Avances analíticos para la mejora de la información sobre seguridad de aceites vegetales y otros productos alimentarios vegetales de alto contenido graso

Memoria presentada por José Luis Hidalgo Ruiz, para optar al grado de Doctor por la Universidad de Almería en el Programa de Doctorado en Química Avanzada con Mención Internacional

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TESIS DOCTORAL

Avances analíticos para la mejora de la información sobre seguridad de aceites vegetales y otros productos alimentarios vegetales de alto contenido graso

Analytical advances for the improvement of information on the safety of vegetable oils and other high-fat vegetable food products

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OBJETIVOS

El objetivo general de esta Tesis, que se realizó en el marco del proyecto "Avances analíticos para la mejora de la información sobre calidad y seguridad de aceites vegetales y otros productos alimentarios vegetales de alto contenido graso" (Referencia RTC-2017-6170-2) es el desarrollo, optimización y validación de métodos fiables para el análisis de residuos y contaminantes de interés en seguridad alimentaria y su control en aceites vegetales, frutos secos y alimentos procesados. Para ello se emplearon diferentes técnicas como la cromatografía de líquidos de (ultra) alta resolución (*(ultra)-high performance liquid chromatography*, (U)HPLC) y la cromatografía de gases (*gas chromatography*, GC) acopladas a espectrometría de masas en tándem (*tandem mass spectrometry*, MS/MS) y detector de ionización de llama (*flame ionization detector*, FID), respectivamente.

Para lograr el objetivo general, se llevaron a cabo las siguientes tareas:

1. Revisión bibliográfica de la presencia de contaminantes en aceites comestibles y semillas oleaginosas y su determinación en los últimos años (Publicación I).

2. Desarrollo y validación de métodos de extracción basados en QuEChERS (acrónimo de sus características en inglés: *Quick, Easy, Cheap, Effective, Rugged and Safe,* Rápida, Fácil, Barata, Efectiva, Robusta y Segura) y un método analítico por UHPLC-MS/MS para la determinación de micotoxinas en aceites vegetales (Publicación II) y frutos secos (Publicación III).

3. Desarrollo y validación de métodos de extracción basados en extracción líquido-líquido (*liquid-liquid extraction*, LLE) para aceites y extracción sólido-líquido (*solid-liquid extraction*, SLE) para alimentos

procesados, aplicando una etapa de limpieza basada en la extracción dispersiva en fase sólida (*dispersive solid phase extraction*, d-SPE) y posterior análisis mediante UHPLC-MS/MS para la determinación directa de ésteres de 3-monocloropropanodiol (*3-monochloropropanediol*, 3-MCPD) y ésteres de glicidilo en aceites vegetales y alimentos procesados (Publicación IV).

4. Desarrollo y validación de métodos de extracción, basados en LLE y QuPPe (*Quick Method for the Analysis of Numerous Highly Polar Pesticides,* Método rápido para el análisis de numerosos plaguicidas altamente polares), y posterior análisis de plaguicidas polares en aceites vegetales y frutos secos mediante HPLC-MS/MS (Publicación V).

5. Desarrollo y validación de un método basado en extracción en fase sólida (*solid phase extraction*, SPE) y GC-FID para la determinación de hidrocarburos de aceite mineral (*mineral oil hydrocarbons*, MOH) en aceites vegetales (Publicación VI).

Los métodos desarrollados se aplicaron a un amplio número de matrices y muestras como aceites de oliva y orujo, incluyendo aceites refinados, girasol, soja y maíz (Publicaciones II, IV, V y VI), frutos secos como almendras, avellanas, cacahuetes, pistachos y nueces (Publicaciones III y V), y alimentos procesados como margarina, galletas y croissants (Publicación IV), mostrándose en todos los casos la aplicabilidad de los métodos desarrollados.

OBJECTIVES

The general objective of this Thesis, which was performed in the framework of the project "Analytical advances for the improvement of information on the quality and safety of vegetable oils and other high-fat vegetable food products" (Reference RTC-2017-6170-2) is the development, optimization and validation of reliable methods for the analysis of significant residues and contaminants in food safety, and their control in vegetable oils, nuts and processed foods. For that purpose, different techniques such as (ultra)-high performance liquid chromatography ((U)HPLC) and gas chromatography (GC) coupled to tandem mass spectrometry (MS/MS) and flame ionization detector (FID), respectively, were employed.

To achieve the general objective, the following tasks were carried out:

1. Bibliographic review of the occurrence of contaminants in edible oils and oilseeds and their determination in recent years (Publication I).
2. Development and validation of extraction methods based on QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) and an analytical method by UHPLC-MS/MS for the determination of mycotoxins in vegetable oils (Publication II) and nuts (Publication III).
3. Development and validation of extraction methods based on liquid-liquid extraction (LLE) for oils and solid-liquid extraction (SLE) for processed foods with dispersive solid phase extraction (d-SPE) clean-up and an analytical method by UHPLC-MS/MS for the direct determination of 3-monochloropropanediol (3-MCPD) esters and glycidyl esters in vegetable oils and processed foods (Publication IV).
4. Development and validation of extraction methods, based on LLE and QuPPe (Quick method for the analysis of numerous highly polar

pesticides), and HPLC-MS/MS determination of polar pesticides in vegetable oils and nuts (Publication V).

5. Development and validation of a method based on solid phase extraction (SPE) and GC-FID for the determination of mineral oil hydrocarbons (MOH) in vegetable oils (Publication VI).

The developed methods were applied to a wide number of matrices and samples such as olive and pomace oils, including refined, sunflower, soy and corn oils (Publications II, IV, V and VI), nuts such as almonds, hazelnuts, peanuts, pistachios and walnuts (Publications III and V), and processed food such as margarine, biscuits and croissants (Publication IV) showing in all cases the applicability of the developed methods.

RESUMEN

Teniendo en cuenta la creciente preocupación por la seguridad alimentaria, y la normativa vigente que establece los límites que no se pueden superar para determinados compuestos en productos alimenticios, en esta Tesis se desarrollaron métodos analíticos reproducibles que permiten la extracción y análisis de contaminantes orgánicos, como las micotoxinas, ésteres de 3-MCPD y ésteres de glicidilo y plaguicidas polares en aceites vegetales y otras matrices grasas mediante cromatografía de líquidos de (ultra) alta resolución (*(ultra)-high performance liquid chromatography*, (U)HPLC) junto con espectrometría de masas en tándem (*tandem mass spectrometry*, MS/MS), e hidrocarburos de aceite mineral (*mineral oil hydrocarbons*, MOH) por cromatografía de gases con detector de ionización de llama (*gas chromatography with flame ionization detector*, GC-FID).

Para ello, se optimizaron diferentes métodos, principalmente basados en técnicas cromatográficas acopladas a analizadores de espectrometría de masas para la determinación de diferentes contaminantes y residuos en aceites vegetales y matrices relacionadas. Estos métodos fueron validados para verificar su robustez, sensibilidad y fiabilidad. Además, se aplicaron a un amplio número de muestras para comprobar su aplicabilidad y aportar información relevante sobre la presencia de estos compuestos en las muestras analizadas.

En primer lugar, se llevó a cabo una revisión exhaustiva de la presencia de contaminantes y los métodos analíticos más recientes para su determinación en aceites y semillas oleaginosas. A partir de esta información, se llevó a cabo la optimización de las condiciones de extracción, cromatográficas y espectrométricas para detectar niveles bajos de concentración de los compuestos objeto de estudio. Finalmente, se

realizó la evaluación de los métodos desarrollados y se aplicaron a diferentes tipos de muestras como aceites, frutos secos y alimentos procesados. Por tanto, los estudios incluidos en esta Tesis son:

1. Revisión bibliográfica de la presencia de contaminantes en aceites comestibles y semillas oleaginosas y su determinación en los últimos años (entre 2010 y 2018). Esta revisión discute métodos de extracción, métodos de limpieza, así como técnicas cromatográficas y de detección más utilizados en este ámbito.

2. Desarrollo y validación de métodos analíticos para la determinación de micotoxinas en aceites vegetales y frutos secos por UHPLC-MS/MS. Para ello, se desarrollaron métodos de extracción basados en QuEChERS (*Quick, Easy, Cheap, Effective, Rugged and Safe,* Rápida, Fácil, Barata, Efectiva, Robusta y Segura). Estos métodos se aplicaron a 194 muestras de aceite vegetal y 36 muestras de frutos secos.

3. Desarrollo y validación de un método analítico para la determinación directa de ésteres de 3-monocloropropanodiol (*3-monochloropropanediol*, 3-MCPD) y ésteres de glicidilo en aceites vegetales y alimentos procesados, basado en extracción líquido-líquido (*liquid-liquid extraction*, LLE) para aceites y extracción sólido-líquido (*solid-liquid extraction,* SLE) para alimentos procesados con limpieza mediante extracción dispersiva en fase sólida (*dispersive solid phase extraction*, d-SPE) y análisis por UHPLC-MS/MS. Este método se aplicó a 20 aceites vegetales y 4 alimentos procesados.

4. Desarrollo y validación de métodos analíticos para la determinación de plaguicidas polares en aceites vegetales y frutos secos por HPLC-MS/MS. Los métodos de extracción se basaron en LLE para aceites vegetales y QuPPe (*Quick Method for the Analysis of Numerous Highly Polar Pesticides,* Método rápido para el análisis de numerosos plaguicidas altamente polares) para frutos secos. Se analizaron un

total de 20 muestras de aceites vegetales y 20 muestras de frutos secos.

5. Desarrollo y validación de un método basado en extracción en fase sólida (*solid phase extraction*, SPE) y GC-FID para la determinación de MOH en aceites vegetales. Se estudiaron un total de 6 muestras previamente analizadas mediante técnicas más complejas en laboratorios acreditados con el fin de comprobar la fiabilidad del método.

ABSTRACT

Taking into account the growing concern for food safety, and the current regulations that establish the limits that cannot be exceeded for certain compounds in food commodities, this Thesis aims to develop reproducible analytical methods for the extraction and analysis of organic contaminants, such as mycotoxins, 3-monochloropropanediol (3-MCPD) esters and glycidyl esters and polar pesticides in vegetable oils and other fatty matrices by (ultra)-high performance liquid chromatography ((U)HPLC) coupled with tandem mass spectrometry (MS/MS), and mineral oil hydrocarbons (MOH) by gas chromatography with flame ionization detector (GC-FID).

Therefore, different methods were developed, mainly based on chromatographic techniques coupled with mass spectrometry analyzers for the determination of different contaminants and residues in vegetable oils and related matrices. These methods were validated to verify their robustness, sensitivity, and reliability. Furthermore, they were applied to a wide number of samples to check their applicability and provide valuable information regarding the presence of these compounds in the analyzed samples.

Firstly, a comprehensive review of the occurrence of contaminants and the most recent analytical methods for their determination in oils and oilseeds was carried out. Consequently, the optimization of extraction, chromatographic and spectrometric conditions was carried out to detect low concentration levels. Finally, the evaluation of the developed methods was performed, and they were applied to different kind of samples such as oils, nuts and processed food. Therefore, the studies included in this Thesis are:

1. Bibliographic review of the occurrence of contaminants in edible oils

and oilseeds and their determination in recent years (between 2010 and 2018). This review includes extraction methods, cleaning methods as well as chromatographic and detection techniques mostly used to detect contaminants and residues in oil and related samples.

2. Development and validation of analytical methods for the determination of mycotoxins in vegetable oils and nuts by UHPLC-MS/MS. For that purpose, extraction methods based on QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) were developed. These methods were applied to 194 vegetable oil samples and 36 samples of nuts.

3. Development and validation of an analytical method for the direct determination of 3-monochloropropanediol (3-MCPD) esters and glycidyl esters in vegetable oils, and processed foods, based on liquid-liquid extraction (LLE) for oils and solid-liquid extraction (SLE) for processed foods with dispersive solid phase extraction (d-SPE) clean-up and analysis by UHPLC-MS/MS. This method was applied to 20 vegetable oils and 4 processed foods.

4. Development and validation of analytical methods for the determination of polar pesticides in vegetable oils and nuts by HPLC-MS/MS. The extraction methods were based on LLE for vegetable oils, and QuPPe (Quick Method for the Analysis of Numerous Highly Polar Pesticides) for nuts. A total of 20 samples of vegetable oils and 20 samples of nuts were analyzed.

5. Development and validation of a method based on solid phase extraction (SPE) and GC-FID for the determination of MOH in vegetable oils. A total of 6 samples previously analyzed by more complex techniques in accredited laboratories were studied in order to check the reliability of the method.

Lista de publicaciones incluidas en la Tesis

A continuación se muestra la lista de publicaciones derivadas de la presente Tesis:

I. Occurrence and determination of contaminants in edible oils and oilseeds.

Hidalgo-Ruiz, J. L.; Romero-González, R.; Martínez Vidal, J. L.; Garrido Frenich, A. *Oil and Oilseed Processing: Opportunities and Challenges*. Ed. Wiley Blackwell. **2021**, 149-181.

II. A rapid method for determination of mycotoxins in edible vegetable oils by ultra-high performance liquid chromatography-tandem mass spectrometry.

Hidalgo-Ruiz, J. L.; Romero-González, R.; Martínez Vidal, J. L.; Garrido Frenich, A. *Food Chemistry*. **2019**, *288*, 22–28.

III. Determination of mycotoxins in nuts by ultra high-performance liquid chromatography-tandem mass spectrometry: Looking for a representative matrix.

Hidalgo-Ruiz, J. L.; Romero-González, R.; Martínez Vidal, J. L.; Garrido Frenich, A. *Journal of Food Composition and Analysis*. **2019**, *82* (March), 103228.

IV. Determination of 3-monochloropropanediol esters and glycidyl esters in fatty matrices by ultra-high performance liquid chromatography-tandem mass spectrometry.

Hidalgo-Ruiz, J. L.; Romero-González, R.; Martínez Vidal, J. L.; Garrido Frenich, A. *Journal of Chromatography A*. **2021**, *1639*, 461940.

V. Monitoring of polar pesticides and contaminants in edible oils and nuts by liquid chromatography-tandem mass spectrometry.

Hidalgo-Ruiz, J. L.; Romero-González, R.; Martínez Vidal, J. L.; Garrido Frenich, A. *Food Chemistry*. **2021**, *343*, 128495.

VI. Off-line solid phase extraction and separation of mineral oil saturated hydrocarbons and mineral oil aromatic hydrocarbons in edible oils, and analysis via GC with flame ionization detector.

Hidalgo-Ruiz, J. L.; Arrebola-Liébanas, J.; Romero-González, R.; Martínez Vidal, J. L.; Garrido Frenich, A. *Journal of Separation Science*. (Submitted for publication).

Contribución del autor en cada publicación

Publicación I → El autor participó en la búsqueda bibliográfica y la redacción del manuscrito.

Publicaciones II, III, IV, V y VI → El autor participó en el diseño de los experimentos, la realización de la parte experimental y la redacción de los manuscritos.

Abreviaturas y acrónimos

1-MN	1-Metilnaftaleno (*1-Methylnaphthalene*)
2,4-D	Ácido 2,4-diclorofenoxiacético (*2,4-Dichlorophenoxiacetic acid*)
2-MCPD	2-Monocloropropanodiol (*2-Monochloropropanediol*)
2-MN	2-Metilnaftaleno (*2-Methylnaphthalene*)
2-Ol	2-Oleoil-3-cloropropanodiol (*2-Oleoyl-3-chloropropanediol*)
2-Pa	2-Palmitoil-3-cloropropanodiol (*2-Palmitoyl-3-chloropropanediol*)
2-Pa-Ol	2-Palmitoil-1-oleoil-3-cloropropanodiol (*2-Palmitoyl-1-oleoyl-3-chloropropanediol*)
3-MBPD	3-Monobromopropanodiol (*3-Monobromopropanediol*)
3-MCPD	3-Monocloropropanodiol (*3-Monochloropropanediol*)
5B	Pentilbenceno (*Pentylbenzene*)
AALLME	Microextracción líquido-líquido asistida por aire (*Air-Assisted Liquid-Liquid Microextraction*)
AFB1	Aflatoxina B1 (*Aflatoxin B1*)
AFB2	Aflatoxina B2 (*Aflatoxin B2*)
AFG1	Aflatoxina G1 (*Aflatoxin G1*)
AFG2	Aflatoxina G2 (*Aflatoxin G2*)
AFM1	Aflatoxina M1 (*Aflatoxin M1*)
AMPA	Ácido Aminometilfosfónico (A*minomethylphosphonic acid*)
AOCS	Sociedad Estadounidense de Químicos del Aceite (*American Oil Chemists' Society*)
ASGM	Micropartículas de amino-sílica gel (*Amino-Silica Gel Microparticles*)
BEA	Beauvericina (*Beauvericin*)

BfR Instituto Federal de Evaluación de Riesgos de Alemania (*Bundesinstitut für Risikobewertunk*)

BSTFA N,O-bis (trimetilsilil) trifluoroacetamida (*N,O-bis (trimethylsilyl) trifluoroacetamide*)

C11 *n*-Undecano (*n-Undecane*)

C13 *n*-Tridecano (*n-Tridecane*)

C18 Octadecasilano (*Octadecasilane*)

C8 Dimetil *n*-octilsilano (D*imethyl n-octylsilane*)

CHO Colestano (*Cholestane*)

CIT Citrinina (*Citrinin*)

CyCy Biciclohexil (*Bicyclohexyl*)

DGF Sociedad Alemana de la Ciencia de la Grasa (*Deutsche Gesellschaft Für Fettwissenschaft (German Society for Fat Science)*)

DLLME Microextracción líquido-líquido dispersiva (*Dispersive Liquid-Liquid Microextraction*)

DMPS Dimetil polisiloxano (*Dimethyl Polysiloxane*)

DMSO Dimetil sulfóxido (*Dimethyl Sulfoxide*)

d-SPE Extracción dispersiva en fase sólida (*dispersive Solid Phase Extraction*)

ECD Detector de captura electrónica (*Electron Capture Detector*)

EDTA Ácido etilendiaminotetraacético (*ethylenediaminetetraacetic Acid*)

ELISA Ensayo por inmunoabsorción ligado a enzimas (*Enzyme-Linked ImmunoSorbent Assay*)

EMR Eliminación de matriz mejorada (*Enhanced Matrix Removal*)

ENA Enniatina A (*Enniatin A*)

ENB Enniatina B (*Enniatin B*)

ENB1 Enniatina B1 (*Enniatin B1*)

ESI-	Ionización por electronebulización en modo negativo (*Electrospray Ionization in negative mode*)
ESI+	Ionización por electronebulización en modo positivo (*Electrospray Ionization in positive mode*)
EVOO	Aceite de oliva virgen extra (*Extra Virgin Olive Oil*)
FAO	Organización de las Naciones Unidas para la Alimentación y la Agricultura (*Food and Agriculture Organization*)
FB1	Fumonisina B1 (*Fumonisin B1*)
FDA	Administración Estadounidense de Alimentos y Medicamentos (*U.S. Food and Drug Administration*)
FID	Detector de ionización con llama (*Flame Ionization Detector*)
FLD	Detector de fluorescencia (*Fluorescence Detector)*
FMOC-Cl	Cloroformiato de 9-fluorenilmetilo (*9-Fluorenylmethyl chloroformate*)
FPS	Sílica totalmente porosa (*Fully Porous Silica*)
GC	Cromatografía de gases (*Gas Chromatography)*
GCB	Carbono grafitizado negro (*Graphitized Carbon Black*)
GE	Éster glicidílico (*Glydidyl Ester*)
GPC	Cromatografía por permeación en gel (*Gel Permeation Chromatography*)
HFBA	Anhídrido heptafluorobutírico (*heptafluorobutyric anhydride*)
HFBI	Heptafluorobutirilimidazol (*heptafluorobutyrylimidazole*)
HILIC	Cromatografía de interacción hidrofílica (*Hydrophilic Interaction Chromatography*)
HPSFC	Cromatografía de fluidos supercríticos de alta resolución (*High Performance Supercritical Fluid Chromatography*)
HRMS	Espectrometría de masas de alta resolución (*High Resolution Mass Spectrometry*)
HS-SPME	Microextracción en fase sólida de espacio en cabeza

	(*Head Space Solid Phase Microextraction*)
IAC	Cromatografía de inmunoafinidad (*Immunoaffinity Chromatography*)
JRC	Centro común de investigación (*Joint Research Centre*)
La-GE	Laurato de glicidilo (*Glycidyl Laurate*)
Lau	1-Lauroil-3-cloropropanodiol (*1-Lauroyl-3-chloropropanediol*)
Lau-GEd5	Laurato de glicidilo-d5 (*Glycidyl Laurate-d5*)
LC	Cromatografía de líquidos (*Liquid Chromatography*)
Lin	1-Linoleoil-3-cloropropanodiol (*1-Linoleoyl-3-chloropropanediol*)
Lin-GE	Linoleato de glicidilo (*Glycidyl Linoleate*)
Lin-Lin	1,2-Dilinoleoil-3-cloropropanodiol (*1,2-Dilinoleoyl-3-chloropropanediol*)
Lin-St	1-Linoleoil-2-estearoil-3-cloropropanodiol (*1-Linoleoyl-2-stearoyl-3-chloropropanediol*)
LLE	Extracción líquido-líquido (*Liquid-Liquid Extraction*)
LLME	Microextracción líquido-líquido (*Liquid-Liquid Microextraction*)
Lnn	1-Linolenoil-3-cloropropanodiol (*1-Linolenoyl-3-chloropropanediol*)
Lnn-GE	Linolenato de glicidilo (*Glycidyl Linolenate*)
LOQ	Límite de cuantificación (*Limit Of Quantification*)
LVI	Inyección de grandes volúmenes (*Large Volume Injection*)
m/z	Relación masa/carga (*Mass to charge ratio*)
MAE	Extracción asistida con microondas (*Microwave Assisted Extraction*)
MCPA	Ácido 4-cloro-2-toliloxiacético (*4-Chloro-2-tolyloxyacetic acid*)
***m*CPBA**	Ácido meta-cloroperbenzoico (*meta-Chloroperbenzoic acid*)

MCPD Monocloropropanodioles (*Monochloropropanodiols*)

MIP Polímeros impresos molecularmente (Molecularly Imprinted Polymers)

MOAH Hidrocarburos aromáticos de aceites minerales (*Mineral Oil Aromatic Hydrocarbons*)

MOHs Hidrocarburos de aceites minerales (*Mineral Oil Hydrocarbons*)

MOSH Hidrocarburos alifáticos de aceites minerales (*Mineral Oil Saturated Hydrocarbons*)

MPS Metil Polisiloxano (*Methyl Polysiloxane*)

MRL Límite máximo de residuo (*Maximum Residue Limit*)

MRM Monitorización de reacciones múltiples (*Multiple Reaction Monitoring*)

MS Espectrometría de masas (*Mass Spectrometry*)

MS/MS Espectrometría de masas en tándem (*Tandem Mass Spectrometry*)

MSPE Extracción en fase sólida magnética (*Magnetic Solid Phase Extraction*)

My 1-Miristoil-3-cloropropanodiol (1-*Myristoyl-3-chloropropanediol*)

My-GE Miristato de glicidilo (*Glycidyl Myristate*)

NP Fase normal (*Normal Phase*)

NP-RP Fase normal-fase reversa (*Normal Phase-Reverse Phase*)

Ol 1-Oleoil-3-cloropropanodiol (*1-Oleoyl-3-chloropropanediol*)

Old5 1-Oleoil-3-cloropropanodiol-d5 (*1-Oleoyl-3-chloropropanediol-d5*)

Ol-GE Oleato de glicidilo (*Glycidyl Oleate*)

Ol-GEd5 Oleato de glicidilo-d5 (*Glycidyl Oleate-d5*)

Ol-Lin 1-Oleoil-2-linoleoil-3-cloropropanodiol (*1-Oleoyl-2-linoleoyl-3-chloropropanediol*)

Ol-Lnn 1-Oleoil-2-linolenoil-3-cloropropanodiol (*1-Oleoyl-2-linolenoyl-3-chloropropanediol*)

Ol-Ol 1,2-Dioleoil-3-cloropropanodiol (*1,2-Dioleoyl-3-chloropropanediol*)

Ol-Old5 1,2-Dioleoil-3-cloropropanodiol-d5 (*1,2-Dioleoyl-3-chloropropanediol-d5*)

Ol-St 1-Oleoil-2-estearoil-3-cloropropanodiol (*1-Oleoyl-2-stearoyl-3-chloropropanediol*)

OPO Aceite de orujo de oliva (*Olive Pomace Oil*)

OTA Ocratoxina A (*Ochratoxin A*)

OTB Ocratoxina B (*Ochratoxin B*)

Pa 1-Palmitoil-3-cloropropanodiol (*1-Palmitoyl-3-chloropropanediol*)

Pa-GE Palmitato de glicidilo (*Glycidyl Palmitate*)

PAH Hidrocarburos aromáticos policíclicos (*Polyciclic Aromatic Hydrocarbons*)

Pa-Lin 1-Palmitoil-2-linoleoil-3-cloropropanodiol (*1-Palmitoyl-2-linoleoyl-3-chloropropanediol*)

Pa-Pa 1,2-Bis-palmitoil-3-cloropropanodiol (*1,2-Bis-palmitoyl-3-chloropropanediol*)

Pa-St 1-Palmitoil-2-estearoil-3-cloropropanodiol (*1-Palmitoyl-2-stearoyl-3-chloropropanediol*)

PBA Ácido fenilborónico (*Phenylboronic Acid*)

PCD Derivatización post-columna (*Post-Column Derivatization*)

PER Perileno (*Perylene*)

PGC Carbono grafítico poroso (*Porous Graphitic Carbon*)

PGR Regulador del crecimiento de la planta (*Plant Growth Regulator*)

PLE Extracción con líquidos presurizados (*Pressurized Liquid Extraction*)

PMPS Fenilmetilpolisiloxano (*Phenylmethyl Polysiloxane*)

PSA Amina primaria secundaria (*Primary Secondary Amine*)

Q Detector de cuadrupolo (*Quadrupole detector*)

QqQ Detector de triple cuadrupolo (*Triple quadrupole detector*)

QTOF Analizador de cuadrupolo acoplado a tiempo de vuelo (*Quadrupole Time-Of-Flight analyzer*)

QTRAP Cuadrupolo con trampa de iones lineal (*Quadrupole-Linear Ion Trap*)

QuEChERS Rápido, Fácil, Barato, Efectivo, Robusto y Seguro (*Quick, Easy, Cheap, Effective, Rugged & Safe*)

QuPPe Método rápido para el análisis de numerosos pesticidas altamente polares (*Quick Method for the Analysis of Numerous Highly Polar Pesticides*)

RASFF Sistema de Alerta Rápida para Alimentos y Piensos (*Rapid Alert System for Food and Feed*)

rGO Óxido de grafeno reducido (*reduced Graphene Oxide*)

RNA Ácido ribonucleico (*Ribonucleic Acid*)

ROO Aceite de oliva refinado (*Refined Olive Oil*)

ROPO Aceite de orujo de oliva refinado (*Refined Olive Pomace Oil*)

RP Fase reversa (*Reverse Phase*)

RP-NP Fase reversa-fase normal (*Reverse Phase-Normal Phase*)

RSD Desviación estándar relativa (*Relative Standard Deviation*)

RTW Ventana de tiempo de retención (*Retention Time Window*)

SAX Intercambio aniónico fuerte (*Strong Anion Exchange*)

SC Citrato de sodio (*Sodium Citrate*)

SCDS Citrato de sodio dibásico sesquihidratado (*Sodium Citrate Dibasic Sesquihydrate*)

SCTD Citrato de sodio tribásico dihidratado (*Sodium Citrate Tribasic Dihydrate*)

SCX	Intercambio catiónico fuerte (*Strong Cation Exchange*)
SfO	Aceite de girasol (*Sunflower Oil*)
SIM	Monitorización de iones seleccionados (*Selected Ion Monitoring*)
St	1-Estearoil-3-cloropropanodiol (*Stearoyl-3-chloropropanediol*)
St-GE	Estearato de glicidilo (*Glycidyl Stearate*)
TBB	1,3,5-tri-tert-Butilbenceno (*1,3,5-tri-tert-Butylbenzene*)
TBME	Tert-butil metil éter (*Tert-Butyl Methyl Ether*)
TFA	Ácido trifluoroacético (*Trifluoroacetic Acid*)
THF	Tetrahidrofurano (*Tetrahydrofuran*)
TMCS	Trimetilclorosilano (*Trimethylchlorosilane*)
VOO	Aceite de oliva virgen (*Virgin Olive Oil*)
WHO	Organización Mundial de la Salud (*World Health Organization*)
ZEA	Zearalenona (*Zearalenone*)
Z-Sep	Dióxido de zirconio (*Zirconium dioxide*)
α-ZOL	α-Zearalenol (*α-Zearalenol*)

ÍNDICE

CAPÍTULO I

INTRODUCCIÓN

1. ANTECEDENTES

En los tiempos actuales, el ciudadano se preocupa cada vez más por la calidad y seguridad de los alimentos que consume, siendo cada vez mayor el número de notificaciones sobre problemas de seguridad alimentaria. En 2012, el Sistema de Alerta Rápida para Alimentos y Piensos (*Rapid Alert System for Food and Feed*, RASFF) recibió un total de 522 notificaciones, mientras que en 2019 se recibieron 4118, clasificándose como alertas 1175 [1]. En otras palabras, en solo 8 años se ha duplicado el número de alertas originales, lo cual da una perspectiva sobre la creciente preocupación por la presencia de contaminantes en alimentos. De estas 4118 notificaciones, 38 pertenecen a aceites y grasas, 173 a cereales y productos de panadería, mientras que 672 pertenecen a semillas y frutos secos, lo cual significa más del 20% de las notificaciones totales. Además, 534 de esas notificaciones corresponden a micotoxinas, 253 a residuos de plaguicidas y 115 de ellas se refieren a posibles adulteraciones o fraudes [1].

En la actualidad, el aceite de oliva es el producto alimentario que más dinero genera en España ya que se producen de media 1,3 millones de toneladas por campaña [2], siendo el líder europeo en exportación de aceite. En cuanto a los frutos secos, en 2019 se produjeron en España 518.700 toneladas de almendras, castañas, nueces y avellanas [3]. Tanto el aceite de oliva virgen como los frutos secos son dos de los principales alimentos de la dieta mediterránea, la cual está relacionada con una serie de beneficios para la salud, que incluyen una reducción del riesgo de mortalidad y una menor incidencia de enfermedades cardiovasculares [4].

Por todos los argumentos esgrimidos es de interés avanzar en información analítica en términos de seguridad, bien soportada científicamente, para la caracterización de estos alimentos.

La contaminación de los alimentos puede producirse tanto en el cultivo de la materia prima, como en el propio alimento y dicha

contaminación puede provenir tanto de fuentes endógenas, cuando se produce dentro del propio alimento, o exógenas, cuando se le ha añadido algún componente ajeno a éste, que puede resultar perjudicial para la salud. Dentro de los compuestos endógenos se encuentran las micotoxinas o los ésteres de 3-monocloropropanodiol (*monochloropropanediol*, 3-MCPD), entre otros, mientras que dentro de los exógenos se pueden citar los contaminantes polares o los hidrocarburos de aceites minerales (*mineral oil hydrocarbons*, MOH).

Debido a que puede existir cierta confusión entre residuo y contaminante, aclarar que residuo se define como una sustancia exógena que no ha llegado a eliminarse por completo, mientras que contaminante es cualquier sustancia que tiene efectos no deseados y afecta negativamente a la calidad o utilidad de un alimento y puede ser tanto de origen endógeno o exógeno. En la presente Tesis, por simplicidad, se va a hablar de contaminantes.

2. CONTAMINANTES ENDÓGENOS

2.1 Micotoxinas

Las micotoxinas son metabolitos secundarios producidos por ciertas especies de hongos durante su proceso de digestión [5]. Éstas afectan a animales vertebrados, pero también a bacterias, como por ejemplo la penicilina, o a plantas [6].

Se pueden clasificar en 6 grupos principales: aflatoxinas, ocratoxina, citrinina, alcaloides ergóticos, patulina y las toxinas *Fusarium*, mostrándose algunos ejemplos en la Figura 1.

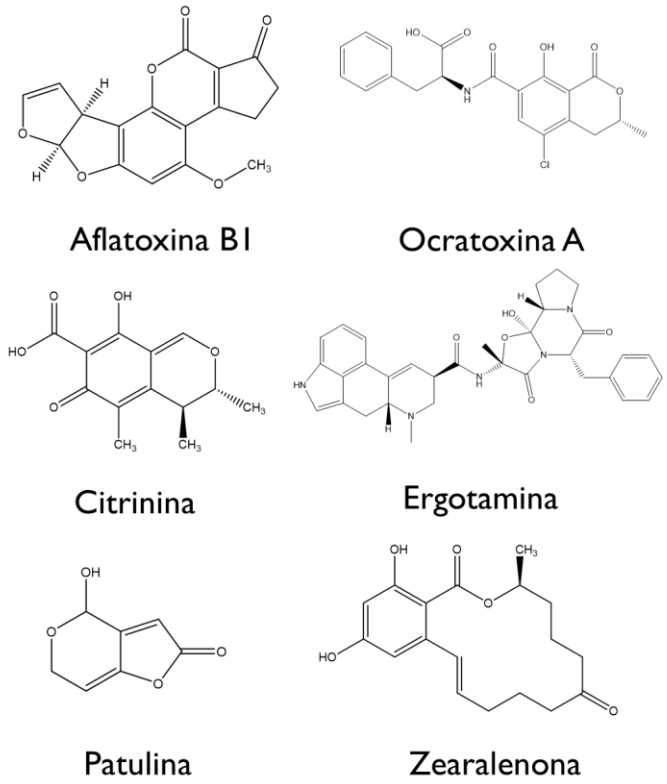

Figura1. Estructura de distintas micotoxinas.

- Las aflatoxinas son producidas por la especies *Aspergillus* como pueden ser *A. flavus* y *A. parasiticus* [7]. El término aflatoxina se debe a 4 de ellas, que son B1, B2, G1 y G2 [8]. De éstas, la aflatoxina B1 se considera la más tóxica, siendo un potente carcinógeno [7]. Están ampliamente asociadas a la contaminación de productos producidos en los trópicos y áreas subtrópicales, como algodón, cacahuetes, especias, pistachos y maíz [7,8].

- La ocratoxina puede aparecer en tres formas, A, B o C, y todas ellas son producidas por las especies *Penicillium* y *Aspergillus*. La ocratoxina A es la forma de las que proceden la B y C, ya que la ocratoxina B es una forma no clorada de la anterior, y la C se

forma por la adición de éster etílico a la ocratoxina A [9]. Se ha encontrado que especies como *A. ochraceus* y *A. carbonarius* contaminan bebidas, como vino y cerveza, o uvas, respectivamente [10]. La ocratoxina A ha sido clasificada como nefrotoxina y carcinógena, estando ligada a tumores en el tracto urinario humano [9,10].

- La citrinina fue inicialmente aislada del hongo *Penicillium citrinum,* aunque más tarde se ha identificado en más de una docena de especies de *Penicillium* y algunas de *Aspergillus*. Determinadas especies se usan para producir alimentos como queso (*Penicillium camemberti*), sake, miso o salsa de soja (*Aspergillus oryzae*). Esta micotoxina se asocia a la enfermedad del arroz amarillento en Japón, y actua como nefrotoxina [6]. Además, puede presentar un efecto sinérgico con la ocratoxina A para reducir la síntesis de ácido ribonucleico (r*ibonucleic acid*, RNA) en riñones de murinos. Aunque se ha relacionado con diversos alimentos, su efecto en la salud humana aún es desconocido [6].

- Los alcaloides ergóticos son compuestos producidos en los esclerocios de especies de *Claviceps*, los cuales son patógenos de especies herbáceas. La ingesta de esclerocios ergóticos de cereales infectados causa ergotismo, ya sea gangrenoso, el cual afecta al riego sanguíneo de las extremidades, o convulsivo, que afecta al sistema nervioso central. Mediante los métodos de limpieza de grano modernos se ha conseguido reducir significativamente el ergotismo como enfermedad humana, aunque sigue siendo un problema veterinario importante [6].

- La patulina es producida por las especies *Aspergillus, Penicillium,* y *Paecilomyces.* Entre ellas, *P. expansum* se asocia

con los mohos de las frutas y verduras, especialmente manzanas e higos [11,12], aunque es destruido durante la fermentación, por lo que no se encuentra en las bebidas de estas frutas, como la sidra. Se ha observado que patulina daña el sistema inmune en animales [11].

- Por último, las toxinas fusarium son producidas por alrededor de 50 especies de *Fusarium* e infectan cereales como trigo y maíz [13,14]. Entre ellas se encuentran un gran rango de micotoxinas, como son las fumonisinas, que afectan al sistema nervioso de caballos y pueden causar cáncer en roedores; los tricotecenos, los cuales se asocian con efectos crónicos en animales y humanos; y zearalenona, que interfiere con la función reproductiva de mamíferos y puede causar inmunosupresión [15]. Otras toxinas fusarium son beauvercina y enniatinas, butenólido, equisetina y fusarinas [16].

Algunas micotoxinas han sido clasificadas como carcinógenos de grupo 1 por la Organización Mundial de la Salud (*World Health Organization*, WHO) [17]. De los cientos de micotoxinas que se han llegado a identificar, las aflatoxinas, citrinina, fumonisinas, ocratoxina A, patulina, tricotecenos, zearalenona o alcaloides ergóticos se encuentran entre las más importantes para la salud humana debido a su toxicidad [6].

En esta Tesis se ha desarrollado un método para el análisis de las aflatoxinas B1, B2, G1 y G2, así como zearalenona y uno de sus metabolitos, α-zearalenol, ya que, como se ha comentado en el párrafo anterior, son algunas de las más tóxicas para el ser humano.

2.1.1 Contaminación en alimentos

Algunas micotoxinas pueden detectarse en cereales como maíz, trigo, cebada, avena o arroz, y en frutos secos o aceitunas [16,18]. De hecho, se ha demostrado que se transfieren entre un 18 y un 47% de las micotoxinas presentes en las aceitunas al aceite de oliva prensado [19].

Se conocen 24 especies tóxicas de *Fusarium* para la salud animal y humana. Por ejemplo, *Fusarium graminearum* produce las enfermedades fusariosis y podredumbre en trigo y maíz, respectivamente, ya que dicho hongo produce varias micotoxinas, entre las que se incluyen deoxinivalenol, zearalenona y fusarina [5]. Por otro lado, *Fusarium sporotrichioides* produce toxina T-2, la cual es la responsable de la mayor toxicosis en Europa del Este conocida, que ocurrió en Rusia entre 1942 y 1948 y afectó al 10% de la población total [20].

Las aflatoxinas afectan normalmente a los cultivos como los cereales, semillas, especias y frutos secos [17].

Determinadas condiciones ambientales, como altas temperaturas y humedad relativa y lluvia, aumentan las posibilidades de que se produzcan este tipo de sustancias, ya que favorecen la proliferación micótica [21,22]. La Organización de las Naciones Unidas para la Alimentación y la Agricultura (*Food and Agriculture Organization*, FAO) estima que más del 25% de los productos agrícolas están contaminados con micotoxinas, debido a la facilidad con que se producen [23]. Por ejemplo, en 2004 murieron en Kenia 125 personas y más de 200 enfermaron debido al consumo de maíz contaminado con estas sustancias [24].

2.1.2 Legislación

En cuanto a los aceites vegetales, el Comité de Expertos en Aditivos Alimentarios conjunto FAO/WHO (*Joint FAO/WHO Expert Committee on*

Food Additives, JECFA) evaluó los riesgos de consumir micotoxinas a través de la alimentación. A raíz de esa evaluación, la Comisión Europea estableció un límite de 400 µg/kg de zearalenona en aceite de maíz refinado [25,26]. Sin embargo, no hay ningún otro límite establecido para alguna otra micotoxina [27].

Respecto a frutos secos, la Comisión Europea estableció un límite de 15 y 10 µg/kg de aflatoxinas totales y un máximo de 8 y 5 µg/kg de aflatoxina B1 en cacahuetes y frutos secos respectivamente si el alimento va a ser tratado físicamente antes de su consumo, y un límite de 4 y 2 µg/kg de aflatoxinas totales y aflatoxina B1 respectivamente si van a ser consumidos directamente [27,28].

Generalmente, muchos países fuera de la Unión Europea, principalmente de Asia, han establecido un límite máximo para cacahuetes de ocratoxina A de 15 µg/kg, de aflatoxina B1 entre 1 y 20 µg/kg y de aflatoxinas totales entre 10 y 35 µg/kg [22].

2.1.3 Métodos de análisis

La extracción y análisis de micotoxinas presenta un desafío debido entre otras razones a los bajos límites máximos de residuos (*maximum residue limit*, MRL) establecidos, así como la complejidad de matrices grasas como aceites vegetales y frutos secos.

2.1.3.1 Técnicas de extracción y limpieza

a) Aceites vegetales

La extracción líquido-líquido (*liquid-liquid extraction*, LLE), como se puede observar en la Tabla 1, se usa en la mayoría de los métodos desarrollados. Mediante LLE se pueden separar dos líquidos inmiscibles, generalmente uno polar y otro apolar. La finalidad de esta separación es

conseguir la transferencia por afinidad del analito de interés desde la matriz a la fase más limpia.

El disolvente más usado para la extracción de micotoxinas de aceites vegetales es una mezcla de metanol:agua en diferentes proporciones (Tabla 1), como (55:45, *v/v*) [29–31], (60:40, *v/v*) [32], (70:30, *v/v*) [33–35], (75:25, *v/v*) [36], (80:20, *v/v*) [37–39] o (90:10, *v/v*) [40,41], todos ellos mostrando recuperaciones aceptables (72-110%). Además, también se han usado otras mezclas como acetonitrilo:agua en distintas proporciones, (80:20, *v/v*) [42] o (84:16, *v/v*) [36], acetonitrilo:ácido fórmico (95:5, *v/v*) [43], acetonitrilo:ciclohexano (50:50, *v/v*) [44], o simplemente acetonitrilo [45]. En todos los casos se han obtenido recuperaciones aceptables, excepto con el uso de acetonitrilo:agua (50:50, *v/v*), que se obtuvieron recuperaciones de hasta 145% para fumonisina B2 en aceite de canola [46].

La extracción en fase sólida (*solid phase extraction*, SPE) es una técnica de extracción por la cual los compuestos disueltos o suspendidos en una mezcla se separan de otros compuestos contenidos en esa mezcla según sus propiedades físicas y químicas [47]. Una modificación de la técnica SPE es la extracción magnética en fase sólida (*magnetic solid phase extraction*, MSPE), donde se dispersan nanopartículas de Fe_3O_4 en la muestra para la extracción de aflatoxinas B1 y B2, fumonisina B1, zearalenona y ocratoxina A [39,48], o el uso de polímeros impresos molecularmente (*molecularly imprinted polymers*, MIP), que presentan una determinada selectividad hacia un analito o un grupo de especies estructuralmente relacionadas, aplicándose para la extracción de aflatoxinas B1 y M1 en aceite de cacahuete [49] con recuperaciones aceptables (83-96%). En estos casos, los MIP actúan como fase estacionaria de la SPE [49,50]. Por último, se han llevado a cabo extracciones mediante hidracina dinámica covalente para la extracción de zearalenona en aceites vegetales obteniendo en todos los casos recuperaciones dentro del rango aceptable (74-107%) [51,52].

Tabla 1: Métodos usados para la extracción de micotoxinas en aceites.[a]

Matriz	Analitos	Método de extracción	Método de limpieza	R (%)	Ref.
Aceites vegetales (10 tipos)	AFB1	Agua alcalina electrolizada	PSA	83-111	[53]
Aceites vegetales (7 tipos)	AFB1 y AFB2	MSPE: Fe_3O_4/rGO	No utilizado	80-106	[48]
Aceites de girasol, cacahuete y sésamo	AFB1, AFB2, AFG1 y AFG2	LLE: CH_3OH:H_2O (80:20, *v/v*) - Derivatización (TFA)	Éter de petróleo	83-96	[37]
Aceites de oliva, cacahuete y sésamo	AFB1, AFB2, AFG1 y AFG2	LLE: CH_3OH:H_2O (55:45, *v/v*)	No utilizado	76-97	[30]
Aceites vegetales (6 tipos)	AFB1, AFB2, AFG1 y AFG2	LLE: CH_3OH:H_2O (70:30, *v/v*)	IAC - Beacon	72-78	[33]
Aceites de cacachuete, algodón y sésamo	AFB1, AFB2, AFG1 y AFG2	LLE: CH_3OH:H_2O (55:45, *v/v*) - Derivatización (TFA)	No utilizado	-	[31]
Aceites de soja, cacahuete, maíz y mezcla	AFB1, AFB2, AFG1 y AFG2	LLE: CH_3OH:H_2O (70:30, *v/v*)	IAC - AflaStar R	82-109	[34]
Aceites de oliva	AFB1, AFB2, AFG1 y AFG2	DLLME: CH_3OH:H_2O (60:40, *v/v*)	IAC - AflaCLEAN	96-110	[32]
Aceites vegetales	AFB1, AFB2, AFG1 y AFG2	LLE: CH_3OH:H_2O (80:20, *v/v*)	IAC - hecha con ASGM	90-104	[38]
Aceites de colza y maíz	ZEA	LLE: CH_3OH:H_2O (90:10, *v/v*)	No utilizado	87-91	[40]
Aceites vegetales	ZEA	Extracción mediante hidracina dinámica covalente	No utilizado	75-107	[51]

Tabla 1 (continuación)

Matriz	Analitos	Método de extracción	Método de limpieza	R (%)	Ref.
Aceites de arroz, maíz y soja	ZEA	SPE (MIP)	No utilizado	71-93	[50]
Aceites vegetales (5 tipos)	Cis-ZEA y trans-ZEA	LLE: $CH_3OH:H_2O$ (90:10, *v/v*)	No utilizado	104	[41]
Aceites de colza, maíz y soja	FB1, ZEA y OTA	LLE: $CH_3OH:H_2O$ (80:20, *v/v*) - MSPE: Fe_3O_4@$nSiO_2$@$mSiO_2$	No utilizado	89-97	[39]
Aceites vegetales (5 tipos)	ZEA y 5 derivados	LLE: CH_3CN:ciclohexano (50:50, *v/v*) - Derivatización (BSTFA+TMCS (99:1 *v/v*))	GPC	80-97	[44]
Aceites de maíz, girasol y palma	7 micotoxinas	LLE: CH_3CN	d-SPE: C18:GCB (3:1 *p/p*), $MgSO_4$	88-107	[45]
Aceites de arroz, maíz y soja	11 micotoxinas	LLE: H_2O y $CH_3CN:CH_2O_2$ (95:5, *v/v*) QuEChERS: $MgSO_4$, NaCl, SCTD, SCDS	d-SPE: $MgSO_4$, C18	52-83	[43]
Aceites vegetales (5 tipos)	12 micotoxinas	LLE: $CH_3CN:H_2O$ (50:50, *v/v*)	No utilizado	80-145	[46]
Aceites vegetales (6 tipos)	16 micotoxinas	LLE: $CH_3CN:H_2O$ (80:20, *v/v*) QuEChERS: $MgSO_4$, NaCl	d-SPE: C18, PSA, Al_2O_3	73-106	[42]
Aceite de cacahuete	AFB1	LLE: $CH_3OH:H_2O$ (70:30, *v/v*)	No utilizado	84-93	[35]
Aceite de cacahuete	AFB1 y AFM1	SPE (MIP)	No utilizado	83-96	[49]

Tabla 1 (continuación)

Matriz	Analitos	Método de extracción	Método de limpieza	R (%)	Ref.
Aceite de coco	AFB1, AFB2, AFG1 y AFG2	IAC - Aflatest	No utilizado	34-101	[54]
Aceite de germen de maíz	ZEA	Extracción mediante hidracina dinámica covalente	SPE *online*: sílica gel SLC1	74-81	[52]
Aceite de maíz	FB1, FB2, DON y ZEA	LLE: CH_3OH:H_2O (75:25, *v/v*) LLE: CH_3CN:H_2O (84:16, *v/v*)	SPE: SAX	74-103	[36]

[a] AFB1: Aflatoxina B1; AFB2: Aflatoxina B2; AFG1: Aflatoxina G1; AFG2: Aflatoxina G2; AFM1: Aflatoxina M1; ASGM: Micropartículas de amino-sílica gel; BSTFA: N,O-bis (trimetilsilil) trifluoroacetamida; C18: Octadecasilano; DLLME: Microextracción dispersiva líquido-líquido; DON: Deoxinivalenol; d-SPE: Extracción dispersiva en fase sólida; IAC: Cromatografía de inmunoafinidad; FB1: Fumonosina B1; FB2: Fumonisina B2; Fe_3O_4/rGO: Óxido ferroso-férrico/óxido de grafeno reducido; Fe_3O_4@nSiO_2@mSiO_2: Óxido ferroso-férrico/nanopartículas de sílica/sílica mesoporosa monodispersada; LLE: Extracción líquido-líquido; MSPE: Extracción en fase sólida magnética; OTA: Ocratoxina A; MIP: Polímeros impresos molecularmente; PSA: Amina primaria secundaria; QuEChERS: Extracción Rápida, Fácil, Barata, Efectiva, Robusta y Segura; SAX: Intercambio aniónico fuerte; SCDS: Citrato de sodio dibásico sesquihidratado; SCTD: Citrato de sodio tribásico dihidratado; SPE: Extracción en fase sólida; TFA: Ácido trifluoroacético; TMCS: Trimetilclorosilano; ZEA: Zearalenona.

Del mismo modo, la técnica de extracción y limpieza QuEChERS (*Quick, Easy, Cheap, Effective, Rugged and Safe*, Rápida, Fácil, Barata, Efectiva, Robusta y Segura) también se ha utilizado ampliamente [42,43]. Esta técnica fue introducida por Anastassiades et al. [55] y consiste en la utilización de sales de extracción que reduzcan el contenido en agua, como puede ser el $MgSO_4$ además de NaCl, usando acetonitrilo como disolvente [42]. Además de éstas, se han empleado otras sales como citrato de sodio tribásico dihidratado y citrato de sodio dibásico sesquihidratado, para generar un tampón, [43] para la extracción de 11 micotoxinas en aceites de arroz, maíz y soja. En este caso, las recuperaciones obtenidas oscilaron entre 52 y 83%.

La etapa de limpieza se puede llevar mediante extracción dispersiva en fase sólida (*dispersive solid phase extraction*, d-SPE) usando distintos sorbentes en función de la matriz que se esté estudiando. Así, en el caso de matrices lipídicas se puede usar C18 combinado con $MgSO_4$ [43], C18 con carbono grafitizado negro (*graphitized carbon black*, GCB) en proporción (3:1, *m/m*), además de $MgSO_4$ [45], o C18, amina primaria secundaria (*primary secondary amine*, PSA) y Al_2O_3 combinados [42].

También en la etapa de limpieza se ha usado cromatografía por permeación en gel (*gel permeation chromatography*, GPC), en la que la separación se produce en función del comportamiento hidrodinámico de las moléculas, empleando una disolución de acetato de etilo:ciclohexano (1:1, *v/v*) para la extracción de zearalenona y 5 derivados en 5 tipos de aceites vegetales [44].

Sin embargo la principal técnica utilizada para la limpieza, o extracción directa, de los extractos ha sido la cromatografía de inmunoafinidad (*immunoaffinity chromatography*, IAC) [32–35,38,54]. Esta técnica consiste en hacer pasar el extracto de la muestra a través de un lecho que contiene anticuerpos o fragmentos de anticuerpos

inmovilizados que retienen el analito [56]. Esta técnica se empezó a usar debido a que ofrece una gran limpieza de la matriz, y por tanto se consigue un buen aislamiento del analito, necesaria ya que los detectores usados, como el detector de fluorescencia (*fluorescence detector*, FLD), no son tan selectivos y fiables como los basados en espectrometría de masas (*mass spectrometry*, MS). En este caso, se han usado diferentes columnas comerciales como la columna Beacon [33], AflaStar R [34], AflaCLEAN [32], Aflatest [54] o la fabricada manualmente con micropartículas de amino-sílica gel (*amino silica-gel microparticles*, ASGM) [38]. La utilización de IAC puede permitir evitar la etapa de limpieza, como es el caso de la extracción de aflatoxina B1, aflatoxina B2, aflatoxina G1 y aflatoxina G2 de aceite de coco [54].

Por otro lado, se ha usado la técnica SPE con cartuchos de sal de intercambio aniónico fuerte (*strong anion exchange*, SAX) [36], o cartuchos de sílica gel SLC1 acoplados a LC [52]. Este es el único caso encontrado en bibliografía en el que se usa SPE *online* para el análisis de micotoxinas en aceites vegetales, obteniendo recuperaciones entre 74 y 81%. En cuanto a la etapa de limpieza, finalmente se ha usado simplemente el sorbente PSA [53] para el análisis de aflatoxina B1 en aceites vegetales o el disolvente éter de petróleo [37] para la limpieza de aflatoxinas en aceites de girasol, cacahuete y sésamo.

b) Frutos secos

La extracción de micotoxinas de frutos secos, según los trabajos consultados durante la redacción de esta Tesis, utilizan principalmente la técnica de extracción sólido-líquido (*solid-liquid extraction,* SLE).

La Tabla 2 refleja un resumen de los procedimientos empleados. Como se puede observar se han utilizado diferentes disolventes, como acetonitrilo [57] o acetonitrilo y agua de manera secuencial [58], obteniendo en el primer caso recuperaciones aceptables (85-112%)

mientras que en el segundo caso se han obtenido algunas recuperaciones altas (74-132%). También se han empleado mezclas de disolventes, tales como la mezcla metanol:agua en diferentes proporciones, (60:40, *v/v*) [59–62], (70:30, *v/v*) [63] ó (80:20, *v/v*), [38,64,65] obteniendo en la mayoría de los casos recuperaciones entre 75-112%, excepto cuando se ha usado metanol:agua (70:30, *v/v*) que ofrece recuperaciones ligeramente inferiores (69-84%) [63]. Otra combinación que se ha utilizado ampliamente es la de acetonitrilo:agua en diferentes proporciones, (80:20, *v/v*) [66], (84:16, *v/v*) [67,68] ó (85:15, *v/v*) [69–72]. En estos casos se han obtenido recuperaciones dentro del rango aceptable (68-104%). También se ha usado metanol y acetonitrilo combinados en proporción (60:40, *v/v*) obteniendo recuperaciones comprendidas entre 72 y 108% [73].

La acidificación del disolvente de extracción se ha utilizado frecuentemente con el fin de mejorar la ionización de los compuestos y por consiguiente su extracción. Así se ha empleado la mezcla acetonitrilo:ácido fórmico (95:5, *v/v*) tras una adición de agua [74] obteniendo recuperaciones aceptables pero por debajo del 100% (70-91%). De igual modo se ha empleado ácido fórmico pero en menor proporción, acetonitrilo:ácido fórmico (99,9:0,1, *v/v*) [75,76], logrando valores de recuperación entre 65 y 104%. También se ha usado la mezcla acetonitrilo:ácido cítrico (99,8:0,2, *v/v*), consiguiendo valores de recuperación entre 81 y 111% [77].

Sin embargo, el ácido más ampliamente utilizado es el acético, usado en las proporciones, acetonitrilo:ácido acético (99:1, *v/v*) [78,79] o (99,5:0,5, *v/v*) [80] obteniendo recuperaciones entre 81 y 129%. Finalmente, la mezcla acetonitrilo:agua con adición de ácido acético se ha utilizado frecuentemente en las proporciones acetonitrilo:agua:ácido acético (79:20:1, *v/v/v*) [81–87] ó (79,5:20:0,5, *v/v/v*) [88], obteniendo puntualmente alguna recuperación excesivamente baja como la obtenida

para citrinina en anacardos (27%) [83] como se puede observar en la Tabla 2.

También se han utilizado disolventes poco habituales para la extracción de ocratoxina A, como son tolueno, ácido clorhídrico 2M y cloruro de magnesio 0,4M, obteniendo recuperaciones algo bajas (73-77%) [89].

Por otro lado, como se puede observar en la Tabla 2, en ocasiones se ha usado la técnica QuEChERS, usando mezclas de disolvente con acetonitrilo como disolvente principal como se ha explicado anteriormente. En este caso solo se han empleado las sales $MgSO_4$ y NaCl [73,74,78,79,90], o bien combinadas con citrato de sodio y citrato de sodio dibásico sesquihidratado, habiéndose utilizado para el análisis de 17 micotoxinas en almendra, cacahuete y pistacho [75].

En algunos estudios tras la extracción QuEChERS se aplica una etapa de limpieza mediante d-SPE. En esta etapa se añade el sobrenadante extraído en la etapa anterior a un tubo que puede contener un sorbente, por ejemplo C18 [90] o una combinación de ellos, como C18 y Z-Sep+ [74], C18 y PSA [75], $MgSO_4$ y C18 [79] o una combinación de más sales como $MgSO_4$, NaCl, C18 y citrato de sodio, empleándose esta combinación para el análisis de 14 micotoxinas en castañas [85].

Asimismo, la técnica IAC se ha utilizado ampliamente para la etapa de limpieza, como se puede observar en la Tabla 2. Como se ha comentado previamente esta técnica es bastante selectiva para los analitos de interés, y debido a eso se pueden separar pocos analitos en una misma extracción. Esta alta especificidad hace que se empleen diferentes columnas incluso para la extracción de las mismas micotoxinas.

Tabla 2: Métodos usados para la extracción de micotoxinas en frutos secos.[a]

Matriz	Analitos	Método de extracción	Método de limpieza	R (%)	Ref.
Varios frutos secos (12 tipos)	AFB1	SLE: CH_3OH:H_2O (60:40, *v/v*)	IAC - AflaTest WB	-	[59]
Almendras, cacahuetes, nueces y pistachos	AFB1	SLE: CH_3CN:H_2O (84:16, *v/v*)	IAC - Aflazon	-	[67]
Varios frutos secos (8 tipos)	Aflatoxinas	SLE: CH_3OH:H_2O (60:40, *v/v*)	IAC - NeoColumn	-	[60]
Varios frutos secos (9 tipos)	AFB1, AFB2, AFG1 y AFG2	SLE: CH_3OH:H_2O (80:20, *v/v*)	IAC - AflaCLEAN	86-112	[64]
Avellanas, anacardos, avellanas, nueces y piñones	AFB1, AFB2, AFG1 y AFG2	QuEChERS: $MgSO_4$, NaCl CH_3OH:CH_3CN (60:40, *v/v*)	-	72-108	[73]
Frutos secos	Aflatoxinas y OTA	SLE: CH_3OH:H_2O (60:40, *v/v*)	No utilizado	75-95	[61]
Almendras, nueces y pistachos	OTA	SLE: CH_3OH:H_2O (70:30, *v/v*)	IAC - OchraTest	69-84	[63]
Almendras, cacahuetes y pistachos	OTA	SLE: tolueno, HCl 2M y $MgCl_2$ 0,4M	IAC - OchraPrep	73-77	[89]
Frutos secos	OTA y OTB	SLE: CH_3OH:H_2O (80:20, *v/v*)	No utilizado	89-95	[65]
Anacardos, nueces, pistachos, y semillas de calabaza	CIT y OTA	QuEChERS: $MgSO_4$, NaCl CH_3CN:CH_3COOH (99:1, *v/v*)	-	81-100	[78]
Varios frutos secos (9 tipos)	BEA, ENA, ENB y ENB1	SLE: CH_3CN	SPE: C18	85-112	[57]

Tabla 2 (continuación)

Matriz	Analitos	Método de extracción	Método de limpieza	R (%)	Ref.
Almendras, cacahuetes y pistachos	11 micotoxinas	SLE: CH_3CN, H_2O	No utilizado	74-132	[58]
Varios frutos secos (10 tipos)	15 micotoxinas	QuEChERS: $MgSO_4$, NaCl CH_3CN:ácido cítrico (99,8:0,2, *v/v*)	d-SPE: C18	81-111	[90]
Varios frutos secos (8 tipos)	16 micotoxinas	QuEChERS: $MgSO_4$, NaCl H_2O, CH_3CN:CH_2O_2 (95:5, *v/v*)	d-SPE: C18, Z-Sep+	70-91	[74]
Almendras, cacahuetes y pistachos	17 micotoxinas	QuEChERS: $MgSO_4$, NaCl, SC, SCDS SLE: CH_3CN:CH_2O_2 (99,9:0,1, *v/v*)	d-SPE: C18, PSA	75-98	[75]
Almendras, avellanas, castañas y nueces	19 micotoxinas	SLE: CH_3CN:CH_2O_2 (99,9:0,1, *v/v*)	No utilizado	65-104	[76]
Almendras, cacahuetes y pistachos	26 micotoxinas	SLE: CH_3CN:H_2O (85:15, *v/v*)	No utilizado	84-104	[69]
Frutos secos	106 micotoxinas	SLE: CH_3CN:H_2O:CH_3COOH (79:20:1, *v/v/v*)	No utilizado	-	[81]
Almendras, avellanas, castañas y pistachos	191 micotoxinas	SLE: CH_3CN:H_2O:CH_3COOH (79:20:1, *v/v/v*)	No utilizado	>50 (94% de ellas)	[82]
Almendras	AFB1, AFB2, AFG1 y AFG2	SLE: CH_3OH:H_2O (60:40, *v/v*)	IAC - AflaTest WB	86-106	[62]
Anacardos	Aflatoxinas y ZEA	SLE: CH_3CN:H_2O:CH_3COOH (79:20:1, *v/v/v*)	IAC - EASI EXTRACT (sólo para ZEA)	27-114	[83]

Tabla 2 (continuación)

Matriz	Analitos	Método de extracción	Método de limpieza	R (%)	Ref.
Cacahuetes	AFB1, AFB2, AFG1 y AFG2	SLE: CH_3OH:H_2O (80:20, *v/v*)	IAC - hecha con ASGM	90-104	[38]
Almendras, avellanas	Aflatoxinas y OTA	SLE: CH_3CN:CH_3COOH (99,5:0,5, *v/v*)	IAC	84-129	[80]
Cacahuetes	AFB1, OTA, FB1 y ZEA	SLE: CH_3CN:CH_3COOH (99:1, *v/v*) QuEChERS: $MgSO_4$, NaCl	d-SPE: $MgSO_4$, C18	84-115	[79]
Nueces de Brasil	AFB1, AFB2, AFG1 y AFG2	SLE: CH_3CN:H_2O (85:15, *v/v*) Derivatización (TFA)	SPE: C18	80-84	[71]
Nueces de pecán	AFB1, AFB2, AFG1 y AFG2	SLE: CH_3CN:H_2O (84:16, *v/v*)	No utilizado	90-92	[68]
Cacahuetes	10 micotoxinas	SLE: CH_3CN:H_2O:CH_3COOH (79:20:1, *v/v/v*)	No utilizado	40-367	[84]
Cacahuetes	12 micotoxinas	SLE: CH_3CN:H_2O:CH_3COOH (79,5:20:0,5, *v/v/v*)	IAC - Myco6in1	71-112	[88]
Castañas	14 micotoxinas	SLE: CH_3CN:H_2O:CH_3COOH (79:20:1, *v/v/v*)	d-SPE: $MgSO_4$, NaCl, C18, SC	74-110	[85]
Avellanas	34 micotoxinas	SLE: CH_3CN:H_2O:CH_3COOH (79:20:1, *v/v/v*)	No utilizado	42-125	[86]
Nueces de Brasil	235 micotoxinas	SLE: CH_3CN:H_2O:CH_3COOH (79:20:1, *v/v/v*)	No utilizado	56-136	[87]
Semillas de girasol	Esterigmato-cistina	SLE: CH_3CN:H_2O:CH_3COOH (80:20, *v/v*)	IAC - EASI-EXTRACT STERIGMATOCYSTIN	68-97	[66]

[a] AFB1: Aflatoxina B1; AFB2: Aflatoxina B2; AFG1: Aflatoxina G1; AFG2: Aflatoxina G2; ASGM: Micropartículas de amino-sílica gel; BEA: Beauvericina; C18: Octadecasilano; CIT: Citrinina; DON: Deoxinivalenol; d-SPE: Extracción dispersiva en fase sólida; ENA: Enniatina A; ENB: Enniatina B; ENB1:Enniatina B1; IAC: Cromatografía de inmunoafinidad; FB1: Fumonosina B1; FB2: Fumonisina B2; OTA: Ocratoxina A; OTB: Ocratoxina B; PSA: Amina primaria secundaria; QuEChERS: Rápida, Fácil, Barata, Efectiva, Robusta y Segura; SC: Citrato de sodio; SCDS: Citrato de sodio dibásico sesquihidratado; SCTD: Citrato de sodio tribásico dihidratado; SLE: Extracción sólido-líquido; SPE: Extracción en fase sólida; TFA: Ácido trifluoroacético; ZEA: Zearalenona.

Por ejemplo, para la extracción de aflatoxinas se han usado las columnas Aflatest WB [59,62], Aflazon [67], AflaCLEAN [64] y NeoColumn [60]. Para la separación de aflatoxinas en cacahuetes, Ma et al. preparó manualmente una columna de IAC con ASGM [38]. Del mismo modo, las columnas OchraTest [63] y OchraPrep [89] se han utilizado para la extracción de ocratoxina A en almendras, cacahuetes, nueces y pistachos. En estos dos últimos casos las recuperaciones obtenidas oscilaron entre 69 y 84% y 73 y 77%, respectivamente. Las columnas EASI EXTRACT [83] y EASI EXTRACT STERIGMATOCYSTIN [66] se han empleado para la extracción de zearalenona y esterigmatocistina en anacardos y semillas de girasol respectivamente, mientras que la columna Myco6in1 se ha utilizado para la extracción de hasta 12 micotoxinas en cacahuetes [88].

Por último, como se puede ver en la Tabla 2, algunos autores han empleado SPE con cartuchos comerciales de C18 para la extracción de aflatoxinas en nueces de Brasil [71,72] o de beauvericina, enniatina A, enniatina B, y enniatina B1 en 9 tipos de frutos secos [57].

2.1.3.2 Técnicas de análisis

La técnica LC es la más ampliamente utilizada para la separación de micotoxinas en aceite y frutos secos, ya sea acoplado a FLD [40], o a detectores más complejos como los de MS de tipo simple cuadrupolo (*single quadrupole*, Q) [53], triple cuadrupolo (*triple quadrupole,* QqQ) [34] o el analizador híbrido cuadrupolo con trampa de iones lineal (*quadrupole linear ion trap*, QTRAP) [51]. Las Tablas 3 y 4 muestran un resumen de las condiciones instrumentales usadas para el análisis de micotoxinas en aceites vegetales y frutos secos, respectivamente.

Como se puede observar se ha usado principalmente LC en fase reversa (*reverse phase*, RP) con una fase estacionaria de octadecasilano (*octadecasilane*, C18), excepto de dimetil n-octilsilano (*dimethyl-n-octylsilane*, C8) que se ha utilizado para la separación de aflatoxinas [68].

Tabla 3: Condiciones de análisis de micotoxinas en aceites por LC.[a]

Analitos	Técnica y analizador	Modo de trabajo y columna	Fase móvil	Tiempo (min)	LOD	Ref.
AFB1	HPLC-Q-MS	RP, C18 150 × 4,6 mm, 3,5 µm	A: 0,1% CH_2O_2 en H_2O B: CH_3CN	12	0,2 µg/L	[53]
AFB1	UHPLC-QqQ-MS/MS	RP, C18 100 × 2,1 mm, 1,7 µm	A: 5 mM $C_2H_7NO_2$ en H_2O B: CH_3CN:CH_3OH (50:50, *v/v*)	7	0,01 µg/kg	[35]
AFB1 y AFM1	HPLC-FLD	RP, C18 250 × 4,6 mm, 5 µm	CH_3CN:H_2O (75:25, *v/v*)	10	0,05 µg/kg	[49]
AFB1 y AFB2	HPLC-FLD	RP, C18 150 × 4,6 mm, 5 µm Derivatización post-columna	A: H_2O B: CH_3OH	20	0,02 µg/kg	[48]
AFB1, AFB2, AFG1 y AFG2	HPLC-FLD	RP, C18 150 × 4,6 mm, 5 µm	H_2O:CH_3OH:CH_3CN (60:20:20, *v/v/v*)	8	0,09-1,5 µg/kg	[37]
AFB1, AFB2, AFG1 y AFG2	HPLC-FLD	RP, C18 250 × 4,6 mm, -	H_2O:CH_3OH:CH_3CN (4:1:1, *v/v/v*)	-	0,1 µg/kg	[31]
AFB1, AFB2, AFG1 y AFG2	HPLC-FLD	RP, C18 250 × 4,6 mm, 4 µm	H_2O:CH_3OH:CH_3CN (6:3:2, *v/v/v*)	12	0,11 – 5,3 ng/L	[32]
AFB1, AFB2, AFG1 y AFG2	HPLC-FLD	RP, C18 150 × 4,6 mm, 5 µm	A: CH_3CN B: H_2O	26	0,03 - 0,09 µg/kg	[38]
AFB1, AFB2, AFG1 y AFG2	HPLC-QqQ-MS/MS	RP, C18 150 × 3,5 mm, 3 µm	A: 0,1% CH_2O_2 en H_2O B: CH_3OH	-	0,02 - 0,09 µg/L	[33]
AFB1, AFB2, AFG1 y AFG2	HPLC-QqQ-MS/MS	RP, - 150 × 2,1 mm, -	A: 0,1% CH_2O_2 en H_2O B: CH_3CN:CH_3OH (50:50, *v/v*)	24	0,02 - 0,09 µg/kg	[34]

Tabla 3 (continuación)

Analitos	Técnica y analizador	Modo de trabajo y columna	Fase móvil	Tiempo (min)	LOD	Ref.
ZEA	HPLC-FLD	RP, C18 250 × 4 mm, 5 µm	A: CH_3CN:H_2O (48:52, *v/v*) B: CH_3CN:H_2O (80:20, *v/v*)	40	20 µg/kg	[40]
ZEA	HPLC-FLD	RP, C18 150 × 4,6 mm, 2,7 µm	A: 2% CH_3COOH en H_2O B: CH_3CN	18	1,2 µg/kg	[50]
ZEA	HPLC-FLD	RP, C18 150 × 3 mm, 3 µm	A: H_2O B: CH_3CN	30	10 µg/kg	[52]
ZEA	HPLC-FLD HPLC-QTRAP-MS/MS	RP, C18 150 × 2 mm, 3 µm	A: 0,1% CH_2O_2 en H_2O B: 0,1% CH_2O_2 en CH_3CN	26 20	10 µg/kg 5 µg/kg	[51]
Cis-ZEA y trans-ZEA	HPLC-QTRAP-MS/MS	RP, C18 150 × 2 mm, 3 µm	A: 0,1% CH_2O_2 en H_2O B: 0,1% CH_2O_2 en CH_3CN	30	0,28 - 0,35 µg/kg	[41]
FB1, ZEA y OTA	UHPLC-QqQ-MS/MS	RP, C18 100 × 2,1 mm, 1,7 µm	2 mM $C_2H_7NO_2$ + 0,2% CH_2O_2 en CH_3CN:H_2O (60:40, *v/v*)	10	0,08 - 1,03 µg/kg	[39]
FB1, FB2, DON y ZEA	HPLC-FLD	RP, C18 250 × 4,6 mm, 5 µm	FB1/FB2: H_2O:CH_3CN:CH_3COOH (53:46:1, *v/v/v*) ZEA: H_2O:CH_3OH:CH_3CN (46:8:46, *v/v/v*) DON: H_2O:CH_3OH:CH_3CN (95:5:5, *v/v/v*)	40 19 15	10 - 70 µg/kg	[36]
FB1, FB2, DON y ZEA	UHPLC-QqQ-MS/MS	RP, C18 250 × 2,1 mm, 1,7 µm	A: 0,1% CH_2O_2 en H_2O B: 0,1% CH_2O_2 en CH_3OH	40	10 - 70 µg/kg	[36]

Tabla 3 (continuación)

Analitos	Técnica y analizador	Modo de trabajo y columna	Fase móvil	Tiempo (min)	LOD	Ref.
7 micotoxinas	HPLC-QqQ-MS/MS	RP, C18 150 × 3 mm, 5 µm	A: CH_3OH B: 0,1% CH_3COOH en H_2O	16	0,01 - 650 µg/kg	[45]
11 micotoxinas	UHPLC-QqQ-MS/MS	RP, C18 100 × 2,1 mm, 1,7 µm	A: 0,1% CH_2O_2 en H_2O B: 0,1% CH_2O_2 en CH_3OH	28	-	[43]
12 micotoxinas	UHPLC-QTRAP-MS/MS	RP, C18 100 × 2,1 mm, 1,6 µm	A: 0,1% CH_2O_2 + 10 mM NH_4HCO_2 en H_2O B: 0,1% CH_2O_2 + 10 mM NH_4HCO_2 en CH_3OH	15	0,03 - 1,5 µg/kg	[46]
16 micotoxinas	UHPLC-QTRAP-MS/MS	RP, C18 150 × 2,1 mm, 1,8 µm	A: H_2O B: CH_3CN	11	0,04 - 2,9 µg/kg	[42]

[a] AFB1: Aflatoxina B1; AFB2: Aflatoxina B2; AFG1: Aflatoxina G1; AFG2: Aflatoxina G2; AFM1: Aflatoxina M1; C18: Octadecasilano; DON: Deoxinivalenol; FB1: Fumonosina B1; FB2: Fumonisina B2; FLD: detector de fluorescencia; HPLC: Cromatografía de líquidos de alta resolución; OTA: Ocratoxina A; Q-MS: Simple cuadrupolo; QqQ-MS/MS: Triple cuadrupolo; QTRAP-MS/MS: Cuadrupolo con trampa de iones lineal; RP: Fase reversa; UHPLC: Cromatografía de líquidos de ultra-alta resolución; ZEA: Zearalenona.

El tiempo de análisis varía dependiendo de la aplicación entre 7 y 61 minutos como se puede observar en las Tablas 3 y 4.

En cuanto a las fases móviles empleadas, en ocasiones se ha utilizado una fase móvil única con mezcla de disolventes en modo isocrático cuando el detector empleado es FLD [31,32,36,37,49,60,62–64,66,67,71,72,83,89], o incluso utilizando QqQ [39], aunque en la mayoría de ocasiones se ha usado una fase móvil binaria, independientemente del detector utilizado. Generalmente se ha usado una fase acuosa y una fase orgánica, adicionando en ocasiones solo a la fase acuosa o a ambas fases ácido acético [45,50], ácido fosfórico [63], ácido cítrico además de ácido fórmico [90] o simplemente ácido fórmico [33,34,36,41,43,51,53,58,75,76,85]. El uso de un ácido en la fase móvil favorece la ionización de los compuestos. Con el mismo fin, se le ha añadido acetato de amonio [35,65] o formiato de amonio [57,68,79,88]. Del mismo modo, para favorecer la separación así como la ionización en modo positivo y negativo, se ha generado un tampón usando formiato o acetato de amonio junto con ácido fórmico [46,69,70,80] o junto con ácido acético [74,78,81,82], respectivamente.

Algunos autores han realizado una derivatización antes del análisis de micotoxinas, usando diferentes reactivos como ácido trifluoroacético (*trifluoroacetic acid*, TFA) [31,37,71,72], o N, O-bis-trimetilsililtrifluoroacetamida, que contiene 1% de trimetilclorosilano (trimethylchlorosilane, TCMS) [44]. Asimismo, se han utilizado diferentes derivatizaciones pre- y post columna. La derivatización pre-columna *online* se utiliza para mejorar la separación cromatográfica [61], mientras que la derivatización post-columna se usa para mejorar la detección de los analitos [29,30,48,60,73,83]. Esto permite mejorar la detección de los compuestos en detectores FLD al ser menos selectivos.

El detector FLD se ha utilizado para el análisis de aflatoxina B1 [67], aflatoxinas [29–32,37,38,48,49,54,60,62,64,71–73,83], zearalenona

[40,50–52] y fumonisina B1, fumonisina B2, deoxinivalenol y zearalenona [36], esterigmatocistina [66], las aflatoxinas B1, B2, G1 y G2 junto con ocratoxina A y citrinina [61], o sólo ocratoxina A [63,89].

También se ha utilizado el detector Q para el análisis de aflatoxina B1 en aceites vegetales [53]. El detector QqQ se usa frecuentemente por su sensibilidad, capacidad de identificación y robustez, y ha sido ampliamente empleado para la detección de aflatoxina B1 [59,91], aflatoxina B1, ocratoxina A, fumonisina B1 y zearalenona [79], aflatoxina B1, B2, G1 y G2 [33,34], aflatoxina B1, B2, G1 y G2 y ocratoxina A [80], aflatoxina B1, B2, G1 y G2 y zearalenona [83], citrinina y ocratoxina A [78], beaubericina, enniatina A, enniatina B y enniatina B1 [57], fumonisina B1, zearalenona y ocratoxina A [39], o para un número mayor de micotoxinas, [43,45,58,74,76,85,90,92], llegándose a analizar hasta un total de 191 micotoxinas [82]. Además, también se ha usado para la confirmación de resultados después de haber empleado FLD [36].

Por último, el analizador QTRAP también se ha empleado para la determinación cuantitativa de dos isómeros de zearalenona [41], aflatoxina B1, aflatoxina B2, aflatoxina G1 y aflatoxina G2 [68], así como para la detección de 10 [84], 12 [46], 16 [42] y hasta 26 micotoxinas [69,70]. Además se ha utilizado para la realizar la confirmación de resultados después de haber usado FLD para el análisis de zearalenona [51]. Asimismo, este analizador se ha empleado para la detección no dirigida o *untarget* de metabolitos, llegando a identificar hasta 235 metabolitos [81,86,87].

En otro estudio se ha usado la combinación de los detectores QqQ y QTRAP para la determinación de 12 micotoxinas [88] en cacahuetes, mientras que el analizador de alta resolución Q-Exactive Orbitrap se ha utilizado para el análisis de 17 micotoxinas [75] en almendras, cacahuetes y pistachos.

Tabla 4: Condiciones de análisis de micotoxinas en frutos secos por LC.[a]

Analitos	Técnica y analizador	Modo de trabajo y columna	Fase móvil	Tiempo (min)	LOD	Ref.
Esterigmato-cistina	HPLC-FLD	RP, C18 150 × 4,6 mm, 3 µm	H_2O:CH_3CN (40:60, *v/v*)	7	0,75 µg/kg	[66]
AFB1	HPLC-QqQ-MS/MS	RP, - 100 × 2,1 mm, 5 µm	A: 10 mM NH_4HCO_2 en H_2O B: CH_3OH	16	-	[59]
AFB1	HPLC-FLD	RP, C18 250 × 4,6 mm, 5 µm	H_2O:CH_3OH:CH_3CN (55:22,5:22,5, *v/v/v*)	-	-	[67]
AFB1, AFB2, AFG1 y AFG2	HPLC-FLD	RP, C18 250 × 4,6 mm, 5 µm	H_2O:CH_3OH:CH_3CN (60:20:20, *v/v/v*)	-	0,06 - 0,46 µg/kg	[62]
AFB1, AFB2, AFG1 y AFG2	HPLC-FLD	RP, C18 150 × 4,6 mm, 5 µm	A: CH_3CN B: H_2O	26	0,03 - 0,09 µg/kg	[38]
AFB1, AFB2, AFG1 y AFG2	HPLC-FLD	RP, C18 150 × 4,6 mm, -	H_2O:CH_3OH:CH_3CN (8:1,5:1,5, *v/v/v*) + 0,1% TFA	-	0,5 µg/kg	[71]
AFB1, AFB2, AFG1 y AFG2	HPLC-FLD	RP, C18 250 × 4,6 mm, 5 µm	H_2O:CH_3OH:CH_3CN (54:29:17, *v/v/v*)	-	0,05 - 0,42 µg/kg	[64]
AFB1, AFB2, AFG1 y AFG2	HPLC-FLD	RP, C18 250 × 4,6 mm, 5 µm Derivatización post-columna	H_2O:CH_3OH:CH_3CN (65:25:10, *v/v/v*)	36	0,05 - 0,35 µg/kg	[73]
AFB1, AFB2, AFG1 y AFG2 ZEA	HPLC-FLD	RP, C18 150 × 4,6 mm, 5 µm Derivatización post-columna	1 mM KBr + 1,4 mM HNO_3 en H_2O:CH_3OH (55:45, *v/v*) H_2O:CH_3CN (55:45, *v/v*)	-	0,01 µg/kg	[83]
AFB1, AFB2, AFG1 y AFG2	HPLC-QTRAP-MS/MS	RP, C8 150 × 4,6 mm, 5 µm	A: CH_3OH:H_2O:NH_4HCO_2 (95:4:1, *v/v/v*) B: H_2O:NH_4HCO_2 (99:1, *v/v*)	-	0,1 - 0,3 µg/kg	[68]

Tabla 4 (continuación)

Analitos	Técnica y analizador	Modo de trabajo y columna	Fase móvil	Tiempo (min)	LOD	Ref.
AFB1, OTA, FB1 y ZEA	HPLC-QqQ-MS/MS	RP, C18 150 × 4,6 mm, 2,7 µm	A: 10 mM NH_4HCO_2 en H_2O B: CH_3OH	12	0,1 - 25 µg/kg	[79]
Aflatoxinas	HPLC-FLD	RP, C18 150 × 4,6 mm, 5 µm Derivatización post-columna	H_2O:CH_3OH:CH_3CN (64:23:13, *v/v/v*)	20	-	[60]
Aflatoxinas OTA, CIT	HPLC-FLD	RP, C18 250 × 4,6 mm, 5 µm Derivatización pre-columna	H_2O:CH_3OH (55:45, *v/v*) H_2O:CH_3CN:CH_3COOH (49,5:49,5:1, *v/v/v*)	-	-	[61]
Aflatoxinas y OTA	UHPLC-QqQ-MS/MS	RP, C18 100 × 5 mm, 1,7 µm	A: 0,15% CH_2O_2 + 10 mM NH_4HCO_2 en H_2O B: 0,05% CH_2O_2 en CH_3OH	10	-	[80]
OTA	HPLC-FLD	RP, C18 250 × 4,6 mm, 5 µm	0,1% H_3PO_4 en H_2O:CH_3CN (35:65, *v/v*)	15	0,08 µg/kg	[63]
OTA	HPLC-FLD	RP, C18 250 × 4 mm, 5 µm	5 mM CH_3COONa en H2O:CH_3OH:CH_3CN:CH_3COOH (40:30:30:1,4, *v/v/v/v*)	10	0,05 µg/kg	[89]
OTA y OTB	HPLC-MS/MS	RP, C18 50 × 2,1 mm, 4 µm	A: 5 mM $C_2H_7NO_2$ en H_2O B: CH_3CN	10	0,089 - 0,092 µg/L	[65]
CIT y OTA	UHPLC-QqQ-MS/MS	RP, C18 100 × 2,1 mm, 1,8 µm	A: 5 mM NH_4HCO_2 + 0,05% CH_3COOH en H_2O B: CH_3OH	10	0,1 - 2,5 µg/kg	[78]

Tabla 4 (continuación)

Analitos	Técnica y analizador	Modo de trabajo y columna	Fase móvil	Tiempo (min)	LOD	Ref.
BEA, ENA, ENB y ENB1	HPLC-QqQ-MS/MS	RP, C18 150 × 4,6 mm, 5 µm	A: 10 mM NH_4HCO_2 en CH_3OH B: CH_3CN	21	0,02 - 0,15 µg/kg	[57]
11 micotoxinas	UHPLC-QqQ-MS/MS	RP, C18 100 × 2,1 mm, 1,7 µm	A: 0,1% CH_2O_2 en H_2O B: 0,1% CH_2O_2 en CH_3CN	8	0,14 - 7,5 µg/kg	[58]
12 micotoxinas	UHPLC-QTRAP-QqQ-MS/MS	RP, C18 100 × 2,1 mm, 1,7 µm	A: 5 mM NH_4HCO_2 en H_2O A: 5 mM NH_4HCO_2 en CH_3OH	10	0,05 - 5 µg/kg	[88]
14 micotoxinas	UHPLC-QqQ-MS/MS	RP, C18 100 × 2,1 mm, 1,7 µm	A: 0,5% CH_2O_2 en H_2O B: CH_3OH	14	0,02 - 1 µg/kg	[85]
15 micotoxinas	UHPLC-QqQ-MS/MS	RP, C18 100 × 2,1 mm, 1,7 µm	A: CH_3CN B: 10 mM ácido cítrico + 0,1% CH_2O_2 en CH_3OH	8	0,05 - 1,00 µg/kg	[90]
16 micotoxinas	HPLC-QqQ-MS/MS	RP, C18 150 × 4,6 mm, 2,6 µm	A: H_2O:CH_3OH:CH_3COOH (94:5:1, *v/v/v*) + 5 mM NH_4HCO_2 B: H_2O:CH_3OH:CH_3COOH (2:97:1, *v/v/v*) + 5 mM NH_4HCO_2	25	0,3 - 3,5 µg/kg	[74]
17 micotoxinas	HPLC-Q-Orbitrap-MS/MS	RP, C18 150 × 7,5 mm, 3 µm	A: 0,1% CH_2O_2 en H_2O B: 0,1% CH_2O_2 en CH_3CN	38	-	[75]
19 micotoxinas	HPLC-QqQ-MS/MS	RP, C18 150 × 2 mm, 3 µm	A: 0,05% CH_2O_2 en H_2O B: CH_3CN	61	0,12 - 34,4 µg/kg	[76]

Tabla 4 (continuación)

Analitos	Técnica y analizador	Modo de trabajo y columna	Fase móvil	Tiempo (min)	LOD	Ref.
26 micotoxinas	HPLC-QTRAP-MS/MS	RP, C18 100 × 2,1 mm, 3 µm	A: 0,1% CH_2O_2 + 10 mM NH_4HCO_2 en H_2O B: 0,1% CH_2O_2 + 10 mM NH_4HCO_2 en CH_3OH	15	0,1 - 8,1 µg/kg	[69]
106 micotoxinas	HPLC-QTRAP-MS/MS	RP, C18 150 × 4,6 mm, 5 µm	A: H_2O:CH_3OH:CH_3COOH (89:10:1, *v/v/v*) + 5 mM NH_4HCO_2 B: H_2O:CH_3OH:CH_3COOH (2:97:1, *v/v/v*) + 5 mM NH_4HCO_2	21	0,04 - 160 µg/kg	[81]
191 micotoxinas	UHPLC-QqQ-MS/MS	RP, C18 150 × 2,1 mm, 1,8 µm	A: H_2O:CH_3OH:CH_3COOH (89:10:1, *v/v/v*) + 5 mM NH_4HCO_2 B: H_2O:CH_3OH:CH_3COOH (2:97:1, *v/v/v*) + 5 mM NH_4HCO_2	21	-	[82]

[a] AFB1: Aflatoxina B1; AFB2: Aflatoxina B2; AFG1: Aflatoxina G1; AFG2: Aflatoxina G2; AFM1: Aflatoxina M1; BEA: Beauvericina; C18: Octadecasilano; C8: dimetil n-octilsilano; CIT: Citrinina; DON: Deoxinivalenol; ENA: Enniatina A; ENB: Enniatina B; ENB1:Enniatina B1; FB1: Fumonosina B1; FB2: Fumonisina B2; FLD: Detector de fluorescencia; HPLC: Cromatografía de líquidos de alta resolución; OTA: Ocratoxina A; OTB: Ocratoxina B; Q-MS: Simple cuadrupolo; QqQ-MS/MS: Triple cuadrupolo; QTRAP-MS/MS: Cuadrupolo con trampa de iones lineal; RP: Fase reversa; UHPLC: Cromatografía de líquidos de ultra-alta resolución; ZEA: Zearalenona.

El uso de cromatografía de gases (*gas chromatography*, GC) para el análisis de micotoxinas es mucho menos frecuente. Por ejemplo, se ha utilizado acoplada a QqQ para la detección de zearalenona y 5 derivados en aceites vegetales [44].

Algunos autores han empleado el ensayo por inmunoabsorción ligado a enzimas (*enzyme-linked immunosorbent assay*, ELISA) para la detección y cuantificación de micotoxinas en aceites vegetales [35,54,93], aunque finalmente se han usado FLD [54] o QqQ [35] para la confirmación de resultados.

2.2 Monocloropropanodioles

El 3-MCPD es un cloropropanol que se forma durante el proceso de refinado de aceites comestibles. Además, puede aparecer 2-monocloropropanodiol (2-MCPD) y sus correspondientes ésteres. El glicidol está asociado a ellos y generalmente forma monoésteres con ácidos grasos durante el proceso de refinado [94]. En la Figura 2 se puede ver un esquema general de estos compuestos.

Se ha demostrado que la exposición oral crónica a 3-MCPD da lugar a nefropatía e hiperplasia tubular y adenomas debido a que este compuesto ataca al hígado [95]. Además, se ha comprobado que este compuesto reduce la fertilidad y provoca infertilidad en ratas así como la supresión de la función inmune [96]. La IARC ha clasificado este compuesto como posible carcinógeno humano (grupo 2B) [97] a partir de que se han proporcionado evidencias de actividad carcinogénica [98]. Por otro lado, los ésteres glicidílicos se han clasificado como posibles agentes carcinógenos (grupo 2A) [97].

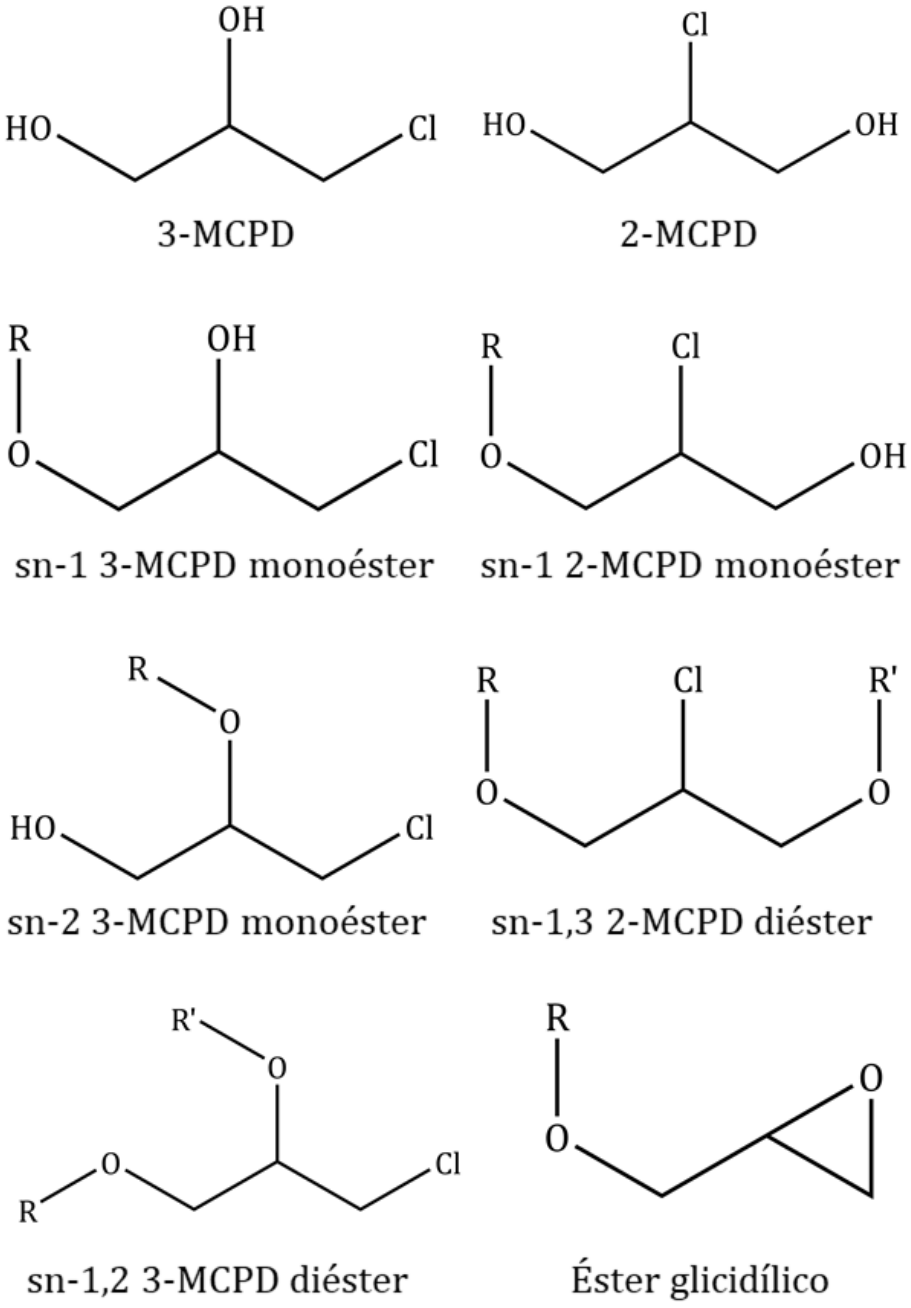

Figura 2. Esquema general de monocloropropanodioles

En la Figura 3 se puede ver un esquema general del mecanismo de formación de ésteres de 2- y 3-MCPD.

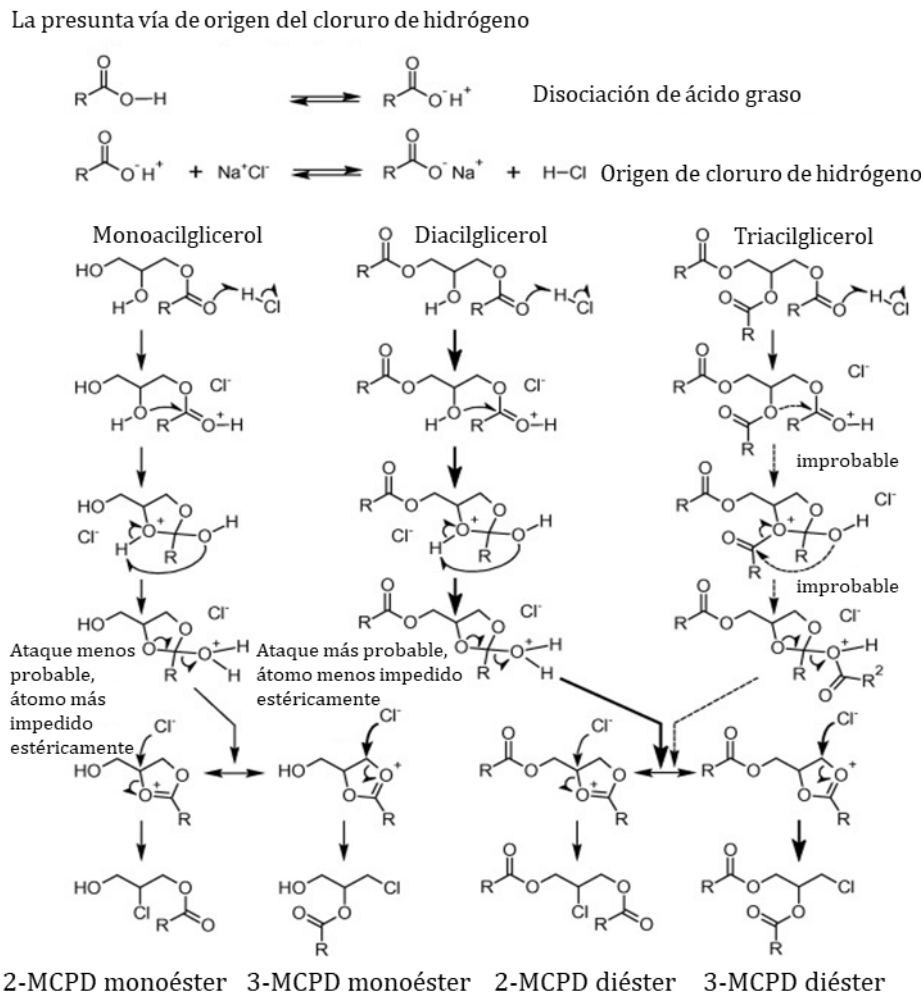

Figura 3. Mecanismo de formación de ésteres de 2- y 3-MCPD [99].

Durante el proceso de refinado, en primer lugar, se establece un equilibrio entre un ácido graso y una forma clorada, generalmente cloruro de sodio para formar cloruro de hidrógeno. Este cloruro de hidrógeno formado reacciona con los acilgliceroles para formar los ésteres de 3-MCPD. Si el cloruro de hidrógeno reacciona con un monoacilglicerol formará monoésteres de 2- y 3-MCPD, mientras que si reacciona con un diacilglicerol o triacilglicerol formará diésteres de 2- y 3-MCPD. Por tanto, la cantidad de ésteres que se van a formar durante el proceso de refinado

es directamente proporcional a la cantidad de ácidos grasos presentes en el aceite [99].

Por otro lado, en la Figura 4 se puede ver un esquema del mecanismo de formación de los ésteres glicidílicos.

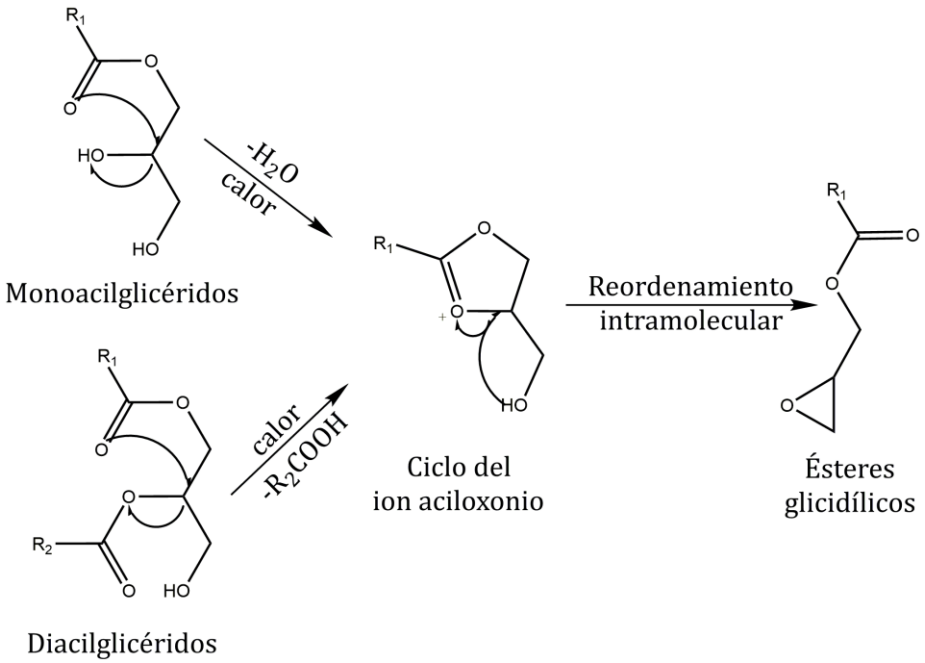

Figura 4. Mecanismo de formación de ésteres glicidílicos [100].

Los ésteres glicidílicos, por otra parte, presentan un mecanismo de formación diferente. Los propios monoacilgliceroles y diacilgliceroles en presencia de calor pierden una molécula de agua o uno de los acilgliceroles junto con un ácido carboxílico respectivamente y al reorganizarse se forma el éster glicidílico. En este caso, no se ha demostrado que los triacilgliceroles sean responsables directos de la formación de estos ésteres [100].

En esta Tesis se ha desarrollado un método de análisis directo para la determinación de 19 ésteres de 3-MCPD y 7 ésteres glicidílicos. La elección de estos ésteres se debe a que se consideran los ésteres mayoritarios que

se producen en aceite de oliva, además de en otros aceites vegetales que podrían estar presentes junto a los aceites de oliva.

2.2.1 Contaminación en aceites vegetales y productos de bollería

El 3-MCPD en los aceites comestibles se forma durante su procesamiento debido principalmente a la reacción del ácido clorhídrico, pero también a otros compuestos que contienen cloruro como $FeCl_3$, $FeCl_2$, $MgCl_2$ y $CaCl_2$, que existen en cantidades de mg/kg en aceite de palma, con triacilgliceroles, fosfolípidos y glicerol [101]. Además, puede aparecer en los procesos térmicos de otros alimentos como productos de panadería, productos derivados de la malta, pescados o carnes, ya sean cocinados o curados. En el caso de la carne, se forman cuando reaccionan los lípidos y el cloruro de sodio, estando estos componentes presentes de forma natural o añadidos al alimento.

Los ésteres de 3-MCPD se pueden formar a temperaturas de 180-200 °C [102], mientras que en un proceso de refinado típico para la eliminación de ácidos grasos libres se alcanzan temperaturas de 240-270 °C [103].

Aunque el refinado se lleve a cabo a temperaturas usualmente por encima de 200 °C, es posible la eliminación de ésteres usando adsorbentes o enzimas adecuadas [104], aunque como se ha demostrado, los esfuerzos para la mitigación de estos compuestos deben empezar con la selección de la materia prima o incluso con el cultivo de la semilla o fruta ya que de esta manera no sería necesario realizar el refinado [105].

2.2.2 Legislación

La Unión Europea estableció recientemente en su Directiva 1322/2020 un límite de 1,00 mg/kg para la suma de ésteres glicidílicos en aceites y grasas comestibles para adultos y de 0,50 mg/kg si se destinan a

la producción de alimentos para bebés o alimentos a base de cereales para lactantes y niños pequeños. Asimismo, se estableció un límite de 1,25 mg/kg (2,50 mg/kg para aceites de orujo) para la suma de ésteres de 3-MCPD y 3-MCPD en aceites y grasas vegetales para adultos, y de 0,75 mg/kg si se destinan a producción de alimentos para bebés o alimentos a base de cereales para lactantes y niños pequeños [106].

2.2.3 Métodos de análisis

Entre los métodos desarrollados para el análisis de ésteres de 3-MCPD y ésteres glicidílicos se pueden distinguir entre métodos directos y métodos indirectos. Generalmente, los métodos indirectos suelen incluir etapas de transesterificación, neutralización y derivatización para cuantificar dichos ésteres como 3-MCPD, 2-MCPD y glicidol libres, lo que supone un tiempo mucho mayor de preparación de muestra que los métodos directos. Por otra parte, un enfoque directo caracteriza y cuantifica cada éster de 3-MCPD, 2-MCPD y glicidol individual en muestras de alimentos, siendo la principal desventaja que presenta este método la cantidad de patrones de ésteres de MCPD necesarios y el coste de los mismos. Aunque no es necesario un patrón para cada éster sí que sería necesario al menos uno representativo de cada familia, o los mayoritarios de cada tipo de aceite. En este sentido habría que contar con los patrones de los ésteres provenientes de los ácidos grasos predominantes en los aceites, como por ejemplo los provenientes de los ácidos oleico, linoleico y linolénico para el caso del aceite de oliva [107]. El estado actual de ambos enfoques se resume a continuación.

2.2.3.1 Técnicas de extracción y limpieza

a) Métodos indirectos

Como se ha comentado anteriormente, los métodos indirectos de análisis de ésteres de 3-MCPD, 2-MCPD y glicidol son aquellos que convierten éstos en 3-MCPD, 2-MCPD y 3-monobromopropanodiol (*3-monobromopropanediol*, 3-MBPD) derivatizados y se cuantifican como 3-MCPD, 2-MCPD y glicidol libres. Estos métodos siguen unos pasos comunes como son la hidrólisis química o enzimática, la neutralización de esta reacción y por último la derivatización de 2-, 3-MCPD y glicidol para un análisis final por GC-MS.

Para el análisis indirecto de ésteres de 2-, 3-MCPD y glicidol existen métodos estandarizados elaborados por diferentes organismos como la Sociedad Estadounidense de Químicos del Aceite (*American Oil Chemists' Society*, AOCS) [108–110] o la Sociedad Alemana de la Ciencia de la Grasa (*Deutsche Gesellschaft Für Fettwissenschaft* (*German Society for Fat Science*, DGF)) [111]. Dentro de estos métodos se diferencia entre hidrólisis ácida o básica. La hidrólisis ácida se realiza en presencia de bromuro de sodio en agua acidificada con ácido sulfúrico, para después realizar una transesterificación utilizando una disolución metanólica con ácido sulfúrico al 1,8%. Esta reacción se produce a 40°C durante 16 horas. Ambas reacciones se detienen usando una disolución de bicarbonato de sodio [108]. En el caso de la hidrólisis básica se emplea hidróxido de sodio en disolución metanólica y se deja reaccionar durante 16 horas entre -22 y -25 °C. Finalmente la reacción se detiene mediante la adición de bromuro de sodio en disolución ácida de ácido sulfúrico [109].

En ambos casos se usa ácido fenilborónico (*phenylboronic acid*, PBA) para realizar la derivatización de los compuestos. Sin embargo, también se han reportado problemas que genera este agente derivatizante, como que se queda retenido en la columna [112]. Es por ello que algunos autores han realizado la derivatización con heptafluorobutirilimidazol (*heptafluorobutyrylimidazole*, HFBI) [113–115].

Los protocolos de la DGF son similares a los de AOCS con la salvedad de que en el método de la hidrólisis básica, el protocolo DGF C-VI 18 (10) [116] utiliza cloruro de sodio en lugar de la disolución acidificada de bromuro de sodio, aunque la utilización de cloruro de sodio puede provocar una sobreestimación de los resultados ya que iones cloruro libres pueden reaccionar para formar 3-MCPD [117].

Ambos métodos son ampliamente aceptados para el análisis de ésteres de 2-, 3-MCPD y glicidol de manera indirecta, y a menudo se emplean como métodos de referencia en el desarrollo de nuevos métodos para el análisis de estos compuestos [112,118–123].

Además de estos métodos, en algunos casos se usa la hidrólisis enzimática. En este caso se emplea una disolución al 1% de Triton-X 100 (t-octilfenoxypolietoxietanol) y la enzima *Candida Antarctica lipasa A* [124,125], la enzima *Candida rugosa lipasa* [126–129] y la enzima *Amano lipasa G* [130].

b) Métodos directos

A diferencia de los métodos indirectos, en los directos se pretende modificar lo menos posible los ésteres, aislándolos al máximo del resto de la matriz para poder cuantificarlos correctamente. Sin embargo, antes de realizar el análisis, se debe eliminar la gran cantidad de acilgliceroles, especialmente triacilgliceroles que contiene el aceite, los cuales afectan negativamente a dicho análisis [131].

Como se puede ver en la Tabla 5, se suele utilizar una LLE usando como disolventes tert-butil metil éter:acetato de etilo (80:20, *v/v*) [132–139], así como cloroformo y acetona [140–144]. En todos los casos se obtienen recuperaciones aceptables excepto un trabajo que ha usado *tert*-butil metil éter:acetato de etilo (80:20, *v/v*), con valores de hasta 163% [139]. Menos frecuente es el uso de otros disolventes como acetonitrilo:2-propanol (50:50, *v/v*) [145,146], la mezcla ciclohexano:acetato de etilo

(50:50, *v/v*) [147], diclorometano para la extracción de monoésteres y *n*-hexano para la extracción de diésteres [114], *n*-hexano y acetonitrilo [148], acetonitrilo [149], acetonitrilo y *n*-heptano [150] o *n*-heptano [151] obteniendo en la mayoría de casos recuperaciones aceptables dentro del rango establecido del 70-120%. También se ha empleado una mezcla compuesta por una disolución 0,6 mM de acetato de sodio en metanol:diclorometano:acetonitrilo (10:80:10, *v/v/v*) para la extracción de 5 ésteres glicidílicos y 20 ésteres de 3-MCPD de aceites vegetales [152].

A diferencia de los disolventes usados para la disolución de la muestra, la parte del método de limpieza está mucho más unificada, ya que la mayoría de autores realizan una doble limpieza mediante SPE, empleando en primer lugar una SPE de RP usando cartuchos C18 para pasar a una SPE de fase normal (*normal phase*, NP) con cartuchos de sílica gel [114,132,133,136–142,144,149,150,153,154]. Además, también se han usado estas mismas etapas de SPE pero en orden inverso, es decir, en primer lugar se hace pasar la muestra por un cartucho de sílica para realizar una SPE de NP para acabar con un cartucho de C18 realizando por último una SPE de RP [139,148]. El hecho de que usualmente se utilicen dos cartuchos de SPE de diferente fase estacionaria se debe a que, tal como se ha indicado, los cartuchos que contienen C18 como fase estacionaria eliminan los triacilgliceroles, mientras que los cartuchos que contienen sílica como fase estacionaria eliminan los diacilgliceroles [139]. Por ello, se han utilizado ambas estrategias según se pretenda extraer monoésteres o diésteres: para la extracción de monoésteres se utiliza la secuencia fase reversa-fase normal (*reverse phase-normal phase*, RP-NP), mientras que para la extracción de diésteres se utiliza la secuencia contraria, fase normal-fase reversa (*normal phase-reverse phase*, NP-RP) [139]. En otro caso, también se ha empleado la secuencia RP-NP para la extracción de monoésteres, mientras que para la extracción de diésteres utiliza solamente SPE en fase normal [114].

Tabla 5: Métodos directos usados para la extracción de ésteres de 3-MCPD y ésteres glicidílicos en aceites.[a]

Matriz	Analitos	Método de extracción	Método de limpieza	R (%)	Ref.
Aceites vegetales	5 ésteres glicidílicos	LLE: CH_3CN	RP-SPE: C18 NP-SPE: Sílica	71-95	[149]
Aceites vegetales	5 ésteres glicidílicos	LLE: $CHCl_3$, acetona	RP-SPE: C18 NP-SPE: Sílica	103-110	[144]
Aceites vegetales	5 ésteres glicidílicos	LLE: TBME:acetato de etilo (80:20, *v/v*)	RP-SPE: C18 NP-SPE: Sílica	96-110	[133]
Aceites vegetales	7 ésteres glicidílicos	LLE: CH_3CN, *n*-heptano	RP-SPE: C18 NP-SPE: Sílica	85-115	[150]
Aceites vegetales	7 ésteres glicidílicos	LLE: ciclohexano:acetato de etilo (50:50, *v/v*)	GPC automatizada	68-111	[147]
Aceites vegetales	7 ésteres de 3-MCPD	LLE: TBME:acetato de etilo (80:20, *v/v*)	d-SPE: Si-SAX, PSA d-SPE: Z-Sep+, PSA	71-123	[135]
Aceites vegetales	14 ésteres de 3-MCPD	LLE: *n*-hexano, CH_3CN	NP-SPE: Sílica RP-SPE: C18	63-109	[148]
Aceites vegetales	18 ésteres de 3-MCPD	LLE: CH_3CN:2-propanol (50:50, *v/v*)	d-SPE: C18, PSA	94-109	[146]
Aceites vegetales	8 monoésteres de 3-MCPD	LLE: CH_2Cl_2	RP-SPE: C18 NP-SPE: Sílica	61-150	[114]
	11 diésteres de 3-MCPD y 1 diéster de 2-MCPD	LLE: *n*-hexano	NP-SPE: Sílica	56-130	
Aceites vegetales	5 ésteres glicidílicos y 20 ésteres de 3-MCPD	LLE: 0,26 mM CH_3COONa en CH_3OH: CH_2Cl_2:CH_3CN (10:80:10, *v/v/v*)	No utilizado	-	[152]

Tabla 5 (continuación)

Matriz	Analitos	Método de extracción	Método de limpieza	R (%)	Ref.
Aceites vegetales	5 monoésteres de 3-MCPD 25 diésteres de 3-MCPD	LLE: TBME:acetato de etilo (80:20, *v/v*)	NP-SPE: Sílica RP-SPE: C18 RP-SPE: C18 NP-SPE: Sílica	78-163	[139]
Aceites refinados	5 ésteres de 2-MCPD y 21 ésteres de 3-MCPD	LLE: *n*-heptano	No utilizado	69-113	[151]

[a] 2-MCPD: 2-monocloropropanodiol; 3-MCPD: 3-monocloropropanodiol; C18: Octadecasilano; d-SPE: Extracción dispersiva en fase sólida; GPC: Cromatografía de permeación en gel; LLE: Extracción líquido-líquido; PSA: Amina primaria secundaria; TBME: *Tert*-butil metil éter; NP-SPE: Extracción en fase sólida normal; RP-SPE: Extracción en fase sólida reversa; Si-SAX: Sílica con intercambio aniónico fuerte.

También se ha usado la técnica d-SPE empleando las sales C18 y PSA [145,146] o dos d-SPE consecutivas utilizando en primer lugar Si-SAX y PSA y en segundo lugar Z-Sep+ y PSA [134,135]. Por último, indicar que Dubois et al. han usado GPC automatizada usando una columna de vidrio rellena con estireno divinilbenceno [147].

Finalmente señalar que otros autores no realizan etapa de extracción y simplemente realizan una dilución con una mezcla de una disolución metanólica 0,26 mM de acetato de sodio:diclorometano:acetonitrilo (10:80:10, *v/v/v*) [152] o *n*-heptano [151] e inyección directa. Esta técnica ofrece la ventaja de que se necesita muy poca preparación de muestra, pero, por otra parte, el instrumento que se usa para realizar el análisis se ensucia más fácilmente. En consecuencia puede existir una posible supresión de señal por coextractos de las muestras de aceite durante la ionización en LC-MS/MS [139].

2.2.3.2 Técnicas de análisis

De nuevo existe una gran diferencia entre el análisis por método directo y por método indirecto. Mientras que por el método indirecto se suele usar GC-MS, por el método directo se suele emplear LC-MS/MS.

a) Métodos indirectos

La cuantificación de 2- y 3-MCPD y glicidol (que ha sido convertido a 3-MBPD en la reacción con NaBr) se lleva a cabo usualmente utilizando GC-MS, con una columna de 5% fenil-metilpolisiloxano de 30 metros de longitud, 0,25 µm de espesor y 0,25 mm de diámetro interno, o de características parecidas. Se usan patrones internos de 2- y 3-MCPD y 3-MPBD deuterados, detectando los iones en modo monitorización de iones seleccionados (*selected ion monitoring*, SIM), empleando los iones *m/z* 147 y 196 para 3-MCPD, *m/z* 196 y 198 para 2-MCPD y *m/z* 146 y 240 para 3-

MBPD, así como los iones *m/z* 150 y 201, *m/z* 201 y 203 y *m/z* 150 y 245 para los mismos compuestos respectivamente deuterados [155].

En la Figura 5 se ve un cromatograma de ejemplo del análisis de 2-MCPD, 3-MCPD y 3-MBPD junto con sus estándares internos correspondientes.

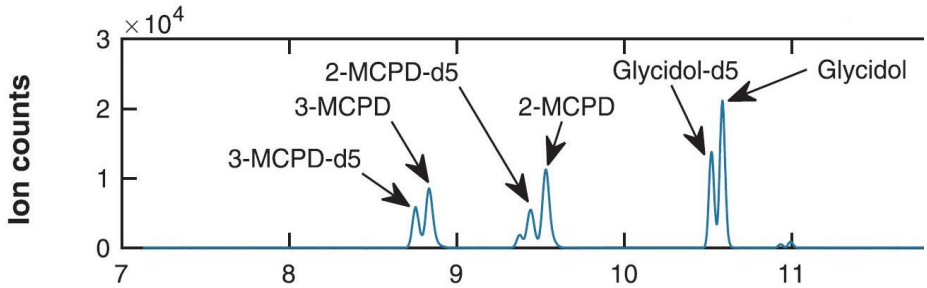

Figura 5. Cromatograma de 2-MCPD, 3-MCPD y 3-MBPD junto con sus estándares internos adaptado de Yan et al. [120].

También se ha realizado la detección de los analitos utilizando espectrometría de masas en tándem (*tandem mass spectrometry*, MS/MS) [120], consiguiendo así una determinación más selectiva que en modo SIM, aunque menos sensible. Comparando ambos métodos se puede comprobar que mientras que en modo SIM se obtienen límites de cuantificación (*limit of quantification*, LOQ) de 0,02 mg/kg [112], en modo monitorización de reacciones múltiples (*multiple reaction monitoring*, MRM) se obtienen LOQ de 0,028, 0,036 y 0,078 mg/kg para 2-, 3-MCPD y glicidol, respectivamente [120].

b) Métodos directos

Como puede observarse en la Tabla 6, todos los estudios se han realizado empleando como fase estacionaria una columna de RP, en su mayoría de C18, excepto algunos trabajos en los que han empleado una columna de NP de sílica [141,142,153,154].

En cuanto a las condiciones de análisis, sólo en un estudio se ha utilizado una fase móvil compuesta por metanol [140], mientras que la mayoría utilizan una fase binaria con un disolvente más polar y otro más apolar. Debido a la alta similitud que presentan los compuestos, es necesario que las fases móviles sean muy parecidas y se vaya modificando el gradiente lentamente para conseguir una buena separación de los compuestos. Es por ello que a menudo se han usado fases acuosas (más polares) y orgánicas (menos polares) compuestas por mezclas de disolventes.

En cuanto a la fase más polar, en la mayoría de los casos se ha empleado una mezcla de metanol:agua (92:8, *v/v*) [134,135,137,138,146,156], aunque en otros casos se ha usado agua [141–143,154], metanol [133], o las mezclas acetonitrilo:metanol:agua (42,5:42,5:15, *v/v/v*) [144,149] y metanol:acetonitrilo (90:10, *v/v*) [152].

Para la fase menos polar, se ha usado simplemente 2-propanol [114,133,144,147,149], una mezcla de 2-propanol y agua (98:2, *v/v*) [134,135,137,138,145,146,156], o bien la mezcla metanol:diclorometano:acetonitrilo (10:80:10, *v/v/v*) [152].

Para favorecer la ionización de los compuestos se ha empleado ácido fórmico [114,141–143,147,154] o acetato de sodio [152], mientras que otros autores trabajan con un tampón mezcla de ácido fórmico y formiato de amonio [134,135,137,138,145,146,156].

Un caso particular es el empleo de una fase ternaria para conseguir una mejor separación empleando una fase de agua, otra de una disolución metanólica de acetato de amonio y otra de metanol [139].

Tabla 6: Condiciones de análisis de ésteres de 2-, 3-MCPD y glicidílicos en aceites por métodos directos en LC.[a]

Analitos	Técnica y analizador	Modo de trabajo y columna	Fase móvil	Tiempo (min)	LOD	Ref.
5 Ésteres glicidílicos	HPLC-Q-MS	RP, C18 150 × 4,6 mm, 5 µm	A: CH_3OH B: 2-propanol	22	25 - 110 µg/kg	[133]
5 Ésteres glicidílicos	HPLC-Q-MS	NP, Sílica 150 × 2,0 mm, 3 µm	A: 0,1% CH_2O_2 en H_2O B: 0,1% CH_2O_2 en CH_3OH	35	28 - 150 µg/kg	[142]
5 Ésteres glicidílicos	HPLC-Q-MS	RP, C18 150 × 4,6 mm, 5 µm	A: CH_3CN:CH_3OH:H_2O (42,5:42,5:15, v/v/v) B: 2-propanol	35	0,67 - 1,0 µg/L	[144]
5 Ésteres glicidílicos	UHPLC-QqQ-MS/MS	RP, C18 100 × 2,1 mm, 1,7 µm	A: CH_3CN:CH_3OH:H_2O (42,5:42,5:15, *v/v/v*) B: 2-propanol	40	1,4 - 3,7 µg/L	[149]
5 Ésteres glicidílicos	HPLC-QqQ-MS/MS	RP, C18 250 × 2,0 mm, 5 µm	CH_3OH	10	1 - 150 µg/kg	[140]
7 Ésteres glicidílicos	UHPLC-QTOF-MS	RP, C18 50 × 2,1 mm, 1,8 µm	A: 0,1% CH_2O_2 en CH_3OH:H_2O (75:25, *v/v*) B: 0,1% CH_2O_2 en 2-propanol	30	-	[147]
7 Ésteres glicidílicos	HPLC-QqQ-MS/MS	RP, C18 50 × 3 mm, 3 µm	A: 0,5% CH_2O_2 en CH_3OH:H_2O (75:25, *v/v*) B: 0,5% CH_2O_2 en 2-propanol	25	-	[147]
7 ésteres de 3-MCPD	HPLC-QqQ-MS/MS	RP, C18 50 × 2,1 mm, 2,6 µm	A: 2 mM NH_4HCO_2 + 0,05% CH_2O_2 en CH_3OH:H_2O (92:8, *v/v*) B: 2-propanol:H_2O (98:2, *v/v*)	15	10 - 25 µg/kg	[135]
14 ésteres de 3-MCPD	UHPLC-QTOF-MS	RP, C18 50 × 2,1 mm, 1,7 µm	A: CH_3OH:H_2O (15:85, *v/v*) B: CH_3OH:H_2O (97,5:2,5, *v/v*)	30	0,16 - 0,86 µg/L	[148]
18 ésteres de 3-MCPD	UHPLC-QqQ-MS/MS	RP, C18 150 × 2,1 mm, 1,7 µm	A: 2 mM NH_4HCO_2 + 0,05% CH_2O_2 en 2-propanol:H_2O (98:2, *v/v*)	45	0,1 - 20 µg/kg	[146]

Tabla 6 (continuación)

Analitos	Técnica y analizador	Modo de trabajo y columna	Fase móvil	Tiempo (min)	LOD	Ref.
			B: 2 mM NH_4HCO_2 + 0,05% CH_2O_2 en CH_3OH:H_2O (92:8, *v/v*)			[146]
18 ésteres de 3-MCPD	HPLC-Q-ORBITRAP-MS/MS	RP, C18 100 × 2,1 mm, 3 µm	A: 2 mM NH_4HCO_2 + 0,05% CH_2O_2 en CH_3OH:H_2O (98:2, *v/v*) B: 2 mM NH_4HCO_2 + 0,05% CH_2O_2 en 2-propanol:H_2O (98:2, *v/v*)	40	0,100 - 53,958 µg/kg	[145]
5 Ésteres glicidílicos y 20 ésteres de 3-MCPD	HPLC-QTOF-MS	RP, C18 50 × 3 mm, 3 µm	A: 0,26 mM CH_3COONa en CH_3OH: CH_3OH:CH_3CN (10:80:10, *v/v/v*) B: 0,26 mM CH_3COONa en CH_3OH: CH_2Cl_2:CH_3CN (10:80:10, *v/v/v*)	24	0,07 - 1,69 mg/kg	[152]
5 ésteres de 2-MCPD y 21 ésteres de 3-MCPD	HPSFC-QTOF-MS	RP, C18 100 × 3 mm, 1,7 µm	CO_2	15	0,5 - 7,5 µg/L	[151]
30 ésteres de 3-MCPD	HPLC-Q-MS	RP, C18 50 × 2,0 mm, 3,2 µm	A: H_2O B: 0,01 mM $NH_4CH_3CO_2$ en CH_3OH C: CH_3OH	40	0,02 - 0,08 mg/kg	[139]
7 Ésteres glicidílicos y 28 ésteres de 3-MCPD	HPLC-QTOF-MS	RP, C18 150 × 2,0 mm, 3,0 µm	A: 2 mM NH_4HCO_2 + 0,05% CH_2O_2 en CH_3OH:H_2O (92:8, *v/v*) B: 2 mM NH_4HCO_2 + 0,05% CH_2O_2 en 2-propanol:H_2O (98:2, *v/v*)	27	30 - 180 µg/kg	[137]

[a] 2-MCPD: 2-monocloropropanodiol; 3-MCPD: 3-monocloropropanodiol; C18: Octadecasilano; GE: Éster glicidílico; GPC: Cromatografía de permeación en gel; HPLC: Cromatografía de líquidos de alta resolución; HPSFC: Cromatografía de fluidos supercríticos de alta resolución; Q-MS: Simple cuadrupolo; QqQ-MS/MS: Triple cuadrupolo; Q-ORBITRAP-MS/MS: Orbitrap; QTOF-MS: Cuadrupolo acoplado a tiempo de vuelo; NP: Fase normal; RP: Fase reversa; UHPLC: Cromatografía de líquidos de ultra-alta resolución.

Por su parte, para el análisis de ésteres de 2-MCPD, 3-MCPD y ésteres glicidílicos se suelen utilizar analizadores de MS. El analizador más simple que se ha usado es el Q, empleándose para un número reducido de compuestos, 5, [132,133,141,142,144,153,154] o 7 [150] aunque algunos autores lo han empleado para el análisis de hasta 30 ésteres de 3-MCPD, con la peculiaridad de que ha empleado una fase móvil ternaria [139].

Por otro lado, QqQ es uno de los analizadores más usados para este tipo de compuestos, habiéndose usado tanto para un número bajo de ésteres glicídílicos, como 5 [140,149] o 7 [147], 7 ésteres de 3-MCPD [134,135], así como para un número elevado de ésteres, habiéndose aplicado para determinar hasta 18 ésteres de 3-MCPD [146].

De igual forma, el analizador QTOF se ha utilizado para el análisis de 7 ésteres glicidílicos [114,147], 14 ésteres de 3-MCPD [148], 14 ésteres de 2-MCPD [138] y 5 ésteres de 2-MCPD y 21 ésteres de 3-MCPD usando en este caso cromatografía de fluidos supercríticos de ultra-alta eficacia, empleando CO_2 como fase móvil [151]. Además, este analizador también se ha utilizado para el análisis combinado de ésteres glicidílicos y ésteres de 3-MCPD [137,152,156].

Por último, se ha utilizado UHPLC acoplada a espectrometría de masas de alta resolución (*high resolution mass spectrometry*, HRMS) con un analizador Q Exactive Orbitrap para el análisis de 18 ésteres de 3-MCPD [145].

3. CONTAMINANTES EXÓGENOS

3.1 Contaminantes polares

Los parásitos son animales o plantas que se consideran dañinos para los seres humanos o los cometidos que realizan los humanos, incluidos cultivos, ganado y silvicultura [157], mientras que los plaguicidas son

sustancias químicas que se utilizan para controlar estos parásitos [158]. Dependiendo del tipo de parásito contra el que actúen estas sustancias se pueden clasificar en herbicidas, insecticidas, nematicidas, molusquicidas, piscicidas, avicidas, raticidas, bactericidas, repelentes de insectos, repelentes de animales, antimicrobianos o fungicidas [159].

Por otro lado, los plaguicidas también se pueden clasificar según sus propiedades químicas en organoclorados, organofosforados y carbamatos. Dentro de los plaguicidas organoclorados se encuentran los diclorodifeniletanos (por ejemplo, dicloro difenil tricloroetano, DDT) y los compuestos de ciclodieno (por ejemplo, clordano), además de otros compuestos relacionados.

En los últimos años, los aminofosfonatos (por ejemplo, glifosato) y carbamatos (por ejemplo, carbofuran) han ido reemplazando a los organoclorados debido a su persistencia y potencial de bioacumulación. Del mismo modo, debido a su toxicidad hacia los organismos vertebrados, algunos organofosforados se han ido sustituyendo por carbamatos menos tóxicos. Dentro de los carbamatos se encuentran los tiocarbamatos y ditiocarbamatos.

Algunos de los compuestos usados habitualmente en la actualidad se identifican como plaguicidas polares, que a veces se definen como plaguicidas con un coeficiente de distribución *n*-octanol-agua (log K_{OW}) menor de 4,5 [160] y son contaminantes preocupantes desde el punto de vista toxicológico [161].

En esta Tesis se ha desarrollado un método para la determinación de etefón, fosetyl-Al, ácido fosfónico, clorato y perclorato. En primer lugar, etefón es empleado como regulador de crecimiento vegetal (p*lant growth regulator*, PGR) y promueve la maduración de los productos vegetales antes y después de su cosecha [162], mientras que fosetyl-Al es un fungicida usado para controlar el moho en una gran variedad de cultivos,

siendo ácido fosfónico su principal producto de degradación [163]. Clorato puede ser causa de contaminación por diversos factores como el empleo de agua de riego desinfectada con cloro, el uso de fertilizantes que contienen clorato o por su presencia natural en el suelo o aguas subterráneas [164], así como que haya podido persistir ya que estuvo permitido como plaguicida hasta 2010 [165]. Por último, perclorato puede aparecer debido a la liberación industrial de esta sustancia en el medio ambiente, ya sea por el uso de perclorato de amonio en propulsores sólidos para cohetes y misiles [166], el uso de fertilizantes con base de nitrato o de la degradación de hipoclorito de sodio empleado para la desinfección del agua [167].

3.1.1 Contaminación en aceites vegetales y frutos secos

Los plaguicidas polares se utilizan en agricultura, tanto a nivel doméstico como industrial. Por tanto, es relativamente fácil que se contaminen los productos agrícolas. Además, estos plaguicidas tienen una persistencia y movilidad diferencial al ser afines al agua, por lo que se transportan muy fácilmente a través de los procesos naturales de movimientos de agua [160].

El transporte de plaguicidas polares a través del medio ambiente puede ser una fuente de contaminación debido a su uso en agricultura, tanto doméstica como industria, ya que pueden entrar en el medio acuoso por diferentes vías, debido a su afinidad higroscópica. Además, los plaguicidas polares tienen diferente persistencia y movilidad ambiental, y la variación temporal en el uso y los procesos naturales (por ejemplo, las precipitaciones) dan como resultado una fluctuación dinámica en las concentraciones acuosas [160]. Esto hace que del medio acuoso puedan acabar en los cultivos. Finalmente, se pueden agregar durante los procesos de postcosecha para mantener la calidad del cultivo si se van a almacenar

por un tiempo prolongado o se van a transportar a grandes distancias [168].

3.1.2 Legislación

El uso de plaguicidas en la Unión Europea está regulado por el Reglamento n° 1107/2009 [169] sobre productos fitosanitarios y soportado en otros reglamentos y directivas de la Unión Europea, por ejemplo, el reglamento sobre MRLs en alimentos, Reglamento (CE) n° 396/2005 [170] y la Directiva sobre el uso sostenible de plaguicidas, Directiva 2009/128/EC [171].

Esta legislación de la UE establece MRLs de casi 650 plaguicidas para 315 productos frescos. Si no existe ninguna legislación para el plaguicida en la matriz estudiada, se establece un MRL por defecto de 0,01 mg/kg. Sin embargo, dependiendo de la combinación de plaguicida/matriz, este MRL podría ser de hasta 20 mg/kg [172].

Algunos de los plaguicidas han sido prohibidos en la Unión Europea, como clorato [170], y por lo tanto, como se ha expresado anteriormente se le aplica un MRL de 0,01 mg/kg. Sin embargo, el clorato es también una sustancia que se forma como subproducto del uso de desinfectantes a base de cloro en la transformación de alimentos y en el tratamiento del agua potable, por lo que se ha establecido un límite de 0,1 mg/kg para almendras, avellanas, pistachos y nueces y 0,05 mg/kg en cacahuetes [173]. Además, perclorato no ha sido incluido como sustancia activa [169]. También se han establecido MRLs para etefón, fosetyl-Al y ácido fosfónico en frutos secos. Por ejemplo, para etefón, se han establecido límites de 0,1, 0,2, 0,1, 0,1, y 0,5 mg/kg en almendras, avellanas, cacahuetes, pistachos y nueces, respectivamente [174]. Del mismo modo se han establecido MRLs para la suma de fosetyl-Al, ácido fosfónico y sus sales en frutos secos: 2 mg/kg en cacahuetes y 500 mg/kg en almendras, avellanas, pistachos y

nueces [175]. Finalmente, cabe resaltar que ninguna de estas sustancias está legislada en aceites comestibles [170], ni están incluidas en la lista de sustancias para las cuales se aplica un factor de procesado cuando se procesa la materia prima [176].

El *Codex Alimentarius* también ha establecido sus propios MRLs y la mayoría de los plaguicidas en aceites y semillas no superan los 0,8 mg/kg, excepto en algunos casos, como el MRL de 5 mg/kg de clormequat en semillas de colza, 70 mg/kg de fluorpyram en semillas de eneldo y de 400 mg/kg de fosetyl-Al en frutos secos [177].

Por otro lado, el Instituto Federal de Evaluación de Riesgos de Alemania (*Bundesinstitut für Risikobewertunk*, BfR) ha proporcionado un listado de factores de procesamiento. Estos factores se deben tener en cuenta a la hora de aplicar la legislación ya que los productos agrícolas a menudo no se consumen crudos, sino que pueden ser sometidos a procesos de transformación, lo cual puede alterar la magnitud de los residuos de plaguicidas que contienen. En este sentido el factor de procesamiento es la relación entre el residuo del producto procesado y el del producto sin procesar. Este factor indica si los residuos de plaguicidas se enriquecen o reducen durante los procedimientos de procesado [176].

3.1.3 Métodos de análisis

Con independencia de que la complejidad de las matrices grasas es un gran desafío en el análisis de residuos de plaguicidas, pocos estudios se centran en el análisis de compuestos polares en matrices de este tipo debido a que la naturaleza polar de estos contaminantes hace poco probable que aparezcan en estas matrices [178]. Todo ello explica que se hayan encontrado pocos trabajos que determinen los referidos compuestos polares en matrices grasas, por lo que se van a referir, a título comparativo, algunos datos relativos a otros plaguicidas polares.

3.1.3.1 Técnicas de extracción y limpieza

El método QuPPe (Método rápido para el análisis de numerosos plaguicidas altamente polares, *Quick Method for the Analysis of Numerous Highly Polar Pesticides*) ha sido desarrollado para, como su nombre indica, el análisis de plaguicidas polares en 4 grupos de alimentos, tales como cereales, legumbres, semillas oleaginosas y por último el grupo de nuestro interés, frutos secos. Para dichas matrices, este método consiste básicamente en la extracción con agua, metanol acidificado al 1% con ácido fórmico, 100 µL de ácido fórmico extra, agitar, y por último añadir una disolución acuosa de ácido etilendiaminotetraacético (*ethylenediaminetetraacetic acid*, EDTA) al 10%. Tras una primera etapa de agitación, se realiza una centrifugación después de congelar o no la muestra, para pasar a una fase de limpieza con acetonitrilo y C18 para eliminar proteínas y lípidos. Se vuelve a agitar y centrifugar y por último se filtra para ser analizado por LC-MS/MS [179]. En la Figura 6 se puede ver un esquema general de este método.

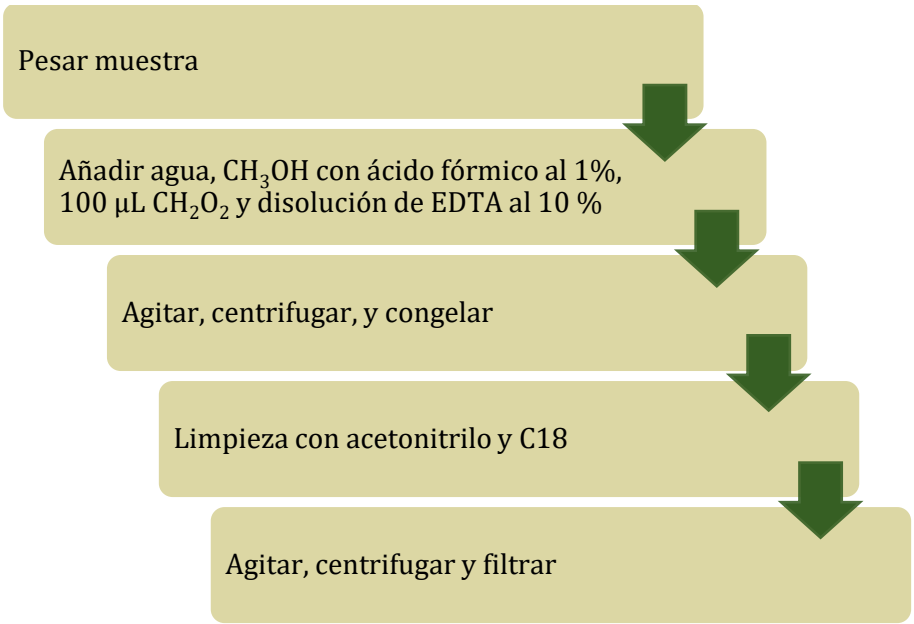

Figura 6. Esquema general del método QuPPe.

Para la extracción de diquat y paraquat, el método QuPPe recomienda realizar una segunda extracción con una mezcla de metanol:agua acidificada con ácido clorhídrico al 0,1M (1:1, *v/v*), agitando durante 1 minuto manualmente y seguidamente agitar durante 15 minutos a 80 °C en un baño de agua [179].

Como se puede observar en la Tabla 7, generalmente se ha adaptado el método QuPPe para el análisis de compuestos polares en aceites. En cuanto a las pequeñas modificaciones realizadas al método QuPPe, en un caso apenas se ha alterado la extracción que usa agua y acetonitrilo con 1% de ácido fórmico para la extracción de 67 plaguicidas en aceite de oliva con recuperaciones entre 40 y 275% [180], mientras que en otros casos se ha usado esta extracción, posteriormente a una con metanol acidificado (1% de ácido fórmico) y agua para la extracción de 7 plaguicidas polares en aceite de oliva, con recuperaciones entre 58 y 121% [181,182]. Algunos autores han optado por extracciones más simples, usando agua para la extracción de glufosinato de aceite de soja con recuperaciones aceptables (80-108%) [183], o agua acidificada con 1% de ácido fórmico para la extracción de glifosato, glufosinato y ácido aminometilfosfónico (*aminomethylphosphonic acid*, AMPA) de aceites vegetales obteniendo recuperaciones entre 81 y 119% [184]. En otro estudio se ha empleado LLE, usando *n*-hexano y dimetil sulfóxido (*dimethyl sulfoxide*, DMSO) como agente extractante para 5 plaguicidas azólicos en aceites vegetales, obteniendo recuperaciones entre 71 y 96% [185]. Por último, comentar la extracción de 19 pesticidas azólicos en aceite de colza en varias fases utilizando *n*-hexano, acetonitrilo acetonitrilo con $HClO_4$ 0,01M, acetonitrilo con HCl 1M, una disolución acuosa de K_2HPO_4 al 10% y diclorometano, obteniendo recuperaciones comprendidas entre 85 y 115%) [186].

Tabla 7: Métodos usados para la extracción de plaguicidas polares en aceites.[a]

Matriz	Analitos	Método de extracción	Método de limpieza	R (%)	Ref.
Aceite de soja	Glufosinato	LLE: H_2O	Derivatización: FMOC-Cl 1%	80-108	[183]
Aceites vegetales	Glifosato, glufosinato y AMPA	LLE: 1% CH_2O_2 en H_2O	No utilizado	81-119	[184]
Aceites vegetales	5 plaguicidas azólicos	LLE: *n*-hexano, DMSO	No utilizado	71-96	[185]
Aceite de oliva	7 plaguicidas polares	LLE: 1% CH_2O_2 en CH_3OH:H_2O (50:50, *v/v*) LLE: 1% CH_2O_2 en CH_3CN	No utilizado	58-121	[181]
Aceite de colza	19 plaguicidas azólicos	LLE: *n*-hexano, CH_3CN, 0,01M $HClO_4$ en CH_3CN, 1M HCl en CH_3CN, 10% K_2HPO_4 en H_2O, CH_2Cl_2	No utilizado	85-115	[186]
Aceite de oliva	67 plaguicidas	LLE: H_2O, 1% CH_2O_2 en CH_3CN QuEChERS: $MgSO_4$, NaCl	a) d-SPE: $MgSO_4$, PSA, C18 b) d-SPE: $MgSO_4$, Z-Sep+ c) d-SPE: EMR, $MgSO_4$, NaCl	40-275	[180]
Almendras	115 plaguicidas	LLE: CH_3CN + IS QuEChERS: $MgSO_4$, NaCl, SCTD, SCDS	a) d-SPE: $MgSO_4$, PSA, C18 b) d-SPE: $MgSO_4$, Z-Sep c) d-SPE: $MgSO_4$, Z-Sep+ d) d-SPE: $MgSO_4$, Sílica	26-111	[187]

[a] AMPA: Ácido aminometilfosfónico; C18: Octadecasilano; d-SPE: Extracción dispersiva en fase sólida; EMR: Eliminación de matriz mejorada; IS: Patrón interno; FMOC-Cl: Cloroformiato de 9-fluorenilmetilo; LLE: Extracción líquido-líquido; PSA: Amina primaria secundaria; QuEChERS: Rápida, Fácil, Barata, Efectiva, Robusta y Segura; SCDS: Citrato de sodio dibásico sesquihidratado; SCTD: Citrato de sodio tribásico dihidratado.

También se han evaluado 4 diferentes limpiezas empleando el método QuEChERS obteniendo los mejores resultados llevando a cabo una extracción con acetonitrilo y $MgSO_4$, NaCl, citrato de sodio tribásico dihidratado y citrato de sodio dibásico sesquihidratado para seguir con una d-SPE usando Z-Sep, ya que se obtienen mejores recuperaciones y además elimina más componentes de la matriz que cuando se ha usado PSA y C18 [187]. En este caso, se han añadido malatión-d_{10} y trifenil fosfato como patrones internos subrogados y dimetoato-d_6 como patrón interno de inyección para el análisis de plaguicidas en almendras [187]. Del mismo modo, en otro trabajo se han evaluado 3 métodos QuEChERS diferentes usando $MgSO_4$, PSA y C18 en uno de los experimentos, $MgSO_4$ y Z-Sep+ en otro caso y una sal de eliminación de matriz mejorada (*Enhanced Matrix Removal*, EMR), $MgSO_4$ y NaCl por último, comprobando que los mejores resultados en términos de eficiencia de extracción y efecto matriz se obtuvieron en el último experimento nombrado, utilizando en primer lugar una extracción QuEChERS con acetonitrilo, $MgSO_4$ y NaCl y después una d-SPE con EMR [180].

En estos dos últimos trabajos mencionados se ha desarrollado un método multirresiduo para el análisis de un elevado número de plaguicidas, entre los que se encuentran compuestos polares y no polares, y los autores señalan que se obtienen las recuperaciones más bajas para los plaguicidas más polares, como por ejemplo un 41% para quinoxifem [187] o un 39% para tiofanato-metilo [180].

3.1.3.2 Técnicas de análisis

Como se ha comentado anteriormente, los plaguicidas polares no suelen estar incluidos en los métodos multirresiduo. Una de las razones es que no suelen ser compatibles con la cromatografía de RP que se emplea para el análisis de la mayoría de plaguicidas. Es por ello que ha aumentado

el interés hacia métodos plurirresiduo de separación para el análisis de compuestos más polares e hidrofílicos, desarrollándose la cromatografía de interacción hidrofílica (*hydrophilic interaction chromatography*, HILIC). En HILIC los analitos polares interactúan con una fase estacionaria polar y se eluyen con una fase móvil mayormente hidrófoba [188].

Para conseguir incluir plaguicidas polares en los métodos multirresiduo, en los últimos años se han intentado desarrollar fases estacionarias mixtas, que funcionen tanto en RP como en modo de intercambio iónico con el fin de poder analizar ambos tipos de analitos, polares y apolares, en un mismo análisis [189]. Por ejemplo, la empresa SIELC Technologies, entre otras, ha desarrollado columnas con funcionalidad de intercambio aniónico y superficie de intercambio catiónico que incorporan partículas modificadas con un ligando hidrófobo (Obelisc R) y su columna análoga con ligando hidrófilo (Obelisc N) [188]. Así, la columna Obelisc N ha permitido el análisis de glifosato en arroz, maíz y soja [190]. A pesar de estas innovaciones, existen pocos métodos que determinen plaguicidas polares en métodos multirresiduo.

Se han comparado diferentes columnas y fases móviles para la determinación de 24 plaguicidas polares en naranja [188]. Las 9 columnas evaluadas son dos HILIC, las dos columnas mixtas Obelisc N y R, tres columnas de NP siendo utilizadas como HILIC y dos columnas C18 de RP. Para realizar los análisis, se ha empleado la técnica de HPLC acoplada a MS/MS con detector QTOF, utilizando modo ESI+ para la determinación de 15 de estos compuestos y el modo ESI- para 7, mientras que 2 compuestos, daminozida e hidrazida maleica, se han analizado tanto en ESI+ como en ESI-. Como conclusión general del trabajo, se extrae que fue imposible encontrar unas condiciones comunes que dieran buenos resultados para el análisis simultáneo de los 24 plaguicidas polares. Además, la columna HILIC de tamaño de partícula 1,8 μm proporcionó los mejores resultados

para todos los compuestos excepto glifosato y AMPA, cuyo análisis mediante la columna Obelisc N en modo ESI- fue óptima [188].

Teniendo en cuenta el resumen de la Tabla 8, en cuanto a las columnas usadas, la de RP de C18 se ha utilizado para el análisis de glufosinato [183], aunque cuando se tiene que separar más de un compuesto se han aplicado columnas de NP como por ejemplo de carbono grafítico poroso para el análisis de glifosato, glufosinato y AMPA [184], o el uso de la columna HILIC para el análisis de 7 plaguicidas polares [181,182].

Como fase móvil acuosa todos los autores usan agua, excepto un trabajo que ha empleado una mezcla de agua y metanol [184]. No ocurre igual con la fase móvil orgánica, ya que mientras que unos autores han usado metanol [180,184], otros han utilizado acetonitrilo [181–183,187]. Para mejorar la ionización de los compuestos, se ha empleado ácido acético [184], ácido fórmico [181,187], formiato de amonio [183] o estos dos últimos reactivos para formar un tampón [181,182].

En la Tabla 8 se puede ver que la técnica y detector más utilizados para el análisis de plaguicidas polares en matrices grasas es HPLC-QqQ-MS/MS [182–184], aunque también se ha usado el analizador QTOF [181].

Por último, en algunos trabajos se ha utilizado GC con detector de ionización con llama (*flame ionization detector*, FID) y con helio como gas portador para analizar 5 plaguicidas azólicos en aceites vegetales [185], así como GC con detector de captura electrónica (*electron capture detector*, ECD) con nitrógeno como fase móvil para el análisis de 19 plaguicidas azólicos en aceite de colza [186].

Tabla 8: Condiciones de análisis de plaguicidas polares en matrices grasas.[a]

Analitos	Técnica y analizador	Modo de trabajo y columna	Fase móvil	Tiempo (min)	LOD	Ref.
Glufosinato	HPLC-QqQ-MS/MS	RP, C18 150 × 4,6 mm, 5 µm	A: CH_3CN B: 5 mM NH_4HCO_2 en H_2O	10	0,6 µg/kg	[183]
Glifosato, glufosinato y AMPA	HPLC-QqQ-MS/MS	NP, PGC 100 × 2,1 mm, 5 µm	A: 1% CH_3COOH en CH_3OH B: H2O:CH_3OH:CH_3COOH (94:5:1, *v/v/v*)	10	-	[184]
7 plaguicidas polares	UHPLC-QTOF-MS/MS	NP, HILIC 100 × 2,1 mm, 1,8 µm	A: 0,1 M NH_4HCO_2 en H_2O a pH 2.85 ajustado con CH_2O_2 B: 0,1% CH_2O_2 en CH_3CN	16	-	[181]
67 plaguicidas	UHPLC-QqQ-MS/MS	RP, C18 50 × 2,1 mm, 1,8 µm	A: 0,1% CH_2O_2 en H_2O B: 0,1% CH_2O_2 en CH_3OH	20	-	[180]
104 plaguicidas (7 polares)	UHPLC-QqQ-MS/MS	NP, HILIC 100 × 2,1 mm, 1,8 µm	A: 0,1 M NH_4HCO_2 en H_2O B: 0,1% CH_2O_2 en CH_3CN	16	-	[182]
115 plaguicidas	HPLC-QqQ-MS/MS	RP, C8 150 × 4,6 mm, 5 µm	A: CH_3CN B: 0,1% CH_2O_2 en H_2O	38	-	[187]
5 plaguicidas azólicos	GC-FID	HP-5MS 30 m × 0,25 mm, 0,25 µm	He	22,8	2,2 – 6,1 µg/kg	[185]
19 plaguicidas azólicos	GC-ECD	HP-5MS 30 m × 0,32 mm, 0,5 µm	N_2	50	1 - 100 µg/kg	[186]

[a] Abreviaturas: 5MS: (5% fenil)-metilpolisiloxano; AMPA: Ácido aminometilfosfónico; C18: Octadecasilano; C8: dimetil n-octilsilano; GC-ECD: Cromatografía de gases con detector de captura electrónica; GC-FID: Cromatografía de gases con detector de ionización con llama; HILIC: Cromatografía interacción hidrofílica; HPLC: Cromatografía de líquidos de alta resolución; NP: Fase normal; PGC: Carbono grafítico poroso; QqQ-MS/MS: Triple cuadrupolo; QTOF-MS: Cuadrupolo acoplado a tiempo de vuelo; RP: Fase reversa; UHPLC: Cromatografía de líquidos de ultra-alta resolución.

3.2 Aceites minerales

Los aceites minerales son productos derivados o producidos sintéticamente a partir de carbón, el gas natural y la biomasa. Están compuestos por MOH, lineales o ramificados (parafinas) [191]. Estos aceites minerales se utilizan como aceites lubricantes para la maquinaria empleada en la industria alimentaria [192].

Dentro de los MOH se pueden distinguir dos categorías, alifáticos, que se denominan hidrocarburos saturados de aceite mineral (*mineral oil saturated hydrocarbons*, MOSH) (Figura 7) o aromáticos, denominados hidrocarburos aromáticos de aceite mineral (*mineral oil aromatic hydrocarbons*, MOAH) (Figura 8). Estos MOSH y MOAH están compuestos por hidrocarburos con un número de carbonos entre C15 y C50.

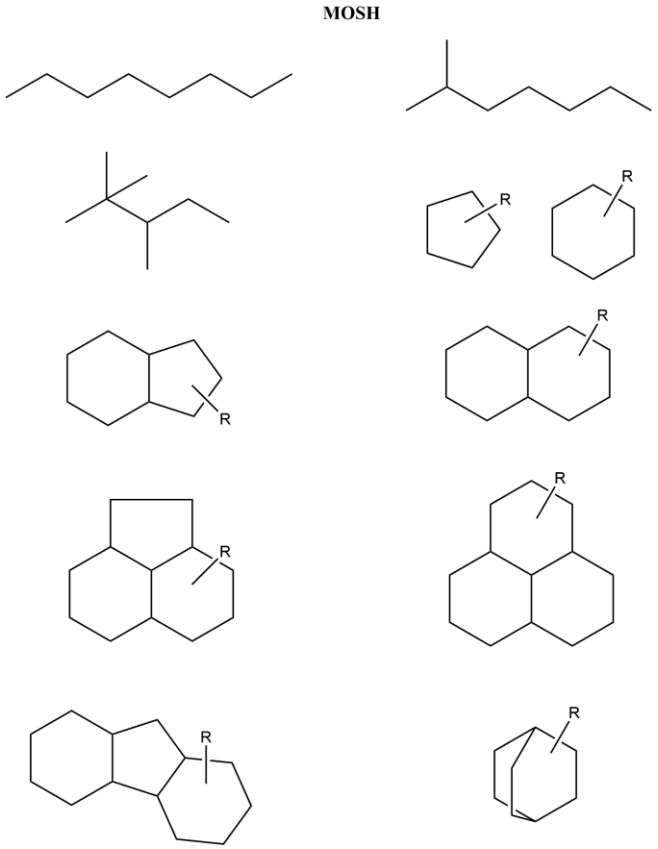

Figura 7. Ejemplos de estructuras de MOSH

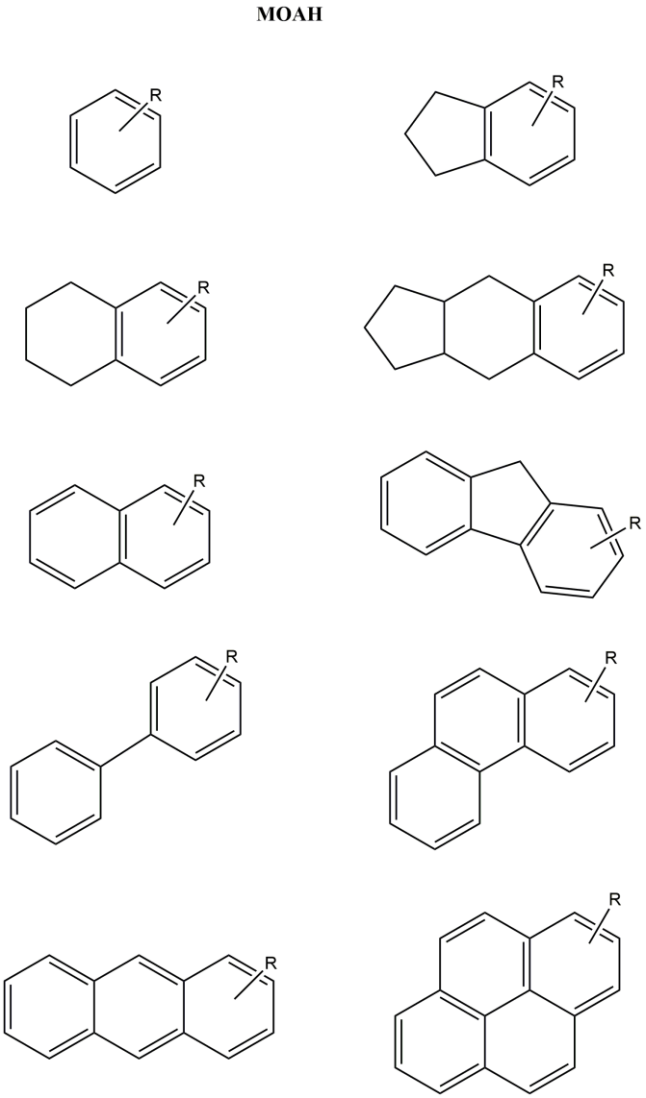

Figura 8. Ejemplos de estructuras de MOAH

Como se ha demostrado en varios estudios, los MOSH se acumulan en el cuerpo humano, especialmente en los tejidos, mientras que los MOAH presentan potencial carcinogénico [193].

3.2.1 Contaminación en aceites vegetales

La contaminación de los alimentos con este tipo de analitos se produce de manera relativamente sencilla y fácil. Solo el contacto de los alimentos con materiales tan simples como el cartón, las tintas, o los aceites minerales utilizados en la maquinaria empleada durante el proceso de fabricación del aceite o incluso los aditivos alimentarios son fuentes de contaminación de MOSH y MOAH [194].

Además, los MOH pueden aparecer en aceites comestibles cuando sus semillas han sido sometidas a procesos de extracción duros, como centrifugación o extracción con disolventes [195]. De hecho, el contenido de estos compuestos en los aceites, que se someten a estos procesos como los aceites de orujo, es considerablemente mayor.

Finalmente, en el caso de los aceites comestibles, el fraude puede ser una fuente importante de contaminación, al agregar intencionalmente aceites minerales a los aceites comestibles, lo que puede suponer un riesgo muy grave para la salud humana.

3.2.2 Legislación

En primer lugar, sería conveniente aclarar que la detección de estos compuestos no se hace de igual manera que la mayoría de analitos, donde en un cromatograma aparece un pico correspondiente al compuesto objetivo, sino que lo que aparece es una "joroba", o elevación de la señal cromatográfica, a lo largo de un rango de hidrocarburos, normalmente entre C15 y C50. Este concepto hay que tenerlo presente cuando se habla de la legislación de estos compuestos. En la Figura 9 se ve un cromatograma típico de una muestra contaminada con MOH entre el minuto 16 y 34 aproximadamente, lo que corresponde al rango de hidrocarburos entre C15 y C35 aproximadamente.

Para la cuantificación de los MOH, según la Guía del Centro Común de Investigación (*Joint Research Centre*, JRC) de la Comisión Europea [196], se integra la "joroba" y se le restan los picos que sobresalen por encima de ella, ya que son hidrocarburos naturales del aceite y no pertenecen a la fracción MOH.

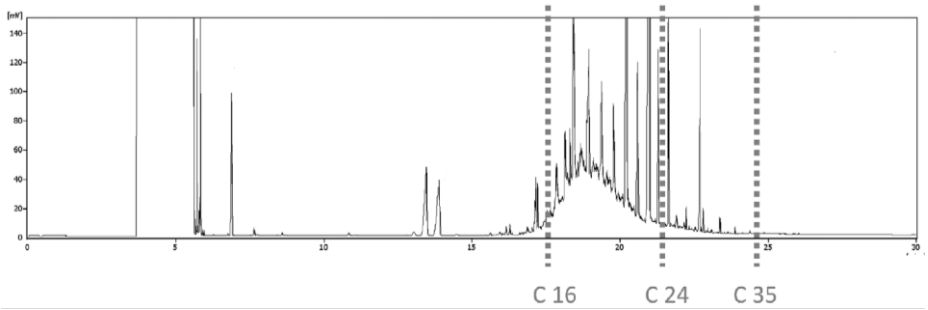

Figura 9. Cromatograma típico de una muestra de aceite contaminada con MOH.

La Comisión Europea ha establecido un límite de 50 mg/kg de MOH para los aceites de girasol crudos y refinados [197], mientras que para el aceite de orujo de oliva crudo, el límite es de 250 mg/kg con la condición de que la relación *n*-alcanos/"joroba" debe ser superior a uno [195].

3.2.3 Métodos de análisis

Se han realizado varios estudios para aislar y analizar MOH, distinguiendo entre métodos que realizan la extracción-separación *offline* y los que lo hacen *online*.

3.2.3.1 Técnicas de extracción y limpieza

El primer método desarrollado consistió en una LLE con *n*-hexano y a continuación una separación por SPE para enriquecer la muestra. El cartucho de SPE se preparó manualmente en el laboratorio con sílica y se recogió el extracto con una disolución de *n*-hexano:CH_2Cl_2 (80:20, *v/v*) [198].

Seguidamente, se llevó a cabo una epoxidación, para conseguir eliminar en la medida de lo posible las olefinas naturales. Esta epoxidación se realizó añadiendo una alícuota de la disolución del aceite con *n*-hexano a un tubo de 15 mL. A continuación, se le añadió diclorometano y una solución del agente epoxidante, ácido *meta*-cloroperbenzoico (*meta-chloroperbenzoic acid*, *m*CPBA). Para detener la reacción se empleó una disolución de carbonato de sodio, y nuevamente se realizó otra LLE, esta vez con agua y diclorometano, recogiendo una alícuota de este último disolvente [198]. A partir de este procedimiento se han desarrollado el resto de métodos publicados realizando pequeñas modificaciones. La Figura 9 muestra un esquema del método desarrollado por Biedermann et al. [198].

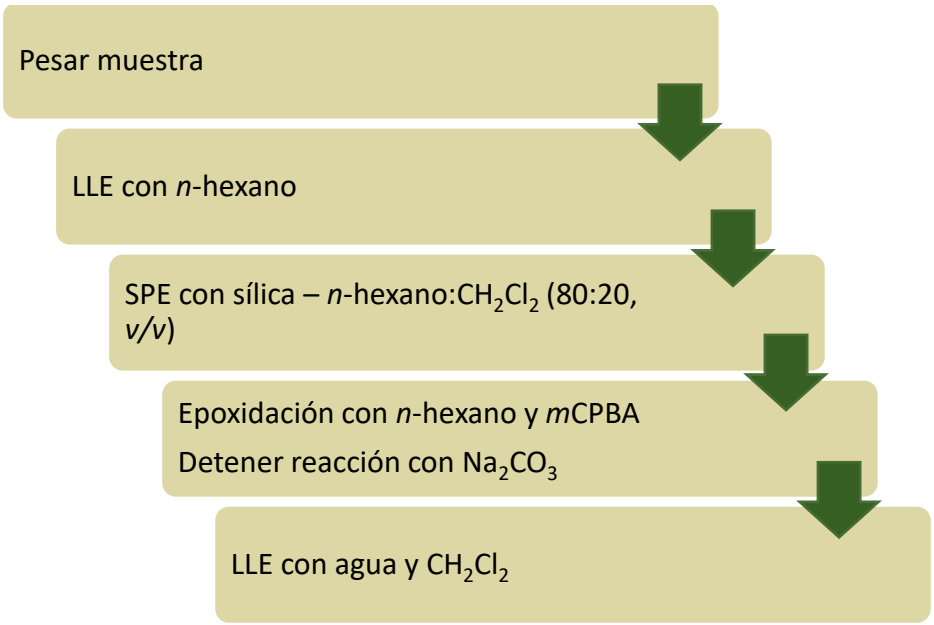

Figura 9. Esquema del método desarrollado por Biedermann et al. [198].

Este método ha sido usado por otros autores [199], pero merece prestar atención a intentos de mejora del mismo como se detalla a continuación. La Tabla 9 muestra un resumen de dichos cambios.

Tabla 9: Métodos usados para la extracción de MOH en aceites vegetales.[a]

Matriz	Analitos	Método de extracción	Método de limpieza	R (%)	Ref.
Aceites vegetales	MOSH	LLE: *n*-hexano	Automatizado *online*	-	[200]
Aceites vegetales	MOSH	LLE: *n*-hexano	SPE: Sílica con 10% Ag	>97	[201]
Aceites vegetales	MOSH	No utilizado	SPE: Sílica con 10% Ag	-	[192]
Aceites vegetales	MOSH	LLE: *n*-hexano	SPE: Sílica con 1% Ag	84-87	[202]
Aceites vegetales	MOAH	Epoxidación: *n*-hexano, *m*CPBA, etanol, $Na_2S_2O_3$	Automatizado *online*	95-102	[203]
Aceite de oliva virgen	MOSH/MOAH	LLE: *n*-hexano Epoxidación: CH_2Cl_2, *m*CPBA, Na_2CO_3 LLE: CH_2Cl_2	Enriquecimiento: SPE: Sílica	-	[198]
Aceites vegetales	MOSH/MOAH	LLE: *n*-hexano Epoxidación: CH_2Cl_2, *m*CPBA, Na_2CO_3	SPE: Sílica y sílica con 0,3% Ag	-	[204]
Aceites vegetales	MOSH/MOAH	LLE: *n*-hexano	Automatizado *online*	-	[205]
Aceites vegetales	MOSH/MOAH	LLE: *n*-hexano:CH_2Cl_2 (70:30, *v/v*) Epoxidación: *n*-hexano:CH_2Cl_2 (70:30, *v/v*), *m*CPBA, ácido ascórbico	SPE: Al_2O_3, Sílica	-	[206]

[a] LLE: Extracción líquido-líquido; *m*CPBA: Ácido *meta*-cloroperbenzoico; MOAH: Hidrocarburos aromáticos de aceites minerales; MOSH: Hidrocarburos saturados de aceites minerales; SPE: Extracción en fase sólida.

En cuanto a la extracción, todos los trabajos han usado LLE con *n*-hexano, excepto uno de ellos, que no ha empleado LLE antes de realizar la SPE [192,195].

Algunos autores han utilizado una epoxidación ligeramente modificada, normalmente usando como disolvente *n*-hexano [203], diclorometano [204] o la mezcla *n*-hexano:diclorometano (70:30, *v/v*) [206]. En cuanto al agente epoxidante, todos han empleado *m*CPBA, aunque sí que hay modificaciones en el uso del reactivo, empleando tiosulfato de sodio [203] o ácido ascórbico [206].

En relación a la limpieza a través del cartucho de SPE, aunque se han empleado sorbentes de óxido de aluminio y sílica [206], en general se ha observado que adicionando plata a la sílica se retienen mejor las olefinas [203]. El porcentaje de plata que se le agrega a la sílica ha sido objeto de estudio variando desde 0,3% [204], 1% [202,207], hasta el 10% [192,195,201].

Alternativamente, como se puede ver en la Tabla 9, para el análisis de MOSH y MOAH, varios autores han realizado una SPE *online* [200,203,205]. Esta técnica ofrece la ventaja de poder usar volúmenes menores de disolventes para obtener los mismos límites de detección y cuantificación, además de disminuir la fuente de error provocada por la preparación manual de cartuchos, pero en cambio requiere una mayor inversión en equipamiento ya que combina en tándem LC-GC-FID.

3.2.3.2 Técnicas de análisis

La mayoría de los artículos consultados han realizado una separación de MOSH y MOAH mediante LC y a continuación el análisis por GC-FID. Debido a la complejidad de las "jorobas" analizadas, se ha concluido que el mejor analizador que proporciona una respuesta selectiva y sensible a los

MOH es FID, no utilizándose de manera habitual MS. El primer autor que propuso esta estrategia fue Biedermann et al., que realizó la separación de MOSH y MOAH empleando una columna de NP de sílica para la separación de los grupos de compuestos en LC, y una columna de 99% de metil polisiloxano (*methyl polisiloxane*, MPS) para el análisis en GC [198]. Esta configuración instrumental fue empleada por otros autores [199,204], aunque se han ido realizando diversas modificaciones, como se detalla a continuación.

Como se puede observar en la Tabla 10, todos los trabajos que han utilizado separación por LC, han empleado columnas de NP de sílica [199,200,203–206]. Cabe destacar que en un estudio se han empleado tres columnas en tándem para la separación de MOSH y MOAH [205]. Además, se han usado las mismas fases móviles que Biedermann et al., es decir, *n*-hexano y diclorometano, excepto en un caso que se ha utilizado solamente *n*-hexano [200].

Como se puede observar en la Tabla 10, usando la técnica LC-GC-FID, la columna de 95% de MPS se ha empleado en la mayoría de los casos ya sea para el análisis de MOSH [200], o el análisis de ambas fracciones, MOSH y MOAH [205,206]. Otros autores han utilizado una columna de 99% de MPS para el análisis de MOSH y MOAH [198,199,204] y, por último, se ha empleado una columna con un 100% de dimetil polisiloxano (*dimethyl polisiloxane*, DMPS) [203].

Por otro lado, algunos trabajos no han utilizado LC, pero a su vez no han separado las fracciones MOSH y MOAH, sino que solamente han analizado la fracción MOSH. Para su análisis, se ha utilizado la técnica GC-FID con columna de 95% de MPS [192,195,201,202,207].

En cuanto a las fases móviles de GC, algunos artículos han empleado helio [198,199,201,204,205], otros hidrógeno [192,195,200,203] y otros nitrógeno [202,207].

Tabla 10: Condiciones de análisis de MOSH y MOAH.[a]

Analitos	Técnica y analizador	Modo de trabajo y columna	Fase móvil	Tiempo (min)	LOD	Ref.
MOSH	LC-GC-FID	LC: NP, Sílica 100 × 3 mm, 5 µm GC: SLB-5ms 15 m × 0,1 mm, 0,1 µm	LC: *n*-hexano GC: H_2	5,4	0,6 mg/kg	[200]
MOSH	GC-FID	PS-255 10 m × 0,25 mm, 0,15 µm	He	14	5 mg/kg	[201]
MOSH	GC-FID	OV-5 10 m × 0,32 mm, 0,10 µm	H_2	29,2	5 mg/kg	[192]
MOSH	GC-FID	DB-5HT 15 m × 0,25 mm, 0,1 µm	N_2	32,4	-	[202]
MOAH	LC-GC-FID	LC: NP, Sílica 250 × 2,1 mm, 5 µm GC: MTX-1 (100% DMPS) 15 m × 0,25 mm, 0,1 µm	A: *n*-hexano B: CH_2Cl_2 H_2	18	-	[203]
MOSH/MOAH	LC-GC-FID	LC: NP, Sílica 250 × 2,1 mm, 5 µm GC: PS-255 (99% MPS) 15 m × 0,25 mm, 0,15 µm	A: *n*-hexano B: CH_2Cl_2 He	23	3 mg/kg	[198]
MOSH/MOAH	LC-GC-FID	LC: NP, Sílica 150 × 3 mm, 5 µm + 250 × 2,1 mm, 5 µm + 150 × 1 mm, 5 µm GC: SLB-5ms 30 m × 0,25 mm, 0,25 µm	A: *n*-hexano B: CH_2Cl_2 He	15,9	0,1 mg/kg	[205]

Tabla 10 (continuación)

Analitos	Técnica y analizador	Modo de trabajo y columna	Fase móvil	Tiempo (min)	LOD	Ref.
MOSH/MOAH	LC-GC-FID	LC: NP, Sílica 250 × 2,1 mm, 5 µm GC: DB-5MS 15 m × 0,25 mm, 0,25 µm	-	-	1,5 mg/kg	[206]

[a] 5MS: (5% fenil)-metilpolisiloxano; AMPA: Ácido aminometilfosfónico; C18: Octadecasilano; C8: Dimetil n-octilsilano; DMPS: Dimetilpolisiloxano; ECD: Detector de captura electrónica; FID: Detector de ionización con llama; GC: Cromatografía de gases; LC: Cromatografía de líquidos; MOAH: Hidrocarburos aromáticos de aceites minerales; MOSH: Hidrocarburos saturados de aceites minerales; MPS: Metilpolisiloxano.

Tras el estudio bibliográfico realizado y considerando la opinión del Comité Oleícola Internacional en el sentido de usar métodos de análisis en aceite que no resulten costosos en su implantación en laboratorios de rutina dado el extenso control que se realiza a este producto agroalimentario a nivel mundial, en la presente Tesis se va a optar por estudiar mejoras del método *offline* para MOSH y MOAH.

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CAPÍTULO II

DETERMINACIÓN DE CONTAMINANTES ENDÓGENOS EN ACEITES VEGETALES Y OTRAS MATRICES DE ALTO CONTENIDO GRASO

1. INTRODUCCIÓN

Los consumidores valoran cada día más la calidad y la seguridad alimentaria. La comercialización de alimentos está frecuentemente regulada por normas de obligado cumplimiento, especialmente en el ámbito de la seguridad alimentaria. Algunas de ellas se aplican internacionalmente, y otras solo en ciertos países, por lo que en ocasiones pueden actuar como barreras comerciales, dificultando la circulación de productos sobre la base de criterios científicos discutibles. Éste pudiera ser el caso de "indicadores de calidad" aplicados al aceite de oliva, producto del que España es el mayor productor a nivel mundial. En consecuencia, es de interés avanzar en información analítica en términos de seguridad, bien soportada científicamente, para la caracterización de productos alimentarios de alto contenido graso.

Como se ha comentado a lo largo de esta Tesis, se puede diferenciar entre contaminación endógena y exógena. Entre los contaminantes endógenos se encuentran las micotoxinas, y ésteres de 3-MCPD y ésteres glicidílicos.

Las micotoxinas son metabolitos secundarios producidos por ciertas especies de hongos durante su proceso de digestión [1], que se producen cuando se dan determinadas condiciones ambientales, como altas temperaturas y humedad relativa y lluvia, ya que se favorece la proliferación micótica [2,3].

Por otro lado, los ésteres de 3-MCPD y ésteres glicidílicos se forman durante el proceso de refinado de aceites comestibles [4] mediante una reacción con cloruro de hidrógeno en el caso de los ésteres de 3-MCPD [5], y mediante un reordenamiento provocado por la alta temperatura en el caso de los ésteres glicidílicos [6].

Debido a la complejidad que presentan las matrices grasas, el desarrollo de los métodos de extracción de los analitos de interés, en este

caso micotoxinas y ésteres de 3-MCPD y ésteres glicidílicos, supone un gran reto. Es por ello que, a pesar de existir diversos métodos de análisis de estos compuestos en aceites, frutos secos y productos elaborados, éstos suelen ser complejos y requieren un tiempo elevado.

El método QuEChERS ha sido empleado en algunos casos para la extracción de micotoxinas en aceites vegetales [7,8] y frutos secos [9–11], aunque los tiempos empleados para el análisis se pueden considerar elevados. Del mismo modo, existen dos tipos de métodos para la cuantificación de ésteres de 3-MCPD y ésteres glicidílicos, los métodos indirectos, que transforman estos ésteres en 3-MCPD y glicidol libres, y los métodos directos, con los que se puede cuantificar individualmente los distintos ésteres presentes en la muestra. En cuanto a los métodos directos, existen métodos para llevar a cabo la extracción de ésteres de 3-MCPD y ésteres glicidílicos, aunque solamente un trabajo analiza ambos tipos de ésteres a la vez, y lo hace mediante LC-QTOF [12], un analizador de alta resolución, que no siempre está disponible en los laboratorios de rutina.

Por todo lo expuesto anteriormente, el primer objetivo de este capítulo es, en primer lugar, realizar una revisión exhaustiva de la presencia de contaminantes, así como de los métodos analíticos empleados recientemente para su determinación en aceites y semillas oleaginosas (Publicación I).

Por otra parte, se pretende aplicar la metodología QuEChERS para la extracción de micotoxinas y su posterior análisis mediante UHPLC-QqQ-MS/MS en aceites vegetales (Publicación II) y en frutos secos (Publicación III). Finalmente se desarrolla un método simple para la extracción de ésteres de 3-MCPD y ésteres glicidílicos, como es la extracción por d-SPE, para realizar su determinación mediante UHPLC-QqQ-MS/MS en aceites vegetales y productos elaborados (Publicación IV).

Las publicaciones incluidas en este capítulo son las siguientes:

I. Occurrence and determination of contaminants in edible oils and oilseeds. **Hidalgo-Ruiz, J. L.**; Romero-González, R.; Martínez Vidal, J. L.; Garrido Frenich, A. *Oil and Oilseed Processing: Opportunities and Challenges*. Ed. Wiley Blackwell. **2021**, 149-181.

II. A rapid method for determination of mycotoxins in edible vegetable oils by ultra-high performance liquid chromatography-tandem mass spectrometry. **Hidalgo-Ruiz, J. L.**; Romero-González, R.; Martínez Vidal, J. L.; Garrido Frenich, A. *Food Chemistry*. **2019**, *288*, 22–28.

III. Determination of mycotoxins in nuts by ultra high-performance liquid chromatography-tandem mass spectrometry: Looking for a representative matrix. **Hidalgo-Ruiz, J. L.**; Romero-González, R.; Martínez Vidal, J. L.; Garrido Frenich, A. *Journal of Food Composition and Analysis*. **2019**, *82* (March), 103228.

IV. Determination of 3-monochloropropanediol esters and glycidyl esters in fatty matrices by ultra-high performance liquid chromatography-tandem mass spectrometry. **Hidalgo-Ruiz, J. L.**; Romero-González, R.; Martínez Vidal, J. L.; Garrido Frenich, A. *Journal of Chromatography A*. **2021**, *1639*, 461940.

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PUBLICACIÓN I

Occurrence and determination of contaminants in edible oils and oilseeds

Hidalgo-Ruiz, J. L.; Romero-González, R.; Martínez Vidal, J. L.; Garrido Frenich, A.

Oil and Oilseed Processing: Opportunities and Challenges. Ed. Wiley Blackwell. **2021**, 149-181.

8

Occurrence and Determination of Contaminants in Edible Oils and Oilseeds

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8.1 Introduction

In 2018, the Rapid Alert System for Food and Feed (RASFF) has received a total of 3699 original notifications about food safety issues, and 1118 have been classified as an alert. This number of original alerts has been increasing since 2012, when 522 of them were received by the RASFF (Rapid Alert System for Food and Feed 2019). This means that in only seven years, the number of original alerts has increased more than seven fold, which gives us a perspective about the increasing concern about contaminants in food. Oils and seeds contribute significantly to these 3699 notifications: 25 were related to oils, while 667 were made for nuts and seeds, which comprise almost 20% of the total number of notifications (Figure 8.1) (Rapid Alert System for Food and Feed 2019).

The contamination of crops, and hence, their harvest, can come from several sources, either natural or anthropogenic. Natural sources are those that are not added willfully, as those substances that appear in production, storage, or transportation processes and these include mycotoxins. On the other hand, there are other contaminants that come from human activities. Within this group there are compounds that are intentionally added to the crops to protect them from infections, such as pesticides used to increase production yields, which can be detected in the samples as residues. There are also other substances that come from food processing processes such as materials that are in contact with food or environmental and/or ubiquitous contaminants, such as polycyclic aromatic hydrocarbons (PAHs), 3-monochloropropane-1,2-diol (3-MCPDs), mineral oils, or phthalates, among others.

Oil and Oilseed Processing: Opportunities and Challenges, First Edition.
Edited by Tomás Lafarga, Gloria Bobo, and Ingrid Aguiló-Aguayo.

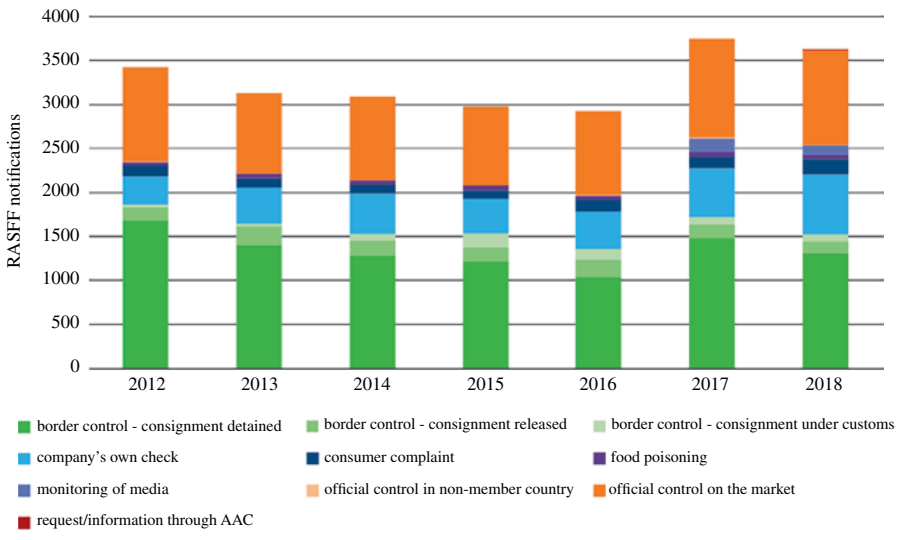

Figure 8.1 Evolution of total RASFF notifications between 2012 and 2018.

The appearance of mycotoxins in crops can happen either before or after harvest. Crops in general, including oils and seeds in this case, can be affected by mycotoxins produced by *Aspergillus* species, such as aflatoxins or *Fusarium* species, such as zearalenone and its derivatives (World Health Organization, and International Agency for Research on Cancer 2010). Oils and seeds can be easily contaminated with mycotoxins due to environmental conditions, such as high temperatures, relative humidity, and rainfall, as these favor fungal proliferation and as a direct consequence, mycotoxin production (Hidalgo-Ruiz et al. 2019a, b).

PAHs can be included in both natural and anthropogenic sources. They can appear during the degradation of vegetal matter carried out by plants and bacteria or formed when organic substances are burnt or exposed to high temperatures under low or no oxygen conditions, which occur in the treatment processes that oils are submitted to, such as refining (Abdel-Shafy and Mansour 2016).

Contaminants can also be produced during food processing similar to those developed during refining, i.e. 3-MCPDs and its glycidyl esters, and PAHs. 3-MCPD is formed in the reaction of hydrochloric acid with triacylglycerols, phospholipids, and glycerol in the refining process in the case of edible oils, but also during thermal processes commonly used during the production of bakery products, malt-derived products, cooked/cured fish, or meat. In the latter, they are formed when lipids react with sodium chloride, which is naturally present or added to foods (Baer et al. 2009). Moreover, other food- processing steps like extractions or even packing can contaminate the product with mineral oils through leaks of lubricating oil or the direct contact of the product with inks, plastics, or paperboard (Brühl 2016). Also, mineral oil hydrocarbons (MOHs) can appear in edible oils when their seeds have been submitted to hard extraction processes such as centrifugation or solvent extraction. Indeed, the content of these compounds in oils, which are submitted to these processes like pomace oils, is considerably high (Gómez-Coca et al. 2016a). Something similar happens with the phthalates present in plastic materials.

They are fat-like and therefore they are easily released from plastic containers and commonly found in fat-containing foods as phthalates are not covalently bonded to the plastic (Rudel and Perovich 2009). Furthermore, oils and oilseeds are also commonly contaminated with pesticides due to the use of these compounds to increase the yields, or during the postharvest processes to maintain quality if the product is going to be stored for a long period (Amvrazi 2011).

Contamination often generates important economic losses due to the risk to the population and therefore, the profit of the product may be reduced. In order to control chemical contaminants present in the final commercial products, governments and international organizations have established maximum residue limits (MRLs) at toxicologically acceptable levels for all the contaminants present in oils or seeds as mycotoxins, PAHs, 3-MCPDs, glycidyl esters, MOHs, phthalates, or pesticides. Among these organizations, the European Union (EU), the Food and Drug Administration (FDA), Ministry of Health of the People's Republic of China, the CODEX Alimentarius Commission of the Food and Agriculture Organization of the United States (FAO) and the World Health Organization (WHO) have set different regulations related to the presence of these substances in oily matrices (Ma et al. 2016). The analysis and monitoring of chemical contaminants in oils and seeds is a challenge because of the complexity of the matrices. Lipid matrices usually require a deeper cleaning because of the high number of interferences that may be coextracted. Thus, many analytical methods employ clean-up procedures. Also, several analytical methods have been developed for the determination of all the contaminants indicated earlier, being chromatographic methods the most widely used.

In this chapter, chemical contaminants such as mycotoxins, PAHs, 3-MCPDs, mineral oils, phthalates, and pesticides present in edible oils and oilseeds will be studied. Furthermore, extraction, analytical techniques, and occurrence of them from 2010 will be reviewed in the target matrices.

8.2 Mycotoxins

Mycotoxins are secondary metabolites produced by certain species of fungi during their digestion processes (Adeyeye 2016). Some of these mycotoxins, such as aflatoxins, have been classified as group 1 human carcinogens by the World Health Organization (World Health Organization, and International Agency for Research on Cancer 2010). Among the mycotoxins that can cause any kind of illness, aflatoxins, citrinin, fumonisins, ochratoxin A, patulin, trichothecenes, zearalenone, or ergot alkaloids are the most important compounds (Bennett and Klich 2003).

8.2.1 Sources of Contamination

Aflatoxins usually affect crops including cereals, oilseeds, spices, and tree nuts (World Health Organization, and International Agency for Research on Cancer 2010). At certain environmental conditions, such as high temperatures, relative humidity, and rainfall, the contamination of the stored product occurs. These conditions favor fungal proliferation, which leads to the mycotoxin production that contaminates foods (Bahrami et al. 2015; Bhat and Reddy 2017; Hidalgo-Ruiz et al., 2019a, b). Because

these compounds are easily produced, the FAO appraises that more than a quarter of all agricultural products are contaminated with them (Marin et al. 2013). To cite an example of the hazard of these compounds, in 2004, 125 people died and more than 200 fell ill in Kenya due to the consumption of contaminated corn (Ongoma 2013).

8.2.2 Legislation

The Joint Expert Committee on Food Additives (JECFA) evaluated the hazards derived from the consumption of these mycotoxins in food. Consequently, the European Commission has stablished a limit of 400 μg/kg of zearalenone in refined corn oil. Regarding the seeds, different criteria have been adopted by different countries around the world. Japan has set a limit of 10 μg/kg of aflatoxin B1 for mustard, rapeseed, soybean, and sunflower seeds, while 5 μg/kg is the limit for the same seeds in Russia. Also, a limit of 35 μg/kg for the sum of aflatoxins has been set for rapeseed, soybean, and sunflower seeds in Malaysia. Some countries have adopted the same limits for soybean and sunflower seeds. These limits are: 50 and 20 μg/kg for the sum of aflatoxins in soybean and sunflower seeds respectively in Brazil, Canada, Egypt, and Iran. Finally, a maximum of 5 μg/kg of aflatoxin B1 is allowed for soybean in China, while a maximum of 10 μg/kg of the same aflatoxin is permitted in Kenya (Bhat and Reddy 2017).

8.2.3 Analysis

8.2.3.1 Sample Treatment

Liquid–liquid extraction (LLE) combined with the QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method are widely used for the extraction of mycotoxins in edible oils and seeds. The most used solvents and salts are acetonitrile : water (4 : 1, v/v) with Na_2SO_4 and NaCl (Hidalgo-Ruiz et al. 2019a; Zhao et al. 2016), water and acetonitrile : formic (95 : 5, v/v) with $MgSO_4$ and NaCl (Eom et al. 2017), or acetonitrile alone with dispersive solid phase extraction (d-SPE) and $MgSO_4$ (Sharmili et al. 2016). Also, other extraction methods have been evaluated, such as magnetic solid phase extraction (MSPE), where magnetic Fe_3O_4 nanoparticles are dispersed into the sample solution to extract the analytes (Yu et al. 2019; Zhao et al. 2017), molecularly imprinted polymers SPE, that are tailor-made polymers with a predetermined selectivity toward a given analyte or a group of structurally related species (Wei et al. 2015), or gel permeation chromatography (GPC) that performs a separation based on the hydrodynamic behavior of the molecules using an ethyl acetate : cyclohexane solution (1 : 1, v/v) used by Qian et al. 2015.

Immunoaffinity chromatography is another widely used technique for the separation of mycotoxins in edible oils. For that, different solvents are used: acetonitrile : water (4 : 1, v/v) and a phosphate buffer saline solution to dissolve the matrix and pass it through the column (Marley et al. 2015), whereas methanol (Bao et al. 2012, 2013) or methanol : acetic acid (9 : 1 v/v) (Wei et al. 2015) were used for the elution of the compounds.

Finally, methods based on gas chromatography (GC) usually require a derivatization step like the silylation performed by Qian et al. (2015), using N,O-bis-trimethylsilyltrifluoroacetamide, containing 1% trimethylchlorosilane. Also, Yu et al. (2019) performed a derivatization by photochemical post-column derivatization. This technique is used

to maintain the natural fluorescence of mycotoxins, avoiding the emission quenching in the aqueous mobile phase (see Table 8.1).

8.2.3.2 Determination

The determination of mycotoxins is usually performed by liquid chromatography (LC) with a simple detector like fluorescence detector (LC-FLD) used in the studies carried out by Wei et al. (2015), Yu et al. (2019), and the interlaboratory study carried out by 17 laboratories for the analysis of 4 aflatoxins in olive, peanut, and sesame oils (Bao et al. 2012, 2013). Also, sterigmastocystin was determined in samples of sunflower seeds by LC with ultraviolet (UV) detector by Marley et al. (2015). However, when a higher number of mycotoxins need to be determined, LC coupled to tandem

Table 8.1 Analytical methods for the determination of mycotoxins.

Matrix	Analytes	Sample extraction/ clean-up	Analysis	References
Vegetable oils	AFB1 and AFB2[a]	MSPE	HPLC-PCD-FLD	Yu et al. (2019)
Olive oils	AFB1, AFB2, AFG1, AFG2, α-ZOL, and ZEA	LLE – QuEChERS	UHPLC-QqQ-MS/MS	Hidalgo-Ruiz et al. (2019a)
Vegetable oils	11 mycotoxins	Liquid-liquid microextraction – QuEChERS	LC-QqQ-MS/MS	Eom et al. (2017)
Vegetable oils	FB1, ZEA, and OTA	LLE – MSPE	UHPLC-QqQ-MS/MS	Zhao et al. (2017)
Vegetable oils	ZEA and 5 derivatives	LLE – GPC – Derivatization	GC-QqQ-MS/MS	Qian et al. (2015)
Sunflower seeds	Sterigmatocystin	Immunoaffinity Chromatography	HPLC-UV/Vis LC-QqQ-MS/MS	Marley et al. (2015)
Vegetable oils	16 mycotoxins	QuEChERS	LC-QTRAP-MS/MS	Zhao et al. (2016)
Vegetable oils	7 mycotoxins	QuEChERS – SPE	LC-QqQ-MS/MS	Sharmili et al. (2016)
Vegetable oils	AFB1, AFB2, AFG1, and AFG2	LLE – Immunoaffinity chromatography	LC-FLD	Bao et al. (2012, 2013)
Peanut oil	AFB1 and AFM1	MISPE – Immunoaffinity chromatography	LC-FLD	Wei et al. (2015)

[a] AFB1: Aflatoxin B1; AFB2: Aflatoxin B2; AFG1: Aflatoxin G1; AFBG2: Aflatoxin G2; AFM1: Aflatoxin M1; FB1: Fumonixin B1; GC-QqQ-MS: Gas chromatography coupled to mass spectrometry with triple quadrupole; GPC: Gel permeation chromatography; HPLC-PCD-FLD: High performance liquid chromatography with post-column photochemical derivatization and fluorescence detector; HPLC-UV/Vis: High performance liquid chromatography with ultraviolet visible detector; LC-FLD: Liquid chromatography with fluorescence detector; LC-QTRAP-MS/MS: Liquid chromatography with ion trap coupled to triple quadrupole detector; LLE: Liquid-liquid extraction; MISPE: Molecularly imprinted solid phase extraction; MSPE: Magnetic solid phase extraction; OTA: Ochratoxin A; ZEA: Zearalenone α-ZOL: α-Zearalenol.

mode mass spectrometry (LC–MS/MS) is used. Within this category, the most used analyzer is triple quadrupole (QqQ); case of Hidalgo-Ruiz et al. (2019a), Eom et al. (2017), Y. Zhao et al. (2017), Marley et al. (2015), Sharmili et al. (2016). Also, QqQ analyzer is used by Qian et al. (2015) in a study carried out for the determination of zearalenone and five derivatives in 40 samples of vegetable oils.

Finally, H. Zhao et al. (2016) used LC coupled to an ion trap (QTRAP) for the determination of 16 mycotoxins in 25 samples of palm, corn, and sunflower oils. This analyzer offers a high sensitivity, accuracy, and an excellent mass resolution maintaining the same acquisition speed (see Table 8.1).

8.2.3.3 Occurrence

Almost 200 oil samples including olive, lampante, pomace, and refined oils were analyzed in the study carried out by Hidalgo-Ruiz et al. (2019a). The authors of that study observed that almost half of the analyzed samples were contaminated. Zearalenone was found at the highest concentration (25.6 µg/kg) but also other mycotoxins like aflatoxin B2 and aflatoxin G2 were present. Figure 8.2a shows the extracted ion chromatogram of a lampante oil sample contaminated with 25.6 µg/kg of zearalenone, while Figure 8.2b shows the extracted ion chromatogram of a crude olive pomace oil sample contaminated with 6.8 µg/kg of aflatoxin G2. Zearalenone was the most detected mycotoxin in other studies that analyzed oil samples, independently of the type of oil analyzed (Eom et al. 2017; Sharmili et al. 2016; Zhao et al. 2016, 2017). Specifically, Eom et al. analyzed nine edible oil samples, including soybean, corn, and rice bran oil and

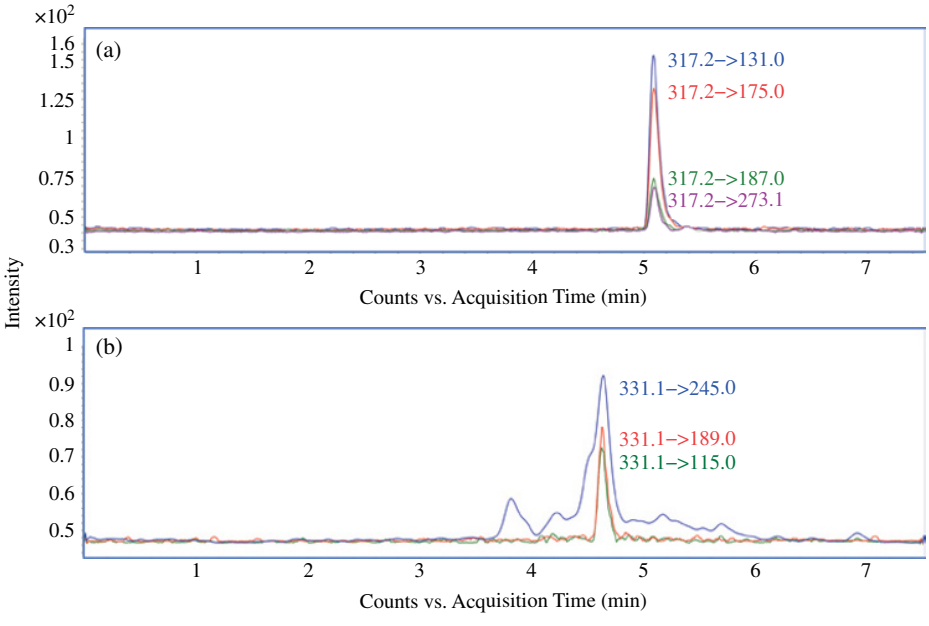

Figure 8.2 (a) Extracted ion chromatogram of a lampante olive oil sample contaminated with 25.6 µg/kg of zearalenone, and (b) extracted ion chromatogram of a crude olive pomace oil sample contaminated with 6.8 µg/kg of aflatoxin G2. *Source:* Hidalgo-Ruiz (2019a). Reprinted with permission from Elsevier.

detected zearalenone in six samples at a maximum concentration of 3.25 μg/kg (Eom et al. 2017). Sharmili et al. analyzed a total of 25 samples including palm, corn, and sunflower oil detecting zearalenone in all the palm oil samples analyzed at a maximum level of 69.1 μg/kg. They also found positives for aflatoxin B1, G1, and G2, while they did not detect aflatoxin B2, ochratoxin A, or deoxynivalenol (Sharmili et al. 2016). The highest number of mycotoxins analyzed in vegetable oils was performed by H. Zhao et al. analyzing 16 mycotoxins in six kinds of oils including sunflower, peanut, soybean, corn, linseed, and olive oil. Only six samples were positive among the 62 samples tested, detecting zearalenone (up to 42.5 μg/kg), aflatoxin B1 (up to 11.0 μg/kg), aflatoxin B2 (up to 4.6 μg/kg), aflatoxin G1 (up to 0.6 μg/kg),and α-zearalenol (up to 1.4 μg/kg) (Zhao et al. 2016). Finally, the highest concentration of zearalenone (111.0 μg/kg) was detected by Y. Zhao et al. in a sample of maize oil in a study carried out with three samples of maize, rapeseed, and soybean oil (Zhao et al. 2017).

8.3 Polycyclic Aromatic Hydrocarbons

PAHs are organic compounds, with two or more condensed benzene rings, produced by the incomplete combustion of organic material such as wood, petroleum products, coal, or food and they have been detected in different types of food (Lacoste 2014; Yao et al. 2015).

8.3.1 Sources of Contamination

There are three types of contamination sources: pyrogenic, petrogenic, and biological. In the first case, pyrogenic PAHs are formed when organic substances are burnt or exposed to high temperatures under low or no oxygen conditions. Secondly, petrogenic PAHs are derived from coal distillation as well as the cracking of petroleum. Also, they can appear during incomplete combustion of fuels, woods, or fuel oils. Finally, biological PAHs are those that come from natural sources; for example they can be synthetized by certain plants and bacteria or formed during the degradation of vegetative matter (Abdel-Shafy and Mansour 2016). PAHs are in the environment mainly due to the incomplete combustion of organic matter either coming from natural or anthropogenic sources, like vehicle exhaust, agricultural fires, or factories (Ravindra et al. 2008; Wang et al. 2013). PAHs can be found in the surroundings of the contamination source or they can be transported through air until they are deposited on the soil, where they can be absorbed by the crops (Cachada et al. 2012). Additionally, cooking of foods is a major source of PAHs as they are generated in situ (Zhao et al. 2012). Thus, edible oils should not have PAHs as they are extracted through cold processes, except pomace oils that are submitted to harder processes (Food and Agriculture Organization of the United Nations, and World Health Organization 2008).

8.3.2 Legislation

A limit of 2.0 μg/kg for benzo(a)pyrene and 10.0 μg/kg for the sum of benzo(a)pyrene, benz(a)anthracene, benzo(b)fluoranthene and chrysene is set for oils and fats by the European Commission (EC), whereas a maximum of 20.0 μg/kg for the sum of these

PAHs, has been set in coconut oil (European Commission 835 2011). The limit of benzo(a)pyrene is the lowest because it is considered the most carcinogenic PAH (United States Environmental Protection Agency 2000). For this reason, in 2003, the WHO set a unit risk of lung cancer of this PAH of 87×10^{-6} ng/m^3 for lifetime exposure (WHO 2003).

The US Agency for Toxic Substances and Disease Registry (ATSDR) and US Environmental Protection Agency (EPA) prepared a list of substances that are most commonly found and which are the most significant potential threat to human health due to their known or suspected toxicity and potential for human exposure. In that list, 16 PAHs were included, as they are frequently found in environmental monitoring samples. These PAHs are acenaphthene, acenaphthylene, anthracene, fluoranthene, fluorene, naphthalene, phenanthrene, pyrene, benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[g,h,i]perylene, benzo[a]pyrene, chrysene, dibenz[a,h]anthracene, and indeno[1,2,3-cd]pyrene (Agency for Toxic Substances and Disease Registry 2017).

8.3.3 Analysis

PAHs are present in edible oils in concentrations of μg/kg (Sun et al. 2019). They are light sensitive, so the manipulation during the different steps of the analysis must be carried out within dark conditions. This will avoid the decomposition by photoirradiation and oxidation of the compounds (Plaza-Bolaños et al. 2010).

8.3.3.1 Sample Treatment

In recent years, the extraction and clean-up steps have been unified in animal and vegetable fats and oils, applying the ISO 15753 standard, where two LLEs are carried out (Spanish Association for Standardization and Certification [AENOR] 2012). To accomplish the extraction, a mixture of acetonitrile : acetone (3 : 2, v/v) is used (Amzad Hossain and Salehuddin 2012; Hao et al. 2016; Yousefi et al. 2018). Sun and Wu (2020) tested the solvent used in ISO 15753 and other combinations and they concluded that the mixture of acetonitrile : acetone (3 : 2 v/v) was the best option to extract PAHs from oils. They also tested acetonitrile : acetone (2 : 1, v/v) and noticed that the recovery was higher for three to four ring PAHs but lower for five to six ring PAHs.

Before ISO 15753, which was published in 2012, other methods were reported. For instance, a LLE with dimethylformamide : water (9 : 1, v/v) was developed by Camargo et al. (2011) for the analysis of PAHs in soybean oil. Also, a magnetic solid-phase extraction method with multiwalled carbon nanotubes was published in 2011 for the extraction of PAHs in edible oils (Zhao et al. 2011). Finally, the most recent method published uses a QuEChERS method, utilizing the same solvent as that in ISO 15753, but employing a new adsorbent material, named enhanced matrix removal to remove lipids that, according to the authors, exhibits better adsorption capacity for lipids in clean-up applications than C18, graphitized carbon black (GCB), Z-Sep, and Z-Sep+ (Sun and Wu 2020).

Moreover, a method in which no sample treatment is used was developed by Hollosi and Wenzl (2011). In this study, the olive oil is directly injected in the instrument after dilution with 30% of isopropanol. Nevertheless, the majority of the methods follow the ISO 15753 for the clean-up step. This consists in two SPE steps. Firstly, a C18 cartridge is used, and then, the extract is transferred to a Florisil cartridge (Yousefi et al. 2018; Zhao et al. 2018) (see Table 8.2).

Table 8.2 Analytical methods for the determination of PAHs.[a]

Matrix	Analytes	Sample extraction/ clean-up	Analysis	References
Edible oils	8 of the 16 EPA PAHs[b]	LLE	GC–MS	Amzad Hossain and Salehuddin (2012)
Edible oils	9 of the 16 EPA PAHs	LLE	HPLC-UV–Vis	Dost and Deli (2012)
WFO and VODD	16 EPA PAHs	QuEChERS – EMR	GC-QqQ-MS	Sun and Wu (2020)
Edible oils	8 of the 16 EPA PAHs	MSPE	GC–MS	Zhao et al. (2011)
Vegetable oils	4 of the 16 EPA PAHs	LLE – SPE	UHPLC-FLD	da Silva et al. (2017)
Soybean oils	13 of the 16 EPA PAHs	LLE – SPE	HPLC-FLD	Camargo et al. (2011)
Edible oils	13 of the 16 EPA PAHs	LLE – SPE	HPLC-FLD	Yousefi et al. (2018)
Edible oils	13 of the 16 EPA PAHs	LLE – SPE	HPLC-FLD	Molle et al. (2017)
Vegetable oils	16 EPA PAHs	LLE – SPE	GC–MS	Zhao et al. (2018)
Edible oils	16 EPA PAHs	LLE – SPE	GC–MS	Wu and Yu (2012)
Edible oils	16 EPA PAHs	LLE – Chromatography column	HPLC-UV	Hao et al. (2016)
Olive oil	16 EPA PAHs	Dilution – Direct injection	LC-QqQ-MS/ MS	Hollosi and Wenzl (2011)
Vegetable oils	16 EPA PAHs	LLE – SPE	GC–MS	Hua et al. (2016)

[a] EMR: Enhanced matrix removal; GC–MS: Gas chromatography coupled to mass spectrometry; GC-QqQ-MS: Gas chromatography coupled to mass spectrometry with triple quadrupole; HPLC-FLD: High performance liquid chromatography with fluorescence detector; LLE: Liquid-liquid extraction; MSPE: Matrix solid phase extraction; VODD: Vegetable oil deodorizer distillate; WFO: Waste frying oil.
[b] 16 EPA PAHs: Acenaphthene, Acenaphthylene, Anthracene, Benz[a]anthracene, Benzo[b]fluoranthene, Benzo[k]fluoranthene, Benzo[ghi]perylene, Benzo[a]pyrene, Chrysene, Dibenz[a,h]anthracene, Fluoranthene, Fluorene, Indeno[1,2,3-cd]pyrene, Naphthalene, Phenanthrene and Pyrene.

8.3.3.2 Determination

Regarding the analysis step, both LC and GC are commonly used. When GC is used, MS is coupled as detector in order to get a reliable analysis (Amzad Hossain and Salehuddin 2012; Hua et al. 2016; Wu and Yu 2012; Zhao et al. 2011, 2018), although only one of them used MS/MS (Sun and Wu 2020). Furthermore, the last study mentioned is the only one that used QqQ for the analysis of PAHs in waste frying oil (WFO) and vegetable oil deodorizer distillate (VODD) (Sun and Wu 2020). Whitin the MS analyzers, the single quadrupole is the most used analyzer. Hua et al. and X. Zhao et al. used it for the analysis of PAHs in soybean and rapeseed oils (Hua et al. 2016; Zhao et al. 2018), Q. Zhao et al. for the analysis of blend, peanut, olive, maize, rapeseed, sunflower, and soybean oils (Zhao et al. 2011) and Wu et al. determined the 16 EPA PAHs in peanut and olive oils (Wu and Yu 2012). On the other hand, Amzad Hossain et al. used an ion trap for the analysis of soybean, mustard, and coconut oils (Amzad Hossain and Salehuddin 2012).

LC can be coupled to a conventional detector as FLD (Camargo et al. 2011; da Silva et al. 2017; Molle et al. 2017; Yousefi et al. 2018) or UV–Vis (Dost and Deli 2012; Hao et al. 2016) for the analysis in edible oils. Only one study was found using LC coupled to MS/MS and QqQ as the analyzer (Hollosi and Wenzl 2011). This method highlights the use of anisole as dopant, utilizing assisted atmospheric pressure photo ionization. The photo ionization initiates the formation of dopant radical cations that react further with the analyte via several routes (McCulloch et al. 2017). Then the sample is analyzed by QqQ analyzer (see Table 8.2).

8.3.3.3 Occurrence

Regarding the real sample analyses, a difference was found between the heated and not heated oils, as it has been evaluated by Sun and Wu (2020) where WFO and VODD were measured. Very high concentrations were found for the 16 PAHs obtained in both categories, but in VODD, concentrations higher than 100 μg/kg were detected for the majority of the analytes, and the maximum concentration was detected for pyrene at 173.68 μg/kg. The difference between the two categories becomes 7.5-fold higher when the sum of all the 16 PAHs is done, obtaining 197.44 μg/kg in the case of WFO and 1482.25 μg/kg in the case of VODD. In addition, 38 out of 40 samples were found contaminated with benzo(a)pyrene in the study carried out by Yousefi et al. (2018) finding concentrations up to 74.89 μg/kg. Additionally, high concentrations of phenanthrene (58.80 μg/kg), anthracene (54.37 μg/kg), and pyrene (50.45 μg/kg) were detected in corn oil. Fluoranthene was also found as the most abundant PAH in edible oils by Dost and Deli (2012) detecting the highest concentration at 76.08 μg/kg in a sample of corn oil. In a different study, Amzad Hossain and Salehuddin (2012) analyzed 8 (naphthalene, anthracene, phenanthrene, fluorene, pyrene, chrysene, benzo(a)pyrene, and benzo(a)anthracene) of the 16 EPA PAHs in three types of edible oils; soybean, mustard, and coconut oils finding contamination of 7 out of the 8 analytes, except chrysene. Wu and Yu (2012) found high concentrations of acenaphthylene (457.12 μg/kg on average) and acenaphthene (222.63 μg/kg on average) in olive oil, finding contamination of all the 16 EPA PAHs in the four samples analyzed with ranges from 0.71 to 457.12 μg/kg on average.

Overall, positive samples were found in all the published studies. PAHs are ubiquitous contaminants that can contaminate oils and oilseeds either from natural sources or be produced during food processing. The analysis of these compounds is extremely necessary for the routine analysis of oils and oilseeds.

8.4 3-MCPD Esters and Glycidyl Esters

3-MCPD is a chloropropanol that can be formed during the refining process of edible oils. Also, 2-MCPD and their esters can appear. Glycidol is associated with them and usually forms monoesters with fatty acids during the refining processes (European Food Safety Authority 2013) (see Figure 8.3).

8.4.1 Sources of Contamination

In edible oils, 3-MCPD is formed during food processing due to the reaction of hydrochloric acid with triacyclglycerols, phospholipids, and glycerol. Furthermore, it can

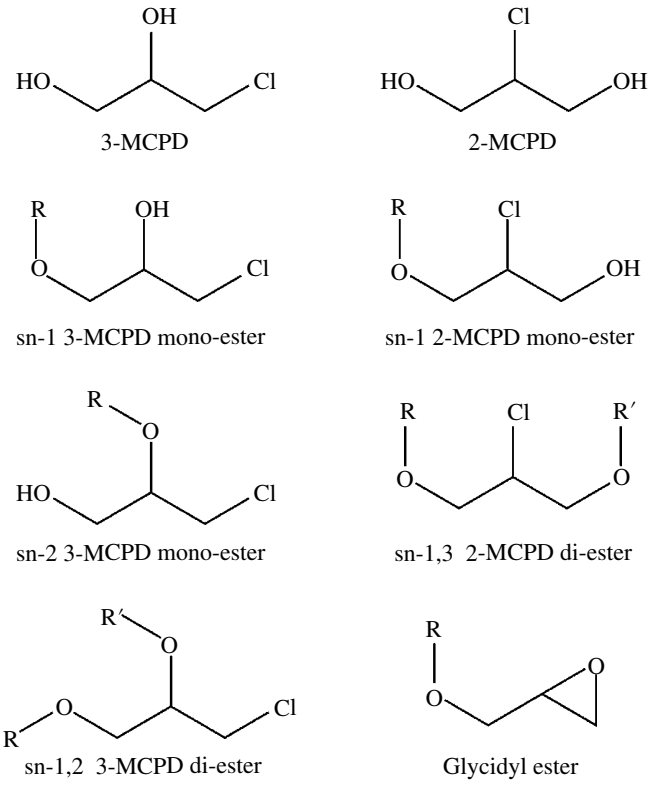

Figure 8.3 Molecules of 3-MCPD, 2-MCPD, their esters, and glycidyl ester.

appear in the thermal processes of other products such as baked goods, malt-derived products, or cooked/cured fish or meat. In the case of meat, it is formed when lipids and sodium chloride react, these components being naturally present or added to the food (Baer et al. 2009).

8.4.2 Legislation

Several criteria about the dairy intake of 3-MCPD and its esters have been adopted by different organizations. The European Food and Safety Authority (EFSA) has established 0.8 μg/kg per body weight/day for the sum of 3-MCPD and its esters (European Food Safety Authority 2016a), while the Joint Committee of FAO and WHO established a limit five times higher than the EFSA value (4.0 μg/kg) (Food and Agriculture Organization of the United Nations (FAO), and World Health Organization (WHO) 2007). The European Union has established a limit of 1000 μg/kg for glycidyl esters in edible oils and fats for adults, and 500 μg/kg if they are destined to baby food production or cereal-based foods for infants and young children. Also, a limit of 1250 μg/kg (2500 μg/kg for pomace oils) was established for the sum of 3-MCPD and 3-MCPD esters in vegetable oils and fats for adults, and 750 μg/kg if they are destined to baby food production or cereal-based foods for infants and young children (European Commission 1322, 2020).

8.4.3 Analysis

Determination of 2- and 3-MCPD esters is based on well-established sample preparation methods in lipid analysis, providing information about the profiles of 2- and 3-MCPD esters (Hori et al. 2012), with 3-MCPD more studied than 2-MCPD. Graziani et al. 2017, Haines et al. 2011, and Li et al. 2015 report the determination of a variable number of mono and diesters of the 2- and 3-MCPD esters.

8.4.3.1 Sample Treatment

Various analytical methods have been developed in recent years for the determination of 3-MCPD and its esters in several matrices, mainly in edible oils (Crews et al. 2013) or foods derived from them (Jędrkiewicz et al. 2017) distinguishing between direct and indirect approaches.

Indirect methods were developed first and they need some steps in order to transform 2- and 3-MCPD esters in free MCPD, performing a transesterification, neutralization, and subsequent quantification after derivatization with phenilboronic acid (Jędrkiewicz et al. 2017) or heptafluorobutyryl imidazole (Dubois et al. 2012). The analysis of glycidyl esters has been incorporated into these methodologies, due to the conversion of these substances into MCPD or bromopropanediol through the use of sodium bromide (Ermacora and Hrncirik 2013). However, a drawback of this approach is the complexity of the sample treatment and the time required to perform all these stages.

In turn, direct methods for the determination of glycidyl esters quantify the level of every species bearing different fatty acyl chains without chemical transformation (Cheng et al. 2017). LLE techniques are commonly used to extract 3-MCPD esters and glycidyl esters from edible oils, and different solvents are used. In fact, between all the revised studies, only two of them used the same, a mixture of cyclohexane : ethyl acetate (1 : 1, v/v) (Dubois et al. 2011, 2012; Weißhaar and Perz 2010). The rest of them use different solvents, as tert-butyl methyl ether : ethyl acetate (4 : 1, v/v) (Blumhorst et al. 2013), acetonitrile and *n*-heptane consecutively (Steenbergen et al. 2013), chloroform (Aniołowska and Kita 2015, 2016a, b, c), tetrahydrofuran (Ermacora and Hrncirik 2013; Steenbergen et al. 2013), acetonitrile (Masukawa et al. 2010, 2011), acetone (Becalski et al. 2012) or *n*-hexane (Hori et al. 2012). Other extraction techniques, such as GPC (Weißhaar and Perz 2010), using a mixture of 0.26 mM methanol–sodium acetate solution (MSA) : methylene chloride : acetonitrile (1 : 8 : 1, v/v/*v*) (Haines et al. 2011), or 20% ethyl acetate in methyl *tert*-butyl ether (MacMahon et al. 2013a, b) were used. An extraction making the oil dissolved in *n*-hexane go through a chromatography column (Song et al. 2015) was also published.

Regarding the clean-up step, most of the methods have followed the same 2-steps SPE method (Cheng et al. 2017), using first a C18 cartridge and then a silica cartridge, except in the case of Hori et al. (2012), who perform the first clean-up step using the silica cartridge and then the C18 one (see Table 8.2).

8.4.3.2 Determination

Both LC and GC are widely used for the determination of 3-MCPD esters and glycidyl esters, always coupled to MS. In the case of GC, all the studies used single quadrupole (Ermacora and Hrncirik 2013; Kuhlmann 2011, 2016; Steenbergen et al. 2013; Weißhaar and Perz 2010). LC–MS (Blumhorst et al. 2013; Shiro et al. 2011) and LC–MS/MS (Aniołowska and Kita 2016a; Becalski et al. 2012; MacMahon and Beekman 2019) are also widely used for the determination of 3-MCPD esters and

glycidyl esters in edible oils. Two studies with time-of-flight analyzer (TOF) have been reported, using LC, for the analysis of glycidyl esters in vegetable oils (Dubois et al. 2011, 2012; Haines et al. 2011). Finally, a proton nuclear magnetic resonance determination method was developed by Song et al. (Song et al. 2015) (see Table 8.3).

Table 8.3 Analytical methods for the determination of 3-MCPDs and glycidyl esters.[a]

Matrix	Analytes	Sample preparation/ extraction	Analysis	References
Vegetable oils	5 GEs	LLE – 2-step SPE	LC–MS	Blumhorst et al. (2013), Shiro et al. (2011)
Extra virgin olive oils	5 GEs	LLE – 2-step SPE	GC–MS	Steenbergen et al. (2013)
Edible oils	Glycidol, 2- and 3-MCPD and 2 3-MCPD diesters	Alkaline transformation with Br^-	GC–MS	Kuhlmann (2016), Kuhlmann (2011)
Frying and refined oils	5 GEs	LLE – 2-step SPE	LC–MS/MS	Aniołowska and Kita (2015, 2016a, b, c),
Palm oil	14 GEs	GPC	GC–MS	Weißhaar and Perz (2010)
Vegetable oils	7 GEs and 20 3-MCPD mono- and diesters	Dilution with MSA/ methylene chloride/ acetonitrile (1 : 8 : 1)	LC-TOF-MS	Haines et al. (2011)
Vegetable oils	Glycidol and 2- and 3-MCPD	LLE – Derivatization	GC–MS	Ermacora and Hrncirik (2013)
Edible oils	6 GEs and 12 3-MCPD mono- and diesters	Dilution with ethyl acetate/methyl *tert*-butyl ether – 2-step SPE	LC–MS/MS	MacMahon et al. (2013a, b)
Edible oils	5 GEs	LLE – 2-step SPE	LC–MS	Masukawa et al. (2010, 2011)
Vegetable oils	7 GEs	LLE – GPC – SPE	LC-TOF-MS	Dubois et al. (2011, 2012)
Edible oils	5 GEs	LLE – 2-step SPE	LC–MS/MS	(Becalski et al. 2012)
Edible oils	5 GEs and 9 3-MCPD mono- and diesters	LLE – 2-step SPE	LC-TOF-MS	Hori et al. (2012)
Edible oils	Intact GEs	Chromatography column	^{1}H-NMR	Song et al. (2015)

[a] GC-FID: Gas Chromatography with Flame Ionization Detector; GC–MS: Gas Chromatography coupled to Mass Spectrometry; GEs: Glycidyl Esters; GPC: Gel Permeation Chromatography; ^{1}H-NMR: Proton Nuclear Magnetic Resonance; LC-TOF-MS/MS: Liquid Chromatography coupled to Mass Spectrometry in tandem with Time Of Flight Detector; LC–MS/MS: Liquid Chromatography coupled to Mass Spectrometry in tandem; LLE: Liquid–Liquid extraction; MCPD: Monochloropropanediol; MSA: 0.26 mM methanol–sodium acetate solution; SPE: Solid Phase Extraction.

8.4.3.3 Occurrence

Many types of oils have been analyzed, such as soybean, corn (Hori et al. 2012), olive (Steenbergen et al. 2013), sesame (Haines et al. 2011), rapeseed, sunflower (Kuhlmann 2016), peanut, almond, grapeseed (MacMahon et al. 2013b), walnut, and coconut oil (Kuhlmann 2011) and concentrations up to 28.0 mg/kg were found in palm oil and 28.8 mg/kg in rice oil (Shiro et al. 2011). Moreover, a collaborative study for the analysis of five glycidyl esters in edible oils has been developed and 17 laboratories around the world participated. In this study, several spiked samples were analyzed in 13 months to evaluate the degradation of the glycidyl esters and it was found that in the first three months, the amount of these compounds decreased by 13% while after 13 months, it had decreased by 24% for all five analytes (Blumhorst et al. 2013). Another interesting study is the one carried out by Aniołowska and Kita (2016a), where 20 refined vegetable oils (rapeseed, sunflower, and palm oils) from retail outlets were analyzed. It was found that refined palm oils showed a high contamination ratio in comparison with other oils, finding the highest contamination at 44.33 mg/kg. In another study, they evaluated the effect of frying on glycidyl esters content in palm oil (Aniołowska and Kita 2016c). They discovered that the content of glycidyl esters decreased when the amount of time and temperature was increased (to a maximum of 180 °C). These results confront the theory that glycidyl esters are formed in thermal processes (Baer et al. 2009). However, EFSA suggests that they appear with temperatures higher than 200 °C (European Food Safety Authority 2016b), while they do not increase until over 180 °C.

8.5 Mineral Oil

Mineral oils are either derived from or produced synthetically from coal, natural gas, or biomass. They are composed of MOHs, linear or branched (paraffins) (European Commission 2018). These mineral oils are used as lubricating oils for the machinery used in the food industry (Gómez-Coca et al. 2016b). Within the MOHs, two categories can be distinguished, depending if they are aliphatic (Mineral Oil Saturated Hydrocarbons, MOSH) (Figure 8.4) or aromatic (Mineral Oil Aromatic Hydrocarbons, MOAH) (Figure 8.5).

8.5.1 Sources of Contamination and Legislation

Direct contact of food or feed with materials containing MOHs is enough to contaminate the product. Also, MOHs can appear in edible oils when their seeds have been submitted to hard extraction processes, such as centrifugation or solvent extraction (Gómez-Coca et al. 2016a). Indeed, the content of these compounds in pomace oils is considerably higher. Finally, in the case of edible oils, fraud can be an important source of contamination, intentionally adding mineral oils to edible oils, which can be a very serious risk for human health.

The European Commission has set a maximum level of contamination for MOHs in edible oils. For crude and refined sunflower oils the limit is 50 mg/kg (Standing Committee on the Food Chain and Animal Health 2008).

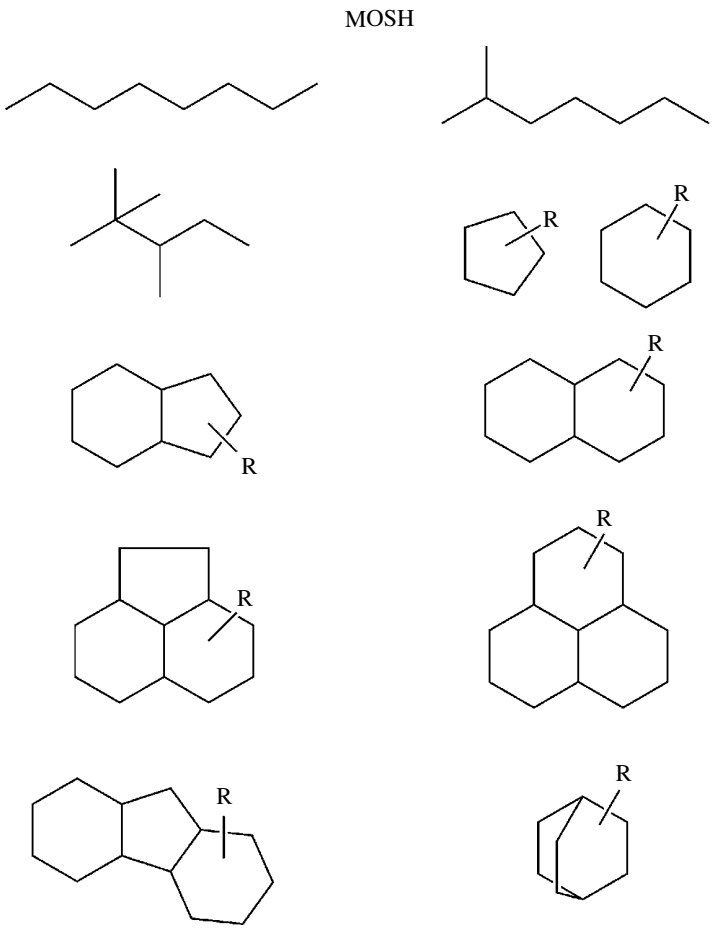

Figure 8.4 Examples of structures of MOSH.

8.5.2 Analysis

8.5.2.1 Sample Treatment

Due to the complexity of the matrices, a pre-treatment step is recommended before the analysis of MOHs. Sometimes only MOSH are analyzed. In these cases, an SPE using Ag-activated silica gel is enough to extract them (Li et al. 2016; Liu et al. 2017; Moret et al. 2011). However, the analysis of MOAH often needs a more sophisticated extraction, as there are compounds like olefins that interfere with the result. In this sense, several authors used the epoxidation reaction in order to eliminate the interferences that olefins produce (DIN EN 16995 2017; Gharbi et al. 2017; Zurfluh et al. 2014). Furthermore, Nestola et al. tried three reactions in order to eliminate those interferences: epoxidation, hydroboration, and bromohydrin formation reactions, finding the best results with epoxidation using 3-chloroperbenzoic acid (*m*CPBA) (Nestola and Schmidt 2017). In addition, Gharbi et al. 2017 used an Abencor extractor composed by a hammer crusher, a mixer, and a pulp centrifuge, which is used to smash and mix

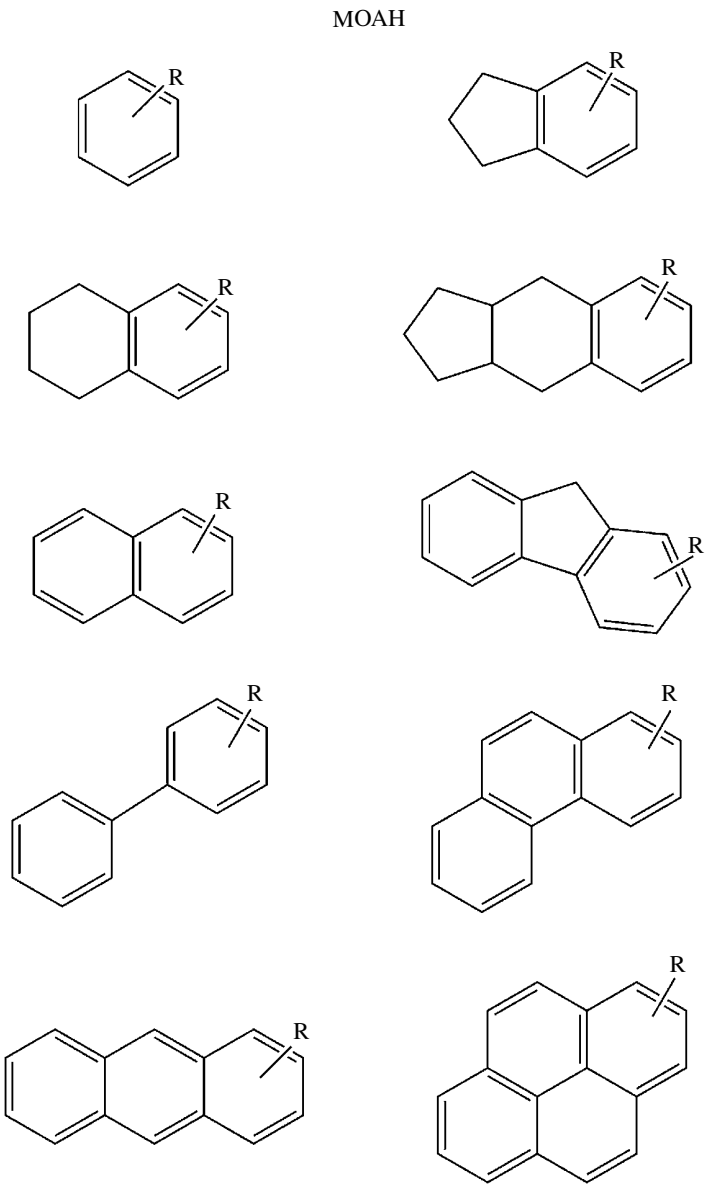

Figure 8.5 Examples of structures of MOAH.

the olives as it is the only study that analyses oilseeds. They also use microwave assisted extraction to speed-up the process and minimize the volume of organic solvents employed.

Overall, in most of the studies where these substances have been analyzed in oils, the separation has been carried out by LC and then analyzed by GC (DIN EN 16995 2017; Gharbi et al. 2017; Nestola and Schmidt 2017; Tranchida et al. 2011; Zoccali et al. 2016; Zurfluh et al. 2014) (see Table 8.4).

8.5.2.2 Determination

Few studies have performed the separation of the MOSH fraction from the MOAH one (Gharbi et al. 2017; Zoccali et al. 2016; Zurfluh et al. 2014). Most of these studies use LC prior to the injection of the sample into the GC. However, this high cost instrument can be skipped if a good epoxidation reaction is done (Nestola and Schmidt 2017) and very precise SPE cartridges are prepared, using 1% Ag-silica gel (Li et al. 2016; Liu et al. 2017), which are not commercially available. Finally, the most used detector is a flame ionization detector (FID) as it presents the advantage of cheapness against the high price of MS (Weber et al. 2018). Also, in the case of these compounds, a hump below the peaks of natural hydrocarbons has to be integrated, and compared to an internal standard, so the MS detection is not very useful. The injection method also plays an important role and different methods have been developed, such as splitless mode (Li et al. 2016), and large volume injection (Liu et al. 2017) when an LC equipment is not coupled to the GC to carry out the separation (see Table 8.4).

8.5.2.3 Occurrence

High concentrations of MOSH and MOAH are often found in oils and seeds. This fact does not mean that the sample is contaminated, as the seeds can have these compounds naturally (Gómez-Coca et al. 2016b). For example, Liu et al. (Liu et al. 2017) found concentrations below 60.9 mg/kg of MOSH in the majority of the samples, except in a blend oil (a mixture of rapeseed, soybean, peanut, corn, sunflower, fish,

Table 8.4 Analytical methods for the determination of mineral oil hydrocarbons (MOHs).[a]

Matrix	Analytes	Sample extraction/clean-up	Analysis	References
Edible oils	MOAH/PAH	Epoxidation	LC-GC-FID	Nestola and Schmidt (2017)
Vegetable oils	MOSH	Hexane, SPE-clean-up(Ag-activated silica gel)	LVI-GC-FID	Liu et al. (2017)
Vegetable oils	MOSH	Hexane	LC-GC-FID	Tranchida et al. (2011)
Virgin olive oil	MOSH/ MOAH/PAH	Epoxidation – Abencor extractor or MAE	LC-GC-FID	Gharbi et al. (2017)
Oils	MOSH/MOAH	Hexane, SPE-clean-up – Epoxidation	LC-GC-FID	DIN EN 16995 (2017)
Vegetable oils	MOSH	Hexane, SPE-clean-up(Ag-activated silica gel)	GC-FID	Li et al. (2016)
Vegetable oils	MOSH/MOAH	Hexane	LC-GC-FID/MS	Zoccali et al. (2016)
Vegetable oils	MOSH	Hexane, SPE-clean-up(Ag-activated silica gel)	GC-FID	Moret et al. (2011)
Edible oils	MOSH/MOAH	Hexane, SPE-clean-up – Epoxidation	LC-GC-FID	Zurfluh et al. (2014)

[a] GC-FID: Gas chromatography with flame ionization detector; GC-FID/MS: Gas chromatography with flame ionization detector coupled to mass spectrometry; LC-GC-FID: Liquid chromatography coupled to gas chromatography with flame ionization detector; LC-GC-FID/MS: Liquid chromatography coupled to gas chromatography with flame ionization detector and mass spectrometry; LVI-GC-FID: Large volume injection gas chromatography; MAE: Microwave assisted extraction; MOAH: Mineral oil aromatic hydrocarbons; MOSH: Mineral oil saturated hydrocarbons; PAH: Polycyclic aromatic hydrocarbons; SPE: Solid phase extraction.

sesame, linseed, and rice oils) that had 259.4 mg/kg. In the case of the extra virgin olive oil samples, the maximum concentration found was 30.3 mg/kg. Zoccali et al. (2016) found low levels of MOSH (below 21.8 mg/kg) and did not found MOAH in extra virgin olive oils, whereas levels up to 444.8 mg/kg of MOSH and 66.1 mg/kg of MOAH in olive pomace oils were found. Tranchida et al. (2011) analyzed 18 samples of extra virgin olive, olive, sunflower, grapeseed, corn, peanut, soybean, and pomace oils, and found concentrations ranging from 7.6 mg/kg in an extra virgin olive oil to 180.6 mg/kg in a pomace oil. Gharbi et al. (2017) analyzed five samples of extra virgin olive oil, detecting concentrations up to 38.0 mg/kg. Li et al. (2016) analyzed nine types of oils, and found that camellia oil contained the highest average contamination (78.49 mg/kg of maximum) compared with the other types of oils.

Overall, results summarized herein agree with the affirmation made by Gómez-Coca et al. (2016b), who indicated that hard extractions, such as centrifugation or solvent extractions used for olive pomace oils, extract more MOHs from the fruit, whereas extra virgin olive oils show the lowest levels of MOHs because they are not submitted to these processes.

8.6 Phthalates

Phthalates or phthalic acid esters are a group of chemical compounds used as plasticizers, and they are added to the plastics to increase their flexibility. The most common use is the conversion of polyvinylchloride (PVC), a rigid plastic, into a more flexible one. The most common phthalates used are bis (2-ethylhexyl) phthalate (DEHP), which represents approximately 50% of the world production of phthalates, dibutyl phthalate (DBP), butyl benzyl phthalate (BBP), diisodecyl phthalate (DIDP) and diisononyl phthalate (DINP). Phthalates are also used as solvents in perfumery and pesticides, in nail polish, adhesives, putties, paint pigments, lubricants, food packaging, plastic wrap, and toys (Lacoste 2014).

8.6.1 Sources of Contamination

These compounds are ubiquitous and atmospheric transport and deposition are the main sources of crops contamination of phthalates (Rakkestad et al. 2007). As there is no covalent bond between phthalates and the plastic, and they have a strong affinity for fat, they are easily released from its containers into fat-containing foods (Rudel and Perovich 2009). Phthalates do not persist in the outdoor environment because they can easily be degraded, either by photo, bio, or anaerobic degradation and in normal conditions, they do not persist more than 15 days in the environment before degradation (Xie et al. 2006).

8.6.2 Legislation

In 2011, the European Commission set a limit for the migration of five phthalates in food. The limit for DBP is 0.3 mg/kg and the limit for DEHP is 1.5 mg/kg. The sum of DINP and DIDP must not be higher than 9 mg/kg and finally, the limit for BBP is 30 mg/kg. The use of them in material containing fatty foods is forbidden (except infant formulae for DINP, DIDP and BBP), and DINP, DIDP, and BBP must be used only in single-use materials while DEHP and DBP can be used in reusable materials.

8.6.3 Analysis

Apart from the fact that usually foods are complex matrices to analyze, as discussed earlir, in this case, the ubiquity of the analytes makes their presence in any laboratory normal, so consequently, their determination is even harder due to background interference (Haji Harunarashid et al. 2017).

8.6.3.1 Sample Treatment

LLE is a very simple method that is used, utilizing different solvents such as chloroform : methanol (2 : 1, v/v) (Ostrovský et al. 2011), *n*-hexane (Guo et al. 2012), and acetonitrile (Xu et al. 2014). Head space solid phase microextraction (HS-SPME) was developed and used by Rios et al. (2010) and Amanzadeh et al. (2016). This last technique offers numerous benefits such as minimum solvent use, the integration of sampling and sample preparation steps, simple operation, low cost, and the possibility of an on-line analytical procedure (Amanzadeh et al. 2016). Rios et al. tested the performance of three fibers for the extraction (85 μm polyacrylate [PA], 100 μm-polydimethylsiloxane [PDMS], and divinylbenzene carboxen PDMS [DVB/CAR/PDMS]) of these compounds, finding the best results when DVB/CAR/PDMS was used (Rios et al. 2010). A great advantage of the HS-SPME extraction is that a clean-up step is not necessary. However, it is necessary when LLE is carried out. For this purpose, an innovative SPE method was developed with a nylon 6 nanofibers mat, which is characterized by small fiber diameters, controllable pore sizes, high porosities, and the consumption of solvent is also minimized. To perform the extraction, only 200 μL of acetone, 600 μL of water, and 20 μL of methanol were used (Cao et al. 2013; Xu et al. 2010) (see Table 8.5).

8.6.3.2 Determination

GC and LC are the most used techniques in liquid samples. In the case of GC, two detectors are used: FID (Amanzadeh et al. 2016; Ostrovský et al. 2011) and MS (Guo et al. 2012; Rios et al. 2010). Rios et al. (2010) used an ion trap analyzer based on the capture of charged particles after the desorption of the fiber. In the case of LC, the UV/Vis detector is commonly used (Cao et al. 2013; Xu et al. 2010) as well as MS (Xu et al. 2014) (See Table 8.5). The two methods that use UV/Vis detection, carry out the separation with the nylon 6 nanofibers mat, which means that a much better separation must be done prior to the analysis (see Table 8.5).

8.6.3.3 Occurrence

Rios et al. (2010) found high concentrations of four phthalates in olive oils stored in plastic bottles, with DEHP the phthalate detected at the highest concentration (840 μg/kg), followed by diisobutyl phthalate (DIBP), which was detected at 216 μg/kg, BBP (211 μg/kg) and DBP (175 μg/kg). Q. Xu et al. (2010) studied the migration of phthalates from plastic to cooking oil, maintaining the oils at 20, 40, and 60 °C for two months in plastic bottles. After the experiment, they found migration values up to 14% for DEHP. Ostrovský et al. (2011) analyzed DMP in four samples of vegetable oils and detected concentrations from 1.5 to 3.2 mg/kg. Guo et al. (2012) found DEHP at 79.0 μg/kg in a sample of cooking oil. Finally, Amanzadeh et al. (2016) did not found any of the studied samples (sunflower and olive oils) contaminated by these compounds either because their concentration was lower than their limit of quantification determined by this method or they were not contaminated by the analytes.

Table 8.5 Analytical methods for the determination of phthalates.[a]

Matrix	Analytes	Sample preparation/extraction	Analysis	References
Olive oil	DMP, DEP, DIBP, BBP, DEHP, DOP, etc.	Headspace solid-phase microextraction	Ion trap; MS	Rios et al. (2010)
Cooking oil	DMP, DEP, BBP, DBP, DEHP, DOP, etc.	Solid-phase extraction by nylon 6 nanofibers mat	HPLC-UV/VIS	Xu et al. (2010)
Edible oil	DMP, DEP, DBP, DEHP, etc.	Liquid-liquid extraction chloroform/metanol (2:1); NaCl	GC-FID	Ostrovský et al. (2011)
Cooking oil	DMP, DEP, DBP, DIBP, etc.	Liquid-liquid extraction with *n*-hexane	GC–MS	Guo et al. (2012)
Edible oil	DMP, DEP, DBP, DEHP, DOP	Solid-membrane extraction	HPLC-UV/VIS	Cao et al. (2013)
Oil	DMP, DEP, DBP, DCHP, DNOP, etc.	Liquid-liquid extraction with acetonitrile	HPLC-MS/MS	Xu et al. (2014)
Vegetable oil	DBP, DEHP, etc.	Graphene/polyvinyl chloride nanocomposite fiber headspace solid-phase microextraction	GC-FID	Amanzadeh et al. (2016)

[a] BBP: Butylbenzyl phthalate; DEHP: Bis (2-ethylhexyl) phthalate; DEP: Diethyl phthalate; DIBP: Diisobutyl phthalate; DMP: Dimethyl phthalate; DOP: Dioctyl phthalate; GC-FID: Gas chromatography with flame ionization detector; GC–MS: Gas chromatography coupled to mass spectrometry; HPLC: High performance liquid chromatography; HPLC-MS/MS: High performance liquid chromatography coupled to mass spectrometry in tandem; HPLC-UV/VIS: High performance liquid chromatography with ultraviolet–visible spectroscopy; MS: Mass spectrometry.

8.7 Pesticides

Pests are animals or plants that are considered harmful for humans or human concerns, including crops, livestock, and forestry (Britannica Online Encyclopedia 2019). Therefore, pesticides are chemical substances used to control pests (US Environmental Protection Agency 2019). Depending on where these substances are going to be used they can be classified as herbicides, insecticides, nematicides, molluscicides, piscicides, avicides, rodenticides, bactericides, insect repellents, animal repellents, antimicrobials, or fungicides (National Association of State Departments of Agriculture (NASDA) n.d.).

8.7.1 Sources of Contamination

According to the EPA report (Kiely et al. 2004), in 2001, more than five billion pounds of pesticides were used, and 76% of this amount was used in the agricultural sector, another 12.5% in the industry/commercial/government sector and finally, the remaining 11.5% was used in the home and garden sector. Transport of pesticides through the environment can also be a source of contamination. Volatile or semi-volatile compounds can be evaporated at high temperatures and condensed in zones where temperatures are lower (Bloomfield et al. 2006). This transportation can cause high levels of persistent bioaccumulative toxicants in areas far from the source of pollution (Blais et al. 2006). Parallel, they can be transported by other mobile particles, by migratory

animals, or by hydrological flows (Blais et al. 2006). Finally, they can be added during the postharvest processes to maintain the quality of the crop if they are going to be stored for a long time or transported through a long distance (Amvrazi 2011).

8.7.2 Legislation

The use of pesticides in the European Union is regulated by Regulation No. 1107/2009 (European Commission 2009a) on Plant Protection Products in cooperation with other EU Regulations and Directives (e.g. the Regulation on MRLs in food; Regulation (EC) No. 396/2005 (European Commission 2010) and the Directive on sustainable use of pesticides, Directive 2009/128/EC (European Commission 2009b). This EU legislation sets MRLs of over 1100 pesticides for 315 fresh products. For any pesticide not specifically named in the legislation that is found in the matrix studied, a default MRL at 0.01 mg/kg is set. However, depending on the combination pesticide/matrix, this MRL could be up to 20 mg/kg (European Commission 2008).

The Codex Alimentarius has also set MRLs and the majority of the pesticides in oils and seeds are not higher than 0.8 mg/kg, except for some cases, as the limit of 5 mg/kg of chlormequat in rape seed, 70 mg/kg of fluorpyran in dill seed and 2800 mg/kg of propiconazole in orange oil (Joint FAO/WHO Expert Committee on Food Additives (JECFA) 2018).

8.7.3 Analysis

Currently, the determination of pesticides from fatty materials is a complex task. In fact, several different techniques have been developed to solve this problem.

8.7.3.1 Sample Treatment

In the majority of the cases, several steps are needed to extract these compounds from food. A solvent partitioning step with acetonitrile is used in the majority of the studies to extract and isolate the pesticides from the matrix (Deme et al. 2014; Moreno-González et al. 2014; Ramli et al. 2012; Ruiz-Medina et al. 2012; Tuzimski and Rejczak 2016). Then, low-temperature precipitation combined with QuEChERS using different salts is used. In the case of Anagnostopoulos and Miliadis (2013), primary secondary amine sorbent (PSA), GCB, and $MgSO_4$ are used. Deme et al. (2014) used PSA, activated charcoal and $MgSO_4$ and Sobhanzadeh et al. (2012) used PSA, C18, and GCB. SPE is widely used as a clean-up technique (Chung and Chen 2015; Han et al. 2016; Muhamad et al. 2012; Ramli et al. 2012; Tuzimski and Rejczak 2014, 2016). Muhamad et al. (2012) tested different SPE sorbents for the extraction of cypermethrin and λ-cyhalothrin in palm oil such as GCB, PSA, C18, silica, and florisil. The results exhibited that the combination GCB/PSA showed the best recoveries, while florisil showed the highest precision in terms of relative standard deviation (RSD) for cypermethrin and GCB for λ-cyhalothrin.

Other techniques that are applied include matrix solid phase dispersion (MSPD) (Liu et al. 2012), GPC for the determination of organophosphorus pesticides in camellia oil (Chung and Chen 2015), and other highly effective extraction techniques, like air-assisted liquid–liquid microextraction (AALLME), which was used for the determination of triazole pesticides in edible oils (Farajzadeh et al. 2015). AALLME uses a few microliters of an extraction solvent, such as dimethyl sulfoxide (DMSO), saving high amounts of solvent compared to a normal LLE (see Table 8.6).

Table 8.6 Analytical methods for the determination of pesticides.[a]

Matrix	Analytes	Sample preparation/ extraction and clean-up	Analysis	References
Camellia oil	Organophosphorus pesticides (15)	Solvent partitioning – MSPD	GC-FPD	Liu et al. (2012)
Palm oil	Pesticides from organophosphates, carbamates, triazines, and phenylureas groups (7)	Solvent partitioning – Low temperature precipitation; QuEChERS	LC-TOF-MS	Sobhanzadeh et al. (2012)
Palm oil	Diuron	Solvent partitioning – Low temperature precipitation; SPE	HPLC-UV	Ramli et al. (2012)
Palm oil	λ-cyhalothrin and cypermethrin	Solvent partitioning – Low temperature precipitation; SPE	GC-ECD	Muhamad et al. (2012)
Olive, sunflower, palm and rapeseed oils	Multiclass pesticides (44)	Solvent partitioning – QuEChERS	LC-QTRAP-MS/MS	Polgár et al. (2012)
Sunflower, rice bran and ground nut oils	Organochlorines, organophosphorus, and synthetic pyrethroids (35)	Solvent partitioning – Low temperature precipitation; QuEChERS	GC-QqQ-MS/MS	Deme et al. (2014)
Extra virgin olive, sunflower, maize, linseed and sesame oils	Carbamates (31)	Solvent partitioning – QuEChERS	UHPLC-QqQ-MS/MS	Moreno-González et al. (2014)
Sunflower, olive, grape and corn oils	Triazole pesticides (5)	AALLME	GC-FID	Farajzadeh et al. (2015)
Olive and grapeseed oils	Multiclass pesticides (21)	Solvent partitioning – SPE; QuEChERS	HPLC-DAD	Tuzimski and Rejczak (2016)
Olive oil and olives	Multiclass pesticides from 32 different groups	Solvent partitioning – Low temperature precipitation; QuEChERS	GC-QqQ-MS/MS LC-QqQ-MS/MS	Anagnostopoulos and Miliadis (2013)

Olive, sunflower oil and corn oils	Carbaryl	Solvent partitioning – QuEChERS	Automated Flow methodologies: Sequential Injection Analysis and Multicommutated Flow Injection Analysis	Ruiz-Medina et al. (2012)
Olive oil and peanuts	Organochlorines (33)	MSPD – GPC; SPE	GC–MS	Chung and Chen (2015)
Soybean and maize oils	Glufosinate	Derivatization – SPE	LC-QqQ-MS/MS	Han et al. (2016)
Sunflower seeds	Multiclass pesticides (10)	Solvent partitioning – SPE; QuEChERS	HPLC-DAD	Tuzimski and Rejczak (2014)
Flaxseeds	Multiclass pesticides (34)	Solvent partitioning – QuEChERS	GC-TOF-MS	Koesukwiwat et al. (2010)

[a] AALLME: Air-assisted liquid–liquid microextraction; GC-ECD: Gas chromatography with electron capture detector; GC-FPD: Gas chromatography with flame photometric detector; GC-TOF-MS: Gas Chromatography coupled to time of flight mass spectrometry; GC–MS: Gas chromatography coupled to mass spectrometry; GC-QqQ-MS/MS: Gas chromatography coupled to mass spectrometry in tandem with triple quadrupole detector; GPC: Gel permeation chromatography; HPLC-DAD: High performance liquid chromatography with diode array detector; HPLC-UV: High performance liquid chromatography ultraviolet; LC–MS: Liquid chromatography coupled to mass spectrometry; LC-QqQ-MS/MS: Liquid chromatography coupled to mass spectrometry in tandem with triple quadrupole detector; LC-QTRAP-MS/MS: Liquid chromatography coupled to mass spectrometry in tandem with ion trap-triple quadrupole detector; MSPD: Matrix solid phase dispersion; QuEChERS: Quick, easy, cheap, effective, rugged, and safe; SPE: Solid phase extraction; UHPLC-QqQ-MS/MS: Ultra performance liquid chromatography coupled to mass spectrometry in tandem with triple quadrupole detector.

8.7.3.2 Determination

The fact that an elevated number of pesticides need to be determined, makes the combination of chromatographic techniques (GC and LC) with MS or MS/MS very important. In this sense, a method for the determination of 33 organochlorines with GC–MS and single quadrupole in olive oil samples (Chung and Chen 2015) was developed. Other methods of MS/MS were applied, like the case of Anagnostopoulos and Miliadis (2013), who used both GC-QqQ-MS/MS and LC-QqQ-MS/MS for the determination of 32 groups of pesticides in 262 samples of olive oil and olives. Also a QqQ analyzer was coupled to GC for the determination of 35 organochlorines, organophosphorus, and synthetic pyrethroids in sunflower, rice bran, and ground nut oils (Deme et al. 2014). QqQ was also used in two studies with LC for the determination of carbamates (Moreno-González et al. 2014) and glufosinate (Han et al. 2016). In addition, the QTRAP analyzer coupled to LC was employed by Polgár et al. (2012) for the determination of 44 pesticides in vegetable oils. In addition, Sobhanzadeh et al. (2012) used a TOF analyzer coupled to LC for the determination of seven pesticides in palm oil. The same analyzer was used by Koesukwiwat et al. (2010) but coupled to GC for the determination of 34 pesticides in flaxseeds. Three different detectors were used apart from the MS cited ones. Flame photometric detection (FPD) was used by Y. Liu et al. (2012) for the determination of 15 organophosphorus pesticides in camellia oil. Muhamad et al. (2012) used an electron capture detector for the determination of λ-cyhalothrin and cypermethrin in samples of palm oil. Finally, Farajzadeh et al. (2015) used FID for the determination of five triazole pesticides in sunflower, olive, grape, and corn oils.

When high sensitivity is not crucial, LC with diode array detector (DAD) (Tuzimski and Rejczak 2014, 2016) can be used. Other detection techniques used are fluorescence spectrophotometry (Chen et al. 2015) and solid-phase spectroscopy (Ruiz-Medina et al. 2012) (see Table 8.6).

8.7.3.3 Occurrence

High concentrations of pesticides were found in samples of olive and olive oil in the study performed by Anagnostopoulos and Miliadis (2013). The authors of that study analyzed a total of 262 samples, of which 21 of olive oil and 17 of olives were found to be positive. The highest concentration was 458 μg/kg of omethoate followed by 184 μg/kg of dimethoate. Dimethoate and malathion were found at 1.5 and 3.5 μg/kg respectively in samples of palm oil in the study carried out by Sobhanzadeh et al. (2012). Deme et al. (2014) analyzed 35 pesticides in edible oils finding a maximum of 2.14 μg/kg of p,p′ -DDD (Dichlorodiphenyldichloroethane). Hexaconazole was found at 22.0 μg/kg in a sample of grape oil analyzed by Farajzadeh et al. (2015). Tuzimski and Rejczak (2016) analyzed olive and grapeseed oils and found α-cypermethrin in extra virgin olive oil at concentrations ranging from 17.45 to 23.05 μg/kg, fenuron, dimethomorph, and propazine in grapeseed oil at concentrations ranging from 2.24 to 3.08, from 2.95 to 3.01, and from 0.46 to 1.12 μg/kg, respectively. Moreover, Muhamad et al. (2012) did not find λ-cyhalothrin and cypermethrin in any of the 30 palm oil samples analyzed.

8.8 Conclusions

The presence of most of the chemical contaminants in foods is controlled and regulated by different agencies around the world. However, there is still a lot of work to do in terms of researching on the different contaminants that can affect oils and seeds, as

not all of them are regulated. For example, there is no MRL for aflatoxins in oils, which is surprising taking into account the tremendous hazard and health risk of these contaminants. When this happens, both institutions and laboratories must work in order to investigate the possible occurrence, toxicity, health effects and risks, and consequently elaborate regulations and analytical methods in order to control those substances.

Very often, the presence of contaminants in oils and seeds can be reduced by submitting food to processes like refining, although sometimes, these methods can enhance the presence of other contaminants like 3-MCPD. Regarding the determination of the contaminant studies, it must be noted that the robustness and reliability of the analysis is becoming higher and higher due to recent advances and the modern equipment that laboratories have nowadays. Furthermore, the automatization of the analysis of samples is also improving as it was seen in some studies where more than 200 samples were analyzed. Overall, research in food contaminants cannot be stopped as every day new dangerous substances and new hazards are discovered.

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PUBLICACIÓN II

A rapid method for determination of mycotoxins in edible vegetable oils by ultra-high performance liquid chromatography tandem mass spectrometry

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Analytical Methods

A rapid method for the determination of mycotoxins in edible vegetable oils by ultra-high performance liquid chromatography-tandem mass spectrometry

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ABSTRACT

An analytical method based on a QuEChERS procedure (quick, easy, cheap, effective, rugged and safe) has been developed for the determination of mycotoxins (α-zearalenol and zearalenone, and aflatoxins B1, B2, G1 and G2) in edible oils. The analysis was performed by ultra-high performance liquid chromatography coupled to triple quadrupole analyser (UHPLC-QqQ-MS/MS). The method was fully validated and the quantification limit is 0.5 μg kg^{-1} for aflatoxins and 1 μg kg^{-1} for α-zearalenol and zearalenone. Suitable recoveries were obtained at low concentration levels (0.5–25 μg kg^{-1} for aflatoxins and 1–25 μg kg^{-1} for α-zearalenol and zearalenone), ranging from 80 to 120%. Intra and inter-day precision values were also evaluated and relative standard deviation was lower than 20%. The expanded uncertainty, U, was also evaluated ant it was below 32% at 25 μg kg^{-1}. The validated method has been applied to monitor the presence of mycotoxins in 194 samples belonging to different types of edible oils (olive oil, sunflower oil, soy oil and corn oil). Zearalenone was detected in 25% of the analysed samples at concentrations up to 25.6 μg kg^{-1}, and aflatoxin G1 and G2 in 3% and 14% of the samples at a maximum concentration of 1.9 and 6.8 μg kg^{-1} respectively.

1. Introduction

Many enzymes digest carbon-containing substances such as cellulose and organic materials that are present in food. These digestion processes produce compounds that fungi use as energy (such as sugars) as well as a large number of secondary metabolites. These metabolites, namely mycotoxins (Adeyeye, 2016), are toxic to both human and animal cells (Nielsen et al., 2014). In this sense, the Food and Agricultural Organization of the United Nations (FAO) estimates that more than 25% of all agricultural products are contaminated with mycotoxins (Marin, Ramos, Cano-Sancho, & Sanchis, 2013).

Within the group of mycotoxins, aflatoxins, including B1, B2, G1 and G2, produced by *Aspergillus* species of fungi (Tosun & Arslan, 2013), are of main concern for producers (Jalili, 2015). Aflatoxins have been classified as group 1 human carcinogens and the most dangerous of them is aflatoxin B1, which has been demonstrated to be related to liver cancer (Castañeda Sánchez, Chirivella Martorell, & Carbonel Baldoví, 2012; World Health Organization, 2002). In 2004, 125 people died and more than 200 became ill in Kenya as a result of consuming aflatoxin-contaminated corn (Ongoma, 2013).

Other mycotoxins are zearalenone (ZEA) and its metabolite α-zearalenol (α-ZOL). ZEA is mainly produced by *Fusarium* species in several kinds of cereals as corn (Li et al., 2014). It can interfere with the reproductive function of mammals as well as it can cause immunosuppression (Abrunhosa et al., 2016).

The processed vegetable oils can easily be contaminated with mycotoxins due to certain environmental conditions, such as high temperatures, relative humidity and rainfall. These favour fungal proliferation, and as a direct consequence, mycotoxin production (Bahrami, Shahbazi, & Nikousefat, 2015; Bhat & Reddy, 2017; Fink-Gremmels, 2008; Mahmoudi & Norian, 2015). Therefore, the quality of the oils may be affected, and for example, a contaminated extra virgin olive oil may leave that category and be forced to be refined. Thus, there is an increase interest in the development of validated analytical methods for the determination of mycotoxins, although up to our knowledge, there are scarce studies that evaluate the presence of these compounds in edible oils (Zhao, Chen, Shen, & Qu, 2017).

The Joint Expert Committee on Food Additives (JECFA), a scientific

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advisory body of the World Health Organization (WHO) and the FAO, has evaluated the hazards posed by the most significant mycotoxins in food (Castañeda Sánchez et al., 2012; Li et al., 2014) and the European Commission has stablished a limit of 400 μg kg^{-1} of ZEA for refined corn oil. However, there are not legal limits for aflatoxins in oil, although they have been set in oilseeds, being 2.0 μg kg^{-1} for aflatoxin B1, and 4.0 μg kg^{-1} for the sum of the four aflatoxins (B1, B2, G1 and G2) (European Commission, 1126, 2007; European Commission 1881, 2006).

The analysis of mycotoxins in edible oils is a difficult task due to the complexity of the matrices and the low concentrations of mycotoxins. Thus immunoaffinity chromatography (MacDonald, Chan, Brereton, Damant, & Wood, 2005) or enzyme-linked immunosorbent assay (ELISA) (Klarić, Cvetnić, Pepeljnjak, & Kosalec, 2009) have been developed for the determination of mycotoxins in food. In addition, thin layer chromatography (TLC) (Welke, Hoeltz, Dottori, & Noll, 2009), high performance liquid chromatography (HPLC) have also been tested. Nevertheless, in the last few years, liquid chromatography-electrospray ionization-tandem mass spectrometry (LC–ESI-MS/MS) and matrix-assisted laser/desorption ionization time of flight mass spectrometry (MALDI-TOF MS) (Jerome Jeyakumar, Zhang, & Thiruvengadam, 2018) have been commonly used.

Triple-quadrupole tandem mass spectrometry (QqQ-MS/MS) is highly used for the determination of mycotoxins in food (Bahrami et al., 2015; Garrido-Frenich, Martínez-Vidal, Romero-González, & Aguilera-Luiz, 2009).

Therefore, the development of a suitable extraction method is necessary. Usually, QuEChERS methods (quick, easy, cheap, effective, rugged and safe) have been used for the extraction of mycotoxins in food due to its cost-effectiveness, ease to use and wide applicability (Wang et al., 2018). Then, the determination is usually performed by gas chromatography coupled to tandem mass spectrometry (GC–MS/MS) (Cunha & Fernandes, 2010), LC-MS/MS (Rasmussen, Storm, Rasmussen, Smedsgaard, & Nielsen, 2010) or HPLC coupled to a fluorescence detector (HPLC-FLD) (Sirhan, Huat Tan, & Wong, 2011). In this study, ultra-high performance liquid chromatography-MS/MS (UHPLC-MS/MS) was chosen as it offers several advantages such as speed, low-cost sample preparation and robust analysis (Seserko, Hendrix, & Marzinke, 2013).

However, there are few research carried out in vegetable oils as it is a complex matrix, whose main components are lipids, containing fatty acids and pigments, and an additional clean up step is commonly necessary (Cunha & Fernandes, 2010). In addition, a maximum of 62 samples are analyzed (Eom et al., 2017; Sharmili, Jinap, & Sukor, 2016; Zhao, Chen, et al., 2017; Zhao, Wan, et al., 2017), whereas in this study almost 200 edible vegetable oils have been monitored. Thus, different types of olive oils (extra virgin olive oil, olive oil, lampante olive oil and refined olive oil), two types of pomace oil (olive pomace oil and crude olive pomace oil), two types of sunflower oil (sunflower oil and crude sunflower oil), soy oil and corn oil, monitoring 6 mycotoxins have been included. For that purpose, a QuEChERS-based method has been used, and UHPLC-QqQ-MS/MS analysis has been developed in order to ensure a fast and reliable determination of α-zearalenol, zearalenone, aflatoxins B1, B2, G1 and G2 in edible oils.

2. Materials and methods

2.1. Reagents and chemicals

Aflatoxin B1, B2, G1 and G2, α-ZOL and ZEA reference standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). All compounds present a purity ≥ 99.7%.

Stock standard solutions of the compounds (200 mg L^{-1}) were individually prepared by exact weighing of the solid substances and dissolving in 50 mL of acetonitrile (LC-MS grade, Honeywell, Morriston, NJ, USA). These solutions were stored in the dark at −18 °C. Then, a working standard solution, containing the 6 mycotoxins, was prepared at 10 mg L^{-1} with acetonitrile and stored in glass tubes with screw cap in the dark at −18 °C. The working standard solutions were prepared every two months, while the stock standard solutions were stable up to one year.

LC-MS grade methanol was purchased from Honeywell, while ultrapure water was obtained by a gradient system Milli-Q water (Millipore, Bedford, MA, USA). Aluminium oxide powder was provided by Bruker (Billerica, MA, USA), anhydrous sodium sulphate, sodium chloride and ammonium formate were purchased from Sigma-Aldrich, while ExtraBond C18 and ExtraBond PSA were purchased from Scharlab (Barcelona, Spain).

Finally, 0.22 μm nylon syringe filters were used and they were obtained from Agilent Technologies (Santa Clara, CA, USA).

2.2. Instrument and apparatus

To extract mycotoxins from the samples, a Reax 2 rotatory shaker from Heidolph (Schwabach, Germany) was used, while for the homogenization, a WX vortex from Velp Scientifica (Usmate, Italy) was utilized. A Centronic-PL II centrifuge from JP Selecta (Barcelona, Spain) was used for centrifugation.

Separation of mycotoxins was carried out with an Agilent series 1290 RRLC instrument (Santa Clara, CA, USA) equipped with an autosampler thermostat (G1330B), a binary pump (G4220A) and a column compartment thermostat (G1316C). A Zorbax Eclipse Plus C18 column (100 × 2.1 mm, 1.8 μm particle size) from Agilent was employed for separation of the target compounds. The RRLC system was coupled to an Agilent triple quadrupole mass spectrometer (6460 A) with a Jet Stream electronic spray ionization (ESI) source (G1958-65138). The MassHunter (Agilent) software was used for optimization and quantification.

2.3. Samples collection

Most of the samples were provided by Laboratorio Tello, from Jaén (Spain) and some other were obtained from local supermarkets located in Almería (Spain). The analysed samples included four types of olive oil: extra virgin olive oil (n = 33 samples), olive oil (n = 35), lampante olive oil (n = 31) and refined olive oil (n = 11); two types of pomace oil: olive pomace oil (n = 15) and crude olive pomace oil (n = 28); two types of sunflower oil: sunflower oil (n = 34) and crude sunflower oil (n = 3); soy oil (n = 3) and corn oil (n = 1). The total number of analysed samples was 194.

2.4. Sample preparation

A simple extraction procedure based on QuEChERS approach (Zhao, Chen, et al., 2017) was employed. Briefly, one gram of sample was mixed with 2 mL of water for 1 min in a vortex. Then, 8 mL of acetonitrile was added and it was mixed in a rotatory agitator for 20 min. Afterwards, 4.00 ± 0.05 g of the pre-weighed Na_2SO_4 anhydrous salt and 1.00 ± 0.01 g of NaCl were added and the tube was capped and shaken immediately. After that, the mixture was centrifuged at 5000 rpm (4136*g*) for 10 min. Then, 3 mL of the supernatant were transferred into a 15 mL tube containing 100 mg of C18. The tube was shaken for 1 min in a vortex and subsequently centrifuged at 5000 rpm (4136*g*) for 10 min. Finally, the sample was filtered into a 0.22 μm nylon syringe filter and injected into the LC system.

2.5. UHPLC-MS/MS analyses

The chromatographic separation was carried out employing a binary mobile phase with methanol (A) and 5 mM ammonium formate aqueous solution (B) at flow rate of 0.2 mL min^{-1}. The gradient elution started at 25% of A and increased to 100% A in 3.75 min. This

Table 1
Retention time windows (RTWs) and MS/MS parameters for the target compounds.

Compound	RTW (min)	Precursor ion (*m/z*)	Product ion (*m/z*)[a]	Ion ratio (%)
Aflatoxin B1	4.13–4.17	313.1 (130)[b]	**241.1** (40)[c]	
			285.1 (20)	96.8
			128.1 (80)	91.0
			115.1 (80)	65.9
Aflatoxin B2	4.04–4.06	315.2 (125)	**259.0** (30)	
			287.0 (30)	95.8
			115.0 (80)	78.1
			243.0 (50)	37.7
Aflatoxin G1	3.87–3.89	329.1 (125)	**115.0** (80)	
			243.0 (30)	87.5
			200.0 (50)	80.6
Aflatoxin G2	3.74–3.78	331.1 (125)	**245.0** (30)	
			189.0 (50)	73.3
			115.0 (80)	69.4
α-ZOL	4.93–4.95	319.2 (125)	**129.9** (40)	
			160.0 (40)	87.1
			174.0 (40)	26.2
ZEA	5.04–5.06	317.2 (185)	**131.0** (25)	
			175.0 (25)	85.5
			187.0 (25)	32.7
			273.1 (25)	23.9

[a] Transition in bold was used for quantification.
[b] Fragmentor voltage (V) is given in brackets.
[c] Collision energy (eV) is given in brackets.

composition was held for 2.25 min and then returned to the initial conditions (25% A) in 0.5 min. Finally, this composition was held for 1 min. The total running time was 7.5 min. Injection volume was 5 μL and column temperature was kept at 25 °C.

Aflatoxins B1, B2, G1 and G2 were ionized at positive ESI mode, while α-ZOL and ZEA were ionized at negative ESI mode. All of them were detected using multiple reaction monitoring (MRM) mode. Source gas temperature and sheath gas temperature were 325 °C and 400 °C, respectively. Source gas flow and sheath gas flow were 5 L min^{-1} and 11 L min^{-1}, respectively. Nebulizer was of 45 psi. Capillary and nozzle voltage were 3500 V and 500 V, respectively. Retention time windows (RTWs) and MS/MS parameters are shown in Table 1.

2.6. Method validation

Validation for the optimized methodology was performed using the European Commission regulations (European Commission, 2002; European Commission 401, 2006). Linearity, matrix effect, trueness in terms of recoveries, intra and inter-day precision, limit of quantification (LOQ) and expanded uncertainty (U) were appraised.

A matrix-matched calibration was performed in order to evaluate the linearity, spiking blank extracted samples at several concentrations: 0, 0.5, 1, 2.5, 5, 10, 25, 50, 100 and 250 μg L^{-1}.

Matrix-matched calibrations in extracted extra virgin olive oil, as representative matrix, as well as in other matrices (olive oil, lampante olive oil, refined olive oil, olive pomace oil, crude olive pomace oil, sunflower oil, crude sunflower oil, soy oil and corn oil), and in solvent were prepared. Concentrations ranged from 0.5 to 250 μg L^{-1}. The slopes were compared in order to assess the matrix effect. To calculate the percentage of matrix enhancement or suppression, Eq. (1) was used:

$$Matrix\ effect = \left[\frac{slope\ in\ matrix}{slope\ in\ solvent} - 1\right] \times 100 \quad (1)$$

Thus, matrix effect can be considered negligible if it is equal to or lower than ± 20%, whereas values higher than 20% indicate strong matrix enhancement and significant matrix suppression can be present at values lower than −20%.

Trueness was investigated through recovery trials, spiking blank samples at five levels (0.5, 1, 5, 10 and 25 μg kg^{-1}), using extra virgin olive oil as representative matrix. Each fortified concentration was repeated five times.

Precision was evaluated, assessing repeatability (intra-day precision) and reproducibility (inter-day precision), and the results were expressed as relative standard deviation (RSD, %). For intra-day precision, five replicates at five concentration levels (0.5, 1, 5, 10 and 25 μg kg^{-1}) for aflatoxins and five replicates at four concentration levels (1, 5, 10 and 25 μg kg^{-1}) for α-ZOL and ZEA were evaluated. For inter-day precision, five replicates at three concentration levels (0.5, 5 and 25 μg kg^{-1}) for aflatoxins and five replicates at two concentration levels (5 and 25 μg kg^{-1}) for α-ZOL and ZEA were tested during 5 days.

The estimation of the expanded uncertainty (U) was carried out using the data derived from the validation of the method (Martínez Vidal, Moreno Frías, Garrido Frenich, Olea-Serrano, & Olea, 2002)

Finally, for the estimation of the LOQ, indications described by SANTE guidelines (SANTE/11813, 2017) were followed. LOQ was set as the lowest concentration of the analyte that has been validated with acceptable trueness (recovery ranging from 70 to 120%) and precision (RSD lower than 20%). In addition, it has been checked that S/N values were always higher than 10. Thus, spiked samples at low concentrations, between 0.5 and 25 μg kg^{-1}, were extracted to estimate this parameter.

3. Results and discussion

3.1. Optimization of UHPLC-QqQ-MS/MS

First, the MS conditions were optimized. To obtain the characteristic ion for each compound, full-scan MS (ESI+ & ESI-) was applied. To achieve a correct optimization of the MS/MS parameters, the target compounds, individually prepared at 2 mg L^{-1} in acetonitrile, were injected into the UHPLC-QqQ-MS/MS system at a flow rate of 0.20 mL min^{-1}. Aflatoxins were ionized using ESI+, whereas α-ZOL and ZEA were ionized using ESI-.

In order to evaluate the intensity of each precursor and product ion, further optimization was carried out, applying different fragmentor voltages (from 50 to 200 V) and collision energies (CE, from 10 to 90 eV) respectively. Finally, for quantification purposes, the most intense transitions for the mycotoxins were chosen, while other transitions were used for confirmation purposes (see Table 1). In order to increase the reliability of the confirmation process more than two transitions were selected for each compound, and thus, four transitions were used for AFB1, AFB2 and ZEA, whereas three transitions were used for AFG1, AFG2 and α-ZOL.

In order to obtain the best peak shape and reduce the analysis time, an optimization of the chromatographic conditions was carried out. First, methanol, acetonitrile, and mixtures of methanol:water (50:50, *v/v*) and acetonitrile:water (50:50, *v/v*), were tested as organic phase, and methanol was selected due to better peak shape was obtained. Several aqueous solutions of ammonium formate (0.1, 5.0 and 10.0 mM) were evaluated. The results showed that the best elution of the target compounds was achieved using an aqueous solution of ammonium formate (5.0 mM) and methanol as mobile phase. Moreover, gradient profile and flow rate (0.1–1.0 mL min^{-1}) were evaluated, selecting the conditions indicated in Section 2.5. Fig. 1 shows the chromatogram of a mixture of the selected mycotoxins at 50 μg L^{-1} in solvent.

3.2. Extraction method optimization

Firstly, a conventional QuEChERS extraction procedure with the extraction and clean-up stages previously developed (Zhao, Chen, et al., 2017) was tested. In this study, three different amounts of oil (1.0, 5.0 and 10.0 g), and three different volumes of acetonitrile (4.0, 8.0 and

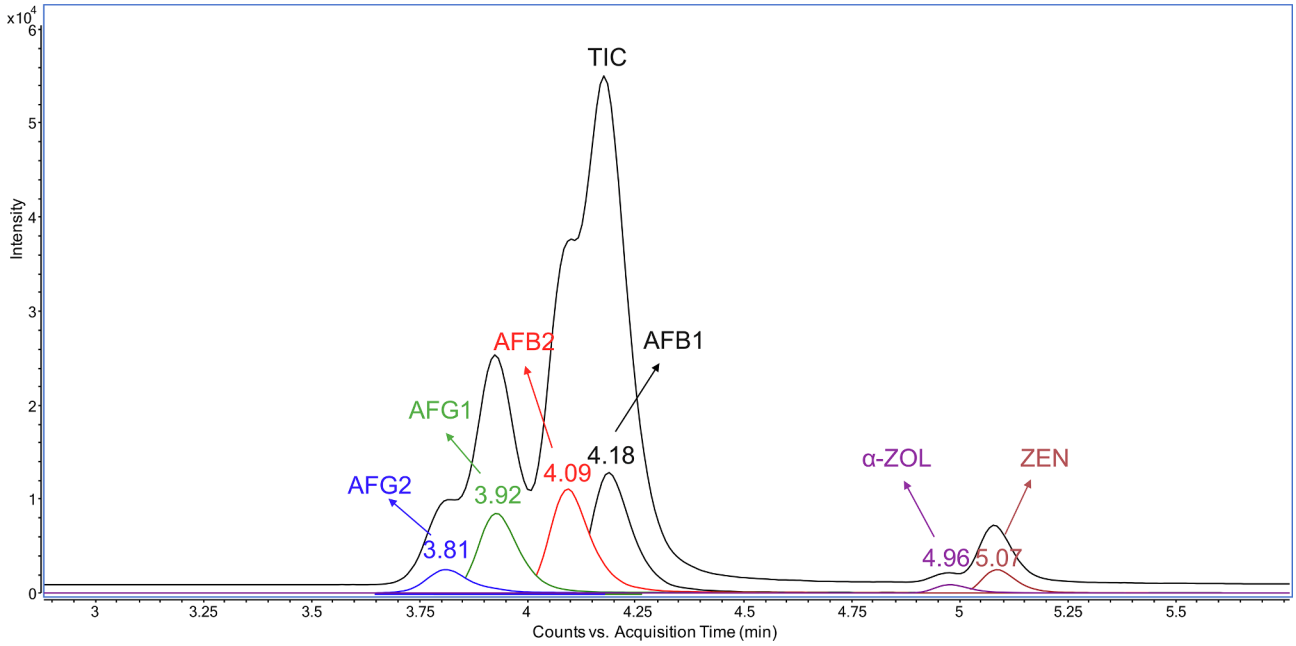

Fig. 1. Extracted ion chromatogram obtained from a standard solution of mycotoxins at 50 μg L^{-1}.

12.0 mL) were assayed in order to evaluate the extraction of mycotoxins. The best results were obtained with 1.0 g of oil and 8.0 mL of acetonitrile.

Due to the complexity of the matrix, a clean-up step based on dispersive solid phase extraction (d-SPE) was needed. In this case, only C18 was used because it was observed that the addition of PSA and Al_2O_3 did not improve the effectiveness of the clean-up step. In addition, three different amounts of C18 (50, 100 and 150 mg) were tested, obtaining the best results when 100 mg of C18 was used.

3.3. Method validation

Linearity was evaluated throughout determination coefficients (R^2) (see Table 2), which are always higher than 0.9968. Intercepts and slopes obtained from the calibration curve are shown in Table 2. In addition, it was observed that standard deviation of the residuals was lower than 20%.

Regarding matrix effect, percentage of enhancement (positive) or suppression (negative) between matrix (extra virgin olive oil) and solvent calibration was 34%, 63%, 54%, 65%, 14% and 4% for the mycotoxins AFB1, AFB2, AFG1, AFG2, α-ZOL and ZEA respectively, which shows that there is a matrix enhancement effect for aflatoxins but it is negligible for α-ZOL and ZEA.

Because several types of edible oils were tested, a representative matrix, extra virgin olive oil, has been selected and the slopes obtained in other types of edible oils were compared to that obtained in extra virgin oil. It has been observed that most of the matrices are comparable to extra virgin olive oil apart from crude olive pomace oil, in which there is suppression effect for aflatoxins (see Table 3). Therefore, this type of edible oils has to be quantified using this matrix if crude olive pomace oil samples must be analysed. Nevertheless, for the other types of edible oils and compounds, extra virgin oil can be used as

Table 2
Validation parameters for the mycotoxins included in this study.

		Mycotoxin					
		AFB1	AFB2	AFG1	AFG2	α-ZOL	ZEA
Regression equation		y = 874.1x (± 23.3) + 200.3 (± 281.0)[a]	y = 540.3x (± 17.5) + 141.6 (± 211.5)	y = 476.2x (± 6.5) + 82.2 (± 78.8)	y = 231.3x (± 5.6) + 51.4 (± 67.6)	y = 9.0x (± 0.3) + 4.1 (± 3.1)	y = 62.3x (± 1.1) + 4.9 (± 13.8)
Determination coefficient (R^2)		0.9979	0.9968	0.9994	0.9982	0.9976	0.9990
Spiked concentration							
0.5 μg kg^{-1}	Recovery (%)	117.1 (5.1)[b]	116.9 (9.0)	119.4 (10.7)	91.5 (13.8)	–	–
	Inter-day RSD (%)	6.4	16.4	12.2	18.2	–	–
1 μg kg^{-1}	Recovery (%)	117.1 (5.8)	99.6 (1.9)	101.1 (5.0)	100.3 (7.7)	109.9 (10.3)	99.2 (4.4)
5 μg kg^{-1}	Recovery (%)	96.0 (3.1)	97.6 (7.2)	106.2 (4.0)	104.3 (5.1)	101.3 (7.3)	95.4 (6.4)
	Inter-day RSD (%)	8.8	9.5	10.2	10.0	18.9	16.9
10 μg kg^{-1}	Recovery (%)	107.9 (1.3)	98.5 (5.3)	99.6 (4.5)	87.5 (3.0)	100.1 (6.5)	94.0 (4.0)
25 μg kg^{-1}	Recovery (%)	100.4 (2.4)	98.3 (5.2)	106.3 (4.1)	100.8 (1.8)	89.4 (6.0)	98.1 (3.9)
	Inter-day RSD (%)	2.8	10.6	11.3	3.9	9.6	12.9
	Expanded Uncertainty (U)	10%	22%	23%	14%	29%	32%

[a] Standard deviation of slope and intercept is given in brackets, obtained when matrix-matched calibration when extra virgin olive oil was used.
[b] Recoveries and RSD were calculated based on n = 5.

Table 3
Matrix effect of each aflatoxin in each type of oil respect to extra virgin olive oil.[a]

Matrix	AFB1	AFB2	AFG1	AFG2	α-ZOL	ZEA
Refined Olive Oil	18%	20%	14%	16%	15%	16%
Crude Olive Pomace Oil	−60%	−73%	−30%	−41%	−6%	−6%
Sunflower Oil	17%	14%	19%	19%	20%	9%
Soy Oil	18%	18%	18%	13%	19%	20%
Corn Oil	11%	19%	13%	13%	10%	10%

[a] Matrix effect was estimated using the following equation: Matrix effect = [(slope in matrix/slope in solvent) − 1] × 100.

representative matrix, simplifying the quantification step.

Recoveries for the mycotoxins included in this study ranged between 91.5 and 119.4%, 99.2–109.9%, 95.4–106.2%, 87.5–107.9% and 89.4–106.3%, for the concentrations 0.5 (except for ZEA and α-ZOL), 1, 5, 10 and 25 μg kg^{-1} respectively as it can be observed in Table 2.

Intra-day precision was always below 13.8%, while inter-day precision was below 18.9% for all the mycotoxins evaluated (see Table 2).

The values of expanded uncertainty at 25 μg kg^{-1} ranged between 10 and 32% (see Table 2), which were within the range allowed by the EURACHEM/CITAC guide (Barwik, 2016)

Finally, the LOQ for AFB1, AFB2, AFG1 and AFG2 were 0.5 μg kg^{-1}, and 1.0 μg kg^{-1} for α-ZOL and ZEA.

3.4. Real samples analysis

The developed method was applied to the determination of the six mycotoxins in 194 oil samples. During the analysis of the samples, an internal quality control was carried out to guarantee the reliability of the results. This includes a blank sample in order to check the absence of interferences, a calibration curve using an extract from extra virgin olive oil, from 0.5 to 250 μg kg^{-1}, to evaluate the sensitivity and perform the quantification of the samples, and fortified samples at 0.5, 5 and 25 μg kg^{-1} to determine the efficiency of the extraction process.

The results obtained show that aflatoxin G1 has only been detected in samples of extra virgin olive oil, mainly in 6 out of the 33 analyzed, at a maximum concentration of 1.9 μg kg^{-1}. However, aflatoxin G2 has been detected in most samples of crude olive pomace oil, 23 out of the 28 samples analyzed, at a maximum concentration of 6.8 μg kg^{-1}. In addition, this compound has been found in all samples of crude sunflower oil at a concentration lower than 2.0 μg kg^{-1}, as well as in one of the samples of refined oil at a concentration of 1.1 μg kg^{-1} (see Table 4).

ZEA was detected in most samples of olive oil, lampante olive oil and refined olive oil. In the first case, concentrations higher than the LOQ were observed in 18 out of the 35 samples analyzed, that is, in more than 50% of them, at a maximum concentration of 21.0 μg kg^{-1}. On the other hand, it has also been detected in 18 out of the 31 samples of lampante olive oil, at a maximum concentration of 25.6 μg kg^{-1}. As example, Fig. 2a and 2b show the chromatogram of the sample containing ZEA at 25.6 μg kg^{-1} and the chromatogram of the sample containing AFG2 at 6.8 μg kg^{-1} respectively. In the case of refined olive oil, this compound has been found in 8 out of the 11 samples analyzed, at a maximum concentration of 20.2 μg kg^{-1}. Finally, it has been detected in one sample of extra virgin olive oil, in one sample of olive-pomace oil, in two samples of crude pomace oil and in one sample of sunflower oil (see Table 4).

Finally, aflatoxin B1 was not detected above the LOQ in any of the samples analyzed, whereas aflatoxin B2 was only detected in a sample of sunflower oil at a concentration of 0.7 μg kg^{-1} whereas, the metabolite of ZEA, α-ZOL, has not been detected above the set LOQ in any of the samples (see Table 4).

Comparing with other studies, it has been found that ZEA is the mycotoxin that was detected in most of the tested samples, and also it is the only one detected in all previous studies (Eom et al., 2017; Sharmili et al., 2016; Zhao, Chen et al., 2017; Zhao, Wan, et al., 2017), and at higher concentrations. Nevertheless, it is far from the limit of 400 μg kg^{-1} stablished by the European Commission. Despite corn and related matrices can be contaminated with *Fusarium* species, producing ZEA, in the sample of corn oil analyzed in this study, this mycotoxin was not detected, suggesting that raw material (corn) was not contaminated with *Fusarium* fungi. On the other hand, almost half of the analyzed olive oil samples have been found positive for one or more mycotoxins, while neither corn nor soy oils has been found contaminated. However, in previous articles, mycotoxins have been detected in corn and soy oils at a maximum of 111 μg kg^{-1} in corn oil (Zhao,Wan, et al., 2017).

In addition, this is the first study that evaluates different types of olive oil. Thus, and taking into account that olive oil should be a higher quality oil than lampante olive oil, it can be noted that the same proportion of both types of olive oil are contaminated with ZEA (51% of olive oil and 55% of lampante olive oil), although the maximum concentration detected in lampante olive oil is slightly higher than the one in olive oil (25.6 μg kg^{-1} vs 21.1 μg kg^{-1}). Moreover, most of the samples of crude olive pomace oil (82%) contain AFG2 (see Table 4). It is remarkable that most of the samples of refined olive oil (73%) contain ZEA, despite it is well-known that the refining process can eliminate or minimize these compounds in the final product.

Regarding the other edible vegetable oils, all crude sunflower oil samples are contaminated with aflatoxin G2 (see Table 4).

4. Conclusions

A rapid and simple method for the determination of 6 mycotoxins in edible oils has been developed using an extraction procedure based on the modified QuEChERS procedure coupled to UHPLC-QqQ-MS/MS, increasing sample throughput. The validation of the method indicates that a reliable determination (suitable precision and recovery) of the targeted mycotoxins at trace levels (LOQs between 0.5 and 1.0 μg kg^{-1}) can be performed. The developed method has been applied to a total of

Table 4
Concentration range (μg kg^{-1}) of mycotoxins detected in the different types of edible oils analysed.

Matrix	Analyzed samples	AFB1	AFB2	AFG1	AFG2	α-ZOL	ZEA
Olive Oil	35	–	–	–	–	–	1.1–21.1 (51%)[a]
Extra Virgin Olive Oil	33	–	–	0.8–1.9 (18%)	–	–	1.3 (3%)
Lampante Olive Oil	31	–	–	–	–	–	0.6–25.6 (55%)
Refined Olive Oil	11	–	–	–	1.1 (9%)	–	0.7–20.2 (73%)
Olive Pomace Oil	15	–	–	–	–	–	0.7 (7%)
Crude Olive Pomace Oil	28	–	–	–	1.4–6.8 (82%)	–	0.6 (7%)
Sunflower Oil	34	–	0.7 (3%)	–	–	–	2.0 (3%)
Crude Sunflower Oil	3	–	–	–	0.5–2.0 (100%)	–	–
Soy Oil	3	–	–	–	–	–	–
Corn Oil	1	–	–	–	–	–	–

[a] Percentages of positive samples are given in brackets.

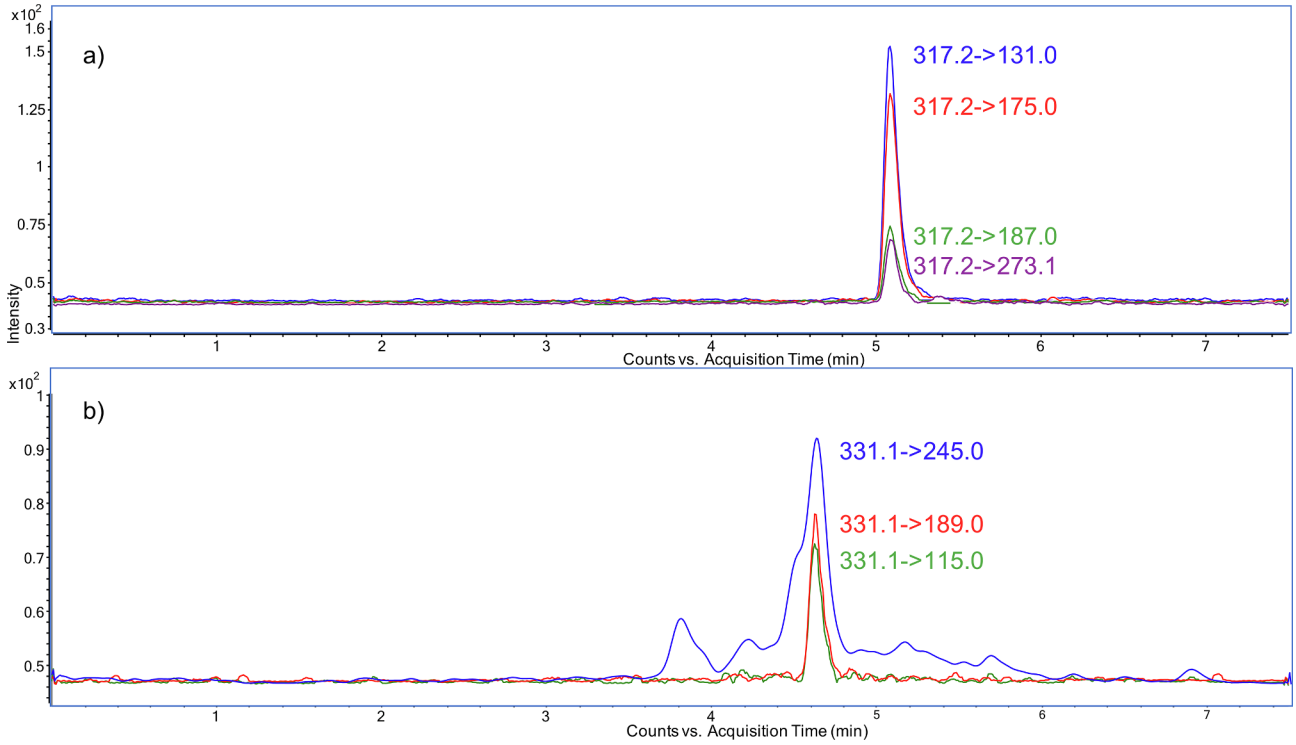

Fig. 2. (a) Extracted ion chromatogram of the lampante olive oil sample contaminated with 25.6 μg kg^{-1} of ZEA; (b) Extracted ion chromatogram of the crude olive pomace oil sample contaminated with 6.8 μg kg^{-1} of AFG2.

194 real samples of different types of vegetable oils, and mycotoxins have been detected in 40% of the total samples. Targeted compounds were detected in all the types of tested edible oils except in soy and corn oil, but few samples of these oils were evaluated. Aflatoxins B1 and G2 have not been detected above the LOQ in any of the samples analyzed, whereas aflatoxins B2 and G1 have been detected at very low concentrations. Nevertheless aflatoxin G2 and ZEA were detected at higher concentrations, up to 6.8 and 25.6 μg kg^{-1} respectively. Considering the high level of positive samples, routine control of these compounds in edible vegetable oils is necessary to ensure food safety in these products.

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Conflict of interest

The authors declare that there is no conflict of interest.

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PUBLICACIÓN III

Determination of mycotoxins in nuts by ultra high-performance liquid chromatography-tandem mass spectrometry: Looking for a representative matrix

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Original Research Article

Determination of mycotoxins in nuts by ultra high-performance liquid chromatography-tandem mass spectrometry: Looking for a representative matrix

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ABSTRACT

A method was developed for the determination of 6 mycotoxins (α-zearalenol and zearalenone, and aflatoxins B1, B2, G1 and G2) in nuts. For that purpose, a QuEChERS based method (quick, easy, cheap, effective, rugged and safe) procedure has been used as well as ultra-high performance liquid chromatography coupled to triple quadrupole tandem mass spectrometry (UHPLC-QqQ-MS/MS). Peanuts was selected as representative matrix after checking several matrices (almonds, hazelnuts, peanuts, pistachios and walnuts). Thus, a quantification strategy of mycotoxins in nuts has been stablished using only one matrix.

The method was also fully validated and the quantification limit was set at 0.5 μg kg^{-1} for the four aflatoxins and 1 μg kg^{-1} for α-zearalenol and zearalenone. Appropriate recoveries were obtained at low concentration levels (0.5, 10 and 25 μg kg^{-1}), ranging from 80 to 120%. Intra and inter-day precision values, expressed as relative standard deviation, were lower than 20%. Expanded uncertainty was also estimated and it was below 42%.

To check the applicability of the method, it has been applied to 36 samples of different types of nuts, such as almonds, hazelnuts, peanuts, pistachios and walnuts. Aflatoxin G2 was detected in 40% of the analysed samples at concentrations up to 6.3 μg kg^{-1}.

1. Introduction

The Mediterranean diet has been related to a number of health benefits, including reduced mortality risk and lower incidence of cardiovascular disease (Trichopoulou et al., 2014). One of the main components of the Mediterranean diet are nuts (Storniolo et al., 2017). They contain a relatively large quantity of calories, essential unsaturated and monounsaturated fats including the fatty acids linoleic and linolenic acid, vitamins, essential amino acids. Moreover, they are considered as source of vitamin E, vitamin B2, folate, fiber, and the essential minerals magnesium, phosphorus, potassium, copper, and selenium (Sabaté and Wien, 2013).

Despite this, nuts can also contain toxic compounds as mycotoxins, and among them, aflatoxins (Eneroth et al., 2017), which are related to liver cancer (Castañeda Sánchez et al., 2012; World Health Organization, 2002). These substances are secondary metabolites produced by fungi, specifically by *Aspergillus* and *Fusarium* species in their digestion processes (Tosun and Arslan, 2013). While *Aspergillus* species mainly produce aflatoxins B1, B2, G1 and G2, *Fusarium* species produce zearalenone (ZEA) (Li et al., 2014) and one of its metabolites, α-zearalenol (α-ZOL).

Due to certain weather conditions, such as high temperatures, relative humidity and rainfall, favour fungal proliferation and in consequence, mycotoxin production, nuts can easily be contaminated with mycotoxins at these conditions (Bahrami et al., 2015; Fink-Gremmels, 2008; Mahmoudi and Norian, 2015). In fact, there are more than 600 alerts for aflatoxins and 2 alerts for ZEA included in the Rapid Alert System for Food and Feed (RASFF) from 2018 to January 2019. Thus, the monitoring of mycotoxins is necessary, and there is an increase in the development of validated analytical methods for their determination.

The European Commission has stablished a limit of 15 and 10 μg kg^{-1} of total aflatoxins, and a maximum of 8 and 5 μg kg^{-1} for aflatoxin B1, in groundnuts and nuts respectively if the samples are

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subjected to be sorted or treated physically before human consumption, and a limit of 4 and 2 μg kg^{-1} for total aflatoxins and aflatoxin B1 respectively if they are intended for direct human consumption (European Commission 1881, 2006European Commission 1881, 2006). On the other hand, it does not exist legislation for ZEA in groundnuts or nuts.

This lack of legislation for ZEA and α-ZOL in nuts may be due to these mycotoxins hardly occur in nuts, although there are some studies that detected ZEA in this type of matrices (Arroyo-Manzanares et al., 2013; Han et al., 2016), and therefore they have been included in this study.

Mycotoxin analysis in nuts is a complex task, due to both the high lipid content and the low concentrations that they may be present in these matrices. Despite this, some methods have been developed to detect mycotoxins in nuts (Arroyo-Manzanares et al., 2013; Cunha et al., 2018; Dong et al., 2019a; Kafouris et al., 2017; Spanjer et al., 2008; Wang et al., 2018) using triple-quadrupole tandem mass spectrometry (QqQ-MS/MS), due to the advantages that this analyzer offers, such as reliability, low-cost sample preparation and robust analysis.

Isotopically labeled compounds are widely used to compensate the matrix effect of the analyte during sample extraction (Yuan et al., 2019, 2016). These isotopically labeled compounds are very expensive. Although the consumption of internal standards can be reduced by using an autosampler program, finding a representative matrix for all the nuts can reduce the cost of the analysis, and furthermore the matrix effect is compensated. Therefore, samples can be quantified using the representative matrix, minimizing the cost in routine laboratories.

However, none of the previous publications has performed a research to find a proper matrix to quantify mycotoxins avoiding the use of isotopically labeled internal standard in each matrix. Therefore, the search of a representative matrix was carried out for the determination of these mycotoxins in nuts, in order to use it as a model for all matrices. In addition, 36 samples, including 5 different matrices that were previously studied in this research, such as almonds, hazelnuts, peanuts, pistachios and walnuts were analyzed using a QuEChERS (quick, easy, cheap, effective, rugged and safe) method. QuEChERS-based methods have been widely used (Dong et al., 2019b; Dong and Xiao, 2017; Xian et al., 2019). This extraction procedure reduces matrix effect (ion suppression or ion enhancement phenomena), which are usually a limitation of MS methods. Therefore, a very rapid QuEChERS method coupled with ultra high liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) has been applied for the determination of aflatoxins, ZEA and α-ZOL in nuts.

2. Materials and methods

2.1. Reagents and chemicals

Aflatoxin B1, B2, G1 and G2, α-ZOL and ZEA reference standards were obtained from Sigma-Aldrich (St. Louis, MO, USA). The purity of the compounds was ≥ 99.7%.

Stock standard solutions (200 mg L^{-1}) were prepared individually by exact weighing of the solid substances and dissolved in 50 mL of acetonitrile (LC–MS grade, Honeywell, Morristown, NJ, USA). They were stored at -18 °C in the dark and these solutions were stable up to one year. Then, a standard solution was prepared at 10 mg L^{-1} with acetonitrile and stored in the dark in screw-capped glass tubes at −18 °C. This working standard solution was prepared every two months.

Ultrapure water was obtained by a gradient system Milli-Q water (Millipore, Bedford, MA, USA), whereas LC–MS grade methanol was purchased from Honeywell. Aluminium oxide powder was purchased from Bruker (Billerica, MA, USA), ammonium formate, sodium chloride and anhydrous sodium sulphate were obtained from Sigma-Aldrich. ExtraBond C18 and ExtraBond PSA were purchased from Scharlab (Barcelona, Spain).

A 0.22 μm nylon syringe filters were used and they were obtained from Agilent Technologies (Santa Clara, CA, USA).

2.2. Instrument and apparatus

A Reax 2 rotatory shaker from Heidolph (Schwabach, Germany) was used to extract the compounds, while a WX vortex from Velp Scientifica (Usmate, Italy) was utilized to homogenize the samples. A Centronic-PL II centrifuge from JP Selecta (Barcelona, Spain) was used for centrifugation.

In order to carry out the detection of mycotoxins, an Agilent series 1290 RRLC instrument (Agilent, Santa Clara, CA, USA) equipped with a binary pump (G4220A), an autosampler thermostat (G1330B) and a column compartment thermostat (G1316C) was employed. A Zorbax plus C18 column (100 × 2.1 mm, 1.8 μm particle size) from Agilent (San Jose, CA, USA) was used for the separation of the compounds. An Agilent triple quadrupole mass spectrometer (6460 A) with a Jet Stream electronic spray ionization (ESI) source (G1958-65138) was coupled to the LC. The MassHunter (Agilent) software was used during optimization and quantification stages.

2.3. Samples collection

Samples were bought in local supermarkets located in Almería (Spain). The analysed samples were almonds (n = 7 samples), hazelnuts (n = 6 samples), peanuts (n = 10 samples), pistachios (n = 6 samples), and walnuts (n = 7 samples).

2.4. Sample preparation

A previously developed QuEChERS method (Hidalgo-Ruiz et al., 2019) was used as reference for the development of the extraction method. First, 10 mL of acetonitrile: water (80:20, *v/v*) were added to 2 g of milled and homogenized sample. Then, it was mixed for 2 min in a vortex and then in a rotatory agitator for 20 min. After that, 4.00 ± 0.05 g of Na_2SO_4 anhydrous salt and 1.00 ± 0.01 g of NaCl were added and the tube was sealed and shaken without delay. Afterwards, the mixture was centrifuged at 5000 rpm (4136 × *g*) for 10 min. Then, 3 mL of the supernatant were added to a 15 mL tube that contains 100 mg of C18. The tube was shaken for 1 min in a vortex and then, it was centrifuged at 5000 rpm (4136 × *g*) for 10 min. Finally, a 0.22 μm nylon syringe filter was used to filter the supernatant and it was injected into the LC system.

2.5. UHPLC-MS/MS analyses

The mobile phase was methanol (A) and an aqueous solution of ammonium formate 5 mM (B) at flow rate of 0.2 mL min^{-1}. The elution gradient started at 25% of A and increased to 100% A in 3.75 min. This composition was maintained during 2.25 min and after that, it was reduced to initial conditions (25% A) in 0.5 min. Finally, this composition was maintained for 1 min, and total running time was 7.5 min. The column temperature was 25 °C and the injection volume was 5 μL.

α-ZOL and ZEA were ionized at negative ESI mode, whereas aflatoxins B1, B2, G1 and G2 were ionized at positive ESI mode. They were detected using multiple reaction monitoring (MRM) mode. Sheath gas temperature and source gas temperature were 400 °C and 325 °C, respectively. Sheath gas flow and source gas flow were 11 L min^{-1} and 5 L min^{-1}, respectively. Nebulizer gas pressure was 45 psi. Capillary and nozzle voltage were 3500 V and 500 V, respectively. Retention time windows and MS/MS parameters are shown in Table 1.

2.6. Method validation

Validation of the optimized methodology was performed according to the European Commission regulations (European Commission, 2002;

Table 1
Retention time windows (RTWs) and MS/MS parameters for the target compounds.

Compound	RTW (min)	Precursor ion (m/z)	Product ion (m/z)[a]	Ion ratio (%)
Aflatoxin B1	4.13-4.17	313.1 (130)[b]	**241.1** (40)[c]	
			285.1 (20)	96.8
			128.1 (80)	91.0
			115.1 (80)	65.9
Aflatoxin B2	4.04-4.06	315.2 (125)	**259.0** (30)	
			287.0 (30)	95.8
			115.0 (80)	78.1
			243.0 (50)	37.7
Aflatoxin G1	3.87-3.89	329.1 (125)	**115.0** (80)	
			243.0 (30)	87.5
			200.0 (50)	80.6
Aflatoxin G2	3.74-3.78	331.1 (125)	**245.0** (30)	
			189.0 (50)	73.3
			115.0 (80)	69.4
α-Zearalenol	4.93-4.95	319.2 (125)	**129.9** (40)	
			160.0 (40)	87.1
			174.0 (40)	26.2
Zearalenone	5.04-5.06	317.2 (185)	**131.0** (25)	
			175.0 (25)	85.5
			187.0 (25)	32.7
			273.1 (25)	23.9

[a] Transition in bold was used for quantification.
[b] Fragmentor voltage (V) is given in brackets.
[c] Collision energy (eV) is given in brackets.

European Commission 401, 2006). The parameters evaluated were: linearity, matrix effect, trueness, intra and inter-day precision, limit of quantification (LOQ) and uncertainty (U).

Linearity was appraised with a matrix-matched calibration, spiking blank extract samples at several concentrations: 0, 0.5, 1, 2.5, 5, 10, 25, 50, 100 and 250 μg L^{-1} in the different matrices.

To evaluate the matrix effect, matrix-matched calibrations in different nuts (almonds, hazelnuts, peanuts, pistachios and walnuts) and in solvent were prepared at concentrations between 0.5–250 μg L^{-1} and the slopes were compared. To calculate the percentage of matrix enhancement or suppression, equation (1) was used:

$$Matrix\ effect = \left[\frac{slope\ in\ matrix}{slope\ in\ solvent} - 1\right] \times 100 \quad (1)$$

A limit of 20% of matrix effect has been set as it has been recommended by SANTE guide (SANTE/11813, 2017SANTE/11813, 2017).

Trueness was investigated through recovery trials, spiking blank samples at five levels (0.5, 1, 5, 10 and 25 μg kg^{-1}), using peanuts as representative matrix. Each fortified concentration was repeated five times.

Precision was performed in terms of repeatability (intra-day precision) and reproducibility (inter-day precision), expressed as relative standard deviation (RSD, %). For intra-day precision, five replicates at three concentration levels (0.5 (1 for α-ZOL and ZEA) 10 and 25 μg kg^{-1}) were evaluated in all the matrices (almonds, hazelnuts, peanuts, pistachios and walnuts). Likewise, for inter-day precision, five replicates at three concentration levels (0.5 (1 for α-ZOL and ZEA), 10 and 25 μg kg^{-1}) were tested for 5 days in peanuts.

The estimation of the expanded uncertainty (U) was carried out using the data obtained from the validation of the method (Martínez Vidal et al., 2002).

Finally, SANTE recommendations (SANTE/11813, 2017SANTE/11813, 2017) were followed for the estimation of the LOQ, and it was set as the lowest concentration of the analyte that has been validated with acceptable trueness (recovery ranging from 70 to 120%) and precision (RSD lower than 20%). Thus, spiked samples at low concentrations between 0.5 to 25 μg kg^{-1} were extracted, and LOQ was evaluated.

3. Results and discussion

A previous extraction method based on QuEChERS method has been tested for the determination of mycotoxins in nuts (Hidalgo-Ruiz et al., 2019). Whereas the LC–MS procedure has been the same, the extraction procedure was slightly modified. Briefly, the amount of sample has increased from 1 g to 2 g, as well as the homogenization time was increased from 10 to 20 min, using the final procedure described in Section 2.4. Then, the method was validated as it has been discussed in the next sections.

3.1. Matrix effect

When several compounds should be determined in different matrices, the evaluation of the matrix effect using matrix-matched calibrations is commonly performed (Huang et al., 2019; Lin et al., 2018; Yang et al., 2017).

Matrix effect has been tested with respect to solvent and to five conventional nut matrices (almonds, hazelnuts, peanuts, pistachios and walnuts), showing the results in Table 2. Additionally, Fig. 1 represents the percentage of matrix effect of each mycotoxin in each matrix studied in relation to the selected matrix.

It can be observed in Table 2 (Fig. 1a) that all the mycotoxins were above or below the ± 20% set, except for α-ZOL. Furthermore, it can be observed that the matrix effect was out of this limit in almost the 75% of the combinations matrix-analyte (Table 2, Fig. 1a). Only for α-ZOL, the matrix effect of all the matrices was lower than 20%. For this reason, a representative matrix was sought, in order to compensate the matrix effect, avoiding the use of isotopically labeled internal standard. Therefore, matrix effect was evaluated, comparing the slopes of the analytical curves obtained for the five matrices, selected one as reference, and Figures b–f show the results.

When almond was selected as representative matrix, it can be observed a different behaviour between aflatoxins and α-ZOL and ZEA. Matrix suppression was observed for aflatoxins, whereas for α-ZOL and ZEA, matrix suppression or enhancement can be observed, but between ± 20%, except for ZEA in walnuts (Table 2, Fig. 1b).

The matrix effect when hazelnuts were selected was close to be within the limits, but there are two values that were out of range: AFG1 in walnuts and α-ZOL in pistachios (Table 2, Fig. 1c).

For peanuts, the matrix effect is similar than when hazelnuts were selected, but in this case, all the values are within ± 20%. The only

Table 2
Matrix effect of mycotoxins in tested nuts in relation to solvent.

Matrix	AFB1	AFB2	AFG1	AFG2	α-ZOL	ZEA
Peanuts	30%	48%	47%	53%	1%	−17%
Almonds	15% (9%)[a]	31% (6%)	22% (12%)	28% (9%)	−4% (6%)	−22% (-4%)
Hazelnuts	28% (−2%)	46% (−2%)	43% (−2%)	48% (−3%)	−12% (−13%)	−28% (−14%)
Pistachios	42% (−1%)	57% (1%)	64% (24%)	67% (16%)	6% (−16%)	−20% (−28%)
Walnuts	29% (−12%)	50% (−12%)	82% (−17%)	77% (−16%)	−16% (−5%)	−40% (−6%)

[a] Matrix effect in each nut using peanut as representaitive matrix are given in brackets.

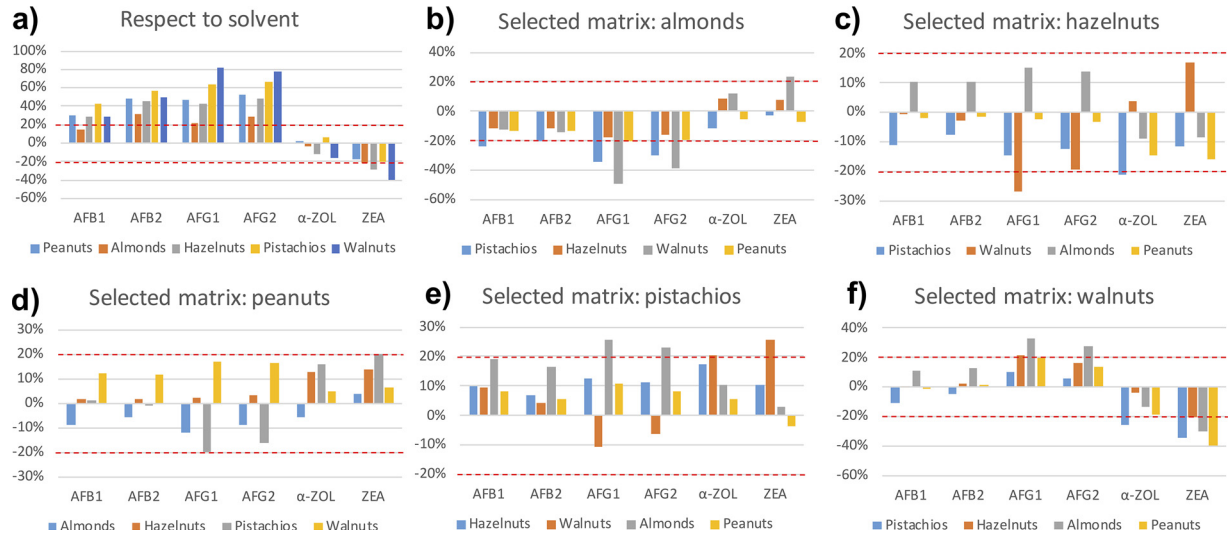

Fig. 1. Comparison of matrix effect selecting as reference matrix (a) solvent, (b) almonds, (c) hazelnuts, (d) peanuts, (e) pistachios and (f) walnuts.

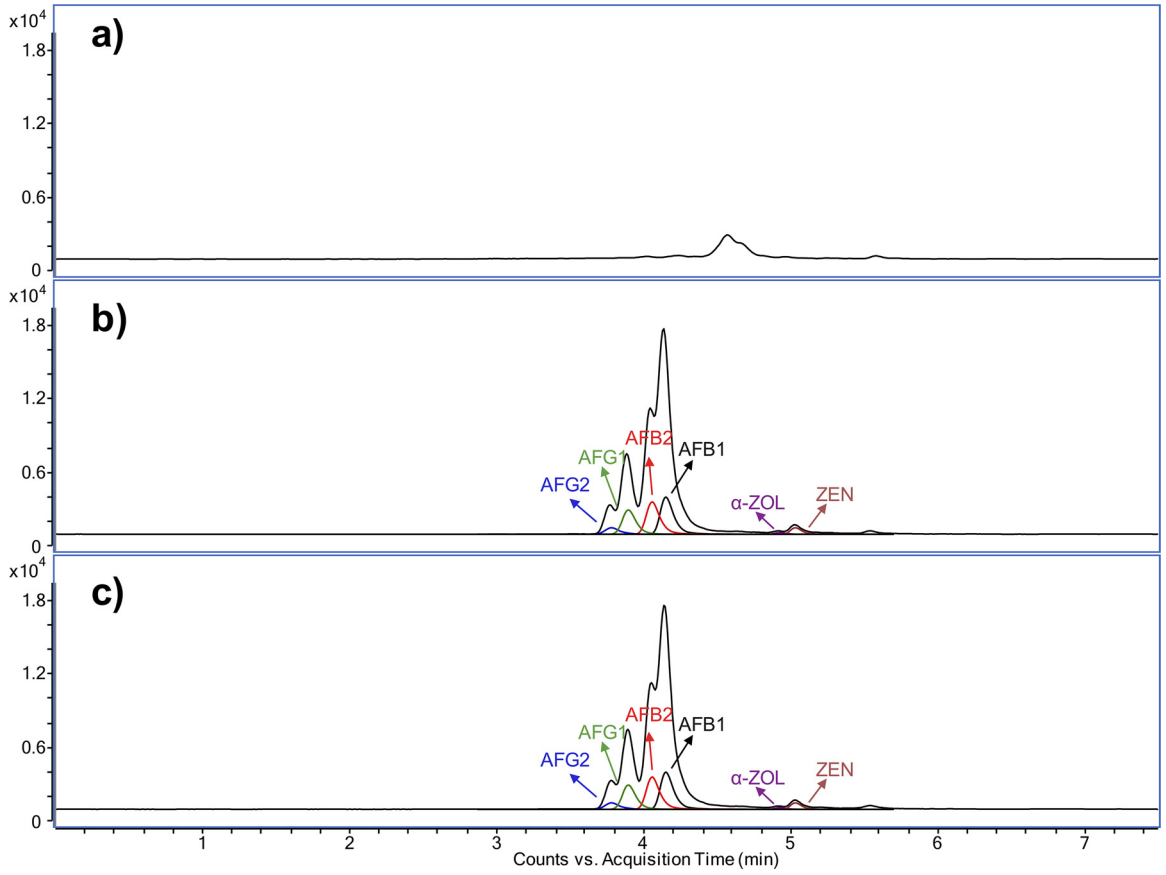

Fig. 2. Total ion chromatograms and extracted ion chromatograms of a (a) blank sample of peanut; (b) Matrix-matched calibration point of peanut at 25 μg kg^{-1}. (c) Spiked peanut sample at 25 μg kg^{-1}.

values that are close to that limit are AFG1 and ZEA in pistachios, which are just in the limit. It is noticeable that all values of matrix effect of almonds in peanuts were below ± 12%. Also the matrix effect values of aflatoxins for hazelnuts in peanuts were very low (3% as maximum) (Table 2, Fig. 1d).

In order to check the matrix effect between different samples of the same matrix, 5 different samples of peanuts were evaluated, and it was found that matrix effect values were always within that limits of 20% for the six analytes.

When the selected matrix is pistachios, matrix enhancement was observed for most of the combinations analyte-matrix, except for aflatoxin G1 and G2 in walnuts and ZEA in peanuts. Nevertheless, matrix effect was always lower than 20%, except for aflatoxin G1 and G2 in almonds and ZEA in walnuts (Table 2, Fig. 1e).

Finally, several values were out of the established limits (± 20%) when walnut was used as representative matrix (Table 2, Fig. 1f), as for AFG1, AFG2, α-ZOL and ZEA.

Therefore, the percentage of enhancement or suppression of the

Table 3
Recovery and precision values obtained for the different compounds in all the matrices.

	Spiked concentration		Mycotoxin					
			AFB1	AFB2	AFG1	AFG2	α-ZOL	ZEA
		LOQ (μg kg^{-1})	0.5	0.5	0.5	0.5	1.0	1.0
Peanuts	0.5 μg kg^{-1}[a]	Recovery (%)	100.6	88.3	94.7	96.7	73.6	88.2
		Intra-day (Inter-day) RSD (%)	6.7 (8.9)[b]	3.8 (11.1)	3.8 (14.5)	10.8 (15.4)	3.5 (18.5)	4.6 (17.0)
		Expanded Uncertainty (U)	20%	25%	39%	32%	42%	39%
	10 μg kg^{-1}	Recovery (%)	89.1	91.8	90.1	92.7	87.1	87.6
		Intra-day (Inter-day) RSD (%)	3.9 (5.9)	2.7 (8.6)	4.1 (5.7)	4.0 (8.8)	6.4 (7.0)	3.8 (9.7)
		Expanded Uncertainty (U)	11%	18%	11%	18%	15%	21%
	25 μg kg^{-1}	Recovery (%)	97.9	99.7	100.3	103.0	116.3	99.9
		Intra-day (Inter-day) RSD (%)	1.3 (3.9)	1.9 (5.0)	0.5 (4.8)	2.7 (4.7)	5.7 (9.6)	4.8 (10.1)
		Expanded Uncertainty (U)	10%	11%	10%	11%	19%	21%
Almonds	0.5 μg kg^{-1}	Recovery (%)	89.4	93.0	97.8	103.5	74.7	78.0
		Intra-day RSD (%)	4.9	4.5	7.3	8.2	6.7	5.4
	10 μg kg^{-1}	Recovery (%)	99.8	101.4	102.1	112.7	99.4	93.6
		Intra-day RSD (%)	2.3	3.3	7.1	5.8	4.2	2.7
	25 μg kg^{-1}	Recovery (%)	97.3	96.4	92.6	97.6	92.3	96.3
		Intra-day RSD (%)	2.4	2.6	4.9	2.0	2.4	4.0
Hazelnuts	0.5 μg kg^{-1}	Recovery (%)	82.9	86.4	97.2	95.8	76.4	72.6
		Intra-day RSD (%)	3.9	4.9	4.5	6.9	4.3	5.5
	10 μg kg^{-1}	Recovery (%)	92.9	92.0	90.8	89.2	97.1	93.7
		Intra-day RSD (%)	2.8	2.3	3.1	4.9	5.7	2.6
	25 μg kg^{-1}	Recovery (%)	92.9	92.0	94.2	89.0	97.1	93.7
		Intra-day RSD (%)	2.8	2.3	2.0	0.9	5.7	2.6
Pistachios	0.5 μg kg^{-1}	Recovery (%)	100.6	115.3	101.8	117.5	97.2	107.0
		Intra-day RSD (%)	7.6	1.5	3.4	12.2	15.1	9.2
	10 μg kg^{-1}	Recovery (%)	86.5	97.4	97.4	98.7	93.3	97.4
		Intra-day RSD (%)	5.3	6.9	5.6	2.9	6.9	1.6
	25 μg kg^{-1}	Recovery (%)	84.5	94.6	90.8	99.3	103.0	85.7
		Intra-day RSD (%)	2.1	2.5	1.8	2.3	9.3	6.1
Walnuts	0.5 μg kg^{-1}	Recovery (%)	89.3	88.0	87.4	70.2	70.1	85.8
		Intra-day RSD (%)	4.4	3.5	6.3	16.3	13.8	7.5
	10 μg kg^{-1}	Recovery (%)	96.2	90.5	90.3	90.6	103.0	95.6
		Intra-day RSD (%)	2.5	1.5	4.9	2.2	6.2	10.4
	25 μg kg^{-1}	Recovery (%)	99.4	100.3	103.2	100.7	105.9	107.1
		Intra-day RSD (%)	0.8	1.2	2.4	1.6	6.4	8.8

Note: Recoveries and precision were calculated based on $n_{sample\ number} = 5$.
[a] 1 μg kg^{-1} for α-ZOL and ZEA.
[b] Inter-day precision is given in brackets.

Table 4
Comparison of main performance characteristics.

Reference	Running time (min)	LOQ (μg kg^{-1})	Recovery (%)	RSD (%)
Proposed method	7.5	0.5-1.0	70.1-117.5 (at 0.5 μg kg^{-1})[a]	< 16.3
Han et al., 2016	16.0	0.1-3.0[b]	88.2-112.5 (at 1 μg kg^{-1})	< 16.7
Cunha et al., 2018	25.0	1.25-5.0[b]	56.0-91.0 (at 5 μg kg^{-1})	< 14.0
Azaiez et al., 2014	32.5	0.2-0.9[c]	60.0-118.0 (at 5 μg kg^{-1})	< 14.0

[a] 1 μg kg^{-1} for α-ZOL and ZEA.
[b] α-ZOL is not analysed.
[c] α-ZOL and ZEA are not analysed in this paper.

aflatoxins was always smaller when peanuts was used as representative matrix (Table 2, Fig. 1d). Regarding these results, it can be seen that all the percentages are below the set limit (± 20%). Consequently, peanut has been selected as representative matrix in order to perform a quantification strategy using only one matrix for the determination of mycotoxins in different types of nuts. In addition, 5 blank samples of each matrix were collected, and matrix effect was estimated using peanut as representative matrix. Then, a *t*-test was used to check if matrix effect is significantly different than 1 or not (no matrix effect) and in all the cases, p-value was always higher than 0.05, indicating that when peanut is used as representative matrix, matrix effect is compensated for these mycotoxins in the tested matrices.

Fig. 2 shows a blank sample of peanut (a), a matrix-matched point of peanut at 25 μg kg^{-1} (b), and a spiked peanut sample at 25 μg kg^{-1} (c). It can be observed that complete resolution for the analytes is not achieved, but MS/MS allows the selective analysis without chromatographic resolution.

3.2. Method validation

The method has been validated, using peanuts as representative matrix (Table 3). Regarding linearity, determination coefficients (R^2) obtained were always between 0.9967 and 0.9999.

Recoveries at 0.5 (1 for α-ZOL and ZEA) μg kg^{-1} ranged between 70.1 and 117.5%, at 10 μg kg^{-1} between 86.5 and 112.7% and at 25 μg kg^{-1} between 84.5 and 116.3%. Therefore, recovery values were between 70 and 120% for all the matrices.

Intra-day precision was always below 15.1% at 0.5 (1 for α-ZOL and ZEA) μg kg^{-1}, lower than 10.4% at 10 μg kg^{-1} and below 9.3% at 25 μg kg^{-1}, while inter-day precision was below 18.5% for all the mycotoxins evaluated in peanuts

The values of expanded uncertainty at 0.5 (1 for α-ZOL and ZEA),

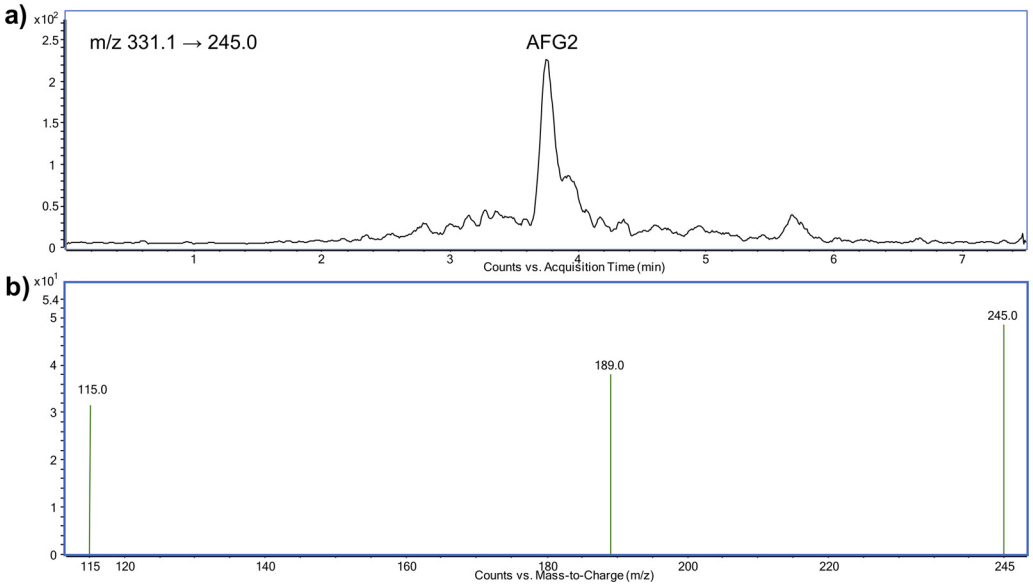

Fig. 3. (a) Extracted ion chromatogram of the pistachio sample contaminated with 0.9 μg kg^{-1} of AFG2. (b) Selected product ions of AFG2 in the same sample.

10 and 25 μg kg^{-1} ranged between 11 and 42% (see Table 3), which were within the range allowed by the EURACHEM/CITAC guide (EURACHEM/CITAC, 2012).

The LOQ for AFB1, AFB2, AFG1 and AFG2 were 0.5 μg kg^{-1}, whereas the LOQ for α-ZOL and ZEA were 1 μg kg^{-1}. These LOQs are comparable to those published in dried fruits (Azaiez et al., 2014; Cunha et al., 2018; Han et al., 2016), as it can be observed in Table 4, as well as other performance characteristics as recovery and precision, which were evaluated at lower concentrations than those published previously.

The total analysis time was also considerably reduced, when it is compared with other methods (Azaiez et al., 2014; Cunha et al., 2018; Han et al., 2016) (see Table 4). Furthermore, no isotopically labelled reference standards were used as in other methods (Yuan et al., 2019, 2016), which reduces the cost of analysis.

3.3. Samples analysis

The validated method was applied to 36 nut samples. In order to guarantee the reliability of the results an internal quality control set was carried each time samples were analysed. The set is formed by a blank sample, a calibration curve in peanut matrix, from 0.5 to 250 μg L^{-1}, and a spiked sample at 10 μg kg^{-1}. In order to confirm the presence of the compounds in positive samples, it has been assured that the signal corresponding to the compound was within the retention time window set during validation, as well as product ions were detected and the ion ratios were similar to those shown in Table 1.

The results obtained show that aflatoxin G2 was detected above the LOQ in 13 out of the 36 samples analyzed (approximately 40% of positive samples) at a concentration ranging from 0.9 μg kg^{-1} to 6.3 μg kg^{-1}, with a standard deviation of 1.5 μg kg^{-1}. Fig. 3a shows the extracted ion chromatogram of a positive sample containing 0.9 μg kg^{-1}, while Fig. 3b shows the selected product ion of AFG2 in the same sample.

Aflatoxin G2 was detected in all the almond and pistachio samples, but it was observed that depending on the way that the pistachios have been prepared, cooked, farmed or stored, they showed different levels of this mycotoxin. For example, the highest concentration, 6.3 μg kg^{-1}, was found in a sample obtained in a street market, while an organic sample, collected from a local market, had the lowest concentration of aflatoxin G2, 0.9 μg kg^{-1}. Besides, almonds contain higher concentrations (2.8–4.4 μg kg^{-1}) when they have not been submitted to any cooking processes, while fried almonds have lower levels of aflatoxin G2 (1.4–1.8 μg kg^{-1}).

Moreover, aflatoxin G1 was detected in one sample of pistachios that was stored for three months in conditions of high humidity at 1.2 μg kg^{-1}.

Finally, aflatoxin B1 and B2, ZEA and its metabolite, α-ZOL, were not detected above the LOQ in any of the samples.

These results are comparable to other studies carried out by different authors. For example, in the study performed by Cunha et al. (Cunha et al., 2018), AFG2 was detected up to 4.0 μg kg^{-1} in a sample of cashew, although they usually find higher percentages of positive samples.

In the case of Wang et al. (Wang et al., 2018), they found two samples of chestnut and dried fig contaminated with AFB1 at a concentration of 39.3 and 410.5 μg kg^{-1} respectively, which is a very high concentration taking into account the current legislation. On the other hand, only 17% of positive samples were found by Wang et al.

Taking into account the current legislation, (European Commission 1881, 2006European Commission 1881, 2006), that stablishes a limit of 10 μg kg^{-1} of total aflatoxins in nuts, none of these results are above the limit but some of them are close to it, so the developed method is a suitable tool to control these mycotoxins.

A striking study is the one carried out by Cheraghali et al. (Cheraghali et al., 2007) because they found AFB1 in 3699 and total aflatoxins in 2852 out of 10,068 samples of pistachio analyzed in Iran. These results contrasts with our study, as no sample has been found contaminated with AFB1.

4. Conclusions

Peanut was selected as representative matrix for the determination of 6 mycotoxins in nuts. The use of the representative matrix reduces the cost of the analysis, due to no isotopically labeled internal standard has to be used. The developed method has allowed to improve both the sensitivity and the analysis time with respect to others previously published.

The method has been applied to a total of 36 real samples of different types of nuts. Taking into account the results, aflatoxin G2 is the main mycotoxin that has been found in the samples at a concentration up to 6.3 μg kg^{-1}. It should be pointed out that all the samples of almonds and pistachios are contaminated with this aflatoxin. Bearing in mind that 40% of the analyzed samples were contaminated with

aflatoxin G2 and G1, the routine control of these compounds in this kind of nuts in necessary.

Conflict of interest

The authors declare that there is no conflict of interest.

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PUBLICACIÓN IV

Determination of 3-monochloropropanediol esters and glycidyl esters in fatty matrices by ultra-high performance liquid chromatography-tandem mass spectrometry

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Determination of 3-monochloropropanediol esters and glycidyl esters in fatty matrices by ultra-high performance liquid chromatography-tandem mass spectrometry

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ABSTRACT

The development and validation of a method for the analysis of traces of 3-monochloropropanediol (3-MCPD) esters (19) and glycidyl esters (7) of fatty acids in vegetable oils, margarine, biscuits and croissants was performed. An extraction method based on the use of solvents (tert–butyl methyl ether (20% ethyl acetate, *v/v*)) was carried out and cleaning of the extract with a mixture of sorbents (Si-SAX, PSA and Z-sep+) was optimized for the elimination of fatty interferents. The analysis of the targeted compounds was carried out by ultra-high-performance liquid chromatography coupled to tandem mass spectrometry, using a triple quadrupole analyzer (UHPLC-MS/MS-QqQ). The validation of the method provided trueness values between 72 and 118% and precision lower than 20%. The limits of quantification ranged from 0.01 to 0.1 mg kg^{-1}, which were below the current legal limits. Twenty samples of vegetable oils as well of 4 samples of margarine, biscuits and croissants were analyzed. Six out of the 24 samples (25%) exceeded the limits set by European legislation, and a maximum contamination of 3-MCPD esters at 2.52 mg kg^{-1} was obtained in a sample of corn oil (being 1-myristoyl-3-MCPD the compound detected at the highest concentration). A maximum concentration of glycidyl esters at 7.84 mg kg^{-1} was determined in a soybean oil sample (glycidyl linoleate as the main compound). Only one sample of olive oil exceeded the maximum allowable limit for 3-MCPD esters with a value of 1.72 mg kg^{-1}, expressed as 3-MCPD.

1. Introduction

The refinement of oils must be done when one or more physicochemical or organoleptic characteristics are not acceptable. This process eliminates unwanted tastes, colors, odors and components that affect negatively to the quality of the oil [1]. In this process, a reaction of hydrochloric acid with triacylglycerols, phospholipids and glycerol is produced and as a direct consequence, 3-monochloropropane-1,2-diol (3-MCPD) is formed. Furthermore, bakery products and butter are submitted to thermal process that produces 3-MCPD, 2-MCPD and their esters [2]. Additionally, glycidol is associated to them and it usually forms monoesters with fatty acids during the refinement process [3].

As it has been shown in several studies, the chronic oral exposure of 3-MPCD results in nephropathy and tubular hyperplasia and adenomas, due to this compound attacks the kidney [4]. Furthermore, 3-MCPD has been proved to reduce fertility, or it provokes infertility in rats and suppression of the immune function [5]. The International Agency for Research on Cancer (IARC) has classified this compound as possible human carcinogen (group 2B) [6], since evidences of carcinogenic activity have been reported [7]. Moreover, glycidyl esters have been classified in group 2A as possible carcinogen agents [6].

The European Union recently set a limit of 1.00 mg kg^{-1} for glycidyl esters in edible oils and fats for adults and 0.50 mg kg^{-1} if they are used for the production of baby food or cereal-based food for infants and small children. Likewise, a limit of 1.25 mg kg^{-1} (2.50 mg kg^{-1} for pomace oils) was established for the sum of 3-MCPD and 3-MCPD esters in vegetable oils and fats for adults, and 0.75 mg kg^{-1} if they are used for the production of baby food or cereal-based foods for infants and young children [8].

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Oily matrices usually offer difficulties in terms of the extraction of contaminants because of their complexity. In that sense, well-established sample preparation methods must be accomplished in order to clean the matrix and extract the targeted compounds.

In the last years, several analytical methods have been developed for the determination of these compounds in edible oils [9] or pastries [10]. These methods are distinguished between underivatized and derivatized ones (also known as direct and indirect methods respectively).

Derivatized methods transform 2- and 3-MCPD esters in free MCPD, and glycidyl esters in free glycidol, and they include several stages such as transesterification, neutralization and derivatization steps before quantification [11], using NaOH for transesterification, sodium bromide for neutralization and phenylboronic acid for derivatization. In relation to the determination step, derivatized methods are simpler than underivatized ones, and they are based on gas chromatography coupled to mass spectrometry. In this case, identification of individual compounds is not carried out and a global overview of the presence of these compounds is achieved. Routine laboratories and official organisms developed methods based on this approach [12] as the American Oil Chemists' Society (Cd 29a-13, Cd 29b-13, and Cd 29c-13) [13–15]. However, these methods present some disadvantages, such as several steps must be carried out prior to the analysis, and therefore, they are more time-consuming. On the other hand, once all the steps are done, only free 3-MCPD and free glycidol can be quantified, and the esters that led to that total value cannot be known (Dubois et al., 2012).

Underivatized methods can detect and quantify all the 3-MCPD esters and glycidyl esters individually, since they are not submitted to any reaction prior to their analysis. This kind of methods provides information about the individual composition of 3-MCPD esters and glycidyl esters of the sample, instead of the total amount of them, as derivatized methods do. However, the large amount of acylglycerols, especially triacylglycerols that are contained in oils negatively affect the analysis, and they must be removed before it [12]. In this case, the analysis is performed using liquid chromatography coupled to mass spectrometry (LC-MS). This technique is used for the analysis of MCPDs present in oily matrices due to its high sensitivity and specificity, allowing the identification and characterization of organic compound as well as providing a "fingerprint" of a specific analyte because the MS spectra, but a higher investment in analytical standards as well as equipment is needed [12]. Nevertheless, few fatty acids are predominant in oils, such as oleic, linoleic and linolenic acid [17], reducing the number of standards that should be purchased.

A liquid-liquid extraction using methanol, 10% ethyl ether/*n*-hexane or 40% dichloromethane/*n*-hexane and two-step solid phase extraction (SPE) with C18 or silica gel cartridges is usually performed [16,18,19], but these approaches consume a high amount of time. Nevertheless, in this study, a much simpler method was developed, reducing the time needed for this stage.

Besides, when a high number of MCPDs is analyzed by an underivatized method, QTRAP [20,21] or QqQ are commonly used [22]. Therefore, in this study an underivatized method was developed using ultra-high performance liquid chromatography tandem mass spectrometry with triple quadrupole analyzer (UHPLC-MS/MS-QqQ) for the analysis of up to 26 glycidyl esters and MCPD mono- and diesters, major in edible oils. Furthermore, the analysis of twenty samples was performed, and the comparison of some samples analyzed by both, underivatized and derivatized, methods was carried out.

2. Materials and methods

2.1. Reagents and chemicals

1-Lauroyl-3-chloropropanediol (Lau), 1-linolenoyl-3-chloropropanediol (Lnn), 1-linoleoyl-3-chloropropanediol (Lin), 2-palmitoyl-3-chloropropanediol (2-Pa), 1-palmitoyl-3-chloropropanediol (Pa), 2-oleoyl-3-chloropropanediol (2-Ol), 1-oleoyl-3-chloropropanediol (Ol), 1-stearoyl-3-chloropropanediol (St), 1-myristoyl-3-chloropropanediol (My), 2-palmitoyl-1-oleoyl-3-chloropropanediol (2-Pa-Ol), 1,2-dilinoleoyl-3-chloropropanediol (Lin-Lin), 1-oleoyl-2-linolenoyl-3-chloropropanediol (Ol-Lnn), 1-palmitoyl-2-linoleoyl-3-chloropropanediol (Pa-Lin), 1-palmitoyl-2-stearoyl-3-chloropropanediol (Pa-St), 1,2-bis-palmitoyl-3-chloropropanediol (Pa-Pa), 1-oleoyl-2-linoleoyl-3-chloropropanediol (Ol-Lin), 1,2-dioleoyl-3-chloropropanediol (Ol-Ol), 1-linoleoyl-2-stearoyl-3-chloropropanediol (Lin-St), 1-oleoyl-2-stearoyl-3-chloropropanediol (Ol-St), glycidyl laurate (Lau-GE), glycidyl myristate (My-GE), glycidyl linolenate (Lnn-GE), glycidyl linoleate (Lin-GE), glycidyl palmitate (Pa-GE), glycidyl oleate (Ol-GE) and glycidyl stearate (St-GE) reference standards, as well as the internal standards glycidyl laurate-d5 (Lau-GEd5), 1-oleoyl-3-chloropropanediol-d5 (Old5), glycidyl oleate-d5 (Ol-GEd5) and 1,2-dioleoyl-3-chloropropanediol-d5 (Ol-Old5) were purchased from Toronto Research Chemicals (Toronto, Canada). All compounds present a purity $\geq$ 99.9% except 2-Ol, which presents a purity of 95%.

Stock standard solutions of the compounds (200–5000 mg L^{-1}) and internal standards (200–500 mg L^{-1}), were individually prepared by exact weighing of the solid substances and dissolved in 10 mL of isopropanol (LC-MS grade, J.T. Baker, Phillipsburg, NJ, USA). These solutions were stored in the dark at −18 °C. Then, a working standard solution, containing the selected 3-MCPD esters and glycidyl esters and another one with the internal standards at the same concentration were prepared at 10 mg L^{-1}, using isopropanol as solvent, and stored in the dark at −18 °C in glass tubes with screw cap. The working standard solutions were prepared every two months, while the stock standard solutions were stable up to one year.

LC-MS grade acetonitrile, methanol, tert–butyl methyl ether and isopropanol were purchased from Honeywell (Charlotte, NC, USA), ethyl acetate was obtained from Chem Lab (Zedelgem, Belgium), and ultrapure water was obtained by a gradient system Milli-Q water (Millipore, Bedford, MA, USA). Formic acid was acquired from Fisher Scientific (Pittsburgh, PA, USA), Si-SAX was acquired from Agilent (Santa Clara, CA, USA), florisil, Z-sep+, GCB and ammonium formate were purchased from Merck-Sigma Group (St. Louis, MO, USA), while ExtraBond C18 and ExtraBond PSA were purchased from Scharlab (Barcelona, Spain).

2.2. Instrument and apparatus

A WX vortex from Velp Scientifica (Usmate, Italy) was employed to homogenize the samples, while a Reax 2 rotatory shaker from Heidolph (Schwabach, Germany) was used for the extraction of the compounds. A S80H Elma ultrasound system (Wetzikon, Switzerland) and a Centronic-PL II centrifuge from JP Selecta (Barcelona, Spain) were utilized for dilution and centrifugation respectively.

An Agilent series 1290 Rapid Resolution Liquid Chromatography (RRLC) instrument equipped with a binary pump (G4220A), a column compartment thermostat (G1316C) and an autosampler thermostat (G4226B) was employed for the separation of the MCPDs. To carry out the separation of the compounds, a Zorbax Eclipse

Plus C18 column (100 × 2.1 mm, 1.8 µm particle size) from Agilent was used. An Agilent triple quadrupole mass spectrometer (6460 A) with a Jet Stream electronic spray ionization (ESI) source (G1958–65,138) was coupled to the chromatographic system. For data acquisition and data processing, the MassHunter (Agilent) software was utilized.

2.3. Samples collection

Some oil samples were provided by Laboratorio Tello, from Jaén (Spain), while other samples of oils, margarine, biscuits and croissants were obtained from supermarkets located in Almería (Spain). The analyzed olive oil samples belong to four types: extra virgin olive oil ($n = 2$ samples), virgin olive oil ($n = 1$), olive oil ($n = 6$) and refined olive oil ($n = 2$); two types of pomace oil: refined olive pomace oil ($n = 2$) and olive pomace oil ($n = 1$); five types of vegetable oils: sunflower oil ($n = 2$), soy oil ($n = 1$), corn oil ($n = 1$), peanut oil ($n = 1$), grapeseed oil ($n = 1$), as well as other matrices as margarine ($n = 1$), biscuits ($n = 1$) and croissants ($n = 2$). The total number of analyzed samples was 24.

2.4. Sample preparation

Briefly, one gram of sample was weighed out in a 5 mL volumetric flask and 50 µL of the internal standard solution at 10 mg kg^{-1} were added. Then, it was flushed with tert–butyl methyl ether (20% ethyl acetate, *v/v*). This solution was homogenized in vortex for 30 s and put in ultrasound system for 10 min. Then, 3 mL of this solution were transferred to a 15 mL tube containing 300 mg of the following mixture of salts: 2 mg of Si-SAX, 148 mg of PSA and 150 mg of Z-sep+ and the tube was capped immediately (a brief shaking by hand was performed immediately after the addition of salts to prevent their agglomeration). To allow the salts work properly, the tube was mixed in a rotatory agitator for 10 min. After that, the mixture was centrifuged at 5000 rpm (4136 × *g*) for 10 min. Finally, the sample was filtered into a 0.22 µm nylon syringe filter and injected into the LC system.

2.5. UHPLC-MS/MS-QqQ analysis

The chromatographic separation was carried out employing a binary mobile phase. Solvent A was methanol:H_2O (90:10, *v/v*) with 2 mM ammonium formate and 0.05% (*v/v*) of formic acid; and solvent B was isopropanol:H_2O (98:2, *v/v*) with 2 mM ammonium formate and 0.05% (*v/v*) of formic acid at flow rate of 0.2 mL min^{-1}. The gradient elution started at 90% of A and it was held for 3 min. Then, it was decreased to 30% during 11.5 min and held for 6 min. Finally, conditions come back to initial composition of mobile phase (90% A) for 3 min and held for 1.5 min. The total running time was 25 min. Injection volume was 5 µL and column temperature was kept at 25 °C.

All the compounds were ionized at positive ESI mode. Dynamic multiple reaction monitoring (dMRM) mode was used. Source gas temperature and sheath gas temperature were 325 °C and 400 °C, respectively. Source gas flow and sheath gas flow were 5 L min^{-1} and 11 L min^{-1}, respectively. Nebulizer gas pressure was 45 psi. Capillary and nozzle voltage were 3500 V and 500 V, respectively. Retention time windows (RTWs), defined as retention time average ± three standard deviation of the retention time when ten standards at 0.1 mg L^{-1} were injected, and MS/MS parameters are shown in Table 1.

2.6. Method validation

The validation for the optimized methodology was carried out using the Analytical Quality Control and Method Validation Procedures for Pesticide Residues Analysis in Food and Feed (SANTE) guide [23]. Thus, linearity, trueness in terms of recoveries, intra and inter-day precision and limit of quantification (LOQ) were calculated.

Firstly, an evaluation of the internal standards was carried out. For that, the estimation of the recoveries of each compound with each internal standard was accomplished in order to select the proper internal standard for each compound. Thus, the analytical signal is the ratio of areas between the analyte and the selected internal standard.

Then, linearity was evaluated performing a matrix-matched calibration, spiking blank extracted samples of extra virgin olive oil at several concentrations: 0, 0.01, 0.02, 0.05, 0.1, 0.2 and 0.5 mg L^{-1}. Furthermore, standard deviation of the back-calculated concentrations of the calibration standards from the true concentrations should be always lower than ± 20% [23], and at least, five levels were used to build the calibration curve.

Recovery trials were carried out in order to evaluate trueness. Extra virgin olive oil, margarine, biscuits and croissant samples were spiked at two levels (LOQ and 0.2 mg kg^{-1}). Five replicates were tested at each concentration level.

Repeatability (intra-day precision) and reproducibility (inter-day precision) were used to evaluate precision. The results were expressed as relative standard deviation (RSD,%). Regarding intra-day precision, five replicates were evaluated at two concentration levels (LOQ and 0.2 mg kg^{-1}) while for inter-day precision, the same procedure was followed, but testing one replicate of each concentration during 5 days.

For the estimation of the LOQ, the SANTE guide [23] was followed, which defines this parameter as the lowest concentration of the analyte that has been validated with acceptable trueness (recovery ranging from 70–120%) and precision (RSD lower than 20%). Thus, samples of extra virgin olive oil were spiked at low concentrations, between 0.01 and 0.2 mg kg^{-1}, and analyzed after extraction.

The validation was carried out in different matrices as extra virgin olive oil, margarine, biscuits and croissants.

In order to express the results according to the current regulation, the 3-MCPD esters are expressed as free 3-MCPD, and the Eq. (1) is applied:

$$3MCPD = \sum \frac{3MCPD\ ester\ result\ x\ Molecular\ weight\ of\ free\ 3MCPD}{Molecular\ weight\ of\ 3MCPD\ ester} \quad (1)$$

Similarly, glycidyl esters are expressed as free glycidol using with Eq. (2):

$$Glycidol = \sum \frac{Glycidyl\ ester\ result\ x\ Molecular\ weight\ of\ free\ glycidol}{Molecular\ weight\ of\ glycidyl\ ester} \quad (2)$$

3. Results and discussion

3.1. Optimization of UHPLC-MS/MS-QqQ parameters

Firstly, the MS conditions were optimized. The characteristic ions of each compound were obtained applying full-scan MS. To achieve a proper optimization of the MS/MS parameters, the target compounds were individually prepared at 10 mg L^{-1} in isopropanol. They were injected into the analyzer utilizing flow injection mode (no chromatographic column was used) at a flow rate of 0.15 mL min^{-1} using H_2O with 0.1% (*v/v*) of formic acid and methanol in isocratic mode at 50% each. All the compounds were ionized using ESI+ mode.

To appraise the intensity of the ions, further optimization was accomplished. Different fragmentor voltages (from 60 to 360 V) and collision energies (CE, from 5 to 60 eV) were applied to achieve optimum conditions of precursor and product ions respectively. The most intense transition of each compound was selected for quantification purposes, while the rest of them were employed

Table 1
UHPLC-MS/MS-QqQ parameters for the target compounds and internal standards used for quantification.

Compound	ISQ	RTW (min)	Precursor ion (*m/z*)	Product ion (*m/z*)[a]			
Lau	La-GEd5	2.96–3.06	293.2 (135)[a]	57.1 (25)[b]	71.1 (15)	108.8 (10)	**183.0**[c] **(5)**
Lnn	Ol-GEd5	3.00–3.10	371.2 (160)	261.1 (15)	267.0 (15)	**354.9 (10)**	
Lin	Ol-Old5	5.19–5.29	390.2 (105)	244.9 (5)	**263.0 (10)**	371.7 (5)	373.1 (5)
2-Pa	Ol-Old5	6.27–6.37	366.2 (110)	108.8 (20)	122.8 (20)	**239.1 (10)**	349.2 (5)
Pa	Ol-GEd5	6.37–6.47	349.2 (75)	109.2 (15)	**239.4 (5)**		
2-Ol	Old5	6.55–6.65	375.3 (135)	135.1 (17)	181.1 (13)	247.2 (5)	**265.2 (5)**
Ol	Old5	6.66–6.76	375.3 (145)	247.3 (9)	**265.2 (9)**		
St	La-GEd5	8.25–8.35	377.3 (155)	109.1 (10)	**267.2 (5)**		
My	Ol-Old5	8.62–8.72	321.2 (150)	57.1 (25)	71.2 (15)	**94.9 (30)**	211.2 (5)
2-Pa-Ol	Ol-GEd5	12.53–12.63	613.5 (60)	220.9 (9)	**357.2 (13)**		
Lin-Lin	Ol-Old5	13.62–13.72	651.2 (275)	221.1 (10)	**355.1 (15)**	429.2 (5)	
Ol-Lnn	La-GEd5	16.55–16.65	652.2 (195)	261.3 (20)	**353.3 (20)**	357.2 (20)	635.4 (10)
Pa-Lin	Old5	17.02–17.12	628.3 (150)	223.1 (20)	267.0 (40)	**355.2 (21)**	371.1 (10)
Pa-St	–	17.22–17.32	563.0 (315)	73.2 (60)	147.1 (35)	475.1 (20)	**563.0 (5)**
Pa-Pa	Old5	17.60–17.70	604.5 (60)	239.3 (20)	**331.1 (20)**	551.6 (20)	
Ol-Lin	Old5	17.60–17.70	654.6 (190)	263.2 (20)	**355.3 (20)**	357.3 (15)	
Ol-Ol	Ol-Old5	18.02–18.12	656.6 (75)	265.3 (20)	**357.3 (20)**		
Lin-St	Old5	18.20–18.30	656.6 (90)	263.2 (20)	355.2 (20)	**359.3 (15)**	
Ol-St	Old5	19.11–19.21	658.6 (200)	267.2 (25)	357.4 (25)	**359.4 (20)**	
Lau-GE	La-GEd5	3.44–3.54	257.2 (118)	103.1 (13)	109.1 (13)	**183.2 (9)**	201.2 (13)
My-GE	La-GEd5	4.62–4.72	285.2 (112)	109.2 (13)	123.1 (13)	211.2 (9)	**229.2 (13)**
Lnn-GE	La-GEd5	4.63–4.73	335.3 (155)	105.1 (25)	107.1 (21)	**109.1 (17)**	121.1 (17)
Lin-GE	Ol-Old5	6.17–6.27	337.3 (155)	107.1 (17)	**109.1 (17)**	121.1 (17)	123.1 (17)
Pa-GE	Ol-Old5	6.70–6.80	313.3 (155)	109.1 (17)	123.1 (17)	239.3 (9)	**257.2 (13)**
Ol-GE	Old5	7.58–7.68	339.3 (140)	109.1 (17)	111.1 (17)	**121.1 (21)**	135.1 (17)
St-GE	Old5	9.21–9.31	341.3 (90)	**57.1 (25)**	71.1 (20)	85.1 (20)	285.1 (10)
Lau-GEd5		3.44–3.54	262.2 (130)	109.1 (13)	123.1 (13)	**183.1 (9)**	202.2 (13)
Old5		6.56–6.66	380.3 (70)	246.7 (9)	**265.3 (9)**		
Ol-GEd5		7.58–7.68	341.1 (360)	73.1 (25)	324.9 (20)	**341.0 (5)**	
Ol-Old5		18.02–18.12	661.6 (90)	265.3 (18)	**362.4 (18)**		

[a] Fragmentor voltage (V) is given in brackets.
[b] Collision energy (eV) is given in brackets.
[c] Transition in bold was used for quantification.

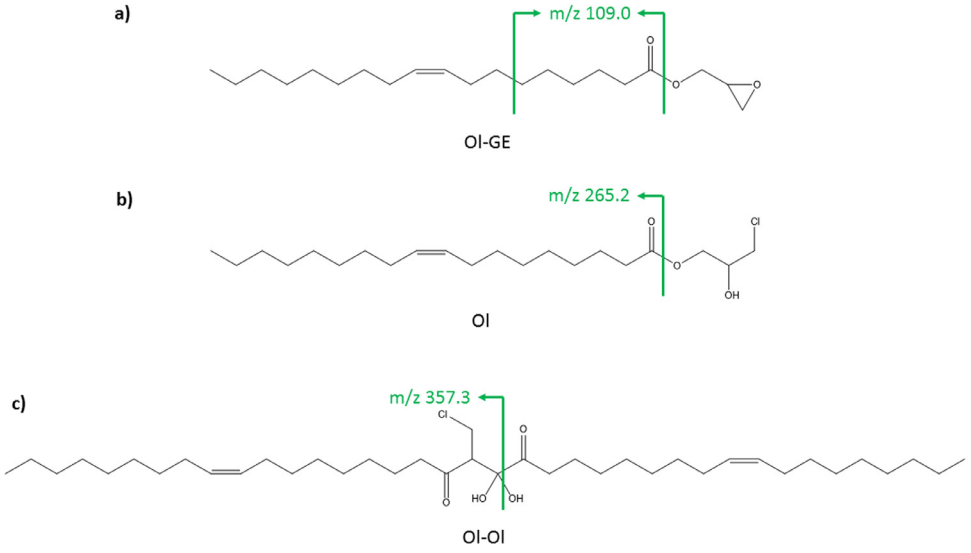

Fig. 1. Characteristic fragmentations of the esters of 3-monochloropropanediol (3-MCPD) and glycidyl esters included in the study. Transitions are indicated in green. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

for confirmation purposes (see Table 1). For the majority of the compounds two or three transitions were used for confirmation purposes, which increases the reliability of the analysis.

The most characteristic transitions are represented in Fig. 1. One product ion that appears frequently is *m/z* 109.0, which comes from the seven carbons closer to the 3-MCPD or glycidol, as it can be observed in Fig. 1a for Ol-GE. Furthermore, the transition of the fatty acid without the carboxylic acid is very common (example: *m/z* 390.2 → *m/z* 263.0 of Lin, *m/z* 375.3 → *m/z* 265.2 of Ol or *m/z* 371.2 → *m/z* 267.0 of Lnn), showing in Fig. 1b the transition of Ol. Finally, a characteristic transition was observed for the 3-MCPD di-esters, such as *m/z* 652.2 → *m/z* 353.3 of Ol-Lnn, *m/z* 654.6 → *m/z* 355.3 of Ol-Lin or *m/z* 656.6 → *m/z* 357.3 of Ol-Ol (see Fig. 1c). In this case, the transition is due the diester losses one of the ester and the another one remains bonded to the 3-MCPD.

Chromatographic parameters were optimized to reduce analysis time and obtain the best peak shapes. Although the use of a three-phase solvent system is becoming more and more used when com-

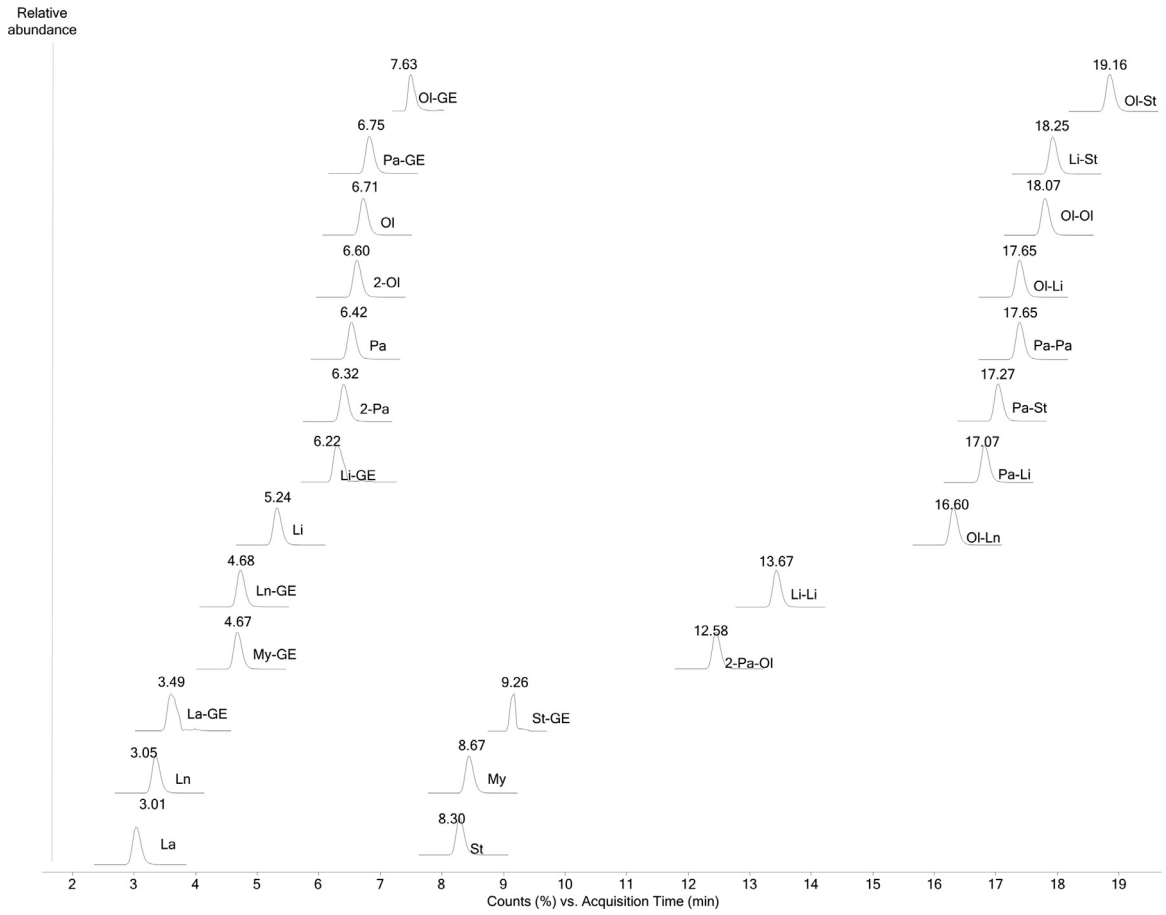

Fig. 2. Extracted ion chromatogram obtained from a standard solution of some representative esters of 3-monochloropropanediol (3-MCPD) and glycidyl esters at 0.05 mg L^{-1}.

pounds with similar chemical-physical properties need to be separated [24], our instrument is equipped with a binary pump and this approach was not tested.

Firstly, the conditions proposed by Custodio-Mendoza et al. [22] were tested. However, a proper separation of the compounds was not reached. Consequently, the mobile phases were modified, using H_2O with 2 mM ammonium formate and 0.05% (*v/v*) of formic acid as phase A and acetonitrile with 2 mM ammonium formate and 0.05% (*v/v*) of formic acid as phase B, and keeping a constant percentage of 2% A. The results showed that a higher percentage of phase A was needed since all the compounds were retained at the beginning of the chromatogram.

Secondly, the same mobile phase was used but the percentage of H_2O was increased until 10%, being the final proportion methanol:H_2O (90:10 *v/v*), and phase B isopropanol:H_2O (98:2 *v/v*). Using the cited mobile phase, a better separation of the peaks was reached.

Moreover, the minimum percentage of phase A tested in the gradient profile was tested, trying 10, 20, 25 and 30%, obtaining the best separation when a minimum percentage of 30% of mobile phase A was used since a lower percentage did not properly allow the elution of the compounds from the chromatographic column.

Finally, in order to reach the best sensitivity of the instrument 5, 10 and 20 μL of extract were injected, obtaining the best peak shapes with 5 μL, since higher quantities could provoke breakthrough of the column. Table S1 summarizes the optimization of the chromatographic conditions.

Finally, the selected chromatographic conditions are indicated in Section 2.5, and Fig. 2 shows the chromatogram of a mixture of some representative 3-MCPD esters and glycidyl esters at 0.05 mg L^{-1} in isopropanol.

3.2. Extraction method optimization

Firstly, the method proposed by Custodio-Mendoza et al. [22] was tested, but the results in terms of recovery were very poor, since the majority of the recoveries were below 37% or above 184% (See Table S2). Consequently, the development of a much simpler extraction method was tested.

In order to simplify the process, an approach of a cleaning step was made testing the same amount (300 mg) of different cleaning salts (C18, PSA, Florisil, GCB, Si-SAX and Z-sep+) flushing a flask of 5 mL with 1 g of sample with two different solvents: ethyl acetate and a mixture of tert–butyl methyl ether:ethyl acetate (80:20, *v/v*) [22]. It was seen that the mixture tert–butyl methyl ether:ethyl acetate (80:20, *v/v*) provided better results.

Additionally several salt mixtures (Mixture 1: 98% of PSA, 2% of Si-SAX; and Mixture 2: 75% of Z-sep+, 25% of PSA) [22] were also tested. In this case, several trials were performed, in order to use the proper amount of salts. On the one hand, these mixtures were tried sequentially performing the procedure as follows for the cleaning step. Once the sample was weighed out, the internal standard was added and flushed and homogenized with the solvent, 3 mL of this solution were transferred to a 15 mL tube containing 100 or 200 mg of Mixture 1. Then, the tube was capped, mixed in a rotatory agitator for 10 min and centrifuged at 5000 rpm for 10 min and finally the procedure was repeated with 100, 200 or 300 mg of Mixture 2. On the other hand, an approach adding 100

or 200 mg of Mixture 1 plus 100, 200 or 300 mg of Mixture 2 at the same time was carried out. In this case only one extraction was needed since the mixtures were added together.

Table S2 shows the results of all these trials. When Mixture 1 and 2 were added sequentially, the recoveries of 3-MCPD monoesters and glycidyl esters were low. For example, when 100 mg of Mixture 1 and 100 mg of Mixture 2 were added, the maximum recovery obtained was 36 and 49% for 3-MCPD monoesters and glycidyl esters respectively, while recoveries of diesters were up to 130% although the recovery of Ol-Lnn was 1%. Likewise, when 100 mg of Mixture 1 and 200 mg of Mixture 2 were added, the maximum recovery obtained was 28 and 50% for 3-MCPD monoesters and glycidyl esters respectively. On the other hand, when 200 mg of Mixture 1 and 200 mg of Mixture 2 were added at the same time, low recoveries were achieved, below 10%, such as Lnn, 2-Pa, 2-Pa-Ol, Lin-Lin, Ol-Lin and St-GE (Table S2).

The best results were obtained when 100 mg of Mixture 1 and 200 mg of Mixture 2 were added at the same time (see Table S2), so the final mixture used in further experiments was: 2 mg of Si-SAX, 148 mg of PSA and 150 mg of Z-sep+.

3.3. Method validation

The evaluation of linearity was carried out through determination coefficients (R^2) of the different calibration curves at 0.01, 0.02, 0.05, 0.1, 0.2 and 0.5 mg L^{-1} (oil, margarine, biscuits and croissants) and the values were always above 0.9964.

The evaluation of the internal standard chosen for the quantification of each compound was carried out assessing the recovery of each compound with each internal standard. When appropriate internal standards (Pa-St) were not available, it was directly quantified by its corresponding peak area. The choice of each one is showed in Table 1. Calibration curves were generated using the ratio of the chromatographic peak area for each analyte to the corresponding internal standard (see Table 1).

Recovery, intra- and inter-day precisions were calculated at 0.01 and 0.2 mg kg^{-1}. The average recoveries ranged between 72 and 119% and 82–120%, in oil (see Table 2), between 72 and 112% and 73–117% in margarine (see Table S3), between 71 and 116% and 71–110% in biscuits (see Table S4) and between 73 and 119% and 71–114% in croissants (see Table S5) for both concentrations respectively.

Intra-day precision was always below 20% (oil, margarine and biscuits) and 19% (croissants), whereas inter-day precision was below 20%, 19%, 20% and 20% for all the compounds assessed in the same matrices respectively (see Tables 2, S2, S3, S4).

Recovery values and intra- and inter-day precisions were similar to those obtained in previous studies [22,25], being the extraction procedure proposed in this study simpler and faster.

The LOQ was 0.01 mg kg^{-1} for all the 3-MCPD esters and glycidyl esters, except Pa and Lin-St, which was set at 0.02 mg kg^{-1}, 0.05 mg kg^{-1} for Lnn-GE and 0.1 mg kg^{-1} for Lin in all the matrices. These LOQs are generally lower than other published previously, like for example, MacMahon et al., who reached limits from 0.02 to 0.18 mg kg^{-1} [26], or Custodio-Mendoza et al., who defined the lowest LOQ at 0.025 mg kg^{-1} [27].

The LOQ values, expressed according to current legislation, were evaluated by calculating the result of each compound as free 3-MCPD or free glycidol (see Eq. (1) and 2). In this way, for the 3-MCPD esters, expressed as free 3-MCPD, a LOQ of 0.08 mg kg^{-1} was obtained, and 0.03 mg kg^{-1} for the glycidyl esters, expressed as free glycidol. These LOQs comply with the current legislation, since the minimum limit needed for the sum of 3-MCPD is 0.75 mg kg^{-1}, which is 10 times higher than the LOQ obtained in this study. Likewise, the minimum limit needed for the sum of glycidyl esters is 0.50 mg kg^{-1}, almost 20 times higher than our LOQ.

3.4. Analysis of samples

The developed method was applied to the determination of 3-MCPD esters and glycidyl esters in the 24 samples analyzed. During the analysis of the samples, an internal quality control was carried out every day samples were analyzed, to guarantee the reliability of the results. This quality control consisted of a blank sample to check the absence of interferences, a matrix calibration line from 0.01 to 0.5 mg L^{-1} to evaluate the sensitivity and perform the quantification of samples, and two samples, which were spiked and analyzed at 0.01 and 0.2 mg kg^{-1}, in order to evaluate the efficiency of the extraction process.

In the analyzed oil samples (see Table S6), the most detected esters are those coming from oleic, linoleic and linolenic acid, since they are the major fatty acids present in oils [28].

Samples of extra virgin and virgin olive oil did not show high concentrations of esters, which is normal since they are not submitted to any refinement process. This is a good example to explain how this kind of oils can be contaminated with these compounds but below the legal limit. For instance, the sample of extra virgin olive oil, which would be acceptable for human consumption according to current legislation, had a concentration of 0.17 mg kg^{-1} of St-GE (see Fig. 3a).

Likewise, olive oils do not usually show high levels of contamination. However, in this case, the sample “olive oil 4” contains high concentrations of 2-Pa-Ol (1.03 mg kg^{-1}), Lin-Lin (2.13 mg kg^{-1}) and Ol-Lin (2.69 mg kg^{-1}). Moreover, the sample “olive oil 6” was contaminated with 1.1 mg kg^{-1} of Lnn-Ge and 2.77 mg kg^{-1} of Ol-GE. Additionally, the two samples of refined olive oil presented high concentrations of Ol-GE, 1.62 and 1.03 mg kg^{-1}, respectively.

The two samples of refined olive pomace oil are contaminated with 2-Ol at concentrations of 2.20 and 1.41 mg kg^{-1}, while the sample of pomace olive oil was contaminated with Pa-St at 5.47 mg kg^{-1}, which may be due to a contamination with other kind of oil, like palm oil. In addition, high levels of Lnn-GE were detected in this kind of samples at concentrations of 1.06, 2.02 and 3.98 mg kg^{-1}, as well as one sample contained 1.56 mg $kg^{-1}$of Ol-GE.

In addition to olive oils, 6 vegetable oils were analyzed. Within this kind of edible oils, the esters detected at higher concentrations were Lin-GE and Ol-GE; Lin-GE was found in the sample of soy oil at 19.24 mg kg^{-1} (see Fig. 3b) and a sample of sunflower oil was contaminated at 17.67 mg kg^{-1}. Ol-GE was found at 11.73 mg kg^{-1} in the same sample of soy oil. The sample of corn oil was contaminated with My at 5.74 mg kg^{-1}.

The levels found in food samples were lower than those found in oils, due to the percentage of fat is lower. However, 0.58 mg kg^{-1} of 2-Pa-Ol was detected in the biscuit sample. Also, in both samples of croissants, Pa-St was detected at 0.24 mg kg^{-1} in croissant 1 and Pa-Pa was detected at 0.13 mg kg^{-1} in croissant 2, which may mean that they were elaborated with palm oil (see Table S7).

Our results agree with those obtained in other studies. For instance, Custodio-Mendoza et al. [27] obtained contamination of Ol-Ol, Lin-Lin and Pa-Ol in almost all the samples of refined and pomace oils, with a maximum of 10.89 mg kg^{-1} in a sample of olive pomace oil. Furthermore, Aniołowska et al. [29] found 2.38 mg kg^{-1} of Ol-GE in a sample of sunflower oil. Finally, Dubois et al. detected a total of 19.36 mg kg^{-1} of the sum Ol-Ol and Lin-St in a sample of sunflower oil.

3.4.1. Conversion to free 3-MCPD and glycidol

The values of MCPDs and glycidol esters were also calculated as sum of 3-MCPD esters and glycidyl esters, since currently, the legislation evaluates these parameters.

Table 2
Recovery and precision values obtained in extra virgin olive oil.

Compound	LOQ[a] (mg kg^{-1})	Linearity (Regression coefficient)	Recovery values		Intra-day precision RSD values	
			0.01 mg kg^{-1}	0.2 mg kg^{-1}	0.01 mg kg^{-1}	0.2 mg kg^{-1}
Lau	0.01	0.9932	78[b]	106	14 (16)[c]	17 (7)
Lnn	0.01	0.9915	83	113	18 (19)	12 (15)
Lin	0.1	0.9912	77[d]	105	20 (18)	15 (13)
2-Pa	0.01	0.9966	95	101	8 (10)	13 (16)
Pa	0.02	0.9909	114[d]	104	9 (14)	14 (11)
2-Ol	0.01	0.9944	100	104	8 (11)	15 (9)
Ol	0.01	0.9935	104	98	13 (17)	12 (18)
St	0.01	0.9987	103	120	20 (17)	15 (18)
My	0.01	0.9950	89	99	7 (9)	19 (16)
2-Pa-Ol	0.01	0.9915	116	105	13 (15)	19 (19)
Lin-Lin	0.01	0.9899	97	113	19 (20)	17 (19)
Ol-Lnn	0.01	0.9991	72	82	19 (16)	17 (14)
Pa-Lin	0.01	0.9981	72	93	20 (18)	16 (12)
Pa-St	0.01	0.9963	111	96	5 (9)	14 (17)
Pa-Pa	0.01	0.9978	114	96	16 (12)	5 (11)
Ol-Lin	0.01	0.9963	113	94	10 (13)	18 (9)
Ol-Ol	0.01	0.9993	119	97	10 (19)	16 (18)
Lin-St	0.02	0.9935	108[d]	112	18 (17)	11 (19)
Ol-St	0.01	0.9989	111	118	19 (17)	18 (18)
Lau-GE	0.01	0.9975	98	96	10 (13)	12 (10)
My-GE	0.01	0.9965	100	102	15 (18)	12 (12)
Lnn-GE	0.05	0.9903	98[d]	99	18 (17)	15 (15)
Lin-GE	0.01	0.9985	98	97	12 (15)	16 (19)
Pa-GE	0.01	0.9975	104	102	12 (14)	15 (17)
Ol-GE	0.01	0.9979	110	107	9 (17)	12 (17)
St-GE	0.01	0.9995	99	112	19 (9)	16 (16)

[a] LOQ: Limit of Quantification.
[b] Note: Recoveries and RSD were calculated based on $n = 5$.
[c] Inter-day precision (RSD values) are given in brackets.
[d] Intra-day and inter-day RSD values are calculated at their LOQ.

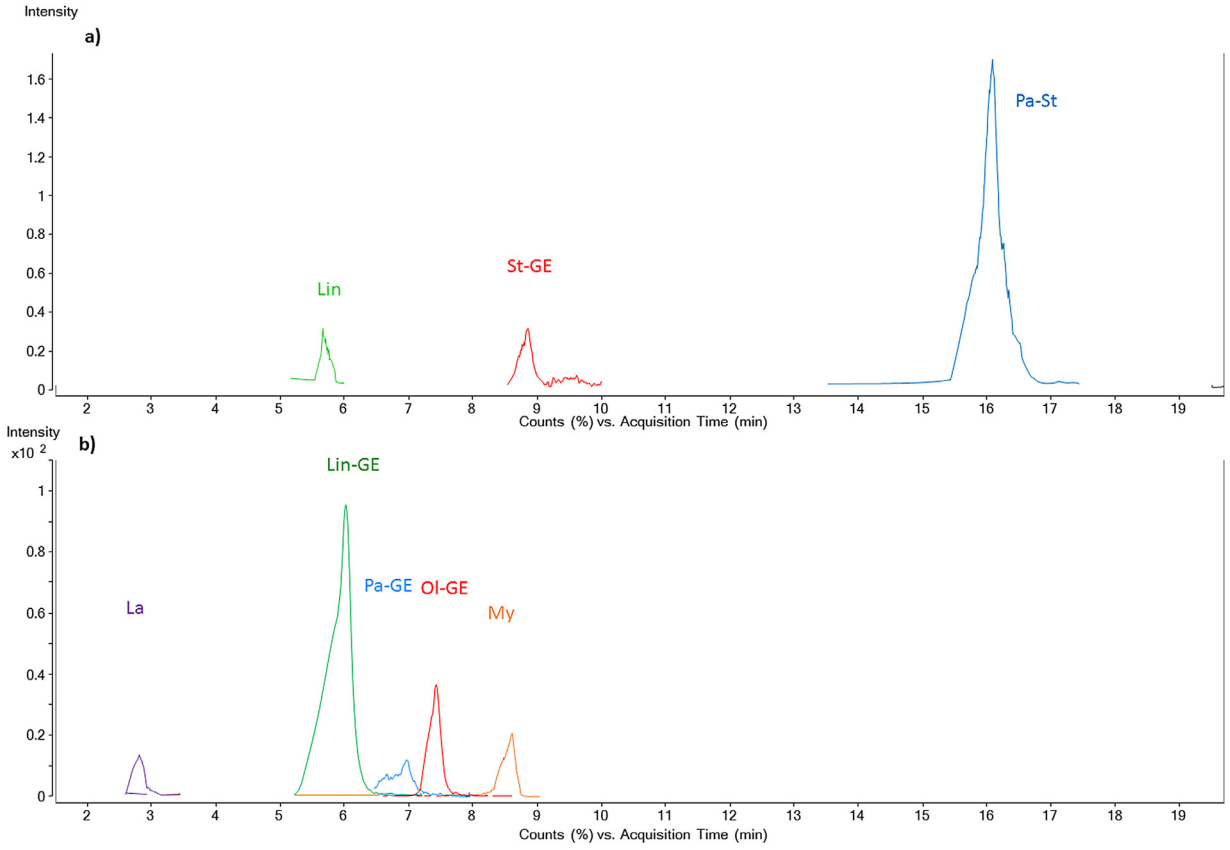

Fig. 3. (a) Extracted ion chromatogram of the sample extra virgin olive oil 1 contaminated with 0.2 mg kg^{-1} of St-GE and (b) Extracted ion chromatogram of the soy oil sample contaminated with 19.2 mg kg^{-1} of Lin-GE. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
Concentrations of the sum of 3-MCPD and glycidyl esters expressed as free 3-MCPD and free glycidol detected in the different types of vegetable oils analyzed.

Sample	Sum of 3-MCPD esters (calculated as free 3-MCPD) mg kg^{-1}	Sum of glycidyl esters (calculated as free glycidol) mg kg^{-1}
Extra virgin olive oil 1	0.14 ± 0.01[a]	0.06 ± 0.01
Extra virgin olive oil 2	0.14 ± 0.02 (<0.15)[b]	0.07 ± 0.01 (<0.15)
Virgin olive oil	0.04 ± 0.01 (<0.15)	0.05 ± 0.01 (<0.15)
Olive oil 1	0.22 ± 0.04	<LOQ
Olive oil 2	0.19 ± 0.02	0.09 ± 0.01
Olive oil 3	0.23 ± 0.03	0.35 ± 0.04
Olive oil 4	1.72 ± 0.29	0.18 ± 0.03
Olive oil 5	0.33 ± 0.06	0.27 ± 0.05
Olive oil 6	0.27 ± 0.03	0.95 ± 0.10
Refined olive oil 1	0.17 ± 0.02	0.60 ± 0.08
Refined olive oil 2	0.11 ± 0.02	0.51 ± 0.08
Olive pomace oil	1.30 ± 0.25 (1.20)	0.45 ± 0.09 (0.46)
Refined olive pomace oil 1	1.24 ± 0.25	0.60 ± 0.12
Refined olive pomace oil 2	0.66 ± 0.09	1.29 ± 0.18
Sunflower oil 1	0.26 ± 0.05	0.15 ± 0.03
Peanut oil	0.08 ± 0.01	1.22 ± 0.15
Grapeseed oil	0.41 ± 0.08	0.46 ± 0.09
Sunflower oil 2	1.06 ± 0.11	4.20 ± 0.62
Soy oil	1.38 ± 0.23	7.84 ± 1.33
Corn oil	2.52 ± 0.30	0.96 ± 0.12
Margarine	0.09 ± 0.02	0.03 ± 0.01
Biscuit	0.26 ± 0.03	<LOQ
Croissant 1	0.16 ± 0.02	0.08 ± 0.01
Croissant 2	0.10 ± 0.01	<LOQ

[a] Mean concentration ± standard deviation ($n = 3$).
[b] Results obtained by derivatized methods are given in brackets.

Regarding the samples of virgin and extra virgin olive oil analyzed, low concentrations were found, between 0.04 and 0.14 mg kg^{-1} of 3-MCPD esters, and between 0.05 and 0.07 mg kg^{-1} of glycidyl esters (see Table 3), far below the maximum permitted concentrations of 1.25 and 1.00 mg kg^{-1} respectively.

On the other hand, only one out of 6 samples from the olive oil category exceeded the limit of 3-MCPD esters with a concentration of 1.72 mg kg^{-1}, being the others below a maximum value of 0.33 mg kg^{-1}. Furthermore, none of these samples exceeds the maximum concentration of glycidyl esters, being the highest concentration found 0.95 mg kg^{-1}, but the rest of the samples between <LOQ and 0.35 mg kg^{-1} (see Table 3).

Within the refined olive oil samples, concentrations above the maximum allowed limit were not obtained, although in both samples, values of glycidyl esters were almost twice (0.51 and 0.60 mg kg^{-1}) than the mean value of the samples of unrefined olive oil (see Table 3).

Finally, refined pomace and pomace oils were studied. In this case, the values are usually higher than in olive oils, being 3-MCPD esters between 0.66 and 1.30 mg kg^{-1} and glycidyl esters between 0.45 and 1.29 mg kg^{-1} (see Table 3), which were below the permitted values for this type of oils (2.50 mg kg^{-1} in the case of glycidyl esters).

Regarding the vegetable oils, as can be seen in Table 3, the results show that a sample of soybean oil was contaminated with up to 7.84 mg kg^{-1} of glycidyl esters, a result that is well above the maximum 1.00 mg kg^{-1} allowed for this type of oils. In this case, we found that 4 out of the 6 samples exceed this limit. On the other hand, a sample of corn oil contaminated with 3-MCPD esters was found up to 2.52 mg kg^{-1}, also exceeding up to double the maximum allowed limit of 1.25 mg kg^{-1}.

The values found in some samples were compared with those obtained by the derivatized method analyzed by an external laboratory [14]. As can be seen in Table 3, the results obtained by both methods are very similar, which shows the robustness of the proposed method. In addition, the LOQ for both groups of compounds in the derivatized method is 0.15 mg kg^{-1}, while in the underivatized method developed in this study it is much lower (0.08 mg kg^{-1} for the sum of the 3-MCPD esters and 0.03 mg kg^{-1} for the sum of the glycidyl esters).

Comparing the results of food samples, our results were higher than the ones obtained by Belkova et al. who found 0.006 mg kg^{-1} of 3-MCPD esters and 0.009 mg kg^{-1} of glycidyl esters in bread [30]. Although it is difficult to compare different types of bakery samples since we do not know how it is been prepared, maybe the quality of the oil used to bake the bread was of higher quality than the one used in the samples of industrial bakery.

4. Conclusions

In this study, a sensitive analytical method was developed for the determination of 26 3-MCPD esters and glycidyl esters in vegetable oils, margarine, biscuits and croissants. A simple and fast extraction method based on the use of solvents and cleaning of the extract was optimized by using fatty interference cleaning salts, reducing the time of extraction of the current methods. Validation parameters were evaluated, and the values obtained met the established criteria by current legislation.

The analysis of the extracts was performed by UHPLC-MS/MS-QqQ with a total analysis time of only 25 min, less than that used by derivatized methods. Using this approach, the identification and quantification of the most significant 3-MCPD esters and glycidyl esters present in the sample is available.

Finally, a total of 24 samples of oils, margarine, biscuits and croissants were analyzed, finding the highest cases of contamination in vegetable oils, detecting Lin-GE at concentrations up to 19.24 mg kg^{-1} in a sample of soy oil. Generally, the less contaminated samples are the extra virgin olive oils while vegetable oils are the most contaminated samples. In total, a 25% of the analyzed oils have values higher that those set by current legislation.

Due to the possible contamination that fatty matrices can suffer from these compounds, the monitoring of these substances seems necessary and this method is a robust and reliable tool to monitor these compounds.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

CRediT authorship contribution statement

José L. Hidalgo-Ruiz: Formal analysis, Investigation, Validation, Writing - original draft, Visualization. **Roberto Romero-González:** Methodology, Software, Data curation, Writing - review & editing. **José Luis Martínez Vidal:** Conceptualization, Supervision, Writing - review & editing. **Antonia Garrido Frenich:** Resources, Writing - review & editing, Funding acquisition, Project administration.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2021.461940.

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CAPÍTULO III

DETERMINACIÓN DE CONTAMINANTES EXÓGENOS EN ACEITES VEGETALES Y OTRAS MATRICES DE ALTO CONTENIDO GRASO

1. INTRODUCCIÓN

Al igual que los contaminantes endógenos, los exógenos pueden provenir de diversas fuentes. Una de éstas puede ser la adición de plaguicidas a los cultivos para protegerlos de infecciones y de esta manera aumentar su productividad, o que estos plaguicidas hayan permanecido en el medio ambiente durante largo tiempo transfiriéndose al alimento, aunque no se le hayan añadido directamente. Por otro lado, los alimentos se pueden contaminar por medio del contacto con materiales que contengan contaminantes como pueden ser los hidrocarburos de aceites minerales, o directamente por la adición de aceites minerales para abaratar el coste de producción.

El control de este tipo de contaminantes, por tanto, es necesario para asegurar la calidad de los productos alimenticios, en este caso de los aceites vegetales y frutos secos.

Dentro de la amplia familia de plaguicidas, los polares presentan una alta movilidad a través del medioambiente mediante los procesos naturales de movimientos de agua al ser afines a ella [1].

Por otro lado, los aceites minerales son productos que derivan del carbón, el gas natural y la biomasa, y están compuestos por MOH [2], y se utilizan como lubricantes en la maquinaria o en la fabricación de tintas, que pueden ser empleadas en la industria alimentaria [3]. Dentro de los MOH se puede distinguir entre alifáticos (MOSH) y aromáticos (MOAH).

El análisis de compuestos polares en matrices grasas es un gran reto debido a la diferente polaridad entre analitos y matrices, lo cual hace improbable que estos contaminantes aparezcan en este tipo de matrices [4], pero no es imposible [5]. Por otro lado, debido a la similitud de los compuestos que componen MOSH y MOAH, su separación supone un gran desafío.

La técnica LLE se ha empleado para la extracción de plaguicidas polares en aceites vegetales [5–10] y frutos secos, aunque en este caso han sido necesarias etapas adicionales de QuEChERS y d-SPE para eliminar lípidos, ya que se analizan un mayor número de compuestos [11]. En cuanto a la extracción de MOH, a pesar de que existen métodos analíticos para la separación de MOSH y MOAH en aceites vegetales, éstos han empleado equipamiento acoplado, como LC-GC-FID [12–15].

En consecuencia, en primer lugar, se pretende desarrollar un método LLE simple, así como modificar el método QuPPe para la extracción de contaminantes polares en aceites vegetales y frutos secos respectivamente para su determinación mediante HPLC-QqQ-MS/MS (Publicación V). A continuación, se desarrolla un método de extracción de MOSH y MOAH en aceites vegetales mediante SPE *offline* para su posterior análisis mediante GC-FID (Publicación VI).

Las publicaciones incluidas en este capítulo son las siguientes:

V. Monitoring of polar pesticides and contaminants in edible oils and nuts by liquid chromatography-tandem mass spectrometry. **Hidalgo-Ruiz, J. L.**; Romero-González, R.; Martínez Vidal, J. L.; Garrido Frenich, A. *Food Chemistry*. **2021**, *343*, 128495.

VI. Off-line solid phase extraction and separation of mineral oil saturated hydrocarbons and mineral oil aromatic hydrocarbons in edible oils, and analysis via GC with flame ionization detector. **Hidalgo-Ruiz, J. L.**; Arrebola-Liébanas, J.; Martínez Vidal, J. L.; Garrido Frenich, A.; Romero-González, R. *Foods*. (Submitted for publication).

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PUBLICACIÓN V

Monitoring of polar pesticides and contaminants in edible oils and nuts by liquid chromatography-tandem mass spectrometry

Hidalgo-Ruiz, J. L.; Romero-González, R.; Martínez Vidal, J. L.; Garrido Frenich, A.

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Analytical Methods

Monitoring of polar pesticides and contaminants in edible oils and nuts by liquid chromatography-tandem mass spectrometry

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ABSTRACT

A single method was developed for the determination of polar pesticides (fosetyl-Al and its metabolite, phosphonic acid, and ethephon) and environmental contaminants (chlorate and perchlorate) in edible oils and nuts. Two extraction methods based on QuPPe-PO approach (Quick Polar Pesticides Method for products of Plant Origin) were optimized. In oils, a single extraction using water acidified with formic acid (1%) was performed, while in nuts, the clean-up step was modified. C18 was used as sorbent and an extra cleaning step with *n*-hexane was added. The extracts were analysed by liquid chromatography coupled to a triple quadrupole mass analyser (LC-QqQ-MS/MS). The method was validated and the limit of quantification was 0.01 mg kg^{-1} for all analyte-matrix combination. Recoveries from 70 to 120%, and intra and inter-day precision values ≤20% were obtained. Forty samples of edible oils and nuts were analysed, detecting phosphonic acid in nuts at concentrations up to 4.6 mg kg^{-1}.

1. Introduction

The need of crop protection entails the use of pesticides to prevent crops from insects and other pests, which would lead to economic losses. Therefore, when pesticides are used, crop productivity is increased (Ma, Wu, Li, & Yu, 2016). However, the incorrect use of pesticides may lead to food contamination.

Both polar and non-polar pesticides are commonly used. Among polar pesticides, fosetyl-Al and ethephon have been widely used (US Environmental Protection Agency, 2017). Ethephon is employed as growth regulator and promotes the maturation of vegetable products before and after their harvest (EPA, 1995), while fosetyl-Al is a fungicide used to control mildew in a great variety of crops, and phosphonic acid is one of its degradation product (Müller, Ackermann, & Margot, 2012). Also, food can be contaminated by chlorate because several factors such as the use of chlorine-disinfected irrigation water, the use of fertilisers that contain certain amounts of chlorate such as potassium nitrate and monopotassium phosphate or its natural presence in soil or groundwater (European Food Safety Authority, 2015), as well as the persistence in the soil since the use of chlorate was permitted until 2010 (European Commission 865, 2008). Finally, the appearance of perchlorate is due to the industrial release of this substance to the environment, particularly, the use of ammonium perchlorate in solid propellants for rockets and missiles (European Food Safety Authority, 2014), the use of nitrate-based fertilizers or from the degradation of sodium hypochlorite used to disinfect water (Spanish Agency for Food Safety and Nutrition, 2020).

Most of pesticides are regulated by European Legislation (European Commission, 2020) and some of them have been forbidden in the European Union (EU), such as chlorate (European Commission 865, 2008). Since chlorate was not approved as active substance (European Commission 1107, 2009), a default maximum residue limit (MRL) of 0.01 mg kg^{-1} for unauthorized substance is accepted (European Commission 396, 2005), and a limit of 0.1 mg kg^{-1} was proposed in almonds, hazelnuts, pistachios and walnuts, and 0.05 mg kg^{-1} in peanuts (European Commission 749, 2020). Additionally, perchlorate has not been included as active substance (European Commission 1107, 2009). MRLs have also been set for ethephon, fosetyl-Al and phosphonic acid in nuts. Specifically, limits of 0.1, 0.2, 0.1, 0.1 and 0.5 mg kg^{-1} were established for ethephon in almonds, hazelnuts, peanuts, pistachios and walnuts respectively (European Commission 1777, 2017). On the other hand, MRLs were set for the sum of fosetyl-Al, phosphonic acids and its salts in peanuts (2 mg kg^{-1} in peanuts and 500 mg kg^{-1} in almonds, pistachios,

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hazelnuts and walnuts) (European Commission 552, 2019). Finally, it is remarkable that none of these compounds is legislated in edible oils (European Commission 396, 2005) nor included in the list of substances for which a processing factor is applied when the raw material is processed (Bundesinstitut für Risikobewertunk, 2019).

Due to the complexity of fatty matrices, the analysis of pesticide residues is a challenging issue. Few studies are focused on the analysis of polar compounds in oily matrices probably due to the complexity of these matrices or because of their polar nature are very unlikely to appear in these matrices, which also depends on the content of water. Therefore, the development of strategies to selectively extract these type of pesticides is really interesting (Madej, Kalenik, & Piekoszewski, 2018).

The Quick Polar Pesticides Method for products of Plant Origin (QuPPe-PO) (Anastassiades et al., 2020) was tested to extract polar compounds from edible oils and nuts. However, this approach was mainly optimized for fruits and vegetables, which are matrices with a null or low-fat content. Thus, the problem appears when the method is applied to an oily matrix, whose behavior is totally different. Specifically, in the case of nuts, the recoveries obtained did not match the SANTE guidelines (Pihlström et al., 2020), which set a minimum value of 70%. Therefore, a proper optimization of the extraction method was carried out in order to avoid this problem in both type of matrices included in this study, edible oils and nuts.

In relation to the determination of pesticides, multi-residue methods include a wide number of pesticides, which allows the proper control of a large number of them in a single analysis (Madej et al., 2018). However, polar pesticides are poorly retained and bad peak shapes were achieved when the reverse phase mode, usually used in these methods, was applied. Therefore, specific methods for these compounds need to be carried out.

The development of a method to detect and quantify this kind of pesticides in oil and nuts is a challenge and there are scarce published methods that analysed these compounds (Madej et al., 2018). Some of them carried out a derivatization of the compounds, such as Han et al. (2016), who determined glufosinate in maize and soybean oils employing 9-fluorenylmethyl chloroformate (FMOC-Cl) as derivatizing agent, and the extract is analysed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

In order to avoid the derivatization step, hydrophilic interaction liquid chromatography (HILIC) column was used (Hanot, Joly, Bonnechère, & Van Loco, 2015; Nortes-Méndez et al., 2016), which is not considered as a reverse phase column (Heaton & Smith, 2012), using MS/MS as detection system. Other techniques such as ion chromatography (Adams et al., 2017; Melton, Taylor, & Flynn, 2019), supercritical fluid chromatography (Ishibashi et al., 2012), which requires high pressure operating conditions, and it is difficult to maintain (Taylor, 2010), or gas chromatography (Takenaka, 2002), which has several limitations (McNally, 2000), are also used.

In this study, the QuPPe-PO method was improved in terms of recoveries of five polar pesticides and environmental contaminants (phosphonic acid, fosetyl-Al, ethephon, chlorate and perchlorate) from oils and nuts. Then, an analytical method based on LC-MS/MS was developed and validated, and finally, 40 samples of different types of oils (extra virgin olive oil, refined olive oil, olive pomace oil, crude olive pomace oil and sunflower oil) and different types of nuts (almonds, hazelnuts, peanuts, pistachios and walnuts) were analysed.

2. Materials and methods

2.1. Reagents and chemicals

Ethephon, chlorate and perchlorate (99.9%) reference standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fosetyl-Al (96.1%) and phosphonic acid (97.5%) were obtained from Dr. Ehrenstorfer (Augsburg, Germany), while isotopically labelled perchloric acid ($Cl^{18}O_4^-$) (90%) was acquired from Cambridge Isotope Laboratories (Tewksbury, MA, USA).

Chlorate and perchlorate stock solutions had a concentration of 1000 mg L^{-1}. To prepare the standard solutions of phosphonic acid, fosetyl-Al, ethephon and isotopically labelled perchloric acid, they were weighed out individually and dissolved in 10 mL of ultrapure water (Millipore, Bedford, MA, USA). The concentration of these stock solutions was 200 mg L^{-1}, and a working standard solution of a mixture of the analytes was prepared at 10 mg L^{-1} in ultrapure water and stored in the dark at −18 °C in glass tubes with screw cap. A solution of the internal standard of 10 mg L^{-1} was also prepared and stored under the same conditions than the working standard solution. Stock standard solutions were stored up to one year whereas working standard solutions were prepared every two months.

LC-MS grade acetonitrile, methanol and *n*-hexane were obtained from Honeywell (Morristown, NJ, USA), ultrapure water was obtained by a gradient system Milli-Q water (Millipore, Bedford, MA, USA), ExtraBond C18 was purchased from Scharlab (Barcelona, Spain), formic acid was acquired from Fisher Scientific (Pittsburgh, PA, USA) while ethylenediaminetetraacetic acid (EDTA), florisil, PSA, Z-sep+, diatomaceous earth, graphene, GCB, SCX and SAX were obtained from Sigma-Aldrich. C18 (6 cc, 200 mg) and OASIS (6 cc, 200 mg) SPE cartridges were obtained from Waters (Milford, MA, USA) while EMR-Lipid (6 cc, 600 mg) SPE cartridges were purchased from Agilent Technologies (Santa Clara, CA, USA).

Finally, 0.45 μm nylon syringe filters were obtained from Agilent Technologies.

2.2. Instrument and apparatus

A Reax 2 rotatory shaker from Heidolph (Schwabach, Germany) was utilized for the extraction of the polar compounds. To achieve a proper homogenization, a WX vortex obtained from Velp Scientifica (Usmate, Italy) was used. For centrifugation, a Centronic-PL II centrifuge from JP Selecta (Barcelona, Spain) was employed.

The analysis was performed with an Agilent series 1290 Rapid Resolution Liquid Chromatography (RRLC) instrument (Santa Clara, CA, USA). This instrument was equipped with a column compartment thermostat (G1316C), a binary pump (G4220A) and an autosampler thermostat (G1330B). The separation of the compounds was achieved with a Hypercarb column (100 × 2.1 mm, 5.0 μm particle size) from Thermo, protected with a drop-in guard cartridge (10 × 4.6 mm, 7.0 μm particle size). The RRLC system was coupled to an Agilent triple quadrupole mass spectrometer (6460 A) with a Jet Stream electronic spray ionization (ESI) source (G1958-65138). For optimization and quantification, the MassHunter (Agilent) software was used.

2.3. Samples collection

Oil samples were provided by Laboratorio Tello, from Jaen (Spain), whereas nut samples were obtained from local supermarkets located in Almería (Spain). Different types of oil were analysed: extra virgin olive oil (n = 4 samples), refined olive oil (n = 4), olive pomace oil (n = 4), crude olive pomace oil (n = 4) and sunflower oil (n = 4); and four types of nuts: almonds (n = 6), hazelnuts (n = 3), peanuts (n = 5), pistachios (n = 4) and walnuts (n = 2). The total number of analysed samples was 40.

2.4. Sample preparation

2.4.1. Olive oil

The method proposed by Chiarello et al. (2019) was employed adding an internal standard in order to carry out the quantification of perchlorate. Briefly, 100 μL of a solution of 1 mg L^{-1} of isotopically labelled perchloric acid was added to ten grams of sample, and they were mixed with 10 mL of an aqueous solution of 1% formic acid in a 50

mL tube. The tube was shaken for 1 min in a vortex and then centrifuged at 5000 rpm (4136 × *g*) for 10 min. Finally, the supernatant was filtered into a 0.45 μm nylon filter and directly injected into the LC system.

2.4.2. Nuts

In this case, the QuPPe method (Anastassiades et al., 2020) was slightly modified and the addition of internal standard was not necessary. First, 5 g of sample were weighed in a 50 mL tube. Then, 9 mL of water, 100 μL of formic acid, 10 mL of a methanol solution (containing 1% of formic acid) and 1 mL of an aqueous solution containing 1% EDTA were added. Afterwards, it was mixed for 15 min in a rotary agitator and centrifuged at 5000 rpm (4136 × *g*) for 10 min. Then, 2 mL of the supernatant were added to a 15 mL tube containing 2 mL of *n*-hexane and 100 mg of C18. The tube was shaken for 1 min in a vortex and centrifuged again at 5000 rpm (4136 × *g*) for 10 min. Finally, the methanolic extract of the sample, which was below the *n*-hexane layer, was filtered into a 0.45 μm nylon filter and injected into the LC system.

2.5. LC-MS/MS analysis

The chromatographic separation was carried out employing a binary mobile phase, consisting of methanol with 1% formic acid (A) and an aqueous solution with 5% methanol and 1% formic acid (B) at a flow rate of 0.3 mL min^{-1}. The gradient elution started at 0% of A and increased to 5% A in 3.0 min. Then, it was increased to 10% in one minute, to 20% for the next minute and to 30% in two minutes. Then, the percentage of mobile phase A drastically increased to 90% in 1 min and this composition was held for 0.5 min. Finally, the system comes back to the initial conditions (0% A) for 0.5 min and held for 11 min. The total running time was 20.0 min. Injection volume was 20 μL and column temperature was kept at 40 °C.

All the compounds were ionized at negative ESI mode and multiple reaction monitoring (MRM) was used. The temperatures of the source gas and the sheath gas were 325 °C and 400 °C, respectively. The flow of both source and sheath gases were 5 L min^{-1} and 11 L min^{-1}, respectively. Nebulizer was 45 psi. Capillary and nozzle voltage were 3500 V and 500 V, respectively. Retention time windows (RTWs) and MS/MS parameters are shown in Table 1.

To ensure a proper performance of the column, 10 injections of an extracted blank sample (almond) were injected before starting each sequence of analysis.

Table 1
Retention time windows (RTWs) and MS/MS parameters for the target compounds.

Compound	RTW (min)	Precursor ion (*m/z*)	Product ion (*m/z*)	Ion ratio (%)
Phosphonic acid	5.3–5.5	81.1 (30)[a]	**79.0**[b] **(20)**[c]	
			63.1 (40)	55.9
Fosetyl-Al	6.6–6.8	109.1 (50)	**81.0 (10)**	
			63.1 (35)	10.0
Ethephon	8.1–8.3	143.0 (50)	**107.0 (5)**	
			79.1 (20)	39.7
Chlorate	11.6–11.8	85.0 (50)	**69.0 (20)**	
		83.0 (50)	67.0 (20)	93.1
			51.0 (35)	11.0
Perchlorate	15.4–15.6	101.0 (110)	**85.0 (30)**	
		99.0 (130)	82.9 (30)	15.0
			67.0 (35)	13.1
Perchloric acid isotopically labelled ($Cl^{18}O_4^-$)	15.4–15.6	107.0 (160)	**89.0 (35)**	
			71.0 (48)	18.0

[a] Fragmentor voltage (V) is given in brackets.
[b] Transition in bold was used for quantification.
[c] Collision energy (eV) is given in brackets.

2.6. Method validation

SANTE guidelines (Pihlström et al., 2020) were used for the validation of the optimized methods. Linearity, matrix effect, trueness in term of recoveries, intra and inter-day precision and limit of quantification (LOQ) were evaluated.

To evaluate linearity, a matrix-matched calibration was carried out. Blank extracted samples were spiked at several concentrations: 0, 10, 25, 50, 100, 250 and 500 μg L^{-1}.

Matrix-matched calibrations in extracted extra virgin olive oil and almonds, as representative matrices, as well as in other matrices (extra virgin olive oil, refined olive oil, olive pomace oil and crude olive pomace oil, sunflower oil, almonds, hazelnuts, peanuts, pistachios and walnuts), and in solvent were prepared at concentrations between 10 and 500 μg L^{-1}. To evaluate the matrix effect, the slopes were compared. The following equation (Eq. (1)) was employed for the calculation of the percentage of matrix effect (enhancement or suppression):

$$Matrix\ \ effect = \left[\frac{slope\ \ in\ \ matrix}{slope\ \ in\ \ solvent} - 1\right] \times 100 \quad (1)$$

If matrix effect is between −20% and +20%, it can be considered negligible. However, values higher than +20% indicate significant matrix enhancement, whereas values lower than −20% indicate matrix suppression.

Recovery trials were carried out in order to evaluate trueness. Extra virgin olive oil and almond blank samples were spiked at three levels (10, 100 and 500 μg kg^{-1}). Five replicates were tested at each concentration level.

Precision was assessed evaluating repeatability (intra-day precision) and reproducibility (inter-day precision), expressing the results as relative standard deviation (RSD, %). For intra-day precision, five replicates at three concentration levels (10, 100 and 500 μg kg^{-1}) were evaluated. For inter-day precision, the same procedure than for intra-day precision was followed, testing one replicate for 5 days.

Finally, LOQ was estimated following the indications described in the SANTE guidelines (Pihlström et al., 2020), defining this parameter as the lowest concentration of the analyte that has been validated with acceptable trueness (recovery ranging from 70 to 120%) and precision (RSD lower than 20%). Consequently, samples were spiked at low concentrations, between 1 and 50 μg kg^{-1}, and extracted.

3. Results and discussion

3.1. Optimization of LC-QqQ-MS/MS

Firstly, the MS conditions were optimized. Full-MS-scan in negative ESI mode was applied in order to obtain the characteristic precursor ion for each compound. The target compounds were individually prepared at 10 mg L^{-1} in water and injected into the LC-QqQ-MS/MS system at a flow rate of 0.15 mL min^{-1}, without a chromatographic column.

Different fragmentor voltages (from 30 to 160 V) and collision energies (CE, from 5 to 50 eV) were respectively applied to optimize the best signal of each precursor and product ions. The most intense transitions were chosen for quantification purposes, whereas another transition was used for confirmation purposes (see Table 1). In the case of chlorate and perchlorate, three transitions were selected, while only two were chosen for the rest of the compounds.

Fragmentation of the molecules during collision induced dissociation (CID) process is shown in Fig. 1. Ethephon loses its chlorine atom (transition *m/z* 143.0 → *m/z* 107.0) and the ethyl group (transition *m/z* 107.0 → *m/z* 79.1), which is the same loss that is observed in the transition *m/z* 109.1 → *m/z* 81.0 of the fosetyl-Al. Then, for fosetyl-Al the transition *m/z* 81.0 → *m/z* 63.1 was also observed, where the hydroxyl group plus the hydrogen bonded to the phosphorus is lost. In the case of phosphonic acid, both hydrogens of the hydroxyl group are lost

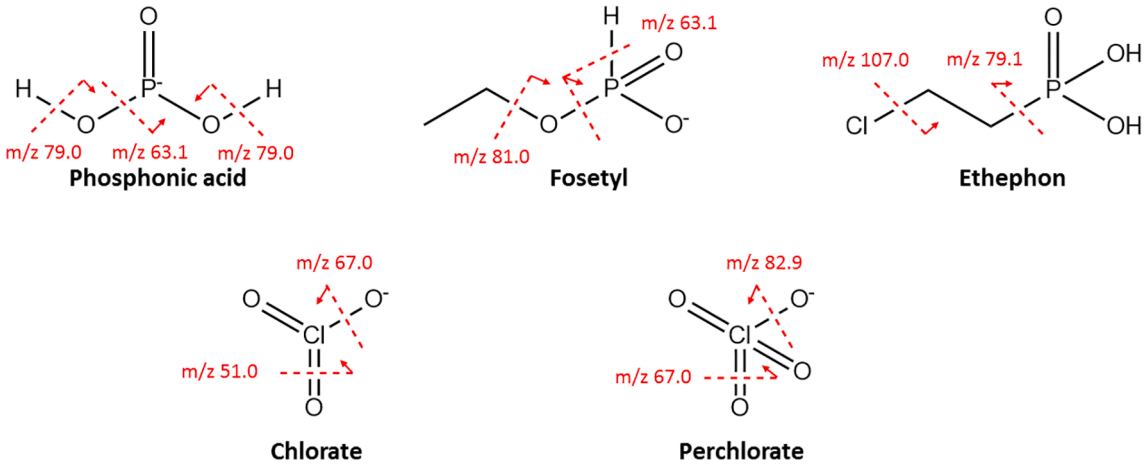

Fig. 1. Fragmentation of the polar pesticides included in the study. Transitions are indicated in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in the transition *m/z* 81.1 → *m/z* 79.0, and one oxygen is lost in the transition *m/z* 79.0 → *m/z* 63.1. Transitions of chlorate and perchlorate are identical due to these molecules are very similar, with the only difference that perchlorate has one more oxygen than chlorate. Transitions *m/z* 83.0 → *m/z* 67.0 (or *m/z* 85.0 → *m/z* 69.0) of chlorate and *m/z* 99.0 → *m/z* 82.9 (or *m/z* 101.0 → *m/z* 85.0) of perchlorate correspond to the loss of the oxygen bonded by the single linkage, while the transitions *m/z* 67.0 → *m/z* 51.0 of chlorate and *m/z* 82.9 → *m/z* 67.0 of perchlorate correspond to the loss of another oxygen double bonded to chlorine.

The sum of two units in the case of the precursor ions of chlorate and perchlorate is due to the ^{37}Cl. In addition to *m/z* 83.0 and *m/z* 99.0, which are the masses of the precursor ions of chlorate and perchlorate respectively, their ions *m/z* 85.0 and *m/z* 101.0, corresponding to ^{37}Cl, can also be used as their abundance is quite high.

An optimization of the chromatographic conditions was also carried out in order to obtain the best separation in the shorter running time, and furthermore, achieving the best peak shapes. Different mixtures of water:methanol were tested for the aqueous phase (water, water:methanol (95:5, *v/v*) and water:methanol (90:10, *v/v*). Water:methanol (95:5, *v/v*) was selected as the best separation was achieved with this mixture, since a higher amount of water does not separate the compounds properly, while a lower percentage of water separates the analytes too much and the analysis time is longer. The amount of formic acid was also optimized, testing 0.1, 0.5, 1.0 and 2.0%. The best elution of the target compounds was obtained with 1% of formic acid.

3.2. Extraction method optimization

3.2.1. Olive oil

Experiments were performed spiking blank extra virgin olive oil at 100 μg L^{-1} of the target compounds. First, the method proposed by Chiarello et al. (2019) was tested, but perchlorate was poorly recovered

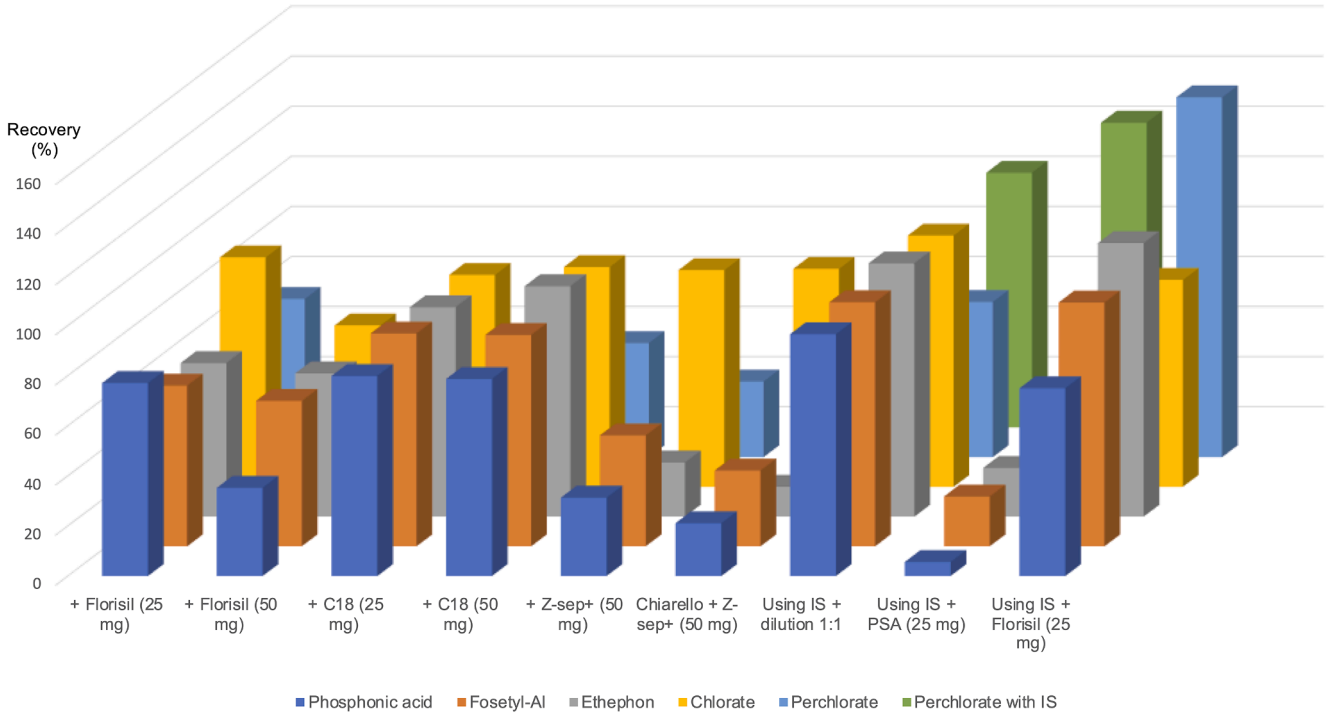

Fig. 2. Comparison of the recovery values obtained for the polar compounds in oils modifying the method proposed by Chiarello et al. (2019). For perchlorate, recoveries are compared with and without internal standard in the last three experiments.

(30–63%), and therefore, some modifications were tested. Different cleaning sorbents, such as C18, florisil, and Z-sep+ were tested adding different amounts of sorbents (25 and 50 mg). As it can be seen in Fig. 2, when Z-sep+ was used recoveries were very low (12–43%) except for chlorate (86–87%). Although recoveries were suitable (70–120%) when other sorbents (C18 and florisil) were tested, they were still low for perchlorate (30–63%).

That is why some experiments such as dilution of the extract with water (1:1 *v/v*), or the addition of PSA and florisil were tested after the extraction. In this case, experiments were performed with and without internal standard and results were compared. It must be noted that when florisil was used, a recovery higher than 200% was obtained for perchlorate (365%) if internal standard was used, and therefore, this value was omitted in Fig. 2. As it can be seen in Fig. 2, the best recovery of perchlorate was obtained without the addition of cleaning sorbents, but adding the internal standard for the quantification of this compound (102%). In the next experiment, it was corroborated that the dilution was not necessary when the quantification was made with the internal standard. The recoveries of the rest of the compounds must be estimated without the use of internal standard.

3.2.2. Nuts

Trials were carried out with blank almond spiked at 100 μg kg^{-1}. An approach similar to the oil extraction method using an aqueous solution of 1% formic acid, described in Section 2.4.1, was appraised because good results were obtained in oils, and nuts are also fatty matrices. Additionally, 50 and 100 mg of a cleaning sorbent (C18) as well as ion exchange salts (SAX (100 mg) and SCX) were added to 2 mL of supernatant. Also, a mixture of C18 and SAX (50 + 50 mg) and a mixture of SCX and SAX (50 + 50 mg) were tested. It can be seen in Figure S1a (see supplementary material) that when 50 mg of C18 was used, suitable results in terms of recovery were achieved, but in all cases the perchlorate recoveries are meaningless (0–6% or 562–905%).

The same procedure mentioned above was tested using a methanol solution of 1% formic acid instead of the aqueous solution and freezing the sample at −20 °C for three hours before its extraction, as Herrera López et al. recommended (Herrera López, Scholten, Kiedrowska, & de Kok, 2019). Also, in this method no clean-up step was used and another experiment was carried out using SCX as well as another one using a dilution of the extract with H_2O (1:10 *v/v*). SCX was tested to get a better cleaning while the dilution was appraised in order to reduce matrix effect. The results shown that neither clean-up nor dilution give good results, as recoveries ranged from 0 to 45% (Figure S1b).

Because no good results were obtained, the QuPPe method was checked (Anastassiades et al., 2020) and some dilutions of the extract with H_2O (1:1, 1:2, *v/v*), as well as several clean-up sorbents (C18, GCB, Z-sep+, florisil, diatomaceous earth and graphene) were tested. In this case, very low sensitivity was obtained due to the matrix suppression, as can be observed Figure S1c where high suppression effect was observed. Since the values were completely out of range (±20%), a new approach needed to be tested.

Therefore, the QuPPe method was tried again including the step of freezing for 3 h as the official method recommends. d-SPE (C18) and SPE (C18 and OASIS, EMR) were also tried in order to remove interferents from the extract. As it can be observed in Figure S1d, results are not conclusive at all. The results using the QuPPe method with methanol show almost suitable recoveries (59–122%) except for perchlorate (843%). In order to get better results, SPE was used. The better recoveries were obtained when OASIS cartridges were used (38–125%) except for perchlorate (278%), while the majority of the values were out of the expected range (70–120%) when EMR cartridges were used (1–64% and 469–840%). Finally, the combination of the QuPPe method using methanol and the clean-up step with C18 in d-SPE was tested, obtaining the best recoveries for all the compounds (43–122%).

Finally, an approach was tried adding *n*-hexane to obtain more clean extracts. This step improved considerably recoveries and matrix effect as it can be observed in Figure S1d.

Finally, Fig. 3a shows the chromatogram of the extraction of an extra virgin olive oil sample spiked with 100 μg kg^{-1} of the polar compounds' while Fig. 3b shows the chromatogram of the extraction of an almond sample spiked at the same concentration.

3.3. Method validation

Determination coefficients (R^2) were used to evaluate linearity in solvent. The results obtained were 0.9826, 0.9975, 0.9963, 0.9969 and 0.9993 for phosphonic acid, fosetyl-Al, ethephon, chlorate and perchlorate respectively. Furthermore, standard deviation of the back-calculated concentrations of the calibration standards from the true concentrations was always lower than 20% (Pihlström et al., 2020).

Matrix effect was between 1% and 9% for phosphonic acid, fosetyl-Al, ethephon, chlorate and perchlorate for oils, and between 3% and 8% in the case of nuts. This percentage can be considered as negligible enhancement as does not exceed 20% which consequently shows that there was not matrix effect neither for oils nor for nuts (see Table 2). Table 2 shows the percentage of matrix effect of each matrix in relation to solvent. It can be observed that matrix effect is between ± 20%, set as limit of matrix effect. Therefore, no matrix effect was observed for the targeted compounds in the tested matrices (extra virgin olive oil, refined olive oil, olive pomace oil, crude olive pomace oil, sunflower oil, almonds, hazelnuts, peanuts, pistachios and walnuts).

Then, extra virgin olive oil was chosen to estimate other validation parameters since it is the most consumed oil in Spain, and almond was chosen as representative matrix for nuts.

The average recoveries in oils ranged between 90.5 and 104.3%, 83.4–94.3% and 75.6–79.6%, for the concentrations 10, 100 and 500 μg kg^{-1} respectively as it can be observed in Table 3. On the other hand, in nuts, recoveries ranged between 70.0 and 89.5%, 72.7–95.8% and 70.7–100.5% for the same concentrations (see Table 3).

Intra- and inter-day precision values were always below 20%. Specifically, the highest RSD values of intra- and inter-day were 15.3% and 19.2% respectively for oils (see Table 3), while for nuts, these values were 19.6% and 19.8% (see Table 3).

The LOQ was set at 10.0 μg kg^{-1} for all the polar pesticides for both matrices, oils and nuts. This LOQ is the same than the MRL allowed for chlorate and ten times lower than the MRL allowed for ethephon in almonds and pistachios, that are the lowest MRLs allowed in nuts (European Commission 1777, 2017). Also, this LOQ is much smaller than the MRL allowed for fosetyl-Al and phosphonic acid that is 2 mg kg^{-1} in peanuts, which is the lowest set in nuts (European Commission 552, 2019).

LOQs presented in the QuPPe method (Anastassiades et al., 2020) are not assayed for oils and nuts or any lipidic matrix, while the more similar food to nuts could be cereals, and the lowest LOQ for the polar compounds was 20.0 μg kg^{-1}, which is double of the LOQ proposed in this study. To the best of our knowledge, there is not any publication that analyses these polar compounds in oily matrices, so this parameter cannot be compared to previous studies.

3.4. Analysis of samples

The method was applied to 20 samples of vegetable oils and 20 samples of nuts. An internal quality control set was carried out each time that samples were analysed. This set consisted of a blank sample, a calibration curve in solvent, from 10 to 250 μg L^{-1}, and a spiked sample at 10 μg kg^{-1}. The presence of positive samples was performed by comparing the retention time with those obtained by standards, and the confirmation was done taking into account that the ion ratios of the product ions were similar to those shown in Table 1.

As can be seen in Table S1, none of the vegetable oil samples were contaminated with the polar compounds, probably due to the polarity of the compounds.

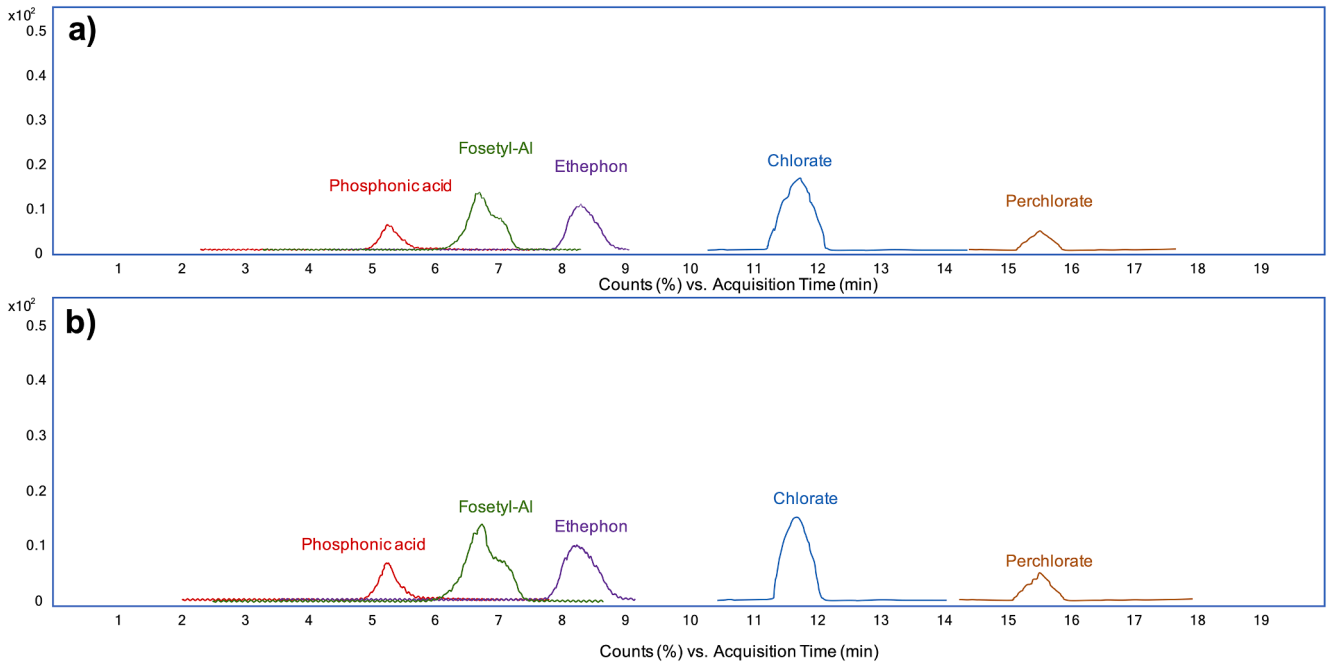

Fig. 3. (a) Extracted ion chromatogram obtained from an extra virgin olive oil sample spiked with 100 μg kg^{-1} of the polar compounds; (b) Extracted ion chromatogram obtained from an almond sample spiked with 100 μg kg^{-1} of the polar compounds.

Table 2
Matrix effect of each polar compound in each type of oil and nut respect to solvent.

	Polar pesticide					
	Spiked matrix	Ethephon	Fosetyl-Al	Phosphonic acid	Chlorate	Perchlorate
Oils	Extra virgin olive oil	3.6	2.8	7.1	−6.0	10.7
	Refined oilve oil	−1.0	0.4	−7.0	−6.1	−4.1
	Olive pomace oil	6.4	2.8	1.6	−4.9	1.7
	Crude olive pomace oil	14.0	−5.4	2.1	−10.4	−5.5
	Sunflower oil	12.2	−0.6	−2.5	−5.4	−7.9
Nuts	Almonds	5.7	9.5	−2.2	2.0	−1.2
	Hazelnuts	5.6	−17.4	9.9	11.6	−14.7
	Peanuts	5.5	16.3	5.3	12.0	−3.3
	Pistachios	5.8	18.4	18.5	10.2	−2.6
	Walnuts	8.8	−18.7	12.5	9.0	8.5

Note: All the experiments were carried out with a spiked concentration between 10 and 500 μg L^{-1}. Matrix effect was estimated using the following equation: Matrix effect = [1 − (slope in matrix/slope in solvent)] × 100.

Table 3
Recovery and precision values obtained for the polar compounds in oils and nuts.

			Polar pesticide				
	Spiked concentration		Phosphonic acid	Fosetyl-Al	Ethephon	Chlorate	Perchlorate
Oils (extra virgin olive oil)	10 μg kg^{-1}	Recovery (%)	93.3[a] (4.5)[b]	97.7 (7.5)	93.4 (4.9)	104.3 (10.3)	90.5 (3.3)
		Inter-day RSD (%)	11.3[c]	15.5	7.3	19.2	13.6
	100 μg kg^{-1}	Recovery (%)	86.2 (9.0)	87.6 (10.2)	86.0 (8.1)	94.3 (11.1)	83.4 (5.3)
		Inter-day RSD (%)	12.4	10.3	9.9	12.0	11.8
	500 μg kg^{-1}	Recovery (%)	77.6 (15.3)	76.9 (13.0)	79.4 (13.8)	75.6 (10.6)	79.6 (7.7)
		Inter-day RSD (%)	8.3	9.6	8.8	8.9	9.7
Nuts (almonds)	10 μg kg^{-1}	Recovery (%)	86.9 (17.0)	89.5 (14.7)	79.7 (10.3)	86.6 (13.1)	70.0 (15.9)
		Inter-day RSD (%)	19.1	17.5	16.7	15.4	19.8
	100 μg kg^{-1}	Recovery (%)	95.8 (15.3)	83.2 (11.5)	78.1 (7.8)	84.2 (17.0)	72.7 (12.6)
		Inter-day RSD (%)	17.0	14.4	13.3	16.7	16.3
	500 μg kg^{-1}	Recovery (%)	100.5 (16.0)	70.7 (8.7)	79.8 (5.5)	85.7 (19.6)	75.9 (9.1)
		Inter-day RSD (%)	18.0	11.2	8.8	19.7	11.3

[a] Recoveries were calculated based on n = 5.
[b] Intra-day precision (RSD values) are given in brackets and were based on n = 5.
[c] Inter-day precision (RSD values) were calculated based on n = 5.

However, nuts retain some polar compounds included in this study, such as phosphonic acid. In fact, all the categories of nuts analysed were contaminated with this compound, and furthermore, half of samples of each nut contain this compound, at concentrations up to 4.6 mg kg^{-1}.

Particularly, a very contaminated sample of almond was found, with more than 4.6 mg kg^{-1}. Also, hazelnuts require special mention bearing in mind that all the analysed samples were positive (30.9–65.7 μg kg^{-1}). These results agrees with EFSA that states that the major residue identified in rotational crops is phosphonic acid (Brancato et al., 2018).

Moreover, one sample of pistachios was contaminated with chlorate at 193.0 μg kg^{-1}.

Chlorate was monitored in nuts, ranging its concentration from 0.01 to 0.07 mg kg^{-1} (Kaufmann-Horlacher, Sherbaum, Stroher-Kolberg, & Wildgrube, 2014) and a method was developed to determine fosetyl-Al and other polar pesticides not included in this study in olive oil (Nortes-Méndez et al., 2016), but to the best of our knowledge, these polar compounds have not been simultaneously determined in these type of matrices as it was performed in this study, providing a valuable tool to be applied in routine laboratories.

4. Conclusions

In this study, a sensitive analytical method was developed by LC-QqQ-MS/MS for the determination of five polar pesticides in edible oils and nuts, being the first method that determines polar pesticides in this type of matrices. Two extraction methods based on the QuPPe-PO method (Quick Polar Pesticides Method for products of Plant Origin) were developed for the extraction of the target compounds in the matrices indicated, and experimental conditions were modified in order to achieve suitable recoveries. Validation parameters were estimated in both matrices and the values obtained conformed the criteria established in SANTE guideline.

The analysis of the extracts was performed by LC-QqQ-MS/MS with a total analysis time of only 20 min, and the proposed method is a step-forward in the analysis of polar compounds in oily matrices, increasing the scope of the analysis in terms of matrices and compounds in relation to previous studies.

A total of 40 samples of five types of oils and five types of nuts were analysed and the results obtained showed that vegetable oils were not contaminated with the polar compounds due to the polarity of them, while nuts mainly contained phosphonic acid, finding a sample contaminated with 4.6 mg kg^{-1}. Also, a sample of pistachio was contaminated with chlorate at 193.0 μg kg^{-1} which may be due to the use of chlorate-containing fertilisers such as potassium nitrate and monopotassium phosphate or its natural presence in soil or groundwater.

Because of the inevitable use of pesticides and the fact that a high number of positives were detected in nuts, the monitoring of these substances seems necessary and this method is a robust and reliable tool to control them.

CRediT authorship contribution statement

José L. Hidalgo-Ruiz: Formal analysis, Investigation, Validation, Writing - original draft, Visualization. **Roberto Romero-González:** Methodology, Software, Data curation. **José Luis Martínez Vidal:** Conceptualization, Supervision. **Antonia Garrido Frenich:** Resources, Writing - review & editing, Funding acquisition, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.128495.

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PUBLICACIÓN VI

Off-line solid phase extraction and separation of mineral oil saturated hydrocarbons and mineral oil aromatic hydrocarbons in edible oils, and analysis via GC with flame ionization detector

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Offline Solid-Phase Extraction and Separation of Mineral Oil Saturated Hydrocarbons and Mineral Oil Aromatic Hydrocarbons in Edible Oils, and Analysis via GC with a Flame Ionization Detector

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Abstract: A method was developed for the determination of mineral oil saturated hydrocarbons (MOSH) and mineral oil aromatic hydrocarbons (MOAH) in edible oils, achieving similar limits of quantification than those obtained by online extraction methodologies, i.e., 0.5 mg/kg. The isolation of MOSH and MOAH was performed in a silver nitrated silica gel stationary phase prior to their analysis by gas chromatography–flame ionization detector (GC-FID). To improve the sensitivity, the simulated on-column injection method, using a suitable liner, was optimized. The method was validated at 0.5, 10.0 and 17.9 mg/kg, and recoveries ranged from 80 to 110%. Intra and inter-day precision were evaluated at the same levels, and relative standard deviation (RSD) was lower than 20%. The method was applied to a total of 27 samples of different types of oil previously analyzed in an accredited laboratory, detecting MOSH up to 79.2 mg/kg and MOAH up to 22.4 mg/kg.

Keywords: edible oils; gas chromatography–flame ionization detector; mineral oil aromatic hydrocarbons; mineral oil saturated hydrocarbons; solid-phase extraction

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1. Introduction

Mineral oil hydrocarbons (MOH) are products obtained from the distillation of petroleum and are mainly composed of hydrocarbons, but they are also synthetically produced from coal, natural gas and biomass [1]. These MOH can usually contaminate food in many different ways, by contact with materials that have mineral oils, such as paperboard or inks, mineral oils used in machinery that are utilized during the oil manufacturing process, or even food additives [2]. Lubricating oils for food use are a complex mixture of these aliphatic saturated hydrocarbons and linear or branched ones (paraffin), ranging from C20 to C54 [3].

Moreover, MOH can also be found in edible oil samples that were submitted to a tougher extraction process, such as a second centrifugation of the olive, or to solvent extraction [4]. For this reason, the content of MOH in pomace oils is considerably higher than in other types of olive oils, such as extra virgin olive oil, where the fat extraction is not intense.

MOH can be either mineral oil saturated hydrocarbons (MOSH), which accumulate in tissues, lymph nodes, spleen and liver, and can cause microgranulomas [2], or mineral oil aromatic hydrocarbons (MOAH), which are considered as possible carcinogenic and mutagenic substances [5].

The contamination of edible oils by MOH is becoming a problem of great importance since a severe case of contamination was detected in Ukrainian sunflower oils in 2008,

which were contaminated with these compounds at concentrations up to 3100 mg/kg [6]. This fact took the Standing Committee on the Food Chain and Animal Health of the European Commission to set a maximum level of contamination for these compounds, and a limit of 50 mg/kg for crude and refined sunflower oils was established [7].

Oils are always complex matrices to analyze, so several methods were developed to isolate MOH. However, to the best of our knowledge, there is not a method that meets the three following requirements: achieving the separation of both MOSH and MOAH by offline solid-phase extraction (SPE), analyzing them by gas chromatography coupled to a flame ionization detector (GC-FID), and, finally, what can be the most difficult, performing it in vegetable oils. Some studies carried out the offline separation of MOSH and MOAH by SPE, but in different matrices, such as dried foods and cardboard [8–11], cosmetic lip products [12], or other less fatty matrices, such as pasta, rice, and icing sugar [13], as well as in cereals, chocolate, sausages, and cocoa powder [14]. Moreover, some studies performed the offline extraction by SPE in oils, but they only analyzed the MOSH fraction [15–17]. Other studies achieved the goal of separating the MOSH fraction from the MOAH one in oils using a multidimensional chromatographic technique, online liquid chromatography (LC)–GC-FID [14,18–24], which is more expensive than the offline methodology. In these cases, LC was used to carry out the separation of MOSH and MOAH.

In this study, an offline column chromatography method is proposed to carry out the separation of MOSH and MOAH due to the accuracy and reliability reached, and furthermore, its lower cost and high accessibility by any laboratory, where the use of online methods is not always possible. Moreover, the preparation of the offline chromatographic columns reduces the cost given the much more expensive equipment needed for LC and will achieve the same goal. In conclusion, offline isolation of MOSH from MOAH, and analyzing them via GC-FID in complex samples, such as edible oils, seems to be a good alternative.

Independently of the MOSH/MOAH separation method used prior to the chromatographic determination, the use of FID has widely increased because of its capability in analyzing hydrocarbons and quantifying them according to the number of carbons of the compound; indeed, it is especially used due to the lack of proper analytical standards for the studied compounds and the large number of compounds considered. Therefore, MOSH and MOAH are considered as the sum of several compounds that are chromatographically unresolved, which provide wide humps in the chromatograms.

One of the problems that these methods has is the potential risk of overestimation of the results due to interferences. In this sense, it must be mentioned that olefins usually interfere. Thus, epoxidation can be used to remove them when offline column chromatographic methods are applied [22]. This procedure is simple and quick, improving the selectivity of the method by eliminating interferences. Different epoxidation procedures were developed [20,22], and it was observed that the one proposed by Nestola et al. [22] had several advantages, such as no cooling is necessary and no evaporation of the solvent is needed [25].

Therefore, the development of a sensitive and reliable method that properly separates both fractions (MOSH and MOAH) but at the same time reduces the cost of the analysis is necessary. For this reason, an offline column chromatography coupled to a GC-FID analysis method was developed for the analysis of both fractions. The proposed methodology was successfully tested analyzing 27 samples.

2. Materials and Methods

2.1. Reagents and Chemicals

The standard mixture of markers and the retention time standard were obtained from Restek (Bellefonte, PA, USA). This standard mixture of markers included (in order of appearance in the chromatogram) *n*-undecane (C11, as a marker for the loss of low molecular mass MOSH), pentylbenzene (5B, as a marker for the loss of low molecular mass MOAH), 1-methylnaphthalnene and 2- methylnaphthalnene (1-MN and 2-MN, as

internal standards for the MOSH fraction), bicyclohexyl (CyCy, as the internal standard for the MOAH fraction), *n*-tridecane (C13, present at half concentration as a marker of good sensitivity), 1,3,5-tri-tert-butylbenzene (TBB, as a marker for the beginning of the elution of MOAH), cholestane (CHO, as a marker for the end of MOSH), and perylene (PER, as a marker for the end of MOAH), while the retention time standards included the *n*-alkanes C10, C11, C13, C16, C20, C24, C25, C35, C40, and C50. The standard mixture of *n*-alkanes, ranging from C11 to C40, sodium thiosulfate, and 3-chloroperoxybenzoic acid (*m*CPBA) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

For validation purposes, an oil sample was analyzed by several accredited laboratories by LC-GC-FID, providing a mean concentration of MOSH (38.8 mg/kg) and MOAH (4.2 mg/kg), which were used as the reference values.

LC-MS grade *n*-hexane, toluene, and ethanol were purchased from Honeywell (Morristown, NJ, USA), while ultrapure water was obtained by a gradient system Milli-Q water (Millipore, Bedford, MA, USA). Silver nitrate was provided by Merck (Darmstadt, Germany) whereas dichloromethane and silica gel 60 Å (particle size 0.063–0.200 mm, 70–230 mesh) were purchased from Sigma-Aldrich.

Fritted glass chromatographic columns with a glass stopcock (1 cm of diameter and 20 cm of length) were used, obtained from Pobel (Madrid, Spain).

2.2. Instrument and Apparatus

To extract MOH from the samples, a WX vortex from Velp Scientifica (Usmate, Italy) was utilized. An R-114 rotary evaporator from Büchi (Flawil, Switzerland) was used for the evaporation of the solvent.

A Scion GC system equipped with an autosampler (Bruker Corporation, Freemont, CA, USA) was used for chromatographic analyses. An ultra-inert liner SPI 0.25/0.32 mm from Agilent was used to simulate on-column injection. A DB-1HT capillary column (15 m × 0.32 mm i.d. × 0.10 µm film thickness) from Agilent (Santa Clara, CA, USA) was utilized for GC separation after an untreated fused silica capillary column used as pre-column (2 m × 0.32 mm) from Supelco (Bellefonte, PA, USA). The two columns were connected with a press-fit column connection from Agilent. Helium was used as carrier gas at a constant flow rate of 3 mL/min (62.2 cm/s linear velocity). Interactive Graphics (Bruker) v8.2.1 software was used for optimization and quantification.

2.3. Sample Collection

The samples were collected from local supermarkets located in Almería (Spain). The total amount of analyzed samples was 27 and they were stored in glass bottles with screw caps in the dark until their analysis.

2.4. Preparation of the Chromatographic Columns

The chromatographic columns for the extraction of MOH and isolation of the MOSH and MOAH fractions were prepared manually as no commercial SPE cartridge with 1% $AgNO_3$ was available. A 15 mL fritted glass column (20 cm × Φ1 cm) with a glass stopcock was used as the cartridge. The packed sorbent was 6 g of activated silica gel impregnated with 1% silver nitrate. The sorbent was prepared as follows: 100 g of silica gel were weighed out and activated at 600 °C for 6 h in a muffle furnace (JP Selecta, Barcelona, Spain), and then cooled down to room temperature. Consequently, 100 mL of a solution of 1% $AgNO_3$ was added drop by drop while shaking into a 1000 mL dark glass bottle. Finally, the sorbent was homogenized on the rotary equipment for 2–3 h and dried in an oven at 125 °C for 12 h. The silica was weekly prepared, and it was maintained in darkness in a dry place at room temperature.

To transfer and pack the sorbent into the column, 12 mL of *n*-hexane were added to the beaker where the silica gel was weighed out. The silica gel with the *n*-hexane was added to the column and then vortexed for 2 min in order to compact the sorbent and let the bubbles go out.

2.5. Epoxidation

To remove olefins from the sample, the epoxidation procedure developed by Nestola et al. [22] was used. Briefly, the sample was prepared by exact weighing of 0.6 g of oil and then 1.3 mL of *n*-hexane and 0.05 mL of the solution of internal standards and markers of Restek were added. Then, 1 mL of the ethanolic solution of *m*CPBA (20%) were added and the tube was shaken in a vortex for 10 min. Afterward, 1 mL of ethanol and 4 mL of an aqueous solution of sodium thiosulfate (10%) were added to facilitate the phase separation and eliminate the excess of *m*CPBA. The tube was shaken in a vortex for 30 s and centrifuged at 5000 rpm (4136× *g*) for 5 min.

2.6. Extraction

After 40 mL of the mixture *n*-hexane:toluene:dichloromethane (40:40:20, *v/v/v*) and 30 mL of *n*-hexane were added to wash and condition the silica gel, the supernatant (*n*-hexane phase, ~1 mL) phase obtained previously (epoxidation step) was loaded onto the Ag-silica gel column. Then, 8 mL of *n*-hexane were added to collect the MOSH fraction, and 9 mL of *n*-hexane:toluene:dichloromethane (40:40:20, *v/v/v*) were added to collect the MOAH fraction. Finally, 0.3 mL of toluene were added to the MOSH fraction to avoid the total evaporation. The solvents were evaporated in a rotary evaporator with a water bath at 40 °C. It is important to avoid a rapid decrease in pressure and that this pressure does not drop below 200 mbar in the case of MOSH and below 190 mbar in the case of MOAH. The fractions were evaporated until 0.3 mL of toluene was left and they were transferred to a vial with an insert for its injection into the GC-FID instrument.

2.7. GC-FID Analysis

In total, 2 µL of the 300 µL of toluene containing the MOSH or MOAH fractions were injected into GC-FID using a liner to simulate on-column injection. The injector temperature was programmed at 100 °C, and directly increased up to 360 °C at a rate of 200 °C/min. Once the temperature was reached, it was held for 10 min. The split valve was closed during the whole analysis.

The oven was at 40 °C and directly increased until 360 °C at a rate of 25 °C/min. Once the maximum temperature was reached, it was held for 15 min. The temperature of the detector was set at 350 °C during the whole analysis. The make-up gas (He), H_2, and air flow for the detector were 27, 35, and 300 mL/min, respectively.

2.8. Integration and Calculation

Integration is a critical part in this study, as it can change the result of the analysis. For this reason, a few instructions must be followed to achieve reliable results.

- A retention time standard mix (C10–C50) must be injected with every set of samples. Thus, the retention times can be properly adjusted.
- The baseline must start at the beginning of the C10 peak and finish at the end of the C50 peak, taking into account the fractions proposed by the JRC guideline (C10–C16, C16–C20, C20–C25, C25–C35, C35–C40, and C40–C50 in the case of MOSH; and C10–C16, C16–C25, C25–C35, and C35–C50 in the case of MOAH) [26].
- Shoulder peaks of the natural hydrocarbons must be subtracted from the hump as they do not belong to the MOH fraction.

To perform the calculations after the integration, Equation (1) was used:

$$\text{HC} = (\text{Area HC} \times \text{ISTD})/(\text{Area ISTD} \times \text{Sample}), \quad (1)$$

where "HC" is the concentration of each group of MOSH or MOAH in mg/kg; "Area HC" is the area of the hump subtracting the peaks of the hydrocarbons; "ISTD" is the amount of internal standard in gram; "Area ISTD" is the area of the peak of the internal standard (CyCy for MOSH and 1-MN or 2-MN (the peak with smallest area) for MOAH); and "Sample" is the amount of sample loaded in the chromatographic column in gram.

2.9. Method Validation

The JRC guide and the European Commission regulations were used for the validation of the methodology [26]. Trueness in terms of recoveries, intra- and inter-day precision, and limit of quantification (LOQ) per range of hydrocarbons were evaluated.

Linearity was evaluated building a calibration curve diluting the reference oil sample with an extra virgin olive oil blank sample until getting concentrations of 0.5, 1.0, 2.5, 5.0, 10.0, and 17.9 mg/kg for every range of hydrocarbons.

Intra- and inter-day precision and recovery parameters were determined using extra virgin olive oil as a blank and the reference oil diluted with extra virgin olive oil until achieving 0.5, 10.0, and 17.9 mg/kg of MOSH and MOAH.

For intra-day precision, a blank of extra virgin olive oil was spiked five times with the reference oil to have 0.5, 10.0, and 17.9 mg/kg of MOAH and MOAH, and spiked samples were analyzed. For inter-day precision, the same spiking procedure was followed, and the samples were analyzed during five consecutive days.

Trueness was evaluated analyzing five times a reference oil sample analyzed by different laboratories and the error (in %) obtained for the MOSH and MOAH fractions was calculated.

Finally, for the estimation of the LOQ, indications described in the SANTE guideline [27] were followed, defining this parameter as the lowest concentration of the analyte (in this case, it was a group of hydrocarbons of MOH) that was validated with acceptable trueness (recovery ranging from 70 to 120%) and precision (RSD lower than 20%).

3. Results and Discussion

3.1. Extraction Method Optimization

The olefins that naturally occur in oils enhanced the hump of the MOAH fraction, which led to overestimating the results, because the interfering compounds were taken into account mistakenly; therefore, the quantities were up to ten times larger than they should be. Consequently, the epoxidation described by Nestola et al. [22] was followed, since it considerably improved the results due to the olefins being removed.

An offline column chromatography method was developed. Firstly, 1% of silver nitrate was added to the silica gel due to it is believed that the silver nitrate remained in a crystalline form, filling the pores of the silica gel; thus, the olefins and triglycerides are better retained in the offline column chromatography [15].

Regarding the solvents used to extract the fractions in the chromatographic column, different mixtures were tested. For the MOSH fraction, only *n*-hexane was used as it is a non-polar solvent that dissolves saturated hydrocarbons. Furthermore, it was widely used in previous studies [15–17]. In the case of MOAH, different mixtures of solvents were evaluated. It was realized that toluene was necessary for the extraction of all the compounds, as the internal standard PER did not elute if this solvent was not used. According to Kantonales Labor Zurich (Zurich, Switzerland) [28], 20% of dichloromethane should be used, and finally, in the remaining 80%, a mixture of *n*-hexane and toluene (50:50, *v/v*) was added as it was observed that this mixture allowed the elution of the MOAH fraction. Thus, the mixture *n*-hexane:toluene:dichloromethane (40:40:20, *v/v/v*) was selected as the most appropriate for further experiments.

Furthermore, different amounts of sorbent were tested (3.0, 4.0, 5.0, and 6.0 g) to achieve a good separation between MOSH and MOAH. In the same way, the amount of *n*-hexane for MOSH and *n*-hexane:toluene:dichloromethane (40:40:20, *v/v/v*) for MOAH was evaluated analyzing fractions of 1 mL until the adequate markers were observed in the GC-FID. This separation was recognized because of the elution order of the markers. After 8 mL, CHO stopped coming out and the MOSH fraction was considered completely eluted from the chromatographic column. Consequently, TBB started eluting and the MOAH fraction was collected until PER eluted from the column completely after 9 mL.

The washing of the column was also important to minimize the noise as much as possible. Thus, two types of washing procedures were tested: washing the silica in

ultrasound or passing a solvent through the column several times. It was checked that the noise did not decrease when the silica was washed in ultrasound. However, when the silica was washed by passing a solvent through the column, the noise was decreasing every 10 mL of solvent until it was checked that 30 mL of the mixture *n*-hexane:toluene:dichloromethane (40:40:20, *v/v/v*) and 40 mL of *n*-hexane were necessary. This solvent washing order was chosen to have in the column the same solvent needed to start the extraction.

The quantity of sample used was also appraised (0.3 and 0.6 g of oil were tested), verifying that 0.6 g of oil was enough to carry out the epoxidation process, having a higher quantity of solvent to transfer the supernatant to the chromatographic column, thus minimizing the risk of collecting the aqueous phase.

Finally, the evaporation conditions were optimized. Different bath temperatures were tested (35, 40, and 45 °C), as well as the minimum vacuum pressures were controlled in order to evaporate the solvents as quickly as possible but avoiding the loss of the most volatile compounds. Thus, in the case of the MOSH fraction, as the solvent was *n*-hexane, the pressure should not be below 200 mbar, while for the MOAH fraction, the minimum pressure should be 190 mbar. When pressures were below these values, the internal standards were lost, as they were low molecular mass compounds, and the analysis was unreliable.

Finally, Figure 1 shows that a proper separation between MOSH and MOAH was achieved, checking the correct appearance of the internal standards in each chromatogram. Thus, in Figure 1a, C11, CyCy, C13, and CHO were detected, while in Figure 1b, 5B, 1-MN, 2-MN, TBB, and PER were monitored.

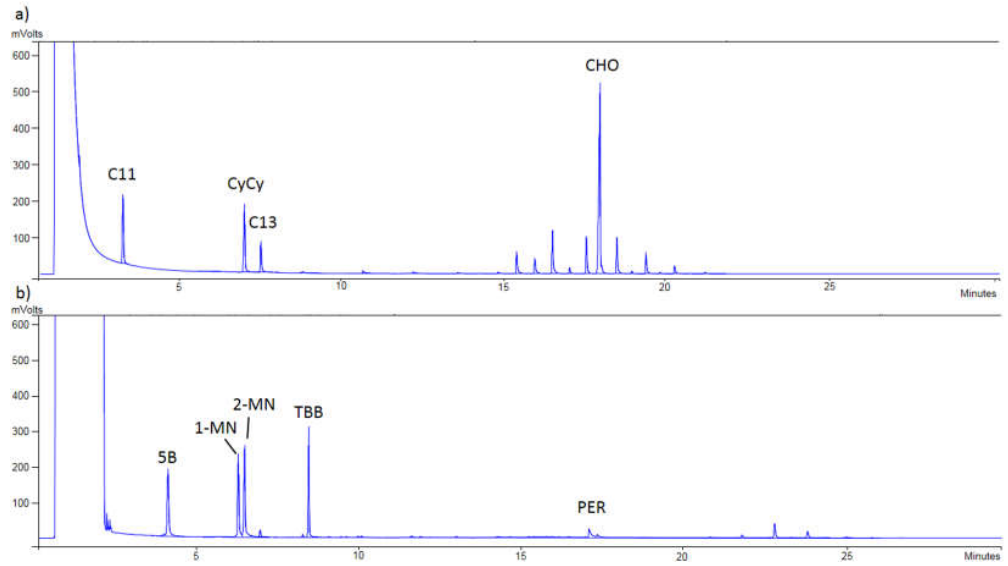

Figure 1. (**a**) Chromatogram of the mineral oil saturated hydrocarbons (MOSH) of an extracted blank sample at 10 mg/kg; (C11: *n*-undecane; CyCy: bicyclohexyl; C13: *n*-tridecane; CHO: cholestane). (**b**) Chromatogram of the mineral oil aromatic hydrocarbons (MOAH) of an extracted blank sample spiked at 10 mg/kg; (5B: pentylbenzene; 1-MN: 1-methylnaphthalene; 2-MN: 2-methylnaphthalene; TBB: 1,3,5-tri-tert-butylbenzene; PER: perylene).

3.2. Optimization of the Chromatographic Conditions

The volatilization of the hydrocarbons can vary depending on several factors, such as the solvent used or the injector and column temperatures. In this study, all the conditions were optimized to obtain the best peaks in terms of peak shape and sensitivity for all the range of the hydrocarbons that are going to be analyzed (C10–C50). For this, 2 µL of a mixture of hydrocarbons at 2 mg/L, ranging from C10 to C50, were injected for on-column simulated injection in order to reduce the amount injected and avoid dirt in the injector. To simulate on-column injection, an ultra-inert liner SPI 0.25/0.32 mm from Agilent was used.

Thus, the column could be introduced from one extreme of the liner and got stuck into it, while the syringe was introduced from the other one until the narrowing of the liner, so the sample was introduced directly into the column passing through an "aisle" of the liner. A scheme of this system is shown in Figure S1.

The optimization of the injection was carried out in order to lose the minimum amount of volatile compounds and comply with the requirement of a ±20% difference between C20 and C50, as required by the JRC guidelines [26]. Thus, a mixture of hydrocarbons at 2 mg/L ranging from C10 to C50 was injected assaying several minimum (60, 100, and 150 °C) and maximum injection temperatures (340, 350, 360, and 370 °C). Figure S2a shows the results obtained for the minimum temperatures tested in the injector while Figure S2b shows the results obtained for the maximum temperatures. Initially, the solvent was evaporated in the injector at 100 °C and a ramp at 200 °C/min was set. Figure 2 shows a chromatogram of the internal standards of Restek, where it can be observed that the peaks of the compounds are perfectly separated, and no loss of the volatile compounds was produced.

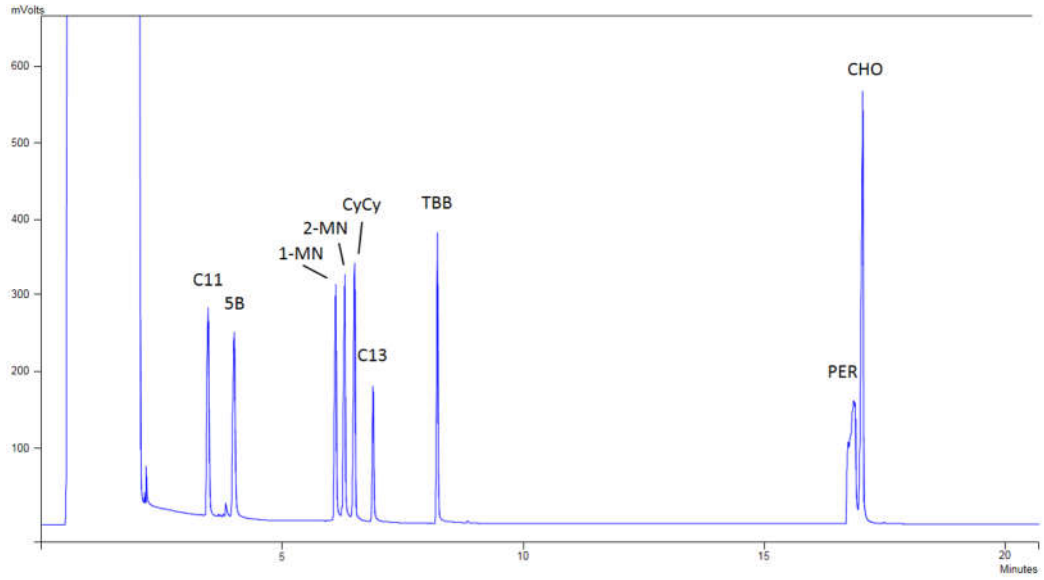

Figure 2. Chromatogram of the mixture of internal standards of Restek spiked at 10 mg/kg in *n*-hexane:toluene (50:50, *v/v*); (C11: *n*-undecane; 5B: pentylbenzene; 1-MN: 1-methylnaphthalene; 2-MN: 2-methylnaphthalene; CyCy: bicyclohexyl; C13: *n*-tridecane; TBB: 1,3,5-tri-tert-butylbenzene; CHO: cholestane; PER: perylene).

The temperature programming of the oven was also optimized. Five ramps were assayed (50, 35, 25, 20, and 15 °C/min), injecting the linear mixture of hydrocarbons at 2 mg L^{-1}. A temperature programming of 25 °C/min provided the correct separation of the lower and higher hydrocarbons. Moreover, the ratio of the lower and higher peaks were tested and the difference was always below 20%, as the JRC guidelines recommend [26].

The temperature of the FID was optimized, injecting the retention time standard at 2 mg L^{-1} at three temperatures (350, 370, and 390 °C). The results showed that at a temperature higher than 350 °C, the noise was higher, and the peak areas did not increase (see Figure S2c), so 350 °C was chosen.

Although MS can also be used [14], this is a more sophisticated detector and less commonly used for this topic.

3.3. Method Validation

The evaluation of linearity was carried out through determination coefficients (R2) of the different calibration curves of each range of hydrocarbons from 0.5 to 17.9 mg/kg, depending on the fraction evaluated, and the values were always above 0.99 (see Table S1).

Intra-day and inter-day precisions and recovery values were determined by using an extra virgin olive oil as a blank, previously verified, and the extra virgin olive oil spiked with a reference oil. The average recoveries for MOSH and MOAH are shown in Table 1 and they were between 80.9% and 114.9%. In terms of RSD, the results for the intra-day and inter-day precision were always below 20% (see Table 1). All these parameters are comparable to those obtained in other studies carried out with more sophisticated equipment, such as LC-GC-FID [18–21].

Table 1. Recovery and precision values obtained during the method validation.

Spiked Concentration	Parameter	MOSH	MOAH
0.5 mg/kg	Recovery (%) [1]	80.9–110.6	105.3–114.9
	Intra-day RSD (%)	15.4	16.4
	Inter-day RSD (%)	18.7	19.3
10.0 mg/kg	Recovery (%)	90.1–105.6	102.3–112.5
	Intra-day RSD (%)	6.4	10.9
	Inter-day RSD (%)	9.7	17.1
17.9 mg/kg	Recovery (%)	92.2–104.3	99.1–108.7
	Intra-day RSD (%)	3.0	6.5
	Inter-day RSD (%)	5.2	8.0

[1] Recoveries and RSDs were calculated based on $n = 5$.

Trueness was evaluated by analyzing five times a reference oil sample analyzed by different laboratories. The theoretical value for MOSH was 48.8 mg/kg while the total result obtained was 41.0 mg/kg, whereas the theoretical value for MOSH was 4.2 mg/kg and the total result obtained was 5.0 mg/kg. The percentage of error obtained for MOSH was below 16% while for MOAH it was below 19%.

The LOQ was set at 0.5 mg/kg for each range of hydrocarbons of both MOSH and MOAH, as the JRC guidelines require [26]. For this purpose, taking into account the results per range of hydrocarbons of the reference oil, a calibration curve per each range of hydrocarbons was built. This could be performed since the results of the range of hydrocarbons were known individually, as this reference oil was analyzed by several accredited laboratories. This oil measured 1.7, 2.2, 3.2, 17.9, 11.0, and 2.8 mg/kg in the ranges C10–C16, C16–C20, C20–C25, C25–C35, C35–C40, and C40–C50, respectively, for MOSH; and 0.8, 1.0, 1.8, and 0.6 mg/kg in the ranges C10–C16, C16–C25, C25–C35, and C35–C50, respectively, for MOAH. It was verified that an adequate trueness was reached at the lowest point of the calibration curve, 0.5 mg/kg, in each range of hydrocarbons in both MOSH and MOAH, for the setting of the LOQ at this value.

As it can be observed in Table 1, it meets the requirements of the JRC guidelines [26], the recoveries always being between 70 and 120%, and the intra- and inter-day precisions below 20% in terms of RSD. This LOQ value is similar or lower than other studies that used similar approaches (2.5 mg/kg) [16] and is also lower than those that used much complex equipment, such as LC-GC-FID (8 mg/kg) [29].

3.4. Sample Analysis

The developed method was applied for the determination of MOSH and MOAH in 27 edible oil samples. A set of internal quality controls (IQC) was injected with every set of samples to guarantee that the analytical procedure was under statistical control. The IQC included a reagent blank, a mixture of the internal standards in a solvent (5, 10, and 20 mg/mL), a mixture of the linear hydrocarbons in a solvent (10 mg/mL), a mineral oil mixture in a solvent (10 mg/mL), and a blank spiked oil sample (10 mg/mL).

Results were reported using the recommended ranges given by the JRC guide [26]. Table 2 sums up the results obtained from the analyzed samples, while in Table S2, the results per range of hydrocarbons can be seen. As can be seen, between C25 and C35, as well as between C35 and C40, the highest concentrations were observed.

Table 2. Concentration by ranges of MOH detected in the samples of edible oils analyzed.

Matrix	MOSH (mg/kg)	MOAH (mg/kg)
Extra Virgin Olive Oil 1	<LOQ	<LOQ
Extra Virgin Olive Oil 2	<LOQ	<LOQ
Extra Virgin Olive Oil 3	<LOQ	<LOQ
Extra Virgin Olive Oil 4	<LOQ	<LOQ
Extra Virgin Olive Oil 5	<LOQ	<LOQ
Extra Virgin Olive Oil 6	<LOQ	<LOQ
Extra Virgin Olive Oil 7	<LOQ	<LOQ
Refined Olive Oil 1	12.2	<LOQ
Refined Olive Oil 2	5.7	3.3
Refined Olive Oil 3	5.4	5.8
Refined Olive Oil 4	1.1	0.9
Refined Olive Oil 5	<LOQ	<LOQ
Refined Olive Oil 6	8.2	1.4
Refined Olive Oil 7	24.2	12.9
Olive Pomace Oil 1	22.4	7.7
Olive Pomace Oil 2	35.3	19.7
Olive Pomace Oil 3	49.9	18.1
Olive Pomace Oil 4	68.3	21.5
Olive Pomace Oil 5	79.2	22.4
Sunflower Oil 1	<LOQ	<LOQ
Sunflower Oil 2	4.4	<LOQ
Sunflower Oil 3	15.0	5.9
Sunflower Oil 4	<LOQ	<LOQ
Sunflower Oil 5	7.3	3.4
Sunflower Oil 6	<LOQ	<LOQ
Corn Oil 1	<LOQ	<LOQ
Corn Oil 2	<LOQ	<LOQ

As expected, the extra virgin olive oil samples were not contaminated, neither with MOSH nor with MOAH. As can be observed in Table 2, olive pomace oils presented the highest concentrations of MOSH and MOAH, with concentrations up to 79.2 mg/kg of MOSH and 22.4 mg/kg of MOAH. Furthermore, a sample of sunflower oil showed a concentration of MOSH of 15.0 mg/kg and a MOAH concentration of 5.9 mg/kg. Furthermore, the analyzed corn oils were not contaminated.

The sample of refined olive oil 6 contaminated with 8.2 mg/kg of MOSH is shown in Figure 3a while in Figure 3b the same chromatogram can be seen but zoomed in, to appreciate the humps of MOSH. Figure 4a shows the contamination with MOAH (12.9 mg/kg) of the sample of refined olive oil 7, while in Figure 4b, the humps of MOAH can be appreciated as the interesting zone is zoomed in.

Comparing our results with those achieved in other studies, similar results were obtained. For example, Liu et al. [16] found concentrations below 60.9 mg/kg of MOSH in the majority of the samples, except in a blend oil, detecting up to 259.4 mg/kg. Zoccali et al. [19] found low levels of MOSH (below 21.8 mg/kg) but MOAH were not detected in extra virgin olive oils. In addition, they found levels up to 444.8 mg/kg of MOSH and 66.1 mg/kg of MOAH in olive pomace oils.

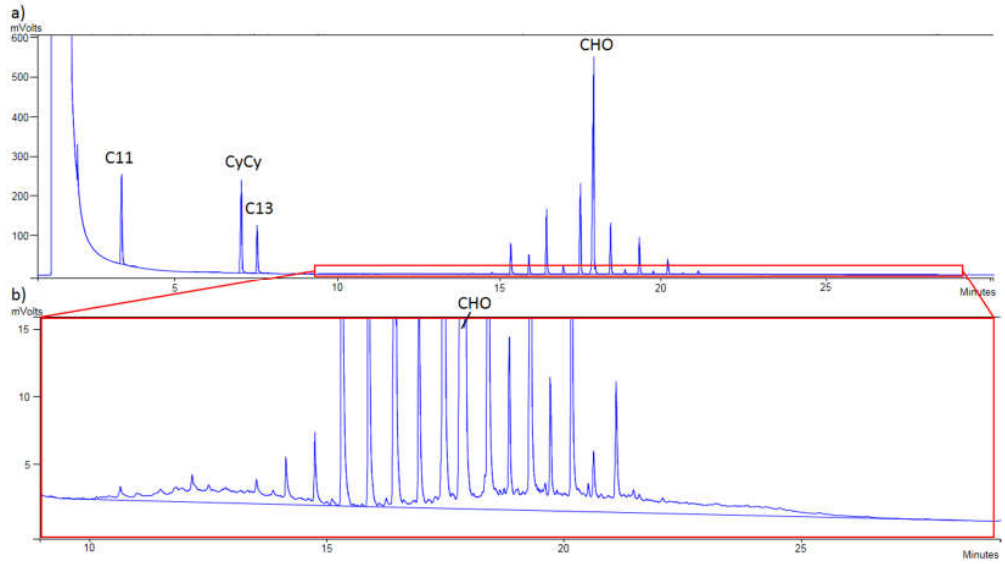

Figure 3. (**a**) Chromatogram of mineral oil saturated hydrocarbons (MOSH) of the sample of refined olive oil 6 contaminated at 8.2 mg/kg; (C11: *n*-undecane; CyCy: bicyclohexyl; C13: *n*-tridecane; CHO: cholestane). (**b**) Zoomed-in from (a) (CHO: cholestane).

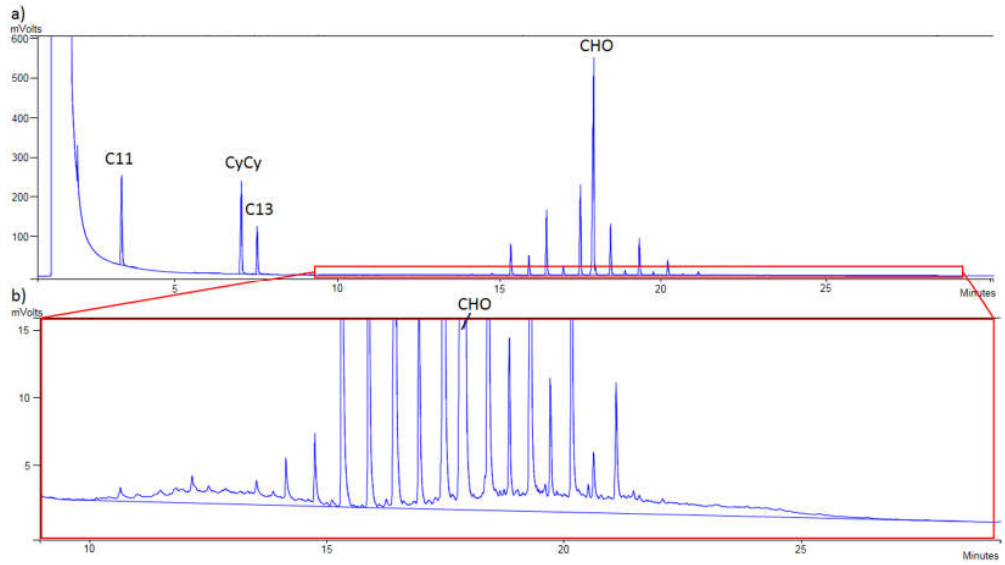

Figure 4. (**a**) Chromatogram of mineral oil aromatic hydrocarbons (MOAH) of the sample of refined olive oil 7 contaminated at 12.9 mg/kg (5B: pentylbenzene; 1-MN: 1-methylnaphthalene; 2-MN: 2-methylnaphthalene; TBB: 1,3,5-tri-tert-butylbenzene; PER: perylene). (**b**) Zoomed in from (a) (PER: perylene).

4. Conclusions

A method for the analysis of MOH was developed using an offline extraction followed by GC-FID. The method was fully validated and the LOQ was 0.5 mg/kg for each group of hydrocarbons. The validation of the method also showed suitable trueness and precision values. The validated method was applied to a total of 27 samples of edible oils, detecting MOSH and MOAH in the majority of them. Taking into account that a high amount of MOH was found in the majority of the samples at high concentrations, the routine control of these compounds in edible vegetable oils is necessary.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/foods10092026/s1, Figure S1: Scheme of the on-column simulation system of injection used, Figure S2: Comparison of the peak areas between the minimum (a) and maximum (b) temperatures of the injector and FID (c) tested. Table S1. Calibration curves and R2 obtained, by ranges, of MOH. Table S2: Concentration by ranges of MOH detected in the samples of edible oils analyzed.

Author Contributions: Conceptualization, J.L.M.V., A.G.F. and J.A.L.; methodology, J.A.L., R.R.G. and J.L.H.R., validation: J.L.H.R., J.A.L. and R.R.G.; formal analysis: J.L.H.R., J.A.L. and J.L.M.V.; data curation: A.G.F., J.L.H.R. and R.R.G.; writing—original draft preparation, J.L.H.R. and J.A.L.; writing—review and editing: J.L.M.V., A.G.F. and R.R.G.; supervision: J.L.M.V. and A.G.F.; project administration: A.G.F.; funding acquisition: J.L.M.V. and A.G.F. All authors have read and agreed to the published version of the manuscript.

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CHAPTER IV

COMPREHENSIVE DISCUSSION

1. INTRODUCTION

As it was shown throughout this Thesis, food safety concern is increasing due to several reasons such as the contaminants that appear, either endogenously or exogenously, in the crops. The endogenous contamination come from the effect of natural organisms or from processing, while the exogenous contamination is provoked by the addition of substances to prevent crops from infections or to increase the production. To face this concern, the development and validation of robust analytical methods for the control of contaminants is necessary.

Currently, the development of methods for the analysis of contaminants in food is a field in constant growth, since new contaminants appear every day. Particularly, the analysis of oils and other fatty matrices is a challenge, and it usually requires a deeper clean-up stage due to the high number of interferers.

Some of those concerning contaminants can be of natural origin such as mycotoxins, which are produced by fungus naturally occurring in food, or MCPDs, which appear when the oils or other fatty matrices are subjected to high temperatures. Furthermore, contaminants can intentionally be added to crops, which can end in food such as polar pesticides, or the final product can be contaminated directly with contaminants like MOH.

Therefore, a comprehensive review of the possible contaminants in edible oils and oilseeds and the analytical techniques mainly used to monitor them was carried out in Publication I. Subsequently, several methods were developed and validated for the determination of endogenous contaminants, like Publications II, III and IV, and for exogenous contaminants, like Publications V and VI. These methods will help to control the presence of these contaminants in food and thus, ensure their safety.

2. OCCURRENCE AND DETERMINATION OF CONTAMINANTS IN EDIBLE OILS AND OILSEEDS

In order to have an overview of the contaminants present in edible oils and oilseeds, and the techniques used to analyze them, a review of the last ten years was carried out in Publication I, mainly focusing on mycotoxins, polycyclic aromatic hydrocarbons (PAH), 3-MCPD esters and glycidyl esters, mineral oils, phthalates and pesticides, paying special attention to the analytical techniques used to monitor them.

For mycotoxins, LLE and QuEChERS were the most used methods for their extraction, as well as immunoaffinity chromatography, while their analysis was frequently carried out by LC, using simple detectors like FLD or more complex MS or MS/MS detectors such as QqQ or QTRAP.

It was found out that PAHs are photosensitive, so the manipulation of the samples needs to be done under dark conditions to avoid the decomposition and oxidation of the compounds. In this case, for the extraction of the compounds, LLE was usually performed with a d-SPE step to remove lipids. Usually, the analysis was performed with LC using a simple detector such as FLD or by GC using MS detector.

As it was explained in Chapter 1, there are two main ways of analyzing 3-MCPD esters and glycidyl esters: direct and indirect methods. Usually, indirect methods, those that analyze these compounds and expressed as the sum of free 3-MCPD and free glycidol, were the most used due to the legislation requires these values. They normally had more steps than the direct methods such as the conversion of glycidyl esters to 3-MBPD esters, transesterification and derivatization and they were often analyzed by GC-MS. On the other hand, direct methods needed at least two clean-up steps prior to the analysis of the 3-MCPD esters and glycidyl esters by LC coupled to MS/MS.

Sometimes, to analyze MOSH and MOAH, an epoxidation reaction may be necessary due to the oil can contain olefins that may interfere with the final result. The majority of the methods for extracting and separating MOSH and MOAH from mineral oils utilized complex techniques such as LC-GC-FID. However, sometimes, the epoxidation step as well as the extraction and separation of the fractions was carried out offline, and GC-FID was only used for the analysis of the fractions. In this case, only the MOSH fraction was analyzed in oils.

For the extraction of phthalates, LLE was used in the majority of the cases. Furthermore, head space solid phase microextraction (HS-SPME) was utilized regarding the numerous benefits that this technique offers such as minimum use of solvent, the integration of sampling and sample preparation steps, simple operation, low cost and the possibility of an online analytical procedure. To separate the compounds, both LC and GC were employed. In the case of LC, UV/Vis detector is often used as well as MS detector, while in the case of GC, both FID and MS detectors were used.

Finally, several approaches for the extraction of pesticides from edible oils and oilseeds were carried out, such as LLE combined with QuEChERS or SPE, as well as some others less common like air-assisted liquid-liquid microextraction (AALLME). A high number of pesticides were simultaneously analyzed. For that, multiresidue methods were developed using both LC and GC and normally using MS or MS/MS with detectors such as Q, QqQ or QTRAP.

3. DEVELOPMENT AND VALIDATION OF ANALYTICAL METHODS FOR ENDOGENOUS CONTAMINANTS

In this section, the research carried out to develop analytical methods for the determination of endogenous contaminants will be discussed. This includes the method developed for mycotoxins such as aflatoxins B1, B2,

G1 and G2, α-zearalenol and zearalenone in vegetable oils (Publication II) and nuts (Publication III) as well as the direct determination of nineteen 3-MCPD esters and seven glycidyl esters in oils and fatty matrices (Publication IV), using UHPLC-MS/MS.

3.1 Determination of mycotoxins in vegetable oils and nuts by UHPLC-MS/MS

3.1.1 Separation and detection by liquid chromatography coupled to QqQ mass detector

To get proper MS conditions of mycotoxins, individual solutions containing the aflatoxins at 2 mg/L were injected. Firstly, full scan MS, including ESI+ and ESI- modes, was applied. Thus, aflatoxins were ionized using ESI+, while α-zearalenol and zearalenone were ionized using ESI-.

In Publication II and Publication III, the chromatographic separation technique was carried out using LC. The flow rate was maintained at 0.2 mL/min and elution gradient is shown in Figure 1, while a summary of the chromatographic conditions can be seen in Table 1.

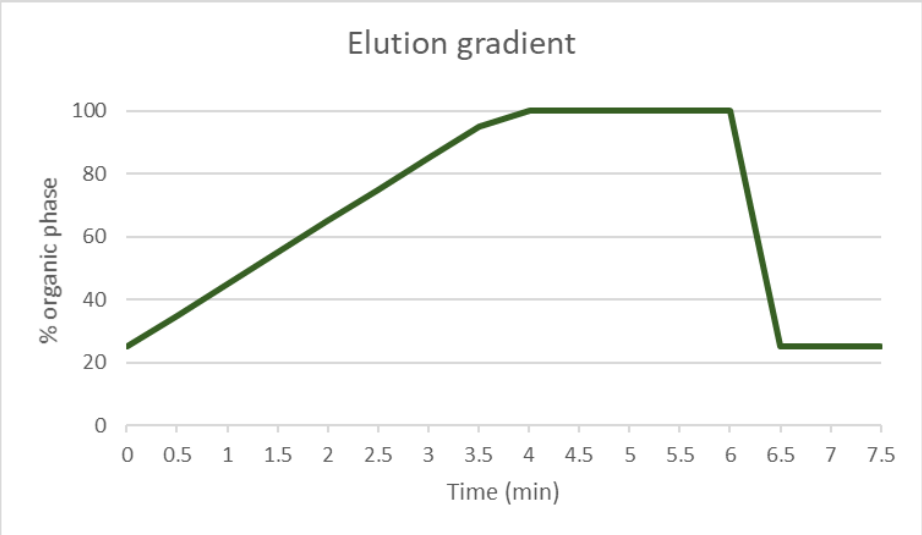

Figure 1. Elution gradient used in Publications II and III.

Table 1: Chromatographic conditions used for the separation of mycotoxins.

LC Parameter	Conditions (Publications II and III)
Aqueous phase	5 mM ammonium formate in water
Organic phase	Methanol
Column	Zorbax plus C18 (100 × 2.1 mm; 1.8 µm)
Column temperature	25 °C
Injection volume	5 µL
Flow rate	0.2 mL/min
Time of analysis	10 min
Elution mode	Gradient

[a] Abbreviations: C18: Octadecasilane; LC: Liquid chromatography.

Since some of the aflatoxins are very similar among them, a proper chromatographic separation could not be achieved, but the separation was good enough to be able to discriminate between them since they have different molecular masses and different ions, as it can be seen in Figure 2.

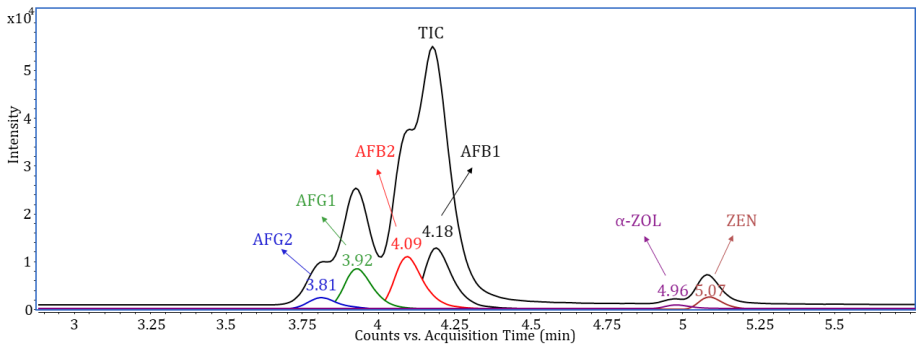

Figure 2. Chromatogram of the mycotoxins analyzed in Publications II and III at 50 µg/L in solvent. AFB1: Aflatoxin B1; AFB2: Aflatoxin B2; AFG1: Aflatoxin G1; AFG2: Aflatoxin G2; TIC: Total ions chromatogram; α-ZOL: α-Zearalenol; ZEA: Zearalenone.

The final conditions such as retention time windows, precursor and product ion *m/z* and ion ratios are summarized in Table 2.

Table 2: Retention time windows (RTW) and MS/MS parameters for the mycotoxins.

Compound	RTW (min) [a]	Precursor ion (*m/z*)	Product ion (*m/z*) [b]	Ion ratio (%)
Aflatoxin B1	4.13-4.17	313.1 (130)[c]	**241.1** (40)[d] 285.1 (20) 128.1 (80) 115.1 (80)	 96.8 91.0 65.9
Aflatoxin B2	4.04-4.06	315.2 (125)	**259.0** (30) 287.0 (30) 115.0 (80) 243.0 (50)	 95.8 78.1 37.7
Aflatoxin G1	3.87-3.89	329.1 (125)	**115.0** (80) 243.0 (30) 200.0 (50)	 87.5 80.6
Aflatoxin G2	3.74-3.78	331.1 (125)	**245.0** (30) 189.0 (50) 115.0 (80)	 73.3 69.4
α-Zearalenol	4.93-4.95	319.2 (125)	**129.9** (40) 160.0 (40) 174.0 (40)	 87.1 26.2
Zearalenone	5.04-5.06	317.2 (185)	**131.0** (25) 175.0 (25) 187.0 (25) 273.1 (25)	 85.5 32.7 23.9

[a] RTW: Retention time windows; [b] Transition in bold was used for quantification; [c] Fragmentor voltage (V) is given in brackets; [d] Collision energy (eV) is given in brackets.

3.1.2 Extraction methods

a) Vegetable oils

After the optimization of the chromatographic and spectrometric conditions, the extraction method of mycotoxins from vegetable oils was optimized. For that purpose, an existing QuEChERS extraction procedure

was tested. The experiments carried out to optimize the extraction consisted on studying the influence on the recovery of the following parameters:

- Quantity of oil
- Volume of extraction
- Cleaning salts
- Quantity of cleaning salts

The tested method used 1.0 g of oil, 2.0 mL of water and 8.0 mL of acetonitrile, and 100 mg of C18, 150 mg of PSA and 200 mg of Al_2O_3 as cleaning salts. In our case, three amounts of oil were tested (1.0, 5.0 and 10.0 g), as well as three volumes of acetonitrile (4.0, 8.0 and 12.0 mL). The experiments were performed spiking extra virgin olive oil blank samples at 50 μg/kg. The best results were obtained using the same amounts than the original method. However, in the case of the cleaning salts, it was observed that the addition of PSA and Al_2O_3 did not improve the results, so only C18 was used. Moreover, three quantities of this sorbent were tested (50, 100 and 150 mg), and the results showed that 50 mg of C18 could not be enough to the cleaning, and 150 mg did not improve the results in terms of recovery.

A scheme of the final extraction method of mycotoxins in vegetable oils in Publication II is shown in Figure 3.

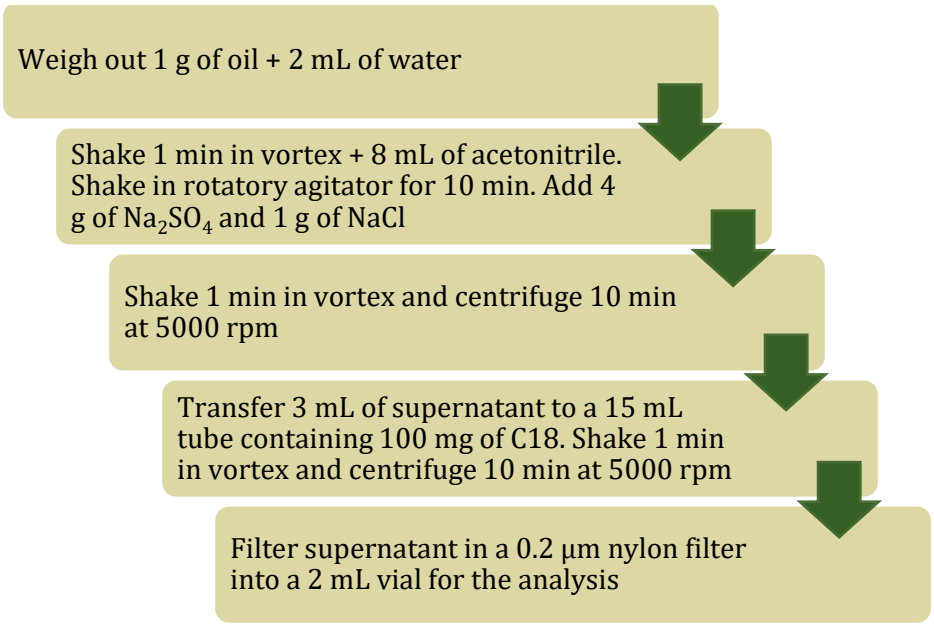

Figure 3. Scheme of the extraction method of mycotoxins in vegetable oils in Publication II. Abbreviations: C18: Octadecasilane.

b) Nuts

Firstly, the method developed for Publication II was performed including some modifications, such as an exhaustive homogenization. The experiments were carried out spiking almond blank samples at 50 µg/kg.

The amount of the sample was also increased from 1.0 g to 2.0 g. Furthermore, the time of homogenization was increased from 10 to 20 minutes since it was believed that the time needed for the transfer of the mycotoxins present in a solid matrix to a liquid extractant should be higher than the time needed for the transfer of the mycotoxins between two liquids.

A scheme of the final extraction method of mycotoxins in nuts in Publication III is shown in Figure 4.

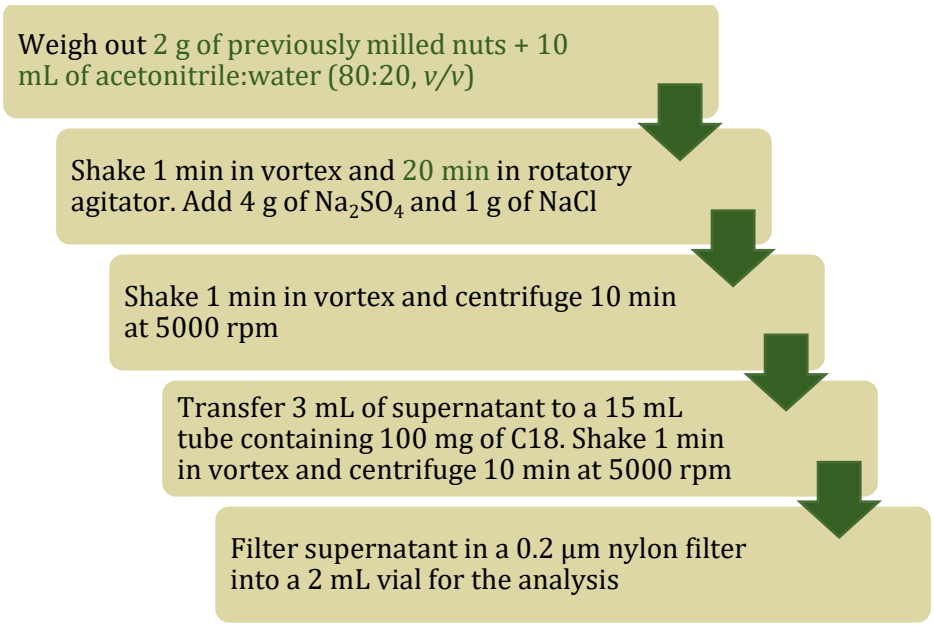

Figure 4. Scheme of the extraction method of mycotoxins in nuts in Publication III. Changes respect to vegetable oils are marked in green. Abbreviations: C18: Octadecasilane.

3.1.3 Validation of the methods

The validation of the methods was carried out using the SANTE guidelines in order to show its applicability and ensure a proper identification and quantification of the target compounds. Parameters evaluated in both methods (Publication II and Publication III) are summarized in Table 3.

In both Publications II and III, linearity was evaluated by preparing calibration curves at several concentrations: 0, 0.5, 1, 2.5, 5, 10, 25, 50, 100 and 250 µg/L, in both, solvent and matrices (extra virgin olive oil in Publication II and peanuts in Publication III), and determining the determination coefficients (R^2) using the area of the signal as analytical response, obtaining R^2 values higher than 0.9968 for all the mycotoxins in vegetable oils, and higher than 0.9967 in nuts.

Table 3: Parameters evaluated for the validation of the method in Publications II and III.[a]

Parameter	Vegetable oils (Publication II)	Nuts (Publication III)
Determination coefficient (R^2)	0.9968-0.9994	0.9967-0.9999
LOQ (µg/kg)	0.5 (1.0 for α-ZOL and ZEA)	
Recovery (%)[b]	88-119	74-116
Intra-day precision, RSD (%)	1-14	1-11
Inter-day precision, RSD (%)	3-19	4-19

[a] Abbreviations: LOQ: Limit of quantification; RSD: Relative standard deviation; α-ZOL: α-Zearalenol; ZEA: Zearalenone.

[b] Note: Recoveries and RSDs were calculated based on n = 5.

Furthermore, a study of the matrix effect was carried out, which consisted on the comparison of the slopes of the calibration curves prepared in solvent and in matrix. It was 34%, 63%, 54%, 65%, 14% and 4% for the aflatoxin B1, aflatoxin B2, aflatoxin G1, aflatoxin G2, α-zearalenol and zearalenone respectively, which shows that there was a matrix enhancement effect for aflatoxins but it was negligible for α-zearalenol and zearalenone. Considering this result, matrix calibration was used for quantification to correct this effect.

In Publication III, the matrix effect was tested comparing solvent and five nut matrices (almonds, hazelnuts, peanuts, pistachios and walnuts) and results are showed in Table 4. Figure 5 represents the percentage of matrix effect of each mycotoxin in each matrix studied in relation to the selected matrix.

Table 4: Matrix effect of mycotoxins in tested nuts in relation to solvent.[a]

Matrix	AFB1	AFB2	AFG1	AFG2	α-ZOL	ZEA
Peanuts	30%	48%	47%	53%	1%	-17%
Almonds	15% (9%)[b]	31% (6%)	22% (12%)	28% (9%)	-4% (6%)	-22% (-4%)
Hazelnuts	28% (-2%)	46% (-2%)	43% (-2%)	48% (-3%)	-12% (-13%)	-28% (-14%)
Pistachios	42% (-1%)	57% (1%)	64% (24%)	67% (16%)	6% (-16%)	-20% (-28%)
Walnuts	29% (-12%)	50% (-12%)	82% (-17%)	77% (-16%)	-16% (-5%)	-40% (-6%)

[a] Abbreviations: AFB1: Aflatoxin B1; AFB2: Aflatoxin B2; AFG1: Aflatoxin G1; AFG2: Aflatoxin G2; α-ZOL: α-Zearalenol; ZEA: Zearalenone; [b] Matrix effect in each nut using peanut as representative matrix are given in brackets.

As it can be observed in Figure 5, for solvent (Figure 5a), almost all the values of matrix effect were above or below the ±20%. This fact made necessary to look for a representative matrix to compensate the matrix effect avoiding the use of isotopically labelled internal standards. Consequently, matrix effect of all the matrices was evaluated comparing the slopes of the analytical curves obtained selecting the matrices one by one.

In Figure 5b it can be seen that when almond was used as representative matrix, a different behavior between aflatoxins and α-zearalenol and zearalenone was observed. While for aflatoxins matrix suppression was observed, α-zearalenol and zearalenone presented both matrix suppression and enhancement within the acceptable ±20% except for zearalenone in walnuts.

The case of hazelnuts (Figure 5c) was close to be within the limits but there were two values out of range: aflatoxin B1 in walnuts and α-zearalenol in pistachios.

Figure 5. Comparison of matrix effect selecting as reference matrix (a) solvent, (b) almonds, (c) hazelnuts, (d) peanuts, (e) pistachios and (f) walnuts. Abbreviations: AFB1: Aflatoxin B1; AFB2: Aflatoxin B2; AFG1: Aflatoxin G1; AFG2: Aflatoxin G2; α-ZOL: α-Zearalenol; ZEA: Zearalenone.

In peanuts (Figure 5d) all the values were within the limits and only two compounds, aflatoxin G1 and zearalenone, were in the limit of ±20% while the majority of the values were below ±12%, and the matrix effect values of aflatoxins for hazelnuts were almost negligible (below 3%).

When pistachios were selected as representative matrix (Figure 5e), matrix enhancement was observed for the majority of combinations analyte-matrix. Something similar happened when walnuts were selected (Figure 5f), since several values were above the ±20%.

In conclusion, as it can be seen in Figure 5, the best results were obtained when peanuts were used as representative matrix (Figure 5d) since all the values were below ±20%.

Recovery was evaluated at three concentration levels (0.5 (1.0 for α-zearalenol and zearalenone), 10 and 25 μg/kg) spiking blank samples of extra virgin olive oil in Publication II and peanuts in Publication III. Recoveries were between 88% and 119% in Publication II and between 74% and 116% in Publication III. Precision was estimated calculating relative standard deviation (RSD) of five samples analyzed the same day for intra-day precision and in five different days for inter-day precision, obtaining values between 1-14% and 1-11%, respectively, for Publication II, and between 3-19% and 4-19%, respectively, for Publication III.

Finally, limits of quantification (LOQs) were calculated as the lowest concentration that has been validated with acceptable accuracy by applying the complete analytical method and identification criteria. LOQ were established as 0.5 μg/kg for aflatoxins and 1.0 μg/kg for α-zearalenol and zearalenone.

3.1.4 Analysis of samples

To test de applicability of the developed methods, different vegetable oils for Publication II, and different kind of nuts for Publication III were analyzed.

An internal quality control was applied with each batch of samples. This included a blank sample to verify the absence of interferents, a calibration curve using an extract from a blank sample of extra virgin olive oil or peanuts, from 0.5 to 250 μg/kg, to appraise the sensitivity and quantify the samples, and blank samples of extra virgin olive oil or peanuts

spiked at 0.5 (1.0 for α-zearalenol and zearalenone), 10 and 25 μg/kg to evaluate the efficiency of the extraction method.

a) Vegetable oils

The developed method was applied to a total of 194 samples of vegetable oils including four types of olive oil: 33 samples of extra virgin olive oil, 35 samples of olive oil, 31 samples of lampante olive oil; two types of pomace oils: 15 samples of olive pomace oil and 28 samples of crude olive pomace oil; two types of sunflower oil: 34 samples of sunflower oil and 3 samples of crude sunflower oil; and finally 3 samples of soy oil and 1 sample of corn oil.

The results are summarized in Table 5. They show that among the aflatoxins, aflatoxin G1 was only found in samples of extra virgin olive oil at a maximum concentration of 1.9 μg/kg. On the other hand, aflatoxin G2 was detected in the majority of the samples of crude olive pomace oil (23 out of 28) at a concentration up to 6.8 μg/kg. Moreover, aflatoxin G2 was found in all samples of crude sunflower oil at concentration lower than 2.0 μg/kg as well as in one sample of refined oil at 1.1 μg/kg.

Zearalenone was detected in 7 out of 10 types of oil, and in most samples of olive oil, lampante olive oil and refined olive oil. In the case of olive oil, concentrations above the LOQ were found in 18 out of 35 samples at concentrations up to 21.0 μg/kg. Furthermore, it was detected in 18 out of 31 samples of lampante olive oil analyzed, which means almost 60% of them, at a maximum concentration of 25.6 μg/kg, showing the extracted ion chromatogram in Figure 6a. Figure 6b shows the chromatogram of the same sample contaminated with 6.8 μg/kg of aflatoxin G2. This compound was present in 8 out of 11 samples of refined olive oil at a maximum concentration of 20.2 μg/kg. Furthermore, aflatoxin G2 was detected in one sample of extra virgin olive oil, one sample of olive pomace oil, two samples of crude pomace oil and one sample of sunflower oil.

Table 5: Concentration range (µg/kg) of mycotoxins detected in the different types of edible oils analyzed.[a]

Matrix	AFB1	AFB2	AFG1	AFG2	α-ZOL	ZEA
Olive oil	<LOQ	<LOQ	0.8-1.9 (18%)	<LOQ	<LOQ	1.1-21.1 (51%)
Extra virgin olive oil	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	1.3 (3%)
Lampante olive oil	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.6-25.6 (55%)
Refined olive oil	<LOQ	<LOQ	1.1 (9%)	<LOQ	<LOQ	0.7-20.2 (73%)
Olive pomace oil	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.7 (7%)
Crude olive pomace oil	<LOQ	<LOQ	1.4-6.8 (82%)	<LOQ	<LOQ	0.6 (7%)
Sunflower oil	<LOQ	0.7 (3%)	<LOQ	<LOQ	<LOQ	2.0 (3%)
Crude sunflower oil	<LOQ	<LOQ	0.5-2.0 (100%)	<LOQ	<LOQ	<LOQ
Soy oil	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Corn oil	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

[a] Abbreviations: AFB1: Aflatoxin B1; AFB2: Aflatoxin B2; AFG1: Aflatoxin G1; AFG2: Aflatoxin G2; LOQ: Limit of quantification; α-ZOL: α-Zearalenol; ZEA: Zearalenone; [b] Percentages of positive samples are given in brackets.

Finally, aflatoxin B1 and α-zearalenol were not detected in any of the samples analyzed while aflatoxin B2 was detected in one sample of sunflower oil at 0.7 µg/kg.

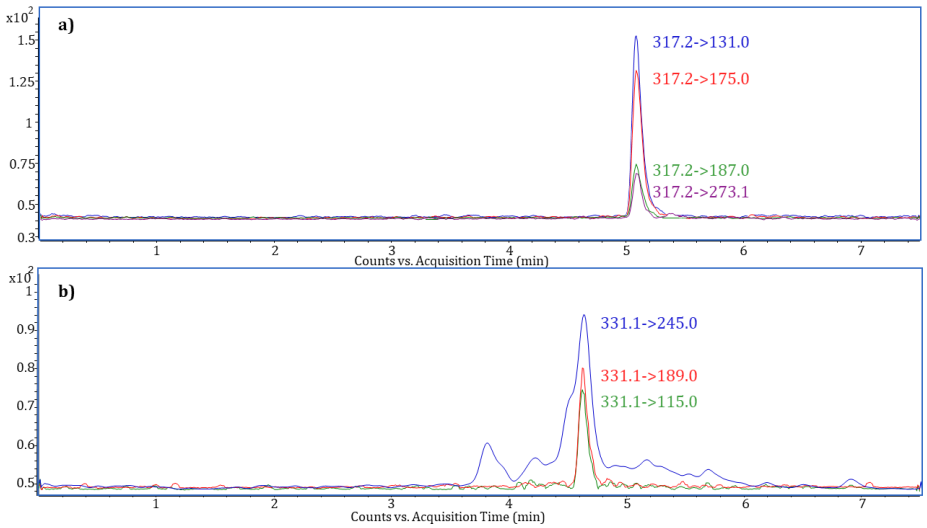

Figure 6. (a) Extracted ion chromatogram of the lampante olive oil sample contaminated with 25.6 µg/kg of zearalenone; (b) Extracted ion chromatogram of the crude olive pomace oil sample contaminated with 6.8 µg/kg of aflatoxin G2.

b) Nuts

In Publication III, the method was applied to 36 samples of five kinds of nuts including 7 samples of almonds, 6 samples of nuts, 10 samples of peanuts, 6 samples of pistachios and 7 samples of walnuts.

The most detected mycotoxin was aflatoxin G2 and it was found in 13 out of 36 samples analyzed, at concentrations ranging from 0.9 µg/kg to 6.3 µg/kg. The chromatogram of the sample of pistachio contaminated with 0.9 µg/kg is showed in Figure 7a, while the selected product ions of aflatoxin G2 in that sample are showed in Figure 7b. It is remarkable that this aflatoxin was detected in all the samples of almond and pistachio, and its concentration varied depending on how the pistachios were treated (i.e. roasted). For example, the lowest concentration, 0.9 µg/kg, was detected in an organic sample, while the highest concentration, 6.3 µg/kg, was found in a sample from the street market. Besides, almonds contained higher concentrations (2.8-4.4 µg/kg) when they were not submitted to

any cooking processes, while fried almonds had lower levels of this aflatoxin (1.4-1.8 µg/kg).

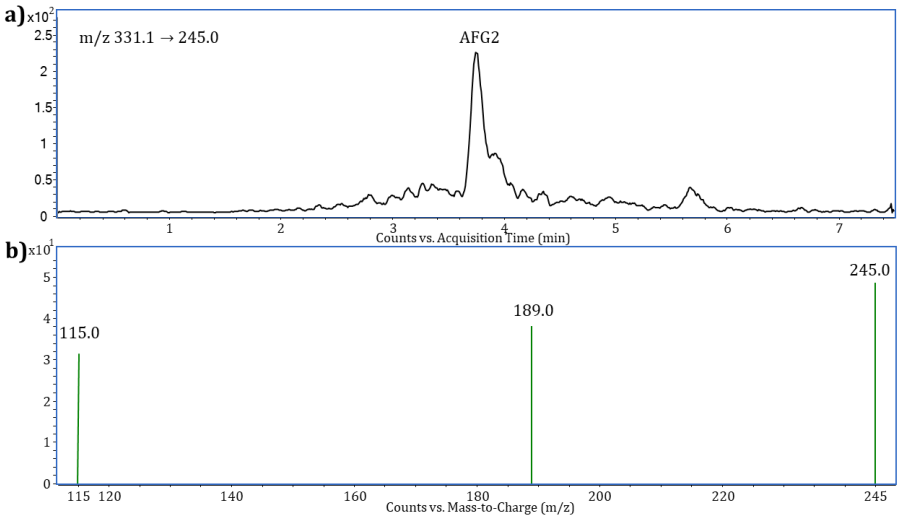

Figure 7. (a) Extracted ion chromatogram of the pistachio sample contaminated with 0.9 µg/kg of aflatoxin G2; (b) Selected product ions of AFG2 in the same sample. Abreviations: AFG2: Aflatoxin G2.

One sample of pistachios that was stored in conditions of high humidity for three months was contaminated at 1.2 µg/kg of aflatoxin G1.

Finally, aflatoxins B1 and B2, α-zearalenol and zearalenone were not detected above the LOQ in any of the samples analyzed.

3.2 Determination of 3-MCPD esters and glycidyl esters in vegetable oils and pastries by UHPLC-MS/MS

3.2.1 Separation and detection by liquid chromatography coupled to QqQ mass detector

Regarding the MS conditions, individual solutions of the 3-MCPD esters and glycidyl esters at 10 mg/L were used. For full MS scan, ESI+ mode was utilized for ionization, applying fragmentor voltages from 60 to

360 V and collision energies from 5 to 50 eV to achieve optimum conditions of precursor and product ions respectively.

Some of the most characteristic transitions are represented in Figure 8. In Figure 8a it can be seen a transition that is usually repeated, *m/z* 109.0. It appears as they are the seven closest carbons to the 3-MCPD or glycidol. In addition, in Figure 8b it can be observed a very common transition, which is the fatty acid without the carboxylic acid, in this case for 1-oleoyl-3-MCPD, *m/z* 375.3 → *m/z* 265.2. Finally, the transition of 1,2-dioleoyl-3-MCPD, *m/z* 656.6 → *m/z* 357.3, in Figure 8c, represents the loss of one of the esters of the diester, while the other ester remains bonded to the 3-MCPD.

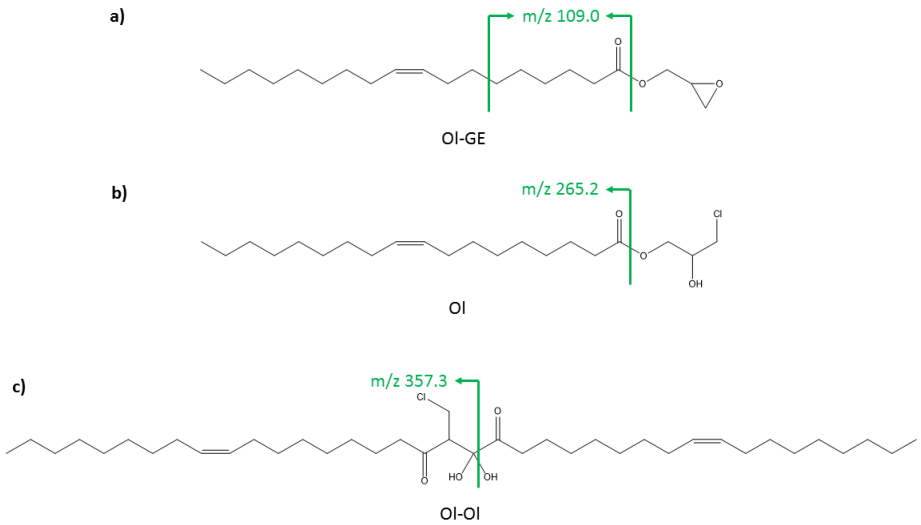

Figure 8. Characteristic fragmentations of the 3-monochloropropanediol (3-MCPD) esters and glycidyl esters included in the study. Transitions are indicated in green. Ol: 1-Oleoyl-3-chloropropanediol; Ol-GE: Glycidyl oleate; Ol-Ol: 1,2-Dioleoyl-3-chloropropanediol;

In Publication IV, the chromatographic conditions were optimized to reach proper peak shapes and separation.

Firstly, methanol:water (92:8, *v/v*) and isopropanol:water (98:2, *v/v*) were tested as mobile phases A and B respectively, but unfortunately, a

proper separation was not reached. Therefore, mobile phases were modified using water with 2 mM ammonium formate and 0.05% (*v/v*) of formic acid as phase A and acetonitrile with 2 mM ammonium formate and 0.05% (*v/v*) of formic acid as phase B, and keeping a constant percentage of 2% A. It was seen that a higher percentage of water in phase A was needed since all the compounds appeared at the beginning of the chromatogram. Thus, the percentage of water was increased until 10% in mobile phase A, so the final composition of mobile phase A was: methanol:water (90:10, *v/v*), and B: isopropanol:water (98:2, *v/v*). A better separation of the compounds was reached using this mobile phase.

Furthermore, the minimum percentage of phase A in the gradient profile was tested, trying 10, 20, 25 and 30%, and the best results, in terms of separation, were obtained using a minimum of 30% of mobile phase A.

In conclusion, the mobile phase was composed of methanol:water (90:10, *v/v*) with 2 mM ammonium formate and 0.05% (*v/v*) of formic acid; and solvent B was isopropanol:water (98:2, *v/v*) with 2 mM ammonium formate and 0.05% (*v/v*) of formic acid. Elution gradient is shown in Figure 9.

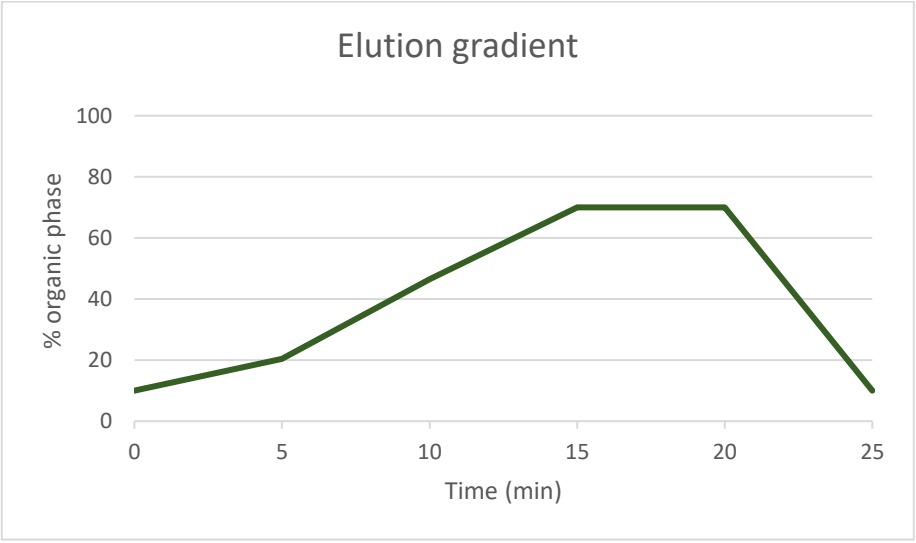

Figure 9. Elution gradient used in Publication IV.

Finally, the injection volume was also appraised to increase the sensitivity, testing 5, 10 and 20 µL, obtaining that injecting volumes higher than 5 µL did not improve peak shapes and could provoke column breakthrough.

Chromatographic conditions are summarized in Table 6.

Table 6: Chromatographic conditions used for the separation of 3-MCPD esters and glycidyl esters.[a]

LC Parameter	Conditions (Publication IV)
Aqueous phase	Methanol:water (90:10, *v/v*) with 2 mM ammonium formate and 0.05% (*v/v*) of formic acid
Organic phase	Isopropanol:water (98:2, *v/v*) with 2 mM ammonium formate and 0.05% (*v/v*) of formic acid
Column	Zorbax plus C18 (100 × 2.1 mm; 1.8 µm)
Column temperature	25 °C
Injection volume	5 µL
Flow rate	0.2 mL/min
Time of analysis	25 min
Elution mode	Gradient

[a] Abbreviations: 3-MCPD: 3-Monochloropropanediol; C18: Octadecasilane; LC: Liquid chromatography.

The chromatographic separation is showed in Figure 10. As it can be seen, a very good separation of the compounds was achieved taking into account that 26 similar esters were analyzed.

The final conditions such as retention time windows, internal standard used for quantification and precursor and product ions *m/z* are summarized in Table 7.

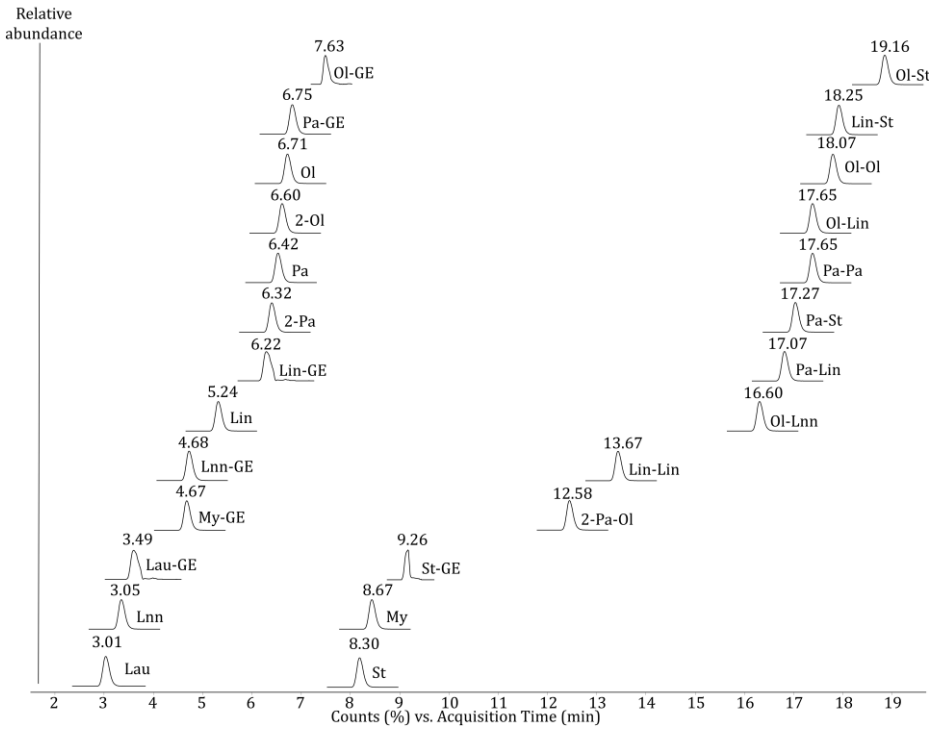

Figure 10. Extracted ion chromatogram obtained from standard solution 3-monochloropropanediol (3-MCPD) esters and glycidyl esters at 0.05 mg/L. 2-Ol: 2-Oleoyl-3-chloropropanediol; 2-Pa: 2-Palmitoyl-3-chloropropanediol; 2-Pa-Ol: 2-Palmitoyl-1-oleoyl-3-chloropropanediol; Lau: 1-Lauroyl-3-chloropropanediol; Lau-GE: Glycidyl laurate; Lau-GEd5: Glycidyl laurate-d5; Lin: 1-Linoleoyl-3-chloropropanediol; Lin-GE: Glycidyl linoleate; Lin-Lin: 1,2-Dilinoleoyl-3-chloropropanediol; Lin-St: 1-Linoleoyl-2-stearoyl-3-chloropropanediol; Lnn: 1-Linolenoyl-3-chloropropanediol; Lnn-GE: Glycidyl linolenate; My: 1-Myristoyl-3-chloropropanediol; My-GE: Glycidyl myristate; Ol: 1-Oleoyl-3-chloropropanediol; Old5: 1-Oleoyl-3-chloropropanediol-d5; Ol-GE: Glycidyl oleate; Ol-GEd5: Glycidyl oleate-d5; Ol-Lin: 1-Oleoyl-2-linoleoyl-3-chloropropanediol; Ol-Lnn: 1-Oleoyl-2-linolenoyl-3-chloropropanediol; Ol-Ol: 1,2-Dioleoyl-3-chloropropanediol; Ol-Old5: 1,2-Dioleoyl-3-chloropropanediol-d5; Ol-St: 1-Oleoyl-2-stearoyl-3-chloropropanediol; Pa: 1-Palmitoyl-3-chloropropanediol; Pa-GE: Glycidyl palmitate; Pa-Lin: 1-Palmitoyl-2-linoleoyl-3-chloropropanediol; Pa-Pa: 1,2-Bis-palmitoyl-3-chloropropanediol; Pa-St: 1-Palmitoyl-2-stearoyl-3-chloropropanediol; St: 1-Stearoyl-3-chloropropanediol; St-GE: Glycidyl stearate.

Table 7: UHPLC-MS/MS-QqQ parameters for the target compounds and internal standards used for quantification.[a]

Compound	ISQ	RTW (min)	Precursor ion (*m/z*)	Product ion (*m/z*)			
Lau	Lau-GEd5	2.96-3.06	293.2 (135)[b]	57.1 (25)[c]	71.1 (15)	108.8 (10)	**183.0[d] (5)**
Lnn	Ol-GEd5	3.00-3.10	371.2 (160)	261.1 (15)	267.0 (15)	**354.9 (10)**	
Lin	Ol-Old5	5.19-5.29	390.2 (105)	244.9 (5)	**263.0 (10)**	371.7 (5)	373.1 (5)
2-Pa	Ol-Old5	6.27-6.37	366.2 (110)	108.8 (20)	122.8 (20)	**239.1 (10)**	349.2 (5)
Pa	Ol-GEd5	6.37-6.47	349.2 (75)	109.2 (15)	**239.4 (5)**		
2-Ol	Old5	6.55-6.65	375.3 (135)	135.1 (17)	181.1 (13)	247.2 (5)	**265.2 (5)**
Ol	Old5	6.66-6.76	375.3 (145)	247.3 (9)	**265.2 (9)**		
St	Lau-GEd5	8.25-8.35	377.3 (155)	109.1 (10)	**267.2 (5)**		
My	Ol-Old5	8.62-8.72	321.2 (150)	57.1 (25)	71.2 (15)	**94.9 (30)**	211.2 (5)
2-Pa-Ol	Ol-GEd5	12.53-12.63	613.5 (60)	220.9 (9)	**357.2 (13)**		
Lin-Lin	Ol-Old5	13.62-13.72	651.2 (275)	221.1 (10)	**355.1 (15)**	429.2 (5)	
Ol-Lnn	Lau-GEd5	16.55-16.65	652.2 (195)	261.3 (20)	**353.3 (20)**	357.2 (20)	635.4 (10)
Pa-Lin	Old5	17.02-17.12	628.3 (150)	223.1 (20)	267.0 (40)	**355.2 (21)**	371.1 (10)
Pa-St	-	17.22-17.32	563.0 (315)	73.2 (60)	147.1 (35)	475.1 (20)	**563.0 (5)**
Pa-Pa	Old5	17.60-17.70	604.5 (60)	239.3 (20)	**331.1 (20)**	551.6 (20)	
Ol-Lin	Old5	17.60-17.70	654.6 (190)	263.2 (20)	**355.3 (20)**	357.3 (15)	
Ol-Ol	Ol-Old5	18.02-18.12	656.6 (75)	265.3 (20)	**357.3 (20)**		
Lin-St	Old5	18.20-18.30	656.6 (90)	263.2 (20)	355.2 (20)	**359.3 (15)**	
Ol-St	Old5	19.11-19.21	658.6 (200)	267.2 (25)	357.4 (25)	**359.4 (20)**	

Table 7 (continued)

Compound	ISQ	RTW (min)	Precursor ion (*m/z*)	Product ion (*m/z*)			
Lau-GE	Lau-GEd5	3.44-3.54	257.2 (118)	103.1 (13)	109.1 (13)	**183.2 (9)**	201.2 (13)
My-GE	Lau-GEd5	4.62-4.72	285.2 (112)	109.2 (13)	123.1 (13)	211.2 (9)	**229.2 (13)**
Lnn-GE	Lau-GEd5	4.63-4.73	335.3 (155)	105.1 (25)	107.1 (21)	**109.1 (17)**	121.1 (17)
Lin-GE	Ol-Old5	6.17-6.27	337.3 (155)	107.1 (17)	**109.1 (17)**	121.1 (17)	123.1 (17)
Pa-GE	Ol-Old5	6.70-6.80	313.3 (155)	109.1 (17)	123.1 (17)	239.3 (9)	**257.2 (13)**
Ol-GE	Old5	7.58-7.68	339.3 (140)	109.1 (17)	111.1 (17)	**121.1 (21)**	135.1 (17)
St-GE	Old5	9.21-9.31	341.3 (90)	**57.1 (25)**	71.1 (20)	85.1 (20)	285.1 (10)
Lau-GEd5		3.44-3.54	262.2 (130)	109.1 (13)	123.1 (13)	**183.1 (9)**	202.2 (13)
Old5		6.56-6.66	380.3 (70)	246.7 (9)	**265.3 (9)**		
Ol-GEd5		7.58-7.68	341.1 (360)	73.1 (25)	324.9 (20)	**341.0 (5)**	
Ol-Old5		18.02-18.12	661.6 (90)	265.3 (18)	**362.4 (18)**		

[a] Abbreviations of 3-MCPD esters and glycidyl esters are given in Figure 10; ISQ: Internal standard of quantification; RTW: Retention time window. [b] Fragmentor voltage (V) is given in brackets; [c] Collision energy (eV) is given in brackets; [d] Transition in bold was used for quantification.

3.2.2 Extraction method

The extraction method of glycidyl and 3-MCPD esters from vegetable oils was optimized. For it, extra virgin olive oil blank samples were spiked at 0.2 mg/kg. Firstly, an extraction method based on LLE using *tert*-butyl methyl ether:ethyl acetate (80:20, *v/v*) and two d-SPE steps with Si-SAX and PSA, and Z-Sep+ and PSA respectively, was tested, but this method gave recoveries below 37% or above 184%, as it can be seen in Table 8.

Table 8: Recovery values obtained using the existing extraction method in extra virgin olive oil spiked at 100 µg/kg.[a]

Compound	Recovery (%)	Compound	Recovery (%)
Lau	20	Pa-St	2733
Lnn	2408	Pa-Pa	7
Lin	732	Ol-Lin	17
2-Pa	316	Ol-Ol	5
Pa	227	Lin-St	3640
2-Ol	19	Ol-St	5
Ol	16	Lau-GE	37
St	82	My-GE	12
My	24	Lnn-GE	30
2-Pa-Ol	2575	Lin-GE	184
Lin-Lin	7825	Pa-GE	31
Ol-Lnn	9	Ol-GE	31
Pa-Lin	8	St-GE	31

[a] Abbreviations of 3-MCPD esters and glycidyl esters are given in Figure 10.

Then, an optimization of the extraction solvent was carried out trying ethyl acetate and separately a mixture of *tert*-butyl methyl ether:ethyl acetate (80:20, *v/v*), finding the best results when this mixture was used. In the same experiment, 300 mg of five different cleaning sorbents were tested (C18, PSA, florisil, GCB, Si-SAX and Z-Sep+). Regarding the cleaning salts employed, bad results were obtained using the cited sorbents, so some trials were accomplished to get proper recoveries.

Then, several sorbent mixtures were tested such as Mixture 1: 98% of PSA and 2% of Si-SAX; and Mixture 2: 75% of Z-Sep+ and 25% of PSA. These mixtures were added to the extract in two ways: sequentially and together. On the one hand, the sequential experiment was performed as follows: once the sample was prepared (with internal standard, flushed and homogenized), 3 mL of this solution were transferred to a 15 mL tube containing 100 or 200 of Mixture 1 to carry out the first clean-up. This tube was capped, mixed in a rotatory agitator for 10 minutes and centrifuged at 5000 rpm (4136×*g*) for 10 minutes. Then, the procedure was repeated with 100, 200 or 300 mg of Mixture 2. Results are shown in Table 9. On the other hand, both Mixtures were added together, testing 100 and 200 mg of Mixture 1 plus 100, 200 and 300 mg of Mixture 2. In this case, only one extraction was needed. Results of these trials are shown in Table 9.

As it can be seen in Table 9, when Mixture 1 and 2 were added sequentially, low recovery values were obtained for 3-MCPD monoesters and glycidyl esters. For example, when 100 mg of Mixture 1 and 2 were added, recoveries were below 36 and 49% for 3-MCPD monoesters and glycidyl esters respectively, while most of the recoveries of the diesters were almost within acceptable terms (52-130%). Likewise, when 100 mg of Mixture 1 and 200 mg of Mixture 2 were added, the recoveries for 3-MCPD monoesters and glycidyl esters were up to 28 and 50% respectively.

Moreover, in Table 9 it can be seen that when 200 mg of each Mixture, 1 and 2, were added together, recoveries below 34% for 3-MCPD monoesters and 63% for glycidyl esters were obtained, and recoveries below 10% were got for 1-linolenoyl-3-chloropropanediol, 2-palmitoyl-3-chloropropanediol, 2-palmitoyl-1-oleoyl-3-chloropropanediol, 1,2-dilinoleoyl-3-chloropropanediol, 1-Oleoyl-2-linoleoyl-3-chloropropanediol and glycidyl stearate. The best results were obtained when 100 mg of Mixture 1 and 200 mg of Mixture 2 were added together,

Table 9: Recovery values obtained using different amounts of cleaning salts together.[a]

Compound	Recovery (%)							
	100 mg M1 + 100 mg M2 sequential	100 mg M1 + 200 mg M2 sequential	100 mg M1 + 300 mg M2 sequential	200 mg M1 + 200 mg M2 sequential	100 mg M1 + 100 mg M2 together	100 mg M1 + 200 mg M2 together	100 mg M1 + 300 mg M2 together	200 mg M1 + 200 mg M2 together
Lau	24	28	44	32	35	106	27	34
Lnn	36	7	95	74	80	113	82	2
Lin	25	14	15	40	72	105	123	15
2-Pa	13	14	17	27	29	101	25	8
Pa	20	19	33	28	43	104	29	22
2-Ol	21	17	26	27	31	104	25	14
Ol	22	17	26	21	24	98	24	15
St	25	18	51	44	28	120	37	10
My	32	24	24	46	35	99	32	21
2-Pa-Ol	41	10	114	152	76	105	76	8
Lin-Lin	21	15	62	61	69	113	92	10
Ol-Lnn	1	2	62	126	187	82	108	97
Pa-Lin	101	85	109	97	145	93	93	70
Pa-St	52	56	88	45	74	96	63	58
Pa-Pa	85	170	111	83	101	96	119	98
Ol-Lin	130	101	67	85	11.7	94	46	8
Ol-Ol	82	77	137	82	97	97	97	113
Lin-St	99	86	123	114	111	112	118	62
Ol-St	104	94	107	103	119	118	121	68

Table 9 (continued)

Compound	Recovery (%)							
	100 mg M1 + 100 mg M2 sequential	100 mg M1 + 200 mg M2 sequential	100 mg M1 + 300 mg M2 sequential	200 mg M1 + 200 mg M2 sequential	100 mg M1 + 100 mg M2 together	100 mg M1 + 200 mg M2 together	100 mg M1 + 300 mg M2 together	200 mg M1 + 200 mg M2 together
Lau-GE	42	28	42	42	46	96	46	25
My-GE	36	44	55	49	77	102	64	22
Lnn-GE	49	50	91	78	84	99	63	63
Lin-GE	46	25	40	51	44	97	47	20
Pa-GE	41	26	31	54	74	102	33	16
Ol-GE	31	22	32	40	35	107	41	15
St-GE	43	22	32	32	24	112	68	9

[a] Abbreviations of 3-MCPD esters and glycidyl esters are given in Figure 10; M1: Mixture 1: 98% of PSA and 2% of Si-SAX; M2: Mixture 2: 75% of Z-Sep+ and 25% of PSA.

so, the mixture used in further experiments was: 2 mg of Si-SAX, 148 mg of PSA and 150 mg of Z-Sep+.

Once the extraction method was optimized for vegetable oils, matrices like margarine, biscuits and croissants were tested giving suitable results. In the case of croissants, they were dried in the oven for 12 h at 105 °C. Furthermore, both biscuits and croissants needed to be crushed and homogenized before weighing them out. Moreover, the process of weighing of these three matrices was slightly different since it could not be done into a volumetric flask, and it was performed into a 15 mL tube. Then 50 µL of the internal standard solution at 10 mg/kg and 5 mL of *tert*-butyl methyl ether:ethyl acetate (80:20, *v/v*) were added. From this point, the process was the same to the used for oils. A scheme of the final extraction method of 3-MCPD esters and glycidyl esters in all the matrices mentioned in Publication IV is shown in Figure 11.

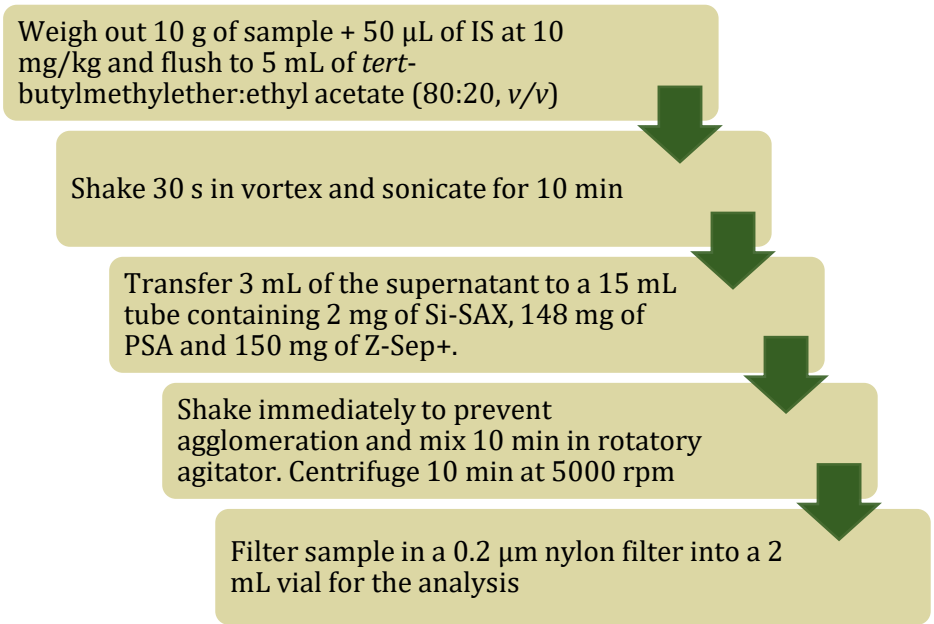

Figure 11. Scheme of the extraction method of 3-MCPD esters and glycidyl esters in vegetable oils, margarine, biscuits and croissants in Publication Abbreviations: IS: Internal standard; PSA: Primary secondary amine; SAX: Strong anion exchange.

3.2.3 Validation of the method

The method was validated in four matrices such as vegetable oils, margarine, biscuits and croissants and the validation was accomplished using the SANTE guidelines. Thus, the identification and quantification of the target compounds is demonstrated and it can be applied to samples in routine analyses. Parameters evaluated are shown in Table 10. Different calibration curves at 0.01, 0.02, 0.05, 0.1, 0.2 and 0.5 mg/kg were prepared for each matrix to check linearity.

Thus, the evaluation of linearity was carried out and as it can be observed in Table 10, it was above 0.9899, 0.9895, 0.9924 and 0.9911 for vegetable oils, margarine, biscuits and croissants respectively.

Table 10: Parameters evaluated for the validation of the method for 3-MCPD and glycidyl esters in Publication IV.[a]

Parameter	Vegetable oils	Margarine	Biscuits	Croissants
Determination coefficient (R^2)	0.9899-0.9995	0.9895-0.9996	0.9924-0.9990	0.9911-0.9994
LOQ (mg/kg)	0.01 (0.02 for Pa and Lin-St, 0.05 for Lnn-GE and 0.1 for Lin)			
Recovery (%)[b]	72-120	72-117	71-116	71-119
Intra-day precision, RSD (%)	5-20	4-20	2-20	4-19
Inter-day precision, RSD (%)	7-20	7-19	4-20	5-20

[a] Abbreviations: Lin: 1-Linoleoyl-3-chloropropanediol; Lin-St: 1-Linoleoyl-2-stearoyl-3-chloropropanediol; Lnn-GE: Glycidyl linolenate; LOQ: Limit of quantification; Pa: 1-Palmitoyl-3-chloropropanediol; RSD: Relative standard deviation. [b] Note: Recoveries and RSDs were calculated based on n = 5.

Then, an evaluation of the internal standard used for the quantification of each compound was accomplished. This was performed assessing the recovery of each compound with each internal standard. The choice of each standard is shown in Table 7. Calibration curves appearing

in Table 10 were calculated using the ratio of the chromatographic peak area for each analyte to the corresponding internal standard. However, for 1-palmitoyl-2-stearoyl-3-chloropropanediol none of the internal standards used were suitable, so this compound was directly quantified by its corresponding peak area.

Recovery, intra- and inter-day precisions were calculated at LOQ and 0.2 mg/kg. As it can be seen in Table 10, the recoveries ranged 72-120%, 72-117%, 71-116% and 71-119% for vegetable oils, margarine, biscuits and croissants respectively.

Intra-day precision was below 20% for vegetable oils, margarine and biscuits, and below 19% for croissants while inter-day precision was below 20% for vegetable oils, biscuits and croissants, and below 19% for margarine for all the compounds studied in Publication IV.

The LOQ were set as the lowest concentration that was validated with acceptable accuracy by applying the complete analytical method and identification criteria. Therefore, they were set at 0.01 mg/kg for all the 3-MCPD and glycidyl esters, except 1-palmitoyl-3-chloropropanediol and 1-linoleoyl-2-stearoyl-3-chloropropanediol, which were set at 0.02 mg/kg, glycidyl linolenate at 0.05 mg/kg and 1-linoleoyl-3-chloropropanediol at 0.1 mg/kg in all the matrices.

The current legislation establishes the MRL as the sum of 3-MCPD esters and glycidyl esters, expressed as free 3-MCPD and glycidol respectively. Thus, Equation 1 was used for the calculation of the result of each compound as free 3-MCPD, while Equation 2 was used for the calculation of the result of each compound as free glycidol. Once the individual results were obtained, the sum of 3-MCPD esters and glycidyl esters were performed. Thus, the LOQ for 3-MCPD esters, expressed as free 3-MCPD was 0.08 mg/kg while the LOQ for glycidyl esters, expressed as free glycidol was 0.03 mg/kg.

$$3MCPD = \sum \frac{3MCPD\ ester\ result \times Molecular\ weight\ of\ free\ 3MCPD}{Molecular\ weigh\ of\ 3MCPD\ ester}$$

Equation 1

$$Glycidol = \sum \frac{Glycidyl\ ester\ result \times Molecular\ weight\ of\ free\ glycidol}{Molecular\ weigh\ of\ glycidyl\ ester}$$

Equation 2

3.2.4 Analysis of samples

The developed method was applied to the analysis of different types of vegetable oils and other fatty samples. For that purpose, an internal quality control was carried out with each batch of samples. This consisted on a blank sample to verify the absence of interferents, a calibration curve, using an extract from a blank sample of extra virgin olive oil, from 0.01 to 0.5 mg/kg, was used to appraise the sensitivity and quantify the samples, and blank samples of extra virgin olive oil spiked at 0.01 and 0.2 mg/kg were injected to evaluate the efficiency of the extraction method.

The developed method was used to analyze 24 samples belonging to four types of olive oil: 2 samples of extra virgin olive oil, 1 sample of virgin olive oil, 6 samples of olive oil; two types of pomace oils: 2 samples of refined olive pomace oil and 1 sample of olive pomace oil; 2 samples of sunflower oil, 1 sample of soy oil, 1 sample of corn oil, 1 sample of peanut oil, 1 sample of grapeseed oil, as well as one sample or margarine, one sample of biscuits and 2 samples of croissants.

Results are shown in Table 11. As it can be seen, the most detected esters are those that came from oleic, linoleic and linolenic acids, the fatty acids that are present in oils at higher concentrations.

Table 11: Results of 3-monochloropropanediol esters and glycidyl esters obtained in analyzed samples (mg/kg).[a]

Compound	EVOOs	Olive oils	ROOs	OPO	ROPOs	Vegetable oils	Margarine	Pastries
Lau	<LOQ-0.34 (2/3)[b]	<LOQ-0.22 (3/6)	0.22-0.23 (2/2)	<LOQ	0.24-0.26 (2/2)	0.02-0.62 (6/6)	<LOQ	<LOQ-0.05 (1/3)
Lnn	<LOQ-0.03 (2/3)	0.01-0.68 (6/6)	0.01 (2/2)	0.05	<LOQ-0.01 (1/2)	<LOQ-0.58 (5/6)	0.04	<LOQ-0.33 (2/3)
Lin	<LOQ-0.23 (1/3)	<LOQ-0.20 (1/6)	<LOQ-0.11 (1/2)	<LOQ	<LOQ-0.15 (1/2)	<LOQ-1.30 (3/6)	0.12	<LOQ
2-Pa	<LOQ	<LOQ-0.23 (2/6)	<LOQ	<LOQ	<LOQ-0.04 (1/2)	<LOQ-0.02 (1/6)	<LOQ	<LOQ
Pa	<LOQ	<LOQ-0.53 (3/6)	<LOQ	<LOQ	<LOQ-0.09 <LOQ	<LOQ-0.30 (3/6)	<LOQ	<LOQ-0.07 (2/3)
2-Ol	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Ol	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
St	<LOQ	<LOQ-0.37 (1/6)	<LOQ	<LOQ	<LOQ-0.02 (1/2)	<LOQ	<LOQ	<LOQ-0.01 (1/3)
My	0.02-0.07 (3/3)	<LOQ-0.64 (4/6)	0.01-0.03 (2/2)	0.20	<LOQ-0.02 (1/2)	0.08-5.74 (6/6)	0.02	<LOQ
2-Pa-Ol	<LOQ	0.01-1.03 (6/6)	<LOQ	0.05	<LOQ	<LOQ-0.30 (5/6)	0.10	<LOQ-0.58 (2/3)
Lin-Lin	<LOQ	<LOQ-2.13 (3/6)	<LOQ-0.01 (1/2)	0.07	<LOQ	0.01-0.02 (4/6)	<LOQ	<LOQ
Ol-Lnn	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Pa-Lin	<LOQ	<LOQ-0.09 (1/6)	<LOQ	<LOQ	0.01-0.02 (2/2)	<LOQ	<LOQ	<LOQ
Pa-St	<LOQ-0.13 (2/3)	<LOQ-0.14 (2/6)	<LOQ-0.02 (1/2)	5.47	<LOQ	0.02 (1/6)	0.02	<LOQ-0.24 (2/3)

Table 11 (continued)

Compound	EVOOs	Olive oils	ROOs	OPO	ROPOs	Vegetable oils	Margarine	Pastries
Pa-Pa	<LOQ	<LOQ-0.02 (1/6)	<LOQ	0.01	<LOQ-0.02 (1/2)	0.05 (1/6)	0.02	<LOQ-0.13 (2/3)
Ol-Lin	<LOQ-0.04 (2/3)	<LOQ-2.69 (3/6)	0.04 (2/2)	0.03	0.04-0.06 (2/2)	0.02-0.08 (6/6)	<LOQ	0.01-0.11 (3/3)
Ol-Ol	<LOQ	<LOQ-0.10 (2/6)	0.02-0.18 (2/2)	0.26	0.02-0.10 (2/2)	<LOQ-0.10 (5/6)	<LOQ	<LOQ
Lin-St	<LOQ	<LOQ-0.76 (3/6)	<LOQ	<LOQ	<LOQ	<LOQ-0.23 (4/6)	<LOQ	<LOQ-0.14 (2/3)
Ol-St	<LOQ-0.05 (1/3)	<LOQ-0.04 (3/6)	0.01-0.03 (2/2)	0.02	0.20-0.31 (2/2)	0.01-0.02 (3/6)	<LOQ	<LOQ-0.06 (2/3)
Lau-GE	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ-0.07 (1/3)
My-GE	<LOQ-0.12 (2/3)	<LOQ-0.04 (3/6)	0.03-0.05 (2/2)	0.15	<LOQ-0.40 (1/2)	0.02-0.06 (3/6)	<LOQ	<LOQ-0.19 (2/3)
Lnn-GE	<LOQ-0.17 (2/3)	<LOQ-1.05 (4/6)	0.32-0.87 (2/2)	1.06	2.11-3.98 (2/2)	0.09-0.36 (3/6)	<LOQ	<LOQ
Lin-GE	<LOQ	<LOQ-0.74 (3/6)	0.04-0.37 (2/2)	0.28	0.03-0.04 (2/2)	0.30-19.24 (6/6)	0.03 ± 0.01	<LOQ-0.01 (1/3)
Pa-GE	<LOQ-0.04 (1/3)	<LOQ-0.29 (3/6)	<LOQ-0.05 (1/2)	<LOQ	<LOQ	0.02-1.84 (6/6)	0.01 ± 0.01	<LOQ-0.02 (1/3)
Ol-GE	<LOQ-0.03 (2/3)	0.05-2.77 (6/6)	1.03-1.62 (2/2)	0.43	0.37-1.56 (2/2)	0.15-11.73 (6/6)	0.06 ± 0.01	<LOQ-0.03 (2/3)
St-GE	<LOQ-0.17 (2/3)	<LOQ-0.78 (5/6)	0.30-0.36 (2/2)	0.09	0.20 (2/2)	0.09-1.17 (6/6)	<LOQ	<LOQ-0.06 (1/3)

[a] Abbreviations of 3-MCPD esters and glycidyl esters are given in Figure 10; EVOOs: Extra virgin olive oils; LOQ: Limit of Quantification; OPO: Olive pomace oil; ROOs: Refined olive oils; ROPOs: Refined olive pomace oils; [b] Number of positive samples out of the total samples analyzed are given in brackets.

As it was expected, samples of extra virgin and virgin olive oil did not provide any contamination since these categories are not submitted to any refinement process. However, certain amounts of some esters were detected, like for instance 0.17 mg/kg of glycidyl stearate in a sample of extra virgin olive oil. The chromatogram of this sample is shown in Figure 12a.

Usually, olive oils are not contaminated with 3-MCPD and glycidyl esters, but in some cases, some of them can be found. For instance, 1.03 mg/kg of 2-palmitoyl-1-oleoyl-3-chloropropanediol, 2.13 mg/kg of 1,2-dilinoleoyl-3-chloropropanediol and 2.69 mg/kg of 1-oleoyl-2-linoleoyl-3-chloropropanediol were found in one sample of olive oil. Also, other sample of olive oil was contaminated with 1.1 mg/kg of glycidyl linolenate and 2.77 mg/kg of glycidyl oleate.

The contamination of refined oils is more common. For example, the two samples of refined olive oil presented high concentrations of glycidyl oleate, 1.62 and 1.03 mg/kg, respectively. Moreover, the two samples of refined olive pomace oil contained 2-oleoyl-3-chloropropanediol at concentrations up to 2.20 and 1.41 mg/kg.

Sometimes low-quality oils are mixed with other vegetable oils to increase their quality. For instance, the olive pomace oil sample presented 5.47 mg/kg of 1-palmitoyl-2-stearoyl-3-chloropropanediol, which may be due to a contamination with palm oil. Furthermore, high levels of glycidyl linolenate were found in olive pomace oil and refined olive pomace oils, at 1.06, 2.02 and 3.98 mg/kg olive pomace oil and two samples of refined olive pomace oil, respectively. Additionally, one of the samples of olive pomace oil was contaminated with 1.56 mg/kg of glycidyl oleate.

Within the vegetable oils analyzed, glycidyl linoleate was detected at 19.24 mg/kg in a sample of soy oil (see Figure 12b) and at 17.63 mg/kg in a sample of sunflower oil, while 11.73 mg/kg of glycidyl oleate were found

in the same sample. The sample of corn oil was contaminated with 5.74 mg/kg of 1-myristoyl-3-chloropropanediol.

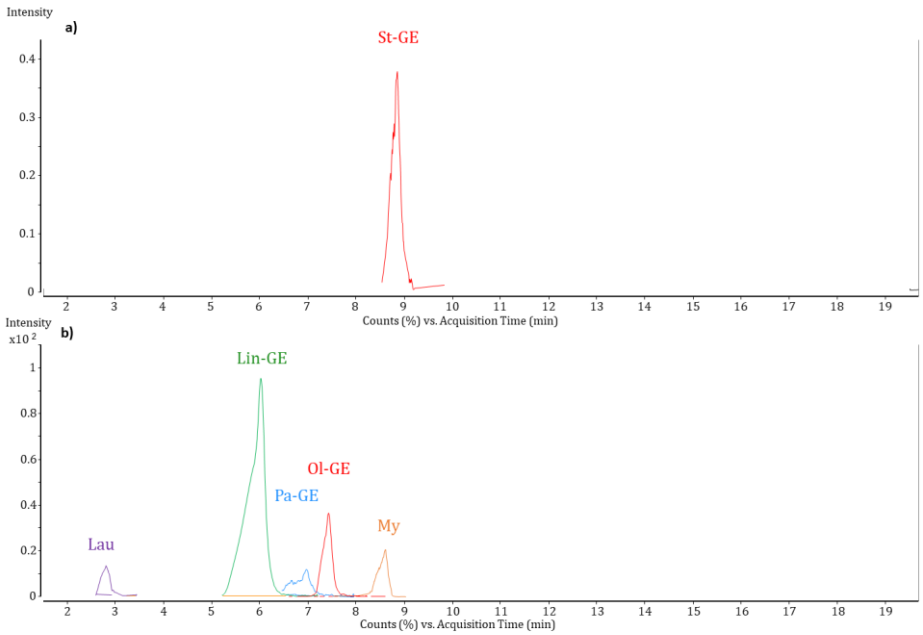

Figure 12. (a) Extracted ion chromatogram of an extra virgin olive oil sample contaminated with 0.17 mg/kg of St-GE and (b) Extracted ion chromatogram of a soy oil sample contaminated with 0.43 mg/kg of Lau, 19.24 mg/kg of Lin-GE, 1.84 mg/kg of Pa-GE, 11.73 mg/kg of Ol-GE and 0.32 mg/kg of My. Abbreviations: Lau: 1-Lauroyl-3-chloropropanediol; Lin: 1-Linoleoyl-3-chloropropanediol; Lin-GE: Glycidyl linoleate; My: 1-Myristoyl-3-chloropropanediol; Ol-GE: Glycidyl oleate; Pa-GE: Glycidyl palmitate; Pa-St: 1-Palmitoyl-2-stearoyl-3-chloropropanediol; St-GE: Glycidyl stearate.

All these results highlighted the necessity of direct analytical methods that analyze the esters individually rather than the sum of them, since knowing the individual ester present in an oil, the source of the contamination could be suggested.

As it can be observed in Table 11, in food samples, the contamination with 3-MCPD esters and glycidyl esters is lower because the percentage of fat is lower. However, 2-palmitoyl-1-oleoyl-3-chloropropanediol was

found at 0.58 mg/kg in a biscuit sample, while in both samples of croissants, 1-palmitoyl-2-stearoyl-3-chloropropanediol was detected at 0.24 and 0.13 mg/kg. The contamination of these compounds may suggest that these samples were elaborated with palm oil.

3.2.4.1 Conversion to free 3-MCPD and glycidol

In order to comply with the current legislation, the sum of 3-MCPD esters and glycidyl esters was calculated and the results are shown in Table 12.

Extra virgin and virgin olive oil samples presented low concentrations of 3-MCPD esters, between 0.04-0.14 mg/kg, and glycidyl esters between 0.05 and 0.07 mg/kg. These values are below the maximum permitted level of 1.25 and 1.00 mg/kg for the sum of 3-MCPD esters and glycidyl esters respectively.

Regarding olive oil samples, only one out of the six analyzed ones presented 3-MCPD esters concentrations of 1.72 mg/kg, while the other five samples were below 0.33 mg/kg and none of the samples exceeded the maximum concentration allowed by legislation, although one sample was close, with 0.95 mg/kg, being the rest of them below 0.35 mg/kg.

Refined oil samples did not exceed the allowed limits in any case, although the values of glycidyl esters were 0.51 and 0.60 mg/kg, almost twice the mean value of the samples of unrefined olive oil.

Generally, olive pomace oils and refined olive pomace oils presented higher concentrations than those obtained from olive oils, obtaining concentrations of 3-MCPD esters between 0.66 and 1.30 mg/kg and glycidyl esters between 0.45 and 1.29 mg/kg. Nevertheless, these values were below the legal limit since a maximum of 2.50 mg/kg of glycidyl esters is permitted for this type of oils.

Table 12: Concentrations of the sum of 3-MCPD and glycidyl esters expressed as free 3-MCPD and free glycidol detected in the different types of vegetable oils analyzed.[a]

Sample	Sum of 3-MCPD esters (calculated as free 3-MCPD) (mg/kg)	Sum of glycidyl esters (calculated as free glycidol) (mg/kg)
Extra virgin olive oil 1	0.14 ± 0.01[b]	0.06 ± 0.01
Extra virgin olive oil 2	0.14 ± 0.02 (<0.15)[c]	0.07 ± 0.01 (<0.15)
Virgin olive oil	0.04 ± 0.01 (<0.15)	0.05 ± 0.01 (<0.15)
Olive oil 1	0.22 ± 0.04	<LOQ
Olive oil 2	0.19 ± 0.02	0.09 ± 0.01
Olive oil 3	0.23 ± 0.03	0.35 ± 0.04
Olive oil 4	1.72 ± 0.29	0.18 ± 0.03
Olive oil 5	0.33 ± 0.06	0.27 ± 0.05
Olive oil 6	0.27 ± 0.03	0.95 ± 0.10
Refined olive oil 1	0.17 ± 0.02	0.60 ± 0.08
Refined olive oil 2	0.11 ± 0.02	0.51 ± 0.08
Olive pomace oil	1.30 ± 0.25 (1.20)	0.45 ± 0.09 (0.46)
Refined olive pomace oil 1	1.24 ± 0.25	0.60 ± 0.12
Refined olive pomace oil 2	0.66 ± 0.09	1.29 ± 0.18
Sunflower oil 1	0.26 ± 0.05	0.15 ± 0.03
Sunflower oil 2	1.06 ± 0.11	4.20 ± 0.62
Peanut oil	0.08 ± 0.01	1.22 ± 0.15
Grapeseed oil	0.41 ± 0.08	0.46 ± 0.09
Soy oil	1.38 ± 0.23	7.84 ± 1.33
Corn oil	2.52 ± 0.30	0.96 ± 0.12
Margarine	0.09 ± 0.02	0.03 ± 0.01
Biscuit	0.26 ± 0.03	<LOQ
Croissant 1	0.16 ± 0.02	0.08 ± 0.01
Croissant 2	0.10 ± 0.01	<LOQ

[a] Abbreviations: 3-MCPD: 3-Monochloropropanediol, LOQ: Limit of quantification; [b] Mean concentration ± standard deviation (n = 3); [c] Results analyzed by indirect method are given in brackets.

Extra virgin and virgin olive oil samples presented low concentrations of 3-MCPD esters, between 0.04-0.14 mg/kg, and glycidyl esters between

0.05 and 0.07 mg/kg. These values are below the maximum permitted level of 1.25 and 1.00 mg/kg for the sum of 3-MCPD esters and glycidyl esters respectively.

As it can be seen in Table 12, four out of the six vegetable oil samples were above the maximum limit of 1.00 mg/kg of glycidyl esters permitted. On the other hand, the most contaminated one with glycidyl esters was the sample of soy oil with 7.84 mg/kg. Likewise, the sample of corn oil presented 2.52 mg/kg of 3-MCPD esters, which is more than the double of the maximum allowed limit of 1.25 mg/kg.

Some of the samples were analyzed by an external laboratory with an indirect method. The results can be seen in Table 12 and as it can be observed, they were very similar, which reveals the robustness of the proposed direct method.

4. DEVELOPMENT AND VALIDATION OF ANALYTICAL METHODS FOR EXOGENOUS CONTAMINANTS

In this section, the research performed to develop methods for the analysis of exogenous contaminants is discussed. In this case, the methods developed for polar contaminants (phosphonic acid, fosetyl-Al, ethephon, chlorate and perchlorate) in vegetable oils and in nuts by HPLC-MS/MS (Publication V) and MOH in oils by GC-FID (Publication VI) are included.

4.1 Determination of polar contaminants in vegetable oils and nuts by HPLC-MS/MS

4.1.1 Separation and detection by liquid chromatography coupled to QqQ mass detector

To get proper MS conditions, solutions with the compounds individually prepared at 10 mg/L were injected. Firstly, full MS scan was applied, and all the compounds were ionized using ESI- mode. Fragmentor voltages, from 30 to 160 V, and collision energies, from 5 to 50 eV, were tested to optimize the best signal of each precursor and product ions.

As it can be observed in Figure 13 in the case of phosphonic acid, the first transition, *m/z* 81.0 → *m/z* 79.0, was due to the loss of both hydrogens of the hydroxyl groups, whereas the second transition, *m/z* 79.0 → *m/z* 63.1, was due to the loss of one oxygen. Regarding fosetyl, the first transition, *m/z* 109.0 → *m/z* 81.0, came from the loss of the ethyl group, while the second transition, *m/z* 81.0 → *m/z* 63.1, was due to the loss of the hydroxyl group plus the hydrogen bonded to the phosphorus, which is a whole molecule of water. Ethephon lost its chlorine for the first transition, *m/z* 143.0 → *m/z* 107.0, while the second one, *m/z* 107.0 → *m/z* 79.1, was the same as the first corresponding to fosetyl, i. e. the loss of the ethyl group.

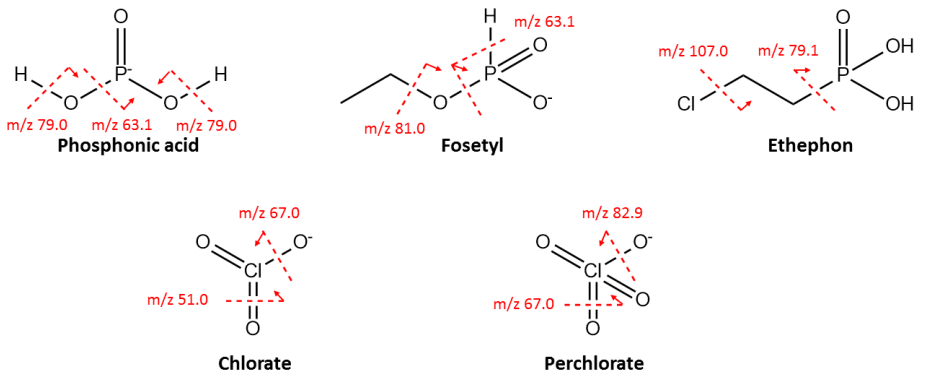

Figure 13. Fragmentation of the polar contaminants included in the study. Transitions are indicated in red.

Finally, transitions of the ion ^{37}Cl were monitored for quantification purposes since their abundance is high. The transitions of chlorate and

perchlorate are identical because these molecules are very similar, except perchlorate has one more oxygen than chlorate. Transitions *m/z* 83.0 → *m/z* 67.0 (or *m/z* 85.0 → *m/z* 69.0 using ion ^{37}Cl) of chlorate and *m/z* 99.0 → *m/z* 82.9 (or *m/z* 101.0 → *m/z* 85.0 using ion ^{37}Cl) of perchlorate came from the loss of the oxygen bonded by the single linkage, while the loss of another oxygen double bounded to chlorine gave the transitions *m/z* 67.0 → *m/z* 51.0 of chlorate and *m/z* 82.9 → *m/z* 67.0 of perchlorate.

In Publication V, the chromatographic separation technique used was LC. The mobile phase composition and the elution gradient were optimized using the Hypercarb column (100 × 2.1 mm; 5.0 µm particle size) coupled to a drop-in guard cartridge (10 × 4.6 mm; 7.0 µm particle size).

To obtain the best separation of the compounds and achieve the best peak shapes in the shorter running time, an optimization of the chromatographic conditions was accomplished. Thus, water, water:methanol (95:5, *v/v*) and water:methanol (90:10, *v/v*) were tested as aqueous phases. This optimization was carried out since the use of only water did not separate the compounds properly, and a higher percentage of methanol separated them too much and the analysis time was too long. Also, the amount of formic acid added to the mobile phases was tested adding 0.1, 0.5, 1.0 and 2.0%, and the best results were obtained with 1% of formic acid.

Figure 14 represents the elution gradient employed.

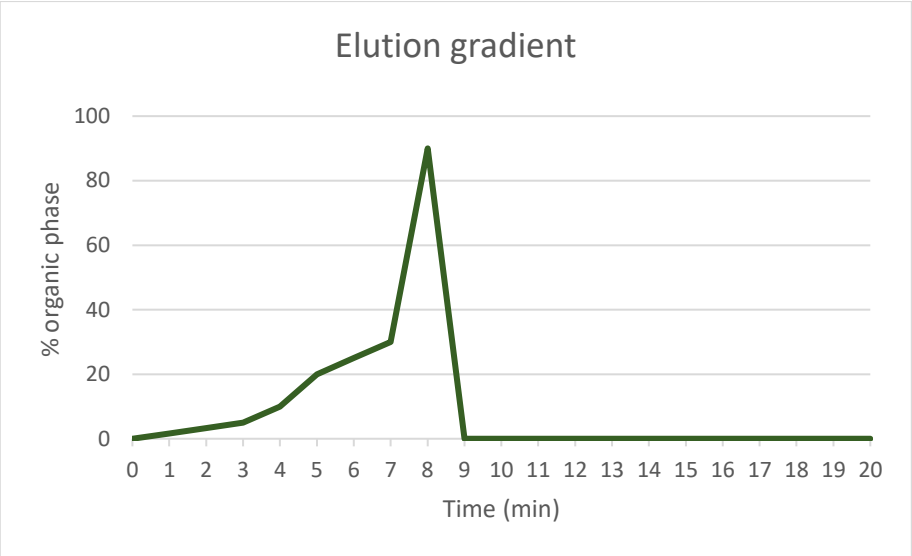

Figure 14. Elution gradient used in Publication V.

Table 13 shows a summary of the chromatographic conditions employed.

Table 13: Chromatographic conditions used for the separation of polar contaminants.[a]

LC Parameter	Conditions (Publication V)
Aqueous phase	1% formic acid in water:methanol (95:5, *v/v*)
Organic phase	1% formic acid in methanol
Column	Drop-in guard cartridge (10 × 4.6 mm; 7.0 µm) Hypercarb (100 × 2.1 mm; 5.0 µm)
Column temperature	40 °C
Injection volume	5 µL
Flow rate	0.3 mL/min
Time of analysis	20 min
Elution mode	Gradient

[a] Abbreviations: LC: Liquid chromatography.

The Hypercarb column needed a proper conditioning, so to ensure a proper performance, ten injections of a blank sample of almond were injected before each sequence of analysis.

Chromatograms in Figure 15 show the separation of the polar compounds in a spiked sample of extra virgin olive oil (Figure 15a) and in a spiked sample of almond (Figure 15b).

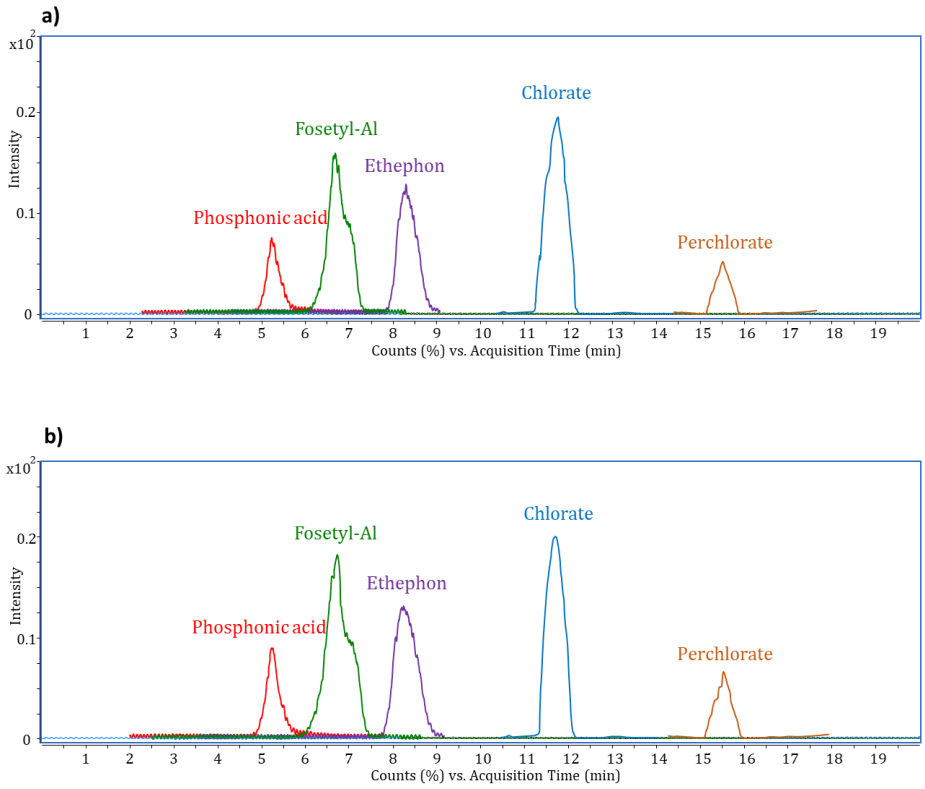

Figure 15. (a) Extracted ion chromatogram obtained from an extra virgin olive oil blank sample spiked with 100 µg/kg of the polar compounds; (b) Extracted ion chromatogram obtained from an almond blank sample spiked with 100 µg/kg of the polar compounds.

The final conditions such as retention time windows, precursor and product ion *m/z* and ion ratios for each compound are summarized in Table 14.

Table 14: Retention time window (RTW) and MS/MS parameters for the polar compounds.

Compound	RTW (min)[a]	Precursor ion (m/z)	Product ion (m/z) [b]	Ion ratio (%)
Phosphonic acid	5.3-5.5	81.1 (30)[c]	**79.0** (20)[d] 63.1 (40)	 55.9
Fosetyl-Al	6.6-6.8	109.1 (50)	**81.0** (10) 63.1 (35)	 10.0
Ethephon	8.1-8.3	143.0 (50)	**107.0** (5) 79.1 (20)	 39.7
Chlorate	11.6-11.8	85.0 (50) 83.0 (50)	**69.0** (20) 67.0 (20) 51.0 (35)	 93.1 11.0
Perchlorate	15.4-15.6	101.0 (110) 99.0 (130)	**85.0** (30) 82.9 (30) 67.0 (35)	 15.0 13.1
Perchloric acid isotopically labelled ($Cl^{18}O_4^-$)	15.4-15.6	107.0 (160)	**89.0** (35) 71.0 (48)	 18.0

[a] RTW: Retention time windows; [b] Transition in bold was used for quantification; [c] Fragmentor voltage (V) is given in brackets; [d] Collision energy (eV) is given in brackets.

4.1.2 Extraction methods

a) Vegetable oils

To optimize the extraction conditions, extra virgin olive oil blank samples were spiked at 100 µg/kg to carry out the experiments. Firstly, an existing method developed by our research group, based on the QuPPe method, for other polar compounds such as glufosinate, glyphosate and AMPA, was tested. The procedure was as follows: 10 g of edible oil were weighed into a 50 mL centrifuge tube. Then, 10 mL of acidified water (1% of formic acid) were added and the tube was shaken for 1 min in vortex and centrifuged for 10 min at 3700 rpm (3060×*g*). Finally, 1 mL of the aqueous phase was filtered through a syringe filter (0.45 µm) prior to the

injection into the LC system. However, recoveries of perchlorate were very low (30-63%), so some modifications were tested.

Firstly, some cleaning sorbents were tried, such as C18, florisil and Z-Sep+ and 25 and 50 mg of each one were tested. As it can be seen in Figure 16, the lowest recoveries were obtained using Z-Sep+ (12-43%) except chlorate (86-87%). For the other sorbents, C18 and florisil, recoveries were suitable (70-120%), but still low for perchlorate (30-63%).

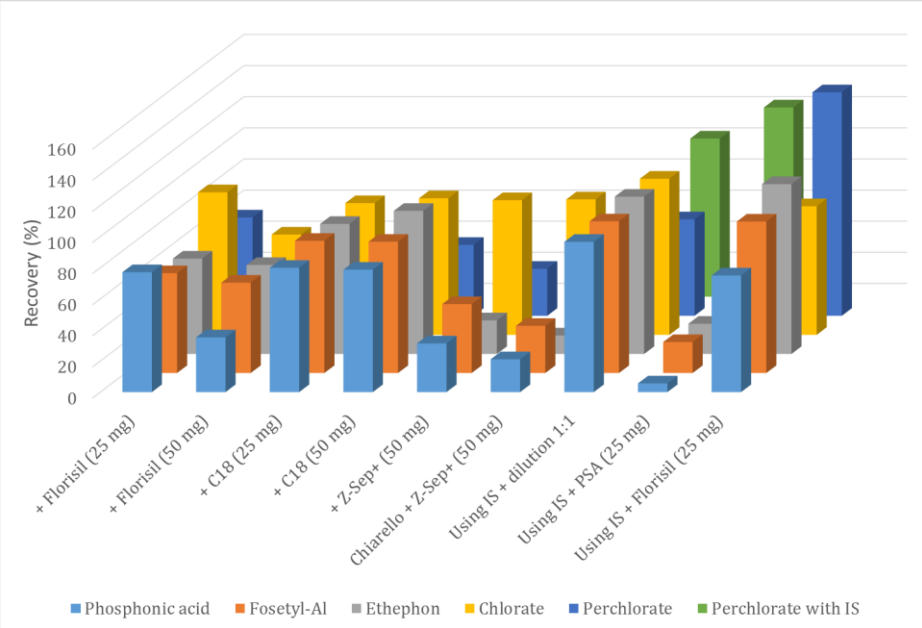

Figure 16. Comparison of the recovery values obtained for the polar compounds in vegetable oils modifying the first method tested. Abbreviations: C18: Octadecasilane; IS: Internal standards; PSA: Primary secondary amine.

To solve this problem some other experiments were carried out such as dilution of the extract with water (1:1, *v/v*) and addition of PSA and florisil. All these experiments were performed with and without the addition of internal standard, perchloric acid isotopically labelled. When florisil was added, a recovery of 365% was obtained for perchlorate, so this value was omitted in Figure 16 to be able to see a proper scale.

As it can be observed in Figure 16, the best recovery for perchlorate was obtained using the internal standard without adding cleaning sorbents (102%). Consequently, the next experiment was performed to confirm that the dilution was not necessary when the quantification of perchlorate was carried out with the internal standard. The recoveries of the other compounds must be calculated without the internal standard.

Consequently, the final extraction method in vegetable oils only differs from the first tested method in the use of perchloric acid isotopically labelled as internal standard for the quantification of perchlorate. Figure 17 shows a scheme of the final extraction method of polar compounds in vegetable oils in Publication V.

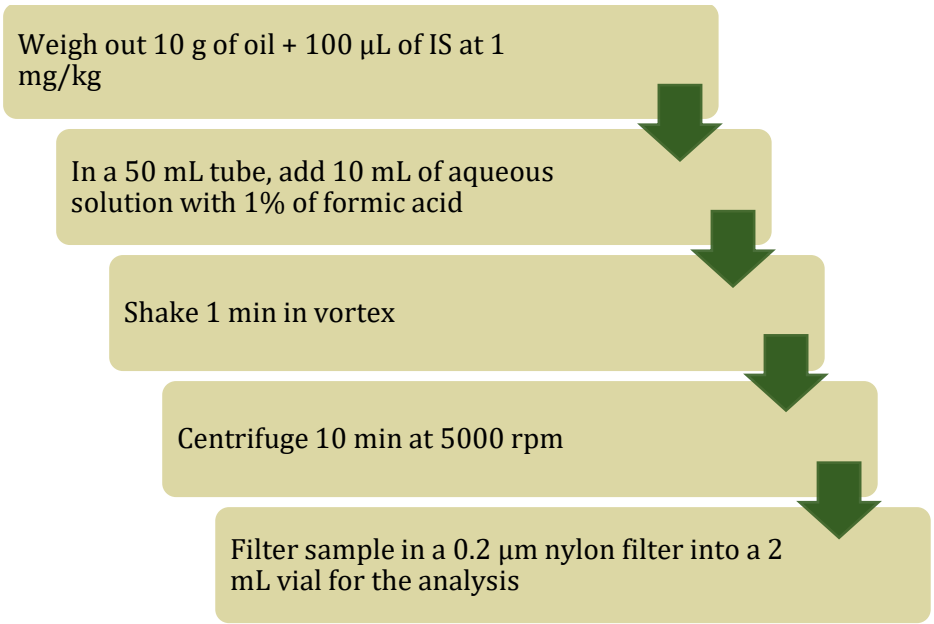

Figure 17. Scheme of the extraction method of polar compounds in vegetable oils in Publication V. Abbreviations: IS: Internal standards.

b) Nuts

For the extraction of nuts, a similar approach was carried out. Additionally, the several experiments such as addition of cleaning sorbents, QuPPe method including freezing, QuPPe method with dilutions,

QuPPe method with d-SPE or SPE, QuPPe method changing solvent were carried out, using almond blank samples spiked at 100 µg/kg. All the details of these experiments are indicated below.

Test 1. Addition of cleaning sorbents

Firstly, different amounts of sorbents were added to 2 mL of supernatant after vortex agitation. The sorbents tested, the quantities of them and the results in terms of recovery are shown in Table 15, and the best recoveries were obtained when 50 mg of C18 were added, but in all cases, bad recoveries of perchlorate were obtained (0-6% or 562-905%).

Table 15: Recovery values obtained after testing the extraction method for oils, in nuts, using different salts.[a]

	Added sorbent				
	C18 (50 mg)	**C18 (100 mg)**	**SAX (100 mg)**	**C18 (50 mg) SAX (50 mg)**	**SCX (50 mg) SAX (50 mg)**
Phosphonic acid	144%	175%	471%	130%	19%
Ethephon	79%	55%	43%	33%	55%
Fosetyl-Al	111%	142%	146%	24%	17%
Chlorate	68%	129%	3%	2%	4%
Perchlorate	562%	905%	4%	0%	6%

[a] Abbreviations: C18: Octadecasilane; SAX: Strong anion exchange; SCX: Strong cation exchange.

Test 2. QuPPe method including freezing

Then, the same extraction for vegetable oils was performed changing the aqueous solution with 1% of formic acid for a methanolic solution with 1% of formic acid and freezing the sample at -20 °C for three hours before

the extraction. Another experiment was carried out with the same conditions and an extra clean-up with SCX to get a better cleaning, as well as another dilution of the extract with water (1:10, *v/v*) to reduce matrix effect. Results showed that none of these experiments gave good results in terms of recovery (0-45%) as it can be seen in Table 16.

Table 16: Recovery values obtained after testing different freezing strategies in nuts.[a]

	Freezing sample	Freezing sample + SCX (100 mg)	Freezing sample + SCX (100 mg) + dilution (1:10, *v/v*)	Freezing sample + dilution (1:10, *v/v*)
Phosphonic acid	9%	1%	6%	7%
Ethephon	27%	10%	1%	2%
Fosetyl-Al	45%	14%	3%	3%
Chlorate	26%	13%	2%	2%
Perchlorate	15%	0%	2%	2%

[a] Abbreviations: SCX: Strong cation exchange.

Test 3. QuPPe method + dilutions

Then, QuPPe method using some dilutions of the extract (1:1, 1:2, *v/v*) and several clean-up sorbents (C18, GCB, Z-Sep+, florisil, diatomaceous earth and graphene) were tested since no good results were obtained. The results of percentage of matrix effect of all these experiments were low because of a lack of sensitivity provoked by matrix suppression effect as it can be observed in Figure 18.

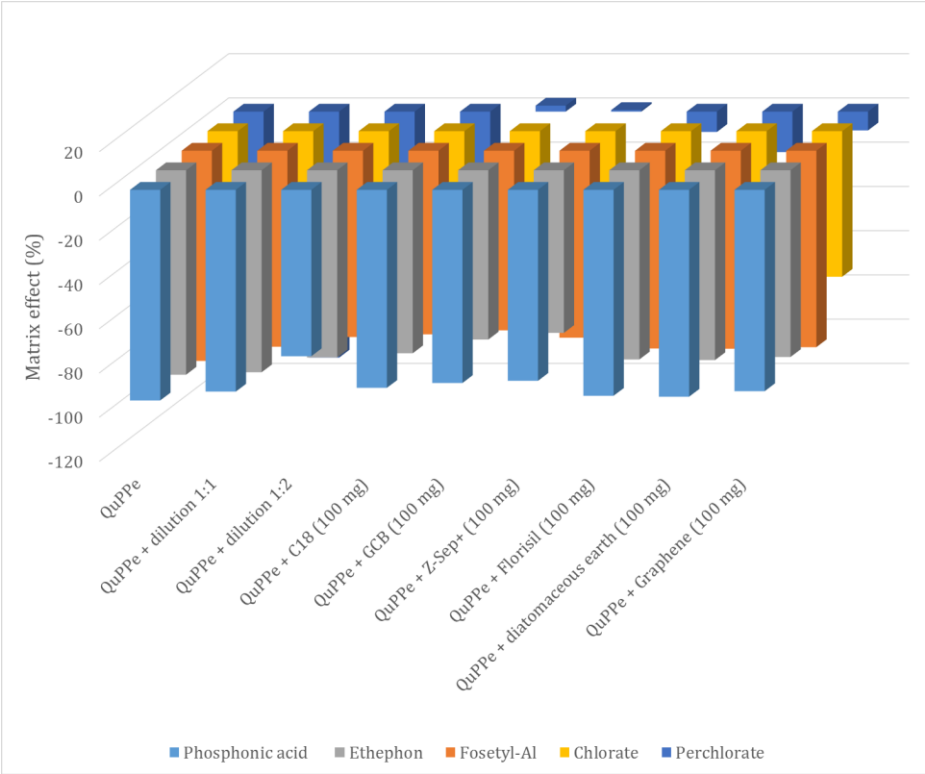

Figure 18. Values of matrix effect obtained after applying the QuPPe method in nuts, plus testing different salts. Abbreviations: C18: Octadecasilane; GCB: Graphitized carbon black; QuPPe: Quick Method for the analysis of numerous highly polar pesticides.

Test 4. QuPPe method + d-SPE or SPE

A new approach based on the QuPPe method was tested using d-SPE with C18 and SPE with C18, OASIS and EMR cartridges to remove interferents and to minimize matrix effect. Figure 19 summarizes the recoveries obtained with all these experiments. Regarding SPE, as it can be observed in Figure 19, OASIS cartridges provided the best results (38-125%) except for perchlorate (278%), while when EMR cartridges were used, the results were out of an acceptable range (1-64% and 469-840%). Likewise, the majority of the recoveries obtained with the C18 cartridge were overestimated, from 129 to 449%, except 81% for ethephon. As it can be seen, the QuPPe method with methanol provided recovery values from

59 to 122% which is almost acceptable, except for perchlorate (843%). Finally, the combination of the QuPPe method and the clean-up step with C18 in d-SPE provided the best recoveries for all the compounds, between 43 and 122%.

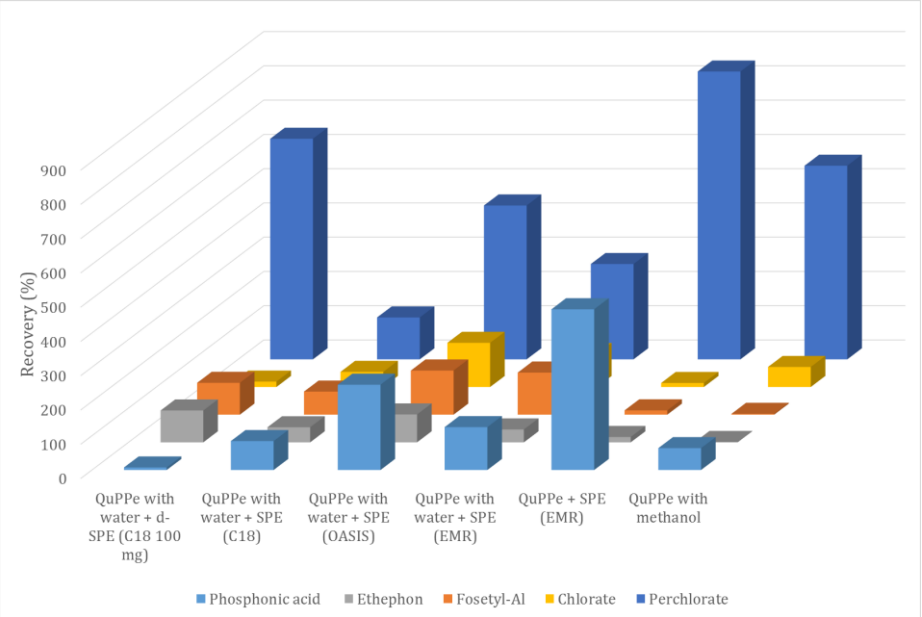

Figure 19. Recovery values obtained after testing the QuPPe method in nuts, plus testing different SPE clean-ups. Abbreviations: C18: Octadecasilane; d-SPE: Dispersive solid phase extraction; EMR: Enhanced matrix removal; QuPPe: Quick Method for the analysis of numerous highly polar pesticides; SPE: Solid phase extraction.

Test 5. QuPPe method changing solvent

The last test performed was the addition of *n*-hexane instead of acetonitrile, in which the extracts were considerably cleaner and therefore, recovery and matrix effect values were improved obtaining recoveries between 73 and 96% for all the compounds studied.

Figure 20 shows a scheme of the final extraction method of polar compounds in nuts in Publication V.

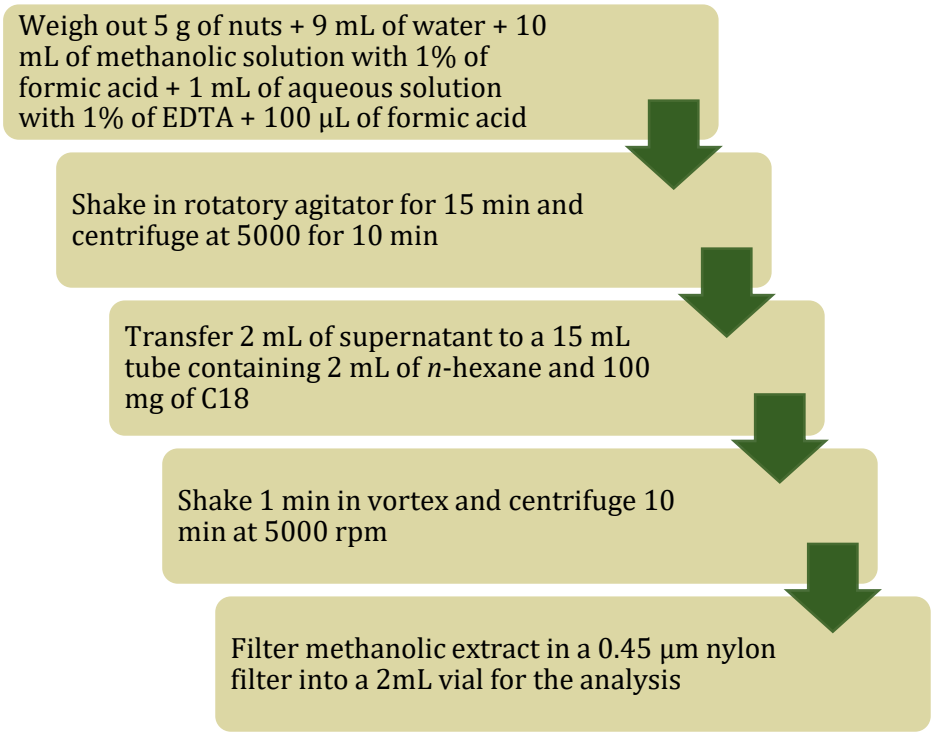

Figure 20. Scheme of the extraction method of polar compounds in nuts in Publication V. Abbreviations: C18: Octadecasilane; EDTA: Ethylenediaminetetracetic acid.

4.1.3 Validation of the method

The validation of the analytical method was performed to comply with the parameters indicated by SANTE guidelines. Thus, the applicability of the method and a proper identification and quantification of the polar compounds was ensured. Parameters evaluated in both methods for the polar compounds studied, in vegetable oils and nuts (Publication V), are summarized in Table 17.

Determination coefficients (R^2) were obtained after the evaluation of the linearity in solvent analyzing a calibration curve at 0, 10, 25, 50, 100, 250 and 500 µg/L. The results obtained were 0.9826, 0.9975, 0.9963,

0.9969 and 0.9993 for phosphonic acid, fosetyl-Al, ethephon, chlorate and perchlorate respectively.

Table 17: Parameters evaluated for the validation of the method for polar compounds in Publication V.[a]

Parameter	Vegetable oils	Nuts
Determination coefficient (R^2)	0.9826-0.9993	
LOQ (µg/kg)	10.0	
Recovery (%)[b]	76-104	70-101
Intra-day precision, RSD (%)	3-15	6-20
Inter-day precision, RSD (%)	7-19	9-20

[a] Abbreviations: LOQ: Limit of quantification; RSD: Relative standard deviation. [b] Note: Recoveries and RSDs were calculated based on n = 5.

Matrix effect was almost negligible since the results obtained were between 1 and 9% for all the polar compounds in vegetable oils and between 3 and 8% in the case of nuts.

The values of recovery, intra- and inter-day precisions were calculated at 10, 100 and 500 µg/kg. As it can be seen in Table 17, the recoveries ranged from 76 to 104% and from 70 to 101% for vegetable oils and nuts respectively.

As it can be seen in Table 17, intra-day and inter-day precisions were below 15% and 19% respectively for vegetable oils, whereas both precisions were below 20% for nuts for all the polar compounds studied in Publication V.

LOQ was established at 10 µg/kg for all the polar compounds, phosphonic acid, ethephon, fosetyl-Al, chlorate and perchlorate in both matrices, vegetable oils and nuts.

4.1.4 Analysis of samples

The application of the method was appraised through the analysis of several samples of different types of vegetable oils and different types of nuts.

Additionally, an internal quality control was applied with each batch of samples that consisted of a blank sample, a calibration curve in solvent, from 10 to 250 µg/L, and a spiked sample at 10 µg/kg to verify the sensitivity of the extraction method.

a) Vegetable oils

The developed method was applied to a total of 20 samples of vegetable oils including four types of olive oil: 4 samples of extra virgin olive oil, 4 samples of refined olive oil, 4 samples of olive pomace oil, 4 samples of crude olive pomace oil and 4 samples of sunflower oil.

None of the samples of vegetable oils were found positive for the polar compounds analyzed. This may be due to that the compounds are polar while oils are non-polar, which hinder the transference to this kind of matrices.

b) Nuts

The method for the extraction of polar compounds in nuts was applied to a total of 20 samples of nuts including 6 samples of almonds, 3 samples of hazelnuts, 5 sample of peanuts, 4 samples of pistachios and 2 samples of walnuts.

In the case of nuts, as it can be seen in Table 18, some polar compounds were detected. For instance, phosphonic acid was found in all the types of nuts. Furthermore, half of the samples of each nut were contaminated, achieving a maximum concentration of 4.6 mg/kg in a sample of almonds.

Table 18: Concentration range of polar compounds detected in the different types of nuts analyzed (µg/kg).

	Almonds	Hazelnuts	Peanuts	Pistachios	Walnuts
Samples analyzed	6	3	5	4	2
Phosphonic acid	19.3-4632.3 (5/6)[a]	30.9-65.7 (3/3)	57.4-306.5 (3/5)	15.6-60.6 (2/4)	37.6 (1/2)
Ethephon	<LOQ[b]	<LOQ	<LOQ	<LOQ	<LOQ
Fosetyl-Al	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Chlorate	<LOQ	<LOQ	<LOQ	193.0 (1/4)	<LOQ
Perchlorate	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

[a] Number of positive samples out of the total samples analyzed are given in brackets. [b] Abbreviations: LOQ: Limit of quantification.

As it can be observed in Table 18, all the samples of hazelnuts were contaminated with phosphonic acid at concentrations between 30.9 and 65.7 µg/kg. According to EFSA, the major residue identified in rotational crops is phosphonic acid, which agrees with the results obtained.

Furthermore, 193 µg/kg of chlorate were found in a sample of pistachios.

4.2 Determination of mineral oil hydrocarbons in vegetable oils by GC-FID

4.2.1 Separation and detection by gas chromatography with flame ionization detector

In Publication VI, the chromatographic separation technique used was GC, using a DB-1HT column (15 m × 0.32 mm i.d.; 0.10 μm film thickness) coupled to an untreated fused silica capillary column (2 m × 4.6 mm). The make-up gas (He), H_2 and air flows for the detector were 27, 35 and 300 mL/min respectively. The flow rate of the carrier gas (He) was constant at 3 mL/min.

Factors like the solvent used, type of injector or column temperatures have a strong influence in the volatilization of hydrocarbons. Thus, all the conditions of the instrument needed to be optimized to obtain the best peak shapes and sensitivities for the whole range of hydrocarbons analyzed (C10-C50).

Firstly, two injection techniques, such as large volume injection (LVI) and on-column simulation, were evaluated. Consequently, a mixture of hydrocarbons from C10 to C50 at 10 mg/L was injected in both injection modes (50 μL for LVI and 2 μL for on-column simulation) and it was seen that LVI injection did not provide good results for the lower hydrocarbons (C10-C13) since they were lost. Therefore, on-column simulation injection mode was selected.

Then, the optimization of the temperatures of the injector was carried out. For that, a mixture of hydrocarbons from C10 to C50 at 10 mg/L was injected starting the temperature ramp of the injector at 60, 100 and 150 °C. Figure 21 represents the peak areas obtained of the hydrocarbons that were used to define the ranges versus the minimum temperature of the injector. As it can be seen, the best results were obtained using a minimum temperature of 100 °C.

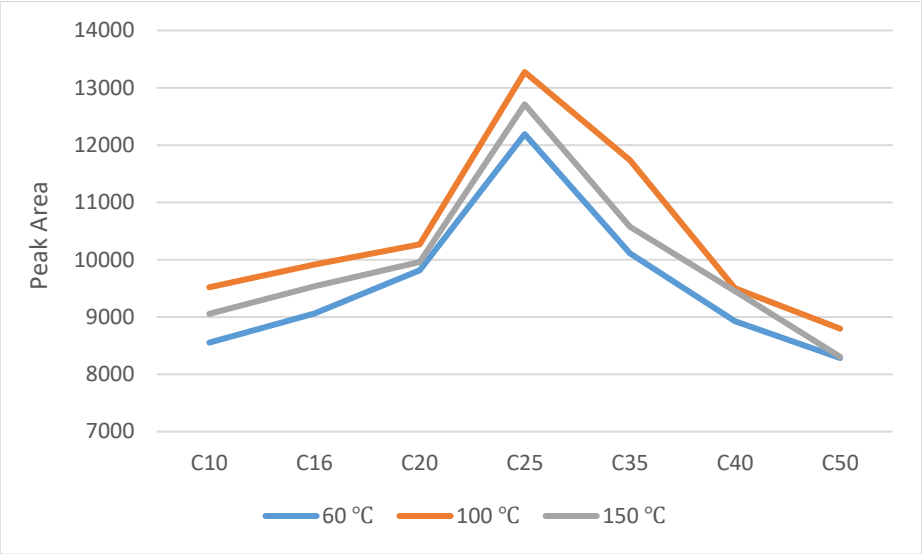

Figure 21. Comparison of the peak areas of the hydrocarbons at different minimum temperatures tested in the injector.

Moreover, the maximum temperature reached in the injector was optimized testing 340, 350, 360 and 370 °C injecting a mixture of hydrocarbons from C10 to C50 at 10 mg/L. Figure 22 represents the peak areas of each hydrocarbon obtained for the different maximum temperatures. Finally, 360 °C was chosen as the optimum maximum temperature of the injector since the results show that the peak area of the hydrocarbons, when 360 °C was reached, was higher than those obtained with other temperatures except for C40. Besides, the peak area of C40 was acceptable with 360 °C with respect to 370 °C, while for the hydrocarbons from C10 to C25 and C50 the results obtained were much better than those obtained with other maximum temperatures.

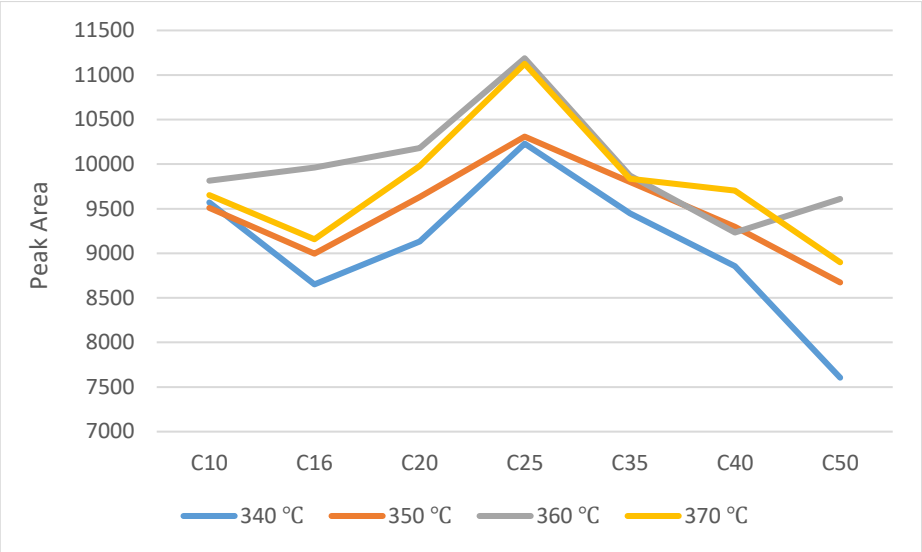

Figure 22. Comparison of the peak areas of the hydrocarbons at different maximum temperatures tested in the injector.

Finally, the temperature programming used in the injector started at 100 °C and it was increased until 360 °C at 200 °C/min. When the final temperature was reached, it was held for 10 minutes.

The temperature programming of the oven was also optimized. For that, four ramps were tested: at 50 °C/min (see Figure 23a), 35 °C/min (see Figure 23b), 25 °C/min (see Figure 23c) and 15 °C/min (see Figure 23d), injecting a mixture of hydrocarbons from C10 to C40 at 10 mg/L. In Figure 23 the peaks of the mixture of hydrocarbons obtained at the four ramps can be seen. As it can be observed in Figure 23c, the ramp of 25 °C/min provided good separation, good peak shapes with acceptable height, and there was not much difference in terms of area between each other. Moreover, it was checked that the ratio between C20 and C50 was not above 20% injecting the mixture of hydrocarbons used to define the ranges.

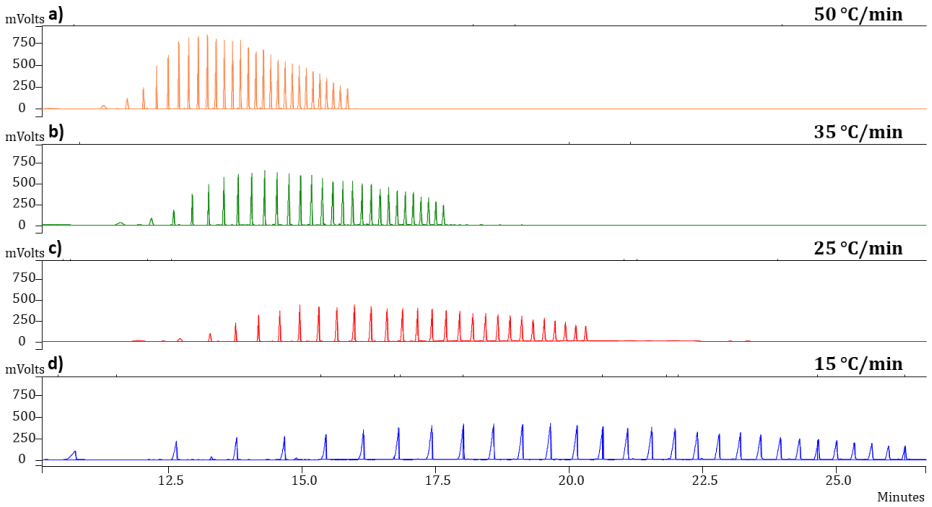

Figure 23. Comparison of the hydrocarbons with different temperature programming in the oven: (a) 50 °C/min; (b) 35 °C/min; (c) 25 °C/min; (d) 15 °C/min.

Finally, the temperature programming started at 40 °C and it was increased to 360 °C at 25 °C/min. When the maximum temperature was reached, it was held for 15 minutes.

In addition, the temperature of the FID was optimized injecting the mix of markers at 10 mg/L (except C13, at 5 mg/L, and cholestane and perylene at 20 mg/L) and setting the temperature of the FID at 350, 370 and 390 °C. As it can be seen in Figure 24, the best peak areas were obtained using 350 °C, and furthermore, the noise increased with higher temperatures. Moreover, the sensitivity of perylene increased considerably at 350 °C and this was a huge advantage detecting the end of the MOAH fraction.

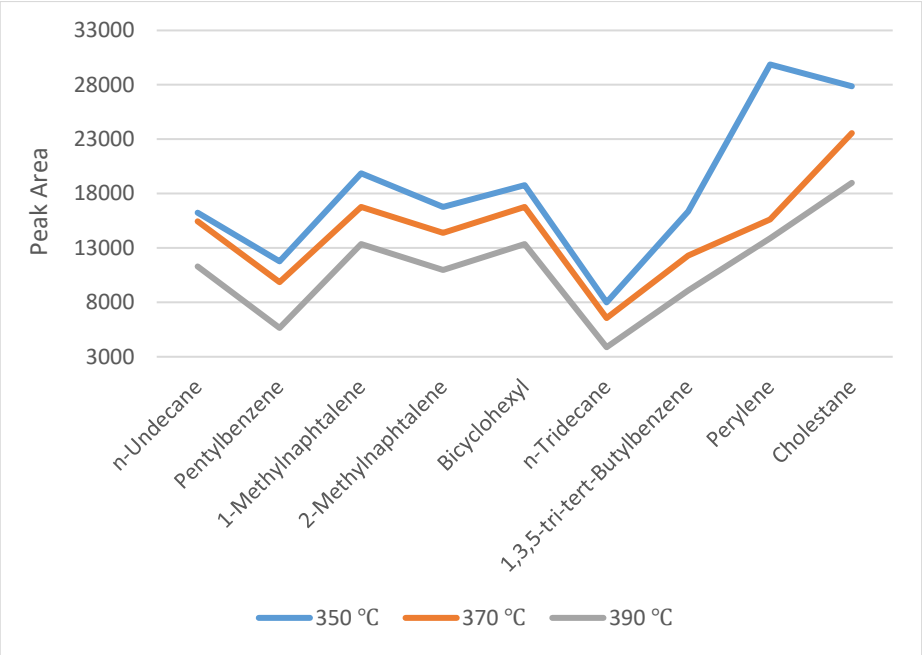

Figure 24. Comparison of the peak areas of the standards used in the analysis at different temperatures in the detector.

Table 19 shows a summary of the chromatographic conditions.

Table 19: Chromatographic conditions used for the separation of MOSH and MOAH fractions.[a]

GC Parameter	Conditions (Publication VI)
Carrier gas	He
Column	DB-1HT (15 m × 0.32 mm i.d.; 0.10 µm)
Column temperature	40 °C → 360 °C
Injection volume	2 µL
Flow rate	3 mL/min
Time of analysis	27.8 min

[a] Abbreviations: GC: Gas chromatography.

The chromatographic separation of the standards can be seen in Figure 25.

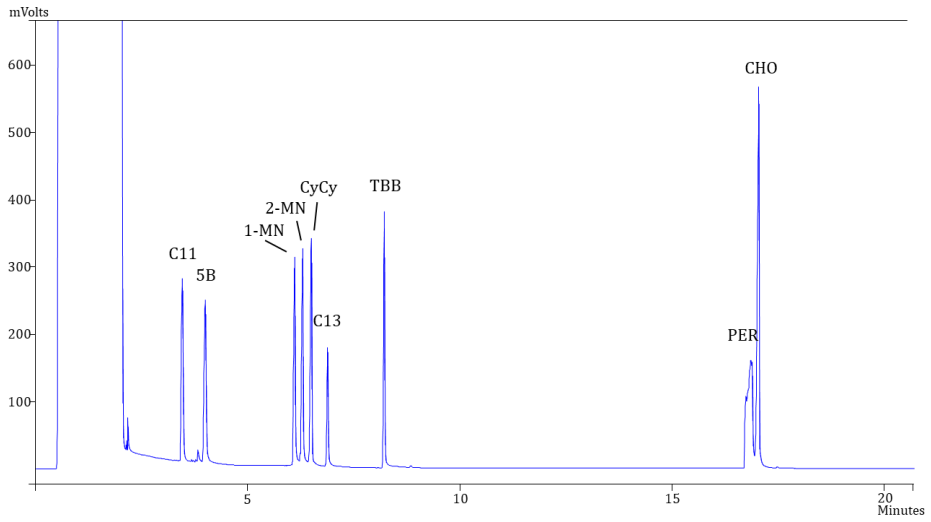

Figure 25. Chromatogram of the mixture of internal standards. Abbreviations: 1-MN: 1-Methylnaphthalene; 2-MN: 2-Methylnaphthalene; 5B: Pentylbenzene; C11: *n*-Undecane; C13: *n*-Tridecane; CyCy: Bicyclohexyl; CHO: Cholestane; PER: Perylene; TBB: 1,3,5-tri-*tert*-Butylbenzene.

4.2.2 Extraction method

All the experiments were carried out adding 50 µL of the mixture of markers at 300 mg/L (except C13, at 150 mg/L, and cholestane and perylene at 600 mg/L) to a blank extra virgin olive oil.

Firstly, the silica gel was prepared following the procedure shown in Figure 26.

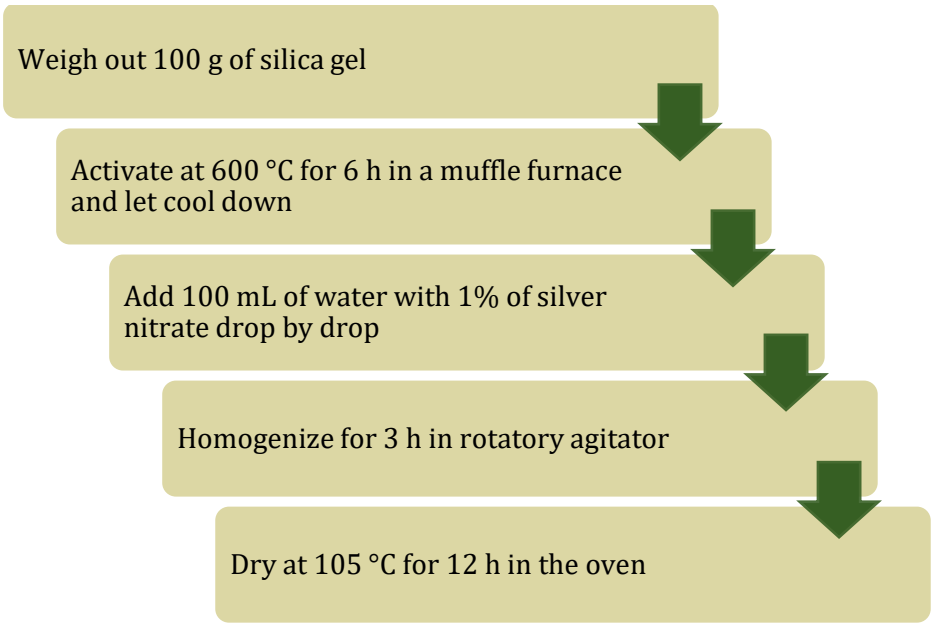

Figure 26. Scheme of the preparation of silica gel for the extraction of MOSH and MOAH in oils used in Publication VI.

Different solvents and mixtures were tested for the extraction of MOAH while for the extraction of the MOSH fraction, only *n*-hexane was used as it is a non-polar solvent, which is appropriate for the dissolution of saturated hydrocarbons. On the other hand, different mixtures were evaluated for the elution of the MOAH fraction. It was realized that perylene did not come out unless toluene was added to the solvent, so a mixture *n*-hexane:toluene (50:50, *v/v*) was used. Furthermore, in the Laboratory BfR (Zurich, Switzerland), a 20% of dichloromethane was used, so the final mixture was *n*-hexane:toluene:dichloromethane (40:40:20, *v/v/*v), which was checked and worked properly.

To avoid interferences and minimize the noise in the chromatogram, two types of washing of the column were tested. The first one was washing the silica gel in an ultrasound system, which did not provide good results in terms of decreasing the noise, so it was discarded. The other washing experiment consisted on passing solvent through the chromatographic

column and it was seen that the noise was decreasing every 10 mL of solvent. Thus, fractions of 10 mL of the mixture *n*-hexane:toluene:dichlorometane (40:40:20, *v/v/v*) were introduced into the column with the silica until it was seen that after 30 mL the signal did not reduce. Afterwards, the same procedure was followed with *n*-hexane and it was checked that 40 mL of this solvent were necessary. This solvent washing order was chosen to leave *n*-hexane inside chromatographic column, that is the solvent that allows the elution of MOSH fraction. Figure 27 shows the procedure followed for the preparation of the offline chromatographic column.

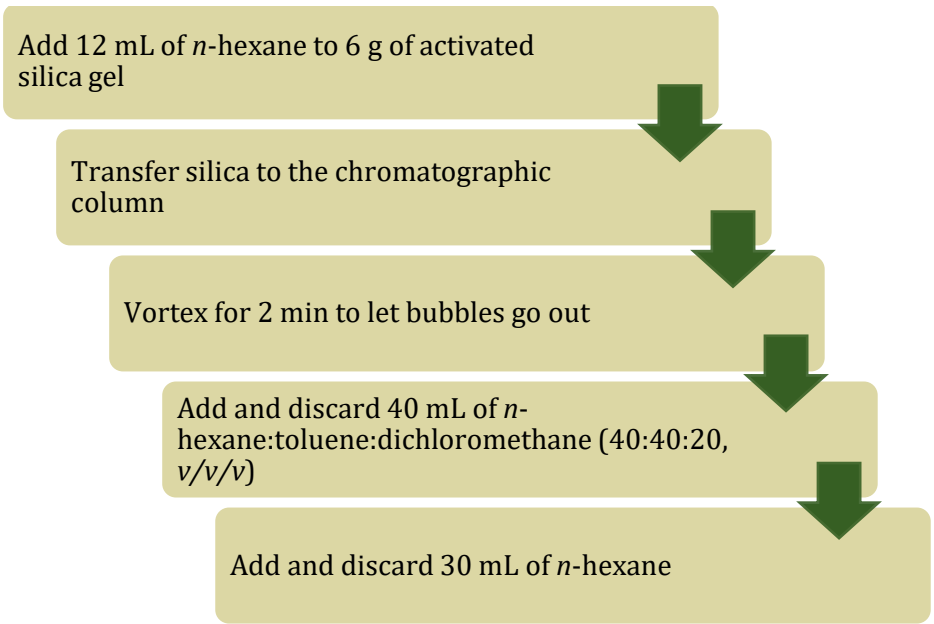

Figure 27. Scheme of the preparation of the offline chromatographic column for the extraction of MOSH and MOAH in oils used in Publication VI.

Olefins that naturally occur in oils led to an overestimation of the results of MOAH. Thus, an epoxidation was performed to clean these olefins and avoid this overestimation.

Figure 28 shows a scheme of the procedure followed for the epoxidation of olefins prior to the MOSH and MOAH extraction.

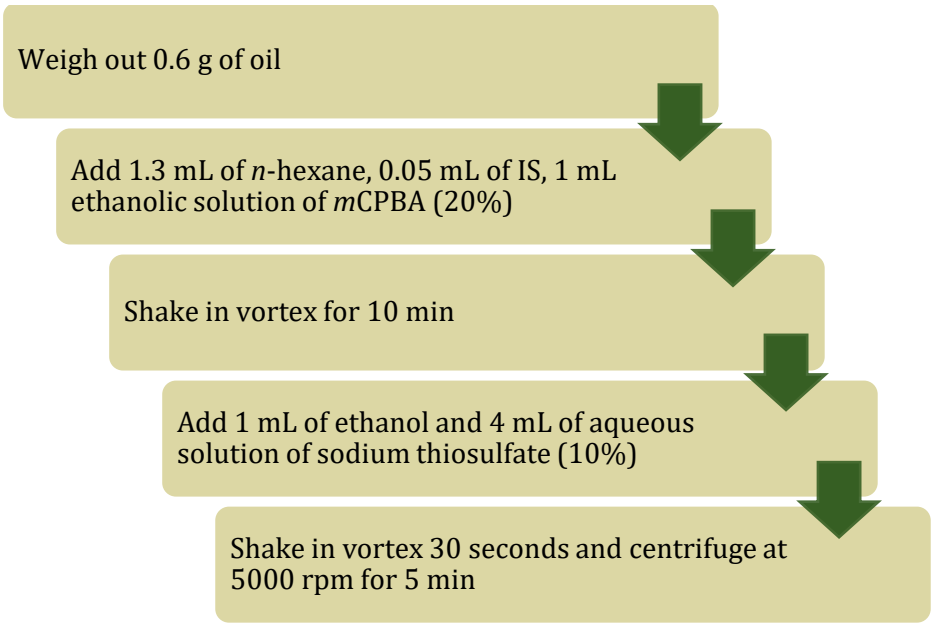

Figure 28. Scheme of the epoxidation previous to the extraction of MOSH and MOAH in oils used in Publication VI. Abbreviations: IS: Internal standards; *m*CPBA: meta-Chloroperbenzoic acid.

Regarding the extraction itself, several experiments were carried out to optimize an offline extraction method. To prepare the offline column manually, 3, 4, 5 and 6 g of sorbent (silica with 1% of silver nitrate) were tested. It was seen that 6 g of sorbent provided the best separation of fractions. This parameter, as well as the quantity of solvent needed to achieve the separation were checked analyzing fractions of 1 mL until the adequate markers were observed in the GC-FID. Thus, after 8 mL of *n*-hexane, the marker of the end of the MOSH fraction, cholestane, was completely eluted, so the MOSH fraction was considered fully eluted from the chromatographic column. Then, a mixture of *n*-hexane:toluene:dichlorometane (40:40:20, *v/v/v*) was added to extract the MOAH fraction, so the marker of the beginning of the MOAH fraction, 1,3,5-tri-*tert*-butylbenzene, started to appear in the fractions and after 9 mL, the marker of the end of the MOAH fraction, perylene, stopped coming out, which meant that the elution of the MOAH fraction had finished.

The amount of sample used, 0.3 and 0.6 g, was evaluated obtaining that 0.6 g of oil were necessary to carry out the epoxidation process without introducing noise into the chromatogram. Thus, the volume of supernatant of the epoxidation was big enough to transfer it to the chromatographic column without introducing the lower phase.

Finally, the evaporation conditions were optimized to minimize the loss of volatile compounds. Different bath temperatures as well as the minimum vacuum pressures necessary for the evaporation of the solvent were assayed. It was seen that in the case of the MOSH fraction, the minimum pressure should be 200 mbar while for the MOAH fraction, the pressure should not be below 190 mbar. In both cases, the temperature of the water bath was set at 40 °C. In Figure 29 it can be seen a scheme of the extraction method of MOSH and MOAH from vegetable oils.

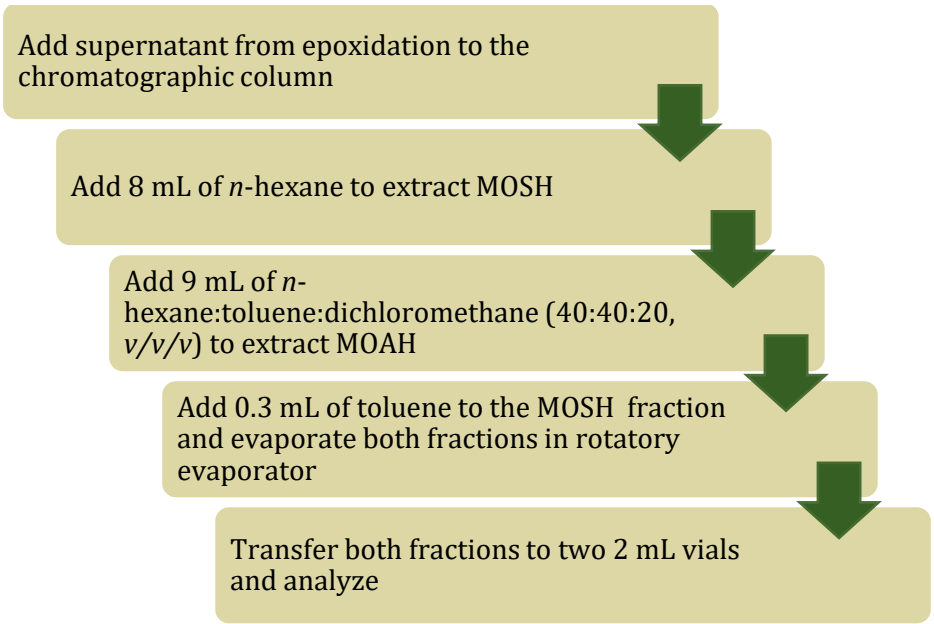

Figure 29. Scheme of the extraction method of MOSH and MOAH in oils used in Publication VI. Abbreviations: IS: Internal standard; MOAH: Mineral oil aromatic hydrocarbons; MOSH: Mineral oil saturated hydrocarbons.

Figure 30 shows the proper separation achieved between MOSH and MOAH, checking the correct appearance of the internal standards in each fraction. Thus, in Figure 30a *n*-undecane, bicyclohexyl, *n*-tridecane and cholestane appeared, whereas in Figure 30b, pentylbenzene, 1-methylnaphthalene, 2-methylnaphthalene, 1,3,5-tri-*tert*-butylbenzene and perylene were detected.

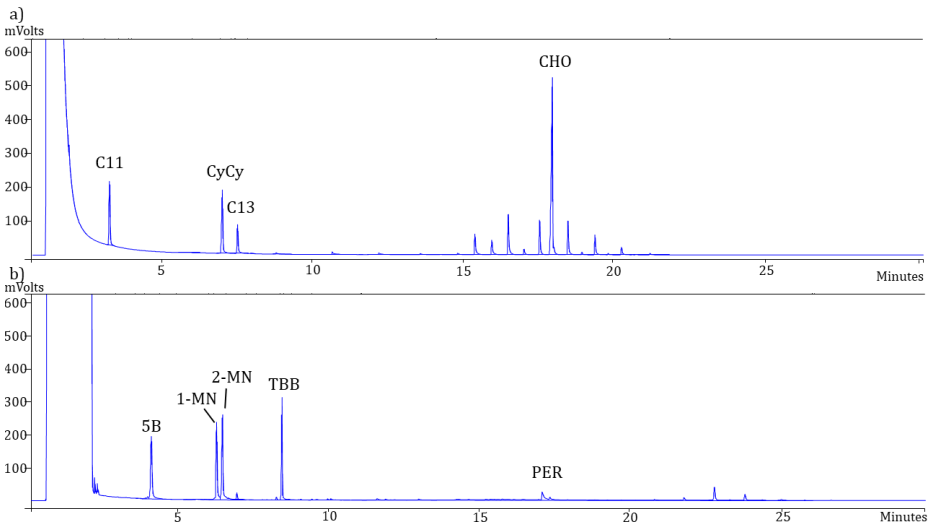

Figure 30. (a) Chromatogram of mineral oil saturated hydrocarbons (MOSH) of an extracted blank sample spiked 10 mg/kg. (b) Chromatogram of mineral oil aromatic hydrocarbons (MOAH) of an extracted blank sample spiked at 10 mg/kg. Abbreviations: 1-MN: 1-Methylnaphthalene; 2-MN: 2-Methylnaphthalene; 5B: Pentylbenzene; C11: *n*-Undecane; C13: *n*-Tridecane; CyCy: Bicyclohexyl; CHO: Cholestane; PER: Perylene; TBB: 1,3,5-tri-*tert*-Butylbenzene.

4.2.3 Validation of the method

In this case, the validation of the methodology was performed taking into account the JRC and the SANTE guidelines, ensuring the applicability of the method. Table 20 summarizes the parameters evaluated for the validation of the method in Publication VI.

Table 20: Parameters evaluated for the validation of the method for mineral oil hydrocarbons in Publication VI.[a]

Parameter	MOSH	MOAH
LOQ (mg/kg)	0.5 for each range of hydrocarbons	
Recovery (%)[b]	81-111	99-115
Intra-day precision, RSD (%)	3-15	7-16
Inter-day precision, RSD (%)	5-19	8-19

[a] Abbreviations: LOQ: Limit of quantification; RSD: Relative standard deviation. [b] Note: Recoveries and RSDs were calculated based on n=5.

All the experiments were carried out at three concentrations: 0.5, 10.0 and 17.9 mg/kg. As it can be observed in Table 20, trueness was evaluated in terms of recoveries and they were between 81 and 111% for MOSH, and between 99 and 115% for MOAH.

As it can be seen in Table 20, intra-day precision in terms of RSD was always below 15% for MOSH and below 16 % for MOAH, while inter-day precision was below 19% for both MOSH and MOAH.

LOQ was appraised diluting an oil sample analyzed by several accredited laboratories with blank extra virgin olive oil, providing a mean concentration of MOSH (38.8 mg/kg) and MOAH (4.2 mg/kg) which was used as reference value. Thus, a LOQ of 0.5 mg/kg for each range of hydrocarbons of both MOSH and MOAH was established.

4.2.4 Analysis of samples

The application of the method was tested through the analysis of 27 samples of vegetable oils. With every set of analysis, an internal quality control was applied to guarantee the reliability of the analytical procedure.

This consisted of a solvent blank, an extra virgin oil blank sample, a mixture of the internal standards in solvent, a mixture of the linear hydrocarbons in solvent, a retention time standard mix in solvent and an extra virgin oil blank sample spiked with 10 mg/kg.

Table 21 summarizes the results of MOHs from the analyzed samples.

Table 21: Concentration by ranges of mineral oil hydrocarbons detected in the samples of vegetable oils analyzed.[a]

Matrix	MOSH (mg/kg)	MOAH (mg/kg)
Extra Virgin Olive Oil 1	<LOQ	<LOQ
Extra Virgin Olive Oil 2	<LOQ	<LOQ
Extra Virgin Olive Oil 3	<LOQ	<LOQ
Extra Virgin Olive Oil 4	<LOQ	<LOQ
Extra Virgin Olive Oil 5	<LOQ	<LOQ
Extra Virgin Olive Oil 6	<LOQ	<LOQ
Extra Virgin Olive Oil 7	<LOQ	<LOQ
Refined Olive Oil 1	12.2	<LOQ
Refined Olive Oil 2	5.7	3.3
Refined Olive Oil 3	5.4	5.8
Refined Olive Oil 4	1.1	0.9
Refined Olive Oil 5	<LOQ	<LOQ
Refined Olive Oil 6	8.2	1.4
Refined Olive Oil 7	24.2	12.9
Olive Pomace Oil 1	22.4	7.7
Olive Pomace Oil 2	35.3	19.7
Olive Pomace Oil 3	49.9	18.1
Olive Pomace Oil 4	68.3	21.5
Olive Pomace Oil 5	79.2	22.4
Sunflower Oil 1	<LOQ	<LOQ
Sunflower Oil 2	4.4	<LOQ
Sunflower Oil 3	15.0	5.9
Sunflower Oil 4	<LOQ	<LOQ
Sunflower Oil 5	7.3	3.4
Sunflower Oil 6	<LOQ	<LOQ
Corn Oil 1	<LOQ	<LOQ
Corn Oil 2	<LOQ	<LOQ

[a] Abbreviations: LOQ: Limit of quantification; MOAH: Mineral oil aromatic hydrocarbons; MOSH: Mineral oil saturated hydrocarbons.

As it was expected, extra virgin olive oil samples were not contaminated neither with MOSH nor with MOAH. As it can be observed in Table 21, olive pomace oils presented the highest concentrations of MOSH and MOAH with concentrations up to 79.2 mg/kg of MOSH and 22.4 mg/kg of MOAH. Furthermore, a sample of sunflower oil showed a concentration of MOSH 15.0 mg/kg and a MOAH concentration of 5.9 mg/kg. Besides, the analyzed corn oils were not contaminated.

Figure 31a shows the chromatogram of the sample of refined olive oil 6, which was contaminated with 8.2 mg/kg of MOSH. In Figure 31b, a zoomed part of the hump of MOSH can be appreciated.

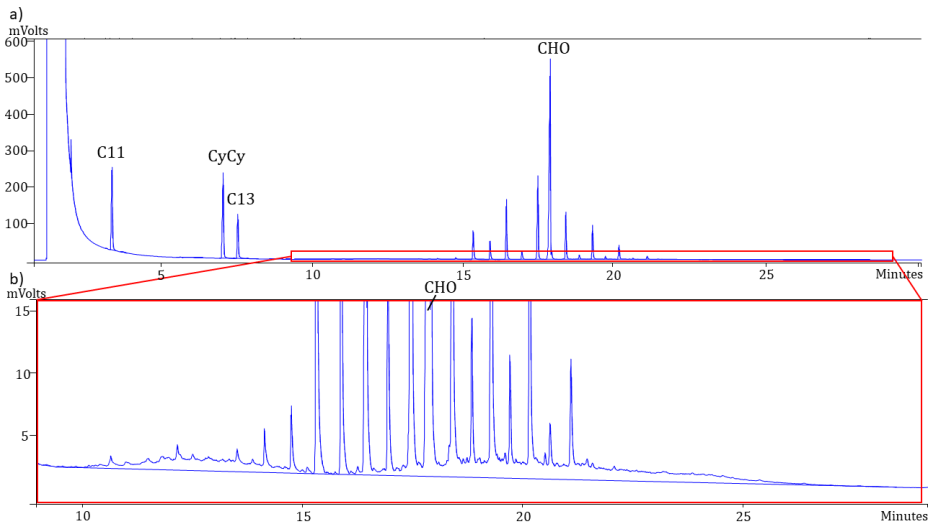

Figure 31. (a) Chromatogram of mineral oil saturated hydrocarbons (MOSH) of the sample of refined olive oil 6 contaminated at 8.2 mg/kg; (b) Zoom of the Figure 31a. Abbreviations: C11: n-undecane, CyCy: bicyclohexyl, C13: n-tridecane, CHO: cholestane.

Likewise, Figure 32a shows the contamination of the sample of refined olive oil 7 containing 12.9 mg/kg of MOAH, whereas in Figure 32b the humps can be seen.

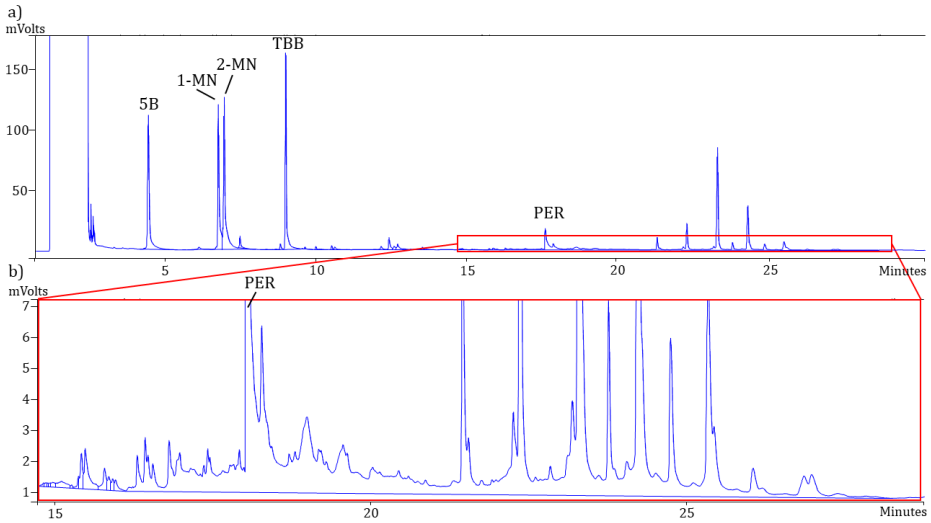

Figure 32. (a) Chromatogram of mineral oil aromatic hydrocarbons (MOAH) of the sample of refined olive oil 7 contaminated at 12.9 mg/kg; (b) Zoom of the Figure 29a. Abbreviations: 5B: Pentylbenzene, 1-MN: 1-Methylnaphthalene, 2-MN: 2-Methylnaphthalene, TBB: 1,3,5-tri-*tert*-Butylbenzene, PER: Perylene.

CHAPTER V

CONCLUSIONS

1. CONCLUSIONS

The general objective of this Thesis was the development of reproducible methods to carry out the analysis of contaminants in oils and other high-fat matrices to move forward in terms of food safety. From the results obtained throughout this Thesis, the following conclusions can be highlighted:

1. QuEChERS-based method coupled to UHPLC-MS/MS provided satisfactory results in terms of recovery and precision for the extraction of different families of mycotoxins from oils and nuts.

2. The study carried out on mycotoxin contamination in 194 samples of vegetable oils and 36 samples of nuts provided that 40% of samples were contaminated at concentrations above the LOQ. In the case of vegetable oils, zearalenone was the most found mycotoxin, mainly in olive, lampante and refined olive oils, with 51, 55 and 73% of the samples contaminated, respectively.

3. The use of LLE and d-SPE combined with UHPLC-MS/MS was used for the extraction and analysis of 3-MCPD esters and glycidyl esters from oils and processed foods, reducing the analysis time generally employed in current methods.

4. The study performed on 20 samples of vegetable oils and 4 samples of processed foods showed that 20% of the analyzed oils presented values of 3-MCPD esters and glycidyl esters expressed as free 3-MCPD and glycidol above the limits set by the legislation, finding that vegetable oils, as well as pomace oils presented the highest concentration levels.

5. For the extraction of polar compounds, both LLE used in vegetable oils, and QuPPe modified utilized in nuts, and their analysis by HPLC-MS/MS provided good results in terms of recovery and precision,

being fast and effective extraction methods.

6. The polar compounds studied were not found in the vegetable oils analyzed due probably to their different polarities, while phosphonic acid was present above the LOQ in 70% of the samples of analyzed nuts (20), which indicates that this compound should be monitored in these matrices.

7. SPE combined with the use of GC-FID provided satisfactory recovery and precision values for the analysis of MOSH and MOAH. Furthermore, the LOQ reached was similar to those obtained with more sophisticated instrumentation.

8. Both MOSH and MOAH were found in half of the samples analyzed (27) at concentrations up to 79.2 mg/kg of MOSH and 22.4 mg/kg of MOAH. These results give a good sight of how important is to make a strict regulation for these compounds and they should be monitored in routine analysis.

ANEXOS

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A rapid method for the determination of mycotoxins in edible vegetable oils by ultra-high performance liquid chromatography-tandem mass spectrometry

Author:
José L. Hidalgo-Ruiz,Roberto Romero-González,José Luis Martínez Vidal,Antonia Garrido Frenich

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Determination of mycotoxins in nuts by ultra high-performance liquid chromatography-tandem mass spectrometry: Looking for a representative matrix

Author:
José L. Hidalgo-Ruiz,Roberto Romero-González,José Luis Martínez Vidal,Antonia Garrido Frenich

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Determination of 3-monochloropropanediol esters and glycidyl esters in fatty matrices by ultra-high performance liquid chromatography-tandem mass spectrometry

Author:
José L. Hidalgo-Ruiz,Roberto Romero-González,José Luis Martínez Vidal,Antonia Garrido Frenich

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Monitoring of polar pesticides and contaminants in edible oils and nuts by liquid chromatography-tandem mass spectrometry

Author:
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