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D. Juan Ignacio Cacho Aparicio

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Memoria presentada para optar al grado de
Doctor en Química

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AUTORIZA:

La presentación de la Tesis Doctoral titulada "TÉCNICAS DE MICROEXTRACCIÓN ACOPLADAS A CROMATOGRAFÍA DE GASES-ESPECTROMETRÍA DE MASAS MEDIANTE DESORCIÓN TÉRMICA. APLICACIONES ANALÍTICAS", realizada por D. Juan Ignacio Cacho Aparicio, bajo mi inmediata dirección y supervisión, en el Departamento de Química Analítica, y que presenta para la obtención del grado de Doctor por la Universidad de Murcia.

Murcia, a 10 de Febrero de 2017

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If I have seen further
it is by standing
on the shoulders of Giants

Sir Isaac Newton

Abbreviations

Abbreviations

AA: acetic anhydride
ACE: acenaphthene
ACN: acetonitrile
ACY: acenaphthylene
ANT: anthracene
AP: alkylphenol
APEO: alkylphenol polyethoxylated
BAA: benz[a]anthracene
BBP: n-butylbenzylphthalate
BE: back-extraction
BF: empty baffled
BHA: butylated hydroxyanisole
BHT: butylated hydroxytoluene
BP: biphenol
BPA: bisphenol A
BPF: bisphenol F
BPs: bisphenols
BPZ: bisphenol Z
BSTFA: N,O-bis(trimethylsilyl)fluoroacetamide
BTEX: benzene, toluene, ethylbenzene and xylene isomers
BuP: butyl paraben
CA: chloroanisole
CAR: carvacrol
CB: chlorobenzene
CCα: decision limit
CCβ: detection capability
CCD: central composite design
CE: capillary electrophoresis
CHR: chrysene
CIA: cold-induced aggregation
CIS: cooled injector system
CP: chlorophenol
CPE: cloud point extraction
Cw: Carbowax
DAD: diode array detector
DBP: di-n-butylphthalate
DBT: dibutyltin
DBTDL: dibutyltin dilaurate
DCA: dichloroanisole
DCB: dichlorobenzene
DCP: dichlorophenol
DEHP: di(2-ethylhexyl) phthalate
DEP: diethyl phthalate
DLLME: dispersive liquid-liquid microextraction
DMP: dimethyl phthalate
DMT: dimethyltin
DOP: di-n-octylphthalate
DOT: dioctyltin
DPhT: diphenyltin
DPO: 2,6-diphenylphenylene oxide
DSI: direct sample introduction
ECD: electron capture detector
EDC: endocrine disruptor chemical
EG: ethylene glycol
EI: electron-impact
EPA: Environmental Protection Agency
EtP: ethyl paraben
EU: European Union
EUC: eucalyptol
EUG: eugenol
FG: fiberglass
FID: flame ionization detector
FLA: fluoranthene
FLD: fluorimetric detector
FLE: fluorene
GC: gas chromatography
GER: geraniol

HAs: haloanisoles
HCB: hexachlorobenzene
HF: hollow-fiber
HS: headspace
HSSE: headspace sorptive extraction
IARC: International Agency for Research on Cancer
iBuP: isobutyl paraben
ICP: inductive coupled plasma
IL: ionic liquid
IS: internal standard
LC: liquid chromatography
LIN: linalool
LLE: liquid-liquid extraction
LLME: liquid-liquid microextraction
LOD: limit of detection
LOQ: limit of quantification
LPME: liquid phase microextraction
LVI: large volume injection
MAE: microwave assisted extraction
MBT: monobutyltin
3M4CP: 3-methyl-4-chlorophenol
MEN: menthol
MeP: methyl paraben
MHSPE: mixed hemimicelles solid phase extraction
MIP-AED: microwave induced plasma-atomic emission detection
MMT: monomethyltin
MNPs: magnetic nanoparticles
MOT: monooctyltin
MS: mass spectrometry
MWCNTs: multiwalled carbon nanotubes
NAP: naphthalene
ND: not detected
NP: nitrophenol
4-NP: 4-nonylphenol
NQ: not quantified
OP: 4-*n*-octylphenol
OTC: organotin compound
PA: polyacrylate
PAH: polycyclic aromatic hydrocarbon
PBD: Plackett-Burman design
PBT: persistent, bioaccumulative, and toxic
PCA: pentachloroanisole
PCB: pentachlorobenzene
PCP: pentachlorophenol
PDMS: polydimethylsiloxane
PE: phthalate ester
PEG: polyethylene glycol
PET: polyethylene terephthalate
PFE: pressurized fluid extraction
PFPD: pulsed flame photometric detection
PHN: phenanthrene
PIL: polymeric ionic liquid
PP: polypropylene
PrP: propyl paraben
PS: polystyrene
PT: polyethylene
P&T: Purge-and-trap
PTV: programmed temperature vaporization
PVC: poly(vinyl chloride)
PYR: pyrene
QuEChERS: quick, easy, cheap, effective, rugged and safe
RSD: relative standard deviation
RT: retention time
RTE: ready-to-eat
SBSE: stir bar sorptive extraction
SCSE: stir cake sorptive extraction
SDME: single drop microextraction
SFODME: solidification of floating organic drop microextraction
SHS: static headspace

SIM: selected ion monitoring
SLE: solid-liquid extraction
SLM: specific migration limit
SPE: solid phase extraction
SPME: solid-phase microextraction
TBA: 2,4,6-tribromoanisole
TBHQ: *tert*-butyl hydroquinone
TC: temperature controlled
TCA: trichloroanisole
TCB: trichlorobenzene
TCP: trichlorophenol
TDU: thermal desorption unit
TeCA: tetrachloroanisole
TeCB: tetrachlorobenzene
THY: thymol
Tn: Tenax
TOF: time of flight
tOP: 4-*tert*-octylphenol
UAE: ultrasound assisted extraction
US: ultrasounds
USAEME: ultrasound assisted emulsification microextraction
UV: ultraviolet
WSVAME: water-contained surfactant-based vortex-assisted microextraction

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Resumen

Resumen

La preparación de la muestra es una de las etapas más complejas y costosas de todo método analítico. En los últimos años, gran parte de la investigación en Química Analítica se ha centrado en el desarrollo y puesta a punto de procedimientos que permitan la miniaturización de la etapa de preparación, con el objetivo fundamental de simplificarla.

La miniaturización de la preparación de la muestra, y su consiguiente preconcentración, permiten aumentar la sensibilidad de la instrumentación comúnmente empleada en el laboratorio analítico. Los métodos de análisis basados en este enfoque resultan mucho más versátiles, y por lo tanto, susceptibles de ser aplicados a una amplia variedad de problemas relacionados con la industria, la alimentación, el medio ambiente o las ciencias forenses.

Adicionalmente, la miniaturización de la preparación de muestra posee como ventaja la reducción en el consumo de disolventes orgánicos, y por tanto, de los residuos generados en el laboratorio. Esta reducción de la huella ecológica de la determinación analítica se enmarca dentro de los principios de sostenibilidad de la Química Analítica Verde.

La miniaturización en la preparación de la muestra ha dado lugar al desarrollo de las técnicas de microextracción, que permiten la extracción y concentración de los compuestos de interés presentes en la muestra en un pequeño volumen de una fase extractante, que puede ser tanto de naturaleza líquida como sólida.

Estas técnicas de microextracción pueden acoplarse a una amplia variedad de técnicas instrumentales, aunque la mayor parte de los métodos publicados hasta el momento han abordado su hibridación con técnicas de separación, como la cromatografía líquida (LC) o la cromatografía de gases (GC). En el caso de esta última, el acoplamiento con las técnicas de microextracción resulta más complejo, y por ello ha sido estudiado en mayor profundidad.

Teniendo en cuenta la relevancia de GC como técnica analítica, y la importancia que las técnicas de microextracción tienen en la Química Analítica actual, resulta conveniente el desarrollo de investigaciones que profundicen en las posibilidades de este acoplamiento.

Por ello, el objetivo fundamental de esta Tesis Doctoral ha sido el desarrollo de métodos analíticos que combinen diferentes técnicas de preparación de la muestra basadas en microextracción, con la determinación instrumental mediante separación por GC y detección por espectrometría de masas (MS).

Para lograr este acoplamiento, se ha empleado un nuevo sistema de introducción de la muestra paraGC, el inyector de desorción térmica (TD). Por sus características, este sistema, formado por la combinación de una unidad de desorción térmica (TDU) y un inyector de temperatura programada

(PTV), ofrece la posibilidad de extender el rango de aplicación y simplificar el acoplamiento de las técnicas de microextracción con determinaciones analíticas basadas en GC-MS.

A lo largo de esta Tesis Doctoral se han desarrollado diecinueve nuevos métodos analíticos. A fin de mostrar la aplicabilidad de las técnicas consideradas, así como reflejar la relevancia que las contribuciones de la Química Analítica pueden tener para nuestra sociedad contemporánea, se han abordado una amplia variedad de problemas analíticos, que incluyen la detección de compuestos de interés nutricional en alimentos, la presencia de contaminantes en muestras de origen medioambiental, o la detección de compuestos nocivos en envases plásticos.

El trabajo desarrollado se ha estructurado en cuatro bloques fundamentales, según las características de la técnica de microextracción empleada:

- Extracción por absorción sobre barras agitadoras (SBSE)
- Extracción por absorción en espacio de cabeza (HSSE)
- Microextracción en fase líquida (LPME) e introducción directa de muestra (DSI)
- Microextracción usando líquido iónicos (IL) e introducción directa de muestra (DSI)

La técnica SBSE implica la microextracción de los compuestos de interés hacia una fase sólida. Se trata de una técnica de preconcentración en la que la fase extractante es un polímero apolar, usualmente polidimetilsiloxano (PDMS), que recubre un agitador magnético, semejante a los empleados usualmente en el laboratorio. La extracción de la muestra se realiza sometiendo ésta a agitación magnética durante un periodo de tiempo determinado, durante el cual los compuestos de interés son extraídos y concentrados en la fase extractante de la barra agitadora. Posteriormente, los compuestos objeto de estudio son analizados por GC usando un inyector TD.

La combinación SBSE-GC ha sido empleada para la:

- Determinación de ftalatos (PEs) y alquilfenoles (APs) en vegetales.
  Estos compuestos, que pueden actuar como disruptores endocrinos (EDCs), son empleados como aditivos en los plásticos destinados al envasado de vegetales. Usando la combinación SBSE-GC se procede a su determinación en diferentes vegetales embolsados, evaluándose asimismo, mediante estudios de migración, su presencia en los alimentos como resultado de su liberación desde el material de envasado.
- Detección de bisfenoles (BPs) en productos enlatados
  Los BPs se emplean usualmente para el recubrimiento del interior de los envases metálicos destinados a alimentación, a fin de evitar la oxidación del material. Estos compuestos pueden migrar al producto final, ya se trate de una bebida refrescante o de un alimento en conserva, pudiendo originar problemas de salud. Debido a la naturaleza polar de estos compuestos, en este trabajo se evalúan dos procedimientos diferentes de derivatización específicos para la microextracción por SBSE.

- Cuantificación de resveratrol y compuestos relacionados en vinos.

  Los estilbenoides, entre los que se incluye el resveratrol, son compuestos presentes en la uva que han sido señalados como responsables de los beneficios que una ingesta moderada de vino puede tener para la salud cardiovascular. Dado que estos compuestos pueden estar presentes en la naturaleza en dos formas isoméricas, *cis-* y *trans-,* el método desarrollado contempla la determinación de ambas, mediante la generación del isómero *cis-* a partir del *trans-* por irradiación ultravioleta una vez preconcentrado en la fase extractante.
- Determinación de antioxidantes fenólicos sintéticos en refrescos.

  Los antioxidantes fenólicos se emplean usualmente como conservantes, a fin de evitar la degradación de los productos alimentarios. Pese a ello, a niveles elevados pueden ser perjudiciales para el consumidor, debido a su potencial como EDCs. Se estudian tres diferentes estrategias de derivatización para lograr la preconcentración de estos compuestos por SBSE.
- Detección de clorofenoles (CPs) y cloroanisoles (CAs) en vino.

  Estos compuestos organoclorados han sido señalados como responsables del olor a tapón, un defecto organoléptico del vino que origina graves pérdidas a la industria vinícola. Por primera vez se emplean recubrimientos extractantes para SBSE de naturaleza polar, como el co-polímero etilenglicol (EG)-silicona o el poliacrilato (PA), para la preconcentración de estos compuestos.
- Determinación de BPs en productos de higiene corporal.

  Debido al carácter polar de los BPs, su derivatización es un requisito previo a su extracción cuando se emplean fases extractantes de naturaleza apolar. Sin embargo, el empleo de fases polares en SBSE permite la determinación de estos compuestos sin necesidad de etapas previas de derivatización. El método desarrollado permite la determinación de estos compuestos en productos cosméticos y de higiene personal, en los que su contenido se encuentra restringido.

HSSE es una técnica de microextracción en fase sólida derivada de la SBSE y que también emplea como dispositivo de extracción una barra agitadora recubierta de una fase polimérica extractante. A diferencia de la SBSE, en HSSE el muestreo se realiza no sumergiendo la fase extractante en la muestra líquida, sino manteniéndola sobre la fracción gaseosa del vial que contiene la muestra. A través de HSSE se consigue extraer compuestos de interés presentes en la muestra líquida que se caractericen por su elevada volatilidad, a la vez que se logra eludir la posible interferencia de otros compuestos menos volátiles presentes en la muestra.

La combinación HSSE-GC ha sido utilizada en la presente Tesis Doctoral para la:

- Detección de compuestos organoestánnicos (OTCs) en aguas.
  Estos compuestos organometálicos se emplean, entre otros usos, como aditivos en tuberías de PVC. Por su toxicidad pueden resultar muy perjudiciales para el medio ambiente. A fin de incrementar su volatilidad, y favorecer su extracción por HSSE, se procede a su derivatización con tetraetilborato.
- Determinación de hidrocarburos aromáticos policíclicos (PAHs) en infusiones.
  Los PAHs son contaminantes prioritarios para la UE, debido a su naturaleza genotóxica. Dado que la ingesta de infusiones es una importante vía de entrada al organismo, se desarrolla un método para la cuantificación de diez de estos compuestos en este tipo de bebidas.
- Determinación de clorobencenos (CBs) en aguas.
  Los CBs se generan durante la etapa de cloración del agua, y a elevadas concentraciones en el agua de consumo pueden originar problemas en la salud. A fin de mejorar los resultados obtenidos mediante HSSE, se desarrolla un nuevo dispositivo para la exposición de la barra extractante, que aumenta la eficiencia de la etapa de preconcentración.
- Cuantificación de terpenoides en muestras de miel.
  Estos compuestos orgánicos volátiles son responsables tanto de las características organolépticas de la miel, como de algunas de sus propiedades beneficiosas para la salud. Debido a su naturaleza polar, se evalúa por primera vez el uso de fases extractantes de naturaleza polar en HSSE, para proceder a su extracción y preconcentración.

El inyector de desorción térmica permite no solo el análisis mediante GC de muestras sólidas, sino que también permite la introducción de muestras líquidas. Este enfoque, conocido como inyección directa, puede ser de gran interés en el caso de las técnicas de microextracción en fase líquida, y ha sido aplicado para la:

- Determinación de nitrofenoles (NPs) en suelos.
  Los NPs son compuestos que no se encuentran usualmente en la naturaleza y cuya presencia es indicativa de contaminación. Mediante el método propuesto se analizaron muestras de suelo de diferentes orígenes, siendo sometidas a una etapa de extracción asistida por ultrasonidos (UAE) a fin de extraer estos compuestos en un disolvente orgánico. Posteriormente, el extracto obtenido es preconcentrado mediante microextracción dispersiva líquido-líquido (DLLME).
- Determinación de CPs y CAs en bebidas alcohólicas.
  La extracción en punto de nube (CPE) supone el uso de tensoactivos para la extracción y preconcentración de los compuestos de interés en las micelas resultantes. Está técnica de microextracción se combina por primera vez con GC para la determinación de compuestos responsables de defectos organolépticos en diferentes bebidas alcohólicas.

Otra posibilidad del inyector de desorción térmica, dentro del enfoque de inyección directa, es el análisis por GC de muestras con matrices de elevada complejidad, sin necesidad de realizar ningún tipo de tratamiento de muestra previo a la determinación. Este enfoque ha sido utilizado para la:

- Detección de hidrocarburos aromáticos tóxicos en aceites vegetales.
  A fin de identificar algunos hidrocarburos volátiles tóxicos, como benceno, tolueno o xilenos, en muestras de aceites vegetales destinados a alimentación, se usó la técnica conocida como desorción térmica de microvial. Se logró así desarrollar un método sencillo, que implicaba una mínima manipulación de la muestra.
- Análisis directo de corchos para la detección de defectos organolépticos.
  La presencia de CAs en el corcho empleado para el embotellado del vino es el origen del defecto organoléptico conocido como olor a tapón. Usando la técnica de introducción directa de muestra en el sistema TDU-GC se desarrolló un método para la monitorización de cuatro CAs en muestras sólidas de corcho.

En los últimos años los líquidos iónicos (ILs) han captado gran atención dentro de la química. La Química Analítica, y especialmente la investigación en preparación de muestra ha sido reflejo de este interés, dando lugar a la adaptación de las técnicas de microextracción al uso de ILs, o al desarrollo de nuevas técnicas basadas en sus propiedades únicas.

Hasta la fecha, estas técnicas de microextracción no eran acoplables con GC, dado que los IL no cumplen el principal requisito de volatilidad necesario para cualquier muestra susceptible de analizarse por GC. Sin embargo, esta limitación puede ser superada de manera sencilla mediante el empleo del inyector de desorción térmica.

Esta posibilidad ha sido evaluada por primera vez en la presente Tesis Doctoral, dando lugar al desarrollo de tres métodos analíticos que combinan técnicas de microextracción basadas en IL y análisis por GC, a fin de llevar a cabo la:

- Determinación de parabenos en aguas.
  La preconcentración de los parabenos presentes en muestras de agua se logró usando dos técnicas diferentes de microextracción basadas en ILs. La muestra resultante se introdujo en el sistema GC usando la técnica de desorción térmica en microvial. El método optimizado se empleó para el análisis de parabenos a niveles traza en aguas de piscina.
- Evaluación de la migración de BPs desde envases alimentarios.
  La migración de BPs desde envases plásticos destinados al almacenamiento de alimentos se evaluó usando la combinación de una técnica de microextracción basada en la generación *in situ* del IL usado como fase extractante, y una introducción en el sistema GC mediante desorción térmica en microvial.

- Estudio de la migración de PEs desde envases destinados a contener aceites.
  Las características únicas de los ILs les hacen inmiscibles tanto en agua como en disolventes orgánicos. Esta propiedad se emplea en el trabajo desarrollado para emplearlos como fase extractante en la preconcentración de los PEs presentes en isooctano, disolvente usado como simulante de aceite en envases destinados a contener este producto alimentario.

A lo largo de la presente Tesis Doctoral se han desarrollado diferentes métodos analíticos, aplicables a una amplia variedad de problemas de interés, basados todos ellos en la combinación de una etapa de preparación de muestra basada en técnicas de microextracción, y su posterior determinación instrumental por GC-MS. Este acoplamiento ha sido posible gracias al empleo del inyector de desorción térmica, que ha mostrado ser un sistema de alta versatilidad, que expande los límites de aplicación de las técnicas disponibles actualmente en Química Analítica.

Summary

Summary

Sample preparation is one of the most complex and costly step of any analytical method. In recent years, much of the analytical chemistry research has focused on the development of procedures that allow the miniaturization of sample preparation, with the aim of simplifying this step.

Miniaturization of sample preparation, and its resulting preconcentration of the analytes, allows increasing the sensitivity of the instrumentation commonly used in the analytical laboratory. Analytical methods based on this approach are more versatile, and therefore may be applied to a wide variety of analytical problems, including those related to industry, food, environment or forensic sciences.

In addition, miniaturization of sample preparation allows a reduction in organic solvents consumption, and therefore of the laboratory residues generated, being an additional advantage. This reduction in the ecological footprint of the analytical determination is in line with the sustainability principles of Green Analytical Chemistry.

Miniaturization in sample preparation has led to the development of microextraction techniques, which allow the extraction and concentration of the compounds of interest present in the sample in a small volume of an extracting phase, which may be either a liquid or a solid.

Microextraction techniques can be coupled to a wide variety of instrumental techniques. However, most of the published analytical methods have used them in combination with separation techniques, such as liquid chromatography (LC) or gas chromatography (GC). In this last case, its coupling with microextraction techniques is more complex, and thus, it has been much less studied.

Taking into account the relevance of GC as an analytical technique, as well as the importance of microextraction techniques in current Analytical Chemistry, new researches deepening in this coupling and its possibilities are required. Therefore, the main aim of this Doctoral Thesis has been the development of new analytical methods that combine different sample preparation techniques based on microextraction and instrumental determination by GC - mass spectrometry (MS).

A new sample introduction system for GC, thermal desorption injector (TD) has been used to achieve this coupling. This system is composed by a Thermal Desorption Unit (TDU) and a Programmed Temperature Injector (PTV). Due to its advantageous characteristics, it simplifies the coupling of microextraction techniques with GC-MS. In this way, analytical methods with an extended range of application may be developed.

Throughout this Doctoral Thesis nineteen new analytical methods have been developed. In order to show the applicability of the techniques considered, as well as reflect the relevance of the contributions of Analytical Chemistry on our contemporary society, a wide variety of analytical

problems have been addressed, including the detection of compounds of nutritional interest in food, the presence of contaminants in samples of environmental origin, or the detection of harmful compounds in plastic containers.

The work developed has been structured in four fundamental blocks, according to the characteristics of the microextraction technique used:

- Stir bar sorptive extraction (SBSE)
- Headspace sorptive extraction (HSSE)
- Liquid phase microextraction (LPME) and direct sample introduction (DSI)
- Microextraction using ionic liquid (IL) and direct sample introduction (DSI)

SBSE is a solid phase microextraction technique, in which the extracting phase is commonly a non-polar polymer, usually polydimethylsiloxane (PDMS), which coats a magnetic stirrer, similar to those usually used in the laboratory. The extraction is performed by subjecting the sample to magnetic stirring for a period of time during which the compounds of interest are extracted and concentrated in the extractant phase of the stir bar. Subsequently, the compounds under study are analysed by GC using a TD.

The SBSE-GC combination has been used for the:

- Determination of phthalate esters (PEs) and alkylphenols (APs) in vegetables.
  These compounds, which can act as endocrine disruptor chemicals (EDCs), are incorporated as additives in the plastics used for the packaging of vegetables. Using the SBSE-GC combination, their determination in different pooled vegetables is evaluated, as well as its possible origin in the packaging by means of migration studies.
- Detection of bisphenols (BPs) in canned products
  BPs are usually added to the inside coating material of the metal containers destined to feed, in order to avoid the oxidation of the material. These compounds can migrate to the canned product, whether it is a refreshing drink or a canned food, which can lead to problems in human health. Due to the polar nature of these compounds, two different derivatisation procedures for SBSE microextraction are evaluated in this work.
- Quantification of resveratrol and related compounds in wines.
  Stilbenoids, including resveratrol, are compounds present in grapes that have been identified as responsible for the benefits that moderate wine intake may have for cardiovascular health. Since these compounds may be present in nature in two isomeric forms, *cis*- and *trans*-, this method contemplates the determination of both, by generating the *cis*- isomer by UV irradiation of the trans- compound once preconcentrated in the extracting phase.

- Analysis of synthetic phenolic antioxidants in soft drinks.
  Phenolic antioxidants are usually used as preservatives in order to avoid degradation of food products. However, at high levels, they may be harmful to the consumer, because of their potential as EDCs. Three different derivatization strategies are studied to achieve the preconcentration of these compounds by SBSE.
- Detection of chlorophenols (CPs) and chloroanisoles (CAs) in wine.
  These organochlorine compounds have been identified as responsible for the cork taint, an organoleptic defect of the wine that causes serious losses to the wine industry. For the first time, polar extractive coatings for SBSE, such as EG-silicone copolymer or PA, are used for the preconcentration of CPs and CAs.
- Determination of BPs in personal care products.
  Due to the polar character of BPs, their derivatisation is a prerequisite for their extraction in non-polar extracting phases. However, the use of polar phases in SBSE allows the determination of these compounds without the need of previous derivatisation steps. The developed method allows the determination of three different BPs in cosmetics and personal care products, in which their content is restricted.

Headspace sorptive extraction (HSSE) is a solid phase microextraction technique derived from SBSE, which also employs as extraction device a stir bar coated with an extracting polymeric phase. Unlike the SBSE, sampling is not performed by immersion of the extracting phase into the liquid sample, but to expose it to the sample headspace. In this way, it is sought to extract compounds of interest present in the liquid sample that are characterized by high volatility, while at the same time avoiding the possible interference of other less volatile compounds present in the sample.

The HSSE-GC combination has been used to:

- Detection of organtin compounds (OTC) in water.
  These organometallic compounds are used as additives in PVC pipes. Due to their toxicity, they can be very harmful to the environment. In order to increase their volatility, and favour the extraction by HSSE, they are derivatised with sodium tetraethylborate.
- Determination of polycyclic aromatic hydrocarbons (PAHs) in infusions.
  PAHs are priority pollutants for the EU because of their genotoxic nature. Since the intake of herbal infusions is an important route of entry into the body, a method is developed for the determination of ten PAHs in these beverages.
- Determination of chlorobenzenes (CBs) in water.
  CBs are generated during the chlorination stage of water, and high concentrations in drinking water can cause health problems. In order to improve the results obtained with

HSSE, a new device is developed for exposing the extracting bar, which increases the efficiency of the preconcentration step.

- Terpenoids quantification in honey.
  These volatile organic compounds are responsible for both the organoleptic characteristics of honey and some of its beneficial properties. Due to their polar nature, the use of extracting phases of polar character in HSSE is evaluated for the first time to extract and preconcentrate seven terpenoid compounds.

The thermal desorption injector allows not only the GC analysis of solid samples, but also allows the introduction of liquid samples. This approach, known as DSI, may be of great interest in the case of LPME techniques, and throughout this Doctoral Thesis it has been used for the:

- Determination of nitrophenols (NPs) in soils.
  NPs are not usually found in nature, and their presence is indicative of contamination. Under the proposed method, soil samples from different sources are subjected to ultrasonic assisted extraction (UAE), in order to transfer these compounds to an organic solvent. Subsequently, the obtained extract is preconcentrated using dispersive liquid-liquid microextraction (DLLME).
- Determination of CPs and CAs in alcoholic beverages.
  The cloud point extraction (CPE) procedure involves the use of surfactants for the extraction and preconcentration of the compounds of interest in the resulting micelles. This microextraction technique is combined for the first time with GC for the determination of compounds responsible for organoleptic defects in different alcoholic beverages.

Another possibility of the thermal desorption injector, within the direct injection approach, is the GC analysis of samples with high complexity matrices, without the need to carry out any kind of sample treatment prior to determination. This approach has been used for the:

- Determination of toxic hydrocarbons in vegetable oils
  In order to identify some toxic volatile aromatic hydrocarbons such as benzene, toluene or xylenes, the technique known as microvial thermal desorption was applied for the analysis of vegetable oil samples intended for feed. It was thus possible to develop a simple method, which involved a minimum sample manipulation.
- Direct analysis of corks for the detection of organoleptic defects.
  The presence of CAs in the cork used for wine bottling is the origin of the organoleptic defect known as cork taint. A method for the monitoring of four CPs in solid cork samples was developed using the DSI technique in the TDU-GC system.

In recent years, ILs have attracted more attention within chemistry. Analytical Chemistry, especially research in sample preparation has been a reflection of this interest, resulting in the adaptation of microextraction techniques to the use of ILs, or the development of new techniques based on their unique properties.

To date, these microextraction techniques have not been coupled to GC, since ILs do not meet the main volatility requirements for any GC susceptible sample. However, this limitation can be overcome simply by the use of the thermal desorption injector.

This possibility has been evaluated for the first time in this Doctoral Thesis, giving rise to the development of three analytical methods combining IL-based microextraction techniques and GC analysis for the:

- Determination of parabens in water.
  The preconcentration of the parabens present in water samples was achieved using two different microextraction techniques based on ILs. The resulting sample was introduced into the GC system using the microvial thermal desorption. The resulting method was applied for the determination of five parabens at trace levels in pool waters.
- Evaluation of BPs migration from food packaging.
  The migration of BPs from plastic containers intended for food storage was evaluated using the combination of a microextraction technique based on the *in situ* generation of the IL used as the extracting phase and its introduction into the GC system by microvial thermal desorption.
- Study of PEs migration from containers destined for edible oils.
  The unique characteristics of ILs make them immiscible with both water and organic solvents. This property is exploited in this work to be used as extracting phase in the preconcentration of the PEs present in isooctane, being this solvent used as an oil-simulant solvent in containers intended to contain these food products.

Throughout the present Doctoral Thesis different analytical methods have been developed, applicable to a wide variety of problems of interest, all based on the combination of a sample preparation stage based on microextraction techniques, and their subsequent instrumental determination by GC-MS. This coupling has been possible by using the thermal desorption injector, which has shown to be a highly versatile system, which expands the limits of application of the techniques currently available in Analytical Chemistry.

Objeto

El trabajo presentado en esta memoria de Tesis Doctoral forma parte de una de las líneas que el grupo de investigación "Métodos Instrumentales Aplicados" viene desarrollando dentro del Departamento de Química Analítica de la Universidad de Murcia.

Esta línea de investigación se centra en el desarrollo y puesta a punto de procedimientos que permitan la miniaturización de la etapa de preparación de muestra en el laboratorio analítico. El objetivo fundamental es simplificar esta etapa, usualmente la más compleja y costosa de todo método analítico.

Los esfuerzos desarrollados en la miniaturización de la preparación de la muestra han dado lugar al desarrollo de las técnicas de microextracción. Estas técnicas permiten extraer los compuestos de interés presentes en la muestra en un pequeño volumen de fase extractante, tanto de naturaleza líquida como sólida. Así, las técnicas de microextracción permiten la preconcentración de los analitos antes de su determinación instrumental.

Las técnicas de microextracción permiten alcanzar una mayor sensibilidad usando la instrumentación comúnmente empleada en el laboratorio. Se logra así la determinación de cantidades de sustancia muchos menores que las detectables usando métodos analíticos convencionales. Por lo tanto, los métodos basados en microextracción resultan mucho más versátiles, pudiendo aplicarse a una gran variedad de problemas analíticos, ya sea en la industria alimentaria o en las ciencias forenses.

Las técnicas de microextracción han sido usualmente acopladas a una amplia variedad de técnicas instrumentales, centrándose el grupo de investigación "Métodos Instrumentales Aplicados" en su hibridación con espectrometría de absorción atómica y técnicas cromatográficas, como la cromatografía líquida. En el caso de los métodos analíticos objeto de estudio en la presente tesis, las técnicas de microextracción han sido acopladas a cromatografía de gases.

El objetivo fundamental de esta Tesis Doctoral ha sido profundizar en el acoplamiento de diferentes técnicas de preparación de la muestra basadas en microextracción con la determinación instrumental mediante separación por cromatografía de gases y detección por espectrometría de masas. A este fin, se empleará un nuevo sistema de introducción de la muestra para cromatografía gaseosa, el inyector de desorción térmica. Por sus características, este sistema, formado por la combinación de una Unidad de Desorción Térmica (TDU) y un Inyector de Temperatura Programada (PTV), ofrece la posibilidad de extender el rango de aplicación y simplificar el acoplamiento de las técnicas de microextracción con determinaciones analíticas basadas en cromatografía de gases.

Este objetivo es el nexo común de los resultados recogidos en esta memoria y que se estructuran en cuatro capítulos:

Capítulo 1. Acoplamiento de la extracción por absorción sobre barra agitadora y cromatografía de gases mediante desorción térmica.
Stir-Bar Sorptive Extraction and Gas-Chromatography coupling by Thermal Desorption.

Capítulo 2. Acoplamiento de la extracción por absorción en espacio de cabeza y cromatografía de gases mediante desorción térmica.
Headspace Sorptive Extraction and Gas-Chromatography coupling by Thermal Desorption.

Capítulo 3. Introducción directa de muestra e inyección de grandes volúmenes en microvial.
Direct Sample Introduction and Microvial insert Large Volume Injection.

Capítulo 4. Inyección de grandes volúmenes en microvial para el acoplamiento de líquidos iónicos y cromatografía de gases.
Microvial insert Thermal Desorption:coupling Ionic Liquids and Gas-Chromatography.

Adicionalmente se incluye en esta memoria una introducción a las técnicas de microextracción, así como al sistema de introducción de muestra por desorción térmica, empleado para su acoplamiento con cromatografía de gases.

Introducción

1. Técnicas de Microextracción

Con objeto de responder a las demandas crecientes de la sociedad, el laboratorio analítico ha experimentado grandes cambios a lo largo de los últimos años. La relevancia de las aplicaciones del análisis químico, desde el control de aditivos o toxinas en productos alimentarios, hasta la determinación de pesticidas o contaminantes en el medio ambiente, ha obligado a esta disciplina científica a centrar sus esfuerzos en un desarrollo permanente, en pos de una mayor sensibilidad y selectividad, a las que cada vez más se añaden mayores requisitos de seguridad y calidad.

Pese a la imagen cada vez más extendida de un laboratorio analítico plenamente automatizado, en el que el analista es un mero colector de resultados, la mayoría de los instrumentos analíticos no pueden proceder a la determinación directa de los compuestos objeto de interés. Es necesaria una etapa previa de preparación en la que se proceda a la separación, extracción y concentración de los analitos del resto de componentes que forman la matriz de la muestra. Tal etapa es fundamental en cualquier procedimiento analítico y no puede estar sujeta a una metodología general, pues dependerá tanto de la naturaleza del analito como de la muestra a analizar.

La complejidad de las muestras objeto de análisis, así como las implicaciones que los resultados a obtener pueden tener en el ámbito de estudio, hacen aún más difícil el desarrollo de técnicas de preparación de la muestra. Buena parte de las investigaciones actuales en el campo de la química analítica se encaminan precisamente hacia la puesta a punto de nuevos procedimientos de preparación de la muestra. En este sentido, las tendencias recientes se centran en el desarrollo de técnicas de microextracción, que permiten una simplificación, miniaturización, y potencial automatización de la preparación de la muestra, limitan el uso de disolventes perjudiciales para el medio ambiente, a la vez que aseguran una buena eficiencia de extracción.

1.1. Características y ventajas generales

Se conocen como técnicas de microextracción (ME) aquellos procedimientos de preparación de la muestra que extraen y concentran los analitos en volúmenes de una fase extractante (de naturaleza sólida o líquida) menores a 100 µL. Desde su introducción hace ya casi dos décadas, estas técnicas han sido empleadas para la extracción, purificación y concentración de una gran variedad de analitos, incluyendo especies volátiles y no volátiles, polares y apolares, iónicas y metálicas en muestras medioambientales, clínicas, farmacéuticas y alimentarias. Las bases de datos de publicaciones científicas recogen más de once mil artículos relativos al uso de técnicas de ME en química analítica, y el número de trabajos publicados se incrementa año tras año.

Las técnicas de ME presentan importantes ventajas cuando se contemplan desde diferentes puntos de vista:

- Respetuosas con el medio ambiente

Las técnicas de microextracción están basadas en las técnicas clásicas de preparación de muestra, como la extracción líquido-liquido o la extracción en fase sólida. Estas metodologías clásicas, además de ser lentas y a veces tediosas, implican el empleo de grandes cantidades de disolventes orgánicos. En los últimos años se ha suscitado un gran interés por el desarrollo de procedimientos analíticos respetuosos con el medio ambiente. Este objetivo se ha plasmado en los conocidos como los doce principios de la Química Verde [1], entre los que se incluye la sustitución o limitación en el empleo de reactivos tóxicos. En esta línea de actuación se enmarcan las técnicas de ME, pues en ellas se reduce el uso de disolventes orgánicos a unos pocos microlitros por ensayo, e incluso en el caso de ME en fase sólida, puede evitarse por completo el uso de disolventes. Se logra así no solo disminuir el impacto medioambiental del procedimiento analítico, sino que se evita la exposición de los trabajadores del laboratorio a productos nocivos para su salud, y se minimiza la necesidad de un tratamiento de residuos, con el incremento de costes asociado al mismo.

- Mejora de la sensibilidad.

Los límites legales para sustancias tóxicas y contaminantes están en continuo descenso, como resultado de una demanda de mayor calidad y seguridad por parte de la sociedad. Existe un requerimiento de métodos analíticos más sensibles, tanto para controlar la presencia de sustancias reguladas dentro de sus límites establecidos, como para detectar la presencia de compuestos contaminantes o que supongan un peligro para la salud, como especies de carácter genotóxico o carcinogénicos. Resulta posible alcanzar mayores sensibilidades sin la necesidad de adquirir nueva y costosa instrumentación de laboratorio. Las técnicas de ME concentran los analitos presentes en grandes volúmenes de muestra en unos pocos microlitros, proporcionando así factores de enriquecimiento del orden de varias centenas de veces, y reduciendo en una magnitud semejante los límites de detección.

- Bajo precio y fácil manejo.

La implementación de las técnicas de ME en cualquier laboratorio resulta sumamente sencilla, ya que usualmente no requieren de aparataje o instrumentación específica. Los elementos empleados en estos procedimientos son normalmente utilizados en todo laboratorio analítico, y en todo caso presentan un coste relativamente bajo. Dado que uno de los objetivos del desarrollo de las técnicas de microextracción es la simplificación del procedimiento analítico y la reducción de su número de etapas, se trata de métodos rápidos, que suponen un considerable ahorro en tiempo. Adicionalmente, las destrezas requeridas para su desarrollo e implementación son fácilmente

adquiribles, y cualquier operario de laboratorio puede llegar a dominar estas técnicas con un poco de práctica.

- Versatilidad en la forma de detección final.

Desde su origen, las técnicas de ME fueron desarrolladas para su hibridación con diferentes técnicas instrumentales. Los pequeños volúmenes obtenidos tras la aplicación de estos procedimientos facilitan su acoplamiento con técnicas de separación como la cromatografía líquida, de gases y la electroforesis capilar. Asimismo, las técnicas de ME han sido empleadas en combinación con espectroscopia visible-ultravioleta y de absorción atómica.

- Posibilidad de automatización.

Debido a su simplicidad y fácil hibridación estas técnicas son susceptibles de ser automatizadas, si no totalmente, al menos en la mayoría de sus etapas, permitiendo un importante ahorro de tiempo.

1.2. Tipos de técnicas para microextracción

Una amplia variedad de técnicas de preparación de la muestra basadas en el empleo de fases extractantes de volumen micrométrico han sido desarrolladas en los últimos años. Estas técnicas de microextracción han mostrado ser baratas, sencillas y rápidas, permitiendo obtener resultados con una gran sensibilidad y fiabilidad. Debido a su versatilidad son fácilmente adaptables a los requerimientos de cualquier problema analítico.

En términos generales, es posible distinguir dos tipos principales de técnicas de ME de acuerdo a la naturaleza de la fase extractante: ME en fase líquida y ME en fase sólida. A continuación, se describen y comentan brevemente algunas de las técnicas más habituales, aunque existe una amplia variedad de modificaciones propuestas sobre estas técnicas generales, para su adaptación a los requisitos específicos de la muestra o los analitos.

1.2.1. Microextracción en fase líquida (LPME)

Las técnicas de LPME derivan de las extracciones líquido-líquido (LLE) tradicionales, que históricamente han sido el método de separación más habitual en química analítica. Las técnicas de LPME combinan la extracción y concentración de los analitos en una única etapa, empleando a este fin unos pocos microlitros de un disolvente inmiscible con el agua, que actuará como fase extractante.

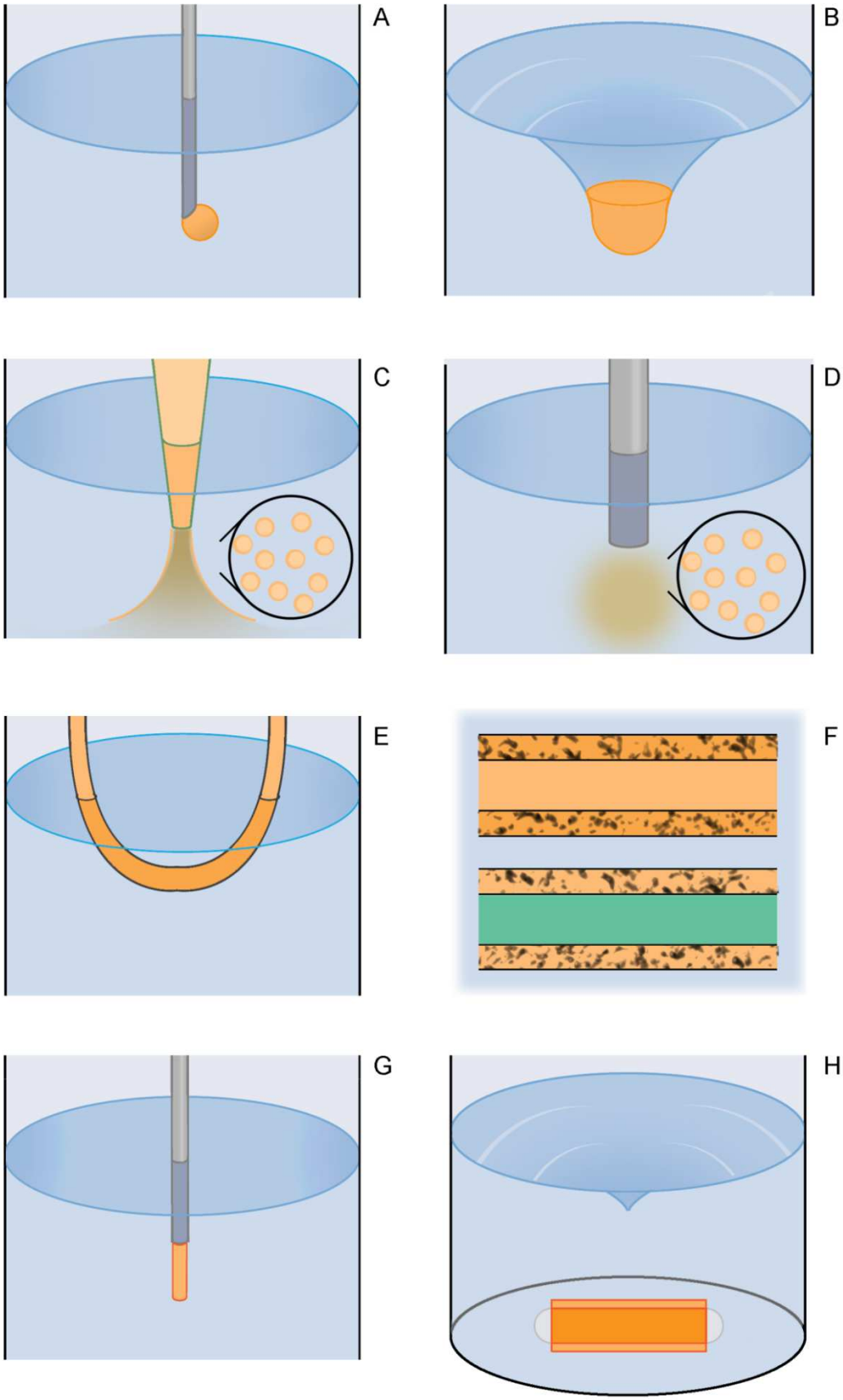

Fig. 1. Representación esquemática de diferentes técnicas de microextracción:
A. SDME, B. DSDME, C. DLLME, D. USAEME, E. F. HF-LPME, G. SPME, H. SBSE.

Existen dos principales categorías de LPME, según si el disolvente orgánico que actúa como extractante se expone directamente a la muestra o se dispone de una membrana para separar la fase dadora (muestra) y la aceptora (extractante).

Exposición directa

La primera técnica de LPME fue la denominada microextracción en gota simple (*single drop microextraction,* SDME) (Fig. 1A), desarrollada en 1996 por Jeannot [2]. En esta técnica los analitos son extraídos hacia una gota de disolvente orgánico (por lo general, unos pocos microlitros) situada en la punta de una microjeringa. Una vez completada la extracción, la gota es retraída hacia el interior de la microjeringa e inyectada en el instrumento analítico [3].

A fin de evitar los problemas derivados de mantener una gota de tan pequeño tamaño en el extremo de la microjeringa, se desarrolló la técnica conocida como microextracción en gota directamente suspendida [4] (*directly suspended droplet microextraction,* DSDME) (Fig. 1B). En esta técnica, un pequeño volumen del disolvente orgánico es añadido a la muestra a fin de extraer los analitos de interés. Al ser inmiscible con la fase acuosa, esta gota de disolvente podrá ser recogida una vez terminada la extracción usando una microjeringa [5].

La difícil recuperación de la gota de fase extractante puede ser simplificada si se emplea un disolvente de punto de fusión cercano a la temperatura ambiente (como 1-undecanol o 2-decanona), dando lugar a la técnica conocida como microextracción por solidificación de la gota orgánica flotante (*solidification of floating organic drop microextraction,* SFODME) [6,7]. Así, una vez completada la extracción bastará con enfriar levemente la mezcla, para que la gota solidifique en la superficie de ésta y pueda ser extraída fácilmente. Un leve calentamiento posterior volverá al disolvente extractante al estado líquido.

Las técnicas de ME expuestas requieren de un cierto tiempo de extracción para que se alcance el equilibrio de reparto de los analitos entre la fase dadora y aceptora. El incremento en la superficie de contacto entre ambas fases puede acelerar este proceso, pasando a completarse en unos pocos segundos. A este fin, es necesario formar una emulsión entre la fase extractante y la muestra acuosa, que después deberá ser rota por un proceso de centrifugación para separar las fases y recuperar el disolvente orgánico conteniendo los analitos.

La formación de esta emulsión puede lograrse empleando un disolvente auxiliar que actúe como dispersante de la fase extractante, y que debe ser soluble tanto en agua como en el disolvente orgánico empleado (ej: mezcla ternaria: tetracloruro de carbono/acetona/agua). Esta técnica es conocida como microextracción dispersiva líquido-líquido (*dispersive liquid-liquid microextraction,* DLLME) (Fig. 1C) [8,9]. A veces se emplean ultrasonidos para formar la emulsión entre la muestra acuosa y el disolvente extractante, que sustituyen al disolvente

dispersante, dando lugar a la técnica conocida como microextracción por emulsificación asistida por ultrasonidos (*ultrasound assisted emulsification microextraction,* USAEME) (Fig. 1D) [10]. La metodología dispersiva es una de las técnicas de ME de más amplia aplicabilidad y su popularidad ha crecido mucho en los últimos años [11].

Fibra hueca

En la técnica llamada LPME con fibra hueca (*hollow fiber - liquid phase microextraction,* HF-LPME) (Fig. 1E) [12,13] se emplea una fibra hueca de alta porosidad y carácter hidrófobo a fin de albergar la fase extractante, evitando así la posible pérdida del disolvente y facilitando su recuperación. Usualmente esta fibra hueca se ubica en un montaje en forma de U, que se sumerge en la disolución conteniendo las especies de interés. Según el número de disolventes empleados en el montaje se distingue entre sistemas de dos y tres fases.

En un sistema de dos fases, el disolvente empleado para impregnar los huecos de la fibra es el mismo que rellena su interior. Posteriormente este disolvente es extraído usando una jeringa, para proceder a su análisis. En un sistema de tres fases la fibra es impregnada con un disolvente orgánico a fin de que éste ocupe los huecos de sus paredes, mientras que otro disolvente diferente e inmiscible con el primero (usualmente agua) rellena el interior de la fibra (Fig. 1F). Así, los analitos serán inicialmente extraídos desde la disolución de la muestra hacia el disolvente orgánico alojado en las paredes de la fibra, para posteriormente ser extraídos de nuevo hacia la fase acuosa del interior de la fibra.

Líquidos iónicos

Las relevantes propiedades de los líquidos iónicos, y en especial la hidrofobicidad que los hace muy adecuados como fase extractante, ha dado lugar en los últimos años a buen número de técnicas de microextracción que involucran su empleo [14,15]. Hasta la fecha, su mayor inconveniente ha sido su incompatibilidad con la cromatografía de gases, debido a su baja volatilidad y alta temperatura de ebullición.

1.2.2. Microextracción en fase sólida

Las técnicas de ME en fase sólida están basadas en la extracción de los analitos desde la muestra hacia una fase por lo general de naturaleza polimérica, que se encuentra unida a un soporte sólido. Los analitos no son únicamente retenidos sobre la superficie del extractante, sino que son absorbidos en la totalidad del volumen de éste. La efectividad de la extracción vendrá dada por el coeficiente de partición de los analitos entre la muestra y la fase extractante.

Microextracción en fase sólida (SPME)

La primera aplicación de la ME empleando una fase extractante sólida fue la técnica conocida con el mismo nombre del grupo, microextracción en fase sólida (*solid phase microextraction,* SPME) (Fig. 1G), desarrollada por Pawliszyn en 1990 [16].

En SPME los analitos son concentrados sobre una pequeña fibra de sílice recubierta de un polímero absorbente, siendo polidimetilsiloxano (PDMS) el más utilizado [17]. Esta fibra se encuentra unida a un sistema tipo jeringa, que permite su exposición al espacio de cabeza del vial conteniendo la muestra (HS-SPME) [18] o su inmersión directa (DI) en ésta, y su posterior retracción. Los analitos retenidos sobre la fibra pueden ser desorbidos empleando pequeños volúmenes de disolventes orgánicos, o bien directamente en el instrumento analítico mediante un tratamiento térmico.

La técnica SPME supuso un gran avance con respecto a las técnicas clásicas de preparación de muestra, dado que se trataba de una técnica simple, fácilmente acoplable a técnicas de separación, y que presentaba una buena respuesta para una gran variedad de analitos, proporcionando resultados de elevada fiabilidad. Sin embargo, presenta algunas desventajas, como la fragilidad de la fibra empleada, o lo limitado de la vida útil de éstas, como resultado de su degradación térmica o por exposición a disolventes.

Extracción por absorción sobre barras agitadoras (SBSE)

La técnica llamada extracción por absorción sobre barras agitadoras (*stir bar sorptive extraction,* SBSE) (Fig. 1H) fue desarrollada por Patrick Sandra en 1999 [19]. Esta técnica se basa en los mismos principios que en SPME, con la salvedad de que la fase absorbente polimérica se encuentra fijada, no ya sobre una fibra de vidrio, sino sobre una barra magnética semejante a las usualmente empleadas para la agitación de muestras.

Este montaje permite un incremento en el volumen de fase extractante, que alcanza de 24 a 126 μL, un volumen de 50 a 250 veces mayor que el usualmente empleado en SPME. Este incremento en el volumen de la fase extractante da lugar a un aumento en la cantidad de analitos susceptibles de ser retenidos, y por lo tanto, de la potencial sensibilidad a obtener [20]. Además, el montaje sobre barra agitadora de la SBSE hace de esta una técnica de ME mucho más robusta, ya que las barras agitadoras tienen mayor vida útil, lo que facilita su aplicación rutinaria en el laboratorio.

Al igual que en el caso de la SPME, la desorción de los analitos absorbidos en la fase extractante puede realizarse empleando pequeños volúmenes de disolventes orgánicos, en los que la barra agitadora recubierta de fase extractante se sumerge y libera los compuestos retenidos. Una alternativa a esta desorción líquida, es la realización de una desorción térmica, en la que los

analitos retenidos en la fase extractante son vaporizados mediante un tratamiento térmico en un inyectores específico (unidad de desorción térmica) [21].

La extracción por absorción desde el espacio de cabeza (*headspace sorptive extraction,* HSSE) [22] es una técnica derivada de SBSE, empleada para la preconcentración de analitos de elevada volatilidad. En esta técnica, el dispositivo de extracción no se sumerge en la muestra, sino que se expone a la atmósfera del vial que la contiene, de forma semejante a como se hace en HS-SPME [23]. Los compuestos de interés, de elevada volatilidad, pasan de la muestra a la atmósfera del vial, y de esta fase gaseosa a la fase extractante. La eficiencia de extracción con esta técnica es semejante a la alcanzada mediante SBSE, limitándose la posible interferencia de especies no volátiles que no son extraídas.

2. Acoplamiento de las técnicas de microextracción con técnicas cromatográficas

Los nuevos procedimientos de preparación de la muestra basados en microextracción han sido acoplados a una gran variedad de técnicas instrumentales, entre las que se incluyen las técnicas cromatográficas, tanto líquida como de gases. Estas técnicas, debido a su capacidad de separar los diferentes componentes de la muestra y aumentar así la especificidad del método analítico, así como a la elevada sensibilidad de los detectores a los que se acoplan, se han convertido en la base del análisis químico actual, sustituyendo a los métodos clásicos de análisis en el trabajo habitual de laboratorio.

Gran parte del éxito de las técnicas cromatográficas es achacable a los magníficos resultados que proporciona la cromatografía de gases (GC). Inicialmente desarrollada en los años cincuenta del siglo pasado, las mejoras en el diseño de las columnas cromatográficas y los avances en el desarrollo electrónico han convertido a la GC en la técnica más utilizada para el análisis cualitativo y cuantitativo de mezclas de compuestos orgánicos de volatilidad media y alta. El empleo de diferentes estrategias, como la derivatización, permite ampliar el campo potencial de aplicación de esta técnica a otros analitos de baja volatilidad que usualmente no podrían ser determinados por GC.

El empleo del espectrómetro de masas (MS) como detector en GC extiende la aplicabilidad de esta técnica instrumental, al combinar la elevada capacidad de separación de GC con la selectividad inherente a la detección espectrométrica, que proporciona no solo una respuesta cuantitativa, sino también información estructural sobre el compuesto detectado. A pesar de los avances en otras técnicas instrumentales, como LC acoplada a MS, GC-MS sigue siendo no solo una instrumentación de referencia, sino también una técnica para la que continuamente se desarrollan nuevas metodologías analíticas.

Pese a ello, la gran mayoría de los métodos analíticos basados en microextracción se han acoplado a sistemas de LC, debido a su mayor sencillez [5]. La naturaleza líquida del extractante en LPME, o la posibilidad en SMPE de realizar una re-extracción hacia una fase líquida compatible con LC, hacen que las técnicas de ME sean fácilmente combinables con esta técnica cromatográfica. A esta semejanza en la naturaleza de sus fases se une el empleo en LC de volúmenes de inyección usualmente mayores que en GC y semejantes a los obtenidos en los procedimientos de microextracción.

Pese a resultar menos directa, una amplia variedad de métodos analíticos también se han basado en la combinación de técnicas de microextracción y GC [24].

2.1 Acoplamiento de técnicas de microextracción y GC usando inyector split/splitless

Por la propia naturaleza de la técnica GC, resulta necesario que la muestra se encuentre en fase gaseosa antes de proceder a su separación. Esta vaporización de la muestra líquida tiene lugar en el inyector, un componente del sistema GC que logra, mediante la aplicación de elevadas temperaturas, el paso a fase gaseosa de los analitos y su transferencia a la columna cromatográfica.

El inyector más usual en un sistema GC es el de tipo con/sin división de flujo (*split/splitless)* (Fig. 2). En este dispositivo, el puerto de inyección se mantiene a una temperatura elevada, superior a la temperatura de ebullición de los componentes de la muestra a analizar. La muestra líquida es introducida usando una microjeringa, y liberada en el interior del *liner* del inyector, que por su geometría y disposición favorece su vaporización, a la vez que limita la degradación de los compuestos de interés.

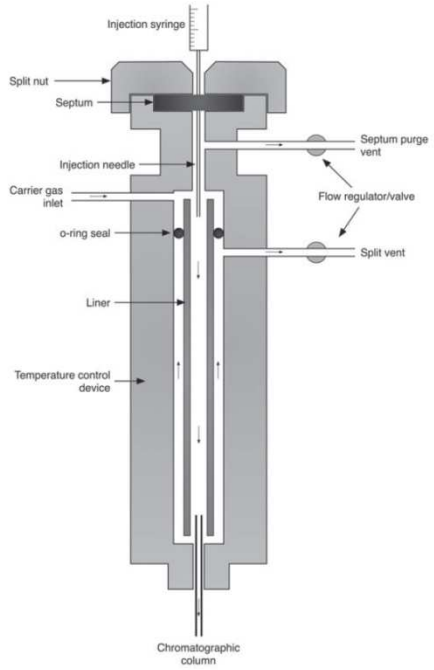

Fig. 2. Inyector *split/splitless*.

El nombre del dispositivo, *split/splitless*, deriva de su capacidad de realizar una selección del flujo que pasa del inyector a la columna cromatográfica. En una inyección *splitless* toda la muestra vaporizada pasa a la columna cromatográfica para su separación, mientras que en una inyección *split* solo una porción de la muestra, definida por una fracción denominada *split ratio* continúa a través del sistema cromatográfico. Esta última opción puede resultar conveniente en muestras de elevada concentración o matrices complejas.

Microextracción en fase líquida

Algunas técnicas de microextracción pueden ser fácilmente acopladas a GC usando inyectores de tipo *split/splitless*. Tal es el caso de LPME, en la que un pequeño volumen de la fase extractante, inferior a los 3-4 μL, es introducido como si se tratase de una muestra convencional. Este volumen solo representa una pequeña parte del volumen total del extracto obtenido tras la aplicación de la técnica de LPME, lo que implica una importante pérdida de muestra, y una pérdida potencial de sensibilidad durante el acoplamiento instrumental.

Los compuestos de interés solo van a suponer una pequeña fracción de la muestra inyectada, dado que la mayor parte de ésta corresponde al disolvente empleado como fase extractante. Así, para que la combinación entre LPME y GC sea factible, resulta necesario que la fase extractante empleada en la microextracción sea compatible con GC, lo que limita la amplia variedad de disolventes orgánicos susceptibles de utilizarse como fases extractantes.

El empleo de fases extractantes de baja volatilidad daría lugar a la retención de estos disolventes en el inyector del equipo, pudiendo causar una contaminación del inyector, originando bloqueos del sistema neumático al impedir el flujo de gas portador, o incluso a una contaminación de la columna en caso de alcanzar ésta, dando lugar a efectos memoria o picos fantasma. El empleo, por parte de algunos autores, de fases extractantes de baja volatilidad directamente inyectadas en el sistema GC ha requerido de modificaciones de este sistema para evitar los problemas descritos [25].

Idealmente, la fase extractante debe ser un disolvente volátil, con un punto de ebullición inferior al de los analitos. De esta manera es posible asegurar su elución a tiempos de retención cortos, que limite su posible solapamiento con las señales de los compuestos de interés. Usualmente, en caso de emplear sistemas de detección por MS, resulta de gran interés esta elución previa de la fase extractante, pues esta diferencia de tiempos de retención permite mantener el sistema de detección apagado durante la elución del disolvente (*solvent delay*), evitando que el disolvente pueda degradar las diferentes partes del sistema de detección.

En ocasiones, no resulta posible el empleo de disolventes de tan baja volatilidad como fases extractantes, resultando la volatilidad de los disolventes semejante a la de las especies

consideradas. En este caso, la señal analítica del disolvente aparecerá entre aquellas correspondientes a los compuestos de interés. Aunque una adecuada selección de los iones monitorizados en el sistema MS puede limitar su impacto en el cromatograma final, la presencia de la señal del disolvente originará un incremento de los niveles de ruido, en caso de que alguno de sus iones minoritarios coincida con los monitorizados por el sistema MS, así como una disminución en la vida útil de los componentes del sistema de detección.

Microextracción en fase sólida

La técnica SPME es fácilmente acoplable a los sistemas GC usando el inyector *split/splitless* [26].

Una vez realizada la extracción, la jeringa modificada que soporta la fibra de SPME es introducida, de forma semejante a una microjeringa para muestras líquidas, en el puerto de inyección. La fibra SPME recubierta de su fase extractante polimérica, en la que han sido extraídos los compuestos de interés, es expuesta al *liner* situado en el interior del inyector. Las altas temperaturas en este dispositivo dan lugar a la desorción térmica de los mismos, y su paso a lafase gaseosa, y posteriormente a la columna cromatográfica para su separación.

Debido al pequeño espesor de la fase extractante, este proceso de desorción es relativamente rápido, usualmente inferior a un minuto. A fin de lograr picos cromatográficos más definidos, la columna se mantiene a una temperatura baja durante los primeros instantes de la separación, de tal manera que los analitos quedan retenidos en la fase estacionaria de los primeros centímetros de la columna [27].

Otras técnicas de microextracción en fase sólida, como la SBSE, no pueden ser acopladas a GC tan fácilmente como la SPME. En el caso de SBSE, la única posibilidad que ofrecen los inyectores convencionales de tipo *split/splitless*, es realizar una re-extracción de los analitos desde la fase extractante hacia un medio líquido compatible con GC, y proceder a su posterior inyección como si de una muestra líquida convencional se tratase [28]. Esta etapa de desorción líquida no solo implica un tiempo adicional de análisis, sino una importante pérdida de sensibilidad, ya sea por la imposibilidad de alcanzar una recuperación total o por la necesidad de inyectar solo una pequeña parte de la muestra líquida re-extraída, apenas unos pocos microlitros de un volumen de re-extracción usualmente un par de órdenes de magnitud mayor.

La imposibilidad de lograr un acoplamiento sencillo y efectivo entre SBSE y GC dio lugar al desarrollo de un nuevo sistema de inyección para GC: el inyector de desorción térmica.

3. Desorción térmica

El inyector de desorción térmica es un sistema de introducción de muestra para GC formado por dos componentes independientes: la unidad de desorción térmica (TDU) y el inyector de temperatura programada (PTV). Aunque pueden ser empleados de forma individual, esta combinación TDU/PTV trabaja como un único sistema de inyección. Pese a tratarse de dos sistemas físicamente diferenciados, se encuentran unidos formando una unión estanca, que asegura una adecuada trasferencia de la muestra entre ambos, y limita cualquier posible contaminación procedente del exterior.

La TDU es un sistema de alta eficiencia diseñado para la introducción de muestras en GC mediante un proceso de desorción térmica.

Originalmente las TDUs se desarrollaron para la introducción en sistemas de GC de los compuestos orgánicos volátiles (VOCs) retenidos en trampas, activas o pasivas, conteniendo un material adsorbente, como las usadas en muestreo atmosférico, ya fuera para el control de exposición en entornos de trabajo o para control de contaminación ambiental [29]. El calentamiento intenso de estos materiales adsorbentes en el interior de la TDU permitía la desorción de los compuestos de interés y su transferencia al inyector del GC a través de una línea de transferencia a una temperatura controlada. La imposibilidad de combinar SBSE con GC dio lugar a la adaptación de la TDU a este fin. Sin embargo, la TDU es un dispositivo de alta versatilidad, que permite adicionalmente la inyección de muestras líquidas o la extracción térmica de sólidos.

El PTV es un sistema de inyección para GC semejante al inyector *split/splitless*. Su principal diferencia con respecto a éste, que opera a temperatura fija, es que permite un preciso y rápido control de la temperatura, programando su enfriamiento o calentamiento según las necesidades analíticas.

Inicialmente los PTVs se desarrollaron para permitir el modo de inyección de gran volumen (LVI) [30]. El objetivo de este tipo de inyección es la introducción en el sistema GC de volúmenes de muestra líquida de hasta centenas de microlitros, varios órdenes de magnitud mayores que los introducidos con inyectores usuales como el *split/splitless*. En un inyector LVI, la muestra es introducida en el inyector a una temperatura cercana a la ambiental. Posteriormente, ésta se somete a un calentamiento rápido hasta una temperatura moderada, que permite la evaporación del disolvente, pero no la vaporización de los compuestos de interés. Una vez eliminado el disolvente, y evacuado a través de la salida de *split*, se produce un segundo calentamiento a una temperatura mayor, que permite la vaporización de los compuestos de interés y su transferencia a la columna cromatográfica para su separación, operando en este caso el inyector en un modo semejante al *splitless*.

3.1 Componentes del inyector de desorción térmica

La muestra a analizar, ya se trate de una barra de extracción SBSE o de un microvial conteniendo una fase extractante líquida, se sitúa en el interior de un tubo de transporte. Este tubo de transporte es un cilindro de vidrio, con unos topes para evitar la caída de la muestra, que actuará a su vez como *liner* en la TDU. Para facilitar su transporte, ya sea por el analista o por un sistema de automuestreo, este tubo dispone de un adaptador metálico que se fija a su parte superior. Éste también incluye un par de juntas tóricas que asegurarán la estanqueidad del inyector durante el proceso de introducción de la muestra.

Una vez posicionado el tubo de transporte en la TDU, un sistema neumático acciona el cierre de la TDU, desplazando unas bolas metálicas que bloquean la posición del tubo y aseguran el cierre hermético del sistema, etapa previa a su presurización.

La TDU va a ser el primer punto presurizado del sistema GC, por lo que en él se sitúa la entrada de gas portador, usualmente helio, al sistema. Adicionalmente, y dado que los flujos de gas en la TDU pueden ser diferentes de los que se den en el PTV y mayores que el flujo en la columna cromatográfica, debe haber una salida de gas, controlada por una válvula que permite una operación tipo *split/splitless*.

Por su naturaleza, la TDU requiere de un sistema de calentamiento rápido, preciso y homogéneo. Éste está formado por un sistema termo-eléctrico que permite el calentamiento del puerto en el que se sitúa la muestra y por un sistema de refrigeración, usualmente de tipo líquido, que consta de una unidad Peltier externa, que asegura el mantenimiento de la temperatura o el enfriamiento del sistema, cuando así convenga.

En la parte inferior de la TDU se sitúa la unión con el PTV. Esta unión debe asegurar que exista una continuidad entre los *liners* de la TDU y el PTV, tal que permita un flujo sin pérdidas ni contaminación de la muestra. Una palanca de bloqueo asegura esta unión, a la vez que permite separar la TDU del PTV para la realización de operaciones de mantenimiento.

El puerto de inyección del PTV también cuenta con un *liner* propio. Este cilindro de vidrio tiene un diámetro inferior al del *liner* de la TDU, y en su interior es posible situar rellenos de diferente naturaleza, que contribuyen a la retención de los compuestos de interés.

El puerto de inyección del PTV dispone de un sistema de calentamiento/enfriamiento semejante al de la TDU. Dado que en ocasiones resulta conveniente que el PTV opere a una temperatura inferior a la ambiental, el enfriamiento líquido con una unidad Peltier externa puede ser sustituido por otros sistemas. La operación a temperaturas inferiores a las ambientales, conocido como *cryofocusing*, hace uso de enfriamiento con CO_2 líquido o N_2 líquido.

El PTV posee también una válvula de tipo *split/splitless*, que permite ajustar las diferencias de flujo de gas portador entre el flujo que entra procedente de la TDU en este sistema y el que finalmente alcanza la columna cromatográfica.

En la parte inferior del PTV se sitúa su unión con la columna cromatográfica. El comienzo de ésta penetra cerca de 1 cm en el PTV, solapándose con la parte final del *liner*. La estanqueidad de la unión entre PTV y columna cromatográfica se asegura mediante una férula de grafito. La Figura 3 muestra un esquema gráfico del acoplamiento TDU/PTV.

La complejidad de la combinación TDU/PTV hace necesaria la participación de componentes externos que aseguren su adecuado funcionamiento. Adicionalmente a las unidades Peltier anteriormente citadas, que permiten el enfriamiento del sistema, existe un módulo externo que regula todo el sistema neumático, así como una conexión de todas las unidades descritas con el equipo informático donde opera el software que controla el instrumento.

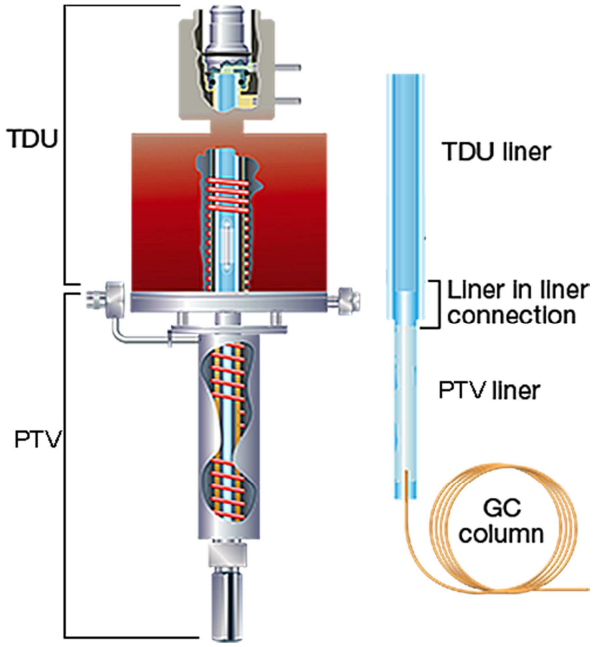

Fig. 3. Representación esquemática de la combinación TDU/PTV.

3.2 Descripción de la etapa de introducción de la muestra

Una vez completada la etapa de extracción, los analitos se encuentran disueltos en el disolvente empleado como fase extractante líquida, o bien retenidos en el polímero empleado como fase extractante sólida, según la naturaleza de la técnica de microextracción empleada.

La posterior etapa de introducción de la muestra puede dividirse en dos pasos diferenciados: la desorción térmica y la transferencia de la muestra hacia la columna cromatográfica. Estas dos etapas tienen lugar de forma consecutiva.

3.2.1 Desorción térmica

La desorción térmica, que tiene lugar en la TDU, es el proceso de transferencia de los analitos desde la fase extractante hacia la fase gaseosa del interior del inyector. La naturaleza de este proceso es independiente del estado físico de la fase extractante empleada, por lo que se describirá de manera general para cualquier tipo de fase extractante: fase sólida polimérica o fase líquida no volátil, como un líquido iónico. En el caso de fases líquidas volátiles, la eliminación del disolvente tiene lugar de manera previa a la desorción térmica de los analitos.

Para su desorción térmica, la muestra se introduce en la TDU dentro de su vial de transporte. Inicialmente la TDU se encuentra a una baja temperatura, cercana a la temperatura ambiental. Una vez introducida la muestra, la unidad se somete a un calentamiento controlado, que permite la desorción térmica de los analitos desde la fase extractante. Un flujo de gas portador arrastra las sustancias desorbidas hacia la segunda parte del sistema de inyección, el inyector de temperatura programada, para su posterior transferencia hacia la columna cromatográfica.

Fundamentos de la desorción térmica

El proceso de desorción térmica está determinado fundamentalmente por la temperatura aplicada y las diferencias de concentración del analito entre la fase gaseosa y la fase extractante.

Un incremento de la temperatura del medio da lugar a un aumento en la presión de vapor de los compuestos de interés. La relación entre presión de vapor y temperatura es de tipo exponencial, y puede ser descrita de forma aproximada usando la ecuación de Antoine [31]. En esta ecuación los parámetros A, B, y C son característicos de cada compuesto:

$$logP_v = A - \frac{B}{C + T}$$

El reparto de los analitos entre la fase extractante y la fase gaseosa vendrá determinado por su coeficiente de reparto ($K_{v/e}$) [18]. La variación de temperatura tendrá un impacto mucho menor en su solubilidad en la fase extractante (C_{ext}) que sobre su presión de vapor, por lo que un aumento de temperatura dará lugar a un incremento del coeficiente de reparto entre la fase gaseosa y la fase extractante:

$$K_{vap/ext} = \frac{C_{vap}}{C_{ext}}$$

Si: $T_1 < T_2$ $C_{vap\,T} \propto P_{vap\,T}$ $C_{vap\,T1} \ll C_{vap\,T2}$

$$C_{ext\,T1} \approx C_{ext\,T2}$$

$$K_{T1 vap/ext} \ll K_{T2 vap/ext}$$

Dado que las temperaturas aplicadas en el interior de la TDU son cercanas o superiores a la del punto de ebullición de los compuestos de interés, los analitos retenidos en la fase extractante tendrán tendencia a abandonar esta fase.

El flujo de analitos (*dM/dt*) entre la fase extractante y la fase gaseosa puede ser descrito de forma aproximada usando la ecuación de evaporación de Langmuir [32]:

$$\frac{dM}{dt} = (P_v - P_p)\sqrt{\frac{m}{2\pi k_B T}}$$

Esta ecuación muestra que el flujo de analitos durante la etapa de desorción térmica es proporcional a la diferencia entre la presión de vapor del analito descrita previamente, y su correspondiente presión parcial (P_p) en la fase gaseosa.

El desplazamiento de los analitos de la fase extractante a la fase gaseosa se ve favorecido por el gradiente de concentraciones entre ambas fases, de un lado una fase extractante saturada o concentrada en los compuestos de interés, y de otra una fase gaseosa donde su presión parcial es nula o muy inferior a la presión de vapor.

El flujo continuo de gas hacia la columna cromatográfica, a fin de mantener la presión en cabeza de la misma, asegura que la presión relativa de los analitos en el interior del inyector sea baja o nula durante toda la desorción.

Este bajo o nulo valor de la presión parcial del analito en la fase gaseosa permite simplificar la ecuación de Langmuir eliminando este término. Esta expresión representa el flujo de desorción resultado de la evaporación de los analitos retenidos en la superficie de la fase extractante.

$$\frac{dM}{dt}_{evap} = P_v\sqrt{\frac{m}{2\pi k_B T}}$$

Sin embargo, dado que las técnicas de microextracción consideradas se basan en absorción y no en adsorción, los compuestos de interés se encuentran distribuidos de forma homogénea en todo el seno de la fase extractante. Así, una vez los compuestos absorbidos superficialmente pasan a la fase gaseosa, aparece un gradiente de concentraciones entre la superficie y el seno de la fase extractante. Este gradiente de concentraciones da lugar a un proceso de difusión, con su correspondiente flujo de analitos.

Durante la mayor parte del proceso de desorción, el flujo total de desorción será igual al flujo de difusión desde el seno de la fase extractante, debido al carácter limitante y necesario de este fenómeno de transporte, así como por tratarse de un proceso más lento que el de evaporación. La difusión viene descrita por la primera ley de Fick, considerando el coeficiente de difusión del analito (D) y su gradiente de concentraciones en el seno de la fase extractante [33,34].

$$\frac{\mathrm{dM}}{\mathrm{dt}}_{dif} = \mathrm{D}\frac{\partial \mathrm{C}_{ext}}{\partial \mathrm{x}}$$

Esta ecuación puede simplificarse al sustituir el gradiente de concentraciones por la diferencia entre la concentración en el seno y en la superficie de la fase extractante, dividida por el tamaño de la capa de difusión. El espesor de la capa de difusión variará a lo largo del proceso de desorción (d(t)) [35]. De igual manera, en los momentos finales de la etapa de desorción, se produce un agotamiento del analito presente en el seno de la fase extractante, disminuyendo su concentración hasta alcanzar la concentración superficial. El bajo valor de la concentración en la superficie de la fase extractante, en equilibrio con la concentración en la fase gaseosa, permite aproximarlo a un valor nulo.

$$\frac{\mathrm{dM}}{\mathrm{dt}}_{dif} = \mathrm{D}\frac{\mathrm{C}_{ext_{sen}}(t) - C_{ext_{sup}}}{\mathrm{d(t)}} \approx \mathrm{D}\frac{\mathrm{C}_{ext_{sen}}(t)}{\mathrm{d(t)}}$$

Una vez descritos ambos flujos, de evaporación y difusión, es posible evaluar la validez de la suposición de que el transporte desde el seno de la disolución es la etapa limitante del proceso de desorción. A este fin es posible igualar ambas expresiones, y despejar a qué valores de capa de difusión este proceso comienza a ser más lento que la evaporación.

$$\mathrm{P_v}\sqrt{\frac{\mathrm{m}}{2\pi \mathrm{k_B T}}} = \mathrm{D}\frac{\mathrm{C}_{ext}}{\mathrm{d}} \qquad \rightarrow \qquad \mathrm{d} = \frac{\mathrm{D\ C}_{ext}}{\mathrm{P_v}}\sqrt{\frac{2\pi \mathrm{k_B T}}{m}}$$

Considerando valores usuales para los compuestos de interés [$D \approx 10^{-12}$ m^2/s (a partir de la ecuación de Stokes-Einstein para T=500 K, η=10 Pa·s, r=0.2 nm) [36], $C \approx 1\% \approx 10$ kg/m^3, $P_v = 2 \cdot 10^5$ Pa, m≈0.2 kg], los valores de capa de difusión obtenidos son muy inferiores al tamaño molecular. Esto indica que el modelo de evaporación de Langmuir solamente es aplicable a la desorción de los analitos localizados inmediatamente en la superficie de la fase extractante, y que el flujo de desorción del resto de los compuestos extraídos se corresponde con el de un modelo de difusión. Por lo tanto, es adecuado considerar que en general el flujo del proceso de desorción será igual al flujo del proceso de difusión.

La cantidad total de analito desorbida durante la etapa de desorción térmica se obtendrá así por la integración de la ecuación de difusión durante el tiempo de desorción considerado (t_f).

$$M = \int_0^{t_f} \frac{dM}{dt}$$

En la Figura 4, se muestra una versión esquematizada de la evolución del gradiente de concentraciones entre la superficie y el seno de la fase extractante durante las diferentes etapas del proceso de desorción. Usando esta representación es posible estimar valores relativos de flujo, y a partir de su integración, obtener el perfil de la cantidad total de analito desorbida frente al tiempo de desorción.

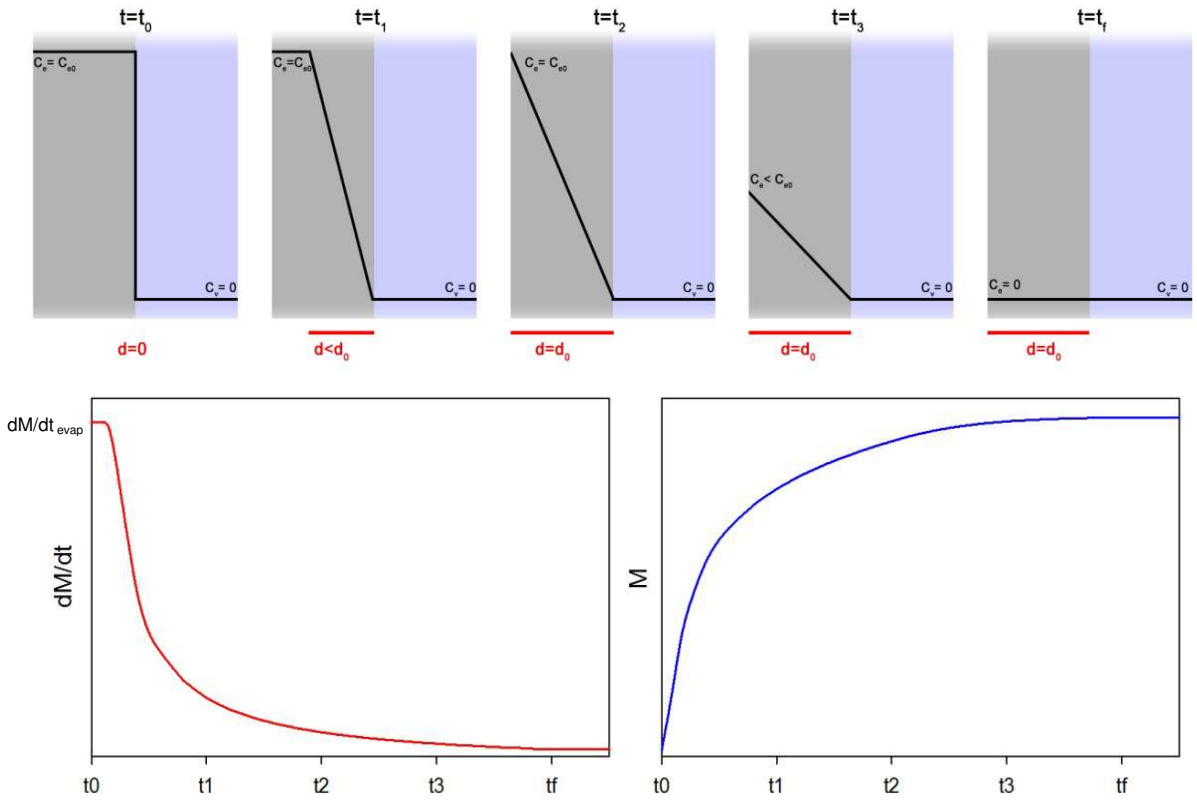

Fig. 4. Gradiente de concentraciones, flujo y cantidad total de analito durante la etapa de desorción térmica.

Como se puede observar, el flujo máximo corresponde al flujo de evaporación, y únicamente se alcanza en los momentos iniciales del proceso de desorción. El flujo disminuye conforme se incrementa el tamaño de la capa de difusión (d), hasta que ésta abarca la totalidad del espesor de la fase extractante ($d=d_0$), y posteriormente, al disminuir la concentración del analito en el seno de la disolución. La desorción térmica se completará al alcanzarse el equilibrio entre la concentración en el seno de la disolución y la concentración en la fase gaseosa.

3.2.2 Transferencia de muestra

Durante la etapa de desorción térmica, un flujo de gas portador arrastra las sustancias desorbidas hacia la segunda parte del sistema de inyección, el PTV. En esta unidad, los compuestos desorbidos son concentrados mientras se completa la etapa de desorción térmica. A este fin se emplean bajas temperaturas, por debajo de su temperatura de ebullición, que permiten su condensación en el *liner* del PTV, y por lo tanto, su retención. Para lograr que este proceso sea más eficiente es posible situar materiales de relleno de gran superficie en el interior del *liner* del PTV. Estos rellenos, como fibra de vidrio, actúan como puntos de nucleación para la etapa de condensación y favorecen la retención. También es posible el empleo de rellenos de naturaleza adsorbente, que no solo actúan como soporte para la condensación, sino que ofrecen una retención química de los compuestos de interés.

Una vez completada la etapa de desorción térmica, se procede a la transferencia de la muestra hacia la columna cromatográfica. Para ello, el PTV se somete a un programa de calentamiento controlado que da lugar a re-volatilización de los compuestos de interés, en un proceso semejante a la desorción térmica descrita previamente. Esta etapa posee una duración mucho menor que la desorción térmica, al no existir limitaciones por fenómenos de transporte, permitiendo obtener picos mucho más definidos. Aun así, el empleo de condiciones de calentamiento moderado, o el empleo de materiales de relleno que no ofrezcan sólo una gran superficie, sino que posean características adsorbentes, puede hacer que este proceso de introducción de la muestra sea más largo, originando picos poco definidos.

Fundamentos de la transferencia de muestra

Como se ha indicado previamente, la etapa de desorción térmica es un proceso relativamente lento. Su velocidad está limitada por el proceso de difusión de los analitos desde el seno de la fase extractante a la superficie de la misma. A diferencia de SPME, donde la desorción requiere sólo unos segundos, este proceso puede durar varios minutos debido al mayor espesor de la capa de difusión. Así, las estrategias empleadas en SPME para la concentración de los analitos sobre la misma columna, actuando como punto frio de condensación, resultan inviables.

El acoplamiento directo de la TDU con el sistema cromatográfico no daría lugar a picos, sino a bandas cromatográficas con varios minutos de anchura, solapadas entre los diferentes compuestos y que limitarían la amplia capacidad de resolución de la técnica GC. Se hace necesario por lo tanto una etapa intermedia de concentración, en la que los analitos desorbidos en el TDU sean retenidos en el PTV, antes de introducirse en la columna cromatográfica.

A fin de evaluar el funcionamiento del PTV, supongamos un flujo de un gas, procedente de la TDU, conteniendo los compuestos de interés desorbidos con una determinada presión parcial (P_p), igual o inferior a la presión de vapor (P_v) en las condiciones de temperatura empleadas durante la desorción térmica.

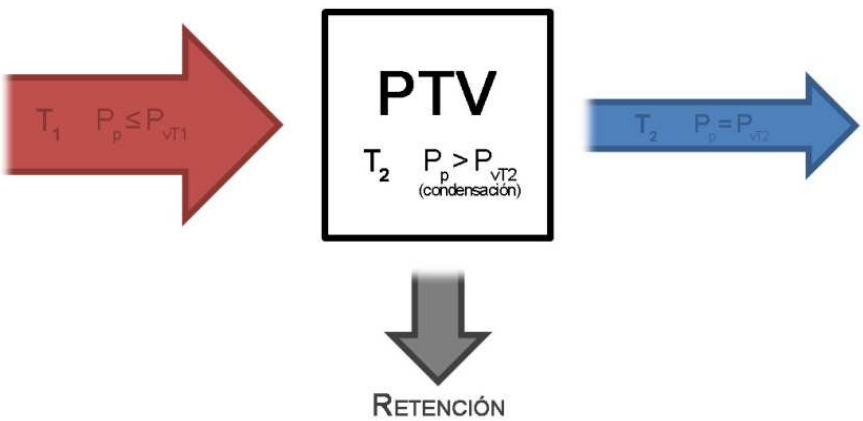

Fig. 5. Diagrama esquemático de flujos, temperaturas y presiones en el PTV.

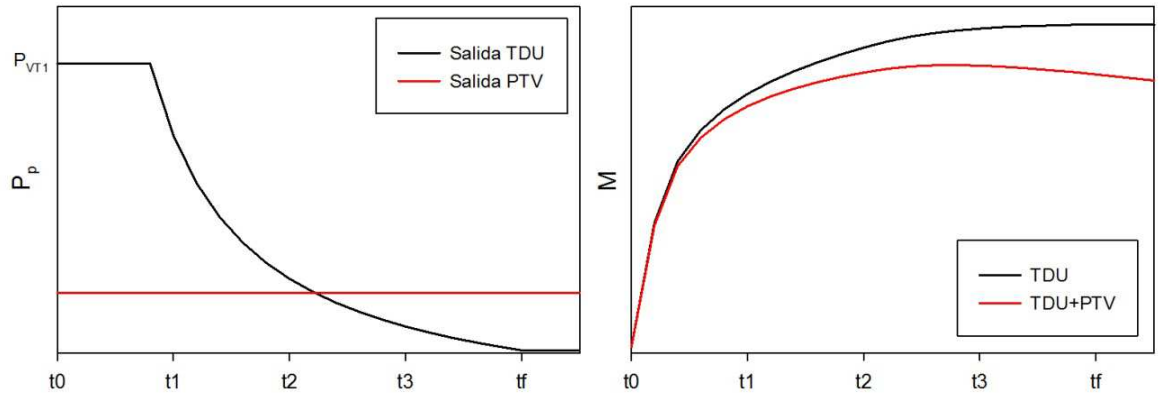

Fig. 6. Presiones parciales en los flujos de salida de TDU y PTV y cantidad total de analito usando TDU o TDU+PTV.

El súbito enfriamiento de este flujo de gas en el interior del PTV originará una disminución de la presión de vapor del analito. En estas condiciones de temperatura, la presión parcial del analito es superior a su presión de vapor, y se producirá la condensación de estos compuestos [37]. Para que esta condensación potencial sea efectiva, el interior del *liner* del PTV debe ofrecer una amplia superficie que favorezca la nucleación, y minimice así las limitaciones cinéticas del proceso de condensación.

Finalmente, el flujo de gas a la salida del PTV se encontrará saturado en los analitos, es decir, tendrá una presión parcial de los analitos igual a su presión de vapor en las condiciones del PTV. Durante gran parte de la etapa de desorción, la presión parcial del analito en el flujo de gas que entra en el PTV es mayor que la presión de vapor en las condiciones de operación de éste, por lo que la condensación está favorecida y existe una retención efectiva de los compuestos de interés.

Sin embargo, en las fases finales de la etapa de desorción, o una vez completada ésta, la presión parcial puede ser menor a la presión de vapor, es decir, la concentración de la especie de interés puede ser mayor en el gas saturado en los analitos que sale del PTV que en el gas que entra procedente del TDU, con lo que habría una pérdida neta de los compuestos de interés retenidos en el PTV (Fig. 5).

De acuerdo con lo descrito, es posible que un incremento del tiempo total de desorción, dé lugar a pérdidas de analitos y origine una disminución en la cantidad total de compuestos de interés introducidos en el sistema cromatográfico, y por lo tanto, a una menor sensibilidad. Así, el tiempo de desorción óptimo siempre será inferior al tiempo necesario para alcanzar una desorción completa de la muestra.

En el primero de los gráficos presentados (Fig. 6) se muestran esquemáticamente los perfiles de presiones parciales en el flujo efluente de los sistemas TDU y PTV. Se puede observar que a tiempos de desorción largos, el efluente del PTV tiene una mayor concentración que el procedente de la TDU, indicando una pérdida de analitos. En la segunda gráfica (Fig. 6) se comparan los perfiles de desorción, mostrando la cantidad total de analito introducida en el sistema frente al

tiempo de desorción, si se considera únicamente la TDU o la combinación TDU/PTV. En este segundo caso, se observa que se alcanza un valor máximo en la cantidad total de analito desorbido a tiempos inferiores al tiempo de desorción total.

3.3 Parámetros del inyector de desorción térmica

La optimización de las condiciones experimentales es la etapa inicial de todo desarrollo analítico. En el caso de la etapa de introducción de la muestra por desorción térmica, y debido a su complejidad, existe un amplio número de variables involucradas. Adicionalmente, la mayoría de estas variables se encuentran interrelacionadas.

Por lo tanto, la optimización de esta etapa requiere de un estudio detallado de la influencia de cada una de estas variables en la respuesta analítica, así como de las posibles interacciones entre ellas. A este fin es posible emplear optimizaciones de tipo univariante, que proporcionan una gran información sobre una única variable con un pequeño número de experiencias, o bien el uso de diseños experimentales que permitan la optimización multivariante.

3.3.1 Parámetros de la TDU

Entre los parámetros más relevantes que se deben considerar en la optimización de la operación de la TDU, destacan los siguientes:

Temperatura inicial de la TDU

Se trata de la temperatura inicial del programa de calentamiento de esta unidad, así como de la temperatura a la que ésta se encuentra durante la introducción de la muestra. Una vez completada la desorción térmica y acabado el programa de calentamiento, la unidad vuelve a esta temperatura inicial. Este enfriamiento puede realizarse de forma no forzada, de manera que la TDU va reduciendo su temperatura lentamente, o bien puede aplicarse un enfriamiento forzado, en el que la temperatura del sistema se reduce hasta la temperatura inicial usando la unidad de enfriamiento Peltier.

Tiempo inicial de la TDU

Es el tiempo durante el cual, una vez introducida la muestra en el sistema TDU, se mantiene la temperatura inicial, antes de proceder a elevar la temperatura. Al igual que la temperatura inicial de la TDU, se trata de un parámetro que no tiene un impacto directo en la eficiencia de la etapa de desorción térmica, pero que, sin embargo, puede tener cierta relevancia en el caso de trabajar con algunas fases extractantes líquidas volátiles, que deben eliminarse antes de proceder a la desorción térmica, o bien para eliminar restos de agua en algunas fases extractantes sólidas.

Programa de calentamiento

La principal característica de la TDU es su posibilidad de aplicar un calentamiento programado a la muestra, con un elevado grado de precisión y reproducibilidad. Este programa de calentamiento puede aplicarse como una única rampa de calentamiento, desde la temperatura inicial hasta la temperatura de desorción, o bien como un programa de dos rampas, en el que entre ambas temperaturas se intercala una mesete a una temperatura intermedia. El calentamiento en dos rampas suele ser empleado para la eliminación de restos de agua o para la evacuación de disolventes.

La velocidad de calentamiento está condicionada por factores instrumentales, no pudiendo ser superior a 720 ºC/min. En el caso de microextracción mediante SBSE es conveniente emplear velocidades de calentamiento inferiores, que aseguren un calentamiento más homogéneo, evitando la fatiga de los materiales y alargando la durabilidad de las barras de extracción. Un calentamiento excesivamente rápido puede provocar el agrietamiento de la superficie del material polimérico extractante, ya sea por tensiones asociadas a la dilatación del material o por la volatilización súbita de los materiales en él absorbidos.

Temperatura de desorción

La temperatura de desorción es el parámetro de mayor relevancia en la etapa de desorción térmica. En general, la efectividad de esta etapa será mayor a temperaturas más elevadas. En el caso de SBSE, el límite de temperatura a aplicar vendrá condicionado por la naturaleza de la fase extractante sólida, no recomendándose temperaturas superiores a 280 ºC para polidimetilsiloxano (PDMS), ni superiores a 220 ºC para etilenglicol (EG) o poliacrilato (PA).

Un incremento de la temperatura de desorción originará un incremento de la presión de vapor del analito, lo que aumentará el coeficiente de reparto de éste entre la fase gaseosa y la fase extractante. El aumento de temperatura dará lugar a la evaporación de los compuestos de interés localizados en la superficie de la fase extractante, y originará un flujo difusivo de estos compuestos desde el seno hacia la superficie. Cuanto mayor es esta temperatura, menor es la concentración superficial de equilibrio, y por lo tanto, mayor es la cantidad de analito que difunde y posteriormente se vaporiza, antes de alcanzar el equilibrio de concentraciones en la fase extractante (Fig. 7).

El incremento en la eficiencia de desorción con la temperatura alcanza un límite superior cuando la presión de vapor del compuesto se iguala a la presión del dispositivo, a una temperatura correspondiente a la temperatura de ebullición del analito en las condiciones de operación de la TDU. Incrementos de temperatura por encima de este valor no dan lugar a un incremento en la eficiencia de desorción, e incluso en ocasiones dan lugar a una disminución de la eficiencia global del proceso. Esta disminución en la eficiencia puede ser resultado de:

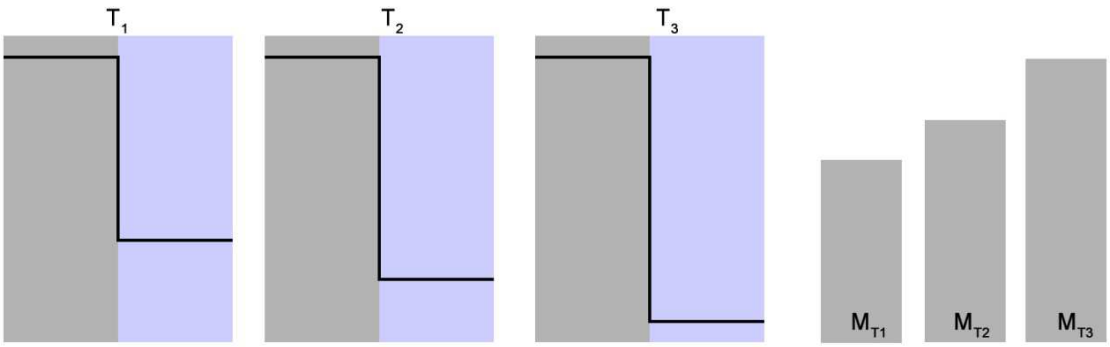

Fig. 7. Influencia de la temperatura en el reparto del analito entre fase extractante (gris) y fase gaseosa (azul), y en la cantidad total desorbida (M) ($T_3>T_2>T_1$).

- El flujo máximo de desorción viene determinado por la ecuación de evaporación de Langmuir. Si el incremento de temperatura no origina un incremento en la presión de vapor, lo que sucede cuando ésta es mayor que la temperatura de operación, existe una disminución del flujo potencial total como resultado del término ($\sqrt{1/T}$).

$$\left(\frac{dM}{dt}\right)_{evap} = P_{TDU}\sqrt{\frac{m}{2\pi k_B T}}$$

- La posible naturaleza termolábil de los compuestos de interés. Una temperatura de desorción alta no solo puede favorecer una desorción térmica más efectiva, sino también una degradación térmica más eficaz.

Tiempo de desorción

Es el tiempo durante el cual se mantiene la temperatura de desorción. Debe ser tal que permita una difusión de los analitos desde el seno de la fase extractante hasta la superficie de la misma, donde se produce la desorción. Tal y como se ha mostrado previamente (Fig. 6), tiempos de desorción excesivamente prolongados no solo suponen un incremento del tiempo de análisis, sino que, lejos de aumentar la sensibilidad del método, pueden disminuirla al favorecer pérdidas de los compuestos de interés en el PTV.

Temperatura de transferencia entre la TDU y el PTV

Se trata de la temperatura de la conexión entre la TDU y el PTV. A fin de limitar pérdidas por condensación en esta unión, la temperatura debe ser entre 20 y 30 ºC superior a la temperatura de desorción térmica. Aunque usualmente esta temperatura se mantiene fija, existe la posibilidad de que varíe a la vez que la temperatura de desorción térmica, siendo una opción que puede ser adecuada para compuestos termolábiles o de elevada volatilidad.

Modos de muestreo

Usualmente, la TDU va a albergar un vial de transporte vacío, que será reemplazado por la muestra cuando comience la desorción de ésta. Una vez completada la desorción, el vial de transporte conteniendo la muestra puede retirarse y sustituirse por este vial vacío, o bien mantenerse en la unidad de desorción hasta ser sustituido por otra muestra en la siguiente inyección. Ambas opciones pueden resultar adecuadas según la naturaleza de la muestra.

Retirar el tubo de transporte conteniendo la muestra puede ser adecuado para muestras que se vayan a re-analizar y en las que no se haya completado la desorción térmica. También se recomienda para fases extractantes de tipo EG y PA, que presentan una menor durabilidad que las de PDMS y para las que la exposición a temperaturas elevadas durante largos periodos degrada rápidamente la fase extractante.

Mantener el tubo puede ser adecuado para compuestos de elevada volatilidad, ya que no implica la ruptura de la presión del sistema entre la etapa de desorción y el inicio de la separación cromatográfica, minimizando las posibles pérdidas.

Como se ha indicado previamente, una vez completada la desorción térmica, y mientras tiene lugar la separación cromatográfica, la TDU se enfría de manera natural o forzada hasta su temperatura inicial. Durante esta etapa, el flujo de gas que atraviesa la TDU es evacuado a través de la válvula de *split*. El empleo de esta opción de muestreo, manteniendo la muestra en el sistema TDU tras la desorción térmica y no aplicar un enfriamiento forzado, puede resultar una opción conveniente, especialmente si se trabaja con SBSE.

Como se ha observado anteriormente, el tiempo óptimo de desorción suele ser menor al tiempo requerido para una desorción total. Así, es posible que una pequeña cantidad de analitos o interferentes permanezcan retenidos sobre la fase estacionaria. A fin de limitar efectos de memoria es necesario realizar un re-acondicionamiento de las fases extractantes antes de proceder a otra determinación. Una forma sencilla de realizar este acondicionamiento, y de forma simultánea al análisis, permitiendo así un importante ahorro de tiempo, es mantener la barra SBSE en el sistema TDU sin aplicar enfriamiento forzado. De esta manera, la fase extractante se mantiene a una temperatura relativamente elevada y se somete a un flujo de gas portador, asegurando la desorción de cualquier compuesto retenido, minimizando la contaminación cruzada entre muestras.

3.3.2 Parámetros del PTV

Entre los parámetros más relevantes que se deben considerar en la optimización de la operación del PTV, destacan los siguientes:

Temperatura de enfriamiento

La temperatura de enfriamiento es el parámetro de mayor relevancia en el funcionamiento del PTV. Tal y como se ha mostrado previamente, el enfriamiento aplicado en este dispositivo originará una disminución en la presión de vapor del analito que permitirá la condensación de los compuestos desorbidos procedentes del sistema TDU. Por lo tanto, temperaturas más bajas asegurarán una retención más efectiva de los compuestos de interés.

El empleo de enfriamiento criogénico (*cryofocusing*), con CO_2 o N_2 líquido, permite alcanzar temperaturas más bajas que con las unidades Peltier. Sin embargo, estas últimas proporcionan un enfriamiento más rápido y con menor costo de operación.

Temperatura final del PTV

Una vez completada la desorción térmica, los analitos retenidos en el PTV deben ser transferidos a la columna cromatográfica mediante un calentamiento intenso y rápido. El proceso de vaporización es semejante al descrito en la TDU. Del mismo modo, temperaturas más altas favorecerán la vaporización, teniendo siempre en cuenta las limitaciones asociadas a la degradación térmica de los analitos. En general, la temperatura de final a emplear en el PTV será igual o ligeramente superior a la temperatura final de la desorción térmica en la TDU.

El relleno del *liner* empleado en el PTV también puede condicionar la temperatura final a alcanzar en este dispositivo. En caso de usar rellenos con características adsorbentes se requerirán usualmente temperaturas más elevadas que en la TDU, debido a la mayor retención de los compuestos de interés. Adicionalmente, cada material de relleno posee una temperatura de operación máxima, debido a su posible degradación térmica.

Velocidad de calentamiento

El proceso de vaporización en el PTV debe ser rápido para lograr picos cromatográficos definidos. Por ello, la velocidad de calentamiento en este dispositivo, desde la temperatura de enfriamiento hasta la temperatura final, debe ser lo más alta posible (16 ºC/s). Por otro lado, con el fin de limitar la anchura de los picos cromatográficos, resulta conveniente mantener la columna cromatográfica a una temperatura baja mientras tiene lugar la etapa de evaporación en el PTV.

Relleno del PTV

La capacidad de retención del PTV puede verse incrementada, no solo mediante la aplicación de bajas temperaturas, sino mediante el empleo de un relleno adecuado en su *liner*. Este relleno debe ofrecer una elevada superficie, que proporcione puntos de nucleación para la condensación de los compuestos de interés. Adicionalmente, una distribución homogénea de los compuestos retenidos sobre la superficie de este soporte favorecerá una evaporación y transferencia hacia la columna cromatográfica más rápida.

En el caso de analitos de elevada volatilidad puede resultar conveniente el uso de rellenos que no solo ofrezcan un soporte para la retención de los compuestos, sino que posean un papel activo en este proceso. A este fin es posible emplear rellenos con características absorbentes, como la espuma de PDMS, o adsorbentes, como polímetros de óxido de 2,6-difenileno (DPO) o formas porosas de carbono como Carbowax (Cw).

3.3.3 Parámetros del conjunto TDU/PTV

Algunos parámetros a considerar en la optimización de la desorción térmica son comunes a los sistemas TDU y PTV, siendo considerados a continuación:

Flujo de gas

Un flujo de gas portador arrastra los analitos que son desorbidos en la TDU hacia el PTV, donde son retenidos; posteriormente, una vez completada la desorción térmica pasan desde el PTV a la columna cromatográfica. Existe un único flujo de gas a través de estas dos unidades, aunque puede ajustarse el flujo efectivo en cada una de ellas usando los diferentes modos de operación.

Un flujo de gas elevado permite asegurar que la presión relativa de los analitos en la fase gaseosa se mantenga baja o nula en la TDU, lo que favorece el proceso de desorción térmica. Sin embargo, flujos elevados disminuyen el tiempo de paso a través del PTV, lo que reduce la posibilidad de enfriamiento de este gas, dificultando la condensación de los analitos, y por lo tanto, restringiendo la retención efectiva de los compuestos de interés.

Los valores del flujo de gas portador deben ser evaluados y establecidos de manera acorde al resto de variables involucradas en la etapa de desorción térmica. Usualmente se emplean valores de flujo de entre 40 y 100 mL/min.

Presión de gas

El sistema TDU/PTV es una unidad presurizada que, en condiciones operativas, siempre se encontrará a una presión superior a la atmosférica. Por su disposición continua, la presión en el sistema TDU/PTV deberá ser igual a la de la cabeza de columna en el sistema cromatográfico.

El sistema TDU/PTV no permite un control directo de su presión, pero ésta se puede establecer mediante un ajuste de la presión en cabeza de columna, de forma semejante a una inyección *pulsed splitless* en un inyector convencional *split/splitless*. Pese a ello, y dado que la presurización del sistema no es inmediata, usualmente la presión no se considera una variable a optimizar en el sistema TDU/PTV.

Adicionalmente, existe un límite inferior de presión alcanzable, en cualquier caso superior a la presión atmosférica, derivado del sistema de bloqueo de la TDU. A fin de asegurar la muestra en el

interior de la TDU, existe un sistema de bloqueo neumático que impide la retirada del vial de transporte. La presión de este sistema de bloqueo es semejante a la empleada para la presurización de la TDU. Presiones inferiores a 3-4 psi no permiten un bloqueo del vial de transporte de la muestra, e impiden el cierre hermético del sistema de inyección. Este límite inferior de presión está condicionado por el estado de los componentes del sistema de bloqueo (juntas, bolas, adaptador del vial de transporte, etc.)

Modo de desorción

Como se ha indicado previamente, los sistemas TDU y PTV comparten un único flujo de gas, pero cada una de estas unidades dispone de su propia válvula *split/splitless*, por lo que es posible ajustar el flujo en cada una de ellas, dando lugar a distintos modos de desorción (Fig. 8).

- TDU *split*: Solo una parte del flujo (*split ratio*) que pasa a través de la TDU se dirige hacia el PTV, el resto abandona el sistema a través de la válvula de *split.*
- TDU *splitless*: Todo el flujo que pasa a través de la TDU se dirige hacia el PTV.
- PTV *split*: Sólo una parte del flujo que pasa a través del PTV se dirige a la cabeza de columna, el resto abandona el sistema por la valvula de *split*. Usualmente se suele operar con un split ratio igual a cero, para evitar la entrada de compuestos indeseados a la columna cromatográfica.
- PTV *splitless*: Todo el flujo que pasa a través del PTV se dirige a la cabeza de columna., teniendo en cuenta las limitaciones de flujo de ésta (<2 mL/min).

Un factor adicional es el momento en el que se produce el cierre de estas válvulas. Tanto en la TDU como en el PTV puede resultar de interés mantener inicialmente la válvula de *split* abierta, para posteriormente proceder a su cierre. Esta opción se suele emplear a fin de eliminar disolventes, por lo que se conoce como venteo de disolvente (*solvent vent*).

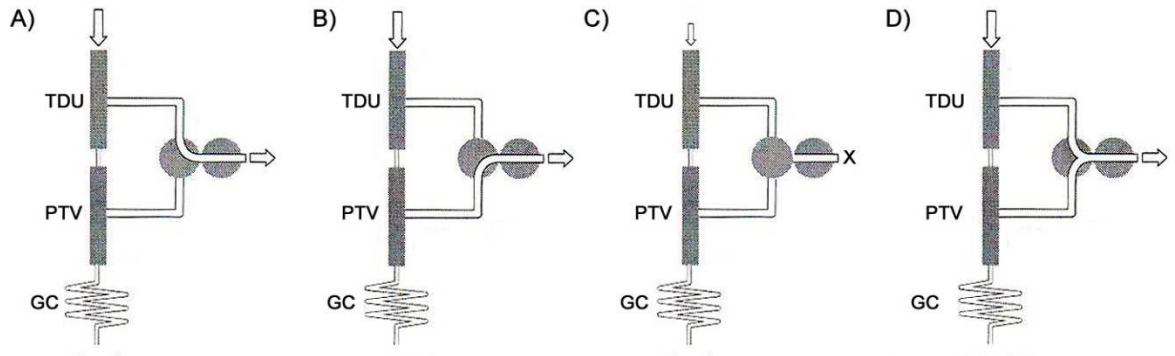

Fig. 8. Modos de desorción: A) TDU *split* + PTV *splitless*, B) TDU *splitless* + PTV *split*, C) TDU *splitless* + PTV *splitless*, D) TDU *split* + PTV *split*.

3.3.4 Parámetros en una desorción térmica de microextracción

A fin de considerar cómo se modifica el conjunto de variables involucradas en la operación del sistema TDU/PTV a lo largo del proceso de introducción de la muestra, se va a considerar la desorción térmica para una microextracción en fase sólida, más específicamente una microextracción SBSE utilizando fases polares. El programa instrumental empleado se detalla en el Capítulo I, dentro del apartado "*Stir bar sorptive extraction polar coatings for the determination of chlorophenols and chloroanisoles in wines using gas chromatography and mass spectrometry*".

Las fases extractantes polares de SBSE son fases poliméricas que por su composición (PA o copolímero EG-silicona) poseen tendencia a retener agua. Adicionalmente, debido a su fragilidad mecánica, estos polímeros se encuentran protegidos detrás de una malla metálica, que dificulta el secado de la barra con papel, y favorece la presencia de restos de agua. La entrada de agua al sistema cromatográfico puede originar sobrepresiones, problemas de picos fantasmas o limitar la vida útil del sistema de detección MS. Por ello, cuando se emplean barras SBSE con fase extractante polar, resulta conveniente realizar una etapa inicial de secado. Esta etapa se incluye dentro del conjunto de etapas de la desorción térmica: desorción térmica, transferencia de la muestra y reacondicionamiento.

En la Figura 9 se muestran las variaciones de temperatura de la TDU, el PTV y la columna cromatográfica a lo largo de la determinación analítica, así como el estado de las válvulas *split/splitless* de la TDU y el PTV, y el valor de flujo total de gas portador en el sistema TDU/PTV. En dicha figura cada una de las diferentes etapas de la desorción térmica ha sido separada con una línea discontinua y etiquetada como:

- Secado (*A*-*B*): Una vez introducida la barra SBSE en el sistema, ésta se mantiene durante medio minuto a 50 ºC. Un flujo de gas portador de 95 mL/min arrastra cualquier resto de humedad presente en la barra SBSE. El flujo resultante de gas húmedo es evacuado por la válvula de *split* de la TDU, sin alcanzar ni el PTV ni la columna.
- Desorción térmica (*B*-*C*): Una vez acabado el secado, la válvula de *split* de la TDU se cierra, y comienza el calentamiento de esta unidad, a una velocidad de 300 ºC/min hasta alcanzar una temperatura final de 220 ºC. Esta temperatura se mantiene durante un tiempo de desorción de 12.7 minutos. Durante este tiempo, un flujo de gas portador de 95 mL/min pasa a través de la TDU, arrastrando los analitos hacia el PTV. En esta unidad, que se encuentra a 15 ºC y posee un *liner* relleno de material adsorbente, los analitos condensan o son adsorbidos. El flujo de gas portador resultante, enfriado y del que se han retenido los compuestos de interés, se evacua a través de la válvula de split del PTV, no alcanzando la columna del GC.

- Transferencia (*C-D*): Una vez completa la desorción térmica en la TDU, este dispositivo deja de mantener su temperatura y comienza a enfriarse de manera natural. A la vez, el PTV comienza a calentarse, a una velocidad superior a 10 ºC/s hasta alcanzar una temperatura final de 330 ºC. Esta temperatura se mantendrá durante 2 minutos, a fin de vaporizar los analitos retenidos. Durante esta etapa de transferencia, las válvulas de los sistemas TDU y PTV permanecen cerradas, y todo el flujo que entra en el sistema TDU/PTV se dirige a la columna cromatográfica. Por tanto, el valor de flujo total se ve drásticamente reducido. Durante esta etapa, la columna se mantiene a una temperatura relativamente baja, para favorecer la concentración de los analitos en su primer tramo, y así la obtención de picos cromatográficos más definidos.

- Reacondicionamiento (*D-E*): Una vez completada la transferencia de la muestra hasta la columna, el PTV comienza a enfriarse de manera espontánea. Un flujo de gas portador, que pasa a través de los sistemas TDU y PTV aún calientes, arrastra cualquier posible compuesto remanente en estas unidades. Dado que las válvulas de la TDU y el PTV se encuentran abiertas, cualquier posible contaminante no alcanzaría la columna cromatográfica. Se logra de esta manera reacondicionar la barra SBSE y el *liner* del PTV para sus siguientes usos.

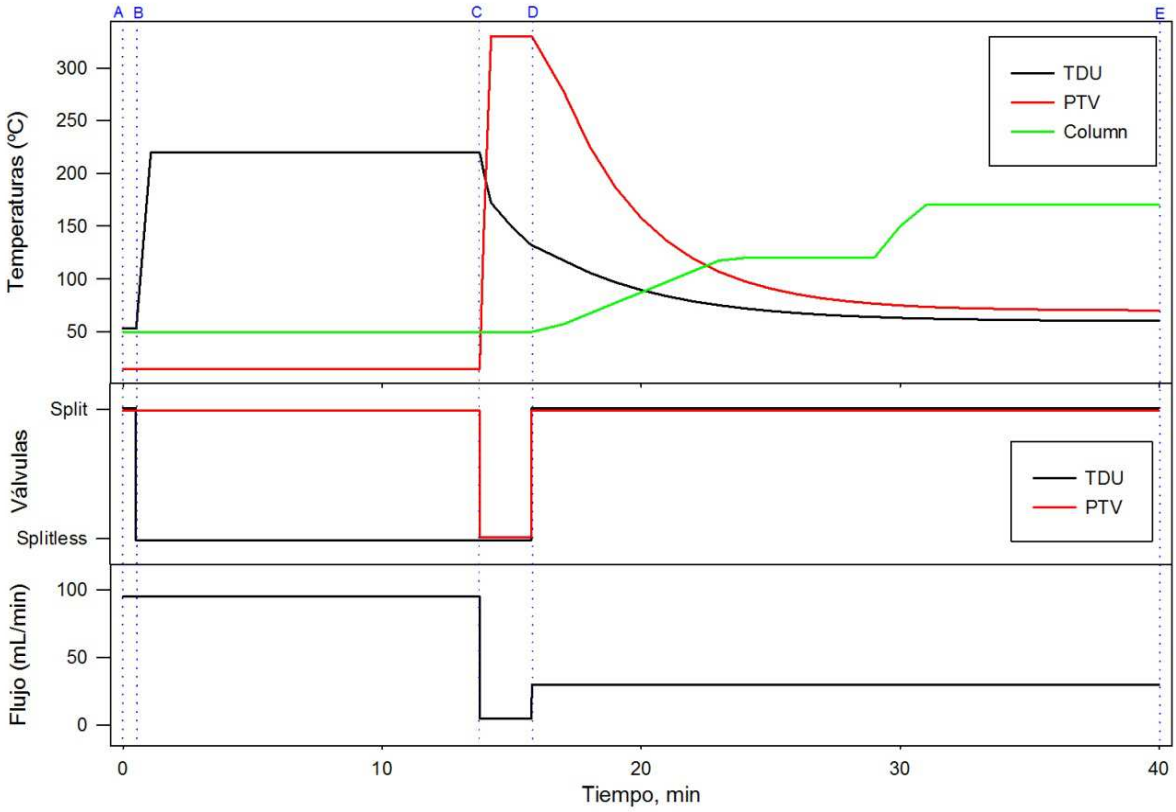

Fig. 9. Evolución de la temperatura, modo de operación en las válvulas y flujo de gas durante la desorción térmica en el sistema TDU/PTV y en la columna cromatográfica.

Programas de desorción térmica semejantes al descrito son empleados para la introducción de muestras tratadas mediante LPME. Dado que en estos casos los volúmenes a introducir en el sistema son superiores al volumen interno del inyector, resulta conveniente, a fin de evitar sobrepresiones y las consiguientes pérdidas de muestras, la evaporación de parte del disolvente antes de proceder a la volatilización de los analitos. Esta eliminación del disolvente se realiza de manera semejante a la etapa de secado descrita, manteniendo la temperatura inicial de la TDU durante el tiempo necesario para vaporizar el disolvente, que es arrastrado por el gas portador y evacuado a través de la válvula de *split*.

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Chapter I

Stir-Bar Sorptive Extraction and Gas-Chromatography coupling by Thermal Desorption

Determination of alkylphenols and phthalate esters in vegetables and migration studies from their packages by means of stir bar sorptive extraction coupled to gas chromatography-mass spectrometry

Abstract

This paper describes a method for the determination of three alkylphenols (APs), 4-tert-octylphenol (tOP), 4-n-octylphenol (OP) and 4-nonylphenol (4-NP), and six phthalate esters (PEs), dimethylphthalate (DMP), diethylphthalate (DEP), di-n-butylphthalate (DBP), n-butylbenzylphthalate (BBP), di-2-ethylhexylphthalate (DEHP) and di-n-octylphthalate (DOP), in vegetables using stir bar sorptive extraction (SBSE) in combination with thermal desorption-gas chromatography-mass spectrometry (TD-GC-MS). Ultrasonic radiation was used to extract the analytes from the solid food matrix, and the extract obtained was preconcentrated by SBSE. The different parameters affecting both stages were carefully optimized. The method was applied to analyze commercial vegetables, in the form of plastic packed salads and canned greens, as well as the corresponding filling liquids of the canned food. Quantification of the samples was carried out against aqueous standards using an internal standard (anthracene). The analysis of a 2 g vegetable sample provided detection limits between 12.7 and 105.8 pg g^{-1} for OP and DEHP, respectively. Migration studies from the plastic packages of the vegetables samples analyzed were carried out. DEP, DBP and DEHP were found to have migrated from the bags to the simulant and the same compounds were quantified in lettuce, corn salad, arugula, parsley and chard, at concentration levels in the 8 – 51 ng g^{-1} range. However, OP and NP were found in only two vegetable samples and one filling liquid, but neither was detected in any package. The proposed method provided recoveries of 83 – 118%.

1. Introduction

Fruits and vegetables play a relevant role in human diet, since their intake ensures an adequate supply of biologically active compounds such as vitamins, minerals, antioxidants and fibers. The importance of a suitable nutrition, along with the growing interest for fresh vegetables, has resulted in the development of a market share for fresh ready-to-eat (RTE) vegetables (bagged salads, pre-packed mixed vegetables, etc.) [1,2]. These products receive some technological processing before their commercial distribution, taking into account that they are usually consumed without any washing or additional preparation [1]. Consequently, the potential chemical contaminants that may be incorporated to the food until its consumption have to be controlled.

Endocrine disrupter chemicals (EDCs) are compounds of known toxicity even at low concentrations, which are able to mimic or block the action of natural hormones affecting the normal biological function in animals and humans [3]. Alkylphenols (APs) and phthalate esters (PEs) are EDCs commonly used in different industrial areas. APs are degradation products of alkylphenol polyethoxylates (APEOs), which are added as non-ionic surfactants to cleaning agents, including food detergents. The use of detergents containing nonylphenolethoxylates or octylphenolethoxylates to wash vegetables prior to their package can led to contamination by their degradation products, whose estrogenic activity has been proved [3-5] and their sale and use legislated in 2005 [6]. PEs are a numerous group of chemicals used in everyday life because of their wide variety of applications, being the most important by far as plasticizer agents in polymer industry [7]. The migration of PEs from plastic materials to the food in contact to has been widely reported [8-12], even in the case of polyethylene film bags [13] similar to those used in RTE vegetables. The residual moisture of vegetables and the large surface / weight ratio may facilitate the migration of these species. Considering the toxicological effects of PEs, they were forbidden in materials to come into contact with foodstuffs [14].

Gas chromatography (GC) and liquid chromatography (HPLC) coupled to mass spectrometry (MS) are the most widely used techniques in trace analysis of APs and PEs. The low quantification limits required for the analysis of these compounds in different types of samples, environmental, biological or food matrices, makes necessary the inclusion of enrichment methods in the whole procedure. Environmentally friendly sample preparation techniques have replaced classical methods. In this sense, the application of solid-phase microextraction (SPME) [15-19], stir bar sorptive extraction (SBSE) [20-28] and several liquid-liquid microextraction techniques (LLME) as dispersive liquid-liquid microextraction (DLLME) [29-32], ultrasound-assisted emulsification-microextraction (USAEME) [33] and solidification of floating organic drop microextraction (SFODME) [34-35] satisfies the primary objectives of green analytical chemistry, and have been employed for the determination of APs or PEs in water or aqueous samples. The determination of

APs and PEs in vegetables requires an extraction step prior to preconcentration. Steam distillation [5,36], Soxhlet extraction [37,38] and bath ultrasound assisted [39-42] have been applied in these complex matrices.

This paper deals with the determination of nine EDCs, three APs, 4-tert-octylphenol (tOP), 4-octylphenol (OP) and 4-nonylphenol (4-NP) and six PEs, dimethylphthalate (DMP), diethylphthalate (DEP), di-n-butylphthalate (DBP), n-butylbenzylphthalate (BBP), di-2-ethylhexylphthalate (DEHP) and di-n-octylphthalate (DOP), in RTE vegetables, which to the best of our knowledge has not been carried out by extracting them by ultrasounds applied by means of a probe directly immersed into the sample mixture and preconcentration and separation by the combination SBSE-TD-GC-MS.

2. Materials and methods

2.1. Reagents

A standard stock solution containing six phthalates esters, dimethylphthalate (DMP), diethylphthalate (DEP), di-n-butylphthalate (DBP), n-butylbenzylphthalate (BBP), di-2-ethylhexylphthalate (DEHP) and di-n-octylphthalate (DOP), in methanol at 200 mg L^{-1} per compound was purchased from Supelco (Bellefonte, PA, USA). 4-Tert-octylphenol (tOP), 4-n-octylphenol (OP) and 4-n-nonylphenol (4-NP), with purities in the range 97-99.7%, were also provided by Supelco. Anthracene (IS), with a purity of 99.5% was supplied by Dr. Ehrenstorfer GmbH (Augsburg, Germany). Stock solutions of the alkylphenols (1000 mg L^{-1}) were prepared by dissolving the commercial products, without previous purification, in methanol. Solutions were kept at -10 ºC in the dark. Working standard solutions were prepared daily by diluting with Milli-Q water.

Sodium chloride was obtained from Sigma (St. Louis, MO, USA). Analytical-reagent grade methanol, acetonitrile and acetone were purchased from Lab-Scan (Dublin, Ireland). Deionized water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Instrumentation

Stir bars (Twisters supplied by Gerstel, Mullheim an der Ruhr, Germany) with PDMS coating film (0.5 mm thick, 10 mm length, 24 μL) were used. Prior to use, the stir bars were conditioned in an empty thermal desorption tube at 275 ºC for 0.5 h with helium at a flow desorption rate of 50 mL min^{-1}. The stir bars could be used more than 50 times after a suitable reconditioning process (as recommended by the manufacturer). All analyses were performed in 15 mL glass vials and the solutions were stirred with a fifteen positions magnetic stirrer (Velp Scientifica, Usmate, Italy). Coated stir bars were thermally desorbed using an automated TDU-2 thermal desorption unit

(Gerstel) connected to a programmed temperature vaporization (PTV) injector CIS-4 (Gerstel) by a heated transfer line. The CIS-4 was equipped with a deactivated empty glass liner with baffles. This injection system was mounted on an Agilent 6890N (Agilent, Waldbronn, Germany) gas chromatograph coupled to an Agilent 5973 quadrupole mass selective spectrometer equipped with an inert ion source.

The analytes were desorbed in the splitless mode and applying the following desorption temperature program: start temperature 50 ºC, increased to 250 ºC at 200 ºC min^{-1} and held 10 min. Meanwhile, the desorbed compounds were trapped on the baffled liner in the CIS-4 injector, which was maintained at 25 ºC by means a Peltier unit. After the thermodesorption step, the PTV CIS-4 was heated at 250 ºC min^{-1} to 275 ºC, which was maintained for 5 min. Other conditions in the TDU were: vent flow 30 mL min^{-1} and vent pressure 13.5 psi. Injection into the GC was performed in the splitless mode (9 min splitless time). Analytes were separated on a DB-17MS (50% diphenyl–50% dimethylpolysiloxane, Agilent) capillary column (30 m × 0.25 mm I.D., 0.25 µm film thickness). The oven temperature was programmed as follows: start at 75 ºC for 0.5 min, increase to 200 ºC at 25 ºC min^{-1}, increase to 275 ºC (held 5 min) at 50 ºC min^{-1}, in a total run time of 12 min**.** The helium carrier gas in column was maintained at a constant flow of 1 mL min^{-1}.

The transfer line, ion source and quadrupole analyzer temperatures were maintained at 300, 230 and 150 ºC, respectively. The mass spectrometer was operated using electron-impact (EI) mode (70 eV). The compounds were quantified in the selected ion monitoring (SIM) mode in order to improve the detection limits using the target ion (Table 1). Identification was confirmed by the retention time of the target ion and the qualifier-to-target ion ratios for each compound.

An ultrasonic probe processor UP 200H (Dr. Hielscher, Germany) was used to extract the analytes from food samples.

2.3. Samples and analytical procedure

A total of twelve vegetable samples, eight packed in plastic bags (lettuce, corn salad, arugula, parsley, red cabbage, carrot and two different samples of chard) and the rest canned (artichokes, mushroom, corn and peas), were obtained from a local supermarket.

All analyses were carried out before the expiry date printed on the package. Vegetables were placed in glass beakers, frozen at -18 ºC and then chopped to produce a homogenized product. The packaging bags were cut with scissors into small pieces (~30 mg).

All glassware used was washed with acetone, rinsed with hexane and dried at 80 ºC for at least 2 hours, in order to avoid phthalate contamination. Direct extraction by immersing the stir bar into a suspension containing the vegetable solid sample is not possible and so a previous extraction step was carried out, submitting the reconstituted liquid extract to the SBSE procedure.

Table 1
Retention times and target and qualifier ions for the analytes.

Compound	RT, min	T	Q_1 (Q_1/T %)	Q_2 (Q_2/T %)
DMP	6.8	163	194 (6.3)	
tOP	7.2	135	107 (13.1)	206 (3.6)
DEP	7.3	149	177 (23.8)	222 (2.3)
OP	8.0	107	206 (14.9)	
IS	8.2	178	152 (9.8)	
4-NP	8.45	107	220 (15.2)	
DBP	8.73	149	223 (6.3)	278 (<1)
DEHP	9.9	149	167 (31.2)	279 (11.2)
BBP	10.5	149	206 (26.1)	312 (<1)
DOP	11.76	149	279 (8.4)	390 (<1)

For this, 10 mL of methanol was added to 2 g of vegetable (1 g for samples with high analytes content) in a 15 mL vial. The mixture was sonicated for 1 min (30% amplitude, 0.5 cycles per second) by means of a probe directly immersed into the sample mixture. The mixture was centrifuged for 5 min at 2000 rpm and the supernatant was concentrated to almost dryness using a rotator vacuum evaporator at 40 ºC and 200 mbar. The dried extract was reconstituted in 1 mL of methanol and added to 10 mL of a 2% (m/v) sodium chloride aqueous solution previously placed in a 15 mL glass vial. A stir bar was introduced into the mixture and stirred at 900 rpm for 4 h. The stir bar was removed from the vial, rinsed with Milli-Q water in order to eliminate salt residues and dried with a lint-free tissue before being introduced into a glass desorption tube. The analytes were thermally desorbed from the stir bar, placing the desorption tube in the TDU-2 connected to the PTV injector, and conducted to the GC-MS system.

Filling liquids of canned vegetables were also analyzed by preconcentrating aliquots of 5 mL diluted to 10 mL with water under the above experimental conditions. To identify possible sources of contamination, the migration of the analytes from plastic bags used to package the vegetables was also studied. For this purpose, 30 mg of ink free plastic bag was maintained in contact with 5 mL of water for 10 days at room temperature. Then 1 mL volume of this solution was diluted to 10 mL with Milli-Q water, and the SBSE procedure previously described was applied.

Spiked samples were prepared by addition of a standard mixture of the analytes at two concentration levels (2 and 5 ng g^{-1}) to two different solid food samples (0.2 – 0.5 µg L^{-1} for filling liquids of two canned vegetables). The samples were allowed to stand at 4 ºC for at least one hour before starting their analysis.

3. Results and discussion

3.1. GC-MS separation

The separation conditions were optimized using a standard mixture of the analytes at 1 μg L^{-1} preconcentrated with SBSE and comparing three capillary columns of 30 m x 0.25 mm x 0.25 μm and different stationary phases: HP-1MS (100% dimethylpolysiloxane), HP-5MS (5% diphenyl-95% dimethylpolydimethylsiloxane) and DB-17MS (50% diphenyl-50% dimethylpolysiloxane). Best separation was achieved with the DB-17MS column, which was selected. Several temperature programs were tested in order to obtain the best separation in the lowest time. The program selected used a helium flow rate of 1 mL min^{-1} and eluted the analytes in 6.8 to 11.8 min.

3.2. Study of derivatization reaction

Derivatization of the hydroxyl group in alkylphenols was studied by using two derivatization reactions: in-situ acetylation with acetic anhydride and in-tube silylation with BSTFA. Derivatization reactions were studied by using 10 mL of an aqueous solution containing the analytes at a concentration of 1 μg L^{-1}, and SBSE sampling was carried out in all cases for 180 min at room temperature.

The acetylation reaction was studied varying the acetic anhydride concentration in the 0.5 – 5.0% (v/v) range and the sodium carbonate between 0.5 – 10% (m/v). In-situ acetylation was discarded because the responses for the three alkylphenols were poor, especially for 4-*n*-octylphenol and nonylphenol, agreeing with the literature [20]. The analyte responses did not increase when in-tube silylation was assayed, using different BSTFA volumes or different experimental conditions in the TDU. Consequently, derivatization was not included in the proposed method.

3.3. Optimization of the extraction step

The EPA procedure for extraction of nonvolatile and semivolatile organic compounds [43] was adapted to the required necessities of the SBSE preconcentration technique to be applied. The studied variables were nature and volume of organic solvent, time and power of ultrasounds and sample mass.

The solid sample was extracted using a suitable solvent, capable of extracting the analytes quantitatively from vegetable samples and also compatible with the need of an aqueous medium for SBSE preconcentration. Three different solvents were assayed: methanol, acetonitrile and acetone, all of which are usually employed as organic modifiers in SBSE preconcentration. As shown in Fig. 1A, better results were achieved with methanol for most compounds, except for DEHP and DOP, which were more effectively extracted with acetone. Therefore, methanol was selected.

Another relevant factor is solvent volume, values from 2 to 10 mL were tested. The higher the solvent volume, the higher the extraction efficiency for all the analytes, so 10 mL was chosen.

The extraction of the analytes from solid matrix to liquid was accelerated by the aid of ultrasounds, by the immersion in the extraction vial of an ultrasonic probe. Different times from 1 to 5 min were applied with ultrasound amplitude of 50% and 0.5 cycles per second. As can be observed in Fig. 1B, 1 min of ultrasounds provided the higher extraction efficiency for four analytes, whereas other three compounds were better extracted with 2 min, no significant differences being observed between 1 and 2 min for BBP and DEP. The application of ultrasounds for 1 min was finally selected. It is noteworthy that similar extraction efficiencies were attained when ultrasounds were applied for 30 min by means of a bath. Moreover, this large extraction time involves the solution heating and losses of the organic solvent by evaporation. When the amplitude and the number of cycles per second in the ultrasonic probe were varied in the ranges 30 – 90% and 0.2 – 0.8, respectively, best results were obtained with 30% amplitude and 0.5 cycles per second.

Different volumes of methanol, as reconstitution solvent of the dried residue obtained after evaporation, from 0.2 to 1 mL were checked (Fig. 1C). Best results were obtained with the use of 1 mL. Higher volumes of methanol were not checked considering that percentages of the organic solvent higher than 10% (v/v) were not recommended.

Finally, the amount of vegetable sample, fortified at 10 ng g^{-1}, was varied from 0.5 to 2.0 g. As shown in Fig. 1D best sensitivity was attained with 2.0 g. Nevertheless, the signals were not proportional to the increased sample mass, therefore the presence of matrix effect was suspected.

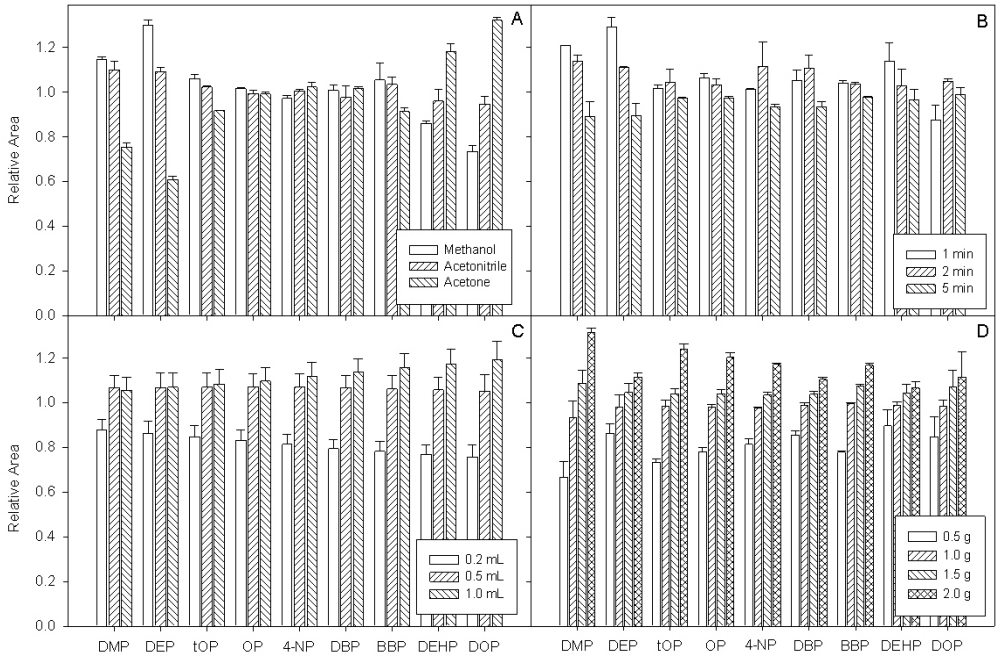

Fig. 1. Influence of (A) extraction solvent, (B) ultrasound time, (C) volume of reconstitution solvent, (C) and (D) sample mass on the sensitivity.

3.4. Optimization of SBSE parameters

To optimize the SBSE procedure, preliminary experiments were carried out using 10 mL of a 10% (v/v) methanolic standard mixture containing 1 μg L^{-1} of each compound, preconcentrated for two hours. All experiments were carried out in duplicate. No significant differences were observed for most compounds when stir bars of different stationary phase volumes (between 24 and 126 μL) were used. Therefore, stir bars of 24 μL were selected in order to reduce equilibration times.

The influence of different ionic strengths of the extraction solution was studied between 0 and 10% (m/v) sodium chloride. The addition of NaCl reduces the water solubility of polar organic analytes and increases their partitioning coefficient between the PDMS coating and water, although, in the case of nonpolar compounds, the water solubility is reduced. Independently of the polarity of the compounds, high salt concentrations may decrease the extraction efficiency because the higher viscosity of the solution hinders the diffusion. All compounds attained maximum extraction efficiency with 2% (m/v) NaCl, except DBP, DEHP and DOP, whose highest sensitivity was attained at 10, 5 and 0% (m/v), respectively. A 2% (m/v) NaCl concentration was selected as a compromise value.

Diffusion from the aqueous phase to the PDMS coating influences the time needed for extraction, which can be shortened by the use of rapid stirring. However, high stirring rates may damage the stir bar coating. The influence of the stirring speed was studied up to 1200 rpm, and, although greater sensitivity was attained for most compounds at 1200 rpm, the lower repeatability due to turbulence effects and the possible deleterious effect on the PDMS polymer led us to select a stirring speed of 900 rpm.

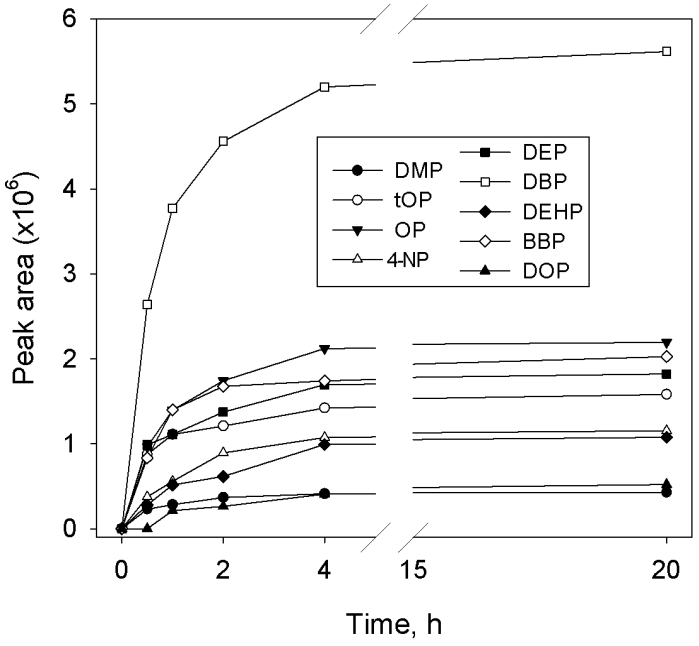

Fig. 2. Effect of the SBSE extraction time on the analytical responses. Sample volume, 10 mL; salt concentration, 2% (m/v); methanol concentration, 10% (v/v). Concentration of the compounds, 1 μg L^{-1}.

The most important parameter affecting SBSE is extraction time. Therefore, once other extraction parameters had been fixed, the optimum extraction time was investigated from 0.5 to 20 h. The extraction time profiles (equilibration curves) are show in Fig. 2. Equilibrium was reached for all compounds at around 4 h, so this time was chosen to ensure high extraction efficiencies.

3.5. Thermal desorption parameters

Desorption temperature, time and flow were studied for the TDU-2, while vent pressure and temperature were the parameters studied for the CIS-4 injector. In general, higher temperatures facilitate desorption of analytes, but may reduce the useful life of the stir bar. When the desorption temperature was evaluated between 250 and 300 ºC, the signals decreased at higher temperatures. Thus, the desorption step was programmed to a final temperature of 250 ºC.

The efficiency of a desorption process generally increases with time and so desorption times were checked between 5 and 15 min. The analytical responses were better at short times, especially in the case of the more volatile species, but when the twisters desorbed for 5 min were reanalyzed, the heavier compounds were seen to be retained (up to 10% in the case of DEP). In order to avoid the carry over effect, 10 min was selected. A carrier gas is necessary to propel the analytes to the PTV injector while they are being thermally desorbed in the TDU. The variation of the gas flow rate between 30 and 50 mL min^{-1} did not affect the responses of the heavier compounds, but lower flow rates favored the more volatile ones, so 30 mL min^{-1} was chosen.

Desorbed compounds were focused in the PTV before entering in the chromatographic column. It was found that losses were less in this device and retention efficiency better at lower temperatures, especially for the more volatile compounds. Since the Peltier unit only allows cooling to temperatures slightly below room temperature, 25 ºC was selected as the focusing temperature. Different liners can be used to facilitate the retention of the analytes in the PTV. Three different liners were tested: empty baffled liners, liners filled with the inert support fiberglass and liners filled with the chemical adsorbent Tenax. The Tenax material provided high noise in blank chromatograms, whereas analytical signals were higher with the baffled liner, which was selected.

Different vent pressures, between 5 and 23 psi, were applied in the PTV injector. High pressure values were not considered because they resulted in column flows higher than 2 mL min^{-1} and possible column bleeding. The highest sensitivity was attained for the more volatile analytes (DMP, tOP and DEP) at the lowest pressure value, whereas high pressures favored the less volatile compounds. A compromise value of 14 psi was chosen. In order to elute the retained compounds in the liner into the chromatographic column, different temperatures, between 250 and 300 ºC, were applied in the PTV, 275 ºC providing the best results. Therefore, a program temperature increasing from 25 to 275 ºC at 250 ºC min^{-1}, with a hold time of 5 min was applied.

3.6. Analytical characteristics of the method

Due to the presence of matrix effect, revealed by comparison of the slopes of aqueous standards calibration graphs with those obtained when the standard additions method was applied to two vegetables (lettuce and red cabbage) and to the filling liquids of two canned food samples (artichokes and peas), the use of an internal standard was assayed, anthacene being selected for this purpose, added to all samples at 10 ng g^{-1}. The use of the IS at 2 ng mL^{-1} allowed the standard additions method to be avoided, because no significant differences were observed when all vegetables and aqueous standards slopes were compared by means of a paired t-test, which provided "p" values in the range 0.07 – 0.32. Table 2 shows the slope values used for quantification purposes.

The optimized method was validated for linearity, detection and quantification limits, selectivity, accuracy and precision. When analyzing blanks of Milli-Q water and blank samples, chromatographic peaks appeared at the retention times of DEP, DBP and DEHP. Because the repeatability of the areas obtained for the blank chromatograms were good under the optimized conditions, the linear range for these compounds was obtained by subtracting the areas obtained in the blank chromatograms (n=5) from the areas obtained for the working solution spiked at different concentration levels. Calibration curves using SBSE-TD-GC-MS were obtained by least-squares linear regression analysis of the ratio of the analyte peak area to the internal standard peak area versus analyte concentration, using eight concentration levels in triplicate. Correlation coefficient values were good (r>0.99), with good linearity responses obtained in the concentration range 0.1 – 5 µg L^{-1} (0.5 – 30 ng g^{-1}, considering a sample mass of 2 g) for all compounds.

Detection limits (LODs) were calculated on the basis of a signal-to noise ratio of 3 (Table 2). The quantitation limits, calculated using a signal-to-noise ratio of 10, ranged between 8.4 and 69 ng L^{-1} for OP and DEHP, respectively. Taking into account that 2 g samples of vegetables were submitted to the procedure, the detection limits obtained ranged between 13 and 106 pg g^{-1} as shown in Table 2.

Table 2
Calibration characteristics of the SBSE-TD-GC-MS method.

Compound	Slope[a], L ng^{-1}	LOD[b], ng L^{-1}	LOD[b], pg g^{-1}	RSD[c], % (n=10)
tOP	1.26±0.03	5.2	25.8	9.4
OP	2.14±0.07	2.5	12.7	4.0
4-NP	1.77±0.06	2.9	14.5	8.2
DMP	2.45±0.06	14	70.7	5.5
DEP	2.65±0.07	9.4	47.0	3.6
DBP	12.3±0.40	15	76.5	3.2
DEHP	3.95±0.09	21	106	3.4
BBP	2.46±0.13	3.2	15.8	4.8
DOP	0.65±0.02	5.0	25.1	5.5

[a] Mean value ± standard deviation (n=10). [b] Corresponding to S/N=3.
[c] Calculated for a spiked vegetable at a concentration level of 2.5 ng g^{-1} for each compound.

To check the repeatability of the method, ten replicate analyses of a fortified vegetable sample were performed at a 2.5 ng g^{-1} concentration level for each compound. Relative standard deviation (RSD) values were lower than 10% in all cases, as shown in Table 2.

3.7. Analysis of samples and recovery studies

Eight different plastic packed vegetables (lettuce, corn salad, arugula, parsley, red cabbage, carrot and two different samples of chard) were analyzed using the SBSE-TD-GC-MS procedure and the results are shown in Table 3. The phthalates, DEP, DBP or DEHP were found in five of the samples at concentration levels in the range 8 – 51 ng g^{-1}, while NP was detected in the arugula sample at 48 ng g^{-1}.

In order to identify a possible source for this contamination, migration studies from the corresponding plastic packages were carried out. Considering the character of the fresh vegetables analyzed, water was used as food simulant to test migration, adopting also the time and temperature established in Council Directive 85/572/EEC [44]. The total mass and volume capacity ratio of the bags employed was considered for selecting the plastic mass and simulant volume. The same PEs found in the food samples were detected at concentration levels in the μg g^{-1} range, in the packages corresponding to the contaminated vegetables, meaning that packages may be one of the main sources of contamination. Nevertheless, no alkylphenols were detected in any package sample.

Figure 3A shows a typical chromatogram obtained under SIM mode for a carrot sample fortified at a 12 ng g^{-1} concentration level, in the selected conditions. Similar chromatograms were obtained for other samples. The analytes were identified comparing the retention time, identifying the target (T) and qualifier ions and qualifier-to-target ratios of the peaks in samples and standard solutions. Figure 3B shows the mass spectra, which confirmed the identity.

Table 3
Contents[a] in vegetable samples (ng g^{-1}), plastic bags (μg g^{-1}) and filling liquid (ng mL^{-1}).

Sample		OP	4-NP	DEP	DBP	DEHP
Lettuce	Food	ND	ND	24.3±0.2	13.8±0.7	ND
	Bag	ND	ND	16.5±3.0	0.35±0.01	ND
Corn salad	Food	ND	ND	24±1	ND	ND
	Bag	ND	ND	7.9±0.4	ND	ND
Arugula	Food	ND	48±2	51±3	ND	ND
	Bag	ND	ND	2.9±0.1	ND	ND
Parsley	Food	ND	ND	8.0±0.2	ND	22±1
	Bag	ND	ND	2.7±0.2	ND	2.2±0.2
Chard	Food	ND	ND	ND	ND	27±1
	Bag	ND	ND	ND	ND	1.2±0.1
Canned artichokes	Food	2.3±0.2	ND	ND	ND	1.5±0.3
	Filling liquid	0.42±0.02	ND	ND	ND	0.71±0.04
Canned mushroom	Food	ND	ND	1.6±0.1	ND	ND
	Filling liquid	ND	ND	0.28±0.01	ND	ND

[a] Mean ± standard deviation (n=3). ND means not detected.

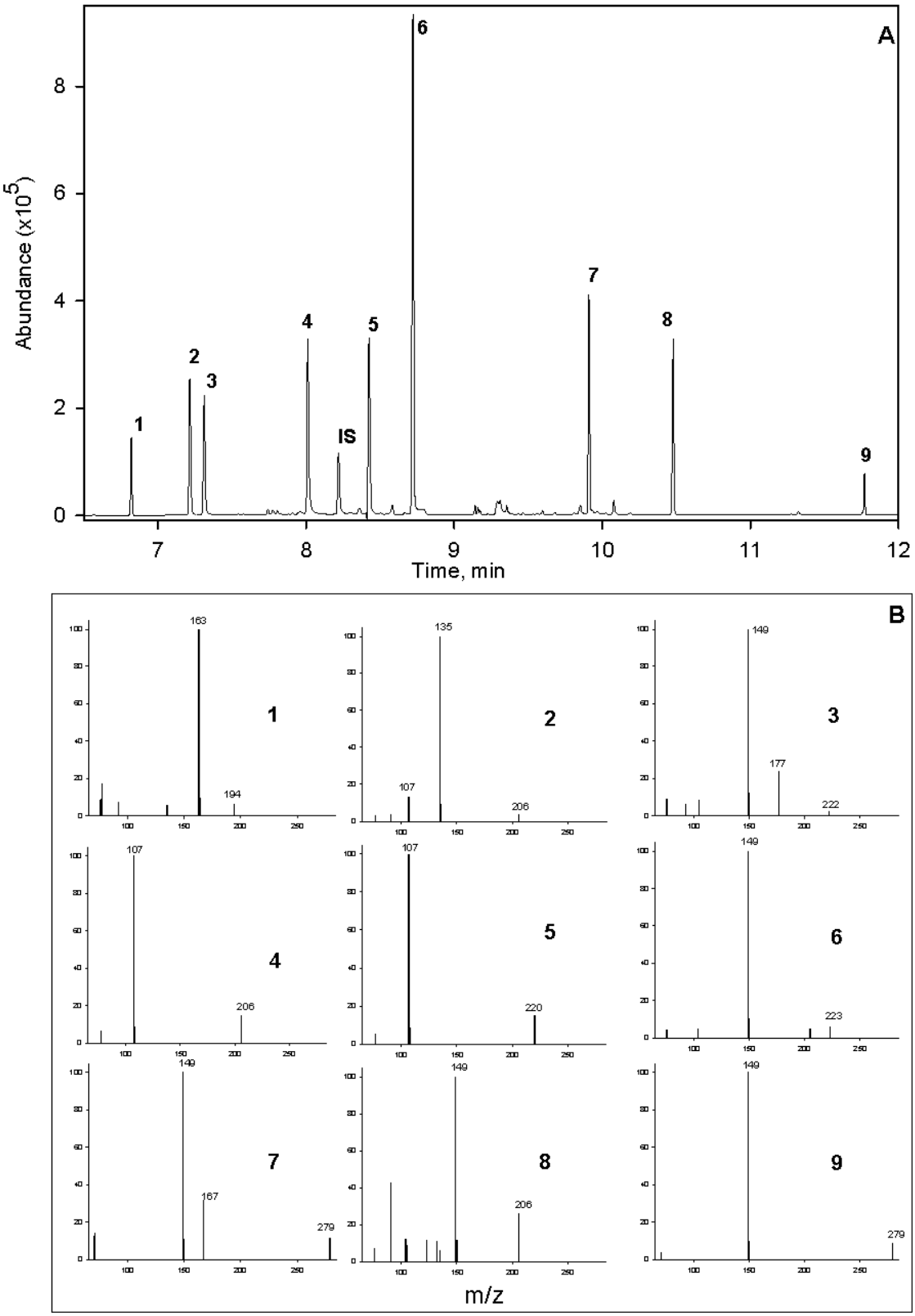

Fig. 3. (A) Elution profile obtained for a spiked carrot sample by SBSE-TD-GC-MS under SIM mode. Peaks correspond to: (1) DMP, (2) tOP, (3) OP, (4) 4-NP, (IS) Naphthalene, (5) DEP, (6) DBP, (7) DEHP, (8) BBP and (9) DOP. Fortification level: 5 ng g^{-1} for each compound. (B) Mass spectra of the compounds.

Both filling liquid and food obtained from four different canned vegetables (artichoke, peas, corn and mushrooms) were analyzed and the results are summarized in Table 3. DEP and DEHP were found in two solid samples (artichokes and mushroom). OP was also found in canned artichoke. For those canned samples which contained the cited compounds, they were also detected in the corresponding filling liquids at concentrations lower than 0.71 ng mL^{-1}. The obtained results are consistent with those presented in previous works, both in food and plastics [4,36-37,40].

Since no reference materials were available for validation of the method, in order to check the accuracy of the proposed method, recovery assays were performed by fortifying two filling liquids of canned vegetables (corn and peas) at two concentration levels corresponding to 0.2 and 0.5 ng mL^{-1}, and two vegetables (lettuce and red cabbage) at 2.0 and 5.0 ng g^{-1}. Each fortification level was carried out by duplicate. The recoveries varied from 87 to 115% (n=72) for the filling liquids and from 83 to 118.5% (n=72) for the vegetable solid samples.

4. Conclusion

A sensitive analytical method for the determination of endocrine disrupter chemicals, three alkylphenols and six phthalate esters, has been developed. The method can be regarded as environmentally friendly given that it uses SBSE for preconcentration purposes as well as thermal desorption prior to GC-MS. The application of this combination to the analysis of vegetables provides several advantages, such as the low consumption of sample and organic solvents, the ease of the extraction using a probe ultrasonic processor and the precise quantification achieved at low concentrations against aqueous standards. The results obtained from migration studies concluded that the phthalate esters contamination observed in the vegetable samples may come from the bags.

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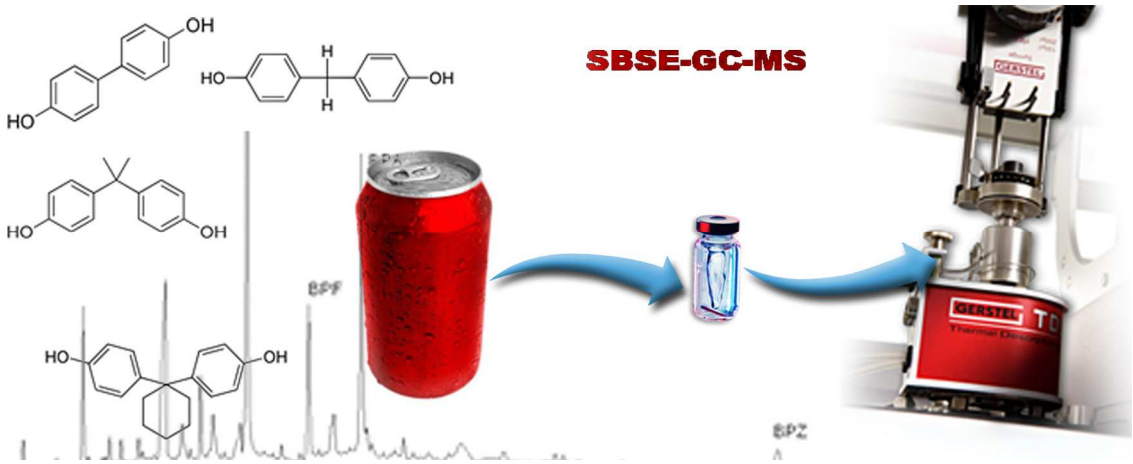
Stir bar sorptive extraction coupled to gas chromatography-mass spectrometry for the determination of bisphenols in canned beverages and filling liquids of canned vegetables

Abstract

This paper describes a method for the simultaneous determination of bisphenol A (BPA), bisphenol F (BPF), bisphenol Z (BPZ) and biphenol (BP), using stir bar sorptive extraction (SBSE) in combination with thermal desorption-gas chromatography-mass spectrometry (TD-GC-MS). Several parameters affecting both extraction and thermal desorption of the SBSE stages were carefully optimized by multivariate designs. SBSE was performed with two derivatization procedures, *in-situ* acetylation and *in-tube* silylation, and the results were compared with those obtained when the analytes were not derivatized. The proposed method, determining the analytes as acyl derivatives, was applied to analyze commercially canned beverages, as well as the filling liquids of canned vegetables, providing detection limits of between 4.7 and 12.5 ng L^{-1}, depending on the compound. The intraday and interday precisions were lower than 6% in terms of relative standard deviation. Recovery studies at two concentration levels, 0.1 and 1 μg L^{-1}, were performed providing recoveries in the 86 – 122% range. The samples analyzed contained higher concentrations of BPA than of the other analytes.



1. Introduction

Bisphenols (BPs) are widely used as modifying monomers of plastics to produce engineering and composite materials with excellent properties. Since most polymerization processes do not provide a 100% yield, the finished plastic material might contain trace residue levels of the monomer that can be released by diffusion or degradation [1]. Bisphenol A (BPA) is an industrially important chemical widely used in the manufacture of polycarbonate plastics and epoxy resins, the latter used in the production of food-contact surface lacquers for cans [2]. Bisphenol F (BPF) is also used in the manufacture of epoxy resins, due to its capacity to reduce viscosity in matrices such as coating linings or impregnations [3]. Bisphenol Z (BPZ) is used in curing highly heat resistant plastic materials or for electrical insulation. The monomer of epoxy resins, between other materials, is modified with an organic intermediate named 2,2'-biphenol (BP), which improves its properties and reduces the presence of rubber and occurrence of plastic oxidation. The heating processes (e.g. sterilisation) involved in the manufacture of foodstuff packages, such as plastic food containers and epoxy food-can coatings, can lead to the migration of these four species into food or beverages, a situation that is aggravated by the presence of sodium chloride, vegetable oils or acids, and long storage times [1,2].

BPs are considered endocrine disruptor chemicals (EDCs). They show estrogenic activity and have been suspected as potential carcinogenic and mutagenic compounds, producing adverse effects in animals, aquatic life and humans [3]. Their content in food has been restricted by the EU Commission to 0.6 mg kg^{-1} of food or food simulant in the case of BPA [4], and the reference dose established by the Environmental Protection Agency (EPA) is 0.05 mg BPA kg^{-1} body weight/day [5].

The extensive use of BP-based polymers, the hazards associated with them, and increasingly restrictive legal regulations underline the need for new efficient and sensitive analytical methods to control human exposure. Gas chromatography (GC) and liquid chromatography (LC), coupled to mass spectrometry (MS), have been widely used for trace analysis of BPs, BPA being the most studied congener [6]. Lower detection limits and higher resolution can be achieved with GC-MS, although not strictly necessary, a derivatization step is recommended in order to increase the volatility of the compounds and the sensitivity of the method. In this sense, silylation and acetylation have been by far the most used derivatization reactions, although each has its own limitations and advantages.

The low quantification limits required for the analysis of these compounds in environmental, biological or food samples make it necessary to include enrichment methods in the procedure. In this respect, environmentally friendly sample preparation techniques have widely replaced classical methods with their need for large amounts of organic solvents and long processing times. Liquid-

liquid extraction (LLE) [7,8] and solid-phase extraction (SPE) [9-12] have been used for BP quantification in beverages and canned foods, as have other SPE variants [13-17]. Nevertheless, the number of references dealing with microextraction techniques applied to the determination of BPs in this type of sample is very scarce, but include solid-phase microextraction (SPME) [18-20] and ultrasound-assisted emulsification-microextraction (USAEME) [21]. USAEME [22], dispersive liquid-liquid microextraction (DLLME) [23,24], single-drop microextraction [25] and others based on ionic liquids [26], have been used to preconcentrate BPs from water and urine samples, as well as for migration studies.

Within the framework of green analytical chemistry, the use of stir bars coated with an adsorbent phase, usually polydimethylsiloxane (PDMS), has led to the development of a clean sample preparation technique, stir bar sorptive extraction (SBSE). This is based on the same mechanism as SPME, but the larger volume of PDMS phase deposited on the stir bar ensures higher recoveries of the compounds of interest [27,28]. New commercially available thermal desorption units facilitate the hyphenation of GC and SBSE, providing higher repeatability and sensitivity as well. SBSE has previously been used for the determination of BPs in waters [29-33], soils [34] and body fluids [29,35], as well as to carry out migration studies from baby bottles [36]. Few of the cited references deal with other bisphenol congeners, apart from BPA [7,14,20,22,24]. As far as we know, no procedures based on the SBSE-TD-GC-MS combination have been reported for the determination of BPs in foods. Consequently, the aim of this work is the optimization of this coupling for the sensitive determination of BPA, BPF, BPZ and BP in beverages and the filling liquids of canned vegetables. Migration studies from food cans are also carried out using the optimized methodology.

2. Materials and methods

2.1. Reagents

Analytical-reagent grade methanol was purchased from Lab-Scan (Dublin, Ireland). Deionized water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Sodium carbonate and sodium chloride were purchased from Probus (Barcelona, Spain) and Panreac (Barcelona, Spain), respectively. The derivatising reagents assayed were acetic anhydride (AA, Fluka, Buchs, Switzerland, >99% purity) and bis(trimethylsilyl)trifluoroacetamide (BSTFA, Supelco, Bellefonte, PA, USA). Commercially available bisphenol A (BPA, 2,2-(4,4-dihidroxydiphenyl)propane, 99% purity), bisphenol F (BPF, 2,2-(4,4-dihidroxydiphenyl)methane, 98% purity), bisphenol Z (BPZ, 1,1-bis(4-hydroxyphenyl)cyclohexane, 99% purity) and biphenol (BP, 2,2'-biphenol, 98% purity) were provided by Sigma (St. Louis, MO, USA). Stock solutions of

1000 mg L^{-1} were prepared in methanol and stored in darkness at −10 °C. Working standard mixed solutions were prepared daily by diluting with Milli-Q water.

2.2. Instrumentation

All analyses were performed in 15 mL glass vials and the solutions were stirred in a 15 positions magnetic stirrer (Velp Scientifica, Usmate, Italy) using commercial stir bars for sorptive extraction coated with a 0.5 mm layer thickness of PDMS (24 µL) obtained from Gerstel (Mullheim an der Ruhr, Germany). Prior to use the stir bars were conditioned in an empty thermal desorption tube at 275 ºC for 0.5 h with helium at a flow desorption rate of 50 mL min^{-1}.

The sample introduction system was composed of a Thermal Desorption Unit (TDU-2) equipped with an autosampler (MPS-2) and a Programmed Temperature Vaporization (PTV) Cooled Injector System (CIS-4) provided by Gerstel. The TDU was operated in splitless mode, with the following desorption programme temperature: start temperature 50 ºC, increase to 275 ºC at 200 ºC min^{-1} and hold for 10 min. Meanwhile the desorbed compounds were trapped on a 1 mm d.i. liner packed with silanized glass wool (Gerstel) in the CIS-4 injector, which was maintained at 25 ºC by means a Peltier unit. After the thermodesorption step, the PTV CIS-4 was heated at a speed of 250 ºC min^{-1} from 25 to 275 ºC, which was maintained for 5 min. Other conditions in the sample introduction system were: vent flow 90 mL min^{-1} and vent pressure 9 psi.

GC analyses were performed on an Agilent 6890N (Agilent, Waldbronn, Germany) gas chromatograph coupled to an Agilent 5973 quadrupole mass selective spectrometer equipped with an inert ion source. The helium carrier gas in the column was maintained at a constant flow of 1 mL min^{-1}. A HP-5MS (5% diphenyl–95% dimethylpolysiloxane, Agilent) capillary column (30 m × 0.25 mm I.D., 0.25 µm film thickness) was used. The GC temperature programme was: start temperature of 75 ºC, increase to 275 ºC at 50 ºC min^{-1} and hold for 6 min, eluting the analytes with retention times between 4.6 and 8.7 min, corresponding to BP and BPZ, respectively. The temperatures of the ion source, transfer line and quadrupole were 230, 300 and 150 ºC, respectively. The mass spectrometer was operated using electron-impact (EI) mode (70 eV). The compounds were quantified in the selected ion monitoring (SIM) mode in order to improve the detection limits using one target and two qualifier ions. Monitorized ions (*m/z*) were 186, 228(28) and 270(<5) for BP; 200, 242(30) and 284(<5) for BPF; 213, 228(25) and 255(20) for BPA and 268, 310(88) and 352(22) for BPZ. The underlined number is the *m/z* of the target, and values in brackets represent the qualifier-to-target ion ratios in percentage. Identification was confirmed by the retention time of the target ion and the qualifier-to-target ion ratios for each compound.

An ultrasonic bath (Selecta, Barcelona, Spain) and an EBA 20 centrifuge (Hettich, Tuttlingen, Germany) were used to degass and eliminate solid particles, respectively, in the samples.

2.3. Samples and analytical procedure

A total of twenty samples of different types of beverages and vegetables sold in cans internally protected with epoxy lacquers were obtained from a local supermarket. Prior to their analysis, the beverage samples were degassed by placing them in an ultrasound bath for one hour [17], in order to prevent the appearance of bubbles on the coating stir bar during the extraction step, which might reduce the efficiency of the sorption process. All samples, degassed beverages and filling liquids were centrifuged for 5 min at 2000 rpm and the liquid portion recovered. Aliquots of 2 mL of the supernatant (1 mL for samples with high analytes content) were diluted to 10 mL with Milli-Q water and placed in a 15 mL glass vial. For *in-situ* derivatization, 200 μL of AA and 300 mg of Na_2CO_3 were added to the vial, and the sample solution was vigorously shaken for a few seconds. After derivatization, the extraction was started by inserting the SBSE stir bar into the mixture, which was stirred at 600 rpm for 3 h until equilibrium was reached. Next, the stir bar was removed from the vial, rinsed with Milli-Q water in order to eliminate salt residues and dried with a lint-free tissue before being introduced into a glass desorption tube. The analytes were thermally desorbed from the stir bar by placing the desorption tube in the TDU.

In order to identify possible sources of sample contamination, migration studies from used cans were performed using food simulants according to Commission Directive 97/48/EC of 29 July 1997 [37]. For this purpose, 10 mL aliquots of an aqueous solution containing a 3% (v/v) acetic acid were maintained in contact with cans for 10 days at 40 ºC, and then subjected to analysis using the above described procedure.

Recovery experiments were carried out using samples which were spiked, with a standard mixture of BPs, at concentration levels of 0.1 and 1 μg L^{-1}. The samples were allowed to stand at 4°C for at least half an hour before being submitted to the procedure above described. Analytes were extracted and quantified by triplicate, and recovery data were calculated.

All glassware used was washed with acetone, rinsed with hexane and dried at 80 ºC for at least 2 h, in order to avoid BPs contamination.

3. Results and discussion

3.1. Study of the derivatization reaction and SBSE extraction

Both the analysis of the underivatized analytes, as well as two different derivatization reactions, *in-situ* acetylation and *in-tube* silylation, have been considered in this work. In order to evaluate the main factors affecting the SBSE procedures different screening multivariate designs, according to the number of variables studied, were used. For this purpose, a statistical package was used to generate the experimental matrices and to evaluate the results. In order to define which variables affected significantly the response of the analytes, an analysis of variance for a 95%

confidence level was carried out. For those factors checked as most important, a more exhaustive study was carried out.

3.1.1. Optimization of the SBSE extraction for non- derivatized BPs

In order to simplify the sample treatment, the SBSE extraction of the free phenolic compounds was considered, taking into account their relatively high values for log $K_{o/w}$ and the low acid character of their phenolic groups, which mean that they show acceptable chromatographic behaviour.

A fractional factorial design was proposed to select the optimal conditions, considering the following main variables at two different levels: pH (3 and 7), NaCl (0 and 5% (m/v)) and methanol (0 and 10% (v/v)). The design consisted of 5 experiments carried out in duplicate and on a random basis. The analysis of the effects obtained for this design, related to variability in centre samples, is summarized in Table 1A. The negative sign means that the change of the factor from the low to the high level produces a decrease in the analytical signal, while a positive sign means that this change increases such signal. These experiments were carried out using 10 mL of aqueous solutions containing the analytes at 10 μg L^{-1} concentration and stirring for 4 h.

According to the analysis (Table 1A), the pH had a positive effect. This behaviour may be attributed to the fact that the optimum extraction pH range for the PDMS coating is 2-10, being pH 3 very close to the lower limit and a neutral pH was adopted. Similar behaviour was observed for sodium chloride, for which a highly positive effect was observed for the four analytes, since the addition of salt reduces the water solubility of polar organic analytes, increasing their partitioning coefficient between the PDMS coating and solution.

Given that high salt concentrations may decrease the extraction efficiency due to the increased viscosity of the solution, which hinders analyte diffusion [27], a more detailed study of this variable was carried out. Maximum sensitivity was attained with the addition of 10% (m/v) NaCl (Figure 1A), which was selected. Methanol was employed as organic modifier to avoid the retention of some analytes on the glass walls of the vials during extraction. As shown in Table 1A, methanol addition had a highly negative effect for all the compounds, which can be explained by the reduction in polarity of the extraction medium in the presence of the organic solvent, which increases analyte solubility. Consequently, the addition of methanol was discarded.

The most important parameter affecting SBSE is extraction time, which was investigated from 0.5 to 24 h using 10 mL aliquots of neutral aqueous solutions containing 10% (m/v) sodium chloride and the analytes at 10 μg L^{-1} concentration level. As shown in Fig. 1B, equilibrium was reached for all compounds close to 4 h, so this extraction time was selected to ensure the highest extraction efficiencies.

Table 1
Analysis of effects obtained for the multivariate designs during the optimization of different steps of the SBSE procedure.

A. Extraction step	BP	BPF	BPA	BPZ
pH	+	+	++	+++
NaCl	+++	+++	+++	+++
Methanol	---	---	---	---
B. *In-tube* silylation derivatization	BP	BPF	BPA	BPZ
BSTFA	+++	+++	+++	+++
BSTFA-Stir bar contact time	+++	+++	+++	+++
TDU initial hold time	---	---	NS	---
TDU initial temperature	NS	NS	++	NS
C. *In-situ* acetylation and extraction	BP	BPF	BPA	BPZ
AA	NS	+++	++	NS
Na_2CO_3	---	---	---	---
Methanol	NS	NS	NS	NS
NaCl	NS	NS	NS	NS
D. Thermal desortion step for acyl derivatives	BP	BPF	BPA	BPZ
TDU maximum temperature	++	+	NS	++
TDU holding time	+	+	NS	++
PTV maximum temperature	++	++	NS	NS
Inlet flow	+	+++	+++	+++
Inlet pressure	+++	NS	NS	NS

Significance: NS not significant, + positive effect, ++ high positive effect, +++ very high positive effect, − negative effect, −− high negative effect, −−− very high negative effect.

3.1.2. Optimization of the in-tube silylation derivatization procedure

Silylation has been the classic derivatization method for BPs, with BSTFA being the most used reagent. The main disadvantage of this approach is the requirement for a water-free environment. The coupling of silylation to SBSE-TD, was first applied for alkylphenols [38] and named as *in-tube* silylation, although only one application of this approach for BPA has been found in the literature [33].

Since derivatization of compounds occurs after they have been extracted onto the stir bar, the variables which provided the highest extraction efficiencies without derivatization, such as salt or methanol addition, pH or extraction time, were adopted.

Relevant factors affecting *in-tube* silylation, such as the volume of BSTFA (0.5 and 2 μL), contact time between the derivatization reagent and the stir bar prior to thermal desorption (0 and 20 min), TDU initial holding time (0 and 10 min) and TDU initial temperature (25 and 75 ºC), were considered in a Plackett-Burman design with 9 experiments carried out in duplicate and on a random basis.

These experiments were carried out by stirring 10 mL of 2 μg L^{-1} aqueous solutions for 4 h and then analysing the effects, related to variability in centre samples, see Table 1B. According to the results, the volume of derivatizing reagent is a very important parameter, so a more detailed study was carried out in order to achieve maximal efficiency.

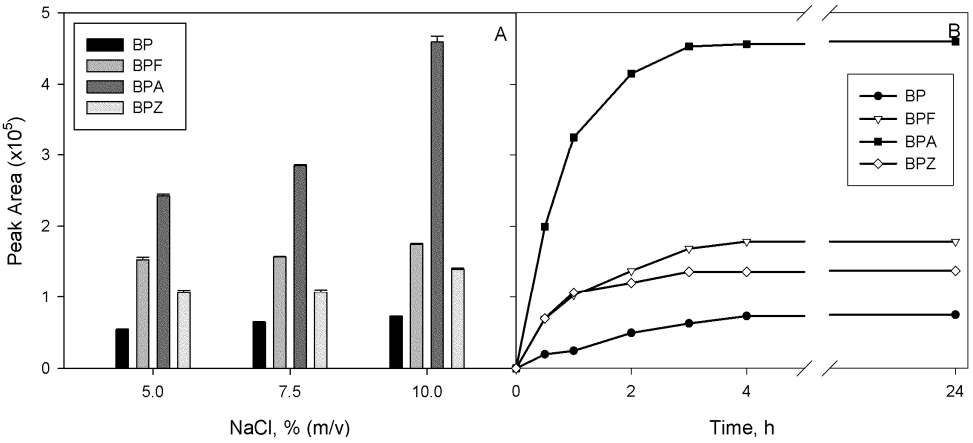


Fig. 1. Effect of sodium chloride (A) and SBSE extraction time (B) on the sensitivity of the underivatized BPs.

Thus, the BSTFA volume was varied between 1 – 3 μL (Figure 2A), the maximal signal for all analytes being obtained when 3 μL were used. Higher volumes were not assayed since they may lead to swelling of the PDMS phase or blockage of the CIS-4 unit, resulting finally in overpressure problems [33].

On the other hand, as expected the contact time between BSTFA and the stir bar prior to thermal desorption had a highly positive effect. This can be explained by the fact that BSTFA gradually vaporizes even at 20 ºC and the reaction takes place in the PDMS phase with the trapped analytes, as has been previously demonstrated for other analytes [39] and for BPs when using on-fiber SPME silylation [20]. The effect of the TDU initial holding time was negligible (Table 1B) for BPA, considering that derivatization had occurred outside the TDU. Nevertheless, a very high negative effect was observed for the other compounds, which can be explained by the losses in the CIS-4 because of the flowing gas. Consequently, no initial holding time was included in the TDU desorption temperature programme, which represents a saving of time when a large number of samples have to be analyzed.

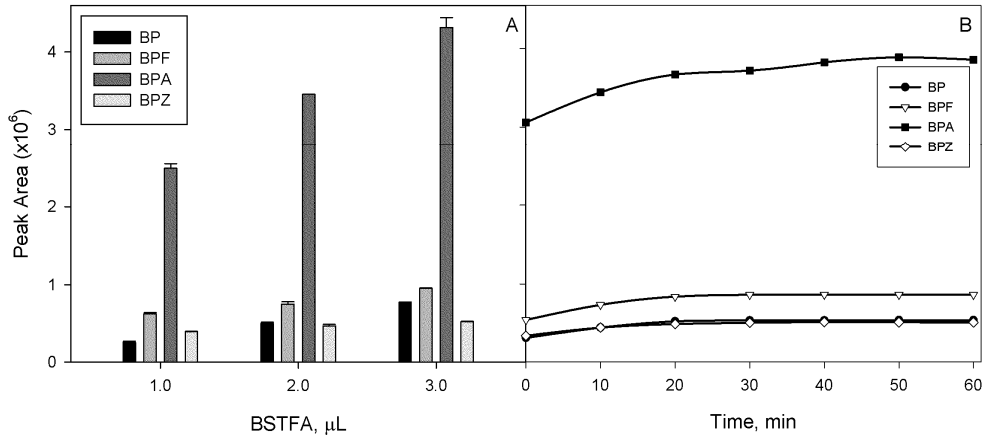


Fig. 2. Influence of BSTFA volume (A) and contact time between BSTFA and the stir bar trapped analytes prior to thermal desorption (B) on the sensitivity.

Although two largely differing temperatures were studied for the TDU initial temperature (below and above the BSTFA boiling point), this factor was seen to have a highly positive effect for BPA, but was negligible for the other analytes. Consequently, the TDU initial programme temperature adopted was 75 ºC.

3.1.3. Optimization of the acetylation derivatization and SBSE extraction

In-situ acetylation prior to SBSE improves not only the chromatographic response but also sample enrichment in the PDMS phase [30]. This derivatization implies less time and effort than other reactions. Extraction and derivatization parameters, such as AA volume (50 and 200 µL) and sodium carbonate (1 and 2 neutralization equivalents, ranging from 0.5 to 4% (m/v)), sodium chloride (0 and 5% (m/v)) and methanol (0 and 10% (v/v)) concentrations, were studied by means of a Plackett-Burman design (9 experiments by duplicate and on a random basis) and using 10 mL aqueous solutions containing 1 µg L^{-1} of each analyte stirred for 4 h. Analysis of the effects results, related to variability in centre samples, is summarized in Table 1C.

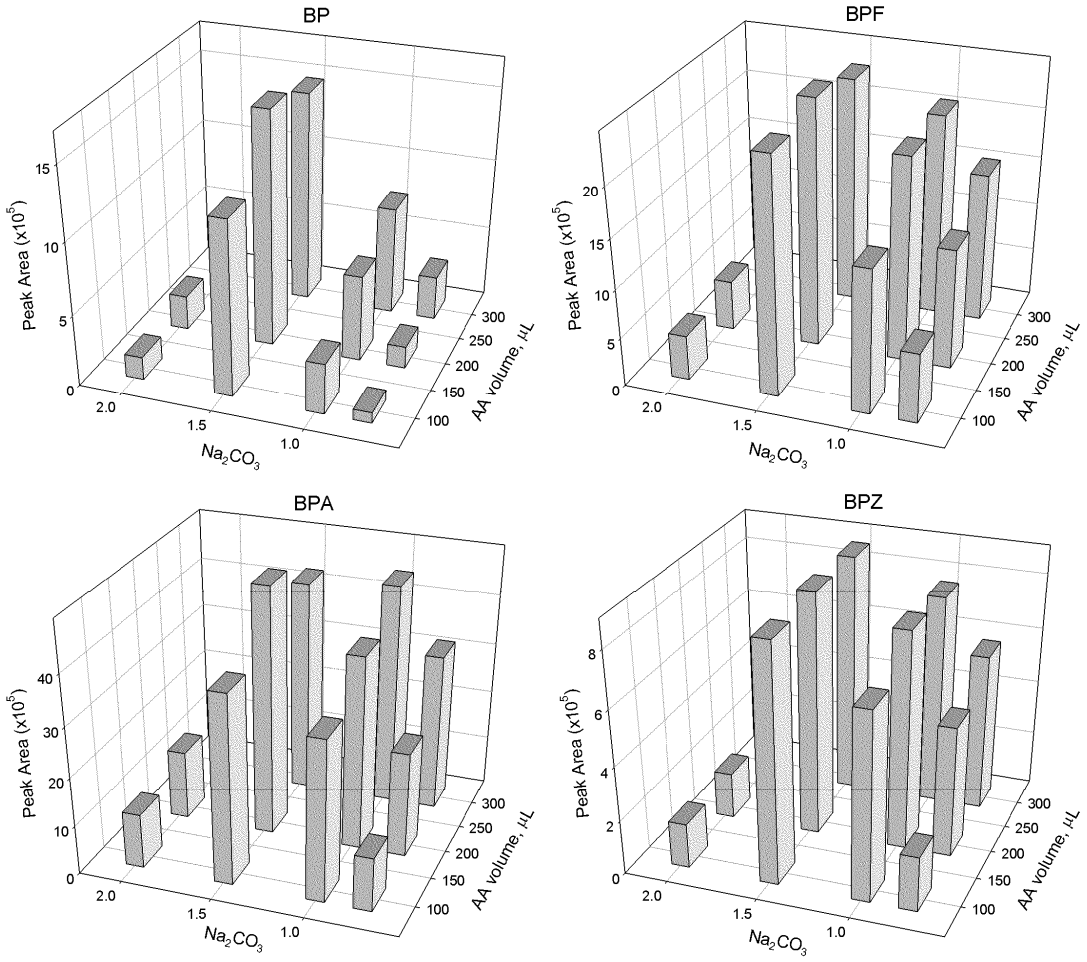


Fig. 3. Responses obtained for the acetylated analytes using different AA volumes and Na_2CO_3 stoichiometric equivalents related to AA.

Salt addition did not seem to be a relevant factor, which can be explained considering the addition of regulating salts, which ensures a high ionic strength in the extraction medium. In the same way, methanol addition does not improve the analytical responses. The volume of the derivatization agent and the concentration of Na_2CO_3 were the variables having the greatest influence on sensitivity. A positive effect of AA volume on BPF and BPA was observed, whereas no significant effect was observed on BP and BPZ. On the other hand, the lowest concentration level of Na_2CO_3 provided increased responses. A more detailed study of these two variables was jointly conducted in the range 100-300 μL for AA and 0.75-2.0 stoichiometric equivalents for Na_2CO_3 related to the AA (n=12), and the responses obtained were represented in Fig. 3. As it shows, the maximum sensitivity was attained with 200 μL of AA and 3% (m/v) of Na_2CO_3, corresponding to a pH of about 8.0.

Since the extracted compounds differed from those of previous sections, SBSE extraction time profiles were obtained for acyl derivatives. In this case, BPs reached equilibrium after approximately 3 h. Of note is the fact that this step took less time than was necessary for the extraction of non-derivatized BPs, probably because of the higher log $K_{o/w}$ of the acyl derivatives.

3.2. Comparison of the three procedures

Applying those optimal experimental conditions found for underivatized compounds, silyl and acyl derivatives, the sensitivity of the three procedures was compared. *In-tube* silylation and *in-situ* acetylation procedures were about 30 and 70 times, respectively, more sensitive than when analyzing underivatized compounds, being these results similar to those previously found [6,19,27]. The lower sensitivity attained for silylated compounds than for acyl compounds can be explained by considering that the latter have higher log $K_{o/w}$ values than underivatized compounds, being this the form in which the silylation procedure extracts the analytes on the stir bar. Consequently, *in-situ* acetylation was selected. In constrast, similar studies carried out previously using SPME and polyacrylate fibers found higher sensitivity with BSTFA than with AA [20]. Since *in-situ* acetylation provided higher sensitivity, thermal desorption parameters were studied for this procedure.

3.3. Thermal desorption conditions for acyl derivatives

Since the number of variables involved in the thermal desorption step is large, their optimization was carried out following a Plackett-Burman design (9 experiments, by duplicate) considering the following variables: desorption time (5 and 10 min), desorption temperature (225 and 275 ºC), desorption flow (30 and 90 mL min^{-1}), PTV heating temperature (225 and 275 ºC) and vent pressure (6 and 12 psi). Analysis of effects results, related to variability in centre samples, is summarized in Table 1D.

Values of 275 ºC and 10 min were selected for the desorption temperature and time, respectively, since they positively affected the sensitivity of all compounds, except BPA. A carrier gas is necessary to propel the analytes to the PTV injector while they are being thermally desorbed in the TDU. Higher desorption flows increased the efficiency of the process, so 90 mL min^{-1} was chosen. The pressure of this gas flow did not seem to be a significant factor in the desorption step for most compounds but was highly positive for BP. A compromise value of 9 psi was selected, which involves a saving of time and gas consumption compared with the use of higher pressures. Desorbed compounds were focused in the PTV before entering the chromatographic column. Lower temperatures in this device mean lower losses and greater retention efficiency, especially for the more volatile compounds. Since the Peltier unit only allows cooling to slightly below room temperature, 25 ºC was selected. In order to facilitate the retention of the analytes in the PTV, a liner filled with silanized glass wool as inert support was used. As regards PTV maximum temperature, Table 1D shows that temperature had no significant effect for the analytes, and so 275 ºC was selected. Therefore, a PTV programme temperature increasing from 25 to 275 ºC (6 ºC s^{-1}), with a hold time of 5 min was applied.

In order to determine whether analyte desorption was complete in the finally selected conditions, second desorptions were carried out on previously desorbed stir bars, and there were no signs of carry over.

3.4. Analytical data

To study the relevance of any matrix effect, the slopes of aqueous standards calibration graphs were compared with those obtained when the standard additions method was applied to four samples (tonic water, beer and filling liquids for asparagus and corn) using a t-test. The absence of a matrix effect was confirmed because "p" values in the range 0.20 – 0.89 were obtained. Consequently, quantification of the samples was carried out against external aqueous standards.

The selected method was validated for linearity, detection and quantification limits, selectivity, accuracy and precision. Even though Milli-Q water was used during the procedure, traces of BPA were detected at concentrations of about 10 ng L^{-1}. Because the repeatability of areas obtained for the blank chromatograms was good under the optimized conditions, this value was taken as background level, and the linear range for this compound was obtained by subtracting the average area obtained in blank chromatograms (n=5) from those obtained in working solutions. Calibration curves using SBSE-TD-GC-MS were obtained by least-squares linear regression analysis of the peak area versus analyte concentration using seven concentration levels in triplicate. The correlation coefficient values were good ($r>0.99$), meaning good linearity responses in the concentration range 20 – 2500 ng L^{-1} for all compounds. Detection limits (Table 2) were calculated taking into account a signal-to noise ratio of 3, except for BPA, whose limit of detection should be

related to the uncertainty of its blank, by 3 times the standard deviation. The detection limits ranged between 0.9 and 2.5 ng L^{-1}, depending on the compound. Taking into account that aliquots of 2 mL of sample were submitted to the analysis procedure, the detection limits for samples ranged between 4.7 and 12.5 ng L^{-1}.

To check the repeatability of the method, twenty-five replicate analyses, ten one day and five each day during the following three days, were performed by using an aqueous standard solution of 0.5 µg L^{-1} for each compound. Intraday and interday relative standard deviation (RSD) values were lower than 6% in all cases, as shown in Table 2.

3.5. Analysis of samples and recovery studies

Ten canned beverages (soda, tonic water, beer, sports drink, tea and cola) and the filling liquids of ten canned vegetables (mushrooms, asparagus, artichoke, olives, pineapple, peas, corn and pepper) were analyzed using the optimized SBSE-TD-GC-MS procedure and the results are shown in Table 3. BPF, BPA or BPZ were found in eight of the beverage samples analyzed at concentration levels in the range 0.08 – 0.68 µg L^{-1}. On the other hand, one or more of the four analytes were detected in all the filling liquids analyzed at concentrations in the 0.5 – 14 µg L^{-1} range. The results obtained are consistent with those presented in previous works, both for food and beverages [7,11,14,20].

In order to identify a possible source of this contamination, migration studies were carried out using four of the used cans corresponding to the samples analyzed. Considering the character of the analyzed foods, a 3% (v/v) acetic acid solution was used as food simulant to test migration studies [37]. The same compounds found previously in the corresponding samples were detected at concentration levels ranging from 0.04 to 0.35 µg L^{-1} (Table 4), with the exception of BPF in pea filling liquid. These results indicated that the packaging may be a source of contamination [40].

Table 2
Analytical characteristics of the selected method.

Compound	Slope, (L mg^{-1})	RSD[a] (%)		LOD[b] (ng L^{-1})	LOQ[c] (ng L^{-1})
		Intraday, n=10	Interday, n=25		
BP	905	2.89	5.28	0.9	3.1
BPF	1239	3.59	5.80	0.9	3.2
BPA	2436	1.90	3.11	2.5[d]	8.4[e]
BPZ	449	3.22	3.57	1.7	5.7

[a] Calculated for a fortified sample at the concentration level of 0.5 µg L^{-1} for each compound.
[b] Corresponding to S/N=3. [c] Corresponding to S/N=10.
[d] Corresponding to $3\sigma_{blank}$. [e] Corresponding to 10 σ_{blank}.

Table 3
BPs contents in the soft drinks and filling liquids (μg L^{-1}).

Sample	BP	BPF	BPA	BPZ
Tonic water 1	ND	ND	0.24±0.01	ND
Tonic water 2	ND	ND	ND	ND
Sports drink 1	ND	ND	0.30±0.05	ND
Sports drink 2	ND	0.26±0.01	0.21±0.02	0.09±0.01
Tea	ND	0.08±0.01	ND	ND
Cola Soda	ND	0.08±0.01	0.67±0.03	ND
Soda 1	ND	ND	0.39±0.01	ND
Soda 2	ND	ND	ND	ND
Beer 1	ND	ND	0.53±0.01	ND
Beer 2	ND	ND	0.68±0.02	ND
Mushroom 1	ND	1.02±0.01	5.67±0.02	ND
Mushroom 2	ND	ND	7.42±0.02	ND
Asparagus 1	ND	ND	4.81±0.12	ND
Asparagus 2	ND	7.07±0.02	13.98±0.3	ND
Artichokes	ND	0.87±0.03	3.83±0.11	ND
Olives	ND	ND	1.51±0.11	0.76±0.10
Pineapple	ND	ND	5.35±0.17	0.92±0.12
Pepper	ND	1.60±0.01	5.68±0.34	ND
Peas	0.58±0.07	4.44±0.22	ND	ND
Corn	ND	ND	2.45±0.04	ND

Values are mean ± standard deviation (n=3). ND means not detected.

Since no reference materials were available for the validation of the method, in order to check the accuracy, recovery assays were performed by fortifying two beverages (beer and tonic water) and two filling liquids (asparagus and corn) at two concentration levels 0.1 and 1 μg L^{-1}, which corresponded to the lowest concentration of the linearity range and an intermediate value. The recoveries varied from 86 to 122% (n=48) at the lowest level and from 97 to 105% (n=48) for the highest level. RSD values for these samples were lower than 10%. Consequently, the method does not seem to be influenced by the type of liquid sample analyzed, if it is subjected to proper dilution.

Table 4
Migration contents to food simulant from cans (μg L^{-1}).

Sample	BP	BPF	BPA	BPZ
Tonic water 1	ND	ND	0.10±0.01	ND
Beer 1	ND	ND	0.22±0.01	ND
Peas	0.04±0.01	ND	ND	ND
Pepper	ND	0.04±0.01	0.35±0.01	ND

Values are mean ± standard deviation (n=3). ND means not detected.

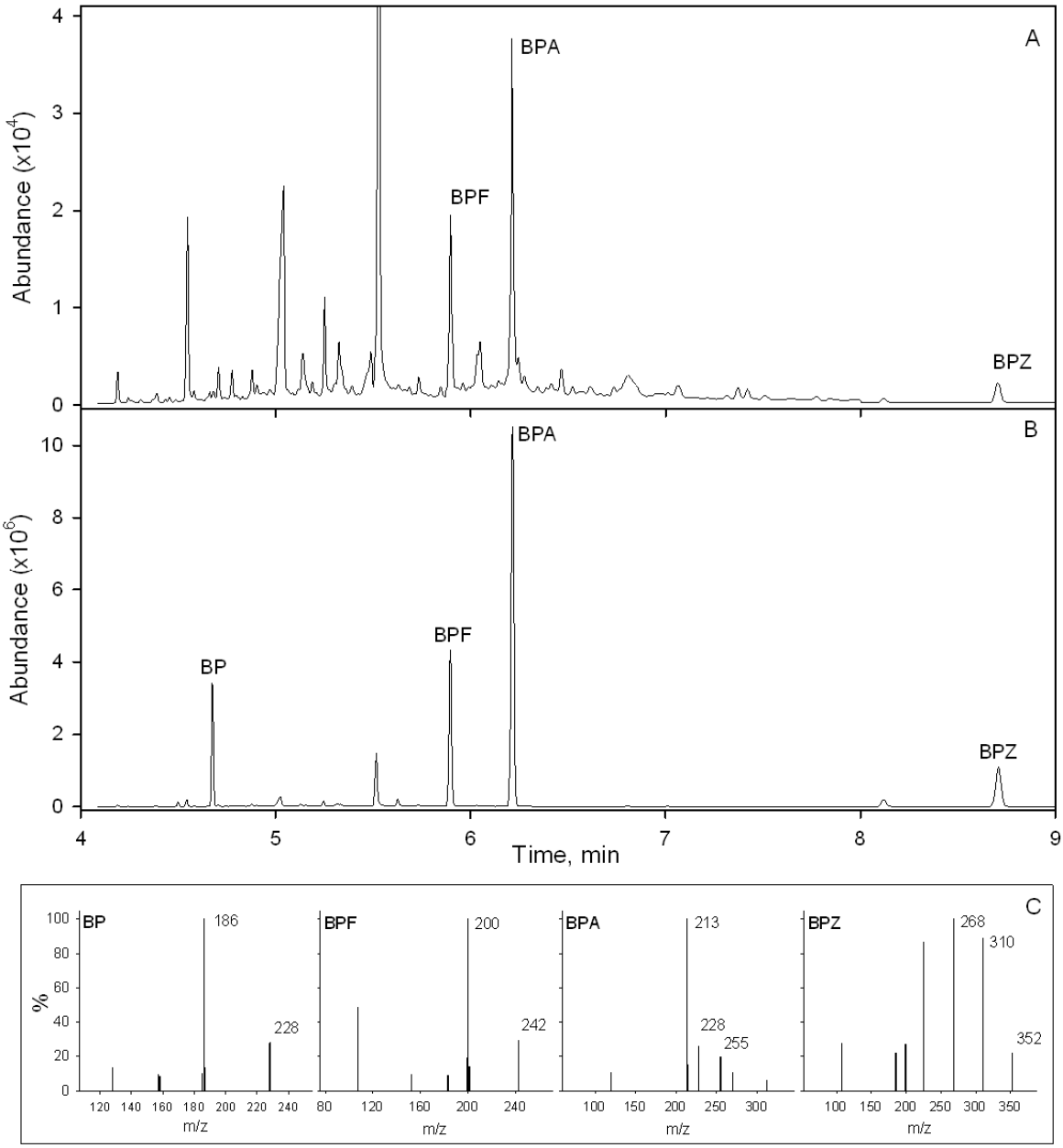


Fig. 4. Elution profiles obtained for sports drink sample 2 (A) and for soda 2 sample fortified at 5 μg L^{-1} concentration level for each compound (B), using the SBSE-TD-GC-MS procedure under selected ion monitoring (SIM) mode. (C) Mass spectra of each compound.

4. Conclusion

A sensitive analytical method for the determination of some bisphenols and biphenol has been developed. Several derivatization alternatives were optimized and compared among themselves and with respect to the analysis of the underivatized compounds. The proposed method, involving *in-situ* acetylation of the analytes, can be categorized as environmentally friendly since SBSE is used

for preconcentration purposes, which was thermally desorbed prior to the chromatographic separation by GC-MS.

The application of the combination SBSE-TD-GC-MS to the analysis of canned beverages and filling liquids has several advantages, including the low consumption of sample and organic solvents and the precise quantification possible at low concentrations against external standards. The results obtained from migration studies from cans suggested that bisphenol contamination in samples may come from the packaging.

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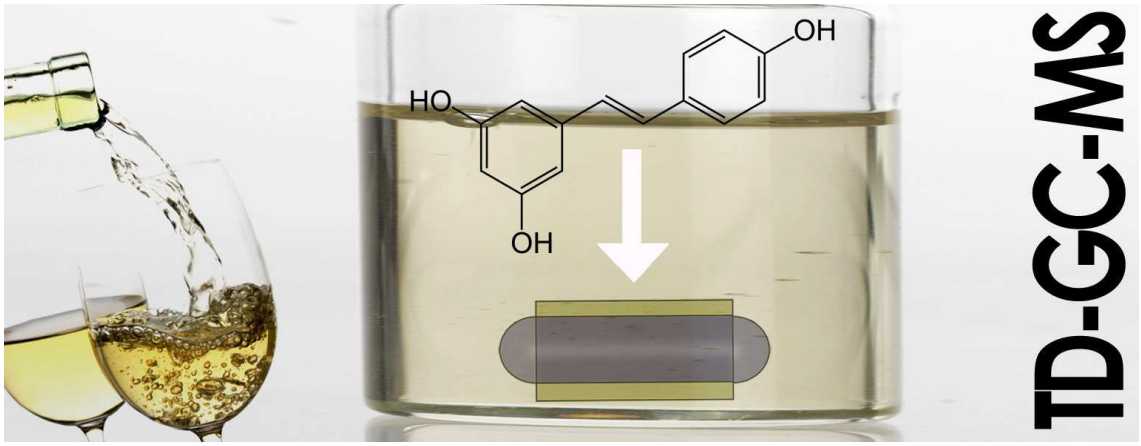
Stir bar sorptive extraction with gas chromatography-mass spectrometry for the determination of resveratrol, piceatannol and oxyresveratrol isomers in wines

Abstract

A simple and highly sensitive procedure based on stir bar sorptive extraction coupled to gas chromatography-mass spectrometry by means of a thermal desorption unit (SBSE-TD-GC-MS) has been optimized for the determination of *cis/trans* isomers of resveratrol, piceatannol and oxyresveratrol in wine samples. Quantification of the *cis*-isomers was carried out by generating the standards from the corresponding *trans*-species once they had been preconcentrated on the SBSE extracting phase. The optimization of the acetylation derivatization, SBSE extraction and thermal desorption steps was investigated using Plackett-Burman designs, taking into account the high number of variables to be considered. The use of bisphenol F as internal standard allowed quantification of the samples against aqueous standards. Repeatability, expressed as relative standard deviation of 10 successive analyses was between 5 and 9%, confirming the high precision attained under the optimized conditions. Satisfactory recovery values of between 79 and 109% were obtained for spiked samples in the 0.2 – 1.0 μg L^{-1} concentration range, depending on the compound. The main compound determined in the analyzed samples was *trans*-resveratrol, with concentrations in the range 3 – 230 μg L^{-1}, depending on the type of wine.



1. Introduction

Stilbenes are polyphenolic phytoalexins produced by plants as defence substances against biotic or abiotic stress. Resveratrol (3,4',5-trihydroxystilbene), piceatannol (3,3´,4´,5-tetrahydroxystilbene) and oxyresveratrol (2,3',4,5'-tetrahydroxystilbene) are the most representative compounds of this group of polyphenols [1]. Due to their antioxidant activity, stilbenes have a wide range of beneficial effects, including anticancer, anti-inflammatory, anti-microbial and estrogenic activities, and they have been proposed as therapeutic agents in humans [1-3]. The frequent consumption of resveratrol-rich products has been associated with longer life expectancy [4]. While resveratrol is the most studied stilbene, some attention has also been paid to piceatannol [5] and oxyresveratrol, in the search for more effective analogue compounds [6]. These stilbenoids exist naturally in two isomeric forms, the more stable *trans*-(E) and the less stable *cis*-(Z), whose isomerization can be achieved by heating or irradiation with intense UV light. The *cis*-resveratrol isomer is sensitive to atmospheric oxidation, and is only stable at neutral pH and when completely protected from light [7].

These compounds have been found in several plants, including peanuts, raspberries, blueberries and tea, although the highest concentrations are reached in grapes and derived products, such as wine, which is one of the main sources of stilbenes in Mediterranean diet [8]. Vines synthetize *trans*-resveratrol and other *trans*-stilbenoids in response to stress situations such as physical injuries, fungal invasion or exposure to ultraviolet light. *Cis*-isomers are produced during the wine making process, while the stilbenoids content in wine depends on the grape variety, as well as climatic conditions [9]. Since the compounds are usually located in grape skin, their concentrations are usually higher in red wine, which is fermented with the skins, unlike white wine [3]. The oenological procedures followed may affect their concentration, and wines produced by carbonic maceration provide considerable lower stilbene contents than those prepared in the traditional way [10].

Taking into account the beneficial properties of these species, efficient and sensitive methods are necessary for their determination [7]. Stilbenoids are generally determined by liquid chromatography (LC) using different detection systems, such as UV [11–14], fluorescence [15–17] or mass spectrometry [11,18–20]. Gas chromatography (GC), especially when it is hyphenated with mass spectrometry (MS), has important advantages over LC, allowing identification and confirmation even at low levels [9,14,21–29]. Nevertheless, since these compounds are non-volatile, the need for a chemical derivatization reaction, such as acetylation [9,25] or silylation [21-24,26-30], prior to GC separation has long been considered the main drawback to this technique.

The low concentration of stilbenoids in some food samples, as well as the complexity of these matrices, mean that sample treatment steps must be included in the analytical procedure. In this

regard, conventional approaches are time-consuming and require large volumes of sample and organic solvents, implying environmental contamination, risks for human health and additional costs for residue treatment, thus being replaced by methods based on solvent-free procedures, which offer automation and are cleaner, more selective, rapid and efficient. The determination of polyphenolic compounds has been achieved by techniques such as solid-phase microextraction (SPME) [19-22,26,27] and different liquid-liquid microextraction (LLME) procedures, including single drop microextraction (SDME) [28] and dispersive liquid-liquid microextraction (DLLME) [25]. The use of stir bars coated with an absorbent phase (usually PDMS) has led to the development of a green sample preparation technique, stir bar sorptive extraction (SBSE) [30]. SBSE is based on the same mechanism as SPME, but the larger volume of PDMS phase on the stir bar ensures higher recoveries of the compounds of interest [30]. New commercially available thermal desorption units (TDU) facilitate hyphenation between GC and SBSE, and provide higher repeatability and sensitivity. However, as far as we know, the literature shows only one application of SBSE, in this case coupled to LC, for the determination of stilbenoids [17].

The present study describes a new solvent-free method for the sensitive determination of both *cis*- and *trans*-isomers of resveratrol, piceatannol and oxyresveratrol in wines, using the SBSE-TD-GC-MS combination.

2. Materials and methods

2.1. Reagents

Commercially available stilbenes and the internal standard were obtained from Sigma (St. Louis, MO, USA): *trans*-resveratrol (>99%), *trans*-piceatannol (>98%), *trans*-oxyresveratrol (>97%) and bisphenol F (BPF, 2,2-(4,4-dihidroxydiphenyl)methane, 98%, IS). Stock solutions (1000 mg L^{-1}) were prepared by dissolving the commercial products in methanol, and were kept at −18 ◦C in darkness. Working standard solutions were prepared by diluting with pure water, while solutions of the *cis*-isomers were obtained by UV irradiation of the corresponding *trans*-isomer using a UV cracker (PS Analytical, Orping, UK) for 1 min. Analytical-reagent grade methanol was purchased from Lab-Scan (Dublin, Ireland). Sodium hydrogen phosphate, sodium phosphate and acetic anhydride (AA) were supplied by Sigma.

2.2. Instrumentation

Commercial stir bars coated with a 0.5 mm thick layer of PDMS (24 μL) were obtained from Gerstel (Mullheim an der Ruhr, Germany). Prior to use, the stir bars were conditioned in an empty thermal desorption tube at 275 ºC for 0.5 h with helium at a flow desorption rate of 50 mL min^{-1}.

The sample introduction system comprised a Thermal Desorption Unit (TDU-2) equipped with an autosampler (MPS-2) and a Programmed Temperature Vaporization (PTV) Cooled Injector System (CIS-4) provided by Gerstel. Experimental conditions used for the sample introduction system are summarized in Table 1.

GC analyses were performed on an Agilent 6890N (Agilent, Waldbronn, Germany) gas chromatograph coupled to an Agilent 5973 quadrupole mass selective spectrometer equipped with an inert ion source. Under the selected conditions (Table 1) the analytes eluted at retention times between 9.7 and 15.8 min, corresponding to *cis*-resveratrol and *trans*-piceatannol, respectively (Table 2).

The compounds were quantified in the selected ion monitoring (SIM) mode in order to improve the detection limits using different ions (Table 2). Identification was confirmed by the retention time of the target ion and the qualifier-to-target ion ratios for each compound.

2.3. Samples and analytical procedure

A total of 15 wines (white, rosé and red) were obtained from local wine merchants. Samples were kept in darkness at 4 °C until analysis.

An SBSE stir bar was placed in a 15 mL glass vial containing a 10 mL aliquot of the sample, 10 ng of the internal standard, 210 µL of AA and 650 mg of Na_2HPO_4. The resulting solution was stirred at 600 rpm for 3 h until extraction equilibrium was reached. Taking into account the high content of some of the analytes in several samples, especially red wines, an appropriate dilution with water, ranging from 1:1 to 1:10, was carried out prior to the SBSE extraction step. Next, the stir bar was removed from the vial, rinsed with water and dried with a lint-free tissue before being introduced into a glass desorption tube. The analytes were thermally desorbed from the stir bar by placing the tube in the TDU.

Calibration curves for the quantification of the *cis*-isomers were obtained by including an isomerization step between the SBSE extraction and the thermal desorption steps. The isomerization was carried out by exposing the enriched stir bars with the *trans*-isomers to an UV lamp for 10 min.

A synthetic wine containing 3.2 g L^{-1} of L-(+)-tartaric acid and 12% (v/v) of ethanol, with the pH adjusted to 3.6 by means of a diluted NaOH solution, was used to optimize the method, in order to ensure similar conditions to that of a real sample [31].

Since no reference materials were available, spiked samples were prepared at two different concentration levels for validation purposes. Two wine samples (white and red) were spiked at concentrations ranging from 0.2 to 1.0 µg L^{-1}, depending on the compound. Three replicates were analyzed in each case.

Table 1
Experimental conditions of the TD-GC-MS procedure.

Thermal Desorption Unit	
Mode	Splitless
Temperature programme	50 – 260 ºC at 210 ºC min^{-1}, held 9.6 min
Desorption flow and pressure	75 mL min^{-1}, 7 psi
Cooled Injector System	
Mode	Solvent Venting
Liner	Packed silanized glass wool, 1 mm i.d.
Temperature programme	15 – 275 ºC (5 min) at 540 ºC min^{-1}
GC-MS	
Capillary column	HP-5MS, 5% diphenyl-95% dimethylpolysiloxane (30 m x 0.25 mm, 0.25 μm)
Carrier gas	Helium (1 mL min^{-1})
Oven programme	80 – 280 ºC at 25 ºC min^{-1}, held 8 min
Transfer line temperature	300 ºC
Quadrupole temperature	150 ºC
Ion source temperature	230 ºC
Ionization	Electron-impact mode (70 eV)

3. Results and discussion

3.1 Study of the derivatization reaction and SBSE extraction

Acetylation of the hydroxyl groups of stilbenoids improves their chromatographic response and increases their partition coefficient, leading to greater enrichment in the PDMS extracting phase. This derivatization reaction can be easily coupled to SBSE extraction in a procedure known as *in-situ* acetylation [32], which involves less time and effort than other derivatization reactions.

Since acetylation reactions take place more effectively in basic medium, a buffer solution should be added to the sample or generated by the addition of neutralizing salts which react with the resulting acetic acid. Sodium carbonate, which has been used for this purpose [9,17], may produce large amounts of CO_2 bubbles that may remain on the stir bar, reducing its extraction efficiency and reproducibility. The use of dibasic and tribasic phosphate salts may avoid this drawback while assuring a basic medium, for which reason Na_2HPO_4 and Na_3PO_4 were assayed. When both salts were added at different amounts in the interval corresponding to 0.5 – 2 neutralizing equivalents, higher sensitivity was obtained for all the analytes with 1 and 2 equivalents of Na_3PO_4 and Na_2HPO_4, respectively. Since stilbenoids are very sensitive to pH, being decomposed at high pH values [7,33], the stability of the analytes in the resulting buffered solution should be considered. The responses of the analytes were monitorized when time gaps, between the end of the derivatization step and the start of the SBSE extraction, of between 0 and 2 h were applied under the most favourable conditions. Na_2HPO_4 was seen to be a more suitable neutralizing reagent, providing higher analyte responses and good stability. It was therefore used as neutralizing salt in all subsequent experiments.

Table 2
Method characteristics.

Compound	RT (min)	Monitorized ions (*m/z*)	Linearity (μg L^{-1})	RSD[a] (%)	LOQ[b] (ng L^{-1})
cis- Resveratrol	9.7	228, 312, 270	0.1 – 15	5.2	14.4
cis-Oxyresveratrol	11.3	328, 286, 244	0.2 – 25	5.7	17.9
cis-Piceatannol	11.6	328,244, 286	0.2 – 30	6.8	13.5
trans-Resveratrol	13.0	228, 312, 270	0.1 – 15	8.4	14.6
trans-Oxyresveratrol	14.9	328, 244, 286	0.3 – 25	6.4	19.5
trans- Piceatannol	15.8	244, 286, 328	0.25 – 30	8.7	49.2

Underlined numbers correspond to *m/z* of the target ion. [a] n=10. [b] Calculated for S/N=10.

Extraction and derivatization parameters, such as AA volume (50 and 200 μL) and Na_2HPO_4 equivalents (1 and 2 neutralization equivalents, ranging from 0.65 to 5.15% (m/v)), sodium chloride (0 and 5% (m/v)) and methanol (0 and 10% (v/v)) concentrations, were studied simultaneously using a Plackett-Burman design (PBD) (12 experiments per duplicate) and using 10 mL aqueous solutions containing 1 μg L^{-1} of each analyte, which were stirred for 2 h. Pareto charts of the standardized effects are presented in Figure 1A. The addition of salt to the sample solution may reduce analyte solubility in the aqueous phase, resulting in an increase in the enrichment capability of the extracting phase. However, as Fig. 1A shows, the effect of this variable was very close to not being statistically significant, and furthermore it had a negative effect on the analytical response obtained. The addition of regulating salts for *in-situ* acetylation ensures a high ionic strength in the extraction medium, although an additional increase of this factor may have a negative effect due to the movement of non-polar compounds to the water surface [34].

The presence of methanol as an organic modifier may avoid analyte losses due to retention on the glass walls [35], but its organic nature reduces the polarity of the extraction medium, increasing the solubility of the compounds and thus reducing the SBSE extraction efficiency. The addition of methanol to the sample solution showed no statistically significant effect.

According to Fig. 1A, the volume of the derivatization agent and the concentration of Na_2HPO_4 were the variables with the greatest influence on sensitivity, increasing the analyte responses obtained at their higher values. A more detailed study of both variables was conducted by means of a central composite design (CCD) in the range 125 – 275 μL for AA and 1.5-2.5 stoichiometric equivalents for Na_2HPO_4 related to the AA (α=1.5, 4 cube points, 4 axial points and 2 central points, in duplicate). The obtained responses were fitted to the quadratic surface response regression represented in Fig. 1B. As can be seen, maximum sensitivity was attained with 210 μL of AA and 2.4 eq. (6.5% (m/v)) of Na_2HPO_4. The salt concentration adopted provided a pH value of 10 for the extraction medium before the addition of AA, and remained neutral once the acetylation reaction had been performed.

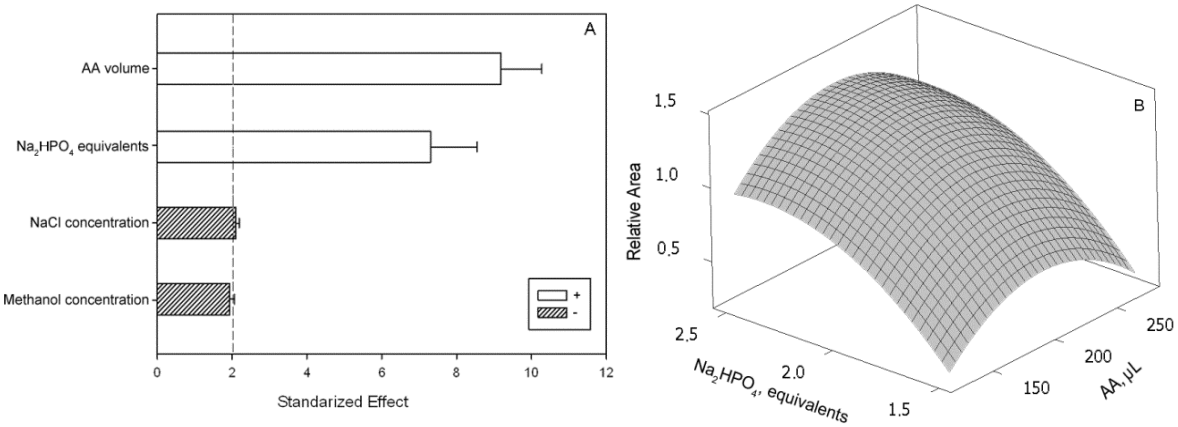


Fig. 1. (A) Pareto Charts obtained of the Plackett-Burman designs for the SBSE extraction step. (B) Response surface showing the effects of AA volume and the amount of Na_2HPO_4 on the relative response.

Once these extraction and derivatization parameters had been optimized, the extraction time, the most relevant SBSE extraction parameter, was separately investigated from 0.5 to 6 h. The resulting time profiles (Fig. 2) show that equilibrium between phases was reached after 2 or 3 h, depending on the compound, and so 3 h was chosen as extraction time.

3.2 Thermal desorption optimization

Due to the high number of variables involved in thermal desorption, the optimization of this step was carried out following a PBD (12 experiments in duplicate), including the following variables: TDU desorption temperature (250 and 300 ºC), TDU desorption time (5 and 10 min), CIS cooling temperature (15 and 25 ºC) and CIS heating temperature (250 and 300 ºC), desorption flow (50 and 100 mL min^{-1}) and vent pressure (6 and 8 psi). A Pareto chart of the standardized effects is presented in Figure 3A.

According to the results, the most relevant parameters for the thermal desorption process were TDU desorption time and temperature, both of which were carefully studied using a CCD (α=1.5, 4 cube points, 4 axial points and 2 central points, in duplicate), which included both variables in the 6.9 – 10.6 min and 240 – 280 ºC ranges, respectively. The obtained response surface was fitted to a quadratic surface response regression (Fig. 3B). A desorption temperature of 260 ºC was selected, so the TDU was heated from 50 to 260 ºC (420 ºC min^{-1}). This TDU temperature was maintained for 9.6 minutes, ensuring the total desorption of the analytes and avoiding the possible carry-over caused by incomplete desorption at lower times, or analyte losses due to excessively long desorption times.

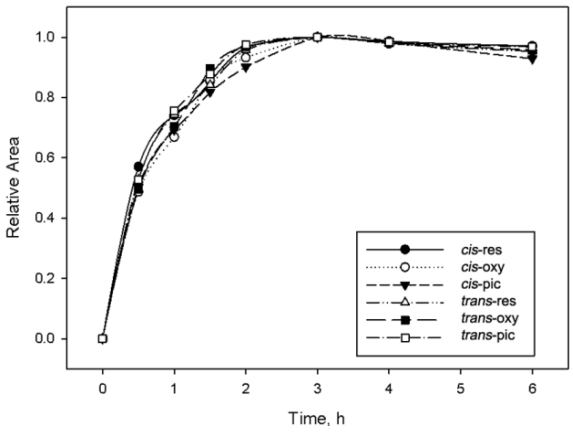


Fig. 2. Extraction time profiles.

An inert gas flow is employed to transfer the analytes from the TDU to the PTV injector. As shown in Fig. 3A, neither the flow-rate nor the pressure of the gas seemed to be significant factors for the desorption step in the studied range, so 75 mL min^{-1} and 7 psi, corresponding to maximum response values in the PBD, were selected.

The desorbed compounds were focused in the PTV by means of a suitable liner. Different liners, empty or filled with different materials, like silanized glass wool, which acts as an inert support, or PDMS foam, which strongly retains non-polar analytes, were assayed. A silanized glass wool filled liner was finally selected.

In general, lower temperatures in the PTV mean lower losses and a greater retention efficiency, but, as Fig. 3 shows, the effect of the focusing temperature was close to not being statistically significant in the range studied, so a compromise value of 20 ºC, at which analytes showed their maximum response in the PBD, was selected.

As regards CIS desorption temperature, Fig. 3A shows that this variable had a significant effect on the analytes response in the studied range. Higher temperatures for the CIS were not assayed due to limitations of the selected PTV liner. The highest suitable temperature, 300 ºC, was selected. Therefore, a PTV heating program increasing from 20 to 300 ºC (9 ºC s^{-1}), with a hold time of 5 min, was applied.

3.3 Generation of the cis-isomers

In previous studies [17, 27, 28] a continuous flow UV cracker was used to obtain a *cis*-isomer solution from the *trans*-isomer, proving to be highly effective in a short time. Despite total conversion, the concentration of the obtained *cis*-resveratrol solution is not equal to the concentration of the original *trans*-resveratrol solution, since UV irradiation may lead not only to their *cis-trans* isomerization, but also to their photodegradation [33].

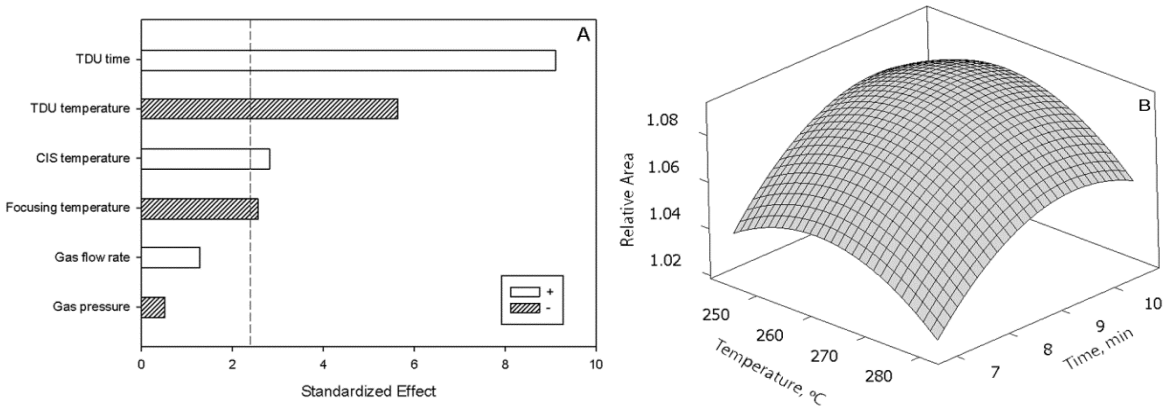


Fig. 3. (A) Pareto Charts obtained of the Plackett-Burman designs for the thermal desorption step. (B) Response surfaces showing the effects of desorption time and temperature on the relative responses.

Fig. 4A shows the influence of irradiation time on the *cis*- generation ratio, using the UV cracker. Fig. 4B represents the combined response of each pair of stilbenoid isomers for different irradiation times. As can be seen, there was a significant decrease in the overall analytical signal, meaning that this in-solution isomerization procedure was not suitable for quantification purposes.

An alternative isomerization procedure was then checked. The rate of isomerization of the stilbenoid depends to a large extent on the surrounding medium, and high viscosity solvents may hinder the *trans*- to *cis*- conversion, thus decreasing the isomerization rate and, additionally, limiting the photodegradation. Thus, it would be possible to obtain a highly effective isomerization without photodegradation by irradiating the stilbenoids after being retained on the surface of the extracting bar. To verify this idea, the *trans*-compounds were firstly extracted on a stir bar and then, while hold with a magnet, it was irradiated with an UV lamp. Fig. 4C and D show the generation percentage of *cis*-isomers and the combined response of each pair of stilbenoid isomers when different irradiation times were applied. Despite the longer times needed for total conversion of the *trans*-isomers, the analytical signals remained almost constant during this isomerization procedure.

The use of this post-SBSE isomerization procedure for quantification purposes is only possible if both *cis*- and *trans*-isomers present similar behaviour during derivatization and SBSE extraction.

3.4 Method performance

The slopes of the aqueous calibration graphs and those obtained from the standard additions to different wine samples (synthetic, white and red) were compared using a paired t-test, and significant differences at the 95% confidence level were found (p values ranging between 0.01 and 0.03). An internal standard was assayed in order to overcome the matrix effect. BPF was used for the purpose because of its chemical similarities to analytes, requiring an acetylation prior to its determination, as well as its similar chromatographic and SBSE extraction behaviour.

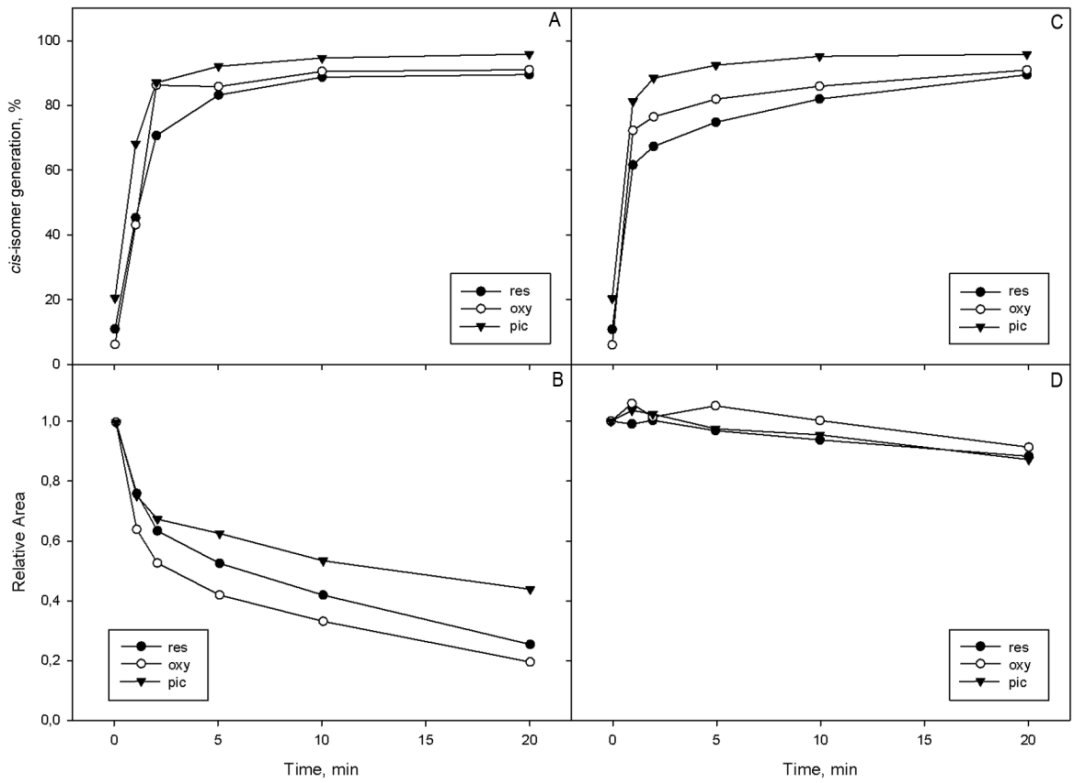


Fig. 4. Influence of time on the generation percentage of *cis*-isomers (A, C) and on the total analytical response (B, D). In-solution isomerization by means of a UV cracker (A, B) and in-coating isomerization by means of a UV lamp (C, D).

The use of this IS, added to samples at a concentration of 1 μg L^{-1}, notably improved the repeatability, calculated as the relative standard deviation from 10 replicate analyses, from 19.6-29.7% to 5.2 – 8.7%, depending on the compound. Moreover, the matrix effect was overcome (p values in the 0.07 – 0.77 range), since no significant differences between the slopes of the aqueous calibration graphs and those obtained from the standard additions to the wine samples were found (Table 3). Consequently, aqueous calibration, providing good regression values ($r^2 > 0.99$) and an excellent linearity for the concentration range studied (Table 3), was used for quantification purposes.

The detection and quantification limits of the method were calculated on the basis of three and ten times signal-to-noise ratio, respectively (Table 2). Detection limits varied between 4 and 14.8 ng L^{-1} for *cis- and trans*-piceatannol, respectively.

When the proposed method is compared with others based in GC-MS and classical sample treatments, such as SPE [9] and LLE [14,29], the main advantages are the organic solvent consumption and the higher sensitivity, which is increased for *trans*-resveratrol [9,29]. If the SBSE optimized method is compared with other GC-MS methods for which sample treatments are based on miniaturized procedures, such as SPME [27], DLLME [25] and DSDME [28], larger times are required for SBSE but lower detection limits are achieved.

Table 3
Slopes[a] (L μg^{-1}) obtained for standard additions to different samples.

Sample	Aqueous	Red wine	White wine
cis-resveratrol	91560±7498	64508±4541	70990±5032
	(0.244±0.006)	(0.233±0.004)	(0.237±0.004)
cis-oxyresveratrol	95133±7931	66223±4466	70519±4032
	(0.254±0.011)	(0.239±0.004)	(0.235±0.005)
cis-piceatannol	316828±21967	215293±14875	230288±14658
	(0.845±0.016)	(0.826±0.014)	(0.838±0.014)
trans-resveratrol	105613±9371	75321±7262	81928±8771
	(0.282±0.008)	(0.272±0.010)	(0.273±0.010)
trans-oxyresveratrol	76999±5192	56203±4135	62033±5320
	0.205±0.003)	(0.203±0.005)	(0.207±0.007)
trans-piceatannol	24072±1633	17217±1283	18632±1709
	(0.064±0.003)	(0.062±0.003)	(0.062±0.005)

(Slopes obtained using internal standard) [a] Mean ± standard deviation (n=6).

3.5 Analysis of wine samples and recovery studies

Fifteen different samples of wine (red, rosé and white) were analyzed using the SBSE-TD-GC-MS procedure. All samples were analyzed in triplicate. The obtained results are summarized in Table 4.

Resveratrol was found to be the most abundant analyte in the studied samples. A clear dominance of its most stable trans-isomer was observed, representing an average of the 73% of the total content.The *trans*-resveratrol levels were higher in red wines, 47.7 – 231.6 $\mu g\ L^{-1}$, than in white wines 0.4 – 13.0 $\mu g\ L^{-1}$. Piceatannol and oxyresveratrol levels were lower than those of resveratrol. Both compounds were higher in red wines, where they were present mostly as *trans*-isomers. The levels of stilbenoids found in wine samples agreed with those reported in the literature [21,25,27], taking into account the possible variations related with climate or light conditions [36].

Fig. 5 shows a typical chromatographic profile obtained for a white wine sample using the described procedure. The analytes were identified by comparing the retention time, mass spectra and relative ratios between the principal ions (Table 2), both in samples and standard solutions.

Since no reference materials were available, a recovery study was carried out to check the accuracy of the proposed method. For this purpose, three wines (white, red and rosé) were fortified with the studied species at two concentrations ranging from 0.20 to 1.0 $\mu g\ L^{-1}$, depending on the compound. The recoveries of the stilbenoids from spiked samples varied from 79 to 97% at the lowest level, and from 99 to 109% at the highest level.

Table 4
Analysis of the samples (μg L^{-1}).

Sample	*cis*-res	*cis*-oxy	*cis*-pic	*trans*-res	*trans*-oxy	*trans*-pic
Red	138±6	NQ	ND	190±15	0.9±0.1	21.7±0.3
Red	206±3	ND	ND	158±1	5.0±0.7	ND
Red	93±3	ND	ND	121±5	NQ	ND
Red	69±1	1.4±0.6	15.2±0.7	87±2	5.2±0.5	8.5±0.1
Red	134±7	ND	ND	231±2	4.4±0.5	23.2±0.3
Red	48±2	NQ	NQ	62±2	1.0±0.3	16.4±0.1
Rosé	4.4±0.4	ND	ND	28±1	ND	ND
Rosé	5.4±0.2	1.6±0.8	1.8±0.9	17.5±0.2	3.1±0.2	4.0±0.2
Rosé	13.0±0.2	3.1±1.5	35±1.6	23±6	5.4±0.3	8.0±0.4
White	8.4±0.4	0.6±0.4	0.6±0.4	13.0±0.1	2.1±0.1	2.7±0.1
White	0.8±0.1	ND	ND	4.7±0.2	ND	ND
White	3.0±0.4	NQ	NQ	8.3±0.2	1.9±0.1	2.5±0.4
White	6.3±0.3	ND	ND	13.0±0.4	ND	ND
White	0.4±0.2	ND	ND	3.4±0.3	ND	ND

Values are mean ± standard deviation (n=3).
ND means not detected. NQ means not quantified.

4. Conclusion

A very sensitive analytical method using the SBSE-GC-MS combination has been proposed for the first time for the simultaneous determination in wine samples of the *cis*/*trans* isomers of the three more representative stilbenoids: resveratrol, piceatannol and oxyresveratrol. A novel and very effective procedure for the generation of the *cis*-isomer standards, in which isomerization takes place by irradiation of the *trans*-isomers once they have been preconcentrated on the SBSE extraction phase, has been employed.

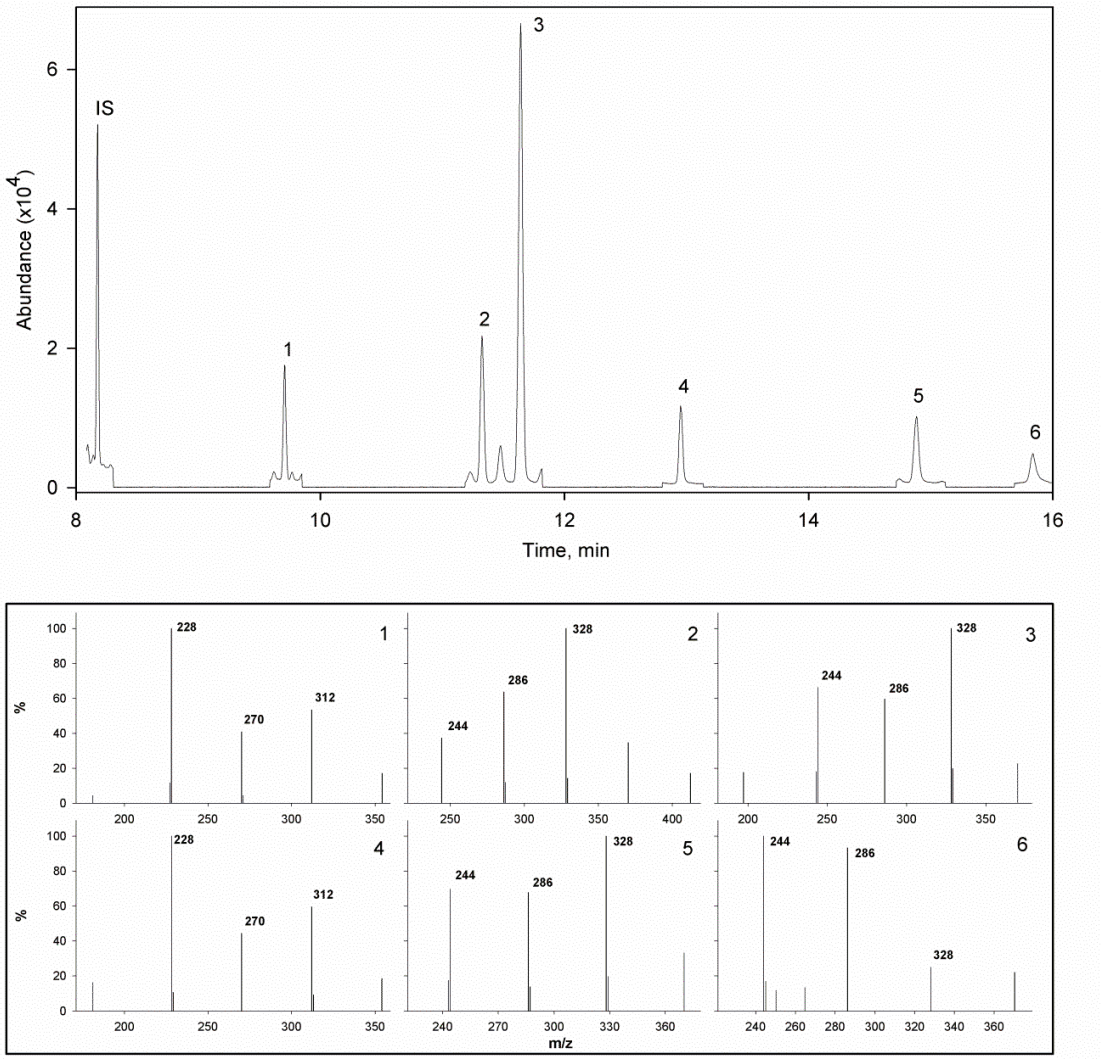


Fig. 5. (A) SBSE-TD-GC-MS chromatogram obtained for a spiked white wine fortified at 2 μg L^{-1} under SIM mode. Peaks correspond to: (1) *cis*-resveratrol, (2) *cis*-oxyresveratrol, (3) *cis*-piceatannol, (4) *trans*-resveratrol, (5) *trans*-oxyresveratrol and (6) *trans*-piceatannol. (B) Mass spectra of each compound.

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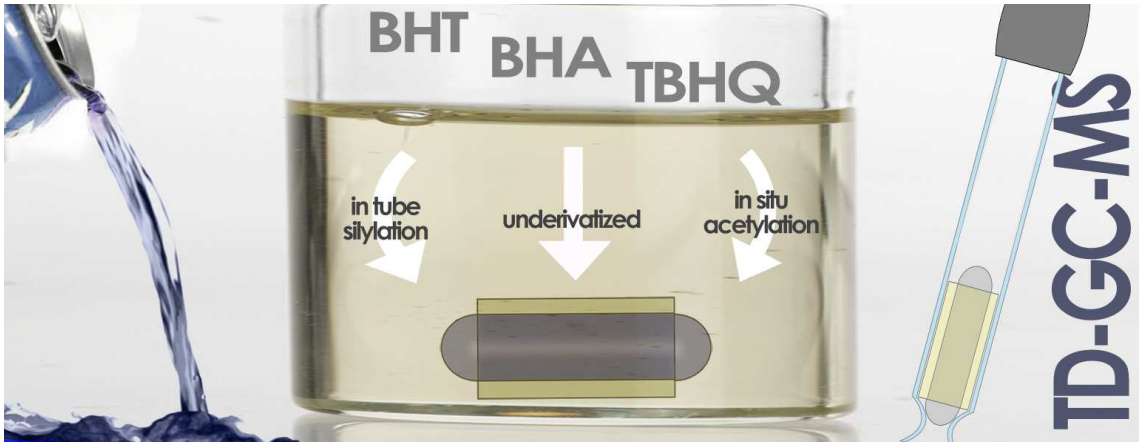
Determination of synthetic phenolic antioxidants in soft drinks by stir bar sorptive extraction coupled to gas chromatography-mass spectrometry

Abstract

The synthetic phenolic antioxidants, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butyl hydroquinone (TBHQ), have been preconcentrated by stir bar sorptive extraction and thermally desorbed before analysis by gas chromatography-mass spectrometry (SBSE-TD-GC-MS). Several parameters affecting the derivatization step and both SBSE extraction and thermal desorption were carefully optimized. When the analyses of BHA and TBHQ in their acetylated, silylated and underivatized forms were compared, the best results were obtained when the *in-situ* derivatization procedure with acetic anhydride was employed. Quantification was carried out using carvacrol as internal standard, providing quantification limits of between 0.11 and 0.15 ng mL^{-1}, depending on the compound. Recovery assays for samples spiked at two concentration levels, 1 and 5 ng mL^{-1}, provided recoveries in the 81 – 117% range. The proposed method was applied to analyze canned soft drinks and the analytes were found in five of the ten samples analyzed.



1. Introduction

Stir bar sorptive extraction (SBSE) has become one of the most useful preconcentration techniques, due to its simplicity and robustness, since its development [1]. In SBSE the analytes are extracted onto a polydimethylsiloxane (PDMS) coating of a stir bar immersed and stirred in the aqueous sample. PDMS is usually employed as extraction phase, but its non-polar nature limits its applicability to hydrophobic compounds with octanol-water partition coefficients (log $K_{o/w}$) higher than 3 [2]. The determination of polar species, such as phenols, requires their previous transformation into less polar compounds by means of derivatization reactions, such as *in-situ* acetylation [3] or *in-tube* silylation [4]. The desorption of trapped compounds can be accomplished by thermal desorption (TD) hyphenated with gas chromatography (GC), or by liquid desorption in conjunction with liquid chromatography (LC). New commercially available TD units facilitate the coupling of GC and SBSE, providing higher repeatability and sensitivity. SBSE has been successfully applied in food analysis [5], and some papers have previously dealt with its employment in beverages and soft drinks, for the determination of pesticide residues [6], preservatives [7], flavouring agents [8] and endocrine disruptors [9].

Synthetic phenolic antioxidants are a group of compounds that include butylated hydroxyanisole (BHA, E320), butylated hydroxytoluene (BHT, E321) and *tert*-butyl hydroquinone (TBHQ, E319), which are usually added to foods. These compounds help prevent fat degradation, for which reason they are mainly added to fat-containing foods. Nevertheless, their presence in fruit juices and beverages has been reported [10,11], probably as result of their addition as antioxidants to the essential oils used as flavouring additives. The safety of these compounds is a great concern, since they may cause allergen reactions, including asthma and hives, in sensitive subjects [12], so their levels in food have been restricted in the European Union [13]. The use of BHA, BHT and TBHQ in soft drinks is not authorized [14] and the maximum permitted level is 1000 mg kg^{-1} in essential oils and 200 mg kg^{-1} in other flavourings, alone or in combination with other antioxidants.

The determination of phenolic antioxidants has been accomplished using LC coupled with different detectors, such as ultraviolet (UV) [10,15,16,17,18,19,20,21,22,23] or mass spectrometry (MS) [16,24,25,26,27]. Gas chromatography (GC) [26,28,29,30,31,32,33,34] has also been used for the determination of these compounds, MS being the most commonly used detector [26,28,29,30,32,34]. Taking into account the low expected concentration of these antioxidants in soft drinks, a preconcentration step is required. Classical enrichment methods, like solid phase extraction (SPE) [26,30] or liquid-liquid extraction (LLE) [27], have been proposed, but in recent years new microextraction procedures, like solid-phase microextraction (SPME) [34] and different modes of liquid-liquid microextraction (LLME) [10,17,18,19], have been applied for the preconcentration of phenolic antioxidants from different sample matrices. These procedures, which

are usually less time-consuming and require lower volumes of organic solvents than classical techniques, are clean, selective, rapid and efficient. Moreover, they are suitable for automatization. SBSE offers a new way to determine phenolic antioxidants, but, as far as we know, it has not previously been applied to these chemicals. In this work, we propose an SBSE-TD-GC-MS method for the determination of three phenolic antioxidants, BHA, BHT and TBHQ, in different soft drinks. Two derivatization procedures as well the extraction of the underivatized compounds were optimized.

2. Materials and methods

2.1. Reagents

Butylated hydroxytoluene (2,6-bis(1,1-dimethylethyl)-4-methylphenol, E321, BHT), butylated hydroxyanisole (3-*tert*-butyl-4-hydroxyanisole, E320, BHA), *tert*-butyl hydroquinone (2-(1,1-dimethylethyl)-1,4-benzenediol, E319, TBHQ) and carvacrol (5-isopropyl-2-methylphenol, IS) were obtained from Sigma (St. Louis, MO, USA). Stock solutions (1000 mg mL^{-1}) were prepared by dissolving the commercial products in methanol, and kept at −18 ºC in darkness. Working standard solutions were prepared by diluting with pure water. Sigma supplied potassium monohydrogen phosphate, acetic anhydride (AA), sodium chloride, and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA).

2.2. Instrumentation

Commercial stir bars coated with a 0.5 mm thick layer of PDMS (24 μL) were obtained from Gerstel (Mullheim an der Ruhr, Germany). The stir bars were conditioned prior to use in an empty thermal desorption tube at 275 ºC for 0.5 h with helium at a flow rate of 50 mL min^{-1}. The sample introduction system comprised a Thermal Desorption Unit (TDU-2) equipped with an autosampler (MPS-2) and a Programmed Temperature Vaporization (PTV) Cooled Injector System (CIS-4) provided by Gerstel. The experimental conditions used for the sample introduction system are summarized in Table 1.

GC analyses were performed on an Agilent 6890N (Agilent, Waldbronn, Germany) gas chromatograph coupled to an Agilent 5973 quadrupole mass selective spectrometer equipped with an inert ion source. Under the selected conditions (Table 1) the compounds eluted at retention times between 6.8 and 9.8 min, corresponding to carvacrol and TBHQ, respectively (Table 2). The compounds were quantified in the selected ion monitoring (SIM) mode in order to improve the detection limits using different ions (Table 2). Identification was confirmed by the retention time of the target ion and the qualifier-to-target ion ratios for each compound.

Table 1
Experimental conditions of the TD-GC-MS procedure.

Thermal Desorption Unit	
Mode	Splitless
Temperature programme	50 – 275 ºC at 225 ºC min^{-1}, held 7.5 min
Desorption flow	40 ml min^{-1}
Cooled Injector System	
Mode	Solvent Venting
Liner	Packed silanized glass wool, 1 mm i.d.
Temperature programme	20 – 275 ºC (5 min) at 540 ºC min^{-1}
GC-MS	
Capillary column	HP-5MS, 5% diphenyl-95% dimethylpolysiloxane (30 m x 0.25 mm, 0.25 µm)
Carrier gas	Helium (1 ml min^{-1})
Oven programme	80 ºC, held 1 min 80 – 230 ºC at 15 ºC min^{-1}, held 1 min
Quadrupole, ion source, transfer line temperatures	150ºC, 230ºC, 300 ºC
Ionization	Electron-impact mode (70 eV)

2.3. Samples and analytical procedure

A total of 10 soft drink samples, including two isotonic drinks, two cold tea, two sodas, two tonic waters, one energy drink and one lemon flavoured soda were obtained from local supermarkets. Samples were kept in darkness at 4 °C until analysis.

A 2 mL-aliquot of sample was placed in a 15 mL glass vial containing 8 mL of water, and 100 µL of AA and 740 mg of K_2HPO_4 were added to the mixture. Carvacrol was incorporated in the sample as internal standard at a concentration of 2 ng mL^{-1}. The resulting solution was manually shaken for 20 seconds while the derivatization reaction proceeded. Then, an SBSE stir bar was placed into the solution and was stirred at 600 rpm for 3 h using a multi-position magnetic stirrer, until extraction equilibrium was reached.

Next, the stir bar was removed from the vial, rinsed with pure water in order to eliminate residues and dried with a lint-free tissue before being introduced into a glass desorption tube. The analytes were thermally desorbed from the stir bar by placing the desorption tube into the TDU.

Since no reference materials were available, spiked samples were prepared at two different concentration levels (1 and 5 ng mL^{-1}) for validation purposes. Three replicates were analyzed in each case.

Table 2
Method characteristics.

Compound	RT (min)	Monitored ions	RSD[a] (%)	LOQ[b] (ng mL^{-1})
IS	6.8	135, 150 (30), 192 (6)	-	-
BHT	7.8	205, 220 (25)	6.6	0.11
BHA	8.9	165, 180 (78), 137 (24), 222 (10)	6.5	0.12
TBHQ	9.8	166, 151 (62), 208 (14), 250 (10)	7.4	0.15

Underlined numbers correspond to *m/z* of the target ion, and values in brackets represent the qualifier-to-target ion ratios as percentage [a] n=10 [b] Calculated for S/N=10.

3. Results and discussion

3.1. Derivatization procedure

Both the analysis of the underivatized analytes, as well as two different derivatization reactions, *in-situ* acetylation and *in-tube* silylation, have been considered. The main factors affecting the derivatization and SBSE procedures were checked. Even though BHT remains underivatized in all cases, due to the steric hindrance of the *tert*-butyl groups, its analytical signal is evaluated to take into account the effect of the variations in the extraction conditions. The experiments were carried out using 10 mL of aqueous solutions containing the analytes at 10 ng mL^{-1} concentration.

In-situ acetylation

Acetylation of the hydroxyl groups of the phenolic compounds improves their chromatographic response and increases their partition coefficient, leading to higher enrichment efficiencies in the PDMS extracting phase. This derivatization reaction can be easily coupled to SBSE extraction through a procedure known as *in-situ* acetylation [3].

The influence of the derivatization agent volume on the analyte responses was tested with AA volumes ranging from 100 to 400 µL. For each AA volume tested, one neutralizing equivalent of K_2HPO_4 was added to the extraction solution to provide the basic medium required for the derivatization reaction to take place and to neutralize the acetic acid generated in the acetylation reaction. The resulting solution was manually shaken for 20 seconds while the derivatization reaction took place. The best response was obtained for BHA when 100 µL of AA were added, while for TBHQ, the highest signal was attained when 300 µL were used (Figure 1A). Taking into account the average response, a 100 µL volume was selected.

A neutralizing salt (K_2HPO_4) was added to the sample to fix the pH prior to the derivatization reaction and also during SBSE extraction once the analytes had been derivatized. Different masses of K_2HPO_4, ranging from 185 to 740 mg (corresponding to 0.5 to 2 neutralizing equivalents) were tested. As shown in Figure 1B, an increase in the analytical signal was observed for BHA up to 1 equivalent and for TBHQ throughout the studied range, while the BHT response remained almost constant. A mass of 740 mg of K_2HPO_4, corresponding to 2 neutralizing equivalents, was selected.

Taking into account that the addition of salt reduces the water solubility of polar organic analytes and increases their partitioning coefficient between the PDMS coating and solution, different amounts of sodium chloride, providing salt concentrations between 0 and 10% (m/v), were added to the extraction medium. Since salt addition did not improve the sensitivity of the studied compounds, its use was discarded. The addition of the neutralizing salt to the extraction medium seems to provide an ionic strength enough to assure an effective salting out effect.

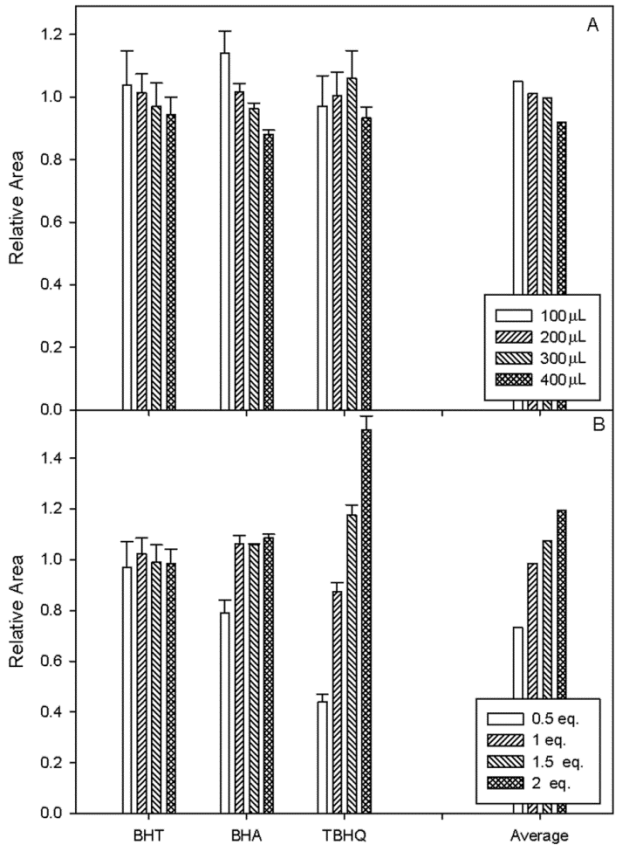


Fig. 1. Effect of acetic anhydride volume (A) and number of K_2HPO_4 neutralizing equivalents (B) on the sensitivity of BHT and the acetylated derivatives of BHA and TBHQ.

The most important parameter affecting SBSE is extraction time, which was investigated from 0.25 to 4 h. SBSE extraction time profiles (Figure 2) were obtained for acyl derivatives, and equilibrium was reached after approximately 3 h, so this extraction time was selected to ensure the highest extraction efficiencies.

Once the SBSE extraction conditions had been selected, thermal desorption parameters were studied in order to ensure complete desorption of the retained compounds and to avoid any carry over effect between injections. Different temperatures were assayed for the thermal desorption unit, ranging from 225 to the highest operating temperature recommended for the PDMS coating (275 ºC), and best results were obtained with desorption temperature of 250 ºC. When this temperature was maintained for different times, ranging from 5 to 10 min, studied compounds response decreased with longer desorption times, so 5 min was selected as desorption time.

During this desorption step a helium gas flow impels the analytes to the PTV injector. Low gas flow rates may hinder analyte thermal desorption, while high gas rates may cause losses through compound retention in the PTV. Different gas flow rates were assayed, ranging from 40 to 80 mL min^{-1}, and best results were obtained with a gas flow-rate of 60 mL min^{-1}.

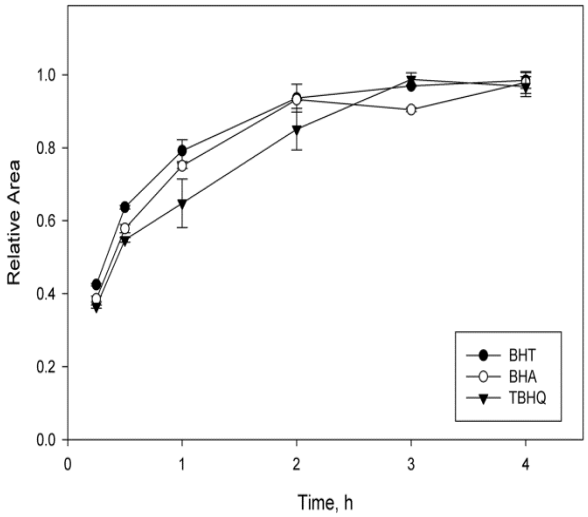


Fig. 2. Influence of the SBSE extraction time on the analytical responses of the acetylated compounds.

In order to retain the analytes during the thermal desorption step, the PTV injector temperature was fixed during thermal desorption at 20 °C by means of a Peltier unit. A fibreglass filled liner was used to facilitate the retention of analytes. Prior to injection into the GC column, PTV must be heated in order to vaporize the compounds. Different temperatures, ranging from 225 °C to the maximum value allowed for the fibreglass-filled liners (275 °C), were assayed, and a PTV temperature of 275 ºC was finally selected.

Without derivatization

In order to simplify the sample treatment, SBSE extraction of the underivatized phenolic antioxidants was considered, since their relatively high log $K_{o/w}$ (2.3 – 5.2) values and the low acid character of their phenolic groups mean that they show acceptable chromatographic behaviour.

Different amounts of sodium chloride, ranging from 0 to 20% (m/v), were added to the extraction solution. An increase in the analytical signal was observed for BHT and BHA within the studied range, while TBHQ achieved its maximum response at 5% (m/v). A value of 10% (m/v) of NaCl was selected as a compromise value.

The extraction efficiency of the analytes may also be influenced by the pH of the medium, not only as a result of variations in the behaviour of the PDMS extracting phase, but also because of the possible deprotonation of phenolic antioxidants at high pH values. The extraction medium was buffered by adding 1 mL of 0.2 M buffer solution of different pH values: citrate/citric acid (pH=3.1), acetate/acetic acid (pH=4.8), hydrogen phosphate/dihydrogen phosphate (pH=7.2) and carbonate/hydrogen carbonate (pH=9.8). The obtained responses increased with the pH throughout the studied range, and consequently, the carbonate buffer was selected. Different pH behaviours have been reported for the studied analytes when other preconcentration techniques were applied [10,35].

SBSE extraction time was investigated from 0.25 to 4 h, being 3 h selected. Different values of desorption temperature (225 – 275 ºC), desorption time (5 – 10 min), desorption flow-rate (40 – 80 mL min^{-1}) and PTV temperature (225 – 275 ºC) were tested. Best results were obtained with desorption temperature of 275 ºC, a gas flow rate of 40 mL min^{-1} and a PTV temperature of 250 ºC, while no significant differences were observed between different desorption times, so 7.5 min was chosen as a compromise value.

In-tube silylation

Silylation has been the classic derivatization method for phenols, being BSTFA one of the most used reagents. The main disadvantage of this approach is the requirement for a water-free environment. The coupling of silylation to SBSE-TD is named *in-tube* silylation [4]. Since derivatization of compounds takes place after they have been extracted onto the stir bar, the conditions which provided the highest extraction efficiencies without derivatization, such as salt addition, pH and extraction time, were adopted.

The volume of derivatizing reagent was studied between 0.5 and 2 μL. The highest signal for BHA and TBHQ was obtained when 1.5 μL were used, and, as expected, BHT was practically not affected by this parameter. Higher volumes were not assayed since they may lead to swelling of the PDMS phase or blockage of the PTV unit, resulting finally in overpressure problems.

For thermal desorption conditions, different values of desorption temperature (225 – 275 ºC), desorption time (5-10 min), desorption gas flow-rate (40 – 80 mL min^{-1}) and PTV temperature (225 – 275 ºC) were tested. Best results were obtained with desorption temperature and time of 250 ºC and 5 min, respectively, a gas flow-rate of 40 mL min^{-1} and a PTV temperature of 225 ºC. As expected, desorption temperatures for the silyl-derivatives were milder than for the original compounds, owing to their lower boiling point and more volatile nature.

Comparison of the studied derivatization procedures

Applying the previously selected experimental conditions for underivatized compounds, silyl and acyl derivatives, the sensitivity of the three procedures was compared by means of the corresponding quantification limits (LOQs), which were calculated taking into account a signal-to-noise ratio of 10 for aqueous standards (Table 3).

Table 3
LOQ (ng L^{-1}) obtained using different derivatization procedures.

Compound	Underivatized	Acetylated	Silylated
BHT	21	22	21
BHA	28	23	70
TBHQ	54	28	114

Minor differences were observed for BHT between the three proposed procedures, since in all cases BHT remains underivatized and the response was only modified by small variations in the extraction conditions. BHA showed slightly higher sensitivity when was extracted in its underivatized form, since its relative high log $K_{o/w}$ value ensured efficient extraction without the need of any further derivatization step. The *in-situ* acetylation procedure increased TBHQ obtained slopes, due to the increase in its log $K_{o/w}$ value, and in addition reduced the peak tailing of this compound, as result of the acetylation of the hydroxyl groups present in its structure.

Despite providing sharper peaks, silylation led to lower responses for BHA and TBHQ, since only a fraction of the extracted analytes is effectively silylated, and thus, lower sensitivities are attained.

Taking into account the global response for the three analytes, the improvement in the obtained chromatogram shape of the acetylated species, as well as the simplicity of the derivatization step, the *in-situ* acetylation procedure was adopted.

3.2. Method performance

The complex matrix of soft drinks, with high levels of sugars and other interfering compounds, may influence not only the partitioning process of the studied species between the solution and the extracting coating, but also their equilibration time among both phases. In order to minimize this matrix effect, a dilution step is usually applied to the sample [6,9] allowing the use of an aqueous calibration curve for quantitation purposes. The standard additions method was applied to different dilution ratios (1:1, 1:2, 1:3, 1:5 and 1:10) of a soda sample, and the slopes of the corresponding calibration graphs were compared with the slopes of an aqueous standard calibration, using a t-test. Significant differences ($p<0.05$) were found for dilutions lower that 1:10.

In order to reduce the loss of sensitivity that such large dilution implies, an internal standard (IS) was tested. Carvacrol, a natural phenolic antioxidant present in oregano, which shows similar chemical and chromatographic behaviour to the analytes, was assayed after confirming that the samples were free of this compound. When the slopes of the aqueous calibration and the standard additions to the previously reported dilutions (1:1, 1:2, 1:3, 1:5 and 1:10) in the presence of the IS were compared using a t-test, no significant differences were found for a 1:5 dilution ($0.3<p<0.86$), which was selected.

On the other hand, when the slopes of the aqueous calibration graphs were compared using a t-test with those obtained by applying the standard additions method to three different samples (isotonic drink, cold tea and soda) diluted 1:5, “p” values ranging from 0.12 to 0.97 were obtained, confirming the absence of statistically significant differences. Consequently, quantification of the samples was carried out against external aqueous standards.

Table 4
Comparison of the proposed method with others previously developed for the determination of phenolic antioxidants in aqueous samples.

Method	Sample	Technique	Sample consumption Treatment time	Linearity (ng mL^{-1})	LOD (ng mL^{-1})	Ref.
LC-UV	Juices	DLLME	40 mL / ~ 20 min	2-2500	0.9-2.5	[10]
GC-MS	Environmental water	SPE	200 mL / ~ 2 h	2-2000	0.2-0.8	[35]
GC-MS	Bottled water	SPME	15 mL / ~ 30 min	13-64	4.2[a]	[37]
GC-MS	Environmental water	SPME	15 mL / ~ 20 min	3-1018	0.7[a]	[38]
GC-MS	Soft drinks	SBSE	2 mL / ~3 h	0.5-20	0.03-0.05	This work

[a] Only for BHT.

In addition, the use of carvacrol as internal standard led to an increase in the repeatability of the studied compound responses. RSD (n=10) values were reduced from 6.4-14.2 without IS to 6.5-7.4% with IS, depending on the compound.

Calibration graphs were found to be linear in the 0.5 – 20 ng mL^{-1} range. QLs for soft drink samples (Table 2) were calculated taking into account a signal-to-noise ratio of 10, and obtained values ranged between 0.11 and 0.15 ng mL^{-1}, depending on the compound. When QLs were calculated as ten times the standard deviation of the calibration intercepts on the y axis, values in the 0.28 – 0.37 ng mL^{-1} were obtained.

A comparison of the proposed method with other methods previously published for the determination of phenolic antioxidants in aqueous samples is summarized in Table 4. The SBSE-TD-GC-MS method provided the best sensitivity achieved to date.

3.3. Analysis of samples

The optimized procedure was applied for the analysis of 10 soft drink samples, including two isotonic drinks, two cold tea, two sodas, two tonic waters, one energy drink and one lemon flavoured soda. Some of the analytes were found in five of the samples, as shown in Table 5. Phenolic antioxidant concentrations found in the samples analyzed were lower than values previously reported in the bibliography for beverage samples, which were 1 mg kg^{-1} and 5 mg kg^{-1} for BHA and BHT, respectively [11].

Even though the presence of these compounds in this kind of sample is forbidden [14], considering the very low concentrations detected, they seem to be not intentionally added to the products by the manufacturers. Their presence may be result of the employment of flavouring additives, that can contain these compounds at concentration as high as 1000 mg kg^{-1}. Other possible source of phenolic antioxidants is migration from packaging material [36].

Table 5
Analysis of the samples (ng mL^{-1}).

Sample	BHT	BHA	TBHQ
Energy drink	ND	ND	ND
Isotonic drink A	ND	ND	ND
Isotonic drink B	NQ	ND	ND
Lemon soda	ND	14.3±0.6	5.2±0.3
Soda A	ND	1.3±0.1	ND
Soda B	1.6±0.1	NQ	ND
Cold tea A	ND	2.6±0.1	1.7±0.2
Cold tea B	NQ	1.7±0.1	ND
Tonic water A	ND	ND	NQ
Tonic water B	ND	ND	NQ

Values are mean ± standard deviation (n=3), ND: not detected, NQ: not quantified.

Since no reference material was available, the method was validated by recovery assays, fortifying all the analyzed samples at two concentration levels (1 and 5 ng mL^{-1}). The results obtained are summarized in Table 6 and ranged from 81 to 117%, confirming the reliability of the method for use in the determination of synthetic antioxidants in soft drinks.

Figure 3 shows a typical chromatogram obtained by SBSE-TD-GC-MS for a soda sample spiked at 1 ng mL^{-1}, confirming the absence of interfering peaks at the retention time of the analytes, which were identified by comparing their retention time and mass spectra in samples and standard solutions.

Table 6
Recovery percentages of the antioxidants in spiked beverages using SBSE-GC-MS method

Sample	Spike level (ng mL^{-1})	BHT	BHA	TBHQ
Energy drink	1	116±5	84±8	91±7
	5	110±8	92±8	97±6
Isotonic drink A	1	111±5	89±7	94±3
	5	106±9	94±9	95±6
Isotonic drink B	1	98±4	105±3	94±3
	5	99±7	102±2	97±7
Lemon soda	1	107±3	111±8	81±14
	5	104±4	106±2	93±7
Soda A	1	105±5	87±2	92±8
	5	102±7	93±4	96±5
Soda B	1	109±2	84±6	102±2
	5	105±3	94±3	104±1
Cold tea A	1	97±3	115±1	91±1
	5	98±8	107±4	96±8
Cold tea B	1	109±3	84±1	97±11
	5	105±4	94±2	99±3
Tonic water A	1	109±1	88±2	99±1
	5	105±3	94±4	100±1
Tonic water B	1	114±2	117±8	109±2
	5	102±14	112±8	100±1

Values are mean ± standard deviation (n=3).

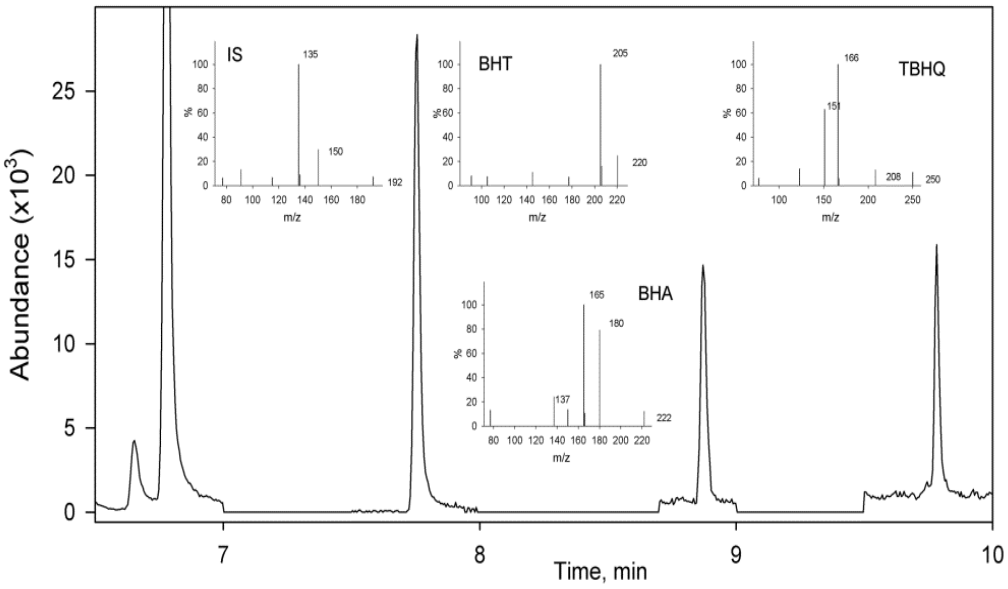


Fig. 3. Elution profile obtained for a soda sample fortified at 1 ng mL^{-1} concentration level for each compound using the SBSE-TD-GC–MS procedure under selected ion monitoring (SIM) mode, and mass spectra of each compound.

4. Conclusion

This is the first time that the SBSE miniaturized technique has been applied for the preconcentration of synthetic phenolic antioxidants, namely BHT, BHA and TBHQ.

The coupling of SBSE with GC-MS by means of a thermal desorption unit provides higher sensitivity than previously reported works, allowing the quantification of very low concentrations of the analytes, below the ng mL^{-1} level. Simple, direct and rapid derivatization procedure by *in-situ* acetylation with acetic anhydride provides best results than *in-tube* silylation with BSTFA and the direct determination of the underivatized analytes. Environmental friendly preconcentration by SBSE involves a minimum sample manipulation, while the analytical characteristics and recovery data demonstrate the reliability of the method.

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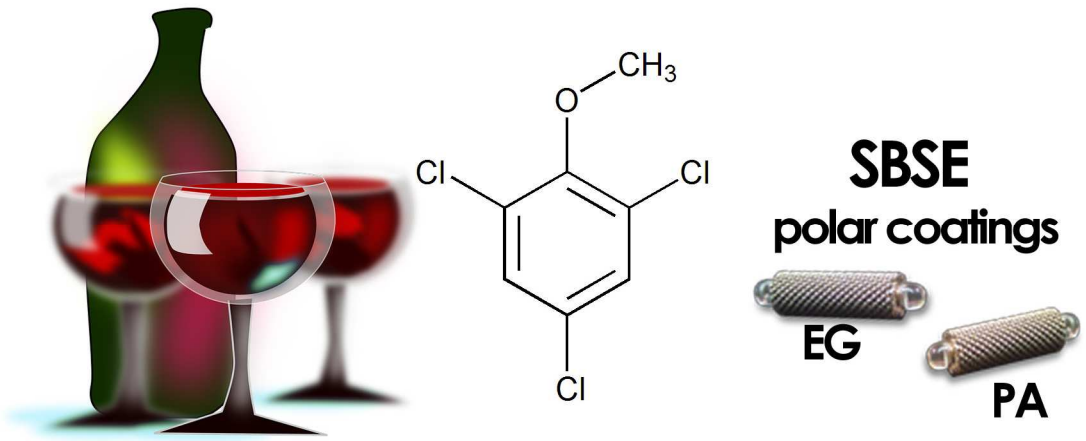
Stir bar sorptive extraction polar coatings for the determination of chlorophenols and chloroanisoles in wines using gas chromatography and mass spectrometry

Abstract

The simultaneous determination of fourteen chlorophenols (CPs) and chloroanisoles (CAs) in wine samples is carried out using stir bar sorptive extraction (SBSE) with thermal desorption and gas chromatography-mass spectrometry (TD-GC-MS), evaluating the preconcentration efficiency of two different polar extracting phases, ethylene glycol-silicone (EG-Silicone) copolymer and polyacrylate, which have recently become commercially marketed. The influence of several extraction variables on the preconcentration capacity of these two novel coatings was tested, as well as the variables affecting the thermal desorption step. The EG-Silicone extraction phase provided the best results, since it allowed the simultaneous preconcentration of both species the non-polar CAs, due to the silicone base, and the polar CPs, because of the ethylene glycol polymer. Consequently, under the finally selected conditions, CPs were determined without any derivatization step, reaching detection limits in the 0.3 – 1.4 ng L^{-1} range, depending on the compound. For CAs the detection limits ranged from 0.2 to 0.5 ng L^{-1}, with good precision and recovery. Five CAs and three CPs were found in several analyzed wines, some of which can be regarded as defective considering their contents in 2,4,6-TCA and 2,6-DCA.



1. Introduction

Stir bar sorptive extraction (SBSE) is a solvent-free sample preparation technique based on the extraction of target compounds from aqueous matrices onto a stationary phase-coated stir bar. For many years, polydimethylsiloxane (PDMS) was the only commercially available coating for stir bars, but its non-polar nature limited the applicability of SBSE to hydrophobic compounds. Since PDMS was unable to extract polar species, they usually showed poor recovery with SBSE, and transformation into less polar species by derivatization reactions, such as *in-situ* acetylation or *in-tube* silylation [1] was the only alternative.

The development of *in-house* coatings for SBSE using more polar extracting phases has extended the applicability of this technique to polar compounds. Several approaches have been successfully applied to species showing low affinity for PDMS coatings [2], such as sol-gel technology [3], monolithic materials [4], molecularly imprinted polymers [5] and polyurethane foams [6]. However, the lack of robustness of *in-house* coatings, which may lead to mechanical or thermal degradation, reducing their useful life and producing high bleeding rates, as well as the difficulties associated with the preparation of such coatings [7], involve significant limitations to their analytical application.

Recently, stir bars coated with polar friendly coatings, like ethylene glycol-polydimethylsiloxane copolymer (EG-Silicone) and polyacrylate (PA) [8] have reached the market, improving SBSE flexibility while maintaining robustness and ease of handling. These new commercial SBSE coatings were assayed to assess their suitability for the determination of the polar compounds, chlorophenols (CPs) and the related chloroanisoles (CAs), which are the main compounds responsible of the mouldy aroma in wines.

Aroma is one of the most important characteristics of wine, since it is related with product quality and consumer acceptance. Thus, the appearance of corky, musty or earthy taints in wines, frequently related to the presence of some CPs and CAs [9], is a concern for the wine industry. The main compound responsible for this defect is 2,4,6-trichloroanisole (2,4,6-TCA), although other CAs, may also contribute to the off-flavours. These compounds are usually synthetized by fungal methylation of the corresponding CPs [10], which usually reaches wine samples by means of the natural cork used as bottle stoppers, or from contact with barrels. These species are generated during the treatment of the cork or wooden barrels with hypochlorite, although other sources, such as wood biocides, may also be responsible for spoilage [11]. Moreover, the control of the CP content in wines is of great importance because of their carcinogenic character and persistence.

Even though immunoassay tests have been used for the determination of CPs and CAs in wines [12,13], a more extensive use of gas chromatography (GC) is reported in the bibliography [11,14-29], coupled to a large variety of microextraction techniques, such as different liquid-liquid

microextraction (LLME) modalities [26-28] and solid-phase microextraction (SPME) [16,18-24] with the aim of reaching the human olfactory and taste threshold ranges for haloanisoles. Although these ranges vary with the age of the wine and grape variety used in production, as well as with the sensitivity and training of judges, an interval of 0.03 – 50 ng L^{-1} has been proposed for 2,4,6-TCA (the TCA concentration considered to produce a defect in wine usually ranges from 10 to 40 ng L^{-1}) [14] and values of around 400 ng L^{-1} for 2,4-DCA, 40 ng L^{-1} for 2,6-DCA and 4 µg L^{-1} for PCA [10,17,26]. SBSE has previously been used for the determination of CP and CA-related taints in wine [11,15,23,25], as well as in cork [30-33] and other sample matrices, such as water [34] and soil [35]

Even though the volatility and thermostability of CAs mean that they are suitable analytes for GC, a previous derivatization step is recommended in the case of CPs in order to improve sensitivity and to reduce peak tailing. These species, have also been determined by GC, without a derivatization step, which represents a saving of time and reagents, using SPME as preconcentration technique and the polar coating PA [19,36-39] and polyethylene glycol (PEG) fibers [40,41]. Similar extraction phases are available in SBSE but they have never been used for the determination of the compounds deemed responsible for cork taint. In this paper, fourteen CPs and CAs were determined in wine samples using SBSE with thermal desorption and gas chromatography-mass spectrometry (TD-GC-MS), comparing the effectiveness of the two novel polar coatings, EG-Silicone and PA.

2. Materials and methods

2.1. Reagents

4-Chloroanisole (4-CA, 99%), 2,6-dichloroanisole (2,6-DCA, 97%), 2,4-dichloroanisole (2,4-DCA, 97%), 2,4,6-trichlorophenol (2,4,6-TCP, 98%), 2,4,6-trichloroanisole (2,4,6-TCA, 99%) and pentachlorophenol (PCP, 98%) were purchased from Aldrich (Steinheim, Germany). 4-Chlorophenol (4-CP, 99.5%), 2,4-dichlorophenol (2,4-DCP, 99.5%), 2,6-dichlorophenol (2,6-DCP, 99.5%), 2,4,5-trichloroanisole (2,4,5-TCA, 99.5%), 2,4,5-trichlorophenol (2,4,5-TCP), 2,3,4,6-tetrachlorophenol (2,3,4,6-TeCP, 98%) and 2,3,4,5-tetrachloroanisole (2,3,4,5-TeCA, 99%) were obtained from Dr. Ehrenstorfer (Ausburg, Germany) and pentachloroanisole (PCA, 99.3%) from Supelco (Bellefonte, PA, USA).

Individual stock solutions of the compounds (1000 µg mL^{-1}) were prepared using HPLC grade methanol and stored in darkness at −20 °C. Working standard solutions were freshly prepared in pure water and stored at 4 °C. Sodium hydroxide (99%) and sodium chloride (99.5%) were purchased from Sigma (St. Louis, MO, USA). L-(+)-Tartaric acid (99.5%) was provided by Merck (NJ, USA). Chromatographic quality methanol and ethanol were obtained from Sigma. Water was

previously purified in a Milli-Q system (Millipore, Bedford, MA, USA) and the carrier gas used for GC was helium (Air Liquide, Madrid, Spain).

A synthetic wine containing 3.2 g L^{-1} of L-(+)-tartaric acid and 12% (v/v) of ethanol, with pH adjusted to 3.6 using a diluted NaOH solution, was used for the development and optimization of the method [15]. All the glass material was soaked with a detergent solution with added ethanol and dried in an oven.

2.2. Instrumentation

Commercial stir bars coated with polyacrilate (PA) and ethylene glycol-polydimethylsiloxane copolymer (EG-Silicone) layers (32 μL) were obtained from Gerstel (Mullheim an der Ruhr, Germany). Prior to use, the stir bars were conditioned in an empty thermal desorption tube at 200 ºC for 0.5 h with helium at a flow desorption rate of 50 mL min^{-1}. The sample introduction system was composed of a Thermal Desorption Unit (TDU-2) equipped with an autosampler (MPS-2) and a Programmed Temperature Vaporization (PTV) Cooled Injector System (CIS-4) provided by Gerstel. The main experimental conditions used in the sample introduction system are summarized in Table 1. GC analyses were performed on an Agilent 6890N (Agilent, Waldbronn, Germany) gas chromatograph coupled to an Agilent 5973 quadrupole mass selective spectrometer equipped with an inert ion source. The total analysis time for one GC run was 27 min, the analytes being eluted with retention times of between 10.1 and 25.4 min, corresponding to 4-CA and PCP, respectively, as shown in Table 2.

Table 1
Experimental conditions of the TD-GC-MS procedure.

Thermal Desorption Unit	
Mode	Splitless
Temperature program	50 (0.5 min) – 220 ºC (12.7 min) at 300 ºC min^{-1}
Gas flow and pressure	95 mL min^{-1}, 7.5 psi
Cooled Injector System	
Mode	Solvent venting
Liner	Poly(2,6-diphenyl-p-phenylene oxide), 2 mm i.d.
Temperature program	15 – 150 ºC at 840 ºC min^{-1} 150 – 330 ºC (5 min) at 630 ºC min^{-1}
GC-MS	
Capillary column	HP-5MS, 5% diphenyl-95% dimethylpolysiloxane 30 m x 0.25 mm, 0.25 μm film thickness
Carrier gas	Helium (1 mL min^{-1})
Oven program	50 (2.5 min) – 120 ºC (6 min) at 10 ºC min^{-1} 120 – 170 ºC (10 min) at 33 ºC min^{-1}
Transfer line, temperature	280 ºC
Quadrupole temperature	150ºC
Ion source temperature	230ºC
Ionisation	Electron-impact mode (70 eV)

The ionization was carried out in the electron-impact (EI) mode (70 eV). The electron multiplier voltage was set automatically. The identification of the compounds was confirmed by injection of pure standards and comparison of the retention time and full MS-spectra. Analytes were quantified using selected ion monitoring (SIM) mode with the most abundant ions (Table 2).

2.3. Samples, analytical procedure and recovery studies

A total of 30 wines (samples 1-8 were white, 9-26 red and 27-30 rosé wines) were obtained from local wine merchants. Taking into account that cork taint is very unusual in large-scale industrial produced wines [26], craft wines, from small local productions and aged in barrels, were chosen for sample selection. Samples were kept at 4 °C until analysis, in order to prevent losses of the most volatile analytes.

An SBSE stir bar was placed in a 15 mL glass vial containing an aliquot of 10 mL of the sample and 1.0 g of NaCl, and stirred at 600 rpm for 2 h until equilibrium was reached. Next, the stir bar was removed from the vial, rinsed with Milli-Q water in order to eliminate salt residues and dried with a lint-free tissue before being introduced into a glass desorption tube. The analytes were thermally desorbed from the stir bar by placing the desorption tube in the TDU.

3. Results and discussion

In order to evaluate the performance of the PA and EG-Silicone SBSE coatings for the preconcentration of CAs and CPs, and the influence of some experimental variables, both during the extraction step and during the desorption process, a synthetic wine spiked at 1 µg L^{-1} was used.

Table 2
Method characteristics.

Compound	RT (min)	Monitorized ions (*m/z*)	RSD[a] (%)	LOD[b] (ng L^{-1})
4-CA	10.1	142, 127	3.4	0.2
2,6-DCP	11.5	162, 126	5.7	0.5
2,6-DCA	11.8	176, 161	4.8	0.4
2,4-DCP	12.1	162, 126	6.0	0.5
4-CP	12.4	128, 100	6.4	1.4
2,4-DCA	14.7	176, 161	3.5	0.4
2,4,6-TCA	16.0	195, 210	4.3	0.4
2,4,5-TCA	16.2	195, 210	5.1	0.3
2,4,6-TCP	16.7	196, 132	5.2	0.4
2,4,5-TCP	16.9	196, 132	6.4	0.3
2,3,4,6-TeCP	20.1	232, 133	5.3	0.3
PCA	24.1	280, 267	3.9	0.5
PCP	25.4	266, 167	5.5	0.5

[a] n=10. [b] Corresponding to S/N=3.

3.1. SBSE extraction parameters

The following variables were studied individually: ionic strength, pH of the extraction medium and extraction time. The addition of chemical modifiers such as methanol, a commonly procedure in SBSE, was discarded since the ethanol content of the samples was high enough to avoid any adsorption of non-polar compounds on the inner walls of the sample vials.

The influence of the ionic strength of the extraction medium was evaluated at different sodium chloride concentrations (0, 2, 5, 10 and 15% m/v). The addition of NaCl usually increases the extraction efficiency of PDMS coatings, since a decrease in the water solubility of polar organic compounds increases their partitioning coefficients between the coating and aqueous extraction medium. In the case of the polar coatings evaluated, the relationship between salt addition and extraction efficiency may not be so clear. However, regardless of the extracting phase, high salt concentrations may decrease the extraction efficiency because the increased viscosity of the solution hinders diffusion. Maximum extraction efficiency for the EG-Silicone coating was attained with 10% (m/v) NaCl, except in the case of PCP, for which the highest sensitivity was attained with 15% (m/v). Because the enhancement of sensitivity (from 10 to 15% m/v) for PCP was not important, a 10% (m/v) NaCl concentration was chosen. For the PA coating, most of the analytes reached their maximum extraction efficiency with a 10% (m/v) NaCl concentration, the salt having little effect in the case of the less polar analytes TeCA, TeCP, PCA and PCP.

Since CPs are weakly acidic, the influence of pH on the extraction efficiency was considered. Wine samples generally show pH values close to 3, and so CPs will probably remain in their neutral form and so be effectively extracted. However, because such a low pH may reduce the extraction efficiency of the tested coatings, the influence of the extraction medium pH was evaluated at two levels by incorporating 1 mL of two different buffer solutions: citric acid/sodium citrate (0.2 M, pH 3.5) or acetic acid/sodium acetate (0.2 M, pH 4.8). Since no significant differences were observed for either coating in the pH range studied, the use of a buffer solution was discarded.

The most important parameter affecting SBSE is extraction time. Therefore, the optimum extraction time was investigated from 0.5 to 6 h. The extraction time profiles (equilibration curves) are shown in Fig. 1. Note that in order to assess the relevance of this variable for the different analytes in the optimization process, regardless of their different sensitivities; the analytical signals were normalized with regard to average areas for each compound. Equilibrium was reached for all the compounds at about 2 h using the two extraction phases tested, so this time was chosen to ensure high extraction efficiencies.

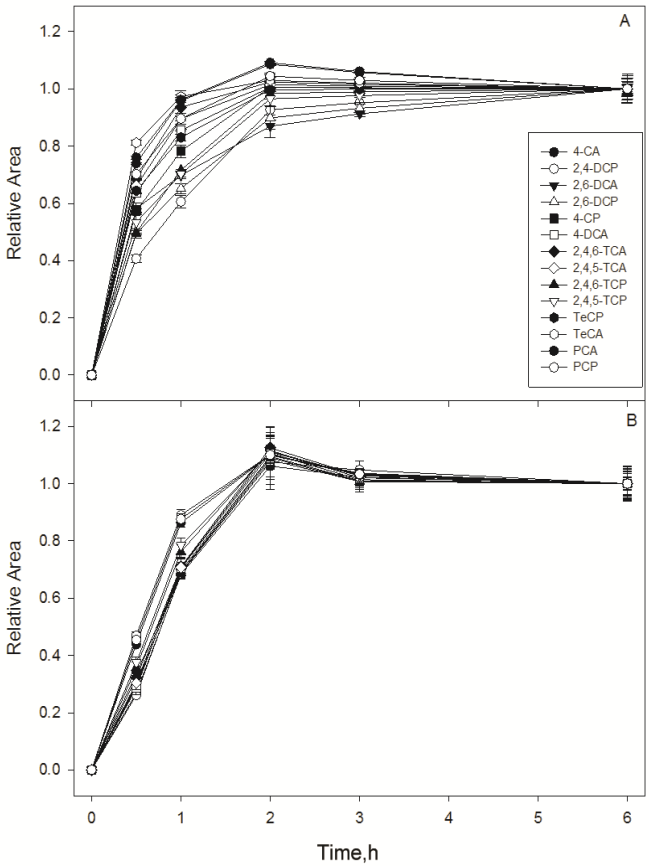


Fig. 1. Influence of the SBSE extraction time on the analytical responses for (A) EG-Silicone and (B) PA coatings.

3.2. Thermal desorption conditions

Since the number of variables involved in the thermal desorption step is large, their effect and significance were tested using a Plackett-Burman multivariate design (PBD). The use of this screening test allows the most important variables to be identified, and the most suitable values to be selected for the rest of the variables assayed. Once the most important parameters had been identified, they were submitted to a Central Composite Design (CCD), which provided the optimum values.

The PBD (12 experiments, in duplicate) was similar for both coatings, and included the following variables: TDU desorption time (5 and 10 min), TDU desorption temperature (200 and 220 ºC), inert gas flow rate (50 and 100 mL min^{-1}), CIS heating temperature (280 and 330 ºC) and inert gas vent pressure (6 and 8 psi). The results of using EG-Silicone or PA are summarized by means of Pareto charts in Fig. 2A and 2B, respectively.

Even though the desorption temperature was identified by the PBD as being the most relevant desorption parameter for EG-Silicone (the higher the temperature, the higher the response as shown in Fig. 2A), the thermolability of the EG-Silicone coating prevented temperatures higher than 220 ºC from being applied, so this variable was not subjected to further study and the maximum assayed value was selected.

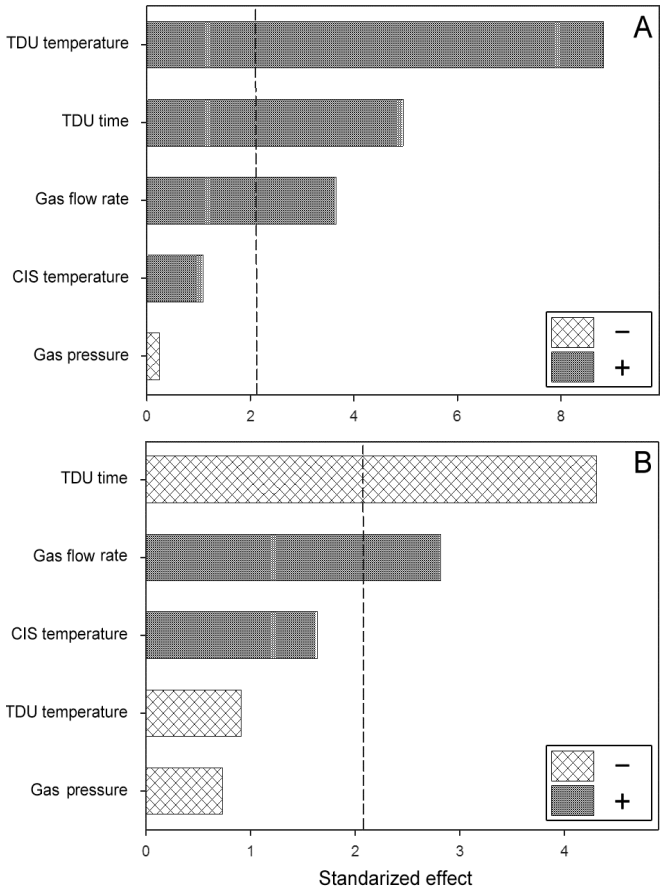


Fig. 2. Pareto Charts obtained for the analysis of effects through the Plackett-Burman designs for thermal desorption step using (A) EG-Silicone and (B) PA extraction phases.

The other relevant parameters for the thermal desorption process when using EG-Silicone coating, according to Fig. 2A, were TDU desorption time and gas flow rate, which were carefully studied in the 6.6 – 13.5 min and 55 – 110 mL min^{-1} ranges, respectively, using a CCD (α=1.5, n=10).

The obtained response surface (Fig. 3A) showed its adequacy to experimental results (r^2>0.95) and the relevance of the assayed variables ($p<0.01$). Therefore, a desorption time of 12.7 min and an inert gas flow rate of 95 mL min^{-1} were adopted. Other less relevant parameter values were set. A pressure of 7.5 psi, corresponding to column pressure, was chosen as gas vent pressure to avoid longer pressure equilibration times, while a temperature of 330 ºC was selected for CIS heating.

The application of a fast heating program to achieve this temperature in the PTV injector provided sharper chromatographic peaks, and thus better peak resolution. Taking account that a high ramp temperature as 840 ºC min^{-1} can be only applied until 150 ºC is attained, a heating program with two ramps at almost the highest heating temperature rates (Table 1) was selected for further experiences.

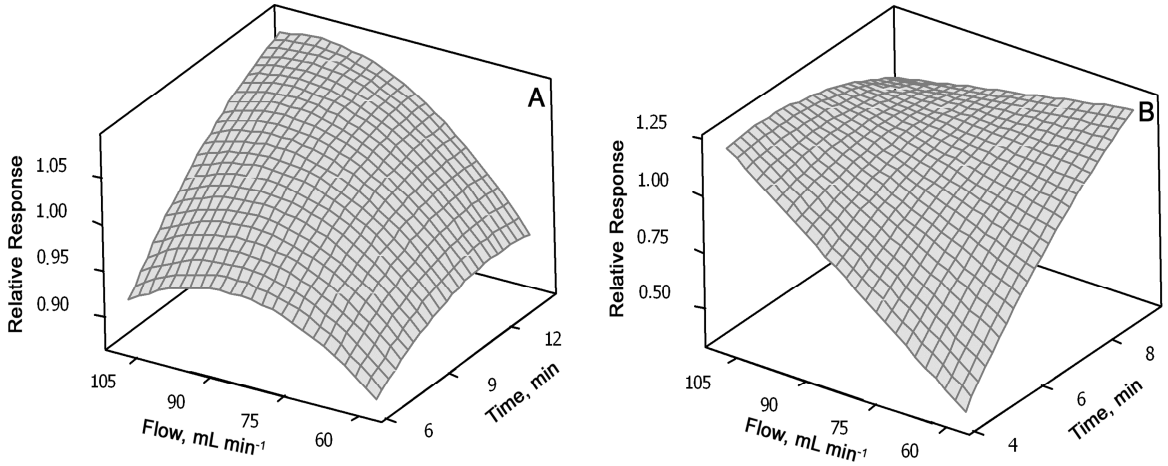


Fig. 3. Response surfaces showing the effects on relative responses obtained with (A) EG-Silicone and (B) PA coatings.

When a PA coating was evaluated, the most relevant thermal desorption parameters were desorption time and inert gas flow rate, as shown by the Pareto charts (Fig. 2B). Consequently, these parameters were optimized by means of a CCD (α=1.5, n=10), between 3.75 and 8 min for desorption time and between 55 and 110 mL min^{-1} for the gas flow. The response surface obtained (Fig. 3B) fitted the experimental results (r^2>0.95) and pointed the relevance of the assayed variables ($p<0.01$). Consequently, the desorption of PA coated stir bars was carried out by heating the TDU at 200 ºC for 5 min, while an inert gas flow of 105 mL min^{-1} impelled the analytes to the PTV. The CIS heating temperature was set to 330 ºC and a 7.5 psi gas pressure was used for the vent gas.

The PTV focusing temperature was set to 15 ºC by means of a Peltier Unit. Different filling materials for the PTV liner were checked in order to facilitate the retention of the analytes: fiberglass, poly(2,6-diphenylphenylene oxide) and polyethylene glycol. As can be observed in Fig. 4, fiberglass retained most of the heavier compounds and so provided low recoveries for the most volatile compounds, whereas polyethylene glycol showed the opposite behaviour. The liner filled with poly(2,6-diphenylphenylene oxide) showed a balanced retention power for the studied compounds, providing good recoveries both for the more volatiles and the heavier analytes, therefore it was selected.

3.3. Coating evaluation

After optimizing the SBSE extraction and desorption conditions, the extraction capabilities of EG-Silicone and PA coatings were evaluated by comparing the slopes of calibration graphs using a synthetic wine.

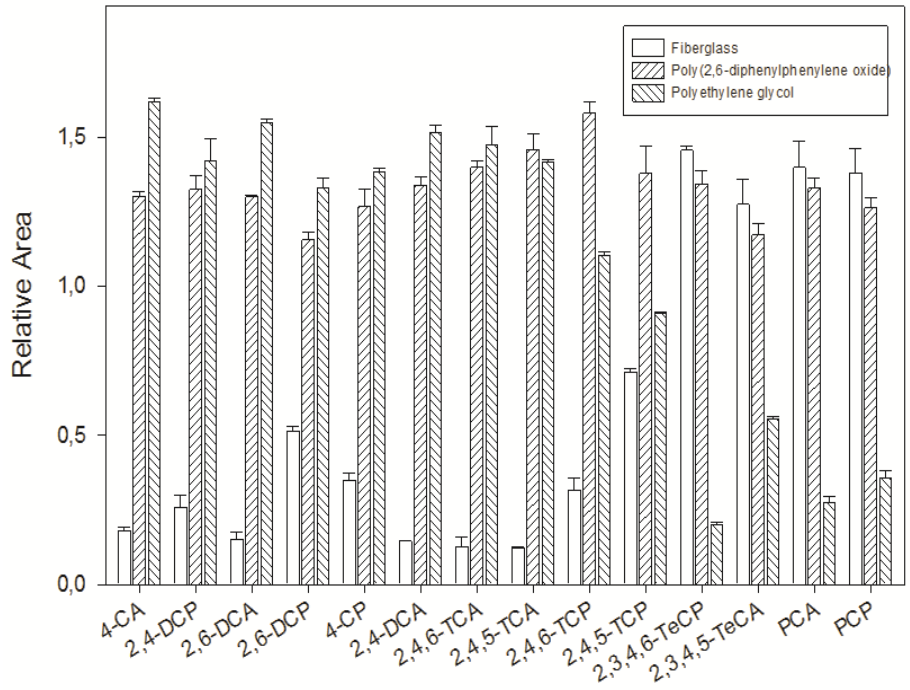


Fig. 4. Influence of CIS liner filling material on the analyte sensitivity.

As shown in Table 3, the use of EG-Silicone was nearly 5 times more sensitive than the PA coating (5.3 times for CAs and 4.5 times for CPs). The higher preconcentration power of the EG-Silicone phase may result from its copolymeric composition, the high extraction efficiency for CPs being due to the hydrogen bond interactions with EG. Moreover, this coating phase also ensures the efficient extraction of CAs due to its silicone base. Considering the results obtained, EG-Silicone coated stir bars were selected for the determination of CPs and CAs in wine samples.

Table 3
Analytical characteristics obtained using EG-Silicone and PA as SBSE extracting phase.

Compound	EG-Silicone		PA	
	Slope[a] (L mg^{-1})	Regression coefficient	Slope[a] (L mg^{-1})	Regression coefficient
4-CA	901±35	0.991	153±6	0.991
2,4-DCP	639±27	0.993	141±7	0.986
2,6-DCA	982±43	0.988	152±6	0.991
2,6-DCP	319±13	0.993	75±3	0.994
4-CP	353±13	0.992	64±2	0.994
2,4-DCA	827±27	0.993	146±4	0.995
2,4,6-TCA	1159±36	0.994	193±5	0.995
2,4,5-TCA	1465±39	0.996	286±7	0.996
2,4,6-TCP	913±22	0.993	175±4	0.996
2,4,5-TCP	1153±40	0.993	258±10	0.990
2,3,4,6-TeCP	1398±38	0.993	357±10	0.995
2,3,4,5-TeCA	1331±28	0.997	385±1	0.996
PCA	1141±40	0.993	261±9	0.992
PCP	1510±48	0.994	315±10	0.995

[a] Mean value ± standard deviation (n=6).

3.4. Analytical characteristics of the method

The standard additions method was applied to three different wine samples (white, rosé and red wine) and also to a synthetic wine, by spiking these samples at six concentration levels, which were submitted in duplicate to the optimized procedure. The representation of peak area *versus* the analyte concentration was linear in the range 25 – 1750 ng L^{-1}, with correlation coefficients higher than 0.99 in all cases.

When the slopes obtained were compared using an analysis of variance (one-way ANOVA), no statistically significant differences were observed ($p>0.05$), calibration by using the synthetic wine solution was used for quantification purposes.

Repeatability tests were performed by submitting to the proposed procedure ten aliquots of a spiked red wine sample at a 500 ng L^{-1} concentration level, providing RSD values of 4.7±0.8%.

The detection (LODs) and quantification (LOQs) limits of the method were calculated as three and ten times the signal-to-noise ratio, respectively (Table 2). QLs in the range 0.7 – 4.7 ng L^{-1}, depending on the compound, were obtained. The LOD values obtained are lower than the sensory thresholds reported in the literature for the analytes studied. A slight increase of sensitivity was obtained for TeCA with EG-Silicone stir bars related to the use of non-polar PDMS phase, whereas an important increase of sensitivity (about 6500 times) was attained for PCP [28].

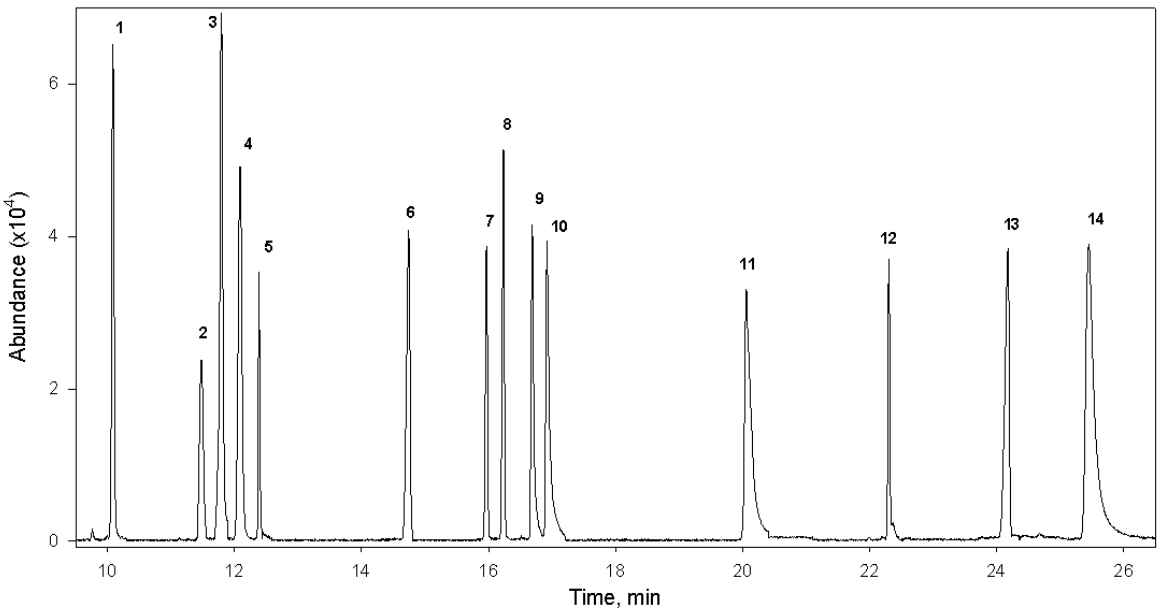


Fig. 5. SBSE-TD-GC-MS chromatogram obtained for a spiked synthetic wine fortified at 0.5 µg L^{-1} under SIM mode. Peaks correspond to: (1) 4-CA, (2) 2,6-DCP, (3) 2,6-DCA, (4) 2,4-DCP, (5) 4-CP, (6) 2,4-DCA, (7) 2,4,6-TCA, (8) 2,4,5-TCA, (9) 2,4,6-TCP, (10) 2,4,5-TCP, (11) 2,3,4,6-TeCP, (12) 2,3,4,5-TeCA, (13) PCA, (14) PCP.

3.5. Analysis of wines and validation of the method

Thirty samples, including red, rosé and white wines, were analyzed using the optimized procedure and, some of the studied compounds were found in seven samples (Table 4). 4-CA and 2,4,6-TCA were the most abundant analytes, being present in six of the samples at concentrations in the range 30 – 106 and 32 – 82 ng L^{-1}, respectively. The content of 2,4,6-TCA in two of these samples was higher than human threshold reported, 50 ng L^{-1}. 2,4-DCA was also found in four samples, at concentrations between 35 and 95 ng L^{-1}, which are lower than its sensory threshold (400 ng L^{-1}), whereas 2,6-DCA was found in three samples at concentrations higher than the corresponding threshold level (40 ng L^{-1}). Other species such as 2,4-DCP, 2,4,6-TCP, 2,3,4,6-TeCP and 2,3,4,5-TeCA were detected in some samples. The presence of these compounds in wine samples at similar concentrations has been reported previously [19,22].

A typical chromatogram obtained by SBSE-TD-GC-MS under SIM mode for a red wine fortified at 500 ng L^{-1} in the selected conditions is shown in Fig. 5. The chromatogram showed the absence of interfering peaks at the analyte retention times. The analytes were identified by comparing their retention times, and by identifying mass spectra of the peaks in samples and standard solutions.

To check the accuracy of the proposed method, and since no reference materials were commercially available for the validation of the method, recovery assays were performed using three different wine samples (red, rosé and white) by fortifying at two concentration levels (100 and 200 ng L^{-1}). The recoveries obtained ranged from 84 to116% (n=126) at the lower level and from 89 to 113% (n=126) for the higher level (Table 5).

Table 4
Results obtained in the analysis of the samples (ng L^{-1}).

Compound	Wine 2	Wine 3	Wine 9	Wine 12	Wine 14	Wine 21	Wine 24
4-CA	97±7	38±2	106±3	30±2	53±3	ND	50±5
2,6-DCP	146±8	ND	ND	52±4	ND	ND	ND
2,6-DCA	ND	162±9	ND	ND	ND	64±7	116±8
2,4-DCA	75±6	35±2	ND	95±13	48±2	ND	ND
2,4,6-TCA	44±5	40±4	57±5	82±9	32±2	ND	38±3
2,4,6-TCP	ND	ND	ND	47±2	ND	ND	ND
2,3,4,6-TeCP	26±2	ND	ND	20±1	ND	ND	ND
2,3,4,5-TeCA	ND	ND	ND	51±5	ND	ND	ND

Values are mean ± standard deviation (n=3). ND means not detected.

Table 5
Results obtained from the recovery assays using the proposed method.

Compound	Spiked level[a] (μg L^{-1})	Concentration found[b] (μg L^{-1})		
		White wine	Rosé wine	Red wine
4-CA	0.1	0.095±0.003	0.103±0.004	0.092±0.006
	0.2	0.194±0.007	0.202±0.007	0.190±0.014
2,6-DCP	0.1	0.097±0.005	0.095±0.005	0.103±0.010
	0.2	0.197±0.011	0.198±0.010	0.203±0.021
2,6-DCA	0.1	0.096±0.004	0.097±0.004	0.094±0.008
	0.2	0.193±0.009	0.196±0.009	0.190±0.015
2,4-DCP	0.1	0.102±0.006	0.091±0.005	0.105±0.013
	0.2	0.202±0.011	0.196±0.011	0.203±0.021
4-CP	0.1	0.104±0.005	0.094±0.005	0.106±0.012
	0.2	0.205±0.011	0.199±0.011	0.206±0.023
2,4-DCA	0.1	0.091±0.004	0.092±0.004	0.088±0.009
	0.2	0.188±0.009	0.190±0.008	0.180±0.016
2,4,6-TCA	0.1	0.089±0.004	0.102±0.004	0.090±0.008
	0.2	0.184±0.009	0.203±0.009	0.185±0.015
2,4,5-TCA	0.1	0.088±0.004	0.095±0.004	0.092±0.008
	0.2	0.183±0.008	0.195±0.008	0.186±0.017
2,4,6-TCP	0.1	0.105±0.005	0.087±0.004	0.107±0.011
	0.2	0.206±0.009	0.195±0.009	0.200±0.018
2,4,5-TCP	0.1	0.108±0.004	0.105±0.005	0.111±0.010
	0.2	0.211±0.009	0.206±0.009	0.218±0.020
2,3,4,6-TeCP	0.1	0.099±0.005	0.095±0.004	0.102±0.010
	0.2	0.201±0.009	0.192±0.009	0.196±0.018
2,3,4,5-TeCA	0.1	0.084±0.003	0.091±0.003	0.093±0.006
	0.2	0.178±0.006	0.186±0.006	0.188±0.012
PCA	0.1	0.086±0.002	0.094±0.002	0.096±0.004
	0.2	0.181±0.004	0.193±0.004	0.200±0.008
PCP	0.1	0.092±0.004	0.113±0.005	0.110±0.009
	0.2	0.209±0.009	0.220±0.010	0.210±0.020

[a] Values in brackets correspond to spiking levels for red wine samples.
[b] Mean value ± standard deviation (n=3).

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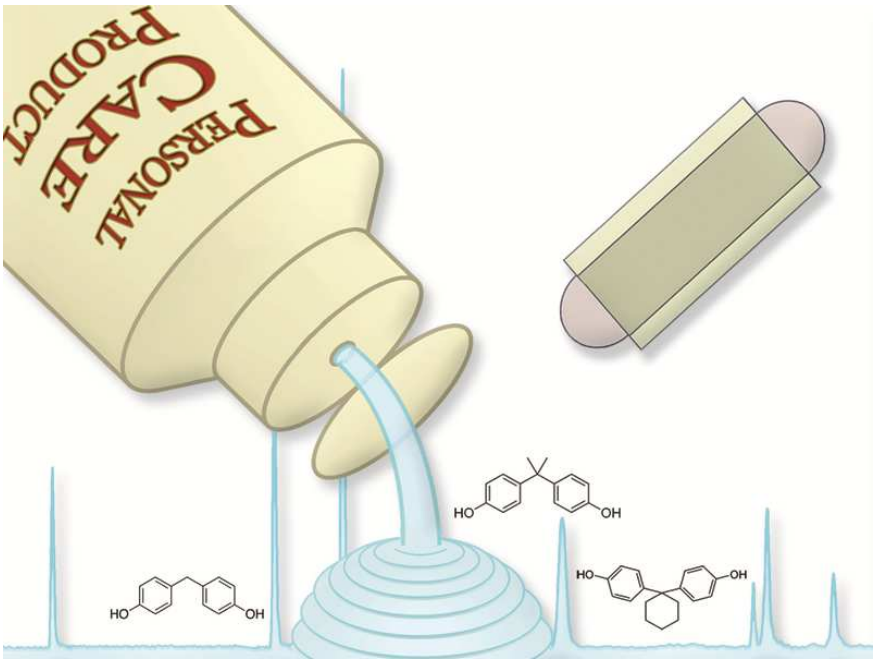
Stir bar sorptive extraction with EG-Silicone coating for bisphenols determination in personal care products by GC-MS

Abstract

An easy to perform analytical method for the determination of three bisphenol compounds (BPs) in commonly used personal care products is presented. Ethylene glycol-silicone (EG-Silicone) coated stir bars, which have recently become commercially available, are evaluated in this study for the simultaneous determination of bisphenol A (BPA), bisphenol F (BPF) and bisphenol Z (BPZ) by stir bar sorptive extraction (SBSE) in combination with thermal desorption-gas chromatography-mass spectrometry (TD-GC-MS). This new sorptive extraction phase allows the analysis of these compounds without any previous derivatization procedure. Different parameters affecting both SBSE extraction and thermal desorption were carefully optimized, using experimental designs based on the Taguchi orthogonal arrays. The procedure was applied to analyzing easily bought personal care products, providing detection limits of about 8 ng g^{-1}, with precisions lower than 11% in terms of relative standard deviation. Recovery studies performed at two different concentration levels provided satisfactory values for all the compounds. The analyzed personal care samples contained BPA at concentration levels ranging from 30.9 to 88.3 ng g^{-1}.



1. Introduction

Bisphenols (BPs) are compounds commonly used in the production of plastics, such as epoxy resins or polycarbonate. Bisphenol A (BPA) has been extensively studied due to its endocrine disrupting properties, acting as xenobiotic with an estrogenic action, and has been related with reproductive alterations, decreased fertility and cancer [1]. Since BPA is used in the manufacture of different products of common use, such as adhesives, protective coatings or paints, the amounts contained in plastics that will come into contact with foods has been subject to legal regulations [2,3]. The presence of BPA in personal care products (such as shampoo, bath lotions or cosmetic creams) has been previously reported [4-6]. Taking into account that this specie can be assimilated through the skin [7], its presence in personal care products has been prohibited appealing the general principle of the responsibility of the manufacturer or the importer [8]. Nevertheless, even though BPA was not added as ingredients, their presence in these samples may be due to their migration from polycarbonate plastic containers [9] or to the degradation of some BPs containing components, as silicones in hair conditioner [10], urethane thickening agents [11] or water-insoluble fine particulate polymer in hair cosmetic [12]. Considering the wide use of personal care products in the actual society, the disposable of rapid and sensitive analytical methods for guarantying their safety to human health is of great interest. Even though only the content of BPA has been restricted in personal care products [8], the present study has been extended to other BPs, bisphenol Z (BPZ) and bisphenol F (BPF), considering their chemical relation with BPA and the possibility of appearance in the studied samples.

Since BPs concentration in personal care products is suspected to be very low, a preconcentration technique should be required prior to their determination. Stir bar sorptive extraction (SBSE) has become since its development in 1999 [13], one of the most popular preconcentration techniques due to its simplicity and robustness. In SBSE, the analytes are extracted into a sorbent coating placed on a magnetic stir bar. Extraction can be carried out by direct immersion of the stir bar into the aqueous sample solution or in the headspace. The trapped compounds can be thermally desorbed prior to gas chromatography (GC), or by liquid desorption when liquid chromatography (LC) is used. SBSE has been successfully applied to environmental, food and biological samples [14-17].

For years, the only commercially available coating for SBSE was the non-polar polymer, polydimethylsiloxane (PDMS), meaning that SBSE was largely unsuitable for the direct extraction and analysis of compounds with polar character, like BPs. Usually, polar compounds with octanol-water partition coefficients ($K_{o/w}$) lower than 1,000 showed poor recovery when using this preconcentration technique [18] and, a derivatization step was necessary in order to improve the $K_{o/w}$ values ensuring a high extraction or preconcentration on the SBSE coating as well as suitable

gas chromatographic behavior. In this sense, *in-situ* acetylation [19-28] and *in-tube* silylation [23,29] reactions are described in the literature to derivatize BPs previously to their determination by SBSE-GC-MS using PDMS stir bar coatings.

In addition, BPA has also been preconcentrated using in-house stir bar coatings. The employment of polar friendly coating materials, like molecularly imprinted polymers [30,31], monolithic materials [32] and sol/gel composites [33] has improved SBSE performance for BPA. Nevertheless, the difficulties involved in the preparation, the lack of stability or the high bleeding rates were some of the disadvantages which limit the extent of their use.

Recently, SBSE stir bars with the polar coating materials polyacrylate (PA) and ethylene glycol-PDMS copolymer (EG-Silicone) have been marketed [34]. This latter coating allows high extraction efficiency for both polar compounds, due to the polar nature of EG, and non-polar compounds, due to its silicone base. The use of this new EG-Silicone stir bars coating may allow the extraction and preconcentration of polar compounds avoiding the derivatization step, so they can be expected to be useful for BPs, an application that is evaluated in this study.

The goal of this work was the development of an analytical method to determine trace levels of different BPs in everyday products in the actual society, by means of SBSE-TD-GC-MS. In this sense, the use of the new available EG-Silicone coated stir bars is tested avoiding the inclusion of a derivatization step in the procedure. As far as we know, despite the inherent advantages of SBSE preconcentration, the literature only mentions two applications of this technique to the analysis of personal care products [35,36] using liquid desorption previously to LC for the determination of triclosan and parabens, therefore its use for determination of BPs in this type of samples was addressed.

2. Materials and methods

2.1. Reagents

Analytical-reagent grade methanol was purchased from Lab-Scan (Dublin, Ireland). Deionized water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Sodium chloride was purchased from Panreac (Barcelona, Spain). Commercially available bisphenol A (BPA, 4,4'-dihidroxy-2,2-diphenylpropane, 99% purity), bisphenol F (BPF, 4,4'-dihidroxy-2,2-diphenylmethane, 98% purity), bisphenol Z (BPZ, 1,1-bis(4-hydroxyphenyl)cyclohexane, 99% purity) and biphenol (BP, 2,2'-biphenol, 98% purity, internal standard, IS) were provided by Sigma. Stock solutions of 1000 mg L^{-1} were prepared in methanol and stored in darkness at −10 °C. Working standard mixed solutions were prepared daily by diluting with Milli-Q water.

2.2. Instrumentation

All analyses were performed in 15 mL glass vials and the solutions were stirred in a 15 position magnetic stirrer (Velp Scientifica, Usmate, Italy) using commercial stir bars for sorptive extraction (Twisters®) coated with with a layer of EG-Silicone (32 µL) on an inert metal grid for mechanical stabilization, obtained from Gerstel (Mullheim an der Ruhr, Germany). Prior to use, the stir bars were conditioned in an empty thermal desorption tube at 200 ºC for 1 h with helium at a flow desorption rate of 50 mL min^{-1}. Reconditioning of stir bars was done after its use in the same way to avoid any matrix retention, allowing its employment over 30 times. The sample introduction system comprised a Thermal Desorption Unit (TDU-2) equipped with an autosampler (MPS-2) and a Programmed Temperature Vaporization (PTV) Cooled Injector System (CIS-4) provided by Gerstel. The TDU was initially operated in solvent vent mode maintaining a temperature of 50 ºC for 0.5 min in order to dry the stir bar coating. Next, a desorption programme starting at 50 ºC and increasing to 220 ºC at 200 ºC min^{-1} and holding for 10 min was applied. The PTV was cooled to 20 ºC by a Peltier Unit while the analytes were desorbed from the stir bar in the TDU. The PTV was equipped with a liner packed with fiberglass wool (Gerstel). The PTV temperature programme was as follows: start at 20 ºC, increase to 275 at 250 ºC min^{-1} and hold for 5 min. A vent flow and pressure of 60 mL min^{-1} and 10 psi, respectively, were applied in the sample introduction system.

The TDU was installed in an Agilent 6890N (Agilent, Waldbronn, Germany) gas chromatograph coupled to an Agilent 5973 quadrupole mass selective spectrometer equipped with an inert ion source. Helium maintained at a constant flow of 1 mL min^{-1} was used as carrier gas. An HP-5MS (5% diphenyl–95% dimethylpolysiloxane, Agilent) capillary column (30 m × 0.25 mm I.D., 0.25 µm film thickness) was used. The GC temperature programme was: start temperature of 75 ºC, increase to 275 ºC at 50 ºC min^{-1} and hold for 6 min. The compounds were eluted at: 4.0 min (BP), 5.0 min (BPF), 5.3 min (BPA) and 7.2 min (BPZ). The temperatures of the ion source, transfer line and quadrupole were 230, 300 and 150 ºC, respectively. The mass spectrometer was operated using electron-impact (EI) mode (70 eV). The compounds were quantified in the selected ion monitoring (SIM) mode in order to improve the detection limits. Monitorized ions (*m/z*) were 186 for BP; 200 for BPF; 213 for BPA and 268 for BPZ. Identification was confirmed by the retention time and scan mass-spectra database for each compound.

An ultrasonic bath (Selecta, Barcelona, Spain) was used to assure a total homogenization of the mixtures.

2.3. Samples and analytical procedure

A total of thirty cosmetic and personal hygiene samples, including shampoo, shower and hair gel, make-up remover, body lotion, face cream and mouthwash were obtained from a local supermarket. For sample extraction and preconcentration, 10 mL of Milli-Q water was added to

about 120 mg of the sample previously weighed in a 15 mL vial containing 1 g sodium chloride. BP was added to the mixture as internal standard (2 ng mL^{-1}), and the mixture was submitted to ultrasounds by means of a bath for 5 min in order to homogenize the solutions. An EG-Silicone stir bar was introduced into the vial, which was stirred for 3 h at 900 rpm. The stir bar was then removed from the vial, rinsed with Milli-Q water in order to eliminate salt residues and dried with a lint-free tissue before being introduced into a glass desorption tube. The analytes were thermally desorbed from the stir bar, placing the desorption tube containing the stir bar in the TDU connected to the PTV injector, and then directed to the GC-MS system.

3. Results and discussion

3.1. Optimization of the SBSE conditions

SBSE preconcentration involves two different sequential stages, the extraction of analytes from the aqueous sample onto stir bar, and their thermal desorption into the GC-MS injector. Because of the high number of variables involved in these processes [17], as well as its interrelationship, experimental designs are usually employed for optimization purposes, such as factorial, Plackett-Burman, Central Composite or Box-Behnket [23,37,38]. In our case, their effect and significance was tested using a mixed level Taguchi multivariate design. In this way, the variables were checked at two or four levels depending on their characteristics. In order to weigh up the relevance of each variable for the different analytes in the optimization process, regardless of their different sensitivities, the peak area values of each compound were normalized relative to average areas for the same compound in the corresponding experimental set. A statistical package was used to generate the experimental matrices and to evaluate the results.

The possible influence of three factors on the performance of the SBSE extraction step was studied, namely ionic strength (four levels: 0, 5, 10 and 20% m/v NaCl), organic modifier concentration (four levels: 0, 5, 10 and 20% v/v methanol) and pH of the extraction medium (two levels: 5 and 7). The experiments were carried out in duplicate, making a total of 32 experiments. An aqueous solution (10 mL) containing the analytes at a concentration level of 5 ng mL^{-1} for each compound was used in the experiments.

The ionic strength was fixed by the addition of sodium chloride, checking the effect of different salt concentrations in the 0 – 20% (m/v) range. Salt reduces the water solubility of polar organic analytes, increasing their extraction efficiency, although high salt concentrations may decrease the extraction efficiency by enhancing solution viscosity, which hinders analytes diffusion. As shown in Fig. 1A, all the compounds attained maximum extraction efficiency with 10% (m/v) NaCl.

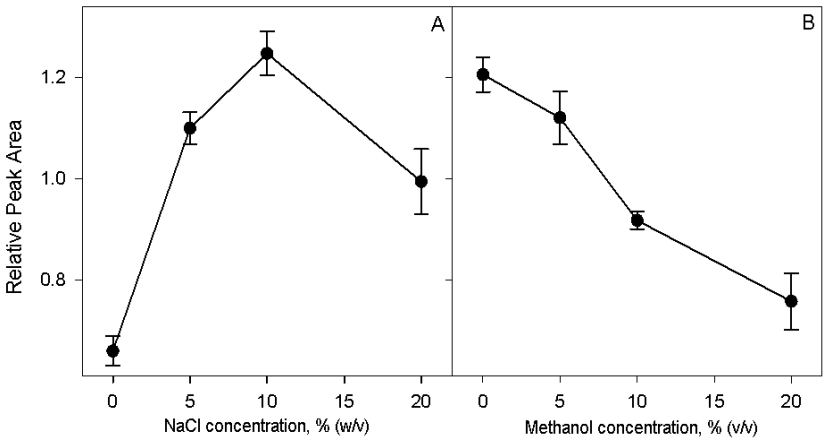


Fig. 1. Effects of factor levels of the salt **(A)** and organic modifier addition **(B)** on the mean relative response for BPs obtained from the Taguchi orthogonal design application for the optimization of the SBSE preconcentration step.

The presence of methanol often can improve the responses obtained, preventing the adsorption of the compounds onto the inner glass walls, while high methanol concentrations may increase analyte solubility in the extraction medium. Nevertheless, when several concentrations of methanol, from 0 to 20% (v/v), were tested, it was found that extraction efficiency for all compounds decreased with increasing methanol concentration (Fig. 1B), so its use was discarded. Two considerations should be taken in the selection of the extraction medium pH. On the one hand, 3.5 is the lower limit pH of EG-Silicone phase, on the other hand, BPs, which are species of low acid character, are deprotonated in basic medium. Two different pH values, 5 and 7, were tried, and best results were achieved at neutral pH.

The most important parameter affecting SBSE is extraction time and this was separately investigated from 0.5 to 24 h, equilibrium being reached for all compounds at around 3 h, which was adopted to ensure greatest extraction efficiency. The selected extraction time is not a serious drawback since the use of the autosampler allows the system to be working overnight.

The performance of the SBSE desorption step was studied by means of a Taguchi experimental design of six factors: desorption temperature and PTV liner filling (studied at two levels) and desorption time, gas flow-rate and pressure and CIS temperature (studied at four levels). Analysis of effects results for the factors related to the desorption stage are summarized in Fig. 2. The maximum working temperature recommended by the manufacturer for the EG-Silicone polymer is lower than that recommended for PDMS, so that, 200 and 220 ºC were the temperatures assayed in the TDU program being attained from 50 ºC at 200 ºC min^{-1}, both of which were maintained for times in the 5 - 12.5 min range (Fig. 2A). Optimal temperature and time of desorption were 220 ºC and 10 min, respectively, except for BPZ, which desorbed earlier, attaining its maximum sensitivity at 5 minutes. Several flow-rates and pressures for the inert gas (in the 30 – 120 mL min^{-1} and 8 – 11 psi ranges, respectively) were applied in the thermal desorption unit. Fig. 2B shows that maximum sensitivity was attained for all the compounds with a 60 mL min^{-1} helium flow-rate.

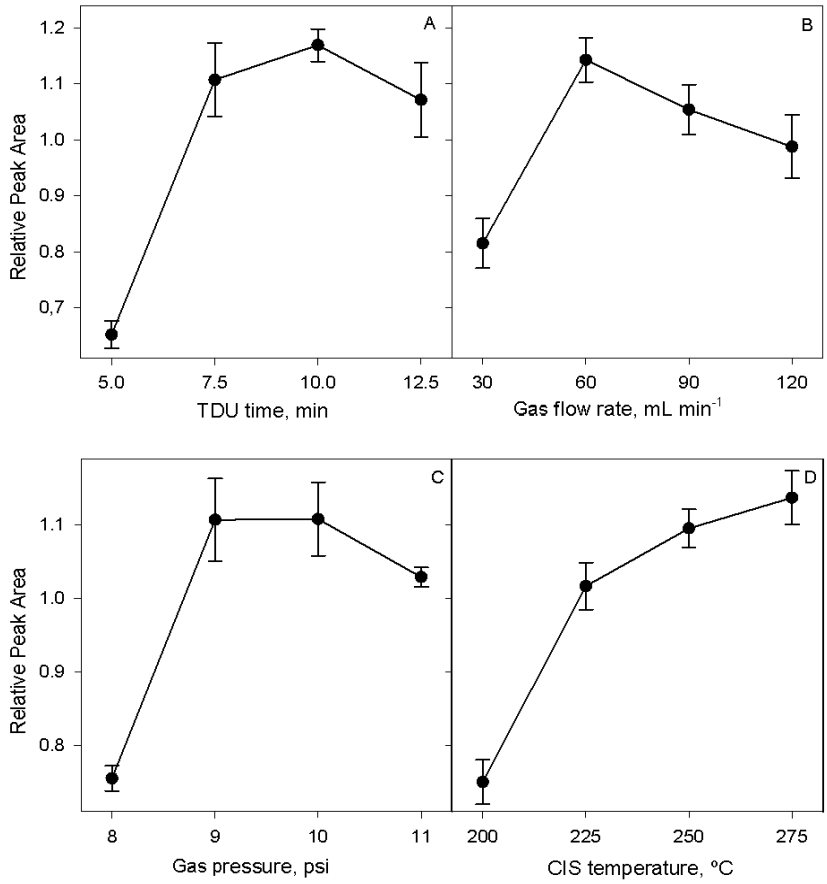


Fig. 2. Effects of factor levels of the desorption time **(A)**, desorption flow **(B)**, desorption flow pressure **(C)** and CIS maximum heating temperature **(D)** on the mean relative response for BPs obtained from the Taguchi orthogonal design application for the optimization of the SBSE desorption step.

As regards the gas pressure, since two of the analytes provided maximum signals with 10 psi and no significant differences were obtained between 9 and 10 for the other analytes (Fig. 2C), 10 psi was selected. These conditions ensure a complete analytes desorption, since no carry over was observed.

The retention of compounds in the PTV injector is heavily influenced by temperature, which was fixed at 20 ºC by means of a Peltier unit. Moreover, trapping of the analytes in the PTV while they are being desorbed from the SBSE stir bar may be influenced by the nature of the filling in the PTV liner. Two different PTV liners, one filled with fiberglass and other empty with baffles, were tested. Best results were obtained with the fiberglass filled liner, probably because the analytes condensed on its large surface. Prior to injection into the GC column, PTV has to be heated in order to vaporize the compounds. Different temperatures, ranging from 200 ºC to the maximum value allowed for the fiberglass-filled liners (275 ºC), were assayed. As can be seen from Fig. 2D, the higher the temperature, the higher the sensitivity, therefore the PTV was heated to 275 ºC.

Table 1
Slopes[a] (L mg^{-1}) obtained for standard additions to different samples.

Sample	BPF	BPA	BPZ
Aqueous	176±1	357±3	143±1
	(1.42±0.01)	(2.89±0.03)	(1.16±0.01)
Shampoo	80±1	169±2	72±1
	(1.36±0.02)	(2.87±0.05)	(1.23±0.02)
Shower gel	109±1	210±2	85±1
	(1.47±0.02)	(2.83±0.04)	(1.15±0.02)
Hair gel	100±2	189±2	81±1
	(1.46±0.03)	(2.77±0.05)	(1.18±0.03)

[a] Mean ± standard deviation (n=7).
Values into brackets correspond to the slopes obtained using the internal standard calibration.

3.2. Method performance

Aqueous calibration curves representing peaks area versus concentration, ranging from 0.5 to 20 ng mL^{-1}, were obtained for all the analytes. Correlation coefficients were higher than 0.99 in all cases. When the slopes of aqueous standards calibration graphs were compared with those obtained when the standard additions method was applied to three different samples (shampoo, shower gel and hair gel) using a t-test, the presence of a matrix effect was detected (Table 1). BP was assayed as IS after checking that all samples were free of this compound, which appeared to be the most suitable for this purpose [39] because it showed similar chromatographic and SBSE extraction behaviour to the rest of the studied compounds. When the internal standard was added at 2 ng mL^{-1} to the aqueous standard solutions (166 ng g^{-1} to the samples), the slopes of the aqueous standard calibration graphs and those obtained by means of the standard additions to the samples showed no significant differences at the 95% confidence level, with "p" values ranging between 0.08 and 0.93 (Table 1). It was concluded that the use of this compound as IS compensated the matrix effect, thus obviating the need to use the tedious standard addition method for quantification purposes. In addition, the use of an IS limited any uncertainty related to the SBSE extraction and desorption steps. To check the performance of the procedure, sample masses up to 120 mg were used, higher masses could not be used since main components of the personal care products sample matrix could foul the ion source and the mass analyzer. Considering that the higher the sample mass, in the studied range, the higher sensitivity, a sample mass of 120 mg was adopted for further experiments.

Considering the use of this internal standard procedure, the method detection limits (LODs) were calculated as three times the standard deviation of the calibration intercept on the y axis, and values between of 0.10 and 0.11 ng mL^{-1} (8.0 – 8.7 ng g^{-1}, considering 120 mg of sample submitted to analysis), were obtained. The obtained values for each compound are summarized in Table 2,

Table 2
Analytical characteristics of the SBSE-TD-GC-MS method.

Compound	RSD[a] (%)	LOD[b] (ng g^{-1})	LOQ[c] (ng g^{-1})
BPF	1.4	8.0	26.7
BPA	2.9	8.7	29.2
BPZ	1.2	8.0	26.7

[a] n=10. [b] Corresponding to $3\sigma_{intercept}$. [c] Corresponding to 10 $\sigma_{intercept}$.

Table 2 also include values obtained for the method quantitation limits (LOQs), calculated as ten times the standard deviation of the calibration intercept on the y axis. To check the repeatability of the method, ten replicate analyses of a standard solution at 5 ng mL^{-1} concentration level for each compound were performed, and RSD values lower than 3% were obtained in all cases (Table 2). RSD data for spiked samples are detailed in Section 3.3, and the data obtained indicated that the precision of the method was satisfactory for control analysis purposes.

In agreement with previous data [21,23], the sensitivity for BPs increased between 5 and 10-fold when using EG-Silicone stir bars compared with the obtained with those coated with PDMS and preconcentrating the analytes not derivatized. Nevertheless, the preconcentration of underivatized BPs using EG-Silicone SBSE stir bars is still less sensitive than those methods that include derivatization, such as *in-tube* silylation or *in-situ* acetylation [19-23,29], although the method described here can be considered simpler than those involving derivatization since derivatization reagents are not used. Even though a shorter lifetime than their PDMS equivalents would be expected for EG-Silicone stir bars, no significant degradation or reduction in its extraction capabilities was observed. Other drawbacks of the use of EG-Silicone stir bars, such as the need for an initial drying step, usually carried out exposing the stir bar to a dry atmosphere during long times, can be overcome using the TDU solvent vent mode, evaporating water at 50 ºC for 0.5 min [34].

Although the whole analytical procedure takes nearly four hours for a single sample, a sequential preparation of them as well as the use of an autosampler, allow a performance of at least twenty determinations each day.

Table 3
Results for the analysis of the samples.

Sample	BPA (ng g^{-1})
Shower gel A	30.9±0.8
Shower gel B	69.0±2.0
Hair gel A	< LOQ
Hair gel B	88.3±0.4
Face cream	31.0±1.0
Make-up remover	77.0±3.0
Mouthwash	86.7±0.5

Values are mean ± standard deviation (n=3).
< LOQ means detected but not quantified.

3.3. Analysis of samples

The determination of BPs traces in different personal care commercial products, using the optimized SBSE without derivatization and TD-GC-MS method, was carried out. The results obtained are shown in Table 3. BPA was the only bisphenol detected, being present in six of the thirty analyzed samples, at concentrations ranging from 30.9 to 88.3 ng g^{-1}.

A typical chromatogram under the selected conditions for a fortified sample at 160 ng g^{-1} is shown in Fig 3A, while the mass spectra of the analyzed compounds used to confirm identity appear in Fig. 3B. Since no reference materials were available for the validation of the method, the precision and accuracy of the evaluated method was assessed by replicate analyses (n=10) of three samples (shampoo, shower and hair gel) spiked at 40 and 160 ng g^{-1} levels. Good recoveries of between 89 and 114% were obtained for all the analytes, with RSD values in the 4.2 – 11% range at the lowest level, and between of 94 and 108% with RSD values in the 2.1 – 8.3 % range at the highest level.

4. Conclusion

A new analytical method has been developed for the quality control of widely used personal care products relating to their content in three bisphenol compounds. The optimized method, a combination of SBSE and TD-GC-MS using recently commercially available EG-Silicone phase stir bars, enables the precise determination of trace amounts of bisphenol A, bisphenol F and bisphenol Z at low concentrations levels (ng g^{-1}). The absence of a previous derivatization step could be a relevant advantage in save of time and reagents over previously reported procedures.

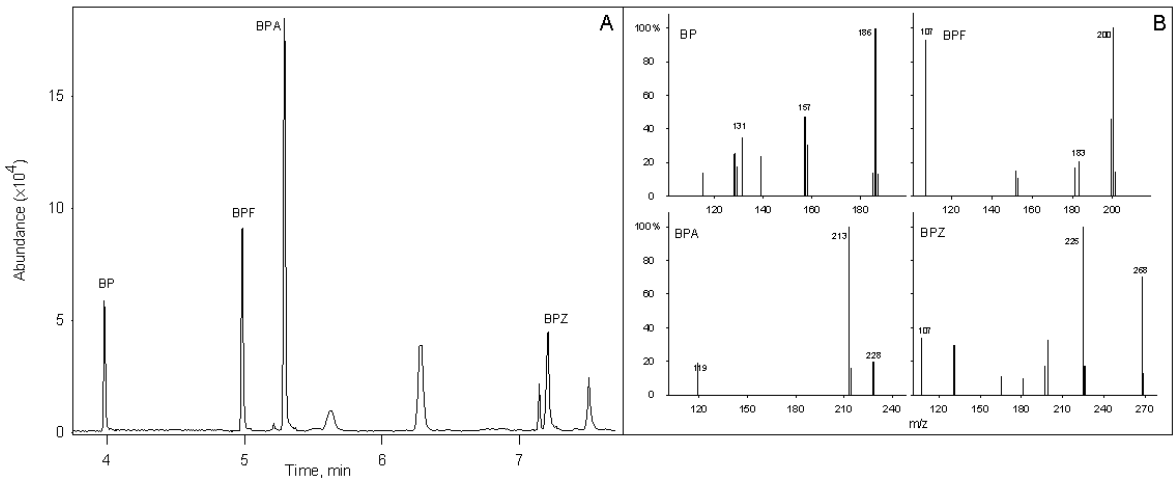


Fig. 3. (A) Elution profile obtained with the optimized SBSE-TD-GC-MS procedure for a fortified shampoo sample at 160 ng g^{-1} concentration level of each analyte. (B) Mass spectra of each compound.

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Chapter II

Headspace Sorptive Extraction and Gas-Chromatography coupling by Thermal Desorption

Headspace sorptive extraction for the analysis of organotin compounds using thermal desorption and gas chromatography with mass spectrometry

Abstract

A method based on headspace sorptive extraction (HSSE) in combination with thermal desorption-gas chromatography-mass spectrometry (TD-GC-MS) has been developed for the simultaneous determination of six organotin compounds (OTCs), corresponding to mono- and di-substituted methyltin, butyltin and octyltin species. Several parameters affecting both the headspace extraction and thermal desorption steps were carefully optimized using multivariate designs. Analytes were derivatized by in-situ ethylation with sodium tetraethylborate. The optimized method was applied to the analysis of water samples of different origins, as well as to checking the migration of the studied compounds from commercially available plastic containers to the adequate liquid simulant. Quantification was carried out against aqueous calibration curves using diphenyltin as internal standard, providing detection limits of between 1.7 and 7.0 ng(Sn) L^{-1}, depending on the compound, and repeatabilities lower than 10% in terms of relative standard deviation. The applicability of the method was assessed by means of recovery studies and satisfactory values for all compounds were attained. The release of OTCs from the tested packages to the liquid simulant was confirmed, concentrations as high as 2.4 μg(Sn) L^{-1} being found for dioctyltin. Even though the proposed method was developed for organotin halides, its application to an organotin ester shows its suitability for determining these compounds in migration assays.

1. Introduction

Organotin compounds (OTCs) are involved in many human activities, mainly as heat and light stabilizers for poly(vinyl chloride) (PVC). A variety of mono- and di-substituted compounds, like methyl, butyl, octyl or dodecyltins, are added to plastic as additives in order to prevent degradation processes. Since they improve thermal stability, these compounds make the plastic easier to recycle [1]. However, their use has been restricted due to the negative effects that OTCs may have for humans, taking into account that they are severely toxic and are considered endocrine disrupting chemicals (EDCs) whose danger is heightened by the possibility of bioaccumulation [2]. So, any improvement in analytical methods susceptible of being applied to OTCs is of great importance.

Legal regulations in this respect have been extended to plastic food contact materials [3], the stabilization of which by means of OTCs is very common, and lower maximum values have been set and a greater number of compounds, especially carboxylates and mercapto esters, included. Since organotin stabilizers may migrate from packaging materials to food owing to high temperatures or long contact times, migration tests are usually carried out using food simulants [4,5]. The EU has established specific migration limits (SML) for methyl, di-n-octyl and mono-n-octyltin compounds as low as 0.006 mg(Sn) kg^{-1} [6], but not yet for the butyltin species.

The most common separation technique used for determining OTCs is gas chromatography (GC), following a derivatization step to increase OTC volatility. The ethylation reaction with sodium tetraethylborate has been widely used for this purpose because it can be applied in an aqueous medium, making it preferable to alternatives such as the Grignard reaction. Usually, GC is coupled with an element-specific detection system, such as microwave induced plasma-atomic emission detection (MIP-AED) [7-11], inductive coupled plasma-mass spectrometry (ICP-MS) [12-15] or pulsed flame photometric detection (PFPD) [16-23]. Nevertheless, hyphenation with mass spectrometry provides complete structural information, high sensitivity and also avoids possible interference from complex matrices [4,7,13,24-28].

Since the OTC concentrations in the samples analyzed were expected to be very low, a preconcentration step was needed before GC. For this, easy and clean microextraction techniques have been successfully applied, such as solid-phase microextraction (SPME) [7,14,16-18,20,22-24,29,30], dispersive liquid-liquid microextraction (DLLME) [19], single drop microextraction (SDME) [13,25,31,32] or stir bar sorptive extraction (SBSE) [12,33].

Headspace sorptive extraction (HSSE) is a microextraction technique derived from the application of SBSE in headspace mode. The stir bar coated with a thick film of polydimethylsiloxane (PDMS) is suspended in the vial headspace, where the analytes are adsorbed on the stir bar coating, which acts as extraction phase. After sampling and prior to GC analysis, the retained compounds are thermodesorbed in a specific injector, comprising a thermal desorption unit

(TDU) and a programmed temperature vaporizing (PTV) injector. This microextraction technique provides higher recoveries and so higher sensitivity than SPME, due to the larger amount of extracting phase involved [34]. In addition, the robustness of the stir bar assembly facilitates its application. These advantages of HSSE have allowed the determination of OTCs in seawater by Prieto et al. [28]. However only butyltin compounds were considered [29] but, as far as we know, no application of this technique to migration studies of OTCs from plastic packages has been reported.

In this work, three types of mono- and di-substitued alkyltin compounds, monomethyltin (MMT), dimethyltin (DMT), monobutyltin (MBT), dibutyltin (DBT), monooctyltin (MOT) and dioctyltin (DOT), in waters of different origin are simultaneously analyzed using HSSE coupled to TD-GC-MS. In addition, in light of the improved sensitivity of the developed procedure compared with those previously used for this purpose, it was applied to evaluating the possible migration of the studied compounds from plastic containers.

2. Materials and methods

2.1. Reagents

Monomethyltin trichloride (MMT, 97%), dimethyltin dichloride (DMT, 97%), monobutyltin trichloride (MBT, 95%), dibutyltin dichloride (DBT, 96%), dibutyltin dilaurate (DBTDL, 98%) and diphenyltin dichloride (DPhT, 96%) were obtained from Aldrich (Steinheim, Germany). Monooctyltin trichloride (MOT, 90%) and dioctyltin dichloride (DOT, 97%) were supplied by Dr. Ehrenstorfer (Augsburg, Germany). Individual stock solutions of these compounds were prepared using HPLC grade methanol (Lab-Scan, Dublin, Ireland) at a concentration of 1000 mg(Sn) L^{-1}, and stored in the dark at -20ºC. Working standard solutions were prepared daily by diluting the standard solutions with Milli-Q water (Millipore, Bedford, MA, USA).

The derivatization reagent was prepared by dissolving 1 g of sodium tetraethylborate ($NaBEt_4$, 98% purity, Strem Chemicals, Newburyport, MA, USA) in 50 mL of a 2% (m/v) sodium hydroxide (Riedel-de Haën) solution. Fractions of this solution were stored in the dark at -20ºC, where they remained stable for one month. The acetate buffer solution (0.2 M) was prepared by dissolving sodium acetate (Riedel-de Haën) in water and then adjusting the pH to 4.8 using acetic acid. Sodium chloride was obtained from Sigma (St. Louis, MO, USA). Helium (99.9999% purity) was supplied by Air Liquide (Madrid, Spain).

2.2. Instrumentation

Commercial stir bars coated with a 0.5 mm thick layer of PDMS (24 μL) were obtained from Gerstel (Mullheim an der Ruhr, Germany). Prior to use the stir bars were conditioned in an empty

thermal desorption tube at 275 ºC for 0.5 h with helium at a flow desorption rate of 50 mL min^{-1}. To control the temperature during the extraction step, a homemade heating system consisting of a drilled block equipped with an electronic temperature control system was used for stirring and heating.

The sample introduction system was composed of a Thermal Desorption Unit (TDU-2) equipped with an autosampler (MPS-2) and a Programmed Temperature Vaporization (PTV) Cooled Injector System (CIS-4) provided by Gerstel. The experimental conditions used for the sample introduction system are summarized in Table 1.

GC analyses were performed on an Agilent 6890N (Agilent, Waldbronn, Germany) gas chromatograph coupled to an Agilent 5973 quadrupole mass selective spectrometer equipped with an inert ion source. Under the selected conditions (Table 1) the analytes eluted at retention times between 5.4 and 13.4 min, corresponding to DMT and DOT, respectively (Table 2). The compounds were quantified in the selected ion monitoring (SIM) mode in order to improve the detection limits using different ions (Table 2). Identification was confirmed by the retention time of the target ion and the qualifier-to-target ion ratios for each compound.

2.3. Samples

Six seawater samples were obtained from different areas of Cartagena Bay (Spain). Additionally, six tap water samples were obtained from different points of the city of Murcia, in the same region of S.E. Spain. Sample aliquots of around 100 mL volume were collected in glass flasks. All samples were kept in the dark at 4 ºC before analysis, which took place within 48 h of arrival at the laboratory. In order to avoid any possible residual contamination from the flasks or adsorption on their inner surfaces, all the glass material employed was previously washed using a 5% (v/v) nitric acid solution [11].

Table 1
Experimental conditions of the TD-GC-MS procedure.

Thermal Desorption Unit (TDU)	
Mode	Splitless
Temperature Programme	50 – 275 ºC at 120 ºC min^{-1}, held 8 min
Desorption Flow and Pressure	35 mL min^{-1}, 9 psi
Programmed Temperature Vaporization (PTV)	
Mode	Solvent Venting
Liner	Silanized glass wool, 1 mm i.d.
Temperature Programme	15 – 250 ºC (5 min) at 6 ºC s^{-1}
GC-MS	
Capillary Column	HP-5MS, 5% diphenyl-95% dimethylpolysiloxane 30 m x 0.25 mm, 0.25 μm
Carrier gas	Helium (1 mL min^{-1})
Oven Programme	75 – 275 ºC at 50 ºC min^{-1}, held 6 min
Quadrupole, ion source and transfer line temperature	150 ºC, 230 ºC, 300 ºC
Ionization	Electron-impact mode (70 eV)

2.4. Analytical procedure

10 mL of the sample were placed in a 15 mL glass vial, and pH adjusted to 4.8 by adding 1 mL of 0.2 M acetate buffer solution. Diphenyltin was added as internal standard, at a concentration of 1 μg(Sn) L^{-1} after confirming that the samples were free of this compound. Next, ethylation was performed by adding 75 μL of the 2% (m/v) $NaBEt_4$ solution. HSSE extraction was carried out by exposing the stir bar to the headspace of the vial, while the sample was stirred and heated at 900 rpm and 90 ºC, respectively.

Eight different plastic containers (three bicycle drinking bottles, two canteens, two water bottles and a cocktail shaker) obtained from local stores were tested for possible organotin migration. Migration tests were performed according to EU regulation [6]. A 20% (v/v) ethanol-water solution was used as food simulant, and all samples were placed in a thermostatic bath at 40 ºC for 10 days. Since most of the selected plastic containers are intended to come into repeated contact, the migration tests were carried out three times whit the same sample using another portion of food simulant on each occasion. After the migration test, the simulants were analyzed according to the procedure described above, being blank test for simulant also included.

3. Results and discussion

HSSE preconcentration implies two different and sequential stages: the extraction of analytes from the aqueous sample to the stir bar coating and their thermal desorption for injection into the GC. Due to the high number of variables involved in these processes, their effect and significance were tested using a Plackett-Burman multivariate design (PBD). This initial screening test allows not only the less important variables to be identified, but also the most suitable conditions among those assayed to be selected. Once the most relevant parameters had been identified, they were considered in more detail using a Central Composite Design (CCD) and response surface analysis by quadratic polynomial regression, which provide linear and quadratic terms and first order interactions, allowing fitting optimum values.

In order to weight the relevance of each variable for the different analytes in the optimization process, regardless of their different sensitivities, peak area values of each one were normalized with respect to their average area in the corresponding experimental set. The Minitab 15.0 statistical package was used to generate the experimental matrices and to evaluate the obtained results.

3.1. Optimization of HSSE parameters

The extraction of the analytes was studied using spiked water samples at 1 μg(Sn) L^{-1} for all compounds and a 2 h extraction time. The variables studied were: the addition of an inert salt

(NaCl, 0 – 10% (m/v)), the addition of an organic modifier (methanol, 0 – 10% (v/v)), sample volume (5 – 10 mL), extraction temperature (25 – 75 ºC) and the concentration of the derivatizing reagent ($NaBEt_4$, 0.01 – 0.02% (m/v)). These five variables were simultaneously tested following a PBD (n=12, in duplicate).

In order to increase the efficiency of the extraction procedure, avoiding the adsorption of non-polar compounds to the inner walls of the sample vials, methanol is often added as an organic modifier to aqueous samples. In the same way, the addition of salt increases the medium ionic strength, facilitating the extraction of non-polar species because of the decrease in their solubilities. Nevertheless, as Fig. 1A shows, neither factor seemed to have a positive effect on the analytical signals obtained. Additionally, even though NaCl concentration appeared as a relevant factor according to Fig. 1A, its negative effect, as well as that provided by methanol, was close to being not statistically significant, so the use of these chemicals was discarded.

The sample amount was investigated by varying the final volume of the fortified sample in a 15 mL vial. Fig. 1A illustrates that this is a relevant factor, and extraction efficiency increased with higher sample amounts, so a final volume of 10 mL was selected. Using higher sample volumes, it was not possible to ensure that the liquid sample and the stir bar would not come into contact. Other vial sizes were not considered due to limitations imposed by the HSSE sampling device.

According to Fig.1A, extraction temperature and concentration of the derivatization reagent solution were also relevant factors in the extraction step, which positively affected the sensitivity of all the OTCs studied. These two variables were studied in more detail using a CCD (α=1.5, 4 cube points, 4 axial points and 2 central points, by duplicate), developed in the range 60 – 90 ºC and 0.015 – 0.025% (m/v). The obtained response surface (Fig. 1B) closely reflected with experimental data (r^2>0.95) and underlined the significance of the tested variables ($p<0.01$). HSSE sampling temperature was the most influential parameter. High extraction temperatures favored vaporization of the ethylated analytes, shifting the equilibrium of these species to the headspace phase from where they were extracted to the HSSE sorptive phase. Taking into account that the highest extraction efficiencies for all the investigated compounds were obtained at 90 ºC, this temperature was selected. Higher temperatures were not considered, due to the limits imposed by the boiling point of water, and no negative effects associated with the thermal release of trapped species from the PDMS to the headspace were observed.

As regards the derivatizing reagent according to the response surface (Fig. 1B), the maximum sensitivity was attained when 75 µL of 2% (m/v) solution was added to the sample, meaning a concentration 0.015% (m/v).

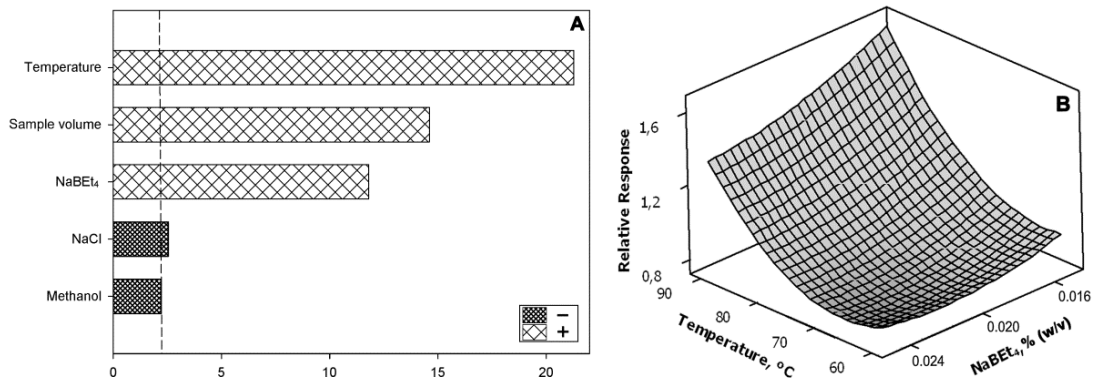

Fig. 1. (A) Pareto Charts obtained for the analysis of effects for the Plackett-Burman designs in HSSE extraction step. (B) Response surfaces obtained for the CCD showing the effects on relative responses of extraction temperature and $NaBEt_4$ derivatizing solution concentration.

The most important parameter affecting HSSE is commonly found to be the extraction time, which was separately investigated from 0.5 to 24 h after optimizing the other extraction parameters. In general, HSSE extraction efficiency increased with extraction time, at least until equilibrium between the liquid sample, headspace and extracting phase was attained. Despite this equilibrium being reached after about 3 h for the more volatile compounds, like DMT or MMT, 4 h was chosen to ensure complete recovery of all the compounds.

3.2. Optimization of thermal desorption parameters

In order to optimize the thermal desorption of the HSSE-trapped analytes, a screening test following a PBD (n=12) was performed. The variables and ranges considered in this initial study were: TDU desorption time (5 – 10 min), TDU desorption temperature (225 – 275 ºC), inert gas flow rate (50 – 100 mL min^{-1}), inert gas pressure (7.5 – 9 psi), PTV focusing temperature (15 – 20 ºC), PTV heating temperature (225 – 275 ºC) and PTV liner inner diameter (1 – 2 mm).

Based on the results of the PBD screening (Fig. 2A), the most relevant parameters for the thermal desorption process, inert gas flow rate and TDU desorption time, were carefully studied using a CCD (α=1.5, 4 cube points, 4 axial points and 2 central points, by duplicate), in the 35 – 80 mL min^{-1} and 3.5 – 8 min ranges, respectively. The obtained response surface (Fig. 2B) showed its adequacy to experimental results (r^2>0.95) and the relevance of the assayed variables (p<0.01).

A TDU desorption temperature of 275 ºC was selected. Consequently, the TDU was heated from 50 to 275 ºC (120 ºC min^{-1}). This relative low heating rate allowed a simultaneous heating of the TDU-PTV interphase, minimizing losses of the most volatile compounds (MMT and DMT). This TDU temperature was maintained for 8 min, ensuring the total desorption of the analytes and avoiding the possible carry-over caused by incomplete desorption at lower times, or analytes losses due to excessively long desorption times.

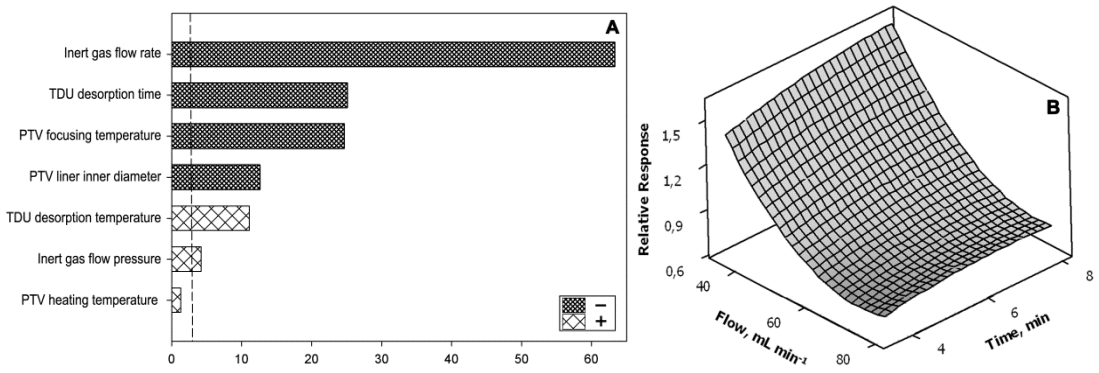

Fig. 2. (A) Pareto Charts obtained for the analysis of effects for the Plackett-Burman designs in thermal desorption step. (B) Response surfaces obtained for the CCD showing the effects on relative responses of desorption time and gas flow rate.

A carrier gas is necessary to propel the analytes towards the PTV injector while they are being thermally desorbed in the TDU. As shown in Figs. 2A and 2B, the lower desorption flow, the greater the efficiency of the process, so 35 mL min^{-1} was chosen.

Lower gas flow rates cannot assure avoid air entrance into the system. The pressure of this gas flow seemed to be a significant factor in the desorption step, so a value of 9 psi was selected, since a higher pressure involves increased gas consumption and may lead to column bleeding as a result of large column flows.

Desorbed compounds were focused in the PTV before entering the chromatographic column. Lower temperatures in this device mean lower losses and greater retention efficiency, as Fig. 2A shows, especially for the more volatile compounds. Since the Peltier unit only allows cooling to slightly below room temperature, 15 ºC was selected. In order to facilitate the retention of the analytes in the PTV, a liner filled with silanized glass wool as inert support was used. The inner diameter of this liner was a relevant factor, according to Fig. 2A, so the narrowest commercially available liner (Φ=1 mm) was used. As regards PTV maximum temperature, Fig. 2A shows that temperature had no significant effect for the analytes in the studied range, and so a compromise value of 250 ºC was selected. Therefore, a PTV programme temperature increasing from 15 to 250 ºC (6 ºC s^{-1}), with a hold time of 5 min was applied.

3.3. Analytical characteristics of the method

For quantification purposes the internal standard method was employed, minimizing the possible uncertainty associated with analyte losses or any possible matrix effect. Since all the samples analyzed were free of diphenyltin, a species with an analytic behaviour similar to that of the analytes, this compound was used as internal standard.

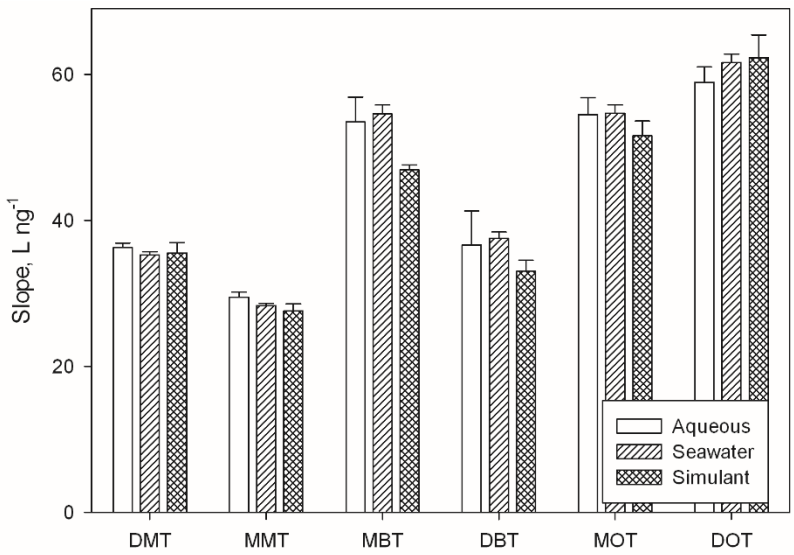

Fig. 3. Slopes of calibration graphs using DPhT as internal standard. Vertical bars indicate standard deviation of the slope (n= 12).

The slopes of aqueous standards calibration graphs were compared with those obtained when the standard addition method was applied to a seawater sample and a 20% (v/v) ethanol-water simulant maintained for 10 days at 40 ºC in a plastic container. In all cases six concentration levels were submitted in duplicate to the optimized procedure. Peak area ratios with respect to internal standard were plotted versus analyte concentration and no statistically significant differences were found when a t-test was used (Fig. 3), confirming the reliability of the use of aqueous standard calibration for quantitation purposes.

Calibration curves were found to be linear between 0.1 – 10 and 0.025 – 2.5 μg(Sn) L^{-1}, depending on the compound (Table 2), with correlation coefficients higher than 0.99 in all cases. The detection limits (LODs) and quantification limits (LOQs) were calculated as three and ten times the standard deviation of the calibration intercept on the y-axis, respectively. The values obtained are summarized in Table 2. The repeatability was calculated using the relative standard deviation (RSD) for a standard mixture (0.1 and 0.4 μg(Sn) L^{-1}, depending on the compound), which was submitted to the proposed procedure 10 times, providing RSD values in the 4.2 – 9.6% range, as shown in Table 2.

A comparison of the main characteristics of the proposed method with previously reported works is summarized in Table 3. An important advantage of the current paper over those based on liquid-liquid extraction (LLE) [4,35] is that toxic organic solvents are avoided, and a high is achieved. In this sense, low LODs have been reported as a consequence of the large sample volume analyzed [35]. Purge-and-trap (P&T) is also a solvent-free preconcentration system, but provides higher LODs than those here reported [11]. As regards SPME, very different LODs are attained depending on the sample matrix analyzed [20,22].

Table 2
Method characteristics.

Compound	Retention time, min	Monitorized ions (*m/z*)	Linearity range, μg(Sn) L^{-1}	RSD, %	LOD, ng(Sn) L^{-1}	LOQ, ng(Sn) L^{-1}
DMT	5.4	193, 165, 135	0.1 – 10	9.6	6.3	20.9
MMT	7.2	179, 151, 135	0.1 – 10	7.7	7.0	23.3
MBT	11.0	179, 235, 149	0.025 – 2.5	5.8	1.8	5.9
DBT	11.6	207, 263, 235	0.025 – 2.5	6.8	2.0	6.5
MOT	12.2	179, 291,149	0.025 – 2.5	4.2	2.2	7.4
DOT	13.4	263, 375, 291	0.025 – 2.5	6.9	1.7	5.5

Underlined numbers correspond to *m/z* of the target ion.

Although the proposed method was developed using organotin chloride compounds, the nature of the derivatization reaction used, replacing halogen or oxygen substituents by ethyl groups [36], would presumable allow the determination of total OTC amounts, regardless their chemical form. If this is so, the total amounts of different OTCs or mixtures authorized for use in plastic food contact materials [6] could be determined. Since the analytical standards of most of these compounds are not commercially available, the suitability of the proposed method was only checked for dibutyltin dilaureate (DBTDL). According to a t-test (p=0.61), the calibration slope for this compound showed no differences with respect to DBT, providing recoveries in the 96-104% range.

3.4. Analysis of samples. Migration and recovery studies

The optimized procedure was applied to different samples: 6 tap waters, 6 seawaters and 24 aqueous solutions resulting from migration assays carried out with eight packages.

Table 3
Comparison of the proposed method with other previously reported.

Analytical technique	Extraction time, h	Sample volume, mL	Linearity ng(Sn)L^{-1}	LODs, ng(Sn) L^{-1}						Ref.
				MMT	DMT	MBT	DBT	MOT	DOT	
HSSE-TD GC-MS	4	10	25-10000	7.0	6.3	1.8	2.0	2.2	1.7	Current work
LLE GC-MS	0.25	10	(3-210)10^4	-[a]	-	1680	2270	2250	1000	[4]
P&T GC-MS	0.3	5	50-3000	-	14	16	18	-	-	[11]
HS-SPME GC-PFPD	0.17	80	0.25-125	0.08	0.03	0.04	0.02	0.03	17	[20]
HS-SPME GC-PFPD	0.17	4-40	1-1000	-	-	2.5	1.7	35	-	[22]
HSSE-TD GC-MS	5	4.5	40-21000	-	-	0.84	0.16	-	-	[28]
LLE GC-ICP-MS	>16	200	0.5-100	2.2	0.9	0.9	0.7	1.0	0.8	[35]

AED – atomic emission detector, GC – gas chromatography, HS – headspace sorptive extraction, ICP – inductively coupled plasma (ICP), LLE – liquid-liquid extraction (LLE), MS – mass spectrometry PFPD – pulsed flame photometric detector, P&T – purge-and-trap, SPME – solid-phase microextraction TD – thermal desorption. [a] Not determined in this work.

Table 4
Results obtained in the analysis of the samples (ng(Sn) L^{-1}).

Sample	DMT	MMT	MBT	DBT	MOT	DOT
Seawater	ND	ND	17±2	81±10	ND	ND
Seawater	ND	ND	29±4	103±9	ND	ND
Tap water	34±3	<QL	ND	ND	ND	ND

Values are mean ± standard deviation (n=3). ND means not detected.

A concentration of 34 ng(Sn) L^{-1} DMT was found in one of the tap water samples analyzed, MMT being detected but not quantified in the same sample (Table 4). This DMT content quantified is higher than values commonly reported in the literature [9,26,37,38], but can be attributed to the recent installation of the water pipe in question and the subsequent migration of PVC additives during first uses [39,40].

With respect to the seawater samples, the two butylated compounds, MBT and DBT, were found in two of them in the 17 – 103 ng(Sn) L^{-1} range (Table 4). Since these compounds may appear as degradation products of tributyltin, which is still used in antifouling paints for large vessels or aluminium boats, they were only found in the samples taken from near a commercial port. Organotin pollution in this area has been reported previously [11,41].

Additionally, the developed method was applied to checking OTC migration from eight different plastic containers to a food simulant. The results obtained are summarized in Table 5. Several analytes were detected in six samples, concentrations reaching as high as 2.4 μg(Sn) L^{-1} for dioctyltin, confirming its release from the tested packages. These results are in accordance with those found by other authors [4]. Due package reuse, three successive migration tests were carried out, showing that the migration of OTC decreases with successive tests, about a 50% decrease for the second and a 70% decrease for the third reuse with respect to the first, depending on the compound and the package. The concentrations found for methyltin and octyltin compounds were, in all cases, lower than the specific migration limits proposed by the EU for these compounds [6].

The compounds were identified by comparing the retention times and identifying the selected ion and ion ratios of the peaks in samples and standard solutions. Fig. 4 shows a typical chromatogram obtained by HSSE–GC–MS in the selected ion monitoring (SIM) mode under the optimized conditions for a sample fortified at 0.1 and 0.4 μg(Sn) L^{-1}, depending on the compound.

As no suitable certified reference materials were available, recovery studies were carried out in order to check the trueness of the proposed method. Three different samples, a tap water, a seawater and a migration solution, all of them free of the analytes, were fortified at two concentrations ranging from 0.1 to 2 μg(Sn) L^{-1} and submitted in duplicate to the previously described procedure. Recoveries ranged from 85 to 115% at the lowest level, and from 91 to 107% at the highest level.

Table 5
Results obtained in the migration studies (ng(Sn) L^{-1}).

Cont.	Time (days)	DMT	MMT	MBT	DBT	MOT	DOT
CB1	10	ND	ND	ND	ND	1011±75	2365±98
	20	ND	ND	ND	ND	326±22	156±21
	30	ND	ND	ND	ND	174±12	72±12
CB2	10	ND	ND	308 27	474±32	997±68	463±58
	20	ND	ND	141±8	141±10	409±28	154±15
	30	ND	ND	113±7	94±6	324±22	89±4
CB3	10	ND	ND	141±9	126 ±9	ND	ND
	20	ND	ND	159±19	141±10	ND	ND
	30	ND	ND	103±6	113±13	ND	ND
CA1	10	ND	ND	ND	ND	160±11	456±91
	20	ND	ND	ND	ND	121±8	56±15
	30	ND	ND	ND	ND	73±5	60±21
WB1	10	288±28	154±12	259±15	364±25	527±36	157±7
	20	134±13	67±5	86±5	174±12	218±15	73±6
	30	65±10	42±3	71±4	114±8	125±16	30±8
CK	10	ND	ND	549±87	421±72	268±34	821±77
	20	ND	ND	27±6	ND	ND	34±4
	30	ND	ND	9±2	ND	ND	ND

Values are mean ± standard deviation (n=3). ND means not detected.
CB – Bicycle drinking bottle, CA – Canteen, WB – Water bottle, CK – Cocktail shaker.

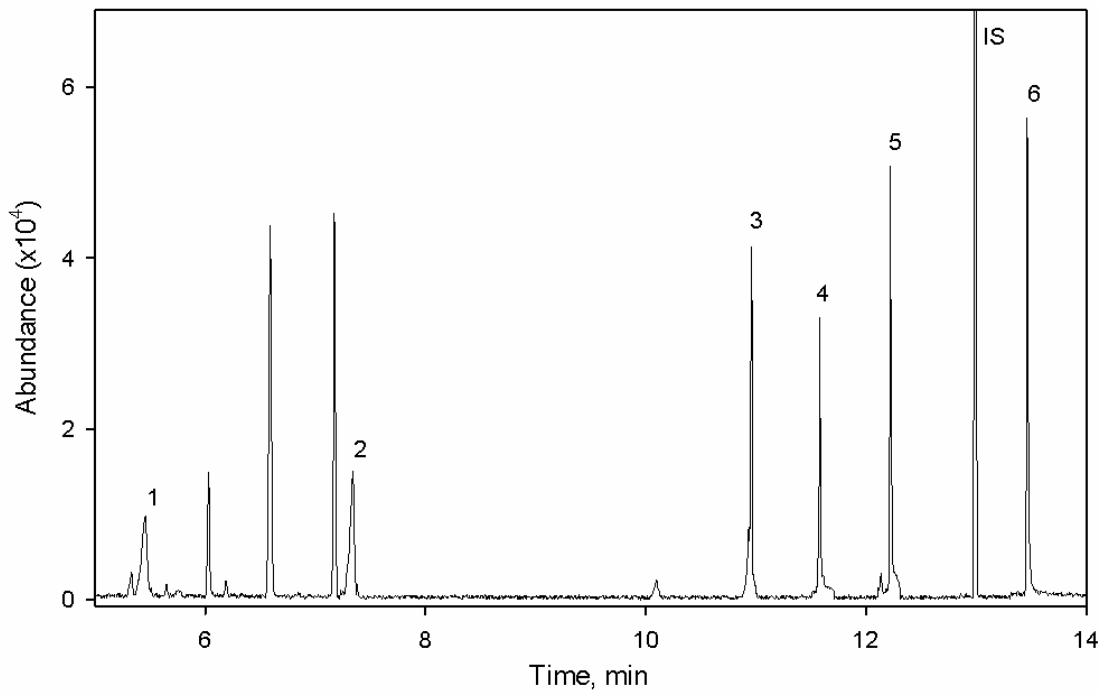

Fig. 4. HSSE-TD-GC-MS chromatogram obtained for a tap water sample fortified with the target compounds at 0.1(3-6) and 0.4(1-2) μg(Sn) L^{-1} under SIM mode. (1) DMT, (2) MMT, (3) MBT, (4) DBT, (5) MOT and (6) DOT.

4. Conclusions

Headspace sorptive extraction (HSSE) was demonstrated to be an effective sampling technique which allowed, in combination with thermal desorption-gas chromatography-mass spectrometry (TD-GC-MS), the simultaneous determination of six OTCs. The optimization of parameters affecting headspace extraction and thermal desorption using multivariate designs led to the development of an analytical method that provides detection limits in the ng(Sn) L^{-1} range. This procedure was successfully applied to the determination of OTC compounds in different water samples as well as to studying OTC migration from plastic containers.

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Use of headspace sorptive extraction coupled to gas chromatography-mass spectrometry for the analysis of volatile polycyclic aromatic hydrocarbons in herbal infusions

Abstract

A solvent-free method is described for the determination of ten volatile polycyclic aromatic hydrocarbons (PAHs), considered as priority pollutants by the EU, in different herbal infusions using headspace sorptive extraction (HSSE) and gas chromatography-mass spectrometry (GC-MS). The parameters affecting both the extraction and thermal desorption steps in the HSSE were optimized by means of Plackett-Burman designs. Ten millilitres of the herbal infusion was submitted to the HSSE preconcentration in the presence of salt for 4 h at 88 ºC. The use of d^{10}-phenanthrene as internal standard not only improved the repeatability of the method but allowed quantification of the samples against external aqueous standards. Detection limits ranged between 11 and 26 ng L^{-1}.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs), a large family of fused-ring aromatic compounds, are persistent and ubiquitous organic pollutants. They are formed in both natural and man-made processes due to the incomplete combustion of organic matter and are therefore found in a wide range of matrices [1]. These species have generated considerable interest, due to their reported carcinogenic and mutagenic capabilities, making them a concern for human health [1,2].

Dietary intake is the main way of exposure to PAHs, since their high molecular weight and lipophilic character facilitate their bioaccumulation in human body, after the ingestion of polluted food [3]. PAH pollution in food is related to their production and processing environment [1,4]. The concern about exposure to these compounds has led the European Union [5] and the US Environmental Protection Agency to regulate their content, and sixteen PAHs are currently listed as priority pollutants.

PAHs have been reported in tea [6–22] and herbal infusions [23–26], and, in light of their worldwide consumption, both are likely to be an important dietary source of these chemicals. Moreover, the frequent intake of tea has been associated with the development of certain kinds of cancer [27]. Tea may become polluted by through direct absorption by the leaves during plant development due to the chemical treatment of crops or as a result of the deposition of PAHs bound to particulate matter in air during their post-harvest treatment, or when, for drying purposes, some tea varieties are exposed to smoke [14]. The transfer of PAHs from tea leaves to infusion is related with other in-house elaboration variables, like brewing time or the tea/water ratio [16]. Given the popularity of these hot drinks, two of the most widely consumed beverages worldwide, there is an obvious need for techniques to measure PAHs in infusions.

The most commonly used analytical methods for this purpose in tea and related drinks are based on liquid chromatography (LC) with fluorimetric (FL) [11,12,16,21,26] and ultraviolet (UV) [6,7,14,15,17,23] detection, and gas chromatography-mass spectrometry (GC-MS) [8–10,18–20,24,25]. However, the low concentrations present in these samples means that it is necessary to include a preconcentration step in the analytical procedure. In this respect, classic preconcentration methods, like solid phase extraction (SPE) [9,22] or liquid-liquid extraction (LLE) [7], have been replaced by new sample preparation microextraction techniques, which are simpler, cleaner and quicker and involve low solvent consumption. Some microextraction procedures, including liquid-liquid microextraction (LLME) [6,23], solid-phase microextraction (SPME) [11,12,17,21,25] and stir bar sorptive extraction (SBSE) [26], have been successfully applied for PAH quantification.

Headspace sorptive extraction (HSSE) is an SBSE-derived microextraction technique [28,29], in which the polydimethylsiloxane (PDMS) coated stir bar is exposed to the headspace sample vial, trapping the analytes into its extracting phase coating. The retained compounds are later

thermodesorbed in a specific injector, composed of a thermal desorption unit (TDU) and a programmed temperature vaporizing (PTV) injector, and submitted to GC separation. Due to the large amount of the PDMS extracting phase compared with that commonly used in SPME, HSSE provides higher recoveries and sensitivity [30], and the robustness of the stir bar assembly facilitates its application. In spite of the advantages of HSSE, as far as we know, no procedure based on this technique has been reported for PAH determination in tea or other infusions. This study presents a procedure for the determination of ten volatile PAHs, included in the sixteen PAHs considered as priority pollutants [5], in different infusion samples using HSSE followed by GC separation and MS detection.

2. Materials and methods

2.1. Reagents

A standard mixture containing ten PAHs (naphthalene (NAP), acenaphthylene (ACY), acenaphthene (ACE), fluorene (FLE), phenanthrene (PHN), anthracene (ANT), fluoranthene (FLA), pyrene (PYR), benz[a]anthracene (BAA) and chrysene (CHR)) in methylene chloride:methanol (1:1) at concentrations ranging from 100 to 2000 μg mL^{-1} and a solution of phenanthrene-d^{10} (2000 μg mL^{-1} in methanol) were supplied by Supelco (Bellefonte, PA, USA). Stock solutions of these compounds were prepared by dilution with HPLC grade acetone (Lab-Scan, Dublin, Ireland) and kept at 4 ºC in dark bottles sealed with PTFE/silicone caps. Working standard solutions were prepared daily by dilution with water. Sodium chloride was obtained from Sigma (St. Louis, MO, USA) and helium (99.9999% purity) from Air Liquide (Madrid, Spain).

Table 1
Experimental conditions of the TD-GC-MS procedure.

Thermal Desorption Unit	
Mode	Splitless
Temperature programme	50 – 275 ºC at 225 ºC min^{-1}, held 5.3 min
Desorption flow	39 mL min^{-1}
Cooled Injector System	
Mode	Solvent Venting
Liner	Packed silanized glass wool, 2 mm i.d.
Temperature programme	15 – 300 ºC (5 min) at 650 ºC min^{-1}
GC-MS	
Capillary column	HP-5MS, 5% diphenyl-95% dimethylpolysiloxane (30 m x 0.25 mm, 0.25 μm)
Carrier gas	Helium (1 mL min^{-1})
Oven programme	75 (held 2 min) – 150 ºC at 25 ºC min^{-1} 150 – 175 ºC at 3.3 ºC min^{-1}, held 3 min 175 – 200 ºC at 5 ºC min^{-1}, held 2 min 200 – 245 ºC at 7.5 ºC min^{-1}, held 5 min.
Transfer line temperature	300 ºC
Quadrupole temperature	150 ºC
Ion source temperature	230 ºC
Ionization	Electron-impact mode (70 eV)

2.2. Instrumentation

Commercial stir bars coated with a 0.5 mm layer thickness of PDMS (24 μL) were obtained from Gerstel (Mullheim an der Ruhr, Germany). Prior to use the stir bars were conditioned in an empty thermal desorption tube at 275 ºC for 0.5 h with helium at a flow rate of 50 mL min^{-1}. The temperature during the extraction step was controlled using a laboratory-made heating system, consisting of a drilled block provided with an electronic temperature control system, constructed in the Central Laboratory Service of the University of Murcia. An RH-KT/C magnetic stirrer (IKA, Staufen, Germany) was used for stirring the sample solutions.

The sample introduction system, provided by Gerstel, was composed of a Thermal Desorption Unit (TDU-2) equipped with an autosampler (MPS-2) and a Programmed Temperature Vaporization (PTV) Cooled Injector System (CIS-4). Experimental conditions used for this system are summarized in Table 1. GC analyses were performed on an Agilent 6890N (Agilent, Waldbronn, Germany) gas chromatograph coupled to an Agilent 5973 quadrupole mass selective spectrometer equipped with an inert ion source.

In the selected conditions (Table 1), the analytes eluted with retention times (Table 2) between 5.8 and 31.7 min, corresponding to NAP and CHR, respectively. The compounds were quantified in the selected ion monitoring (SIM) mode in order to improve the detection limits using different ions (Table 2). The identification of the compounds was confirmed by injection of pure standards and comparison of the retention times and full MS-spectra.

2.3. Samples and analytical procedure

Samples of the different beverages, including different blends of green, red and black tea, pennyroyal, lime flower, chamomile, lemon balm and blends of different plants (1: green tea and mate, 2: dandelion, boldo and mint, 3: fennel, sage, eucalyptus and thyme), were obtained from local supermarkets. In order to simulate as closely as possible the conditions in which these infusions are usually prepared, the content of a bag (about 1.5 g of dried leaves) was poured into 50 mL of boiling water, filtered after 5 min and cooled to room temperature. A seaweed sample (Fucus sp. IAEA-140/OC) supplied by the International Atomic Energy Agency (Austria), which was employed as a reference material, was also submitted to the previously described infusion preparation procedure. Spiked samples were prepared using two herbal samples, pennyroyal and green tea, being the corresponding infusions prepared by applying the above described procedure. The analytes were added to the infusions at two spiking levels, in the 0.5 – 22 μg L^{-1} concentration range, close to the quantification limits. Samples were vigorously shaken to homogenize the mixture and then submitted to the HSSE extraction procedure. Three replicates were analyzed at each fortification level.

Table 2
PAHs identification parameters.

Compound	Retention time (min)	Monitored ions (*m/z*)
Naphthalene (NAP)	5.8	128, 127 (10)
Acenaphthylene (ACY)	8.8	152, 151 (13)
Acenaphthene (ACE)	9.5	154, 153 (72)
Fluorene (FLE)	10.8	166, 165 (83)
Phenanthrene (PHN)	15.1	178, 176 (8)
IS	15.1	188, 186 (8)
Anthracene (ANT)	15.6	178, 176 (8)
Fluoranthene (FLA)	22.3	202, 101 (9)
Pyrene (PYR)	23.5	202, 101 (10)
Benz(a)anthracene (BAA)	31.2	228, 114 (8)
Chrysene (CHR)	31.7	228, 114 (9)

Underlined numbers correspond to *m/z* of the target ion, and values in brackets represent the qualifier-to-target ion ratios in percentage.

Ten millilitres of the liquid infusion were placed in a 15 mL glass vial with 1.5 g of NaCl. Isotopically labelled phenanthrene-d^{10} was added to the samples as internal standard, at a concentration of 1 μg L^{-1}. HSSE extraction was carried out by exposing the PDMS-coated stir bar to the headspace vial, while the sample was stirred at 900 rpm and heated at 88 ºC, for 4 hours. After analyte extraction to the polymeric phase, the stir bars were rinsed with Milli-Q water, dried with a lint-free tissue and placed in a desorption tube for analysis.

3. Results and discussion

HSSE preconcentration involves two sequential stages: extraction of the analytes from the sample headspace to the stir bar coating and thermal desorption for injection into the GC-MS system. Due to the high number of variables involved in these processes, their effect and significance were first tested using a factorial multivariate design (Plackett-Burman). This design acts as a screening test, which identifies less relevant variables and selects the most suitable conditions among those assayed. Once the most relevant parameters had been identified, they were considered in more detail using a Central Composite Design (CCD) whose results were fitted to a response surface by quadratic polynomial regression, providing linear and quadratic terms as well as first order interactions.

In order to weight the relevance of each variable for the different analytes in the optimization process, regardless of their different sensitivities, peak area values of each one were normalized with respect to their average area in the corresponding experimental set. The Minitab 15.0 statistical package was used to generate the experimental matrices and to evaluate the results obtained.

3.1. HSSE parameters

The extraction of the analytes was studied using 10 mL of an aqueous standard solution containing the analytes in 1-20 ng mL^{-1} concentration range, depending on the compound, and 2 h as extraction time. The variables studied were: the addition of an inert salt (NaCl: 0, 5 and 10% (m/v)), the addition of an organic modifier (methanol: 0, 5 and 10% (v/v)), extraction temperature (60, 75 and 90 ºC) and the pH of the extraction medium, which was adjusted by adding 1 mL of a buffer solution (0.2 M acetic acid/acetate buffer pH=4.8; 0.2 M dihydrogen phosphate/hydrogen phosphate pH=7.2). These four variables were simultaneously tested following a two level fractional factorial design (n=18, in duplicate, including central points).

The addition of a buffer solution did not seem to be a relevant factor for HSSE extraction, since no statistically significant differences were observed between the pH values assayed (Fig. 1A), so its use was discarded. In the absence of buffer, the pH was in the 4.4 – 5.1 range for all the infusions studied.

In order to increase the efficiency of the extraction procedure, avoiding the adsorption of non-polar compounds to the inner walls of the sample vials, methanol is often added to aqueous samples as an organic modifier. As Fig. 1A shows, the addition of methanol was a relevant factor in the analytes response, but had a negative effect, reducing the HSSE extraction efficiency. The presence of the organic solvent in the extraction medium increases PAH solubility and reduces their partition coefficient with PDMS. Taking into account these results, its use was discarded.

According to Fig. 1A, extraction temperature and salt concentration were also relevant factors in the extraction step, both affecting positively the sensitivity of all the PAHs. These two variables were studied in more detail using a CCD (α=1.5, 4 cube points, 4 axial points and 2 central points, in duplicate), developed in the range 65 – 95 ºC and 7.5 – 22.5% (m/v). The obtained response surface for each analyte fitted experimental data ($0.92<r^2<0.96$) and underlined the significance of the tested variables ($p<0.01$).

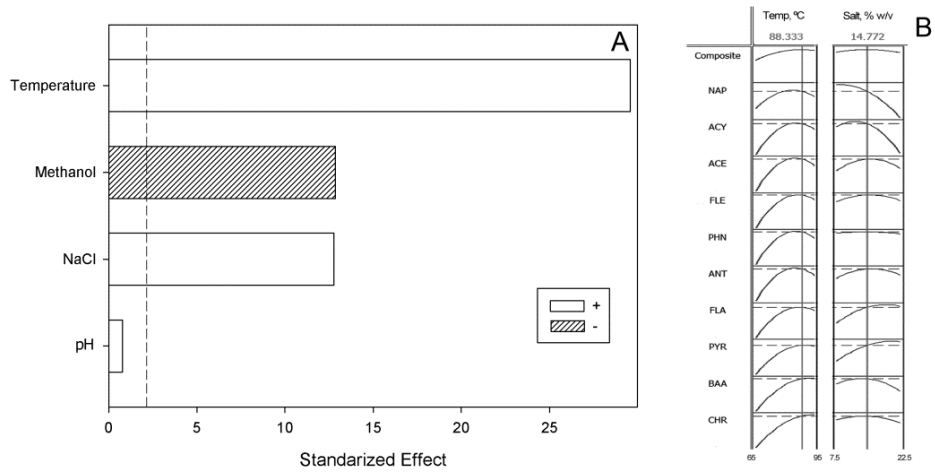

Fig. 1. (A) Pareto charts obtained of the Plackett–Burman designs for the HSSE extraction step and (B) response plots showing the effects of extraction temperature and the amount of NaCl on the relative response of each analyte.

HSSE sampling temperature was the most influential parameter. High extraction temperatures favoured vaporization of the analytes, shifting the equilibrium of these species to the headspace phase, from where they were extracted to the HSSE coating.

However, high temperatures may lead to the thermal release of trapped species from the PDMS back to the headspace. The obtained responses reflected this behaviour, showing a maximum response for the optimum temperature. Fig. 1B, representing the effect of each studied factor (columns) on the composite desirability and analytes responses (rows), shows that the optimum temperature for each analyte is related with its molecular weight and boiling point, so lighter compounds reach their maximum temperature at lower values than heavier compounds. Although CHR does not reach its maximum extraction temperature in the studied range, higher temperatures were not considered, due to the limits imposed by the boiling point of water. The evaluation of the obtained quadratic regressions shows that the optimum extraction temperature was 88.3 ºC, so the closest integer value, 88 ºC, was chosen as extraction temperature.

The addition of salt increases the ionic strength of the medium, generally facilitating the extraction of non-polar species to the polymeric coating because of the decrease in water solubility. However, high salt concentrations may decrease the extraction efficiency, although the mechanism involved is not clear [31]. As Fig. 1B shows, the average maximum response was attained with a 14.8% (m/v) salt concentration, so the closest integer value, 15% (m/v) was selected.

Once other extraction parameters had been optimized, extraction time, which is the most relevant parameter affecting HSSE, was investigated from 0.5 to 20 h. Extraction time profiles (Fig. 2) show that equilibrium was reached for all compounds at around 4 h, so this time was chosen to ensure high extraction efficiencies.

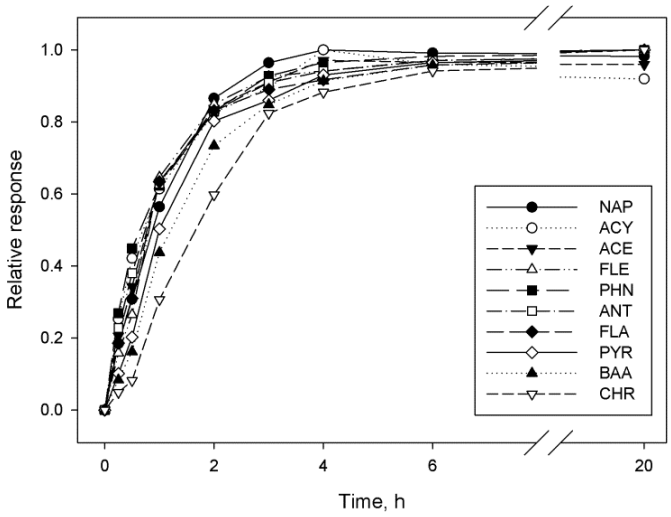

Fig. 2. Extraction time profiles.

3.2. Thermal desorption conditions

In order to optimize the thermal desorption of the HSSE-trapped analytes, a screening test following a two level fractional factorial design (n=18, in duplicate, including central points) was performed. The variables and ranges considered in this initial study were: TDU desorption time (5, 7.5 and 10 min), TDU desorption temperature (225, 250 and 275 ºC), inert gas flow rate (50, 75 and 100 mL min^{-1}), inert gas pressure (6, 8 and 10 psi), PTV focusing temperature (15, 20 and 25 ºC) and PTV heating temperature (250, 275 and 300 ºC). Based on the results of the screening (Fig. 3A), the statistically relevant parameters for the thermal desorption process were PTV focusing temperature, inert gas flow rate, TDU desorption time and CIS heating temperature.

Taking into account their relevance, inert gas flow rate and TDU desorption time, were carefully studied using a CCD (α=1.5, 4 cube points, 4 axial points and 2 central points, by duplicate), in the 35 – 65 mL min^{-1} and 4.375 – 8.125 min ranges, respectively. The obtained response surface, whose optimization plots are summarized in Fig. 3B, showed its adequacy to experimental results (r^2>0.93) and the relevance of the assayed variables (p<0.01). The average maximum responses were 38.94 mL min^{-1} and 5.32 min.

TDU desorption temperature was not a statistically significant variable, so a temperature of 275 ºC was selected. Consequently, the TDU was heated from 50 to 275 ºC (225 ºC min^{-1}). In accordance with the CCD results, this TDU temperature was maintained for 5.3 min, ensuring the total desorption of the analytes and avoiding the possible carry-over caused by incomplete desorption at lower times, or analyte losses due to excessively long desorption times.

A carrier gas is necessary to propel the analytes towards the PTV injector while they are being thermally desorbed in the TDU. As shown in Fig. 3B, the efficiency of the desorption process was maximum when a gas flow rate of 39 mL min^{-1} was employed, so this value was chosen. The pressure of this gas flow did not seem to be a significant factor in the desorption step, so a value of 9 psi, corresponding to head column pressure at the start of the heating programme was selected, minimizing any delay related with pressure acquisition requirements.

Before entering the chromatographic column the desorbed compounds were focused in the PTV, which was equipped with a packed silanized glass wool liner. Especially for the more volatile compounds, the lower the temperature in this device, the lower the losses and the greater the retention efficiency, as Fig. 3A shows. Since the Peltier unit only allows cooling to slightly below room temperature, the lowest achievable temperature, 15 ºC, was selected. As regards PTV temperature, Fig. 3A shows this parameter had a significant effect for the analytes in the studied range, so the highest suitable value for the liner used (300 ºC) was selected. Therefore, a PTV programme temperature increasing from 15 to 300 °C (10.8 °C s^{-1}), with a hold time of 5 min, was applied.

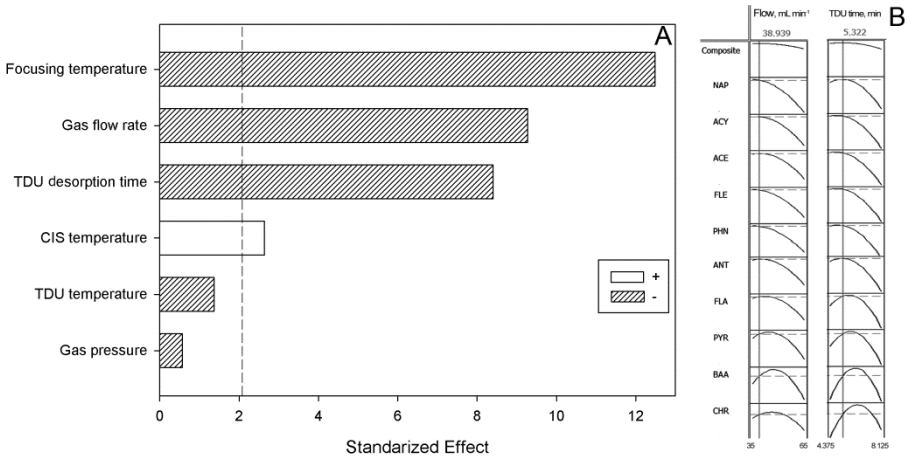

Fig. 3. (A) Pareto charts obtained of the Plackett–Burman designs for the HSSE desorption step and (B) response plots showing the effects of desorption gas flow rate and TDU desorption time on the relative response of each analyte.

3.3. Analytical characteristics of the method

A deuterated PAH (phenanthrene-d^{10}) was employed as internal standard (IS) for quantification purposes. This compound, which is analogous to those studied, was used to compensate any losses during the analytical procedure or any possible matrix effect, allowing the quantification of trace amounts of PAHs with high precision.

Calibration graphs for PAHs in two different samples (green tea and pennyroyal infusions) were obtained by standard addition procedure using the optimized experimental conditions for HSSE extraction followed by TDU-GC-MS by least-squares linear regression analysis of the analyte peak area related to the IS peak area versus analyte concentration using six levels in duplicate experiments. Calibration graphs assessed the linearity of the studied PAHs responses in the evaluated range: 0.2 – 4.5 to 5 – 90 μg L^{-1}, depending on the compound, and provided regression coefficients higher than 0.99 in all cases.

The response of the analytes can be affected by the presence of substances co-extracted from the sample, so the possibility of a matrix effect was tested by comparison of the obtained slopes for aqueous calibration and standard additions to the samples. A statistical study was carried out using a t-test, and the presence of a matrix effect was discarded since "p" values (in the 0.15 – 0.89 range) showed no significant differences. Consequently, calibration and analysis of the samples was carried out against aqueous standards.

The repeatability of the method was calculated using the average relative standard deviation (RSD) of ten replicate analyses of a tea infusion sample spiked with the analytes at concentrations ranging from 0.5 to 11 μg L^{-1}, depending on the compound. RSD values ranged from 9 to 13.2% (Table 3) when only the analyte response was evaluated, and were in the 3.5 – 7% (Table 3) range when the responses were related to the IS signal, indicating that the precision of the method was satisfactory.

Table 3
Method characteristics.

Compound	RSD[a] (%)	RSD[b] (%)	LOD[c] (ng mL^{-1})	LOD[d] (ng mL^{-1})
Naphthalene (NAP)	9.0	5.7	0.101	0.026
Acenaphthylene (ACY)	12.3	7.0	0.181	0.023
Acenaphthene (ACE)	10.3	4.9	0.114	0.019
Fluorene (FLE)	13.2	3.5	0.036	0.014
Phenanthrene (PHN)	9.2	4.8	0.023	0.011
Anthracene (ANT)	9.2	4.7	0.023	0.011
Fluoranthene (FLA)	12.3	5.8	0.049	0.023
Pyrene (PYR)	10.5	6.2	0.026	0.015
Benz(a)anthracene (BAA)	11.4	5.7	0.029	0.021
Chrysene (CHR)	11.9	6.3	0.030	0.022

[a] Without IS n=10. [b] With IS n=10. [c] Calculated for σ_{oo}=3. [d] Calculated for S/N=3.

The sensitivity of the method was evaluated using detection limits, which were calculated taking into account two different criteria. When they were calculated on the basis of three times the standard deviation of the intercept of the aqueous calibration graphs, values ranged from 23 ng L^{-1}, corresponding to PHN and ANT, to 181 ng L^{-1}, for ACY. If the limits were calculated taking into account a signal to noise ratio of three, values ranged from 11 ng L^{-1}, for PHN and ANT, to 26 ng $L^{-1,}$ corresponding to NAP (Table 3). In spite of the differences in the vapour pressure for the studied compounds, their sensitivity are similar, since HSSE extraction efficiency, and thus sensitivity, is related with analytes partition coefficients among water and PDMS extracting phase.

3.4. Analysis of real samples

The optimized procedure was applied to 15 different infusion samples, including different blends of green, red and black tea, pennyroyal, lime flower, chamomile tea, dandelion and lemon balm, prepared according to procedure described in section 2.3.

Some of the studied PAHs were found in 8 samples, at variable concentrations, ranging from 0.18 to 11 µg L^{-1} (Table 4). The highest level of PAHs was found in a camomile sample, with a total PAH concentration of about 30 µg L^{-1}. The most widespread PAHs in the analyzed infusions were ACY and ACE, which are three-ring PAH with less toxic character than those containing higher number of condensed rings.

Fig. 4 shows the chromatograms obtained for a spiked pennyroyal infusion and a non-spiked lime flower infusion sample. Similar chromatograms were obtained for the other samples, showing the absence of interfering peaks. Compounds were identified comparing their retention time, target ions and ion ratios with those obtained by injection of the PAH standards.

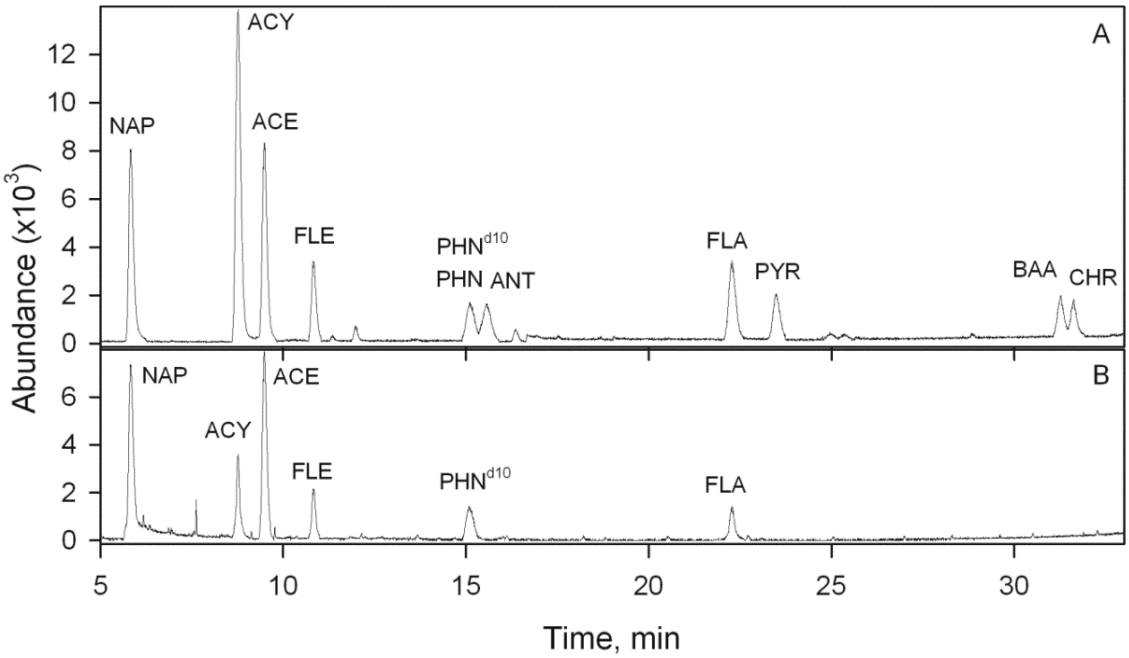

Fig. 4. Elution profiles obtained for (A) a pennyroyal infusion sample spiked in the 0.5-11 µg L^{-1} range, depending on the compound, and (B) non-spiked lime flower infusion sample using the HSSE-TD-GC–MS procedure under selected ion monitoring (SIM) mode.

In order to check the trueness of the proposed method, recovery studies were carried out using two different infusion samples, pennyroyal and green tea, spiked at two concentration levels. For the lowest concentration, ranging from 0.5 to 11 µg L^{-1}, depending on the compound, recoveries were between 87.5 and 108% for pennyroyal and between 88.4 and 104.8% for green tea.

For the highest spiked concentration level, which ranged from 1 to 22 µg L^{-1}, depending on the compound, recoveries were between 99.2 and 101.3% for pennyroyal and between 96.7 – 101.7% for green tea.

Table 4
Results[a] obtained in the analysis of the infusion samples (µg L^{-1}).

	Black tea	Green tea	Lime flower	Chamomile	Red tea	Blend 1	Blend 2	Blend 3
NAP	ND	ND	4.8±0.3	5.1±0.4	11±1	ND	1.2±0.1	5.6±0.6
ACY	3.0±0.3	2.5±0.3	2.7±0.1	4.1±0.5	3.7±0.4	4.9±0.1	2.9±0.2	2.6±0.2
ACE	1.83±0.05	1.5±0.1	5.2±0.1	6.6±0.2	2.1±0.2	3.5±0.2	1.5±0.2	1.7±0.2
FLE	0.73±0.01	0.77±0.03	0.68±0.04	5.1±0.3	1.5±0.1	0.86±0.05	0.76±0.05	0.84±0.05
PHN	0.39±0.01	ND	ND	1.8±0.2	ND	0.82±0.02	ND	0.44±0.01
ANT	0.28±0.01	0.34±0.03	ND	4.0±0.1	ND	0.32±0.02	0.32±0.02	0.26±0.01
FLA	0.59±0.02	0.50±0.02	0.43±0.01	1.6±0.1	1.3±0.2	0.59±0.01	0.23±0.01	0.62±0.05
PYR	0.27±0.02	0.24±0.01	ND	1.4±0.2	0.7±0.1	0.30±0.01	0.49±0.01	0.32±0.04
BAA	ND	ND	ND	ND	ND	ND	0.23±0.01	ND
CHR	ND	ND	ND	ND	ND	ND	0.18±0.01	ND

Mean value ± standard deviation (n=3). ND means not detected.

The reliability of the method was further established by analyzing the certified reference material, seaweed (*Fucus* sp.) (IAEA-140/OC), which was submitted to the same procedure as the infusion samples, and analyzed using the proposed HSSE method. The results obtained for the analysis of this reference material, as well as the recommended values, are summarized in Table 5.

It should be noted that the values provided by the supplier are not certified but merely indicative of the range of concentrations found by different laboratories and referred to the solid samples, while those given here are calculated taking into account the volume of the prepared infusion.

4. Conclusions

The HSSE-TD-GC-MS combination has proven to be a useful tool for the determination of ten volatile PAHs in herbal infusions. MS detection provided unequivocal confirmation of the presence of the studied compounds. The data obtained by recovery studies and the analysis of an infusion prepared with a certified reference material proved the accuracy of the proposed procedure. The reliable control and quantification of these compounds can be carried out using this solvent-free and simple method.

Table 5
Analysis of the IAEA-140/OC certified reference material.

Compound	Concentration[a] found (ng g^{-1}) by HSSE-GC-MS	Concentration range (ng g^{-1}) recommended
NAP	9.5±0.6	9 – 43
ACY	5.8±0.2	3.3 – 7.0
ACE	27.7±0.5	4.6 - 1600
FLE	103±3	40 – 110
PHN	42±2	4 – 93
ANT	53±2	57 – 110
FLA	32±1	46 – 79
PYR	7.0±0.2	14 – 32
BAA	10.5±0.2	25 – 49
CHR	9.5±0.6	9 – 43

[a] Mean value ± standard deviation (n=3).

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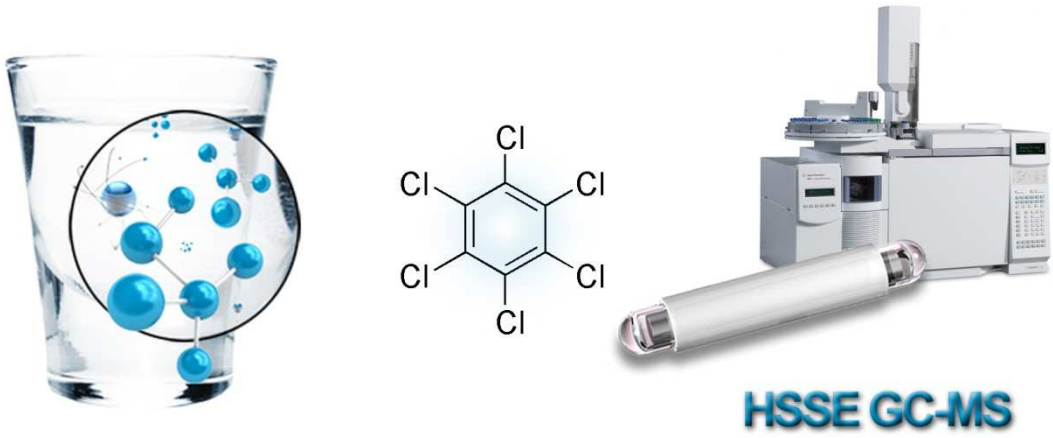
A simple device for headspace sorptive extraction in gas chromatography-mass spectrometry: application to the determination of very low concentrations of chlorobenzenes

Abstract

The adverse effects which chlorobenzenes (CBs) may have on human health point to the need for easy to carry out methods to monitor them, particularly in waters. In this paper, a device for the practice of the headspace sorptive extraction (HSSE) technique combined with gas chromatography-mass spectrometry (GC-MS) is proposed. The device is based on a simple magnetic disk that permits the stir bar to be placed at the top of the sample vial. The complete surface of the coated stir bar is exposed to the headspace, and the movement of the bar caused by the magnetic stirrer facilitates the equilibrium of the extraction process to be reached. The final consequence is an increase in sensitivity. The determination of ten chlorobenzenes in waters is optimized and used as a test bench to check the performance of the device. Under the finally selected extraction conditions (8 mL sample containing 5% (w/v) sodium chloride and 1% (v/v) metanol, and maintained at 90 ºC during 1h while magnetically stirred), the detection limits varied between 0.4 and 1.4 ng L-1, corresponding to 1,2,4-trichlorobenzene and hexachlorobenzene, respectively.



1. Introduction

Headspace sorptive extraction (HSSE) is a stir bar sorptive extraction (SBSE) derived technique [1] in which the bar, usually coated with polydimethylsiloxane (PDMS), is exposed to the headspace in a sample vial, trapping the analytes into the coating extracting phase. This technique was first applied by Bicchi et al. [2,3] under the name of high-capacity HSSE. The exposition of the stir bar to the sample headspace is usually carried out by holding it suspended by means of a stainless steel wire, with one end inserted into the stir bar coating and the other end inserted through a GC injection septum [3-9]. A commercially available special glass insert has also been used for this purpose [10-12]. A simpler device which maintains the stir bar in the headspace by magnetic forces was developed in our lab and has previously demonstrated its effectiveness [13,14]. Such a homemade device is here explained in detail, and compared with the commercialized gadget. Retained compounds are later thermodesorbed in a specific injector, comprised of a thermal desorption unit (TDU) and a programmed temperature vaporizing (PTV) injector, and submitted to GC separation.

The comparison of HSSE with other headspace extraction techniques has shown its higher concentration capacity, but requiring longer extraction times, respect to headspace solid phase microextraction (HS-SPME), which is based on the same principle, and besides, the robustness of the stir bar assembly makes easier its application [4,5,12,15].

In this manuscript, chlorobenzenes (CBs) are used as a test bench to study the behaviour and performance of the above mentioned homemade magnetic device and the possible advantages over the commercial arrangement. CBs, a wide group or organic compounds considered environmental pollutants, are harmful for human health. They are used as industrial solvents in a large variety of processes and, also as intermediates in the production of certain chemicals, like aniline or phenol, and a great number of pesticides. Due to their widespread usage, they are released into the environment in solid and liquid effluents and atmospheric discharges. In addition, some CBs are directly employed as deodorizers, fumigants, degreasers, insecticides, herbicides and defoliants [16]. Hexachlorobenzene (HCB) shows the highest toxicity and represents the greatest danger to the environment, being classified as carcinogenic by the International Agency for Research on Cancer (IARC) [17]. The American Environmental Protection Agency (EPA) has catalogued CBs as persistent, bioaccumulative, and toxic (PBT) pollutants, and severe restrictions have been imposed on their presence in environmental waters [18].

Most commonly used analytical methods for CBs determination in waters are based on gas chromatography with mass spectrometry detector (GC-MS) [16,19-26], but also with flame ionization detector (GC-FID) [27-29], or electron capture detector (GC-ECD) [30-37]. Liquid chromatography-diode array detector (LC-DAD) [38-41] has also been applied for this purpose.

The low concentrations expected for these analytes in water samples make necessary the inclusion of a preconcentration step in the analytical procedure. Classic sample preparation methods, such as solid-phase extraction (SPE) [38] or static headspace (SHS) [28,35], have been replaced by new microextraction techniques, which are simpler, cleaner, quicker and with low solvent consumption. Some microextraction procedures, including liquid-liquid microextraction (LLME) [20,23,27,30-33,39-41] or solid-phase microextraction (SPME) [16,21,26,29,34,37] have been successfully applied for CBs sample extraction and clean-up purposes.

In spite of the advantages of HSSE for preconcentration purposes, as far as we know, no procedures based on this technique have been reported for CBs determination in water samples. A headspace sampling method using silicone tubes instead of PDMS coated stir bars has been proposed [22], and HSSE has been used for the study of the PDMS-air partition coefficients of p-dichlorobenzene [42].

Since the interest of determining low CBs concentrations is clear, and HSSE-GC-MS is an appropriate way for the purpose, they were considered as suitable candidates to test the performance of the homemade device and compare it with the commercial glass holder.

2. Materials and methods

2.1. Reagents

1,2-Dichlorobenzene (99%, 1,2-DCB), 1,3-dichlorobenzene (98%, 1,3-DCB), 1,4-dichlorobenzene (≥99%, 1,4-DCB), 1,2,3-trichlorobenzene (99%, 1,2,3-TCB), 1,2,4-trichlorobenzene (≥99%, 1,2,4-TCB), 1,3,5-trichlorobenzene (99%, 1,3,5-TCB), 1,2,3,4-tetrachlorobenzene (98%, 1,2,3,4-TeCB), 1,2,3,5-tetrachlorobenzene (1,2,3,5-TeCB), pentachlorobenzene (98%, PCB) and hexachlorobenzene (HCB) were provided by Sigma (St. Louis, MO, USA). Isotopically labelled 1,4-dichlorobenzene-d^4 was also obtained from the same supplier and used as internal standard (IS). Stock solutions of these compounds were prepared by dilution with HPLC grade acetone (Lab-Scan, Dublin, Ireland) and kept at 4 ºC in dark bottles sealed with PTFE/silicone caps. Working standard solutions were prepared daily by dilution with water. Sodium chloride was obtained from Sigma (St. Louis, MO, USA). Helium (99.9999% purity) was supplied by Air Liquide (Madrid, Spain).

2.2. Instrumentation

Commercial stir bars coated with a 0.5 mm layer thickness of PDMS and 20 mm in length (48 μL) were obtained from Gerstel (Mullheim an der Ruhr, Germany). Prior to use the stir bars were conditioned in an empty thermal desorption tube at 275 ºC for 0.5 h with helium at a flow rate of 50 mL min^{-1}. A laboratory-made heating system, constructed in the Central Laboratory Service of

the University of Murcia and consisting of a drilled block provided with an electronic temperature control system, was used to control the temperature during the extraction step. An RH-KT/C magnetic stirrer (IKA, Staufen, Germany) was used for stirring the sample solutions.

The sample introduction system was composed of a Thermal Desorption Unit (TDU-2) equipped with an autosampler (MPS-2) and a Programmed Temperature Vaporization (PTV) Cooled Injector System (CIS-4) provided by Gerstel. The experimental conditions used for the sample introduction system are summarized in Table 1. GC analyses were performed on an Agilent 6890N (Agilent, Waldbronn, Germany) gas chromatograph coupled to an Agilent 5973 quadrupole mass selective spectrometer equipped with an inert ion source. In the selected conditions (Table 1) analytes eluted with retention times (Table 2) of between 5.2 and 9.1 min, corresponding to 1,3-DCB and HCB, respectively. The compounds were quantified in the selected ion monitoring (SIM) mode in order to improve the detection limits using different ions (Table 2). Identification of the compounds was confirmed by injection of pure standards, samples and fortified samples and comparing the retention times and full MS-spectra.

2.3. Holder assembly

A scheme of the homemade holder assembly evaluated is shown in Fig. 1. As can be seen in the graph, the arrangement of the magnetic stir bar allows it to be maintained totally exposed to sample headspace. A video showing the movement of the bar during the extraction stage is available online (https://dl.dropboxusercontent.com/u/44515633/hsse_holder.mp4).

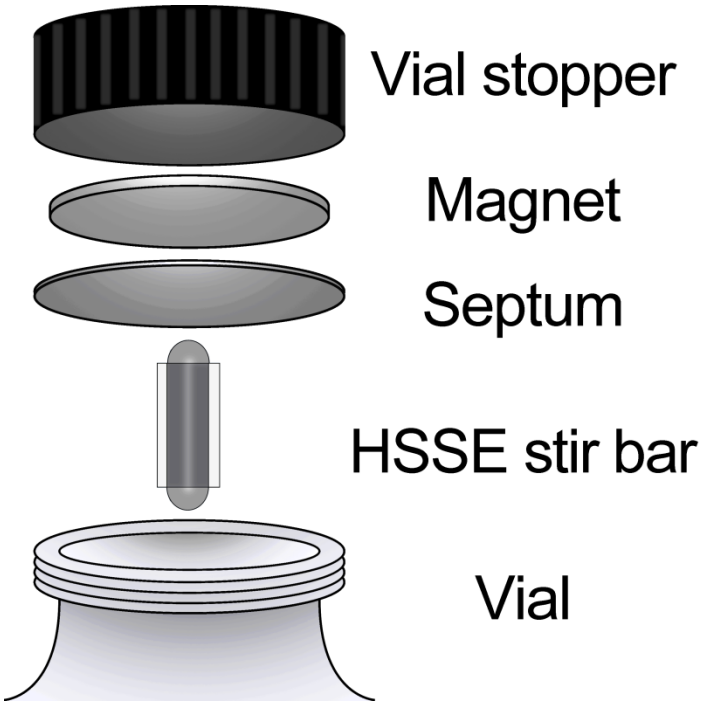


Fig.1 Schematic diagram of the HSSE homemade holder

Table 1
Experimental conditions of the TD-GC-MS procedure.

Thermal Desorption Unit	
Mode	Splitless
Temperature programme	40 – 245 ºC at 205 ºC min^{-1}, held 2 min
Desorption flow	45 mL min^{-1}
Cooled Injector System	
Mode	Solvent Venting
Liner	Tenax, 2 mm i.d.
Temperature programme	22 – 280 ºC (5 min) at 650 ºC min^{-1}
GC-MS	
Capillary column	HP-5MS, 5% diphenyl-95% dimethylpolysiloxane (30 m x 0.25 mm, 0.25 μm)
Carrier gas	Helium (1 mL min^{-1})
Oven programme	40 ºC, held 1 min 40 – 130 ºC at 20 ºC min^{-1} 130 – 175 ºC at 30 ºC min^{-1} 175 – 255 ºC at 40 ºC min^{-1}, held 1 min
Transfer line temperature	300 ºC
Quadrupole temperature	150 ºC
Ion source temperature	230 ºC
Ionization	Electron-impact mode (70 eV)

2.4. Samples and analytical procedure

Different water samples, including bottled water, tap water and water from ornamental and drinking fountains were obtained from the south-east of Spain. Samples were kept refrigerated in totally filled sealed opaque glass bottles until their analysis. Eight millilitres of the water sample were placed in a 15 mL glass vial containing 0.4 g of NaCl. Isotopically labelled 1,4-DCB was added to the samples as IS, at a concentration of 40 ng L^{-1}. HSSE extraction was carried out by exposing the PDMS coated stir bar to the headspace vial, while the sample was stirred and heated at 900 rpm and 90 ºC, respectively, for 2 h. After the analyte had been extracted into the polymeric phase, the stir bars were rinsed with Milli-Q water, dried with a lint free tissue and placed in a desorption tube for analysis in the experimental conditions summarized in Table 1. For comparison purposes, the same conditions were used for the experiments involving the commercial device.

3. Results and discussion

HSSE preconcentration involves two different sequential stages: the extraction of the analyte from the sample headspace into the stir bar coating and its thermal desorption for injection into the GC-MS system. The effect of different variables involved in these processes was evaluated, and their value was selected in order to maximize the obtained response. To determine the relevance of the different variables for each analyte, regardless of their different sensitivities, peak area values of each compound were normalized with respect to their average area in the corresponding experimental set.

3.1. HSSE parameters

The extraction of the CBs was studied using an aqueous standard solution containing the analytes at 2 μg L^{-1} placed in a 15 mL glass vial and submitted to extraction for 2 h. The variables studied were: volume of the sample, extraction temperature, and the addition of an inert salt and organic modifier. Once these variables were optimized, the performance of the holder to decrease the time required to extract, when compared with the commercial holder, was studied (Section 3.3).

As a first step, a sample volume of 5 to 10 mL was submitted to extraction. According to HS sampling theory, high sample volumes lead to low HSSE recoveries, but over a higher total amount of CBs. Fig. 2 illustrates how the CB responses increased up to a volume of 8 mL, but decreased for larger values; so, a final sample volume of 8 mL was selected. If volumes higher than 10 mL are used, liquid sample and stir may come into contact. Vials of higher capacity than 15 mL were not considered due to limitations imposed by the HSSE sampling device.

The extraction temperature is one of the most influential parameters in HSSE extraction efficiency. High temperatures favour vaporization of the analytes, shifting the equilibrium of these species to the headspace phase from where they are extracted to the HSSE coating. However, high temperatures may also lead to the thermal release of trapped species from the PDMS back to the headspace, or to interference related with water vapour condensation on the HSSE device. Extraction temperatures ranging from 50 to 90 ºC were tested, and maximum responses were obtained at the highest temperature (Fig. 2), which was selected. The increase in sensitivity with extraction temperature was especially relevant for CBs of high molecular weight and boiling point, such as TeCBs, PCB and HCB, while the responses of DCBs remained practically constant above 70 ºC.

Table 2
Retention times and monitored ions.

Compound	Retention time (min)	Monitored ions (*m/z*)
1,3-Dichlorobenzene (1,3-DCB)	5.2	146, 148 (75), 111 (45), 75 (29)
1,4-Dichlorobenzene-d^4 (IS)	5.4	150, 152 (60), 115 (40)
1,4-Dichlorobenzene (1,4-DCB)	5.4	146, 148 (72), 111 (38), 75 (26)
1,2-Dichlorobenzene (1,2-DCB)	5.6	146, 148 (66), 111 (37), 75 (23)
1,3,5-Trichlorobenzene (1,3,5-TCB)	6.3	182, 180 (99), 145 (26), 109 (18)
1,2,4-Trichlorobenzene (1,2,4-TCB)	6.6	182, 180 (99), 145 (25), 109 (19)
1,2,3-Trichlorobenzene (1,2,3-TCB)	6.9	182, 180 (99), 145 (28), 109 (20)
1,2,4,5-Tetrachlorobenzene(1,2,4,5-TeCB)	7.5	216, 214 (80), 181 (17), 179 (16)
1,2,3,4-Tetrachlorobenzene (1,2,3,4-TeCB)	7.9	216, 214 (76), 181 (19), 179 (18)
Pentachlorobenzene (PCB)	8.4	250, 252 (66), 248 (63), 215 (19)
Hexachlorobenzene (HCB)	9.1	284, 286 (81), 249 (24), 142 (24)

Underlined numbers correspond to *m/z* of the target ion, and values in brackets represent the qualifier-to-target ion ratios as percentage.

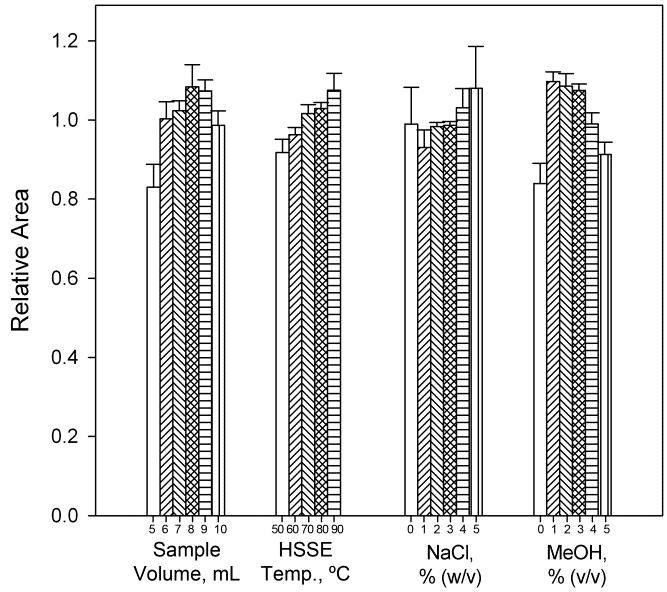


Fig. 2. Effect of different HSSE sampling variables on the average CB responses obtained.

The addition of salt increases the ionic strength of the medium, generally facilitating the extraction of non-polar species to the polymeric coating because of the decrease in their water solubility. The addition of different NaCl amounts, ranging from 0 to 5% (m/v), was tested, and average responses are shown in Fig. 2. While the addition of higher amounts of salt increased the response for the more polar CBs, including DCBs and TCBs, the extraction efficiency decreased for those of lower polarity, such as TeCBs, PCB and HCB, whose response was higher in the absence of salt. Since the maximum average response was attained with a 5% (m/v) salt concentration, this value was selected.

In order to increase the efficiency of the extraction procedure by avoiding the adsorption of non-polar compounds to the inner walls of the glass vial, methanol is often added as organic modifier in the case of aqueous samples. However, the presence of this solvent in the extraction medium may increase CB solubility, reducing their partition coefficient with PDMS. The addition of methanol assayed was in the 0 – 5% (v/v) range, and the obtained responses are summarized in Fig. 1. All the studied compounds showed similar behaviour, providing maximum response with the addition of 1% (v/v) MeOH, which was selected.

3.2. Thermal desorption conditions

In order to optimize the thermal desorption of the trapped analytes, different variables were optimized, including: TDU desorption temperature, TDU desorption time, inert gas flow rate and pressure, PTV liner filling, PTV focusing temperature and PTV heating temperature.

After the extraction step, the HSSE device is heated to desorb the trapped analytes. In most cases, the higher the desorption temperature, the greater the obtained responses. However, high desorption temperatures may lead to thermal degradation of the preconcentrated compounds, as well as a reduction in the focusing of the species in the PTV. Different TDU desorption temperatures, ranging from 200 to the maximum recommended for the PDMS coating (275 ºC), were assayed and the average obtained responses are shown in Fig. 3. The optimal desorption temperature depended on the CB, ranging from 245 ºC for DCBs to 260 ºC for HCB. The maximum average response was obtained at 245 ºC, which was selected.

The TDU temperature is maintained for a period which ensures the total desorption of the analytes, while avoiding any possible carry-over. Excessively long desorption times may lead to analyte losses. Different desorption times, ranging from 0.5 to 7 min were tested (Fig. 3). Most of the studied compounds attained maximum sensitivity with a desorption time of 2 min, except 1,3-DCB and HCB, which showed maximum signals after 1 and 3.5 min, respectively. Taking into account the average response, 2 min was selected as desorption time.

A carrier gas is needed to propel the analytes towards the PTV injector while they are being thermally desorbed in the TDU. High carrier gas flow rates usually facilitate thermal desorption in the TDU, reducing the concentration of the desorbed compounds in the gas phase. However, high flow rates may decrease the focusing efficiency in the PTV, increasing the rate at which they pass through this device and reducing the contact time with the retaining PTV filling material. Different values ranging from 30 to 105 mL min^{-1} were tested (Fig. 3). The gas flow rate was especially relevant in the case of the more volatile compounds, such as DCBs, which showed their maximum sensitivity at 45 mL min^{-1}, a value that was selected.

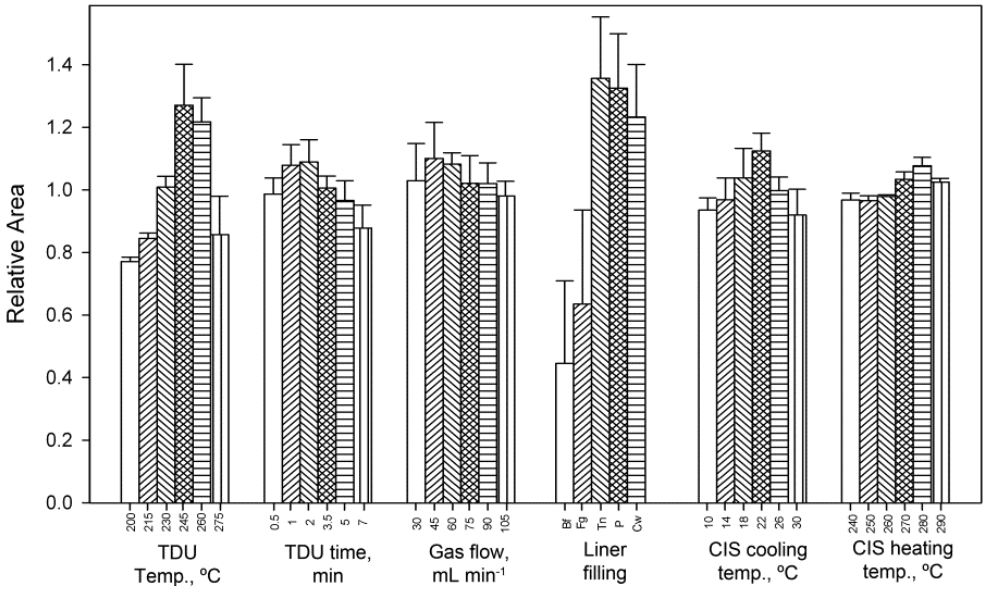


Fig. 3. Effect of different TD variables on the average CB responses obtained.

Before entering the chromatographic column, the compounds are focused in the PTV, which is equipped with a glass liner filled with a retaining material to increase the surface available for CBs focusing. Empty baffled liners (BF) and those filled with fiberglass (FG), or other materials with absorbent or adsorbent properties, such as PDMS, Tenax (Tn) or Carbowax (Cw), were tested. The obtained responses showed that the Tn filling provided the best results (Fig. 3).

Low temperatures applied in the PTV usually prevent losses and lead to greater retention efficiency, especially of the more volatile compounds. A Peltier unit, which only allows cooling to slightly below room temperature, was used for this purpose. Fig. 2 shows the responses obtained for temperatures ranging from 10 to 30 ºC. CB responses were maximum at 22 ºC, which was selected. This relatively high value indicates that Tn is an efficient PTV liner filling material that does not require such low temperatures as other fillings.

After the desorption step, the PTV is heated, allowing to the entrance of the focused compounds into the GC column. Different CIS heating temperatures, in the 240-290 ºC range, were studied (Fig. 3), and the best CB responses were obtained for 280 ºC. So, a heating program from 22 to 280 ºC at 650 ºC min^{-1}, which was held for 5 min, was applied in the PTV after the thermal desorption step.

3.3. Holder assembly

HSSE stir bars are usually exposed to the vial headspace using a commercial glass holder. From our previous experience, such a device has major drawbacks that may reduce the full capabilities of HSSE. The commercial holder only has a small hole in its base, which is the only way in which the sample headspace can come into contact with the coating phase. This hinders the diffusion of compounds, leading to low recoveries and long extraction times. In addition, as result of the condensation of water vapour over the holding device, this slit may become be total or partially occluded, reducing the repeatability of the extraction procedure.

In the light of this, our research group has developed an alternative to the HSSE commercial holder (Fig. 1). This device, based on magnetic holding, ensures total exposure and full contact of the PDMS stir bar coating and the sample headspace, increasing the extraction efficiency. In addition, this allows the continuous movement of the coated stir bar, facilitating the homogenization of the gas phase, reducing the extraction time and avoiding the condensation of water vapour over the HSSE coating, thus improving the repeatability of the extraction step.

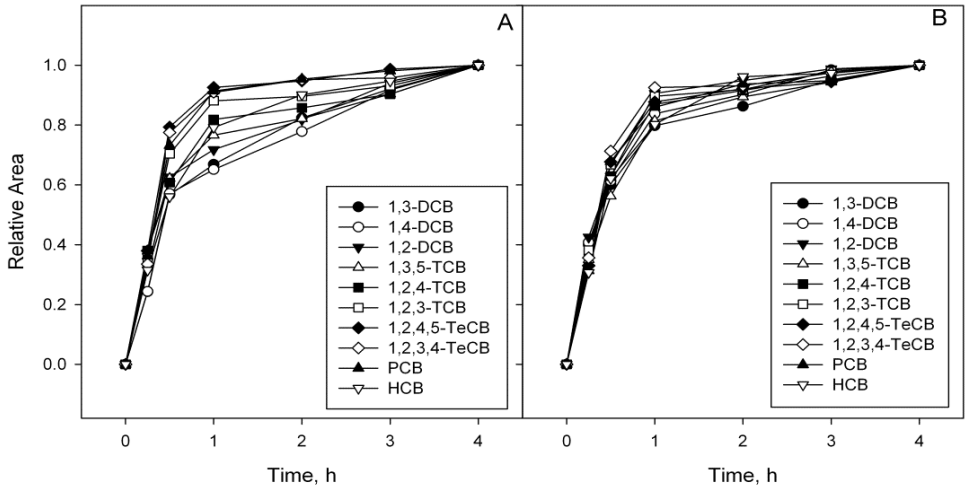


Fig. 4. Extraction profiles obtained using the commercial (A) and the homemade (B) HSSE holder.

Once the main parameters of the HSSE extraction step had been optimized, extraction time, which is the most important parameter affecting HSSE, was investigated using the two holders (Fig. 4). The extraction time profiles show that the effect on the extraction time was not as relevant as expected, but higher extraction efficiencies were reached using the homemade magnetic holder, which was particularly advantageous in the case of heavier compounds with lower diffusion coefficients.

For comparison purposes, the quantification limit (QL) and repeatability were obtained for each analyte using both holders (Table 3). Sensitivity was evaluated according to two criteria. In the case of the slopes obtained using both holders, the magnetic holder provided higher values. The ratio between the slopes obtained with the magnetic/commercial holders ranged from 2.4 for HCB to 5.6 for 1,3-DCB, with an average value of 3.7. In the same way, the LOQs (calculated as the concentration corresponding to a signal-to-noise ratio of 10) were on average 5.4 times higher using the commercial holder, confirming the increase in sensibility possible when the magnetic holder is used. Repeatability was evaluated using the relative standard deviation (RSD) of the replicate analysis (n=10, 80 ng L^{-1}) of a spiked water sample. The lower RSD values obtained using the magnetic holder (2.1 times lower on average) demonstrated its potential for improving repeatability of the HSSE step.

3.4. Analytical characteristics of the method

An isotopically labelled CB (1,3-DCB-d^4) was used as IS for quantification purposes, to compensate for any losses during the whole analytical procedure or any possible matrix effect, allowing the quantification of trace amounts of CBs with high precision.

The standard additions method was applied to three water samples of different origins (bottled, tap and fountain) under the selected conditions. The possible matrix effect was tested by

comparison of the obtained slopes, using an ANOVA statistical test. Slopes of the standard additions calibration graphs were similar to those obtained for external aqueous standards, with "p" values higher than 0.05, confirming the absence of interfering matrix components, and the quantification was carried out against external aqueous standards. The calibration graphs showed good linearity in the studied range (10 to 200 ng L^{-1}, depending on the compound), with correlation coefficients higher than 0.99 in all cases.

The repeatability of the method was calculated using the average RSD of ten replicate analyses of a bottled water sample spiked with the analytes at a concentration of 80 ng L^{-1}. RSD values ranged from 6 to 10% when only the analyte response was evaluated (Table 3), and were in the 3-7% range when the responses were related to the IS signal. Detection limits (LODs) and LOQs were calculated taking into account a signal-to-noise ratio of three and ten, respectively. The resulting values were LODs and LOQs ranged in the 0.4 – 1.4 and 1.4 – 4.7 ng L^{-1}, the lowest and highest values corresponding to 1,2,4-TCB and HCB, respectively (Table 4).

A comparison of the proposed method with other previously published studies for the determination of CBs in water samples using GC-MS is presented in Table 4. The proposed method shows very high sensitivity, which was only improved when CBs were preconcentrated by SPME based on ZnO nanostructures [26].

3.5. Analysis of real samples

The optimized procedure was applied to 15 different water samples, including bottled water, tap water and water obtained from ornamental and drinking fountains. In none of these samples were CBs found to be present, at least above their corresponding LODs.

Table 3
Method characteristic obtained with two different HSSE holders.

	Glass holder		Magnetic holder	
Compound	RSD[a] (%)	LOQ[b] (ng L^{-1})	RSD[a] (%)	LOQ[b] (ng L^{-1})
1,3-DCB	11.4	20.4	7.1	2.9
1,4-DCB	21.0	24.8	7.3	2.7
1,2-DCB	19.2	31.7	8.7	4.1
1,3,5-TCB	8.9	9.4	6.1	2.4
1,2,4-TCB	20.5	10.7	6.3	1.4
1,2,3-TCB	16.8	17.7	7.4	3.4
1,2,4,5-TeCB	12.2	8.5	8.6	2.6
1,2,3,4-TeCB	15.1	8.2	6.5	2.0
PCB	13.5	7.3	6.8	2.1
HCB	11.8	12.3	10.2	4.7

[a] n=10. [b] Calculated for S/N=10.

Table 4
Comparison of the proposed method with other previously developed methods for the determination of CBs in waters by GG-MS.

Technique	Sample volume (mL)	Treatment time (min)	LOD (ng L^{-1})	RSD (%)	Reference
HS-SDME	10	5	3 – 31	2.1 – 13.2	[20]
IL-HS-SDME	10	37	2 – 4	2 – 17	[23]
HS-SPME	10	30	0.01 – 0.1	4.3 – 7.6	[26]
HS-SPME	5	30	3 – 6	1.2 – 8.2	[19]
MEPS[a]	1.75	18	0.3 – 70	2.8 – 12	[24]
HSSE	50	60	2 – 12	5 – 10	[22]
HSSE	8	120	0.4 – 1.4	3 – 7	This work

[a] Microextraction by packed sorbent.

To check the accuracy of the proposed method, recovery studies were carried out. Three different water samples, corresponding to bottled, tap and fountain water, were spiked at two concentration levels (40 and 140 ng L^{-1}), and submitted to the developed method in triplicate. Relative recoveries were determined as the ratio of the concentration found in these water samples compared to the spiked level. The obtained recovery values, summarized in Table 5, confirmed the applicability of the proposed method.

Fig. 5 shows a typical chromatogram obtained for a fountain water sample spiked at 40 ng L^{-1} using the proposed procedure Similar chromatograms were obtained for the other samples, pointing to the absence of interfering peaks at the retention times of the analytes. Compounds were identified comparing their retention time, target ion and ion ratios with those obtained by injection of the CBs standards.

Table 5
Relative recovery[a] percentages of the ten CBs studied in real water samples.

	Bottled		Tap		Fountain	
Spiking level (ng L^{-1})	40	140	40	140	40	140
1,3-DCB	92±4	100±8	86±9	107±7	100±1	101±5
1,4-DCB	103±7	101±5	89±9	96±10	101±2	103±6
1,2-DCB	111±4	99±2	87±	96±4	100±5	101±3
1,3,5-TCB	83±8	102±8	81±8	108±1	94±4	98±5
1,2,4-TCB	112±2	97±1	107±3	97±2	107±3	100±1
1,2,3-TCB	106±3	106±5	106±4	96±6	107±2	93±6
1,2,4,5-TeCB	96±1	108±9	91±9	97±7	107±11	94±2
1,2,3,4-TeCB	90±10	108±6	89±9	103±1	90±6	108±9
PCB	113±11	95±6	89±7	107±8	86±5	106±7
HCB	97±10	98±4	84±7	94±9	85±8	90±9

[a]Mean value ± standard deviation (n=3).

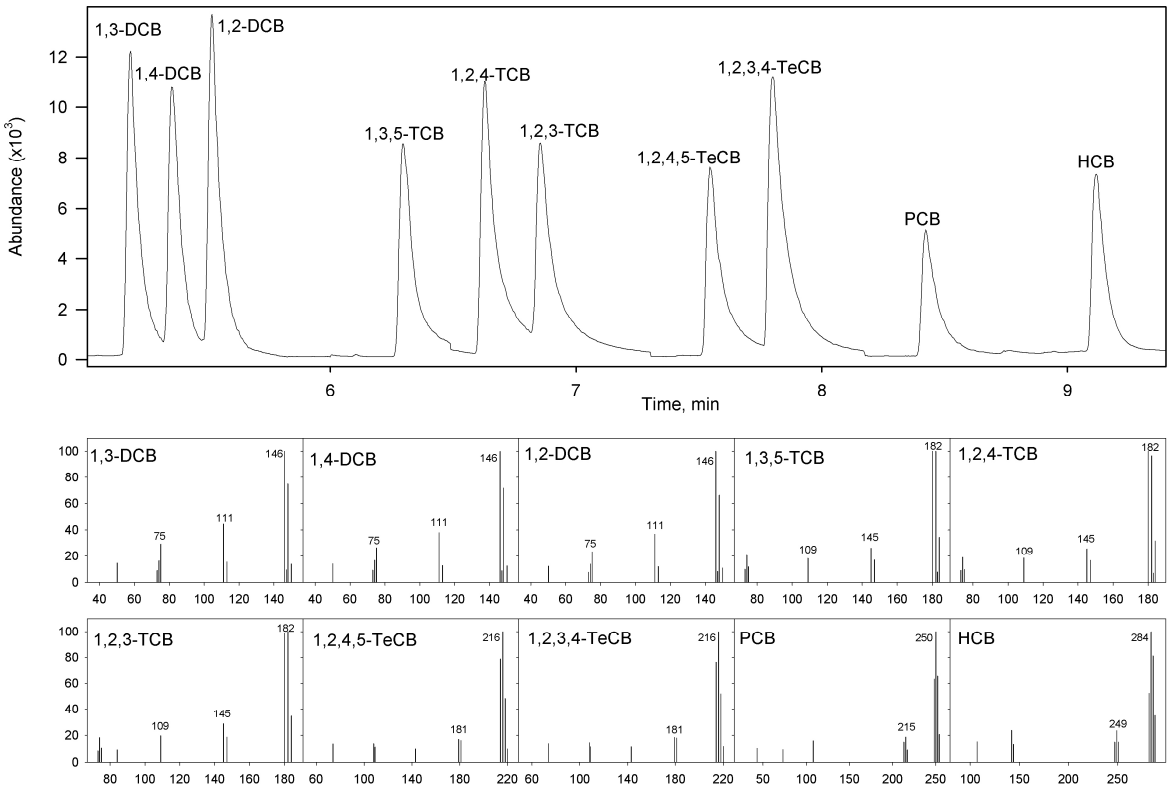


Fig. 5. Chromatogram obtained for fountain water sample spiked at 40 ng L^{-1} for each compound by HSSE-TD-GC-MS under SIM mode, and mass spectra of each CB.

4. Conclusions

The new homemade magnetic holder increased the sensitivity compared with commercial devices, allowing a highly sensitive determination of ten CBs in waters. The holder is simple, easy to arrange, cheap and can be adapted to any common sample vial. This solvent-free microextraction technique, coupled with GC-MS determination by means of thermal desorption, means a highly automatized analytical method with minimal sample handling.

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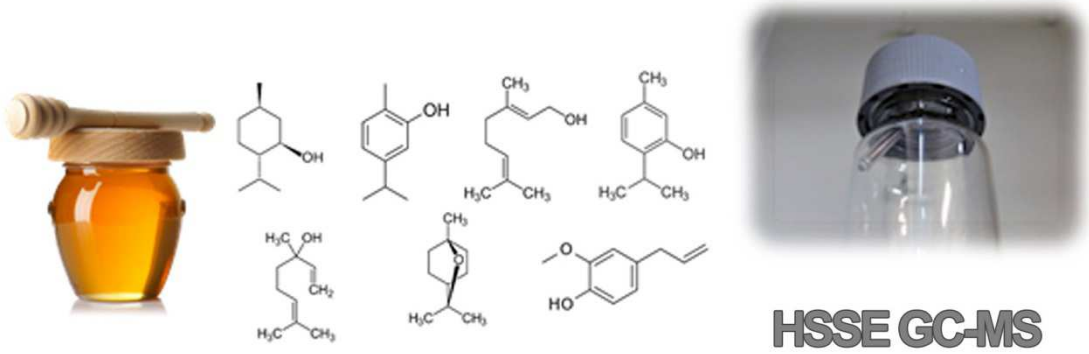
Evaluation of three headspace sorptive extraction coatings for the determination of volatile terpenes in honey using gas chromatography-mass spectrometry

Abstract

Headspace sorptive extraction (HSSE) was used to preconcentrate seven monoterpenes (eucalyptol, linalool, menthol, geraniol, carvacrol, thymol and eugenol) for separation by gas chromatography and mass spectrometry (GC-MS). Three commercially available coatings for the stir bars, namely polydimethylpolisiloxane (PDMS), polyacrilate (PA) and ethylenglycol-Silicone (EG-Silicone), were tested, and the influential parameters both in the adsorption and the thermal desorption steps were optimized. PDMS provided the best sensitivity for linalool, geraniol, menthol and eucalyptol, whereas EG-Silicone was best for extracting the phenolic monoterpenes studied. Considering the average obtained slopes from all compounds, PDMS pointed as the best option, and the analytical characteristics for the HSSE-TD-GC-MS method using this coating were obtained. Quantification of the samples was carried out by matrix-matched calibration using a synthetic honey. Detection limits ranged between 0.007 and 0.032 ng g^{-1}, depending on the compound. Twelve honey samples of different floral origins were analyzed using the HSSE-GC-MS method, the analytes being detected at concentrations up to 64 ng g^{-1}.



1. Introduction

Headspace sorptive extraction (HSSE) is a stir bar sorptive extraction (SBSE) derived technique [1], in which the stir bar coated with a polymeric extracting phase is exposed to the headspace of the sample vial, trapping the volatile analytes. Thermo-desorption of the retained compounds in a specific injector composed of a thermal desorption unit (TDU) and a programmed temperature vaporizing (PTV) injector is the best option when the analytes are to be submitted to gas chromatography (GC) separation. Due to the larger amount of extracting phase than when solid-phase microextraction (SPME) is used, HSSE provides higher recoveries and sensitivities [2]; moreover, the robustness of the stir bar assembly facilitates its application.

Unlike SPME, for which a wide range of extracting phases is available, PDMS has long been the only choice in HSSE. Polar compounds, with octanol-water partition coefficients ($K_{o/w}$) lower than 1,000, usually show poor recovery when PDMS is used as extracting phase, due to the non-polar nature of this polymer [2]. In these cases, a derivatization step is usually needed in order to improve $K_{o/w}$ values and thus extraction efficiency. The importance of some hydrophilic species, such as polar pesticides, alcohols, esters or phenolic compounds, has led to the development of stir bars coated with polar friendly coatings [3].

In this sense, different in-house procedures for stir bar coatings based on sol-gel technology, monolithic materials, new materials such as PDMS/polypyrrole, and more selective materials based on restricted access materials and molecular imprinted polymers have been developed and evaluated [4]. Nevertheless, difficulty in the preparation, a lack of stability, high bleeding rates and the low extraction efficiencies are just some of the disadvantages of these coatings. In addition, these materials are usually degraded at high temperature, and are therefore unstable for thermal desorption, requiring liquid desorption, which takes longer and is less effective. Recently, stir bars coated with polar materials, such as polyacrylate (PA) or ethylene glycol-silicone (EG-Silicone) have become available commercially. EG-Silicone and PA coatings have been applied for the determination of pharmaceuticals [5] and personal care products [6] in waters, volatiles from vegetable matrices [3] and cork taint-related compounds in wine [7]. Considering the obtained results, these coatings might be expected to be useful for the determination of other volatile polar compounds, which is why their application for the HSSE preconcentration of volatile monoterpenes from honey is evaluated herein.

Honey volatile compounds comprise a complex mixture of different chemical families, originated from various biosynthetic pathways, including terpenes, norisoprenoids, and other compounds, that are responsible for characteristics taste, smell and flavour of honey [8]. The volatile composition of honey is determined by origin of the nectar and processing and storage conditions, and is similar to that of many plants natural essential oils. Since flowers of many plant

species contain significant amounts of these compounds, the sucking of nectar by bees leads to their incorporation into honey [9]. Essential oils have been used for thousands of years as pharmaceuticals, and, recently, a great interest has been developed in the potential use of some of their components for the treatment of diseases. The essential oils present in honey have been identified as responsible for some of its biological and health related values [10].

Monoterpenes are isoprene-derived chemicals that are common in essentials oils obtained from plants. These compounds have a volatile nature and strong odour, being widely used in perfumery industry. They form the major volatile fraction in flower organs, where they attract pollinators, acting as both guide and stimulating agents. Monoterpenes include species like linalool (common floral scent in *Freesia* plants) or geraniol (from geranium and roses). In addition, these compounds have other interesting properties, since they act as wound healing [11], analgesic [12], anti-inflammatory [13], antibacterial and antifungal [14] agents. The frequent intake of honey has been claimed to provide benefits for human health as result of its monoterpene content among many other components [8].

Monoterpenes are usually analyzed using GC and mass spectrometry (MS) coupled to different extraction and preconcentration techniques. In this sense, different methodologies have been used for the extraction of the volatile fraction of honey [15], such as dynamic headspace [16,17], purge and trap (P&T) [18–20] and SPME [21–26]. These extraction techniques have demonstrated to be more efficient than classical ones, such as hydrodistillation or simultaneous distillation extraction, which use heat that can affect the composition of the volatile fraction [8].

In the present study, the use of HSSE, through three commercially available extraction coatings, is evaluated for the determination of seven volatile monoterpenes, namely eucalyptol (EUC), linalool (LIN), menthol (MEN), geraniol (GER), carvacrol (CAR), thymol (THY) and eugenol (EUG), in honey using GC-MS.

2. Materials and methods

2.1. Reagents

Eucalyptol (EUC), linalool (LIN), menthol (MEN), geraniol (GER), carvacrol (CAR), thymol (THY), eugenol (EUG) and 3-methyl-4-chlorophenol (3M4CP) were supplied by Sigma (St. Louis, MO, USA). Stock solutions of these compounds were prepared by dilution with pure acetone (Lab-Scan, Dublin, Ireland) and kept at 4 ºC in dark bottles sealed with PTFE/silicone caps. Working standard solutions were prepared daily by dilution with water. Sodium chloride was obtained from Sigma. Helium was supplied by Air Liquide (Madrid, Spain).

2.2. Instrumentation

Commercial stir bars coated with a 0.5 mm layer thickness of PDMS (24 μL), EG-Silicone (32 μL) and PA (25 μL) were obtained from Gerstel (Mullheim an der Ruhr, Germany). Stir bars were conditioned prior to use according to the instructions of the supplier. In order to control the temperature during the extraction step, a laboratory-made heating system, constructed in the Central Laboratory Service of the University of Murcia and consisting of a drilled block provided with an electronic temperature control system, was used. An RH-KT/C magnetic stirrer (IKA, Staufen, Germany) was used for stirring the sample solutions.

The sample introduction system was composed of a thermal desorption unit (TDU-2) equipped with a multipurpose autosampler (MPS-2) and a programmed temperature vaporization (PTV) cooled injector system (CIS-4), from Gerstel. The optimized experimental conditions used for the sample introduction system are summarized in Table 1. GC analyses were performed on an Agilent 6890N (Agilent, Waldbronn, Germany) gas chromatograph coupled to an Agilent 5973 quadrupole mass selective spectrometer equipped with an inert ion source. Under the selected conditions (Table 1), the analytes eluted with retention times (Table 2) between 5.3 and 10.2 min, corresponding to EUC and EUG, respectively. The compounds were quantified in the selected ion monitoring (SIM) mode in order to improve the detection limits using different ions (Table 2). The identification of the compounds was confirmed by injection of pure standards and comparison of the retention times and full MS-spectra.

Table 1
Experimental conditions of the TD-GC-MS procedure.

Thermal Desorption Unit	
Mode	Splitless
Temperature programme	75 – 250 ºC at 350 ºC min^{-1}, held 7.5 min
Desorption flow	50 mL min^{-1}
Cooled Injector System	
Mode	Solvent venting
Liner	Packed silanized glass wool, 2 mm i.d.
Temperature programme	15 – 275 ºC (5 min) at 650 ºC min^{-1}
GC-MS	
Capillary column	HP-5MS, 5% diphenyl-95% dimethylpolysiloxane (30 m x 0.25 mm, 0.25 μm)
Carrier gas	Helium, 1 mL min^{-1}
Oven programme	60º C, held 0.5 min 60 – 115 ºC at 10 ºC min^{-1} 115 – 155 ºC at 20 ºC min^{-1}, held 1.5 min 155 – 235 ºC at 40 ºC min^{-1}, held 0.5
Transfer line temperature	300 ºC
Quadrupole temperature	150 ºC
Ion source temperature	230 ºC
Ionization	Electron-impact mode (70 eV)

Table 2
Method characteristics.

Compound	Retention time (min)	Monitored ions (*m/z*)	RSD[a] (%)	LOQ[b], (ng g^{-1})
Eucalyptol (EUC)	5.3	108, 139, 154	6.1	0.053
Linalool (LIN)	6.2	71, 93, 121	10.3	0.035
Menthol (MEN)	7.3	71, 81, 95	8.4	0.029
Geraniol (GER)	8.2	69, 93, 123	5.9	0.022
Carvacrol (CAR)	9.2	135, 150	6.3	0.036
Thymol (THY)	9.4	135, 150	6.7	0.030
3M4CP (IS)	9.9	142, 107	–	–
Eugenol (EUG)	10.2	164, 149, 77	7.2	0.108

[a] n=10. [b] Calculated for S/N=10.

2.3. Samples and analytical procedure

Samples of honeys from different geographical and botanical origin, including orange blossom, rosemary, eucalyptus, thyme, lavender, heather, chesnut and multifloral, were obtained from local supermarkets. In order to simulate as closely as possible the characteristics of a typical honey sample during the optimization procedure, a synthetic honey was elaborated. This mixture contained 30% (m/m) glucose, 50% (m/m) fructose and 20% (m/m) water [27], and was heated to 50 ºC to achieve the complete dissolution of the sugars.

Two grams of honey were weighed into a 15 mL glass vial and dissolved in 2 mL acetate/acetic (0.2 M) buffer solution. 3M4CP was added as internal standard, at a concentration of 5 ng g^{-1}. HSSE extraction was carried out by exposing the coated stir bar to the vial headspace, while the sample was stirred at 900 rpm and heated at 50 ºC for 2 h until extraction equilibrium was reached. After analyte extraction into the polymeric phase, the stir bar was rinsed with pure water, dried with a lint-free tissue and placed in a desorption tube for analysis.

3. Results and discussion

Two sequential steps are involved in HSSE preconcentration: extraction of the analytes from the sample headspace to the stir bar coating and their thermal desorption to be injected into the GC-MS system. Different parameters affecting these steps were optimized so as to achieve the best conditions. In order to ascertain the relevance of each variable for the different analytes in the optimization process, regardless of their different sensitivities, peak area values of each were normalized with respect to their average area in the corresponding experimental set.

3.1. HSSE parameters

The extraction of the analytes was studied using a synthetic honey containing the analytes at 10 ng g^{-1}. Some essential parameters of the extraction procedure, such as the honey sample dilution rate, pH of the extraction solution, salt concentration and amount of honey sample analyzed, were

initially optimized using PDMS coated stir bars. Since the influence of these variables in the extraction efficiency is not related with the nature of the extracting phase, the values selected for these variables were applied to other coatings in further optimization assays.

Prior to extraction, honey is usually diluted to improve homogenization [28]. In addition, this dilution reduces sample viscosity allowing a more efficient transfer of the studied compounds to the vial headspace, and shortening HSSE extraction times. However, dilution implies a decrease in analyte concentration in the extraction medium, in the vial headspace, and thus in the obtained analytical response. The influence of the dilution proportion (honey:water) was checked by diluting 1 g of spiked synthetic honey with different volumes of water, ranging from 0 to 2 mL. As shown in Fig. 1, the best response was obtained for a 1:1 dilution, which was selected. Because no significant differences were observed in the behaviour of each compound, mean relative area is represented.

Some of the studied terpenes have hydroxyl phenolic groups, whose deprotonation in basic media implies the loss of their volatile nature. To avoid this, different buffer solutions were assayed for sample dilution: 0.2 M citrate/citric acid (pH=3.1), 0.2 M acetate/acetic acid (pH=4.8), 0.2 M hydrogen phosphate/dihydrogen phosphate (pH=7.2) and 0.2 M carbonate/hydrogen carbonate (pH=9.8). The obtained responses are shown in Fig. 1. Although the pH of the extraction medium had little effect on most of the studied compounds, a decrease in the obtained signal was observed for THY, CAR and GER at pH 9.8. Taking into account the average response, a pH=4.8 acetate/acetic acid buffer solution was therefore used for diluting the sample.

The addition of salt increases the ionic strength of the aqueous phase, generally facilitating the extraction of non-polar species to the polymeric coating because of the decrease in water solubility. However, high salt concentrations may decrease the extraction efficiency, although the mechanism involved is not clear. The addition of NaCl in the 0 – 12% (m/m) range was assayed. As Fig. 1 shows, salt addition leads to a decrease in the obtained response for some compounds (EUC and LIN), and an increase at medium levels for others (MEN and GER). Since the average maximum response was attained with a 4% (m/v) salt concentration, this value was selected.

Even though an increase in the sample amount implies an increase in the total amount of the studied compounds and thus in the achievable detection limits, the use of larger honey samples also increases possible matrix interference in the sample extraction by HSSE, related with suspended matter as well as dissolved compounds present in the sample [29]. The amount of honey submitted to the extraction procedure was optimized by testing different values in the 1 to 4 g range. The obtained responses are summarized in Fig. 1, where it can be seen that increasing the honey mass over 2 g did not lead to any significant increase in the obtained response. Therefore, 2 g of honey sample was employed.

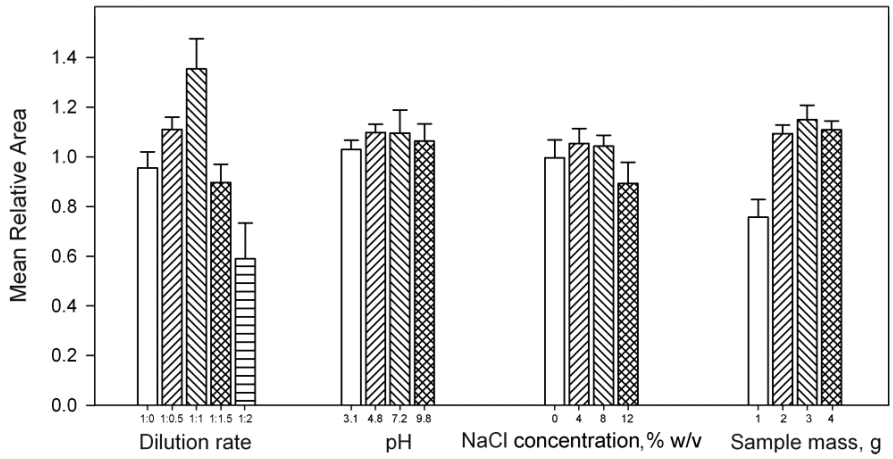


Fig. 1. Effect of dilution rate, pH, salt concentration and honey sample mass in the extraction solution on the mean analyte response. Vertical segments correspond to standard deviation (n=21).

Some other variables in the HSSE extraction step, including extraction temperature and extraction time, may be related with the partition coefficients among headspace and the extracting phase or with total volume and thickness of the coating. Therefore these variables are related with the nature of the polymeric phase used, and they must be optimized in each case.

Extraction temperature is generally a very influential parameter in HSSE. High extraction temperatures favour vaporization of the analytes, shifting the equilibrium of these species to the headspace phase, from where they are extracted to the HSSE coating. However, high temperatures may lead to the thermal release of trapped species from the extracting phase back to the headspace. The extraction temperature was evaluated in the 50-90 ºC range; higher temperatures were not considered due to the limits imposed by the boiling point of water. Fig. 2 shows that the optimum temperature for the PDMS coating is 50 ºC, while for the EG-Silicone and PA the maximum response was attained at 70 ºC.

The extraction time, which is the most relevant parameter affecting HSSE, was investigated from 15 min to 4 h. Extraction time profiles show that equilibrium was reached for all the compounds for all coatings at around 2 h, so this time was chosen to ensure high extraction efficiencies.

3.2. Thermal desorption conditions

As in the case of the HSSE extraction step, some of the variables involved in the thermal desorption of the stir bars may be related with the nature of the coating used. Desorption temperature, desorption time and gas flow rate were optimized for each coating phase.

In general, higher TDU temperatures facilitate the release of analytes retained in the extracting phase, but may lead to degradation of the polymeric coating reducing the useful life of the stir bar. Extraction temperature was evaluated in the 225 – 275 ºC range for PDMS, and in the 180 – 220 ºC

range for EG-Silicone and PA. The highest value of these ranges was imposed by the maximum recommended temperature for the used coating. As Fig. 2 shows, maximum responses were attained at 250 ºC for PDMS. For EG-Silicone and PA, even though maximum signals were attained at 220 and 200 ºC, respectively, no great differences were obtained for any analyte between these two temperature values.

This TDU temperature has to be maintained for sufficiently long time to ensure the total desorption of the analytes and to avoid the possible carry-over caused by incomplete desorption. However, long desorption times may lead to analyte losses in the PTV. When the desorption time was evaluated in the 5 – 10 min range, the best results were obtained at 7.5 min for PDMS, and 10 min for EG-Silicone and PA (Fig. 2).

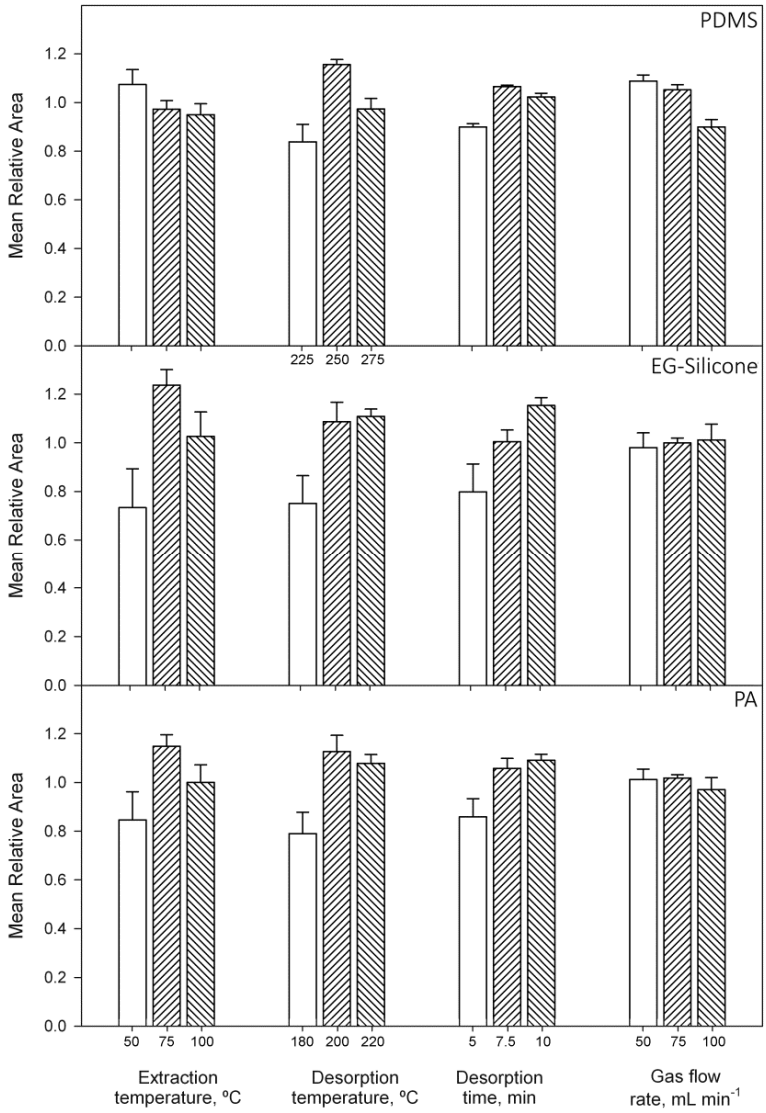


Fig. 2. Effect of HSSE sampling temperature, desorption temperature and time, and gas flow-rate on the mean analyte response for the three evaluated coatings. Vertical segments correspond to standard deviation (n=21).

A carrier gas is necessary to propel the analytes towards the PTV injector while they are being thermally desorbed in the TDU. Values in the 50 – 100 mL min^{-1} were tested, and as shown in Fig. 2, the efficiency of the desorption process was maximum when a gas flow rate of 50, 100 and 75 mL min^{-1} for PDMS, EG-Silicone and PA, respectively.

Before entering the chromatographic column, the desorbed compounds were focused into the PTV, which was equipped with a packed silanized glass wool liner. The lower the temperature in this device, the lower the losses and the greater the retention efficiency, especially for the more volatile compounds. Since the Peltier unit only allows cooling to slightly below room temperature, the lowest achievable temperature, 15 ºC, was selected. Once the TDU desorption step is over, compounds are introduced into the GC column by heating the PTV, up to the highest suitable value for the liner used (275 ºC). Therefore, a PTV programme temperature increasing from 15 to 275 °C (650 °C min^{-1}), with a hold time of 2 min, was applied.

3.3. Comparison among extracting phases

Applying the previously selected experimental conditions for each extracting coating, the sensitivity achieved was tested by comparing the slopes of the standard addition calibration graphs obtained using honey samples spiked at six concentration levels.

For lighter compounds, from EUC to GER, maximum sensitivity was attained using the PDMS coated stir bar, followed by EG-Silicone and PA. Despite their volatile nature, these compounds have relative high $K_{o/w}$ values, ranging from 2.7 to 3.5, demonstrating high affinity for this non-polar phase.

For phenolic compounds, from CAR to EUG, maximum sensitivity is attained using EG-Silicone coating, EG maximizes H-bond interactions with hydroxyl phenolic groups, while maintaining dispersive interactions among aryl chains and its silicone components.

Taking into account the overall response for all the analytes, PDMS was seen to be the most suitable extracting phase for volatile monoterpene extraction from honey samples, so the use of PDMS coated stir bars was adopted for the quantification of real samples.

3.4. Analytical characteristics of the method

For quantification purposes, 3M4CP was employed as internal standard (IS). This compound, which showed similar behavior in HSSE and GC to the analytes, was used to compensate any losses during the analytical procedure or any possible matrix effect, allowing the precise quantification of trace amounts of volatile terpenes.

The standard additions method was applied to three different honey samples (orange blossom, rosemary and eucalyptus) and the synthetic honey using the optimized experimental conditions.

The possibility of a matrix effect was tested by comparison of the obtained slopes using a t-test. Slopes of the standard addition calibration graphs were similar, with "p" values higher than 0.05, both when no IS was used and when IS was added to the sample, confirming that the matrix of the samples did not produce any interference. The quantification was carried out using matrix-matched calibration using a synthetic honey. Calibration graphs showed good linearity in the studied range: 1 – 50 ng g^{-1}, with correlation coefficients higher than 0.99 in all cases.

The repeatability of the method was calculated using the average relative standard deviation (RSD) of ten replicate analyses of a honey sample spiked with the analytes at 10 ng g^{-1}. RSD values ranged from 9 to 17% when only the analyte response was evaluated, and in the 6-10% range when the responses were related to the IS signal.

Detection and quantification limits were calculated on the basis of a signal to noise ratio of three and ten, respectively, and values in the 0.007 – 0.032 and 0.022 – 0.108 ng g^{-1} ranges were obtained, depending on the compound.

3.5. Analysis of real samples

The optimized procedure was applied to 12 different honey samples. Some of the studied compounds were found in the samples, at concentrations ranging from 0.6 to 64 ng g^{-1} (Table 3). As expected, there was an agreement between the high levels of some of the main terpenes (LIN, CAR and THY) in the honey samples labelled as coming from green plants like lavender and thyme.

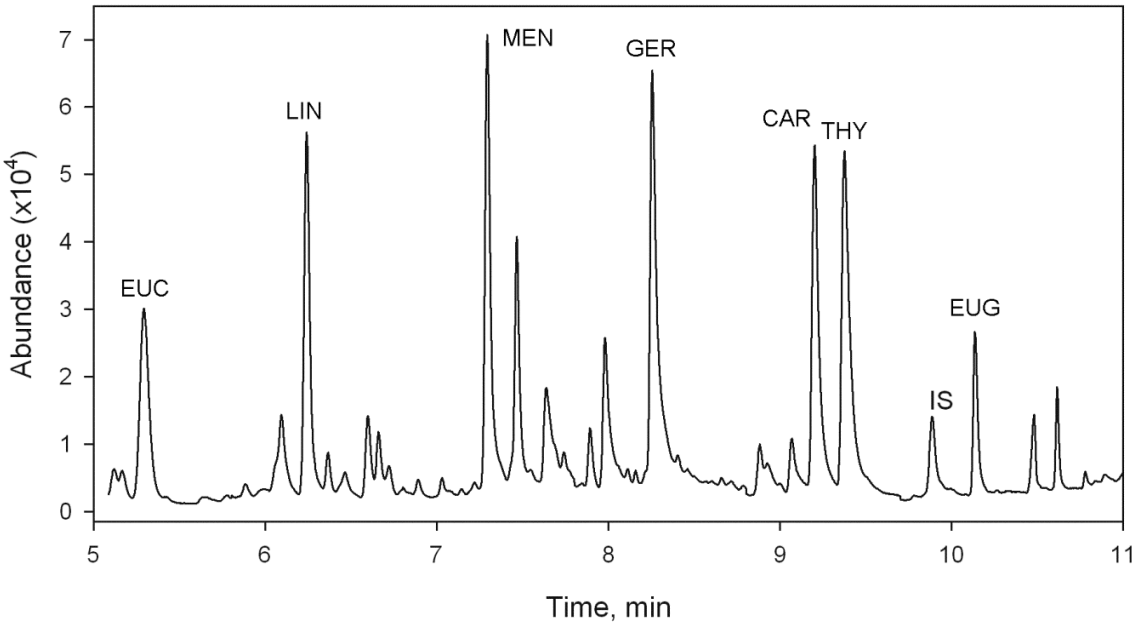


Fig. 3. HSSE-GC-MS chromatogram obtained for a multifloral honey sample spiked with the studied compounds at 10 ng g^{-1}.

Table 3
Results obtained in the analysis of the honey samples (ng g^{-1}).

Honey	EUC	LIN	MEN	GER	CAR	THY	EUG
Orange blossom	1.9±0.1	15.2±1.0	12.4±0.9	0.4±0.1	NQ	NQ	1.5±0.3
Rosemary	1.6±0.1	6.4±0.3	0.6±0.1	0.4±0.1	0.7±0.1	NQ	11.8±0.8
Eucalyptus	63.5±5.0	0.8±0.1	6.6±0.5	0.7±0.0	3.5±0.1	1.2±0.1	7.7±0.4
Thyme	8.0±1.3	19.6±1.2	7.7±0.6	1.4±0.1	26.5±0.7	38.6±1.8	2.8±0.2
Lavender	2.6±0.3	24.9±2.6	2.0±0.2	1.1±0.1	3.7±0.3	1.0±0.1	25.1±1.5
Heather	1.0±0.1	12.3±0.6	1.0±0.1	1.7±0.3	1.5±0.1	0.6±0.1	24.9±0.7
Chestnut	1.1±0.1	24.0±1.4	1.8±0.2	24.8±1.3	6.5±0.2	ND	2.8±0.3
Multifloral A	14.0±3.8	9.3±0.9	1.4±0.1	2.6±0.7	2.3±0.1	1.3±0.1	2.1±0.6
Multifloral B	6.8±1.0	8.0±0.6	9.3±0.3	2.0±0.1	2.4±0.2	1.7±0.1	NQ
Multifloral C	1.6±0.3	14.0±1.4	36.0±2.9	3.4±0.3	1.6±0.2	1.4±0.2	1.1±0.1
Multifloral D	0.7±0.0	2.3±0.2	2.1±0.1	2.7±0.3	4.1±0.2	1.3±0.3	NQ
Multifloral E	1.6±0.1	4.7±0.5	3.2±0.4	1.4±0.1	1.4±0.1	0.6±0.1	2.6±0.1

Mean value ± standard deviation (n=3). NQ means not quantified. ND means not detected.

Fig. 3 shows a typical chromatogram obtained for a spiked multifloral honey at 10 ng g^{-1}. Similar chromatograms were obtained for the other samples, demonstrating the absence of interfering peaks. Compounds were identified by comparing the retention time, target ion and ion ratios with those obtained by injection of the standards.

In order to check the trueness of the proposed method, recovery studies were carried out using two different honey samples (orange blossom and rosemary), spiked at two concentration levels. For the lowest concentration, at 5 ng g^{-1}, recoveries were between 90 – 110%. For the highest spiked concentration level, at 20 ng g^{-1}, recoveries of between 94 – 107% were obtained.

4. Conclusions

HSSE-TD-GC-MS is applied for the first time for the determination of seven volatile monoterpenes in honey samples, providing a simple and solvent free method for the reliable control and quantification. In addition to PDMS, new commercially available polymeric coatings, EG-Silicone and PA, are applied for the first time to this technique. Despite their polar nature, they did not show any improvement over PDMS, at least for the extraction of the evaluated volatile compounds.

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Chapter III

Direct Sample Introduction and Microvial insert Large Volume Injection

Direct sample introduction gas chromatography and mass spectrometry for the determination of phthalate esters in cleaning products

Abstract

A method using direct sample introduction (DSI) coupled to gas chromatography – mass spectrometry (GC-MS) is developed for the determination of six phthalate esters (dimethyl, diethyl, dibutyl, butylbenzyl, diethylhexyl and dioctyl phthalate) in cleaning products. The different variables involved in the DSI step, including venting time and temperature, vaporization time and temperature, injector heating temperature and gas flow rate and pressure, were evaluated and optimized using Taguchi orthogonal arrays. The proposed method, using calibration against methanolic standards, showed good linearity in the 0.05 – 15 μg g^{-1} range and good repeatability, with RSD values ranging from 3.5 to 5.7%. Quantification limits between 0.010 and 0.041 μg g^{-1}, depending on the compound, were attained, while recovery assays provided values from 83 to 115%. Twenty seven cleaning products were analyzed using the DSI-GC-MS method, being four phthalates (dimethyl, diethyl, dibutyl and diethylhexyl phthalate) found in fourteen of them at concentration levels in the 0.1 – 21 μg g^{-1} range. Compared with the most common GC injection technique, which uses the split/splitless injector, the proposed DSI procedure provided larger peak areas and lower detection limits, as result of the greater injected volume and reduction in noise.

1. Introduction

Phthalate esters (PEs) comprise a wide group of compounds, firstly synthetized in the 1920, whose large scale use began in the 1950s. More than 60 of these compounds have industrial applications, di(2-ethylhexyl) phthalate (DEHP) being the most produced [1]. PEs, especially those with long chains, are mainly employed as plasticizers, due to their high solubility in polymeric materials and low volatility, which provides more flexible and malleable materials. They are widely used in plastics, like PVC, epoxy resins or polyesters, which are used in a broad range of products. Since these compounds are not linked to the polymeric matrix, they can migrate from their original containers to the surrounding environment. This fact, along with their extensive use, has led to them becoming widespread contaminants, since they are found in a wide range of matrices [2]. Dimethyl (DMP) and diethyl (DEP) phthalate are employed as insect repellents, while other PEs are used as additives and lubricants in cosmetic formulations, soaps and detergents.

Great concern has arisen in recent years, since PEs are considered estrogenic compounds. Alterations in the reproductive capacity, related to lower testosterone levels have been reported [3]. In the same way, they may cause alterations in endocrine regulation, leading to problems in foetal development, with low birth weights and higher prenatal mortality [4]. Even though PEs do not cause direct genetic damage, some of them are considered carcinogenic compounds and have been included in the International Agency for Research on Cancer (IARC) 2B and 3 lists [5]. Human exposure to PEs may take place by ingestion of contaminated water or food, by inhalation of polluted air as well as by dermic contact with phthalate-containing plastic products. The addition of PEs to plastics for the manufacture of baby toys [6] and medical material [7] has been restricted, and severe limitations have been imposed for their presence in plastic food containers [8].

Cleaning products are widely used because of the reduction in time and effort required to clean and achieve hygienic conditions in the home. The daily use of these products has been one of the factors contributing to improving human health and increasing life expectancy in the last century. The large volume of cleaning products used, reaching 26.5 kg/person/year [9] in some countries, means that these products may be one of the main routes of exposure of the population to harmful chemicals compounds. Taking this into account, cleaning products need to be approved to make them safe for users, especially if they are used inappropriately.

Although the presence of PEs in cleaning products has been assessed [2,10], their determination in this kind of household products has not been yet reported. The determination of PEs has mainly been carried out by GC-MS [11–16]. Taking into account the complexity of cleaning product matrices, a clean-up step has to be introduced in the whole analytical procedure. Classical clean-up procedures, like solid phase extraction (SPE) [17] or liquid-liquid extraction (LLE) are time- and solvent-consuming, while the intensive labour needed makes their use in

routine quality control analysis uneconomic. Direct sample introduction (DSI), first developed by Amirav and col. [18,19], is a rapid, sensitive, simple and inexpensive procedure related with large volume injection (LVI). The liquid or solid sample is placed in a disposable glass microvial that is submitted to a heating step programme in a programmed temperature vaporization (PTV) injection port. In the automated version, the sample is injected into the microvial placed in a liner, which is inserted into a thermal desorption unit (TDU) attached to the GC inlet. The sample solvent is separated from the analytes through split vent mode, next the volatile compounds are vaporized and transferred to the GC column for separation, while non-volatile interfering matrix components are retained on the inner surface of the vial, is discarded which after each assay [20]. DSI usually implies a minimal sample manipulation, simplifying the analytical procedure. This technique has been successfully applied for the determination of furaneol in fruit juices [21], or pesticides in rice [22] and cereal grains [23].

In the present work, a method using DSI coupled to GC-MS is developed for the determination of six PEs in cleaning products. In addition, the DSI injection mode is compared with the most common injection mode, isothermal split/splitless.

2. Materials and methods

2.1. Reagents

A standard stock solution containing six phthalate esters, dimethylphthalate (DMP), diethylphthalate (DEP), di-n-butylphthalate (DBP), n-butylbenzylphthalate (BBP), di-2-ethylhexylphthalate (DEHP) and di-n-octylphthalate (DOP), in methanol at 200 μg mL^{-1} per compound was purchased from Supelco (Bellefonte, PA, USA). Anthracene is used as internal standard (IS), with a purity of 99.5% was supplied by Dr. Ehrenstorfer (Ausburg, Germany). Solutions were kept at -10 ºC in the dark. Working standard solutions were prepared daily by diluting with Milli-Q water.

Analytical-reagent grade methanol was purchased from Lab-Scan (Dublin, Ireland), while deionized water was obtained from a Milli-Q water purification system (Millipore, Bedford, USA).

2.2. Instrumentation

GC analyses were performed on an Agilent 6890 (Agilent, Waldbronn, Germany) gas chromatograph coupled to an Agilent 5973 quadrupole mass selective spectrometer equipped with an inert ion source. The helium carrier gas was maintained at a constant flow of 1 mL min^{-1}. An HP-5MS (5% phenyl – 95% dimethylpolysiloxane, Agilent) capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) was used.

The GC temperature was programmed as follows: start temperature of 75 °C (held 0.5 min) and increase to 275 °C at 20 °C min^{-1} (held 4.5 min). The total analysis time for one GC run was 15 min. The ionization was carried out in the electron-impact (EI) mode (70 eV). The identity of the compounds was confirmed by comparison of retention time and MS-spectra with respect to pure standards. The analytes were quantified under the selected ion monitoring (SIM) mode using the target ions (Table 1).

Samples and standards were injected into disposable glass microvials (15 mm long, 1.9 mm i.d., 2.5 mm o.d., Gerstel, Germany) and then placed in the TDU (Gerstel) by means of a multipurpose autosampler (MPS2, Gerstel).

2.3. Samples

A total of 27 cleaning products, including surface, kitchen and floor cleaners as well as laundry and dishwashing liquid detergents, were obtained from a local supermarket. Due to the viscous nature of most of the analyzed samples, and in order to facilitate their manipulation and introduction into the injection system, a dilution step was carried out. For sample dilution, 5 mL of methanol were added to about 200 mg of the sample previously weighed in a 15 mL glass vial. Anthracene was added as internal standard (80 ng mL^{-1}), and the mixture was submitted to ultrasounds in a bath for 5 min in order to homogenize the solution.

2.4. Procedures

2.4.1. Direct sample introduction

An aliquot of the diluted sample (50 μL) was placed in a 200 μL glass microvial and then transferred to the TDU by means of the MPS2. The TDU was firstly operated in the solvent vent mode, in order to evaporate the sample solvent, with a temperature of 40 ºC which was maintained for 2 min, while a helium gas-flow of 125 mL min^{-1} at 9 psi flowed through the GC inlet during this solvent drying step. Next, the TDU was set to splitless mode and heated to 300 ºC with a ramp temperature of 260 ºC min^{-1} with a 2 min holding time, in order to evaporate the analytes, which were retained in a 1 mm fiberglass packed liner in the PTV-CIS 4. During the evaporation of the analytes, the PTV-CIS was maintained at 15 ºC, and then ramped at a rate of 10 ºC s^{-1} to a final temperature of 275 ºC with a 5 min holding time.

2.4.2. Classic splitless injection

A 2 μL volume of the diluted sample was injected into the split/splitless injector equipped with a single taper liner. The injection port was held at 275 °C and used in the pulsed splitless mode, applying a pressure pulse of 30 psi during the first 0.5 min of the injection.

Table 1
Phthalates and analytical characteristics of both injection methods.

Compound	RT (min)	Monitorized ions (*m/z*)	DSI			Splitless injection		
			Slope (L pg^{-1})	RSD[a] (%)	LOQ (µg g^{-1})	Slope (L pg^{-1})	RSD[a] (%)	LOQ (µg g^{-1})
DMP	6.7	163, 194, 135	4.9	4.1	0.041	0.17	5.1	0.119
DEP	7.5	149, 177, 105	5.8	3.5	0.019	0.22	6.7	0.123
IS	8.8	178, 152		-	-		-	-
DBP	9.4	149, 223, 205	13.5	4.0	0.010	0.46	6.9	0.041
BBP	11.3	149, 91, 206	5.1	3.1	0.025	0.18	7.7	0.091
DEHP	12.2	149, 167, 279	7.7	4.4	0.017	0.29	7.6	0.074
DOP	13.7	149, 279	9.2	4.4	0.017	0.41	6.5	0.050

Underlined numbers correspond to *m/z* of the target ion. [a] n=10.

3. Results and discussion

3.1. Optimization of the GC sample injection

3.1.1. Direct sample introduction

Taking into account the high number of variables and their possible interaction that may influence the efficiency of the DSI in the chromatographic system, experimental designs based on Taguchi orthogonal arrays were used to test their effect and significance. In order to assess the relevance of each variable for the different analytes, regardless of their different sensitivities, the peak area values of each compound were normalized relative to average areas for each one in the corresponding experimental set. The Minitab 15 statistical package was used to generate the experimental matrices and to evaluate the results.

The possible influence on the performance of the DSI method of seven different factors (solvent drying time and temperature, desorption time and temperature, PTV heating temperature and gas flow-rate and pressure) were initially tested using a Taguchi at two levels with a set of 24 experiments. For this purpose, 50 µL of the diluted detergent containing the analytes at about 5 ng mL^{-1} were placed in the microvial and injected into the gas chromatograph using the TDU system.

The methanol used to dilute the samples is removed by heating, thus preventing its entry into the analytical column, where large amounts of solvent may damage the stationary phase or even MS detector components. Two different drying temperatures (40 and 50 ºC) and times (1 and 2 min) were assayed. The obtained responses (Fig. 1A and 1B) were higher for most compounds at 40 ºC and 2 min. DOP was the only compound that provide a higher signal at 1 min, perhaps because of the volatilization of the compound.

To vaporize the analytes, thermal desorption time and temperature were studied at 1 and 2 min and 225 and 275 ºC, respectively. In general, higher temperatures and longer desorption times may facilitate the vaporization of the analytes, but may reduce the overall efficiency due to retention losses in the PTV injector. According to the results (Fig. 1C), desorption time does not seem not to

be a relevant factor for most of the compounds, but since a slight increase was obtained for BBP at 2 min, this time was selected. On the other hand, the analytical signal increased significantly with temperature for the heavier compounds (Fig. 1D) like DEHP, DOP and BBP. Since this variable was reported in the ANOVA statistical analysis as the second most relevant of those considered (F = 119.8, p = 0.058), it was optimized in more detail using a second Taguchi design.

A carrier gas is necessary to propel the analytes to the PTV injector while they are being thermally vaporized. The increasing of the gas flow-rate from 50 to 100 mL min^{-1} provided a significant increase in the analytical response for most compounds (Fig. 1E), especially for the heavier ones, like DEHP, BBP and DOP. This variable was the most relevant of the seven considered in this initial design according to ANOVA statistical analysis (F = 724.8, p = 0.024), so its value was optimized thoroughly using a second Taguchi. The gas pressure, assayed at 8 and 10 psi, did not affect significantly the responses of the compounds (Fig. 1F), showing to be the least relevant variable studied, so 9 psi was chosen as a compromise value.

The desorbed compounds were focused in the PTV before entering the chromatographic column. Lower temperatures in this device increased the retention efficiency and minimized losses of the more volatile compounds. Since the Peltier unit only allows cooling to temperatures slightly below room temperature, 20 ºC was selected as the focusing temperature. Different liners can be used to facilitate the retention of the analytes in the PTV. Generally, deactivated packing material may cause analyte degradation. When an empty baffled liner and a liner filled with the inert support fibreglass were tested, the resulting analytical signals were higher with that containing fibreglass, especially for the lighter compounds, such as DMP and DEP. The liner filled with fibreglass was therefore selected.

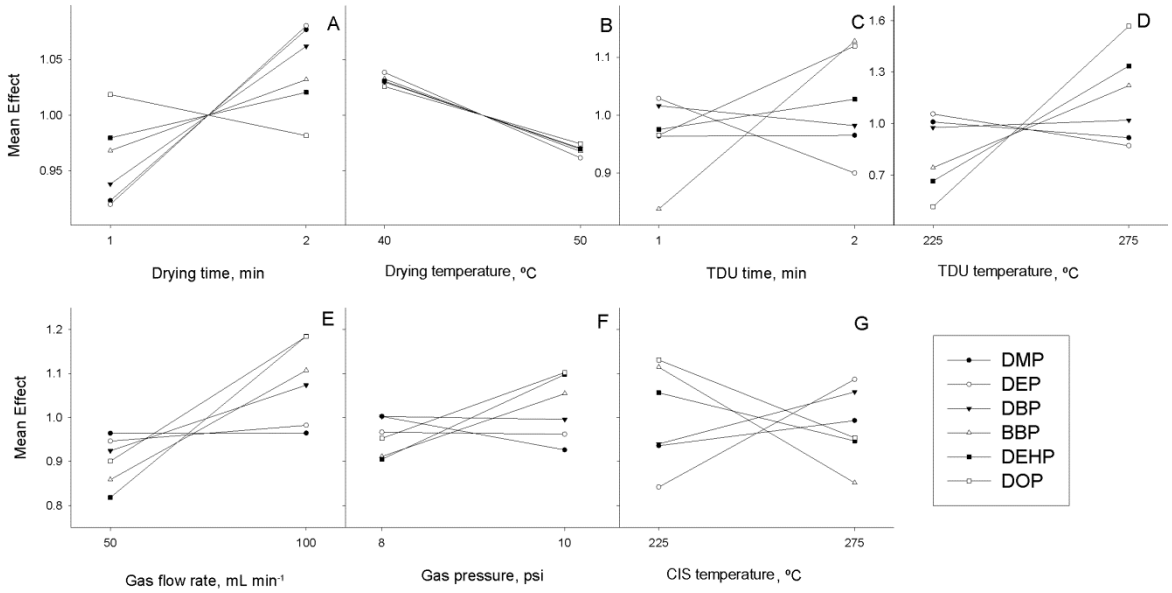

Fig. 1. Effects of factor levels of the drying time (A) and temperature (B), time (C) and temperature (D) of the TDU heating, gas flow rate (E) and pressure (F) and CIS heating temperature (G) on the mean relative response of PEs.

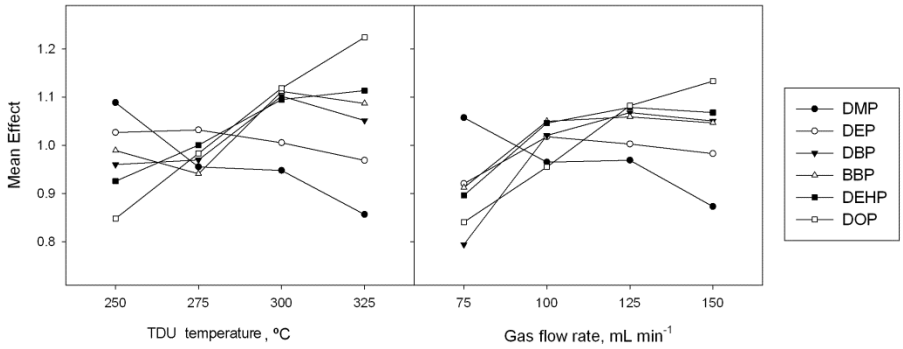

Fig. 2. Effects of factor levels of the TDU temperature (A) and gas flow-rate (B) on the mean relative response of PEs.

In order to elute the compounds retained in the liner to the chromatographic column, two different temperatures, 225 and 275 ºC, were applied. BBP and DOP showed maximum sensitivity at 225 ºC, while 275 ºC provided best results for DEP (Fig. 1G). Taking into account the average response, 275 ºC was selected. Therefore, a programme temperature increasing from 20 to 275 ºC at 600 ºC min^{-1}, with a hold time of 5 min, was applied.

The two variables with the greatest effect on the sensitivity according to the first multivariate study (TDU temperature and gas flow rate) were optimized in detail in a second Taguchi design, with four levels for each in a set of 16 experiments. Temperatures of 250, 275, 300 and 325 ºC were assayed: DBP, BBP and DEHP showed their maximum response at 300 ºC, whereas DMP, DEP and DOP reached their maximum signal at 250, 275 and 325 ºC, respectively (Fig. 2A). A temperature of 300 ºC was finally selected and therefore, a TDU heating program increasing from 40 to 300 ºC at 260 ºC min^{-1}, with a hold time of 2 min, was applied. Gas flow rates of 75, 100, 125 and 150 mL min^{-1} were tested. Except DMP and DOP which showed their highest responses at 75 and 150 mL min^{-1}, respectively, the rest of the compounds showed maximum sensitivity at 125 mL min^{-1} (Fig 2B). A gas flow rate of 125 mL min^{-1} was chosen.

3.1.2. Split/splitless injection

To select the optimum conditions for injecting the samples by means of the common split/splitless injector, a detergent solution containing analyte concentrations of around 20 ng mL^{-1} was used. Given the nature of the solvent used, methanol, and taking into account the inner volume of the liner, a 2 μL volume of the sample was injected in the splitless mode. The effect of the injection temperature was studied at 225, 250 and 275º C. The highest analytical signals were attained for all the compounds at 275 °C, and this value was adopted.

When injecting high volumes in splitless mode, the application of a pressure pulse can improve sensitivity and repeatability, leading to a quicker and more effective sample introduction into the chromatographic column. Pressure pulses of 10, 20 and 30 psi were assayed, the highest sensitivity being attained with 30 psi for all compounds. This pressure pulse was maintained for 0.5 min.

3.2. Method characteristics

3.2.1. Direct sample introduction

Calibration curves using DSI-GC-MS were obtained by plotting the ratio between analyte peak area and IS peak area *versus* analyte concentration, using spiked samples at six concentration levels, in triplicate. The least-squares linear regression analysis provided good correlation coefficient values ($r > 0.99$), meaning good linearity responses in the studied concentration range 0.05 – 15 μg g^{-1} for all the compounds. The use of the standard addition calibration procedure using the IS avoided any possible matrix effect, since no significant differences were observed when the slopes of methanolic standards calibration graphs and those obtained for the standard additions calibration graphs for three different samples were compared by means of a paired *t*-test ($p>0.05$).

In order to check the repeatability of the method, ten replicate analyses were performed using spiked surface cleaner samples at 0.5 μg g^{-1} for each compound. RSD values ranged from 3.5 to 5.7% (Table 1), using the IS, and from 7.0 to 9.5%, when no IS was considered, depending on the compound. Quantification limits (LOQs) were calculated taking into account a signal-to-noise (S/N) ratio of 10, and values between 10 and 41 ng g^{-1} (Table 1), depending on the compound, were obtained. In order to check the accuracy of the method, recovery assays were performed by fortifying two cleaning product samples (dishwashing and surface cleaner) at two concentration levels 0.5 and 1.25 μg g^{-1}. The recoveries varied from 83 to 115% (n = 36) at the lowest level and from 84 to 106% (n = 36) at the highest level.

3.2.2. Method characteristics of classic injection

Calibration curves using the IS procedure were found to be linear between 0.3 and 30 μg g^{-1}, with correlation coefficients higher than 0.99 in all cases. The absence of a matrix effect was confirmed by comparison of the obtained slopes for methanolic standards and standard additions to three samples using a t-test, which provided "p" values higher than 0.05. The repeatability of the method was studied for a surface cleaner sample fortified at 3 μg g^{-1} which provided RSD values (n=10) in the 4.5 – 7.7% range (Table 1). LOQ values, calculated for a signal-to noise-ratio of 10, of between 41 and 123 ng g^{-1} (Table 1), depending on the compound, were obtained. To check the accuracy of the method, two samples were fortified at two concentration levels (3 and 6 μg g^{-1}) and an average recovery ± SD (n = 72) of 101 ± 13% was obtained.

3.2.3. Comparison of DSI and classical splitless injection modes

Applying the optimized experimental conditions found for the two procedures, their sensitivity was compared. When the slopes obtained for standards addition calibrations were examined, the DSI procedure provided slopes between 21 and 30-fold larger (Table 1), depending on the

compound. In terms of LOQ, evaluated according to S/N ratios, DSI provided values between 3 and 8 times lower (Table 1). The higher sensitivity of the DSI procedure was related to the greater amount of diluted sample injected in the GC-MS system (50 µL *versus* 2 µL), which is reflected in the obtained increase in slopes, and to the fact that most of the non-volatile matrix components were retained in the microvial, leading to an increase in S/N ratios.

3.3. Analysis of real samples

Twenty seven cleaning products were analyzed using the DSI-GC-MS procedure. Table 2 shows the results obtained for the 14 samples in which some of the target PEs were detected. These compounds were found at concentration levels between 0.1 and 21 µg g^{-1}, corresponding to DEHP in a kitchen cleaner and DEP in a surface detergent, respectively.

Fig. 3A shows a typical chromatogram obtained under SIM mode for a floor detergent sample fortified at 1.25 µg g^{-1} concentration level, in the selected conditions.

The specificity of the proposed procedure was assured by identification of the analytes by comparing the retention time, target (*T*) and qualifier ions, and qualifier-to-target ratios of the peaks in samples and standard solutions. Fig. 3B shows the mass spectra, which confirmed the identity.

Table 2
Analysis of the samples (µg g^{-1}).

Cleaning product	Splitless injection				DSI			
	DMP	DEP	DBP	DEHP	DMP	DEP	DBP	DEHP
Dish Washing 1	0.25±0.02	ND	ND	ND	0.23±0.01	ND	ND	ND
Laundry 1	0.25±001	ND	1.59±0.07	ND	0.27±0.01	ND	1.39±0.11	ND
Dish Washing 2	0.61±0.02	ND	ND	0.34±0.02	0.58±0.02	ND	ND	0.31±0.01
Laundry 2	0.34±0.02	ND	ND	1.38±0.04	0.29±0.03	ND	ND	1.47±0.11
Floor 1	ND	1.28±0.08	ND	ND	ND	1.43±0.06	ND	ND
Floor 2	ND	13.80±0.41	ND	ND	ND	12.99±0.44	ND	ND
Bathroom 1	ND	0.40±0.03	ND	ND	ND	0.47±0.04	ND	ND
Surfaces 1	ND	19.79±0.98	0.89±0.03	ND	ND	21.03±0.62	0.88±0.06	ND
Laundry 3	ND	3.29±0.11	ND	1.53±0.10	ND	3.49±0.14	ND	1.61±0.07
Surfaces 2	ND	3.38±0.13	ND	0.14±0.01	ND	3.08±0.17	ND	0.13±0.01
Dish Washing 3	ND	1.76±0.08	ND	0.39±0.02	ND	1.96±0.08	ND	0.33±0.04
Kitchen 1	ND	ND	0.44±0.02	0.09±0.01	ND	ND	0.48±0.01	0.07±0.01
Laundry 4	ND	ND	3.74±0.17	3.20±0.14	NQ	ND	3.45±0.18	3.47±0.17
Kitchen 2	ND	ND	ND	0.14±0.01	ND	ND	ND	0.12±0.01

Mean value±standard deviation (n=3). ND means not detected. NQ means not quantified.

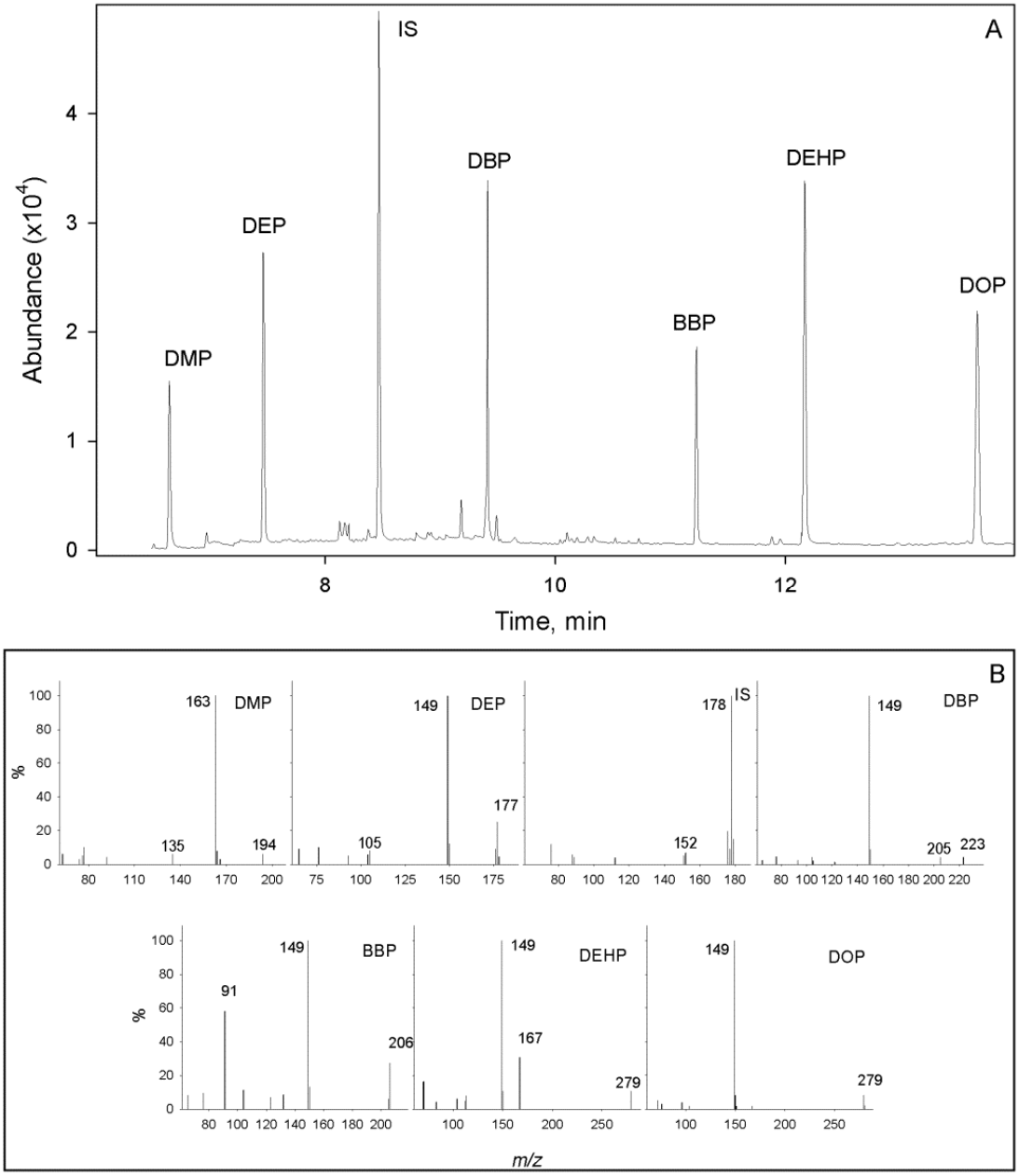

Fig. 3. Elution profile obtained for a floor detergent fortified at 1.25 μg g^{-1} concentration level for each compound using DSI-GC-MS under SIM mode (A), and mass spectra of each compound (B).

4. Conclusion

A sensitive analytical method for the determination six PEs implying a minimal sample manipulation has been developed, applying the DSI technique by means of a TDU coupled to GC-MS. This combination allowed an important increase in sensitivity attained with respect to the classical splitless injection mode, considering that large masses of the analytes can be injected into the GC system. In addition, most of the sample matrix components remained in the microvial, thus not damaging the chromatographic stationary phase or the spectrometer components.

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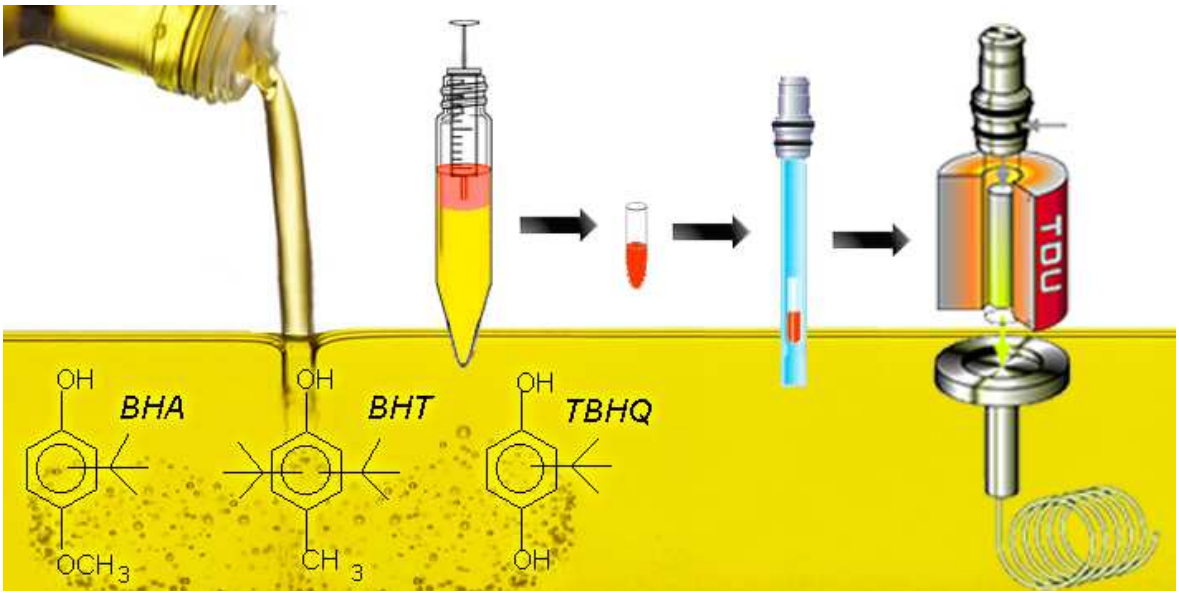
Determination of synthetic phenolic antioxidants in edible oils using microvial insert large volume injection gas-chromatography

Abstract

Three synthetic phenolic antioxidants, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butyl hydroquinone (TBHQ), were determined in different edible vegetable oil samples. The analyses were carried out by gas chromatography and mass spectrometry (GC-MS) using microvial insert large volume injection (LVI). Several parameters affecting this sample introduction step, such as temperatures, times and gas flows, were optimized. Quantification was carried out by the matrix-matched calibration method using carvacrol as internal standard, providing quantification limits between 0.08 and 0.10 ng g^{-1}, depending on the compound. The three phenolic compounds were detected in several of the samples, BHT being the most frequently found. Recovery assays for oil samples spiked at two concentration levels, 2.5 and 10 ng g^{-1}, provided recoveries in the 86 – 115% range.



1. Introduction

The growth of the modern food industry has been accompanied by the development of new food preservatives that ensure longer product shelf lives. One of the main problems in food conservation is rancidity, which is related to the oxidation of unsaturated fatty acids, a process that takes place through the formation of free radicals by oxygen, leading to chain reactions. In this way, small portions of oxygen may cause the decomposition of great fat amounts. Rancid fats provide a bad taste to foodstuffs, since they contain certain undesirable compounds (aldehydes, ketones and organic acids). While the use of modified atmospheres in the manufacturing process or vacuum packs for storage purposes may limit such degradation phenomena, it is impossible to exclude all traces oxygen in food [1].

In order to ensure the proper conservation of fat-containing food products, compounds that are able to stop radical chain reactions are needed. It is for this reason that synthetic antioxidants such as butylated hydroxyanisole (BHA, E320), butylated hydroxytoluene (BHT, E321) and *tert*-butyl hydroquinone (TBHQ, E319) are added to food as antioxidant additives. These phenolic compounds are fat-soluble and reactive against radicals and some of their resulting degradation intermediates, thus being able to stop degradation by means of their reaction with the free radicals, limiting the propagation of their chain reaction [2]. The radicals formed as a result of these reactions are quite stable, due to the presence of their aromatic ring as well as the steric hindrance imposed by the large *tert*-butyl group substituents [3].

Phenolic antioxidants are quite effective in preventing fat degradation, being added to fat-containing foods, like oils, fried potatoes or nuts. In addition, these compounds have an antimicrobial effect against Gram positive bacteria [4]. BHA is especially resistant against pH variations, while TBHQ is mostly used in foods submitted to thermal treatments because of its good thermal stability. When used together, BHA and BHT have a synergic effect, which further increases their protective potential [5].

However, the safety of these compounds has been of great concern, since they may cause allergic reactions, including asthma and hives, in sensitive subjects [6]. Animal laboratory tests have demonstrated their enzymatic induction capability, and there is some evidence that at high diet doses BHT may act as a carcinogenic agent, leading to the development of lung and liver cancer; indeed, its presence in the body can increase the effect of other carcinogenic compounds [7]. BHA is not a mutagenic agent *in vivo* or *in vitro* systems, but may affect the metabolism of some mutagenic compounds [8]. Both, BHA and BHT may act as endocrine disruptors [9], whose longer-term effects can include infertility and growth problems. The use of synthetic antioxidants and the levels added to food are subject to regulations in the European Union, being the maximum

permitted concentration in edible oils 200 mg kg^{-1}, for any synthetic phenolic antioxidant or their combinations [10].

Vegetable oils are complex chemical mixtures, whose health benefits and composition depend on the vegetable, seed or nut from which it is extracted. Being this food-type the major source of lipids and micronutrients in the human dietary intake, its quality control is of great concern [11]. The determination of phenolic antioxidants in edible oils has been accomplished using specific electrochemical probes [12,13] and different separation techniques, like micellar electrokinetic capillary chromatography [14] and liquid chromatography (LC) coupled with different detection methods, such as fluorescence [15,16], UV-Visible [17-19] or mass spectrometry (MS) [20,21]. Gas chromatography-mass spectrometry (GC-MS) [20,22-24] has also been used.

Methods for the determination of these antioxidants in edible oils frequently include a clean-up step using liquid-liquid extraction (LLE) [15,21-23]. Novel microextraction procedures, such a cloud point extraction (CPE) [17,18], or water-containing surfactant-based vortex assisted microextraction [15] have also been proposed as greener alternatives to classic LLE. Usually, only a few microliters of the extraction solvent are finally introduced in the instrumental set-up for the determination of the analytes, which, considering the low expected concentration of these compounds in oil samples, results in a great loss of the attainable sensitivity.

Amirav and Dagan [25] developed direct sample introduction (DSI), a rapid, sensitive, simple and inexpensive procedure in the context of large volume injection (LVI). When this technique is applied to liquid samples is also known as microvial insert thermal desorption or microvial insert large volume injection [26]. The liquid sample, with a volume up to 150 µL, is placed in a glass microvial and introduced into the programmed temperature vaporizator (PTV) or into a thermodesorption unit (TDU) attached to the PTV inlet. Non-volatile interfering matrix components are retained in the vial, which can be removed and discarded after the assay, while volatile compounds are vaporized and transferred to the GC column for separation [27]. DSI has previously been used for the determination of pesticides [28,29] and odour related compounds [26,30]. The use of microvial insert large volume injection allows the introduction into the chromatographic system of larger volumes of the edible oil extract, and, in addition, avoids contamination problems related to the co-extracted oil matrix components.

In this work, we present the results obtained in the analysis of twelve vegetable edible oil samples, obtained from different plants, for the determination of three synthetic phenolic antioxidants, BHA, BHT and TBHQ, using a microvial insert large volume injection GC-MS method.

2. Materials and methods

2.1. Chemicals and reagents

Butylated hydroxytoluene (2,6-bis(1,1-dimethylethyl)-4-methylphenol, BHT), butylated hydroxyanisole (3-*tert*-butyl-4-hydroxyanisole, BHA), *tert*-butyl hydroquinone (2-(1,1-dimethylethyl)-1,4-benzenediol, TBHQ) and carvacrol (5-isopropyl-2-methylphenol) were obtained from Sigma (St. Louis, MO, USA). Stock solutions (1000 mg L^{-1}) were prepared by dissolving the commercial products in methanol, and kept at −18 ºC in darkness. Acetonitrile (ACN), hexane and methanol were obtained from Sigma.

2.2. Instrumentation

The sample introduction system was composed of a Thermal Desorption Unit (TDU-2) equipped with an autosampler (MPS-2) and a Programmed Temperature Vaporization (PTV) Cooled Injector System (CIS-4) provided by Gerstel (Mullheim an der Ruhr, Germany). The experimental conditions used for the sample introduction system are summarized in Table 1.

GC analyses were performed on an Agilent 6890N (Agilent, Waldbronn, Germany) gas chromatograph coupled to an Agilent 5973 quadrupole mass selective spectrometer equipped with an inert ion source. Under the selected conditions (Table 1) the compounds eluted at retention times between 6.2 and 8.5 min, corresponding to carvacrol (IS) and TBHQ, respectively (Table 2). The compounds were quantified in the selected ion monitoring (SIM) mode in order to improve the detection limits using different ions (Table 2). Identification was confirmed by the retention time of the target ion and the qualifier-to-target ion ratios for each compound.

Table 1
Experimental conditions of the TD-GC-MS procedure.

Thermal Desorption Unit	
Mode	Solvent vent
Venting time	5 min
Venting pressure	2 MPa
Temperature	85 ºC held 5 min
programme	85 – 250 ºC at 190 ºC min^{-1}, held 1 min
Desorption flow	100 mL min^{-1}
Cooled Injector System	
Mode	Solvent Venting
Liner	Packed sylanized glass wool, 2 mm i.d.
Temperature programme	15 – 275 ºC (5 min) at 540 ºC min^{-1}
GC-MS	
Capillary column	HP-5MS, 5% diphenyl-95% dimethylpolysiloxane (30 m x 0.25 mm, 0.25 μm)
Carrier gas	Helium (1 mL min^{-1})
Oven programme	80 ºC, held 1 min 80 – 230 ºC at 15 ºC min^{-1}, held 1 min
Transfer line temperature	300 ºC
Quadrupole temperature	150 ºC
Ion source temperature	230 ºC
Ionization	Electron-impact mode (70 eV)

An ultrasonic processor UP 200 H (Dr. Hielscher, Teltow, Germany), with an effective output of 200 W in liquid media equipped with a titanium sonotrode (7 mm i.d.), was used for oil sample extraction. An EBA 20 centrifuge (Hettich, Tuttlingen, Germany) was used to disrupt the sample emulsions formed.

2.3. Samples and analytical procedure

A total of 12 edible oil samples, including corn, olive, sunflower, sesame and peanut, were obtained from local supermarkets. Samples were kept in darkness at 4 °C until analysis.

A 1 mL-aliquot of oil sample spiked with the IS, 0.25 mL of hexane, 0.25 mL of ACN were placed in a glass centrifuge tube. Carvacrol was added as internal standard to the oil, at a concentration of 25 ng mL^{-1} after confirming that the samples were free of this compound. The resulting mixture was submitted to ultrasounds by means of a probe for 20 cycles of 0.6 s at 60% amplitude. The resulting emulsion was centrifuged for 2 min at 3000 rpm, and the upper ACN phase was collected using a microsyringe. A 50 µL sample of the recovered ACN phase was placed in a 150 µL glass microvial and introduced into a glass desorption tube. The analytes were submitted to the microvial insert LVI procedure by placing the desorption tube in the TDU.

For validation purposes, three oil samples (sunflower, corn and olive) were spiked at two different concentrations, 2.5 and 10 ng g^{-1}, and submitted by triplicate to the analytical procedure.

3. Results and discussion

3.1. Sample extraction procedure

The analytes must be firstly extracted into a medium compatible with the procedure applied, removing as far as possible the main interfering matrix components. Ultrasound assisted liquid-liquid extraction was used for this purpose. Different parameters affecting the extraction efficiency of the target compounds, discussed below, were studied using 1 mL of an oil sample spiked at 50 ng g^{-1} for each antioxidant. Each assay was performed in triplicate.

Part of the oil matrix may be co-extracted into the solvent during extraction, leading to pollution in the GC system. Solvents which show low miscibility with edible oils minimize the complexity of the injected extracts, providing clean chromatograms and extending the useful life of the GC column. Aliquots of 1 mL of different extraction solvents, including ACN, methanol, acetone and ethanol, were assayed to select the most suitable. Acetone totally mixed with edible oil, while the other assayed solvents were partially miscible with the sample. ACN, which showed to be the less miscible one, provided best responses for all compounds. This organic solvent has previously been used for the extraction of phenolic antioxidants from fat matrices [21, 22], showing

adequate extraction capabilities and minimum miscibility with the oil samples, thus providing clean extracts. Therefore, ACN was used for the extraction of the studied compounds.

In order to reduce the amount of organic solvent used and to provide higher preconcentration ratios, different volumes of ACN, ranging from 0.25 to 1 mL, were tested with 1 mL of the spiked edible oil. Due to the lower density of ACN compared with edible oil, the extraction solvent remains as an upper phase, which hinders the collection of volumes lower than 0.25 mL. As Fig. 1A shows, an increase in the extraction volume resulted in a decrease in the obtained analytical signals due to dilution effect, while lower volumes assure an efficient extraction. Taking this into account, 0.25 mL was selected as the extraction volume. Successive extractions did not improve the obtained response.

The addition of hexane to oil samples before extraction with more polar solvents has been proposed as a way to reduce oil viscosity, increase the contact surface between phases, and facilitate phase separation [14]. When different volumes of hexane, in the 0 – 1 mL range, were added to 1 mL of the spiked oil prior to extraction, the best results were obtained using 0.25 mL of hexane (Fig. 1B).

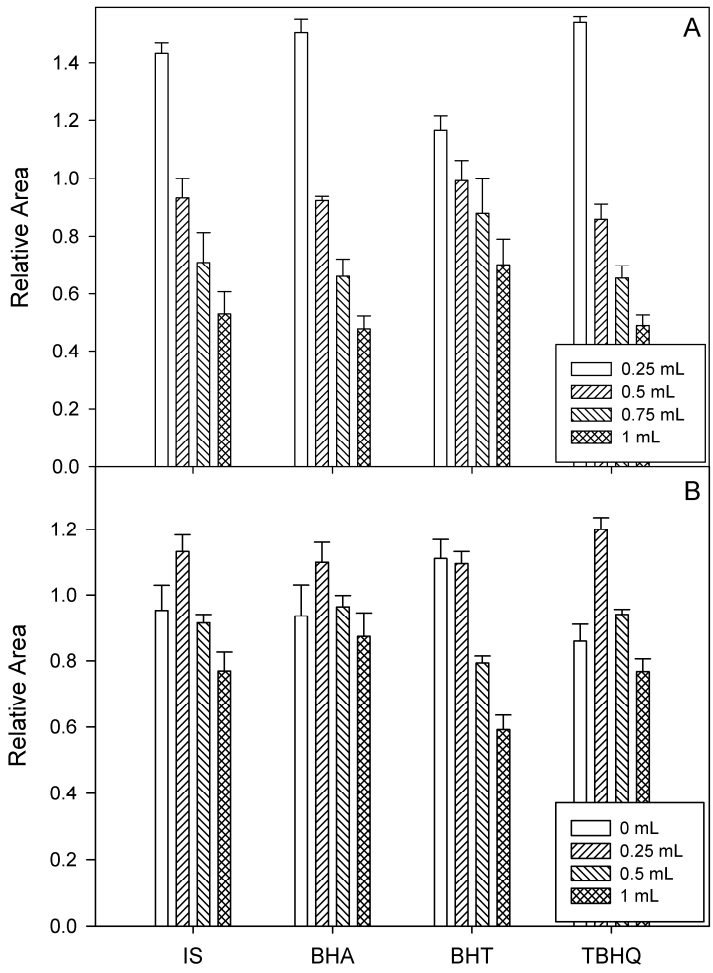


Fig. 1. Effect of the (**A**) ACN and (**B**) hexane volume in the sample extraction step.

An ultrasonic probe processor was used to create an emulsion between the oil sample and the ACN, increasing the interface area and reducing the extraction time of the analytes. Different parameters affecting the efficiency of this ultrasound (US) extraction step, including duration, amplitude and duration of the US cycle, were optimized using a Taguchi orthogonal design (Fig. 2). Each variable was considered at three levels, leading to a total of 9 experiments, which were carried out in duplicate.

Time values ranging from 5 to 20 s, were tested for the duration of the US cycle. Times longer than 20 s were not assayed because the resulting heating of the sample may cause antioxidant degradation and solvent losses. Amplitude was assayed at values ranging 70 – 105 µm, and the duration of ultrasounds per cycle was tested between 0.2 and 0.6 s. Times higher than 0.6 s led to the detachment of the sonotrode. Best results were obtained when 20 pulses of ultrasounds were applied with a duration of 0.6 s and an amplitude of 105 µm.

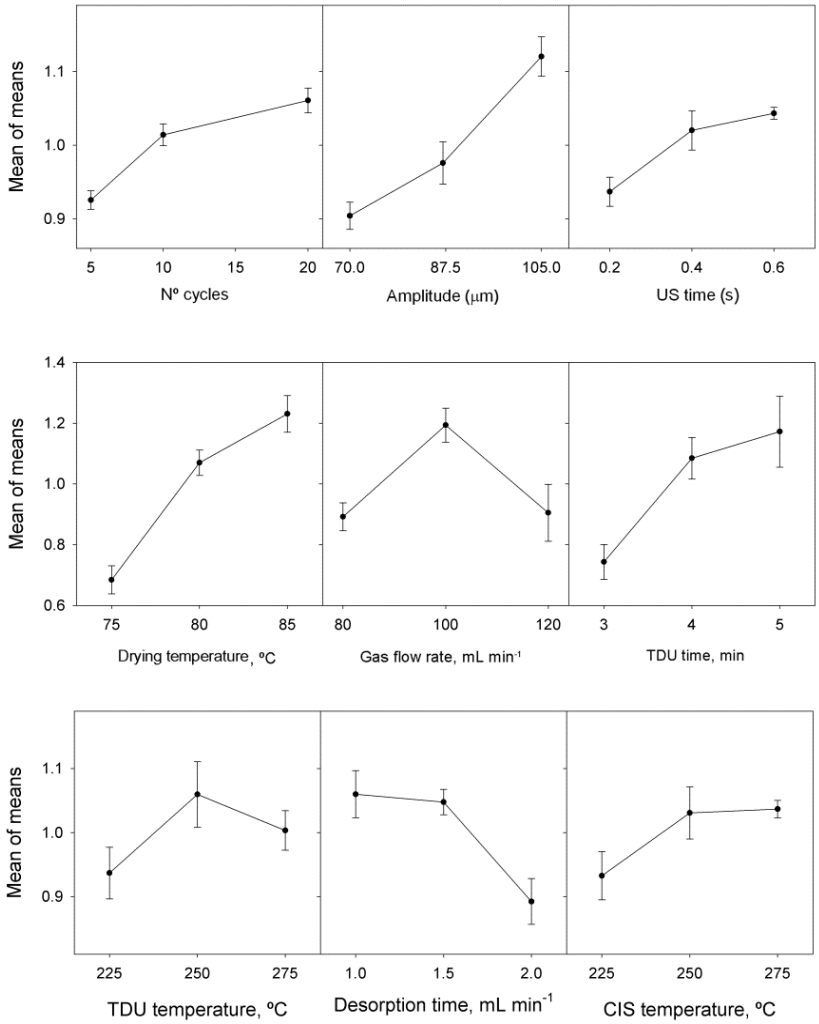


Fig. 2 Effects of Taguchi factor levels: US time, US amplitude, US cycle duration, drying temperature, gas flow rate, drying time, TDU desorption temperature, desorption time and CIS heating temperature; on mean relative response.

In order to break the fine droplets of the extracting solvent and edible oil emulsion, a centrifugation step at 3000 rpm for 2 min was applied, which efficiently separated both phases, and allowed the recovery of the ACN layer.

The efficiency of the liquid-liquid extraction procedure can be checked from the ratio of the slopes of the calibration graphs obtained by the standard additions method applied to an edible oil sample and those obtained by direct injection of ACN solutions of the antioxidant standards at the same concentrations. These values were 0.70, 0.78 and 0.83, for TBHQ, BHA and BHT, respectively.

3.2. Direct sample introduction by microvial insert large volume injection

The ACN employed for sample extraction was removed by heating, avoiding its entry to the chromatographic system, where large solvent amounts may damage the column coating or components of the mass spectrometer.

GC, and therefore TDU/CIS, usually operates over atmospheric pressure to ensure an adequate gas carrier flow through the capillary column. This increase in pressure leads to a higher boiling point of the extracting solvent that may hinder its evaporation. Since this overpressure is used for the TDU pneumatic blockage, an excessive reduction of its value may lead to instrumental problems. For the described system, a value of 0.02 MPa seems to be the minimum achievable value for ensuring a normal operation, so this pressure value was maintained during the drying step.

The Taguchi experimental method allows the optimization of experimental conditions in an efficient way. Multiple factors can be considered together by a balanced orthogonal array design according to the number of parameters, identifying the optimum conditions and levels of all the considered parameters. Three different drying temperatures (75, 80 and 85 ºC), times (3, 4 and 5 min) and venting gas flow rates (80, 100 and 120 mL min^{-1}) were tested following the Taguchi design, with a set of 9 experiments carried out in duplicate. For this purpose, a 50 µL ACN solution containing analyte concentrations of 5 ng mL^{-1} was submitted to the described procedure. The use of this volume assures no ACN leaking to the CIS, while maintains a high sensibility, and allows, if necessary, further injections of the obtained extract. Although evaporation of the extraction solvent is facilitated at higher temperatures, the application of high gas flow rates for lengthy times may also increase analytes losses, leading to a lack of sensitivity. The obtained responses increased for the studied compounds at higher drying temperatures and times, and intermediate values of vent flow, so 85 ºC, 100 mL min^{-1} and 5 min were chosen. These values, which ensure effective drying and total elimination of the ACN, are significantly higher than the values provided by theoretical models of LVI [27], perhaps as a result of the evaporation area limitation imposed by the microvials size.

Once the drying step was complete, thermal desorption of the compounds was carried out. Three different thermal desorption temperatures (225, 250 and 275 ºC), desorption times (1, 1.5 and 2 min) and CIS heating temperatures (225, 250 and 275 ºC) were assayed following a Taguchi design with a set of 9 experiments carried out in duplicate. In general, higher temperatures and longer desorption times may facilitate the vaporization of analytes from the sample, but reduce the overall efficiency due to retention losses in the PTV injector. The obtained responses were maximal at a desorption temperature of 250 ºC, which was selected, while the analytical signal decreased significantly at longer desorption times, so the lowest assayed value, 1 min, was selected.

Desorbed compounds were focused in the PTV before entering the chromatographic column. Lower temperatures in this device increase their retention efficiency and minimize losses of the more volatile compounds. Since the Peltier unit only allows cooling to temperatures slightly below room temperature, 15 ºC was selected as focusing temperature. A liner filled with the inert support fiberglass was used to facilitate the retention of the analytes in the PTV. Once the desorption step is over, the PTV is heated in order to elute the compounds retained in the liner into the chromatographic column. Taking into account the average response obtained using different temperatures, 275 ºC was selected. Therefore, a temperature programme increasing from 15 to 275 ºC at 540 ºC min^{-1}, with a hold time of 5 min, was applied.

As stated previously, the main advantage of microvial insert LVI over conventional LVI is that even though both techniques allow the injection of large volumes of the edible oil extract into the GC, only microvial insert LVI avoids contamination problems related to the co-extracted oil matrix components. Once optimized, a comparison of these two techniques was carried out using the same spiked oil extract. Evaporation of 50 µL of ACN using conventional LVI over fibreglass liner took, as in microvial insert LVI, some minutes. While obtained responses for the studied compounds were quite similar in both techniques, oil matrix interfering compounds peaks were much higher using conventional LVI, and at least twice in case of linolenic acid, main component of sunflower oil. Microvial insert LVI assures minimal PTV liner and column contamination, minimizing the required maintenance, while providing similar responses to conventional LVI.

Table 2
Method characteristics.

Compound	RT (min)	Monitored ions	RSD[a] (%)	LOD[b] (ng g^{-1})	LOQ[c] (ng g^{-1})
IS	6.2	135, 150 (30), 91(12)	-	-	-
BHT	7.0	205, 220 (25), 206 (10)	4.5	0.04	0.10
BHA	7.9	165, 180 (78), 137 (42)	2.8	0.03	0.08
TBHQ	8.5	151, 166 (52), 123 (32)	2.9	0.04	0.09

Underlined numbers correspond to *m/z* of the target ion, and values in brackets represent the qualifier-to-target ion ratios in percentage. [a] n=10. [b] Calculated for S/N=3. [c] Calculated for S/N=10.

3.3. Method performance

Calibration graphs using the standard additions method were obtained for the three samples (sunflower, olive and corn oils), with good correlation coefficient ($r > 0.99$), and being linear in the 1 to 50 ng g^{-1} range (6 concentration levels in duplicate). The statistical comparison (*t-test*) of the resulting slopes proved the suitability of a matrix matched calibration method, no significant differences being obtained at the 95% confidence level. However, the repeatability of this procedure provided relatively high relative standard deviation (RSD) values, concretely 5.5, 9.5 and 11.9% (n=10) for BHT, BHA and TBHQ, respectively. The use of carvacrol, a natural phenolic antioxidant usually present in oregano and with similar behaviour to the analytes, as IS was tested in order to increase repeatability. In this way, the repeatability was enhanced, with RSD values of 2.8, 2.8 and 4.5% (n=10), corresponding to TBHQ, BHA and BHT spiked in the oil sample at 10 ng g^{-1}. The absence of statistically significant differences between the slopes of standard additions calibrations using IS was newly checked by means of a *t-test*, with "p- value > 0.05".

Detection and quantification limits (LOD and LOQ, respectively) were calculated taking into account a signal-to-noise ratio of 3 and 10 (Table 2). LOD and LOQ values obtained were 0.03 and 0.08 ng g^{-1} for BHA, 0.04 and 0.09 ng g^{-1} for TBHQ and 0.04 and 0.10 ng g^{-1} for BHT, respectively.

Since no reference material was available, the method was validated by recovery assays, fortifying three different oil samples (sunflower, corn and olive) a two concentrations (2.5 and 10 ng g^{-1}). Recoveries were between 86 – 115% and 94 – 108% for the low and the high spiked levels, respectively.

Table 3
Comparison of the proposed method with other previously developed methods for the determination of BHA, BHT and TBHQ in edible oils.

Method	Sample treatment method	Sample consumption and treatment time	Linearity (µg mL^{-1})	LOD (ng mL^{-1})	Ref.
LC-UV	WSVAME[a]	5 mL / 2 min	0.2 – 200	20 – 26	[15]
LC-UV	CPE	2 g / 50 min	1 – 500	1.1 – 5.9	[17]
LC-UV	CPE	2 g / 30 min	1 – 500	2 – 9	[18]
LC-DAD	Dilution with isopropanol-hexane, filtration	~ 0.02 mL / ~0.5 min	8 – 25	180 – 1620	[19]
LC-TOF-MS	Extraction with ACN saturated in hexane	0.25 g / ~25 min	0.05 – 0.8[b]	30[c]	[21]
GC-MS	Extraction with ACN:ethanol and SPE	15 g / ~80 min	0.01 – 20	10[c]	[20]
GC-MS	Microextraction with ACN	0.05 g / ~10 min	0.01 – 20	1 – 4	[22]
GC-MS	Extraction with ethanol	1 g / 35 min	0.1 – 20	0.92 – 11.5	[23]
GC-FID	Dilution with diethyl ether	0.0 3– 0.06 g / ~0.5 min	No data	3300	[24]
Microvial LVI-GC-MS	Extraction with ACN/hexane	1 mL / ~3 min	1 – 50[c]	0.03 – 0.04[c]	This work

[a] Water-contained surfactant-based vortex-assisted microextraction. [b] µg g^{-1}. [c] ng g^{-1}.

A comparison of the proposed method with other methods previously published for the determination of BHA, BHT and TBHQ in edible oils is summarized in Table 3. The proposed method provided the best sensitivity achieved to date, with a relatively low sample consumption and a rapid sample treatment.

3.4. Analysis of samples

The optimized procedure was applied for the analysis of 12 edible oil samples (olive, sunflower, peanut, sesame and corn), so as to cover a statistically representative sample of the most commonly employed edible oils.

Some of the analytes were found in ten of the samples, as shown in Table 4. BHT was the most common phenolic antioxidant in the analyzed samples, being found in nine of them, at concentration levels from 0.64 to 12.7 ng g^{-1}. None of the analyzed samples exceeded the maximum doses established by legal regulations, 200 mg kg^{-1}, for any synthetic phenolic antioxidant or their combinations [10]. The phenolic antioxidant concentrations found in the samples analyzed were lower than values previously reported in the bibliography for other edible oil samples [20,22,23].

Fig. 3 shows a typical chromatogram obtained using the described procedure for a spiked oil sample, illustrating the absence of interfering peaks at the retention time of the analytes, which were identified by comparing their retention times and mass spectra in samples and standard solutions.

Table 4
Analysis of the samples (ng g^{-1}).

Oil type	BHT	BHA	TBHQ
Corn	1.51 ± 0.14	ND	ND
Olive	0.64 ± 0.23	ND	1.12 ± 0.05
Olive	3.66 ± 0.14	10.8 ± 0.18	13.8 ± 0.73
Olive	5.00 ± 0.83	73.7 ± 0.52	25.2 ± 1.11
Olive	1.42 ± 0.10	ND	ND
Peanut	0.68 ± 0.06	ND	1.11 ± 0.25
Sesame	0.75 ± 0.09	2.17 ± 0.05	ND
Sunflower	12.7 ± 0.6	ND	ND
Sunflower	ND	3.86 ± 0.59	ND
Sunflower	1.95 ± 0.16	4.65 ± 0.67	ND

Values are mean ± standard deviation (n=3). ND means not detected.

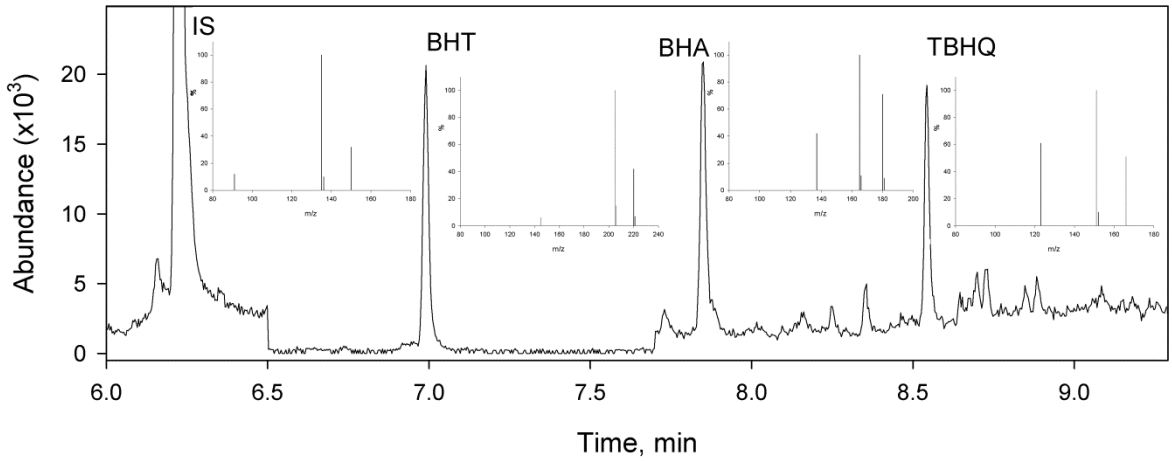


Fig. 3. Elution profile obtained for a sunflower oil sample fortified at 5 ng g^{-1} concentration level for each compound using the proposed procedure under selected ion monitoring (SIM) mode, and mass spectra of each compound.

4. Conclusion

Microvial insert large volume injection coupled to GC-MS greatly increases sensitivity in the determination of three synthetic phenolic compounds in edible oils. The samples are previously submitted to a rapid and simple LLE extraction step using an acetonitrile/hexane mixture assisted by ultrasounds applied by a probe. The repeatability and recovery data obtained demonstrate the suitability of the method for quality control of edible vegetable oils.

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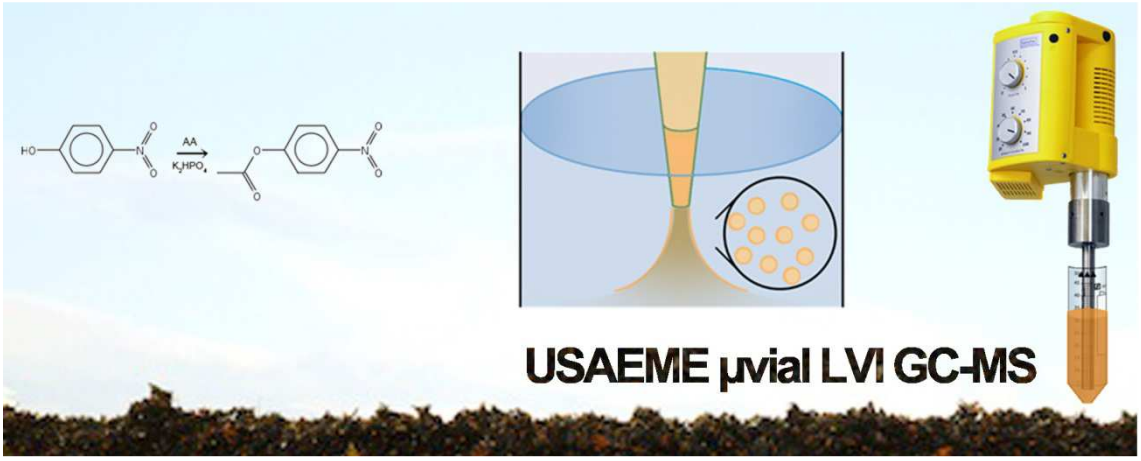
Dispersive liquid-liquid microextraction for the determination of nitrophenols in soils by microvial insert large volume injection-gas chromatography-mass spectrometry

Abstract

A rapid and sensitive procedure for the determination of six nitrophenols (NPs) in soils by gas chromatography and mass spectrometry (GC-MS) is proposed. Ultrasound assisted extraction (UAE) is used for NP extraction from soil matrices to an organic solvent, while the environmentally friendly technique dispersive liquid-liquid microextraction (DLLME) is used for the preconcentration of the resulting UAE extracts. NPs were derivatized by applying an "*in-situ*" acetylation procedure, before being injected into the GC-MS system using microvial insert large volume injection (LVI). Several parameters affecting UAE, DLLME, derivatization and injection steps were investigated. The optimized procedure provided recoveries of 86 – 111% from spiked samples. Precision values of the procedure (expressed as relative standard deviation, RSD) lower than 12%, and limits of quantification ranging from 1.3 to 2.6 ng g^{-1}, depending on the compound, were obtained. Twenty soil samples, obtained from military, industrial and agricultural areas, were analyzed by the proposed method. Two of the analytes were quantified in two of the samples obtained from industrial areas, at concentrations in the 4.8 – 9.6 ng g^{-1} range.



1. Introduction

Nitrophenols (NPs) are not usually found in nature, and their presence in the environment is related with their production or usage. NPs are used in paints, adhesives, explosives, pesticides and pharmaceutical manufacturing [1]. They may also be generated as hydrolysis products of some pesticides, which are alkyl- or cycloalkyl- NPs derivatives, such as dinoseb, 4,6-dinitro-o-cresol or parathion [2]. These pesticides are frequently applied on fruit trees, due to their efficient insecticide action and low toxicity. They can be found in air, due to car exhausts, mainly diesel engine particulate emissions, or be present as secondary contaminants, via further atmospheric chemistry of traffic-emitted precursors [3]. Most remain in groundwater or soil, where they are slowly degraded by microorganisms. Different methyl-NPs may also be found in the environment as degradation products of pesticides, or be generated in atmospheric pollution processes [1].

NPs are poisonous compounds that may irritate the eyes, skin and respiratory tract in humans. In addition, they may lead to the development of methaemoglobinemia [4], a blood disorder that reduces the oxygen transportation capability, affecting organs and tissues. Exposure to NPs mainly results from breathing polluted air in work-place atmospheres, or through drinking polluted water from contaminated areas [1]. NP pollution is particularly serious near former explosives factories and military plants, but low concentrations may be found in waste disposal sites or fungicide-treated farmed fields [1].

Because of their health related relevance, NPs have been determined in air [5–10], water [6,11–21] and soil [4,10,20,22–24], using a variety of analytical techniques, including liquid chromatography (LC) coupled to mass spectrometry (MS) [6,7,22] or to diode array detector (DAD) [4,5,12,14,16,21,23,25], gas chromatography-mass spectrometry (GC-MS) [11,13,17–20,24] and capillary electrophoresis-mass spectrometry (CE-MS) [9].

The determination of NPs in soils is of significance. Soil analysis requires a previous extraction step, in which chemicals are isolated from the soil matrix. Several approaches, including Soxhlet extraction [22], microwave assisted extraction (MAE) [20,22], QuEChERS [24] and ultrasound-assisted extraction (UAE) [4,10], can be found in the literature.

Taking into account the complexity of soil matrices, as well as the low expected concentration of NPs, and thus in the resulting extracts, clean-up and preconcentration steps must be included in the analytical procedure, for which purpose, solid-phase extraction (SPE) [4,10,22] has been previously proposed. Classic preconcentration techniques have been replaced in recent years by new microextraction techniques, which are simpler, cleaner and quicker, and which also involve low solvent consumption. Solid-phase microextraction (SPME) [11,14,19], stir bar sorptive extraction (SBSE) [17], and different liquid phase microextraction (LPME) techniques, such as single drop microextraction (SDME) [18], hollow-fiber (HF-LPME) [9,20,21] or ultrasound

assisted emulsification microextraction (USAEME) [25] have been successfully applied in NP determination.

Dispersive liquid–liquid microextraction (DLLME) [26], in which the organic solvent used as extractant is mixed with a disperser solvent and the mixture is rapidly injected into an aqueous phase, presents the advantage of a great contact surface area between donor and acceptor phases, in such a way that extraction equilibrium is reached almost instantaneously [27]. After extraction, the mixture is centrifuged, the dispersion is disrupted and the enriched phase can be collected for analysis.

When LPME techniques are applied for GC analysis, conventional split/splitless injectors only allow the introduction of a small fraction of the resulting extracting phase, implying a great loss of sensitivity. The use of large volume injection (LVI) systems may overcome this drawback [28], although conventional LVI not only introduces larger volumes of the sample in the GC system, but also, larger amounts of non-volatile matrix compounds. As the injection sequence progresses, sample residues dirty the injector liner, decreasing reproducibility; they may even block the capillary column, which will require frequent maintenance. Microvial insert LVI, whereby the sample is placed in a glass microvial insert which is then introduced in a thermal desorption unit exposed to a programmed temperature vaporization combination (TDU/PTV), may help avoid these problems [29]. Heating the microvial leads to the analytes being vaporized and transferred to the GC, while non-volatile components remain in the glass insert, which is later discarded [30].

In this work, we propose an analytical procedure for the determination of three NPs and three methyl-NPs in soils. Sample treatment is based on UAE and DLLME followed by microvial insert LVI–GC–MS.

2. Materials and methods

2.1. Reagents

2-Nitrophenol (2-NP, 98%), 3-nitrophenol (3-NP, 99%), 4-nitrophenol (4-NP, >99%), 4-methyl-2-nitrophenol (4-M-2-NP, 99%), 5-methyl-2-nitrophenol (5-M-2-NP, 97%), 2-methyl-4-nitrophenol (2-M-4-NP, 97%) and 4-fluoro-2-nitrophenol (4-F-2-NP, 99%, internal standard, IS) were provided by Sigma (St. Louis, MO, USA). Individual stock solutions of the compounds (1000 μg mL^{-1}) were prepared in ultrapure grade methanol, and stored in darkness at −18 °C. Working standard solutions were freshly prepared by diluting the stock solutions with pure water before being stored at 4 °C. Acetic anhydride (AA), sodium chloride and dipotassium hydrogen phosphate were purchased from Fluka (Buchs, Switzerland). Chromatographic quality carbon tetrachloride, chloroform, dichloromethane, acetone, acetonitrile, ethanol and methanol were

obtained from Sigma. Water used was previously purified in a Milli-Q system (Millipore, Bedford, MA, USA). The carrier gas used for GC was helium (Air Liquide, Madrid, Spain).

2.2. Instrumentation

The sample introduction system comprised a thermal desorption unit (TDU-2) equipped with a multipurpose autosampler (MPS-2) and a programmed temperature vaporization (PTV) cooled injector system (CIS-4) provided by Gerstel (Mullheim an der Ruhr, Germany). The experimental conditions used for the sample introduction system are summarized in Table 1.

GC analyses were performed on an Agilent 6890N (Agilent, Waldbronn, Germany) gas chromatograph coupled to an Agilent 5973 quadrupole mass selective spectrometer equipped with an inert ion source. Under the selected conditions (Table 1), the compounds eluted at retention times of between 7.8 and 12.1 min, corresponding to 4-F-2-NP and 2-M-4-NP, respectively (Table 2). In order to improve the limits of detection (LODs), compounds were quantified in the selected ion monitoring (SIM) mode using the ions appearing in Table 2. Identification was confirmed by the retention time of the target ion and the qualifier-to-target ion ratios for each compound. For identification purposes, tolerances of 1 and 10% were applied for retention time and ion ratios, respectively.

An UP 200 H ultrasonic processor (Dr. Hielscher, Teltow, Germany), with an effective output of 200 W in liquid media equipped with a titanium sonotrode (7 mm i.d.), was used for emulsification. An EBA 20 centrifuge (Hettich, Tuttlingen, Germany) was used to disrupt the emulsions.

Table 1
Experimental conditions of the TD-GC-MS procedure.

Thermal Desorption Unit	
Mode	Solvent Venting
Temperature programme	50 ºC, held 0.5 min 50 – 280 ºC at 230 ºC min^{-1}, held 0.5 min
Desorption flow	100 mL min^{-1}
Cooled Injector System	
Mode	Solvent Venting
Liner	Silanized glass wool, 2 mm i.d.
Temperature programme	15 – 300 ºC (5 min) at 540 ºC min^{-1}
GC-MS	
Capillary column	HP-5MS, 5% diphenyl-95% dimethylpolysiloxane (30 m x 0.25 mm, 0.25 μm)
Carrier gas	Helium (1 mL min^{-1})
Oven programme	80 ºC, held 0.5 min 80 – 150 ºC at 10 ºC min^{-1}, held 4 min 150 – 275 ºC at 50 ºC min^{-1}, held 1 min
Transfer line temperature	300 ºC
Quadrupole temperature	150 ºC
Ion source temperature	230 ºC
Ionization	Electron-impact mode (70 eV)

Table 2
Studied compounds characteristics.

Compound	Molecular formula	Molecular mass	log K_{OW}	RT (min)	Monitored ions (*m/z*)
4-Fluoro-2-nitrophenol (4-F-2-NP)	$C_6H_4FNO_3$	157.1	1.75	7.8	43, 157 (35), 82 (26)
2-Nitrophenol (2-NP)	$C_6H_5NO_3$	139.1	1.61	8.8	43, 139 (46), 63 (35)
3-Nitrophenol (3-NP)	$C_6H_5NO_3$	139.1	1.61	9.9	43, 63 (32), 139 (22)
4-Nitrophenol (4-NP)	$C_6H_5NO_3$	139.1	1.61	10.5	43, 63 (32), 139 (28)
4-Methyl-2-nitrophenol (4-M-2-NP)	$C_7H_7NO_3$	153.1	2.12	10.9	43, 153 (53), 77 (38)
5-Methyl-2-nitrophenol (5-M-2-NP)	$C_7H_7NO_3$	153.1	2.12	11.3	43, 153 (81), 77 (42)
2-Methyl-4-nitrophenol (2-M-4-NP)	$C_7H_7NO_3$	153.1	2.12	12.1	43, 153 (78), 77 (36)

Underlined numbers correspond to *m/z* of the target ion, and values in brackets represent the qualifier-to-target ion ratios in percentage. [a] n=10. [b] Calculated using S/N=10.

2.3. Samples and analytical procedure

A total of twenty soil samples were obtained from different agricultural, industrial and military areas from the south-east of Spain. Samples were collected from the surface (top 20 cm), dried overnight at room temperature, manually ground and passed through a 2 mm sieve. To optimize the sample procedure three soil samples were used (soil samples A, B and C). These were characterized as 29.1, 32.9 and 31.7% clay, 33.4, 30.2 and 23% silt, 37.5, 36.9 and 45.3% sand, 0.22, 0.51 and 0.17% total nitrogen, 0.9, 1.8 and 1.8% total organic carbon, 7.9, 7.3 and 7.7 for the pH measured in aqueous (1:1) extracts, and 1300, 7300 and 6400 μS cm^{-1} electrical conductivity for the extracts measured at 25 ºC, respectively [31].

For the extraction of the analytes from the solid matrices, 3 mL of methanol were added to 1 g soil previously weighed in a centrifuge tube and to which the IS has been added at 20 ng g^{-1}. The mixture was submitted to UAE by means of a probe directly immersed into the suspension for 20 s with 0.5 s pulses of 105 μm amplitude. Centrifugation for 1 min at 3000 rpm provided a methanolic supernatant (~2.5 mL), which was recovered and used as dispersant solvent in the subsequent DLLME step. To this methanolic phase, 300 μL of dichloromethane and 150 μL of acetic anhydride were added as DLLME extracting solvent and derivatization reagent, respectively. The resulting mixture was rapidly injected by means of a syringe into a conical bottomed glass centrifuge tube containing 10 mL water and 280 mg of K_2HPO_4, used as neutralizing salt for the derivatization step. A cloudy solution was obtained consisting of very fine droplets of CH_2Cl_2 dispersed through the aqueous phase. After centrifugation for 2 min at 3000 rpm, the extracting phase sedimented at the bottom of the tube (about 20 μL), was collected and transferred to a glass insert microvial placed inside a desorption tube for injection into the chromatograph by means of the TDU/PTV combination. The whole analytical procedure is schematically represented in Fig. 1.

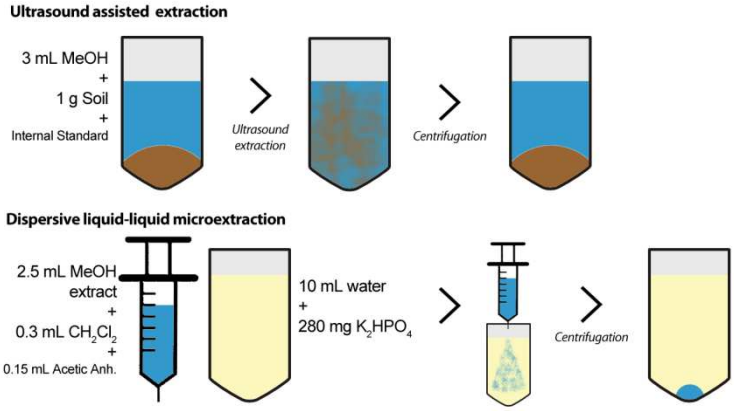


Fig. 1. Schematic diagram of the analytical procedure.

The different variables involved in the extraction step were optimized using soil samples spiked at 100 ng g^{-1}. Linearity was evaluated using samples fortified at levels ranging from 5 to 200 ng^{-1}, while for recovery studies, fortification levels of 10, 20 and 100 ng g^{-1} were employed.

3. Results and discussion

3.1. Ultrasound assisted soil extraction

The application of ultrasounds facilitates and accelerates mass transfer between solid and liquid phases, providing high recoveries in short times [32]. Consequently, ultrasound assisted extraction (UAE) was assayed to extract NPs from soil matrices. The different variables involved in this extraction step were optimized using 1 g of soil spiked at 100 ng g^{-1}.

As could be expected, NPs were extracted into aqueous media, especially at basic pH that lead to deprotonation of their hydroxyl group. Moreover, the relatively low polar character of these organic compounds, with log K_{ow} values ranging from 1.6 to 2.2, enables their extraction into organic solvents. Different extracting phases (1 mL), including several organic solvents, such as acetone, acetonitrile, methanol and ethanol, as well as different aqueous 0.2 M buffer solutions, including acetic acid/acetate (pH 4.8), dihydrogenphosphate/hydrogen phosphate (pH 7.2) and hydrogencarbonate/carbonate (pH 9.8), were tested. The results obtained showed the advantage of using organic solvents rather than aqueous buffer solutions, methanol providing the highest responses (Fig. 2) and thus the highest recoveries from soil samples for all the compounds. Therefore, methanol was selected as extraction solvent.

The volume of the extracting solvent may not only influence UAE efficiency, but may also be relevant during the DLLME step. Taking this into account this parameter was optimized simultaneously with DLLME optimization.

Anhydrous sodium sulfate (Na_2SO_4) is usually added to soil samples during the extraction step to remove any remaining water [10]. The addition of 0-1 g of this salt to 1 g sample was tested (Fig. 2). For all compounds, the obtained response decreased with the addition of Na_2SO_4, probably due to retention of the analytes by this salt, so its use was discarded.

Ultrasounds energy was applied by means of a probe directly immersed into the soil/solvent mixture. Different ultrasound application times, ranging from 10 to 30 s, were assayed. Extraction equilibrium between solid and liquid extracting phases was reached after 20 s, so this time was selected (Fig. 2).

3.2. Acetylation procedure

Due to the acid character of their phenolic group, NPs show poor chromatographic behaviour, with tailing peaks. In addition, their relative hydrophilicity, with low K_{ow} values, may lead to low extraction efficiencies into non-polar extractants. The inclusion of a derivatization step in the analytical procedure helps to overcome these disadvantages. Among the different derivatization reaction possibilities, *in-situ* acetylation was selected, since the widely used silylation technique [8,11,17–20] requires water-free media.

Acetylation of NPs improves their peak shape and chromatographic separation and, in addition, reduces their polarity, increasing their affinity for the DLLME extraction solvent, leading to greater recoveries and higher enrichment factors. The acetylation reaction, which is an efficient, simple and fast derivatization procedure, can be easily carried out using acetic anhydride (AA) in basic medium. However, this derivatization is only suitable for mono-NPs and does not provide satisfactory results when applied to di-NPs [18].

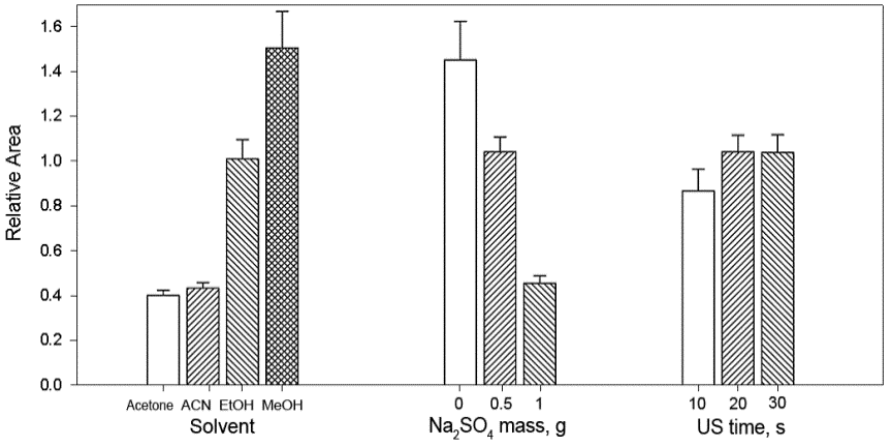


Fig. 2. Influence of different UAE parameters on NP mean responses. Error bars indicate standard dev. n=3.

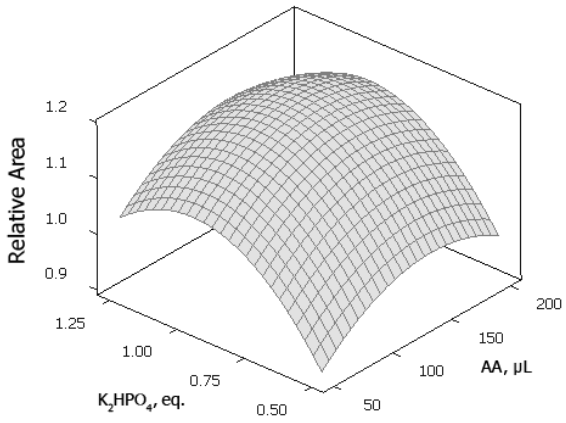


Fig. 3. Response surface obtained in the optimization of acetylation conditions.

Dipotassium hydrogen phosphate was added in order to ensure the basic medium required for the derivatization reaction, as well as to fix the sample pH at a neutral value during the extraction, once the analytes have been derivatized. Since the derivatization agent volume and the neutralizing equivalents of K_2HPO_4 added to the sample are related experimental variables, their influence on the NP responses was evaluated using a Central Composite Design (CCD) and response surface analysis by quadratic polynomial regression, which provide linear and quadratic terms and first order interactions, allowing fitting optimum values.

This CCD experimental set ($\alpha = 1.5$, 4 cube points, 4 axial points and 2 central points, in duplicate) was developed with AA volumes ranging from 50 to 200 µL and different masses of K_2HPO_4, in the 140-350 mg range (corresponding to 0.5 to 1.25 neutralizing equivalents for 150 µL of AA). The resulting mixture was manually shaken for 20 seconds while derivatization takes place, and afterwards submitted to microextraction. The obtained response surface (Fig. 3) closely reflected the experimental data ($r^2 > 0.95$) and underlined the significance of the tested variables ($p < 0.01$). Best responses for all the analytes were obtained when 150 µL of AA were used as derivatization agent volume and 280 mg of K_2HPO_4, corresponding to 1 neutralizing equivalent, were added to the sample.

3.3. Dispersive liquid-liquid microextraction

The conventional DLLME procedure must be adapted to the nature of the sample containing the NPs, a methanol extract resulting from the UAE of the soil. In this modified DLLME procedure, the analytes present in a methanol solution must be concentrated in an organic solvent, thus dispersing the mixture of both solvents in an aqueous phase. The influence of the nature and volume of the extractant solvent as well as disperser volume on the extraction efficiency was studied.

Three different solvents (dichloromethane, chloroform and carbon tetrachloride) were tested as extracting phase. All of them are miscible with methanol, immiscible with water and have higher density than water, allowing their recovery as a settled drop after centrifugation of the cloudy solution. Different volumes were assayed in order to obtain a settled drop of 50 µL after DLLME. The extraction efficiency for most compounds was higher when dichloromethane was used, so this was selected (Fig. 4).

The volume of dichloromethane as well as the volume of methanol, the latter used as extracting solvent in UAE and as disperser solvent in DLLME, were optimized simultaneously using a CCD. The experimental set ($\alpha = 1.5$, 4 cube points, 4 axial points and 2 central points, in duplicate) was developed using CH_2Cl_2 and methanol volumes in the 300–400 µL and 1–3 mL range, respectively.

As expected, the higher the dichloromethane volume, the lower the sensitivity, due to the dilution effect in larger drops. Volumes lower than 300 µL were not assayed because less than 20 µL were recovered after centrifugation, hindering drop collection when using a microsyringe. On the other hand, methanol volumes lower than 1 mL may lead to low UAE extraction efficiency and poor DLLME emulsion formation, while large methanol volumes may dilute UAE extracted compounds, increase analyte solubility in the aqueous medium, and thus reduce DLLME extraction efficiency. The obtained responses provided the response surface plot ($r^2 > 0.95$, $p < 0.01$) shown in Fig. 5, which reflected the described behavior. Consequently, 300 µL and 3 mL were the volumes selected for CH_2Cl_2 and methanol, respectively.

In order to aggregate the fine droplets of the extractant solvent, a centrifugation step at 3000 rpm for 2 min was applied, which efficiently allowed the recovery of the settled phase.

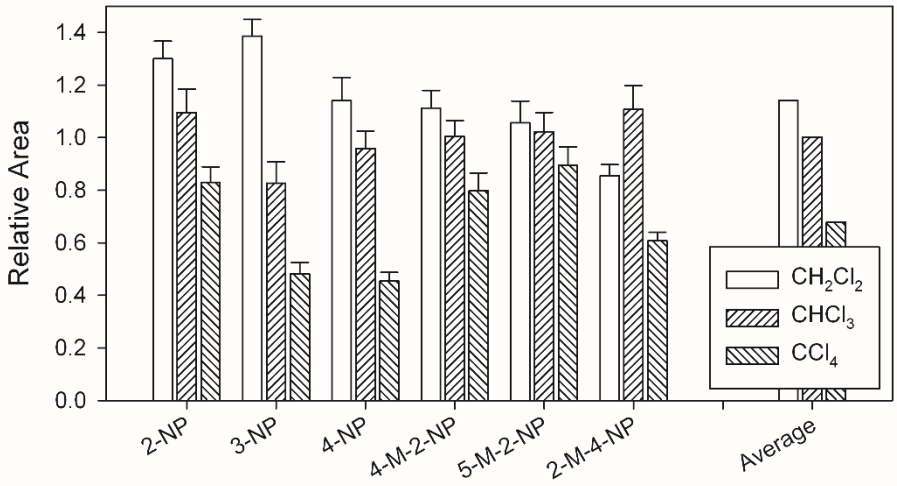


Fig. 4. Influence of DLLME extractant solvent nature on analyte responses. Error bars indicate standard deviation for n=3.

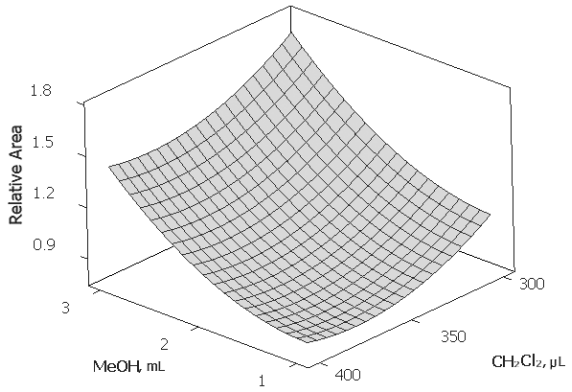


Fig. 5. Response surface obtained in the optimization of DLLME conditions.

3.4. Microvial insert large volume injection

The dichloromethane employed for DLLME preconcentration was removed by heating, preventing its entry into the chromatographic system, where large solvent amounts may damage the column coating or components of the mass spectrometer. For this purpose, the microvial placed inside the TDU was heated at 50 ºC for 0.5 min while a 100 mL min^{-1} helium gas flow was applied.

After the drying step, thermal desorption of the analytes was carried out. The Taguchi experimental method allowed efficient optimization of the experimental conditions. Multiple factors can be considered together in a balanced orthogonal array design depending on the number of parameters, permitting the optimum conditions and levels of all the considered parameters to be identified. Three different thermal desorption temperatures (260, 280 and 300 ºC), desorption times (0.5, 1 and 2 min), gas flow rates (50, 75 and 100 ºC), and CIS heating temperatures (260, 280 and 300 ºC) were assayed following a Taguchi design with a set of 9 experiments carried out in duplicate. Generally, higher temperatures and longer desorption times may facilitate analyte vaporization, but could reduce the overall efficiency due to retention losses in the PTV injector. The obtained responses were maximal with a desorption temperature of 280 ºC, which was selected, while the analytical signal decreased significantly at longer desorption times, so the lowest assayed value, 0.5 min, was selected.

A carrier gas is necessary to propel the analytes to the PTV injector while they are being thermally vaporized. TDU vaporization and subsequent PTV focusing of the NPs may be influenced by this flow-rate gas. High flow rates led to efficient propulsion of the vaporized compounds, but increased their speed through the PTV, reducing their residence time, and thus the retention efficiency. Best responses were obtained when a flow of 100 mL min^{-1} was applied.

Low temperatures in the PTV increase the retention efficiency and minimize losses of the more volatile compounds. Since the Peltier unit only allows cooling to temperatures slightly below room temperature, 15 ºC was selected as focusing temperature. A liner filled with the inert support fiberglass was used to facilitate the retention of the analytes in the PTV. After the desorption step,

the PTV is heated in order to elute the compounds towards the chromatographic column. Taking into account the average response obtained using different temperatures, 300 ºC was selected.

3.5. Method performance

For quantification purposes, 4-F-2-NP was used as internal standard (IS). The presence of this compound in soils has not previously been reported, and confirmation of its absence was checked in the analyzed samples.

The standard additions method was applied to three different soil samples (soils A, B and C), whose characteristics are reported in section 2.3, using the selected experimental conditions. An ANOVA statistical test was applied to compare the obtained slopes in order to test the presence of a matrix effect in the samples. The slopes of the standard additions calibration graphs were similar, with "p" values higher than 0.05 when IS was used, but not when IS was not added to the samples, confirming that the use of this compound for calibration purposes reduces the interferences related with the sample matrix (Table 3). The quantification was carried out by matrix-matched calibration by plotting peak area ratios of each analyte with respect to the IS versus analyte concentration. Calibration graphs showed good linearity in the studied range, 5–200 ng g^{-1}, with correlation coefficients higher than 0.99 in all cases.

In order to check the repeatability of the method, ten replicate analyses were performed using a soil sample spiked at 20 ng g^{-1} for each compound. RSD values ranged from 4.2 to 11.1% (Table 4), using the IS, and from 12 to 19%, when no IS was considered, depending on the compound.

Limits of detection (LODs) and limits of quantification (LOQs) were calculated using a signal-to-noise ratio of 3 and 10, respectively. LOD and LOQ values in the 0.4 – 0.8 and 1.3 – 2.6 ng g^{-1} ranges, depending on the compound, were obtained, respectively (Table 4).

The sensitivity using the optimized experimental conditions was compared with a conventional splitless injection mode. When the slopes obtained for standard additions calibration graphs were compared, the microvial insert LVI procedure provided slopes between 6.5 and 7.5-fold higher, depending on the compound.

Table 3
Slopes[a] (g ng^{-1}) of the standard additions calibration graphs obtained for different soils in the presence and in the absence of the internal standard.

	Soil A		Soil B		Soil C	
Compound	Without IS	With IS	Without IS	With IS	Without IS	With IS
2-NP	5335 ± 579	0.096 ± 0.012	6880 ± 646	0.107 ± 0.013	7469 ± 295	0.113 ± 0.010
3-NP	4948 ± 372	0.086 ± 0.003	4940 ± 323	0.077 ± 0.005	5272 ± 292	0.080 ± 0.004
4-NP	5052 ± 484	0.090 ± 0.007	5323 ± 519	0.083 ± 0.010	5167 ± 383	0.078 ± 0.003
4-M-2-NP	6996 ± 630	0.125 ± 0.009	9134 ± 916	0.143 ± 0.019	9018 ± 777	0.136 ± 0.005
5-M-2-NP	8086 ± 406	0.145 ± 0.007	9433 ± 870	0.147 ± 0.013	10365 ± 910	0.157 ± 0.008
2-M-4-NP	6810 ± 494	0.122 ± 0.006	7586 ± 595	0.118 ± 0.007	7538 ± 398	0.114 ± 0.009

[a] Mean value ± standard deviation (n=6).

Table 4
Method characteristics.

Compound	RSD[a] (%)		Recovery [b] (%)			LOD[c] (ng g^{-1})	LOQ[d] (ng g^{-1})
	Without IS	With IS	10 ng g^{-1}	20 ng g^{-1}	100 ng g^{-1}		
2-NP	12.2	4.2	98 ± 6	105 ± 3	101 ± 2	0.6	2.1
3-NP	15.3	7.0	98 ± 3	94 ± 7	97 ± 2	0.8	2.5
4-NP	17.8	11.1	111 ± 6	103 ± 5	105 ± 2	0.8	2.6
4-M-2-NP	18.5	8.1	99 ± 3	104 ± 2	101 ± 1	0.4	1.4
5-M-2-NP	17.2	5.9	86 ± 3	107 ± 3	98 ± 1	0.5	1.6
2-M-4-NP	16.0	8.2	100 ± 4	101 ± 3	100 ± 1	0.4	1.3

[a] n=10. [b] Mean value ± standard deviation (n=9) [c] Calculated using S/N=3. [d] Calculated using S/N=10.

The higher sensitivity of the microvial insert large volume injection procedure was due to the greater amount of sample injected in the GC-MS system (20 µL *versus* 2 µL), which is reflected in the obtained increase in the slopes. In addition, since most of the non-volatile matrix components were retained in the microvial during the desorption step, an increase in the signal-to-noise ratio of the chromatographic peaks (between 3.6 - 4.9 times higher) was also obtained.

A comparison of the proposed method with others previously published for the determination of nitrophenols in soil samples is summarized in Table 5. The DLLME-LVI-GC-MS method provided the best sensitivity achieved to date.

3.6. Analysis of samples and recovery studies

The proposed method was applied to the analysis of twenty different soil samples, which had been obtained from military, industrial and agricultural areas. In two of these samples, corresponding to an industrial zone, 2-NP and 4-M-2-NP were found. The concentrations found were 5.5±0.6 and 9.6±0.3 ng g^{-1} for 2-NP, and 4.8±0.3 ng g^{-1} for 4-M-2-NP.

Table 5
Comparison of the proposed method with others previously developed for the determination of nitrophenols in soil samples.

Method	Extraction technique	Preconcentration technique	Sample consumption and treatment time	Linearity (ng g^{-1})	LOD (ng g^{-1})	Ref.
GC-MS	SLE	SPE	5 g / ~ 30 min	200 - 20000	200 - 1000	[10]
GC-MS	MAE	HF-LPME	1 g / ~ 45 min	20 - 2000	2 - 4	[20]
GC-MS/MS	QuEChERS	---	10 g / ~ 1 hour	50 - 300	20 - 50	[24]
GC-MS	UAE	DLLME	1 g / ~ 10 min	5 - 200	0.4 - 0.8	This work

DLLME: Dispersive liquid-liquid microextraction; HF-LPME: hollow-fiber liquid-phase microextraction; MAE: Microwave-assisted solvent extraction; QuEChERS: quick, easy, cheap, effective, rugged and safe; SLE: Solid-liquid extraction; SPE: Solid-phase extraction; UAE: Ultrasound assisted extraction.

In order to check the accuracy of the method, recovery assays were performed by fortifying three soil samples at three concentration levels of 10, 20 and 100 ng g^{-1}. Recoveries varied from 86 to 111% at the lowest level and from 97 to 105% at the highest level (Table 4).

Fig. 6A shows a typical chromatogram obtained under SIM mode for a soil sample fortified at 20 ng g^{-1}, in the selected conditions; while Fig. 6B shows the chromatogram obtained for one of the polluted samples.

The analytes were identified by comparing the retention time, identifying the target (*T*) and qualifier ions and qualifier-to-target ratios in the mass spectra of the peaks (Fig. 4), in samples, spiked samples and standard solutions. The major ions were attributed to acetyl and phenyl fragments for *m/z* values of 43 and 77, respectively.

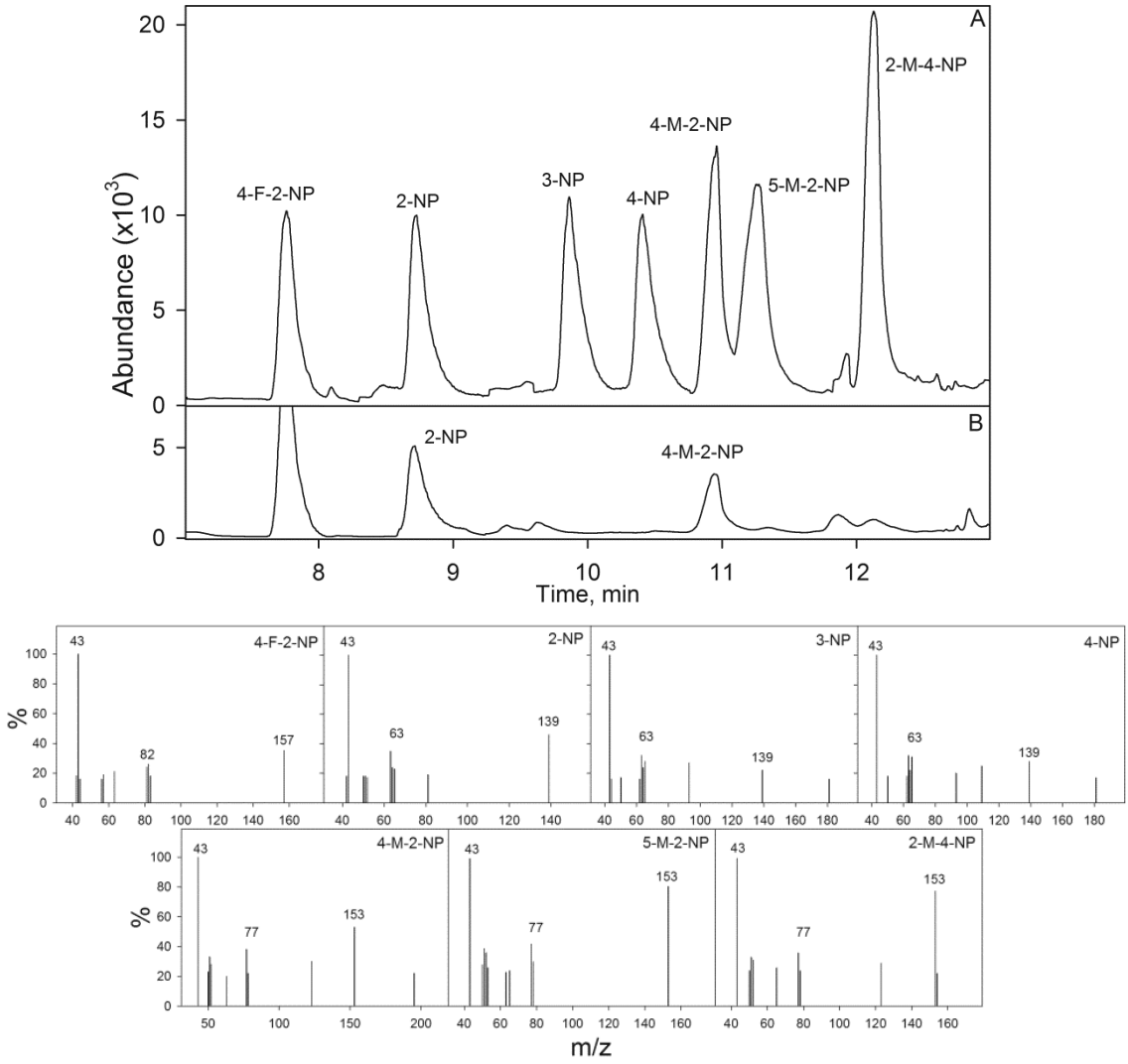


Fig. 6. Chromatogram obtained by UAE combined with DLLME and microvial insert LVI-GC-MS for a spiked soil sample (20 ng g^{-1}) (A) for a non-spiked soil. Mass spectra of the studied compounds.

4. Conclusion

Microvial insert LVI facilitated the GC-MS analysis of the whole extract obtained by DLLME. UAE by means of a probe provided efficient extraction of the analytes from the soil matrices with a low volume of organic solvent. Consequently, the combination UAE and DLLME with microvial insert LVI led to LOD at the sub ng g^{-1} levels to be obtained for the analysis of six NPs in soil samples.

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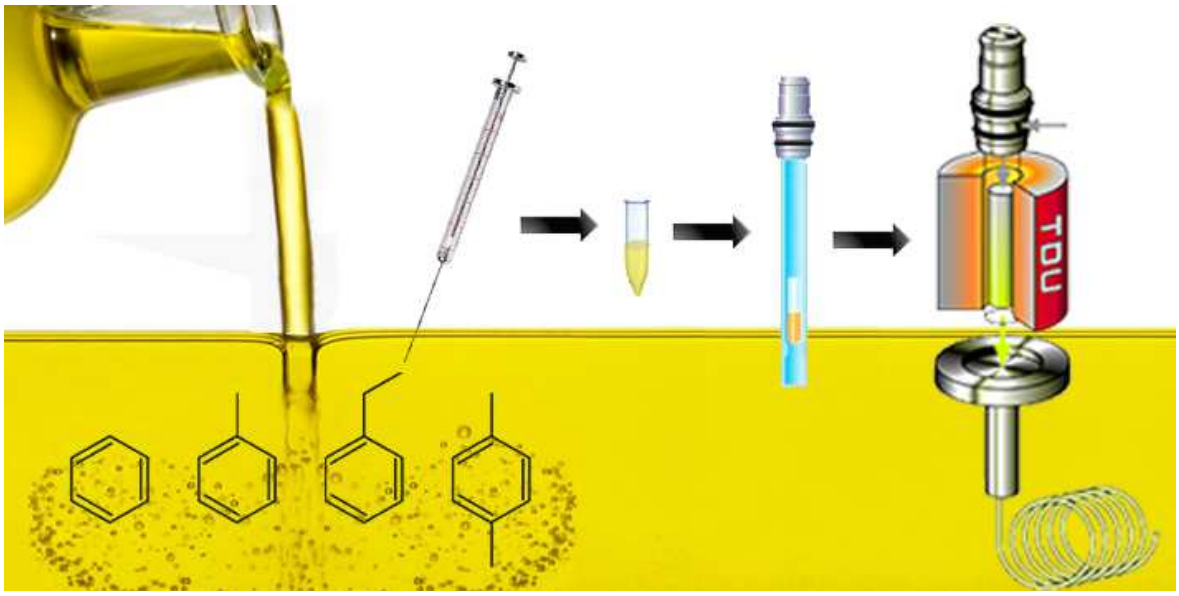
Gas chromatography-mass spectrometry using microvial insert thermal desorption for the determination of BTEX in edible oils

Abstract

Microvial insert thermal desorption is evaluated as a sample introduction system for gas chromatography-mass spectrometry (GC-MS) determination of benzene, toluene, ethylbenzene and xylene isomers (BTEX) in vegetable oil samples. Under the optimized conditions, the BTEX contained in a 50 µL oil sample placed in the microvial insert were thermally vaporized at 140 ºC for 5 min while helium at a flow of 120 mL min^{-1} and pressure of 4.3 psi propelled the analytes to the programmable temperature vaporizator (PTV), where they were focused at 15 ºC. The compounds were finally injected into the GC column by rapidly heating the PTV at 270 ºC. The proposed method provided good linearity between 10 and 200 µg L^{-1}. High sensitivity, with detection limits in the 0.7 – 1.2 µg L^{-1} range, depending on the compound, was achieved with a low sample volume and minimum sample handling. Fourteen edible vegetable oil samples were analyzed and the analytes were found in a wide concentration range.



1. Introduction

Benzene, toluene, ethylbenzene and xylene isomers, commonly known as BTEX, are volatile organic compounds commonly found in a wide range of petroleum derivatives. BTEX are emitted to the environment during a large variety of processes, including the combustion of wood and fuels, or as consequence of their use as additives in industrial adhesives, degreasing agents, dyes, resins, detergents or paints [1]. The sum of the concentrations of these chemicals is used as an indicator of contamination, particularly in the case of soil or groundwater. Apart from harming the environment, they tend to accumulate in foodstuffs because of their lipophilic nature, especially in fatty products and edible oils [2]. A study of the origin of BTEX in edible vegetable oils concluded that both contamination and biosynthetic reactions taking place during vegetable maduration are responsible [3].

BTEX are health hazards due to their toxic characteristics. Benzene has been identified as a carcinogenic agent, involved in the development of leukaemia, for which reason it has been included in the International Agency for Research on Cancer (IARC) Group 1. Ethylbenzene has also been classified as a possible carcinogenic agent and is included in IARC Group 2, while toluene and xylene, neither of which has shown any carcinogenic effect to date, are included in IARC Group 3. In addition, toluene, ethylbenzene and xylenes have harmful effects on the central nervous system. When chronically ingested, all BTEX may cause liver and kidney damage [4]. Such toxicological data, joined to the fact that BTEX have been detected in edible oil samples, led the European Union Commission Scientific Committee on Food to express concern about dietary exposure to these compounds through olive oil [5]. Nevertheless, no regulations have been introduced related to the presence of BTEX in these food products.

Analysis of the headspace (HS) of samples by gas chromatography-mass spectrometry (GC-MS) [3,6-10] is the analytical strategy most widely used for the determination of BTEX in edible oils, although direct HS-MS coupling [9,11] has also been used for the global determination of BTEX. In order to minimize matrix interference and to improve the separation of the analytes from oil sample, some authors include a step prior to HS, for example liquid-liquid extraction (LLE) [6] or distillation [10].

HS sampling, despite being a clean and simple technique, usually requires an equilibrium time of between 15 and 30 min. This implies that sensitivity is limited by the relative vapour pressure of the studied compound, meaning that the technique is only applied to volatile analytes at relative high concentrations. Solid-phase microextraction (SPME) in the headspace mode has also been proposed for the determination of all or some BTEX compounds in oils [12-14]. From those published SPME procedures, only Vichi et al. [14] provide the method performance.

Amirav and Dagan developed the named direct sample introduction (DSI) injection GC technique [15], which, among large volume injection (LVI) techniques, can be catalogued as rapid, sensitive, simple and inexpensive. When applied to liquid samples, the technique is also known as microvial insert thermal desorption or microvial insert large volume injection [16]. The liquid sample, with a volume of up to 150 μL, is placed in a glass microvial and introduced into the programmed temperature vaporizator (PTV) or a thermodesorption unit (TDU) attached to the PTV inlet. Non-volatile interfering matrix components remained in the vial, which can be removed and discarded after each assay, while volatile compounds are vaporized and transferred to the GC column for separation [17]. DSI has previously been used for the determination of pesticides in eggs and vegetables [18,19], odour-related compounds in wine and fruits [16, 20] and phthalate esters in cleaning products [21]. The literature mentions the application of DSI for the determination of volatile compounds in oils [22], but only toluene is included in the characterization and no quantitative data are provided.

In this work, we propose a microvial insert thermal desorption GC-MS method for the determination of BTEX in different vegetable oil samples. The use of microvial insert large volume injection allows introduction of the edible oil into the chromatographic system and the selective vaporization of the BTEX, while avoiding any contamination problem related to the oil matrix components.

2. Experimental

2.1 Chemicals and reagents

A benzene, toluene, ethylbenzene, o-xylene, m-xylene and p-xylene solution, each at 200 mg L^{-1} in methanol, was obtained from Sigma (St. Louis, MO, USA). Working solutions (2 mg L^{-1}) were prepared by diluting the commercial products with acetone, and kept at −18 ºC in darkness. Pure grade acetone was also provided by Sigma. Helium (99.9999% purity) was supplied by Air Liquide (Madrid, Spain)

2.2 Instrumentation

The sample introduction system was composed of a Thermal Desorption Unit (TDU-2) equipped with an autosampler (MPS-2) and a Programmed Temperature Vaporization (PTV) Cooled Injector System (CIS-4) provided by Gerstel (Mullheim an der Ruhr, Germany). The experimental conditions used for the sample introduction system are summarized in Table 1.

Table 1
Experimental conditions of the TD-GC-MS procedure

Thermal Desorption Unit	
Mode	Splitless
Temperature programme	40 – 140 ºC at 200 ºC min^{-1}, held 5 min
Desorption flow	120 mL min^{-1}
Desorption flow pressure	4.3 psi
Cooled Injector System	
Mode	Solvent venting
Liner	Graphitized carbon black, 2 mm i.d.
Temperature programme	15 – 270 ºC (5 min) at 540 ºC min^{-1}
GC-MS	
Capillary column	HP-5MS, 5% diphenyl-95% dimethylpolysiloxane (30 m x 0.25 mm, 0.25 µm)
Carrier gas	Helium (1 mL min^{-1})
Oven programme	40 ºC, held 5 min 40 – 50 ºC at 2.5 ºC min^{-1}, held 1 min 50 – 240 ºC at 50 ºC min^{-1}, held 1 min
Transfer line temperature	300 ºC
Quadrupole temperature	150 ºC
Ion source temperature	230 ºC
Ionization	Electron-impact mode (70 eV)

GC analyses were performed on an Agilent 6890N (Agilent, Waldbronn, Germany) gas chromatograph coupled to an Agilent 5973 quadrupole mass selective spectrometer equipped with an inert ion source. Under the selected conditions (Table 1) the compounds eluted at retention times between 3 and 10 min, corresponding to benzene and p-xylene, respectively (Table 2).

The compounds were quantified in the selected ion monitoring (SIM) mode in order to improve the detection limits using different ions (Table 2). Identification was confirmed by the retention time of the target ion and the qualifier-to-target ion ratios for each compound.

2.3 Samples and analytical procedure

A total of 14 edible oil samples, including corn, olive, sunflower, sesame and peanut, were obtained from local supermarkets. All of the edible oils were sold in plastic (PET) or glass bottles. Once opened, samples were kept in darkness at 4 °C until analysis.

A 50 µL-aliquot of oil was placed in a 150 µL glass microvial insert using a microsyringe, and then introduced into a glass desorption tube. The whole assembly was submitted to the thermal desorption procedure by placing the desorption tube in the TDU and using the experimental conditions given in Table 1.

For validation purposes, three oil samples (corn, olive and sunflower) were spiked at two different concentrations, 20 and 140 µg L^{-1}, and allowed to stand for 1 h before being submitted in triplicate to the analytical procedure.

Table 2
Method characteristics

Compound	RT (min)	Monitored ions (*m/z*)	RSD[a] (%)	LOD[b] (μg L^{-1})
Benzene	3.0	78, 77 (16), 52 (15)	9.9	0.9
Toluene	5.0	91, 92 (78), 65 (12)	6.5	0.7
Ethylbenzene	8.4	91, 106 (37), 51 (9)	7.7	0.9
m-Xylene / p-Xylene	8.9	91, 106 (69), 77 (12)	7.0	1.0
o-Xylene	10.0	91, 106 (63), 77 (10)	6.5	1.2

Underlined numbers correspond to *m/z* of the target ion, and values in brackets represent the qualifier-to-target ion ratios as percentage.
[a] n=10. [b] Calculated using S/N=3

3. Results and discussion

3.1 Optimization of GC-MS conditions

Different temperature programmes were tested to separate the analytes, and the best resolution was achieved when an initial temperature of 40 ºC was maintained for 5 min, which allowed the elution of benzene and toluene; next a ramp of 2.5 ºC min^{-1} was applied to increase the column temperature to 50 ºC, ethylbenzene eluting at 8.4 min and both m- and p-xylene 0.5 min later. This temperature was maintained 1 min to elute o-xylene. All the experiments carried out to separate m- and p-xylene isomers were unsuccessful, as other authors have found [6-8, 13]. Consequently, m- and p-xylene were therefore quantified as the sum of both isomers, taking into account that there are no differences between their ions. The GC-MS analysis of oil samples in the scanning mode (SCAN) in the 40 – 300 *m/z* range allowed identification of the analytes and confirmation of the need to increase the column temperature beyond the elution of o-xylene to elute matrix components of the oil (Fig. 1A). Quantification was carried out in the SIM mode in order to increase sensitivity (Table 2).

3.2 Selection of microvial insert thermal desorption conditions

Microvial insert thermal desorption is a complex step that may be influenced by a large number of experimental variables. The effect of seven of these variables (desorption time and temperature, gas flow-rate and pressure, PTV liner filling and focusing and heating temperature) in the BTEX response and in its ratio *versus* the matrix response was evaluated at different levels. For this purpose, 50 μL of olive oil spiked with each analyte at 100 μg L^{-1} was placed in the microvial and introduced into the gas chromatograph using the TDU system.

Therefore, a temperature programme increasing from room temperature to 140 ºC at 200 ºC min^{-1}, with a hold time of 5 min, was applied in the TDU for vaporization purposes. The behaviour of each analyte with the seven TDU variables studied is shown in Fig. 2.

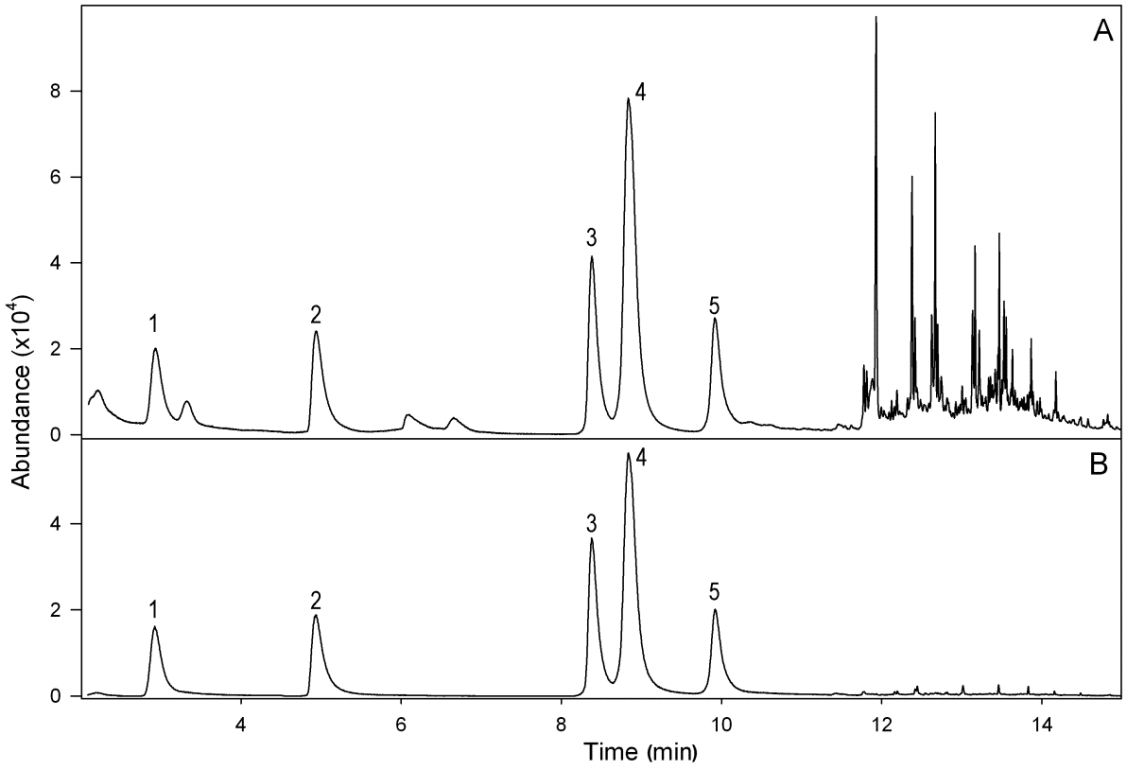


Fig. 1. Microvial insert TD-GC-MS chromatogram obtained for an olive oil sample spiked with the studied compounds at 40 µg L^{-1} in SCAN (A) and SIM (B) modes. (1) Benzene, (2) toluene, (3) ethylbenzene, (4) m,p-xylene and (5) o-xylene.

Five different thermal vaporization temperatures (from 80 to 160 ºC) and vaporization times (from 1 to 5 min) were tested. In general, higher temperatures and longer vaporization times may facilitate the release of analytes from the sample, but may reduce the overall efficiency due to retention losses in the PTV injector, as well as increase interference from the oil matrix. Maximum sensitivity was attained for all compounds, except for toluene, when 140 ºC was applied as desorption temperature, while all analytes increased their responses with at a desorption time of 5 min (Fig. 3).

A carrier gas is necessary to propel the analytes to the PTV injector while they are being thermally vaporized. TDU vaporization and subsequent PTV focusing of the BTEX may be influenced by the flow-rate and pressure of this gas. High flow rates generally lead to the effective propulsion of the vaporized compounds, but increase their speed through the PTV, reducing their residence time, and thus the retention efficiency in this device. Low pressures during the vaporization step facilitate the transfer of the BTEX from the oil sample to the gas phase. Five different flow-rates (ranging from 40 to 120 mL min^{-1}) and pressures (from 4.3 to 10 psi) were tested and, as expected, best responses were obtained for all BTEX when 120 mL min^{-1} and 4.3 psi were applied (Fig. 3).

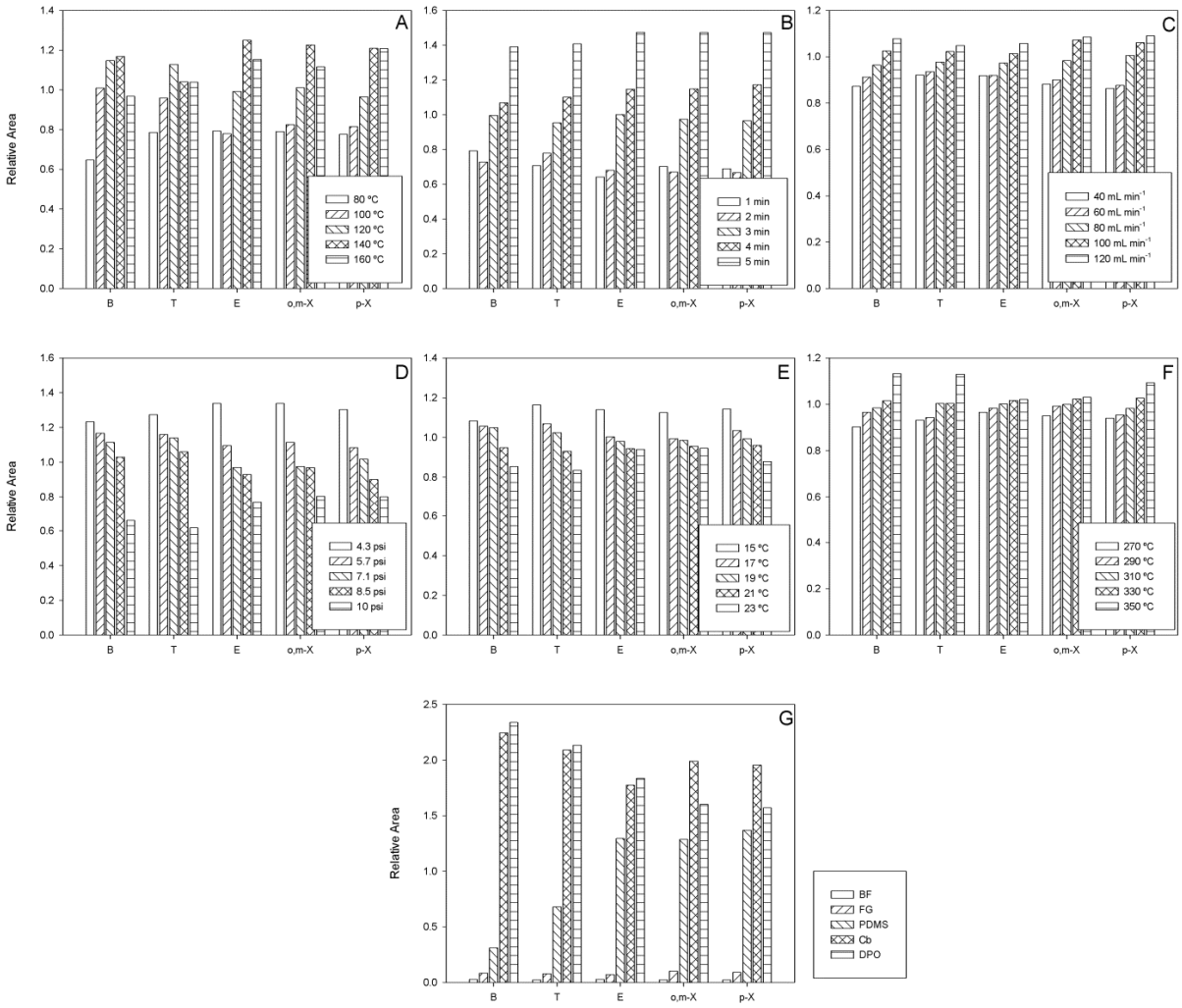


Fig. 2. Effect of TDU temperature (A), TDU heating time (B), gas flow rate (C), gas pressure (D), PTV cooling temperature (E), PTV heating temperature (F) and PTV liner filling (G) on the response of each analyte.

The PTV liner is usually filled with different inert supports to facilitate the retention of the analytes. Several liner filling materials, including fibreglass (FG), polydimethylsiloxane (PDMS) foam, graphitized carbon black adsorbent (Cb) or 2,6-diphenylphenylene oxide (DPO), and an empty baffled (BF) liner were tested for this purpose. Best responses were obtained for benzene, toluene and ethylbenzene with Cb filling, while the response of xylene was maximum with DPO. Taking into account the average response (Fig. 3), as well as the oil matrix response, which was higher with DPO, Cb was selected as PTV liner filling material.

Once the vaporization step is over, the PTV is heated in order to elute the BTEX retained in the liner into the chromatographic column. Five different PTV heating temperatures, ranging from 270 ºC to the maximum recommended for the Cb filling, 350 ºC, were evaluated. This factor did not significantly affect the responses of the studied compounds (Fig. 3). Nevertheless, the temperature increase led substantially enhanced the matrix response; taking into account the BTEX/matrix response ratio, 270 ºC was adopted. Therefore, a temperature programme increasing

from 15 to 270 ºC at 540 ºC min^{-1}, with a hold time of 5 min, was applied in the PTV after the vaporization step.

3.3 Method performance

Calibration graphs using the standard additions method were obtained for three edible oil samples of different vegetable origin (sunflower, olive and corn oils). The graphs were linear in the 10 – 200 μg L^{-1} range ($r > 0.99$). The statistical comparison of the resulting slopes by means of an ANOVA test confirmed the suitability of using the matrix matched calibration method ($0.09<p<0.87$), and a single edible oil calibration graph for the quantification of different oil samples (see Table 3).

The repeatability of the method was calculated using the average relative standard deviation (RSD) of 10 replicate analyses of an olive oil sample spiked with the analytes at a concentration of 40 μg L^{-1}. RSD values ranged from 6.5 to 9.9%, depending on the compound (Table 2).

The sensitivity was evaluated from the detection limits (LODs) (Table 2), which were calculated considering a signal-to-noise ratio of 3. The LOD values ranged between 0.7 and 1.2 μg L^{-1}, depending on the compound.

A comparison of the proposed method with other methods previously published for the determination of BTEX in edible oils is summarized in Table 4. The method here optimized provided good sensitivity, with very low sample consumption and a rapid sample treatment, allowing proposing it for the routine monitoring of BTEX in oil samples

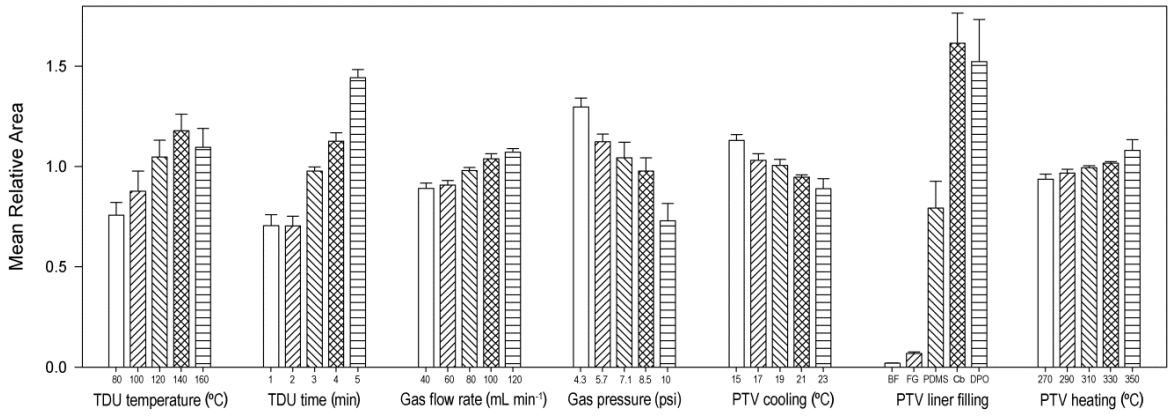


Fig. 3. Effect of TDU temperature, TDU heating time, gas flow rate, gas pressure, PTV cooling temperature, PTV liner filling and PTV heating temperature on mean analyte responses. Error bars indicate standard deviation for n=15.

Table 3
Slopes[a] of standard additions calibration graphs for three edible oils

Compound	Slope (mL μg^{-1})		
	Sunflower	Olive	Corn
Benzene	20.5±1.5	20.9±1.7	20.7±1.5
Toluene	9.8±0.9	9.9±1.2	9.8±1.0
Ethylbenzene	8.7±0.9	9.9±0.8	9.3±0.7
m-Xylene / p-Xylene	19.6±1.9	23.8±1.6	21.7±1.6
o-Xylene	5.4±0.6	6.5±0.8	5.9±0.7

[a] Mean value ± standard deviation (n= 6)

Since no reference material was commercially available, the method was validated by recovery assays, fortifying three different oil samples (sunflower, olive and corn) at two concentration levels (20 and 140 μg L^{-1}). Recoveries in the 88 – 112% range were obtained for the lowest level (n=45), while recoveries of 96 – 101% were obtained for the highest one (n=45).

3.4 Analysis of samples

The optimized procedure was applied for the determination of BTEX in commercial vegetable edible oil samples. Fourteen real samples, including a wide variety of oil types (olive, sunflower, peanut, sesame and corn), from different brands and with different packages, were analyzed in triplicate. As shown in Table 5, BTEX were found in all of the samples. Benzene was the most commonly found compound, with an average concentration of 108 μg L^{-1}, while the highest total BTEX concentration was found in an olive oil sample. None of the samples exceeded the maximum recommended total BTEX concentration of 2000 μg L^{-1} [6]. The concentrations of the studied compounds found in the edible oils analyzed were in concordance with previously reported values for other edible oil samples in the bibliography [6-8].

Fig. 1 shows a typical chromatogram obtained by microvial insert TD-GC-MS for a spiked oil sample at 40 μg L^{-1} using SCAN and SIM modes. This figure illustrates the absence of interfering peaks at the retention time of the analytes, which were identified by comparing their retention times and mass spectra in samples and standard solutions.

Table 4
Comparison of the proposed method with other previously developed methods based on GC-MS for the determination of BTEX in edible oils.

Sample treatment	Sample consumption	Treatment time min)	Approximate total time analysis (min)	Linearity (μg L^{-1})	LOD (μg L^{-1})	Ref.
MWCNTs-LLE-HS	9 mL	~ 15	35	1-200	0.25-0.43	6
HS	15 mL	30	60	12.5-625	0.6-5.5	7
HS	10 mL	25	40	10-1000	2.8-7.4	8
SPME	2 g	30	75	1-70[a]	0.4-0.7[a]	14
Microvial insert TD	50 μL	5	20	10-200	0.7-1.2	This work

MWCNTs, Multiwalled carbon nanotubes. [a] ng g^{-1}.

Table 5
Analysis of oil samples (µg L^{-1}).

Sample	Benzene	Toluene	Ethylbenzene	m,p-Xylene	o-Xylene	Total
Corn	177.6±5.1	56.3±7.5	11.6±0.8	14.3±0.9	32.1±5.2	291.9
Olive	38.3±2.3	ND	2.3±0.2	ND	NQ	44.6
Olive	13.6±2.1	ND	2.7±0.4	ND	22.8±2.7	39.1
Olive	267±16	46.4±3.7	17.0±1.4	5.3±0.3	32.3±0.7	368
Olive	ND	ND	65.6±0.6	ND	ND	65.6
Olive	96.3±7.8	28.8±3.6	5.1±0.3	7.2±0.5	15.7±0.9	153.1
Olive	24.6±3.1	55.5±8.2	ND	7.4±0.4	32.3±4.2	119.8
Peanut	17.4±2.3	13.9±1.2	ND	NQ	7.4±0.6	39.9
Sesame	ND	16.5±1.2	ND	NQ	24.0±0.5	42.0
Sunflower	233±15	71.0±4.6	3.3±0.2	3.8±0.2	ND	311.1
Sunflower	85.5±5.8	10.3±1.1	6.5±0.4	ND	14.0±0.8	116.3
Sunflower	155±11	15.1±2.1	8.1±0.4	5.7±0.4	43.6±8.4	227.5
Sunflower	86.5±7.4	23.5±1.5	NQ	8.4±1.2	47.8±3.9	169.2
Sunflower	ND	5.5±0.5	2.6±1.0	ND	54.9±4.5	63.0

Values are mean ± standard deviation (n=3), ND means not detected, NQ means not quantified.

4. Conclusion

The microvial insert thermal desorption procedure for GC-MS analysis allows a rapid, robust and reliable method for the quantification of BTEX in vegetable oils. The high sensitivity attained, joined to the low sample volume consumption, minimum sample handling, as well as the low time required, allow us to propose the optimized procedure for the routine monitoring of BTEX in oil samples.

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Direct sample introduction-gas chromatography-mass spectrometry for the determination of haloanisole compounds in cork stoppers

Abstract

A solventless analytical method is proposed for analyzing the compounds responsible for cork taint in cork stoppers. Direct sample introduction (DSI) is evaluated as a sample introduction system for the gas chromatography-mass spectrometry (GC-MS) determination of four haloanisoles (HAs) in cork samples. Several parameters affecting the DSI step, including desorption temperature and time, gas flow rate and other focusing parameters, were optimized using univariate and multivariate approaches. The proposed method shows high sensitivity and minimises sample handling, with detection limits of 1.6 – 2.6 ng g^{-1}, depending on the compound. The suitability of the optimized procedure as a screening method was evaluated by obtaining decision limits (CCα) and detection capabilities (CCβ) for each analyte, which were found to be in 6.9 – 11.8 and 8.7 – 14.8 ng g^{-1}, respectively, depending on the compound. Twenty-four cork samples were analysed, and 2,4,6-trichloroanisole was found in four of them at levels between 12.6 and 53 ng g^{-1}.

1. Introduction

Cork taint is a wine defect related with a musty, mouldy or earthy aroma and off-flavours, leading to a poor quality wine and a decrease in consumer acceptance. The number of wine bottles affected by this taint has been estimated in the 1 – 5% range [1], which represents large economic losses to a winery, whose reputation may also suffer. The compound suggested as being mainly responsible for this defect is 2,4,6-trichloroanisole (TCA) [1], although other haloanisoles (HAs), such as 2,4,6-tribromoanisole (TBA), 2,3,4,6-tetrachloroanisole (TeCA) or pentachloroanisole (PCA) may also contribute to the off-flavour [2]. Other compounds have been suggested as being co-responsible for cork taint, including 1-octen-3-ol, 1-octen-3-one, 2-methylisoborneol, geosmin and guaicol [3]. HAs are generated as a result of fungal activity in cork, through methylation of their corresponding halophenols [4], which may be produced during the chlorine bleaching of the bark cork, or may already be in this material as a consequence of their addition as biocides to wood [5] or as cleaning agents during manufacture of cork stoppers and oak barrels [6].

A wide range of analytical methods have been proposed for the determination of cork taint-related compounds in corks. Most imply their separation by gas chromatography (GC) and detection by mass spectrometry (MS) [7–17] or electron capture detector (ECD) [18–26], although biosensors [27] have also been proposed.

Different sample preparation techniques have been applied for the extraction and preconcentration of HAs present in corks. Extraction from the solid matrix is usually carried out by means of solid-liquid extraction (SLE), or different modifications of this procedure such as pressurized fluid extraction (PFE) [12] or microwave assisted extraction (MAE) [17,21,28], followed by preconcentration by liquid-liquid extraction (LLE) [19], or solid phase extraction (SPE) [16,18]. SLE is usually time-consuming and requires large amounts of sample and organic solvents, which brings with it a risk of environmental contamination, resulting in additional costs for the treatment of residues. In recent years, this technique has been replaced by others which are solvent-free, offer automation and are cleaner, more selective, rapid and efficient. For example, headspace solid-phase microextraction (HS-SPME) [7,10,11,22–26,29] and headspace sorptive extraction (HSSE) [14] have been used for the direct HA extraction and preconcentration from corks. Other miniaturized techniques have been used to preconcentrate the obtained extracts, such as SPME [15,18] and stir bar sorptive extraction (SBSE) [8,17] under direct immersion modes, as well as dispersive liquid-liquid microextraction (DLLME) [21].

In order to avoid the use of organic solvents for extracting the analytes, Amirav and Dagan developed direct sample introduction (DSI) [30], a rapid, sensitive, simple and inexpensive procedure in the context of large volume injection (LVI). In DSI, the solid sample, with a diameter up to 5 mm and a length of 30 mm, is placed in a glass desorption tube and introduced into the

programmed temperature vaporizator (PTV) or into a thermodesorption unit (TDU) attached to the PTV inlet. The solid matrix components remaining in the desorption tube are removed and discarded after the assay, while volatile compounds are vaporized and transferred to the GC column for separation [31]. DSI has previously been used for the determination of pesticides in eggs, milk and vegetables [32,33] after conventional SLE and to determine odour-related compounds other than HAs in juices and wines, after extraction with SPE [34,35]. Despite the interesting advantages of DSI, to the best of our knowledge, this methodology has not previously been applied to the determination of HAs in corks that, as already pointed, is especially relevant in the winery industry.

In the present work, we develop an analytical method using DSI coupled to GC-MS for the determination of HAs responsible for cork taint (TCA, TBA, TeCA and PCA) in different types of cork stoppers.

2. Materials and methods

2.1. Reagents

2,4,6-Trichloroanisole (TCA, 99%) and 2,4,6-tribromoanisole (TBA, 99%) were supplied by Aldrich (Steinheim, Germany). 2,3,4,6-Tetrachloroanisole (TeCA, >95%) and pentachloroanisole (PCA, >95%) were provided by Ultra Scientific (Middlesex, UK) and Chem Service (West Chester, PA, USA), respectively. 5-Bromo-2-chloroanisole (97%) was used as internal standard (IS), being supplied by Aldrich. Individual stock solutions of the compounds (1000 μg mL^{-1}) were prepared using pure grade acetone as solvent, and stored in darkness at −10 °C. Working standard solutions were freshly prepared in the same solvent and stored at 4 °C. The carrier gas used for GC was helium (Air Liquide, Madrid, Spain).

2.2. Instrumentation

The sample introduction system was composed of a Thermal Desorption Unit (TDU-2) equipped with an autosampler (MPS-2) and a Programmed Temperature Vaporization (PTV) Cooled Injector System (CIS-4) from Gerstel (Mullheim an der Ruhr, Germany). The experimental conditions used for the sample introduction system are summarized in Table 1. GC analyses were performed on an Agilent 6890N (Agilent, Waldbronn, Germany) gas chromatograph coupled to an Agilent 5973 quadrupole mass selective spectrometer equipped with an inert ion source. The instrumental conditions are summarized in Table 1.

Table 1
Experimental conditions of the TD-GC-MS procedure.

Thermal Desorption Unit	
Mode	Splitless
Temperature programme	40 – 141 ºC at 200 ºC min^{-1}, held 2.1 min
Desorption flow	35 mL min^{-1}
Cooled Injector System	
Mode	Solvent Venting
Liner	Fiberglass, 2 mm i.d.
Temperature programme	15 – 260 ºC (5 min) at 540 ºC min^{-1}
GC-MS	
Capillary column	HP-5MS, 5% diphenyl-95% dimethylpolysiloxane (30 m x 0.25 mm, 0.25 μm)
Carrier gas	Helium (1 mL min^{-1})
Oven programme	80 ºC, held 0.6 min 80 – 180 ºC at 25 ºC min^{-1}, held 0.6 min 180 – 210 ºC at 25 ºC min^{-1}, held 0.8 min 210 – 300 ºC at 50 ºC min^{-1}, held 1.4 min
Transfer line temperature	300 ºC
Quadrupole temperature	150 ºC
Ion source temperature	230 ºC
Ionization	Electron-impact mode (70 eV)

Retention times (Table 2) between 5.0 and 7.4 min, corresponding to TCA and PCA, respectively, were obtained. Compounds were identified by comparing their retention time and relevant MS-spectra with those obtained for the injection of pure standards (Table 2). The analytes were quantified under the selected ion monitoring (SIM) mode using the target ion.

2.3. Samples and analytical procedure

Twenty-one used corks were obtained from wine bottles from different origins, while three different batches of unused cork were obtained from a local cellar. All stoppers were also classified according to their manufacturing process in natural (7 stoppers) or agglomerated (17 stoppers), made of cork granulated material mixed with chemical agglutinating compounds. A cork borer was used for sampling purposes, and cylindrical portions (5 x 10 mm) were obtained from each stopper. Prior to analysis each cork portion was weighed on an analytical balance, and was seen to weigh, on average, 20 mg.

Table 2
Retention time and monitored ions.

Compound	RT (min)	Monitored ions (*m/z*)
2,4,6-Trichloroanisole (TCA)	5.0	169 (61), <u>195</u>, 197 (98), 210 (63), 212 (60)
5-Bromo-2-chloroanisole (IS)	5.3	179 (59), <u>222</u>
2,3,4,6-Tetrachloroanisole (TeCA)	6.2	201 (69), 229 (80), <u>231</u>, 233 (48), 244 (58), 246 (71)
2,4,6-Tribromoanisole (TBA)	6.8	301 (45), 303 (42), 329 (77), 331 (72), <u>344</u>, 346 (91)
Pentachloroanisole (PCA)	7.4	235 (61), 237 (66), 263 (52), <u>265</u>, 278 (37), 280 (58)

Underlined numbers correspond to *m/z* of the target ion, and values in brackets represent the qualifier-to-target ion ratios in percentage.

Spiked samples were prepared by applying different volumes of the standard solution mixture, to the bored cork, using a microsyringe at different points of the sample, including its surface and the inner part. The IS was also added at a concentration level of 100 ng g^{-1}. Samples were set aside for 60 min at room temperature in 1.5 mL microcentrifuge tubes to allow the solvent to evaporate while the analytes were retained in the cork before being submitted to the DSI procedure.

3. Results and discussion

3.1. Direct sample introduction

Direct sample introduction is a complex step that may be influenced by a large number of experimental variables. The effect of five of these variables (desorption time and temperature, gas flow-rate, PTV liner filling, and heating temperature) in the response of the HAs was evaluated at different levels. For this purpose, cork cylinders of about 20 mg containing the analytes at 100 ng g^{-1} were placed in the desorption tube and introduced into the gas chromatograph using the TDU-CIS system.

Five different desorption temperatures (from 100 to 180 ºC) and times (from 0.5 to 4 min) were tested. In general, higher temperatures and longer times facilitated the vaporization of analytes from the cork sample, but tended to reduce the overall efficiency due to retention losses in the PTV injector, and to increase matrix interference. Temperatures over 180 ºC were not assayed since they led to the charring of the cork and a substantial increase in the chromatographic background. As shown in Fig. 1, maximum sensitivity was attained for all the analytes with a desorption time of 2 min, whereas 140 ºC provided the highest signals, except in the case of TCA and PCA, for which maximum sensitivity were attained at 120 and 160 ºC, respectively.

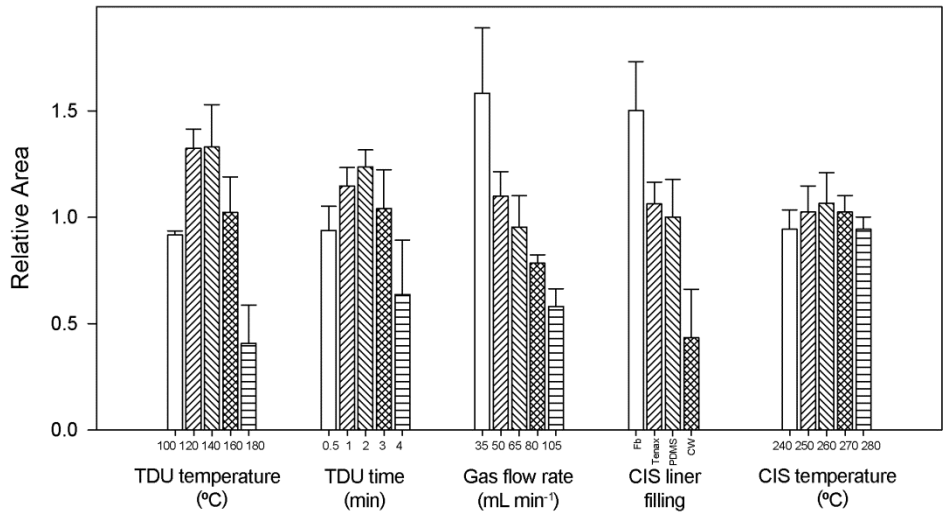

Fig. 1. Effect of different thermal desorption variables on average analyte responses.

In order to evaluate thermal vaporization temperatures and vaporization times in more detail, the multivariate procedure central composite design (CCD) (α = 1.5, 4 cube points, 4 axial points and 2 central points, in duplicate) was developed in the 120 – 160 ºC and 1 – 3 min range, corresponding to the maximum sensitivity ranges reported in previous experiments. The responses showed that the optimal conditions were a desorption temperature of 140.6 ºC applied for 2.1 min (Fig. 2). Therefore, a temperature programme increasing from room temperature to 141 ºC at 200 ºC min^{-1}, with a hold time of 2.1 min, was applied in the TDU for vaporization purposes.

A carrier gas is necessary to propel the analytes from TDU to the PTV injector while they are being thermally vaporized. The TDU vaporization and subsequent PTV focusing of the HAs may be influenced by the flow-rate of this gas. High flow rates led to the efficient propulsion of the vaporized compounds, although the speed at which they passed through the PTV was also increased, with the subsequent decrease in the retention efficiency of this device. Five different flow-rates (ranging from 35 to 105 mL min^{-1}) were tested and best responses (Fig. 1) were obtained for all the compounds, especially for the heavier ones, at 35 mL min^{-1}.

The vaporized compounds were focused in the PTV before entering the chromatographic column, where low temperatures increased their retention efficiency and minimized losses. This device is cooled by a Peltier unit, which only allows cooling to slightly below room temperature, so a focusing temperature of 15 ºC was selected.

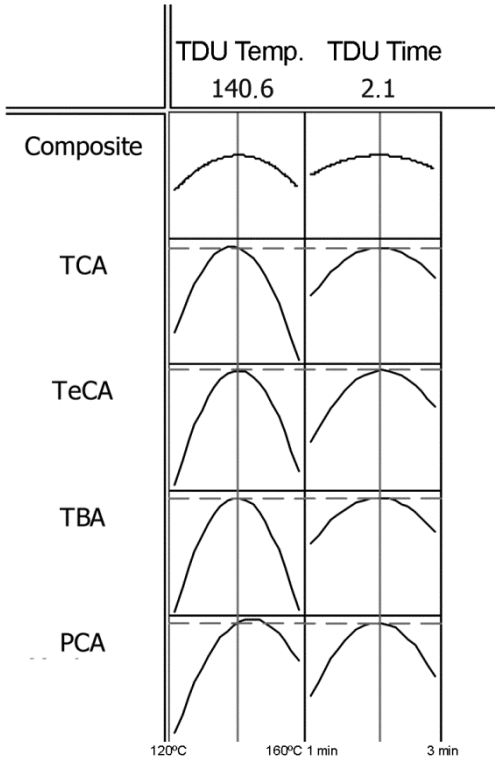

Fig. 2. Response plots showing the effects of TDU desorption temperature and time desorption time on the relative response of each haloanisole.

The PTV liner is usually filled with different inert supports in order to facilitate the retention of the analytes. Different liner filling materials, including fibreglass (FG), polydimethylsiloxane foam (PDMS), graphitized carbon black adsorbent (CB) and 2,6-diphenylene oxide resin (DPO), were tested for this purpose. Best responses for all compounds were obtained with FG filling, while TCA responses were maximum with PDMS, due to its low vapour pressure. Taking into account the average response (Fig. 1), FG was selected as PTV liner filling material.

After the vaporization step, the PTV is heated in order to elute the compounds retained in the liner into the chromatographic column. Five different PTV heating temperatures, ranging from 240 ºC to the maximum recommended for the FG filling, 280 ºC, were evaluated. This variable did not significantly affect the responses of the studied compounds (Fig. 1), but, taking into account the average response, 260 ºC was selected. Therefore, a temperature programme increasing from 15 to 260 ºC at 540 ºC min^{-1}, with a hold time of 5 min, was applied in the PTV after vaporization step.

3.2. Analytical characteristics of the method

Firstly, the standard addition method was used to check the possible presence of a matrix effect. To this purpose, the samples were spiked at six concentration levels and submitted in duplicate to the optimized procedure. The slopes of the calibration graphs obtained for the different cork samples were seen to be significantly different, according to the comparison made using an ANOVA statistical test. According to the nature of the cork sample, the obtained slopes can be grouped into those corresponding to natural cork stoppers and those resulting from cork agglomerate stoppers (Table 3).

Having checked that all samples were free of 5-bromo-2-chloroanisole, its use as IS in order to minimize the matrix effect was tested, by adding at 100 ng g^{-1}. When calibration graphs were obtained by plotting peak area ratios of each analyte with respect to the IS versus analyte concentration, no statistically significant differences between the cork samples were found when an ANOVA comparison was carried out, as shown in Table 3. Consequently, a matrix-matched calibration using 5-bromo-2-chloroanisole as IS is proposed for quantification purposes.

Table 3
Slopes[a] (g ng^{-1}) of the standard additions calibration graphs obtained for different types of corks and by the internal standard method.

	Natural corks		Agglomerate corks		Ratio	
	Without IS	With IS	Without IS	With IS	Without IS	With IS
TCA	5638±252	0.0166±0.0012	4302±321	0.0160±0.0007	1.31	1.04
TeCA	3583±217	0.0113±0.0009	2957±242	0.0103±0.0006	1.21	1.10
TBA	2471±119	0.0077±0.0006	2015±146	0.0071±0.0003	1.23	1.08
PCA	3135±204	0.0100±0.0007	2601±184	0.0090±0.0006	1.21	1.11

[a] Mean value ± standard deviation (n=6).

Calibration curves were found to be linear between 10 and 250 ng g^{-1}, with correlation coefficients higher than 0.99 in all cases (Table 4).

The detection (LODs) and quantification (LOQs) limits were calculated as the concentration corresponding to a signal-to-noise ratio of 3 and 10, respectively. The LOQ values obtained, which ranged from 5.4 to 8.8 ng g^{-1}, are summarized in Table 4.

The repeatability was calculated using the relative standard deviation (RSD) for a batch of ten cork samples spiked at 50 ng g^{-1}, providing RSD values ranging from 5.5 to 8.2 (Table 4).

In many cases, the aim of HAs analysis is not to determine their concentration in cork samples, but to check whether these compounds are present in a given batch of corks. The proposed DSI procedure can be used as a rapid and sensitive screening method to establish the presence or absence of these compounds in cork stoppers.

In order to evaluate the characteristics of the proposed procedure as a screening method, the parameters decision limit (CCα) and detection capability (CCβ) were obtained for each analyte according to the EU Commission Decision 2002/657/EC [36].

Taking into account that no maximum permitted limits have been established for HAs, CCα was calculated by the calibration curve procedure, using a blank material fortified at and above the LOD level in equidistant steps.

The decision limit (CCα) is equal to the corresponding concentration at the y-intercept plus 2.33 times the standard deviation of the intercept. CCβ was calculated by analyzing at least 20 blank materials fortified with the analytes at their corresponding decision limit. The value obtained for CCα plus 1.64 times the corresponding standard deviation is equal to the detection capability (β=5 %). Table 4 summarized the calculation of CCα and CCβ.

A comparison of the proposed method with others previously published for the determination of haloanisoles in cork stopper samples is summarized in Table 5.

Table 4
Method characteristics.

Parameter	TCA	TeCA	TBA	PCA
RSD[a] (%)	8.2	6.1	6.2	5.5
LOD[b] (ng g^{-1})	1.6	2.6	1.7	5.7
LOQ[c] (ng g^{-1})	5.4	8.8	5.7	8.5
R^2	0.989	0.991	0.998	0.991
Validation of the DSI-GC-MS procedure according with the criteria of the Commission Decision 2002/657/EC				
S_{y0} (ng g^{-1})	3.0	5.1	3.3	4.5
CCα (ng g^{-1})	6.9	11.8	7.6	10.4
Added (ng g^{-1})	10.0	10.0	10.0	10.0
Found (ng g^{-1})	9.7 ± 1.1	10.3 ± 1.8	9.2 ± 1.1	9.1 ± 2.0
CCβ (ng g^{-1})	8.7	14.8	9.4	13.6

[a] n=10. [b] Calculated using S/N=3. [c] Calculated using S/N=10.

Table 5
Comparison of the proposed method with others previously reported for CAs determination in cork samples

Analytical technique	Extraction	Preconcentration	Sample consumption, preparation time	Linearity range (ng g^{-1})	LOD (ng g^{-1})	Ref.
GC-MS	LSE	SBSE	2 g, 25 h	15 – 120 (ng L^{-1})	1.2 – 23.0	[9]
GC-MS	UAE	DLLME	2.5 – 3 g, 30 min	-	0.03 – 0.22	[10]
GC-TOF-MS	CF-HS-SPME		0.2 g, 20 min	5 – 100	0.25 – 0.5	[11]
GC-MS	PFE	-	0.75 g, ~55 min	-	0.3	[13]
GC-MS	HSSE		whole cork, 90 min	1 – 70	2.7 – 30.6	[15]
GC-MS	MAE	SBSE	~ 2g, 3.5 h	Up to ~12	0.01	[18]
GC-MS	LSE		1 g, 90 min	-	2.2 – 4.1	[19]
GC-ECD	MAE	DLLME	0.5 g, 90 min	0.06 – 100	0.03 – 0.09	[22]
GC -ECD	LSE HS-SPME		0.2 g, 25 h	0.3 – 500	0.2	[23]
GC-ECD	MAE	-	0.5 g, 90 min	1 – 600	0.3 – 0.68	[29]
GC-MS	DSI		0.02 g, 5 min	10 – 250	1.6 – 2.6	This paper

CF, cold fiber; DLLME, dispersive liquid-liquid microextraction; DSI, direct sample introduction; HSSE, headspace sorptive extraction; HS-SPME, headspace solid-phase microextraction; LSE, liquid-solid extraction; PFE, pressurized fluid extraction; SBSE, stir bar sorptive extraction; UAE, ultrasound assisted extraction.

3.3. Analysis of corks and recovery studies

A typical chromatogram obtained by DSI-GC–MS under SIM mode for a 25 ng g^{-1} spiked cork sample under the selected conditions is shown in Fig. 3A. Similar chromatograms were obtained for the other samples, showing the absence of interfering peaks at the analyte retention times. The studied compounds were identified by comparing the retention time, identifying the target (*T*) and qualifier ions (*Q*) and comparing the qualifier-to-target ratios (*Q*/*T,* %) of the peaks in both unspiked and spiked samples.

The proposed method was applied to the analysis of twenty-four different cork samples, obtained and sampled according to the described experimental procedure. TCA was found in four of samples, corresponding to agglomerate corks, at concentrations ranging from 12.6 to 53 ng g^{-1} (Table 6). These values are well over the reported olfactory threshold level for this compound in wine samples, 5.6 pg mL^{-1}, so the use of these cork stoppers in wine production could have led to the appearance of cork taints in the resulting product.

Table 6
Contents of TCA in corks.

Sample	Concentration[a] (ng g^{-1})
1	19.7 ± 0.4
2	12.6 ± 1.3
3	52.9 ± 7.9
4	23.0 ± 1.0

[a] Mean value ± standard deviation (n=3).

Since no reference materials were available for the verification of the accuracy of the method, spiked cork samples were analysed and the recoveries obtained. Two different corks stoppers, one natural and the other made from a cork agglomerate, were spiked with the studied compounds at two levels, namely 25 and 125 ng g^{-1}, and submitted to the described procedure in triplicate. As shown in Table 7, the recoveries were in the 85 – 113% range, for the lowest level and in the 97 – 110% range, for the highest one.

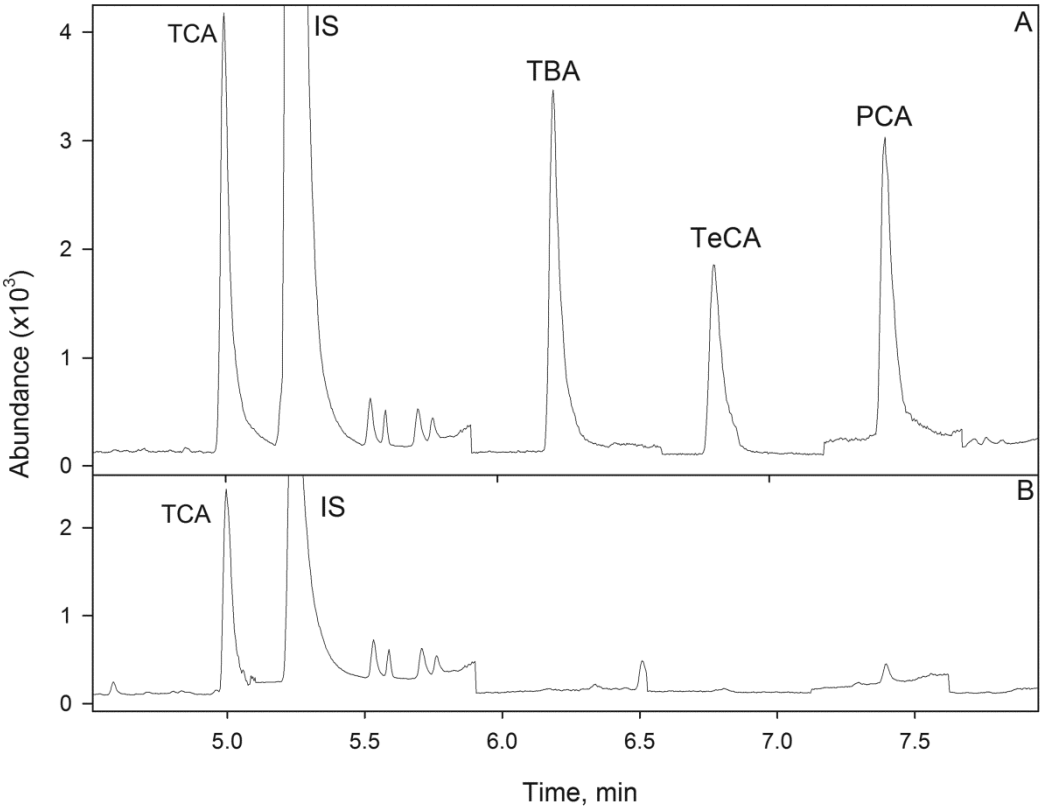

Fig. 3. Elution profile obtained for: A) cork sample spiked at 25 ng g^{-1}, B) contaminated cork sample (12.6 ± 1.3 ng g^{-1}), using the DSI-GC–MS procedure under selected ion monitoring (SIM) mode.

Table 7
Recovery[a] percentages obtained for two types of cork.

Compound	Spike level (ng g^{-1})	Natural cork	Agglomerate cork
TCA	25	91 ± 5	97 ± 6
	125	97 ± 7	110 ± 7
TeCA	25	102 ± 6	85 ± 6
	125	109 ± 8	98 ± 7
TBA	25	97 ± 1	113 ± 7
	125	99 ± 3	106 ± 5
PCA	25	104 ± 4	92 ± 6
	125	109 ± 6	99 ± 8

[a] Mean value ± standard deviation (n=3).

4. Conclusion

DSI has proven to be a simple and effective sample introduction system for the GC-MS determination of HAs in cork samples. This technique involves minimal sample handling, reducing the analysis time, while providing high sensitivity. The described DSI-GC-MS method was validated not only for quantitative purposes, but also as a screening method, and was seen to be a fast and accurate method for detecting contaminated cork stopper batches.

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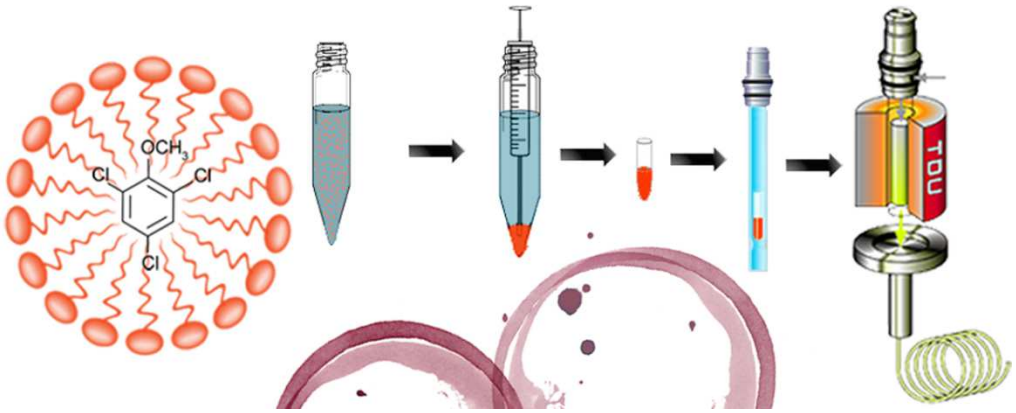
Cloud point extraction and gas chromatography with direct microvial insert thermal desorption for the determination of haloanisoles in alcoholic beverages

Abstract

A sensitive analytical procedure for the determination of four haloanisoles related with cork taint defects in wines (2,4,6 trichloroanisole, 2,4,6-tribromoanisole, 2,3,4,6-tetrachloroanisole and pentachloroanisole), in different types of alcoholic beverages has been developed. The analytes were extracted from the matrix samples by cloud point extraction (CPE) using Triton X-114 heated to 75 ºC, and the surfactant rich phase was separated by centrifugation. By means of direct microvial insert thermal desorption, 20 µL of the CPE obtained extract was submitted to gas chromatography-mass spectrometry (GC-MS) analysis. The parameters affecting the CPE and microvial insert thermal desorption were optimized. Quantification was carried by matrix-matched calibration using an internal standard. Detection limits ranged between 12.9 and 20.8 ng L^{-1}, depending on the compound, for beer and wine samples, whereas for whiskies values in the 46.3 – 48 ng L^{-1} range were obtained, since these samples were diluted for analysis. Recoveries for alcoholic beverages were in the 89 – 111% range, depending on the analyte and the sample.



1. Introduction

In recent years, cloud point extraction (CPE) has been widely used as a sample preparation technique in analytical chemistry [1]. CPE is based on the fact that a solution of non-ionic surfactants leads to the formation of micelles when it is heated above a certain temperature, known as cloud point temperature. In this way, a homogeneous solution provides a two phase disperse system, which can be separated [2]. Some compounds can be concentrated in the surfactant-rich fraction (coacervate), which is recovered by centrifugation. CPE is similar to other liquid–liquid microextraction (LLME) techniques, its main advantage being that it avoids the use of organic solvents, which mean it can be catalogued as an environmental friendly procedure [3].

CPE has been successfully applied for the determination of both inorganic [1,3-6] and organic species [2,7–9]. The enriched extracts obtained by CPE have usually been analysed by atomic spectroscopic techniques for elementary determinations, and by liquid chromatography (LC) or electrophoresis (CE) [7] in the case of organic species. However, relatively few works have dealt with the coupling of this extraction technique with gas chromatography (GC) [11–20]. The low volatility of the coacervate makes its direct introduction into the GC system impossible using conventional injectors, since the injection of CPE extracts may not only pollute the inlet, but also cause capillary column blockage. However, two approaches have been proposed for this purpose, the most widely used being back-extraction (BE) [11–13,16,17,20], whereby the analytes preconcentrated in the surfactant rich phase are back extracted into an organic solvent immiscible with the CPE extract and compatible with GC. The efficiency and speed of this process is usually increased by the application of ultrasounds [12,16,20] or microwaves [13,16]. The other approach involves the derivatization of the surfactant contained in the coacervate [18,19] prior to its introduction into the GC. Silylation derivatization with N,O-bis(trimethylsilyl)fluoroacetamide (BSTFA) is usually applied, increasing the volatility of the surfactant, which is not retained in the injector or column. However, large quantities of surfactants are still introduced into the GC system, leading to large peaks that may overlap the analyte responses, or foul the ion source if MS is used as detector. Separation of the analytes from the surfactant has also been achieved using cation exchange columns [15].

In order to overcome this limitation, direct microvial insert thermal desorption [21] has been tested, thus avoiding additional steps after the CPE step. Such an approach is based on the use of a commercial thermal desorption unit (TDU) as interface to transfer the extracted analytes from the surfactant rich phase to the GC system. For this purpose, glass inserts containing up to 150 μL of liquid sample are placed in the thermal desorption tube. Microvial insert thermal desorption has previously been applied for the GC analysis of IL drops obtained through a microextraction [22].

In this paper GC analysis of the CPE extracts using microvial insert thermal desorption is proposed. The surfactant rich phase drop is placed in a glass microvial by microsyringe. The microvial is introduced inside the TD tube, and the whole assembly is submitted to thermal desorption. A carrier gas propels the analytes to a programmed temperature vaporizator (PTV) injector, where they are focused before entering the chromatographic column. Next, the PTV is heated, and the retained compounds enter the GC system to be separated. In this way, the surfactant hardly reaches the GC system, and, even if some vapours are dragged by the gas flow, they are retained in the disposable PTV liner, and so do not enter the chromatographic column.

Cork taint is a defect related with musty, mouldy or earthy aromas and off-flavours, which can be detected in wine, but also in other alcoholic beverages, such as beer or whisky [23]. 2,4,6-Trichloroanisole (TCA) has been suggested as being mainly responsible for this defect [24], although other haloanisoles (HAs), such as 2,4,6-tribromoanisole (TBA), 2,3,4,6-tetrachloroanisole (TeCA) and pentachloroanisole (PCA), may contribute to the off-flavours [25]. These species are generated by fungal methylation of their corresponding halophenols [26], which may be produced during the chlorine bleaching of the wood, or reach this material as a consequence of their use as biocides [27].

In the present work, a CPE using direct microvial insert thermal desorption coupled to GC-MS procedure is developed for the determination of four HAs in different alcoholic beverages.

2. Experimental

2.1. Standards and reagents

2,4,6-Trichloroanisole (TCA, 99%) and 2,4,6-tribromoanisole (TBA, 99%) were supplied by Aldrich (Steinheim, Germany). 2,3,4,6-Tetrachloroanisole (TeCA, >95%) and pentachloroanisole (PCA, >95%) were provided by Ultra Scientific (Teddington, England) and Chem Service (West Chester, PA, USA), respectively. 5-Bromo-2-chloroanisole (97%), supplied by Aldrich, was used as internal standard (IS). Individual stock solutions of the compounds (1000 μg mL^{-1}) were prepared using pure grade acetone as the solvent, and stored in darkness at −10 °C. Working standard solutions were prepared daily by diluting with ultrapure water.

The non-ionic surfactant Triton X-114, which was used as a 30% (w/v) aqueous solution, was provided by Fluka (Buchs SG, Switzerland). Sodium chloride, potassium dihydrogen phosphate and dipotassium hydrogen phosphate were obtained from Fluka. The water used was previously purified in a Milli-Q system (Millipore, Bedford, MA, USA). The carrier gas used for GC was helium (Air Liquide, Madrid, Spain).

Table 1
Experimental conditions of the TD-GC-MS procedure.

Thermal Desorption Unit	
Mode	Solvent Venting
Temperature programme	75 ºC, held 0.5 min 75 – 225 ºC at 300 ºC min^{-1}, held 5 min
Desorption flow	50 mL min^{-1}
Cooled Injector System	
Mode	Solvent Venting
Liner	Tenax, 2 mm i.d.
Temperature programme	15 – 250 ºC (5 min) at 540 ºC min^{-1}
GC-MS	
Capillary column	HP-5MS, 5% diphenyl-95% dimethylpolysiloxane (30 m x 0.25 mm, 0.25 µm)
Carrier gas	Helium (1 mL min^{-1})
Oven programme	80 ºC, held 0.6 min 80 – 180 ºC at 25 ºC min^{-1}, held 0.6 min 180 – 210 ºC at 25 ºC min^{-1}, held 0.8 min 210 – 300 ºC at 50 ºC min^{-1}, held 1.4 min
Transfer line, quadupole and ion source temperature	300, 150, 230 ºC
Ionization	Electron-impact mode (70 eV)

2.2. Instrumentation

Samples were placed in disposable glass microvials (15 mm long, 1.9 mm i.d., 2.5 mm o.d., Gerstel, Mullheim an der Ruhr, Germany). The sample introduction system was composed of a Thermal Desorption Unit (TDU-2) equipped with an autosampler (MPS-2) and a Programmed Temperature Vaporization (PTV) Cooled Injector System (CIS-4) (Gerstel).

The experimental conditions used for the sample introduction system are summarized in Table 1. GC analyses were performed on an Agilent 6890N (Agilent, Waldbronn, Germany) gas chromatograph coupled to an Agilent 5973 quadrupole mass selective spectrometer equipped with an inert ion source. In the selected conditions (Table 1), the analytes eluted with retention times between 5.0 and 7.4 min, corresponding to TCA and PCA, respectively (Table 2). The identification of the compounds was confirmed by comparison of retention times and MS-spectra with respect to pure standards. The analytes were quantified under the selected ion monitoring (SIM) mode using the target ions (Table 2). A domestic microwave oven was used in the sample treatment.

Table 2
Retention times, monitored ions and enrichment factors

Compound	RT (min)	Monitored ions (*m/z*)	EF
2,4,6-Trichloroanisole (TCA)	5.0	167, 195 (98), 210 (63)	73.2
5-Chloro-2-bromoanisole (IS)	5.3	222, 179 (89)	68.0
2,3,4,6-Tetrachloroanisole (TeCA)	6.2	203, 231 (84), 131 (80), 246 (64)	78.8
2,4,6-Tribromoanisole (TBA)	6.7	344, 346 (95), 329 (78), 301 (50)	76.0
Pentachloroanisole (PCA)	7.4	237, 265 (76), 280 (68), 167 (58)	73.2

Underlined numbers correspond to *m/z* of the target ion, and values in brackets represent the qualifier-to-target ion ratios in percentage.

2.3. Samples and analytical procedure

Different wood-aged alcoholic beverages, including white and red wine, beer and whisky, were obtained from local supermarkets. Once opened, all samples were kept at 4 °C until analysis, in order to prevent losses of the more volatile analytes. Prior to analysis, a 1:4 sample:water dilution was applied for whisky samples, to decrease the alcohol content. For CPE, a 5 mL volume of beverage sample, in the presence of the IS at 0.25 ng mL^{-1}, was placed in a 10-mL screw cap glass tube with conical bottom, into which 0.2 g of NaCl had previously been weighed, and 0.5 mL of phosphate buffer solution (0.2 M, pH 7.2) was added. Next, 100 µL of 30% (w/v) Triton X-114 aqueous solution were injected into the sample solution using a microsyringe, and the mixture was gently shaken manually for several seconds. The resulting solution was heated at 75 ºC in a microwave oven for 40 s, leading to the formation of a cloudy solution.

HAs were extracted into the fine droplets of the coacervate dispersed through the sample solution. After centrifugation for 2 min at 3000 rpm, the surfactant rich phase sedimented at the bottom of the conical tube (drop volume of about 25 µL) was collected using a microsyringe. The recovered drop was placed in a microvial and then transferred to the TDU by means of the MPS2. The TDU was operated in the solvent vent mode. First, in a drying step, the split mode allowed any remaining water to evaporate and then the splitless mode was activated to transfer the analytes to the PTV and the GC system.

A synthetic alcoholic beverage containing 3.2 g L^{-1} of L-(+)-tartaric acid and 12% (v/v) of ethanol, with the pH adjusted to 3.6 by means of a diluted NaOH solution, was used to optimize the method.

3. Results and discussion

3.1. Optimization of the cloud point extraction

The effect of different variables on the CPE step was studied in order to maximize extraction efficiencies and enrichment factors, using a 5 mL volume of synthetic alcoholic beverage. Among the available non-ionic surfactants, Triton X-114 was selected for its relatively low cloud point temperature (23 ºC), which means low heating temperatures.

The concentration of the surfactant is a key parameter in CPE, since it must be sufficiently high to allow the molecules to give rise to a coacervate, thus providing the extractant phase, but not too high since that would lead to large settled phases and dilution of the corresponding extracted analytes. Surfactant concentrations ranging from 0.4 to 1.0% (m/v), corresponding to 70 – 170 µL of a 30% (m/v) Triton X-114 solution, were evaluated. As Fig. 1A shows, maximum response was obtained for most analytes with 0.6% (m/v) surfactant, except for TCA, which reached its

maximum sensitivity at 0.8% (m/v). Taking into account the average response, 0.6% (m/v) was chosen as surfactant concentration.

The addition of salt to the extraction medium, thus increasing its ionic strength, may improve phase separation and facilitate recovery of the settled drop. Different amounts of NaCl, ranging from 0 to 0.3 g, corresponding to 0 and 6% (m/v), were tested. As shown in Fig. 1B, a 4% (m/v) salt concentration provided the highest responses for all the compounds, and was selected.

The pH of the extraction medium was adjusted by adding 0.5 mL of 0.2 M buffer solutions, including acid/citrate (pH 3.1), acetic acid/acetate (pH 4.8), $H_2PO_4^-/HPO_4^{2-}$ (pH 7.2) and NH_4^+/NH_3 (pH 9.2). Despite the non-ionic nature of the surfactant and the analytes, for most of the HAs, lower signals were observed at acid pH, while no significant differences in sensitivity were obtained at pH values of 7.2 and 9.2 (Fig. 1C). Considering the average signals, the phosphate buffer solution was selected to adjust pH of the extraction medium.

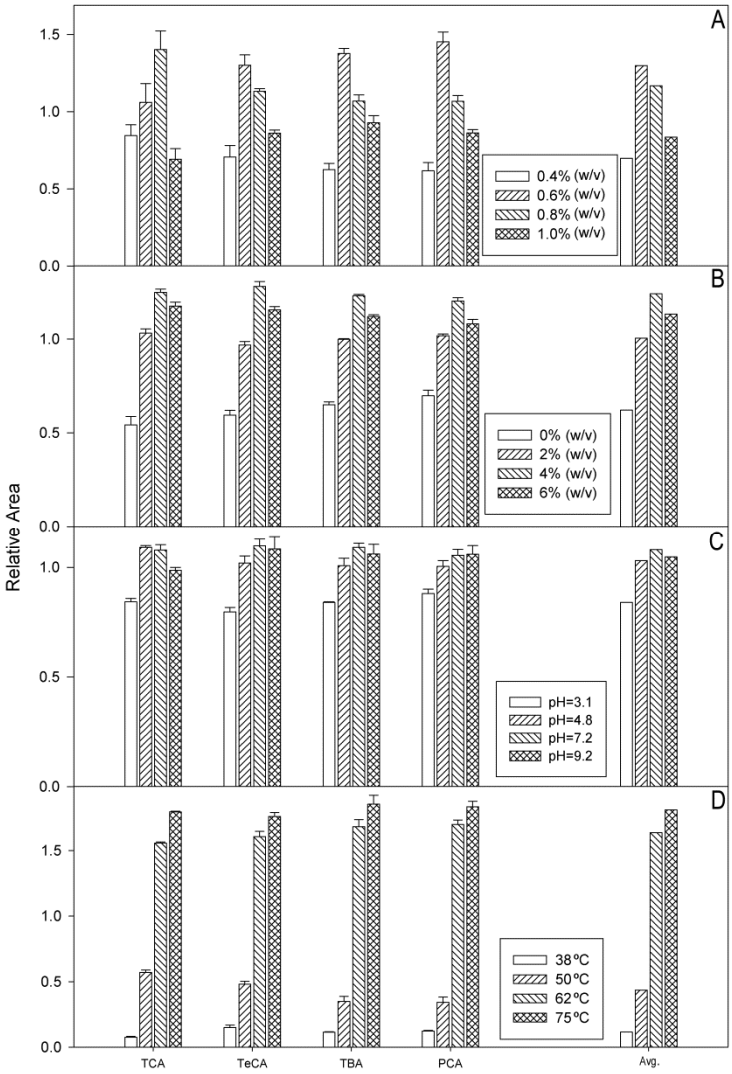


Fig. 1. Effect of (A) surfactant concentration, (B) salt concentration, (C) sample pH, and (D) heating temperature on the sensitivity of the analytes. Vertical segments correspond to standard deviation (n=3).

When the surfactant-sample mixture was heated above the cloud point temperature, the formation of a coacervate resulted in a cloudy solution. Increasing the temperature led to more efficient dehydration of the micelles and a decrease in the surfactant rich phase volume, thus enhancing the extraction efficiency. For this purpose, a microwave oven was used allowing the quick and simultaneous heating of a large number of samples, while ensuring reproducible conditions. Different heating times, ranging from 10 to 40 s, which corresponded to temperatures of 38 and 75 ºC, respectively, were assayed. As shown in Fig. 1D, sensitivity increased with the temperature in all the studied intervals. Consequently, a heating time of 40 s, corresponding to 75 ºC, was applied.

3.2. Optimization of microvial insert thermal desorption

The possible influence of different factors on microvial insert thermal desorption performance was evaluated using the analytical signals obtained for the studied compounds, as well as their ratio to the background signals corresponding to the surfactant. For this purpose, 20 µL of the surfactant rich phase containing the extracted analytes were placed in a glass microvial and injected into the gas chromatograph using the TDU system.

Due to the way in which the coacervate is obtained, it still contains water, that has to be removed to prevent it entering the chromatographic column. A drying step at 75 ºC for 0.5 min was included in the TDU heating program, to allow the helium gas flow to expel any remaining water.

To vaporize the analytes from the glass microvial, different hold temperatures and times were applied in the TDU. Higher temperatures and longer times may facilitate analyte vaporization, but may also reduce the overall efficiency due to retention losses in the PTV injector. Vaporization times and temperatures in the 1 – 5 min (Fig. 2A) and 150 – 225 ºC (Fig. 2B) ranges, respectively, were tested. The analytical signals increased with higher temperatures and longer desorption times, especially for TBA and PCA. Taking into account the average response and the analyte-to-surfactant signal ratio, and considering that no dramatic increase in the surfactant signal was observed in the studied conditions, a vaporization temperature of 225 ºC for 5 min was finally selected.

A carrier gas is necessary to propel the analytes to the PTV injector while they are being thermally vaporized. Different values ranging from 50 to 125 mL min^{-1} were tested. This variable was especially relevant for the more volatile compounds, and there was an increase in the obtained response at lower gas flow rates (Fig. 2C). Thus, 50 mL min^{-1} was adopted.

The desorbed compounds were focused in the PTV before entering the chromatographic column. Lower temperatures in this device increased the retention efficiency and minimized losses of the more volatile compounds. Since the Peltier unit only allows cooling to temperatures slightly

below room temperature, 15 ºC was adopted. Different filling materials, including fiberglass, Carbotrap, Tenax and PDMS, can be placed in the PTV liner to facilitate the retention of the analytes once vaporized. As shown in Fig. 2D, Tenax and PDMS provided best results for all compounds. Considering the average response, Tenax filling was selected.

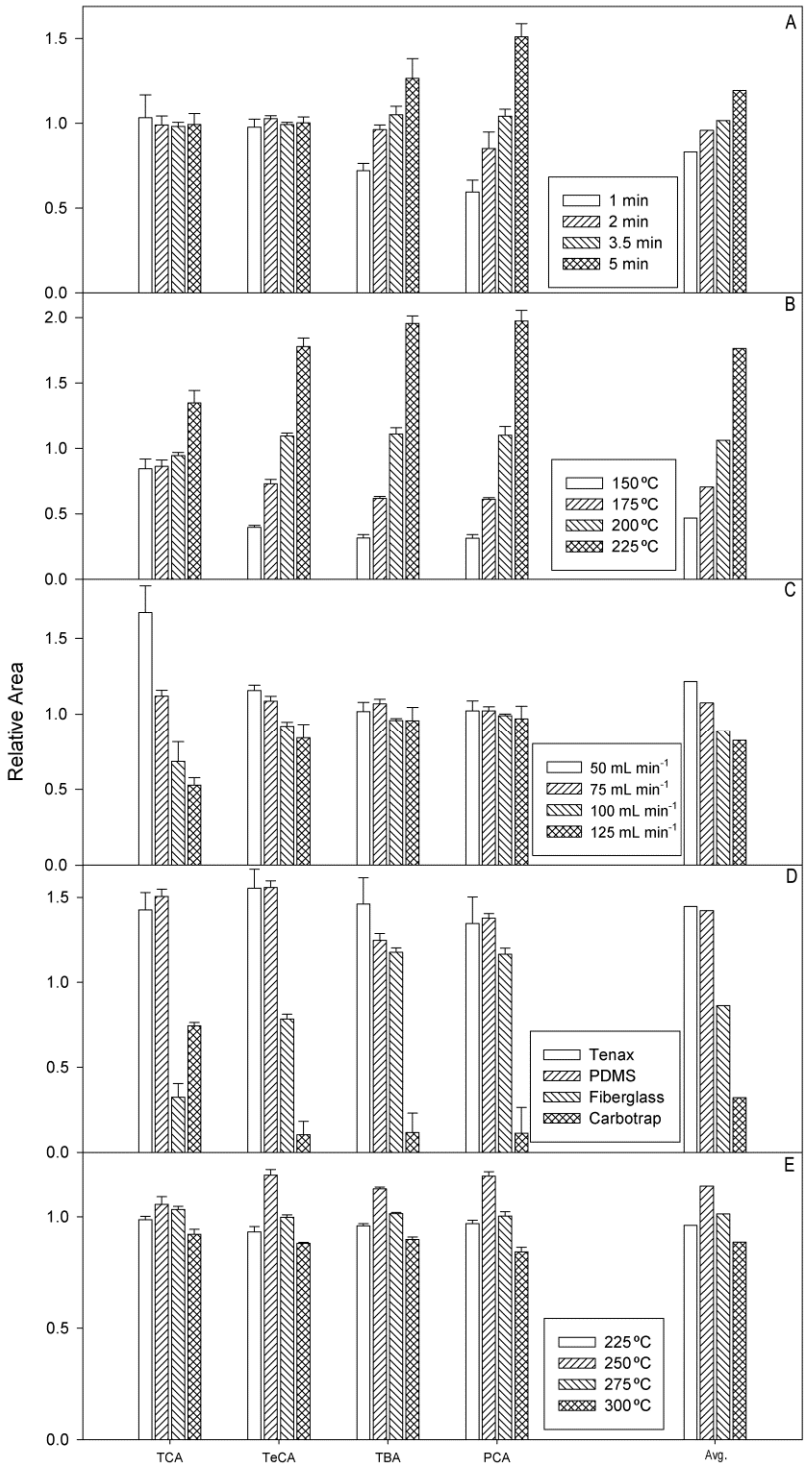


Fig. 2. Effect of (A) vaporization time, (B) vaporization temperature, (C) gas flow rate, (D) PTV liner filling and (E) PTV heating temperature on the average analyte responses. Vertical segments correspond to standard deviation (n=3).

In order to elute the retained compounds in the PTV liner, different temperatures, from 225 to 300 ºC, were applied. As shown in Fig. 2E, the responses were in all cases higher at 250 ºC, so this temperature was selected. Therefore, a PTV programme temperature increasing from 15 to 250 ºC at 540 ºC min^{-1}, with a hold time of 5 min, was applied.

3.3. Method characteristics

The standard additions method was applied to the three different samples of the four types of wood-aged alcoholic beverages studied (red wine, white wine, beer and diluted whisky samples) using the optimized method and 5-chloro-2-bromoanisole (0.25 ng mL^{-1}) as IS.

For this purpose, the ratio between analytes and IS peak areas were plotted *versus* analyte concentration, using spiked samples at six concentration levels, in triplicate. Least-squares linear regression analysis provided good correlation coefficient values ($r > 0.99$), meaning good linearity responses for all the compounds in the studied concentration ranges: 0.05 – 2 μg L^{-1} for beers and wines, and 0.2 – 8 μg L^{-1} for whiskies.

The use of the IS calibration procedure provided no significant differences between the slopes obtained for samples of the same type of alcoholic beverage, "p" values higher than 0.05 being obtained when a paired *t*-test was used for the comparison of the resulting slopes.

However, significant differences were still found between the slopes of different types of alcoholic beverages when an ANOVA statistical procedure was used for comparison purposes ($p<0.001$). Consequently, matrix matched calibration was adopted, since it differed for each type of sample: white wines, red wines, beers and diluted whiskies. Table 3 shows the slope obtained for one sample of each type of beverage.

Table 3
Calibration parameters obtained for different sample matrices.

Sample	Parameter	TCA	TeCA	TBA	PCA
Beer	Slope[a] (L μg^{-1})	3.24±0.06	1.63±0.04	2.03±0.05	1.18±0.03
	LOD (ng L^{-1})	13.9	13.0	13.1	14.0
Whiskey	Slope[a] (L μg^{-1})	1.17±0.05	0.72±0.03	0.77±0.03	0.53±0.03
	LOD (ng L^{-1})	39.4	36.3	35.9	48.0
White wine	Slope[a] (L μg^{-1})	3.83±0.04	2.14±0.06	2.51±0.03	1.77±0.02
	LOD (ng L^{-1})	20.8	14.9	19.1	16.9
Red wine	Slope[a] (L μg^{-1})	4.29±0.09	3.16±0.09	3.53±0.03	1.85±0.04
	LOD (ng L^{-1})	16.3	18.2	17.8	12.9

[a] Mean value ± standard deviation (n=6).

Limits of detection (LODs) and quantification (LOQs) were calculated taking into account signal-to-noise ratios of 3 and 10, respectively. LOD values between 12.9 and 20.8 ng L^{-1}, depending on the compound, were obtained for beer and wine samples, and between 36.3 and 48 ng L^{-1}, for whisky (Table 3). LOQs ranged between 43 and 160 ng L^{-1}, depending on the compound and the sample.

In order to check the accuracy and repeatability of the method, ten replicate analyses were performed using different beverages spiked at two different levels. Beer and wine samples (red and white) were spiked at 0.2 and 1 μg L^{-1} for each compound, while whisky samples were spiked at 0.8 and 4 μg L^{-1}.

As shown in Table 4, recoveries varied from 89 to 111% (n=160) at the lowest level and from 96 to 107% (n=160) at the highest level. RSD values ranged from 4 to 11% for the lowest level, and from 3 to 6% for the highest level, depending on the compound (Table 4).

3.4. Analysis of real samples

Seventeen wood aged alcoholic beverages, including four whiskies, five white wines, four red wines and four beers were analyzed by CPE using the microvial insert thermal desorption coupled to GC–MS procedure.

The results showed that all the analyzed beverages were free of the compounds under study, or at least at levels above the corresponding LODs. Only TCA was found in one red wine sample (176±19 ng L^{-1}) in which a taint defect had been detected by sensory analysis.

Fig. 3A shows a typical chromatogram obtained under SIM mode for a white wine sample fortified at a 0.2 μg L^{-1} concentration level, in the selected conditions. The analytes were identified by comparing the retention time, identifying the target (*T*) and qualifier ions and qualifier-to-target ratios of the peaks in samples and spiked samples.

Table 4
Recovery studies.

Sample	Spike level (μg L^{-1})	TCA	TeCA	TBA	PCA
Beer	0.2	108 (11)	106 (5)	97 (4)	88 (8)
	1	96 (4)	96 (3)	101 (3)	107 (3)
Whiskey	0.8	103 (5)	108 (4)	95 (6)	94 (4)
	4	100 (5)	99 (4)	102 (3)	99 (3)
White wine	0.2	107 (4)	104 (4)	93 (5)	95 (5)
	1	97 (4)	101 (3)	102 (3)	100 (6)
Red wine	0.2	103 (7)	89 (4)	96 (10)	111 (5)
	1	96 (3)	105 (5)	101 (4)	99 (4)

Values in brackets correspond to RSD values (%, n=10).

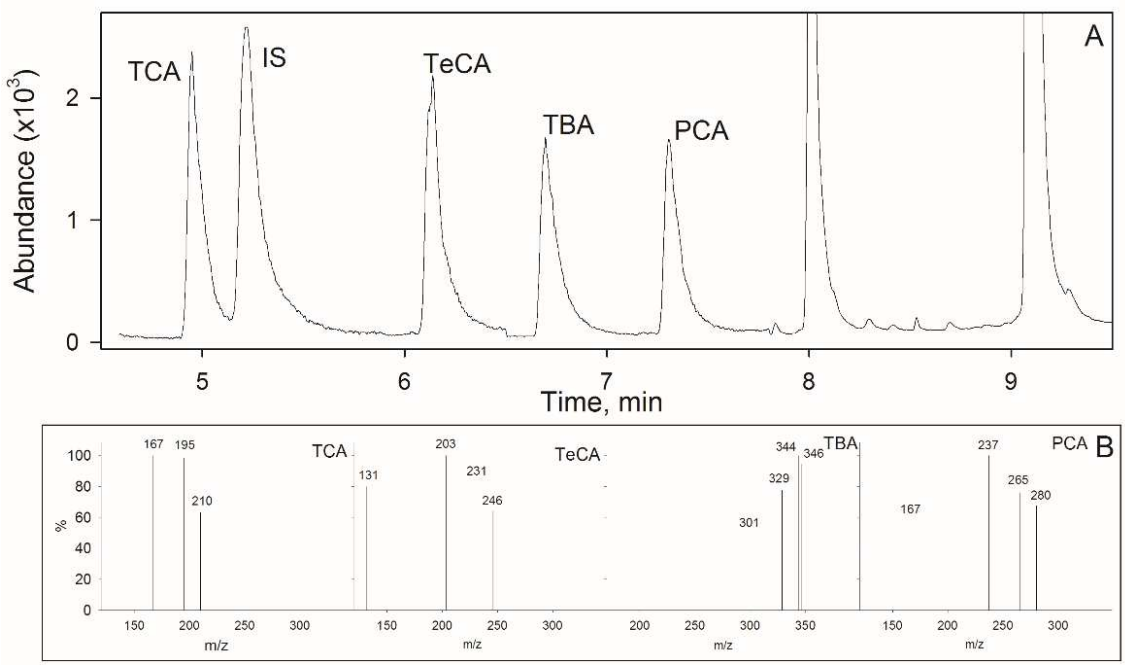


Fig. 3. (A) Elution profile obtained for a spiked (0.2 μg L^{-1}) white wine sample by CPE microvial insert thermal desorption GC–MS under SIM mode. (B) Mass spectra of the compounds.

4. Conclusion

The incompatibility of surfactant rich phases with GC is here successfully overcome by microvial direct thermal desorption. High volumes of the obtained extract can be analysed while avoiding back extraction or surfactant derivatization steps. The sensitivity, repeatability and recovery values obtained shows that the CPE-GC-MS method can be used for routine analysis of alcoholic beverages, avoiding the use of toxic organic solvents and with minimal sample handling.

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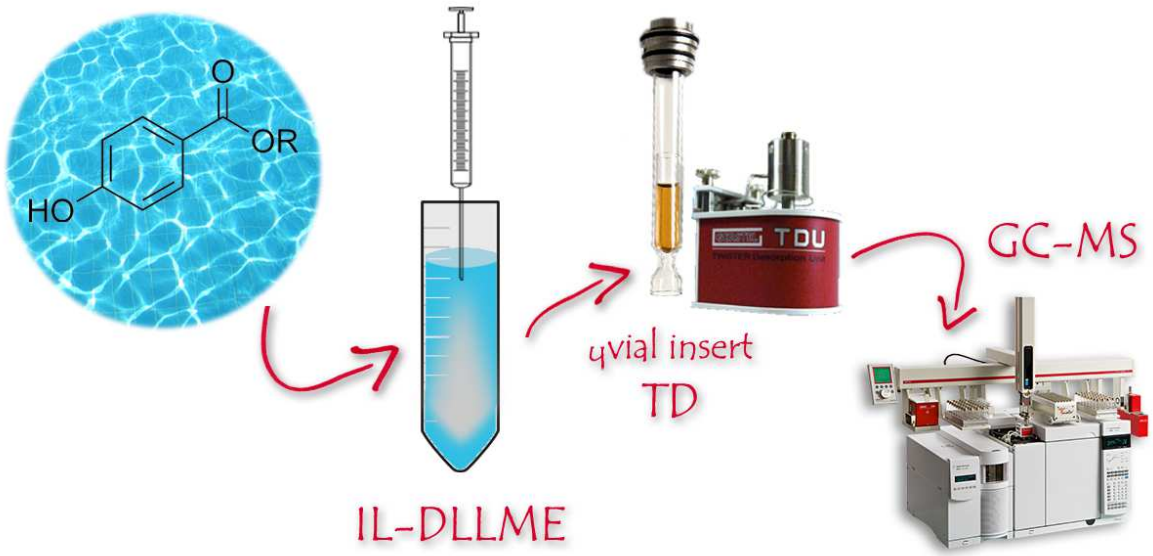
Chapter IV

Microvial insert Thermal Desorption: coupling Ionic Liquids and Gas-Chromatography

Improved sensitivity gas chromatography-mass spectrometry determination of parabens in waters using ionic liquids

Abstract

A new procedure for the introduction of ionic liquid samples in gas chromatography (GC) is proposed. This procedure, based on microvial insert thermal desorption, allows the direct analysis of the compounds preconcentrated by ionic liquid based liquid-liquid microextraction (IL-LLME) using the combination of a thermal desorption unit (TDU) and a programmed temperature vaporization injector (PTV). Two different IL-LLME methodologies, one based on the formation of a microdroplet emulsion by dispersive liquid-liquid microextraction (DLLME) and other through ultrasound-assisted emulsification microextraction (USAEME) were studied and evaluated. IL-DLLME proved advantageous and consequently, it was adopted for preconcentration purposes. This easy to apply approach was used for the determination of five parabens (methyl-, ethyl-, propyl-, butyl- and isobutyl paraben) in swimming pool waters, after *in situ* acetylation. The optimized conditions of TDU/PTV allowed the analytes contained in 20 µL of the enriched IL to be transferred to the capillary column. Quantification of the samples was carried out against aqueous standards, and quantification limits of between 4.3 and 8.1 ng L^{-1} were obtained, depending on the compound. Concentrations of between 9 and 47 ng L^{-1} for some analytes were obtained in the analysis of ten samples.



1. Introduction

The valuable characteristics shown by ionic liquids (ILs) have made them one of the most investigated topics in analytical chemistry in recent years [1,2]. Due to their low vapour pressure and high thermal stability, as well as their great capacity to dissolve a wide range of organic compounds, ILs have been employed as stationary phases in gas chromatography (GC) [3] or as extracting coatings in solid-phase microextraction (SPME) [4]. Moreover, ILs have been proposed as an alternative to conventional organic solvents in liquid-liquid microextraction (LLME) techniques [5–8], including those based on the dispersion of the extractant [9], like IL dispersive LLME (IL-DLLME) and IL ultrasound-assisted emulsification microextraction (IL-USAEME), or other new strategies for DLLME, such as *in situ* ionic liquid formation or cold induced aggregation.

Liquid chromatography (LC) is commonly used as the separation technique when dealing with IL-LLME processes [7], since the direct injection of the IL used as extractant is not compatible with common GC sample introduction systems. Although ILs are thermally stable at high temperatures in inert atmospheres, their low volatility leads to them accumulating in the injector and column, resulting in unsatisfactory separation performance and distorted chromatograms [5]. However, the use of IL extracts in GC may be advantageous, since their low volatility avoids the interference of organic solvent peaks, as well as the degradation related with the introduction of large solvent volumes into the system. Besides, their high solubility allows the GC determination of a wide range of analytes with different polarities and boiling points [6].

GC analysis of compounds extracted by ILs usually requires a previous back extraction into a GC-compatible organic solvent after water dilution of the IL phase. This time consuming procedure may lead to sample contamination and a decrease in the overall efficiency of the extraction process [7]. While direct injection of small volumes of IL extract without further manipulation provides good analytical performance, IL contamination within the GC liner requires frequent cleaning and maintenance [10]. Zhao et al. [11,12] proposed the direct exposure of the extracting drop (IL-SDME) in the GC injector, where high temperatures lead to the thermal desorption of analytes, while the IL remains unaltered. The use of a micro-syringe allows the exposure of the drop and the recovery from the injector, without introducing the IL into the GC system. Due to the unstable nature of this hanging drop, some modifications have been assayed, including larger diameter injection liners to avoid drop losses [11], or the use of a small glass tube inside the injector to prevent the entry of IL into the chromatographic column [12].

Although difficult, IL-LLME and GC can be coupled using external interfaces that facilitate the transfer of the analytes extracted from the IL microdrop to the GC column, while preventing the contamination of the GC system by IL. Aguilera-Herrador et al. proposed a self-made interface

[13], in which target analytes were volatilized by heating before being transferred to the GC column by helium gas. This system was applied for the determination of BTEX [14] and THM [15] in water samples. Canals et al. proposed the use of a commercially-available thermodesorption unit, in which a removable insert containing the IL drop was placed inside the desorption tube to determine chlorobenzenes [16] and organotin compounds [17].

New thermal desorption devices allow the introduction of glass inserts capable of containing 150 μL of liquid sample into the thermal desorption tubes, in a method known as direct microvial insert thermal desorption [18]. This technique is adapted here to inject larger volumes of the IL extract into the GC system, while ensuring no IL contamination of the GC system.

Parabens are p-hydroxybenzoic esters with different alkyl chains, which are employed as antibacterial, antifungal and preservative agents in food, cosmetic, personal care and pharmaceutical products. Their antimicrobial activity increases with the length of the alkyl chain whereas their water solubility decreases. Some studies have suggested that parabens act as possible endocrine disruptors, so that exposure to them may be related with breast cancer [19]. Because these compounds are widely used in cosmetics and personal care products, they can reach the water in swimming pools through bathers. Parabens can also be found in swimming pool water as chlorinated by-products [20]. Their determination has been accomplished mainly by GC-MS [21], using preconcentration techniques like solid-phase extraction (SPE) [22], solid-phase microextraction (SPME) [23–25], stir bar sorptive extraction (SBSE) [26–29], membrane assisted liquid-liquid extraction [29], extraction with nanoparticles [20,30], and different LLME techniques, including hollow fiber LLME (HF-LLME) [31], DLLME [32–37], or packed sorbent ME [38]. IL-LLME has been proposed for parabens determination using direct spectrometric detection [39] or LC-UV [40]. Other IL related techniques, like IL coated SPME coupled to GC with flame ionization detection (FID) [41], and polymerized IL in stir cake sorptive extraction (SCSE) [42] for LC-UV have also been used for this purpose. Here, the determination of five parabens, methyl paraben (MeP), ethyl paraben (EtP), propyl paraben (PrP), isobutyl paraben (iBuP) and butyl paraben (BuP), in water samples has been tackled using direct microvial insert thermal desorption for IL-DLLME-GC-MS.

2. Materials and methods

2.1. Chemicals and reagents

Methyl paraben (methyl 4-hydroxybenzoate, 99%, MeP), ethyl paraben (ethyl 4-hydroxybenzoate, 99%, EtP), propyl paraben (propyl 4-hydroxybenzoate, >99%, PrP), butyl paraben (butyl 4-hydroxybenzoate, >99%, BuP), isobutyl paraben (isobutyl 4-hydroxybenzoate, 97%, iBuP), and 4'-hydroxyacetophenone (internal standard, 99%) were obtained from Sigma (St.

Louis, MO, USA). Stock solutions (1000 mg L^{-1}), prepared by dissolving the commercial products in methanol, were kept at −18 ºC in darkness. The water-immiscible ionic liquids 1-methyl-3-hexylimidazolium bis(trifluoromethyl sulfonyl)imide ($[C_6MIm][NTf_2]$), 1-methyl-3-octylimidazolium bis(trifluoromethyl sulfonyl)imide ($[C_8MIm][NTf_2]$) and 1-decyl-3-methylimidazolium bis(trifluoromethyl sulfonyl)imide ($[C_{10}MIm][NTf_2]$) were supplied by IOLITEC (Heilbronn, Germany). The derivatising reagents assayed were acetic anhydride (AA, Fluka, Buchs, Switzerland, >99% purity) and bis(trimethylsilyl)trifluoroacetamide (BSTFA, Supelco, Bellefonte, PA, USA). Dipotassium hydrogen phosphate was purchased from Fluka (Buchs, Switzerland).

2.2. Instrumentation

The sample introduction system comprised a thermal desorption unit (TDU-2) equipped with a multipurpose autosampler (MPS-2) and a programmed temperature vaporization (PTV) cooled injector system (CIS-4) provided by Gerstel (Mullheim an der Ruhr, Germany). The experimental conditions used for the sample introduction system are summarized in Table 1.

GC analyses were performed on an Agilent 6890N (Agilent, Waldbronn, Germany) gas chromatograph coupled to an Agilent 5973 quadrupole mass selective spectrometer equipped with an inert ion source. Under the selected conditions (Table 1), the compounds eluted at retention times between 8.1 and 10.9 min, corresponding to MeP and BuP, respectively (Table 2). In order to improve the detection limits, compounds were quantified in the selected ion monitoring (SIM) mode using different ions (Table 2). Identification was confirmed by the retention time of the target ion and the qualifier-to-target ion ratios for each compound.

Table 1
Experimental conditions of the TD-GC-MS procedure.

Thermal Desorption Unit	
Mode	Splitless
Temperature programme	80 – 280 ºC at 200 ºC min^{-1}, held 4 min
Desorption flow	100 mL min^{-1}
Cooled Injector System	
Mode	Solvent venting
Liner	Packed sylanized glass wool, 2 mm i.d.
Temperature programme	15 – 280 ºC (2 min) at 530 ºC min^{-1}
GC-MS	
Capillary column	HP-5MS, 5% diphenyl-95% dimethylpolysiloxane (30 m x 0.25 mm, 0.25 μm)
Carrier gas	Helium, 1 mL min^{-1}
Oven programme	80 ºC, held 0.5 min 80 – 170 ºC at 20 ºC min^{-1} 170 ºC, held 3.25 min 170 – 265 ºC at 20 ºC min^{-1}
Transfer line, quadrupole and ion source temperature	300 ºC, 150 ºC, 230ºC
Ionization	Electron-impact mode (70 eV)

Table 2
Method characteristics.

Compound	RT, min	Monitored ions	RSD[a], %	LOQ[b], ng L^{-1}
MeP	8.1	121, 152, 194	4.0	8.1
EtP	9.0	121, 166, 138	4.4	7.7
PrP	10.0	138, 121, 179	6.2	6.5
iBuP	10.4	138, 121, 163	7.7	5.8
BuP	10.9	138, 121, 194	6.0	4.3
IS	8.3	121, 136, 178		

Underlined numbers correspond to *m/z* of the target ion. [a] n=10.
[b] Calculated for S/N=10.

An UP 200 H ultrasonic processor (Dr. Hielscher, Teltow, Germany), with an effective output of 200 W (24 kHz) in liquid media equipped with a titanium sonotrode (7 mm i.d.), was used for emulsification. An EBA 20 centrifuge (Hettich, Tuttlingen, Germany) was used to disrupt the emulsions.

2.3. Samples and analytical procedure

A total of 10 samples were obtained from indoor swimming pools in south-eastern Spain with the kind permission of the local authorities. Samples were kept in darkness at 4 °C until analysis.

For IL-DLLME extraction, a 10 mL aliquot of the water sample was placed in a 15 mL glass tube with conical bottom and spiked with the IS (4'-hydroxyacetophenone) at a concentration of 200 ng L^{-1}. Then, 70 mg of K_2HPO_4 and 50 µL of acetic anhydride were added as neutralizing and derivatizing agents, respectively, and the mixture was shaken for 20 seconds. A volume of 400 µL of methanol (disperser) containing 50 µL of the IL $[C_6MIm][NTf_2]$ (extractant) was rapidly injected into the mixture, leading to the formation of a cloudy solution consisting of very fine droplets of IL dispersed through the aqueous phase, which extract the acetylated parabens. This emulsion was centrifuged for 2 min at 3000 rpm, and the sedimented IL phase was collected using a microsyringe. A 20 µL aliquot of the recovered phase was poured in a 150 µL glass microvial, which was introduced into a glass desorption tube. The analytes were submitted to the vaporization step by placing the desorption tube in the TDU.

Since no reference materials were available, spiked samples were prepared at two different concentration levels for validation purposes. Three water samples were spiked with the analytes at 50 and 350 ng L^{-1} and three replicates were analyzed in each case.

3. Results and discussion

As previously stated, direct IL injection into the GC system is not possible, and tedious procedures are usually necessary before the sample can be introduced. In order to overcome this limitation, the use of a commercial thermal desorption unit as interface to transfer the extracted analytes from the IL to the GC system was evaluated.

This approach has already been proposed by Canals et al. [16,17], who placed the IL drop in a small glass tube, which was inserted into the TD tube. However, this configuration cannot guarantee that no IL is propelled by the carrier gas from the TD tube to GC column, so that the glass tube has to be surrounded by glass wool. In addition, only IL volumes lower than 5 μL can be placed in the glass tube, meaning that a small fraction of the resulting IL extraction phase is effectively employed.

TDUs have been extensively used in SBSE-GC hyphenation, since they are versatile tools that allow the thermal desorption of liquid samples placed in a glass microvial inside the desorption tube. Direct microvial insert thermal desorption [18] may be employed for introducing IL extracted compounds into the GC system. The proposed assembly is schematically represented in Fig. 1. The IL is placed in a glass microvial introduced inside the TD tube, and the whole assembly is submitted to thermal desorption. When the IL is heated, extracted compounds are thermally desorbed (vaporized), while less volatile IL remains in the glass microvial. A carrier gas drags the analytes to a PTV injector where they are focused before entering the chromatographic column. After the desorption step, this PTV is heated, and the retained compounds enter the GC system for their separation. This assembly has two main advantages: (i) the whole IL drop resulting from LLME (~20 μL) can be submitted to thermal desorption, since the glass micro-vials can hold large volumes (which simply require longer times for complete thermal desorption); (ii) the tube shape of the glass microvial assures no IL entry to the GC system, and even if some IL vapours are dragged by the carrier flow, they would be retained in the disposable PTV liner, not reaching the GC column.

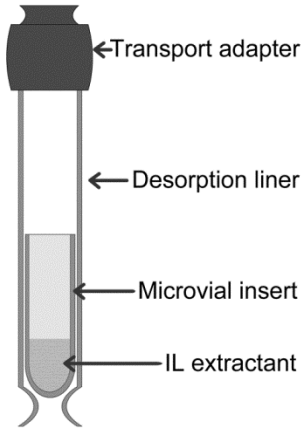


Fig. 1. Scheme of the assembly used for the GC injection of IL by means of a thermal desorption unit.

3.1. Ionic liquid extraction procedure

Due to the acid character of their phenolic group, parabens show poor chromatographic behaviour, providing tailing peaks. In addition, their relative hydrophilicity, with low log $k_{o/w}$ values (1.7 – 3), could lead to low extraction efficiencies into nonpolar extractants. The inclusion of a derivatization step in the analytical procedure helps to overcome these disadvantages. With this in mind, both the analysis of the underivatized compounds, as well as two different derivatization reactions (*in situ* acetylation and *in tube* silylation) were considered.

Silylation is the classic derivatization method for phenols, BSTFA being one of the most used reagents. The main disadvantage of this approach is the need for a water-free environment, which means that derivatization can only be carried out once the parabens have been extracted into the IL. For silylation, 2 μL of BSTFA were placed in the TDU desorption tube inside a glass microvial positioned over the microvial containing the extracting IL, in an assembly similar to that used for the in tube silylation of SBSE stir bars [43].

The acetylation of hydroxyl groups of the phenolic compounds improves their chromatographic response and increases their partition coefficient, leading to higher enrichment efficiencies in the extraction step. This derivatization reaction can be easily performed in the sample medium prior to extraction, by adding an acetylating agent (100 μL of acetic anhydride (AA)) and a neutralizing salt (0.35 g of K_2HPO_4), which react with the acetic acid, resulting in the hydrolysis of the acetic anhydride.

According to the obtained results, (Fig. 2A), acetylation of the studied compounds led to an increase in the analytical responses obtained, especially for MeP, the most polar of the studied parabens. Despite the significant improving in peak shapes, silylation provided lower responses for all the analytes, as result of the lower extraction efficiency of the underivatized parabens and the incomplete silylation reaction.

The influence of the derivatization agent volume on the paraben responses was tested with AA volumes ranging from 50 to 200 μL. For each AA volume tested, one neutralizing equivalent of K_2HPO_4 was added to the extraction solution. The resulting mixture was manually shaken for 20 seconds while derivatization took place, and afterwards submitted to extraction. As shown in Fig. 2B (n=3), best responses for all the analytes were obtained when 50 μL of AA were used.

A neutralizing salt (K_2HPO_4) was added to the sample in order to ensure the basic medium required for the derivatization reaction to take place, as well as to fix the sample pH at a neutral value during the extraction, once the analytes had been derivatized and the neutralizing salt had reacted with the resulting acetic acid. Different masses of K_2HPO_4, ranging from 35 to 185 mg (corresponding to 0.2 to 1 neutralizing equivalents for 50 μL of AA) were tested. As shown in Fig. 2C, the higher the amount of K_2HPO_4 used, the stronger the MeP analytical signal. The opposite

effect was observed for BuP due to the different polarity as result of the alkyl chain. Taking into account the average response, the addition of a mass of 70 mg of K_2HPO_4, corresponding to 0.4 neutralizing equivalents, was selected.

Three ILs of low water solubility and higher density than water, $[C_6MIm][NTf_2]$, $[C_8MIm][NTf_2]$ and $[C_{10}MIm][NTf_2]$, were assayed as extraction solvents, adding 80 µL of each to the aqueous phase containing the derivatized analytes. Even though the differences obtained were not statistically significant, best results were provided by $[C_6MIm]$, especially for the less polar compounds (Fig. 3A).

The IL volume was tested in the 50–80 µL range. As shown in Fig. 3B, the higher the volume, the lower the sensitivity, due to a dilution effect. Volumes lower than 50 µL were not assayed because less than 20 µL were recovered after centrifugation, hindering the recovery of the bottom drop when using a microsyringe. Consequently, 50 µL was adopted.

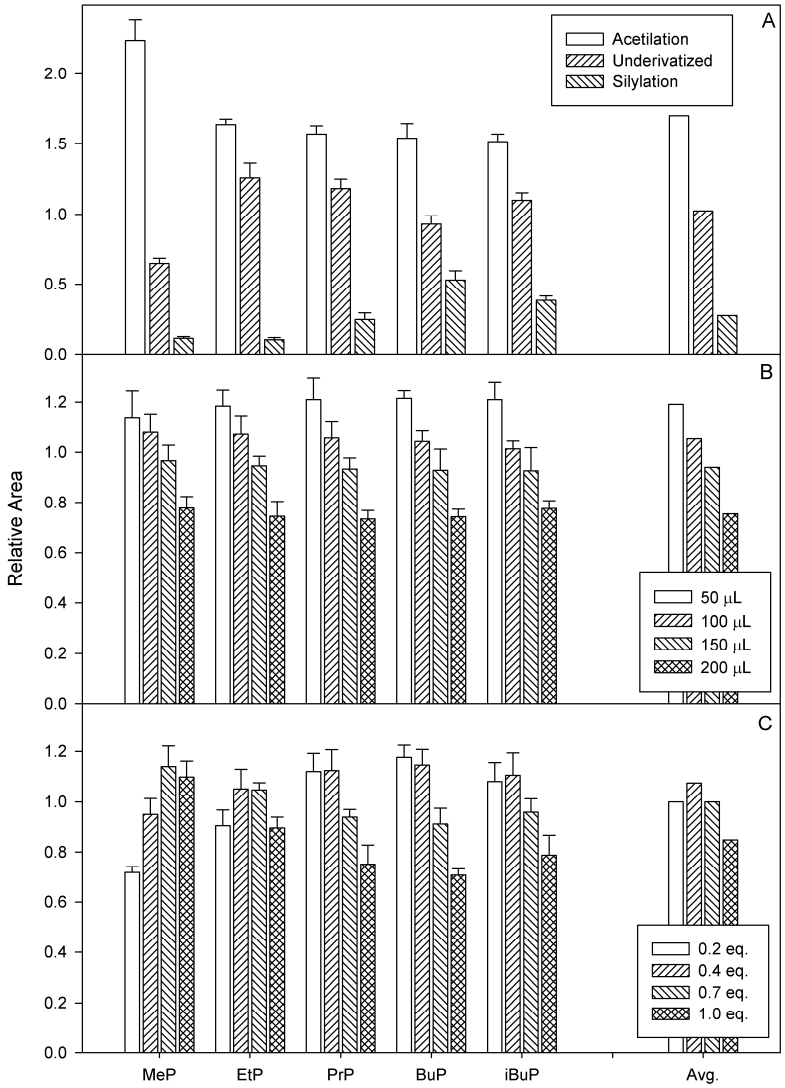


Fig. 2. (A) Responses obtained using different derivatization procedures (n=3). Influence of (B) volume of acetic anhydride and (C) neutralizing equivalents on the sensitivity (n=3).

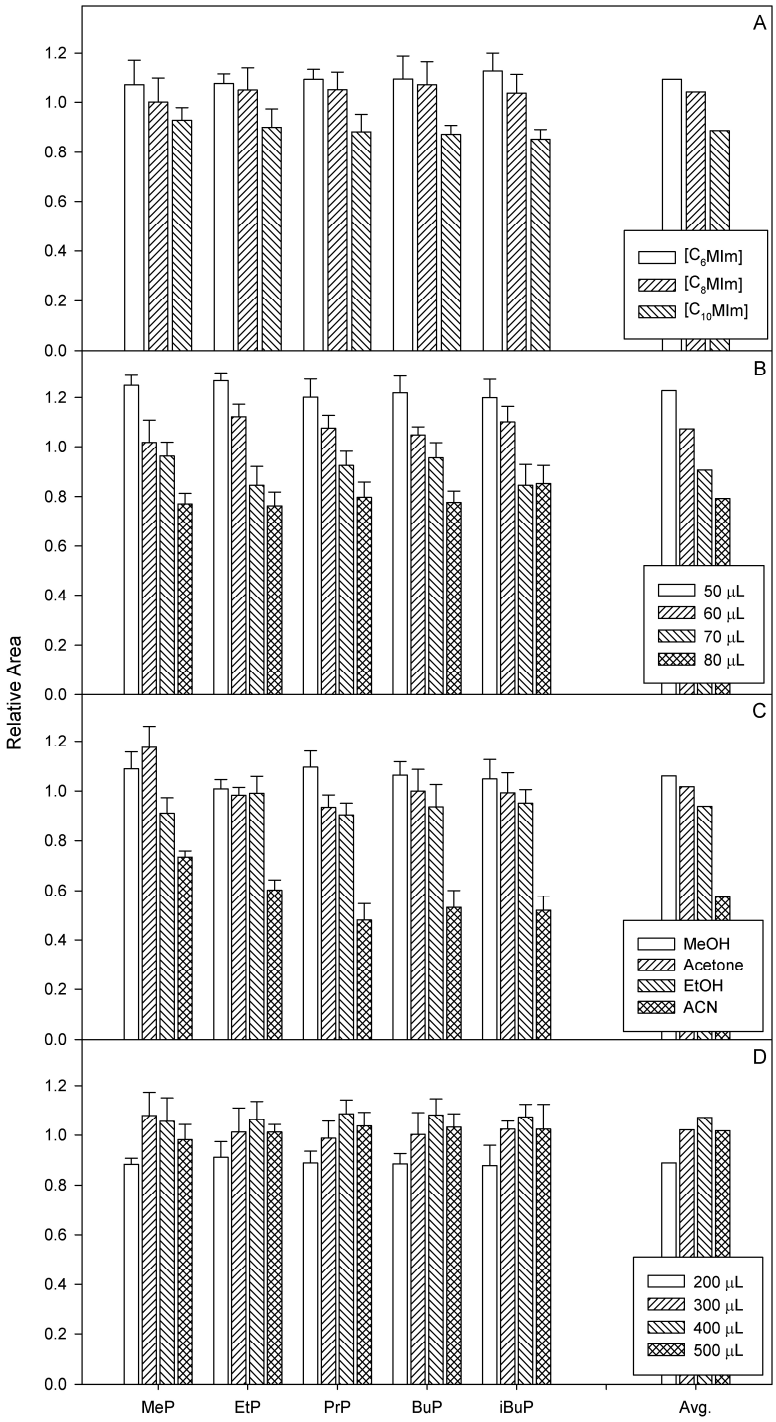


Fig. 3. (A) Extraction efficiency (n=3) of different ILs and (B) influence of the volume of [C_6MIm]. Effect of (C) different disperser solvents and (D) volume of methanol as disperser on the sensitivity of the paraben responses (n=3).

A relative long extraction time is required to reach equilibrium between the water sample and the IL extracting phase. An increase in the contact surface area of these two phases through the formation of an emulsion can speed up the extraction process, which can be completed in a few seconds. The formation of this emulsion can be achieved using a third solvent, as in DLLME. This dispersive solvent must be soluble both in water and in the IL. The application of ultrasounds to the water sample and IL mixture may also lead to the formation of a cloudy solution, as in USAEME.

In both cases, the resulting emulsion is broken by centrifugation, allowing separation of the two phases and recovery of the IL containing the analytes. These two different dispersive extraction approaches were assayed.

For IL-DLLME, the two variables involved in the extraction step, namely disperser solvent nature and disperser solvent volume, were studied. Four different solvents (acetone, acetonitrile, ethanol and methanol) were tested, mixing 0.5 mL of each solvent with 50 μL of the IL, and rapidly injecting into the sample solution. As shown in Fig. 3C, extraction efficiency for most of the studied compounds was higher when methanol was used.

The volume of methanol was tested in the 200-500 μL range. Low disperser volumes may lead to poor emulsion formation, hindering an effective and quick extraction, while large volumes may increase analyte solubility in the aqueous sample medium, thus reducing extraction efficiency. A disperser volume of 400 μL provided the best responses (Fig. 3D), except in the case of MeP, which attained its maximum sensitivity when 300 μL was used. Taking into account the average response, 400 μL was selected.

For IL-USAEME, variables involved in the sonication step, including number of cycles, duration of each cycle and amplitude, were studied. Each of these variables was evaluated at three levels. The optimal conditions for US extraction of parabens were 30 cycles of 0.6 s duration and 105 μm amplitude.

The two dispersive extraction approaches were applied to spiked water samples and the results were compared (Fig. 4). DLLME provided higher responses than USAEME for all the studied compounds, and thus, the former was selected.

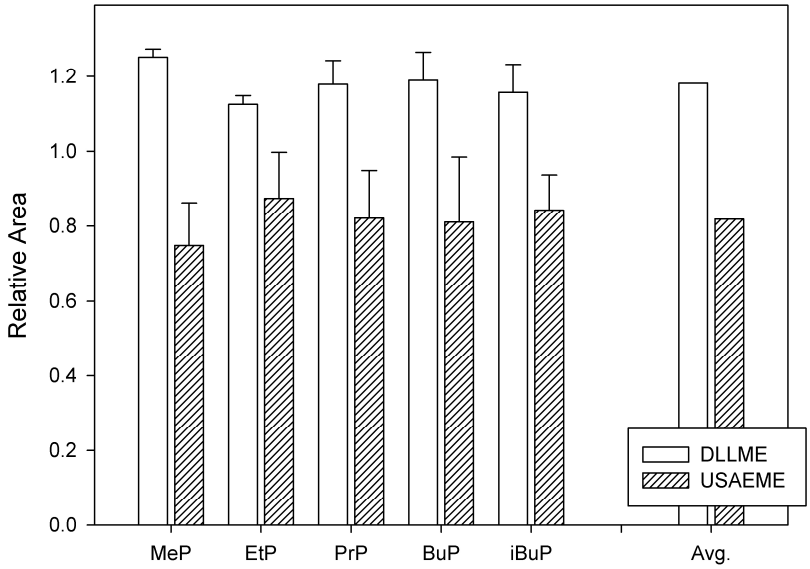


Fig. 4. Comparison of the responses obtained using IL-DLLME-GC-MS and IL-USAEME-GC-MS.

3.2. Thermal desorption

Aliquots (20 µL) of the enriched IL were placed in a glass microvial using a microsyringe, and submitted to thermal desorption to vaporize the analytes. Three parameters involved in this step, desorption temperature and time and gas flow rate, were considered.

The desorption temperature was studied in the 220 – 280 ºC range. Although higher temperatures may facilitate analyte vaporization, values higher than 280 ºC were not applied owing to a significant increase in the chromatographic background related with the thermal decomposition of the IL. Maximum sensitivity was obtained for all the compounds at desorption temperature of 280 ºC (Fig. 5A).

As result of the relatively large volume of IL placed in the glass microvial (20 µL), the analytes are not immediately vaporized from the extracting phase, and so heating should be maintained. When desorption times of 1 – 4 min were tested, the analytical signal increased significantly for all compounds at longer desorption times (Fig. 5B), so the highest assayed value, 4 min, was selected.

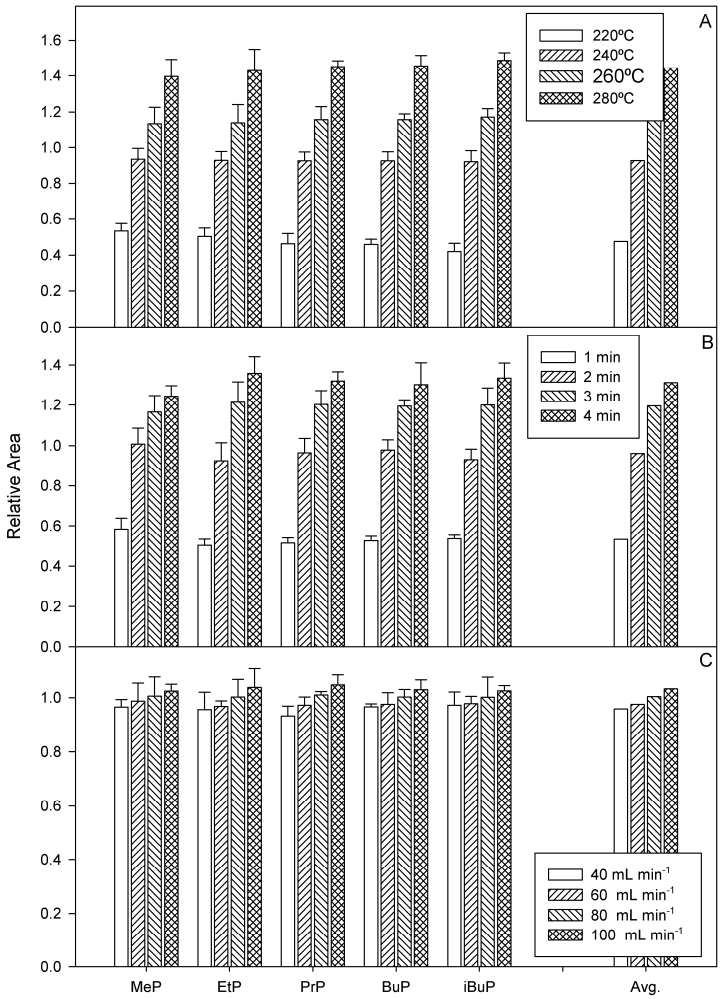


Fig. 5. Influence of (A) desorption temperature, (B) desorption time and (C) gas flow rate on obtained responses (n=3).

A helium carrier gas flow should be maintained during the thermal desorption step, so as to propel the vaporized analytes from the heated TDU to the cooled PTV. Values from 40 to 100 mL min^{-1} were tested (Fig. 5C), and a slight increase in the obtained responses was observed at the highest value.

Vaporized compounds were focused on the PTV before they enter the chromatographic column. Lower temperatures in this device increase its retention efficiency and minimize losses of the more volatile compounds. Since the Peltier unit only allows cooling to temperatures slightly below room temperature, 15 ºC was selected as focusing temperature. A liner filled with the inert support fibreglass was used to facilitate the retention of the analytes in the PTV. After the desorption step, the PTV was heated to 280 ºC in order to elute the retained compounds to the chromatographic column. Therefore, a program temperature of 15 to 280 ºC at 530 ºC min^{-1}, with a hold time of 2 min, was applied.

3.3. Method performance

The use of an internal standard (IS) to increase repeatability was assayed. 4'-Hydroxyacetophenone [44], which has a similar chemical and chromatographic behaviour towards the analytes, was tested for this purpose. The use of the IS, added to the samples at a concentration of 200 ng L^{-1}, produced an enhancement in the repeatability of the response of the studied compounds. RSD values, obtained at a concentration of 350 ng $L^{-1,}$ were reduced from 5 – 11% to 4 – 8%, depending on the compound (Table 2).

Calibration graphs using the standard additions method were obtained for distilled water and four water samples from different origins (tap and swimming pool), which were spiked with the analytes in the 25 – 1000 ng L^{-1} range, at six levels in duplicate, providing good correlation coefficient values ($r > 0.99$). The slopes of the standard additions method for the four cited samples were evaluated using an ANOVA test. "P" values in the 0.20 – 0.95 range were obtained, pointing to the absence of statistically significant differences among the samples, and demonstrating the suitability of single aqueous calibration for the quantification of water samples of different origins.

Quantification limits (LOQs) were calculated taking into account a signal-to-noise ratio of 10 and the obtained values ranged between 4.3 and 8.1 ng L^{-1}, depending on the compound (Table 2). The proposed procedure provides sensitivity of the same order as other previously published methods based on SPME [25] and SBSE [28], which need longer analysis times, and others based on LPME [25,32].

Table 3
Analysis of the samples, ng L^{-1}.

Sample	MeP	EtP	PrP	iBuP
Pool A	15±2	ND	ND	ND
Pool B	ND	ND	28±4	ND
Pool C	ND	22±2	ND	27±4
Pool D	20±3	9±1	ND	ND
Pool F	9±2	NQ	ND	ND
Pool G	33±14	ND	47±11	ND
Pool H	16±3	ND	29±4	ND
Pool I	9±3	ND	ND	ND

Values are mean ± standard deviation (n=3).
ND means not detected. NQ means not quantified.

3.4. Analysis of the samples

The optimized procedure was applied to the analysis of 10 swimming pool water samples. Some of the analytes were found in eight of the samples, as shown in Table 3. MeP was the most widespread paraben in the analyzed samples, being found in six of them, at concentration of 9 to 33 ng L^{-1}. The concentrations of the analytes were low since the water samples were obtained from indoor swimming pools, and bathers would not use the cosmetics and sunscreens to the extent that would usually apply in outdoor swimming pools.

Fig. 6 shows a typical chromatogram obtained by IL-DLLME-GC-MS for a spiked swimming pool water sample, demonstrating the absence of interfering peaks at the retention time of the analytes, which were identified by comparing their retention times and mass spectra in samples and standard solutions.

Since no reference material was available, the method was validated by recovery assays, fortifying three different swimming pool water samples at two concentration levels (50 and 350 ng L^{-1}). Recoveries in the 85 – 120% range were obtained for the lowest level, while recoveries of 95 – 105% were obtained for the highest level.

4. Conclusion

Direct microvial insert thermal desorption is shown to be an easy and efficient sample introduction procedure for GC when the analytes are extracted in ILs. The sensitivity attained for the parabens is comparable to that of other previously proposed procedures based on microextraction to a solid phase, which involve longer analysis times, or a liquid phase using chlorinated extractant solvents. The inherent characteristics of ILs allow the proposed procedure to be catalogued as a green analytical method. The analytical characteristics obtained for the IL-DLLME procedure are better than those for IL-USAEME. The proposed procedure showed its robustness and reliability for the determination of parabens in swimming pool waters.

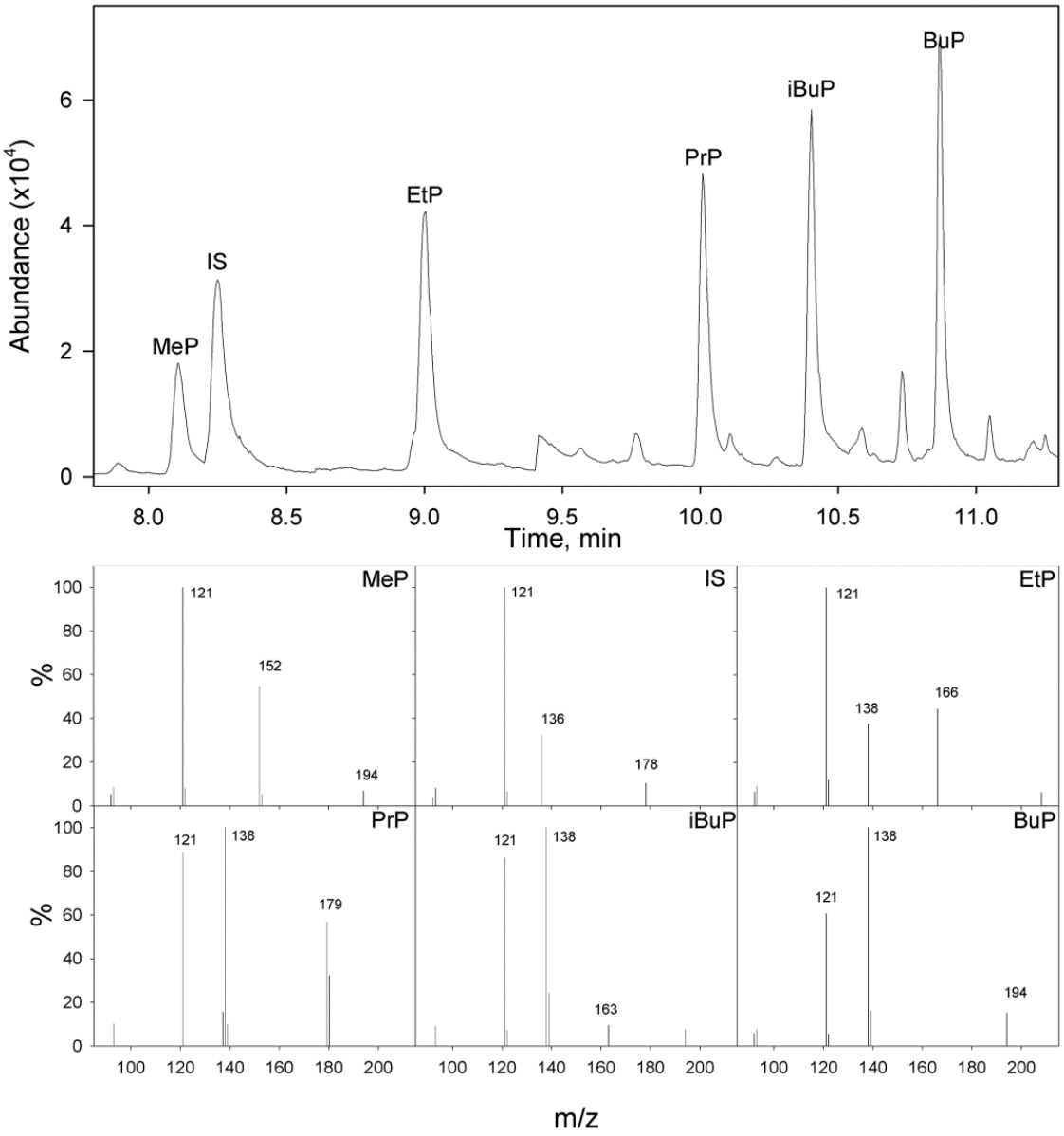


Fig. 6. (A) IL-DLLME-GC-MS chromatogram of a spiked swimming pool sample (350 ng L^{-1}) showing peaks corresponding to 1-MeP, 2-IS, 3-EtP, 4-PrP, 5-iBuP, 6-BuP. (B) Mass spectra of the studied compounds.

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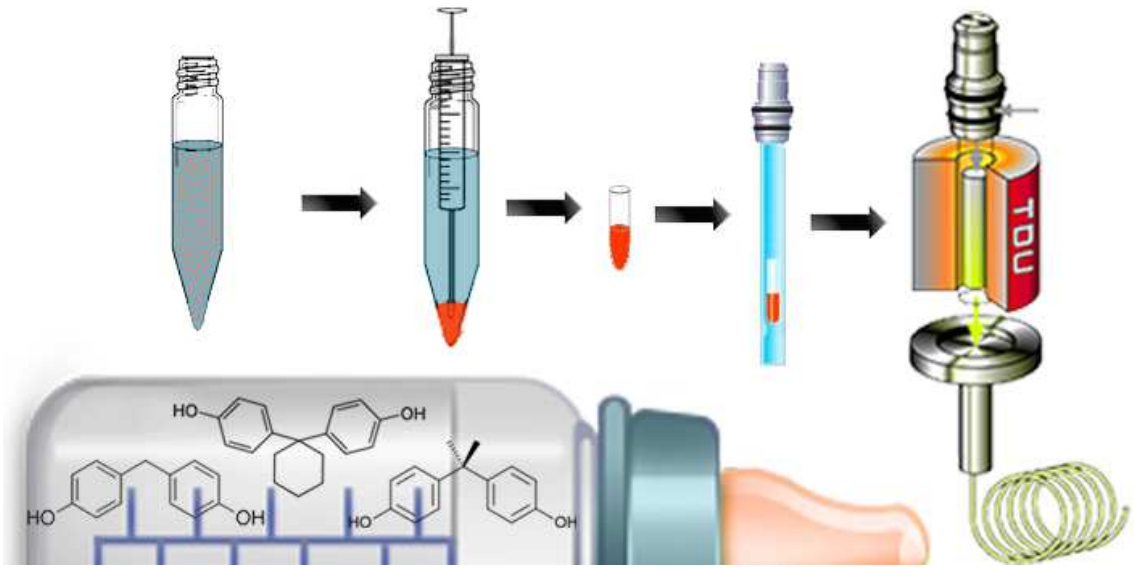
In situ ionic liquid dispersive liquid-liquid microextraction and direct microvial insert thermal desorption for gas chromatographic determination of bisphenol compounds

Abstract

A new procedure based on direct insert microvial thermal desorption injection allows the direct analysis of ionic liquid extracts by gas chromatography and mass spectrometry (GC-MS). For this purpose an *in situ* ionic liquid dispersive liquid-liquid microextraction (*in situ* IL DLLME) has been developed for the quantification of bisphenol A (BPA), bisphenol Z (PBZ) and bisphenol F (BPF). Different parameters affecting the extraction efficiency of the microextraction technique and the thermal desorption step were studied. The optimized procedure, determining the analytes as acetyl derivatives, provided detection limits of 26, 18 and 19 ng L^{-1} for BPA, BPZ and BPF, respectively.

The release of the three analytes from plastic containers was monitored by this new developed analytical method. Analysis of the migration test solutions for fifteen different plastic containers in daily use identified the presence of the analytes at concentrations ranging between 0.07 and 37 µg L^{-1} in six of the samples studied, BPA being the most commonly found and at higher concentrations than the other analytes.



1. Introduction

Bisphenols (BPs) are industrially important chemicals widely used in the manufacture of plastics and epoxy resins, the latter employed in the production of food-contact surface lacquers for cans. Trace residue levels of BPs can be released by diffusion or degradation into food or beverages. BPs are considered endocrine disruptor chemicals (EDCs) and may even act as carcinogenic and mutagenic compounds. Consequently, the European Union (EU) has established a specific migration limit (SLM) of 0.6 mg kg^{-1} for food-contact plastic materials [1], more specifically for bisphenol A (BPA), which is the most widely used of the BPs. Moreover, in 2011 the use of BPA was prohibited for the manufacture of polycarbonate feeding bottles intended for infants of up to 12 months of age [2]. The extensive use of BP-based polymers, the hazards associated with their use, and restrictive legal regulations, underline the need for efficient and sensitive analytical methods to control their exposure to humans.

The high sensitivity required for BP determination means that enrichment methods are necessary during the sample preparation step. Environmentally friendly sample preparation techniques based on microextraction approaches have replaced classical methods in the last years. Ionic liquids (ILs), due to their low vapour pressure, high thermal stability, and great capacity to solubilize a wide range of organic compounds, have been proposed as an alternative to conventional organic solvents in liquid-liquid microextraction (LLME) techniques [3]. Indeed, ILs have been used in a great variety of LLME techniques [4–6], including those based on the dispersion of the extractant [7], IL dispersive LLME (IL-DLLME). In addition, ILs offer new strategies for DLLME, such as *in situ* IL formation or vortex assisted IL-DLLME [7].

In the particular case of BPs, microextraction techniques based on ILs, such as IL-DLLME [8–14], hollow fibre (HF–LPME) [15, 16], magnetic nanoparticles dispersive solid phase extraction [17], or solid phase microextraction (SPME) [18-20], have previously been employed. Usually these techniques are coupled to liquid chromatography (LC) using ultraviolet (UV) [8–9, 11–12, 15–17, 20], mass spectrometry (MS) [10] or fluorimetric (FLD) [13] detectors, or capillary electrophoresis (CE) [14]. The direct GC analysis of enriched ILs is only possible when SPME [18, 19] or SDME [22] are employed, since ILs used as extractants are not compatible with common GC sample introduction techniques, leading to injector and column blockage due to their low volatility.

This disadvantage may be overcome by using of thermal desorption units and removable glass microvials, in which the IL extract may be placed. When the IL containing the analytes is heated in this assembly, extracted compounds are vaporized and introduced into the GC column, while the IL remains in the glass microvial. This technique, known as direct microvial insert thermal desorption or microvial insert large volume injection [23, 24] allows large volumes of the solvent extract to be

introduced into the GC system, avoiding no stationary phase column contamination. As far as we know, direct microvial insert thermal desorption has not been applied for the direct analysis of IL enriched phases by GC.

Consequently, the aim of this work was the optimization of *in situ* IL-DLLME using direct microvial insert thermal desorption and GC-MS for the determination of bisphenol A (BPA), bisphenol F (BPF) and bisphenol Z (BPZ) in studies of the migration that may occur from plastic containers.

2. Materials and methods

2.1. Reagents

Bisphenol A (BPA, 4,4'-dihidroxy-2,2-diphenylpropane, 99% purity), bisphenol F (BPF, 4,4'-dihidroxy-2,2-diphenylmethane, 98% purity), bisphenol Z (BPZ, 1,1-bis(4-hydroxyphenyl)cyclohexane, 99% purity) and biphenol (BP, 2,2'-biphenol, 98% purity) were obtained from Sigma (St. Louis, MO, USA). Stock solutions (1000 mg L^{-1}) were prepared by dissolving the commercial products in methanol, and kept at −18 ºC in darkness. Acetic anhydride (AA, >99% purity) and dipotassium hydrogen phosphate were purchased from Fluka (Buchs, Switzerland) and Panreac (Barcelona, Spain), respectively.

The ILs 1-hexyl-3-methylimidazolium chloride ([C_6MIm]Cl), 1-octyl-3-methylimidazolium chloride ([C_8MIm]Cl), 1-decyl-3-methylimidazolium chloride ([C_{10}MIm]Cl), 1-dodecyl-3-methylimidazolium ([C_{12}MIm]Cl) and litium bis(trifluoromethyl)sulfonylimide (Li[NTf_2]) were supplied by IOLITEC (Heilbronn, Germany). Ultrapure water was used to prepare 1 M solutions of these ILs.

2.2. Instrumentation

The sample introduction system was composed of a Thermal Desorption Unit (TDU-2) equipped with an multipurpose autosampler (MPS-2) and a Programmed Temperature Vaporization (PTV) Cooled Injector System (CIS-4) provided by Gerstel (Mullheim an der Ruhr, Germany). The experimental conditions used for the sample introduction system are summarized in Table 1.

GC analyses were performed on an Agilent 6890N (Agilent, Waldbronn, Germany) gas chromatograph coupled to an Agilent 5973 quadrupole mass selective spectrometer equipped with an inert ion source. Under the selected conditions (Table 1) the compounds eluted at retention times of between 6.1 and 10.4 min, corresponding to BP and BPZ, respectively (Table 2).

Table 1
Experimental conditions of the TD-GC-MS procedure

Thermal Desorption Unit	
Mode	Splitless
Temperature program	80 – 280 ºC at 400 ºC min^{-1}, held 7.6 min
Desorption flow	78 mL min^{-1}
Cooled Injector System	
Mode	Solvent Venting
Liner	Packed sylanized glass wool, 2 mm i.d.
Temperature programme	15 – 280 ºC (2 min) at 530 ºC min^{-1}
GC-MS	
Capillary column	HP-5MS, 5% diphenyl-95% dimethylpolysiloxane (30 m x 0.25 mm, 0.25 μm)
Carrier gas	Helium (1 mL min^{-1})
Oven programme	90 ºC held 0.5 min 90 – 275 ºC at 25 ºC min^{-1}, 275 – 295 ºC at 40 ºC min^{-1}, held 2.1 min
Transfer line, quadrupole and ion source temperature	300, 150 and 230 ºC
Ionization mode	Electron-impact mode (70 eV)

In order to improve the detection limits (LODs), compounds were quantified in the selected ion monitoring (SIM) mode using different ions (Table 2). Identity was confirmed by the retention time of the target ion and the qualifier-to-target ion ratios for each compound.

An EBA 20 centrifuge (Hettich, Tuttlingen, Germany) was used to disrupt the sample emulsions formed.

2.3. Samples and analytical procedure

Fifteen different plastic containers, including spout cups, feeding bottles, or different varieties of cups, were obtained from local stores or kindly donated by individuals. Migration tests were performed according to EU regulation [1]. The containers were totally filled with distilled water as food simulant and placed in a thermostatic bath at 40 ºC for 10 days. Next, the simulants were kept in darkness at 4 °C until analysis. Blank tests for the simulant were obtained by using glass vessels of 250 mL capacity, being maintained at 40 ºC for 10 days and analyzed.

Table 2
Method characteristics.

Compound	RT, min	Monitorized ions, *m/z*	RSD[a], %	RSD[b], %	Calibration slope[c], L μg $^{-1}$	LOD[d], ng L^{-1}	LOQ[e], ng L^{-1}
BPF	8.1	<u>200</u>, 242, 284	4.7	13.8	0.490±0.024	5.7	19
BPA	8.4	<u>213</u>, 228, 255	7.3	10.5	0.346±0.017	7.8	26
BPZ	10.4	<u>268</u>, 310, 352	5.9	13.2	0.423±0.021	5.4	18
BP (IS)	6.1	<u>186</u>, 228, 270	-			-	-

Underlined numbers correspond to *m/z* of the target ion.
[a] n=10 (Intraday), [b] n=10 (Interday), [c] Mean value±standard deviation (n=6).
[d] Calculated for S/N=3, [e] Calculated for S/N=10.

For *in situ* IL-DLLME extraction, a 10 mL-aliquot of the simulant was placed in a 15 mL conical bottom glass tube and spiked with the internal standard (BP) at a concentration of 2 ng L^{-1}. Next, 210 mg of K_2HPO_4 and 115 µL of acetic anhydride were added as neutralizing and derivatizing agents, respectively, and the mixture was shaken for 10 seconds. Then, 80 µL of $[C_6MIm]Cl$ and 102 µL of $Li[NTf_2]$ solutions were injected into the sample solution, leading to the formation of a cloudy dispersion consisting of very fine droplets of IL dispersed through the sample solution, which extracted the acetylated BPs. This emulsion was centrifuged for 2 min at 3000 rpm, and the sediment IL phase was collected using a microsyringe. 20 µL of the recovered phase were placed in a 150 µL glass microvial and introduced into a glass desorption tube. The analytes were submitted to the vaporization step by placing the desorption tube in the TDU.

Since no reference materials were available, migration test solutions obtained from three different containers, which had been shown to be free from the analytes, were spiked at two different concentration levels (0.35 and 2 µg L^{-1}) for validation purposes. Three replicates were analyzed in each case.

3. Results and discussion

3.1. Ionic liquid extraction procedure

Due to the acid character of their phenolic group, BPs show poor chromatographic behaviour, providing tailing peaks. In addition, their relative hydrophilicity, with low values of log $k_{o/w}$ (1.7-3), may lead to low extraction efficiencies with respect to apolar extractant solvents. The inclusion of a derivatization step in the analytical procedure may overcome these disadvantages. The acetylation of hydroxyl groups of the phenolic compounds improves their chromatographic response and increases their partition coefficients, leading to higher enrichment factors in the extraction step. This derivatization reaction can be easily performed in aqueous medium prior to extraction, by means of the addition of AA and a neutralizing salt (K_2HPO_4). This salt reacts with the acetic acid, resulting in the hydrolysis of AA in aqueous medium, ensuring the basic medium required for the derivatization reaction to take place, as well as fixing the pH at a neutral value during the extraction once the analytes have been derivatized.

The influence of the AA volume and the amount K_2HPO_4 on the response of BPs was tested using a central composite design (CCD, α = 1.5, 4 cube points, 4 axial points and 2 central points, by duplicate), developed in the range 50 – 200 µL of AA and 1 – 4 neutralizing equivalents of K_2HPO_4. The resulting mixture was manually shaken for 20 seconds while derivatization reaction takes place, and afterwards submitted to extraction. The obtained responses showed that areas of the acetylated compounds were maximum using 115 µL of AA and 210 mg of K_2HPO_4, corresponding to 1 neutralizing equivalent.

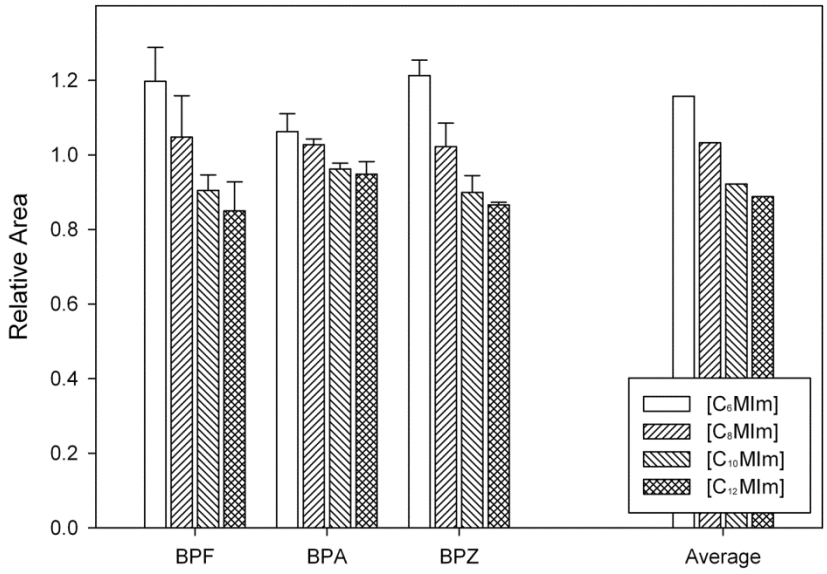


Fig. 1 Influence of the nature of the IL cationic component on the response of each analyte.

A relatively long extraction time is required for reaching equilibrium between the water sample and IL extractant phase when ILs are used in the conventional form. On the contrary, increasing the contact surface between both phases by the formation of an emulsion can speed up the extraction process, which can be completed almost instantaneously (less than 10 s). The formation of this emulsion can be achieved using *in situ* IL formation. In this approach a metathesis reaction between the anionic and cationic components of the IL leads to the formation of insoluble IL in the media, the nanodroplets acting as extracting phase of the acetylated BPs. The resulting emulsion is broken by centrifugation, allowing the aggregation of the IL drops, the separation of the two phases and the recovery of the IL containing the analytes.

Several ILs, $[C_6MIm][NTf_2]$, $[C_8MIm][NTf_2]$, $[C_{10}MIm][NTf_2]$ and $[C_{12}MIm][NTf_2]$, were assayed as extraction solvent. For these tests, 100 μL of the cationic part of each IL, followed by 100 μL of the anionic part, were added to the sample, leading to the formation of a cloudy solution. After centrifugation, different volumes were recovered, IL solubility increasing as alkyl chain length decreased. The relative responses of the analytes, corrected by taking into account that only 20 μL of the recovered volume was submitted to TD, are represented in Fig. 1. The combination $[C_6MIm][NTf_2]$ provided the most efficient IL for extracting acetylated BPs.

The ratio between the anionic and cationic parts, as well as the combined volumes may influence the response obtained. IL formation, and thus recovery, may be fomented if there is a stoichiometric excess of the anionic part, while higher IL extracting volumes may decrease sensitivity, due to dilution effect. These two variables were studied using a CCD (α = 1.5, 4 cube points, 4 axial points and 2 central points) in duplicate. Volumes of the $[C_6MIm]Cl$ solution ranging from 80 to 140 μL, and $Li[NTf_2]/[C_6MIm]Cl$ ratios in the 0.9 – 1.5 range were included in

this test. Volumes lower than 80 µL were not assayed because less than 20 µL could be recovered after centrifugation, hindering the recovery of the sedimented drop. As shown in Fig. 2A the obtained responses were maximum using 80 µL of $[C_6MIm]Cl$ solution and 102 µL of and $Li[NTf_2]$ solution, corresponding to a 1.27 $Li[NTf_2]/[C_6MIm]Cl$ ratio. These conditions led to the recovery of 23 µL of the enriched phase.

3.2. Thermal desorption

After the extraction step, 20 µL of the sedimented IL were placed into glass microvials using a microsyringe, and submitted to thermal desorption in order to vaporize the BPs. This step may be influenced by desorption temperature, desorption time, and the gas flow rate. While high desorption temperatures may facilitate the vaporization of the analytes, values higher than 280 ºC lead to a significant increase in the chromatographic background related with the thermal decomposition of the IL, and so were not applied. Vaporization of the analytes from the extracting phase does not take place immediately as a result of the relatively large volume of IL placed in the glass microvial (20 µL), and so the desorption temperature should be maintained for a short period of time. While this process takes place, a helium carrier gas flow drags the vaporized analytes from the heated TDU to the cooled PTV. These three parameters involved in vaporization were carefully optimized using a CCD (α = 1.5, 8 cube points, 6 axial points and 2 central points, by duplicate). The obtained response surfaces appear in Fig. 2B, showing that sensitivity was enhanced using 280 ºC as desorption temperature, 7.6 min as desorption time and 78 mL min^{-1} as carrier gas flow.

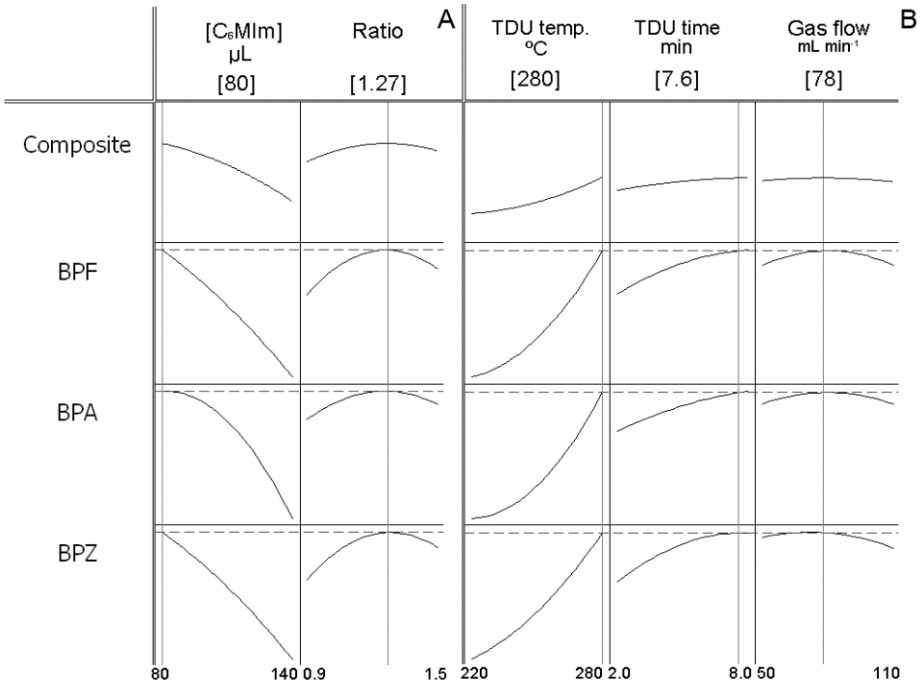


Fig. 2. Response plots showing the effects of (A) IL volume and cationic/anionic ratio of IL components and (B) desorption temperature and time, and gas flow rate on the relative responses.

Vaporized compounds were focused into the PTV before entering into the chromatographic column. Lower temperatures in this device increase its retention efficiency and minimize losses of the more volatile compounds. Since the Peltier unit only allows cooling to temperatures slightly below room temperature, 15 ºC was selected as focusing temperature. A liner filled with the inert support fibreglass support was used to facilitate the retention of the analytes in the PTV. After the desorption step, PTV is heated to 280 ºC, in order to elute the retained compounds in the liner to the chromatographic column. Therefore, a program temperature increasing from 15 to 280 ºC at 530 ºC min^{-1}, with a hold time of 2 min, was applied.

3.3. Method performance

The use of an IS, in order to increase repeatability, was assayed. BP, which shows a relatively similar chemical and chromatographic behaviour to the analytes, was tested for this purpose. Its use led to an enhancement of the repeatability of the procedure, since the RSD values decreased from 8 – 13% (n=10) when no IS was added, to 5 – 7% (n=10), depending on the compound, when IS was added (Table 2).

Calibration graphs were obtained by plotting the ratio between analyte peak area and IS peak area versus analyte concentration, using six aqueous standard solutions prepared in the 0.05 – 5 μg L^{-1} concentration range, which provided good correlation coefficient values ($r > 0.99$). The slopes of three different aqueous calibrations obtained in different days were evaluated using an ANOVA test. "P" values in the 0.11 – 0.40 range were obtained, showing the absence of statistically significant differences among calibrations, and confirming the good reproducibility for the optimized procedure.

Table 3
Comparison of the proposed method with others previously developed, for the determination of BPA in aqueous samples, based on microextraction techniques using ionic liquids.

Preconcentration technique	Method	Sample volume, mL	Treatment time, min	LOD, μg L^{-1}	Ref.
IL-CIA-DLLME	LC-UV	5	70	0.58	8
In situ IL-DLLME	LC-UV	10	9	4.8	11
In situ IL-DLLME	LC-UV	10	5.5	14.7	12
In situ IL-DLLME	LC-MS	30	10	0.024	10
IL-Three phase HF-LPME	LC-UV	20	30	0.2	16
TC-IL-DLLME	LC-FLD	10	60	0.23	13
IL-MNPs-MHSPE	LC-UV	5	35	0.03	17
PIL-SPME	LC-UC	30	45	0.2	20
IL-DLLME-BE	CE-UV	10	10	5.0	14
PIL-SPME	GC-MS	20	60	2.1	18
PIL-SPME	GC-FID	20	60	7.0	19
In situ IL-DLLME	TD-GC-MS	10	3	0.026	This work

BE, back extraction; CIA, cold-induced aggregation; MHSPE, mixed hemimicelles solid phase extraction; MNPs, magnetic nanoparticles; PIL, polymeric ionic liquid; TC, temperature controlled.

LODs and quantification limits (LOQs) were calculated taking into account a signal-to-noise ratio of 3 and 10, respectively. Values within the 5.4-7.8 ng L^{-1} and 18-26 ng L^{-1} ranges were obtained for LOD and LOQ, respectively (Table 2). The sensitivity attained was intermediate between other previously obtained values using more expensive and more time-consuming preconcentration methodologies, such as solid-phase microextraction [25] and stir bar sorptive extraction [26] coupled to GC-MS.

A comparison of the proposed method with other previously published for the determination of BPA using microextraction techniques based on ILs is presented in Table 3.

3.4. Analysis of samples and recovery studies

The optimized procedure was applied to fifteen different plastic containers, in order to evaluate the possible migration of the BPs to a food simulant. The results obtained are summarized in Table 4. Some BPs were detected in six of the fifteen samples. A high concentration of BPA (36.8 μg L^{-1}) was found in a polycarbonate feeding bottle, which had been donated by an individual, who bought it several years before BPA was prohibited in this type of container [2]. This sample had to be diluted in the 1:10 ratio before being analyzed. The other feeding bottle from which BPA release was demonstrated was made of polypropylene. Other results agree with those previously found [27]. The concentrations found for the studied compounds were, in all cases, lower than the SMLs suggested by the EU [1], 600 mg kg^{-1}.

Fig. 3 shows a typical chromatogram obtained by *in situ* DLLME direct microvial thermal desorption in combination with GC-MS for the migration test applied to the spout cup, which provided signals for the three analytes (Table 4). The blank tests analysis confirmed the absence of interfering peaks at the analyte retention times, which were identified by comparing their retention time and mass spectra in samples and standard solutions.

Recovery assays, fortifying three different samples at two concentration levels, 0.35 and 2 μg L^{-1}, provided values in the 98 – 113% range for the lowest level, and in the 95 – 100% for the highest one (Table 5).

Table 4
Analysis of the samples (μg L^{-1}).

	BPF	BPA	BPZ
Spout cup	ND	0.09±0.01	ND
Spout cup	0.21±0.04	1.3±0.1	0.17±0.01
Feeding bottle	ND	36.8±2.5	ND
Feeding bottle	ND	2.1±0.3	ND
Insulated cup	ND	2.9±0.1	ND
Straw cup	0.07±0.02	0.97±0.07	0.15±0.01

Values are mean ± standard deviation (n=3).
ND means not detected.

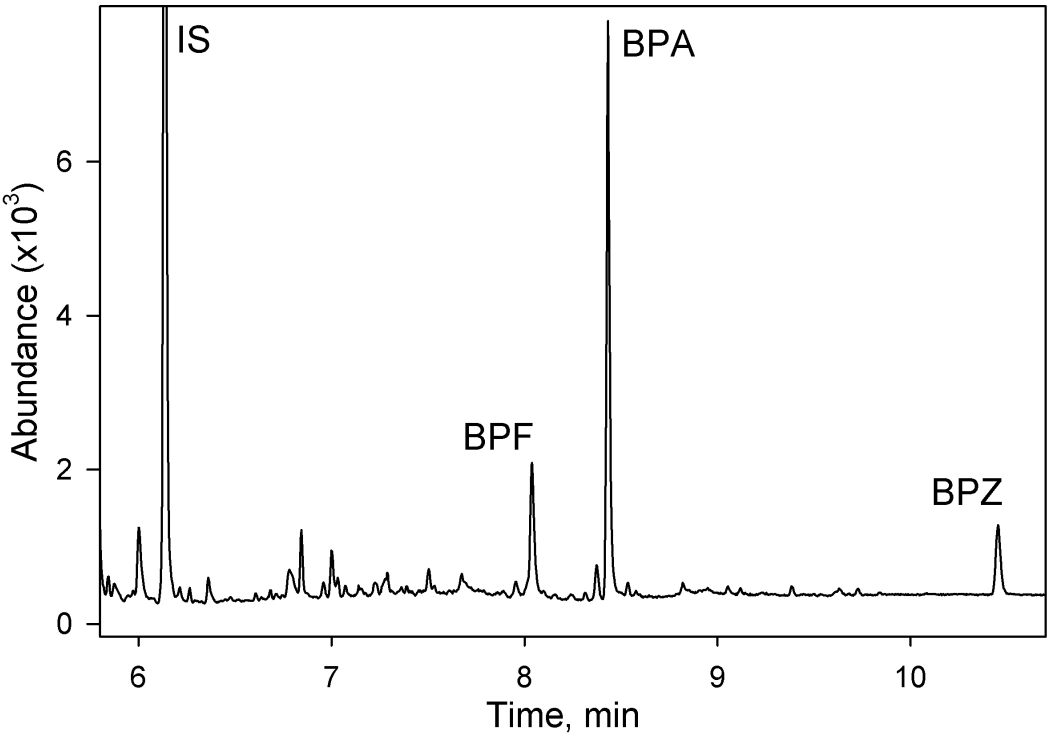


Fig. 3. Elution profile obtained for a migration test solution obtained from a spout cup, using the optimized procedure under selected ion monitoring (SIM) mode.

Table 5
Spiked and found levels in recoveries studies.

Sample	Spiked level (μg L^{-1})	Found level[a] (μg L^{-1})		
		BPF	BPA	BPZ
Spout cup	0.35	0.36±0.03 (102±9%)	0.34±0.02 (98±4%)	0.35±0.04 (99±13%)
	2	1.89±0.17 (95±9%)	2.01±0.04 (100±2%)	1.98±0.19 (99±10%)
Feeding bottle	0.35	0.39±0.06 (113±16%)	0.35±0.04 (99±13%)	0.39±0.03 (112±9%)
	2	1,99±0,18 (100±9%)	2.00±0.15 (100±8%)	1.91±0.17 (95±8%)
Cup	0.35	0.39±0.04 (112±12%)	0.36±0.02 (104±7%)	0.39±0.03 (111±9%)
	2	1.92±0.15 (96±8%)	1.97±0.17 (99±9%)	2.01±0.15 (100±8%)

4. Conclusion

The use of ILs generated *in situ* coupled to direct microvial insert thermal desorption offers different advantages over the conventional use of these reagents. Firstly, this technique allows the injection of the enriched IL extracting phase into the GC-MS avoiding a back extraction into a compatible solvent. The emulsion formed with the sequential addition of the IL ionic parts into the aqueous phase provides the formation of an emulsion which efficiently extracts the analytes at high speed. This is the first application of the IL-direct microvial insert-TD-GC-MS combination to the quantification of BPs in migration tests. The approach has shown good analytical characteristics related to sensitivity, precision, accuracy and selectivity.

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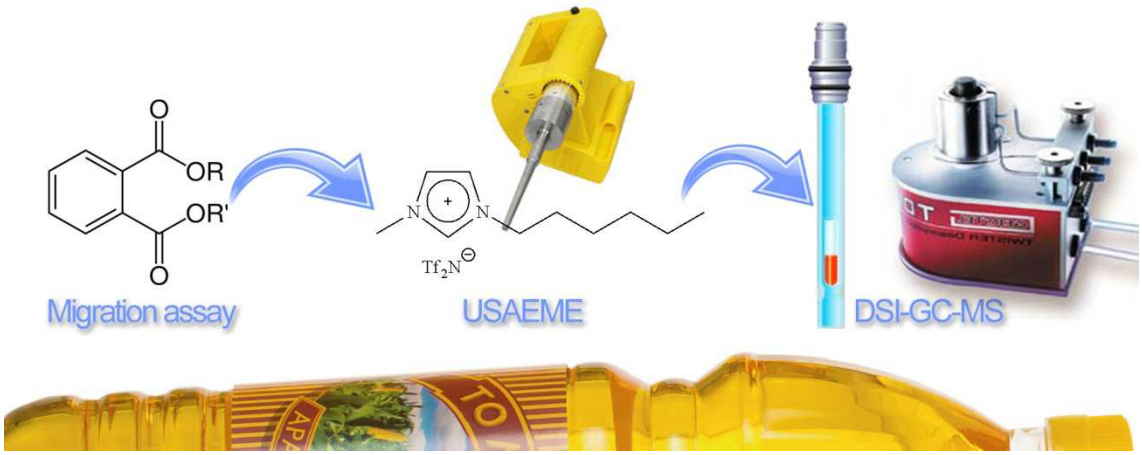
Microvial Insert Thermal Desorption and Ionic Liquid Microextraction for the Control of Phthalate Esters Release from Food Containers

Abstract

A new procedure is proposed for the analysis of isooctane migration test solutions obtained from plastic bottles used in the packaging of edible oils. Ultrasound assisted emulsification microextraction (USAEME) with ionic liquids (ILs) was applied for the preconcentration of six phthalate esters (PEs): dimethyl- (DMP), diethyl- (DEP), di-*n*-butyl (DBP), *n*-butylbenzyl (BBP), di-2-ethylhexyl (DEHP) and di-*n*-octylphthalate (DOP). The enriched IL was directly analyzed by gas chromatography and mass spectrometry (GC-MS) using direct insert microvial thermal desorption. The different factors affecting the microextraction efficiency and the thermal desorption step were studied. Under the selected conditions, detection limits for the analytes were in the 0.012 – 0.18 µg L^{-1} range, while recovery assays provided values ranging from 80 to 112%. When the release of the six PEs from the tested plastic bottles to liquid simulants was monitored using the optimized procedure, analyte concentrations of between 1.0 and 273 µg L^{-1} were detected.



1. Introduction

A wide range of different materials has been used over the years for food packaging, but plastics are the most commonly used material for this purpose at present. Among the high number of plastics commercially available, only a small group, including polyethylene (PT), polypropylene (PP), polystyrene (PS) and polyethylene terephthalate (PET), is used in food packaging. In addition to their malleability and low cost, these plastics have excellent properties, providing containers that show high resistance against water, acid or basic media, and some organic solvents. Their properties remain stable for long storage times, and are not reactive in the face of a wide variety of chemicals [1].

Phthalates or phthalate esters (PEs), are the main constituents of PET wrappings, and are also used as plasticizers to improve the flexibility of other plastics. These compounds can migrate from the containers to the surrounding environment [2, 3]. This, along with their extensive use, has led to them becoming widespread contaminants, and they are found in a wide range of products [4].

Concern about PEs has grown in recent years, since they are considered estrogenic compounds that may lead to alterations in the reproductive capacity or in endocrine regulation [5-7]. Even though PEs may not cause direct genetic damage, some of them are considered carcinogenic and have been included in the International Agency for Research on Cancer (IARC) 2B and 3 lists [8]. Although human exposure to PEs is mainly the ingestion of water or food, the addition of PEs to plastics used to manufacture baby toys [9] and medical material [10] as well as the presence of PEs in plastic containers for food [11] is restricted. Thus, European Union establishes specific migration limits (SML) for some plastic constituents, including dibutylphthalate (DBP), butylbenzylphthalate (BBP) and diethylhexylphthalate (DEHP): 0.3, 30 and 1.5 mg kg^{-1}, respectively [11]. However, no particular indications about the analytical methods to be used for the quantification of such migration are provided by the authorities, and a wide range of approaches has been suggested for the purpose [12, 13].

Migration tests carried out under normalized conditions, related to time and temperature according to the expected use of the package, are the commonest way to monitorize the risk posed by the package. For oily foods, whose nature may facilitate the migration due to the lipophilicity of the plastic monomers, solvents such as ethanol or isooctane are used to simulate contact [11, 14]. PE migration studies to water miscible food simulants [15-18] and to those of oily solvent nature [19; 20; Li et al. 2013) have been carried out. These latter being analysed by liquid chromatography (LC) in normal phase [19] and previously submitted to liquid-liquid extraction (LLE) [20] or solid-phase extraction [21] in order to transfer the analytes to solvents compatible with reverse phase LC system.

The release of PEs from plastic materials to isooctane simulants has already been evaluated by gas chromatography (GC) using splitless injection [22], an approach that presents practical difficulties. In addition to the low volume injected, the relative high boiling point of isooctane may lead to large solvent peaks, requiring large solvent delays, which could lead to the loss of chromatographic information concerning the more volatile compounds. The high boiling point also hinders the use of large volume injection approaches. An alternative may be the application of microextraction procedures, which have been already used for PE determination [23]. Conventional liquid-liquid microextraction (LLME) procedures use a microvolume of an organic solvent to concentrate from aqueous samples [24]. The analyte transfer speed into the acceptor phase can be enhanced by using a third solvent (termed dispersant) or by the application of ultrasounds, giving rise to dispersive liquid-liquid microextraction (DLLME) and ultrasound assisted emulsification microextraction (USAEME) techniques, respectively. Several approaches using reverse systems, with aqueous phases to extract the analytes from oily matrices, have been proposed [25], but such LLME procedures are not useful for PE extraction from isooctane due to their apolar nature.

Ionic liquids (ILs) have recently been employed as extracting phase in LLME techniques [26]. These compounds offer a non-polar environment that may efficiently extract and preconcentrate the organic compounds present in isooctane.

In this work, we propose the use of USAEME with ILs for the extraction and preconcentration of six PEs (dimethyl- (DMP), diethyl- (DEP), DBP, BBP, DEHP and di-*n*-octylphthalate (DOP)) present in the isooctane simulants obtained from plastic bottles used to contain edible oils. Analysis of the enriched IL phase is carried out by GC-MS, after introduced into the chromatographic system by direct microvial insert thermal desorption.

2. Material and methods

2.1. Reagents

A standard stock solution containing six phthalate esters, dimethylphthalate (DMP), diethylphthalate (DEP), di-*n*-butylphthalate (DBP), *n*-butylbenzylphthalate (BBP), di-2-ethylhexylphthalate (DEHP) and di-*n*-octylphthalate (DOP) in methanol at 200 mg L^{-1}, was purchased from Supelco (Bellefonte, PA, USA). Butyl benzoate (internal standard, IS), with a purity of 99% was supplied by Sigma (St. Louis, MO, USA). Stock solutions (10 mg L^{-1}), prepared by diluting the commercial products in acetone, were kept at −18 ºC in darkness. The following ILs were supplied by IoLiTec (Heilbronn, Germany): 1-butyl-3-methylimidazolium bis{(trifluoromethyl)sulfonyl}imide ($[C_4MIm][NTf_2]$), 1-hexyl-3-methylimidazolium bis{(trifluoromethyl)sulfonyl}imide ($[C_6MIm][NTf_2]$), 1-methyl-3-octylimidazolium bis{(trifluoromethyl)sulfonyl}imide ($[C_8MIm][NTf_2]$), 1-decyl-3-methyimidazolium

bis{(trifluoromethyl)sulfonyl}imide ($[C_{10MIm}][NTf_2]$) and 1-dodecyl-3-methylimidazolium bis{(trifluoromethyl)sulfonyl}imide ($[C_{12}MIm][NTf_2]$). Pure grade isooctane was supplied by Sigma (St. Louis, MO, USA).

2.2. Instrumentation

The sample GC introduction system was composed of a multipurpose autosampler (MPS-2), a thermal desorption unit (TDU-2) and a programmed temperature vaporization (PTV) cooled injector system (CIS-4) provided by Gerstel (Mullheim an der Ruhr, Germany), whose experimental conditions are summarized in Table 1.

An Agilent 6890N (Agilent, Waldbronn, Germany) gas chromatograph coupled to an Agilent 5973 quadrupole mass selective spectrometer equipped with an inert ion source was used. The studied compounds eluted at retention times between 4.4 and 9.2 min, corresponding to DMP and DOP, respectively (Table 2). In order to improve the detection limits (DLs), quantification was carried out in the selected ion monitoring (SIM) mode using different ions (Table 2). Identification was confirmed by the retention time, target and qualifier ions, as well as the qualifier-to-target ion ratios, for each compound.

An UP 200 H ultrasonic processor (Dr. Hielscher, Teltow, Germany), with an effective output of 200 W in liquid media equipped with a titanium sonotrode (7 mm i.d.), was used for emulsification, while an EBA 20 centrifuge (Hettich, Tuttlingen, Germany) was used to disrupt the resulting emulsions.

Table 1
Experimental conditions of the TD-GC-MS system.

Thermal Desorption Unit	
Mode	Splitless
Temperature programme	40 – 280 ºC at 240 ºC min^{-1}, held 5 min
Desorption flow	60 mL min^{-1}
Cooled Injector System	
Mode	Solvent venting
Liner	Fibreglass, 2 mm i.d.
Temperature programme	15 – 280 ºC (5 min) at 530 ºC min^{-1}, held 2 min
GC-MS	
Capillary column	HP-5MS, 5% diphenyl-95% dimethylpolysiloxane (30 m x 0.25 mm, 0.25 μm)
Carrier gas	Helium (1 mL min^{-1})
Oven programme	
Transfer line temperature	300 ºC
Quadrupole temperature	150 ºC
Ion source temperature	230 ºC
Ionization	Electron-impact mode (70 eV)

Table 2
Method characteristics.

Compound	RT (min)	Monitored ions (*m/z*)	RSD[a] (%)	LOD[b] (µg L^{-1})	LOQ[c] (µg L^{-1})
Dimethylphthalate (DMP)	4.4	163, 194, 135	3.4	0.180	0.60
Diethylphthalate (DEP)	5.0	149, 177, 105	6.6	0.060	0.20
Butyl benzoate (IS)	5.7	178, 152	-		-
Dibutylphthalate (DBP)	6.3	149, 223, 205	7.1	0.012	0.04
Butylbenzylphthalate (BBP)	7.6	149, 91, 206	7.1	0.012	0.04
Diethylhexylphthalate (DEHP)	8.2	149, 167, 279	4.4	0.012	0.04
Dioctylphthalate (DOP)	9.2	149, 279	3.9	0.180	0.60

Underlined numbers correspond to *m/z* of the target ion. [a] n=10. [b] Calculated using S/N=3. [c] Calculated using S/N=10.

2.3. Samples and analytical procedure

Due to the ubiquitous presence of PEs in the environment, any analysis of these compounds is hindered by the possibility of contamination. Therefore, special precaution was taken regarding experimental control: laboratory glassware was sequentially rinsed with ethyl acetate, isooctane, and purified water two times before use [20]. In addition, possible contamination arising from the employed solvents was limited applying a dispersive solid-phase extraction [27] with a solid adsorbent, namely silica, before their use.

Ten different plastic bottles for containing edible oil were obtained from local suppliers. These containers were made of different types of plastics: three of high-density polyethylene (HDPE) (P1-P3), three of low-density polyethylene (LDPE) (P4-P6), two of polystyrene (PS) (P7-P8) and two of PET (P9-P10). All of them were labeled with the food safe symbol used for marking food contacting materials. According to EU regulations [11, 14], migration tests were carried out by filling the bottles with isooctane before sealing and keeping at 40 ºC for 10 days. The resulting migration liquids were placed in glass bottles, and stored at 4 ºC in darkness until analysis.

To quantify the PEs, 10 mL of the isooctane were placed in a 15 mL glass conical tube, spiked with 40 µg L^{-1} of the IS, before adding 30 µL of the IL $[C_6MIm][NTf_2]$ to the sample by microsyringe. The resulting mixture was submitted to ultrasounds by means of a probe directly immersed into the liquid phase for 25 s and applying 0.5 s pulses with 105 µm of amplitude. The resulting cloudy emulsion was disrupted by centrifugation at 3000 rpm for 2 min. The IL drop (25 – 30 µL), settled in the bottom of the conical tube, was recovered using a mycrosyringe and an aliquot of 20 µL placed in a glass microvial inside a desorption tube. This whole assembly was submitted to thermal desorption in the TDU-PTV injector.

3. Results and discussion

3.1. Ionic liquid extraction procedure

A relatively long extraction time is required to reach transfer equilibrium between the sample and the IL extracting phase if the microextraction technique is applied in the conventional way. By increasing the contact surface area of these two phases through the formation of an emulsion, obtained by application of ultrasounds, the extraction process can be speeded up and completed in a few seconds. The resulting cloudy solution is broken up by centrifugation into two phases, allowing the enriched IL to be recovered.

Three variables involved in the USAEME extraction step, namely the nature and volume of the extracting IL, and ultrasounds application time, were optimized. For this purpose, 10 mL isooctane containing the analytes at a concentration of 20 μg L^{-1} were used.

Five different ILs were assayed as extraction solvents: $[C_4MIm][NTf_2]$, $[C_6MIm][NTf_2]$, $[C_8MIm][NTf_2]$, $[C_{10}MIm][NTf_2]$ and $[C_{12}MIm][NTf_2]$. Fifty microliters of each IL were added to the isooctane phase, and submitted to USAEME. The selected ILs differ in the length of their alkyl chain, and thus in their polarity. All of them were immiscible with isooctane, but showed different extraction capabilities. The results, summarized in Fig. 1A, showed that all compounds were more effectively extracted with $[C_6MIm][NTf_2]$, so it was selected as extraction solvent.

The volume of the IL is a relevant parameter in USAEME efficiency. Low extractant volumes may lead to poor emulsion formation, hindering effective and rapid extraction, while large volumes may dilute the extracted analytes in larger drops, and thus reduce the overall response. Volumes in the 30 – 60 μL range were tested. Lower values were not assayed because the resulting settled drop after USAEME was too small. As shown in Fig. 1B, the obtained responses matched the described behaviour, extraction efficiency being maximum at the lowest assayed volume, so 30 μL was selected.

Since extraction can be speeded up through the formation of an emulsion by ultrasounds, the application time is a relevant parameter in USAEME. Short ultrasounds application times may lead to poor emulsion formation and thus low extraction efficiency, while long ultrasounds application times may heat the sample or facilitate the degradation of the studied compounds. Different times, ranging from 10 to 25 s, were tested. Best responses were obtained (Fig. 1C) with longer times, especially for heavier PEs, so 25 s was selected.

The resulting emulsion was disrupted by centrifugation at 3000 rpm for 2 min, allowing recovery of the settled IL drop.

3.2. Thermal desorption

Aliquots (20 μL) of the recovered IL were placed in a glass microvial using a microsyringe, and submitted to thermal desorption to vaporize the studied compounds. Three parameters involved in this step, desorption temperature and time, and gas flow rate, were optimized.

Desorption temperature was studied in the 250 – 280 ºC range. Although higher temperatures may facilitate analyte vaporization, values higher than 280 ºC were not applied due to a significant increase in the chromatographic background related with thermal decomposition of the IL. Maximum sensitivity was obtained for all the studied PEs at 280 ºC (Fig. 2A), especially for heavier ones so this temperature was selected.

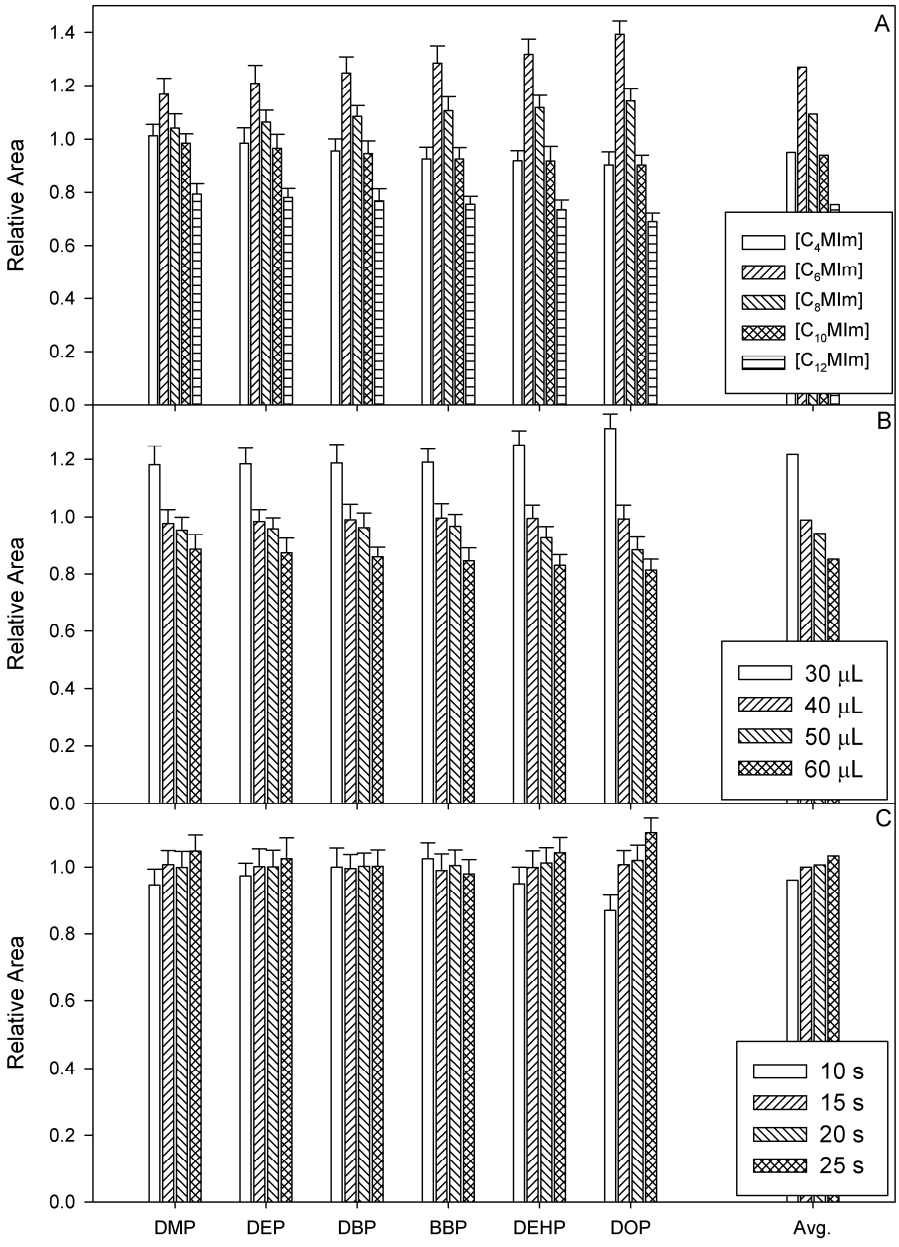


Fig. 1. Influence of the: IL nature (A), $[C_6MIm][NTf_2]$ volume (B) and ultrasounds application time (C), on the mean analyte response.

As a result of the relatively large volume of IL placed in the glass microvial (20 µL), the analytes are not immediately vaporized from the extracting phase, and so heating should be maintained for a specified time. When times ranging from 2 to 5 min were tested (Fig. 2B), the analytical signal increased significantly for all compounds at longer vaporization times, so the highest assayed value, 5 min, was selected.

A helium carrier gas flow is maintained during the thermal desorption step, to propel the vaporized analytes from the heated TDU to the cooled PTV. Values from 40 to 100 mL min^{-1} were tested (Fig. 2C). DMP and DEP showed higher responses at low flow rates, while BBP, DEHP and DOC attained their maximum sensitivity at high flow rates. As a compromise value, 60 mL min^{-1} was selected.

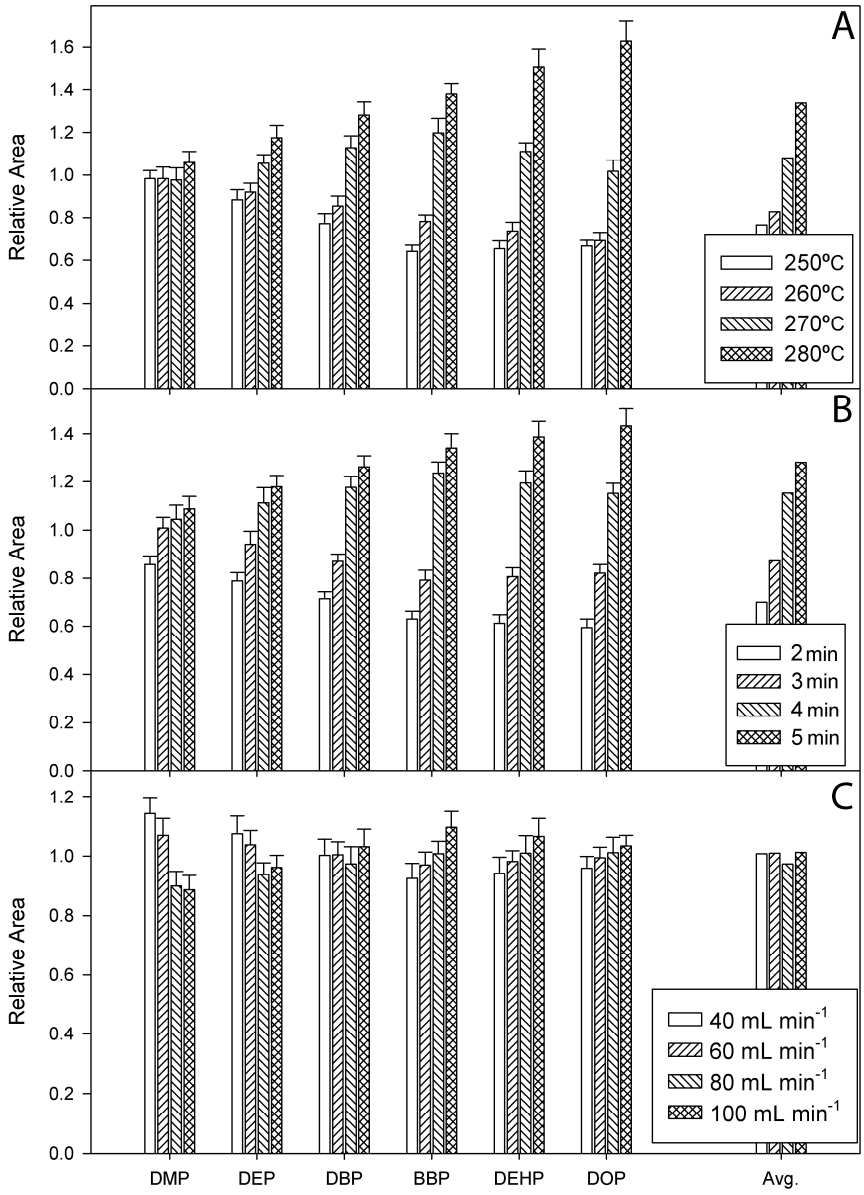


Fig. 2. Influence of the: TDU temperature (A), desorption time (B) and gas flow rate (C), on the mean analyte response.

Vaporized compounds were focused in the PTV before entering the chromatographic column. Low temperatures in this device increase retention efficiency and minimize losses of the more volatile compounds. Since the PTV is cooled by a Peltier unit, which only allows cooling to slightly below room temperature, 15 ºC was selected as focusing temperature. A liner filled with fibreglass as inert support was used to facilitate the retention of the analytes in the PTV. After the desorption step, the PTV was heated to 280 ºC in order to elute the retained compounds to the chromatographic column. Therefore, a heating programme from 15 to 280 ºC at 530 ºC min^{-1}, with a hold time of 2 min, was applied.

3.3. Method performance

For quantification purposes, butyl benzoate was employed as IS, and added to the samples at a concentration of 40 µg L^{-1}. This compound, whose absence was confirmed in the analyzed samples, shows chemical and chromatographic behaviour similar to the PEs studied, allowing the compensation for any loss during the analytical procedure or any possible matrix effect [28].

Calibration graphs were obtained using standard solutions prepared in isooctane at six different concentration levels and submitted in duplicate to the optimized procedure, and plotting peak area ratios of each analyte with respect to the IS peak area *versus* analyte concentration. The resulting graphs were found to be linear in the 1 – 200 µg L^{-1} range, with correlation coefficients higher than 0.99 in all cases.

The use of an IS led increased the repeatability of the procedure, which was calculated using the relative standard deviation (RSD) for a batch of ten spiked samples at 20 µg L^{-1}. RSD values ranged from 7 to 13% when no IS was added to the samples, and fell to 3 and 7% when IS was added (Table 2).

The sensitivity of the method was evaluated using detection (LODs) and quantification limits (LOQs), which were calculated taking into account a signal-to-noise ratio of three and ten, respectively. LOQ values ranged from 0.2 to 0.6 µg L^{-1}, corresponding to DEP and DOP, respectively (Table 2). The proposed procedure greatly improves the sensitivity respect other methods dealing with PE determination in oily migration solvents [20, 21].

3.4 Analysis of samples and recovery studies

The proposed procedure was applied to the determination of PEs in the isooctane simulants obtained from the migration tests carried out for ten plastic oil bottles. The obtained results, summarized in Table 3, showed the widespread presence of PEs in the considered containers, nine out of the ten studied samples being at least above the reported LODs.

Table 3
Results[a] obtained in the migration studies (μg L^{-1}).

Sample	Material	DMP	DEP	DBP	DEHP	BBP	DOP
P1	HDPE	29.4±1.0	59.3±3.7	99.3±8.7	3.3±0.5	26.2±2.4	1.3±0.3
P2	HDPE	7.2±0.6	55.4±1.4	177±8	3.6±0.2	8.0±1.4	1.0±0.1
P3	HDPE	3.0±0.4	40.0±1.0	38.1±1.6	2.3±0.3	ND	NQ
P4	LDPE	NQ	2.8±0.2	28.9±2.7	ND	9.3±1.3	ND
P5	LDPE	1.5±0.2	85.6±2.1	254±13	8.2±0.9	63.0±4.2	10.4±0.6
P6	LDPE	12.2±1.8	55.6±2.1	273±11	ND	22.2±3.1	2.6±0.3
P7	PS	ND	4.1±0.4	11.2±2.2	ND	ND	ND
P8	PS	NQ	ND	ND	NQ	16.9±0.1	NQ
P9	PET	ND	4.5±0.8	ND	21.4±2.8	ND	ND

[a] Mean ± standard deviation (n=3). ND means not detected. NQ means not quantified.

DEP was the most widespread PE in the analyzed containers, while higher levels corresponded to DBP, reaching a maximum concentration of 273 μg L^{-1}. The highest total PE level (420 μg L^{-1}) was found in the isooctane resulting from the migration test applied to a LDPE bottle (P7). None of the studied packages exceeded the European SMLs [11].

Fig. 3A shows a typical chromatogram obtained under SIM mode for a standard solution containing 10 μg L^{-1} concentration level, in the selected conditions, while Fig. 3B shows the elution profile obtained for a plastic bottle migration test (P1, HDPE). Similar chromatograms were obtained for other samples. The analytes were identified by comparing the retention time, identifying the target and qualifier ions and qualifier-to-target ratios of the peaks in samples and standard solutions.

Since no reference materials were available for validating the method, in order to check its accuracy, recovery assays were performed by fortifying two isooctane samples obtained from migration tests at two concentration levels: 4 and 40 μg L^{-1}. Each fortification level was carried out in triplicate. The recoveries varied from 80 to 112% (n = 36) for the lowest level and from 86 to 104% (n =36) for the highest one.

4. Conclusion

The preconcentration of the PEs studied from isooctane simulants used to test their release from plastic bottles manufactured to contain edible oils, by means of USAEME with IL as extractant, was seen to increase the sensitivity of the applied technique. The incompatibility of ILs with GC split-splitless injection devices was successfully overcome by using direct microvial insert thermal desorption, allowing the analytes contained in the whole enriched phase to be analysed by GC-MS.

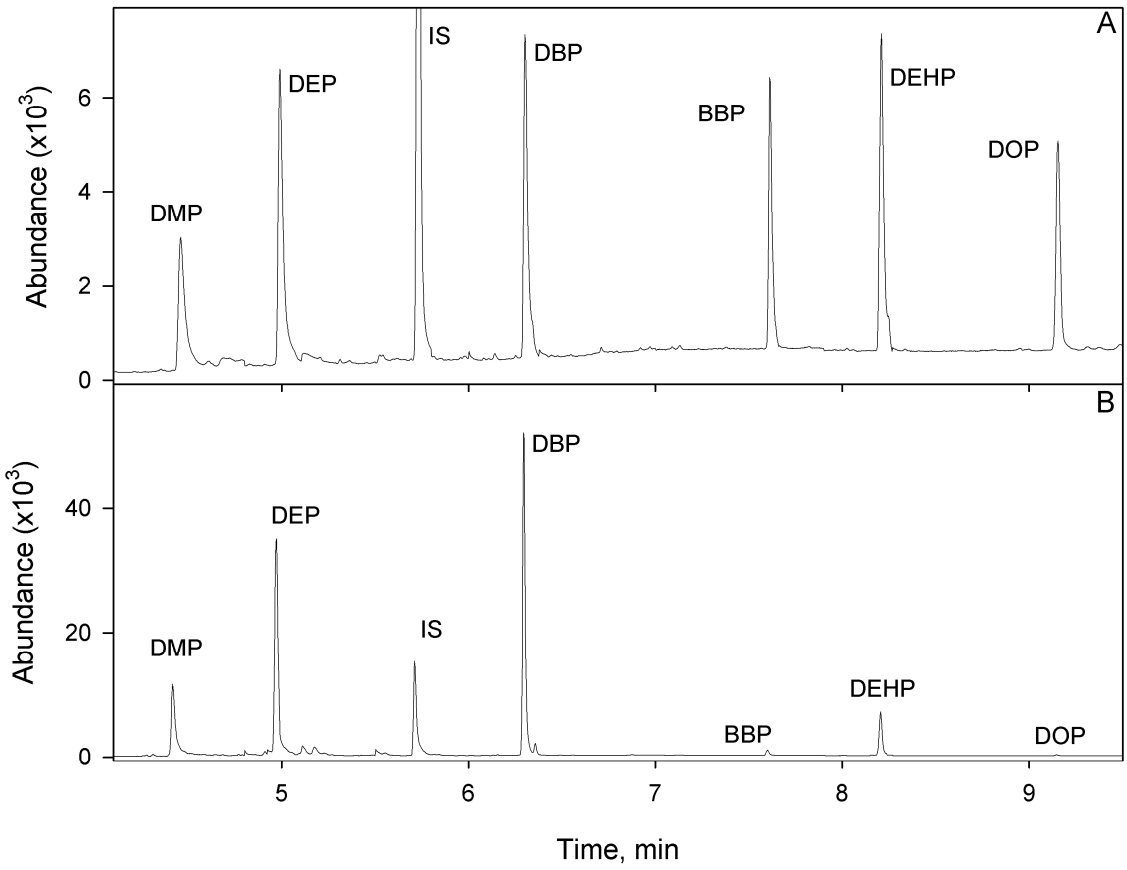


Fig. 3. IL-USAEME-TD-GC-MS chromatograms obtained from a standard solution of 10 µg L^{-1} (A) and a non-spiked migration solution test obtained from HDPE sample P1 (B).

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Conclusiones

Conclusiones

Como conclusión general de esta Tesis Doctoral, es posible destacar es que el inyector de desorción térmica es un dispositivo adecuado para el acoplamiento de diferentes técnicas de preparación de la muestra basadas en microextracción, con la determinación instrumental mediante separación por cromatografía de gases (GC) y detección por espectrometría de masas (MS).

Este sistema de introducción de muestra, formado por la combinación de una Unidad de Desorción Térmica (TDU) y un Inyector de Temperatura Programada (PTV), ha permitido ampliar el rango de aplicación y simplificar el acoplamiento de una amplia variedad de técnicas de microextracción con determinaciones analíticas basadas en GC.

Esta conclusión se ve corroborada por el trabajo experimental incluido en esta Tesis, que comprende el desarrollo de diferentes métodos analíticos basados tanto en microextracciones en fases líquidas y sólidas, enfocados a la resolución de problemas analíticos en una amplia variedad de campos, y usando en todos ellos el inyector de desorción térmica para el acoplamiento con cromatografía de gases.

Las siguientes conclusiones concretas pueden ser destacadas del trabajo desarrollado en esta Tesis Doctoral:

1. La microextracción por absorción en barra agitadora (SBSE) ha mostrado ser una técnica versátil y robusta para la determinación de una amplia variedad de analitos. A pesar de sus tiempos de extracción relativamente largos, la posibilidad de realizar un amplio número de extracciones de forma simultánea y su fácil automatización incrementan su aplicabilidad en el análisis de rutina.

2. El empleo de fases extractantes de naturaleza polar en SBSE, como el poliacrilato o el etilenglicol, aunque limitado por la falta de robustez de estas nuevas fases extractantes, permite la aplicación de esta técnica de microextracción a compuestos polares sin la necesidad de etapas adicionales de derivatización.

3. La microextracción por absorción en espacio de cabeza (HSSE) resulta una técnica de microextracción de gran utilidad para la preconcentración de analitos de naturaleza volátil, permitiendo una elevada selectividad en la etapa de preparación de la muestra, y limitando las interferencias asociadas a la matriz. Su eficiencia puede verse incrementada por el uso de nuevos dispositivos de exposición, como el soporte magnético desarrollado en esta Tesis a este fin.

4. El inyector de desorción térmica permite la introducción en el sistema cromatográfico de muestras líquidas, como pueden ser las procedentes de microextracciones líquido-líquido. Este sistema de inyección permite el uso de volúmenes semejantes a los empleados en un inyector de gran volumen, pero limita las posibilidades de contaminación cruzada y *carry-over* debido a la naturaleza desechable de los microviales empleados para contener la muestra.

5. Debido a las características del inyector de desorción térmica, éste permite el acoplamiento con cromatografía de gases de técnicas de microextracción que han sido hasta ahora no compatibles con esta técnica instrumental, como las microextracciones basadas en líquidos iónicos o en surfactantes.

6. El uso de inyector de desorción térmica permite reducir al mínimo la etapa de preparación de la muestra, mediante el empleo de la técnica de introducción directa de la muestra. Este enfoque permite el análisis de compuestos de interés en muestras de elevada complejidad, tanto de naturaleza líquida como sólida, como aceites vegetales o corchos; sin necesidad de ningún tratamiento previo de la muestra.

La presente Tesis Doctoral ha dado lugar a las siguientes publicaciones, que se han presentado en Congresos Nacionales e Internacionales.

- Determination of alkylphenols and phthalate esters in vegetables and migration studies from their packages by means of stir bar sorptive extraction coupled to gas chromatography–mass spectrometry.
  J.I. Cacho, N. Campillo, P. Viñas, M. Hernández-Córdoba
  Journal of Chromatography A, *1241* (2012) 21-27.
  Presentado como comunicación poster en las 13as Jornadas de Análisis Instrumental, Barcelona (2011)

- Stir bar sorptive extraction coupled to gas chromatography–mass spectrometry for the determination of bisphenols in canned beverages and filling liquids of canned vegetables.
  J.I. Cacho, N. Campillo, P. Viñas, M. Hernández-Córdoba
  Journal of Chromatography A, *1247* (2012) 146-153.
  Presentado como comunicación oral en 4th EMUNI Research Souk multi-conference (2012)

- Headspace sorptive extraction for the analysis of organotin compounds using thermal desorption and gas chromatography with mass spectrometry.
  J.I. Cacho, N. Campillo, P. Viñas, M. Hernández-Córdoba
  Journal of Chromatography A, *1279* (2013) 1-6.
  Presentado como comunicación poster en International Symposium on Chromatography ISC, Torun, Polonia (2012)

- Stir bar sorptive extraction with EG-Silicone coating for bisphenols determination in personal care products by GC–MS.
  J.I. Cacho, N. Campillo, P. Viñas, M. Hernández-Córdoba
  Journal of pharmaceutical and biomedical analysis, *78* (2013) 255-260.
  Presentado como comunicación oral en XXIII Reunión Nacional de Espectroscopía - VII Congreso Ibérico de Espectroscopía, Córdoba (2012)

- Stir bar sorptive extraction with gas chromatography–mass spectrometry for the determination of resveratrol, piceatannol and oxyresveratrol isomers in wines.
  J.I. Cacho, N. Campillo, P. Viñas, M. Hernández-Córdoba
  Journal of Chromatography A, *1315* (2013) 21-27.

Presentado como comunicación poster en XXIV Reunión Nacional de Espectroscopía - VIII Congreso Ibérico de Espectroscopía, Logroño (2014)

- Stir bar sorptive extraction polar coatings for the determination of chlorophenols and chloroanisoles in wines using gas chromatography and mass spectrometry.
  J.I. Cacho, N. Campillo, P. Viñas, M. Hernández-Córdoba
  Talanta, *118* (2014) 30-36.

  Presentado como comunicación poster en XVIII Reunión Nacional de la Sociedad Española de Química Analítica, Úbeda (2013)

- Use of headspace sorptive extraction coupled to gas chromatography–mass spectrometry for the analysis of volatile polycyclic aromatic hydrocarbons in herbal infusions.
  J.I. Cacho, N. Campillo, P. Viñas, M. Hernández-Córdoba
  Journal of Chromatography A, *1356* (2014) 38-44.

  Presentado como comunicación poster en Euro Food-2015 Food & Beverages, Alicante, (2015)

- Direct sample introduction gas chromatography and mass spectrometry for the determination of phthalate esters in cleaning products.
  J.I. Cacho, N. Campillo, P. Viñas, M. Hernández-Córdoba
  Journal of Chromatography A, *1380* (2015) 156-161.

  Presentado como comunicación poster en 14as Jornadas de Análisis Instrumental, Barcelona (2014)

- Determination of synthetic phenolic antioxidants in soft drinks by stir-bar sorptive extraction coupled to gas chromatography-mass spectrometry.
  J.I. Cacho, N. Campillo, P. Viñas, M. Hernández-Córdoba
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- Evaluation of three headspace sorptive extraction coatings for the determination of volatile terpenes in honey using gas chromatography–mass spectrometry.
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- Gas chromatography-mass spectrometry using microvial insert thermal desorption for the determination of BTEX in edible oils.
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- Determination of synthetic phenolic antioxidants in edible oils using microvial insert large volume injection gas-chromatography.
  J.I. Cacho, N. Campillo, P. Viñas, M. Hernández-Córdoba
  Food chemistry, *200* (2016) 249-254.
  Presentado como comunicación en Virtual Symposium on Applied Separation Science (2015)

- Dispersive liquid-liquid microextraction for the determination of nitrophenols in soils by microvial insert large volume injection-gas chromatography-mass spectrometry.
  J.I. Cacho, N. Campillo, P. Viñas, M. Hernández-Córdoba
  Journal of Chromatography A, *1456* (2016) 27-33
  Presentado como comunicación poster en European Geoscience Union General Assembly 2015, Viena, Austria (2015)

- Cloud point extraction and gas chromatography with direct microvial insert thermal desorption for the determination of haloanisoles in alcoholic beverages.
  J.I. Cacho, N. Campillo, P. Viñas, M. Hernández-Córdoba
  Talanta, *160* (2016) 282-288.

- Direct sample introduction-gas chromatography-mass spectrometry for the determination of haloanisole compounds in cork stoppers.
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Conclusions

Conclusions

As general conclusion of this Doctoral Thesis, it is possible to highlight that thermal desorption injector is a suitable device for the coupling of different sample preparation techniques based on microextraction with instrumental determination using GC-MS.

This sample introduction system, a combination of a TDU and a PTV, has been used for the coupling of a wide variety of microextraction techniques with GC. In this way, simple and applicable to a wider range of analytical challenges analytical methods have been developed.

This conclusion is supported by the experimental work included in this doctoral dissertation. Different analytical methods, based on liquid and solid phase microextraction coupled with GC using a thermal desorption injector, have been developed and applied to the quantification of a wide range of analytes in very different samples.

The following specific conclusions, reached over this Doctoral Thesis, could be highlighted:

1. SBSE has shown to be a versatile and robust technique for the determination of a wide variety of analytes. Despite its relatively long extraction times, the applicability in the routine analysis of this technique is enhanced by the possibility of simultaneously carrying out a large number of extractions, as well as its easy automation.

2. SBSE polar extracting phases, such as PA or EG-silicone, allow the application of SBSE technique to polar compounds without the need for additional derivatisation steps. However, the applicability of these new extracting phases is limited by their lack of robustness.

3. HSSE is a useful microextraction technique for the preconcentration of volatile nature analytes. HSSE provides high selectivity during the sample preparation stage, and it limits any possible matrix interfering effect. HSSE extraction efficiency can be increased by the use of new exposure devices, such as the magnetic holder developed for this purpose in this Thesis.

4. Thermal desorption injector allows the introduction of liquid extracts, such as those obtained by LPME, into the chromatographic system. Volumes similar to those used in a LVI can be used with this injection system. In addition, thermal desorption injector limits any possible carry-over, due to the disposable nature of the microvials used as sample container.

5. Thermal desorption injector, due to its unique characteristics, allows the coupling of microextraction techniques based in non-volatile liquid extracting phases, such as IL microextraction or CPE, with GC.

6. Thermal desorption injector can be employed for DSI, a technique that minimizes the required sample preparation. In this way, high complexity samples, both liquids and solids, as vegetable oils or corks, can be analyzed without any previous sample treatment.

Experimental work during this Doctoral Thesis has led to the following publications in high impact scientific journals, and communication in national and international scientific meetings

- Determination of alkylphenols and phthalate esters in vegetables and migration studies from their packages by means of stir bar sorptive extraction coupled to gas chromatography–mass spectrometry.
  J.I. Cacho, N. Campillo, P. Viñas, M. Hernández-Córdoba
  Journal of Chromatography A, *1241* (2012) 21-27.
  Poster communication in 13th Instrumental Analysis Conference, Barcelona (2011)

- Stir bar sorptive extraction coupled to gas chromatography–mass spectrometry for the determination of bisphenols in canned beverages and filling liquids of canned vegetables.
  J.I. Cacho, N. Campillo, P. Viñas, M. Hernández-Córdoba
  Journal of Chromatography A, *1247* (2012) 146-153.
  Oral presentation in 4th EMUNI Research Souk multi-conference (2012)

- Headspace sorptive extraction for the analysis of organotin compounds using thermal desorption and gas chromatography with mass spectrometry.
  J.I. Cacho, N. Campillo, P. Viñas, M. Hernández-Córdoba
  Journal of Chromatography A, *1279* (2013) 1-6.
  Poster communication in International Symposium on Chromatography ISC, Torun, Polonia (2012)

- Stir bar sorptive extraction with EG-Silicone coating for bisphenols determination in personal care products by GC–MS.
  J.I. Cacho, N. Campillo, P. Viñas, M. Hernández-Córdoba
  Journal of pharmaceutical and biomedical analysis, *78* (2013) 255-260.
  Oral presentation in XXIII National Spectroscopy Meeting - VII Iberian Conference on Spectroscopy, Córdoba (2012)

- Stir bar sorptive extraction with gas chromatography–mass spectrometry for the determination of resveratrol, piceatannol and oxyresveratrol isomers in wines.
  J.I. Cacho, N. Campillo, P. Viñas, M. Hernández-Córdoba
  Journal of Chromatography A, *1315* (2013) 21-27.

Poster communication in XXIV National Spectroscopy Meeting - VIII Iberian Conference on Spectroscopy, Logroño (2014)

- Stir bar sorptive extraction polar coatings for the determination of chlorophenols and chloroanisoles in wines using gas chromatography and mass spectrometry.
  J.I. Cacho, N. Campillo, P. Viñas, M. Hernández-Córdoba
  Talanta, *118* (2014) 30-36.
  Poster communication in XVIII Meeting of the Spanish Society of Analytical Chemistry, Úbeda (2013)

- Use of headspace sorptive extraction coupled to gas chromatography–mass spectrometry for the analysis of volatile polycyclic aromatic hydrocarbons in herbal infusions.
  J.I. Cacho, N. Campillo, P. Viñas, M. Hernández-Córdoba
  Journal of Chromatography A, *1356* (2014) 38-44.
  Poster communication in Euro Food-2015 Food & Beverages, Alicante, (2015)

- Direct sample introduction gas chromatography and mass spectrometry for the determination of phthalate esters in cleaning products.
  J.I. Cacho, N. Campillo, P. Viñas, M. Hernández-Córdoba
  Journal of Chromatography A, *1380* (2015) 156-161.
  Poster communication in 14th Instrumental Analysis Conference, Barcelona (2014)

- Determination of synthetic phenolic antioxidants in soft drinks by stir-bar sorptive extraction coupled to gas chromatography-mass spectrometry.
  J.I. Cacho, N. Campillo, P. Viñas, M. Hernández-Córdoba
  Food Additives & Contaminants: Part A, *32* (2015) 665-673.
  Poster communication in 14th Instrumental Analysis Conference, Barcelona (2014)

- Evaluation of three headspace sorptive extraction coatings for the determination of volatile terpenes in honey using gas chromatography–mass spectrometry.
  J.I. Cacho, N. Campillo, P. Viñas, M. Hernández-Córdoba
  Journal of Chromatography A, *1399* (2015) 18-24.
  Poster communication in XV Scientific Meeting of the Spanish Society of Chromatography and Related Techniques, Castellón de la Plana (2015)

- Improved sensitivity gas chromatography–mass spectrometry determination of parabens in waters using ionic liquids.
  J.I. Cacho, N. Campillo, P. Viñas, M. Hernández-Córdoba
  Talanta, *146* (2015) 568-574.
  Oral presentation in XX Meeting of the Spanish Society of Analytical Chemistry, Santiago de Compostela (2015)

- In situ ionic liquid dispersive liquid–liquid microextraction and direct microvial insert thermal desorption for gas chromatographic determination of bisphenol compounds.
  J.I. Cacho, N. Campillo, P. Viñas, M. Hernández-Córdoba
  Analytical and bioanalytical chemistry, *408* (2016) 243-249.

- Gas chromatography-mass spectrometry using microvial insert thermal desorption for the determination of BTEX in edible oils.
  J.I. Cacho, N. Campillo, P. Viñas, M. Hernández-Córdoba
  RSC Advances, *6* (2016), 20886-20891.

- Determination of synthetic phenolic antioxidants in edible oils using microvial insert large volume injection gas-chromatography.
  J.I. Cacho, N. Campillo, P. Viñas, M. Hernández-Córdoba
  Food chemistry, *200* (2016) 249-254.
  Communication in Virtual Symposium on Applied Separation Science (2015)

- Dispersive liquid-liquid microextraction for the determination of nitrophenols in soils by microvial insert large volume injection-gas chromatography-mass spectrometry.
  J.I. Cacho, N. Campillo, P. Viñas, M. Hernández-Córdoba
  Journal of Chromatography A, *1456* (2016), 27-33
  Poster communication in European Geoscience Union General Assembly 2015, Viena, Austria (2015)

- Cloud point extraction and gas chromatography with direct microvial insert thermal desorption for the determination of haloanisoles in alcoholic beverages.
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