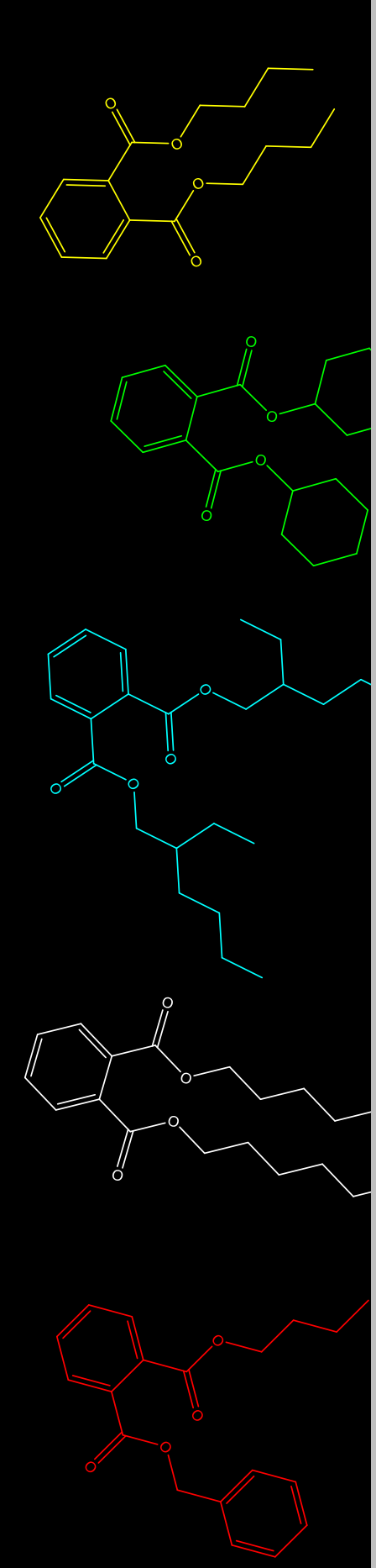

Development of dispersive liquid-liquid microextraction-based analytical methodologies. Application to real samples.

PhD Thesis
Jessica Pérez Outeiral

Donostia / San Sebastián
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Supervisors: Rosa Garcia Arrona

Maider Vidal Postigo

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Summary

Summary

The main objective of the present thesis was the development of new reliable dispersive liquid-liquid microextraction-based methodologies coupled to different analytical instrumentation for the determination of diverse analytes in different matrices.

Although sample preparation has been overlooked for years, it is a crucial step that affects in a high extent the quality of the results of a given analytical process and, at the present time, it is one of the main research trends. Interest on sample preparation is nowadays specially focused on miniaturized extraction procedures based on previously existing extraction techniques. These new procedures are generally simpler, faster and greener. Amongst all the new extraction techniques, liquid-phase microextraction is a widely extended method, and from its introduction, several branches have arisen.

In the introduction to this thesis, the different branches are explained and advantages and disadvantages of all of them are briefly commented. In addition, some details regarding the different variables affecting the microextraction processes are given, followed by a short explanation of the coupling of dispersive liquid-liquid microextraction to instrumental analysis. As experimental design was used in some of the methods to search for the best achievable experimental conditions, its basis is also explained in this section. Moreover, the used method validation parameters are described, and finally, some basic principles of multi-way data analysis, specifically of PARAFAC and PARAFAC2, are given.

After introduction, main **objectives** of the thesis are defined, and then, the main block of the thesis follows. In this block, in Chapters 3, 4 and 5 three different methodologies are developed, validated and applied to different real samples. In **Chapter 3,** a procedure of dispersive liquid-liquid microextraction and UV-Vis spectrophotometry for cadmium determination in water samples is explained; In **Chapter 4,** ultrasound-assisted dispersive liquid-liquid microextraction followed by solidification of the floating organic drop and GC-FID are used for phthalate determination in food simulants and liquid samples; and in **Chapter 5,** a method based on ultrasound-assisted emulsification microextraction followed by the

solidification of the floating organic drop and HPLC-DAD for the determination of 18 fragrance allergens in cosmetic and water samples is described.

In summary, in this main block of the thesis, the versatility of dispersive liquid-liquid microextraction-based methodologies is probed. The coupling of the methodologies to relatively affordable diverse analytical instrumentation is studied. At the same time, the applicability of the techniques to different types of analytes (organic compounds and a heavy metal) is assessed and, due to the different nature of the analytes, microextraction is therefore accomplished in a wide range of sample matrices.

In an additional chapter, **Chapter 6**, multi-way data analysis, particularly PARAFAC2, is proposed to determine the concentration of several fragrance allergens from the overlapped HPLC-DAD chromatographic peaks obtained when the method developed in Chapter 5 is applied. Different models have been built to model the different sets of data created, with the purpose of obtaining reliable results for the quantification of the analytes that otherwise could not be determined by the conventional univariate approach. In univariate analysis only the peak area or height at a defined wavelength is considered and, when there is a severe overlapping, the quantification can be completely senseless.

At the end of the thesis, main general **conclusions** are detailed.

Index

List of abbreviations

Abbreviation	Definition
AAS	Atomic absorption spectroscopy
AES	Atomic emission spectrometry
AFS	Atomic fluorescence spectroscopy
ANOVA	Analysis of variance
AOAC	Association of analytical communities
BBP	Benzyl butyl phthalate
BBW	Baby bath water
BM	Body milk
CC	Core-consistency
CFME	Continuous flow microextraction
CTFA	Cosmetic, toiletries and fragrance association
DAD	Diode array detector
DBP	Dibutyl phthalate
DC	Digital colorimetry
DCHP	Dicyclohexyl phthalate
DDME	Drop-to-drop microextraction
DEHP	Bis(2-ethylhexyl)phthalate
DI	Direct immersion
DLLME	Dispersive liquid-liquid microextraction
D-LPME	Dynamic liquid-phase microextraction
DNOP	Di-n-octyl phthalate
DSDME	Directly-suspended drop microextration
DW	Drinking water
EB	Emulsification-based
EC	*Eau de cologne*
ECHA	European chemicals agency
ECPI	European council for plasticizers and intermediates
EF	Enrichment factor
EP	*Eau de parfum*
ET	*Eau de toilette*
ETAAS	Electro thermal atomic absorption spectroscopy
EV	Explained variance
FAAS	Flame atomic absorption spectroscopy
FI	Flow injection
FID	Flame ionization detector
FO	Fiber optic

GC	Gas chromatography
GF	Graphite furnace
HF	Hollow-fiber
HPLC	High-performance liquid chromatography
HS	Headspace
ICP	Inductively coupled plasma
IR	Infrared
IS	Internal standard method
LADS	Linear array detection spectrophotometry
LC	Liquid chromatography
LLE	Liquid-liquid extraction
LLLME	Liquid-liquid-liquid microextraction
LOD	Limit of detection
LOF	Lack of fit
LOQ	Limit of quantification
LPME	Liquid-phase microextraction
MEEKC	Microemulsion electrokinetic chromatography
MEKC	Micellar electrokinetic chromatography
MISPE	Molecularly-imprinted solid-phase extraction
MS	Mass spectrometry
MSPD	Matrix solid-phase dispersion
MSPE	Magnetic solid-phase extraction
OES	Optical emission spectrometry
OVAT	One-variable-at-a-time
PAE	Phthalate ester
PARAFAC	Parallel factor analysis
PARAFAC2	Parallel factor analysis 2
PAS	Potentially allergenic substances related to fragrances
PLE	Pressurized liquid extraction
QuEChERs	Quick, easy, cheap, effective, rugged and safe
REP	Relative error of prediction
RMSEP	Root mean square error of prediction
RSD	Relative standard deviation
RW	River water or water from an irrigation channel
SB	Solid-based
SBME	Solvent-bar microextraction
SCCNFP	Scientific committee on cosmetic products and non-food products
SDME	Single-drop microextraction
SFOD	Solidified floating organic drop

SML	Specific migration limit
SPD	Solid-phase dispersion
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
SPW	Swimming pool water
SSME	Solvent-supported microextraction
SW	Snow water
TSIL	Task specific ionic liquid
TW	Tap water
TXRF	Total reflection x-ray spectrometry
UA	Ultrasound-assisted
UASEME	Ultrasound-assisted surfactant-enhanced emulsification microextraction
USAEME	Ultrasound-assisted emulsification microextraction
USEPA	United states environmental protection agency
UV	ultraviolet
Vis	Visible
VWD	Variable wavelength detector
WHO	World health organization

Introduction

Introduction

Analytical chemistry is the branch of chemistry that develops, optimizes and applies methods and tools to get information on the chemical composition of the matter (Harvey, 2000). Generally, analytical methods involve various steps such as sampling, sample preservation, sample preparation and instrumental and data analysis. Each of these steps is crucial for obtaining accurate and precise information.

The importance of sample preparation has been overlooked for years, probably due to the huge advances in analytical instrumentation. However, despite of the great improvements achieved, instruments cannot normally handle directly with the original sample matrix and even when they can do it, additional clean-up or preconcentration of the analytes is frequently necessary for accurate and precise determination. In general words, the objectives of sample preparation are to extract, isolate and concentrate the analytes of interest from matrices, making possible or easier their subsequent measurement by analytical instruments.

Sample preparation step affects the quality of the results obtained after all the analytical process at a high extent, and it is known that it is the main source of systematic error. Likewise, it is probably the most time-consuming step. Indeed, it has been calculated that more than the 60% of analysis time is spent in sample preparation, compared to only about 7% for the measurement, and about 33% for sample collection and data handling (Majors, 1991; Fritz, 1999). Thus, nowadays efforts are being focused on the improvement of existing sample preparation techniques and in the development of new ones.

Recent research trends in sample preparation techniques include miniaturized extraction procedures based on previously existing extraction techniques. Thus, special attention has been paid to the development of liquid-phase microextraction techniques during the last years, which are inspired in a well-known sample preparation technique, the liquid-liquid extraction (LLE).

Liquid-liquid extraction, also known as “solvent extraction”, is based on the distribution equilibrium of analytes between two immiscible liquid phases in contact (generally water and an organic solvent). It is normally performed by

rigorously shaking the two immiscible liquids together for some time in a separating funnel. In this way, a suspension of small droplets of the organic phase into the aqueous phase is formed, enhancing the transfer of the solutes from one phase to another due to the increment of the superficial area between phases. After agitation, funnel is let stand until the two phases are separated. Then, the layer that remains below is drained through the tap at the base of the funnel (Higson, 2003).

Liquid-liquid extraction has been widely used for years probably because it is possible to get efficient cleanup and high enrichment factors in many applications with this technique. However, it presents some drawbacks: large volumes of samples and toxic organic solvents, lack of safety when handling with those organic solvents (risk of explosion, flammability, toxicity and carcinogenity), possible analyte adsorption on glassware, potential solvent mediation decomposition and presence of some impurities in the solvent that could cause interferences. This type of extraction technique is also labour-intensive, difficult to automate and connect in-line with analytical instruments, and, in addition, sometimes emulsion break-up after extraction can be difficult and slow (Pawliszyn, 2002).

Due to the disadvantages of LLE and other previously used extraction techniques, and especially to the necessity to avoid, or at least reduce, the use of toxic organic solvents, miniaturized extraction procedures based on conventional extraction techniques are being developed in the last years. These new techniques are generally simpler, faster and they use fewer amounts of solvents than the traditional ones.

1. Liquid-phase microextraction

Liquid-phase microextraction (LPME) emerged as a solvent-minimized version of the classic LLE in which only several microliters of extractant are used to extract the target analytes. Initial development of LPME techniques started in the mid-1990s (Liu and Dasgupta, 1995A; Liu and Dasgupta, 1995B, Cardoso and Dasgupta, 1995; Jeannot and Cantwell, 1996; Liu and Dasgupta, 1996), and since them, this technique has evolved into different branches. Fundamentally, LPME is performed by putting in touch a small amount of extraction solvent with the sample or with the headspace. In that way, analytes are distributed between the two phases in contact according to a distribution coefficient. After extraction, extractant is collected and conducted to instrumental analysis.

Ideally, a microextraction process finishes when the equilibrium of the analytes between the phases is achieved. At this point, concentration of the analyte in the organic phase is proportional to the initial analyte concentration in the aqueous phase and depends on the ratio of organic phase/ water volumes, as it is described in Equation 1.1. (Jeannot and Cantwell, 1996).

$$C_{eq,o} = \kappa\, C_{eq,aq} = \frac{\kappa C_{0,aq}}{1+\kappa\frac{V_o}{V_{aq}}} \qquad \text{(Equation 1. 1.)}$$

Where $C_{eq,o}$ and $C_{eq,aq}$ are the concentrations in the equilibrium of the organic and aqueous phase respectively, $C_{0,aq}$ is the initial aqueous phase concentration, V_o and V_{aq} are the organic and aqueous phase volumes, and κ is the distribution coefficient, which is defined as the ratio between organic and aqueous analyte concentrations in the equilibrium.

In headspace LPME techniques, or when analyte concentration in the headspace is important due to the volatility of analytes, a new term due to the distribution of analytes between the sample and the headspace must be taken into account, as described in Equation 1. 2. (Jeannot *et al*., 2010).

$$C_{eq,o} = \frac{\kappa C_{0,aq}}{1+\kappa\frac{V_o}{V_{aq}}+\kappa'\frac{V_{HS}}{V_{aq}}} \qquad \text{(Equation 1. 2.)}$$

Where κ' is the headspace-water distribution constant and V_{HS} the headspace volume.

As it can be deduced from the last equation, a very small headspace volume or a very small κ' (related to the volatility of analytes) would convert Equation 1.2. into Equation 1.1.

The enrichment factor (EF) gives an approximation of how effective the extraction is, and it is defined as the ratio between the analyte concentration in the organic phase after the extraction, when the equilibrium has been reached ($C_{eq,o}$), and the initial concentration of the analyte in the water sample ($C_{0.aq}$), as expressed in Equation 1.3. (Rezaee *et al.*, 2006).

$$EF = \frac{C_{eq,o}}{C_{0,aq}} \qquad \text{(Equation 1.3.)}$$

In general, $C_{0,aq}$ is directly known by sample preparation and $C_{eq,o}$ is obtained from a suitable calibration curve.

From LPME introduction in the mid-1990s, the technique has evolved and now different variants of the technique are of common use (Pena-Pereira *et al.*, 2010; Mahugo-Santana *et al.*, 2011; Han and Row, 2012). These can be classified into three main categories according to the way the extraction is done: single-drop microextraction (SDME), hollow-fiber liquid-phase microextraction (HF-LPME) and dispersive liquid-liquid microextraction (DLLME) (Sarafraz-Yazdi and Amiri, 2010; Asensio-Ramos *et al.*, 2011; Cabaleiro *et al.*, 2013; Spietelun, 2014). General main advantages of these three different approaches of LPME are that they are low cost, almost solvent-free, rapid, environmentally friendly and simple, and the possibility of in situ derivatization or complexation (Mahugo-Santana *et al*., 2011; Spietelun *et al.*, 2014).

In the following lines the most important variations in the three different categories are presented and their advantages and disadvantages are discussed. Then, a description of some solvents used as alternative for the commonly first used ones will follow. The use of these solvents define a new type of techniques which can be termed together as LPME methods based on solidification of a floating organic drop.

Single drop microextraction (SDME)

This technique implies the distribution of analytes between a single drop of the extraction solvent (typically few microliters) and the sample. Several variations can be considered depending on the way the organic drop is exposed to the sample (Asensio-Ramos *et al.*, 2011; Kokosa, 2015). The most commonly used variations are schematized in Figure 1.1.

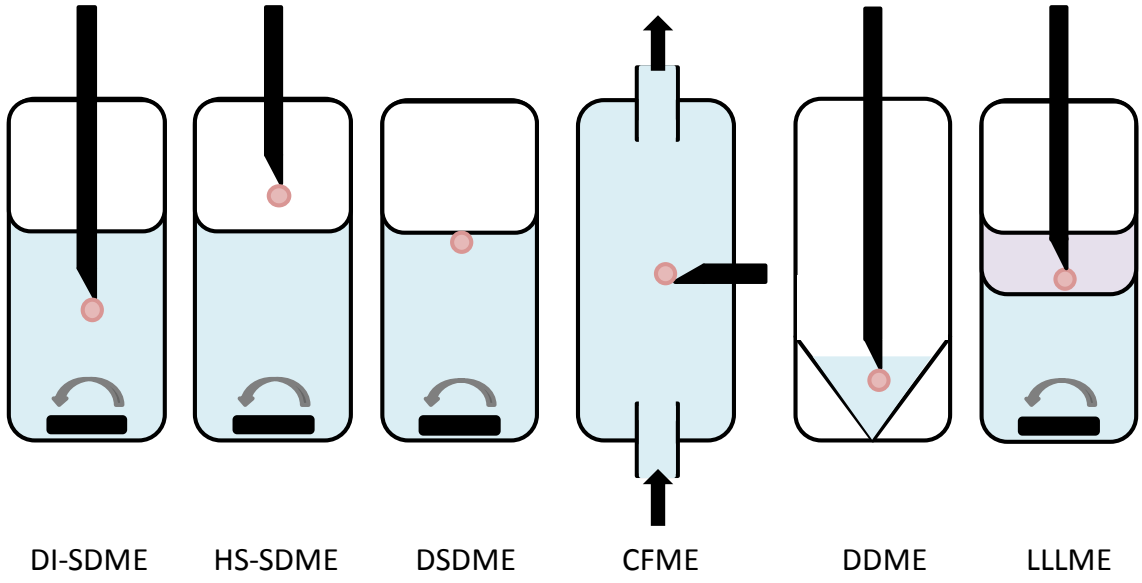


Figure 1.1. Schematic representation of the main approaches of single drop microextraction.

In direct immersion DI-SDME, the extraction solvent is exposed to the liquid sample by the suspension of a drop from the needle of a microsyringe, and when the extraction is finished, the drop is retracted and led to the analytical instrument. If extractant is exposed to the headspace (HS) instead to the liquid, the technique is called HS-SDME. In directly suspended droplet microextraction (DSDME) the drop rests directly in the vortex of a stirred liquid sample solution, without the aid of external supports. In continuous flow microextraction (CFME) extraction solvent is continuously put in contact with fresh flowing sample solution. Drop-to-drop microextraction (DDME) is a micro-version of DI-SDME, where volumes as small as 7 µL of sample and 0.3 µL of extraction solvents are used. Liquid-liquid-liquid microextraction (LLLME) is the three-phase variation of SDME, where analytes are extracted into an extraction solvent placed at the vortex of the aqueous sample, and then, a microdrop of a polar solvent (the final acceptor phase) at the end of a syringe needle is used to extract ionisable analytes from the second phase. Finally, in some

cases, a solid matrix is used to support the extraction solvent in the solvent-supported microextraction (SSME). This last variation can also be performed by direct contact of the drop with the sample or with the headspace, and it is not represented in the figure due to the wide range of different used support means.

In general, SDME is easy to operate, no special equipment is required (except for CFME) and with this technique high enrichment factors are obtained. However, its main disadvantages are the instability of the drop at high stirring rates and temperatures, the restrictions on the selection of the extraction solvent and the limited drop surface (which leads to higher extraction times) (Mahugo-Santana *et al.*, 2011; Spietelun *et al.*, 2014).

Hollow-fiber liquid-phase microextraction (HF-LPME)

Hollow-fiber liquid-phase microextraction was first introduced in 1999 by Pedersen-Bjergaard and Rasmusen (Pedersen-Bjergaard and Rasmusen, 1999). In this technique analytes are initially extracted into a supported liquid membrane sustained in the pores of a hydrophobic porous hollow-fiber, and later into an acceptor solution located inside the lumen of the fiber. When a unique organic solvent is used to fill the fiber pores and the lumen, this technique is known as two-phase-HF-LPME. When different organic solvents fill the lumen and the pores, the technique is called three-phase-HF-LPME or HF-LLLME. If extraction takes place into a short piece of a porous hollow-fiber sealed at both ends and introduced into a stirred sample, the technique is called solvent-bar microextraction (SBME) (Pena-Pereira *et al.*, 2010; Asensio-Ramos *et al.*, 2011). Figure 1.2 graphically represents these three variants.

The main advantages of HF-LPME techniques are the major stability of the drop of extractant (as it is mechanically protected) and the higher clean-up efficiency (high molecular mass compounds cannot go through the membrane barrier). Nevertheless, with this technique memory effects can be observed, special equipment is required, a previous step of preconditioning of membranes is necessary and higher extraction times and temperatures must be used (Mahugo-Santana *et al.*, 2011; Spietelun *et al.*, 2014).

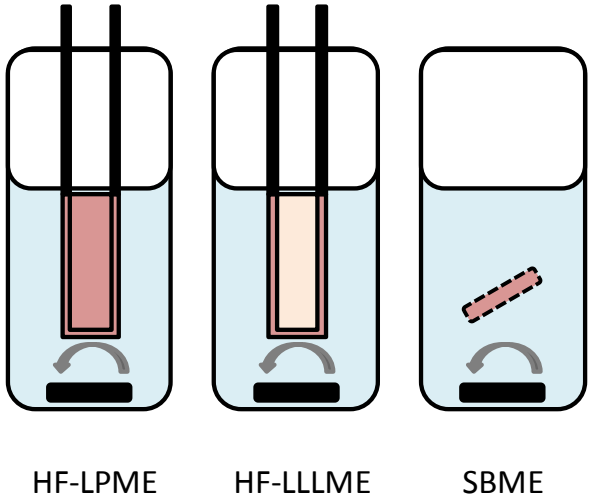


Figure 1.2. Schematic representation of the main approaches of hollow-fiber liquid-phase microextraction.

1. 1. Dispersive liquid-liquid microextraction (DLLME)

Dispersive liquid-liquid microextraction was firstly presented by Rezaee and co-workers in 2006 (Rezaee *et al.*, 2006). In this technique dispersion of the extractant is achieved by the addition of a third solvent miscible with both phases (the aqueous and the organic phase), known as dispersant or disperser solvent. When the appropriate mixture of extraction and disperser solvent is rapidly injected into a sample, a high turbulence is produced, which causes the formation of small droplets dispersed into the sample. Once this "cloudy solution" is formed, surface area between the extractant and the sample is larger and, thus, the extraction is faster. After extraction, samples are centrifuged in order to separate the two phases. Originally, this microextraction technique is performed using extraction solvents with higher density than water, such as chlorobenzene, chloroform, tetrachloromethane, tetrachloroethylene and carbon disulfide, and thus, after centrifugation, a sedimented phase is obtained at the bottom of the vial (Rezaee *et al.*, 2010). Tipically, the re-formed organic drop is collected with the aid of a microsyringe and led to the analytical instrumentation. Figure 1.3. schematically represents the extraction process until the obtaining of the sedimented drop in DLLME techniques.

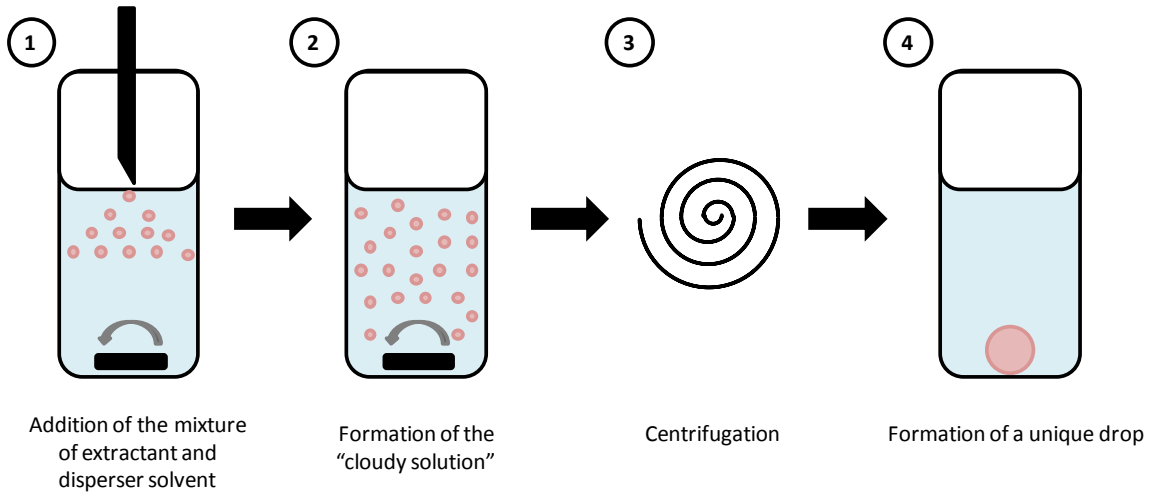


Figure 1.3. Schematic representation of the process in dispersive liquid-liquid microextraction.

Over the last 10 years several new variations on this technique have appeared, leading to different dispersive liquid-liquid microextraction-based techniques with different characteristics and, in some cases, nomenclature (Leong *et al.*, 2014; Šandrejová *et al.*, 2016). In classical DLLME mechanical agitation is frequently used in order to help the disperser solvent to form the "cloudy solution". Ultrasonic energy has been widely used in sample preparation processes (Priego-Capote and Luque de Castro, 2004), as it is known to cause cavitation, enhancing chemical reactions and mass transfer (Pena-Pereira *et al.*, 2010). In DLLME, therefore, ultrasound energy has also been used for the acceleration of the mass transfer process; the technique that combines the action of the disperser solvent and the ultrasound energy is known as ultrasound assisted dispersive liquid-liquid microextraction (UA-DLLME) (Lv *et al.*, 2014). On the other hand, in another well-known technique called ultrasound-assisted emulsification microextraction (USAEME) (Regueiro *et al.*, 2008), the "cloudy solution" is already caused by just ultrasonic radiation, which plays the role of the dispersant. These and other dispersive liquid-liquid microextraction methods have found application in very different fields (El-Shahawi and Al-Saidi, 2013; Hongyuan and Wang, 2013; Saraji *et al.*, 2014; Viñas *et al.*, 2014; Ahmad *et al.*, 2015; Jain and Singh, 2016)

Main advantages of DLLME techniques are the enormous contact area between the phases, and in consequence, faster extraction kinetics, and higher enrichment factors are reached. The main disadvantages are the addition of a third solvent (which can be avoided by applying ultrasonic energy in USAEME), the restrictions on the selection of the extraction solvent (as in SDME techniques) and

the additional step for disrupting the emulsion (Mahugo-Santana *et al*., 2011; Spietelun *et al*., 2014).

In this thesis, three different methods, each based on each of the three DLLME variants presented in this section, will be developed: dispersive liquid-liquid microextraction (DLLME), ultrasound-assisted dispersive liquid-liquid microextraction (UA-DLLME) and ultrasound-assisted emulsification microextraction (USAEME).

1. 2. Liquid-phase microextraction methods based on the solidification of a floating organic drop

LPME avoids the problem of the large solvent volumes in classical liquid-liquid extraction, but commonly used extraction solvents in these techniques are still generally toxic. In addition, the extraction of analytes of different polarities is limited. Thus, the search of new alternatives for extraction solvents has been of increasing importance during the last years (Kocúrová *et al.*, 2012; Yan and Wang, 2013).

Amongst all the new types of less toxic organic solvents, the ones with a melting point near to room temperature have become very popular. These solvents are more environmentally friendly than the originally used extraction solvents and, in addition, they provide a new way of collection of the drop after the extraction. In this way, the use of sophisticated extraction devices or collecting strategies necessary with other lighter-than-water extraction solvents is avoided. Techniques using these types of solvents are known as microextraction techniques based on the solidification of a floating organic drop (SFOD) (Kocúrová *et al.*, 2012; Yan and Wang, 2013).

The first liquid-phase microextraction method based on the solidification of an organic drop was proposed by Khalili-Zanjani and co-workers in 2007 (Khalili-Zanjani *et al.*, 2007). In this work, the typical procedure of the DSDME was used, but with an important variation, the use of an extraction solvent with a melting point near to room temperature (in the range of 10-30^{o}C) and higher density than water. Thanks to the new characteristics of the solvent, after extraction, the organic droplet was solidified by placing the extraction vial in an ice bath for 5 min. Then, the solidified floating solvent drop was collected with a spatula, transferred into a

conical vial and let melt at room temperature. After that, extraction solvent was ready for being conducted to analytical measurements.

From then on, this type of solvents has been used in different LPME techniques (Viñas *et al.*, 2015). In this way, different combined techniques evolved, such as DLLME-SFOD (Leong and Huang, 2008), UA-DLLME-SFOD (Wang *et al.*, 2014) and USAEME-SFOD (Bordagaray *et al.*, 2014). Those techniques combine the advantages of former explained microextraction techniques with being environmentally friendly, due to the use of low volumes of practically non toxic solvents.

1. 3. Variables affecting liquid-phase microextraction processes

In the development of a new quantitative analytical method all the variables influencing the measurement process must be carefully studied in order to select the parameters that provide the desired sensitivity, selectivity and precision. In this section, some of the more influent factors in LPME processes in general, and in DLLME and SFOD processes in particular, are presented.

Extractant

The type of extractant is one of the most important parameters in microextraction methods. Thus, the selection must be carefully done. Each approach to microextraction has its own criteria for extraction solvent selection depending on the specific requirements of each technique (Dadfarnia and Haji-Shabani, 2014). Generally, extraction solvents must satisfy the following criteria (Zang *et al.*, 2009; Wang *et al.*, 2010; Ghambarian *et al.*, 2013):

1. They should show good extraction efficiency for the analytes of interest.

2. They must have low water solubility.

3. They must have low volatility in order to prevent the loss of solvent during the extraction process.

4. The density should be appropriate for the selected technique: In conventional DLLME techniques, a higher density than water´s is desired in order to be able to collect the sedimented phase easier. In SFOD techniques, a lower density than water´s is required so that the solvent remains in the surface of the aqueous phase after the extraction, and it is more easily collected with the spatula.

5. They should be compatible with the selected detection technique and they should not interfere with the signal of the analyte. In addition, the solvent should be totally removed from the instrument from one measurement to the following.

6. When SFOD techniques are selected, solvents should have a melting point near room temperature (in the range of 10°C – 30°C).

According to these considerations, halogenated hydrocarbons, such as chlorobenzene, chloroform, carbon tetrachloride and tetracloroethylene are usually used in conventional DLLME techniques (Zang *et al.*, 2009). Table 1.1. shows the boiling points and the densities of the most commonly used solvents in DLLME processes (Lide, 2009).

Table 1.1. Boiling points, in °C, and densities, in g/mL, of common used extractants in DLLME techniques.

Extraction solvent	Boiling point (°C)	Density (g/mL)
Chlorobenzene	132	1.11^{20*}
Chloroform	61	1.48^{25*}
Carbon tetrachloride	77	1.59^{20*}
Tetracloroethylene	121	1.62^{20*}

*Temperature at which density has been measured

In liquid-phase microextraction methods based on the solidification of a floating organic drop, solvents that solidify at a little bit higher temperatures than water are used. Table 1.2. shows the most commonly used solvents in SFOD techniques, their melting and boiling points and their densities (Ghambarian *et al.*, 2013).

Table 1.2. Common used extractants in SFOD techniques, melting and boiling points, in ^{o}C, and densities, in g/mL.

Extraction solvent	Melting point (^{o}C)	Boiling point (^{o}C)	Density (g/mL)
1-Undecanol	13-15	243	0.83
1-Dodecanol	22-24	259	0.83
2-Dodecanol	17-18	249	0.80
n-Hexadecane	18	287	0.77
1-Bromohexadecane	17-18	190	0.99
1,10-Diclorodecane	14-16	167	0.99
1-Chlorooctadecane	20-23	157	0.85

The volume of the extractant is also an important factor and the optimal volume depends, on a high extent, on the type of microextraction technique (Krylov *et al.*, 2011). In general, the volume of the extraction solvent influences the surface area of the drop, and thus, the mass transfer process of the analyte from the sample to the extractant. An increase in the extraction solvent volume has also impact on the final organic phase volume, leading to the dilution of the analytes. Too little extraction solvent volumes, however, could lead to instability of the drop or to problems in its collection. In practice, solvent volumes that provide high preconcentration factors allowing a comfortable and reproducible collection of the drop should be selected (Krylov *et al.*, 2011; Ghambarian *et al.*, 2013).

Disperser solvent

The disperser solvent assists the dispersion of droplets of the extraction solvent in the aqueous phase, increasing the surface area between the phases in this way, and accordingly improving the extraction efficiency. The most important requirement is that it should be soluble in the extraction solvent and miscible in water. According to these characteristics methanol, ethanol, acetonitrile, acetone and tetrahydrofuran are the most commonly used disperser solvents (Zang *et al.*, 2009).

The volume of the disperser solvent directly affects the formation of the "cloudy solution" in the dispersive techniques, and consequently, the extraction efficiency. On one hand, too low volumes of disperser solvent could be ineffective in the formation of the dispersion of extractant but, on the other hand, too high volumes of disperser solvent lead to dilution of the sample. For all this, the lowest

volume of disperser solvent that is effective for the "cloudy solution" formation is normally selected. Experimentally, volumes between 0.5 and 1.5 mL are usually used (Zang *et al.*, 2009; Krylov *et al.*, 2011).

Extraction time

The extraction time in LPME is dependent on the mass transfer rate of the analytes from the sample into the extractant. In theory, in order to obtain the highest extraction efficiency and the highest repeatability, the equilibrium between the aqueous and the organic phase should be reached. However, often, it is not practical to wait for the equilibrium and extractions are performed under non-equilibrium conditions. Nevertheless, in dispersive techniques equilibrium is very rapidly attained due to the infinitely large surface area between both phases after the formation of the "cloudy solution". In consequence, these techniques are considered almost time independent, which is probably their major advantage (Ganjali *et al.*, 2010; Ghambarian *et al*., 2013).

Extraction temperature

High temperatures facilitate the mass transfer of the analytes from the aqueous solution into the extractant and, in this way, the time required to reach the equilibrium is decreased. However, at high temperatures, solubility of the extractant in the aqueous phase increases and an over-pressurization of the sample vial could make the extraction system unstable. Therefore, in LPME techniques temperatures should not exceed 60°C (Ghambarian *et al.*, 2013).

Salt addition

Salt addition may have several and opposite effects on microextraction methods. In general, an increase in the ionic strength leads to a decrease in the solubility of the analytes in the aqueous phase due to the salting-out effect, and in this way, extraction efficiency is improved. However, at the same time, solubility of the extraction solvent in the aqueous phase is decreased, and the obtained volume of organic phase after extraction is increased, resulting in the dilution of the extractated analytes. In addition, the presence of high concentrations of salt in

samples could change the physical properties of the Nernst diffusion film and reduce the diffusion rate of the analytes into the organic phase accordingly (Wang *et al*., 2010; Ghambarian *et al*., 2013).

2. Analytical instrumentation and coupling

In the typical analytical workflow, after sample preparation, instrumental data analysis is performed. In this thesis, three different methods were developed for the determination of three different types of analytes by three different analytical techniques. The types of analytes were: a heavy metal, phthalates and potentially allergenic substances related to fragrances. The techniques were: Ultraviolet-visible spectrophotometry, gas chromatography coupled to flame ionization detector and high-performance liquid chromatography with diode array detector.

Nowadays, UV-Vis spectrophotometry is doubtlessly one of the most important analytical techniques in any laboratory. Its success is probably due to its simplicity, versatility, high availability, speed, low cost and effectiveness. However, its main disadvantage is possibly its lack of selectivity. Nevertheless, sample preparation techniques can be used prior to spectrophotometric determination not only to improve selectivity but also sensitivity (Abadi *et al.*, 2012).

Although LPME techniques have been widely coupled with different detection techniques, the coupling with spectrophotometry is not straightforward and it has been only recently achieved. The difficulty lies in the adaptation of the microvolumes of extraction solvents obtained after extraction to the necessary volumes (2-3 mL) for conventional UV-Vis spectrophotometry. Amongst all the microextraction approaches, DLLME and SDME are the most widely used in combination with UV-Vis spectrophotometry (Abadi *et al.*, 2012). In those techniques the coupling problem has been mostly solved by evaporation and/or dilution of the sedimented phase after extraction and by the use of special instrumentation, such as cuvetteles micro-volume spectrophotometers, self-constructed miniaturized spectrophotometers, fiber optic-linear array detection spectrophotometry or digital colorimetry (Andruch *et al.*, 2012). However, adapting the organic phase to larger volumes tends to lead to a decrease in the determination capability of the analytes at low concentrations, and the use of special instrumentation tends to raise the price of the technique. In this thesis, the coupling

between the dispersive liquid-liquid microextraction and the spectrophotometry was achieved by the use of a simple microlitters-capacity cuvette. This way of connection avoided price rise and loss of determination capability related to other ways of coupling.

For the determination of phthalates and fragrances, as the objective was the simultaneous determination of various compounds, more sophisticated analytical instruments were necessary. In both cases, chromatographic methods were selected, due to their known potential in the separation of sample components. In particular, gas chromatography (GC) and high-performance liquid chromatography (HPLC) were applied. In gas chromatography a flame ionization detector (FID) and in HPLC a diode array detector (DAD) were used. Both, GC-FID and HPLC-DAD are relatively affordable analysis techniques by most of the routine analysis laboratories.

Gas chromatography is the preferred analytical technique to be used in combination with liquid microextraction methods (Abadi *et al.*, 2012). In general, the coupling with this technique is straightforward. In this thesis, the coupling was performed by simply injecting the obtained extractant into the instrument. In HPLC the coupling can be more problematic because the injected solvent must be soluble in the mobile phase, and thus, the selection of the extraction solvent is more restricted. Nevertheless, in this work, the compatibility was achieved by a slight dilution of the used extraction solvent in methanol (soluble in both, extractant and mobile phase).

3. Chemometrics

"*Chemometrics* is a chemical discipline that uses mathematics, statistics and formal logic (a) to design or select optimal experimental procedures; (b) to provide maximum relevant chemical information by analyzing chemical data; and (c) to obtain knowledge about chemical systems" (Massart *et al.*, 1997).

3. 1. Experimental design

In the development of a new quantitative analytical method, all the variables affecting the determination process need to be carefully studied. In the three developed methods of this thesis, dispersive liquid-liquid microextraction techniques were applied and thus, the variables previously explained in Section 1.3. needed to be considered.

Study of the influence of the different variables on the final signal has been traditionally done by the 'one variable at a time' (OVAT) approach. This is a simple method in which the influence of a specific variable is studied by changing its levels while the levels of all the remaining variables are maintained constant. In this thesis, when OVAT approach was used, results were visualized using bar plots. From those plots the conditions that gave the best results were selected. When a variable seemed to have no effect, analysis of variance (ANOVA) was used to confirm it.

ANOVA analysis compares variances in order to check if there are significant differences between the means of two or more groups of measurements. In the procedure the variance observed between groups is compared with the variance observed within groups by an F-test, and in this way, it is studied whether all the groups are part of one larger group or, if, on the contrary, there are different groups. The p-values in ANOVA table express the probability that the observed difference occurred by pure chance, and in many areas the considered borderline is p=0.05. This means that if a variable has a p-value lower than 0.05, the variation in the response induced by this variable is not by chance and the variable can be considered significant (Hill and Lewicki, 2007).

Although OVAT approach is a widely used method, sometimes the number of experiments is too high (especially when many variables are involved) and so it is the cost and time consumption of the optimization process. In addition, with this technique interaction between factors is not considered (Massart *et al.*, 1997).

Experimental design allows the simultaneous study of various parameters at the same time, and that simplifies the optimization procedure. With that purpose a specific set of experiments is constructed to simultaneously evaluate the effect produced by several factors at different levels. In order to clarify concepts, some of the most used terms in experimental design are defined below (Massart *et al.*, 1997; Bezerra *et al.*, 2008).

a) Factors or independent variables: experimental variables that can be changed independently of each other and in a controlled way to study their effect on the studied process. These can be qualitative (categorical) or quantitative (numerical). The variables affecting the microextraction processes seen in Section 1.3. are factors or independent variables.

b) Levels of a variable: the values at which a variable is studied in the experiments defined in the experimental design. Usually levels are coded: For quantitative factors the high level is indicated as "+1" (or "+") and the low level as "-1" (or "-") ("0", "+ α" and "- α" represent the central point and the star points respectively when necessary); for qualitative factors each code represents just a different option.

c) Experimental domain: experimental field delimited by the extreme levels of the studied factors.

d) Responses or dependent variables: measured values in the experiments. Examples of typical responses are the analytical signal (peak area, absorbance...), the recovery of an analyte or the resolution of chromatographic peaks. In general the highest or the lowest value of responses is searched. Sometimes more than one response may be studied, and results can be in conflict with each other: in this case the optimum value is a compromise between them.

e) Factor effect: change in the response caused by a variation in the level of a variable.

f) Interaction: Change in the response as a result of the combined effect of two or more variables.

In general terms, there are two main types of experimental designs, sequential and simultaneous designs. In sequential procedures a few initial experiments are performed and the obtained results are used to define the following experiments. The best known sequential model is the *Simplex* method. In simultaneous procedures, however, the experiments are well-defined from the beginning. These are the most commonly used and they can be divided into two types of designs (Massart *et al.*, 1997):

- Designs with the purpose of detecting the factors that have an influence or of estimating that influence: *Screening designs.*

- Designs with the purpose of modelling: *Optimization designs.*

When a new analytical method needs to be developed, different strategies are followed in order to optimize all the factors involved (Dejaegher and Vander Heyden, 2011). The different strategies combine univariate and multivariate approaches and are represented in Figure 1.4.

In summary, when a microextraction method is being developed, first of all, all the variables that possibly have influence on the extraction should be kept in mind. Then, the levels at which those variables are normally used in similar techniques need to be considered. Afterwards, the procedure schematized in Figure 1.4. has to be followed. That is, the variables that really affect the extraction process are selected either by univariate or by multivariate techniques. Finally, the optimization of these variables should be done either by OVAT approach or by multivariable procedures depending on the previous results and on the system characteristics.

In the following lines screening designs, and in particular full factorial designs and fractional factorial designs, are explained, as these are the ones used in this thesis.

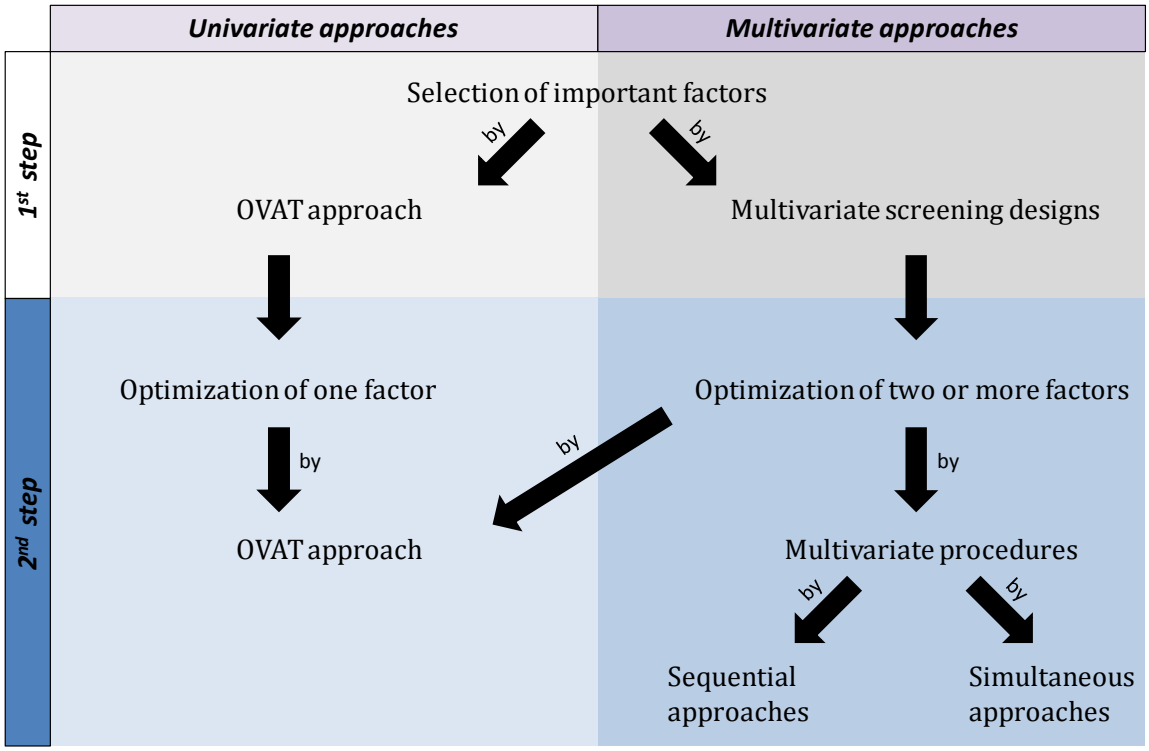


Figure 1.4. Different optimization strategies in method development.

3. 1. 1. Screening designs

Screening designs are employed to differentiate the relevant factors amongst all the factors that could be considered important at the beginning. That is, the factors that lead to a significant change in the response when their levels are changed.

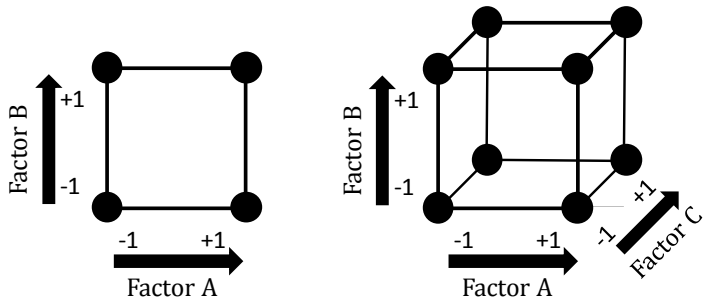
3. 1. 1. 1. Full factorial design

Full factorial designs include all the possible combination of the levels of the studied variables. Normally, two levels are considered in screening full factorial designs. Table 1.3 presents the experimental full factorial design matrix for two and three factors. Figure 1.5. shows a symbolic representation of the designs.

Table 1.3. Experimental full factorial design matrix at two levels for two and three factors.

	Two factor design		Three factor design		
Run	Factor A	Factor B	Factor A	Factor B	Factor C
1	+1	+1	+1	+1	+1
2	+1	-1	+1	+1	-1
3	-1	+1	+1	-1	+1
4	-1	-1	+1	-1	-1
5			-1	+1	+1
6			-1	+1	-1
7			-1	-1	+1
8			-1	-1	-1

+1: high level; -1: low level.



Two factors Three factors

Figure 1.5. Symbolic representation of two and three factors full factorial designs.

In these types of designs all main effects and interaction effects between the considered factors are estimated. The general mathematical model that describes the relationship between factors and responses in a three factor full factorial design, including only linear terms, is expressed in Equation 1.4.

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{1,2}x_1x_2 + b_{1,3}x_1x_3 + b_{2,3}x_2x_3 + b_{1,2,3}x_1x_2x_3 \quad \text{(Equation 1.4.)}$$

Where *y* represents the response, x_1 to x_3 represents the three factors and the different *b* (b_1, b_2, b_3, $b_{1,2}$, $b_{1,3}$, $b_{2,3}$, $b_{1,2,3}$) symbolize the coefficients. Similar mathematical models can be described when two or more than three factors are studied. In some cases high-order interactions are neglected and the polynomial is simplified (Stojanović, 2013).

Usually, analysis and model fitting with these designs is done based on the coded factor levels (and not on the actual values) because in this way the model

coefficients are dimensionless and, in consequence, directly comparable, which makes easier the determination of the relative size of factor effects (Wang and Wan, 2009).

After the construction of the model a graphical and/or statistical interpretation of the effects is done in order to determine their significance. Usually, ANOVA is used. Thanks to that, the variability of the effect is compared to the error by the use of an F-test. The Pareto chart of effects is often used to illustrate the obtained results. In this chart, the magnitude of each effect is represented by a bar and a line that crosses all the bars indicates how large an effect has to be to be considered statistically significant. In addition, the sign of the magnitude of each bar indicates the preferred coded value either to the high (if the sign is positive) or to the low level (if the sign is negative) (Hill and Lewicki, 2007).

A weakness of full factorial designs is the large amount of experiments to be performed, which increases exponentially with the number of factors considered, as expressed in Equation 1.5. (Brereton, 2003).

$$N = l^k \qquad \text{(Equation 1.5.)}$$

Where l is the number of levels considered for each variable (usually two in screening designs) and k is the number of variables. Table 1.4. shows how the number of experiments increases with the number of factors.

Table 1.4. Number of experiments in full factorial designs depending on the number of factors for two levels.

Number of factors	2	3	4	5	6	7	8	9	10
Number of experiments	4	8	16	32	64	128	256	512	1024

Sometimes, due to the high cost of reagents and/or due to time restrictions, it is not feasible to perform so many different experiments. In those cases, alternative designs that use less experimental runs are used, such as fractional factorial, Plackett-Burman and Taguchi designs (Brereton, 2003). Each of these types of designs reduces the number of experiments by building different matrices. The fractional factorial design is explained in the following lines as it is the one used in the development of the methods in this thesis.

3. 1. 1. 2. Fractional factorial design

A fractional two level factorial design examines *k* factors at two levels using 2^{k-v} experiments instead of 2^k (which is $\frac{1}{2^v}$ of the total number of experiments of the full factorial design) (Dejaegher and Vander Heyden, 2011) .

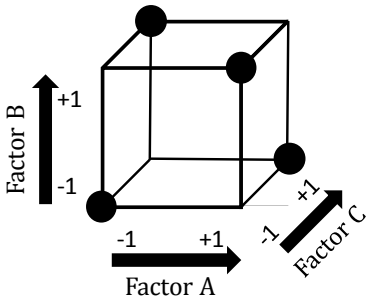
With full factorial designs models are built to estimate all the effects concerning main factors and interactions. With fractional factorial designs, as only a fraction of the full factorial design experiments are performed, some information is lost, and thus, some effects are confounded (which means that they are estimated together) (Dejaegher and Vander Heyden, 2011) . However, high-order interactions are usually considered unimportant and this lost of information is usually preferred to the elevated number of experiments of the full factorial designs (Massart *et al.*, 1997).

The description of the quality (the extent of the confounding) in fractional factorial designs is given by the resolution (*R*), which is expressed in Roman numbers by convention. The meaning of III, IV and IV resolution is as follows (Massart *et al.*, 1997):

- Resolution III: main effects are not confounded with each other, but they are confounded with two-factor interactions.
- Resolution IV: main effects are confounded neither with each other nor with two-factor interactions. However, two-factor interactions are confounded with each other.
- Resolution V: Both main effects and two-factor interactions are not confounded with each other.

In all the previous cases both main effects and two-factor interactions are confounded with high-order interactions.

As an example, Figure 1.6 presents a 2^{3-1} factorial fractional design.



	Three factor design		
Run	Factor A	Factor B	Factor C
1	+1	+1	-1
2	+1	-1	+1
3	-1	+1	+1
4	-1	-1	-1

Figure 1.6. Symbolic representation and experimental design matrix of a 2^{3-1} factorial fractional design.

This design consists of half of the experiments of the full factorial design. In addition, experiments are chosen in a balanced way in which every column in the experimental matrix is different; in each column an equal number of – and + levels are found, and for each experiment at level + for a factor, there are equal number of experiments for the other factors at levels + and – (Brereton, 2003). This allows the determination of all main factor effects if two factor interactions are neglected (Resolution III).

After the construction of fractional factorial design models, graphical and/or statistical interpretation of the estimated effects for determination of their significance is frequently done by ANOVA and Pareto charts, as in the case of full factorial designs.

3. 2. Method validation

After the development of a new analytical method, a validation study has to be performed in order to document its quality. The validation of an analytical method gives information about if the method is reliable, precise and remains completely under operator control. Parameters that should be determined during the validation process depend on the requirements needed for the developed analytical method, the final purpose and the time and cost desired to spend in the validation process. The parameters usually considered in a validation process are described below (Konieczka and Namieśnik, 2009).

Linearity and Range

Linearity of an analytical method is the ability that the method has to obtain output signals that correlate linearly with the analyte concentration within a given range. The most used method to estimate linearity is to construct a calibration curve and to obtain the regression parameters. Generally, it is enough to calculate the coefficient of regression (*R*) (Konieczka and Namieśnik, 2009; Rambla-Alegre *et al.*, 2012). Commonly, the measurement of linearity is expressed as the square of the correlation coefficient, as defined in Equation 1.6. (Harris, 2010).

$$R^2 = \frac{[\sum(x_i-\bar{x})(y_i-\bar{y})]^2}{\sum(x_i-\bar{x})^2 \sum(y_i-\bar{y})^2} \qquad \text{(Equation 1.6.)}$$

Where $\bar{x}$ and $\bar{y}$ are the mean of all *x* and *y* values in the regression, and x_i and y_i the individual x and y values in the regression. A R^2 of 1 indicates that all the observations fit perfectly a straight line, and therefore, the total variation in the data is explained by the regression. However, this never happens with real data and it is expected that R^2 values are as close to 1 as possible (Harris, 2010).

The linear range of a given analytical procedure is defined as the interval from the lower to the upper concentration of analytes for which it has been probed that the analytical procedure has a value of R^2 not significantly different from 1.

Limit of detection and Limit of quantification

In general, the detection limit (LOD) can be defined as the concentration, given by an instrument, which is significantly different from the blank (y_B). Due to the fact that "significantly different" can be interpreted in several ways, there are several modes to define the LOD. Statistically, the difference between the detection limit and the blank has been traditionally described by Equation 1.7.

$$LOD - y_B = 3S_B \qquad \text{(Equation 1.7.)}$$

Where S_B is the standard deviation of repeated measurements of the blank. This difference entails a probability for both, a false negative and a false positive, of 7% (Massart *et al.*, 1997).

Then, the calculation of the LOD can be accomplished in different ways. For example, to determine S_B in Equation 1.7., several independent measurements of the blank are usually made (a number of 10 determinations are normally considered enough) and the S_B is calculated accordingly. When there are difficulties to measure the blank, as it is a sample with no analyte present, this blank can be spiked with a certain amount of analyte, and then, S_B can be calculated from repeated measurements of the spiked sample (also usually 10). The added concentration of analyte should be on a level close to the expected LOD. Therefore, once the LOD has been calculated, this assumption has to be checked, and the procedure repeated if too little or too much analyte was added. In general, the following conditions should be fulfilled:

$$10 \cdot LOD > C_{min} \qquad \text{(Equation 1.8.)}$$

$$LOD < C_{min} \qquad \text{(Equation 1.9.)}$$

Where C_{min} is the concentration of analyte in the spiked sample (Konieczka and Namieśnik, 2009).

This method has been used to calculate the LOD in Chapters 4 (determination of phthalates) and 5 (determination of fragrance-allergens).

On the other hand, an alternative to calculate the LOD is very frequently used when a linear calibration has been previously built. In this alternative LOD is calculated as in Equation 1.10.

$$LOD = \frac{3\,S_{xy}}{b} \qquad \text{(Equation 1.10.)}$$

Where S_{xy} is the standard deviation estimated by the regression line and b is the slope of the regression. This expression is supported by the assumption that each point of the linear calibration plot, obtained by the unweighted least-squares method, has a normally distributed variation in the y-direction.

This method has been used to calculate the LOD in Chapter 3 (determination of cadmium) and in the determination of fragrance-allergens by multi-way analysis, in Chapter 6.

The limit of quantification (LOQ) is the lowest concentration of an analyte that can be determined with an acceptable accuracy, precision and uncertainty. Its calculation is made similarly to the calculation of the detection limit, but in this case, standard deviations are multiplied by a factor of 10 (instead of 3).

Precision

Precision is defined as the agreement between measured values for replicated measurements. There are different ways of measuring precision depending on the conditions it is measured, but all of them give a measurement of random errors and they are usually expressed as relative standard deviations (RSD %)(Konieczka and Namieśnik, 2009; Rambla-Alegre *et al.*, 2012).

- Repeatability: Precision measured under the same measurement conditions (analyst, instrument, reagents ...). If measurements are made in the same day, it is also known as intra-day precision.

- Intermediate precision: Precision measured within a given laboratory over a long time or using other different conditions (analyst, instrument...). If the precision is measured at different days maintaining the remaining conditions, it is also denoted as inter-day precision.

- Reproducibility: Precision measured by different analysts in different laboratories using the same procedure. Also called inter-laboratory precision.

Evaluation of precision requires the performance in the same specific conditions of a minimum number of independent replicates, which usually varies between 6 and 15 depending on the protocol (Magnusson and Örnemark, 2014).

Accuracy

Accuracy measures how close the measured value by an analytical procedure is to the accepted real value. This characteristic can be determined using real samples spiked with analytes, and it can be expressed in terms of recovery (%). By doing this, the presence of systematic errors is considered (Konieczka and Namieśnik, 2009).

3. 3. Multi-way analysis

One of the main purposes of chemometrics is to provide the maximum relevant chemical information of a system by analyzing the obtained data provided by the analytical instrument. Nevertheless, the nature of the obtained data can vary in a high extent from one analytical instrument to another.

Some instruments, such as pH-meters, give a unique response for each measurement, and thus, when several samples are measured, a sequence of numbers is obtained (a vector), which can be analyzed with one-way tools. However, some other instruments, such as the ones used in this thesis, deliver multiple responses from a single measurement. For example, absorbance at different wavelengths can be measured for each sample in UV-Vis spectrophotometers, and thus, data for several samples can be arranged in a two-way structure (a matrix). GC-FID provides two-way data, as well, as one electric signal is measured at each retention time for all the samples in the FID detector. In HPLC, however, a complete UV-Vis spectrum is obtained for each retention time, and thus, a three-way structure (a cube) is necessary to arrange all the obtained data: a value of absorbance is recorded at each retention time and each wavelength for each sample.

Multivariate data analysis involves the investigation of many variables simultaneously for getting a thorougher knowledge of the data. Multi-way analysis is the extension of multivariate analysis to three- or higher way arrays (Bro, 1998). In general, multivariate methods try to reduce mathematically the large amount of variables by creating new variables or components that describe all the interesting system information. This can be done by applying different methodologies.

Before explaining the methodologies, the nomenclature used in this thesis needs to be clarified. Lower case letters are used to denote scalars (x), vectors are shown with bold lower case letters (**x**), two-way matrices are expressed with bold capital letters (**X**) and three-way cubic arranges of data are named with bold capital letters underlined (**$\underline{\mathbf{X}}$**). In graphical representations vectors are described with lines, squares represent matrices and cubes symbolize 3-way data. Each dimension of an array is normally called “mode” and the dimension of each mode should be given. Thus, an array of dimensions (I x J x K) means that it has I variables in the 1st mode, J variables in the 2nd mode and K variables in the 3rd mode. Lastly, the superscript T is used to indicate the transpose of a matrix ($\mathbf{X}^{\mathbf{T}}$).

3. 3. 1. Parallel Factor Analysis (PARAFAC)

PARAFAC is a decomposition method for multi-way data in which the data array is decomposed as a sum of triple products of vectors (Bro, 1997). These vectors are called loadings, and each triplet of loading vectors is called "component". When the data can be well-modelled by a small number of components, the interpretation of the variations in the data can be done by these components instead of by the raw data, and thus, the analysis is simplified (Smilde *et al.*, 2004). Figure 1.7. shows the typical graphical representation of PARAFAC decomposition of data into a model with R components.

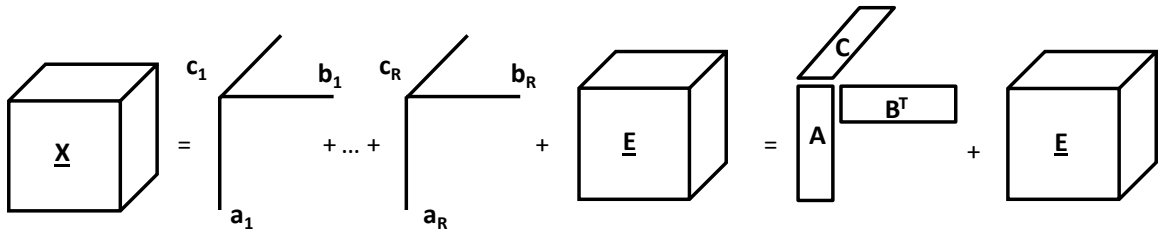


Figure 1.7. Graphical representation of a PARAFAC model with R components.

As it can be seen in Figure 1.7., the loading vectors for all components can also be arranged into three matrices, one for each mode. These matrices are frequently named as *loading matrices*. The loading matrix of samples mode is also usually called *score matrix*.

PARAFAC is then a trilinear model that can also be mathematically described as in Equation 1.11. (Smilde *et al.*, 2004).

$$x_{ijk} = \sum_{r=1}^{R} a_{ir} b_{jr} c_{kr} + e_{ijk} \qquad \text{(Equation 1.11.)}$$

Where x_{ijk} are the elements part of the $\underline{\mathbf{X}}$ three-way array, R is the number of components describing the PARAFAC model, and e_{ijk} is a residual term describing all the remaining unexplained variation.

As in the graphical representation (Figure 1.7.), Equation 1.11. can also be described with matrices as in Equation 1.12. (Smilde *et al.*, 2004).

$$\boldsymbol{X_K = AD_K B^T + E_K} \qquad \text{(Equation 1. 12.)}$$

Where $\boldsymbol{X_K}$ is the kth (I x J) slice of the $\underline{\mathbf{X}}$ array (I x J x K), $\boldsymbol{D_k}$ is the diagonal matrix with the kth row of **C** (K x R) on its diagonal (elements c_{k1} ..... c_{kr}), $\boldsymbol{A}$ (I x R) and $\boldsymbol{B}$ (J x R) collect the elements a_{ir} and b_{ir} respectively, and $\boldsymbol{E_k}$ is again a residual term.

In consequence, it can be clearly seen that each $\mathbf{X_k}$ can be modelled using the same components **A** and **B**, but with different weights, represented by $\mathbf{D_k}$. This fact is graphically represented in Figure 1.8.

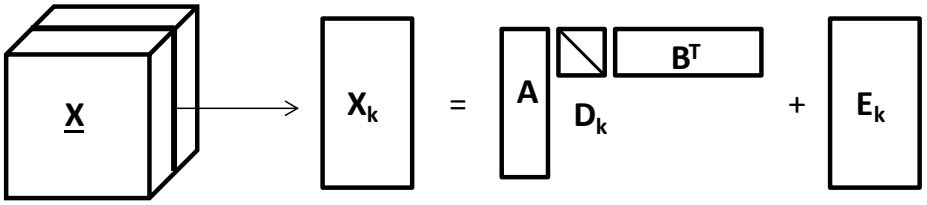


Figure 1.8. Description of each $\mathbf{X_k}$ in a PARAFAC model.

If the model is properly constructed, the score matrix (**D**) contains the relative concentration of each component in each sample. This fact allows the use of PARAFAC decomposition for calibration purposes.

One of the main advantages of PARAFAC models is the uniqueness of the solution, that is, it gives unique estimates and thus, **A**, **B** and **C** cannot be changed without changing the fit of the model (Smilde *et al.*, 2004).

PARAFAC has been successfully used with chromatographic data, although some data pretreatments are frequently necessary (Bylund *et al.*, 2002). In chromatography, a defined elution profile with stable retention times and well-defined peak shapes are only ideally obtained, and in the practice, retention time shifts (amongst other artifacts) are frequently observed between different runs. This problem is frequently solved by alignment before multi-way models are applied. However, there is also a modified version of PARAFAC, called Parallel Factor Analysis 2 (PARAFAC2) which can handle with retention time shifts and peak shapes changes (Skov and Bro, 2008; Amigo *et al.*, 2010).

3. 3. 2. Parallel Factor Analysis 2 (PARAFAC2)

PARAFAC2 also decomposes three-way data into different contributions (elution profile, spectral profile and concentrations in the case of chromatography), but its main difference with PARAFAC is that PARAFAC2 is able to extract a different elution profile for each component in each of the analyzed samples (Bro *et al.*, 1999; Kiers *et al.*, 1999; Amigo *et al.*, 2008). Figure 1.9. shows a graphical representation of PARAFAC2 decomposition of data into a model with R components.

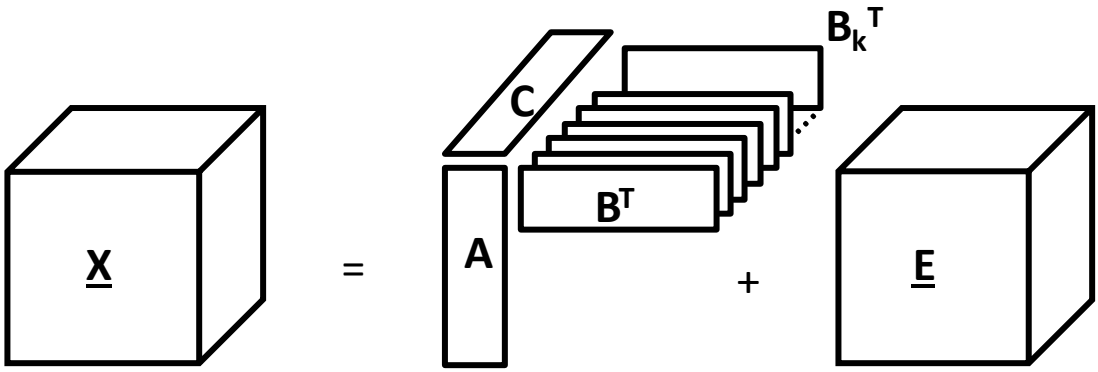


Figure 1.9. Graphical representation of a PARAFAC2 model with R components.

PARAFAC2 can be mathematically described in a similar way as PARAFAC (see Equation 1.12.) as shown in Equation 1.13.

$$\boldsymbol{X_K = AD_K B_K^T + E_K} \qquad \text{(Equation 1.13.)}$$

Although in PARAFAC2 we can obtain a different **B** for each sample, the possible **B**s are restricted, as the cross-product of $\mathbf{B_k}$ has to be constant over all the samples. This implies that the elution profiles of the different samples may differ but should still be somehow related. Thanks to this constraint, PARAFAC2, as PARAFAC, is also unique (Bro *et al.*, 1999; Amigo *et al.*, 2008).

In this thesis, PARAFAC2 has been applied to the data obtained from an HPLC-DAD instrument in order to estimate the concentration values of overlapped analytes in the obtained chromatogram.

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Objectives

Objectives

The main objective of the present thesis was the development of new reliable analytical methodologies combining different types of dispersive liquid-liquid microextraction techniques and diverse analytical instrumentation for the determination of several analytes in a variety of matrices.

This main objective can be divided into the following specific objectives:

1. The development of procedures based on dispersive liquid-liquid microextraction techniques. This type of techniques was selected because they are more environmentally-friendly and faster than other microextraction techniques.

2. The study of the versatility of the dispersive liquid-liquid microextraction techniques. With that purpose, the coupling of the techniques to diverse, and relatively affordable, analytical instrumentation was studied. Furthermore, the application of the methodologies to different, but commonly analyzed, matrices was accomplished for the determination of diverse analytes. Selected analytical instrumentation was UV-Vis spectrophotometry, GC-FID and HPLC-DAD. Analyzed matrices were water samples, food simulants, food liquid samples and cosmetics. Determined compounds were cadmium, phthalates and potentially allergenic substances related to fragrances.

3. The use of different available chemometric tools. First of all, to select the best experimental conditions for each methodology, experimental design and one variable at a time approaches were applied. Then, to validate the developed methods, different analytical figures of merit were calculated. And finally, multi-way analysis was used to overcome several problems that univariate analysis cannot solve.

4. The application of the optimized and validated procedures to different real samples to check compliance with the current legislation for each of the analyzed compounds.

Development of a method for cadmium determination in water samples using dispersive liquid-liquid microextraction and UV-Vis spectrophotometry

Development of a method for cadmium determination in water samples using dispersive liquid-liquid microextraction and UV-Vis spectrophotometry

1. Introduction

Heavy metals are natural elements with high atomic weight and a relatively high density. Some of them are essential nutrients necessary for diverse biochemical and physiological functions, and others are considered nonessential, as they have no biological function. Inadequate supply of both types of metals can lead to different diseases. Amongst all the heavy metals, arsenic, cadmium, chromium, lead and mercury present the greatest environmental and health hazard due to their wide use, toxicity and extensive distribution. In general, although these metals are naturally found on the Earths´s crust, most of the environmental contamination and human exposure results from their multiple industrial, domestic, agricultural, and technological applications (Baird and Cann, 2005; Tchounwou *et al.*, 2012).

In this chapter, determination of cadmium (Cd) is proposed. This is a very toxic heavy metal with no known biological function, which can cause very severe and irreversible adverse effects on human health. These effects include nephrotoxicity, osteotoxicity, carcinogenicity, teratogenicity and endocrine and reproductive toxicities (Goering *et al.*, 1995; Casas *et al.*, 2012). Moreover, due to its long biological half-life in human body (between 10 and 30 years), cadmium is efficiently retained once it is absorbed. In consequence, even minor intakes can lead to an important cadmium accumulation in the organism (Casas *et al.*, 2012).

Adverse effects of human exposure to cadmium were revealed in the late 1940s, when Friberg reported incidence of emphysema and proteinuria in workers exposed to cadmium dust (Goering *et al.*, 1995). Historically, the most important problem related to cadmium environmental contamination occurred in the Jintsu River Valley region of Japan. There, local rice was grown with water from a river contaminated with cadmium coming from a zinc mining and smelting plant upstream. Hundreds of people, especially women with various children and poor diets, fell ill with a degenerative bone disease called "*Itai-itai*" ("ouch-ouch") that causes severe pains in the joints. In this disease, Ca^{2+} ions in bones are replaced by

Cd^{2+} due to the fact that they have same charge and similar size, and therefore, bones slowly become porous and suffer from fractures and collapses (Baird and Cann, 2005).

Cadmium is uniformly distributed in the Earth´s crust at an average concentration of 0.15-0.20 ppm in the form of various inorganic compounds and complexes with natural chelating agents (Naja and Volesky, 2009). Cadmium has fundamentally +2 oxidation state. Some compounds with cadmium +1 are also known, but they are not important from the toxicological and analytical point of view (Burriel *et al*., 1985; Casas *et al.*, 2012).

Cadmium is usually found in natural deposits, like ores containing other elements as well (Naja and Volesky, 2009). Indeed, most cadmium is produced as a by-product of zinc smelting (Baird and Cann, 2005; Casas *et al.*, 2012). For this reason, environmental cadmium contamination frequently happens in areas where there are surrounding zinc mainly but also lead and copper smelters. The burning of coal and the incineration of waste materials containing cadmium are also significant sources of cadmium in the environment (Baird and Cann, 2005).

The main application of cadmium is in the electroplating of other metals, process in which approximately the 50% of the world production is inverted. In addition, it is also used in nickel-cadmium batteries, in alloys, in pigments, in PVC establishers and in nuclear fission control rods (Baird and Cann, 2005; Casas *et al.*, 2012).

All the previously mentioned industrial activity is the main source of cadmium pollution in the environment. Its presence in water and soils makes the incorporation of cadmium to plants quite easy, and from them, to the food chain. Tobacco leaves also absorb cadmium from soils and irrigation water, and then, the metal is again released to the environment into the smokestream. In summary, cadmium especially accumulates in human body via ingestion of contaminated food, inhalation of contaminated air and dust particles, and through cigarette smoke (Baird and Cann, 2005; Johri *et al*., 2010; Casas *et al.*, 2012).

According to the United States Environmental Protection Agency (USEPA) the level of cadmium in drinking water considered not risky is below 0.005 mg/L (USEPA, 2016). WHO, however, establishes the limit at 0.003 mg/L for a lifetime of consumption (WHO, 2011). In Europe legislation, environmental quality standards

criteria are also established for cadmium (and its compounds) in surface water; the annual average and the maximum allowable concentrations of cadmium are regulated depending on the type of water and its hardness, but values range from 0.08 to 0.25 μg/L and from 0.45 to 1.5 μg/L respectively (Directive 2008/105/EC).

Determination of cadmium in aqueous samples is therefore interesting, in order to control water for human and animal consumption and for irrigation of vegetable products. Due to the fact that low concentration levels cause adverse effects and to the complexity of the matrix samples, a preconcentration and separation step is normally necessary prior to cadmium determination. As previously stated in the introduction chapter, the field of sample pre-treatment is nowadays focused on miniaturized extraction procedures based on LLE. Amongst all the liquid-phase microextraction techniques, dispersive procedures seems to be one of the most attracting for cadmium determination, as they represent more than half of the cadmium determination procedures using liquid-phase microextraction techniques found in literature.

The different dispersive liquid-phase microextraction approaches have been coupled to different determination techniques for cadmium analysis. Amongst all, atomic absorption spectroscopy (AAS) techniques, either by flame (FAAS) (Sánchez Rojas *et al.*, 2011; Rahimi-Nasrabadi *et al.*, 2013; Rosa *et al.*, 2015) or graphite furnace (GF) AAS (Jahromi *et al.*, 2007; Rivas *et al.*, 2009; López-García *et al.*, 2013; Arain *et al.*, 2015; Pirsaheb and Fattahi, 2015; Ataee *et al.*, 2016) are the most widely used. Other techniques used include inductively coupled plasma (ICP) coupled to optical emission spectrometry (OES) (Silva *et al.*, 2012; Lemos and Vieira, 2014), atomic emission spectrometry (AES) (Sereshti *et al.*, 2014) or mass spectrometry (MS) (Jia *et al.*, 2010; Ramos and Borges, 2014), total reflection x-ray spectrometry (TXRF) (Marguí *et al.*, 2013), and atomic fluorescence spectroscopy (AFS) (Zhou *et al.*, 2011).

The coupling of liquid-phase microextraction techniques to UV-Vis spectrophotometry has only been recently achieved, as it has been difficult to adapt the microvolumes after the extraction to the necessary volumes in conventional UV-Vis spectrophotometers. This problem has been mainly solved by either evaporation and/or dilution of the sedimented phase after extraction or by the use of special instrumentation, such as fiber optic-linear array detection spectrophotometry (FO-LADS) or digital colorimetry (DC) (Abadi *et al.*, 2012; Andruch *et al.*, 2012); but the dilution to larger volumes decreases the determination capability of the analytes at

low concentrations, and the use of special instrumentation increases the price of the technique. In cadmium determination, in particular, UV-Vis spectrophotometry has been applied by conventional DLLME followed by either dilution of the sample (Wen *et al.*, 2011)or by using a donor-active solvent instead of a disperser solvent (Kocúrová *et al.*, 2013).

In recent years, the coupling of DLLME to UV-Vis spectrophotometers by means of microcapacitty-cuvettes has been widely studied for the determination of different compounds; some of them metals (Alexovič et al., 2012; Sereshti and Aliakbarzadeh, 2013; Rastegarzadeh et al., 2014; Peng *et al.*, 2015; Pourreza *et al.*, 2015). This has been proved to be an efficient way of coupling, which avoids problems related to dilution and/or evaporation of the sample and to the necessity of special equipments.

For extraction and spectrophotometric determination of cadmium, the use of a chelating agent is needed in order to make the metal more soluble in the organic phase and to make it determinable by UV-Vis spectrometry. There are several chelating agents that can be used in the extraction and spectrophotometric determination of cadmium, such as the 2-(5-Bromo-2-pyridylazo)-5-diethylaminophenol, 4-(2-pyridilazo)resorcinol or diphenylcarbazone in the presence of 1,10-phenantroline. Amongst all the possible chelating agents, dithizone has been widely used and its efficacy has been extensively probed (Sandell and Onishi, 1978; Marczenko, 1986). This reagent was first prepared by Emil Fischer, but it remained in oversight of analytical chemists for almost 50 years until 1925, when Hellmut Fischer showed its exceptional value for trace metals analysis. Even when the final determination of metals is not performed by photometric methods, dithizone is widely used for trace metal separations (Sandell and Onishi, 1978). Figure 3.1. shows the structure of dithizone.

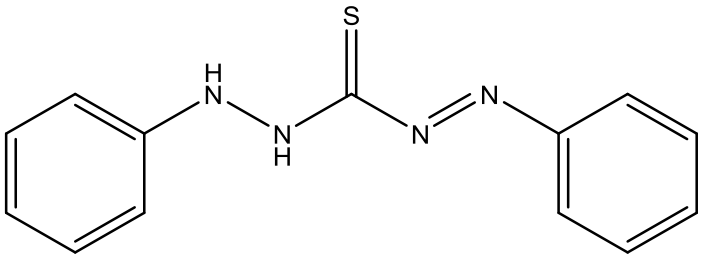

Figure 3.1. Chemical structure of dithizone.

As it can be seen, dithizone contains two active hydrogen atoms, each of which could be replaced by an equivalent of a metal. Primary dithizonates are those in which only an active hydrogen is replaced by the corresponding metal; in secondary dithizonates, however, both hydrogens are replaced. Furthermore, secondary dithizonates are only formed by some specific metals, and they do not have spectrophotometric applications (Sandell and Onishi, 1978; Marczenko, 1986).

Dithizone reacts with cadmium forming primary dithizonates. Figure 3.2. shows the chemical structure of general metal primary dithizonates.

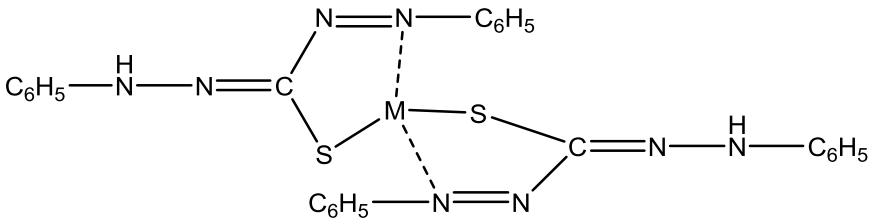

Figure 3.2. Chemical structure of primary metal dithizonates.

UV-Vis spectrophotometry is a commonly used technique in analytical chemistry. Its main advantages are its simplicity, speed, high availability and low cost, in addition to its versatility and effectiveness. The coupling of this technique to dispersive liquid-liquid microextraction techniques can develop simple, fast and low cost analytical methods. However, as previously said, the coupling of UV-Vis spectrophotometry with liquid-phase microextraction techniques has not been straightforward, and some problems have commonly arisen. In this chapter, the coupling of both techniques for cadmium determination was accomplished by means of a microcapacitty-cuvette to avoid both, a loss in the determination capability of the analytes at low concentrations due to the dilution of the organic phase, and an increase in the price due to special instrumentation.

2. Objectives of the chapter

The main objective of the present chapter was the development of a simple, fast and inexpensive analytical method for cadmium determination in aqueous samples by coupling dispersive liquid-liquid microextraction to UV-Vis spectrophotometry.

The principal objective of the chapter was accomplished with the following specific objectives:

1. The extension of the use of dispersive liquid-liquid microextraction techniques coupled to UV-Vis spectrophotometry. This equipment was selected because it is fast, simple and inexpensive, and due to its availability in most laboratories.

2. The solving of problems of previous ways of coupling liquid-phase microextraction techniques and UV-Vis spectrophotometry. With the use of microlitters-capacity cuvettes, dilutions and the need to use special equipment are avoided.

3. The study of the best conditions for the determination of cadmium. The principal parameters affecting extraction and determination of the metal were studied in detail by combination of `one variable at a time´ (OVAT) approach and experimental design.

4. The validation of the optimized procedure by means of the appropriate quality parameters. Studied properties were linearity, limits of detection, precision, accuracy and enrichment factors.

5. The application of the optimized procedure to different types of water samples.

3. Experimental

3. 1. Reagents, standards and materials

All the following analytical grade reagents and solvents used were purchased from Panreac Química S.A (Barcelona, Spain): cadmium standard stock solution (1000 mg/L), dithizone used as chelating agent, tricloromethane employed as extractant, methanol and ethanol used as disperser solvents, sodium hydroxide and potassium chloride utilized in the preparation of the solution for adjusting the pH, and sodium chloride. Exception was tetrachloromethane, also used as extraction solvent, which was obtained from VWR International Eurolab S.L. (Barcelona, Spain).

Working solutions of cadmium were obtained daily by appropriate stepwise dilution from standard stock solution in 0.5 M nitric acid. Working solution of dithizone was also daily prepared in the corresponding extraction solvent. pH of the metal solutions was adjusted just before the measurement with the aid of a weekly prepared NaOH/KCl solution. All solutions were preserved in the fridge while not using. Doubly distilled water was used throughout the whole work.

3. 2. Samples

All the samples collected for cadmium determination were aqueous samples. In particular, the following samples were analyzed: Two commercial differently demineralised drinking water of different brands (DW1, DW2), tap water (TW), snow water (SW), river water (RW1) and water from an irrigation channel (RW2). Tap water was collected after letting it run for 10 minutes. Waters from river, irrigation channel or snow were collected a few days before analysis. All samples were filtered through a 0.45 μm micropore filter and subjected to proper dilutions (from 0.5 to 2.5 mL of sample into 10 mL of solution). pH in samples was adjusted with the NaOH/KCl solution.

3. 3. Instrumentation

The spectrophotometer 8453 UV-Vis Diode Array System (Agilent Technologies, Madrid, Spain) with the software "UV-Visible Chemstation Rev. A. 10.01" was used for obtaining the spectra with a 80 µL-capacity cuvette (Hellma GmbH & Co. KG, Müllheim, Germany).

The extractions were made in a vessel with a jacket joined to a Lauda Ecoline Re104 E100 thermostatic bath (Lauda GmbH & Co. KG, Lauda-Königshofen, Germany). Agitation was provided by a HI 190M magnetic agitator (Hanna instruments, Eibar, Spain) and centrifugation was performed on a 2600 Nahita centrifuge (Auxilab, Beriáin, Spain). The used pH-meter was a "pH & Ion-Meter GLP 22+" (Crison, Barcelona, Spain).

Values of cadmium for the calculus of the enrichment factor were obtained by measurements in the 7700X ICP-MS equipment (Agilent Technologies, Madrid, Spain).

Experimental design was performed and the results were evaluated by the use of Statistica Software (StatSoft,Tulsa, USA).

3. 4. DLLME procedure

Microextraction was accomplished in a test tube containing 7.5 mL of a sample (or of a solution of cadmium) adjusted at pH 12.8 ± 0.2 and 1.5 mL of disperser solvent (methanol). The mixture sample/dispersant was brought to 40°C, and then, 150 µL of a 34 mg/L solution of dithizone (chelating agent) in chloroform (extraction solvent) was added. Everything was stirred at 1000 rpm and 40°C for 3 minutes to form a cloudy solution in which chloroform was dispersed into fine droplets that extract the complex cadmium-dithizone, formed during the extraction. Then, the solution was centrifuged at 3500 rpm for 1 minute and the dispersed droplets were deposited together at the bottom of the test tube. Finally, an amount of 60 µL of the resultant sedimented phase was transferred to a microcuvette for the determination in the spectrophotometer. The extraction procedure is schematically shown in Figure 3.3.

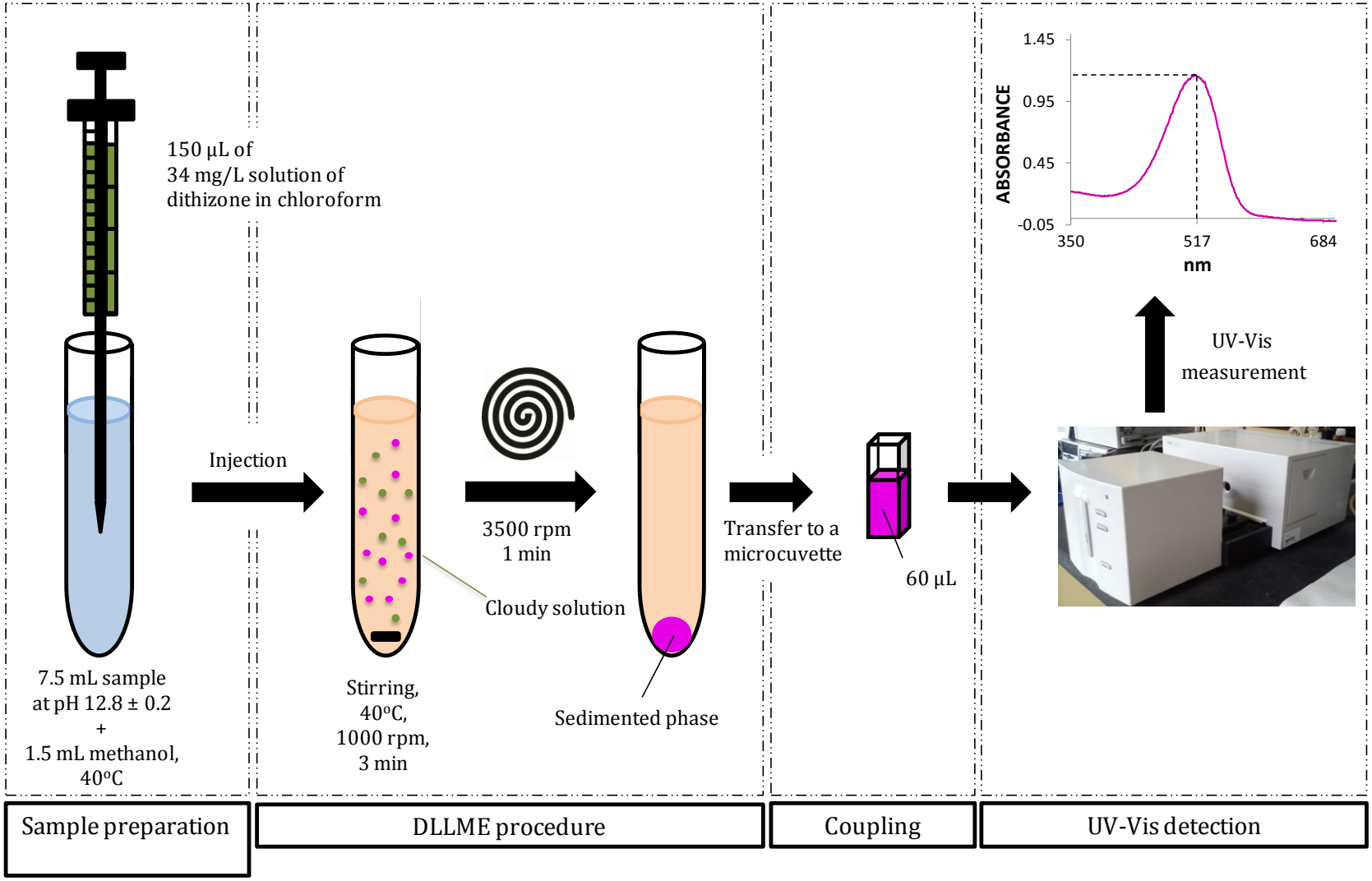

Figure 3.3. Schematic representation of the DLLME procedure divided in four steps: Sample preparation, DLLME procedure, coupling and UV-Vis detection.

4. Results and discussion

The principal objective of the present chapter was to develop a new reliable microextraction method for cadmium determination by coupling a dispersive liquid-liquid microextraction technique to UV-Vis spectrophotometry.

With that purpose, the absorption spectra of all the species affecting the determination process were firstly studied. Secondly, some considerations regarding the stability of the used solutions were considered. After that, the most appropriate conditions for the DLLME-UV-Vis process were studied. Finally, the developed method was validated and applied to different real samples.

4. 1. Study on the absorption spectra

Due to its high efficiency, dithizone was selected as chelating agent for the extraction and determination of cadmium by UV-Vis spectrophotometry (Marczenko, 1986; Sandell and Onishi, 1978). Thus, the first step for the development of the method was to study the absorption spectra of dithizone and cadmium dithizonate.

For studying the spectrum of dithizone, solutions in carbon tetrachloride and chloroform were directly measured in the spectrophotometer. For measuring the spectrum of dithizonates, previous mentioned solutions were subjected to the extraction of cadmium, and the resultant organic phase containing the dithizonate was measured.

In both cases, results according with bibliography were obtained; dithizone has an absorption spectrum with two maximums, at 450 and 610 nm, respectively. Alternatively, the spectrum of the complex ($Cd(HDz)_2$) has a maximum around 515 nm (516 nm in chloroform and 517 nm in carbon tetrachloride), where the dithizone spectrum has a minimum. Both spectra are shown in Figure 3.4.

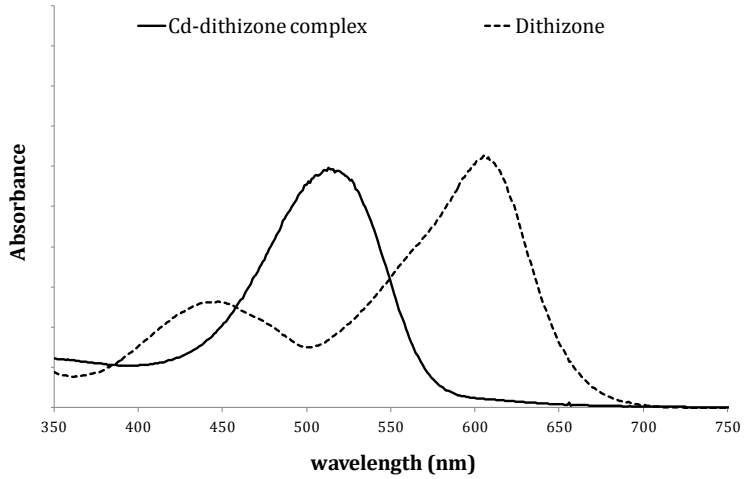

Figure 3.4. Dithizone and Cd-dithizone complex spectra

It could seem at first sight that the presence of dithizone can interfere in the cadmium analysis due to the spectral overlapping. During the extraction process from aqueous solutions at high pHs, however, the excess of dithizone passes completely to the aqueous phase (Marczenko, 1986). Hence, in those cases, only the signal due to the dithizonate is observed in the organic phase.

4. 2. Initial considerations

Dithizone can be oxidized by air, by products formed in the photodecomposition of the organic solvents in contact with it, and by the possible oxidants present in the sample solution (Sandell and Onishi, 1978). Thus, dithizone solutions are not totally stable. In this case, it was observed that solutions of dithizone gave decreasing values of absorbance from one day to the other, while values of absorbance made during the same day were considerably reproducible. In consequence, it was decided to prepare working solutions of dithizone daily.

In the same way, it was observed that cadmium diluted samples at high pH values were also not stable because decreasing values were obtained if solutions were let stand for some time. The reason is that cadmium hydroxide precipitates above pH 8 (Burriel *et al*, 1985). Therefore, it was decided to prepare cadmium solutions at the moment of measuring.

4. 3. Selection of the most suitable experimental conditions

With the purpose of obtaining a proper method for cadmium determination by dispersive liquid-liquid microextraction and UV-Vis spectrophotometry, the most suitable experimental conditions affecting the microextraction process had to be found.

Firstly, the effect of stirring rate and centrifugation conditions were visually evaluated. Then, other variables that needed a more thorough study were considered univariately. These variables were the pH of the sample, the effect of salt addition and the extraction time. After that, the extraction solvent to be used in the determination process was considered. Finally, the rest of the variables that may also affect the determination process, that is: temperature, dithizone concentration and type and volume of disperser solvent, were evaluated simultaneously by experimental design in order to save time and resources. According to the obtained results, the most appropriate conditions for the determination of cadmium using DLLME and UV-Vis spectrophotometry were defined.

Concentration of cadmium during all the optimization process was 0.075 mg/L. The runs to study the variables univariately were made under fixed conditions of the others. Therefore, initial conditions of the different variables, unless otherwise specified, were the following: extraction solvent: 100 μL of carbon tetrachloride; concentration of dithizone: four times the stoichiometrically necessary; disperser solvent: 0.4 mL of methanol; extraction temperature: 25^{o}C; extraction time: 3 minutes; and sample volume: 7.5 mL.

4. 3. 1. Stirring rate and centrifugation conditions

In dispersive microextraction techniques, efficient formation of the cloudy solution is necessary in order to increase the superficial area of contact between both phases and to fasten the mass transfer process. Then, the dispersion must be disrupted in order to obtain a unique well-formed organic phase. In this case, the dispersion was caused by mechanical agitation with a magnetic stirrer, and then, it was disrupted by means of centrifugation.

The rate of the magnetic agitator has direct impact on the formation efficiency of the dispersion. Its effect was visually evaluated in a range from 500 to 1250 rpm, and it was observed that at low rates (around 500 rpm) dispersion was not formed, at medium rates (around 750 rpm), dispersion was only partially formed, and at very high rates (above 1250 rpm), the magnetic stirrer used to raise up in the test tube avoiding the dispersion of the organic drop. In consequence, a rate of 1000 rpm was selected for further experiments.

The conditions of the centrifugation process were also considered. In general, a fast and efficient process is desirable in order to not extend the process time too much. In this work, vials were usually broken at high centrifugation rates. That is why, the highest centrifugation rate that did not break the vials was selected, that is, 3500 rpm. At that rate, 1 minute was enough for obtaining a well formed sedimented-phase.

4. 3. 2. Effect of pH

Extraction efficiency of metal complexes is generally closely related to the pH of the system. The extraction of cadmium with dithizone has traditionally been done at a high pH values (Sandell and Onishi, 1978; Marczenko, 1986). Indeed, it has been reported that extraction of cadmium with dithizone into carbon tetrachloride occurs around pH 10, and then, it increases with the pH to reach a maximum between pH 12 and 13 (Harris, 2010).

The solubility of dithizone is also highly affected by the pH. Dithizone is not soluble in water under pH 7; and it is soluble in basic aqueous solutions, where it forms orange solutions of dithizonate ions. Figure 3.5. shows the curves of dithizone distribution between aqueous phase and carbon tetrachloride and chloroform (Marczenko, 1986). From then, it can be concluded that only at high values of pH (above 11 for carbon tetrachloride and above 12.5 for chloroform) all the dithizone present in the system can be in the aqueous phase. For the determination of cadmium it is interesting that the dithizone remains in the aqueous phase so that it does not interfere in the analysis.

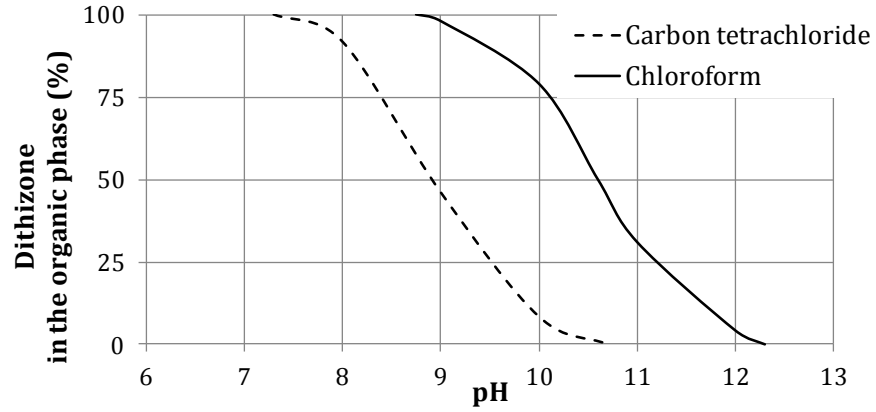

Figure 3.5. Dithizone distribution between aqueous and carbon tetrachloride or chloroform phases.

Experimentally, the effect of the pH in the signal was investigated in solutions with and without cadmium at pHs ranging from 4.0 to 13.0. Results for the blank at some of the studied pHs are shown in Figure 3.6. As it was expected, dithizone gave a remarkable signal when pHs below 12 were used. Therefore pH 12.8 ± 0.2 was selected for further experiments.

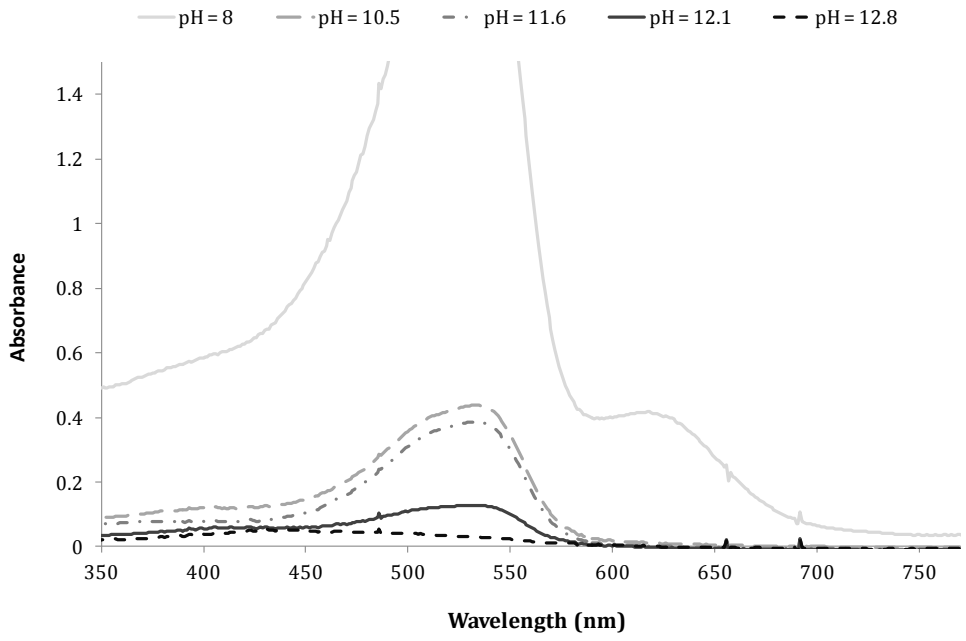

Figure 3.6. Blank signals obtained for the determination process at different pHs.

4. 3. 3. Selection of salt addition

Salt addition is known to be one of the main parameters affecting extraction processes and it is commonly employed to improve extraction efficiency due to the salting out effect. However, as previously said in the Introduction Chapter (Section 1.3.), it can also have opposite effects on microextraction processes, like the reduction of the diffusion rate of the analytes into the organic phase or the negative influence in the solubility of the extraction solvent in the aqueous phase.

In this case, addition of 100 g/L of salt was compared with no addition of salt and results are shown in Figure 3.7. As it can be observed, obtained signal was better when no salt was added. Furthermore, it was observed that when salt was added, a higher volume of organic phase after extraction was obtained. In consequence, it can be concluded that salt addition affects to the system by lowering the solubility of the extraction solvent in water and in consequence, by causing a higher dilution of the cadmium dithizonate in the organic phase.

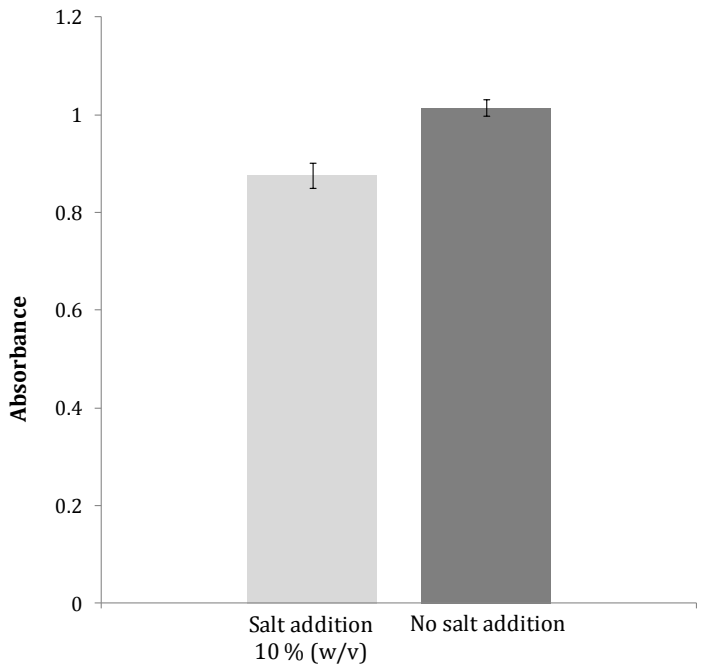

Figure 3.7. Comparison of the signals obtained after all the microextraction process with salt addition and without salt addition and fixed levels of the other variables.

4. 3. 4. Selection of extraction time

Extraction time is a very important factor in LPME techniques, as in general, a certain period of time is needed for reaching the equilibrium of the distribution of analytes between phases. However, in dispersive techniques equilibrium is attained almost immediately, and extraction is considered almost time-independent (Ghambarian *et al*., 2013).

In this work, time effect was evaluated at 1, 3 and 5 min. In this case, due to technical reasons, extraction time was compared using in all cases 140 μL of chloroform and 0.56 mL of methanol. Results are presented in Figure 3.8.

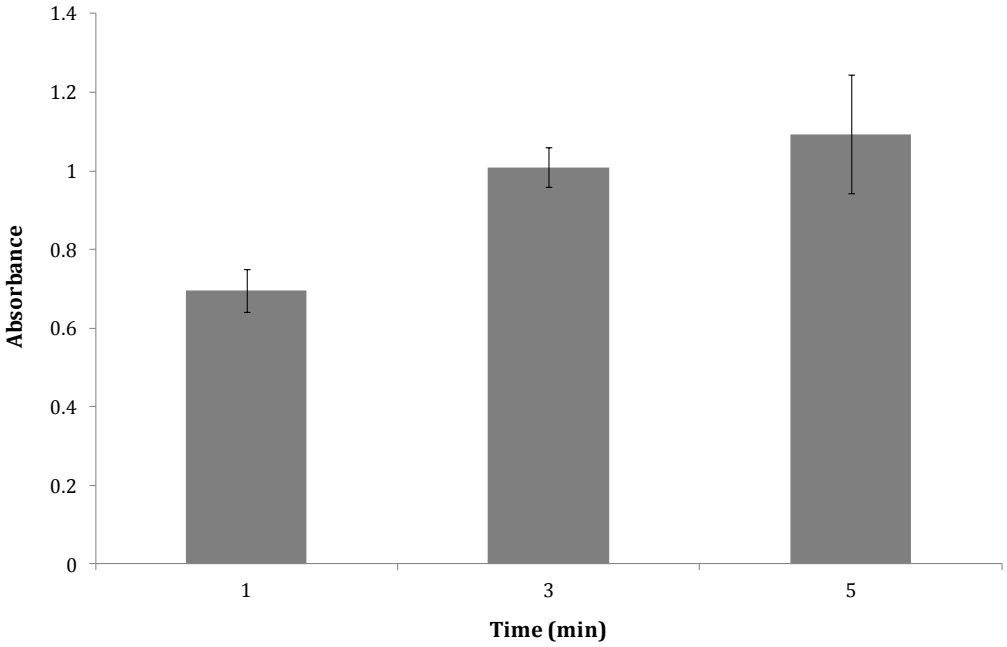

Figure 3.8. Comparison of the signals obtained after all the determination process with different extraction times (1, 3 and 5 minutes) and fixed levels of the other variables.

As it can be seen, signal obtained after all the microextraction process increases when 3 minutes are used instead of 1 minute, but then it remains similar at 5 minutes. This fact was confirmed by ANOVA analysis (see Introduction Chapter, Section 3.1.), which showed that there were statistical differences between the signals obtained at 1 and 3 minutes ($p = 0.027$), but there were not statistical differences between the signals at 3 and 5 minutes ($p = 0.530$). Thus, it can be concluded that equilibrium is achieved after only 3

minutes of extraction. In consequence, an extraction time of 3 minutes was used in the following experiments.

4. 3. 5. Selection of extractant

The selection of an appropriate extraction solvent is really important for the DLLME process. Halogenated hydrocarbons are usually selected as extraction solvents because of their higher density than water, their extraction capability, and their low solubility in water (Xiao-Huan *et al.*, 2009). Among them, chloroform ($CHCl_3$) and carbon tetrachloride (CCl_4) were selected for the extraction of cadmium for their better properties.

Due to the different characteristics of the two extraction solvents (different boiling points, solubilities in water and interactions with different disperser solvents, specifically methanol, ethanol, acetone and acetonitrile), extraction efficiency of both could not be compared in the same conditions, so it was decided to make a different experimental design for each solvent, and to compare the results of the better conditions of each.

Regarding the volume of extraction solvent, if it is too low, the volume of the sedimented phase is not enough to carry out the analysis. On the other hand, if the volume is too high, the organic phase after extraction is too large, the analytes are more diluted, and therefore, the analyte detection capability of the method is lower. Thus, it was decided to use the less volume of extraction solvent which still gave manageable sedimented phase volume (100 µL CCl_4 and 150 µL for $CHCl_3$). In this way, waste is minimised and unnecessary dilution resulted from larger volumes is avoided.

4. 3. 6. Experimental design

After having fixed univariately some of the variables affecting the determination process, experimental design was used to find the relative significance amongst the remaining variables, in order to save time and resources. As it has been previously said, the best extraction conditions using independently both, CCl_4 and $CHCl_3$ have to be found.

In particular, in the present work, full factorial designs at two levels were selected, as they are one of the most useful designs when the number of factors is relatively limited (Araujo and Brereton, 1996).

As previously commented, a different full factorial design was run for each of the selected extraction solvents. The levels of each factor in the designs were chosen combining information found in the literature and preliminary studies as follows (Xiao-Huan *et al*., 2009):

i. Methanol and ethanol were selected as dispersants due to their capability to form the dispersion. Other very used disperser solvents such as acetone and acetonitrile were also tried in preliminary studies, but they were finally discarded because they showed low signals.

ii. Disperser solvent volume directly affects the efficiency of the formation of the cloudy solution and therefore, extraction efficiency. Usually, values between 0.5 and 1.5 mL are used in dispersive microextraction processes, and thus, these were the values selected.

iii. Normally, high temperatures facilitate the transfer of analytes from the sample solution to the organic phase. However, it also affects the solubility of the solvents in the aqueous phase, and the volatilization of the different components. In this case, the boiling temperatures of the different extraction solvents (chloroform: 61^{o}C; carbon tetrachloride: 77^{o}C) and different disperser solvents (methanol: 65^{o}C; ethanol, 78^{o}C) were considered (Lide, 2009). In the chloroform design, higher level was fixed at 40^{o}C, due to its lower boiling point. In carbon tetrachloride, the boiling point of methanol was the restricting condition, and a temperature of 50^{o}C was selected as the higher level of temperature. In both cases low level was set at 25^{o}C.

iv. Dithizone should be in excess during the process, to make sure that all the cadmium present in the sample reacts with the dithizone available. Hence, an amount of dithizone double to the necessary, according to the stoichiometry, was selected as low level, whereas 6 times the necessary amount was selected as high level. Higher concentrations of dithizone lead to blanks with high signals. As dithizone is added dissolved in the extractant, and different extractant volumes are used depending on the extraction solvents, different net concentrations of dithizone were defined for each design. For carbon tetrachloride, 52 and 154 mg/L were selected and for chloroform 34 and 102 mg/L (low and high level respectively).

Table 3.1 summarizes all the variables and the levels considered in both screening experimental designs.

Table 3.1. Variables and levels considered in the experimental designs.

Variables	CCl_4 Variable levels		$CHCl_3$ Variable levels	
	Low	High	Low	High
Temperature (°C)	25	50	25	40
Dithizone concentration (mg/L)	52	154	34	102
Volume of dispersant (mL)	0.5	1.5	0.5	1.5
Type of dispersant	Ethanol	Methanol	Ethanol	Methanol

Tables 3.2. and 3.3. show experimental runs and results obtained for the carbon tetrachloride and chloroform designs respectively. In both cases 2^4 full factorial designs were selected, and each of the planned experiments were performed twice. Therefore, each experimental design consisted of 32 experiments in total.

Table 3.2. 2^4 full factorial experimental design matrix and obtained absorbance signal at 517 nm for cadmium determination using CCl_4 as extraction solvent.

Experiment	Replicate	Dithizone conc (mg/L)	Disp	V disp (mL)	T (°C)	Abs (517 nm)
1	2	52	MeOH	1.5	50	0.6442
2	2	154	EtOH	0.5	50	0.9565
3	2	52	EtOH	0.5	25	0.4532
4	2	154	MeOH	1.5	25	0.4550
5	1	154	EtOH	0.5	50	0.9957
6	1	154	MeOH	1.5	25	0.5170
7	1	52	EtOH	0.5	25	0.3718
8	1	52	MeOH	1.5	50	0.6947
9	2	154	MeOH	0.5	25	0.4658
10	1	52	MeOH	0.5	50	0.8113
11	1	154	MeOH	0.5	25	0.4579
12	2	52	EtOH	1.5	25	0.4140
13	1	52	EtOH	1.5	25	0.4554
14	2	52	MeOH	0.5	50	0.7253
15	1	154	EtOH	1.5	50	0.9129
16	2	154	EtOH	1.5	50	0.8549
17	2	154	MeOH	0.5	50	0.8368
18	1	52	MeOH	0.5	25	0.3939
19	1	52	EtOH	1.5	50	0.5996
20	2	154	EtOH	1.5	25	0.4757
21	2	52	EtOH	1.5	50	0.6142
22	2	52	MeOH	0.5	25	0.4209
23	1	154	EtOH	1.5	25	0.3562
24	1	154	MeOH	0.5	50	0.7372
25	1	52	EtOH	0.5	50	0.9782
26	2	52	EtOH	0.5	50	1.0345
27	2	154	EtOH	0.5	25	0.4354
28	1	154	MeOH	1.5	50	0.9886
29	2	154	MeOH	1.5	50	1.0177
30	2	52	MeOH	1.5	25	0.4040
31	1	154	EtOH	0.5	25	0.3916
32	1	52	MeOH	1.5	25	0.3940

Conc: concentration; Disp: disperser solvent; V disp: volume of disperser solvent; T: temperature; Abs: absorbance; MeOH: methanol; ETOH: ethanol.

Table 3.3. 2^4 full factorial experimental design matrix and obtained absorbance signal at 516 nm for cadmium determination using $CHCl_3$ as extraction solvent.

Experiment	Replicate	Dithizone conc (mg/L)	Disp	V disp (mL)	T (°C)	Abs (516 nm)
1	1	34	EtOH	0.5	25	0.4650
2	2	102	MeOH	1.5	25	0.5194
3	2	34	EtOH	0.5	25	0.5017
4	1	34	MeOH	1.5	40	0.8964
5	1	102	MeOH	1.5	25	0.5007
6	2	102	EtOH	0.5	40	0.6833
7	1	102	EtOH	0.5	40	0.7754
8	2	34	MeOH	1.5	40	0.8745
9	2	102	EtOH	1.5	40	1.1081
10	1	34	MeOH	0.5	40	0.9479
11	2	34	EtOH	1.5	25	0.4672
12	1	102	EtOH	1.5	40	1.2654
13	2	34	MeOH	0.5	40	0.8205
14	2	102	MeOH	0.5	25	0.3262
15	1	34	EtOH	1.5	25	0.5384
16	1	102	MeOH	0.5	25	0.3174
17	1	102	EtOH	1.5	25	0.1773
18	2	34	EtOH	1.5	40	0.9652
19	2	102	MeOH	0.5	40	0.8808
20	1	34	EtOH	1.5	40	0.9335
21	2	34	MeOH	0.5	25	0.3886
22	1	34	MeOH	0.5	25	0.3970
23	1	102	MeOH	0.5	40	0.9492
24	2	102	EtOH	1.5	25	0.1375
25	2	102	EtOH	0.5	25	0.2944
26	1	34	EtOH	0.5	40	0.6253
27	1	102	EtOH	0.5	25	0.2916
28	2	102	MeOH	1.5	40	1.0590
29	1	34	MeOH	1.5	25	0.5266
30	1	102	MeOH	1.5	40	1.1468
31	2	34	MeOH	1.5	25	0.5338
32	2	34	EtOH	0.5	40	0.7544

Conc.: concentration; Disp: disperser solvent; V disp.: volume of disperser solvent; T: temperature; Abs.: absorbance; MeOH: methanol; ETOH: ethanol.

The obtained data were evaluated by analysis of variance (ANOVA) (Table 3.4.) and main effects were visualized in Pareto Charts (Figure 3.9.). These graphical and statistical representations of the results were explained in detail in the Introduction Chapter (Section 3.1.). Interactions were not considered significant, and in consequence, they were not taken into account in the models.

In brief, the significance of the factors in ANOVA is evaluated by means of an F-test, and fast interpretation can be made considering the associated p-values. p-values lower than 0.05 indicate that the factor considered is significant. On the contrary, p-values higher than 0.05 denote not-significant factors.

Table 3.4. ANOVA results obtained in experimental designs for each extraction solvent. In black: significant factors ($p < 0.05$).

CCl_4					
	SS	df	MS	F	p
T	**1.336899**	**1**	**1.336899**	**1123.9063**	**0.000000**
Dithizone conc	**0.065321**	**1**	**0.065321**	**6.0541**	**0.020559**
V disp	0.013944	1	0.013944	1.2924	0.265603
Disp	0.003517	1	0.003517	0.3260	0.572769
Error	0.291319	27	0.010790		
Total SS	1.711001	31			
$CHCl_3$					
	SS	df	MS	F	p
T	**2.154280**	**1**	**2.154280**	**118.6714**	**0.000000**
Dithizone conc	0.001294	1	0.001294	0.0713	0.791473
V disp	**0.155574**	**1**	**0.155574**	**8.5700**	**0.006859**
Disp	0.037888	1	0.037888	2.0871	0.160054
Error	0.490140	27	0.018153		
Total SS	2.839176	31			

Parameters: SS: sum of squares; df: degress of freedom; MS: mean square effect; F: fischer coefficient; p: probability.

Variables: T: temperature; Conc: concentration; V disp: volume of disperser solvent; Disp: disperser solvent.

In Pareto Charts, the magnitude of each standardized estimated effect is represented by a horizontal column and a vertical line indicates the value from which an effect is considered statistically significant (at a 95% confidence level). Moreover, the sign of each effect reflects at which level of the variables the response is higher (positive: high level, negative: low level).

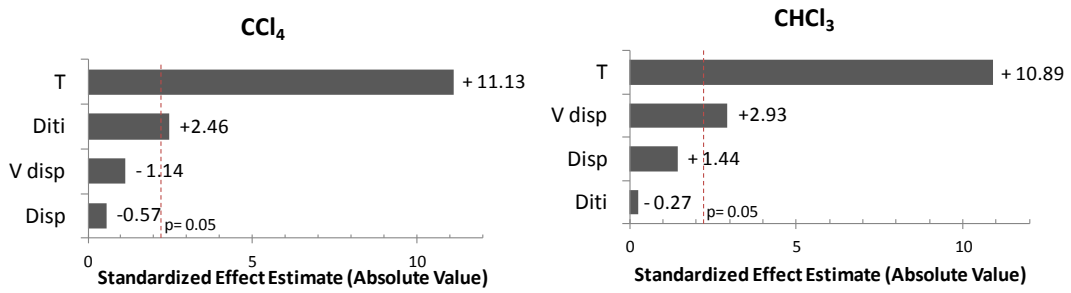

Figure 3.9. Pareto charts obtained in the full factorial designs using carbon tetrachloride and chloroform. *Variables:* T: temperature; Diti: dithizone concentration; V disp: volume of disperser solvent; Disp: type of disperser solvent.

According to ANOVA and Pareto charts the effect of temperature was positive and significant when both extraction solvents were used. Volume of dispersant was not significant when carbon tetrachloride was used but when chloroform was used it was significant and high levels were preferred. Dithizone concentration had a significant effect when carbon tetrachloride was used, and higher signals were obtained at higher dithizone concentrations; for chloroform, however, it was not significant. Finally, the type of dispersant was not significant in none of the designs. Table 3.5. summarises the significance of each variable and the sign of its effect.

Table 3.5. Summary of the results obtained in the performed designs.

CCl_4				
Factor	Low level	High level	S	E
Temperature (ºC)	25	50	Yes	+
Volume of dispersant (mL)	0.5	1.5	No	-
Dithizone concentration (mg/L)	52	154	Yes	+
Type of dispersant	Ethanol	Methanol	No	-

$CHCl_3$				
Factor	Low level	High level	S	E
Temperature (ºC)	25	40	Yes	+
Volume of dispersant (mL)	0.5	1.5	Yes	+
Dithizone concentration (mg/L)	34	102	No	-
Type of dispersant	Ethanol	Methanol	No	+

S: significant; E: effect.

In concordance with the results the levels of the non-significant variables were fixed at the values which gave better responses. That is, 0.5 mL of dispersant and ethanol for carbon tetrachloride and 34 mg/L of dithizone and methanol for chloroform. Temperature was fixed at the corresponding high level in both cases because a further increase of the temperature than the higher level led to problems in the collection of the drop after extraction. For the same reason, when chloroform was used, the disperser solvent volume was fixed at high level. When carbon tetrachloride was used the concentration of dithizone was fixed at 154 mg/L.

Absorbances in the best conditions for both extraction solvents were compared and results are shown in Table 3.6.

Table 3.6. Absorbance in the best conditions of each extraction solvent.

Extractant	T (ºC)	V disp (mL)	Disp	Dithizone conc (mg/L)	Abs
CCl_4	50	0.5	Ethanol	154	0.98±0.03
$CHCl_3$	40	1.5	Methanol	34	0.89±0.02

T: temperature; V disp: volume of disperser solvent; Disp: disperser solvent; Conc: concentration; Abs: absorbance.

As it can be seen, absorbance when CCl_4 is used is higher than when $CHCl_3$ is used. However, it is not sufficiently higher to compensate for its higher toxicity and cost. Moreover, due to its elevated toxicity, CCl_4 was sometimes difficult to purchase. That is why chloroform was selected for further experiments.

In the chloroform design, significant variables were temperature and volume of dispersant, and both of them gave higher signals at the high level. However, further optimization of those variables is not possible as none of them can be further increased, because it would lead to problems in the collection of the drop and, in the case of the temperature, in the volatilization of the extraction solvent.

4. 3. 7. Summary of the most suitable experimental conditions

The following table (Table 3.7.) summarises which are, in conclusion, the most appropriate extraction conditions to obtain the best response for the determination of cadmium by DLLME coupled to UV-Vis measurement according to the previous results.

Table 3.7. The most suitable extraction conditions for cadmium determination by DLLME-UV-Vis.

Variable	Condition
Extraction solvent	
• Type	Chloroform
• Volume	150 μL
Disperser solvent	
• Type	Methanol
• Volume	1.5 mL
Dithizone	
• Concentration	34 mg/L
Centrifugation	
• Time	1 min
• Rate	3500 rpm
Extraction	
• Time	3 min
• Agitation	1000 rpm
• Temperature	40°C
Sample	
• Volume	7.5 mL
• pH	12.8 ± 0.2
• Salt addition	No

4. 4. Method validation

After fixing all the conditions that define the optimized method for cadmium determination by means of DLLME and UV-Vis spectrophotometry, the developed method needs to be validated. Validation of an analytical method is the process of testing some important parameters with the purpose of being sure that every future measurement performed with the method will provide reliable and precise results. In this case, the evaluated statistical parameters were: Linearity, limit of detection, precision, and accuracy. In addition, enrichment factor and the possible interferences will also be studied in this section.

Linearity of the method was studied in the range from 10 to 100 µg/L. Calibration curve was constructed using seven different levels of concentrations, and each of them was measured twice. Correlation coefficient (R^2= 0.9947) showed a good linearity in the selected range. Calibration curve is presented in Figure 3.10. In this case, the limit of detection (LOD) was calculated based in the residual standard deviation of the calibration curve (SD_{Xy}), as previously explained in the Introduction Chapter (Section 3.2.), and LOD was 8.5 µg/L. This value is considered high for cadmium determination according to the levels established by USEPA (5 µg/L), WHO (3 µg/L) and European legislation for environmental water (0.08-1.50 µg/L) (Directive 2008/105/EC; WHO, 2011; USEPA, 2016). However, it allows cadmium determination in highly contaminated water samples.

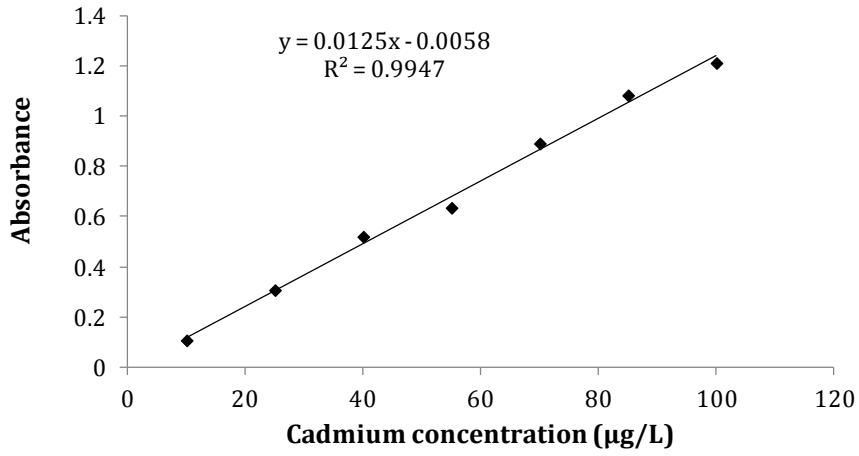

Figure 3.10. Calibration curve for cadmium obtained with the optimized method.

The precision of the method was evaluated in order to have an overview of the deviation in the measurements for the same cadmium concentration. Evaluation was made at two concentration levels (20 and 80 μg/L) and the types of precision studied were intra-day and inter-day precision. According to EURACHEM recommendations, at least 10 independent measurements should be made for precision evaluation at each level and results should be given as standard deviation (EURACHEM, 1998). In this case, intra-day precision was evaluated by means of 10 measurements carried out the same day and inter-day precision by means of 12 experiments performed in three different days during two weeks. Results show relative standard deviations (RSD, %) of 13.3 and 9.0% for intra-day precision and 10.9 and 9.0 % for inter-day precision at the low (20 μg/L) and high (80 μg/L) levels respectively.

Acceptable values for precision studies of an analytical method according to the AOAC recommendations are presented in Table 3.8. (AOAC, 2012). All the values obtained in precision studies were below 15, and therefore, they were considered adequate.

Table 3.8. Expected precision values for a tested method according to AOAC.

Analyte fraction	Unit	RSD (%)
1	100%	1.3
10^{-1}	10%	1.9
10^{-2}	1%	2.7
10^{-3}	0.1%	3.7
10^{-4}	100 ppm	5.3
10^{-5}	10 ppm	7.3
10^{-6}	1 ppm	11
10^{-7}	**100 ppb**	**15**
10^{-8}	**10 ppb**	**21**
10^{-9}	1 ppb	30

In bold concentration levels used in this method.

Enrichment factor (EF) is not normally considered a validation parameter. However, when microextraction techniques are developed this value is normally given in order to provide with an approximation of how effective the process is. As it was shown in the Introduction Chapter (Section 1.), EF is defined as the ratio obtained dividing the value of the concentration in the organic phase after the extraction and the initial concentration of the

analyte in the water sample. In this case, because it was very difficult to construct a calibration curve of determination of cadmium in water using UV-Vis spectrophotometry without using a preconcentration technique, the measurements of the concentrations were made by inductively coupled plasma-mass spectroscopy (ICP-MS). A value of 73 was obtained for EF.

Another important property of the developed method is selectivity, as it is one of the weak points of UV-Vis spectrophotometry. Selectivity is the extent to which a method can determine a specific analyte in a complex mixture without interferences from other components (Rambla-Alegre *et al.*, 2012). In our case, dithizone can form complexes with other metals, so interferences with them may occur. The effect of potentially interfering ions or metals was considered by calculating the concentration of cadmium in a solution containing 80 μg/L of cadmium and different molar ratios of the metals using the developed procedure. Tolerable limit was taken when the interfering ion/cadmium molar ratio did not cause a relative error in the signal higher than 10%. The tolerable limit for each metal is presented in Table 3.9.

Table 3.9. Tolerable limits to different metal ions.

Tolerable limits*	Metal ions
250	Al^{3+}
100	Ca^{2+}
10	Pb^{2+}
1	Mg^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}
0.1	Fe^{3+}, Ni^{2+},Cu^{2+}

*Units of tolerable limits: interfering ion/cadmium molar ratio.

Table 3.10. shows a summary of the most important analytical figures of merit of the developed method together with the ones found in literature for cadmium determination in aqueous samples by combining dispersive liquid-liquid microextraction techniques with different detection methods.

Table 3.10. Several important analytical parameters in this work compared with the found in bibliography.

	R^2	LOD (µg/L)	RSD (%)	EF	Reference
DLLME-FAAS	0.9898	0.4	1.9-2.7	55*	Sánchez Rojas *et al*., 2011
EB-DLLME-FAAS		0.03	4.6	151*	Rahimi-Nasrabadi *et al*., 2013
DLLME-GF AAS	0.9991	0.0006	3.5	125*	Jahorimi *et al*., 2007
DLLME-ETAAS	0.9990	0.004	2.9	115*	Rivas *et al*., 2009
DLLME-ICP-OES		0.3	0.9	13*	Silva *et al*., 2012
TSIL-DLLME-ICP-AES	0.9991	0.22	1.0-3.0		Sereshti *et al*., 2014
DLLME-FI-ICP-MS	0.9994	0.0005	2.6	460	Jia *et al*., 2010
DLLME-TXRF		0.04	5		Marguí *et al*., 2013
DLLME-UV-Vis		7	1.8-5.0		Kocúrová *et al*., 2013
DLLME-UV-Vis	0.9947	8.5	9.0-13.3	73	**This work** (Pérez-Outeiral *et al.*, 2014)

NOTE: The explanation of the methods abbreviations is included in the abbreviation list.
*Enhancement factor is given instead of enrichment factor.
R^2: correlation coefficients; LOD: limit of detection; RSD: relative standard deviation; EF: enrichment factor.

As it can be seen, all the reported methods had an appropriate linearity and precision. Obtained LOD in this method was considerably higher than some of those obtained when more sensitive techniques such as AAS, ICP-MS or TXRF are coupled to DLLME (Jahorimi *et al.*, 2007; Rivas *et al.*, 2009; Jia *et al.*, 2010; Marguí *et al.*, 2013; Rahimi-Nasrabadi *et al.*, 2013). However, it was only an order of magnitude higher than the ones obtained with DLLME coupled to ICP-OES (Silva *et al.*, 2012; Sereshti *et al.*, 2014) or in another study using DLLME coupled to FAAS (Sánchez Rojas *et al.*, 2011). In addition, the LOD in the developed method was similar to the one obtained in another work that applied UV-Vis spectrophotometry coupled to DLLME (Kocúrová *et al.*, 2013).

EF value obtained in the developed method was also similar to the ones found in literature. It is remarkable that in some articles enhancement factor was given instead of enrichment factor. Enhancement factor has a similar definition to EF, but it is calculated as the ratio between the slope of a calibration curve prepared from standards submitted to the recommended procedure and the slope obtained for a calibration curve for solutions not submitted to pre-concentration processes.

4. 5. Application to real samples

Finally, the proposed method was applied for cadmium determination in different real samples. The samples were: two differently demineralised brands of drinking water (DW1, DW2), tap water (TW), river water (RW1), water from an irrigation channel (RW2) and snow water (SW). However, no cadmium was found in any of the analyzed water samples.

Hence, in order to evaluate the applicability of the developed method, the accuracy was studied by spiking the samples with known concentrations of analyte. The accuracy of a given analytical method is defined as the agreement between the measured value of analyte content and the real value, and it can be expressed in terms of recovery (Rambla-Alegre *et al.*, 2012). In this case, the accuracy was checked by comparing the added concentration with the concentration obtained after triplicate measurements of the cadmium content. Table 3.11. shows the obtained values of recovery for each of the samples

Table 3.11. Recovery (mean of triplicates) of Cd in water samples.

Sample	Spiked (μg/L)	Found (μg/L)	Recovery (%)	RSD (%)
DW1	20.0	19.9	99.5	9.3
DW1	80.0	82.3	102.8	9.0
DW2	40.0	43.4	108.6	3.1
TW	40.0	38.0	95.1	7.1
RW1	40.0	35.9	89.7	5.7
RW2	40.0	40.3	100.9	9.4
SW	40.0	43.3	108.2	4.6

DW: drinking water; TW: tap water; RW: river water; SW: snow water, RSD: relative standard deviation.

According to AOAC recommendations, acceptable recovery values, depending on the analyte content, are presented in Table 3.12. (AOAC international, 2012).

Table 3.12. Expected recovery values for a tested method according to AOAC.

Analyte fraction	Unit	Recovery (%)
1	100%	98-102
10^{-1}	10%	98-102
10^{-2}	1%	97-103
10^{-3}	0.1%	95-105
10^{-4}	100 ppm	90-107
10^{-5}	10 ppm	80-110
10^{-6}	1 ppm	80-110
10^{-7}	***100 ppb***	***80-110***
10^{-8}	***10 ppb***	***60-115***
10^{-9}	1 ppb	40-120

In bold concentration levels used in this method

In this case, results showed recoveries ranging from 90 to 109% and RSDs between 3 and 9% between the replicates, and thus, they were considered satisfactory. Therefore, it can be concluded, that the proposed method can be successfully applied to the determination of cadmium in real water samples giving accurate and reproducible results.

5. Conclusions

A simple and inexpensive method for the determination of cadmium has been developed by coupling DLLME and UV-Vis spectrophotometry. DLLME has some advantages in comparison with other preconcentration techniques, because it does not use high volume of solvents or very exclusive reagents. UV-Vis spectrophotometry, on the other hand, shows low cost and simplicity, although its sensitiveness is more limited than in other techniques. As a result, this method can be considered affordable for many analytical laboratories.

The coupling of these two techniques has been successfully achieved by the use of a microlitters-capacity cuvette. This form of connection avoids the inconveniences of previous DLLME-UV-Vis coupling ways, such as the loss of detection capability due to dilution, or the increment in the price of the technique when special instrumentation is used.

The best conditions for the determination of cadmium by combining DLLME and UV-Vis spectrophotometry have been successfully obtained by OVAT approach and experimental design.

Once the method was defined, analytical validation showed that the proposed method has acceptable LOD and good precision, repeatability and reproducibility. Thus, the method can be applied to determination of cadmium in highly contaminated water.

Finally, the developed method was successfully applied to real samples spiked with cadmium. Accuracy was then calculated, which showed satisfactory results.

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7. PUBLISHED PAPER:

USE OF DISPERSIVE LIQUID-LIQUID MICROEXTRACTION AND UV-VIS SPECTROPHOTOMETRY FOR THE DETERMINATION OF CADMIUM IN WATER SAMPLES.

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Research Article

Use of Dispersive Liquid-Liquid Microextraction and UV-Vis Spectrophotometry for the Determination of Cadmium in Water Samples

J. Pérez-Outeiral, E. Millán, and R. Garcia-Arrona

Department of Applied Chemistry, Faculty of Chemistry, University of the Basque Country (UPV/EHU), P.O. Box 1072, 20800 San Sebastian, Spain

Correspondence should be addressed to R. Garcia-Arrona; rosa.garcia@ehu.es

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A simple and inexpensive method for cadmium determination in water using dispersive liquid-liquid microextraction and ultraviolet-visible spectrophotometry was developed. In order to obtain the best experimental conditions, experimental design was applied. Calibration was made in the range of 10–100 μg/L, obtaining good linearity ($R^2 = 0.9947$). The obtained limit of detection based on calibration curve was 8.5 μg/L. Intra- and interday repeatability were checked at two levels, obtaining relative standard deviation values from 9.0 to 13.3%. The enrichment factor had a value of 73. Metal interferences were also checked and tolerable limits were evaluated. Finally, the method was applied to cadmium determination in real spiked water samples. Therefore, the method showed potential applicability for cadmium determination in highly contaminated liquid samples.

1. Introduction

Cadmium is one of the most toxic heavy metals [1, 2]. Due to its low damaging concentration levels and to the complexity of the matrix of samples, a prior preconcentration and separation step is frequently necessary in its determination. Liquid-liquid microextraction (LLME) emerged from the classic liquid-liquid extraction (LLE) overcoming one of its major drawbacks by reducing the amount of organic solvents. From the LLME introduction, different approaches have been developed [3]; among them is dispersive liquid-liquid microextraction (DLLME) presented by Rezaee et al. in 2006 [4]. In this method, a cloudy solution is formed after the fast injection of a suitable mixture of extraction and disperser solvents into the aqueous phase. Thus, due to the large superficial area in contact between the phases, analytes in the aqueous sample are rapidly extracted into the fine droplets of extraction solvent. After extraction, phase separation is achieved by centrifugation. This technique offers several advantages such as high recoveries, high enhancement factors, and rapidity. Moreover, it may be considered environmentally friendly, due to the use of reduced amount of organic solvents. In addition, it is simple and inexpensive, since neither very specific reagents nor costly laboratory equipment is required [5, 6].

For cadmium determination DLLME has been coupled to different spectrometry detection techniques, such as atomic absorption (AAS), atomic fluorescence, inductively coupled plasma with optical emission (ICP-OES), and total reflection X-ray [7–11]. Due to the difficulty in adapting the microvolume of extraction solvent to the necessary volume in conventional UV-Vis spectrophotometers, the coupling to UV-Vis spectrophotometry has only been recently achieved. This problem has been sometimes solved by evaporation and/or dilution of the organic phase or by using special instrumentation [12, 13].

The goal of this work was to develop a simple and inexpensive method for determination of cadmium in aqueous samples by coupling DLLME and UV-Vis spectrophotometry with a microcapacity cuvette. In this way, loss of sensitivity linked to the dilution of the organic phase and increase of the price due to the special instrumentation may be avoided. In

order to select the best experimental conditions, a planned experimental design was run. The chosen procedure was validated and applied to the determination of cadmium in different water samples.

2. Materials and Methods

2.1. Reagents and Apparatus. All analytical grade reagents and solvents used were purchased from Panreac Química S.A. (Barcelona, Spain), except for carbon tetrachloride that was obtained from VWR International Eurolab S.L. (Barcelona, Spain). Working solutions of cadmium and dithizone were daily prepared. The pH of the extraction was adjusted just before the use with a weekly prepared NaOH/KCl solution. All solutions were preserved in the fridge while not being used. Doubly distilled water was used throughout the whole work.

A Spectrophotometer 8453 UV-Vis Diode Array System (Agilent Technologies, Spain) with the software "UV-Visible Chemstation Rev. A. 10.01" and an 80 μL cuvette (Hellma GmbH & Co. KG, Germany) were used.

The extractions were made in a vessel with a thermostatic jacket joined to a Lauda Ecoline Re 104 E100 thermobath (Lauda, Germany). Values of cadmium concentration for the enrichment factor evaluation were obtained by measurements in the 7700X ICP-MS (Agilent Technologies, Spain). Experimental design was performed and the results were evaluated using Statistica Software (StatSoft, Tulsa, USA).

2.2. Procedure. Microextraction was accomplished in a tube containing 7.5 mL of sample at pH 12.8 ± 0.2 and 1.5 mL of disperser solvent (methanol). The mixture sample/dispersant was brought to 40°C. Then, 150 μL of a 34 mg/L solution of dithizone (chelating agent) in chloroform (extraction solvent) was added. The mixture was stirred at 1000 rpm for 3 min to form a cloudy solution, in which chloroform was dispersed as fine droplets to extract the complex cadmium dithizone. This solution was centrifuged at 3500 rpm for 1 min, and the dispersed droplets were deposited at the bottom of the tube. 60 μL of the sedimented phase was transferred to the microcuvette for determination in the spectrophotometer. The extraction procedure is schematically shown in Figure 1.

2.3. Water Samples. Two commercial drinking mineral water samples (DW1, DW2), tap water (TW), snow water (SW), river water (RW1), and irrigation channel water (RW2) were analyzed. All samples were filtered through a 0.45 μm micropore filter, and different volumes (from 0.5 to 2.5 mL) were diluted up to 10 mL with the NaOH/KCl solution.

3. Results and Discussion

3.1. Study on the Absorption Spectra. Due to its high efficiency, dithizone was selected as chelating agent for extraction and determination of cadmium by UV-Vis spectrophotometry [14]. Absorbance of the complex ($Cd(HDz)_2$) was measured in the wavelength of the maximum absorption (516 nm for chloroform, 517 nm for carbon tetrachloride), which corresponded to the wavelength of the minimum absorption for dithizone.

3.2. Selection of Working Conditions. There are several parameters affecting the extraction process. Some of the experimental conditions were fixed according to working characteristics.

In stirring step, agitation speed and time were high enough to form the cloudy solution (1000 rpm, 3 min). In the sedimentation step, centrifugation rate and time were the minimum that allowed collecting the cloudy solution into a sedimented drop (3500 rpm, 1 min).

Other variables were univariately studied. pH was studied in the range of 4.0–13.0, obtaining better results at high levels of pH, where extraction efficiency is higher and the dithizone does not interfere in the analysis [15]. Effect of salt addition was investigated at two levels, without salt and at 10% (W/V) addition. The best results were obtained without salt addition.

The remaining considered experimental variables were studied using experimental design. Taking into account their high density, extraction capacity, and low solubility in water, chloroform and carbon tetrachloride were selected as extraction solvents [6]. Due to their different characteristics (boiling points, water solubility, and interactions with the different dispersants) efficiency of both solvents could not be compared in the same conditions. Hence, two different designs for finding the better experimental conditions for each solvent were done. Regarding the volume of extraction solvent, the minimum volume which gave manageable sedimented phase (100 μL for carbon tetrachloride and 150 μL for chloroform) was chosen.

A 2^4 full factorial design with temperature, volume and type of dispersant, and dithizone concentration was made for each extraction solvent [16]. Levels of the factors were chosen based on previous experiments. Concentration of dithizone was selected in the way that in both cases the net amount of chelating agent was the same. A summary of experimental design and its results are included in Table 1.

The levels of the nonsignificant variables were fixed at the values which gave better responses, 0.5 mL of dispersant and ethanol for carbon tetrachloride and 34 mg/L concentration of dithizone and methanol for chloroform. Temperature was fixed at the high level in both cases because a further increase of the temperature led to problems in the collection of the sedimented phase. For the same reason, when chloroform was used, the disperser solvent volume was fixed at high level. When carbon tetrachloride was used the concentration of dithizone was fixed at 154 mg/L.

Absorbance in the best conditions of both extraction solvents was compared. Carbon tetrachloride gave a slightly higher value of absorbance but not enough to compensate its higher toxicity and cost compared with those ones of chloroform. Hence, chloroform was selected for further experiments.

Taking into account the experimental design, 1.5 mL of methanol, 150 μL of a 34 mg/L solution of dithizone in chloroform, and 40°C of temperature were the chosen experimental

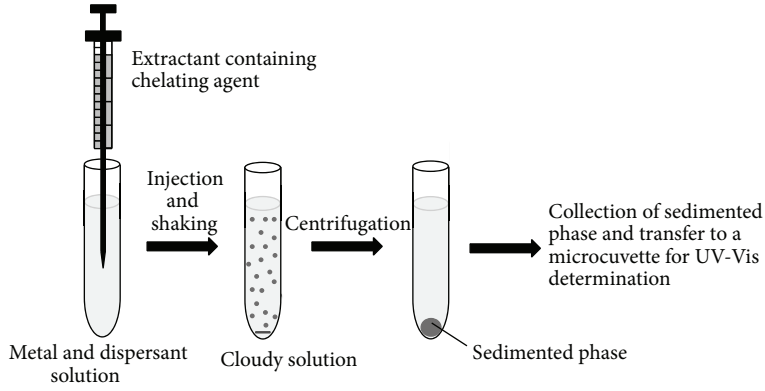

Figure 1: Extraction procedure scheme for determination of cadmium.

Table 1: Resume of the experimental designs' conditions and results for determination of cadmium with two extraction solvents using DLLME and UV-Vis spectrophotometry.

Factor	Low level	High level	Factor significance	Sign of effect
		Carbon tetrachloride		
Temperature (°C)	25	50	Significant	Positive
Volume of dispersant (mL)	0.5	1.5	Nonsignificant	Negative
Dithizone concentration (mg/L)	52	154	Significant	Positive
Type of dispersant	Ethanol	Methanol	Nonsignificant	Negative
		Chloroform		
Temperature (°C)	25	40	Significant	Positive
Volume of dispersant (mL)	0.5	1.5	Significant	Positive
Dithizone concentration (mg/L)	34	102	Nonsignificant	Negative
Type of dispersant	Ethanol	Methanol	Nonsignificant	Positive

Screening designs.
2^4 full factorial designs.
Total number of runs: 32 (with duplicates, in four blocks).

conditions. Those levels, 7.5 mL of aqueous phase, pH 12.8 ± 0.2, no salt addition, 3 min of extraction at 1000 rpm, and 1 min of centrifugation at 3500 rpm were the final working conditions.

3.3. Analytical Characteristics. The correlation coefficient (R^2 = 0.9947) showed a good linearity in the studied range (10–100 μg/L). Limit of detection (LOD) was calculated based on the residual standard deviation of the calibration curve [17]. The obtained value (8.5 μg/L) was little higher than some of those obtained when more sensitive techniques such as FAAS and ICP-OES are coupled to DLLME [8, 10]. Precision of the method was evaluated at two concentration levels (20 and 80 μg/L). For intraday repeatability ten measurements were carried out in the same day. For interday repeatability twelve experiments were performed in three days during two weeks. The relative standard deviation percentages (RSD) ranged from 9.0 to 13.0% for intraday repeatability and from 9.0 to 10.9% for interday repeatability.For these analyte concentrations, RSDs between 15 and 21% are acceptable [18]. Hence, the obtained results showed satisfactory precision.

Enrichment factor (EF) was calculated as the relation between the concentration of cadmium in the sedimented phase obtained after the extraction and the initial concentration in the sample. Both concentrations were evaluated by inductively coupled plasma-mass spectroscopy (ICP-MS). EF had a value of 73, which is among the EFs obtained in the previous mentioned works [7, 8, 10].

3.4. Interferences. Due to the fact that dithizone can form complexes with other metals, interferences from other present cations in samples may occur frequently. The effect of potentially interfering ions in the developed method was studied in an 80 μg/L cadmium solution. Tolerable limit was taken when the interfering ion/cadmium molar ratio did not cause a relative error in the signal higher than 10%. Molar ratio tolerable limits were the following: 250 for Al^{3+}, 100 for Ca^{2+}, 10 for Pb^{2+}, 1 for Mg^{2+}, Mn^{2+}, Co^{2+}, and Zn^{2+}, and 0.1 for Fe^{3+}, Ni^{2+}, and Cu^{2+}.

3.5. Application to Real Samples. No cadmium was found in the collected water samples. All of them were spiked at 40 μg/L, except for DW1 that was spiked at 20 and 80 μg/L. Each determination was made in triplicate and the results were evaluated on the basis of recovery (R, %) and repeatability (RSD, %). The results showed recoveries

ranging from 90 to 109% and RSDs between 3 and 9%. Thus, the proposed method can be successfully applied to the determination of cadmium in real water samples giving accurate and reproducible results.

4. Conclusions

A simple and inexpensive method for determination of cadmium has been developed by coupling DLLME and UV-Vis. DLLME confers some advantages since it is rapid, simple, and environmentally friendly. On its behalf, UV-Vis spectrophotometry, in spite of its lower sensitiveness compared with other techniques for determination of cadmium, grants the mentioned benefits. Coupling of these two techniques has been successfully achieved by the use of a microliter-capacity cuvette. This form of connection allows avoiding the inconveniences of previous DLLME-UV-Vis coupling ways.

The proposed method showed good precision, repeatability, and reproducibility, and it was successfully applied to real spiked samples. This method is especially suitable when the analytical laboratories have no funds for acquisition of high cost equipment. In conclusion, the proposed method could be applied to the determination of cadmium in highly contaminated water samples.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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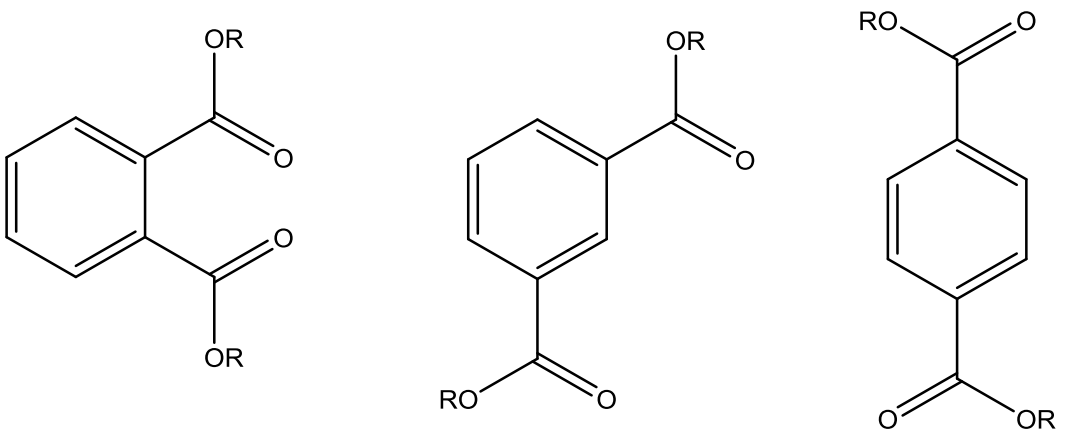
Development of a method for phthalates simultaneous determination in food simulants and liquid samples using ultrasound-assisted dispersive liquid-liquid microextraction followed by the solidification of the floating organic drop and GC-FID

Development of a method for phthalates simultaneous determination in food simulants and liquid samples using ultrasound-assisted dispersive liquid-liquid microextraction followed by the solidification of the floating organic drop and GC-FID

1. Introduction

Plastics are nowadays undoubtedly ubiquitous. Additives such as plasticizers, fillers, antioxidants, flame retardants, stabilisers, lubricants and colouring agents impart desired functionalities to polymers creating hundreds of different varieties of plastic materials of diverse properties. Those different properties make their huge spectrum of applications possible. However, due to their extensive use and to the high content of additives, plastics present some potential health and environmental risks. Nowadays, the group of additives of greatest health concern are probably phthalates (Halden, 2010).

Structurally, phthalate esters (PAEs) are the diesters of the benzenedicarboxilic acids. There are three isomeric forms of this acid, thus, there are three different types of phthalates: orthophthalates, isophthalates and terephthalates. The structure of the different types of phthalates is shown in Figure 4.1.



Orthophthalates Isophthalates Terephthalates

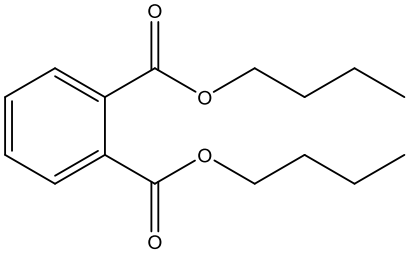
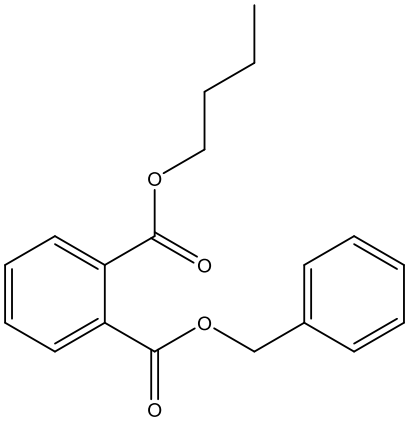
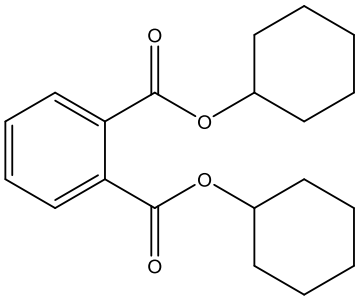
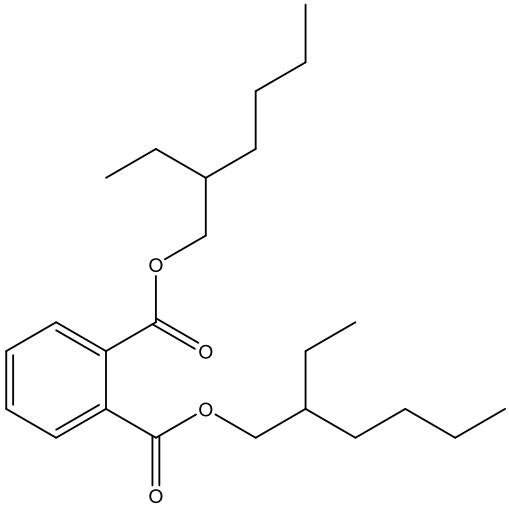
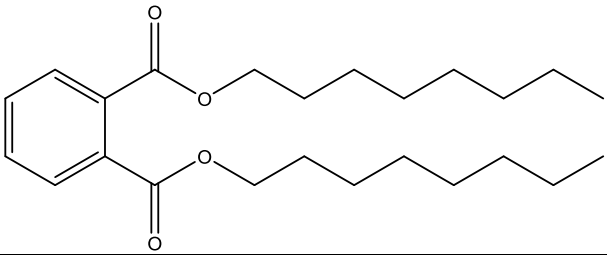
Figure 4.1. Structures of the different families of phthalates.

The group of ortophthalates is the most known and used, probably due to its role as plasticizers. In Europe, for example, orthophthalates comprise about 80% of the entire plasticizers market (ECPI, 2014). That is why in literature the term "phthalate" is frequently used to designate only this type of phthalate. In consequence, the term phthalate has been used to mention only ortophthalates also throughout this text.

The industrial synthesis of phthalates involves the esterification of phthalic anhydride with oxo alcohols in the presence of an acid catalyst such as sulphuric acid or p-toluene sulfonic acid. Then, PAEs differ from the nature and the length of the oxo alcohols from which they come from. The structural differences in the ester side chains from one phthalate to another is what will define its different properties and, in consequence, its different applications. Due to the high number of alcohols able to react with the phthalic anhydride, there are a lot of different PAEs. However, only about 60 of them have industrial applications. Amongst them, only the ones with alkyl chains from 1 to 13 carbons are produced in large scale, being bis(2-ethylhexyl) phthalate (DEHP) the most widely produced phthalate (and plasticizer) in the world (Llompart *et al.,* 2005; ECPI, 2014). Table 4.1 collects the name, CAS and structure of the PAEs determined in this chapter

High molecular weight phthalates are principally used as plasticizers in the plastic industry, where they are used to increase the flexibility and softness of the polymeric materials by weak secondary molecular interactions with the polymer chains. Approximately 95% of the European production of phthalates is used as plasticizer in PVC-made products, but they are also used in non-PVC materials, such as epoxy and polystyrene resins, chlorinated, natural and synthetic rubbers, polysulfide, nitrocellulose, ethylcellulose and polyurethane. Plastics and resins containing phthalates have several applications including toys, rainwear, shower curtains, food packaging, carpets, wall coverings, shoes, cable and medical tubing and automobile and furniture upholstery (Llompart *et al.,* 2005; Cao, 2010; ECPI, 2014).

Table 4.1. Name, CAS and structure of the phthalates determined in this chapter.

	Name	Abbreviation	CAS	Structure
1	Dibutyl phthalate	DBP	84-74-2	
2	Benzyl Butyl phthalate	BBP	85-68-7	
3	Dicyclohexyl phthalate	DCHP	84-61-7	
4	Bis(2-ethylhexyl) phthalate	DEHP	117-81-7	
5	Di-n-octyl phthalate	DNOP	117-84-0	

In addition to their main application as plasticizers, phthalates (mainly low molecular phthalates) have also some other applications: they are used as industrial solvents, as additives in the textile industry, as components of dielectric fluids, lubricants, fragrances, hairsprays, nail polish, deodorants, paints, glues, pesticide formulations, adhesives, inks, waxes, etc (Llompart *et al.,* 2005; Cao, 2010; ECPI, 2014).

Due to the wide range of applications, general population is exposed to phthalates. Ingestion, inhalation, intravenous injection and skin absorption are potential pathways of exposure. Amongst all of them, ingestion of contaminated food is probably the largest single source of phthalate exposure in the general population (Schettler, 2006). The massive use of phthalates in the packaging industry may be one of the main causes of food contamination. Other sources include the contamination of environment, especially water, and the use of PVC tools used to manipulate food (globes, tubing, etc.) (Cao, 2010).

Food packaging is nowadays necessary during the transportation and storage of food products until their end use in order to protect them from tampering and from contamination by physical, chemical and biological sources. Quality and safety is maintained in this way. Contamination with PAEs may occur in the food and beverages in contact with food packaging (Hongyuan *et al.*, 2010; Hongyuan *et al.*, 2011; Cacho *et al.*, 2012; Hongyuan *et al.*, 2012). One of the reasons is that when PAEs act as plasticizers, they are only physically bound to polymer chains, and temperature and other factors can cause their release. Moreover, in food packaging related products, phthalates are also used as adhesives, offset printing inks, lacquers and so on, which constitute an additional source of contamination.

Migration of PAEs to food has received considerable attention in recent years because of their effect in human health as they act as endocrine disruptors and possible carcinogens, amongst others (Batlle and Nerín, 2004; USEPA, 2005; Halden, 2010; Ventrice *et al.,* 2013). Indeed, due to their ubiquity and to their potential risk for human health and environment, several of them have been included in the priority list of pollutants of the United States Environmental Protection Agency (USEPA, 2013) and in the current candidate list of substances of very high concern for Authorisation of the European Chemical Agency (ECHA). In the latter, they are catalogued as "Toxic for reproduction" (ECHA, 2016).

In Europe, in order to protect human health, restrictions on the quantities of substances that could migrate to the food are imposed on food packaging materials (Commision regulation (EU) Nº 10/2011, 2011). These restrictions, known as specific migration limits (SML), are defined as "the maximum permitted amount of a given substance released from a material or article into food or food simulants" and they are expressed in legislation in mg substance per kg food. In this regulation, the SMLs of the esters studied in this chapter, DBP, DEHP and BBP are defined as 0.3, 1.5 and 30 mg substance per kg food respectively. For compounds not specifically indicated, a generic SML of 60 mg/kg is defined.

This limit is defined with the purpose of assuring that food contact materials do not pose a risk to health and it should be checked by manufacturers in the worst foreseeable conditions. As food is a really complex matrix, and, consequently, analysis of food samples may be difficult, several food simulants are used as models of different food types in order to check compliance with the limits in the regulation. Table 4.2. collects the list of food simulants assigned with this purpose. For selecting the most appropriate food simulant for a specific food, chemical composition and physical properties have to be considered. A detailed table with food description and the type of simulant that has to be used in each case is found in the original text of the regulation (Commision regulation (EU) Nº 10/2011, 2011). In general terms, food simulants A to C are assigned for foods with hydrophilic character. Between them, simulant B shall be used for food with pH below 4.5 and simulant C for alcoholic foods with an alcohol content below 20% and for foods with relevant amounts of organic ingredients. On the other hand, D simulants are assigned for foods with lipophilic character, being D1 more specifically for foods with alcohol content above 20% and for oil in water

emulsions and D2 for foods with free fats in the surface. Food simulant E is assigned for dry foods.

Table 4.2. List of food simulants in Commision regulation (EU) Nº 10/2011, 2011.

Food simulant	Abbreviation
Ethanol 10 % (v/v)	Food simulant A
Acetic acid 3% (w/v)	Food simulant B
Ethanol 20 % (v/v)	Food simulant C
Ethanol 50 % (v/v)	Food simulant D1
Vegetable oil*	Food simulant D2
Poly(2,6-diphenyl-p-phenylene oxide), particle size 60-80 mesh, pore size 200 nm	Food simulant E

*In the original reference, some specifications for the type of vegetable oil that should be used are given.

The determination of PAEs in food simulants is important because it allows checking the quantity of a specific PAE that could migrate into the food from the food packaging material. But the determination of PAES in products which are already in the market, such as bottled water, beverages and different food samples is also necessary. These are the products arriving at the consumers for sure and the assays performed with food simulants in some cases do not totally reflect the real conditions (Fasano *et al.*, 2012).

Analytical methods for determination of PAEs are mainly based on chromatographic techniques, such as gas chromatography (GC) (Farahani *et al*. 2007; Xu *et al.*, 2007; Farahani *et al.*, 2008) or high pressure liquid chromatography (HPLC) (Pérez-Feás *et al.*, 2008; Kamarei *et al.*, 2011). Among them, the first one is the most commonly used due to the semivolatile nature of phathalates and to their thermal stability (Cao, 2010; Farajzadeh *et al.*, 2015). Mass spectrometry-based detectors have been widely applied for the PAEs determination by these two techniques (Ma *et al.*, 2010; Lv *et al.*, 2013; Cacho *et al.*, 2015; Yang et *al.*, 2015), but less sensitive and more affordable techniques such as HPLC-UV (Liang *et al.*, 2008; Xue *et al.*, 2014) and GC-FID (Batlle and Nerin, 2004; Hongyuan *et al.*, 2012) have also been used.

Owing to the low concentration of PAEs in real samples and to the complexity of sample matrices, a preconcentration and separation step is often required prior to analysis. A wide range of combinations of chromatography with different pre-treatment methods have been used for phthalate determination in

food or water samples (Farajzadeh *et al.*, 2015). From 2010, conventional solid-phase extraction (SPE), molecularly-imprinted SPE (MISPE) and magnetic SPE (MSPE) represent more than the 60% of the total extraction techniques for the analysis of PAEs in food. Dispersive liquid-liquid microextraction (DLLME) represents a 10%, quick, easy, cheap, effective, rugged and safe method (QuEChERs) the 8% and solid-phase microextraction (SPME) the 7% (Yang *et al.*, 2015).

To our knowledge, only the bis-(2-ethylhexyl) phthalate has been determined by a technique based on the solidification of the organic drop and GC-FID (Yamini *et al.*, 2009). For the determination of the rest of PAEs by gas chromatography and dispersive techniques, however, highly toxic chlorinated solvents have been frequently used (Hongyuan *et al.*, 2011; Hongyuan *et al.*, 2012; Cinelli *et al.*, 2013). Hence, the use of typical solvents for techniques based in the solidification of the organic drop (SFOD) is interesting to avoid toxicity. In addition, combination of DLLME and SFOD in the UA-DLLME-SFOD technique shows several advantages; it is rapid, due to the high superficial area between phases, and it is environmentally friendly, due to the used solvents.

2. Objectives of the chapter

The principal objective of the present chapter was the development of a simple, low cost and reliable analytical method for the simultaneous determination of 5 PAEs (dibutyl phthalate, benzyl butyl phthalate, dicyclohexyl phthalate, bis(2-ethylhexyl) phthalate and di-n-octyl phthalate) in food simulants, liquid food and water samples using ultrasound-assisted dispersive liquid-liquid microextraction followed by GC-FID.

The main objective of the chapter was accomplished with the following specific objectives:

1. The development of a new procedure based on dispersive liquid-liquid microextraction techniques followed by the solidification of the floating organic drop. Due to the characteristics of the former techniques, the procedure is expected to be fast and environmentally friendly.

2. The extension of the use of microextraction techniques coupled to GC-FID. This equipment was selected because it is a commonly used inexpensive technique.

3. The study of the best conditions for the determination of PAEs. The main parameters that affect extraction and determination of PAEs have been considered in detail with the aid of both, `one variable at a time (OVAT)´ method and experimental design.

4. The validation of the optimized procedure by obtaining the typical analytical figures of merit. Studied properties were linearity, limits of detection and quantification, precision, accuracy and enrichment factors.

5. The application of the optimized procedure to food simulants and to different water and liquid food samples. In this way, it is demonstrated that the developed method can be applied for checking the compliance with the PAEs regulation at the required levels.

3. Experimental

3. 1. Reagents, standards and materials

Phthalates, namely, dibutyl phthalate (DBP, 99%), benzyl butyl phthalate (BBP, 98%), dicyclohexyl phthalate (DCHP, 99%), bis(2-ethylhexyl) phthalate (DEHP, 99.5%), and di-n-octyl phthalate (DNOP, 99.5%) were purchased from Sigma-Aldrich (Barcelona, Spain).

Solvents for SFOD techniques, specifically, 1-undecanol (99%), 2-dodecanol (99%), *n*-hexadecane (99%), Br-hexadecane (97%), 1-chlorooctadecane (96%) and 1,10-dichlorodecane (99%) were also purchased from Sigma-Aldrich (Barcelona, Spain). Exception was 1-dodecanol (98%), which was supplied by Panreac (Madrid, Spain).

Other common reagents such as methanol (99.8%), acetonitrile (99.7%), ethanol absolute (99.5%), acetone (99.5%) and sodium chloride (99.5%) were supplied by Panreac (Madrid, Spain) too. Doubly distilled water, taken from an Aquatron A4D system (J. Bibby Sterilin Ltd., Staffordshire, United Kingdom), was used throughout this work.

Individual stock solutions of PAEs and a mixed stock solution (1 g/L of each analyte) were prepared in methanol and stored in amber-colored vials in the refrigerator. Working solutions were prepared weekly by dilution of the stock with methanol, and they were also preserved in the refrigerator when not used. All solutions were warmed at room temperature before use.

All the glassware used in this research was previously soaked and washed with distilled water first and with acetone later. Then, it was dried at 240°C for at least 4 hours.

3. 2. Samples

Food simulants were prepared in the laboratory as described in regulation (Commission regulation (EU) No 10/2011, 2011): Simulant B (3% (w/v) acetic acid/water solution) and simulant C (20% (v/v) ethanol/water solution).

The commercial samples were purchased from one local shopping centre and they were the following: three different mineral waters, three different vinegars (two wine vinegars and a cider vinegar), four wines (two red wines and two white wines; one of each class being contained in a glass bottle and the other one in a Tetrapak box), three different soft drinks and a sample of sangria. Recovery tests in commercial samples were carried out using appropriate dilutions. Samples were spiked by adding 50 µL of a working solution containing all the analytes to the final solution.

3. 3. Instrumentation and chromatographic conditions

Chromatographic analyses were performed on a HP 6890N (Agilent Technologies, Wilmington, DW, USA) gas chromatographer equipped with a split/splitless injector used in splitless mode and a flame ionization detector (FID). Injector temperature was 300°C and splitless time lasted for 3 min. The column used was a HP-5 (30 m x 0.250 mm x 0.25 µm film thickness) capillary column (Agilent Technologies). The carrier gas was helium at a flow of 1.3 mL/min. The oven temperature was as follows: 160°C for 1 min, increased to 200°C at a rate of 10°C/min, and then a ramp of 2°C/min to 255°C. Detector temperature was set at 300°C. After the analysis, a post-run program of 5 min at 315°C was run, in order to clean the column.

Extractions were carried out in a Bandelin Sonorex Digitec DT100H ultrasound bath (ALLPAX, Papenburg, Germany) at a 35 kHz ultrasound frequency. Centrifugation was performed on a Selecta centrifuge (Barcelona, Spain). The cooling bath was a Julabo F26 (Augsburg, Germany) and the heating bath was a Lauda ecoline re104. Experimental design was performed and evaluated with Statistica software (StatSoft, Tulsa, USA).

3. 4. UA-DLLME-SFOD procedure

Firstly, 10 mL of sample solution containing 25 g/L NaCl was placed in a 40 mL glass vial. Secondly, 0.75 mL of acetronitrile and, when necessary, a mixed solution of PAEs standards were spiked, and the resulting solution was placed in a thermostatic bath for 5 minutes at 35°C. After that, 15 μL of *n*-hexadecane (extraction solvent) were added to the solution, it was gently shaken by hand and placed into an ultrasonic bath for sonication at 35 kHz and 35 ± 1°C during 5 min. As a result, extraction solvent was entirely dispersed into the aqueous phase. Then, dispersion was disrupted by centrifugation at 4500 rpm for 10 min and the vial was placed into a thermostatic bath at 3°C for 10 min. The solidified organic drop was subsequently collected with a spatula and transferred to a 0.9 mL clear glass vial where it was let melt at room temperature. Once melted, 3 μL of the organic phase were collected and directly injected in the GC-FID for analysis. The entire UA-DLLME-SFOD experimental procedure is schematically presented in Figure 4.2.

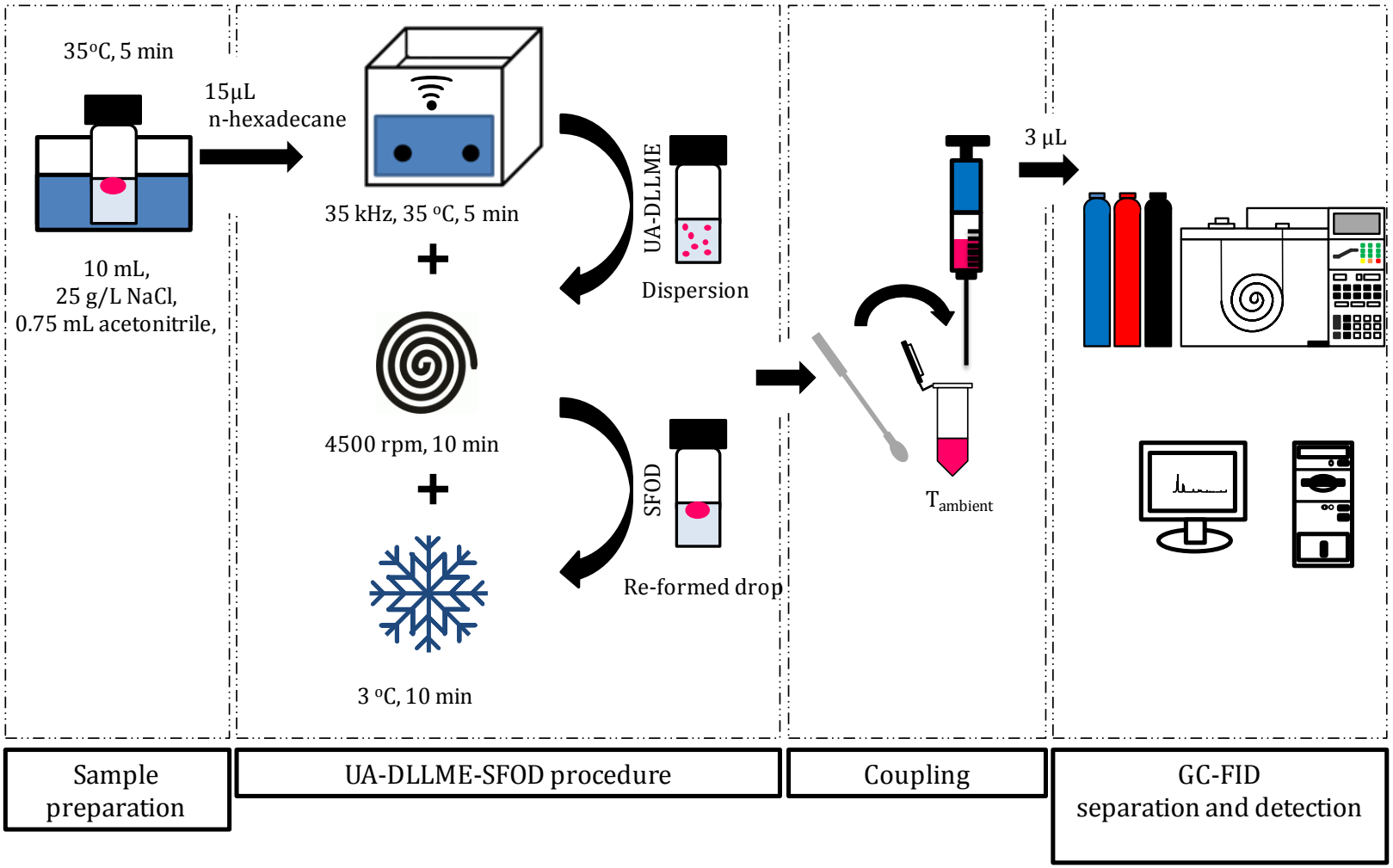


Figure 4.2. Schematic representation of the UA-DLLME-SFOD procedure divided in four steps: Sample preparation, UA-DLLME-SFOD procedure, coupling and GC-FID separation and detection.

4. Results and discussion

The main purpose of the present chapter was the development of a new reliable microextraction method for the determination of phthalates in different liquid samples by coupling ultrasound-assisted dispersive liquid-liquid microextraction and GC-FID. Selected phthalates were DBP, BBP, DCHP, DEHP and DNOP.

Firstly, the best conditions for the chromatographic determination of PAEs were investigated. Secondly, some initial considerations regarding a common problem found in the determination of phthalates were considered. Afterwards, the most suitable conditions for the UA-DLLME-SFOD process were studied. Once the method was optimized, it was validated and applied to different real samples.

4. 1. Optimization of the chromatographic conditions and peak identification

First of all, the separation of all the analytes by chromatography was studied. In gas chromatography, separation depends on the intensity of interactions between the solutes and the stationary phase, and on the solutes vapour pressure, which is dependent on the temperature (Poole, 2003; Harris, 2010; Kenkel, 2013). In consequence, to obtain the best separation, different stationary phases, and different elution conditions (flow of the carrier gas and temperature programming) can be used.

In this case, a HP-5 capillary column from Agilent was used, as it had been successfully used for phthalate determination (Chen *et al.*, 2005). This is a non-polar column made of 5%-phenyl-methylpolysiloxane. Injector and detector temperature were fixed at 300°C to maintain analytes in vapour phase. Splitless injection was selected because as much sensitivity as possible is needed and little fractionation of the sample is desired. Time (3 min) and volume (3 µL) for splitless were selected according to previous experiences.

Optimization of the chromatographic program started with a unique ramp from 120°C to 285°C at 10°C/min and a flow rate of 1 mL/min. With these conditions, not an analyte eluted during the first minutes of the chromatogram,

and although a good separation was achieved for DBP and BBP, several analytes co-eluted at the end of the chromatogram.

Thus, the initial temperature of the program was raised to 160°C, a ramp of 10°C/min was maintained at the beginning of the chromatogram (until 200°C were reached) and temperature gradient was then reduced to 2°C/min until a temperature of 255°C (there are not analytes that elute later). Consequently, the time to complete the chromatogram was reduced. Flow was also slightly increased to 1.3 mL/min to achieve better resolution. A post-run program of 5 min at 315°C was used in order to clean the column after each run. After fixing all the chromatographic conditions, good separation of all the analytes was achieved (See Figure 4.3.).

The identification of peaks was carried out by injecting single reference standards and by comparing the retention times of each obtained chromatogram with the peaks observed in the chromatogram with all the analytes. Figure 4.3. represents the chromatogram obtained following the UA-DLLME-SFOD optimized procedure for a standard sample (50 µg/L of each analyte) and for a blank (with no content of analytes).

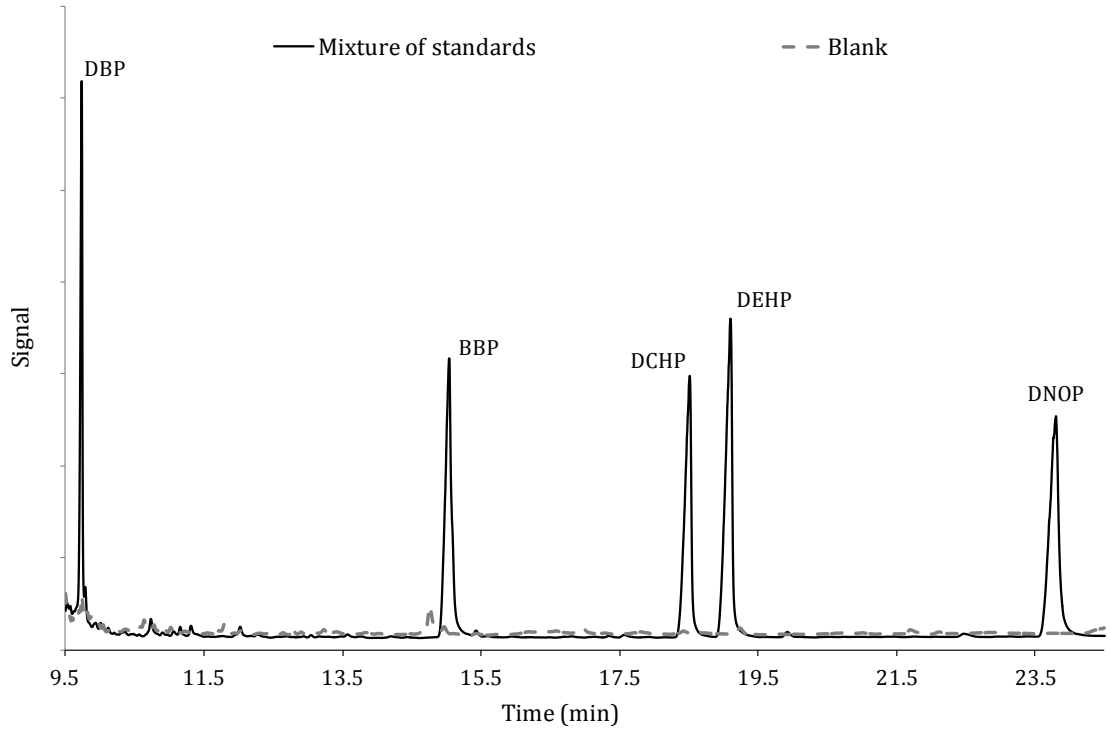


Figure 4.3. GC-FID chromatogram of a standard mixture of PAEs (50 µg/L of each analyte) and a blank using the optimized procedure. DBP: dibutyl phthalate; BBP: benzyl butyl phthalate; DCHP: dicyclohexyl phthalate; DEHP: Bis(2-ethylhexyl)phthalate; DNOP: di-n-octyl phthalate.

4. 2. Initial considerations

Due to the spreading of the phthalates, they can be found almost everywhere, including laboratory equipment and reagents. For this reason, one of the main problems in phthalate analysis is external contamination (Garcia-Jares *et al.*, 2009). In this case, as it is recommended, plastic materials were avoided due to the probable presence of PAEs in them. In addition, glassware was deeply soaked and washed with distilled water and acetone, and then it was dried up at 240°C for at least 4 hours. While not used, material was also stored in closed containers and wrapped in aluminium foil. Moreover, blanks were periodically run. In our case, thanks to the taken precautions, no evidences of contamination were found. A typical blank obtained is shown in Figure 4.3.

4. 3. Selection of the most suitable experimental conditions

In order to obtain a suitable method for phthalate determination by a liquid phase microextraction technique coupled to GC-FID, the best experimental conditions for all the parameters significant to the microextraction process have to be found.

Firstly, the extraction solvent was selected. Then, a comparison of UA-DLLME with other liquid microextraction techniques was also accomplished. Once the microextraction technique was chosen, some other parameters, in which a wide range of levels needs to be studied, were univariately considered. These parameters included the type of dispersant, the volume of extraction solvent and the pH. During the univariate approach, analysis of variance (ANOVA) was used to compare signals under different conditions when necessary. Extraction time, extraction temperature, dispersant volume and salt addition were investigated by experimental design and, amongst them, influential factors were outlined.

Concentration of all the analytes during all the optimization process was around 500 ng/mL. Initial conditions of the different variables were the following: sample volume: 10 mL; extraction solvent: 20 µL; dispersant: acetonitrile; volume of dispersant: 1 mL; extraction temperature: 35°C; extraction time: 10 min; centrifugation time: 10 min; centrifugation speed: 4500 rpm; drop freezing: 3°C; cooling time: 10 min; and no salt addition. All the runs for the study of one single variable were performed at fixed levels of the other variables.

4. 3. 1. Selection of extractant

The selection of an appropriate extraction solvent is of major importance for the optimization process of solidification of the organic drop-based microextraction methods. In summary, as thoroughly explained in the Introduction Chapter (Section 1.3.), the extraction solvent should satisfy the following requirements: it should be able to extract the analytes of interest, it should be not miscible in water and it should have low volatility, lower density than water and a melting point near to room temperature. Moreover, it is desirable that the peaks of the solvent in the chromatogram are sufficiently separated from those of the analytes. Taking into account these characteristic, extraction solvents normally chosen in SFOD techniques are 1-undecanol, 1-dodecanol, 2-dodecanol, *n*-hexadecane, 1-bromohexadecane, 1, 10-dichlorodecane and 1-chlorooctadecane.

Alcohols, that is, 1-undecanol, 1-dodecanol and 2-dodecanol were rapidly discarded because extra peaks in the chromatogram suggested reaction between the extractant and some of the phthalates. 1, 10-dichlorodecane was also discarded because a baseline full of small peaks was obtained with this extractant and analytes signals are confused over it.

1-bromohexadecane, 1-chlorooctadecane and *n*-hexadecane seem adequate for PAEs determination. However, 1-bromohexadecane and 1-chlorooctadecane partially or totally co-eluted with some of the selected analytes, making their determination impossible. In consequence, *n*-hexadecane was selected for further experiments as it was the only extractant that gave a good signal separated from all the analytes.

4. 3. 2. Comparison with other liquid-phase microextraction techniques.

Selection of the best extraction technique is not always straightforward. Although, in general, the use of dispersants and ultrasonic energy help to the transfer process, due to the increment of superficial contact area between aqueous and organic phases, sometimes LPME can work better due to different reasons; for example, if the concentration of analytes is high in the headspace, the contact of the extractant with the liquid surface can be an advantage. Hence, a general study of the suitability of the techniques is advisable before the final choice is made.

In order to select the best SFOD technique, the following procedures were compared: Liquid-phase microextraction (LPME), dispersive liquid-liquid microextraction (DLLME), ultrasound-assisted emulsification microextraction (USAEME) and ultrasound-assisted dispersive liquid-liquid microextraction (UA-DLLME). All the conditions defined in Section 4.3. and *n*-hexadecane, as extractant, were used. In LPME and DLLME agitation is necessary; in these cases rates of 400 and 1125 rpm were respectively used. In LPME no centrifugation was needed.

Results obtained by applying the four different SFOD techniques are shown in Figure 4.4. In general, dispersion of the extractant improved extraction efficiency of the analytes. Ultrasound energy (USAEME) greatly enhanced the extraction of DEHP and DNOP, but it was not so efficient in improving the extraction of the rest of the analytes. The use of dispersant (DLLME) increased especially the efficiency in DBP, BBP and DCHP, but it was not as efficient as ultrasound energy for the rest of the analytes. Combination of ultrasonication and disperser solvent (UA-DLLME) provided the best results. The reason could be that the addition of a dispersant was very convenient to improve the microextraction of some analytes, whereas the application of ultrasound energy was more appropriate to improve the microextraction of the remainder. Consequently, the combination of the two effects was, in general, the best choice.

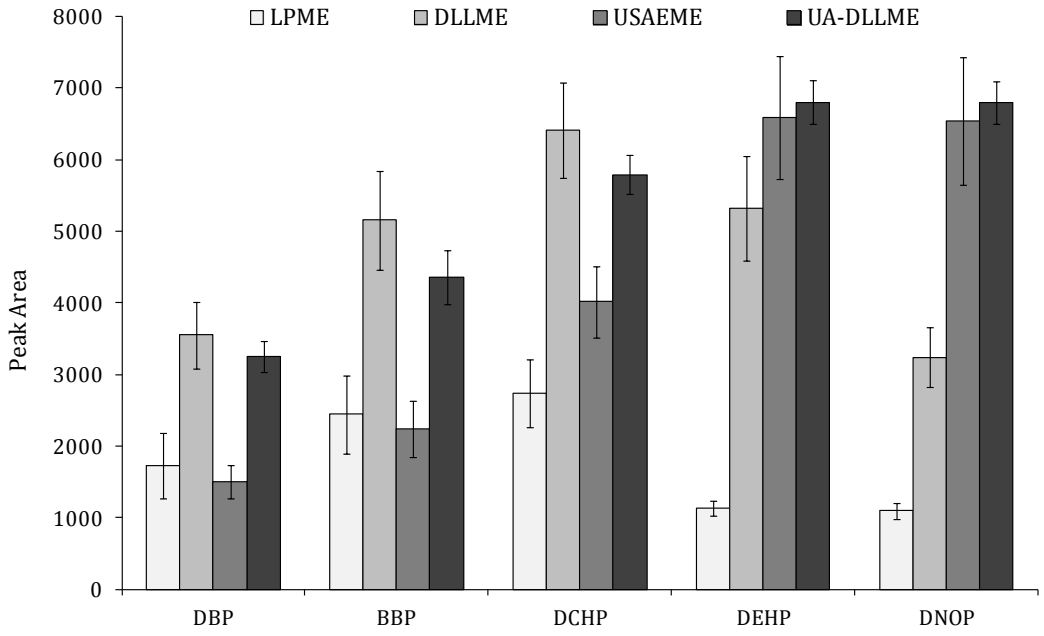


Figure 4.4. Comparison of the signals obtained by the four techniques: LPME, DLLME, USAEME and UA-DLLME, all of them followed by SFOD for DBP, BBP, DCHP, DEHP and DNOP phthalates.

4. 3. 3. Selection of centrifugation and cooling conditions

In dispersive techniques, after extraction, a step for recollecting the organic phase into a unique organic drop is necessary. This is normally achieved by centrifugation, and frequently a fast process is desirable in order to save time. In this case, centrifugation conditions were fixed according to previous experience in our laboratory, and the maximum recommended centrifugation rate for the centrifuge, 4500 rpm, was selected. At these conditions, 10 minutes were necessary in order to obtain a well-formed solidified drop in all the extractions.

After the re-formation of the drop, it must be frozen so that it is possible to gather it with a spatula. Cooling temperature was fixed at 3^{o}C, as it was the lowest secure temperature achievable with the thermostatic bath. At that temperature, 10 minutes were enough to obtain a well formed solidified drop. In some cases, slight agitation helps to the maintenance of the drop while it is freezing; in this case, however, agitation produced the break-up of the drop, so it was discarded.

4. 3. 4. Selection of disperser solvent

In the dispersive techniques, the disperser solvent plays an active role by assisting the formation of a dispersion of droplets of the extraction solvent into the aqueous phase. In this way, the surface area between the phases is increased and, accordingly, extraction efficiency is also increased. As explained in the Introduction Chapter (Section 1.3.), the main requisites for disperser solvents are: elevated solubility in the extraction solvent and high miscibility with water. Due to low toxicity and low cost, acetone, methanol, ethanol and acetonitrile are generally used as dispersants (Sarafraz-Yazdi and Amiri, 2010).

The changes in the response of different analytes caused by the change of the type of disperser solvent are shown in Figure 4.5. As it can be seen at first glance, the best results were obtained with both, ethanol and acetonitrile, for all the analytes. Moreover, very similar results were procured by these two disperser solvents. Thus, ANOVA was used to compare their signals, using the p-values (see Introduction Chapter, Section 3.1.). In this way, it was showed that there were not significative differences between the signals obtained with both dispersants for none of the analytes (p-values for DBP, BBP, DCHP, DEHP and DNOP were 0.209, 0.795, 0.648, 0.991 and 0.883 respectively). Nevertheless, values obtained with

acetonitrile were slightly higher than values obtained with ethanol, and therefore, acetonitrile was selected for further experiments.

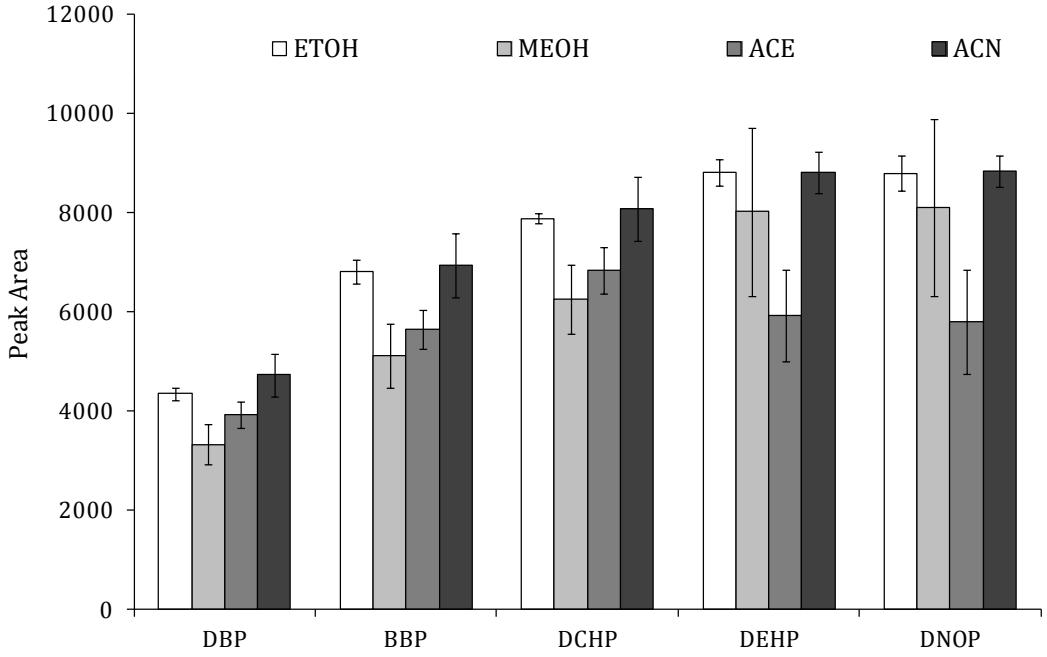


Figure 4.5. Comparison of obtained signals using different dispersants for DBP, BBP, DCHP, DEHP and DNOP phthalates. ETOH: ethanol; MeOH: methanol; AC: acetone; ACN: acetonitrile.

4. 3. 5. Selection of volume of extractant

In general, an increase in the extraction solvent volume leads to a higher volume of the organic phase after centrifugation, resulting in a decrease in the concentration of the analytes of interest due to dilution (Wang *et al.*, 2010; Ghambarian *et al.*, 2013). On the other hand, too low volume of extractant can lead to lack of reproducibility when collecting the drop. Hence, the lowest volume of extractant that allows a reproducible collection is normally used.

In this work extractions with volumes of 10, 13, 15 and 20 µL of *n*-hexadecane were carried out. Results (Figure 4.6.) show that the lower the extraction volume the higher the signal. However, when 10 and 13 µL were used, reproducibility was not good enough (for some analytes RSDs near 30% were obtained) and collection of enough volume after the extraction was not always possible. Reproducibility was better when 15 µL were used (RSDs below 15%) and

the collection of a sufficient amount of extractant was always possible. That is the reason why 15 µL of *n*-hexadecane was selected for further experiments.

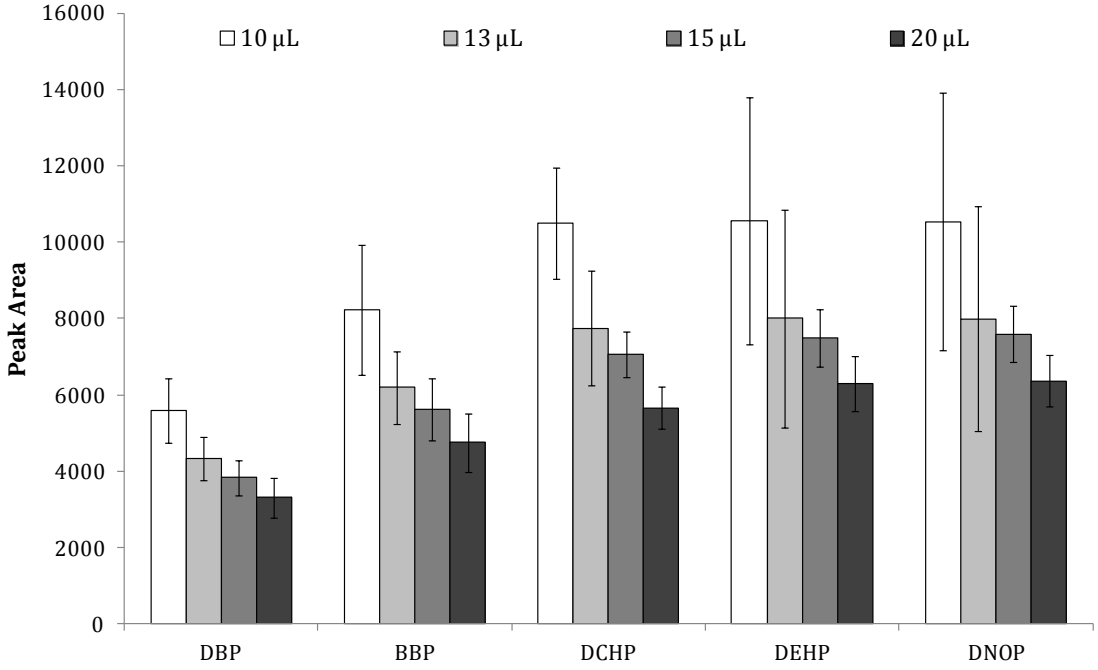


Figure 4.6. Comparison of obtained signals using different volumes of extraction solvent for DBP, BBP, DCHP, DEHP and DNOP phthalates.

4. 3. 6. Effect of pH

The pH of the sample is another important factor for extraction because it has influence over some characteristics of the microextraction process. On one hand, it affects the hydrolysis status and the solubility of the analytes in the aqueous phase. On the other hand, it can also have impact on the solubility of the extractant in the aqueous phase. Therefore, the pH effect must be carefully evaluated. In this case, the evaluation was accomplished in the range of pH 2-12 and the results are shown in Figure 4.7.

In this case, the results show similar extraction efficiency within the pH range, which was confirmed by an ANOVA analysis (p-values= 0.626, 0.474, 0.179, 0.934 and 0.975 for DBP, BBP, DCHP, DEHP and DNOP, respectively). In consequence, samples were not subjected to pH adjustment in further experiments.

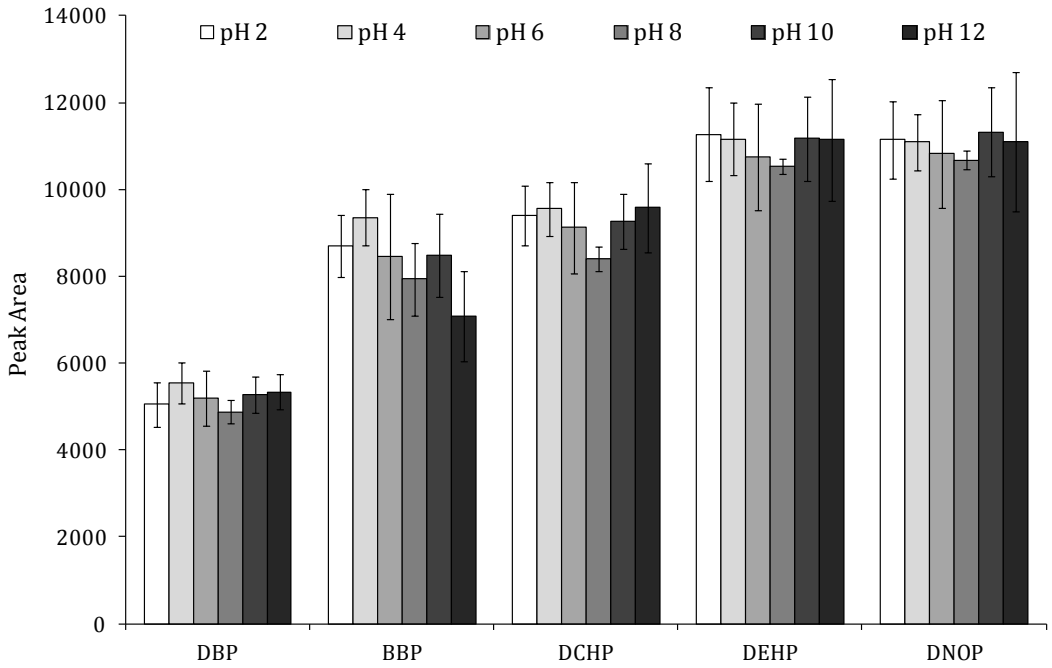


Figure 4.7. Comparison of obtained signals at different pHs for DBP, BBP, DCHP, DEHP and DNOP phthalates.

4. 3. 7. Experimental design

After the fixing of the variables whose study require more deep consideration at more than two levels, screening experimental design was used in order to check the importance of the remaining variables possibly affecting the determination process. These variables were extraction time, extraction temperature, volume of dispersant and salt addition. Levels of these variables were chosen based on previous experience in the laboratory as follows:

- Short extraction times were selected (5 and 10 minutes) because dispersive microextraction techniques are almost time-independent (Ghambarian *et al.*, 2013).

- Temperature values of 35 and 45°C were studied as temperatures below room temperature are difficultly reproduced in the ultrasonic bath, and too high temperatures make the extraction system unstable (Ghambarian *et al.*, 2013).

- For salt addition, concentration levels were 0 and 100 g/L because it was experimentally observed that higher ionic strengths made difficult the recollection of the drop after the microextraction.

- Volumes of dispersant of 0.5 and 1.5 mL were studied as these are the most commonly used volumes in DLLME applications (Asensio-Ramos *et al.*, 2011).

Table 4.3 summarizes the variables and levels taken into account in the screening experimental design.

Table 4.3. Variables and levels considered in screening experimental design.

Variables	Levels	
	Low	High
Time (min)	5	10
Temperature (°C)	35	45
Volume of dispersant (mL)	0.5	1.5
Salt addition (g/L)	0	100

The selected experimental design was a 2^{4-1} fractional factorial design. Resolution for this design is IV, that is, no main effects are confounded either with each other or with two-factor interactions. The latter, however, are confounded with each other. Even though three or more factor interactions could be confounded with the main effects, these are not normally considered important (Massart *et al.*, 1997). With this fractional design, furthermore, the number of experiments is reduced by half. In consequence, the design consisted of 8 experiments, and each one was performed twice (16 experiments). All the experiments were performed in a random order. Variables individually fixed (Sections 4.4.1 to 4.4.6) were used at their optimum value. Table 4.4. shows details of the experimental runs and their results.

Table 4.4. 2^{4-1} fractional factorial design experimental matrix and obtained peak areas for the analytes under study.

Experiment No.	Replicate	t (min)	T (°C)	Salt (g/L)	V disp (mL)	DBP	BBP	DCHP	DEHP	DNOP
1	1	5	35	0	0.5	4843	7059	8433	10775	10776
2	1	10	35	0	1.5	3914	5466	7543	8974	8711
3	1	5	55	0	1.5	4232	5947	8439	10788	10439
4	1	10	55	0	0.5	5263	7066	8517	10976	11126
5	1	5	35	100	1,5	5988	9329	10693	12231	12183
6	1	10	35	100	0.5	6874	11045	11021	12451	12407
7	1	5	55	100	0.5	6320	10139	10511	12229	12287
8	1	10	55	100	1.5	5144	7653	8958	10426	10395
9	2	5	35	0	0.5	4504	6466	7715	9854	9913
10	2	10	35	0	1.5	4368	6288	8332	10298	10292
11	2	5	55	0	1.5	3641	5084	7622	10646	10266
12	2	10	55	0	0.5	5416	7658	9231	12547	12757
13	2	5	35	100	1.5	5256	8145	9660	11158	11167
14	2	10	35	100	0.5	6245	10178	10243	11610	11610
15	2	5	55	100	0.5	6386	10325	10368	11858	11813
16	2	10	55	100	1.5	4852	7661	9037	10538	10574

t: extraction time; T: extraction temperature; Salt: salt concentration; V disp: volume of dispersant.

The obtained data were evaluated by analysis of variance (ANOVA) (Table 4.5.) and main effects were visualized in Pareto Chart (Figure 4.8.). Both ways of expressing the results were previously explained in the Introduction Chapter (Section 3.1.). In general, interactions were not considered significant, and therefore, they were not taken into account in the models.

In summary, in ANOVA, a statistical interpretation of the results is done by comparing the effect of each factor with the error using a F test. The p-value given in the ANOVA table represents the probability of a variability to have occurred by pure chance, and in many areas, p-values lower than 0.05 are considered indicative of a significant effect. In this case, the numbers corresponding to significant variables are marked in bold.

As it can be seen in Table 4.5., the salt addition and the volume of dispersant were the most important factors. The salt addition was a significant parameter for all the analytes, and the volume of dispersant was significant for all the analytes except for DEHP, which had a p-value of 0.053, and therefore, it was considered not significant.

Table 4.5. ANOVA results obtained for each analyte in the experimental design.

DBP					
	SS	df	MS	F	p
V disp	**4470264**	**1**	**4470264**	**28.57141**	**0.000236**
Salt	**7402753**	**1**	**7402753**	**47.31422**	**0.000027**
T	34207	1	34207	0.21863	0.649213
t	51234	1	51234	0.32746	0.578665
Error	1721053	11	156459		
Total SS	13679511	15			
BBP					
	SS	df	MS	F	p
V disp	**12891511**	**1**	**12891511**	**32.96325**	**0.000130**
Salt	**34335205**	**1**	**34335205**	**87.79419**	**0.000001**
T	373168	1	373168	0.95418	0.349651
t	16881	1	16881	0.04316	0.839215
Error	4301962	11	391087		
Total SS	51918726	15			
DCHP					
	SS	df	MS	F	p
V disp	**2069498**	**1**	**2069498**	**5.39912**	**0.040321**
Salt	**13434241**	**1**	**13434241**	**35.04862**	**0.000100**
T	57133	1	57133	0.14905	0.706804
t	19537	1	19537	0.05097	0.825523
Error	4216333	11	383303		
Total SS	19796742	15			
DEHP					
	SS	df	MS	F	p
V disp	3278363	1	3278363	4.704532	0.052870
Salt	**3652590**	**1**	**3652590**	**5.241557**	**0.042819**
T	440730	1	440730	0.632459	0.443277
t	185008	1	185008	0.265490	0.616569
Error	7665372	11	696852		
Total SS	15222063	15			
DNOP					
	SS	df	MS	F	p
V disp	**4690365**	**1**	**4690365**	**6.282603**	**0.029167**
Salt	**4156196**	**1**	**4156196**	**5.567100**	**0.037849**
T	421818	1	421818	0.565012	0.468026
t	58964	1	58964	0.078980	0.783900
Error	8212203	11	746564		
Total SS	17539545	15			

Parameters: SS: sum of squares; df: degrees of freedom; MS: mean square effect; F: fischer coefficient; p: probability.
Variables: V disp: volume of dispersant; Salt: salt concentration; T: extraction temperature; t: extraction time.

In Pareto Charts, the magnitude of each standardized estimated effect is represented by a horizontal column, and the vertical line indicates the criteria to consider an effect statistically significant (at a given level of confidence) (Figure 4.8.). Furthermore, the sign in Pareto Charts reflect if the response is increased (positive sign) or decreased (negative sign) when passing from the lowest to the highest level of a factor (Hill and Lewicki, 2007).

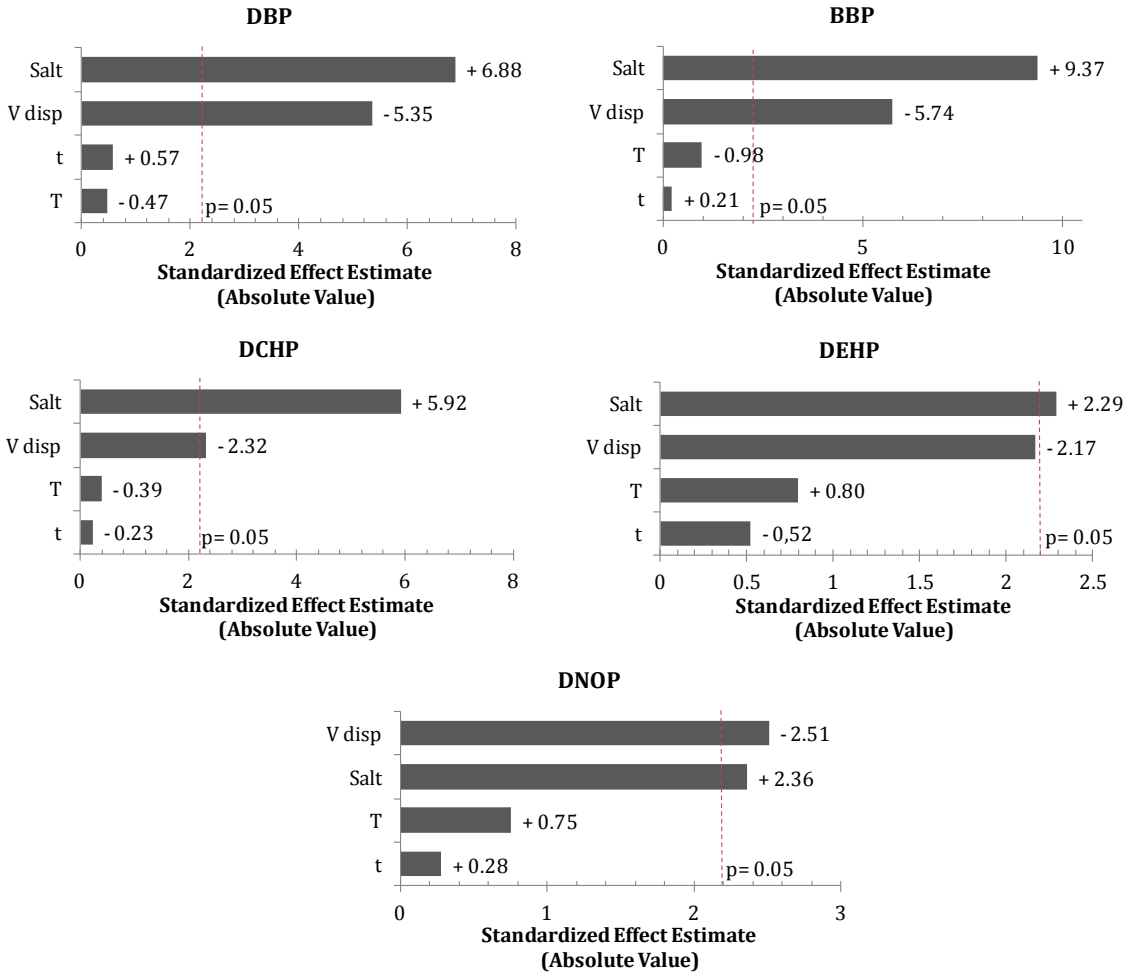


Figure 4.8. Parecto charts obtained in the 2^{4-1} fractional factorial design for DBP, BBP, DCHP, DEHP and DNOP. Red broken line represents p=0.05.

According to the ANOVA and Pareto results, salt addition was a significant parameter with a positive effect. This means that salt addition enhances extraction efficiency due to the salting out effect. Therefore, its concentration has to be optimized.

The volume of the dispersant was also a significant factor for DBP, BBP, DCHP and DNOP with a negative effect in all of them, suggesting that the addition of a low volume of dispersant helps to create the dispersion, but the addition of high volume of dispersant causes the dilution of the sample. For DEHP, the volume of dispersant was close to be significant and its effect was also negative. In consequence, this parameter was also proposed for optimization studies.

Time and extraction temperature were significant effects in none of the analytes. This result was expected as dispersive techniques are considered almost time-independent due to the large superficial area between phases. In addition, as previously commented in the Introduction Chapter (Section 1.3.), high temperatures usually facilitate the mass transfer process of the analytes from the sample into the organic phase, but in dispersive techniques this process is already enhanced. Although these variables are not significant, for each of the analytes slightly better results are obtained with one of the levels studied, and the sign in Pareto Chart reflects if the level giving slightly better results is the high level (positive sign) or the low level (negative sign). In this case, the time effect was positive for DBP and BBP and negative for DCHP, DEHP and DNOP, and the temperature effect was negative for DBP, BBP and DCHP and positive for DEHP and DNOP. As in both cases 3 analytes out of 5 had a negative effect, the low level of both variables was selected as optimum. That is, time was fixed at 5 minutes and temperature at 35°C.

Table 4.6 summarises the significance of each variable and the sign of the effect for each analyte.

Table 4.6. Summary of the results obtained in the 2^{4-1} fractional factorial design.

Compound	Time		Temperature		Volume of dispersant		Salt addittion	
	S	E	S	E	S	E	S	E
DBP	No	+	No	-	Yes	-	Yes	+
BBP	No	+	No	-	Yes	-	Yes	+
DCHP	No	-	No	-	Yes	-	Yes	+
DEHP	No	-	No	+	Yes	-	Yes	+
DNOP	No	-	No	+	Yes	-	Yes	+

S: significant; E: effect.

In summary, the salt addition and the volume of dispersant were proposed for optimization studies by univariate approaches after screening.

The disperser solvent volume was studied around the low level, specifically, between 0.25 and 1.00 mL, due to the results in the screening experimental design. All the previously fixed variables, including the ones fixed after experimental design, were used at their optimum level and salt concentration was set at 100 g/L. As it can be seen in Figure 4.9., the best results were obtained using 0.75 mL of acetonitrile. With this volume, the dispersion was well-formed and the dilution of the sample was not excessive.

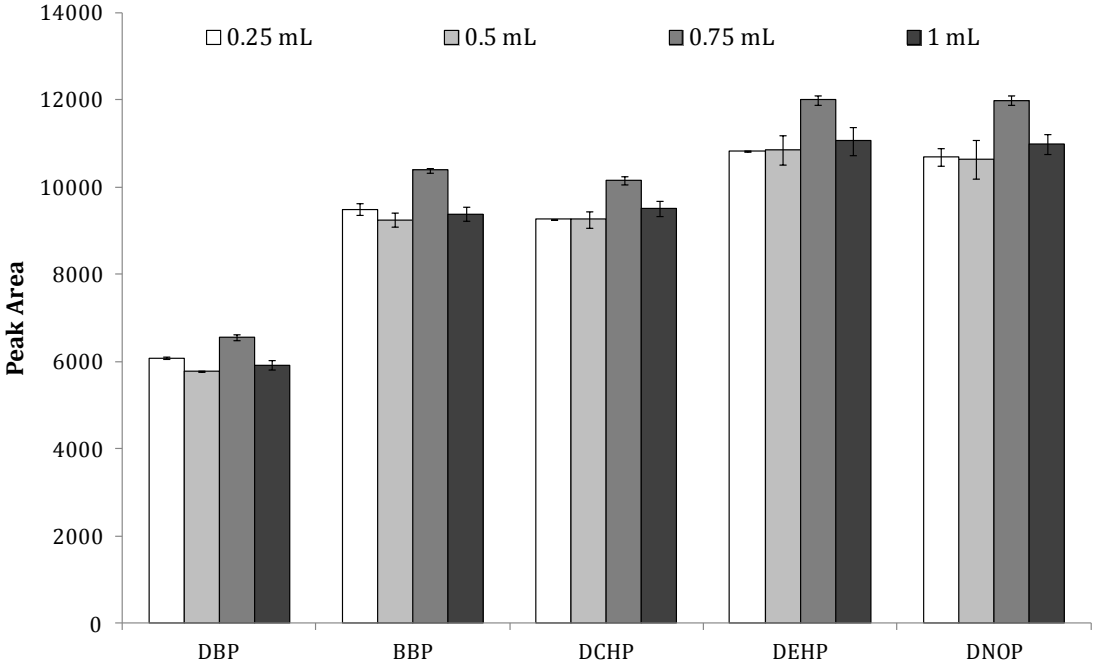


Figure 4.9. Comparison of the obtained signals with different volumes of dispersant for DBP, BBP, DCHP, DEHP and DNOP phthalates.

Salt addition decreases the solubility of analytes in aqueous phase and enhances their partitioning into the organic phase. Nevertheless, the presence of high concentrations of salt changes physical properties of the Nernst diffusion film and this can reduce the diffusion rate of the compounds into the organic phase (Wang *et al*., 2010; Ghambarian *et al.*, 2013).

In experimental design it was shown that presence of salt improves the extraction efficiency, and in consequence, the effect of salt concentration was studied for a wide range of concentrations, between 5 and 125 g/L. All the previously fixed variables were used at their optimum levels. Results (Figure 4.10.) showed that extraction efficiency increased from 5 to 25 g/L probably due to the salting out effect, but then remained practically constant in all the studied range. ANOVA was used to confirm that they were not significant differences between the signals obtained using salt concentrations from 25 to 125 g/L for each analyte (p-values= 0.882, 0.978, 0.946, 0.964 and 0.766 for DBP, BBP, DCHP, DEHP and DNOP, respectively). In addition, the higher the salt concentration, the worse the organic droplet was formed. Hence, 25 g/L of salt were chosen.

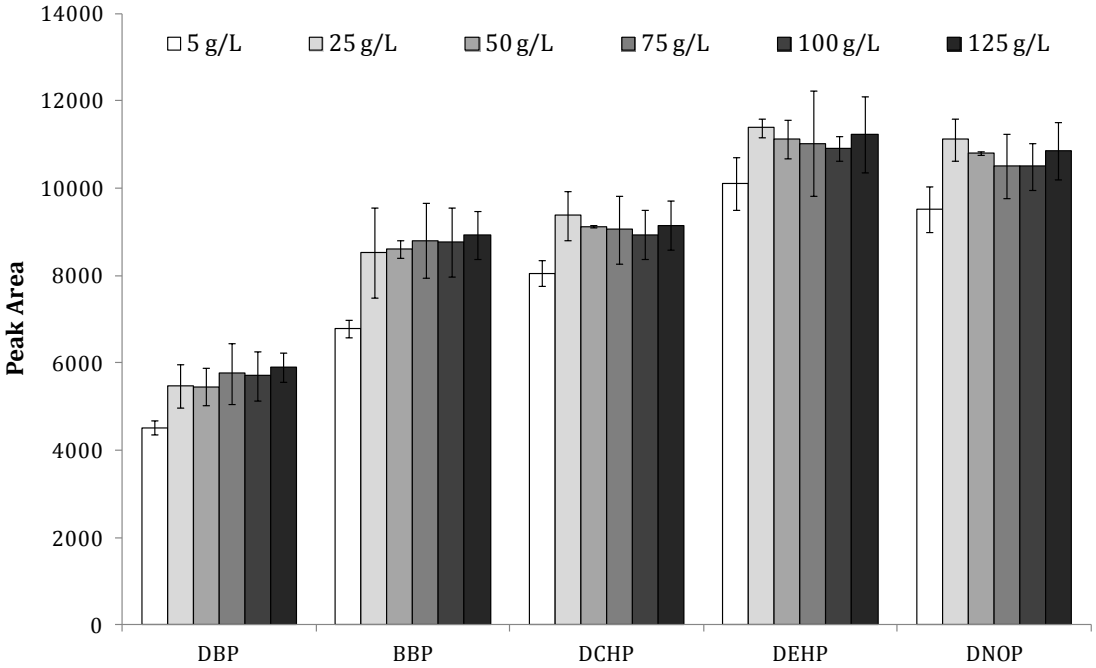


Figure 4.10. Comparison of obtained signals with samples with different concentrations of salt for DBP, BBP, DCHP, DEHP and DNOP phthalates.

4. 3. 8. Summary of the most suitable experimental conditions

Table 4.7. summarises all the best extraction conditions to obtain the best response for the determination of PAEs by UA-DLLME-SFOD-GC-FID according to all the previously carried out analysis.

Table 4.7. The most suitable extraction conditions for PAEs determination by UA-DLLME-SFOD-GC-FID.

Variable	Condition
Extraction solvent	
• Type	*n*-hexadecane
• Volume	15 µL
Disperser solvent	
• Type	Acetonitrile
• Volume	0.75 mL
Centrifugation	
• Time	10
• Rate	4500
Cooling	
• Time	10 min
• Temperature	3°C
• Agitation rate	-
Extraction	
• Time	5 min
• Temperature	35°C
Sample	
• Volume	10 mL
• Salt addition	25 g/L

4. 4. Method validation

The method validation is an important requirement in the development of a new analytical method. Its final objective is to ensure that future measurements of analyte concentrations in a sample can be close enough to the true value. The validation process is based on the acquirement of some statistical parameters of the procedure, including the following (Rambla-Alegre *et al.*, 2012): range, linearity, limit of detection, limit of quantification, precision and accuracy. All this parameters have been extensively explained in the Introduction Chapter (Section 3.2.).

The UA-DLLME-SFOD-GC-FID method developed in this chapter was evaluated according to those statistical parameters. But, before obtaining the analytical figures of merit of the method, internal standard addition method (IS) was considered.

Internal standard addition method is desirable when the quantity of sample or the instrument response varies meaningfully from run to run. In literature, examples of determination of phthalates combining microextraction techniques with GC-FID analysis in splitless mode with and without IS have been found (Batlle and Nerín, 2004; Xu *et al.,* 2007). In brief, the IS method consists of adding a known amount of a compound (different to the analyte of interest) to all the standards and samples. In this way, comparing the signal of each analyte with the signal of the internal standard in each chromatogram, variations in the response due to uncontrolled factors that affect similarly the signals of all the components are compensated.

Anthracene, a polycyclic aromatic hydrocarbon (PAH), has previously been used as internal standard for phthalate determination by microextraction and gas chromatography (Cinelli *et al.,* 2013). In this case, different PAHs, such as anthracene, phenanthrene, fluoranthene, pyrene, benz[a]anthracene and chrysene, were added to standard solutions containing all the PAEs and the mixtures were measured by GC-FID analysis. It was observed that most of the PAHs overlapped with other peaks in the chromatogram, with the exception of fluoranthene. Therefore, fluoranthene was proposed as internal standard.

The entire optimized UA-DLLME-SFOD-GC-FID procedure was applied to 3 different standards containing concentrations of 50, 200 and 400 μg/L of each

analyte and 200 g/L of the internal standard. Each measurement was performed 3 times. The reproducibility for each standard (RSD, %) and the regression coefficients (R^2) of the calibration curves, either considering or not the internal standard, were checked. Results are summarized in Table 4.8. As it is observed, reproducibility and regression coefficients when internal standard was used were worse than the ones obtained in the direct determination of the analytes. In consequence, the internal standard addition method was discarded.

Table 4.8. Reproducibility values for different standards and regression coefficients of the calibration curves obtained by direct determination and by the internal standard method.

	50 µg/L		200 µg/L		400 µg/L		R^2	
	D. D. RSD (%)	I. S. RSD (%)	D. D. RSD (%)	I. S. RSD (%)	D. D. RSD (%)	I. S. RSD (%)	D. D.	I. S.
DBP	4.2	12.0	1.2	7.9	7.0	8.0	0.988	0.985
BBP	3.1	8.0	3.8	6.1	5.2	10.6	0.992	0.979
DCHP	5.8	2.0	8.5	6.5	4.7	11.2	0.993	0.976
DEHP	5.3	2.4	2.8	7.3	5.8	8.3	0.989	0.984
DNOP	4.8	2.9	2.8	7.2	6.5	7.8	0.988	0.986

D. D.: direct determination; I. S.: internal standard method; RSD: relative standard deviation.

After the discarding of the IS method, analytical figures of merit of the determination of phthalates by UA-DLLME-SFOD-GC-FID were obtained, and they are summarized in Table 4.9. Linearity was studied using seven different levels of concentration at similar ranges for all the analytes (5-500 µg/L), except for BBP and DEHP, whose LOQ was above 5 µg/L and linear range was studied from 10 to 500 µg/L. Determination with each standard was performed three times.

Table 4.9. Analytical figures of merit of the proposed UA-DLLME-SFOD-GC-FID method for PAEs determination.

Compound	Linear range (µg/L)	R^2	LOD (µg/L)	LOQ (µg/L)
DBP	5.29-529	0.995	0.78	2.35
BBP	10.5-524	0.993	2.57	7.71
DCHP	5.03-503	0.994	0.92	2.77
DEHP	10.5-523	0.995	2.82	8.47
DNOP	5.46-546	0.994	0.64	1.93

R^2: correlation coefficient; LOD: Limit of detection; LOQ: limit of quantification.

All analytes showed good linearity in the studied range with correlation coefficients (R^2) ranging from 0.993 to 0.995. LODs were calculated based on 10 determinations of samples in which the analyte concentration was close to the

expected LOD (Introduction Chapter, Section 3.2.) and they ranged from 0.64 to 2.82 μg/L. For the calculation of LODs, the used samples had a concentration higher than the LOD but lower than 10 times the concentration of the LOD. LOQs were calculated as 3 x LOD and they ranged from 1.93 to 8.47 μg/L.

AOAC defines precision as "the closeness of agreement between independent test results obtained under stipulated conditions" and explains that "the measure of precision is usually expressed in terms of imprecision and computed as a standard deviation of the rest results" (AOAC international, 2012). The specific conditions in which replicates are performed, determine the type of precision obtained. Evaluation of precision requires the performance of a minimum number of replicates which varies between 6 and 15 depending on the protocol (Magnusson and Örnemark, 2014). In this case, 6 replicates of spiked samples at two concentration levels on the same day were performed to define intra-day precision. On the other hand, 9 replicates at the same two concentrations on three different days were accomplished for assessing inter-day precision. Results are expressed in Table 4.10. Obtained RSDs were in the range 2.7-9.3% for intra-day precision and within 3.2-8.1% for inter-day precision.

Table 4.10. Precision results at different levels obtained applying the proposed UA-DLLME-SFOD-GC-FID method for PAEs determination.

Compound	Intraday precision (RSD, %)		Interday precision (RSD, %)	
	Low[a]	High[b]	Low[a]	High[b]
DBP	3.1	6.7	5.4	6.1
BBP	7.7	9.3	6.0	7.5
DCHP	3.4	8.4	5.5	6.8
DEHP	2.8	7.4	4.1	8.1
DNOP	2.7	7.4	3.2	7.3

[a]Low level: around 50 μg/L.
[b]High level: around 400 μg/L.
RSD: relative standard deviation.

Table 4.11. presents the acceptable values for precision studies of an analytical method according to the AOAC recommendations (AOAC international, 2012). Considering this, obtained precision results were considered satisfactory, as the highest RSD in this study was 9.3%.

Table 4.11. Expected precision values for a tested method according to AOAC.

Analyte fraction	Unit	RSD (%)
10^{-5}	10 ppm	7.3
10^{-6}	***1 ppm***	***11***
10^{-7}	***100 ppb***	***15***
10^{-8}	***10 ppb***	***21***
10^{-9}	1 ppb	30

In bold concentration levels used in this method.
RSD: relative standard deviation.

The accuracy of the developed method was checked by means of extraction recoveries (ER). According to AOAC, recovery is the "fraction or percentage of the analyte that is recovered when the test sample is analyzed using the entire method" (AOAC international, 2012). In this case, as the samples intended to be analyzed were water and different food liquid samples (wine, vinegar and soft drinks), simulants B and C were selected for recovery studies in agreement with the current legislation (Commission regulation (EU) Nº 10/2011, 2011). Furthermore, recoveries in distilled water were also checked. In all cases, recovery was studied by adding a concentration of 200 µg/L of each PAE. Results are shown in Table 4.12.. They ranged 92-111%, except for DBP and BBP in simulant C whose values were 63 and 55% respectively.

Table 4.12. Recovery (mean of triplicates) of PAEs in distilled water and food simulants.

Compound	Distilled water		Simulant B		Simulant C	
	ER (%)	RSD (%)	ER (%)	RSD (%)	ER (%)	RSD (%)
DBP	97	2	92	6	63	5
BBP	94	2	93	3	55	7
DCHP	100	2	109	5	95	6
DEHP	98	4	103	5	109	4
DNOP	97	3	106	5	111	4

ER: extraction recovery; RSD: relative standard deviation.

AOAC recommendations of acceptable values for recovery of a given analytical method are shown in Table 4.13.. As it is can be seen, recoveries between 80 and 110 % are adequate at the studied concentration levels, and most of the obtained results are within the range.

Table 4.13. Recovery values for a tested method according to AOAC.

Analyte fraction	Unit	Recovery (%)
10^{-5}	10 ppm	80-110
10^{-6}	***1 ppm***	***80-110***
10^{-7}	***100 ppb***	***80-110***
10^{-8}	10 ppb	60-115

In bold concentration levels used in this method

Enrichment factor (EF) is defined as the ratio between analyte concentration in the organic phase after extraction and the initial concentration of the analyte in the water sample. In this case, the entire procedure was applied to samples with concentrations of 200 μg/L of all the analytes. For the calculation of the concentration in the organic phase after extraction, an external calibration curve was constructed by direct injection of the prepared standard solutions. Obtained values ranged from 854 to 1893 and are shown in Table 4.14.. These values were considered satisfactory as they mean that a significant preconcentration is achieved with the developed microextraction process.

Table 4.14. Values of enrichment factor for each PAE.

Compound	EF
DBP	854
BBP	1608
DCHP	1416
DEHP	1662
DNOP	1893

EF: enrichment factor

After validation, the performance of the proposed method was compared with different methods that have also coupled different microextraction techniques to GC-FID and DLLME based-techniques with different detectors. Table 4.15. summarizes the general characteristics of the developed method and some of the ones found in literature.

The obtained LODs are comparable to the ones obtained in literature with GC-FID detector and dynamic liquid-phase microextraction (Xu *et al.*, 2007), ultrasound-assisted dispersive liquid-liquid microextraction (Hongyuan *et al.*, 2010; Hongyuan *et al.*, 2011) or ultrasound-assisted surfactant-enhanced emulsification microextraction (Hongyuan *et al.*, 2012). On the other hand, they are higher than the ones obtained by single-drop microextraction (Batlle and Nerín, 2004) or with solid-based disperser LPME (Farajzadeh *et al.*, 2014). Compared to the latter, the method developed in this work, however, presents higher extraction recoveries in most samples and analytes.

By comparison with different detection procedures, the LODs of the present study are similar to the ones obtained in a previous work that uses DLLME-HPLC with variable wavelength detector (Liang *et al*., 2008). The LODs are, however, higher than the ones obtained by the sophisticated and expensive DLLME-GC-MS technique (Farahani *et al.*, 2007).

All the reported methods presented, in general, appropriate and similar linearity, precision and accuracy. Enrichment factors of the method developed in this work were higher than the ones obtained in the studied references.

As commented in the introduction to this chapter, the maximum quantity of each phthalate that could migrate into the food from food packaging is regulated in the current legislation by means of specific migration limits (SML). Values of these limits for DBP, DEHP and BBP are 0.3, 1.5 and 30 mg of substance per kg food respectively. For the other analytes, as they are not specifically indicated in the regulation, the generic value, 60 mg/kg, is considered. Taking into account that the density of aqueous samples is approximately 1 kg/L, it can be seen that the detection limits of the developed method are far below these requirements. It can be assumed, therefore, that the developed method has LODs low enough to check compliance with the regulation.

Table 4.15. Several important analytical parameters in this work compared with the found in bibliography.

	Matrix	Nº PAEs	R^2	LOD (μg/L)	RSD (%)	Recovery (%)	EF	Reference
D-LPME-GC-FID	Water	3	1.000	0.43-4.3	5.3-6.4	84-102	28-95	Xu *et al.*, 2007
UA-DLLME-GC-FID	Water	4	0.999	1.0-1.1	1.4-3.7	85-105	490-530	Hongyuan *et al.*, 2010
UA-DLLME-GC-FID	Milk	6	0.999-1.000	0.64-0.79 (ng/g)	2.8-4.0	93-106	220-270	Hongyuan *et al.*, 2011
UASEME-GC-FID	Beverages	5	0.999-1.000	0.41-0.79	2.4-5.8	89-100	230-288	Hongyuan *et al.*, 2012
SDME-GC-FID	Food simulants	7	-	0.02-0.4	3.5-17	75-118	-	Batlle and Nerín, 2004
SB-DLLME-GC-FID	Water, Beverages, Liquid food	5	0.996-0.999	0.10-0.25	2.5-5.2	52-100	286-556	Farajzadeh *et al.*, 2014
DLLME-HPLC-VWD	Water	3	0.999-1.000	0.64-1.8	4.3-5.9	84-113	44-196	Liang *et al.*, 2008
DLLME-GC-MS	Water	6	0.990-0.996	0.002-0.008	4.6-6.8	81-117	681-889	Farahani *et al.*, 2007
UA-DLLME-GC-FID	Water, Food simulants, Liquid food	5	0.993-0.995	0.64-2.82	2.7-9.3	55-111	854-1893	**This work** (Pérez-Outeiral et al., 2016)

NOTE: The explanation of the methods abbreviations is included in the abbreviation list.
R^2: correlation coefficients; LOD: Limit of detection; RSD: Relative standard deviation.

4. 5. Application to real samples

The proposed method was applied for the determination of PAEs in three different bottled drinking mineral water, three vinegars, four wines, three soft drinks and one bottle of sangria (alcoholic fruit beverage).

No analyte content was found in real samples, except for one wine, in which DBP was present at a concentration higher than the LOD but lower than the LOQ.

In order to facilitate phthalate migration from a plastic bottle to its contents, a bottle, in which water had been previously analyzed, was subjected to more extreme conditions than the ambient ones. Specifically, one bottle containing 0.5 L of water was kept in an oven at 40°C for two days. After that, water was again analyzed, and no phthalate content was found. Then, the same bottle was heated up in a domestic microwave for 6 minutes, and even though the bottle was totally deformed, no phthalate content was neither found in the contained water. This procedure showed that the studied bottle would not release phthalate content above the allowed SMLs even in the worst foreseeable conditions, when it is totally deformed and therefore it would not be sold in the market.

Hence, as no analyte was found in the studied samples, a real sample of each type was spiked with a mixture of phthalate standards to check precision and accuracy of the developed method for the phthalate determination in real samples. Recovery values and their precision were evaluated as in the previous section (Section 4.4.) and they are shown in Table 4.16.

Table 4.16. Recovery (mean of triplicates) of PAEs in liquid food samples.

Compound	Water		Vinegar		Soft drink		Wine	
	ER (%)	RSD (%)	ER (%)	RSD (%)	ER (%)	RSD (%)	ER (%)	RSD (%)
DBP	96	8	103	6	88	3	95	9
BBP	95	5	100	8	86	1	87	12
DCHP	109	4	110	6	92	1	98	10
DEHP	106	5	104	6	89	13	78	6
DNOP	105	5	103	7	72	7	75	6

ER: extraction recovery; RSD: relative standard deviation.

In water and vinegar samples, all the recovery values ranged from 95 to 110%, and in consequence, they were considered satisfactory. In soft drink and wine samples, most of the analytes also presented adequate recoveries. Exception was DNOP, which presented values of 72 and 75 %, reflecting a small matrix effect. DEHP recovery was also slightly low in the case of the wine sample, but it was close to the accepted limit.

As examples, Figure 4.11 a) and b) show typical chromatograms obtained by applying the entire procedure to a) a raw vinegar sample and the same sample after spiking and b) a raw soft drink and the same sample after spiking. As it can be seen, chromatograms without spiking were very similar to the blank shown in Figure 4.3.

In conclusion, the study in real samples demonstrates that the developed procedure can be satisfactorily applied to real samples giving accurate and precise results, although, it is difficult to find real samples containing measurable amount of phthalates. The most important problem of the method application to real samples was the difficulty in the drop formation after extraction, which was not the case with standard samples. This difficulty has been solved by a slight dilution of samples when necessary. Although this causes a decrease in the concentration of analytes in the samples, the quantification was possible due to the low LODs of the method.

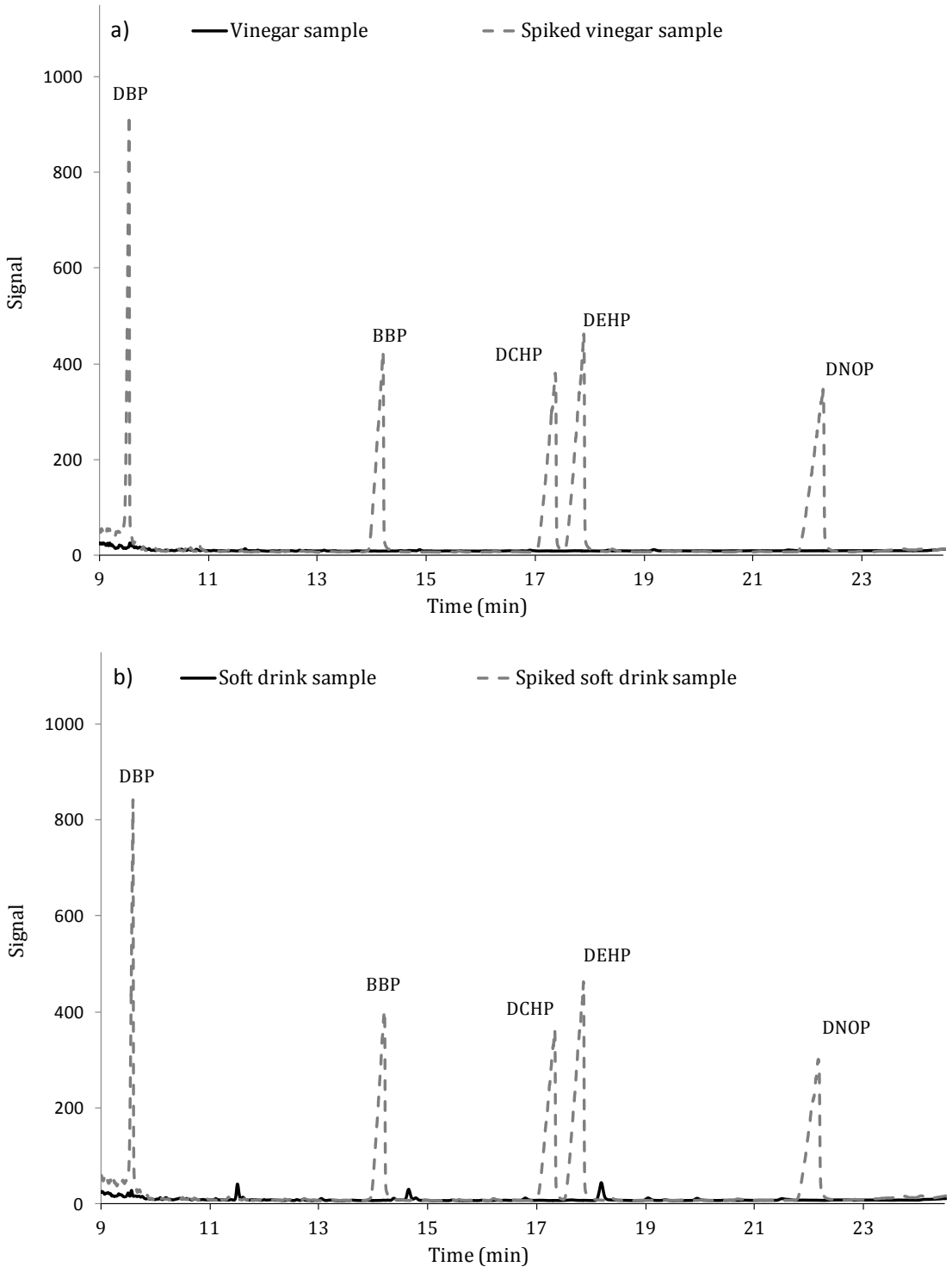


Figure 4.11. GC-FID chromatogram of a) a vinegar sample before and after spiking with analytes and b) a soft drink sample before and after spiking with analytes. DBP: dibutyl phthalate; BBP: benzyl butyl phthalate; DCHP: dicyclohexyl phthalate; DEHP: Bis(2-ethylhexyl)phthalate; DNOP: di-n-octyl phthalate.

5. Conclusions

In this chapter, a UA-DLLME-SFOD-GC-FID method has been developed for simultaneous determination of PAEs in food simulants and liquid samples. *n*-hexadecane has replaced the highly toxic organic solvents used previously with DLLME techniques, spreading out the applications of a widely used and affordable technique such as GC-FID.

OVAT approach and experimental design were satisfactorily combined for the definition of the best experimental conditions for the determination of PAEs by the UA-DLLME-SFOD-GC-FID procedure.

During method validation, the developed procedure was shown to be simple, precise, accurate and reproducible. Finally, it was also shown that the method can be applied to phthalate determination in different liquid food real samples and in food simulants.

Among all the samples, only dibutyl phthalate at very low concentration has been found in a wine sample. For all the regulated analytes, the LODs of the method have been low enough for a reliable determination at lower levels than the SMLs established by the European Union Regulation.

Thus, it can be concluded that the developed method can be applied to check the compliance with the European Union Regulation, and that no analyte content higher than the regulated one was present in the analyzed samples.

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7. PUBLISHED PAPER

DETERMINATION OF PHTHALATES IN FOOD SIMULANTS AND LIQUID SAMPLES USING ULTRASOUND-ASSISTED DISPERSIVE LIQUID-LIQUID MICROEXTRACTION FOLLOWED BY SOLIDIFICATION OF FLOATING ORGANIC DROP.

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Determination of phthalates in food simulants and liquid samples using ultrasound-assisted dispersive liquid–liquid microextraction followed by solidification of floating organic drop

Jessica Pérez-Outeiral, Esmeralda Millán, Rosa Garcia-Arrona*

Department of Applied Chemistry, Faculty of Chemistry, University of the Basque Country (UPV/EHU), P.O. Box 1072, 20080 San Sebastián, Spain

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ABSTRACT

A simple, inexpensive, reliable and environmentally friendly method based on ultrasound-assisted dispersive liquid–liquid microextraction followed by solidification of floating organic drop and gas chromatography–flame ionization detector was developed for the simultaneous determination of five phthalates in food simulants and different food and water samples. Parameters affecting the extraction process were studied and optimized by univariate analysis and experimental design. Under optimum conditions, method showed good linearity in the selected range (R^2 from 0.993 to 0.995). Limits of detection (LOD) ranged from 0.64 to 2.82 μg L^{-1} and enrichment factors from 854 to 1893. Precision of the method, expressed as relative standard deviation, was checked at two levels obtaining good results (2.7–9.3%). Accuracy of the method was checked in food simulants also obtaining good results. The method allowed determination of phthalates in food simulants at lower concentrations than the migration limits established by the European Union. The developed method was also applied to real water, wine, vinegar and soft drink samples obtaining acceptable results.

1. Introduction

Phthalate esters (PAEs) are used in a wide range of industrial and domestic applications. Particularly, they are widely used as plasticizers in polymeric materials to increase their flexibility through weak secondary molecular interactions with polymer chains. Since PAEs are only physically bound to the polymer chains, they can be easily released from products and migrate into the food, beverages or water in direct contact (Hongyuan, Baomi, Jingjing, & Kyung, 2010; Hongyuan, Xiaoling, & Baomi, 2011; Hongyuan, Xiaoling, & Kuo, 2012). In addition, in the food packaging industry, they are not used only as plasticizers, but also as adhesives, offset printing inks and lacquers. Migration of PAEs has received considerable attention in recent years because of their effect in human health, being considered endocrine disruptors and possible carcinogens among others (Batlle & Nerín, 2004; United States Environmental Protection Agency, 2005). Indeed, due to their ubiquity and their potential risk for human health and environment, several of them have been included in the priority list of pollutants of the United States Environmental Protection Agency (2013). In Europe, restrictions on the quantities of substances able to migrate into the food are imposed on materials used for food packaging (Commision regulation (EU) N° 10/2011, 2011). These restrictions are known as specific migration limits (SML) and they are defined as "the maximum permitted amount of a given substance released from a material or article into food or food simulants" and expressed in mg substance per kg food. Compliance with these limits has to be checked in food simulants as models for different food categories. In addition, determination of the PAEs in real water, beverages and food samples arriving at consumers is also important.

Owing to the low concentration of PAEs and to the complexity of sample matrices, a preconcentration and separation step is often required prior to analysis. Recent trends in sample preparation include miniaturization of classical extraction techniques, getting generally simpler, faster and greener techniques. In this way, liquid phase microextraction (LPME) emerged as a solvent-minimized version of the classic liquid–liquid extraction in which only several microliters of extractant are used. From LPME introduction, different approaches classifiable into three main categories have been developed: single drop microextraction (SDME), dispersive liquid–liquid microextraction (DLLME) and

* Corresponding author.
E-mail address: rosa.garcia@ehu.eus (R. Garcia-Arrona).

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hollow-fiber liquid-phase microextraction (HF-LPME) (Asensio-Ramos, Ravelo-Pérez, González-Curbelo, & Hernández-Borges, 2011).

In DLLME (Rezaee et al., 2006), dispersion of the extractant is achieved by the addition of a third solvent (dispersant), miscible with both phases. Due to the formed cloudy solution, superficial area in contact between these two phases is larger, and thus, extraction faster. After extraction, sample must be centrifuged in order to separate both phases. Ultrasonic radiation is used for the acceleration of mass transfer process, in ultrasound-assisted dispersive liquid–liquid microextraction (UA-DLLME) (Lv et al., 2014). The cloudy solution can also be only caused by ultrasound radiation in the called ultrasound-assisted emulsification microextraction (USAEME) (Regueiro, Llompart, Garcia-Jares, Garcia-Monteagudo, & Cela, 2008).

LPME avoids the problem of the large solvent volumes in classical liquid–liquid extraction, but extraction solvents used in this technique are still generally toxic. In this respect, a new approach based on the solidification of floating organic drop (SFOD) was proposed, in which an extractant with lower density than water, low toxicity and proper melting point (10–30 °C) was used (Khalili Zanjani, Yamini, Shariati & Jönsson, 2007). In that way, after extraction, the organic droplet is solidified in an ice bath and then, easily collected with a spatula, melted and conducted to analytical determination. These type of solvents have been used in different LPME techniques, giving rise to different combined techniques (Viñas, Campillo, & Andruch, 2015), such as DLLME-SFOD (Leong & Huang, 2008), UA-DLLME-SFOD (Wang, Zhu, Cui, Miao, & Chen, 2014) and USAEME-SFOD (Bordagaray, Garcia-Arrona, & Millán, 2014). Those techniques combine the advantages of both former techniques, being all of them environmentally friendly due to the use of low volumes of practically non toxic solvents.

Analytical methods for determination of the PAEs are mainly based on chromatographic techniques, such as gas chromatography (GC) or high pressure liquid chromatography (HPLC). Mass spectrometry based detectors have been widely applied for the PAEs determination by these two techniques, but less sensitive and more affordable techniques such as HPLC-DAD and GC-FID have also been used (Lv, Hao, & Jia, 2013; Yang et al., 2015). A wide range of combinations of these detection techniques with different pretreatment methods have been used for the phthalate determination in food or water samples (Farajzadeh, Sorouraddin, & Afshar Mogaddam, 2015).

The aim of this work was to develop a simple, low cost and reliable analytical method for simultaneous determination of five PAEs in food simulants and liquid food and water samples using UA-DLLME-SFOD as a preconcentration technique followed by GC-FID. Up to our knowledge, amongst phthalates, only di-(2-ethylhexyl) phthalate has been determined combining the solvents used in SFOD techniques and GC-FID (Yamini, Ghambarian, Khalili-Zanjani, Faraji, & Shariati, 2009) and often determination of the PAEs using a dispersive technique is carried out using highly toxic chlorinated solvents (Cinelli, Avino, Notardonato, Centola, & Russo, 2013; Hongyuan et al., 2012; Xue, Zhang, Wang, Wang, & Du, 2014). The UA-DLLME-SFOD technique combines advantages of both DLLME and SFOD techniques; it is rapid due to the high superficial area between phases and it is environmentally friendly due to the solvents used. In this work, influence of different parameters in extraction was investigated with the aid of experimental design. After optimization, procedure was validated and it was finally applied to the determination of the PAEs in food simulants, and different water and liquid food samples.

2. Experimental

2.1. Reagents and standards

Dibutyl phthalate (DBP, 99%), benzyl butyl phthalate (BBP, 98%), dicyclohexyl phthalate (DCHP, 99%), bis(2-ethylhexyl) phthalate (DEHP, 99.5%), di-n-octyl phthalate (DNOP, 99.5%), 1-undecanol (99%), 2-dodecanol (99%), n-hexadecane (99%), Br-hexadecane (97%), 1-chlorooctadecane (96%) and 1,10-dichlorodecane (99%) were purchased from Sigma–Aldrich (Barcelona, Spain). 1-Dodecanol (98%), methanol (99.8%), acetonitrile (99.7%), ethanol absolute (99.5%), acetone (99.5%) and sodium chloride (99.5%) were supplied by Panreac (Madrid, Spain). Doubly distilled water was used throughout this work.

Individual stock solutions of PAEs and a mixed stock solution (1 g L^{-1} of each analyte) were prepared in methanol and stored in amber-colored vials in the refrigerator. Working solutions were prepared weekly by dilution of the stock one with methanol, and they were preserved in the refrigerator.

All the glassware used in this research was previously soaked and washed with acetone and dried at 240 °C for at least 4 h.

2.2. Samples

Food simulants were prepared in the laboratory as described in regulation (Commission regulation (EU) No 10/2011, 2011): Simulant B (3% (w/v) acetic acid/water solution) and simulant C (20% (v/v) ethanol water solution).

Different commercial samples (three mineral water, three vinegars, four wines (2 packed in glass bottles and 2 in Tetrapak box), three soft drinks and one sangria) were purchased from one local shopping center. Recovery tests in commercial samples were carried out using appropriate dilutions. Samples were spiked adding 50 μL of a working solution containing all the analytes to the final solution.

2.3. Instrumentation and chromatographic conditions

Chromatographic analyses were performed on a HP 6890N (Agilent Technologies, Wilmington, DW, USA) gas chromatographer equipped with a split/splitless injector used in splitless mode and a flame ionization detector (FID). Injector temperature was 300 °C and splitless time was 3 min. The column used was a HP-5 (30 m × 0.250 mm × 0.25 μm film thickness) capillary column (Agilent Technologies). The carrier gas was helium with a 1.3 mL min^{-1} flow. The oven temperature program was: 160 °C for 1 min, increased to 200 °C at a rate of 10 °C min^{-1}, and then a ramp of 2 °C min^{-1} to 255 °C. Detector temperature was 300 °C.

Extractions were carried out in a Bandelin Sonorex Digitec DT100H ultrasound bath (ALLPAX, Papenburg, Germany) with 35 kHz ultrasound frequency. Centrifugation was performed on a Selecta centrifuge (Barcelona, Spain). The cooling bath was a Julabo F26 (Augsburg, Germany). The heating bath was a Lauda ecoline re104. Experimental design was performed and evaluated with Statistica software (StatSoft, Tulsa, USA).

2.4. UA-DLLME-SFOD procedure

10 mL sample solution containing 25 g L^{-1} NaCl was placed in a 40 mL glass vial. 0.75 mL of acetronitrile and a mixed solution of PAEs standards were spiked, and the resulting solution was placed in a thermostatic bath for 5 min at 35 °C. Then, 15 μL of n-hexadecane (extraction solvent) was added to the solution, it was gently shaken by hand and placed into an ultrasonic bath for sonication at 35 kHz and 35 °C ± 1 °C during 5 min. As a result,

extraction solvent was entirely dispersed into the aqueous phase. Then, dispersion was disrupted by centrifugation at 4500 rpm for 10 min and the vial was placed into the thermostatic bath at 3 °C for 10 min. The solidified organic drop was then collected with a spatula and transferred to a 0.9 mL clear glass vial where it was let melt at room temperature. Once melted, 3 μL of the organic phase was collected and directly injected in the GC-FID for analysis. A chromatogram obtained following the UA-DLLME-SFOD procedure with a standards sample (50 μg L^{-1} of each analyte) is shown in Fig. 1.

3. Results and discussion

3.1. Selection of working characteristics

In this study, a method using UA-DLLME-SFOD coupled to GC-FID was developed for phthalate determination in different liquid samples. A comparison of UA-DLLME-SFOD with other liquid microextraction techniques was also accomplished. Once the technique was selected, in order to obtain the best experimental conditions, the influence of different parameters in the signal was evaluated. Some parameters, in which a wide range needs to be studied, were univariately studied. Other factors were investigated by experimental design, with which influential factors were distinguished. Concentration of analytes during the optimization process was around 500 ng mL^{-1}.

3.1.1. Selection of extraction solvent

Selection of an appropriate extraction solvent is important in the optimization process of a microextraction method based on solidification of the organic drop. The extraction solvent should satisfy the following requirements: It should be immiscible with water and have low volatility, lower density than water and present a melting point near to room temperature. It should also be able to extract the target analytes, and, in the case of chromatography, its peaks should be well separated from those of the analytes. Taking into account these characteristics 1-undecanol, 1-dodecanol, 2-dodecanol, n-hexadecane, 1-bromohexadecane, 1,10-dichlorodecane and 1-chlorooctadecane are normally chosen in SFOD techniques as extraction solvents (Ghambarian, Yamini, & Esrafili, 2013; Wang et al., 2010).

1-Undecanol, 1-dodecanol and 2-dodecanol were rapidly discarded because when they were used the occurrence of non-assigned peaks took place. 1, 10-Dichlorodecane was also discarded because baseline obtained was not good and analytes could be confused. 1-Bromohexadecane and 1-chlorooctadecane were adequate for determination of some PAEs but their peak was too near other peaks and in some cases, even covered them. n-Hexadecane gave a good signal, making determination of all analytes possible. Therefore, n-hexadecane was selected for further experiments.

3.1.2. Comparison with other liquid microextraction techniques

In order to select the best SFOD technique for the process, LPME, DLLME, USAEME and UA-DLLME were compared and results are shown in Fig. 2. As it can be seen, dispersion of the extraction solvent improved the extraction of analytes. Ultrasound improved the extraction in DEHP and DNOP, but it was not so efficient in the extraction of the rest of the analytes. Use of dispersant increased especially DBP, BBP and DCHP, but it was not so efficient in the rest of the analytes. Combining ultrasonication and disperser solvent, the best results were obtained.

3.1.3. Selection of centrifugation and cooling conditions

These experimental conditions were fixed according to previous experience in our laboratory (Pérez-Outeiral, Millán, & Garcia-Arrona, 2015). Centrifugation rate and time were the minimum that allowed collecting the cloudy solution into a sedimented drop (4500 rpm, 10 min). Cooling conditions were fixed to obtain a well formed solidified drop (3 °C, 10 min). In this case agitation was not necessary in solidification.

3.1.4. Selection of dispersant

Disperser solvent should assist the formation of a dispersion of the droplets of extraction solvent in the aqueous phase increasing in that way the surface area between the phases and accordingly improving extraction efficiency. In this study, methanol, ethanol, acetone and acetonitrile were studied, and, as it can be seen in Fig. 3, best results were obtained with ethanol and acetonitrile. Although there was not statistical difference between the obtained values with these two dispersants, values obtained with acetonitrile were slightly higher, that is why it was selected for further experiments.

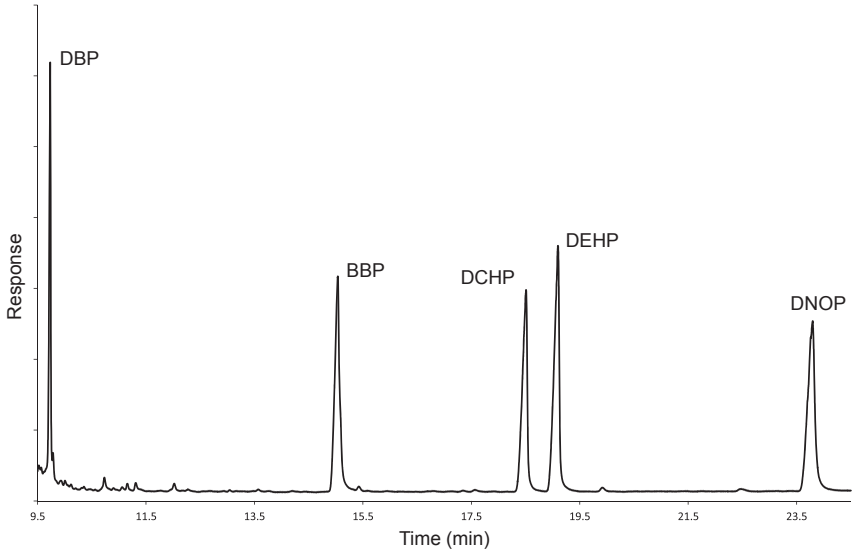

Fig. 1. Chromatogram of the five PAEs obtained following the optimized UA-DLLME-SFOD-GC-FID process with a standard sample (50 μg L^{-1}).

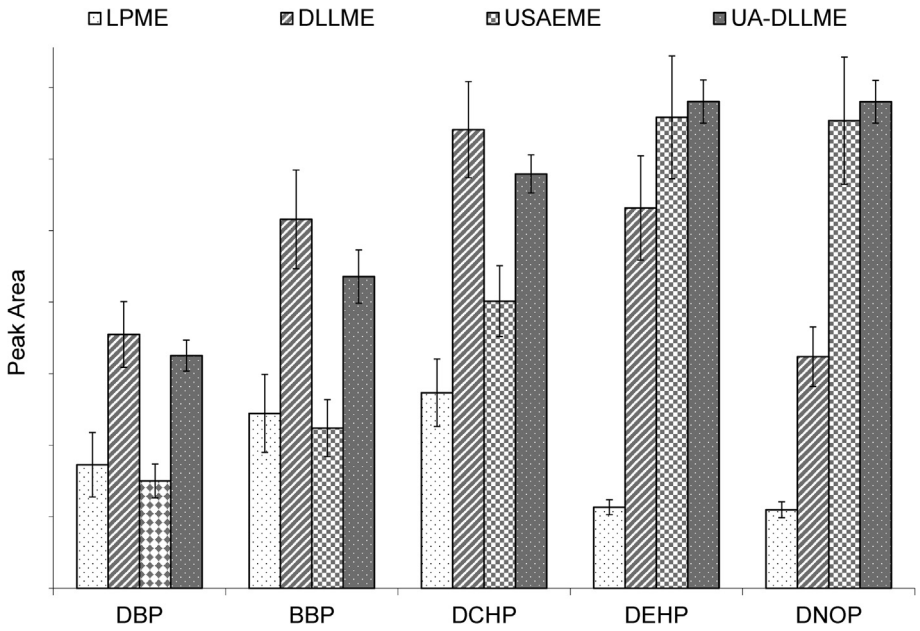

Fig. 2. Comparison of LPME, DLLME, USAEME and UA-DLLME techniques, all of them followed by solidification of floating organic drop. Experimental conditions were: sample volume, 10 mL; extraction solvent, 20 μL of n-hexadecane; extraction temperature, 35 °C; extraction time, 10 min; drop freezing, 3 °C and 10 min and no salt adittion. Agitation in LPME and DLLME was 450 and 1125 rpm respectively. 1 mL of acetonitrile was used in DLLME and UA-DLLME.

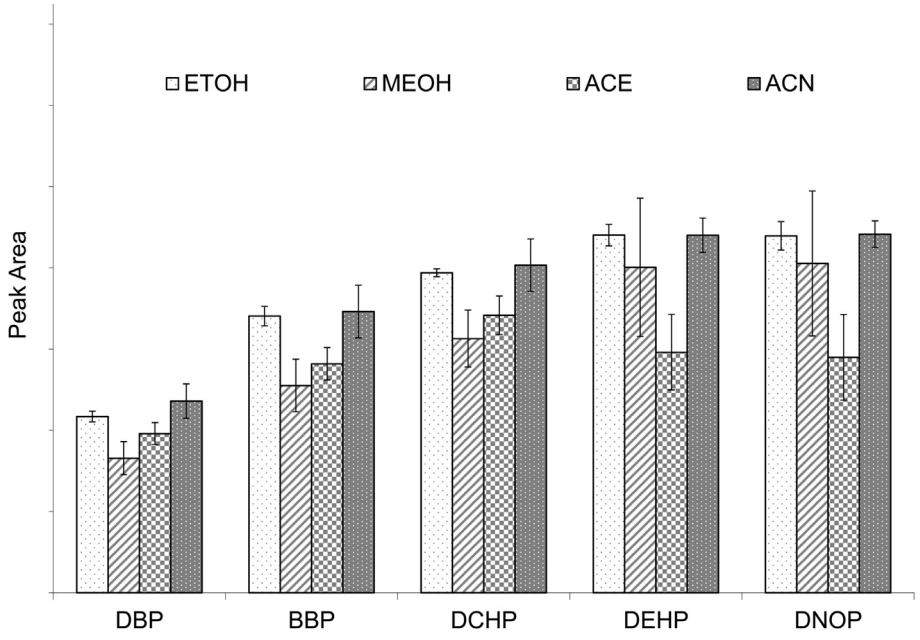

Fig. 3. Comparison of the obtained signals using different types of dispersants. Other extraction conditions were kept the same in all cases.

3.1.5. Selection of volume of extraction solvent

In general, an increase of the extraction solvent volume leads to a higher volume of the organic phase after centrifugation, resulting in a decrease of the concentration of target compounds due to the dilution (Ghambarian et al., 2013; Wang et al., 2010). So, normally, as low volume of extractant as possible is used. In this work extractions with volumes of 10, 13, 15 and 20 μL of n-hexadecane were carried out and average values and standard deviation were compared. As expected, it was observed that signal was higher when extraction volume was lower. However, when 10 and 13 μL was used, reproducibility was not good enough (in some analytes RSDs near 30% were obtained) and collection of enough volume of extractant after the extraction was not always possible. Using 15 μL reproducibility was better (RSDs below 15%) and collection of a sufficient amount of extractant was always possible. That is why, 15 μL of n-hexadecane was selected for further experiments.

3.1.6. Effect of pH

pH of the sample is another important factor that might affect extraction efficiency because it affects the hydrolysis status and the solubility of the analytes in the aqueous phase. Therefore, its effect was evaluated in the range of pH 2–12. As it can be seen in Fig. 4

results showed constant extraction efficiency within the pH range, so samples were not subjected to pH adjustment.

3.1.7. Experimental design

Experimental design was used to find the significant experimental variables amongst the remaining variables to study. The considered variables were extraction time, extraction temperature, volume of dispersant and salt addition. Levels of variables were chosen based on previous experience and they were 5 and 10 min, 35 and 45 °C, 0.5 and 1.5 mL of dispersant and no addition and 100 g L^{-1} salt addition, respectively. The 2^{4-1} fractional factorial design consisted of 8 experiments, each of one was performed twice (16 experiments in total).

Obtained data was evaluated by analysis of variance (ANOVA) and main effects were visualized via Pareto chart. In ANOVA, significance of factors is evaluated by *F* test. In Pareto Chart, the magnitude of each effect is represented by a horizontal column, and a vertical line indicates when the magnitude of an effect has to be considered statistically significant (usually at 95% confidence). In addition, signs in Pareto Charts reflect if the response is enhanced or reduced when passing from the lowest to the highest level of a factor. Table 1 summarizes the results obtained in experimental design.

Results showed that time and temperature were not significant in the studied analytes. The time effect was positive for DBP and BBP and negative for DCHP, DEHP and DNOP, and the temperature effect was negative for DBP, BBP and DCHP and positive for DEHP and DNOP. Thus, time was fixed at 5 min and temperature at 35 °C.

Salt addition and volume of dispersant resulted significant, having in all cases salt addition a positive effect and volume of dispersant a negative effect. Thus, these two variables were considered for further studies. Respecting the volume of dispersant, commonly, as little dispersant as possible is preferred to achieve the lowest toxicity to the environment. However, at low volumes of disperser solvent, dispersion may not be effective. On the other hand, too high volumes of dispersant could favor solubility of analytes in water and cause dilution of the sample, lowering the extraction efficiency. In this work, the disperser solvent volume was studied between 0.25 and 1.00 mL, and it was found that the best results were obtained with 0.75 mL acetonitrile, where dispersion was well formed and dilution of the sample was not excessive.

The addition of salt decreases the solubility of analytes in aqueous phase and enhances their partitioning into the organic phase. Nevertheless, the presence of high concentrations of salt could change physical properties of the Nernst diffusion film and thus reduce the diffusion rate of the target compounds into the organic phase (Ghambarian et al., 2013; Wang et al., 2010). In this work, effect of salt concentration was studied between 5 and 125 g L^{-1} and it was observed that extraction efficiency increased considerably from 5 to 25 g L^{-1} probably because of the salting out effect, but then remained practically constant in the studied range. In addition, at higher concentration of salt organic droplet was worse formed. Hence, 25 g L^{-1} of salt was chosen.

3.2. Method validation

Linear range, limits of detection (LODs), limits of quantification (LOQs), precision and accuracy were considered for the method validation. The obtained analytical figures of merit of the proposed UA-DLLME-SFOD method are summarized in Table 2. All analytes showed good linearity throughout the studied range with squared correlation coefficients (R^2) ranging from 0.993 to 0.995. LODs were calculated based on ten determinations of samples in which the analyte concentration was close to the expected LOD (Konieczka & Namieśnik, 2009, chap. 7) and ranged from 0.64 to 2.82 μg L^{-1}. LOQs were calculated as 3 × LOD and they ranged from 1.93 to 8.47 μg L^{-1}.

Intra and interday precision were evaluated by analyzing six replicates of spiked samples at two concentration levels on the same day, and nine replicates on three different days respectively. Low level was around 50 μg L^{-1} for all analytes and high level around 400 μg L^{-1}. The relative standard deviations (RSDs) were in the range of 2.7–9.3% for intraday precision and of 3.2–8.1% for interday precision. Enrichment factors, defined as the ratio between analyte concentration in the organic phase after extraction and the initial concentration of the analyte within the water sample, ranged from 854 to 1893.

Accuracy was checked by means of extraction recoveries (ER). B and C simulants were selected according to the current legislation

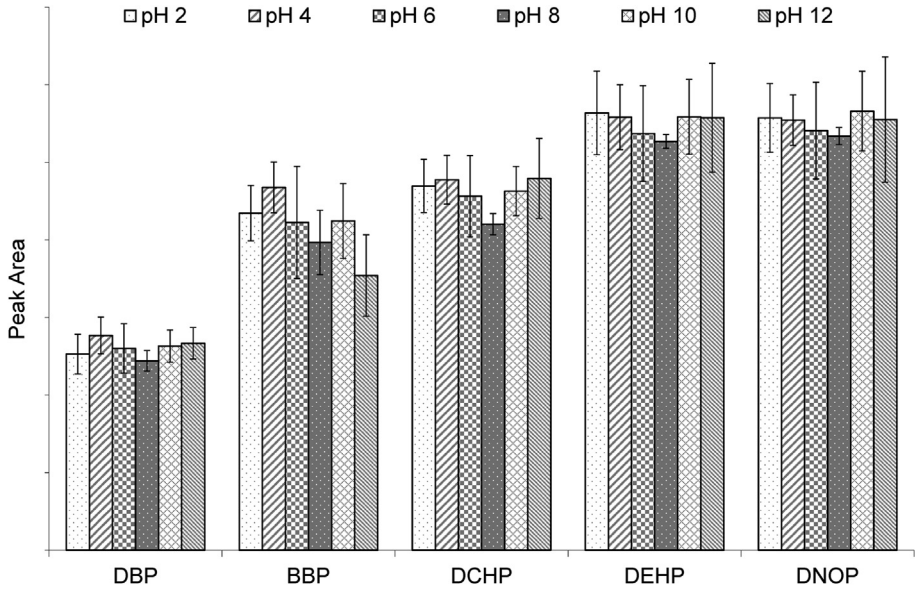

Fig. 4. Comparison of the obtained signals when extractions are made at different pHs. Other extraction conditions were kept the same in all cases.

Table 1
Summary of the results obtained in experimental design.

Compound	Time		Temperature		Volume of dispersant		Salt addittion	
	Significance	Effect	Significance	Effect	Significance	Effect	Significance	Effect
DBP	No	+	No	−	Yes	−	Yes	+
BBP	No	+	No	−	Yes	−	Yes	+
DCHP	No	−	No	−	Yes	−	Yes	+
DEHP	No	−	No	+	Yes	−	Yes	+
DNOP	No	−	No	+	Yes	−	Yes	+

Table 2
Analytical figures of merit of the proposed UA-DLLME-SFOD-GC-FID method for PAEs determination.

Compound	Linear range (μg L^{-1})	R^2	LOD (μg L^{-1})	LOQ (μg L^{-1})	EF	Intraday precisión (RSD, %)		Interday precisión (RSD, %)	
						Low	High	Low	High
DBP	5.29–529	0.995	0.78	2.35	854	3.1	6.7	5.4	6.1
BBP	10.5–524	0.993	2.57	7.71	1608	7.7	9.3	6.0	7.5
DCHP	5.03–503	0.994	0.92	2.77	1416	3.4	8.4	5.5	6.8
DEHP	10.5–523	0.995	2.82	8.47	1662	2.8	7.4	4.1	8.1
DNOP	5.46–546	0.994	0.64	1.93	1893	2.7	7.4	3.2	7.3

(Commission regulation (EU) N° 10/2011, 2011) and to the type of samples intended to be analyzed. In addition, recoveries in distilled water were also checked. Results are shown in Table 3 and they are adequate, so the developed method can be satisfactorily applied for the phthalates determination in food simulants.

The performance of the proposed method was compared with other GC-FID methods, and it was found that obtained LODs are in the range of other studies in which dynamic liquid-phase microextraction (Xu, Liang, & Zhang, 2007), ultrasound-assisted dispersive liquid–liquid microextraction (Hongyuan et al., 2010; Hongyuan et al., 2011) or ultrasound-assisted surfactant-enhanced emulsification microextraction were used (Hongyuan et al., 2012). On the other hand, they are higher than results obtained with single-drop microextraction (Batlle & Nerín, 2004) or with solid-based disperser LPME (Farajzadeh, Khorram, & Nabil, 2014). However, comparing to the latter, the method developed in this work presented higher extraction recoveries in most samples and analytes.

Considering other methods using different detection techniques, LODs were in the range of the study using DLLME-HPLC with variable wavelength detector (Liang, Xu, & Li, 2008) but were higher than LODs found in the work using a more sophisticated and expensive technique such as DLLME-GC-MS (Farahani, Norouzi, Dinarvand, & Ganjali, 2007).

On the other hand, looking at the previous commented legislation, it can be found that SML of some of the analytes are regulated, being the SML of DBP, BBP and DEPH 0.3, 30 and 1.5 mg substance per Kg food respectively. Considering that the density of aqueous samples is approximately 1 kg L^{-1}, it can be seen that the detection limits of the developed method are far below these requirements. So, it can be assumed that the developed method has enough sensitivity for checking compliance with this regulation.

3.3. Analysis of real samples

Three bottled drinking mineral water, three vinegars, four wines, three soft drinks and one sangria were analyzed using the proposed method. No analyte content was found in real samples, except for one wine, in which DBP was present at a concentration higher than LOD but lower than LOQ. In order to facilitate phthalate migration from a plastic bottle to water, one of the bottles of water was kept in an oven at 40 °C for two days. After that, water was again analyzed, and no phthalate content was found. Following, the same water was heated up in a domestic microwave for 6 min, and bottled was totally deformed, but still no phthalate content was found in the water. Thus, to evaluate the applicability of the developed method, a real sample of each type was spiked with analytes and recoveries in those samples are presented in Table 3. Obtained results were considered acceptable.

4. Concluding remarks

In the present study, a UA-DLLME-SFOD-GC-FID method was developed for determination of PAEs in food simulants and liquid food and water samples. Until now, solvents used in SFOD techniques had not been found for simultaneous application of the phthalate determination by GC-FID. In this work, hexadecane replaces the highly toxic organic solvents used in other DLLME techniques. The developed method is simple, precise, accurate, reproducible and it can be applied to phthalate determination in different liquid real samples and in food simulants. Only a slight amount of dibutyl phthalate was found in a wine sample. For all the

Table 3
Recovery (mean of triplicates) of PAEs in distilled water, food simulants and liquid food samples.

Compounds	Distilled water		Simulant B		Simulant C		Water		Vinegar		Soft drink		Wine	
	ER (%)	RSD (%)	ER (%)	RSD (%)	ER (%)	RSD (%)	ER (%)	RSD (%)	ER (%)	RSD (%)	ER (%)	RSD (%)	ER (%)	RSD (%)
DBP	97	2	92	6	63	5	96	8	103	6	88	3	95	9
BBP	94	2	93	3	55	7	95	5	100	8	86	1	87	12
DCHP	100	2	109	5	95	6	109	4	110	6	92	1	98	10
DEHP	98	4	103	5	109	4	106	5	104	6	89	13	78	6
DNOP	97	3	106	5	111	4	105	5	103	7	72	7	75	6

regulated analytes, the sensitivity of the method was good enough for reliable determination of analytes at lower levels than the SMLs established by European Union Regulation.

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Development of a method for fragrance allergens simultaneous determination in cosmetic and water samples using ultrasound-assisted emulsification microextraction followed by the solidification of the floating organic drop and HPLC-DAD

Development of a method for fragrance allergens simultaneous determination in cosmetic and water samples using ultrasound-assisted emulsification microextraction followed by the solidification of the floating organic drop and HPLC-DAD

1. Introduction

Nowadays cosmetic products are worldwide used every day. The use of cosmetics can be traced back to ancient times, when different civilizations used different products to clean themselves, to enhance their beauty or to modify to some extent their physical appearance. There are several examples of the use of cosmetics across the history: Egyptians used scented oils and ointments to clean and soften their skin and mask their body odour. Claudius Galen, an ancient Greek physician, invented cold cream in the second century. Romans used oil-based perfumes. In the seventeenth century in Europe dandy gentlemen used cosmetics in abundance, often to hide that they usually did not bathe themselves. European ladies of the eighteenth-century used to whiten their face with lead carbonate (and lots of them died because of poisoning with lead). During the nineteenth-century, chemicals were used to replace more expensive natural ingredients making cosmetics even more widely used (Hill and Kolb, 1999; Kumar, 2005; Cosmetics info, 2016).

Later, innovation and technologies in producing cosmetics in twentieth-century boosted the cosmetic industry, which has continued growing until the present days. Indeed, nowadays, the cosmetic industry is a huge business that moves more than 200 billion euro only considering its seven more important markets (Europe, United States, China, Japan, Brazil, India and South Korea)(Cosmetics Europe, 2014).

Few people, apart from the ones working with cosmetics, are aware of the extremely wide range of cosmetics currently available. According to the current legislation, a cosmetic product is *"*(...) 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"(Regulation (EC) No. 1223/2009, 2009).

According to this definition, cosmetics comprise an extensive range of products such as creams, emulsions, lotions, gels, soaps, oils, perfumes, bath and shower preparations, depilatories, deodorants and anti-perspirants, hair care products, shaving products, products for making-up and products for removing make-up, products for care of the teeth and the mouth, products for nail and lips care, products for external intimate hygiene, sunbathing and related products, anti-wrinkle products and so on.

Cosmetic products can be classified according to different characteristics: to their principal use (make-up, hygiene...), to their area of application (skin, mucous membranes...), to their format (creams, lotions...), to the person who normally applies it (professional use, no-professional use...), etc. But perhaps the most used classification in current documentation is the one that divides cosmetic products in "rinse-off products" and "leave-on products". The first ones are those cosmetic products intended to be removed after the application, and the second ones those intended to stay in prolonged contact with the site of application.

Cosmetics are complex mixtures of numerous chemical compounds. According to the Personal Care Product Council (previously known as the Cosmetic, Toiletries and Fragrance Association, CTFA) there are approximately 17500 different ingredients and ingredient mixtures that can be used in cosmetic products (Michalun and Dinardo, 2014). The large quantity of commercially accessible ingredients has led to the creation of a huge variety of cosmetic formulations, which are used everyday around the entire world.

In past times, cosmetic safety received very little attention, probably due to their external use and to the lack of information about the effect of the compounds used in cosmetics. However, with the growth of the cosmetic industry and the advance in science, a general concern for the cosmetic´s effects on health has arisen. In addition, due to the further knowledge, people have also started wondering about the efficacy of cosmetics. Thus, legislation has become mandatory to ensure safety and efficacy of cosmetic products.

In spite of the fact that the cosmetic industry is a global industry, regulatory frameworks vary greatly between different countries and that makes practically

impossible to sell the same product without any change on all the markets. In the last decade of the twentieth century the main markets were regulated by very different models, but in recent years the European model of regulation has become an international model.

In Europe, the first cosmetic regulation was the European Council Directive 76/768/EEC, adopted on 27 July 1976. The main objective of that directive was to ensure a high level of consumer protection. For that purpose, among other provisions, a safety assessment for human health of each final product done by a qualified professional was required as a part of the product development. Other important purpose was the free circulation of cosmetic products through the European market (Gagliardi and Dorato, 2007).

Current legislation in Europe is defined in the regulation (EC) No. 1223/2009 of the European Parliament and of the council of 30 November 2009 and in its amendments and corrections. This regulation became effective on July 11, 2013 (with the exception of some articles) completely replacing the previous directive. Its main scopes were to clarify the legislation (due to the significant number of amendments done to the previous legislation), to simplify procedures and terminology (reducing administrative burden and ambiguities), to ensure the free movement of goods through Europe in the same way as the previous directive and a high consumer's health protection. The new regulation is expressed in a compilation of 40 articles structured in 10 chapters that contain information about restrictions for specific substances, animal testing and the information that must be delivered to the consumer´s, amongst others.

Nowadays, analytical official methods for cosmetic analysis do not guarantee the rigorous control of the total number of substances that are prohibited or restricted according to the current legislation. Furthermore, the analytical control of ingredients in the batches of finished products is necessary to guarantee that the contents are the expected according to the designed formulation, and, in concordance, the efficacy is the desired and the safety is preserved. Thus, methods to detect and/or determine those components are necessary. In the last times, different procedures have been developed (Salvador *et al.,* 2007).

The annexes of the current legislation and the published procedures of the most analyzed substances, give an overview of the different families of compounds

whose determination is more interesting. The substances determined in the published procedures can be classified into the following different families according to their purpose: UV-filters, colouring agents, preservatives, fragrance ingredients, surfactants and other actives (Salvador *et al*., 2007).

Normally, cosmetic products on the market meet the legislation and, therefore, it can be assumed that they are safe. Preservatives and fragrances, however, have some high risk of allergic reaction (Smith and Wilkinson, 2016).

Perfumes in cosmetics

Perfumes are strongly related to cosmetic products, as they are frequently added to modify their olfactory properties. Each perfume is composed of hundreds of aromatic chemicals, known as fragrances, which together give its characteristic aroma. Perfume type and its content in the final cosmetic product depend on the type of cosmetic and its scope. A division of the cosmetics depending on their perfume content can be found in Table 5.1.

The cosmetic directive in force establishes that the list of the ingredients used in a cosmetic may be indicated on the packaging preceded by the term "ingredients". However, it is almost impossible to specify all the ingredients of a perfume and the total composition is usually referred by the terms 'parfum' or 'aroma'. Exception is the presence of potentially allergenic fragrance-related substances, which must be additionally declared, if any, according to the current legislation.

Table 5.1. Perfume contents usually found in cosmetic products (Chisvert and Salvador, 2007).

Cosmetic product	Perfume content (%)
Fine fragrances	
- Baby cologne	1 - 2
- Cologne	2 - 3
- Eau de cologne	3 - 4
- Eau Fraiche	4 - 5
- Eau de Toilet	5 - 8
- Eau de Parfum	8 - 12
- Parfum	12 - 18
Skin care products	0.01 - 0.5
Hair care products	0.01 - 1
Bath preparations	0.1 - 3
Toothpastes	0.5 - 1

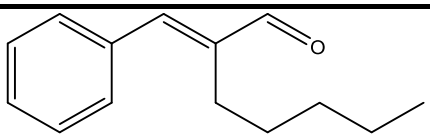
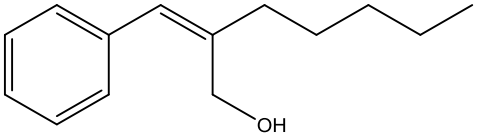
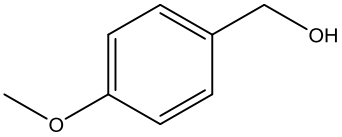
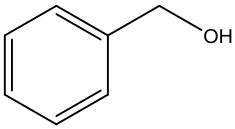
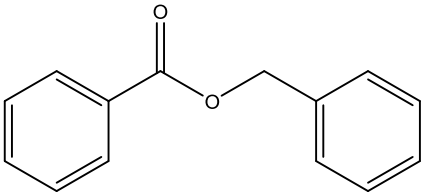
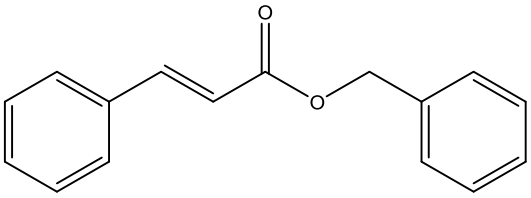
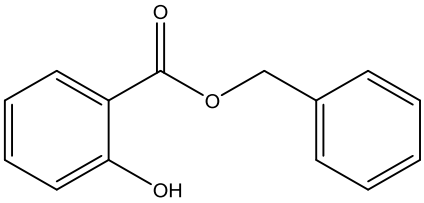
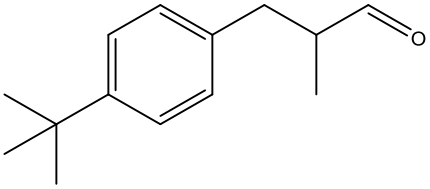
Potentially allergenic fragrance-related substances in cosmetics

Fragrances are generally innocuous, but they can sometimes cause skin irritation or allergic reactions. In 1999, a list of 24 fragrance-related substances with a well recognized potential to cause allergy was identified by the Scientific Committee on Cosmetic Products and Non-Food Products intended for consumers (SCCNFP/0017/98). A year later, in 2000, two natural moss extracts were also added to this list (SCCNFP/0421/00). Currently there are, therefore, 26 potentially allergenic substances (PAS) known in literature. Table 5.2. shows the name, CAS and structure (when available) of the 26 PAS.

In 2003, the European Union published an amendment that modified the in force directive (Directive 2003/15/EC) about the conditions of use of the fragrance-related substances. Thus, current directive requires that these substances are specifically declared when concentration exceeds 0.001% in leave-on products and 0.01% in rinse-off products.

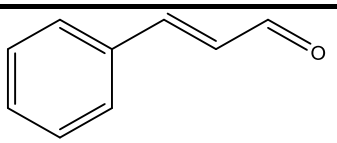
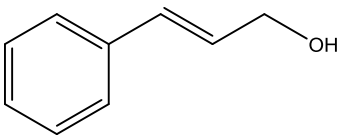
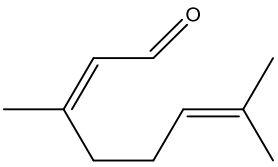
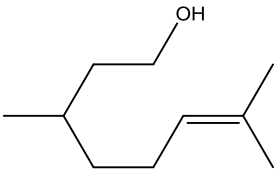
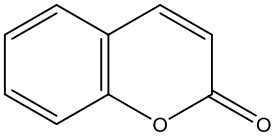
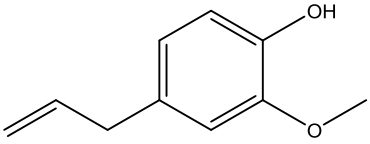
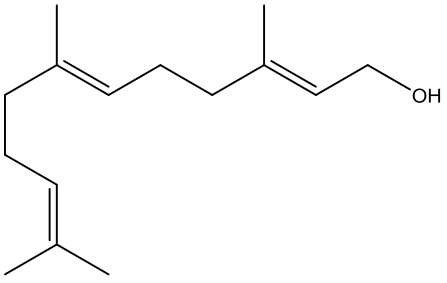
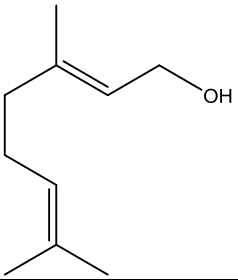
The main route of exposure to these ingredients is the direct application of cosmetics, but the contact of human body with contaminated water must also be considered. As PAS are present in all kind of cosmetics they can be easily transferred to bathtubs, swimming pools and other recreational waters. Special attention has been paid to the presence of PAS in baby bathwater as babies use to spend a long time playing in the bath (Lamas *et al.*, 2009). In addition, these compounds are continuously introduced in the environment via urban wastewater effluents and they have been detected in wastewater treatment plants (Becerril-Bravo *et al.*, 2010; Godayol *et al.*, 2015). In consequence, the development of new analytical methods for the determination of PAS in cosmetics and different water samples is of high interest.

Table 5.2. Name, CAS and structure of the 26 PAS.

Name	CAS	Structure
Amyl cinnnamal*	122-40-7	
Amylcinnamyl alcohol*	101-85-9	
Anise alcohol	105-13-5	
Benzyl alcohol	100-51-6	
Benzyl benzoate	120-51-4	
Benzyl cinnamate*	103-41-3	
Benzyl salicylate	118-58-1	
Butylphenyl methylpropional (Lilial)	80-54-6	

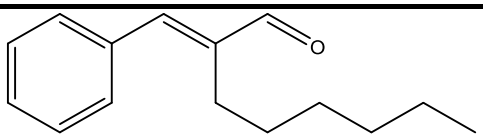
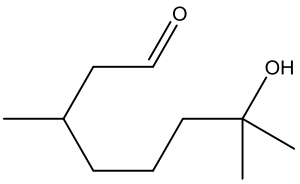
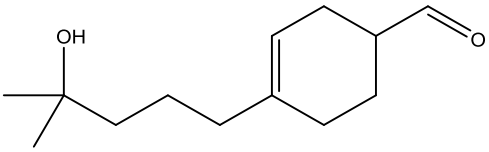
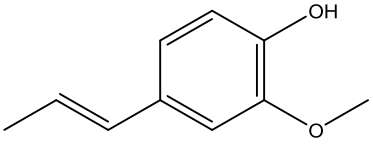
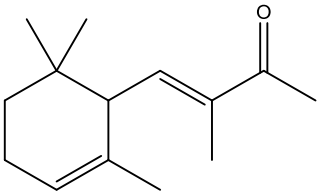
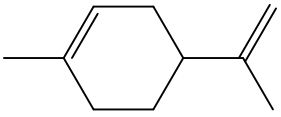
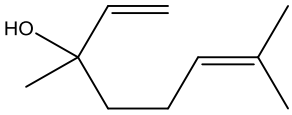
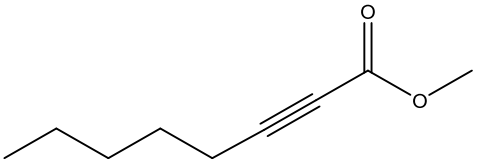
* E/Z isomeric mixtures.

Table 5.2. (Cont) Name, CAS and structure of the 26 PAS.

Name	CAS	Structure
Cinnamal*	104-55-2	
Cinnamyl alcohol*	104-54-1	
Citral*	5392-40-5	
Citronellol	106-22-9	
Coumarin	91-64-5	
Eugenol	97-53-0	
Farnesol*	4602-84-0	
Geraniol	106-24-1	

* E/Z isomeric mixtures.

Table 5.2. (cont) Name, CAS and structure of the 26 PAS.

Name	CAS	Structure
Hexyl cinnamal*	101-86-0	
Hydroxycitronellal	107-75-5	
Hydroxyisohexyl 3-cyclohexene carboxaldehyde (Lyral)	31906-04-4	
Isoeugenol*	97-54-1	
Alpha-isomethyl ionone*	127-51-5	
Limonene	5989-27-5	
Linalool	78-70-6	
Methyl 2-octynoate	111-12-6	
Evernia prunastri extract	90028-68-5	Oak moss extract
Evernia furfuracea extract	90028-67-4	Treemoss extract

* E/Z isomeric mixtures.

To the date, the usual analytical methods for the determination of PAS are mainly based on GC-MS systems (Chaintreau *et al*., 2003; Mondello *et al.*, 2007; Sanchez-Prado *et al.*, 2011B). The volatility of these compounds and the high separation capability of the technique make the GC a good choice for the analysis of PAS. On the other hand, the variety of different functional classes makes mandatory the use of a universal detector system, such as the MS detector. To enhance the separation capability of the technique in complex samples, two dimensional gas chromatography has also been sometimes used. In this technique fractions that have been heart-cut from a first column are further separated and analyzed on a second column (Dunn *et al.*, 2006; Cordero *et al.*, 2007). Also other chromatographic techniques, such as size exclusion chromatography coupled to GC/MS have been used (Niederer *et al.*, 2006). Focusing on the detector system, tandem mass spectrometry (MS/MS) has also been applied to the determination of PAS with the aim of improving selectivity, sensitivity and applicability of the methods (Lv *et al.*, 2013; Celeiro *et al*., 2014).

Furthermore, GC-MS systems have been widely coupled to diverse sample pre-treatment techniques including liquid extraction (Lamas *et al.*, 2010A; Desmedt et *al.*, 2015), matrix solid-phase dispersion (MSPD) (Sanchez-Prado, 2011A), micro-MSPD (Celeiro *et al.*, 2014), SPME (Lamas *et al.*, 2009), headspace (HS) SPME (Becerril *et al.*, 2010; Lamas *et al.*, 2010C; Godayol *et al.*, 2015) pressurized liquid extraction (Lamas *et al.*, 2010B; Celeiro *et al.*, 2015), dispersive liquid–liquid microextraction (Tsiallou *et al*., 2012) and ultrasound-assisted emulsification microextraction (Becerril-Bravo *et al.*, 2010).

Apart from gas chromatography, other determination techniques such as microemulsion electrokinetic chromatography (MEEKC) (Furlanetto *et al.*, 2010), micellar electrokinetic chromatography (MEKC) (Lopez-Gazpio *et al.*, 2014), attenuated total reflectance IR spectroscopy (Wang *et al.*, 2014) and HPLC (Villa *et al.*, 2007) have also been used for determination of PAS, but in those cases, up to our knowledge, no pretreatment technique has been reported.

HPLC–DAD is a very widespread instrumentation in most routine analysis laboratories; and, in consequence, this option is a good alternative to the more sophisticated and expensive techniques used to the date. In addition, the use of a preconcentration technique prior to HPLC-DAD determination can improve the limits of detection of the method and, therefore, spread out its applications. Amongst all the sample preparation techniques, in recent years, liquid phase

microextraction methods have received a growing attention due to their inexpensive, rapid, environmentally friendly and simple character. From the different approaches, dispersive techniques are the fastest because of the enormous contact area between phases. The use of special solvents, such as the ones used in SFOD techniques, not only provides a new and easy form of recollecting the organic phase after extraction, but also avoids the use of the toxic solvents used in first dispersive liquid-liquid microextraction methods.

2. Objectives of the chapter

The main objective of the present chapter was the development of a simple, low cost and reliable analytical method for simultaneous determination of 18 PAS (amyl cinnamal, amylcinnamyl alcohol, anise alcohol, benzyl alcohol, benzyl benzoate, benzyl cinnamate, bencyl salicylate, lilial, cinnamal, cinnamyl alcohol, citral, geraniol, hexyl cinnamal, isoeugenol, alpha-isomethyl ionone, limonene, linalool and methyl-2-octynoate) in cosmetic and water samples using ultrasound-assisted emulsification microextraction followed by the solidification of the floating organic drop and HPLC coupled to diode array detection.

The main objective of the chapter was accomplished with the following specific objectives:

1. The development of a procedure based on dispersive liquid-liquid microextraction techniques followed by the solidification of the floating organic drop. This is a green technique due to the use of low volume of solvents with a lower toxicity than the traditional ones.

2. The extension of the use of microextraction techniques coupled to HPLC-DAD. This equipment was selected because of its inexpensive character which makes the instrument affordable by most of routine analysis laboratories.

3. The study of the best conditions for the determination of PAS. The main parameters influencing extraction and determination of PAS were considered in detail in this chapter by `one-variable-at-a-time (OVAT)´ approach.

4. The validation of the optimized procedure by obtaining the appropriate quality parameters. Studied characteristics were linearity, limits of detection and quantification, precision, accuracy and enrichment factors.

5. The application of the optimized procedure to different water and cosmetic samples. These samples were selected because a method for PAS determination is necessary to check compliance with current legislation in cosmetic samples, and to study the possible presence of PAS in different recreational waters.

3. Experimental

3. 1. Reagents, standards and materials

Anise alcohol (98%), benzyl alcohol (99%), cinnamyl alcohol (98%), cinnamal (95%), isoeugenol (98%), geraniol (98%), linalool (97%), citral (96%), methyl-2-octynoate (99%), benzyl benzoate (99%), benzyl salicylate (99%), benzyl cinnamate (99%), lilial (98%), amyl cinnamal (97%), alpha-isomethyl ionone (85%), hexyl cinnamal (95%) and limonene (96%) were purchased from Sigma–Aldrich (Barcelona, Spain). Amylcinnamyl alcohol (45.5%) was supplied by Dr. Ehrenstorfer (Augsburg, Germany).

2-Dodecanol (99%), 1-undecanol (99%), n-hexadecane (99%) and 1-bromohexadecane (97%) were acquired from Sigma–Aldrich (Madrid, Spain). 1-Dodecanol (98%) was supplied by Panreac (Barcelona, Spain).

The acetonitrile (HPLC grade) used for the mobile phase was obtained from Romil (Cambridge, UK). The methanol and the acetonitrile used for the stock solutions were obtained from Panreac (Barcelona, Spain). Doubly distilled water, taken from an Aquatron A4D system (J. Bibby Sterilin Ltd., Staffordshire, United Kingdom), was used throughout this work.

Individual stock solutions of PAS were prepared in acetonitrile and stored in amber-colored vials at –23°C. From them, working solutions were prepared weekly by dilution in acetonitrile. All solutions were preserved in the freezer and they were warmed at room temperature before use.

3. 2. Samples

Different cosmetic and personal care products including four *eau de toilettes* (ET), one *eau de cologne* (EC), one *eau de parfum* (EP) and one body milk (BM) were purchased from a local shopping center. Swimming pool water (SPW) was obtained from a public local swimming pool before closing at the end of the day. The average number of people bathing in that swimming pool is 500 persons per day. Baby bath waters (BBW) were taken from different baby bathtubs after the bath of different babies. All samples were stored in glass bottles until the analysis.

Cosmetic and personal care products were subjected to dilution before analysis. Agitation and ultrasonication were applied when necessary to dissolve the sample. Given the wide range of concentration of fragrance allergens even inside the same sample, different dilutions were applied to each sample to be able to determine all the present analytes. Dilutions ranged from 0.0029 g to 1.0077 g in 50 mL. In the case of the different types of water no dilution was applied.

3. 3. Instrumentation and chromatographic conditions

Chromatographic analyses were performed in a LC-20AD liquid chromatographer equipped with a SPD-M20A Diode Array Detector (Shimadzu Corporation, Duisburg, Germany) and a manual sample injector (20 µL injection volume). Data were collected and processed using LC Solution software (2.1. version). Separations were carried out using a XDB-C18 column (250 mm x 4.6 mm x 5µm) from Agilent (Wilmington, DW, USA) at ambient temperature.

Gradient elution with water and acetonitrile was used for separation of target compounds. The best separation was achieved by the gradient elution and the flow profile summarized in Table 5.3. UV-Vis spectra were recorded during all the chromatogram from 190 to 800 nm.

Table 5.3. HPLC Chromatograhic conditions.

Time (min)	Acetonitrile (%)	Water (%)	Flow (mL/min)
0	50	50	0.5
6.5	50	50	0.5
15	60	40	0.5
16	-	-	1
32	80	20	1
32.5	90	10	1
33.5	90	10	1
34	50	50	1
41	50	50	1

Extractions were carried out in a Bandelin Sonorex Digitec DT100H ultrasound bath (ALLPAX, Papenburg, Germany) with 35 kHz ultrasound frequency. Centrifugation was performed on a Selecta centrifuge (Barcelona, Spain). The cooling bath was a Julabo F26 (Augsburg, Germany).

3. 4. USAEME-SFOD procedure

Firstly, 10 mL of a 150 g/L NaCl solution were placed in a 40 mL glass vial and the appropiate mixed solution of fragrance allergens standards was spiked when necessary. Then, 50 µL of 2-dodecanol as a extraction solvent were added to the solution, and it was gently shaken by hand and placed into the ultrasonic bath, previously heated, for sonication at 35 kHz and 35 ± 1°C during 5 min. As a result, emulsion of extraction solvent in water was formed. Emulsion was subsequently disrupted by centrifugation at 4500 rpm for 15 min, forming again a unique floating organic phase of extractant. Next, the vial was placed into the cooling thermostatic bath at 3°C for 15 min. The formed solidified organic drop was then carefully collected with a spatula and transferred to an Eppendorf vial where it was let melt at room temperature. After that, 25 µL of the organic phase were collected and mixed with 20 µL of methanol to make it compatible with the HPLC mobile phase. Finally, this mixture was injected in the HPLC and subjected to analysis. The entire USAEME-SFOD procedure is schematically presented in Figure 5.1.

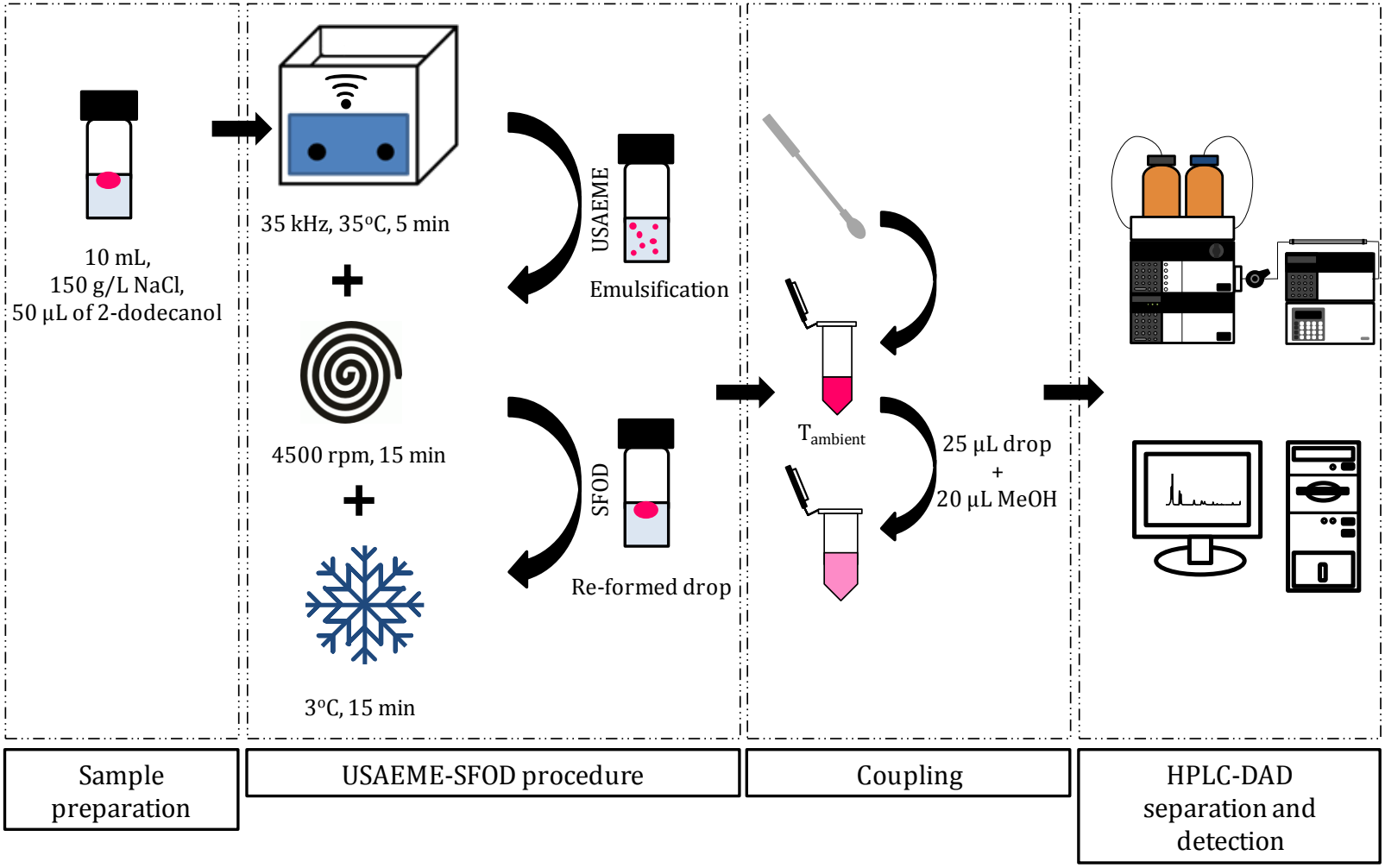


Figure 5.1. Schematic representation of the USAEME-SFOD procedure divided in four steps: Sample preparation, USAEME-SFOD procedure, coupling and HPLC-DAD separation and detection.

4. Results and discussion

The main goal of the present chapter was the development of a new reliable microextraction method for the determination of 18 PAS using HPLC-DAD. Selected PAS were amyl cinnamal, amylcinnamyl alcohol, anise alcohol, benzyl alcohol, benzyl benzoate, benzyl cinnamate, bencyl salicylate, lilial, cinnamal, cinnamyl alcohol, citral, geraniol, hexyl cinnamal, isoeugenol, alpha-isomethyl ionone, limonene, linalool and methyl-2-octynoate.

In the first step, the best conditions for the chromatographic determination of PAS were studied. Once they were selected, best working characteristics of the microextraction process were considered. Once all the method was defined, it was validated and, afterwards, it was applied to different real samples.

4. 1. Optimization of the chromatographic conditions

First of all, as univariate determination was proposed, separation of all the analytes along the chromatogram was necessary. In HPLC instruments, separation is achieved based on the relative affinity of the analytes for the stationary phase (contained in a chromatographic column) and the mobile phase (the eluent). Thus, for obtaining the best separation, different stationary phases, different mobile phases and different elution conditions can be used. However, normally, due to technical reasons, when a method is being developed, the stationary phase is selected from the beginning attending to the type of the analytes wanted to be separated, and then, the other variables are changed until the best conditions are achieved.

In this case, a XDB-C18 column of Agilent was used. This column packing is constituted by a monolayer of dimethyl-n-octadecylsilane stationary phase chemically bonded to a specially prepared ultra-high purity Zorbax Rx-SIL porous silica support, and the bonded-phase packing is doubly endcapped to get the highest deactivation of the silica surface.

In reversed-phase chromatography the stationary phase is non-polar (or weakly polar) and the eluent is more polar. Elution can be isocratic or gradient elution can be used. In isocratic elution a constant solvent mixture is used. In gradient elution, there is a continuous change of solvent composition to increase

the eluent strength. In this case, with the purpose of making the eluent less polar. Commonly, the mobile phase composition is formed by an aqueous phase, which can contain a buffer or not, and an organic solvent. Probably, the most commonly used organic solvents in reversed chromatography are methanol and acetonitrile. In addition to the mobile phase composition, its flow rate can also affect the separation process.

It is remarkable that the first attempt in this work was to separate the 24 regulated PAS corresponding to defined substances. However, complete separation was not achieved. Therefore, it was decided to determine the maximum number of analytes by a univariate method and the remainder by multivariate methods. At the end, 18 analytes were adequate for univariate determination, and the development of the method for determining them is described in this chapter. The other 6 analytes (hydroxycitronellal, coumarin, lyral, eugenol, citronellol and farnesol) were only taken into account for the optimization of the separation process, but their determination will be later explained in Chapter 6. The reason is that they required chemometric tools.

The optimization of the separation process started with the conditions used in a previous work for determination of PAS using HPLC-DAD (Villa *et al.*, 2007). In that work, the initial conditions (flow = 0.7 mL/min; acetonitrile/H_2O composition = 50%/50%) were held for 5 minutes. Then, flow and acetonitrile composition were raised to reach 1.0 mL/min and 60%, respectively, at minute 15. After, these conditions were maintained until minute 24 and, finally, acetonitrile composition was increased to reach 90% at the end of the 40 minutes chromatogram.

In the chromatogram obtained, analytes exiting before minute 15 were not separated with satisfactory resolution. Moreover, further separation by using mobile phases with a higher content of water was considered not adequate as extractants were not totally soluble in them, leading to problems of compatibility between the microextraction process and the detection technique. In consequence, flow was decreased during the first 15 minutes (0.5 mL/min). On the other hand, it was observed that after minute 15, the content of acetonitrile in the mobile phase could be faster increased leading to a shorter chromatogram with still satisfactory resolution. Use of acetate and phosphate buffers was also tried, but they did not affect the separation process, so use of water without buffer was maintained. Use of acetonitrile was also maintained because methanol has a higher cuttoff point which could hinder determination of some analytes. The cutoff point

is defined as the wavelength below which a solvent absorbs strongly and it is 205 nm for methanol and 190 nm for acetonitrile and water (Harris, 2010). The chromatographic conditions which lead to the best achievable separation were summarized in Table 5.3.. As previously said, total separation of the 24 PAS was not achieved but separation was enough to allow precise and accurate determination of 18 PAS.

4. 2. Peak identification and selection of working wavelengths

Data provided by a diode array detector is tridimensional data. Figure 5.2., shows a typical chromatogram obtained using the optimized procedure. As it can be seen, a complete UV-VIS spectrum is obtained at each elution time. For accurate and precise determination of each analyte, the selection of the wavelength of determination according to analyte´s specific characteristics is necessary, but for having a general view of the separation of the analytes, the chromatogram at 200 nm will be frequently showed, as most of the existing coumpounds absorbe at that wavelength. Figure 5.3. shows a chromatogram containing all the PAS measured at 200 nm.

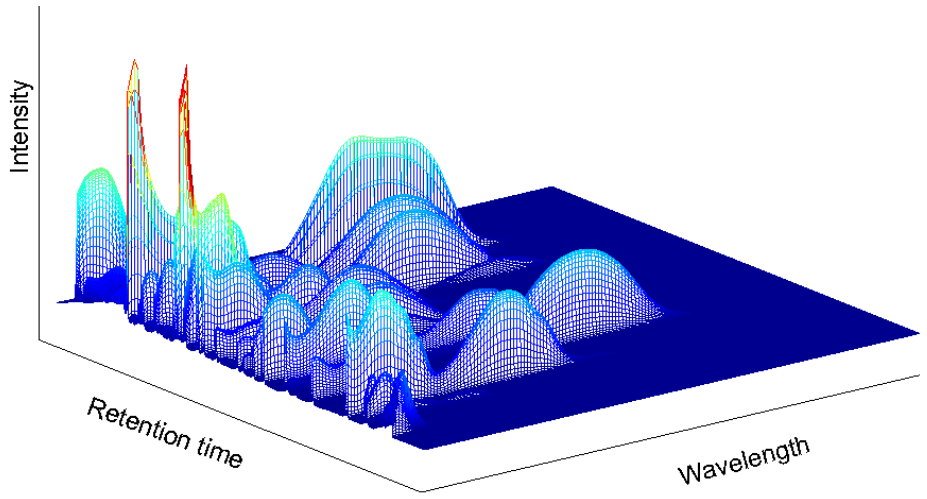


Figure 5.2. Typical 3D Chromatogram obtained for the determination of the 18 PAS using the optimized procedure.

Once separation of the analytes was performed, identification of peaks was carried out by injecting single reference standards and by comparing the retention times and the UV-Vis spectra of each obtained chromatogram with the peaks observed in the chromatogram including all the analytes. The observed PAS sequence and their elution time was the following: anise alcohol (6.5 min), benzyl alcohol (6.8 min), cinnamyl alcohol (8.8 min), cinnamal (12.8 min), isoeugenol (15.6 min), geraniol (18.0 min), linalool (19.2 min), citral (20.4 y 21.0 min), methyl-2-octynoate (23.4 min), amylcinnamyl alcohol (24.8 min), benzyl benzoate (25.5 min), benzyl salicylate (27.5 min), benzyl cinnamate (27.9 min), lilial (30.3 min), amyl cinnamal (31.9 min), alpha-isomethyl ionone (32.3 min), hexyl cinnamal (35.2 min) and limonene (38.3 min).

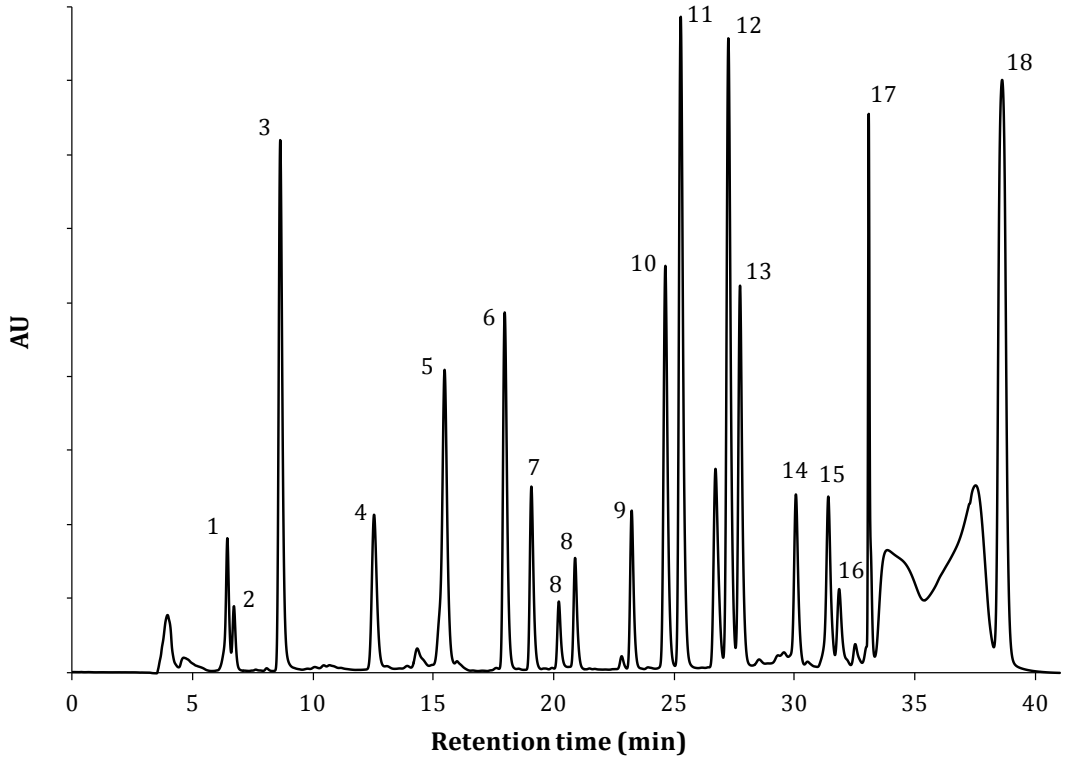


Figure 5.3. HPLC–DAD chromatogram at 200 nm of a standard mixture of PAS using the optimized procedure. Assignation of peaks: 1: anise alcohol; 2: benzyl alcohol; 3: cinnamyl alcohol; 4: cinnamal; 5: isoeugenol; 6: geraniol; 7: linalool; 8: citral; 9: methyl-2-octynoate; 10: amylcinnamyl alcohol; 11: benzyl benzoate; 12: benzyl salicylate; 13: benzyl cinnamate; 14: lilial; 15: amyl cinnamal; 16: alpha-isomethyl ionone; 17: hexyl cinnamal; 18: limonene.

Appropriate wavelength for the determination of each analyte was selected according to spectra characteristics; spectra of all the analytes in elution order is shown in Figure 5.4.. In general, the wavelength at the maximum absorbance and above the cut-off wavelength of the mobile phase is recommended. In this case, mobile phase was composed by water and acetonitrile and both of them have a cut-off wavelength of 190 (Harris, 2010). Therefore, for analytes with the maximum of absorbance near the cut-off point (anise alcohol, benzyl alcohol, cinnamyl alcohol, geraniol, linalool, amylcinammyl alcohol, benzyl benzoate, benzyl salicylate, lilial and limonene) selected suitable wavelength was 200 nm, and for the rest of the analytes: 210 nm for isoeugenol and methyl-2-octynoate, 237 nm for citral and alpha-isomethyl ionone, 276 nm for benzyl cinnamate and 282 nm for hexyl cinnamal, amyl cinnamal and cinnamal. As it can be seen (Figure 5.3.), citral isomers presented two different peaks (peaks 8), and in consequence, the sum of the area of the both peaks at 237 nm was used in the optimization and the quantification of citral.

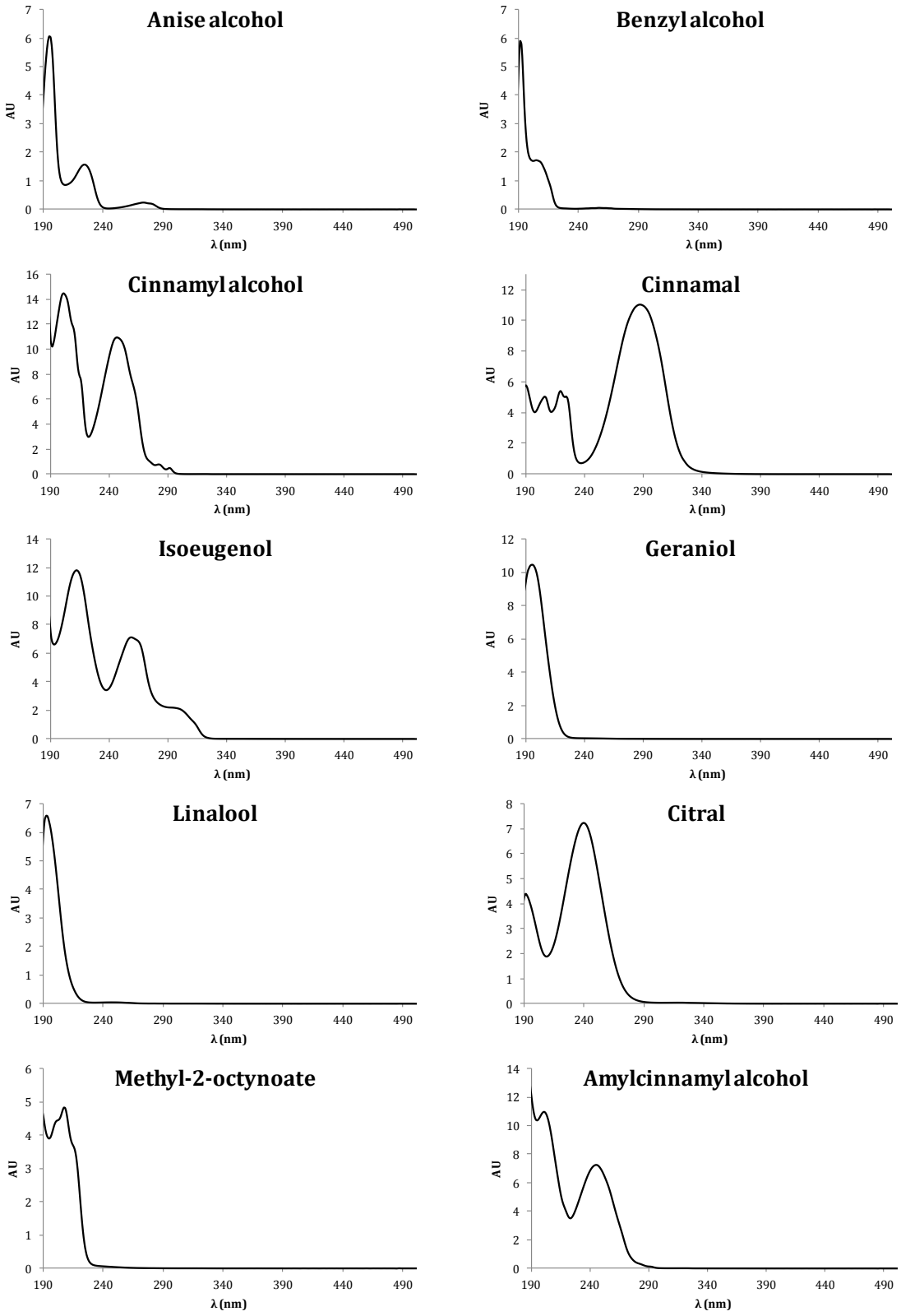


Figure 5.4. UV-Vis spectra obtained from the maximum of each of the peaks in Figure 5.3.

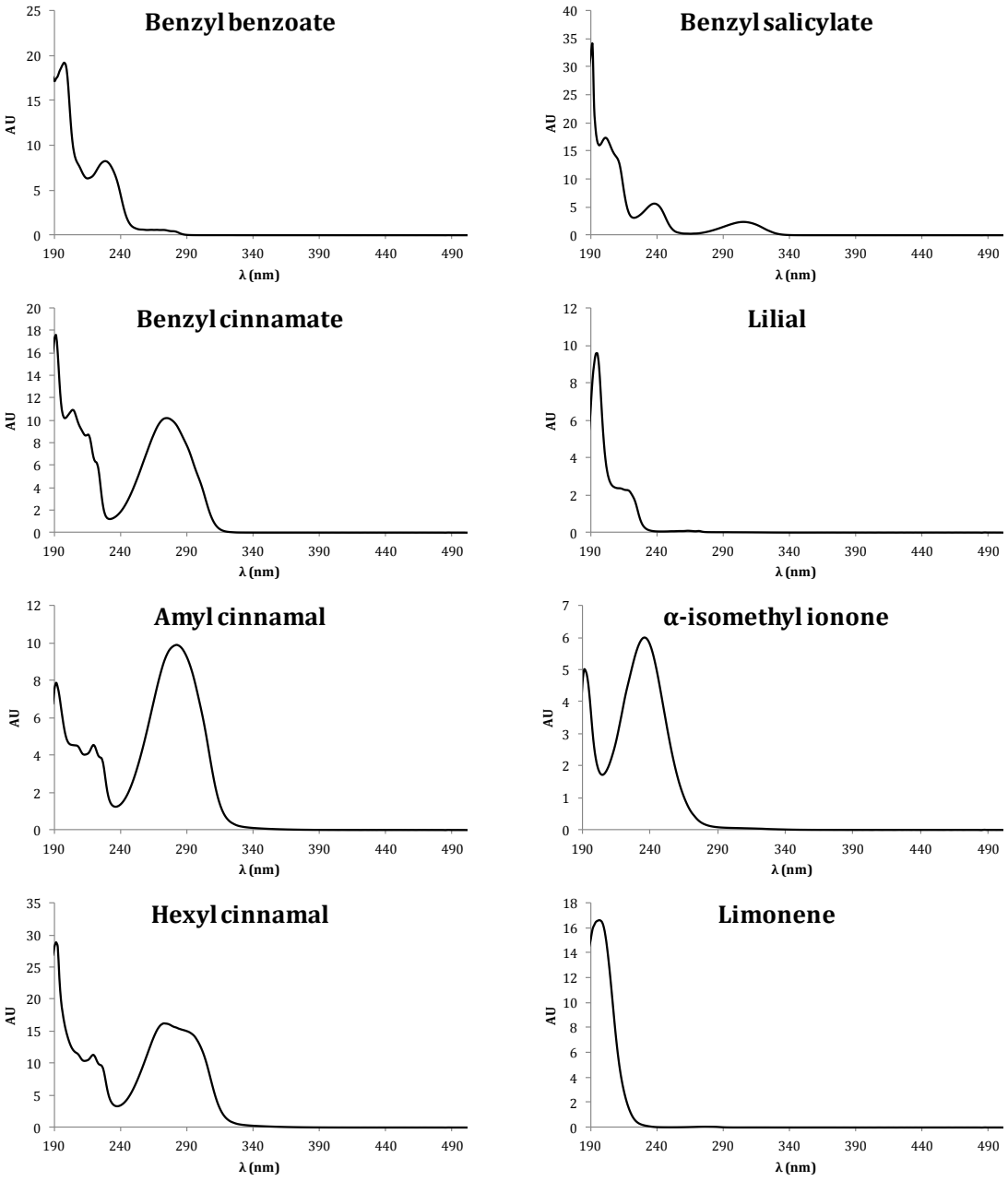


Figure 5.4. (cont). UV-Vis spectra obtained from the maximum of each of the peaks in Figure 5.3.

4. 3. Selection of the most suitable experimental conditions

In order to obtain an appropriate method for the determination of PAS by liquid-phase microextraction followed by HPLC-DAD, the best experimental conditions need to be found.

With this purpose, the influence in the signal of different parameters that could affect the microextraction process was checked using the OVAT approach. The studied parameters were the following: type and volume of extraction solvent, volume of sample, extraction time and temperature and salt addition. A comparison of USAEME-SFOD with other liquid microextraction techniques was also accomplished to guarantee that the best technique was selected. Analysis of variance (ANOVA) was used to compare the signals obtained under different conditions when necessary.

Concentration of all the analytes during the optimization processes was between 0.1 and 2.6 μg/mL depending on the analyte. Initial levels of the different variables were the following: sample volume: 10 mL; extraction solvent: 50 μL; extraction temperature: 30°C; extraction time: 10 min; centrifugation time: 15 min; centrifugation speed: 4500 rpm; drop freezing: 3°C and 15 min; and no salt addition. For the study of one single variable all the runs were performed with fixed levels of the other variables.

4. 3. 1. Selection of extractant

In this chapter, extractants to be used in techniques based on the solidification of the organic drop were chosen due to their lower toxicity and because they provide with an easy way of collecting the drop after extraction. The type of extraction solvent is one of the most important parameters in SFOD based microextractions. Thus, its selection must be carefully done. As it is detailed in the Introduction Chapter (Section 1.3.), the used extractant must satisfy some restrictions: it should have low water solubility and low volatility, its density should be lower than the one of water, and it should present a melting point near room temperature. In addition, it should be able to extract efficiently the analytes and it should exhibit a good behaviour for the chromatographic analysis. Considering all these requirements, 1-undecanol, 1-dodecanol, 2-dodecanol, 1-

bromohexadecane and n-hexadecane were chosen as candidates for being the extraction solvent.

Initially, compatibility of the extracting solvent with the HPLC mobile phase was checked. At this point, 1-bromohexadecane and n-hexadecane were rapidly discarded due to their insolubility in the mobile phase. In consequence, alcohols (1-undecanol, 1-dodecanol and 2-dodecanol) were considered in the following experiments even if 1-dodecanol showed some slight problems of solubility.

Extractions using the different alcoholic extractants were performed under the same conditions. As shown in Figure 5.5., some of the analytes (amyl cinnamal, alpha-isomethyl ionone and hexyl cinnamal) overlapped when 1-undecanol was used and their determination was not possible with this extractant.

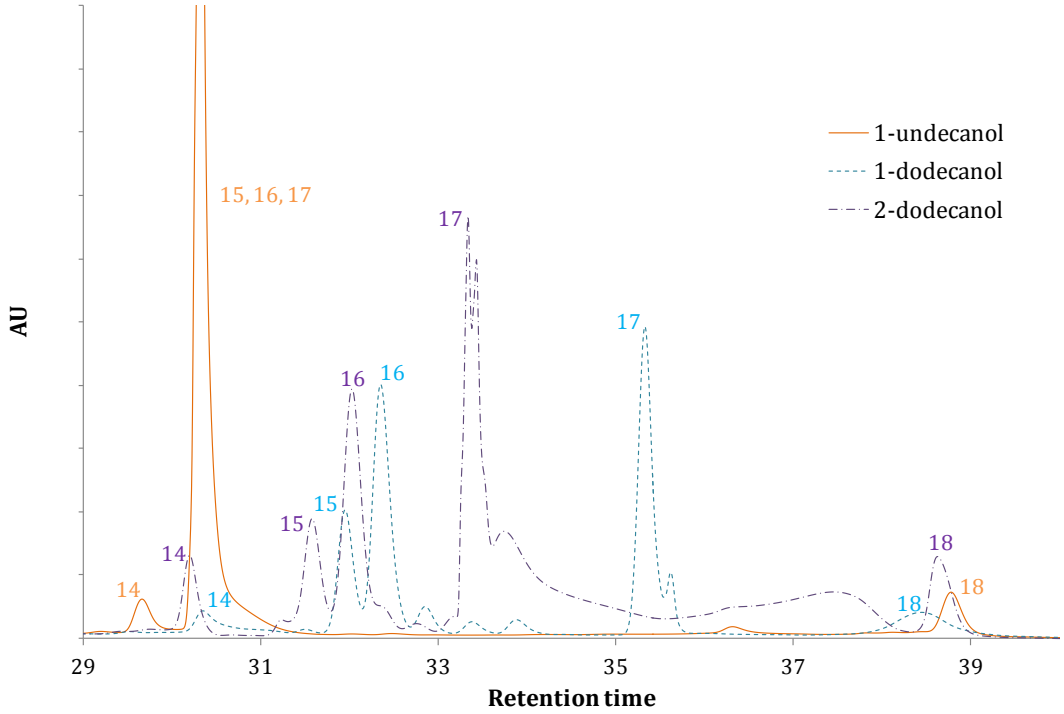


Figure 5.5. HPLC–DAD chromatogram (at 200 nm) obtained after the extraction of the 18 PAS using the different proposed extractants. Assignation of peaks: 14: lilial; 15: amyl cinnamal; 16: alpha-isomethyl ionone; 17: hexyl cinnamal; 18: limonene.

Table 5.4. summarizes obtained results with each extractant in terms of compatibility with HPLC mobile phase and overlapping of the measured analytes. As it can be seen, the unique extractant with proper compatibility with the mobile phase and with no overlapping analytes was 2-dodecanol. Moreover, signals obtained with this extractant were higher than the ones obtained with the other

HPLC compatible extractants. Therefore, further experiments were carried out using 2-dodecanol as extraction solvent.

Table 5.4. Summary of the results obtained with the different extractants.

	Compatibility with HPLC mobile phase	Analytes overlapping with extraction solvent peak
1-Undecanol	✓	Amyl cinnamal, alpha-isomethyl ionone and hexyl cinnamal
1-Dodecanol	~	None
2-Dodecanol	✓	None
1-Hexadecane	***X***	-
1-Bromohexadecane	***X***	-

✓: totally compatible.
~: Slight problems of solubility.
X: not compatible.

4. 3. 2. Comparison with other liquid-phase microextraction techniques

In the Introduction Chapter (Section 1.) a brief general description of the advantages and disadvantages of each type of microextraction technique was presented. As it was explained, dispersants and ultrasonic energy use to help the transfer of the analytes between phases. However, those are general points, and sometimes, depending on the nature of the analytes and its characteristics, a technique can work better or worse than expected. Thus, in this case, before final election of the technique, USAEME was compared with both LPME and DLLME, followed by solidification of the organic drop.

The previously defined microextraction conditions were used in all the techniques to compare. In LPME, slight agitation is necessary in order to help the microextraction process, and a rate of 625 rpm was selected. Moreover, in this technique, centrifugation was not needed. In DLLME, faster agitation and presence of dispersant solvent are necessary in order to achieve the dispersion of the extractant, and 1125 rpm and 1 mL of acetonitrile or ethanol were used. Furthermore, extraction time was 10 min in USAEME and 15 min in LPME and DLLME.

As it is shown in Figure 5.6. in general, DLLME offered better extraction recoveries than LPME. Using USAEME the highest extraction recoveries were obtained for all analytes even when shorter extraction times were used with this

method. This can be explained by the fact that the application of ultrasonic energy facilitates the emulsification phenomenon and accelerates the mass-transfer process that leads to an increment in the extraction efficiency in a very short time (Ghambarian *et al*., 2013).

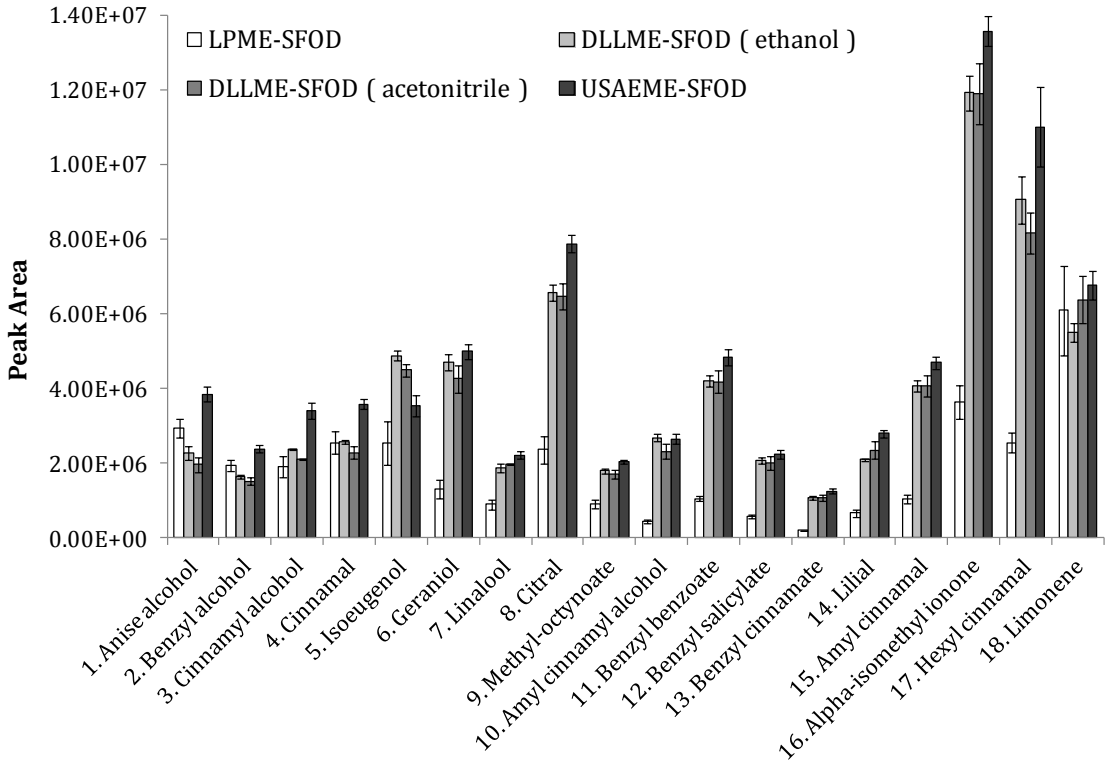


Figure 5.6. Comparison of three techniques: LPME, DLLME and USAEME, all of them followed by SFOD.

4. 3. 3. Selection of centrifugation and cooling conditions

In USAEME-SFOD technique, centrifugation is necessary after extraction in order to recollect the organic phase into a unique organic drop. In general, an efficient and fast centrifugation process is desirable in order to not stretch out the time of the process unnecessarily. Therefore, the fastest centrifugation rate recommended for the centrifuge was selected, that is, 4500 rpm. At these conditions, 15 minutes were necessary to obtain a well-formed organic drop in all the extractions.

After centrifugation, the next step is the freezing of the drop to make possible to collect it easily with a spatula. The cooling temperature was set at 3°C with a thermostatic bath. At this temperature, 15 minutes were necessary for the total freezing of the drop.

In some cases slight agitation can help to the formation of the drop. This agitation must be high enough to avoid the adhesion of the solvent to the walls of the vial, but low enough to not break the drop and to make easy its recollection. In this case, setting up the agitation at 250 rpm, the drop was well formed and easy to collect.

4. 3. 4. Selection of volume of extractant

As explained in the Introduction Chapter (Section 1.3.), volume of the extraction solvent directly affects the extraction efficiency. The reason is that an increase of the extractant volume leads to a higher volume of the organic phase after centrifugation, resulting in a decrease of the concentration of target compounds due to the dilution. Therefore, in general, the lowest volume of the extraction solvent that allows comfortable and reproducible collection of the drop after centrifugation is preferred in order to avoid unnecessary the dilution of the analytes.

In this work, after extraction, the amount of 25 µL of extractant were collected to carry out the measurement. In consequence, higher extractant volumes needed to be considered, as part of the initial solvent is usually lost due to different reasons, such as partial solubilisation and attatchment to the vial walls. Thus, extraction with volumes of 50, 75 and 100 µL of 2-dodecanol were tried to study the effect of the volume of the extraction solvent. As expected (Figure 5.7.), extraction was remarkably better when volume of extractant was lower. Moreover, reproducibility with the lowest studied volume was not worse than with the other volumes. Hence, 50 µL was selected for further experiments.

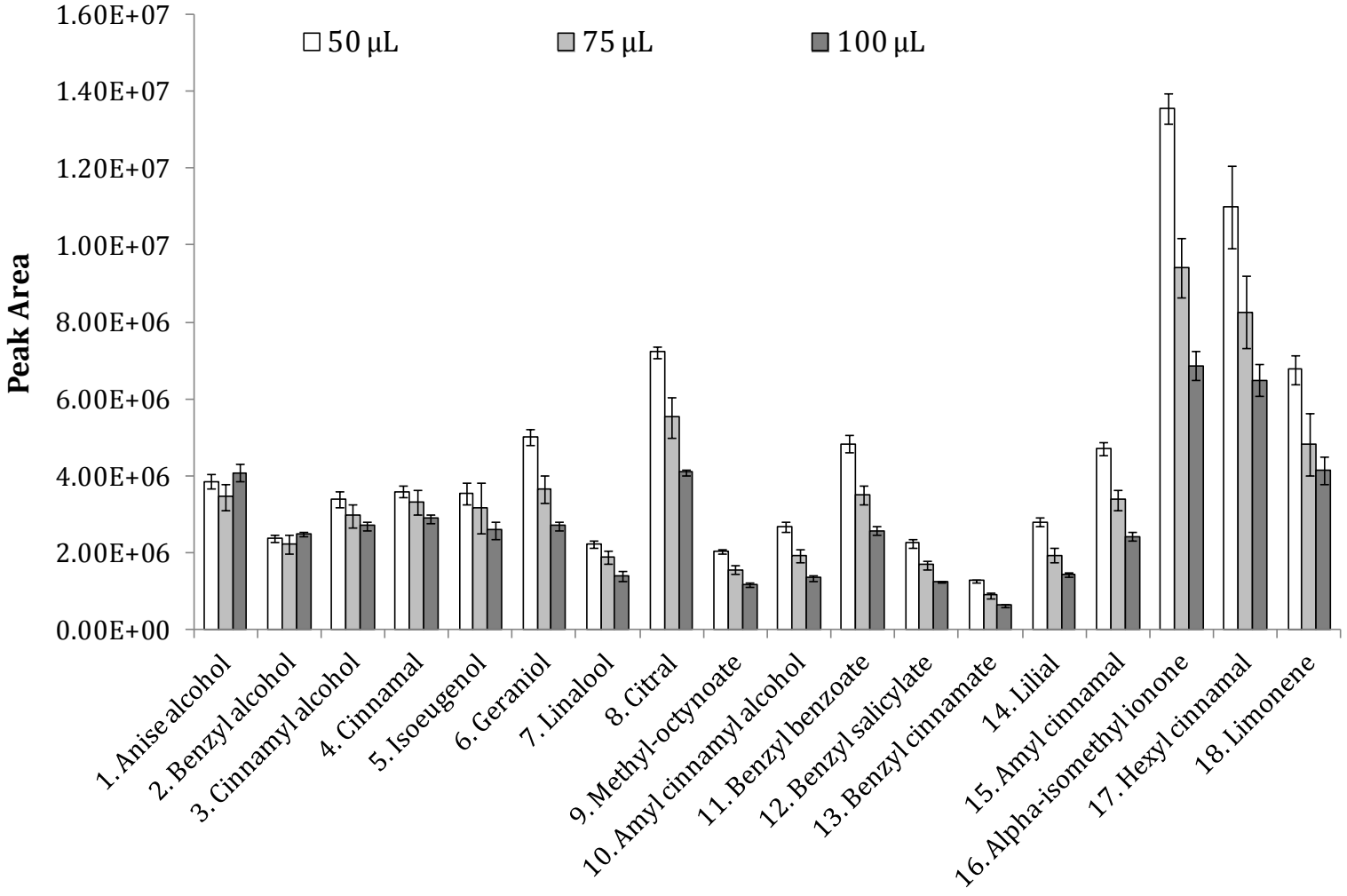


Figure 5.7. Comparison of obtained signals using different volumes of extraction solvent.

4. 3. 5. Selection of sample volume

In general, if same sample concentration is used, higher sample volumes led to higher signals due to the fact that there is more analyte readily to be extracted. However, this volume also affects the extraction efficiency by affecting other characteristics of the process such as the final volume of the organic phase and the way the dispersion is formed. In this case, the effect of using 5, 10 and 15 mL was compared.

Initially, an amount of 50 µL of extractant, as defined in the previous section, was consided. In these conditions, 10 mL of sample gave higher signals than 5 mL, and the determination with 15 mL of sample was not possible because the higher sample volume led to a higher solubilisation of the extraction solvent and the volume of organic phase after extraction was not enough for carrying the measurement. Thus, an amount of 100 µL of extractant was used in order to compare the use of 10 and 15 mL of sample. In this case, it was also observed that the higher the sample volume, the higher the signal. Nevertheless, when 15 mL of sample were used, vials were often broken in the centrifugation process.

In Figure 5.8., all the conditions considered in these experiments are compared together. As it can be clearly seen, the higher signals obtained for almost all the analytes are the ones obtained with 10 mL of sample and 50 µL of extractant. This means that the effect of using a lower volume of extractant is more important than using a higher volume of sample. Thus, 10 mL of sample and 50 µL of extractant was maintained as optimal for further experiments.

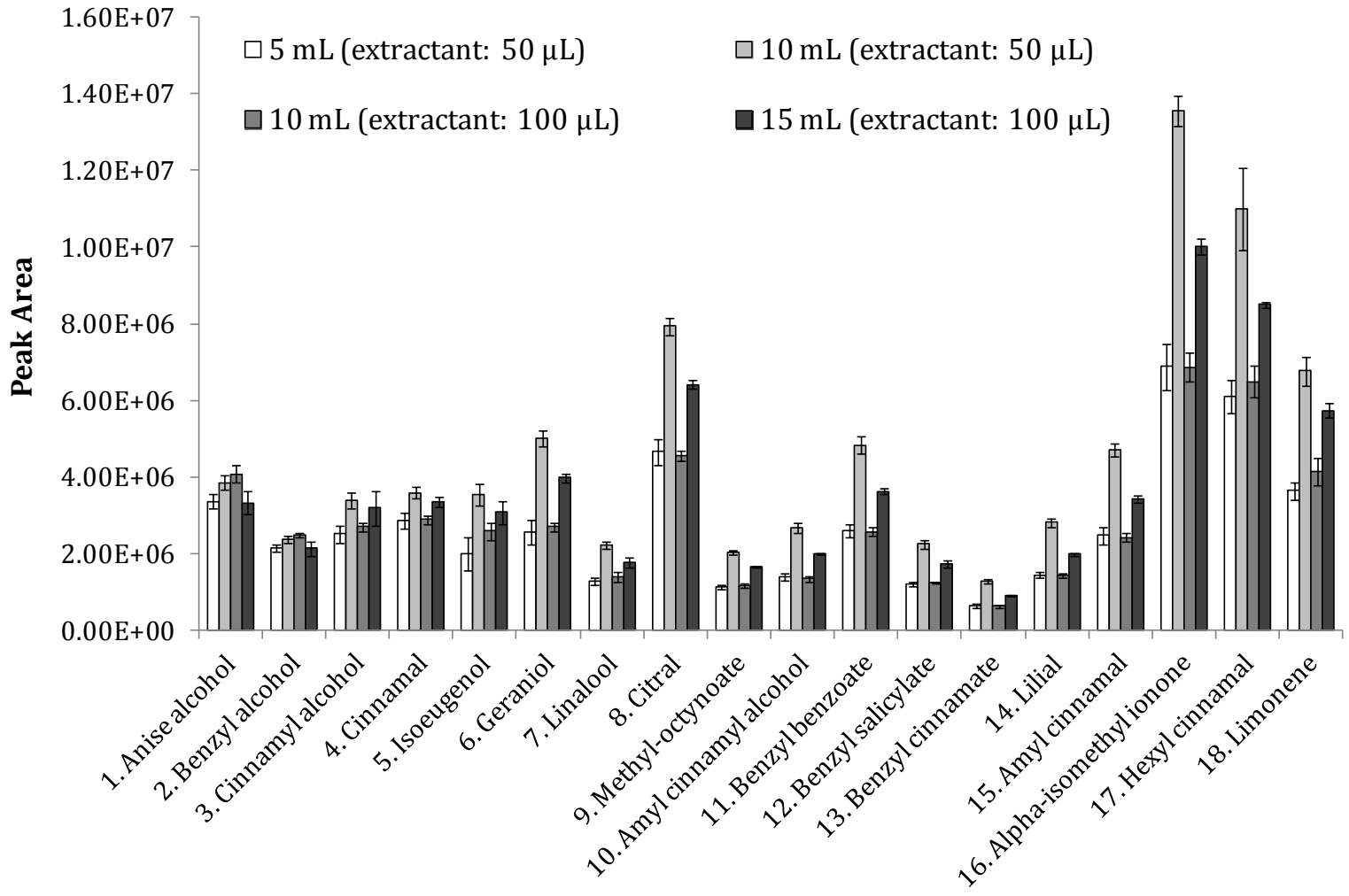


Figure 5.8. Comparison of obtained signals using different volumes of aqueous phase (5, 10 and 15 mL) and different volumes of extractant (50 and 100 µL).

4. 3. 6. Selection of extraction time

Extraction time is a significant variable in LPME methods. These methods are non-exhaustive (they extract only a portion of the analyte in the sample) and, thus, they require a certain period of time to reach the equilibrium between aqueous and organic phases. However, the main advantage of DLLME based-techniques is that they are almost time-independent because of the large superficial area that there is in contact between the extractant and the aqueous phases, which allows the target compounds to diffuse quickly from one phase into the other (Ghambarian *et al.*, 2013).

The effect of the extraction time was evaluated at 1, 5 and 15 min and results are graphically represented in Figure 5.9. In this case, visual evaluation suggested that there was no difference in the signals obtained by using different extraction times.

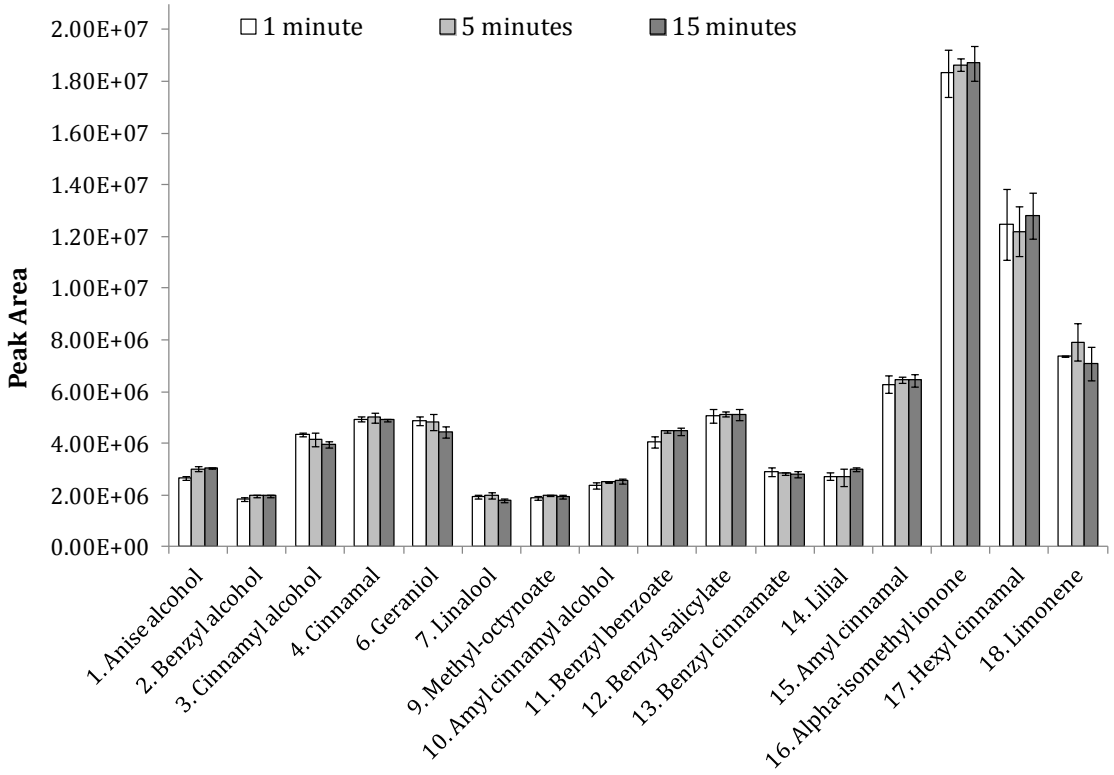


Figure 5.9. Comparison of obtained signals using different extraction times.

In order to confirm this, ANOVA analysis was used. The purpose of ANOVA in this case was to test for significant differences between the mean signals obtained using the different extraction times. ANOVA analysis compares the variance observed between and within the experimental groups and calculates the p-value, which is the probability that the observed difference occurs by pure chance. In many areas the borderline is considered p= 0.05. This means that if a p-value lower than 0.05 is obtained, the variation in the response induced by the effect (of the change in extraction time) is not by chance and it is significant (Hill and Lewicki, 2007).

ANOVA analysis of the results presented in Figure 5.9. was performed and it confirmed that in general there were not statistically significant differences between signals obtained using the three different extraction times. Exceptions were only found when comparing individually signals of anise alcohol (p=0.001) and benzyl benzoate (p= 0.028). In these cases, statistical difference was observed between the signals using 1 and 5 min for both analytes (p=0.006 and p=0.035, respectively) but not between the signals using 5 and 15 min (p=0.630 and p=0.988). These results revealed that thanks to the emulsification formed by ultrasound energy, equilibrium was reached in 1 min for the majority of the analytes, and in only 5 min for all the analytes. Therefore, 5 min was selected for further experiments.

4. 3. 7. Selection of extraction temperature

In general, in most extraction processes, higher temperatures facilitate the diffusion and mass transfer process of the analytes from the sample solution to the extractant, and the time required to reach the equilibrium is decreased. However, at high temperatures solubility of the solvent in the aqueous phase is increased, and, in addition, the possible over-pressurization of the sample vial can make the extraction system unstable. That is why; normally sample solution temperature should not exceed 60^{o}C (Ghambarian *et al*., 2013).

In this work, temperature was examined at 35, 45 and 55°C. Higher temperatures were not recommended to avoid the over-presurization of the vial. Lower temperatures were not practical as it was very difficult to maintain them stable due to the ambient temperature and to the heating effect of the ultrasonic radiation. As in the previous case, visual evaluation suggested that extraction temperature did not affect the obtained signals (Figure 5.10.). In order to confirm this, ANOVA was used. ANOVA results showed that effect of this parameter was not significant except for anise alcohol (p=0.029), where better extraction results were obtained at 35°C. Therefore, 35°C was chosen for further experiments.

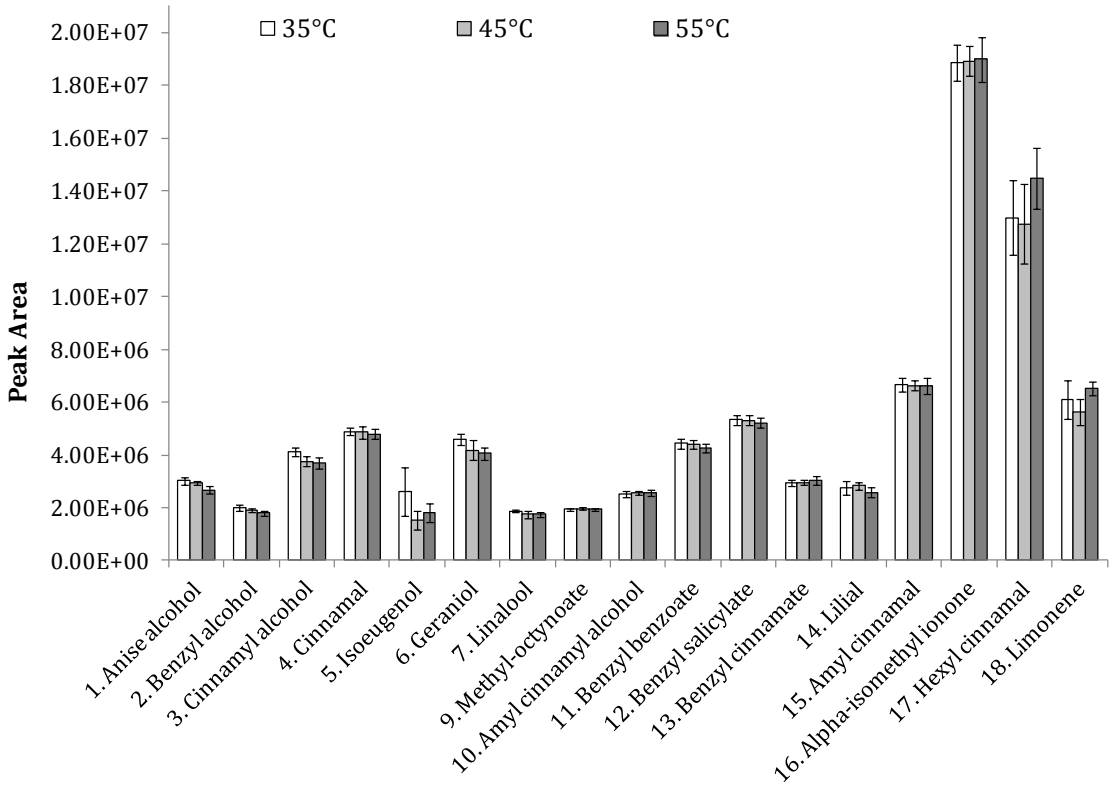


Figure 5.10. Comparison of obtained signals using different extraction temperatures.

4. 3. 8. Selection of salt addition

Addition of salt into the sample solution may have opposite effects on microextraction methods. On the one hand, it can improve the extraction efficiency due to the salting out effect. That is, addition of salt can make the solubility of the analytes in the aqueous phase lower, and, in that way, enhance their partition into the organic phase. But, on the other hand, the presence of high concentrations of salt could change the physical properties of the Nernst diffusion film and

consequently reduce the diffusion rate of the target compounds into the organic phase (Wang *et al*., 2010; Ghambarian *et al*., 2013).

Experiments at salt concentrations of 0, 50, 100 and 150 g/L were performed in order to study the influence of salt addition and results are represented in Figure 5.11. Higher concentrations of salt were not possible because they made difficult the recollection of the organic drop after microextraction.

Graphical and ANOVA analysis showed that salt addition had a significant effect on anise alcohol, benzyl alcohol, cinnamyl alcohol, cinnamal, isoeugenol, geraniol, linalool and citral, and a not significant effect in the rest of the analytes. Moreover, in all the cases where the effect was significant 150 g/L of salt was the level giving higher responses. In consequence, salt addition at a final concentration of 150 g/L was chosen for the final microextraction procedure.

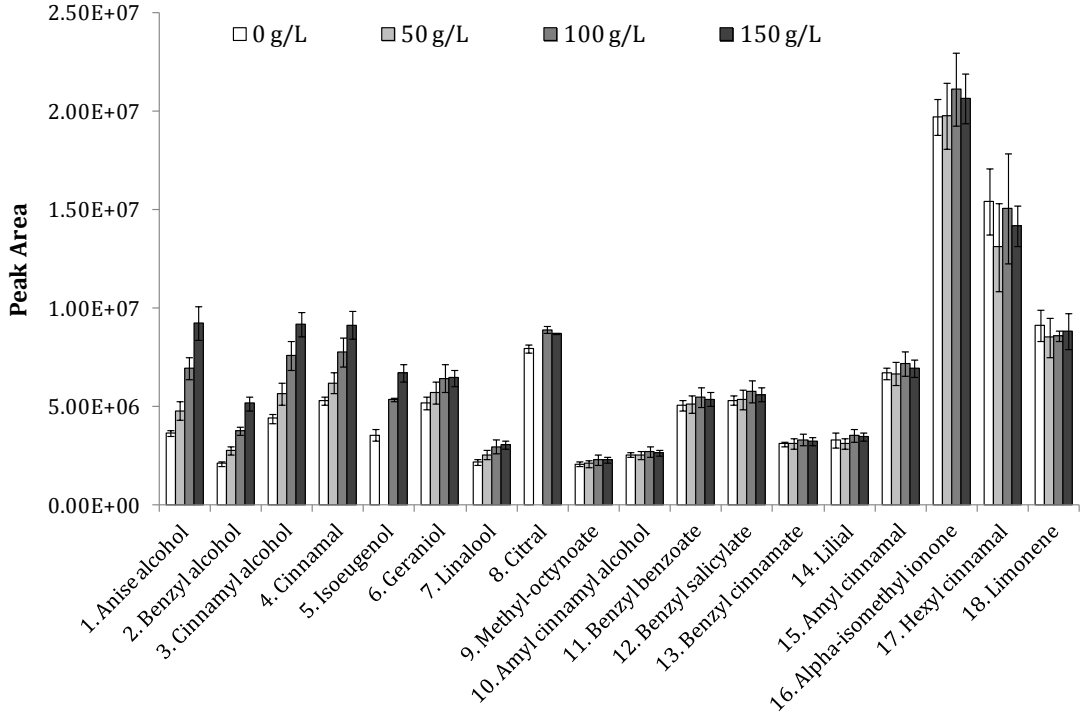


Figure 5.11. Comparison of obtained signals using different salt concentration in aqueos phase.

4. 3. 9. Summary of the most suitable experimental conditions

The following table (Table 5.5.) summarises all the most appropriate extraction conditions to obtain the best response for the determination of the selected PAS by USAEME-SFOD-HPLC-DAD according to the univariate studies.

Table 5.5. The most suitable extraction conditions for PAS determination by USAEME-SFOD-HPLC-DAD.

Variable	Condition
Extraction solvent	
• Type	2-dodecanol
• Volume	50 μL
Centrifugation	
• Time	15 min
• Rate	4500 rpm
Cooling	
• Time	15 min
• Temperature	3°C
• Agitation rate	250 rpm
Extraction	
• Time	5 min
• Temperature	35°C
Sample	
• Volume	10 mL
• Salt addition	150 g/L

4. 4. Method validation

After the development of the method, several studies were carried out in order to verify that the analysis using the developed method was reliable and precise. The evaluated properties were the following: Linearity, limits of detection, limits of quantification, precision and accuracy.

Linearity was studied in similar levels of concentration for all the analytes (0.01-1.00 μg/mL), except for limonene, whose determination was done at a higher order of magnitude (0.50-10.00 μg/mL) due to its higher limit of quantification. Standards at 5 and 10 μg/mL levels were used in preliminary studies but due to saturation they were finally discarded. Standards at concentrations below 0.01 μg/mL were also tried, but they were under the limit of quantification. Calibration curves were constructed using between six and nine levels of concentrations, depending on the limit of quantification, and each determination was performed three times. Table 5.6. shows the calibration characteristics of the developed method for PAS. All analytes showed good linearity in the studied range with squared correlation coefficients ranging from 0.948 to 0.999.

Table 5.6. Analytical figures of merit of the proposed USAEME-SFOD-HPLC-DAD method for PAS determination.

	Analyte	Linear range (μg/mL)	Equation	R^2
1	Anise alcohol	0.10-1.09	Y= 3192875X + 61228	0.988
2	Benzyl alcohol	0.07-1.00	Y= 1583345X + 34862	0.990
3	Cinnamyl alcohol	0.01-0.99	Y= 13176385X + 18467	0.993
4	Cinnamal	0.05-0.96	Y= 16826101X + 6365	0.992
5	Isoeugenol	0.11-1.07	Y= 12961880X - 1149029	0.961
6	Geraniol	0.06-1.14	Y= 7321416X + 643846	0.948
7	Linalool	0.01-0.99	Y= 5019340X + 8963	0.995
8	Citral	0.04-1.01	Y= 9623444X + 182591	0.999
9	Methyl-2-octynoate	0.01-1.08	Y= 4142138X + 37620	0.997
10	Amylcinnamyl alcohol	0.05-1.00	Y= 8665353X + 271962	0.986
11	Benzyl benzoate	0.05-0.99	Y= 15925779X + 1326384	0.979
12	Benzyl salicylate	0.01-1.01	Y= 17274508X + 197427	0.997
13	Benzylcinnamate	0.01-1.00	Y= 11147238X + 33284	0.999
14	Lilial	0.03-0.93	Y= 5350699X - 39553	0.986
15	Amyl cinnamal	0.01-1.02	Y= 11781234X + 226960	0.998
16	Alpha-isomethyl ionone	0.01-0.88	Y= 8816725X + 60633	0.998
17	Hexyl cinnamal	0.19-0.96	Y= 8312463X + 2358829	0.963
18	Limonene	0.48-9.64	Y= 3687318X + 1649868	0.965

In this case, LODs were calculated using 10 samples spiked with analyte concentrations near the expected LOD. The correctness of the concentration of those samples was checked by verifying that it was higher than the concentration of the LOD but lower than ten times the concentration of the LOD. Obtained LODs ranged from 1 to 154 ng/mL. LOQ is frequently calculated as three times the LOD, and in this case values ranged from 4 to 463 ng/mL. LOD and LOQ values for all the analytes are presented in Table 5.7.

Table 5.7. LOD and LOQ for each analyte in the proposed USAEME-SFOD-HPLC-DAD method for PAS determination

	Analyte	LOD (ng/mL)	LOQ (ng/mL)
1	Anise alcohol	26	79
2	Benzyl alcohol	22	65
3	Cinnamyl alcohol	3	8
4	Cinnamal	10	31
5	Isoeugenol	20	61
6	Geraniol	8	23
7	Linalool	2	5
8	Citral	6	17
9	Methyl-2-octynoate	3	8
10	Amylcinnamyl alcohol	9	28
11	Benzyl benzoate	7	21
12	Benzyl salicylate	2	8
13	Benzylcinnamate	1	4
14	Lilial	7	20
15	Amyl cinnamal	3	9
16	Alpha-isomethyl ionone	1	4
17	Hexyl cinnamal	56	168
18	Limonene	154	463

Precision of the method was also evaluated by checking the agreement between the measured values for replicated measurements. According to EURACHEM recommendations, at least 10 independent measurements should be performed for precision assessment and results should be given as standard deviations (EURACHEM, 1998). In this case, precision was evaluated in terms of intra and interday precision; for the first one, 10 measurements were performed in the same day, for the second one, 12 experiments were carried out on three days over two weeks. For both of them, validation was performed at two levels of concentrations.

Results, expressed as RSD in percentages, ranged between 3.3 and 12.4% for intraday precision and 3.9 and 14.4% for interday precision and are represented in Table 5.8.

Table 5.8. Precision data obtained applying the proposed USAEME-SFOD-HPLC-DAD method for PAS determination.

	Compound	Intraday precision (RSD, %, n=10)		Interday precision (RSD, %, n=12)	
		Low[a]	High[b]	Low[a]	High[b]
1	Anise alcohol	8.7	4.7	8.2	5.1
2	Benzyl alcohol	12.3	4.0	11.6	4.2
3	Cinnamyl alcohol	6.0	4.1	7.9	4.2
4	Cinnamal	6.6	3.3	7.7	4.0
5	Isoeugenol	12.4	10.7	7.6	14.4
6	Geraniol	5.3	4.5	8.4	3.9
7	Linalool	4.6	3.7	5.7	6.0
8	Citral	4.3	3.3	6.3	4.5
9	Methyl-2-octynoate	4.3	3.9	5.8	4.9
10	Amylcinnamyl alcohol	3.7	4.1	6.9	4.2
11	Benzyl benzoate	6.3	4.3	7.6	4.7
12	Benzyl salicylate	5.7	4.1	6.0	5.8
13	Benzylcinnamate	5.7	3.9	6.2	5.8
14	Lilial	3.3	6.0	7.3	5.4
15	Amyl cinnamal	4.0	3.6	4.9	4.9
16	Alpha-isomethyl ionone	3.4	3.7	4.5	5.0
17	Hexyl cinnamal	4.1	4.4	4.3	4.5
18	Limonene	6.1	7.9	4.7	6.8

[a]Low level: around 0.2 μg/mL for anise alcohol and isoeugenol, around 0.3 μg/mL for hexyl cinnamal, around 0.7 μg/mL for limonene and around 0.08 μg/mL for the rest of the analytes

[b]High level: around 8 μg/mL for limonene and around 0.8 μg/mL for the rest of the analytes.

Acceptable values for precision of a given analytical method according to AOAC recommendations are the ones presented in Table 5.9. (AOAC international, 2012) and accordingly precision results were considered satisfactory. The highest RSD values for almost all the analytes were below 10%, which is considered more than acceptable according to Table 5.9. Exceptions were only benzyl alcohol and isoeugenol with values of 12.3 and 14.4, but still in the range of the tabulated acceptable values.

Table 5.9. Expected precision values for a tested method according to AOAC.

Analyte fraction	Unit	RSD (%)
10^{-5}	10 ppm	7.3
10^{-6}	***1 ppm***	***11***
10^{-7}	***100 ppb***	***15***
10^{-8}	***10 ppb***	***21***
10^{-9}	1 ppb	30

In bold concentration levels used in this method.

Recovery studies were carried out in order to evaluate the accuracy of the entire procedure in different matrices. With that purpose two different samples were spiked with a known content of analyte, and the observed concentration value was compared with the real one. The two selected real samples were baby bath water (BBW) and *eau de cologne* (EC). In EC, extractions were performed dissolving approximately 1 g of each sample in 50 mL, and in the case of BBW, no dilution was necessary. Acceptable values for recovery of a given analytical method also according to AOAC recommendations are the ones presented in Table 5.10.

Table 5.10. Expected recovery values for a tested method according to AOAC.

Analyte fraction	Unit	Recovery (%)
10^{-5}	10 ppm	80-110
10^{-6}	***1 ppm***	***80-110***
10^{-7}	***100 ppb***	***80-110***
10^{-8}	10 ppb	60-115

In bold concentration levels used in this method

Recovery values (Table 5.11.) were also considered adequate. In general values from 90 to 110% were obtained, which are inside the acceptable limits. Exceptions were isoeugenol (138%), amylcinnamyl alcohol (121%), benzyl benzoate (128%) and lilial (112%) in EC and isoeugenol (154%), geraniol (125%) and lilial (136%) in BBW. In both matrices, values of RSDs remained satisfactory.

Table 5.11. Recovery data obtained applying the proposed USAEME-SFOD-HPLC-DAD method for PAS determination.

	Compound	Recovery in EC sample (n=3)		Recovery in BBW sample (n=3)	
		ER, %	RSD, %	ER, %	RSD, %
1	Anise alcohol	93	8.1	98	9.0
2	Benzyl alcohol	98	11.1	106	11.1
3	Cinnamyl alcohol	109	6.1	110	8.3
4	Cinnamal	90	1.9	94	3.8
5	Isoeugenol	138	4.7	154	7.9
6	Geraniol	104	7.4	125	5.3
7	Linalool	105	1.6	110	6.2
8	Citral	104	2.8	98	2.6
9	Methyl-2-octynoate	110	6.4	100	1.1
10	Amylcinnamyl alcohol	121	8.1	109	0.8
11	Benzyl benzoate	128	7.1	108	0.7
12	Benzyl salicylate	109	5.9	96	1.2
13	Benzylcinnamate	103	5.5	99	1.5
14	Lilial	112	3.6	136	3.4
15	Amyl cinnamal	99	5.4	99	0.8
16	Alpha-isomethyl ionone	108	7.6	97	1.0
17	Hexyl cinnamal	95	13.9	90	2.6
18	Limonene	100	15.7	97	5.1

ER: Extraction recovery; RSD: relative standard deviation.

Although enrichment factor (EF) is not normally considered a validation parameter, in microextraction processes it is usually calculated to give an idea of the extent of the microextraction process. In this chapter, it was calculated as the ratio between analyte concentration in the organic phase after extraction and the initial concentration of the analyte within the water sample. Results are expressed in Table 5.12. and they show EFs from 9 to 237 depending on the analyte.

Table 5.12. Enrichment factors obtained applying the proposed USAEME-SFOD-HPLC-DAD method for PAS determination.

	Compound	EF
1	Anise alcohol	9
2	Benzyl alcohol	11
3	Cinnamyl alcohol	43
4	Cinnamal	39
5	Isoeugenol	118
6	Geraniol	103
7	Linalool	117
8	Citral	117
9	Methyl-2-octynoate	220
10	Amylcinnamyl alcohol	190
11	Benzyl benzoate	197
12	Benzyl salicylate	226
13	Benzylcinnamate	194
14	Lilial	149
15	Amyl cinnamal	203
16	Alpha-isomethyl ionone	237
17	Hexyl cinnamal	183
18	Limonene	122

After all the validation process, the performance of the proposed USAEME-SFOD-HPLC-DAD method was compared with some other reported methods for PAS determination in different matrices. Table 5.13. summarizes the general characteristics of the developed method and the ones found in the literature.

As it can be seen, the best LODs were obtained with SPME followed by GC using MS detector (Lamas *et al.*, 2009). To date, GC with MS has been the most sensitive and used technique, and it has been coupled to different sample pre-treatment procedures lowering the LODs of the technique (Becerril *et al.*, 2010; Becerril-Bravo *et al.*, 2010; Tsiallou *et al.*, 2012; Godayol *et al.*, 2015). However, other less sensitive techniques for determination of these compounds have emerged as an alternative (Cordero *et al.*, 2007; Furlanetto *et al.*, 2010; Lopez-Gazpio *et al.*, 2014), HPLC–DAD being one of them (Villa *et al.*, 2007). This technique coupled to a preconcentration method, as the proposed USAEME-SFOD, provides similar detection limits to those methods that use GC–MS without a sample pretreatment technique (Cordero *et al.*, 2007; Sánchez-Prado *et al.*, 2011B).

Table 5.13. Comparison of the presented work with other methods reported in the literature.

	Matrix	Nº of PAS	R^2	LOD (ng/mL)	RSD (%)	Recovery (%)	Reference
GC-MS	Perfumes	24	0.996-1.000	0.26-29	0.38-7.7	89 -106	Sanchez-Prado *et al.*, 2011B
Fast-GC-MS	Fragrances	24	> 0.999	0.8-8.8 (mg/kg)	0.1-5.0		Mondello *et al.*, 2007
GC-MS/MS	Toys	24	0.985-1.000	0.02-5.00(mg/kg)	3.1-13.4	80-107	Lv *et al.*, 2013
GCxGC-MS	Fragrances	24	0.973-1.000	1-10			Cordero *et al.*, 2007
GCxGC-FID	Fragrances	24	0.984-1.000	4-8	0.51-10.61		Cordero *et al.,* 2007
MSPD-GC-MS	Cosmetics	24	0.999-1.000	1-60	0.2-10	75-118	Sanchez-Prado *et al.*, 2011A
SPME-GC-MS	Baby bath water	14	0.993-1.000	0.001-0.300	0.4-21	74 -124	Lamas *et al.*, 2009
HS-SPME-GC-MS	Water	24	0.994-1.000	0.001-1.100	0.6-17	73-136	Becerril *et al.*, 2010
HS-SPME-GC-MS	Wastewater treatment plants	15	0.985-0.999	0.01-1.70	4-33	40-100	Godayol *et al.*, 2015
SPD-PLE-GC-MS	Leave on cosmetics	24	0.996-0.999	0.83-25	0.2-9.7	85 -114	Lamas *et al.,* 2010B
DLLME-GC-MS	Water	21	0.981-0.996	0.007-1.000	3-16	29 -112	Tsiallou *et al.*, 2012
USAEME-GC-MS	Water	24	0.997-1.000	0.006-1.15	0.2-23	18 -116	Becerril-Bravo *et al.*, 2010
MEEKC-DAD	Commercial rinse-off products	18	0.990-0.999	-	0.5-6.0	93-107	Furlanetto *et al.*, 2010
MEKC-DAD	Personal care products	8	0.994-0.999	1150-11040	2.4-19.2	89-115	Lopez-Gazpio *et al.*, 2014
HPLC-DAD	Commercial scented products	24	0.990-1.000	20 – 10880	0.6-3.5	90-105	Villa *et al.*, 2007
USAEME-SFOD-HPLC-DAD	Water and cosmetic samples	18	0.948-0.999	1.3 -154.3	3.3-14.4	90-145	**This work** (Pérez-Outeiral *et al.*, 2015)

NOTE: The explanation of the methods abbreviations is included in the abbreviation list.
R^2: correlation coefficients; LOD: Limit of detection; RSD: Relative standard deviation.

Also in some studies with sample pre-treatment procedures, other than microextraction, prior to GC-MS analysis, similar LODs have been obtained (Lamas *et al.*, 2010B; Sanchez-Prado *et al.*, 2011A).

Recovery studies in the literature were made in both different and analogous matrices. Comparison of obtained recovery data with results of similar matrices was acceptable. Moreover, regression coefficients and relative standard deviation ranges found in this method are also similar to the ones found in the literature.

4. 5. Application to real samples

Finally, the developed method was applied to the determination of PAS in several real samples. These samples included two different samples of water taken from two different bathtubs after the bath of two babies (BBW1 and BBW2), water from a local swimming pool (SPW), one body milk (BM), one *eau de cologne* (EC), one *eau de parfum* (EP) and four different *eau de toilette*s (ET1, ET2, ET3, ET4). Given the wide range of PAS concentration even inside the same sample, different dilutions were applied to each sample to be able to determine all the present analytes. Dilutions of cosmetics ranged from 0.0029 g to 1.0077 g in 50 mL. In the case of waters no dilution was applied.

In SPW and in BBW1 no analyte of interest was found. In BBW2 presence of anise alcohol was observed but this could not be quantified as it was under LOQ. Table 5.14. shows the results of quantitative analysis in the EC, EP and BM samples and Tables 5.15 and 5.16. show the results for the different ETs. As it is observed some analytes were not found in the samples.

Table 5.14. Concentration (mean of triplicates) of PAS in different real samples.

		EC		EP		BM	
		C (mg/kg)	RSD (%)	C (mg/kg)	RSD (%)	C (mg/kg)	RSD (%)
2	Benzyl alcohol	n.d.	n.d.	212±11	5.2	n.d.	n.d.
3	Cinnamyl alcohol	n.d.	n.d.	n.d.	n.d.	67±5	7.5
6	Geraniol	98±3	3.2	5626±344	6.1	253±14	5.5
7	Linalool	323±10	3.0	2467±371	15.0	272±14	5.1
8	Citral	966±38	3.9	n.d.	n.d.	7±1	14.3
11	Benzyl benzoate	n.d.	n.d.	32±3	9.3	n.d.	n.d.
12	Benzyl salicylate	n.d.	n.d.	4±1	24.0	54±1	1.8
14	Lilial	n.d.	n.d.	193±10	5.2	67±11	16.8
16	Alpha-isomethylionone	n.d.	n.d.	592±47	7.9	31±1	3.3
18	Limonene	n.d.	n.d.	n.d.	n.d.	219±9	4.1

C: concentration; RSD: relative standard deviation; n.d.: not detected.

Table 5.15. Concentration (mean of triplicates) of PAS in ET1 and ET2 .

		ET1		ET2	
		C (mg/kg)	RSD (%)	C (mg/kg)	RSD (%)
2	Benzyl alcohol	n.d.	n.d.	16±1	6.2
3	Cinnamyl alcohol	n.d.	n.d.	n.d.	n.d.
6	Geraniol	n.d.	n.d.	1607±55	3.4
7	Linalool	329±5	1.5	9094±219	2.4
8	Citral	n.d.	n.d.	286±39	13.6
11	Benzyl benzoate	172±14	8.1	n.d.	n.d.
12	Benzyl salicylate	n.d.	n.d.	n.d.	n.d.
14	Lilial	n.d.	n.d.	n.d.	n.d.
18	Limonene	2291±236	10.3	5456±334	6.1

C: concentration; RSD: relative standard deviation; n.d.: not detected.

Table 5.16. Concentration (mean of triplicates) of PAS in ET3 and ET4.

		ET3		ET4	
		C (mg/kg)	RSD (%)	C (mg/kg)	RSD (%)
2	Benzyl alcohol	n.d.	n.d.	1002±71	7.1
3	Cinnamyl alcohol	n.d.	n.d.	2.1±0.1	4.8
6	Geraniol	n.d.	n.d.	n.d.	n.d.
7	Linalool	258±17	6.5	2751±7	0.6
8	Citral	n.d.	n.d.	n.d.	n.d.
11	Benzyl benzoate	n.d.	n.d.	292±30	10.3
12	Benzyl salicylate	n.d.	n.d.	206±15	7.3
14	Lilial	1288±154	11.9	894±31	3.5
18	Limonene	n.d.	n.d.	n.d.	n.d.

C: concentration; RSD: relative standard deviation; n.d.: not detected.

As example, Figures from 5.12. a) to e) show typical chromatograms for BBW2, BM, EC, EP and ET2, respectively. In the different samples, depending on the analyte to quantify, a different dilution was required.

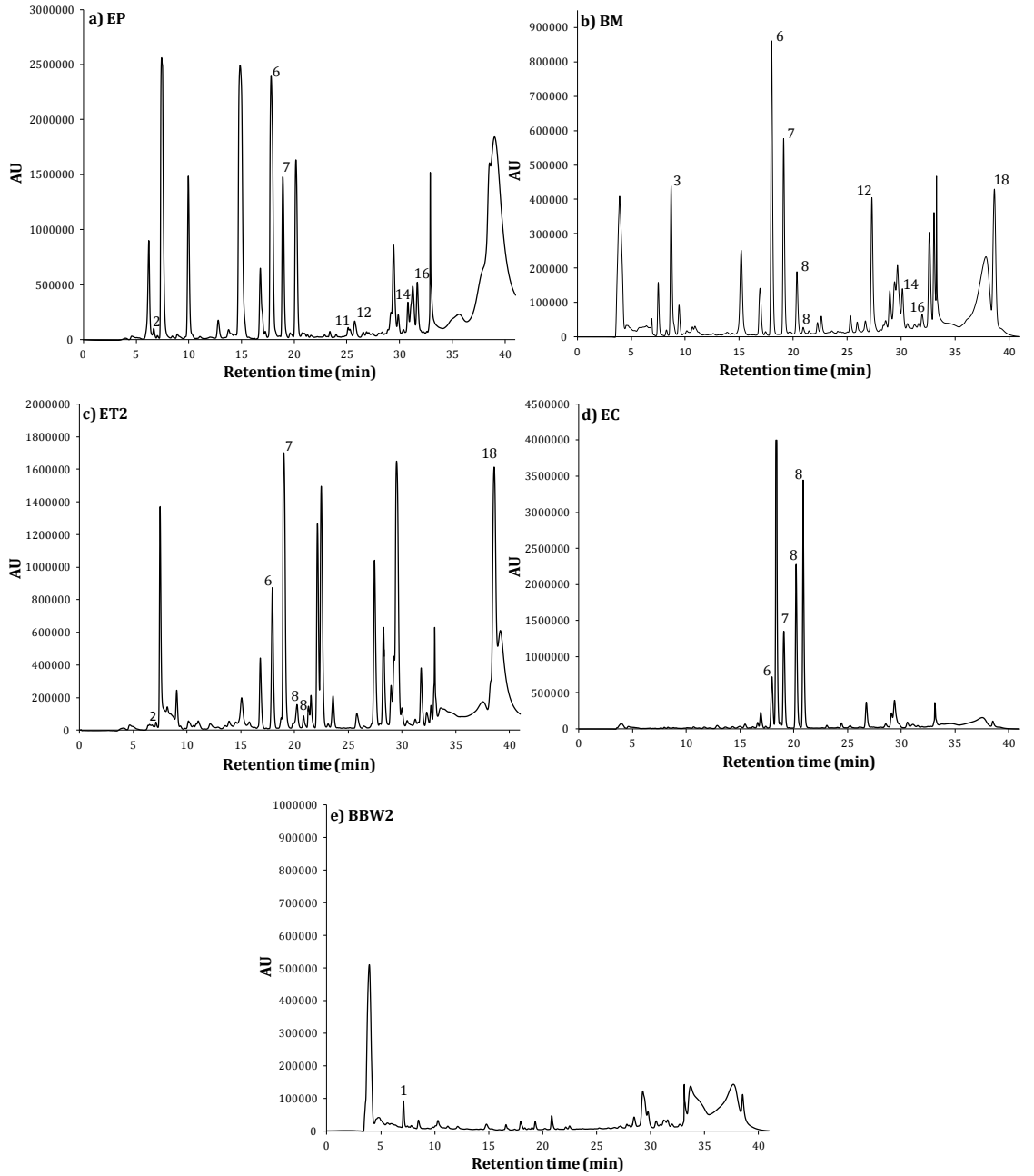


Figure 5. 12. Sample chromatogram at 200 nm corresponding to a) EP, b) BM, c) ET2, d) EC and e) BBW2. Analytes present in the samples: 1: anise alcohol; 2: benzyl alcohol; 3: cinnamyl alcohol; 6: geraniol; 7: linalool; 8: citral; 11: benzyl benzoate; 12: benzyl salicilate; 14: lilial; 16: alpha-isomethylionone and 18: limonene.

As it can be observed, benzyl alcohol, cinnamyl alcohol, geraniol, linalool, citral, benzyl benzoate, benzyl salicilate, lilial, alpha-isomethyl ionone and limonene were the analytes found in the analyzed cosmetic samples. Between them, linalool was the most commonly found PAS. Indeed, it was found in all the analyzed cosmetics. Geraniol and lilial are also common, being present in more than in the half of the cosmetic samples. It is remarkable that values above and below the ones required in the European Union Regulation were found in the cosmetics, but presence of PAS was correctly informed in the label when required.

On the other hand, the presence of PAS in SPW and BBW was not significant, probably due to the dilution of the cosmetics in the high volume of water. Therefore, it cannot be concluded that swimming pool and bathtubs are important routes of exposure to these substances.

The most important difficulty in the application of the method to real cosmetic samples was that in some cases problems in the formation of the drop after extraction were observed, probably due to the presence in the cosmetics of other types of ingredients. However, these difficulties were successfully solved by further dilution when necessary. Although this causes a decrease in the amount of analyte that the method is able to detect in each sample, in this method LODs were enough for analyte quantification at the required levels even with the necessary dilutions.

5. Conclusions

In this chapter, a method based on USAEME-SFOD-HPLC-DAD was developed for simultaneous determination of 18 fragrance allergens in cosmetic and water samples. Previously only a method had been developed for quantification of PAS using HPLC–DAD, and it did not include any sample preparation technique. In this study, sample preparation has been accomplished by USAEME-SFOD, which has been found to be an efficient, environmentally friendly and non-expensive technique that allows decreasing LODs of the determination technique spreading out its applications.

The procedure has been developed using the OVAT approach with statistical tests when necessary. In this way, the optimum levels of the different variables affecting the determination process have been found and the method has been performed under the best possible conditions.

The developed method has been evaluated in terms of linear range, LOD, LOQ, precision and accuracy. All the checked characteristics gave satisfactory results.

Finally, the proposed method has been successfully applied to the analysis of several real samples, where the presence of 11 analytes out of 18 has been detected. Levels of analytes below and above the levels included in the current legislation have been found, but they were specifically indicated when necessary.

As a general conclusion, the proposed method is competitive and valid for checking compliance with current legislation in the levels needed for the studied 18 PAS.

The main drawback of the method was the impossibility to apply it to the 24 PAS corresponding to defined substances. Nevertheless, it was valid for 18 out of the 24 PAS. Chemometrics will be used in Chapter 6 with the objective of determining the six remaining PAS (hydroxycitronellal, coumarin, lyral, eugenol, citronellol and farnesol).

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7. PUBLISHED PAPER

ULTRASOUND-ASSISTED EMULSIFICATION MICROEXTRACTION COUPLED WITH HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE SIMULTANEOUS DETERMINATION OF FRAGRANCE ALLERGENS IN COSMETICS AND WATER.

Pérez-Outeiral, Jessica, Millán, Esmeralda, Garcia-Arrona, Rosa.

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Jessica Pérez-Outeiral
Esmeralda Millán
Rosa Garcia-Arrona

Department of Applied Chemistry, Faculty of Chemistry, University of the Basque Country (UPV/EHU), San Sebastian, Spain

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Research Article

Ultrasound-assisted emulsification microextraction coupled with high-performance liquid chromatography for the simultaneous determination of fragrance allergens in cosmetics and water

A simple, inexpensive, and environmentally friendly method based on ultrasound-assisted emulsification microextraction followed by solidification of floating organic drop and high-performance liquid chromatography coupled to diode array detection was developed for the simultaneous determination of 18 potentially allergenic fragrance substances. Several parameters affecting the microextraction process were investigated in detail by the "one-variable-at-a-time" approach. Optimal conditions were the following: 50 μL of 2-dodecanol as extraction solvent, 10 mL of sample containing 150 g/L of salt, and 5 min of sonication at 35°C. Under the optimized conditions, method showed good linearity in the selected ranges, with squared correlation coefficients ranging from 0.948 to 0.999. Limits of detection ranged from 0.001 to 0.154 μg/mL and enrichment factors from 9 to 237. Precision of the method, expressed as relative standard deviation, was checked at two levels obtaining good results (3.3–14.4%). Recovery studies were made in baby bath water and in eau de cologne showing acceptable accuracy. Finally, the developed method was successfully applied to different commercial cosmetic and water samples. The most commonly found analyte was linalool followed by cinnamal and lilial. Most of the analyzed samples contained at least one of the target compounds.

Keywords: Cosmetics / Fragrance allergens / High-performance liquid chromatography / Ultrasound-assisted microextraction / Water
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1 Introduction

Fragrances are important components of many formulations present in our daily life such as perfumes, cosmetics, personal care items, household, and laundry products. Although the fragrances are generally innocuous, they can sometimes cause skin irritation or allergic reactions. In 1999, the Scientific Committee on Cosmetic Products and Non-Food Products intended for consumers identified a list of 24 fragrance related substances with a well-recognized potential to cause allergy [1]. In 2000, two natural moss extracts were also added to the list [2]. Currently, the above-mentioned 24 compounds and the two natural moss extracts are known in literature as potentially allergenic substances (PAS). In 2003, the European Union published an amendment to the Annex III of its Cosmetic Directive in which requires the declaration of the above-mentioned PAS on product labels if concentration is >0.001% in leave-on products and 0.01% in rinse-off products [3]. The main route of exposition to these ingredients is the direct application of cosmetics in the skin, but contact with contaminated water must be also considered. Thus, nowadays interest is not being focused only in cosmetic samples but also in water samples.

It is necessary to develop reliable analytical methods for quantification of PAS. Sample preparation is a critical step in analytical methods as it has direct impact on their quality and sensitivity. Recent research trends in sample preparation include miniaturized extraction procedures based on classical extraction techniques. These new techniques are generally simpler, faster, and use fewer amounts of solvents than the traditional ones.

LPME emerged as a solvent-minimized version of the classic LLE in which only several microliters of a water-immiscible solvent (extractant) are put in contact with an aqueous phase (sample) to extract the compounds of interest. From LPME introduction, different approaches have

Correspondence: Dr. Rosa Garcia-Arrona, Department of Applied Chemistry, Faculty of Chemistry, University of the Basque Country (UPV/EHU), P.O. Box 1072, 20080 San Sebastian, Spain
E-mail: rosa.garcia@ehu.es

Abbreviations: ANOVA, analysis of variance; **BBW**, baby bath waters; **BM**, body milk; **DAD**, diode array detection; **DLLME**, dispersive liquid–liquid microextraction; **EC**, eau de cologne; **EF**, enrichment factor; **PAS**, potentially allergenic substances; **SFOD**, solidification of floating organic drop; **SPW**, swimming pool water; **USAEME**, ultrasound-assisted emulsification microextraction

been developed, which can be classified into three main categories: single drop microextraction, dispersive liquid–liquid microextraction (DLLME), and hollow-fiber liquid microextraction [4, 5].

DLLME was presented by Rezaee et al. [6]. In this technique, dispersion of the extractant is achieved by the addition of a third solvent, miscible with both phases, known as dispersant. As a result, a cloudy solution is formed and the superficial area in contact between the two phases is larger and, thus, extraction is faster. After the extraction, samples are centrifuged to separate the two phases. DLLME has several advantages: simplicity, efficiency, low sample volume, low cost, and high enrichment factor (EF). However, DLLME has also a disadvantage, the necessity of using a disperser solvent. Ultrasound-assisted emulsification microextraction (USAEME) emerged to solve this problem by the use of ultrasound radiation for generating the emulsion and accelerating the mass-transfer process [7].

Although volume of extraction solvents is diminished in LPME, extraction solvents used are still generally toxic. In the last years, there has been a thorough attempt to improve this aspect within the green chemistry framework [8]. Khalili-Zanjani et al. proposed a new approach of LPME based on the solidification of floating organic drop (SFOD) [9]. In this method, an extractant with lower density than water, low toxicity, and proper melting point (in the range of 10–30°C) is used. After extraction, organic droplet is solidified in an ice bath and then, it is easily collected with a spatula, melted and finally conducted to analytical determination. Leong et al. used this type of solvents in DLLME, developing a new method called DLLME based on SFOD [10]. Finally, USAEME-SFOD was introduced by Kamarei et al. [11]. This method possesses all the advantages of both USAEME and SFOD; analyte extraction is very efficient and fast due to the high superficial area between phases, it is an environmentally friendly technique as it only uses low volumes of practically nontoxic solvents and, in addition, it is simple and leads to high EFs.

Analytical methods for the determination of PAS are mainly based on GC–MS [12–18]. This technique has been coupled in several studies to sample pretreatment techniques, including matrix solid-phase dispersion [19] SPME [20], headspace (HS) SPME [21, 22], solid-phase dispersion pressurized liquid extraction [23], DLLME [24] and USAEME [25]. Other determination techniques such as microemulsion electrokinetic chromatography [26], MEKC [27], attenuated total reflectance IR spectroscopy [28], and HPLC [29] have been also used for determination of PAS, but in those cases no pretreatment technique was used.

The aim of the present work was to develop a simple, low cost, and reliable analytical method for simultaneous determination of eighteen PAS using USAEME-SFOD as a preconcentration technique followed by HPLC coupled to diode array detection (DAD). The use of a preconcentration technique, as USAEME-SFOD, could improve the sensitivity of the method and, in consequence, spread out its applications. Also, HPLC–DAD is a very widespread instrumentation in most routine analysis laboratories; and therefore, this option could be a good alternative to other instrumental techniques. The main parameters influencing extraction and determination of PAS were considered in detail by "one-variable-at-a-time" approach. Then, the optimized procedure was validated and applied to the determination of these fragrance allergens in different water and cosmetic samples.

2 Materials and methods

2.1 Reagents and materials

Anisyl alcohol (98%), benzyl alcohol (99%), cinnamyl alcohol (98%), cinnamal (95%), isoeugenol (98%), geraniol (98%), linalool (97%), citral (96%), methyl-2-octynoate (99%), benzyl benzoate (99%), benzyl salicylate (99%), benzyl cinnamate (99%), lilial (98%), amyl cinnamal (97%), α-isomethyl ionone (85%), hexyl cinnamal (95%), and limonene (96%) were purchased from Sigma–Aldrich (Barcelona, Spain). Amylcinnamyl alcohol (45.5%) was supplied by Dr. Ehrenstorfer (Augsburg, Germany).

Acetonitrile (HPLC grade) was obtained from Romil (Cambridge, UK). 2-Dodecanol (99%), 1-undecanol (99%), *n*-hexadecane (99%) and 1-bromohexadecane (97%) were acquired from Sigma–Aldrich (Madrid, Spain). 1-Dodecanol (98%) was supplied by Panreac (Barcelona, Spain). Methanol was obtained from Panreac. Doubly distilled water was used throughout this work.

Individual stock solutions of PAS were prepared in acetonitrile and stored in amber-colored vials at –23°C. Working solutions were prepared weekly by dilution of the stock ones with acetonitrile and also preserved in the freezer and warmed at room temperature before use.

Four eau de toilettes, one eau de cologne (EC), one eau de perfume, and one body milk (BM) were purchased from local shopping center. The swimming pool water (SPW) was obtained from a local swimming pool. The two baby bath waters (BBW) were taken from bathtubs after babies had bathed. All samples were stored in glass bottles.

2.2 Instrumentation and chromatographic conditions

Chromatographic analyses were performed with a LC-20AD system equipped with a SPD-M20A Diode Array Detector (Shimadzu Corporation, Duisburg, Germany) and a manual sample injector (20 μL injection volume). Data were collected and processed using LC Solution (2.1 version) software. Separations were carried out using XDB-C_{18} column (250 × 4.6 mm, 5 μm) from Agilent (Wilmington, DW, USA) at ambient temperature.

Gradient elution with water and acetonitrile was used for separation of target compounds. The initial mobile phase was held for 6.5 min with 50% acetonitrile, followed by an increase to 60% acetonitrile from 6.5 to 15 min and after that, to reach 80% acetonitrile from 15 to 32 min. The acetonitrile

composition was raised to 90% from 32 to 32.5 min and kept for 1 min. Finally, the acetonitrile composition was decreased for 0.5 min to reach the starting conditions again. These conditions were maintained from 34 to 41 min to obtain the last peaks and homogenize the system. Regarding the flow, it was maintained at 0.5 mL/min for 15 min, from 15 to 16 min was raised to 1 mL/min and, lastly, it was kept until the end. UV-Vis spectra were recorded from 190 to 800 nm.

Extractions were carried out in a Bandelin Sonorex Digitec DT100H ultrasound bath (ALLPAX, Papenburg, Germany) with 35 kHz ultrasound frequency. Centrifugation was performed on a Selecta centrifuge (Barcelona, Spain). The cooling bath was a Julabo F26 (Augsburg, Germany).

2.3 USAEME-SFOD procedure

First, 10 mL of a 150 g/L solution of NaCl was placed in a 40 mL glass vial and a mixed solution of fragrance allergens standards was spiked. Then, 50 μL of 2-dodecanol (extraction solvent) was added to the solution, and it was gently shaken by hand and placed into the ultrasonic bath for sonication at 35 kHz and 35 ± 1°C during 5 min. As a result, emulsion of extraction solvent in water was formed. Emulsion was then disrupted by centrifugation at 4500 rpm for 15 min and the vial was placed into the thermostatic bath at 3°C for 15 min. The solidified organic drop was then collected with a spatula and transferred to an Eppendorf vial where it melted at room temperature. Once melted, 25 μL of the organic phase was collected and mixed with 20 μL of methanol to make it compatible with the HPLC mobile phase. Finally, this mixture was injected in the HPLC and subjected to analysis.

Given the wide range of concentrations in which the compounds appear in samples, convenient dilutions were made when required.

3 Results and discussion

3.1 Peak identification and selection of working wavelengths

Identification of peaks was carried out by comparing the retention times and the UV-Vis spectra of each peak with those of the single reference standards. The PAS sequence and elution time was anisyl alcohol (6.5 min), benzyl alcohol (6.8 min), cinnamyl alcohol (8.8 min), cinnamal (12.8 min), isoeugenol (15.6 min), geraniol (18.0 min), linalool (19.2 min), citral (20.4 and 21.0 min), methyl-2-octynoate (23.4 min), amylcinnamyl alcohol (24.8 min), benzyl benzoate (25.5 min), benzyl salicylate (27.5 min), benzyl cinnamate (27.9 min), lilial (30.3 min), amyl cinnamal (31.9 min), α-isomethyl ionone (32.3 min), hexyl cinnamal (35.2 min), and limonene (38.3 min). Working wavelength for determination of each analyte was selected according to spectra characteristics. A real chromatogram at 200 nm obtained injecting a standard mixture of the allergens is shown in Fig. 1A. This wavelength, 200 nm, was the working wavelength for the most of the analytes except for isoeugenol (210 nm), methyl-2-octynoate (210 nm), citral (237 nm), α-isomethyl ionone (237 nm), benzyl cinnamate (276 nm), hexyl cinnamal (282 nm) amyl cinnamal, and cinnamal (282 nm).

3.2 Selection of working characteristics

To obtain the best experimental conditions for determination of PAS by USAEME-SFOD along with HPLC–DAD, the influence of different parameters in the obtained signal was tested using the "one-variable-at-a-time" approach. A comparison of USAEME-SFOD with other liquid microextraction techniques was also accomplished. To compare signals, analysis of variance (ANOVA) was used. Concentration of the analytes in the experiments used to fix the experimental conditions was between 0.1 and 2.6 μg/mL depending on the analyte.

3.2.1 Selection of extraction solvent

Selection of an appropriate extraction solvent is important in the optimization process of a microextraction method based on the solidification of organic drop. The extraction solvent should satisfy the following requirements: It should be immiscible with water and have low volatility, low density, and present a melting point near to room temperature. It should also be able to extract the target analytes, and, in the case of chromatography, its peaks should be well separated from those of the analytes [30, 31]. Taking into account these characteristics, 1-undecanol, 1-dodecanol, 2-dodecanol, 1-bromohexadecane, and *n*-hexadecane were chosen as extraction solvents.

First, the compatibility of the extracting solvent with the HPLC mobile phase was checked. 1-Bromohexadecane and *n*-hexadecane were discarded due to their insolubility in some of the compositions of the HPLC mobile phase. Hence, only the alcohols were considered in the following experiments. 1-Dodecanol gave slight problems of solubility, the peak of 1-undecanol overlapped with peaks of amyl cinnamal and α-isomethyl ionone, making their determination not possible, and 2-dodecanol gave higher and better results. Therefore, further experiments were carried out considering 2-dodecanol as the extraction solvent.

3.2.2 Comparison with other liquid microextraction techniques

USAEME was compared with both LPME and DLLME, all of them followed by solidification of the organic drop. In all runs, the following extraction conditions were considered: concentrations of PAS, between 0.1 and 2.6 μg/mL; sample volume, 10 mL; extraction solvent, 2-dodecanol; volume of extraction solvent, 50 μL; extraction temperature 30°C; and no salt addition. For LPME followed by SFOD, 625 rpm agitation during extraction was used. For DLLME followed by SFOD,

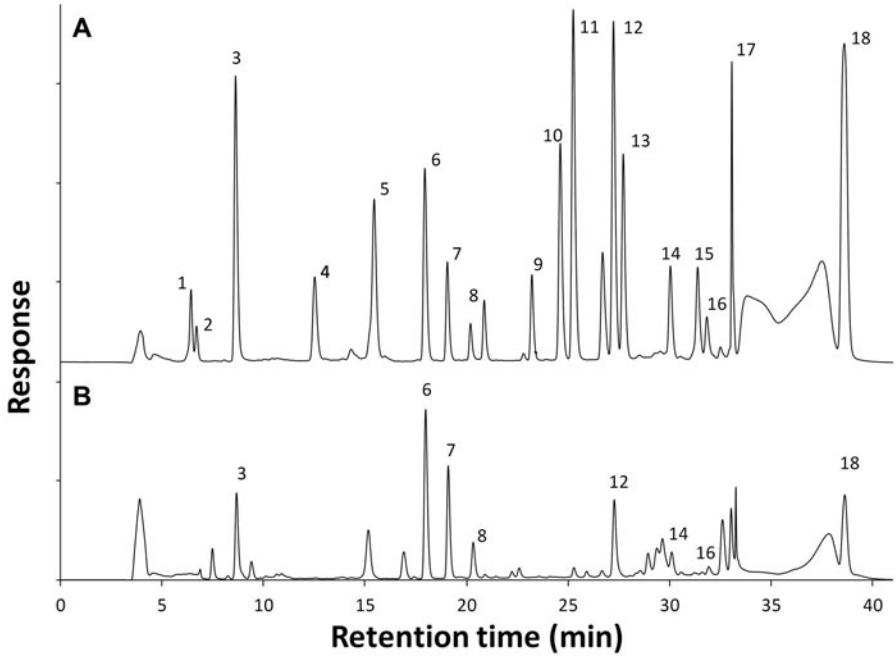

Figure 1. HPLC–DAD chromatograms at 200 nm of (A) the standard mixture of PAS and (B) of BM real sample using the optimized procedure. Assignation of peaks: (1) anisyl alcohol, (2) benzyl alcohol, (3) cinnamyl alcohol, (4) cinnamal, (5) isoeugenol, (6) geraniol, (7) linalool, (8) citral, (9) methyl-2-octynoate, (10) amylcinnamyl alcohol, (11) benzyl benzoate, (12) benzyl salicylate, (13) benzyl cinnamate, (14) lilial, (15) amyl cinnamal, (16) α-isomethyl ionone, (17) hexyl cinnamal, (18) limonene. Chromatogram B is an example of sample chromatograms at 200 nm, for determination of all the analytes using different dilutions.

1 mL of acetonitrile or ethanol was used as dispersants. The solidification conditions (3°C cooling temperature for 15 min) were similar in all cases. For DLLME and USAEME centrifugation was necessary after extraction (10 min at 4500 rpm). Extraction time was 10 min in USAEME and 15 min in LPME and DLLME. As it is shown in Fig. 2A, in general, DLLME offered better extraction recoveries than LPME. Using USAEME, the highest extraction recoveries were obtained for all analytes even when shorter extraction times were used with this method. The application of ultrasonic energy facilitates the emulsification phenomenon and accelerates the mass-transfer process that leads to an increment in the extraction efficiency in a very short time [31].

3.2.3 Selection of centrifugation and cooling conditions

These experimental conditions were fixed according to previous experience in our laboratory [32]. Centrifugation rate and time were the minimum that allowed collecting the cloudy solution into a sedimented drop (4500 rpm, 15 min). Cooling conditions were fixed to obtain a well-formed solidified drop (3°C, 15 min, and 250 rpm of agitation).

3.2.4 Selection of volume of extractant

An increase of the extractant volume leads to a higher volume of the organic phase after centrifugation, resulting in a decrease of the concentration of target compounds due to the dilution [30, 31]. In this work, extractions with volumes of 50, 75, and 100 μL of 2-dodecanol were carried out. As expected, it was observed that extraction was remarkably better when volume of extractant was lower, as it can be seen in Fig. 2B, Hence 50 μL was selected as the lower volume that allowed the collection of the drop after centrifugation.

3.2.5 Selection of the extraction time

Extraction time is a significant variable in LPME methods, as these procedures require a period of time to reach the equilibrium between aqueous and organic phases. Nevertheless, extraction time is not as important in DLLME technique. The reason is that this type of technique is almost time-independent due to the large superficial area between extractant and sample, which allow the target compounds to diffuse quickly into the organic phase [31]. The extraction time was evaluated at 1, 5, and 15 min. ANOVA showed that taken into account the signals of all the analytes at a time, there were no significant differences between signals obtained after different extraction times. These results revealed that owing to the emulsification formed by ultrasound energy, equilibrium was reached in only 5 min for all the analytes. Therefore, 5 min was selected for further experiments.

3.2.6 Selection of the extraction temperature

In general, in LPME, higher temperatures facilitate the diffusion and mass transfer of the analytes from sample solution to the extractant, and time required to reach the equilibrium is decreased. However, at high temperatures, solubility of the organic solvent increases and the possible over-pressurization of the sample vial could make the extraction system unstable; normally, sample solution temperature should not exceed 60°C [31]. In this work, temperature was examined at 35, 45, and 55°C. ANOVA results showed that effect of this parameter was not significant except for anisyl alcohol, where better

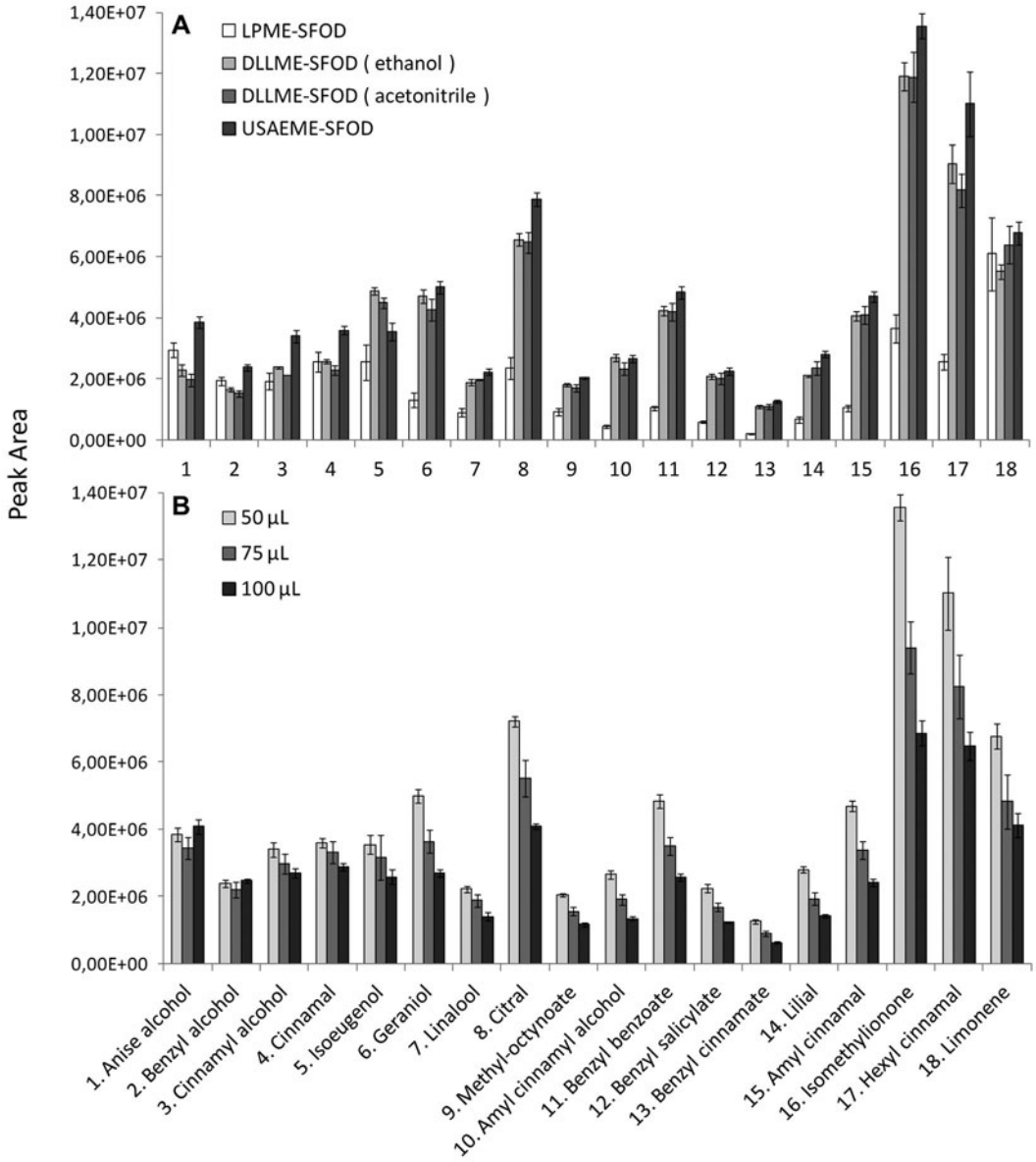

Figure 2. (A) Comparison of three techniques: LPME, DLLME, and USAEME, all of them followed by SFOD. Extraction conditions are expressed in Section 3.2.2. (B) Comparison of obtained signals using different volumes of extraction solvent. Other extraction conditions were the ones explained before.

extraction results were obtained at 35°C. Therefore, 35°C was chosen for further experiments.

3.2.7 Selection of salt addition

Addition of salt into the sample solution may have several effects on microextraction methods. On the one hand, it can improve the extraction efficiency due to the salting-out effect. But, on the other hand, the presence of higher concentrations of salt could change the physical properties of the Nernst diffusion film and thus reduce the diffusion rate of the target compounds into the organic phase [30, 31]. To investigate the influence of ionic strength, experiments at salt concentrations of 0, 50, 100, and 150 g/L were performed. ANOVA showed that addition of salt was a significant parameter. Looking closely at the results, it was observed that in the most of analytes higher extraction efficiency was reached at 150 g/L of salt. Thus, 150 g/L of salt was chosen.

3.3 Method validation

Analytical figures of merit of the proposed method were obtained under optimal conditions and summarized in Table 1. All analytes showed good linearity in the studied range with squared correlation coefficients (R^2) ranging from 0.948 to

Table 1. Analytical figures of merit of the proposed USAEME-SFOD-HPLC-DAD method for PAS determination

	Compound	Linear range (μg/mL)	R^2	LOD (μg/mL)	LOQ (μg/mL)	EF	Intraday precision (RSD, %) Low[a)]	Intraday precision (RSD, %) High[b)]	Interday precision (RSD, %) Low[a)]	Interday precision (RSD, %) High[b)]	Recovery (EC) (%)	Recovery (BBW) (%)
1	Anisyl alcohol	0.10–1.09	0.988	0.026	0.079	9	8.7	4.7	8.2	5.1	93	98
2	Benzyl alcohol	0.07–1.00	0.990	0.022	0.065	11	12.3	4.0	11.6	4.2	98	106
3	Cinnamyl alcohol	0.01–0.99	0.993	0.003	0.008	43	6.0	4.1	7.9	4.2	1090	110
4	Cinnamal	0.05–0.96	0.992	0.010	0.031	39	6.6	3.3	7.7	4.0	90	94
5	Isoeugenol	0.11-1.07	0.961	0.020	0.061	118	12.4	10.7	7.6	14.4	138	154
6	Geraniol	0.06–1.14	0.948	0.008	0.023	103	5.3	4.5	8.4	3.9	104	125
7	Linalool	0.01–0.99	0.995	0.002	0.005	117	4.6	3.7	5.7	6.0	105	110
8	Citral	0.04–1.01	0.999	0.006	0.017	117	4.3	3.3	6.3	4.5	104	98
9	Methyl-2-octynoate	0.01–1.08	0.997	0.003	0.008	220	4.3	3.9	5.8	4.9	110	100
10	Amylcinnamyl alcohol	0.05–1.00	0.986	0.009	0.028	190	3.7	4.1	6.9	4.2	121	109
11	Benzyl benzoate	0.05–0.99	0.979	0.007	0.021	197	6.3	4.3	7.6	4.7	128	108
12	Benzyl salicylate	0.01–1.01	0.997	0.002	0.008	226	5.7	4.1	6.0	5.8	109	96
13	Benzylcinnamate	0.01–1.00	0.999	0.001	0.004	194	5.7	3.9	6.2	5.8	103	99
14	Lilial	0.03–0.93	0.986	0.007	0.020	149	3.3	6.0	7.3	5.4	112	136
15	Amyl cinnamal	0.01–1.02	0.998	0.003	0.009	203	4.0	3.6	4.9	4.9	99	99
16	α-Isomethyl ionone	0.01–0.88	0.998	0.001	0.004	237	3.4	3.7	4.5	5.0	108	97
17	Hexyl cinnamal	0.19–0.96	0.963	0.056	0.168	183	4.1	4.4	4.3	4.5	95	90
18	Limonene	0.48–9.64	0.965	0.154	0.463	122	6.1	7.9	4.7	6.8	100	97

a) Low level is around 0.2 μg/mL for anysil alcohol and isoeugenol, around 0.3 μg/mL for hexyl cinnamal, around 0.7 μg/mL for limonene, and around 0.08 μg/mL for the rest of analytes.
b) High level is around 8 μg/mL for limonene and around 0.8 μg/mL for the rest of analytes.

Table 2. Comparison of the presented work with other methods reported in the literature

	Matrix	Number of PAS	R^2	LOD (ng/mL)	RSD (%)	Recovery (%)	Reference
GC-MS	Perfumes	24	0.996–1.000	0.26–29	0.4–7.7	89–106	13
GC-MS	Fragrances	24	>0.990	0.8–8.8 (mg/Kg)	0.1–5.0		14
GC-MS/MS	Toys	24	0.9850–0.9999	0.02–5.00(mg/Kg)	3.1–13.4	80–107	15
GC × GC-MS	Fragrances	24	0.9734–0.9996	1–10			17
GC × GC-FID	Fragrances	24	0.9796–0.9997	4–8	0.51–10.61		17
MSPD-GC-MS	Fragrances	24	0.9970–1.0000	1–12	<10	75–118	19
SPME-GC-MS	Baby bath water	14	0.993–1.000	0.001–0.300	0.4–21	74–124	20
HS-SPME-GC-MS	Water	24	0.9944–0.9998	0.001–1.100	0.6–17	73–136	21
HS-SPME-GC-MS	Wastewater treatment plants	15	0.9851–0.9989	0.01–1.70	4–33	40–100	22
SPD-PLE-GC-MS	Leave on cosmetics	24	0.997–0.999	0.83–25	0.2–9.7	85–114	23
DLLME-GC-MS	Water	21	0.9807–0.9959	0.007–1.000	3–16	29–112	24
USAEME-GC-MS	Water	24	0.9974–1.0000	0.006–1.15	0.2–23	18–116	25
MEEKC	Commercial rinse-off products	18	0.9903–0.9990	–	0.5–6.0	93–107	26
MEKC	Personal care products	8	0.9941–0.9994	1150–11040	2.4-19.2	89–115	27
HPLC	Commercial scented products	24	0.990–0.999	20–10880	0.6–3.5	90–105	29
USAEME-SFOD-HPLC-DAD	Water and cosmetic samples	18	0.948–0.999	1.3–154.3	3.3–14.4	90–145	This work

MEEKC, microemulsion electrokinetic chromatography; MSPD, matrix solid-phase dispersion.

0.999. LODs were calculated based on ten determinations of samples in which the analyte concentration was close to the expected LOD, and then the LODs were calculated as 3 × SD, where SD is the SD of concentration. [33]. The LODs were from 0.001 to 0.154 μg/mL. LOQs were calculated as 3 × LOD and ranged from 0.004 to 0.463 μg/mL.

The method was also evaluated considering inter and intraday precision, all of them at two different concentrations for each analyte. Low level was around 0.2 μg/mL for anysil alcohol and isoeugenol, around 0.3 μg/mL for hexyl cinnamal, around 0.7 μg/mL for limonene, and around 0.08 μg/mL for the other analytes. High level is around

Table 3. Concentration of PAS in real samples

Samples	Compounds[a] 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
EC						98 ± 3 (3.2%)	323 ± 10 (3.0%)	966 ±3 8 (3.9%)										
EP		212 ± 11 (5.2%)				5626 ± 344 (6.1%)	2467 ± 371 (15.0%)				32 ± 3 (9.3%)	4 ± 1 (24.0%)		193 ± 10 (5.2%)		592 ± 47 (7.9%)		
ET1							329 ± 5 (1.5%)				172 ± 14 (8.1%)							2291 ± 236 (10.3%)
ET2		16 ± 1 (6.2%)				1607 ± 55 (3.4%)	9094 ± 219 (2.4%)	286 ± 39 (13.6%)										5456 ± 334 (6.1%)
ET3							258 ± 17 (6.5%)							1288 ± 154 (11.9%)				
ET4		1002 ± 71 (7.1%)	2.1 ± 0.1 (4.8%)				2751 ± 7 (0.6%)				292 ± 30 (10.3%)	206 ± 15 (7.3%)		894 ± 31 (3.5%)				
BM			67 ± 5 (7.5%)			253 ± 14 (5.5%)	272 ± 14 (5.1%)	7 ± 1 (14.3%)				54 ± 1 (1.8%)		67 ± 11 (16.8%)		31 ± 1 (3.3%)		219 ± 9 (4.1%)

Mean of triplicates (in mg/kg) ± SD (RSD%). EP, Eau de perfume; ET, Eau de toilette.
a) Compounds are numbered as shown in Table 1.

8 μg/mL for limonene and around 0.8 μg/mL for the other analytes.

For intraday precision, ten measurements were performed in the same day. For interday precision, 12 experiments were carried out on three days over two weeks. Values, expressed as RSD in percentages, ranged between 3.3 and 12.4% for intraday precision and 3.9 and 14.4% for interday precision.

Recovery studies were carried out in one BBW and one EC. EC was performed diluting approximately 1 g of each sample in 50 mL, and in the case of BBW no dilution was necessary. The obtained values were in general satisfactory, ranging from 90 to 110%, except for isoeugenol (137.5%), amylcinnamyl alcohol (121.1%), benzyl benzoate (128.5%), and lilial (112.4%) in EC and for isoeugenol (154.0%), geraniol (125.1%), and lilial (136.3%) in BBW.

The EF was defined as the ratio between the analyte concentration in the organic phase after the extraction and the initial concentration of analyte within the water sample. EFs ranged from 9 to 237.

The performance of the proposed USAEME-SFOD method was compared with other reported extraction methods for PAS in different matrices that are listed in Table 2. The best LODs were obtained with SPME followed by GC using MS detector. To date, GC with MS has been the most sensitive and used technique. However, other less sensitive techniques for determination of these compounds have emerged as an alternative, HPLC–DAD being one of them. This technique coupled to a preconcentration method, as USAEME-SFOD, could provide similar results to those methods that use GC–MS without a sample pretreatment technique. The RSDs obtained with the proposed procedure were <14.4%. The results of recovery studies taken from consulted reference were made in different and analogous matrices. The comparison of obtained recovery data with results of similar matrices was acceptable.

3.4 Analysis of real samples

The developed method was applied to the determination of PAS in several samples, including two BBW, one SPW, one BM, one EC, one eau de perfume, and four different ETs. Given the wide range of concentrations of fragrance allergens even inside the same sample, different dilutions were applied to each sample to be able to determine all the present analytes. Dilutions of scented products ranged from 0.0029 to 1.0077 g in 50 mL. In case of water no dilution was applied. In SPW and in BBW1 no analyte content was found. In BBW2 presence of anisyl alcohol was observed but this could not be quantified as it was under LOQ. Table 3 shows the results of quantitative analysis of the samples. Data are presented as mean of three independent experiments ± SD (RSD, in %). A chromatogram obtained from BM sample is presented in Fig. 1B showing eight PAS. In this real sample different dilutions were needed for analytes quantification. Linalool was the most commonly found PAS in the samples. Benzyl

alcohol, geraniol, citral, benzyl benzoate, benzyl salicilate, lilial, α-isomethyl ionone and limonene were also found in these samples. These reported values are above or below, and are also in accordance with the European Union regulation [34].

4 Concluding remarks

In the present work, a method based on USAEME-SFOD–HPLC–DAD was developed for determination of fragrance allergens. Until now, only one method had been developed for quantification of these compounds using HPLC–DAD and it did not include any sample preparation technique. In this study, sample preparation has been accomplished by USAEME-SFOD, which has been found to be an efficient, environmentally friendly, and nonexpensive technique that allows decreasing LODs of the determination technique.

The developed method has been evaluated in terms of linear range, precision, and accuracy obtaining satisfactory results. Finally, the proposed method has been successfully applied to the analysis of several real samples. In conclusion, the proposed method can be applied for the determination of these fragrance allergens in different water and cosmetic samples.

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The authors have declared no conflict of interest.

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Application of PARAFAC2 for the determination of PAS by HPLC-DAD. Resolution of overlapped peaks.

Application of PARAFAC2 for the determination of PAS by HPLC-DAD. Resolution of overlapped peaks.

1. Introduction

Ideally, in chromatography, well-resolved and not-overlapped peaks are expected for the analytes present in a sample. If that happens, the development of methods for the determination of analytes is relatively simple and easily achieved by univariate analysis. However, this situation is not always possible and it is frequent to have partially (or even totally) overlapped peaks due to the complexity of the sample matrices, to technical limitations or simply due to the fact that faster analysis times are preferred. In those cases, if proper data are available, multi-way data analysis can be used in order to obtain quantitative and qualitative information (Bro, 2006).

Hyphenated chromatographic systems combine online separation techniques and spectroscopic detection methods, creating complex data structures, which make the data analysis more challenging. Furthermore, these systems allow to solve problems that otherwise could not be solved (Amigo *et al.*, 2010B). Examples of hyphenated methods are the commonly used GC-MS and LC-MS techniques, and the HPLC-DAD system used in Chapter 5. Thanks to them, each sample can be visualized as a chromatographic landscape in which a complete spectral profile is obtained for each elution time and vice-versa. Thus, data obtained from these techniques can be arranged into a three-way data array. This type of data is known as "second-order" data, and it may show the so-called "second-order advantage" if it is adequately modelled. This property allows the quantification of analytes even if there are unknown interfering compounds not previously modelled (Booksh and Kowalski, 1994; Ortiz and Sarabia, 2007; de Juan and Tauler, 2007; Goicoechea *et al.*, 2011).

Three-way data arrays can be studied using a family of resolution models called three-way methods (Bro, 2006; Ortiz and Sarabia, 2007). Amongst all the possible techniques, PARAFAC has been frequently used to model multi-way chromatographic data (Bylund *et al.*, 2002; Johnson et *al.*, 2004; Bro, 2006). As it is thoroughly detailed in the Introduction Chapter (Section 3.3.), the idea behind

PARAFAC is that all the samples can be modelled by the same loadings (chromatographic and spectral profiles for each factor or component) but with different weights, which can be related to the relative concentration of each component in each sample. However, retention time-shifts are frequent in chromatography as well as peak-shape changes and baseline drifts (Amigo *et al.*, 2010B), and therefore, all samples cannot be explained by the same chromatographic loadings. In these cases, in order to make the data analyzable by PARAFAC, proper data pre-processing must be done. For example, correlation optimized warping (COW) can be used for peak alignment (Nielsen *et al.*, 1998; Tomasi *et al.*, 2004). Nevertheless, data pre-processing can be complex and time consuming. An alternative to face with the problem is to use PARAFAC2, which obtains different elution profiles for each of the analyzed samples (Bro *et al.*, 1999; Kiers *et al.*, 1999; Skov and Bro, 2008).

In a previous chapter (see Chapter 5) a new method was developed for the determination of 18 potentially allergenic fragrance-related substances (PAS) in cosmetic and water samples using ultrasound-assisted emulsification microextraction coupled to HPLC-DAD (Pérez-Outeiral *et al.*, 2015). The initial purpose was to separate completely, and determine, the entire list of 24 regulated fragrance-related allergens corresponding to well-defined chemical compounds (Regulation (EC) No. 1223/2009, 2009). However, a suitable and complete separation in a single chromatographic run was not possible, and thus, only 18 PAS were finally determined by a univariate method.

In this chapter, multi-way analysis is considered in order to determine the PAS whose determination was not possible univariately. These analytes are 6: hydroycitronellal, coumarin, lyral, eugenol, citronellol and farnesol. The use of both, univariate and multivariate calibration methods will allow to check if the maximum allowable concentration of allergenic fragrance-related substances is in compliance with the regulation.

2. Objectives of the chapter

The main objective of the present chapter was the study of the applicability of PARAFAC2 for the extension of the ultrasound-assisted emulsification microextraction method coupled to HPLC-DAD, already explained in Chapter 5, to determine hydroxycitronellal, coumarin, lyral, eugenol, citronellol and farnesol when the other 18 regulated PAS corresponding to well-defined chemical compounds are present. These analytes show overlapped peaks and their quantification cannot be made univariately.

This main objective was fulfilled by the accomplishment of the following more specific objectives:

1. The building of proper PARAFAC2 models to quantify several PAS from their overlapped signals measured by HPLC-DAD. The goodness of the models was evaluated in terms of quality parameters of model performance such as lack of fit (LOF, %), core consistency (CC, %), and percentage of explained variance (EV, %), and in terms of the chemical meaning of the obtained results.

2. The validation of the obtained PARAFAC2 models with different standard samples. Similar errors to the ones obtained with calibration samples should be expected.

3. The application of the PARAFAC2 models to quantify the analytes in different water and cosmetic real samples. These samples may contain interferences that are not in the standards.

4. The validation of the obtained PARAFAC2 models by measuring different real samples spiked with the analytes. The obtained errors in the quantification of analytes in real samples will then be analyzed.

3. Experimental

3. 1. Reagents, standards and samples

Hydroxycitronellal (95%), coumarin (99%), eugenol (99%), citronellol (95%) and farnesol (98%) were all purchased from Sigma-Aldrich (Barcelona, Spain). An exception was lyral (97%), which was supplied by Dr. Ehrenstorfer (Augsburg, Germany). The remainder compounds and materials used are the ones of Chapter 5 (Section 3).

The experimental procedure is detailed elsewhere (Chapter 5, Section 3.4.). In brief, the experimental procedure for fragrance allergens determination consisted on an ultrasound-assisted emulsification microextraction technique followed by the solidification of the floating organic drop and the coupling to HPLC-DAD. Chemometric tools have been applied in this chapter for extracting useful information from the data, with the purpose of quantifying the analytes that cannot be studied by classical chromatographic data treatment procedures (in which only the area or the height of a peak at a defined wavelength is usually used for calibration purposes).

Figure 6.1. shows a typical chromatogram, obtained at 200 nm, by applying the experimental procedure to a mixed standard sample that contains the 24 fragrance allergens regulated in the current legislation. As it can be seen in the chromatogram, there are four regions with problems of overlapped peaks (from interval 1 to interval 4). In Figure 6.2., a zoom-in of each interval has been made, and it is presented together with the UV-Vis spectrum of each compound. Spectral differences will be used to estimate the contribution of each analyte to the overlapped peaks in the chromatogram.

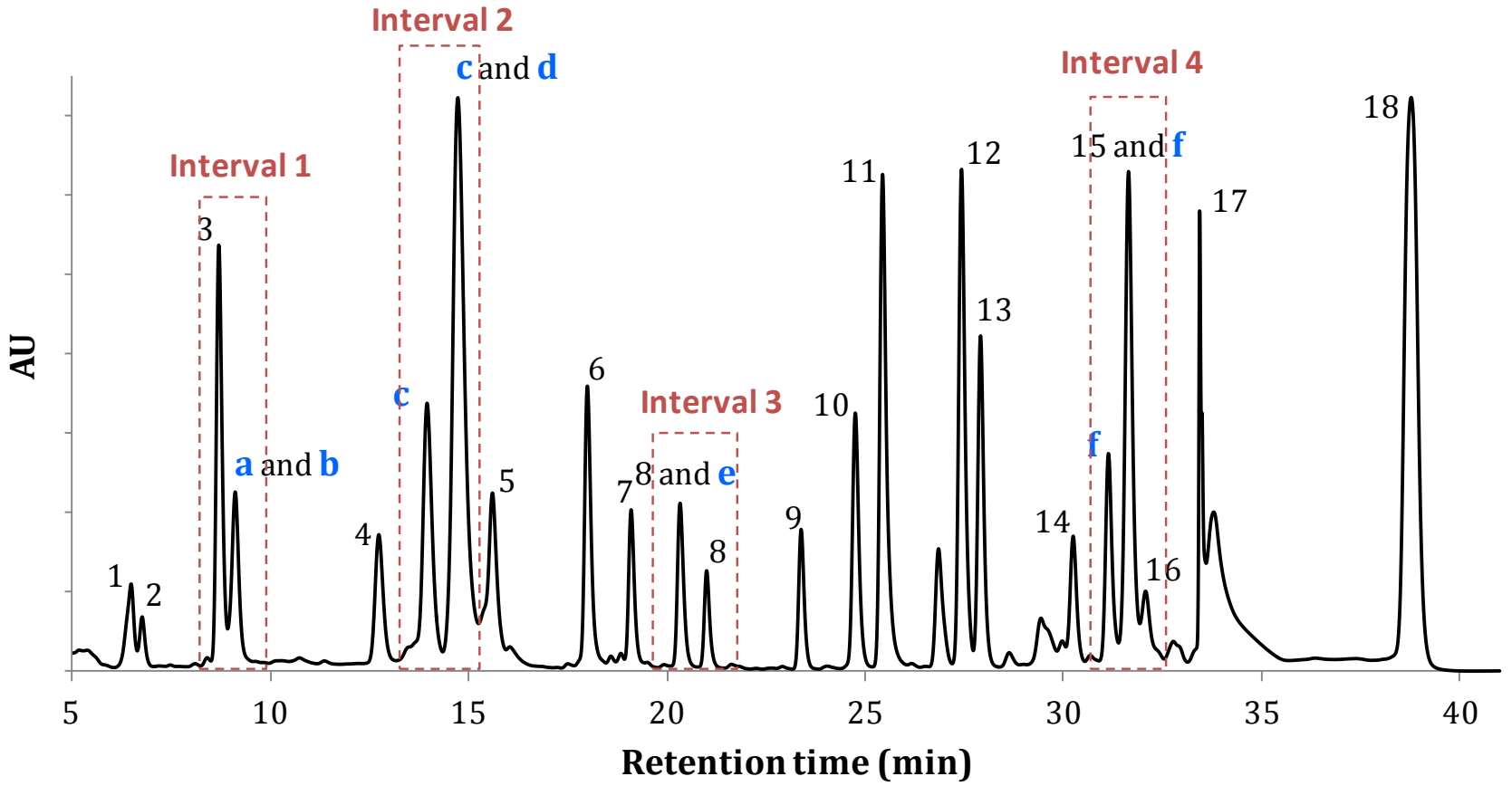

Figure 6.1. HPLC–DAD chromatogram at 200 nm of a standard mixture of the 24 regulated PAS corresponding to well-defined chemical compounds. Assignation of peaks: 1: anise alcohol; 2: benzyl alcohol; 3: cinnamyl alcohol; 4: cinnamal; 5: isoeugenol; 6: geraniol; 7: linalool; 8: citral; 9: methyl-2-octynoate; 10: amylcinnamyl alcohol; 11: benzyl benzoate; 12: benzyl salicylate; 13: benzyl cinnamate; 14: lilial; 15: amyl cinnamal; 16: alpha-isomethyl ionone; 17: hexyl cinnamal; 18: limonene; **a: hydroxycitronellal; b: coumarin; c: lyral; d: eugenol, e: citronellol; f: farnesol.**

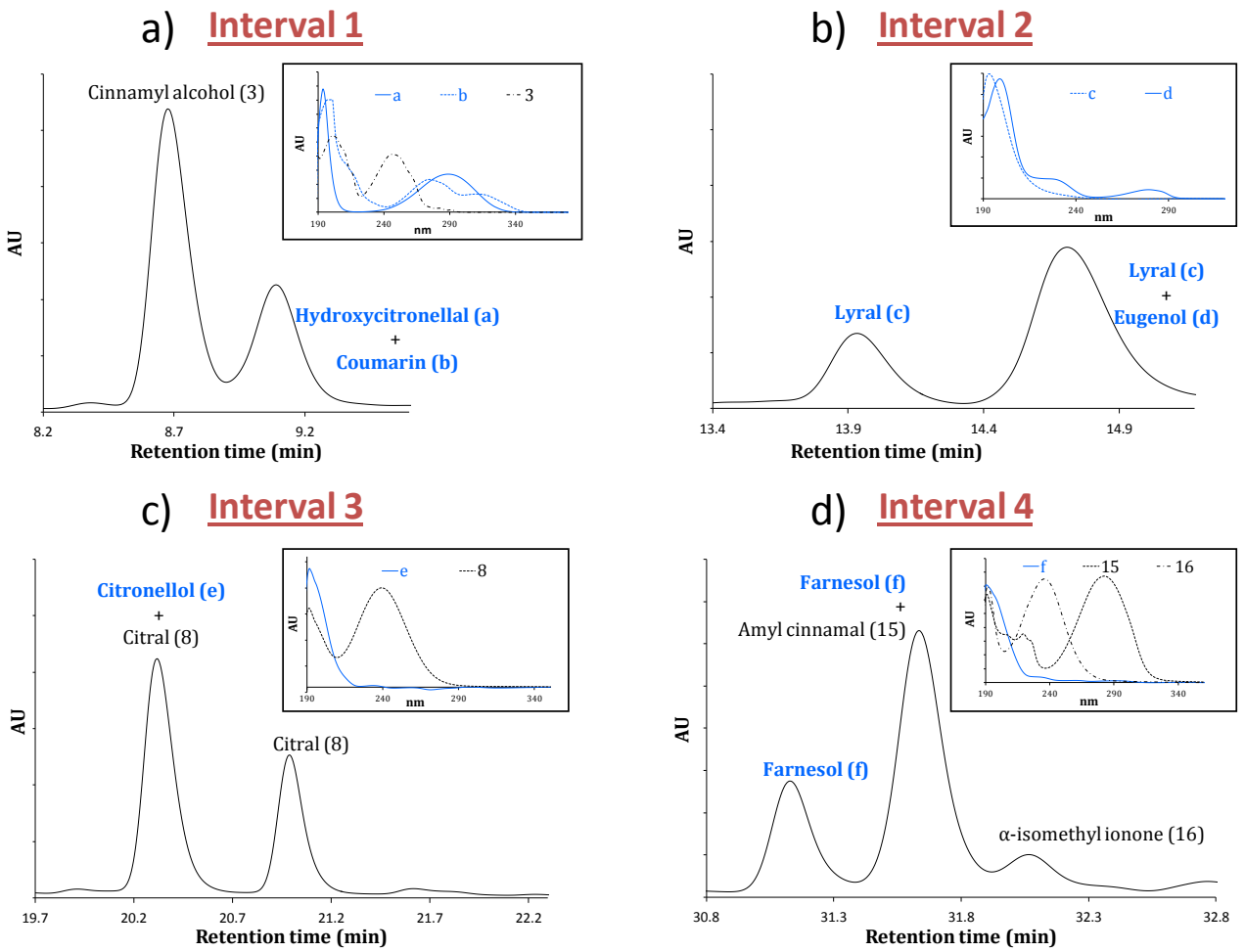

Figure 6.2. Zoom-in of the intervals with problems of overlapped peaks and the related UV-Vis spectra obtained are presented for a) Interval 1; b) Interval 2; c) interval 3; d) interval 4. 3: cinnamyl alcohol; 8: citral; 15: amyl cinnamal; 16: alpha-isomethyl ionone; **a: hydroxycitronellal; b: coumarin; c: lyral; d: eugenol, e: citronellol; f: farnesol.**

A number of 10 out of the 24 PAS show severe overlappings (see intervals in Figure 6.1.). These 10 analytes have been divided in 2 groups (Table 6.1.): Target analytes (T) and non-target analytes (NT). Target analytes are the PAS whose determination needed multi-way analysis. Non-target analytes, however, are the PAS that can be determined properly by univariate methods even if their chromatographic signal is overlapped. The main difference between target and non-target analytes is in the fact that the former show extensive spectral overlapping whereas the latter show only partial spectral overlapping (see Figure 6.2.). That makes possible the univariate determination of the non-target analytes.

Table 6.1. Analytes classification depending on if multivariate analysis is necessary.

Interval	Target analytes (T)	Non-target analytes (NT)
1	Hydroxycitronellal Coumarin	Cinnamyl alcohol
2	Lyral Eugenol	
3	Citronellol	Citral
4	Farnesol	Amyl cinnamal Alpha-isomethylionone

In interval 1, there are two chromatographic peaks, one corresponding to cinnamyl alcohol (NT) which is partially overlapped with a peak containing both hydroxycitronellal (T) and coumarin (T).

In interval 2, lyral (T) gives a double chromatographic signal. The first one shows no overlappings. The second one is totally overlapped with the peak of eugenol (T). Later on the peak of isoeugenol arises (minute 15.6, Figure 6.1.) and it is partially overlapped to the second peak of this interval (that contains both lyral and eugenol), but it has not been considered for analysis of interval 2.

In interval 3, citronellol (T) gives a chromatographic peak totally overlapped with one of the signal contributions of citral (NT).

Finally, in interval 4, farnesol (T) gives two chromatographic peaks, the second one totally overlapped with the peak of amyl cinnamal (NT). Moreover, alpha-isomethyl ionone (NT) is also present, whose peak is partially overlapped to the one containing both farnesol and amyl cinnamal.

As it can be seen in intervals 2 and 4, two target analytes (lyral (c) and farnesol (f)) give two chromatographic signals, one of which is completely overlapped to the peak of another analyte (see Figures 6.2. b) and d)). Although the determination of lyral and farnesol could be done by using only the signal of the not overlapped peak, only a part of the total real signal would be considered in this way. This could possibly lead to limit the capability of determining these analytes at the lowest concentrations. In addition, the appearing of two peaks could be an indicative of the presence of two isomers of the same analyte, and a change in the isomers ratio in any of the samples could lead to a high error in quantification if the two peaks were not considered.

3. 2. Experimental setup

The entire HPLC-DAD procedure to obtain a complete chromatogram lasted for 40 minutes. A complete spectrum between 190 and 800 nm every 1.2 nm and every 0.01 minutes was recorded. Finally, only wavelengths longer than 197 nm (200 nm for interval 3) and shorter than 355 nm for interval 1, 336 for interval 2, 405nm for interval 3 and 335 for interval 4 were considered. The reason was to avoid the cut-off point of the mobile phase (shorter wavelengths) (Harris, 2010) and the zone of the spectra with no absorbance (longer wavelengths) in each case. Regarding the chromatogram, only the intervals with overlappings (interval 1-interval 4) were considered. The efficiency of PARAFAC2 in terms of both interpretability and computation time is increased when only local intervals are taken (Amigo *et al.*, 2010A). All this created data matrices of 133x128, 158x113, 140x165 and 188x113 variables for each interval (Table 6.2.).

Table 6.2. Selected variables in each interval.

Interval	1	2	3	4
Retention time (min)	8.2-9.6	13.5-15.2	19.8-21.3	30.5-32.5
Wavelengths (nm)	197-355	197-336	201-405	197-335
X size (I x J)	133x128	158x113	140x165	188x113

X: Matrix obtained for each sample for I retention times and J wavelengths.

Different datasets were measured in order to build the models and apply them to different kind of samples. Experimental details of the measured sets of samples are given in the following.

3. 2. 1. Calibration set samples

A set of 10 standard calibration samples was measured in triplicate (30 data matrices). The lowest and the highest concentrations of the analytes (regardless of whether they are target or non-target analytes) were around 0.01 and 1.00 μg/mL respectively. Exception were hydroxycitronellal (T), lyral (T) and farnesol (T), which were evaluated at concentrations of 15.94 and 153.05 μg/mL, 0.09 and 10.47 μg/mL and 0.04 and 3.96 μg/mL, respectively.

3. 2. 2. Validation set samples

For intervals 1, 2 and 4, a set of 36 samples was prepared at two different concentration levels, the low and the high. The levels were around 0.08 μg/mL (the low) and 0.80 μg/mL (the high) for all the analytes, with the exception of hydroxycitronellal (T), studied at 59.95 and 135.85 μg/mL, lyral (T) examined at 1.04 and 8.37 μg/mL and farnesol (T) determined at 0.39 and 1.98 μg/mL respectively.

For interval 3, a set of 54 samples was prepared at three concentration levels. Low level was around 0.08 μg/mL for both analytes (citral (NT) and citronellol (T)), medium level was 0.46 μg/mL for citronellol and 0.26 μg/mL for citral and high level was around 0.80 μg/mL for both analytes.

3. 2. 3. Real set samples

Real samples were: four *eau de toilettes* (ET1, ET2, ET3 and ET4), one *eau de cologne* (EC), one *eau de parfum* (EP), one body milk (BM), one swimming pool water (SPW) and two different baby bath waters (BBW1 and BBW2). Given the different concentration of fragrance allergens even inside the same sample, different dilutions were carried out in order to be able to determine all the present analytes. Dilutions ranged from 0.0029 g to 1.0077 g in 50 mL. No dilution was applied for SPW, BBW1 and BBW2 samples.

For each interval, the samples under suspicion of having the corresponding target analytes were selected for further analysis. The initial selection was made visually by observing the obtained chromatograms, as there were samples with not clear analyte content and with lots of interferences. That produced unsatisfactory

results. Then, the applicability of the models to the discarded samples was checked one by one.

3. 2. 4. Recovery set samples

Recovery samples are real samples in which analytes have been artificially added. This dataset consisted of six samples, all spiked with analytes: three of them corresponding to a baby bath water (BBW1) and the other three samples corresponding to *eau de cologne* (EC).

For intervals 1, 2 and 4, EC samples were prepared by dissolving 1 g of EC in 50 mL with the appropriate salt content and then spiked with a certain amount of the corresponding analytes. In interval 3, however, due to the fact that EC contains quantifiable amount of citral, further dilution was needed and 0.01 g of EC were solved in 50 mL. Then, extra amount of citral (NT) and citronellol (T) was added. Salt addition but no dilution was necessary for BBW1 samples in all the intervals. All samples were spiked to have final concentrations around 0.5 μg/mL for all the analytes, except for hydroxycitronellal (T), lyral (T) and farnesol (T), which were spiked to have concentrations of 110.30, 5.83 and 0.89 μg/mL, respectively.

4. Data treatment

4. 1. Data structure

HPLC-DAD data collected from the different sets of samples can be combined in different ways depending on the type of information desired. Generally, the way to proceed was the following: in a first step (step 1) the calibration dataset was studied in order to obtain an approximation to the models that better describe the experimental data (calibration errors and limits of detection can be calculated in this step). Secondly, calibration and validation datasets were examined together (step 2) to calculate how the error is extended to new standard samples similar to the ones used in calibration. After that, the calibration dataset was modelled together with the set of real samples (step 3) in order to get the concentration of the target analytes in these real samples, in the presence of various interfering compounds. Finally, recovery dataset was studied together with the calibration dataset (step 4) with the purpose of studying the error of analytes quantification in real spiked samples. Figure 6.3. schematizes the

different data arrangements considered (step 1- step 4). For each step, the analysis was done at the, previously defined, 4 intervals.

Step 1	Step 2	Step 3	Step 4
Calibration set	Calibration set + Validation set	Calibration set + Real set	Calibration set + Recovery set

Figure 6.3. Representation of the different data arrangements considered in each step of the analysis.

An additional problem, when data coming from different sets of samples are analyzed together, was the retention time-shifts observed between chromatograms, some of them measured over a 6-months period of time. The reason is probably due to either time passage or to column resolution loss between the early and the late data.

4. 2. Method

PARAFAC2 is a powerful tool for the resolution of chromatographic data with varying retention time profiles (Amigo *et al.*, 2008). The basis of this model was explained in the Introduction Chapter (Section 3.3.), and thus, only some considerations for the practical application will be given here.

To apply PARAFAC2 to chromatographic data with spectral detection, data have to be organized into three-way data arrays, where shifted profiles have to be located in the first mode, spectra in the second mode and sample concentrations in the third mode. Thus, in this case, a three-way data array $\underline{\mathbf{X}}$ (I x J x K) was arranged for the construction of each model, where I symbolizes the number of elution times considered, J the number of wavelengths and K the number of samples.

The application of constraints to PARAFAC2 models can be helpful in order to find stable solutions with real chemical meaning (Bro, 1997, Eigenvector Research incorporated, 2015). In this case, one of the most often used constraints, that is, nonnegativity, was applied. When this constraint is applied to one of the modes, it implies that the loadings in that mode cannot have negative values. In our case, this constraint was applied to the second (spectra) and third (samples)

modes, since values of absorbance and concentration cannot be negative. Although elution profiles can neither be negative, the application of the nonnegativity constraint to this mode is not possible with the used PARAFAC2 algorithm.

In all the models, the convergence criteria used was a relative change in fit of 10^{-9} or an absolute change in fit of 10^{-12} (by default). The maximum allowed iterations and computational time to complete the analysis were modified in order to achieve these criteria, when necessary. For initialization, the best fitting model of 20 small runs was used.

Through this Chapter, graphical representations of the loadings (meaning both chromatographic and spectral profiles) obtained in different models for different number of components will be given when necessary. In any case, the first PARAFAC2 component is presented in blue, the second in green, the third in red, the fourth in orange and the fifth in magenta.

The correct determination of the number of components required in order to achieve the best model is a critical aspect when PARAFAC2 is applied. There are several criteria for assessing the right number of components, but normally a combination of some of them is recommended. These criteria include the core consistency test (CC, %), the percentage of variance explained by the model (EV, %), the lack of fit (LOF, %), the visual analysis of the residuals, the chemical meaning of the loadings obtained and the correlation between the loadings and the real measured signals (Amigo *et al.*, 2008; Kamstrup-Nielsen *et al.*, 2013; Elcoroaristizabal *et al.*, 2014).

Core-consistency, percentage of variance explained and lack of fit are statistical parameters used to mathematically validate the model performance. In general words, core consistency measures the "appropriateness" of the structural model (Bro and Kiers, 2003) and it is expressed as a percentage. A low core-consistency value for a model might indicate that the number of components considered is too high and the model is overfitted. This value, together with the EV (%) given by the model (Equation 6.1.) are direct outputs when PARAFAC2 analysis is performed. LOF can be calculated in a similar way to EV (Equation 6.2.).

$$EV(\%) = 100\, x\, \left(1 - \frac{SSE}{SSX}\right) \qquad \text{(Equation 6.1.)}$$

$$LOF\ (\%) = 100\ x \sqrt{\frac{SSE}{SSX}} \qquad \text{(Equation 6.2.)}$$

In both equations *SSE* is the sum of the squares of the residuals and *SSX* is the sum of the squares of the elements in the original array.

The quality of the loadings can be checked according to their chemical meaning. Visual evaluation was used to assess if loadings in the first mode (retention times) looked similar to the measured chromatograms. Correspondence of the spectral loadings with the measured spectra can be assessed by calculating the correlation coefficient (r). Regarding r values, the closer to 1 the better. In this work, loadings and real spectra were scaled before any comparison.

Once a suitable PARAFAC2 model has been built, the score matrix contains the relative concentration of each component in each sample. This allows us to use the PARAFAC2 decomposition for calibration purposes, and then, to predict the concentration of the analytes in samples with unknown analyte content. In order to study the predictive capability of the constructed models, when real concentrations are known, predicted values can be compared with real values. For that purpose, root mean square errors of prediction (RMSEP) (Equation 6.3.) and relative errors of prediction (REP, %) (Equation 6.4.) can be used.

$$RMSEP = \sqrt{\frac{\sum(c_i - \hat{c}_i)^2}{n}} \qquad \text{(Equation 6.3.)}$$

$$REP(\%) = 100 \ \frac{RMSEP}{\bar{c}} \qquad \text{(Equation 6.4.)}$$

Where n is the number of samples, c_i the reference concentration value of the i sample, $\hat{c}_i$the predicted concentration value of the i sample, and $\bar{c}$ the mean reference value of the concentration in all the samples considered.

4. 3. Software

All PARAFAC2 analyses were performed using the PLS-Toolbox (Version 8.1) (Eigenvector Research, Wenatchee, USA) under MATLAB environment (Mathworks, Natick, USA).

5. Results and discussion

5. 1. Calibration

Calibration models using PARAFAC2 were constructed at the defined intervals with the calibration samples in order to assess if the data can be adequately modelled by this algorithm.

Different PARAFAC2 models with increasing number of components were used to fit the data at each interval. The optimum number of components in each interval was initially assessed attending mainly to the chemical meaning of the obtained loadings in spectral and elution time profiles. For that, elution time profile-loadings were visually inspected to check if they looked like the raw chromatographic data. In the case of spectral profile-loadings, the correlation coefficient between the spectral loading of each component and the reference spectrum, experimentally obtained, was used. Moreover, the core consistency test, the percentage of explained variance and the lack of fit of the different constructed models were also evaluated.

Best fitting models in intervals 3 and 4 were those in which the number of PARAFAC2 components was exactly the same as the number of analytes. In those cases, the addition of an extra component divided the single contribution of a single analyte into two different components, making the determination senseless.

In intervals 1 and 2, however, due to the presence of unexpected contributions, an additional component in relation to the number of analytes was needed to appropriately model the data (a 4-component model for 3 analytes in interval 1 and a 3-component model for 2 analytes in interval 2). In interval 1, in particular, the reason was possibly the presence of an absorbing interference, because the addition of the extra component in the model allowed the modelling of a little peak, not attributable to any of the present compounds. Furthermore, the spectral loading of hydroxycitronellal was more similar to the real measured spectrum when the 4-component model was used compared to what it was obtained with the 3-component model (correlations of 0.996 and 0.983 respectively) and the drift of the baseline was better explained. The 4-component model had a high core-consistency (98%), suggesting that the model was not overfitted. Besides, looking at the residuals in the 3-component model, it was clear

that the interfering peak not attributable to any of the analytes remained unexplained.

In interval 2, in a similar way, a third component not attributable to any of the two present analytes was identified. In this case, this additional and unknown contribution was overlapped with the common peak of eugenol and lyral. As before, the suitability of the addition of an extra component to the model was confirmed by measuring the correlations and the core-consistency. The spectral loading of eugenol (T) was slightly more similar to the real measured spectrum when the 3-component model was used compared to what it was obtained with the 2-component model (correlations of 0.997 and 0.994 respectively). In addition, the 3-component model had a high core consistency (93%). The additional component was identified as a residual influence coming from isoeugenol. In Chapter 5 Figure 5.3., the chromatogram of the univariately-determined 18 PAS is shown, and just a little bit sooner than the peak of isoeugenol (approximately at 14.2 min, where the eugenol is located in Figure 6.1.) an additional little peak is observed. This could be related to the third additional component in this model.

Figure 6.4. shows a representation of the original elution time profiles and the loadings obtained after applying PARAFAC2 to each interval. The best PARAFAC2 model has been selected in each case. As it can be seen, models used to describe the data give loadings that look similar to the original chromatographic (raw) data in all cases.

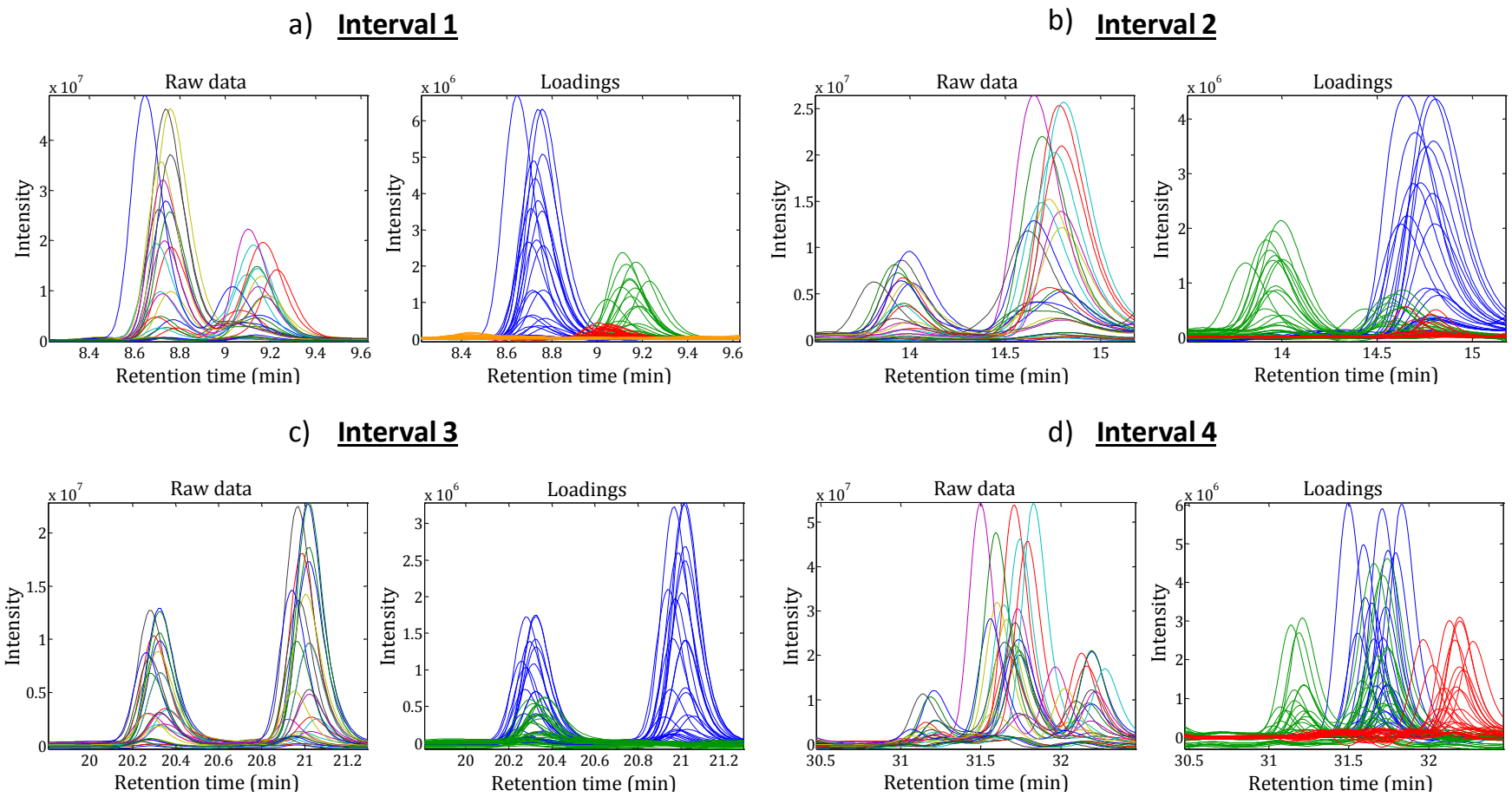

Figure 6.4. Raw elution profile data and PARAFAC2 chromatographic loadings of the a) interval 1 modelled with 4 components, b) interval 2 modelled with 3 components, c) interval 3 modelled with 2 components and d) interval 4 modelled with 3 components. In all cases, the first PARAFAC2 component is presented in blue, the second in green, the third in red and the fourth in orange.

Table 6.3. shows the most important performance characteristics of the best models obtained for each interval. As it can be seen, suitable values of core-consistency were obtained in all the cases (between 93 and 100%). The obtained relatively high lack of fit value in the interval 4 (11.56%) means that part of the data variance was left out from the model. One of the main reasons could be the high influence of the baseline in this interval. A way to check that could be the modelling of the baseline before PARAFAC2 is applied, and an alternative could be the addition of an extra component to the model. The latter was demonstrated to be not adequate because this new component divided the contribution of a single analyte into two different components. In consequence, this problem was noted down and saved for further consideration in the following steps.

Table 6.3. Quality parameters of the best PARAFAC2 models obtained in each interval for the calibration dataset.

	Components	LOF (%)	EV (%)	CC (%)
Interval 1	4	1.31	99.98	98
Interval 2	3	1.74	99.97	93
Interval 3	2	4.35	99.81	100
Interval 4	3	11.56	98.66	100

LOF: Lack of fit; EV: Explained variance;
CC: core-consistency

In PARAFAC2 models, the score matrix in the third mode contains information about the relative concentrations of each component in each sample. When information of the real concentrations is available, regression curves can be constructed relating both values. In this case, the curves were built for each analyte present in each interval. Table 6.4. shows the regression parameters obtained from these representations. Not only the results of target analytes, but the ones of non-target analytes are also presented, because they can be indicative of an adequate modelling even if these non-target analytes were satisfactorily determined in Chapter 5 by univariate methods.

Table 6.4. Regression parameters from the regression curves constructed for each analyte and interval.

	R^2	r	LOD (µg/mL)	LOQ (µg/mL)
Interval 1				
Hydroxycitronellal (T)	0.988	0.996	6.20	18.78
Coumarin (T)	0.989	0.974	0.03	0.10
Cinnamyl alcohol (NT)	0.995	1.000	0.02	0.07
Interval 2				
Eugenol (T)	0.995	0.997	0.02	0.06
Lyral (T)	0.984	0.999	0.39	1.19
Interval 3				
Citronellol (T)	0.943	0.996	0.07	0.22
Citral (NT)	0.998	0.999	0.01	0.04
Interval 4				
Farnesol (T)	0.978	0.998	0.14	0.42
Amyl cinnamal (NT)	0.997	0.994	0.02	0.05
Alpha-isomethyl ionone (NT)	0.997	0.989	0.01	0.04

(T): target analyte; (NT): non-target analyte.
R^2: regression coefficient;
r: correlation coefficient between the spectral loading and the measured spectrum.
LOD: limit of detection; LOQ: limit of quantification; LOD and LOQ were calculated as in Equation 1.10 (Introduction chapter).

As it can be seen, linear regression coefficients (R^2) above 0.943 were obtained in all the cases (the closer to 1, the better). This fact, together with the high correlation observed between the spectral loadings and the measured spectra (r) (in all the cases above 0.974), confirms that the raw data variability was adequately modelled. Figure 6.5. shows the obtained spectral loadings (L) and their corresponding real spectra (R) for each interval. As it can be seen, obtained loadings were very similar to the corresponding real spectra, just like it was suggested by the *r* values.

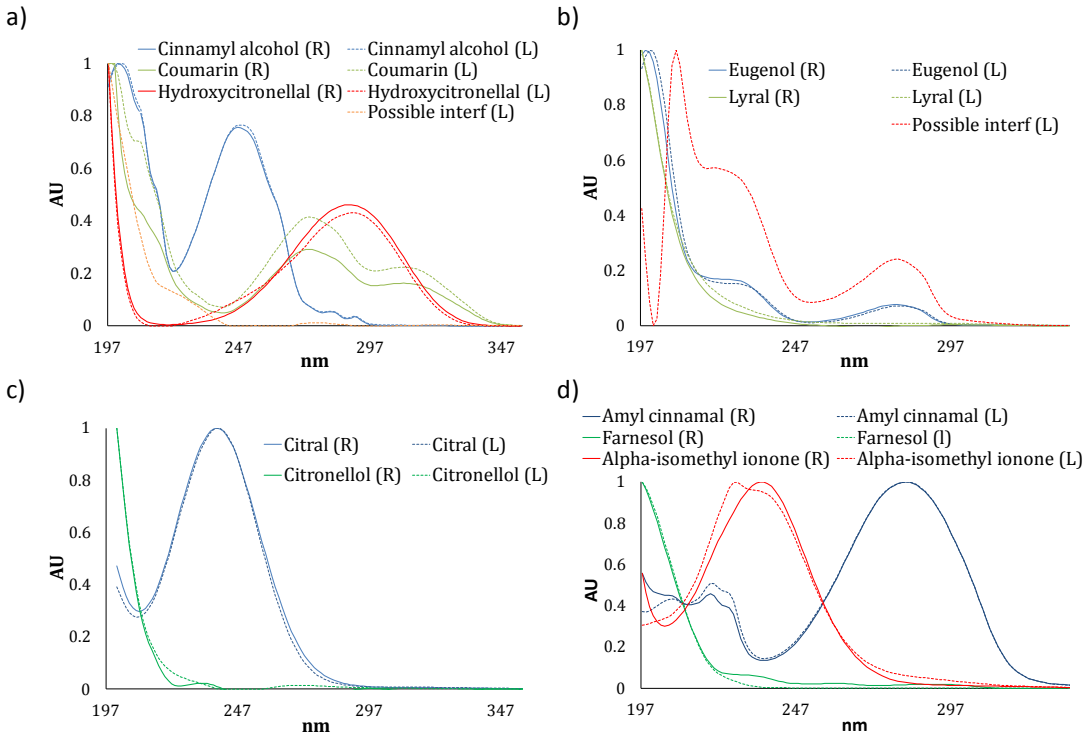

Figure 6.5. Comparison between the spectral loadings obtained by PARAFAC2 and the measured real spectra in a) interval 1, b) interval 2, c) interval 3 and d) interval 4. L: loadings, R: real measured spectra. In all cases, the first PARAFAC2 component is presented in blue, the second in green, the third in red and the fourth in orange.

The limits of detection (LOD) and limits of quantification (LOQ) were also obtained for both target and non-target analytes (Table 6.4.). They were calculated based on the standard deviation of the constructed linear regression curves (Sxy) as it is explained in the Introduction Chapter (Section 3.2.). In general, the LODs of the non-target analytes were an order of magnitude higher than the obtained when these analytes were analyzed by univariate methods (Chapter 5). However, the way to calculate the standard deviation for the determination of the LODs in both cases has been different. It is widely known that the obtained LODs are usually lower when the standard deviation is determined by the repeated measurements of a sample spiked with concentrations near to the expected LOD (this methodology was used in Chapter 5). In any way, the LODs obtained with both methods could be considered comparable.

5. 2. Validation

In this step, the data in calibration and validation sets were modelled together in order to check the prediction ability of PARAFAC2 in similar samples to the ones used in calibration.

Although the number of components was expected to be the same than in the previous step, when only the calibration set was considered, this resulted to be not always true. For intervals 1 to 3, the optimum number of components was effectively the same. For interval 4, the lack of fit value (LOF) showed to decrease from a value of 8.42% in the 3-component model to a value of 5.76% for the 4-component model and 1.24% for the 5-component model. Furthermore, the insertion of two additional components demonstrated to explain the behaviour of the baseline drift better, as it can be seen in Figure 6.6. The core consistency value of the model with five components (99%) was indicative of no-overfitting, and apart from that, meaningful loadings in retention time profiles and spectral profiles were obtained. In consequence, the 5-component model was selected as the best for interval 4. The difference in the number of necessary components for interval 4 compared to the calibration step can be due either to the higher number of samples considered or to the fact that validation samples provide more proper information about the drift of the baseline, or both.

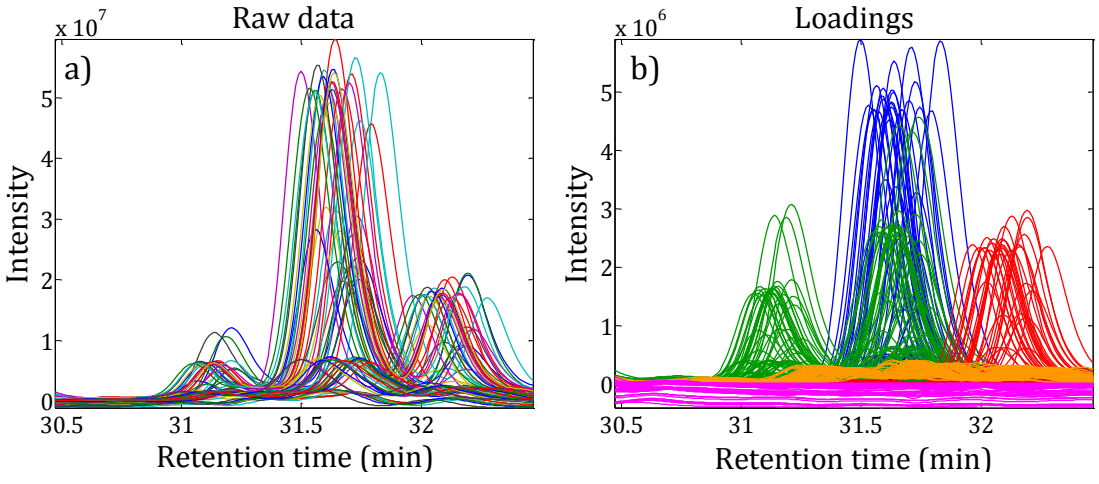

Figure 6.6. a) Raw elution profile data for interval 4 and b) loadings obtained after applying PARAFAC2 model with 5 components. The first PARAFAC2 component is presented in blue, the second in green, the third in red, the fourth in orange and the fifth in magenta.

Table 6.5. shows the quality parameters of the best obtained models for each interval when calibration and validation sets were modelled together. Suitable values of LOF (1.23–3.08%), EV (99.90-99.98%) and CC (91-100%) were obtained for all the intervals. Regression parameters obtained from the regression curves that relate score values and real concentrations were also studied. Similar values to the ones obtained when only calibration samples were considered in the model were found. As expected, the higher differences were observed in interval 4, probably due to the fact that two extra components have been added to the model when calibration and validation datasets are considered together.

Table 6.5. Quality parameters of the best PARAFAC2 models obtained in each interval when calibration and validation datasets are modelled together.

	Components	LOF (%)	EV (%)	CC (%)
Interval 1	4	1.23	99.98	91
Interval 2	3	1.49	99.98	96
Interval 3	2	3.08	99.90	100
Interval 4	5	1.24	99.98	99

LOF: Lack of fit; EV: Explained variance;
CC: core-consistency

The errors (RMSEP and REP) obtained for the validation samples when both the calibration and the validation standards are included in the PARAFAC2 model are summarized in Table 6.6. for each analyte at each interval. Apart from target analytes, non-target analytes have been also included because they can be indicative of the model global performance. The errors have been computed at a low concentration level (low level) and at a high concentration level (high level). The values of concentrations at each level for each analyte are specified in Section 3.2.2.

Table 6.6. Errors in the determination of the different analytes for validation samples.

	High level		Low level	
	RMSEP (µg/mL)	REP (%)	RMSEP (µg/mL)	REP (%)
Interval 1				
Hydroxycitronellal (T)	9.33	6.9	6.36	10.6
Coumarin (T)	0.06	6.9	0.02	15.3
Cinnamyl alcohol (NT)	0.04	4.7	0.01	10.7
Interval 2				
Eugenol (T)	0.05	6.4	0.01	14.3
Lyral (T)	1.20	14.3	0.19	18.3
Interval 3				
Citronellol (T)	0.08	10.0	0.06*	13.0*
Citral (NT)	0.08	9.9	0.01	7.0
Interval 4				
Farnesol (T)	0.22	11.2	0.04	10.1
Amyl cinnamal (NT)	0.05	6.5	0.00	5.0
Alpha-isomethyl ionone (NT)	0.04	6.1	0.00	5.5

(T): target analyte; (NT): non-target analyte.
RMSEP: root mean square error of prediction; REP: relative error of prediction.
* Errors calculated at the medium level for citronellol (0.46 µg/mL) because the low concentration value (0.08 µg/mL) was under the LOD.

As it can be seen, good prediction ability was achieved for all the analytes, with relative errors below 15% in all cases except for the low levels of coumarin (T) (15.3%) and lyral (T) (18.3 %). It has to be remarked that the low level was very close to the LOQ for coumarin and slightly lower to the LOQ for lyral. Anyway, the errors remain acceptable.

An acceptable relative error of 10.1% was obtained for farnesol (T) considering that its low level concentration, 0.39 µg/mL, is under the LOQ (see Table 6.4.). The reason could be that this error has been calculated from the 5-component model obtained when calibration and validation datasets have been modelled together. This model presumably gives lower errors in prediction than the 3-component model built with the calibration dataset because its quality parameters are better.

As a general conclusion for this section, it can be seen that PARAFAC2 is able to model the data adequately when calibration and validation samples are jointly examined.

5. 3. Application to real samples

In a third step, PARAFAC2 was used to predict the concentration of target analytes in real samples where interferences may be possibly present (See Figure 6.3.). In this step, only the real samples that were suspicious of containing the target analytes for each interval were considered *(*EP and ET2 for interval 1, EP, ET2, ET3 and ET4 for interval 2, BM, EC, EP, ET1, ET2, ET3, ET4 for interval 3 and EP for interval 4). As the number of interferences in real samples for each interval was not known, different models were constructed with increasing number of components.

In interval 2 and interval 4, no real samples with a measurable content of the target analytes were found. In intervals 1 and 3, however, a measurable content of several analytes was found in some samples. In both intervals, a higher number of components than the necessary in the calibration and in the calibration plus validation steps were required due to the presence of interferences. Specifically, interval 1 needed 5 components, and interval 3, 4 components. Table 6.7. shows the quality parameters of the best obtained models for intervals 1 and 3 when calibration and real samples datasets are modelled together. As it can be seen, all parameters showed acceptable values.

Table 6.7. Quality parameters of the best PARAFAC2 models obtained in intervals 1 and 3 when calibration and real samples datasets are modelled together.

	Components	LOF (%)	EV (%)	CC (%)
Interval 1	5	1.90	99.96	98
Interval 3	4	3.24	99.89	98

LOF: Lack of fit; CC: core-consistency;
EV: Explained variance;

As an example, Figure 6.7. shows the comparison between raw retention profiles and the corresponding loadings of the PARAFAC2 model with 4 components in interval 3.

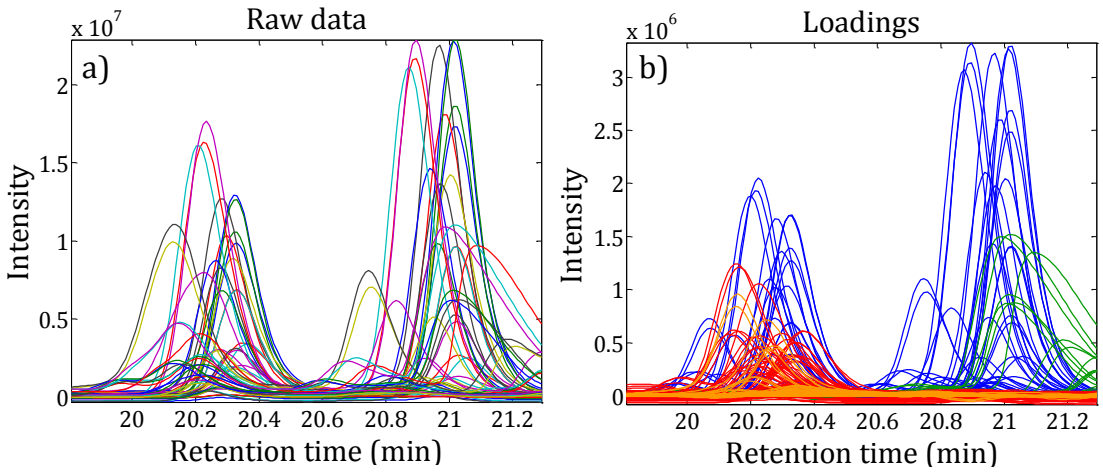

Figure 6.7. a) Raw elution profile data for interval 4 and b) loadings obtained after applying PARAFAC2 model with 4 components. The first PARAFAC2 component is presented in blue, the second in green, the third in red and the fourth in orange.

Regarding the concentrations in real samples, analytes were found in only two samples (EP and ET2) in interval 1, and the obtained concentrations are shown in Table 6.8. Although in ET4 some peaks probably corresponding to the target analytes were visually detected, the presence of interferences made the determination of the analytes in this sample not possible.

Table 6.8. Concentrations (mean of triplicates) and RSDs for hydroxycitronellal, coumarin and cinnamyl alcohol in real samples (Interval 1).

	Hydroxycitronellal (T)		Coumarin (T)		Cinnamyl alcohol (NT)	
	C (mg/kg)	RSD (%)	C (mg/kg)	RSD (%)	C (mg/kg)	RSD (%)
EP	<LOQ	<LOQ	11±1	8.9	<LOQ	<LOQ
ET2	<LOQ	<LOQ	299±15	4.9	n.d.	n.d.

(T): target analyte; (NT): non-target analyte.
C: concentration; RSD: relative standard deviation.
n.d.: not detected; <LOQ: below limit of quantification.

All real samples considered for analysis in interval 3 showed measurable concentration of either citronellol (T) or citral (NT) or both. Table 6.9. shows the obtained concentration values for both analytes. Except for EC, citronellol was present in all the samples. In a previous work citronellol was determined in the 90% of the analyzed perfume samples as well. In that work, citronellol showed higher concentrations in some samples than in this work. The reason could be that samples used in the previous work had a higher perfume content than the ones used here (see Table 5.1. in Chapter 5) (Sanchez-Prado *et al.*, 2011).

Table 6.9. Concentrations (mean of triplicates) and RSDs for citronellol and citral in real samples (Interval 3).

	Citronellol (T)		Citral (NT)	
	C (mg/kg)	RSD (%)	C (mg/kg)	RSD (%)
EC	n.d.	n.d.	914±37	4.1
EP	5145±1559	30.3	n.d.	n.d.
BM	96±3	3.0	9±1	9.4
ET1	76±13	16.9	<LOQ	<LOQ
ET2	248±43	17.3	224±32	14.5
ET3	1324±275	20.8	n.d.	n.d.
ET4	1247±49	3.9	4±0	7.5

(T): target analyte; (NT): non-target analyte.
C: concentration; RSD: relative standard deviation.
n.d.: not detected; <LOQ: below limit of quantification.

Although citral is a non-target analyte, its concentration determined by PARAFAC2 can be indicative of the good prediction capability of the multiway-model if a similar value to the obtained by univariate calibration is found. Table 6.10 shows the concentration of citral in the samples given by both univariate and multi-way approaches.

Table 6.10. Concentrations (mean of triplicates) and RSDs for citral in the same real samples measured by univariate and multi-way approaches.

	Univariate analysis		Multi-way analysis	
	C (mg/kg)	RSD (%)	C (mg/kg)	RSD (%)
EC	966±38	3.9	914±37	4.1
EP	n.d.	n.d.	n.d.	n.d.
BM	7±1	14.3	9±1	9.4
ET1	n.d.	n.d.	<LOQ	<LOQ
ET2	286±39	13.6	224±32	14.5
ET3	n.d.	n.d.	n.d.	n.d.
ET4	n.d.	n.d.	4±0	7.5

(T): target analyte; (NT): non-target analyte.
C: citral concentration; RSD: relative standard deviation.
n.d.: not detected; <LOQ: below limit of quantification.

As it can be seen, concentrations measured in both ways are in concordance. In ET1 and ET4, where multi-way analysis gives low concentrations of citronellol (in ET1 below LOQ), the analyte concentration was under the LOD in univariate analysis. However, this could be due to the different capability of detection of both approaches.

5. 4. Application to real spiked samples

In a final step, PARAFAC2 was applied to calibration and recovery samples together with the purpose of checking the prediction ability of PARAFAC2 in real spiked samples. Recovery studies were made in the baby bath water (BBW1) and in the *eau de cologne* (EC) samples, spiked with known concentration of analytes as explained in Section 3.3.3. BBW1 was selected because it did not show appreciable interferences at any of the intervals and EC showed to contain only citral (NT).

The quality parameters as well as the number of components obtained for the optimum models in this new step are summarized in Table 6.11. As it can be seen, all parameters showed acceptable values (LOF (%) ≤ 4.51; EV (%) ≥ 99.80 and CC (%) ≥ 84).

Table 6.11. Quality parameters of the best PARAFAC2 models obtained in each interval when calibration and recovery datasets are modelled together.

	Components	LOF (%)	EV (%)	CC (%)
Interval 1	4	2.20	99.95	84
Interval 2	4	2.23	99.95	97
Interval 3	2	4.51	99.80	100
Interval 4	5	4.26	99.82	97

LOF: Lack of fit; EV: Explained variance;
CC: core-consistency

The same number of components as in the models for only calibration (step 1) and calibration plus validation samples (step 2) were found for intervals 1 and 3. The number of components for interval 2, however, differed from the previous results. The reason could be that there is a shift in the chromatogram retention times when comparing recovery set samples to calibration and validation set samples. This shift can be attributable either to column resolution loss between early data (calibration and validation) and late data (recovery) or to time passage (6 months). The shift causes that the peak of isoeugenol is now shown up in interval 2 (see interval 2 in Figure 6.1.). Therefore, an additional component to explain the new extra variability due to this peak is needed when the model is built for interval 2. Figure 6.8. shows the raw data, where the shift in the retention times can be appreciated, and the loadings obtained when a PARAFAC2 model with 4 components is considered for interval 2.

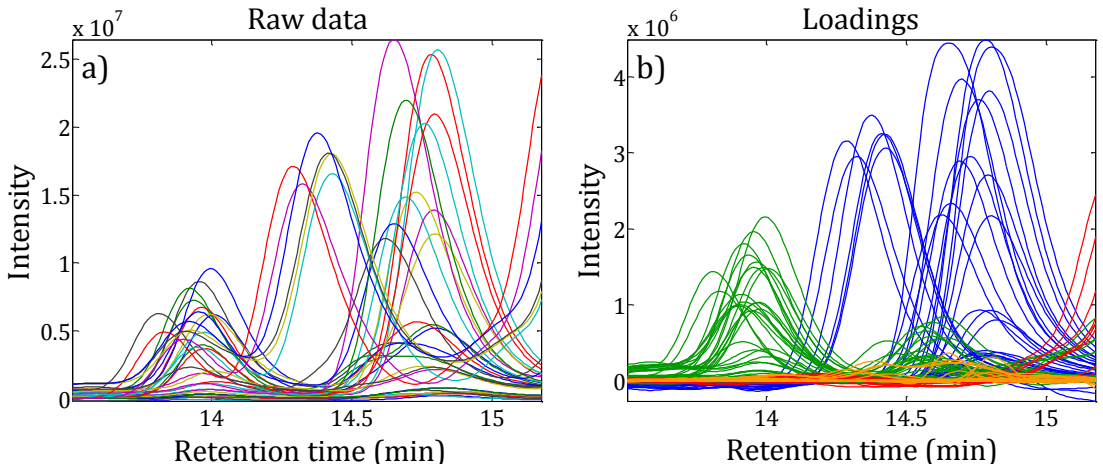

Figure 6.8. a) Raw elution profile data for interval 2 and b) loadings obtained after applying a PARAFAC2 model with 4 components. The first PARAFAC2 component is presented in blue, the second in green, the third in red and the fourth in orange.

Regarding interval 4, and due to the discrepancy in the number of components between the different considered combinations of datasets, a PARAFAC2 model including calibration, validation and recovery datasets for interval 4 was built. A 5-component model was considered the best. Lack of fit was very acceptable (4.26%), core consistency was suitable (97%) and spectral loadings had chemical meaning. Three components of the model showed a high correlation with the three compounds of that interval and the other two acceptably explained the behaviour of the baseline drift.

The concentrations for the samples in the recovery set at each interval were calculated and the obtained values were compared to the added concentrations. In this case, results are expressed in recovery terms, in which the percentage of the analyte recovered is given (Table 6.12.). In the case of the EC sample, the quantity of citral present in the raw sample plus the spiked concentration have to be taken into account to calculate the recovery.

Table 6.12. Recovery values obtained applying the optimum PARAFAC2 model to calibration and recovery datasets.

	Recovery in BBW sample (n=3)		Recovery in EC sample (n=3)	
	ER (%)	RSD (%)	ER (%)	RSD (%)
Interval 1				
Hydroxycitronellal (T)	103.3	7.6	110.6	6.4
Coumarin (T)	83.1	6.7	95.5	4.2
Cinnamyl alcohol (NT)	99.6	6.2	104.1	6.4
Interval 2				
Eugenol (T)	119.2	2.8	129.5	4.5
Lyral (T)	96.5	10.7	98.33	7.4
Interval 3				
Citronellol (T)	117.2	11.2	105.6	5.6
Citral (NT)	99.9	2.3	101.8	2.7
Interval 4*				
Farnesol (T)	109.6	4.1	157.6	8.9
Amyl cinnamal (NT)	93.3	3.5	99.8	4.2
Alpha-isomethyl ionone (NT)	82.3	2.5	100.4	7.8

(T): target analyte; (NT): non-target analyte.
ER: Extraction recovery; RSD: relative standard deviation.
*In interval 4 the model was built including calibration, validation and recovery datasets.

The obtained recovery values were very acceptable for all the analytes except for eugenol (T) in both samples and farnesol (T) in EC, where tolerable values were obtained (119.2, 129.5 and 157.6% respectively). RSD (%) values were satisfactory as well (≤ 11.2%), which is indicative not only of the high precision of the method but also of the good model performance.

6. Conclusions

This chapter shows the feasibility of using multi-way analysis techniques for the extension of the method developed in Chapter 5 for determining simultaneously hydroxycitronellal, coumarin, lyral, eugenol, citronellol and farnesol when the other 18 regulated PAS are also present. Due to co-elution problems in the chromatograms, these analytes cannot be adequately determined by univariate methods. Thanks to PARAFAC2, co-elution problems have been solved.

PARAFAC2 has been used to decompose the data into different contributions, some of them directly related to the chemical compounds. The relative concentration of each analyte in each sample given in the score matrix has been used to construct satisfactory regression curves with the real concentration values. In some cases, these have been even built in the presence of interferences.

Validation of the obtained PARAFAC2 models has been made using standard and real spiked samples. In both cases satisfactory results have been obtained. In addition, PARAFAC2 models have been satisfactorily used for the determination of the analytes in several real samples, even in the presence of interferences not previously modelled in the calibration dataset.

Thanks to PARAFAC2, the problem of retention time shifts between chromatographic measurements has not been restrictive. Furthermore, the baseline drift has been successfully modelled by adding new components to the models when necessary. However, a higher number of samples with proper information about the drift of the baseline were required for this purpose.

In summary, in this chapter, the determination of the 6 target analytes has been possible despite co-elution profiles, overlapping spectra, unknown interfering compounds, retention time shifts and baseline drifts thanks to the use of PARAFAC2. The application of different multivariate techniques, such as multivariate curve resolution (MCR), could be a good idea in order to compare and check the reliability of the obtained results.

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Conclusions

Conclusions

Considering the initial objectives of the present thesis, the general conclusions that can be outlined are the following:

1. Development of dispersive liquid-liquid microextraction-based methodologies

Dispersive liquid-liquid microextraction-based techniques were selected especially due to the fact that they are environmentally friendly and faster compared to other sample pretreatment techniques. Three different methodologies have been developed in this thesis combining the following techniques: 1. dispersive liquid-liquid microextraction and UV-Vis spectrophotometry for cadmium determination in water samples (Chapter 3), 2. ultrasound-assisted dispersive liquid-liquid microextraction followed by solidification of the floating organic drop for phthalate determination in food simulants and liquid samples (Chapter 4) and 3. ultrasound-assisted emulsification microextraction followed by the solidification of the floating organic drop and HPLC-DAD for the determination of 18 fragrance allergens in cosmetic and water samples (Chapter 5). In Chapter 6, the procedure developed in Chapter 5 was extended to the 24 fragrance allergens corresponding to well-defined chemical compounds contemplated in the current legislation with the aid of multi-way analysis.

The entire number of the developed procedures posse the different advantages of dispersive liquid-liquid microextraction techniques. On one hand, all of them are environmentally friendly due to the low amounts of organic solvents used. In addition, in Chapters 4 and 5, the different dispersive liquid-liquid microextraction techniques were combined with the solidification of the floating organic drop, which uses alternatives to the use of toxic organic solvents. On the other hand, all the procedures have shown that extraction time is not an important factor for these types of techniques, and extraction has been completed in all cases in less than five minutes.

The dispersion of the extractant has been achieved in different ways: with the aid of a disperser solvent (Chapter 3), by means of ultrasonic energy (Chapter

4) and by the combination of both of them (Chapter 5). The three ways showed similar advantages, and the best approximation depends on the specific analytes.

2. Versatility of dispersive liquid-liquid microextraction-based techniques

The versatility of dispersive liquid-liquid microextraction-based techniques has been probed by the development of procedures using diverse analytical instrumentation for the determination of different analytes in several sample matrices.

The coupling of dispersive liquid-liquid microextraction techniques to UV-Vis spectrophotometry was done by a simple micro-capacity cuvette: This is an inexpensive way of coupling that avoids the dilution of the extractant after extraction and the use of more sophisticated equipments. The coupling to GC-FID was straightforward, as a part of the organic phase obtained after the extraction was directly injected into the chromatograph. Finally, the coupling to HPLC-DAD was expected to be more problematic, because the injected sample has to be compatible with the mobile phase, but not compatibility problems arose in this work. *n*-hexadecane was used as extractant and it was perfectly compatible after slight dilution in methanol.

The applicability of dispersive liquid-liquid microextraction techniques to different analytes has been probed as well. Among the analytes, two types of organic compounds (phthalates and fragrance allergens) and a heavy metal (cadmium) were determined.

3. Use of different chemometric tools

✓ Experimental design and one variable at a time (OVAT) approach

There are several variables affecting the dispersive liquid-liquid microextraction-based processes, and the selection of the most appropriate values in each specific methodology is an important task. This selection can be done either by a univariate approach (OVAT) or by simultaneous study of various variables (experimental design). In this thesis, a combination of experimental design and OVAT approach in Chapters 3 and 4, and only OVAT approach in

Chapter 5, have been satisfactorily used for the selection of the best experimental conditions in each of the developed procedures. When OVAT approach was applied, ANOVA analysis was used to confirm the lack of statistical significance between levels when necessary.

In Chapter 3, OVAT approach has been used for searching the best levels of variables such as salt addition, the pH of the sample and extraction time. The type of extraction solvent was considered to be an important factor; however, the selected extraction solvents (chloroform and carbon tetrachloride) could not be compared at the same conditions due to their different characteristics. Therefore, a different experimental full factorial design was used in order to find an approximation to the most appropriate experimental conditions for each solvent. The studied variables were the following: The type and volume of disperser solvent, extraction temperature and dithizone (chelating agent) concentration. Then, absorbances at the best studied conditions were compared for both extraction solvents. In spite of its lower signal, chloroform was finally selected as extraction solvent due to technical reasons. The final experimental conditions were fixed according to the levels that gave better responses in the experimental design. The significant variables in the design with chloroform were: extraction temperature and volume of dispersant, and both gave better responses at higher levels. However, a further optimization at those levels was not possible because higher values of these variables led to problems in the collection of the drop.

In Chapter 4, OVAT approach was successfully used for searching the best conditions of the variables whose study at more than two levels was considered interesting (the type and volume of extraction solvent, extraction technique, disperser solvent and pH). The relative significance amongst the remaining variables (extraction time and temperature, salt addition and disperser solvent volume) was studied by fractional factorial design. This allowed saving time by directly fixing the variables with little influence in the process (extraction time and temperature). After, OVAT approach was effectively used for the univariate optimization of the relevant variables (salt addition and disperser solvent).

In Chapter 5 only OVAT approach was used for searching the best levels of variables such as type and volume of extractant, extraction technique, sample volume, extraction time, extraction temperature and salt addition.

- ✓ **Method validation**

After the finding of the most appropriate experimental conditions for the developed methodologies, method validation was performed in order to assess their quality. This was accomplished by calculating some quality parameters such as linearity, limits of detection, limits of quantification, precision and accuracy. In all cases, satisfactory results were obtained. Limits of detection were adequate to check compliance with the current legislation of phthalates and fragrance allergens. For cadmium, however, LODs were higher than desired and the developed method could only be applied to highly contaminated waters. In all cases, satisfactory precision and accuracy results were in general obtained, according to the studied levels of analyte concentrations.

- ✓ **Multi-way data analysis**

The use of multivariate analysis became necessary due to the presence of overlapping peaks in the HPLC-DAD chromatogram that contained all the potentially allergenic substances related to fragrances (PAS) that correspond to well-defined chemical compounds. PARAFAC2 was satisfactorily applied in Chapter 6 to extend the method developed in Chapter 5 to the determination of all the defined PAS with restrictions in the current legislation. In this way, multi-way data analysis has been shown to be able to use information not used in univariate methods to solve problems that otherwise could not be solved. However, the application of alternative multivariate techniques to the same data would be interesting in order to compare the obtained results, and to try to overcome the problems found in the quantification of some real samples.

4. Application of the procedures to real samples

The application of the different developed dispersive liquid-liquid microextraction techniques to the analysis of the target analytes in different real sample matrices has been successfully performed. These matrices included aqueous samples, food simulants, liquid food samples and cosmetics.

No one of the real samples measured showed a quantifiable amount of cadmium or phthalates. This was expected, as these compounds are banned above certain limits in the current legislation. Therefore, in order to check the

applicability of the developed methods for cadmium and phthalate determination in real samples, samples were spiked with the analytes of interest and their quantification was accomplished. In general, satisfactory results were obtained.

Several samples with measurable fragrance allergen content were found (Chapters 5 and 6). In this case, the presence of target analytes in cosmetic products is not totally banned, but their concentration must be indicated on the label when it exceeds certain limits. In all cases, the compliance with the regulation was verified, and the presence of the allergens was confirmed to be adequately labelled.

General conclusion: Dispersive liquid-liquid microextraction-based techniques have shown a fast and environmentally friendly character in all the developed procedures, in addition to a high versatility in their combination with different analytical instrumentation for the determination of various analytes in diverse sample matrices. Chemometric tools have shown high usefulness in different steps of the method development: method optimization, validation and data analysis.

Resumen

Resumen

La preparación de la muestra es una etapa importante en el desarrollo de los métodos analíticos, ya que influye directamente en la calidad de los resultados. En la actualidad el interés se centra especialmente en la aplicación de versiones miniaturizadas de las técnicas de preconcentración ya existentes porque son, por lo general, más simples, rápidas y ecológicas. De entre todas ellas, la microextración en fase líquida (Liquid-phase microextraction, LPME) es la que se empleará en este trabajo.

La LPME emerge como una versión miniaturizada de la clásica extracción líquido-líquido. En ella, la extracción tiene lugar entre un pequeño volumen (del orden de microlitros) de un disolvente inmiscible en agua (extractante) y la muestra acuosa conteniendo los analitos de interés. Desde su primera aplicación se han desarrollado diferentes variantes que difieren en la forma en la que el extractante es puesto en contacto con la muestra. Entre esas variantes destaca la microextracción en fase líquida dispersiva (Dispersive liquid-liquid microextraction, DLLME), por su rapidez de extracción. En la DLLME, desarrollada en el año 2006, se consigue la dispersión del extractante en la fase acuosa mediante la adición de un tercer disolvente orgánico miscible en ambas fases, el dispersante. De esta forma, el área superficial entre ambas fases se ve aumentada, y con ello, la velocidad de extracción. Tras el proceso, las dos fases deben ser separadas, generalmente mediante centrifugación. Una vez separada, la fase orgánica es conducida al correspondiente análisis instrumental. Una nueva variante, denominada microextracción líquido-líquido dispersiva asistida por ultrasonidos (Ultrasound-assisted-DLLME, UA-DLLME) incorpora el uso de ultrasonidos para acelerar la extracción. En la microextracción por emulsificación asistida por ultrasonidos (Ultrasound-assisted emulsification microextraction, USAEME), por otro lado, la dispersión se produce únicamente por el uso de ultrasonidos, en ausencia de dispersante.

A pesar de que en la LPME se reduce considerablemente el uso de disolventes, los extractantes utilizados siguen siendo por lo general tóxicos. En 2007, se introdujo por primera vez una variedad de disolventes, que aparte de tener una menor toxicidad, tiene una menor densidad que el agua, y un punto de fusión cercano a la temperatura ambiente (10-30^{o}C). Esto permite que tras la

extracción, la gota orgánica flote en la superficie de la muestra, y se pueda solidificar introduciendo la muestra en un baño de hielo. Posteriormente se podrá recoger la gota solidificada mediante una espátula. El uso de estos disolventes en las diferentes variantes de LPME ha dado lugar a las técnicas de solidificación de gota orgánica flotante (solidified floating organic drop, SFOD)

Teniendo en cuenta lo comentado, se consideró de interés en esta tesis desarrollar, validar y aplicar nuevas metodologías analíticas basadas en la microextracción por DLLME acoplada a diferente instrumentación. El objetivo fue determinar diferentes tipos de analitos que necesitaban una etapa previa de preconcentración y separación de la matriz. Además, algunos objetivos adicionales de la tesis fueron la utilización de diferentes técnicas quimiométricas para el desarrollo de los métodos (diseño experimental, parámetros metrológicos adecuados en la validación del método o análisis multivariable) y la aplicación de las metodologías desarrolladas a diferentes muestras para comprobar que los niveles observados de los analitos en ellas cumplen con la legislación vigente.

La tesis está formada por siete capítulos. El primer y el segundo capítulo corresponden a **la introducción** y a **los objetivos**. A continuación viene el bloque principal, constituido por los capítulos 3, 4 y 5, donde se desarrollan, validan y aplican las diferentes metodologías analíticas propuestas.

En el **Capítulo 3**, la DLLME y la espectrofotometría UV-Vis se combinan para la determinación de cadmio en diferentes muestras acuosas. El cadmio es un metal pesado de elevada toxicidad, incluso a bajas concentraciones, que puede ser encontrado en gran variedad de matrices. Su presencia en aguas de consumo y de riego podría suponer un serio riesgo para la salud.

Para la extracción y detección espectrofotométrica del cadmio es necesario utilizar un agente quelante. De entre todos, la ditizona ha sido extensamente utilizada por su eficacia. Una vez seleccionado el agente quelante, se estudiaron los espectros de absorción tanto de la ditizona como de sus complejos con cadmio, y se definió la longitud de onda para la determinación. La medida espectrofotométrica se llevó a cabo gracias al uso de una microcubeta que permitió obtener una señal apreciable con solo 60 μL de disolución. De esta forma, además, se resolvió la dificultad de adaptar los pequeños volúmenes obtenidos en LPME a las medidas en espectrofotómetros convencionales.

El proceso de determinación puede verse afectado por diversas variables. Algunas de ellas fueron fijadas previamente atendiendo a que la dispersión se formara y se rompiera correctamente: velocidad de agitación (1000 rpm), velocidad y tiempo de centrifugación (3500 rpm y 1 minuto) y volumen de muestra (7,5 mL). Además, la adición de sal (no adición), el tiempo de extracción (3 minutos) y el pH (pH 12,8 ± 0,2) se fijaron de forma univariante.

Las características principales para ser un buen extractante en DLLME son tener mayor densidad que el agua y una baja solubilidad en ella, además de una alta capacidad de extracción. En base a ello, el cloroformo ($CHCl_3$) y el tetracloruro de carbono (CCl_4) fueron seleccionados. Sin embargo, debido a sus diferentes características, su eficiencia no pudo ser comparada en las mismas condiciones, y se buscaron las mejores condiciones con cada uno de los extractantes mediante la aplicación de dos diseños experimentales.

Los diseños planteados fueron factoriales completos (2^4). Las variables fueron: temperatura (25°C y 40°C para el $CHCl_3$, y 25°C y 50°C para el CCl_4, debido a sus diferentes puntos de fusión), volumen de dispersante (0,5 y 1,5 mL), tipo de dispersante (etanol y metanol) y concentración de ditizona (34 y 102 mg/L para el $CHCl_3$ y 52 y 154 mg/L para el CCl_4). Las concentraciones de ditizona fueron seleccionadas de forma que la cantidad neta de agente quelante en ambos casos fuera la misma, ya que esta se añade disuelta en el volumen de extractante, siendo en ambos casos el necesario para obtener una fase orgánica manejable tras la extracción (100 µL para CCl_4 y 150 µL para el $CHCl_3$).

Se compararon las absorbancias en las mejores condiciones de cada diseño, observando que la absorbancia obtenida con el CCl_4 era ligeramente superior, pero no lo suficiente para compensar su mayor coste y toxicidad. Por ello se decidió utilizar $CHCl_3$, con las mejores condiciones obtenidas en su correspondiente diseño (40°C, 1,5 mL de metanol, y 34 mg/L de ditizona).

Una vez definido el método experimental, se procedió a su validación. El rango de estudio fue de 10-100 µg/L, obteniendo una buena linealidad (R^2=0,9947) y un límite de detección (LOD) de 8,5 µg/L. La precisión se estudió a dos niveles de concentración, obteniendo resultados aceptables (RSDs: 9-13,3%). El efecto matriz se estudió en diferentes muestras acuosas dopadas obteniendo recuperaciones entre el 99 y el 103%. Además se estudió la posible interferencia de otros metales (Al^{3+}, Ca^{2+}, Pb^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, Fe^{3+}, Ni^{2+}, Cu^{2+}), y también

se calculó el factor de enriquecimiento (EF, 73). Finalmente, se llevó a cabo la determinación de cadmio en diferentes muestras acuosas, demostrando que el método desarrollado puede ser aplicado satisfactoriamente a la determinación de cadmio en estas matrices.

En el **Capítulo 4**, la UA-DLLME se combina con cromatografía de gases con detector de ionización en llama (GC-FID) para la determinación de ftalatos en simulantes alimentarios y diferentes muestras acuosas alimentarias. Los ftalatos son utilizados en gran variedad de aplicaciones industriales y domésticas, destacando su uso en la industria del empaquetado. Debido al potencial riesgo para la salud de los ftalatos, la cantidad máxima de éstos que debería migrar desde el empaquetado a la comida está legislada en Europa por el reglamento (UE) N^{o} 10/2011.

Se seleccionaron cinco ftalatos para el estudio: ftalato de dibutilo (DBP), de bencilo y butilo (BBP), de diciclohexilo (DCHP), de bis(2-etilhexilo) (DEHP) y de di-n-octilo (DNOP).

El desarrollo del método comenzó con el estudio de la separación de los analitos mediante GC-FID. Una vez obtenida la mejor separación, se procedió a la búsqueda del extractante que, debido a su menor toxicidad, se centró en los extractantes normalmente utilizados en las técnicas SFOD. De entre todos ellos, el *n*-hexadecano se eligió por sus mejores características. Después, en esta ocasión, la aplicación de diversas técnicas fue estudiada, incluyendo las siguientes: LPME, DLLME, USAEME y UA-DLLME. Así, se observó que los mejores resultados se obtenían cuando la dispersión se provocaba con una combinación de dispersante y ultrasonidos; por ello, se seleccionó la UA-DLLME.

A continuación, se definieron las mejores condiciones experimentales para una correcta formación de la gota tras la extracción, que facilitase su recogida: condiciones de centrifugación (4500 rpm, 10min) y congelación de la gota (3^{o}C, 10 min). Además, el efecto del pH de la muestra (pH natural), el volumen de extractante (15 µL) y el tipo de dispersante (acetonitrilo) fue fijado mediante el estudio univariante a varios niveles.

El resto de variables se estudió mediante un diseño experimental factorial fraccionado (2^{4-1}), siendo las variables estudiadas (y los niveles) el tiempo de extracción (5 y 10 min), la temperatura de extracción (35 y 45^{o}C), el volumen de

dispersante (0,5 y 1,5 mL) y la adición de sal (0 y 100 g/L). Los resultados mostraron que el tiempo y la temperatura de extracción no eran variables significativas, y por eso sus niveles se fijaron en los niveles a los que la mayoría de los analitos daban eficiencias ligeramente mayores (5min y 35°C). Los otros dos factores, el volumen de dispersante y la adición de sal, resultaron estadísticamente significativos, y un análisis univariante para cada uno de ellos mostró que los niveles óptimos correspondían a 0,75 mL de acetonitrilo y 25 g/L de adición de sal respectivamente.

Una vez obtenidas las condiciones óptimas, el método fue validado mediante la obtención de los parámetros metrológicos adecuados. La linealidad observada en el rango de estudio fue buena (R^2 =0,993-0,995). Los LODs fueron de 0,64 a 2,82 μg/L y los límites de cuantificación (LOQs) de 1,93 a 8,47 μg/L, lo que permite la determinación de los ftalatos a límites inferiores a los límites de migración establecidos en la regulación europea. La precisión del método fue evaluada a dos niveles obteniendo buenos resultados (RSDs entre 2,7 y 9,3%). La exactitud del método se evaluó mediante ensayos de recuperación llevados a cabo en simulantes de alimentos y en agua destilada obteniendo también resultados aceptables. Los valores de los factores de enriquecimiento variaron entre 854 y 1893. Por último, el método fue aplicado a diferentes muestras acuosas y alimentarias (agua embotellada, vinagres, vinos, refrescos y sangría) sin encontrarse ftalatos en ninguna de ellas. Además, con la intención de evaluar la aplicabilidad del método a muestras reales, algunas de ellas se doparon y fueron evaluadas obteniendo buenos resultados.

En el **Capítulo 5**, la USAEME se combina con la cromatografía de líquidos de alta eficacia con detector de diodos (HPLC-DAD) para la determinación de sustancias potencialmente alérgenas relacionadas con las fragancias (potentially allergenic substances, PAS) en diferentes muestras acuosas y cosméticas. Actualmente, la legislación europea limita el uso de 26 PAS (24 compuestos químicamente bien definidos y 2 extractos naturales) y su presencia debe declararse en las etiquetas de los productos cuando su concentración supera ciertos límites (Reglamento (CE) No 1223/2009). Además, el uso de gran cantidad de cosméticos, hace que las aguas recreacionales (piscinas, jacuzzis, etc.) sean consideradas importantes fuentes de exposición. Por todo ello, deben desarrollarse métodos analíticos fiables para la cuantificación de PAS en diferentes muestras cosméticas y en aguas.

En este caso, el desarrollo del método comenzó con el estudio de la separación de los analitos por HPLC. En las mejores condiciones, se consiguió una buena separación de 16 de los analitos y estos fueron determinados mediante el método tradicional, en el que el área o la altura del pico del analito a una determinada longitud de onda se utilizan como señal. El resto de los analitos aparecían solapados dos a dos. De los analitos solapados, dos se determinaron mediante el procedimiento tradicional, ya que poseen un máximo de absorbancia a una longitud de onda donde los analitos con los que se solapan no absorben. Este capítulo se centra en el desarrollo de un método para determinar los 18 PAS cuantificables mediante medios tradicionales: Amyl cinnamal, amylcinnamyl alcohol, anise alcohol, benzyl alcohol, benzyl benzoate, benzyl cinnamate, bencyl salicylate, butylphenyl methylpropional, cinnamal, cinnamyl alcohol, citral, geraniol, hexyl cinnamal, isoeugenol, alpha-isomethyl ionone, limonene, linalool y methyl-2-octynoate (nombres comunes recogidos en el glosario).

Para la medida de estos 18 analitos se procedió a la optimización del proceso de microextracción. La primera variable a estudiar fue el extractante. Como en el caso anterior, se estudiaron los extractantes habitualmente utilizados en las técnicas SFOD; de todos ellos, el 2-dodecanol fue seleccionado. A continuación se compararon diferentes variantes de LPME, concretamente las técnicas LPME, DLLME y USAEME. La eficiencia de la extracción fue mayor en el caso de la última técnica, y por ello, ésta fue la elegida. El resto de variables fueron estudiadas de forma univariante. Las variables consideradas (y sus valores óptimos) fueron las siguientes: volumen de extractante (50 µL), volumen de muestra (10 mL), tiempo de extracción (5 min), temperatura de extracción (35°C) y adición de sal (150 g/L). Por otro lado, las condiciones de centrifugación (4500 rpm, 15 min), congelación de la gota (250 rpm, 15 min, 3°C) e inyección en HPLC (recogida de 25 µL de fase sedimentada y dilución con 20 µL de metanol) fueron elegidas en base a ensayos previos.

A continuación, el método se validó para los 18 PAS. La linealidad en el rango de estudio (R^2= 0,999-0,948) fue buena. Los LODs tuvieron valores entre 0,001 y 0,154 µg/L y los LOQs entre 0,004 y 0,463 µg/L, lo que permitió la cuantificación de los PAS estudiados a los niveles requeridos en la legislación. La precisión del método se estudió a dos niveles obteniendo buenos resultados para todos los analitos (RSDs <15%). La exactitud se midió en base a estudios de recuperación realizados en un agua de bañera tras el baño de un bebé y en un agua

de colonia, con resultados aceptables. Los factores de enriquecimiento obtenidos variaron entre 9 y 237. Por último, el método propuesto fue aplicado a diferentes muestras reales incluyendo diferentes tipos de cosméticos y aguas recreacionales.

En el **Capítulo 6**, se propone la utilización de técnicas de análisis multivariable para la extensión del método desarrollado en el capítulo 5 a la cuantificación de los analitos que presentaban problemas de solapamiento y cuya determinación no fue posible mediante análisis univariante. En concreto los analitos a estudiar fueron: hydroxycitronellal, coumarin, eugenol, hydroxyisohexyl-3-cyclohexene carboxaldehyde (lyral), citronellol y farnesol. La técnica multivariable que se utiliza para la resolución es PARAFAC2.

El PARAFAC es una técnica de análisis multivariable que descompone una matriz tridimensional de datos en la suma de triples productos de vectores. Los vectores son conocidos como "loadings" y cada triplete de loadings se conoce como "componente". Así, por ejemplo, la matriz tridimensional de datos obtenida en un HPLC-DAD (para cada muestra se recoge un perfil cromatográfico en el que se obtiene un espectro UV-Vis entero para cada tiempo de retención), puede ser expresada como la suma de varios componentes, cada uno de los cuales tiene un perfil cromatográfico (loading cromatográfico) y un perfil espectral definido (loading espectral), cuyo peso relativo en cada muestra está recogido en los "scores" (tercer loading) y puede ser relacionado con la concentración del componente.

El problema que surge al aplicar PARAFAC en cromatografía es que a menudo la variabilidad de los datos no puede ser explicada con un único perfil cromatográfico para cada componente debido, entre otras causas, a variaciones en los tiempos de retención o cambios en las formas de los picos. PARAFAC2 soluciona este problema mediante la definición de un loading cromatográfico diferente para cada muestra.

Para la cuantificación de los analitos solapados, se escogieron únicamente los 4 intervalos del cromatograma donde se encontraron problemas de solapamiento. Para cada intervalo se construyeron modelos adecuados de PARAFAC2 con muestras de calibración, que permitieron descomponer adecuadamente los datos en diferentes contribuciones, algunas de las cuales estaban relacionadas con los analitos estudiados. Las rectas de regresión obtenidas relacionando la concentración de los analitos con los "scores" fueron buenas,

obteniéndose regresiones en todos los casos superiores a 0,978 y LODs para los 6 analitos entre 0,02 y 6,20 μg/mL.

La validación se realizó mediante la construcción de un segundo modelo para cada rango con las muestras de calibración y otros patrones similares a los de las muestras de calibración (muestras de validación). Los modelos permitieron calcular la concentración en las muestras de validación con errores relativos de predicción de entre 6,4 y 18,3%, a pesar de los bajos niveles de concentración utilizados para algunos analitos. Además, se construyó un tercer modelo para cada rango, conteniendo muestras de calibración y muestras reales, mostrándose la aplicabilidad de PARAFAC2 para la determinación de los analitos en muestras con algunas interferencias. Por último, un cuarto modelo para cada rango incluyó las muestras de calibración y diferentes muestras reales dopadas, permitiendo calcular los errores cometidos en la determinación de analitos en matrices diferentes a las de los estándares. Estos errores fueron considerados adecuados.

El capítulo 7 recoge **las conclusiones** generales de la tesis, las cuales se pueden resumir en una: Todas las técnicas basadas en la microextracción en fase líquida dispersiva desarrolladas en este trabajo han demostrado un carácter rápido y ecológico, además de una gran versatilidad para su combinación con diferente instrumentación analítica y para la determinación de analitos de diversa naturaleza en sus correspondientes matrices. Por su parte, las herramientas quimiométricas utilizadas han demostrado una gran utilidad en varias etapas del desarrollo de los métodos: en la optimización, validación y en el análisis de los datos obtenidos.

Publications

Research articles

Pérez-Outeiral, Jessica, Millán, Esmeralda, Garcia-Arrona, Rosa; Use of dispersive liquid-liquid microextraction and UV-Vis spectrophotometry for the determination of cadmium in water samples. **2014**. Journal of Spectroscopy.

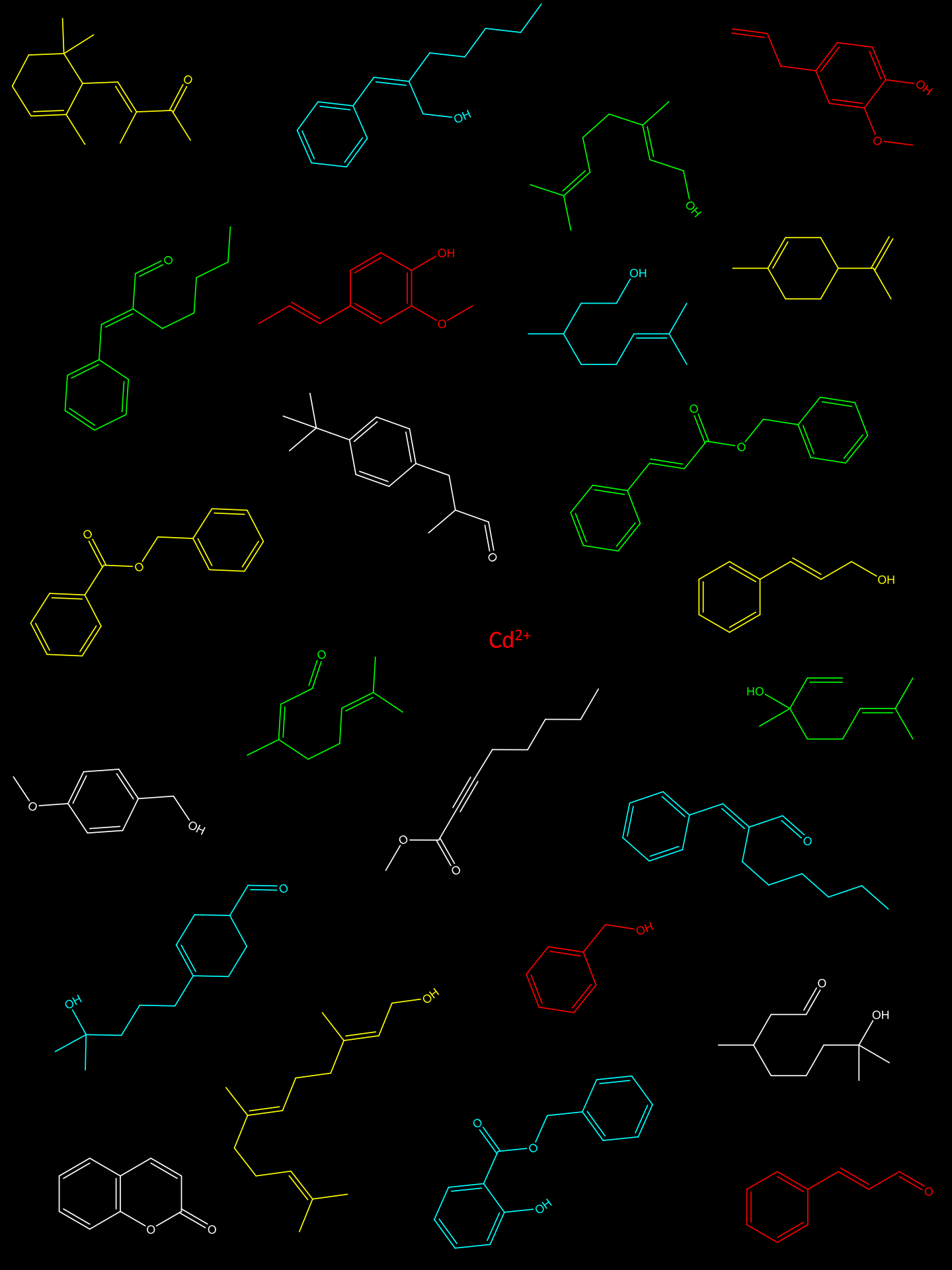
Pérez-Outeiral, Jessica, Millán, Esmeralda, Garcia-Arrona, Rosa; Ultrasound-assisted emulsification microextraction coupled with high-performance liquid chromatography for the simultaneous determination of fragrance allergens in cosmetics and water. **2015**. Journal of Separation Science, 38, 1561-1569.

Pérez-Outeiral, Jessica, Millán, Esmeralda, Garcia-Arrona, Rosa; Determination of phthalates in food simulants and liquid samples using ultrasound-assisted dispersive liquid-liquid microextraction followed by solidification of floating organic drop. **2016**. Food Control, 62, 171-177.

Contributions to conferences

Pérez Outeiral, Jessica, Millán Martín, Esmeralda, García Arrona, Rosa; Desarrollo de un método para la determinación de cadmio basado en la microextracción en fase líquida dispersiva (DLLME) y espectroscopía UV-Vis. XVIII Reunión de la Sociedad Española de Química Analítica, Úbeda (Spain), **16-18 June 2013**.

Pérez Outeiral, Jessica, Millán Martín, Esmeralda, García Arrona, Rosa; Aplicación de la microextracción USAEME-SFOD acoplada con HPLC para la determinación de fragancias potencialmente alérgenas en agua y cosméticos. XX Reunión de la Sociedad Española de Química Analítica, Santiago de Compostela (Spain), **1-3 July 2015**.


Cd2+