DEVELOPMENT OF ADVANCED ANALYTICAL METHODOLOGIES BASED ON GAS CHROMATOGRAPHY COUPLED TO MASS SPECTROMETRY FOR THE DETERMINATION OF POPs AND VOCs IN THE FOOD AND ENVIRONMENTAL FIELD

Doctoral Thesis

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"*This thesis has been accepted by the co-authors of the publications listed above that have waved the right to present them as a part of another PhD thesis*"

Doctors **Joaquim Beltrán Arandes**, **Tania Portolés Nicolau** full professor and professor of Analytical Chemistry at University Jaume I of Castellón, and **Adrian Covaci**, full professor of Toxicological Department at University of Antwerp,

HEREBY CERTIFY: That the doctoral thesis "DEVELOPMENT OF ADVANCED ANALYTICAL METHODOLOGIES BASED ON GAS CHROMATOGRAPHY COUPLED TO MASS SPECTROMETRY FOR THE DETERMINATION OF POPs AND VOCs IN THE FOOD AND ENVIRONMENTAL FIELD" has been developed under their supervision at the Research Institute for Pesticides and Water, Physical and Analytical Chemistry department of the University Jaume I and at the Toxicological Department of University of Antwerp, by **Carlos Sales Martínez**.

Which we certify for the record at Castellón de la Plana, on 15th of October of 2019

Dr. Joaquim Beltrán Arandes

Dr. Tania Portolés Nicolau

Dr. Adrian Covaci

This thesis corresponds to the compromise with the University Jaume I by the concession of a predoctoral grant for the formation of research personnel, since 1st of March of 2017

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This thesis has been developed and will be defended according with the requisites required to obtain the mention of International PhD and Joint PhD.

Prior to the defense of this thesis, this work has been evaluated by two external reviewers related to the research area. Dr. Alexander Van Nuijs (Toxicological Center, University of Antwerp, Belgium) and Dr. Nikolaos S Thomaidis (Department of Chemistry, University of Athens, Greece).

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Summary

Nowadays, with the vast quantity of chemicals released into the environment due to human activities in different ways, there is a stringent need to detect and quantify them, especially those chemicals with high toxicity. Chemicals can migrate from the products they are added to and end up in the environment. Once in the environment, they can bioaccumulate in the rophic chain and finally contaminate food. Among all contaminants, the most interesting to quantify are those which are persistent as they do not degrade easily. These contaminants are called persistent organic pollutants (POPs) and they are often also the most toxic ones. Among them, hallogenated flame retardants and dioxin-like polychlorinated biphenyls (DL-PCBs), with toxicological properties similar to dioxins, are one of the most dangerous ones. These toxicological effects include effects on immune and nervous system and endocrine and reproductive disruption. With such hazardous properties, the monitoring of these contaminants is a necessity, especially at trace levels. In this scenario, the development of advanced analytical methodologies based on gas chromatography coupled to mass spectrometry to quantify these contaminants is imperious.

In the food field, monitoring of POPs is not the only analytical problem to solve. Food fraud and adulteration is, unfortunately, a common practice worldwide, usually pursuing to sell bad quality products at high prizes. A special case is related to olive oil, not only because it may be adultered with low quality oils, but because a simple mislabelling or misclassification of its quality can result in huge economical profits or

losses. Quality classification of olive oils is made by PANEL TESTs, which are well trained people who determine the quality of oils according to their organoleptic properties. However, such technique is considerably expensive and time-consuming, as only a limited number of samples per day can be analysed by the panelists. Accordingly, the development of alternative analytical methodologies to complement the PANEL TEST could be of a high value to reduce quality classification costs and times, as well as to introduce an instrumental, objective and unbiased value to the classification process.

The work in the present thesis has been clearly divided in three parts:

In the **first part**, efforts were devoted to obtain a trace-level quantification method for halogenated flame retardants using large volume injections and gas chromatography coupled to tandem mass spectrometry GC-MS/MS. The methodology used the soft chemical ionization source (CI) in electron capture negative ionization (ECNI) mode to reach sub-parts per trillion quantification levels. Dechloranes, polybrominated diphenyl ethers (PBDEs) and novel brominated flame retardants (NBFRs) were determined in a single injection in food and serum.

In the **second part**, the potential of GC-MS/MS equipped with the novel atmospheric pressure chemical ionization source (APCI) was evaluated for the quantification of PBDEs, NBFRs (including hexabromocyclododecane (HBCD)) and DL-PCBs at trace levels in food and environmental samples. The findings, resulting in three different papers, showed an enhanced sensitivity and specificity of the methodology compared with traditional methods based on electron impact (EI) sources. The soft ionization provided by APCI promoted the

formation of highly abundant molecular $M^{+\cdot}$ and quasi-molecular $[M+H]^+$ ions, which were selected as precursor ions for SRM transitions. These highly specific transitions allowed the decrease of the detection and quantification limits around 10 times compared with traditional EI methodologies.

In the **third part,** two different untargeted GC-MS methodologies were developed as complementary tools to the PANEL TEST for the quality classification of olive oil. In the first methodology, GC-APCI-MS, with a hybrid quadrupole time of flight (QTOF) analyser, was used in combination with purge and trap (P&T) with solvent elution extraction. The metabolomics workflow developed included automatic spectral deconvolution using MzMINE2 and multivariate statistical analysis using Ez-Info. The new classification model not only achieved an accuracy of 70% compared to the PANEL TEST method, but also allowed the identification of compounds responsible for this quality. In the second methodology efforts were directed to optimize a methodology more easy to use, with reduced analysis times and costs which could be effectively applied in routine laboratories. Accordingly, the high sensitivity of P&T with thermal desorption was used, together with a state-of-the-art metabolomics workflow, to enhance the classification results as well as compound identification. To do so, GC-EI-MS data were processed with the novel software PARADISe2, with enhanced peak picking and tentative identification capabilities. The methodology allowed the creation of a classification method with an overall accuracy of 85%, as well as the identification of around 30 compounds responsible of defects in non-extra olive oils.

Resumen

Hoy en día, con la gran cantidad de productos químicos relacionados con las actividades del ser humano liberados al medio ambiente de diferentes maneras, existe la necesidad de detectarlos y cuantificarlos, especialmente los de alta toxicidad. Los productos químicos pueden migrar de los productos a los que se agregan y terminar en el medio ambiente. Una vez en el medio ambiente, pueden bioacumularse en la cadena trófica y finalmente contaminar los alimentos. Entre todos los contaminantes, los más interesantes para cuantificar son aquellos que no se degradan con facilidad. Este tipo de contaminantes se denominan contaminantes orgánicos persistentes (POPs) y a menudo también son los más tóxicos. Entre ellos, los retardantes de llama halogenados y los policlorobifenilos similares a las dioxinas (DL-PCB), con propiedades toxicológicas similares a las dioxinas, son unos de los más peligrosos. Estos efectos toxicológicos incluyen efectos sobre el sistema inmune y nervioso y la disrupción endocrina y reproductiva. Esto convierta al monitoreo de estos contaminantes en imprescindible, especialmente a niveles de trazas. En este escenario, es necesario el desarrollo de metodologías analíticas avanzadas basadas en la cromatografía de gases acoplada a la espectrometría de masas (GC-MS) para cuantificar estos contaminantes.

En el campo alimentario, el monitoreo de los POPs no es el único problema analítico a resolver. El fraude alimentario y la adulteración de productos de consumo son, desafortunadamente, una práctica común en todo el mundo, que generalmente busca vender productos de mala calidad a precios elevados. El aceite de oliva es un caso especial, no solo

porque es adulterado adulterado con aceites de baja calidad, sino porque un etiquetado incorrecto o una clasificación errónea de su calidad puede generar enormes ganancias o pérdidas económicas. La clasificación de los aceites de oliva acorde a su calidad se realiza mediante los *PANEL TEST*, compuestos por personas bien entrenadas que determinan la calidad de los aceites según sus propiedades organolépticas. Sin embargo, dicha técnica es considerablemente costosa y requiere mucho tiempo, ya que los panelistas solo pueden analizar un número limitado de muestras por día.

En consecuencia, el desarrollo de metodologías analíticas alternativas para complementar al PANEL TEST podría ser de gran valor para reducir los costes y tiempos de clasificación de los aceites, así como para introducir un valor instrumental e imparcial en el proceso de clasificación.

El trabajo en la presente tesis se ha dividido claramente en tres partes:

En la **primera parte**, los esfuerzos se dirigieron a la obtención de un método de cuantificación a nivel de trazas para retardantes de llama halogenados utilizando inyecciones de gran volumen y cromatografía de gases acoplada a espectrometría de masas en tándem GC-MS/MS. La metodología emplea la fuente de ionización química suave (CI) en el modo de ionización negativa de captura de electrones (ECNI) para alcanzar niveles de cuantificación por debajo de las partes por trillón. Decloranos, difenil éteres polibromados (PBDE) y nuevos retardantes de llama bromados (NBFR) se cuantificaron en alimentos y suero en una sola inyección.

Resumen

En la **segunda parte**, se evaluó el potencial de la técnica GC-MS/MS junto con la nueva fuente de ionización química a presión atmosférica (APCI) para la cuantificación de PBDEs, NBFRs (incluido el hexabromociclododecano (HBCD)) y DL-PCBs a niveles de traza en alimentos y muestras ambientales. Los resultados, publicados en tres artículos diferentes, demostraron una mayor sensibilidad y especificidad de la metodología en comparación con los métodos tradicionales basados en fuentes de impacto de electrones (EI). La ionización suave proporcionada por la fuente de APCI favoreció la formación de iones moleculares $M^{+\cdot}$ y cuasi-moleculares $[M+H]^+$ altamente abundantes que fueron seleccionados como iones precursores para las transiciones SRM. Estas transiciones altamente específicas permitieron disminuir los límites de detección y cuantificación alrededor de 10 veces en comparación con las metodologías tradicionales basadas en la fuente de EI.

En la **tercera parte**, se desarrollaron dos metodologías de análisis no dirigido por GC-MS como herramientas complementarias de la PRUEBA DE PANEL para la clasificación del aceite de oliva de acuerdo a su calidad. En la primera metodología, basada en GC-APCI-MS, con un analizador híbrido cuadrupolo- tiempo de vuelo (QTOF), se usó en combinación con purga y trampa (P&T) elución con solvente. El flujo de trabajo metabolómico desarrollado incluyó la deconvolución automática de espectros usando MzMINE2 y el análisis estadístico multivariante utilizando Ez-Info. El modelo de clasificación creado no solo logró una precisión del 70% en comparación con el método de PANEL TEST, sino que también permitió identificar los compuestos responsables de la calidad de los aceites. En la segunda metodología, los esfuerzos se dirigieron a optimizar una metodología más fácil de usar, con tiempos y costes de análisis reducidos que podría aplicarse efectivamente en

laboratorios de rutina. En consecuencia, se utilizó la alta sensibilidad de la P&T con desorción térmica, junto con un flujo de trabajo metabolómico de última generación, para mejorar los resultados de clasificación, así como la identificación de compuestos. Para ello, los datos de GC-EI-MS se procesaron con el nuevo software PARADISe2, con capacidades de selección de picos e identificación tentativa de compuestos mejoradas. La metodología permitió la creación de un método de clasificación de los aceites con una precisión general del 85%, así como la identificación de alrededor de 30 compuestos responsables de defectos en los aceites de oliva no extra.

Overzicht

Voor de meeste chemicaliën die gerelateerd kunnen worden aan menselijke activiteiten en die tegenwoordig op verschillende manieren in het milieu terecht komen is er de noodzaak om deze te detecteren en kwantificeren, in het bijzonder de chemicaliën met hoge toxiciteit. Stoffen kunnen migreren uit produkten, waar zij aan zijn toegevoegd, en zo in het milieu terecht komen. Eenmaal in het milieu kunnen ze ophopen en bioaccumuleren en uiteindelijk ook in de voedselketen terecht komen. Van alle contaminanten, zijn persistente stoffen de meest interessante stoffen om te kwantificeren, omdat deze niet makkelijk degraderen. Dit soort contaminanten worden in het engels *persistent organic pollutants* (POPs) genoemd en zijn vaak ook het meest toxisch. Onder hen zijn de gehalogeneerde brandvertragers en polychloorbifenylen (DL-PCBs), die met dioxine-achtige toxicologische eigenschappen het meest gevaarlijk zijn. De toxische eigenschappen hebben onder andere effect op het immuunsysteem en het zenuwstelsel. Met zulke gevaarlijk eigenschappen, is het van groot belang om deze stoffen te monitoren en controleren, met name ook op lage concentratie niveaus. Om deze reden is de ontwikkeling van een analytische methode, gebasseerd op gas chromatografie en gekoppeld aan massa spectrometrie, om deze contaminanten te kunnen kwantificeren een noodzaak.

Het controleren van POPs in voedsel is niet het enige analytische problem wat opgelost moet worden. Voedsel fraude en vervalsing is helaas een gebruikelijke praktijk wereldwijd, meestal word dit gedaan om produkten van slechte kwaliteit te verkopen tegen een hoge prijs. Dit

wordt met name gedaan bij olijf olie, hierbij wordt de olie niet alleen vervalst en aangelengd met olie van lage kwaliteit, maar ook wordt simple weg het label vervalst of wordt de olie verkeerd gekwalificeerd, dit met grote economische winsten of verliezen als gevolg. Kwaliteit classificering van olijf olie wordt uitgevoerd met panel testen, deze bestaan uit goed getrainde mensen door wie de kwaliteit van olie wordt bepaald aan de hand van organoleptische eigenschappen. Deze technieken zijn echter behoorlijk duur en kost veel tijd, omdat enkel een gelimiteerd nummer van stalen per dag kunnen worden geanalyseerd door het test panel. De ontwikkeling van een alternatieve analytische methode die de panel test complementeerd is daarom van grote waarde om kosten en tijd te besparen, maar ook om een instrumentele waarde wat meetbaar is toe tevoegen aan het kwalificatie proces.

Het werk gepresenteerd in dit promotie proefschrift is verdeeld in drie delen:

In het **eerste deel** is er een kwantitatieve methode ontwikkeld om gehalogeneerde brandvertragers te meten op zeer lage concentratie niveaus met behulp van groot volume injectie en gaschromatografie gekoppeld aan tandem massa spectrometrie GC-MS/MS. De methode maakt gebruik van een zachte chemische ionizatie bron (CI) dat werkt in electron capture negatieve ionizatie mode (ECNI) om de lage parts per trillion (ppt) niveaus te kunnen kwantificeren. Dechloranen, polygebromeerde difenylethers (PBDEs) en nieuwe gebromineerde brandvertragers (NBFRs) werden in een enkel injectie in voedsel en serum bepaald.

In het **tweede deel** is het potentieel van GC-MS/MS met een innovatieve chemische ionizatie bron dat werkt onder atmosferische druk (APCI)

geevaluaeerd voor de kwantificatie van PBDEs, NBFRs (inclusief hexabroomcyclododekaan (HBCD)) en DL-PCBs op ppt niveau in voedsel en milieu stalen. De bevindingen, die geresulteerd hebben in drie artikelen, lieten een betere gevoeligheid en specificiteit zien ten opzichte van traditionele methoden gebasseerd op ionizatie bronnen met electron impact (EI). De zachte ionizatie verkregen met APCI bevorderde de vorming van de zeer gevoelige moleculair $M^{+\cdot}$ en quasi-moleculair $[M+H]^+$ ionen, welke als precursor ionen werden geselecteerd voor SRM transities. De detectie en kwantificatie limieten konden met deze zeer specifieke transities tot ongeveer 10 keer verlaagd worden in vergelijking met de tradtionele EI methoden.

In het **derde deel** werden twee verschillende GC-MS methoden ontwikkeld waarbij er geen doelstoffen niet worden geselecteerd. Deze methoden worden toegepast als extra hulpmiddel voor de panel test, voor de kwaliteit classificering van olijf olie. De eerste GC-APCI-MS methode, met hybrid quadrupole time of flight (QTOF) massa analysator, werd gebruikt in combinatie met purge and trap (P&T) en solvent elutie extractie. De ontwikkelde metabolomische werkwijze bedroeg een automatische spectrale deconvolutie gebruikmakend van MzMINE2 en een statistische multivariatie analyse gebruikmakend van Ez-Info. Niet alleen werd er een juistheid van 70% verkregen met het gecreëerde classificatie model ten opzichte van de panel test, maar ook konden de stoffen verantwoordelijk voor deze kwaliteit worden geidentificeerd. De tweede methode werd geoptimaliseerd voor een makkelijker gebruik, met een snellere analyze tijd en lagere kosten, en welke efficienter kan worden toegepast in routine laboratoria. Hiervoor werd de gevoeligere P&T met thermale desorptie gebruikt, samen met een geavanceerde

metabolomische werkwijze, om de classificatie van olijf olie en identificatie van componenten te verbeteren. Om dit doel te bereiken werd de GC-EI-MS data geprocest met de nieuwe PARADISe2 software, wat verbeterde peak picking en identificatie mogelijkheden heeft. Dit heeft het mogelijk gemaakt om een classificatie methode te ontwikkelen met een juistheid 85%, als mede de identificatie van ongeveer 30 stoffen, verantwoordelijk voor defecten in non-extra olijf olie.

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Objectives
Objetivos

Objectives

This doctoral thesis has been developed in the Research Institute for Pesticides and Water (IUPA) at University Jaume I and the Toxicological Center at University of Antwerp, as a Joint PhD, with the collaboration with several national and international research groups. These collaborations have been crucial for the successful completion of the different studies undertaken. **Chapter 2** has been carried out mainly at the Toxicological Department of University of Antwerp, Belgium. The PhD student performed two three-months stays in the framework of the Joint PhD thesis, with Prof. Adrian Covaci as co-supervisor. **Chapter 3** has been completed thanks to the collaboration of three different entities. Scientific Article 2 and Scientific Article 3 were developed with the partnership of Dr. Belén Gómara and her research group at General Organic Chemistry Institute (IQOC) from CSIC, Madrid. Scientific Article 3 was also developed with the collaboration of Dr. Esteban Abad, and his research group at Institute of Environmental Assessment and Water Research (IDAEA) of CSIC in Barcelona and with the help of Dr. Heidelore Fiedler from MTM Research Centre at Orebro University, Sweden. Scientific Article 4 was developed also with the help of IDAEA researchers. Scientific Article 6 was undertaken thanks to the collaboration with Dr. Morten Danielsen and Dr. Lea Johnsen, from MS-Omics, Denmark. All these collaborations and works were developed with the following general objectives in mind:

- Evaluation of the potential of LVI with GC-ECNI-MS/MS for the trace determination of POPs in food and blood samples.

- Evaluation of GC-APCI-QqQ MS/MS for the trace quantification of legacy and novel flame retardants, as well as of dioxin like PCBs (DL-PCBs) in complex food and environmental samples.
- Development of advanced analytical methodologies based on GC-MS for the quality classification of olive oils using different volatile organic compounds extraction techniques and metabolomics-based statistical analysis.

As a consequence of the three main objectives of the thesis, more specific objectives can be stated.

- Evaluation of the application of different gas chromatography inlets, and configurations to reach sub-ppt quantification levels using different mass spectrometers (single quadrupole (Q), triple quadrupole (QqQ) and hybrid quadrupole-time of flight (Q-TOF)).
- Development of GC-QqQ MS/MS advanced methodologies using soft ionization sources to obtain specific and sensitive transitions for the quantification of legacy and novel POPs at trace level in complex matrices. Special emphasis has been placed in the atmospheric pressure chemical ionization source, revived in the last years for GC.
- Development and evaluation of different purge and trap (P&T) methodologies (solvent elution and thermal desorption) for preconcentration of VOCs present in olive oil.
- Study the applicability of different automatic deconvolution software (xcms, MzMINE2 and PARADISe2) to obtain relevant information from olive oil sample data acquired using untargeted analyses by GC-APCI-QTOF HRMS and GC-EI-Q MS.

- Development of multivariate statistical methodologies to determine olive oil quality based on untargeted GC-MS data using Ez-Info.

Identification of relevant compounds in olive oil responsible of overall quality and single defects making use of the mass accuracy provided by GC-APCI-QTOF HRMS and the use of spectral libraries on GC-EI-MS data.

Objectivos

Esta tesis doctoral ha sido desarrollada en el Instituto de Investigación de Pesticidas y Agua (IUPA) de la Universidad Jaume I y el Centro Toxicológico de la Universidad de Amberes, en la modalidad de Joint PhD, con la colaboración de múltiples grupos de investigación nacionales e internacionales. Estas colaboraciones han sido cruciales para completar con éxito los diferentes estudios realizados. El **Capítulo 2** se ha llevado a cabo principalmente en el Departamento de Toxicología de la Universidad de Amberes, Bélgica. El estudiante de doctorado realizó dos estancias de tres meses en el marco de la tesis de doctorado en la modalidad de Joint PhD, con el Prof. Adrian Covaci como co-supervisor. El **Capítulo 3** se ha completado gracias a la colaboración de tres entidades diferentes. El Artículo Científico 2 y el Artículo Científico 3 se desarrollaron con la colaboración de la Dra. Belén Gómara y su grupo de investigación en el Instituto General de Química Orgánica (IQOC) del CSIC, Madrid. El Artículo Científico 3 también se desarrolló con la colaboración del Dr. Esteban Abad y su grupo de investigación en el Instituto de Evaluación Ambiental e Investigación del Agua (IDAEA) del CSIC en Barcelona y con la ayuda del Dr. Heidelore Fiedler del Centro de Investigación MTM de la Universidad de Orebro, Suecia. El Artículo Científico 4 también fue desarrollado con la ayuda de investigadores del IDAEA. El Artículo Científico 6 se llevó a cabo gracias a la colaboración con el Dr. Morten Danielsen y la Dra. Lea Johnsen, de MS-Omics, Dinamarca. Todas estas colaboraciones y trabajos se desarrollaron con los siguientes objetivos generales en mente:

- Evaluación del potencial de LVI con GC-ECNI-MS/MS para la determinación de trazas de POPs en muestras de alimentos y sangre.

- Evaluación de la metodología de GC-APCI-QqQ MS / MS para la cuantificación de trazas de retardantes de llama tradicionales y novedosos, así como PCBs tipo dioxina (DL-PCBs) en muestras complejas de alimentos y medioambientales.

- Desarrollo de metodologías analíticas avanzadas basadas en GC-MS para la clasificación de aceites de oliva de acuerdo con su calidad utilizando diferentes técnicas de extracción de compuestos orgánicos volátiles y análisis estadísticos basados en metabolómica.

Como consecuencia de los tres objetivos principales de la tesis, se pueden establecer objetivos más específicos.

- Evaluación de la aplicación de diferentes inyectores para cromatografía de gases y sus configuraciones para alcanzar niveles de cuantificación sub-ppt utilizando diferentes espectrómetros de masas (cuadrupolo simple (Q), triple cuadrupolo (QqQ) y analizador híbrido cuadrupolo-tiempo de vuelo (Q-TOF)).

- Desarrollo de metodologías avanzadas basadas en GC-QqQ MS/MS que utilizan fuentes de ionización suave para obtener transiciones específicas y sensibles para la cuantificación de POPs tradicionales y nuevos a nivel de trazas en matrices complejas. Se ha puesto especial énfasis en la fuente de ionización química a presión atmosférica, revivida en los últimos años para su acoplamiento a GC.

- Desarrollo y evaluación de diferentes metodologías de purga y trampa (P&T) (elución con solvente y desorción térmica) para la preconcentración de VOCs presentes en el aceite de oliva.

- Estudio de la aplicabilidad de diferentes softwares de desconvolución automática de espectros (xcms, MzMINE2 y PARADISe2)

para obtener información relevante de los datos de muestras de aceite de oliva adquiridos mediante análisis no dirigidos por GC-APCI-QTOF HRMS y GC-EI-Q MS.

- Desarrollo de metodologías estadísticas de análisis multivariante para determinar la calidad del aceite de oliva a partir de datos de GC-MS adquiridos de forma no dirigida utilizando Ez-Info.

- Identificación de compuestos relevantes en el aceite de oliva responsables de la calidad general y defectos únicos utilizando la precisión de masa proporcionada por GC-APCI-QTOF HRMS y el uso de bibliotecas espectrales en los datos de GC-EI-MS.

Chapter 1

General Introduction

1.1 Strategies in analytical chemistry

Analytical chemistry is a metrological science that helps to solve problems of society, in most cases, through studies that rely on determination of substances in a specified matrix in order to get answers to some questions relatives to the studied system. There are several approaches to define the problem and to get the information relative to the system. The most classical approach is typically focused on the sample and quantitation of individual compounds as a way to define the system characteristics. Nowadays, there is a more holistic approach that considers the whole system information focusing on the population and the overall fingerprint of samples, which is well known as metabolomics. Both approaches are quite different and require analytical and working strategies that need to be correctly addressed, not only considering time and efforts but also analytical instrumentation. It is important to define the right strategy to achieve the analytical purpose defined to solve the initial problem, thus determining the validity of the experimental data and the final conclusions.

As indicated above, the most classical approach is devoted to determination of individual compounds in real samples in a target working approach based on quantitative determinations. Among the analytical techniques and procedures capable of producing quantitative results for organic compounds, chromatographic techniques are the most widely used, especially coupled to mass spectrometry if reliable identification of analytes is expected.

Chromatography has evolved considerably since its appearance. First, it was only able to separate a few components, with poor or inexistent resolution. With technological advances not only in separation, but also in computation, chromatography started to be controlled by microprocessors and coupled to more complex detectors. Among all detection techniques, mass spectrometry is, by far, the analytical technique which provides the most abundant, and of highest quality information about the compounds of interest. It allows not only to quantify well known compounds, but also to identify unknown components.

In general, the analysis of one to several compounds by chromatography coupled to mass spectrometry can be approached in three different ways: *pre-target, post-target* and *non-target* mode.

In pre-target analysis the compounds of interest are known and selected from the beginning, so each analytical step is optimized and directed towards obtaining the best results for the selected analytes. Pre-target analysis constitute up to 90% of the analyses performed globally. By applying a pre-target analysis, only the information regarding the compounds of interest is acquired, while the rest of the sample information is lost. When performing pre-target analysis, standards must be injected during the sequence, allowing the quantification of the analytes. The main drawback of target analysis is the limited amount of compounds determined at once.

Post-target analysis consists in looking back to already acquired data searching for new discovered compounds. Post-target needs a more generic determination, as the information regarding unknown compounds must be acquired simultaneously. This may imply that the compounds of

interest are determined in conditions that are not optimal, in contrast to the pre-target analysis, but the acquired data is of a higher value, and can be reprocessed several times looking for newly considered *targets*. Nowadays, there are thousands of compounds of interest, including toxic contaminants, precious flavours or compounds which presence is indicative of a disease or attributable to some specific characteristic of the sample.

Together with quantitative procedures, non-target or screening methods devoted to a more qualitative approach to describe the samples can also be considered, not only as a previous step of quantitative determinations, but as a characterization methodology in itself.

Metabolomics is defined as ‘‘the unbiased, global screening approach to classify samples based on metabolite patterns or fingerprints that change in response to disease, environmental or genetic perturbations with the ultimate goal to identify discriminating metabolites” (Cevallos-Cevallos, Reyes-De-Corcuera, Etxeberria, Danyluk, & Rodrick, 2009). Omics techniques application is increasing permanently, with hundreds of methodologies being optimized in very different fields including genomics, lipidomics, proteomics, and foodomics, among others.

In all cases, relatively generic methodologies are necessary, being gas chromatography (GC) and liquid chromatography (LC) coupled to high resolution mass spectrometry (HRMS) as one of the preferred technologies. The universality of the techniques coupled to their sensitivity and capability of provide molecular and structural information of the compounds detected make them perfect for the purpose. Both GC-HRMS and LC-HRMS produce a high amount of 3D chemical information, as samples are characterized in terms of retention time, mass spectra and

intensity of the ions which reach the detector. In order to widen the already described determination capabilities of target methodologies, non-target or *untargeted* methods, able to determine up to thousands of compounds at once have been increasingly developed in recent years. Non-targeted analysis relies in generic sample treatments and determination methods to obtain the maximum sample information possible. Untargeted analysis require of state-of-the-art instruments as well as computers and software able to deal with such amount of chemical information. After detection of relevant compounds, their identification can be performed using spectral libraries or structural identification based on the fragments observed. Non-target analysis can be useful to identify individual compounds, but also to highlight relevant components of a determined population.

Dealing manually with such amount of information manually is time-consuming and barely impossible to accomplish, as the number of ions to check is regularly close to hundreds of thousands per sample. In this context, automatic spectral deconvolution software is being continuously developed to deal with more and more complex HRMS data. Each software works differently and has a different output, with some of them giving a relationship of absolute area for individual ions at a determined retention time (as R and MzMine) (Díaz, Pozo, Sancho, & Hernández, 2014; Kind, Tolstikov, Fiehn, & Weiss, 2007), and some other giving a relationship of absolute intensity per compound at a retention time, as does the novel software PARADISe (Johnsen, Skou, Khakimov, & Bro, 2017). In both cases, the output, while being less difficult to handle than raw 3D data produced by the instrument, contains plenty of chemical information for a person to handle it manually. Here, the use of statistical tools, mainly multivariate

analysis, is crucial to distinguish the relevant chemical information from the noise of the matrix.

When performing multivariate analysis, there are three statistical methods widely used for group classification. Principal component analysis (PCA), provides a fast, unsupervised distribution of the samples according to linear combinations of their features called principal components (PCs) (Hu et al., 2014). PCA is also useful to reduce data matrix and check the correct position of the quality controls (QC) if used (Gil-Solsona et al., 2016). When PCA is not powerful enough to distinguish between groups, supervised analyses can be applied. Partial least squares-discriminant analysis (PLS-DA) and orthogonal PLS-DA (OPLS-DA) are useful multivariate techniques to enhance group differentiations. They work similar to PCA, creating linear combinations of features called latent variables (LVs). While PCs are created to explain the maximum variance possible in the dataset, LVs are generated to maximize group differences (Ballabio & Consonni, 2013). OPLS-DA works similar to PLS-DA, focusing on the differences between only two groups of samples. Its most interesting feature is the creation of a plot known as S-Plot, to identify which features are more relevant in each group (Gil-Solsona et al., 2016). These statistical tools can reveal the compounds *(markers)* responsible for a disease, a specific good flavour, or adulteration of a sample meant for consumption, also named *markers*.

Once identified through omics-techniques, these markers are normally quantified to assess the quality of a sample or a patient disease. Tough quantitative metabolomics methodologies are rising, especially with the apparition of data independent acquisition (DIA) techniques (Meyer & Schilling, 2017), such methodologies require the latest instrumentation, as

well as highly trained personal. In addition, untargeted techniques often have several sensitivity limitations. Therefore, markers found by untargeted metabolomics are often quantified in routine methods using target methodologies.

In the present thesis, both approaches are investigated using GC-MS; in one hand the use of targeted methodologies for the quantification of ultra-trace levels of POPs. On the other hand, the development of untargeted metabolomics methods for the classification of olive oil according to quality.

1.2 Gas Chromatography-Mass Spectrometry

Gas chromatography (GC) is the most suitable technique for the separation of volatile, semi-volatile and thermostable compounds, able to resolve a huge amount of species in a single analysis. Coupled with mass spectrometry (MS) it becomes one of the most powerful techniques for the identification and quantification of a broad range of non-polar and semi-polar compounds. It takes advantage of the capabilities of MS to obtain information about the composition and structure of the detected analytes. Due to this fact, GC-MS is widely used in environmental, toxicological and food fields, among others.

1.2.1 Injection

The way the sample is introduced in the GC system is crucial to obtain good results. Nowadays, there are several inlets available, which allow us to introduce more or less sample volume depending on the concentration of the compounds, and to do it at different inlet temperatures. Accordingly,

there are 3 basic different inlets for GC, each of them with their special features and working modes.

On column

The on column inlet is the most basic one. As its name suggests, the sample is directly introduced inside the column, condensing solvent and compounds of interest and avoiding discrimination due to the boiling temperature of different compounds. **Figure 1.1** shows the diagram for an *On Column* inlet.

The amount of sample introduced on an on column inlet is limited, and there is no possibility of splitting the extract in case of high compound concentration. To do so, there is another inlet with such capabilities, known as *split/splitless* inlet.

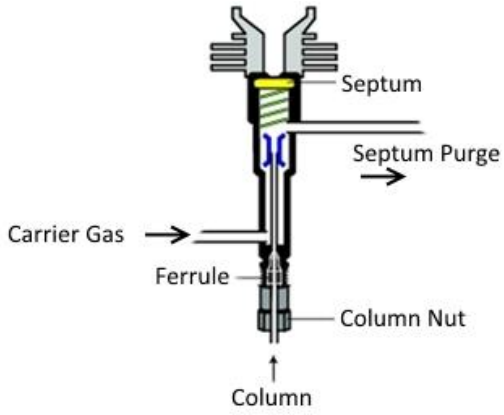

Figure 1.1. Diagram of an on column inlet

Split/Splitless

The split/splitless inlet is shown in **Figure 1.2.** It has a glass tube known as liner and an additional tubbing which goes from the inlet to the outside, known as split line, which flow can be adjusted depending on the purpose of the analysis and the characteristics of the extract.

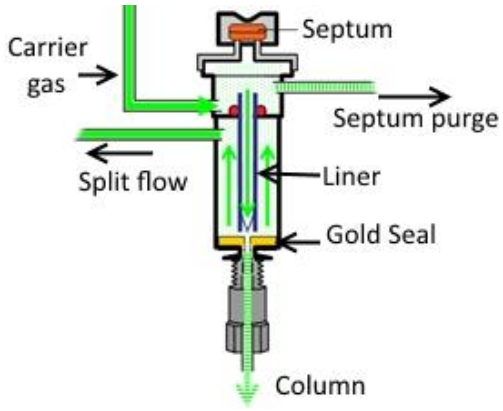

Figure 1.2. Diagram of a split/splitless inlet.

If the extract has high compound concentration or is dirty because of the matrix, the split can be set to a high value, which means working in *split* mode. This value determines the ratio of extract that will actually enter the column. For example, a 10:1 split ratio means that only a 10% of the injected extract will enter the column. If there is no problem with the sample concentration or cleanliness, the inlet can be set into *splitless* or *pulsed splitless* mode, in which the entire extract enters into the column in vapor phase. The pulse can be useful to avoid transfer discrimination due to the different boiling points of the compounds.

On the contrary, sometimes there is a need of preconcentrating the extract in the inlet due to low compound concentration in the sample, or a need of applying a ramped temperature program in the inlet to avoid

compound discrimination. In these cases, the *multi-mode inlet* (MMI) which is a type of programmable temperature vaporizer (PTV), is the best choice to use.

Multimode

Figure 1.3. shows a diagram of the multimode inlet. The main difference with the split/splitless inlet relies in the possibility to apply a temperature gradient. MMI allows working in split, (cold) splitless, (cold) pulsed splitless and solvent vent mode. In split mode it behaves exactly as a split/splitless inlet. In cold splitless or cold pulsed splitless, it acts in the same way than a split/splitless with the difference of the injection temperature. With this mode, all the injected volume is condensed in the liner and then evaporated with the ramp of temperature, achieving total transfer of the compounds into the GC column.

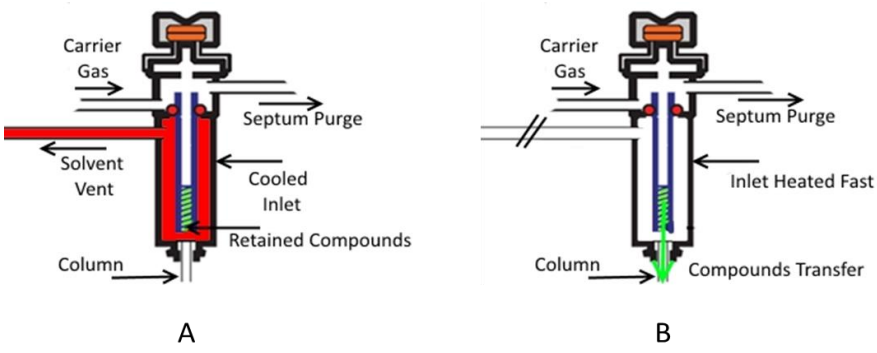

Figure 1.3. Diagram of MMI. A) Solvent venting and sample concentration at low inlet temperature. B) Heated inlet transfers compounds to the column.

The most interesting working mode of the MMI inlet is the solvent vent mode. It is applied when using a large volume of injection (LVI). In this mode, the extract is introduced at a temperature below the boiling point of the solvent, with the split line open with a high flow. In that way, the

compounds are retained in the liner while the solvent is evaporated. Once the solvent has been evaporated, the split vent and the septum purge line are closed, the inlet temperature is raised fast, and the compounds enter the column, where they are refocused due to the low column temperature. In this way, a high preconcentration can be achieved, while avoiding discrimination of the low boilers.

1.2.2 Chromatographic column

The hearth of GC is the chromatographic column, which has evolved from packed, unreliable columns to state-of-the-art capillary columns, with impressive resolving power and unlimited stationary phase compositions. Choosing the right column is crucial in order to obtain the best parameters for the chromatographic analysis. In the present thesis, several column configurations have been tested and applied depending on the compounds of interest and the purpose of the works. **Table 1** shows a list of the main columns used for each type of analysis.

1.2.3 Ionization sources in mass spectrometry

After an effective chromatographic separation, molecules must be ionized in order to become detectable on a MS as mass over charge (*m/z*) ions. GC ionization started with electron ionization (EI) early in 1918. EI operates at high temperature and high vacuum, and provides very energetic ions, which induce extensive fragmentation as a result of the internal energy transmitted by the kinetic energy of electrons. When the molecule of interest enters in the ion source, an electron beam, coming from a tungsten filament, impacts the gas-phase molecules, which are converted into a radical cation $M^{+\bullet}$.

Table 1. List of common chromatographic stationary phases for GC, together with their applications and commercial names.

Stationary phase	Polarity	Application	GC columns
100% Dimethylpolysiloxane	Low	PBDEs, PAHs, non-polar organic compounds	SPB-1 Rtx-1 OV-1 DB-1
(5% Phenyl)-methylpolysiloxane	Low	Alkaloids, drugs, FAMEs, halogenated compounds, aromatic compounds	Rtx-5 SPB-5 DB-5 ZB-5
50% Phenyl 50% dimethyl polysiloxane	Medium	Drugs, glycols, pesticides, steroids	Rtx-50 SPB-50 DB-17 AT-50
Polyethylene glycol	High	Alcohol, ester and aldehyde analysis, highly volatile compounds	DB-WAX Supelcowax 10 BP-20 Rtx-WAX
PLOT	High	Room temperature gases	HP-PLOT Molesieve Rt-Msieve 5A MXT-Msieve 5A

In terms of energy transfer, if the electron interacts the molecule efficiently, the energy transferred may be greater than the ionization energy (IE) of the molecule. EI is the most universal and robust ionization source, as its rough operating conditions permit the same fragmentation for each molecule with independence of the instrument and the laboratory of the analysis. This allows the construction of spectral libraries, which are very helpful in routine analyses. However, its extensive molecule fragmentation results, in some cases, in the total fragmentation of the molecular ion, which may result in complex spectra for elucidation of unknown molecules.

In an attempt to solve the problems linked to this excessive fragmentation, in 1966 the chemical ionization (CI) source was developed. In this case, the source makes use of a reagent gas, normally ammonia or methane, which is charged by the ion beam. In chemical ionization new ionized species are formed when gaseous molecules interact with ions. Chemical ionization may involve the transfer of an electron, proton, or other charged species

between the reactants, which can be the neutral analyte (M) and ions from the reagent gas. As the energy transmitted to the molecule of interest is lower, the fragmentation in CI sources is generally limited. As a consequence, the molecular ion is easier to identify, though less structural information is acquired, in comparison with EI sources. CI has demonstrated huge sensitivity capabilities, especially for the analysis of halogenated compounds working in negative ionization mode, but it is not sufficiently soft to obtain the molecular ion in all cases, nor enough universal to be used as an alternative to EI.

In this scenario, in 1979, the capability of using chemical ionization at atmospheric pressure was explored (Lovett, Reid, Buckley, French, & Cameron, 1979) with the development of the atmospheric pressure chemical ionization (APCI) source. This novel source was effectively coupled to GC during the last decade, and produces a really soft ionization with the use of nitrogen as reagent gas. As no vacuum is needed, a high stream of N_2 pushes the analytes from the column end to the source, with higher ionization efficiency than regular CI. As ionization occur at atmospheric pressure, there is always a presence of water in the source. With these conditions, APCI produces two different simultaneous ionization pathways; charge-transfer, in which N_2 losses an electron and transfers the positive charge to the molecule of interest, producing the $M^{+\bullet}$ molecular ion, and proton transfer, in which the water gets first ionized as H_3O^+ and then transfers a proton to the target molecule, producing the quasimolecular ion, or protonated molecule $[M+H]^+$. Detailed ionization mechanisms are displayed in **Figure 1.4.** Both ionization pathways provide molecular information about the analyte, which can be of a high value when performing non-target analysis and in

order to obtain highly specific transitions in SRM methods for target analysis. Both APCI benefits will be discussed in detail in chapters 3 and 4 of the present thesis. APCI allows to work at higher carrier flow rates, which permits to produce fast chromatographic analysis, with shorter elution times and enhanced peak shapes.

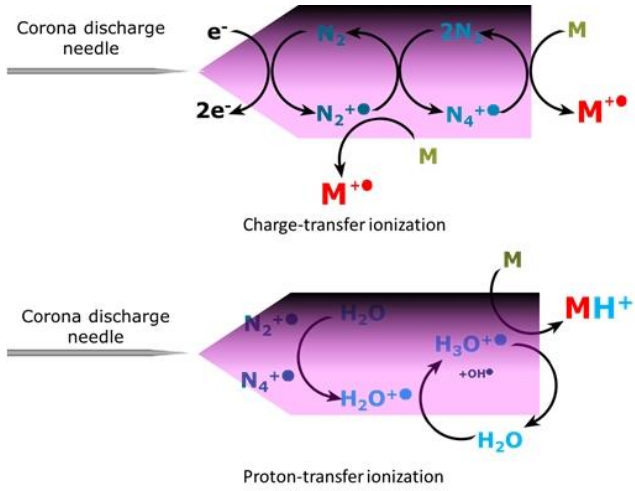

Figure 1.4. Ionization mechanisms in APCI source.

1.2.4 Analysers in MS

Once the charged molecule leaves the ionization source, it enters into the mass analyser, which basically discriminates between different mass-over-charge (*m/z*) ions to allow only the selected ones to reach the detector.

Single Quadrupole

There are several types of mass analysers, depending on the way they work and their purpose. The most basic of them is the single quadrupole (single quad or just *Q*), which consists in four metallic cylindrical bars

placed in parallel two versus two. Different radiofrequency and continuous current are applied in opposite bars to determine the *m/z* relations which are allowed to move through them and reach the detector. **Figure 1.5** represents the functioning of a single quadrupole.

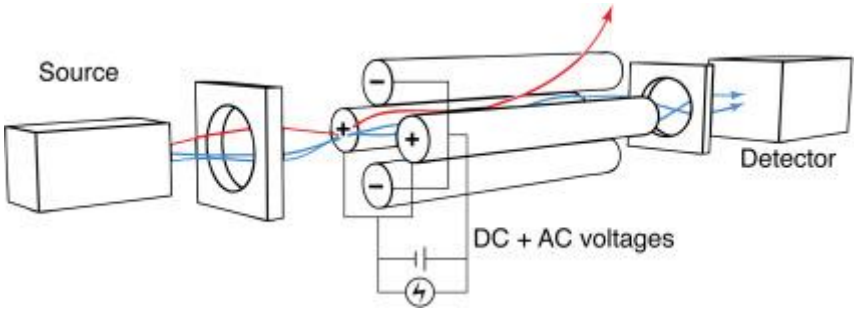

Figure 1.5. Schematic representation of a single quadrupole. Ions with *m/z* selected (in blue) go through the analyser into the detector while ions with different *m/z* are repelled out from the path to the detector.

With this configuration, two different data acquisition modes can be applied; *full scan*, in which all *m/z* ions over a selected range are screened by the detector, by application of a set of different potential, and *selected ion monitoring* (SIM) in which only a specific *m/z* ion is allowed to reach the detector. Full scan is preferred when looking for unknowns as the whole spectrum of a molecule is acquired. Due to the large number of ions that the detector has to monitor in a short period of time, sensitivity can be sometimes compromised in this working mode. SIM sensitivity is by far higher than full-scan, as only one *m/z* (or a small group of ions) is monitored in each scan. SIM methodologies can only be applied for pre-target methodologies, as no other chemical information is saved for post-target purposes.

Single quadrupole has a main drawback: when working in full scan mode, the obtained spectra has unitary resolution, so there are a huge number of compounds giving exactly the same fragmentation, which can lead to errors when looking in spectral libraries. When working in SIM mode, in a similar way, there are thousands of interferences that can co-elute with the compound of interest and which produce an in-source (fragment) ion identical (in unitary resolution) to the quantification ion of an analyte.

To improve the identification and quantification issues of the single quadrupole analyser, tandem mass spectrometry was developed, which mainly consists in combining two analysers and adding a collision cell in the middle to fragment the ions selected in the first *m/z* filter. There are several tandem MS configurations available in the market, but in the present thesis the efforts have been directed in the capabilities of two of them for identification and quantification purposes. On one side, the hybrid quadrupole time-of-flight (QTOF) analyser to identify molecules confidently. On the other side the tandem MS/MS triple quadrupole for more sensitive and more specific quantification.

QTOF

The QTOF hybrid mass spectrometer makes use of the abovementioned properties of a single quadrupole together with the enhanced resolution of a time-of-flight analyser (TOF) to obtain high accuracy spectra in full spectrum acquisition mode and high accuracy product ion spectra in tandem MS mode.

TOF analysers impulses the ions by applying an orthogonal electric potential and accelerating them towards the detector. The same amount of energy is conferred to each ion in form of kinetic energy. With this

kinetic energy, the ions with less mass acquire more velocity, reaching the detector faster (an inferior time-of-light) than the ions with higher mass. TOF analysers can be completely linear or can be equipped with one reflector (or even two), which reflects the ions back, achieving more resolution (more flight distance) with a shorter tube. Both configurations are displayed in **figure 1.6.**

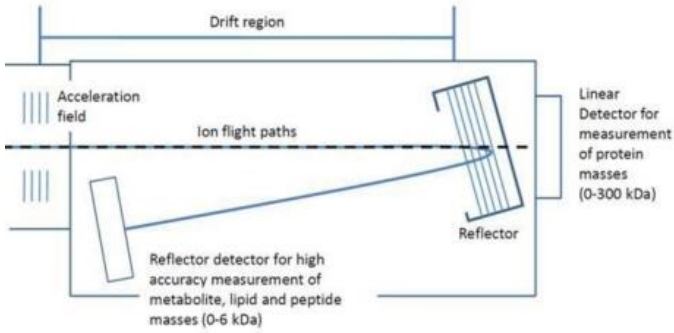

Figure 1.6. Schematic representation of a TOF analyser

Novel QTOF systems can work in MS^E mode, which implies the simultaneous acquisition of the spectra at two different collision energies. In MS^E mode, the quadrupole acts as an ion transmitter, without filtering any *m/z*. In the collision cell, which consist normally on another quadrupole or a hexapole, full of inert gas (Ar or N_2), ions accelerate with a determined energy, with possibility of additional fragmentation. This permits the acquisition of two simultaneous spectra; one obtained applying around 4eV of collision energy, known as low energy (LE) function, and another one, applying a collision energy ramp, generally between 15 and 60 eV, known as high energy (HE) function. In GC-APCI, minimal fragmentation is observed in the LE function, obtaining the molecular $M^{+\bullet}$ and/or quasi-molecular $[M+H]^+$ ion as base peak. In the high

energy function, the higher collision energy applied produces the fragmentation of this molecular ion or protonated molecule, together with all other ions that reach the collision cell.

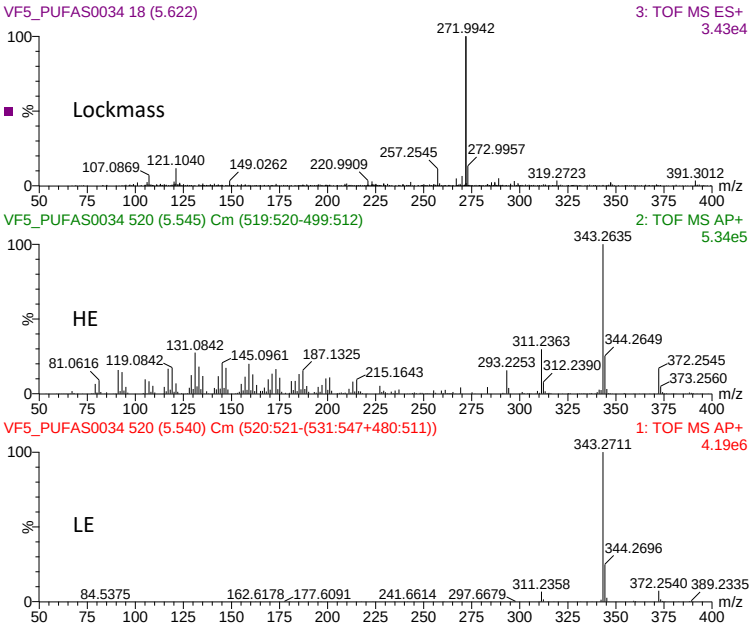

Figure 1.7. GC-APCI-MS^E mass spectra for Docosahexaenoic Acid (DHA) methyl ester.

Figure 1.7 illustrates this acquisition mode with the spectra obtained for docosahexanoic acid (DHA) methyl ester. The bottom spectrum corresponds to LE function. As it can be appreciated, the $[M+H]^+$ ion 343.2711 is the base peak, followed by ion 344.2696 (C_{13} pattern). Minimal fragmentation is observed. However, in the HE function, the typical fatty acid fragmentation pattern is observed as a consequence of the higher collision energy applied. The last spectra corresponds to lockmass ion 271.9942 (Octafluoronaphtalene). This MS^E mode from Waters Corp®, which is also known as *All ions MS/MS* (Agilent Technologies ®) and *All ions fragmentation* (Thermo Scientific®) allows to obtain both molecular

information and structural information of a molecule in one single run. It is important to note that, though this acquisition mode has some advantages, it may not be sufficiently sensitive for some applications, as three functions have to be acquired simultaneously (LE, HE and lockmass). Additionally, in cases of excessive in-source fragmentation and chromatographic coelution of different compounds, the HE spectra does not permit to predict which ion is coming from the molecular ion, so an actual MS/MS experiment must be performed to confirm the identity of one compound. In MS/MS mode, selected ions are filtered in the quadrupole and further fragmented in the collision cell, to finally enter the TOF and reach the detector. This working mode permits to obtain high accuracy mass spectra corresponding to the fragments of a specific *m/z*, typically the molecular ion of the analyte of interest. This mode is of a high value for elucidation purposes, as it permits to assign fragments with a mass accuracy in the order of a few mDa to a specific ion, cleaning spectral noise that could interfere in the elucidation in regular full-scan experiments.

Triple Quadrupole

As indicated above, single quadrupole is not usually sufficiently sensitive to reach the quantification needs for some specific compounds, especially for toxic POPs and emerging contaminants in the environment. Though QTOF and other analysers (as ion trap) can perform MS/MS experiments, they need of high monitoring (or cycle) times to acquire each transition. So, these tyoe of analysers cannot be effectively used for multiresidue methods, in which tens to hundreds of compounds are quantified in one single run. In this scenario, the triple quadrupole analyser is the preferred choice. As its name indicates, a triple quadrupole consists of three

consecutive quadrupoles, though the one in the middle, which acts as collision cell, has been lately replaced by hexapoles and other configurations. **Figure 1.8** shows a scheme of a typical QqQ analyser configuration.

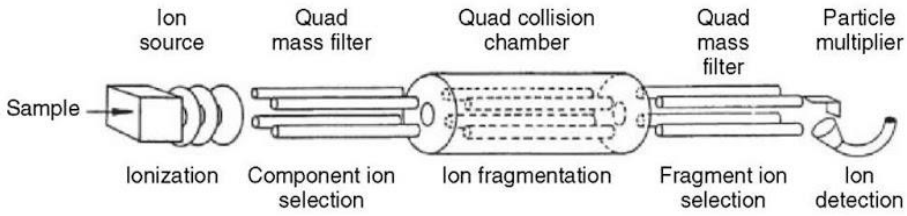

Figure 1.8. Scheme of a triple quadrupole analyser.

Triple quadrupoles allow several working modes, which include:

- Full scan (SCAN): In this acquisition mode, the first Q and the collision cell are switched off, and the Q2 acts as a single quadrupole, screening all ions in a selected *m/z* range.
- Selected ion monitoring (SIM): Here, Q1 and the collision cell are switched off, and the Q2 acts as a single quadrupole, filtering selected *m/z* ions.
- Product ion scan: In this mode, the Q1 filters the desired ions, which are further fragmented in the collision cell. All the product ions coming from the precursor ion filtered in the Q1 are screened in the Q2, recording all fragments coming from this precursor (or parent) ion.
- Selected Reaction Monitoring (SRM): In this mode, Q1 and collision cell act as in product ion scan mode. Q2 now is also used to filter only a determined fragment ion, obtaining which is called

a transition, which is the monitoring of one single fragment coming from a single precursor ion.

SRM is the preferred mode to deal with quantification purposes. The possibility of selecting a specific transition for each compound enhances specificity, as the number of compounds which share a transition with the same intensity is much lower than the number of compounds that fragment into a single ion. This higher specificity also allows reducing noise drastically, as column bleed ions are not acquired. As a result, absolute signal of the transitions is lower than intensity of SIM functions, but signal to noise ratio is enhanced several orders of magnitude. This leads to really low detection and quantification limits, with state-of-the-art instruments capable to reach sub ppt (fg/µL) levels. This is especially notable in GC instruments equipped with the novel APCI source, as the ionization at atmospheric pressure permits to choose highly specific transitions coming from a high intensity molecular $M^{+\bullet}$ or quasi molecular $[M+H]^+$ ion.

In the present thesis, the capabilities of GC-QqQ (MS/MS) have been explored for the quantification of POPs in different complex matrices. In chapter two, GC-ECNI-MSMS and GC-APCI-MSMS have been tested, together with the use of large volume injection, for trace level determination of PBDEs, PCBs, OCPs and novel flame retardants in biological samples. **Scientific article 1** covers the use of GC-NICI-QqQ (MS/MS) for the quantification of PBDEs and novel flame retardants in serum using LVI.

In chapter 3, the enhanced quantification capabilities of GC-APCI-QqQ are applied to a wider number of PBDEs in **Scientific Article 2**, to hexabromocyclododecane in **Scientific Article 3** and to dioxin-like PCBs **Scientific Article 4**.

In chapter 4, the use of GC-APCI-QTOF MS^E is explored for quality classification of olive oils, making use of a non-target metabolomics approach, which results are shown in **Scientific Article 5**. Finally, the discoveries of **Scientific Article 5** were translated to a much affordable instrument, to approach the same problem using a GC-EI-Q MS with state-of-the-art sample treatment and metabolomics data treatment, which lead to the publication of **Scientific Article 6**.

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Chapter 2

Potential of large volume injection together with improved GC-MS/MS for contaminants biomonitoring at ultra-trace levels.

2.1 Introduction

In this chapter, the analytical capabilities of GC-MS/MS with triple quadrupole analyser combined with the latest sample introduction inlets for GC, have been explored for the targeted quantification of trace contaminants in food and human and animal serum.

Biomonitoring has become an indispensable tool for studying occupational and environmental exposure to chemicals, including persistent organic pollutants (POPs). Among POPs, there is a family of compounds whose properties and toxicity make them of special interest to monitor; they are known as brominated flame retardants (BFRs) and have been used since 1960s (Siddiqi & Clinic, 2003). BFRs are used in a wide variety of products, such as housing and wiring of TV sets, computers and mobile phones, in electrical kitchen appliances, upholstery, building materials, and many plastic products, to reduce their flammability. Exposure to these contaminants may occur as a result of direct contact with the products that contain them (BFRs are used in % weight in some furniture and car parts) or via ingestion of contaminated food or inhalation of dust. Most BFRs are persistent, can bioaccumulate in the food chain, especially in seafood and large fish, and have a wide and dangerous variety of toxic and neurotoxic effects (Zezza, Tait, Della, Amorena, & Merola, 2019). Thus, European Union (EU) has taken several actions in regard to the use and applications of these compounds, banning the use of (low) polybrominated diphenyl ether (PBDEs) and their corresponding mixtures (Penta-BDE and to Octa-BDE mixtures) in 2004 and Deca-BDE mixture in electric and electronic products in 2009. As a result of these bans, alternative flame retardants, including novel BFRs, such as

decabromodiphenyl ethane (DBDPE), 1,2-bis(2,4,6-tribromophenoxy)-ethane (BTBPE) and dechloranes (Syn- and Anti-Dechlorane PLUS (DP), Dechlorane 602 and 603, as well as organophosphorus flame retardants, were considered as a replacement of the PBDE mixtures (Cequier, Marcé, Becher, & Thomsen, 2013; Law et al., 2014).

Biomonitoring has been expanded to become the primary tool for human and animal exposure characterization for POPs. The same physical and chemical characteristics that lead to persistence in the environment lead to persistence in biological systems, with limited metabolism, slow elimination, resulting in bioaccumulation. Thus, very low ongoing exposure rates, which may be difficult to detect or quantify analytically, can lead to accumulation of concentrations of POPs in tissues over time to more easily quantifiable levels. However, quantification of these substances in some animals and young people, including new-borns, can be difficult, as the expected levels are in the femtogram per microliter level.

In this scenario, state-of-the-art highly sensitive quantitative techniques are necessary. As stated in the Introduction section, GC-QqQ MS/MS is the technique of choice to reach femtogram per microliter level determinations of GC-amenable compounds. In this chapter, the soft chemical ionization source (CI) has been tested, working in electron capture negative ionization (ECNI) mode in order to obtain the highest sensitivity possible for Dechlorane Plus isomers in SRM mode and for the rest of compounds in SIM mode. Despite the potential of soft ionization to enhance the detection levels compared to traditional EI ionization (Cristale, Quintana, Chaler, Ventura, & Lacorte, 2012; González-Gago et

al., 2011), sometimes even more sensitivity is needed, especially when monitoring new contaminants on young patients.

In such cases, the use of Large Volume Injection (LVI) must be considered as an additional way to gain sensitivity. Programmable temperature vaporization (PTV) based LVI consists in the introduction of a large sample extract volume (more than the typical 1-2 µL) at low injector temperature and then the use of a high temperature ramp to evaporate the compounds condensed in the liner. Depending on the sample type, concentration and the nature of the extract, the inlet can be set in splitless, split or solvent vent mode. **Figure 2.1** represents a regular split/splitless inlet (a) and a PTV inlet (b). As can be appreciated, the main difference relies in the thermocouple and the cooling and heating system inside the PTV inlet, which permits to modify the temperature in a fast and reliable way.

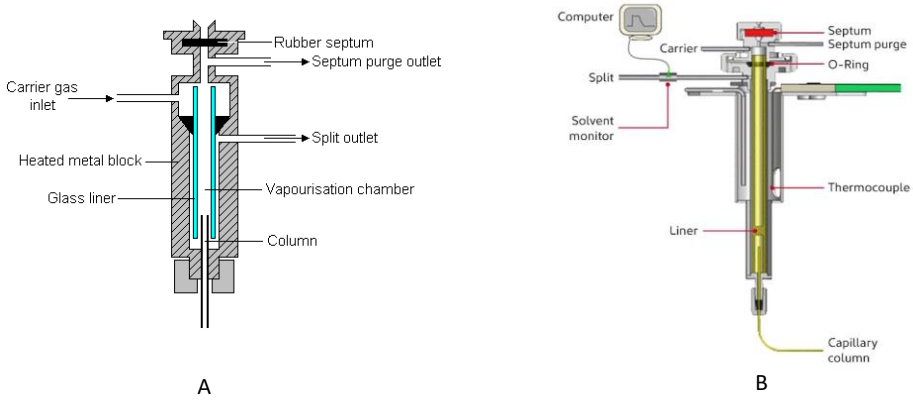

Figure 2.1. Split/splitless inlet (A) and PTV inlet (B).

PTV permits the injection up to 50 µL of sample extract, allowing a significant signal enhancement without the need of additional pre-concentration steps, which is crucial in ultra-trace determinations.

In the present chapter, the capabilities of GC-QqQ MS/MS have been explored for the determination of POPs in animal and serum samples using PTV injection of large sample volumes. The use of this technique, together with chemical ionization source, permitted the determination of POPs, including Dechlorane Plus isomers, at fg/g level in serum samples. Findings are summarized in **Scientific Article 1**.

2.2 Scientific Article 1

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RAPID COMMUNICATION

Simultaneous determination of dechloranes, polybrominated diphenyl ethers and novel brominated flame retardants in food and serum

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Abstract A sensitive method for the simultaneous quantification of dechloranes, polybrominated diphenyl ethers (PBDEs) and novel brominated flame retardants (NBFRs) has been developed for gas chromatography (GC) coupled to tandem mass spectrometry operating in electron capture negative ionization (ECNI) mode. The major advance has been achieved by combining selected ion monitoring (SIM) and multiple reaction monitoring (MRM) modes in well-defined time windows, to determine dechloranes, PBDEs and NBFRs at picogram per gram level in one single analysis in complex matrix biological samples. From the chromatographic point of view, efforts were devoted to study several injection modes using multimode inlet (MMI) in order to obtain low instrumental detection limits, necessary for trace compounds such as Dechlorane Plus (DP) isomers. Method performance was also evaluated: calibration curves were linear from 20 fg μL^{-1} to 100 pg μL^{-1} for the studied compounds, with method detection limits at levels of 50 fg g^{-1} for DPs. Repeatability and reproducibility, expressed as relative standard deviation, were better than 5% even in solvent vent mode for the injection of standards. The application to a wide range of complex samples (including food, human and animal serum samples) indicated a sensitive and reliable way to quantify at the picogram per gram level 4 halogenated norbornenes (HNs), Dechlorane Plus (*anti*-DP and *syn*-DP) and 2 of their homologues (Dechlorane-602 and Dechlorane-603), 11 PBDE congeners (no. 28, 47, 49, 66, 85, 99, 100, 153, 154, 183 and 209) and 5 novel BFRs, i.e. decabromodiphenyl ethane (DBDPE), 1,2-bis(2,4,6-tribromophenoxy)ethane (BTBPE), hexabromobenzene (HBB), 2,3,4,5-tetrabromo-ethylhexyl-benzoate (TBB) and tetrabromophthalate (TBPH).

Keywords Chemical ionization · Gas chromatography · Brominated flame retardants · Dechloranes · Large volume injection · Biological matrices

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Introduction

Halogenated flame retardants (HFRs), including chlorinated and brominated compounds, are used to prevent ignition and combustion of flammable materials, widely employed in furniture, plastics, foams and textile upholstery, among other products [1]. HFRs have been detected in various environmental and food samples as they are released into air, soil and water due to manufacture, improper handling and disposal of HFR-containing products and materials [2]. Among them, polybrominated diphenyl ethers (PBDEs) have been extensively investigated, as a consequence of their past usage, toxicity and persistence in the food chain [3, 4]. As a result of bans applied to commercial PBDE mixtures, there is an increasing production and use of alternative HFRs [5, 6]. Nevertheless, apart from monitoring these HFRs, the determination of PBDEs is still necessary for monitoring purposes and to assess their replacement efficiency [7, 8]. A scheme displaying the different structures of the investigated HFRs is shown in Fig. 1.

There is a large amount of literature regarding the analysis of PBDEs and novel brominated flame retardants (NBFRs) by gas chromatography–mass spectrometry (GC-MS) and GC-

Simultaneous determination of dechloranes, polybrominated diphenyl ethers and novel brominated flame retardants in food and serum

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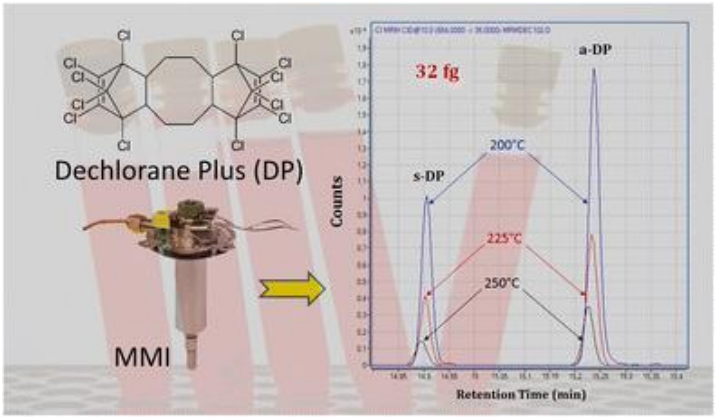
2.2.1 Abstract

A sensitive method for the simultaneous quantification of dechloranes, polybrominated diphenyl ethers (PBDEs) and novel brominated flame retardants (NBFRs) has been developed for gas chromatography (GC) coupled to tandem mass spectrometry operating in electron capture negative ionization (ECNI) mode. The major advance has been achieved by combining selected ion monitoring (SIM) and multiple reaction monitoring (MRM) modes in well-defined time windows, to determine dechloranes, PBDEs and NBFRs at picogram per gram level in one single analysis in complex matrix biological samples. From the chromatographic point of view, efforts were devoted to study several injection modes using multimode inlet (MMI) in order to obtain low instrumental detection limits, necessary for trace compounds such as Dechlorane Plus (DP) isomers. Method performance was also evaluated: calibration curves were linear from 20 fg µL−1 to 100 pg µL−1 for the studied compounds, with method detection limits at levels of 50 fg g−1 for DPs. Repeatability and reproducibility, expressed as relative standard deviation, were better than 5% even in solvent vent mode for the injection of standards. The application to a wide range of complex samples (including food, human and animal serum samples) indicated a sensitive and reliable way to quantify at the picogram per gram level four halogenated norbornenes (HNs), Dechlorane Plus (anti-DP and syn-DP) and two of their homologues (Dechlorane-602 and Dechlorane-603), 11 PBDE congeners (no. 28, 47, 49, 66, 85, 99, 100, 153, 154, 183 and 209) and 5 novel BFRs, i.e. decabromodiphenyl ethane (DBDPE), 1,2-bis(2,4,6-tribromophenoxy)ethane (BTBPE), hexabromobenzene (HBB), 2,3,4,5-tetrabromo-ethylhexyl-benzoate (TBB) and tetrabromophthalate (TBPH).



GC-ECNI-MS/MS chromatograms showing the most sensitive transition for DPs when injecting 2 µL of a 16 fg/µL standard solution of s-DP and a-DP at three different source temperatures

2.2.2 Keywords

Chemical ionization, Gas chromatography, Brominated flame retardants, Dechloranes, Large volume injection, Biological matrices

2.2.3 Introduction

Halogenated flame retardants (HFRs), including chlorinated and brominated compounds, are used to prevent ignition and combustion of flammable materials, widely employed in furniture, plastics, foams and textile upholstery, among other products [1]. HFRs have been detected in various environmental and food samples as they are released into air, soil and water due to manufacture, improper handling and disposal of HFR-containing products and materials [2]. Among them, PBDEs have been extensively investigated, as a consequence of their past usage, toxicity and persistence in the food chain [3, 4]. As a result of bans applied to commercial PBDE mixtures, there is an increasing production and use of alternative HFRs [5, 6]. Nevertheless, apart from monitoring these HFRs, the determination of PBDEs is still necessary for monitoring purposes and to assess their replacement efficiency [7, 8]. A scheme displaying the different structures of the investigated FRs is shown in **Fig. 1.**

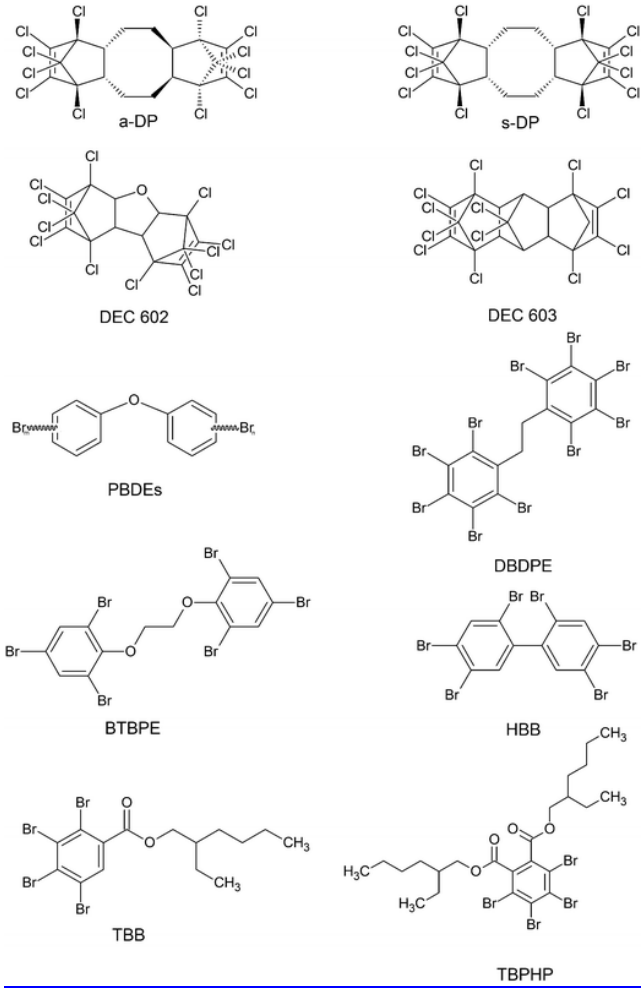


Fig. 1 Scheme of the structures of the main compounds selected for the study

There is a large amount of literature regarding the analysis of PBDEs and novel brominated flame retardants (NBFRs) by gas chromatography–mass spectrometry (GC-MS) and GC-MS/MS using electron capture negative ionization (ECNI) and electron ionization (EI) [9]. More recently, atmospheric pressure chemical ionization (APCI) [10, 11] has also been reported for the analysis of brominated FRs. Both ECNI and APCI offer good sensitivity when compared to EI, while the specificity when using APCI and EI in MS/MS experiments is higher than the obtained by monitoring bromide ions in ECNI [10]. For the determination of

dechloranes, the majority of studies performed so far used EI-MS(/MS), with insufficient detection limits in some cases [12, 13] or ECNI-MS(/MS) with the need of an additional injection, separated from PBDEs [14, 15, 16]. GC-EI-MS/MS methods monitor transitions derived from the molecular ion to *m*/*z* 237 and *m*/*z* 228 [16]. Similar to PBDEs, the analysis of DPs can benefit of selecting more specific transitions coming from the molecular ion by using softer ionization sources. DP isomers constitute a special case study, as they have quite a particular fragmentation behaviour. Several studies have investigated the different fragmentation of *anti*- and *syn*-DP isomers under variable ECNI source temperatures, either in full-scan [17] or in selected ion monitoring (SIM) experiments [15], but not yet in multiple reaction monitoring (MRM) experiments.

Human biomonitoring data on dechloranes is not extensive, but there are few articles on studies investigating their presence and levels in serum, e.g. from China, where the highest levels have been reported near e-waste recycling plants [18, 19, 20], and from Canada [21], Norway [22, 23] or Germany [24]. In all these studies, the limits of detection for DPs were in the picogram per gram level.

Against this background, the availability of a method with the benefits of sensitivity and specificity for dechloranes and sensitivity for PBDEs and other flame retardants in a single analysis using a chemical ionization (CI) source in negative mode could be beneficial for monitoring laboratories. The aim of this work was the development of a methodology for the simultaneous analysis of HFRs of high concern at low picogram per gram levels in a wide range of complex samples, such as food, human and animal serum samples. Such improvement of the analytical methods will be useful in the currently running biomonitoring schemes, such as the Flemish Environment and Health study.

2.2.4 Materials and methods

Chemicals and reagents

Standards of BDE-28, BDE-49, BDE-47, BDE-99, BDE-100, BDE-153, BDE-154, BDE-138, BDE-183 and BDE-209, 1,2-bis(2,4,6-tribromophenoxy)ethane (BTBPE), *syn*-DP and *anti*-DP isomers, 2-ethylhexyl-2,3,4,5 tetrabromo-benzoate (TBB), 2,3,4,5-tetrabromophthalate (TBPH), hexabromobenzene (HBB), dechlorane-602 (Dec-602), dechlorane-603 (Dec-603), isotopically labelled internal standards (IS) ^{13}C-BDE-209, ^{13}C-TBPH, ^{13}C-TBB, ^{13}C-*syn*-DP and ^{13}C-*anti*-DP were purchased from Wellington Laboratories (Guelph, ON, Canada). Recovery standard (RS) CB-207 was purchased from Dr. Ehrenstorfer Laboratories (Augsburg, Germany). Polypropylene (PP) tubes (15 mL) were obtained from Greiner Bio-one (Belgium). Empty PP cartridges (25 mL) were purchased from Grace (Lokeren, Belgium), while Florisil® cartridges (500 mg, 3 mL) and empty PP cartridges (6 mL) were purchased from Supelco (Bellefonte, PA, USA). Silica gel, anhydrous sodium sulfate (Na_2SO_4) and concentrated sulfuric acid (H_2SO_4, 98%) were purchased from Merck (Darmstadt, Germany). All solvents were of chromatography grade: *n*-hexane was purchased from Acros Organics (Belgium); dichloromethane (DCM), iso-octane, toluene and acetonitrile (ACN) were purchased from Merck.

Sample treatment

Food samples (including biscuits, smoked salmon and chicken eggs) were treated as indicated in a previous work [25]. Briefly, samples were homogenized, freeze-dried and stored at −20 °C until analysis. The samples were weighted in pre-washed 15 mL PP tubes, and spiked with the IS mixture. After spiking, samples were extracted by solid-liquid extraction (SLE) with ACN/toluene (9:1, *v/v*). After a two-step clean-up (performed on Florisil® and acidified silica 5%), the samples were evaporated to dryness and reconstituted in 100 µL of the RS (CB-207 in iso-octane/toluene; 9:1, *v/v*) and transferred to amber injection vials for GC-ECNI-MS(/MS) analysis.

Serum samples including hyena, cheetah and lion (Zoo Antwerp, Belgium), sea eagle plasma (Trondheim, Norway) and human cord blood (Flemish Environment and Health study II-Flemish newborns) were extracted according to the method described elsewhere [26], with slight modifications. Solid-phase extraction (SPE) on OASIS HLB cartridges was used followed by clean-up on 1 g of acidified silica 44% and eluted with 10 mL *n*-hexane/dichloromethane (1:1, *v/v*). The cleaned extract was evaporated to incipient dryness and re-dissolved in 100 µL iso-octane.

2.2.5 GC-(ECNI)-MS(/MS)

The chromatographic analysis was performed using an Agilent 7890B gas chromatograph, equipped with an Agilent 7693A autosampler with multimode inlet (MMI), coupled to a triple quadrupole mass spectrometer, 7000C (Agilent Technologies Inc., Palo Alto, CA, USA), with a CI source working in electron capture negative ionization mode. Methane was used as reagent gas at a flow of 2 mL min^{-1}. The GC separation was performed using a fused silica a ZB-semivolatiles capillary column (5% phenyl-arylene-95% dimethyl-polysiloxane) with a length of 20 m × 0.18 mm ID and a film thickness of 0.18 µm (Phenomenex, Torrance, CA, USA) working at a ramped flow from 1 mL min^{-1} (14 min) with 10 to 2 mL min^{-1} (10.9 min) of helium (99.999%; Air Liquide, Liège, Belgium). The oven program was set as follows: 90 °C (1.25 min); 30 °C min^{-1} to 240 °C; then 10 °C min^{-1} to 325 °C, stay 10.4 min with a total run time of 25 min. The injection of 2 µL of sample extracts was performed in cold pulsed splitless mode at a temperature of 80 °C and a pulse time of 1.25 min. The pulse pressure was set to 50.0 psi, with a split purge flow of 50 mL min^{-1} and purge time of 1.25 min.

2.2.6 Results and discussion

MS optimization

Optimal *m/z* values for SIM of each compound were selected according to [27], while the optimal MRM transitions for DPs were taken from reference [28], also considering the common fragmentation pattern for every compound, usually leading to bromide ions. To achieve maximum

sensitivity, different collision energies were tested to study the fragmentation of *syn*- and *anti*-DPs in the collision cell. Two ions from the isotopic pattern corresponding to $M^{-\cdot}$ (M + 4 and M + 6) were selected in the first quadrupole and fragmentation was performed using a range of collision energies between 5 and 35 eV. A collision energy of 5 eV was optimal for the ^{13}C-labelled DPs, while 10 eV was selected for the native *syn*- and *anti*-DPs. Accordingly, the selected transitions were 654➔35; 654➔37 and 652➔35 corresponding to the fragmentation of the precursor *m/z* ions $[M + 6]^-$ and $[M + 4]^-$ for the native DPs and 664➔35 and 664➔37 taking $[M + 6]^-$ *m/z* ion as precursor for the ^{13}C-DPs.

The source temperature was also optimized pursuing the maximum response for every analyte. Previous studies [15, 17], demonstrated that low source temperatures favour the detection of the molecular ion cluster, while higher temperatures (250 °C) had different effects on both isomers. According to De la Torre et al. [17], a temperature of 150 °C provided similar spectra for both isomers, with the most abundant cluster being the one corresponding to the molecular ion $[M]^{-\cdot}$. However, at 250 °C, the two isomers showed a different pattern. In the case of *syn*-DP, the cluster corresponding to the ion $[M\text{-}6Cl]^-$ became the most abundant. Summarizing, higher temperature source provided more energy, hence favouring the dissociative electron capture process and increasing the abundance of fragment ions, whereas lower temperatures enhanced molecular ion abundance.

Accordingly, after obtaining low collision energies as the optimal for the determination of DPs, we theorized that low source temperatures might enhance the formation in the ion source of the parent ions for the DPs transitions. Hence, source temperatures of 250, 225 and 200 °C were tested. An increase in the response of DPs was seen at lower source temperatures (Fig. 2), while too low temperatures could affect the sensitivity for PBDEs, for which detection relies in the fragmentation to the bromide ion *m/z* 79. The temperature of 200 °C was hence chosen as a compromise for these experiments. Selected quantification and qualification transitions and ions for each analyte are summarized in **Table 1**. These findings add to the previous studies on the behaviour of DPs at different source temperatures, as in MRM experiments, the formation of

an abundant molecular pattern to be selected as a parent ion has been proved more sensitive than a high in-source fragmentation, which leads to larger losses of chlorine atoms before entering in the first quadrupole.

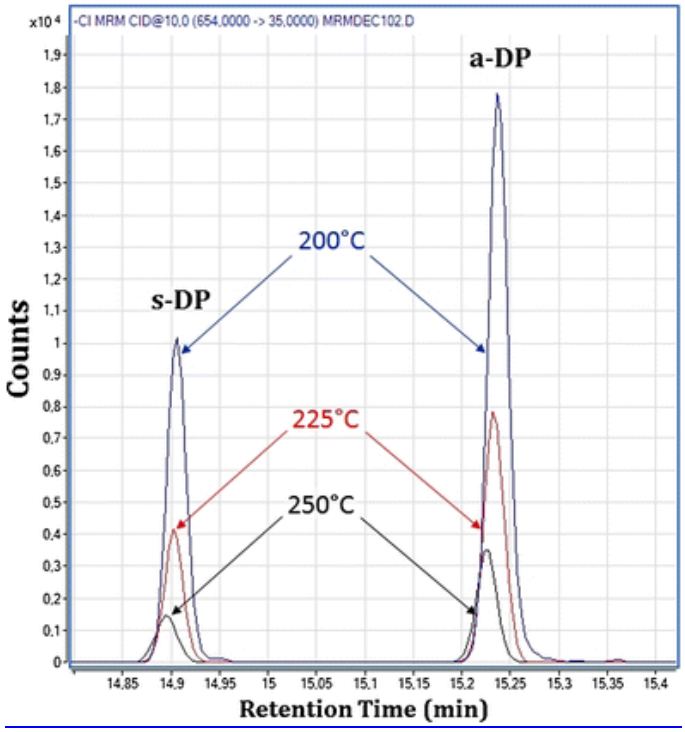


Fig. 2 Variation in the peak area for the most sensitive MRM transition for DPs, for the injection of a standard mixture at 16 fg μL^{-1} in iso-octane at different source temperatures (200, 225 and 250 °C)

Table 1. Analytical performance of the method including MS quantitation parameters

Compounds	RT	Q	q	iLOD (fg µL−1)		
				2 µL	5 µL SV	10 µL SV
BDE 28	8.05	79	81	20	10	5
BDE 49	9.06	79	81	20	10	5
BDE 47	9.26	79	81	10	5	2.5
BDE 66	9.51	79	81	20	10	5
BDE 100	10.36	79	81	20	10	5
BDE 99	10.72	79	81	20	10	5
BDEs 85	11.40	79	81	20	10	5
BDE 154	11.69	79	81	20	10	5
BDE 153	12.24	79	81	20	10	5
BDE 138	12.95	79	81	20	10	5
BDE 183	13.74	79	81	20	10	5
BDE 209	21.04	487	489	200	100	50
HBB	9.06	79	81	20	10	5
TBB	10.65	357	359	200	100	50
DEC602	10.70	612	35	250	120	60
DEC603	13.30	638	35	100	50	25
BTBPE	14.18	79	81	20	10	5
TBPH	14.60	384	515	100	50	25
s-DP	14.90	654 > 35	654 > 37	1	0.5	0.25
a-DP	15.22	654 > 35	654 > 37	0.5	0.25	0.125
DBDPE	23.95	79	81	1000	500	250

2.2.7 Analytical parameters

To maximize the signal obtained for each analyte, the use of the multimode inlet in large volume injection mode was considered. The possibility of starting at a low inlet temperature allowed the injection of a higher volume of extract. Therefore, several injection configurations were tested: cold pulsed splitless (2 µL), and solvent vent (5 µL, 2 × 5 µL and 3 × 5 µL). **Figure 3** highlights the response enhancement for the DP congeners when working at the three selected working conditions. Although solvent vent injections enhance the sensitivity for DPs as well as for the rest of the selected compounds, reproducibility and overloading issues were noticed when injecting extracts from fatty matrices, so the

injection of 2 µL in cold pulsed splitless mode was selected as optimal. To test the reliability of the method, the repeatability of absolute area was studied in five repeated injections of standards at five different levels (20 fg μL^{-1}, 100 fg μL^{-1}, 1 pg μL^{-1}, 20 pg μL^{-1} and 100 pg μL^{-1}). The relative standard deviation was below 5%. Linearity of the relative response of the different compounds (to their ^{13}C isotopically labelled or BDE internal standards) was studied by analysing standard solutions, in triplicate (five levels), in the range of 20 fg μL^{-1} to 100 pg μL^{-1}. The correlation coefficients (r^2) were higher than 0.99 for every compound, with residuals lower than 2%. Special attention has to be paid to the method sensitivity for DPs, which can be derived from **Fig. 2** (injection of a 16 fg μL^{-1} standard solution in iso-octane). Instrumental limits of detection (iLODs) were calculated as the lowest concentration level giving a signal-to-noise ratio (S/N) of 3. These iLODs were determined to be around 1 fg μL^{-1} for *syn*-DP and 0.5 fg μL^{-1} for *anti*-DP, when injecting 2 µL in cold pulsed splitless mode. The iLODs were even lower when using solvent vent mode, as can be seen in **Fig. 3**. Obtained iLODs are summarized in **Table 1**. LODs and LOQs in real samples were estimated using the same criteria, by extrapolation from the lowest responses (detectable and quantifiable) of every compound within the analysed samples. These results are relevant especially for DP isomers, as their LODs and LOQs have been lowered sensibly in comparison to previous studies. **Table 2** lists the majority of previous studies performed to detect and quantify DP isomers, indicating the systems used and the achieved performance in each case in terms of LOD and LOQ.

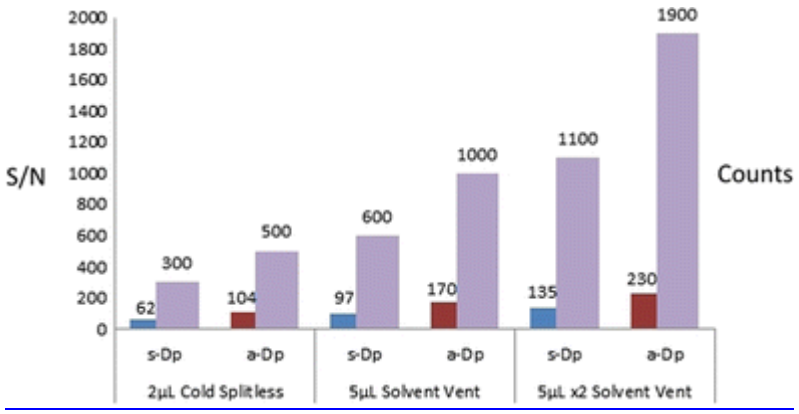


Fig. 3 Graphical comparison of the methodology performance for the injection of a DP mixture (16 fg μL^{-1}). S/N = signal to noise ratio

2.2.8 Analysis of real samples

The enhanced capabilities of the presented method were finally tested using extracts of samples of food and human and animal serum previously analysed by GC-ECNI-MS, according to the method used for routine analysis and described elsewhere [25]. The developed methodology allowed the determination of trace quantities (below pg g^{-1} range) of the selected PBDEs in several samples. In these samples, NBFRs could also be evidenced. A good agreement was found when comparing the quantification results of the new methodology with those given by the validated reference method [25] (at the levels achievable by the reference method).

Table 2. Techniques and conditions previously used for the determination of DP isomers and their performance in terms of LOD and LOQ

No.	Technique	Column	Separation	*m/z*	LOD	LOQ	Ref
1	GC-ECNI-MS	DB-5 (15 m × 0.25 mm × 0.10 μm)	90 °C (1.5 min); 10 °C/min to 300 °C (3 min); 40 °C/min to 310 °C (5 min)	$[M\text{-}H]^-$; 650, 652	n.a.	2 ng g^{-1} (dust)	[6]
2	GC-APCI-HRqTOFMS	DB-5 HT (15 m × 0.25 mm × 0.10 μm)	110 °C; 40 °C/min to 200 °C; 10 °C/min to 280 °C; 30 °C/min to 330 °C (5 min)	$[M]^{-\cdot}$; 653.711	0.16 pg μL^{-1}	n.a.	[11]
3	GC-EI-HRMS	DB-5 (15 m × 0.25 mm × 0.10 μm)	120 °C (1 min); 30 °C/min to 240 °C; 5 °C/min to 275 °C; 40 °C/min to 320 °C (3 min)	$[M\text{-}C_{13}H_{12}C_{16}]^{+\cdot}$; 271.8102; 273.8072	0.5 pg g^{-1} (sediment), 15 pg g^{-1} (fish)	n.a.	[13]
4	GC-ECNI-MS	DB-5 (30 m × 0.25 mm × 0.25 μm) DB-35 (30 m × 0.25 mm × 0.25 μm) (confirmation)	80 °C (2 min); 10 °C/min to 285 °C (5 min)	$[M\text{-}H]^-$; 650.652	30 pg g^{-1} (sediment)	n.a.	[14]
5	CZC-GC/ECNI-TOFMS	Rtx- PCB (15 m × 0.25 mm × 0.25 μm) plus Rxi-17 (1 m × 0.18 mm × 0.18 μm)	140 °C (2 min); 30 °C/min to 280 °C; 5 °C/min to 300 °C (10 min)	$[M\text{-}H]^-$; 650, 652	3 pg (iLOD)	n.a.	[16]
6	GC-ECNI-MS	DB-XLB (30 m × 0.25 mm × 0.25 μm)	110 °C (1 min); 8 °C/min to 180 °C (1 min); 2 °C/min to 240 °C (5 min); 2 °C/min to 280 °C (15 min; 10 °C/min to 310 °C (5 min)	$[M]^{-\cdot}$; 653.8 and 651.8	n.a.	3.08 pg g^{-1} fat (serum) (s-DP), 1.29 pg g^{-1} fat (serum) (a-DP)	[19]
7	GC-ECNI-MS	DB-1MS (30 m × 0.25 mm × 0.25 μm)	120 °C (1 min), 10 °C/min to 300 °C (8 min); 10 °C/min to 310 °C (12 min)	$[M]^{-\cdot}$; 651.7 and 653.7	40 pg g^{-1} l.w. (s-DP), 120 pg g^{-1} l.w. (a-DP), (serum)	n.a.	[21]
8	GC-ECNI-MS	DB-5 (15 m × 0.25 mm × 0.10 μm)	50 °C, 25 °C/min to 300 °C (5 min)	$[M]^{-\cdot}$; 653.8 and 651.8	1.1 pg mL^{-1} (serum) (s-DP), 3.3 pg mL^{-1} (serum) (a-DP)	3.5 pg mL^{-1} (serum) (s-DP), 10 pg mL^{-1} (serum) (a-DP)	[22]

n.a. not available

Special emphasis was made on the capability of the methodology to detect DPs in most of analysed samples. Due to the presence of these compounds in the procedural blanks, only the samples with DPs relative area higher than 10 times their corresponding relative area in the blank were quantified. The most remarkable results to highlight are as follows: a pool of four cord blood human serum samples with 0.13 and 0.19 pg g^{-1} of *syn*-DP and *anti*-DP, respectively; a sea eagle plasma sample with 1.95 pg g^{-1} of *syn*-DP and 26 pg g^{-1} of *anti*-DP, a chicken egg with 9 ng g^{-1} of *syn*-DP and 29 ng g^{-1} of *anti*-DP, and a hyena serum sample with 0.33 pg g^{-1} of *syn*-DP. Dec-603 and Dec-602 were also quantified in human/animal serum ranging from 5 to 66 pg g^{-1}. Chromatograms with the quantification transition of DP isomers in the mentioned samples can be seen in **Fig. 4** (4A for a procedure blank, biscuits, smoked salmon, chicken egg and hyena serum extracts, and 4B showing a cheetah serum, human cord blood (pool), sea eagle serum and two chicken egg extracts). **Table 3** summarizes the concentration found for each analyte in the samples.

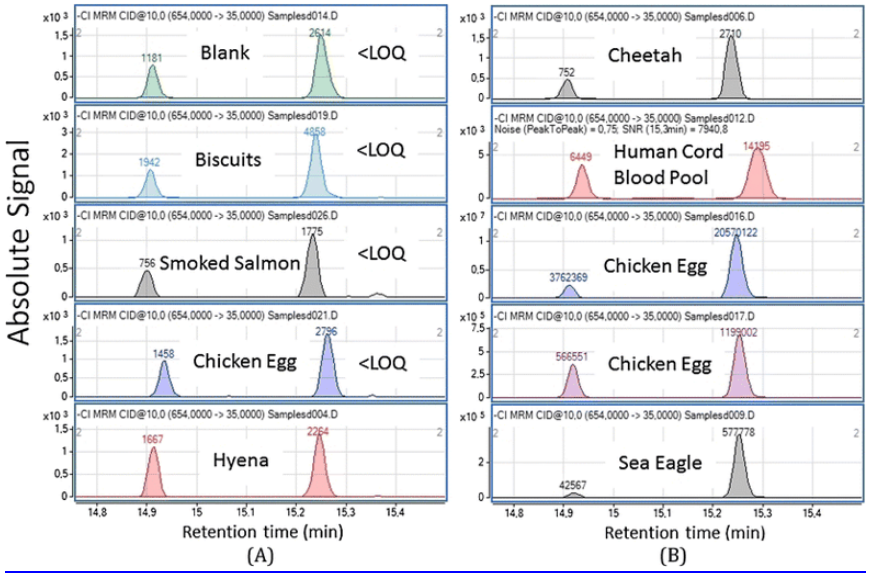


Fig. 4. Chromatograms corresponding to the quantification transition of DPs for the injection of **A** procedural blank, biscuits, smoked salmon, chicken egg and hyena serum extracts; and **B** cheetah serum, human cord blood serum, chicken egg and sea eagle serum extracts

Table 3. Concentrations of PBDEs and other HFRs (pg g^{-1}) in the analysed samples

Compounds	LOD (pg g^{-1})	LOQ (pg g^{-1})	Biscuits	Smoked salmon	Chicken egg	Hyena serum	Cheetah serum	Lion serum	Human cord blood pool	Blank	Chicken egg	Albumin	Chicken egg	Sea eagle plasma	Sea eagle plasma
DEC602	1	2.5	<1	<1	<1	<1	66	12	<1	<1	5	<1	8	<1	14
DEC603	1	2.5	<1	<1	<1	8	19	8	<2.5	<1	<2.5	<1	<2.5	9	<1
syn-DP	0.03	0.10	<0.03	<0.03	<0.03	0.33	2.5	13	0.13	<0.03	9000	4	2700	1.95	<0.03
anti-DP	0.05	0.15	<0.05	<0.05	<0.05	<0.15	6	18	0.19	<0.05	29,000	6	3500	22	<0.05
ΣDPs			<0.03	<0.03	<0.03	0.33	9	31	0.32	<0.003	38,000	10	6000	24	<0.003
fAnti			–	–	–	–	0.71	0.58	0.59	–	0.76	0.40	0.56	0.92	
BDE 28	0.8	20	<0.8	<0.8	<0.8	<0.8	<20	<0.8	<0.8	<0.8	<0.8	<0.8	26	<0.8	<0.8
BDE 49	0.3	1	<0.3	35	<1	<0.3	<0.3	<1	<0.3	<1	220	<1	70	1	12
BDE 47	0.7	3	41	233	44	<3	11	4.5	<0.7	<0.7	540	6	455	3	16
BDE 66	1	10	<1	<1	<10	<1	<1	<1	<1	<1	<1	<10	46	<1	<1
BDE 100	0.4	4	9	51	6	<0.4	<0.4	4	<0.4	<0.4	440	7	270	4	4
BDE 99	0.5	2	18	7	5	8	8	2	<2	<0.5	1400	26	600	3	6
BDE 85	3	9	<9	9	<9	<3	<3	<3	<3	<3	20	<3	21	<3	<3
BDE 154	0.05	0.22	1	22	<0.22	3	56	9	0.22	<0.05	340	2	126	1.4	2.7
BDE 153	0.8	2	35	13	4	16	18	2	<0.8	<2	870	5	635	4	3
BDE 138	0.3	1	1	20	2	<0.3	13	42	<0.3	<0.3	50	<0.3	58	<0.3	<1
BDE 183	0.7	2	<0.7	<0.7	<0.7	<0.7	<2	<2	<0.7	<0.7	1900	4	1700	<0.7	3.5
BDE 209	6	20	92	84	92	58	610	<6	<6	<6	12,000	55	6700	26	1100

Table 3. Concentrations of PBDEs and other HFRs (pg g^{-1}) in the analysed samples

Compounds	LOD (pg g^{-1})	LOQ (pg g^{-1})	Biscuits	Smoked salmon	Chicken egg	Hyena serum	Cheetah serum	Lion serum	Human cord blood pool	Blank	Chicken egg	Albumin	Chicken egg	Sea eagle plasma	Sea eagle plasma
ΣPBDEs			197	467	153	88	723	63	1.3	2	18,000	105	11,000	42	1200
DBDPE	13	30	<13	<13	<13	<13	<13	<13	<13	<13	<13	<13	<13	<13	<13
HBB	5	15	<15	<15	<15	<5	<5	<5	<5	<5	250	<5	50	<5	<5
TBB	10	40	<40	<10	<10	<10	<10	<10	50	<10	<10	124	<10	<10	<10
BTBPE	0.24	1	8	3	5	8	34	7.2	<1	<1	300	14	1990	4	4
TBPH	5	15	72	32	53	<5	<5	<5	<5	<5	45	290	290	<5	159
ΣNBFRs			94	44	60	8	34	7.2	50	–	600	427	2300	4	163

*< xx: below the respective LOQ or LOD

The most contaminated samples corresponded, as expected, to captive animals from the Antwerp Zoo and the eggs of wild birds. It is also important to consider the differences found in the f-anti value. *Anti*-DP has been found to degrade faster than *syn*-DP at high temperatures and at e-waste sites [18], so the differences measured with this methodology, for example in the hyena sample, could help to assess for the degradation of these compounds in areas close to recycling facilities and monitor their presence in animals and humans.

2.2.9 Conclusions

The use of a method combining SIM and MRM acquisition modes in an ECNI source has demonstrated high sensitivity for a wide range of HFRs, specifically for DP isomers, which have been detected in most of analysed samples, including procedural blanks. This combination of acquisition modes together with large volume injections allowed decreasing the LODs for DPs to femtogram per gram levels, which constitutes a significant advancement compared to previous methodologies monitoring the molecular ion in SIM mode or less sensitive transitions in EI-MS/MS. Nevertheless, the use of large volume injections can be an issue for some fatty matrices and has to be carefully applied to selected samples. The method was applied to a wide range of complex matrices and was able to quantify DP isomers at low picogram per gram levels in serum samples. This methodology is an important tool for the determination of HFRs at ultra-trace levels in food and biological samples, helping to monitor the release and occurrence of PBDEs, HNs and NBFRs in the environment.

2.2.10 Acknowledgments

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2.2.11 Conflict of interest

The authors declare that they have no conflict of interest.

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2.3 Discussion

Results obtained in this paper demonstrate the enhanced capabilities of GC-QqQ MS/MS together with the soft ionization CI source in ECNI mode for the determination of POPs at ultra-trace levels in food and serum.

For POPs in serum, especially for DP isomers, detection and quantification limits were lowered significantly compared with traditional methodologies, relying in SIM acquisition instead of SRM transitions coming from the molecular ion. Results reveal the potential of the technique to simultaneously monitor DP isomers and traditional PBDEs in samples with sub-ppt concentrations, which could be of a high value for POPs biomonitoring in newborn samples.

The results obtained for food samples demonstrate a large linear range of the technique for most of selected compounds, from sub-ppt to ppb concentrations. This technique could be effectively applied to food control routines for a wide range of products without the need of sample treatment modifications or extract dilutions.

The optimization of CI source temperatures for the first time for the determination of DP isomers in SRM mode has permitted, together with the use of MMI inlet, to reach sub-ppt LOQs. The sensitivity of this technique suggests its potential application in human fluids analysis, especially considering reduction of blood sample extraction and treatment. In this way, this technique could be well suited for application together with a non-invasive sample collection system, with low sample volume, known as known as Dried Blood Spots (DBS). DBS has been used for more than 50 years for biomonitoring purposes, as it is a non-invasive

technique which allows transporting the extracted samples easily. However, DBS collects only a few microliters of sample, which could be a limitation for ultra-trace biomonitoring. So, the combined use of LVI and GC-MS/MS methodologies with soft ionization sources could be a useful technique to monitor trace contaminants in humans.

2.3.1 Dried Blood Spots

The analysis of blood spotted and dried on a matrix (i.e., "dried blood spot" or DBS) has been used since the 1960s in clinical chemistry; mostly for neonatal screening. Since then, many clinical analytes, including nucleic acids, small molecules and lipids, have been successfully measured using DBS (Lehmann, Delaby, Vialaret, Ducos, & Hirtz, 2013). DBS collection can be done by patients themselves by a simple puncture with a lancet after disinfection with alcohol and the blood is placed in a pre-marked circle on a DBS card. The blood-spotted card is allowed to dry at room temperature and then stored at then frozen until analysis, tough it can be kept at room temperature depending on the analyte. In this context, a new methodology based on DBS sample collection and GC-APCI-(QqQ) MS/MS determination has been already tested for POPs determination in human blood in our research group. For this purpose, a sample extraction procedure based on the work from B. L'Homme (L'Homme & Focant, 2012), with some modifications has been used to determine PBDEs (BDE-28, -49, -47, -99, -100, -153, -154, -138, -183, and -209), PBCs (PCB- 28, -52, -101, -118, -153, -138, -180), OCPs (HCH isomers. DDD, DDE, DDT and HCB) and Chlorpyriphos methyl. Briefly, DBS cards were spiked with 10 µL of a mixture of PCB 143 at 20 ng/µL and BDE 77 at 4 ng/µL as surrogates. Blood spots were accurately measured using a volumetric capillary of 20 µL. Thus, 40 µL were deployed in each card spot. The cards were then

allowed to dry at room temperature and stored in a zipped bag with silica gel at -20°C prior to extraction. Dried samples were cut (16 mm for 40 µL of blood or serum) and placed in 1.5 mL polypropylene Eppendorf® with 150 µL 95% formic acid. After pre-soaking, samples were liquid-liquid extracted (LLE) using a mixture of n-Hexane/Acetone (3:1). The solution was hand-shaked and the extraction repeated three times with 300 µL of the hexane mixture each. Each extraction was performed applying 1 min vortex and 5 min ultrasonication. The three organic layers were directly collected in a new Eppendorf. The extract was then evaporated to dryness and reconstituted in 50 µL of nonane and transferred to amber injection vials with glass inserts for GC-APCI-MS/MS analysis. Optimal SRM transitions for were taken from previous works (Pitarch et al., 2016; Portolés et al., 2015).

Preliminary results show impressive detection capabilities, with LODs below 1 fg/µL of blood in cold pulsed splitless and LVI. As an example **Figure 2.2.** shows the chromatograms for 4,4'-DDE, PCB-180 and BDE-209 injecting 2 µL of a 0,1 pg/µL standard in cold pulsed splitless mode (A) and (B) injection of 2x5 µL of a 0.1 pg/µL standard in solvent vent mode.

However, some issues with recovery (around 30%), and quantification results using BDE-77 and PCB-143 as internal standards and other chromatographic issues due to the matrix need still to be addressed. We are working in applying slight improvements, as the use of dedicated solvent vent liners, deactivated guard columns and isotopically labelled internal standard.

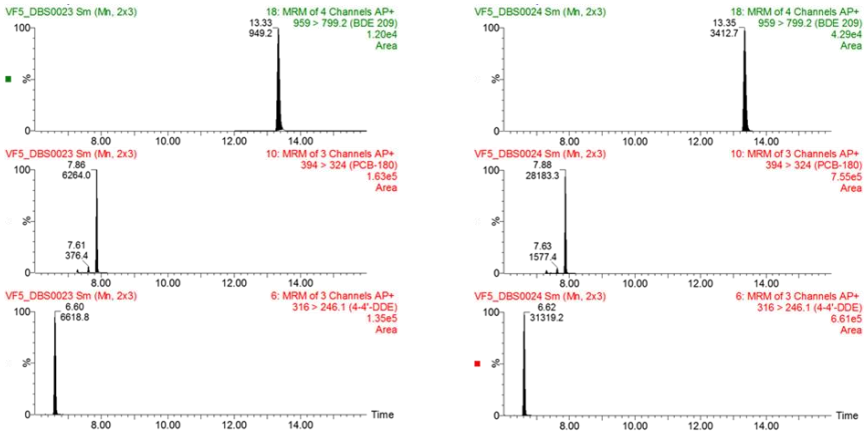

Fig 2.2. Chromatograms for 4,4'-DDE, PCB-180 and BDE-209 corresponding to: (A) injection of 2 µL of a 0,1 pg/µL standard in cold pulsed splitless mode and (B) injection of 2x5 µL of a 0,1 pg/µL standard in solvent vent mode.

In a near future, DBS together with LVI could be a useful and powerful biomonitoring tool combined with GC-MSMS determination. Until that moment, we have proved the potential of GC-CI-MS(/MS) with LVI for biomonitoring, not only of traditional POPs, but also for emerging contaminants. The outputs of the preliminary results shown, also suggest the potential of coupling the benefits of LVI with the known sensitivity of GC-APCI-MSMS (which will be discussed in depth in next chapter) in order to benefit from both, the availability of molecular ions to perform more specific SRM transitions and the capability of introducing more sample in the system, preconcentrating ultra-trace compounds in the inlet.

The potential of soft ionization sources with state-of-the-art tandem mass spectrometry instruments will be discussed in detail in **Chapter 3**.

2.4 References

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Chapter 3

Potential of APCI source for the GC-MS/MS determination of persistent organic pollutants at ultra-trace levels

3.1 Introduction

In this chapter, the analytical capabilities of GC-MS/MS with triple quadrupole analyser combined with the recently introduced atmospheric pressure chemical ionization (APCI) source for GC, have been explored for the targeted quantification of trace contaminants in complex environment and food matrices.

Compounds detected in the environment can, often, be related to human activities. As a consequence of worldwide industrial development, there are many chemicals that migrate from the products they are added to, and end up in organisms, soil, and water bodies. In this chapter, two different types of contaminants will be considered. Brominated flame retardants (BFRs) belong to a wider class of purpose-made chemicals and whose function in many products is to delay the spreading of the fire. The other group is composed by dioxin-like polychlorobiphenyls (DL-PCBs) which are a sub-group among PCBs with toxicological properties similar to dioxins.

BFRs problematic was earlier discussed in Chapter 2. As mentioned, due to their toxicity and persistence, PBDEs have been banned over the years, with the need of introducing new compounds with the same functionality. In this scenario, there is another BFR to be considered for monitoring, introduced in 1960 as an alternative to PBDEs, the Hexabromo cyclododecane (HBCD). However, HBCD was also banned by the Stockholm Convention later in 2013 (Koch & Schmidt-k, 2015) and its production has decreased consequently. Despite the consecutive bans, it will take some time for the industry to seek qualification and re-certification of polystyrene bead and foam products for fire-rating using alternative

compounds. In this scenario, the availability of ultra-trace determination techniques for FRs including PBDEs, and their alternatives is necessary, to assess the decrease in its production and to monitor its spreading worldwide.

On their side, due to their chemical stability and electrical insulating properties, PCBs were commonly used in the past in hundreds of industrial and commercial applications, including its use as electrical insulating fluids in transformers and also as hydraulic, heat transfer, and lubricating fluids. In other applications, PCBs were commonly blended with other chemicals as plasticizers and fire retardants and used in a range of products including caulks, adhesives, plastics, and carbonless copy paper (Erickson & Kaley, 2011). Amongst the total 209 possible PCB congeners, there are some that can take a planar conformation, giving them an extremely high toxic potential, so close to that of dioxins (polychlorinated dibenzo-p-dioxins and polychlorinated di-benzofurans (PCDD/Fs)) that they are commonly named as dioxin-like PCBs. These include PCB congeners 77, 81, 105, 114, 118, 123, 126, 156, 157, 167, 169, and 189.

Due to the physicochemical characteristics of these two groups of contaminants, especially in terms of non-polarity, volatility and thermal stability, and the low levels found in environmental and food samples, traditional methods for their determination commonly rely in gas chromatography (GC) coupled to mass spectrometry (MS). Several reviews have reported the analytical methods commonly used for the determination of PBDEs and PCBs in environmental and food matrices (Pietroń & Małagocki, 2017; van Leeuwen & de Boer, 2008). In summary, GC-MS operating in both electron ionization (EI) and electron capture negative ionization (ECNI) modes using quadrupole (Q), triple quadrupole

(QqQ), or ion trap (IT) analyzers are the most widely used techniques (Cristale, Quintana, Chaler, Ventura, & Lacorte, 2012; Gómara, Herrero, & González, 2007). GC-EI-MS(/MS) methods are readily suitable for quantifying PBDEs in environmental and biological matrices, but their sensitivity can be compromised when analysing highly brominated congeners (Gómara et al., 2007; Wang & Li, 2010). Due to the high number of PCB congeners that can be present in real samples, GC coupled to high resolution MS (HRMS) magnetic sectors has been the gold standard technique for their quantification , in particular that of DL-PCBs (Ábalos et al., 2016; E. Eljarrat & Barceló, 2002). GC-EI-HRMS instruments also offer high sensitivity for the highly brominated PBDE congeners and an increased specificity, as they are able to eliminate interferences between the different halogenated compounds. To this aim, it is necessary to increase the resolution to over 20000 FWHM, which, unfortunately, is translated into a significant loss in sensitivity. Additionally, their high acquisition and maintenance costs make GC-EI-HRMS a technique not much accessible for most routine laboratories, as well as they increase the overall sample analysis cost.

When considering the use of ECNI ionization sources, the sensitivity for all types of halogenated POPs can be increased typically by a factor of 10 (Gómara et al., 2007), especially when monitoring Br^- ions. This increase in sensitivity generally comes with a loss on specificity, as the signal is indistinct for all brominated species which can be present in the sample. As a consequence, up-to-date sample treatment techniques must be applied in order to get rid of any possible interference, and a baseline chromatographic resolution is required for the correct determination of the selected analytes, which can result in long chromatographic runs.

In this scenario, the availability of a novel ionization GC source, the atmospheric pressure chemical ionization source (APCI), offers a chance to apply the benefits of a soft ionization of CI sources and the specificity and universality of EI sources. APCI ionization is based on the use of N_2 as collision gas, together with the application of a low voltage to produce a soft ionization and a really low in-source fragmentation. In this conditions, there are two coexisting ionization processes; the first one known as charge-transfer, as a result of the collision of N_2 generated plasma containing $N_2^{+\bullet}$ and $N_4^{+\bullet}$ ions, with the compound molecules. The second one, due to the presence of H_2O particles inside the atmospheric pressure source, as a result of the reaction between charged $H_3O^{+\bullet}$ molecules and the analyte, resulting in a proton-transfer reaction obtaining the $[M+H]^+$ quasi-molecular ion. **Figure 3.1** represents the reaction mechanisms which take place in the APCI source.

Both mechanisms take place simultaneously, which can lead to mixed ionization patterns depending on the nature of the compound. The addition of modifiers in the source to promote the proton-transfer reaction was also studied in **Scientific article 2**, and will be discussed in more detail in the discussion section.

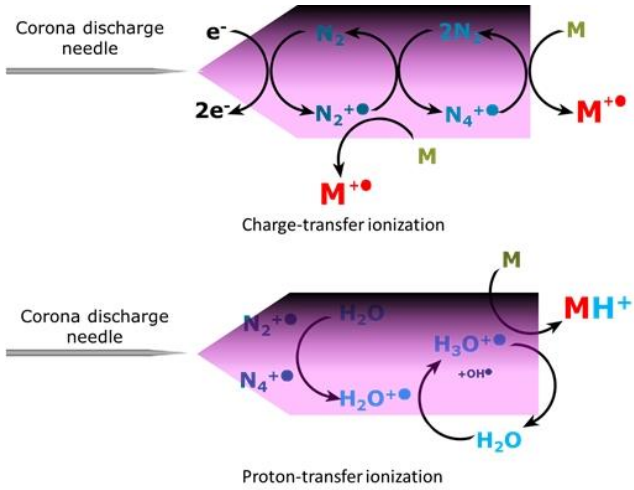

Figure 3.1 Ionization mechanisms in APCI source.

APCI has already been satisfactorily applied to the determination of GC-amenable compounds, such as pesticides (Cervera, Portolés, Pitarch, Beltrán, & Hernández, 2012), polycyclic aromatic hydrocarbons (PAHs) (Portolés, Garlito, Nácher-Mestre, Berntssen, & Pérez-Sánchez, 2017), non dioxin-like PCBs (Pitarch et al., 2016), and dioxins (Van Bavel et al., 2015), among others. In this chapter, the use of APCI source is presented, coupled to GC-MS/MS (QqQ) instruments, looking for enhanced limits of detection, reliability in the determination, and shorter analytical methodologies applied to 17 flame retardants and 12 dioxin-like PCBs.

In a first approach, a methodology for the determination for 16 BFRs, including low and high brominated congeners and two novel BFRs has been developed and optimized. Efforts were devoted towards reaching the lowest possible detection levels with enhanced sensitivity due to the possibility of choosing an abundant molecular ion for the selection of SRM transitions. The findings of this work are presented in the **Scientific article 2.** In a second work, following the advances reached in the first article, a

method for a rapid screening and quantification of total HBCD in a wide variety of complex samples, including polyurethane foam disks (PUFs), food, and marine samples was developed. HBCD commonly elutes in the same fraction as the traditional BFRs treated in article 2, but quantification of its individual isomers is typically performed by LC-MS procedures. In this case, the use of GC-(APCI)MS/MS allowed to determine if samples had HBCD concentrations high enough to be worth quantifying further by LC-MS, while simultaneously determining the concentrations of other BFRs. This work is summarized in **Scientific article 3.** In the last approach, to improve the detection capabilities of other POPs, the same workflow and strategy was applied for the determination of DL-PCBs in complex-matrix food samples. The outputs of this work are described in **scientific article 4.**

These three research articles share some characteristics, paying special attention to the optimization of the instrumental parameters and the fragmentation conditions of the different compounds in the APCI source in order to ensure the correct identification/confirmation of the contaminants in the samples to avoid reporting false positives. All methodologies fulfil the requirements for the identification and quantification of pollutants, according to the newest European Union (EU) guidance, such as SANTE/11813/2017.

In all cases, the sample treatment was performed in external accredited laboratories, belonging to the Spanish National Research Council (CSIC) (Madrid and Barcelona) leading, on one hand, to the publication of highly cited interdisciplinary articles and, on the other hand, to extremely reliable results, in terms of limits of detection/quantification and recovery values.

The developed methodologies were tested analysing reference materials and/or interlaboratory samples to demonstrate their robustness and reliability.

3.2 Scientific Article 2

analytical chemistry

Article

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Novel Analytical Approach for Brominated Flame Retardants Based on the Use of Gas Chromatography-Atmospheric Pressure Chemical Ionization-Tandem Mass Spectrometry with Emphasis in Highly Brominated Congeners

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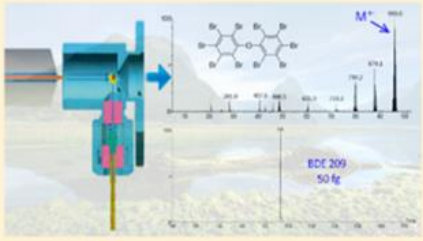
Supporting Information

ABSTRACT: The analysis of brominated flame retardants (BFRs) commonly relies on the use of gas chromatography coupled to mass spectrometry (GC-MS) operating in electron ionization (EI) and electron capture negative ionization (ECNI) modes using quadrupole, triple quadrupole, ion trap, and magnetic sector analyzers. However, these brominated contaminants are examples of compounds for which a soft and robust ionization technique might be favorable since they show high fragmentation in EI and low specificity in ECNI. In addition, the low limits of quantification (0.01 ng/g) required by European Commission Recommendation 2014/118/EU on the monitoring of traces of BFRs in food put stress on the use of highly sensitive techniques/methods. In this work, a new approach for the extremely sensitive determination of BFRs taking profit of the potential of atmospheric pressure chemical ionization (APCI) combined with GC and triple quadrupole (QqQ) mass analyzer is proposed. The objective was to explore the potential of this approach for the BFRs determination in samples at pg/g levels, taking marine samples and a cream sample as a model. Ionization and fragmentation behavior of 14 PBDEs (congeners 28, 47, 66, 85, 99, 100, 153, 154, 183, 184, 191, 196, 197, and 209) and two novel BFRs, decabromodiphenyl ethane (DBDPE) and 1,2-bis(2,4,6-tribromophenoxy)ethane (BTBPE), in the GC-APCI-MS system has been investigated. The formation of highly abundant (quasi) molecular ion was the main advantage observed in relation to EI. Thus, a notable improvement in sensitivity and specificity was observed when using it as precursor ion in tandem MS. The improved detectability (LODs < 10 fg) achieved when using APCI compared to EI has been demonstrated, which is especially relevant for highly brominated congeners. Analysis of samples from an intercomparison exercise and samples from the marine field showed the potential of this approach for the reliable identification and quantification at very low concentration levels.



Brominated flame retardants (BFRs) are used in a variety of products, such as housing and wiring of TV sets, computers and mobile phones, in electrical kitchen appliances, textiles, building materials, and many plastic products, to reduce their flammability. Thus, exposure to BFRs may occur in many situations in daily life. Due to the fact that most BFRs are persistent, bioaccumulative, and toxic or neurotoxic, the European Union (EU) has taken actions in regard to the use and applications of BFRs, banning the use of polybrominated diphenyl ether (PBDEs) mixtures (Penta-BDE and Octa-BDE mixtures) in 2004 and Deca-BDE mixtures in electric and electronic products in 2009. In addition, limits have been set for Penta-BDE and Octa-BDE in products placed on the market. As a result of bans, the use of novel BFRs, such as decabromodiphenyl ethane (DBDPE) and 1,2-bis(2,4,6-tribromophenoxy)ethane (BTBPE), used in replacement of Deca-BDE and Octa-BDE mixtures, respectively, and the use of organophosphorus flame retardants is increasing.[1−3] Several reviews have reported the analytical methods commonly used for the determination of PBDEs in environmental and biological matrices.[4−10] Gas chromatography (GC) coupled to mass spectrometry (MS) operating in both electron ionization (EI) and electron capture negative ionization (ECNI) modes using quadrupole (Q), triple quadrupole (QqQ), or ion trap (IT) analyzers are the techniques most widely used.[2,11−15] GC-EI-MS methods are readily suitable for quantifying Br_{1-7} BDEs in environmental and biological

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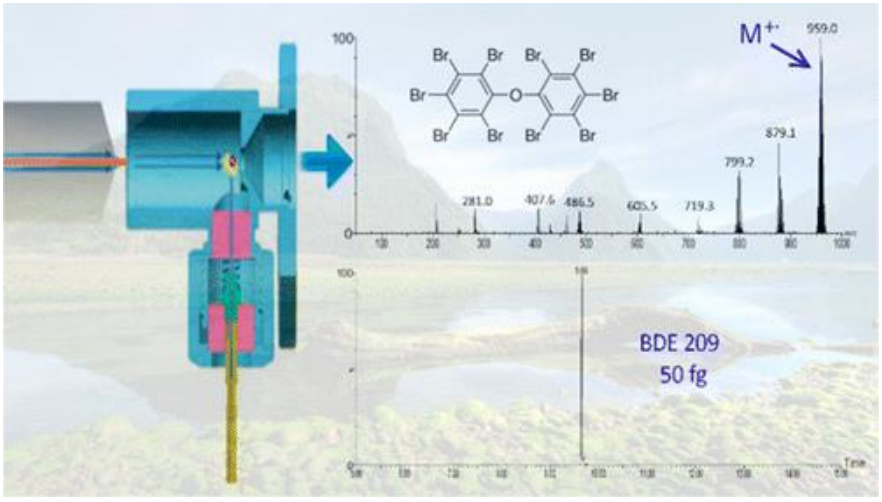
3.2.1 Abstract

The analysis of brominated flame retardants (BFRs) commonly relies on the use of gas chromatography coupled to mass spectrometry (GC-MS) operating in electron ionization (EI) and electron capture negative ionization (ECNI) modes using quadrupole, triple quadrupole, ion trap, and magnetic sector analyzers. However, these brominated contaminants are examples of compounds for which a soft and robust ionization technique might be favorable since they show high fragmentation in EI and low specificity in ECNI. In addition, the low limits of quantification (0.01 ng/g) required by European Commission Recommendation 2014/118/EU on the monitoring of traces of BFRs in food put stress on the use of highly sensitive techniques/methods. In this work, a new approach for the extremely sensitive determination of BFRs taking profit of the potential of atmospheric pressure chemical ionization (APCI) combined with GC and triple quadrupole (QqQ) mass analyzer is proposed. The objective was to explore the potential of this approach for the BFRs determination in samples at pg/g levels, taking marine samples and a cream sample as a model. Ionization and fragmentation behavior of 14 PBDEs (congeners 28, 47, 66, 85, 99, 100, 153, 154, 183, 184, 191, 196, 197, and 209) and two novel BFRs, decabromodiphenyl ethane (DBDPE) and 1,2-bis(2,4,6-tribromophenoxy)ethane (BTBPE), in the GC-APCI-MS system has been investigated. The formation of highly abundant (quasi) molecular ion was the main advantage observed in relation to EI. Thus, a notable improvement in sensitivity and specificity was observed when using it as

precursor ion in tandem MS. The improved detectability (LODs < 10 fg) achieved when using APCI compared to EI has been demonstrated, which is especially relevant for highly brominated congeners. Analysis of samples from an intercomparison exercise and samples from the marine field showed the potential of this approach for the reliable identification and quantification at very low concentration levels.



Graphical Abstract

3.2.2 Introduction

Brominated flame retardants (BFRs) are used in a variety of products, such as housing and wiring of TV sets, computers and mobile phones, in electrical kitchen appliances, textiles, building materials, and many plastic products, to reduce their flammability. Thus, exposure to BFRs may occur in many situations in daily life. Due to the fact that most BFRs are persistent, bioaccumulative, and toxic or neurotoxic, the European Union (EU) has taken actions in regard to the use and applications of BFRs, banning the use of polybrominated diphenyl ether (PBDEs) mixtures (Penta-BDE and Octa-BDE mixtures) in 2004 and Deca-BDE mixtures in electric and electronic products in 2009. In addition, limits have been set for Penta-BDE and Octa-BDE in products placed on the market. As a result of bans, the use of novel BFRs, such as decabromodiphenyl ethane (DBDPE) and 1,2-bis(2,4,6-tribromophenoxy)ethane (BTBPE), used in

replacement of Deca-BDE and Octa-BDE mixtures, respectively, and the use of organophosphorus flame retardants is increasing.(1-3) Several reviews have reported the analytical methods commonly used for the determination of PBDEs in environmental and biological matrices.(4-10) Gas chromatography (GC) coupled to mass spectrometry (MS) operating in both electron ionization (EI) and electron capture negative ionization (ECNI) modes using quadrupole (Q), triple quadrupole (QqQ), or ion trap (IT) analyzers are the techniques most widely used.(2, 11-15) GC-EI-MS methods are readily suitable for quantifying $Br_{1–7}$ BDEs in environmental and biological samples, but are not on purpose for higher brominated analogues ($Br_{8–10}$ BDEs), and further research should be directed toward the improvement of accuracy and sensitivity for highly brominated BDE congeners. GC-EI-MS is prone to interferences from other chemicals with similar structures (especially polychlorinated biphenyls, PCBs), which may affect the quality of the analytical data. GC-ECNI-MS shows higher sensitivity than with EI (especially for high brominated PBDEs) and can avoid chlorinated interferences. However, ECNI mass spectra are mainly dominated by the two ions of bromine (*m*/*z* 79 and 81), which leads to difficult discrimination of PBDE congeners from different homologue groups and among any bromo-compounds.(2)

The higher selectivity and sensitivity offered by GC-EI-MS/MS with QqQ and IT might be a good alternative to the other MS modes, as less matrix effects and interferences from PCBs are commonly observed. However, despite the higher detection specificity, coeluting PBDE congeners with higher bromine content could still rise to isobaric precursor and fragment ions, which would contribute false signals to the MS/MS channel monitored for a specifically targeted PBDE. In addition, EI ionization produces high fragmentation of compounds, making it difficult to select specific and abundant precursor ions. This fact compromises the selectivity and sensitivity of the MS/MS method.(4)

GC coupled with high resolution MS (GC-HRMS) using magnetic sectors has better sensitivity for the higher brominated congeners and can eliminate interferences between chlorinated and brominated compounds and also among brominated congeners. To this aim, it is necessary to increase the resolution to 24000 fwhm, which is not appropriate from the sensitivity

point of view due to the significant loss of sensitivity. Moreover, the high acquisition and maintenance costs of these instruments make this technique not very accessible for most laboratories.(4)

Recently, the European Commission has published a recommendation urging the European Member to perform monitoring on the presence of brominated flame retardants in a wide variety of individual foodstuffs at limits of quantification extraordinarily low, mostly setup at 0.01 ng/g.(16) This will imply great difficulties for the laboratories to get the sensitivity required to reach such a low concentration in complex-matrix samples as eggs, milk, meat, animal and vegetable fats and oils, fish, and food for infants and small children. Difficulties will be even higher for highly brominated congeners (e.g., BDE 209). The recently revived atmospheric pressure chemical ionization (APCI) source has been satisfactorily applied for GC-amenable compounds such as pesticides, polycyclic aromatic hydrocarbons (PAHs), PCBs, and, very recently, dioxins, dioxin-like PCBs, and flame retardants.(17-21) The soft ionization generated by this source promotes the formation of the molecular ion and the protonated molecule, commonly as the base peak of the spectrum, with poor fragmentation in comparison to the high fragmentation degree generally observed under EI. This is particularly relevant for compounds that suffer extensive fragmentation in EI and for which the molecular ion used is present with low abundance (e.g., most PBDEs). The use of GC-APCI-MS/MS allows the selection of sensitive and specific precursor ion for selected reaction monitoring (SRM) transitions, which leads to a notable increase in sensitivity and selectivity of the analytical method.

In the present study, the potential of GC-MS/MS using QqQ with APCI source has been explored for the extreme sensitive determination of brominated flame retardants such as PBDEs, BTBPE, and DBDPE in complex matrices. Special attention has been paid to the optimization of ionization and the selection of appropriate SRM transitions to improve both sensitivity and selectivity. The developed approach has been tested in real-world samples in order to illustrate the possibilities of this technique in the near future.

3.2.3 Experimental Section

Reagents

All reagents used for the analysis of PBDEs were of trace analysis grade. Sulfuric acid (95–97%) and silica gel were supplied by Merck Co. (Darmstadt, Germany). Acetone, *n*-hexane, toluene, and granular anhydrous sodium sulfate were obtained from J.T. Baker (Deventer, The Netherlands). All standards were purchased from Wellington Laboratories (Ontario, Canada). A total of 14 PBDE congeners from tri- to deca-BDEs, numbers 28 (2,4,4′-tri-BDE), 47 (2,2′,4,4′-tetra-BDE), 66 (2,3′,4,4′-tetra-BDE), 85 (2,2′,3,4,4′-penta-BDE), 99 (2,2′,4,4′,5-penta-BDE), 100 (2,2′4,4′,6-penta-BDE), 153 (2,2′,4,4′,5,5′-hexa-BDE), 154 (2,2′,4,4′,5,6′-hexa-BDE), 183 (2,2′,3,4,4′,5′,6-hepta-BDE), 184 (2,2′,3,4,4′,6,6′-hepta-BDE), 191 (2,3,3′,4,4′,5′,6-hepta-BDE), 196 (2,2′,3,3′,4,4′,5′,6-octa-BDE), 197 (2,2′,3,3′,4,4′,6,6′-octa-BDE), and 209 (2,2′,3,3′,4,4′,5,5′,6,6′-deca-BDE) and two novel BFRs (BTBPE and DBDPE) were considered. Three $^{13}C_{12}$-labeled standards, PBDEs 47, 99, and 153, were used as surrogate standards for the quantification of samples.

Real Samples

A cream sample belonging to the “Interlaboratory Comparison on Dioxins in Food” organized by the Norwegian Institute of Public Health in 2008 and 11 samples from the marine field (1 dolphin, 4 fish of different sizes, 2 prawns, 2 squids, and 2 zooplankton) were used for the evaluation of the applicability of the developed method.

Sample Treatment

Sample extraction and purification procedures have been previously described in the literature, where recoveries of the extraction procedure for labeled BDE congeners $^{13}C_{12}$–BDEs 47, 99, and 153, added to samples before the extraction step, were found to be higher than 65%.(22, 23) Briefly, extraction involved matrix solid-phase dispersion (MSPD); 6–200 g fresh sample homogenized with 4:1 (w/w) silica gel/anhydrous sodium sulfate powder, and spiked with $^{13}C_{12}$–PBDEs 47, 99, and 153, was ground to a fine powder, loaded onto a column, and extracted with 400 mL of 1:1

(v/v) acetone/*n*-hexane mixture. For the cleanup of the extracts, two multilayer columns filled with neutral silica, silica modified with sulfuric acid (44%, w/w), and silica modified with KOH (56%, w/w) were employed using *n*-hexane as elution solvent. When required, the final extract containing the target compounds was subjected to further fractionation on Supelclean ENVI -Carb SPE cartridges, as described elsewhere(24) to separate *ortho*-substituted PCBs plus PBDEs from PCDD/Fs and non-*ortho*-substituted PCBs.

3.2.4 Instrumentation

GC-APCI-MS/MS

Data were acquired using a GC system (Agilent 7890A, Palo Alto, CA, U.S.A.) equipped with an autosampler (Agilent 7693) and coupled to a triple quadrupole (QqQ) mass spectrometer (Xevo TQ-S, Waters Corporation, Manchester, U.K.), operating in positive APCI mode. A fused silica capillary column of 15 m × 0.25 mm i.d. and a film thickness of 0.1 µm (J&W Scientific, Folson, CA, U.S.A.), and using a stationary phase of 100% methyl polysiloxane (DB-1HT), which can hold temperatures higher than the 300 °C required to elute high brominated PBDEs, was used for GC separation. The injector was operated in pulsed splitless mode (50 psi, 1 min), injecting 1 µL at 280 °C. The oven temperature was programmed as follows: 140 °C (1 min); 10 °C/min to 200 °C; 20 °C/min to 300 °C; and 40 °C/min to 350 °C (1 min). Helium was used as carrier gas in constant flow mode (4 mL/min). In the SRM method, automatic dwell time (9–46 ms) was applied in order to obtain 15 points per peak.

The interface temperature was set to 340 °C using N_2 as auxiliary gas at 250 L/h, makeup gas at 300 mL/min, and cone gas at 170 L/h. The APCI corona discharge pin was operated at 1.6 µA. The ionization process occurred within an enclosed ion volume, which enabled control over the protonation/charge transfer processes. Water used as modifier when working under proton-transfer conditions was placed in an uncapped vial, which was located within a specially designed holder placed in the source door. Targetlynx (a module of MassLynx) was used to process the acquired data.

GC-EI-MS/MS

EI analyses were performed on a TRACE GC Ultra gas chromatograph (Thermo Fisher Scientific) coupled triple quadrupole analyzer (TSQ Quantum XLS, Thermo Fisher Scientific).

3.2.5 Results and Discussion

Ionization and Fragmentation Behavior of PBDEs, BTBPE, and DBDPE in GC-APCI and GC-EI Coupled to MS with Triple Quadrupole Analyzer

In EI, the major ions formed from PBDEs are normally the $[M]^{+\bullet}$ and $[M\text{-}Br_2]^{+\bullet}$, which are useful for identification purposes. Abundances of $[M]^{+\bullet}$ and $[M\text{-}Br_2]^{+\bullet}$ decrease with increasing bromine substituents being this effect more drastic in the case of BDE 209 (**Figure 1**, bottom). For tri- and tetra-BDE congeners, the most abundant ions correspond to the molecular ion cluster and low fragmentation is observed. Moreover, the EI spectra for penta- to deca-BDEs are generally dominated by the cluster corresponding to the loss of two bromine atoms, except for non-*ortho*-brominated congeners, which have a particularly abundant $[M]^{+\bullet}$, but almost no $[M\text{-}Br_2]^{+\bullet}$.(2, 4, 11, 25, 26)

In this work, the "soft" ionization behavior of the APCI interface was tested using PBDEs, BTBPE, and DBDPE standards in solvent. Two mechanisms of ionization were observed: (i) charge transfer in which the nitrogen plasma created by the corona discharge needle promotes the formation of $M^{+\bullet}$, and (ii) proton transfer, where the presence of water vapor traces in the source favors the formation of the $[M + H]^+$ ion.

The PBDEs studied showed a mixture of two isotopic patterns corresponding to $M^{+\bullet}$ and $[M + H]^+$ as base peak of the spectrum. **Figure 1** (up) shows the APCI spectrum of BDE 209, where a cluster corresponding to both isotopic clusters of $M^{+\bullet}$ and $[M + H]^+$ can be observed as base peak of the spectrum, although water had not been added in the source. The bromination degree affected to the behavior of the compound under APCI. In the case of $Br_{3–4}$ BDEs, the $[M + H]^+$ intensity was higher than $M^{+\bullet}$. In contrast, for $Br_{5–10}$ BDEs, the intensity of $M^{+\bullet}$ was slightly higher than $[M + H]^+$ and increased with increasing bromine substituents. APCI mass

spectrum of BTBPE also showed a mixture of the isotopic patterns corresponding to $M^{+\bullet}$ and $[M + H]^+$ (although in this case not as base peak). For DBDPE ($C_{14}H_4Br_{10}$), the base peak was a fragment corresponding to the ion $C_8H_4Br_5^{+\bullet}$ and neither $M^{+\bullet}$ nor $[M + H]^+$ was observed.

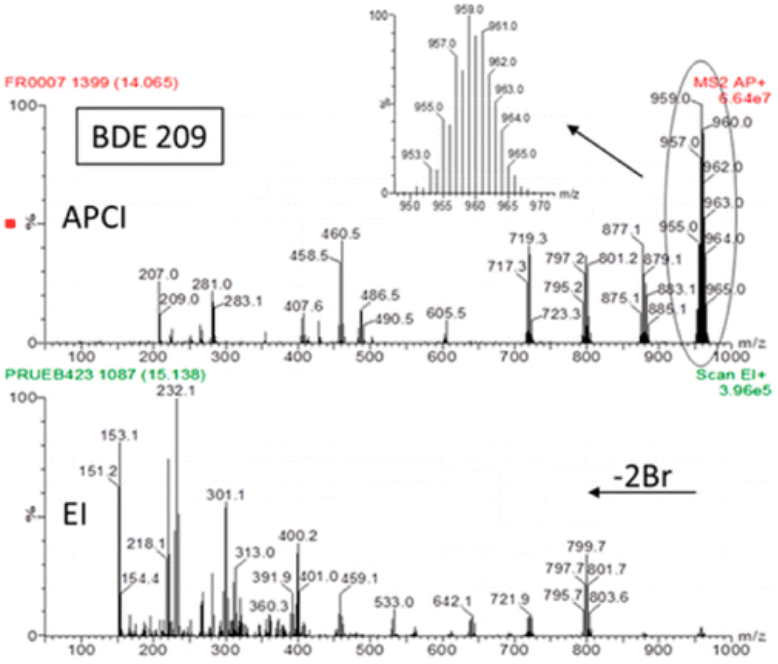


Figure 1. APCI (top) and EI (bottom) spectrum of BDE 209.

Cone voltage between 5 and 50 V was tested in order to select the optimum value for each compound considering the possibility that in-source fragmentation can occur, especially at higher cone voltages. No significant differences on in-source fragmentation pattern were observed, but voltages higher than 40 V generally led to a loss of abundance of the molecular ion and protonated molecule without increasing the abundance of mass fragments. For each compound, the cone voltage that gave the highest intensity for the quasi-molecular ion (typically 20 V) was selected for further experiments.

Finally, the fragmentation of the PBDEs, BTBPE, and DBDPE in the collision cell was studied. $[M]^{+\bullet}$, $[M + 2]^{+\bullet}$, and $[M + 4]^{+\bullet}$ were selected as precursor ions for $Br_{3–4}$ BDEs; $[M + 2]^{+\bullet}$, $[M + 4]^{+\bullet}$, and $[M + 6]^{+\bullet}$ for $Br_{5–6}$ BDEs; [M +

$4]^{+\bullet}$, $[M + 6]^{+\bullet}$, and $[M + 8]^{+\bullet}$ for $Br_{7–10}$ BDEs. Fragmentation was performed at collision energies between 5 and 60 eV. The losses of Br_2 and Br_2 + CBrO were the most abundant and common to all PBDEs studied. Moreover, the loss of Br_4 was also observed for $Br_{5–10}$ BDEs, Br_4 + CO for $Br_{4–5}$ BDEs, and Br_5 + CO for $Br_{5–6}$ BDEs. BTBPE showed the losses of $C_6H_2Br_3O$, $C_6H_2Br_4O$, and $C_6H_2Br_4O_2$ from the ions $[M + 2]^{+\bullet}$, $[M + 4]^{+\bullet}$, and $[M + 6]^{+\bullet}$. DBDPE showed the losses of Br, Br_2, and Br_3 from the fragment ion ($C_8H_4Br_5^{+\bullet}$) selected as precursor ($[F + 2]^{+\bullet}$, $[F + 4]^{+\bullet}$, and $[F + 6]^{+\bullet}$). As an illustrative example, **Figure S-1A** shows the APCI product ion spectra for $M^{+\bullet}$ (charge-transfer conditions) of BDE 47 (top) and BDE 209 (down) at different collision energies.

Effect of Added Modifiers on the Ionization and Fragmentation Behavior of PBDEs, BTBPE, and DBDPE in GC-APCI-MS/MS with QqQ

As explained above, both $[M + H]^+$ and $M^{+\bullet}$ were observed on APCI spectra of all PBDEs, even under "dry" conditions. This fact encouraged us to introduce water as a modifier to promote the formation of the protonated molecule. Water was added on purpose, and the presence/absence and improvement on the signal of the protonated molecule was evaluated. The modifier was placed in an uncapped vial, located within a specially designed holder in the source door.

The use of water as modifier favored the formation of the $[M + H]^+$ and the $M^{+\bullet}$ disappeared in most cases, increasing the proportion of $[M + H]^+$. These occurred for all the PBDEs studied, except for the $Br_{7–10}$ BDEs and BTBPE for which a low percentage of $M^{+\bullet}$ still appeared in their spectra. Protonation degree was improved for the BTBPE and $Br_{7–10}$ BDEs adding aqueous 1% HCOOH as modifier. The spectrum of DBDPE did not change when adding modifiers to the source, maintaining the fragment indicated before as base peak.

Next, the PBDEs fragmentation in the collision cell was studied, after selection of the protonated molecule as precursor ion. The quasi-molecular ion $[M + H]^+$, $[M + H + 2]^+$, and $[M + H + 4]^+$ were selected as precursor ions for $Br_{3–4}$ BDEs; $[M + H + 2]^+$, $[M + H + 4]^+$, and $[M + H + 6]^+$ for $Br_{5–6}$ BDEs; and $[M + H + 4]^+$, $[M + H + 6]^+$, and $[M + H + 8]^+$ for $Br_{7–10}$

BDEs. Again, fragmentation was performed at collision energies in the range 5–60 eV. The losses of Br and Br_3 were the most abundant and common to all PBDEs. The loss of $CHBr_3O$ was observed for tri-BDEs, and the loss of CBr_4O for tetra- and penta- BDEs. BTBPE showed losses of $C_6H_2Br_3O$, $C_6H_2Br_3O$ + Br, $C_6H_2Br_3O$ + COBr, and $C_6H_2Br_3O$ + Br + COBr from $[M + H + 2]^+$, $[M + H + 4]^+$, and $[M + H + 6]^+$. DBDPE showed the losses of CH_2, Br, Br_2, and Br_3 from the fragment ion selected $C_8H_4Br_5^{+\bullet}$ ($[F + 2]^{+\bullet}$, $[F + 4]^{+\bullet}$, and $[F + 6]^{+\bullet}$). As an example, **Figure S-1B** shows the APCI product ion spectra for $[M + H]^+$ (proton-transfer conditions, 1% HCOOH as modifier) of BDE 47 (top) and BDE 209 (down) at different collision energies. The selected reaction monitoring (SRM) transitions optimized for each compound are shown in **Table 1**.

Sensitivity and Repeatability

The sensitivity and repeatability of the GC-MS/MS signals for PBDEs, BTBPE, and DBDPE were evaluated for all the transitions selected (those coming from $M^{+\bullet}$ as well as from $[M + H]^+$) under the different conditions (with and without the use of modifiers). Under charge-transfer conditions (without adding water), all compounds showed better sensitivity for $M^{+\bullet}$ transitions being in most cases among 2 and 7 times higher than for $[M + H]^+$ (see **Figure 2A**). As expected, the sensitivity for all compounds dramatically decreased (around 20×) for the $M^{+\bullet}$ transitions when adding water as modifier, while at the same time, the intensity for $[M + H]^+$ transitions improved around 1.5×. This occurred for all the compounds except for $Br_{6–10}$ BDEs and DBDPE for which a 2-fold decrease was observed for $[M + H]^+$. When adding HCOOH 1%, the sensitivity of $[M + H]^+$ transitions remained almost the same as with only water. In any case, all the compounds showed the highest sensitivity under charge-transfer conditions for the $M^{+\bullet}$ transitions.

The repeatability of the response (n = 10 at 25 ng/mL) was also evaluated under the different working conditions (**Figure 2B**). Data showed a slightly lower repeatability for $M^{+\bullet}$ transitions when working under charge-transfer conditions (relative standard deviations, RSD, between 10 and 20% for most compounds) being a bit better for $[M + H]^+$ transitions (RSD 5–20%). This parameter improved under proton-transfer conditions using

water as modifier (RSD 5–15%) and became even better when the protonation was promoted adding HCOOH 1% (RSD < 10%). Therefore, under charge-transfer conditions, a reproducible mixture of both molecular ion and protonated molecule occurred for most PBDEs, with acceptable RSD.

Specificity of the SRM Transitions

Despite the soft ionization occurring in the APCI source, some in-source fragments were observed in the full scan spectra of PBDEs. These mainly came from losses of bromine that also correspond to precursor ions of SRM transitions selected for PBDEs showing a lower brominated degree. This is important, because potential losses of bromine that also correspond to precursor ions of SRM transitions selected for PBDEs showing lower brominated degree. This is important, because potential interferences from PCBs can be eliminated, as it already occurred with GC-EI-MS/MS experiments with QqQ and IT and the GC-ECNI-MS. However, this fact might led to loss of specificity of the transitions in case of coelution of PBDEs with different bromine degrees, as PBDEs with higher bromination might interfere in the determination of the lower ones, as it already occurred with GC-EI-MS/MS experiments with QqQ and IT. This fact only occurred for experiments performed under charge-transfer conditions and using the SRM transitions resulting from the use of $M^{+\bullet}$ as precursor ion. On the contrary, if precursor ions were $[M + H]^+$, this overlapping did not occur (see **Figure S-2**). This implies that ion-source fragmentation mainly comes from the $M^{+\bullet}$; thus, when it is removed by favoring the formation of $[M + H]^+$, its in-source fragments are also eliminated.

Overall, the better sensitivity expected for a SRM method with $M^{+\bullet}$ transitions under charge-transfer conditions together with acceptable repeatability seemed to be a good option for determination of PBDEs at low concentrations. However, the better specificity and repeatability resulting from $[M + H]^+$ transitions encouraged us to not discard an analytical method based on this option yet. Therefore, further experiments were carried out using both approaches in order to select the best one.

Linearity and LODs

Linearity of relative response of analytes was established by analyzing standards solutions, in triplicate, in the range of 0.2–100 ng/mL (for $Br_{3–8}$ BDEs and BTBPE), 1–500 ng/mL (BDE 209), and 2–1000 ng/mL (DBDPE). Data were obtained using both approaches: $M^{+\bullet}$ transitions under charge-transfer conditions and $[M + H]^+$ transitions under proton-transfer conditions. The correlation coefficients (*r*) were higher than 0.99, with residuals lower than 20% for all the compounds. LODs (signal-to-noise ratio 3) were extremely low, between 1 and 10 fg for the method under charge-transfer conditions, and slightly higher under proton-transfer conditions, 1–25 fg. These values were lower than those previously reported using other ionization sources (40–41000 fg by GC-EI-MS/MS with QqQ, 280–5200 fg by GC-EI-MS/MS with ITD, 6–507 fg by GC-ECNI-MS, or 20–50 fg by GC-EI-HRMS (in this last case, only for low brominated PBDEs)).(11, 13, 22, 27) In contrast to EI-based techniques, in our work the sensitivity for these compounds seemed not to be related with the brominating degree, as detectability was similar for all congeners independent of the number of bromine atoms.

Table 1. Experimental Conditions of the Optimized GC-APCI-QqQ (MS/MS) Method for PBDEs and Novel BFRs under Both Charge- and Proton-Transfer Conditions

		charge-transfer conditions				proton-transfer conditions			
compound	t_R (min)	precursor ion (m/z)	product ion (m/z)	collision energy (eV)	loss	precursor ion (m/z)	product ion (m/z)	collision energy (eV)	loss
BDE 28	3.7	403.8	246	20	$-Br_2$	404.8	167.9	30	$-Br_3$
			139	30	$-CBr_3O$	406.8	327.9	10	$-Br$
		405.8	246	20	$-Br_2$		168.1	30	$-Br_3$
			139	30	$-CBr_3O$	408.8	327.9	10	$-Br$
		407.8	248	20	$-Br_2$		168.0	30	$-Br_3$
			139	30	$-CBr_3O$				
$^{13}C_{12}$-BDE 47	*4.82*	*495.5*	*335.8*	*20*		*496.7*	*258*	*30*	
BDE 47	4.82	481.6	323.8	20	$-Br_2$	484.7	405.8	20	$-Br$
BDE 66	4.97	483.6	323.8	20	$-Br_2$		246	30	$-Br_3$
			217	60	$-CBr_3O$	486.7	405.8	20	$-Br$
		485.6	325.8	20	$-Br_2$		246	30	$-Br_3$
			219	50	$-CBr_3O$		139.1	60	$-CBr_4O$
			138	60	$-CBr_4O$				
$^{13}C_{12}$-BDE 99	*5.81*	*575.5*	*415.7*	*30*		*578.6*	*337.9*	*30*	
BDE 100	5.6	561.5	244	50	$-Br_4$	562.6	483.6	20	$-Br$
BDE 99	5.81		137.1	60	$-CBr_3O$	564.6	485.6	20	$-Br$
BDE 85	6.14	563.5	403.7	30	$-Br_2$		325.9	30	$-Br_3$
			296.9	50	$-CBr_3O$	566.6	485.6	20	$-Br$
		565.5	405.7	30	$-Br_2$		325.9	30	$-Br_3$
			296.9	50	$-CBr_3O$				
$^{13}C_{12}$-BDE 153	*6.67*	*655.4*	*495.6*	*30*		*656.5*	*415.8*	*30*	
BDE 154	6.39	639.4	372.8	50	$-CBr_3O$	640.4	561.6	20	$-Br$
BDE 153	6.67		321.8	60	$-Br_4$	642.4	563.6	20	$-Br$
		641.4	481.6	30	$-Br_2$		403.8	30	$-Br_3$
			374.8	50	$-CBr_3O$	644.5	563.5	20	$-Br$
		643.4	483.6	30	$-Br_2$		403.8	30	$-Br_3$
			217	60	$-CBr_3O$				
BDE 184	7.3	719.3	452.7	60	$-CBr_3O$	720.4	641.4	20	$-Br$
BDE 183	7.44		401.8	60	$-Br_4$	722.3	643.4	20	$-Br$
BDE 191	7.63	721.3	561.7	30	$-Br_2$		483.6	30	$-Br_3$
			454.7	60	$-CBr_3O$	724.3	643.4	20	$-Br$
		723.3	563.5	30	$-Br_2$		483.6	30	$-Br_3$
			454.7	60	$-CBr_3O$				
BTBPE	7.69	683.5	355	10	$-C_6H_2Br_5O$	684.5	354.9	10	$-C_6H_2OBr_5$
			275.9	30	$-C_6H_2Br_4O$	686.5	356.8	10	$-C_6H_2OBr_5$
		685.5	357.1	10	$-C_6H_2Br_5O$	688.5	357	10	$-C_6H_2OBr_5$
			249.9	40	$-C_7H_2Br_4O_2$		277.9	30	$-C_6H_2OBr_4$
		687.5	357.1	10	$-C_6H_2Br_5O$		251.8	50	$-C_7H_2O_2Br_4$
			278.3	30	$-C_6H_2Br_4O$				
BDE 197	8.3	797.2	530.6	60	$-CBr_3O$	798.2	719.7	20	$-Br$
BDE 196	8.47		479.9	60	$-Br_4$	800.3	721.3	20	$-Br$
		799.2	639.5	30	$-Br_2$		561.8	40	$-Br_3$
			532.5	60	$-CBr_3O$	802.3	721.6	20	$-Br$
		801.3	641.8	30	$-Br_2$		561.5	40	$-Br_3$
			534.5	60	$-CBr_3O$				
BDE 209	9.69	955	795.2	30	$-Br_2$	956.1	877.1	20	$-Br$
			637.4	60	$-Br_4$	958.1	879.3	20	$-Br$
		957	797.2	30	$-Br_2$		719.3	40	$-Br_3$
			637.4	60	$-Br_4$	960.1	879.2	20	$-Br$
		959	799.2	30	$-Br_2$		719.3	40	$-Br_3$
			639.3	60	$-Br_4$				
DBDPE	9.94	496.4	417.5	20	$-Br$	496.4	417.5	20	$-Br$
			338.6	30	$-Br_2$		338.6	30	$-Br_2$
			257.7	50	$-Br_3$		257.7	50	$-Br_3$
		498.4	419.6	20	$-Br$	498.4	419.6	20	$-Br$
			338.7	30	$-Br_2$		338.7	30	$-Br_2$
			259.9	50	$-Br_3$		259.9	50	$-Br_3$

The improvement in sensitivity obtained is more crucial for the highly brominated BDEs, as the analytical chemistry commonly find problems in reaching the low concentrations required in the legislation. **Figure 3A** shows the excellent sensitivity that can be reached by GC-APCI-MS/MS (charge-transfer conditions). At 0.05 ng/mL (0.05 pg BFRs on-column) S/N was still around 100 for all BFRs, except BTBPE (approximately 10× less sensitive) for which S/N was around 20 at 0.1 ng/mL. Similarly, **Figure 3B** illustrates the good sensitivity that can also be reached by GC-APCI-MS/MS (proton-transfer conditions), although being a bit lower than in the other option. Under the conditions established in this work, the disadvantages when using other reported techniques can be avoided. In addition, working under GC-APCI-MS/MS, the typical interferences coming from PCBs (e.g., in GC-EI-MS) do not exist. The high sensitivity observed for all PBDEs, especially for the higher brominated PBDE congeners (even better than that obtained with GC-ECNI-MS and GC-EI-HRMS), is also a key aspect in the present approach.

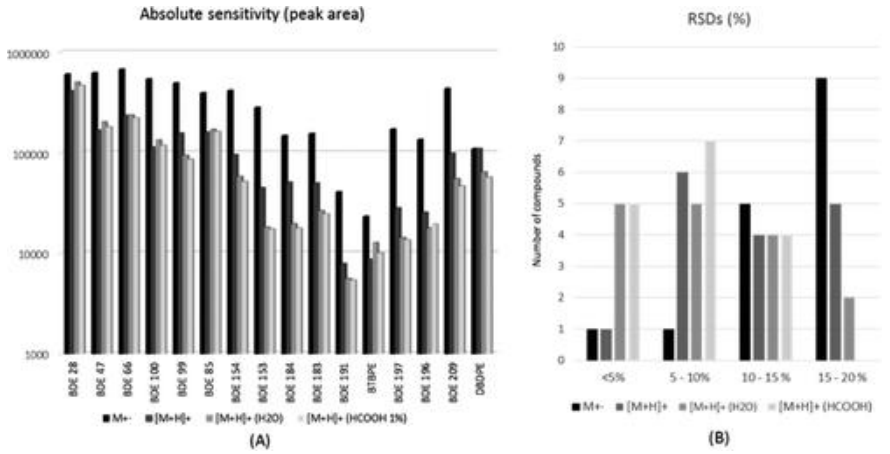


Figure 2. (A) Absolute sensitivity (logarithmic scale) of PBDEs, BTBPE, and DBDPE under different ionization conditions using different modifiers. (B) Repeatability of the response (% RSD, *n* = 10) for PBDEs, BTBPE, and DBDPE under different conditions using different modifiers.

Application to the Analysis of Interlaboratory Exercise and Marine Samples

The two approaches evaluated in this work were finally applied to the analysis of the endogenous PBDE levels in samples belonging to the "Interlaboratory Comparison on Dioxins in Food" organized by the Norwegian Institute of Public Health in 2008. A cream sample was selected since it was contaminated with low levels of PBDEs (from 0.55 to 45 pg/g fresh weight). The concentrations obtained using the developed methodology were all within the range ($|z\text{-score}| < 2$) for all the congeners detected. It is important to note that only a small number of participants in the interlaboratory comparison exercises reported PBDEs data, compared with those that reported PCBs and PCDD/Fs. This fact, together with the high variability of the reported data (for some PBDEs with deviations up to 100%), illustrates the present difficulties in performing determinations of PBDE congeners in biological and food samples and the need for accurate, selective, sensitive, and low-cost methods, such as those that can result from the present work. Unfortunately, novel BFRs are still not included as target compounds in this type of interlaboratory exercise, and thus, it was not possible to verify method accuracy for them.

In addition, our methodology was tested for PBDEs and novel BFRs in samples from the marine field. Quantification of the samples was carried out by using calibration curves with standards in solvent, using relative responses to three internal labeled standards added as surrogates to the samples: $^{13}C_{12}$–BDE 47 for tritetra BDEs, $^{13}C_{12}$–BDE 99 for penta-BDEs, and $^{13}C_{12}$–BDE 153 for the rest. The results were compared with those obtained by GC-EI-MS/MS (see **Table S-1**). For the major congeners (PBDEs 28, 47, 99, 100, 153, and 154) present in the samples at concentrations above the lowest calibration level (LCL), data were rather similar for both techniques with mean relative errors around 10%, except for a few cases where errors were up to 20 and 30%.

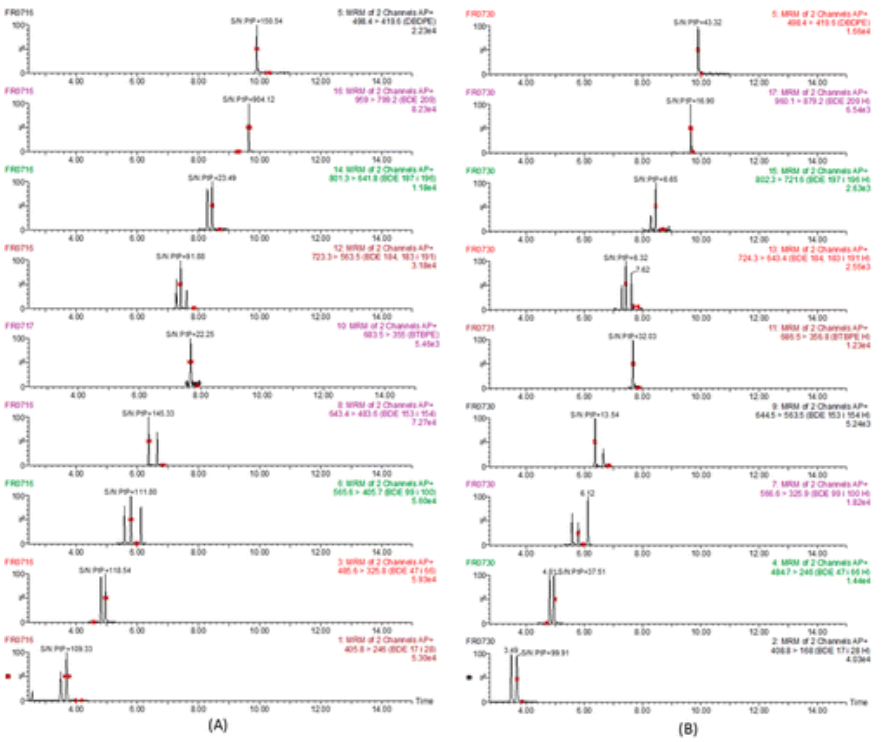


Figure 3. GC-APCI-MS/MS chromatogram of all BFRs for the concentration of 0.05 µg/L (except 0.1 µg/L for BTBPE) under (A) charge-transfer conditions and (B) proton-transfer conditions. S/N:PtP, signal-to-noise peak-to-peak ratio.

For BDE 209, the differences between APCI and EI were notable. While in EI, BDE 209 was detected in 6 out of 11 samples (quantified in 4 of them); the better sensitivity of APCI allowed to detect this compound in 9 out of 11 samples (quantified in 7 of them). The different concentrations reported by the two techniques applied revealed that BDE-209 was more problematic than the other PBDEs analyzed. The fact that none of the internal standards was the labeled BDE-209 surely affected to quantification, although not to the signal intensity, which was notably higher in APCI. More research is required to understand these differences, which were probably due to unknown matrix effects that could not be corrected using an appropriate internal standard. **Figure 4A** shows a positive finding of BDE 209 detected in fish that was not detected under GC-EI-MS/MS.

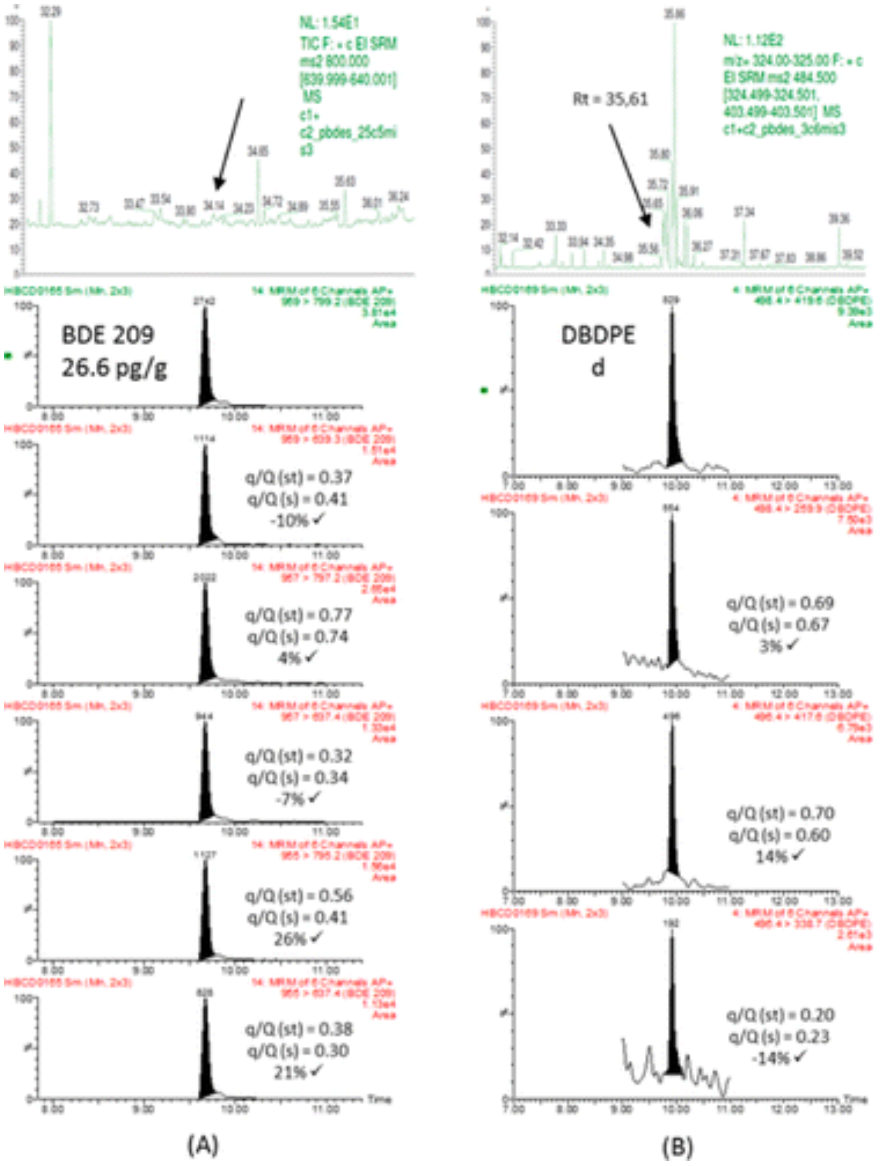


Figure 4. Positive findings in fish of (A) BDE 209 and (B) DBDPE detected and identified by applying GC-APCI-MS/MS (down) previously reported as not detected by GC-EI-MS/MS (top): d, detected, below LCL.

On the contrary, analysis by GC-APCI-MS/MS allowed identification of this compound using up to six SRM transitions (one for quantification and five for confirmation), giving an estimated concentration as low as 26.6 pg/g (corresponding to 7 µg/L in the extract). The improved method sensitivity

for real samples in comparison to previous reported approaches was evident.

For the minor congeners (PBDEs 66, 85, 183,184, 191, 196, and 197) and for the novel BFRs (BTBPE and DBDPE), the higher sensitivity resulting from GC-APCI-MS/MS allowed the detection and identification of many of these compounds that had not been detected by GC-EI-MS/MS (see **Table S-1**). Especially for some BDE congeners (numbers 183, 196, and 197) and also for DBDPE, most of the samples had previously resulted as non-detected, but after reanalysis by GC-APCI-MS/MS, they could be identified in the samples.

Figure 4B shows a positive finding of DBDPE in a fish sample that had been previously reported as negative sample by GC-EI-MS/MS with QqQ. Up to four SRM transitions were observed by APCI showing a concentration level near or below the lowest calibration point, which illustrates the improvement in sensitivity in the analysis of real samples.

No relevant differences were found either in the detection/identification or quantification of the samples when using the two GC-APCI approaches evaluated in this paper. As an example, **Figure S-3** shows a positive finding of BDE 209 in dolphin using both methodologies: charge-transfer mode (left) and proton-transfer mode (right).

3.2.6 Conclusions

The use of APCI has been evaluated as alternative source for GC-MS/MS analysis of BFRs. In contrast to the EI source, (quasi)molecular ions ($M^{+\bullet}$ and/or $[M + H]^+$) were obtained as base peak of the spectrum for most compounds. Commonly, a mixture of $M^{+\bullet}$ and $[M + H]^+$ was observed, and both ions might be selected as precursors in a MS/MS method. The best sensitivity was reached working under charge-transfer conditions and using $M^{+\bullet}$ as precursor ion. This fact strongly improved sensitivity (LODs, lower than 10 fg on-column) and selectivity compared to GC-EI-MS/MS analysis. Moreover, working under charge-transfer conditions would allow the investigation of other brominated flame retardants, for example, hexabromocyclododecane (data not shown), which elutes in the same cleanup fraction during sample treatment of PBDEs and whose ionization

was dramatically affected under proton-transfer conditions Under the conditions established in this work, some disadvantages when using other reported techniques for the sensitive determination of BFRs can be avoided. Thus, the high sensitivity observed for all PBDEs, especially for the higher brominated PBDE congeners (even better than with GC-ECNI-MS and GC-EI-HRMS), is a key aspect in the present approach. Not less important is the fact that this technique is not so expensive and requires lower maintenance costs and specialization than GC-HRMS. This paper lays the foundations for the subsequent development of an analytical procedure, which will be properly validated to set up the most relevant analytical characteristics (accuracy, precision, and limits of quantification). Hopefully, the future GC-APCI-MS/MS method will notably improve the performance of the traditional GC-EI-MS/MS methodology.

3.2.7 Acknowledgments

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3.3 Scientific Article 3

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PAPER IN FOREFRONT

Potential of gas chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry for screening and quantification of hexabromocyclododecane

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Abstract A fast method for the screening and quantification of hexabromocyclododecane (sum of all isomers) by gas chromatography using a triple quadrupole mass spectrometer with atmospheric pressure chemical ionization (GC-APCI-QqQ) is proposed. This novel procedure makes use of the soft atmospheric pressure chemical ionization source, which results in less fragmentation of the analyte than by conventional electron impact (EI) and chemical ionization (CI) sources, favoring the formation of the $[M - Br]^+$ ion and, thus, enhancing sensitivity and selectivity. Detection was based on the consecutive loses of HBr from the $[M - Br]^+$ ion to form the specific $[M - H_5Br_6]^+$ and $[M - H_4Br_5]^+$ ions, which were selected as quantitation (Q) and qualification (q) transitions, respectively. Parameters affecting ionization and MS/MS detection were studied. Method performance was also evaluated; calibration curves were found linear from 1 pg/μL to 100 pg/μL for the total HBCD concentration; instrumental detection limit was estimated to be 0.10 pg/μL; repeatability and reproducibility, expressed as relative standard deviation, were better than 7 % in both cases. The application to different real samples [polyurethane foam disks (PUFs), food, and marine samples] pointed out a rapid way to identify and allow quantification of this compound together with a number of polybrominated diphenyl ethers (BDE congeners 28, 47, 66, 85, 99, 100, 153, 154, 183, 184, 191, 196, 197, and 209) and two other novel brominated flame retardants [i.e., decabromodiphenyl ethane (DBDPE) and 1,2-bis(2,4,6-tribromophenoxy)ethane (BTBPE)] because of their presence in the same fraction when performing the usual sample treatment.

Keywords Atmospheric pressure chemical ionization · Gas chromatography · Hexabromocyclododecane · Brominated flame retardants · Polyurethane foam (PUF) disks

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Introduction

Hexabromocyclododecane (HBCD) is a highly lipophilic brominated flame retardant (BFR) most commonly used in expanded polystyrene (EPS) and extruded polystyrene (XPS) foams produced for the building and construction industry to meet fire safety standards (approximately 96 % of total production). HBCD also has minor uses as a flame retardant in textile back coatings and high-impact polystyrene (HIPS) used in electronics housings. Its use has increased concurrently to restrictions on polybrominated diphenyl ethers (PBDEs) [1]. HBCD may be released into air, water, soil, and sediment during manufacture, processing, improper handling, improper storage or containment, product usage, and disposal of HBCD-containing products and materials. Because of its toxicity, persistence, and tendency for bioaccumulation and biomagnification in food chains, HBCD is classified by REACH as a substance of very high concern. At its sixth meeting in 2013, the Conference of the Parties of the Stockholm Convention adopted the listing of HBCD to Annex A to the

Potential of gas chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry for screening and quantification of hexabromocyclododecane

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3.3.1 Abstract

A fast method for the screening and quantification of hexabromocyclododecane (sum of all isomers) by gas chromatography using a triple quadrupole mass spectrometer with atmospheric pressure chemical ionization (GC-APCI-QqQ) is proposed. This novel procedure makes use of the soft atmospheric pressure chemical ionization source, which results in less fragmentation of the analyte than by conventional electron impact (EI) and chemical ionization (CI) sources, favoring the formation of the $[M - Br]^+$ ion and, thus, enhancing sensitivity and selectivity. Detection was based on the consecutive loses of HBr from the $[M - Br]^+$ ion to form the specific $[M - H_5Br_6]^+$ and $[M - H_4Br_5]^+$ ions, which were selected as quantitation (Q) and qualification (q) transitions, respectively. Parameters affecting ionization and MS/MS detection were studied. Method performance was also evaluated; calibration curves were found linear from 1 pg/µL to 100 pg/µL for the total HBCD concentration; instrumental detection limit was estimated to be 0.10 pg/µL; repeatability and reproducibility, expressed as relative standard deviation, were better than 7 % in both cases. The application to different real samples [polyurethane foam disks (PUFs), food, and marine samples] pointed out a rapid way to identify and allow quantification of this compound together with a number of polybrominated diphenyl ethers (BDE congeners 28, 47, 66, 85, 99, 100, 153, 154, 183, 184, 191, 196, 197, and 209) and two other novel brominated flame retardants [i.e., decabromodiphenyl ethane

(DBDPE) and 1,2-bis(2,4,6-tribromophenoxy)ethane (BTBPE)] because of their presence in the same fraction when performing the usual sample treatment.

3.3.2 Keywords

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Nevertheless, the availability of a highly sensitive and selective screening method for HBCD by GC-MS could be interesting as this compound elutes

in the same fraction as PBDEs when applying conventional sample treatment for persistent organic pollutants (POPs) analysis as used in the majority of routine laboratories. Thus, only in those cases where the concentration of total HBCD is above established limits in the GC-MS screening, quantitative analysis of individual isomers would be carried out using the isomer selective LC methods [5, 6]. Additionally, HBCD debromination in negative ion chemical ionization (NICI) [7, 8, 9] could yield overlapping signals with some PBDE congeners when monitoring ions at *m/z* 79 and 81 for quantitation. The determination of HBCD by GC-HRMS in positive EI mode has demonstrated greater selectivity, as the different fragmentation allows selecting higher, more specific ions for quantification. However, the high fragmentation on EI sources compromises sensitivity, leading to limits of detection (LODs) of approximately 0.04 ng on column [10].

In this scenario, the availability of a softer ionization source in GC could render higher-mass precursor ions more specific, avoiding the potential interferences. The new soft atmospheric pressure chemical ionization (APCI) source has already been satisfactorily applied for GC-amenable compounds such as pesticides, polycyclic aromatic hydrocarbons (PAHs), PBDEs, and, very recently, dioxins/furans (PCDDs/PCDFs) [11, 12]. The aim of this work is to study the potential of GC coupled to a triple quadrupole mass spectrometer using APCI source (GC-APCI-QqQ) for the determination of total HBCD in complex matrices, such as polyurethane foam (PUF) disks used for passive air sampling, and marine samples (dolphin, fish, prawn, squid, and zooplankton) and two Standard Reference Materials (SRMs 2974a and 1954; i.e., mussel tissue and human milk).

3.3.4 Materials and Methods

Chemicals and reagents

HBCD standard (γ-1,2,5,6,9,10-Hexabromocyclododecane) as well as isotopically labeled HBCD (γ-1,2,5,6,9,10-hexabromo[$^{13}C_{12}$]cyclododecane) was purchased from Wellington Laboratories (Guelph, ON, Canada) with a purity higher than 98 % as 50 ng/µL solution in toluene. All reagents used for the sample treatment were of trace analysis grade. Sulphuric acid (95–97 %) and silica gel were supplied by J.T. Baker, Deventer, The Netherlands (for the analysis of PUF disks) and by Merck Co., Darmstadt, Germany (for the rest of the samples).

Basic alumina, EcoChrom MP Alumina B–Super I was purchased from MP Biomedicals Germany GmbH. Sodium hydroxide was from Carlo Erba, Milan, Italy. Acetone, n-hexane, dichloromethane, toluene, and granular anhydrous sodium sulfate were obtained from J.T. Baker, Deventer, The Netherlands.

Samples

Samples analyzed in this work consisted of several PUF disks (140 mm ø, 13.5 mm thickness, and approximately 5.6 g weight) used for passive air sampling (after 3 mo exposure) in four different countries (Mali, Kenya, Fiji, and Uruguay), a blank (nonexposed PUF) was also analyzed.

Extracts from two different NIST standard reference materials, SRM 2974a (mussel tissue) and SRM 1954 (human milk); and 11 sample extracts coming from the marine field (one dolphin, four different fish species, two prawns, two squids, and two zooplankton) previously analyzed for PBDE [13] determination, were also used in this work to investigate the presence of HBCD.

Sample treatment

Analytical procedure for PUFs was based on that optimized at Laboratory of Dioxins (IDAEA, CSIC, Barcelona, Spain). It consisted in a Soxhlet extraction with toluene for 24 h, cutting the disk in several pieces beforehand, and adding a known amount of $^{13}C_{12}$-HBCD. Subsequent clean-up was carried out by a multilayer acid–base silica column eluted with n-hexane. Then, the concentrated extract was added onto a basic alumina column and HBCD was collected in the n-hexane:dichloromethane (80:20) fraction.

Marine samples extraction and purification procedures were previously described in the literature [14, 15]. Briefly, extraction involved matrix solid-phase dispersion (MSPD) of the samples. Six to 200 g of fresh sample homogenized with 4:1 (w/w) silica gel/anhydrous sodium sulfate powder, and spiked with $^{13}C_{12}$-BDEs 47, 99, and 153, was ground to a fine powder, loaded onto a column, and extracted with 400 mL of 1:1 (v/v) acetone/n-hexane mixture. For the clean-up of the extracts, two multilayer columns filled with neutral silica, silica modified with sulfuric acid (44 %, w/w), and silica modified with KOH (56 %, w/w) were employed using n-hexane as

elution solvent. When required, the final extract containing the target compounds was subjected to further fractionation on Supelclean ENVI-Carb SPE cartridges (Supelco, Palo Alto, CA, USA), as described elsewhere [16] to separate *ortho*-substituted PCBs plus PBDEs from PCDD/PCDF and non-*ortho*-substituted PCBs. HBCD remains in the PBDEs fraction [17].

GC-(APCI)QqQ

Data were acquired using a GC system (Agilent 7890A; Palo Alto, CA, USA) equipped with an autosampler (Agilent 7693) and coupled to a triple quadrupole (QqQ) mass spectrometer (Xevo TQ-S; Waters Corporation, Manchester, UK), operating in APCI positive mode. A fused silica DB-1HT capillary column, 15 m × 0.25 mm i.d. and a film thickness of 0.1 µm (J&W Scientific, Folsom, CA, USA) was used. The oven temperature was programmed as follows: 140 °C (1 min); 20 °C/min to 285 °C; 40 °C/min to 350 °C (1 min). Pulsed splitless injections (100 psi) of 1 µL sample extract were carried out at 280 °C. Helium was used as carrier gas at 4 mL/min. To establish the Selected Reaction Monitoring (SRM) conditions, automatic dwell time (35 ms to 58 ms) was applied in order to obtain 15 points per peak. The interface temperature was set to 350 °C using N_2 as auxiliary gas at 250 L/h, as make-up gas at 300 mL/min, and as cone gas at 170 L/h. The APCI corona discharge pin was operated at 1.6 µA. The ionization process occurred within an enclosed ion volume, which enabled control over the protonation/charge transfer processes. TargetLynx (a module of MassLynx) was used to handle and process the acquired data. Final MS conditions selected to determine HBCD included a cone voltage of 20 V, collision energy of 20 eV, and the transitions 560.4 → 129.0; 560.4 → 157.1 and 560.4 → 237 for native HBCD and 572.4 → 169 for the labeled HBCD.

3.3.5 Results and Discussion

Ionization and in-source fragmentation of HBCD under NICI, EI and APCI

The typical HBCD determination by GC-MS is performed either using NICI [7, 8, 9] or EI coupled to HRMS [10, 18]. When using NICI, the molecular ion is not observed, being the $[M - HBr]^{-\bullet}$ ion, the one with the highest mass (Fig. 1a). The fragmentation in negative mode only permits monitoring either the ion at *m/z* 160 ($[M - H_2Br_6]^{-\bullet}$) or the bromide ion, which is preferred because of its higher sensitivity. However, the use of

these nonspecific ions is an impediment for the use of isotopically labeled HBCD standards [19]. On the other hand, EI source generates a spectrum with a high fragmentation pattern for HBCD (**Fig. 1b**). The $[M]^{+\bullet}$ ion is completely fragmented in the source being again nonspecific ions at *m/z* 67 and 79, the most intense peaks of the spectrum. The ions with the highest mass correspond to the loss of one Br atom, followed by the subsequent losses of HBr. Under these conditions, literature shows that the limit of detection is high [around 2 ng/g lipid weight (lw)] and not enough to detect HBCD in several expected positive samples [18]. Table 1 enlists the majority of attempts performed so far in order to detect and quantify HBCD, indicating the systems used and the achieved performance in each case in terms of limit of detection (LOD) and limit of quantification (LOQ). The results evidence the need of an improvement of the analytical performance in samples with low HBCD concentrations (lower than 2 ng/g) as foods and environmental samples are expected to be low-contaminated, as a result of the HBCD use in the past [4, 18].

At this point, APCI source was explored, revealing its softer ionization and the much lower in-source fragmentation of the HBCD in comparison with NICI and EI. Although $[M]^{+\bullet}$ was absent in the APCI spectrum, the loss of one Br atom, followed by subsequent losses of HBr were observed. Nevertheless, the relative abundances of these ions were significantly higher to those observed in EI, being the ion corresponding to the loss of $[H_3Br_4]^-$, the base peak of the APCI spectrum (Fig. 1c). The abundant presence (50 % of the base peak) of the ion corresponding to the loss of one single bromine atom ($[M - Br]^+$) was selected for the required application. It was considered to be a good candidate as precursor ion for MS/MS experiments because of its high *m/z* value and specificity. During these experiments, cone voltage values between 5 and 50 V were tested in order to select the optimum value pursuing a low in-source fragmentation and maximum response. Finally, 20 V was selected as optimum.

Fragmentation of HBCD in the collision cell

In order to study the fragmentation of HBCD in the collision cell, two ions from the isotopic pattern corresponding to $[M - Br]^{+\bullet}$ (M + 4 and M + 6)

were selected in the first quadrupole, and fragmentation was performed using collision energies between 10 and 30 eV. A collision energy of 20 eV was selected as optimum for all the aforementioned transitions (Fig. 2). Accordingly, the selected transitions tested were 560.4 → 129.0; 560.4 → 157.1, and 560.4 → 237 corresponding to the fragmentation of the precursor ion $[M + 4 - Br]^+$ and 562.6 → 129.0, 562.6 → 157.1, and 562.6 → 236.9 taking $[M + 6 - Br]^+$ ion as precursor. The transition 560.4 → 157.1 demonstrated the highest sensitivity and was, hence, chosen as Q transition for further experiments. The transition 572.4 → 169 was selected for monitoring the $^{13}C_{12}$-labeled standard as its fragmentation pattern was comparable to the native HBCD.

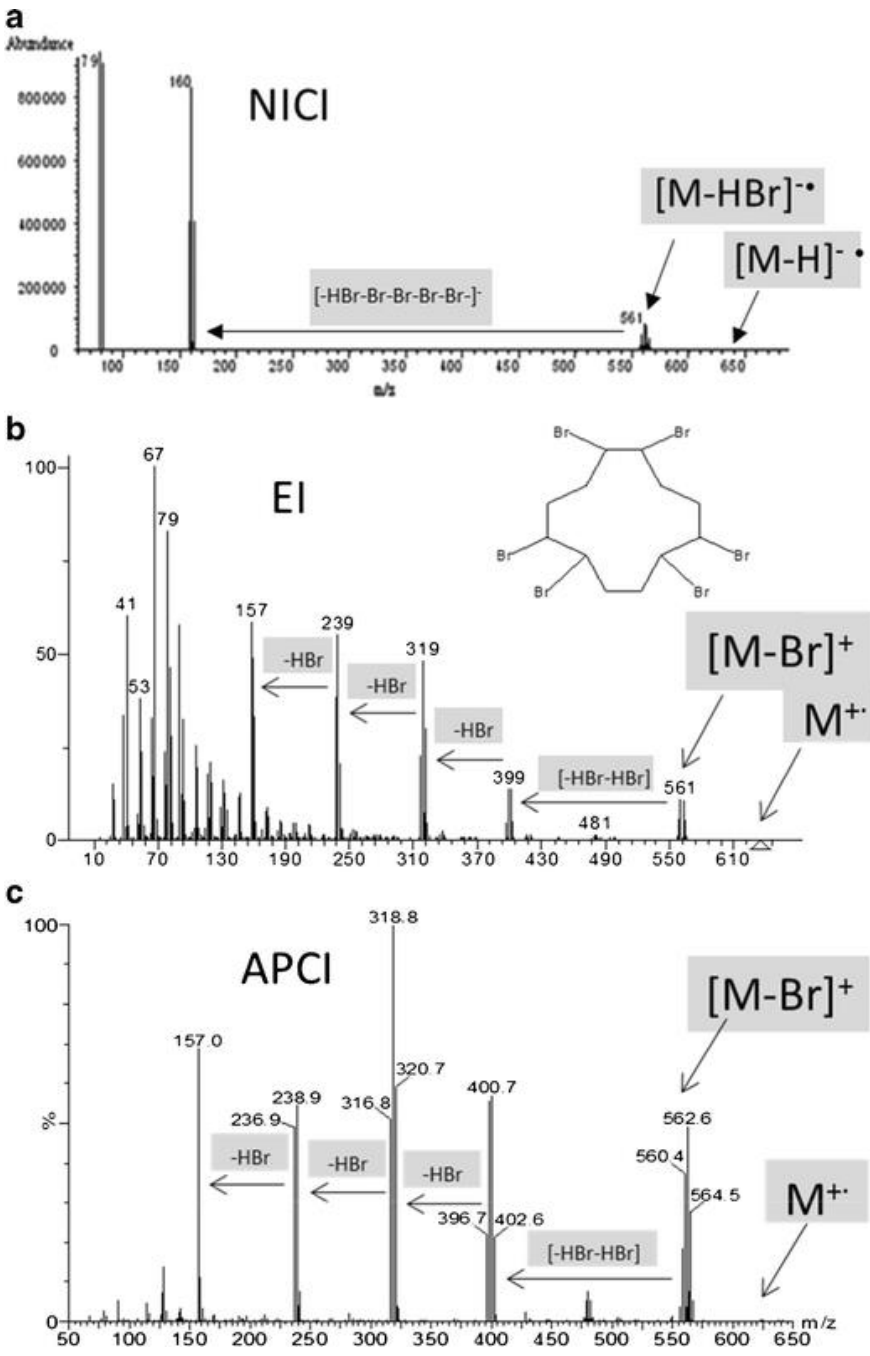

Fig. 1 a NICI, **b** EI, and **c** APCI spectra of γ-HBCD

Table 1 Techniques and conditions previously used for the determination of HBCD and their performance in terms of LOD and LOQ

Technique	Column	Gradient	Ionic species and *m/z*	LOD	LOQ	Reference
GC-HRMS (EI)	DB-1 (10 m×0.28 mm×0.1 μm)	–	$[M-Br]^-$;560.7289; 562.7269	–	30–50 pg on column	[10]
LC-MS (APCI-)	C18-RP (125 mm×4 mm)	Methanol/water (80:20); 1 mL/min	$[M-H]^-$; 638.6; 640.6; 642.6	–	500–1000 pg on column [a]	
LC-MS/MS (ESI-)	C30 YMC Carotenoid S-5 (4.6×250 mm)	Water/methanol (20:80); to 100 % methanol over 35 min; 0.5 mL/min	$[M-H]^- \rightarrow Br^-$;640.8→78.8	5–25 pg on column [a]	300 pg/g (ww)	[17]
LC-MS/MS (ESI-)	NUCLEODEX β-PM (200 mm×4 mm, 5 μm)	Water/methanol (50:50); 500 μL/min	$[M+Cl]^- \rightarrow [M-H]^-$;676.6→640.6	1.5–4.3 pg/μL	5–14 pg/μL [b]	[14]
GC-MS (NICI)	HP-5MS (30 m×0.25 mm×0.25 μm)	110 °C (1 min); 8 °C/min to 180 °C (1 min); 2 °C/min to 240 °C (5 min); 2 °C/min to 265 °C (6 min)	Br^-; 79	–	2000 pg/g dry wt.	[8]
LC-MS/MS (ESI-)	Symmetry C18 (2.1 mm×150 mm, 5 μm) preceded by C18 guard column (2.1×10 mm)	H_2O:MeOH (3:1 v/v); 0.25 mL/min; 8 min to MeOH 100 %; 17 min to MeOH 97.5 %; 3 min to initial cond (15 min)	$[M-H]^- \rightarrow Br^-$;638.7→78.9, 638.7→80.9	30–60 pg/g	110–200 pg/g [a]	[3]
GC-MS (NICI)	DB-5MS (30 m×0.25 mm×0.25 μm)	80 °C (2 min); 25 °C /min to 240 °C; 4 °C/min to 315 °C (50 min)	Br^-; 79, 81	50–100 ng/g	–	[9]
LC-MS/MS (ESI-)	Symmetry C18 (2.1×150 mm, 5 μm) [a]	Water/ methanol/acetonitrile (60:30:10); 250 μL/min; 5 min to methanol/acetonitrile (50:50) (6 min)	$[M-H]^- \rightarrow Br^-$; 640.6→79	0.5–5 pg on column	15 to 75 pg/g (ww)	[6]
	NUCLEODEX β-PM (200 mm×4 mm, 5 μm) [b]	water/ methanol/acetonitrile (40:30:30) (0.5 min); 500 μL/ min; 8 min to methanol/acetonitrile (30:70) (14 min)				
LC/LC-MS/MS (ESI-)	Eclipse Plus-C18 RP (250 mm×4.6 mm, 5 μm) coupled to a Zorbax Eclipse XDB-C8 RP (150 mm×4.6 mm, 5 μm)	MeOH (A)/ACN (B)/H_2O (C); 0.5 mL/min. A/B/C (3:87:10) (25 min), 1 min to 100 % B (9 min), 3 min to initial cond. (10 min)	$[M-H]^- \rightarrow Br^-$; 640.7→78.8	0.4–0.8 pg on column	1–2.5 pg/μL [a]	[5]
GC-MS (NICI)	DB-5 (15 m×0.25 mm×0.10 μm)	90 °C (1.5 min); 15 °C/min to 295 °C (15 min)	Br^-; 79, 81	–	100 pg/g (dry wt) 0.4 ng/g (lw)	[7]
GC-HRMS (EI)	DB-5 (30 m×0.25 mm×0.1 μm)	–	Isotope ratio and RT identification	–	2400 pg/g (lw)	[18]
GC-MS (EI)	DB-5 (30 m×0.25 mm×0.25 μm)	–	Full scan	200,000 pg/g	–	[20]
	DB-1 (30 m×0.25 mm×0.25 μm)	–	Full scan	200,000 pg/g	–	

[a] Diastereoisomers
[b] Enantiomers

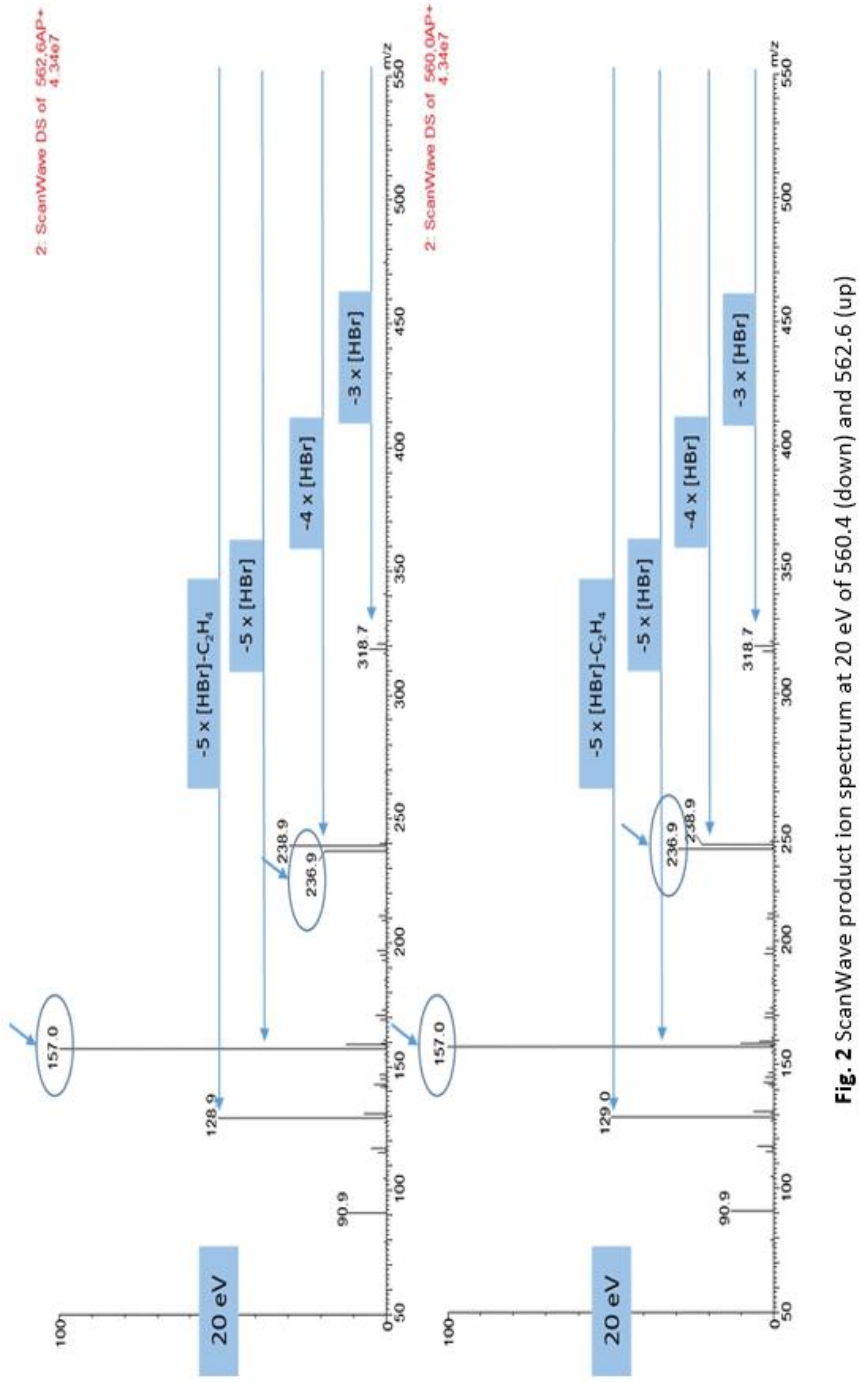

Fig. 2 ScanWave product ion spectrum at 20 eV of 560.4 (down) and 562.6 (up)

Chromatographic optimization

Chromatographic conditions for the determination of HBCD were taken from a previous work [13], as the main goal of the developed method was to detect and/or quantitate the total amount of HBCD in the same sample extracts prepared for PBDEs determination in a single injection. Taking into account the literature regarding HBCD determination by GC (**Table 1**), two columns, a HP-5MS 30 m × 0.25 mm, 0.25 µm and a DB-1HT 15 m × 0.25 mm, 0.10 µm, were tested in order to determine the suitability of both stationary phases for the determination of HBCD. The results showed poor linearity and reproducibility for the HP-5MS column compared with the DB-1HT column. An important decay of the signal with the increasing number of runs was observed in the HP-5MS column. This column-dependency and the fact that signal to noise (S/N) ratios were five times lower when using the 5MS column confirmed the DB-1HT column as the proper one. This kind of comparison between columns has been previously performed but at concentration levels of ng/µL, finding that the DB-1HT column provided sharper signals and better separation from egg matrix peaks, making the DB-1HT column more suitable for HBCD analysis [20]. It is worth mentioning that the film thickness was different, which could also influence the column performance in the case of HBCD.

Analytical parameters

In order to test the reliability of the method, repeatability of response was studied by 10 repeated injections of HBCD standards at 1 pg/µL level. The relative standard deviation was clearly lower than 10 %. Linearity of the relative response of the HBCD (to its corresponding $^{13}C_{12}$ isotopically labeled standard) was studied by analyzing standard solutions, in triplicate, in the range of 1–100 pg/µL. The correlation coefficient (r^2) was higher than 0.999, with residuals lower than 2 %. Sensitivity of the method can be derived from Fig. 3a (1 pg/µL standard solution in nonane). LOD was determined to be around 100 fg injected in pulsed splitless mode.

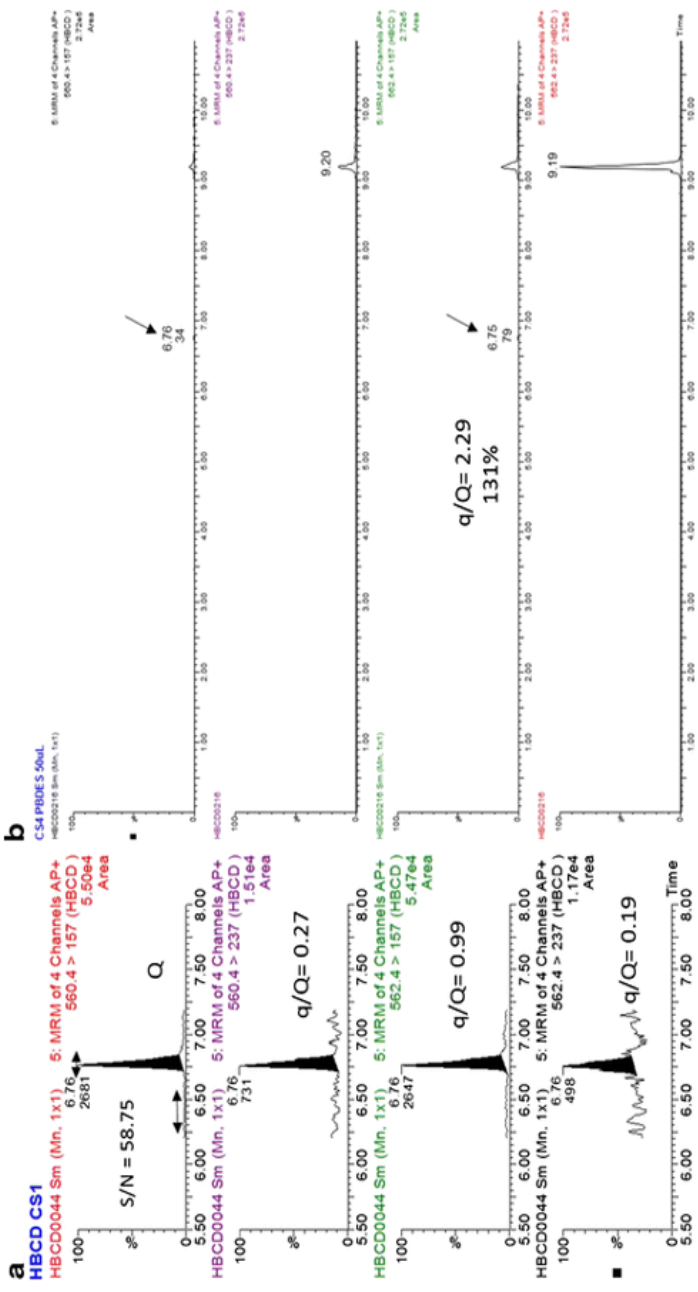

Fig. 3 a GC-APCI-QqQ chromatogram for 1 pg HBCD standard. **b** GC-APCI-QqQ chromatogram for 50 pg PBDE standard mixture acquired with the transitions of HBCD. S/N: signal-to-noise ratio; Q: Quantification transition; q: qualification transition

With the purpose of evaluating the specificity provided by the use of the selected HBCD transitions, a CS4 (50 pg/µL) PBDEs mixture standard was injected, monitoring the HBCD transitions through the whole chromatogram. Figure 3b shows the result, in which only two signals appeared in the whole chromatogram; one "interferent" eluting 2.5 min later but one eluting at the same time as HBCD, showing a completely different q/Q ratio. The signal produced by the 50 pg/µL PBDEs mixture in the Q transition was around 1 % of the signal for the 1 pg/µL HBCD standard (see **Fig. 3**). Consequently, possible interferences coming from PBDEs that would lead to false positives when determining HBCD are discarded and only quantifications over around 1 % of the area of the native HBCD could appear in regular analysis, which is more than acceptable.

Conversely, the possible interferences of HBCD in PBDEs determination were also studied by monitoring the transitions of PBDEs in an injection of the most concentrated HBCD standard available (CS5, 100 pg/µL). Degradation of HCBD to lower brominated analogues at injection port temperatures above 240 °C has been reported [21, 22]. These degradation products are an important PBDE quantitation issue when acquiring the bromide ion as quantitation ion. This situation is much less an issue when using the GC-APCI-QqQ method. **Figure 4a** shows that the signal generated by thermal degradation product(s) originating from 100 pg of HBCD in the transitions of BDE 47 is only of about 1 % of the corresponding 1 pg BDE 47 signal (**Fig. 4b**). This would have practically no negative effect on the quantification of this PBDE and constitutes another advantage of the APCI source and its associated low fragmentation, which allows minimizing the mutual interferences among brominated compounds.

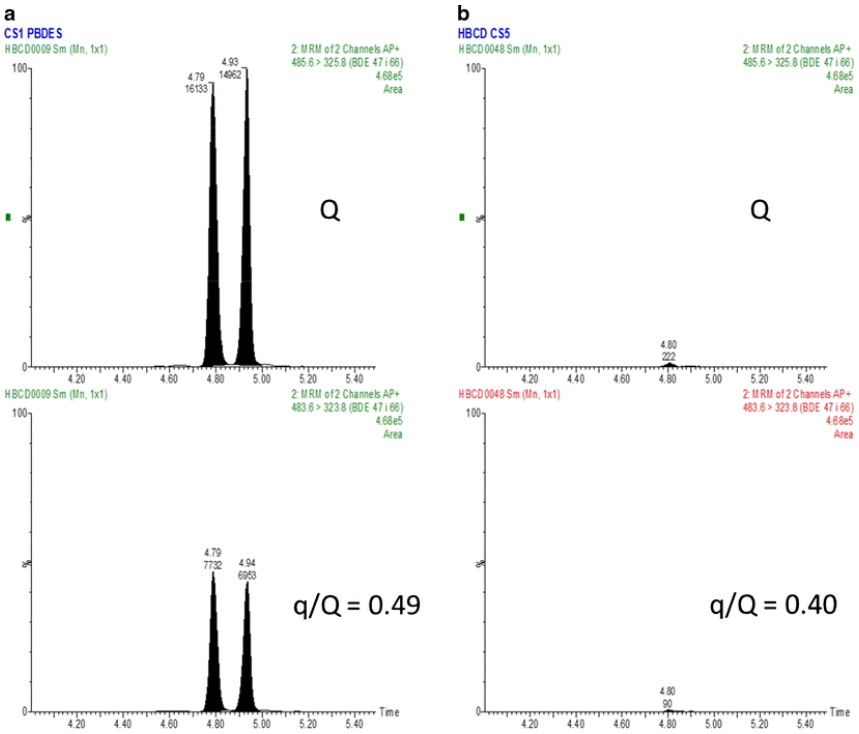

Fig. 4 a GC-APCI-QqQ chromatogram for 1 pg PBDE standard solution. **b** GC-APCI-QqQ chromatogram for 100 pg of γ-HBCD acquired with the transitions of BDE 47

Analysis of real samples

The usability/suitability of this new procedure was tested against real/naturally contaminated samples by analyzing PUF extracts from a UNEP project having been exposed for 3 mo in Suva (Fiji), Montevideo (Uruguay), Nairobi (Kenya), and Bamako (Mali). Quantification of the samples was carried out by using calibration curve with standard in solvent, using relative responses to internal labeled $^{13}C_{12}$ γ-HBCD standard added as surrogate to the sample.

Total HBCD concentrations obtained using the GC-APCI-QqQ method ranged from 190 to 400 pg/µL (in the extract), which correspond to 4 ng/PUF and 8 ng/PUF, respectively. In Fig. 5a, we can observe the detection and identification of HBCD in one of the PUF extracts (3.68 ng/PUF) by the presence of its 4 SRM transitions at expected retention time and the q/Q ratios within stablished tolerances.

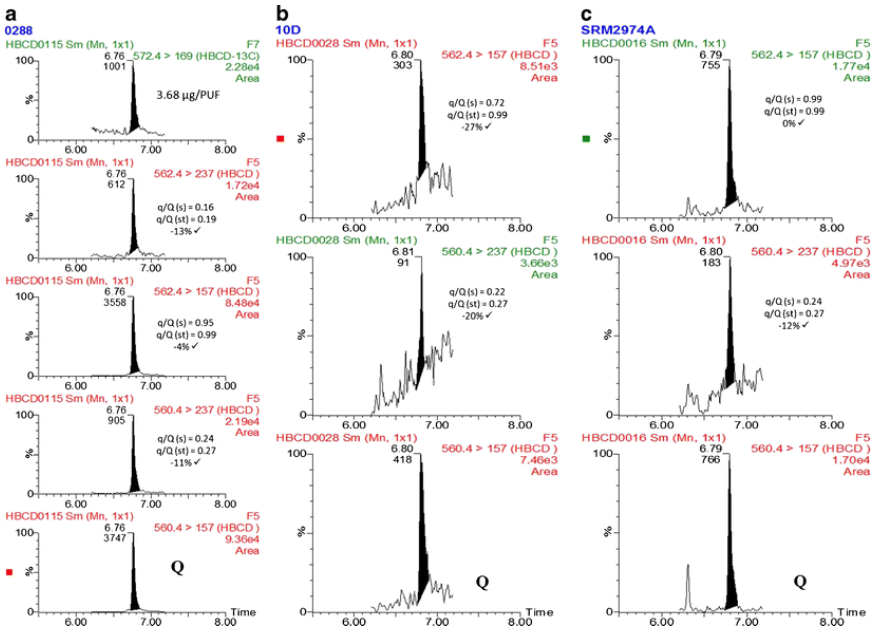

Fig. 5 **a** GC-APCI-QqQ chromatogram for a PUF extract. **b** GC-APCI-QqQ chromatogram for a fish extract. **c** GC-APCI-QqQ chromatogram for a mussel tissue reference material extract

Finally, the 11 marine samples and the two reference materials were reinjected into the system under optimized conditions for HBCD. HBCD was detected in 10 out of the 13 samples analyzed. Figure 5 shows the chromatograms for a fish sample, Fig. 5b, and the mussel tissue reference sample, Fig. 5c, respectively.

3.3.6 Conclusions

The use of the atmospheric pressure chemical ionization source has been proven to render better sensitivity and specificity than commonly used EI/CI sources, thanks to the acquisition of several transitions coming from a selective $[M - Br]^{+\bullet}$ precursor ion and leading to specific HBCD product ions instead of the common bromide ion at *m/z* 79/81. This fact allowed decreasing the LOD for total HBCD down to 100 fg/µL, which implies a significant advance compared with traditional previous methodologies, based in GC or LC coupled to different sources and analyzers (**Table 1**) resulting in a limit of detection around 100 times lower in most of the cases. Additional specificity has been found for the simultaneous

determination of PBDEs and HBCD, as the fact of monitoring different transitions for each compound instead of the common bromide atom has shown to minimize their mutual interferences.

Although LC-MS/MS methods allow isomer-specific determination at levels of few ppb, the developed methodology has demonstrated the ability to detect and quantify total concentration of HBCD in PBDE extracts without additional treatment or analysis. It could be effectively applied as a screening methodology to select positive samples to be processed by LC-MS/MS if individual isomer information is required, saving time and budget analysis.

3.3.7 Acknowledgments

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3.3.8 Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Human and animal rights and informed consent

No research involving human participants or animals has been carried out in development of this work.

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3.4 Scientific Article 4

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Evaluation of the capabilities of atmospheric pressure chemical ionization source coupled to tandem mass spectrometry for the determination of dioxin-like polychlorobiphenyls in complex-matrix food samples

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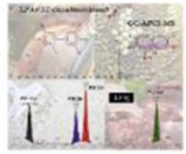
HIGHLIGHTS

- GC-(APCI)MS/MS with QqQ: a suitable alternative to GC-(EI)HRMS for DL-PCBs determination.
- LODs and LOQs as low as 0.0025 and 0.005 pg μL^{-1} respectively achieved for each DL-PCB congener.
- Enhanced sensitivity and specificity of APCI in comparison with EI source in QqQ instruments.

GRAPHICAL ABSTRACT



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ABSTRACT

The use of the novel atmospheric pressure chemical ionization (APCI) source for gas chromatography (GC) coupled to triple quadrupole using tandem mass spectrometry (MS/MS) and its potential for the simultaneous determination of the 12 dioxin-like polychlorobiphenyls (DL-PCBs) in complex food and feed matrices has been evaluated.

In first place, ionization and fragmentation behavior of DL-PCBs on the APCI source under charge transfer conditions has been studied followed by their fragmentation in the collision cell. Linearity, repeatability and sensitivity have been studied obtaining instrumental limits of detection and quantification of 0.0025 and 0.005 pg μL^{-1} (2.5 and 5 fg on column) respectively for every DL-PCB. Finally, application to real samples has been carried out and DL-PCB congeners (PCB 77, 81, 105, 114, 118, 123, 126, 156, 157, 167, 169, 189) have been detected in the different samples in the range of 0.40–10000 pg g^{-1}. GC-(APCI)MS/MS has been proved as a suitable alternative to the traditionally accepted confirmation method based on the use of high resolution mass spectrometry and other triple quadrupole tandem mass spectrometry techniques operating with electron ionization. The development of MS/MS methodologies for the analysis of dioxins and DL-PCBs is nowadays particularly important, since this technique was included as a confirmatory method in the present European Union regulations that establish the requirements for the determination of these compounds in food and feed matrices.

1. Introduction

Polychlorinated biphenyls (PCBs) belong to a broad family of anthropogenic organic chemicals known as chlorinated

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Evaluation of the capabilities of atmospheric pressure chemical ionization source coupled to tandem mass spectrometry for the determination of dioxin-like polychlorobiphenyls in complex-matrix food samples

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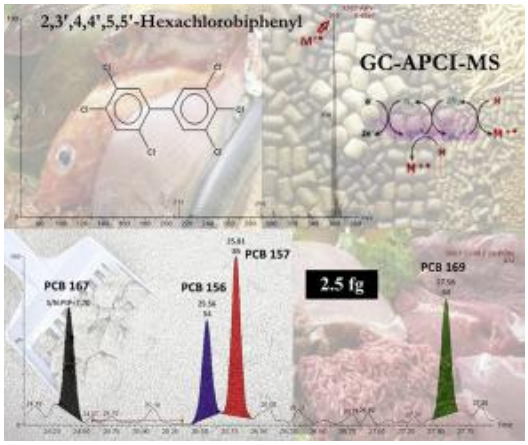
3.4.1 Abstract

The use of the novel atmospheric pressure chemical ionization (APCI) source for gas chromatography (GC) coupled to triple quadrupole using tandem mass spectrometry (MS/MS) and its potential for the simultaneous determination of the 12 dioxin-like polychlorobiphenyls (DL-PCBs) in complex food and feed matrices has been evaluated.

In first place, ionization and fragmentation behavior of DL-PCBs on the APCI source under charge transfer conditions has been studied followed by their fragmentation in the collision cell. Linearity, repeatability and sensitivity have been studied obtaining instrumental limits of detection and quantification of 0.0025 and 0.005 pg μL^{-1} (2.5 and 5 fg on column) respectively for every DL-PCB. Finally, application to real samples has been carried out and DL-PCB congeners (PCB 77, 81, 105, 114, 118, 123, 126, 156, 157, 167, 169, 189) have been detected in the different samples in the range of 0.40–10000 pg g^{-1}. GC-(APCI)MS/MS has been proved as a suitable alternative to the traditionally accepted confirmation method based on the use of high resolution mass spectrometry and other triple quadrupole tandem mass spectrometry techniques operating with electron ionization. The development of MS/MS methodologies for the analysis of dioxins and DL-PCBs is nowadays particularly important, since this technique was included as a confirmatory method in the present European Union regulations that establish the requirements for the determination of these compounds in food and feed matrices.



Graphical Abstract

3.4.2 Keywords

Dioxin-like polychlorinated biphenyls, Persistent organic pollutants, Atmospheric pressure chemical ionization, Food and feed samples

3.4.3 Introduction

Polychlorinated biphenyls (PCBs) belong to a broad family of anthropogenic organic chemicals known as chlorinated hydrocarbons. Due to their non-flammability, chemical stability and electrical insulating properties, PCBs were commonly used in the past in hundreds of industrial and commercial applications [1]. PCBs have been demonstrated to cause a variety of adverse effects on the immune, reproductive, nervous and endocrine systems of the living organisms, as well as other health effects [2]. As a result of their structure, PCBs are lipophilic and persistent, expected to be bioaccumulated (specially the coplanar ones) in the environment and biological matrices. Recently, a Working Group of the International Agency for Research on Cancer (IARC) has evaluated PCBs as carcinogenic for humans (Group 1) [3].

Among the total number of 209 PCB congeners, there are congeners that can take a planar conformation, which could confer on them toxicological properties, the same as those observed for dioxins (polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans (PCDD/Fs)) These PCBs congeners are called dioxin-like PCBs (DL-PCBs). Even though DL-PCBs are usually present at levels quite lower than other PCBs, they have

demonstrated to be harmful to living organism at these very low levels like PCDD/Fs [4]). Consequently sensitive and selective analytical methodologies are needed, to demonstrate foods are safe. The analysis of DL-PCBs has been traditionally close related to that of dioxins. These PCBs have been assigned with toxicity equivalency factors (TEFs), taking the toxicity of the 2,3,7,8-tetraclorodibenzo-*p*-dioxin as a reference, similarly to what was previously done for all the toxic PCDD/Fs. Since 2006, maximum levels for the sum of PCDD/Fs and DL-PCBs in food and feedstuff products are listed, together with the maximum levels for PCDD/Fs in the same matrices, in the corresponding European Union (EU) regulations and directives. Furthermore, in 2002 the European Commission had already laid down methods of analysis for the official control of dioxins that also referred to the determination of DL-PCBs.

In these EU regulations and directives, confirmatory methods were based on high resolution gas chromatography coupled to high resolution mass spectrometry (GC-HRMS). The HRMS technique allowed to totally fulfill most of the basic requirements (i.e. high sensitivity and low detection limits, high selectivity and specificity and high accuracy). Alternatively, other techniques have been explored, in particular for the analysis of DL-PCBs: GCxGC-μECD [5], ECNI–LRMS (for non-ortho PCBs) [6], [7] and ITMS/MS [8]. For real samples, accuracy, precision and LOQs obtained with these techniques in the analysis of food samples are in the same range (fish oil and fish), or slightly worse (milk and pork) compared to GC–HRMS results [8], [9], [10], confirming their potential for DL-PCB determination.

To complete the scenario, GC-MS/MS techniques have recently been approved as valid techniques for confirmatory methods for the determination of PCDD/Fs and DL-PCBs, according to EU Regulations No 589/2014 and 709/2014 of June 2014 [11], [12]. However, specific criteria are applied to these techniques; in particular it is mandatory to monitor at least 2 specific precursor ions. Although from a theoretical point of view with ITMS/MS it is possible to monitor the product ions coming from a precursor cluster, from a practical perspective this would lead to an increment of the scan time and quite compromised sensitivity and peak shape. This would be even worse when monitoring the isotopically labelled internal standards. On the contrary, mass spectrometry instruments with a triple quadrupole configuration (QqQ) can perform multiple reaction monitoring, allowing to acquire various specific transitions (with different precursor ions) simultaneously. In addition, other criteria has to be fulfilled both for GC-HRMS and GC-MS/MS, such as

those related to the sensitivity of the method. It is important to have appropriate LOQs since most food and feed samples showed low levels of PCDD/Fs and DL-PCBs, far below the maximum established. Quantitation at around one fifth of the level of interest has to be feasible.

Regarding available sources for GC-MS/MS, electron impact ionization (EI) have been the most widely used in this field. However, EI sources usually give a considerable fragmentation of the molecules, due to the high energy transferred to them during the ionization process which could affect selectivity in some samples as a consequence of a higher matrix effect. Considering these limitations related with EI sources, it is clear that MS/MS methods with QqQ could benefit from the use of soft and universal ionization techniques able to provide more abundant molecular ions and less in-source fragmentation, thus allowing to reach higher sensitivity. Atmospheric pressure chemical ionization (APCI) source has already demonstrated its efficacy in obtaining the molecular ion (or the protonated molecule) and enhancing sensitivity in many applications, mainly coupled to HPLC [13], [14], [15] or GC-(Q)TOF [16], [17], [18]. The GC-(APCI)MS/MS coupling with QqQ has not been fully tested, but it is showing promising results in terms of sensitivity when compared to GC-(EI)MS/MS methods [19], [20].

This work follows up the pioneer contribution to the analysis of polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans (PCDDs/PCDFs) by GC-(APCI)MSMS which proved the capabilities of this technique as a real alternative to the HRMS instruments [21]. In the present work, a method for the determination of DL-PCBs in different food and feed complex matrices has been optimized and compared with the widely accepted GC-HRMS technique.

3.4.4 Materials and methods

Chemicals and reagents

Solvents for organic trace analysis (cyclohexane, dichloromethane, *n*-hexane and toluene) were from J.T. Baker (Deventer, The Netherlands), ethanol was from Merck (Darmstadt, Germany) and nonane was purchased from Fluka Chemie (St. Gallen, Switzerland). Silica and basic alumina for clean-up and fractionation were obtained from J.T. Baker and MP Biomedicals (Eschwege, Germany), respectively. Sulfuric acid (Merck)

and sodium hydroxide (Carlo Erba, Milano, Italy) were also used to prepare modified silica.

Standard solutions of ^{13}C-labelled DL-PCBs for quantification (WP-LCS) and analytical recovery of the samples (WP-ISS), as well as calibration standards (WP-CS1 to WP-CS7), were from Wellington Laboratories Inc. (Guelph, Ontario, Canada).

Samples

Nine samples archived from Proficiency Test (PT) organized by the European Union Reference Laboratory (EU-RL) for Dioxins and PCBs in Feed and Food (2 pork meat, 2 lard, 1 whole egg and 1 egg yolk powder, 1 milk powder and 1 milk fat, and 1 mineral (sepiolite) together with 3 additional samples (1 fish, 1 spiked feed and 1 milk powder) were used for the evaluation of the applicability of the developed method.

Sample preparation

Matrices with high water content (pork meat, egg, fish) were freeze-dried as a pre-treatment step. Lyophilized samples and dry samples (milk powder, feed, egg yolk powder, mineral) were then spiked with a working standard solution, containing the 12 ^{13}C-labelled DL-PCBs in nonane, and extracted in a Soxhlet for aprox. 24 h with a mixture of cyclohexane:toluene (50:50) or ethanol:toluene (70:30) (in the case of milk powder and mineral matrices). Next, extracts were concentrated in a rotary evaporator and transferred to *n*-hexane for purification on a multilayer (acid/basic) silica column. If needed, fat content was gravimetrically determined after keeping the dry residue coming from the rotary evaporator overnight in the oven (105 °C). Fat samples (lard, milk fat) were directly dissolved in *n*-hexane, spiked with the working standard solution and added to the multilayer silica column without previous extraction. Further fractionation of the extracts was carried out on a basic alumina column (14 g), with all the DL-PCBs been eluted in a 75 mL hexane:dichloromethane fraction (90:10). Finally, this fraction was concentrated to dryness in a vial and a standard mixture in nonane, containing ^{13}C-labelled PCBs for analytical recovery evaluation, was added.

3.4.5 Instrumentation

GC-(APCI) MS/MS

The chromatographic analysis were performed using an Agilent 7890A gas chromatograph (Agilent Technologies Inc., Palo Alto, CA, USA), equipped with an Agilent 7693A autosampler, coupled to a triple quadrupole mass spectrometer, Xevo TQ-S (Waters Corporation, Manchester, UK), with an APCI source. The GC separation was performed using a fused silica DB-5MS capillary column with a length of 60 m × 0.25 mm ID and a film thickness of 0.25 µm (J&W Scientific, Folson, CA, USA) working at a constant flow of 2 mL min^{-1} of Helium (99.999%; Praxair, Valencia, Spain). The oven program was set as follows: 140 °C; 20.00 °C min^{-1} to 200 °C (1.00 min); 3.00 °C min^{-1} to 270 °C; 50.00 °C min^{-1} to 300 °C (1.33 min) with a total runtime of 30 min. The injections of 1 µL of sample extracts were performed in pulsed splitless mode with at a temperature of 280 °C and a pulse time of 1.00 min. The pulse pressure was set to 35.0 psi, with a purge flow of 80.0 mL min^{-1} and purge time of 1.00 min.

The interface temperature was set to 310 °C using N_2 as auxiliary gas at a flow rate of 250 L h^{-1}, the make-up gas flow rate was set to 320 mL/min and the cone gas flow rate to 170 L h^{-1}. APCI corona pin operated at 1.4 µA. Mass spectrometer was operated in SRM mode, acquiring one quantification transition and one confirmation transition for both, native and ^{13}C-labelled compounds. In the SRM method, automatic dwell time (values ranging from 20 to 60 ms) was applied in order to obtain at least 15 points per peak. Targetlynx (a module of MassLynx) was used to handle and process the acquired data.

GC-(EI)HRMS and GC-(EI)MS/MS

Sample extracts were also analyzed on a 6890 N Agilent gas chromatograph (Agilent Technologies Inc., Palo Alto, CA, USA) coupled to an Autospec NT high resolution mass spectrometer (EBE geometry) (Micromass, Manchester, UK), using a EI source and operating in the SIM mode.

Additionally, sensitivity and specificity for selected compounds with traditional GC-(EI)MS/MS methodology was tested. For that purpose, standards were injected on a 6890 N Agilent gas chromatograph (Agilent

Technologies Inc., Palo Alto, CA, USA) equipped with an autosampler (Agilent 7683) coupled to a triple quadrupole mass spectrometer, Quattro Micro GC (Micromass, Boston, MA) operating in EI mode. Chromatographic conditions in both instruments were the same as in GC-(APCI)MS/MS analysis.

3.4.6 Results and discussion

GC-(APCI)MS/MS optimization

DL-PCBs commercial standard mixture WP-CS5 (40 ng mL^{-1}) was used to study the ionization of the 12 DL-PCB congeners and their corresponding ^{13}C-labelled internal standards under APCI source. As expected, all studied DL-PCBs were ionized under charge-transfer conditions due to the presence of only C, H and Cl atoms in their structure, without any protonable groups. Consequently, $M^{+\cdot}$ was formed as base peak of the spectrum for all the compounds. Additionally, as a consequence of the soft ionization character of the APCI source, low in-source fragmentation was observed if compared with their corresponding EI spectra. These aspects will have important consequences in sensitivity of the obtained transitions. As an illustrative example, **Fig. 1** shows the difference between fragmentation degrees generated by EI and APCI sources for the PCB 156 where the fragment ion corresponding to the loss of 2 Cl atoms represents the 60% of the base peak under EI, while only the 2% under APCI.

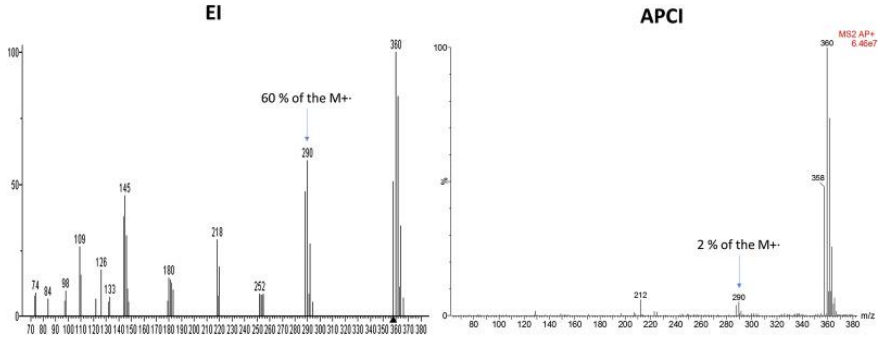


Fig. 1. Comparison of the in-source fragmentation for the 2,3,3',4,4',5 - Hexachlorobiphenyl DL-PCB in EI (left) and APCI (right) sources.

For developing the SRM method, up to three different precursor ions were studied, i.e. $M^{+\bullet}$, $[M+2]^{+\bullet}$ and $[M+4]^{+\bullet}$. Values between 20 and 70 V were tested, pursuing the optimal ionization for each DL-PCB. After selecting the precursor ions, daughter scan experiments at different collision energies, (5–50 eV) were conducted in order to determine the most sensitive and specific transitions. DL-PCBs commercial standard mixture WP-CS7 diluted to a final concentration of 80 ng mL^{-1} was used. For tetra- and penta-DL-PCBs, the most sensitive transitions were the ones which came from the corresponding $M^{+\bullet}$ ions losing two ^{35}Cl atoms, i.e. $[M-{}^{35}Cl_2]^{+\bullet}$, so the selected quantification (Q) transitions were 290 > 220 for tetra-DL-PCBs and 324 > 254 for the penta-DL-PCBs. On the contrary, the most sensitive transitions for hexa- and hepta-DL-PCBs were the ones which came from the corresponding $[M+2]^{+\bullet}$ ions losing two ^{35}Cl atoms, i.e. $[M+2-{}^{35}Cl_2]^{+\bullet}$, so the selected Q transition was 360 > 290 and 394 > 324, respectively. Their corresponding ^{13}C-labelled internal standards showed a correlative fragmentation behavior. As a summary, experimental MS/MS parameters for each compound are shown in **Table 1**.

Table 1. Experimental conditions of the optimized GC-(APCI)-MS/MS method

Compound	Rt (min)	Cone Voltage (V)	Precursor Ion	Collision Energy (eV)	Product Ion
13C-PCB 81	17.73	50	302	35	232
			304	35	234
PCB 81	17.73	50	290	35	220
			292	35	222
13C-PCB 77	18.22	50	302	35	232
			304	35	234
PCB 77	18.22	50	290	35	220
			292	35	222
13C-PCB 123	19.18	30	336	35	266
			338	35	268
PCB 123	19.17	30	324	35	254
			326	35	256
13C-PCB 118	19.36	30	336	35	266
			338	35	268
PCB 118	19.37	30	324	35	254
			326	35	256
13C-PCB 114	19.86	30	336	35	266
			338	35	268
PCB 114	19.87	30	324	35	254
			326	35	256
13C-PCB 105	20.61	30	336	35	266
			338	35	268
PCB 105	20.61	30	324	35	254
			326	35	256
13C-PCB 126	22.29	30	336	35	266
			338	35	268
PCB 126	22.28	30	324	35	254
			326	35	256
13C-PCB 167	23.22	30	372	35	302
			370	35	300
PCB 167	23.22	30	360	35	290
			358	35	288
13C-PCB 156	24.37	30	372	35	302
			370	35	300
PCB 156	24.36	30	360	35	290
			358	35	288
13C-PCB 157	24.62	30	372	35	302
			370	35	300
PCB 157	24.63	30	360	35	290
			358	35	288
13C-PCB 169	26.37	30	372	35	302
			370	35	300
PCB 169	26.36	30	360	35	290
			358	35	288
13C-PCB 189	28.27	30	406	40	336
			408	40	338
PCB 189	28.28	30	394	40	324
			396	40	326

Table 2. Target compounds with the selected quantification and validation parameters

Compound name	Rt (min)	r^2	RF mean	RF RSD (%)	Inst. precision RSD (%)	Inst. LOD (pg μL^{-1})	Inst. LOQ (pg μL^{-1})
PCB 81	17.73	0.999	1.14	8	2	0.0025	0.005
PCB 77	18.22	1.000	1.11	9	6	0.0025	0.005
PCB 123	19.18	0.999	1.13	12	5	0.0025	0.005
PCB 118	19.36	0.999	1.09	15	3	0.0025	0.005
PCB 114	19.86	0.999	1.11	12	8	0.0025	0.005
PCB 105	20.61	0.999	1.13	6	8	0.0025	0.005
PCB 126	22.29	0.999	1.14	11	2	0.0025	0.005
PCB 167	23.22	1.000	1.07	7	4	0.0025	0.005
PCB 156	24.36	0.999	1.10	9	6	0.0025	0.005
PCB 157	24.63	0.999	1.09	6	0	0.0025	0.005
PCB 169	26.36	0.999	1.08	10	6	0.0025	0.005
PCB 189	28.27	0.999	1.05	11	6	0.0025	0.005

*Inst. = Instrumental

Linearity, repeatability and limits of detection

In order to test the reliability of the instrumental method, parameters as linearity, repeatability, specificity, limits of detection (LODs) and quantification (LOQs) were evaluated (see **Table 2**). Linearity was tested with calibration curves at 10 different concentration levels ranging from 0.001 pg μL^{-1} to 40 pg μL^{-1} and analyzed by triplicate. The solvent calibration curves generated by plotting relative response versus concentration (pg μL^{-1}) of each standard showed an acceptable correlation coefficient (r^2) > 0.999 for all the DL-PCBs. Repeatability of the response (n = 10) of relative peak areas ranged from 2% to 12% for standards at concentration levels as low as 0.005 pg μL^{-1}. Instrumental LOQs were calculated as the lowest calibration point with a response factor (RF) deviation lower than 30% related to the mean response factor of the different standards of the calibration curve. For all the DL-PCBs, concentration level of 0.005 pg μL^{-1} gave RF values with deviations ranging from 3 to 19% analyzed by triplicate so this concentration was set as LOQ (5 fg on column). Additionally, to test the reproducibility of this LOQ level, calibration point at 0.005 pg μL^{-1} was injected at the beginning and at the end of the sequence, showing RSDs below 20% in all cases. Instrumental LODs were calculated as the lowest concentration level giving a signal-to-noise ratio (S/N) of at least 3. The calibration point at 0.0025 pg μL^{-1} (2.5 fg on column) was estimated as the LOD for every DL-PCB. Instrumental LOQs and LODs reported in the bibliography for DL-PCBs by GC-(EI)MS/MS range

from 0.030 to 2.1 pg μL^{-1} and from 0.05 to 0.6 pg μL^{-1} respectively [22], [23].

Analysis of real samples

Once the method was fully instrumentally validated, analysis of 3 real samples (1 fish, 1 spiked feed and 1 milk powder) and nine samples belonging to proficiency test studies (two replicates of a pork meat sample, two replicates of a lard sample, egg, egg yolk powder, milk powder, milk fat, and one mineral) were performed. All these samples had been previously analyzed by the Laboratory of Dioxins from the Institute of Environmental Assessment and Water Research (IDAEA, CSIC, Barcelona) by using the standard methodology by HRMS [24].

For the first three real samples analyzed, a good agreement was found. Relative errors below 15% were obtained for all the studied DL-PCBs in the different samples. Considering the complexity of these matrices, this kind of errors are assumable, validating the use of the developed GC-(APCI)MS/MS methodology as an alternative to traditional GC-(EI)HRMS. **Fig. 2** represents the comparison between HRMS and APCI for the three mentioned matrices in pg g^{-1} of product for every single DL-PCB.

In order to fully confirm the goodness of the determination by using the developed methodology, nine additional sample extracts coming from different EU-RL Proficiency Tests [25], [26], [27], [28] were also analyzed by the GC-(APCI)MS/MS method after their analysis by GC-(EI)HRMS. As in the other samples, results showed a good concordance between methods. Relative errors were lower than 20% for most of DL-PCBs in every sample, except for PCB 114 in the mineral sample; in that case the highest deviation was obtained (about 50%) (**Table 3**). The comparison with the HRMS methodology is graphically displayed in **Fig. 3**, in which the different box-plots represent the residuals for each matrix (**Fig. 3A**) and DL-PCB congener (**Fig. 3B**) considering the HRMS as the reference value. It can be noticed that the matrices which present a larger variability are milk fat and mineral (**Fig. 3A**) as their corresponding boxplots are wider than the rest. Milk fat matrix also has the highest median relative error, close to −20%. For individual congeners (**Fig. 3B**) PCB 77 presents the largest positive bias, as well as the greatest relative error. This behavior should be further studied to see if the differences in ionization can have this kind of effects in the analysis of DL-PCBs using alternative ionization techniques.

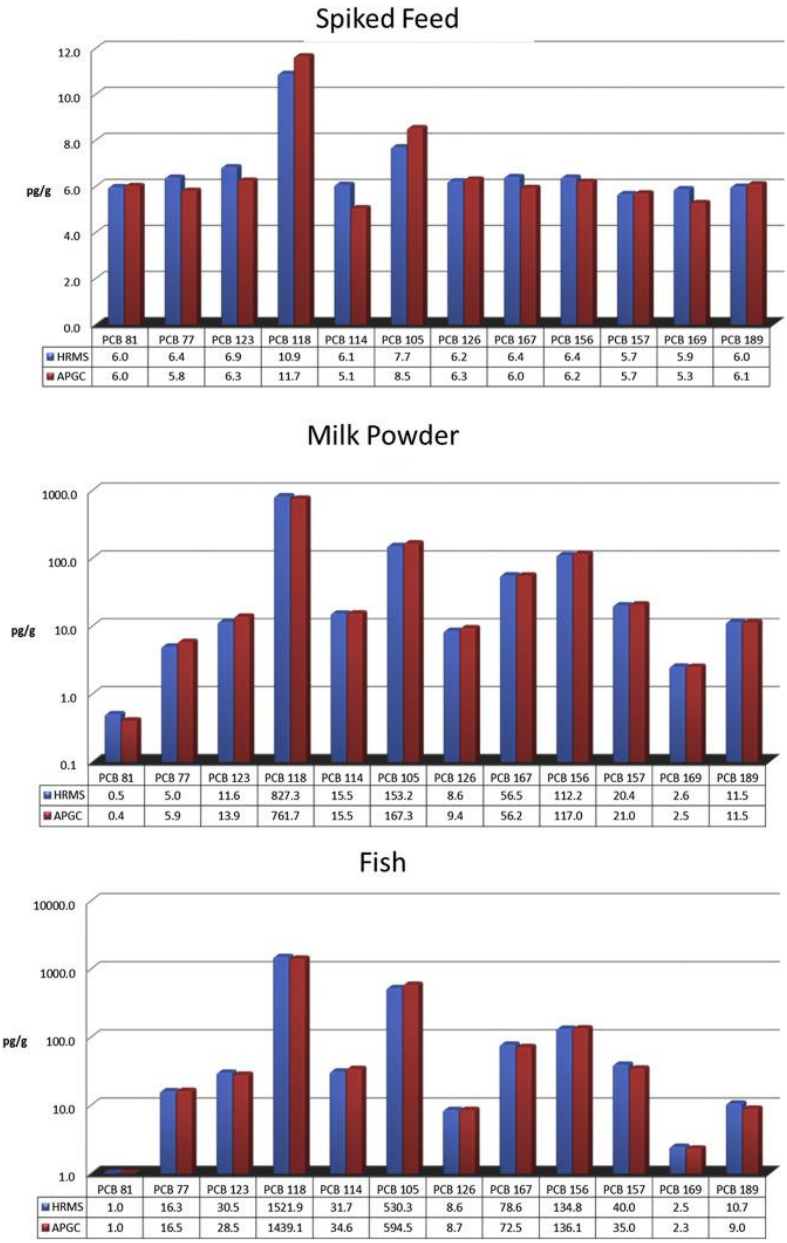


Fig. 2. DL-PCB profile for three different complex matrices in pg g^{-1}. The results for every single DL-PCB are shown comparing the GC-(EI)HRMS determination (blue) and GC-(APCI)MS/MS determination (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3. Concentrations of DL-PCBs (pg g -1 fat) determined in the Proficiency test (PT) samples analyzed by GC-(EI)HRMS and GC-(APCI)-MS/MS together with the Proficiency test assigned values [25-28]. The relative error (RE) between HRMS and MS/MS is also shown.

Compound	Pork Meat			RE	Pork Meat			RE	Lard			RE
	HRMS	APCI	PT		HRMS	APCI	PT		HRMS	APCI	PT	
PCB 81	0.61	0.66	n.r.	8%	0.4	0.41	n.r.	2%	6.52	7.54	8.36	16%
PCB 77	9.67	7.61	n.r.	-21%	8.02	7.7	n.r.	-4%	185	128	184	-31%
PCB 123	18.52	14.13	n.r.	-24%	14.54	16.05	n.r.	10%	145	147	131	1%
PCB 118	656	719	444	10%	630	680	444	8%	7377	7676	7428	4%
PCB 114	10.42	9.63	n.r.	-8%	9.64	9.63	n.r.	0%	235	251	243	7%
PCB 105	131.26	123.76	79.2	-6%	122.5	112.2	79.2	-8%	3414	3413	3483	0%
PCB 126	7.23	8.32	5.34	15%	6.86	9.26	5.34	35%	6.84	7.9	7.2	16%
PCB 167	98	89.6	88.7	-9%	100.65	90.6	88.7	-10%	297.5	276	290	-7%
PCB 156	398	407	382	2%	401	407	382	2%	876	917	906	5%
PCB 157	68.3	64.4	63.8	-6%	69.5	69.1	63.8	-1%	205	197	202	-4%
PCB 169	5.75	4.88	4.86	-15%	5.63	5.28	4.86	-6%	<LOQ	<LOQ	n.r.	
PCB 189	89.0	85.4	84.6	-4%	89.6	78.3	84.6	-13%	32	38.1	34	16%

Table 3. (Cont.) Concentrations of DL-PCBs (pg g -1 fat) determined in the Proficiency test (PT) samples analyzed by GC-(EI)HRMS and GC-(APCI)-MS/MS together with the Proficiency test assigned values [25-28]. The relative error (RE) between HRMS and MS/MS is also shown

Compound	Lard			RE	Egg			RE	Egg Yolk Powder			RE
	HRMS	APCI	PT		HRMS	APCI	PT		HRMS	APCI	PT	
PCB 81	7.70	7.82	8.36	2%	1.38	1.09	0.94	-21%	37.1	22.6	34.6	-39%
PCB 77	197	133	184	-33%	34.2	38.1	19.8	12%	821	536	711	-35%
PCB 123	157	144	131	-8%	14.89	22.63	n.r.	52%	196	238	170	21%
PCB 118	7671	8917	7428	16%	908	968	755	7%	9541	11167	7960	17%
PCB 114	241	251	243	4%	7.08	7.23	n.r.	2%	337	297	304	-12%
PCB 105	3667	3415	3483	-7%	193	182	166	-5%	4776	4670	4160	-2%
PCB 126	7.2	7.2	7.2	0%	56.6	50.5	47.1	-11%	18.0	16.8	15.2	-7%
PCB 167	310	281	290	-9%	302	275	260	-9%	297	253	256	-15%
PCB 156	930	949	906	2%	463	452	408	-2%	874	853	759	-2%
PCB 157	212	211	202	0%	81.0	82.1	73.1	1%	193	199	167	3%
PCB 169	<LOQ	<LOQ	n.r.		6.03	5.56	5.12	-8%	<LOQ	<LOQ	n.r.	
PCB 189	35	33.6	34	-5%	72.2	70.9	65.1	-2%	37.39	32.59	32	-13%

Table 3. (Cont.) Concentrations of DL-PCBs (pg g -1 fat) determined in the Proficiency test (PT) samples analyzed by GC-(EI)HRMS and GC-(APCI)-MS/MS together with the Proficiency test assigned values [25-28]. The relative error (RE) between HRMS and MS/MS is also shown

Compound	Milk Powder			RE	Milk fat			RE	Mineral (*)			RE
	HRMS	APCI	PT		HRMS	APCI	PT		HRMS	APCI	PT	
PCB 81	8.6	9.2	13.3	7%	14.3	9.6	18.8	-33%	13.2	9.7	15.8	-27%
PCB 77	267	182	302	-32%	352	255	404	-28%	297	203	293	-32%
PCB 123	193	191	165	-1%	297	241	232	-19%	35.9	33.8	34.3	-6%
PCB 118	8451	8701	8670	3%	10501	11651	12100	11%	1267	1559	1250	23%
PCB 114	248	256	275	3%	341	361	405	6%	62.3	92.8	66.2	49%
PCB 105	4085	3677	4110	-10%	4980	4130	5730	-17%	798	751	770	-6%
PCB 126	11.0	9.3	12.6	-16%	13	10	16	-26%	3.99	3.09	4.08	-23%
PCB 167	354	311	345	-12%	447	383	462	-14%	32.6	29.2	30.6	-11%
PCB 156	954	989	1000	4%	1222	1286	1370	5%	96.5	102.5	94.1	6%
PCB 157	201	222	224	10%	269	274	310	2%	22.3	22.4	20.8	1%
PCB 169	0.97	0.95	n.r.	-2%	0.85	0.83	n.r.	-2%	<LOQ	<LOQ	n.r.	
PCB 189	36.1	35.2	41.2	-2%	44.7	46.2	52.3	3%	3.6	3.3	3.5	-10%

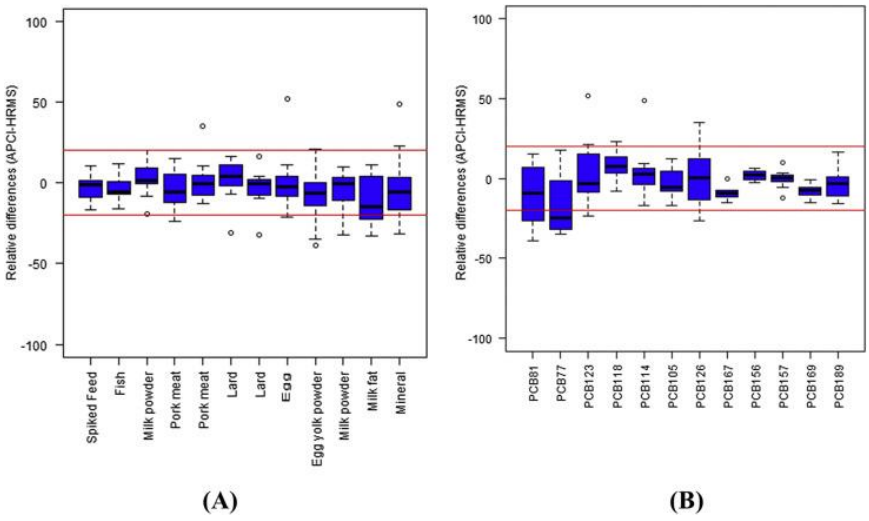


Fig. 3. Box-plots for the comparison of the results given by GC-(APCI)MS/MS and GC-(EI)HRMS by type of matrix (A) and type of analyte (B). The red lines indicate a difference higher than 20% between both techniques.

Z-scores were calculated taking into account the congener reference values, included in the corresponding reports of the EU-RL Proficiency Tests [25], [26], [27], [28], and a standard deviation of 20%. In general, calculated Z-scores (**Table 4**) are below 2 in most of the cases, with only a few values above 3, but in these specific cases deviations from the reference values could be most likely attributed to problems related to previous steps of the sample treatment (extraction and purification/fractionation) rather to a differences from the instrumental analysis. These findings highlight the good agreement between techniques, as well as the suitability of APCI for the determination of DL-PCBs in these complex samples.

Dioxins and furans

Prior to the development of the proposed method, another fraction of some of the samples had been analyzed looking for the presence of dioxins and furans, making use of a recently validated GC-(APCI)MS/MS method [21] in a pioneer work where the potential of this technique was proved in PCDD/Fs analysis field. The obtained results, also compared to HRMS techniques, are summarized in **Table 5** where a concordance in the quantification results with both techniques and the similarity of estimated

LODs can be observed. Taking that method as a reference, the fundamental idea of this work was to test the quantification capabilities of the APCI source not only for the analysis of dioxins and furans but also for DL-PCBs.

The results of the two studies highlight the suitability of the novel atmospheric pressure chemical ionization source coupled to last generation GC-MS/MS instruments for the analysis of these pollutants, which gives comparable results with enhanced limits of detection for the majority of the compounds.

Table 4. Z-scores for the samples analyzed by GC-(EI)HRMS and GC-(APCI)MS/MS relative to the Proficiency Test assigned values [25-28].

Compound name	Pork Meat		Pork Meat		Lard		Lard		Egg	
	HRMS	APCI	HRMS	APCI	HRMS	APCI	HRMS	APCI	HRMS	APCI
PCB 81					-1,1	-0,49	-0,39	-0,32	2,37	0,82
PCB 77					0,04	-1,51	0,36	-1,38	**3,64**	**4,64**
PCB 123					0,55	0,63	1,01	0,51		
PCB 118	2,4	**3,11**	2,1	2,67	-0,03	0,17	0,16	1	1,01	1,41
PCB 114					-0,16	0,17	-0,03	0,17		
PCB 105	**3,29**	2,81	2,74	2,08	-0,1	-0,1	0,27	-0,1	0,8	0,49
PCB 126	1,77	2,79	1,42	**3,67**	-0,25	0,51	0	0,01	1,01	0,36
PCB 167	0,52	0,05	0,67	0,11	0,13	-0,24	0,35	-0,15	0,81	0,3
PCB 156	0,21	0,33	0,25	0,34	-0,16	0,07	0,13	0,24	0,68	0,54
PCB 157	0,35	0,05	0,45	0,42	0,07	-0,1	0,26	0,24	0,54	0,61
PCB 169	0,92	0,02	0,79	0,43					0,89	0,43
PCB 189	0,26	0,05	0,3	-0,37	-0,18	0,61	0,23	-0,05	0,55	0,45

Compound name	Egg Yolk Powder		Milk Powder		Milk fat		Mineral (*)	
	HRMS	APCI	HRMS	APCI	HRMS	APCI	HRMS	APCI
PCB 81	0,36	-1,73	-1,76	-1,53	-1,19	-2,45	-0,84	-1,94
PCB 77	0,78	-1,23	-0,58	-1,99	-0,64	-1,85	0,07	-1,54
PCB 123	0,77	1,99	0,83	0,8	1,4	0,19	0,23	-0,08
PCB 118	0,99	2,01	-0,13	0,02	-0,66	-0,19	0,07	1,24
PCB 114	0,55	-0,1	-0,49	-0,34	-0,79	-0,54	-0,29	2,01
PCB 105	0,74	0,61	-0,03	-0,53	-0,65	-1,4	0,18	-0,12
PCB 126	0,92	0,54	-0,63	-1,33	-0,88	-1,96	-0,11	-1,21
PCB 167	0,81	-0,07	0,12	-0,5	-0,16	-0,86	0,33	-0,23
PCB 156	0,76	0,62	-0,23	-0,06	-0,54	-0,31	0,13	0,44
PCB 157	0,78	0,97	-0,51	-0,05	-0,67	-0,58	0,36	0,39
PCB 169								
PCB 189	0,84	0,09	-0,62	-0,73	-0,72	-0,59	0,23	-0,28

Table 5. Comparison of quantification results for dioxins and furans in the analyzed samples by GC-(EI)HRMS and GC-(APCI)MS/MS analyses by previously developed method[21]. Comparison of estimated LODs by both techniques.

Congener	Powdered Milk				Fish				Spiked fish feed			
	Conc (pg g^{-1})		LOD (pg g^{-1})		Conc (pg g^{-1})		LOD (pg g^{-1})		Conc (pg g^{-1})		LOD (pg g^{-1})	
	HRMS	APCI	HRMS	APCI	HRMS	APCI	HRMS	APCI	HRMS	APCI	HRMS	APCI
2,3,7,8-TCDF	0.07	0.05	0.003	0.002	1.50	1.33	0.011	0.001	0.07	0.08	0.001	0.002
1,2,3,7,8-PeCDF	0.07	0.06	0.010	0.002	0.13	0.13	0.013	0.001	0.28	0.26	0.004	0.002
2,3,4,7,8-PeCDF	1.74	1.68	0.011	0.002	0.38	0.37	0.025	0.001	0.26	0.26	0.004	0.002
1,2,3,4,7,8-HxCDF	0.95	0.92	0.006	0.003	0.03	0.03	0.009	0.001	0.25	0.28	0.004	0.001
1,2,3,6,7,8-HxCDF	1.11	1.02	0.007	0.003	0.04	0.04	0.009	0.001	0.26	0.27	0.004	0.001
2,3,4,6,7,8-HxCDF	1.05	0.97	0.007	0.003	0.05	0.04	0.019	0.002	0.24	0.24	0.004	0.001
1,2,3,7,8,9-HxCDF	0.01	0.01	0.009	0.004	-	-	0.015	0.001	0.25	0.26	0.004	0.001
1,2,3,4,6,7,8-HpCDF	0.59	0.54	0.008	0.002	0.02	0.02	0.005	0.001	0.29	0.30	0.003	0.002
1,2,3,4,7,8,9-HpCDF	0.06	0.05	0.011	0.003	-	-	0.008	0.001	0.26	0.26	0.003	0.002
OCDF	0.20	0.16	0.012	0.003	0.04	0.03	0.005	0.001	0.53	0.52	0.004	0.003
2,3,7,8-TCDD	0.27	0.27	0.011	0.001	0.05	0.04	0.005	-	0.05	0.05	0.005	0.001
1,2,3,7,8-PeCDD	0.79	0.82	0.010	0.002	0.07	0.06	0.011	-	0.26	0.26	0.003	0.001
1,2,3,4,7,8-HxCDD	0.42	0.40	0.010	0.002	-	-	0.007	0.001	0.25	0.27	0.003	0.001
1,2,3,6,7,8-HxCDD	0.93	0.93	0.011	0.002	0.06	0.06	0.007	0.001	0.25	0.27	0.003	0.001
1,2,3,7,8,9-HxCDD	0.34	0.36	0.017	0.003	0.01	0.01	0.007	0.001	0.25	0.30	0.002	0.001
1,2,3,4,6,7,8-HpCDD	3.77	3.68	0.021	0.003	0.05	0.04	0.013	0.001	0.37	0.35	0.005	0.002
OCDD	18.03	17.79	0.009	0.007	0.16	0.10	0.013	0.002	0.98	0.73	0.001	0.005

3.4.7 Conclusions

A GC-(APCI)MS/MS with QqQ method has been developed for the determination of DL-PCBs in a wide range of complex matrices which including fish, feed, milk powder, pork meat, lard, egg, egg yolk powder, milk fat and mineral (sepiolite). The comparison with the standard GC-(EI)HRMS methodology demonstrates the capabilities of the method, which relies on the use of the novel APCI with last generation GC-MS/MS instruments, achieving performances almost identical to GC-(EI)HRMS in the determination of the selected POPs. The obtained instrumental LODs and LOQs, are between 6 and 200 times lower than those attained by previously developed analytical methodologies based on GC-(EI)MS/MS with QqQ, and in the same range than those provided by GC-(EI)HRMS. These results are in agreement with the last outputs from APCI-related articles, in which a clear improvement of LODs for POPs has been achieved when analyzing this type of contaminants by GC-(APCI)MS/MS instead of GC-(EI)-HRMS [29]. Additionally, QqQ instruments are less expensive, and generally easy to use and maintain, making them an interesting addition to commercial laboratories that otherwise would not be able to perform these types of analyses. The low fragmentation provided by the APCI source has demonstrated to be capable of enhancing the performance of triple quadrupole analyzers applied to the determination of DL-PCBs, dioxins and furans, which may constitute a real alternative to the use of HRMS for the analysis of these compounds in food samples.

The demonstrated capabilities of this revived technique in the field of POPs open a wide range of possibilities for further studies, which could include the analysis of DL-PCBs and dioxins and furans in one single injection.

3.4.8 Acknowledgements

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3.5 Discussion

Results obtained in these three articles demonstrate the enhanced capabilities of GC-APCI-MS/MS coupling for the determination of several POPs in food and environmental samples. Efforts were devoted towards promoting the formation of highly abundant molecular (or quasi molecular) ions that could then be selected as precursor ions in order to obtain highly specific MS/MS transitions.

Among the chemical families studied, PBDEs have a particular behaviour, as they can protonate easily to give the $[M+H]^+$ species together with $M^{+\cdot}$ ions, even without the addition of in-source modifiers. Therefore, the ionization and fragmentation behavior of PBDEs, 1,2-Bis(2,4,6-tribromophenoxy)ethane (BTBPE) and decabromodiphenyl ethane (DBPDE) was carefully studied in **scientific article 2.** As an example, the ionization and in-source fragmentation of BDE-209 is presented in **Figure 3.2**. Under the soft ionization provided by APCI, the base peak is the $M^{+\bullet}$ ion at m/z 958.1691 together with its molecular cluster.

The full MS spectrum acquired using GC-APCI-QTOF with a resolution of 12000 (FWHM) reveals that both proton transfer and charge transfer reactions occur simultaneously when working in ”dry” conditions as a result of the moisture present in air. “Dry” conditions imply that no modifier is added to the source to enhance charge transfer ionization. As both $[M+H]^+$ and $M^{+\bullet}$ clusters were observed on APCI spectra of all PBDEs, even under “dry” conditions, different modifiers were added inside the APCI source housing, in order to induce the formation of the desired ions.

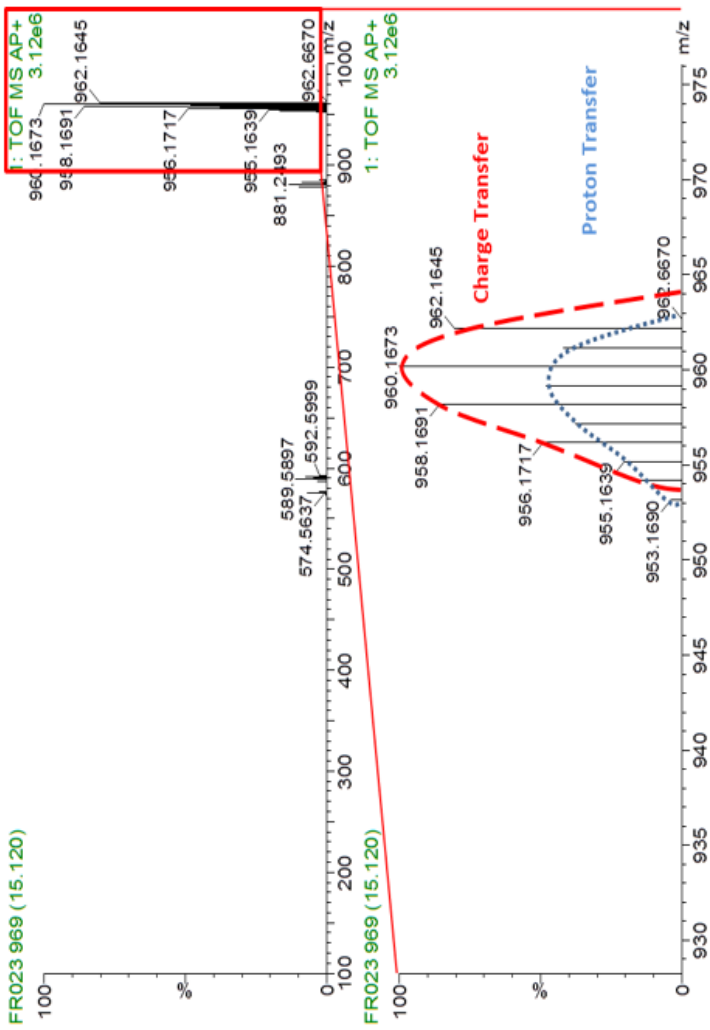

Figure 3.2. Ionization and in-source fragmentation spectra of BDE-209 in an APCI source under charge transfer conditions acquired in a QTOF HRMS system.

In this case, proton-donor modifiers, e.g. water and HCOOH (1%), were added to the source atmosphere to maximize the formation of the protonated molecule. The use of water as modifier favored the formation of the $[M+H]^+$ and the drastic reduction in the $M^{+\bullet}$ intensity, increasing the proportion of $[M+H]^+$, as displayed in **Figure 3.3** for BDE 66.

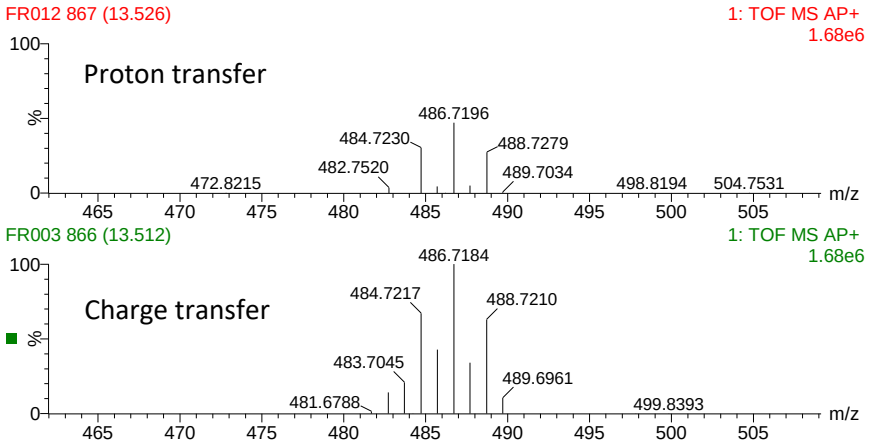

Figure 3.3. Ionization spectra of BDE-66 (tetra-BDE) in APCI source under charge transfer and proton transfer conditions using water as modifier.

This cleared the spectra in all cases, where the presence of water gives a molecular cluster with much lower intensity of charge transfer ionization products. Considering this, both $M^{+\bullet}$ and $[M+H]^+$ as well as the rest of the ions from the isotopic cluster (+2, +4) were selected as potential precursor ions for the development of SRM transitions.

This behavior was observed for all studied PBDEs, except for the highly brominated (Br7–10) PBDE congeners and BTBPE, for which a low percentage of $M^{+\bullet}$ was still present in their spectra. DBDPE spectrum did not change by adding water to the source, maintaining the fragment $C_8H_4Br_5^{+\bullet}$ as base peak. For the highly brominated compounds,

protonation could also be improved by the addition of HCOOH at 1% in water.

Figure 3.4 (S-1) highlights the different fragmentation behavior of BDE-47 and BDE-209 (Tetra-BDE and Deca-BDE, respectively) under charge-transfer and proton-transfer conditions. For low brominated congeners, as BDE-47, the addition of water produced the formation of ions with a higher specificity. In this case, the first loss at CE of 20 eV is that of one Br instead of Br_2 in charge transfer conditions. When applying a higher collision energy (60 eV), the fragmentation in the presence of water was slightly more gentle, producing the more specific ion $[M+H-Br_3]^+$, while the charge transfer reaction fragmentation produced the ion $[M-CBr_3O]^+$.

The addition of modifiers revealed another interesting APCI feature, the suppression of cross-talking between congeners. As PBDEs share a common structure with different bromination degrees, coeluting highly brominated congeners may interfere with lower brominated BDEs, as in-source fragments of the highly brominated are in fact the precursor ions of the lower brominated congeners. This was reported for EI sources and it was also found in APCI (but to a much lower degree) when working under charge transfer conditions. Surprisingly, these interferences were reduced significantly when working with protonation modifiers, which implies that in-source fragmentation mainly comes from the $M^{+\cdot}$.

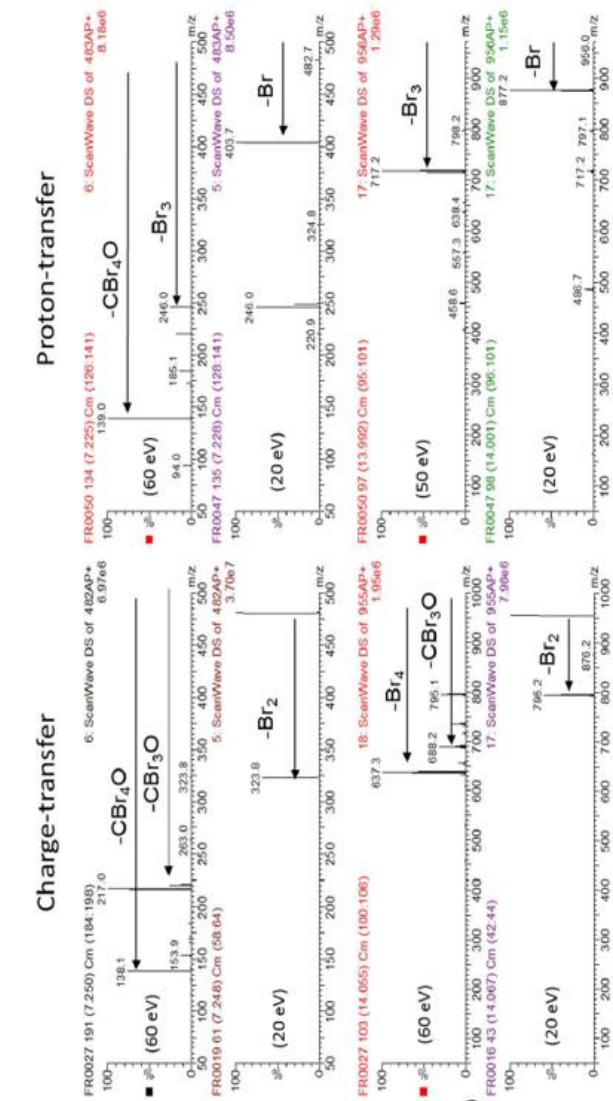

Figure 3.4. Product ion spectra of $M^{\bullet+}$ under charge-transfer conditions (left) and $[M+H]^+$ under proton-transfer conditions (right) at different collision energies for BDE 47 and BDE 209.

This increased selectivity is displayed in **Figure 3.5**. (S-2). Higher bromination degree congeners are not the only compounds likely to co-elute when performing PBDE analyses. As reported by (Korytár, Covaci, Leonards, De Boer, & Brinkman, 2005) and (Eljarrat, De la Cal, & Barceló, 2003), depending on the type and the length of the column, several other compounds may interfere with the selected analytes, as shown in **Table 3.1.**

As much as 66 PBDEs and 27 other BFRs coelutions can occur using 30-meter columns in GCxGC methodologies, and the number increases for traditional analyses. Additionally, other POPs that often coelute with the selected analytes, such as PCBs, MeO-PBDEs and PCTs/PCNs, producing interference ions (Eljarrat et al 2003). With such a high number of interferences, the use of APCI in proton transfer conditions would be of high relevance for future works. This may be emphasized in reducing analysis time of PDBEs and related substances, as different congeners coelutions would not increase the signal of the selected PBDEs.

Table 3.1. An assessment of PBDE coelution from seven gas chromatography columns as tested and reported by Korytár et al.

Column	DB-1	DB-5	HT-5	DB-17	DB-XLB	HT-8	CP-Sil 19
Dimension (m × mm × μm)	30 × 0.25 × 0.25	30 × 0.25 × 0.25	30 × 0.25 × 0.10	30 × 0.25 × 0.25	30 × 0.25 × 0.25	25 × 0.22 × 0.25	17 × 0.15 × 0.30
Number of coeluting BDEs	62	63	66	67	56	62	72
Number of coelutions with flame retardants	24	26	27	30	22	26	29
Co-elution with major BDE congeners							
Major BDE							
28	16, 33	16, 33	16, 33, 38			16, 33, 38	16, 33
47							
49	68, 80	68	68	62	42, 48, 68, 71	68	51, 75
85				155			114
99			116	127			
100			109	101		109, 120	
138			166		HBCD		166
153			HBCD	168			
154	MTBBP-A, BB-153	MTBBP-A, BB 153		105		126	BB 153
183	BB 169	BB 169					

MTBBP-A dimethylated tetrabromobisphenol-A, HBCD hexabromocyclododecane

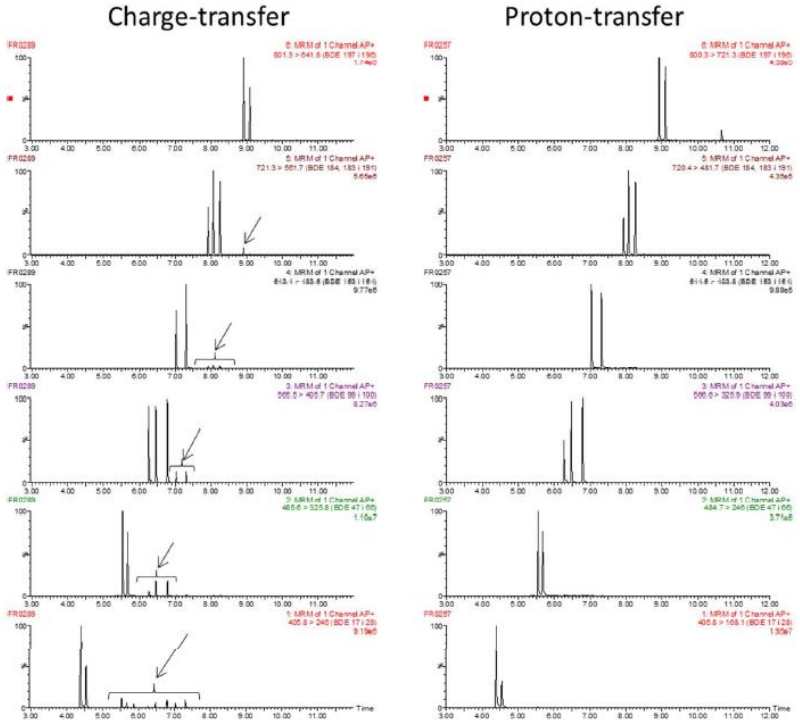

Figure 3.5. GC-APCI-QqQ(MS/MS) chromatograms (left) under charge-transfer conditions where crosstalking can be observed and (right) under proton-transfer conditions

Although both $M^{+\bullet}$ and $[M+H]^+$ ions might have been selected as precursor ions in a MS/MS method, the best sensitivity was reached working under charge-transfer conditions and using $M^{+\bullet}$ as precursor ion. As an example, **Figure 3.6** (S-3) shows the positive findings of BDE-209 in a dolphin sample with the developed SRM transitions in both, charge transfer and proton transfer conditions. This behavior correlates well with the physicochemical properties of PBDEs, which have low protonation affinity and the fact that the in-source humidity is not constant, even with the addition of the abovementioned modifiers. A higher amount of in source particles may

also lead to lower ionization efficiency, as the modifier molecules compete for the ionization with the analyte molecules.

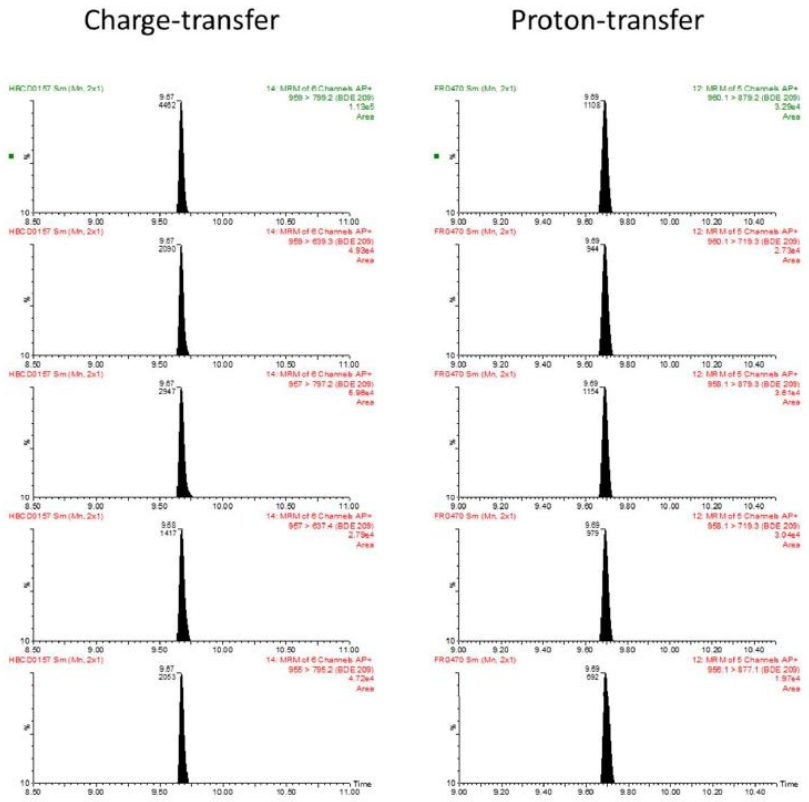

Figure 3.6. Positive detection and identification of BDE-209 in dolphin by applying GC-APCI-QqQ(MS/MS) under charge-transfer conditions (left) and proton-transfer conditions (right).

Apart from the novel ionization and the related advantages that APCI presents, there is another interesting feature that the design and function of the APGC source permits in order to increase sensitivity and peak shape. The carrier gas flow, typically set between 1 and 2 mL/min when working with EI sources, as limited by vacuum related issues, can be set to values only limited by the maximum inlet pressure and column stability when working with APCI. This is possible due to two main APCI source

particularities; in one hand, as the source works at atmospheric pressure, there is no pumping system needed to eliminate the high He flows (more than 2 mL/min). There is practically no limit in the volume of He which gets into the source. On the other hand, the ionization in APCI is efficient at high flows (Portolés, 2012), opposite to what happens in EI sources. For the method development, carrier gas flows between 1 mL/min and 6 mL/min were tested. **Figure 3.7** highlights the evolution of the BDE-209 peak, monitored by the $[M+H+10]^+$ -> $[M+H-^{79}Br]^+$ transition. As the carrier gas flow increases, there is a peak width reduction (from 15 s to 6 s) together with an increase in sensitivity. Although 6 mL/min of He as carrier gas flow was found optimal in terms of BDE-209 sensitivity, it was a bit excessive for the early eluting compounds, and thus 4 mL/min was selected as the best compromise between good sensitivity, enhanced peak shape, and a significantly reduced analysis times.

Finally, the developed methodology was validated by analyzing marine samples previously characterized by the traditional GC-EI-HRMS technique. The results, shown in **Table 3.2** (ST-1) demonstrate a good agreement between both methodologies, while in the cases in which EI was not enough sensitive, APCI was able to detect peaks. Choosing the molecular ion as precursor strongly improved sensitivity (leading to LODs lower than 10 fg on-column) and selectivity compared to GC-EI-MS/MS analysis. Samples already analyzed by GC-EI-MS/MS with negative results, showed to have concentrations in the low ppb range, quantifiable by this APCI-based approach.

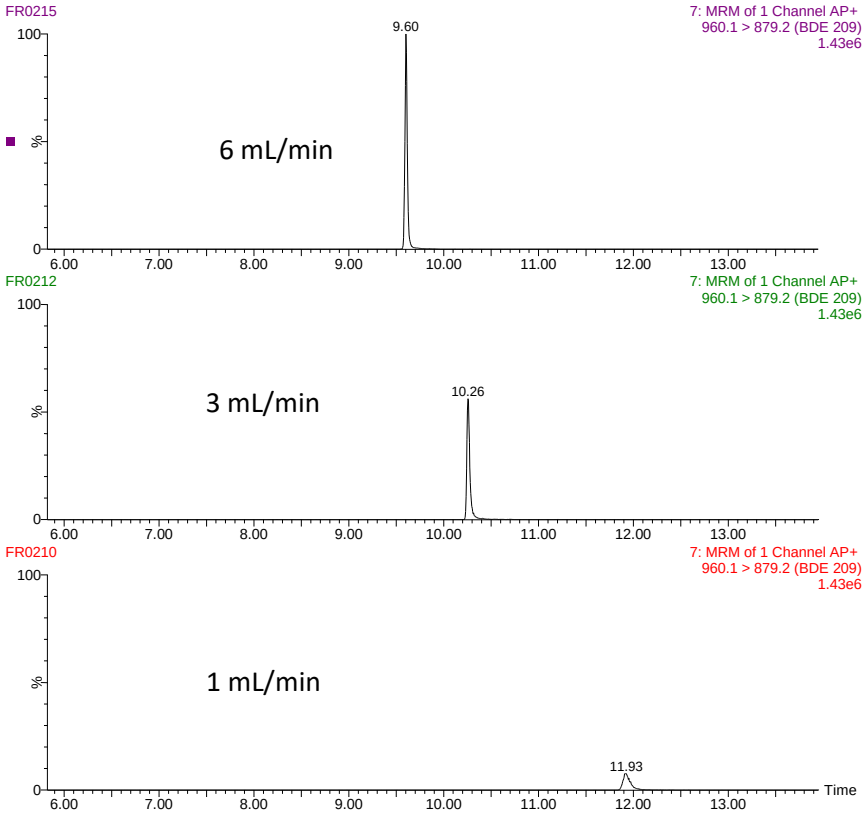

Figure 3.7. Chromatograms for BDE-209 main transition at different carrier gas flows (1 mL/min, 3 mL/min and 6 mL/min). Injection of 25 pg/µL.

Table 3.2. BFRs detected in marine samples (concentrations expressed in pg/g fresh weight) by GC-EI-MS/MS and GC-APCI-MS/MS (charge transfer conditions).

		BDE 28		BDE 47		BDE 66		BDE 100		BDE 99		BDE 85		BDE 154		BDE 153	
	weight(g)	EI	APCI	EI	APCI	EI	APCI	EI	APCI	EI	APCI	EI	APCI	EI	APCI	EI	APCI
dauphin	33.86	5.0	3.8	1087.2	1184.0	6.3	5.4	601.5	703.6	198.3	184.1	6.4	8.2	624.3	794.3	369.1	372.8
zooplankton	198.71	d	d	1.90	1.8	-	-	d	d	0.7	0.6	-	-	d	d	d	d
zooplankton	39.07	d	d	15.8	16.3	d	d	3.4	3.6	5.7	5.3	-	d	d	d	-	d
fish	51.08	5.1	4.1	91.3	76.1	5.3	5.3	36.2	37.4	12.1	9.0	d	d	52.3	38.4	d	d
fish	27.28	16.1	11.7	174.6	142.6	18.1	17.2	162.4	188.2	33.5	27.3	13.02	10.2	298.7	248.1	17.4	15.1
small fish	19.15	d	d	10.3	10.9	d	d	9.4	9.1	d	d	d	d	31.7	37.2	d	d
small fish	7.31	d	d	22.7	22.6	-	d	d	d	d	d	-	d	d	d	-	d
prawn	12.41	27.9	21.2	455.9	477.1	23.3	27.4	149.6	184.6	85.8	66.5	d	d	200.2	212.5	42.9	38.5
prawn	10.42	d	d	145.3	144.0	d	d	35.1	41.7	41.1	38.0	d	d	60.3	69.4	d	d
squid	9.43	d	d	72.3	72.2	d	d	17.8	16.4	20.7	19.9	-	d	18.1	21.1	d	d
squid	22.31	d	d	5.7	5.5	-	d	0.7	0.9	0.9	1.1	-	d	d	d	-	d

		BDE 184		BDE 183		BDE 191		BTBPE		BDE 197		BDE 196		BDE 209		DBDPE	
	weight(g)	EI	APCI	EI	APCI	EI	APCI	EI	APCI	EI	APCI	EI	APCI	EI	APCI	EI	APCI
dauphin	33.86	3.3	22.4	7.4	24.1	-	-	d	d	-	-	-	-	d	d	-	-
zooplankton	198.71	-	d	-	d	-	-	-	-	-	1.5	-	2.1	d	69.2	-	-
zooplankton	39.07	-	-	-	d	-	-	-	-	-	d	-	3.7	12.6	107.7	-	d
fish	51.08	-	-	-	d	-	-	-	-	-	-	-	-	-	20.1	-	-
fish	27.28	-	15.7	-	-	-	-	d	d	-	d	-	d	-	26.5	-	d
small fish	19.15	-	-	-	d	-	-	d	d	-	d	-	d	30.5	376.2	d	d
small fish	7.31	-	-	-	d	-	-	d	d	-	d	-	d	23.5	727.3	-	d
prawn	12.41	-	-	-	d	-	-	-	-	-	-	-	-	-	-	-	-
prawn	10.42	-	-	-	d	-	-	d	d	-	d	-	d	-	d	-	d
squid	9.43	-	-	-	d	-	-	d	d	-	d	-	d	106.6	521.5	-	d
squid	22.31	-	-	-	d	-	-	d	d	-	-	-	-	-	-	-	-

d: detected, below lowest calibration level

Given the extraordinary results of the methodology, and taking into account that many other halogenated compounds elute in the same fraction as PBDEs in regular sample treatments, the following step was to study the behavior of HBCD, which was described in **Scientific Article 3**.

HBCD, as other HFRs, has been typically determined by GC-MS using NICI or EI coupled to HRMS. Both approaches produced significant fragmentation, being $[M - Br]^+$ and $[M - H_2Br_6]^{-\bullet}$ the ions with the highest m/z in positive and negative CI, respectively, and the nonspecific ions at m/z 67 and 79 the most intense peaks when working in EI. This high fragmentation led, traditionally, to high limits of detection [around 2 ng/g lipid weight (lw)], which is insufficient to monitor HBCD.

In this scenario, the application of the softer ionization achieved with APCI, led to a lower in-source fragmentation, which allowed a significant increase in terms of sensitivity and specificity when using MS/MS experiments using quasi-molecular ion as precursor ion. Under these conditions, LOD was determined to be in the order of 100 fg of HBCD injected in pulsed splitless mode, while no cross-talking with any BDE congener was found, due to the highly specific transitions acquired, taking the $[M+4 - Br]^{+\bullet}$ and $[M+6 - Br]^{+\bullet}$ ions as precursor ions.

The developed method allowed determining the total HBCD concentration in very particular samples, as PUF extracts obtained worldwide (Fiji, Uruguay and Kenya), different marine samples and reference materials. Findings revealed both the necessity of HBCD monitoring in remote areas, and the potential of GC-(APCI)-MS/MS for the determination of halogenated substances. The developed methodology would be a very efficient screening tool for HBCD, especially in samples from remote areas, where the concentration levels are lower than LODs achievable by traditional techniques.

The promising results obtained for BFRs brought into consideration the application of GC-(ACPI)-MS/MS to another type of contaminants, with a similar structure and also with compromised limits of detection by GC-EI-HRMS method. In **Scientific article 4**, efforts were focused on the determination of DL-PCBs in different food and feed samples.

In EU regulations and directives, confirmatory methods for these compounds have been traditionally based on GC-(EI)-HRMS due to the strict requirements demanded on sensitivity together with specificity and accuracy, in order to correctly distinguish the 209 different PCB congeners. In addition, DL-PCBs, as a consequence of their high toxicity, have the lowest LODs required (in particular non-ortho substituted congeners), in the low picogram levels.

The developed methodology based on GC-APCI-MS/MS allowed to comply with the strong sensitivity requirements, with LOD of 2.5 fg for all studied compounds around 4 times lower than recommended by EU regulations. LODs were calculated as the concentration giving a signal of 3 times the background noise. To achieve these results, the combination of an accurate GC separation, together with the exploitation of APCI capabilities applied to these chlorinated chemicals was needed.

Thus, the use of a 60m column permitted to obtain baseline resolution for the studied congeners and avoid interferences coming from the non-dioxin-like congeners potentially present in samples. Working with this column limited the carrier gas flow (as in the previous studies) so a He flow of 2 mL/min was selected as a good compromise to achieve high resolution while maintaining the analysis time to the minimum.

It was checked that DL-PCBs do not protonate, even in presence of water as modifier, due to their chemical structure. Thus, APCI was used under

dry source conditions. Fragmentation of DL-PCBs was carefully studied looking for highly specific and sensitive transitions, applying different collision energies. Similar to PBDEs and HBCD, APCI fragmentation of DL-PCBs was significantly lower than under EI, obtaining a much more intense molecular ion $M^{+\bullet}$, and a considerably lower in-source fragmentation, which favored to design very sensitive SRM transitions. **Figure 1** in **Scientific article 4** clearly displays the difference between EI and APCI fragmentation for 2,3,3',4,4',5-Hexaclhorobiphenyl.

When studying each compound separately, a different MS/MS fragmentation behavior was observed depending on the chlorination degree. For low chlorination congeners, PCB 81 and PCB 77, with 4 chlorine atoms in their structure, a higher collision energy was needed (50 eV), while for the rest of congeners, the value was set to an optimum value of 30 eV. When studying the optimal collision energy for the SRM transitions, the value was set to 35 eV for all the compounds, but PCB 189, the only heptachlorinated DL-PCB, needed 50 eV to achieve similar responses. The different chlorination degree also has an impact on the SRM transitions intensity. While for the low chlorination congeners (i.e. tetra- and penta-chlorinated) the most intense transition was with $M^{+\bullet}$ ion as a parent ion, compounds containing a higher number of chlorine atoms (i.e. hexa- and heptachlorinated) had the most sensitive transitions using $[M+2]^{+\bullet}$ as the precursor ion. **Figure 3.8.** shows the in-source fragmentation of DL-PCBs in the APCI source.

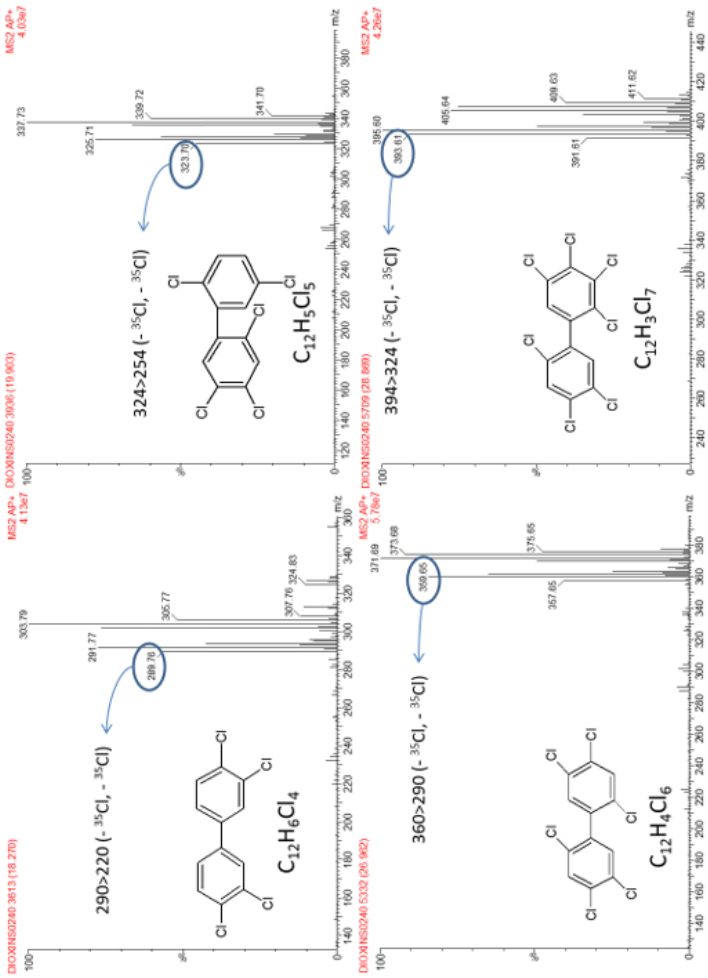

Figure 3.8. In-source fragmentation of DL-PCBs according to their chlorination degree.

Specificity of the selected transitions was also carefully studied, looking for cross-talking as in the case of PBDEs in **Scientific article 1**. The signal coming from fragmentation of higher chlorination degree congeners was studied in the transitions corresponding to the lower chlorination degree PCBs. This experiment was performed using GC-EI-MS/MS and GC-APCI-MS/MS. Although the GC-EI-MS/MS methodology was not completely optimized in terms of sensitivity, APCI was able to provide 1000 times more sensitivity and a high specificity, with signals coming from the high chlorination degree congeners below 1% of the signal corresponding to the lower chlorinated compounds when acquiring the transitions of the last ones. **Figure 3.9.** shows the results, highlighting the specificity of the SRM transitions obtained by APCI, as a result of the lower in-source fragmentation.

Although the developed methodology was proved to be extremely sensitive and specific, its accuracy had to be determined in order to validate GC-APCI-MS/MS for the analysis of dioxin-like PCBs. Accordingly, nine samples coming from an interlaboratory study, previously analyzed by routine GC-EI-HRMS methods, were included in the tests. The results were really promising, as for most compounds the concentration determined for individual congeners was in good agreement between the two techniques. Z-scores were calculated in order to visualize the accuracy of the values obtained (**Figure 3.10).** There was only one value with a z-score over 2 and only one value higher than 3. In both cases, they correspond to tetra-PCBs in fatty matrices, in which more interferences are expected. Further investigations should be performed to check if this behavior is due to the different APCI fragmentation or due to specific matrices. In the latter case, additional sample treatments could solve these problems.

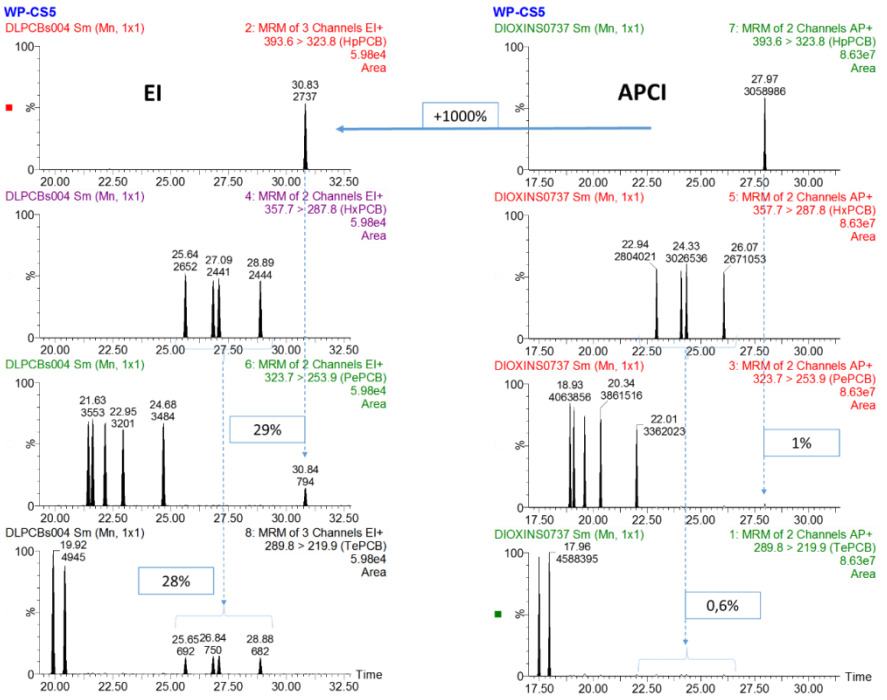

Figure 3.9. Comparison of the chromatograms corresponding to the injection of a 100 pg/µL standard in EI (A) and APCI (B). The lower chromatograms correspond to the Q transition monitoring for the Tetra-and Penta-PCBs in the window in which Hexa- and Hepta-PCBs transitions are controlled (upper chromatograms).

It is also important to highlight that the total DL-PCBs concentration in each matrix (i.e. the sum of the concentrations of each congener in a matrix) is in good agreement when using both methods for all analyzed matrices.

That implies that, when calculating the Toxic Equivalent (TEQ) according to equation 3.1, and considering that tetra-PCBs have the lowest toxic equivalent factor (TEF)(van der Berg, 2005), the calculated TEQ will be exactly the same using individual concentrations calculated by GC-APCI-MS/MS and GC-EI-MS.

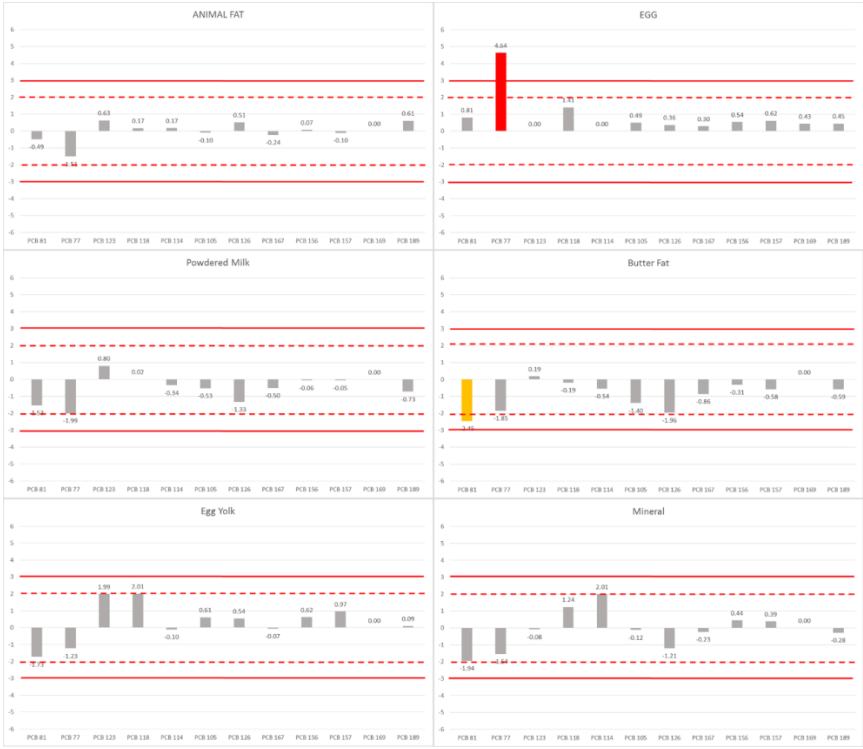

Fig 3.10. Z-score graphs for six of the nine different interlaboratory samples analyzed.

$$TEQ = \sum_{n1}[PCDD_i\, xTEF_i] + \sum_{n2}[PCDF_i\, xTEF_i] + \sum_{n3}[PCB_i\, xTEF_i] \quad \text{(eq 3.1)}$$

In conclusion, APCI has demonstrated to be a powerful ionization source in GC-MS/MS systems for the determination of persistent organic pollutants. The results obtained in these studies complement the already good results of the technique applied to dioxins, furans and pesticides. APCI enhances sensitivity and specificity when compared to traditional EI sources, and permits to obtain good results in the analysis of difficult compounds in complex samples using a triple quadrupole instead of HRMS systems. Triple quadrupole systems are less expensive and require lower

maintenance costs and specialization than GC-HRMS, an additional benefit which can reduce the cost of the analysis of such compounds in a near future, as more laboratories could have access to the technique.

Apart from the outcomes of the studies presented in this thesis, APCI for GC is in a continuous development as can be seen with the recently introduced transfer line (APGC v2.0) from Waters (Corporation, n.d.) and the very last trials with a flow modulator at the exit of the GC column (Karl J. Jobst, LCGC 2018). APGC v2 enhances sensitivity due to a better transmission of the analytes into the ion chamber, while flow modulation is able to produce sharper peaks (<200 ms wide) with a significant improvement in signal intensity compared to unmodulated peaks. This will surely have very positive implications in quantitative analysis, allowing reducing sample treatment and matrix effect as less pre-concentration will be required.

3.6 References

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Chapter 4

Development of metabolomics-based methodologies for quality classification of Spanish Olive Oils

4.1 Introduction

In this chapter, the potential of metabolomics, and more specifically foodomics with the use of different GC-MS configurations, has been explored for the untargeted identification of volatile organic compounds (VOCs) responsible of the quality of food.

Food quality is a matter of concern for consumers and producers. This quality is, in most cases, connected to the origin of the products, as it is the case for French wine (Arvanitoyannis, Katsota, Psarra, Soufleros, & Kallithraka, 1999) or Spanish ham (Sánchez-Peña, Luna, García-González, & Aparicio, 2005), and contributes to raise their prices. For this reason and to avoid fraud, guarantee of the genuine quality is a critical step from an economical point of view.

In the specific case of Olive oils, quality class characterization is performed by testers who establish, by tasting it, if an olive oil should be classified as extra virgin, virgin or lampante (not recommended for consumption) (Council, 2016). This strategy is known as "PANEL TEST", and classifies the oils according to two main properties: defects and goods. **Table 4.1** shows the maximum defect values allowed to assign the quality of an olive oil. Note that to be classified as virgin or extra virgin, an olive oil must have a fruity intensity superior to 0. On the other hand, depending on the amount

of the greatest defect, this olive oil is tagged as virgin (good quality but with some minor flaws) or lampante (not recommended for consumption).

Table 4.1. Summary of PANEL TEST parameters limits used to qualify olive oil.

Category	Intensity of the greatest defect	Fruity intensity
Extra	0	> 0
Virgin	≤ 2.5 ± (1.0)	> 0
Lampante	> 2.5 ± (1.0)	Indistinct
	≤ 2.5 ± (1.0)	0

Major sensory defects in olive oil are rancid, fusty/muddy sediment, musty/humid/earthy, acetone, burnt/heated, frozen/wet wood and winey/vinegary. The positive attributes can be fruity (specifying green attribute), bitter and spicy. According to the literature (Kalua, Allen, Bedgood Jr, Bishop, Prenzler & Robards, 2007; Luna, Morales, & Aparicio, 2006), the organic compounds responsible of these flavours are, from a chemical point of view, typically esters, ketones, aldehydes, alcohols, terpenes, phenols and their derivatives, in concentrations ranging from few ng L^{-1} to hundreds of mg L^{-1} and with different odour thresholds. **Figure 4.1** graphically represents the tasting process, in which VOCs present in olive oil arrive to olfactory bulbs by nasal olfaction and retro-nasal olfaction (volatilization in contact with the tongue).

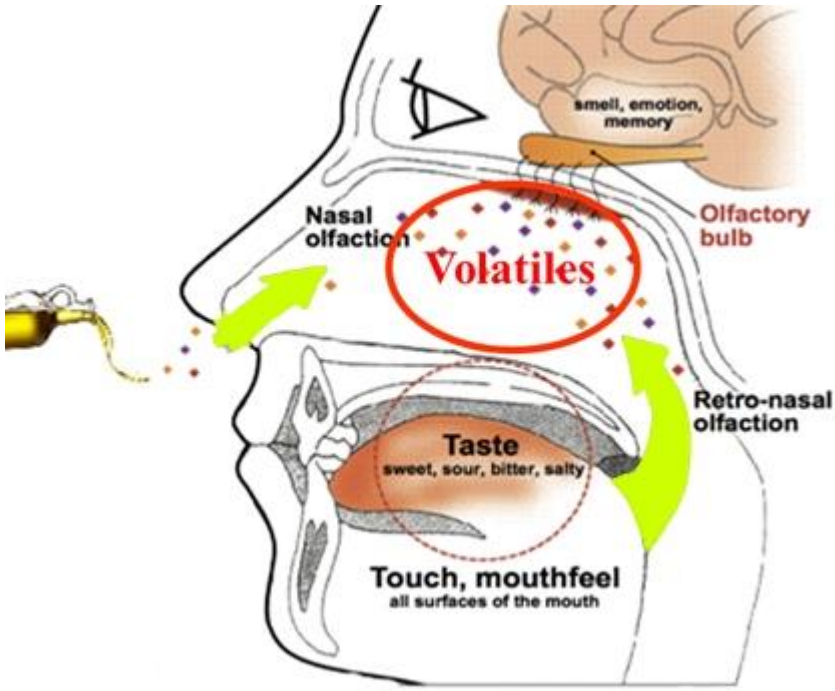

Figure 4.1. Graphical description of the detection of VOCs from olive oil in a regular tasting procedure.

PANEL TEST methodology has some important drawbacks. It requires really well trained testers and it is considerably expensive and time-consuming, as only a limited number of samples per day can be analysed by panelists. In this scenario, alternative methodologies based on the use of chromatographic techniques coupled to mass spectrometry (MS), complementary to PANEL TESTs, could be of great interest, allowing the objective determination of olive oil quality. Taking into account the properties of the compounds indicated before as volatile components of olive oils, as a first approach, the most adequate analysis technique seems

to be gas chromatography coupled to mass spectrometry (GC-MS) (Angerosa et al., 2004; Flath, Forrey, & Guadagni, 1964). Choosing a specific extraction technique for volatiles is also an important issue in order to perform a suitable separation from the matrix. From several studies on determination of VOCs in different matrices (Barco-Bonilla et al., 2011; Jiménez, Aguilera, Beltrán, & Uceda, 2006; Lam & Proctor, 2003; Salemi, Lacorte, Bagheri, & Barceló, 2006; Serrano, Beltrán, & Hernández, 2009), there is an evidence of the extended use of trapping processes of the volatilized compounds in various sorbents; either by forcing the VOCs to pass through the sorbent bed (purge and trap (P&T)) (Barco-Bonilla et al., 2011; Salemi et al., 2006) or letting them to establish an equilibrium between the vapor phase and the adsorbent in a closed place (SPME) (Serrano et al., 2009). Other techniques also used for VOCs extraction include: direct headspace (HS) injection (López-Feria, Cárdenas, García-Mesa, & Valcárcel, 2008), stir bar sorptive extraction (SBSE) (Bicchi, Iori, Rubiolo, & Sandra, 2002), or liquid phase microextraction (LPME) (Lee, Lee, Rasmussen, & Pedersen-Bjergaard, 2008). P&T, also known as dynamic headspace (DHS), provides determination of compounds at much lower concentrations than static HS, can be the best sampling method (Boczkaj, Makoś, & Przyjazny, 2016). DHS involves purging the headspace with a large known volume of inert gas to force the extraction of VOCs

from the matrix into a trap, resulting in a high preconcentration of analytes.

Figure 4.2 graphically highlights the preconcentration capabilities of some of the sample treatment techniques listed above. As it can be appreciated, DHS offers a higher preconcentration factor than HS or SPME, especially when thermal desorption in the GC system is carried out.

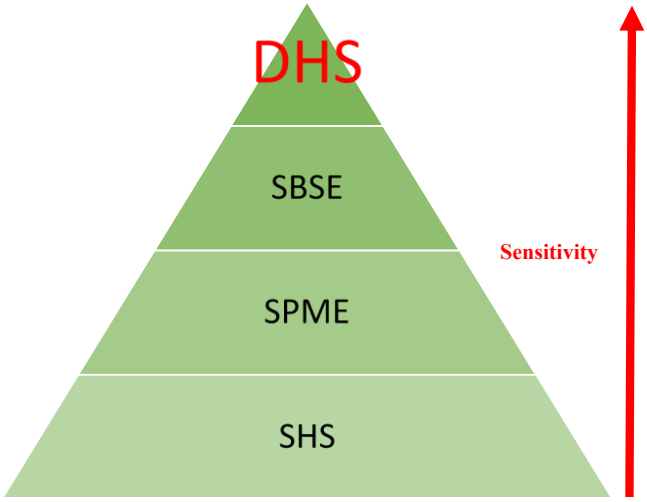

Figure 4.2. Graphical comparison of the preconcentration factor achievable by the different VOC extraction techniques.

Studies based on the determination of VOCs in vegetable matrices has been traditionally carried out by targeted analyses (Beltran et al., 2006; Fredes et al., 2016; Kalogiouri, Aalizadeh, & Thomaidis, 2017), making use of expensive standards and with limited information, as the chemical information acquired is selected *a priori*.

The last advances in analytical instrumentation together with the growth of massive data treatment technologies has permitted to explore the advantages provided by the use of untargeted methodologies. In this kind of analyses, all chemical information is acquired and registered, which allows to reprocess data obtained, “unlimitedly” looking for new compounds. In this context, metabolomics, which is defined as "the unbiased, global screening approach to classify samples based on metabolite patterns or fingerprints that change in response to disease, environmental or genetic perturbations with the ultimate goal to identify discriminating metabolites" (Cevallos-Cevallos, Reyes-De-Corcuera, Etxeberria, Danyluk, & Rodrick, 2009), can be an interesting approach to solve complex classification problems. When looking at the full picture of a sample with hundreds of VOCs (Angerosa et al., 2004; Stilo et al., 2019) the use of MS and, more specifically, HRMS can be of a great help, due to their accurate mass measurements and extraordinary sensitivity and identification capabilities, providing good results for the identification of non-target compounds. Furthermore, the use of APCI, which allows to obtain high intensity peaks for the molecular ion ($M^{+\cdot}$) and/or the protonated molecule ($[M+H]^+$) (Portolés, Sancho, Hernández, Newton, & Hancock, 2010) can be really convenient for the identification step. In some cases the molecular ion is absent in EI due to its high fragmentation,

and also in terms of sensitivity. Additionally, the lower fragmentation produced by APCI has the benefit of simplifying the peak peaking process in metabolomics analysis, as there are less ions tentative of being markers.

In complex matrices, the observation of sample chromatograms does not give easily significant information about differences between samples, with hundreds of peaks in several samples to be compared. Specialized software is required to extract chromatographic peaks and their corresponding instrumental response from raw data in a non-directed approach. For this purpose, different software solutions can be used to get the information needed from the chromatograms, such as XCMS package of R (Díaz, Pozo, Sancho, & Hernández, 2014), MetAlign (Tikunov et al., 2005), MzMine 2.0 (Kind, Tolstikov, Fiehn, & Weiss, 2007) or Paradise (Johnsen, Skou, Khakimov, & Bro, 2017).

In this chapter the use GC-MS and has been explored, together with different state-of-the-art data treatment methodologies, looking to obtain objective, fast and robust analytical approaches, to complement PANEL TEST analyses for the determination of quality in Spanish olive oils.

In a first approach, a methodology based on GC-APCI-QTOF MS, making use of the novel APCI source, fully described in **Introduction** and **Chapter 3** was tested. The capabilities of APCI, which produces highly abundant $M^{+\cdot}$

and $[M+H]^+$ ions, together with the power of the HRMS provided by the use of a QTOF, were explored to determine the quality of 425 olive oils using purge and trap as sampling technique for VOCs. The findings of this work are presented in the **scientific article 5**. In a second work, following the advances reached in the first article, a method for the determination of quality of olive oils together with the compounds responsible of defects and good flavour was developed. In this work, sample treatment was enhanced by using dynamic headspace entrainment with thermal desorption, which allowed to achieve a preconcentration factor for VOCs around 1000 times higher than with Purge and Trap followed by solvent desorption. The desorbed VOCs were analysed with a regular GC-MS (single quadrupole), which reduced significantly the analysis cost. Finally, the application of a novel state-of-the-art deconvolution software (PARADISe) based on PARAFAC2 allowed a significant enhancement in terms of accuracy of the classification models and permitted to identify the main components responsible of the quality in 108 Spanish olive oils. This work is summarized in **scientific article 6.**

4.2 Scientific Article 5

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Quality classification of Spanish olive oils by untargeted gas chromatography coupled to hybrid quadrupole-time of flight mass spectrometry with atmospheric pressure chemical ionization and metabolomics-based statistical approach

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ABSTRACT

The novel atmospheric pressure chemical ionization (APCI) source has been used in combination with gas chromatography (GC) coupled to hybrid quadrupole time-of-flight (QTOF) mass spectrometry (MS) for determination of volatile components of olive oil, enhancing its potential for classification of olive oil samples according to their quality using a metabolomics-based approach. The full-spectrum acquisition has allowed the detection of volatile organic compounds (VOCs) in olive oil samples, including Extra Virgin, Virgin and Lampante qualities. A dynamic headspace extraction with cartridge solvent elution was applied. The metabolomics strategy consisted of three different steps: a full mass spectral alignment of GC–MS data using MzMine 2.0, a multivariate analysis using Ez-Info and the creation of the statistical model with combinations of responses for molecular fragments. The model was finally validated using blind samples, obtaining an accuracy in oil classification of 70%, taking the official established method, "PANEL TEST", as reference.

1. Introduction

The determination of olive oil quality is typically performed by testers who establish if an olive oil must be labelled as Extra Virgin, Virgin or Lampante (not recommended for consumption) (Council, 2016). This strategy is known as "PANEL TEST", which classifies the oils according to two main properties: defects and positive attributes. The classification requirements are established by the International Council of Olive Oil (Council, 2016). Defects can be fusty, mouldy, sour and woody, and positive attributes can be fruity (green), bitter and spicy. According to the literature (Kalua et al., 2007; Luna, Morales, & Aparicio, 2006), the organic compounds responsible for olive oil flavour are typically esters, ketones, aldehydes, alcohols, terpenes, phenols and their derivatives, in concentrations ranging from a few ng L^{-1} to hundreds of mg L^{-1} and with different odour thresholds. PANEL TEST methodology could be considered slightly subjective, as the opinion of testers may vary. This may lead to misclassification of an oil, causing considerable economical losses, commercial problems and fraud. A more objective alternative could be based on the use of chromatographic techniques coupled to mass spectrometry (MS), which could allow the determination of chemical composition of the volatile fraction of olive oil samples, even at really low concentration levels. In this sense, the most by adequate analysis technique is gas chromatography coupled to mass spectrometry (GC–MS) (Angerosa et al., 2004; Flath, Forrey, & Guadagni, 1973).

According to the volatile characteristics of the compounds of interest, a specific extraction technique is also an important issue in order to perform a suitable separation from the matrix. From several studies on determination of VOCs in different matrices (Barco-Bonilla et al., 2011; Jiménez, Aguilera, Beltrán, & Uceda, 2006; Lam & Proctor, 2003; Salemi, Lacorte, Bagheri, & Barceló, 2006; Serrano, Beltrán, & Hernández, 2009), there is evidence for the extended use of trapping processes of the compounds in some kind of sorbent, either by forcing them to pass through the sorbent bed (P&T) (Barco-Bonilla et al., 2011; Salemi et al., 2006) or letting them to establish an equilibrium between the vapour phase and the adsorbent in a closed place (SPME) (Pouliarekou et al., 2011; Serrano et al., 2009). Other techniques also used for VOCs extraction include: direct headspace (HS) injection (Hu et al., 2014), stir bar sorptive extraction (SBSE) (Bicchi, Iori, Rubiolo, & Sandra, 2002), or liquid phase micro extraction (LPME) (Lee, Lee, Rasmussen, & Pedersen-Bjergaard, 2008).

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Quality classification of Spanish olive oils by untargeted gas chromatography coupled to hybrid quadrupole-time of flight mass spectrometry with atmospheric pressure chemical ionization and metabolomics-based statistical approach

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4.2.1 Abstract

The novel atmospheric pressure chemical ionization (APCI) source has been used in combination with gas chromatography (GC) coupled to hybrid quadrupole time-of-flight (QTOF) mass spectrometry (MS) for determination of volatile components of olive oil, enhancing its potential for classification of olive oil samples according to their quality using a metabolomics-based approach. The full-spectrum acquisition has allowed the detection of volatile organic compounds (VOCs) in olive oil samples, including Extra Virgin, Virgin and Lampante qualities. A dynamic headspace extraction with cartridge solvent elution was applied. The metabolomics strategy consisted of three different steps: a full mass spectral alignment of GC–MS data using MzMine 2.0, a multivariate analysis using Ez-Info and the creation of the statistical model with combinations of responses for molecular fragments. The model was finally validated using blind samples, obtaining an accuracy in oil classification of 70%, taking the official established method, “PANEL TEST”, as reference.

4.2.2 Keywords

Virgin olive oil, Atmospheric pressure chemical ionization, Gas chromatography, Mass spectrometry, Metabolomics,Purge and trap

4.2.3 Introduction

The determination of olive oil quality is typically performed by testers who establish if an olive oil must be labelled as Extra Virgin, Virgin or Lampante

(not recommended for consumption) (Council, 2016). This strategy is known as "PANEL TEST", which classifies the oils according to two main properties: defects and positive attributes. The classification requirements are established by the International Council of Olive Oil (Council, 2016). Defects can be fusty, mouldy, sour and woody, and positive attributes can be fruity (green), bitter and spicy. According to the literature (Kalua et al., 2007, Luna et al., 2006), the organic compounds responsible for olive oil flavour are typically esters, ketones, aldehydes, alcohols, terpenes, phenols and their derivatives, in concentrations ranging from a few ng L^{-1} to hundreds of mg L^{-1} and with different odour thresholds. PANEL TEST methodology could be considered slightly subjective, as the opinion of testers may vary. This may lead to misclassification of an oil, causing considerable economical losses, commercial problems and fraud. A more objective alternative could be based on the use of chromatographic techniques coupled to mass spectrometry (MS), which could allow the determination of chemical composition of the volatile fraction of olive oil samples, even at really low concentration levels. In this sense, the most by adequate analysis technique is gas chromatography coupled to mass spectrometry (GC–MS) (Angerosa et al., 2004, Flath et al., 1973).

According to the volatile characteristics of the compounds of interest, a specific extraction technique is also an important issue in order to perform a suitable separation from the matrix. From several studies on determination of VOCs in different matrices (Barco-Bonilla et al., 2011, Jiménez et al., 2006, Lam et al., 2003, Salemi et al., 2006, Serrano et al., 2009), there is evidence for the extended use of trapping processes of the compounds in some kind of sorbent, either by forcing them to pass through the sorbent bed (P&T) (Barco-Bonilla et al., 2011, Salemi et al., 2006) or letting them to establish an equilibrium between the vapour phase and the adsorbent in a closed place (SPME) (Pouliarekou et al., 2011, Serrano et al., 2009). Other techniques also used for VOCs extraction include: direct headspace (HS) injection (Hu et al., 2014), stir bar sorptive extraction (SBSE) (Bicchi, Iori, Rubiolo, & Sandra, 2002), or liquid phase micro extraction (LPME) (Lee, Lee, Rasmussen, & Pedersen-Bjergaard, 2008).

The determination of the chemical fingerprint of food samples is an interesting well-known approach for characterization of food products, for example in oil (Reboredo-Rodríguez et al., 2016, Reboredo-Rodríguez et al., 2012) or tomato samples (Vaz-Freire, Da Silva, & Freitas, 2009). Chemical fingerprint, normally obtained using targeted analyses, can be

further used to classify unknown samples, but due to the fact that a limited number of compounds must be selected *a priori*, some information regarding the samples is lost. In this context, metabolomics, which is defined as "the unbiased, global screening approach to classify samples based on metabolite patterns or fingerprints that change in response to disease, environmental or genetic perturbations with the ultimate goal to identify discriminating metabolites" (Cevallos-Cevallos, Reyes-De-Corcuera, Etxeberria, Danyluk, & Rodrick, 2009), can be an interesting approach to solve complex classification problems. One of the main drawbacks of metabolomics occurs when the relations or discrepancies between samples are just determined by a compound or group of compounds which are present at very low levels in complex matrices and can be easily dismissed. High resolution (HR) MS systems, such as time of flight (TOF) (Kind, Tolstikov, Fiehn, & Weiss, 2007) or magnetic sector (Kieken et al., 2009), enhance the detection of molecules in complex matrices at very low levels (Salihovic, Nilsson, Hagberg, & Lindström, 2013) due to their accurate mass measurements and extraordinary sensitivity, providing good results for the determination of non-target compounds. Furthermore, the use of atmospheric pressure chemical ionization (APCI), a very promising ionization source in GC, which is softer than the common electron ionization (EI), allowing the acquisition of high intensity peaks for the molecular ion ($M^{+\bullet}$) and/or the protonated molecule ($[M+H]^+$), depending on the nature of the compounds (Portolés, Sancho, Hernández, Newton, & Hancock, 2010). This information is really convenient as in some cases the molecular peak is absent in EI, due to its high fragmentation and in terms of sensitivity, the peak intensity can be reduced. GC coupled to hybrid quadrupole time of flight (QTOF) MS equipped with APCI allows the acquisition of accurate-mass full scan spectra at low and at high collision energy (MS^E mode) being a very useful tool for elucidation purposes.

Data processing, together with data acquisition, are the mainstays of metabolomics. The direct observation of sample chromatograms does not give significant information about the difference between sample quality, and thus specialized software is required to obtain chromatographic peaks and masses from raw data. In literature, metabolomics studies use different software to get the information needed from the chromatograms, such as XCMS package of R (Díaz, Pozo, Sancho, & Hernández, 2014), MetAlign (Tikunov et al., 2005) or MzMine 2.0 (Kind et al., 2007).

The aim of this work has been the development of a GC-(APCI)QTOF MS methodology to obtain the chemical profile/fingerprint of olive oil volatile compounds, in order to establish differences between virgin olive oil qualities using P&T extraction and through the use of metabolomics techniques, in order to give a more objective decision in their classification, compared with that provided by the "PANEL TEST".

4.2.4 Materials and methods

Chemicals and reagents

Internal standard triphenyl phosphate (TPP) $\geqslant$ 99% was purchased from Sigma Aldrich (Germany). Diethyl ether (residue analysis quality GC) and hexane (trace analysis quality (AT) GC) were provided by Scharlau (Barcelona, Spain).

Supelclean ENVI-Carb® SPE tubes 500 mg, volume 6 mL, 120–400 mesh, surface area 100 $m^2\ g^{-1}$, used as traps, were purchased from Supelco (Barcelona, Spain).

Olive oil samples

A total of 425 olive oil samples was provided by the "Interprofesional del Aceite de Oliva Español" Organization (INTERPRO, Spain), the "Agencia para el Aceite de oliva del Ministerio de Agricultura, Alimentación y Medio Ambiente" and the official control services from the "Consejería de Agricultura, Pesca y Desarrollo Rural de la Junta de Andalucía".

Oil samples were taken from different regions of Spain and included 300 quality characterized samples (120 Extra Virgin, 120 Virgin and 60 Lampante) and 125 blind samples (the quality was unknown during analysis). Samples were stored at −22 °C until use. Samples were characterized by means of pH measurements and physicochemical and organoleptic properties by the official participating laboratories (Laboratorio Arbitral Agroalimentario del MAGRAMA, Laboratorios Agroalimentarios de Córdoba y Atarfe de la Junta de Andalucía) and their corresponding certified "PANEL TESTs".

Sample treatment

Olive oil samples were allowed to defrost at room temperature before analysis. Then, they were aliquoted in 4 different 10-mL vials. One aliquot was used to perform the extraction and the remaining ones were stored at 4 °C.

Olive oil (5 g) was weighed into a 150-mL flask before inserting a magnetic stirrer. The flask was rapidly closed with a glass tap with a nitrogen entrance and the exit connected to the sorbent trap (Envi-Carb cartridge). The cartridge was conditioned with 2 × 5 mL of a mixture of hexane:diethyl ether (50/50; *v/v*) and vacuum dried for 10 min. Sample extraction was then carried out for 60 min at 40 °C with a nitrogen flow of 1 L min^{-1} and with stirring at 300 rpm. After extraction, Envi-carb® cartridges were eluted by gravity with 5 mL of the hexane:diethyl ether mixture (50/50 *v/v*), into a glass tube previously weighed. A 50-µL aliquot of TPP solution at 5 mg L^{-1} (in hexane) was added as internal standard. The entire extract was then concentrated under vacuum conditions with a MiVac Duo Concentrator (Genevac, Italy), until total removal of diethyl ether, i.e. until a final volume of approximately 0.5 mL. Finally, in order to adjust the final volume of the extract (0.5 mL of hexane), several drops of hexane were added until adjusted mass (0.3274 g). An aliquot of 100 µL of this extract was transferred to a 20-mL vial in order to generate a pool of extracts to prepare the quality control (QC) sample. The remaining extract was divided into two different vials with 200 µL inserts, sealed and stored in freezer at −20 °C until their analysis by GC-QTOFMS. In each extraction batch, 2 Extra Virgin, 2 Virgin and 2 Lampante oil samples were processed simultaneously.

GC–(APCI)QTOFMS

The chromatographic analyses were performed using an Agilent 7890A gas chromatograph, equipped with an Agilent 7693 autosampler, coupled to a quadrupole/time-of-flight mass spectrometer, Xevo G2 QTOFMS (Waters Corporation, Manchester, UK), with APCI source. The GC separation was performed using a fused silica HP-5MS capillary column with (30 m × 0.25 mm ID; film thickness 0.25 µm (J&W Scientific, Folsom, CA). The oven program was set as follows: 40 °C (3 min); 5 °C/min to 160 °C (1 min); 50 °C/min to 300 °C (2.2 min); total runtime 33 min. Injections of 1 µL of sample extracts were performed using pulsed splitless mode

(50 psi) at a temperature of 270 °C with a pulse time of 0.90 min. Helium (99.999%; Praxair, Valencia, Spain) was used as the carrier gas at a constant flow rate of 3 mL min^{-1}.

The interface temperature was set to 310 °C using N_2 as auxiliary gas at a flow rate of 150 L h^{-1}, the make-up gas flow rate was set at 300 mL min^{-1} and the cone gas flow rate at 16 L h^{-1}. The APCI corona pin was operated at 1.7 µA with a cone voltage of 20 V. The ionization process occurred within an enclosed ion volume, which enabled control over the protonation/charge transfer processes. Water placed in an uncapped vial, which was located within a special designed holder placed in the source door, was used as modifier. Xevo QTOFMS was operated with a scan time of 0.4 s, acquiring over the mass range *m/z* 50–650. TOFMS resolution was approximately 18,000 (FWHM) at *m/z* 614. For MS^E measurements, two alternating acquisition functions were used applying different collision energies; 4 eV for low energy function (LE), and a collision energy ramp (10–40 eV) for high energy function (HE). Heptacose (PFTBA) was used for the daily mass calibration. Mass accuracy calibration was continuously performed using a background ion coming from the GC-column bleed as lock mass (protonated molecule of octamethylcyclotetrasiloxane, *m/z* 297.0830).

In order to avoid bias in the methodology, samples were analyzed in batches of 60 (20 pertaining to each class, Extra Virgin, Virgin and Lampante) randomly distributed. In addition, in each analysis batch, 10 QCs were injected along the sequence (one QC every 10 samples and 2 QCs at the beginning and at the end of the sequence).

Data processing

GC–QTOF MS data were converted from ".raw" to ".cdf" format using Databridge software provided by MassLynx v4.1 (Waters Corporation). Data mining was carried out using MzMine 2.0 software with the conditions indicated in **Fig. 1.** The first part of the procedure (including cropping, mass detection, building chromatograms and baseline cut-off) led to a data matrix of areas corresponding to the markers detected in the form of combination of *m/z* value and retention time *(m(m/z in Da))t(time in* sec*)*). This matrix was then filtered to remove too low intensity peaks (could be considered noise) and unpaired markers (which were only present in a low proportion: less than 20% of samples). The last step consisted in a "forced integration" of the considered markers in all

samples, in order to eliminate the presence of zeroes in the final data matrix. The forced-integrated area was established as 1% of the background absolute signal at the considered RT, as this was the normalization step which led to best results. Finally a filter was applied to eliminate duplicated signals that correspond to the same marker.

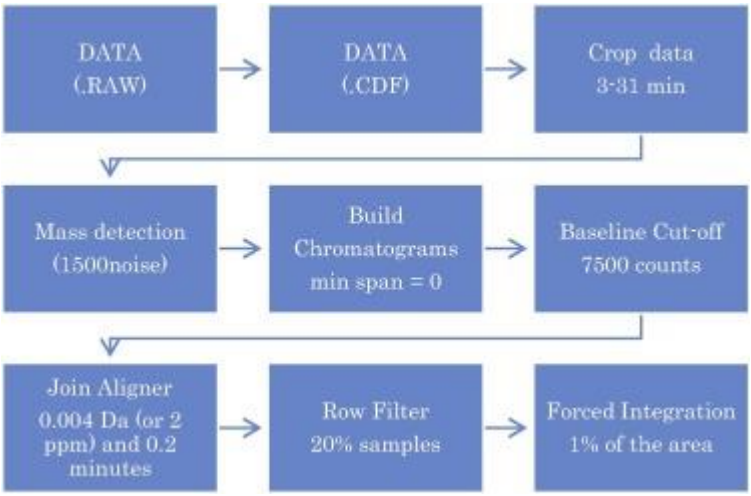

Fig. 1. Workflow followed for chromatographic data treatment using MzMine 2.0.

The data matrix obtained after applying MzMine 2.0 was exported to a text file (.csv). Using Microsoft Excel absolute areas were converted to relative areas using the area of one of the TPP *m*/*z* ions (*m*/*z* 327), which was quantified in each sample using TargetLynx® software. The relative areas were corrected with the nearest QC and then pareto scaled to minimize the impact of the peaks with higher standard deviation. In order to carry out the statistical analysis, EzInfo software (U-Metrics) was applied as it is easy to use and, from our previous experience, it provides valid results (Díaz et al., 2014).

4.2.5 Results and discussion

GC–(APCI)QTOFMS

Chromatographic analysis of sample extracts was carried out by GC–(APCI)QTOFMS working in MS^E mode, in order to get both maximum

sensitivity and full information. From previous experiences in our laboratory (Cervera, Portolés, López, Beltrán, & Hernández, 2014) it was expected that the soft ionization provided by the APCI source combined with low energy acquisition function, would reveal the molecular ion or the protonated molecule ($M^{+\cdot}$ or $[M+H]^+$), as only low fragmentation occurs. Additionally, the information acquired at high energy function (collision energy ramp of 10–40 eV) provided a fragmentation pattern, which was very useful for elucidation purposes. As an example, Fig. S1 shows the accurate-mass full spectra obtained for a sesquiterpene, α-farnesene, acquired by GC–APCI-QTOFMS (at low and high energy) comparing them with its corresponding EI (70 eV) spectrum from a commercial library (NIST). The information about the protonated molecule in the low energy function together with the fragmentation obtained in the high energy one was of great help in the elucidation process, which followed the steps detailed in a previous work (Portolés, Pitarch, López, Hernández, & Niessen, 2011). All instrumental parameters, selected according to previous screening (Cervera et al., 2014, Portolés et al., 2014), were found to be reproducible and robust, basic requirements of metabolomics. An example of the instrument performance is shown in **Fig. S2**, a total ion chromatogram obtained for the injection of a QC using the detailed parameters.

Extraction procedure optimization

Taking into account the properties of the potential analytes, especially their volatility and considering the advantages of obtaining a final liquid extract (that can be injected several times, even in different chromatographic instruments) purge and trap (P&T) with solvent trap elution was selected as the extraction technique. This approach requires low economical investment and provides high reproducibility with the advantage of keeping a second extract vial for further analysis. In previous work a similar P&T method with subsequent GC–MS analysis was developed in our research group for the determination of volatiles in tomato and melon samples (Beltran et al., 2006, Fredes et al., 2016), and it has been used as a starting point for method development, although some changes were considered to improve its performance in application to olive oil samples.

Efficiency of two different sorbent materials was tested in the P&T step: Tenax TA® SPE and non-porous carbon Envi-Carb cartridges. For this purpose, aliquots of an extra virgin olive oil sample were extracted using

Tenax (*n* = 3) and Envi-Carb (*n* = 3) with the same extraction conditions. The comparison of peak areas of some selected analytes in these chromatograms showed that both cartridges provided almost the same performance, as seen previously in melon samples (Fredes et al., 2016). Although Tenax can be a bit more efficient for some compounds (20% more area) both sorbents extracted quantitatively the majority of the volatiles from the sample. Moreover, compared to Tenax, Envi-Carb cartridges are commercially available (while Tenax cartridges are produced on demand, with longer supply times) and have an analysis cost of almost 3 times lower. Finally, Envi-Carb was selected for further development.

Metabolomics optimization

After optimization of extraction and chromatographic procedures, 425 oil samples (including 300 samples for model generation and 125 samples for model validation) were analyzed in several batches, both for extraction (6 samples for each extraction batch) and chromatographic analysis (including samples, 10 QC and 10 blank samples in each sequence). Firstly considering a completely unbiased analysis, a principal component analysis (PCA) was applied over the training data matrix corresponding to 300 olive oil samples. Fig. 2a illustrates the PCA obtained.

This PCA analysis seemed to be fairly promising, as at first sight a rough distinction between Extra Virgin and Lampante can be seen, although Virgin samples appeared in the middle and overlapped the two other groups. Additionally, this step checks the correct normalization of the samples by analyzing the behavior of the QC samples, which have to be clustered in the centre of the score plot. For a better visualization of the 300 samples, the QCs have been removed from the PCA plot.

The next step consisted in performing a partial least squares-discriminant analysis (PLS-DA), which considers additional information about groups during classification. It is a biased PCA which provides a better separation between groups based on some parameters. Thus, a PLS-DA was applied to construct a statistical model to find differences in volatile compounds composition between samples (see **Fig. 2b**). As it can be seen, the PLS-DA analysis separated the three groups of samples slightly better, though some group overlapping was still present.

Finally, in order to get information relative to the relevant compounds allowing sample classification, an orthogonal partial least squares-discriminant analysis (OPLS-DA) was applied to highlight the differences between groups comparing two different groups at a time, regarding different compound concentrations in samples. For this purpose, S-Plot graphics for all two-classes comparisons, Virgin *vs* Extra, Extra *vs* Lampante and Virgin *vs* Lampante, were obtained (**Fig. 3**) and studied looking for endpoints. Theoretically, in an S-Plot the points with a p-corr value between 0.9 and 1 are closely related to the compounds characterizing the samples of one group, which constitute important class markers. Thus the most significant ions, i.e. the further from the centre in the S-Plot and with a p-corr close to 1, were selected as tentative markers. According to this, a total of 15 markers (corresponding to combination of an *m*/*z* ion and retention time) was selected and the corresponding chromatographic peaks, processed with TargetLynx® for a better precision of peak areas. **Table 1** shows the data for the 15 markers, together with the assigned elemental compositions for the protonated molecule and for the main fragment ions. In most cases they are labelled as *markers*, as the high number of possible compounds with the same fragments made it unfeasible to unequivocally identify them. We have been able to completely identify some of the markers even by confirmation through injection of reference standards.

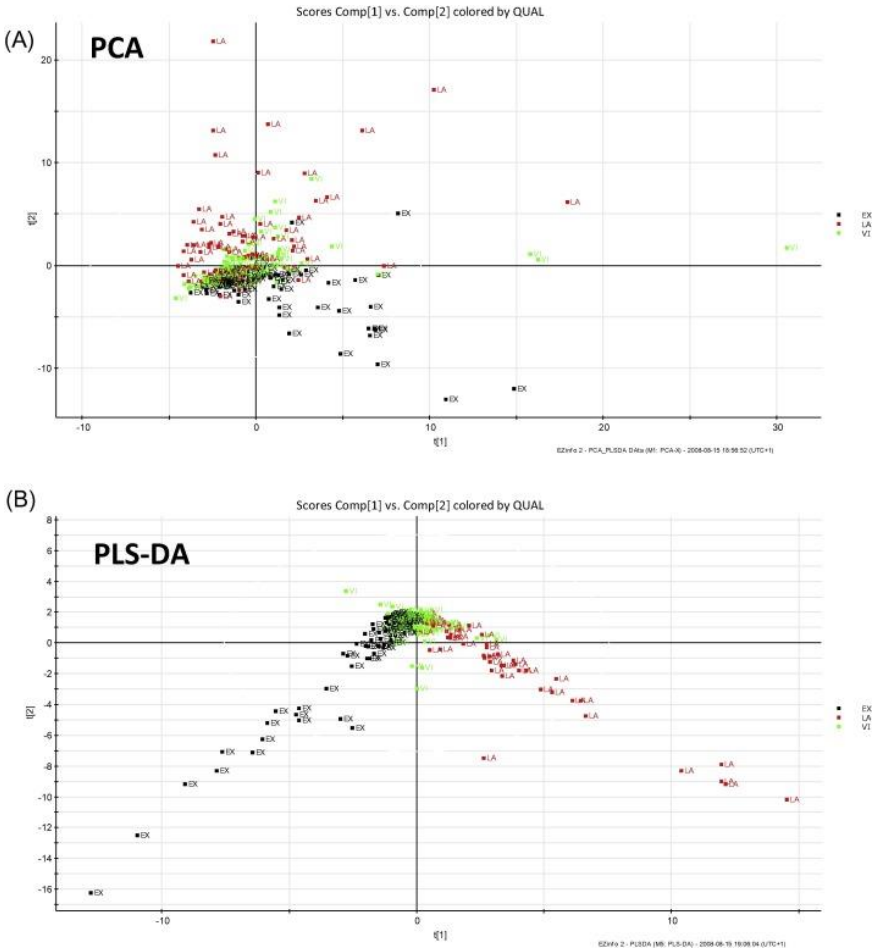

Fig. 2. PCA (a) and PLS-DA (b) figures corresponding to the samples analyzed to create the model.

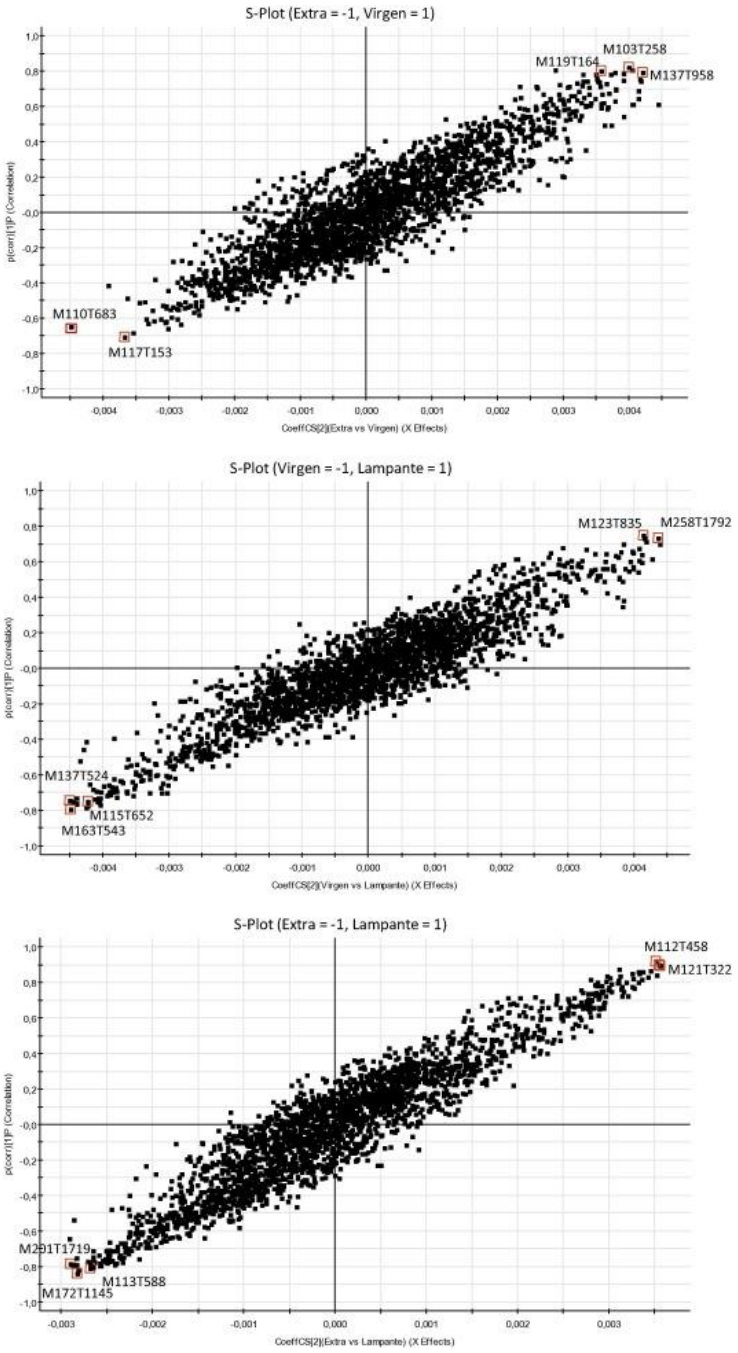

Fig. 3. SPLOTs Extra Virgin *vs* Virgin, Virgin *vs* Lampante and Extra Virgin *vs* Lampante. Tentative markers are highlighted with red squares and designated by their corresponding M(*m*/*z*)T(Rt).

Table 1. GC–QTOFMS accurate mass measurements for selected compounds for olive oil classification.

Compounds	Elemental composition $[M+H]^+$	*m/z*	mass error (mDa)[a]	fragment ion 1 (loss)	*m/z*	mass error (mDa)	fragment ion 2 (loss)	*m/z*	mass error (mDa)	fragment ion 3 (loss)	*m/z*	mass error (mDa)
Methyl-2-methyl butyrate	$C_6H_{13}O_2$	117.0916	0	C_5H_9O $M+H-CH_4O$	85.0653	-0.2	-	-	-	-	-	-
Diethyl carbonate	$C_5H_{11}O_3$	119.0708	0.1	$C_3H_7O_3$ $M+H-C_2H_4$	91.0395	-0.1	CH_3O_3	63.0082	-0.1	-	-	-
Ethyl-2-methyl butyrate	$C_7H_{15}O_2$	131.1072	-0.5	$C_5H_{11}O_2$ $[M+H-C_2H_4]^+$	103.0759	-0.4	C_5H_9O $[M+H-C_2H_4-H_2O]^+$	85.0653	-0.2	-	-	-
Marker 4	C_8H_9O	121.0649	0	C_8H_9 $[M+H-H_2O]^+$	105.0704	-0.2	C_7H_7 $[M+H-H_2O-CH_2]^+$	91.0548	-0.2	C_6H_5 $[M+H-H_2O-C_2H_4]^+$	77.039	-0.2
Marker 5	$C_6H_9O_2$	113.0603	0	C_5H_9O $[M+H-CO]^+$	85.0653	-0.1	-	-	-	-	-	-
Marker 6	$C_{10}H_{17}O$	153.1279	0.1	$C_{10}H_{17}$	137.133	-0.2	-	-	-	-	-	-
Marker 7	$C_{12}H_{21}$	165.1643	-0.3	C_8H_{11}	83.0861	0.4	-	-	-	-	-	-

Table 1. GC–QTOFMS accurate mass measurements for selected compounds for olive oil classification.

Compounds	Elemental composition $[M+H]^+$	*m/z*	mass error (mDa)[a]	fragment ion 1	*m/z*	mass error (mDa)	fragment ion 2	*m/z*	mass error (mDa)	fragment ion 3	*m/z*	mass error (mDa)
Marker 8	$C_6H_9O_2$	113.0603	-0.3	C_6H_7O $[M+H-H_2O]^+$	95.0497	0.4	C_5H_7 $[M+H-CO]^+$	67.0548	0	-	-	-
Marker 9	$C_6H_{11}O_2$	115.0759	-0.2	C_6H_9O $[M+H-H_2O]^+$	97.0653	-0.2	C_6H_7 $[M+H-(H_2O)_2]^+$	79.0548	-0.2	C_5H_9 $[M+H-H_2O-CO]^+$	69.0704	0
Guaiacol	$C_7H_9O_2$	125.0603	-0.2	$C_6H_6O_2$ $[M+H-CH_3]^+$	110.0368	0.3	C_6H_5O $[M+H-CH_3-H_2O]^+$	93.034	0.1	-	-	-
Marker 11	$C_8H_{11}O$	123.081	-0.4	C_7H_7O $[M+H-CH_3]^+$	107.0497	0.1	C_6H_7O $[M+H-C_2H_3]^+$	95.0497	-0.3	C_6H_5 $[M+H-C_2H_3-H_2O]^+$	77.0391	0.1
Marker 12	$C_9H_{15}O_2$	155.1072	-0.5	$C_9H_{13}O$ $[M+H-H_2O]^+$	137.0966	-1.6	C_8H_{13} $[M+H-H_2O-CO]^+$	109.1017	0	-	-	-
Marker 13	$C_{10}H_{22}NO$	172.1701	-0.5	$C_9H_{18}NO$ $[M^+-CH_3]^+$	156.1388	-0.2	$C_8H_{20}N$ $[M^+-CH_3-CH_2O]^+$	130.1596	0.1	$C_6H_{14}NO$ $[M^+-C_4H_7]^+$	116.1075	-0.1
Marker 14	$C_{12}H_{26}NO$	200.2014	0.1	$C_5H_{11}NO$ $[M-C_7H_{13}]^+$	102.092	-0.2	C_4H_9NO $[M-C_8H_{15}]^+$	88.0762	0.4	C_3H_7NO $[M-C_9H_{17}]^+$	74.0607	0.2
Marker 15	$C_{16}H_{33}O_2$	257.2481	-0.2	-	-	-	-	-	-	-	-	-

a Calculations of mass errors have been performed from the sample with the highest amount of the compound.

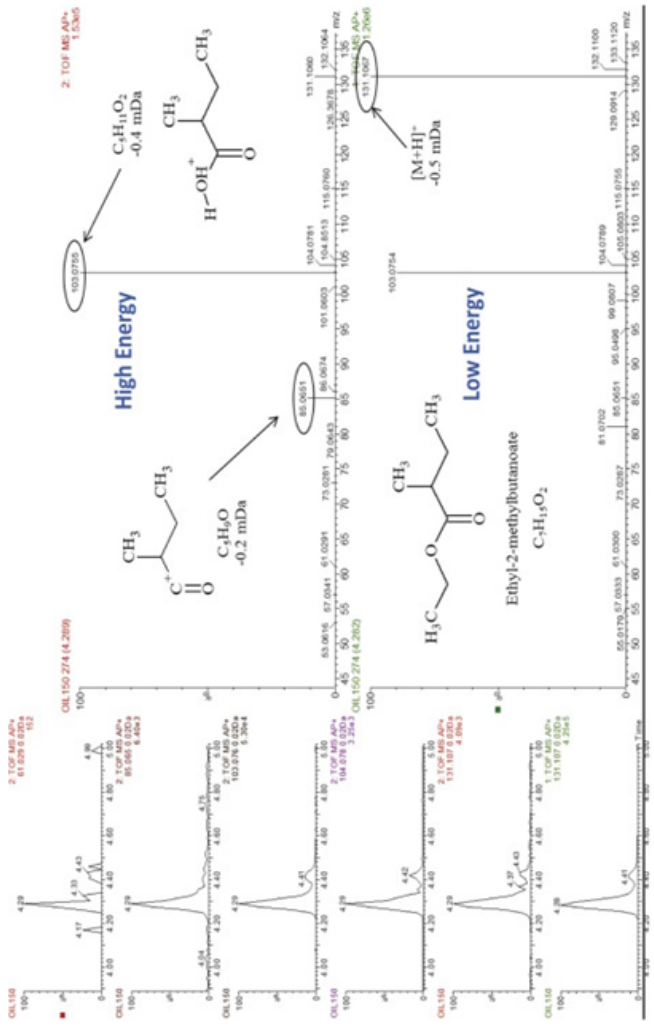

Fig. 4. GC–(APCI) QTOFMS narrow-window extracted ion chromatogram (mass window 0.02 Da) showing the detection of a possible marker, tentatively identified as ethyl 2-methylbutanoate – Experimental APCI accurate-mass spectra for the high collision energy function and for the low collision energy function are also shown with the chemical structures proposed for the most abundant fragment ion together with the experimental mass errors (in mDa).

An example of the elucidation process of a marker can be found in **Fig. 4**, with the experimental APCI accurate-mass spectra obtained by GC–QTOFMS for the high and low collision energy functions and with the possible chemical structures proposed for the most abundant fragment ions together with the experimental mass errors (in mDa). The structure was assigned to ethyl-2-methylbutanoate and was then confirmed by the injection of its standard. This compound has been already identified by some authors in olive oil samples (Dierkes et al., 2012, Kiralan et al., 2012) and has been reported as related to fruity, sweet and green apple flavour.

Finally, considering the 15 markers selected, a series of linear combinations of these ions were studied, in order to achieve the best separation possible between groups, and a model for the classification of the three groups was built. This model was based in a combined double classification, first differentiating between Extra Virgin/Virgin oils and Virgin/Lampante oils and then distinguishing among the three different classes.

As an efficiency pre-test, the samples used to build the model were classified using it, comparing the model classification results with the classes assigned by the corresponding "PANEL TEST", which was considered as reference. After the first classification, which distinguishes between only two classes, Extra Virgin and Lampante oils, nearly 90% of oils were correctly classified, considering that Virgin oils can be present in both groups. Then, a second classification was applied to both groups, allowing a final distinction between the three different classes. After the application of these series of binary classifications, around 70% of the samples were correctly classified as Extra Virgin, Virgin or Lampante. In all cases, the worst classification efficiency corresponded to Virgin samples, which are always the most difficult to assign.

In order to take the maximum advantage possible from the samples, PLS-DA and OPLS-DA analysis considering the defects perceived by the PANEL TEST was performed. As can be seen in **Fig. S3a**, the samples cannot be easily separated by defects with a PLS-DA, as the overlapping of samples with different defects is quite important. From **Fig. S3b**, obtained comparing samples with each kind of defect with the flawless Extra Virgin samples in several OPLS-DA analyses, a clearer distinction is observed, which points out the fact that, from an analytical point of view, the Extra Virgin samples are easier to distinguish from the rest due to the absence of defects rather than the presence of positive attributes. This preliminary

approach to the study of the defects shows potential for the refining of the classification methodology, and will be further studied in depth with an appropriate design to maximize the outputs of the research, but will not be analyzed in more detail here.

Model validation

Once the model was created with the 300 training samples, it was validated using the 125 blind samples, to evaluate its efficiency. Results obtained for the ternary classification of the blind samples were sent to the project coordinating centre, in order to evaluate the accuracy and the performance of the model. As seen in **Fig. 5**, Extra Virgin samples were always correctly classified as Extra Virgin or, in some few cases, as Virgin, but never as Lampante, which would lead them to be discarded with a high economic loss. In addition, there was a low proportion of virgin oils which got classified as Lampante and a small quantity of Lampante samples which were overrated. It is important to highlight that the method is able to distinguish the samples which have clear defects and poor fruity properties, as the match in the first binary classification is around 90%. It is also remarkable that almost none of the Lampante samples (only a residual 5%) are overclassified as Extra Virgin samples. This can be very useful in discarding clear Lampante samples and reducing the amount of samples which have to be tested by the test panel, being an important complement to it. The distinction between Lampantes and Virgins, as they usually share defects, has shown to be the most difficult, with a considerable number of Lampantes classified as Virgin. This is also in agreement with the preliminary results of the PLSDA and OPLS-DAs, considering the defects found in the samples (**Fig. S3**). Future work will focus on refining the methodology from these two perspectives.

OBJECTIVE

	Extra	Virgin	Lampante
Extra	100	0	0
Virgin	0	100	0
Lampante	0	0	100

RESULTS

	Extra	Virgin	Lampante
Extra	80	20	0
Virgin	35	60	5
Lampante	5	45	50

	Extra	Non Extra
Extra	80	20
Virgin	35	65
Lampante	5	95

Fig. 5. Confusion matrix showing the comparison between the objective (up) of getting every blind sample classified as "PANEL TEST" classification and the results (down) after processing these unknown samples through the entire developed procedure.

4.2.6 Conclusions

A simple and efficient methodology has been developed for the analysis of volatile compounds in olive oil samples, with a prior extraction by P&T technique and subsequent analysis by GC–(APCI)QTOFMS.

The use of GC–(APCI)QTOFMS has allowed the use of MS^E mode, which provides information at low and high collision energies and has been proved to be an important added benefit in order to elucidate the markers. The developed method is sensitive enough and capable of obtaining a considerable number of resolved chromatographic peaks for volatile compounds. The use of the APCI source with water as modifier has favoured the formation of protonated molecules ($[M+H]^+$) which, added to the soft ion source fragmentation, enhanced the sensitivity for most of the compounds when compared to the traditional EI source.

The methodology was evaluated in terms of accuracy, obtaining an overall accuracy near to 70% on 125 blind samples, discarding only Lampante samples and a low percentage of Virgin samples, which makes it perfect as

a complementary analysis together with the "PANEL TEST" method. The preliminary results obtained for the distinction of samples according to their defects look promising, and will be taken as a starting point to enhance the classification of Lampante and Virgin samples in a future work, with a refined experimental design to maximize the difference between samples of different quality with the same defect. The further study of these data will also promote the determination and exhaustive elucidation of the compounds responsible for each type of defect in these samples.

The metabolomics-based approach allowed the classification of the samples with considerable performance and minimal data treatment after the method was built. Among the different software used, the combination of MzMine 2.0 and Ez-Info gave the best results. MzMine 2.0 allowed the inclusion of a large number of samples in order to build the model, and the addition of new samples to expand the population taken to create it. In the same way, Ez-Info provided reliable results with a really simple user interface and was preferred to software used in previous work.

In order to improve the classification method, a higher number of samples is required, to include every possible combination of defects and positive attributes. Considering the huge variety of positive and negative attributes that can give a specific classification to the samples, the overall accuracy of 70% achieved enables the developed methodology to be an important complement to the official PANEL TEST.

4.2.7 Conflict of interest

The authors declare no conflict of interest.

4.2.8 Acknowledgements

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4.3 Scientific Article 6

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Olive oil quality classification and measurement of its organoleptic attributes by untargeted GC–MS and multivariate statistical-based approach

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ABSTRACT

The capabilities of dynamic headspace entrainment followed by thermal desorption in combination with gas chromatography (GC) coupled to single quadrupole mass spectrometry (MS) have been tested for the determination of volatile components of olive oil. This technique has shown a great potential for olive oil quality classification by using an untargeted approach. The data processing strategy consisted of three different steps: component detection from GC-MS data using novel data treatment software PARADISe, a multivariate analysis using EZ-Info, and the creation of the statistical models. The great number of compounds determined enabled not only the development of a quality classification method as a complementary tool to the official established method "PANEL TEST" but also a correlation between these compounds and different types of defect. Classification method was finally validated using blind samples. An accuracy of 85% in oil classification was obtained, with 100% of extra virgin samples correctly classified.

1. Introduction

Olive oil quality is a matter of concern for consumers and producers, and the complete characterization of olive oil is an important aim where analytical chemists can be of great support. Apart from physicochemical parameters that can determine the quality (such as the acidity and turbidity of a sample), olive oil classification, as established by Spanish legislation (COI/T.20/Doc. No 15/Rev. 9 2017) and European Legislation (EEC No 2568/91), is performed by testers, who establish if an olive oil should be labelled as extra virgin, virgin or lampante (not recommended for consumption). This strategy is known as "PANEL TEST", which classifies the oils according to two main properties: defects (negative factors) and positive attributes (positive factors). The major sensory defects are *rancid, fusty/muddy sediment, musty/humid/earthy, acetone, burnt/heated, frozen/wet wood* and *winey/vinegary*, and the positive attributes can be fruity (specifying green attribute), bitter and spicy. An extra-virgin oil must have positive attributes and no defects, while the presence and amount of defects determines if an olive oil must be classified as virgin or lampante. According to the literature (Kalua et al., 2007; Luna, Morales, & Aparicio, 2006) and based on previous work (Sales et al., 2017), the organic compounds responsible for these flavour are predominantly volatiles, including esters, ketones, aldehydes, alcohols, terpenes, phenols and their derivatives, with different concentrations and odor thresholds. To this extent, qualitative and quantitative analysis of volatile organic compounds (VOCs) has been an important issue of scientific interest for the organoleptic characterization of olive oil. Although PANEL TESTs are quite well trained in distinguishing these many differences with an impressive precision, such methodology is rather expensive, and remarkably time-consuming. In this scenario, a more objective methodology, based on instrumental responses, could be presented as cheaper and faster alternative approach to PANEL TESTs and could also be useful as a complementary tool, to prevent fraud due to sample adulteration or mislabeling.

Dynamic headspace with sorbent trapping (DHS) together with gas chromatography (GC) coupled to mass spectrometry (MS) in full scan mode is a well-known technique that has been used in our laboratory for the determination of VOCs in food products (Beltran et al., 2006; Fredes et al., 2016), including olive oils (Sales et al., 2017), at low to trace levels, as it allows concentration of most of the volatile compounds present in the sample with a good efficiency and significantly low cost. When coupled to thermal desorption, results improve considerably. This volatile-focused extraction technique makes use of no solvents, which helps to cut analysis costs and time while enhancing the sensitivity due to its high pre-concentration factor. Additionally, Mass spectra obtained when applying this sampling technique have been demonstrated to be cleaner than those obtained by traditional sampling methods, as the lack of solvents reduces column bleed and overloading

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Olive oil quality classification and measurement of its organoleptic attributes by untargeted GC–MS and multivariate statistical-based approach

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4.3.1 Abstract

The capabilities of dynamic headspace entrainment followed by thermal desorption in combination with gas chromatography (GC) coupled to single quadrupole mass spectrometry (MS) have been tested for the determination of volatile components of olive oil. This technique has shown a great potential for olive oil quality classification by using an untargeted approach. The data processing strategy consisted of three different steps: component detection from GC–MS data using novel data treatment software PARADISe, a multivariate analysis using EZ-Info, and the creation of the statistical models. The great number of compounds determined enabled not only the development of a quality classification method as a complementary tool to the official established method "PANEL TEST" but also a correlation between these compounds and different types of defect. Classification method was finally validated using blind samples. An accuracy of 85% in oil classification was obtained, with 100% of extra virgin samples correctly classified.

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Dynamic headspace with sorbent trapping (DHS) together with gas chromatography (GC) coupled to mass spectrometry (MS) in full scan mode is a well-known technique that has been used in our laboratory for the determination of VOCs in food products (Beltran et al., 2006, Fredes et al., 2016), including olive oils (Sales et al., 2017), at low to trace levels, as it allows concentration of most of the volatile compounds present in the sample with a good efficiency and significantly low cost. When coupled to thermal desorption, results improve considerably. This volatile-focused extraction technique makes use of no solvents, which helps to cut analysis costs and time while enhancing the sensitivity due to its high pre-concentration factor. Additionally, Mass spectra obtained when applying this sampling technique have been demonstrated to be cleaner than those obtained by traditional sampling methods, as the lack of solvents reduces column bleed and overloading issues (Marquez, Serratosa, Merida, Zea, & Moyano, 2014).

Other automatable alternatives to this approach rely on headspace (HS) coupled to MS or GC–MS, with high detection limits and no pre-

concentration factor (Arrebola et al., 2005, Garrido-Delgado et al., 2011) or headspace-solid phase micro-extraction (HS-SPME) coupled to GC–MS, which has shown good performance in extraction of volatiles and has even been used for the determination of defect-related compounds in olive oils (Benelli et al., 2015, Dierkes et al., 2012, Zhu et al., 2016). Most studies carried out on oil characterization are based on a targeted approach, which can produce biased classification models that could lead to important misclassification of the samples if the compounds responsible for a specific type of defect have not been considered in advance. Alternatively to target analyses for the determination of the chemical fingerprint of food samples, in the last years and together with the advance of data treatment technology, novel non-targeted methodologies have started to gain importance. Despite its great potential, only a few applications are found in the olive oil analysis field (Gerhardt et al., 2017, Gil-Solsona et al., 2016, Sales et al., 2017).

Metabolomics, defined as an "unbiased, global screening approach to classify samples based on metabolite patterns or fingerprints that change in response to disease, environmental or genetic perturbations with the ultimate goal to identify discriminating metabolites" (Cevallos-Cevallos, Reyes-De-Corcuera, Etxeberria, Danyluk, & Rodrick, 2009), has already demonstrated great capabilities to solve this problem. Application of a non-targeted approach based on analytical techniques to determine chemical fingerprints in food leads to a new field known as foodomics (Herrero, García-Cañas, Simo, & Cifuentes, 2010). Data processing together with data acquisition has to be carefully optimized through the use of specialized software to automatically obtain valuable markers (chromatographic peaks and masses) from raw data. As no compounds are selected in advance, chromatography must be robust and has to pursue the best peak resolution possible. Also, data acquisition has to be performed in full-scan mode, in order to obtain maximum information (Cevallos-Cevallos et al., 2009, Garcia and Barbas, 2011). After data acquisition, automatic deconvolution of spectra is needed, to resolve relevant signals through the whole chromatogram (Meyer, Peters, & Maurer, 2010).

Foodomics studies make use of different software to get this information, such as the XCMS package of R (Díaz, Pozo, Sancho, & Hernández, 2014), MetAlign (Tikunov et al., 2005) or MZmine 2.0 (Kind, Tolstikov, Fiehn, & Weiss, 2007). These software detect the relevant *m*/*z* values at a specific time and automatically integrate the signal (area or total intensity), in a

procedure known as peak picking. Normally these approaches lead to different features detected in the same samples depending on their specifications and use (Myers, Sumner, Li, Barnes, & Du, 2017). Recently, PARADISe (Johnsen, Skou, Khakimov, & Bro, 2017), which makes use of the algorithm PARAFAC2 ((Elcoroaristizabal et al., 2015, Johnsen et al., 2017, Lenhardt et al., 2015) has emerged as a really promising tool for treatment of GC–MS data. This software possesses a major difference compared with the others, which is the detection of compounds instead of singular ions. This reduces the data matrix and makes the statistical analyses easier and faster.

The aim of this work has been the development of a quality classification model for olive oil through the application of a novel untargeted methodology. For this purpose, the potential of GC–MS with DHS-TD has been exploited together with the use of the recently developed PARADISe software for peak deconvolution purposes. As an additional aim, the correlation of detected compounds with the major defects reported by the PANEL test has been explored.

4.3.3 Materials and methods

Chemicals and reagents

Internal standard toluene-d_8 (tol-d8) ≥99% was purchased from Sigma-Aldrich (Germany).

Tenax® TA glass desorption tubes 60/80 mesh, OD 6.00 mm × 4 mm ID × L 60 mm, used as traps were purchased from Gerstel (Mülheim an der Ruhr, Germany).

External standards of volatile compounds used for signal deviation correction ((*Z*)-3-hexenal, hexanal, (*E,E*)-2,4-hexadienal, 6-methyl-5-hepten-2-one, 6-methyl-5-hepten-2-ol, (*E,E*)-2,4-heptadienal, (R)-limonene, 2-isobutylthiazole, guaiacol, (*E*)-2-octenal, linalool, 2-phenylethanol, methyl salicylate, α-terpineol, β-cyclocitral, (*Z*)-citral, (*E*)-citral, (*E*,*E*)-2,4-decadienal, diphenyl ether, geranylacetone, β-ionone, phenylacetaldehyde, benzaldehyde) were supplied by Supelco (Sigma-Aldrich and Fluka; Barcelona, Spain) as pure compounds (92–99.5%).

Olive oil samples

A total of 108 olive oil samples was provided by the Spanish Olive Oil Interprofessional Organization (INTERPRO, Spain), the Olive Oil Agency of the Ministry of Agriculture and Fisheries, Food and Environment (AAO, MAPAMA) and the official control services from the Council of Agriculture and Fisheries of Andalucia.

Oil samples were obtained from several Spanish cultivar regions during the 2015 season, which were fully quality characterized by the official participating laboratories (Agricultural Laboratory from MAPAMA, Cordoba and Atarfe) using the official COI method (COI/T.20/Doc. No 15/Rev. 9 2017), by the corresponding panels accredited under EU Regulation 2017/625. Eighty-seven samples were used for the training set of the models (18 extra virgin, 48 virgin and 19 lampante), and 21 were analysed as blind samples (the quality was unknown during analysis and classification) and were used for validation of the created models. Samples were stored at −22 °C until use. They were characterized by means of pH measurements and physicochemical and organoleptic properties (including defects, positive attributes and quality classification). Each sample was analysed once, due to limited sample volume and due to the fact that, after desorption, the sample has to be re-extracted in case a replicate is needed.

Sample treatment

Olive oil samples were allowed to defrost at room temperature before analysis. Then, they were aliquoted in 4 different 10-mL vials. One aliquot was immediately used to perform the extraction and the remaining ones were stored at 4 °C.

Three grams of oil were weighed on a precision balance directly into a 150-mL Erlenmeyer flask. The general procedure was based on previous work (Fredes et al., 2016, Sales et al., 2017), but improving the trapping and desorption steps. The trap consisted of a Tenax® TA TDU tube (ID 4 mm, 60 mm length), previously conditioned by applying a desorption step (300 °C for 8 min with a flow of high purity helium of 55 mL/min). Prior to the extraction procedure, the trap was spiked with 10 µL of a solution of 50 ng/µL of toluene-d_8 to correct for extraction deviations.

For the extraction step, the sample was maintained at 40 °C (by immersion of the flask in a water bath) with magnetic agitation at 300 rpm and the headspace was purged with a flow of 100 mL/min of pure N_2 for 1 h onto a Tenax tube trap. **Fig. S1** shows the experimental set up used. After the extraction, the traps were directly transferred to the GC/MS autosampler to automatically carry out the thermal desorption on the TDU. In each sample extraction batch, 6 samples (when possible, 2 extra virgin, 2 virgin and 2 lampante oils) were processed simultaneously.

In order to avoid bias in the methodology, samples were analyzed in batches of 18 tubes, randomly distributed. To ensure stability of the system and correct instrument deviation, replicate thermal desorptions of traps spiked with 10 µL of a mixture of 50 ng/µL of standards corresponding to volatile compounds present in vegetable matrices (and specific for tomato (Serrano, Beltrán, & Hernández, 2009)) were performed. These VOCs were used as they were already available in the laboratory and are present in many different vegetable matrices, including olive oil (Uriarte, Goicoechea, & Guillen, 2011). They are also used in volatile metabolomics studies (Gómez-Cortés, Brenna, & Sacks, 2012). These desorptions were planned at the beginning and at the end of each sequence batch, as well as every 6 samples.

GC–MS

The chromatographic analyses were performed using an Agilent 6890A gas chromatograph, equipped with a Gerstel MPS2 autosampler (Gerstel, Linthicum, MD), coupled to a single quadrupole mass spectrometer, Agilent 5973 N MSD (Agilent Technologies, Santa Clara, CA), operating in EI mode. The GC separation was performed using a fused silica Supelcowax 10 capillary column with a length of 30 m × 0.25 mm ID and a film thickness of 0.25 µm (Sigma Aldrich, Germany). The oven program was as follows: 40 °C (3 min); 5 °C/min to 160 °C (1 min); 40 °C/min to 260 °C (1.5 min). The injection system consisted of two units; the thermal desorption unit (TDU) and the programmable temperature vaporizing (PTV) cooled injection system (CIS4) (Gerstel). The TDU parameters were set as follows: sample removal mode, splitless at an initial temperature of 40 °C (1 min equilibrium time); 60 °C/min to 260 °C (4 min), transfer line temp 260 °C. The CIS4 PTV was equipped with a Tenax® TA packed liner, CIS4 temperature program: 40 °C (1 min equilibrium time); 12 °C/s to 260 °C (4 min). A summary of the different temperature programs is displayed in **Fig. S2**.

Data processing

GC–MS data were converted to netCDF format using the Chemstation® (Version G1701CA; Agilent Technologies) *export to .AIA* function. Data mining was carried out using PARADISe. After importing the netCDF data to the PARADISe software, the regions of interest (ROIs), which are the time intervals where software applies the deconvolution, were selected manually along the full chromatogram. A total of 118 intervals were selected, paying attention to peak shape (when visible in the TIC) and leaving no empty spaces between intervals. Modeling options were set to a maximum of 8 compounds per interval and 50,000 maximum iterations per interval. After the modeling step, models created for each interval were carefully optimized attending to: model fitting over 95%, model consistency over 95%, background removal, and avoiding model overfitting (in this order). The data matrix obtained after applying PARADISe consisted of an *.xls* file which could be opened with Microsoft Excel for future data transformations. The areas provided by PARADISe were divided by the area of tol-d8 to correct the differences between extraction batches and TDU tubes. The relative areas were corrected with the nearest external standard and then scaled applying mean-centering. Statistical analyses were performed using EzInfo software (U-Metrics, Version 2.0.0.0; Waters Corporation, Wilmslow, UK).

4.3.4 Results and discussion

Extraction procedure optimization

Considering our previous experiences (Beltran et al., 2006, Fredes et al., 2016, Sales et al., 2017), efforts were devoted to optimize and apply static headspace-stir bar sorptive extraction-thermal desorption (SHS-SBSE-TD) and dynamic headspace entrainment followed by thermal desorption (DHS-TD). Although SPME has been extensively used for the analysis of olive oils (Arrebola et al., 2005, Benelli et al., 2015, Gómez-Cortés et al., 2012, Oliver-Pozo et al., 2015, Uriarte et al., 2011, Zhu et al., 2016), SPME automatically coupled to GC–MS was not available in our laboratory.

The first step was to compare the performance of the two considered extraction methods, to select one for further development; accordingly, a selected extra virgin olive oil sample was extracted in triplicate by HS-SBSE-TD and DHS-TD under the same conditions. Additionally, an aliquot

of the same oil sample, spiked with the above mentioned mixture of VOCs (see experimental section), was extracted (*n* = 3 for each method) with the same two methodologies. The analysis was performed in full-scan mode and then integrating the areas for the specified ions. Results obtained unequivocally demonstrated the higher performance of DHS-TD. On one hand, N_2 current (dynamic process) and the larger surface area of Tenax® TA tubes enhanced the extraction from 4 to 10 times for most components and up to 1000 times for the most volatile compounds, when compared to Twister (SBSE) extraction. Furthermore, to test the reliability of the DHS-TD extraction procedure, 15 replicates of an extra virgin olive oil spiked with the IS mixture were extracted. This test gave RSD values below 15% for most compounds, and permitted the detection of all the spiked compounds, together with a huge amount of additional VOCs present in the olive oil sample. As an example, box plots for a number of selected spiked compounds are shown in **Fig. S3**. The plots show no outliers for the selected compounds, highlighting the repeatability of the methodology.

Data analysis optimization

Many different deconvolution software packages can be used for the automatic detection of chromatographic peaks in non-targeted approaches. There is plenty of literature regarding the use of xcms package of R (Fernández-Varela et al., 2015, Gil-Solsona et al., 2016) and MZmine (Hung et al., 2014, Sales et al., 2017). More recently PARADISe, integrating the algorithm PARAFAC2, has emerged as an efficient alternative (Elcoroaristizabal et al., 2015, Khakimov et al., 2016, Lenhardt et al., 2015, Vegge et al., 2016). From our previous knowledge on the application of these deconvolution tools to GC–MS data, and specifically related to the analyses of VOCs in olive oil, PARADISe has been recently revealed as a potential tool in this field. It provides more robust integrations while removing a huge amount of interfering and ghost peaks. Additionally, it gives an additional benefit due to its ease of use and peak visualization. PARAFAC2 algorithm (Harshman, 1972) performs peak deconvolution attending to the intensity and the spectra of the signals, so it is extremely powerful when resolving co-eluting peaks, even with unit mass resolution MS.

During the optimization of PARADISe models for peak deconvolution, 118 individual intervals were obtained from the entire chromatogram, cropping the last 3 min to avoid ghost peaks from column bleed at elevated temperatures. This step reduced data complexity and weight

before the model validation. PARADISe model validation was performed as previously described elsewhere (Khakimov et al., 2016), testing the model fitting for each interval with one to eight components. Each model was carefully evaluated to find the optimal number of components, looking for a good model fit (over 95%), noise removal and low residuals, with a core consistency over 95%. Also model overfitting was avoided while obtaining well resolved peaks. As an example, the capabilities of PARADISe for compound detection and noise reduction are displayed in **Fig. 1**. In **Fig. 1(A)**, the total ion chromatogram shows a very complex interval, with three presumed compounds.

The residuals in this case were up to 10^6. After selecting 5 different components **(Fig. 1(B))**, residuals were lowered by two orders of magnitude, and the algorithm detected four different signals and noise (the red component). The model fit increases from 60% for one component to 100% with the selected compounds. At the same time, consistency is kept higher than 95%, ensuring the goodness of the selected model. Among the components selected, the green component was identified by using NIST08 as 3-(methylthio)propanal, and confirmed with the injection of its standard. **Fig. S4** highlights the potential of PARADISe for spectra deconvolution, as it is able to distinguish the signals coming from two different co-eluting components and column bleed. This capability results in a higher number of components detected (green and blue in **Fig. 1B**) with cleaner spectra, which results in better tentative identifications when using NIST. With the final selected model for each interval, all the samples were processed. Data exported from PARADISe led to a *.xls* file containing a total of 230 different compounds, a number significantly lower than those obtained by other peak picking software commonly used, often close to ten thousand different features (Li et al., 2018). This step reduces the data matrix, which simplifies the statistical analysis. All these compounds were processed by dividing each compound peak area by the area of the internal standard (tol-D_8) in each sample to correct for instrument deviation. Then they were corrected by nearest external standard and finally mean centered to enhance the differences between groups.

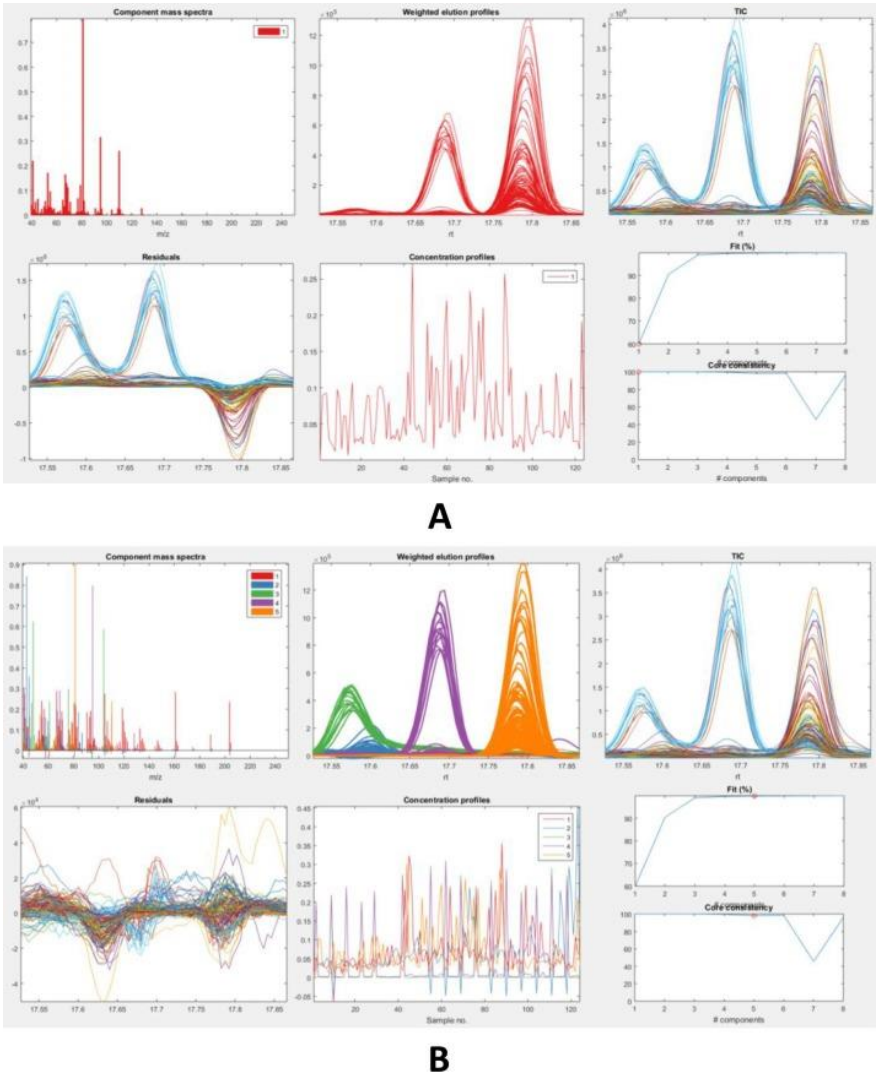

Fig. 1. Evolution of PARADISe model at interval 57 (17.53–17.87 min) for 1 component (A) and 5 components (B) finally selected.

The whole dataset was divided in two groups: one for method training, containing 87 samples (20 lampante, 48 virgin and 19 extra) and a smaller subset of 21 blind samples for model validation. **Fig. 2** shows the evolution of the different principal component analyses (PCA) applied depending on different data corrections applied. As can be appreciated, the use of surrogate tol-D_8 for data correction helps to minimize

deviation in groups when compared to the raw data. Furthermore, the use of the response of the nearest external standard for data correction enhances the differences between groups, and consequently, was selected for further method development.

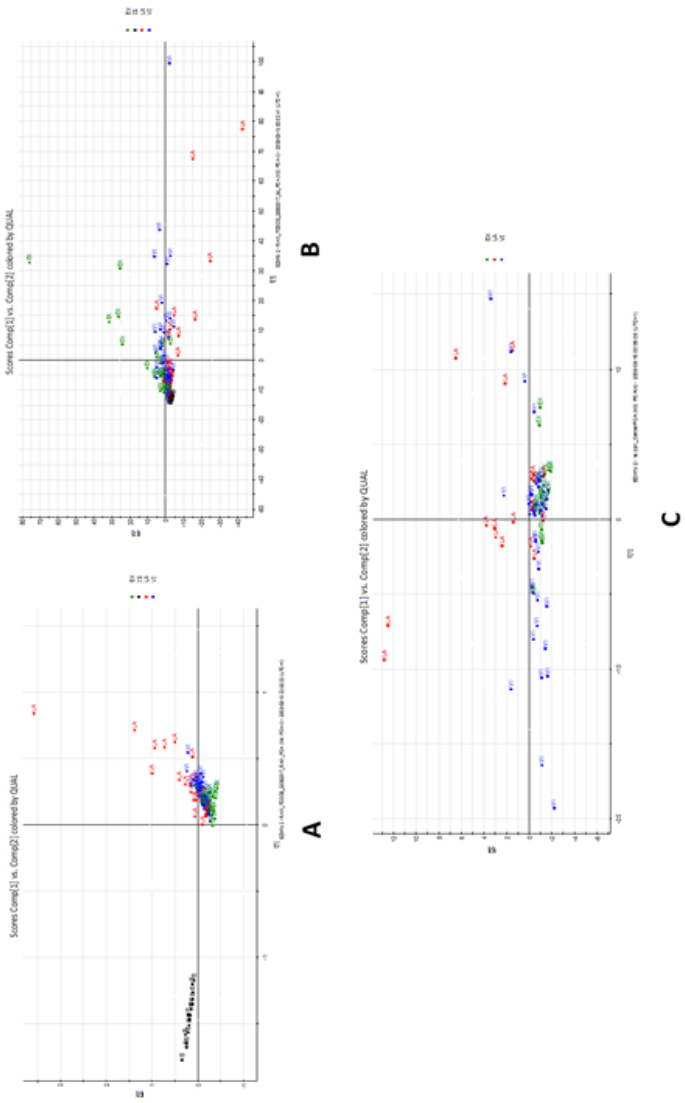

Fig. 2. Evolution of PCA plots after each data treatment step (mean centered in all cases): **A** Raw data, **B** corrected dividing by TOL-D_8 area, **C** corrected with respect to the nearest standard.

Classification model validation

At this point, the development of a quality classification model of olive oils by DHS-TD was studied. Subsequently, after aforementioned data transformations and having checked the PCA for goodness of data, a partial least squares discriminant analysis (PLS-DA) was constructed according to the quality of each group (extra virgin, virgin and lampante groups (see **Fig. 3**). The PLS-DA showed a clear distinction between lampante and extra samples, while the virgin samples, with both positive attributes and defects, were in the middle. In order to verify the accuracy of the model, it was validated with the analysis of blind samples, i.e. with *a priori* unknown quality. **Fig. S5** shows a confusion matrix presenting the results for the training and the validation set of samples classified by PLS-DA. One of the greatest outputs was the capability of the developed methodology to correctly classify 100% of extra virgin olive oil samples. Another output was the great differentiation achieved between extra and lampante samples, which avoided any misclassification between these two extreme groups. Finally, in order to determine the compounds responsible for extra and lampante qualities, two different orthogonal partial least squares-discriminant analysis (O-PLSDA) models were created. Firstly, the flawless extra virgin samples were compared to the virgin and lampante ones; and secondly, lampante samples were compared to the rest. From them, two S-PLOT graphs were obtained and inspected for endpoints. **Table 1** lists the main compounds responsible for the positive attributes of extra samples and also those responsible for the lampante quality.

Defect-related compounds identification

As a final step, PARADISe automatically compares deconvoluted spectra with the NIST library (in this case NIST08 (NIST, Gaithersburg, MD)), giving the best fitted candidate for each peak. In order to add more confidence to the identification, the retention index for each compound was calculated using a C_7–C_{30} alkane mixture which was injected along with the rest of the sequence.

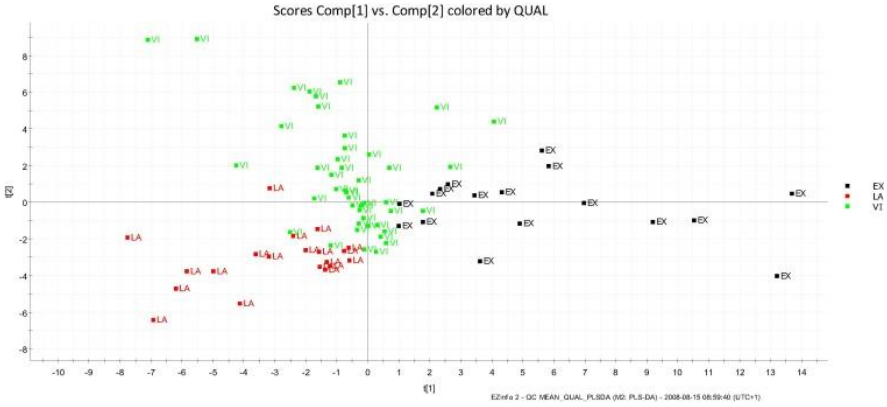

Fig. 3. PLS-DA plot for the training set used in the construction of the classification model.

Although all the deconvoluted features were used in the creation of the statistical model, only the compounds with a match over 850 and a RI match ± 20 (Chemspider) were considered as tentatively identified compounds. Compounds given as completely identified were confirmed by the injection of their corresponding standards.

Table 1. Compounds responsible for Extra and Lampante qualities in olive oils.

Compound	RT	RI	RMF*	p[1]P	p(corr)[1]P	FACTOR OF CHANGE Extra	No Extra
3-Hexenal, (Z)- ++	8,89	1143	837	-0,14	-0,70	98,7	-25,8
2(5H)-Furanone, 5-ethyl-	21,12	1608	850	-0,14	-0,73	91,2	-20,7
2-Hexenal ++	10,63	1206	891	-0,14	-0,72	88,9	-23,6
Hexanoic acid, 2-oxo-, methyl ester	28,43	1981	815	-0,13	-0,67	75,2	-16,8
2,4-Hexadienal, (E,E)- ++	16,16	1410	889	-0,15	-0,75	68,0	-19,8
3-Ethyl-1,5-octadiene +	5,87	1026	942	-0,15	-0,78	65,5	-18,3
Compound				**p[1]P**	**p(corr)[1]P**	**Lampante**	**Virgin**
1-Octen-3-ol +	17,35	1456	939	-0,11	-0,38	28,8	-7,7
Furan, 2-pentyl- +	11,46	1236	839	-0,11	-0,40	26,6	-8,3
Butanoic acid, 2-methyl-, ethyl ester ++	6,58	1055	915	-0,13	-0,47	3,7	-12,8
Benzene, 1-methyl-3-(1-methylethyl)- +	12,48	1273	924	-0,12	-0,44	2,9	-0,3

*NIST Library Reverse Match Factor.

+ Tentatively identified; RMF over 900; RI + –20.

++ Identified with the injection of the standard.

p[1]P : Loadings for each compound in the component 1 of OPLS-DA model.

p(corr)[1]P: Predictive loading values. They indicate if the compound is at higher levels in Extra in comparison with No Extra, and in Lampante in comparison with Virgin (Including Extra and regular Virgin), respectively.

Factor of change: Times the mean value for the compound is higher (or lower) in one quality than in the average value for all the samples.

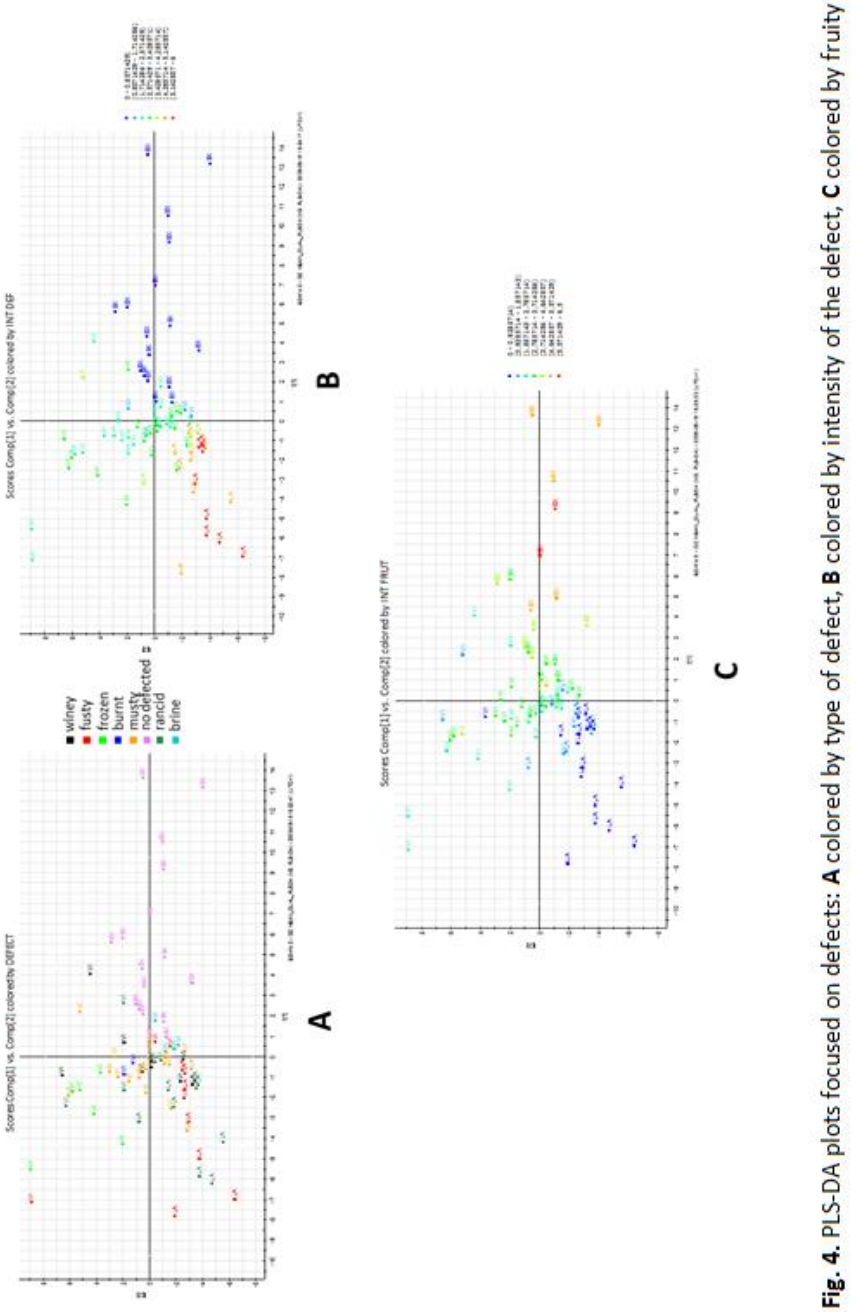

Fig. 4. PLS-DA plots focused on defects: **A** colored by type of defect, **B** colored by intensity of the defect, **C** colored by fruity intensity.

Table 2. Defect-related compounds in olive oils, resulting from comparing all the Extra (non-defective) samples against Virgin and Lampante samples with each major defect, in several O-PLSDA models.

Musty defect						**FACTOR OF CHANGE**	
Compound	**RT**	**RI**	**RMF***	**p[1]P**	**p(corr)[1]P**	**No defect**	**Musty**
2,6-Octadiene, 4-methyl-	7,22	1081	834	0,15	0,79	-749,2	349,5
2-Octene, 2-methyl-6-methylene-	5,50	1011	798	0,14	0,74	-127,9	99,7
Octane ++	2,5	800	924	-0,12	-0,61	45,6	25,6
Hexane ++	1,86	600	929	0,09	0,57	-10,7	20,4
Butanoic acid, 2-methyl-, ethyl ester ++	6,58	1055	915	0,12	0,78	-38,7	19,3
Hexanal ++	7,34	1086	915	0,10	0,65	-37,4	3,7
Winey-vinegary defect							
Compound	**RT**	**RI**	**RMF**	**p[1]P**	**p(corr)[1]P**	**No defect**	**Winey**
2-Octene, 2-methyl-6-methylene-	5,50	1011	798	0,19	0,70	-127,9	87,7
2,6-Octadiene, 4-methyl-	7,22	1081	834	0,18	0,68	-749,2	13,9
1-Butanol, 3-methyl-	10,76	1211	845	0,16	0,80	-39,9	8,1
2-Butenoic acid, ethyl ester, (E)-	9,56	1168	857	0,13	0,63	-36,8	23,1
Pentanoic acid, ethyl ester +	8,78	1139	900	0,12	0,59	-32,5	15,1
Formic acid, heptyl ester +	17,46	1460	924	0,12	0,63	-39,0	5,7
Benzoic acid, ethyl ester +	22,69	1675	939	0,13	0,64	-41,9	0,4
3-Ethyl-1,5-octadiene +	5,87	1026	951	-0,19	-0,72	246,6	-142,5

Table 2. Defect-related compounds in olive oils, resulting from comparing all the Extra (non-defective) samples against Virgin and Lampante samples with each major defect, in several O-PLSDA models.

Fusty defect

Compound	RT	RI	RMF	p[1]P	p(corr)[1]P	No defect	Fusty
2-Octene, 2-methyl-6-methylene-	5,50	1011	798	0,12	0,47	-127,9	62,1
Bicyclo[4.2.0]octa-1,3,5-triene +	12,19	1263	939	0,11	0,44	-157,1	53,0
Phenol, 4-ethyl- +	29,94	2197	961	0,12	0,50	-2,0	9,5
1-Heptanol +	17,46	1460	910	0,11	0,46	-24,4	9,2
Octanal ++	13,05	1294	903	0,10	0,42	-13,3	3,1
2-Decenal, (E)- +	22,09	1649	968	0,11	0,42	-11,9	2,6

Frozen defect

Compound	RT	RI	RMF	p[1]P	p(corr)[1]P	No defect	Frozen
Acetic acid, hexyl ester	12,64	1279	846	-0,09	-0,71	-94,1	280,2
Benzyl Alcohol ++	27,30	1889	939	-0,10	-0,72	-23,9	71,1
Heptanal ++	10,15	1189	914	-0,11	-0,81	-8,9	30,5
2-Octanol ++	16,53	1424	882	-0,09	-0,68	-3,0	27,1
1-Heptanol +	17,46	1460	910	-0,11	-0,83	-24,4	23,8
2,4-Heptadienal, (E,E)- ++	17,79	1473	917	-0,11	-0,85	-6,3	18,5

Table 2. Defect-related compounds in olive oils, resulting from comparing all the Extra (non-defective) samples against Virgin and Lampante samples with each major defect, in several O-PLSDA models.

Burnt defect

Compound	RT	RI	RMF	p[1]P	p(corr)[1]P	No defect	Burnt
Ethylbenzene ++	8,49	1129	944	-0,18	-0,83	-25,5	236,9
1-Butanol, 3-methyl-, acetate +	8,44	1127	926	-0,17	-0,79	-0,7	26,6
1-Butanol, 3-methyl-	10,76	1211	831	-0,16	-0,72	-0,4	0,4
2-Butenoic acid, ethyl ester, (E)-	9,55	1167	857	0,14	0,66	0,5	-15,7
1,3-Pentadiene, 3-methyl-, (E)-	13,97	1328	837	0,17	0,76	522,6	-2324,2

Rancid defect

Compound	RT	RI	RMF	p[1]P	p(corr)[1]P	No defect	Rancid
2-Decenal, (E)- +	22,09	1649	968	-0,16	-0,74	-11,9	1,9

Brine defect

Compound	RT	RI	RMF	p[1]P	p(corr)[1]P	No defect	Brine
Butanoic acid, 2-methyl-, ethyl ester ++	6,58	1055	915	0,16	0,69	32,7	13,0

*NIST Library Reverse Match Factor

+ Tentatively identified; Nist Match over 900; RI +-20

++ Identified with the injection of the standard

In a previous work (Sales et al., 2017), it was demonstrated that the distinction between flawless extra samples and samples with a specific defect was larger than the difference between the three quality classes. Continuing with that work, our efforts were devoted towards the complete identification of the compounds responsible for each kind of defect or negative attribute. To that extent, a PLS-DA was constructed according to the main defect of each sample (or the absence of it). **Fig. 4** shows the results for the PLS-DA, grouping the samples by quality and colored by: considering the predominant (main) defect (**A**), defect intensity (**B**) and main fruitiness intensity (**C**). From the first PLS-DA, distinguishing by the type of defect, several O-PLSDAs were performed comparing samples with one defect against the flawless extra, one defect at a time. The next step was to obtain the corresponding S-PLOT graph for each case and to inspect them looking for endpoints, especially in the part of the defect, to see which compounds were highly related to each negative attribute. Applying this methodology to each defect, a group of compounds were considered as responsible for the poor quality of virgin/lampante olive oils, which are summarized in **Table 2**. The results show the great potential of this technique for the identification of defect-related compounds, as well as for the discrimination of samples according to their defect. These results correlate well with previous works in the field of defect identification using targeted approaches. Especially interesting are (*E*)-2-decenal and (*E*)-2-heptenal, with odor thresholds at the low ppb level, which have been reported by many authors in different olive oils to be related with distinct major defects (Morales et al., 2005, Zhu et al., 2016). Our approach, additionally, shows that their presence has stronger impact than other compounds when labelling an olive oil with *rancid* or *fusty* defects and *frozen* defect, respectively, and that their presence correlates normally with the label virgin rather than lampante. In a similar way, octanal, which in our results is indicative of *fusty* defect, and octane, with a stronger presence in defective oils (Table S1) are also reported as present in defective oils in previous literature (Morales et al., 2005, Oliver-Pozo et al., 2015).

As complementary information, an overview of the signals (relative areas) for all the detected and unequivocally identified compounds in olive oils and their relation to quality and defects are also shown in **Tables S1** and **S2**. Data shown in these tables highlight the potential of the combination of DHS-TD together with PARADISe for the detection of high numbers of relevant compounds in untargeted analysis. Apart from this, the use of this state-of-the art workflow for the determination of VOCs using EI GC–MS

together with NIST library matching, allows tentative identification of several compounds detected in a previous work using atmospheric pressure chemical ionization source (APCI) (Sales et al., 2017). Also, RI values from the non-polar column used previously in the GC-APCI-QTOF MS system, and RI from the polar Supelcowax 10 used in the current work were compared and compounds were tentatively correlated when considering the molecular ion and the molecular fragments found by both methodologies. **Table S3** summarizes the results. Special attention must be paid to 4-ethylphenol and 5-ethyl-2(5*H*)-furanone, which have been detected by both methodologies and have been found to be responsible for *fusty* defect and extra quality, respectively. It is also notable that *rancid* and *brine* defects are poorly characterized by volatiles, as only one compound has been linked to each defect.

4.3.5 Conclusions

A methodology coupling an advanced sample treatment technique for VOCs analyses, with promising powerful deconvolution software for non-targeted analyses, has been developed for the quality classification of Spanish olive oils. This classification has been studied from an untargeted point of view, a novel contribution in a field where normally target approaches are applied. Also, this approach has determined a wide number of compounds related to the main defects found in olive oils.

The high pre concentration factor obtained by DHS-TD has allowed the detection of a huge number of volatile compounds in olive oil at trace levels. PARADISe has demonstrated capabilities for robust peak detection. Thanks to its special algorithm (PARAFAC2), extremely clean mass spectra have been provided. This has been useful for tentative identification of unknown compounds, when matching their spectra with NIST libraries and also for resolving co-eluting peaks.

The developed methodology has resulted in an enhanced quality classification model, with a 100% discrimination of extra samples, and an overall 86% accuracy for the three different classes, which reveals it as an important complement to the PANEL TEST. As a final remark, the method has allowed to putative identification and completely identification (when standards were available) of the main compounds responsible for each type of organoleptic defect in virgin olive oils. This work presents an affordable solution for olive oil classification thanks to the use of state-of-

the-art sample treatment and data treatment methodologies for untargeted foodomics. It shows that DHS-TD methodology is a powerful technique for the identification and quantitation of volatiles. Also it shows potential for the classification of samples through untargeted analysis not only in oils, but in any sample with a complex volatile composition.

4.3.6 Conflict of interest

The authors declare no conflict of interest.

4.3.7 Acknowledgements

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4.4 Discussion

Results obtained in these two articles demonstrate the capabilities of MS-based foodomics for the quality classification of olive oils and the identification of the compounds responsible of each quality.

The capabilities of GC coupled to QTOF HRMS with APCI source to overcome olive oil quality classification were studied in **scientific article 5.** Taking into account previous works on VOCs determination in vegetable matrices as tomato and melon (Fredes et al., 2016; Serrano et al., 2009) and the potential of omics-based techniques to differentiate between groups of samples (Díaz et al., 2016; Gil-Solsona et al., 2016) untargeted GC-MS was selected as the methodology to be applied.

For the effective separation of VOCs from complex vegetable matrices, and based on previous experiences in our group, P&T with solvent elution (P&T-SE), SPME, SBSE and DHS-thermal desorption were tested in order to maximize both, the number of compounds detected and the overall analyte response. With that purpose, several replicates of an extra virgin olive oil spiked with a mixture of 42 VOCs present in tomato (Beltran et al., 2006) at 5 ppb level were extracted (n=3) applying each sample technique, and analysed by GC-EI-MS in scan mode. Attention was paid to the number of compounds detectable by each technique and their absolute signal in each case.

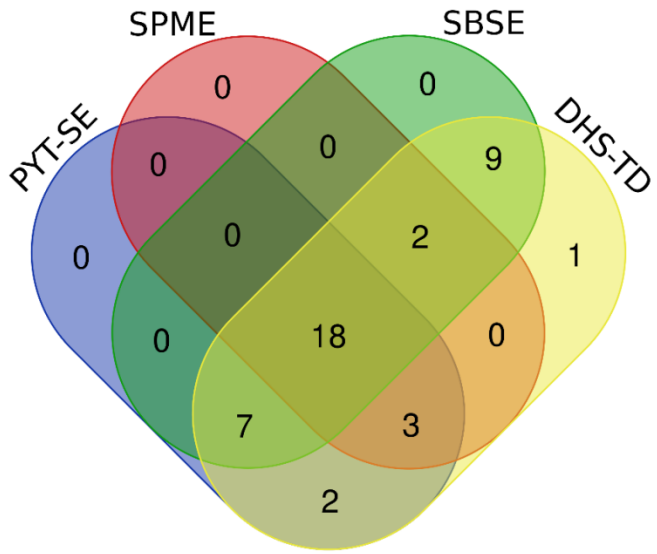

Figure 4.3. Venn diagram showing the number of compounds detected by each VOCs sampling technique.

Results, shown in **Figure 4.3.** demonstrate that DHS-TD is the optimal sample treatment technique for VOCs, as it was able to detected all compounds at 5ppb level. SBSE performed in a similar way, but since the stir bars could not be placed directly into the olive oil, was an important (experimental) drawback that forced us to discard it for further development. Both DHS-TD and SBSE require of a thermal desorption unit to desorb the trapped VOCs into the GC system for direct transfer of all extracted analyte amount. As efforts were focused on obtaining a method based on GC-APCI-QTOF, and due to the lack of a thermal desorption unit coupled to that instrument. P&T-solvent elution procedure was selected as the sample treatment technique. An in-house made system allowed to extract up to 6 samples at once, decreasing analysis times when compared to manual or automated SPME. The extraction set-up can be seen in **Figure 4.4.** This extraction set-up is fully scalable, which implies an added benefit

in the case the methodology was to be implemented as a routine test, with almost unlimited samples to be extracted at a time.

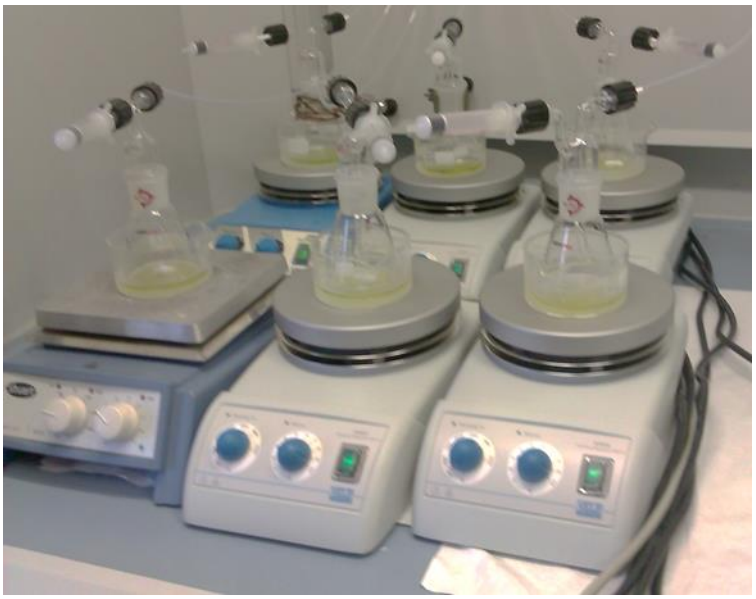

Figure 4.4. In-house set-up for the extraction of VOCs from olive oil.

Extraction conditions, including extraction time, oil mass and N_2 flow were optimized using experimental design software (DesignExpert®). The selected design was based on surface response using a randomized central composite model, which used just 20 runs divided in 2 analysis blocks to find the optimal values for these 3 variables. A linear combination of responses of the compounds present in the mentioned tomato mix (Beltran et al., 2006) was used to evaluate the suitability of each combination of parameters. As it can be appreciated in **Figure 4.5.** the response increased with the addition of more olive oil and the use of a higher flow. However, the results suggested a reduction in the response at flows higher than 1.3 L/min, and extraction times higher than 80 minutes. This could be atributable to the fact that an overall N_2 flow higher than 1.3

L /min for more than 1 h, or around 60L per cartridge, could start eluting (breakingthrough) the more volatile (less retained) compounds attached to the Envicarb stationary fase, thus decreasing the overall response.

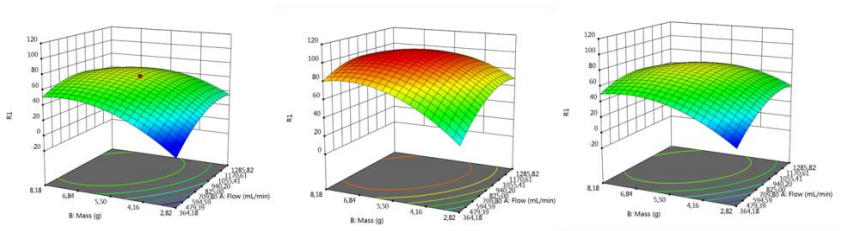

Figure 4.5. Response surfaces obtained from the randomized Central Composite optimization design. (A) Section at 30 min extraction time, (B) at 60 min and (C) at 90 min.

After data evaluation with Design Expert®, in which parameters were forced to minimum values to save analysis time and cost, extraction time was set at 60 min, N_2 flow at 1L/h and sample weight at 5g.

Chromatographic analysis of sample extracts were carried out by GC–(APCI)QTOF MS working in MS^E mode, in order to get both, maximum sensitivity and full compound information. As introduced earlier in Chapter 3 the soft ionization provided by the APCI source combined with low energy acquisition function was useful to enhance the presence of the molecular cluster ($M^{+\bullet}$or $[M+H]^+$), really important when determining unknown compounds. Additionally, the information acquired at high energy function (collision energy ramp of 10–40 eV) provided a high fragmentation pattern, which was crucial in order to elucidate the identity of the markers. As an example, **Figure 4.6.** (supplementary Figure S-1 of **scientific article 5**) shows the accurate-mass full spectra obtained for α-farnesene, comparing the obtained LE and HE spectra with its

corresponding EI (70 eV) spectrum from NIST library. As can be appreciated, the base peak of the LE spectra corresponded to the $[M+H]^+$ ion from the compound, which exhibited a really low fragmentation, with only 4 fragments visible in this acquisition function. The high energy function revealed much more information, and allowed to annotate the different fragmentation products with mass errors below 1mDa. EI in-source fragmentation is clearly higher, provoking the absence of the $M^{+\cdot}$ and producing a high number of low *m/z* ions, more unspecific, which can lead to errors in identification when coelution with similar compounds occurs.

The chromatographic conditions were established looking for good peak separation and a relatively short analysis time. As no information about the compounds was known, a regular HP-5MS column (30 m × 0.25 mm ID; film thickness 0.25 µm, J&W Scientific) was selected as GC column due to its versatility. The oven program, detailed in the article, permitted a good peak separation of the compounds visible in the TIC, within a total run of 33 min. As indicated in **Chapter 3**, APCI permits to efficiently work at higher carrier flow rates, so 2 mL/min were selected to improve peak shape and sensitivity without losing resolution. Figure **4.7.** (Supplementary Figure S-2 of **scientific article 5**) shows the instrumental chromatographic performance of the methodology for a QC extract.

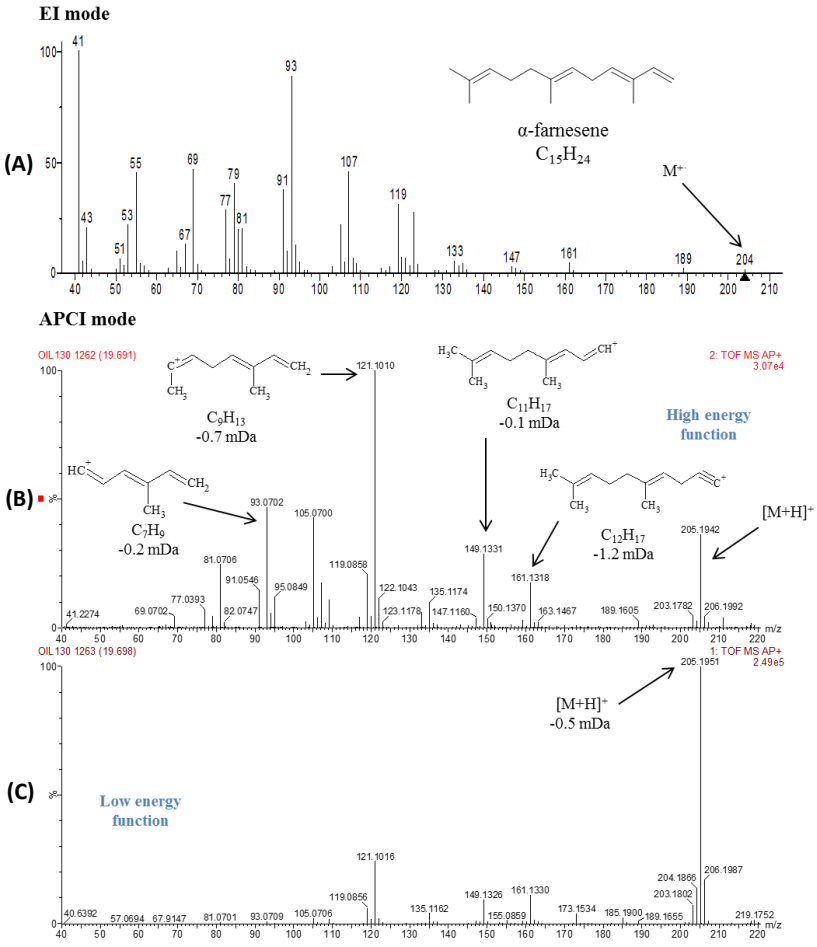

Figure 4.6 Comparison between the MS spectrum of α-farnesene obtained by (A) electron ionization (NIST library), and by APCI-QTOF (MS^E mode), (B) high energy function and (C) low energy function.

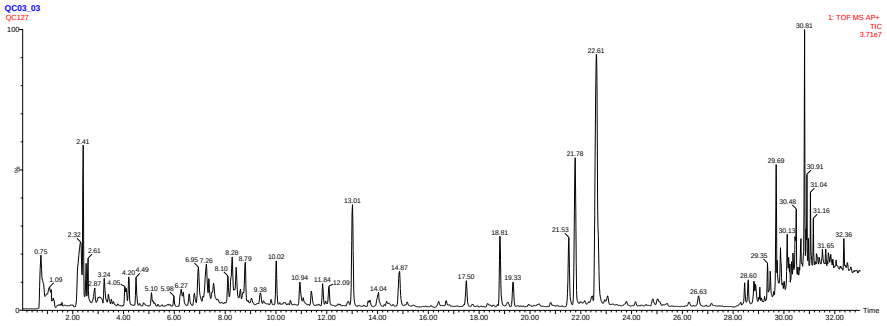

Figure 4.7. Full-scan total ion chromatogram (TIC) obtained by the injection of a QC sample.

Ionization behavior and response under APCI source had already been studied for different types of analytes, including pesticides, PBDEs and dioxins (Cervera, Portolés, Pitarch, Beltrán, & Hernández, 2012; Portolés et al., 2015; Tienstra, Portolés, Hernández, & Mol, 2015; Van Bavel et al., 2015) in targeted, suspect and non-targeted analysis. However, GC-APCI-MS^E had never been used for omics-based purposes before. The metabolomics approach makes use of automatic spectral deconvolution software to extract the relevant information from the sample, looking for every single ion found at any retention time with an abundance determined by the user. In this scenario, the low fragmentation of APCI LE function spectra has an added value, as the lower number of ions produced can lead to a smaller data matrix to work with, still maintaining the complete information of the sample. In order to perform the peak picking stage, in other words to scan among all acquired data to obtain the abundance of each ion at any point of the chromatogram, several automatic spectra deconvolution software can be used. As omics-based research has been increasingly conducted in last years, as can be seen in **Figure 4.8,** more and more tools for preprocessing, data treatment and post-processing of metabolomics data are available (Table 4.2).

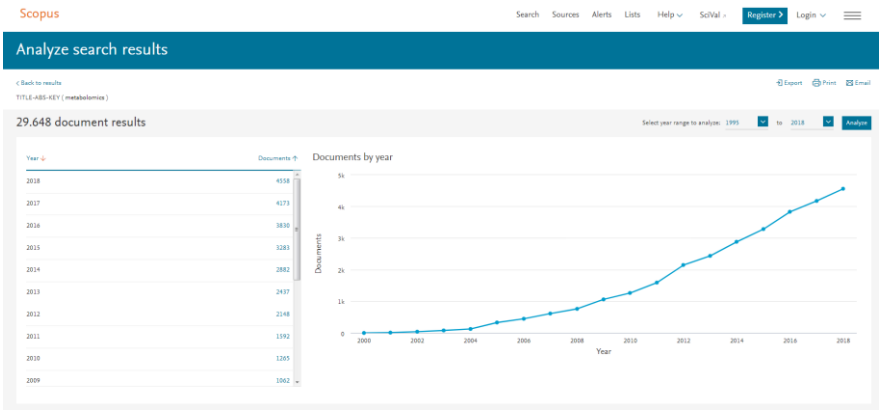

Figure 4.8. Capture showing articles containing the word "metabolomics" indexed in Scopus® database until 2018.

Table 4.2 lists some of the data processing tools available to extract information from LC-MS and GC-MS data. Although all software could be suitable for the purpose, the main objective when applying automatic deconvolution is to obtain reliable peak integration and a limited number of peaks.

Several software were tested in order to conduct the experiments: XCMS (R-based package), mzMine2 and metAlign. MetAling required an unreachable computing power, so it was discarded earlier. Between XCMS and mzMine2, XCMS was found to be least robust in terms of repeatability when extracting features from repeated QC injections and produced peak lists with a larger number of features. This was also in agreement with other studies (Kind et al., 2007; Myers, Sumner, Li, Barnes, & Du, 2017) which indicated mzMine2 as the software which produces more reliable values. Accordingly, mzMine2 was selected for the peak peaking step.

Table 4.2. Some of the data treatment tools available for LC-MS and GC-MS metabolomics data (Gardinassi, Xia, Safo, & Li, 2017; Misra, 2018)

Name	Main application	Name	Main application
ADAP	Data preprocessing	MetExplore	Pathway analysis
AntDAS	Data preprocessing	MetPA	Pathway analysis
apLCMS	Data preprocessing	Metscape	Pathway analysis
Babelomics	Statistical analysis	MIDcor	Data preprocessing
Baitmet	Data preprocessing	M-IOLITE	Data preprocessing
CAMERA	Annotation	mixOmics	Data integration
Galaxy-M	Analysis workflow	MS-DIAL	Data preprocessing
KEGG	Metabolic models	Mummichog	Pathway and network analysis
KPIC2	Data preprocessing	mzCloud	Spectral search, metabolite annotation
LIPID MAPS	Lipid annotation	MZedDB	Annotation
LipidHome	Lipid annotation	mzMine2	Data preprocessing
Lipostar	Data preprocessing	NIST	Spectral search, metabolite annotation
LIQUID	Data preprocessing	OpenMS	Data preprocessing
MAIMS	Data preprocessing	PARADISe	Data preprocessing
Mass Studio	Data preprocessing	Pathomx	Analysis workflow
MassBank	Spectral search, metabolite annotation	PiMP	Data preprocessing
MAVEN	Data preprocessing	R2DGC	Data preprocessing
Metab	Analysis workflow	RAMclust	Annotation
MetaboAnalyst	Statistical analysis and visualization	RECON2	Metabolic models
MetaboloDerivatizer	Data preprocessing	SIMCA-P	Statistical analysis
MetaboQC	Data preprocessing	SMART	Statistical analysis
MetaboSearch	Annotation	Specmine	Statistical analysis
Metabox	Analysis workflow	WGCNA	Data integration
MetaCyc and BioCyc	Metabolic models	Workflow4Metabolomics	Analysis workflow
metaP-server	Pathway analysis	XCMS	Data preprocessing
MetAssign	Annotation	XCMS online	Data preprocessing and downstream data analysis
MetDAT	Analysis workflow	xMSannotator	Annotation

Data treatment with mzMine2 produces a data matrix with the samples in columns and the features in rows, giving the absolute intensity as a response. It was found that correction to the internal standard signal (*m/z* 327.0786 of $[M+H]^+$) was more efficiently applied manually, as mzMine2 failed to detect the internal standard peak in some samples.

After IS correction, all samples were corrected by dividing their signal by the signal of the nearest QC, to correct instrumental deviations along the different sequences injected. Finally, data were pareto scaled in order to minimize the effect of the variables with high standard deviation. **Table 4.3.** shows some of the data pre-treatment that can be applied in order to scale the data (van den Berg, Hoefsloot, Westerhuis, Smilde, & van der Werf, 2006).

Table 4.3. Overview of data pre-treatment methods commonly used in metabolomics.(van den Berg et al., 2006)

Class	Method	Formula	Unit	Goal	Advantages	Disadvantages
I	Centering	$\tilde{x}_{ij} = x_{ij} - \bar{x}_i$	0	Focus on the differences and not the similarities in the data	Remove the offset from the data	When data is heteroscedastic, the effect of this pretreatment method is not always sufficient
II	Autoscaling	$\tilde{x}_{ij} = \frac{x_{ij} - \bar{x}_i}{s_i}$	(-)	Compare metabolites based on correlations	All metabolites become equally important	Inflation of the measurement errors
	Range scaling	$\tilde{x}_{ij} = \frac{x_{ij} - \bar{x}_i}{\left(x_{i_{max}} - x_{i_{min}}\right)}$	(-)	Compare metabolites relative to the biological response range	All metabolites become equally important. Scaling is related to biology	Inflation of the measurement errors and sensitive to outliers
	Pareto scaling	$\tilde{x}_{ij} = \frac{x_{ij} - \bar{x}_i}{\sqrt{s_i}}$	0	Reduce the relative importance of large values, but keep data structure partially intact	Stays closer to the original measurement than autoscaling	Sensitive to large fold changes
	Vast scaling	$\tilde{x}_{ij} = \frac{\left(x_{ij} - \bar{x}_i\right)}{s_i} \cdot \frac{\bar{x}_i}{s_i}$	(-)	Focus on the metabolites that show small fluctuations	Aims for robustness, can use prior group knowledge	Not suited for large induced variation without group structure
	Level scaling	$\tilde{x}_{ij} = \frac{x_{ij} - \bar{x}_i}{\bar{x}_i}$	(-)	Focus on relative response	Suited for identification of e.g. biomarkers	Inflation of the measurement errors
III	Log transformation	$\tilde{x}_{ij} = {}^{10}\log\left(x_{ij}\right)$ $\tilde{x}_{ij} = \tilde{x}_{ij} - \bar{\tilde{x}}_i$	Log 0	Correct for heteroscedasticity, pseudo scaling. Make multiplicative models additive	Reduce heteroscedasticity, multiplicative effects become additive	Difficulties with values with large relative standard deviation and zeros
	Power transformation	$\tilde{x}_{ij} = \sqrt{\left(x_{ij}\right)}$ $\tilde{x}_{ij} = \tilde{x}_{ij} - \bar{\tilde{x}}_i$	√0	Correct for heteroscedasticity, pseudo scaling	Reduce heteroscedasticity, no problems with small values	Choice for square root is arbitrary.

Depending on the data acquired, data pre-treatment can affect diversely, so different pre-treatments should always be checked (including no treatment at all).

Curated data must be analyzed in order to obtain the compounds responsible for each type of quality, and the optimal way to do so in studies with hundreds to thousands of variables is to use multivariate analysis. Principal component analysis (PCA) is the most widely used chemometric technique for the analysis of GC-MS based metabolomics data. PCA is very useful to obtain a general view of huge data sets. In this study, as PCA was not powerful enough for discriminating among qualities, it was only used to check for validity of data matrix, with QCs grouped in the center. To be able to distinguish between oil qualities, a more powerful multivariate method was however needed. The solution was to move from non-directed PCA to directed PLS-DA and OPLSDA methods. PLS-DA allows a better differentiation between groups, as each specific group is determined when choosing the model. In most of chemometrics tests, PLS-DA already provides a perfect separation between groups, and is sufficient for validating the model (Ballabio & Consonni, 2013; Szymańska, Saccenti, Smilde, & Westerhuis, 2012). In this case, given the complexity of the samples and the characteristics of the different groups, with virgin samples similar to extra and lampante samples and with lampante samples that can have a really high concentration of some extra-related compounds together with a singular defect, an extra chemometric method was needed. OPLS-DA provides differences between two groups of samples. Taking into consideration the quality requirements for olive oils listed in **Table 4.1.** a double binary classification, making use of three different OPLS-DA combined, was applied in order to improve the

classification methodology. Accordingly, samples were firstly classified as Extra Virgin vs Non-Extra Virgin (including virgin and Lampantes) and then separated as Virgin olive oils or Lampantes in a second model (lampante vs non-lampante). Thus, the first separation discriminated flawless EVOOs from the rest, taking into account predominantly compounds responsible of defects, using a linear combination from the responses of the markers obtained in the 2nd and 3rd SPLOTs in **Figure 3** from the **scientific article 5**. One of the advantages of this approach is that the threshold for differentiating between classes can be set according to the industry's preferences. In this case, efforts were devoted towards the correct classification of extra samples as EVOO or VOO (in the worst case), so no extra sample would be classified as lampante, which would discard it from the panel test (and from the market). As an example, the criteria selection is shown in **Figure 4.9.** Setting a threshold of 8 in the marker combination selection allowed to correctly classify 90% of EVOO/VOO samples and 83% of VOO/Lampante samples, and an overall accuracy of 70%. The first separation between EVOO/VOO and VOO/Lampante samples was more efficient than the separation between Lampante vs EVOO/VOO and then the differentiation between extra and virgin. This suggested that the developed method was more sensitive to defects than to good attributes, as it was easier to distinguish flawless samples (with greater or softer positive flavors, EVOO and VOO) from the VOO and lampante samples with some defect.

Taking this into account, OPLS-DAs differentiating between defects were developed, and the results are displayed in **Figure 4.10.** (Supplementary figure 3 of **scientific article 5**)**.** These results confirm the hypothesis that the methodology is more sensitive to any kind of defect, being able to

clearly distinguish between the flawless extra samples and samples with a specific type of defect, without easily differentiating between virgin and lampante.

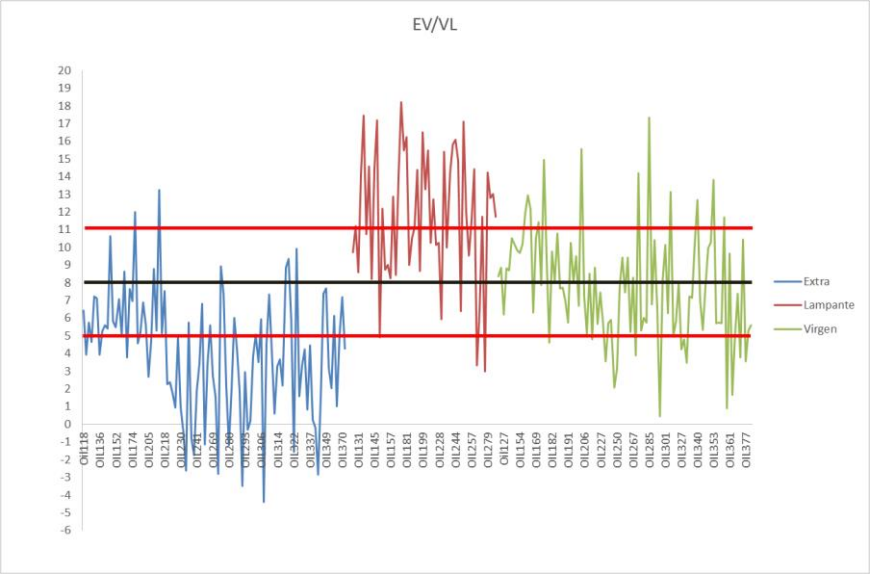

Figure 4.9. Criteria selection threshold for the differentiation between EVOO/VOO and VOO/Lampante Olive oil.

That was in good agreement with the overall classification method, as the separation between virgin and lampante samples was, in all cases, the most difficult. It has to be highlighted that EVOO and lampante classes are well defined by the presence or absence of defects, while VOO class is a mixture that can have some minor defects together with good flavor. So, it fits well as being the class more poorly classified by linear models.

Results obtained confirm GC-APCI-QTOF as a suitable technique for quality classification of olive oils, and for the elucidation of volatile markers responsible of this quality, as it can be appreciated in **Figure 4.6**. However, the technique presented some limitations, such as the difficult and time-

consuming extraction technique, the cost of the instrument and the lack of commercial libraries, which can make the elucidation process time-consuming. On the other hand, considering technology transfer to the industry, this approach is not the most adequate possibility, as it requires a quite expensive instrument and a complex data treatment.

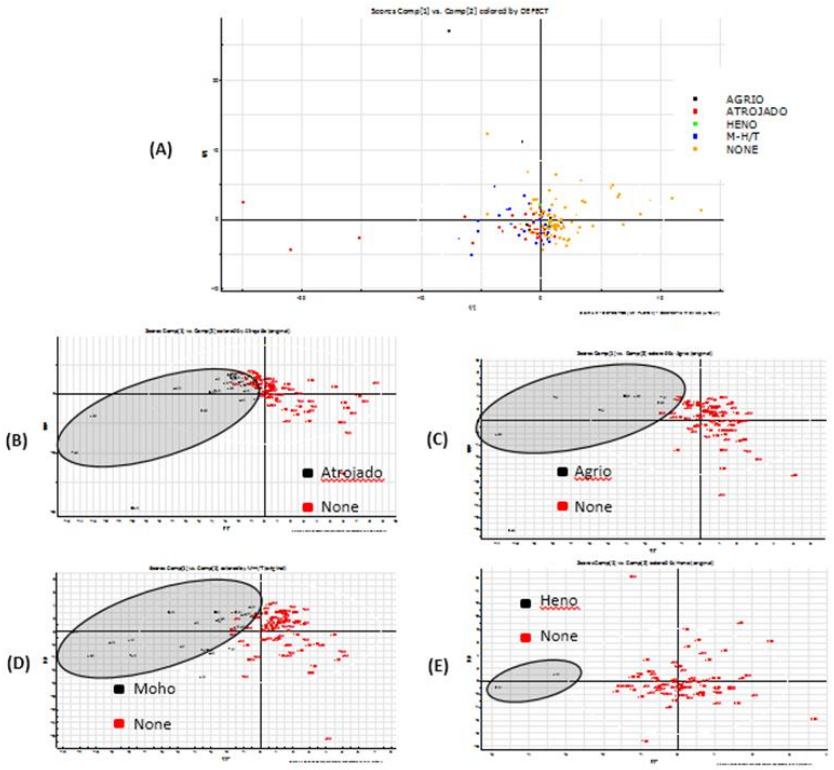

Figure 4.10 (A) PLS-DA analysis based on the defects determined by the PANEL TEST in a subset of 150 samples used for the creation of the model. OPLS-DA analysis facing (B) "Atrojado" defect versus no defect, (C) "Agrio" defect versus no defect, (D) "Moho" defect versus no defect and (E) "Heno" defect versus no defect. Darkened area highlights the region of major predominance of the defect.

Accordingly, in **Scientific Article 6** efforts were directed towards obtaining a more affordable technique, with an easier and more effective sample

treatment technique more easily implementable as routine method in control laboratories.

As previously displayed in **Figure 4.3,** DHS coupled to thermal desorption is considered the optimal sample treatment technique for volatiles, as it has a high pre-concentration factor, is automatable and requires no solvent to elute the VOCs from the cartridge. Using a set up similar to that of **scientific article 5**, the extraction procedure was performed forcing the transfer of the VOCs present in the oil sample to a Tenax® TA trap using a N_2 flow. A picture of the extraction procedure is shown in **Figure 4.11. (Supplementary S.1)**. The setup permitted to extract 6 samples at once, with the advantage of storing the cartridges until their desorption on the GC-MS, without any additional pretreatment step.

Figure 4.11: Picture of the final configuration for sample extraction (DHS-TD).

TenaxTA® was selected as it has proved to have good performance as sorbent for a wide range volatiles and semi-volatiles, with breakthrough volumes higher than carbon-based sorbents and good thermal stability (up to 300°C). A comparison of the performance of different sorbents is displayed in **Figure 4.12.** (Manura, 1999)

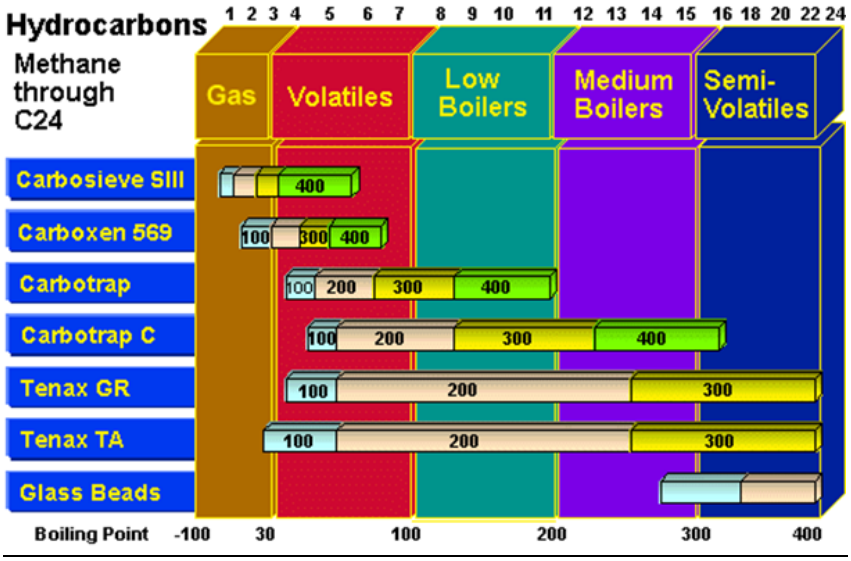

Figure 4.12. Comparison of sorbents depending on their affinity for volatiles. The colored horizontal bars indicate the desorption temperature for each kind of compound. (Manura, 1999)

The determination by DHS-TD was then carried out by desorbing the volatiles directly into a GC-EI-MS, using a trap desorption system (Gerstel TDU) combined with a PTV injector installed on a GC-MS equipped with single quadrupole analyzer. The desorption step includes many variables, for the thermal desorption unit (TDU) as well as for the PTV (CIS 4). These variables were optimized through a multivariate optimization design making use of Design Expert 9 ® being the final optimum desorption configuration as summarized in **Figure 4.13. (Supplementary Figure S2)**

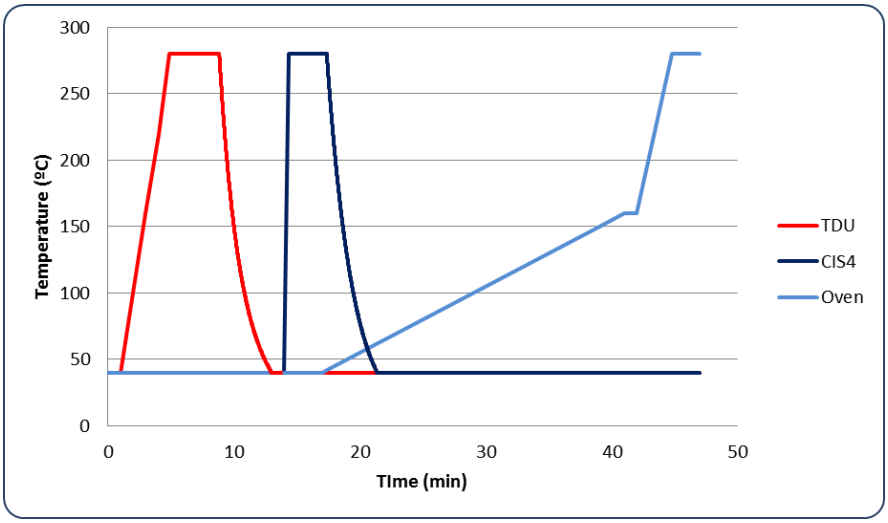

Figure 4.13. Graphic representation of the thermal conditions for TDU, CIS4 and the chromatograph oven along one single run.

The detection and resolution limitations of the single quadrupole were overcome by the high pre-concentration factor of DHS-TD system. Additionally, the thermal desorption, without the use of solvents, produces very clean extracts, without the need of applying a solvent delay in the instrument. This permits the acquisition of the full spectra, which is translated in being able to detect even the early eluting compounds (time < 5min). The performance of DHS-TD can be seen in **Figure 4.14**, where a TIC chromatogram corresponding to an EVOO extract is shown.

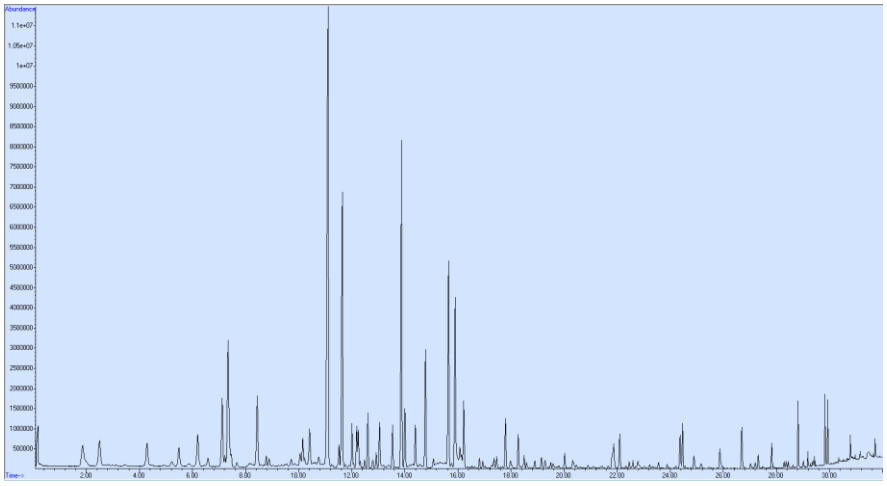

Figure 4.14. TIC chromatogram for the injection of a EVOO extract.

A fast look at the TIC reveals a large number of peaks, spread all over the chromatogram. The high cleanness of the extracts was better noticeable in the spectra **(Figure 4.15a)** obtained from the full scan acquisition. This permits almost a 100% of match when searching for spectra in commercial libraries, as NIST®. **Figure 4.15** shows the comparison between the experimental spectrum for E-2-Hexenal and its spectrum stored in NIST. Note that the experimental record does not show *m/z* values lower than 40, as lower masses were not acquired in order to avoid interferences from N_2, CO and O_2.

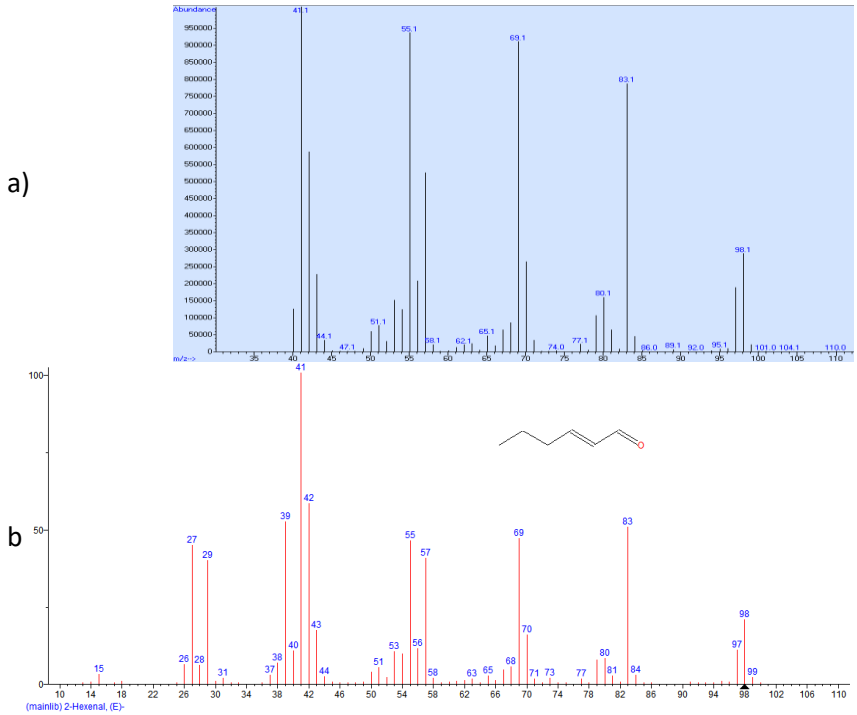

Figure 4.15. A) Experimental spectra for E-2-Hexenal. B) NIST spectrum for E-2-Hexenal.

Additionally, DHS-TD was tested for replicability. To do so, 15 replicates of an EVOO spiked with the VOCs mixture above mentioned (Beltran et al., 2006) were analyzed obtaining RSD values below 15% for most compounds. As an example, box plots for a number of selected spiked compounds are shown in **Figure 4.16.** (Supplementary S3). The plots show no outliers for the selected compounds, highlighting the repeatability of the methodology.

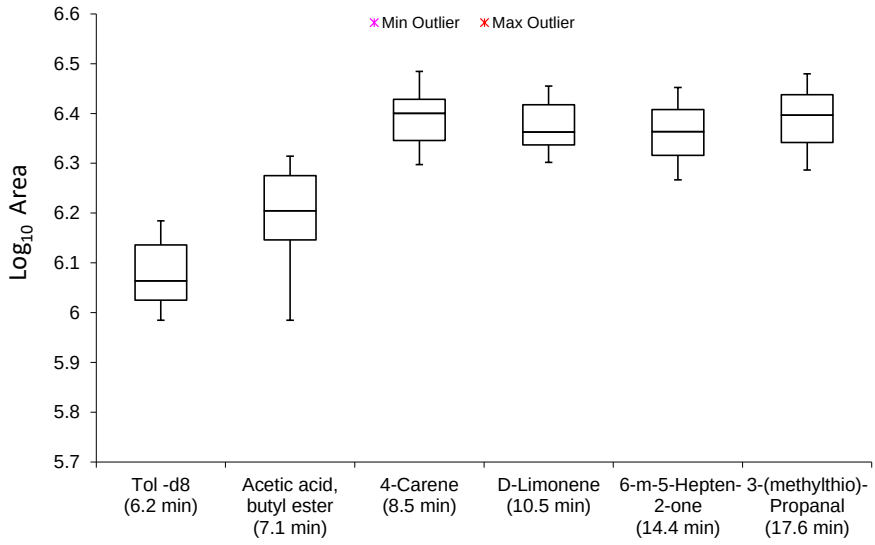

Figure 4.16. Boxplots for 5 selected external standards and the toluene-d_8 created with data of 15 replicates.

When developing the methodology described earlier in this chapter, mzMine2 was selected as the optimal data treatment due to its easiness of use and performance, compared to other software and tools tested (XCMS, metAlign…) However, mzMine2 provides a higher volume of data, with thousands of features to work with (as it works with ions at a given retention time). Also mzMine2 has some issues in repeatability, as described in other works (Kind et al., 2007; Myers et al., 2017). Here, the application of other software, with the best properties of mzMine2 and adding more robustness and reduced data matrices could be of help. In this article, PARADISe software (based on PARAFAC2 algorithm) was applied to extract the relevant information from the samples. PARADISe is a freely available software incorporating a number of newly developed algorithms in a coherent framework. It requires only few inputs to process GC–MS data and subsequently converts raw netCDF data files into a

compiled peak table, were absolute intensity for each compound is presented. This is of a high importance, as the number of features is comprised normally between 0 and a few hundreds, which makes the further data processing much easier. Furthermore, the method is generally robust towards minor variations in the input parameters. The method automatically performs peak identification based on deconvoluted mass spectra using integrated NIST search engine and generates an identification report. This step is an advantage compared to mzMine2 and many other software, as the analyst has immediately a temptative identification for each marker, without the need of time consuming manual elucidation (Díaz et al., 2016; Gil-Solsona et al., 2016). Additionally, PARADISe can handle more overlapping signals, and lower signal-to-noise peaks within a short time regardless of the number of samples (Johnsen et al., 2017). Moreover, there are no non-detects in PARADISe, meaning that all compounds are detected in all samples. This is very important, as there are no zeros in the matrix which could lead to wrong outputs when applying multivariate analysis. The enhanced noise-reduction and identification capabilities of PARADISe are shown in **Figure 4.17. (Supplementary S4).** As it can be appreciated, the direct library search for the peak at 17.56 min **(Figure 4.17 A)** gives a relatively poor reverse match factor (RMF) of 800 when comparing the experimental spectrum to NIST® spectrum. After PARADISe deconvolution **(Figure 4.17 B)**, three different spectra are observed; one corresponding to noise, another one corresponding to a coeluting compound, and the spectra of 3-(methylthio)-propanal, now with a RMF of 950.

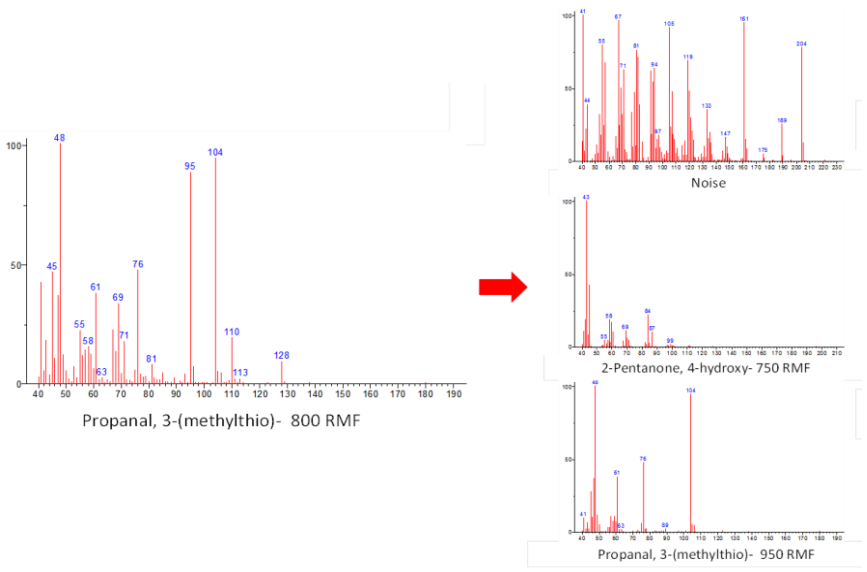

Figure 4.17. Mass spectrum for the TIC at time 17.56 min considering only one component in the corresponding PARADISe model (A) and (B) mass spectra for the three different compounds at that time (after selecting 5 components in the interval). Below each spectrum, the best NIST search result and the RMF is indicated.

After deconvolution using PARADISe, data was treated by means of multivariate analysis in order to create a quality classification methodology. In this case, PLSDA was powerful enough to correctly classify most of the studied samples without the need of creating linear combinations of class markers, as needed in **scientific article 5**. The classification capabilities of the developed procedure are displayed in **Figure 4.18. (Supplementary S5)**. The methodology based in DHS-TD-GC-MS was able to correctly classify 100% of EVOOs, with only 15% of VOOs overclassified as EVOOs. A very interesting output is that no EVOO or VOO was misclassified as Lampante. This is very positive thinking about the

developed technique being used as complementary tool to the PANEL test, as more than 72% of the lampante samples could be already discarded from the PANEL test, thus reducing times and costs in the classification of oils. It also could help to determine if a VOO must be labelled as EVOO, as no EVOO was misclassified during the validation test

Objective

	Extra	Virgin	Lampante
Extra	100	0	0
Virgin	0	100	0
Lampante	0	0	100

Trainning Set

	Extra	Virgin	Lampante
Extra	100	0	0
Virgin	0	100	0
Lampante	0	5	95

Validation Set

	Extra	Virgin	Lampante
Extra	100	0	0
Virgin	14	86	0
Lampante	0	28	72

Figure 4.18. Confusion matrix showing the percentage of samples belonging to training set and validation set which have been correctly classified according to "PANEL TEST".

The enhanced capabilities of the new developed technique, highlighted its potential, not only for quality classification of olive oils, but also to identify the compounds responsible of each defect. Accordingly, several O-PLSDAs were developed facing EVOOs and samples corresponding to one defect each time, looking at the corresponding S-PLOTs in order to determine the endpoints. The results, summarized in **Table 4.4 (Supplementary S1)** and **Table 4.5 (Supplementary S2)** show the responses for detected compounds in the different qualities and defects, respectively.

Results showed a good agreement with previous studies (Luna et al., 2006; Morales, Luna, & Aparicio, 2005; Uriarte, Goicoechea, & Guillen, 2011), as well as with the methodology developed in **scientific article 5**, as can be

appreciated in **Table 4.6 (Supplementary S3)**, which compares the performance of both techniques.

The developed techniques have demonstrated a good performance for the determination of quality in Olive oils, as well as for the identification of compounds responsible of quality and defects. While APCI coupled to HRMS offers the advantage of detecting the molecular mass of the compounds with low mass errors (less than 1 mDa), it lacks from commercial libraries, which makes elucidation step really time-consuming. DHS-TD has demonstrated really high pre-concentration capabilities, which permits to enhance the capabilities of much simply and more affordable instruments as GC-EI-MS equipped with single quadrupole analyzers.

PARADISe has emerged as a very interesting deconvolution tool, as its capabilities allow the use of single quadrupole, with unit mass resolution, for elucidation purposes in untargeted studies with incomparable precision, as demonstrated by the match factor obtained in **scientific article 6**.

We strongly believe that the developed methodologies will help in a near future in the field of food authentication, not only as complementary to PANEL TEST for olive oil quality evaluation, but in many other applications with complex samples.

Table 4.4 Mean relative area (to the highest peak) for the compounds found in each quality.

Compound	rt (min)	Extra	Virgin	Lampante
Hexane	1.86	0.0563	0.0792	0.0576
Octane	2.50	0.0179	0.0654	0.1029
Butanoic acid, 2-methyl-, ethyl ester	6.58	0.0318	0.0655	0.0836
Acetic acid, butyl ester	7.12	0.0035	0.0042	0.0449
Hexanal	7.34	0.0456	0.0883	0.1086
Ethylbenzene	8.50	0.0450	0.0801	0.0544
(+)-4-Carene	8.52	0	0.0011	0.0012
1-Butanol, 3-methyl-, acetate	8.44	0.0073	0.0247	0.0679
2-Carene	9.00	0.0028	0.0065	0.0070
3-Hexenal, (Z)-	9.05	0.1275	0.0232	0.0098
3 Carene	9.56	0.0297	0.0490	0.0512
Heptanal	10.16	0.0390	0.0917	0.1007
R-Limonene	10.42	0.0113	0.0247	0.0308
2-Hexenal	10.63	0.1216	0.0274	0.0151
Gamma Terpinene	11.76	0.0017	0.0029	0.0037
2-Octanone	12.93	0.0063	0.0158	0.0377
Octanal	13.06	0.0353	0.0791	0.1170
2-Heptenal, (Z)-	14.05	0.0257	0.0619	0.1007
2-Hexen-1-ol, acetate	14.33	0.0075	0.0081	0.0068
1-Hexanol	14.82	0.0745	0.0952	0.0914
3-Hexen-1-ol	15.69	0.1039	0.0848	0.0426
Nonanal	15.95	0.0467	0.0919	0.0958
2,4-Hexadienal, (E,E)-	16.16	0.1185	0.0487	0.0313
2-Hexen-1-ol, (E)-	16.27	0.0433	0.0726	0.0796
2-Octanol	16.54	0.0139	0.0314	0.1047
2-Octenal, (E)-	16.83	0.0068	0.0165	0.0283
Propanal, 3-(methylthio)-	17.58	0.0003	0.0005	0.0002
2,4-Heptadienal, (E,E)-	18.50	0.0072	0.0253	0.0244
Camphor	18.95	0.0035	0.0046	0.0059
1-Octanol	20.04	0.0177	0.0354	0.0651
2,4-Decadienal, (E,E)-	25.88	0.0041	0.0120	0.0171
Phenol, 2-methoxy-	27.01	0.0009	0.0030	0.0199
Benzyl Alcohol	27.29	0.0498	0.0636	0.1152
Phenylethyl Alcohol	27.82	0.0147	0.0283	0.0564
Eugenol	29.91	0.0008	0.0005	0.0005

Table 4.5 Mean relative area for the compounds found in each type of defect in the total of virgin and lampante samples analyzed

Compound	rt (min)	Winey	Rancid	Musty	Frozen	Fusty
Hexane	1.86	0.0463	0.0377	0.0675	0.0412	0.0358
Octane	2.50	0.0404	0.0971	0.0626	0.0921	0.1092
Butanoic acid, 2-methyl-, ethyl ester	6.58	0.0854	0.0684	0.0804	0.0348	0.0662
Acetic acid, butyl ester	7.12	0.0052	0.0444	0.0049	0.0017	0.0337
Hexanal	7.34	0.0721	0.1237	0.0862	0.1288	0.1206
Ethylbenzene	8.50	0.0774	0.0588	0.0804	0.0713	0.0682
(+)-4-Carene	8.52	0	0.0019	0.0006	0.0038	0.0024
1-Butanol, 3-methyl-, acetate	8.44	0.0251	0.0564	0.0291	0.0145	0.0494
2-Carene	9.00	0.0048	0.0061	0.0066	0.0087	0.0077
3-Hexenal, (Z)-	9.05	0.0205	0.0120	0.0239	0.0063	0.0194
3 Carene	9.56	0.0419	0.0487	0.0503	0.0581	0.0610
Heptanal	10.16	0.0519	0.1186	0.0774	0.1618	0.1066
R-Limonene	10.42	0.0159	0.0221	0.0268	0.0320	0.0393
2-Hexenal	10.63	0.0215	0.0213	0.0277	0.0205	0.0287
Gamma-Terpinene	11.76	0.0024	0.0029	0.0030	0.0035	0.0041
2-Octanone	12.93	0.0136	0.0176	0.0152	0.0276	0.0206
Octanal	13.06	0.0448	0.0983	0.0772	0.1189	0.1056
2-Heptenal, (Z)-	14.05	0.0354	0.1104	0.0503	0.1068	0.0898
2-Hexen-1-ol, acetate	14.33	0.0076	0.0085	0.0053	0.0145	0.0101
1-Hexanol	14.82	0.1042	0.0955	0.0935	0.1076	0.0946
3-Hexen-1-ol	15.69	0.0767	0.0660	0.0904	0.0568	0.0608
Nonanal	15.95	0.0568	0.1010	0.0873	0.1381	0.1066
2,4-Hexadienal, (E,E)-	16.16	0.0336	0.0476	0.0384	0.0750	0.0438
2-Hexen-1-ol, (E)-	16.27	0.0619	0.1023	0.0446	0.1357	0.1021
2-Octanol	16.54	0.0216	0.0373	0.0285	0.0564	0.0427
2-Octenal, (E)-	16.83	0.0083	0.0275	0.0145	0.0258	0.0258
Propanal, 3-(methylthio)-	17.58	0.0004	0.0004	0.0004	0.0005	0.0002
2,4-Heptadienal, (E,E)-	18.50	0.0106	0.0417	0.0138	0.0541	0.0282
Camphor	18.95	0.0031	0.0059	0.0051	0.0050	0.0049
1-Octanol	20.04	0.0236	0.0375	0.0405	0.0395	0.0432
2,4-Decadienal, (E,E)-	25.88	0.0047	0.0211	0.0077	0.0230	0.0183
Phenol, 2-methoxy-	27.01	0.0017	0.0243	0.0020	0.0058	0.0065
Benzyl Alcohol	27.29	0.0465	0.0787	0.0498	0.1056	0.0977
Phenylethyl Alcohol	27.82	0.0232	0.0374	0.0237	0.0443	0.0345
Eugenol	29.91	0.0005	0.0009	0.0006	0.0004	0.0003

Table 4.6 Comparison of markers found by GC-APCI-HRMS and DHS-TD-GC-EI-MS

Elemental composition $[M+H]^+$	*m/z*	Fragment ion 1	*m/z*	Fragment ion 2	*m/z*	RI (5MS)	RI (WAX)	Compound	NIST Reverse Match Factor
C_8H_9O	121.0649	C_8H_9	105.0704	C_7H_7	91.0548	1006	1654	Acetophenone	900
$C_6H_9O_2$	113.0603	C_5H_9O	85.0653	-	-	1079	1633	3-Hexene-2,5-dione	915
$C_{10}H_{17}O$	**153.1279**	**$C_{10}H_{17}$**	**137.1330**	-	-	**1114**	**1518**	**Camphor**	**907**
$C_6H_9O_2$	**113.0603**	**C_6H_7O**	**95.0497**	**C_5H_7**	**67.0548**	**1149**	**1769**	**2(5H)-Furanone,5-Ethyl**	**841**
$C_6H_{11}O_2$	115.0759	C_6H_9O	97.0653	C_6H_7	79.0548	1181	1711	2(3H)-Furanone,5-ethyldihydro	897
$C_7H_9O_2$	**125.0603**	**$C_6H_6O_2$**	**110.0368**	**C_6H_5O**	**93.034**	**1199**	**1876**	**Guaiacol**	**922**
$C_8H_{11}O$	**123.0810**	**C_7H_7O**	**107.0497**	**C_6H_7O**	**95.0497**	**1285**	**2197**	**Phenol, 4-ethyl-**	**961**
$C_9H_{15}O_2$	155.1072	$C_9H_{13}O$	137.0966	C_8H_{13}	109.1017	1356	1769	1,7-Dioxaspiro[5.5]undec-2-ene	774

Those compounds satisfactorily identified by both techniques are highlighted in bold

4.5 References

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Chapter 5

Conclusions

5.1 Conclusions

Studies undertaken along this doctoral thesis have permitted the evaluation of novel analytical methodologies for their application to two main areas of study: i) the quantification of trace POPs by GC-QqQ MS/MS using soft ionization sources and ii) the quality classification of olive oils by untargeted metabolomics. The main conclusions inferred from results obtained can be summarized as:

- The use of soft ionization sources for GC, including CI and APCI, together with state-of-the-art QqQ analysers, allows achieving sub parts-per-trillion limits of detection and quantification for POPs in complex samples.
- Untargeted metabolomics is a useful technique for quality classification of olive oil and could be effectively used as a complementary tool to PANEL TEST classification.

Additionally, there are specific conclusions for each chapter worth mentioning.

- The use of large volume injections, together with GC-ECNI-QqQ determination, allows decreasing detection limits for flame retardants compared to traditional methods, and could be effectively applied to other halogenated pollutants.
- Large volume injections have great pre-concentration capabilities, but its use with complex and fatty samples requires of careful optimization and the use of guard columns and isotopically labelled standards to achieve a consistent and reproducible quantification.

- GC-APCI-QqQ MS/MS has been proved as a highly sensitive and specific technique for the quantification of PBDEs, novel flame retardants and DL-PCBs in complex samples. This is specially remarkable for highly brominated PBDE congeners, traditionally difficult to quantify using EI sources.
- The soft ionization provided by APCI permits to obtain abundant molecular ions for the compounds selected, which allows to monitor more specific and sensitive transitions working in SRM mode.
- The use of modifiers inside the APCI source can have a great impact on the sensitivity and repeatability of the monitored signals, and must be optimized for each analyte.
- APCI provides better results in terms of sensitivity and peak shape when working at high carrier gas flow rates, which makes it ideal for fast-GC analyses.
- GC-APCI-QTOF HRMS has demonstrated its potential to be applied in untargeted metabolomics, with trace detection capabilities due to its soft ionization and high mass accuracy.
- The MS^E acquisition allows developing the metabolomics model with the LE function and permits to obtain enough fragmentation on the HE function to elucidate compounds without the need of additional injections.
- Purge and Trap with thermal desorption has demonstrated a great potential for preconcentrating analytes, enabling to reach ppt detection limits using an old generation GC single quadrupole MS.
- PARADISe 2, has demonstrated a great performance for peak picking capabilities, providing reliable results, enhanced

compound differentiation and direct tentative identification making use of NIST spectral library.

5.2 Future Work

The results found in the present thesis highlight the potential of the developed techniques, which could be further evolved and applied to other compounds and fields.

-Further development of the DBS technique for the effective biomonitoring of POPs in human blood using GC-APCI-QqQ MS/MS for quantification and GC-APCI-HRMS for screening purposes.

-Review of old methodologies enhancing their detection and quantification capabilities by the use of curated LVI. This could be applied to the methods developed in Chapter 3 with GC-APCI-MS/MS but also to other methodologies based in GC with other detector configurations.

-Exploration of the use of purge and trap with thermal desorption together with GC-APCI MS intruments (QqQ, QTOF and IMS) for the ultra-trace determination of VOCs in complex matrices, taking profit of the preconcentration capabilities of P&T and the sensitivity provided by APCI source.

-Application of the metabolomics-based methodologies developed to the classification of other matrices according to their VOCs fingerprint.

5.3 Conclusiones

Los estudios realizados a lo largo de esta tesis doctoral han permitido la evaluación de nuevas metodologías analíticas para su aplicación en dos áreas principales de estudio: i) la cuantificación de trazas de POPs mediante GC-QqQ MS/MS utilizando fuentes de ionización suave y ii) la clasificación de aceites de oliva de acuerdo a su calidad organoléptica mediante técnicas metabolómicas no dirigidas. Las principales conclusiones inferidas de los resultados obtenidos pueden resumirse como:

- El uso de fuentes de ionización suave para GC, incluiyendo CI y APCI, junto con analizadores QqQ de última generación, permite alcanzar límites de detección y cuantificación por debajo de partes por trillón para POPs en muestras complejas.

- La metabolómica no dirigida es una técnica útil para la clasificación de aceite de oliva de acuerdo a su calidad organoléptica y podría utilizarse eficazmente como una herramienta complementaria para la clasificación mediante PANEL TEST.

Además, hay conclusiones específicas para cada capítulo que cabe resaltar:

- El uso de LVI, junto con la determinación por GC-ECNI-QqQ, permite disminuir los límites de detección de retardantes de llama en comparación con los métodos tradicionales, y podría aplicarse de manera efectiva a otros contaminantes halogenados.

- La inyección de grandes volúmenes de muestra posee elevadas capacidades de preconcentración, pero su uso con muestras complejas y grasas requiere una optimización cuidadosa y el uso de columnas de protección y estándares marcados isotópicamente para lograr una cuantificación consistente y reproducible.

- GC-APCI-QqQ MS/MS ha demostrado ser una técnica altamente sensible y específica para la cuantificación de PBDEs, nuevos retardantes de llama y DL-PCBs en muestras complejas. Esto es especialmente notable para los congéneres de PBDE altamente bromados, tradicionalmente difíciles de cuantificar utilizando fuentes EI.

- La ionización suave proporcionada por APCI permite obtener abundantes iones moleculares para los compuestos seleccionados, lo que permite obtener transiciones más específicas y sensibles trabajando en modo SRM.

- El uso de modificadores dentro de la fuente APCI puede tener un gran impacto en la sensibilidad y la repetibilidad de las señales monitorizadas, y debe optimizarse para cada analito.

- La fuente APCI para GC proporciona mejores resultados en términos de sensibilidad y forma de pico cuando se trabaja con altas velocidades de flujo de gas portador, lo que la hace ideal para análisis rápidos de por GC, denominados *fast GC*.

- La técnica GC-APCI-QTOF HRMS ha demostrado su potencial para ser aplicada en metabolómica no dirigida, con capacidades de detección de trazas debido a su ionización suave y alta precisión de masa.

- La adquisición en modo MS^E permite desarrollar el modelo de metabolómica con la función LE y permite obtener suficiente fragmentación en la función HE para elucidar compuestos sin la necesidad de inyecciones adicionales.

- La técnica de purga y trampa con desorción térmica ha demostrado un gran potencial para preconcentrar analitos, lo que permite alcanzar límites de detección de partes por trillón para VOCs utilizando un analizador the GC-MS de tipo cuadrupolo simple de generación antigua.

- El software PARADISe 2, ha demostrado un gran rendimiento para la deconvolución automática de espectros, proporcionando resultados confiables, diferenciación de compuestos mejorada e identificación tentativa de forma automática utilizando la librería espectral NIST.

5.4 Trabajo Futuro

Los resultados obtenidos en la presente tesis resaltan el potencial de las técnicas desarrolladas, que podrían continuar evolucionándose para ser aplicadas a otros compuestos y campos.

- Desarrollo adicional de la técnica DBS para la monitorización efectiva de POPs en sangre humana usando GC-APCI-QqQ MS/MS para su cuantificación y GC-APCI-HRMS para la detección de nuevos contaminantes.

- Revisión de metodologías antiguas que podrían mejorar sus capacidades de detección y cuantificación mediante el uso de LVI. Esto podría aplicarse a los métodos desarrollados en el Capítulo 3 con GC-APCI-

MS/MS pero también a otras metodologías basadas en GC con otras configuraciones de detectores.

- Exploración del uso de purga y trampa con desorción térmica junto con instrumentos GC-APCI-MS (QqQ, QTOF e IMS) para la determinación ultra traza de VOCs en matrices complejas, aprovechando las capacidades de preconcentración de la P&T y la sensibilidad proporcionada por la fuente de APCI.

- Aplicación de las metodologías metabolómicas desarrolladas para la clasificación de otras matrices de acuerdo con su huella dactilar de VOCs.

Annex

UNIVERSITAT JAUME I
Escola de Doctorat · ED

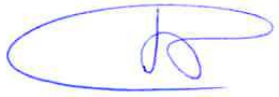
Tania Portolés Nicolau, como coautor/coautora doy mi **autorización** a **Carlos Sales Martínez** para la presentación de las siguientes publicaciones como parte de su tesis doctoral.

Relación de publicaciones:

1. Sales, C., Poma, G., Malarvannan, G., **Portolés, T.**, Beltrán, J., Covaci, A. Simultaneous determination of dechloranes, polybrominated diphenyl ethers and novel brominated flame retardants in food and serum. (2017) Analytical and Bioanalytical Chemistry, 409 (19), pp. 4507-4515.
2. **Portolés, T.**, Sales, C., Gómara, B., Sancho, J.V., Beltrán, J., Herrero, L., González, M.J., Hernández, F. Novel Analytical Approach for Brominated Flame Retardants Based on the Use of Gas Chromatography-Atmospheric Pressure Chemical Ionization-Tandem Mass Spectrometry with Emphasis in Highly Brominated Congeners. (2015) Analytical Chemistry, 87 (19), pp. 9892-9899. DOI: 10.1021/acs.analchem.5b02378
3. Sales, C., **Portolés, T.**, Sancho, J.V., Abad, E., Ábalos, M., Sauló, J., Fiedler, H., Gómara, B., Beltrán, J. Potential of gas chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry for screening and quantification of hexabromocyclododecane. (2016) Analytical and Bioanalytical Chemistry, 408 (2), pp. 449-459.
4. **Portolés, T.,** Sales, C., Abalos, M., Sauló, J., Abad, E. Evaluation of the capabilities of atmospheric pressure chemical ionization source coupled to tandem mass spectrometry for the determination of dioxin-like polychlorobiphenyls in complex-matrix food samples. (2016) Analytica Chimica Acta, 937, pp. 96-105.
5. Sales, C., Cervera, M.I., Gil, R., **Portolés, T.**, Pitarch, E., Beltran, J. Quality classification of Spanish olive oils by untargeted gas chromatography coupled to hybrid quadrupole-time of flight mass spectrometry with atmospheric pressure chemical ionization and metabolomics-based statistical approach. (2017) Food Chemistry, 216, pp. 365-373.
6. Sales, C., **Portolés, T.**, Johnsen, L.G., Danielsen, M., Beltran, J. Olive oil quality classification and measurement of its organoleptic attributes by untargeted GC–MS and multivariate statistical-based approach. (2019) Food Chemistry, 271, pp. 488-496.

Asimismo, **renuncio** a poder utilizar estas publicaciones como parte de otra tesis doctoral.

Y para que conste firmo el presente documento,



Castelló de la Plana, 2 de septiembre de 2019

Todo ello, atendiendo al artículo 23 de la Normativa de los Estudios de Doctorado, regulados por el RD 99/2011, en la Universitat Jaume I (Aprobada por el Consejo de Gobierno núm. 19 de 26 de Enero de 2012, modificada por el Consejo de Gobierno núm. 29 de 27 de Noviembre de 2012 y con posterior modificación por el Consejo de Gobierno núm. 37 de 25 de Julio de 2013):

"(...)

"Aquellas tesis doctorales que opten por la incorporación de artículos (compendio de publicaciones) deben de ajustarse, en la medida de lo posible, a la siguiente estructura: -Introducción/objetivos - Un capítulo por artículo incorporado - Discusión general de los resultados - Conclusiones. -Aceptación de los coautores de que el doctorando presente el trabajo como tesis y renuncia expresa de estos a presentarlo como parte de otra tesis doctoral."

UNIVERSITAT JAUME I
Escola de Doctorat · ED

Joaquim Beltrán Arandes, como coautor/coautora doy mi **autorización** a **Carlos Sales Martínez** para la presentación de las siguientes publicaciones como parte de su tesis doctoral.

Relación de publicaciones:

1. Sales, C., Poma, G., Malarvannan, G., Portolés, T., **Beltrán, J.**, Covaci, A. Simultaneous determination of dechloranes, polybrominated diphenyl ethers and novel brominated flame retardants in food and serum. (2017) Analytical and Bioanalytical Chemistry, 409 (19), pp. 4507-4515.
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3. Sales, C., Portolés, T., Sancho, J.V., Abad, E., Ábalos, M., Sauló, J., Fiedler, H., Gómara, B., **Beltrán, J.** Potential of gas chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry for screening and quantification of hexabromocyclododecane. (2016) Analytical and Bioanalytical Chemistry, 408 (2), pp. 449-459.
4. Sales, C., Cervera, M.I., Gil, R., Portolés, T., Pitarch, E., **Beltran, J.** Quality classification of Spanish olive oils by untargeted gas chromatography coupled to hybrid quadrupole-time of flight mass spectrometry with atmospheric pressure chemical ionization and metabolomics-based statistical approach. (2017) Food Chemistry, 216, pp. 365-373.
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UNIVERSITAT JAUME I

Escola de Doctorat · ED

Félix Hernández Hernández, como coautor/coautora doy mi **autorización** a **Carlos Sales Martínez** para la presentación de las siguientes publicaciones como parte de su tesis doctoral.

Relación de publicaciones:

1. Portolés, T., Sales, C., Gómara, B., Sancho, J.V., Beltrán, J., Herrero, L., González, M.J., **Hernández, F.** Novel Analytical Approach for Brominated Flame Retardants Based on the Use of Gas Chromatography-Atmospheric Pressure Chemical Ionization-Tandem Mass Spectrometry with Emphasis in Highly Brominated Congeners. (2015) Analytical Chemistry, 87 (19), pp. 9892-9899. DOI: 10.1021/acs.analchem.5b02378

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UNIVERSITAT JAUME I

Escola de Doctorat · ED

Elena Pitarch Arquimbau, como coautor/coautora doy mi **autorización** a **Carlos Sales Martínez** para la presentación de las siguientes publicaciones como parte de su tesis doctoral.

Relación de publicaciones:

1. Sales, C., Cervera, M.I., Gil, R., Portolés, T., **Pitarch**, E., Beltran, J. Quality classification of Spanish olive oils by untargeted gas chromatography coupled to hybrid quadrupole-time of flight mass spectrometry with atmospheric pressure chemical ionization and metabolomics-based statistical approach. (2017) Food Chemistry, 216, pp. 365-373.

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UNIVERSITAT JAUME I

Escola de Doctorat · ED

Juan V. Sancho Llopis, como coautor/coautora doy mi **autorización** a **Carlos Sales Martínez** para la presentación de las siguientes publicaciones como parte de su tesis doctoral.

Relación de publicaciones:

1. Portolés, T., Sales, C., Gómara, B., **Sancho, J.V.**, Beltrán, J., Herrero, L., González, M.J., Hernández, F. Novel Analytical Approach for Brominated Flame Retardants Based on the Use of Gas Chromatography-Atmospheric Pressure Chemical Ionization-Tandem Mass Spectrometry with Emphasis in Highly Brominated Congeners. (2015) Analytical Chemistry, 87 (19), pp. 9892-9899. DOI: 10.1021/acs.analchem.5b02378

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UNIVERSITAT JAUME I
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Rubén Gil Solsona, como coautor/coautora doy mi **autorización** a **Carlos Sales Martínez** para la presentación de las siguientes publicaciones como parte de su tesis doctoral.

Relación de publicaciones:

1. Sales, C., Cervera, M.I., **Gil, R.**, Portolés, T., Pitarch, E., Beltran, J. Quality classification of Spanish olive oils by untargeted gas chromatography coupled to hybrid quadrupole-time of flight mass spectrometry with atmospheric pressure chemical ionization and metabolomics-based statistical approach. (2017) Food Chemistry, 216, pp. 365-373.

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UNIVERSITAT JAUME I
Escola de Doctorat · ED

María Inés Cervera Vidal, como coautor/coautora doy mi **autorización** a **Carlos Sales Martínez** para la presentación de las siguientes publicaciones como parte de su tesis doctoral.

Relación de publicaciones:

1. Sales, C., **Cervera, M.I.**, Gil, R., Portolés, T., Pitarch, E., Beltran, J. Quality classification of Spanish olive oils by untargeted gas chromatography coupled to hybrid quadrupole-time of flight mass spectrometry with atmospheric pressure chemical ionization and metabolomics-based statistical approach. (2017) Food Chemistry, 216, pp. 365-373.

Asimismo, **renuncio** a poder utilizar estas publicaciones como parte de otra tesis doctoral.

Y para que conste firmo el presente documento,

MARIA INES| CERVERA| VIDAL

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Castelló de la Plana, 2 de septiembre de 2019

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UNIVERSITAT JAUME I
Escola de Doctorat · ED

Antwerp, 02-Sep-2019

I, **A. Covaci,** hereby authorise **Carlos Sales Martínez** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

1. Sales, C., Poma, G., Malarvannan, G., Portolés, T., Beltrán, J., **Covaci, A.** Simultaneous determination of dechloranes, polybrominated diphenyl ethers and novel brominated flame retardants in food and serum. (2017) Analytical and Bioanalytical Chemistry, 409 (19), pp. 4507-4515.

Signed,

In accordance with article 23 of the Regulation of Doctoral Studies, regulated by RD 99/2011, at the Universitat Jaume I (Approved by the Governing Council No. 19 of January 26, 2012, modified by the Governing Council no. 29 of November 27, 2012 and subsequent amendment by the Governing Council No. 37 of July 25, 2013):"(...) "Those doctoral theses that opt for the incorporation of articles (compendium of publications) must include..... the acceptance of the co-authors of the publications that have waived the right to present them as a part of another PhD thesis"

UNIVERSITAT JAUME I
Escola de Doctorat · ED

Antwerp, 02-Sep-2019

I, **G. Poma,** hereby authorise **Carlos Sales Martínez** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

1. Sales, C., **Poma, G**., Malarvannan, G., Portolés, T., Beltrán, J., Covaci, A. Simultaneous determination of dechloranes, polybrominated diphenyl ethers and novel brominated flame retardants in food and serum. (2017) Analytical and Bioanalytical Chemistry, 409 (19), pp. 4507-4515.

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UNIVERSITAT JAUME I
Escola de Doctorat · ED

Antwerp

02nd September 2019

I, **Govindan Malarvannan,** hereby authorise **Carlos Sales Martínez** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

1. Sales, C., Poma, G., **Malarvannan, G.**, Portolés, T., Beltrán, J., Covaci, A. Simultaneous determination of dechloranes, polybrominated diphenyl ethers and novel brominated flame retardants in food and serum. (2017) Analytical and Bioanalytical Chemistry, 409 (19), pp. 4507-4515.

Signed,

Govindan Malarvannan

In accordance with article 23 of the Regulation of Doctoral Studies, regulated by RD 99/2011, at the Universitat Jaume I (Approved by the Governing Council No. 19 of January 26, 2012, modified by the Governing Council no. 29 of November 27, 2012 and subsequent amendment by the Governing Council No. 37 of July 25, 2013):"(...) "Those doctoral theses that opt for the incorporation of articles (compendium of publications) must include..... the acceptance of the co-authors of the publications that have waived the right to present them as a part of another PhD thesis"

UNIVERSITAT JAUME I

Escola de Doctorat · ED

Esteban Abad Holgado, como coautor/coautora doy mi **autorización** a **Carlos Sales Martínez** para la presentación de las siguientes publicaciones como parte de su tesis doctoral.

Relación de publicaciones:

1. Sales, C., Portolés, T., Sancho, J.V., **Abad, E.**, Ábalos, M., Sauló, J., Fiedler, H., Gómara, B., Beltrán, J. Potential of gas chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry for screening and quantification of hexabromocyclododecane. (2016) Analytical and Bioanalytical Chemistry, 408 (2), pp. 449-459.
2. Portolés, T., Sales, C., Abalos, M., Sauló, J., **Abad, E.** Evaluation of the capabilities of atmospheric pressure chemical ionization source coupled to tandem mass spectrometry for the determination of dioxin-like polychlorobiphenyls in complex-matrix food samples. (2016) Analytica Chimica Acta, 937, pp. 96-105.

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UNIVERSITAT JAUME I
Escola de Doctorat · ED

Manuela Ábalos Navarro, como coautor/coautora doy mi **autorización** a **Carlos Sales Martínez** para la presentación de las siguientes publicaciones como parte de su tesis doctoral.

Relación de publicaciones:

1. Sales, C., Portolés, T., Sancho, J.V., Abad, E., **Ábalos, M.**, Sauló, J., Fiedler, H., Gómara, B., Beltrán, J. Potential of gas chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry for screening and quantification of hexabromocyclododecane. (2016) Analytical and Bioanalytical Chemistry, 408 (2), pp. 449-459.
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UNIVERSITAT JAUME I
Escola de Doctorat · ED

Jordi Sauló Dalmau, como coautor/coautora doy mi **autorización** a **Carlos Sales Martínez** para la presentación de las siguientes publicaciones como parte de su tesis doctoral.

Relación de publicaciones:

1. Sales, C., Portolés, T., Sancho, J.V., Abad, E., Ábalos, M., **Sauló, J.,** Fiedler, H., Gómara, B., Beltrán, J. Potential of gas chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry for screening and quantification of hexabromocyclododecane. (2016) Analytical and Bioanalytical Chemistry, 408 (2), pp. 449-459.

Asimismo, **renuncio** a poder utilizar estas publicaciones como parte de otra tesis doctoral.

Y para que conste firmo el presente documento,

Castelló de la Plana, 2 de septiembre de 2019

Todo ello, atendiendo al artículo 23 de la Normativa de los Estudios de Doctorado, regulados por el RD 99/2011, en la Universitat Jaume I (Aprobada por el Consejo de Gobierno núm. 19 de 26 de Enero de 2012, modificada por el Consejo de Gobierno núm. 29 de 27 de Noviembre de 2012 y con posterior modificación por el Consejo de Gobierno núm. 37 de 25 de Julio de 2013):

"(...)

"Aquellas tesis doctorales que opten por la incorporación de artículos (compendio de publicaciones) deben de ajustarse, en la medida de lo posible, a la siguiente estructura: -Introducción/objetivos - Un capítulo por artículo incorporado - Discusión general de los resultados - Conclusiones. -Aceptación de los coautores de que el doctorando presente el trabajo como tesis y renuncia expresa de estos a presentarlo como parte de otra tesis doctoral."

UNIVERSITAT JAUME I
Escola de Doctorat · ED

Belén Gómara Moreno, como coautor/coautora doy mi **autorización** a **Carlos Sales Martínez** para la presentación de las siguientes publicaciones como parte de su tesis doctoral.

Relación de publicaciones:

1. Portolés, T., Sales, C., **Gómara, B.**, Sancho, J.V., Beltrán, J., Herrero, L., González, M.J., Hernández, F. Novel Analytical Approach for Brominated Flame Retardants Based on the Use of Gas Chromatography-Atmospheric Pressure Chemical Ionization-Tandem Mass Spectrometry with Emphasis in Highly Brominated Congeners. (2015) Analytical Chemistry, 87 (19), pp. 9892-9899. DOI: 10.1021/acs.analchem.5b02378
2. Sales, C., Portolés, T., Sancho, J.V., Abad, E., Ábalos, M., Sauló, J., Fiedler, H., **Gómara, B.**, Beltrán, J. Potential of gas chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry for screening and quantification of hexabromocyclododecane. (2016) Analytical and Bioanalytical Chemistry, 408 (2), pp. 449-459.

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Y para que conste firmo el presente documento,

Madrid, 2 de septiembre de 2019

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UNIVERSITAT JAUME I
Escola de Doctorat · ED

María José González Carlos, como coautor/coautora doy mi **autorización** a **Carlos Sales Martínez** para la presentación de las siguientes publicaciones como parte de su tesis doctoral.

Relación de publicaciones:

1. Portolés, T., Sales, C., Gómara, B., Sancho, J.V., Beltrán, J., Herrero, L., **González, M.J.**, Hernández, F. Novel Analytical Approach for Brominated Flame Retardants Based on the Use of Gas Chromatography-Atmospheric Pressure Chemical Ionization-Tandem Mass Spectrometry with Emphasis in Highly Brominated Congeners. (2015) Analytical Chemistry, 87 (19), pp. 9892-9899. DOI: 10.1021/acs.analchem.5b02378

Asimismo, **renuncio** a poder utilizar estas publicaciones como parte de otra tesis doctoral.

Y para que conste firmo el presente documento,

Mª José González

Castelló de la Plana, 2 de septiembre de 2019

Todo ello, atendiendo al artículo 23 de la Normativa de los Estudios de Doctorado, regulados por el RD 99/2011, en la Universitat Jaume I (Aprobada por el Consejo de Gobierno núm. 19 de 26 de Enero de 2012, modificada por el Consejo de Gobierno núm. 29 de 27 de Noviembre de 2012 y con posterior modificación por el Consejo de Gobierno núm. 37 de 25 de Julio de 2013):

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UNIVERSITAT JAUME I
Escola de Doctorat · ED

Laura Herrero Collantes, como coautor/coautora doy mi **autorización** a **Carlos Sales Martínez** para la presentación de las siguientes publicaciones como parte de su tesis doctoral.

Relación de publicaciones:

1. Portolés, T., Sales, C., Gómara, B., Sancho, J.V., Beltrán, J., **Herrero, L.**, González, M.J., Hernández, F. Novel Analytical Approach for Brominated Flame Retardants Based on the Use of Gas Chromatography-Atmospheric Pressure Chemical Ionization-Tandem Mass Spectrometry with Emphasis in Highly Brominated Congeners. (2015) Analytical Chemistry, 87 (19), pp. 9892-9899. DOI: 10.1021/acs.analchem.5b02378

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Y para que conste firmo el presente documento,

Castelló de la Plana, 2 de septiembre de 2019

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UNIVERSITAT JAUME I
Escola de Doctorat · ED

Vedbaek, 3 September 2019

I, **M. Danielsen**, hereby authorise **Carlos Sales Martínez** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

1. Sales, C., Portolés, T., Johnsen, L.G., **Danielsen, M.**, Beltran, J. Olive oil quality classification and measurement of its organoleptic attributes by untargeted GC–MS and multivariate statistical-based approach. (2019) Food Chemistry, 271, pp. 488-496.

Signed,

Morten Danielsen, PhD
CEO
MS-Omics ApS
Denmark

In accordance with article 23 of the Regulation of Doctoral Studies, regulated by RD 99/2011, at the Universitat Jaume I (Approved by the Governing Council No. 19 of January 26, 2012, modified by the Governing Council no. 29 of November 27, 2012 and subsequent amendment by the Governing Council No. 37 of July 25, 2013):"(...) "Those doctoral theses that opt for the incorporation of articles (compendium of publications) must include..... the acceptance of the co-authors of the publications that have waived the right to present them as a part of another PhD thesis"

UNIVERSITAT JAUME I
Escola de Doctorat · ED

Farum, 3rd of September 2019

I, **L.G. Johnsen**, hereby authorise **Carlos Sales Martínez** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

1. Sales, C., Portolés, T., **Johnsen, L.G.**, Danielsen, M., Beltran, J. Olive oil quality classification and measurement of its organoleptic attributes by untargeted GC–MS and multivariate statistical-based approach. (2019) Food Chemistry, 271, pp. 488-496.

Signed,

Lea Johnsen, PhD
Senior scientist
MS-Omics-ApS
Denmark

In accordance with article 23 of the Regulation of Doctoral Studies, regulated by RD 99/2011, at the Universitat Jaume I (Approved by the Governing Council No. 19 of January 26, 2012, modified by the Governing Council no. 29 of November 27, 2012 and subsequent amendment by the Governing Council No. 37 of July 25, 2013):"(...) "Those doctoral theses that opt for the incorporation of articles (compendium of publications) must include..... the acceptance of the co-authors of the publications that have waived the right to present them as a part of another PhD thesis"