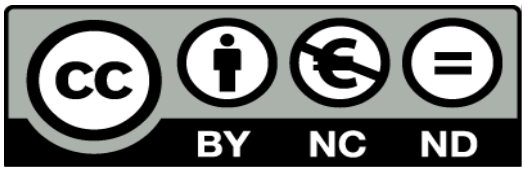
UNIVERSIDAD DE LA RIOJA

TESIS DOCTORAL

Título
Analytical methods for the determination of endocrine disruptors, UV filters and plastic additives in packaging and pharmaceuticals
Autor/es
Cristina Moreta Sánchez
Director/es
María Teresa Tena Vázquez de la Torre
Facultad
Facultad de Ciencias, Estudios Agroalimentarios e Informática
Titulación
Departamento
Química
Curso Académico
2014-2015

Tesis presentada como compendio de publicaciones. La edición en abierto de la misma NO incluye las partes afectadas por cesión de derechos



publicaciones.unirioja.es
E-mail: publicaciones@unirioja.es

Analytical methods for the determination of endocrine disruptors, UV filters and plastic additives in packaging and pharmaceuticals

PhD Thesis 2015

Cristina Moreta Sánchez

Department of Chemistry
Area of Analytical Chemistry
University of La Rioja

ANALYTICAL METHODS FOR THE DETERMINATION OF ENDOCRINE DISRUPTORS, UV FILTERS AND PLASTIC ADDITIVES IN PACKAGING AND PHARMACEUTICALS

A dissertation presented in candidacy for the degree of

International Doctor

CRISTINA MORETA SÁNCHEZ

July 2015

María Teresa Tena Vázquez de la Torre, Catedrática en Química analítica de la Universidad de La Rioja,

Certifica:

Que la memoria titulada "Analytical methods for the determination of endocrine disruptors, UV filters and plastic additives in packaging and pharmaceuticals" que presenta Cristina Moreta Sánchez como compendio de publicaciones, ha sido realizada bajo su dirección en el Departamento de Química de la Universidad de La Rioja y reúne las condiciones exigidas para conseguir el título de doctor internacional tras la estancia realizada en Albany (NY) en el Wadsworth Center bajo la dirección del profesor Kurunthachalam Kannan.

Logroño a 22 de julio de 2015

Fdo: María Teresa Tena Vázquez de la Torre

Para poder realizar una tesis doctoral es imprescindible contar con apoyo económico.

La elaboración de esta tesis no habría sido posible sin la beca de Formación de Personal Investigador y la ayuda para estancias breves en el extranjero que me concedió la Comunidad Autónoma de La Rioja.

Asimismo, este trabajo ha sido realizado gracias a la financiación recibida por AMCOR Flexibles a través del contrato OTEM090908 y por el Ministerio de Educación y Ciencia a través del proyecto CTM 2010-16935 dentro del Plan Nacional de Investigación Científica y Desarrollo e Innovación Tecnológica cofinanciada con fondos FEDER (Fondo Europeo de Desarrollo Regional). También quiero agradecer a AMCOR Flexibles y a Constantia el suministro de muestras y ciertos reactivos, así como a la Universidad de La Rioja por las ayudas para la realización de tesis doctorales subvencionadas por el banco Santander.

Finalmente, quiero también agradecer a la Universidad de La Rioja, a AMCOR Flexibles y al Wadsworth Center el uso de sus instalaciones y equipos.

AGRADECIMIENTOS

¡Hay tantas personas que han formado parte de este proyecto directa o indirectamente! ¡Hay tantas personas que me han ayudado a superar todos los obstáculos, y no han sido pocos, que han ido apareciendo durante la elaboración de esta tesis! Más de uno y una sabéis que realmente llegué a pensar que no podría terminarla. Pero sí, si leéis esto es porque ¡lo conseguí!

Quiero dar primero las gracias a mi directora de tesis, Teresa. Siempre he querido trabajar contigo porque eres ante todo trabajadora, rigurosa, metódica, honrada y leal. Gracias por ofrecerme esta oportunidad, por confiar en mí, por ser exigente, por enseñarme tantas cosas, por "rescatarme" de aquella empresa que tanto me estaba minando. Gracias a eso comprendí que el doctorado me podía ofrecer todo lo que en aquel momento necesitaba. Muchas gracias porque has conseguido que me sienta mucho más segura de lo que hago, porque aunque al principio considero que fuiste muy dura conmigo, me has ayudado y levantado siempre que lo he necesitado, porque me has inculcado valores y principios de trabajo muy valiosos para mí como química analítica. Gracias.

Gracias a David y a Álvaro. Vosotros estuvisteis conmigo en mis primeros pasos. David, trabajador, dulce, constante, SIEMPRE dispuesto a ayudarte y con la mayor paciencia que haya visto jamás. Contigo aprendí que la investigación requiere mucho amor y que da muchos quebraderos de cabeza, pero que cuando sale bien, vale la pena, y todos los agobios y malos ratos parecen lejanos. Álvaro, siempre contento y con mil y una bromas para amenizar el día. Tú me enseñaste lo que no está escrito en los libros, y aunque estemos de acuerdo en mayor o menor medida, el concepto de practicidad que me inculcaste ha sido muy valioso.

Sin embargo, la persona que más aparece en mi mente cuando recuerdo este intenso periodo, es Pilar. Sólo la gente que ha estado contigo, codo con codo, puede llegar a imaginar lo grande que eres. Sin ti no habría superado tantas dificultades. Siempre me has trasmitido tranquilidad y alegría. Tú has sido un pilar indispensable.

Asimismo, Ernesto ocupa también un lugar muy importante. Resolutivo, audaz, positivo y paciente. Siempre me has ayudado y has estado ahí cuando algún equipo se "revelaba". ¡Qué fea manía que a veces tienen de pararse, no estabilizarse, dar cosas raras...! En fin… ¡Qué habría sido de mí sin ti! Yo te hacía doctor *honoris causa* 😉.

ACKNOWLEDGEMENTS

Por el laboratorio y el despacho han pasado también muchas más personas, y con todas ha habido momentos de risas inolvidables, así como momentos duros o de debilidad en los que tanto unos como otros nos hemos desahogado recibiendo siempre palabras de aliento. No voy a decir que esto no suceda en "la empresa", pero este apoyo desinteresado que he visto muchas veces entre los becarios no es tan habitual "ahí fuera". Pilar (necesitaba repetirte), José Miguel, Patricia, Ana J., David, Diego, María, Sofía, Cristina, Nuria, Marivel, Laura, Elisa, Crespo, Ana G., Marcela y Tao. Muchas gracias a todos.

Otros muy buenos momentos que siempre me han alegrado el día han sido con el gran equipo multidisciplinar químico analítico-físicos : Rodrigo, Txino, Judith, Puri y Mayte. Muchas gracias. Asimismo, siempre los becarios de cualquier área nos hemos unido cuando ha hecho falta, sobre todo en reuniones nocturnas… Y eso es también de agradecer . Quiero mencionar en especial a Víctor. Juntos comenzamos el doctorado y hemos luchado por nuestros derechos como becarios FPI-CAR y estudiantes de doctorado. No nos harán ni caso, pero sabemos que no estamos solos .

No podía olvidarme de Pilar Blanco y de todo el servicio de laboratorios: Montse, Amaya, Jorge, Arturo y Sara. Habéis sido también esenciales durante todo el proceso. Vuestra organización y efectividad es de admirar.

I also want to thank to all lab mates from the Wadsworth Center (Albany, NY), and especially to Prof. Dr. Kannan. Thank you for welcoming me into your lab group and for the opportunity you gave me to work with you. It was a complete honor. You are a great person and a great scientist. Thanks also to Ariana, Masato, Xiaohua, Karl, Ying and Ethan (JungKeun) for your friendship and these nice conversations.

Finalmente, también quiero agradecer de forma especial a todos aquellos que fuera del ámbito universitario me han apoyado y motivado durante este trayecto. Muchas gracias a mi familia, en especial a mi padre. Eres la definición de apoyo incondicional. Y también a Roberto. Cariño, tu optimismo y concepto de la vida me han sido imprescindibles. Creo que vosotros dos sois los que más habéis sufrido mis malos momentos y preocupaciones. Gracias por vuestra infinita paciencia. Gracias también a mis amigos. Por suerte sois muchos pero todos habéis sufrido mi falta de tiempo. En especial quiero mencionar a "mis niñas", porque no habéis entendido "una mierda" de lo que he hecho, pero casi ha parecido que era también triunfo vuestro que yo llegara a acabar esto. También a Andrea, eras tú la que siempre me

decía lo “bueno” que era hacer un doctorado cuando empezamos la carrera. ¡Quién me lo iba a decir en ese momento! 😂 Gracias a todos.

Ahora empezaré una nueva etapa y quiero que sigáis formando parte de ella. Y repito una vez más…

¡GRACIAS!

… y nos vemos en los bares 😆…

INDEX

Acronyms/ Abbreviations

ACRONYMS AND ABBREVIATIONS

ACN	Acetonitrile
AO	Antioxidants
APs	Alkylphenols
APPI	Atmospheric pressure photoionization
APCI	Atmospheric pressure chemical ionization
ASAP	Atmospheric solid analysis probe
BDEs	Brominated diphenyl ethers
BDM	INCI: Butyl methoxydibenzoylmethane / INN: Avobenzone
BHA	3-Tert-butyl-4-hydroxyanisole or butylated hydroxyanisole.
BHT	2,6-Di-tert-butyl-4-methyl phenol or butylated hydroxytoluene
BPA	Bisphenol A
BuP	Butylparaben
BZ3	INCI: Benzophenone-3 / INN: Oxybenzone
BzP	Benzylparaben
CCD	Central composite design
CE	Capillary electrophoresis
DAD	Diode array detector
DART	Direct analysis in real time
DCM	Dichloromethane
DESI	Desorption electrospray ionization
DOE	Design of Experiments
DLLME	Dispersive liquid–liquid
EDCs	Endocrine disrupting compounds
EDP	INCI: 2-Ethylhexyl dimethyl PABA / INN: Padimate O
ELSD	Evaporative light scattering detector
EMC	INCI: Ethylhexyl methoxycinnamate / INN: Octinoxate
EMT	INCI: Bis-ethylhexyloxyphenol methoxyphenyl triazine / INN: Bemotrizinol
Eru	Erucamide
ES	INCI: Ethylhexyl salicylate / INN: Octisalate
ESI	Electrospray ionization
ESI(+)	ESI in positive ion mode
ESI(-)	ESI in negative ion mode
EtOH	Ethanol
EtP	Ethylparaben
FID	Flame ionization detection
FTOHs	Fluorotelomers
FUSLE	Focused ultrasonic solid-liquid extraction

GC	Gas chromatography
GM	Geometric mean
GPC	Gel permeation chromatography
HF-LPME	Hollow fiber liquid phase microextraction
^{1}H-NMR	Proton nuclear magnetic resonance
HP136	HP 136: Xylyl dibutylbenzofuranone (reaction product between 5,7-di-tert-butylfuran-2-one and o-xylene)
HpP	Heptylparaben
HRMS	High resolution mass spectrometry
HS	INCI & INN: Homosalate
INCI	International Nomenclature of Cosmetic Ingredients
INN	International nonproprietary names (recommended use in cosmetics by the WHO)
IPE	Ion pair extraction
IR	Infrared spectroscopy
Is126	Irgafos 126
Is168	Irgafos 168
Is126ox	Oxidized Is126
Is126ox2	Double oxidized Is126
Is168ox	Oxidized Is168
IT	Ion tramp
Ix1010	Irganox 1010
Ix1076	Irganox 1076
Ix1024	Irganox MD 1024
Ix3114	Irganox 3114
LC	Liquid chromatography
LLE	Liquid-liquid extraction
LODs	Limits of detection
LOQs	Limits of quantification
LPME	Liquid-phase microextraction
LRM	Linear regression model
LS	Light stabilizers
MAE	Microwave-assisted extraction
MALLE	Membrane-assisted liquid–liquid extraction
MAO	Microwave assisted oxidation
MBC	INCI: 4-Methylbenzylidene camphor / INN: Enzacamene
MBP	INCI: Methylene bis-benzotriazolyl tetramethylbutyl phenol / INN: Bisoctrizole
MED	Minimal erythema dose
MEKC	Micellar electrokinetic chromatography

MeOH	Methanol
MeP	Methylparaben
MIP	Molecularly imprinted polymer
MISPE	Molecular imprinted polymer solid-phase extraction
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MSPD	Matrix solid-phase dispersion
MTBE	Methyl-tert-butyl-ether
NMR	Nuclear magnetic resonance
NPs	Nonylphenols
OCPs	Organochlorine pesticides
OCR	INCI & INN: Octocrylene
OH-MeP	Methyl protocatechuate
OH-EtP	Ethyl protocatechuate
Ole	Oleamide
OTC	Over-the counter
PABA	Para-aminobenzoic acid
PAHs	Polycyclic aromatic hydrocarbons
PAPs	Polyfluoroalkyl phosphate surfactants
PBDEs	Polybrominated Diphenyl Ethers
PCBs	Polychlorinated biphenyls
PE	Polyethylene
PEs	Phthalate esters
PET	Polyester
PFAAs	Perfluoroalkyl acids
PFBA	Perfluorobutanoic acid
PFCs	Perfluorinated compounds
PFCAs	Perfluorocarboxylic acids
PFDA	Perfluorodecanoic acid
PFDoA	Perfluorododecanoic acid
PFHpA	Perfluoroheptanoic acid
PFHxA	Perfluorohexanoic acid
PFNA	Perfluorononanoic acid
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctanesulfonic acid
PFPeA	Perfluoropentanoic acid
PFASs	Perfluoroalkyl substances
PFSAs	Perfluoroalkyl sulfonic acids
PFUnA	Perfluoroundecanoic acid

PLE	Pressurized liquid extraction
^{31}P-NMR	Phosphorus nuclear magnetic resonance
POP	Persistent Organic Pollutants
PP	Polypropylene
PrP	propylparaben
QqQ	Triple quadrupole
QRM	Quadratic regression model
RAM	Restricted access material
REACH	Registration, evaluation, authorization and restriction of chemicals
RRT	Relative retention time
RSD	Relative standard deviation
RT	Retention time
SA	Slip agents
SBSE	Stir bar sorptive extraction
SD	Standard deviation
SDME	Single-drop microextraction
SFE	Supercritical fluid extraction
SLE	Solid-liquid extraction
SPE	Solid-phase extraction
SPF	Sun protection factor
SPME	Solid phase microextraction
T328	Tinuvin 328
T770	Tinuvin 770
TBA	Tetrabutylammonium
TDA	Tetradecanamide
TDS	Total dissolved solids
THF	Tetrahydrofuran
TOF	Time of fight
UAE	Ultrasound-assisted extraction
UV	Ultraviolet
VWD	Variable wavelength detector
WHO	World health organization
^{1}H-NMR	Proton nuclear magnetic resonance
^{31}P-NMR	Phosphorus nuclear magnetic resonance
2-PrOH	2-Propanol
3,4-DHB	3,4-Dihydroxybenzoic acid or protocatechuic acid
4-HB	4-Hydroxybenzoic acid

Summary

SUMMARY

This thesis entitled *"Analytical methods for the determination of endocrine disruptors, UV filters and plastic additives in packaging and pharmaceuticals"* is presented as a compilation of publications.

Most of these research papers deal with the development, optimization and validation of different analytical methods to determine ultraviolet (UV) filters, plastic additives or perfluoroalkyl acids (PFAAs) in packaging. In each work, a focused ultrasonic solid-liquid extraction (FUSLE), a fast, simple and relatively new extraction technique, is developed and optimized to extract these analytes. Moreover, the efficiency of the FUSLE method is evaluated in each work by comparing it with other extraction techniques. Finally these methods are also applied to the analysis of several real samples. In the case of the method for UV filters, it was also applied to evaluate the migration and delamination capacities of these analytes through different contact layers in multilayer packaging.

On a line parallel to these works, an analytical method for the analysis of parabens in pharmaceuticals was also developed, however, in this case only a traditional solid-liquid extraction (SLE) was used. Moreover, the optimized parabens method was also applied to the analysis of more than one hundred samples in order to evaluate if the use of both oral and topical medicines may be a source of exposure to parabens. This research was carried out during a six-month predoctoral stay in the Wadsworth Center (Albany, NY, USA).

A brief and comprehensive summary of each chapter as well as the main results of each work are presented below.

C**hapter 1** is an introduction that describes the families of compounds studied and the more characteristic methodologies employed during this thesis: FUSLE technique and design of experiments (DOE).

Chapter 2 is devoted to UV filters. In **chapter 2.1**, a FUSLE and high performance liquid chromatography (HPLC) coupled to a diode array detector (DAD) method is proposed for the determination of ten liposoluble UV filters in multilayer flexible packaging. This original method was developed because UV filters showed to be some of the most active cosmetic ingredients involved in the deterioration of multilayer packaging in recent studies.

The FUSLE-HPLC-DAD method was validated and applied to the analysis of polyethylene-based multilayer packaging samples. It provided satisfactory features

for most UV filters in polyethylene (PE) film, such as limits of detection (LODs) between 0.4 and 8.5 ng/mg (but for Avobenzone 66 ng/mg), and repeatability and reproducibility values below 5 and 14%, respectively, for most of them.

This study demonstrated that FUSLE is a useful, fast and simple extraction methodology for UV filters due to the complete extraction achieved with just 6 ml of tetrahydrofuran (THF) in only one cycle of 30 s. Furthermore, the proposed method was evaluated with respect to THF extraction under reflux for 2.5h and FUSLE was more efficient for all the analytes except for Bemotrizinol (58%) and Avobenzone (74%).

This work was published in the Journal of Chromatography A.

In **chapter 2.2**, the analytical method previously developed was used to evaluate the UV-filters absorption capacity through the inner layer of promotional multilayer sachets in contact to several creams. Moreover, the loss of adhesion between the layers caused by these migration processes was also evaluated by the T-peel test. For this purpose, different sachets with polyethylene (PE), polypropylene (PP), polyester (PET) and Barex as contact layers were filled with creams containing or not these compounds. The filled sachets were monitored for three months keeping them in an oven at 40ºC in order to accelerate the absorption and delamination processes.

The study revealed that the four packaging suffered significant loss of adhesion strength. Moreover, PE and PP absorbed similar amounts of UV filters, while PET absorbed a much higher amount of UV filters and Barex absorbed them very poorly.

This work was performed under a research contract with the company Tobepal, and the results could not be published due to a confidentiality clause.

In **chapter 3**, a simple and sensitive analytical method for the determination of several plastic additives in multilayer flexible packaging based on solid-liquid extraction (SLE) and ultra-high performance liquid chromatography (UHPLC) coupled to UV and time of flight mass spectrometry (TOF-MS) detectors is presented. This method was developed in order to simplify and enhance the quality control of these materials.

The developed method allowed the simultaneous determination in only 9 min of two slip agents, two light stabilizers, ten antioxidants and two oxidation products with LODs until 5000 times more sensitive than other GC-FID and HPLC-UV methods previously reported.

FUSLE was optimized and evaluated to extract plastic additives from packaging. Moreover, extraction results obtained by FUSLE and SLE were compared to those obtained by pressurized liquid extraction (PLE). All extraction methods showed excellent extraction efficiencies for slip agents, however a quantitative recovery of all analytes was achieved only by SLE with just 5 ml of hexane for 10 hours.

The selected SLE-UHPLC-UV-TOF-MS method was validated. LODs between 0.03 and 0.7 μg/g, matrix effect for only one additive and excellent repeatability and intermediate precision values (less than 6 and 8%, respectively) were obtained using TOF-MS. Finally, the selected method was applied to the analysis of several packaging samples.

Chapter 4 includes two works on PFCs. In **chapter 4.1**, a FUSLE and UHPLC coupled to quadrupole-time of flight mass spectrometry (QTOF-MS/MS) method is proposed to determine perfluorooctanesulfonate (PFOS) and six perfluorocarboxylic acids (PFCAs), from seven to twelve carbon chain lengths, in food-contact packaging. This novel method was developed due to the estrogenic effects of these substances and their use in food-contact packaging as paper coatings.

The chromatographic separation took place in less than 4 min and the optimized FUSLE was carried out with just 8 ml of ethanol in one cycle of only 10 s. The FUSLE-UHPLC-QTOF-MS/MS method provided good LODs between 0.5 and 2.2 ng/g, repeatability and intermediate precision values below 11% and 15%, respectively, and recovery values around 100% in all cases. Moreover, FUSLE efficiency was evaluated against pressurized liquid extraction (PLE) and no significant differences were found between them.

Finally, several real food-contact packaging samples were analyzed. They showed PFAAs concentrations between 4 and 29 ng/g, being PFHpA (perfluorooctanoic acid) by the far the most abundant of them.

However, apart from PFHpA, the PFAAs levels found were very close to the limits of quantification (LOQs) or detected but not quantifiable. Moreover, qualitative analysis of some microwave popcorn bags showed very high concentrations of PFCAs with carbon chain lengths between four and six.

Therefore, in **chapter 4.2**, this method was improved in order to decrease the LODs, to include the PFCAs with carbon chain lengths from four to six and to apply it for not only microwave popcorn bags but also the corn inside before and after cooking.

The new PFAAs method was validated in the three matrices. It showed recovery values around 100% except for the lowest chain length PFCAs, reproducibility with RSDs under 16%, and LODs between 0.2 and 0.7 ng/g.

Finally, this method was applied to the analysis of six microwave popcorn bags and the corn inside before and after cooking. PFCAs contents between 3.50 ng/g and 750 ng/g were found in microwave bags, being PFHxA (perfluorohexanoic acid) the most abundant of them. However, no PFAAs were detected either corn or popcorn, and therefore no migration was assumed.

The works presented in chapter 4 have been published in two research papers of the Journal of Chromatography A.

In **chapter 5**, a method based on SLE or liquid-liquid extraction (LLE), depending on sample state, and HPLC coupled to triple quadrupole mass spectrometry (QqQ-MS/MS) was developed for the determination of the six most frequently used parabens and four paraben derivatives in pharmaceuticals. This method was developed due to the estrogenic activity of parabens and the little knowledge of their presence in pharmaceuticals.

A clean-up step by solid-phase extraction (SPE) had to be included before HPLC injection due to the high matrix effect observed for solid pharmaceuticals. Two SPE methods using two different sorbents were successfully optimized. The HLB-SPE method was selected because it decreased more the signal suppression than the NH_2-SPE one. The SLE-HLB-HPLC-QQQ-MS/MS method provided recoveries from 89% to 98% and RSDs lower than 13%.

Finally, the developed method was applied for the analysis of 128 different drugs collected from the USA and a few other countries in Europe and Asia. Although the majority of medicines analyzed did not contain parabens, concentrations up to 2 mg/g were found in some samples. Methyl- and propyl-parabens were the more frequently detected compounds and the 4-hydroxybenzoic acid was the major metabolite found in pharmaceuticals.

Finally, the general conclusions of all works are exposed in **conclusions** section.

List of papers

LIST OF PAPERS

The following research papers have been included in this thesis:

Chapter 2. UV filters in packaging.

2.1. *Determination of UV filters in packaging by focused ultrasonic solid-liquid extraction and liquid chromatography.*

C. Moreta and M.T. Tena, Journal of Chromatography A 1218 (2011) 3392-3399.

2.2. *Multilayer packaging delamination caused by UV filters: Influence of contact layer on chemical resistance and absorption capacity of sachets.*

C. Moreta and M.T. Tena, Contract OTRI OTEM090908 (2010).

Chapter 3. Plastic additives in packaging.

3.1. *Determination of plastic additives in packaging by liquid chromatography coupled to high resolution mass spectrometry.*

C. Moreta and M.T. Tena, (submitted) Journal of Chromatography A, 2015.

Chapter 4. PFAAs in packaging and food samples.

4.1. *Fast determination of perfluorocompounds in packaging by focused ultrasound solid-liquid extraction and liquid chromatography coupled to quadrupole-time of flight mass spectrometry.*

C. Moreta and M.T. Tena, Journal of Chromatography A 1302 (2013) 88-94.

4.2. *Determination of perfluorinated alkyl acids in corn, popcorn and popcorn bags before and after cooking by focused ultrasound solid-liquid extraction, liquid chromatography and quadrupole-time of flight mass spectrometry.*

C. Moreta and M.T. Tena, Journal of Chromatography A 1355 (2014) 211-218.

Chapter 5. Parabens in pharmaceuticals.

5.1. *Parabens and their derivatives in pharmaceuticals.*

C. Moreta, M.T. Tena and K. Kannan, (submitted) Environmental Research, 2015.

Objectives

OBJECTIVES

The purpose of this thesis was to develop, optimize, validate and apply fast and simple analytical methods for the quality control of multilayer flexible packaging and for determining endocrine disrupting compounds (EDCs). To do this, the main extraction technique employed was the focused ultrasound solid-liquid extraction (FUSLE), a relative new, fast and low cost extraction technique.

Accordingly, the research works collected in this PhD thesis were carried out with the following objectives:

1. To study the advantages of using a new extraction technique as FUSLE for the analysis of multilayer flexible packaging.
2. To optimize the main FUSLE continuous variables by design of experiments (DOE).
3. To compare the efficacy of the developed FUSLE methods with others techniques such as pressurized liquid extraction (PLE), reflux extraction or traditional solid-liquid extraction.
4. To apply the optimized FUSLE methods to packaging quality control, especially to study migration and delamination processes.
5. To determine simultaneously several kind of polymer additives in plastic-based multilayer packaging by liquid chromatography (LC) coupled to high resolution mass spectrometry (HRMS).
6. To develop new methods for the determination of EDCs, such as parabens and perfluoroalkyl acids (PFAAs), in packaging and pharmaceuticals.
7. To determine the levels of the above mentioned EDCs in pharmaceuticals and food-contact packaging.

1.

Introduction

1. INTRODUCTION

In order to introduce the research papers presented in this thesis, the main issues discussed are described in this chapter.

Firstly, in order to obtain a general overview, a scheme of the relationship between the works that make up this dissertation is shown in Figure 1.1 As may be seen, this thesis principally deals with the use of the focused ultrasound liquid-solid extraction (FUSLE) technique for the extraction of UV filters, plastic additives and perfluoroalkyl acids (PFAAs) from packaging. To do this, the principal variables of each FUSLE method were optimized by design of experiments (DOE).

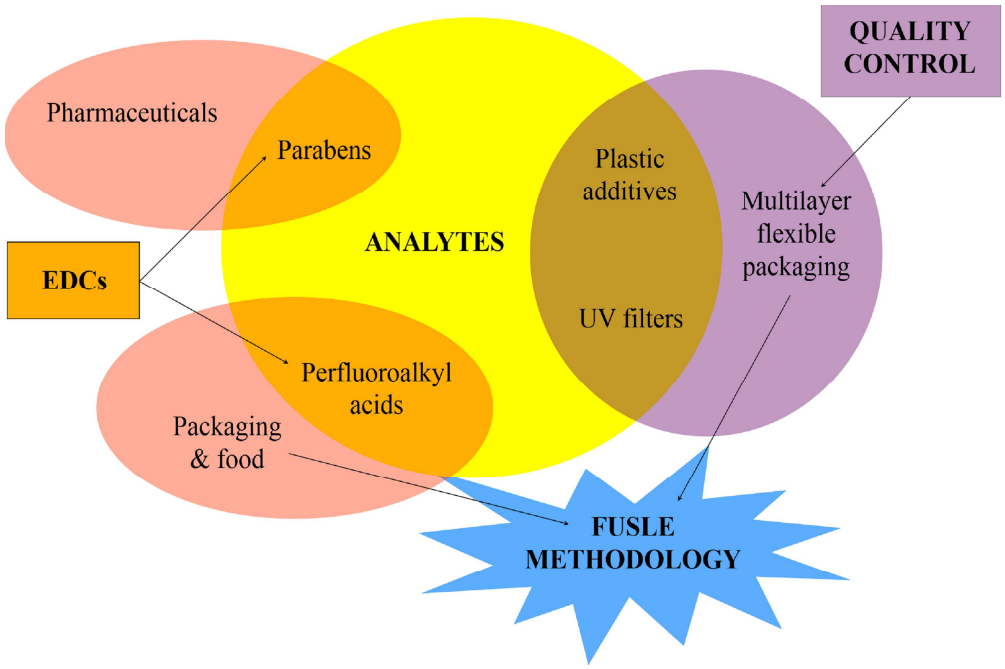


Figure 1.1. Relationship between the works carried out in this thesis.

Figure 1.1 also shows that the analytical methods to determine UV filters and plastic additives in multilayer flexible packaging were developed for quality control of this kind of material. However, a quantitative method for determining PFAAs was developed due to the estrogen-like properties of them. Precisely because of the estrogenic nature of the PFAAs and some UV filters, a work on the determination of the endocrine disrupting parabens in pharmaceuticals was performed during my stay in the Wadsworth Center under the supervision of the Dr. Kurunthachalam Kannan.

However, the extraction of parabens were performed only by traditional solid-liquid extraction (SLE) using a shaker.

Finally, due to the polar character and relatively high molecular weight of most analytes, the chromatographic separations were always carried out by high or ultra high performance liquid chromatography (HPLC or UHPLC). In addition, single or tandem mass spectrometry (MS or MS/MS) was the main detection technique used. Different types of mass spectrometers were used: quadrupole-time of flight (QTOF), time of flight (TOF) or triple quadrupole (QqQ). An UV detector was also used for the determination of UV filters due to their high UV absorbance and their relatively high concentration in the samples.

1.1 Quality control in multilayer flexible packaging

1.1.1. Multilayer flexible packaging

Multilayer flexible packaging are made of several flexible layers perfectly joined together. The layers used are generally different kind of polymers such as polyethylene, polypropylene, etc., aluminum foil or paper. These layers can be joined in two ways: by adhesive lamination, where an adhesive film is applied between the layers, or by extrusion coating lamination, where an extruded molten polyethylene film binds the layers.

Several multilayer flexible laminates before forming the final package are shown in Figure 1.2. These types of packaging are commonly used to contain a huge number of food, cosmetic and pharmaceutical products providing a warranty seal to preserve the product unaltered for promotion and sale at low cost.

Figure 1.2. Different kinds of multilayer flexible packaging.

The layers that make up each multilayer flexible packaging are determined by the final features required for proper storage of the product. In this way, the

properties of a laminate is much better than each layer material by itself. Moreover, the addition of plastic additives can enhance or protect the properties of each polymeric layer. Therefore, the versatility and potential of this type of packaging are vast. During this thesis, different multilayer plastic packaging and microwave popcorn bags were studied (Figure 1.3).

On one hand, in the case of multilayer flexible sachets for promotion and sale, one or two sort of polymers and an aluminum foil are often joined. Polymers provide excellent qualities for use in packaging because they are flexible, relatively inexpensive and a water barrier. However, they normally offer poor oxygen and light barriers. Hence, an aluminum foil is usually added between the plastic layers to overcome these problems obtaining a packaging with a totally hermetic barrier.

Figure 1.3. Promotional multilayer sachets of a cosmetic cream and a microwave popcorn bag.

On the other hand, in the case of microwave popcorn bags, paperboard is coated with plastics to combine the mechanical properties of the paperboard with the barrier and sealing properties of plastics.

Packaging - product interaction: migration

The main downside of multilayer flexible materials is the interaction between the packaging and the product through the inner layer, especially when the moisture and fat content in the product is high. Migration processes from the continent to the content and vice versa may occur due to a concentration gradient between them.

On one hand, toxic substances to human health or compounds involved in packaging quality may migrate from packaging to the product. This process can endanger the human health, the quality of the product and the quality of the

laminate. This is an especially big concern when the content is food because it is the most direct route of human exposure to harmful substances.

A possible example would be the presence of perfluoroalkyl substances used in microwave popcorn bags as paper coatings in food contact packaging. Unfortunately, the perfluoroalkyl substances have shown an estrogenic activity and they could migrate to the popcorn during the microwave cooking.

On the other hand, essential ingredients or aggressive compound from the product may also migrate to the packaging. This process can also endanger the quality of the product but, above all, the greatest concern is that aggressive compounds may reach the interfaces and weaken the adhesion strength between the layers. It is extremely important that the packaging maintains a strong and stable bond between the layers to fulfill its purpose safely and effectively. When the loss of adhesion strength adversely affects the quality of the laminate, it is called delamination.

For example, some cosmetics ingredients such as UV filters, perfumes, etc. may present a negative compatibility with traditional multilayer flexible packaging affecting the stability between the layers due to migration processes [1-4].

Delamination

As commented before, it is essential that the layers remain perfectly joined together in order to ensure the quality and shelf life of the packaged products. Delamination is an extremely undesired process where layers perfectly joined at the beginning are separated. An example of a delaminated sachet is shown in Figure 1.4.

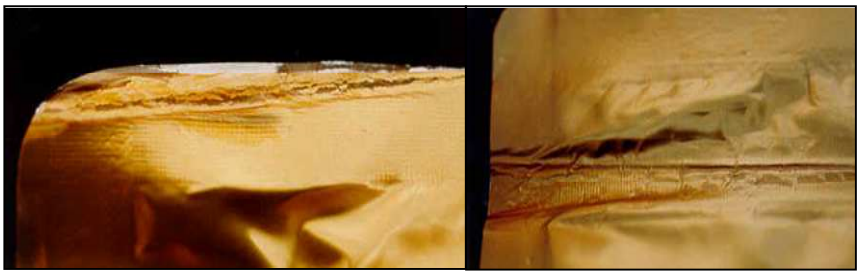


Figure 1.4. Delaminated sachet.

Studies of several mechanisms and physical phenomena related to delamination can be found in literature [2, 5, 6]. However, it is still necessary to perform laminate quality controls prior to bring the packaging onto the market in

order to avoid not only a possible product spoilage but also the negative publicity of the product and even the loss of millions.

The most common way to assess the chemical resistance of a packaging in contact with a specific product is through trial and error. To do this, the packaged product is kept at 40ºC for three months. After that, the laminate should not show any sign of deterioration and the adhesion strength between layers of the material must be higher than 100 N/m (75 g/15mm). This adhesion strength is normally measured by the T-peel test based on a 180º orientation. This experimental method generally reflects very well how the packaging will be affected by the product. A machine to measure the adhesion strength is shown in Figure 1.5.

Figure 1.5. Peel Strength Testing Machine. T-peel test.

1.1.2. UV filters

Ultraviolet (UV) emission comes from the sun. UV light is an electromagnetic radiation between 400 nm and 100 nm. As may be seen in Figure 1.6, there are three kind of UV radiations depending on their wavelengths: UVA, UVB and UVC and the lower the wavelength is (higher energy), the more dangerous is the radiation. Fortunately, the ozone molecules of the stratosphere absorb completely the UVC radiation and most of the UVB radiation. Moreover, UVA radiation, the less dangerous, is able to penetrate into the deeper layers of the skin activating the production of melanin and therefore the tan. Nevertheless, the exposure to high levels of UV radiation remains harmful to a greater or lesser extent and it can intensify or cause erythema (sunburn), skin aging, wrinkles, loss of elasticity, irritation, spots and even skin cancer among others (Figure 1.6). Unfortunately, due

to the trend for people to sunbathe, the number of cases of skin cancer has increased significantly in recent years.

In order to prevent or minimize these negative effects, the use of UV filters is recommended. In accordance with the European Regulation on cosmetic products, UV filters are substances which are exclusively or mainly intended to protect the skin against certain UV radiation by absorbing, reflecting or scattering UV radiation [7]. Nowadays, these UV protectors are added not only in sunscreens but also in a wide variety of day personal care products such as daily skin or hair care products.

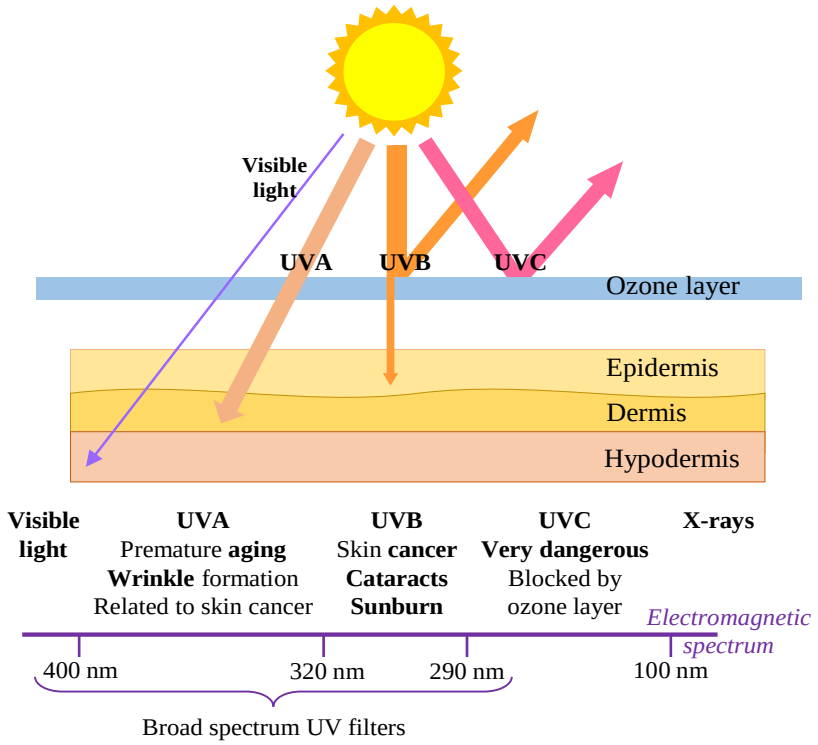


Figure 1.6. Description of the penetration of the three solar UV radiations in the earth and in the skin, their wavelengths and their negative effects on the skin.

UV filters are classified in two group depending on how interact with the UV radiation, chemical or physical UV filters, and depending on their chemical nature, organic or inorganic UV filters (Figure 1.7). Organic or chemical UV filters are aromatic compounds often with double bonds and/or carbonyl groups that absorb the UV light and convert it into a small amount of heat. However, inorganic or physical UV filters are oxide metals that scatter and/or reflect the UV radiation as a protective shield. The organic UV filters are the most commonly used. However, an

inorganic UV filter is also often added into creams in order to increase the efficacy. Likewise, a combination of several different organic UV filters is frequently used to provide a good broad spectrum of protection (UVA + UVB) because each UV filter is more effective at different UV wavelengths (maximum absorbance).

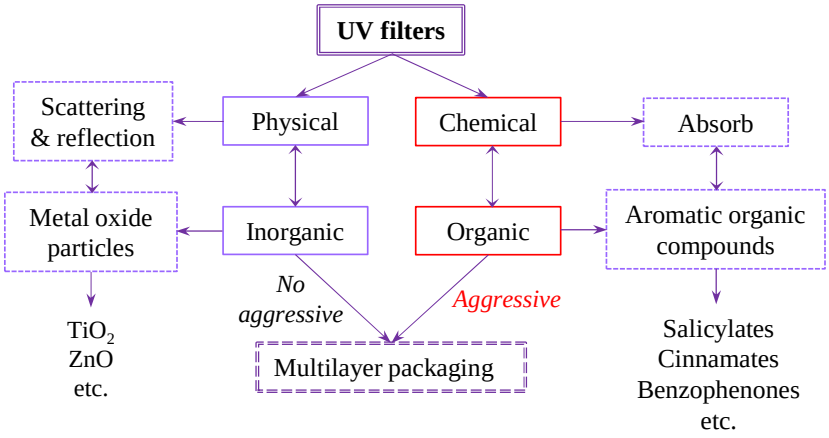


Figure 1.7. Classification of UV filters. Behavior in multilayer flexible packaging.

The efficacy and protection level against the UV light of a cosmetic is evaluated and estimated experimentally. This protection level is commonly expressed by the sun protection factor (SPF). The SPF is the ratio between the amount of UV energy required to produce a minimal erythema dose (MED) on protected and unprotected skin. Many times, SPF is also defined as the ratio between the sun exposure time that it takes to burn with and without sunscreen protection. This latter is more intuitive although it is not rigorous. It must be clear that a higher SPF implies more protection against the UV radiation but no longer time of protection.

The safety, maximum allowed concentrations and efficacy of UV filters in personal care products are regulated and approved by national and international health authorities. Until now, only 26 UV filters are allowed in cosmetic products in Europe [7] (PABA was banned at the end of 2008 although it can still be observed in the list). Only one is an inorganic UV filter (TiO_2). Seven of the 25 organic UV filters are hydrophilic while the other 18 are lipophilic [8]. The latter are much more commonly used in cosmetic formulations due to their water resistance. In addition, both kind of organic UV filters require separate chromatographic conditions due to the different nature characteristics between them [9, 10]. Therefore, the lipophilic UV filters were the selected ones in this thesis to evaluate their migration and

delamination capacity in multilayer flexible packaging. The chemical structure of the ten fat soluble UV filters analyzed is shown in Figure 1.8.

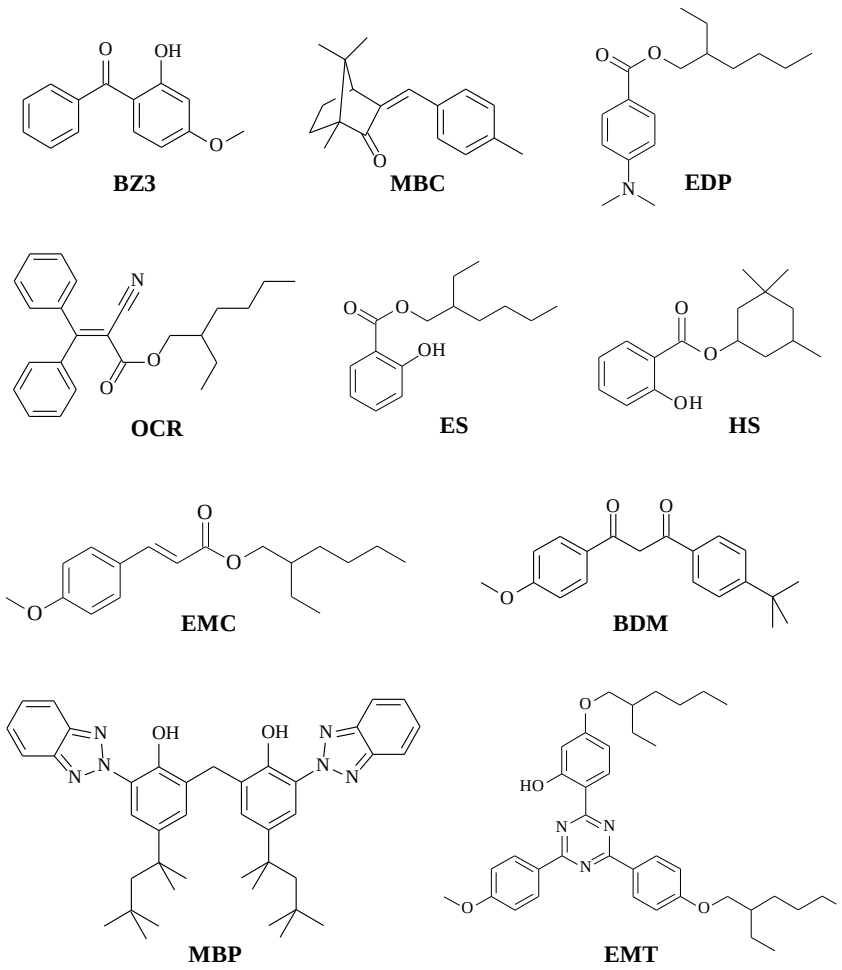


Figure 1.8. Chemical structure of the ten UV filters studied. International nonproprietary names (INN): BZ3, Oxybenzone; MBC, Enzacamene; EDP, Padimate O; EMC, Octinoxate; ES, Octisalate; MBP, Bisoctrizole; OCR, Octocrylene; BDM, Avobenzone; HS, Homosalate; EMT, Bemotrizinol.

The different reasons why UV filters have been analyzed in several matrices in recent years are shown Figure 1.9. The development of analytical methods for determination of UV filters [8-12] have been mainly performed in personal care products due to the regulation of their presence and maximum concentration in cosmetics. However, due to the excessive use of cosmetics containing these compounds in recent years, the UV filters have reached the aquatic environment

mostly through recreational activities. Then, they have been being accumulated especially in water as well as fat soluble UV filters in aquatic biota. Hence, they are currently considered as emerging contaminants and there are not yet enough reliable data to understand the global distribution and the effect of UV filters in the ecosystems. Therefore, they have been analyzed in several environmental matrices [8, 13-15] (Figure 1.9).

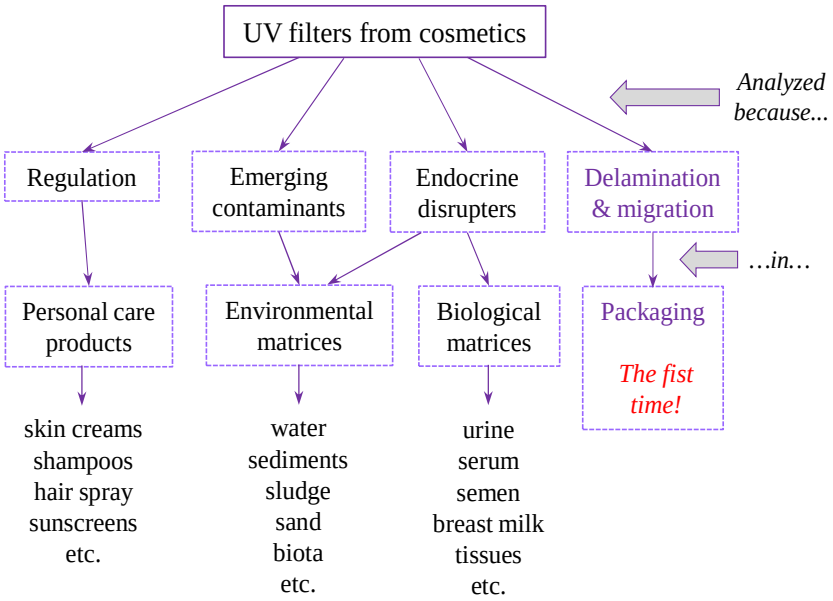


Figure 1.9. Reasons for the analysis of organic UV filters and matrices analyzed.

In addition, the estrogenic activity related to the absorption thorough the skin of some UV filters such as benzophenone derivatives, MBC and EMC present in sunscreens [8, 16, 17] has raised the public alarm and consequently, the number of studies in biological samples. Between them, BZ3 and its metabolites have been by far the most compounds studied in biological matrices [18, 19]. Nevertheless, this has been the first time that UV filters have been analyzed in packaging.

In order to analyze UV filters in cosmetics, the sample has been frequently dissolved with the aid of an ultrasonic bath and injected directly into the chromatographic system. In other matrices, liquid-liquid extraction (LLE), solid-phase extraction (SPE) and SLE are the common techniques to extract UV filters [18]. However, a broad variety of different extraction methods can be found in literature such as Soxhlet, pressurized liquid extraction (PLE), microwave-assisted

extraction (MAE), different liquid-liquid microextraction methods, solid-phase microextraction (SPME), etc [13, 18].

In addition, complex and dirty samples have been usually cleaned and/or concentrated using different solid-phase extraction (SPE) methods. Likewise, gel permeation chromatography (GPC) has been also used for cleaning rich-lipid biological matrices [13, 18].

Regarding the chromatographic separation and detection, liquid chromatography (LC) coupled to a UV detector or diode array detector (DAD) has been by far the most used analytical method for the determination of UV filters due to their polarity and high UV absorbance. LC provides the analysis of all organic UV filters and their metabolites without a derivatization step, contrary to gas chromatography (GC) analysis because of the low volatility of most UV filters. However, GC has been also quite used for the analysis of environmental and biological samples [13] probably because a MS detector has been required in those cases and a GC-MS equipment has been more affordable than a LC-MS for many years. MS or MS/MS are the key technique when environmental and biological samples are analyzed because of their complex matrices, low concentrations and the need for a more selective detector [13, 18]. In these cases, the use of UV has been unsuitable. Hence, nowadays it is increasingly common to find LC-ESI-MS/MS methods to determine UV filters in current literature.

1.1.3. Plastic additives

Plastic additives are mainly incorporated into polymers in order to modify or enhance their properties, to increase their shelf life or even to cut the price of the final product. The variety of polymer additives to get this is overwhelming as can be seen in Figure 1.10. Additives can be classified in three groups based on its purpose or function: stabilizers, modifiers and fillers.

Stabilizers are added to conserve original polymer properties such as strength, flexibility, color, etc. in order to increase its lifetime in good conditions. Therefore, stabilizers protect the valuable features when the polymer is exposed to harmful conditions such as high temperatures, light, oxygen, etc.

However, modifiers are incorporated to improve or alter the physical and chemical properties as well as the performance of the polymer. Finally, fillers are principally used to dilute the matrix with something less expensive but without compromising the properties of the final polymer. Some fillers can also improve

strength of the plastic. Both, modifiers and fillers, provide additional qualities or lower the price of the polymer in order to increase its final value.

Polymer additives may need to be monitored in order to identify stability problems in a polymer, for production control, to compare products from different manufacturers and for regulatory reasons. Therefore, it is very important to develop analytical methods to determine those substances and so ensure that the plastic packaging is adjusted to its purpose safely.

Antioxidants (AO), light stabilizers (LS) and slip agents (SA) were the plastic additives analyzed in this thesis because these are the most often added into the polymeric layers in flexible multilayer packaging (Figure 1.10, written in purple).

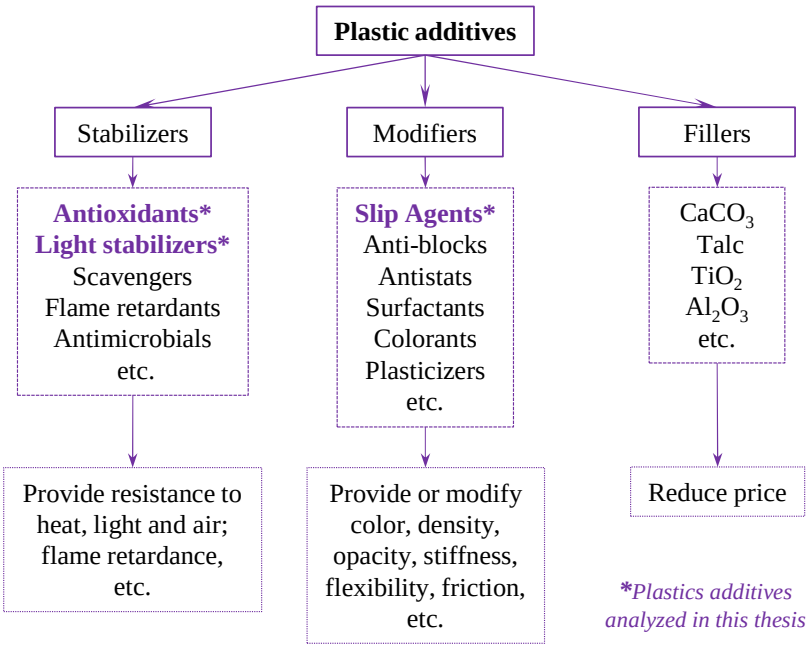


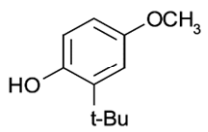
Figure 1.10. Classification of plastic additives.

The chemical structures of the additives studied are shown in Figure 1.11.

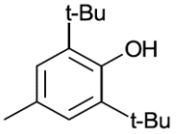
Antioxidants are added to slow down oxidation processes. For example, hindered phenolic antioxidants scavenge free radicals and phosphite antioxidants decompose peroxides that provides protection to melt flow and color during the thermal processing of polymers. Both antioxidants are usually used together to achieve synergistic performance. Their use avoid possible loss of strength, breakdown, discoloration, scratching, flexibility, stiffness or gloss.

Light stabilizers such as benzophenones, hindered amines and benzotriazoles, protect the polymer against photodegradation from UV or sunlight that may cause discoloration, cracking or brittleness among others.

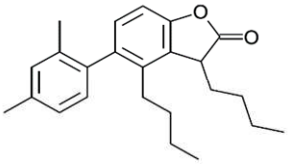
Slip agents are long chain fatty acid amides that produce a lubricating effect reducing the coefficients of friction (resistance of a film to sliding) for post-processing operations.



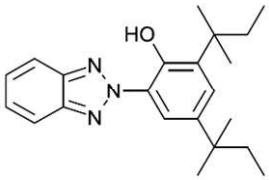
BHA (AO.1)
Butylated hydroxyanisole



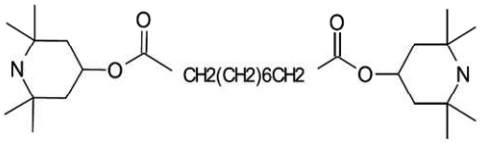
BHT (AO.1)
Butylated hydroxytoluene



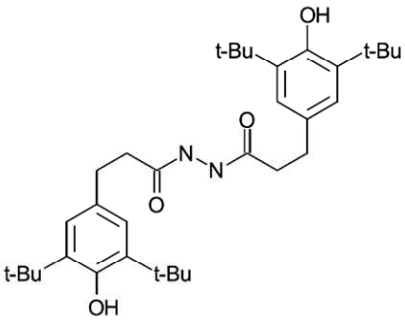
HP136 (AO.3)
Xylyl dibutylbenzofuranone



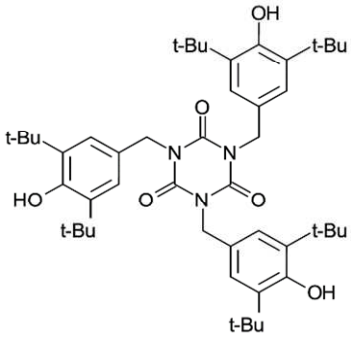
Tinuvin 328 (LS)



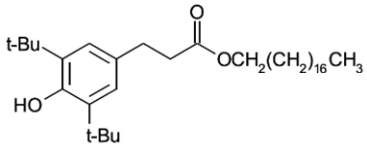
Tinuvin 770 (LS)



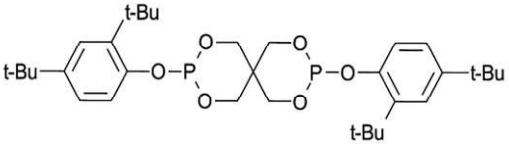
Irganox MD 1024 (AO.1)



Irganox 3114 (AO.1)

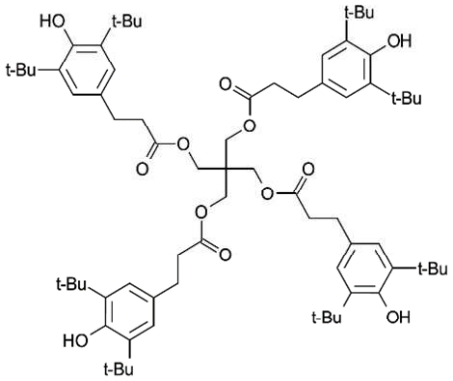


Irganox 1076 (AO.1)

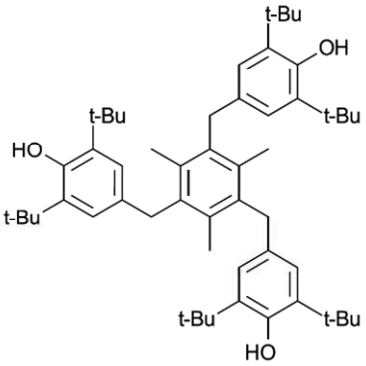


Irgafos 126 (AO.2)

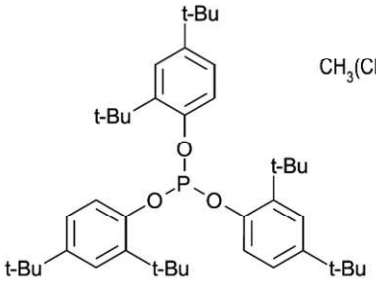
Figure 1.11. Chemical structures of the additives studied in this thesis. AO.1-hindered phenolic antioxidant, AO.2-phosphite antioxidant, AO.3-lactone antioxidant, LS–light stabilizer, SA-Slip agent.



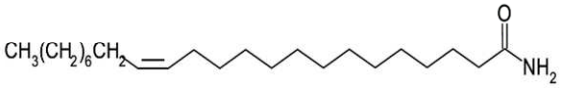
Irganox 1010 (AO.1)



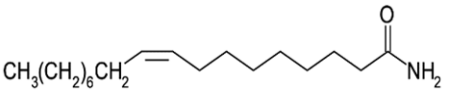
Irganox 1330 (AO.1)



Irgafos 168 (AO.2)



Erucamide (SA)



Oleamide (SA)

Figure 1.11. Cont. Chemical structures of the additives studied in this thesis. AO.1-hindered phenolic antioxidant, AO.2-phosphite antioxidant, AO.3-lactone antioxidant, LS–light stabilizer, SA-Slip agent.

The extraction and determination of plastic additives from polymers have been reviewed by Vandenburg at al. and Reingruber et al. [20, 21]. A scheme of the principal techniques used for quantitative analysis of additives in plastic is shown in Figure 1.12. On one hand, the extraction of additives has been traditionally performed by dilution-precipitation, Soxhlet or reflux [21]. However, since the nineties these extractions have been mainly carried out by ultrasonic assisted extraction (UAE) and other contemporaneous strong extraction methods.

On the other hand, due to the direct contact of plastic packaging to the content, the migration of additives to consumer products, principally food, is a concern for the health or safety of the population and some studies have been performed [22-24]. The methodology for these cases is different. After the migration process, the main objective is to concentrate the extract or food simulant. For this, SPE and different microextraction techniques have been employed (Figure 1.12).

After extraction, additives have been generally separated chromatographically by LC because the high molecular weight and low volatility of the majority of plastic additives makes GC generally unsuitable. Moreover, plastic additives have been mainly determined using a UV detector because the majority of these compounds have aromatic groups or double bond conjugated that absorb UV light. However, others additives such as slips agents do not present this characteristic. Therefore, these additives that also present low molecular weight have been separated preferably by GC because it offers better facilities for their detection [25, 26].

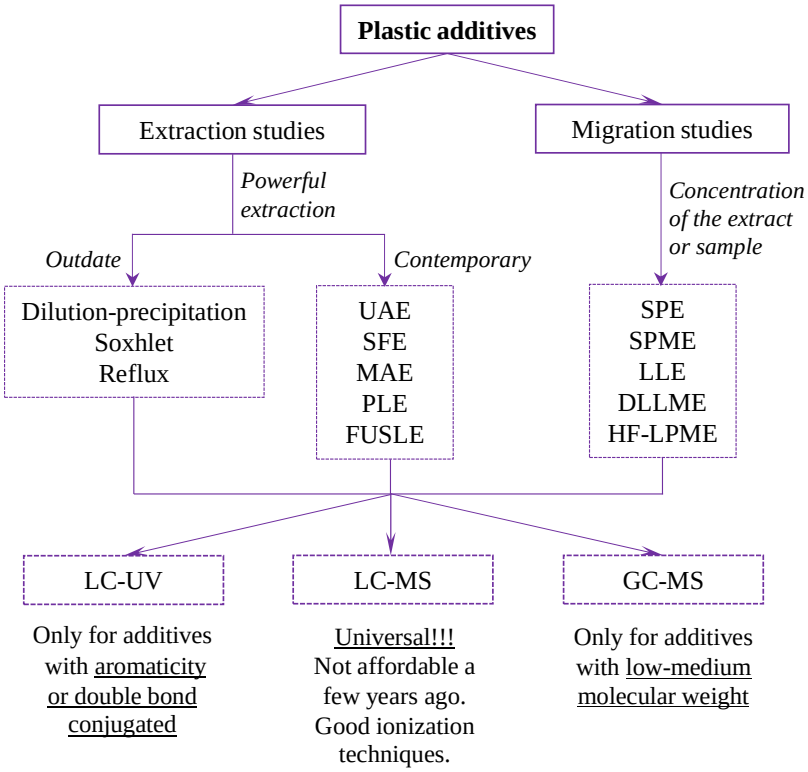


Figure 1.12. Scheme for quantitative analysis of additives in plastic.

Some years ago, the arrival of new ionization techniques such as atmospheric pressure photoionization (APPI), atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI), etc. turned the MS into the more universal detector for all additives. Therefore, liquid chromatography coupled to mass spectrometry has been recently reported for additive determination [21-23, 27, 28].

Finally, it is also worth mentioning that the qualitative and semi-quantitative determination of additives plays also an important role for industrial quality control.

In these cases direct methods such as infrared spectroscopy (IR), evaporative light scattering detector (ELSD), total dissolved solids (TDS), MS, etc. have been also used [21].

1.2. Analysis of endocrine disrupting compounds

The endocrine disruptors are compounds that can act as hormones or interfere with the synthesis, secretion, transport, action or secretion of hormones due to their similar structures to natural chemicals of the body.

According to laboratory studies, endocrine disrupting compounds (EDCs), at certain doses, are associated with several important human diseases such as reduced fertility, obesity, different kind of cancers, etc. [29]. Unfortunately, there are a wide and diverse range of this kind of dangerous substances that cause imbalances in the endocrine system. For example, it's well-known in society the dangers of dioxins and bisphenol A (BPA) among others.

Due to their hazardous, human and environmental exposure to EDCs is nowadays a big concern. There are thousands of works where analytical methods are developed and/or applied for determining some endocrine disruptor compound in all kinds of matrices: from solid, liquid and gaseous samples to consumer products and biological and environmental samples. However, unfortunately, there is still a lot to be done.

A regulation for the approval of marketing and use of new chemical compounds has been established by the EU legislation based on their potential as endocrine disruptors [30, 31]. In addition, the use of substances suspected of being endocrine disruptors must be approved by the REACH regulation of the European Union (registration, evaluation, authorization and restriction of chemicals) [32].

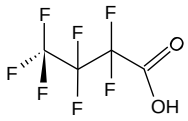
During the course of this thesis, two kind of compounds classified as endocrine disruptors were analyzed: perfluoroalkyl substances (PFASs) and parabens. A brief introduction of each one is presented below.

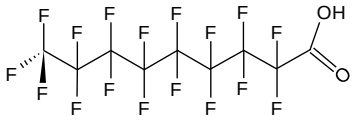
As commented before, some UV filters have been also considered as EDCs, however this was not the feature that leaded us to analyze them but for its possible migration and delamination capacities in packaging.

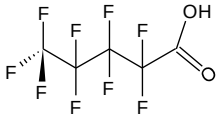
1.2.1. Perfluoroalkyl substances

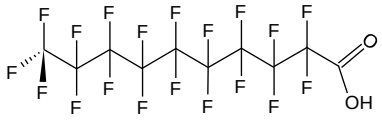
The PFASs, also referred to as perfluorinated compounds (PFCs), are simple synthetic organic chemicals formed by a carbon chain where all the hydrogen atoms are replaced with fluorine atoms. They differ from each other only by the carbon

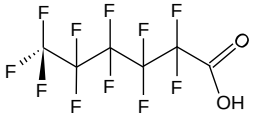
chain length and the functional group attached to it. The PFASs studied in this thesis were the perfluoroalkyl acids (PFAAs), where a carboxylic or sulfonic acid is attached to the chain: the perfluorocarboxylic acids (PFCAs) and the perfluoroalkyl sulfonic acids (PFSAs), respectively. These kind of acids were selected because they are the most common discussed PFASs contaminants. The chemical structures of the PFAAs studied in this thesis are shown in Figure 1.13.

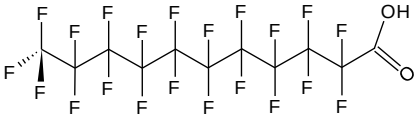

Perfluorobutanoic acid (PFBA)

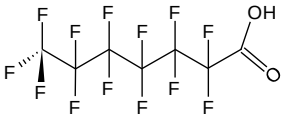

Perfluorononanoic acid (PFNA)

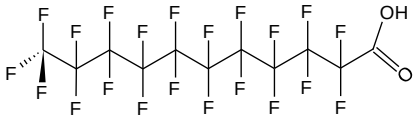

Perfluoropentanoic acid (PFPeA)

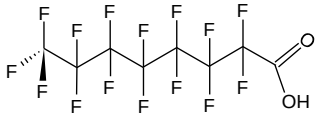

Perfluorodecanoic acid (PFDA)


Perfluorohexanoic acid (PFHxA)


Perfluoroundecanoic acid (PFUnA)


Perfluoroheptanoic acid (PFHpA)


Perfluorododecanoic acid (PFDoA)


Perfluorooctanoic acid (PFOA)

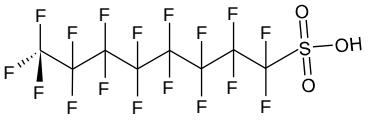

Perfluorooctanesulfonic acid (PFOS)

Figure 1.13. Chemical structures of the PFASs determined in this thesis.

A scheme of the worldwide distribution of products containing PFASs, their properties and their negative effect is shown in Figure 1.14. As may be seen, PFASs have been widely used for over 50 years due their excellent stability and surfactant

properties. Initially PFASs were the alternative for the banned brominated flame retardants [40]. However, it has been observed in the last few years that the PFASs are persistent, bioaccumulative and toxic compounds. Therefore, there is growing concern about their use as well as the exposure to them and potential harmful effects of PFASs on humans and their environment presence [40, 41]. Hence, PFASs have been determined in an extremely wide variety of biological matrices in human and wildlife [42-46], environmental matrices [43, 44, 46-52] as well as consumer products [43, 44, 52, 53] (Figure 1.14).

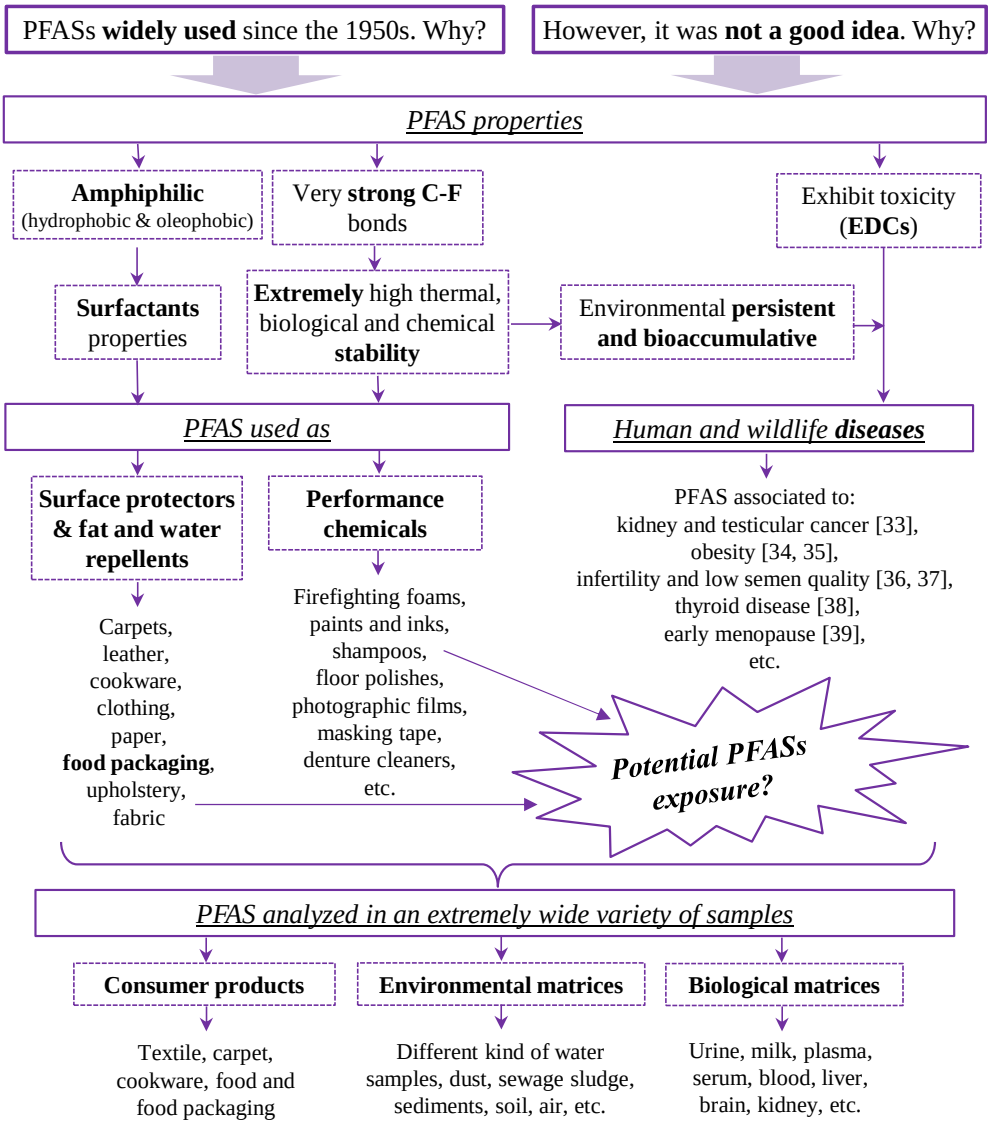


Figure 1.14. Scheme of the worldwide distribution of PFASs and their negative effect.

To analyze PFASs, obviously, it is worth bearing in mind that any material made of fluorotelomers, such as PTFE plastic, must be avoided during the analysis to prevent blank signals. PFASs have been often extracted with methanol, acetonitrile or tetrahydrofuran by traditional solid-liquid extraction using an ultrasonic bath (UAE) or a shaker (SLE) [43, 46-50]. However, other techniques have also played an important role in the extraction of PFASs from biological samples such as ion pair extraction (IPE) with methyl-tert-butyl-ether (MTBE) followed by tetrabutylammonium (TBA), solid phase extraction (SPE) to concentrate and clean the extracts using Oasis WAX, Oasis HLB, Envi-Carb etc., and alkaline digestion with KOH or NaOH [43, 45, 49-51].

Furthermore, in recent years, other new extraction techniques such as PLE or focused ultrasound solid-liquid extraction (FUSLE) were used for the analysis of PFASs in environmental samples, food and packaging [52, 54-56].

It is also worth mentioning that beyond the traditional SPE clean-up for biological samples, it was also found in literature a simple clean up step after extraction where the extract is frozen at -20ºC to favor protein and lipid precipitation [47]. Moreover, Liu et al. have developed a restricted access material (RAM) called Fluorous solid phase extraction where magnetic mesoporous microspheres can absorb small molecules like PFASs but macromolecules like proteins are excluded from the mesopore channels as a result of a size exclusion effect [42].

Regarding PFASs chromatographic separation, it has been normally carried out by LC-MS [43, 49, 50]. Their determination can be also performed by GC after derivatization [43, 57] however, this procedure is more time-consuming and may adversely affect the recovery and reproducibility values. In addition, PFOS does not normally give rise to stable derivatives [58]. Finally, an ESI in negative mode followed by a triple quadrupole mass spectrometer (QqQ-MS/MS) is by the far the most often technique used to PFASs detection due to its sensitivity in the selective multiple reaction monitoring (MRM) although the use of other good detectors such as time of fight (TOF) or ion tramp (IT) have been also described [43, 49, 50].

1.2.2. Parabens

Parabens are alkyl esters of the 4-hydroxybenzoic acid that differ in the chain length of the ester group. As can be seen in Figure 1.15, parabens have been widely used for nearly 100 years as preservatives due to their excellent features such as broad antimicrobial spectrum, solubility, stability, low allergenicity and low cost. Hence, they have been principally added into all kind of cosmetics, foodstuffs and

pharmaceuticals to prevent microbial growth or product degradation. Furthermore, a mixture of parabens (often methylparaben and propylparaben) are usually added to the product to achieve synergistic effects because parabens with longer alkyl chain possess more antimicrobial activity but less water solubility and vice versa (Figure 1.15).

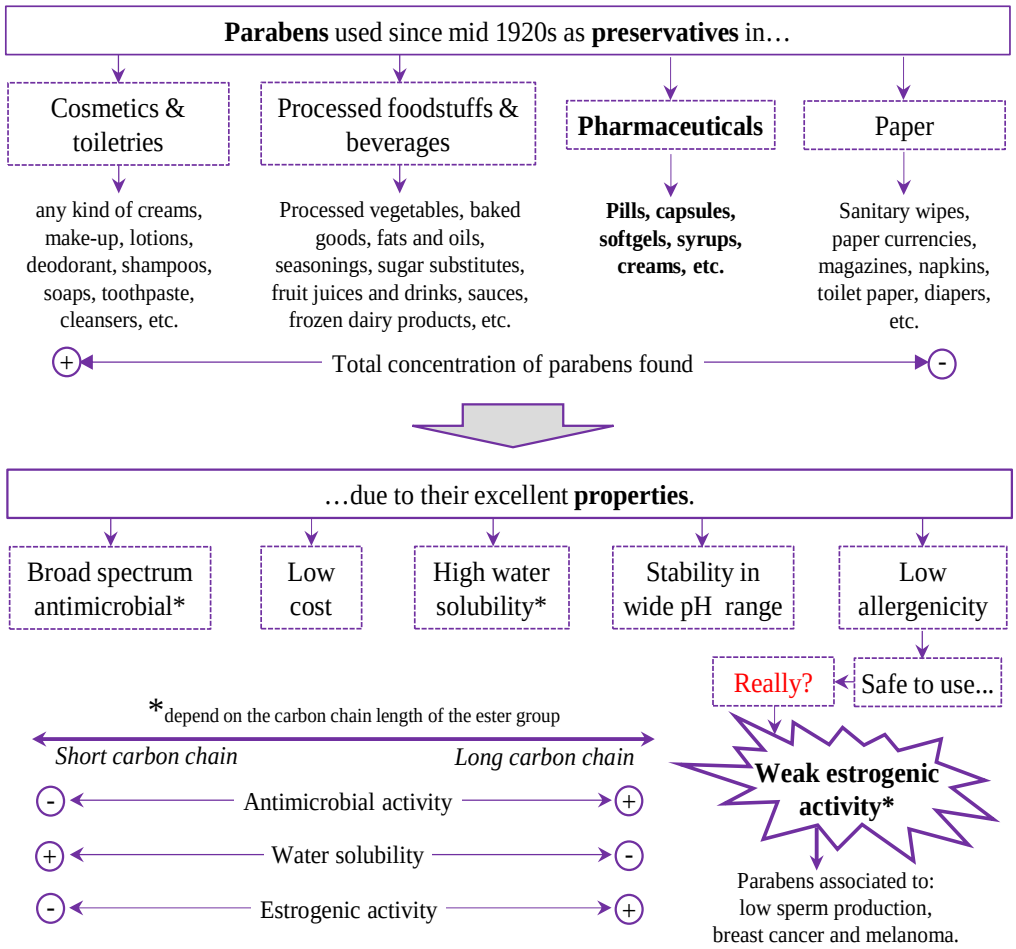


Figure 1.15. Uses and characteristics of parabens.

However, in recent years, parabens are suspected of having a weak estrogenic activity due to their presence in cancerous samples and laboratory studies with animals [59, 60]. It has also been observed that the paraben estrogenic activity increases with the length of the alkyl chain [60]. Hence, human exposure to parabens is a concern because it can originate significant public health problems and the occurrence of them must be fully characterized.

The parabens most commonly used are the methyl, ethyl, propyl, butyl, benzyl and heptyl parabens. Thus, these six parabens were selected in this thesis in order to analyze them in pharmaceuticals. Their chemical structures are shown in Figure 1.16.

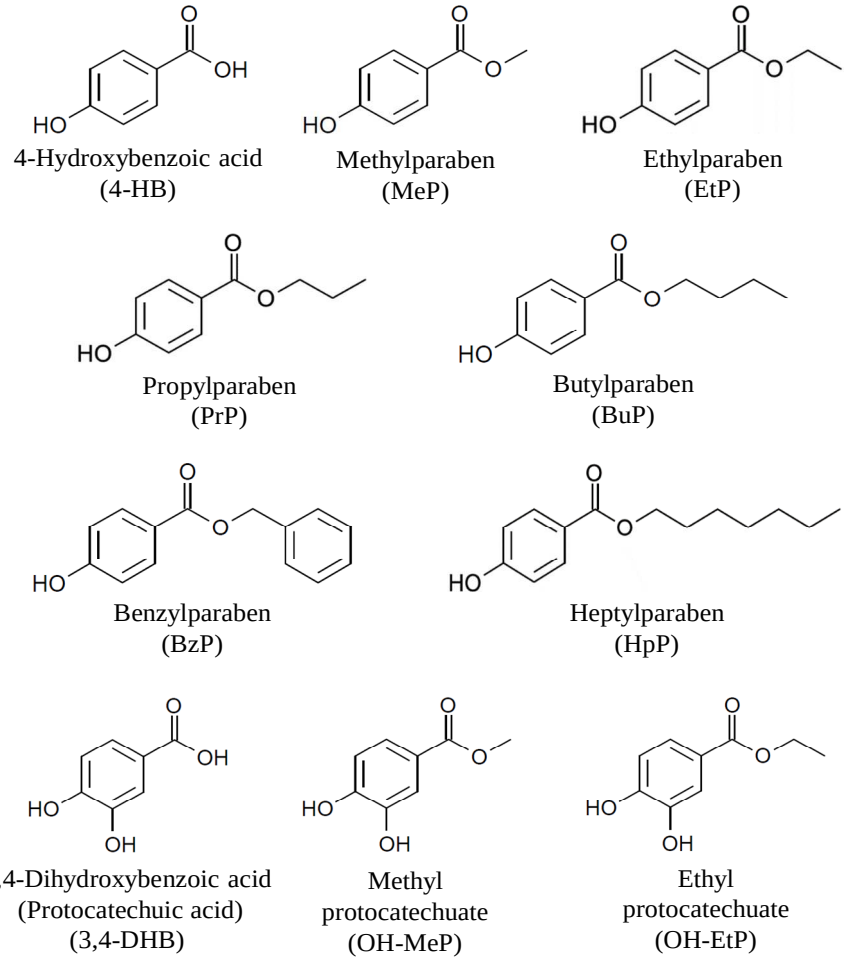


Figure 1.16. Chemical structures of the parabens and their metabolites studied in this thesis.

In addition, although parabens are stable, they may be metabolized as shown in Figure 1.2.5. Some studies have proved the presence of parabens metabolites in biological samples [61, 62]. The ester group can be hydrolyzed by esterases to give rise to the p-hydroxybenzoic acid (4-HB) [63, 64]. Another degradation pathway for parabens is the oxidative hydroxylation. Light–induced hydroxylation of methyl paraben to methyl protocatechuate has been also reported [65]. Therefore, the four

metabolites 4-HB, 3,4-dihydroxybenzoic acid and methy- and ethyl-protocatechuates were also analyzed in this thesis as possible parabens by-products produced during storage of the consumer products (Figure 1.17).

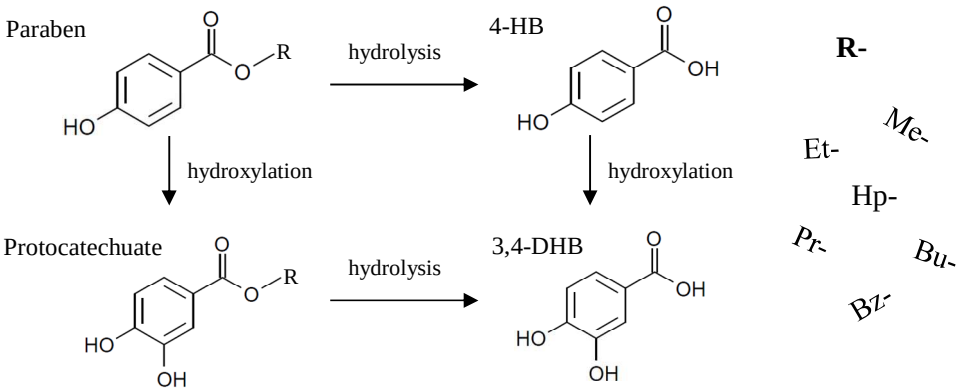


Figure 1.17. Possible metabolic reactions of parabens.

Due to the endocrine disrupting effects associated to parabens and their potential effects as emerging pollutants, the number of analytical methods to determine them has increased strongly in the last decade. Parabens analysis have been carried out extensively in any kind of personal care products followed by environmental samples such us water, biota, sediments, sewage sludge, dust, etc. [66-69]. However, only a few studies have reported the occurrence of parabens in food [69-72] and biological samples such as urine, serum and saliva [61, 62, 69, 73] and the studies of the presence of parabens in pharmaceuticals is scarce [74, 75].

The variety of paraben extraction methods is overwhelming as can be seen in Figure 1.18. However, LLE, traditional SLE by shaking and UAE are the common methods employed for the extraction of parabens [66-68] due to their simplicity, low cost and efficiency although those techniques are more time-consuming. It is worth mentioning that FUSLE has not been employed yet for parabens extraction. It is probable that this technique will provide the same efficiency with lower extraction times.

Regarding the chromatographic separation, parabens have been mainly determined by LC coupled to UV detector followed by GC coupled to MS. Other techniques such as LC-MS or capillary electrophoresis (CE) coupled to UV have also played an important role [66-68]. Nevertheless, it is very probable that LC will finally prevail against GC and CE because GC needs a derivatization step for parabens with long carbon chain and paraben metabolites and CE is not automated.

Regarding the detector, although MS is more expensive, it offers a more secure peak identification than UV or FID where peaks can be overlapped or ambiguous.

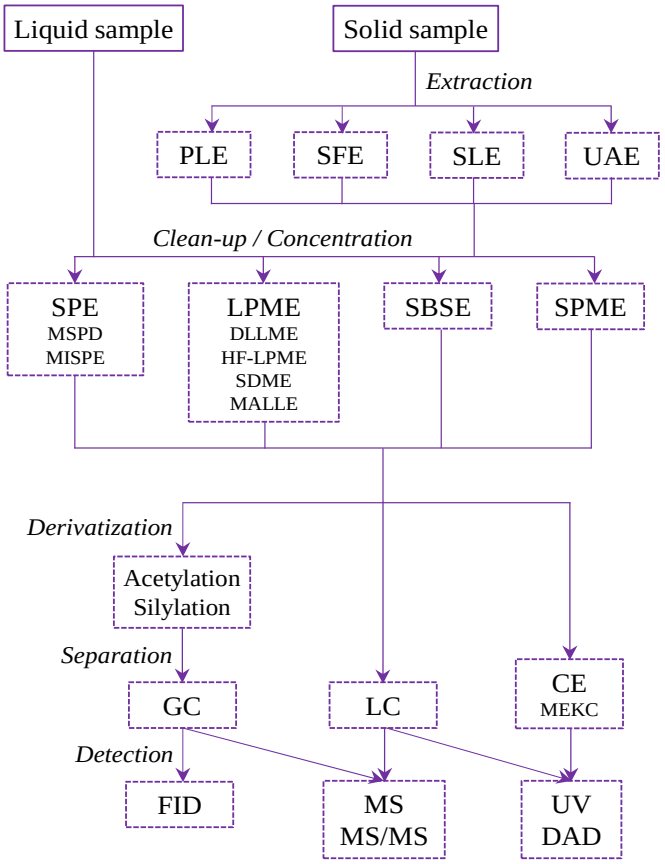


Figure 1.18. Diagram of the different analytical techniques used for the determination of parabens.

1.3. Focused ultrasound solid-liquid extraction

The ultrasounds, frequencies waves above 20 kHz, have been widely used in analytical chemistry to extract different compounds of interest from solid samples due to the versatility and the low cost of this methodology. Moreover, ultrasounds have also been comprehensively used for cleaning, degassing, dispersion, etc. so that it is extremely common to find an ultrasonic bath in each laboratory nowadays.

There are two kind of extraction procedures depending on whether the ultrasonic power is applied indirectly introducing the extraction mixture into an ultrasonic bath, UAE, or if the ultrasonic power is applied directly immersing partially an ultrasonic probe or micro-tip into the extraction mixture, FUSLE.

UAE allows the processing of high sample volumes of around 10-30 g efficiently. However, the extraction times are high (30-60 min or even hours) and the reproducibility is often low. This is probably because ultrasonic waves have to go through the bath water, then the beaker or vessel wall, and finally the extraction solvent before reaching the sample. Thus, the wave energy applied decreases along the way and the performance of this technique is limited.

On the contrary, FUSLE overcomes these disadvantages. The probe immersed is normally made of titanium material which is more powerful and resistant to corrosion. Due to the direct introduction of this probe into the extraction mixture, FUSLE provides an ultrasound power higher and is up to 100 times more effective than the traditional ultrasonic bath [76, 77]. In Figure 1.19, a picture describing the FUSLE equipment used during the realization of this thesis is shown.

Figure 1.19. Ultrasound sonicator SONOPULS HD 2070. Description of the equipment parts: 1) Ultrasonic converter, 2) Booster horn, 3) Probe - Titanium flat micro-tip, 4) Ice bath, 5) Sound-proof box, 6) HF-generator.

The high extraction capacity of FUSLE is based on the cavitation phenomena. A scheme of the process is shown in Figure 1.20. The ultrasonic waves produced go through the liquid generating compression and rarefaction waves at very high speeds that compress and extend the space between the solvent molecules. Due to this, microbubbles filled with a mixture of gas and vapor are formed during the high negative pressure changes. Then, the bubbles intermittently suffer expansion and compression processes. During the expansion processes, gases and vapor enter into the bubbles accumulating potential energy. However, during the compression

processes (positive pressure changes) the bubbles reduce their volume concentrating the energy stored. This cycle is repeated until the cavitational bubbles become unstable during the compression cycle of the ultrasonic wave and finally they implode expelling an extremely large amount of energy [77-80].

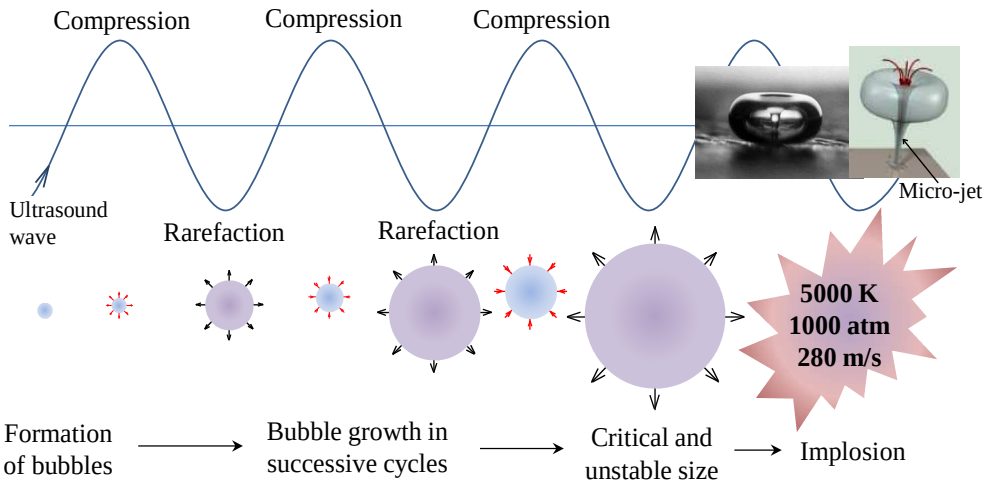


Figure 1.20. Scheme of the cavitational process.

As a result of the cavitational bubbles collapse, very high temperatures (up to 5,000 K) and pressures (up to 2,000 atm) are reached briefly in a microscopic level, and the implosion also generates a powerful solvent micro-jet speed up to 280 m/s [77, 79]). Thus, the tiny bubbles imploded can be considered as microreactors. When the implosion occurs near a sample particle, the micro-jets propagated toward the surface at high velocities can cause pitting, mechanical erosion of the surface and even solid disruption [77-80]. Therefore, the sample size is reduced and the solid surface in contact with the extracting solvent is increased. Therefore, all these features together favor solvent penetration, help to increase the extraction efficiency and reduces the extraction time.

Regarding the enormous temperature values reached, it is important to bear in mind that the size of the bubbles are extremely tiny compared to the total liquid volume. Therefore, the heat produced is dissipated very quickly and negligible changes are observed in the surrounding area. Due to this, ultrasonic cavitation is also known as the “cold boiling” [81]. This does not mean that the extract temperature is kept constant all the time, but the increment of the temperature is very small and slow.

In addition, normally the use of high temperatures increase the extraction efficiency because it favors solvent diffusion in the sample and disruption of the analyte-matrix interactions. However, contrary to expectations, the extraction efficiency using FUSLE increases at low temperatures because this favors cavitation processes. Therefore, the focused ultrasound extraction should be carried out at 0ºC in an ice bath [76, 77, 80, 82]. It is for this reason that this technique is of special interest to extract thermolabile compounds which may be decomposed with other techniques such as PLE, MAE and SFE.

In Table 1.1, different studies where FUSLE was employed to extract organic compounds are summarized. As can be seen, FUSLE is a relatively new extraction technique and the number of studies where this methodology has been applied, has increased rapidly in recent years.

Moreover, thanks to the excellent FUSLE characteristics, this method has shown a high speed extraction (Table 1.1). Only five minutes or less were needed to extract quantitatively organic compounds in all works, except for the extraction of metabolites from orange peel (7.5 min). Furthermore, this technique has showed to be faster than other recent techniques such as MAE [76, 82 vs 83, 84, 85], PLE [56 vs 55, 85, 86] or SFE [86-89] showing similar results, except for the determination of BPA and alkylphenols in sewage sludge where FUSLE showed worse recoveries than PLE [90]. It is also worth mentioning that FUSLE is less expensive than the other techniques mentioned, making its introduction and use in routine analysis laboratories easier.

All in all, due to the advantageous features and good results obtained in most studies, FUSLE is a promising extraction technique for developing new solid-liquid extraction methods.

1.4. Design of Experiments

Normally, there are several variables or factors during the development of an analytical method that need to be optimized to achieve the best possible signal or result.

A univariate analysis, also known as one-factor-at-a-time method, optimizes each factor one by one and frequently involves a larger number of experiments requiring more time, material, staff and thereby, more money. In addition, the information provided by this method can often be irrelevant because possible interactions between the factors are not considered.

Table 1.1. Analytical studies where FUSLE was employed as extraction technique.

Samples	Analytes [a]	FUSLE time	Year	Ref.
Sediment	PAHs	2 x 90 s	2005	[91]
Marine biota	PAHs	120 s	2006	[82]
Sediment	PAHs, PCBs, PEs, NPs	120 s	2008	[76]
Leaf	PAHs	120 s	2010	[86]
Sediment, mussel	APs, 17β estradiol	120 s	2010	[81]
Packaging	UV filters	30 s	2011	[92]
Sewage sludge	BPA, APs	20 s	2011	[90]
Indoor dust	BDEs	20 s	2012	[93]
Packaging	Bisphenols	2 x 5 s	2012	[94]
Sewage sludge	PFASs	2 x 20 s	2013	[85]
Packaging	PFASs	10 s	2013	[95]
Marihuana	Marihuana extracts	5 min	2013	[87]
Citrus peel	Volatile oils / phenols	5 min	2013	[88]
Aromatic plants	Volatile oils / phenols	5 min	2013	[89]
Kidney	Proteins	4 x 10 s	2014	[96]
Packaging, corn	PFASs	10 s	2014	[56]
Vegetables, soil	PBDEs	120 s	2014	[84]
Fish, vegetables, soil	PFASs	2 x 150 s	2014	[52]
Orange peel	Peel metabolites	7.5 min	2015	[97]
Feed & related products	Caffeoylquinic acids	60 s	2015	[98]
Carrot and soil	PFASs	2.5 min	2015	[99]
Carrot, lettuce and soil	EDCs	5 min	2015	[100]
Soil	PCBs, OCPs	2 x 60 s	2015	[101]

[a] Analytes abbreviation are described in the section acronyms and abbreviations.

A design of experiments (DOE) is a key tool to evaluate the influence and effect of each factor as well as their interactions and optimize the response variable efficiently with the least possible number of experiments. To sum up, the main objective is to obtain the maximum information with the minimum effort and time.

Therefore, the DOE was employed in this thesis to optimize the principal variables affecting the FUSLE and (microwave assisted oxidation) MAO processes.

Usually, the first step is to perform a two level full or fractional factorial design when the number of factors are more than four. Both are especially useful for an initial screening to reduce the number of factors to consider. Those methods evaluate the influence and significance of the factors and their interaction on the response in order to be able to reject the non-significant factors.

However, only three variables were always selected as the main factors to optimize during this thesis. Therefore, a factorial design was not necessary and directly a central composite design was carried out in each study.

Central composite design

A central composite design (CCD) is a second-order design to quantify the influence and significance of the factors by response surfaces and desirability approach. This methodology was first proposed by Box and Wilson in 1951 in the article "On the Experimental Attainment of Optimum Conditions" and it is the most widely used design in second order response surfaces (102-104).

It is worth mentioning that theoretically there are no limitations on the number of factors to study. However, a CCD is often performed with only two or three factors due to the high complexity and large number of experiments for a design with four or more factors.

In order to design the model, a series of mathematical and statistical techniques are employed to achieve the best possible approximation of the relationship or dependence between the response variable and the factors. Hence, it will be obtained from this model the optimal conditions of the factors to maximize, minimize or carry out some specific conditions in the response variable to provide the best result.

To do this, one of the premises of this methodology to simplify the design, decrease the number of experiments and obtain response surfaces is that the three order or higher interactions among the factors are negligible or insignificant compared to those of first and second order. Therefore, the function of this quadratic model is as follows:

$$y = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{\substack{i \\ i<j}} \sum_{j} \beta_{ij} x_i x_j + \sum_{i=1}^{n} \beta_{ii} x_i^2 + e$$

where y is the response, x the factors, β the coefficients and e the error associated with the response.

First of all, it is necessary to establish the factors and their experimental domain (the range of values for those factors). Those parameters are defined and selected based mainly on literature, experimental limitations, previous experiences and equipment parameters.

A CCD is actually a 2^k factorial design with 2^k factorial points complemented with 2k axial or star points and several central points, where k is the number of factors.

As in a 2^k factorial design, each factor has two levels that represent the extremes of the experimental domain: the upper one (+1) and the lower one (-1). The factorial points corresponds to all possible combinations of these two levels with all factors. The axial points are all the positions where all coordinates are zero except one which is ± α, for example (± α, 0, 0, ... 0), (0, ± α, 0, ... 0) ... (0,0,0, ... ± α), and the central point is the central position (0, 0, 0 … 0).

Each point has their purpose. The factorial points estimate the effects of the factors and their interactions. The axial points evaluate the effect of the quadratic terms of the model together with the central point that detects the effect of nonlinearity for each factor. The replicates of the center point showed the variance in the response or the experimental error of the predictive model.

Moreover, it is important for a CCD model possesses the characteristics of orthogonality and rotatability.

On one hand, rotatability provide a stable distribution throughout the experimental design region. A CCD is rotatable if the variance of the predicted response of the model at one point depends only on the distance between this point and the center point and not on the direction. This means that the variance is uniform on spherical models and any estimation is equally accurate at any point of this spherical region. In order to create this geometric shape passing through all factorial and axial points, the α value it is given by the equation [105]:

$$\alpha = (2^k)^{1/4}$$

On the other hand, orthogonality simplify the calculation and avoid uncorrelated estimates of the response model coefficients. A CCD is orthogonal if

the effects of any factor sum to zero across the effects of the other factors. This means that design parameters are estimated independently without any correlation. In order to obtain orthogonality, the number of replicates of the center point (N_0) is based on the number of factors (k) as follows [105]:

$$N_0 \approx 4 \times (2^k)^{1/2} + 4 - 2k$$

Finally, it is worth mentioning that the experiments should be randomized in order to avoid false conclusions.

Taking into account all the requirements, a three-factors CCD is a good example to observe the complete spatial domain of a design. As can be seen in Figure 1.21, the factor points correspond to the vertices of a cube (8 points) and the axial or star points are in the midpoints of the cube (6 points) at a distance α=1.682 times higher than the experimental domain to establish the rotatability condition. Taking into account that 9 replicates of the center point are needed to orthogonality, a total of 23 experiments is required for a 2^3 CCD.

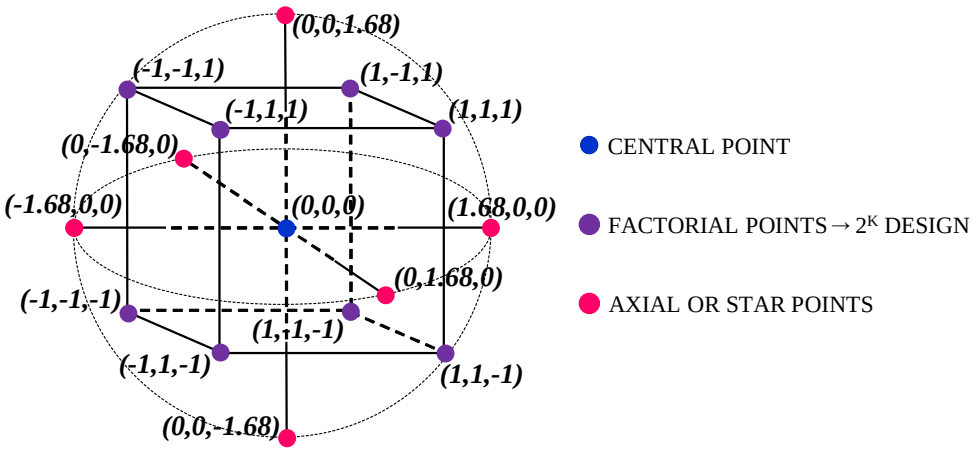


Figure 1.21. 3D representation of a 2^3 CCD: position of the experimental points of the special domain normalized to achieve the rotatability and orthogonality conditions.

REFERENCES

[1] Á. Garrido-López, A. Santa-Cruz, E. Moreno, J. Cornago, M.C. Cañas and M.T. Tena, Determination of cosmetic ingredients causing extrusion-coated and adhesive joint multilayer packaging delamination, Packag. Technol. Sci. 22 (2009) 415-429.

[2] Á. Garrido-López (2009), Doctoral thesis: Innovaciones analíticas para el control de calidad del embalaje flexible multicapa. Determinación de aditivos en polietileno y estudio de los procesos de deslaminación, University of La Rioja.

[3] G. Ortiz, M.T. Tena, Headspace solid-phase microextraction gas chromatography–mass spectrometry method for the identification of cosmetic ingredients causing delamination of packagings, J. Chromatogr. A, 1101 (2006) 32–37.

[4] G. Ortiz, Á. Garrido-López, M.T. Tena, Delamination of multilayer packaging caused by exfoliating cream ingredients, Packag. Technol. Sci. 20 (2007) 173–182.

[5] M. Fraldi, A. Cutolo, L. Esposito, G. Perrella, M.G. Pastore-Carbone, L. Sansone, G. Scherillo, G. Mensitieri, Delamination onset and design criteria of multilayer flexible packaging under high pressure treatments, Innov. Food Sci. Emerging Technol. 23 (2014) 39–53.

[6] Á. Garrido-López, M.T. Tena, Study of multilayer packaging delamination mechanisms using different surface analysis techniques, Appl. Surf. Sci. 256 (2010) 3799–3805.

[7] Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products, Annex VI list of UV filters allowed in cosmetic products.

[8] I. Tarazona (2015), Doctoral thesis: Desarrollo de métodos analíticos para la determinación de filtros UV: de los productos cosméticos al cuerpo humano y al medio ambiente, University of Valencia.

[9] A. Salvador, A. Chisvert, Sunscreen analysis: A critical survey on UV filters determination, Anal. Chim. Acta 537 (2005) 1-14.

[10] A. Chisvert, A. Salvador, Determination of water-soluble UV-filters in sunscreen sprays by liquid chromatography, J. Chromatogr. A 977 (2002) 277-280.

[11] A. Chisvert, I. Tarazona, A. Salvador, A reliable and environmentally-friendly liquid-chromatographic method for multi-class determination of fat-soluble UV filters in cosmetic products, Anal. Chim. Acta 790 (2013) 61-67.

[12] M. Vila, J. P. Lamas, C. Garcia-Jares, T. Dagnac, M. Llompart, Optimization of an analytical methodology for the simultaneous determination of different classes of ultraviolet filters in cosmetics by pressurized liquid extraction–gas chromatography tandem mass spectrometry, J. Chromatogr. A (2015) http://dx.doi.org/10.1016/j.chroma.2015.05.061.

[13] P. Gago-Ferrero, M.S. Díaz-Cruz, D. Barceló, An overview of UV-absorbing compounds (organic UV filters) in aquatic biota, Anal. Bioanal. Chem. 404 (2012) 2597–2610.

[14] A.L. Capriotti, C. Cavaliere, S. Piovesana, R. Samperi, S. Stampachiacchiere, S. Ventura, A. Lagana, Multiresidue determination of UV filters in water samples by solid-phase extraction and liquid chromatography with tandem mass spectrometry analysis, J. Sep. Sci. 37 (2014) 2882–2891.

[15] I. Tarazona, A. Chisvert, A. Salvador, Development of a gas chromatography-mass spectrometry method for the determination of ultraviolet filters in beach sand samples, Anal. Methods 6 (2014) 7772-7780.

[16] S. Maipas, P. Nicolopoulou-Stamati, Sun lotion chemicals as endocrine disruptors, Hormones 14 (2015) 32-46.

[17] I. Jiménez-Díaz, J.M. Molina-Molina, A. Zafra-Gómez, O. Ballesteros, A. Navalóna, M. Real, J.M. Sáenz, M.F. Fernández, N. Olea, Simultaneous determination of the UV-filters benzyl salicylate, phenyl salicylate, octyl salicylate, homosalate, 3-(4-methylbenzylidene) camphor and 3-benzylidene camphor in human placental tissue by LC-MS/MS. Assessment of their in vitro endocrine activity, J.Chromatogr. B 936 (2013) 80-87.

[18] A. Chisvert, Z. León-González, I. Tarazona, A. Salvador, D. Giokas, An overview of the analytical methods for the determination of organic ultraviolet filters in biological fluids and tissues, Anal. Chim. Acta 752 (2012) 11-29.

[19] I. Tarazona, A. Chisvert, A. Salvador, Determination of benzophenone-3 and its main metabolites in human serum by dispersive liquid liquid microextraction followed by liquid chromatography tandem mass spectrometry, Talanta 116 (2013) 388-395.

[20] H.J. Vandenburg, A.A. Clifford, K.D. Bartle, J. Carroll, I. Newton, L.M. Garden, J.R. Dean, C.T. Costley, Critical Review: Analytical Extraction of Additives From Polymers, Analyst 122 (1997) 101-116.

[21] E. Reingruber, W. Buchberger, Analysis of polyolefin stabilizers and their degradation products, J. Sep. Sci. 33 (2010) 3463-3475.

[22] C. Pouech, F. Lafay, L. Wiest, R. Baudot, D. Léonard, C. Cren-Olivé, Monitoring the extraction of additives and additive degradation products from polymer packaging into solutions by multi-residue method including solid phase extraction and ultra-high performance liquid chromatography-tandem mass spectrometry analysis, Anal. Bioanal. Chem. 406 (2014) 1493-1507.

[23] Z. Bodai, B.S. Szabó, M. Novák, S. Hámori, Z. Nyiri, T. Rikker, Z. Eke, Analysis of potential migrants from plastic materials in milk by liquid chromatography-mass spectrometry with liquid-liquid extraction and low-temperature purification, J. Agric. Food Chem 62 (2014) 10028-10037.

[24] E. Canellas, P. Vera, C. Nerin, Risk assessment derived from migrants identified in several adhesives commonly used in food contact materials, Food Chem. Toxicol. 75 (2015) 79-87.

[25] G. Lv, L. Wang, J. Liu, S. Li, Method for determination of fatty acid amides in polyethylene packaging materials—Gas chromatography/mass spectrometry, J. Chromatogr. A 1216 (2009) 8545–8548.

[26] Z. Fekete, T. Rófusz, V. Angyal, P. Szabó-Révész, Z. Aigner, Gas chromatographic-mass spectrometric analysis and subsequent quality improvement of plastic infusion packaging materials, J. Pharm. Biomed. Anal. 97 (2014) 111-115.

[27] S. Beißmann, M. Stiftinger, K. Grabmayer, G. Wallner, D. Nitsche, W. Buchberger, Monitoring the degradation of stabilization systems in polypropylene during accelerated

aging tests by liquid chromatography combined with atmospheric pressure chemical ionization mass spectrometry, Polym. Degrad. Stab. 98 (2013) 1655-1661.

[28] R.E. Duderstadt, S.M. Fischer, Effect of organic mobile phase composition on signal responses for selected polyalkene additive compounds by liquid chromatography-mass spectrometry, J. Chromatogr. A 1193 (2008) 70-78.

[29] WHO and UNEP, State of the science of endocrine disrupting chemicals. 2012.

[30] Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC. Official Journal of the European Community, L309/1.

[31] Regulation (COM 2009/267) of the European Parliament and of the Council of 12th June 2009 replacing Directive 98/8/EC concerning the placing of biocidal products on the market. Official Journal of the European Community, L276.

[32] Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18th December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. Official Journal of the European Union, L396/1.

[33] V. Barry, A. Winquist, K. Steenland, Perfluorooctanoic Acid (PFOA) exposures and incident cancers among adults living near a chemical plant, Environ. Health Perspect. 121 (2013) 1313-1318.

[34] A. Janesick, B. Blumberg, Endocrine disrupting chemicals and the developmental programming of adipogenesis and obesity. Birth Defects Res. C Embryo Today 93 (2011) 34-50.

[35] W. Holtcamp, Obesogens: an environmental link to obesity, Environ. Health Perspect. 120 (2012) 62-68.

[36] C. Fei, J.K. McLaughlin, L. Lipworth, J. Olsen. Maternal levels of perfluorinated chemicals and subfecundity. Hum. Reprod. 24 (5) (2009) 1200-1205.

[37] U.N. Joensen, R. Bossi, H. Leffers, A.A. Jensen, N.E. Skakkebaek, N. Jørgensen, Do perfluoroalkyl compounds impair human semen quality? Environ. Health Perspect. 117 (2009) 923-927.

[38] D. Melzer, N. Rice, M.H. Depledge, W.E. Henley, T.S. Galloway, Association between serum perfluorooctanoic acid (PFOA) and thyroid disease in the US national health and nutrition examination survey, Environ. Health Perspect. 118 (2010) 686-692.

[39] S.S. Knox, T. Jackson, B. Javins, S.J. Frisbee, A. Shankar, A.M. Ducatman, Implications of early menopause in women exposed to perfluorocarbons. J. Clin. Endocrinol. Metab. 96 (2011) 1747-1753.

[40] Å. Bergman, J.J. Heindel, S. Jobling, K.A. Kidd, R.T. Zoeller, State of the science of endocrine disrupting chemicals, an assessment of the state of the science of endocrine disruptors prepared by a group of experts for the United Nations Environment Program and World Health Organization, (2012) http://unep.org/pdf/9789241505031_eng.pdf.

[41] T. Wang, P. Wang, J. Meng, S. Liu, Y. Lu, J. S. Khim, J.P. Giesy, A review of sources, multimedia distribution and health risks of perfluoroalkyl acids (PFAAs) in China, Chemosphere 129 (2015) 87–99.

[42] X. Liu, Y. Yu, Y. Li, H. Zhang, J. Ling, X. Sun, J. Feng, G. Duan, Fluorocarbon-bonded magnetic mesoporous microspheres for the analysis of perfluorinated compounds in human serum by high-performance liquid chromatography coupled to tandem mass spectrometry, Anal. Chim. Acta 844 (2014) 35–43.

[43] A. Jahnke, U. Berger, Trace analysis of per- and polyfluorinated alkyl substances in various matrices-How do current methods perform? J. Chromatogr. A 1216 (2009) 410-421.

[44] S.D. Richardson, Environmental mass spectrometry: Emerging contaminants and current issues, Anal. Chem. 84 (2012) 747-778.

[45] S. Dagnino, Chapter 2: Analysis of PFASs in biological tissues and fluids, Ed. Springer, 2015 pp 23-50.

[46] X.M. Wu, D.H. Bennett, A.M. Calafat, K. Kato, M. Strynar, E. Andersen, R.E. Moran, D.J. Tancredi, N.S. Tulve, I. Hertz-Picciotto, Serum concentrations of perfluorinated compounds (PFC) among selected populations of children and Adults in California, Environ. Research 136 (2015) 264-273.

[47] M.D. Malinsky, C.B. Jacoby, W.K. Reagen, Determination of perfluorinated compounds in fish fillet homogenates: Method validation and application to fillet homogenates from the Mississippi River, Anal. Chim. Acta 683 (2011) 248-257.

[48] N. Luque, A. Ballesteros-Gómez, S. van Leeuwen, S. Rubio, Analysis of perfluorinated compounds in biota by microextraction with tetrahydrofuran and liquid chromatography/ion isolation-based ion-trap mass spectrometry, J. Chromatogr. A 1217 (2010) 3774-3782.

[49] S. Valsecchi, M. Rusconi, S. Polesello, Determination of perfluorinated compounds in aquatic organisms: a review, Anal. Bioanal. Chem. 405 (2013) 143-157.

[50] O.S. Arvaniti, A.S. Stasinakis, Review on the occurrence, fate and removal of perfluorinated compounds during wastewater treatment, Sci. Total Environ. 524-525 (2015) 81-92.

[51] J.L. Reiner, B.J. Place, Chapter 5: Perfluorinated alkyl acids in wildlife, Ed. Springer, 2015 pp 127-150.

[52] I. Zabaleta, E. Bizkarguenaga, A. Iparragirre, P. Navarro, A. Prieto, L.Á. Fernández, O. Zuloaga, Focused ultrasound solid-liquid extraction for the determination of perfluorinated compounds in fish, vegetables and amended soil, J. Chromatogr. A 1331 (2014) 27-37.

[53] H.Y. Kim, S.K. Kim, D.M. Kang, Y.S. Hwang, J.E. Oh, The relationships between sixteen perfluorinated compound concentrations in blood serum and food, and other parameters, in the general population of South Korea with proportionate stratified sampling method, Sci. Total Environ. 470-471 (2014) 1390-1400.

[54] E. Zafeiraki, D. Costopoulou, I. Vassiliadou, E. Bakeas, L. Leondiadis, Determination of perfluorinated compounds (PFCs) in various foodstuff packaging materials used in the Greek market, Chemosphere 94 (2014) 169–176.

[55] M.P. Martínez-Moral, M.T. Tena, Determination of perfluorocompounds in popcorn packaging by pressurised liquid extraction and ultra-performance liquid chromatography-tandem mass spectrometry , Talanta 110 (2012) 104-109.

[56] C. Moreta, M.T. Tena, Determination of perfluorinated alkyl acids in corn, popcorn and popcorn bags before and after cooking by focused ultrasound solid-liquid extraction, liquid chromatography and quadrupole-time of flight mass spectrometry, J. Chromatogr. A 1355 (2014) 211-218

[57] F. Orata, N. Quinete, R.D. Wilken, Long chain perfluorinated alkyl acids derivatisation and identification in biota and abiota matrices using gas chromatography, Bull. Environ. Contam. Toxicol. 83 (2009) 630-635.

[58] C.A. Moody, J.A. Field, Perfluorinated surfactants and the environmental implications of their use in fire-fighting foams, Environ. Sci. Technol. 34 (2000) 3864-3870.

[59] P.D. Darbre, P.W. Harvey, Paraben esters: review of recent studies of endocrine toxicity, absorption, esterase and human exposure, and discussion of potential human health risks, J. Appl. Toxicol. 28 (2008) 561–578.

[60] E.J. Routledge, J. Parker, J. Odum, J. Ashby, J.P. Sumpter, Some alkyl hydroxy benzoate preservatives (parabens) are estrogenic, Toxicol. Appl. Pharmacol. 153 (1998) 12–19.

[61] J. Boberg, C. Taxvig, S. Christiansen, U. Hass, Possible endocrine disrupting effects of parabens and their metabolites, Reprod. Toxicol. 30 (2010) 301–312.

[62] L. Wang, K. Kannan, Alkyl protocatechuates as novel urinary biomarkers of exposure to p-hydroxybenzoic acid esters (parabens), Environ. Int. 59 (2013) 27-32.

[63] S. Abbas, H.Greige-Gerges, N. Karam, M.H. Piet, P. Netter, J. Magdalou, Metabolism of parabens (4-hydroxybenzoic acid esters) by hepatic esterases and UDP-glucuronosyltransferases in man, Drug Metab. Pharmacokinet. 25 (2010) 568-577.

[64] N. Aubert, T. Ameller, J.J. Legrand, Systemic exposure to parabens: Pharmacokinetics, tissue distribution, excretion balance and plasma metabolites of [14C]-methyl-, propyl- and

butylparaben in rats after oral, topical or subcutaneous administration, Food Chem. Toxicol. 50 (2012) 445-454.

[65] Y. Okamoto, T. Hayashi, S. Matsunami, K. Ueda, N. Kojima, Combined activation of methyl paraben by light irradiation and esterase metabolism toward oxidative DNA damage, Chem. Res. Toxicol. 21 (2008) 1594-1599.

[66] C. Haman, X. Dauchy, C. Rosin, J.F. Munoz, Occurrence, fate and behavior of parabens in aquatic environments: A review, water research 68 (2015) 1-11.

[67] J.A. Ocaña-González, M. Villar-Navarro, M. Ramos-Payán, R. Fernández-Torres, M.A. Bello-López, New developments in the extraction and determination of parabens in cosmetics and environmental samples. A review, Anal. Chim. Acta 858 (2015) 1–15.

[68] N. Cabaleiro, I. de la Calle, C. Bendicho, I. Lavilla, An overview of sample preparation for the determination of parabens in cosmetics, Trends Anal. Chem. 57 (2014) 34–46.

[69] C. Piao, L. Chen, Y. Wang, A review of the extraction and chromatographic determination methods for the analysis of parabens, J. Chromatogr. B 969 (2014) 139–148.

[70] U. Alshana, N. Ertas, N.G. Göger, Determination of parabens in human milk and other food samples by capillary electrophoresis after dispersive liquid–liquid microextraction with back-extraction, Food Chem. 181 (2015) 1–8.

[71] C. Liao, L. Chen, K. Kannan, Occurrence of parabens in foodstuffs from China and its implications for human dietary exposure, Environ. Int. 57-58 (2013) 68-74.

[72] C. Liao, F. Liu, K. Kannan, Occurrence of and dietary exposure to parabens in foodstuffs from the United States, Environ. Sci. Technol. 47 (2013) 3918-3925.

[73] Y.D. Park, J.H. Jang, J.E. Park, J.H. Kim, E.C. Kim, Y.J. Song, H.J. Kwon, Analysis of parabens in dentifrices and the oral cavity, Biomedical Chromatography, 28 (2014) 1692-1700

[74] I. Baranowska, I. Wojciechowska, N. Solarz, E. Krutysza, Determination of preservatives in cosmetics, cleaning agents and pharmaceuticals using fast liquid chromatography, J. Chromatogr. Sci. 52 (2014) 88-94.

[75] J.K. Lokhnauth, N.H. Snow, Determination of Parabens in Pharmaceutical Formulations by Solid-Phase Microextraction-Ion Mobility Spectrometry, Anal. Chem. 77 (2005) 5938-5946.

[76] A. Errekatxo, A. Prieto, O. Zuloaga, A. Usobiaga, N. Etxebarria, L.A. Fernández, Simultaneous extraction of several persistent organic pollutants in sediment using focused ultrasonic solid-liquid extraction, Anal. Bioanal. Chem. 392 (2008) 1471-1478.

[77] F. Priego Capote, M.D. Luque de Castro. Analytical applications of ultrasound. Elsevier Science (2007) 1-35.

[78] T.Y. Wu, N. Guo, C.Y. Teh, J.X.W. Hay, Chapter 2: Theory and Fundamentals of Ultrasound (Advances in Ultrasound Technology for Environmental Remediation), Springer (2013) 5-12.

[79] K.S. Suslick, Encyclopedia of Chemical Technology vol. 26J, Kirk-Othmer, Wiley & Sons (1998) 517–541.

[80] H. Miguel-Santos, C. Lodeiro, J.L. Capelo-Martínez, Ultrasound in Chemistry. Wiley-VCH (2009).

[81] P. Navarro, J. Bustamante, A. Vallejo, A. Prieto, A. Usobiaga, S. Arrasate, E. Anakabe, E. Puy-Azurmendi, O. Zuloaga, Determination of alkylphenols and 17β-estradiol in fish homogenate. Extraction and clean-up strategies, J. Chromatogr. A 1217 (2010) 5890-5895.

[82] J. Sanz-Landaluze, L. Bartolome, O. Zuloaga, L. González, C. Dietz, C. Cámara, Accelerated extraction for determination of polycyclic aromatic hydrocarbons in marine biota, Anal. Bioanal. Chem. 384 (2006) 1331-1340.

[83] L. Bartolomé, E. Cortazar, J.C. Raposo, A. Usobiaga, O. Zuloaga, N. Etxebarria, L.A. Fernández, Simultaneous microwave-assisted extraction of polycyclic aromatic hydrocarbons, polychlorinated biphenyls, phthalate esters and nonylphenols in sediments, J. Chromatogr. A 1068 (2005) 229–236.

[84] E. Bizkarguenaga, A. Iparragirre, I. Zabaleta, A. Vallejo, L.A. Fernández, A. Prieto, O. Zuloaga, Focused ultrasound assisted extraction for the determination of PBDEs in vegetables and amended soil, Talanta 119 (2014) 53-59.

[85] M.P. Martínez-Moral, M.T. Tena, Focused ultrasound solid-liquid extraction of perfluorinated compounds from sewage sludge, Talanta 109 (2013) 197-202.

[86] J. Sanz-Landaluze, M. Bocanegra-Salazar, D. Ortiz-Pérez, C. Cámara, Miniaturisated method for the analysis of polycyclic aromatic hydrocarbons in leaf samples, J. Chromatogr. A 1217 (2010) 3567-3574.

[87] J. Omar, M. Olivares, M. Alzaga, N. Etxebarria, Optimisation and characterisation of marihuana extracts obtained by supercritical fluid extraction and focused ultrasound extraction and retention time locking GC-MS, J. Sep. Sci. 36 (2013) 1397-1404.

[88] J. Omar, I. Alonso, A. Garaikoetxea, N. Etxebarria, Optimization of Focused Ultrasound Extraction (FUSE) and Supercritical Fluid Extraction (SFE) of Citrus Peel Volatile Oils and Antioxidants, Food Anal. Method. 6 (2013) 1244-1252.

[89] J. Omar, I. Alonso, A. Garaikoetxea, N. Etxebarria, Optimization of Focused Ultrasound Extraction and Supercritical Fluid Extraction of Volatile Compounds and Antioxidants from Aromatic Plants, Food Anal. Method. 6 (2013) 1611-1620.

[90] M.P. Martínez-Moral, M.T. Tena, Focused ultrasound solid-liquid extraction and selective pressurised liquid extraction to determine bisphenol A and alkylphenols in sewage sludge by gas chromatography-mass spectrometry, J. Sep. Sci. 34 (2011) 2513–2522.

[91] J.L. Capelo, M.M. Galesio, G.M. Felisberto, C. Vaz, J.C. Pessoa, Micro-focused ultrasonic solid-liquid extraction (μFUSLE) combined with HPLC and fluorescence detection for PAHs determination in sediments: Optimization and linking with the analytical minimalism concept, Talanta 66 (2005) 1272-1280.

[92] C. Moreta, M.T. Tena, Determination of UV filters in packaging by focused ultrasonic solid-liquid extraction and liquid chromatography, J. Chromatogr. A 1218 (2011) 3392-3399.

[93] M.P. Martínez-Moral, M.T. Tena, Focused ultrasound solid-liquid extraction and gas chromatography tandem mass spectrometry determination of brominated flame retardants in indoor dust, Anal. Bioanal. Chem. 404 (2012) 289-295.

[94] D. Pérez-Palacios, M.A. Fernández-Recio, C. Moreta, M.T. Tena, Determination of bisphenol-type endocrine disrupting compounds in food-contact recycled-paper materials by focused ultrasonic solid-liquid extraction and ultra performance liquid chromatography-high resolution mass spectrometry, Talanta 99 (2012) 167-174.

[95] C. Moreta, M.T. Tena, Fast determination of perfluorocompounds in packaging by focused ultrasound solid-liquid extraction and liquid chromatography coupled to quadrupole-time of flight mass spectrometry, J. Chromatogr. A 1302 (2013) 88-94.

[96] J.E. Araújo, E. Oliveira, A. Otero-Glez, J. Santos Nores, G. Igrejas, C. Lodeiro, J.L. Capelo, H.M. Santos, A comprehensive factorial design study of variables affecting protein extraction from formalin-fixed kidney tissue samples, Talanta 119 (2014) 90-97.

[97] M. Molina-Calle, F. Priego-Capote, M.D. Luque de Castro, Ultrasound-assisted emulsification-extraction of orange peel metabolites prior to tentative identification by LC-QTOF MS/MS, Talanta 141 (2015) 150-157.

[98] M.T. Tena, M.P. Martínez-Moral, P.W. Cardozo, Determination of caffeoylquinic acids in feed and related products by focused ultrasound solid-liquid extraction and ultra-high performance, 1400 (2015) 1-9.

[99] E. Bizkarguenaga , I. Zabaleta, A. Iparraguirre, J. Aguirre, L.Á. Fernández, U. Berger, A. Prieto, O. Zuloaga, Enrichment of perfluorinated alkyl substances on polyethersulfone using 1-methylpyperidine as ion-pair reagent for the clean-up of carrot and amended soil extracts, Talanta, 143 (2015) 263-270.

[100] L. Mijangos, E. Bizkarguenaga, A. Prieto, L.A. Fernández, O. Zuloaga, Simultaneous determination of a variety of endocrine disrupting compounds in carrot, lettuce and amended soil by means of focused ultrasonic solid-liquid extraction and dispersive solid-phase extraction as simplified clean-up strategy, J. Chromatogr. A, 1389 (2015) 8-18.

[101] R. Flores-Ramirez, S.E. Medellín-Garibay, C.G. Castillo, C.A. Ilizaliturri-Hernández, B.A. Zuki-Orozco, L. Batres-Esquivel, F. Díaz-Barriga, Application of Focused Ultrasound-Assisted Extraction to the Determination of Persistent Organic Pollutants (POPs) in Soil Samples, Bull. Environ. Contam. Toxicol. DOI 10.1007/s00128-015-1545-1.

[102] S. Park, H.J. Kim, J.I. Cho, Optimal Central Composite Designs for Fitting Second Order Response Surface Linear Regression Models, (2008) 323-339, in Recent Advances in Linear Models and Related Areas, Springer.

[103] D.C. Montgomery, Diseño y análisis de experimentos, (1991) Limusa Willey.

[104] G.E.P. Box, K.B. Wilson, On the experimental attainment of optimum conditions, J. Roy. Statist. Soc. 13 (1951) 1-45.

[105] T. Hill, P. Lewicki, Experimental design (Industrial DOE), (2006) 179-230, in Statistics: methods and applications: a comprehensive reference for science, industry, and data mining, StatSoft.

2.

UV filters in packaging

2.1.

Determination of UV filters in packaging by focused ultrasonic solid-liquid extraction and liquid chromatography

Journal of Chromatography A, 1218 (2011) 3392-339

Determination of UV filters in packaging by focused ultrasound solid-liquid extraction and liquid chromatography

Cristina Moreta and María Teresa Tena

Department of Chemistry. University of La Rioja. C/ Madre de Dios 51, E-26006. Logroño (La Rioja). Spain.

ABSTRACT: A focused ultrasound solid-liquid extraction (FUSLE) and high performance liquid chromatography (HPLC) with a diode array detector (DAD) is proposed for the determination of ten fat-soluble UV filters in packaging. FUSLE technique is relatively new and has been used for the extraction of a few analytes; such as polycyclic aromatic hydrocarbons and other organic pollutants. In this work, it has been demonstrated that FUSLE is a useful, fast and simple extraction methodology for UV filters because the complete extraction was carried out with just 6 ml of tetrahydrofuran and in only one cycle of 30 s. The developed method has been validated and applied to the analysis of polyethylene-based multilayer packaging samples. The FUSLE-based method allows the sensitive detection of most of the UV-filters in polyethylene, with limits of detection between 0.4 and 8.5 ng mg/l (except for BDM). Intra and inter-day relative standard deviation values were below 5 and 14%, respectively, except for MBP. In addition, the proposed method was more efficient than tetrahydrofuran extraction under reflux for 2.5 h for all the analytes except for EMT and BDM. Therefore, the developed method can be used to establish the absorption capability of different types of packaging and this information will be very useful in packaging selection.

Keywords: FUSLE, UV filters, multilayer packaging.

1. INTRODUCTION

Nowadays UV filters are commonly used in many cosmetic products in order to protect us from over-exposure to sunlight which promotes skin ageing as well as other harmful effects on human health, such as skin tumors [1]. UV filters are divided into two basic groups, chemical or organic and physical or inorganic protectors. The organic filters, which are used most commonly, absorb the ultraviolet light (UVA and/or UVB rays) and convert it into a small amount of heat, and inorganic filters can reflect and scatter the UV light [2]. In the European Union (EU), 26 organic compounds have been approved to be used as UV filters in

2.1

personal care products with maximum individual concentrations of up to 10%, but for dometrizole trisiloxane with a maximum permissible concentration of 15% [3], and the usual concentrations in these products are between 0.1 and 10% [4, 5, 6]. The UV filters investigated in this paper (see Table 1 and Figure 1) are fat-soluble compounds. The organic UV filters can be classified in two groups: the most fat-soluble, and the easily water-soluble, which are determined under different chromatographic conditions [7]. In this study, fat-soluble UV filters determination has been carried out because they are more common and numerous in creams available on the European market.

Table 1. List of the target UV filters.

INCI name[a]	Abb.	λ_{max}[b] (nm)	MAC[c] (%)	Absorption
Benzophenone-3	BZ3	290	10	UVA + UVB
4-methylbenzylidene camphor	MBC	303	4	UVB
Octocrylene	OCR	306	10	UVA + UVB
2-ethylhexyl dimethyl PABA	EDP	315	8	UVB
Ethylhexyl methoxycinnamate	EMC	312	10	UVB
Butyl methoxydibenzoylmethane	BDM	360	5	UVA
Ethylhexyl salicylate	ES	306	5	UVB
Homosalate	HS	306	10	UVB
Methylene bis-benzotriazolyl tetramethylbutylphenol	MBP	305/347	10	UVA + UVB
Bis-ethylhexyloxyphenol methoxyphenyltriazine	EMT	343	10	UVA + UVB

[a] INCI (International Nomenclature of Cosmetic Ingredients)
[b] Wavelength of maximum absorption
[c] MAC (maximum authorized concentration (%w/w)) by EU Cosmetic Directive

UV filter determinations have increased in recent years, not only in personal care products [4, 5, 7-15] but also in water [6, 16], wastewater [17], sea water [18], sludge [19], dust [20], fish [21] urine [22] and semen [23]. This is because recent studies have indicated that some UV filters can accumulate in biota and act as endocrine disruptors which have estrogenic effects [24-27] hence many personal care product ingredients, such as UV filters, have been included in the so-called emerging contaminants.

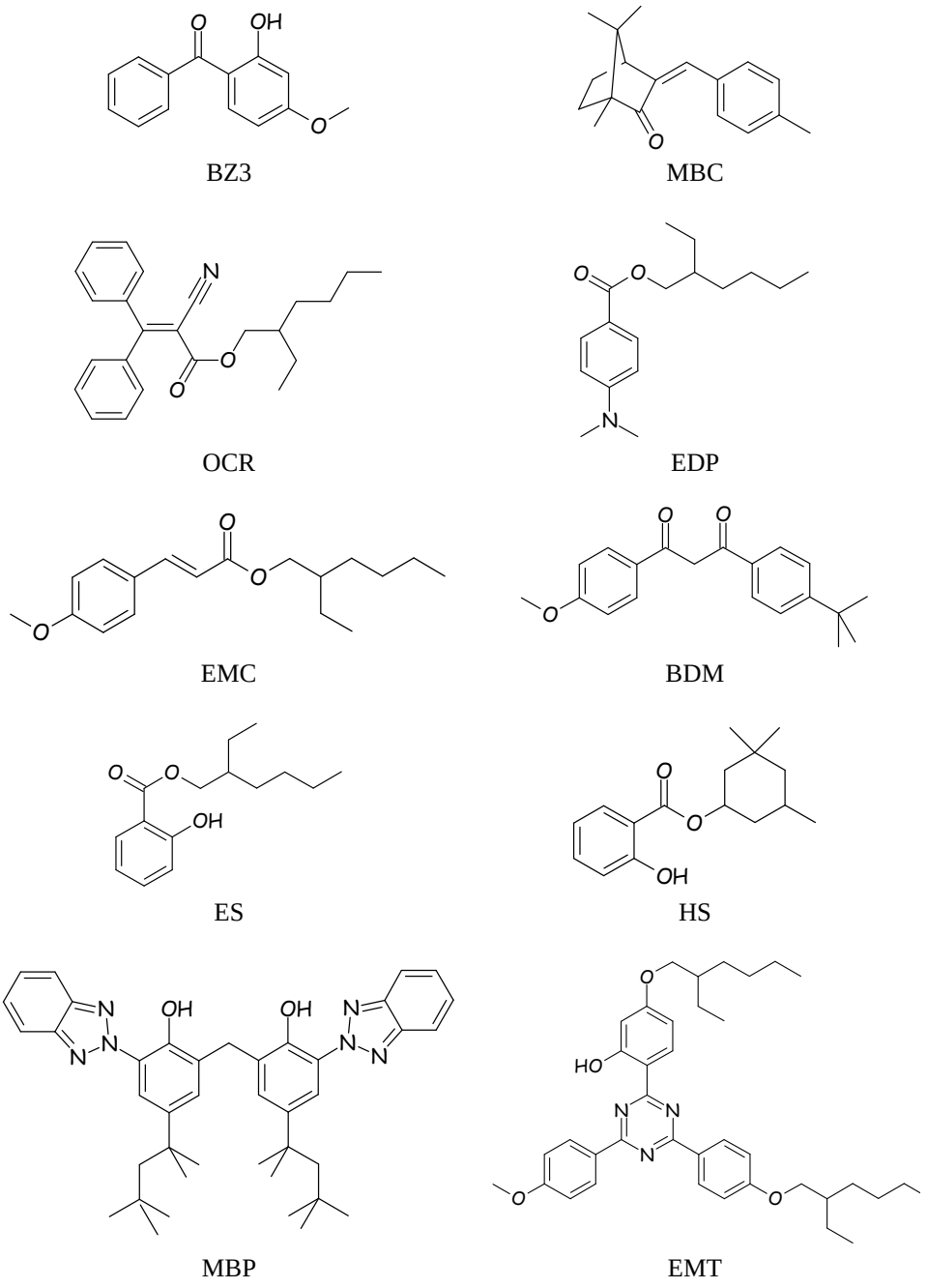


Figure 1. Chemical structures of the ten target UV filters.

Often new cosmetic formulations are promoted in multilayer packaging sachets which consist of several layers fixed together by extrusion or by an appropriate adhesive. The materials normally used in this packaging are polymers

(polyethylene, polyester, polypropylene, etc.) and thin aluminum foils used to provide a hermetic barrier. The advantages of these multilayer packaging materials are their impermeability, good external appearance, flexibility and versatility. However, the main disadvantage of them is their interaction with the product. For instance, certain ingredients of personal care products or food are able to pass through the inner layer (a polymer), causing a loss of adhesion followed by delamination. Personal care products are in a constant evolution, with the development of new formulations and applications. Several investigations have been carried out to identify these aggressive compounds such as 2-phenoxyethanol, benzyl-3-hydroxypropanoate, dihydromyrcenol, menthol, 3,7-dimethyl-3-octanol and p-propenylanisole [28-30] and in recent studies (under confidentiality contract) we have found that UV filters are some of the most active cosmetic ingredients involved in the deterioration of multilayer packaging.

Different methods have been used to determine this kind of UV filters in sunscreen products and other matrices. The most used technique to determine them has been HPLC-UV because they are polar and UV-absorbing compounds, however their chromatographic separation by gas chromatography and detection by single or tandem mass spectroscopy (MS or MS/MS) have also been reported [16, 18, 20, 31].

In order to determine compounds absorbed in packaging coming from the migration from the product, HS-SPME-GC has been the most appropriate and chosen method when they are volatile and have low molecular weight. However, in the case of UV filters because of their polarity and low volatility, chromatographic separation by HPLC was selected.

In some of the reported methods to determine UV filters, the isolation and pre-concentration of sunscreen agents from matrices has been required prior to chromatographic analysis. For instance supercritical fluid extraction (SFE) [8] has been used for cosmetic samples; and solid-phase extraction (SPE) [32], solid-phase microextraction (SPME) [16, 33], dispersive liquid-liquid microextraction (DLLME) [18] and membrane-assisted liquid–liquid extraction (MALLE) [6] for liquid samples such as water. Traditionally, extraction from polymers has been carried out by Soxhlet extraction or by boiling under reflux [34], and more recently by microwave assisted extraction (MAE) [35], supercritical fluid extraction (SFE) [36], pressurized fluid extraction (PFE) [37], headspace solid-phase microextraction (HS-SPME) [38, 39] and ultrasound assisted extraction using an ultrasonic bath [40-44]; but this is the first time that focused ultrasound solid-liquid extraction (FUSLE) has been used to sample preparation of packaging samples.

FUSLE is a fast and low-cost technique, relatively new, that has shown similar results to other extractions, such as MAE in the determination of polycyclic aromatic hydrocarbons, polychlorinated biphenyls, phthalate esters and nonylphenols from environmental matrices [45-47]. In addition, FUSLE is expected to be a more efficient extraction technique than others.

FUSLE is based on the cavitation phenomena: ultrasonic waves crossing a liquid cause the generation, growth, oscillation, splitting and implosion of numerous tiny gas bubbles (cavitation bubbles) [48]. As a result of cavitational bubble implosion, very high temperatures (up to 5000 K) and pressures (up to 2000 atm) are reached locally, and the implosion of the cavitation bubble also results in liquid jets of up to 280 m/s velocity [49]. These features favor extraction efficiency. Furthermore, the size of the bubbles is very small relative to the total liquid volume, so the heat they produce is rapidly dissipated with no appreciable change in the environmental conditions; this is why cavitation is also known as “cold boiling” [46]. It is worth mentioning, that the focused ultrasound microtip is immersed directly in the extracting solution and this, together with the higher ultrasound power, makes the power of the focused ultrasound technique 100 times higher than that of the traditional ultrasonic bath [45]. Therefore, the focused ultrasound approach is very useful for developing new solid-liquid extraction procedures.

In this work, a fast and simple method based on FUSLE has been developed for the determination of ten fat-soluble UV filters sorbed in different polyethylene-based flexible multilayer packaging. The extraction was carried out with only 6 ml of tetrahydrofuran in one cycle of 30 s and the extract analysis was performed by HPLC-UV. The method can be very useful to study the migration of UV filters to the layer of packaging contact; and this information is important for packaging selection.

2. EXPERIMENTAL

2.1. Materials and reagents

Benzophenone-3 (oxybenzone) (BZ3) 98%, 4-methylbenzylidene camphor (enzacamene) (MBC) ≥98.0%, octocrylene (octocrilene) (OCR) 97%, 2-ethylhexyl dimethyl PABA (padimate O) (EDP) 98%, ethylhexyl methoxycinnamate (octinoxate) (EMC) 98%, butyl methoxydibenzoylmethane (avobenzone) (BDM) ≥99.0%, ethylhexyl salicylate (octisalate) (ES) 99% and methylene bis-benzotriazolyl tetramethylbutyl phenol (bisoctrizole) (MBP) 99% were supplied by Sigma-Aldrich (St. Louis, MO, USA). OCR, BDM, homosalate (homosalate) (HS)

and bis-ethylhexyloxyphenol methoxyphenyl triazine (bemotrizinol) (EMT) were also supplied by Beiersdorf (Eimsbüttel, Hamburg, Germany).

2.1

Polyethylene (PE) film and multilayer packaging samples were obtained from AMCOR Flexibles. Multilayer packaging consisted of several layers of different materials, including aluminum, polyethylene (PE) and polyester (PET) fixed together by extrusion or by different polyurethane adhesives.

Ethanol (HPLC grade) and tetrahydrofuran (THF) were provided by Scharlab (Barcelona, Spain). A 1% (v/v) acetic acid aqueous solution was prepared from acetic acid supplied by Scharlab (Barcelona, Spain) in Milli-Q deionized water (Bedford, MA, USA).

Cream samples containing known concentration of analytes were prepared in a base cream containing 20% NeoPCL® Autoemulsionable O/W (oil in water) from Acofarma (Terrassa, Spain) and 80% Milli-Q deionized water (Bedford, MA, USA).

2.2. Solutions and samples preparation

Individual standard solutions containing 10 mg/ml of the UV filter were prepared in ethanol for all UV filters but for MPB and EMT prepared in THF. A multicomponent standard solution was prepared containing 80 µg/ml in ethanol from individual standard solutions and subsequently diluted as necessary.

Base cream was prepared from Milli-Q deionized water and NeoPCL. They were heated separately to 90°C. The water was added slowly to the oily mixture while stirring. It was necessary to continue stirring until the emulsion was cooled to room temperature to obtain a homogeneous mixture. Then, sunscreen agents were incorporated into this emulsion at different levels: 5% (w/w), for determining the absorption in the sachets, 7% (w/w), in order to study the influence of the number of cycles, and 10% (w/w), for the study of the rest of FUSLE variables.

In order to study the influence of FUSLE variables, PE film samples containing UV-filters were prepared. These treated PE samples were prepared by immersing 3 cm^2 of PE film in 1 g of cream formulation containing UV filters between 7 and 10% (w/w) in a NeoPCL base, for 15 days at 40ºC, to favor the absorption, and protected from the light. It is worth mentioning that two cream formulations were made to attain these concentrations for the ten UV filters. The first cream formulation contained OCR, BDM, HS, ES and EMT; and the second contained the remaining UV filters. Therefore, 6 cm^2 of fortified PE film were used to the study FUSLE variables.

The determination of UV filter sorption in PE-based multilayer packaging was carried out using 6x8 and 10x10 cm^2 sachets containing 1.5 and 3.0 g of 5% (w/w) UV filter cosmetic formulation, respectively. In all cases the cosmetic mass-packaging surface ratio was around 30 mg/cm^2. Sachets were thermosealed at 190ºC and were kept in an oven at 40ºC for 23 days to favor the sorption.

PE film and packaging samples were washed with water, dried with paper towel and stored at 4ºC protected from light before their analysis.

2.3. FUSLE procedure

All FUSLE processes were performed at 0ºC in an ice-water bath, using a SONOPLUS 2070 focused ultrasound system equipped with a 3 mm titanium microtip and sound proof box (Bandelin Sonoplus, GmbH & Co. KG). Samples were cut in small pieces of around 6 mm^2 before FUSLE. Around forty-two mg (6 cm^2) of PE film were extracted with a volume of an organic solvent (THF, ethanol or acetone) ranging from 2 to 10 ml for a period of time between 30 and 300 s, at an ultrasound power from 20 to 90%, once to four times, at 50% pulsed cycle, depending on the experiment. Microtip was immersed into a cylindrical glass vessel with flat-bottom, about 5 mm above the bottom of the vessel. Extracts were evaporated up to ~0.5 ml under a nitrogen stream using a Turbo Vap II concentrator (Zymark, Hopkinton. MA, USA). The extracts were transferred to 5 ml volumetric flask, made up to 5 ml with ethanol and filtered through a 0.45 nylon filter before HPLC injection.

2.4. Chromatographic separation

HPLC analysis was performed with an Agilent modular 1100/1200 liquid chromatograph system (Agilent Technologies, Palo Alto, CA, USA) equipped with a G1379A degasser, a G1311A HPLC quaternary pump, a G1329A Automatic Liquid Sampler (ALS) and a G1315D diode array detector (DAD). A Scharlau Nucleosil 120-C18 (5 µm packing, 250 mm × 4 mm i.d.) column protected with a precolumn of the same material (Scharlab, Barcelona, Spain) was used. The temperature of the column was set at 45°C with a Waters column heater module and a temperature control module (Milford, MA, USA). A 1% (v/v) acetic acid aqueous solution and ethanol mixture mobile phase at a flow rate of 1.0 ml/min was used for RP-HPLC. The mobile phase gradient started at 70% of ethanol and was maintained for 17 min, then increased to 100% in 1.5 min and maintained for 7.5 min. Finally, it was decreased to 70% of ethanol in 1 min and was maintained for 4 min in order to attain the initial gradient conditions for the next injection. The injection volume was 30 µl

and the chromatogram was recorded at 305 nm for all analytes, except for BDM, which was detected at 360 nm, its absorption maximum, and because the interference by the coeluting HS isomer was avoided at this wavelength. The chromatograms of a standard solution are shown in Figure 2.

2.1

2.5. Software for statistical analysis

Experimental designs and statistical analysis were performed using Statgraphics Centurion XV (Statpoint, Herndon, VA, USA), and Microsoft Excel was used for drawing response surfaces and plots.

3. RESULTS AND DISCUSSION

3.1. Chromatographic separation of UV filters

3.1.1 Preliminary experiments

In order to quantify the ten UV filters, any wavelength between 305 and 315 nm provided good sensitivity for all analytes, except for BDM. Therefore, BDM was measured at 360 nm the wavelength corresponding to its maximum of absorbance, while the rest of analytes were determined at 305 nm. The chromatographic method used to separate the ten UV filters of this study was a modification of that reported by Salvador and Chisvert [7].

Preliminary experiments on the chromatographic separation of the ten UV filters carried out by injecting the individual standard solutions showed that HS was a mixture of two isomers. This has been already reported [7]. In this work, the quantification of HS was carried out using the peak area of the most abundant isomer which represented 83.22% (RSD = 0.03%) of total HS. It is worth mentioning that although retention time of the minority isomer was very close to that of BDM, it did not pose a problem because the latter was detected at a wavelength at which HS does not absorb at all. In order to select the chromatographic condition, different mobile phase compositions were tested. Methanol and ethanol as organic modifiers at different percentages, acetic acid and AcOH/AcO^- buffer aqueous solutions, temperatures between 25 and 45ºC, and flow-rate values from 0.7 to 1.1 ml/min. However, no improvement of the BDM separation was achieved and this compound showed a significant peak tailing which spoils its determination.

3.1.2 Features of HPLC-UV method

The HPLC-UV method was characterized in terms of linearity, limit of detection (LOD) and quantification (LOQ), and repeatability (RSD, %). Results are

shown in Table 2. The features of the HPLC-UV method were established using standard solutions of the UV filters in ethanol.

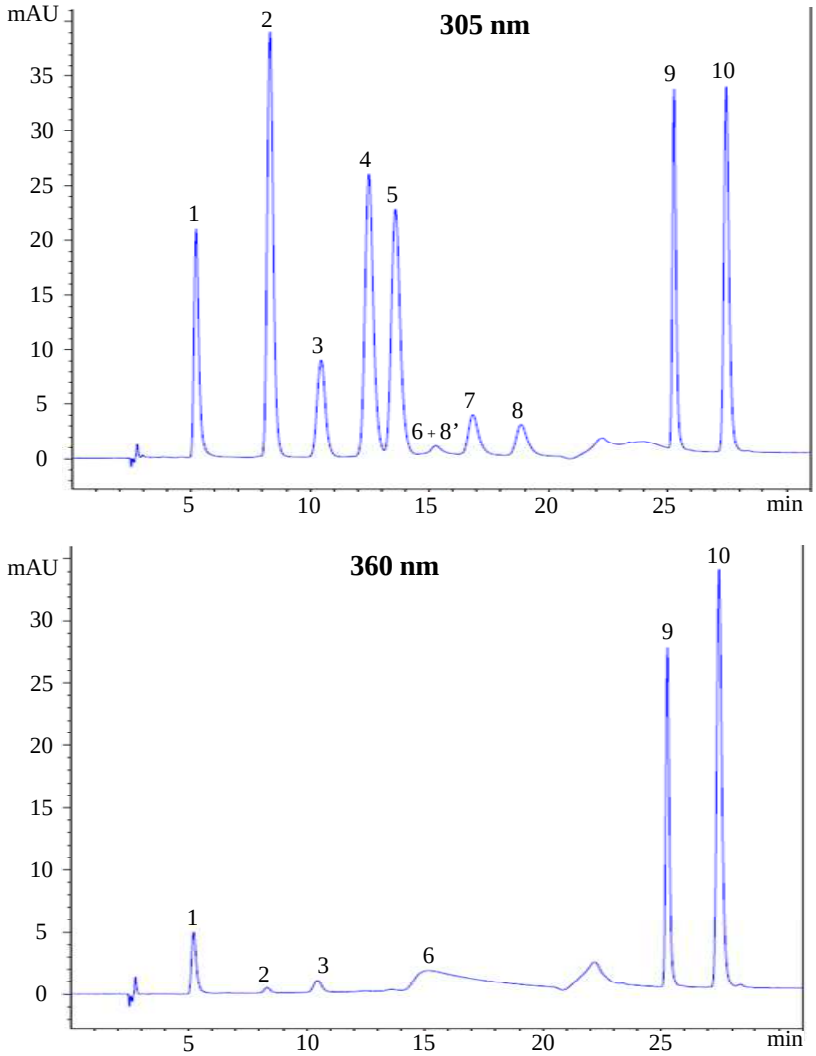


Figure 2. Chromatograms corresponding to the separation of the ten UV filters in ethanol recorded at 305 and 360nm. 1) BZ3, 2) MBC, 3) OCR, 4) EDP, 5) EMC, 6) BDM, 7) ES, 8) HS majority isomer, 8') HS minority isomer, 9) MBP, 10) EMT. Chromatographic conditions are reported in the experimental Section 2.

The linear range of all compounds was studied between the limit of quantification, estimated as ten times the standard deviation of a blank divided by the slope, and an upper limit of 80 µg/ml. The limit of detection was estimated as three times the standard deviation of a blank divided by the slope.

Table 2. Features of the HPLC-UV method

Compound	Retention time (min)	Slope (ml/µg) ± SD	Intercept ± SD	R^2	LOD (ng/ml)[a]	LOQ (ng/ml)[b]	RSD (%)[c]
BZ3	5.188±0.022	77.1±0.3	10±9	0.99990	21	68	0.28
MBC	8.27±0.04	173.3±0.6	22±18	0.99990	3	11	0.17
OCR	10.40±0.06	52.74±0.19	9±6	0.99990	6	19	0.17
EDP	12.40±0.07	154.2±0.5	24±15	0.99990	3	9	0.24
EMC	13.51±0.08	148.0±0.5	14±14	0.99992	10	33	0.25
BDM	14.63±0.13	106.6±1.2	-116±43	0.9993	278	926	1.95
ES	16.72±0.10	26.81±0.11	6±4	0.99990	36	119	0.60
HS	18.74±0.11	23.71±0.08	3±3	0.99992	21	69	0.34
HS	18.74±0.11	23.71±0.08	3±3	0.99992	21	69	0.34
EMT	27.51±0.06	129.2±0.4	10±10	0.99993	2	6	0.24

[a] Estimated as three times the standard deviation of a blank divided by the slope.

[b] Estimated as ten times the standard deviation of a blank divided by the slope.

[c] Relative standard deviation (n=10) at 4 µg/ml.

As can be seen, BDM offered worse features than the rest of UV filters, even the wavelength selected for its detection (360 nm) corresponds to its absorption maximum, and this could be because BDM showed a significant peak tailing which reduces sensibility and precision. Therefore, this HPLC-UV method is not suitable for quantifying BDM. The other UV filters showed good features. The correlation coefficients R^2 were higher than 0.99990. LOD and LOQ ranged from 2 to 36 ng/ml and from 6 to 119 ng/ml, respectively. The relative standard deviation (obtained at 4 µg/ml concentration level) was less than 0.60% therefore the results showed to be precise, even for BDM (1.95%).

3.2. Study of FUSLE variables

The main objective of this study was to select the best FUSLE conditions to extract UV filters from multilayer packaging.

3.2.1 Preliminary considerations

Variables affecting the FUSLE process include: ultrasonic irradiation power, extraction time, solvent volume, composition of the extraction solvent, number of cycles of extraction, sample mass, particle size, extraction temperature, pulse time and vessel shape.

The analyte amount extracted depends on the distribution constant, given by the analyte solubility in the solvent and sample matrix-analyte interaction, as well as the solvent-sample phase ratio. Therefore, solvent and sample volumes are correlated variables, and so they were studied as one, by testing different volumes while the sample amount was maintained constant at a value of 42 mg.

The influence of particle size on extraction of multilayer packaging was already studied elsewhere [30]. According to the results of this previous work, scissor cutting was selected to reduce particle size. Thus, 6 cm^2 of sample (to get 42 mg of sample mass) were cut in small pieces of around 6 mm^2 before FUSLE.

In order to select the extraction temperature, it should be taken into account that higher temperatures increase analyte solubility in the solvents and favor the disruption of analyte-matrix interactions, but also increased temperatures negatively affect the cavitation phenomena. As temperatures increase, the cavities immediately fill with liquid vapor which cushions the implosive action which extracts. [49]. The optimal temperature of the extraction solvent was investigated elsewhere by Sanz-Landaluze et al. [47], who found that the compromise between temperature and

cavitation was achieved at 0ºC so it was decided to keep the solvent temperature at 0ºC during all the extraction, immersing vessels into an ice bath.

2.1

In the case of the pulse time, it is worth mentioning that Henglein [50, 51] proposed that during cavitation there are two different time periods: "Activation time" which is the time required to produce chemically active bubbles with a sufficient size to allow the implosion to be effective, and ''Deactivation time'' which is the interval between pulses; and there is a compromise between the two. If the pulse time is too short, cavitation bubbles will not have enough time to grow to the suitable size to collapse; and if the pulse interval is too long, growth and collapse of bubbles disappear slowly and the following pulse will have to reactivate a new extraction system. This compromise was studied by Ping Sun et al. [52], who found that irradiation for 50% of the time offered the best results. Thus, in this work, it was decided to set the pulse time at a 50% pulsed cycle.

The vessel shape is quite important because "dead zones" where there is no cavitation, and therefore no implosion of the bubbles and no extraction, should be avoided during the extraction. The extraction vessel should be as narrow as possible to avoid this problem [48]. It is worth mentioning that the titanium microtip of the probe must be immersed into the vessel 1-2 cm from the upper surface of the slurry according to manufacturer's recommendations, and about 5 mm above the bottom of the vessel to minimize "dead zones". For this reason, it was decided to use a different vessel to be able to cover the whole volume range to optimize (2-10 ml) using at all times the narrower vessel. Then a 5 ml vessel (9 mm i.d.) was used for solvent volumes between 2 and 4 ml, while 10 ml (18 mm i.d.) and 20 ml (23 mm i.d.) vessels were employed for ranges 4-7 and 7-10 ml, respectively.

The rest of FUSLE variables, including the ultrasonic irradiation power, the extraction time, the solvent volume, the composition of the extraction solvent and the number of extraction cycles were the chosen parameters to study.

In addition, the UV filter stability under strong FUSLE conditions was studied. Six ml of a UV filter solution containing 20 µg/ml of each in THF was subjected to FUSLE under extreme conditions (at 90% ultrasound power and 50% pulsed cycle for 300 s) in triplicate. The solutions were evaporated to ~0.5 ml under a nitrogen stream, reconstituted in 5 ml of ethanol and filtered before HPLC injection. Differences between analyte signal for solutions subjected to FUSLE and the control (untreated solution) were less than 1.5%. Therefore, it can be concluded that UV filters are stable during FUSLE.

3.2.2. Solvent selection

First, in order to find the best extraction conditions, the influence of extraction solvent was studied. Usual solvents reported in literature for dissolving UV filters were tested for FUSLE: ethanol, acetone and tetrahydrofuran (THF). Six cm^2 of spiked PE film were extracted with 10 ml of each organic solvent for 300 s, at 50% of ultrasound power and at 50% pulsed cycle. Experiments were performed in triplicate. After FUSLE, extracts were evaporated to dryness under nitrogen and transferred with ethanol to a 5 ml volumetric flask and filtered through a 0.45 nylon filter before HPLC injection.

Results, presented in the supplementary material, showed that THF extracted the highest amounts of UV filters in all cases, followed by acetone. BZ3 was also well extracted with acetone, while this solvent extracted the same OCR, MBP and EMT amounts as ethanol. Therefore, THF was selected for further extractions.

It is also worth mentioning that the injection of the UV filters dissolved in THF decreased the efficiency and resolution of the chromatographic separation. Therefore, a change of solvents was mandatory and extracts were evaporated and reconstituted in ethanol before HPLC injection. The final THF percentage in the extract was studied; the evaporation process was carried out to dryness, up to 0.5 ml and up to 1.0 ml. No significant differences were observed between evaporation to dryness and up to 0.5 ml in the resolution peaks. Therefore, the extracts were reconstituted containing 10% of THF in ethanol, and the evaporation time was 50% shorter and peaks showed the same resolution as that for ethanolic extracts.

3.2.3. Central composite design

Once THF was selected as extraction solvent, a composite central design (CCD) was carried out to study the influence of the ultrasonic irradiation power, extraction time and solvent volume.

The central composite design consisted of a 23 factorial design with six star points located at ±α from the center of the experimental domain. The axial distance α for this design was 1.68 in order to establish the rotatability condition. The design was also completed with nine replicates of the central point to obtain an orthogonal design. Therefore, the complete design consisted of 23 randomly performed experiments.

All the experiments were carried out using 6 cm^2 of spiked films PE (prepared as described under experimental section). The pulsed cycle was set at 50%, the titanium microtip was immersed about 5 mm above the bottom of the vessel and the

vessels were immersed into an ice bath. Ultrasonic irradiation power values ranged from 20 to 90%, including the following levels: 20, 34, 55 (central value), 76 and 90% of ultrasound power. Extraction time was studied between 30 and 300 s and the levels were 30, 85, 165 (central value), 245 and 300 s. THF volume used in extractions was between 2 and 10 ml with levels of 2, 3.62, 6 (central value), 8.38 and 10 ml.

The ANOVA test of the results (data not presented) showed that only seven of the coefficients were significant (p-value <0.05). No first order coefficients were statistically significant and only second order coefficients were statistically significant. Pareto-charts of the standardized effects for the six UV filters affected significantly by some of FUSLE variables are included in the supplementary material. However, in order to determine the optimal values for the variables, the coefficients which became significant by eliminating the non-significant ones, because they were close to 0.05 (p value <0.08), have also been taken into account. Then, the effects considered were eleven: the time-ultrasound power interaction for MBC, the quadratic effects of ultrasound power and volume for OCR, BDM, ES and HS, the effect of volume for ES, and the effect of ultrasound power for MBP.

Response plot/surfaces for these compounds (included in supplementary material) showed that the highest responses for most of the compounds (OCR, BDM, ES and HS) were attained at 55% of ultrasonic power and at about 6 ml of THF. However, in the case of MBC and MBP, the maximum of the response surface was located at 90% and 20% of ultrasound power respectively. Therefore, an ultrasound power value of 55% was selected as a compromise. Finally, the extraction time effect was only significant in the MBC which attained its maximum at 30 s. According to these results, the optimal conditions selected for the FUSLE step were as follows: 30 s of extraction at 55% of ultrasound power with 6 ml of THF.

3.2.4. Study of number of extraction cycles

Once the best FUSLE conditions were established, the number of extraction cycles required for complete extraction was determined. The effect of a different number of FUSLE steps, from one to four, was studied. Extractions were performed in triplicate using the treated film (prepared as described under experimental section). No significant differences were observed using more than one cycle for all analytes. MBP seemed to be better extracted using three cycles but results obtained for three cycles were statistically equal to those for one or two cycles (F-value of

3.366 lower than the critical value 5.143). Therefore, it can be concluded that one extraction cycle was enough to extract all the UV filters from PE. Further experiments were performed using one extraction cycle.

3.3. Features of the FUSLE-HPLC-UV method

The whole analytical method including FUSLE and HPLC determination was characterized for the ten UV filters, in terms of limit of detection (LOD) and quantification (LOQ), repeatability (intra-day RSD, %), intermediate precision (inter-day RSD, %) and recovery. Results are listed in Table 3.

Table 3. Features of the FUSLE-HPLC-UV method

Compound	LOD [a] (ng UV filter /mg PE)	LOQ [b] (ng UV filter /mg PE)	Repeatability [c] (RSD, %)	Intermediate precision [d] (RSD, %)	Recovery ± SD (%) [e]
BZ3	4.9	16.3	3.3	3.9	113 ± 12
MBC	0.8	2.7	1.8	3.3	199 ± 42
OCR	1.4	4.6	4.4	5.4	179± 14
EDP	0.6	2.2	1.5	4.8	99 ± 3
EMC	2.3	7.8	1.7	1.5	-
BDM	66.1	220.4	2.5	2.2	74± 5
ES	8.5	28.2	2.3	3.9	100 ± 4
HS	4.9	16.4	2.2	3.2	100 ± 3
MBP	2.2	7.3	34.8	47.7	132 ± 4
EMT	0.4	1.4	4.7	13.4	58 ± 2

[a] Estimated as three times the standard deviation of a blank divided by the slope.

[b] Estimated as ten times the standard deviation of a blank divided by the slope.

[c] Intra-day relative standard deviation (n = 3replicates x 3days).

[d] Inter-day relative standard deviation (n = 3replicates x 3days).

[e] Recovery values have been calculated using the results obtained by THF extraction under reflux for 150 min.

The limits of detection and limits of quantification were estimated as three and ten times the standard deviation of a blank (a FUSLE extract of a PE film free from UV filters) divided by the slope, respectively, and expressed as nanograms of

analyte per milligram of film. The limits of detection and quantification were below 10 and 30 ng/mg of PE film, respectively, for all of the analytes except for BDM. The BDM detection was less sensitive than that of the other analytes even the wavelength selected for its detection (360 nm) corresponds to a maximum of its UV spectrum, but it shows a notable peak broadening as it was explained above.

The repeatability and intermediate precision of the method were calculated by processing 9 replicates of spiked films PE (three days x three replicates per day). ANOVA was used to obtain repeatability and intermediate precision. As can be seen in Table 3, repeatability and intermediate precision were satisfactory for all analytes (RSDs less than 5 and 14%, respectively), but for MBP. The RSD values for this compound were too high. Since the HPLC repeatability was good for this compound, there must be a problem during the extraction of MBP. Therefore, the proposed method cannot be used for quantifying MBP until this problem was solved.

In order to check the accuracy of the method, a treated PE sample was extracted by using the FUSLE method and with 20 ml of THF for 2.5 h under reflux. Recovery values, calculated using the concentrations found by extraction under reflux as reference values, were close or higher than 100% for most analytes except for EMT (58%) and BDM (74%). EMC recovery could not be calculated because it was poorly extracted in THF under reflux.

3.4. Analysis of samples

The method was applied to determine the UV filter sorption in different PE contact layer packaging. Packaging samples containing UV-filters were obtained from sachets of multilayer packaging filled with the same amount of a cream, containing the ten UV-filters, and stored for 23 days. UV-filter concentration in the cosmetic preparation was the same for the five samples. Sachets were made of different multilayer complexes, all of them with a polyethylene contact layer, but different number of layers and including both extrusion-coated and adhesive-joint packaging. In the case of adhesive-joint complexes different adhesives were used.

Although external layer was printed, the whole multilayer packaging sample can be processed without layer separation, because no co-extracted compounds were found in the chromatograms. A typical chromatogram is showed in Figure 3. The concentrations found, expressed as μg of compound per milligram of packaging, are given in Table 4.

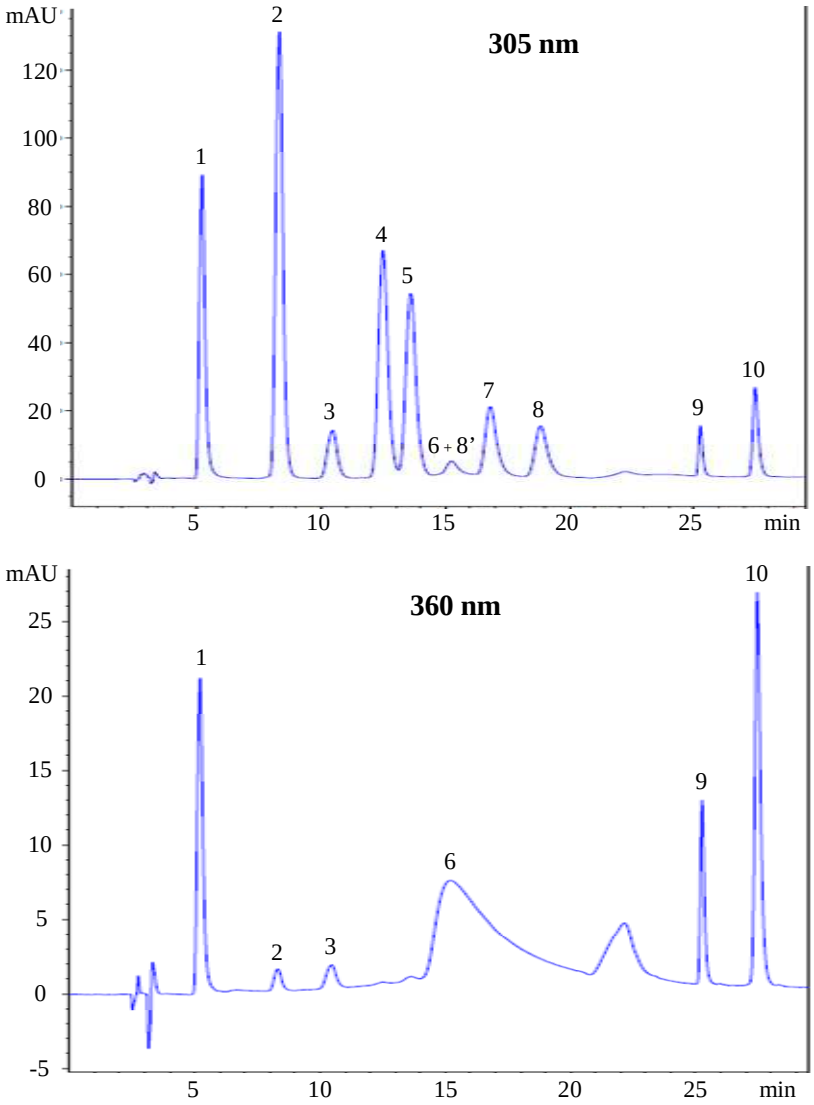


Figure 3. Chromatogram of a multilayer packaging extract containing UV-filters (sample 5 in Table 4). 1) BZ3, 2) MBC, 3) OCR, 4) EDP, 5) EMC, 6) BDM, 7) ES, 8) HS majority isomer, 8') HS minority isomer, 9) MBP, 10) EMT. Chromatographic conditions are reported in the experimental Section 2.

4. CONCLUSIONS

A fast and simple FUSLE method has been developed to determine the sorption in polyethylene-based multilayer packaging of seven of the main compounds authorized and used as UV filters in Europe nowadays to offer a sun protection factor (SPF).

2.1

Table 4. Concentration of each UV filter found in PE-based multilayer packaging (µg UV filter/mg packaging) [a]

UV filters	Samples				
	1	2	3	4	5
BZ3	2.74±0.04	3.12±0.13	1.87±0.03	3.39±0.03	2.57±0.07
MBC	2.24±0.03	2.18±0.05	1.88±0.06	2.59±0.05	2.12±0.03
OCR	1.05±0.01	1.13±0.03	0.72±0.03	1.74±0.07	0.99±0.05
EDP	1.65±0.02	1.62±0.03	1.41±0.04	2.07±0.06	1.59±0.04
EMC	1.52±0.03	1.51±0.02	1.26±0.04	2.00±0.06	1.47±0.03
BDM	1.501±0.006	1.55±0.05	1.05±0.04	2.05±0.04	1.56±0.05
ES	3.47±0.03	3.05±0.08	3.01±0.11	3.63±0.01	3.26±0.04
HS	3.23±0.05	2.88±0.07	2.75±0.11	3.46±0.02	3.05±0.04
MBP	0.099±0.004	0.17±0.02	0.08±0.01	0.13±0.04	0.23±0.10
EMT	0.43±0.07	0.36±0.05	0.258±0.004	0.63±0.02	0.44±0.04

[a] Concentration ± SD; n = 3. Samples 1 and 5: PE/Al/PET extrusion-coated complex, and 2-4: PE/PET/Al/PET adhesive-joint complex.

FUSLE was carried out with just 6 ml of tetrahydrofuran in only one cycle of 30 s. The proposed method allows the sensitive detection of most of the UV-filters in polyethylene, with limits of detection between 0.4 and 8.5 ng mg/l (except for BDM). Intra and inter-day relative standard deviation values were below 5 and 14%, respectively, except for MBP. In addition, the proposed method was more efficient than tetrahydrofuran extraction under reflux for 2.5 h for all the analytes except for EMT and BDM. In addition, the whole packaging can be processed without layer separation, which simplifies the analysis. Therefore FUSLE has shown to be faster and easier to implement than other extraction techniques such as microwave-assisted extraction (MAE) or pressurized liquid extraction (PLE). As well FUSLE is a low-cost technique versus other extraction techniques.

ACKNOWLEDGEMENTS

This work has been financed by the contract OTEM090908 with AMCOR Flexibles.

REFERENCES

[1] National Institutes of Health Consensus Statement (online). Sunlight, Ultraviolet Radiation and the Skin. 7(8) (1989) 1-29

[2] F.P. Gasparro, M. Mitchnick, J.F. Nash, Photochem. Photobiol. 68 (1998) 243.

[3] EU Cosmetic Directive 76/768/EEC, ANNEX VII

[4] D. De Orsi, G. Giannini, L. Gagliardi, R. Porrà, S. Berri, A. Bolasco, I. Carpani, D. Tonelli, Chromatographia 64 (2006) 509

[5] A. Salvador, A. Chisvert, Anal. Chim. Acta 537 (2005) 1

[6] R. Rodil, S. Schrader, M. Moeder, J. Chromatogr. A 1216 (2009) 4887

[7] A. Salvador, A. Chisvert, Anal. Chim. Acta 537 (2005) 15

[8] S. Scalia, J. Chromatogr. A 870 (2000) 199

[9] A. Chisvert, M.-C. Pascual-Martí, A. Salvador, Anal. Bioanal. Chem. 369 (2001) 638

[10] A. Chisvert, A. Salvador, J. Chromatogr. A, 977 (2002) 277

[11] A. Salvador, M.D. de la Ossa, A. Chisvert, Int. J. Cosmet. Sci. 25 (2003) 97

[12] S. Simeoni, R. Tursilli, A. Bianchi, S. Scalia, J. Pharm. Biomed. Anal., 38 (2005) 250

[13] E.R.M. Kedor-Hackmann, M.L. De Lourdes Pérez González, A.K. Singh, M.I.R.M. Santoro, Int. J. Cosmet. Sci. 28 (2006) 219

[14] I. Dencausse, A. Galland, J.L. Clamou, J. Basso, Int. J. Cosmet. Sci. 30 (2008) 373

[15] M. Nyeborg, M. Pissavini, Y. Lemasson, O. Doucet, Int. J. Cosmet. Sci. 32 (2010) 47

[16] N. Negreira, I. Rodríguez, M. Ramil, E. Rubí, R. Cela, Anal. Chim. Acta 638 (2009) 36

[17] M. Pedrouzo, F. Borrull, R. M. Marcé, E. Pocurull, J. Chromatogr. A 1216 (2009) 6994

[18] I. Tarazona, A. Chisvert, Z. León, A. Salvador, J. Chromatogr. A. 1217 (2010) 4771

[19] R. Rodil, S. Schrader, M. Moeder, J. Chromatogr. A 1216 (2009) 8851

[20] N. Negreira, I. Rodríguez., E. Rubí, R. Cela, J. Chromatogr. A 1216 (2009) 5895

[21] M. Meinerling, M. Daniels, Anal. Bioanal. Chem. 386 (2006) 1465

[22] Z. León, A. Chisvert, Á. Balaguer, A. Salvador, Anal. Chim. Acta 664 (2010) 178

[23] Z. León, A. Chisvert, I. Tarazona, A. Salvador. Anal. Chim. Acta 398 (2010) 831

[24] P.Y. Kunz, K. Fent, Aquat. Toxicol. 79 (2006) 305

[25] M. Schlumpf, B.Cotton, M. Conscience, V. Haller, B. Steinmann, W. Lichtensteiger, Environ. Health Perspect. 109 (2001) 239

[26] M. Schlumpf, P. Schmid, S. Durrer, M. Conscience, K. Maerkel, M. Henseler, M. Gruetter, I. Herzog, S. Reolon, R. Ceccatelli, O. Faass, E. Stutz, H. Jarry, W. Wuttke, W. Lichtensteiger, Toxicology 205 (2004) 113

[27] R. Ma, B. Cotton, W. Lichtensteiger, M. Schlumpf, Toxicol. Sci. 74 (2003) 43

[28] G.Ortiz, M.T. Tena, J. Chromatogr. A 1101 (2006) 32

[29] G.Ortiz, A. Garrido-López, M.T. Tena, Packag. Technol. Sci. 20 (2007) 173

[30] A. Garrido-López, A. Santa-Cruz, E. Moreno, J. Cornago, M.C. Cañas, M.T. Tena, Packag. Technol. Sci. 22 (2009) 415

[31] A. Zenker, H. Schmutz, K. Fent, J.Chromatogr. A 1202 (2008) 64

[32] M.E. Balmer, H.R. Buser, M.D. Müller, T. Poiger, Environ. Sci. Technol. 39 (2005) 953

[33] D.A. Lambropoulou, D.L. Giokas, V.A Sakkas, T.A. Albanis, M.I Karayannis, J. Chromatogr. A 967 (2002) 243

[34] Y. Kawamura, M. Miura, T. Sugita, T. Yamada, J. Food Hyg. Soc. Jpn. 38 (1997) 27

[35] C.A. Shuler, A.V. Janorkar, D.E. Hirt, Polym. Eng. Sci. 44 (2004) 2247

[36] H.L. Safa, F. Bourelle, Packag. Technol. Sci.12 (1999) 37

[37] A. Garrido-López, M.T. Tena, J. Chromatogr. A 1099 (2005) 75

[38] Ó. Ezquerro, B. Pons, M.T. Tena, J. Chromatogr. A 985 (2003) 247

[39] Ó. Ezquerro, B. Pons, M.T. Tena, J. Chromatogr. A 963 (2002) 381

[40] N. Haider, S. Karlsson, Pol. Deg. Stab. 74 (2001) 103

[41] X. Li, Z. Zeng, Y. Chen, Y. Xu, Talanta 63 (2004) 1013

[42] Y.S. Song, H. J. Park,V. Komolprasert, J. Agric. Food Chem. 48 (2000) 5856

[43] G. Lv, L. Wang, J. Liu, S. Li, J. Chromatogr. A, 1216 (2009) 8545

[44] S. Mingwu, W. Chao, J. Yongjuan, D. Xinhua, F. Xiang, Anal. Chem. 82 (2010) 5154

[45] A. Errekatxo, A. Prieto, O. Zuloaga, A. Usobiaga, N. Etxebarria, L.A. Fernández, Anal. Bioanal. Chem. 392 (2008) 1471

[46] P. Navarro, N. Etxebarria, G. Arana, Anal. Chim. Acta 648 (2009) 178

[47] J. Sanz-Landaluze, L. Bartolome, O. Zuloaga, L. González, C Dietz, C. Cámara, Anal. Bioanal. Chem. 384 (2006) 1331

[48] J.L. Capelo, M.M. Galesio, G.M. Felisberto, C. Vaz, J. Costa Pessoa, Talanta 66 (2005) 1272

[49] K.S. Suslick, in: Kirk-Othmer, Encyclopedia of Chemical Technology, J. Wiley & Sons, New York, 1998, vol. 26, 517-541

[50] A. Henglein, R. Ulrich, J. Lilie, J. Am. Chem. Soc. 111 (1989) 1974

[51] A. Henglein, Advances in Sonochemistry, JAI Press Ltd, Hampton Hill, 1993, vol. 3, 17-83

[52] P. Sun, L.K. Weavers, Chemosphere 65 (2006) 2268.

2.2.

Multilayer packaging delamination caused by UV filters: Influence of contact layer on chemical resistance and absorption capacity of sachets

Chapter excluded in the publishable version
because it contains confidential information

3.

Plastic additives in packaging

3.1.

Determination of plastic additives in packaging by liquid chromatography coupled to high resolution mass spectrometry

(Submitted) Journal of Chromatography A, 2015

Determination of plastic additives in packaging by liquid chromatography coupled to high resolution mass spectrometry

Cristina Moreta and María Teresa Tena

Department of Chemistry. University of La Rioja. C/ Madre de Dios 51, E-26006. Logroño (La Rioja). Spain.

ABSTRACT: A simple and sensitive analytical method for the determination of several plastic additives in multilayer packaging based on solid-liquid extraction (SLE) and ultra-high performance liquid chromatography (UHPLC) coupled to variable wavelength (VWD) and time of flight mass spectrometry (TOF-MS) detectors is presented. The proposed method allows the simultaneous determination of fourteen additives belonging to different families such as antioxidants, slip agents and light stabilizers, as well as two oxidation products in only 9 min. The developed method was validated in terms of linearity, matrix effect error, detection and quantification limits, repeatability and intermediate precision. The instrumental method showed satisfactory repeatability and intermediate precision at concentrations closed to LOQ with RSDs less than 7 and 20%, respectively, and LODs until 5000 times more sensitive than other GC-FID and HPLC-VWD methods previously reported. Also, focused ultrasound solid-liquid extraction (FUSLE) was optimized and evaluated to extract plastic additives from packaging. Extraction results obtained by FUSLE and SLE were compared to those obtained by pressurized liquid extraction (PLE). All extraction methods showed excellent extraction efficiency for slip agents, however quantitative recovery of all analytes was achieved only by SLE with just 5 ml of hexane for 10 hours. Finally, the selected method was applied to the analysis of packaging samples where erucamide, Irgafos 168, oxidized Irgafos 168, Irganox 1076 and Irganox 1010 were detected and quantified.

Keywords: Plastic additives; Focused Ultrasound Solid-Liquid Extraction; Time of Flight Mass Spectrometry; slip agents; antioxidants.

1. INTRODUCTION

Additives are incorporated into plastic polymers in order to modify or enhance their properties as well as to increase their shelf life. The addition of those compounds to plastic products can provide them color, density, opacity, stiffness, flexibility, resistance to heat, light or air, flame retardant, and improve processing

properties during pellet creation and final product fabrication. Depending on the additive function they can be stabilizer, modifier or filler. Modifiers additives, such as slip or anti-blocking agents, improve and alter the polymeric properties. However, stabilizer additives such as light stabilizers or antioxidants, preserve the original features of the polymer manufactured [1].

For quality and regulatory reasons, it is very important to determine the level of these additives in polymers by both manufacturers and regulators in order to ensure that plastic packaging is adjusted to its purpose safely.

The determination of polyolefin stabilizers and their degradation products was reviewed by Reingruber and Buchberger in 2010 [2]. The extraction from polymers has been traditionally performed by Soxhlet extraction or by boiling under reflux [3]. Since the nineties these extractions have also been carried out by ultrasonic assisted extraction (UAE), supercritical fluid extraction (SFE), microwave assisted extraction (MAE) or pressurized liquid extraction (PLE) [3-9]. To our knowledge, this is the first time that FUSLE has been tested to extract additives from packaging. Those techniques used heretofore are more time-consuming, more complicated and/or more expensive than FUSLE. In addition, this simple and fast extraction method is proposed in this work due to the excellent results obtained in previous works determining UV filters as well as perfluorinated and bisphenol type endocrine disrupting compounds in multilayer flexible packaging based on plastics or paper [10-12].

Regarding by-product of plastic additives, preliminary studies and literature showed that Irgafos 126 and 168, phosphite-type antioxidant additives, are prone to oxidize in solution over time as a function of solvent and temperature [13-15]. All extraction techniques applied heretofore, except UAE, use high extraction temperatures that caused partial or total oxidation of these additives, making impossible the determination of the oxidized Irgafos-to-Irgafos ratio in the sample. However, a traditional or ultrasonic solid-liquid extraction can permit to assess the amount of the Irgafos oxidized in the sample over time.

Stabilizer additives are generally separated chromatographically by LC [3, 4, 6-8] because the high molecular weight and low volatility of the majority of this sort of additives makes gas chromatography generally unsuitable. Although, oleamide and erucamide can be separated chromatographically by both GC and LC, slip agents have been separated preferably by gas chromatography, mainly because GC offers better facilities for detection [5, 9, 16]. Moreover, the majority of additives have been detected normally by ultraviolet visible spectroscopy (UV) because light

stabilizers and antioxidants present normally aromatic groups. However, traditional slips agents are based on unsaturated fatty acid amides, for instance, oleamide and erucamide; and they have no aromatic groups, so they cannot be detected by UV. More recently, liquid chromatography coupled to mass spectrometry has also been reported for additive determination in polymers [2, 17-19] and in migration studies [20, 21]. Among MS and tandem MS instruments, mainly quadrupole [18-22] and ion-trap [17, 23] analyzers have been used. Time-of-flight instruments have also been proposed but for the direct solid analysis of polymers [24-28].

Himmelsbach et al [23] have compared different ion sources, such as atmospheric pressure photoionization (APPI), atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI), for the ionization of polymer additives in liquid chromatography coupled to ion trap mass spectrometry. They concluded that the advantages of the APPI source over an ESI source are huge when analytes are less polar compounds like polymer additives, and that APPI source provided a better performance than APCI in terms of signal-to-noise ratio, and thus lower detection limits.

The direct analysis of solid polymer samples for the rapid identification of additives has been carried out using different instruments: ASAP (atmospheric solid analysis probe)-QTOFMS [24] and DESI (desorption electrospray ionization) [25], DART (direct analysis in real time) [26, 27], and MALDI-TOFMS [28]. In the former instrument, the tip of the ASAP was dipped into the sample and subsequently the analytes were thermally volatilized from the tip with a curtain of desolvation gas flow at high temperature. The volatilized substances were ionized by corona discharge and finally analyzed by QTOFMS [24]. DESI source used for identification and semi-quantitative determination of polymer additives was a combination of an in-house modified standard ESI source and an also modified nano-spray interface [25]. MALDI-TOFMS has also been proposed for the qualitative determination of light stabilizers and antioxidants directly from polypropylene, without any pretreatment. In this case the polymer powder was only mixed with dihydroxybenzoic acid as matrix [28].

The aim of this study was to develop a simple method to determine simultaneously both slip agents and stabilizer additives. To our knowledge, the simultaneous quantitative determination of stabilizer and modifier additives have been reported in only one work [20] where the migration of additives from polymer granules into solution was studied using a triple quadrupole mass detector (QqQ). However, this is the first time that a high-resolution time-of-flight (TOF) mass

spectrometry, that offers a mass accuracy and resolution higher than 5 ppm and 17500, respectively, has been employed to the simultaneous quantitative determination of these two types of additives. Moreover, this technique is much more sensitive than the GC-FID and the HPLC-VWD methods previously reported [4, 5, 9].

In this work, a straightforward method based on SLE and UHPLC-VWD-(TOF) MS has been developed for the quantification of 14 commonly used additives and 2 by-products (see the chemical structures in the Supplementary Material, Figure S1) in multilayer packaging. In addition, PLE, FUSLE and SLE extraction efficiencies have been contrasted and the whole method has also been applied to the analysis of real samples.

2. EXPERIMENTAL

2.1. Standards and materials

3-tert-butyl-4-hydroxyanisole (butylated hydroxyanisole, BHA, ≥ 98.0%) and 2,6-di-tert-butyl-4-methyl phenol (butylated hydroxytoluene, BHT, ≥ 99.0%) were purchased from Sigma-Aldrich (Madrid, Spain). Xylyl dibutylbenzofuranone (reaction product between 5,7-di-tert-butylfuran-2-one and o-xylene, commercial name HP 136, HP136); 2',3-bis (3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionyl) propionohydrazide (commercial name Irganox MD 1024, Ix1024); pentaerythritol tetrakis (3-(3',5'-di-tertbutyl-4'-hydroxyphenyl) propionate (commercial name Irganox 1010, Ix1010); octadecyl-3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate (commercial name Irganox 1076, Ix1076); 3,3',3'',5,5',5''-hexa-tert-butyl-α,α',α''-(mesitylene-2,4,6-triyl) tri-p-cresol (commercial name Irganox 1330, Ix1330); 1,3,5-tris (3,5-di-tert-butyl-4-hydroxybenzyl) 1,3,5-triazine-2,4,6 (1H, 3H, 5H) trione (commercial name Irganox 3114, Ix3114); bis (2,4-di-tert-butylphenyl) pentaerythriol diphosphite (commercial name Irgafos 126, Is126); tris (2,4-di-tert-butylphenyl) phosphite) (commercial name Irgafos 168, Is168); 2-(2H-benzotriazole-2-yl) 4,6-di-tert-pentylphenol (commercial name Tinuvin 328, T328) and bis (2,2,6,6-tetramethyl-4-piperidyl) sebacate (commercial name Tinuvin 770, T770) were provided by Ciba-Geigy Corporation (now BASF Corporation), Additives Division (Barcelona, Spain). Cis-9, 10-octadecenoamide (≥ 99%, oleamide, Ole) and cis-13-docosenoamide (≥ 97%, erucamide, Eru) were supplied by Sigma-Aldrich (Madrid, Spain). Tetradecanamide (≥ 98%, TDA) used as internal standard for all target analytes was purchased from Alfa Aesar (Karlsruhe, Germany).

According to ^{1}H-NMR (Proton Nuclear Magnetic Resonance) (see Figure S2 in the Supplementary Material), the purity of all additives was ≥ 95%, but for Is126 that showed a little impurity (enlarged image). Due to this, ^{31}P-NMR (Phosphorus NMR) for Is126 was performed (see Figure S2). The Is126 ^{31}P-NMR spectrum showed two signals and the little one was around 3% of the high one. The signal close to 100 ppm matched with a phosphite group, while the little signal (enlarged image) close to 0 ppm matched with a phosphate group. Therefore, it was assumed that the impurity was the oxidized or double oxidized Is126 at a level of about 3-6%. As mentioned in the introduction, this is consistent with the degradation of Is126 give rise to their by-products: the oxidized and double oxidized Is126 (Ix126ox and Is126ox2, respectively).

Sodium formate 99.998%, ammonium formate ≥ 99.0%, ammonium persulfate ≥ 98.0% and washed sea sand were purchased from Scharlab (Barcelona, Spain).

LC–MS grade acetonitrile (ACN) and formic acid, and HPLC grade 2-propanol (2-PrOH), hexane, tetrahydrofurane (THF) and dichloromethane (DCM) were purchased from Scharlab (Barcelona, Spain). Chromasolv® Plus grade hexane and DCM were supplied by Sigma-Aldrich (Madrid, Spain). LC–MS grade Methanol (MeOH) was purchased from Fisher Scientific (Madrid, Spain).

Milli-Q deionized water (Bedford, MA, USA) and LC–MS grade methanol were used to prepare solutions.

Polyethylene film and multilayer packaging samples were provided by Constantia Flexibles Tobepal (Logroño, Spain). All samples were kept in paper envelopes at room temperature and were cut approximately to 5-10 mm^2 using scissors before extraction.

No Is126ox2 and Is168ox standards were found. Then, Is126ox2 and Is168ox solutions for calibration were obtained from standards solutions of Is126 and Is168 in MeOH by microwave assisted oxidation (MAO) using a Mars X-press microwave system with a solvent sensor (CEM, Vertex Technics, Barcelona, Spain). The procedure previously used by Garrido-López et al. [13] was modified in order to avoid degradation of Is126ox2 and to replace sodium persulfate by ammonium persulfate because sodium salts are not recommended in MS determinations. The main MAO parameters were optimized using a central composite design (CCD). Is126ox was an intermediate product observed during the oxidation of Is126 that could not be isolated for its use as a standard. All oxidations were performed at 1200

W and the temperature was increased up to the selected value in 3 min. The selected experimental conditions were: 8 ml of a 10 µg/ml additive standard solution containing 1 mM and 0.5 mM ammonium persulfate oxidant for Is126ox2 and Is168ox, respectively, microwaved at 50ºC for 10 min and at 150ºC min for 30 min for Is126ox2 and Is168ox, respectively.

Calibration curves for the two oxidized additives were carried out separately in order to avoid that the non-consumed oxidant excess could oxidize the Is126 and Is168 contained in the calibration solutions.

2.2. SLE procedure

The solid-liquid extraction was carried out as follows: 0.20 g of sample were placed into a 20 ml glass vial and 0.2 ml of 25 µg/ml TDA as internal standard in MeOH and 4.8 ml of hexane were added. The sample was in contact with the extracting solvent at room temperature for at least 10 hours followed by vortex mixing for 15 seconds at 3000 rpm with a vortex mixer type 37600 (Barnstead Thermolyne, Dubuque, IA, USA). The extract was transferred to a 50 ml vessel using a Pasteur pipette. The glassware used and the extracted sample were washed twice with 1 ml of hexane and the two aliquots were also transferred. The final extract was evaporated to around 0.1 ml under a nitrogen stream in a 25ºC water bath using a Calliper Turbo Vap II concentrator (Zymark, Hopkinton. MA, USA). After that, extracts were reconstituted with 10 ml of LC–MS grade MeOH and filtered through a 0.22 µm nylon filter (Scharlau, Barcelona, Spain) before UHPLC injection.

2.3. FUSLE procedure

A SONOPLUS 2070 focused ultrasound system with a power of 70 W and a 20 kHz frequency equipped with a 3 mm titanium microtip and a sound proof box (Bandelin Sonoplus, GmbH & Co. KG, Berlin, Germany) was used.

In order to optimized the main FUSLE parameters, the ultrasonic irradiation power, the extraction time, the extraction solvent (MeOH, 2-PrOH, ACN, hexane, DCM or polar/nonpolar solvent mixtures) and the number of extraction cycles were tested.

Finally, the optimal extraction conditions were as follows: 0.20 g of sample were placed into a 10 ml glass vial and 0.2 ml of 25 µg/ml internal standards in MeOH and 3.8 ml of hexane were added. Then, the probe was immersed in the mixture. The extractions were performed in an ice-water bath and the sample was

exposed to a 50% ultrasonic irradiation power at 50% of pulsed cycle for 90 seconds. Liquid phase was removed using a Pasteur pipette and the extraction was repeated with an additional 4 ml of extraction solvent. Liquid phase was removed again and then, the glassware used and the extracted sample were washed with 1 ml of extraction solvent. The microtip was rinsed with ~0.3 ml of extraction solvent between samples. Blank analysis between samples showed the absence of carry-over contamination for the FUSLE method.

The three liquid portions were joined and evaporated to around 0.1 ml under a nitrogen stream in a 30ºC water bath using a Calliper Turbo Vap II concentrator (Zymark, Hopkinton. MA, USA). After that, the extract was reconstituted with 10 ml of LC–MS grade MeOH.

Finally, all extracts were filtered through a 0.22 µm nylon filter (Scharlau, Barcelona, Spain) before UHPLC injection.

2.4. Pressurized fluid extraction (PLE) procedure

In order to compare the results of the two extraction procedures (SLE and FUSLE), samples were also extracted using pressurized liquid extraction. The PLE conditions were described elsewhere [4, 5].

PLE was carried out using an ASE200 accelerated solvent extractor from Dionex (Sunnyvale, CA, USA), furnished with 11-ml stainless-steel extraction cells. Two cellulose filters were placed at the bottom of the cell, followed by about 1 g of sand. Then a mixture of 0.20 g of sample and sand was added. Finally the cell was completely filled with sand, and a cellulose filter was placed on the top.

The extractions were carried out twice with 2-PrOH/hexane (92.5:7.5, v/v) as solvent extraction at 105ºC and 1500 psi for 15 minutes. The percentage of cell filled with fresh solvent after extraction (flush volume) was 100% and the purge time was set at 120 s. A volume 0.2 ml of 25 µg/ml internal standards in MeOH were added to the PLE extracts (around 20 ml) before they were evaporated to around 0.5 ml under a nitrogen stream in a 30ºC water bath using a Turbo Vap II concentrator (Zymark, Hopkinton. MA, USA). The residues were reconstituted with 10 ml of LC-MS grade methanol and filtered through a 0.22 µm nylon filter before UHPLC injection.

Clean-up of the PLE apparatus was performed automatically. The PLE system was rinsed with 8 ml of extraction solvent when the start button was pressed and it was rinsed with 1 ml of extraction solvent after each sample extraction. Blank analysis between samples showed no carry-over contamination.

2.5. UHPLC-VWD-(TOF) MS analysis

A Waters Acquity UHPLC chromatograph (Milford, MA, USA) equipped with a 100 mm × 2.1 mm i.d., 1.6 µm particle size Waters Cortecs UHPLC C18 column and a Waters VanGuard pre-column of the same material, coupled to a variable wavelength detector followed by a Microtof-Q (Q-TOF) mass spectrometer from Bruker Daltonik (GMBH, Germany) with an electrospray interface, was employed for the separation and quantification of plastic additives. The ultraviolet and mass spectrometry data were acquired with the software Data Analysis Version 4.0 from Bruker Daltonik (GMBH, Germany).

The chromatographic separation conditions selected were as follows. Mixtures of a 1mM ammonium formate and 0.5% formic acid solution in methanol (solvent A) and a 1mM ammonium formate and 0.5% formic acid aqueous solution (solvent B) were used as mobile phase. The chromatographic separation took place in less than 10 min. The mobile phase composition was varied according to a linear gradient that was increased from 65% to 72.5% A in 1.30 min, then to 80% A in 0.10 min; later it was increased to 90% A in 1.10 min, then to 95% A in 0.50 min and further increased to 100% A in 4.00 min. Finally, the mobile phase was maintained at 100% A for 2.00 min and it was returned to the initial conditions. The flow rate was set at 0.40 ml/min and the injection volume was 5 µl. The sample tray was held at 10 °C and the column was maintained at 50 °C.

UV chromatograms were acquired at 279 nm. Electrospray ionization was carried out in positive mode using a capillary voltage of +4.5 kV. A coaxial N_2 gas flow of 9.0 l/min at 180 °C and 4.0 bar around the ESI emitter was used as nebulizer and drying gas to assist ion generation. The mass spectrometer system was calibrated across the mass range of 50–1200 m/z using internal references (sodium formate clusters ions) leading to mass accuracies < 5 ppm. The concentration of the calibrant had to be increased five times in order to detect clearly the clusters due to the presence of ammonium formate in the mobile phases that also formed clusters. At beginning of each chromatographic run, 10 µl of sodium formate at a concentration of 11 mM were injected, immediately before the sample injection. Quantification was performed by scan mode with a summation ratio of 3000. Retention times and quantification ions or wavelength used for the analytes are listed in Table 1. Ion extraction was carried out with a ± 10 mDa m/z precision. The chromatograms of a standard solution of the analytes and the internal standard (a), and an extract of a multilayer packaging sample obtained by SLE with hexane (b) are shown in Figure. 1.

2.6. Software for statistical analysis

The Statgraphics Centurion XVI.I software (Statpoint Technologies, USA) was used to generate the matrices of experimental designs and to estimate the effect of each factor on the extraction efficiency. The rest of statistical analysis were carried out using Microsoft excel and SPSS statistics 19 (IBM, USA).

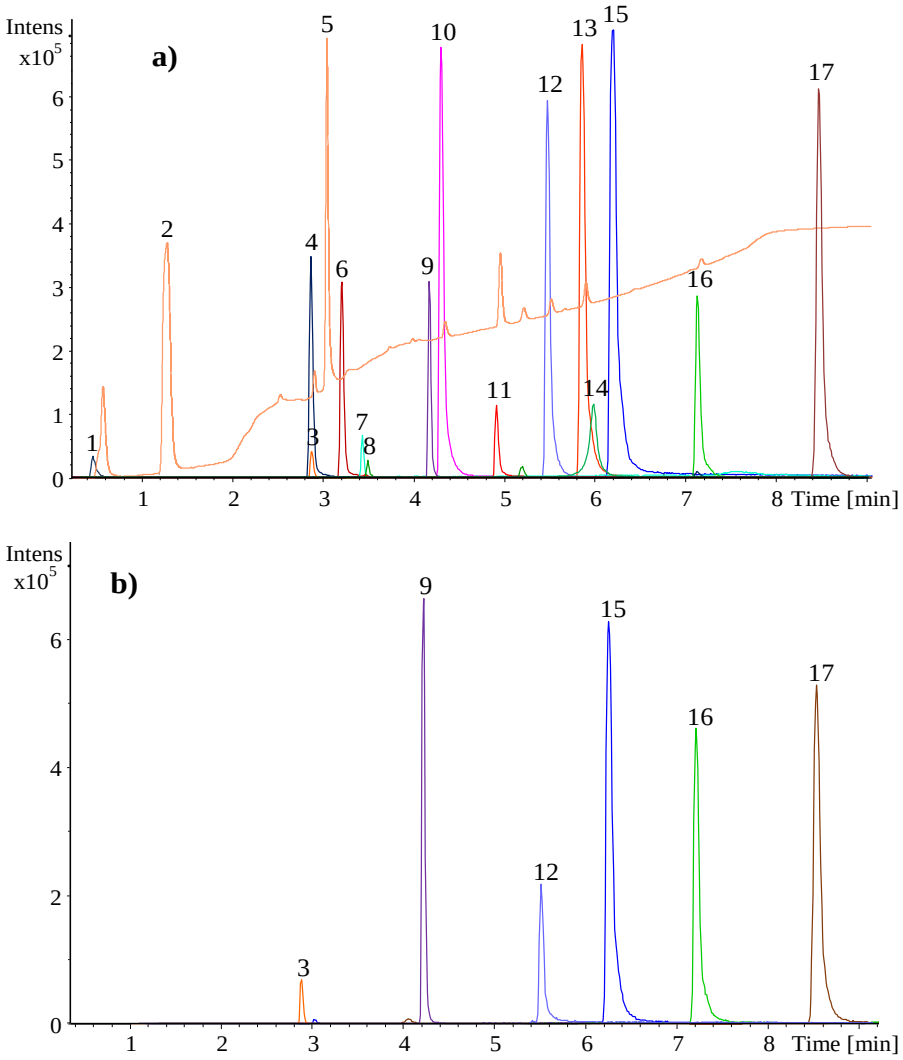


Figure 1. Chromatograms of additives obtained from (a) a methanolic standard solution at 500 ppb (5 ppm for BHA and BHT) and (b) a multilayer packaging extracted by SLE. Peak identification: 1) T770; 2) BHA; 3) TDA; 4) Ix1024; 5) BHT; 6) Is126ox2; 7) Ole; 8) HP136; 9) Eru; 10) Ix3114; 11) T328; 12) Ix1010; 13) Ix1330; 14) Is126; 15) Is168ox; 16) Ix1076; 17) Is168ox.

Table 1. Peak identification, retention times and quantification ions.

Peak identification [a]	Compounds	Retention time (min)	Quantification ions (m/z)
1	T770	0,47	481.40
2	[b] BHA	1.29	-
3	TDA-IS	2.87	455.46
4	Ix1024	2.87	570.42
5	[b] BHT	3.05	-
6	Is126ox2	3.21	654.33
7	Ole	3.44	563.55
8	HP136	3.5	351.23
-	Is126ox	4.02	621.31
9	Eru	4.18	675.68
10	Ix3114	4.3	801.55
11	T328	4.92	352.24
12	Ix1010	5.48	1194.82
13	Ix1330	5.85	792.63
14	Is126	5.96	605.32
15	Is168ox	6.21	663.45
16	Ix1076	7.13	548.50
17	Is168	8.46	647.459

[a] Peak number identification in the chromatograms shown in Fig. 1
[b] Not detected by MS. Detected by UV at 279 nm

3. RESULTS AND DISCUSSION

3.1. Study of the additive detection conditions

The ionization of additives was verified by direct infusion. All additives were ionized by ESI in positive mode except for BHA and BHT. ESI in negative mode and APCI in both positive and negative mode were also tested without success for these two compounds. The addition of sodium or silver ions was as well tested in order to favor the ionization process; however, neither BHA nor BHT could be detected by MS. Hence, UV detection was used for these two compounds.

BHT and BHA showed its absorption maximum at a wavelength of 279 and 291 nm, respectively. A single wavelength of 279 nm was selected as a compromise

to quantify both additives because the BHT absorbance at 291 nm decreased strongly and setting both wavelengths did not improve the sensitivity.

The MS detector should be optimized for quantify different m/z ranges: low (until ~ 500 m/z), medium (from ~ 400 to ~ 900 m/z) or high (from ~ 700 m/z). Low and medium ranges were tested due to the wide m/z range of additives. Only Ix1010 was out of these two ranges. The optimized parameters to detect ions in the medium m/z range showed the best results. The sensitivity of Ix1010 in the low range decreased drastically. Additionally, the sensitivity of Ole and Eru in the medium range was also good when $2M+H^+$ ion instead of $M+H^+$ ion was monitored; also, and the blanks were considerably reduced. Only HP136, T328 and T770 showed less intensity signal when medium m/z range was selected.

3.2. Chromatographic separation

Different organic solvents, buffers and formic acid and buffer concentrations were tested as mobile phase in order to obtain the best chromatographic conditions for additive determination.

In first experiments 0.1% formic acid was always added to the mobile phases in order to favor the ionization of the analytes. Variations of the mobile phase composition reported by Pouech et al [20] were tested. Firstly, MeOH and ACN were tested as organic solvents. MeOH was chosen because this solvent provided higher analytical signals than ACN for all additives except for T770 whose analytical signals were similar in both solvents.

The addition of 1 mM of ammonium formate and sodium formate to the mobile phase solvents was also tested. It was observed that sodium formate gave rise to clusters that suppressed additives ionization and signal values were lower whereas this did not happen for ammonium formate. Therefore, this salt was selected for buffer preparation.

Finally, the majority of combinations of 0 mM, 1 mM, 2.5 mM and 10 mM ammonium formate concentrations and, 0.1%, 0.5% and 1.0% formic acid concentrations in the mobile phases were also tested. A diagram of the results is shown in Table S1 in the Supplementary Material. In general, no significant differences in the analytical signals were found at different formic acid concentrations in absence of ammonium formate. However, when 1 mM ammonium formate was used in the mobile phases, all areas obtained were higher than in the absence of buffer, and this increment was even greater at acid concentrations of 0.5% and 1.0%, that showed similar results. Regarding the buffer, ammonium

formate concentrations above 1 mM showed similar or even lower areas. Therefore, 1mM ammonium formate and 0.5% formic acid in MeOH or aqueous solutions were selected for the mobile phase preparation.

Lastly, the mobile phase composition gradient was optimized in order to resolve BHT and Ix2014 peaks and to achieve a compromise between length of the chromatogram and peak resolution for the rest of analytes. Initially BHT and Ix2014 compounds gave rise to overlapped peaks in the UV spectrum making impossible their quantification with this detector. However, the quantification of overlapped compounds in the mass spectrum such as TDA and Ix1024 or Ix1330 and Is126 (see Figure 1.a) were performed without interference because a chromatogram was obtained for each compound at their corresponding m/z ratio (extracted ion chromatogram). The final selected conditions were those described in Section 2.5.

3.3 Blanks

Plastic additives are present in all kind of plastic material. Micropipette tips contaminated blanks with additives even after washing them by sonication for 30 min in MeOH. Thereby, the use of any material made of plastic must be avoided in order to minimize the blanks and only glassware was employed in this work. Furthermore, signals at m/z 338.34 (Eru, $M+H^+$) and 663.45 (Is168ox, $M+H^+$) from the blank of an empty vial were indicative that even the chromatographic system was a source of contamination of these additives. The injection of LC-MS and HPLC grade solvents showed high blank signals for m/z ions such as 282.28 (Ole, $M+H^+$), 553.46 (Ix1076, $M+Na^+$) and 647.46 (Is168, $M+H^+$). Moreover, blanks can change from one solvent bottle to another. It might be caused by solvent filtration through plastic filters. Therefore, every time a new bottle was open, a blank was measured. In order to decrease blanks, the distillation of the solvents was tested as proposed Pouech et al [20]. Nevertheless, no significant differences were found between blanks obtained from LC-MS grade MeOH and Milli-Q grade water before and after distillation and therefore, this procedure was rejected.

Filters were the only material that could not be found made of glass or metal. Methanol, milliliter by milliliter, from 1 ml to 6 ml was filtered through nylon (13 mm and 0.22 µm) and Teflon (17 mm and 0.22 µm) membrane syringe filters in order to test them. The first, second, fourth and sixth filtrates were injected. The results are shown in Figure S3 in the Supplementary Material. The Ole signal was constant and similar using both filters. Contrary to nylon filters, Teflon filters contaminated the solvent with Is126ox2 and Eru (signal at m/z 675.676, $2M+H^+$).

The Is168ox contamination was higher employing Nylon than Teflon membrane filters, however they become similar after the fourth washes and the blank signal did not decrease more. Therefore, nylon membrane syringe filters were selected and they were washed at least with 4 ml of MeOH before filtering any sample.

It was also observed that the use of new vials and glass Pasteur pipettes increased the signal of m/z 338.34, which corresponds to $M+H^+$ ion of Eru. However, the $M+H^+$ signals of Eru and Ole in blanks was not a problem because $2M+H^+$ ions were selected for Eru and Ole quantification in view of the fact that those signals were not found in blanks.

3.4. Extraction of additives from PE film

Initially, FUSLE was proposed to additive extraction from packaging due to the excellent results obtained in previous works with this extraction method. In order to provide the highest extraction efficiency, the FUSLE parameters studied were the extraction solvent, the ultrasonic irradiation power, the extraction time, the percentage of swelling solvent in the extraction solvent and finally the number of the extraction steps.

All FUSLE experiments were performed at 0ºC in an ice-water bath at 50% of pulsed cycle as well as all samples were cut around 25 mm^2 using scissor before extraction.

3.4.1. Preliminary solvent selection

There is a wide range of solvents to extract plastic additives. Traditionally the usual solvents reported in literature for extracting additives from plastic were nonpolar solvents such us chloroform, dichloromethane (DCM), tetrahydrofurane (THF), hexane, heptane or diethyl ether [3] because they can swell the polymer and this favors the additive extraction. However, polar solvents such as 2-PrOH, MeOH and ACN were selected because this kind of solvents are “greener” and less harmful, and they can be injected directly in reverse-phase liquid chromatography without solvent exchange, thus reducing risks, analysis time and labor. However, the effect of the presence of swelling solvents such us hexane and THF in the extraction solvent was also tested.

Regarding to the compatibility of nonpolar swelling solvent with the further reverse phase chromatographic determination, preliminary tests confirmed that the addition of up to 10% of hexane to the final solution did not affect peak shape and it caused very slight changes in the retention times.

Therefore, based on literature and our previous experience, the following solvents were tested: 2-PrOH, 2-PrOH/Hexane (95:5, v/v), 2-PrOH/THF (95:5, v/v), MeOH, MeOH/Hexane (95:5, v/v) and ACN. Two blanks were carried out for each kind of solvent and experiments were performed in triplicate. For this study a polyethylene (PE) film was used. Half a gram of sample was extracted with 4 ml of each solvent for 60 s at 50% of ultrasound power. Figure 2. Shows the normalized peak areas obtained for each solvent. Relative areas were normalized to the highest one for each compound because Ix1010 signal in the sample was 2 orders of magnitude lower than the other additive signals.

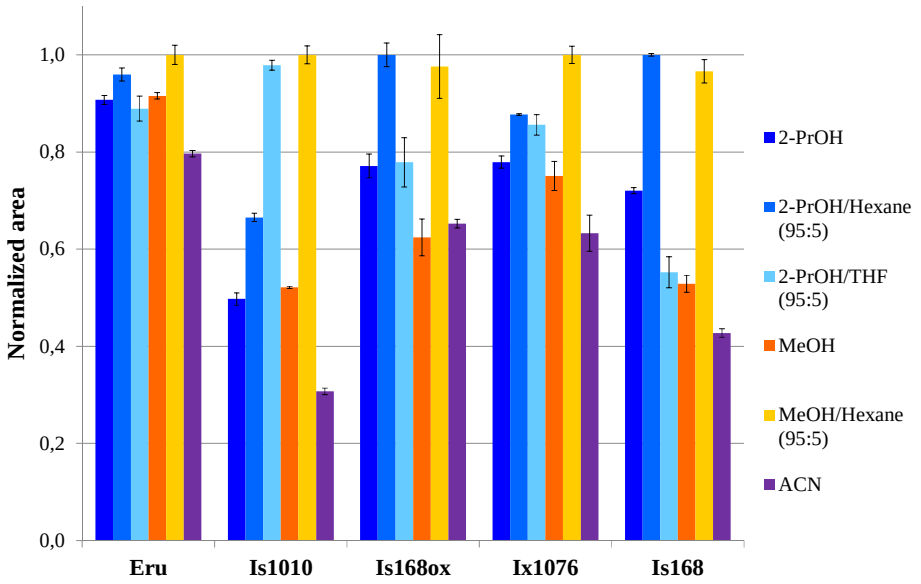


Figure 2. Influence of solvent on the FUSLE of additives from multilayer packaging. Peak area normalized to the highest value of each additive. FUSLE conditions: 4.0 ml of solvent, 50% of ultrasonic power irradiation, 60 s and 50% of pulsed cycle.

In general, MeOH/Hexane (95:5) extracted the highest amount of additives and ACN the lowest one in all cases, as may be seen in Figure 2. Therefore, MeOH/Hexane (95:5) was selected for further extractions. Figure 2 also shows that the presence of the swelling solvent was very important to extract all additives except for Eru. For this compound, differences between extraction efficiencies were less marked. This can be explained because Eru is a slip agent and this kind of compounds bloom to the surface once the film has been produced to reduce friction coefficients in post-processing. Additionally, slip agents are also less branched than

the other additives and thus the diffusion is less hindered. That makes slip agents easier to extract. However for the rest of additives, higher concentrations were obtained employing a mix of polar and nonpolar solvents instead of a pure polar solvent in all cases.

Although MeOH showed the same or less extraction efficiency than 2-PrOH, when 5% of hexane is added to these solvents, the MeOH/hexane mixture provided similar or higher analytical signals than the 2-PrOH/hexane mixture. Likewise, 2-PrOH/THF (95:5) showed also similar or less extraction efficiency than the MeOH/hexane.

3.4.2. Additive stability under FUSLE conditions

It is also worth mentioning that both Is168 and 168ox were found in the PE film used in the study of variables. Preliminary studies and literature [13-15] showed that Is126 and Is168 (phosphite-type antioxidant additives) are prone to oxidize in solution over time as a function of solvent and temperature. The presence of Is168ox in the extract can be caused by its oxidation in the sample over time or by an analytical artifact due to oxidation during the extraction process. Therefore, the stability of the analytes under focused ultrasonic irradiation was studied. Four ml of a standard solution containing 5 µg/ml of each additive was subjected to FUSLE at 100% ultrasonic irradiation power for 0 s, 60 s or 120 s (data not shown). It was observed that all additives were stable after 120 s under extreme FUSLE conditions and thus it was assumed that the presence of Is168 in the PE film was due to a previous oxidation over time. Therefore, unlike PLE [4, 13], FUSLE allows the determination of Is126ox2-to-Is126 and Is168ox-to-Is168 ratios present in plastic samples.

3.4.3. Central composite design: time, power, and percentage of swelling solvent

The influence of extraction time, ultrasonic irradiation power and percentage of hexane as swelling solvent in MeOH were optimized by a CCD. All the experiments were carried out extracting 0.20 g with 4 ml of extraction solvent. Ultrasonic irradiation power was studied from 20 to 80%, extraction time between 10 and 120 s and percentage of hexane from 2 to 10%. This upper value was selected bearing in mind not to exceed a 10% percent of hexane in the extract to inject it the directly into the chromatograph.

The ANOVA of the CCD experiments showed that only ten of the coefficients were significant (p-value <0.05), and they were the only ones considered to obtain the mathematical models. Pareto charts are included in Supplementary Materials

(Figure S4). The effect of the percentage of hexane as swelling solvent showed a positive effect for all additives. However, the time-irradiation power interaction for Ix1076 and Is168, the time-percentage of hexane interaction for Is168ox and the quadratic effect of percentage of hexane for Ix1076 showed a negative effect. The highest percentage of hexane was the optimum for all additives. However, the optimum extraction time value was the lowest (10 s) for Is168ox and Is168 and the highest one (120 s) for Ix1076. Similarly, the optimum ultrasonic irradiation power was the highest one (80%) for Is168 but the lowest one (20%) for Ix1076. Therefore, the desirability function was obtained in order to find the compromise conditions (see in Figure S5 of Supplementary Materials); and it showed that the overall optimum was achieved at 10 s, 75% of ultrasound irradiation power and 10% hexane as swelling solvent. Therefore, these FUSLE conditions were selected for further experiments.

3.4.4. Number of extraction cycles

The number of extraction cycles required for complete extraction was determined. One, two and three extraction steps were tested in quadruplicate. After each extraction step, the solvent was removed and 4 ml of fresh solvent (MeOH/Hexane (90:10)) were added to the extraction vial and the sample was extracted again under the same conditions. The results (in terms of normalized area for each additive) are shown in Figure S6 of the Supplementary Material. Significant differences were observed only between one and three cycles for Ix1076 and Is168. However, the results obtained for two and three cycles were statistically equal. Therefore, two extraction cycles were selected for further experiments.

3.4.5. Evaluation of FUSLE extraction efficiency

In order to evaluate the extraction efficiency of the developed FUSLE method, the results from the determination of plastic additives in packaging by FUSLE were compared with those obtained by PLE and vortex mixing. The latter was used for comparison in order to check the real effect of US irradiation. Therefore, vortex mixing was applied for 10 seconds at 3000 rpm instead of sonicating the sample. PLE conditions were described elsewhere [4, 5] and summarized in Section 2.4. Extracts were analyzed by UHPLC-VWD-(TOF) MS as described in Section 2.5, and all experiments were performed in triplicate.

On one hand, contrary to expectations, significant differences between the results obtained by FUSLE and vortex mixing were found only for Ix1010 and Is168, where FUSLE extracted a 23% and 18% more, respectively. On the other

hand, with respect to PLE, only Eru and Ix168 were quantitatively recovered with recovery values of 98 ± 2% and 94 ± 4%, respectively. Recovery values for other additives were low (64 ± 2% for Ix1076) or very low (9 ± 1% for Ix1010 and 28.9 ± 0.7% for Is168ox). It is important to bear in mind that the partial oxidation of Is168 to Is168ox during PLE at 105ºC does not occur during FUSLE. Therefore, the real Is168 recovery was not close to 100% and the authentic Is168ox recovery was higher than 29%. Then, FUSLE showed the same extraction capacity than PLE only for Eru, probably because slip agents are mostly on the surface and are less branched. Nevertheless, FUSLE was less efficient than PLE for the antioxidant and light stabilizer additive extraction. PLE was probably more efficient because the high temperature used. It is also worth mentioning that the extraction of Ix1010 by FUSLE provided the worst results compared to PLE (only 9%). This is probably because Ix1010 is the largest compound and presents four bulky 3,5-di-tert-butyl-4-hydroxyphenyl groups. Hence, this makes Ix1010 diffusion through the polymer matrix more difficult. Likewise, it was also reported by Vandenburg et al [3] that this additive needed ten times more ultrasound time to extract it than the rest of additives in LDPE and PP.

3.4.6. Influence of nonpolar solvent percentage in extraction solvent

Due to the need of swelling the polymer to increase the recovery of antioxidant and light stabilizer additives by FUSLE, the use of higher concentration of a nonpolar solvent in the extraction solvent was tested. Hexane and DCM were selected because are relatively greener and safer than other good swelling solvents like toluene or chloroform. THF was discarded because showed lower swelling and extraction capacity than hexane during the solvent selection carried out initially.

DCM, Hexane and the following solvent mixtures: DCM/MeOH (50:50), DCM/MeOH (10:90), Hexane/MeOH (30:70) and Hexane/MeOH (10:90) (v/v) were tested. Hexane percentages higher than 30% were not tested because of lack of MeOH miscibility. All experiments were performed in triplicate under the previous optimal FUSLE conditions.

The results (as normalized areas) are shown in Figure 3. As may be seen, the extraction capacity of FUSLE improved significantly when the concentration of the nonpolar solvent increased in all cases. As was expected, no significant difference was found for Eru. In most cases, the best recovery values were obtained when 100% hexane or 100% DCM were used. This improvement was more marked for Ix1010 and 100% hexane as expected.

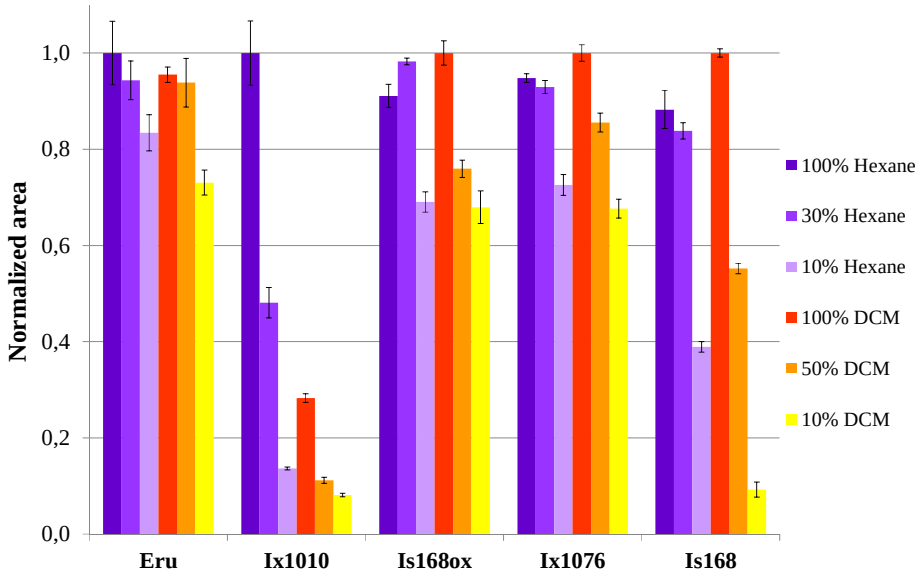


Figure 3. Influence of the nonpolar solvent percentage in methanol on extraction efficiency by FUSLE.

3.4.7. Central composite design: time, power and hexane:DCM ratio

The two principal FUSLE variables and the hexane:DCM ratio were optimized again by a CCD. Ultrasonic irradiation power was studied from 20 to 80%, extraction time between 10 and 90 s and percentage of DCM in hexane from 0 to 100%. The ANOVA results of the CCD (Pareto charts) are shown in the Supplementary Material (Figure S7). The response surface methodology was used to find the optimal conditions (Figure S8 of the Supplementary Material). Only the response surfaces of compounds with significant effects were drawn in Figure S8. DMC percentage showed a negative effect on Ix1010, Is168 and Is168ox responses, as well as the time-DCM percentage interaction on Ix1010 and Is168ox and the quadratic effect of time on Ix1010 response. The optimum was set at the highest hexane percentage and extraction time values.

Taking into account that the time was a very important parameter to extract Ix1010 and the optimal value of the extraction time was the upper limit of the experimental domain studied, this parameter was studied again. Extractions for 90s, 120s, 150s and 180s were carried out in triplicate, however no significant differences were found between any of them. However, additive recovery was not complete.

Moreover, the effect of a maceration period before FUSLE was tested. Sample and hexane were maintain in contact for 1, 2 and 3 hours before FUSLE. Also, an aliquot of sample was vortex mixed at 3000 rpm after maceration in order to check the effect of US irradiation on extraction efficiency. There was no difference between FUSLE and vortex mixing extraction efficiency when 100% hexane was employed and therefore, it was concluded that US irradiation did not improve extraction of additives from packaging. Additionally, the higher the static extraction time in hexane was, the higher was the amount of Ix1010 extracted increasing more than two times from 1 to 180 min. Therefore, extraction time values equal to or greater than three hours were required to obtain a quantitative recovery for Ix1010.

3.4.8. Optimization of SLE

Once FUSLE was discarded for the quantitative extraction of additives, SLE was chosen for sample preparation. Then, the influence of the extraction time was studied from 10 min to 24 hours (see Figure 4). All experiments were carried out in triplicate. As can be seen, recoveries higher than 90% were obtained with 10 min for all additives except for Ix1010. No significant differences between the analytical signals for Ix1010 were found after 10 h, and therefore this value was selected for a complete extraction of Ix1010.

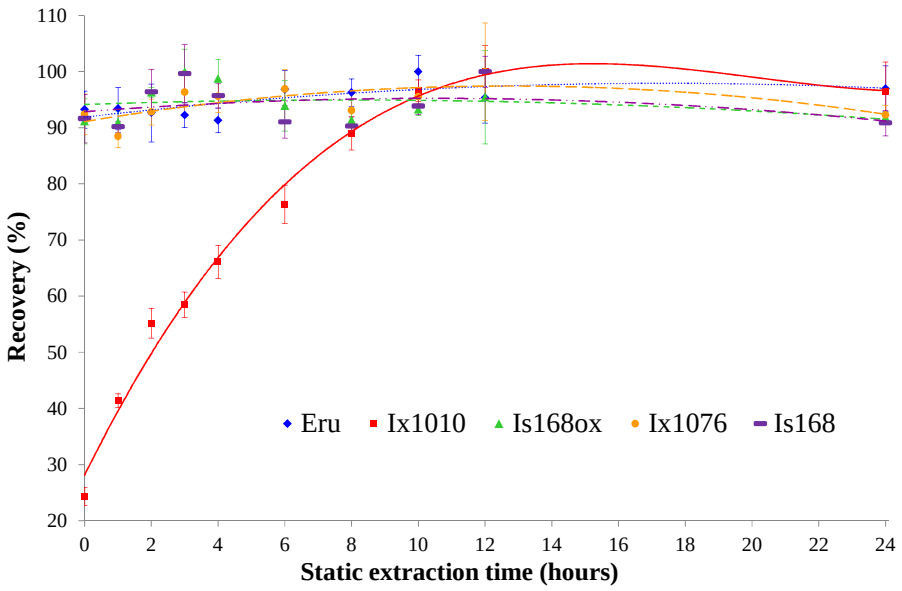


Figure 4. Influence of extraction time on the additive recovery by SLE.

3.5. Features of the UHPLC-VWD-(TOF) MS method

The UHPLC-VWD-(TOF) MS method was characterized in terms of limits of detection (LODs) and quantification (LOQs), linearity, repeatability (intra-day RSD, %) and intermediate precision (inter-day RSD, %). The features of the method shown in Table 2 were established using standard solutions of the additives in MeOH and the analytical signal used for calibration and quantification was the analyte-to-internal standard peak area ratio.

Table 2. Features of the UHPLC-VWD-(TOF) MS method.

Compounds	LOD [a] (ng/ml)	LOQ [b] (ng/ml)	Repeatability [c] (%)		Intermediate precision [c] (%)	
			~LOQ ng/ml	250 ng/ml	~LOQ ng/ml	250 ng/ml
BHA	14	41	2	3	14	2
BHT	25	75	3	3	10	3
T770	12	35	6	8	16	11
Ix1024	2.0	6	3	3	6	4
Is126ox2	0.7	2	2	3	10	7
Ole	6	17	3	4	7	10
HP136	3	8	7	5	20	5
Eru	3	8	3	4	7	9
Ix3114	0.7	3	3	4	10	3
T328	1.6	5	6	5	20	11
Ix1010	1.0	3	2	4	10	6
Ix1330	0.8	3	2	4	11	7
Is168ox	11	34	2	3	12	4
Ix1076	0.20	0.6	7	3	8	6
Is126	1.3	4	3	2	13	9
Is168	0.3	0.7	3	2	11	8

[a,b] Estimated as 3.3 and 10 times standard deviation of the intercept divided by the slope, respectively.
[c] Relative standard deviation calculated by ANOVA (4 replicated x 3 days).
~LOQ: concentration near LOQ (between 2 and 4 times LOQ).

LODs and LOQs were estimated as 3.3 times ($\alpha = \beta = 5\%$) and 10 times the standard deviation of the intercept, respectively, divided by the slope. The intercept and the slope used were obtained from a linear regression at low-concentration

levels, from 7.5 to 75 ng/ml, for T770, Ole and Is168ox, and from 1 to 10 ng/ml for the rest of additives. As can be seen in Table 2, LODs determined by mass spectrometry were between 0.2 ng/ml (Ix1076) and 3 ng/ml (HP136 and Eru) for the most of the compounds. However, T770, Ole and Is168ox showed significantly higher LODs: 12, 6 and 11 ng/ml, respectively. Regarding Is168ox, the high LOD value was due to high blank signal. Finally, as was expected, the additives determined by ultraviolet detection showed the highest LODs: 14 and 25 ng/ml for BHA and BHT, respectively.

Linearity was studied from LOQ to 5000 ng/ml ($N \geq 8$; plots are shown in the Supplementary Material, Figure S5). Only BHA, BHT, T770 and T328 showed a linear fit. Ix1024, HP136, Ix1010, Ix1076 and Is126 data were best fitted by a quadratic curve and Is168, Is126ox2, Is168ox, Ix3114, Ix1330, Ole and Eru data were best fitted by a cubic equation. Therefore, the upper limit of the range was set at 500 ng/ml (5000 ng/g for the additives detected by UV) in order to obtain linear calibration graphs. Then, the presence of significant differences between the residuals obtained for a linear regression model (LRM) and a quadratic regression model (QRM) was checked using the Mandel's fitting test with a 95% confidence level [29]. Differences between the residual variances of both regressions were not significant for most of the analytes except for Ole, Eru, Ix168ox, Ix1330 and Ix3114. Ole and Eru showed concave plots, while Ix168ox, Ix1330 and Ix3114 showed convex plots. R^2 values were between 0.9979 and 0.9996 in the linear fits and from 0.9991 to 0.9995 in the quadratic fits. It is also worth mentioning that checking the residual analysis of the graphs it was observed that all no linear models showed perfect quadratic regression model up to 2500 ng/ml, but for Ole and Eru. These latter showed a good quadratic regression model setting the upper limit of the range at 1000 ng/ml.

Repeatability and intermediate precision were calculated by ANOVA at low levels (between 2 and 4 times LOQ) and at medium levels (250 ng/ml) (see Table 2). Four replicates per day over three different days were measured. RSD values for repeatability were less than 8% in both levels. However, RSD values for intermediate precision were in general higher at low levels ($\leq$ 20%) than at medium levels ($\leq$ 11%).

3.6. Features of the SLE-UHPLC-VWD-(TOF) MS method

The whole analytical method SLE-UHPLC-VWD-(TOF) MS was characterized in terms of matrix effect error, LODs, LOQs, repeatability and

intermediate precision (intra and inter-day RSD, %), and recoveries. See Tables 3 and 4.

Table 3. SLE-UHPLC-VWD-(TOF) MS method sensitivity.

Compounds	LOD [a] (µg/g)	LOQ [b] (µg/g)
BHA*	0.5	1.5
BHT*	1.7	5
T770	0.4	1.2
Ix1024	0.14	0.4
Is126ox2	0.17	0.5
Ole	0.09	0.3
HP136	0.3	1.0
Eru	0.17	0.5
Ix3114	0.04	0.12
T328	0.08	0.25
Ix1010	0.12	0.4
Ix1330	0.06	0.19
Ix168ox	0.7	2.0
Ix1076	0.03	0.10
Is126	0.20	0.6
Is168	0.16	0.5

[a,b] Estimated as 3.3 and 10 times the standard deviation of the intercept divided by the slope, respectively.
* UV detection. MS detection for the rest of analytes.

First, the matrix effect was studied by standard addition on sample extracts extracted for 12 h. The calibration slopes in MeOH and MeOH/hexane (90:10) as well as in sample extract in MeOH/hexane (99:1) and (90:10) were compared at a confidence level of 95%. Eight concentration levels were added from 30 to 400 ng/ml (300 to 4000 for the additives detected by UV). The extract of the sample pull was diluted ten times for Eru and four times for Ix168ox, Ix1076 and Ix168 in order to not exceed a final concentration of 500 ng/ml. The calibration curves in presence and absence of matrix components showed for each additive the same behavior (linear or quadratic fit). R^2 values obtained ranged from 0.9957 to 0.9997 in presence of matrix. No significant differences between the slopes were found for all

analytes when there was an equal or very similar hexane percentage in the standard solutions and the extract, but for Ix1024. Therefore, matching the hexane percentage of calibration solutions and extracts, only the Ix1024 calibration had to be carried out by standard addition.

LODs and LOQs (Table 3) were estimated as in previous section. The intercept and slope standard deviation values were obtained from the regression equation of five low concentration levels (between 6 and 60 ng/ml) in a 1% hexane methanolic extract of a sample pull. The extract of the sample pull was diluted twelve times for Eru and five times for Ix168ox, Ix1076 and Ix168 in order not to exceed a final concentration of around 150 ng/ml. As may be seen in Table 3, BHT showed the highest LODs (1.7 μg/g). The rest of additives showed LODs between 0.03 and 0.7 μg/g.

Table 4. Repeatability and intermediate precision of the SLE-UHPLC-(TOF) MS method.

Compounds	Repeatability [a] (%)		Intermediate precision [a] (%)	
	30 min	12 h	30 min	12 h
Eru	5	4	6	5
Ix1010	6	4	16	7
Ix168ox	4	5	6	6
Ix1076	3	6	7	7
Is168	3	4	6	8

[a] Calculated by ANOVA, 3 replicates x 3 days

Repeatability and intermediate precision of the method were calculated by ANOVA (three replicates × three days) of the whole method for extraction times of 30 min and 12 h (complete extraction) (See Table 4). They were calculated only for the additives present in the sample pull (Eru, Ix1010, Is168ox, Ix1076 and Is168). Excellent repeatability was obtained with RSD ranged from 3 to 6%. Likewise, intermediate precision was lower than 8% RSD for all compounds, except for Ix1010. This additive presented an intermediate precision of 16% for a 30-min extraction, probably due to its incomplete extraction in 30 min.

3.7. Analysis of samples

The method was applied to determine additives in a polyethylene (PE) film (no final product, sample 0) and eight flexible multilayer packaging made of PE,

aluminum foil, polyester and/or paper (samples 1-8). All extracts were injected undiluted and 10-40 times diluted. Results are given in Table 5. All samples showed the presence of the same additives and by-products in a concentration range between 142 µg/g and 910 µg/g, being Ix1076 the predominant compound, followed by Is168ox. Ix1010 was the less abundant. However, the concentrations of each compound varied widely between some samples, especially for Is168 and Is168ox.

Table 5. Analysis of multilayer packaging samples by 12-h SLE and UHPLC-TOFMS.

Sample	Concentration ± SD (µg/g)[a]					
	Eru	Ix1010	Ix1076	Is168	Is168ox	Total [b]
0	13.2 ± 0.7	11.2 ± 0.6	99.5 ± 2.2	123 ± 6	352 ± 5	599 ± 8
1	183 ± 9	25.9 ± 0.6	236 ± 11	25.9 ± 0.6	208 ± 11	679 ± 18
2	87 ± 4	7.2 ± 1.2	92 ± 3	<LOQ	2.04 ± 0.06	188 ± 5
3	57 ± 9	10.81 ± 0.23	77 ± 3	11.7 ± 0.4	38.3 ± 2.0	195 ± 10
4	136 ± 4	27.0 ± 1.4	341 ± 9	19.7 ± 2.2	311 ± 4	835 ± 11
5	126 ± 4	27.5 ± 1.0	275.1 ± 1.2	49.8 ± 1.0	432 ± 5	910 ± 7
6	142 ± 4	14.6 ± 0.6	353 ± 10	24.2 ± 0.5	233 ± 9	767 ± 14
7	69 ± 5	18.18 ± 0.15	204 ± 5	17.21 ± 0.03	85.7 ± 1.9	394 ± 7
8	74.3 ± 0.7	2.08 ± 0.10	38.2 ± 1.0	8.5 ± 0.3	18.81 ± 0.09	141.9 ± 1.3

[a] Standard deviation (N = 3).
[b] Total plastic additives calculated by the sum of quantified additives.
<LOQ: concentration below the quantification limit. Detected but not quantifiable.

No Tinuvin additive (light stabilizer) was found in any sample and only Eru was found as a slip agent. About antioxidants, three additives were quantified in all samples: Is168, Ix1076 and Ix1010. In addition, Is168ox was also found in all samples with concentrations between 2 and 16 times higher than the Is168 concentration. This can be probably due to oxidation during the manufacturing process and the packaging storage.

4. CONCLUSIONS

A simple and sensitive analytical method to determine 14 plastic additives and 2 by-products in packaging based on SLE and UHPLC-VWD- (TOF) MS has been developed and validated.

The chromatographic method proposed in this work allows the simultaneous determination of slip agents and other additives in less than 10 min. Slip agents have been usually determined by GC-FID, while the others by LC. Furthermore, this method is between 21 and 5000 times more sensitive than the GC-FID and the HPLC-VWD methods previously reported showing LOQ between 0.6 and 8 ng/ml for the majority of additives. In addition, good repeatability and intermediate precision values were achieved for all of them even at concentrations close to LOQ with RSDs below 7% and 20%, respectively.

PLE, FUSLE and SLE techniques were evaluated for the extraction of additives in packaging. All extraction methods showed excellent extraction efficiency for slip agents because those additives bloom to the surface once the film is produced and are less branched. However, the Ix1010 extraction was the most troublesome due to its large size. FUSLE was rejected in this work due to its ineffectiveness since it did not show significant acceleration of the extraction process. Regarding PLE, this method makes impossible the determination of Is126ox2-to-Is126 and Is168ox-to-Is168 ratios in packaging because the high temperatures used during the extraction oxidizes partially or completely the phosphite-group of these antioxidant additives. Moreover, PLE showed a partial recovery for Ix1010 around 25-30%. However, an exhaustive extraction of all analytes was achieved after traditional static SLE for 10 hours with just 5 ml of hexane. Actually, only the presence of swelling solvents significantly increased the recovery values, especially for Ix1010, and therefore it seems that the extraction efficiency depends more on the packaging swelling than on the extraction force. Consequently, although a 10-hour SLE is a very time-consuming process, this technique was selected due to its low cost, simplicity and excellent recovery values, as well as, because it preserves phosphite stability during extraction.

Acknowledgments

C. Moreta thanks the Government of La Rioja for her FPI fellowship. Constantia Tobepal is thanked for providing the samples. E. Garrido and F. Rodríguez are also thanked for his help with the UHPLC-(TOF) MS equipment and for the performance of all NMR analysis, respectively.

REFERENCES

[1] K. Keck-Antoine, E. Lievens, J. Bayer, J. Mara, D.S. Jung, S.L. Jung, Chapter 4 – Additives to design and improve the performance of multilayer flexible packaging, J.R.

Wagner (Ed.), Multilayer Flexible Packaging, Elsevier Publishing, Burlington (MA), 2011, pp 37–56.

[2] E. Reingruber, W. Buchberger, Analysis of polyolefin stabilizers and their degradation products, J. Sep. Sci. 33 (2010) 3463-3475.

[3] H.J. Vandenburg, A.A. Clifford, K.D. Bartle, J. Carroll, I. Newton, L.M. Garden, J.R. Dean, C.T. Costley, Critical Review: Analytical Extraction of Additives From Polymers,Analyst 122 (1997) 101-116.

[4] Á. Garrido-López, M.T. Tena, Experimental design approach for the optimisation of pressurised fluid extraction of additives from polyethylene films, J. Chromatogr. A 1099 (2005) 75–83.

[5] Á.Garrido-López, V. Esquiu, M.T. Tena, Determination of oleamide and erucamide in polyethylene films by pressurised fluid extraction and gas chromatography, J. Chromatogr. A 1124 (2006) 51-56.

[6] L. Sternbauer, I. Hintersteiner, W. Buchberger, A. Standler, E. Marosits, Evaluation of a microwave assisted extraction prior to high performance liquid chromatography for the determination of additives in polyolefins, Polym. Test. 32 (2013) 901–906.

[7] M. Arias, I. Penichet, F. Ysambertt, R. Bauza, M. Zougagh, Á. Ríos, Fast supercritical fluid extraction of low- and high-density polyethylene additives: Comparison with conventional reflux and automatic Soxhlet Extraction, J. Supercrit. Fluids 50 (2009) 22–28.

[8] B Marcato, M Vianello, Microwave-assisted extraction by fast sample preparation for the systematic analysis of additives in polyolefins by high-performance liquid chromatography, J. Chromatogr. A 869 (2000) 285–300.

[9] G. Lv, L. Wang, J. Liu, S. Li, Method for determination of fatty acid amides in polyethylene packaging materials—Gas chromatography/mass spectrometry, J. Chromatogr. A 1216 (2009) 8545–8548.

[10] C. Moreta, M.T. Tena, Determination of UV filters in packaging by focused ultrasonic solid-liquid extraction and liquid chromatography, J. Chromatogr. A 1218 (2011) 3392-3399.

[11] D. Pérez-Palacios, M.A. Fernández-Recio, C. Moreta, M.T. Tena, Determination of bisphenol-type endocrine disrupting compounds in food-contact recycled-paper materials by focused ultrasonic solid-liquid extraction and ultra performance liquid chromatography-high resolution mass spectrometry, Talanta 99 (2012) 167-174.

[12] C. Moreta, M.T. Tena, Determination of perfluorinated alkyl acids in corn, popcorn and popcorn bags before and after cooking by focused ultrasound solid-liquid extraction, liquid chromatography and quadrupole-time of flight mass spectrometry, J. Chromatogr. A 1355 (2014) 211-218

[13] A. Garrido-López, I. Sancet, P. Montaño, R. González, M.T. Tena, Microwave-assisted oxidation of phosphite-type antioxidant additives in polyethylene film extracts, J. Chromatogr. A 1175 (2007) 154-161.

[14] H. El Mansouri, N. Yagoubi, D. Ferrier, Extraction of polypropylene additives and their analysis by HPLC, Chromatographia 48 (1998) 491-496.

[15] M.S. Dopico-García, J.M. López-Vilariño, M.V. González-Rodríguez, Determination of antioxidant migration levels from low-density polyethylene films into food simulants, J. Chromatogr. A 1018 (2003) 53-62.

[16] A.S. Rawls, D.E. Hirt, M.R. Havens, W.P. Roberts, Evaluation of surface concentration of erucamide in LLDPE films, J. Vinyl Add. Tech. 8 (2002) 130-138.

[17] S. Beißmann, M. Stiftinger, K. Grabmayer, G. Wallner, D. Nitsche, W. Buchberger, Monitoring the degradation of stabilization systems in polypropylene during accelerated aging tests by liquid chromatography combined with atmospheric pressure chemical ionization mass spectrometry, Polym. Degrad. Stab. 98 (2013) 1655-1661.

[18] C. Block, L. Wynants, M. Kelchtermans, R. De Boer, F. Compernolle, Identification of polymer additives by liquid chromatography-mass spectrometry. Polym. Degrad. Stab. 91 (2006) 3163-3173.

[19] R.E. Duderstadt, S.M. Fischer, Effect of organic mobile phase composition on signal responses for selected polyalkene additive compounds by liquid chromatography-mass spectrometry, J. Chromatogr. A 1193 (2008) 70-78.

[20] C. Pouech, F. Lafay, L. Wiest, R. Baudot, D. Léonard, C. Cren-Olivé, Monitoring the extraction of additives and additive degradation products from polymer packaging into solutions by multi-residue method including solid phase extraction and ultra-high performance liquid chromatography-tandem mass spectrometry analysis, Anal. Bioanal. Chem. 406 (2014) 1493-1507.

[21] Z. Bodai, B.S. Szabó, M. Novák, S. Hámori, Z. Nyiri, T. Rikker, Z. Eke, Analysis of potential migrants from plastic materials in milk by liquid chromatography-mass spectrometry with liquid-liquid extraction and low-temperature purification, J. Agric. Food Chem 62 (2014) 10028-10037.

[22] Y. Zhang, Z. Du, A. Li, A. Tu, W. Yu, J. Zou, Rapid qualitative and semi-quantitative determination of polymer additives in polymer matrix by electrospray ionization-triple quadrupole mass spectrometry with accelerated solvent extraction (ASE-ESI-MS/MS), Anal. Methods 5 (2013) 5112-5120.

[23] M. Himmelsbach, W. Buchberger, E. Reingruber, Determination of polymer additives by liquid chromatography coupled with mass spectrometry. A comparison of atmospheric pressure photoionization (APPI), atmospheric pressure chemical ionization (APCI), and electrospray ionization (ESI), Polym. Degrad. Stab. 94 (2009) 1213-1219.

[24] Z. Du, Y. Zhang, A. Li, S. Lv, Rapid identification of polymer additives by atmospheric solid analysis probe with quadrupole time-of-flight mass spectrometry, Rapid Comm. Mass Spectrom. 28 (2014) 2035-2042.

[25] S.M. Reiter, W. Buchberger, C.W. Klampfl, Rapid identification and semi-quantitative determination of polymer additives by desorption electrospray ionization/time-of-flight mass spectrometry, Anal. Bioanal. Chem. 400 (2011) 2317-2322.

[26] M. Haunschmidt, C.W. Klampfl, W. Buchberger, R. Hertsens, Rapid identification of stabilisers in polypropylene using time-of-flight mass spectrometry and DART as ion source, Analyst 135 (2010) 80-85.

[27] K. Fouyer, O. Lavastre, D. Rondeau, Direct monitoring of the role played by stabilizer in a solid simple of polymer using direct analysis in real time mass spectrometry: The case of Irgafos 168 in polyethylene, Anal. Chem. 84 (2012) 8642-8649.

[28] C. Schwarzinger, S. Gabriel, S. Beißmann, W. Buchberger, Quantitative analysis of polymer additives with MALDI-TOF using an internal standard approach, J. Am. Soc. Mass Spectrom. 23 (2012) 1120-1125.

[29] L. Brüggemann, W. Quapp, R. Wennrich, Test for non-linearity concerning linear calibrated chemical measurements, Accredit. Qual. Assur. 11 (2006) 625-631.

4.

PFAAs in packaging and food samples

4.1.

Fast determination of perfluorocompounds in packaging by focused ultrasound solid-liquid extraction and liquid chromatography coupled to quadrupole-time of flight mass spectrometry

Journal of Chromatography A, 1302 (2013) 88-94

Fast determination of perfluorocompounds in packaging by focused ultrasound solid-liquid extraction and liquid chromatography coupled to quadrupole-time of flight mass spectrometry

Cristina Moreta and María Teresa Tena

Department of Chemistry. University of La Rioja. C/ Madre de Dios 51, E-26006. Logroño (La Rioja). Spain.

ABSTRACT: A focused ultrasound solid-liquid extraction (FUSLE) and liquid chromatography (HPLC) coupled to quadrupole-time of flight mass spectrometry (QTOF-MS/MS) based method is proposed to determine six perfluorocarboxylic acids (PFCAs) and perfluorooctanesulfonate (PFOS) in food-contact packaging. FUSLE, a simple, inexpensive and fast extraction technique, has been carried out with just 8 ml of ethanol in one cycle of only 10 s. The whole method presented good repeatability and intermediate precision, with RSDs bellow 11 and 15%, respectively; limits of detection, with values between 0.5 and 2.2 ng/g, and successful recovery values, around 100% in all cases. The developed method has been validated and applied to the analysis of real food-contact packaging samples. FUSLE results have been compared to those obtained with pressurized liquid extraction (PLE) and no significant differences between them have been found. PFAAs were detected in all the packaging samples analyzed, in a concentration range between 4 and 29 ng/g, being PFHpA (perfluorooctanoic acid) the most abundant of them.

Keywords: Perfluorinated alkyl acids, Focused Ultrasound Solid-Liquid Extraction, Liquid Chromatography, Quadrupole - Time of Flight Mass Spectrometry, Packaging.

1. INTRODUCTION

Perfluorinated alkyl substances (PFASs) are amphiphilic compounds that show high thermal, biological and chemical inertness. They can resist degradation by acids, bases, oxidants, reductants, microbes, photolytic and metabolic processes because carbon-fluorine is the strongest existing covalent bond (450 KJ/mol) [1, 2].

As perfloroalkyl chains are oleophobic and hydrophobic and exhibit surface tension lowering properties, PFASs have been widely used in different commercial and industrial applications such as stain-resistant coatings for textiles, leather and

carpets, lubricants, grease-proof coatings for paper food-contact packaging, fire-fighting foams, insecticides and floor polishes [1,3,4]

Unfortunately, PFASs are environmentally persistent, bioaccumulative and, in addition, potentially harmful; and they have been widely distributed in the environment due to extensive industrial application and consumer use [5]. It has been proved that PFOS (perfluorooctanesulfonate) and PFCAs (perfluorocarboxylic acids) exhibit toxicity in laboratory animals causing developmental diseases, liver cancer, affect the lipid metabolism and disturb the immune system [5]. Moreover, other perfluorinated alkyl substances, such as polyfluoroalkyl phosphate surfactants (PAPs) and fluorotelomers (FTOHs), may be atmospherically or metabolically degraded to perfluorinated alkyl acids (PFAAs), increasing their concentration in the environment [6-8].

Therefore, PFASs have in a trend in a wide variety of matrices, from liquid and solid matrices to air [9]. Methods for the determination of PFASs in environmental and biological samples such as sewage sludge [10-15], water [15-18], sediments [15, 19, 20], biota [21] or even air [22-24] have been developed. Likewise, the accumulation in humans has been studied through the analysis of blood [25-27], tissue [28] or even hair and nail [29]. In order to assess the sources and pathways of human exposure to PFASs, dust [30, 31], articles of commerce [32], food [33-38], drinking water [38] and food packaging [39-46] have also been examined.

One of the two main applications of perfluorinated alkyl substances in food contact materials is as additives in paper coatings to provide oil and moisture resistance to paper food packaging [47]. Begley et al. [45] and Matínez-Moral et al. [45] have found levels up to 290 and 198 ng/g of PFOA (perfluorooctanoic acid), respectively, in microwave popcorn packaging [45, 46] and the US FDA considered treated paper as the greatest potential source of fluorochemicals in 2005 [48]. Therefore, it is important defining possible routes of exposure such as food-contact packaging.

The most reported technique to determine PFASs is LC/MS because with the use of other techniques employed as LC-UV or GC/MS, many perfluorocompounds are not detectable or the measurement of these compounds at low levels is not possible [47, 49].

Ultrasound assisted extraction (UAE) and pressurized liquid extraction (PLE) have been used to extract PFASs from food-contact packaging [39-46], and this is

the first time that focused ultrasonic solid- liquid extraction (FUSLE) has been proposed. The analytes were extracted quantitatively for 30 min by classical solid-liquid extraction [44], for 1-2 hours by UAE [40-42, 45] and for 6-30 min by PLE [39, 43, 46] from different kinds of samples. However, organic pollutants can be extracted by FUSLE in several seconds or in a few minutes [50, 56]. FUSLE has already been used in the determination of polychlorinated biphenyls [53], phthalate esters [53], nonylphenols [53], polycyclic aromatic hydrocarbons [52-55], brominated diphenyl ethers [56] and metals [57] from environmental matrices, and UV filters [50] and bisphenols [51] from packaging.

FUSLE is a relatively novel, simple, inexpensive and fast extraction technique in comparison to others as pressurized liquid extraction (PLE), supercritical fluid extraction (SFE) or microwave assisted extraction (MAE). Its only disadvantage is the lack of automation.

FUSLE technique is based on the cavitation phenomenon, and is carried out by immersing the focused ultrasound microtip directly in the extracting solution and this, together with the higher ultrasound power, makes the irradiation power of the focused ultrasound technique more reproducible and 100 times higher than that of the traditional ultrasonic bath [54, 55].In this study, a fast and simple method based on FUSLE and HPLC-QTOF(MS/MS) has been developed for the quantification of the most commonly determined PFAAs, PFOA and PFOS, and also other PFCAs in food-contact packaging. Moreover, it has also been applied to the analysis of real samples.

2. EXPERIMENTAL

2.1. Standards and materials

Individual standards of perfluoroheptanoic acid (PFHpA) 99%, perfluorooctanoic acid (PFOA) 98%, perfluorononanoic acid (PFNA) 97%, perfluorodecanoic acid (PFDA) 98%, perfluoroundecanoic acid (PFUnA) 95%, perfluorododecanoic acid (PFDoA) 95% and perfluorooctanesulfonic acid (PFOS) 98%, were provided by Sigma Aldrich (Madrid, Spain). The isotopically labelled perfluoro-n-[$^{13}C_8$]octanoic acid and sodium perfluoro-1 [1,2,3,4-$^{13}C_4$]octanesulfonate standards (MPFOA and MPFOS, both >99%), used as an internal standard for perfluorocarboxylic acids and perfluorooctanesulfonic acid, respectively, were purchased from Wellington Laboratories Inc. (Guelph, ON, Canada) as 50 µg/ml solution in methanol.

LC-MS grade acetonitrile (ACN), methanol (MeOH), formic acid and HPLC grade ethanol (EtOH) were obtained from Scharlau (Barcelona, Spain). Aqueous solutions were prepared in Milli-Q deionized water (Bedford, MA, USA).

Standard solutions were prepared in LC-MS grade MeOH using glass volumetric flasks and stored in glass vials protected from light at -18ºC. During preparation and storage, solution or sample contact with perfluorinated materials such as PTFE (polytetrafluoroethylene) and PVDF (polyvinylidene fluoride) was avoided in order to prevent from contamination.

Anhydrous sodium sulphate, for GC residue analysis (min. 99.5%) was obtained from Scharlau (Barcelona, Spain).

2.2. Samples

Different food-contact packaging like microwave popcorn bag, ice cream tub and cardboard cup were obtained from different local supermarkets. The six microwave popcorn bags analyzed were of different brands, all different generic brands but for a name brand (sample 2). There were three types of microwave popcorn: salty (samples 1, 2 and 3), salty and buttered (samples 4 and 5), and sweet popcorn (sample 6). The bags were purchased between late 2011 and early 2012. The cardboard cup tested was made of printed cardboard and lined with polymer layer, and the ice cream tub of printed cardboard and lined inside and outside with a polymer layer.

The samples were ground using an IKA A10 Analytical Mill purchased from IKA-Werke GmbH & Co. KG, (Staufen, Germany). The ground samples were stored protected from the light at 4ºC in cylindrical plastic wide-mouth containers purchased from Lin Lab Rioja (La Rioja, Spain).Containers of 1l and 250 ml were made of PP (polypropylene) and HDPE (high-density polyethylene), respectively.

A pull of microwave popcorn bags samples was used for the method optimization and validation. Spiked samples at a concentration level of 800 ng/g of each analyte were used to study the influence of FUSLE conditions. The features of the method were established using spiked samples containing 15, 40 or 200 ng/g of each analyte. These spiked samples were prepared by adding a standard solution of the perfluorocompounds in ethyl acetate to the packaging. The mixture was thoroughly homogenized and maintained at room temperature until the solvent was completely evaporated, and then it was triturated again to ensure proper homogenization of the sample. Then the samples were aged in polypropylene plastic containers, protected from light at 4ºC, for at least two weeks before use.

2.3. Focused Ultrasound Solid-liquid Extraction (FUSLE)

A SONOPLUS 2070 focused ultrasound system, with a power of 70 W and a 20 kHz frequency, equipped with a 3 mm titanium microtip and a sound proof box (Bandelin Sonoplus, GmbH & Co. KG, Berlin, Germany) was used.

The optimal extraction conditions were as follows: 0.5 g of ground sample were deposited into a 34 x 100 mm 50-ml centrifuge glass tube with round bottom (Scharlau, Barcelona, Spain). , and then 8 ml of ethanol were added.

Before each extraction, 100 µL of the 300 ng/ml internal standard solution was also added. Then, the probe was immersed in the mixture. The extractions were performed at 0ºC in an ice-water bath and the sample was exposed once to a 30% ultrasonic irradiation power for 10 seconds at 50% of pulsed cycle.

The microtip was rinsed with 1.5 ml of fresh MeOH between samples. Blanks between samples showed the absence of carry-over contamination. In addition, 10 ml of acetone were sonicated for 10 s at 30% power at the beginning and at the end of working session.

Liquid phase was removed using a Pasteur pipette and the solid phase was then washed twice with 2.5 ml of extraction solvent (ethanol). FUSLE extracts were evaporated to dryness under a nitrogen stream using a Caliper Turbo Vap II concentrator furnished with 50 ml vessels (Zymark, Hopkinton. MA, USA). The extracts were reconstituted with 2 ml of LC-MS grade methanol and filtered through a Scharlau (Barcelona, Spain) 25-mm diameter, 0.22 µm nylon filter before HPLC injection.

2.4. Pressurized liquid extraction

In order to contrast FUSLE results, the food-contact packing samples were also extracted using pressurized liquid extraction as described by Martinez-Moral et al [46].

PLE was carried out using an ASE200 accelerated solvent extractor from Dionex, furnished with 11-ml stainless-steel extraction cells. Two cellulose filters (20-mm diameter, Restek, Bellefonte, PA, USA) were placed at the bottom of the cell, followed by a layer of 1 g of anhydrous sodium sulphate. Then a mixture of 0.5 g of sample and 1 g of anhydrous sodium sulphate was added. Finally the cell was completely filled with anhydrous sodium sulphate, and a cellulose filter was placed on top. The extractions were carried out once with methanol at 100ºC and 1500 psi for 6 minutes.

Clean-up of the PLE apparatus is performed automatically. The PLE system is rinsed with 8 ml of extraction solvent when the start button is pressed and is rinsed with 1 ml of extraction solvent after each sample extraction. Blank analysis showed no carry-over between samples.

One hundred µL of a 300 ng/ml internal standard solution were added to the PLE extracts (around 15 ml) before they were evaporated to dryness under a nitrogen stream using a Caliper Turbo Vap II concentrator furnished with 50 ml vessels (Zymark, Hopkinton. MA, USA). The residues were reconstituted with 2 ml of LC-MS grade methanol and filtered through a 25-mm diameter, 0.22-µm nylon filter (Scharlau, Barcelona, Spain) before HPLC injection.

2.5. UHPLC-(QTOF) MS/MS

4.1

A Waters Acquity UPLC chromatograph (Milford, MA, USA) equipped with a 50 mm× 2.1 mm i.d., 1.7 µm particle size Waters Acquity BEH C18 column and a Waters Van Guard pre-column of the same material, and coupled to a Microtof-Q (Q-TOF) mass spectrometer from Bruker Daltonik (GMBH, Germany) with an electrospray interface, was employed for the separation and quantification of PFAAs. The chromatographic and mass spectrometry data were acquired with the software Data Analysis Version 4.0 from Bruker Daltonik (GMBH, Germany).

The chromatographic separation conditions used were reported elsewhere [46]. A 0.1% formic acid-acetonitrile mixture (solvent A) and a 0.1% formic acid aqueous solution (solvent B) were used as mobile phases. The chromatographic separation took place in less than 4 min. The mobile phase composition was varied according to a linear gradient that increased from 35% to 55.7% A in 1.84 min, then increased until 58% A in 0.43 min; increased again until 65.7% in 0.5 min and 100% A is reached in 0.23 min, at minute 3.00, and held for 1.5 min. Finally, the mobile phase composition returned to the initial conditions. The flow rate was set at 0.45 ml/min and the injection volume was 5 µl. The sample tray was held at 20ºC and the column was maintained at 35ºC.

Chromatograms of a) the mixture of the analytes and internal standards and b) a fortified microwave popcorn bag at 40 ng/g extracted by FUSLE, are shown in Figure 1. It is worth mentioning that although MPFOA and PFOA, and MPFOS, PFOS and PFDA peaks overlapped; their quantification could be performed without interference because chromatograms were obtained for each compound at their corresponding m/z ratio.

Electrospray ionization was carried out in negative mode using a capillary voltage of 3.5 kV. A coaxial nebulizer N_2 gas flow (9.0 l/min) at 200ºC and 3.0 bar of pressure around the ESI emitter was used to assist the generation of ions. The mass spectrometer was calibrated across the mass range of 50–1500 m/z using internal references. Quantification was performed by multiple reaction monitoring (MRM) and ion extraction. As can be seen in Table 1, the collision energies were set between 8 eV (minimum possible) and 14 eV to achieve the most abundant fragmentation. Retention times, quantification ions and summation ratios used for the analytes are also listed in Table 1.

Table 1. Analyte retention times, quantification ions, collision energy and summation ratio values.

Compounds	Retention time (min)	Quantification ions (m/z)		Collision Energy (eV)	Summation ratio
		MS	MRM		
PFHpA	1.16	362.90	318.98	10	5000
PFOA	1.56	413.00	368.95	12	3750
MPFOA[a]	1.55	421.00	375.99	12	3750
PFNA	2.00	463.00	418.94	14	5000
PFOS	2.35	498.90	498.90	8	2500
MPFOS[b]	2.34	502.90	502.91	8	2500
PFDA	2.47	513.00	468.94	14	2500
PFUnA	3.03	563.00	518.94	12	5000
PFDoA	3.44	613.00	568.92	12	5000

[a] Internal standards for all PFCAs

[b] Internal standards for PFOS

Screening of short chain PFAAs were carried out by multiple reaction monitoring (MRM) and ion extraction (See Figure 1.c).Collision energy was set at 8 eV and transitions monitored were the followings: m/z 312.97→ 268.98 for perfluorohexanoic acid (PFHxA), m/z 262.98→ 218.99 for perfluoropentanoic acid (PFPeA), m/z 212.98→ 168.99 for perfluorobutanoic acid (PFBA) and m/z 162.98→ 118.99 for perfluoropropanoic acid (PFPrA); all of them corresponding to the loss of the CO_2 group.

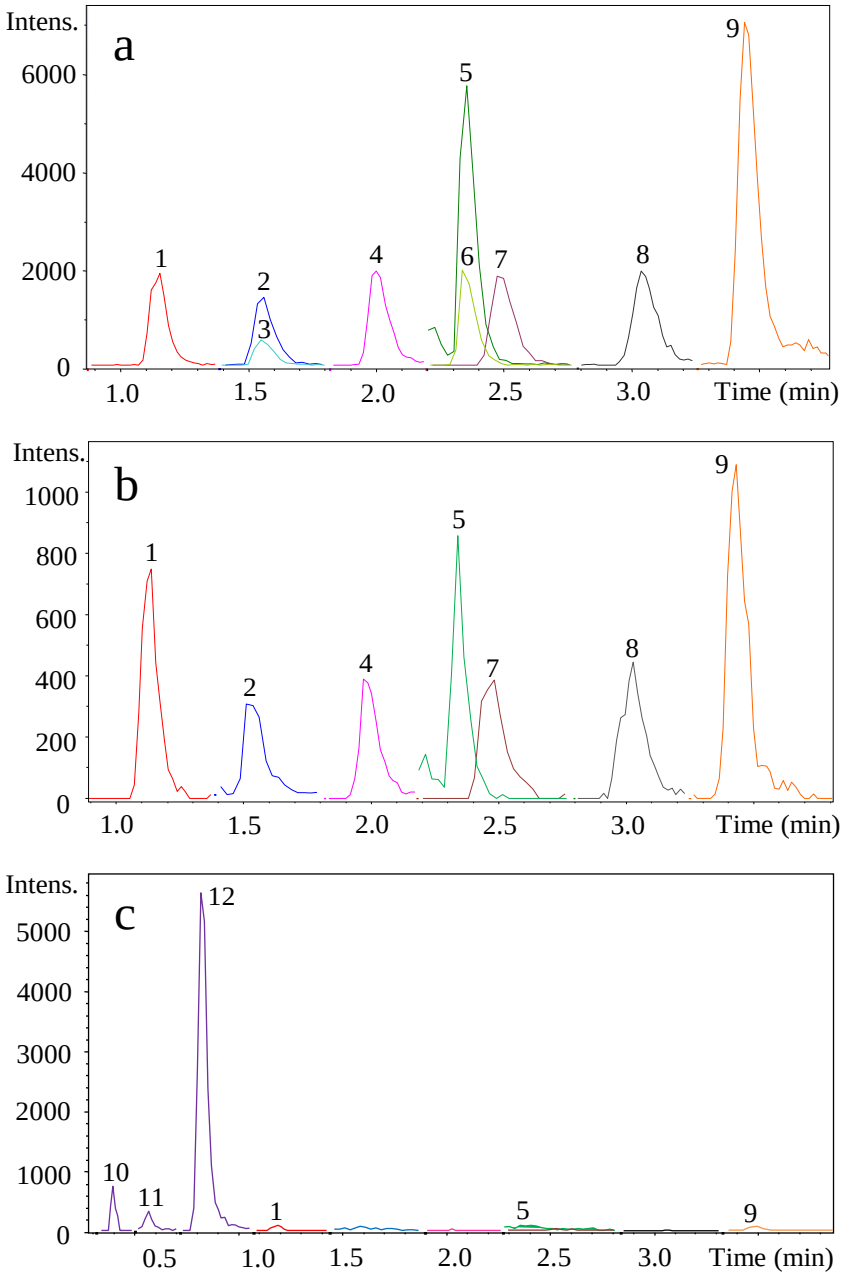


Figure 1. Chromatograms of a) a methanolic standard solution of PFAAs b) a 40 ng/g fortified microwave popcorn bag sample extracted by FUSLE c) a microwave popcorn bag sample extracted by FUSLE. Peak identification: 1) PFHpA, 2) PFOA; 3) MPFOA; 4) PFNA; 5) PFOS; 6) MPFOS; 7) PFDA, 8) PFUnA, 9) PFDoA, 10) PFBA, 11) PFHxA and 12) PFHpA.

2.6. Software for statistical analysis

The experimental design and their statistical analysis were performed using Statgraphics Centurion XV software (Statpoint Technologies, USA) to generate the matrix of experiments and to estimate the effect of each factor on the efficiency of the extraction. The rest of statistical analysis were carried out using Microsoft excel and SPSS statistics 19 (IBM, USA).

3. RESULTS AND DISCUSSION

3.1. Study of FUSLE variables

The aim of this study was to select the FUSLE conditions that provide a high extraction efficiency. The influence of several FUSLE variables have already been reported and some of them are correlated [50, 51]. Therefore, in this work, the FUSLE parameters studied were only the composition of the extraction solvent, the ultrasonic irradiation power, the extraction time, the solvent volume and the number of the extraction cycles.

3.1.1. Solvent selection

The influence of the extraction solvent was studied. Methanol (MeOH) is the most common solvent to extract PFASs according to literature; however another four solvents have been tested in this study because they have also been reported for solid-liquid extractions of PFASs [13, 29, 40, 41, 43]. They were ethanol (EtOH), acetonitrile (ACN), the 1:1 MeOH:ACN mixture, and 1% formic acid in MeOH (FA-MeOH). Extractions were carried out under the following conditions: 13.0 ml of extraction solvent were added to the sample and the mixture was exposed once to a 75% ultrasonic irradiation power for 60 seconds at 50% of pulsed cycle. Results are shown in Figure 2. As can be seen in Figure 2, ACN was the worse solvent for PFDA, PFUnA and PFDoA. Increasing over nine-carbon chain length causes decreasing capacity extraction of ACN. Likewise, EtOH extracted a higher amount of PFDoA than the others. Therefore, EtOH was selected for further extractions.

3.1.2. Stability analyte under focused ultrasonic irradiation

Once EtOH was selected as extraction solvent, the stability of the analytes under focused ultrasonic irradiation was studied. In order to check that the FUSLE process do not cause analyte degradation, the effect of 100% irradiation power and long irradiation times was examined. An ethanolic solution of all analytes and internal standards at a concentration of 15 ng/ml was subjected to FUSLE at 100% ultrasonic irradiation power and 50% of pulsed cycle for 10, 30, 60 and 120 s.

ANOVA (analysis of variance) of the results showed that there are not significant differences for all the analytes (p-value >0.05), but for PFUnA and MPFOS (p-value of 0.002 for both). However, for these analytes the signal decrease was below 10%.

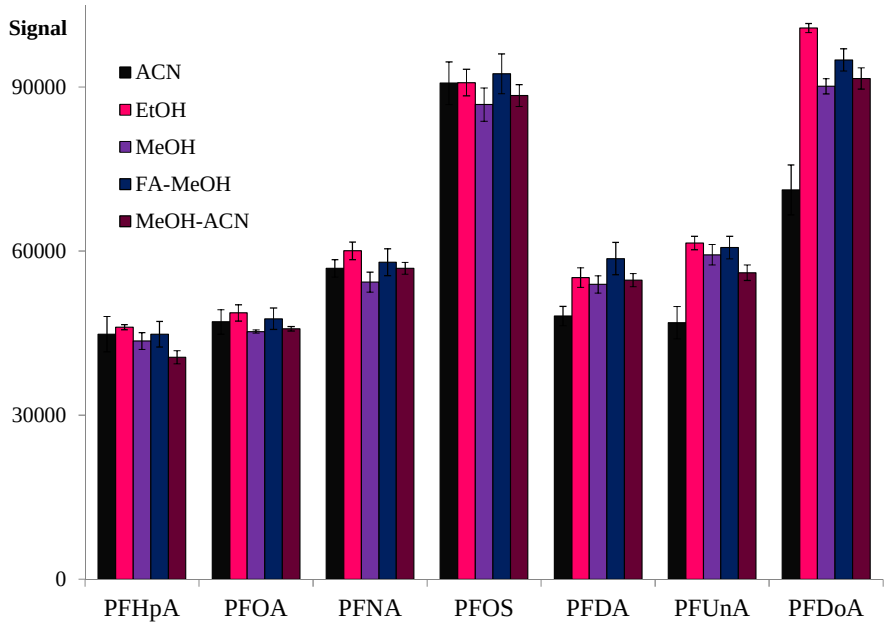


Figure 2. Influence of solvent in FUSLE of PFAAs from food-contact packaging. Extraction condition: 13.0 ml of solvent, 75% of ultrasonic power irradiation, 60 s and 50% of pulsed cycle.

3.1.3. Irradiation power, time and solvent volume

The influence of ultrasonic irradiation power, extraction time and solvent volume were optimized by a central composite design (CCD).

The central composite design consisted of a 23 factorial design with six star points located at ± α from the center of the experimental domain and nine replicates of the central point. An axial distance α of 1.68 was selected in order to establish the rotatability condition. Therefore, the design consisted of 23 randomly performed experiments.

All the experiments were carried out using 0.5 g of a spiked samples containing 800 ng/g of each analyte. The titanium microtip was immersed about 5 mm above the bottom of the vessel, the vessel was immersed into an ice bath and the pulsed cycle was set at 50%.

The experimental domain was selected bearing in mind technical limitations, literature and our previous experience in FUSLE. Ultrasonic irradiation power was studied from 30 to 90%, including the following levels: 30, 42, 60 (central value), 78 and 90%. Extraction time was studied between 10 and 120 s and the levels were 10, 32, 65 (central value), 98 and 120 s. Ethanol volume used in extractions was between 8 and 18 ml with levels of 8, 10, 13 (central value), 16 and 18 ml.

The ANOVA test of the results (data are presented in Table S1. Supplementary Material) showed that the only factor with a significant effect (p-value <0.05) was time for PFHpA (p-value 0.0474 considering all the factors, and p-value 0.0220 when factors with non-significant effects were not considered in the statistical treatment) showing a negative effect. Thus, the lower the extraction time is, the higher PFHpA signal is obtained. Therefore, 10 s was the time value selected. However, this result cannot be explained by a PFHpA degradation because its stability was shown previously.

No statistically significant effects (α=0.05) were found for ultrasonic irradiation power or solvent volume parameters, therefore, the lowest values were chosen (30% and 8 ml) in order to lengthen the life time of the microtip and shorten analysis time, respectively.

3.1.4. Study of the number of extraction steps

Finally, the number of extraction steps required for complete extraction was studied. See Figure S1 in the Supplementary Material.

Experiments were carried out using a different number of steps (1, 2 and 3), and each experiment was carried out in triplicate. After each extraction step, the solvent was removed and 8 ml of fresh solvent (ethanol) were added to the remaining sample in the extraction tube. The sample was extracted again under the same conditions. The two or three collected fractions were joined, evaporated to dryness and processed as described in Section 2.3. The ANOVA test of the results showed that no significant differences were observed between 1, 2 or 3 cycles (p-values between 0.891 and 0.050 for PFOS and PFNA, respectively), therefore, it can be concluded that no additional PFAAs were extracted with more than one extraction step.

3.2. Features of the FUSLE-HPLC-(QTOF) MS/MS method

The whole analytical method FUSLE-HPLC-(Q-TOF)MS/MS for the determination of PFAAs was characterized in term of linearity, matrix effect error,

limit of detection (LOD) and quantification (LOQ), repeatability (intra-day RSD, %) and intermediate precision (inter-day RSD, %) at two concentration levels and recoveries at three concentration levels.

The linearity study was carried out with standard solutions (in methanol) and by standard addition (to a methanolic sample extract) in order to check the absence of matrix effect in the extract. The linearity was studied from 1 to 100 ng/ml and 12 concentration levels were measured in triplicate, except for PFOS which calibration was carried out from 2 to 100 ng/ml, and only 11 levels were included.

The presence of significant differences between the residuals obtained for a linear regression model (LRM) and a quadratic regression (QRM) was checked using the Mandel's fitting test [58]. As can be seen in Table 2, statistical differences were found in the residual variances, for, PFOS, PFDA, PFUnA and PFDoA. Therefore, the calibration curves for these compounds were fitted better by a quadratic function than by a linear one. Once the linearity was studied, the possibility of matrix effect was examined. No significant differences, at a confident level of 95%, were found between the slopes in both matrices for all analytes, except for PFOS and PFDoA (see Table 2). Therefore, the quantification of these compounds was carried out by standard addition.

The limits of detection and quantification were estimated as 3.3 (corresponding to the $\alpha = \beta = 5\%$ guideline) and 10 times the standard deviation of the intercept, respectively, divided by the slope. The intercept and the slope used for the calculation were obtained for a low-concentration level addition standard calibration graph. Results are listed in Table 3. As can be seen, the limits of detection and quantification were below 2.2 and 7 ng/g of packaging, respectively.

The repeatability and intermediate precision of the method were calculated by processing 15 replicates of spiked packaging (five replicates x three days) at two concentration levels of 15 and 40 ng/g. ANOVA was used to obtain repeatability and intermediate precision. As can be seen in Table 3, repeatability and intermediate precision were satisfactory (RSDs were less than 11 and 15%, respectively).

Recovery values were obtained at a low (15 ng/g), low/medium (40 ng/g) and medium (200 ng/g) concentration levels (Table 3). As can be seen, they were close to 100%, at both levels, ranging from 94 to 118%.

Table 2. Linearity and matrix-effect study

	F_{cal} [a]		R^2	Calibration curve		
				$b_0 \pm CI_0$	$b_1 \pm CI_1$	$b_2 \pm CI_2$
PFHpA	3.474	1)	**0.9958**	**-0.06 ± 0.08**	**0.0632 ± 0.0016**	-
		2)	0.9924	-0.12 ± 0.10	0.0657 ± 0.0021	-
PFOA	1.933	1)	**0.9933**	**-0.08 ± 0.09**	**0.0535 ± 0.0018**	-
		2)	0.9918	-0.03 ± 0.09	0.0499 ± 0.0018	-
PFNA	0.510	1)	**0.9946**	**-0.01 ± 0.10**	**0.0700 ± 0.0020**	-
		2)	0.9952	-0.19 ± 0.12	0.0656 ± 0.0024	-
PFOS[b,c]	5.895	1)	0.9958	-0.10 ± 0.12	0.047 ± 0.007	0.00024 ± 0.00007*
		2)	**0.9933**	**0.20 ± 0.11**	**0.049 ± 0.006**	**0.00008 ± 0.00006***
PFDA[b]	25.188	1)	**0.9974**	**0.02 ± 0.12**	**0.070 ± 0.007**	**0.00022 ± 0.00007**
		2)	0.9969	0.05 ± 0.12	0.066 ± 0.007	0.00027 ± 0.00007
PFUnA[b]	25.101	1)	**0.9985**	**0.03 ± 0.09**	**0.074 ± 0.005**	**0.00022 ± 0.00005**
		2)	0.9963	0.05 ± 0.13	0.067 ± 0.007	0.00025 ± 0.00007
PFDoA[b,c]	79.304	1)	0.9982	-0.02 ± 0.26	0.183 ± 0.016*	0.00100 ± 0.00016*
		2)	**0.9976**	**0.06 ± 0.20**	**0.124 ± 0.011***	**0.00068 ± 0.00012***

[a] Mandel's fitting test. For Fcal> Fα;1;N−3 = Fcrit H0 is rejected. Fcrit=4.171 for all compounds, but for, PFOA (Fcrit=4.210)

[b] The calibration curve is fitted better by a quadratic function than by a liner function.

[c] Compounds affected by matrix effect.

bo: intercept.

b1: slope or lineal coefficient.

b2: quadratic coefficient.

CI: Confidence interval.

1) Calibration curve of PFAAs in methanol

2) Calibration curve of PFAAs in methanolic sample extract

* Significant statistical differences between methanol and methanolic sample extract calibration parameters

Bold numbers: selected calibration curve.

Table 3. Features of the FUSLE-HPLC-(Q-TOF) MS/MS method.

	LOD [a] (ng/g)	LOQ [b] (ng/g)	Repeatability [c] (%)		Intermediate precision [c] (%)		Recovery ± 95% CI (%), N=4		
			15 ng/g	40 ng/g	15 ng/g	40 ng/g	15 ng/g	40 ng/g	200 ng/g
PFHpA	1.2	4	10	8.5	11	12	97 ± 10	112 ± 31	97 ± 9
PFOA	2.2	7	10	8.7	12	12	104 ± 12	110 ± 10	96 ± 16
PFNA	0.9	3	8	6.9	10	10	106 ± 16	96 ± 21	102 ± 12
PFOS	0.9	3	6	6.6	13	12	96 ± 8	108 ± 15	103 ± 22
PFDA	0.5	1.6	10	8.2	12	12	102 ± 19	105 ± 7	101 ± 14
PFUnA	1.1	3	11	7.9	13	13	118 ± 32	107 ± 27	99 ± 13
PFDoA	0.5	1.4	9	7.0	10	15	94 ± 8	114 ± 19	97 ± 22

[a] Estimated as 3.3 times the standard deviation of the baseline divided by the slope.

[b] Estimated as 10 times the standard deviation of the baseline divided by the slope.

[c] Calculated by ANOVA (5replicates x 3days).

15, 40 and 200 ng/g: concentration levels studied.

3.3. Analysis of samples

The developed method was applied to determine the studied PFAAs in different matrix as microwave popcorn bag, ice cream tub and cardboard cup; and the results were compared with those obtained by PLE-HPLC-(Q-TOF) MS/MS in order to contrast FUSLE efficiency versus PLE. Results are given in Table 4. Broadly, all samples contained PFAAs, mainly PFHpA, at levels of between 4 and 29 ng/g total PFAAs. PFHpA showed the highest concentrations and PFNA and PFUnA were not detected in any sample. It is worth mentioning that, opposite our expectations, PFOA was found in only two samples. The samples 1, 2 and 4, corresponding to microwave popcorn bags, showed the highest levels of PFAAs, near 30 ng/g, whereas that microwave popcorn bag 6 and ice cream tub samples showed the lowest, around 5 ng/g

PFHpA has been reported as the most abundant PFAA found in articles of commerce such as nylon carpet, carpet protector concentrate, spot removal kit and tire shine [32]. In addition, it has been found in non-stick cookware and microwave popcorn bag [44] and surface soils [59] following PFOA and PFOS as most abundant PFAAs.

Short chain PFAAs were also detected in the FUSLE and PLE extracts of the samples. A chromatogram of a FUSLE extract for a popcorn packaging sample is shown in Figure1.c. These compounds could not be quantified, but the highest peak area was obtained for PFHxA followed by PFBA, PFPeA and PFPrA (not detected). This seems to indicate that compounds with an even number of carbon atoms and with a longer chain are more abundant. The presence of short chain PFAAs in food and the increase of PFHxA levels in prepared food has been reported by Ullah [34] and Gebbink [37], respectively.

No significant differences were found for the results obtained by FUSLE and PLE, except for sample 1 where PLE extracted more PFHpA than FUSLE. On the contrary, PFDA from popcorn bag 2 and PFOA from popcorn bag 3 were extracted with FUSLE, but it could not be quantified after PLE.

CONCLUSIONS

A FUSLE-HPLC-(Q-TOF) MS/MS method has been developed to determine PFAAs in food-contact packaging. FUSLE has shown to be a fast, simple and efficient extraction method for PFAAs. The proposed method allows a quantitative extraction (around 100% recovery values) of PFAAs from this kind of packaging in a very short time (10 s extraction step).

Table 4. Concentration of PFAAs in food-contact packaging.

Samples		Concentration ± SDa (ng/g) [a]					
		PFHpA	PFOA	PFOS	PFDA	PFDoA	Total PFAAs[b]
Microwave popcorn bag 1	FUSLE	14.1 ± 0.8*	n.d.	5.9 ± 0.9	n.d.	n.d.	20.0 ± 1.4
	PLE	21.5 ± 0.7*	n.d.	7.7 ± 0.3	n.d.	n.d.	29.2 ± 0.5
Microwave popcorn bag 2	FUSLE	10.9 ± 0.3	14.0 ± 1.2	<LOQ	1.8 ± 0.4	n.d.	26.7 ± 1.6
	PLE	11.6 ± 1.0	15.2 ± 1.5	<LOQ	<LOQ	<LOQ	27 ± 3
Microwave popcorn bag 3	FUSLE	n.d	7.2 ± 0.9	n.d.	4.6 ± 0.9	2.5 ± 0.3	14.3 ± 1.7
	PLE	n.d	<LOQ	n.d.	4.7 ± 0.7	2.9 ± 0.3	7.2 ± 0.5
Microwave popcorn bag 4	FUSLE	23.1 ± 2.1	n.d.	5.0 ± 0.3	n.d.	n.d.	28 ± 4
	PLE	20.5 ± 1.6	n.d.	5.4 ± 0.6	n.d.	<LOQ	26 ± 3

[a] Standard deviation (N=3)

[b] Total PFAAs calculated by the sum of quantified PFAAs.

n.d.: No detected. Concentration below the detection limit. PFNA and PFUnA were not detected in any sample.

<LOQ: Concentration below the quantification limit. Detected but not quantifiable.

***** Significant statistical differences between FUSLE and PLE methods.

Table 4(cont.). Concentration of PFAAs in food-contact packaging.

Samples		Concentration ± SDa (ng/g) [a]					
		PFHpA	PFOA	PFOS	PFDA	PFDoA	Total PFAAs[b]
Microwave popcorn bag 5	FUSLE	7.1 ± 0.3	n.d.	4.6 ± 0.7	n.d.	n.d.	11.7 ± 0.5
	PLE	8.8 ± 0.9	n.d.	5.6 ± 1.1	n.d.	n.d.	14.3 ± 1.9
Microwave popcorn bag 6	FUSLE	4.3 ± 0.3	n.d.	<LOQ	n.d.	n.d.	4.3 ± 0.3
	PLE	4.9 ± 0.3	n.d.	<LOQ	n.d.	n.d.	4.9 ± 0.3
Cardboard cup	FUSLE	9.76 ± 0.16	n.d.	5.7 ± 0.6	n.d.	<LOQ	15.5 ± 0.4
	PLE	9.2 ± 0.7	n.d.	7.2 ± 1.2	n.d.	<LOQ	16.4 ± 2.5
Ice cream tub	FUSLE	n.d.	n.d.	6.1 ± 0.7	n.d.	<LOQ	6.1 ± 0.7
	PLE	n.d.	n.d.	6.9 ± 0.4	n.d.	<LOQ	6.9 ± 0.4

[a] Standard deviation (N=3)

[b] Total PFAAs calculated by the sum of quantified PFAAs.

n.d.: No detected. Concentration below the detection limit. PFNA and PFUnA were not detected in any sample.

<LOQ: Concentration below the quantification limit.Detected but not quantifiable.

***** Significant statistical differences between FUSLE and PLE methods.

Therefore, FULSE may increase the sample throughput for packaging screening in comparison to classical solid-liquid extraction (extraction times around 30 min), classical UAE (1-2 hours) and PLE (6-30 min). Fast extracts may also be available for analysis of FTOHs, which have very often been quantified in paper based packaging.

ACKNOWLEDGEMENTS

The Spanish Ministerio de Educación y Ciencia is thanked for supporting this work through the CTM 2010-16935 project (within the Plan Nacional de InvestigaciónCientífica y Desarrollo e InnovaciónTecnológica co-financed with FEDER funds). C. Moreta thanks to the Government of La Rioja for the FPI fellowship. E. Garrido-Nájera and M.A. Martínez-Sáenz are also thanked for their help with the HPLC-(Q-TOF) MS/MS equipment.

REFERENCES

[1] J.R.Parsons, M. Sáez, J. Dolfing, P. de Voogt, Rev. Environ. Contam.Toxicol. 196 (2008) 53

[2] M.M. Schultz, D.F. Barofsky, J.A. Field, Environ. Eng. Sci. 20 (2003) 487

[3] R. Renner, Environ. Sci. Technol. 35 (2001) 154A

[4] K. Prevedouros, I.T. Cousins, R.C. Buck, S.H. Korzeniowski, Environ. Sci. Technol. 40 (2006) 32

[5] M.E. Andersen, J.L. Butenhoff, S.C. Chang, D.G. Farrar, G.L. Kennedy, C. Lau, G.W. Olsen, J. Seed, K.B.Wallace, Toxicol. Sci. 102 (2008) 3

[6] J.C. D'Eon, S.A. Mabury, Environ. Sci. Technol. 41 (2007) 4799

[7] J.W. Martin, S.A. Mabury, P.J. O'Brien, Chem. Biol. Interact. 155 (2005) 165

[8] W.J. Fasano, S.C. Carpenter, S.A. Gannon, T.A. Snow, J.C. Stadler, G.L. Kennedy, R.C. Buck, S.H. Korzeniowski, P.M. Hinderliter, R.A. Kemper, Toxicol. Sci. 91 (2006) 341

[9] A. Jahnke, U. Berger, J. Chromatogr. A 1216 (2009) 410

[10] I. Navarro, P. Sanz, M.Á. Martínez, Anal. Bioanal. Chem. 400 (2011) 1277

[11] T. Zhang, H. Sun, A.C. Gerecke, K. Kannan, C.E. Müller, A.C. Alder, J. Chromatogr. A 1217 (2010) 5026

[12] M.S. Díaz-Cruz, M.J. García-Galán, P. Guerra, A. Jelic, C. Postigo, E. Eljarrat, M. Farré, M.J. López de Alba, M. Petrovic, D. Barceló, Trends Anal. Chem. 28 (2009) 1263

[13] H. Yoo, J.W. Washington, T.M. Jenkins, E. Laurence Libelo, J. Chromatogr. A 1216 (2009) 7831

[14] M. Llorca, M. Farré, Y. Picó, D. Barceló, J. Chromatogr. A 1218 (2011) 4840

[15] X. Esparza, E. Moyano, J. De Boer, M.T. Galceran, S.P.J. Van Leeuwen, Talanta 86 (2011) 329

[16] C. Kunacheva, S. Tanaka, S. Fujii, S.K. Boontanon, C. Musirat, T. Wongwattana, B.R. Shivakoti, Chemosphere 83 (2011) 737

[17] V. Dufková, R. Čabala, D. Maradová, M. Štícha, J. Chromatogr. A 1216 (2009) 8659

[18] J. Hu, J. Yu, Chromatographia 72 (2010) 411

[19] R. Alzaga, C. Salgado-Petinal, E. Jover, J.M. Bayona, J. Chromatogr. A 1083 (2005) 1

[20] C.P. Higgins, J.A. Field, C.S. Criddle, R.G. Luthy, Environ. Sci. Technol. 39 (2005) 3946

[21] N. Luque, A. Ballesteros-Gómez, S. van Leeuwen, S. Rubio, J. Chromatogr. A 1217 (2010) 3774

[22] B. Boulanger, A.M. Peck, J.L. Schnoor, K.C. Hornbuckle, Environ. Sci. Technol. 39 (2005) 74

[23] J.L. Barber, U. Berger, C. Chaemfa, S. Huber, A. Jahnke, C. Temme, K.C. Jones, J. Environ. Monit. 9 (2007) 530

[24] S. Huber, L.S. Haug, M. Schlabach, Chemosphere 84 (2011) 1686

[25] N. Luque, A. Ballesteros-Gómez, S. van Leeuwen, S. Rubio, J. Chromatogr. A 1235 (2012) 84

[26] L.W.Y. Yeung, S. Taniyasu, K. Kannan, D.Z.Y. Xu, K.S. Guruge, P.K.S. Lam, N. Yamashita, J. Chromatogr. A 1216 (2009) 4950

[27] W.K. Reagen, M.E. Ellefson, K. Kannan, J.P. Giesy, Anal. ChimicaActa 628 (2008) 214

[28] C.L. Tseng, L.L. Liu, C.M. Chen, W.H. Ding, J. Chromatogr. A 1105 (2006) 119

[29] J. Li, F. Guo, Y. Wang, J. Liu, Z. Cai, J. Zhang, Y. Zhao, Y. Wu, J. Chromatogr. A 1219 (2012) 54

[30] T. Zhang, H.W. Sun, Q. Wu, X.Z. Zhang, S.H. Yun, K. Kannan, Environ. Sci. Technol. 44 (2010) 3572

[31] J.A. Björklund, K. Thuresson, C.A. De Wit, Environ. Sci. Technol. 43 (2009) 2276

[32] X. Liu, K. Krebs, Z. Guo, N. Roache, J. Chromatogr. A 1216 (2009) 3910

[33] A. Ballesteros-Gómez, S. Rubio, S. van Leeuwen, J. Chromatogr. A 1217 (2010) 5913

[34] S. Ullah, T. Alsberg, R. Vestergren, U. Berger, Anal. Bioanal. Chem. 404 (2012) 2193

[35] A.S. Lloyd, V.A. Bailey, S.J. Hird, A. Routledge, D.B. Clarke, Rapid Commun. Mass Spectrom. 23 (2009) 2923

[36] I. Ericson, R. Martí-Cid, M. Nadal, B. Van Bavel, G. Lindström, J.L. Domingo, J. Agric. Food Chem. 56 (2008) 1787

[37] W.A.Gebbink, S.Ullah, O.Sandblom, U.Berger, Environ. Sci. Pollut. Res. DOI: 10.1007/s11356-013-1596-y[38] Y.C. Chang, W.L. Chen, F.Y. Bai, P.C. Chen, G.S. Wang, C.Y. Chen, Anal. Bioanal. Chem. 402 (2012) 1315

[39] S. Poothong, S.K. Boontanon, N. Boontanon, J. Hazard. Mater. 205-206 (2012) 139

[40] S. Dolman, M. Pelzing, J. Chromatogr. B Biomed. Sci. Appl. 879 (2011) 2043

[41] X. Trier, K. Granby, J.H. Christensen, Environ. Sci. Pollut. Res. 18 (2011) 1108

[42] X. Trier, N.J. Nielsen, J.H. Christensen, Environ. Sci. Pollut. Res. 18 (2011) 1422

[43] G. Lv, L. Wang, S. Liu, S. Li, Anal. Sci. 25 (2009) 425

[44] E. Sinclair, S.K. Kim, H.B. Akinleye, K. Kannan, Environ. Sci.Technol. 41 (2007) 1180

[45] T.H. Begley, K. White, P. Honigfort, M.L. Twaroski, R. Neches, R.A. Walker, Food. Addit.Contam. 22 (2005) 1023

[46] M.P. Martínez-Moral, M.T. Tena, Talanta 101(2012) 104

[47] European Food Safety Authority. Opinion of the Scientific Panel on Contaminants in the Food chain. The EFSA Journal 653 (2008) 1-131

[48] J. Raloff, Science News 168 (2005) 341

[49] F. Gosetti, U. Chiuminatto, D. Zampieri, E. Mazzucco, E. Robotti, G. Calabrese, M.C. Gennaro, E. Marengo, J. Chromatogr. A 1217 (2010) 7864

[50] D. Pérez-Palacios, M.A. Fernández-Recio, C. Moreta, M.T. Tena, Talanta 99 (2012) 167

[51] C. Moreta, M.T. Tena, J. Chromatogr. A 1218 (2011) 3392

[52] P. Navarro, N. Etxebarria, G. Arana, Anal. Chim. Acta 648 (2009) 178

[53] A. Errekatxo, A. Prieto, O. Zuloaga, A. Usobiaga, N. Etxebarria, L.A. Fernández, Anal. Bioanal. Chem. 392 (2008) 1471

[54] J. Sanz-Landaluze, L. Bartolome, O. Zuloaga, L. González, C. Dietz, C. Cámara, Anal. Bioanal. Chem. 384 (2006) 1331

[55] J.L. Capelo, M.M. Galesio, G.M. Felisberto, C. Vaz, J.C. Pessoa, Talanta 66 (2005) 1272

[56] M.P. Martínez-Moral, M.T. Tena, Anal. Bioanal. Chem. 404 (2012) 289

[57] S. Fdez-Ortiz de Vallejuelo, A. Barrena, G. Arana, A. de Diego, J.M. Madariaga, Talanta 80 (2009) 434

[58] L. Brüggemann, W. Quapp, R. Wennrich, Accredit. Qual. Assur. 11 (2006) 625

[59]M.J.Strynar,A.B. Lindstrom, S.F. Nakayama, P.P.Egeghy, L.J.Helfant, Chemosphere 86 (2012) 252.

4.2.

Determination of perfluorinated alkyl acids in corn, popcorn and popcorn bags before and after cooking by focused ultrasound solid-liquid extraction, liquid chromatography and quadrupole-time of flight mass spectrometry

Journal of Chromatography A, 1355 (2014) 21-218

Determination of perfluorinated alkyl acids in corn, popcorn and popcorn bags before and after cooking by focused ultrasound solid-liquid extraction, liquid chromatography and quadrupole-time of flight mass spectrometry

Cristina Moreta and María Teresa Tena*.
Department of Chemistry. University of La Rioja. C/ Madre de Dios 51, E-26006. Logroño (La Rioja). Spain.

ABSTRACT: An analytical method is proposed to determine ten perfluorinated alkyl acids (PFAAs) [nine perfluorocarboxylic acids (PFCAs) and perfluorooctane sulfonate (PFOS)] in corn, popcorn and microwave popcorn packaging by focused ultrasound solid–liquid extraction (FUSLE) and ultra high performance liquid chromatography (UHPLC) coupled to quadrupole-time of flight mass spectrometry (QTOF-MS/MS). Selected PFAAs were extracted efficiently in only one 10-second cycle by FUSLE, a simple, safe and inexpensive technique. The developed method was validated for microwave popcorn bags matrix as well as corn and popcorn matrices in terms of linearity, matrix effect error, detection and quantification limits, repeatability and recovery values. The method showed good accuracy with recovery values around 100% except for the lowest chain length PFAAs, satisfactory reproducibility with RSDs under 16%, and sensitivity with limits of detection in the order of hundreds picograms per gram of sample (between 0.2 and 0.7 ng/g). This method was also applied to the analysis of six microwave popcorn bags and the popcorn inside before and after cooking. PFCAs contents between 3.50 ng/g and 750 ng/g were found in bags, being PFHxA (perfluorohexanoic acid) the most abundant of them. However, no PFAAs were detected either corn or popcorn, therefore no migration was assumed.

Keywords: Perfluorinated alkyl acids; Focused Ultrasound Solid-Liquid Extraction; Liquid Chromatography; Quadrupole-Time of Flight Mass Spectrometry; Packaging; Popcorn.

1. INTRODUCTION

Perfluoroalkyl and polyfluoroalkyl substances (PFASs) have been broadly used since the late 1940s in different industrial and commercial applications due to their effect of the reduction of the surface tension and their hydrophobic and oleophobic properties [1, 2]. Hence, they have been extensively distributed in the environment.

However, perfluorinated compounds show high thermal, biological and chemical inertness owing to carbon-fluorine is the strongest existing covalent bond (450 KJ/mol) [3]. Moreover, it has also been proved that perfluoroalkyl acids (PFAAs) exhibit toxicity in laboratory animals causing developmental diseases, liver cancer, affect the lipid metabolism and disturb the immune system [4]. Additionally, these compounds may come from the degradation of other PFASs, such as polyfluoroalkyl phosphate surfactants (PAPs) and fluorotelomers (FTOHs), which may be atmospherically or metabolically degraded to them, increasing the PFAAs concentration, such as perfluorocarboxylic acids (PFCAs) and perfluorooctane sulfonate (PFOS), in the environment and the human exposure [5-7].

Due to their hazardous, PFASs have been determined over the last few years in a wide variety of matrices, such as human and wildlife biological ones (urine, milk, plasma, serum, blood, liver, brain and kidney), environmental liquid (river water, seawater and wastewater) and solid matrices (dust, sewage sludge, sediments and soil), consumer products (textile, carpet, cookware and food packaging), food and even in indoor and outdoor air [1, 8].

One of the main applications of the PFASs has been as additives in food-contact packaging due to their ability to make the covering oil, stain and water resistant [9]. In previous studies, PFOA (perfluorooctanoic acid) has been found at levels up to 198 ng/g and 290 ng/g in microwave popcorn packaging [10, 11], but fortunately, the concentration of long chain PFASs in packaging have decreased in recent years [12, 13] because the manufacture of PFOS and other PFASs have been banned in the USA and in Europe. However, these compounds can still be present in food contact packaging due to the acquisition of products that can still contain PFASs from other countries outside the USA or Europe.

PFASs have been typically extracted quantitatively by classical solid–liquid extraction (SLE) [12, 14], by ultrasound assisted extraction (UAE) [10, 15-17] and by pressurized liquid extraction (PLE) [11, 13, 18, 19] from different kinds of food-contact packaging samples. However, the focused ultrasound solid–liquid extraction (FUSLE) has offered an efficient extraction in only several seconds [20]. FUSLE is a low-cost, fast, simple and safe extraction technique based on the cavitation phenomenon. It is more reproducible and more efficient than traditional ultrasonic bath extraction (USE) due to its 100 times higher ultrasonic power and the immersion of the ultrasound microtip directly in the extracting solution [21, 22].

FUSLE has also been used for the fast extraction (seconds or few minutes) of organic analytes, such as UV filters [23] and bisphenols [24] from packaging, as

well as, polychlorinated biphenyls [25], phthalate esters [25], nonylphenols [25], polycyclic aromatic hydrocarbons [21, 22, 25, 26] and brominated diphenyl ethers [27] from environmental matrices. However, longer extraction times were needed for the extraction of metals from sediments [28] using FUSLE.

Regarding to extract PFASs from food, this matrix has been more widely studied than packaging. The most commonly used extraction methods have been based on SLE using an orbital shaker [29-32] and USE [12, 33, 34]. Ion pair extraction (IPE) [35, 36], alkaline digestion [36, 37], PLE [38] and QuEChERS methods [40] have also been employed. However, any of these techniques are more time-consuming or difficult to implement than FUSLE technique, and this is the first time that this extraction has been used to sample preparation of food samples.

In this study, a fast and simple method based on FUSLE and UHPLC–(QToF) MS/MS has been developed, validated and applied for the detection and quantification of ten PFAAs in six different microwave popcorn bags and the popcorn inside them, before and after microwave cooking. Thereby, the absence of migration from packaging to food has been shown and the effect of the microwaving process on PFAAs has also been studied.

2. EXPERIMENTAL

2.1. Materials and reagents

Individual standards of perfluorooctanesulfonic acid tetraethylammonium salt (PFOS) 98%, perfluorobutanoic acid (PFBA) 98%, perfluoropentanoic acid (PFPeA) 97%, perfluorohexanoic acid (PFHxA) > 97%, perfluoroheptanoic acid (PFHpA) 99%, perfluorooctanoic acid (PFOA) 98%, perfluorononanoic acid (PFNA) 97%, perfluorodecanoic acid (PFDA) 98%, perfluoroundecanoic acid (PFUnA) 95% and perfluorododecanoic acid (PFDoA) 95%, were provided by Sigma Aldrich (Madrid, Spain).

Isotopically labelled internal standards of sodium perfluoro-1-[$^{13}C_8$]-octanesulfonate (M8PFOS) isotopic purity > 99%, perfluoro-n-[3,4,5-$^{13}C_3$]-pentanoic acid (M3PFPeA) isotopic purity > 99%, perfluoro-n-[$^{13}C_8$]-octanoic acid (M8PFOA) isotopic purity > 97.9% and perfluoro-n-[1,2-$^{13}C_2$]-dodecanoic acid (MPFDoA) isotopic purity > 99%, were purchased from Wellington Laboratories Inc. (Guelph, ON, Canada) as 50 µg/ml solutions in methanol. M8PFOS was used as internal standard for PFOS; M3PFPeA was used for PFBA and PFPeA; MPFDoA was used for PFDoA; and M8PFOA was used for the rest of PFCAs.

LC–MS grade acetonitrile (ACN), methanol (MeOH) and formic acid, and HPLC grade ethanol (EtOH) were obtained from Scharlau (Barcelona, Spain). Aqueous solutions were prepared in Milli-Q deionized water (Bedford, MA, USA).

Sodium formate 99.998% and ammonium formate ≥ 99.0% were obtained from Scharlau (Barcelona, Spain).

2.2. Samples

Microwave popcorn bags of six different types were obtained from local supermarkets in mid-2013. There were three different brands (A, B and C) among which were four types of flavors (salty (ST), butter (B), sweet (SW) and with no added fats (NF)). A and C were generic brands and B was a name brand.

Before analysis, fat, salt and/or sugar were thoroughly removed from packaging and corn samples with the aid of paper towel. All samples were ground using an IKA A10 Analytical Mill purchased from IKA-Werke GmbH & Co. KG (Staufen, Germany) and then corn and popcorn were sieved through a 0.5 mm mesh sieve. The ground samples were stored protected from light at 4°C in polyethylene plastic containers purchased from Lin Lab Rioja (La Rioja, Spain).

Three pulls of samples (one for each kind of sample): uncooked microwave popcorn bags, corn and popcorn samples were prepared to be used during method validation. The three pulls were spiked at a concentration level of 20 ng/g of each analyte. The microwave popcorn bag pull was also spiked at a concentration level of 2.5 times the limit of quantification of each analyte.

These spiked samples were prepared by adding a methanolic PFAAs standard solution to the grounded matrix (packaging or food) dispersed in ethyl acetate. The mixture was thoroughly homogenized and maintained at 45°C water bath until the solvent was completely evaporated, and then it was triturated again to ensure proper homogenization of the sample. Then the samples were aged in polyethylene plastic containers protected from light at 4°C for at least two weeks before use.

2.3. FUSLE procedure

A SONOPLUS 2070 focused ultrasound system, with a power of 70 W and a 20 kHz frequency, equipped with a 3 mm titanium microtip and a sound proof box (Bandelin Sonoplus, GmbH & Co. KG, Berlin, Germany) was used.

The optimal extraction conditions were as follows: 1.5 g of ground sample was placed into a 34x100 mm centrifuge glass tube, and then 24 ml of EtOH were added. Before each extraction, 100 µl of the 300 ng/ml internal standards solution

was also added. Then, the probe was immersed in the mixture. The extractions were performed in an ice-water bath and the sample was exposed once to a 30% ultrasonic irradiation power for 10 seconds at 50% of pulsed cycle.

Extracts were filtered through a 50 ml capacity and 35 mm disc diameter filter funnel porosity 3 (16-40 µm nominal max. pore size) (DURAN Produktions GmbH & Co. KG, Mainz, Germany) using a vacuum pump. The glassware and the extracted sample were washed twice with 2 ml of extraction solvent. The three liquid portions were transferred to a 50 ml vessel in order to be evaporated to dryness under a nitrogen stream using a Calliper Turbo Vap II concentrator (Zymark, Hopkinton. MA, USA). However, for corn and popcorn samples an oily residue remained. Therefore, a micro-scale liquid-liquid extraction (LLE) of the highly viscous yellow liquid was performed for these matrices. LLE was carried out twice with 1.0 ml MeOH. It is worth mentioning that salt had to be added as an additive in order to keep immiscible the two phases in the case of sweet popcorn extract. The two methanolic layers were transferred to a 50 ml vessel in order to be evaporated to dryness under a nitrogen stream using a Calliper Turbo Vap II concentrator.

Extracts were reconstituted with 1 ml of LC–MS grade MeOH and filtered through a 0.22 µm nylon filter (Scharlau, Barcelona, Spain) before UHPLC injection.

2.4. UHPLC-(QTOF) MS/MS

A Waters Acquity UPLC chromatograph (Milford, MA, USA) equipped with a 50 mm × 2.1 mm i.d., 1.7 µm particle size Waters Acquity BEH C18 column and a Waters VanGuard pre-column of the same material, coupled to a Microtof-Q (Q-TOF) mass spectrometer from Bruker Daltonik (GMBH, Germany) with an electrospray interface, was employed for the separation and quantification of PFAAs. The chromatographic and mass spectrometry data were acquired with the software Data Analysis Version 4.0 from Bruker Daltonik (GMBH, Germany).

The chromatographic separation conditions were chosen and developed based on those reported in previous works [11, 20, 39]. A 0.8% formic acid–ACN mixture (solvent A) and a 0.8% formic acid aqueous solution (solvent B) were used as mobile phases. The chromatographic separation took place in 4 min. The mobile phase composition was varied according to a linear gradient that was increased from 28% to 50% A in 1.50 min, then increased until 52% A in 1.20 min; increased again until 72% A in 0.5 min and maintained for 0.5 min, then increased to 100% A in

0.10 min and held for 1 min. Finally, the mobile phase composition was returned to the initial conditions.

It is worth mentioning that 100% ACN mobile phase is passed through the column during 1 min in order to clean the column for the next injection. Likewise, cleaning the column with 100% ACN during 5 min every 10-15 injections is also recommended.

The flow rate was set at 0.50 ml/min and the injection volume was 7.5 µl. The sample tray was held at 20°C and the column was maintained at 35°C.

Electrospray ionization was carried out in negative mode using a capillary voltage of 3.5 kV. A coaxial nebulizer N_2 gas flow (9.0 l/min) at 200°C and 3.0 bar of pressure around the ESI emitter was used to assist the generation of ions. The mass spectrometer was calibrated across the mass range of 50–1500 m/z using internal references. Quantification was performed by multiple reaction monitoring (MRM) and ion extraction. The collision energies were set between 8 eV (minimum possible) and 14 eV to achieve the most abundant fragmentation (see Table S1 in Supplementary Material). Regarding to PFOS, collision energies from 8 eV to 80 eV were tested. PFOS began to fragment at collision energy of 35 eV. Nevertheless, no stable and abundant fragment was found for PFOS when the voltage was increased. Therefore, the precursor ion for PFOS was selected. Retention times, quantification ions and summation ratios used for the analytes are also listed in Table S1 in Supplementary Material.

The chromatograms of a mixture of the analytes and the internal standards, an extract of a microwave popcorn bag sample and an extract of a fortified popcorn sample obtained by FUSLE are shown in Figure 1.

2.5. Software for statistical analysis

The statistical analysis was performed using Microsoft Excel and SPSS Statistics 19 (IBM, USA).

3. RESULTS AND DISCUSSION

3.1. UHPLC-(QToF) MS/MS method

3.1.1. Preliminary experiments

In order to select the optimal chromatographic conditions, different mobile phases and flow rates were tested (data not shown).

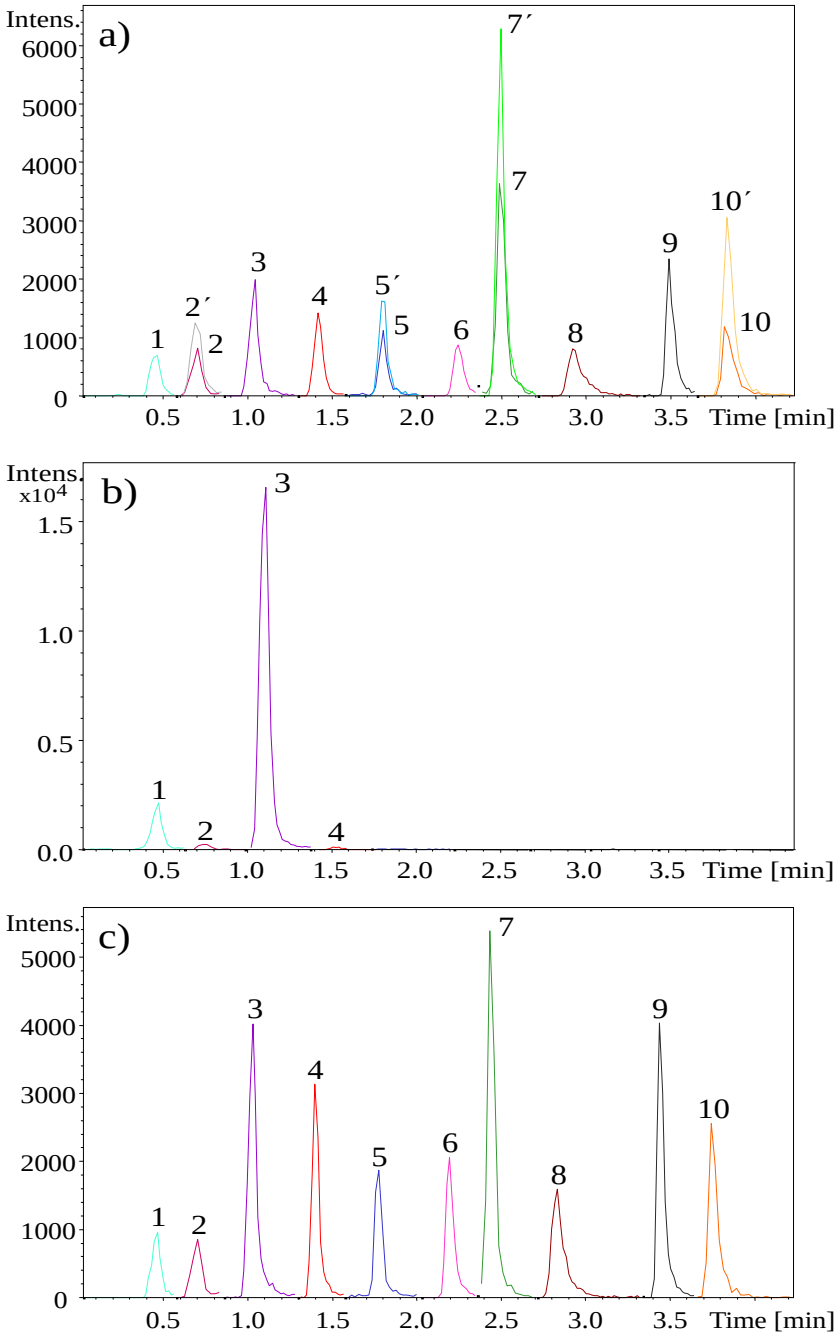


Figure. 1. Selected ion chromatograms of PFAAs obtained for a) a methanolic standard solution at 20 ng/ml b) a microwave popcorn bag sample extracted by FUSLE and c) a 20 ng/g fortified popcorn sample extracted by FUSLE. Peak identification: 1) PFBA; 2) PFPeA; 2´) MPFPeA; 3) PFHxA; 4) PFHpA; 5) PFOA; 5´) MPFOA; 6) PFNA; 7) PFOS; 7´) MPFOS; 8) PFDA; 9) PFUnA; 10) PFDoA and 10´) MPFDoA.

Formic acid concentrations between 0.1% and 1.0% in the mobile phase were studied. PFOS and PFDA cannot be separated chromatographically employing 0.1% formic acid mobile phase, but when formic acid concentration was increased up to 1.0%, PFOS retention time decreases in such a way that PFNA and PFOS began to overlap. Accordingly, a 0.8% formic acid–ACN mixture and a 0.8% formic acid aqueous solution were selected in order to obtain the best chromatographic separation.

Two buffers consisting of formic acid and ammonium formate or sodium formate were also tested. The PFAAs peaks intensity decreased with both buffers. All areas decreased until around two - three times using the ammonium formate buffer and more than 15 times using sodium formate buffer. Therefore, the use of those buffers was discarded.

Flow rates from 0.45 to 0.60 ml/min were checked. The chromatographic separation took place faster increasing this factor. However, a flow rate of 0.5 ml/min was selected because higher flow rates worsened the chromatographic resolution.

Moreover, the injection volume and the summation ratio were studied in order to obtain a sensitive and reproducible method. Injection volume values between 5 and 10 µl were tested. A value of 7.5 µl was selected because higher values spoiled the PFAAs peak shape even showing up peaks with shoulders.

Summation ratio was checked at three different levels (low, medium and high); corresponding to values of 2500, 3750 and 5000. A summation ratio of 5000 showed the highest sensibility for all compounds. However, at the high summation value fewer data are acquired per second and chromatographic peaks are less defined worsening peak area repeatability. A summation ratio of 5000 was set except for PFPeA, PFOA, PFDoA and PFOS because they are detected at the same retention times as their internal standard. A summation ratio of 3750 was set for them.

3.1.2. Features of UHPLC-(QTOF) MS/MS method

The UHPLC–(QTOF)MS/MS method was validated in terms of linearity, limits of detection (LODs) and quantification (LOQs), repeatability (intra-day RSD, %) and intermediate precision (inter-day RSD, %). The features of the method are shown in Table 1. The analytical signal used for calibration and quantification was the analyte-to-internal standard peak area ratio.

Table 1. Features of the UHPLC-(Q-ToF) MS/MS method.

	LOD [a] (ng/ml)	LOQ [b] (ng/ml)	Repeatability [c] (%)			Intermediate precision [c] (%)		
			2 ng/ml	10 ng/ml	45 ng/ml	2 ng/ml	10 ng/ml	45 ng/ml
PFBA	0.4	1.3	9	7	4	10	9	11
PFPeA	0.3	0.8	9	6	3	12	7	5
PFHxA	0.3	0.9	11	4	5	15	8	9
PFHpA	0.3	0.8	8	5	4	15	8	9
PFOA	0.5	1.4	11	7	4	15	10	9
PFNA	0.4	1.1	10	4	4	14	9	10
PFOS	0.3	0.9	7	4	2	11	7	10
PFDA	0.3	0.8	8	3	4	14	8	7
PFUnA	0.2	0.6	10	5	5	13	8	9
PFDoA	0.3	0.8	9	5	6	14	8	6

2, 10 and 45 ng/ml: concentration levels studied.

[a] Estimated as 3.3 times the standard deviation of the intercept divided by the slope.

[b] Estimated as 10 times the standard deviation of the intercept divided by the slope.

[c] Calculated by ANOVA (5 replicates x 3 days).

Linearity was studied from 2 ng/ml to 100 ng/ml and 12 concentration levels were measured in duplicate. The presence of significant differences between the residuals obtained for a linear regression model (LRM) and a quadratic regression model (QRM) was checked using the Mandel's fitting test [40]. Differences between the residual variances of both regressions were not significant for all analytes up to a 45 ng/ml concentration (9 concentration levels) where F values obtained ranged from 0.75 to 4.13 (critical F-value 6.20), so that a LRM was selected for all PFAAs up to that concentration level. R^2 values were between 0.9968 and 0.9991.

LODs and LOQs were estimated as 3.3 times ($\alpha = \beta = 5\%$) and 10 times the standard deviation of the intercept, respectively, divided by the slope. The intercept and the slope used for the calculation were obtained at low-concentration levels, from 0.5 ng/ml to 5 ng/ml. As can be seen in Table 1, LODs were between 0.19 ng/ml (PFUnA) and 0.5 ng/ml (PFOA).

Repeatability and intermediate precision were calculated by ANOVA at three levels: 2 ng/ml, 10 ng/ml and 45 ng/ml (see Table 1). Concentrations levels were measured in five replicates per day over three different days. RSD values for repeatability and intermediate precision were less than 7% and 11%, respectively, at medium and high levels. However, at low levels, RSD values increased until 11% and 15 %, respectively.

3.2. FUSLE-UHPLC-(QTOF) MS/MS method

3.2.1. Preliminary experiments

In order to enhance the sensitivity of the previously developed FUSLE-UHPLC-(QTOF) MS/MS method [20], an increase of the sample amount and a decrease of the final extract volume were tested. It is worth mentioning that solvent and sample volumes are correlated variables, and so the ratio of mass to volume was maintained constant. Accordingly, the sample amount was tested in triplicate at 0.5 g, 1.0 g and 1.5 g using 8 ml, 16 ml and 24 ml of extraction solvent, respectively, and the extracts were evaporated to dryness and reconstituted with 2 ml of MeOH. Spiked microwave popcorn bags at a concentration level of 20 ng/g of each analyte were used. No significant differences were found between the final concentrations obtained. Therefore, the FUSLE procedure was equally efficient in all cases.

Finally, the final extract volume (reconstitution volume) was tested at 2.0 ml, 1.0 ml and 0.5 ml; extracting 1.5 g of spiked bags at the same spiking level, above mentioned. No significant differences were found for 1.0 and 2.0 ml. Nevertheless, PFAAs compounds with longer carbon-chains showed a significant peak tailing and

even a noteworthy change in retention times which spoiled their determination when a reconstitution volume of 0.5 ml was used. This may be caused by the presence a higher amount of matrix components in the more concentrated extract. Consequently, 1.0 ml was the reconstitution volume selected.

It also worth mentioning that when corn and popcorn extracts were evaporated to dryness a highly viscous yellow liquid immiscible with MeOH (probably oil from samples) was observed. A LLE was checked in order to achieve a complete extraction of PFAAs from this phase. From one to three step extraction with 1 ml of MeOH were tested. Two MeOH extractions were enough to ensure at least a 95% extraction of the PFAAs from the viscous liquid.

3.2.2. Features of the FUSLE–UHPLC-(QTOF) MS/MS method for microwave popcorn packaging

The whole analytical method FUSLE–UHPLC–(Q-TOF) MS/MS for the determination of PFAAs in microwave popcorn packaging was characterized in terms of linearity, matrix effect error, LODs and LOQs, repeatability and intermediate precision (intra and inter-day RSD, %) and recovery values.

At the same time, linearity and matrix effect were studied by standard addition on a methanolic sample extract. Eight concentration levels, from 2 ng/ml to 45 ng/ml, were measured in duplicate.

As in Section 3.1.2, linearity was verified by Mandel's fitting test with a 95% confidence level. A LRM was selected for all PFAAs due to the absence of significant differences between the residuals obtained for LRM and QRM. R^2 values obtained ranged from 0.9961 to 0.9990 and F values were between 0.98 and 5.84 (critical F-value 6.41).

Once the linearity was checked, the possibility of matrix effect was examined by comparing the calibration slopes in MeOH and in sample extract. Calibration curves were constructed by plotting both the analyte to internal standard peak area ratio and the analyte peak area (y) against the analyte concentration (x) in order to also check if possible matrix effects can be compensated by using the selected internal standards.

Matrix effect was also evaluated for the different packaging amounts used: 0.25 g. (previous work [20]), 0.50 g, 1.00 g and 1.5 g (present work); reconstituting the extracts with 1 ml of MeOH. Four calibration curves from 4 ng/ml to 45 ng/ml (one for each sample amount) were made by standard addition. Their slopes were compared with those obtained by external standard calibration in MeOH (See Table

S2 in Supplementary Material). As can be seen, the number of analytes affected by matrix effect increased with increased sample amount until 1.00 g.

For packaging, significant differences (at a confidence level of 95%) between the slopes of calibration graph in presence and absence of matrix components were found for all analytes when the internal standard is not used, except for PFHxA, PFHpA, PFOA and PFNA (See Table S2 in Supplementary Material). The matrix effect was compensated for all compound when the internal standards were used, except for PFUnA that showed a negative matrix effect of -39% (signal suppression) that neither MPFDoA nor MPFOA compensated. Therefore, the quantification of PFUnA in microwave popcorn bags was carried out by standard addition.

LODs and LOQs were estimated as in Section 3.1.2. These parameters were acquired at low concentration levels, between 1.0 and 7 ng/ml, by standard addition to extracts that presented a low PFAAs concentration (a pull of samples NF-B and ST-C). As can be seen in Table 2, this improved method is between 3 and 7 times more sensitive than the previous one [20]. PFOA showed the highest LODs and LOQs: 0.5 ng/g and 1.6 ng/g, respectively; and PFCAs with longer carbon chain showed the lowest one: between 0.13-0.19 ng/g and 0.4-0.6 ng/g, respectively.

Repeatability and intermediate precision of the method were calculated by ANOVA (four replicates × three days) at two concentration levels of 2.5 times LOD and 20ng/g. As can be seen in Table 2, repeatability and intermediate precision near the LOQ of the method were higher than at medium concentration levels. However, satisfactory RSDs (less than 12% and 16%, respectively) were obtained.

Recovery values were also acquired at two concentration levels: 2.5 times LOD and 20 ng/g (see Table 2). Good recovery values, ranging from 90% to 106%, were obtained for all analytes at both levels, but for PFBA and PFPeA that showed recovery values of 80% and 84%, respectively, at the low concentration level.

3.2.3. Features of the FUSLE–UHPLC-(QTOF) MS/MS method for corn and popcorn samples

The FUSLE–UHPLC-(QTOF)MS/MS method for the determination of PFAAs in corn and popcorn was also characterized in terms of linearity, matrix effect error, LODs and LOQs, repeatability (intra-day RSD, %) and recovery values for both matrices.

Table 2. Features of the FUSLE-UHPLC-(Q-TOF)MS/MS method for microwave popcorn packaging.

	LOD a (ng/g)	LOQ b (ng/g)	Repeatability c (%)		Intermediate precision c (%)		Recovery (%) ± DE, N=5	
			2.5 LOQ ng/g	20 ng/g	2.5 LOQ ng/g	20 ng/g	2.5 LOQ ng/g	20 ng/g
PFBA	0.4	1.1	12	8	15	8	80 ± 10	103 ± 10
PFPeA	0.3	0.8	11	5	13	9	84 ± 9	91 ± 6
PFHxA	0.3	0.8	10	4	12	8	101 ± 2	105 ± 8
PFHpA	0.2	0.6	2	4	9	6	91 ± 3	99 ± 3
PFOA	0.5	1.6	2	2	11	6	99 ± 5	102 ±6
PFNA	0.3	0.9	6	4	14	5	90 ± 9	104 ± 6
PFOS	0.3	0.9	6	4	7	5	99 ± 9	106 ±5
PFDA	0.2	0.6	8	4	10	5	91 ± 9	103 ± 4
PFUnA	0.2	0.5	8	5	16	15	90 ± 6	96 ± 5
PFDoA	0.2	0.4	2	3	5	7	96 ± 4	104 ± 3

2.5 times the LOQ and 20 ng/g: concentration levels studied.

a Estimated as 3.3 times the standard deviation of the intercept divided by the slope.

b Estimated as 10 times the standard deviation of the intercept divided by the slope.

c Calculated by ANOVA (4 replicates x 3 days).

The linearity of the calibration curves was verified using the Mandel's fitting test [40] as in Section 3.1.2. Calibration curves were constructed by standard addition to methanolic corn and popcorn extracts. Six concentration levels, from 2 ng/ml to 45 ng/ml, were measured in duplicate. The method was shown to be linear in the range tested because no differences between the residuals obtained for LRM and QRM were found at a 95% confidence level. F values obtained ranged from 0.98 to 5.01 (critical F-value 7.21) and R^2 values were between 0.9924 and 0.9995.

The absence of matrix effect was checked by comparing the calibration slopes in MeOH and in sample extract with a confidence level of 95%. In order to check if possible matrix effects can be compensated by using internal standards, both, the analyte to internal standard peak area ratio and the analyte peak area, were employed (See Table S3 in Supplementary Material).

Without internal standard calibration, the corn extracts showed matrix effect for only PFNA and PFDoA with sensitivity enhancements of 23% and 26%, respectively. Moreover significant differences were found in popcorn extracts for PFBA, PFPeA, PFHxA, PFHpA, PFNA and PFDoA showing slope relative standard errors between –21% and 22%. Only PFBA and PFPeA showed signal suppression. However, the use of the internal standard selected compensated the matrix effect error of all PFCAs in both matrices.

LODs and LOQs were estimated as in Section 3.1.2. Intercept and slope were obtained at low concentration levels from 1.0 ng/ml to 7 ng/ml by standard addition. As can be seen in Table 3, PFOA showed the highest LODs in both matrix extracts with values of 0.6 ng/g and 0.7ng/g. LODs for PFAAs were similar in both matrices, but for PFPeA whose LODs and LOQs in popcorn (0.5 ng/g and 1.5 ng/g) were more than twice those in corn (0.2 ng/g and 0.6 ng/g).

Repeatability and recovery values of both methods were calculated at a concentration level of 20 ng/g (see Table 3). Repeatability for corn and popcorn was below 9% and 7%, respectively.

Recovery values in popcorn were close to 100% ranging from 94% to 109%. However, in general terms, recovery values of lower chain length PFAAs in corn were lower than those with longer chains showing values from 65% to 101%. In addition, recovery values from corn are lower than those obtained from popcorn. One reason could be that analyte penetration in corn during spiking process was deeper than in popcorn because popcorn floats and it does not absorb analytes as well as corn does. Furthermore, smaller molecules can penetrate deeply in the

Table 3. Features of the FUSLE-UHPLC-(Q-TOF)MS/MS method for corn and popcorn.

	LOD [a] (ng/g)		LOQ [b] (ng/g)		Repeatability [c] (%)		Recovery [c] (%) ± SD	
	Corn	Popcorn	Corn	Popcorn	Corn	Popcorn	Corn	Popcorn
PFBA	0.4	0.4	1.4	1.3	7	6	65 ± 5	94 ± 6
PFPeA	0.2	0.5	0.6	1.5	7	4	74 ± 5	105 ± 4
PFHxA	0.3	0.3	1.0	0.9	9	5	69 ± 6	109 ± 5
PFHpA	0.3	0.4	1.0	1.3	8	3	65 ± 5	103 ± 3
PFOA	0.7	0.6	2.0	1.8	9	4	82 ± 7	101 ± 4
PFNA	0.3	0.3	0.7	1.0	5	7	101 ± 5	104 ± 7
PFOS	0.2	0.2	0.6	0.7	7	3	85 ± 6	104 ± 3
PFDA	0.4	0.4	1.3	1.3	2	7	97 ± 2	108 ± 7
PFUnA	0.4	0.4	1.2	1.2	4	6	92 ± 3	100 ± 6
PFDoA	0.5	0.4	1.5	1.3	6	4	92 ± 6	105 ± 4

[a] Estimated as 3.3 times the standard deviation of the intercept divided by the slope.
[b] Estimated as 10 times the standard deviation of the intercept divided by the slope.
[c] N=5, Concentration level studied: 20 ng/g.

sample matrix during spiking because they have less sterical hindrance and higher diffusion capacity than higher chain PFAAs. Therefore, lower chain PFAAs are more difficult to recover than higher chain ones and also from corn than from popcorn.

3.3. Analysis of samples

The developed methods were applied to determine the presence of the selected PFAAs in six different microwave popcorn bags as well as their content before and after microwave cooking.

Results of the microwave popcorn bags are given in Table 4. Broadly, all bag samples contained PFCAs at levels of between 3.50 ng/g and 750 ng/g total selected PFCAs. PFHxA and PFHpA were quantified in all samples. Nevertheless, no PFOS or PFCAs with a carbon chain length higher than seven were detected in any sample.

PFPeA and PFHpA showed similar concentration levels before and after microwave cooking unlike PFBA and PFHxA. The concentration of PFBA was decreased after cooking, but for Salty B sample, which did not present significant differences. This decrease could be a result of the microwave cooking but the reason is indeterminate. On the contrary, the concentration of PFHxA was increased after cooking, except Salty C sample, which neither presented significant differences. This increase in the concentration of PFHxA could be explained by degradation pathways of other PFASs such as FTOHs or PAPs which could be present in the bag. For example, 6:2/6:2 diPAP, 6:2 FTOH or 8:2 FTOH could be degraded to PFHxA [6, 7] and due to the stability of PFCAs, these are the likely final degradation products of different PFASs.

There are clearly two different groups of PFCAs concentration levels: Salty A, Butter A and Salty B samples that showed high levels between 329 and 750 ng/g total PFCAs whereas Sweet A, Non-added fats B and Salty C showed much lower levels between 3.50 and 10.0ng/g.

As can be seen in Table 4, compounds with an even number of carbon atoms are more abundant.

A comparison of the results obtained in this work and a previous work [20] showed that the PFAAs concentration has substantially decreased in the microwave popcorn bags acquired between late 2011 and mid-2013. The PFHpA concentration values found for this period have been reduced between 65 and 80%. Moreover, PFOS, PFOA, FPDA, PFDoA were found in some microwave popcorn bag samples from late 2011 whereas that none of them has been detected in the present study.

Similar results were found by other authors for popcorn packaging before and after microwave cooking. Sinclear et al. [14] and Zafeiraki et al. [13] analysed popcorn bags acquired in New York in 2005 and in Greece in 2012, respectively. Sinclear found all PFCAs with carbon chain lengths until twelve in one of the two samples, whereas years later Zafeiraki detected only PFCAs chain length from 4 to 7. In general no differences were found before and after cooking in the first study while on the contrary in the Zafeiraki's study all PFCAs concentration increased after microwave cooking except for PFBA and, as in the present work, PFHxA followed by PFBA showed the highest levels reaching up to 681.35 ng/g of PFHxA after cooking.

Table 4. Analysis of microwave popcorn packaging before and after microwave process.

		Concentration ± SD (ng/g)[a]				
		PFBA	**PFPeA**	**PFHxA**	**PFHpA**	**Total PFCAs[b]**
Salty A (ST-A)	1	**256 ± 8**	36.8 ± 1.0	405 ± 13	7.5 ± 1.3	705 ± 15
	2	**208 ± 3**	37 ± 3	497 ± 32	7.6 ± 1.0	750 ± 32
Butter A (B-A)	1	280 ± 27	37 ± 3	**272 ± 35**	3.32 ± 0.17	592 ± 44
	2	236 ± 8	43 ± 4	**453 ± 32**	4.06 ± 0.16	736 ± 33
Sweet A (SW-A)	1	n.d.	n.d.	2.24 ± 0.06	1.26 ± 0.03	3.50 ± 0.07
	2	n.d.	n.d.	3.4 ± 0.3	1.18 ± 0.18	4.6 ±0.3
Salty B (ST-B)	1	158 ± 6	26.6 ± 2.3	**142 ± 4**	2.74 ± 0.20	329 ± 8
	2	158 ± 21	30.3 ± 1.2	**303 ± 21**	2.18 ± 0.16	493 ± 30
No added fats B (NF-B)	1	n.d.	n.d.	**3.79 ± 0.17**	1.47 ± 0.13	5.26 ± 0.21
	2	<LOQ	n.d.	**8.0 ± 0.5**	1.7 ± 0.3	9.7 ± 0.6
Salty C (ST-C)	1	n.d.	n.d.	7.4 ± 0.6	1.91 ± 0.15	9.3 ± 0.6
	2	n.d.	n.d.	7.6 ± 0.7	2.4 ± 0.3	10.0 ± 0.8

1: Microwave popcorn bags before cooking / 2: Microwave popcorn bags after cooking.

n.d.: not detected. Concentration below the detection limit. PFOA, PFNA, PFDA, PFUnA, PFDoA and PFOS were not detected in any sample.

<LOQ: concentration below the quantification limit. Detected but not quantifiable.

[a] Standard deviation (N = 3).

[b] Total PFCAs calculated by the sum of the selected PFCAs

Regarding to the analysis of corn and popcorn, no PFAAs have been detected in any of analyzed samples. Therefore, no migration of PFAAs to food could be detected.

Gebbink et al [12] carried out a similar study with corn acquired in Sweden in 2012. They found levels between 0.4 and 35.7 pg/g of PFCA in corn samples before and after cooking, being PFHxA, PFOA and PFHpA the most abundant in this order, but no significant differences were found after cooking. It is worth mentioning that those PFCAs levels cannot be detected with the present method. In general terms, the Q-ToF used in the present work is more selective (up one ten-thousandth of Dalton) but less sensitive than the QqQ used in the Gebbink's study.

4. CONCLUSIONS

A FUSLE–UHPLC–(Q-TOF) MS/MS method has been developed to determine nine PFCAs and PFOS in microwave popcorn packaging, corn and popcorn.

4.2

An efficient and simple extraction of PFAAs has been carried out by FUSLE in only one cycle of 10 s. Additionally, the chromatographic separation of the ten PFAAs took place in only 4 min. Therefore, this method allows a fast screening for these emerging pollutants in microwave bags and their content.

The whole method has been validated for the three matrices, showing good repeatability with RSDs below 12% and LODs below 0.7 ng/g. Satisfactory recovery values between 80% and 108% have been obtained in all cases but for corn that showed recovery values between 65% and 74% for PFAAs with slower carbon chains (from PFBA to PFHpA).

The validated method has been applied to the analysis of six different microwave popcorn bags and the content of each bag before and after cooking. PFCAs were found in all the microwave bags samples in a concentration range between 3.50 ng/g and 750 ng/g, being PFHxA followed by PFBA the most abundant of them. On the contrary, PFOS and PFCAs with carbon chain length higher than seven were not found in any bag. However, the presence of low carbon chain PFAAs is still significant.

Regarding to the analysis of corn and popcorn, no PFAAs were detected in any of them. Therefore, it seems that no migration of PFAAs to food happens during the microwave cooking.

ACKNOWLEDGEMENT

The Spanish Ministerio de Educación y Ciencia is thanked for supporting this work through the CTM 2010-16935 project (within the Plan Nacional de Investigación Científica y Desarrollo e Innovación Tecnológica co-financed with FEDER funds). C. Moreta also thanks the Government of La Rioja for the FPI fellowship.

REFERENCES

[1] A. Jahnke, U. Berger, Trace analysis of per- and polyfluorinated alkyl substances in various matrices-How do current methods perform?, J. Chromatogr. A 1216 (2009) 410-421.

[2] R. Renner, Growing concern over perfluorinated chemicals, Environ. Sci. Technol. 35 (2001) 154A-160A.

[3] M.M. Schultz, D.F. Barofsky, J.A. Field, Fluorinated alkyl surfactants, Environ. Eng. Sci. 20 (2003) 487-501.

[4] M.E. Andersen, J.L. Butenhoff, S.C. Chang, D.G. Farrar, G.L. Kennedy, C. Lau, G.W. Olsen, J. Seed, K.B. Wallace, Perfluoroalkyl acids and related chemistries - Toxicokinetics and modes of action, Toxicol. Sci. 102 (2008) 3-14.

[5] J.C. D'eon, S.A. Mabury, Exploring indirect sources of human exposure to perfluoroalkyl carboxylates (PFCAs): Evaluating uptake, elimination, and biotransformation of polyfluoroalkyl phosphate esters (PAPs) in the Rat, Environ. Health Perspect. 119 (2011) 344-350.

[6] H. Lee, J. Deon, S.A. Mabury, Biodegradation of polyfluoroalkyl phosphates as a source of perfluorinated acids to the environment, Environ. Sci. Technol. 44 (2010) 3305-3310.

[7] C.M. Butt, D.C.G. Muir, S.A. Mabury, Biotransformation pathways of fluorotelomer-based polyfluoroalkyl substances: A review, Environ. Toxicol. Chem. 33 (2014) 243-267.

[8] S.D. Richardson, Environmental mass spectrometry: Emerging contaminants and current issues, Anal. Chem. 84 (2012) 747-778.

[9] European Food Safety Authority, Opinion of the Scientific Panel on Contaminants in the Food chain, The EFSA Journal 653 (2008) 1-131.

[10] T.H. Begley, K. White, P. Honigfort, M.L. Twaroski, R. Neches, R.A. Walker, Perfluorochemicals: Potential sources of and migration from food packaging, Food. Addit. Contam. 22 (2005) 1023-1031.

[11] M.P. Martínez-Moral, M.T. Tena, Determination of perfluorocompounds in popcorn packaging by pressurised liquid extraction and ultra performance liquid chromatography-tandem mass spectrometry, Talanta 101(2012) 104-109.

[12] W.A. Gebbink, S. Ullah, O. Sandblom, U. Berger, Polyfluoroalkyl phosphate esters and perfluoroalkyl carboxylic acids in target food samples and packaging-method development and screening, Environ. Sci. Pollut. Res. 20 (2013) 7949-7958.

[13] E. Zafeiraki, D. Costopoulou, I. Vassiliadou, E. Bakeas, L. Leondiadis, Determination of perfluorinated compounds (PFCs) in various foodstuff packaging materials used in the Greek market, Chemosphere 94 (2014) 169-176.

[14] E. Sinclair, S.K. Kim, H.B. Akinleye, K. Kannan, Quantitation of gas-phase perfluoroalkyl surfactants and fluorotelomer alcohols released from nonstick cookware and microwave popcorn bags, Environ. Sci. Technol. 41 (2007) 1180-1185.

[15] X. Trier, K. Granby, J.H. Christensen, Polyfluorinated surfactants (PFS) in paper and board coatings for food packaging, Environ. Sci. Pollut. Res. 18 (2011) 1108-1120.

[16] S. Dolman, M Pelzing, An optimized method for the determination of perfluorooctanoic acid, perfluorooctane sulfonate and other perfluorochemicals in different matrices using liquid chromatography/ion-trap mass spectrometry, J. Chromatogr. B 879 (2011) 2043-2050.

[17] X. Trier, N.J. Nielsen, J.H. Christensen, Structural isomers of polyfluorinated di- and tri-alkylated phosphate ester surfactants present in industrial blends and in microwave popcorn bags, Environ. Sci. Pollut. Res. 18 (2011) 1422-1432.

[18] S. Poothong, S.K. Boontanon, N. Boontanon, Determination of perfluorooctane sulfonate and perfluorooctanoic acid in food packaging using liquid chromatography coupled with tandem mass spectrometry, J. Hazard. Mater. 205-206 (2012) 139-143.

[19] G. Lv, L. Wang, S. Liu, S. Li, Determination of perfluorinated compounds in packaging materials and textiles using pressurized liquid extraction with gas chromatography-mass spectrometry, Anal. Sci. 25 (2009) 425-429.

[20] C. Moreta, M.T. Tena, Fast determination of perfluorocompounds in packaging by focused ultrasound solid-liquid extraction and liquid chromatography coupled to quadrupole-time of flight mass spectrometry, J. Chromatogr. A 1302 (2013) 88-94.

[21] J. Sanz-Landaluze, L. Bartolome, O. Zuloaga, L. González, C. Dietz, C. Cámara, Accelerated extraction for determination of polycyclic aromatic hydrocarbons in marine biota, Anal. Bioanal. Chem. 384 (2006) 1331-1340.

[22] J.L. Capelo, M.M. Galesio, G.M. Felisberto, C. Vaz, J.C. Pessoa, Micro-focused ultrasonic solid-liquid extraction (μFUSLE) combined with HPLC and fluorescence detection for PAHs determination in sediments: Optimization and linking with the analytical minimalism concept, Talanta 66 (2005) 1272-1280.

[23] C. Moreta, M.T. Tena, Determination of UV filters in packaging by focused ultrasonic solid-liquid extraction and liquid chromatography, J. Chromatogr. A 1218 (2011) 3392-3399.

[24] D. Pérez-Palacios, M.A. Fernández-Recio, C. Moreta, M.T. Tena, Determination of bisphenol-type endocrine disrupting compounds in food-contact recycled-paper materials by focused ultrasonic solid-liquid extraction and ultra performance liquid chromatography-high resolution mass spectrometry, Talanta 99 (2012) 167-174.

[25] A. Errekatxo, A. Prieto, O. Zuloaga, A. Usobiaga, N. Etxebarria, L.A. Fernández, Simultaneous extraction of several persistent organic pollutants in sediment using focused ultrasonic solid-liquid extraction, Anal. Bioanal. Chem. 392 (2008) 1471-1478.

[26] P. Navarro, N. Etxebarria, G. Arana, Development of a focused ultrasonic-assisted extraction of polycyclic aromatic hydrocarbons in marine sediment and mussel samples, Anal. Chim. Acta 648 (2009) 178-182.

[27] M.P. Martínez-Moral, M.T. Tena, Focused ultrasound solid-liquid extraction and gas chromatography tandem mass spectrometry determination of brominated flame retardants in indoor dust, Anal. Bioanal. Chem. 404 (2012) 289-295.

[28] S. Fdez-Ortiz de Vallejuelo, A. Barrena, G. Arana, A. de Diego, J.M. Madariaga, Ultrasound energy focused in a glass probe: An approach to the simultaneous and fast extraction of trace elements from sediments, Talanta 80 (2009) 434-439.

[29] A. Ballesteros-Gómez, S. Rubio, S. van Leeuwen, Tetrahydrofuran-water extraction, in-line clean-up and selective liquid chromatography/tandem mass spectrometry for the quantitation of perfluorinated compounds in food at the low picogram per gram level, J. Chromatogr. A 1217 (2010) 5913-5921.

[30] A.S. Lloyd, V.A. Bailey, S.J. Hird, A. Routledge, D.B. Clarke, Mass spectral studies towards more reliable measurement of perfluorooctanesulfonic acid and other perfluorinated chemicals (PFCs) in food matrices using liquid chromatography/tandem mass spectrometry, Rapid Commun. Mass Spectrom. 23 (2009) 2923-2938.

[31] I. Ericson, R. Martí-Cid, M. Nadal, B. Van Bavel, G. Lindström, J.L. Domingo, Human exposure to perfluorinated chemicals through the diet: Intake of perfluorinated compounds in foods from the Catalan (Spain) market, J. Agric. Food Chem. 56 (2008) 1787-1794.

[32] J.L. Domingo, I.E. Jogsten, U. Eriksson, I. Martorell, G. Perelló, M. Nadal, B.V. Bavel, Human dietary exposure to perfluoroalkyl substances in Catalonia, Spain. Temporal trend, Food Chem. 135 (2012) 1575-1582.

[33] S. Ullah, T. Alsberg, R. Vestergren, U. Berger, Determination of perfluoroalkyl carboxylic, sulfonic, and phosphonic acids in food, Anal. Bioanal. Chem. 404 (2012) 2193-2201.

[34] Y.C. Chang, W.L. Chen, F.Y. Bai, P.C. Chen, G.S. Wang, C.Y. Chen, Determination of perfluorinated chemicals in food and drinking water using high-flow solid-phase extraction and ultra-high performance liquid chromatography/tandem mass spectrometry, Anal. Bioanal. Chem. 402 (2012) 1315-1325.

[35] R. Vestergren, S. Ullah, I.T. Cousins, U. Berger, A matrix effect-free method for reliable quantification of perfluoroalkyl carboxylic acids and perfluoroalkane sulfonic acids at low parts per trillion levels in dietary samples, J. Chromatogr. A 1237 (2012) 64-71.

[36] M. Llorca, M. Farré, Y. Picó, D. Barceló, Development and validation of a pressurized liquid extraction liquid chromatography-tandem mass spectrometry method for perfluorinated compounds determination in fish, J. Chromatogr. A 1216 (2009) 7195-7204.

[37] P. Labadie, M. Chevreuil, Partitioning behaviour of perfluorinated alkyl contaminants between water, sediment and fish in the Orge River (nearby Paris, France), Environ. Pollut. 159 (2011) 391-397.

[38] O. Lacina, P. Hradkova, J. Pulkrabova, J. Hajslova, Simple, high throughput ultra-high performance liquid chromatography/tandem mass spectrometry trace analysis of perfluorinated alkylated substances in food of animal origin: Milk and fish, J. Chromatogr. A 1218 (2011) 4312-4321.

[39] H. Yoo, J.W. Washington, T.M. Jenkins, E. L. Libelo, Analysis of perfluorinated chemicals in sludge: Method development and initial results, J. Chromatogr. A 1216 (2009) 7831-7839.

[40] L. Brüggemann, W. Quapp, R. Wennrich, Test for non-linearity concerning linear calibrated chemical measurements, Accredit. Qual. Assur. 11 (2006) 625-631.

5.

Parabens in pharmaceuticals

5.1.

Parabens and their derivatives in pharmaceuticals

(Submitted) Environmental Research, 2015

Parabens and their derivatives in pharmaceuticals

Cristina Moreta, María Teresa Tena and Kurunthachalam Kannan

Department of Chemistry. University of La Rioja. C/ Madre de Dios 51, E-26006. Logroño (La Rioja). Spain.

ABSTRACT: Exposure of humans to parabens is a concern due to the estrogenic activity of these compounds. Parabens are widely used as preservatives in some personal care products, foodstuffs and pharmaceuticals owing to their low cost, high water solubility and broad spectrum antimicrobial properties. Despite this, little is known on the occurrence of parabens in pharmaceutical products. In this study, a method based on solid-liquid or liquid-liquid extraction (SLE or LLE), and high performance liquid chromatography (HPLC) coupled with triple quadrupole mass spectrometry (QqQ-MS/MS) was developed for the determination of six most frequently used parabens and four paraben derivatives (methyl- and ethyl- protocatechuates, and mono- and di-hydroxybenzoic acids) in pharmaceuticals. A sample-purification step involving solid-phase extraction (SPE) was optimized for the analysis of solid and lipid-rich pharmaceuticals. To our knowledge, this is the first comprehensive report on the occurrence of parabens in pharmaceuticals. The developed method was applied for the analysis of 128 liquid/syrup, cream, solid, prescription or over-the counter (OTC) drugs collected from the USA and a few other countries in Europe and Asia. Although majority of the drugs analyzed in the study did not contain parabens, concentrations as high as 2 mg/g were found in some drugs. Methyl- and propyl- parabens were the frequently detected compounds. 4-Hydroxybenzoic acid was the major metabolite found in pharmaceutical products.

Keywords: Parabens, Pharmaceuticals, Liquid chromatography, Mass spectrometry, Human exposure.

1. INTRODUCTION

Parabens are alkyl esters of 4-hydroxybenzoic acid (4-HB) and are widely used as preservatives in consumer products including processed foodstuffs [1, 2], cosmetics [3, 4, 5], toiletries [3, 4, 5], paper products [6] and pharmaceuticals [5] due to their low cost, effectiveness over a wide range of pH, high stability, water solubility and a broad spectrum antimicrobial activity. In particular, preservatives are added to pharmaceuticals to prevent any microbial growth and/or degradation of the drug (i.e., to increase the shelf-life).

Studies have shown that parabens possess a weak estrogenic activity [7]. The most commonly used parabens are methyl-, ethyl-, propyl-, butyl-, benzyl- and heptyl- parabens and the estrogenic activity of these compounds increases with the length of the alkyl chain [7]. Parabens are considered as endocrine disrupting compounds. Some studies have associated a decrease in sperm production or an increase in the incidence of breast cancer and malignant melanoma to paraben exposures [8-12].

Concomitant with an increase in the understanding of toxicological properties of parabens, the European Union has lowered allowable maximum concentrations of propyl- and butyl- parabens in cosmetics from 0.4% when used individually and 0.8% when mixed with other esters, to 0.14% when used individually or in mixture [13]. In addition, the use of propyl- and butyl- parabens is banned in cosmetics intended for children under three years of age [14].

Human exposure to parabens is a concern, and sources of human exposure to parabens are not fully characterized. A few studies have reported the occurrence of parabens in consumer products including processed foods and personal care products [5, 15-17], as well as in environmental and biological samples including water, sediments, sewage sludge, soil, indoor dust, saliva, serum and urine [18-22]. However, to our knowledge, little is known on the occurrence of parabens in pharmaceuticals [5, 15-17]. The two earlier studies that measured parabens in pharmaceuticals involved a small sample size of 2 to 17 liquid pharmaceuticals and these studies analyzed only for 4-HB, MeP and PrP [5, 16].

Parabens have been typically analyzed by high-performance liquid chromatography (HPLC) coupled with ultraviolet (UV) detector or mass spectrometry (MS) [20, 23] and gas chromatography (GC) coupled with MS [15, 20, 23, 24]. Liquid-liquid extraction (LLE), solid-liquid extraction (SLE) and ultrasound-assisted extraction (UAE) are the common methods employed in the extraction of parabens from sample matrices. In addition, pressurized liquid extraction (PLE), dispersive liquid-liquid microextraction (DLLME), supercritical fluid extraction (SFE) and stir bar sorptive extraction (SBSE) [20] have been reported for the extraction of parabens. LLE and UAE have been employed in the analysis of parabens in liquid syrup pharmaceuticals [5, 15-17]. However, no earlier studies have determined parabens in pharmaceutical tablets/capsules.

Although parabens are stable, they can be metabolized by esterases [25]. In addition, hydrolytic transformation of several parabens to p-hydroxybenzoic acid has been reported [26]. Another degradation pathway for parabens is oxidative

hydroxylation. Light–induced hydroxylation of methyl paraben to methyl protocatechuate has been reported [27]. Therefore, hydrolysis and hydroxylation of parabens can occur in pharmaceutical formulations during production and storage.

In this study, a method comprising SLE / LLE and HPLC-MS/MS was developed for the determination of six parabens and four paraben derivatives (methyl- and ethyl- protocatechuates, and mono- and di- hydroxybenzoic acids) in pharmaceuticals (Figure S1) encompassing liquid/syrup, cream, gel, and solid capsules (pills) collected from pharmacies in the USA and a few other countries. The method was applied in the determination of parabens in 128 pharmaceuticals.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Methyl- (MeP), ethyl- (EtP), propyl- (PrP), butyl- (BuP), benzyl- (BzP), and heptyl- (HpP) parabens were purchased from AccuStandard Inc (New Haven, CT, USA) in methanol (MeOH) at 100 µg/ml (purity ≥98%). 4-Hydroxybenzoic acid (4-HB) and 3,4-dihydroxybenzoic acid (i.e., protocatechuic acid; 3,4-DHB) were also purchased from AccuStandard in acetonitrile (ACN) at 100 µg/ml (>99.5%). Methyl protocatechuate (OH-MeP; 97%) and ethyl protocatechuate (OH-EtP; 97%) were purchased from Sigma-Aldrich (St Louis, MO, USA).

Isotopically labelled internal standards, $^{13}C_6$-MeP, $^{13}C_6$-BuP and $^{13}C_6$-4-HB, were purchased from Cambridge Isotope Laboratories (Tewksbury, MA, USA) as individual standard solutions in methanol at 1 mg/ml (99%).

Formic acid (ACS grade; 88%), hexane (ultra-residue grade; 95% n-hexane) and ethyl acetate were purchased from J.T.Baker® (Center Valley, PA, USA). Acetone and dichloromethane (DCM) (ACS grade) were purchased from Macron Fine Chemicals™ (Center Valley, PA, USA). LC–MS grade methanol (MeOH) was purchased from Fisher Scientific (Pittsburgh, PA, USA). Milli-Q water was prepared by an ultrapure water system (Barnstead International, Dubuque, IA, USA). All standards and solutions were prepared in LC–MS grade MeOH and were stored at -20ºC until analysis.

Strata® NH_2 (55 µm, 70Å, 200 mg/3ml) and Oasis® HLB (3cc, 60 mg) cartridges were used for solid-phase extraction (SPE) and were obtained from Phenomenex (Torrance, CA, USA) and Waters Corporation (Milford, MA, USA), respectively.

2.2 Sample collection and preparation

A total of 128 pharmaceuticals were collected from July to November 2014. Over-the-counter (OTC) drugs were purchased in local stores and prescription drugs were obtained from volunteers who donated a small amount (<1 g) for this research. Name of the drug (commercial name), expiration date, manufacturer, and sampling location were recorded. Some of the prescription drugs analyzed had passed the expiration date.

The pharmaceutical samples originated from the USA (New York and New Jersey) (n=104), Italy (n=2), Poland (n=2), Spain (n=5), China (n=7), India (n=5), and Japan (n=3). The pharmaceuticals collected from the USA were grouped into three categories: solid samples (capsules/pills; n=58), liquid/syrup or cream samples (n=32) and softgels (n=14). Samples were also categorized as over-the-counter (OTC) and prescription medicines, as well as, by their therapeutic effects.

Solid samples (tablets, caplets or capsules) and softgels were kept at room temperature in the dark. Tablets and caplets were homogenized with a solvent-rinsed ceramic mortar prior to extraction. The exterior shell of softgels and capsules were cut into small pieces (1-2 mm^2) using scissors. Liquid/syrup, cream and homogenized samples were stored at 4ºC in polypropylene (PP) tubes until analysis.

2.3. Extraction and clean-up

Between 0.05 and 0.10 g of pharmaceutical sample was placed in a 15 ml polypropylene conical tube (PP tube), followed by the addition of 200 µL of 1 µg/ml internal standard mixture and 4.5 ml of MeOH. The extraction was performed by shaking the mixture in a reciprocal shaker for 30 min at 280 ± 5 osc/min (Eberbach Corporation, Ann Arbor, MI, USA). The sample was centrifuged at 5000 x g for 15 min (Centrifuge 5804 Eppendorf, Hamburg, Germany) and the supernatant was transferred into another PP tube. Liquid/syrup, cream and non-oily softgel samples were analyzed by HPLC-MS/MS.

Solid and oily softgel (e.g., fish oil supplements) samples required further SPE clean-up before instrumental analysis. The supernatant was concentrated to 2 ml under a gentle nitrogen stream using a multivap nitrogen evaporation system (Organomation Associates Inc., Berlin, MA, USA). After vortex mixing, 6 ml of water acidified with 0.1% formic acid were added. The final mixture was vortexed and centrifuged at 5000 x g for 15 min, and the MeOH/water (25:75 v/v) mixture was transferred into another PP tube.

The sample purification was accomplished by use of a 24-port solid phase glass block vacuum manifold (Burdick & Jackson, Muskegon, MI, USA). The extract was purified by passage through Oasis HLB 3cc extraction cartridge (60 mg and 30 µm particle size), that was previously conditioned with 3 ml of MeOH and 3 ml of water. The cartridge was washed with 3 ml of 10% MeOH in water and 3 ml of water. The cartridge was dried under vacuum for 10 min and then the analytes were eluted with 3 ml of MeOH. All of the SPE steps were carried out at atmospheric pressure, although a vacuum pump was employed to load some samples, at a rate not exceeding 20 drops /minute. The extract was made up to 4 ml and centrifuged at 5000 x g for 15 min before HPLC-MS/MS analysis.

2.4. HPLC-ESI-(QqQ) MS/MS analysis

An Agilent 1100 Series HPLC system (Agilent Technologies Inc., Santa Clara, CA, USA) connected to a (100 mm × 2.1 mm i.d., 3 µm particle size) Betasil ® C18 column and a (20 mm long) VanGuard pre-column (Thermo Electron, Bellefonte, PA, USA) coupled to an API 2000 electrospray triple quadrupole mass spectrometer (ESI-QqQ; MS/MS) (Applied Biosystems, Foster City, CA, USA) was employed for the separation and quantification of six parabens and four paraben derivatives. The data were acquired with the Analyst® software.

The HPLC column was kept at room temperature. The mobile phase flow rate was set at 0.30 ml/min and the sample injection volume was 10 µL. The mobile phases were MeOH (solvent A) and 0.02% formic acid aqueous solution (solvent B). The mobile phase composition started isocratic for 3 min at 5% A and then increased linearly from 5% to 85% A in 2 min and maintained at 85% A for 1 min. Then the gradient was increased from 85% to 98% A in 2 min and maintained at 98% A for 7 min. The mobile phase composition was then returned to the initial conditions in 1 min and held for 7 min.

The quantification was performed by multiple reaction monitoring (MRM) and the transitions for quantification and confirmation are shown in Table S1 and Figure S2 (Supplementary Material). Electrospray ionization was carried out in negative ion mode using an ion spray voltage of -4.5 kV. Other mass spectrometric conditions used in the analysis are shown in Table S1. Nitrogen was used as both curtain and collision gas. The curtain gas was set at 20 psi and the collision cell gas was set at 2 psi. The ESI source temperature was maintained at 450ºC. Finally, the data acquisition was set at 100 msec for scan speed.

3. RESULTS AND DISCUSSION

3.1. Optimization and validation of HPLC-ESI-(QqQ) MS/MS method

The QqQ parameters were optimized by infusion of individual analytes at 250 ng/ml in MeOH into the mass spectrometer through a flow injection system (see Table S1 Supplementary Material). The confirmation and quantitation ions of parabens and protocatechuates were 136>92 and 152>108, respectively. The mass spectrometric fragments of parabens monitored are shown in Figure S2. Quantitation ion corresponded to the loss of the ester group or the COO^- group for the acids. Confirmation ion was from the loss of the alkyl group. Acids did not fragment considerably even with increasing collision energy.

The mobile phase containing formic acid (up to 0.50%) or ammonia (up to 0.025%) was tested to further improve the signal of the target analytes. Whereas the intensity of alkyl protocatechuates increased, the intensity of parabens and acids (i.e., 4-HB and 3,4-DHB) decreased with a decrease in pH. Conversely, the intensity of parabens and acids increased and protocatechuates diminished at higher pH values. Nevertheless, the presence of ammonia in the mobile phase distorted the peak shapes (fronting) of acids and short chain parabens (possibly due to secondary interactions). These analytes eluted at the beginning of the chromatographic separation at a concentration of 0.025% ammonia. Nevertheless, a mobile phase concentration of 0.02% formic acid yielded optimal conditions for sensitivity and resolution of the target chemicals.

We examined optimal sample injection volume (injections between 10 and 50 µL) and found that volumes above 10 µL yielded poor peak shapes (due to fronting). Therefore, a sample injection volume of 10 µL was used.

The limits of detection (LODs) and limits of quantification (LOQs), repeatability (intra-day RSD, %) and inter-day precision (RSD, %) were determined (Table 1). Quantification was based on the ratios of analyte-to-internal standard peak area. $^{13}C_6$-MeP was used as the internal standard for the quantification of MeP, EtP, OH-MeP and OH-EtP; $^{13}C_6$-BuP for PrP, BuP, BzP and HpP, and $^{13}C_6$-4-HB for 4-HB and 3,4-DHB.

The LODs and LOQs were estimated as 3.3 and 10 times the standard deviation of the intercept, respectively, divided by the slope of the calibration curve injected at concentrations of 0.1 ng/ml to 5 ng/ml. The LODs and LOQs were below 0.06 and 0.18 ng/ml, respectively, for all targets except for 4-HB and 3,4-DHB, and MeP (Table 1). The LODs for these analytes were higher (between 0.21 and 0.4

ng/ml) due to the presence of a background signal. The procedural blanks contained two peaks that eluted close to 4-HB with relative retention times (RRT) of 1.04 and 1.10, although they that did not interfere with the quantification of this acid (RRT = 1.00).

Table 1. Limit of detection (LOD), limit of quantitation (LOQ), retention time (RT), product ion ratio, and precision of the LC-MS/MS method for the analysis of parabens and their derivatives in pharmaceuticals.

Analytes	LOD [a] (ng/ml)	LOQ [b] (ng/ml)	$RT_{analyte}/RT_{IS}$ [c]	Product ion ratio [d]	Repeatability [e] (%)	Inter-day precision [e] (%)
3,4-DHB	0.21	0.7	0.957 ± 0.002	-	2.2	8
4-HB	0.4	1.2	1.00 ± 0.001	-	3.8	6.4
OH-MeP	0.05	0.13	0.965 ± 0.002	27.4 ± 1.0	3.2	3.2
OH-EtP	0.06	0.18	0.994 ± 0.001	32.7 ± 1.0	3.3	5.1
MeP	0.3	0.8	1.00 ± 0.001	18.4 ± 0.6	2.8	4.9
EtP	0.04	0.12	1.03 ± 0.002	9.1 ± 0.3	4.3	4.6
PrP	0.06	0.18	0.972 ±0.002	5.2 ± 0.1	3.8	4.6
BuP	0.019	0.06	1.00 ± 0.001	9.6 ± 0.3	2.7	3.9
BzP	0.04	0.12	0.996 ± 0.001	13.0 ± 0.7	3.8	2.2
HpP	0.03	0.08	1.10 ± 0.003	16.7 ± 0.4	2.4	4.5

[a,b] Estimated as 3.3 and 10 times the standard deviation of the intercept divided by the slope, respectively.

[c] Analyte and internal standard retention times ratio (N=9: 3 replicates x 3 days).

[d] Quantification product ion and qualification product ion peak areas ratio (N=10).

[e] Calculated by ANOVA (4 replicated x 3 days) at a concentration of 20 ng/ml.

All calibration curves were linear from 0.1 to 500 ng/ml with R^2 values between 0.9992 and 0.9997. The repeatability and inter-day precision were calculated by the analysis of variance (ANOVA) at 20 ng/ml (measured in four replicates per day over three different days) and the RSDs were below 8% for all analytes. Select chromatograms of the mixture of all target analytes (standard), and a liquid and solid pharmaceutical samples are shown in Figure S3.

3.2. Optimization and validation of extraction and purification procedures

MeOH was the most frequently used solvent in the extraction of parabens from sample matrixes [1, 2, 4-7]. We selected 3.8 ml of MeOH and 0.2 ml of internal standards in MeOH (at a concentration of 1 µg/ml) for the extraction of 0.1 g of sample. Liquid syrups yielded recoveries of between 37 and 94%; however solid samples exhibited a strong matrix effect of up to 95% for $^{13}C_6$-MeP and $^{13}C_6$-4-HB. Therefore, a sample purification step was needed in the analysis of solid pharmaceutical samples prior to instrumental analysis.

3.2.1. Purification by solid-phase extraction method

An extract from a solid pharmaceutical sample was purified in triplicate by LLE with ethyl acetate/water (80:20 v/v) similar to that reported earlier [28]. However, this procedure did not improve the recoveries of the internal standards (Table S2).

Therefore, two SPE based purification methods employing NH_2 and HLB cartridges, similar to that reported earlier [1], were optimized for the analysis. All tests were performed in triplicate. Four ml of a standard solution containing 100 ng/ml of a mixture of target analytes and 50 ng/ml for the internal standards were transferred into a PP tube prior to purification.

For NH_2 cartridges, the extract was concentrated to near-dryness (less than 0.2 ml) under a gentle nitrogen stream and re-dissolved in 2 ml of DCM/hexane (30:70 v/v) and transferred onto a Strata-NH_2 cartridge. The cartridge was previously conditioned with 4 ml of acetone/MeOH (20:80, v/v) and 4 ml of hexane. After loading the extract, the cartridge was washed with 4 ml of hexane and dried under vacuum for 10 min. The analytes were eluted with 3 ml of acetone/MeOH (20:80, v/v).

We also tested the HLB cartridges for the purification of sample extracts. The extract was concentrated to 2 ml under a gentle nitrogen stream and 6 ml of Milli-Q water were added. The HLB cartridges were conditioned with 3 ml of MeOH and 3 ml of water. After loading the sample, the cartridge was washed with 3 ml of 10% MeOH in water and 3 ml of water. The cartridge was then dried under vacuum for 10 min and the analytes were eluted with 3 ml of MeOH.

The recoveries of target analytes through HLB and NH_2 cartridges are shown in Table 2. The recoveries of all parabens and protocatechuates through the HLB cartridges ranged from 84 to 103%. With the NH_2 cartridges, only parabens showed

good recoveries (86-105%). 4-HB and 3,4-DHB were found to sorb onto glass surface.

Table 2. Recoveries (%) of parabens and their derivatives through the solid-phase extraction (SPE) procedure (N=3).

	HLB cartridge				NH_2 cartridge		
	Not Acidified		0.1% Ac.	0.2% Ac.	Not Acidified		0.2% Ac.
	1st elution	Load	1st elution	1st elution	1st elution	Wash glassware	1st + 2nd elution
MeP	100 ± 3	1.3 ± 0.4	90 ± 10	92 ± 6	91 ± 7	n.d.	98 ± 11
$^{13}C_6$-MeP	101 ± 8	n.d.	93 ± 7	94 ± 4	89 ± 9	n.d.	100 ± 9
EtP	103 ± 6	n.d.	95 ± 7	96 ± 4	88 ± 7	n.d.	97 ± 11
PrP	103 ± 5	1.1 ± 0.2	98 ± 8	91 ± 11	101 ± 8	n.d.	95 ± 14
BuP	103 ± 9	n.d.	98 ± 5	88 ± 12	105 ± 13	n.d.	102 ±5
$^{13}C_6$-BuP	102 ± 4	n.d.	96 ± 3	92 ± 2	103 ± 9	n.d.	103 ± 11
BzP	103 ± 8	n.d.	104 ± 6	97 ± 11	100 ± 12	n.d.	102 ± 13
HpP	103 ± 5	n.d.	98 ± 3	97 ± 6	86 ± 7	n.d.	96 ± 7
4-HB	2.6 ± 0.5	108 ± 5	99 ± 6	99 ± 4	n.d.	32 ± 11	92 ± 5
$^{13}C_6$-4-HB	2.0 ± 0.6	101 ± 4	97 ± 3	95 ± 2	n.d.	34 ± 12	88 ± 3
3,4-DHB	1.2 ± 0.4	101 ± 12	91 ± 3	90 ± 2	n.d.	37 ± 8	84 ± 6
HO-MeP	84 ± 8	n.d.	88 ± 2	92 ± 4	47 ± 9	3.0 ± 1.4	92 ± 3
HO-EtP	89 ± 9	n.d.	89 ± 3	92 ± 3	47 ± 9	2.0 ± 0.8	88 ± 3

All recoveries were below 3% in cartridge wash eluant.

The influence of sample pH on the recoveries through HLB and NH_2 cartridges was tested by employing DCM/hexane (30:70 v/v) acidified with 0.2% of formic acid or water acidified with 0.1% and 0.2% of formic acid (Table 2). In both cases, excellent recoveries of between 80% and 104% were obtained for all target analytes. Because 9 to 11% of the acids and their internal standards were eluted in the second fraction, an elution volume of 6 ml was selected for NH_2 cartridges. Moreover, no significant differences were found between water acidified at 0.1% or 0.2% of formic acid. Therefore, water acidified at 0.1% of formic acid and

DCM/hexane (30:70 v/v) acidified at 0.2% of formic acid were selected for analysis using the HLB and NH_2 cartridges, respectively.

Select solid pharmaceutical samples were analyzed by both the SPE methods. Recoveries of the internal standards spiked into the samples are shown in Table S2. Purification by HLB cartridges yielded higher recoveries for all internal standards than that by NH_2 cartridges. Hence, HLB cartridges were selected for purification of solid pharmaceuticals prior to HPLC-MS/MS analysis.

The purification method was further validated by spiking 1 ml of a standard solution containing 400 ng/ml for all target analytes and 200 ng/ml for all internal standards to a solid pharmaceutical sample extract (n=3). All target analytes showed satisfactory recoveries of between 89% and 98% with RSDs <13%.

3.2.2. Sample extraction cycle

Samples were extracted up to three times to evaluate the extraction efficiency, as described below: 4.5 ml of MeOH and 0.2 ml of internal standards (at 1 µg/ml) were added to 0.10 g sample. The sample was extracted by shaking for 30 min, and centrifuged at 5000 x g for 15 min. For the second and third extractions, 4.5 ml of MeOH was added to the residue and extracted as above. Recoveries of MeP, EtP, PrP, 4-HB, 3,4-DHB, OH-MeP and the internal standards ranged from 95.1% to 98.9% in the first extraction (Figure S4), which suggested that a single extraction can adequately extract the target chemicals from the pharmaceutical samples.

3.3. Quality assurance and quality control (QA/QC)

Quantification of target analytes was performed using linear regressions generated from eleven- or twelve-point calibration standards at concentrations ranging from 0.5/1.0 to 500 ng/ml. The standard calibrations were performed daily at the beginning and at the end of the sample analysis. Duplicate analysis was performed after every 10 samples and the RSD for all duplicate samples was ≤ 18%. A six-point calibration curve was generated after every 20 samples. In addition, a methanol blank was injected between each sample and a procedural blank was processed every day when samples were analyzed. Dilutions were carried out when the analyte concentration was above the calibration range. Some samples required dilution of up to 100 times in MeOH.

Although oily softgel samples showed acceptable recoveries (even without SPE purification), those extracts required further purification to protect the instrument from oily deposits. It is also worth to mention that some samples showed

peaks with a same MRM transition for quantification and confirmation ion, but at different retention times. The ratios of retention time of analyte to internal standard were different; for MeP (0.96 instead of 1.00), EtP (1.00 instead of 1.03) and PrP (0.94 instead of 0.97) (see Table 1). These peaks were not considered as target compounds because it was observed in some cases that the final concentration with the quantification and confirmation ions were very different between them. They are compounds that are very similar to parabens with the same parent and two daughter ions or derivatives of parabens attached to other functional groups, leading to changes in retention times. Further studies are needed to identify these compounds in pharmaceuticals.

3.4. Analysis of pharmaceutical samples

Physical state, therapeutic effect, active ingredients, expiration date and concentrations of parabens and their derivatives in individual sample are shown in Tables S3-S7 (Supplementary Material). Because most of the samples were from the USA, they were categorized by physical state as liquid/syrup or cream (henceforth referred to as liquid), softgel (oily or aqueous) and solid samples (Table 3).

Solid samples were further grouped as over-the-counter (OTC) or prescription medicines (Table 4). All softgel and liquid samples analyzed in this study were OTC medications. Solid and liquid OTC medications were also grouped by their therapeutic purpose: a) solid pharmaceuticals: dietary supplements, antifungal/antibacterial, pain/fever, cold/flu, digestive disorder and allergy medications (Table 4); b) liquid pharmaceuticals: skin, pain/fever, cold/flu, digestive disorder, antifungal/antibacterial and others (eye drops, allergy and hair loss treatment) (Table 5). Softgel and solid prescription medications were not categorized by their purpose due to the low frequency of samples that contained parabens. Pharmaceuticals collected from a few other countries were only classified based on the origin (Table 6).

The results were evaluated by taking into account the geometric mean (GM), median, and maximum as well as the detection frequency (expressed as percentage and number of positive samples) (Tables 3-6).

Regardless of the categories mentioned above, no correlation was found between expiration date and the concentration or detection frequency of any of the target analyte. However, it appeared that the presence of some parabens was related to the nature of active ingredients in medications.

Table 3. Concentrations of parabens and their derivatives (μg/g) in pharmaceuticals collected from the USA.

	MeP	EtP	PrP	BuP	BzP	4-HB	3,4-DHB	OH-MeP	OH-EtP	∑ PB	∑ Total
1. Softgel (N=14)											
Geometric mean	0.16	-	-	-	-	0.14	-	-	-	0.16	0.30
Median	0.16	-	-	-	-	0.14	-	-	-	0.16	0.30
Maximum	0.16	n.d.	n.d.	n.d.	n.d.	0.14	n.d.	n.d.	n.d.	0.16	0.30
Detection frequency (%) [a]	7 (1)	0	0	0	0	7 (1)	0	0	0	7 (1)	7 (1)
2. Liquid / cream (N=32)											
Geometric mean	7.89	2.94	19.0	2.13	1.16	5.90	1.48	0.12	0.08	28.66	16.0
Median	0.86	0.72	105	24.0	1.16	3.04	1.48	0.14	0.08	158.99	4.52
Maximum	2000	394	695	140	1.16	128	1.48	0.30	0.09	2689	2693
Detection frequency (%) [a]	44 (14)	13 (4)	38 (12)	13 (4)	3 (1)	34 (11)	3 (1)	9 (3)	6 (2)	44 (14)	53 (17)
3. Solid (N=58)											
Geometric mean	0.37	-	0.16	-	-	0.96	0.47	0.11	0.11	0.38	0.63
Median	0.41	-	0.16	-	-	0.98	0.35	0.15	0.12	0.41	0.63
Maximum	1.10	n.d.	0.16	n.d.	n.d.	3.38	1.36	0.29	0.12	1.25	4.89
Detection frequency (%) [a]	10 (6)	0	2 (1)	0	0	16 (9)	7 (4)	7 (4)	5 (3)	10 (6)	28 (16)

[a] Figures in parenthesis refer to the number of positive samples

n.d.: not detected / HpP was not detected in any sample

On the one hand, no parabens or their metabolites were detected in any pharmaceuticals with actives ingredients such as acetaminophen / dextromethorphan / doxylamine succinate, loratadine or diphenhydramine hydrochloride, among others. On the other hand, similar concentration and composition pattern were found in samples of different brands with actives ingredients such as hydrocortisone, aluminum hydroxide / magnesium hydroxide / simethicone or vitamin C.

In general, MeP was the predominant compound, in all those samples that contained parabens, followed by 4-HB (Table 3). Concentrations and detection frequencies of parabens in U.S. pharmaceutical samples (n=104) were: 21 samples (20%) contained one or more parabens (of which 67% were liquid samples) and 30 samples (29%) contained the acid or alkyl protocatechuates.

Liquid pharmaceuticals presented the highest frequency of occurrence (44%) and concentration of parabens with a maximum of total paraben concentration of 2689 µg/g. The target analytes were detected in at least one liquid sample, except for HpP. The paraben with the longest carbon chain was not found in any of the sample analyzed. In contrast to the liquid samples, softgels showed low concentration and frequency of parabens. MeP was found only in one of the 14 softgel samples analyzed (7%) at a concentration of 0.16 µg/g. Similarly, 6 solid samples contained MeP (10%) and 1 sample contained PrP (2%) at a maximum concentration of 1.10 µg/g and 0.16 µg/g, respectively. EtP, BuP and BzP were not found in any of the 58 solid samples analyzed.

Taking into account that parabens are used primarily for their bactericidal and fungicidal properties, the low detection frequency and concentration observed in softgel and solid samples can be explained by that these formulations do not require antimicrobials, as the chances for microbial growth in these formulations are limited. Softgel medications are protected by the polymer shell while the moisture content in solid samples is very low to favor microbial growth.

In liquid pharmaceuticals, the composition pattern of parabens was similar to those reported previously in personal care products [3, 4, 6, 29] (i.e., MeP>PrP>EtP>BuP>>BzP). The concentrations of parabens found in liquid samples can be grouped into two categories (see Table S4 in Supplementary Material): samples with concentrations < 1 µg/g and those with hundreds of µg/g. 4-HB showed a similar pattern that some samples contained concentrations in the range of 0.54-3.45 µg/g whereas the others showed concentrations in the range of 22.7-128 µg/g. The sources of 4-HB in pharmaceuticals is not known and further studies are needed.

Table 4. Concentration of parabens and their derivatives (µg/g) in solid pharmaceuticals collected from the USA.

	MeP	EtP	PrP	4-HB	3,4-DHB	OH-MeP	OH-EtP	∑ PB	∑ Total
1.1. Prescription **(N=17)**									
Geometric mean	1.10	-	0.16	0.57	-	-	-	1.25	0.85
Median	1.10	-	0.16	0.57	-	-	-	1.25	0.91
Maximum	1.10	n.d.	0.16	0.57	n.d.	n.d.	n.d.	1.25	1.25
Detection frequency (%) [a]	6 (1)	0	6 (1)	6 (1)	0	0	0	6 (1)	12 (2)
1.2. OTC (N=41)									
GM	0.30	-	-	1.03	0.47	0.11	0.11	0.30	0.60
Median	0.39	-	-	0.98	0.35	0.15	0.12	0.39	0.57
Maximum	0.53	n.d.	n.d.	3.38	1.36	0.29	0.12	0.53	4.89
Detection frequency (%) [a]	12 (5)	0	0	20 (8)	10 (4)	10 (4)	7 (3)	12 (5)	34 (14)

[a] Figures in parenthesis refer to the number of positive samples
n.d.: not detected / HpP was not detected in any sample

Table 4. Cont. Concentration of parabens and their derivatives (μg/g) in solid pharmaceuticals collected from the USA.

	MeP	EtP	PrP	4-HB	3,4-DHB	OH-MeP	OH-EtP	∑ PB	∑ Total
2.1. Dietary supplement (N=8)									
Geometric mean	0.28	-	-	0.68	0.47	0.10	-	0.28	0.54
Median	0.34	-	-	1.44	0.35	0.16	-	0.34	0.39
Maximum	0.53	n.d.	n.d.	2.71	1.36	0.29	n.d.	0.53	4.89
Detection frequency (%) [a]	25 (2)	0	0	25 (2)	50 (4)	25 (2)	0	25 (2)	63 (5)
2.2. Pain / fever reliever (N=10)									
Geometric mean	0.41	-	-	1.49	-	-	0.10	0.41	0.58
Median	0.41	-	-	0.99	-	-	0.10	0.41	1.37
Maximum	0.43	n.d.	n.d.	3.38	n.d.	n.d.	0.12	0.43	3.38
Detection frequency (%) [a]	20 (2)	0	0	30 (3)	0	0	20 (2)	20 (2)	50 (5)
***2.3. Cold / flu reliever* (N=10)**									
Geometric mean	-	-	-	0.88	-	0.07	0.12	-	0.40
Median	-	-	-	0.88	-	0.07	0.12	-	0.53
Maximum	n.d.	n.d.	n.d.	0.88	n.d.	0.07	0.12	n.d.	0.88
Detection frequency (%) [a]	0	0	0	10 (1)	0	10 (1)	10 (1)	0	20 (2)

[a] Figures in parenthesis refer to the number of positive samples.

n.d.: not detected / BuP, BzP and HpP were not detected in any sample.

Table 4. Cont. Concentration of parabens and their derivatives (µg/g) in solid pharmaceuticals collected from the USA.

	MeP	EtP	PrP	4-HB	3,4-DHB	OH-MeP	OH-EtP	∑ PB	∑ Total
2.4. Digestive disorder reliever (N=8)									
Geometric mean	0.18	-	-	1.98	-	-	-	0.18	2.16
Median	0.18	-	-	1.98	-	-	-	0.18	2.16
Maximum	0.18	n.d.	n.d.	1.98	n.d.	n.d.	n.d.	0.18	2.16
Detection frequency (%) [a]	13 (1)	0	0	13 (1)	0	0	0	13 (1)	13 (1)
***2.5. Allergy reliever* (N=4)**									
Maximum	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2.6. Antifungal / antibacterial (N=1)									
Maximum	n.d.	n.d.	n.d.	0.46	n.d.	0.22	n.d.	n.d.	0.69

[a] Figures in parenthesis refer to the number of positive samples.

n.d.: not detected / BuP, BzP and HpP were not detected in any sample.

Alkyl protocatechuates were not found in any softgels. 4-HB was found in one sample (the sample that had MeP) at a concentration of 0.14 µg/g. The four paraben derivatives analyzed in this study were found in liquid and solid samples at frequencies of 3-34% and 5-16%, respectively. Among the four derivatives, 4-HB, followed by 3,4-DHB, were the dominant ones. The alkyl protocatechuates (OH-MeP and OH-EtP) were relatively less abundant than the acids (i.e., 4-HB>>3,4-DHB>OH-MeP~OH-EtP). The concentrations of 4-HB and 3,4-DHB in liquid samples were higher than those found in solid pharmaceuticals and hydrolysis during storage of medications is suggested as a possible reason for the occurrence of 4-HB and 3,4-DHB.

The detection frequencies of parabens in solid OTC and prescription medications were similar (Table 4). However, the detection frequencies of paraben metabolites in solid OTC samples (7-20%) were higher than in prescription medicines. MeP was the only paraben found with a detection frequency of up to 25%. Cold/flu, allergy and antifungal/antibacterial samples did not contain any parabens (Table 4). However, dietary supplements, followed by pain/fever relievers, presented the highest respective detection frequency of 25% and 20%, and the maximum concentrations of 0.53 µg/g and 0.43 µg/g. 4-HB was found in all groups, except for allergy medications.

Among liquid pharmaceuticals, skin medications contained the highest concentrations of MeP, EtP and PrP. The Highest concentrations of BuP, BzP and 4-HB were found in digestive disorder medications (Table 5). Pain/fever relievers contained the lowest concentrations of parabens at 0.11 µg/g. The composition pattern of parabens in skin medications, cold/flu relievers and antifungal/antibacterial drugs was similar (MeP>PrP>EtP) whereas that in digestive disorder medications was Bu>Pr.

All 9 samples collected from Europe showed a paraben concentration pattern similar to that found in the USA (Table 6). Pharmaceuticals from Spain and Italy did not contain parabens, but 4-HB was detected at concentration as high as 0.46 µg/g. Although pharmaceuticals from Japan showed patterns similar to that found for the USA, the maximum concentrations and frequencies of the two acids in Chinese pharmaceuticals were very high: 50.0 µg/g for 4-HB and 152 µg/g for 3,4-DHB and 86% detection rates for both, respectively. The small concentrations of parabens, and the elevated concentration of acids in Chinese samples could not be explained currently and more investigation is needed. Majority of the pharmaceuticals from India showed very high concentrations of MeP, EtP, PrP and

Table 5. Concentrations of parabens and their derivatives (µg/g) in liquid pharmaceuticals collected from the USA.

Liquid samples from USA	MeP	EtP	PrP	BuP	BzP	4-HB	3,4-DHB	OH-MeP	OH-EtP	∑ PB	∑ Total
For skin (N=8)											
Geometric mean	111	394	43.4	-	-	2.89	-	0.20	0.09	172	173
Median	1270	394	270	-	-	2.78	-	0.22	0.09	1740	1743
Maximum	2000	394	695	n.d.	n.d.	3.45	n.d.	0.30	0.09	2689	2693
Detection frequency (%) [a]	50 (4)	13 (1)	50 (4)	0	0	38 (3)	0	25 (2)	13 (1)	50 (4)	50 (4)
Pai n/ fever reliever (N=3)											
Geometric mean	0.07	-	-	0.04	-	-	-	-	-	0.11	0.11
Median	0.07	-	-	0.04	-	-	-	-	-	0.11	0.11
Maximum	0.07	n.d.	n.d.	0.04	n.d.	n.d.	n.d.	n.d.	n.d.	0.11	0.11
Detection frequency (%) [a]	33 (1)	0	0	33 (1)	0	0	0	0	0	33 (1)	33 (1)
Cold / flu reliever (N=9)											
Geometric mean	1107	-	230	-	-	6.32	1.48	0.05	0.07	1343	31
Median	1108	-	241	-	-	3.04	1.48	0.05	0.07	1349	629
Maximum	1160	n.d.	314	n.d.	n.d.	29.8	1.48	0.05	0.07	1474	1476
Detection frequency (%) [a]	22 (2)	0	22 (2)	0	0	33 (3)	11 (1)	11 (1)	11 (1)	22 (2)	44 (4)

[a] Figures in parenthesis refer to the number of positive samples.
n.d.: not detected / HpP was not detected in any sample

Table 5. Cont. Concentrations of parabens and their derivatives (μg/g) in liquid pharmaceuticals collected from the USA.

Liquid samples from USA	MeP	EtP	PrP	BuP	BzP	4-HB	3,4-DHB	OH-MeP	OH-EtP	∑ PB	∑ Total
Digestive disorder reliever (N=3)											
Geometric mean	0.18	1.01	61.1	81.9	1.16	14.3	-	-	-	145	32
Median	0.18	1.01	63.8	93.9	1.16	42.3	-	-	-	159	222
Maximum	0.19	1.01	82.2	140	1.16	128	n.d.	n.d.	n.d.	224	267
Detection frequency (%) [a]	67 (2)	33 (1)	67 (2)	67 (2)	33 (1)	100 (3)	0	0	0	67 (2)	100 (3)
Antifungal / antibacterial (N=5)											
Geometric mean	2.29	0.43	4.59	-	-	22.7	-	-	-	3.17	3.21
Median	0.15	0.43	64.1	-	-	22.7	-	-	-	0.30	0.30
Maximum	593	0.43	128	n.d.	n.d.	22.7	n.d.	n.d.	n.d.	722	745
Detection frequency (%) [a]	60 (3)	20 (1)	40 (2)	0	0	20 (1)	0	0	0	60 (3)	60 (3)
Others (N=4)											
Geometric mean	0.86	0.43	0.39	0.07	-	0.75	-	-	-	1.47	1.72
Median	0.86	0.43	0.43	0.07	-	0.75	-	-	-	1.54	1.91
Maximum	0.88	0.43	0.61	0.07	n.d.	0.75	n.d.	n.d.	n.d.	2.00	2.75
Detection frequency (%) [a]	50 (2)	13 (1)	50 (2)	13 (1)	0	13 (1)	0	0	0	50 (2)	50 (2)

[a] Figures in parenthesis refer to the number of positive samples.

n.d.: not detected / HpP was not detected in any sample

4-HB. These high concentrations can be explained by the need for antimicrobials in drugs because of the prevailing warm and moist climate which is conducive for microbial growth in that country. Two pain/fever reliever OTC medications from India contained total concentrations of all parabens at 1168 µg/g and 1369 µg/g, whereas the three antibiotics contained concentrations of 0.37, 0.63 and 521 µg/g. This can be explained by the fact that OTC samples require longer storage (shelf-life) time, which requires the use of antimicrobials.

4. CONCLUSIONS

An analytical method for simultaneous determination of six parabens and four of their metabolites in in liquid, solid and softgel pharmaceuticals was developed. Solid samples showed matrix effects which required the need for a SPE clean–up step. The SPE clean-up step involving HLB and NH_2 cartridges was optimized to obtain acceptable recoveries of the target compounds. Recoveries of between 84 and 104% and relative standard deviations <15% were achieved for all analytes through these methods. HLB clean-up was applied in the analysis of solid pharmaceuticals and oily softgels. One hundred and twenty eight pharmaceuticals collected from the USA and a few other countries in Europe and Asia were analyzed by the established method. The concentration pattern of parabens in pharmaceuticals was: MeP >PrP>EtP>BuP>>BzP. Paraben derivatives were also found in some samples on the order of, 4-HB>>3,4-DHB>OH-MeP>OH-EtP. HpP was not detected in any sample. Hydrolysis of parabens during storage of pharmaceuticals is an explanation for the presence of 4-HB. Overall, 20% of the pharmaceuticals analyzed in this study contained at least one of the parabens, and elevated concentrations and detection frequencies were found in liquid/syrup or cream samples (concentrations of up to 2000 µg/g and frequency of up to 44%). Solid and softgel samples contained low concentrations of parabens (up to 1.10 µg/g and 10%). Pharmaceuticals can be a source of human exposure to parabens and the exposure dose depends on the type, frequency and amount of pharmaceuticals ingested. Further studies are needed to assess the extent of human exposure to parabens through pharmaceuticals.

ACKNOWLEDGEMENTS

C. Moreta thanks to the Government of La Rioja for the FPI and international stay fellowships.

Table 6. Concentrations of parabens and their derivatives (µg/g) in pharmaceuticals collected from several countries.

Samples other countries	MeP	EtP	PrP	4-HB	3,4-DHB	OH-MeP	OH-EtP	∑ PB	∑ Total
India (N=5)									
Geometric mean	112	13.2	27.91	4.18	-	0.31	0.10	150	45.5
Median	615	109	110	11.4	-	0.31	0.10	833	521
Maximum	1123	216	233	23.3	n.d.	0.31	0.10	1357	1369
Detection frequency (%) [a]	80 (4)	40 (2)	80 (4)	60 (3)	0	20 (1)	20 (1)	80 (4)	100 (5)
China (N=7)									
Geometric mean	0.18	-	-	6.01	31.2	-	-	-	19.2
Median	0.18	-	-	6.14	18.4	-	-	-	23.8
Maximum	0.18	n.d.	n.d.	49.97	152	n.d.	n.d.	n.d.	202
Detection frequency (%) [a]	14 (1)	0	0	86 (6)	86 (6)	0	0	0	86 (6)
Japan (N=3)									
Geometric mean	0.22	-	-	0.53	1.51	-	-	-	0.44
Median	0.22	-	-	2.17	1.51	-	-	-	0.22
Maximum	0.22	n.d.	n.d.	4.28	1.51	n.d.	n.d.	n.d.	5.80
Detection frequency (%) [a]	33 (1)	0	0	67 (2)	33 (1)	0	0	0	67 (2)

[a] Figures in parenthesis refer to the number of positive samples.
n.d.: not detected / BuP, BzP and HpP were not detected in any sample

Table 6. Cont. Concentrations of parabens and their derivatives (µg/g) in pharmaceuticals collected from several countries.

Samples other countries	MeP	EtP	PrP	4-HB	3,4-DHB	OH-MeP	OH-EtP	∑ PB	∑ Total
Spain (N=5)									
Geometric mean	-	-	-	0.35	-	-	-	-	0.35
Median	-	-	-	0.36	-	-	-	-	0.36
Maximum	n.d.	n.d.	n.d.	0.46	n.d.	n.d.	n.d.	n.d.	0.46
Detection frequency (%) [a]	0	0	0	40 (2)	0	0	0	0	40 (2)
Polan (N=2)									
Maximum	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Italy (N=2)									
Geometric mean	-	-	-	0.28	-	-	-	-	0.28
Median	-	-	-	0.28	-	-	-	-	0.28
Maximum	n.d.	n.d.	n.d.	0.28	n.d.	n.d.	n.d.	n.d.	0.28
Detection frequency (%) [a]	0	0	0	50 (1)	0	0	0	0	50 (1)

[a] Figures in parenthesis refer to the number of positive samples.

n.d.: not detected / BuP, BzP and HpP were not detected in any sample

REFERENCES

[1] C. Liao, L. Chen, K. Kannan, Occurrence of parabens in foodstuffs from China and its implications for human dietary exposure, Environ. Int. 57-58 (2013) 68-74.

[2] C. Liao, F. Liu, K. Kannan, Occurrence of and dietary exposure to parabens in foodstuffs from the United States, Environ. Sci. Technol. 47 (2013) 3918-3925.

[3] Y. Guo, K. Kannan, A survey of phthalates and parabens in personal care products from the United States and its implications for human exposure, Environ. Sci. Technol. 47 (2013) 14442-14449.

[4] Y. Guo, L. Wang, K. Kannan, Phthalates and parabens in personal care products from China: Concentrations and human exposure, Arch. Environ. Contam. Toxicol. 66 (2014) 113-119.

[5] I. Baranowska, I. Wojciechowska, N. Solarz, E. Krutysza, Determination of preservatives in cosmetics, cleaning agents and pharmaceuticals using fast liquid chromatography, J. Chromatogr. Sci. 52 (2014) 88-94.

[6] C. Liao, K. Kannan, Concentrations and composition profiles of parabens in currency bills and paper products including sanitary wipes, Sci. Total Environ. 475 (2014) 8-15.

[7] E.J. Routledge, J. Parker, J. Odum, J. Ashby, J.P. Sumpter, Some alkyl hydroxy benzoate preservatives (parabens) are estrogenic, Toxicol. Appl. Pharmacol. 153 (1998) 12–19.

[8] S. Oishi, Effects of propyl paraben on the male reproductive system, Food Chem. Toxicol. 40 (2002)1807-1813.

[9] S. Oishi, Effects of butylparaben on the male reproductive system in rats, Toxicol. Ind. Health 17 (2002) 31-39.

[10] P.D. Darbre, A. Aljarrah, W.R. Miller, N.G. Coldham, M.J. Sauer, G.S. Pope, Concentrations of parabens in human breast tumors, J. Appl. Toxicol. 24 (2004) 5–13.

[11] P.D. Darbre, P.W. Harvey, Paraben esters: review of recent studies of endocrine toxicity, absorption, esterase and human exposure, and discussion of potential human health risks, J. Appl. Toxicol. 28 (2008) 561–578.

[12] R.J. Witorsch, J.A. Thomas, Personal care products and endocrine disruption: a critical review of the literature, Crit. Rev. Toxicol. 40 (2010) 1–30.

[13] SCCS/1514/13: Opinion on Parabens - Updated request for a scientific opinion on propyl and butylparaben (Colipa n° P82) (2013).

[14] SCCS/1446/11: Clarification on Opinion SCCS/1348/10 in the light of the Danish clause of safeguard banning the use of parabens in cosmetic products intended for children under three years of age (2011).

[15] J.K. Lokhnauth, N.H. Snow, Determination of Parabens in Pharmaceutical Formulations by Solid-Phase Microextraction-Ion Mobility Spectrometry, Anal. Chem. 77 (2005) 5938-5946.

[16] M. Jaworska, Z. Szulińska, M. Wilk, Application of a capillary electrophoresis method for simultaneous determination of preservatives in pharmaceutical formulations, J. Sep. Sci. 28 (2005) 137-143.

[17] M. Thomassin, E. Cavalli, Y. Guillaume, C. Guinchard, Comparison of quantitative high performance thin layer chromatography and the high performance liquid chromatography of parabens, J. Pharm. Biomed. Anal. 15 (1997) 831-838.

[18] L. Wang, C. Liao, F. Liu, Q. Wu, Y. Guo, H.B. Moon, H. Nakata, K. Kannan, Occurrence and human exposure of p-hydroxybenzoic acid esters (Parabens), bisphenol a diglycidyl ether (BADGE), and their hydrolysis products in indoor dust from the United States and three east Asian countries, Environ. Sci. Technol. 46 (2012) 11584-11593.

[19] L. Wang, Y. Wu, W. Zhang, K. Kannan, Characteristic profiles of urinary p -hydroxybenzoic acid and its esters (Parabens) in children and adults from the United States and China, Environ. Sci. Technol. 47 (2013) 2069-2076.

[20] C Piao, L. Chen, Y. Wang A review of the extraction and chromatographic determination methods for the analysis of parabens, J. Chromatogr. B, 969 (2014) 139–148.

[21] A. Azzouz, E. Ballesteros, Trace analysis of endocrine disrupting compounds in environmental water samples by use of solid-phase extraction and gas chromatography with mass spectrometry detection, J. Chromatogr. A, 1360 (2014) 248–257.

[22] C. Liao, S. Lee, H.B. Moon, N. Yamashita, K. Kannan, Parabens in sediment and sewage sludge from the United States, Japan, and Korea: Spatial distribution and temporal trends, Environ. Sci. Technol. 47 (2013) 10895-10902.

[23] N. Cabaleiro, I. de la Calle, C. Bendicho, I. Lavilla, An overview of sample preparation for the determination of parabens in cosmetics, Trends Anal. Chem. 57 (2014) 34–46.

[24] M. Haunschmidt, W. Buchberger, C.W. Klampfl, R. Hertsens, Identification and semi-quantitative analysis of parabens and UV filters in cosmetic products by

direct-analysis-in-real-time mass spectrometry and gas chromatography with mass spectrometric detection, Anal. Methods 3 (2011) 99-104.

[25] S. Abbas, H.Greige-Gerges, N. Karam, M.H. Piet, P. Netter, J. Magdalou, Metabolism of parabens (4-hydroxybenzoic acid esters) by hepatic esterases and UDP-glucuronosyltransferases in man, Drug Metab. Pharmacokinet. 25 (2010) 568-577.

[26] N. Aubert, T. Ameller, J.J. Legrand, Systemic exposure to parabens: Pharmacokinetics, tissue distribution, excretion balance and plasma metabolites of [14C]-methyl-, propyl- and butylparaben in rats after oral, topical or subcutaneous administration, Food Chem. Toxicol. 50 (2012) 445-454.

[27] Y. Okamoto, T. Hayashi, S. Matsunami, K. Ueda, N. Kojima, Combined activation of methyl paraben by light irradiation and esterase metabolism toward oxidative DNA damage, Chem. Res. Toxicol. 21 (2008) 1594-1599.

[28] L. Wang, K. Kannan, Alkyl protocatechuates as novel urinary biomarkers of exposure to p-hydroxybenzoic acid esters (parabens), Environ. Int. 59 (2013) 27-32.

[29] T. Wu, C. Wang, X. Wang, Q. Ma, Simultaneous determination of 21 preservatives in cosmetics by ultra performance liquid chromatography, Int. J. Cosmet. Sci. 30 (2008) 367–372.t. Qual. Assur. 11 (2006) 625-631.

6.

Conclusions

CONCLUSIONS

The main conclusions of this thesis are briefly listed below:

1. Focused ultrasound solid-liquid extraction (FUSLE) for packaging analysis

* FUSLE is a faster, simpler and more economical extraction method than other respected techniques as pressurized liquid extraction (PLE) or microwave assisted extraction (MAE).
* All efficient FUSLE extractions were carried out extremely fast, in only 10-30 s.
* FUSLE showed to be an effective technique for the extraction of UV filters and perfluorinated alkyl acids, but not for plastic additives.
* All UV filter recovery values using FUSLE were similar or higher than using reflux for 2.5 h, except for Bemotrizinol (EMT) and Avobenzone (BDM).
* FUSLE efficiency was similar to PLE for perfluorinated alkyl acids extraction.
* Contrary to PLE due to the use of solvents at elevated temperatures, FUSLE allowed the determination of the ratio between the oxidized and unoxidized phosphite-type antioxidant additives present in packaging.

2. UV filters in multilayer flexible packaging

* A fast and simple FUSLE–HPLC–DAD method was developed and validated to determine the ten main organic UV filters allowed and used in Europe, in different multilayer flexible packaging without layer separation.
* The adhesion strength and the absorption of the ten UV filters in four multilayer packaging with polyethylene (PE), polypropylene (PP), polyester (PET) and Barex as contact layers were monitored for 94 days after being in contact with creams at 40ºC.
* All laminates showed the capacity of absorb the ten UV filters studied and the concentration pattern was PET>>>PP>PE>>>Barex.
* UV filters were involved in multilayer packaging deterioration although they did not cause delamination by themselves.
* PP sachets provided the highest chemical stability and resistance to creams.
* PE sachets followed by PET ones showed the higher adhesion strengths throughout the study.
* Barex showed to be an unstable laminate by itself.

3. Polymer additives in plastic-based multilayer flexible packaging

- A simple and sensitive SLE-UHPLC-ESI$^{(-)}$-VWD-TOF-MS method was developed and validated to determine 14 plastic additives and 2 by-products in packaging.
- This work allowed the simultaneous determination of slip agents and antioxidants additives in less than 10 min.
- PLE, FUSLE and SLE techniques showed excellent extraction efficiency for slip agents because they are mostly in the polymer surface and less branched.
- Antioxidants extraction efficiency depended more on the packaging swelling than on the extraction force. Incomplete recovery for Ix1010 was obtained using FUSLE and PLE with polar solvents.
- An exhaustive extraction was achieved only after traditional static SLE for 10 hours with just 5 ml of hexane. Although the optimized SLE method was very time consuming, it was exceptionally economical and simple as well as it preserved the stability of phosphite-type antioxidant additives during extraction.

4. Perfluorinated alkyl acids in packaging

- A FUSLE–UHPLC–ESI(-)-QTOF-MS/MS method was developed and validated to determine nine PFCAs and PFOS in food-contact packaging.
- The chromatographic separation of the ten PFAAs took place in only 4 min.
- PFCAs were found in all samples in a concentration range from 3.50 ng/g to 750 ng/g, being PFHxA the most abundant of them, followed by PFBA.
- PFHpA concentrations in samples from 2013 were reduced between 65 and 80% with respect to those ones from 2011. In addition, contrary to the first study, none of the most dangerous PFAAs (PFOS and PFCAs with the higher carbon chain lengths) were found in any bag in the last study. However, the presence of PFAAs with lower carbon chains remained very significant.

Perfluorinated alkyl acids in corn and popcorn

- The same analytical method for the determination of PFAAs in packaging was successfully used and validated in corn and popcorn analysis by adding a micro-scale liquid-liquid extraction clean-up step before injection.
- No migration of PFAAs to food was identified because they were not detected in any sample.

5. Parabens in pharmaceuticals

- A SLE-HPLC-ESI(+)-QqQ-MS/MS method was developed and validated to determine six parabens and four parabens derivatives in pharmaceuticals.
- Two SPE clean-up methods, with HLB and NH_2 sorbents, were optimized to decrease the extremely high matrix effect observed in solid pharmaceuticals. HLB cartridges showed better results than NH_2 ones.
- The ten analytes were analyzed in 128 pharmaceuticals collected from USA and a few other countries in Europe and Asia.
- The concentration pattern of parabens and parabens derivatives in positive samples was MeP >PrP>EtP>BuP>>BzP and 4-HB>>3,4-DHB>OH-MeP>OH-EtP, respectively. HpP was not found in any sample.
- The relatively high presence of 4-HB may be explained by the hydrolysis of parabens during storage.
- Concentrations up to 2 mg/g and detection frequencies up to 44% were found in liquid/cream pharmaceuticals. On the contrary, only up to 1.1 µg/g and frequencies up to 44% were found in solid and softgel samples. The reason might be because the high moisture content and the less protection from the outside in the liquid/cream samples.
- Pharmaceuticals can be a source of human exposure to the controversial parabens.

Supplementary material

SUPPLEMENTARY MATERIAL

CHAPTER 2.1

Determination of UV filters in packaging by focused ultrasonic solid-liquid extraction and liquid chromatography

Figure S1. Influence of the solvent in the FUSLE of UV filters from polyethylene film.

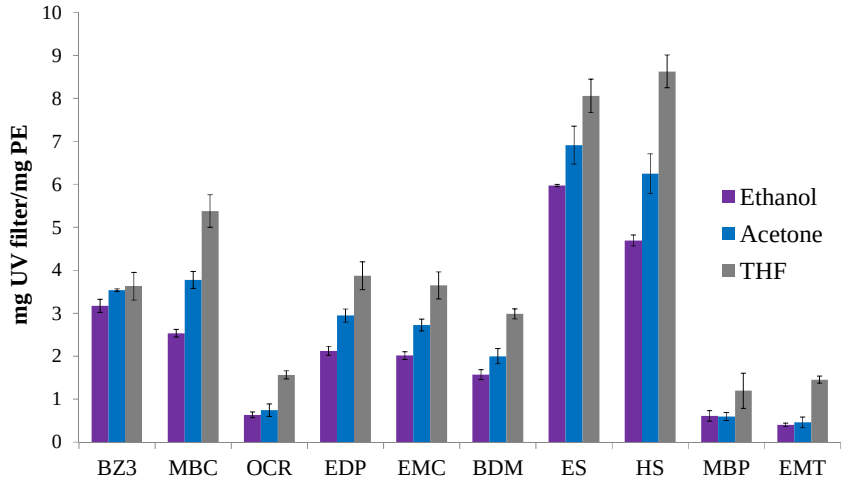
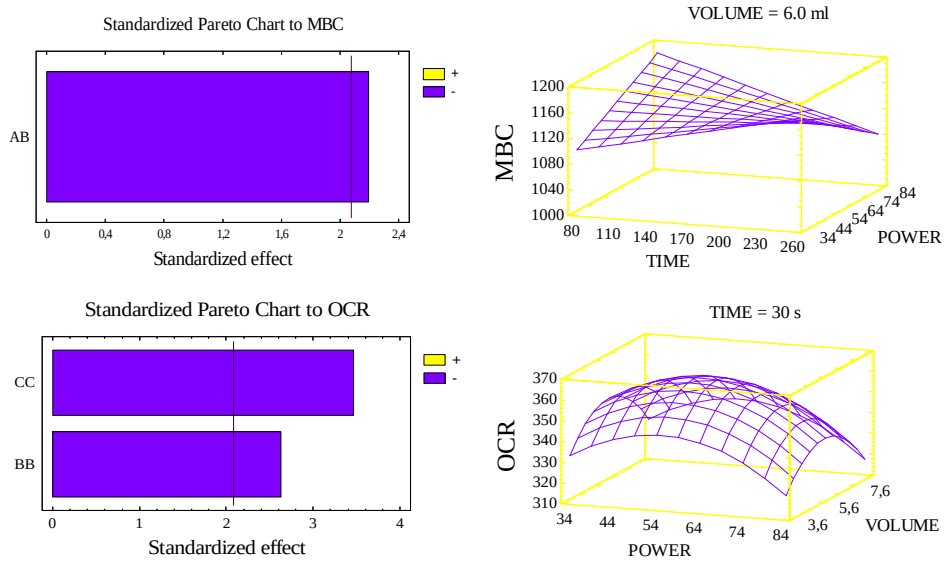
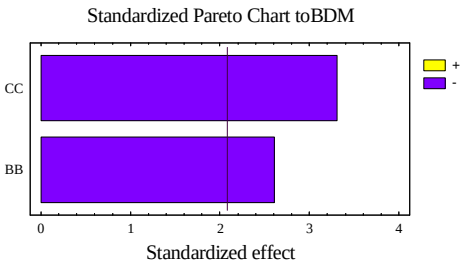
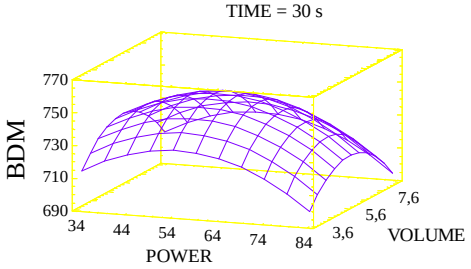
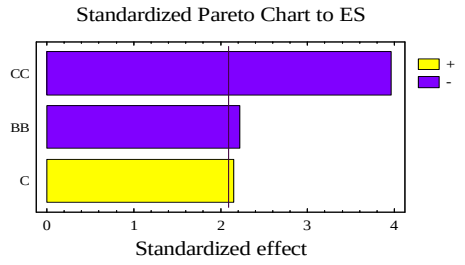


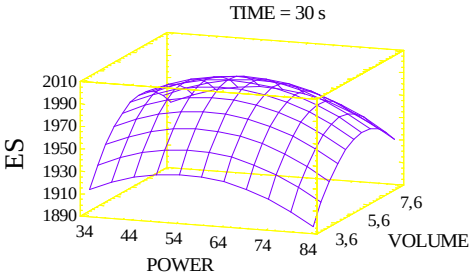
Figure S2. Pareto-charts and response surfaces of significant effects obtained from the central composite experimental design. A: Time, B: Power, C: Volume.

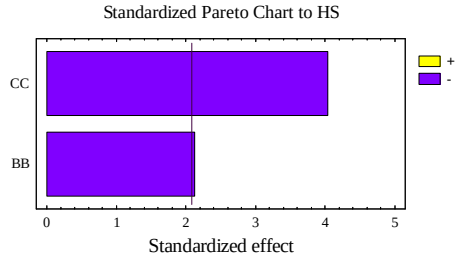


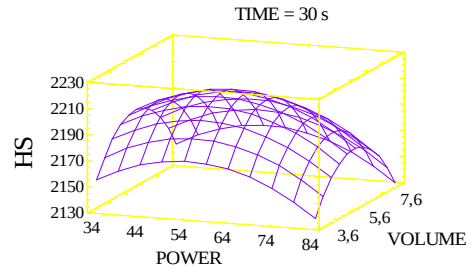

Standardized Pareto Chart toBDM
CC
BB
+
-
0
1
2
3
4
Standardized effect

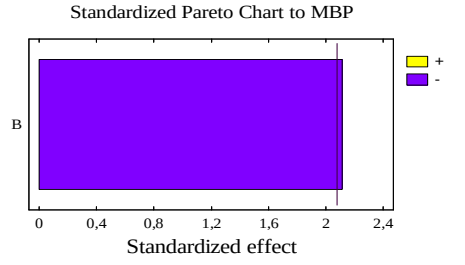

TIME = 30 s
BDM
770
750
730
710
690
34
44
54
64
74
84
POWER
3,6
5,6
7,6
VOLUME

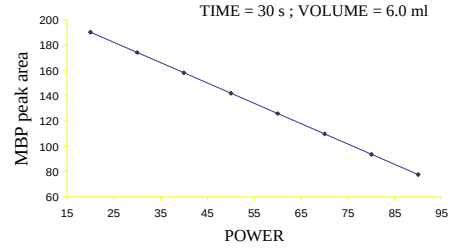

Standardized Pareto Chart to ES
CC
BB
C
+
-
0
1
2
3
4
Standardized effect


TIME = 30 s
ES
2010
1990
1970
1950
1930
1910
1890
34
44
54
64
74
84
POWER
3,6
5,6
7,6
VOLUME


Standardized Pareto Chart to HS
CC
BB
+
-
0
1
2
3
4
5
Standardized effect


TIME = 30 s
HS
2230
2210
2190
2170
2150
2130
34
44
54
64
74
84
POWER
3,6
5,6
7,6
VOLUME


Standardized Pareto Chart to MBP
B
+
-
0
0,4
0,8
1,2
1,6
2
2,4
Standardized effect


TIME = 30 s ; VOLUME = 6.0 ml
MBP peak area
200
180
160
140
120
100
80
60
15
25
35
45
55
65
75
85
95
POWER

CHAPTER 3.1

Determination of plastic additives in packaging by liquid chromatography coupled to high resolution mass spectrometry

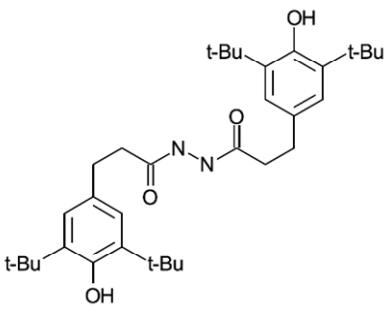
Table S1. Comparison of the overall analytical signal obtained for different combinations of formic acid and ammonium formate concentrations in the mobile phase.

Formic acid concentration (v/v)					Ammonium formate concentration (mM)
0.1%		**0.5 %**		**1.0%**	
x_1	≈	x_2	≈	x_3	**0**
∧		∧		∧	
x_4	<	$\mathbf{x_5}$	≈	x_6	**1**
≈		∨			
x_7	<	x_8			**2.5**
∨					
x_9					**10**

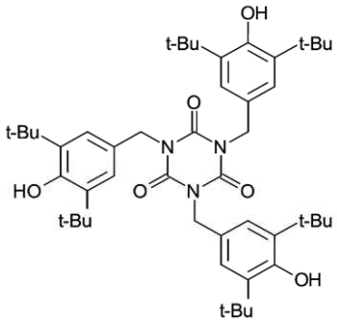
x: Overall analytical signal of the targets obtained from each mobile phase composition.

≈: Not significant differences between the analytical signals obtained for the majority of the additives in two different mobile phases compositions.

< or >: significant differences between the analytical signals obtained for the majority of the additives in two different mobile phases compositions.

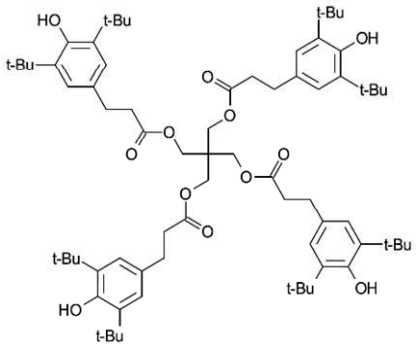


Irganox MD 1024

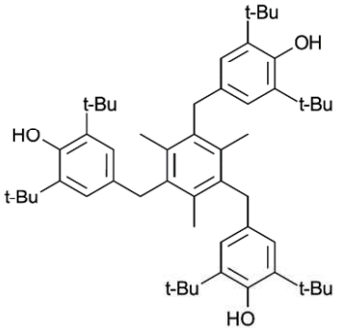


Irganox 3114

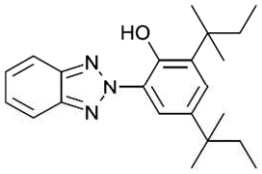
Figure S1. Chemical structures of the additives determined in this study.



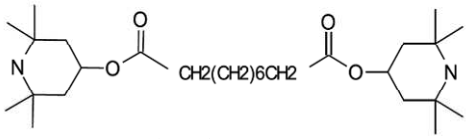
Irganox 1010



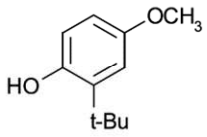
Irganox 1330



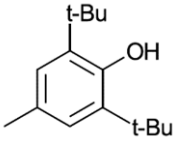
Tinuvin 328



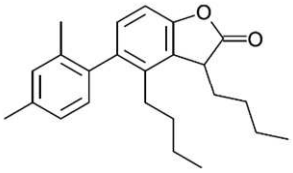
Tinuvin 770



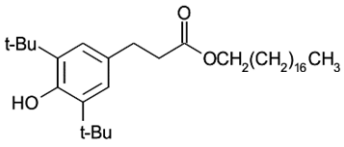
BHA
Butylated hydroxyanisole



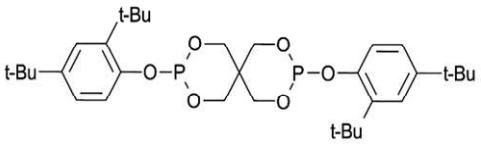
BHT
Butylated hydroxytoluene



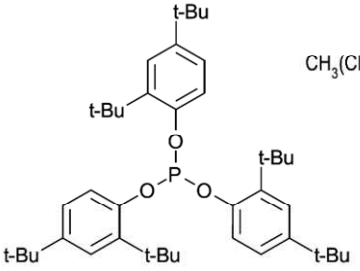
HP136
Xylyl dibutylbenzofuranone



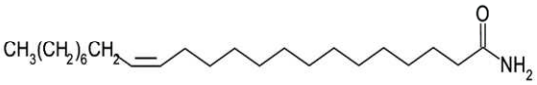
Irganox 1076



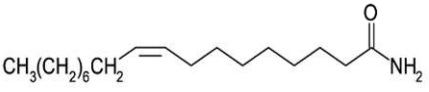
Irgafos 126



Irgafos 168



Erucamide

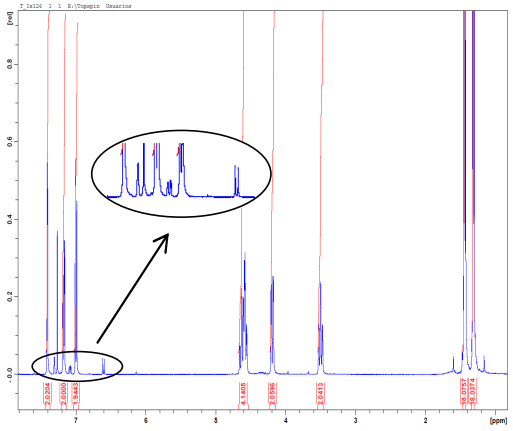


Oleamide

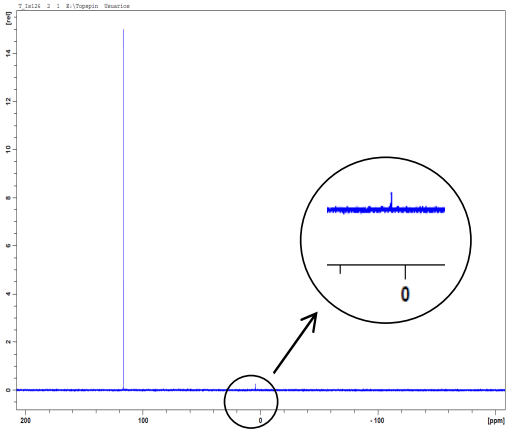
Figure S1. Chemical structures of the additives determined in this study.

Figure S.2. NMR spectra of the additives dissolved in deuterated chloroform except for the Ix1024 which was dissolved in deuterated methanol.

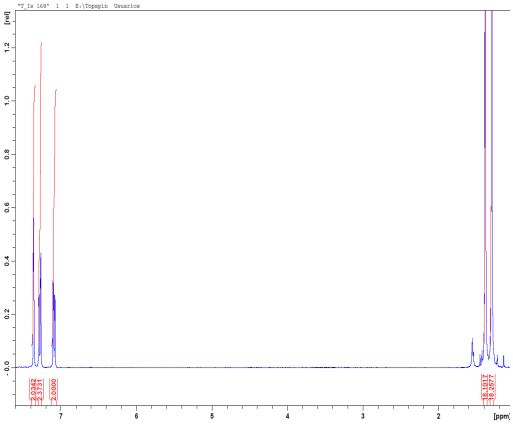
Is126 - ^{1}H-NMR



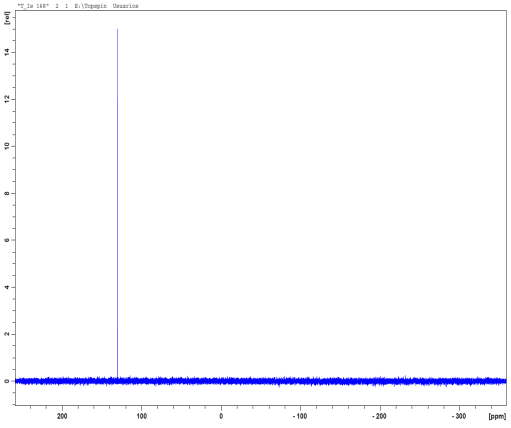
Is126 - ^{31}P-NMR



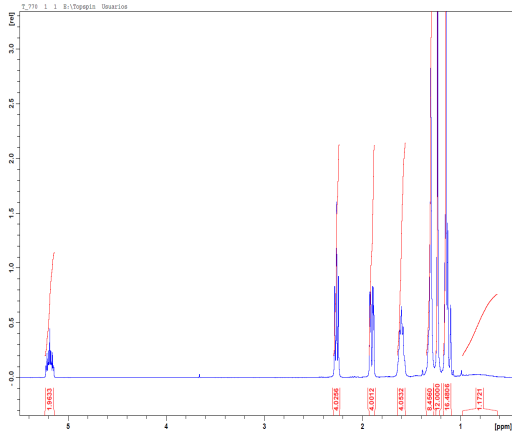
Is168 - ^{1}H-NMR



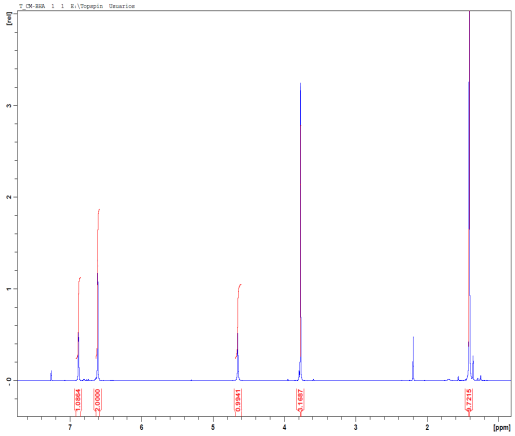
Is168 - ^{31}P-NMR



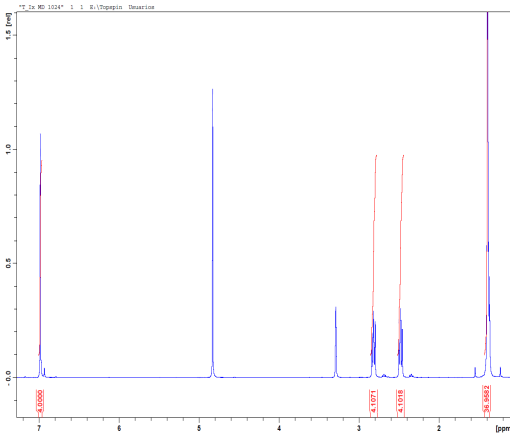
T770 - ^{1}H-NMR



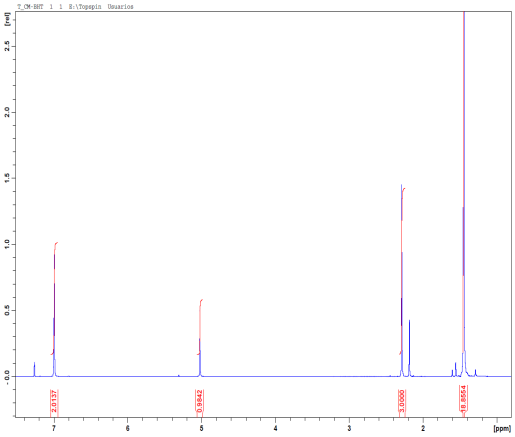
BHA - ^{1}H-NMR



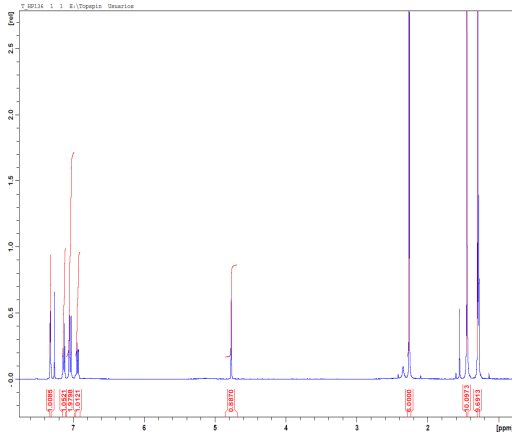
Ix1024 - ^{1}H-NMR



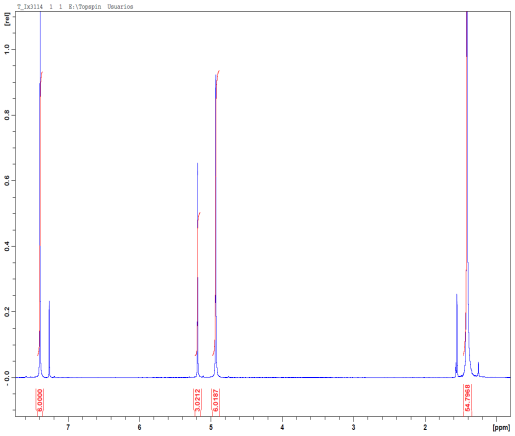
BHT - ^{1}H-NMR



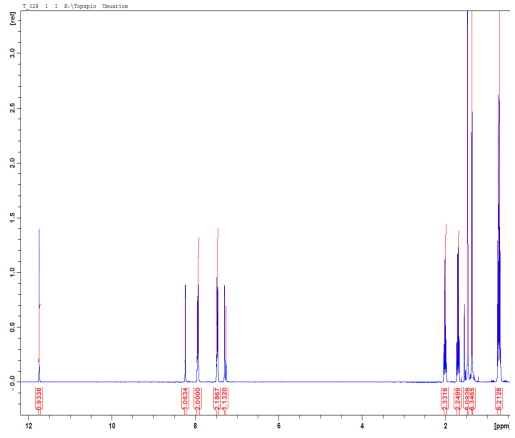
HP136 - ^{1}H-NMR



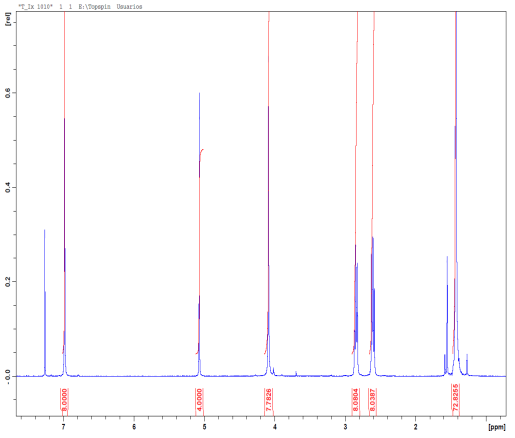
Ix3114 - ^{1}H-NMR



T328 - ^{1}H-NMR



Ix1010 - ^{1}H-NMR



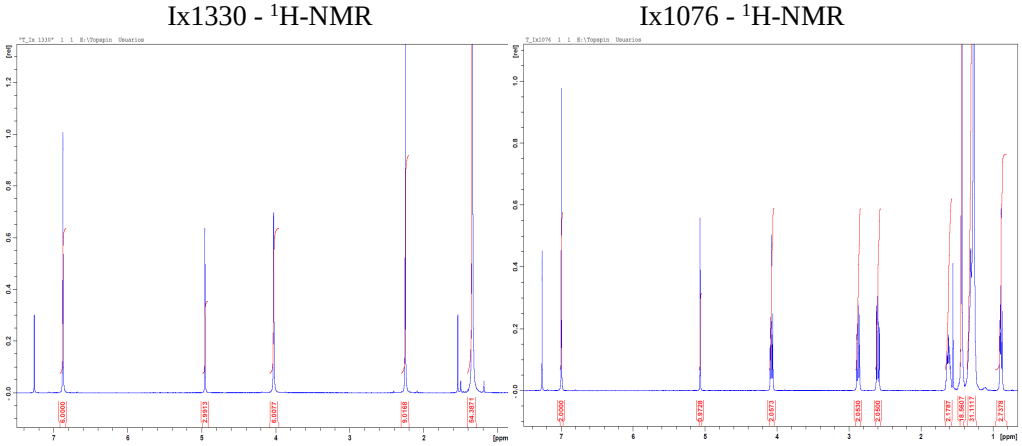


Figure S3. Normalized blank signals for unfiltered LC-MS grade methanol and for the first, second, fourth and sixth millilitre of LC-grade methanol obtained by filtration through Nylon and Teflon membrane filters.

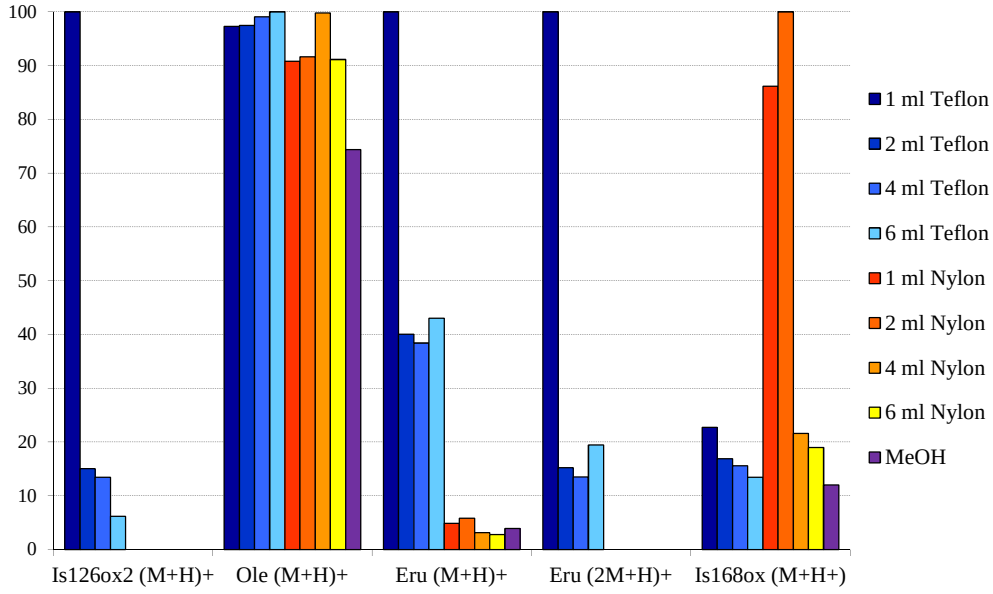
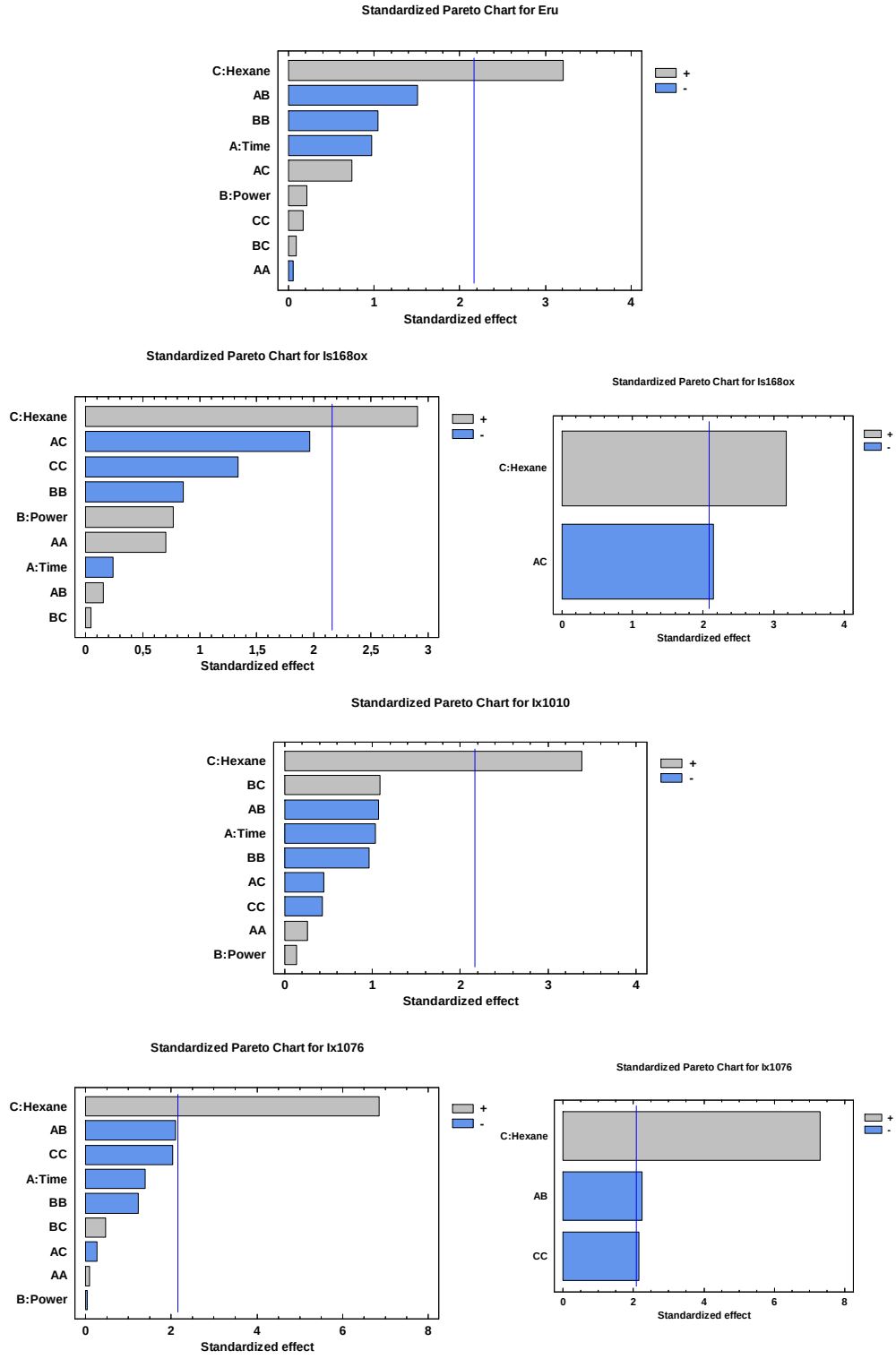


Figure S4. Pareto charts obtained from the ANOVA of the CCD of additive extraction by FUSLE.



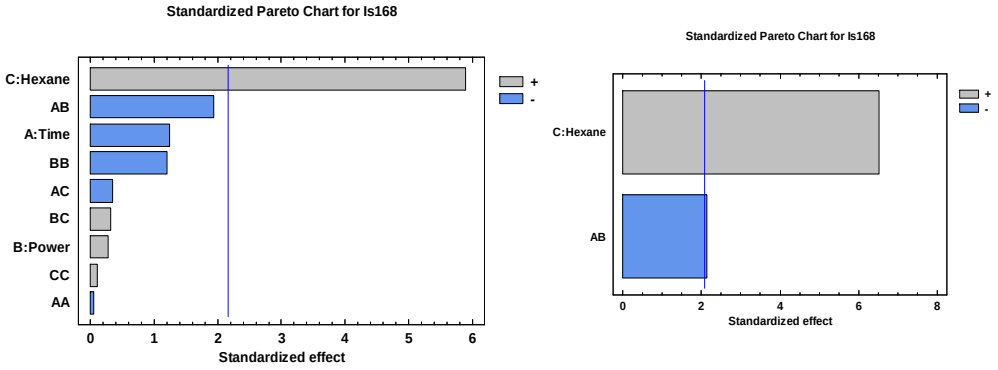


Figure S5. Desirability function for FUSLE conditions.

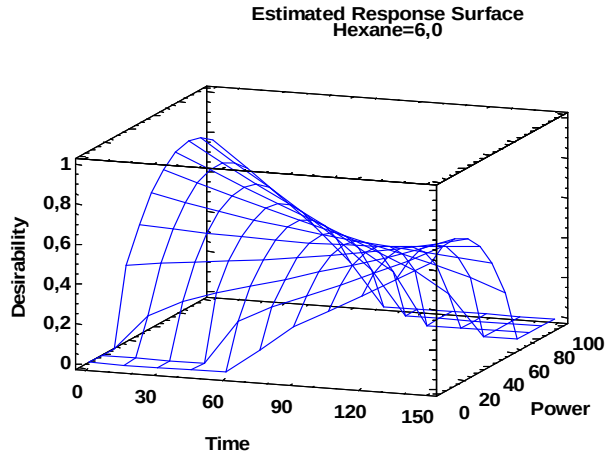


Figure S6. Influence of the number of FUSLE cycles.

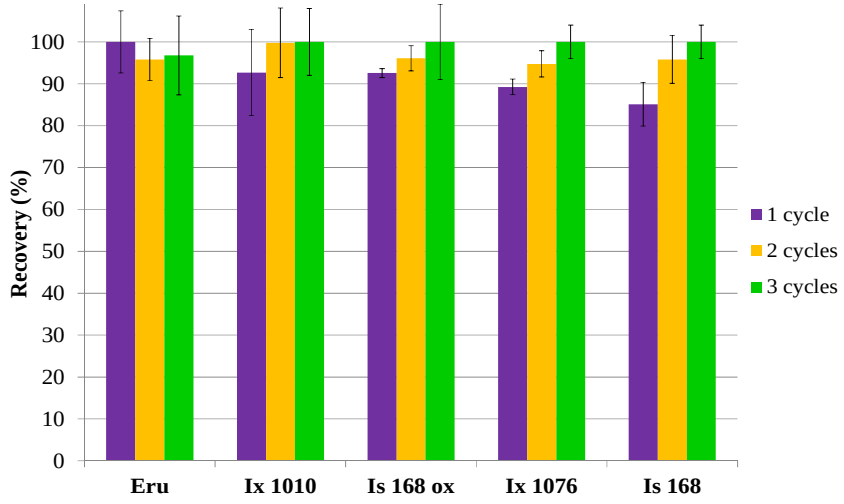


Figure S7. Pareto charts obtained from the ANOVA of the CCD of additive extraction by FUSLE using hexane-DCM mixtures.

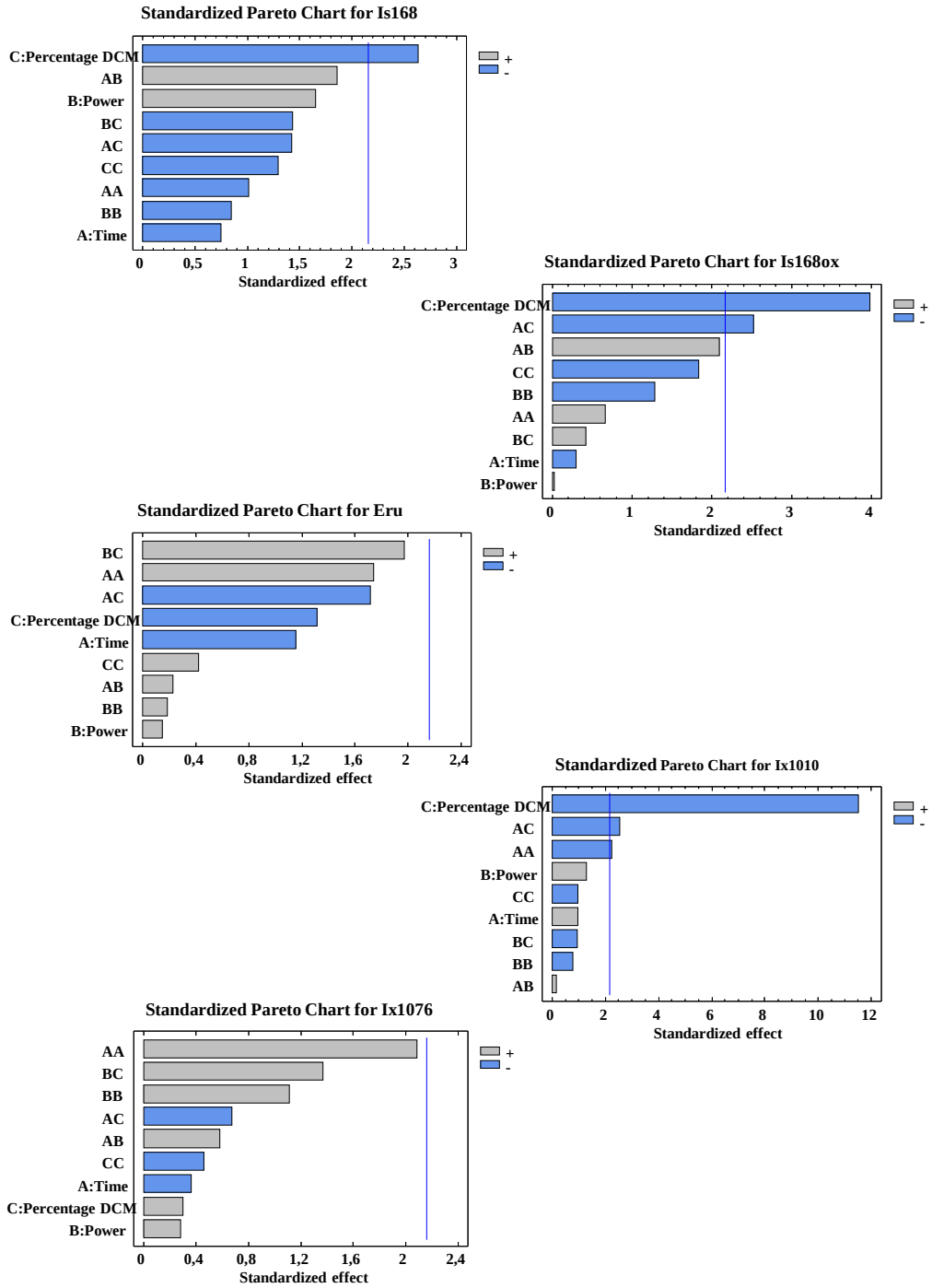
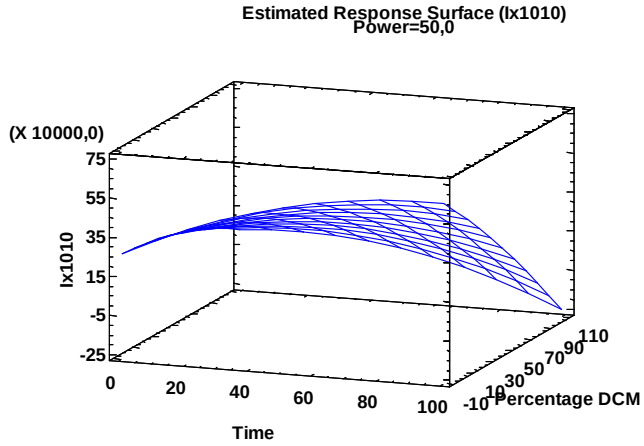
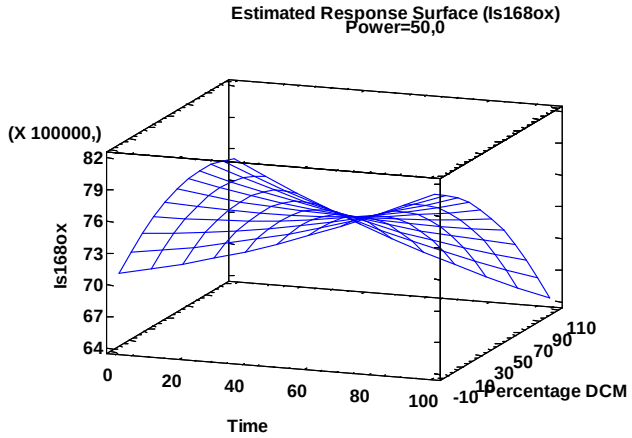


Figure S8. Response surfaces obtained for the FUSLE extraction of additives using hexane-DCM mixtures.





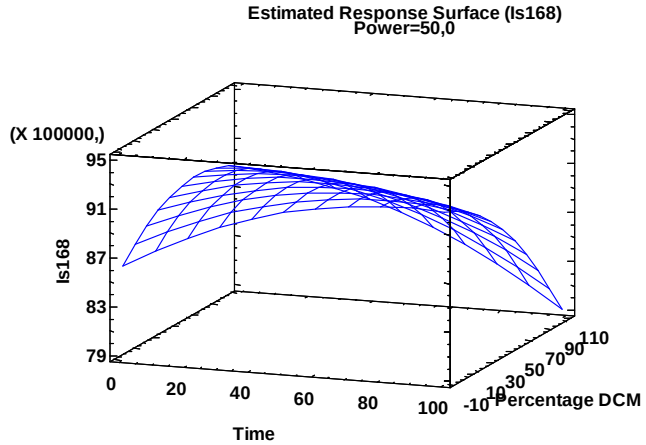
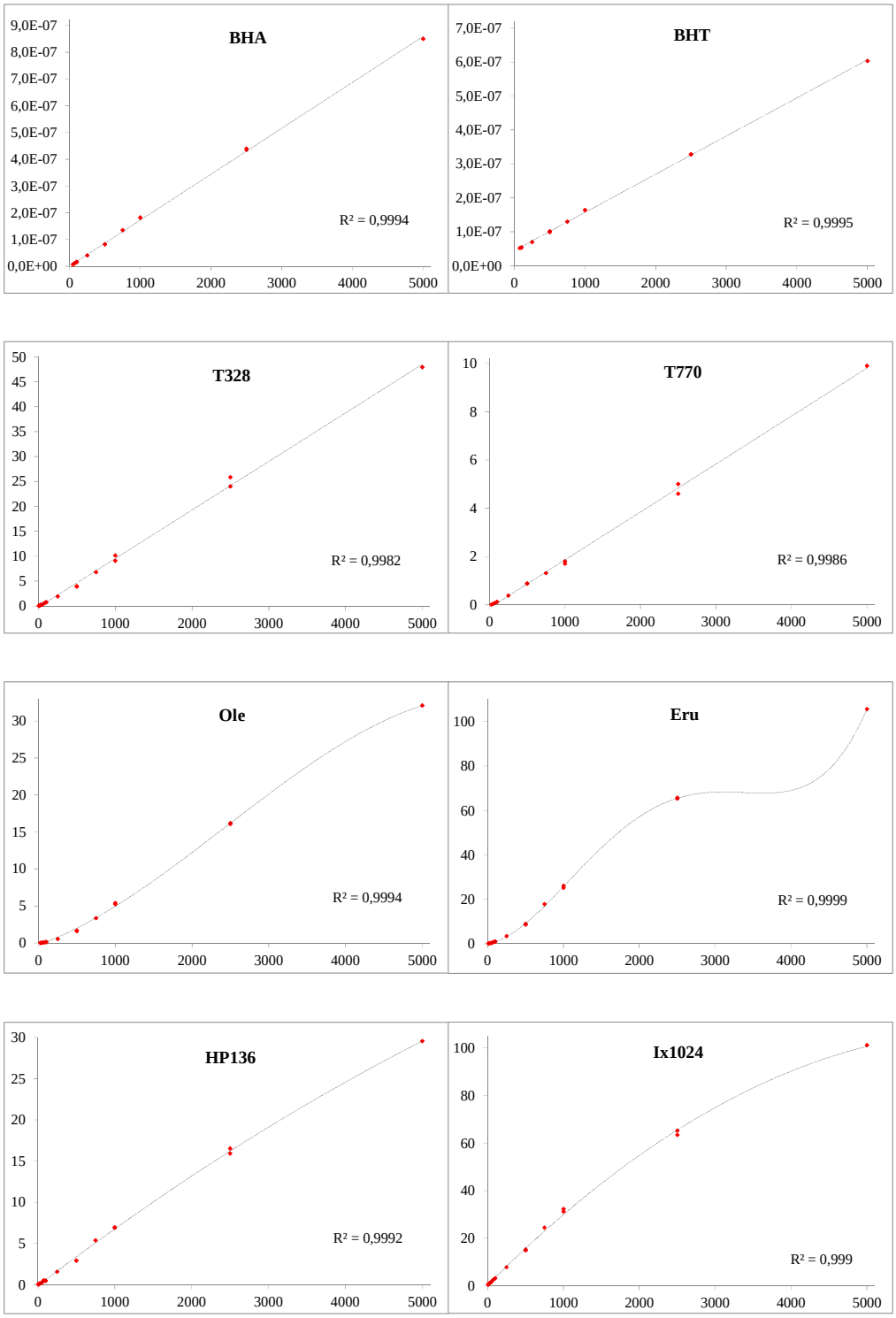
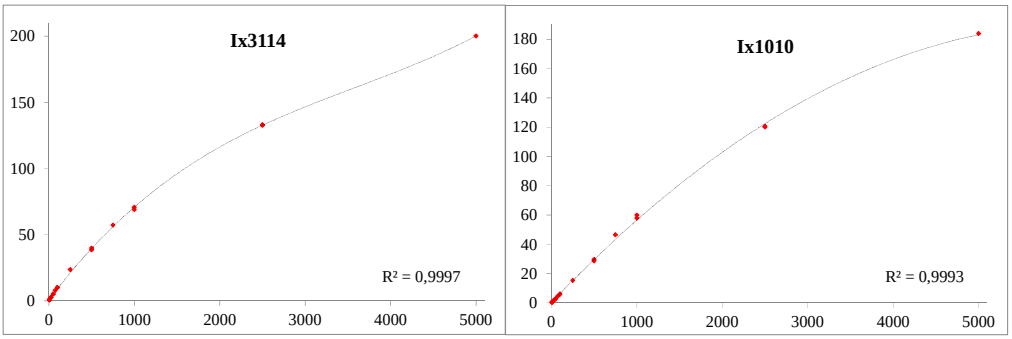
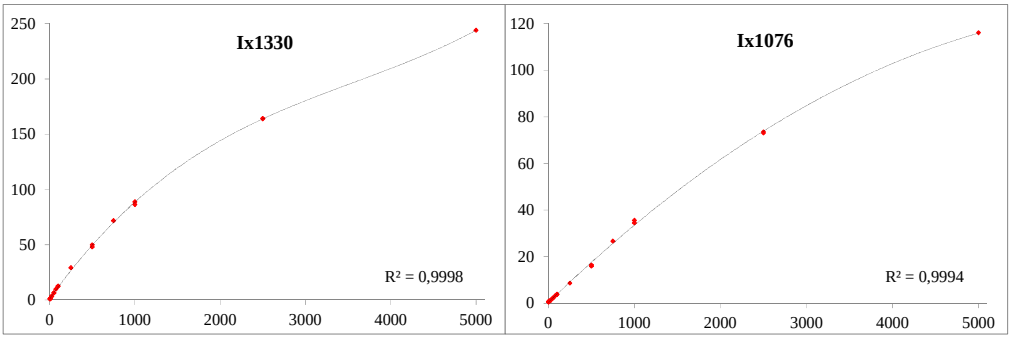
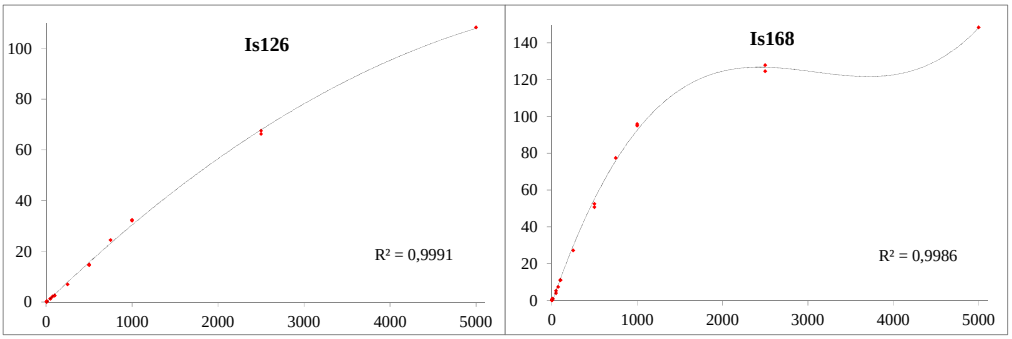


Fig. S9. Calibration curves from LOQ to 5000 ppb for each additive in MeOH.




Ix3114
R² = 0,9997
Ix1010
R² = 0,9993


Ix1330
R² = 0,9998
Ix1076
R² = 0,9994


Is126
R² = 0,9991
Is168
R² = 0,9986

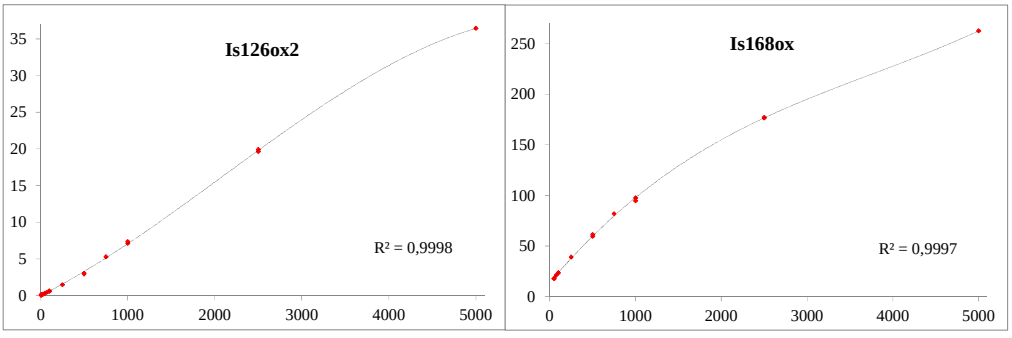
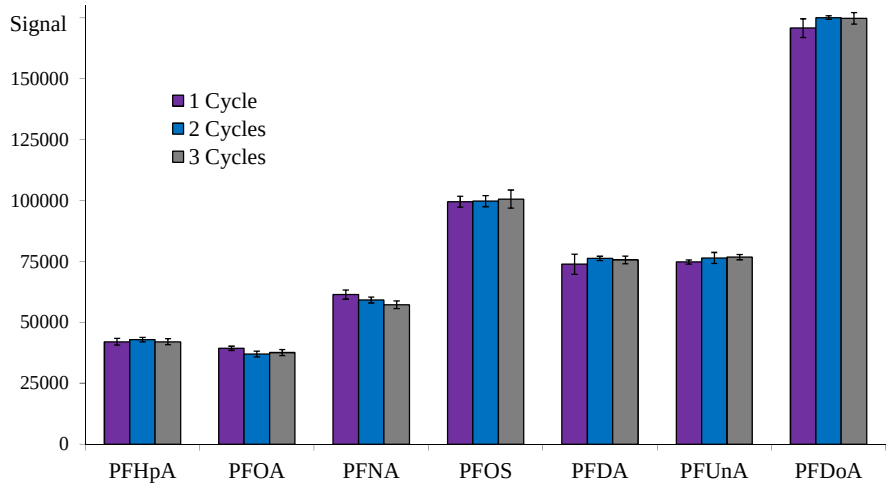

Is126ox2
R² = 0,9998
Is168ox
R² = 0,9997

Table. S1. p-Values of ANOVA of the Central Composite Design (CCD) results.

	A	B	C	AA	AB	AC	BB	BC	CC
PFHpA	0.0474*	0.8787	0.8531	0.8934	0.7721	0.6285	0.2848	0.9410	0.5511
PFOA	0.9468	0.9621	0.0675	0.1214	0.1899	0.9353	0.0717	0.4166	0.4048
PFNA	0.7316	0.9607	0.4721	0.1525	0.1244	0.9791	0.4924	0.5138	0.8651
PFOS	0.2310	0.5536	0.4274	0.7990	0.1000	0.3926	0.7142	0.1783	0.9201
PFDA	0.6022	0.3551	0.1727	0.6324	0.1857	0.3805	0.3411	0.7183	0.7006
PFUnA	0.3748	0.9015	0.2266	0.1862	0.0926	0.6590	0.9009	0.7467	0.7475
PFDoA	0.1992	0.8212	0.4995	0.3183	0.2021	0.3917	0.9483	0.6297	0.8430

A: Extraction time
B: Ultrasonic irradiation power
C: Solvent volume
* Significant statistical: p-valor < 0.05

Figure S1. Influence of the number of FUSLE cycles.



CHAPTER 4.2

Fast determination of perfluorocompounds in packaging by focused ultrasound solid-liquid extraction and liquid chromatography coupled to quadrupole-time of flight mass spectrometry

Table S1. Analyte retention times, quantification ions, collision energy and summation ratio values.

Compounds	Retention time (min)	Quantification ions (m/z)		Collision Energy (eV)	Summation ratio
		MS	MRM		
PFBA	0.44	212.98	168.99	8	5000
PFPeA	0.69	262.97	218.99	8	3750
MPFPeA[a]	0.69	265.98	221.99	8	3750
PFHxA	1.01	312.97	268.98	8	5000
PFHpA	1.38	362.97	318.98	10	5000
PFOA	1.75	412.97	368.98	12	3750
MPFOA[b]	1.75	420.97	375.98	12	3750
PFNA	2.16	462.96	418.97	14	5000
PFOS	2.41	498.93	498.93	8	3750
MPFOS[c]	2.41	506.93	506.93	8	3750
PFDA	2.80	512.96	468.97	10	5000
PFUnA	3.42	562.96	518.97	12	5000
PFDoA	3.75	612.95	568.96	14	3750
MPFDoA[d]	3.75	614.95	569.96	14	3750

[a] Internal standards for PFBA and PFPeA.
[b] Internal standards for PFHxA, PFHpA, PFOA, PFNA, PFDA and PFUnA.
[c] Internal standard for PFOS.
[d] Internal standard for PFDoA

Table S2. Matrix effect of microwave popcorn packaging when no internal standard is used.

	Error of the regression slope (%)			
	0.25 g	**0.50 g**	**1.00 g**	**1.50 g**
PFBA	n.s.	-18	-17	-16
PFPeA	n.s.	-13	-16	-14
PFHxA	n.s.	n.s.	n.s.	n.s.
PFHpA	n.s.	n.s.	n.s.	n.s.
PFOA	n.s.	n.s.	n.s.	n.s.
PFNA	n.s.	n.s.	n.s.	n.s.
PFOS	28	19	24	25
PFDA	n.s.	n.s.	-10	-11
PFUnA	n.s.	-22	-40	-39
PFDoA	21	22	14	16

n.s.: not statistically significant.

Table S3. Matrix effect of corn and popcorn when no internal standard is used.

	Error of the regression slope (%)	
	Corn	**Popcorn**
PFBA	n.s.	-21
PFPeA	n.s.	-21
PFHxA	n.s.	18
PFHpA	n.s.	10
PFOA	n.s.	n.s.
PFNA	23	13
PFOS	n.s.	n.s.
PFDA	n.s.	n.s.
PFUnA	n.s.	n.s.
PFDoA	26	22

n.s.: not statistically significant.

CHAPTER 5.1

Parabens and their derivatives in pharmaceuticals

Figure S.1. Chemical structures of parabens and their derivatives (target chemicals analyzed in this study).

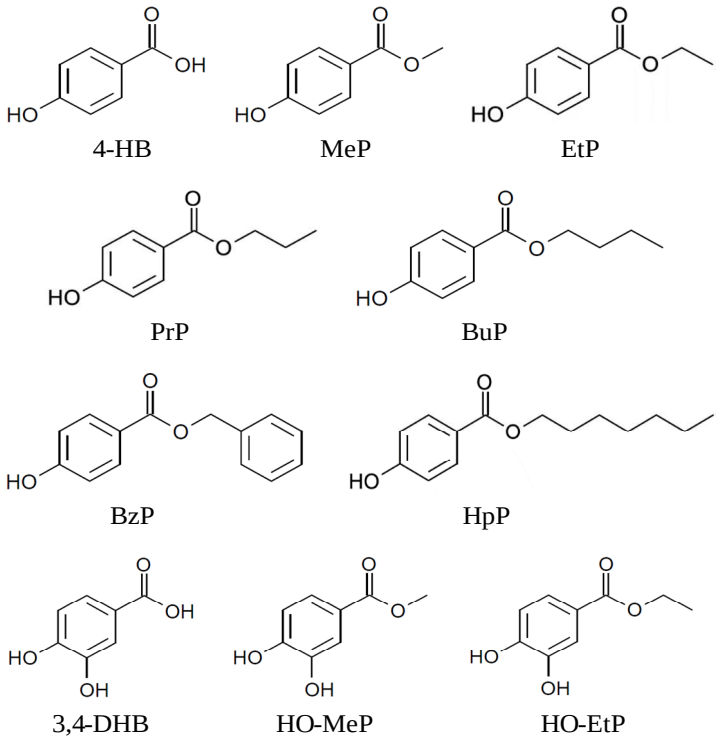


Figure S.2. Loss of the functional groups of target analytes in the collision cell of triple quadrupole mass spectrometer.

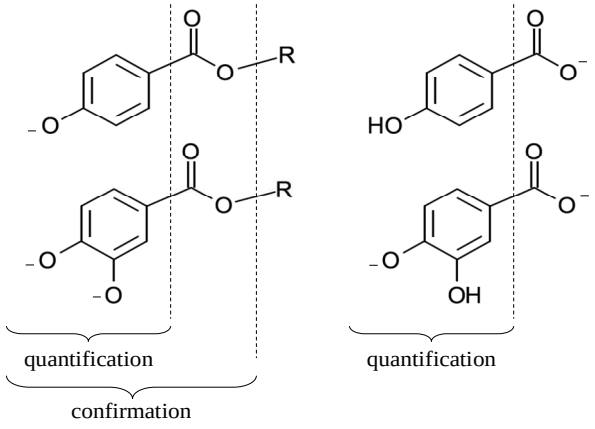


Figure S.3. Chromatograms of a) a 150 ppb methanolic standard solution of parabens b) a liquid pharmaceutical sample extracted by LLE c) a solid pharmaceutical sample extracted and cleaned by SLE-SPE. Peak identification: 1) 3,4-DHB, 2) 4-HB, 3) OH-MeP, 4) OH-EtP, 5) MeP , 6) EtP , 7) PrP , 8) BzP, 9) BuP and 10) HpP.

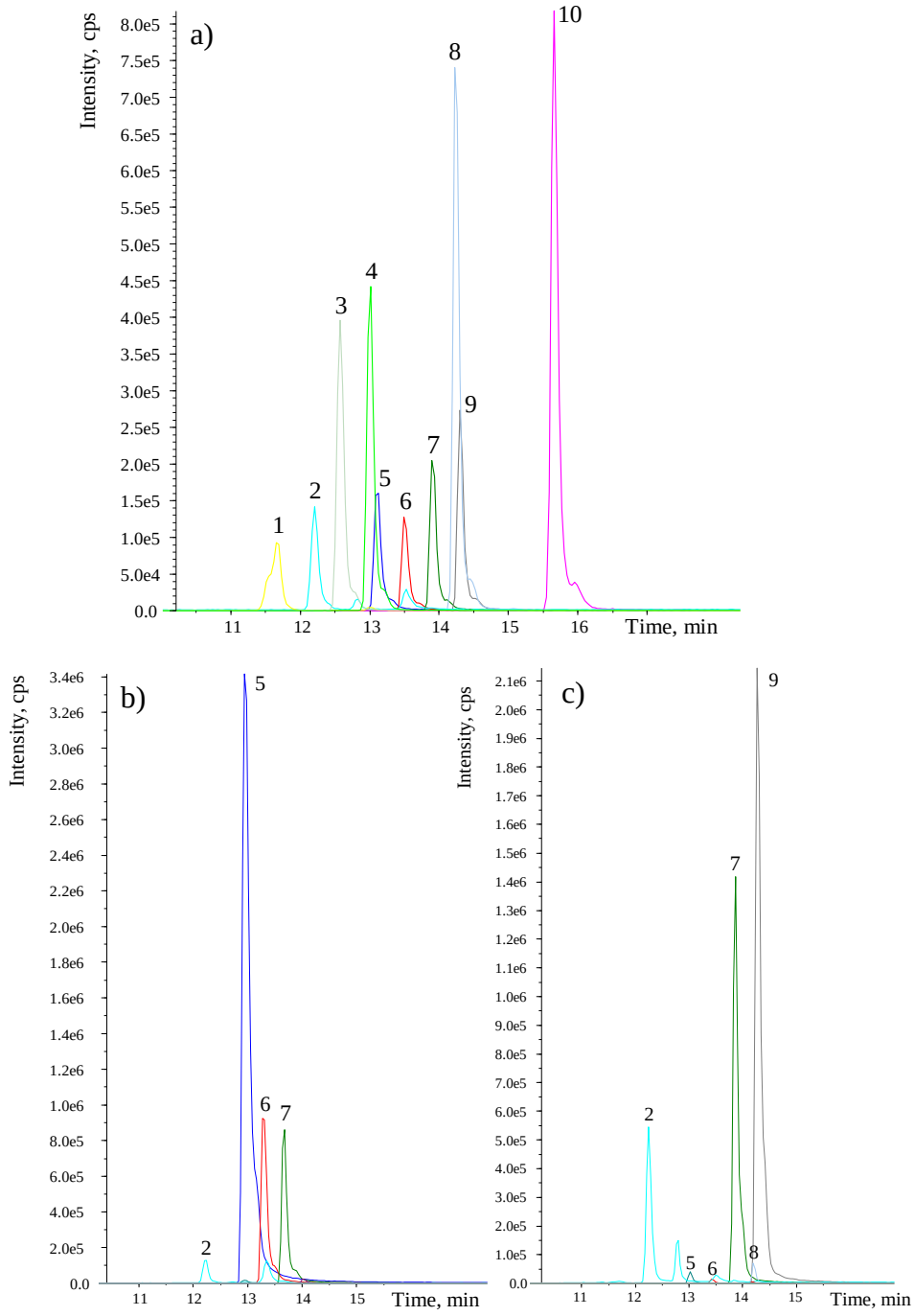


Figure S.4. Number of extraction cycles for parabens in pharmaceuticals and extraction efficiency (%).

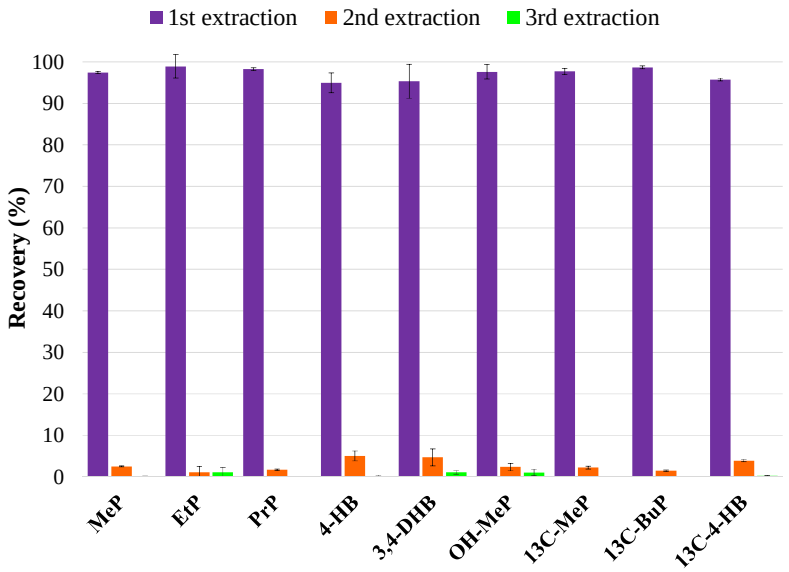


Table S.1. Transitions and MS/MS parameters optimized for analysis of the six parabens, the four derivatives and the four internal standards in pharmaceuticals.

Analytes	Q_1 (amu)	Q_3 (amu)	$Q_{3'}$.(amu)	DP (V)	FP (V)	EP (V)	CEP (V)	CE (eV)	CXP (V)
3,4-DHB	153	109	-	-18	-330	-6	-10	-21	-8
4-HB	137	93	-	-13	-300	-6	-10	-18	-6
OH-MeP	167	108	152	-20	-350	-8	-10	-27	-6
OH-EtP	181	108	152	-21	-380	-10	-10	-27	-7
MeP	151	92	136	-18	-370	-10	-9	-27	-7
EtP	165	92	136	-25	-350	-7	-12	-25	-6
PrP	179	92	136	-25	-390	-8	-18	-25	-6
BuP	193	92	136	-19	-350	-10	-17	-29	-7
BzP	227	92	136	-17	-300	-7	-10	-30	-7
HpP	235	92	136	-22	-350	-8	-11	-35	-7
C^{13}/4-HB	143	99	-	-13	-300	-6	-10	-18	-6
C^{13}/MeP	157	98	-	-18	-370	-10	-9	-27	-7
C^{13}/BuP	199	98	-	-19	-350	-10	-17	-29	-7

Q_1: Parent ion; Q_3: Product ions for quantification; $Q_{3'}$: Product ions for confirmation; DP: Declustering potential (to form ions); FP: Focusing potential (to form ions); EP: Entrance potential (to focus and accelerate ions to Q1); CEP: Collision cell entrance potential (to focus and accelerate ions to Q2); CE: Collision energy (to broke parent ions); CXP: Collision cell exit potential (to focus and accelerate the ions to Q3).

Table S2. Percentage of internal standard peak area in methanolic extract of a solid sample (pain/fever reliever) relative to internal standard peak area in MeOH (N=3).

Internal Standard	No clean-up	LLE	HLB	NH_2
^{13}C-MeP	3.0 ± 0.3	2.5 ± 0.3	26.3 ± 2.1	8.0 ± 1.1
^{13}C-BuP	12.3 ± 1.3	14 ± 3	39 ± 3	26.4 ± 1.6
^{13}C-4HB	4.2 ± 0.3	4.1 ± 0.2	18.3 ± 1.4	13.2 ± 1.6

LLE: liquid solid extraction clean-up.

HLB: solid phase extraction clean-up employing Hydrophilic-lipophilic balance cartridges.

NH_2: solid phase extraction clean-up employing amino cartridges.

Table S3. Concentration of parabens (µg/g) in softgel pharmaceuticals collected from the USA.

USA (New York / New Jersey)		Active compound	Exp.	MeP	4-HB	PB	Total
Softgel (OTC)	***Dietary supplement***	Vitamin D3	08-16	0.16	0.14	0.16	0.30
		Fish Oil	01-16	-	-	-	-
		Vitamin E	08-17	-	-	-	-
		Extract Saw Palmetto	03-17	-	-	-	-
		Chromium Picolinate	03-17	-	-	-	-
		Beta Carotene	05-17	-	-	-	-
		Fish Oil	08-16	-	-	-	-
		Vitamin D2	05-15	-	-	-	-
	Pain / fever reliever	Naproxen Sodium	10-15	-	-	-	-
		Naproxen sodium	08-15				
	Cold / flu reliever	Acetaminophen / Dextromethorphan / Doxylamine succinate	07-14	-	-	-	-
		Acetaminophen / Dextromethorphan / Phenylephrine	02-16	-	-	-	-
		Acetaminophen / Dextromethorphan / Phenylephrine	02-15	-	-	-	-
		Acetaminophen / Dextromethorphan / Doxylamine succinate	05-14	-	-	-	-

"-": Not detected / Only MeP and 4-HB were detected and quantified in the analyzed softgel samples.

Table S4. Concentration of parabens (µg/g) in liquid pharmaceuticals collected from the USA.

New York / New Jersey		Active compound	Exp.	MeP	EtP	PrP	BuP	BzP	4-HB	3,4-DHB	OH-MeP	OH-EtP	PB	Total
Liquid (OTC)	***For skin***	Fluocinonide	04/09	0.05	-	0.07	-	-	-	-	-	-	0.12	0.12
		Antibiotic Ointment - Bacitracin / Neomycin / Polymyxin	05/10	-	-	-	-	-	-	-	-	-	-	-
		Fluocinonide	06/15	-	-	-	-	-	-	-	-	-	-	-
		Diphenhydramine / Zinc Acetate	04-15	**939**	-	**245**	-	-	2.52	-	-	-	**1185**	**1187**
		Hydrocortisone	11-13	**2000**	**394**	**295**	-	-	3.45	-	0.30	0.09	**2689**	**2693**
		Hydrocortisone	07-16	**1600**	-	**695**	-	-	2.78	-	0.14	-	**2295**	**2298**
		Vitamin A and D	06/07	-	-	-	-	-	-	-	-	-	-	-
		White petrolatum USP	02/10	-	-	-	-	-	-	-	-	-	-	-
	Pain / fever reliever	Acetaminophen / Dextromethorphan / Doxylamine succinate	03/14	-	-	-	-	-	-	-	-	-	-	-
		Ibuprofen / acetaminophen	04/16	-	-	-	-	-	-	-	-	-	-	-
		Benzocaine	11/14	0.07	-	-	0.04	-	-	-	-	-	0.11	0.11
	Antifungal / antibacterial	Benzalkonium / Lidocaine	02/13	0.14	-	0.16	-	-	-	-	-	-	0.3	0.3
		Nystatin	11/14	**593**	0.43	**128**	-	-	**22.7**	-	-	-	**722**	**745**
		Clotrimazole 1%	08/06	0.15	-	-	-	-	-	-	-	-	0.15	0.15
		Terbinafine Hydrochloride	10/09	-	-	-	-	-	-	-	-	-	-	-
		Clotrimazole/ betamethasone dipropionate	06/13	-	-	-	-	-	-	-	-	-	-	-

"-": Not detected / HpP was not detected in any liquid or cream sample.

Table S4. Cont. Concentration of parabens (µg/g) in liquid pharmaceuticals collected from the USA.

New York / New Jersey		Active compound	Exp.	MeP	EtP	PrP	BuP	BzP	4-HB	3,4-DHB	OH-MeP	OH-EtP	PB	Total
Liquid (OTC)	***Cold / flu reliever***	Acetaminophen / Dextromethorphan / Doxylamine succinate	10/03	-	-	-	-	-	-	-	-	-	-	-
		-	11/16	**1160**	-	**314**	-	-	2.78	-	-	-	**1474**	**1476**
		Fluticasone Propionate Nasal	12/14	-	-	-	-	-	-	-	-	-	-	-
		Dextromethorphan	06/16	**1056**	-	**169**	-	-	**29.8**	-	-	-	**1224**	**1254**
		Acetaminophen / Dextromethorphan / Doxylamine succinate	08/15	-	-	-	-	-	-	-	-	-	-	-
		Acetaminophen / Dextromethorphan / Doxylamine succinate	04/15	-	-	-	-	-	-	-	-	-	-	-
		Acetaminophen / Dextromethorphan / Doxylamine succinate	06/11	-	-	-	-	-	-	-	0.05	0.07	-	0.12
		Natural ingredients (honey…)	01/15	-	-	-	-	-	3.04	1.48	-	-	-	4.52
		Acetaminophen / Dextromethorphan / Doxylamine succinate	04/15	-	-	-	-	-	-	-	-	-	-	-
	Digestive disorder reliever	Aluminum Hydroxide / Magnesium Hydroxide / Simethicone	06/10	0.19	-	**45.4**	**48.0**		**128**	-	-	-	**93.6**	**222**
		Aluminum hydroxide / Magnesium hydroxide / Simethicone	01/14	0.18	1.01	**82.2**	**140**	1.16	**42.3**	-	-	-	**224**	**267**
		Bismuth Subsalicylate / Salicylate	09/04	-	-	-	-	-	0.54	-	-	-	-	0.5
	For eyes	-	-	-	-	-	-	-	-	-	-	-	-	-
		Light mineral oil / Mineral oil	06/12	0.88	0.43	0.61	0.07	-	0.75	-	-	-	2.00	2.75
	Allergy reliever	Ketotifen	02/04	0.83	-	0.24	-	-	-	-	-	-	1.08	1.08
	Hair loss treatment	Minoxidil.	02/06	-	-	-	-	-	-	-	-	-	-	-

"-": Not detected / HpP was not detected in any liquid or cream sample.

Table S5. Concentration of parabens (µg/g) in solid prescription pharmaceuticals collected from the USA.

USA (New York / New Jersey)		Active compound	Exp.	MeP	PrP	4-HB	PB	Total
Solid (Prescription)	*High cholesterol treatment*	Atorvastatin	02/14	-	-	-	-	-
		Rosuvastatin calcium	04/13	-	-	-	-	-
		Niacin	12/12	-	-	-	-	-
	Blood/heart	Propranolol	06/15	-	-	0.57	-	0.57
		Amlodipine besylate	12/13	-	-	-	-	-
		Clopidogrel	03/15	-	-	-	-	-
		Carvedilol	01/14	-	-	-	-	-
		Cyclobenzaprine	10/14	-	-	-	-	-
	Erectile dysfunction treatment	Sildenafil	01/13	-	-	-	-	-
		Vardenafil	04/12	-	-	-	-	-
	Hypertension treatment	Amlodipine besylate	06/15	-	-	-	-	-
	Glucocorticoids	Prednisone	01/08	1.10	0.16	-	1.25	1.25
	Muscle relaxant	Cyclobenzaprine Hydrochloride	03/15	-	-	-	-	-
	To stop smoking	Varenicline tartrate	12/14	-	-	-	-	-
	Hypotiroidism treatment	Levothyroxine	06/15	-	-	-	-	-
	Arthritis treatment	Acetaminophen / Hydrocodone	2010	-	-	-	-	-
	Antibiotic	Amoxicillin	09/11	-	-	-	-	-

-: Not detected / Only MeP, PrP and 4-HB were detected and quantified in the analyzed prescription solid samples.

Table S6. Concentration of parabens (µg/g) in solid OTC pharmaceuticals collected from the USA.

New York / New Jersey		Active compound	Exp.	MeP	4-HB	3,4-DHB	OH-MeP	OH-EtP	PB	Total
Solid (OTC)	*Dietary supplement*	Natural Zinc	01/16	-	-	-	-	-	-	-
		Ca, Mg, Zn, Vitamin D3	02/16	-	-	-	-	-	-	-
		Psyllium Fiber	-	-	0.17	0.29	-	-	-	0.46
		Complete vitamin	03/15	-	-	-	-	-	-	-
		Selenium	11/16	0.53	2.71	1.36	0.29	-	0.53	4.89
		Vitamin C	05/17	-	-	0.39	-	-	-	0.39
		Vitamin C	08/14	-	-	0.31	0.04	-	-	0.35
		Seleno-Methionine	08/11	0.15	-	-	-	-	0.15	0.15
	Pain / fever reliever	Acetylsalicylic acid	07/15	-	-	-	-	-	-	-
		Acetaminophen	07/10	-	-	-	-	-	-	-
		Ibuprofen	12/14	-	-	-	-	-	-	-
		Acetylsalicylic acid	01/16	-	3.38	-	-	-	-	3.38
		Naproxen sodium	04/15	-	-	-	-	-	-	-
		Ibuprophen	10/14	-	-	-	-	0.12	-	0.12
		Ibuprophen	12/16	-	-	-	-	-	-	-
		Acetylsalicylic acid	09/13	0.39	0.98	-	-	-	0.39	1.37
		Naproxen sodium	11/16	-	-	-	-	0.09	-	0.09
		Acetylsalicylic acid	11/11	0.43	0.99	-	-	-	0.43	1.42
	Antifungal / antibacterial	Undecylenate	-	-	0.46	-	0.22	-	-	0.69

"-": Not detected / EtP, PrP, BuP, BzP and HpP were not detected in any solid OTC sample.

Table S6. Cont. Concentration of parabens (µg/g) in solid OTC pharmaceuticals collected from the USA.

New York / New Jersey		Active compound	Exp.	MeP	4-HB	3,4-DHB	OH-MeP	OH-EtP	PB	Total
Solid (OTC)	*Cold / flu reliever*	Pseudoephedrine	02/17	-	-	-	-	-	-	-
		Benzocaine	05/13	-	-	-	0.07	0.12	-	0.19
		Guaifenesin / Dextromethorphan	09/15	-	-	-	-	-	-	-
		Acetaminophen / Diphenhydramine / Pseudoephedrine	10/01	-	-	-	-	-	-	-
		Acetaminophen / Chlorpheniramine maleate / Dextromethorphan	09/10	-	-	-	-	-	-	-
		Diphenhydramine	02/10	-	-	-	-	-	-	-
		Pseudoephedrine	12/16	-	-	-	-	-	-	-
		Acetaminophen / Diphenhydramine	04/15	-	-	-	-	-	-	-
		Aspirin / Chlorpheniramine maleate / Dextromethorphan / Phenylephrine bitartrate	04/13	-	0.88	-	-	-	-	0.88
		Guaifenesin	08/14	-	-	-	-	-	-	-
	Digestive disorder reliever	Omeprazole	11/11	-	-	-	-	-	-	-
		Sodium / Lactase Enzyme	02-11	-	-	-	-	-	-	-
		Famotidine	04-09	-	-	-	-	-	-	-
		Lansoprazole	04-14	-	-	-	-	-	-	-
		Polyethylene Glycol 3350	06-14	-	-	-	-	-	-	-
		Aspirin / Citric acid / Sodium bicarbonate	12-16	-	-	-	-	-	-	-
		Bismuth subsalicylate	01-13	0.18	1.98	-	-	-	0.18	2.16
		Betaine	06-11	-	-	-	-	-	-	-
	Allergy reliever	Loratadine	04-15	-	-	-	-	-	-	-
		Loratadine	05-14	-	-	-	-	-	-	-
		Diphenhydramine hydrochloride	11-14	-	-	-	-	-	-	-
		Diphenhydramine hydrochloride	-	-	-	-	-	-	-	-

"-": Not detected / EtP, PrP, BuP, BzP and HpP were not detected in any solid OTC sample.

Table S7. Concentration of parabens (µg/g) in solid pharmaceuticals collected from Asia and Europe.

State	Origin	Availability	Purpose	Active compound	Exp.	MeP	EtP	PrP	4-HB	3,4-DHB	OH-MeP	OH-EtP	PB	Total
Solid	India	OTC	*Pain/fever*	Paracetamol	02/15	808	216	121	23.3	-	-	-	1145	1168
				Chlorpheniramine maleate / Phenylephrine hydrochloride	-	1123	0.80	233	11.45	-	0.31	-	1357	1369
		Prescription	*Antibiotic*	Amoxicillin	-	422	-	99.6	-	-	-	-	521	521
				Azithromycin	06-13	-	-	-	0.27	-	-	0.10	-	0.37
				Amoxicillin	11-13	0.42	-	0.22	-	-	-	-	0.63	0.63
	China	OTC	*Cold / flu reliever*	Acetaminophen / Chlorpheniramine Maleate	01-13	-	-	-	5.53	18.4	-	-	-	23.9
				-	05-14	-	-	-	6.75	16.8	-	-	-	23.6
				-	02-14	-	-	-	3.95	4.12	-	-	-	8.07
			Pain / fever reliever	-	05-15	-	-	-	-	-	-	-	-	-
			Digestive disorder reliever	-	10-12	0.18	-	-	20.2	152	-	-	-	172
			Vertigo calming	-	04-14	-	-	-	50.0	152	-	-	-	202
		Prescription	*To sleep at night*	Estazolam	06-17	-	-	-	0.32	-	-	-	-	0.32
	Japan	OTC	*Cold / flu reliever*	-	04-16	0.22	-	-	-	-	-	-	-	0.22
			Digestive disorder reliever	-	09-18	-	-	-	4.28	1.51	-	-	-	5.80
			Pain / fever reliever	-	08-17	-	-	-	-	-	-	-	-	-

"-": Not detectable / BuP, BzP and HpP were not detected in any sample from Asia and Europe.

Table S7. Cont. Concentration of parabens (µg/g) in solid pharmaceuticals collected from Asia and Europe.

State	Origin	Availability	Purpose	Active compound	Exp.	MeP	EtP	PrP	4-HB	3,4-DHB	OH-MeP	OH-EtP	PB	Total
Solid	Spain	OTC	Digestive disorder reliever	Loperamida	09-15	-	-	-	-	-	-	-	-	-
				Cetrizina dihidrocloruro	10-13	-	-	-	0.46	-	-	-	-	0.46
			Pain / fever reliever	Ibuprofeno	01-17	-	-	-	0.27	-	-	-	-	0.27
			For skin	Dimetilideno maletao	03-16	-	-	-	-	-	-	-	-	-
		Prescription	Depression treatment	Triptizol	11-16	-	-	-	-	-	-	-	-	-
	Poland	OTC	Pain / fever reliever	Sodium metamizol	05-17	-	-	-	-	-	-	-	-	-
			Dietary supplement	Calcium / vitamin C	07-16	-	-	-	-	-	-	-	-	-
	Italy	OTC	Vertigo calming	Dimenidrato	11-18	-	-	-	-	-	-	-	-	-
			Digestive disorder reliever	Probiotics lactic ferments / Vitamins B6, B12	04-16	-	-	-	0.28	-	-	-	-	0.28

"-": Not detectable / BuP, BzP and HpP were not detected in any sample from Asia and Europe.