 6	89 ± 6
Perfume 3	120 ± 7	71 ± 3
Perfume 4	146 ± 9	62 ± 8

[a] (Ratio of standard calibration slopes and conventional calibration) x 100

Matrix effects can be observed. Thus, standard addition combined with internal standard is recommended.

Analytical signal

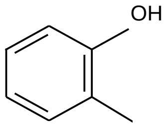
$y = A_i/A_{sur}$

A_i = the peak area of the target analyte
A_{sur} = the peak area of the surrogate

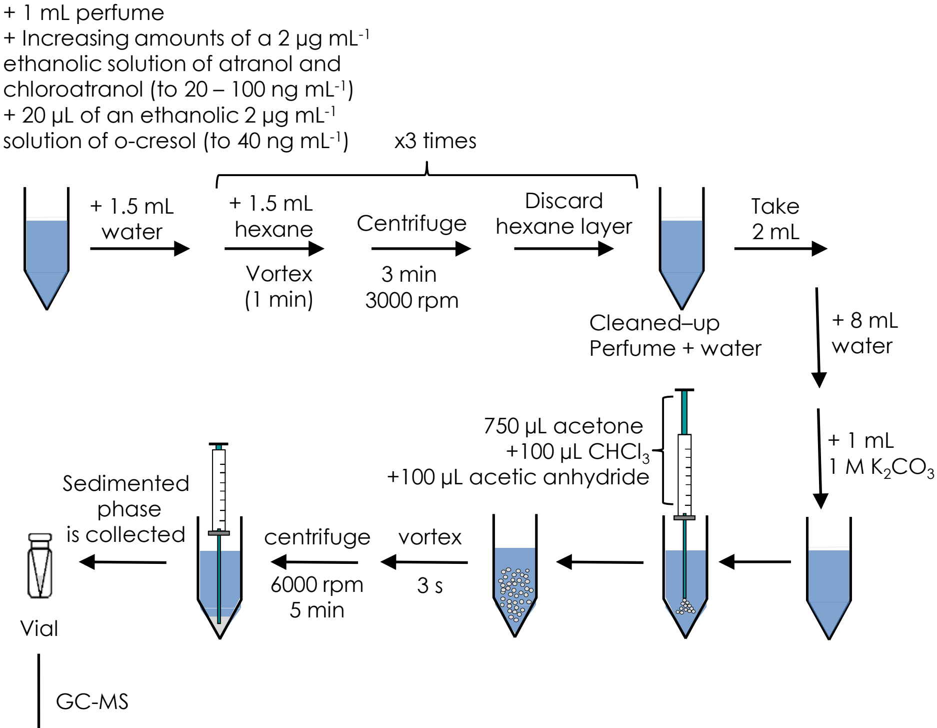
Selected surrogate

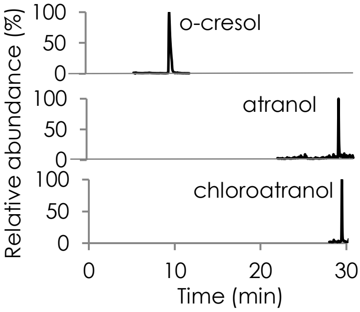
O-cresol

- Suitable for GC-MS.
- Extractable by DLLME in $CHCl_3$.
- Has a very similar log P than the target compounds, so its solubility features are expected to be also similar.
- It is, as the target compounds, a phenol. Thus, it could correct the deviations produced not only in the extraction processes and the injection but also in the derivatization process.
- Structure:



PROPOSED METHOD





GC-MS Conditions :

Injection volume	1 µL (Splitless time 1 min)
Inlet temperature	280 °C
Transfer line temperature	280 °C
Ion source temperature	250 °C
Helium flow rate	1 mL/min
Column	HP-5MS Ultra Inert (30m, 0.25mm, 0.25µm)
Oven temperature	80 °C (1 min) 4 °C/min to 200 °C 20 °C/min to 280 °C (5min)

Acquisition intervals

Compound	Quantification ions[1]	Time (min)
O-cresol (surrogate)	108	5.0 - 11.0
Atranol	152	20.0 – 28.0
Chloroatranol	186	28.0 – 32.0

[1] A full scan mode (m/z 40 – 350) was simultaneously recorded from minute five for identification purposes.

ANALYTICAL FIGURES OF MERIT

Enrichment factor

$$EF = \frac{C_f}{C_0} = \frac{A_f}{A_0} = \frac{V_0}{V_f} R$$

C = Concentration

A = Area

V = Volume

R = Extraction yield

f = Final

0 = Initial

Since the analytes are derivatized simultaneously to DLLME, the pure derivatized compounds were needed. As these compounds were not commercially available they were synthesised in the laboratory. A ^1H NMR of the product showed that the dyacetilated product had been successfully synthesised and no other significant side products or reagents were left. These products were used as standards to calculate the EF of the whole method:

Analyte	EF
Atranol	15
Chloroatranol	15

Calculated taking into account the loss in the LLE step and the concentration in the DLLME step

Working range

20 - 100 ng mL^{-1}

Linearity to, at least, 1000 ng mL^{-1}

Limits of detection and quantification of the method

LOD: 3 times the signal-to-noise ratio

LOQ: 10 times the signal-to-noise ratio

Analyte	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
Atranol	3	9
Chloroatranol	2	6

Repeatability

Evaluated applying the proposed method to five replicates of a standard solution that contained 40 ng mL^{-1} of the target compounds

Analyte	RSD (%)
Atranol	7
Chloroatranol	10

Analysis of real samples

Determined in four different perfumes: a woman *eau de toilette*, a woman *eau de parfum*, a man *eau de parfum* and a children *eau de cologne*. (names are not shown for confidentiality reasons)

Results showed that both target compounds were below the limits of detection in the four samples.

Recovery studies

The proposed method was applied to the free analyte samples previously spiked with the target analytes at two levels: 20 and 100 ng L^{-1}

Sample	Recovery (%)			
	Atranol		Chloroatranol	
	20 ng mL^{-1}	100 ng mL^{-1}	20 ng mL^{-1}	100 ng mL^{-1}
1	94	103	95	98
2	96	101	79	97
3	86	103	104	100
4	106	98	110	98

CONCLUSIONS

■ Although quality control of commercial perfumes and raw materials is necessary little has been done in this field regarding the potent allergens atranol and chloroatranol.

■ The low number of publications dealing with their determination do not obtain accurate results.

■ In this work a method based on LLE followed by simultaneous derivatization and DLLME and GC-MS is presented to determine atranol and chloroatranol in perfumes, allowing their determination in the ng mL^{-1} range with good accuracy and precision.

Chapter 5.

Development of a chromatochemometric approach for evaluating and selecting the perfume maceration time of a perfume

Based on:

M. Lopez-Nogueroles, A. Chisvert, A. Salvador,
A chromatochemometric approach for evaluating and selecting the perfume maceration time,
Journal of Chromatography A 1217 (2010) 3150-3160.

AIM OF THE WORK

Aim

Development of an analytical method to detect any significant change in the process of perfume maceration, and thus to establish the perfume maceration time.

Proposed method

The chromatograms of perfume samples subjected to different maceration times obtained using gas chromatography with a flame ionization detector (GC-FID) are used as a fingerprint.

Linear discriminant analysis (LDA) enables the classification of samples by comparing them to a set of samples which were known to be macerated or not.

The significant GC-FID peaks in each one of the studied perfumes were tried to be identified by means of a gas chromatography mass spectrometry (GC-MS).

Interest

Manufactures usually keep perfumes in maceration cubes for several weeks, even months, in order to assure that the maceration process has been completed and thus, the perfume has achieved a steady-state. This process takes time and money.

The final purpose of this work is to propose a method to find the minimum time necessary for macerating a perfume preserving its organoleptic characteristics. As the perfume will not remain more time than the needed before being packed and ready to put on the market this work can help avoiding important losses of time in the perfume manufacture and the subsequent resulting economic cost.

No papers about the study and/or the establishment of the perfumes maceration time were found in the literature before the publication of this work.

EXPERIMENTAL PROCEDURE

Preliminary aspects

The perfume maceration time used by the manufacturer before the study was of 30 days. Thus, this time was considered as maceration completed.

Calibration objects

Samples considered as not macerated Time: 0, just prepared (10 replicates)	Group I
Completely macerated samples Time: 30 days after preparation (10 replicates)	Group II

Unknown objects

Samples from days 1 to 29, with an unknown degree of maceration

(5 of 10 replicates randomly selected were analysed per day)

Data treatment

The differences in the GC-FID chromatographic profiles of the just prepared perfume and the same perfume after 30 days of maceration (calibration objects) were studied using an LDA model.

The *Statistical Package for the Social Sciences (*SPSS) software allows us to identify those variables (chromatographic peaks) that differentiates the 2 groups and builds the function that better distinguishes between both of them.

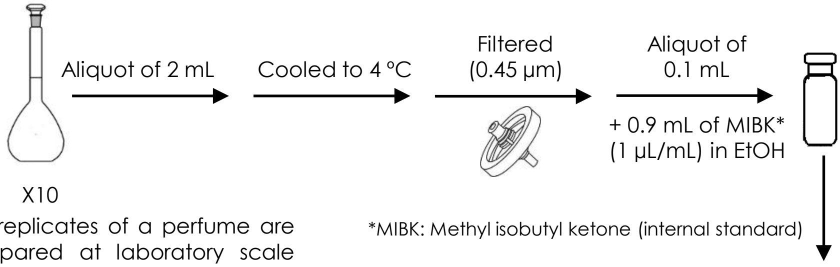
Once this function is acknowledged it can be used to found out if the samples in-between days (unknown objects) are classified as macerated or not macerated samples.

This allows to find out the minimum time in which the changes in the maceration process have already occurred and therefore the perfume can be bottled and market.

The significant GC-FID peaks in each one of the studied perfumes were tried to be identified by means of a gas chromatography mass spectrometry (GC-MS).

PROPOSED METHOD

Days 0 (not macerated) and 30 (macerated): All 10 perfume replicates are treated
Days in-between 0 and 30 (unknown): Only 5 of the 10 (randomly selected) are treated



Aliquot of 2 mL → Cooled to 4 °C → Filtered (0.45 µm) → Aliquot of 0.1 mL + 0.9 mL of MIBK* (1 µL/mL) in EtOH

X10

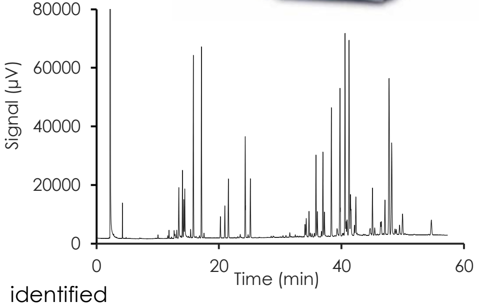
10 replicates of a perfume are prepared at laboratory scale according to manufacturers protocol (Considered day 0)

*MIBK: Methyl isobutyl ketone (internal standard)

GC-FID Conditions

Injection volume	1 µL
Split mode	Split 1:30
Inlet temperature	250 °C
Detector temperature	280 °C
Nitrogen flow rate	1 mL/min
Column	TRB-5 (30m, 0.32mm, 0.50µm)
Oven temperature	70 °C (4 min) 3 °C/min to 200 °C (12 min)

Example of a typical GC – FID chromatogram of a perfume :



The significant compounds (peaks) are identified by GC-MS :

GC-MS Conditions

Acquisition interval	Full Scan m/z 20 – 500 from minute 5
Injection volume	1 µL
Split mode	Split 1:30
Inlet temperature	250 °C
Transfer line temperature	280 °C
Ion source temperature	225 °C
Helium flow rate	1 mL/min
Column	TR-5MS (30m, 0.25mm, 0.25µm)
Oven temperature	70 °C (4 min) 3 °C/min to 200 °C (12 min)

DATA TREATMENT PROCEDURE

Construction of the LDA model

All well resolved chromatographic peaks are integrated

Area is normalized (dividing by the internal standard)

Area is standardized to mean zero and variance one. This will make all variables have the same weight. To standardize:

$$z = \frac{x - \mu}{\sigma}$$

z : The standardized variable

x : Variable (normalized peak area)

μ : Mean of the variable (of the peak area of the ten replicates)

σ: Standard deviation of the variable (of the peak area of the ten replicates)

Two matrices are constructed, containing the normalized-standardized areas (variables) of the chromatographic profiles, first one at time 0, and second one at time 30.

An LDA model is constructed using these two calibration matrices. A software algorithm choses those significant variables to construct the model and avoids the ones that do not change significantly or are redundant (a variable is considered significant when it causes a significant reduction of the λ_{wilks} value if included in the model). This way the linear discriminant function, a linear combination of those significant variables, is obtained.

Assignment of the unknown objects

Only the variables used to construct the LDA model are integrated in the chromatographic profiles of the unknown objects.

These variables are normalized and standardized.

These variables are replaced in the linear discriminant function to obtain the scores.

Each unknown object is assigned to one or other group based on the position of their scores on either side of the cut-off value.

RESULTS OBTAINED IN THE CONSTRUCTION OF THE LDA MODEL

Selected peaks

Perfume	nº peaks in chromatogram	nº peaks selected by the LDA model
1	54	5
2	45	3
3	45	5
4	30	3
5	29	3
6	33	3
7	33	3

Linear discriminant function (LDF)

Obtained for each one of the 7 perfumes under study

LDF_i: the linear discriminant function for perfume i.

P_{ij}: Normalized-standardized area of the peak with a retention time of j (in minutes) in the chromatogram of perfume i.

$LDF_1 = 4.6P_{1,18.1} - 4.7P_{1,20.7} + 6.6P_{1,27.2} - 2.3P_{1,29.5} - 1.8P_{1,34.9}$

$LDF_2 = -0.6P_{2,10.4} + 2.0P_{2,11.6} + 1.1P_{2,20}$

$LDF_3 = -5.4P_{3,18.1} + 14.2P_{3,32.6} - 11.4P_{3,42.7} + 2.8P_{3,49.4} + 2.6P_{3,49.9}$

$LDF_4 = 2.1P_{4,23.3} - 15.2P_{4,30.8} + 12.7P_{4,32.6}$

$LDF_5 = 2.8P_{5,7.5} + 8.2P_{5,14.7} - 3.9P_{5,27.6}$

$LDF_6 = 3.9P_{6,16.3} - 6.2P_{6,41.5} + 2.4P_{6,46.7}$

$LDF_7 = 8.6P_{7,31.5} - 12.5P_{7,36.4} - 5.9P_{7,55.7}$

The extent and sign of the different weighting factors of each LDA model shows that not only there are different compounds changing in the maceration process, but also these changes are different depending on the compound, and also depending on the perfume.

λ_{Wilks}

Calculated as the sum of squares of the distances between objects belonging to the same group divided by the total sum of squares in an LDF

$0<\lambda_{Wilks}<1$

Being values close to 0 very well resolved groups and close to 1 overlapped groups

Perfume	λ_{Wilks}
1	0.022
2	0.118
3	0.013
4	0.067
5	0.015
6	0.037
7	0.085

These λ_{Wilks} values show that the two groups are well resolved.

Scores of the calibration objects

Individual scores (lower part) and average of the scores and standard deviation (upper part) of the calibration objects

🔷 Completely macerated samples

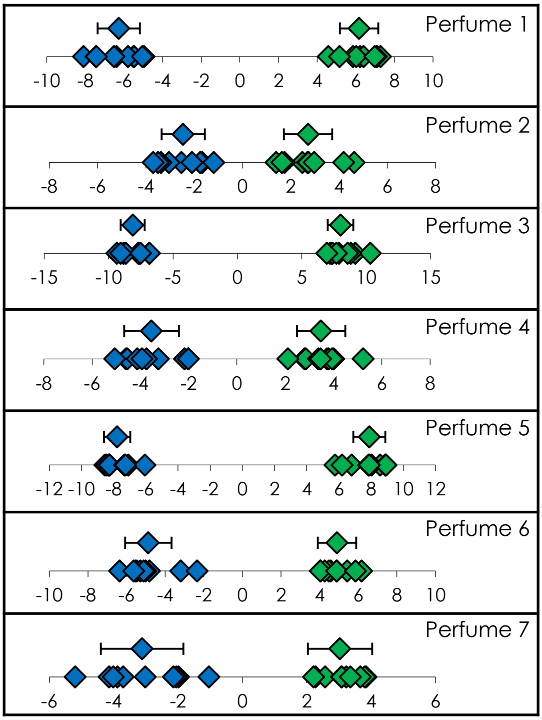
Time: 30 days after preparation

(10 replicates)

◆ Not macerated samples

Time: 0, just prepared

(10 replicates)



An excellent distinction between the two groups is observed, which is consistent with the obtained λ_{Wilks} values.

For the 7 perfumes under study, all the objects of the calibration matrix were correctly classified by leave-one-out cross-validation.

RESULTS OBTAINED FOR THE ASSIGNMENT OF THE PERFUME SAMPLES SUBJECTED TO DIFFERENT MACERATION TIMES (UNKNOWN OBJECTS)

Chart

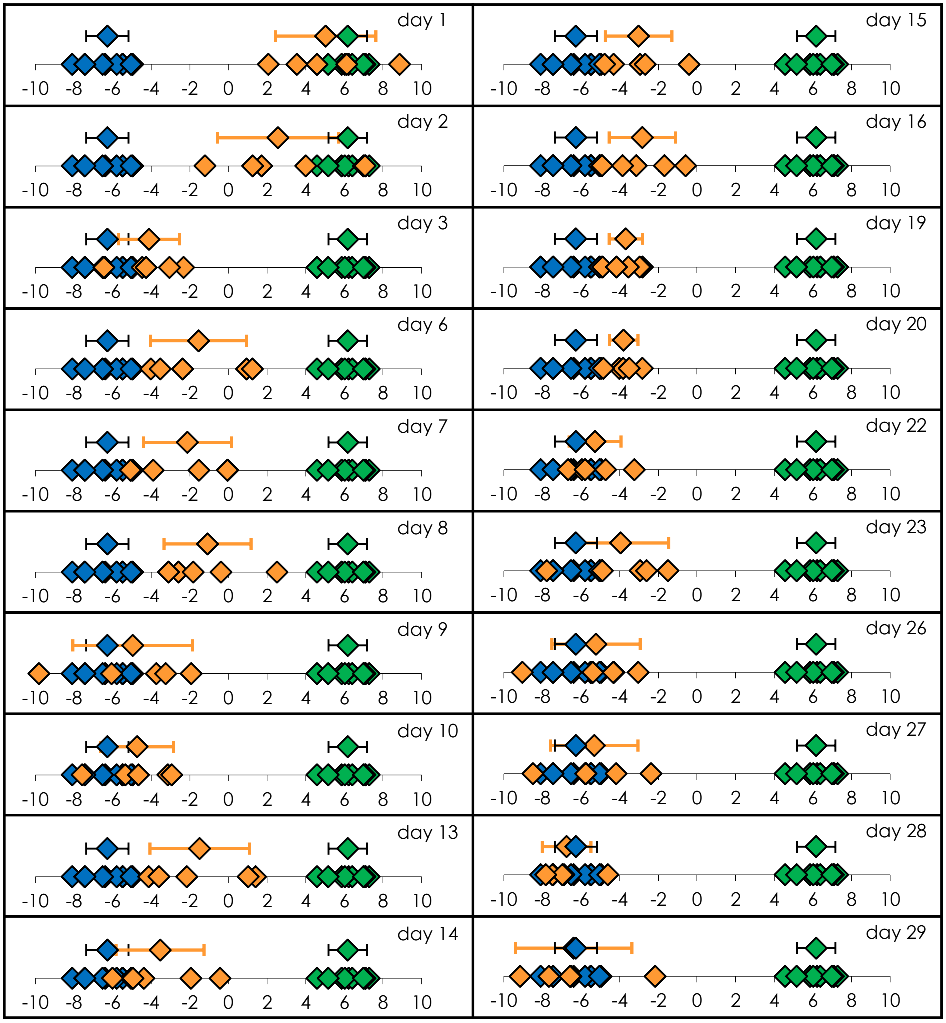
🔷 Completely macerated samples

◆ Not macerated samples

🔶 Unknown samples (In-between maceration time)

The individual scores and average of the scores and standard deviation of the calibration objects (10 replicates) together with that of the unknown objects (5 replicates) were plotted for the seven perfumes under study.

Assignment of the unknown objects of Perfume 1



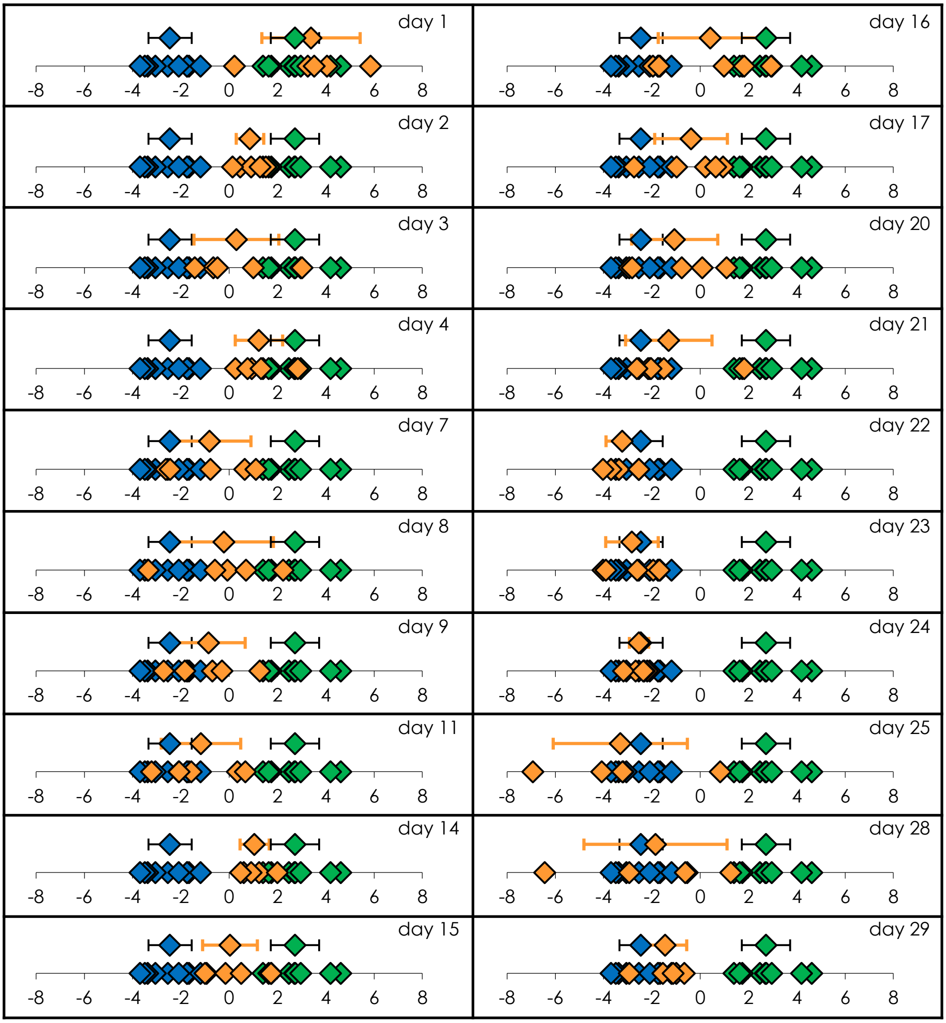
Days 1 and 2: Similar scores to the not macerated group

Days 3 to 13: The replicates have generally similar scores to the macerated group. However, some of the replicates have a similar score to the not macerated group.

Days 14 to 29: All replicates can be assigned to the macerated group, what means that they have a similar chromatographic profile to these calibration objects.

Conclusion: The maceration process is completed after 14 days

Assignment of the unknown objects of Perfume 2



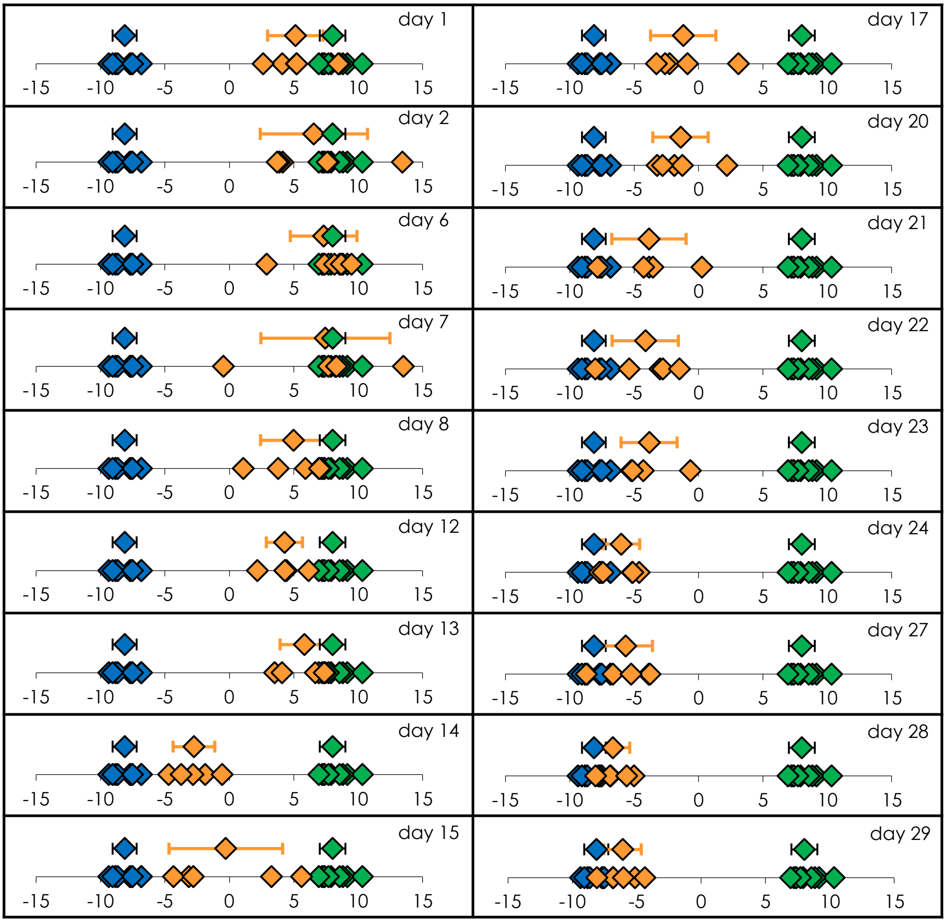
Days 1 to 4: The replicates have generally similar scores to the not macerated group.

Days 7 to 21: Some of the replicates have similar scores to the macerated group and others to the not macerated group.

Days 22 to 29: The replicates have similar scores to the macerated group.

Conclusion: The maceration process is completed on day number 22.

Assignment of the unknown objects of Perfume 3



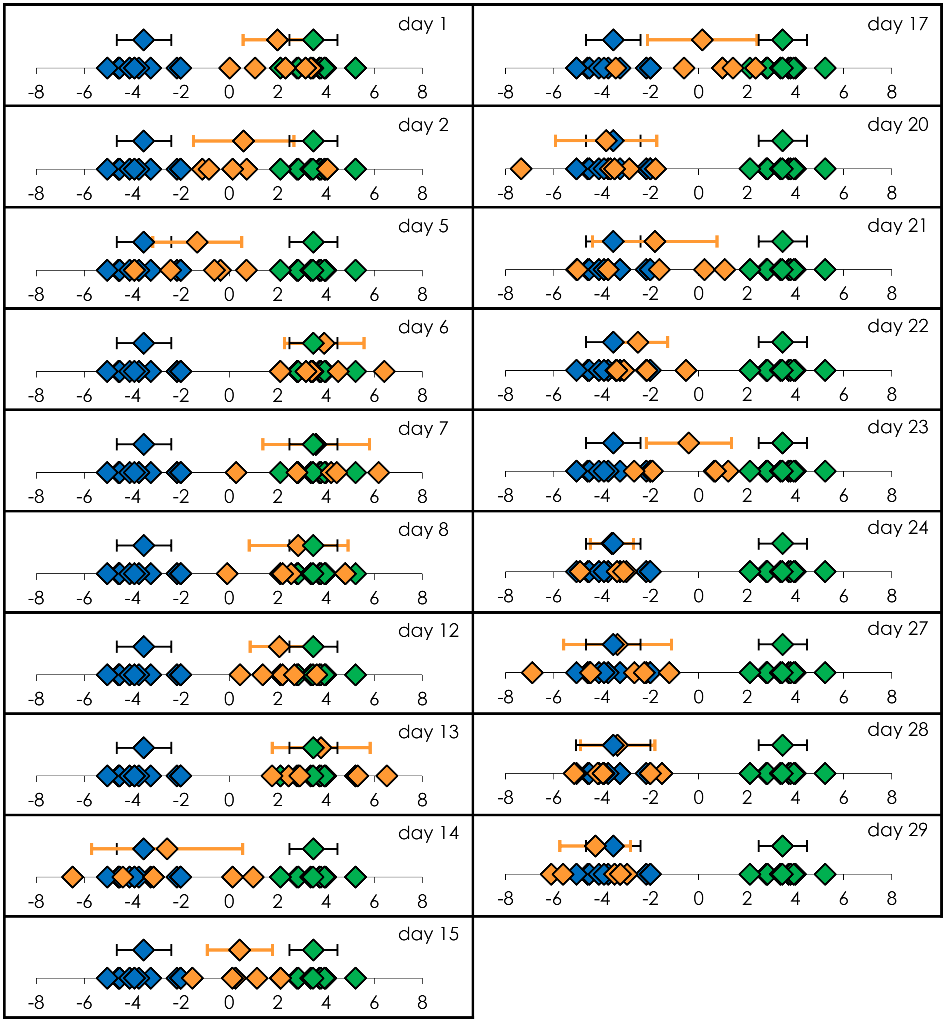
Days 1 to 13: The replicates are classified in the not macerated group.

Days 14 to 20: Some changes have already occurred and some of the replicates have similar scores to the macerated group and others to the not macerated group.

Days 21 to 29: All the replicates have similar scores to the macerated group.

Conclusion: The maceration process is completed on day 21.

Assignment of the unknown objects of Perfume 4



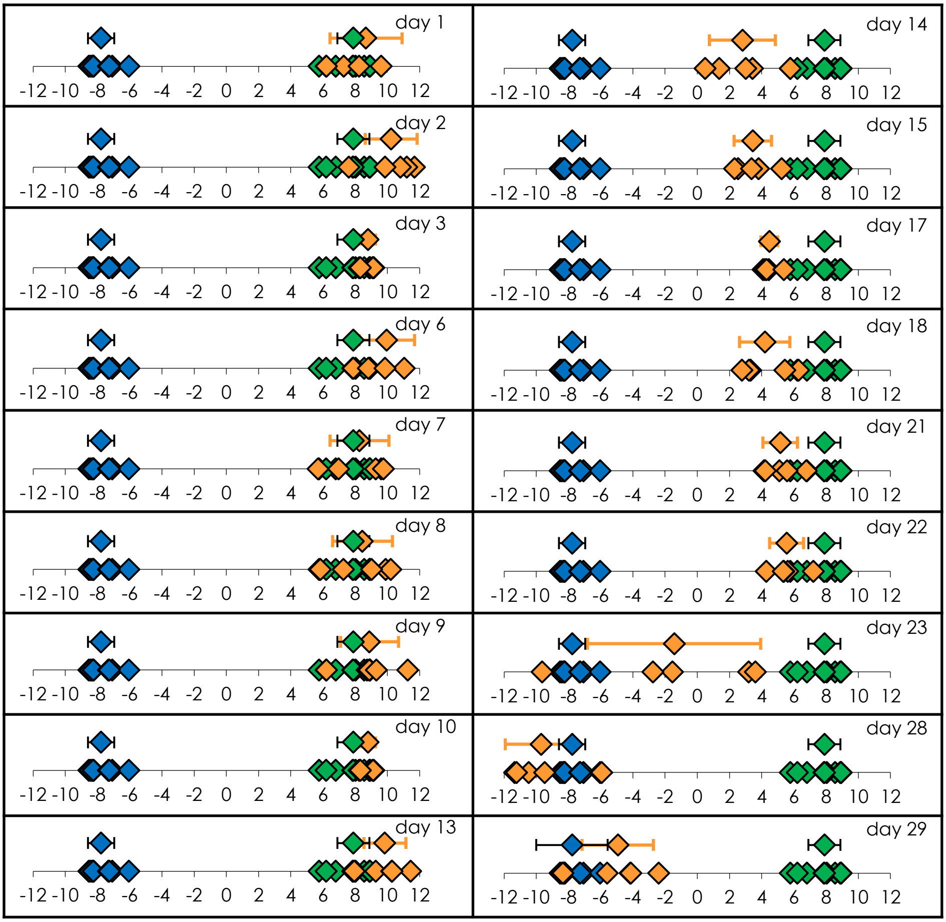
Days 1 to 13: The replicates are classified in the not macerated group.

Days 14 to 23: Changes are still occurring and some of the replicates have still similar scores to the not macerated group.

Days 24 to 29: All the replicates have similar scores to the macerated group.

Conclusion: The maceration process is completed on day 24.

Assignment of the unknown objects of Perfume 5



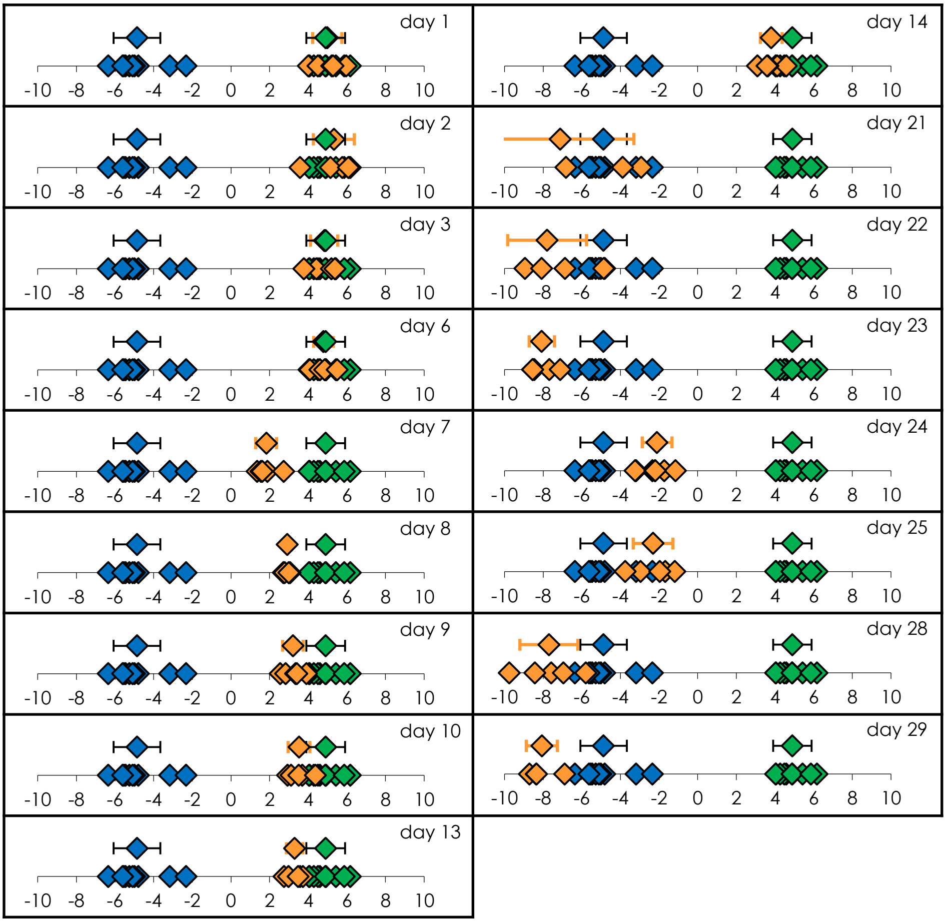
Days 1 to 22: The replicates are classified in the not macerated group.

Day 23: The changes in the maceration process is shown in some of the scores of the replicates

Days 28 and 29: The chromatographic profile is similar to the macerated group on days 28 and 29.

Conclusion: The maceration process is completed on day 28.

Assignment of the unknown objects of Perfume 6

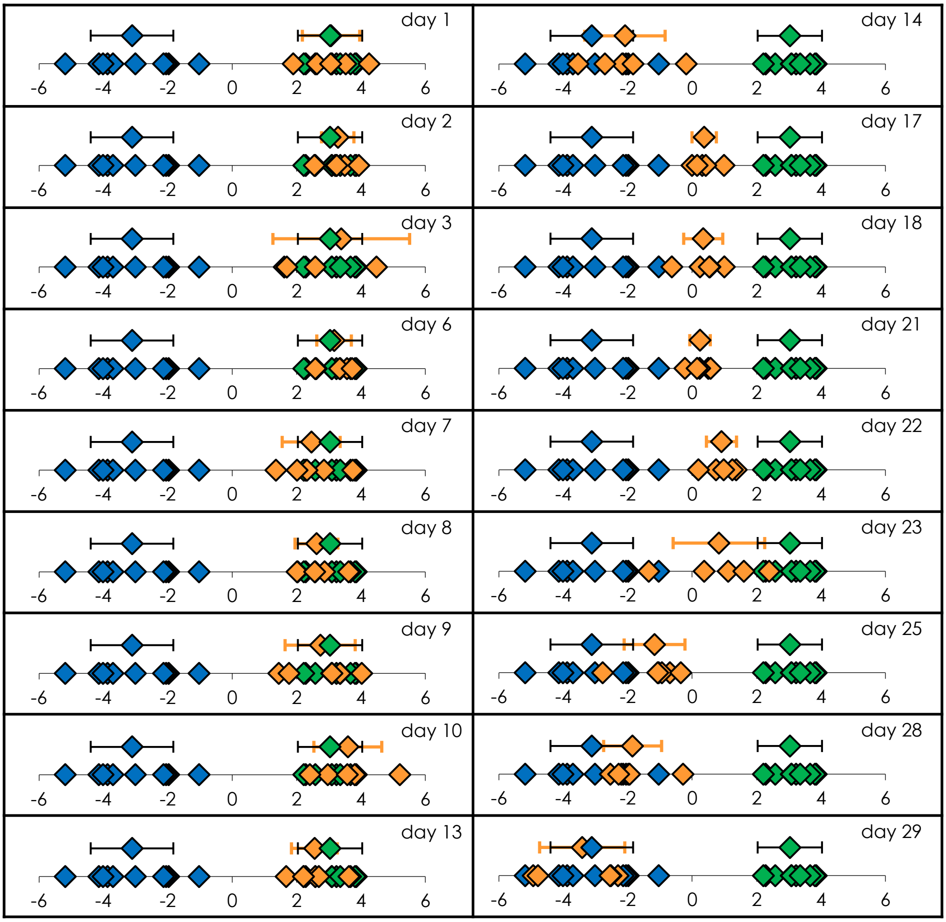


Days 1 to 14: The replicates are classified in the not macerated group.

Days 21 to 29: All the replicates have similar scores to the macerated group

Conclusion: The maceration process is completed on day 21.

Assignment of the unknown objects of Perfume 7



Days 1 to 13: The replicates are classified in the not macerated group.

Days 14 to 23: The replicates obtain intermediate scores. The maceration process has not concluded.

Days 25 to 29: The replicates are classified in the macerated group.

Conclusion: The maceration process is completed on day 25.

MACERATION TIME

Estimated maceration time and reduction

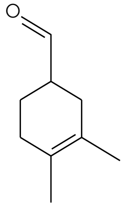
Perfume	Estimated maceration time (days)	Reduction * (%)
1	14	53
2	22	27
3	21	30
4	24	20
5	28	7
6	21	30
7	25	17

*Reduction over the manufacturer time, initially established in 30 days.

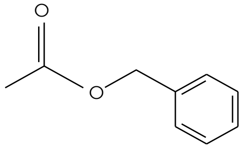
MS IDENTIFICATION OF THE SIGNIFICANT GC-FID PEAKS

Perfume 1

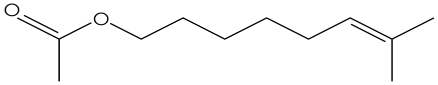
Four of five significant compounds were identified.



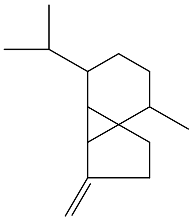
Triplal
(has a powerful smell of citrus).



Benzyl acetate
(found naturally in many flowers. It has a sweet and pleasant aroma that reminds to jasmine).



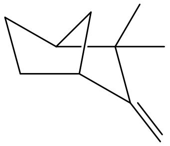
Citronellyl acetate
(widely used in perfumery, has a fruity, sweet smell reminiscent of roses and certain fruits like plums or lychees).



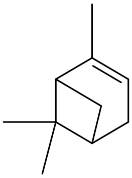
β-Cubenene
(also a common essential oil compound due to its characteristic odor of grass and wood).

Perfume 2

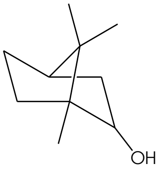
All three significant compounds were identified.



Camphene
(used in the manufacture of essential oils and flavouring, due to its smell to herbs, camphor and wood).



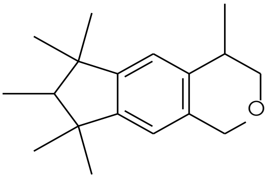
β-Pinene
(naturally found in the resin of pine trees and other plants. It has a hard smell, reminiscent of pine).



Borneol
(widely used in perfumery because of its aroma, reminiscent of conifers. It is also used as a natural insect repellent).

Perfume 3

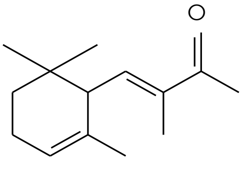
Only one significant compound of five was identified.



Hexahydrohexamethyl cyclopentabenzopyran, also known as *Musk Galaxolide*
(a very common ingredient in perfumes. It is a cheap synthetic musk that smells good).

Perfume 4

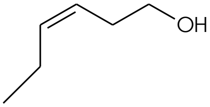
One of three significant compounds were identified.



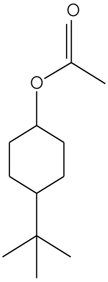
a-isomethylionone
(related to fragrances. It has a sweet fruity odor reminiscent of jasmine).

Perfume 5

Two of three significant compounds were identified.



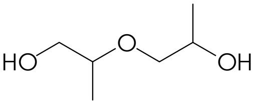
(Z)-3-hexen-1-ol
(a colourless oily liquid with a strong smell of freshly cut grass. Is produced in small amounts in most plants).



4-tert-butylcyclohexyl acetate
(an oil with floral scent. Obtained from fruit plants).

Perfume 6

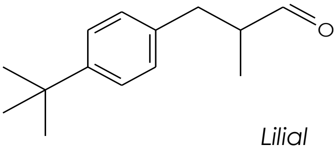
Only one of three significant compounds was identified.



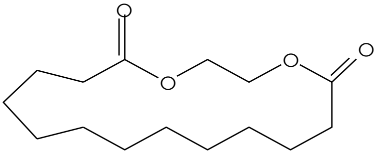
2-(2-hydroxypropoxy)-1-propanol.

Perfume 7

Two of three significant compounds were identified.



Lilial
(linked to perfumes, presents a floral odour).



Ethylene Brassilate
(also known as *Musk T*, smells of wood).

CONCLUSIONS

■ The developed GC-FID methodology combined with LDA data treatment has enabled us to establish the maceration time involved in the perfume manufacture from a chemical standpoint.

■ This methodology has been applied successfully to 7 different perfumes, and up to a reduction of 57% has been achieved with respect to the maceration time initially established by the manufacturer.

■ The obtained results by the proposed methodology were further correlated with those obtained by a panel of olfactory experts. Similar conclusions to those obtained by this work were obtained.

Chapter 6.

Determination of nitro musks in surface water samples using dispersive liquid-liquid microextraction followed by gas chromatography-mass spectrometry

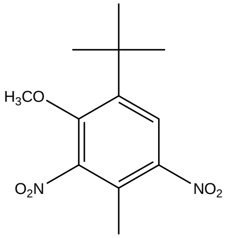
Based on:

M. Lopez-Nogueroles, A. Chisvert, A. Salvador, A. Carretero,
Dispersive liquid-liquid microextraction followed by gas chromatography-mass spectrometry for the determination of nitro musks in surface water and wastewater samples,
Talanta 85 (2011) 1990-1995.

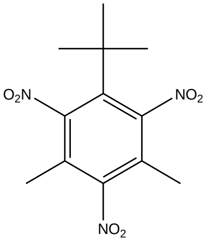
AIM OF THE WORK

Aim	Development of a simple, fast and high sensitive analytical method to determine nitro musks in wastewater and surface water samples.
Proposed method	Dispersive liquid–liquid microextraction (DLLME) followed by gas chromatography–mass spectrometry (GC-MS).
Interest	Considered persistent pollutants. Related to health risks. They could reach the aquatic environment via swimming activities or wastewater treatment plants. The high potential for preconcentration of the DLLME has never been used before for the determination of the complete family of the nitro musk compounds.

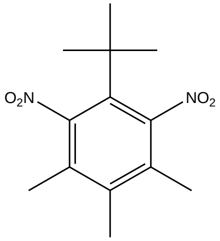
TARGET COMPOUNDS



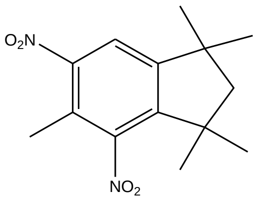
Musk Ambrette
(MA)



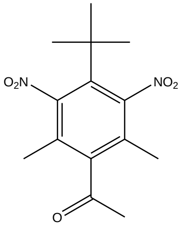
Musk Xylene
(MX)



Musk Tibetene
(MT)



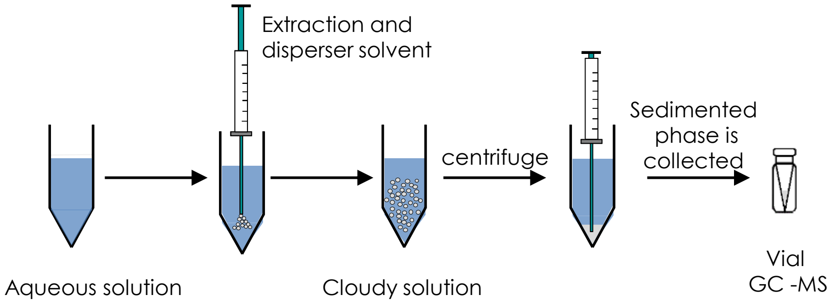
Musk Moskene
(MM)



Musk Ketone
(MK)

GENERAL PROCEDURE FOR DLLME-GC ANALYSIS

To carry out the DLLME, the extraction and disperser solvents are added to the aqueous solution (sample or standard) to form a cloudy solution. After centrifugation, the sediment phase (at the bottom) is collected and analysed by GC-MS.



SELECTION OF DLLME CONDITIONS

DLLME variables to be studied	A) Nature of extraction and disperser solvents. B) Volume of extraction solvent. C) Volume of disperser solvent. D) Ionic strength of the aqueous donor phase. E) pH of the aqueous donor phase.
Response function	GC-MS peak area of each target analyte.
Solution used in the studies	Standard aqueous solution of the five target analytes at 10 ng mL^{-1}.

A) SELECTION OF EXTRACTION AND DISPERSER SOLVENT

Required features for the extraction solvent	Immiscibility in the aqueous phase. Higher density than the aqueous donor phase. Good extraction of the target compounds. Good behaviour as GC solvent.
Assayed extraction solvents	Dichloromethane Chloroform
Required features for the disperser solvent	Formation of the cloudy solution. Miscibility in the aqueous donor phase and the extraction solvent.
Assayed disperser solvents	Acetone Acetonitrile Ethanol
Experimental procedure	1000 µL of each disperser solvent previously mixed with 50 µL of each extraction solvent were rapidly injected into 5 mL of the aqueous standard solutions.
Results	Dichloromethane did not form cloudy solution in none of the three combinations tested. A cloudy solution was also not obtained when the chloroform–ethanol combination was tested. When comparing the other two options (chloroform as extraction solvent and acetone or acetonitrile as disperser solvent) similar analytical signals were obtained.
Selected value	Extraction solvent: chloroform Disperser solvent: acetone

B) SELECTION OF THE VOLUME OF EXTRACTION SOLVENT

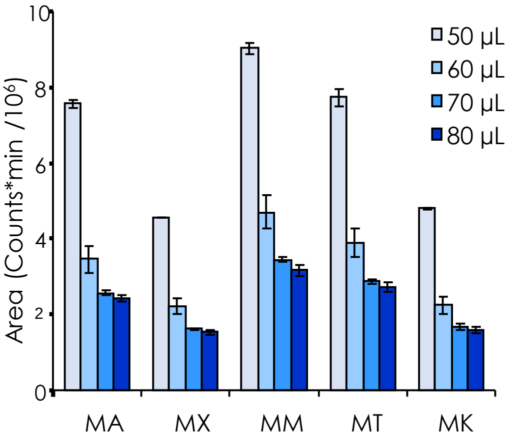
Starting hypothesis Higher volumes will increase the extracted amount but it will also dilute it.

Assayed extraction volumes 40 to 80 µL

Experimental procedure 1000 µL of disperser solvent previously mixed with 40-80 µL of extraction solvent were rapidly injected into 5 mL of the aqueous standard solutions.

Results

Results are the average of 3 replicates, and the error bars show the standard deviation.



40 µL was discarded because the obtained sedimented phase volume was not enough to handle and inject into the GC.

The signal decreases with the increase of the extraction solvent volume.

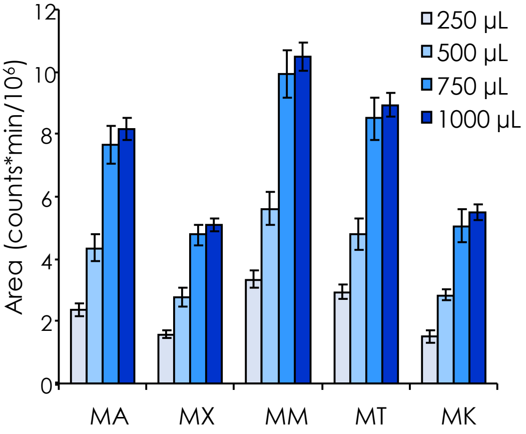
Selected value 50 µL of extraction solvent.

C) SELECTION OF THE VOLUME OF DISPERSER SOLVENT

Starting hypothesis	Low volumes of disperser solvent will not produce a well-formed cloudy solution.
Assayed disperser volumes	250 to 1000 µL
Experimental procedure	250-1000 µL of disperser solvent previously mixed with 50 µL of extraction solvent were rapidly injected into 5 mL of the aqueous standard solutions.

Results

Results are the average of 3 replicates, and the error bars show the standard deviation.



The signal increases with the disperser solvent volume and tends to stabilize after 750 µL.

Selected value

1000 µL of disperser solvent.

D) SELECTION OF THE IONIC STREGTH OF THE AQUEOUS DONOR PHASE

Starting hypothesis

The increase of the ionic strength could improve the extraction by the salting-out effect but could also increase the sedimented phase volume and dilute the analytes.

Assayed % NaCl

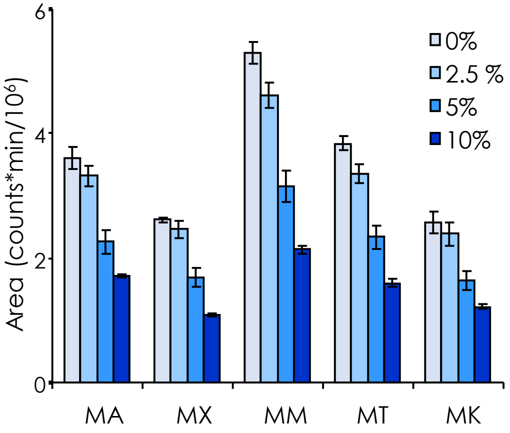
0 to 10 %

Experimental procedure

1000 µL of disperser solvent previously mixed with 50 µL of extraction solvent were rapidly injected into 5 mL of the aqueous standard solution containing different amounts of NaCl.

Results

Results are the average of 3 replicates, and the error bars show the standard deviation.



The addition of salt reduces the signal obtained. This is probably due to the dilution effect as the volume of sedimented phase increases considerably with the ionic strength.

This effect should be taken into account in the analysis of high salt content samples (see calibration mode).

Selected value

No addition of salt to the aqueous donor phase.

E) SELECTION OF THE pH OF THE AQUEOUS DONOR PHASE

Starting hypothesis

The five target nitro musks do not present ionisable moieties, thus it is expected that pH does not affect the extraction.

Assayed pHs

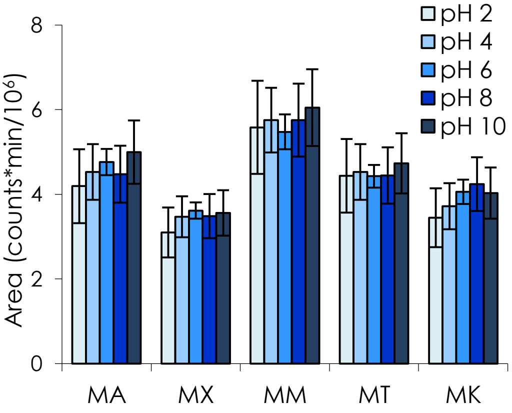
pH 2 to 10

Experimental procedure

1000 μL of disperser solvent previously mixed with 50 μL of extraction solvent were rapidly injected into 5 mL of the aqueous standard solution at different pHs (buffered with phosphate buffer 10^{-3} M).

Results

Results are the average of 3 replicates, and the error bars show the standard deviation.



Results confirmed, as expected, that there was no difference in the extraction of the target compounds at the different pH values studied.

Selected value

pH adjustment of the aqueous donor phase is not necessary.

CALIBRATION MODE

Selected calibration mode

The use of a surrogate and internal calibration was considered in order to reduce the variability of the measurements.

Analytical signal

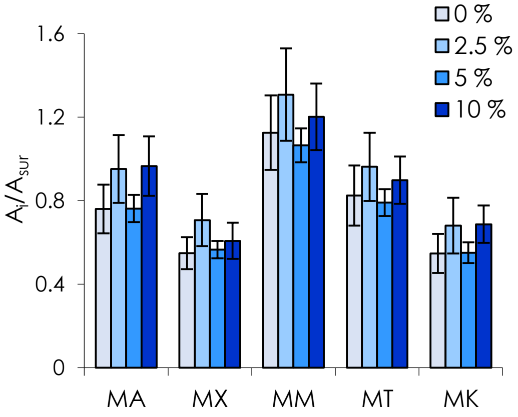
$y = A_i/A_{sur}$

A_i = the peak area of the target analyte
A_{sur} = the peak area of the surrogate

Selected surrogate

Deuterated benzophenone (BZ-d10).

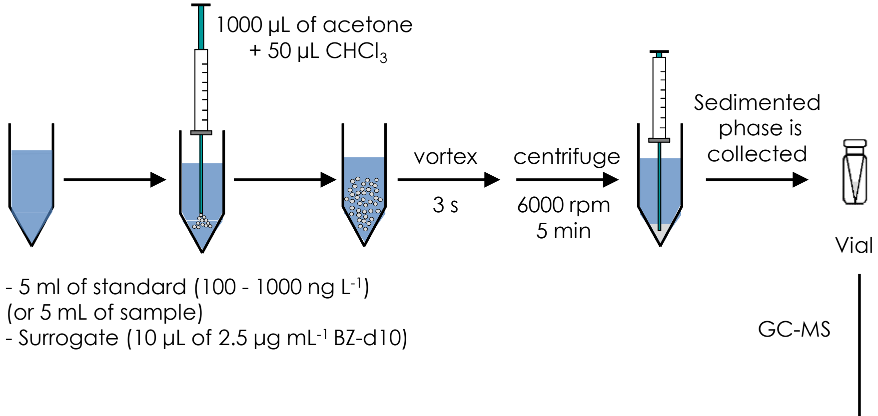
- Suitable for GC-MS.
- Extractable by DLLME in $CHCl_3$.
- No possible presence in environmental samples.
- No ionisable functional groups. Thus, pH does not affect its extraction.
- Affected by the ionic strength in the same way as analytes. Results when performing the method with different % of NaCl:



When A_i/A_{sur} was used as analytical signal, the content of salt (ionic strength) did not affect the extraction.

Since no salt is added in standard preparation this is very useful in the case of high salt content samples.

PROPOSED METHOD

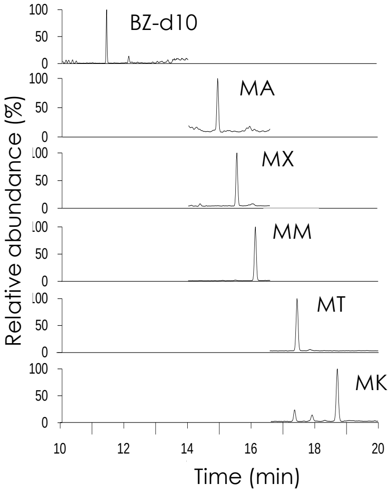


GC-MS Conditions :	
Injection volume	1 µL (Splitless time 1 min)
Inlet temperature	280 °C
Transfer line temperature	280 °C
Ion source temperature	250 °C
Helium flow rate	1 mL/min
Column	HP-5MS Ultra Inert (30m, 0.25mm, 0.25µm)
Oven temperature	60 °C (1 min) 20 °C/min to 120 °C 10 °C/min to 185 °C 1°C/min to 195 °C 25 °C/min to 280 °C (5min)

Acquisition intervals

Compound	Quantification ions[1]	Time (min)
$BZ-d_{10}$ (Surrogate)	110	10.0 - 14.0
MA	253	14.0 – 16.6
MX	263	
MM	282	
MT	251	16.6 – 20.0
MK	279	

[1] A full scan mode (m/z 40 – 300) was simultaneously recorded from minute ten for identification aims.



ANALYTICAL FIGURES OF MERIT

Enrichment factor

$$EF = \frac{C_f}{C_0} = \frac{A_f}{A_0} = \frac{V_0}{V_f} R$$

C = Concentration

A = Area

V = Volume

R = Extraction yield

f = Final

0 = Initial

Analyte	EF
MA	289
MX	263
MM	248
MT	230
MK	314

Working range

100 - 1000 ng L^{-1}

Linearity to, at least, 20 ng mL^{-1}

Limits of detection and quantification of the method

LOD: 3 times the signal-to-noise ratio

LOQ: 10 times the signal-to-noise ratio

Analyte	LOD (ng L^{-1})	LOQ (ng L^{-1})
MA	33	109
MX	19	63
MM	6	19
MT	4	14
MK	7	24

Repeatability

Evaluated applying the proposed method to five replicates of a standard aqueous solution that contained 500 ng L^{-1} of the target compounds

Analyte	RSD (%)
MA	3
MX	3
MM	4
MT	3
MK	1

Analysis of real samples

Determined in different water samples: sea, river, irrigation channel and waters treatment plant.

Results showed that all of the target compounds were below the limits of detection.

The full scan mode also showed that no amino-musks derivatives, which can be formed in the processes of the wastewater treatment plants, were detected in the analyzed samples.

Recovery studies

The proposed method was applied to the free analyte samples previously spiked with the target analytes at 500 ng L^{-1}

Analyte	Recovery (%)				
	Sample 1[a]	Sample 2[b]	Sample 3[c]	Sample 4[d]	Sample 5[e]
MA	93 ± 4	95 ± 5	106 ± 4	98 ± 13	95 ± 14
MX	88 ± 6	105 ±8	103 ± 6	107 ± 9	96 ± 10
MM	87 ± 5	95 ± 6	103 ± 6	109 ±11	94 ± 11
MT	87 ± 6	93 ± 7	102 ± 6	105 ± 9	93 ± 10
MK	88 ± 6	92 ± 8	99 ± 8	107 ± 3	116 ± 5

[a] Sample 1: Sea water (Valencia)
[b] Sample 2: River water (Montanejos)
[c] Sample 3: Irrigation water (La Eliana)
[d] Sample 4: Wastewater treatment plant influent (Gandia)
[e] Sample 5: Wastewater treatment plant effluent (Gandia)

Recoveries ranging between 87 and 116 %.

These results demonstrate that these water matrices have no significant effect on the extraction process.

CONCLUSIONS

■ A simple, fast and high sensitive analytical method is presented in this work in order to determine the complete family of nitro musk compounds in environmental water samples.

■ This method is based on DLLME as extraction technique prior to analysis by GC-MS and presents good analytical features, especially high enrichment factors that allow their determination in the ng L^{-1} range.

■ Previous analytical methods were based on more time-consuming extraction techniques which make the proposed method a good alternative for treating a lot of samples, as environmental surveillance demands.

■ Moreover, the presented method is a good alternative to other methods as it consumes very low levels of solvents.

Chapter 7.

Determination of nitro musks in surface water samples using solid phase extraction with molecular imprinted sorbents followed by gas chromatography-mass spectrometry

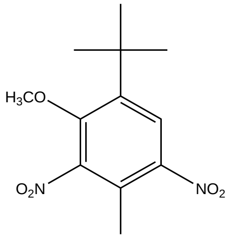
Based on:

M. Lopez-Nogueroles, S. Lordel-Madeleine, A. Chisvert, A. Salvador, V. Pichon,
Development of a selective solid phase extraction method for nitro musk compounds in environmental waters using a molecularly imprinted sorbent,
Talanta 110 (2013) 128-134.

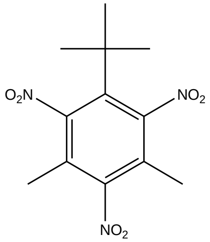
AIM OF THE WORK

Aim	Development of a selective and sensitive analytical method to determine nitro musks in wastewater and surface water samples. Finding a molecularly imprinted material to be used as SPE sorbent for the simultaneous selective extraction of the nitro musk family in wastewater and surface water samples.
Proposed method	Solid phase extraction (SPE) using a molecularly imprinted silica sorbent (MIS) followed by gas chromatography–mass spectrometry (GC-MS).
Interest	Considered persistent pollutants. Related to health risks. They could reach the aquatic environment via swimming activities or wastewater treatment plants. Molecularly imprinted sorbents have never been used before for the determination of nitro musk compounds.

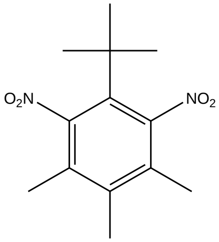
TARGET COMPOUNDS



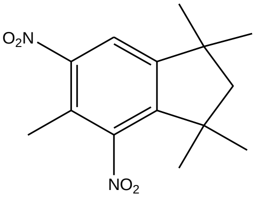
Musk Ambrette (MA)



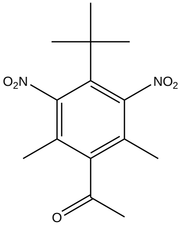
Musk Xylene (MX)



Musk Tibetene (MT)



Musk Moskene (MM)



Musk Ketone (MK)

SYNTHESIS OF THE MIS

Preliminary aspects

The MIS was synthesized on a sol-gel approach according to a procedure previously described by some of the authors of this work for the extraction of nitroaromatic explosives.

Reagents

Role	Compound*	Mass or volume used
Template	2,4-DNT	0.25 mmol
Monomer	PTMS	1 mmol
Cross-linker	TEOS	7.5 mmol
Solvent	5.3 % ammonia aqueous solution	3.4 mL

* 2,4-DNT: 2,4-dinitrotoluene
PTMS: Phenyltrimethoxysilane
TEOS: Tetraethoxysilane
Template/Monomer/cross-linker ratio 1/4/30

Experimental procedure for MIS synthesis

The reagents were mixed in a tube and placed in a thermostated oil bath at 40 °C.

The solutions were stirred, to mixture the reagents, during 24 h and the resulting sorbents were dried in an oven at 120 °C during 18 h.

After polycondensation, monoliths were obtained and these sorbents were ground and sieved to obtain particles in the 25 – 36 μm size range.

A sedimentation with MeOH/water mixture (80/20, v/v) was performed to remove fine particles.

50 mg of each MIS were then packed into a 1 mL cartridge between two frits.

The template was extracted from the sorbent by the percolation of methanol (70 mL), acetonitrile (60 mL) and dichloromethane (20 mL).

Experimental procedure for NIS synthesis

Non imprinted silica (NIS) sorbent were obtained by performing the overall procedure in the absence of template.

SELECTION OF EXTRACTION CONDITIONS

Aim of the study

Provide a selective sample pretreatment (a high difference between the extraction efficiencies of MIS and NIS).

Preliminary aspects

The optimization is generally performed by studying the extraction profiles that must be different when using the MIS and the NIS.

Since aqueous samples cannot be injected on the GC-column, only the elution fractions were quantified. Moreover, it was not possible to evaporate the aqueous fractions without losing the nitro musks.

5 % of MeOH was added in the percolated water sample so that the highly hydrophobic nitro musks did not adsorb to any of the surfaces on which they came in contact.

Used leyend

C: Conditioning step

P: Percolation step

W: Washing step

E: Elution step

Assays:

1.) Volume of the percolation step

C: 5 mL of water

P: 200 mL of a water/MeOH (95/5, v/v) standard solution 500 ng L^{-1}

E: 1 mL of ACN

Extraction efficiencies (%)	
MIS	NIS
75 – 100 %	55 – 70 %

Higher retention on the MIS than on the NIS.

This demonstrates the presence of cavities on the MIS allowing a slight selectivity.

Lower volumes did not change extraction efficiencies. 200 mL was chosen to increase the enrichment factor. Higher volumes than 200 mL were not tested as it was too time consuming.

2.) Composition with highest selectivity

C: 5 mL of water

P: 200 mL of a water/MeOH (40/60, v/v) standard solution 500 ng L^{-1}

E: 1 mL of ACN

Various water/MeOH mixtures were percolated.

The best results (most difference between MIS and NIS) were obtained when the percolation was carried out with water/MeOH (40/60, v/v).

Extraction efficiencies (%)	
MIS	NIS
80 – 92 %	28 – 63 %

This difference confirms again the presence of cavities in the MIS that strongly retain the nitro musks.

This composition was used as that one of the washing step.

3.) Volume of the washing step

V = 1 mL

C: 5 mL of water

P: 200 mL of a water/MeOH (95/5, v/v) standard solution 500 ng L^{-1}

W: 1 mL of a 40/60 water/MeOH solution

E: 1 mL of ACN

The effect of the volume of the washing mixture (water/MeOH, 40/60, v/v) on the efficiencies were studied.

Extraction efficiencies on the MIS using a washing fraction of up to 1 mL are the same as those obtained without applying a washing solution while they do gradually reduce for the NIS.

Extraction efficiencies (%)	
MIS	NIS
76 – 98 %	34 – 54 %

These results, once again, confirm the presence of cavities in the MIS that enable a selective retention of the nitro musks.

V = 1.3 mL

C: 5 mL of water

P: 200 mL of a water/MeOH (95/5, v/v) standard solution 500 ng L^{-1}

W: 1.3 mL of a 40/60 water/MeOH solution

E: 1 mL of ACN

In order to reduce the non-specific interactions, higher volumes of washing solution were considered.

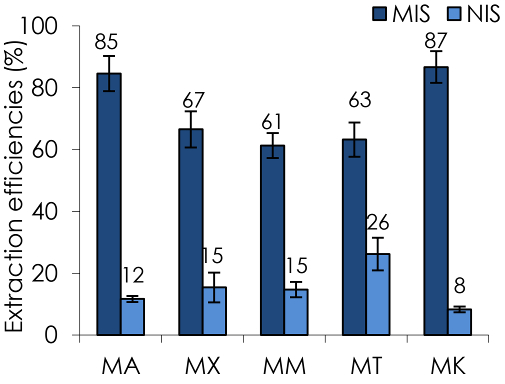
The best results were obtained for volumes of 1.3 mL of water/MeOH (40/60, v/v):

Extraction efficiencies (%)	
MIS	NIS
61 – 87 %	8 – 26 %

(A more detailed graph of extraction efficiencies is shown below)

This process allowed the highest selectivity, when comparing MIS and NIS, with satisfying extraction efficiencies using the MIS.

It is important to note that the cavities accept the nitro musks but only when applying specific conditions of extraction.



Extraction efficiencies otained on MIS and NIS after the percolation of 200 mL of a water/MeOH (95/5, v/v) standard solution containing 500 ng L^{-1} of the five nitro musks and a washing step of 1.3 mL of a MeOH/water mixture (60/40, v/v) (n=3).

CALIBRATION MODE

Selected calibration mode

Internal standard calibration is recommended.

An internal standard is added just before the chromatography step in order to correct possible variability of the injection volume.

Analytical signal

$y = A_i/A_{is}$

A_i = the peak area of the target analyte
A_{is} = the peak area of the internal standard

Selected surrogate

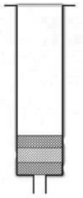
Hexachlorobenzene (HClB)

- Suitable for GC-MS.
- High volatility.
- High sensitivity.

PROPOSED METHOD

A calibration curve of standards is constructed using different multicomponent standard solutions in ACN ranging from 10 – 50 ng mL^{-1}.

Regarding samples, once the MIS is synthesized, 50 mg are packed between two frits into a 1 mL cartridge and the template is extracted. Then, the optimized procedure is:

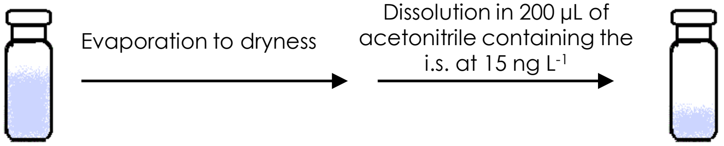


C: 5 mL of water

P: 200 mL of a water/MeOH (95/5, v/v) standard solution or 200 mL of the water sample containing 5% of methanol

W: 1.3 mL of a 40/60 water/MeOH solution

E: 1 mL of ACN



Evaporation to dryness

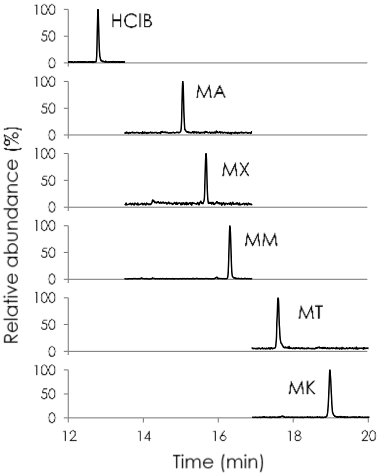
Dissolution in 200 µL of acetonitrile containing the i.s. at 15 ng L^{-1}

GC-MS Conditions	
Injection volume	1 µL (Splitless time 1 min)
Inlet temperature	280 °C
Transfer line temperature	280 °C
Ion source temperature	250 °C
Helium flow rate	1 mL/min
Column	DB-5MS Ultra Inert (30m, 0.25mm, 0.25µm)
Oven temperature	60 °C (1 min) 20 °C/min to 120 °C 10 °C/min to 185 °C 1°C/min to 195 °C 25 °C/min to 280 °C (5min)

Acquisition intervals

Compound	Quantification ions[1]	Time (min)
HCIB (Internal standard)	284	11.0 - 13.5
MA	253	13.5 – 16.9
MX	263	
MM	282	
MT	251	16.9 – 21.0
MK	279	

[1] A full scan mode (m/z 40 – 350) was simultaneously recorded from minute five for identification aims.



ANALYTICAL FIGURES OF MERIT

Enrichment factor

$$EF = \frac{C_f}{C_0} = \frac{A_f}{A_0} = \frac{V_0}{V_f} R$$

C = Concentration

A = Area

V = Volume

R = Extraction yield

f = Final

0 = Initial

Analyte	EF
MA	808
MX	637
MM	580
MT	599
MK	827

Calculated taking into account:
the dilution of 5% with MeOH
the SPE extraction efficiencies
the evaporation step

Tests showed that there was no loss of compounds during the ACN evaporation process.

Working range

10 - 50 µg L^{-1}

Note that this working range does not take into account the EFs as calibration standards are directly injected and the proposed method is not performed.

Instrumental limits of detection and quantification

Calculated with a calibration curve of the standards from 8 – 20 ng mL^{-1} in ACN. Estimated as:

LOD: 3 $S_{y/x}$/b

LOQ: 10 $S_{y/x}$/b

$S_{y/x}$: Residual standard deviation of the calibration curve

b: Slope of the curve

Analyte	IDLs (µg L^{-1})	IQLs (µg L^{-1})
MA	2	7
MX	2	6
MM	1	4
MT	1	4
MK	1	4

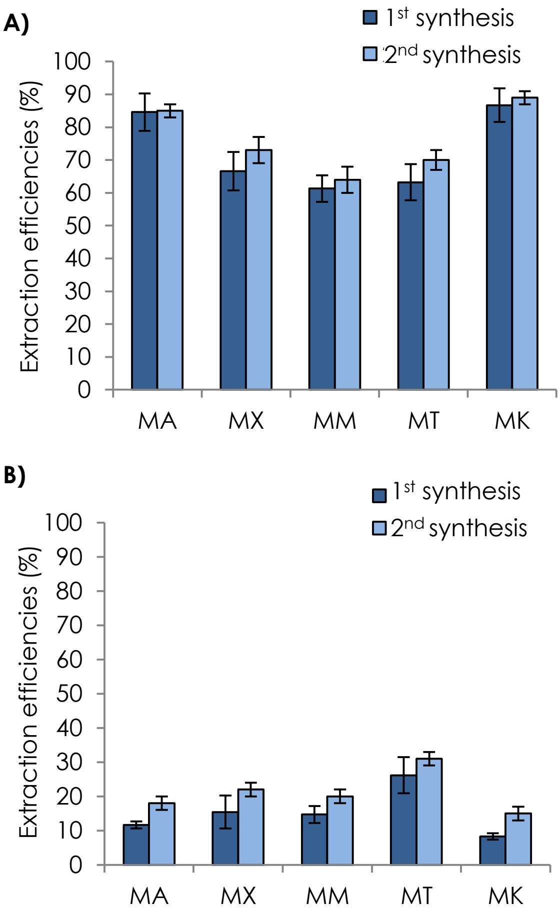
Limits of detection and quantification of the method

Estimated taking into account the IDL or IQL and the EFs of each nitro musk.

Analyte	LOD (ng L^{-1})	LOQ (ng L^{-1})
MA	3	8
MX	3	8
MM	2	7
MT	2	6
MK	2	5

Repeatability

A second synthesis was carried out at a different time, by another manipulator and evaluated applying the previously optimized procedure to compare the average extraction efficiencies obtained after three extractions carried out on each MIS (A) and each NIS (B)



The high selectivity of each synthesized MIS is shown again.

Efficiency deviation values lower than 6 % show the repeatability of the extraction procedure on a given MIS and NIS.

Efficiencies obtained from different syntheses are very close, specially for MISs. This confirms that the imprinted sorbents present similar performances.

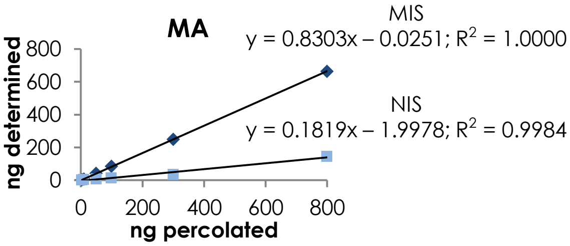
Additional syntheses will be required to confirm the real reproducibility of the MIS synthesis.

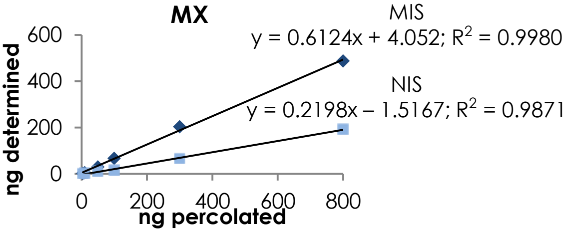
Capacity of the sorbent

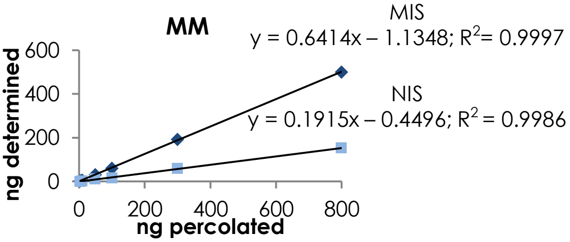
The proposed method was applied to a 95/5 water/MeOH (v/v) standard mixture containing increasing amounts of the five nitro musk compounds.

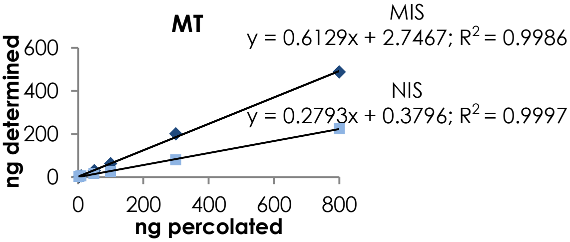
A curve was plotted with the percolated amount and the amount quantified after the application of the method.

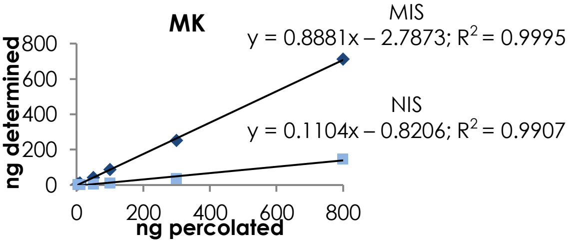
The objective was to verify that the extraction efficiencies kept constant in a wide range of percolated analyte mass. Thus, the analytes could be quantified in all this range.











Analysis of real samples

Determined in different water samples: sea, river, and water from a treatment plant.

Results showed that all of the target compounds were below the limits of detection.

Recovery studies

The proposed method was applied to the free analyte samples previously spiked with the target analytes at 100 ng L^{-1}

Analyte	Recovery (%)		
	Sample 1[a]	Sample 2[b]	Sample 3[c]
MA	91 ± 4	90 ± 6	81 ± 4
MX	101 ± 7	94 ± 1	87 ± 4
MM	104 ± 2	93 ± 5	85 ± 1
MT	102 ± 2	90 ± 4	95 ± 8
MK	106 ± 2	100 ± 3	100 ± 3

[a] Sample 1: River water (Seine River, Paris, France)
[b] Sample 2: Sea water (Villajoyosa, Spain)
[c] Sample 3: Wastewater treatment plant effluent (Valencia)

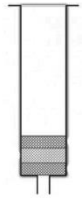
Recoveries ranging between 81 and 106 %.

These results demonstrate that these water matrices have no significant effect on the extraction process.

Comparison with a conventional sorbent

To prove the potential of the imprinted sorbent to selectively extract the analytes, a similar method with a conventional sorbent was developed to compare the results in real samples

An extraction procedure was developed using an Oasis HLB sorbent:



C: 5 mL of water

P: 200 mL of a water/MeOH (95/5, v/v) standard solution 500 ng L^{-1}

W: 1 mL of a 80/20 water/MeOH solution

E: 1 mL of ACN

The extraction efficiencies obtained were:

Extraction efficiencies (%)				
MA	MX	MM	MT	MK
93 ± 4	75 ± 6	72 ± 5	69 ± 2	105 ± 9

Similar data to those obtained with the MIS

The proposed method was applied to the samples previously spiked with the target analytes at 100 ng L^{-1}. The recoveries obtained are:

Analyte	Recoveries (%)		
	Sample 1[a]	Sample 2[b]	Sample 3[c]
MA	82 ± 3	42 ± 6	50 ± 8
MX	74 ± 3	42 ± 3	34 ± 1
MM	77 ± 3	41 ± 2	38 ± 3
MT	91 ± 4	46 ± 1	44 ± 4
MK	111 ± 5	72 ± 3	87 ± 7

[a] Sample 1: River water (Seine River, Paris, France)
[b] Sample 2: Sea water (Villajoyosa, Spain)
[c] Sample 3: Wastewater treatment plant effluent (Valencia)

Recoveries, using the Oasis HLB sorbent, are very low, especially in the case of sea water and wastewater.

When using a conventional sorbent there are matrix effects resulting in a large decrease of the extraction recoveries. However, with the imprinted sorbent no significant matrix effects are observed.

These shows that molecularly imprinted sorbents, with a retention mechanism based on molecular recognition, enable a better clean-up of the sample than conventional sorbents, in which the co-extraction of interfering compounds can interfere in the subsequent determination.

CONCLUSIONS

■ A high sensitive analytical method, with great clean-up potential, is presented in this work in order to determine the complete family of nitro musk compounds in environmental water samples.

■ The sensitivity achieved by the proposed method improves that obtained in our previous work on the same analytes and type of samples.

■ To the best of our knowledge this is the first time an imprinted sorbent is used to selectively extract the nitro musk compounds.

■ The selective extraction of the target compounds from real samples by the MIS was easily succeeded.

■ A high degree of selectivity was obtained allowing a good quantification of the nitro musks after the MIS extraction compared to a conventional sorbent.

Final conclusions

In this PhD thesis analytical methods for the detection and determination of fragrances and fragrance-related substances have been developed.

As far as possible, the principles of Green Chemistry have been followed. In this sense, the use of highly toxic organic solvents has been avoided or minimized.

The contributions of the developed analytical methods are:

A) Regarding quality control in the cosmetic industry sector:

- A method for the determination of those phthalates banned in the EU Cosmetic Regulation *(dibutyl phthalate, bis(2-ethylhexyl) phthalate, bis(2-methoxyethyl) phthalate, n-pentyl-isopentyl-phthalate, di-n-pentyl-phthalate, diisopentylphthalate* and *benzyl butyl phthalate)* in perfumes has been developed. These compounds may be present in the finished product due to migration from packaging or to direct violation of the law (for example if used as denaturants of the perfume alcohols).

  The sample preparation is simple. It consists in the evaporation of the sample in a stream of nitrogen (using an internal standard to improve the accuracy), followed by dilution with ethanol.

  GC-MS was used as analytical technique. Low LOQ are achieved, what enables trace analysis (between 10 and 113 ng ml^{-1} for four of the analytes and between 250 and 980 ng ml^{-1} for the other two).

  There was no study on the simultaneous determination of these analytes in perfumes or any cosmetic product prior to the realization of this work.

- A method for the determination of *atranol* and *chloratranol* in perfumes has been developed. These substances are known for their allergenic potential and could be present in perfumes when natural plant extracts are used in its composition. Both compounds are in the process of inclusion as prohibited substances of the EU Cosmetic Regulation.

  The sample preparation is performed using LLE followed by DLLME and simultaneous derivatization of the compounds.

  GC-MS is used as analytical technique. Low LOQ, that allow trace analysis, are achieved (about 9 and 6 ng ml^{-1} for *atranol* and *chloroatranol* respectively).

  Very few records on the determination of these analytes in perfumes or other cosmetic products could be found before the publication of this work, and the results obtained in this works were not quantitative.

- A method to detect changes during the maceration process of perfumes has been developed. This method is used to set the minimum necessary time for this important stage in the preparation of perfumes.

  Sample preparation is very simple, requiring only dilution with ethanol (using internal standard to improve precision).

  GC-FID was used as analytical technique and the main peaks were identified by GC-MS.

  LDA is used to classify the samples by comparison with a series of samples of the same perfume that are known to be completely macerated or non-macerated.

  There was no records of methods for estimating the maceration time of perfumes before the publication of this work.

B) Regarding the determination of fragrance-related compounds in environmental samples:

- Two methods for the determination of the complete family of the nitro musks (musk ambrette, musk xylene, musk tibetene, musk ketone and musk moskene) in environmental water and wastewater have been developed. These substances have been classified as persistent pollutants with a strong tendency to bioaccumulate due to its lipophilic character.

  Both methods include a treatment step of the sample, before the measure, to concentrate and clean-up the analytes.

  One of the methods use DLLME as treatment step.

  The other method uses SPE, taking advantage of the selectivity provided by molecularly imprinted sorbents.

  GC-MS was used as analytical technique.

  Both methods achieve low LOQ that enable trace analysis. In the case of the first method, they are about 14-109 ng L^{-1} and of the second of about 5-8 ng L^{-1}.

  Note that although the second method achieves lower LOQ it is also a more laborious and time consuming method. Therefore, the choice of either method depends on the analytical needs.

Conclusiones finales

En la Tesis Doctoral presentada se han desarrollado métodos analíticos para la detección y determinación de fragancias y sustancias relacionadas con los perfumes.

En la medida de lo posible se ha intentado evitar o minimizar el uso de disolventes orgánicos de elevada toxicidad, siguiendo los principios de la Química Verde.

Las aportaciones de los métodos analíticos desarrollados son las siguientes:

A) En relación al control de calidad en las industrias del sector cosmético:

- Se ha desarrollado un método para la determinación en perfumes de una serie de ftalatos (*dibutyl phthalate, bis(2-ethylhexyl) phthalate, bis(2-methoxyethyl) phthalate, n-pentyl-isopentyl-phthalate, di-n-pentyl-phthalate, diisopentylphthalate and benzyl butyl phthalate*) que están prohibidos en el Reglamento Europeo de productos cosméticos, y que sin embargo podrían estar presentes en el producto acabado generalmente debido a su migración desde los envases, pero también en casos de incumplimiento de la legislación, si se utilizan como desnaturalizantes de los alcoholes utilizados en fabricación de los perfumes.

  La preparación de la muestra es muy simple, solo se requiere la evaporación de la muestra en corriente de nitrógeno (empleando patrón interno para mejorar la precisión) seguida de la dilución con etanol.

  Se ha utilizado la CG-MS como técnica analítica. Se consiguen LOQ bajos lo que permite el análisis de trazas (entre 10 y 113 ng ml^{-1} para cuatro de los analitos y entre 250 y 980 ng mL^{-1} para los otros dos).

  No existía ningún estudio sobre la determinación simultánea de estos analitos en perfumes ni en otros productos cosméticos antes de la realización de este trabajo.

- Se ha desarrollado un método para la determinación en perfumes de *atranol* y *chloratranol*, sustancias de conocido potencial alérgeno y que podrían estar presentes en los perfumes cuando se utilizan extractos vegetales naturales en su composición. Estas sustancias están en vía de inclusión en el listado de sustancias prohibidas del Reglamento Europeo.

  La preparación de la muestra se lleva a cabo empleando LLE seguida de DLLME y derivatización simultánea de los compuestos.

Se ha utilizado la CG-MS como técnica analítica. Se consiguen LOQ bajos lo que permite el análisis de trazas (del orden de 9 y 6 ng ml^{-1} para el *atranol* y *chloroatranol* respectivamente).

Existían muy pocos antecedentes sobre la determinación de estos analitos en perfumes o en otros productos cosméticos antes de la publicación correspondiente a este trabajo, y los resultados obtenidos en estos trabajos no era cuantitativa.

- Se ha desarrollado un método para detectar los cambios en el proceso de maceración de perfumes, que permitan establecer el tiempo mínimo necesario para esta importante etapa de la elaboración de los perfumes.

La preparación de la muestra es muy simple, solo se requiere la dilución con etanol (empleando patrón interno para mejorar la precisión).

Se ha utilizado la CG-FID como técnica analítica y se han identificado los picos más importantes mediante CG-MS.

Mediante LDA se consigue clasificar las muestras cuyo control de calidad se quiere realizar, mediante su comparación con una serie de muestras del mismo perfume completamente maceradas y otras no maceradas.

No existía ningún antecedente de métodos para la estimación del tiempo de maceración de los perfumes antes de la publicación correspondiente a este trabajo.

B) En relación a la determinación de sustancias relacionadas con las fragancias en muestras medioambientales:

- Se han desarrollado dos métodos para la determinación de la familia completa de los nitro almizcles (*musk ambrette, musk xylene, musk tibetene, musk moskene* y *musk ketone*) en aguas medioambientales y en aguas provenientes de plantas de tratamiento. Estas sustancias han sido catalogadas como contaminantes persistentes con una fuerte tendencia a la bioacumulación, debido a su carácter lipófilo.

Ambos métodos tienen una etapa de tratamiento de la muestra para conseguir la concentración y limpieza de los analitos antes de la etapa de medida.

En uno de los métodos se emplea la DLLME como etapa de tratamiento.

En el otro método se emplea SPE, aprovechando la selectividad que aportan los sorbentes molecularmente impresos.

Se ha utilizado la CG-MS como técnica analítica.

En ambos métodos se consiguen LOQ bajos lo que permite el análisis de trazas. En el caso del primer método son del orden de 14-109 ng L^{-1} y en el segundo del orden de 5-8 ng L^{-1}.

Cabe señalar que aunque el segundo método permite alcanzar LOQ más bajos también es un método más laborioso y lento. Por tanto, la elección de uno u otro método, dependerá de las necesidades analíticas.

Annex

(Publications)

Essential oils: Analytical Methods to Control the Quality of Perfumes

Published in:

A. Chisvert, M. Lopez-Nogueroles, A. Salvador,
Essential Oils: Analytical Methods to Control the Quality of Perfumes,
in: M.J. Ramawat (Ed.), **Handbook of Natural Products**,
Springer-Verlag Berlin Heidelberg, 2013, pp. 3287-3310.

Essential Oils: Analytical Methods to Control the Quality of Perfumes

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Alberto Chisvert, Marina López-Nogueroles, and Amparo Salvador

Contents

Abstract

Cosmetic products, including perfumes, are regulated by a single law concerning important aspects, such as composition or labeling, in order to protect public health. A revision on the regulatory aspects for fragrance chemicals in cosmetics and household products is exposed here.

Quality control of both, commercial perfumes and raw materials, is important for perfume manufacturers to assure that the finished perfume is the one that was formulated. On the other hand, analytical methods are necessary to assure, for safety purposes, that there are no undesired or banned compounds present in the finished product. Methods for perfume analysis are compiled and revised with special emphasis on the potentially allergenic fragrance-related substances and some other groups of substances such as musks and phthalates, being some of them restricted or forbidden.

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K.G. Ramawat, J.M. Mérillon (eds.), *Natural Products*,
DOI 10.1007/978-3-642-22144-6_142,

Keywords

Analytical chemistry • essential oil • fragrances • musks • perfume • phthalates • potentially allergenic substances • quality control

Abbreviations

AHTN	6-acetyl-1,1,2,4,4,7-hexamethyltetralin
AITI	5-acetyl-3-isopropyl-1,1,2,6-tetramethylindane
ALEX	Automated liner exchange
ANN	Artificial neuronal networks
BBP	Benzyl butyl phthalate
CE	Capillary electrophoresis
DBP	Dibutyl phthalate
DEHP	Bis(2-ethylhexyl) phthalate
DEP	Diethyl phthalate
DIBP	Diisobutyl phthalate
DIPP	Diisopentyl phthalate
DMEP	Bis(2-methoxyethyl) phthalate
DMP	Dimethyl phthalate
DNOP	Di-n-octyl-phthalate
DNPP	Di-n-pentyl-phthalate
DPP	Dipropyl phthalate
ECD	Electron capture detector
EESI	Electrospray ionization mass spectrometry
EIC	Extracted ion chromatogram
FDA	Food and Drug Administration
FID	Flame ionization detector
FS	Full scan
GC	Gas chromatography
HHCB	1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta(g)-2-benzopyran
HS	Head space
IFRA	International Fragrance Association
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
MA	Musk ambrette
MD	Multidimensional
MEEKC	Microemulsion electrokinetic chromatography
MK	Musk ketone
MM	Musk moskene
MS	Mass spectrometry
MSPD	Matrix solid-phase dispersion
MT	Musk tibetene
MX	Musk xylene

NPIPP	n-pentyl-isopentylphthalate
PAS	Potentially allergenic substance
PCA	Principal component analysis
PLE	Pressurized liquid extraction
PTV	Programmed temperature vaporizing
RIFM	Research Institute for Fragrance Materials
SBSE	Stir bar sorptive extraction
SIM	Selected ion monitoring
SPD	Solid-phase dispersion
SPE	Solid-phase extraction
TIC	Total ion chromatogram
TLC	Thin-layer chromatography
TOF	Time-of-flight
UV	Ultraviolet

1 Introduction

"Scent is one of the most powerful of senses." This phrase can be read in the website of one of the more remarkable associations related to perfumes, the International Fragrance Association (IFRA), and we are all aware that this is true. Scent can alter mood, stress, or pain. We are not always aware of them, but odors are around us all the time and affect our daily life. People, food, plants, and everything have a particular odor, and we even add aromatic substances to many other things. Cosmetic products, such as shampoos, deodorants, soaps, and fine fragrances, or household products such as laundry detergents, cleaners, and bleaches contain perfumes (Fig. 107.1) which are responsible for their characteristic odors. Moreover, we sometimes scent the air with air fresheners or fragrance candles.

It is worth mentioning that the use of perfumes is not new, as they have been used for thousands of years since the Egyptian civilization, when they were used in religious rites. After the roman civilization, they became an oriental art, and it was not until the thirteenth century that all this knowledge arrived to Europe due to Arabic influences. Louis XV, in the eighteenth century, used it so much that his court was called *"le cour parfumee."* Nowadays, France is still the heart of the European perfume industry. Since then, the perfume market has become very important, with sales of billions of dollars.

Perfumes are composed of hundreds of aromatic chemicals, namely, fragrance chemicals, or just simply fragrances. Among these compounds, it is very common to find compounds with the characteristic five-carbon isoprene unit, giving them the names of terpenes, such as terpene aldehydes, monoterpene alcohols, sesquiterpene alcohols, terpene ketones, terpene esters, monoterpene hydrocarbons, or sesquiterpene hydrocarbons. In addition, other compounds, such as aldehydes, alcohols, ketones, esters, phenols, or lactones, are also very common.

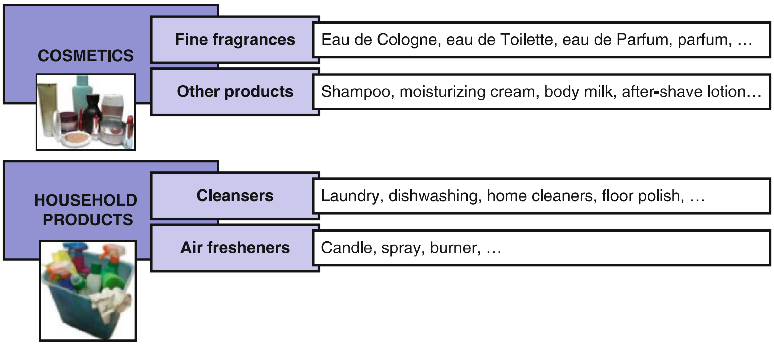


Fig. 107.1 Containing perfume products

Perfumes can be classified according to their nature, in *natural* or *synthetic*. *Natural perfumes* are obtained from plants (e.g., lavender, geranium) or from some of its parts, like flowers (e.g., jasmine, rose, gardenia), fruits (e.g., lemon, orange, vanilla), roots (e.g., vetiver, cistus, angelica), leaves (e.g., violet, patchouli, peppermint), wood (e.g., vetiver, sandalwood, cedarwood), bark (e.g., cinnamon, nutmeg), resin (e.g., benjui, tolu, galbanum), and seeds (e.g., angelica, celery, anis). Also, they may be obtained from animal glands and organs, for example, musk, which is obtained from the testicles of the musk deer; civet, which is a secretion from glands of the civet cat; ambergris, which is obtained from a secretion from the intestine of the sperm whale; and finally, castoreum, which is obtained from glands near the reproductive organs of the beaver. These natural perfumes, or essential oils, are extracted by several methods, which depend on the raw material and the chemical fragrances to be extracted [1].

Thus, *steam distillation* consists in passing water vapor through the raw material to perform the extraction. Once the steam condenses, the oil is easily separated from the water as it floats on the top. In *hydrodistillation*, the raw material is boiled in water, and the volatile essential oil is also obtained from the water vapor by condensation. In the case of *solvent extraction*, raw materials are submerged in organic solvents, such as hexane, ethanol, or petroleum ether. After extraction, the solvent is eliminated by distillation resulting in the extract known as "concrete." The concrete can be further purified by a second extraction with ethanol. In this case, the resulting extract is known as "absolute." When the extraction solvent is animal fat, the process is known as *enfleurage*. This is a very time-consuming and expensive way of extraction. *Maceration* is similar to enfleurage, but in this case, the raw material is immersed in warmed oil. In *rectification*, the raw materials are directly heated without a solvent. This method is used when a toasted scent is wanted. Another way of extraction is named *expression* and consists in manually or mechanically pressing the plants. This is very suitable for thermolabile compounds.

Finally, a more modern way of extraction is *supercritical fluid extraction*. In this process, carbon dioxide in supercritical conditions is used to extract the hydrophobic aromatic compounds from the raw material. It does not alter the nature of the aromatic compounds as it takes place at a low temperature and CO_2 vaporizes after depressurization. However, it is an expensive process and needs special instrumentation [2].

In the case of *synthetic perfumes*, they are obtained by mixing synthetic fragrance chemicals, which are synthesized in the laboratory, usually searching for a similar odor of a natural fragrance and sometimes searching for something new and original. In practice, this type of fragrances has many advantages, specially its lower cost compared to natural perfumes. The quantity and quality of natural source supplies are in many ways unpredictable, due to their dependence on crop quality or weather. Thus, synthetic perfumes do not have this inconvenience, but unfortunately, they have some others. For example, a natural essential oil is made up by hundreds or thousands of different compounds, which makes difficult to reproduce the desired perfume exactly by just mixing different synthetic fragrances. Moreover, all the compounds contribute with their characteristic odors, and then, not only the main components but also those minor components, including those at trace levels, can affect considerably the odor of the perfume. In addition, in those cases where two isomeric forms of a compound smell different, chiral synthesis is needed.

On the other hand, perfumes itself, and also the pure fragrance chemicals that compose the perfumes, are classified according to the note they provide, that is, according to the fragrance type. So, one can find different types, like floral, which reminds one of scents similar to jasmine, rose, heliotrope, etc.; citrus, which are aromas reminiscent of lemon, orange, lime, grapefruit, etc.; fruity, based on non-citrus fruity odors like peach, apple, banana, etc.; green, which creates the sensation of smelling recently cut grass and leaves; woody, which reminds one of dry wood and trees; oriental refers to those sweet strong fragrances reminiscent of vanilla, ambergris, etc.; spice, giving off a redolence coming from clove, cinnamon, thyme, pepper, etc.; animal refers to scents provided by musk, civet, and castoreum; and leather, which tries to reproduce the characteristic smell of leather, tobacco, smoke, etc.

It should be also emphasized that the perfume content depends of the product. Fine fragrances, which are hydroethanolic solutions of perfumes, are usually classified according to their perfume content in eau de cologne (1–5 %), eau de toilet (5–8 %), eau de parfum (8–15 %), and parfum (>15 %). Other cosmetic-products present lower perfume content. Moisturizing creams and body milks (0.1–0.5 %), aftershave (0.5–2 %), toothpastes (0.5–1 %), and hair care products (0.01–0.1 %) are just some examples. Regarding cleansing household products, nearly all, as, for example, laundry or dishwashing products and bathroom or furniture cleaners, contain perfume, in variable amounts, to make them smell clean and mask unpleasant odors from other components of the product.

Thus, perfumers rarely use pure fragrance chemicals, but they usually mix different raw materials, natural or synthetic, to create a perfume that fulfills the market expectations, that is, type of product to which it is to be added, product image, target consumer, originality, fashion, etc. For fine fragrances, high content of pungent and elegant perfumes are used, whereas more sweet and refreshing ones are found in eau de toilets and eau de colognes. For skin and hair care cosmetic products, sweet, tenuous, and refreshing perfumes are combined, whereas high refreshing notes are employed in toothpastes or mouthwashes. In the case of household products, detergents and softeners contain refreshing notes, while different home cleaners and air fresheners can be found with refreshing or on the contrary with pungent and elegant or sweet notes.

2 Regulatory Aspects for Fragrance Chemicals in Cosmetics and Household Products

The establishment of the single market in the European Union meant the free movement of goods in the member states. Cosmetic products were not an exception, but the great exposure of the population to these products enhanced the need of a single law concerning important aspects, such as cosmetic composition or labeling, in order to protect public health. As a result of many discussions between experts, Council Directive 76/768/EEC was adopted on the 27th of July of 1976. Since then, many amendments and adaptations have been made. Last recast of all these changes as one single text is Regulation 1223/2009 [3]. Among other aspects, this regulation controls and regulates the substances banned in cosmetics, as well as the maximum allowable amount of certain restricted substances according to their intended use. Moreover, there is also the so-called European Inventory of Cosmetic Ingredients [4] which lists the substances usually employed in cosmetic products. This inventory is divided into two sections where Sect. 2 deals with those ingredients used in perfume and aromatic raw materials.

Regarding the USA, cosmetics are regulated by the US Food and Drug Administration (FDA), but regulation is much more permissive [5].

A cosmetic product is defined by the European regulation as “any substance or mixture intended to be placed in contact with the 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, protecting them, keeping them in good condition or correcting body odors” and by the FDA as “an article intended to be applied to the human body for cleansing, beautifying, promoting attractiveness, or altering the appearance without affecting the body’s structure or functions.” Therefore, fragrances and perfumes are closely related to cosmetic products as, besides being a cosmetic product itself, they are many times an ingredient added to make the product smell nice or mask any undesirable odor of the product.

Both regulations specify that all ingredients of cosmetic products should be indicated on the label. In the case of perfumes, they are either elaborated by mixing different natural raw materials which could contain hundreds or thousands of different compounds or based on complexes synthetic formulations. This is why perfume components do not necessarily have to be specified, but they can be grouped and labeled under the word "perfume" or "aroma" in the EU or "fragrance" in the USA.

Nevertheless, in 2003, the 2003/15/EC Directive [6] listed 26 fragrances classified as potentially allergenic substances (PASs). Since then, any cosmetic product containing any of these 26 substances have to declare its presence in the label when present at a higher concentration than 10 $\mu g\ mL^{-1}$ in those products to remain on the skin or 100 $\mu g\ mL^{-1}$ in those intended to be rinsed off. This information should improve the diagnosis of contact allergies among consumers and should enable them to avoid the use of cosmetic products which they do not tolerate.

On the other hand, the European regulation also prohibits and restricts the amount used of more than 50 fragrance substances, like some extracts or synthetic musks, in cosmetic products. Other substances related to fragrances, such as phthalate esters, commonly known as phthalates, which have been used as solvents and vehicles for fragrance ingredients, are also restricted as a consequence of their undesirable side effects.

Regarding the US FDA's list of prohibited ingredients, it bans only a few compounds, but none of them are used as fragrance ingredients. Nevertheless, the USA and other countries, such as Canada, will probably adopt the recommendations given in the EU regulation in the future [7].

The EU also regulates household products, and detergents are regulated by Regulation 648/2004 [8] where similar labeling to cosmetic products is required in the case of perfume content. A perfume or an essential oil shall be considered to be a single ingredient and will be labeled as "parfum" listing none of the substances that it contains. Additionally, as in the case of cosmetic products, there is an exception for those allergenic fragrance substances that appear on Annex III of the cosmetic regulation. If their concentrations exceed 0.01 % by weight, the allergenic fragrances must be labeled separately.

Despite the regulations in law in each country, the fragrance industry is in some way self-regulated by some independent entities. The Research Institute for Fragrance Materials (RIFM) evaluates and distributes scientific data on the safety assessment of fragrance substances found in cosmetics and other products. On the other hand, the aforementioned IFRA establishes usage guidelines for fragrance ingredients based on RIFM evaluation results. Recommendations to avoid many ingredients are given, although this organization has no legal authority, and therefore, the final decision is mostly conditioned on legislation [7].

3 Analytical Aspects of Fragrance-Related Compounds

Quality control of commercial perfumes and raw materials is very important for perfume manufacturers. This is the way to assure that the amount of perfume is the one that was initially formulated or that there are no undesired or banned compounds. In addition, characterizing an essential oil is also very interesting.

Different tests, depending on the instrumentation available, are performed in the industry. There are many easy and cheap quality control techniques that provide overall information about their quality, as measurements of physical (density, color, optical rotation, etc.) and chemical properties (acidity or carbonyl indexes). Spectrometric techniques, such as ultraviolet/visible or infrared spectrometry can also provide overall qualitative, or even quantitative, useful information for the quality control of a perfume, by comparing the obtained spectra with those previously recorded and kept in the databases. However, all these techniques do not provide information about each component separately, but overall information. For these purposes, more selective techniques are needed. As most of the perfume ingredients are volatile or semi-volatile compounds, gas chromatography (GC) is, by far, the technique of choice [9]. The identification of the individual compounds is then made by using the Kovats indexes and comparing them with those reported in the databases for the same chromatographic columns, although it can sometimes be a difficult task taking into account the high number of compounds that can be present in the perfume composition. In this sense, the mass spectrometry (MS) detector can be a useful tool since the identity of a compound can be elucidated from the MS spectra obtained.

The so-called electronic noses are also very useful. This device mimics human olfactory system and consists of a sample system in head space, a sensor array as detection system, and a computer for data treatment. It is not a separative mechanism; it just gives signal patterns for a particular odor. In a way, it measures smells. It is worth mentioning that an individual sensor is unspecific and could give exactly the same signal with two different odors. Thus, an array of sensors has to be used to reduce the probability of this happening [10]. In order to use an electronic nose, a database of reference has to be firstly built. To do so, a set of known samples would have to be analyzed and the data statistically treated, for example, with pattern recognition methods, such as principal component analysis (PCA), artificial neural network (ANN), or clusters. New samples could then be recognized by comparing them with the database. This technology is commonly used in the food and beverage industry [11]. It has also been used for medical or environmental applications and, of course, in the cosmetic and fragrance industry. In the last years, some works that use electronic nose in fragrance analysis have been published. For example, Carrasco et al. [12] discriminated between different families of Yves Saint Laurent perfumes by an electronic nose. Branca et al. [13] developed an electronic nose device combined with PCA and ANN to detect the presence of a perfume note called mangone in fragrances. Recently, Cano et al. [14] developed a PCA method to detect between original and counterfeit perfumes. Ye et al. [15] also used an electronic nose combined with PCA to discriminate between natural musk and adulterated. Although this is not an electronic nose, Chingin et al. [16] used extractive electrospray ionization mass spectrometry (EESI-MS) for the same purpose in a very simple and sample-preparation-free method which could be applied for quality control.

3.1 Determination of the Potentially Allergenic Fragrance-Related Substances

As mentioned before, 26 substances were declared as potentially allergenic substances (PASs) by the European regulation [6]. Of these 26 substances, listed in Table 107.1, 24 are chemically defined volatile compounds, whereas the other two are natural moss extracts, thus not defined chemicals, but a natural mixture of many of them.

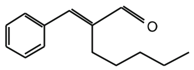
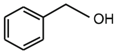
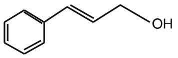
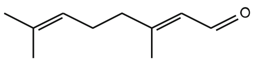
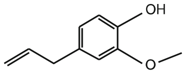
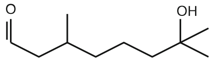
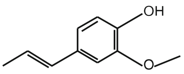
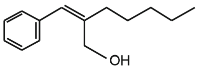
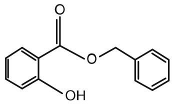
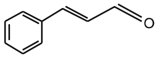
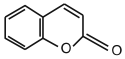
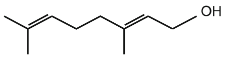
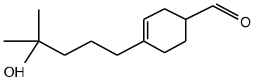
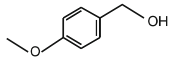
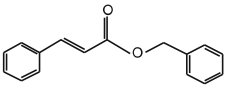
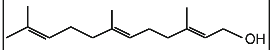
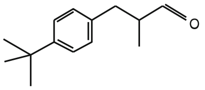
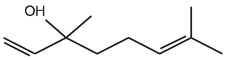
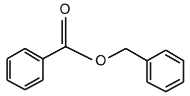
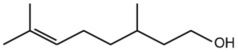
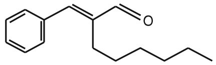
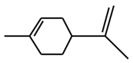
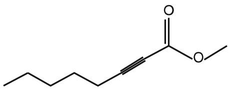
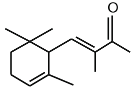
Since this declaration took place in 2003, the determination of these skin-sensitizing ingredients has become a challenge. Literature deals separately with the determination of the 24 chemically defined PASs and the two natural moss extracts.

The group of the 24 chemically defined allergens belongs to very different classes of compounds. These diverse groups include alcohols (such as farnesol), carbonyl compounds (such as citral), esters (such as benzyl benzoate), lactones (such as coumarin), cyclic hydrocarbons (such as limonene), or phenols (such as eugenol). Their volatile characteristics make gas chromatography (GC) the better technique of analysis. Although universal detectors like flame ionization detector (FID) have been used, a more selective detector, like a mass spectrometer (MS), is also a necessity, as one of the biggest difficulties to face in their determination is the coelution between the target compounds and other of the many components of the perfume. The literature deals with these using different solutions, operating either in selected ion monitoring (SIM) or extracted ion chromatogram (EIC) mode, but it is difficult to completely eliminate false positives and negatives, due to coelution. Other solutions are the use of comprehensive GC or clean up steps before the determination to extract the target compounds [17].

Being aware of the problematic skin-sensitizing chemicals in perfumes, Rastogi proposed for the first time, even before the approval of the regulation, a solid-phase extraction (SPE) followed by GC method for the determination of 11 of these allergens [18]. GC-MS was used for previous identification, while GC-FID was chosen for quantification. Some years later, the author published another work [19] using the same GC-MS method, with some modifications, to survey the content of the 24 PASs in cleaning products and other consumer products such as cosmetics or toys. In this method, the sample treatment depended on the type of sample, but in summary, samples were shaken with methanol, and the lixiviated fraction was purified with a silica gel column before its injection into the GC port. Detection was performed in full scan (FS) and quantification in EIC mode. In the same way, Ellendt et al. [20] did also use GC-MS, FS mode for identification and SIM mode for quantification, for the determination of the 24 PASs in deodorants. However, coelution problems were encountered in complex fragrance mixtures.

Later, Chaintreau et al. [21] made a significant progress presenting a method to determine PASs in perfume concentrates, with direct injection of the samples. This work delves in the coelution problem between the target compounds and other components of the perfume. Four different columns were used to try to solve this problem. SIM mode of three different ions per compound (i.e., one ion

Table 107.1 List of the 26 potentially allergenic substances according to the European regulation

 Amyl cinnamal [CAS 122-40-7]	 Benzyl alcohol [CAS 100-51-6]	 Cinnamyl alcohol [CAS 104-54-1]
 Citral [CAS 5392-40-5]	 Eugenol [CAS 97-53-0]	 Hydroxycitronellal [CAS 107-75-5]
 Isoeugenol [CAS 97-54-1]	 Amylcinnamyl alcohol [CAS 101-85-9]	 Benzyl salicylate [CAS 118-58-1]
 Cinnamal [CAS 104-55-2]	 Coumarin [CAS 91-64-5]	 Geraniol [CAS 106-24-1]
 Hydroxyisohexyl 3-cyclohexene carboxaldehyde [CAS 31906-04-4]	 Anise alcohol [CAS 105-13-5]	 Benzyl cinnamate [CAS 103-41-3]
 Farnesol [4602-84-0]	 Butylphenyl methylpropional [80-54-6]	 Linalool [78-70-6]
 Benzyl benzoote [CAS 120-51-4]	 Citronellol [CAS 106-22-9]	 Hexyl cinnamal [CAS 101-86-0]
 Limonene [CAS 5989-27-5]	 Methyl 2-octynoate [CAS 111-12-6]	 Alpha-isomethyl ionona [CAS 127-51-5]
Oakmoss extract (*Evernia prunastri* extract) [CAS 90028-68-5]	Treemoss extract (Evernia furfuracea extract) [CAS 90028-67-4]	

for quantification and the other two as qualifiers) was also used and their relative abundances taken into account for correct identification, thus minimizing false positives and false negatives, by comparing the relative abundances with respect to those of the reference compound. Even though all this helped, false positives, due to coelution, still occurred. Moreover, false negatives can occur as a consequence of retention time shifts, that is, that a target compound shifts out of the acquisition SIM window. The work also claims ethanol is not a good candidate as solvent of the standard solutions since protic solvents react with some of the allergens. Thus, isooctane and o-fluorotoluene were chosen. This is an important fact which not all later works take into account. Lastly, in this paper, Chaintreau and coworkers highlighted the need for cleanup techniques as the nonvolatile parts of the cosmetic products remain in the injector disturbing future injections. It should be emphasized that this work was accepted as the official IFRA method for the quantification of potential fragrance allergens in fragrance compounds [22], with two recommendations: (1) in case of suspected coelutions, one of the qualifier ions should be used as quantization ion; (2) in case of retention time shifts, a qualitative FS run should be done before each sample in order to adjust the time windows. These recommendations were applied in a paper published some years later [23], which concluded that coelution problems could still occur and recommended to inject each sample in two different polarity columns as well as to monitor three ions per compounds to assure about the positive identification/quantification. A ring test was conducted using this strategy, and the results were published years later [24], in which the investigation of an automated data treatment procedure to aid the analyst during the interpretation of the analytical results was also proposed.

A similar strategy was followed by Leijs et al. [25] for the analysis of these 24 compounds in fragrance raw materials and perfume oils. These authors used a chromatograph with two injectors and connected each one to a different polarity column. Both columns were connected to a MS interface using a dual-hole ferrule, thus carrying out a sequential dual-column analysis for each sample. However, they preferred operating in FS mode and quantifying in EIC mode, to avoid additional acquisition in case of coelutions and to prevent false negatives as a result of retention time shift. Obviously, this mode of acquisition significantly increases the limit of quantification (LOQ).

Another work, from Mondello et al. [26], determined the PASs using fast GC-MS, by employing shorter and narrower columns besides high-speed MS detector. The MS operated in FS mode, and quantification was performed in EIC mode. The main novelty of the work basically consisted in a very short run time (less than 5 min). However, the same above-mentioned problems regarding coelutions are expected to occur.

Taking into account the Chaintreau and coworkers' recommendation dealing with cleanup steps to separate the target compound from the matrix and thus avoid dirtiness of the system, several works can be found in the literature describing cleanup steps before the determination of the 24 restricted allergens. For example, as mentioned before, Rastogi [19] used a silica gel column for purifying the extracts. Niederer et al. [27] proposed a cleanup step using size-exclusion chromatography prior to GC-MS for the determination of the 24 PAS in different cosmetic products, such as creams, body lotions, and oils. David et al. [28, 29] classified samples into four different groups based on matrix characteristics. Simple samples, with no presence of nonvolatile material, like fragrances, were diluted and injected.

This is not possible with dirtier matrixes, where a programmed temperature vaporizing (PTV) inlet with an automated liner exchange (ALEX) was proposed. Thus, fractioning of the target compounds from this nonvolatile matrix took place in the liner. When handling aqueous samples with the target compounds at very low concentrations, a stir bar sorptive extraction (SBSE) step was added before the PTV approach. Regarding cleanup steps, Chen et al. [30] used solid-phase microextraction (SPME) followed by GC-FID for the quantitative analysis of geraniol, a restricted allergen, and other flavor and perfume compounds in shampoo. Lamas et al. [31] proposed the use of solid-phase dispersion-pressurized liquid extraction (SPD-PLE) followed by GC-MS in EIC mode for the determination of the 24 regulated fragrance allergens and two other fragrance compounds in cosmetic products (creams and lotions). A year later, the same authors presented a method based on matrix solid-phase dispersion (MSPD) as concentration and cleanup step followed by GC-MS in SIM mode for PASs determination in cosmetic products [32] and a GC-MS method with no pretreatment of samples other than dilution for their determination among other fragrance-related components in perfumes [33].

Development in the field of comprehensive GC (GCxGC) also seems a good way to handle the coelution problem. In this sense, Chaintreau and coworkers [34] presented a GCxGC-FID method for the determination of the 24 regulated substances plus two other compounds. The method could not completely eliminate component overlap, leading to similar results than using 1D-GC coupled to MS. Later, the same authors [35] presented the first work dealing with the quantification of the PASs based on comprehensive GC coupled to a quadrupole MS (GCxGC-MS). As obviously expected, selectivity is improved compared to GC-MS and GCxGC-FID. Comprehensive GC can overcome coelution problems but is very time-consuming during the interpretation of the analytical results. The same authors published another paper [36] presenting a software prototype to reprocess the data which shortens this step. Leco [37] presented a method for allergens in perfumes using GCxGC-(TOF)MS (time-of-flight mass spectrometer). Identification was better achieved than using a simple quadrupole, but the equipment is too expensive to be used in routine analysis. Dunn et al. [38] compared two approaches for this analysis, GCxGC and multidimensional gas chromatography (MDGC), in which selected portions of the primary separation, rather than the whole sample, are transferred to a secondary column. MDGC seems to be a better option as data processing for the output is almost identical to a single column analysis, while the data of a GCxGC experiment is still relatively labor intense. Finally, Cordero et al. [39] published a work where determination by GCxGC-qMS and GCxGC-FID is accomplished.

Only two papers dealing with the determination of these compounds with a different technique than gas chromatography have been found. Villa et al. [40] propose the first work where liquid chromatography and UV detection (LC-UV) are used for the determination of the 24 suspected allergens in different cosmetic products. A C18 column and a gradient acetonitrile/water were used. Samples are basically weighted, diluted, sonicated, and filtered before injection. This can be a useful method for routine analysis in cosmetic industries where more expensive equipment is not available. However, coelution problems are present, not only between compounds but also for matrix components. In this sense, Furlanetto et al. [41] published a method for 18 of the 24 allergens by microemulsion electrokinetic chromatography (MEEKC) and UV detection. The method was applied to two rinse-off cosmetics, a shampoo, and a bath gel.

On the other hand, the number of publications regarding the determination of the two natural moss extracts restricted as potentially allergenic – treemoss and oakmoss – is rather scarce. The industrial processing varies considerably, but basically, harvested lichens, from oak trees in the case of oakmoss and pine and cedar trees in the case of treemoss, are extracted, usually with hexane or more polar solvents. Then, they are further diluted in ethanol and usually submitted to physical treatments intended to remove their original color. Taking into account their natural conditions and that industrial processing is not standardized, it is not surprising to find variability in the chemical composition of these extracts. Joulain et al. published two comprehensive reviews on the composition of these extracts. One hundred seventy constituents are identified in oakmoss extracts and 90 in the case of treemoss [42, 43].

The nature of these extracts makes them unsuitable to GC although Bernard et al. [44] searched for allergenic or sensitizing molecules in oakmoss using GC-MS after chemical fractionation of the extract by gel permeation chromatography (GPC). Some other works can be found in the literature dealing with the determination of some of the oakmoss components that show allergenic properties, like atranorin and chloroatranorin, a lichen depside, or atranol and chloroatranol, formed by transesterification and decarboxylation of atranorin and chloroatranorin during industrial processing. Hiserodt et al. [45] used LC-MS/MS to identify atranorin and some related potential allergens in oakmoss absolute. No quantification was performed in this work. Bossi et al. [46] used this method to quantify atranol and chloroatranol in perfumes. Rastogi et al. [47] also quantified the presence of these two allergenic compounds in different commercial perfumes based on this method. It is worth mentioning that these individually allergenic components of oakmoss are not listed by the European regulation, which only restricts total concentration of oakmoss and treemoss extracts.

3.2 Determination of Musks

Musk compounds have been widely used as fragrance chemicals in many consumer products such as cosmetics, detergents, food additives, or household products [48, 49]. They are valuable compounds not only for their unique odor but also for their fixative properties.

Natural musk, itself, was already used in ancient times and was obtained as a secretion produced by a gland of the musk deer. Traditionally, musk deer are killed to remove the musk gland, although musk can be extracted through the external orifice of the musk gland without the need of killing the animal. These facts and its difficult availability make this natural product very expensive [50].

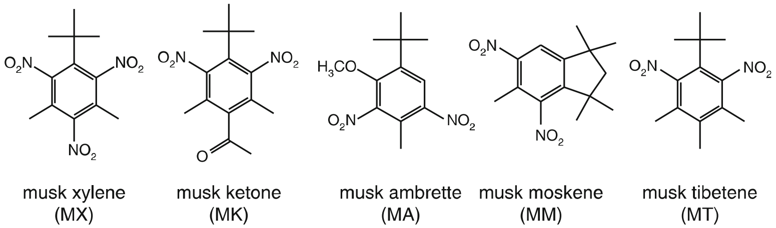


Fig. 107.2 Chemical structure of nitro musk compounds

On the other hand, the term musk also refers to other compounds, with totally different chemical structure but possessing musk-like odor properties. These are commonly named synthetic musks and appeared as a response to economical and ethical motives. Artificial musks have been generally divided in three subgroups: nitro musks, polycyclic musks, and macrocyclic musks.

The *nitro musks*, which are characterized by a nitro-aromatic moiety, were accidentally discovered by Baur in 1888 [51]. This group is basically formed by five compounds, that is, musk ambrette (MA), musk xylene (MX), musk moskene (MM), musk tibetene (MT), and musk ketone (MK). Figure 107.2 shows the chemical structure of these compounds.

Despite their pleasant aroma, nitro musks are believed to be persistent pollutants due to their strong tendency to bioaccumulate [52]. Many papers have been written about their health risks, showing they are related with different types of dermatitis, carcinogenic effects, and endocrine disruption [49, 53–58]. In fact, the use in cosmetic products of MA, MT, and MM is banned in the European Union, while the use of MX and MT is restricted. Nevertheless, its use is permitted in North America.

Regarding the *polycyclic musks*, these are composed of several cycles and do not have nitro substituents. Figure 107.3 shows the structure of some of the most commonly used polycyclic musks, such as 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta(g)-2-benzopyran (HHCB) commonly known as galaxolide®, 6-acetyl-1,1,2,4,4,7-hexamethyltetralin (AHTN) commonly known as tonalide®, 5-acetyl-3-isopropyl-1,1,2,6-tetramethylindane (AITI) commonly known as traseolide®, and 1,1,2,3,3-pentamethyl-2,5,6,7-tetrahydroinden-4-one commonly known as cashmeran.

These musks were developed in 1950 and have slowly replaced the nitro musk compounds in Europe, since they are believed to have less harmful and toxic effects. However, in the later years, many studies show their bioaccumulation in the aquatic system and living beings [52, 59–61]. Nonpolycyclic musk is banned in the European Union, and only some of them are restricted. The rest can be freely used in cosmetic products. Thus, polycyclic musks are more used in Europe, while nitro musks are the common ones in North America [62].

Finally, *macrocyclic musks*, which are much larger compounds than the other two groups, have been developed in the recent years. Even though they have synthetic nature, they are synthesized trying to imitate the natural musk

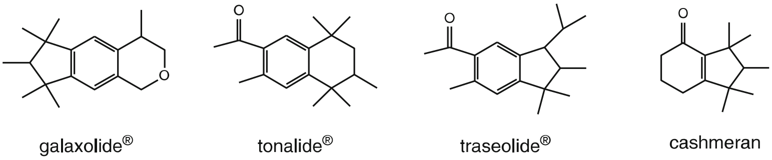


Fig. 107.3 Chemical structure of polycyclic musk compounds

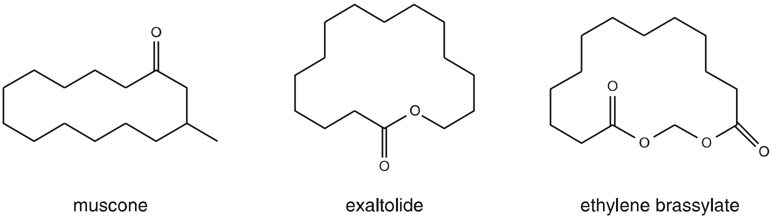


Fig. 107.4 Chemical structure of macrocyclic musk compounds

compounds. As these compounds are not restricted by law and seem not to have biodegradability problems, their use has increased considerably and is replacing the polycyclic musk compounds [50]. Some of the most important macrocyclic musks are shown in Fig. 107.4.

Many articles about the determination of artificial musks in different matrixes can be found in the literature. The most abundant are environmental samples such as air, water, and sediments [52, 59], but other matrixes such as biological fluids (blood [60, 63] or human milk [51]) or aquatic species [64, 65] are also available. However, the literature dealing with the determination of musks in cosmetic samples is rather scarce. The preferred technique is GC, with MS or electron capture detector (ECD) in the case of nitro musks.

Sommer [66] presented a method for quantification of nitro musks in cosmetics and detergents using GC with both, ECD and MS. Wineski et al. [67] determined MA in fragrance products with an internal standard addition technique by GC-ECD. The same author published another paper [68] some years later with a similar method to determine MA, MX, and MK. Struppe et al. [69] proposed a headspace solid-phase microextraction (HS-SPME) and GC with atomic-emission detection method for the determination of MX, MK, and MA in cosmetic samples. Four different commercially available fiber coatings were tested, and a 100-μm polydimethylsiloxane gave the best results. To the best of our knowledge, Eymann et al. [70] published the first paper where the determination of polycyclic musks in cosmetics was achieved, together with nitro musks. GC was used, with ECD in the case of nitro musks and MS for polycyclic musks. Some years later, Sommer and Juhl [71] proposed a GC with FID and MS detection to determine macrocyclic musks in alcohol-containing cosmetics.

More recently, several papers have been published on the determination of musks in cosmetic products. Roosens et al. [72] determined AHTN, HHCB, MX, and MK in 82 personal care products, 19 of them perfumes, with GC-MS and combined these results with the average usage to estimate exposure profiles through dermal application concluding that exposure to the polycyclic musks is much higher than to the nitro musk compounds. Ma et al. [73] proposed a SPE-isotope dilution-GC-MS/MS for the determination of MX in cosmetics. Martinez-Girón [74] achieved the enantiomeric separation of four chiral polycyclic musks using capillary electrophoresis (CE). The method was applied to determine these enantiomers in perfumes. Finally, as already mentioned before, Sanchez-Prado et al. [33] also developed a method that allows the simultaneous determination of many compounds, including nitro musks and polycyclic musks, in perfumes.

3.3 Determination of Phthalates

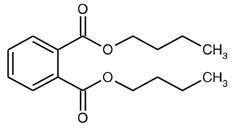
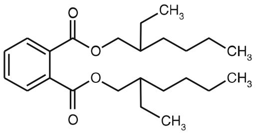
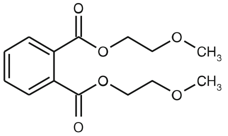
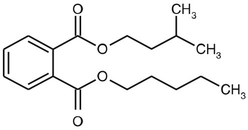
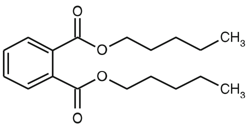
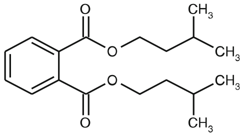
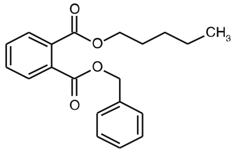
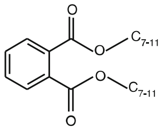
The term phthalate is used to designate the dialkyl or alkyl aryl esters of phthalic acid. These compounds are used in the cosmetic industry mainly as denaturants of the alcohols used in their manufacture, as fixatives or solvents for some fragrances (e.g., synthetic musks) and as film formers. Thus, it is usual to find them in nail polish, hairsprays, and perfumes [75]. Moreover, these substances are mainly used in the manufacture of plastics to increase their flexibility. Therefore, they can be found in different manufactured products, not only as part of its formulation but as a result of migration during manufacture or storage [1].

Phthalates have been proven to be harmful to living organisms [76–78]. These lipophilic compounds are rapidly metabolized by humans and biotransformed in their respective monoesters and other oxidative products, which are excreted in urine and feces [79]. Studies show that some phthalates and their metabolites are potentially toxic in animals due to endocrine-disruptive effects, which can cause adverse health effects, particularly for fertility and reproduction [80–82]. Moreover, some of them cause cancer in rats and mice [83]. Therefore, the presence of some phthalates is forbidden by the European legislation in some vulnerable products, such as toys [84] or personal care products [3]. Specifically, current European regulation of cosmetic products forbids the presence in cosmetic products of the phthalates shown in Table 107.2.

Other phthalates like diethyl phthalate (DEP), dimethyl phthalate (DMP), or di-n-octyl-phthalate (DNOP) are allowed in cosmetic products in Europe without any restriction, even though they are defined as pollutants by the US Environmental Protection Agency [85] and are also related to adverse health effects [86, 87].

Concerning the phthalate determination, the number of publications dealing with these compounds shows their great interest and their widespread presence in many consumer products and the environment. Specifically, in the case of the determination of phthalates in cosmetic products, several analytical methods for the identification and determination at the level of μg mL^{-1} and ng mL^{-1} have been published. The most commonly used techniques are liquid chromatography (LC)

Table 107.2 List of the forbidden phthalates in cosmetics by the European regulation

 dibutyl phthalate (DBP) [CAS 84-74-2]	 bis(2-ethylhexyl) phthalate (DEHP) [CAS 117-81-7]
 bis(2-methoyethyl) phthalate (DMEP) [CAS 117-82-8]	 1,2-benzenedicarboxylic acid, dipentylester, branched and linear (NPIPP) [CAS 84777-06-0]
 di-n-pentyl phthalate (DNPP) [CAS 131-18-0]	 diisopentylphthalate (DIPP) [CAS 605-50-5]
 benzyl butyl phthalate (BBP) [CAS 85-68-7]	 1,2-benzenedicarboxylic acid, di-$C_{7\text{-}11}$, branched and linear alkyl esters (DHNUP) [CAS 68515-42-4]

combined with UV-visible and gas chromatography (GC) with flame ionization detector (FID) or coupled to mass spectrometry (MS). Most of the publications deal with the determination of dibutyl phthalate (DBP), bis(2-ethylhexyl) phthalate (DEHP), benzyl butyl phthalate (BBP), and di-n-pentyl-phthalate (DNPP) within other non-prohibited phthalates. Less common are studies that include bis (2-methoxyethyl) phthalate (DMEP), the most polar prohibited phthalate. Finally, no analytical methods for the determination of all three isomers, DNPP, diisopentyl phthalate (DIPP), and n-pentyl-isopentylphthalate (NPIPP), or the mix of C_{7-11} branched and linear alkyl esters have been found.

The determination of phthalates in cosmetic samples started many years ago, as shown in a work dated in 1973 where different phthalates were determined by GC-FID [88]. Some years later, Markovic et al. [89] published a work where the phthalate DMP and N,N-diethyl-m-toluamide, both used as insect repellent compounds, were determined in cosmetic samples by thin-layer chromatography (TLC) and UV scanning densitometry. Koo and Lee [90] estimated the median daily human exposure to phthalates by determining the presence of the phthalates DEHP, DEP, DBP, and BBP by HPLC in a hundred different cosmetic samples, like perfumes, deodorants, or nail polishes. Samples were weighted, dissolved in methanol, vortexed, and centrifuged before injection. A run time of 50 min in isocratic elution was needed. As expected, DEP, the non-banned phthalate, was found at high concentrations. Nevertheless, the other phthalates were also present in rather high concentrations in many samples.

Chen et al. [91] developed the first method for the determination of six phthalate esters, DMP, DEP, DBP, BBP, DEHP, and DNOP, in cosmetics by GC-FID. MS was also used but just to qualitatively confirm the presence of the target compounds. Sample pretreatment, similar to Koo and Lee's work, consisted in weighting the samples, dissolving them in methanol, sonicating, and centrifuging. Once water from the upper clean layer was eliminated with anhydrous sodium sulfate it was directly injected. Compared to the previous works, this method showed better sensitivity, with lower limits of detection (LOD). De Orsi et al. [92] continued with LC coupled to UV detection and presented a method where DMP, DEP, dipropyl phthalate (DPP), diisobutyl phthalate (DIBP), BBP, DBP, and DEHP were determined in nail cosmetics. This time, a gradient was used, and run time was reduced to 30 min. Shen et al. [93] studied the determination of seven phthalates and four parabens in different kinds of cosmetic samples, using both LC-UV and GC-MS in SIM mode. A cleanup step using C18 SPE was carried out, which improved the LOD. However, the method is time-consuming and uses high volumes of methanol, so it could not be considered a green method suitable for routine analysis. The IFRA has a phthalate quantification procedure, available in its website [94]. The determination is performed in a GC-MS in SIM mode, with both a quantifier and a qualifier ion per compound.

Regarding the sample pretreatment step, traditional extraction techniques that involve the use of high amounts of toxic solvents should be avoided. In this sense, Chingin et al. [95] presented a novel procedure based on extractive electrospray ionization mass spectrometry (EESI-MS) for the detection of DEP in perfumes with no need of sample pretreatment. Su et al. [96] determined DMP, DBP, dicyclohexyl phthalate (DCHP), and DNOP in perfumes and lacquer removers based on polymer monolith microextraction (PMME) and LC-UV. The conditions for extraction were optimized leading to a sensitive method.

Recently, Koniecki et al. [97] determined the phthalate levels in 252 cosmetic products from the Canadian market by GC-MS to estimate the dermal exposure. Sanchez-Prado et al. [33] also developed a method that allows the simultaneous determination of many compounds, including phthalates, in perfumes. The method consists in diluting the sample in ethyl acetate and injecting it directly into a GC-MS system.

All studies with real perfume samples agree that the presence of prohibited phthalates like DBP, BBP, and DEHP, even in several hundred parts per million, is very common. In this sense, the US Consumers Association [98] conducted a study with eight different brands of perfumes where basically all tested perfumes contained at least DEP and the prohibited phthalate DEHP. A Greenpeace study [75] of different commercial perfumes by GC-MS also found prohibited phthalates in the mg L^{-1} range. Cosmetic industry should try to avoid this issue using free phthalate plastics and solvents.

4 Conclusions

This work resumes the most important regulatory aspects for fragrance chemicals. Different legislations concerning cosmetic products or household products can be found all over the world. This should encourage analytical chemists to develop new methods that enable the determination of all the substances mentioned in these regulations. This would surely help manufacturers and authorities to perform quality control and to assure that no restricted substances are present in the product. Different methods for perfume analysis have been revised here, especially those on restricted or forbidden perfume-related substances, such as potentially allergenic fragrance-related substances, musks, and phthalates. All these works are a very good start, but much more work can be done in perfume analysis.

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Published in:

M. Lopez-Nogueroles, J.L. Benede, A. Chisvert, A. Salvador,
A rapid and sensitive gas chromatography-mass spectrometry method for the quality control of perfumes: simultaneous determination of phthalates,
Analytical Methods 5 (2013) 409-415.

Analytical
Methods

RSCPublishing

PAPER

Cite this: *Anal. Methods*, 2013, **5**, 409

A rapid and sensitive gas chromatography-mass spectrometry method for the quality control of perfumes: simultaneous determination of phthalates†

M. López-Nogueroles, J. L. Benedé, A. Chisvert and A. Salvador*

A rapid and sensitive analytical gas chromatography-mass spectrometry (GC-MS) method for perfume analysis to determine the phthalates banned by the European Union Regulation on cosmetic samples is presented. This method has been tested in commercial alcoholic perfume samples for the determination of the following seven phthalates: dibutyl phthalate, bis(2-ethylhexyl) phthalate, bis(2-methoxyethyl) phthalate, *n*-pentyl-isopentylphthalate, di-*n*-pentyl phthalate, diisopentylphthalate and benzyl butyl phthalate. Sample evaporation and redissolution in ethanol is carried out before GC-MS analysis, with no dilution of the sample. External calibration and standard addition calibration are compared to detect possible matrix effects. External calibration could provide good results in some cases but standard addition calibration is recommended as matrix effects are observed in many cases in the determination of the target phthalates. The accuracy of the method has been proven by the analysis of quality control samples prepared from commercial free phthalate perfumes after spiking with known concentrations of the analytes. Accurate results were obtained with limits of detection in the ng mL^{-1} range and good repeatability (relative standard deviations lower than 6%). The method was satisfactorily applied to the analysis of commercial perfume samples, and the results revealed considerable amounts of dibutyl phthalate and bis(2-ethylhexyl) phthalate in some of the analyzed samples.

Received 11th August 2012
Accepted 5th October 2012

DOI: 10.1039/c2ay26130h

www.rsc.org/methods

1 Introduction

The term phthalate is used to designate the dialkyl or alkyl aryl esters of phthalic acid. These compounds are mainly used in the manufacture of plastics to increase their flexibility. Consequently, they can be present in different manufactured products as part of its formulation or as a result of migration during manufacture or storage.

It has been demonstrated that some phthalates can be harmful to living organisms.[1–3] They are metabolized by humans and biotransformed into their respective monoesters and other oxidative products, which are excreted in urine and faeces.[4] Studies in animals show that some phthalates and their metabolites can be toxic as they cause endocrine-disruptive effects, with adverse health effects, mostly for fertility and reproduction.[5–7] In addition, some of them cause cancer in rats and mice.[8] As a result of all these concerns, the presence of some phthalates is forbidden by the European Union (EU) legislation in some products, such as toys[9] or cosmetic products.[10]

Specifically, current legislation on cosmetic products[10] forbids the presence of dibutyl phthalate (DBP), bis(2-ethylhexyl) phthalate (DEHP), bis(2-methoxyethyl) phthalate (DMEP), *n*-pentyl-isopentylphthalate (NPIPP), di-*n*-pentyl phthalate (DNPP), diisopentylphthalate (DIPP), benzyl butyl phthalate (BBP), and 1,2-benzenedicarboxylic acid, di-C_{7-11}, branched and linear alkyl esters (DHNUP). Table 1 shows some features of these compounds.

Within cosmetic products, phthalates are mainly found in nail polish, hairsprays and perfumes.[11] Their presence in perfumes is mainly due to their use as denaturants of the alcohols used in their manufacture,[12] fixatives or solvents for some fragrances (*e.g.* synthetic musks) or due to their migration from the plastic parts of the container or the spray system.[13]

The number of published articles dealing with the determination of phthalates shows the interest of these compounds and their widespread presence in the environment. Phthalates have been determined in different matrices such as food,[14,15] beverages,[16,17] environmental samples,[18] urine[5] or cosmetic products.[11,19–30]

Specifically in the case of the determination of the phthalates in perfumes, several analytical methods for the identification and determination at the level of μg mL^{-1} and ng mL^{-1} have been published. The most commonly used techniques are liquid chromatography (LC) combined with a UV-Visible detector[20,23–25,27,30] and gas chromatography (GC) with a flame ionization detector (FID)[19] or coupled to a mass spectrometry

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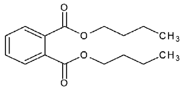
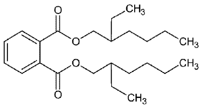
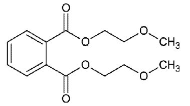
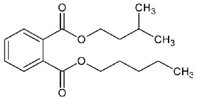
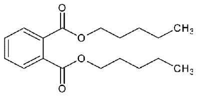
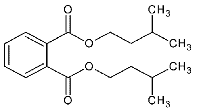
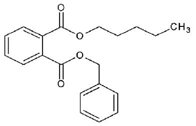
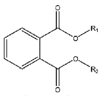
† This article is part of a themed issue on Cosmetic Ingredients, Guest Edited by Alberto Chisvert and Amparo Salvador.

Table 1 Forbidden phthalates in cosmetic products according the EU Regulation[10]

Reference number[a]	Compound	Acronym	CAS no.	Molecular structure
675	Dibutyl phthalate	DBP	84-74-2	
677	Bis(2-ethylhexyl) phthalate	DEHP	117-81-7	
678	Bis(2-methoxyethyl) phthalate	DMEP	117-82-8	
1151	*n*-Pentyl-isopentyl phthalate	NPIPP	84777-06-0	
1151	Di-*n*-pentyl phthalate	DNPP	131-18-0	
1151	Diisopentylphthalate	DIPP	605-50-5	
1152	Benzyl butyl phthalate	BBP	85-68-7	
1153	1,2-Benzenedicarboxylic acid, di-C_{7-11}, branched and linear alkyl esters	DHNUP	68515-42-4	 R = C_7H_{15} or C_8H_{17} or C_9H_{19} or $C_{10}H_{21}$ or $C_{11}H_{23}$

[a] This number correspond to the reference used for each compound in Annex II of the EU Regulation.[10]

(MS) detector.[24,29] Regarding the sample preparation, direct dilution,[19,29] vortex extraction[20] or ultrasonic extraction treatment[21,22,24,27] using organic solvents such as methanol or hexane was the most common. Recently, Kamerai *et al.*[30] have used ultrasound-assisted emulsification microextraction with solidification of an organic droplet as a sample preparation technique.

Most of these publications deal with the determination of the banned DBP, DEHP, BBP and DNPP and also other non-prohibited phthalates as diethyl phthalate (DEP) or di-*n*-octyl-phthalate (DOP). Less common are studies that include DMEP.[29] To our knowledge, no analytical methods for the determination of all three isomers, DNPP, DIPP and NPIPP, or the mixture of DHNUP have been published.

Although it is a need in quality control of perfumes, no study for the simultaneous determination of all prohibited phthalates has been found in the literature. In the present work, GC-MS has been selected for the simultaneous determination of phthalate traces due to the chemical properties of these compounds, the good resolution provided by the GC separation and the analytical features provided by the MS detection that allows a good identification of the analytes with high sensitivity.

The method uses ethanol as a solvent and no dilution of the samples is performed. It has been assayed in the determination of all the currently banned phthalates (with the only exception of DHNUP, as it is a mixture of several compounds).

2 Experimental

2.1 Reagents and samples

For the present study, standards of DBP, BBP and DEHP all 99% from Supelco (Bellefone, USA), DMEP and DNPP ≥99% from Fluka (Steinheim, Germany), DIPP 99.5% and a mixture of DIPP 22%, NPIPP 56% and DNPP 21% from Dr Ehrenstorfer (Augsburg, Germany), all analytical reagent grade, were purchased. Hexachlorobenzene 99% of Sigma-Aldrich (Steinheim, Germany) was used as an internal standard.

LC grade absolute ethanol (EtOH) from Scharlau (Barcelona, Spain) was used as a solvent. High purity helium (99.9999%) from Carburos Metalicos S.A. (Paterna, Spain) was used as a carrier gas in the GC-MS system.

Different commercial alcoholic perfumes (samples A to F) from different brands were purchased and stored at room temperature until analysis. For reasons of confidentiality, the names are not shown.

2.2 Apparatus

The GC-MS system consisted of a Focus GC gas chromatograph coupled to a DSQII mass spectrometry detector equipped with an AI3000 autosampler, all from Thermo Fisher Scientific (Austin, TX, USA).

The chromatographic separations were made using a HP-5MS Ultra Inert (95% dimethyl–5% diphenylpolysiloxane, 30 m length, 0.25 mm i.d., and 0.25 μm film thickness) analytical fused-silica capillary column from Agilent Technologies (Palo Alto, CA, USA).

A gentle stream of nitrogen obtained by a Nitrogen N_2FLO Generator from Claind (Lenno, Italy) was used for evaporation in sample preparation.

2.3 Analytical procedure

2.3.1 Preparation of samples and standards

2.3.1.1 External calibration

Preparation of standards. A multicomponent stock standard solution of the analytes in ethanol was prepared containing 8 μg mL^{-1} of DBP, 20 μg mL^{-1} of DEHP and DMEP and 1 μg mL^{-1} of NPIPP, DNPP, DIPP and BBP. Five standard solutions were prepared by introduction of increasing amounts of the multicomponent standard solution in 1 mL volumetric flasks that contained 20 μL of a 50 μg mL^{-1} ethanolic solution of the internal standard and finally taking to the line with ethanol. The working range for each analyte was: 200 to 1000 ng mL^{-1} for DBP; 500 to 2500 ng mL^{-1} for DEHP and DMEP; 25 to 125 ng mL^{-1} for NPIPP, DNPP, DIPP and BBP. A blank, which only contained the internal standard, was also prepared.

Preparation of samples. In 1 mL volumetric flasks, 20 μL of the 50 μg mL^{-1} internal standard solution and 0.8 mL of the commercial perfume samples were introduced and taken to the line with ethanol. The solutions were evaporated under a stream of nitrogen to dryness. Then, the residue was dissolved in ethanol and taken back to the initial volume with the same solvent.

2.3.1.2 Standard addition calibration. Four solutions were prepared for each commercial perfume using the following procedure. In 1 mL volumetric flasks, 0.8 mL of the commercial perfume sample, 20 μL of the 50 μg mL^{-1} internal standard solution as well as increasing amounts of the multicomponent stock standard solution (described in Section 2.3.1.1) were introduced and taken to the line with ethanol. The spiked concentrations of each analyte were 0 to 800 ng mL^{-1} for DBP; 0 to 2000 ng mL^{-1} for DEHP and DMEP; 0 to 100 ng mL^{-1} for NPIPP, DNPP, DIPP and BBP. The solutions were evaporated under a stream of nitrogen to dryness. Then, the residue was dissolved in ethanol and taken back to the initial volume with the same solvent.

2.3.2 GC-MS analysis. 1 μL of the target solutions was injected into the GC system coupled to a mass spectrometry detector operated in positive electron ionisation mode at an ionisation energy of 70 eV and with a multiplier voltage set at 1500 V. The inlet temperature was 280 °C and the injection was accomplished in splitless mode (splitless time: 1 min). The separation was run at a 1 mL min^{-1} helium constant flow rate. The oven temperature program was: from 50 °C (1 min) to 205 °C at 10 °C min^{-1}, then to 217 °C (0 min) at 1 °C min^{-1} and finally to 280 °C (10 min) at 20 °C min^{-1}. The transfer line and ion source temperatures were set at 280 and 250 °C, respectively. The chromatograms were recorded in selected ion monitoring (SIM) mode. Table 2 shows the retention times, identification and quantification ions for each analyte and acquisition time intervals in which they were recorded.

Examples of the obtained chromatograms are shown in Fig. 1 and 2. Fig. 1 shows a chromatogram of a standard

Table 2 GC-MS features of the target compounds

Compound	Retention time (min)	Selected ions[a]	Acquisition time intervals (min)
Internal standard	16.7	**284**	15.00–18.00
DBP	19.8	**149**, 150 (9), 223 (5)	18.00–21.40
DMEP	20.6	58 (67), **59**, 149 (11)	18.00–21.40
DIPP	21.9	**149**, 150 (10), 237 (6)	21.40–27.00
NPIPP	22.8		
DNPP	23.9		
BBP	29.7	91 (72), **149**, 206 (23)	27.00–31.00
DEHP	31.9	**149**, 167 (50), 279 (32)	31.00–33.00

[a] Quantification ion is represented in bold. The relative ion abundances (%) of the qualifier ions with respect to the quantifier ion are included between brackets. Dwell time: 100 ms per ion.

solution containing the seven target phthalates and the internal standard, subjected to the described GC-MS method. Fig. 2 shows the chromatograms of a sample solution (a) of commercial perfumes that contains DBP and DEHP (perfume D) prepared as described in Section 2.3.1.1 and a sample solution (b) of the same perfume but spiked with the seven analytes as described in Section 2.3.1.2.

3 Results and discussion

3.1 Sample preparation

Alcoholic perfume samples usually contain water of the order of 10%. In some published articles dealing with the determination of phthalates in perfumes by GC-MS, a high dilution of the sample in an organic solvent was carried out, thus avoiding the introduction of a high content of water in the GC-MS system. However, this leads to a decrease of the analytical signal, which is not desirable for trace analysis. Therefore, in this work, some experiments for the sample preparation step were performed. The best results were obtained by evaporation under a stream of nitrogen to dryness. The residue was dissolved in ethanol to the initial volume, thus avoiding the sample dilution.

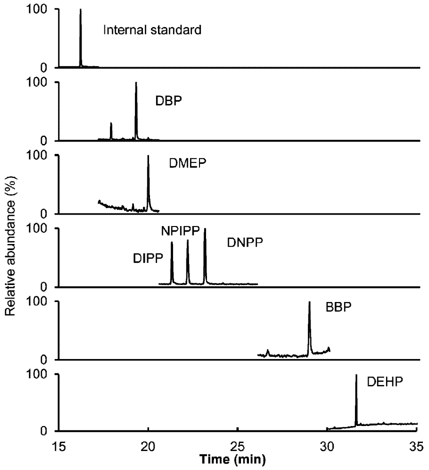


Fig. 1 Chromatogram of a standard solution (containing 25 ng mL^{-1} of the seven target phthalates and 5 ng mL^{-1} of the internal standard).

3.2 Matrix effect

Studies to evaluate the matrix effect were carried out by comparing the slopes of the calibration curves obtained by external calibration (see Section 2.3.1.1) and those obtained by standard addition calibration (see Section 2.3.1.2) of six commercial perfumes.

Firstly, the deviations of the slopes for each analyte, obtained by external calibration and by standard addition calibration, were compared by Fisher's F-test. At a 95% confidence interval most of the deviations were comparable. Next, the slope values were compared by Student's t-test at a 95% confidence interval. Table 3 summarizes the obtained data, according to the critical (t_{crit}) and experimental (t_{exp}) values. The slope values were not comparable in many cases. This means that perfumes cause a matrix effect and it would be advisable to perform standard addition calibration to quantify the analytes. It is worth mentioning that this does not seem to have been considered in some of the published articles, in which external calibration is used without previous matrix effect studies.

3.3 Limits of detection

Table 4 shows the limits of detection (LODs) in both external calibration and standard addition calibration (for the six different commercial samples). LODs were calculated as $3S_{y/x}/b$, where $S_{y/x}$ is the residual standard deviation of the calibration curve and b is the slope of the curve.

It should be noted that the signal to noise ratio was not at all the same for standard solutions (external calibration) and for standard addition solutions (with perfume as a matrix). For this

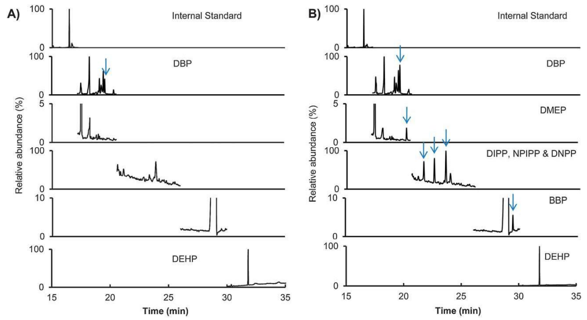


Fig. 2 Chromatogram of a commercial perfume sample (sample D) (A) and the same sample spiked with the seven analytes (B).

Table 3 Matrix effect study on the determination of the target compounds

		External calibration		Standard addition calibration		Student's *t*-test for slope comparison	
Analyte	Perfume	Slope (mL ng^{-1})	Deviation (mL ng^{-1})	Slope (mL ng^{-1})	Deviation (mL ng^{-1})	t_{exp}	Equality?
DBP	A	5700	100	4900	200	3.5^{a}	No
	B	5700	100	4200	200	6.9^{a}	No
	C	5700	100	4400	100	7.0^{a}	No
	D	5000	100	4900	200	0.4^{a}	Yes
	E	7800	200	8200	300	1.0^{a}	Yes
	F	7800	200	7000	200	2.9^{a}	No
DEHP	A	3810	30	3000	200	4.4^{c}	No
	B	3810	30	3100	200	4.5^{c}	No
	C	3810	30	2810	90	10.7^{b}	No
	D	2960	80	3640	70	5.6^{a}	No
	E	4400	100	4600	30	0.5^{b}	Yes
	F	4400	100	4900	30	1.7^{b}	Yes
DMEP	A	1070	30	1060	70	0.16^{a}	Yes
	B	1070	30	1280	20	4.9^{a}	No
	C	1070	30	1430	30	8.0^{a}	No
	D	1570	50	2200	100	5.6^{a}	No
	E	3070	60	3940	90	8.1^{a}	No
	F	3070	60	3500	100	3.1^{a}	No
NPIPP	A	4.01	0.06	3.8	0.1	1.7^{a}	Yes
	B	4.01	0.06	3.6	0.1	3.5^{a}	No
	C	4.01	0.06	3.7	0.2	0.2^{a}	Yes
	D	3.3	0.1	4.1	0.2	3.9^{a}	No
	E	5.8	0.2	6.4	0.3	1.7^{a}	Yes
	F	5.8	0.2	8	1	1.8^{c}	Yes
DNPP	A	5.1	0.1	5.4	0.2	1.4^{a}	Yes
	B	5.1	0.1	5.4	0.4	0.7^{b}	Yes
	C	5.1	0.1	5.3	0.1	1.1^{a}	Yes
	D	4.7	0.1	5.9	0.1	7.0^{a}	No
	E	8.4	0.2	8.9	0.5	1.1^{a}	Yes
	F	8.4	0.2	10.3	0.5	4.3^{a}	No
DIPP	A	3.65	0.07	3.0	0.2	4.0^{a}	No
	B	3.65	0.07	3.9	0.2	1.6^{a}	Yes
	C	3.65	0.07	3.1	0.1	4.3^{a}	No
	D	2.8	0.1	3.3	0.2	2.4^{a}	Yes
	E	5.0	0.1	6.9	0.2	7.8^{a}	No
	F	5.0	0.1	3.9	0.3	3.9^{a}	No
BBP	A	1.52	0.06	1.89	0.05	4.0^{a}	No
	B	1.52	0.06	1.84	0.05	3.4^{a}	No
	C	1.52	0.06	1.43	0.06	0.9^{a}	Yes
	D	1.8	0.1	2.1	0.1	1.7^{a}	Yes
	E	3.2	0.1	3.93	0.06	3.9^{a}	No
	F	3.2	0.1	4.3	0.2	4.3^{a}	No

[a] Homocedastic deviations: $t_{\mathrm{crit}} = 2.5$. [b] No homocedastic deviations: $t_{\mathrm{crit}} = 4.2$. [c] $t_{\mathrm{crit}} = 4.3$.

Table 4 Limits of detection for external calibration and standard addition calibration

		LOD (ng mL^{-1}) for standard addition calibration^{b}					
Compound	LOD (ng mL^{-1}) for external calibration^{a}	A	B	C	D	E	F
DBP	54	75	^{c}	^{c}	^{c}	74	^{c}
DEHP	45	^{c}	^{c}	^{c}	^{c}	^{c}	^{c}
DMEP	122	294	75	92	220	106	184
NPIPP	5	6	8	10	9	12	34
DNPP	6	6	15	6	4	12	10
DIPP	6	13	9	7	12	6	17
BBP	12	6	6	9	14	3	12

[a] Calibration solutions prepared as described in Section 2.3.1.1. [b] Calibration solutions prepared as described in Section 2.3.1.2. [c] Not calculated because the analyte was present in the sample at high concentration.

Table 5 Study of the method accuracy

		Concentration		
Analyte	Perfume	Spiked (ng mL^{-1})	Obtained (ng mL^{-1})	Recovery (%)
DBP	A	400	401	100
	E	400	416	104
DMEP	A	1000	885	88
	B	1000	980	98
	C	1000	978	98
	D	1000	916	92
	E	1000	1027	103
	F	1000	974	97
NPIPP	A	50	51	102
	B	50	47	93
	C	50	55	109
	D	50	47	93
	E	50	53	106
	F	50	64	128
DNPP	A	50	47	95
	B	50	48	97
	C	50	51	101
	D	50	49	99
	E	50	51	102
	F	50	53	106
DIPP	A	50	46	92
	B	50	54	108
	C	50	51	101
	D	50	47	94
	E	50	52	103
	F	50	46	92
BBP	A	50	50	100
	B	50	49	99
	C	50	48	96
	D	50	45	91
	E	50	50	100
	F	50	49	99

reason, in the cases in which free analyte samples were available, LODs were estimated using the $S_{y/x}$ value of the standard addition calibration line. This resulted in higher LODs than those obtained from the external calibration line (especially in the case of DMEP, due to its relatively low mass (m/z 59) quantification ion), but the obtained values are more realistic.

It should be emphasized that the LODs were obtained from the $S_{y/x}$ values of the regression, which results in higher values than those obtained from the standard deviation of a blank. Moreover, those LODs corresponding to standard addition calibration were obtained using real samples, which increases the signal to noise ratio. In spite of these two facts, the obtained values are similar to those found in previously published articles and adequate for trace analysis of perfumes.

3.4 Calibration working range

The final working range (indicated in Section 2.3.1) for external calibration and for standard addition calibration was selected for each analyte according to both LODs and contents of the samples (as no dilution of perfumes was done).

3.5 Repeatability

The repeatability of the method was estimated in terms of relative standard deviation (RSD) and was obtained from the peak areas of five standard solutions of the seven analytes at a concentration of 100 ng mL^{-1} that were treated as samples (evaporation and redissolution) prior to their injection in the GC-MS. The obtained values were: DBP 5.7%, DMEP 5.5%, DIPP 5.6%, NPIPP 5.6%, DNPP 5.1%, BBP 6.3% and DEHP 6.5%.

3.6 Accuracy

Sample solutions prepared from six commercial perfumes, previously spiked with known amounts of the analytes, were prepared as described in Section 2.3.1.2 and analyzed by the proposed GC-method. The recoveries of the determinations of the target analytes were obtained in the perfumes that were free of them. Then, all the analytes were assayed, except DEHP that was not assayed in any perfume because it was present in all of them, and DBP that was assayed only in perfumes A and E, due to its presence in the other four samples. The obtained recoveries (Table 5), all proximate to 100%, show the accuracy of the method.

3.7 Analysis of perfumes

Six commercial perfumes were analyzed by the proposed GC-MS method using standard addition calibration. Data are given in Table 6. DMEP, DIPP, NPIPP, DNPP and BBP were not present in any sample. Although all analytes are banned in cosmetics, DBP was present in four samples (B, C, D and F) with values ranging between 100 and 760 ng mL^{-1} and DEHP was present in all the analyzed samples with values ranging between 520 and 1400 ng mL^{-1}.

Table 6 Analysis of commercial perfumes[a]

Perfume	DBP	DEHP	DMEP	NPIPP	DNPP	DIPP	BBP
A	<LOD	1400 ± 200	<LOD	<LOD	<LOD	<LOD	<LOD
B	760 ± 50	1200 ± 100	<LOD	<LOD	<LOD	<LOD	<LOD
C	150 ± 20	660 ± 60	<LOD	<LOD	<LOD	<LOD	<LOD
D	660 ± 50	520 ± 40	<LOD	<LOD	<LOD	<LOD	<LOD
E	<LOD	800 ± 100	<LOD	<LOD	<LOD	<LOD	<LOD
F	100 ± 10	800 ± 100	<LOD	<LOD	<LOD	<LOD	<LOD

[a] Results are given as concentration ± S_{XE}, with S_{XE} being the standard deviation of the concentration obtained by extrapolation in the standard addition regression line. Concentrations are expressed in ng mL^{-1}.

4 Conclusions

The GC-MS method proposed enables the determination of the banned phthalates DBP, DEHP, DMEP, NPIPP, DNPP, DIPP and BBP, at a trace level with accuracy and precision.

To our knowledge, no other study for the determination of all these prohibited phthalates in perfumes or other cosmetic products had been previously published.

The need to study matrix effects in perfume analysis is worth mentioning, especially when low LODs are required and no dilution of sample is desired, as in phthalate determination.

The proposed method is accurate, rapid, and sensitive and does not require other organic solvents than ethanol, being useful for perfume analysis with quality and safety purposes.

Acknowledgements

The authors acknowledge the financial support of the Spanish Ministry of Science and Innovation (MICINN) (Project CTQ2009-12709). M. Lopez Nogueroles and J. L. Benedé would also like to thank the Spanish Ministry of Science and Innovation and the Generalitat Valenciana, respectively, for their pre-doctoral grant.

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Determination of atranol and chloroatranol in perfumes using simultaneous derivatization and dispersive liquid-liquid microextraction followed by gas chromatography-mass spectrometry

Published in:

M. López-Nogueroles, A. Chisvert, A. Salvador, *Determination of atranol and chloroatranol in perfumes using simultaneous derivatization and dispersive liquid–liquid microextraction followed by gas chromatography–mass spectrometry*,
Analytica Chimica Acta 826 (2014) 28-34.

Analytica Chimica Acta 826 (2014) 28–34

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Determination of atranol and chloroatranol in perfumes using simultaneous derivatization and dispersive liquid–liquid microextraction followed by gas chromatography–mass spectrometry

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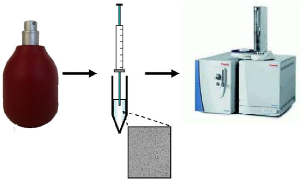
HIGHLIGHTS

- The potent allergens atranol and chloroatranol are determined in perfumes by GC–MS.
- LLE is used to clean up the sample solution from lipophilic substances.
- In situ derivatization and DLLME is used to remove polar compounds and concentrate.
- The method presents good analytical features.
- The method has been assayed in four real perfume samples.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 16 September 2013
Received in revised form 21 March 2014
Accepted 30 March 2014
Available online 4 April 2014

Keywords:
Dispersive liquid–liquid microextraction
Simultaneous derivatization
Gas chromatography–mass spectrometry
Atranol
Chloroatranol
Allergens

ABSTRACT

A new analytical method based on simultaneous derivatization and dispersive liquid–liquid microextraction (DLLME) followed by gas chromatography–mass spectrometry (GC–MS), for the determination of the allergenic compounds atranol and chloroatranol in perfumes, is presented. Derivatization of the target analytes by means of acetylation with anhydride acetic in carbonate buffer was carried out. Thereby volatility and detectability were increased for improved GC–MS sensitivity. In addition, extractability by DLLME was also enhanced due to a less polar character of the solutes. A liquid–liquid extraction was performed before DLLME to clean up the sample and to obtain an aqueous sample solution, free of the low polar matrix from the essential oils, as donor phase. Different parameters, such as the nature and volume of both the extraction and disperser solvents, the ionic strength of the aqueous donor phase or the effect of the derivatization reagent volume, were optimized. Under the selected conditions (injection of a mixture of 750 μL of acetone as disperser solvent, 100 μL of chloroform as extraction solvent and 100 μL of anhydride acetic as derivatization reagent) the figures of merit of the proposed method were evaluated. Limits of detection in the low ng mL^{-1} range were obtained. Matrix effect was observed in real perfume samples and thus, standard addition calibration is recommended.

1. Introduction

A list of 26 fragrance chemicals classified as potentially allergenic substances (PASs) was first included in the European Union (EU) Directive on cosmetic products in 2003 [1] and they are also compiled in the current EU regulation [2]. It was established

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http://dx.doi.org/10.1016/j.aca.2014.03.042
0003-2670/

that, unlike other fragrance chemicals, their presence in any cosmetic product has to be declared on the label when present at a higher concentration than 0.001% in those products to remain on the skin or 0.01% in those intended to be rinsed off. 24 of these PASs are chemically defined volatile compounds while the other two are natural moss extracts, i.e., oakmoss extract (*Evernia prunastri* extract) and treemoss extract (*Evernia furfuracea* extract), which are not a defined compound, but a complex mixture of natural products.

Since then, many works dealing with the determination of these skin-sensitizing ingredients can be found in the literature, especially regarding those 24 chemically defined PASs [3–8]. However, the number of publications regarding the determination of the two natural moss extracts is rather limited, and surely it presents an important challenge. Indeed, Joulain et al. published two comprehensive reviews on the composition of these two extracts identifying 170 different constituents in oakmoss extracts and 90 in the case of treemoss [9,10]. It should be noted that their natural conditions and a not standardized industrial processing makes the composition of these extracts variable. The industrial processing of these two natural extracts usually consists on the extraction with hexane or other solvents of the harvested lichens followed by dilution with ethanol and submission to physical treatments to remove their original colour.

From all the different compounds present in oakmoss and treemoss extracts, atranol and chloroatranol are very potent allergens [11–13]. These two compounds are degradation products, formed after transesterification and decarboxylation of the lichen depsides atranorin and chloroatranorin, and are formed in the moss absolute production [14]. It is worth mentioning that the EU regulation, which only restricts total concentration of oakmoss and treemoss extracts, does not list these individually allergenic components of mosses and their quantification is not indicative of the total amount of the regulated moss. However, the EU Commission has recently proposed to amend the EU Cosmetic Regulation according to the opinion of the Scientific Committee on Consumer Products (SCCP) [15]. This amendment includes the prohibition of atranol and chloroatranol in cosmetic products. This modification is expected to come into force in the near future. Therefore, works dealing with their determination could be useful in the future, specially taking into account the lack of analytical methods available for this purpose.

In this sense, Bernard et al. [11] searched for allergenic molecules in oakmoss extract using GC–MS after chemical fractionation of the extract by gel permeation chromatography. David et al. [16] semiquantitatively determined chloroatranol in moss extracts by GC–MS using a programmed temperature vaporizing inlet with an automated liner exchange. The liner was packed with polydimethylsiloxane that retained non-volatile material. Hiserodt et al. [14] developed a qualitative method using liquid chromatography–tandem mass spectrometry (LC–MS/MS) to identify atranorin and some related potential allergens, including atranol and chloroatranol, in oakmoss absolute. Bossi et al. [17] further developed this method to quantify atranol and chloroatranol in perfumes. The method was based on direct injection of the sample and limits of detection (LOD) of 5 ng mL^{-1} and 2.4 ng mL^{-1} for atranol and chloroatranol, respectively, were obtained. The recovery of chloratranol from spiked perfumes was 89 ± 10%. Low recoveries (49 ± 6%) were observed for atranol in spiked perfumes, indicating ion suppression caused by matrix components. Same authors also applied this method to quantify the presence of these two allergenic compounds in different commercial perfumes [18,19].

It is worth mentioning that perfumes are elaborated by mixing different natural raw materials or complex synthetic formulations that commonly contain hundreds or thousands of different compounds. This fact complicates the determination of compounds in this matrix. Thus, an effective sample preparation, that enables a significant clean-up, is crucial to obtain a good accuracy. The aim of this work is to develop a method to determine atranol and chloroatranol in perfumes with a good accuracy.

In this work, liquid–liquid extraction (LLE) is used to clean up the sample solution from lipophilic substances. This step is followed by a simultaneous derivatization and dispersive liquid–liquid microextraction (DLLME) step that enables to change the solvent (for further GC–MS analysis) and to concentrate the target compounds. Moreover, it provides a second, less crucial, clean up from possible components that would have remained in the polar phase with the analytes after the LLE process.

It should be pointed out that the derivatization step is proposed as atranol and chloroatranol are relatively polar compounds, and without it, a poor extraction efficiency would be achieved in the DLLME process. Moreover, the derivatization allows volatility and thus increases sensitivity in GC–MS. The chosen approach was an in situ derivatization via acetylation of the hydroxyl groups under basic aqueous conditions, using acetic anhydride as acetylating agent. The derivatization is performed at the same time as DLLME, allowing the simplification of the procedure and a decrease in the time of analysis. This one step derivatization and DLLME was first introduced by Fattahi et al. to determine clorophenols in waters [20] and has been later used by other authors to determine phenols in a similar approach although also performing other types of derivatization reactions such as silylation [21,22] and *O*-alkoxycarbonylation [23,24].

2. Experimental

2.1. Reagents and samples

Atranol (97%) and chloroatranol (98.9%), purchased from Ambinter (Orleans, France), were used as standards. O-cresol, purchased from Aldrich, was used as surrogate. Fig. 1 shows the chemical structure of the three of them.

Liquid chromatography grade absolute ethanol from Scharlau (Barcelona, Spain), was used as solvent to prepare dilute solutions of the standards. Deionised water (resistivity ≥18 MΩ cm^{-1}) obtained by means of a Nanopure II water purification system from Barnstead (Boston, MA, USA) was used to prepare the working standard solutions.

Analytical reagent grade chloroform and dichloromethane, from Scharlau Chemie (Barcelona, Spain), were tested as extraction solvents. Ethanol, ultrapure acetone and liquid chromatography grade acetonitrile, all from Scharlau Chemie, were tested as disperser solvents.

Analytical reagent grade sodium chloride 99% from Scharlau Chemie was used to adjust the ionic strength. Acetic anhydride extra pure and anhydrous diethyl ether from Scharlau Chemie, and potassium carbonate analysis grade from Panreac (Barcelona, Spain) were used in the derivatization of the compounds.

High purity helium (99.9999%) from Carburos Metalicos S.A. (Paterna, Spain) was used as carrier gas in the GC–MS system.

Four different commercial perfumes from different brands (a women *eau de toilette* and *eau de parfum*, a man *eau de parfum* and a

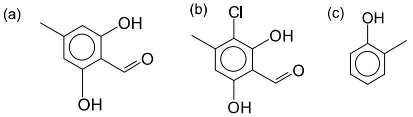

Fig. 1. Chemical structure of (a) atranol (b) chloroatranol and (c) o-cresol.

children *eau de cologne*) were purchased and stored at room temperature until analysis. For reasons of confidentiality, the manufacturers are not shown.

2.2. Apparatus

The GC–MS system used consisted of a Focus GC gas chromatograph coupled to a DSQII mass spectrometry detector equipped with an AI3000 autosampler, all from Thermo Fisher Scientific (Austin, TX, USA). The chromatographic separations were made using an HP-5MS Ultra Inert (95% dimethyl-5% diphenyl polysiloxane, 30 m length, 0.25 mm i.d., 0.25 μm film thickness) analytical fused-silica capillary column from Agilent Technologies (Santa Clara, CA, USA).

An 8452A Hewlett Packard diode array UV–vis spectrophotometer with a 1 cm path length quartz cell was used to determine the losses in the LLE process.

An Avance 300 spectrometer from Bruker (Madrid, Spain) was employed to run ^{1}H nuclear magnetic resonance (NMR) spectra at 300 MHz, using residual non-deuterated chloroform as internal standard (δ 7.26 ppm).

An EBA 21 centrifuge from Hettich (Tuttlingem, Germany) was also used.

2.3. Method

2.3.1. Internal standard calibration

Standards preparation: 1 mL of each one of the ethanolic standard solutions containing the analytes (atranol and chloroatranol) in a concentration between 20 and 100 ng mL^{-1} and the surrogate (o-cresol) in 40 ng mL^{-1} were transferred to different 15 mL polyethylene centrifuge tubes. 1.5 mL of water and 1.5 mL of hexane were added and the mixture vortexed during 1 min. Then, the tubes were centrifuged for 3 min at 3000 rpm and the upper layers of hexane were collected with a syringe and discarded. The liquid–liquid extraction (LLE) process and subsequent discard of hexane was repeated two more times. After this, 2 mL of the remaining aqueous solutions were taken and transferred to other polyethylene centrifuge tubes. 8 mL of water and 1 mL of a 1 M potassium carbonate solution were added. A simultaneous derivatization and DLLME process was then performed injecting different previously mixed solutions of 100 μL of chloroform (extraction solvent), 100 μL of acetic anhydride (derivatization reagent) and 750 μL of acetone (disperser solvent) to these solutions. The tubes were then centrifuged for 5 min at 6000 rpm. The sedimented phases were collected with a 100 μL Hamilton syringe and transferred into 100 μL inserts placed inside different injection vials, which were then ready to be injected into the chromatographic system.

Samples preparation: 3 replicates of 1 mL of perfume sample were submitted to the same procedure.

2.3.2. Standard addition combined with internal standard calibration

Several aliquots of 1 mL of perfume sample were spiked, with a 2 μg mL^{-1} ethanolic solution of the target analytes, in a concentration between 20 and 100 ng mL^{-1}, and with a 2 μg mL^{-1} ethanolic solution of the surrogate, in 40 ng mL^{-1}, and they were submitted to the procedure described in Section 2.3.1.

(As will be seen later, the results showed that standard addition calibration is recommended).

2.3.3. GC–MS analysis

1 μL of each one of the aforementioned sedimented phases was injected into the GC system coupled to a mass spectrometry detector operated in positive electron ionisation mode at ionisation energy of 70 eV and with a multiplier voltage set at 1300 V. The inlet temperature was 280 °C and the injection was accomplished in splitless mode (splitless time: 1 min). The separation was run at a 1 mL min^{-1} helium constant flow rate. The oven temperature program was: from 80 °C (1 min) to 200 °C at 4 °C min^{-1} and finally to 280 °C (5 min) at 20 °C min^{-1}. The transfer line and ion source temperatures were set at 280 and 250 °C, respectively. The chromatograms were recorded in selected ion monitoring (SIM) mode at the following mass/charge (m/z) ratios: m/z 108 from minute 5.0 to 11.0 for o-cresol, m/z 152 from minute 20.0 to 28.0 for atranol and m/z 186 from minute 28.0 to 32.0 for chloroatranol. A full scan mode (m/z from 50 to 350) was simultaneously recorded from minute 5.0 to the end of the analysis time to confirm the identification of the analytes in real samples.

Calibration was perfomed by plotting A_i/A_{sur} (where A_i is the peak area of the target analyte and A_{sur} that of the surrogate (i.e., o-cresol), each one obtained by its quantifier ion) versus target analyte concentration.

2.4. Synthesis of the diacetylated compounds

The diacetylated compounds were not commercially available and thus, they were synthesised in the laboratory in order to calculated the enrichment factor, based on a synthesis of a similar product already published [25]. Briefly, a flask equipped with a stir bar and a nitrogen line containing the target compound (atranol or chloroatranol), 3 equivalents of potassium carbonate, 6 equivalents of anhydride acetic and 4 mL of dry diethyl ether was stirred overnight. The product reaction was filtered and evaporated under reduced pressure. Then, the solid was rinsed thrice with hexane and evaporated again.

3. Results and discussion

3.1. Liquid–liquid extraction

Ethanol content in perfumes is around 80%. The other remaining 20% mostly corresponds to the essential oils, water and keepers. Terpenes are oily, non-polar primary constituents of essential oils. Thus, a good clean-up is important to avoid interfering compounds in the subsequent chromatography. In this sense, if the perfume is directly injected, the injector port and the column head could suffer from dirtiness and thus jeopardize the results [5]. Moreover, as the DLLME used to preconcentrate de target analytes requires an aqueous donor phase, an increase of the water content was needed in the sample solution. The direct dilution of the perfume with water is not possible since a slight increase in the percentage of water is enough to make the solution an opaque white dispersion, due to the presence of the non-polar components of the perfumes. However, a previous liquid–liquid extraction provides a good clean-up and enables the required water dilution before DLLME.

Taking advantage of the relatively high polarity of the compounds of interest (with a log *P*, where *P* is the partition coefficient, of 1.99 and 2.99 for atranol and chloroatranol, respectively), conventional liquid–liquid extraction (LLE) could be a useful technique in the case of having two immiscible phases (an organic and an hydro-alcoholic phase). The analytes could theoretically stay in the hydro-alcoholic phase unlike most of fragrance compounds, which are highly non-polar, and therefore will migrate to the organic phase. Indeed, preliminary tests showed that atranol and chloroatranol remained in the hydro-alcoholic phase and moreover, the injection of the rejected phases in the GC–MS showed that an important clean-up was performed. The clean-up was evidenced when even after adding 8 mL of water to 2 mL of the remaining hydro-alcoholic phase it still remained a completely transparent solution, that allowed the subsequent DLLME.

Three extractions with aliquots of 1.5 mL of hexane were observed to be enough to eliminate most of these fat-soluble interferences of the perfume that made the solution used for DLLME not clear. Chromatograms in both full scan and SIM modes (i.e., at *m*/*z* 152 and 186) of the hexane rejected phases of Perfume 2 and Perfume 3 are shown, as example, as Supplementary Data to prove the clean-up performed.

The recovery of atranol and chloroatranol in the LLE step was then quantified. To do so, the LLE process was performed to a hydroethanolic standard solution of each one of the separate compounds. The compounds were measured in the hydroethanolic standard solution with an UV spectrophotometer after 1, 2 and 3 repetitions of the LLE (all in triplicate) and compared to that of the hydroethanolic solution before performing the clean-up process. Its spectrum was recorded and the absorbance at λ_{max} (i.e., 280 nm) was compared to that of the hydroethanolic solution before performing the LLE process to study how much target analyte was lost to the rejected phase in the process. In the case of atranol a total of 17.9 ± 0.4% is lost after the 3 extractions (12% in the first one and 3% in each of the other 2). Therefore, the recovery would be of 82.1 ± 0.4%. In the case of chloroatranol a total of 31.3 ± 0.8% is lost after the 3 extractions (13% in the first and second one and 5% in the third one). Therefore, the recovery is of 68.7 ± 0.8%. Although this may seem excessive, it is important to keep in mind that it is worthy compared to the clean-up performed.

3.2. Simultaneous dispersive liquid–liquid microextraction and derivatization

The simultaneous DLLME and derivatization parameters needed to be optimized in order to achieve the highest extractability as possible and thus, the highest sensitivity.

Thus, to study the different parameters, the DLLME procedure was carried out to 1 mL of a 50 ng mL^{-1} ethanolic solution of the two target analytes, to which 9 mL of water and 1 mL of a 1 M solution of potassium carbonate had been previously added (standard hydro-ethanolic solution).

The peak area of each analyte was plotted (Figs. 2–6) as the average of 3 replicates with the error bars showing the standard deviation.

3.2.1. Selection of the kind of extraction and disperser solvents

In order to select the optimum extraction and disperser solvents it is important to take into account those features they need to have. The disperser solvent has to form the cloudy solution and has to be miscible in both the aqueous donor phase and the extraction solvent. The extraction solvent has to be immiscible in the aqueous donor phase and should also extract the compounds of interest and have a good behaviour for the further analytical technique going to be used (in this case GC).

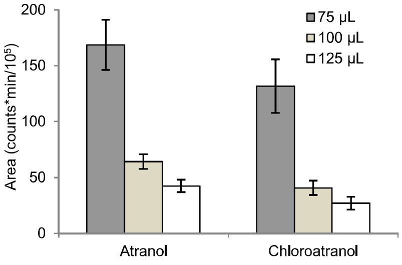

Fig. 2. Effect of the volume of extraction solvent. Extraction conditions: standard solution volume: 10 mL; disperser solvent volume: 1000 µL; K_2CO_3 1 M volume: 1 mL; acetic anhydride volume: 100 µL.

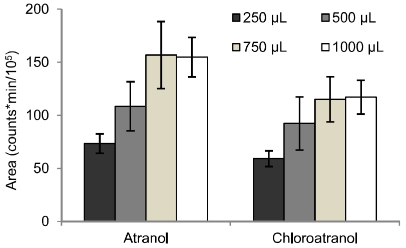

Fig. 3. Effect of the volume of disperser solvent. Extraction conditions: standard solution volume: 10 mL; extraction solvent volume: 100 µL; K_2CO_3 1 M volume: 1 mL; acetic anhydride volume: 100 µL.

Regarding the extraction solvent, dichloromethane and chloroform were studied and in the case of the disperser solvents, acetone, acetonitrile and ethanol. One millilitre of each disperser solvent was mixed with 100 µL of each extraction solvent and 100 µL of acetic anhydride. Then, each mixture was rapidly injected into 10 mL of the standard hydro-ethanolic solution. No phase separation after centrifugation was observed in any of the combinations that used dichloromethane as extraction solvent. Results using chloroform showed that the volume of the sedimented phase varied a lot depending on the disperser solvent. Although higher areas were achieved using ethanol as disperser solvent, it was not chosen as the volume was very low to handle easily. Therefore, chloroform and acetone were chosen as extraction and disperser solvent for further experiments.

3.2.2. Effect of the extraction solvent volume

In order to study the effect of the extraction solvent volume, different volumes of chloroform, ranging from 50 to 125 µL, were mixed with 1 mL of acetone and 100 µL of acetic anhydride and injected into 10 mL of the standard hydro-ethanolic solution (Fig. 2). When 50 µL of chloroform were used, the volume of the obtained sedimented phase was very low to inject it in the GC–MS and when 75 µL were used the total volume of the sedimented phase was only 13 ± 5 µL, very low to handle, which caused a poor repeatibiliy in the results. Thus, although less sensitivity is

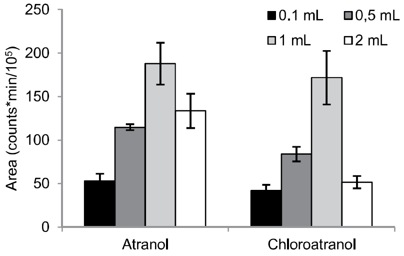

Fig. 4. Effect of the volume of K_2CO_3. Extraction conditions: standard solution volume: 10 mL; disperser solvent volume: 1000 µL; extraction solvent volume: 100 µL; acetic anhydride volume: 100 µL.

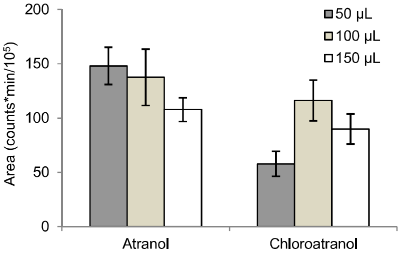

Fig. 5. Effect of the volume of acetic anhydride. Extraction conditions: standard solution volume: 10 mL; disperser solvent volume: 1000 µL; extraction solvent volume: 100 µL; K_2CO_3 1 M volume: 1 mL.

obtained, the subsequent studies were carried out using 100 µL of extraction solvent.

3.2.3. Effect of the disperser solvent volume

To study this effect, different volumes of acetone, ranging from 250 to 1000 µL, were tested. Fig. 3 shows that the signal increases with the disperser solvent volume and tends to stabilize after 750 µL. Low volumes of disperser solvent could mean poor extraction efficiencies, as the extraction solvent needs to be correctly dispersed (cloudy solution). Thus, 750 µL of disperser solvent were selected for further experiments.

3.2.4. Effect of the potassium carbonate amount

An aqueous solution 1 M of potassium carbonate was used to perform the derivatization reaction in a basic aqueous media. Different volumes, ranging from 0.1 to 2 mL, of this solution were tested to study its effect on the simultaneous derivatization and DLLME process. Fig. 4 shows that the best results were obtained when 1 mL of the solution was added. These results could be explained by the fact that there are two effects involved. On one hand, the alkaline pH is needed to perform the reaction in a reasonable yield. On the other hand, potassium carbonate is a salt and it increases the ionic strength of the solution which could affect the results (as described in Section 3.2.6). In this sense, 1 mL of 1 M potassium carbonate solution is chosen for further experiments.

3.2.5. Effect of the acetic anhydride volume

Acetic anhydride was mixed with the extraction (chloroform) and disperser (acetone) solvents in order to inject the mixture and perform the simultaneous derivatization and DLLME process. Different volumes, ranging from 50 to 150 µL, of acetic anhydride were tested while all other parameters were kept constant. Fig. 5 shows that, when comparing 50 and 100 µL, similar results were obtained for atranol. However, in the case of chloroatranol the best results were obtained when 100 µL were used. Thus, 100 µL of derivatizating agent were chosen for further experiments. The signal was expected to increase with the volume of anhydride acetic, as it would be desirable that it was in excess. However, the slight decrease of signal when 150 µL are used could be explained as a raise in the acidity.

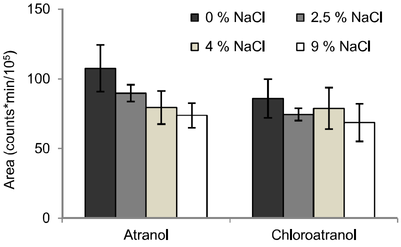

Fig. 6. Effect of the ionic strength. Extraction conditions: standard solution volume: 10 mL; disperser solvent volume: 1000 µL; extraction solvent volume: 100 µL; K_2CO_3 1 M volume: 1 mL; acetic anhydride volume: 100 µL.

Both, potassium carbonate and acetic anhydride amount, are in agreement with other published works where an acetylation of phenols in basic aqueous media is performed and similar proportions are used [26].

3.2.6. Effect of the ionic strength of the donor phase

With the purpose of studying this effect, the simultaneous derivatization and DLLME procedure was performed to different hydro-ethanolic standard solutions containing different concentrations of NaCl ranging from 0 to 9% (m/v). It should be noted that as 1 mL of a 1 M potassium carbonate solution was previously added, there were other salts that already contributed to the ionic strength of the donor phase.

On one hand, the increase of the ionic strength decreases the solubility of the extraction solvent (chloroform) in the aqueous phase, due to the salting out effect, and therefore, the volume of the sedimented phase increases. On the other hand, the salting out effect is expected to favour the extraction of the target compounds from the aqueous phase to the organic phase. Fig. 6 shows that the best results in the case of atranol were obtained when no NaCl was added, whereas, no significant differences were obtained in the case of chloroatranol. Therefore, in subsequent experiments, no NaCl was added to the donor phase.

3.2.7. Effect of the pH of the donor phase

The extraction of compounds can be considerably affected depending on the pH of the donor phase. In this case, the donor phase needed to present an alkaline pH in order to perform the derivatization reaction (potassium carbonate is added just before, resulting on a pH of 11.5 ± 0.2). Thus, different pH values were not studied.

3.2.8. Effect of the extraction and derivatization time

The extraction and derivatization time is defined as the period between the injection of the mixture (i.e., the derivatization reagent and both the disperser and the extraction solvents) and the centrifugation step.

Different times, ranging from 0 to 60 min, were studied. As expected, extraction and derivatization time does not affect the results obtained in the DLLME. As already stated for this technique, the cloudy solution generates a very large surface area between the sample, the extraction solvent and the derivatization reagent, what enables the achievement of the equilibrium state almost instantaneously.

3.2.9. Enrichment factor of the DLLME process

The enrichment factor (EF) is defined as the ratio between the concentration of the compound in the extraction solvent phase and the initial concentration in the aqueous donor phase. The maximum EF value, which would correspond to the total transfer of the target analytes to this sedimented phase, can be calculated as V_0/V_{sed}, where V_0 is the sample volume and V_{sed} the sedimented phase volume. In this case, the final volume of sedimented phase after the optimization is approximately 20 µL and the initial volume of sample is 1 mL. Thus, the maximum EF possible would be 50.

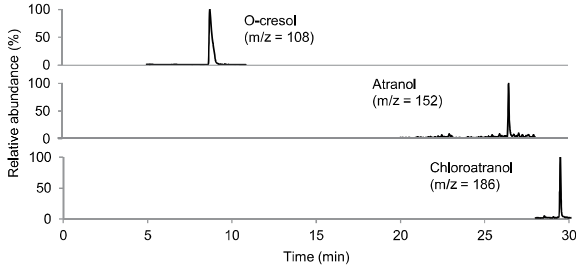

Fig. 7. A chromatogram obtained applying the proposed method to an ethanolic standard solution containing the target compounds at 60 ng mL^{-1} and o-cresol as surrogate at 40 ng mL^{-1}.

Taking into account that analytes are derivatized simultaneously to DLLME, to calculate the real EF of the DLLME process the pure derivatized compounds were needed. Unfortunately, these compounds were not commercially available and thus, they were synthesised in the laboratory as described in Section 2.4. A ^{1}H NMR of the product showed that the dyacetilated product had been successfully synthesised and no other significant side products or reagents were left.

To calculate them the area of a standard solution of atranol and chloroatranol submitted to the DLLME process and that of a solution of derivatized atranol and chloroatranol in the same concentration in chloroform (not extracted) were compared. An EF of 19 and 22 were obtained for atranol and chloroatranol, respectively.

3.3. Use of surrogate

O-cresol was chosen as surrogate in order to increase the repeatability, not only of the GC injections but of the whole method procedure (i.e., LLE, DLLME, derivatization and injection). Thus, A_i/A_{sur} (where A_i is the peak area of the target analyte and A_{sur} that of the surrogate) was used as response function for quantification purposes.

O-cresol was selected as it presented a very similar log *P* than atranol so its solubility features were expected to be similar to those of the analyte. Furthermore it is, as the target compounds, a phenol, what is important when looking for a compound that could act as surrogate not only of the extraction processes but also of the derivatization process.

3.4. Analytical figures of merit of the proposed method

Once the simultaneous derivatization and DLLME was optimized, different quality parameters were evaluated using standard solutions.

An internal standard calibration curve was constructed and linearity reached at least 1000 ng mL^{-1}. The working range employed was set from 20 to 100 ng mL^{-1}. All the solutions contained 40 ng mL^{-1} of o-cresol as surrogate. Fig. 7 shows the chromatogram obtained for an ethanolic standard solution containing the target analytes at 60 ng mL^{-1} and the surrogate, submitted to the proposed LLE followed by derivatization and DLLME and GC–MS method.

The repeatability, expressed as relative standard deviation (RSD), was evaluated by applying the proposed LLE followed by derivatization and DLLME and GC–MS method to five replicates of a standard solution containing 40 ng mL^{-1} of the target compounds and the surrogate. RSD of 7 and 10% were obtained for atranol and chloratranol, respectively. The EFs of the whole method were also calculated, taking into account the loss in the LLE process and the EFs of the DLLME process, resulting in a value of 15 for both, atranol and chloroatranol.

In order to evaluate matrix effects when internal standard calibration is used, the slopes of the calibration curves obtained by this method and those obtained by standard addition calibration combined with internal standard of four commercial perfumes were compared. In this sense, the proposed LLE followed by derivatization and DLLME and GC–MS method was applied to four perfume samples that had been previously spiked with the target analytes at 20–100 ng mL^{-1} (working range). The characteristics of the calibration equations obtained for each standard addition are shown in Table 1. This table also shows the study of matrix effect obtained in percentage calculated with the ratio of the slope of the standard addition calibration of each sample to that of an internal standard calibration performed the same day. Matrix effects can be observed, especially in the case of chloroatranol. Thus, standard addition is recommended. It is worth mentioning that even though matrix effects were observed in some previous published articles [12,13], no correction of the data with standard addition was done. Table 2 shows the recoveries obtained after spiking blank perfume

Table 1
Study of matrix effects.

Analyte	Atranol				Chloroatranol			
	Slope[a] (ng mL^{-1})$^{-1}$	Intercept[a]	Regression coefficient[a]	Calibration slopes ratio (%)[b]	Slope[a] (ng mL^{-1})$^{-1}$	Intercept[a]	Regression coefficient[a]	Calibration slopes ratio (%)[b]
Perfume 1	$(280 \pm 9) \times 10^{-4}$	-0.05 ± 0.05	0.996	116 ± 5	$(56 \pm 2) \times 10^{-4}$	-0.0002 ± 0.008	0.997	72 ± 6
Perfume 2	$(297 \pm 7) \times 10^{-4}$	-0.04 ± 0.03	0.998	112 ± 6	$(65 \pm 4) \times 10^{-4}$	-0.006 ± 0.02	0.990	89 ± 6
Perfume 3	$(315 \pm 12) \times 10^{-4}$	-0.10 ± 0.07	0.994	120 ± 7	$(52 \pm 1) \times 10^{-4}$	0.003 ± 0.003	0.998	71 ± 3
Perfume 4	$(336 \pm 15) \times 10^{-4}$	0.13 ± 0.09	0.992	146 ± 9	$(41 \pm 4) \times 10^{-4}$	0.03 ± 0.02	0.98	62 ± 8

[a] Obtained by means of the internal standardization calibration. Working range: 20–100 ng mL^{-1}. Number of calibration points: 6.

[b] (Standard addition calibration slope/internal standard calibration slope) × 100.

Table 2
Study of the recovery of the proposed method (standard addition calibration).

Real samples	Recovery[a] (%)			
	Atranol		Chloroatranol	
	20 ng mL^{-1}	100 ng mL^{-1}	20 ng mL^{-1}	100 ng mL^{-1}
Perfume 1	94	103	95	98
Perfume 2	96	101	79	97
Perfume 3	86	103	104	100
Perfume 4	106	98	110	98

[a] Recovery obtained after spiking free-analytes samples with 20 and 100 ng mL^{-1}, estimated as ((final concentration – initial concentration)/spiked concentration) × 100.

samples with 20 and 100 ng mL^{-1} of the target compounds and performing the proposed method. Good recovery values are obtained when using standard addition calibration.

As standard addition is proposed, limits of detection were calculated as 3 times the signal-to-noise ratio of a sample spiked with 20 ng mL^{-1} of the analytes. They were of the order of 3 and 2 ng mL^{-1} for atranol and chloratranol, respectively, in the initial perfume samples. Thus, the limits of quantification obtained were of the order of 9 and 6 ng mL^{-1} for atranol and chloratranol, respectively. Repeatability was re-evaluated using the same experiment but this time to the four perfumes spiked instead of the standard solution. The mean of the four RSD resulted in a total RSD of 9% for both atranol and chloroatranol. This shows that there is no significant difference between the internal standard and the standard addition method on repeatability.

3.5. Assay of the proposed method to the analysis of real perfume samples

The target analytes were determined in four different perfume samples. The results indicated that both analytes were not present in the analyzed samples or that they were below the limits of detection of the method. As the number of samples is rather limited these results could be taken as satisfactory preliminary results and a higher number should be analysed to assure the method is applicable to all market perfumes.

4. Conclusions

Quality control of commercial perfumes and raw materials is necessary. Analytical methods are required to guarantee there are no undesired compounds in the finished product. Little has been done in this field regarding the potent allergens atranol and chloroatranol.

In this work a method based on LLE followed by simultaneous derivatization and DLLME and GC–MS is presented to determine atranol and chloroatranol in perfumes.

Since matrix effects were observed when internal standardization is used, standard addition calibration is recommended. In this sense, the method allows the determination of atranol and chloroatranol successfully in the ng mL^{-1} range with good accuracy and precision.

Acknowledgements

The authors acknowledge the financial support of the Spanish Ministry of Science and Innovation (Project CTQ2009-12709), especially Marina López-Nogueroles for her FPI predoctoral grant, and the financial support of University of Valencia (Project UV-INV-AE13-137567).

Authors also acknowledge the advice given concerning the derivatization reaction, by Prof. Gonzalo Blay, Prof. Isabel Fernandez and Ms. Amparo Sanz from the Department of Organic Chemistry of the University of Valencia.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aca.2013.12.001.

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A chromatochemometric approach for evaluating and selecting the perfume maceration time

Published in:

M. Lopez-Nogueroles, A. Chisvert, A. Salvador,
A chromatochemometric approach for evaluating and selecting the perfume maceration time,
Journal of Chromatography A 1217 (2010) 3150-3160.

Journal of Chromatography A, 1217 (2010) 3150–3160

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

A chromatochemometric approach for evaluating and selecting the perfume maceration time[☆]

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ARTICLE INFO

Article history:
Received 10 December 2009
Received in revised form 25 February 2010
Accepted 26 February 2010
Available online 4 March 2010

Keywords:
Cosmetic
Gas chromatography
Linear discriminant analysis
Perfume maceration time
Perfume

ABSTRACT

A chemometric treatment of the data obtained by gas chromatography (GC) with flame ionization detector (FID) has been proposed to study the maceration time involved in perfumes manufacture with the final purpose of reducing this time but preserving the organoleptic characteristics of the perfume that is being elaborated. In this sense, GC–FID chromatograms were used as a fingerprint of perfume samples subjected to different maceration times, and data were treated by linear discriminant analysis (LDA), by comparing to a set of samples known to be macerated or not, which were used as calibration objects. The GC–FID methodology combined with the treatment of data by LDA has been applied successfully to seven different perfumes. The constructed LDA models exhibited excellent Wilks' lambdas (0.013–0.118, depending on the perfume), and up to a reduction of 57% has been achieved with respect to the maceration time initially established.

1. Introduction

Odours play a crucial role in human behaviour. While a pleasant scent can have a calming effect or make one feel better, unpleasant odours can alter our mood negatively and produce anxiety and discomfort [1]. Perfumes are the substances responsible for providing us with pleasant redolence that we smell everyday. Each perfume is made up of hundreds of aromatic chemicals (also referred to as fragrance chemicals), each of which contributes with its characteristic odour, together giving the characteristic aroma of the perfume [2].

The manufacture of a perfume consists of several stages, which can include the obtaining of the perfume raw material, named as extract or essential oil (in case it is of natural origin) or as concentrate (in general terms).

Once the concentrate is obtained, it is mixed up with the other components that make part of the perfume, such as ethanol, water, antioxidants, UV filters and colouring agents among others. After the mixture has been elaborated it must be left some time, in which concentration changes in some components can occur due to the transference of insoluble substances from the concentrate to the solution or the precipitation of cerate components. These changes occur until a steady-state is achieved, in which the organoleptic characteristics of the whished perfume are obtained. This stage is known as the perfume maceration and it is carried out inside vats. Once the perfume maceration has concluded, the mixture is cooled and any sediment formed is removed by filtration. Finally, the perfume can be bottled and put on the market. It should be pointed out that the described perfume maceration process should not be confused with a procedure which is sometimes used for the obtaining of the concentrate also named maceration but referred to the raw material and not to the final product [2].

Manufacturers usually use perfume maceration times of several weeks, even months, in order to assure that the process has been completed. This period frequently exceeds the time that would be really necessary to obtain the organoleptic characteristics of the wished perfume, since the steady-state is generally reached before. This carries important losses of time and its resulting economic cost as the perfumes remain in the vats of maceration more time than the necessary before being packed and ready to put on the market. However, it should be mentioned, that according to bibliographic databases, no papers about the study and/or the establishment of the perfumes maceration time are found.

From a chemical standpoint, perfume analysis is focused on the determination of compounds of interest, such as fragrance allergens or prohibited musks and phthalates, among others. As it has been reviewed recently [2], owing to the volatility of the compounds that constitute the perfumes, gas chromatography (GC) either with flame ionization detector (FID) or with mass spectrometry detector (MS) is the most preferred analytical technique. Moreover, the perfume industry uses preferably this technique both to elucidate the complex composition of a perfume as to carry out quality control analysis [3,4].

[☆] Presented at the 9th Scientific Meeting of the Spanish Society of Chromatography and Related Techniques, San Sebastián, Spain, 28–30 October 2009.

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0021-9673/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2010.02.078

Nevertheless, it should be mentioned that to study the perfume maceration process from a chemical point of view, not only analytical sensitive and selective techniques are needed, such as the above-mentioned GC, but also multivariant treatment of the data is compulsory, provided that not only a compound might be altered during the maceration process.

Linear discriminant analysis (LDA) is a supervised pattern recognition method, which describes, on the basis of a mathematical model, the behaviour of a qualitative dependent variable with the information of different quantitative independent variables. In this sense, LDA recognizes behaviour patterns within previously labelled groups or categories. Once the groups are established – the model is constructed – an unknown object can be assigned to one of the established groups (i.e., qualitative variable) on the basis of the values of a series of predictors (i.e., quantitative variables) [5].

As can be read elsewhere, LDA has been used in many fields of analytical chemistry as a classificatory tool, especially in food analysis, in order to discriminate between samples where the qualitative variable can be geographical origin, genetic variety, cultivar origin, botanical origin, genuine or adulterated, etc. [6]. Regarding perfume analysis assisted with LDA, only two articles have been found in the literature. The first one deals to model the relationship between the structure and fragrance properties of different chemicals, which belong to three categories (apple, pineapple and rose) [7]. In the second one, five terpenes are discriminated based on the use of different serum albumins acting as low-selectively receptors [8]. Neither applications of LDA nor other chemometric approaches for the study of the perfumes maceration process have been found in the literature. However, LDA has been applied to study some other time-dependant procedures. For example, LDA has been used to classify cheeses of different ripening ages (fresh, medium and/or old) based on water extract profiles obtained by liquid-chromatography [9], different proteolysis parameters [10], ethanol–water protein fraction profile obtained by capillary zone electrophoresis [11], or fat, protein, dry matter and different metals contents [12]. LDA has also been used to classify Spanish hams of different curing times (6, 8 and 12 months) based on peptide profiles obtained by capillary zone electrophoresis [13], or to establish the different age of wines based on their concentrations of some inorganic anions [14], or to study the influence of the storage time of young white wines on the phenolic composition and colour [15].

The aim of this work is to develop an analytical method which enables us to study whether there are significant changes in the process of perfume maceration, and thus to establish the perfume maceration time, considered as the time in which the perfume that is being elaborated has achieved a steady-state, with the final purpose of minimizing this time but preserving the organoleptic characteristics of the perfume. In this sense, GC–FID chromatograms are used as a fingerprint of perfume samples subjected to different maceration times, and data are treated by LDA, by comparing to a set of samples which were known to be macerated or not. The significant GC–FID peaks in each one of the studied perfumes and selected from the LDA results were tried to be identified by means of GC–MS.

2. Experimental

2.1. Reagents and samples

High-purity nitrogen (99.999%) and helium (99.999%) from Carburos Metálicos S.A. (Paterna, España) were used as carrier gases in the GC–FID and GC–MS systems, respectively. Hydrogen (99.999%) and synthetic air (99.999%) both GC-grade, also from Carburos Metálicos S.A., were used as auxiliary gases for FID.

4-Methyl-2-pentanone (methyl isobutyl ketone, MIBK) 99% from Panreac (Barcelona, Spain) was used as internal standard (IS) to minimize possible deviations occurred during the GC injection process.

LC-grade ethanol (EtOH) from Scharlau Chemie (Barcelona, Spain) was used as solvent to prepare the measuring solutions.

Ethylenediaminetetraacetic acid dissodium salt (EDTA), butylhydroxytoluene (BHT), vitamine E, UV filters such as 2-ethylhexyl salicylate (ES), butyl methoxydibenzoylmethane (BDM) and 2-ethylhexyl methoxycinnamate (EMC) and 7 perfume concentrates (the name of which are not shown for reasons of confidentiality), all of unknown purity and origin, were kindly provided by RNB S.L. (Valencia, Spain) as raw materials for the manufacture of the 7 studied perfumes. RNB S.L. acted in this study as a collaborator enterprise with our laboratory. LC-grade ethanol from Scharlau Chemie (Barcelona, Spain) and deionized water obtained using a NANOpure II ultrapure water system from Barnstead (Boston, USA), were also used as raw materials.

2.2. Manufacture of the perfume samples

Seven perfumes were prepared at laboratory scale according to an adaptation of the manufacture protocol provided by RNB S.L., by using their own raw materials, with the exception of using LC-grade ethanol and ultrapure water instead of cosmetic-grade ethanol and osmotized water, as originally was stated. Moreover, it should be taken into account that the weighting of volatile liquids (e.g., ethanol), is subjected to imprecision. Thus, in order to reduce possible variations between different replicates of the same perfume, and thus, minimize variability in the results due to the manufacturing process, the protocol was adapted to work on volume and not on weight, but maintaining the original proportions of the supplied protocol. The composition and the adapted protocol followed in the laboratory for each one of the 7 perfumes under study are shown in Table 1. As can be seen, the procedure consists basically in the mixture of the perfume concentrate with ethanolic and aqueous solutions of the rest of the components.

For each of the 7 perfumes under study, 10 replicates were prepared, kept in glass topaz colour bottles to shelter from light and at room temperature during 30 days. In order to emulate the original protocol followed in RNB S.L., the 10 bottles containing each one of the replicates were daily opened and shaken in order to be oxygenated.

2.3. Preparation of measuring perfume replicates

In days 0 and 30, and for each one of the 7 perfumes under study, a 2 mL aliquot was taken from each 1 of the 10 replicates, which was cooled to 4 °C and filtered through a 0.45-μm membrane nylon filter. For every other day, only 5 of the 10 replicates (randomly selected) were treated in the same way.

An aliquot of 0.1 mL of the filtrate was diluted to 1 mL by adding 0.9 mL of an ethanolic solution of MIBK (1 μL/mL), and subjected to GC–FID analysis within the same day, as described below. This process was repeated during 30 days, and for the 7 perfumes under study.

2.4. GC–FID analysis

The GC–FID system consisted of a Focus GC gas chromatograph equipped with a flame ionization detector and an AI 3000 autosampler, all from Thermo Fisher Scientific (Austin, TX, USA).

For each one of the 7 perfumes under study, 1 μL of each one of the previously described solutions was injected into the GC injection port set at 250 °C in split mode (1:30), and run at 1 mL/min nitrogen constant flow rate by using a TRB-5 (95%

Table 1
Composition of the seven perfumes under study (the protocol followed for preparation of each perfume is shown on the footnote).

	%(v/v)						
	Perfume 1 [a]	Perfume 2 [a]	Perfume 3 [b]	Perfume 4 [a]	Perfume 5 [a]	Perfume 6 [c]	Perfume 7 [a]
Phase A							
EtOH	50.0	50.0	50.0	50.0	50.0	50.0	50.0
Ethanolic solution of 1 g/100 mL BHT	4.0	4.0	2.5	4.0	4.0	–	4.0
Perfume concentrate 1[d]	9.0	–	–	–	–	–	–
Perfume concentrate 2[d]	–	9.0	–	–	–	–	–
Perfume concentrate 3[d]	–	–	4.5	–	–	–	–
Perfume concentrate 4[d]	–	–	–	9.0	–	–	–
Perfume concentrate 5[d]	–	–	–	–	9.0	–	–
Perfume concentrate 6[d]	–	–	–	–	–	9.0	–
Perfume concentrate 7[d]	–	–	–	–	–	–	9.0
Ethanolic solution of UV filters and vitamin E[e]	–	–	–	–	–	8	–
Phase B							
EtOH	Up to 100	Up to 100	Up to 100	Up to 100	Up to 100	Up to 100	Up to 100
Phase C							
Deionized water	–	–	15.0	–	–	9.0	–
Aqueous solution of 0.08 g/100 mL EDTA	1.0	1.0	–	1.0	1.0	–	1.0
Phase D							
Deionized water	10.0	10.0	–	10.0	10.0	–	10.0

[a] Perfumes 1, 2, 4, 5 and 7: firstly, to 4 mL of BHT ethanolic solution add the rest of EtOH of phase A, and then the corresponding essence. Secondly, add slowly phase C to phase A. Next, add slowly phase D, and finally add phase B. After maceration, the mixture is cooled to 4 °C and filtered in cool.

[b] Perfume 3: firstly, to 2.5 mL of BHT ethanolic solution add the rest of EtOH of phase A, and then the corresponding essence. Secondly, add slowly water of phase C to phase A, and finally add phase B. After maceration, the mixture is cooled to 4 °C and filtered in cool.

[c] Perfume 6: firstly, to 8 mL of an ethanolic solution of ES, BDM, EMC and vitamine E add the rest of EtOH of phase A, and then the corresponding essence. Then, add slowly phase C to phase A, and finally add phase B. After maceration, the mixture is cooled to 4 °C and filtered in cool.

[d] The real names of the perfume concentrates are not shown for reasons of confidentiality.

[e] Ethanolic solution containing 0.45 g/100 mL of ES, 0.45 g/100 mL of BDM, 2.10 g/100 mL and 2.00 g/100 mL of vitamin E.

dimethyl–5% phenylpolysiloxane, 30 m, 0.32 mm i.d., 0.50 μm film thickness) analytical fused-silica capillary column from Teknokroma (Barcelona, Spain). The oven temperature program was from 70 °C (4 min) at 3 °C/min to 200 °C (12 min), and the detector was set at 280 °C.

2.5. *Linear discriminant analysis (LDA)*

The perfume maceration time used by RNB S.L. before the present study was 30 days. Thus, time 0 replicates (just prepared) were considered as not-macerated and the 30 days replicates were considered to have completed the process of maceration.

In this sense, for each studied perfume, prepared solutions from all the replicates (i.e., 10) in days 0 and 30 of maceration were analyzed by GC–FID and were used as calibration objects to build the model, based on linear discriminant analysis (LDA), using the SPSS (Statistical Package for the Social Sciences Inc., Chicago, IL, USA) 15.0 software package. Every other day only prepared solutions of 5 of the 10 replicates (randomly taken), considered as unknown objects, were analyzed by GC–FID.

Thus, each of the well-resolved GC–FID peaks (original variables), excluding antioxidants and UV filters, from each one of the obtained chromatograms were integrated, and the area of each peak was normalized considering the area of the internal standard. Next, data for each normalized peak was standardized considering the mean and standard deviation coming from the 20 calibration objects, and thus obtaining the normalized-standardized variables.

The 20 calibration objects were used to construct two calibration matrices, each one containing the normalized-standardized variables of 10 chromatographic profiles, corresponding to the measures at time 0 (just prepared) and after 30 days of maceration, respectively. The first of these matrices, containing the data from replicates at time 0 (just prepared) was labelled as *Group 1 not-macerated*, while the other matrix containing data from replicates at time 30 were labelled as *Group 2 macerated*.

The SPSS stepwise algorithm was used with the default probability values of F input (F_{in}) and F output (F_{out}) of 0.05 and 0.10, respectively, to construct the LDA model. After the model was constructed, only the normalized-standardized variables that take part in the model were considered. In this sense, the LDA model was rebuilt considering only those significant normalized-standardized variables.

In the case of the chromatographic profiles of the perfume samples subjected to different maceration times, which were considered as unknown objects, only the original variables (i.e., chromatographic peaks), corresponding to the significant normalized-standardized variables, were integrated. Next, the unknown objects were classified within *Group 1 not-macerated* or *Group 2 macerated* by using the LDA model.

2.6. *GC–MS analysis*

The GC–MS system consisted of a Focus GC gas chromatograph equipped with a DSQII mass spectrometric detector (operated in positive electron ionization mode at ionization energy of 70 eV, with a multiplier voltage set at 1400 V) and an AI 3000 autosampler, all from Thermo Fisher Scientific (Austin, TX, USA).

For each studied perfume, 1 μL of one of the solutions used as calibration objects was injected into the GC injection port set at 250 °C in split mode (1:30), and run at 1 mL/min helium constant flow rate by using a TR-5MS (95% dimethyl–5% phenylpolysiloxane, 30 m, 0.25 mm i.d., 0.25 μm film thickness) analytical fused-silica capillary column from Thermo Fisher Scientific (Austin, TX, USA). The oven temperature program was from 70 °C (4 min) at 3 °C/min to 200 °C (12 min). Transfer line and ion source temperatures were set at 280 and 225 °C, respectively. The records were obtained in full-scan mode with an m/z range from 20 to 500 at 500 amu/s scan rate.

The mass spectra of the original variables (i.e., chromatographic peaks) corresponding to the significant normalized-standardized variables of each LDA model of each one of the studied perfumes were recorded, and compounds tried to be identified by means of the comparison of their mass spectra with those found in the NIST Mass Spectral Library.

3. Results and discussion

3.1. Preliminary considerations

Perfumes are made up of hundreds of chemicals that contribute to their organoleptic characteristics [2], and as said before, the concentration of which may change during the process of maceration. In this sense, it is desirable to study the evolution in the concentration of each one of them as variable of interest in the study of the time maceration process.

On the other hand, taking advantage of the volatility of these compounds, GC was selected as the analytical technique, and a study of the chromatographic profile was carried out, studying the variation in the area of the chromatographic peaks.

To do this, firstly it was necessary to study the chromatographic variables involved, in order to obtain the maximum number of chromatographic peaks and obtain as much information as possible about the system under study. The best results were obtained using those conditions described above, in which the majority of the components of the perfumes under study were separated. Fig. 1 shows a chromatogram of each studied perfume, obtained under optimized conditions.

3.2. Linear discriminant analysis (LDA)

As said before, LDA is a supervised pattern recognition method, which describes, on the basis of a mathematical model, the behaviour of a qualitative dependent variable with the information of different quantitative independent variables throughout the so-called linear discriminant functions (LDF). The LDF are linear combinations of the original variables and up to $N-1$ LDF can be obtained, where N is the lowest value for either the number of variables or the number of groups. Among the many possibilities, those LDF that provide the highest resolution between objects belonging to different groups and the lowest between objects belonging to the same group, are obtained. This can be evaluated with the Wilks' lambda (λ_{Wilks}), which is calculated as the sum of squares of the distances between objects belonging to the same group divided by the total sum of squares in an LDF, and has values between 0 and 1. LDF with λ_{Wilks} values close to 1 indicate that the categories are overlapped in this LDF (i.e., no separation between groups), whereas a value near zero indicate that the groups are well-resolved in this LDF and therefore this LDF is capable of distinguishing groups [5].

In the case under study, only two groups (qualitative dependent variables) are possible: not-macerated or macerated, and different GC–FID peaks (quantitative independent variables), of the order of 30–50 depending of the studied perfume, are considered. Thus, based on the above-mentioned considerations, only one LDF is necessary to build the LDA model for each studied perfume, which accumulates the total (100%) variance.

Moreover, on one hand, it should be noted that not all of the perfume compounds undergo significant changes during the maceration process, thus the chromatographic area of these compounds do not change significantly, and therefore its contribution in the LDA model can be ignored. On the other hand, there may even be compounds that may vary in the same magnitude and consequently their chromatographic areas are correlated, which results in redundant information, and thus part of them can be also avoided in the LDA model.

Table 2
Number of variables used to construct the LDA model for each one of the perfumes under study and their λ_{Wilks}.

Perfume	No. peaks in chromatogram	No. peaks used in the LDA model	λ_{Wilks}
1	54	5	0.022
2	45	3	0.118
3	45	5	0.013
4	30	3	0.067
5	29	3	0.015
6	33	3	0.037
7	33	3	0.085

3.2.1. Construction of the calibration matrices and LDA models

The maceration time used by RNB S.L. for the manufacture of the perfumes under study was 30 days. Thus, those replicates just prepared and belonging to the same perfume were considered as not-macerated (*Group 1 not-macerated*) and the same replicates after 30 days of maceration were considered to have completed the process of maceration (*Group 2 macerated*).

Each of the well-resolved GC–FID peaks (original variables), excluding antioxidants and UV filters, from each one of the GC–FID profiles were integrated, and the area of each peak was normalized considering the area of the internal standard, minimizing possible deviations occurred during the GC injection process. Next, data for each normalized peak were standardized considering the mean and standard deviation of the 20 calibration objects.

After that, and based on the above-mentioned considerations, the LDA model was built using the SPSS stepwise algorithm, which considers one by one all the variables (in this case, normalized-standardized variables coming from the original variables, i.e. chromatographic peaks) so that a variable is considered significant when it causes a significant reduction in the λ_{wilks} value after its inclusion in the model. This is confirmed by an ANOVA (Analysis of Variance), which is reflected by a Fisher's F-test of comparison of variances. It should be pointed out that the inclusion of a new variable in the model can modify the signification of those which have been previously included in the model, so it is also required a significance value of rejection, which helps to decide whether a variable can be removed from the model without significant loss of information. This iterative process is completed when there are no variables entering or being eliminated from the model. The default probability values provided by SPSS, i.e. an entrance threshold (F_{in}) of 0.05 and a rejection threshold (F_{out}) of 0.10, were used.

At this point, it should be mentioned that retention time alignment, e.g. dividing the area of each chromatographic peak by its retention time, could have been carried out in order to improve the LDA model. By using this approach, the possible variations in the peak area caused by possible variations in retention time are avoided. Nevertheless, no apparent improvement was observed, since good repeatability in the retention time variation from chromatogram-to-chromatogram is achieved.

After the LDA model was constructed for each perfume under study, only the normalized-standardized variables that take part in the model were considered. In this sense, the LDA model was rebuilt considering only those significant normalized-standardized variables. Table 2 shows the number of peaks that were chosen to build the LDA model for each of the studied perfumes, along with the number of peaks that originally were present in the corresponding chromatogram. The obtained λ_{Wilks} for each model, also shown in Table 2, proves that an excellent resolution between the two groups (*Group 1 macerated* and *Group 2 not-macerated*) was obtained for each one of the 7 studied perfumes. The LDF obtained for each of the 7 perfumes under study were:

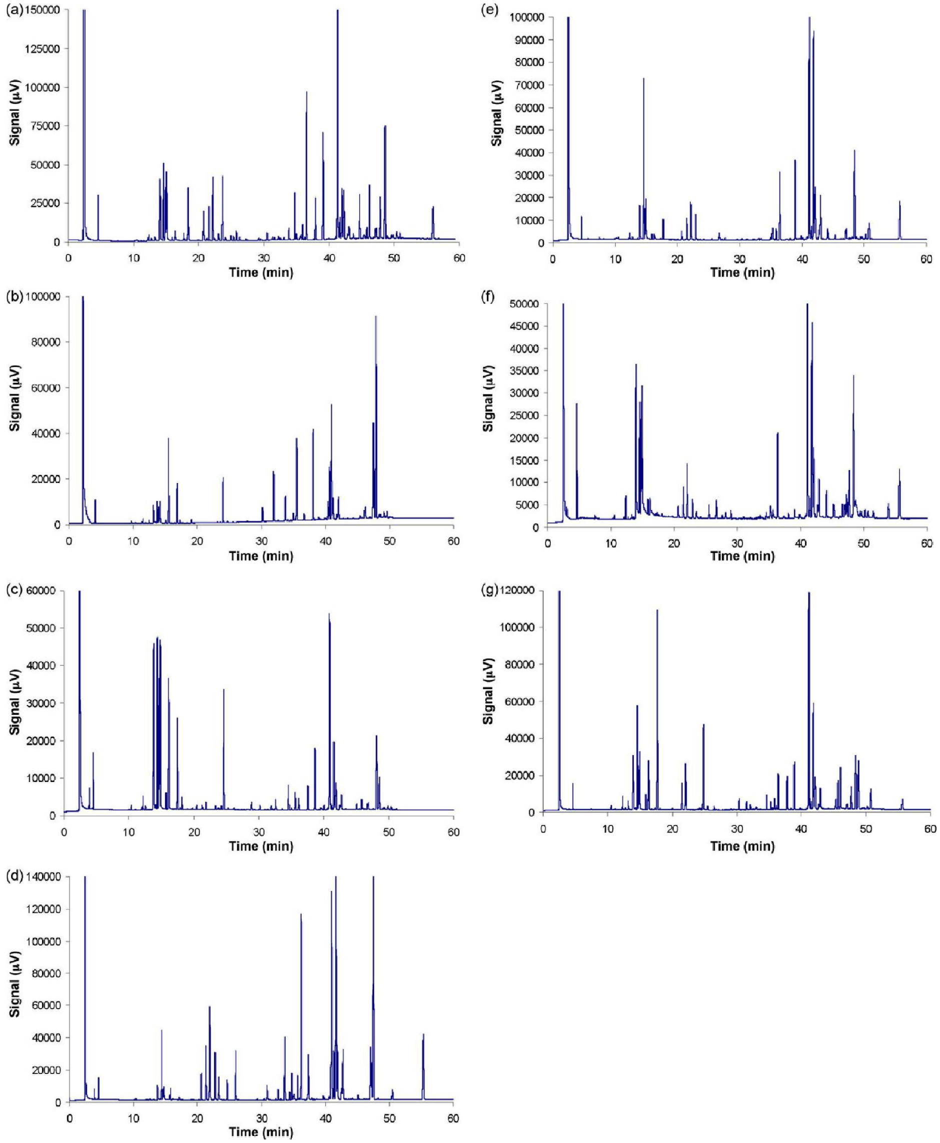

Fig. 1. GC–FID chromatograms of each 1 of the 7 perfumes under study, obtained under optimized conditions. (a) Perfume 1, (b) Perfume 2, (c) Perfume 3, (d) Perfume 4, (e) Perfume 5, (f) Perfume 6 and (g) Perfume 7.

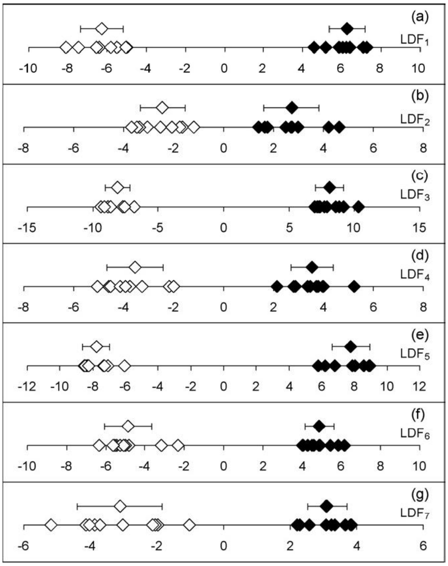

Fig. 2. Individual scores (lower part) and average of the scores with its standard deviation (upper part) of the calibration objects. (a) Perfume 1, (b) Perfume 2, (c) Perfume 3, (d) Perfume 4, (e) Perfume 5, (f) Perfume 6 and (g) Perfume 7 (♦ = *Group 1 not-macerated*; ◇ = *Group 2 macerated*; the cut-off value is 0).

Perfume 1: $LDF_1 = 4.6P_{1,18.1} - 4.7P_{1,20.7} + 6.6P_{1,27.2} - 2.3P_{1,29.5} - 1.8P_{1,34.9}$
Perfume 2: $LDF_2 = -0.6P_{2,10.4} + 2.0P_{2,11.6} + 1.1P_{2,20.1}$
Perfume 3: $LDF_3 = -5.4P_{3,18.1} + 14.2P_{3,32.6} - 11.4P_{3,42.7} + 2.8P_{3,49.4} + 2.6P_{3,49.9}$
Perfume 4: $LDF_4 = 2.1P_{4,23.3} - 15.2P_{4,30.8} + 12.7P_{4,32.6}$
Perfume 5: $LDF_5 = 2.8P_{5,7.5} + 8.2P_{5,14.7} - 3.9P_{5,27.6}$
Perfume 6: $LDF_6 = 3.9P_{6,16.3} - 6.2P_{6,41.5} + 2.4P_{6,46.7}$
Perfume 7: $LDF_7 = 8.6P_{7,31.5} - 12.5P_{7,36.4} - 5.9P_{7,55.7}$

where LDF_i is the linear discriminant function for perfume *i*, and $P_{i,j}$ is the normalized-standardized area of peak with a retention time of *j* (in minutes) in the corresponding chromatogram of perfume *i*. As mentioned before, the LDF in each one of the models accumulates the total (100%) of the variance.

The extent and sign of the different weighting factors of each LDA model shows that not only there are different compounds changing in the maceration process, but also these changes are different depending on the compound, and also depending on the perfume.

Fig. 2 shows the scores of the calibration samples in the corresponding LDF. An excellent distinction between the two groups is observed, which are consistent with the λ_{Wilks} values shown above.

Finally, it should be emphasised, that for the 7 perfumes under study, all the objects of the calibration matrix were correctly classified by leave-one-out cross-validation.

3.2.2. Classification of the perfume samples subjected to different maceration times

Once the LDA models were constructed, only the original variables (i.e., chromatographic peaks) corresponding to the significant normalized-standardized variables were integrated from the chromatographic profiles of the perfumes subjected to different maceration times, and thus, they were treated as unknown objects. So, the LDA models were used to assign to one or the other group these unknown objects, and then evaluate whether the maceration process was completed or not in the different days of study. Fig. 3 shows the scores of the calibration samples besides the scores of those samples at different time of maceration for each one of the 7 studied perfumes.

Based on these results, we can assume that during the maceration process of the 7 perfumes under study there are significant changes in the composition of the mixture, which are reflected by changes in the chromatographic profile obtained by GC–FID, which have been manifested in the scores on the corresponding LDF.

In this way, Fig. 3 shows that the replicates subjected to different maceration times have different scores depending on this time, so they can be classified in one or the other group (*Group 1 not-macerated*, *Group 2 macerated*) based on these ratings, depending of their position on either side of the cut-off value, which is set at 0. Maceration time is considered to be over when the scores of the perfumes subjected to different maceration times are classified as *Group 2 macerated*. This time is different for each one of the perfumes being studied.

So, regarding Perfume 1, as it is shown in Fig. 3(a), on days 1 and 2 of maceration, replicates have similar scores to those of *Group 1*, which indicates that they present a similar chromatographic profile to the calibration objects from *Group 1 not-macerated*. From day 3 to day 13 of maceration, the replicates have generally similar scores to *Group 2 macerated*, which indicates that they present a similar chromatographic profile to the calibration objects in this group. However, in some cases, some of the 5 replicate samples had slightly different scores, which makes them similar to those of *Group 1 not-macerated*. From day 14 to day 30 of maceration all replicates can be assigned to *Group 2 macerated*. Therefore, for Perfume 1 it can be concluded that changes occur during the first 13 days of the maceration process, being more pronounced during the first 2 days. From day 14 of maceration, and until day 30, no significant changes in the chromatographic profile are observed, so it can be concluded that the maceration process is completed after 14 days.

In a similar way, regarding Perfume 2, and as can be concluded from Fig. 3(b), there are changes during the first 20 days of the maceration process, and from this day there are no significant changes in the chromatographic profile, so it can be concluded that Perfume 2 has completed its maceration process after 21 days.

Regarding Perfume 3 and as can be concluded from Fig. 3(c), changes occur during the first 20 days of the maceration process, being more pronounced during the first 14 days. Therefore, we conclude that for Perfume 3, the maceration process ended on day 20.

Regarding Perfume 4, Fig. 3(d) shows that changes occur during the first 24 days of the maceration process, being more pronounced during the first 13 days. From day 24 of maceration, and until day 30, no significant changes in the chromatographic profile are observed. So, the maceration process of Perfume 4 can be established in 24 days.

Regarding Perfume 5, and as it is shown in Fig. 3(e), changes occur during the first 28 days of the maceration process, and from this day there are no significant changes in the chromatographic profile, so it can be concluded that Perfume 5 has completed its maceration process after 28 days.

As can be seen in Fig. 3(f), data of Perfume 6 show similar scores to those of *Group 1 not-macerated* in days between 1 and 14 of maceration. From day 21 of maceration all replicates, with some exceptions, can be assigned to *Group 2*. So, the maceration process of Perfume 6 can be established in 21 days.

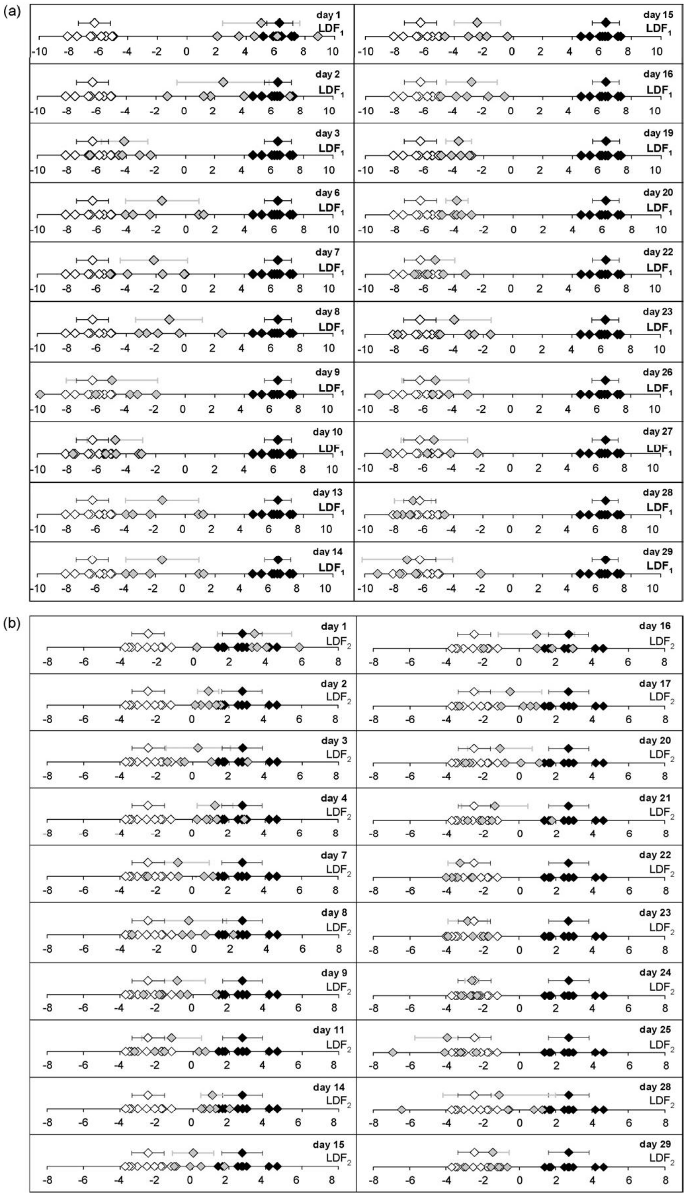

Fig. 3. Individual scores (lower part) and average of the scores with its standard deviation (upper part) of (a) Perfume 1, (b) Perfume 2, (c) Perfume 3, (d) Perfume 4, (e) Perfume 5, (f) Perfume 6 and (g) Perfume 7 at different days (♦ = *Group 1 not-macerated*; ◇ = *Group 2 macerated*; ◈ = replicates at different maceration times; the cut-off value is 0).

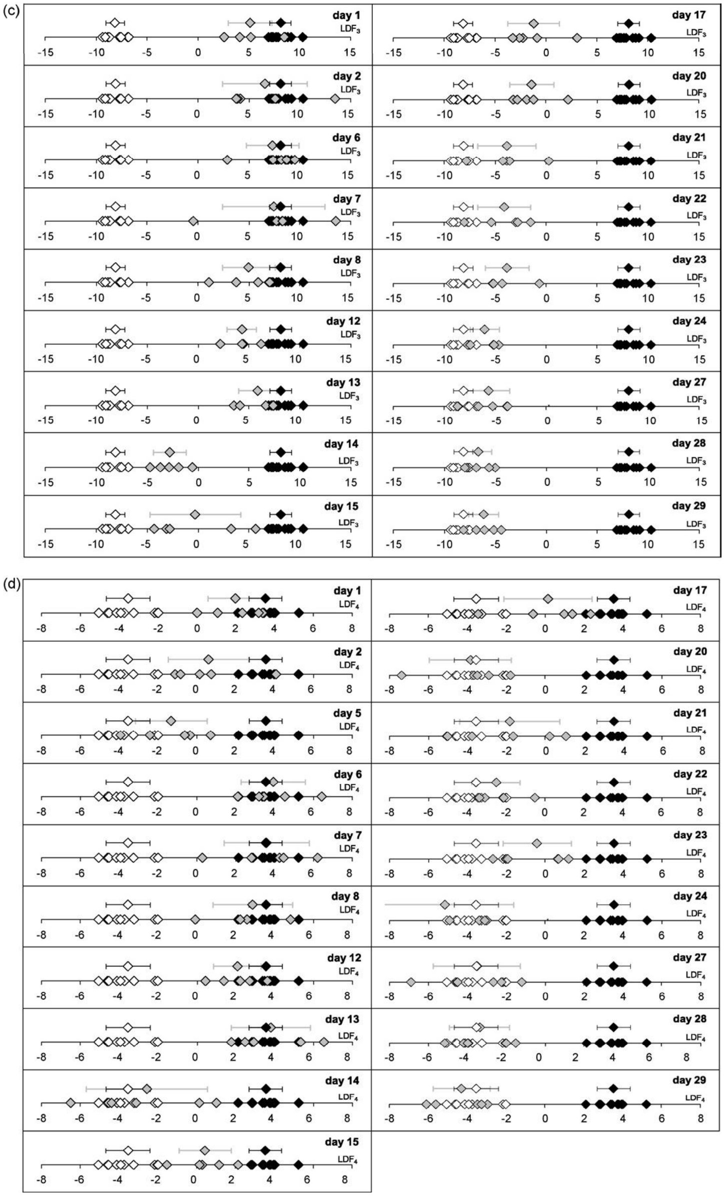

Fig. 3. (*Continued*)

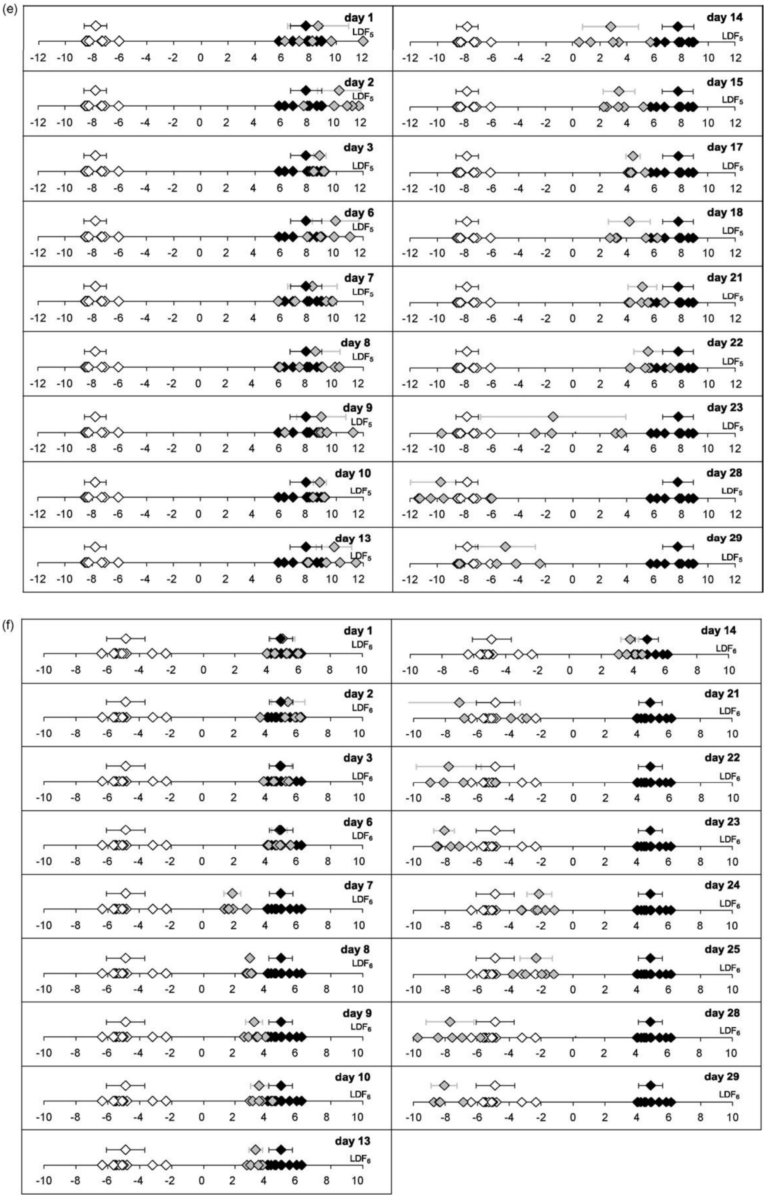

Fig. 3. (*Continued*)

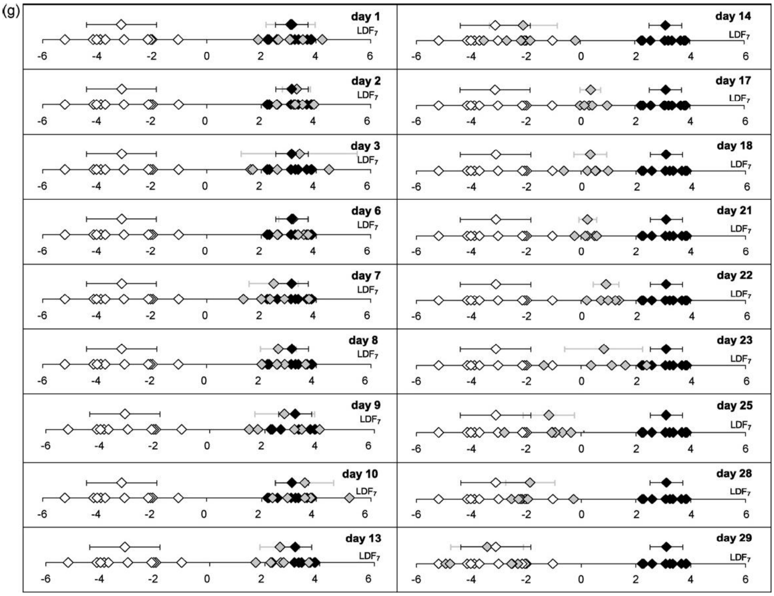

Fig. 3. (*Continued*).

Finally, for Perfume 7, Fig. 3(g) shows that changes occur during the first 25 days of the maceration process, being more pronounced during the first 13 days. Therefore, we conclude that for Perfume 7, the maceration process ended on day 25.

As summary, Table 3 shows the estimated maceration time of the 7 perfumes under study, with the percentage of reduction with respect to the initial maceration time set by the manufacturer (30 days). As can be seen, up to a reduction of 57% has been achieved with respect to the maceration time initially established.

3.3. *MS identification of the significant GC–FID peaks*

The compounds corresponding to the significant GC–FID peaks in each LDA model corresponding to each one of the 7 perfumes under study were tried to be elucidated by means of their mass spectrum acquired under conditions shown in Section 2.6. With only few exceptions, the fragrances were identified by means of their MS spectra, and they were closely related to perfumes, by providing different characteristic notes. For example, benzyl acetate, α-isomethylionone, 4-tert-butylcyclohexyl acetate, lilial, among others, which provide floral notes were identified as significant peaks in some of the perfumes under study. Green notes, like borneol, camphene, β-cubenene, β-pinene and (Z)-3-hexen-1-ol, related with notes that create the sensation of smelling recently cut grass and leaves, were also identified. Finally, triplal, citronellyl acetate and brassilate ethylene, which provide citrus, fruity and woody notes, respectively, were also identified.

Table 3
Estimated maceration time of the 7 perfumes under study and the reduction this represents over the initial maceration time set by the manufacturer (30 days).

Perfume	Estimated maceration time (days)	Reduction (%)
1	14	57
2	20	34
3	20	34
4	24	20
5	28	7
6	21	30
7	25	17

4. Conclusions

The developed GC–FID methodology combined with the treatment of data by LDA has enabled to establish the maceration time, from a chemical standpoint, involved in perfume manufacture.

This methodology has been applied successfully to 7 different perfumes, and up to a reduction of 57% has been achieved with respect to the maceration time initially established.

The obtained results by the proposed methodology are being further correlated with those obtained by a panel of olfactory experts.

Acknowledgements

Authors would like to thank RNB S.L. (Project n. UV 20080516) to have entrusted the accomplishment of this study to our research group and the financial support. We would like to thank especially Pascual Cuadrado, Manuel Alonso and Ángel Balaguer from RNB S.L. for their collaboration in this study. Marina López-Nogueroles is also grateful to RNB S.L. and the University of Valencia for her research grant.

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Published in:

M. Lopez-Nogueroles, A. Chisvert, A. Salvador, A. Carretero,
Dispersive liquid-liquid microextraction followed by gas chromatography-mass spectrometry for the determination of nitro musks in surface water and wastewater samples,
Talanta 85 (2011) 1990-1995.

Talanta 85 (2011) 1990–1995

Contents lists available at ScienceDirect

Talanta

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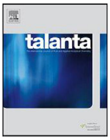

Dispersive liquid–liquid microextraction followed by gas chromatography–mass spectrometry for the determination of nitro musks in surface water and wastewater samples

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ARTICLE INFO

Article history:
Received 20 April 2011
Received in revised form 1 July 2011
Accepted 9 July 2011
Available online 19 July 2011

Keywords:
Dispersive liquid–liquid microextraction
Fragrance chemicals
Gas chromatography–mass spectrometry
Nitro musk
Water analysis

ABSTRACT

A new, simple, fast and high sensitive analytical method based on dispersive liquid–liquid microextraction (DLLME) followed by gas chromatography–mass spectrometry (GC–MS) for the simultaneous determination of nitro musks in surface water and wastewater samples is presented. Different parameters, such as the nature and volume of both the extraction and disperser solvents and the ionic strength and pH of the aqueous donor phase, were optimized. Under the selected conditions (injection of a mixture of 1 mL of acetone as disperser solvent and 50 μL of chloroform as extraction solvent, no salt addition and no pH adjustment) the figures of merit of the proposed DLLME–GC–MS method were evaluated. High enrichment factors, ranging between 230 and 314 depending on the target analyte, were achieved, which redound to limits of detection in the ng L^{-1} range (i.e., 4–33 ng L^{-1}). The relative standard deviation (RSD) was below 5% for all the target analytes. Finally, the recoveries obtained for different water samples of diverse origin (sea, river, irrigation channel and water treatment plant) ranged between 87 and 116%, thus showing no matrix effects.

1. Introduction

Musk compounds have been widely used as fragrance chemicals in many consumer products such as cosmetics, detergents, herbicides, food additives or household products [1,2]. Natural musk, obtained as a secretion produced by the testicles of the musk deer, was already used in ancient times but due to economical and ethical motives synthetic musks were developed and are the ones used at present. Synthetic musks have been generally divided in three subgroups: nitro musks, polycyclic musks and macrocyclic musks [3].

The nitro musk group is formed by five compounds, which structure is shown in Fig. 1. They are considered persistent pollutants due to their strong tendency to bioaccumulate [4] and many papers have been written about their health risks, showing they are related with different types of dermatitis, carcinogenic effects and endocrine dysfunction [2,5–10]. In fact, the use in cosmetic products of musk ambrette (MA), musk tibetene (MT) and musk moskene (MM) is banned in the European Union while the use of musk xylene (MX) and musk ketone (MK) is restricted [11]. Nevertheless, its use is permitted in North America [12]. Moreover, the removal of nitro musks in municipal sewage treatment plants is not quantitative (in fact, it has been estimated to be between 60 and 80% [13]) and in some cases they are transformed to amino derivatives [4,14], which may be even more dangerous than their parent compounds [15]. This highlights the need of better wastewater treatments. Thus, nitro musks indirectly reach the aquatic environment via wastewater treatments plants. Additionally, owing to their presence in cosmetic products, they can directly reach the aquatic environment from swimming activities in seas, rivers and lakes. Therefore, it is important to develop new analytical methods to evaluate its potential for bioaccumulation on aquatic ecosystems.

Some articles dealing with the determination of nitro musks in environmental and biological samples can be found in the literature [4,16]. They have been identified in very diverse samples, such as air [17], water and sediment [18–27], fish [12,28], blood [29,30], human adipose tissue [31] or human milk [32,33], thus showing their bioaccumulation potential. They have also been determined in cosmetic products [34,35]. As nitro musks are found at trace level in the environment, extraction techniques to clean up and concentrate are needed. The most commonly used are liquid–liquid extraction (LLE) [21,22,24], solid phase extraction (SPE) [19,26] and solid-phase microextraction (SPME) [20], and in a minor extent stir

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doi:10.1016/j.talanta.2011.07.048

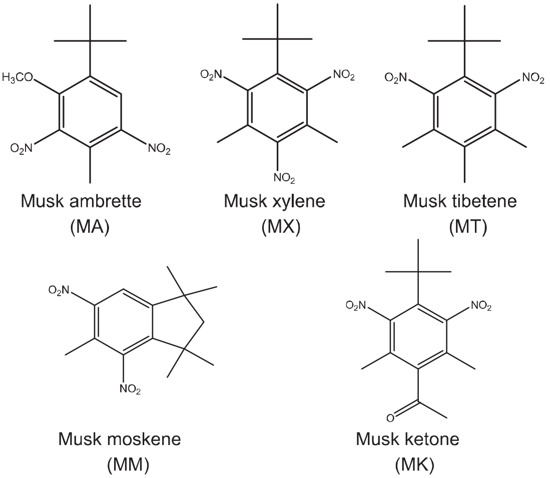

Fig. 1. Chemical structure of the target compounds.

bar sorptive extraction (SBSE) [27], followed by gas chromatography with electron capture (ECD), flame ionisation (FID) or mass spectrometry (MS) detection [4,16].

Recently, a very simple and rapid technique, called dispersive liquid–liquid microextraction (DLLME) was developed [36] in order to reduce the volume of solvent and time required in other extraction techniques while achieving high enrichment factors. The good results in terms of concentration levels are due to the fact that only a few μL of organic extracting solvent are used. On the other hand, the low extraction times are due to the fact that the equilibrium state is immediately achieved since the contact area between the extracting solvent and the sample solution is infinitely large. The principles of this extraction technique can be read elsewhere [36,37].

DLLME has been used to concentrate in environmental water samples different substances found in cosmetics, such as hydroxylated benzophenones [38], used as UV-filters, some phthalates [39], used as solvents in perfumery, and even polycyclic musks [39]. A further modification of DLLME based on the use of ultrasounds as emulsifying system was also used for the determination of some phthalates, besides of some nitro (MX, MK and MM) and polycyclic musks [40]. Nevertheless, as far as we know, the high potential for preconcentration of the DLLME has never been used before for the determination of the complete family of the nitro musk compounds.

In this sense, the aim of this paper is to develop a simple, fast and high sensitive analytical method to determine all the above mentioned nitro musks in wastewater and surface water samples, which could be useful for environmental surveillance purposes. This method is based on DLLME as extraction technique followed by GC–MS analysis.

2. Experimental

2.1. Reagents and samples

1-Tert-butyl-3-methyl-2,4-dinitroanisole (MT) 99%, 1,1,3,3,5-pentamethyl-4,6-dinitroindane (MM) 99% and a cyclohexane solution of 100 mg mL^{-1} of 1-tert-butyl-3,5-dimethyl-2,4,6-trinitrobenzene (MX) 99.5%, purchased from LGS standards (Lancashire, United Kingdom) and 6-tert-butyl-3-methyl-2,4-dinitroanisole (MA) 99% and 4-tert-butyl-2,6-dimethyl-3,5-dinitro-acetophenone (MK) 98% purchased from Dr. Ehrenstorfer (Augsburg, Germany) were used as standards. Deuterated benzophenone (benzophenone-d_{10} (BZ-d_{10})) 99% from Sigma–Aldrich (Miamisburg, OH, USA) was used as surrogate.

LC grade absolute ethanol (EtOH) from Scharlau (Barcelona, Spain), was used as solvent to prepare dilute solutions (1 and 10 μg mL^{-1}) of the standards. Deionised water (resistivity $\geq$ 18 MΩ cm) obtained by means of a Nanopure II water purification system from Barnstead (Boston, MA, USA) was used to prepare the working standard solutions.

Analytical reagent grade chloroform and dichloromethane, from Scharlau Chemie (Barcelona, Spain), were tested as extraction solvents. EtOH, ultrapure acetone and LC grade acetonitrile, all from Scharlau Chemie (Barcelona, Spain), were tested as disperser solvents.

Analytical reagent grade sodium chloride (NaCl) 99% from Scharlau Chemie (Barcelona, Spain) was used to adjust the ionic strength. Analytical reagent grade sodium hydroxide (NaOH) and phosphoric acid (H_3PO_4), also from Scharlau Chemie (Barcelona, Spain), and analytical reagent grade sodium dihydrogen phosphate monohydrate ($NaH_2PO_4{\cdot}H_2O$) from Panreac (Barcelona, Spain) were used to adjust the pH.

High purity helium (99.9999%) from Carburos Metalicos S.A. (Paterna, Spain) was used as carrier gas in the GC–MS system.

Water samples were all collected from different sources in the province of Valencia (Spain): sea water from the *Malvarrosa Beach* (Valencia), river water from the *Mijares River* (Montanejos), irrigation water from a local irrigation channel (La Eliana) and influent and effluent wastewater from a treatment plant (Gandia). All samples were stored in the dark at 4 °C and filtered through 0.45 μm nylon membrane filters before the analysis.

2.2. Apparatus

The GC–MS system consisted of a Focus GC gas chromatograph coupled to a DSQII mass spectrometry detector equipped with an AI3000 autosampler, all from Thermo Fisher Scientific (Austin, TX, USA).

The chromatographic separations were made using a HP-5MS Ultra Inert (95% dimethyl-5% diphenylpolysiloxane, 30 m length, 0.25 mm i.d., 0.25 μm film thickness) analytical fused-silica capillary column from Agilent Technologies (Palo Alto, CA, USA).

An EBA 21 centrifuge from Hettich (Tuttlingem, Germany) and a Micro-pH 2000 pHmeter from Crison (Alella, Spain) were also used.

2.3. Proposed method

2.3.1. DLLME procedure

5 mL of an aqueous standard solution containing the analytes in a concentration between 100 and 1000 ng L^{-1} or an environmental water sample (in triplicate) were transferred to a polyethylene 15 mL centrifuge tube, and 10 μL of a 2.5 μg mL^{-1} solution of BZ-d$_{10}$ (as surrogate) in EtOH were added. Then, 1 mL of acetone previously mixed with 50 μL of chloroform was injected to this solution. After shaking with a vortex mixer (ca. 3 s), the tube was centrifuged for 5 min at 6000 rpm. Thus, separation of two phases occurred, and approximately 10 μL of the sedimented phase were collected with a 50 μL Hamilton 1705 RNR syringe (Bonaduz, Switzerland) and transferred into a 100 μL insert placed inside a 1.5 mL injection vial, which was then ready to be injected into the chromatographic system.

2.3.2. GC–MS analysis

1 μL of the aforementioned sedimented phase was injected into the GC system coupled to a mass spectrometry detector operated in positive electron ionisation mode at ionisation energy of 70 eV and with a multiplier voltage set at 1400 V. The inlet temperature was 280 °C and the injection was accomplished in splitless mode (splitless time: 1 min). The separation was run at a 1 mL min^{-1} helium constant flow rate. The oven temperature program was: from 60 °C (1 min) to 120 °C at 20 °C min^{-1}, then to 185 °C (0 min) at 10 °C min^{-1}, then to 195 °C (0 min) at 1 °C min^{-1} and finally to 280 °C (5 min) at 25 °C min^{-1}. The transfer line and ion source temperatures were set at 280 and 250 °C, respectively. The chromatograms were recorded in selected ion monitoring (SIM) mode at the following mass/charge (m/z) ratios: m/z 110 from 10.0 to 14.0 min for BZ-d$_{10}$, m/z 253, 263 and 282 from 14.0 to 16.6 min for MA, MX and MM, respectively, and m/z 251 and 279 from 16.6 to 20.0 min for MT and MK, respectively. A full scan mode (m/z from 40 to 300) was simultaneously recorded from 10.0 min to the end of the analysis time.

Fig. 2 shows a chromatogram of an aqueous standard solution containing the five target analytes at 500 ng L^{-1} (and BZ-d$_{10}$ as surrogate at 5 ng mL^{-1}) subjected to the described DLLME–GC–MS procedure.

3. Results and discussion

The optimum DLLME parameters, such as the nature and volume of both the extraction and disperser solvent or the ionic strength and pH of the aqueous donor phase, need to be selected in order to achieve high enrichment factors (EF), defined as the ratio between the concentration of the compound in the organic sedimented phase and the initial concentration in the aqueous donor phase.

In DLLME, the extraction time is defined as the period between the injection of the binary mixture of disperser and extraction solvents and the centrifugation.

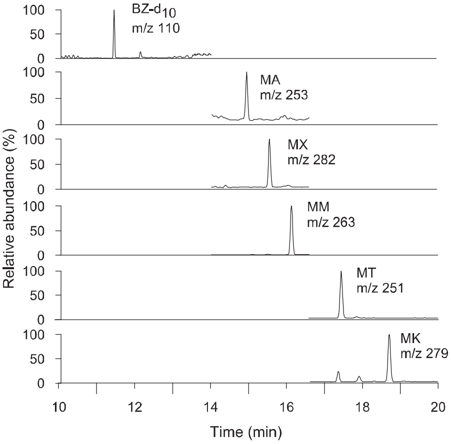

Fig. 2. A chromatogram obtained applying the proposed DLLME–GC–MS method to an aqueous standard solution containing the five target analytes at 500 ng L^{-1} and BZ-d$_{10}$ as surrogate at 5 ng mL^{-1}.

In this work, the effect of extraction time has not been studied because, as stated in the literature, it does not affect the results in DLLME [36,37]. This is, in fact, one of the major advantages of DLLME and is due to the fact that the surface area between the extraction solvent and the aqueous phase is extremely large, reaching equilibrium state almost instantaneously.

Thus, to study the different parameters, the DLLME procedure was carried out to 5 mL of a standard aqueous solution of the five target analytes at 10 ng mL^{-1}. The peak area of each target analyte (A_i) was used as response function.

3.1. Selection of the extraction and disperser solvents

The selection of the extraction and disperser solvents is, probably, one of the most important parameters in DLLME. The selected extraction solvent has to be immiscible and have higher density than the aqueous donor phase so it can settle after centrifugation. It should also extract the compounds of interest and have a good behavior for the further analytical technique going to be used (in this case GC). Regarding the disperser solvent, it has to form the cloudy solution and has to be miscible both in the aqueous donor phase and the extraction solvent.

In this sense, dichloromethane (density 1.25 g mL^{-1}) and chloroform (density 1.48 g mL^{-1}) were studied as extraction solvents, and acetone, acetonitrile and ethanol as disperser solvents. Carbon tetrachloride is usually used as extraction solvent in DLLME [37,41] but its use is banned by the Montreal Protocol from 2010. Thus, 1 mL of each disperser solvent previously mixed with 50 μL of each extraction solvent were rapidly injected into 5 mL of the aqueous standard solution. Dichloromethane did not form cloudy solution in none of the three combinations tested. A cloudy solution was also not obtained when the chloroform–ethanol combination was tested. When comparing the other two options (chloroform as extraction solvent and acetone or acetonitrile as disperser solvent) similar results were obtained. The chloroform–acetone combination was chosen because of low toxicity and cost.

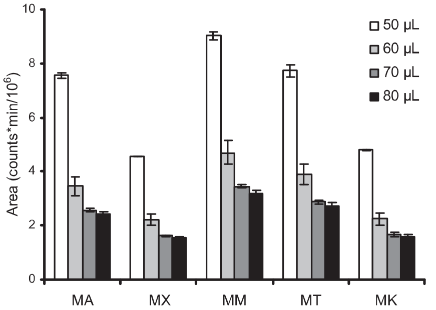

Fig. 3. Effect of the extraction solvent volume on the DLLME process. Extraction conditions: standard aqueous solution volume: 5 mL; disperser solvent volume: 1000 μL. Results are the average of 3 replicates, and the error bars show the standard deviation.

3.2. Effect of the extraction solvent volume

In order to study the effect of the extraction solvent volume, different volumes of chloroform, ranging from 40 to 80 μL, were mixed with 1 mL of acetone and injected into 5 mL of the standard aqueous solution. 40 μL of chloroform was discarded because the obtained sedimented phase volume was not enough to handle and inject into the GC. According to the data (Fig. 3), the best results were obtained when the extraction solvent volume was 50 μL.

3.3. Effect of the disperser solvent volume

In this case, different volumes of acetone, ranging from 250 to 1000 μL, were tested while all other parameters were kept constant. Fig. 4 shows that the signal increases with the disperser solvent volume and tends to stabilize after 750 μL. Thus 1000 μL of disperser solvent was selected for further experiments.

3.4. Effect of the ionic strength of the aqueous donor phase

To study this effect, the DLLME procedure was performed to different aqueous standard solutions, all containing 10 ng mL^{-1} of the target compounds but different concentrations of NaCl (ranging from 0 to 10% (m/v)). It should be noted that the addition of salt could have different effects in the extraction procedure. On one hand, the increase of the ionic strength decreases the solubility of the extraction solvent in the aqueous phase, due to the salting out effect, and therefore, the volume of the sedimented phase increases. On the other hand, the salting out effect is expected to favour the extraction of the target compounds from the aqueous phase to the organic phase. In this case, Fig. 5 shows how the addition of salt reduces the signal obtained, due to the fact that the dilution caused by the higher volume is more important on the target compounds than the salting out effect. In fact, volume did increase considerably with the ionic strength, as volumes of 8 ± 2, 17 ± 2, 23 ± 4 and 42 ± 2 μL for 0, 2.5, 5 and 10% (m/v) of NaCl concentration in the aqueous donor phase, respectively, were collected. Therefore, in subsequent experiments, no salt was added to the donor phase.

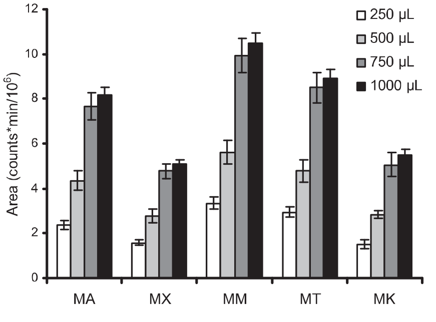

Fig. 4. Effect of the disperser solvent volume on the DLLME process. Extraction conditions: standard aqueous solution volume: 5 mL; extraction solvent volume: 50 μL. Results are the average of 3 replicates, and the error bars show the standard deviation.

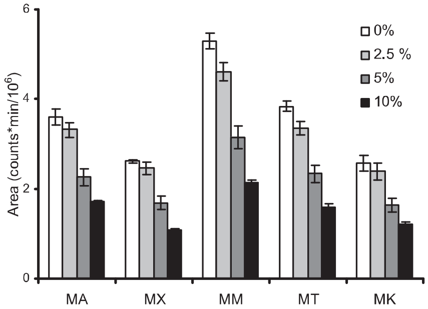

Fig. 5. Effect of the ionic strength of the donor phase on the DLLME process. Extraction conditions: standard aqueous solution volume 5 mL; extraction solvent volume: 50 μL; disperser solvent volume: 1000 μL. Results are the average of 3 replicates, and the error bars show the standard deviation.

3.5. Effect of the pH of the aqueous donor phase

The pH of the donor phase can considerably affect the extraction of compounds with potentially ionisable functional groups. In this case, the five target nitro musks do not present ionisable moieties, thus it is expected that pH does not affect the extraction. Nevertheless, it was studied using different aqueous standard solutions buffered at different pH values (ranging from 2 to 10) with 10^{-3} M phosphate buffer.

Results confirmed, as expected, that there was no difference in the extraction of the target compounds at the different pH values studied. Thus, in subsequent experiments the pH was not adjusted.

3.6. Use of surrogate

In order to reduce the variability of the measurements, especially caused by the handling of low volumes in the DLLME process, the use of deuterated benzophenone, i.e., benzophenone-d$_{10}$ (BZ-d$_{10}$), as surrogate was considered. Thus, A_i/A_{sur} (where A_i is the peak area of the target analyte and A_{sur} that of the surrogate) was used as response function for quantification purposes.

BZ-d$_{10}$ was selected for various reasons: (1) it is extractable in chloroform by the DLLME proposed method; (2) its volatility is suitable to be measured by GC; (3) as it is a deuterated compound, its possible presence in the environmental samples is nil, on the contrary of its non-deuterated homologous; and (4) it does not present ionisable functional groups in its structure, and thus, its extraction is not expected to be influenced by the pH.

Table 1
Main parameters of the proposed DLLME–GC–MS method.

Analyte	Slope[a] $(\text{ng L}^{-1})^{-1}$	Intercept[a]	Regression coefficient[a]	LOD[b] (ng L^{-1})	LOQ (ng L^{-1})[c]	Enrichment factor	RSD (%)[d]
MA	$(94 \pm 2) \times 10^{-5}$	0.12 ± 0.03	0.9992	33	109	289	3.2
MX	$(613 \pm 9) \times 10^{-6}$	0.11 ± 0.02	0.9993	19	63	263	3.5
MM	$(129 \pm 3) \times 10^{-5}$	0.22 ± 0.06	0.998	6	19	248	4.3
MT	$(107 \pm 2) \times 10^{-5}$	0.14 ± 0.05	0.998	4	14	230	3.1
MK	$(62 \pm 1) \times 10^{-5}$	0.04 ± 0.02	0.9991	7	24	314	1.4

[a] Working range: 100–1000 ng L^{-1}. Number of calibration points: 5.
[b] Limit of detection, calculated as 3 times the signal-to-noise ratio.
[c] Limit of quantification, calculated as 10 times the signal-to-noise ratio.
[d] Relative standard deviation (RSD) obtained in the analysis of five replicates of an aqueous standard solution containing 500 ng L^{-1} of the target compounds.

Since the pH of the samples is not adjusted (see Section 3.5), it is mandatory to check that the extraction of BZ-d_{10} is not influenced by the pH of the samples. In this sense, different aqueous solutions buffered at different pH values (ranging from 2 to 10) containing 5 ng mL^{-1} of BZ-d_{10} were prepared and analyzed according to the DLLME–GC–MS proposed method. As expected, results revealed that the extraction of BZ-d_{10} was not affected by the pH.

On the other hand, and as concluded before (see Section 3.4), the ionic strength is not adjusted in the samples. This could involve serious problems from the accuracy standpoint if sea water samples are analyzed, since their salt content can reach up to 3.5% (m/v) and could affect the extraction of the target compounds. However, when the influence of the ionic strength was studied on the extraction of BZ-d_{10}, results revealed that it was affected in the same way as analytes, owing to the increase in the sedimented phase volume. Thus, when A_i/A_{sur} was used as response function, the effect of the ionic strength was negligible.

3.7. Analytical figures of merit of the proposed DLLME–GC–MS method

Under optimized conditions different quality parameters were evaluated and are summarized in Table 1.

Calibration curve was constructed and linearity reached at least 20 ng mL^{-1}. The working range employed was set from 100 to 1000 ng L^{-1}. Table 1 shows the equations obtained for all the target compounds and its regression coefficient.

Table 1 also shows the limits of detection (LOD) and quantification (LOQ) calculated as 3 or 10 times the signal-to-noise ratio, respectively. These values are ranged in the ng L^{-1} level. Nevertheless, analytical methods providing lower LOD can be found in the analytical literature, but they are based on more time-consuming extraction techniques, such as SPME and SBSE. In any case, the LOD obtained by the proposed method are suitable to determine these compounds at ultratrace levels in water.

The EF ranged between 230 and 314 depending on the analyte. As concentration is proportional to the signal obtained, the EF were calculated comparing the areas obtained when measuring the sedimented phase after applying the proposed DLLME–GC–MS method to a standard aqueous solution and a standard solution of the target analytes in chloroform.

The repeatability, expressed as relative standard deviation (RSD), was evaluated by applying the proposed DLLME–GC–MS method to five replicates of a standard aqueous solution containing 500 ng L^{-1} of the target compounds and 5 ng mL^{-1} of the surrogate and resulted below 5% in all cases.

3.8. Application of the proposed DLLME–GC–MS method to the analysis of environmental water samples

The target analytes were determined in different wastewater and surface water samples of different matrix composition (sea, river, irrigation channel and water treatment plant) (see Section 2.1). The results showed that all of them were below the limits of detection. Moreover, the full scan mode also showed that no amino-musks derivatives, which can be formed in the processes of the wastewater treatment plants [4], were detected in the analyzed samples.

Table 2
Recovery values obtained in the analysis of five water samples by using the proposed DLLME–GC–MS method.

Analyte	Recoveries (%)[a]				
	Sample 1[b]	Sample 2[c]	Sample 3[d]	Sample 4[e]	Sample 5[f]
MA	93 ± 4	95 ± 5	106 ± 4	98 ± 13	95 ± 14
MX	88 ± 6	105 ± 8	103 ± 6	107 ± 9	96 ± 10
MM	87 ± 5	95 ± 6	103 ± 6	109 ± 11	94 ± 11
MT	87 ± 6	93 ± 7	102 ± 6	105 ± 9	93 ± 10
MK	88 ± 6	92 ± 8	99 ± 8	107 ± 3	116 ± 5

[a] Spiking level: 500 ng L^{-1}.
[b] Sample 1: Sea water (Valencia).
[c] Sample 2: River water (Montanejos).
[d] Sample 3: Irrigation water (La Eliana).
[e] Sample 4: Wastewater treatment plant influent (Gandia).
[f] Sample 5: Wastewater treatment plant effluent (Gandia).

3.9. Recovery studies

In order to evaluate matrix effects, recovery studies were carried out. In this sense, the proposed DLLME–GC–MS method was applied to the five above-mentioned samples that had been previously spiked with the target analytes at 500 ng L^{-1}. The recoveries obtained (Table 2) ranged between 87 and 116%, depending on the analyte. These results demonstrate that these water matrices have no significant effect on the extraction process.

4. Conclusions

A simple, fast and high sensitive analytical method is presented in this paper in order to determine the complete family of nitro musk compounds in environmental water samples.

This method is based on DLLME as extraction technique prior to analysis by GC–MS and presents good analytical features, especially high enrichment factors that allow their determination in the decade ng L^{-1} range. Although analytical methods providing lower LOD can be found in the analytical literature, these are based on more time-consuming extraction techniques, which make the proposed method a good alternative for treating a lot of samples, as environmental surveillance demands.

Finally, it should be emphasized that the present method is a good alternative to other methods as it consumes very low levels of solvents and is very fast.

Acknowledgements

The authors acknowledge the financial support of the Spanish Ministry of Science and Innovation (Project CTQ2009-12709), especially Marina López-Nogueroles for her FPI predoctoral grant.

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Development of a selective solid phase extraction method for nitro musk compounds in environmental waters using molecular imprinted sorbents

Published in:

M. Lopez-Nogueroles, S. Lordel-Madeleine, A. Chisvert, A. Salvador, V. Pichon,
Development of a selective solid phase extraction method for nitro musk compounds in environmental waters using a molecularly imprinted sorbent,
Talanta 110 (2013) 128-134.

Talanta 110 (2013) 128–134

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Development of a selective solid phase extraction method for nitro musk compounds in environmental waters using a molecularly imprinted sorbent

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ARTICLE INFO

Article history:
Received 31 October 2012
Received in revised form
1 February 2013
Accepted 11 February 2013
Available online 18 February 2013

Keywords:
Musk
Nitro musk
Molecular imprinted silica (MIS)
Environmental analysis
Fragrance chemicals
Gas chromatography–mass spectrometry
Water analysis

ABSTRACT

A powerful analytical method for the determination of the family of the nitro musk compounds at trace level in environmental waters—river, sea, and water from a treatment plant—is presented. The method is based on the use of molecularly imprinted silica (MIS) as sorbent for solid phase extraction (SPE) used for the clean-up and the concentration step of the target analytes previous to their determination by gas chromatography–mass spectrometry. The optimized extraction procedure allowed extraction recoveries between 61% and 87% using the MIS. The comparison with a non-imprinted silica (NIS) sorbent, for which extraction recoveries between 8% and 26% were obtained, showed the high selectivity of the MIS for the nitro musks. Moreover, high enrichment factors, ranging between 580 and 827, were achieved. The imprinted sorbent was compared to a conventional polymeric SPE sorbent for the extraction of the target compounds from environmental waters, showing high selectivity of the MIS and its clean-up potential. For the first time, the five nitro musk compounds were selectively extracted with an imprinted material.

1. Introduction

Musk compounds are valuable fragrance chemicals used in many consumer products such as cosmetics, detergents, herbicides, food additives or household products. Natural musk, a product from natural origin, is no longer used due to economical and ethical reasons. Nowadays synthetic musks, other compounds with totally different chemical structures but possessing musk-like odor properties, have replaced it [1]. Synthetic musks have been generally divided in three subgroups: nitro musks, polycyclic musks and macrocyclic musks. Polycyclic musks are the most used in Europe while nitro musks are the ones that dominate in North America [2,3].

The nitro musks group is basically formed by five compounds i.e., musk ambrette (MA), musk xylene (MX), musk moskene (MM), musk tibetene (MT) and musk ketone (MK), all of them possessing a nitro-aromatic moiety. Table 1 shows the chemical structure of these compounds and some of their relevant characteristics. Nitro musks are hazardous compounds as they are related with different types of dermatitis, carcinogenic effects and endocrine dysfunction [4–10]. They are also considered persistent pollutants as they present a strong bioaccumulation potential [11]. Moreover, nitro musks can indirectly reach the aquatic environment, in which they have been found at trace levels [12,13]. Therefore, it is important to develop new analytical methods that allow carrying out their determination on the ecosystems. To do so, due to the low concentration at which they are usually found, it is necessary to use suitable extraction techniques to concentrate the compounds and to remove interfering substances.

Nitro musks have been determined in different samples, such as cosmetic products [14,15], air [16], water and sediment [17–33], blood [34,35], fish [36,37], human adipose tissue [38] or human milk [39,40]. Different techniques for the extraction of nitro musks in environmental water samples have been reported in the literature. Liquid–liquid extraction (LLE) [18,19,26], liquid–liquid microextraction (LLME) [29,31], solid phase extraction (SPE) [20–22,24,25] and solid-phase microextraction (SPME) [17,23,27,30,32] have been used as clean-up and preconcentration techniques. The most commonly used analytical technique for their determination is by far gas chromatography (GC) either with electron capture (ECD), flame ionization (FID) or mass spectrometry (MS) detection.

SPE has been broadly used as a clean-up step. However, this technique presents some disadvantages as the co-extraction of interfering compounds with a similar polarity to the analytes, which interfere in the subsequent determination of the compounds of interest. An option to improve selectivity in SPE is the use of molecularly imprinted sorbents, which contain cavities

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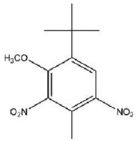
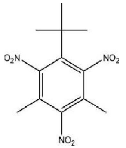
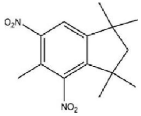
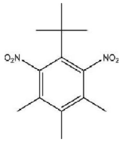
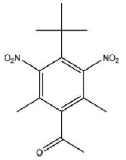
http://dx.doi.org/10.1016/j.talanta.2013.02.023

Table 1
Main characteristics of the target compounds.

Common name	CAS N°	Structure	Log Kow (25 °C)
Musk ambrette (MA)	83-66-9		4.039 ± 0.318
Musk xylene (MX)	81-15-2		4.369 ± 0.295
Musk moskene (MM)	116-66-5		4.839 ± 0.337
Musk tibetene (MT)	145-39-1		4.931 ± 0.295
Musk ketone (MK)	81-14-1		2.510 ± 0.472

designed for a target compound [41]. This provides a retention mechanism based on molecular recognition, in which great potential in selectivity has been largely demonstrated [42–45].

Molecular imprinted polymers (MIPs), synthesized by radical polymerization of organic monomers, are the most commonly used imprinted sorbents. However, it has been shown that the crucial interactions between the template and the monomer are difficult to achieve in the case of templates with a nitro group [46,47]. A strong retention by hydrogen bonds is unlikely and taking advantage of hydrophobic interactions by using the styrene as monomer is neither an option as nitro aromatic compounds inhibit the radical polymerization of the styrene. A different approach is the synthesis of imprinted sorbents by sol–gel polymerization that results in a hybrid material (inorganic–organic) known as molecularly imprinted silica (MIS). In this case, organically modified silica is used as a monomer that commonly forms polar or hydrophobic interactions with the template, and an alkoxysilane is used as cross-linking [46,47].

Unfortunately, as the template is usually not completely removed, residual leaking during the elution is common. This leads to a lack of accuracy in the determination, especially in trace analysis. The simplest and most common way of avoiding this problem is the use of a structural analog of the target compound as template, known as dummy molecule, in the synthesis of the material [41]. Residual leaking of the template is still present but is no more a problem as it elutes at a different retention time than the target compound in the subsequent chromatographic technique. This procedure is also a good option when the use of the target compound as template is not feasible due to its high price or that the conditions used in polymerization may cause degradation in undesired compounds.

The aim of this work was to find a molecularly imprinted material to be used as SPE sorbent for the simultaneous selective extraction of the five compounds from the nitro musk family in wastewater and surface water samples. A MIS synthesized using the same procedure as the one described by our group for nitro explosives using 2,4-dinitrotoluene (2,4-DNT) as template was used because of its structural analogy with the target nitro musks [47]. Then, an extraction procedure different from the procedure developed for nitroaromatic explosives but well adapted to the selective extraction of nitro musk was optimized and applied to their extraction from wastewater and surface water samples.

2. Experimental

2.1. Reagents, materials and samples

1-Tert-butyl-3-methyl-2,4-dinitroanisole (Musk Tibetene, MT) 99%, 1,1,3,3,5-pentamethyl-4,6-dinitroindane (Musk Moskene, MM) 99% and a cyclohexane solution of 100 mg mL^{-1} of 1-tert-butyl-3,5-dimethyl-2,4,6-trinitrobenzene (Musk Xylene, MX) 99.5%, purchased from LGS standards (Lancashire, United Kingdom) and 6-tert-butyl-3-methyl-2,4-dinitroanisole (Musk Ambrette, MA) 99% and 4-tert-butyl-2,6-dimethyl-3,5-dinitro-acetophenone (Musk Ketone, MK) 98% purchased from Dr. Ehrenstorfer (Augsburg, Germany) were used as standards. Hexachlorobenzene (HClB) 99% from Aldrich (Steinheim, Germany) was used as an internal standard.

HPLC grade methanol (MeOH) and acetonitrile were from Carlo Erba (Val de Reuil, France). Deionised water (resistivity ≥ 18 MΩ cm) obtained by means of a Milli-Q purification system (Millipore, Saint-Quentin en Yvelines, France) was used to prepare the working standard solutions.

To synthesize the MIS, 2,4-DNT from Sigma Aldrich (St. Quentin Fallavier, France) was used as a template, phenyltrimethoxysilane (PTMS) and tetraethoxysilane (TEOS), both from Sigma Aldrich, were used as monomer and cross-linker respectively. Lastly, a 32% commercial ammonia solution provided by Merck was used as solvent.

High purity helium (99.9995%) from Messer (Puteaux, France) was used as carrier gas in the GC–MS system

Oasis HLB cartridges (phase of N-vinylpyrrolidone and divinylbenzene) 60 mg sorbent, 3 mL cartridges from Waters were also used to compare the results of the MIS with a conventional cartridge.

Water samples were all collected from different sources: sea water from La Vila Joiosa (Alicante, Spain), river water from the Seine River (Paris, France) and effluent wastewater from a treatment plant (Valencia, Spain). All samples were stored in the dark at 4 °C and filtered through 0.45 μm nylon membrane filters before the analysis.

2.2. Analytical conditions

The GC–MS system used consisted of a trace GC gas chromatograph coupled to an ISQ mass spectrometry detector equipped with a triplus autosampler, all from Thermo Fisher Scientific (Austin, TX, USA). The chromatographic separations were made using a DB-5MS Ultra Inert (95% dimethyl-5% diphenylpolysiloxane, 30 m length, 0.25 mm i.d., 0.25 μm film thickness) analytical fused-silica capillary column from Agilent Technologies (Palo Alto, CA, USA).

The separation was run at a 1 mL min^{-1} helium constant flow rate. The oven temperature program was from 60 °C (1 min) to 120 °C at 20 °C min^{-1}, then to 185 °C (0 min) at 10 °C min^{-1}, then to 195 °C (0 min) at 1 °C min^{-1} and finally to 280 °C (5 min) at 25 °C min^{-1}. The injection volume was 1 μL in splitless mode (splitless time: 1 min). The inlet, transfer line and ion source temperatures were set at 280 °C, 280 °C and 250 °C, respectively. The chromatograms were recorded in selected ion monitoring (SIM) mode at the following mass/charge (*m*/*z*) ratios: *m*/*z* 284 from minute 11.0 to 13.50 for HClB, *m*/*z* 253, 263 and 282 from minute 13.50 to 16.90 for MA, MX and MM, respectively, and *m*/*z* 251 and 279 from minute 16.90 to 21.0 for MT and MK, respectively. A full scan mode (m/z from 50 to 350) was simultaneously recorded from minute 5.0 to the end of the analysis time.

2.3. Evaluation of the imprinted sorbent

The MIS was synthesized according to the procedure previously described by some of the authors of this work for the extraction of nitroaromatic explosives [47]. In summary, it was synthesized using 2,4-DNT as template, PTMS as monomer and TEOS as crosslinking in a 1/4/30M ratio (template/monomer/cross-linker). A nonimprinted silica (NIS) sorbent synthesized simultaneously with the MIS without introduction of the template molecule was also used during the evaluation of the MIS to ensure the development of a selective extraction procedure.

The extraction procedure was optimized by studying the composition and the volume of the percolated and the washing fractions. The conditioning of both, MIS and NIS, consisted in the percolation of 5 mL of water. The optimized procedure consisted in the percolation of 200 mL of a water/MeOH (95/5, v/v) standard solution containing 500 ng L^{-1} of the five nitro musks followed by 1.3 mL of a MeOH/water mixture (60/40, v/v) as washing step. Finally, 1 mL of ACN was chosen for the elution step. In order to quantify the nitro musks only the elution fractions were analyzed by GC–MS. For this, these fractions were directly injected after the addition of the internal standard or evaporated to dryness under a nitrogen stream and dissolved in 200 μL of ACN containing the internal standard at 15 ng L^{-1}.

2.4. Application to environmental samples

Three water samples from different sources—sea water, river water and wastewater—were collected. All samples were stored in the dark at 4 °C and filtered through 0.45 μm nylon membrane filters before the analysis.

The optimized extraction procedure on MIS was applied for each one of them. For this, a conditioning step of 5 mL of water was carried out and it was followed by the percolation step of 200 mL of the water sample containing 5% of methanol and spiked with 100 ng of each nitro musk, a washing step of 1.3 mL of a MeOH/water mixture (60/40, v/v) and an elution step with 1 mL of ACN. After that, the elution fraction was evaporated to dryness and dissolved in 200 μL of ACN containing the internal standard at 15 ng L^{-1}.

To prove the potential of the imprinted sorbent to selectively extract the analytes, a similar method with a conventional sorbent was developed to compare the results in real samples. The conventional sorbent chosen was an Oasis HLB, (60 mg). The procedure consisted in a conditioning step with 2 mL of acetonitrile, 2 mL of methanol and 2 mL of water. The loading step consisted in the percolation of 200 mL of the water sample containing 5% of MeOH spiked with 100 ng of each nitro musk. Washing was accomplished with 1 mL of water/MeOH (80/20, v/v) mixture and compounds were finally eluted with 1 mL of acetonitrile. The elution fraction was evaporated to dryness and dissolved in 200 μL of ACN containing the internal standard at 15 ng L^{-1}.

3. Results and discussion

3.1. Selectivity of the imprinted sorbent

The aim of this study was to provide a selective sample pretreatment by using a MIS to analyze the nitro musks in water samples. The structure of these compounds is reported in Table 1. Indeed, the analysis of the nitro musks in these samples requires the clean-up of the matrix and a high preconcentration in order to detect trace level of these compounds. In these conditions, the use of a dummy molecule during the synthesis of the MIS was necessary to avoid the risk of residual template leaking from the polymer and causing erroneous results. Because of its structural analogy with the nitro musks, 2,4-DNT was used as a template and the synthesis of the MIS was achieved in the same conditions as for the MIS previously synthesized for nitro explosives [47].

First, the selective retention of the nitro musk compounds on the MIS synthesized with the 2,4-DNT as template was studied by the development of an optimized extraction procedure for these compounds. The optimization of the extraction procedure is generally performed by studying the extraction profiles that must be different when using the MIS and the NIS [41]. This comparison necessitates the quantification of the analytes in the percolation, washing and elution fractions. This approach cannot be applied in this study because the percolation and the washing fractions contain mainly water and aqueous samples cannot be injected on the GC-column. Moreover, it was not possible to evaporate the aqueous fractions without losing the nitro musks. So, only the elution fractions were injected for the quantification. Therefore, the comparison of both sorbents only consists in measuring the recovery yields in the elution fraction of the MIS/NIS that must be lower for the NIS than for the MIS.

The retention on both sorbents was then studied after the percolation of 200 mL of pure water sample spiked with nitro musks followed directly by the elution step. Five per cent of MeOH was added in the percolated water sample in order to ensure that the highly hydrophobic nitro musks did not adsorb to any of the surfaces on which they came in contact. In this case, extraction recoveries for MIS ranged between 75% and 100% for the MIS and between 55% and 70% for NIS. So, these results show the retention of the nitro musks on the MIS applying this condition of percolation, with a high volume of water, to obtain a high enrichment factor. Moreover, the higher retention on the MIS than on the NIS demonstrates the presence of cavities on the MIS allowing this slight selectivity, which could be probably increased by modifying the proportion of MeOH in the sample and in the washing solution. Thus, various water/MeOH mixtures containing the nitro musks were percolated through the MIS and the NIS and followed by the elution with ACN. The best results were obtained when the percolation was carried out with water/MeOH (40/60, v/v). Indeed, the extraction recoveries were about 80% and 92% on the MIS and about 28% and 63% on the NIS for all the compounds. This difference in recoveries confirms again the presence of cavities in the MIS that are able to retain more strongly the nitro musks than the NIS.

The volume of the washing fraction was optimized after the percolation of 200 mL of water/MeOH (95/5, v/v) solution spiked with the nitro musks, the effect of various volumes of the washing mixture (water/MeOH, 40/60, v/v) on the recoveries were studied, the elution being carried out with ACN. First, the extraction recoveries reported for the MIS in Fig. 1, for a washing fraction of 1 mL, are the same as those obtained without applying a washing solution while, recovery values for NIS did reduce to values between 34% and 54% (instead of 55% and 70%) depending on the nitro musks.

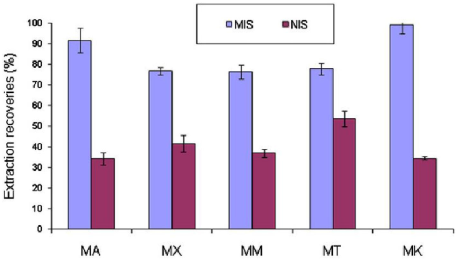


Fig. 1. Extraction recoveries obtained on MIS and NIS after the percolation of 200 mL of a water/MeOH (95/5, v/v) standard solution containing 500 ng L^{-1} of the five nitro musks and a washing step of 1 mL of a MeOH/water mixture (60/40, v/v) (n=3).

In order to reduce the non-specific interactions, higher volumes of washing solution were considered and the best results were obtained for the percolation of 1.3 mL of water/MeOH (40/60, v/v). Fig. 2 shows a chromatogram corresponding to the analysis of the eluate after the percolation of 200 mL of a 95/5 water/MeOH solution spiked with five target compounds at 50 ng L^{-1} and subjected to the described procedure.

The extraction recoveries obtained after the accomplishment of this procedure in triplicate are shown in Fig. 3. This optimized extraction procedure allowed extraction recoveries between 61% and 87% using the MIS while recoveries for the non-imprinted sorbent ranged between 8% and 26%. This process allowed the highest selectivity, when comparing MIS and NIS, with satisfying extraction recoveries using the MIS. Relative standard deviations lower than 6% for the MIS and for the NIS show the good repeatability of the extraction procedure.

Finally, the procedure with a washing step of 1.3 mL, was chosen for all the next experiments. This procedure allowed the selective extraction of the nitro musks using a MIS synthesized with the 2,4-DNT as a template with a high selectivity. So, the cavities are sufficiently large to accept the nitro musks but when applying specific conditions of extraction. The extraction procedure

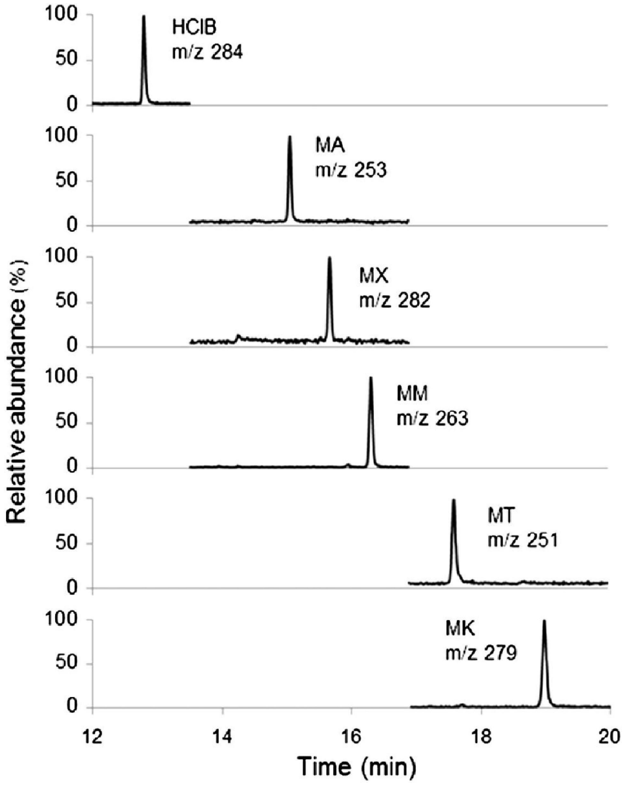


Fig. 2. A chromatogram obtained applying the proposed method to a water/MeOH (95/5, v/v) standard solution containing 50 ng L^{-1} of the five nitro musks.

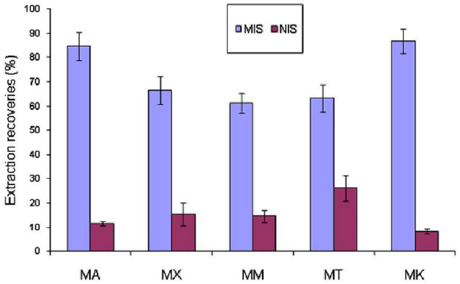


Fig. 3. Extraction recoveries obtained on MIS and NIS after the percolation of 200 mL of a water/MeOH (95/5, v/v) standard solution containing 500 ng L^{-1} of the five nitro musks and a washing step of 1.3 mL of a MeOH/water mixture (60/40, v/v) (n=3).

developed previously for the nitro explosives cannot be used for these compounds. Indeed, a washing step with a water/ACN mixture (75/25, v/v) used for the selective extraction of the nitro explosives led to the loss of the nitro musks before the elution fraction. Moreover, it was observed that the use of MeOH and water in the washing step gave rise to a lowest selectivity for the extraction of nitro explosives on the same MIS than an acetonitrile/water mixture thus highlighting again the necessity to adapt the nature and the volume of the solvent constituting the washing fraction to the nature of the studied compounds.

3.2. Evaluation of the analytical method

The enrichment factors for all the nitro musks were calculated with the optimized extraction procedure. After the extraction, and in order to lower the limits of detection, an evaporation step was performed. The 1 mL elution fraction was evaporated to dryness under a nitrogen stream and re-dissolved in 200 µL of ACN containing the internal standard at 15 ng L^{-1}. Before this, a test, evaporating to dryness in triplicate and redissolving to ensure no nitro musks were lost in the evaporation, was performed. Results showed that there was no loss of compounds during this step.

The enrichment factors achieved by the method were calculated taking into account the dilution of 5% with MeOH, the extraction recoveries of the SPE process and the evaporation step. These factors ranged between 580 and 827 depending on the nitro musk studied, thus showing the high enrichment of this procedure. The enrichment factors are reported in Table 2.

A calibration curve of the standards in ACN was constructed for each nitro musk in the range 8–20 ng mL^{-1}. Then, the instrumental limit of detection (IDL) and quantification (IQL) was calculated as 3 or 10 times the standard deviation of the residuals divided by the slope of the calibration curve. These results are presented in Table 2. The IDL and the IQL obtained range between 1.1 and 2.1 µg L^{-1} and between 3.6 and 7.0 µg L^{-1}, respectively.

The limits of detection (LOD) and quantification (LOQ) of the method were then estimated taking into account the IDL or IQL and the enrichment factors of each nitro musk. All these parameters are summarized in Table 2. The development of this procedure allowed a large decrease of the LOD and the LOQ of the method which ranged between 1.5 and 2.7 ng L^{-1} and between 4.7 and 8.4 ng L^{-1} respectively.

The proposed method provides good analytical features with LODs comparable to other methods that use very sensitive techniques, such as SPME [27,32] or LLME [29,31], and presents a high selectivity.

Table 2
Main parameters of the method.

Analyte	IDLs (µg L^{-1})	IQLs (µg L^{-1})	Enrichment factor	LOD (ng L^{-1})	LOQ (ng L^{-1})
MA	2.1	7.0	808	2.6	8.2
MX	1.7	5.6	637	2.7	8.4
MM	1.3	4.2	580	2.2	7.0
MT	1.1	3.6	599	1.8	5.8
MK	1.2	4.1	827	1.5	4.7

3.3. Evaluation of the repeatability of the synthesis

To confirm the potential of the MIS for the selective extraction of the nitro musks, a second synthesis was carried out at a different time, by another manipulator and evaluated applying the previously optimized procedure. Fig. 4 presents the comparison of the average extraction recoveries obtained after three extractions carried out on each MIS and each NIS. These results show again the high selectivity of each synthesized MIS. The repeatability of the extraction procedure on a given MIS and NIS was also observed with RSD values lower than 6%. Finally, recoveries obtained on MISs resulting from different syntheses are very close, thus confirming that the imprinted sorbents present similar performances. Additional syntheses will be required to confirm the real reproducibility of the MIS synthesis but these first results confirm the selectivity of the MIS for the nitro musks.

3.4. Evaluation of the capacity

The proposed method was applied to a 95/5 water/MeOH (v/v) standard mixture containing increasing amounts of the five nitro musk compounds. This test is usually used for the MIS capacity study, which is the maximum amount of nitro musk that can be selectively retained by the sorbent. Previous studies [47] already showed that this MIS presented a very high capacity that was not possible to reach due to the limit of solubility in water of the 2,4-DNT. The objective in this work was to verify that the extraction recoveries kept constant at the working range i.e. mass range between the limit of quantification to a higher concentration than that found in real samples in the bibliography for the nitro musks. Table 3 shows the curves and regression coefficient obtained. The linearity obtained for the calibration curves shows the possibility to quantify the nitro musks after the extraction on the MIS in the mass range tested. The slopes obtained for each curve, ranged between 0.61 and 0.89, confirm the average extraction recoveries ranged between 61% and 87% for the MIS. The selectivity of the extraction procedure is again demonstrated by the average extraction recoveries between 11% and 27% on NIS.

3.5. Application for real water samples

The selective extraction procedure on MIS was applied to three real samples of different sources: sea water, river water and effluent water from a treatment plant (see Section 2.1), in order to determine the five nitro musk compounds.

The water samples were first analyzed applying the optimized extraction procedure before the analysis by GC–MS. The results showed that all of the target compounds were below the limits of detection. Then, recovery studies were performed with the purpose to evaluate the matrix effect. So, the proposed method was applied to the above-mentioned real samples, previously spiked with 100 ng L^{-1} of the target compounds using the MIS.

In order to compare the results obtained using the MIS with an extraction using a conventional sorbent, an extraction procedure was developed using an Oasis HLB sorbent. The extraction recoveries

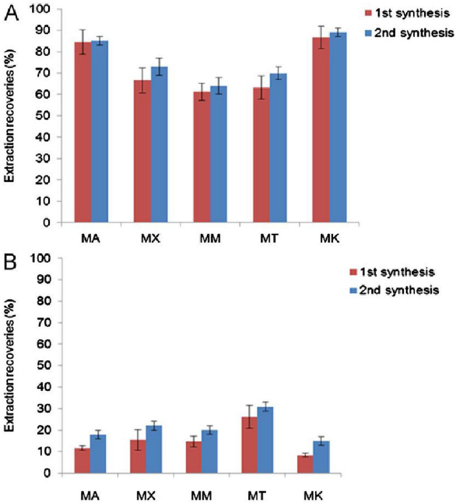


Fig. 4. Extraction recoveries obtained after the application of the optimized procedure to two different syntheses on (A) MIS and (B) NIS ($n=3$).

Table 3
Calibration curves and regression coefficient obtained after the percolation of 200 mL of a water/MeOH mixture (95/5, v/v) containing increasing amounts of the five nitro musks on the MIS and the NIS.

Analyte	MIS		NIS	
	Calibration curve	Regression coefficient	Calibration curve	Regression coefficient
MA	$y=0.8303x-0.0251$	1.0000	$y=0.1819x-1.9978$	0.9984
MX	$y=0.6124x+4,052$	0.9980	$y=0.2198x-1.5167$	0.9871
MM	$y=0.6414x-1.1348$	0.9997	$y=0.1915x-0.4496$	0.9986
MT	$y=0.6129x+2.7467$	0.9986	$y=0.2793x+0.3796$	0.9997
MK	$y=0.8881x-2.7873$	0.9995	$y=0.1104x-0.8206$	0.9907

This data is extracted from a calibration curve where the percolated amount (in ng) and the amount obtained (in ng) after the application of the method was plotted. Number of calibration points: 6 (4, 10, 50, 100, 300, and 800 ng).

obtained after applying, in triplicate, the percolation of a water/MeOH (95/5, v/v) standard solution containing 500 ng L^{-1} of the five nitro musks followed by a washing step with 1 mL of water/MeOH (80/20, v/v) and an elution with 1 mL of ACN, using Oasis HLB sorbents were 93 ± 4%, 75 ± 6%, 72 ± 5%, 69 ± 2% and 105 ± 9% for MA, MX, MM, MT and MK respectively. These extraction recoveries obtained for pure water with the Oasis HLB sorbent are very close to those obtained with the MIS.

Finally, recovery studies for the real samples using the Oasis HLB sorbent and the method developed were also carried out. The recoveries obtained are shown in Table 4 for the MIS and Oasis HLB. First, similar extraction recoveries are obtained using the MIS for the extraction of the nitro musks in pure water and in the real samples. However, the extraction recoveries obtained after the extraction of the nitro musks in the real samples using the Oasis HLB sorbent are very different from those obtained in pure water, especially in the case of sea water and wastewater, where most of the recoveries are lower than 50%. From these results it can be concluded that the imprinted sorbent does not present significant matrix effects while in the case of the conventional sorbent, there are matrix effects resulting in a large decrease of the extraction recoveries.

Table 4
Extraction recoveries (%) obtained in the analysis of three water samples ($n=3$) (spiking level 100 ng L^{-1}).

	Standard		River water		Sea water		Wastewater	
	MIS	OASIS	MIS	OASIS	MIS	OASIS	MIS	OASIS
MA	85 ± 6	93 ± 4	77 ± 4	76 ± 3	77 ± 5	40 ± 5	69 ± 3	47 ± 7
MX	67 ± 6	75 ± 6	68 ± 5	56 ± 2	63 ± 1	32 ± 2	59 ± 3	25 ± 1
MM	61 ± 4	72 ± 5	63 ± 1	55 ± 2	57 ± 3	30 ± 1	52 ± 1	27 ± 3
MT	63 ± 6	69 ± 2	64 ± 1	63 ± 3	57 ± 3	32 ± 1	55 ± 6	31 ± 3
MK	87 ± 5	105 ± 9	92 ± 2	116 ± 5	87 ± 2	76 ± 3	87 ± 3	92 ± 8

Finally, data show that in most of the cases the recovery values for real samples are better for the imprinted sorbent than for the conventional sorbent. So, the MIS presents a higher retention of the nitro musks than the Oasis HLB for real samples. Moreover, the extraction recoveries are similar for all the tested matrices using the MIS showing the repeatability of the procedure on complex samples.

As previously mentioned, these shows that molecularly imprinted sorbents, with a retention mechanism based on molecular recognition, enable a better clean-up of the sample than conventional sorbents, in which the co-extraction of interfering compounds can interfere in the subsequent determination.

4. Conclusions

A high sensitive analytical method, with great clean-up potential, is presented in this paper in order to determine the complete family of nitro musk compounds in environmental water samples. To the best of our knowledge this is the first time an imprinted sorbent is used to selectively extract the nitro musk compounds.

The selective extraction of the target compounds from real samples by the MIS easily succeeded. A high degree of selectivity was obtained allowing a good quantification of the nitro musks after the MIS extraction compared to a conventional sorbent.

Acknowledgments

M. Lopez-Nogueroles would like to acknowledge the financial support of the Ministerio de Economia y Competitividad for her predoctoral grant (Project CTQ2009-12709) and her fellowship to work at the ESPCI in Paris, which allowed the achievement of this work.

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