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NUEVOS DESARROLLOS ANALÍTICOS PARA LA DETERMINACIÓN DE COMPUESTOS ORGÁNICOS EN MATRICES MEDIOAMBIENTALES Y ALIMENTARIAS

Inmaculada Carpinteiro Botana

Memoria para optar al grado de Doctora en Química
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Autorizan:

A la licenciada **Dña. M^a Inmaculada Carpinteiro Botana** a la presentación del trabajo recogido en la memoria titulada **“NUEVOS DESARROLLOS ANALÍTICOS PARA LA DETERMINACIÓN DE COMPUESTOS ORGÁNICOS EN MATRICES MEDIOAMBIENTALES Y ALIMENTARIAS”**, que ha realizado bajo su dirección en el departamento de Química Analítica, Nutrición y Bromatología de la Facultad de Química de la Universidad de Santiago de Compostela , para optar al grado de Doctora en Química.

Y para que así conste, firmamos la presente autorización en Santiago de Compostela en abril de 2012.

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Summary

This chapter of the Ph.D. memory contains (1) the basic aims of the developed work, (2) an introduction section focused on the different groups of studied analytes, including a brief overview of existing sample preparation methodologies; and (3) a summary of research activities and results derived from the experimental work. General conclusions are given at the end of this dissertation.

1. AIMS

An increasing number of organic compounds, present at trace levels in matrices with different complexities, have become the target of Analytical Chemistry because of their potential chronic toxicities, bio-accumulation risks or, simply, undesirable sensorial characteristics. Thus, the development of analytical methodologies displaying suitable features in terms of limits of quantification, accuracy and precision is required. Furthermore, such methodologies have to be sustainable in terms of cost, wastes generation, organic solvents consumption, simplicity and robustness.

The aims of this Ph.D. have been the development, validation and application of new sample preparation methods, fulfilling the above requirements, for the determination of three different groups of organic compounds in solid and liquid matrices. Those techniques were combined with gas (GC) and liquid chromatography (LC) determination methods using, in all cases, mass spectrometry (MS) or tandem MS (MS/MS) detection. Selected analytes cover a group of emerging pollutants (Tinuvins), scarcely investigated in the literature but displaying a high persistence and bio-accumulation trend; fungicides, which represent one of the most important classes of pesticides applied in agriculture; and, finally, volatile ethylphenols, naturally occurring in some foodstuff products and responsible for consumers' rejection, when their concentrations overpass certain thresholds.

Water (surface and wastewater) and wine were considered as liquid matrices, whereas sediments, agriculture soils and dust were the processed solid samples. Sample preparation approaches were based on solid and liquid microextraction techniques, solid-phase extraction (SPE) on mixed-mode sorbents, matrix solid-phase dispersion (MSPD) and pressurized liquid extraction (PLE). Performance of developed methods was systematically compared with that offered by already reported procedures, highlighting

the major achievements in terms of cost reduction, improved selectivity, accuracy (particularly in the case of microextraction techniques) and operational simplicity.

Application to real samples has provided new data regarding the presence of Tinuvin in environmental and indoor matrices, persistence of fungicides in vine soils and existence of residues in wine and levels of volatile ethylphenols in the same matrix.

2. INTRODUCTION

2.1. Tinuvin

Organic UV filters are high volume production chemicals, systematically incorporated in personal care products and industrial goods in order to prevent undesirable reactions enhanced by UV wavelengths of solar radiation. Derivatives of 2-hydroxybenzotriazol (Tinuvin) constitute one of the most relevant groups of UV filter used as additives in plastics, polymeric materials and paints, to avoid light induced degradation reactions and yellowing [1-3]. Conversely to those UV filters incorporated in sunscreens, and other personal care products, their environmental fate and occurrence have received a very limited attention.

Although multi-residue methods had confirmed the long-term persistence of Tinuvin in sediments collected in the vicinity of chemical manufacturing plants [3], the distribution and fate of these compounds in water, sediments and biota have been only recently addressed by Nakata's group [4,5]. Obtained data, restricted to Japan and surrounding areas, have confirmed the stability of this family of compounds in sediments, under anaerobic conditions, and the existence of detectable residues in organisms located at different levels of the food chain, from fish to birds and marine mammals. These findings justify the development of alternative analytical methodologies which overcome the inherent limitations of conventional liquid-liquid extraction (LLE) [4] and Soxhlet [5], which were employed in these earlier studies. Also, there is a need to investigate the presence of Tinuvin in other regions of the planet and also in indoor areas to assess human exposure through inhalation and/or accidental dust ingestion.

2.2. Fungicides

Fungicides constitute one of the most important groups of agrochemicals sprayed in agriculture fields. Their consumption is particularly relevant in vineyards, to prevent and to control fungal diseases (e.g. powdery and downy mildew) responsible for important economical losses to this sector. Their use has a special relevance in regions with high humidity and warm temperatures, as it is the case of the Northwest of Spain. Factors such as type and concentration of fungicides applied to vines, time period and climatic conditions from the last spraying until vintage, determine the occurrence and the concentration of fungicide residues in grapes. Thereafter, the characteristics of each pesticide and the wine-making operations affect the transference of fungicides from grapes to wine [6-9]. The European Union (EU) has established the maximum permissible concentrations of several fungicides in vinification grapes, expressed as Maximum Residue Limits (MRLs) [10]. Nevertheless, with a few exceptions [11,12], the presence of fungicides in wine is not regulated being an issue of concern for consumers and producers. Thus, determination of fungicide residues in wine is necessary for food safety monitoring and future regulatory purposes. The chemical complexity of wine samples and differences among wines justify the development of more selective analytical methodologies, whose performance remain unaltered independently of the particular characteristics of each wine.

In addition to health implications, the intensive use of fungicides in vineyards may lead to other problems related with (1) migration from soil to surface and ground water [13] and (2) development of resistant fungal strains, as a consequence of prolonged exposure to sub-lethal concentrations of fungicide species, a fact which has already been reported for azolic compounds [14]. Obviously, above risks show a higher probability in the case of vineyards, which are fumigated during decades with pools of different fungicides. Thus, there is a need for simple, low cost and effective sample preparation methodologies, covering a large number of compounds from different chemical families, in order to assess the persistence and stability of fungicides in vineyard soils. Up to now, most of the available methods require moderate to high solvent volumes, providing extractions yields, which sometimes vary among soil samples with different characteristics (e.g. carbon content).

2.3. Volatile ethylphenols

Ethylphenols (4-ethylphenol, 4-ethylguaiacol and 4-ethylcatechol) are aromatic compounds, naturally existing in wine. They are generated from hydroxycinnamic acids through enzymatic reactions consisting of a decarboxylation step followed by reduction of the intermediate vinylphenols [15]. Such reactions are enhanced by certain yeasts and take place during wine elaboration and maturation, particularly when ageing in wood barrels. Thus, their levels are usually higher in red than in white wines. Although, at low concentrations, ethylphenols contribute positively to the complexity of wine aroma, they are considered as responsible for organoleptic defects (Brett character) when present at levels above a given threshold, which varies from a few ng mL^{-1} for 4-ethylguaiacol (EG) up to several hundreds of ng mL^{-1} for 4-ethylphenol (EP) [15,16]. In addition to EP and EG, the presence of significant amounts of 4-ethylcatechol (EC) has been also correlated with negative aromatic notes (spicy or medicinal) in wine [17]. Understanding the mechanisms controlling the formation of these off-flavours, and monitoring their evolution during wine maturation, requires sensitive analytical methods, able to provide reliable data at concentrations further below their sensorial thresholds.

Economical losses caused by ethylphenols to wine producers have led to the development of a relevant number of methods, based on GC or LC techniques. In the first case, analytes derivatization is highly advisable, or just mandatory for EC, to improve the quality of chromatographic separations and, even, to increase the efficiency of the extraction step. During last years, several SPME (solid-phase microextraction) based methods have been successfully applied to EP and EG determination in wine samples [18-20]. Moreover, Carrillo and co-workers [21] have combined *in-situ* analytes acetylation and SPME for the simultaneous determination of above compounds and EC in wine samples.

Despite analytical improvements reported in the literature, there are some issues which justify further research, such as (1) reduction of sample preparation costs, (2) verification of accuracy provided by microextraction approaches and (3) design of new microextraction modalities which overcome the slow kinetics associated with SPME and SBSE (stir-bar sorptive extraction) techniques.

3. RESEARCH ACTIVITIES AND RESULTS

3.1. Tinuvin

Within the framework of this Ph.D., novel analytical methodologies have been proposed for the determination of several derivatives of 2-hydroxybenzotriazol in water, sediments and dust samples. In all cases, target species were selectively determined by GC-MS/MS. A brief description of working sample preparation conditions, and their most relevant figures of merit, are summarized below, grouped under the title of the corresponding scientific publication.

Headspace solid-phase microextraction followed by gas chromatography tandem mass spectrometry for the sensitive determination of benzotriazole UV-stabilizers in water samples

In previous works, the presence of trace levels of Tinuvin in water samples was investigated using LLE followed by GC-MS [4,22,23]. The main drawback of such procedures was the excessive consumption of organic solvents (up to 200 mL per sample) and the limited selectivity of GC-MS, since most of these compounds display a single intense ion in their electronic impact MS spectra.

With the aim of assessing the role of wastewater in the spread of Tinuvin in the environment, SPE and SPME, followed by GC-MS/MS, were investigated as alternative sample preparation methods. The SPE approach was unsuccessful since, for the most lipophilic species (Tinuvin 328 and 327), quantitative recoveries could not be achieved due to sorption losses. Thus, SPME, which minimizes sample handling, was proposed as enrichment technique.

Optimization of the microextraction process was carried out using a factorial experimental design, type $3^1 \times 2^2$, to evaluate the effects of fibre coating, sampling mode and temperature in the yield of the process. Other variables, such as ionic strength, sample pH and extraction time, were optimized following a univariant approach. Generally, the behaviour of the more polar analytes (Tinuvin P and Allyl-bzt) was different to that displayed by the more lipophilic species (Tinuvin 328, 327 and 326). For example, Tinuvin P and Allyl-bzt were better extracted in the direct sampling mode, after increasing

the ionic strength of samples with sodium chloride. On the other hand, higher responses were attained in the headspace (HS) mode, without salt, for the other 3 species. Combination of headspace extraction, with a sample temperature of 100 °C, and addition of 150 mg of NaCl per mL of sample, rendered the best compromise in terms of extraction efficiency for all species. Considering a sampling time of 30 min, with a PDMS-DVB (Polydimethylsiloxane-Divinylbenzene) coated SPME fibre, limits of quantification (LOQs) below 2 ng L⁻¹ and relative standard deviations (RSDs, %) between 5 and 12% were achieved for 15 mL water samples. The efficiency of the HS SPME method remained unchanged among ultrapure, surface and treated wastewater; however, it varied considerably for raw wastewater. Three of the five species included in this research were determined in this latter matrix with a maximum concentration of 57 ng L⁻¹ for the Tinuvin 326 UV-absorber.

Matrix solid-phase dispersion followed by gas chromatography tandem mass spectrometry for the determination of benzotriazole UV absorbers in sediments

In the bibliography, methods used for extraction of Tinuvin from sediments were liquid-solid extraction (SLE) [24,25] or Soxhlet [5]. These protocols consume a high amount of organic solvents and time, and need subsequent clean-up steps. Thus, we have investigated the applicability of the MSPD technique to obtain a single step procedure for Tinuvin extraction from sediment samples.

During optimization of sample preparation conditions, variables such as type and quantity of dispersant and elution solvent were evaluated. Another relevant parameter was the composition of the clean-up layer, placed at the bottom of MSPD cartridges, and its deactivation grade with water. Under optimal conditions, 0.5 g of sediment were homogenized with 1 g of diatomaceous earth and transferred to a polypropylene syringe containing 0.5 g of silica deactivated to 10%. Analytes were recovered using just 5 mL of dichloromethane and this extract was concentrated and exchanged to 1 mL of isooctane. Further removal of co-extracted sulphur was achieved adding activated copper powder to final extracts, which were stored overnight before injection in the GC-MS/MS system.

The accuracy of the method was assessed with river and marine sediment samples showing different carbon contents and spiked at concentrations in the range from 40 to 500 ng g⁻¹. Recoveries varied between 78 and 110% with associated standard

deviations below 14%. The limits of quantification (LOQs) of the method stayed between 3 and 15 ng g⁻¹. Levels of target compounds in sediment samples ranged from not detected up to a maximum of 56 ng g⁻¹ for Tinuvin 328.

Pressurized solvent extraction followed by gas chromatography tandem mass spectrometry for the determination of benzotriazole light stabilizers in indoor dust

Dust analysis has been proposed to evaluate human exposure to indoor pollutants in confined environments. Since Tinuvin is used as additives in paints, polymers, furniture, upholstery and building materials, it was decided to investigate if these species are components of the burden of pollutants existing in dust. In this case, no sample preparation methods had been previously reported for this combination of matrix-analytes. Thus, the experience of the research team with dust analysis was used to select PLE as sample preparation technique.

Under optimal conditions, 0.5 g of dust sample, previously mixed with 1 g of sand, was transferred to a PLE cell containing a layer of activated silica. Extractions were carried out in 3 cycles, at 90°C during 10 min each cycle. Analytes were recovered with a hexane/dichloromethane (7/3) mixture. Deactivation of silica was not necessary in PLE, unlike the aforementioned MSPD protocol, because high pressure and high temperature can break hydrogen bounds between analytes and the normal phase sorbent; however, an excessive amount of clean-up sorbent negatively affected the extraction efficiency. Achieved recoveries were between 82 and 122%, and limits of quantification between 4 and 10 ng g⁻¹. Analysis of dust samples from indoor environments (private house, vehicle cabins and public buildings) reflected, for first time, the ubiquity of four of the five investigated Tinuvin in this matrix, with average concentrations between 71 and 780 ng g⁻¹.

3.2. Fungicides

As in the case of any other pesticide, the maximum levels of fungicides in raw vegetable foodstuffs are legislated in the European Union (EU). However, there is a great concern regarding their potential transference to elaborated foods, where their levels are

usually not regulated. A good example is the pair grapes-wine. Furthermore, there is a need to assess the impact of pesticides, arising from agriculture sources, in soil biota and the aquatic environment. Within this Ph.D., two novel sample preparation methods, combined with LC-MS/MS and GC-MS, have been developed and validated for the determination of fungicides in wine and vineyard soils. Their basic characteristics are briefly described below.

Determination of fungicides in wine by mixed-mode solid-phase extraction and liquid chromatography coupled to tandem mass spectrometry.

LC-MS/MS is one the most powerful techniques for the selective determination of pesticides in foodstuff commodities. However, efficiency of electrospray ionization (ESI) interfaces is prone to changes depending on the characteristics of each sample. Although those effects can be compensated using isotopic labelled species, this option is not suitable when dealing with many analytes from different chemical families. Furthermore, non volatile species present in wine samples, e.g. tartaric acid, may lead to salt precipitates in the cones of the LC-MS interface. Thus direct application of LC-MS/MS to wines analysis is not advisable.

In this study, SPE was used as sample preparation (extraction and clean-up) technique for the LC-MS/MS determination of nine fungicides in red and white wines. Efficiency of reversed-phase and mixed-mode (anion exchange and reversed-phase) sorbents were compared in terms of retention efficiency and selectivity. Under optimized conditions, 10 mL of wine were firstly diluted 1:1 with ultrapure water and then passed through the mixed-mode SPE cartridge at a flow of ca. 5 mL min⁻¹. After a washing step with 5 mL of an aqueous NH₄OH (5%, w/v) solution, to fix pigments and anionic interferences existing in wine samples, analytes were recovered with just 1 mL of methanol and injected in the LC-MS/MS system, without any additional purification. The selective extraction process avoided significant changes in the ionization efficiency for red and white wine extracts in comparison with pure standards in methanol. Moreover, absolute recoveries were between 72 and 97% with standard deviations below 9%. Thus, external calibration can be used as quantification strategy. LOQs remained in the range of 0.01 to 0.8 ng mL⁻¹, which are far below the EU MRLs for fungicides in vinification grapes.

Metalaxyl and azoxystrobin residues were found in most wine samples produced in different geographic areas.

Optimization of matrix solid-phase dispersion conditions for organic fungicides determination in soil samples

The availability of low cost, high throughput and sustainable sample preparation methodologies is a basic requirement to investigate the fate and potential accumulation of fungicides in vineyards soils. Procedures reported in the literature are either based on high solvent consuming solid-liquid extraction methodologies [13,26] or in the use of techniques involving the acquisition of expensive equipment, e.g. PLE [27].

In this work, the suitability of matrix solid-phase dispersion (MSPD) for the extraction of 15 fungicides, belonging to different chemical classes, from soil samples was evaluated using GC-MS as determination technique. Particular attention was focussed on accuracy evaluation considering spiked and real life polluted agriculture soils.

Samples (0.5 g) were blended and dispersed with 2 g of C18 and transferred to a polypropylene syringe containing 1 g of diatomaceous earth. Analytes were recovered using 10 mL of ethyl acetate, this extract was concentrated to 1 mL and fungicides determined by GC-MS, without additional clean-up. The method provided recoveries in the range from 74 to 122% for soils with different total carbon contents (1.8- 5.5%) and it allowed the use of external standard as quantification technique. Inter-day precision, given as relative standard deviations, stayed between 3 and 13%, and the limits of quantification were comprised between 0.6 ng g⁻¹ and 15 ng g⁻¹. Analysis of polluted soils, using PLE extraction and the MSPD protocol developed in this study, produced results in good agreement.

The top layer of vineyard soils contained significant levels of several fungicides mainly used against mildew and botrytis infections. The most often detected species were iprovalicarb and metalaxyl, reflecting their intensive use in viticulture and matching with their presence in commercial wines [28].

3.3. Ethylphenols

Determination of ethylphenol species in wine samples has been the aim of many research groups. Within this Thesis, two different microextraction procedures have been proposed, optimized and validated. They consider analytes acetylation followed by GC-MS analysis. Operational conditions and basic figures of merits of both methods are summarized below.

Sorptive extraction with in-sample acetylation for gas chromatography mass spectrometry determination of ethylphenol species in wine samples

In this work, a cost effective solid-phase microextraction method for ethylphenols determination in wine samples is proposed. Analytes were in-situ acetylated and concentrated in a technical grade silicone disc (0.5 mm diameter × 0.5 mm thickness) immersed into diluted samples. Optimization included derivatization, extraction and desorption steps. Under final conditions, 2 mL of wine were diluted with 15 mL of an aqueous solution of potassium bicarbonate (5%, w/v) in a 22 mL vessel, containing 2 g of sodium chloride and 90 μ L of acetic anhydride. The extraction was carried out at room temperature (20 ± 2 °C) during 2 hours. Analytes concentrated in silicone discs were desorbed using just 0.2 mL of ethyl acetate. As the silicone sorbent was considered as a single use trap due to its low cost (c.a. 0.05 €/unit.), the method avoids cross-contamination problems, which might occur in SPME and SBSE procedures.

The achieved limits of quantification (LOQs), between 5 and 15 ng mL⁻¹, remained below sensorial thresholds of target species. Moreover, normalized responses (analytes/internal standard peak areas) did not vary among different wines. This fact facilitates obtaining quantitative data, since the use of the standard addition quantification technique is not required. A further advantage of the developed methodology is that acetylated ethylphenols show an excellent stability once they have been incorporated into silicone discs. Thus, sample preparation can be performed in wineries and then sorbent units transported to the laboratory for desorption and analysis.

Mixed-mode solid-phase extraction followed by dispersive liquid-liquid microextraction for the sensitive determination of ethylphenols in red wines

Despite the increasing number of applications described for the dispersive liquid-liquid microextraction (DLLME) technique, works focused on complex matrices, such as it is the case of wine, are still scarce. In fact, some previous studies have reported difficulties to directly apply this technique to wine [29] and, moreover, the previous application of DLLME to ethylphenols determination did not provide limits of quantification below their sensorial thresholds [30].

In this work, selectivity of mixed-mode SPE was combined with the concentration power of DLLME to obtain a sensitive and low solvent consumption method for gas chromatography-mass spectrometry determination of ethylphenol off-flavours in red wines. Analytes were acetylated after SPE and previously to DLLME concentration to enhance the performance of their GC-MS determination. Thus, the extract from the SPE cartridge was diluted with an aqueous basic solution and mixed with acetic anhydride. Thereafter, the DLLME step was performed with a binary mixture of 1,1,1-trichloroethane (TCE) and acetone. Using just 5 mL of wine and 0.06 mL of TCE, as extractant in the DLLME step, the method achieved limits of quantification below 0.8 ng mL⁻¹ for all species, enrichment factors above 60-times and excellent relative recoveries (from 89 to 109%), considering matrix-matched standard quantification. Despite being a multi-step sample preparation approach, the method still provides a high sample throughput because of the fast kinetics of acetylation and DLLME process. Application to wine samples has demonstrated the presence of low levels of target species in red wines, independently of their quality and maturation time.

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CAPÍTULO I

Justificación y objetivos

Un gran número de compuestos orgánicos de interés por su potencial toxicidad crónica, bioacumulación o simplemente características organolépticas no deseables, se encuentra a niveles traza en matrices de mayor o menor complejidad. Por ello, para su determinación se requieren metodologías analíticas con bajos límites de cuantificación, buena repetibilidad y exactitud, que además resulten sostenibles, atendiendo al consumo de disolventes y la generación de residuos, y aplicables a efectos prácticos en base a su robustez y su coste. Muchos de los problemas anteriores se abordan empleando técnicas cromatográficas, sobre todo combinadas con espectrometría de masas, después de una etapa de preparación de muestra con una mayor o menor complejidad. Tradicionalmente, se emplea la extracción líquido-líquido y la extracción Soxhlet para la extracción/concentración de los analitos de interés de matrices acuosas o sólidas, respectivamente, lo que supone un alto consumo de disolventes orgánicos y/o largos tiempos de análisis. Alternativas para muestras acuosas son la **extracción en fase sólida (SPE)** y las diferentes modalidades de **microextracción** que suponen una reducción drástica del consumo de disolventes y, en ocasiones, la completa eliminación de los mismos. Para muestras sólidas, incluyen la extracción con **disolventes presurizados (PLE)** y la **dispersión de la matriz en fase sólida (MSPD)** que reducen el consumo de disolventes orgánicos y tiempo.

Para la realización de esta Tesis se han seleccionado tres grupos de analitos de naturaleza química diferente: derivados del 2-hidroxibenzotriazol (Tinuvines), fungicidas y fenoles volátiles. Los **Tinuvines** pueden ser introducidos en el medio ambiente por vertidos industriales, domésticos o de depuradoras de aguas residuales, llegando a los ríos y mares, donde se acumulan en sedimentos y biota debido a su marcado carácter lipofílico [1,2]. Además, debido a su amplio uso como fotoprotectores de plásticos, pinturas o recubrimientos, existe la posibilidad de que migren desde estos materiales hacia el polvo, representando una vía de exposición para el ser humano por inhalación o ingestión inadvertida en ambientes cerrados. Como consecuencia de este uso extensivo, los Tinuvines pueden constituir un problema de contaminación a escala mundial que, sin embargo, no ha recibido la atención dedicada a otros contaminantes emergentes durante los últimos años.

El segundo grupo de analitos considerados en esta memoria son los **fungicidas**, que representan una de las familias más importantes de pesticidas empleados en agricultura. Su aplicación es particularmente intensa en viñedos, para prevenir y tratar enfermedades causadas por hongos y mejorar la producción de uva. Sin embargo, este

uso no está exento de riesgos. Considerando que los fungicidas no se eliminan completamente durante el proceso de vinificación [3], el consumo de vino supone una vía más de exposición continuada a niveles residuales de estos compuestos. Antes de desarrollar una legislación específica para este alimento, es necesario disponer de metodología analítica con unas características adecuadas para monitorizar los niveles de residuos existentes y evaluar el comportamiento de diferentes fungicidas en las etapas de vinificación. Por otro lado, los fungicidas también pueden ejercer efectos negativos en el ecosistema del suelo de los viñedos, provocando en ocasiones la aparición de cepas de hongos resistentes; y/o suponer un problema medioambiental derivado de la contaminación de acuíferos subterráneos y/o aguas superficiales [4].

El tercer grupo de analitos son los **etilfenoles volátiles** que, si bien no constituyen un peligro para la salud ni el medio ambiente, se relacionan con defectos organolépticos en el vino, causando severas pérdidas económicas debido al rechazo por parte de los consumidores. Los etilfenoles estudiados (4-etilfenol, 4-etilguayacol y 4-etilcatecol) son producidos por levaduras que se encuentran de forma natural en el vino [5], y que pueden multiplicarse durante determinadas operaciones de vinificación y/o elaboración del vino (ej. envejecimiento), de forma que cuando sobrepasan un determinado umbral provocan el defecto conocido, entre otros, con el nombre de “Brett”.

La finalidad global de esta Tesis ha sido explorar, optimizar y aplicar nuevas metodologías analíticas, incidiendo básicamente en la etapa de preparación de las muestras, de interés para la determinación de los compuestos anteriormente descritos, englobados bajo los epígrafes genéricos de contaminantes emergentes, pesticidas y compuestos de origen natural, en muestras sólidas y líquidas de tipo ambiental y alimentario. La reducción en los costes, tiempo de preparación de muestra y generación de residuos han sido directrices comunes a todas las actividades realizadas. A ellas se han sumado una validación rigurosa de los métodos propuestos y su comparativa con otros descritos previamente en la bibliografía. La combinación de estas metodologías con **cromatografía de gases o de líquidos y espectrometría de masas (MS o tándem MS)**, como técnicas de determinación, ha permitido abordar el análisis de muestras reales aportando información relevante en relación con la presencia y distribución de Tiunvines en muestras ambientales, la existencia de residuos de diferentes fungicidas en vinos comerciales y suelos de viñedo y, por último, los niveles de etilfenoles en vino.

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CAPÍTULO II

Introducción

II.A.

Compuestos estudiados



1. DERIVADOS DEL 2-HIDROXIBENZOTRIAZOL (TINUVINES)

1.1. Definición, estructura y propiedades

Los filtros solares son compuestos capaces de absorber o reflejar la radiación solar. Se introducen en cremas, lociones y otros cosméticos para proteger al ser humano de la radiación solar ultravioleta (UV). Además, los filtros solares se emplean como aditivos en pinturas, adhesivos, recubrimientos de diversas superficies y materiales poliméricos, con objeto de evitar reacciones de descomposición y/o decoloración catalizadas o inducidas por la radiación UV procedente del sol [1-3]. Dentro de este grupo de filtros UV se encuentran los derivados del 2-hidroxibenzotriazol. Estos compuestos, también llamados Tinuvines, poseen un grupo hidroxifenólico en su estructura que puede estar sustituido en las posiciones orto y para, **Figura II.A.1.**

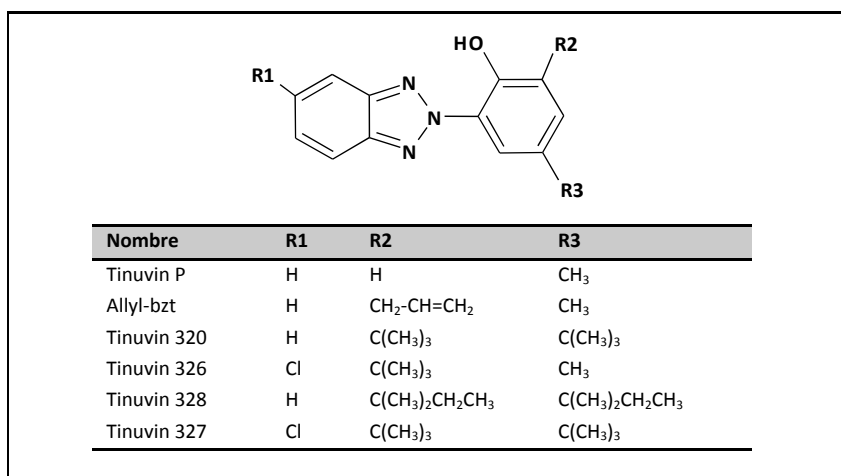


Figura II.A.1. Estructura del 2-hidroxifenilbenzotriazol y sus derivados.

En la **Tabla II.A.1** se muestran las propiedades físico-químicas de los Tinuvines objeto de estudio en esta Tesis. Son compuestos térmica y químicamente estables, poco volátiles (presiones de vapor entre 9.8×10^{-9} y 1.7×10^{-8} Torr) y de elevado carácter lipofílico. Se observa que este último aumenta al aumentar su peso molecular, alcanzando valores para el logaritmo del coeficiente de partición octanol-agua ($\log K_{ow}$) entre 4.3 y 8.3. Son débilmente ácidos con valores de pK_a comprendidos entre 8.2 y 9.4 unidades.

Tabla II.A.1. Nombre y propiedades de los estabilizadores UV estudiados en esta Tesis.

Nombre	Abv	CAS	Peso molecular	log K _{ow} *	Punto de ebullición (°C) (760 Torr)*	Presión de vapor (Torr)*	Solubilidad (g L ⁻¹) (pH 7)*	pKa (25°C)*
Tinuvin P	TP	2440-22-4	225	4.3	424	8.8×10 ⁻⁸	0.018	8.2
Allyl-bzt	Allyl-bzt	2170-39-0	265	5.3	450	9.8×10 ⁻⁹	4.5×10 ⁻³	8.5
Tinuvin 320	T320	3846-71-7	323	6.8	444	1.7×10 ⁻⁸	6.8×10 ⁻⁴	9.4
Tinuvin 326	T326	3896-11-5	315	6.8	460	4.2×10 ⁻⁹	3.5×10 ⁻⁴	9.3
Tinuvin 328	T328	25973-55-1	352	7.9	469	2.0×10 ⁻⁹	1.7×10 ⁻⁴	8.8
Tinuvin 327	T327	3864-99-1	358	7.5	469	2.0×10 ⁻⁹	1.5×10 ⁻⁴	9.2

*Valores obtenidos usando el software V11.02 Advanced Chemistry Development (ACD/Labs) (© 1994-2011 ACD/Labs)

1.2. Usos y producción

Los Tinuvinos son compuestos que se emplean a nivel industrial como aditivos fotoestabilizadores en una gran cantidad de polímeros, tales como policarbonatos, poliésteres, poliestirenos, acrílicos, polivinilos y poliacetales [4]. Sólo en Estados Unidos en el año 2005, se fabricaron y/o importaron de 227 a 453 toneladas de Tinuvin P y de 453 a 4536 toneladas de Tinuvin 328, de Tinuvin 234¹ y de Tinuvin 329² [2]. En Japón se produjeron 549 toneladas de Tinuvin 327 al año en 2006 [5], y 717 toneladas de Tinuvin 320 entre 2002 y 2005 [6].

De forma general los polímeros contienen entre el 0.1 y el 10% en peso de diferentes aditivos [7]. En el caso de los estabilizadores UV, se han encontrado niveles de 0.2-0.3% en poliestireno y 0.01% en botellas de PET para Tinuvin P [1,7], 0.65% de Tinuvin 234 en resina acrílicas [8], 0.97% de Tinuvin 1130³ [8] en pinturas de coche y 0.082% de Tinuvin 326 en otros materiales plásticos [9].

¹ 2-(2H-benzotriazol-2-il)-4,6-bis(2-fenilpropan-2-il) fenol (CAS 70321-86-17)

² 2-(2-Hidroxí-5-tert-octilfenil) benzotriazol (CAS 3147-75-9)

³ α-[3-[3-(2H-benzotriazol-2-il)-5-(1,1-dimetil-4-hidroxifenil)-1-oxopropil]-ω-hidroxí- Poli(oxi-1,2-etanedil)] (CAS 104810-48-2)

1.3. Toxicidad y legislación

La información toxicológica relativa a la exposición a Tinuvines es escasa. La toxicidad aguda de estos compuestos es baja, tanto en mamíferos [2] como en organismos acuáticos [2,5]. Sin embargo, el contacto con superficies tratadas con Tinuvin P puede provocar reacciones alérgicas tales como dermatitis e irritación en la piel [2,10-12].

Los Tinuvines no mostraron actividad estrogénica en levaduras [13], ni genotóxica en bacterias [2], y tampoco mostraron efectos en la reproducción, carcinogénicos o genotóxicos en roedores cuando se administraron por vía oral [2,14]. Los principales daños parecen que se focalizan en el hígado, siendo el Tinuvin 320 el más hepatotóxico [14]. Cuando se administró por vía oral el Tinuvin 320 a ratas, durante 28 días, se observó hipertrofia y necrosis del hígado, degeneración de hepatocitos, anemia, degeneración e hipertrofia en el corazón e hipertrofia en el riñón a concentraciones inferiores a 0.5 mg kg⁻¹ en los machos y 12.5 mg kg⁻¹ en las hembras [15]. Teniendo en cuenta los valores NOAL (*Non observed adverse effect level*), se observa una diferencia de hasta 25 veces en la toxicidad del Tinuvin 320 para ratas hembra (2.5 mg Kg⁻¹ d⁻¹) y ratas macho (0.1 mg Kg⁻¹ d⁻¹) [16]. Esta diferencia de toxicidad por sexo, también ha sido descrita en el caso de Tinuvin P, Tinuvin 328, Tinuvin 234 [2] y Tinuvin 327 [17].

Debido a su potencial bioacumulativo y persistencia, el Tinuvin 320 ha sido prohibido por el gobierno japonés [18] en 2007 y el Tinuvin 327 y el Tinuvin 350 forman parte de la Clase I de sustancias químicas monitorizadas en Japón [19,20]. En cambio, la Unión Europea ha aprobado el uso del Tinuvin 327, junto a Tinuvin P, Tinuvin 326 y Tinuvin 900⁴, como aditivos en envases alimentarios [21], con límites de migración específica de 30 mg Kg⁻¹ para la suma de los 3 primeros y de 1.5 mg kg⁻¹ para el Tinuvin 900.

1.4. Distribución ambiental

La elevada estabilidad de los Tinuvines es una ventaja a nivel industrial, de cara a su uso como absorbentes de radiación UV, pero puede suponer un problema

⁴ 2-(2H-benzotriazol-2-il)-4,6-bis(1-metil-1-feniletil) fenol (CAS 70321-86-7)

medioambiental. Su presencia en el medioambiente se debe principalmente a vertidos industriales [22], domésticos y de plantas de tratamiento de agua residual [23].

Los niveles de estos filtros solares se encuentran en los pocos ng L^{-1} en influentes de estaciones depuradoras de aguas residuales (EDARs). Una vez las aguas residuales son tratadas, los niveles de Tinuvines disminuyen debido a su facilidad para retenerse sobre la materia orgánica particulada [24,25]. Los niveles en lodo varían desde los pocos ng g^{-1} hasta los $\mu\text{g g}^{-1}$, con concentraciones medias de 116 ng g^{-1} para Tinuvin 234, de 50 a 1100 ng g^{-1} para Tinuvin 326, de 3.7 a 170 ng g^{-1} para Tinuvin 327 y de 57 a 1300 ng g^{-1} para Tinuvin 328 [24,26,27]. Aunque los estudios realizados con lodos aún son escasos, en China se ha observado una correlación entre Tinuvin 328 y 326 y entre Tinuvin 234 y 329 [28], lo que implica que presentan fuentes de contaminación comunes, es decir, se usan de forma conjunta. Mientras que el Tinuvin 320 no ha sido detectado en las muestras de lodo procesadas en el estudio anterior [28].

Las descargas de efluentes domésticos, o de EDARs, en ríos están correlacionadas con la presencia de Tinuvin 234, 326, 327 y 328 en aguas superficiales. Sin embargo, esta correlación no se ha observado en el caso de Tinuvin P y 329 [23]. Se detectaron concentraciones desde 1 ng L^{-1} para el Tinuvin 327 hasta 4780 ng L^{-1} para el Tinuvin 328 en ríos de Japón que recibían estos vertidos [23]. También se observaron bajas concentraciones en ríos y lagos que no reciben aguas residuales, por lo que su presencia en estos compartimentos se puede deber a fenómenos de deposición seca y/o húmeda (como partículas procedentes de carreteras o zonas de ocio) [23].

Además, los efluentes procedentes de industrias que manufacturaban estos compuestos pueden contribuir a la contaminación de los ríos. Por ejemplo, en los años 1978 y 1980 se detectaron Tinuvines en los sedimentos y en el agua del río Pawtuxet (Rhode Island, USA) que recibía los efluentes de varias industrias químicas [23,29]. Los niveles encontrados variaban desde $6 \mu\text{g L}^{-1}$ para Tinuvin P hasta $85 \mu\text{g L}^{-1}$ para Tinuvin 328 en agua, y desde $0.5 \mu\text{g g}^{-1}$ para Tinuvin 327 hasta $800 \mu\text{g g}^{-1}$ para Tinuvin P en sedimentos.

Incluso 15 años después de cerrar la planta de producción, los niveles de los benzotriazoles seguían siendo elevados, determinándose concentraciones de Tinuvin 327 de hasta $5000 \mu\text{g g}^{-1}$ en los sedimentos de este río [30]. En otras zonas del planeta, las concentraciones en sedimentos varían desde los pocos ng g^{-1} hasta los bajos $\mu\text{g g}^{-1}$, encontrándose los mayores valores en sedimentos procedentes de ríos altamente

contaminados y con elevados contenidos de materia orgánica [6], siendo Tinuvin 328 y 234 los compuestos más abundantes [23]. Por ejemplo, para el Tinuvin 328 se encuentran concentraciones de 2.06-7.0 ng g⁻¹ en China [27], 0.76-224 ng g⁻¹ en USA [27] y 2.8-1735 ng g⁻¹ en Japón [18,23]. Al igual que ocurría en lodo, existe una correlación entre los niveles de Tinuvin 328 y 326 y además con el Tinuvin 327 [6,23].

Los Tinuvines pueden incorporarse en las cadenas tróficas debido a su alta persistencia y baja degradación [31]. Sus niveles varían desde los pocos ng g⁻¹ hasta los cientos de ng g⁻¹, en organismos del medioambiente marino, desde pequeños gasterópodos y almejas hasta tiburones o patos [31-33], siendo Tinuvin P, 327 y 328 los más predominantes. Algunos Tinuvines muestran preferencia por acumularse en unos organismos más que en otros, como el Tinuvin 328 por el salmonete [31].

Por lo tanto, la ingesta de pescado es una ruta potencial de exposición a los Tinuvines. En el caso del Tinuvin 320, la ingesta diaria calculada (entre 0.04 ng d⁻¹ y 0.02 µg d⁻¹) es muy inferior a la dosis tolerable (6 µg d⁻¹) por lo que no habrá efectos adversos para la salud humana a corto plazo [32]. No obstante, no existen estudios que evalúen los efectos de la exposición crónica a estos compuestos, bien a través de la alimentación o de otras vías.

Otra fuente de exposición es el polvo ya que estos analitos son liberados de los materiales plásticos que los contienen y se adsorben a la materia particulada en suspensión y al polvo depositado, al igual que sucede con otros contaminantes como compuestos perfluorados, productos de cuidado personal, ftalatos, retardantes de llama, etc. [34-37]. Estudios realizados en Filipinas indican que Tinuvin 328 y 234 son los más predominantes, llegando a alcanzar concentraciones de 304 ng g⁻¹ y 817 ng g⁻¹ respectivamente; mientras que Tinuvin P y Allyl-bzt no fueron detectados en polvo [38]. En cambio, en esta Tesis se han observado mayores concentraciones para Tinuvin P y 326 alcanzando niveles de 657 ng g⁻¹ y 4883 ng g⁻¹, respectivamente [39]. Estas diferencias pueden ser debidas a los diferentes usos y aplicaciones de estos fotoestabilizadores en cada región del planeta. Así pues, son necesarios más estudios para evaluar sus niveles en los diferentes compartimentos ambientales y en las diferentes regiones del planeta.

1.5. Determinación de Tinuvines en muestras acuosas

Como se ha comentado anteriormente, los derivados del 2-hidroxibenzotriazol se encuentran a niveles de los pocos ng L^{-1} en agua. Para monitorizar estos niveles se requieren metodologías con bajos límites de cuantificación, que normalmente conllevan una etapa de concentración y purificación de la muestra. Se han utilizado técnicas de extracción clásicas como la extracción líquido-líquido (LLE) y la extracción en fase sólida (SPE) para la determinación de estos compuestos junto con otros tipos de contaminantes emergentes (filtros UV tipo benzofenonas [23], fragancias [24], alcanos, hidrocarburos policíclicos aromáticos (PAHs) [29], etc.), aunque la bibliografía específica para Tinuvines es escasa.

Una de las desventajas que tiene LLE es el gran consumo de disolventes orgánicos. Jungclaus y col. [29] y López-Avila y col. [22] emplearon hasta 0.5 L de diclorometano para la extracción de Tinuvines de 2 L de agua de río y 200 mL de agua residual, obteniendo límites de detección (LODs) de 0.1 ng mL^{-1} y 1 ng mL^{-1} , respectivamente. Más recientemente, Nakata y col. [24] utilizaron un total de 200 mL de hexano para la extracción de Tinuvin 326, 327, 328 y de las fragancias Galaxolide y Tonalide de 0.5 L de agua residual. Además de la extracción, realizaron una etapa de purificación con sílice, desactivada al 5% con agua Milli-Q. Consiguieron LODs de 2.1 a 8.7 ng L^{-1} utilizando la cromatografía de gases (GC) seguida de la espectrometría de masas (MS) para su determinación.

Liu y col. [25,26] utilizaron adsorbentes de fase reversa, tipo Oasis HLB, para la extracción de benzotriazoles y otros filtros UV de muestras de agua subterránea y efluente. Observaron que se obtenían recuperaciones cuantitativas al eluir el cartucho con 6 mL de diclorometano/metanol 1:1. Por otro lado, Kameda y col. [23] utilizaron dos cartuchos en tándem, uno del tipo C18 y otro un intercambiador aniónico débil, para realizar la extracción simultánea de diversos filtros UV, y 10 mL de diclorometano como disolvente de extracción. Ambos autores consiguieron LODs inferiores a los 6 ng L^{-1} para los estabilizadores UV empleando GC seguida de MS [25,26] o MS/MS (espectrometría de masas en tándem) [23].

En esta Tesis se han investigado dos metodologías de preparación de muestra para la determinación de estabilizadores UV en muestras de aguas. Con objeto de reducir el consumo de tiempo y disolventes, se propone el uso de la microextracción en fase

sólida (SPME) para la determinación de estos compuestos en muestras de agua residual. En comparación con SPE se evitan pérdidas por adsorción y se mejora la precisión de los resultados.

1.6. Determinación de Tinuvines en muestras sólidas

Los Tinuvines son compuestos lipofílicos y de baja solubilidad que tienden a acumularse en matrices sólidas, como en lodo procedente de plantas de tratamientos de aguas residuales, en sedimentos de ríos, lagos y bahías donde se vierten aguas residuales industriales o domésticas, e incluso en los organismos que viven en estas zonas (almejas, peces, pájaros, etc.). Además, estos compuestos se pueden encontrar asociados a la materia particulada y polvo en ambientes confinados.

La monitorización de niveles traza de compuestos orgánicos en muestras sólidas requiere metodologías de preparación de muestra que permitan romper la interacción analito-matriz e incluyan una etapa de limpieza para eliminar las interferencias. Para la extracción de Tinuvines de lodo [24], polvo [38], sedimentos [6], peces y otros organismos marinos [6,18,31-33], se han utilizado Soxhlet y HSSE (extracción con disolventes a alta velocidad, *High speed solvent extraction*) (Tabla II.A.2). En ambos casos, se empleó como extractante hexano con acetona [32,33] o con diclorometano [6,18,24,31]. Aunque en ambos casos se consuman hasta 300 mL de disolvente, HSSE [32,33,38] presenta menores tiempos de extracción (30 min) que Soxhlet (6 h) [6]. En el caso de muestras de sedimentos, en la mayoría de las aplicaciones se ha utilizado la extracción sólido-líquido (SLE), con agitación mecánica [27,30], o con ayuda de ultrasonidos (US) [23], empleando diclorometano, acetona, acetato de etilo, hexano o mezclas de estos disolventes como extractantes. En el caso de lodo, además de SLE [27], también se ha utilizado extracción presurizada con disolventes (PLE), empleando mezclas de hexano/diclorometano [26,28], en combinación con altas temperaturas y presiones. Es un método automatizado, con menor consumo de disolventes que Soxhlet o HSSE, en el que se puede incorporar una etapa de limpieza en la extracción [26], o bien realizarla posteriormente [28].

Todas las técnicas anteriores proporcionan extractos muy complejos por lo que se necesita una etapa de limpieza, basada en polímeros de SPE, para la mayoría de las aplicaciones. El absorbente más utilizado es la sílice [6,18,24,27,31-33], aunque también

se ha descrito el uso de carbón grafitizado [23], Florisil [28] y alúmina [38]. Otros autores utilizan la cromatografía de permeación de gel (GPC) para la eliminar los lípidos, y otras especies de elevado peso molecular, co-extraídos de muestras de lodo y biota [6,18,24,28,31].

Tabla II.A.2. Resumen de aplicaciones para la determinación de Tinuvines en sedimentos, lodos, polvo y pescado.

Muestra	Técnica de extracción (disolvente)	Limpieza (Adsorbente)	Determinación	%Recuperación	LOQs (ng g ⁻¹)	Ref.
4 g sedimento	SLE asistida por US (DCM/Acet)	SPE (Florisil, Carbón, amina)	GC-MS	70-91	0.17-3.3 ^a	[23]
5 g sedimento	SLE (AcOEt/DCM)	SPE (sílice)	GC-MS	82-106	0.06-0.33	[27]
0.5-5 g de sedimento	SLE (ACN/Hexano)	LLE (agua pH 4)	GC-MS	90-110	66.7 ^a	[30]
0.1 g polvo	HSSE (Acet/hexano)	SPE (alúmina desactivada 6%)	LC-MS/MS	67-109	2-12.7 ^a	[38]
1 g lodo	PLE (Hexano/DCM)	on-line (sílice, Cu)	GC-MS/MS	81-147	1.1-27.4	[26]
2 g lodo	Soxhlet (DCM/Hexano)	GPC + SPE (sílice)	GC-MS/MS	-	-	[24]
1 g lodo	PLE (hexano/DCM)	GPC + SPE (Florisil)	LC-MS/MS	83-100	0.15-0.77	[28]
1 g lodo	SLE (AcOEt/DCM)	SPE (sílice)	GC-MS/MS	82-106	0.3-1.65	[27]
2-20 g de pescado o sedimento	Soxhlet (DCM/Hexano)	GPC+SPE (sílice)	GC-MS	110-114	0.16-0.5 ^a	[6]
5 g pescado	HSSE (Hexano/Acet)	SPE (sílice desactivada 5%)	LC-MS/MS	71-112	0.001-0.027 ^b	[32]

a: Límites de cuantificación (LOQs) estimados a partir de LODs, b: peso lípidico, DCM: Diclorometano, Acet: Acetona, AcOEt: acetato de etilo, ACN: acetonitrilo, LC: cromatografía líquida

Dentro de esta Tesis doctoral se han desarrollado dos metodologías para la determinación de Tinuvines en muestras sólidas. Una de ellas está basada en el uso de PLE para muestras de polvo y la otra se ha centrado en la extracción con dispersión de la matriz en fase sólida (MSPD) aplicada a muestras de sedimento. En ambos casos, las etapas de extracción y limpieza están integradas en un único paso.

2. FUNGICIDAS

2.1. Definición, estructura y propiedades

Los fungicidas son sustancias tóxicas que se usan para eliminar o impedir el desarrollo de hongos perjudiciales para plantas, animales o para el hombre. Tienen una gran importancia en agricultura donde se aplican sobre semillas, hojas, frutos o plantas. Según su modo de acción, se pueden clasificar como:

- Sistémicos: se incorporan al interior de la planta, son de acción preventiva y curativa.
- De contacto: se depositan sobre la superficie de la planta.

Según su naturaleza química, los fungicidas se pueden clasificar en inorgánicos u orgánicos, presentando estos últimos una acción más específica para combatir diversos hongos que provocan enfermedades a las plantas. Dentro de los fungicidas orgánicos, existe una gran variedad de clases según su estructura química. En esta Tesis se han considerado hasta 15 compuestos diferentes, correspondientes a 8 grupos químicos: fenilamidas, azólicos, estrobilurinas, fenilpirroles, carbamatos, aminopiridinas, hidroxianilidas y dicarboximidas. En la **Figura II.A.2** y en la **Tabla II.A.3** se muestran las estructuras y propiedades físico-químicas de los fungicidas estudiados en esta Tesis.

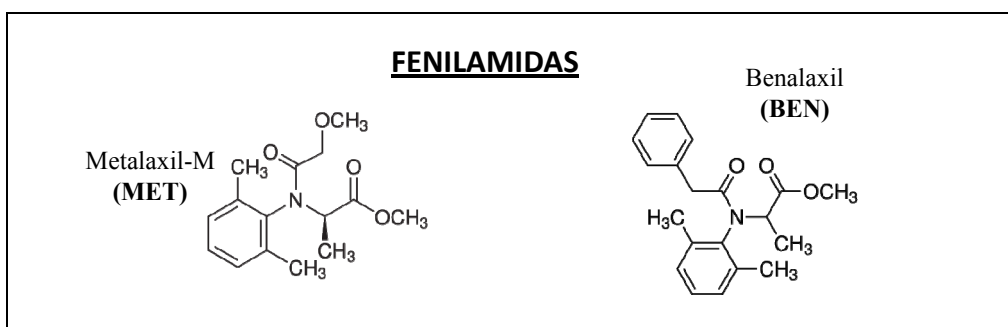


Figura II.A.2. Estructura de los fungicidas estudiados.

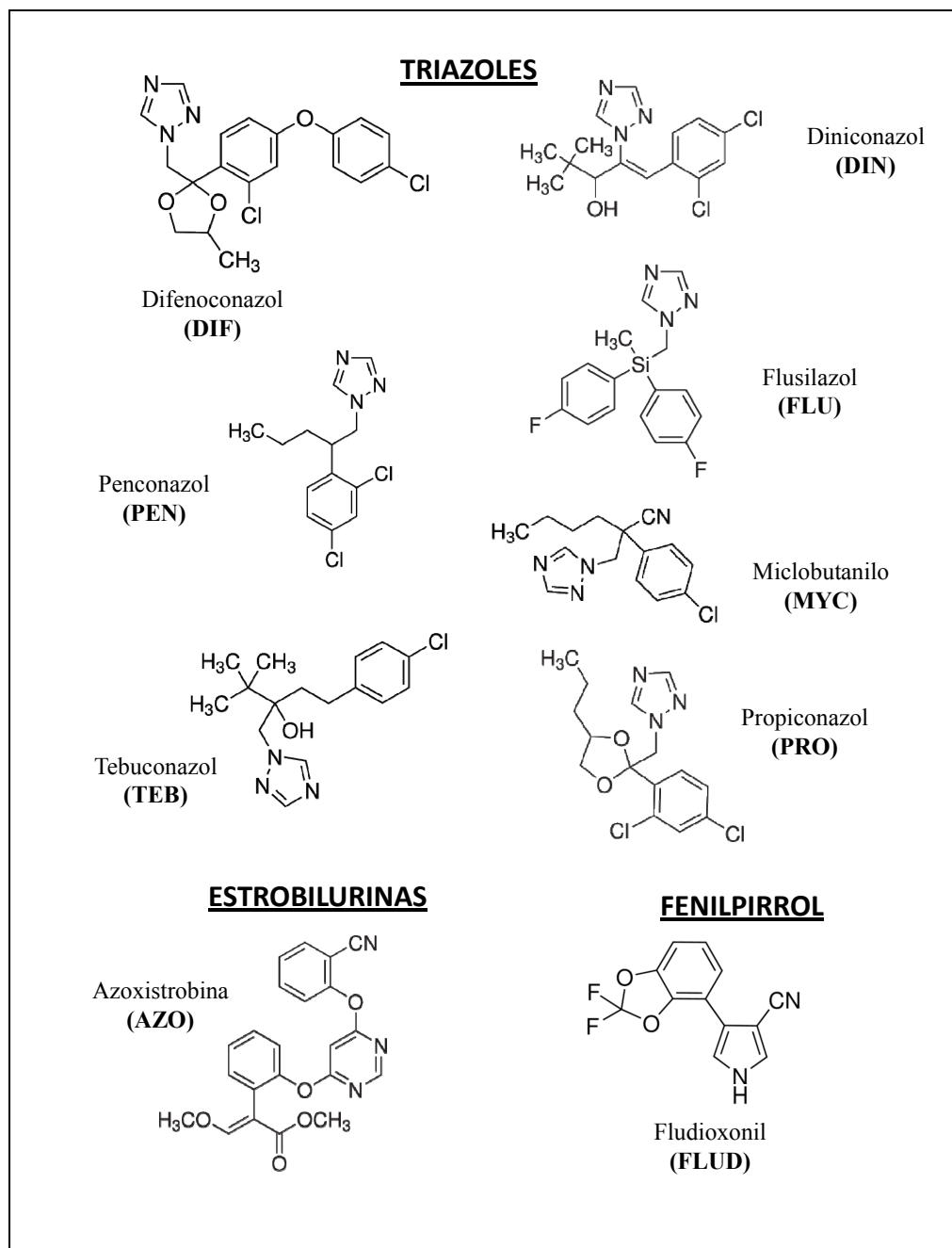


Figura II.A.2. Estructura de los fungicidas estudiados (continuación).

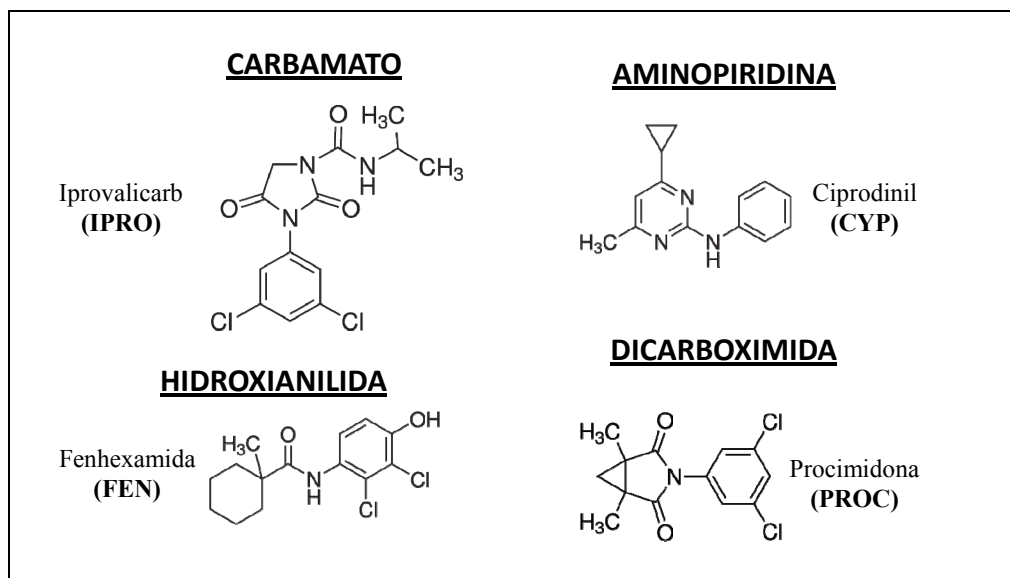


Figura II.A.2. Estructura de los fungicidas estudiados (continuación).

Tabla II.A.3. Nombre y propiedades de los fungicidas considerados en esta Tesis.

Fungicida	Nº CAS	Peso molecular	Punto de ebullición (°C) (760 Torr)*	Log K _{ow} *	Solubilidad (g L ⁻¹) (pH 7)*	pK _a *	Presión de vapor (Pa)*
AZO	131860-33-8	403.39	581.3	5.13	1.5x10 ⁻⁴	-0.67 ^b	2.23x10 ⁻¹¹
BEN	71626-11-4	325.4	468.8	3.26	0.078	1.52 ^b	7.733x10 ⁻⁷
CYP	121552-61-2	225.29	406	3.01	0.015	4.22 ^b	1.12x10 ⁻⁴
DIF	119446-68-3	406.26	547	4.92	4.5x10 ⁻⁴	3.06 ^b	6.79x10 ⁻¹⁰
DIN	70217-36-6	326.22	501.1	4.2	4.9x10 ⁻³	12.89 ^a /2.19 ^b	9.80x10 ⁻⁹
FEN	126833-17-8	302.2	457.9	3.86	0.026	7.73 ^a /0.0 ^b	7.013x10 ⁻⁷
FLU	85509-19-9	315.39	392.5	3.84	0.015	3.02 ^b	3.04x10 ⁻⁴
FLUD	131341-86-1	248.18	420.4	3.59	0.011	-9.13 ^b /14.1 ^a	3.77x10 ⁻⁵
IPRO	140923-17-7	320.43	497.8	4.09	0.048	11.41 ^a /-0.86 ^b	6.399x10 ⁻⁸
MET	70630-17-0	279.33	394.3	2.15	0.31	1.41 ^b	2.67x10 ⁻⁴
MYC	88671-89-0	288.78	465	2.82	0.16	2.30 ^b	1.04x10 ⁻⁶
PEN	66246-88-6	284.18	415.3	3.66	0.016	2.83 ^b	5.57x10 ⁻⁵
PRO	60207-90-1	342.22	479.9	3.88	4.8x10 ⁻³	3.06 ^b	3.01x10 ⁻⁷
PROC	32809-16-8	284.14	477.9	2.93	5.1x10 ⁻³	-2.67 ^b	3.6x10 ⁻⁷
TEB	107534-96-3	307.82	476.9	3.58	0.025	13.70 ^a /3.39 ^b	8.92x10 ⁻⁸

*Valores obtenidos usando el software V11.02 Advanced Chemistry Development (ACD/Labs) (© 1994-2011 ACD/Labs). ^agrupo ácido, ^bgrupo básico

2.2. Usos y aplicaciones

Actualmente, un amplio grupo de insecticidas y fungicidas se usan para proteger a los cultivos de insectos y hongos. En la Unión Europea se utilizan 220 mil toneladas de pesticidas al año, de los cuales el 49% son fungicidas, que se aplican principalmente en el cultivo de la uva [40].

Su principal uso es el de prevenir o curar enfermedades causadas por hongos que atacan a los cultivos, como el oídio, el mildiú o la botritis, en el caso de la vid. La acción de los fungicidas puede afectar a un gran número de patógenos o ser específica para un determinado tipo de hongo. En cualquier caso, inhiben el crecimiento de microorganismos por diferentes vías (inhibición de síntesis del ergosterol, respiración mitocondrial, etc.) [41,42] evitando, de esta manera, grandes pérdidas económicas.

Además de su aplicación en el sector hortofrutícola, los fungicidas tienen otros usos. Así, se utilizan como protectores de madera [43] o conservantes de recubrimientos [44] por su acción biocida, como medicamentos antifúngicos en humanos y en veterinaria [45], y algunos se usan en la terapia contra el cáncer [46].

2.3. Toxicidad

Debido al uso masivo de los fungicidas y a su persistencia, éstos pueden llegar a las aguas superficiales o pueden estar presentes en los alimentos, por lo que pueden originar un riesgo para el medioambiente y para la salud de los consumidores.

Los fungicidas triazólicos están considerados como tóxicos agudos. Afectan a la funcionalidad del hígado, pueden disminuir el peso del riñón, alterar la estructura de la vejiga y tener efectos perjudiciales sobre el sistema nervioso central [47]. Además, tienen efectos teratogénicos [48] y pueden actuar como disruptores endocrinos [49,50].

También se observó una elevada toxicidad para AZO, CYP y quinoxifen en organismos acuáticos, mientras que PROC y viclozolin presentaron toxicidades moderadas y MET resultó menos tóxico [51].

Además, los fungicidas pueden tener efectos negativos sobre los microorganismos que viven en suelos contaminados, tales como una reducción de la

biomasa microbiana [51] o la aparición de fenómenos de resistencia [52,53], resultado de una exposición crónica a concentraciones no letales de estas especies químicas.

Dado que la población está expuesta a residuos de estos compuestos presentes en la comida y en el aire de las áreas fumigadas y que los fungicidas parecen tener varios modos de acción, sus efectos pueden ser sinérgicos si la población se expone a varios fungicidas de forma simultánea y crónica [50]. Desde el punto de vista alimentario, el consumo de vino constituye una de las vías más probables de ingesta de fungicidas sobre todo en regiones con climas húmedos, donde los viñedos se tratan de forma intensiva con diferentes fungicidas durante primavera y verano.

2.4. Legislación

Actualmente, un amplio número de herbicidas, insecticidas y fungicidas se usan en la producción agrícola para evitar problemas causados por plagas que provocarían pérdidas económicas inadmisibles. La contrapartida es que estos productos fitosanitarios pueden ser transferidos a los alimentos frescos o elaborados [54], además de al medio ambiente. Por tanto, pueden tener efectos no deseables sobre la salud humana, la flora o la fauna.

Con objeto de prevenir tales efectos, gobiernos y organismos nacionales y supranacionales, como la EPA (Agencia de Protección Ambiental), la WHO (Organización Mundial de la Salud) y la FAO (Organización de las Naciones Unidas para la Alimentación y Agricultura), publican recomendaciones, directrices, o especificaciones para numerosos alimentos, principalmente aquellos procedentes de la agricultura. Concretamente, en España se aplica la directiva 91/414/CEE [55] que indica que productos fitosanitarios se pueden comercializar para su uso agrícola. A continuación, una vez que se permite el uso de un determinado compuesto fitosanitario, se establecen los niveles máximos de residuos (MRLs) para numerosas frutas y verduras según el reglamento (EC) Nº 396/2005 [56]. Sin embargo, no se han fijado límites para pesticidas en vino, excepto en algunos países. En la **Tabla II.A.4** se indican los MRLs en uva de los diferentes fungicidas considerados en esta Tesis según la legislación europea [56], y para vino según las legislaciones italiana [57] y suiza [58]. Por otro lado, la *Organisation Internationale de la Vigne* (OIV) sugiere como MRL en vino la décima parte del valor establecido para uva [59].

Tabla II.A.4. Límites máximos de residuos (MRLs) para fungicidas en uva y vino.

Fungicida	MRL uva (mg Kg ⁻¹)	MRL vino (µg mL ⁻¹)	MRL vino (µg mL ⁻¹)
	EU	Italia	Suiza
AZO	2	0.5	0.5
BEN	0.3	-	-
CYP	5	0.5	0.5
DIF	0.5	-	-
DIN	0.2	-	-
FEN	5	1.5	-
FLU	0.2	-	-
FLUD	2	0.5	0.5
IPRO	2	1.0	1.0
MET	1	0.2	0.6
MYC	1	5	-
PEN	0.2	-	-
PRO	0.05	-	-
PROC	0.02	0.5	-
TEB	2	-	0.3

Por otro lado, como resultado del amplio uso, los residuos de fungicidas pueden llegar a contaminar las aguas superficiales y subterráneas debido a vertidos incontrolados y a la fumigación de parcelas agrícolas. Estas aguas son la principal fuente de agua potable [60]. Por ello, la Unión Europea estableció un límite de 0.1 µg L⁻¹ para cada pesticida individual, o metabolito de importancia, y de 0.5 µg L⁻¹ para la suma de pesticidas y metabolitos en muestras de agua de consumo humano [61]. Este criterio es también extrapolable a fungicidas.

2.5. Distribución medioambiental

Como consecuencia de su uso extensivo, los fungicidas pueden llegar al medioambiente. La principal fuente de esta contaminación es su aplicación en áreas agrícolas ya que pueden ser arrastrados por el aire o la lluvia, filtrarse a las aguas subterráneas a través del suelo o salir de las tierras tratadas por el agua de tormenta o de riego pudiendo recorrer largas distancias desde los lugares de aplicación [45,62,63]. Así, se ha detectado fungicidas a niveles desde los ng L⁻¹ hasta los µg L⁻¹ en ríos, arroyos y lagos [45,64-67], observándose en algunos casos que en las áreas donde el consumo de fungicidas es mayor, se encuentran también las concentraciones más elevadas en el medio acuático [45].

Además de su aplicación a nivel agrícola, los fungicidas tienen otros usos como biocidas a nivel doméstico, por lo que la contaminación de las aguas superficiales puede deberse a la descarga de aguas residuales, matriz en la que se han detectado niveles de los bajos ng L^{-1} [45].

Las propiedades físico-químicas de los fungicidas afectan, además de a sus usos, a su distribución en el medioambiente [63,64,68]. Por ejemplo, se espera una alta movilidad para la PROC por su lenta degradación (tiempo de vida media $t_{1/2}$ =24-84 días en suelo [51]), llegando a alcanzar concentraciones de $57 \mu\text{g Kg}^{-1}$ en sedimentos [63]. Mientras el MET, con una alta solubilidad en agua y menor tiempo de vida media ($t_{1/2}$ =10-40 días en suelo [51]), se encuentra a niveles más bajos en sedimentos (no detectado - $10 \mu\text{g Kg}^{-1}$) [63].

Los niveles de fungicidas encontrados en los sedimentos, arroyos y suelos de áreas agrícolas están sujetos a variaciones estacionales, con un aumento de sus concentraciones en verano [63,64]. En el caso de suelos de viñedo, en primavera y verano se produce una contaminación directa durante la fumigación ya que es inevitable que una pequeña fracción se deposite en él; mientras otra fracción se deposita y/o es absorbida por la planta para la protección de la misma. Posteriormente, esta última fracción puede ser liberada al suelo, entre días y meses más tarde, por acción de la lluvia, el viento, la caída de las hojas en otoño, etc. [51]. Bermúdez-Couso y col. [63] observaron que las mayores concentraciones de PEN ($411 \mu\text{g Kg}^{-1}$) y MET ($1002 \mu\text{g Kg}^{-1}$) en suelos de viñedo se encontraban en primavera y de los antibotróxicos PROC ($1124 \mu\text{g Kg}^{-1}$), FLUD ($349 \mu\text{g Kg}^{-1}$) y CYP ($462 \mu\text{g Kg}^{-1}$) al final del verano y principios del otoño, coincidiendo con sus periodos de aplicación.

Por otro lado, el nivel de fungicidas en suelo puede disminuir por degradación química y biológica, fotodescomposición, lixiviado hacia las aguas subterráneas, volatilización o asimilación por las plantas [62], aunque en algunas ocasiones no desaparece del todo. A modo de ejemplo, las concentraciones encontradas de FLUD y CYP fueron de $991 \mu\text{g Kg}^{-1}$ y de $260 \mu\text{g Kg}^{-1}$, respectivamente, en muestras de suelo de viñedo recogidas a principios del otoño (1 mes después de la última aplicación) [69]. Sin embargo, 9 meses antes de empezar esta campaña, se encontraron concentraciones de hasta $52 \mu\text{g Kg}^{-1}$ para FLUD, lo que apunta a una ligera persistencia desde la campaña anterior.

La persistencia de fungicidas en suelo también depende de otros factores, como las características de éste (pH, contenido de materia orgánica y arcilla) [70]. Además, se

observó que la presencia de cobre aumenta la retención y estabilidad del PEN por formación de complejos [71]. En el caso del MET, se observó que la aplicación de la formulación comercial (mezcla de MET y oxiclورو de cobre) aumentaba la adsorción en suelos un 30% respecto al uso de fitosanitarios que no incluyen cobre en su formulación [72].

2.6. Niveles en vino

La presencia de fungicidas en vino se debe a que los procesos físico-químicos y biológicos implicados en las operaciones de vinificación no son capaces de eliminar completamente los residuos presentes en uvas. Se han encontrado niveles de 0.13 a 0.47 mg kg⁻¹ para PEN, de 0.07 a 0.21 mg kg⁻¹ para MET y de 0.09 a 0.4 mg kg⁻¹ para AZO [73,74] en uvas de vinificación. Evidentemente, estos residuos dependen de los tratamientos aplicados en los viñedos, de variables meteorológicas y, por supuesto, del tiempo transcurrido entre las aplicaciones de fitosanitarios y la vendimia [75]. Así, se observó que la concentración de TEB en uva se reducía de 4.84 a 0.42 mg kg⁻¹ tres semanas después de su aplicación [77].

Durante la elaboración del vino, los niveles de fungicidas se pueden reducir debido a su hidrólisis en medio ácido, la degradación mediante levaduras durante la fermentación o la adsorción sobre residuos sólidos y lías [76,77]. En la **Tabla II.A.5** se muestran los factores de transferencia de los residuos de fungicidas desde la uva al vino. Destacan entre otros los elevados valores encontrados para AZO, FEN, IPRO y MET. Así pues, la existencia de residuos de estos cuatro compuestos en uva en el momento de su recolección, implica necesariamente su presencia en vino.

La forma de elaboración del vino también es un factor a considerar. Por ejemplo, Cabras y col. [80] observaron que el residuo de AZO se redujo un 60% con la elaboración con maceración respecto a sin maceración, mientras que TEB, CYP y pirementanil no mostraron diferencias, probablemente debido a que AZO puede ser adsorbido de forma más efectiva en los hollejos.

Otro mecanismo por el cual se reducen los niveles en vino es la adición de clarificadores, como el carbón, bentonita o gelatina [75,82].

Tabla II.A.5. Factores de transferencia de fungicidas de la uva al vino.

Fungicida	Factor de transferencia (%)	Ref.
AZO	40	[76]
BEN	4.7	[78]
CYP	1.4	[78]
CYP	15	[76]
FEN	65	[79]
FLUD	4.4	[78]
FLUD	38	[76]
FLUD	27	[80]
IPRO	56	[78]
MET	42	[81]
MYC	<4	[76]
PEN	<6	[78]
PRO	29	[82]
PROC	1.3	[79]
PROC	35	[76]
TEB	31	[80]

En la **Tabla II.A.6** se muestra un resumen de los niveles de fungicidas en vino encontrados en la bibliografía. La mayoría de las muestras contienen más de un fungicida, incluso algunas tienen hasta 10 pesticidas diferentes (8 fungicidas y 2 insecticidas) [40].

Tabla II.A.6. Residuos de fungicidas en vino.

Fungicida	Número de muestras	Incidencia (%)	Rango de concentraciones ($\mu\text{g L}^{-1}$)	Ref.
AZO	40	10	1.2-13.2	[40]
Clozolinato	8	12.5	94	[59]
Cicloproconazol	103	1.9	<0.75	[83]
Epoxiconazol	103	2.9	6.5-30	[83]
FLU	103	8.7	0.5-0.75	[83]
MET	6	100	6.9-31.8	[84]
MYC	103	24.3	0.5-35	[83]
PEN	103	25.2	0.25-1.2	[83]
PRO	2	100	0.4-1.7	[85]
TEB	103	53.4	0.25-33	[83]
Triadimenol	15	20	8.5-163.6	[54]
Triadimefon	103	32	0.5-2.2	[83]
IPRO	30	23.3	9-58	[86]
CYP	15	80	0.9-15	[87]
FLUD	15	73.3	2.5-28.6	[87]
FEN	34	5.9	3.6-450	[40]
PROC	33	33.3	1-110	[40]

2.7. Determinación de fungicidas en vino [8,8990,919293,94,95]

Las metodologías analíticas más extendidas para la determinación de fungicidas en vino son la extracción líquido-líquido (LLE) y la extracción en fase sólida (SPE).

En LLE, entre 5 y 50 mL de vino son extraídos con diferentes volúmenes de disolventes como hexano [88,89], o combinaciones de hexano con acetato de etilo [75,90,91] o con acetona [77,92], o acetona/diclorometano [93-95]. En el caso de SPE, los absorbentes más utilizados son el C18 [96,97] y los poliméricos, como el Oasis HLB [98,99] (**Tabla II.A.7**). En ocasiones, los extractos obtenidos pueden ser demasiado complejos debido a la coextracción de interferencias y pigmentos [90]. Por lo tanto, en algunas aplicaciones, se lleva a cabo una etapa posterior de limpieza, basada de nuevo en SPE utilizando sílice [42], Florisil [96] o carbón grafitizado, así como adsorbentes con grupos amina primaria/secundaria (PSA) [90,91] y, en algunos casos, basada en técnicas de microextracción [84,86]. Por otro lado, Trosken y col. [83] obtienen límites de cuantificación (LOQs) aceptables sin etapa de preparación de muestra (**Tabla II.A.7**) aplicando LC (cromatografía líquida)-MS/MS (espectrometría de masas en tándem) directamente a la determinación de estos compuestos en vino.

Tabla II.A.7. Metodologías analíticas aplicadas a la determinación de fungicidas en vino.

Analitos	Técnica de extracción	Técnica de determinación	LOQs ($\mu\text{g L}^{-1}$)	Ref.
13 fungicidas	LLE+SPE	GC-MS	1-329	[87]
3 fungicidas	LLE+SPE	LC-UV	146-362	[42]
6 pesticidas	LLE	GC-ECD	1-12	[88]
7 fungicidas	SPE+DLLME	GC-MS	0.03-0.12	[84]
160 pesticidas	LLE+dSPE	GC-MS/MS	10-50	[86]
8 fungicidas azólicos	SPE	LC-MS	9-31	[99]
13 fungicidas	SPE	LC-MS/MS	0.1-0.7	[100]
46 pesticidas	SPE	LC-MS/MS	1.1-9.9	[101]
5 fungicidas	SPME	LC-UV	5-27	[102]
4 fungicidas	SPME	GC-MS	0.01-0.33	[85]
24 pesticidas	SPME	GC-MS/MS	0.64-219	[103]
13 fungicidas azólicos	-	LC-MS/MS	0.25-7.5	[83]

dSPE: extracción en fase sólida dispersiva, DLLME: microextracción líquido-líquido dispersiva, UV: detector ultravioleta, ECD: detector de captura electrónica.

En los últimos años han aparecido numerosas publicaciones donde se utiliza SPME para la determinación de fungicidas en vino. En estos casos, la fibra de PDMS (polidimetilsiloxano) [103], PDMS-DVB (divinilbenceno) [102], DVB-PDMS-CAR (carboxeno)

[87] o PA (poliacrilato) [85] se introduce directamente en la muestra de vino. Además, se han encontrado aplicaciones donde se utilizan otras técnicas de microextracción para la determinación de fungicidas, como la extracción con barras agitadoras (SBSE) recubiertas de PDMS [59,104], concentración sobre adsorbentes de silicona [105], microextracción - emulsificación asistida por ultrasonidos (USAEME) utilizando líquidos iónicos como agentes extractantes [106] o la microextracción asistida por membranas (MASE) [59].

Algunos autores observaron que la eficacia de las técnicas de microextracción (sobre todo SPME) disminuía con el porcentaje de etanol en el vino [102,107]. Por lo tanto, se opta por diluir la muestra para intentar reducir la pérdida de la eficacia de extracción y las variaciones entre vinos con distintos contenidos de etanol [102,108,109]. Otro problema asociado a la SPME, es el efecto matriz. Así, Urruty y col. [107] observaron diferencias significativas en el comportamiento de pesticidas en una disolución etanólica al 10% respecto a muestras de vino real.

En esta Tesis se ha desarrollado una metodología analítica basada en extracción en fase sólida para la determinación de fungicidas en vino. Se han utilizado adsorbentes poliméricos funcionalizados con grupos iónicos para conseguir una mayor selectividad, reduciendo así la complejidad de los extractos obtenidos y, por tanto, los efectos de matriz durante la ionización de los compuestos usando una interfase de electrospray (ESI) en LC-MS/MS.

2.8. Determinación de fungicidas en suelo

La monitorización de niveles traza de residuos de pesticidas en suelo requiere metodologías analíticas capaces de romper la interacción analito-matriz sólida, que sean lo suficientemente sensibles para poder cuantificar las bajas concentraciones a las que se encuentran, que tengan en cuenta el amplio rango de polaridades de los pesticidas y que posean cierta selectividad para eliminar las interferencias.

La metodología más utilizada para la extracción de fungicidas de muestras de suelo es la extracción sólido-líquido (SLE) con disoluciones acuosas y/o con disolventes orgánicos. En esta línea de trabajo, Rial Otero y col. [69] estudiaron los parámetros que afectaban a la extracción de 8 fungicidas en suelos de viñedo. Para SLE, sonicaban la muestra de suelo con una disolución acuosa, tamponada a pH 8, y a continuación agitaban

con acetato de etilo para la recuperar los analitos. Observaron que el tipo de suelo afectaba a las recuperaciones obtenidas, por lo que la cuantificación se realizó con el método de adiciones estándar. Estos autores obtuvieron LOQs inferiores a 50 ng g^{-1} utilizando GC-MS como técnica de determinación.

Deng y col. [110] utilizaron una mezcla de agua/metanol, seguida de la adición de diclorometano, para la extracción de hexaconazole, MYC y TEB de suelo. Además, llevaron a cabo una limpieza posterior de los extractos orgánicos mediante SPE con cartuchos de PSA. Utilizando GC-NPD (detector de nitrógeno-fósforo) consiguieron LODs inferiores a 3 ng g^{-1} .

Hu y col. [111] sintetizaron polímeros de impronta molecular (MIP) con los que recubrieron barras agitadoras de SBSE. Primero, sonicaban el suelo con hexano y después realizaban la SBSE para la concentración/purificación de fungicidas triazólicos. Utilizando GC-NPD, consiguieron LODs inferiores a 0.34 ng g^{-1} .

Otros autores utilizaron técnicas más enérgicas en la etapa de extracción, tales como PLE. Así, Schreck y col. [112] obtuvieron LOQs inferiores a 19.4 ng g^{-1} para la extracción de 7 pesticidas, incluyendo MET, folpet y MYC, usando PLE y determinación mediante GC-MS. Aunque PLE es una técnica fácilmente automatizable, presenta el inconveniente de que requiere la compra y mantenimiento de equipamiento especial.

Como alternativa a métodos más tradicionales, Li y col. [113] proponen la MSPD para la determinación de 8 pesticidas, incluyendo los fungicidas DIN, PRO y uniconazole, en suelo. Estudiaron los factores que afectan a la eficacia y selectividad de extracción: cantidad de dispersante, grado de desactivación del dispersante y *ratio* hexano/acetato de etilo. Observaron que la adición de agua, cuando se mezcla Florisil (dispersante) y suelo, favorece la extracción ya que disminuye la capacidad del Florisil para retener los analitos y compuestos interferentes por interacciones polares, como enlace de hidrógeno, dipolo-dipolo, etc. En condiciones óptimas, 4 g de suelo se mezclan con 3 mL de agua y 6 g de Florisil proporcionando mejores recuperaciones y mayor selectividad frente a otras cantidades de agua y Florisil estudiadas. A continuación, estos compuestos se eluían con una mezcla hexano/acetato de etilo (3:1 v/v) mientras los interferentes más polares eran retenidos por el Florisil. Consiguieron LODs inferiores a 50 ng g^{-1} usando LC con detector ultravioleta (UV).

En esta Tesis se propone también el uso de MSPD para la determinación en suelo de 15 fungicidas que son aplicados frecuentemente a los viñedos. La metodología propuesta supone un menor coste y consumo de disolventes que las anteriormente descritas en la bibliografía. Además, las recuperaciones obtenidas no se han visto afectadas por las características particulares de cada suelo.

3. ETILFENOLES

3.1. Definición, estructura y propiedades

Los etilfenoles son compuestos orgánicos volátiles producidos por levaduras presentes en las etapas de elaboración del vino. En la **Figura II.A.3** y en la **Tabla II.A.8** se muestran las estructuras y propiedades de estos analitos. Su presencia en el vino puede contribuir de forma positiva o de forma negativa a su aroma, dependiendo entre otros factores de sus niveles de concentración. En este último caso, el defecto organoléptico producido se describe como fenólico, “olor a caballo” o *Brett*.

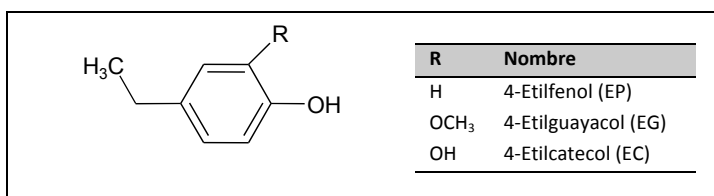


Figura II.A.3. Estructura de los fenoles volátiles estudiados en esta Tesis.

Tabla II.A.8. Nombre y propiedades de los etilfenoles estudiados en esta Tesis.

Abv	CAS	Peso molecular	log K _{ow} *	Punto de ebullición (°C) (760 Torr)*	Presión de vapor (Torr)*	Solubilidad (g L ⁻¹) (pH 7)*	pKa (25°C)*
EP	123-07-9	122	2.47	219.0	0.0828	6.6	10.2
EG	2785-89-9	152	2.18	246.5	0.0173	4.1	10.3
EC	1124-39-6	138	1.87	273.3	3.36x10 ⁻³	11	9.70

*Valores calculados usando el software V11.02 Advanced Chemistry Development (ACD/Labs) (© 1994-2011 ACD/Labs)

3.2. Origen y efectos en el aroma del vino

Las características visuales y las propiedades organolépticas cambian durante la maduración y envejecimiento del vino, especialmente, en los vinos tintos. Este cambio (que en la mayoría de los casos tiene efectos positivos), se produce debido a fenómenos físicos, como la migración de compuestos aromáticos del vino a las barricas de madera

[114] o al corcho [115], o viceversa; químicos, como oxidación o hidrólisis; y microbiológicos, como la formación de etilfenoles [116].

La formación de etilfenoles en vino se debe a la acción de dos enzimas que se encuentran en las levaduras del género *Brettanomyces/Dekkera* (**Figura II.A.4**). En primer lugar, los ácidos hidroxicinámicos se transforman en vinilfenoles por acción de la enzima hidroxicinamato descarboxilasa. A continuación, los vinilfenoles son reducidos a etilfenoles por la vinilfenol reductasa [117].

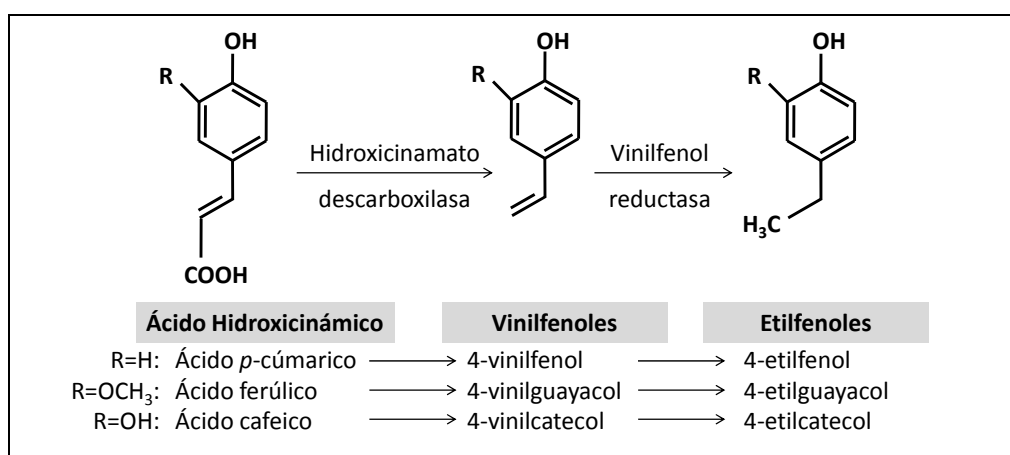


Figura II.A.4. Mecanismo de formación de etilfenoles.

Estas levaduras están presentes en todas las etapas de la elaboración del vino (desde las uvas hasta en el equipamiento de las bodegas) y pueden crecer durante el envejecimiento, e incluso después de que el vino ha sido embotellado. Su lento desarrollo se puede ver favorecido debido al bajo contenido de dióxido de azufre, pH elevado, altas temperaturas, el uso de ciertas técnicas como la microoxigenación, el contacto de los hollejos de las uvas con el mosto/vino, por el uso de barricas de madera, especialmente de barricas viejas que son difíciles de limpiar, y por el propio paso del tiempo desde la elaboración del vino hasta su consumo. Por lo tanto, los vinos tintos serán más susceptibles de presentar problemas asociados a la presencia de etilfenoles que los blancos [116-118].

Para conocer el tamaño de las poblaciones de *Brettanomyces/Dekkera* se pueden utilizar métodos microbiológicos combinados con microscopía de fluorescencia, o técnicas

de PCR (reacción en cadena de polimerasa), o bien metodologías analíticas para la determinación de sus metabolitos, siendo el EP uno de sus principales indicadores [118].

Los etilfenoles son compuestos volátiles que contribuyen al aroma del vino de forma positiva a bajos niveles pero si superan un determinado umbral provocan, junto con 4-vinilfenol y 4-viniguayacol, el defecto organoléptico conocido como *carácter Brett*. Este defecto se describe como “aroma de caballo”, fenólico, especiado, etc. [117,119]. Los umbrales de percepción de EP, EG y EC encontrados en la bibliografía son de 430, 33 y 50 $\mu\text{g L}^{-1}$, respectivamente; aunque estos valores pueden variar en función del tipo de matriz [120]. Sin embargo, la presencia de EC a nivel de 100-400 $\mu\text{g L}^{-1}$ [121] o de EP a nivel inferior a 2 mg L^{-1} [120] todavía puede tener efectos positivos en el aroma del vino, dependiendo de la variedad de uva, de los niveles de otros compuestos aromáticos y/o factores culturales.

Los niveles encontrados en los vinos varían desde $\mu\text{g L}^{-1}$ hasta los mg L^{-1} , observándose que la concentración de EP es superior a la de EG y EC, con un *ratio* medio de 10:1. En la **Tabla II.A.9** se muestran los valores encontrados en diferentes vinos y los *ratios* EP/EG y EP/EC.

Tabla II.A.9. Concentraciones máximas ($\mu\text{g L}^{-1}$) de EP, EG y EC encontradas en vino tinto.

Número de muestras	EP	EG	EC	EP/EG	EP/EC	Ref.
153	1800	1610	430	3.7:1	2.6:1	[121]
10	1344	137	227	10:1	6:1	[122]
10	2708	204	344	13:1	8:1	[123]
6	1120	120	n.d.	9:1		[124]
2	3536	280	n.d.	13:1		[125]
18 ^a	2150	295	n.d.	10:1		[126]
21 ^b	2660	350	n.d.	9:1		[126]
9 ^c	2200	437	n.d.	8:1		[126]
13 ^d	1560	421	n.d.	3.5:1		[126]

Variedad de vino tinto: ^a*Cabernet sauvignon*, ^b*Shiraz*, ^c*Merlot*, ^d*Pinot Noir*

n.d.: no disponible

Además, se observan diferencias entre los niveles de etilfenoles en vino tinto y vino blanco. Esto se debe a que la propia elaboración del vino tinto favorece el crecimiento de *Brettanomyces*, por la presencia de la piel de la uva durante la elaboración y por el mayor tiempo de maduración. Mientras en vino tinto las concentraciones de EP pueden llegar a alcanzar los mg L^{-1} , en vino blanco las concentraciones suelen ser inferiores. Lacher y col. [121] observaron que el 25% de las muestras de vino tinto tenían

niveles de 228-1800 $\mu\text{g L}^{-1}$ de EP, mientras que sólo el 10% de las muestras de vino blanco presentaba niveles de 23-476 $\mu\text{g L}^{-1}$. Sin embargo, en vino blanco se detectan cientos de $\mu\text{g L}^{-1}$ de sus precursores, los vinilfenoles [127].

3.3. Determinación de etilfenoles en vino

La determinación de etilfenoles en vino se basa, en la mayor parte de las aplicaciones, en una etapa de preparación de muestra seguida de cromatografía de gases (GC) combinada con espectrometría de masas (MS), o un detector de ionización de llama (FID). En la **Tabla II.A.10** se muestran algunos ejemplos de aplicaciones para la determinación de etilfenoles en vino.

Tabla II.A.10. Ejemplos de aplicaciones desarrolladas para la determinación de etilfenoles en muestras de vino.

Analitos	Técnica de extracción	Volumen de muestra (mL)	Determinación	%RSD	LOQ ($\mu\text{g L}^{-1}$)	Ref.
EP, EG, VP,VG	SPE	10	GC-FID	<7.2	1600-3770	[128]
30 compuestos volátiles	SPE	50	GC-MS	<10	0.026-1.1	[129]
EP,EG	LLE	5		<8	0.1-0.5	[130]
EP,EG,EC	HS-SPME ^a	4	GC-MS	<3.5	3-30	[122]
EP,EG	HS-SPME	25	GC-FID	<10	5	[131]
EP, EG	HS-SPME	5	GC-MS	<35	9.1-25.1	[132]
EP, EG, VP,VG	HS SPME	5	GC-MS	<11.5	0.06-0.12	[133]
EP, EG, VP,VG	SBSE	3.75	GC-MS	<4.7	0.021-1.244	[134]
EG,EP	DLLME	5	GC-MS	<3.8	95-147	[124]
EP, EG, VP,VG	USAEME	5	GC-MS/MS	<12	0.107-0.190	[135]
EC	-	-	HPLC-CEAD	<3	1.1	[121]
EP	-	-	HPLC-F	<10	13.3 ^b	[127]

a: SPME con acetilación *in situ*. b: LOQ estimado a partir de LOD. VP:vinilfenol, VG:vinilguayacol, CEAD: detector electroquímico coulombimetrico multicanal, F: detector de fluorescencia

Para la determinación de EP y EG se han utilizado técnicas clásicas de extracción como LLE con éter dietílico [125] o éter-pentano [126], o como SPE utilizando como adsorbente LiChrolut-EN y como disolvente de elución diclorometano [114,128,129,136].

En los últimos años, la mayoría de las aplicaciones se centran en SPME, en la modalidad de espacio de cabeza (HS), con fibras de DVB-CAR-PDMS [122,132] y CW (carbowax-DVB [133,137], aunque PDMS [131] y PA [125] también han sido utilizadas. Al

comparar HS-SPME y LLE, Monje y col. [125] concluyeron que HS-SPME era más sensible y presentaba menores %RSDs a bajas concentraciones ($50 \mu\text{g L}^{-1}$). Como alternativa a SPME, Díez y col. [134] proponen el uso de SBSE que permite la extracción de varias muestras simultáneamente y proporciona menores LOQs al utilizar mayor cantidad de absorbente.

Al igual que ocurre en otras técnicas de microextracción, al aplicarla a muestras complejas, como el vino, HS-SPME sufre de efectos matriz por lo que se requiere el uso del método de adiciones estándar para la cuantificación. Para compensar este efecto, Pizarro y col. [133] proponen el uso de HS-SPME múltiple, consistente en la extracción cuantitativa del analito, al realizar varios muestreos sucesivos sobre la misma disolución problema. Otros autores optan por utilizar DMP (3,4-dimetilfenol) como patrón interno [125,137] para compensar el efecto matriz. En otros trabajos se utiliza la dilución isotópica que minimiza las diferencias de comportamiento entre el patrón interno y el analito [126,130].

También se han utilizado nuevas técnicas de microextracción, de bajo coste, para la determinación de EP y EG como la microextracción líquido-líquido dispersiva (DLLME) [124,138] o USAEME [135], ver **Tabla II.A.10**. DLLME presenta límites de detección más altos que otras técnicas. Además, algunos autores señalan la formación de un precipitado en el fondo del tubo de extracción, sobre todo cuando se aplica DLLME sobre vino tinto utilizando disolvente clorados [84,139], que impide la correcta recogida de la fase orgánica.

Por otro lado, la determinación del EC ha sido escasamente investigada debido a que su elevada polaridad provoca picos anchos y difícilmente cuantificables por GC, incluso en columnas polares. En 2004, Hesford y col. [119] publicaron el primer trabajo donde se determinaba EC después de LLE. Estos autores realizaron una derivatización con anhídrido acético para reducir su polaridad y poder así determinarlo por GC-MS. En el año 2007, Carrillo y col. [122] proponen combinar SPME y acetilación, de forma simultánea, obteniendo límites de detección de $6 \mu\text{g L}^{-1}$ para el EC con GC-MS. Posteriormente, se propone el uso de la cromatografía líquida, sin etapa de extracción ni derivatización previa, acoplada a un detector de ultravioleta [120] y a un detector electroquímico [121] para la determinación de EC en sidra y vino, respectivamente.

En esta Tesis se han desarrollado dos metodologías analíticas para la determinación conjunta de EP, EG y EC en vino por GC-MS con una etapa de acetilación previa. La primera metodología se basa en el uso de dispositivos de silicona de bajo coste

como adsorbentes y la segunda en el uso de la combinación SPE-DLLME, que aprovecha la alta selectividad de los cartuchos de modo mixto (eliminando componentes interferentes de la matriz) y el alto poder de concentración de DLLME.

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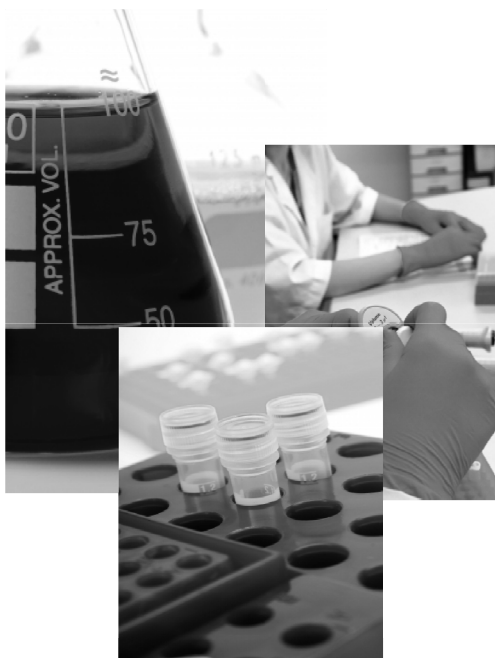
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II.B.

Metodología de preparación de muestra



1. MUESTRAS ACUOSAS

Uno de los pasos más importantes dentro del procedimiento analítico es la preparación de la muestra. Se caracteriza por ser la etapa que más tiempo consume y donde se cometen los mayores errores. Sus objetivos básicos son aislar los analitos y eliminar interferencias, concentrar los analitos y presentarlos en un medio compatible con la técnica de determinación.

Las técnicas convencionales de extracción de muestras acuosas son la extracción líquido-líquido (LLE) y la extracción en fase sólida (SPE). Además, en los últimos años, las técnicas de microextracción han experimentado un gran desarrollo. En esta sección de la memoria de Tesis se hace una revisión de estas técnicas, incidiendo en los fundamentos de aquellas, con mayor grado de novedad y que han sido aplicadas en la parte experimental del trabajo realizado.

1.1. Extracción líquido-líquido (LLE)

La extracción líquido-líquido (LLE) se basa en la distribución o reparto de los analitos entre una fase acuosa y otra orgánica inmiscibles entre sí. Estos procesos son controlados por el coeficiente de reparto k_D , que viene dado por la relación $[A]_o/[A]_a$, donde $[A]_o$ y $[A]_a$ son las concentraciones en la fase orgánica y en la fase acuosa, respectivamente [1]. El rendimiento y la selectividad de la extracción dependen de este coeficiente que a su vez es función del pH, del disolvente orgánico y de la fuerza iónica. Además, la formación de complejos o pares iónicos puede mejorar la eficacia de la extracción.

LLE es una técnica extremadamente versátil por su facilidad de uso y la disponibilidad de disolventes orgánicos de alta pureza. Sin embargo, presenta un elevado consumo de disolventes, por lo que se requiere una etapa de evaporación para la concentración de los extractos, problemas por la formación de emulsiones y una importante manipulación de la muestra, lo que puede conllevar problemas de contaminación o pérdidas de analito.

1.2. Extracción en fase sólida (SPE)

La extracción en fase sólida (SPE) se basa en la retención de un analito, presente en una muestra líquida o gaseosa, sobre un adsorbente apropiado y su posterior elución [1]. Surge como alternativa a LLE ya que reduce el consumo de disolventes, evita la formación de emulsiones, es fácilmente automatizable, disminuye la manipulación de la muestra y aumenta el factor de concentración. Además, puede eliminar interferencias de la matriz, lo que aumenta la sensibilidad y selectividad del procedimiento analítico.

El formato más popular en SPE son los cartuchos o jeringas [2] que contienen una determinada cantidad de adsorbente. La muestra pasa a través de este material (previamente acondicionado) con ayuda de una bomba de vacío, por gravedad o centrifugación. A continuación se realiza un lavado para eliminar las interferencias, se seca el adsorbente y se eluyen los analitos con un disolvente apropiado.

1.2.1. Tipos de adsorbentes

Además de evaluar la capacidad del adsorbente¹ y el volumen de rotura², la selección del adsorbente es uno de los aspectos más importantes en SPE. Las fases adsorbentes en SPE son muy similares a las utilizadas en cromatografía líquida (LC). Entre las más populares se encuentran: sílice enlazada, carbón grafitizado, resinas poliméricas, Florisil y alúmina [1,2]. A continuación se describen brevemente algunas de sus características:

- ❖ **Óxidos inorgánicos**, como la sílice, la alúmina o el Florisil. Son adsorbentes de naturaleza polar que se suelen utilizar para la limpieza de extractos orgánicos procedentes de matrices complejas.
- ❖ **Sílice enlazada**, sintetizada por la unión con organosilanos a través de un enlace sililéter. Se clasifican en fase normal, como el CN (cianopropilsilano); en fase reversa, como el ampliamente usado C18 (octadecilsilano); y en fases de

¹ *Capacidad del adsorbente*: es la máxima cantidad de analito/interferentes que pueden ser retenidos por unidad de masa de adsorbente.

² *Volumen de rotura*: máximo volumen de muestra que se puede pasar a través de un adsorbente sin eluir el analito

intercambio iónico, como el SAX (trimetilamino propilsilano) o SCX (bencenosulfonil propilsilano).

- ❖ **Carbón grafitizado**, es un adsorbente no específico capaz de extraer analitos polares y no polares de muestras acuosas. En ocasiones, es el único adsorbente capaz de retener analitos altamente polares. Sin embargo, puede presentar una retención excesiva de los mismos, lo que haría necesario la elución del cartucho en sentido inverso a la carga para su recuperación. Aún así, muchas veces los fenómenos de retención no son reversibles, resultando prácticamente imposible recuperar los analitos de manera cuantitativa.
- ❖ **Adsorbentes poliméricos**. Tradicionalmente, los adsorbentes poliméricos más utilizados han sido los copolímeros de poliestireno-divinilbenceno (PS-DVB). Presentan ciertas ventajas frente a las sílices enlazadas, como estabilidad en un amplio rango de pH, no poseen grupos silanoles que puedan dar lugar a interacciones secundarias no deseables y ofrecen mayor retención de compuestos polares, pero sin ocasionar los problemas del carbón grafitizado.

En 1996, aparecen en el mercado los adsorbentes basados en el copolímero N-vinilpirrolidona-divinilbenceno (VP-DVB), conocidos como Oasis® HLB. Es un relleno con balance hidrofílico-lipofílico que favorece la retención de compuestos polares y apolares. Posee las mismas ventajas que el PS-DVB, y además proporciona buenas recuperaciones para un gran número de analitos dentro de un amplio espectro de polaridades, incluso si el lecho del adsorbente se seca durante el acondicionamiento o carga de la muestra (lo que no ocurre en fases como el C18).

Además, estos adsorbentes pueden estar funcionalizados de forma que se combinan los mecanismos de retención de fase reversa de Oasis HLB con el intercambio aniónico fuerte (MAX) o débil (WAX), o catiónico fuerte (MCX) o débil (WCX), ver **Figura II.B.1** [3]. Benito Peña y col. [4] compararon cartuchos de C18 con Oasis MAX para la extracción de penicilinas de 250 mL de agua residual. Obtuvieron mejores recuperaciones con cartuchos Oasis MAX (76-100%) que con los basados en C18 (4-73%). Dado que las muestras estaban a un pH 7-8, las penicilinas ($pK_a=2.7-2.8$) se encontraban en su forma iónica y, por lo tanto, se favorecía su retención por mecanismos de intercambio iónico, lo que sucedía en los tipo MAX, en lugar de interacciones únicamente de fase reversa que ocurrían en los tipo C18.

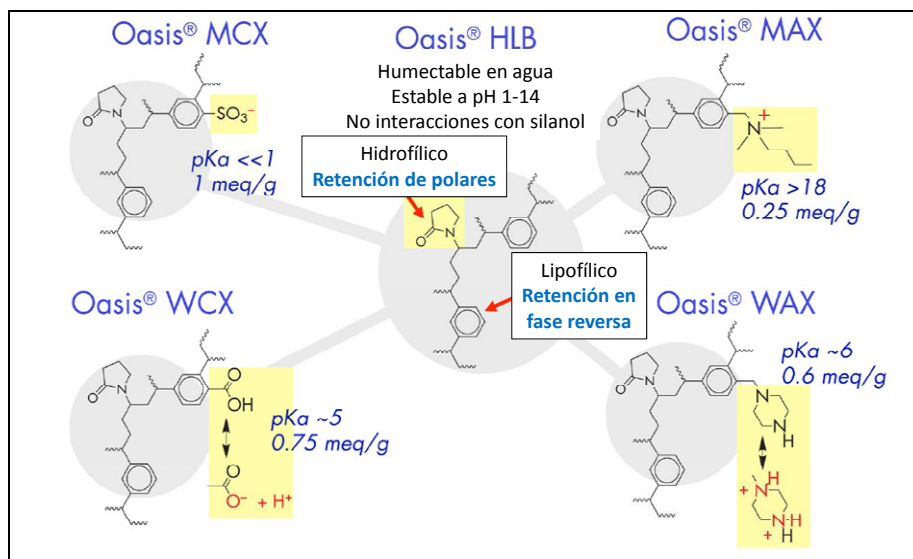


Figura II.B.1. Tipos de adsorbentes con balance hidrofílico-lipofílico derivados de polímeros VP-DVB.

Al modificar el pH se puede variar el factor de retención de los analitos según sus características ácidas/básicas, como se muestra en la **Figura II.B.2** para Oasis MCX y MAX, y conseguir métodos más selectivos. De esta forma, Laven y col. [5] han conseguido separar en tres fracciones fármacos ácidos, básicos y neutros de una muestra de agua residual, ajustada a pH 2, al combinar cartuchos Oasis MCX y MAX. En primer lugar, pasaban 25 mL de agua residual diluida con 25 mL de agua MilliQ por un cartucho Oasis MCX. Los analitos neutros y ácidos (en su forma neutra) son retenidos por mecanismos de fase reversa y eluidos con metanol; y los básicos se retienen por mecanismos de intercambio iónico y son eluidos, al convertirse en su forma neutra, con 2 mL de hidróxido amónico al 2% en metanol. La fracción de metanol fue diluida con 40 mL de agua, se pasó por un cartucho Oasis MAX y se realizó un lavado del adsorbente con una disolución acuosa de hidróxido amónico al 2%. A continuación, los compuestos neutros se eluyen en una primera fracción empleando metanol, mientras que los ácidos permanecen retenidos en el adsorbente debido a interacciones electroestáticas. En una etapa posterior, se recuperan con 2 mL de metanol conteniendo un 2% de ácido fórmico. Consiguieron recuperaciones medias del 85% y límites de cuantificación (LOQs) entre 15 y 940 ng L⁻¹ mediante cromatografía líquida y espectrometría de masas basada en un sistema analizador cuadrupolo-tiempo de vuelo (LC-QTOF-MS).

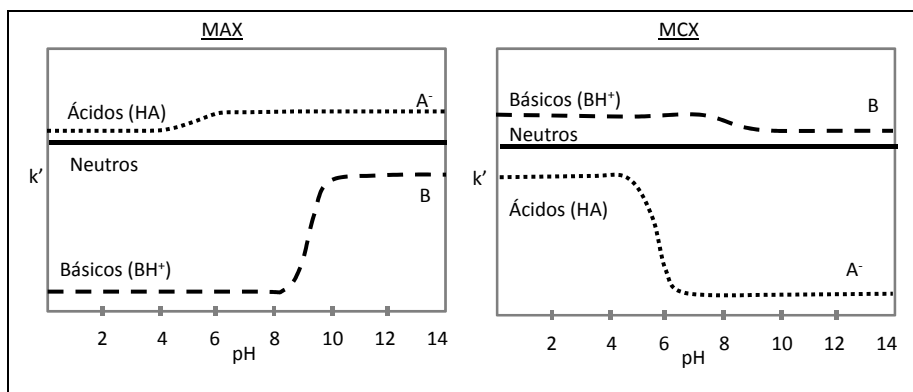


Figura II.B.2. Factor de retención (k') en función del pH para dos tipos de adsorbentes, Oasis MCX y MAX.

Esta variación del factor de retención según la acidez del entorno y de la ionización de los compuestos, se puede aprovechar para separar los analitos de compuestos interferentes presentes en muestras de vino. Montes y col. [6] observaron que el resveratrol y algunos interferentes presentes en muestras de vino tinto, como pigmentos, se retenían en los cartuchos Oasis MAX cuando se concentraba 10 mL de vino diluido (1:1, v/v). Al realizar un lavado con agua se eliminaban azúcares y al eluir con metanol se recuperaba el resveratrol (92-108%), mientras que los pigmentos ácidos se retenían por interacciones electroestáticas. De esta forma, se conseguían extractos más limpios que con Oasis HLB. Peña-Gallego y col. [7] también se aprovecharon de esta selectividad para el análisis de putrescina, cadaverina, tiramina y histamina en muestras de vino utilizando adsorbentes tipo Oasis MCX. Realizaron un lavado en condiciones ácidas para eliminar los compuestos polares, uno básico para eliminar los fenoles y pigmentos, y otro salino para eliminar compuestos que coeluyen con la histamina. Consiguieron recuperaciones entre 64 y 120% y LOQs entre 50 y 166 $\mu\text{g L}^{-1}$ utilizando HPLC (Cromatografía líquida de alta eficacia)-Fluorescencia.

- ❖ **Adsorbentes de acceso restringido**, donde se produce un proceso de exclusión molecular debido a que las partículas del material poseen pequeños poros, de un diámetro determinado, en los que sólo pueden entrar moléculas pequeñas, o debido a una red de polímero que sólo permite el paso de las mismas hacia la parte retentiva del material. Así, moléculas de gran tamaño, como las proteínas, pueden ser eliminadas de muestras de plasma o orina, o como los ácidos húmicos de muestras de agua [2,8].

- ❖ **Inmunoadsorbentes**, la retención se basa en la interacción analito (antígeno)-anticuerpo, inmovilizado en un soporte sólido (agarosa o sílice). Debido a la alta selectividad de esta interacción, se consiguen extractos más limpios que con otras fases, por lo que son muy utilizados en el análisis de muestras biológicas y de alimentos [8]. Por otro lado, entre las principales limitaciones de estos materiales destacan la necesidad de desarrollar anticuerpos específicos para cada analito o clase de analitos, que no siempre están disponibles comercialmente, su reducida estabilidad y su elevado coste.
- ❖ **Polímeros de impresión molecular (MIP)**, son polímeros altamente entrecruzados con propiedades de reconocimiento molecular, similares a las interacciones antígeno-anticuerpo. Se sintetizan en presencia de una molécula plantilla de estructura similar al analito, de forma que, al separarla, pueden unirse de forma selectiva al analito o a otros compuestos químicos de estructura similar [9]. Son más fáciles de preparar que los inmunoadsorbentes y tienen un menor coste.

1.3. Técnicas basadas en microextracción en fase sólida

En lo referente a la preparación de muestras, se constata una demanda de nuevos métodos más rápidos, más fáciles de automatizar, con menos etapas y un consumo mínimo de disolventes. Por ello, en los últimos años, las técnicas de microextracción han experimentado un desarrollo significativo. Aunque normalmente la extracción no es cuantitativa, proporcionan precisión y linealidad aceptables en un gran número de aplicaciones (medioambientales, clínicas, alimentarias, etc.) [10,11].

La microextracción en fase sólida (SPME) fue la primera de las técnicas de microextracción, introducida por Belardi y Pawliszyn en 1989 [12]. Se basa en el uso de una fase polimérica, de unos pocos microlitros, que extrae y concentra a los analitos cuando se expone a la muestra. Desde entonces, esta técnica ha sufrido un gran desarrollo, desde las diferentes fases poliméricas utilizadas como extractantes hasta los diferentes formatos que pueden ser:

- comerciales, como la microextracción en fase sólida (SPME) que se basa en el uso de fibras de sílice fundida recubiertas de fase extractante, o la extracción con

barras agitadoras (SBSE) recubiertas de fase extractante (también denominados *Twister*®).

- no comerciales, basados en el uso de polímeros adsorbentes de grado técnico (siliconas y polietersulfonas), considerados como materiales de un solo uso, y empleados en combinación con desorción térmica o mediante disolventes orgánicos.

Las técnicas basadas en microextracción en fase sólida constan de dos etapas:

- *Extracción*, por adsorción cuando la fase extractante es sólida o por absorción cuando es líquida, como el polidimetilsiloxano (PDMS). Para realizar la extracción, la fibra de SPME (u otro formato) se puede introducir directamente en la muestra (DI), exponerla al espacio de cabeza (HS) o protegerla con una membrana. La cantidad de analito extraída depende de su coeficiente de partición y es proporcional a su concentración en la muestra. Además, factores como el tipo y volumen de fase, volumen de muestra, fuerza iónica, temperatura, agitación, etc. pueden modificar la masa de analito incorporada en el polímero adsorbente.
- *Desorción*, que puede ser térmica, directamente en el puerto de inyección de un cromatógrafo de gases, en el caso de las fibras de SPME, o en una unidad de desorción térmica para otros formatos; o por disolución en un disolvente apropiado.

1.3.1. Fundamento teórico

Para generalizar el tratamiento teórico correspondiente a la eficacia de extracción en SPME, consideramos un sistema de tres fases: muestra, espacio de cabeza y fibra. Las expresiones que se representan son también válidas para SBSE y cualquier otra modalidad de microextracción en fase sólida, basada en fenómenos de absorción. Cuando el polímero extractante se expone a la muestra, o al espacio de cabeza, el analito se reparte entre las diferentes fases hasta alcanzar el equilibrio [1]. Por lo tanto, teniendo en cuenta que la cantidad de analito extraído por la fase polimérica está relacionado con la cantidad que permanece en las otras fases, y haciendo un balance de masa se puede escribir:

$$C_0 V_s = C_f^\infty V_f + C_h^\infty V_h + C_s^\infty V_s \quad (\text{Ecuación II.B.1})$$

donde C_0 , C_f^∞ , C_h^∞ y C_s^∞ son las concentraciones del analito en la matriz inicialmente, en la fase polimérica, en el espacio de cabeza y en la muestra en el equilibrio. Por otra parte, V_f , V_h y V_s son los volúmenes de la fase estacionaria, del espacio de cabeza y de la muestra, respectivamente.

Las constantes de distribución del analito entre la fase polimérica y el espacio de cabeza K_{fh} , entre el espacio de cabeza y la muestra K_{hs} , y entre la fase estacionaria y la muestra K_{fs} , se definen³ como:

$$K_{fh} = \frac{C_f^\infty}{C_h^\infty} \text{ (Ecuación II.B.2), } K_{hs} = \frac{C_h^\infty}{C_s^\infty} \text{ (Ecuación II.B.3), } K_{fs} = \frac{C_f^\infty}{C_s^\infty} = K_{fh}K_{hs} \text{ (Ecuación II.B.4)}$$

Por lo tanto, la masa de analito absorbido por la fase polimérica $n^\infty = C_f^\infty V_f$ vendría dada por:

$$n^\infty = \frac{K_{fs} V_f C_0 V_s}{K_{fs} V_f + K_{hs} V_h + V_s} \text{ (Ecuación II.B.5)}$$

En consecuencia, la cantidad de analito extraída en el equilibrio es proporcional a la concentración inicial en la muestra y a los volúmenes de las fases que intervienen en el equilibrio.

En la **Figura II.B.3** se muestra la recuperación teórica obtenida para diferentes volúmenes de fase en función de K_{fs} , considerando un volumen de muestra de 25 mL y sin espacio de cabeza para simplificar los cálculos. De esta forma, las recuperaciones obtenidas con SPME convencional, correspondiente a una fibra de PDMS de 1 cm de longitud y 100 μm de espesor (volumen de fase de 0.5 μL), serán menores que las obtenidas por ejemplo con un *Twister*[®] (volumen de fase entre 24-126 μL), siendo las diferencias más importantes para los analitos de polaridades medias [13].

³ Se usan concentraciones y no actividades, porque se supone matrices puras y analitos a nivel traza.

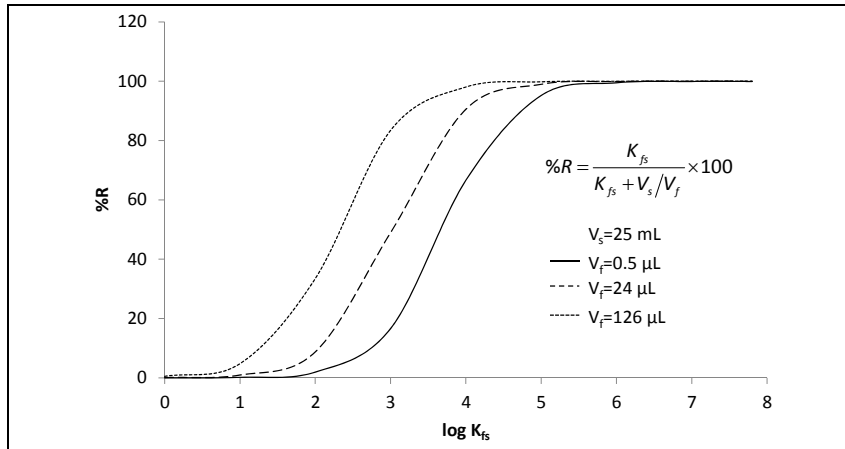


Figura II.B.3. Recuperación teórica, en función de la contante de distribución de un analito entre la fibra y la muestra (sin espacio de cabeza), para diferentes volúmenes de fase extractante.

La ecuación II.B.5 es válida para situaciones en las que el sistema ha alcanzado el equilibrio. En muchas aplicaciones se emplean tiempos de extracción menores al tiempo de equilibrio, es decir, se trabaja en condiciones cinéticas. En este caso, para un sistema de tres fases (muestra, espacio de cabeza y fibra) el analito debe difundirse a través de dos interfases: muestra/espacio de cabeza y espacio de cabeza/fibra. Estos procesos se pueden describir de acuerdo con la primera Ley de Fick según la Ecuación II.B.6, donde F es el flujo másico de analito, n la cantidad de analito en un tiempo t y A el área de la fibra.

$$F = -\frac{1}{A} \frac{\partial n}{\partial t} \quad (\text{Ecuación II.B.6})$$

Así, si consideramos que la difusión en fase gas es muy rápida, se puede describir el flujo másico de analito de la fase gas a la fibra según la ecuación II.B.7 y de la muestra a la fase gas según la ecuación II.B.8, siendo D_2 el coeficiente de difusión del analito a la fase polimérica, $\partial C_f / \partial x$ el gradiente de concentración en la fibra, m_2 el coeficiente de transferencia de masa del analito en la fibra, C'_f la concentración en la superficie interna de la fibra y k la constante de evaporación.

$$F = -D_2 \frac{\partial C_f}{\partial x} = m_2 (K_{fh} C_h - C'_f) \quad (\text{Ecuación II.B.7})$$

$$F = k (K_{hs} C_s - C_h) \quad (\text{Ecuación II.B.8})$$

Si asumimos que el analito tiene un gradiente lineal de concentración en la fase polimérica, la concentración en la muestra C_s será:

$$C_s = C_0 - \frac{n + C_h V_h}{V_s} \quad (\text{Ecuación II.B.9})$$

Combinando las ecuaciones II.B.6 – II.B.9, se obtiene una expresión diferencial que se resuelve al considerar como condición inicial ($t=0$) que la cantidad de analito en la fibra es nula ($n=0$). Por lo tanto, la cantidad de analito extraída en un instante viene dado por la siguiente ecuación:

$$n = (1 - e^{-at}) \frac{K_{fs} V_f C_0 V_s}{K_{fs} V_f + K_{hs} V_h + V_s} \quad (\text{Ecuación II.B.10})$$

$$\text{donde } a = 2Am_2 \frac{kK_{fs}V_f + kK_{hs}V_h + kV_s}{2m_2K_{fh}V_fV_s + kK_{hs}V_fV_h + kV_fV_s}$$

Esta ecuación demuestra la existencia de una relación lineal entre la cantidad de analito retenida sobre la fibra de SPME (n) y la concentración inicial en la muestra (C_0), siempre y cuando el tiempo de extracción, la velocidad de agitación y los volúmenes de muestra, espacio de cabeza y fibra permanezcan constantes.

Cuando el tiempo de muestreo aumenta hasta alcanzar el equilibrio, el término exponencial se anula y la ecuación II.B.10 se convierte en la ecuación II.B.5.

A nivel práctico, la extracción en condiciones de no-equilibrio suele estar controlada bien por la evaporación de los analitos de la muestra hacia el espacio de cabeza (k), o por su difusión en el interior de la fibra (m_2).

1.3.2. Variables experimentales que afectan a la eficacia de la extracción

- *Naturaleza y cantidad del polímero extractante*

La naturaleza del polímero es un factor que influye en el valor de K_{fs} . Así, a mayor afinidad del analito por el polímero, mayor será K_{fs} y mayor será la cantidad de analito extraído, según la Ecuación II.B.5. En el caso de SPME, existen en el mercado varios tipos de recubrimientos. Algunas de sus características se muestran en la **Tabla II.B.1**.

La masa de analito extraída también aumenta con el espesor del recubrimiento y con su volumen. Aunque utilizar mayores volúmenes de fase, como en los *Twister*®, supone cinéticas más lentas y, en consecuencia, mayores tiempos de extracción. Por otro lado, estos dispositivos tienen la ventaja de poder realizar varias extracciones de forma simultánea.

Tabla II.B.1. Tipos y características de los recubrimientos de fibras de SPME comercializados por *Supelco*.

Recubrimiento	Espesor (μm)	Polaridad	Temperatura de desorción recomendada (°C)
PDMS	100	No polar	200-280
	30	No polar	200-280
	7	No polar	220-320
PDMS/DVB	65	Dual	200-270
PA	85	Polar	220-300
CAR/PDMS	75	Dual	250-310
PEG	60	Polar	200-250
DVB/CAR/PDMS	50/30	Dual	230-270
Carbopack	15	Dual	250-340
CW/DVB	70	Polar	200-240

PDMS: Polidimetilsiloxano, DVB: Divinilbenceno, PA: Poliacrílico, CAR: Carboxeno, PEG: Polietilenglicol, CW: Carbowax

- *Tiempo de extracción*

El tiempo de equilibrio es el tiempo a partir del cual la cantidad de analito extraída permanece constante. La agitación, la temperatura o el espesor de fase son parámetros que afectan al tiempo que se tarda en alcanzar el equilibrio. En la práctica, no siempre es necesario esperar a alcanzar el equilibrio, si se controlan los parámetros que afectan a la cinética de extracción. En otras palabras, es posible obtener resultados repetibles, en condiciones de no equilibrio, con un buen control de la temperatura de las muestras y de la velocidad de agitación. En estas condiciones el valor del parámetro α en la ecuación II.B.10 es constante y la cantidad de analito extraída es función de su concentración inicial en la muestra (C_0).

- *Temperatura*

La temperatura tiene un doble efecto. Un aumento de la temperatura favorece la difusión de los analitos hacia la fase extractante acelerando la cinética de extracción. Por otro lado, la extracción es un proceso exotérmico, según la Ecuación II.B.11. Al aumentar

la temperatura, disminuyen las constantes de distribución y, por lo tanto, la cantidad de analito extraído en condiciones de equilibrio.

$$K_{fs} = K_0 e^{-\frac{\Delta H}{R} \left(\frac{1}{T} - \frac{1}{T_0} \right)} \quad (\text{Ecuación II.B.11})$$

siendo K_0 la constante de distribución entre la fibra y la muestra a una temperatura dada, T_0 (°K); ΔH la variación de entalpía cuando el analito pasa de la muestra a la fibra y R la constante de los gases.

- Efecto salino

La adición de sales (NaCl , KCl , Na_2SO_4) a muestras acuosas provoca un aumento de la fuerza iónica de la disolución, tendiendo las moléculas de agua a solvatar a los iones presentes en la muestra y no a las moléculas neutras de los analitos. Se produce así una disminución de la solubilidad de los analitos en agua, forzando su paso a otras fases del sistema (polímero extractante y espacio de cabeza) y aumentando la eficacia de extracción. Pero, también puede producir el efecto contrario debido quizás a un aumento de la viscosidad de la disolución que da lugar a cinéticas más lentas o a interacciones sal-soluto o “efecto aceite”⁴ que no favorecen la interacción analito-polímero [14].

- Volumen de muestra

Al aumentar el volumen de muestra, la cantidad de analito aumenta (según la ecuación II.B.12 para un sistema de dos fases: muestra y fibra), hasta que éste sea tan elevado que $K_{fs}V_f \ll V_s$. A partir de este punto, la cantidad de analito extraído no aumentaría más, siendo independiente del volumen de muestra.

$$n = \frac{K_{fs} V_f C_0 V_s}{K_{fs} V_f + V_s} \quad (\text{Ecuación II.B.12})$$

⁴ “Efecto aceite” (*oil effect*): efecto que favorece el movimiento de compuestos no polares hacia la superficie del agua con adición de sal.

- *Volumen de espacio de cabeza*

Cuando se expone la fase extractante al espacio de cabeza, la cantidad de analitos volátiles extraída es mayor para volúmenes de espacio de cabeza pequeños ya que la concentración de compuestos volátiles en él es mayor.

- *Agitación*

Favorece la difusión del analito desde la muestra a la fase extractante, o al espacio de cabeza, consiguiendo cinéticas más rápidas. Normalmente, el efecto de este parámetro es más acusado en las modalidades de muestreo directo que en espacio de cabeza.

- *pH de la muestra*

Como los analitos se extraen en la forma neutra, al igual que en LLE, el pH puede variar los equilibrios ácido-base afectando a la eficacia de extracción. A nivel práctico, se recomienda trabajar a ± 2 unidades del pK_a y evitar los pH extremos para no deteriorar la fibra, cuando ésta se introduce directamente en la muestra.

- *Adición de un disolvente*

La adición de un disolvente orgánico, normalmente metanol o acetonitrilo, aumenta la solubilidad de los analitos en agua reduciendo la eficacia de extracción. A efectos prácticos, si los analitos son muy lipofílicos, éste incremento de la solubilidad en agua evita, o al menos reduce, la adsorción sobre las paredes de vidrio del vial de extracción y, entonces, mejora la eficacia de extracción.

- *Derivatización del analito*

La derivatización puede tener lugar antes, durante o después de la extracción para mejorar la determinación cromatográfica en base a una mejor separación, más

información estructural en MS, mayor estabilidad térmica, etc. Además, cuando la derivatización se realiza antes o durante la extracción, puede aumentar la eficacia de extracción debido a que la nueva especie presenta mayor afinidad por la fase extractante o mayor volatilidad, lo que es importante para la extracción en modo HS [15]. La derivatización se usa principalmente para analitos polares, entre los que se encuentran algunas de las especies fenólicas consideradas en este trabajo.

1.3.3. Dispositivos comerciales

Para la llevar a cabo la SPME, se emplea el dispositivo comercializado por *Supelco*. Éste consiste en una fibra de sílice fundida, recubierta con un polímero adsorbente/absorbente (típicamente 0.5 μL) y situada en el interior de una aguja de acero inoxidable que forma parte de una jeringa (**Figura II.B.4**). Es un material inerte y estable a altas temperaturas. Sin embargo, las fibras tienen un tiempo limitado de vida, son relativamente frágiles y existe la posibilidad de contaminación entre análisis ya que una misma fibra se utiliza para el análisis de varias muestras. Otra limitación observada para algunos recubrimientos comerciales de SPME es que se hinchan en contacto con agua. A continuación, al retraer la fibra, el recubrimiento roza con la aguja protectora, lo que provoca su deterioro mecánico.

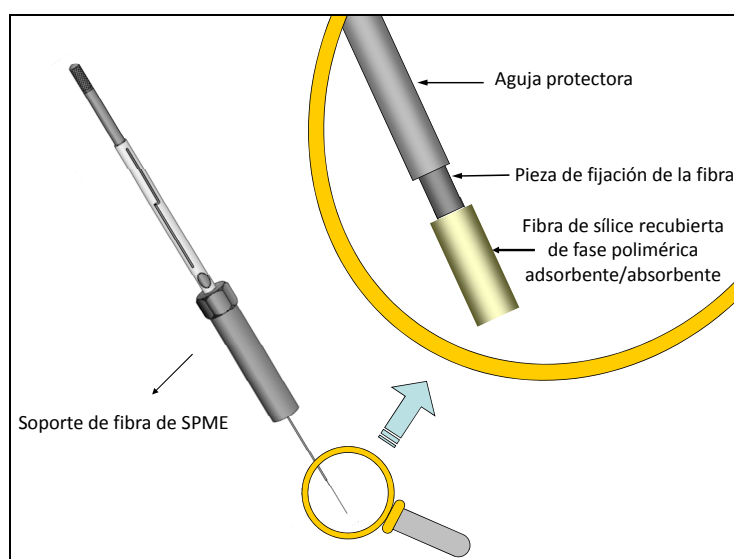


Figura II.B.4. Dispositivo comercial empleado en SPME [16]. [1718,19,20](#)

Además del soporte mostrado en la **Figura II.B.4**, existe la posibilidad de realizar la SPME de forma automatizada, por ejemplo con los automuestradores tipo CTC CombiPAL para lo cual se emplea un soporte de SPME modificado [17].

Este formato de microextracción es muy utilizado para el análisis tanto de matrices acuosas (agua, vino, orina, etc.) como sólidas (suelos, alimentos, etc.) y para un gran número de analitos. Por ejemplo, en el análisis ambiental se han determinado bencenos sustituidos, pesticidas, herbicidas, fungicidas, compuestos orgánicos volátiles, explosivos, nitrosaminas, aromas, parabenos, filtros solares y otros contaminantes emergentes [18-20]. En la **Tabla II.B.2** se muestran algunos ejemplos de aplicaciones en el análisis de agua.

Tabla II.B.2. Aplicaciones de SPME para el análisis de agua.

Analito	Volumen (mL)	Fibra	Modo de muestreo	Tiempo (min)	Método	LOQs (ng L ⁻¹)	Ref.
Pesticidas organoclorados	4	DVB/CAR/PDMS	DI	45	GC-MS	0.005-1.5	[21]
Insecticidas organofosforados	5	PDMS	HS	45	GC-MS	66-133*	[22]
PAHs	30	PDMS	DI	45	GC-MS	1-29	[23]
Haloanisoles y clorofenoles		DVB/CAR/PDMS	DI	60	GC-ECD	0.1-0.8	[24]
Ftalatos	5	CAR/DVB	DI	40	GC-MS	16-133*	[25]
Parabenos	10	PA	DI	40	GC-MS/MS	1-25	[26]
Retardantes de llama bromados	10	PDMS	HS	30	GC-MS/MS	0.008-0.190	[27]
Compuestos orgánicos volátiles	5	DVB/CAR/PDMS	HS	2	GC-MS	15-477	[28]
Fármacos antiinflamatorios	6	PDMS	HS	45	GC-MS	1-9.7	[29]

*LOQs estimados a partir de límites de detección.

Para llevar a cabo la SBSE, *Gerstel* comercializa los denominados *Twister*®. Son barras agitadoras de vidrio recubiertas de una capa de polidimetilsiloxano (PDMS), con un espesor entre 0.5 y 1 mm, (**Figura II.B.5**) en la mayoría de las aplicaciones.

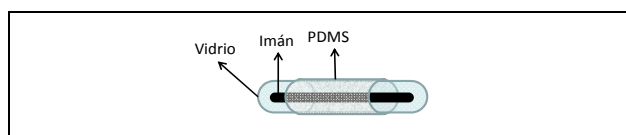


Figura II.B.5: Dispositivo comercial *Twister*® empleado en SBSE [14].

Al tener un volumen mayor de fase extractante (24-126 μL) que la fibra de SPME, la cantidad de analito extraído puede ser mayor y, como resultado, se puede conseguir mayor sensibilidad. Como principal limitación de SBSE señalar que sólo se comercializan *Twister*[®] de PDMS y más recientemente de Etilenglicol (EG)/silicona. Además, para realizar la desorción se requiere una unidad de desorción térmica (TDU) [30], lo que supone una inversión económica adicional en comparación a la desorción de las fibras de SPME. La alternativa es usar disolventes orgánicos en la etapa de desorción. La limitación de esta opción suele ser una disminución en los factores de concentración alcanzados. Desde que Batulssen y col. [31] utilizaron por primera vez en 1999 este tipo de dispositivos, el número de aplicaciones ha crecido mucho, desde análisis de aguas o fluidos biológicos hasta alimentos o suelos [14].

1.3.4. Dispositivos no comerciales

Debido al elevado coste de los *Twister*[®] y las fibras de SPME, en los últimos años han aparecido aplicaciones que utilizan formatos no comerciales. El polímero más utilizado es el PDMS o silicona, aunque también existen trabajos con otros materiales como polipropileno (PP) [32,33] y polietersulfona (PES) [34]. Las siliconas, constituidas por PDMS y aditivos, presentan las mismas ventajas que el PDMS puro, como estabilidad frente a altas temperaturas, disolventes acuosos y orgánicos. No obstante, debido a la presencia de estas impurezas/aditivos, el ruido de fondo en los correspondientes cromatogramas puede ser mayor que el registrado para fibras de PDMS y SBSE. En la práctica, este hecho no tiene porque presentar un problema por ejemplo si se trabaja con espectrometría de masas (MS) en modo SIM (monitorización de único ión) como sistema de detección.

Los polímeros adsorbentes de grado técnico están disponibles en diferentes formatos [35]: tubo, cuerda, láminas, etc. La extracción con estos materiales se rige por las mismas reglas y fundamentos teóricos que SBSE y SPME, siendo una de las principales ventajas su bajo coste (entorno a 0.05 € por pieza). Por lo tanto, se pueden considerar de un solo uso evitando problemas de contaminación cruzada entre muestras. Además, su tamaño y/o forma se puede ajustar a cada aplicación.

Popp y col. [36] concentraron PAHs de muestras de agua con un cilindro de polisiloxano (10 $\times$ 1 mm, 8 μL). Llevaron a cabo la extracción en modo directo durante 3

horas, seguida de la desorción en agua-acetonitrilo. Obtuvieron LODs de 0.1 a 1.2 ng L⁻¹ con LC-F. Montes y col. [37] también utilizaron cilindros de PDMS (20 × 2 mm, 31 µL) que colocaban en el espacio de cabeza del vial durante 150 min para la determinación haloanisoles en vino. Una vez transcurrido este tiempo, realizaban la desorción de los cilindros de PDMS en pentano que concentraban a 50 µL de isooctano. Consiguieron LOQs entre 0.5-1.5 ng L⁻¹ por GC-MS/MS.

Burger y col. [38] utilizaron una sonda con un tubo de PDMS (15 mm × 1 mm I.D., 1.75 mm O.D., 97 µL) en su extremo para la extracción de alcanos de una muestra de gas, aromas de plantas secas de té y PAHs de agua. Al finalizar cada extracción, realizaron la desorción térmica del tubo en el inyector modificado de un sistema de cromatografía de gases (GC) con detector de ionización de llama (FID).

Bichi y col. [39] utilizaron láminas de PDMS (15 × 4 × 0.5 mm, 30 µL) para el estudio de la variación de la composición de aromas emitidos por plantas. Realizaban el muestreo por contacto directo con la hoja de la planta o en HS. Estas láminas también fueron utilizadas por Sisalli y col. [40], colocándolas directamente sobre la piel, para estudiar la cantidad de sebo en su superficie. En este trabajo, realizaban la desorción térmicamente en un sistema GC-MS.

Prieto y col. [34] emplearon tubos de PES (2 mg, aprox. 1.77 µL) para la extracción de 15 contaminantes orgánicos, con valores de log K_{ow} comprendidos entre -0.07 y 6.88, de muestras de agua. Utilizaron dos tubos para cada muestra: un tubo se introduce en una alícuota de 75 mL conteniendo un 30% NaCl para favorecer la extracción de los analitos más polares y el otro tubo se introduce en otra alícuota, de igual volumen, conteniendo un 20% de metanol para favorecer la extracción de los analitos apolares. Después de 5 horas de muestreo, los adsorbentes fueron desorbidos conjuntamente en acetato de etilo. Consiguieron LOQs entre 0.1 y 85 ng L⁻¹ mediante GC-MS. Además, compararon el uso de PES con PDMS o PP observando que el primero proporcionaba mejores eficacias de extracción y menor efecto matriz, sobre todo para compuestos más polares.

1.4. Técnicas basadas en microextracción en fase líquida (LPME)

Si las técnicas basadas en microextracción en fase sólida se consideran una miniaturización de SPE, las diferentes modalidades de microextracción en fase líquida

(LPME) emulan a LLE. LPME es una técnica que surge en la década de los 90s [41] para solventar los problemas asociados a SPE y LLE, por ejemplo: alto consumo de disolventes y tiempo, y otros inherentes a SPME, como el sangrado del material polimérico a altas temperaturas o cuando se expone a ciertos disolventes orgánicos, así como el elevado coste de las fibras.

En LPME, la fase extractante, o aceptora, es un pequeño volumen de disolvente (acuoso u orgánico), del orden de microlitros, que concentra los analitos de una muestra de varios mililitros. En la práctica, la disolución aceptora se expone a la muestra en forma de gota suspendida, de dispersión/emulsión o a través de una membrana. Después de la extracción, se recoge y se inyecta directamente en un cromatógrafo de gases, líquidos o en un equipo de electroforesis capilar, sin necesidad de un paso adicional de desorción como en las técnicas de microextracción en fase sólida.

LPME amplía el rango de polaridades de la fase extractante en comparación con SPME, tiene un menor coste, el consumo de disolventes es muy pequeño y se obtienen altos factores de concentración. Sin embargo, en algunos formatos, la metodología empleada es ligeramente más complicada que en SPME, o menos robusta, lo que implica limitaciones a la hora de automatizar los métodos.

1.4.1. Microextracción con gota suspendida (SDME)

En la microextracción con gota suspendida (SDME), la fase aceptora es una microgota de disolvente orgánico (1-8 μL) sumergida en la muestra acuosa (1-5 mL), o expuesta al espacio de cabeza del vial que la contiene. El principal problema de SDME es la falta de reproducibilidad debido a la inestabilidad de la gota.

A nivel práctico, en la inmersión directa la fase aceptora debe ser un disolvente altamente inmiscible en agua. En espacio de cabeza, se pueden utilizar otros disolventes, incluso agua, con la precaución de que posean bajas presiones de vapor, para evitar la evaporación de fase extractante durante la extracción, y que sean compatibles con el sistema de determinación [41].

1.4.2. Microextracción líquido-líquido dispersiva (DLLME)

La microextracción líquido-líquido dispersiva (DLLME) fue introducida por Assadi y col. [42] en 2006. Una mezcla homogénea de un disolvente extractante (inmiscible en agua y, normalmente, más denso que ella) y un disolvente dispersante (miscible en agua y en el extractante) se añade a una muestra acuosa formándose una emulsión de pequeñas gotas. Como consecuencia, se establece un equilibrio entre las microgotas y la muestra en unos pocos segundos, debido a que la interfase entre ambas tiene una superficie prácticamente infinita. Después se centrifuga y se recoge la gota sedimentada de disolvente extractante (**Figura II.B.6**) [43].

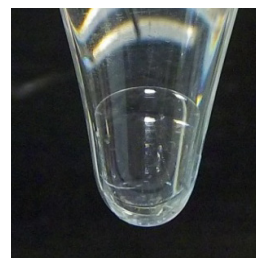


Figura II.B.6. Gota sedimentada en DLLME.

DLLME es una técnica rápida, simple, de bajo coste, con bajo consumo de disolventes y material, que proporciona alto poder de concentración y altas recuperaciones. Sus principales limitaciones son el uso frecuente de disolventes clorados como extractantes, no es adecuada para el análisis de compuestos polares o ionizables, y puede presentar problemas típicos de LLE cuando se aplica a muestras complejas que impidan conseguir una separación nítida entre fases después de la etapa de centrifugación.

1.4.2.1. Fundamento teórico

En DLLME, se define el factor de enriquecimiento (EF) como la relación entre la concentración en la gota (C_{sed}) y la concentración inicial en la muestra acuosa (C_0) para cada analito, según la ecuación II.B.13.

$$EF = \frac{C_{sed}}{C_0} \text{ (Ecuación II.B.13)}$$

La recuperación de la extracción (%R) se define como la cantidad de analito encontrado en la gota en condiciones de equilibrio (n_{sed}) y la cantidad inicial en la muestra (n_0) (ecuación II.B.14). Teniendo en cuenta la ecuación II.B.13, se deduce que en la

recuperación influyen los factores de concentración (EFs) y los volúmenes de gota y de muestra, V_{sed} y V_s respectivamente (ecuación II.B.15).

$$\%R = \frac{n_{sed}}{n_0} \times 100 \text{ (Ecuación II.B.14)}$$

$$\%R = \frac{V_{sed}}{V_s} \times EF \times 100 \text{ (Ecuación II.B.15)}$$

1.4.2.2. Parámetros que afectan a la eficacia de extracción en DLLME

- *Disolvente extractante*

Debe tener una densidad diferente a la del agua, ser insoluble en ella, poseer buen comportamiento cromatográfico y buena capacidad de extracción. Los más utilizados son el clorobenceno, tricloroetano, tetracloruro de carbono, disulfuro de carbono y cloroformo. Como alternativa, algunos autores utilizan agentes extractantes más seguros como los líquidos iónicos, recogiendo la gota sedimentada [44]. Otros, optan por utilizar disolventes menos tóxicos y menos densos que el agua, como hexanol o ciclohexano, recogiendo la gota que queda en la superficie, o como undecanol, dodecanol o hexadecano que solidifican por enfriamiento, facilitando la recolección de la fase extractante solidificada. Con excepción de los líquidos iónicos, la mayoría de estos disolventes no son adecuados para el análisis del extracto por cromatografía líquida (LC), utilizando fases móviles acuosas.

- *Disolvente dispersante*

Debe ser soluble en agua y en el extractante, como el metanol, acetona y acetonitrilo. Este disolvente ayuda a formar la emulsión de agente extractante. Regueiro y col. [45] prescindieron de este disolvente y utilizaron ultrasonidos para favorecer la emulsión. En este caso, esta técnica se podría considerar una variante de DLLME y es conocida como microextracción-emulsificación asistida por ultrasonidos (*Ultrasound-assisted emulsification-microextraction*, USAEME). Estos autores emplearon esta técnica para la extracción de fragancias sintéticas, ftalatos y lindano en agua. Consiguieron límites de detección (LODs) entre 6 y 133 ng L⁻¹ utilizando GC-MS como técnica de determinación. Pizarro y col. [46] también emplearon USAEME para la extracción de clorofenoles y cloroanisoles de vino. Consiguieron LODs de 1.9-4.9 ng L⁻¹ con GC-MS.

- *Volumen de extractante*

Debe ser lo suficientemente grande como para poder recoger y manipular la gota formada. Por otro lado, si es demasiado grande, la concentración de los analitos es menor y disminuye el factor de concentración (EF). Estos volúmenes varían entre 5 y 200 μL para la mayoría de las aplicaciones, dependiendo de la solubilidad del extractante en agua y del factor de concentración requerido [43,44].

- *Volumen de dispersante*

Ayuda a la formación de la emulsión de extractante/dispersante/agua, y cuanto más eficaz sea este proceso, mayor será la recuperación. Por ello es considerado de forma conjunta con el volumen del extractante durante la optimización de los métodos de DLLME. Los valores óptimos suelen variar entre 0.5 y 2 mL para volúmenes de muestra de hasta 20 mL [43,44].

- *Tiempo de extracción*

Es el tiempo que transcurre entre la adición de la mezcla dispersante/extractante a la muestra y la centrifugación. Su efecto es muy pequeño ya que se establece un equilibrio muy rápido entre las microgotas del extractante y la muestra.

- *Tiempo de centrifugación*

Debe ser lo suficientemente elevado para poder separar el extractante del resto del sistema. Normalmente con 5 min es suficiente.

- *Otros parámetros*

La adición de sal, el ajuste del pH o la adición de derivatizantes pueden incrementar la afinidad del analito por la fase extractante y, por lo tanto, conseguir mejores recuperaciones. A modo de ejemplo, Fattahi y col. [47] han propuesto realizar de

forma simultánea la extracción y la derivatización (con anhídrido acético) de clorofenoles en muestras de agua seguida de su determinación por cromatografía de gases (GC) con detector de captura electrónica (ECD). Con este trabajo [47], se abre la vía para combinar DLLME con procesos de derivatización en las etapas de preparación de muestra.

1.4.2.3. Combinación de DLLME con SPE

DLLME se ha aplicado a muestras ambientales de agua para la determinación de contaminantes emergentes y persistentes [43,44] y, en algunas ocasiones, a matrices más complejas (plasma, orina, miel, etc.). En este último caso, la interacción entre los componentes de la matriz y el disolvente orgánico puede impedir la correcta formación de la gota (separación de fases) después de la etapa de centrifugación. Para evitar este problema, algunos autores optan por diluir la muestra, incluso 10 veces, lo que provoca una pérdida de sensibilidad. Otros, emplean una etapa de SPE previa a DLLME para simplificar la matriz y/o aumentar los factores de concentración del método.

Para el análisis de clenbuterol en 1 g de tejido de cerdo, Liu y col. [48] realizaron una primera extracción con etanol y, posteriormente, para purificar el extracto emplearon cartuchos de intercambio catiónico eliminando así proteínas y fosfolípidos. Por último, realizaron una microextracción dispersiva con el extracto metanólico procedente del cartucho de SPE (dispersante) y 150 μL de 1,1,2,2-tetracloroetano (extractante) que añadieron sobre agua. El extracto fue evaporado y reconstituido con 20 μL de una disolución agua/metanol para su análisis por HPLC-UV. Consiguieron factores de enriquecimiento de hasta 62 veces y LODs de $0.07 \mu\text{g Kg}^{-1}$. En el caso de las muestras de vino, algunos autores han aplicado DLLME directamente al vino sin diluir. Otros, por ejemplo Montes y col. [49], observaron la formación de precipitados en el fondo del tubo cónico, especialmente en muestras de vino tinto. Para evitarlo, realizaron una SPE previa del vino, utilizando cartuchos de fase reversa Oasis HLB. El extracto de SPE se usó como dispersante en DLLME. Consiguieron factores de enriquecimiento para fungicidas entorno a 200 veces y LOQs de 30 a 120 ng L^{-1} con GC-ECD, partiendo de sólo 5 mL de vino. En general, en las referencias anteriores [48,49] se obtuvo una mejor selectividad utilizando la combinación SPE-DLLME frente al uso de SPE únicamente.

Por otro lado, SPE permite concentrar grandes volúmenes de muestra aumentando el factor de concentración cuando se combina con DLLME alcanzando LODs

más bajos (**Tabla II.B.3**) para matrices acuosas, sin necesidad de concentrar por evaporación el extracto obtenido del cartucho de SPE.

Tabla II.B.3. Comparación de SPE y DLLME con la combinación SPE-DLLME.

Analito	Método	LOD (ng L ⁻¹)	Volumen de agua (mL)	Ref.
Pesticidas organofosforados	DLLME-GC-FPD	3-20	5	[50]
	SPE-DLLME-GC-MS	0.038-0.23	500	[51]
	SPE-DLLME-GC-FPD	0.2-1.5	100	[52]
Clorofenoles	SPE-GC-ECD	3-110	75	[53]
	DLLME-GC-ECD	10-1000	5	[47]
	SPE-DLLME-GC-ECD	0.5-100	100	[54]
Herbicidas	DLLME-GC-MS	3-40	10	[55]
	SPE-DLLME-GC-MS	2-6	100	[56]

1.4.3. Extracción en fase líquida asistida por membranas

Para solucionar el problema de la inestabilidad de la gota observada en la modalidad de SDME se desarrollaron dos técnicas: una, utiliza membranas porosas y otra, no porosas, para separar el disolvente extractante de la muestra acuosa. Además, el uso de membranas evita la co-extracción de moléculas de gran tamaño molecular haciendo la función de filtro.

Cuando se utilizan membranas porosas, las disoluciones se encuentran a ambos lados de la membrana y están en contacto físico entre sí a través de los poros. Su configuración más conocida es la fibra hueca (HF), normalmente constituida por polipropileno. Estas fibras se caracterizan por un tamaño de poro de 0.2 μm , un 70% de porosidad, tener una longitud desde 1 a 10 cm y un diámetro desde 600 a 1200 μm [57]. En HF-LPME, se puede trabajar en un sistema de dos o tres fases, si la fibra hueca contiene en su interior la fase aceptora orgánica o acuosa, respectivamente, y en sus poros un disolvente orgánico a través del cual los analitos difunden hasta el interior. Además, el disolvente orgánico puede estar sólo impregnando los poros, o contenido en éstos y también en el interior de la fibra (sistema de dos fases). Por otro lado, la fibra hueca puede estar en disposición de U o con un extremo sellado [57] (**Figura II.B.7**).

Uno de los principales inconvenientes de la fibra hueca es que se corta o sella manualmente, lo que puede dar lugar a una reproducibilidad poco satisfactoria. Además, está la dificultad de controlar el volumen de disolvente que se introduce en el interior de la misma, debido a la resistencia que ofrece el aire que contiene.



Figura II.B.7. Dispositivos de extracción más comunes para la extracción mediante HF-LPME: en disposición U (A,B), en extremo cerrado (C,D) y con (A,D) o sin (B,C) disolvente en su interior.

Como alternativa a HF-LPME, surge la microextracción con disolvente asistida por membranas no porosas (MASE). Las membranas más utilizadas son de polietileno de baja densidad, o de polipropileno no poroso, con forma de bolsa en la configuración comercializada por *Gerstel* [58]. El disolvente se sitúa en el interior de esta bolsa a través de la cual difunden los analitos, desde la muestras hasta él. Tienen la ventaja de poder almacenar hasta 1 mL de disolvente orgánico (**Figura II.B.8**). Por otro lado, como los analitos deben difundir a través de un sólido, la cinética de extracción en MASE es más lenta que con la fibra hueca (HF-LPME). Para acelerar la transferencia de masa se recomienda trabajar a temperaturas elevadas (40-50°C). Además, las bolsas nuevas necesitan un acondicionamiento previo a su uso y, en general, MASE no se considera una técnica adecuada para analitos de polaridades altas y medias, con baja capacidad para difundirse a través de membranas apolares [59].



Figura II.B.8. Dispositivo comercial utilizado para extracción con disolventes asistida por membranas (MASE).

2. MUESTRAS SÓLIDAS

Al igual que sucede para las muestras líquidas, el objetivo de la preparación de la muestra para matrices sólidas es aislar y concentrar el analito de la matriz. En este caso, la interacción analito-matriz es más fuerte que la existente en muestras acuosas y se requieren condiciones más enérgicas para romperla, lo cual disminuye la selectividad del proceso y hace necesario etapas posteriores de limpieza, sobre todo en el caso de muestras con elevado contenido orgánico, por ejemplo, lodos y polvo.

A continuación, se presentan, de forma resumida, los fundamentos de diferentes técnicas de preparación de muestra aplicables a matrices sólidas. La discusión realizada se centra, sobre todo, en PLE y MSPD por ser las utilizadas en esta memoria de Tesis para la extracción de Tinuvines y fungicidas de sedimentos, polvo y suelos agrícolas.

2.1. Extracción sólido-líquido tradicional

Dentro de las técnicas tradicionales de extracción sólido-líquido, se encuentran la extracción Soxhlet y la extracción con agitación mecánica o por ultrasonidos. La técnica Soxhlet se basa en poner en contacto la muestra sólida, pulverizada con porciones nuevas de disolvente a una temperatura elevada. De esta forma, se obtienen excelentes eficacias de extracción. Los inconvenientes son que requiere elevados tiempos de extracción (6-24 h), grandes cantidades de disolvente (50-300 mL) y se necesita una etapa posterior de concentración del mismo.

La extracción sólido-líquido (SLE) clásica se basa en poner en contacto un disolvente orgánico con la muestra sólida, normalmente, pulverizada. La extracción se favorece con la agitación mecánica y/o mediante ultrasonidos. El extracto orgánico ha de separarse por centrifugación o filtración y concentrarse para su posterior análisis. Aunque el tiempo de extracción es menor, SLE muestra menor reproducibilidad que Soxhlet y elevado consumo de disolventes. SLE es una de las técnicas utilizadas para la extracción de fungicidas, una de las familias consideradas en esta Tesis, en suelo. En este caso, el disolvente debe poder disolver los conglomerados de la materia húmica, presente en el suelo, y liberar la fracción de fungicidas asociados a ella [60]. Normalmente, el suelo es sonicado con agua [61], disoluciones acuosas básicas [62-64], o conteniendo un

determinado porcentaje de metanol [65], durante 10-30 minutos. Así, Rial-Otero y col. [61] consiguieron recuperaciones de 79 a 115% para pirimetanil, vinclozolin, MET, CYP, PEN, PROC, FLUD y TEB, y de un 20% para diclofluanid que se hidroliza durante SLE, de muestras de suelo con disoluciones acuosas, seguidas de re-extracción líquido-líquido empleando acetato de etilo.

2.2. Extracción presurizada con disolventes (PLE)

La extracción presurizada con disolventes (PLE), o extracción acelerada con disolventes (ASE), se basa en el uso de disolventes a elevadas temperaturas (50-200°C) y presiones (1500-2000 psi) para extraer de forma rápida y eficaz analitos de matrices sólidas. Las altas temperaturas favorecen la difusión de los analitos, su solubilidad en el disolvente y la ruptura de la interacción analito-matriz. Mientras, las altas presiones mantienen el disolvente en estado líquido, a temperaturas por encima de su punto de ebullición, y ayudan a que penetre en la muestra. Bajo estas condiciones, se acelera la cinética de extracción en comparación con otras técnicas como Soxhlet, utilizando además menor volumen de disolvente (del orden de 20 mL por muestra).

Normalmente, la eficacia de extracción es elevada, los extractos no necesitan ser filtrados, como en SLE, y los dispositivos comerciales permiten la extracción simultánea o secuencial de varias muestras de forma automática. Obviamente, PLE también tiene inconvenientes como la inversión económica que supone la compra del equipamiento necesario para realizar la extracción, el tiempo que requiere el montaje de la celda de extracción y el lavado de las líneas del equipo entre muestras lo que aumenta el consumo de disolventes.

2.2.1. Aspectos prácticos

En la mayoría de las aplicaciones se utiliza un equipo comercializado por la marca Dionex[®] (**Figura II.B.9**) [66], que consta de una bomba para impulsar el disolvente a altas presiones a través de la celda de extracción que contiene la muestra, un horno para calentarla, un vial para recoger el disolvente y un gas de purga. Durante la extracción, la

celda se calienta en el horno hasta la temperatura deseada y se mantiene a una presión determinada, con ayuda de una válvula. Después, se recoge el disolvente en un vial colector, se bombea un cierto volumen de disolvente nuevo, conocido como *flush volume* (expresado en tanto por ciento del volumen de celda) y, tras el último ciclo de extracción, se purga el disolvente que hubiese quedado en la celda con nitrógeno presurizado.



Figura II.B.9: Extractor ASE 200 de Dionex.

La extracción se puede realizar en modo estático, dinámico o ambos. En modo dinámico, el disolvente pasa a través de la celda a un flujo controlado; mientras que en modo estático, se llena la celda y el disolvente permanece en su interior durante el tiempo de extracción. El modo dinámico es menos utilizado y presenta la desventaja de requerir mayores cantidades de disolvente y, en consecuencia, diluir el analito [93].

En lo referente a la muestra, es importante que esté seca ya que la presencia de agua reduce la eficacia de extracción para compuestos orgánicos por lo que se puede añadir sulfato sódico anhidro. Normalmente, la muestra es tamizada ya que un menor tamaño de partícula aumenta la superficie de contacto matriz-disolvente. Por este motivo, frecuentemente, la muestra es también dispersada con arena o tierra de diatomeas evitando su aglomeración.

Relativo a la celda, se colocan filtros de celulosa y/o lana de vidrio para evitar que se obstruyan las fritas y conexiones del equipo. Además, se rellena el volumen muerto de la celda con un material inerte (arena, tierra de diatomeas, etc.) para reducir el consumo de disolventes [67]. Además, en la propia celda se pueden introducir adsorbentes para realizar la extracción de los analitos y la purificación del extracto en una sola etapa [68].

2.2.2. Variables que afectan a la extracción en PLE

Las altas temperaturas ayudan a romper la interacción analito-matriz y favorecen que el disolvente penetre en la matriz ya que reducen su viscosidad. En consecuencia, se mejora la eficacia de extracción. Además, aumenta la velocidad de difusión, lo que permite extracciones más rápidas. Pero, también aumenta la cantidad de compuestos

co-extraídos proporcionando extractos más complejos y, una temperatura demasiado alta, puede provocar la degradación de compuestos termolábiles.

Las altas presiones (1500-2000 psi) ayudan a mantener el disolvente en estado líquido a temperaturas por encima del punto de ebullición y se fuerza al disolvente a penetrar en los poros de la matriz e interactuar con los analitos. Sin embargo, el efecto de la presión es, normalmente, poco significativo en la eficacia de la extracción [69]. De hecho, en la mayoría de optimizaciones de métodos de PLE suele trabajarse a un valor de presión constante y prefijado.

La polaridad del disolvente debe ser adecuada para favorecer la solubilidad de los analitos pero no la de la matriz. Los disolventes más utilizados en PLE son la acetona, diclorometano, hexano, metanol, o mezclas de éstos, e incluso agua [69,93]. La utilización del agua como extractante evita el uso de disolventes orgánicos, es barata y capaz de extraer compuestos polares y moderadamente apolares [69]. Evidentemente, el extracto es una disolución acuosa que requiere una etapa de concentración posterior mediante LLE, SPE, etc., sobre todo cuando se emplea GC como técnica de determinación. A nivel práctico, se pueden utilizar disoluciones ácidas o básicas débiles, en porcentajes inferiores al 10%, y se recomienda no utilizar ácidos y bases fuertes porque son corrosivos y pueden dañar las válvulas del equipo.

También, pueden afectar otros parámetros como el tiempo de extracción, el número de ciclos de extracción o el *flush volume*. Aunque, normalmente, su influencia en el rendimiento de la extracción es inferior a la que presentan el tipo de disolvente y la temperatura.

2.2.3. Extracción selectiva mediante etapas de limpieza en línea

PLE es una técnica que utiliza altas temperaturas y presiones para realizar la extracción, lo que se traduce en extractos complejos. Para eliminar las interferencias, muchos autores optan por realizar una limpieza, después de la PLE, mediante SPE [70]. Esta opción puede implicar el uso de adsorbentes tanto en fase normal como en fase reversa. En este caso, suelen usarse extractantes polares y miscibles con agua. Así, el extracto primario se diluye con agua antes de su paso a través del cartucho de SPE de fase reversa. Otros autores realizan la limpieza en línea, es decir, introducen un adsorbente

dentro de la propia celda de extracción. Esta última configuración fue la seleccionada en el trabajo desarrollado en esta Tesis para la extracción de Tinuvines en muestras de polvo. Así, se reduce el consumo de tiempo, disolventes y material.

En las **Tabla II.B.4** y **Tabla II.B.5** se muestran algunos ejemplos de aplicaciones de PLE selectiva, principalmente, en matrices ambientales.

Tabla II.B.4: Ejemplos de aplicaciones de PLE utilizando una etapa de limpieza en línea seguida de determinación por GC-MS.

Analito	Matriz	Limpieza	Temp. (°C)	Nº ciclos	Tiempo (min)	Disolvente	LODs (ng g ⁻¹)	Ref.
Pesticidas	5 g vegetales + 10 g sulfato sódico	12 g de carbón grafitizado	100	1	10	AcOEt	3-8	[76]
Estrógenos	0.5 g suelo	3 g sílice	60	1	5	Acetona/DCM (1:3, v/v)	0.06-0.37	[71]
Filtros UV	0.5 g lodo+ 2 g tierra de diatomeas	0.5 g carbón grafitizado	75	1	5	Hexano/DCM (8:2, v/v)	5.1-18 ^a	[77]
PBDEs	0.5 g polvo	0.5 g Florisil	100	3	5	Hexano/DCM (1:1, v/v)	0.1	[72]
Filtros UV	4-5 g sedimento + 2 g sulfato sódico	2 sílice y 2 g Cu en polvo	160	4	5	AcOEt/hexano (8:2, v/v)	1-6	[75]

a:LODs estimados a partir de LOQs, AcOEt: Acetato de etilo, DCM: Diclorometano, PBDEs: Difeniléteres polibromados

Tabla II.B.5: Ejemplos de aplicaciones de PLE utilizando una etapa de limpieza en línea seguida, principalmente, de determinación por GC-MS/MS.

Analito	Matriz	Limpieza	Temp. (°C)	Nº ciclos	Tiempo (min)	Disolvente	LODs (ng g ⁻¹)	Ref.
Fármacos y productos de cuidado personal	2.5 g pescado + 60 g sulfato sódico	14 g sílice	80	2	5	AcOEt/DCM (1:1, v/v)	0.5-120	[73]
PDBEs	1 g pescado + 3 g sulfato sódico	15 g sílice acidificada con H ₂ SO ₄ (44%, p/p)	100	3	5	Hexano	0.03-0.16	[74]
Parabenes y triclosan	0.5g polvo+1 g sulfato sódico + 3g Florisil	1 g Florisil	103	3	1	AcOEt	0.5-1.1 ^a	[80]
PBDEs	0.5 g polvo + 4 g Florisil	-	40	1	2	Hexano/DCM (1:1, v/v)	0.06-2	[78]
Nicotina y nitrosaminas ^b	0.5 g polvo +1 g sílica	-	100	3	10	AcOEt	2.5-15.8	[79]

a:LODs estimado a o partir de LOQs, b: determinación por GC-NCD (detector nitrógeno-quimioluminiscente), PBDEs: Difeniléteres polibromados, AcOEt: Acetato de etilo, DCM: Diclorometano

Los adsorbentes de *clean-up* utilizados en PLE son Florisil, sílice ácida, alúmina, C18, carbón grafitizado, etc. que se seleccionan en función de la naturaleza de los analitos, la complejidad de la muestra y el disolvente de extracción. Por ejemplo, Rodil y col. [75]

utilizaban cobre para eliminar el azufre elemental en la extracción de filtros ultravioleta (UV) de sedimentos. Para eliminar pigmentos de extractos vegetales, Tanaka y col. [76] colocaron una capa de carbón grafitizado en el fondo de la celda de PLE, mientras que los extractos tenían color verde oscuro con alúmina, Florisil o sílice. De la misma forma, Negreira y col. [77] consiguieron extractos incoloros de muestras de lodo. Además, observaron que necesitaban una etapa posterior de limpieza, utilizando cartuchos de PSA, para eliminar los ácidos grasos. No integraron esta etapa en el proceso de PLE porque observaron la pérdida de eficacia del PSA cuando se introducía en la celda de extracción. La diferencia entre el comportamiento del PSA en el modo *on-line* y *off-line* se atribuye a la elevada temperatura de la celda de PLE. En el análisis de PBDEs (Difeniléteres polibromados), PCBs (Bifenilos policlorados) y otros contaminantes persistentes, se utiliza sílice impregnada en ácido sulfúrico, en combinación con disolventes apolares, para la retención u oxidación de la grasa [68].

Por otro lado, algunos autores homogenizan la muestra con el adsorbente, en lugar y/o además de colocar una capa de éste en el fondo de la celda de PLE [78,79]. Así, Canosa y col. [80] homogenizaron la muestra con Florisil y también colocaron una capa del mismo material adsorbente en el fondo de la celda para la extracción de parabenos y triclosán en polvo. Adicionalmente, para eliminar los compuestos apolares, no deseables, aplicaron un programa de limpieza, previo a la extracción, utilizando hexano y un ciclo de extracción a 40°C durante 10 min. Los analitos fueron extraídos en una segunda etapa con un programa más enérgico (3 ciclos de 1 min a 103°C) y usando un disolvente más polar (acetato de etilo).

2.3. Dispersión de la matriz en fase sólida (MSPD)

La dispersión de la matriz en fase sólida (MSPD) es una técnica sencilla que se basa en mezclar la muestra con un adsorbente sólido, que actúa como abrasivo, en un mortero de ágata o vidrio [81]. De esta forma, se consigue romper la estructura de la muestra y una distribución homogénea de ésta alrededor de las partículas de adsorbente y, por lo tanto, una transferencia de masa eficaz que es responsable de las buenas recuperaciones obtenidas en condiciones suaves (presión atmosférica y temperatura ambiente) con un consumo moderado de disolventes. La mezcla muestra/dispersante se transfiere a un cartucho, normalmente de polipropileno, con una frita en el fondo, y se

coloca otra frita para comprimir la mezcla. Los analitos se eluyen con el disolvente adecuado por gravedad.

La extracción por MSPD se puede aplicar a matrices sólidas, semisólidas o viscosas [82], con poca manipulación de muestra. Es una técnica flexible, de bajo coste, no laboriosa y no necesita instrumentación adicional. No obstante, es difícil de automatizar.

2.3.1. Variables que afectan a la extracción

Las variables que pueden afectar a la extracción mediante MSPD son [81,82]:

- *Tamaño de partícula*, afecta al tiempo de elución. Con tamaños de partículas muy pequeños se necesita aplicar presión o vacío para tener un flujo adecuado de disolvente a través del cartucho o columna de MSPD.
- *Proporción muestra/dispersante*, se encuentra entre 0.25 y 1 en la mayoría de las aplicaciones.
- *Modificación química de la matriz*, por adición de agentes quelatantes, ácidos, bases, etc. A modo de ejemplo, para liberar hidrocarburos policíclicos aromáticos (PAHs) del suelo y mejorar las recuperaciones mediante MSPD, Pena y col. [83] añaden 500 µL de una disolución de hidróxido potásico saturada en metanol.
- *Naturaleza del adsorbente y disolvente de elución*, que afectan a la selectividad de la extracción. El adsorbente más utilizado es el C18. Teóricamente, las cadenas lipofílicas ayudan a disolver los componentes de la matriz, obteniendo extractos más limpios para matrices grasas [82]. Además, los adsorbentes basados en aminas primarias y secundarias, con base de sílice (PSA), son cada vez más utilizados para conseguir extractos libres de grasa. También se pueden utilizar adsorbentes de fase normal no enlazados, como la sílice, alúmina o Florisil. Su interacción con la muestra es diferente a la de las fases enlazadas, sólo se produce la adsorción de las moléculas orgánicas sobre las partículas. Su capacidad de adsorción puede variar en función de la cantidad de agua o carácter ácido o básico. Incluso se utilizan materiales inertes, como la arena o la tierra de diatomeas, como agentes dispersantes. Estos últimos, tienen la ventaja de ser materiales de menor coste que los adsorbentes anteriores, aunque no permiten incrementar la selectividad de la extracción. A nivel práctico, se añade sulfato sódico para eliminar el agua y favorecer la

extracción de los analitos lipofílicos y, en otras ocasiones, los adsorbentes en fase normal son desactivados con agua para que los analitos polares puedan eluirse más fácilmente.

Además de la polaridad de los analitos, el tipo del adsorbente también influye en la selección del disolvente, siendo los más utilizados el hexano, diclorometano, acetonitrilo, acetona o acetato de etilo.

- *Presencia de un co-adsorbente en el fondo del cartucho*, permite la purificación en línea al retener las interferencias. Se utiliza el C18, alúmina, Florisil, sílice y carbón grafitizado.

- *Lavado con disolvente previo a la extracción*, elimina las interferencias mientras los analitos permanecen retenidos en la mezcla dispersante/extractante o en el co-adsorbente.

De los comentarios anteriores, es obvio que el desarrollo de métodos de MSPD presenta un gran número de analogías con PLE, con la diferencia básica de que no se controlan ni la temperatura ni la presión de trabajo.

2.3.2. Aplicaciones de MSPD a muestras sólidas ambientales

Desde su introducción en 1989, MSPD se ha utilizado para la extracción de numerosos analitos principalmente en muestras biológicas y también para muestras de alimentos sólidas y semi-sólidas. Además, en los últimos años se ha demostrado también su aplicabilidad a matrices sólidas de tipo medioambiental. En la **Tabla II.B.6** se resumen las condiciones de trabajo más relevantes empleadas en estos estudios.

Como las condiciones de extracción en MSPD son suaves, en algunas aplicaciones no se realiza una limpieza adicional, simplemente se dispersa la muestra con un adsorbente y se eluyen los analitos. Para conseguir unas condiciones más enérgicas, Sanchez-Brunete y col. [84] introducen el cartucho de MSPD, con disolvente en su interior, en un baño de ultrasonidos para la extracción de pesticidas de muestras de lodo. Además de dispersar la muestra con alúmina, utilizaron cobre activado para eliminar el azufre elemental que interfiere en las determinaciones mediante GC. Esta última estrategia ha sido considerada dentro de las actividades realizadas en esta Tesis, aunque los resultados obtenidos no han sido satisfactorios.

Tabla II.B.6. Ejemplos de aplicaciones de MSPD a muestras sólidas ambientales.

Analito	Muestra + Dispersante	Co-adsorbente	Lavado	Elución	Detección	LODs (ng g ⁻¹ o ng mL ⁻¹)	%R	Ref.
Bactericidas	0.5 g polvo + 1.25 g C18	2 g Florisil	10 mL DCM	10 mL ACN	GC-MS/MS	0.2-0.8	80-114	[91]
Filtros UV	0.5 g polvo + 2 g C18	2 g Sílice	5 mL Hexano	4 mL ACN	GC-MS/MS	3-12	77-120	[92]
PAHs	0.5 g suelo + 1 g Florisil	-	-	6 mL Hex/Acet 1/1	LC-F	0.003-0.18	94-103	[83]
Fentoato	4 g suelo + 6 g Florisil + 3 mL agua	-	-	Hex/AcOEt 7/1	LC-UV	-	74-94	[85]
PBDEs	0.1 g suelo + 10 mg grafeno	50 mg Florisil	-	0.5 mL Hex/DCM 1/1	GC-ECD	0.006-0.21	69-126	[86]
Cloranfenicol	0.5 g suelo + 1 g MIP	0.2 g MIP	6 mL MeOH/agua 3/7	10 mL MeOH	LC-MS/MS	4.1	91	[87]
Estrógenos y similares	1 g sedimento + 4 g C8	alúmina ácida (cartucho)	5 mL MeOH	5 mL MeOH y Acet (2 veces)	UHPLC-MS/MS	0.12-9.7	-	[88]
Triclosán y metiltriclosán	0.5 g lodo o sedimento + 1 g diatomeas	1 g sílice ácida 15% H ₂ SO ₄	5 mL Hexano	10 mL DCM	GC-MS	6-7	99-113	[89]
Pesticidas	2 g lodo +3 g alúmina (3%) + 3 g Cu activado	2 g sílice	-	6 mL DCM (2 veces)	GC-MS	0.03-0.7	84-104	[84]
PAHs	0.2 g lodo +1 g Florisil	-	-	6 mL DCM/MeOH 9/1	LC-F	0.03-2	76-104	[90]

ACN: acetonitrilo, Hex: Hexano, DCM: diclorometano, Acet: acetona, MeOH: metanol, AcOEt: acetato de etilo

Para aumentar la selectividad de la extracción algunos autores combinan adsorbentes de fase reversa y de fase normal [91,92]. Negreira y col. [92] utilizaron el C18 como agente dispersante y la sílice como co-adsorbente de limpieza. De esta forma, los analitos son retenidos en la sílice mientras los compuestos apolares son eliminados con hexano. En una etapa posterior, para recuperar los analitos utilizaron disolventes más polares como el acetonitrilo.

Recientemente, se han utilizado materiales alternativos a los adsorbentes clásicos, como los MIPs [87] que proporcionan una elevada selectividad, o el grafeno [86] que produce una menor rotura de la muestra pero mejora la dispersión de algunos componentes de la matriz.

2.4. Otras técnicas de extracción

En la extracción con fluidos supercríticos (SFE), la muestra sólida se extrae con un disolvente en estado supercrítico. Cuando este disolvente supera la temperatura y presión críticas, sus propiedades son intermedias entre las de un gas y las de un líquido. Su densidad es elevada, como la de los líquidos, su viscosidad es similar a la de los gases y su coeficiente de difusión es intermedio entre gases y líquidos. El resultado es un fluido, con alto poder penetrante y de solubilización de los solutos, capaz de extraer los analitos de forma rápida. El disolvente más utilizado es el CO₂, que alcanza su estado supercrítico a 72.9 atm y a 31.3 °C. Se caracteriza por ser barato, no tóxico, no corrosivo, químicamente inerte y no polar; aunque, su polaridad puede ser modificada con la adición de acetona o metanol. Sin embargo, SFE muestra dificultad para la extracción de compuestos polares e iónicos y mayor complejidad técnica que los métodos convencionales [1]. Además, no aporta ventajas en relación a otras técnicas con similar grado de automatización, como el caso de PLE.

La extracción asistida por microondas (MAE) utiliza ondas de alta frecuencia, microondas, que calientan la muestra rápidamente pudiendo incluso llegar a sobrepasar el punto de ebullición del disolvente. En algunas aplicaciones, donde la muestra contiene un gran contenido de agua, como muestras vegetales, animales, etc., se usa un disolvente que no absorbe esta energía. Así, cuando se realiza la extracción, el agua provoca un sobrecalentamiento y, en consecuencia, la ruptura de las membranas celulares. De esta forma, el disolvente accede al interior de la muestra para extraer los analitos. MAE es una

técnica más rápida y con menor consumo de disolvente si la comparamos con Soxhlet. Pero, los extractos requieren ser filtrados y una etapa adicional de limpieza [93].

La extracción con disolvente a alta velocidad (HSSE) se basa en el contacto de una muestra sólida, homogenizada con sulfato sódico, con un flujo constante y relativamente elevado de un disolvente orgánico, como acetona, hexano, etc. El inconveniente principal de esta técnica es el elevado uso de disolventes, por ejemplo, si se trabaja a un flujo de 10 mL min^{-1} durante 30 min, el extracto resultante tendrá un volumen de 300 mL [94]. Teóricamente, equivaldría a un proceso de PLE en modo dinámico, aunque las diferencias entre ambas técnicas no aparecen suficientemente especificadas en la bibliografía.

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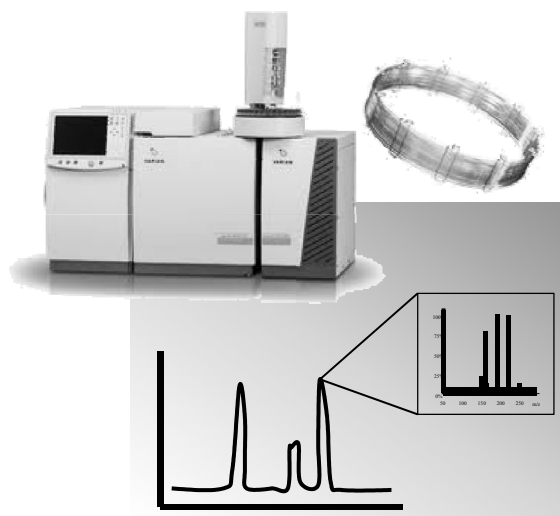
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II.C.

Técnicas de determinación



1. CROMATOGRAFÍA DE GASES (GC)

La cromatografía de gases (GC) es una técnica que permite la separación de los componentes de una mezcla debido a su diferente volatilidad y su distribución entre la fase estacionaria, normalmente líquida, y un gas portador inerte que actúa como fase móvil. Es el método de elección para la determinación de especies volátiles o semivolátiles (con puntos de ebullición inferiores a 450°C) abarcando un gran número de compuestos orgánicos y organometálicos. Su principal limitación es que los analitos deben ser térmicamente estables ya que, normalmente, se introducen en cabeza de columna en fase vapor. Para aumentar el campo de aplicación de GC, se recurre a la derivatización de las especies para mejorar su volatilidad o estabilidad térmica [1].

Existen cuatro modos de trabajo básicos para la inyección de muestras líquidas en columnas capilares: volatilización a temperatura constante con o sin división de flujo, también conocidos como *split* y *splitless*, respectivamente; inyección *on-column* y vaporización con programación de temperatura (PTV).

En los modos *split* y *splitless* se introduce un volumen de 1-2 µL de muestra líquida en el inyector del cromatógrafo que se encuentra a una temperatura constante y normalmente 50°C por encima de la temperatura de ebullición del componente menos volátil. Si se aplica un pulso de presión durante la inyección, la expansión del disolvente en el *liner* es menor [2] y, en consecuencia, disminuye el ancho de banda en cabeza de columna mejorando la eficacia de la separación. Además, como la transferencia de la muestra del inyector a la columna es más rápida, se reduce la probabilidad de pérdidas de analito por degradación o adsorción sobre el *liner* [3,4].

El modo *on-column* se puede utilizar para el análisis de una gran cantidad de analitos, incluso los térmicamente lábiles [1] ya que la muestra se deposita en la columna a baja temperatura. Pero, no es adecuado para muestras complejas o sucias al provocar la contaminación de la columna.

Para la inyección de grandes volúmenes de disolvente, se utiliza un inyector de temperatura programable o PTV. Es similar al inyector convencional *split/splitless* pero está equipado con un sistema de control de temperatura para calentarlo rápidamente o enfriarlo. En la mayoría de las aplicaciones se sigue el esquema que se muestra en la **Figura II.C.1**, conocido como *solvent vent*. En primer lugar, la muestra se introduce a baja temperatura con la válvula de *split* abierta [2]. El disolvente es eliminado con ayuda del

gas portador a través de la válvula de *split*, mientras los analitos se retienen en el *liner*. Después, se cierra la válvula de *split* y se transfieren los analitos a la columna al aumentar rápidamente la temperatura del inyector. Como la columna está a baja temperatura, los analitos se concentran en cabeza de columna para formar una banda estrecha. Finalmente, se vuelve a abrir la válvula de *split* para eliminar interferencias poco volátiles y se continúa la separación cromatográfica en la columna [1,2]. El éxito de la inyección de grandes volúmenes con un sistema PTV está condicionado por la diferencia entre el punto de ebullición del disolvente y del más volátil de los analitos considerados. Este ha sido el modo de inyección empleado en una de las aplicaciones recogidas en esta memoria de Tesis.

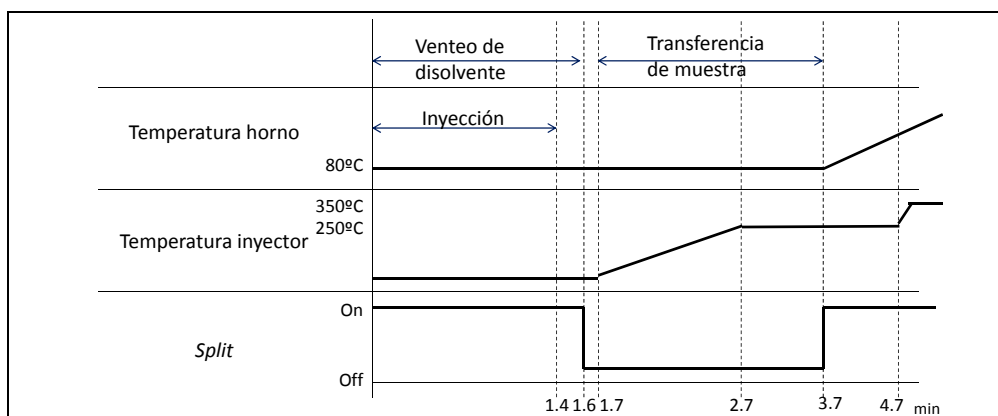


Figura II.C.1: Parámetros de un inyector PTV y temperatura del horno frente a tiempo.

Para la determinación de las tres familias de compuestos estudiadas en esta Tesis, Tinuvines, fungicidas y etilfenoles, los principales detectores acoplados a la cromatografía de gases, encontrados en la bibliografía, son el detector de captura electrónica (ECD), el detector de nitrógeno-fosforo (NPD), el detector de ionización de llama (FID) y el detector de espectrometría de masas simple (MS) o de espectrometría de masas en tándem (MS/MS).

En ECD se registra la disminución de la corriente eléctrica (creada por la emisión de partículas β por un isótopo de ^{63}Ni que colisionan con el gas portador creando una nube de electrones) debido a la llegada al detector de analitos que contienen átomos electronegativos, como por ejemplo halógenos mientras los compuestos con oxígeno, nitrógeno, fósforo o anillos aromáticos son prácticamente transparentes al detector.

En el FID se registra la señal eléctrica originada por los iones generados a partir de la combustión de los compuestos orgánicos, procedentes de la columna, en una llama de hidrógeno/aire. Es un detector universal, poco selectivo y su respuesta es proporcional a los átomos de carbono.

El NPD, o detector termoiónico, ha evolucionado desde los detectores de FID que se dopaban con sales alcalinas. La presencia de estos iones en la llama favorece la ionización de los compuestos que contienen nitrógeno y fósforo (aunque también azufre, boro y halógenos) y minimiza la ionización de los grupos C-H. Se consigue así una elevada selectividad hacia los compuestos con nitrógeno o fósforo en su estructura.

A continuación se describen con más detalle las combinaciones GC-MS y GC-MS/MS.

1.1. Cromatografía de gases acoplada a espectrometría de masas (GC-MS) y espectrometría de masas en tándem (GC-MS/MS)

Esta técnica híbrida consta de un cromatógrafo de gases conectado directamente al espectrómetro de masas, que a su vez está constituido por una fuente de ionización, un analizador de masas, un detector (generalmente un multiplicador de electrones) y un sistema de vacío. Tanto la adquisición como el tratamiento de datos se controlan mediante un ordenador. La columna cromatográfica se introduce directamente en el espectrómetro gracias al uso de columnas capilares que utilizan flujos lo suficientemente bajos, $1\text{-}2\text{ mL min}^{-1}$, para que el espectrómetro pueda mantener las condiciones de alto vacío necesarias para evitar el reagrupamiento de los iones [5].

Una de las fuentes de ionización más utilizadas es el impacto electrónico (EI). Los iones se generan por colisión de las moléculas, que salen de la columna, con electrones procedentes de un filamento de wolframio. El patrón de ionización y fragmentación es característico de cada molécula y es utilizado para su identificación. Existen en el mercado diferentes librerías que recopilan los espectros de EI de un gran número de compuestos, como la librería suministrada por el NIST (*National Institute of Standards and Technology*).

Estos iones son dirigidos hacia el analizador donde son separados y expulsados hacia el detector (un multiplicador de electrones) que genera una señal eléctrica que es

procesada y registrada para dar lugar al pico cromatográfico. En esta Tesis se ha utilizado una trampa de iones (IT) como analizador de masas. Las trampas de iones tienen un electrodo anular y dos colectores que forman una cavidad de sección hiperbólica. Al aplicar unas radiofrecuencias determinadas, unos iones quedan atrapados en su interior en una órbita circular estable mientras los iones de orbitas inestables salen de la trampa. Modulando estas radiofrecuencias, los iones pueden ser expulsados en orden creciente a su relación masa/carga (m/z) [6]. De esta forma, se obtiene un espectro de masas completo.

Además, la trampa de iones ofrece la posibilidad de trabajar en MS/MS sin coste adicional. Para ello, el ión precursor seleccionado se aísla en el interior de la trampa, se vuelve a fragmentar, por colisión con moléculas de helio, registrándose los iones producto generados. Así, se consigue mayor selectividad y, al reducir el ruido de fondo aumenta la relación señal/ruido, mayor sensibilidad. Esta mejora de los límites de cuantificación es más evidente cuanto mayor es la complejidad de los extractos considerados.

Otro tipo de analizador de masas muy utilizado es el cuádruplo (Q), consta de cuatro barras cilíndricas paralelas que actúan como electrodos. Las barras opuestas se conectan eléctricamente, un par está unido a un polo positivo y el otro par al negativo. Se aplican potenciales variables de corriente alterna en cada par de barras pero que están desfasados 180°C. Los iones se aceleran entre las barras y sólo aquellos que tengan un determinado valor de m/z incidirán en el detector ya que, viajaran a lo largo del analizador, mientras el resto de los iones serán desviados y eliminados por el sistema de vacío.

Con los analizadores cuadrupolares se puede trabajar en dos modos de adquisición de datos: el modo barrido completo (*full scan*) y el modo SIM (*Selected Ion Monitoring*). Este último, sólo registra las relaciones masa/carga (m/z) definidas previamente por el usuario, que suelen corresponder a los iones más abundantes del analito. Por lo tanto, las interferencias no modifican la línea de base mejorando la sensibilidad.

2. CROMATOGRAFÍA DE LÍQUIDOS (LC)

La cromatografía de líquidos (LC) es una de las técnicas más utilizadas para el análisis de una gran variedad de compuestos. Especies polares o ionizables, térmicamente inestables o de muy baja volatilidad se pueden separar sin necesidad de derivatizarlos mediante LC. Por ejemplo, Alder y col. [7] realizaron un estudio comparativo de GC-(EI)-MS y LC-(ESI)-MS/MS para 500 pesticidas y observaron que de los 500 pesticidas analizados, 135 no podían ser detectados por GC-MS y sólo 47 por LC-MS. Por otro lado, aun considerando que el número de especies determinables mediante LC es superior al que se puede abordar por GC, el carácter complementario de ambas técnicas es indiscutible.

Un cromatógrafo de líquidos consta de los siguientes elementos [5] (**Figura II.C.2**):

- *Bombas*: impulsan la fase móvil a través del sistema.
- *Inyector* (manual o automático): inserta la muestra, líquida, en la fase móvil.
- *Columna cromatográfica*: medio de separación de los componentes de la muestra.
- *Detector*: monitoriza el paso de los componentes de la muestra separados por la columna.
- *Registrador o software de cromatografía*: reciben la señal y la transforman en resultados (cromatogramas).

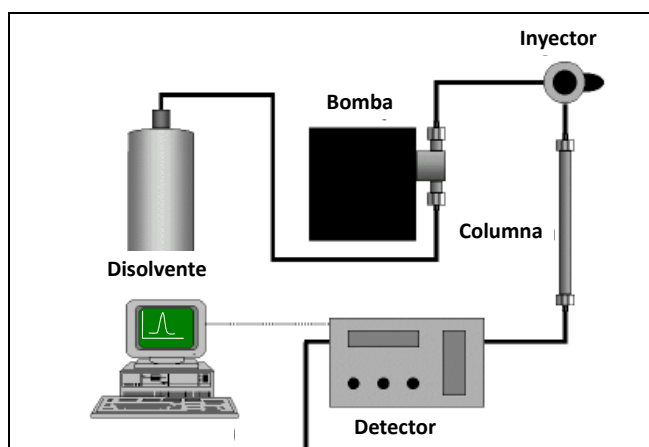


Figura II.C.2: Componentes de un cromatógrafo de líquidos.

El tipo de columna (dimensiones y relleno) influirá en la capacidad de separación de los compuestos, el tiempo de análisis, la presión del sistema y el volumen de inyección.

En la mayoría de las aplicaciones centradas en la separación de compuestos orgánicos, se han utilizado columnas de fase reversa, del tipo C18, con tamaño de partícula de 2-5 μm . En la cromatografía de líquidos de ultra eficacia (UPLC), el tamaño de partícula es inferior a 2 μm mejorando la sensibilidad, la resolución y reduciendo el tiempo de análisis a menudo a 10 min [8] con la desventaja de que el sistema alcanza mayor presión y, por tanto, existe mayor riesgo de bloqueos u obturaciones. En este caso (UPLC), es preciso reducir los volúmenes muertos en el cromatógrafo y disponer de suficiente velocidad de adquisición de datos para registrar los picos cromatográficos.

Las fases móviles más utilizadas en LC-MS son mezclas de metanol-agua y acetonitrilo-agua que pueden contener, o no, modificadores como acetato amónico o ácido fórmico compatibles con el uso de interfases de ionización a presión atmosférica empleadas en LC-MS.

2.1. Cromatografía de líquidos acoplada a espectrometría de masas en tándem (LC-MS/MS)

La detección en modo MS/MS permite la identificación de especies no resueltas por la columna de LC (reduciendo el esfuerzo cromatográfico) y aumenta la confianza en la identificación gracias a la selectividad proporcionada por MS/MS en modo MRM (*Multiple Reaction Monitoring*).

Los sistemas LC-MS requieren una interfase para que los compuestos separados por la columna se ionicen para su detección por MS. Las interfases basadas en técnicas de ionización a presión atmosférica (API), logran compatibilizar con éxito la eliminación de la fase móvil procedente de la columna y la ionización de los analitos con las condiciones de alto vacío necesarias para su determinación mediante MS. Las más empleadas en la actualidad son ionización por electrospray (ESI), ionización química a presión atmosférica (APCI) y fotoionización a presión atmosférica (APPI).

La ionización por electrospray (ESI), utilizada en este estudio, está ampliamente extendida al análisis de compuestos polares de peso molecular bajo-medio. El eluyente cromatográfico es nebulizado en una cámara a presión atmosférica bajo un campo eléctrico al pasar a través de la aguja de nebulización. El resultado es la formación de una niebla de finas gotitas cargadas. A medida que estas gotas pierden volumen, al evaporarse

el disolvente con ayuda de un gas caliente, la carga electrostática se concentra. Cuando las fuerzas de repulsión entre cargas superan las de cohesión, los iones son desorbidos en la fase gas y son atraídos hacia el capilar de entrada al analizador de masas.

Una vez formados los iones, éstos son conducidos hacia el analizador para ser separados. En esta Tesis se ha utilizado un sistema LC-MS equipado con un triple cuadrupolo (QqQ) donde el primer y tercer cuadrupolo actúan como analizadores de masas, mientras el segundo cuadrupolo funciona como celda de colisión (**Figura II.C.3**). El segundo cuadrupolo sólo enfoca a los iones. En él se introduce un gas inerte (Argón en nuestro caso) que choca con los iones. Como consecuencia, se produce una fragmentación que da lugar a los denominados iones producto. Este fenómeno se conoce como fragmentación inducida por colisión o CID (*collision-induced dissociation*).

Esta técnica permite operar en cuatro modos de adquisición [6]:

- MS/MS de iones producto (*product ion scan, fragment ion scan*). Sólo un ión precursor es seleccionado por Q1 al operar en modo SIM, mientras los iones producto son monitorizados por Q3 en modo barrido (*scan*).

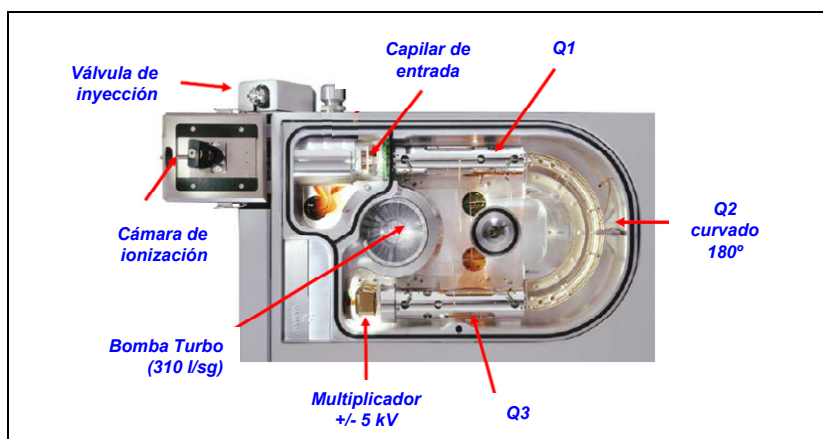


Figura II.C.3: Esquema del espectrómetro de masas QqQ utilizado en este estudio.

- MS/MS de iones precursores (*parent scan o precursor ion scan*). Q1 escanea sobre un rango de posibles precursores y Q3 monitoriza un único ión producto en modo SIM.
- Pérdida de fragmento neutro (*Neutral loss scan (NL)*). Consiste en seleccionar un fragmento neutro y detectar todos los compuestos que pierden este fragmento neutro.

Los barridos de Q1 y Q3 están sincronizados de tal forma que la diferencia entre Q1 y Q3 corresponde a la masa del fragmento eliminado.

- *Multiple reaction monitoring (MRM)*. Tanto Q1 como Q3 trabajan en modo SIM. Permite seleccionar uno o varios iones precursores y uno o varios iones producto. Es el modo de operación más sensible y el más utilizado en procedimientos cuantitativos como el desarrollo de esta Tesis para la determinación de residuos de fungicidas en vino.

3. BIBLIOGRAFÍA

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CAPÍTULO III

Metodología desarrollada

1. Determinación de Tinuvines



Los Tinuvines son compuestos usados como fotoprotectores en materiales plásticos, recubrimientos poliméricos, pinturas, embalajes de comida, lentes de contacto, etc. [1-3]. Se introducen en el medioambiente acuático por descargas de efluentes industriales, domésticos y de EDARs, encontrándose niveles de Tinuvines del orden de los ng L^{-1} en agua. La metodología existente al inicio de este trabajo, para la determinación de Tinuvines en muestras acuosas, utilizaba LLE como técnica de preparación de muestra seguida de GC-MS [4,5]. Además de requerir grandes volúmenes de muestra, los métodos anteriores consumen hasta 0.5 L de disolventes orgánicos consiguiendo LODs de 1 ng mL^{-1} en agua residual [4]. En esta Tesis, en primera instancia, se contempló la posibilidad de utilizar SPE para la extracción de Tinuvines en agua. No obstante, los compuestos más lipofílicos se adsorbían al material utilizado dando lugar a recuperaciones no cuantitativas. Como alternativa, se ha propuesto una metodología más eco-compatible, libre de disolventes orgánicos, al utilizar SPME como técnica de preparación de muestra. Con esta aproximación se ha conseguido detectar estas especies a niveles de los bajos ng L^{-1} en agua residual. Además, el uso de GC-MS/MS mejora la selectividad y sensibilidad de la determinación, en comparación con los métodos basados en GC-MS.

Debido a su elevado carácter lipofílico, los Tinuvines se encuentran asociados a la materia particulada, como sedimentos, donde se acumulan y pueden persistir durante años [6]. Las metodologías descritas en la bibliografía para esta matriz eran SLE y Soxhlet, seguidas de etapas de limpieza posteriores mediante LLE, SPE con adsorbentes en fase normal, o cromatografía de permeación de gel [7-9]. En esta Tesis se propone el uso de MSPD seguida de GC-MS/MS para la extracción y determinación de Tinuvines en sedimentos. MSPD permite integrar las etapas de extracción y de limpieza en único paso al incluir el adsorbente de limpieza en el fondo del cartucho de MSPD. La presencia de azufre en los sedimentos supuso un pequeño inconveniente ya que interfería en la determinación del Tinuvin P, utilizando un espectrómetro de masas de baja resolución. Como solución, se propone la adición de cobre en polvo a los extractos. Una vez optimizado el procedimiento analítico se aplicó a muestras reales, obteniendo los primeros datos relativos a la presencia de Tinuvines en muestras de sedimentos tomadas en Europa.

Además de producir una contaminación global en el medioambiente, los Tinuvines también se pueden encontrar en atmósferas interiores. Al igual que ocurre con otros aditivos, tales como retardantes de llama o ftalatos, los Tinuvines se liberan de los materiales plásticos de los que forman parte para adsorberse sobre las partículas de polvo

en suspensión o depositado. En el momento de abordar este trabajo, no se había encontrado ninguna publicación describiendo la presencia de Tinuvines en polvo. En esta Tesis se propone el uso de PLE, seguida de GC-MS/MS, como metodología analítica para investigar la presencia de Tinuvines en esta matriz. De nuevo, se integró la extracción y la limpieza en un solo paso al colocar el adsorbente de *clean-up* en el fondo de la celda de extracción.

A continuación, se muestran los esquemas de las metodologías desarrolladas para la determinación de Tinuvines en agua (**Figura III.1.1**), sedimentos (**Figura III.1.2**) y polvo (**Figura III.1.3**).

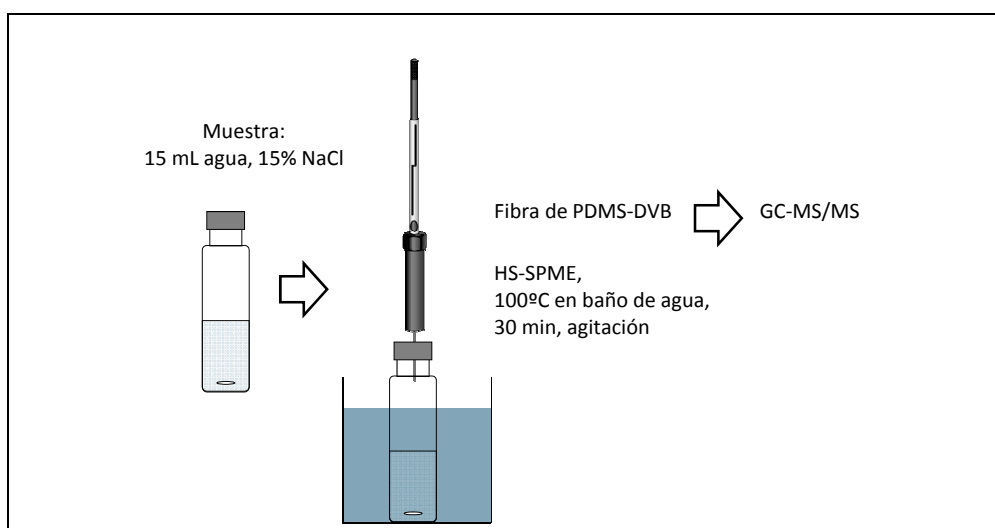


Figura III.1.1. Esquema de preparación de muestra para la determinación de Tinuvines en agua mediante SPME y GC-MS/MS.

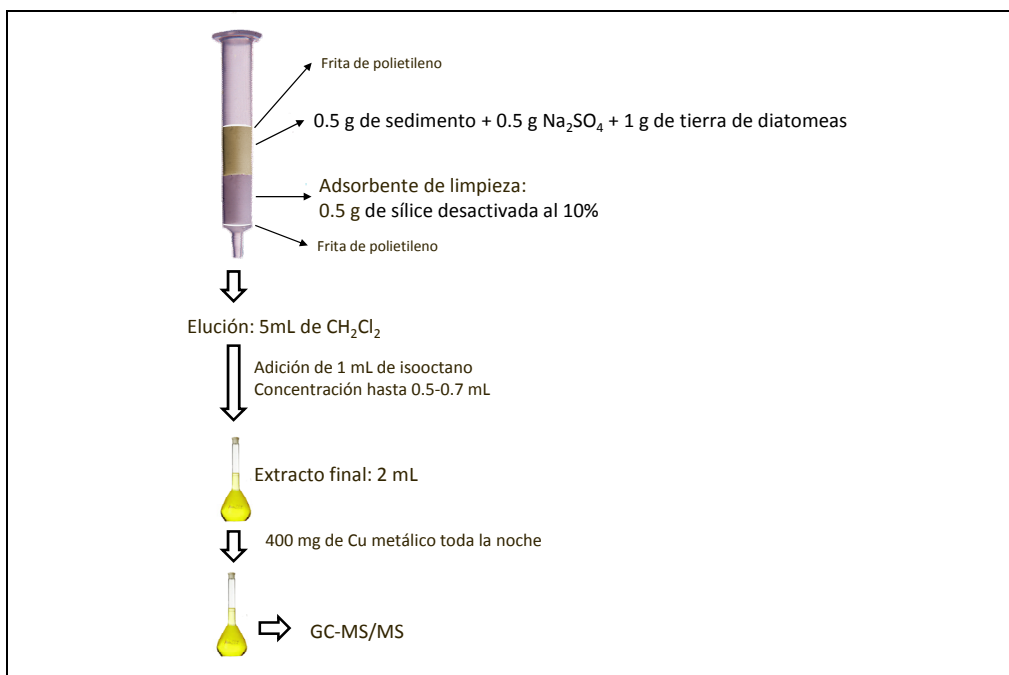


Figura III.1.2. Esquema de preparación de muestra para la determinación de Tinuvines en sedimentos mediante MSPD y GC-MS/MS.

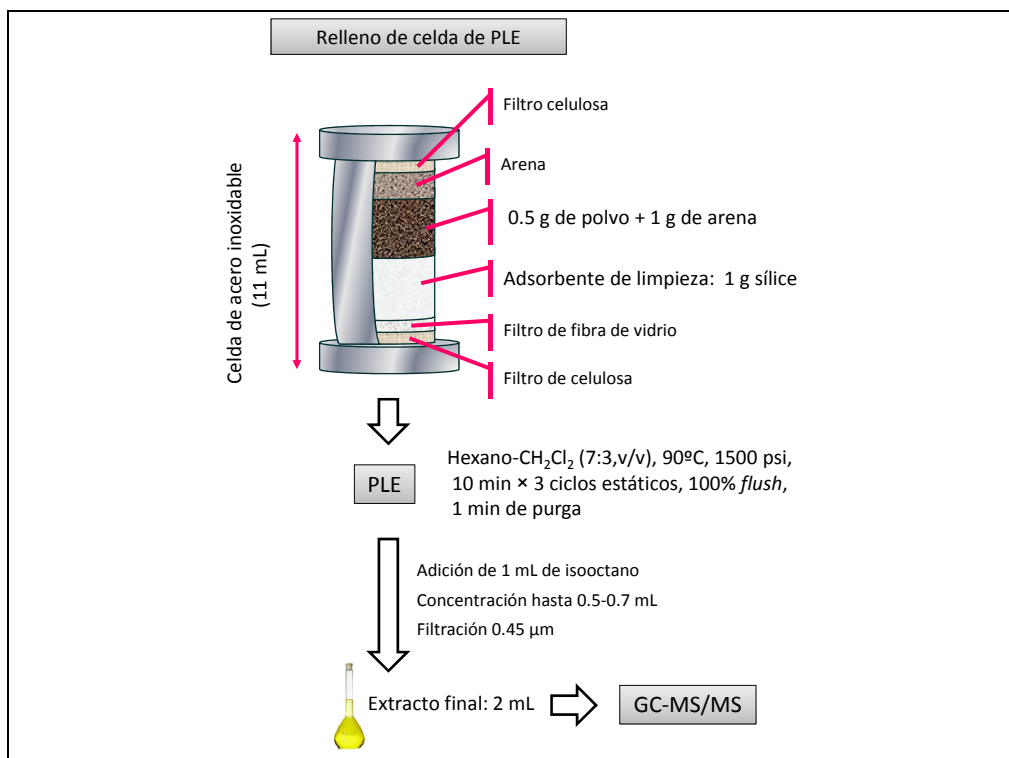


Figura III.1.3. Esquema de trabajo para la determinación de Tinuvin en polvo mediante PLE y GC-MS/MS.

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Publicación III.1.1:

**Headspace solid-phase microextraction followed by gas chromatography
tandem mass spectrometry for the sensitive determination of
benzotriazole UV-stabilizers in water samples**

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Headspace solid-phase microextraction followed by gas chromatography tandem mass spectrometry for the sensitive determination of benzotriazole UV-stabilizers in water samples

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Abstract

A sensitive procedure for the determination of five ultraviolet (UV) absorbers, belonging to the benzotriazole class, in environmental water samples is proposed. Analytes were first extracted and concentrated from the matrix and then selectively determined by gas chromatography in combination with tandem mass spectrometry detection. The high lipophilic character of some of the investigated species resulted in a strong trend to remain sorbed on solid surfaces, even after addition of considerable percentages of methanol (up to 30%) to water. Thus, minimizing sample handling during the enrichment step is mandatory in order to obtain acceptable accuracy and precision. Solid-phase microextraction (SPME), as sample preparation approach, fulfilled the above requirement and provided acceptable figures of merit for the determination of target species in environmental water samples, including raw wastewater. Optimization of SPME conditions showed that the combination of headspace extraction, with a sample temperature of 100 °C and addition of 150 mg of NaCl per milliliter of sample rendered the best compromise in terms of extraction efficiency for all species. Considering a sampling time of 30 min with a poly(dimethylsiloxane)-divinylbenzene-coated SPME fiber, limits of quantification below 2 ng L⁻¹ and relative standard deviations between 5 and 12% were achieved. Three of the five species included in this research were determined in raw wastewater with a maximum concentration of 57 ng L⁻¹ for the Tinuvin 326 UV absorber.

Keywords: UV-stabilizers, benzotriazoles, water samples, solid-phase microextraction, gas chromatography, mass spectrometry

1. Introduction

Environmental and toxicological aspects related to anthropogenic compounds designed to absorb ultraviolet (UV) wavelengths of solar radiation are receiving an increased attention. Most of the available information concerns the so-called UV filters, which are included in the formulation of solar screens and other personal care products applied directly on the human skin [1,2]. Some of the most often employed UV filters, e.g. certain cinnamates, camphors, and octocrylene, have been detected at relevant concentrations, not only in water samples but also in other solid matrices, such as sediments, sludge, and even in the tissues of fish living in recreational areas or aquatic environments affected by urban wastewater discharges [3-6]. In vitro studies indicate that some of the above compounds behave as endocrine disrupters [7].

In addition to personal care products, UV absorbers are also added to polymeric materials and paints (particularly car paints) to improve their stability and prevent light-induced degradation and yellowing [8]. These additives are referred to under the generic name of UV stabilizers. In terms of production and use, the 2-hydroxyphenyl derivatives of benzotriazole constitute one of the most important families of UV stabilizers [9]. In contrast to the polar benzotriazole species, its phenolic derivatives show medium to extremely high hydrophobic character and thus, as happens with the less polar UV filters, a potential to be accumulated in solid environmental matrices and even to be magnified through the trophic chain. Multiresidue studies, focused on aquatic environments heavily impacted by industrial sewage water discharges, demonstrated the accumulation and persistence of several benzotriazole-type UV stabilizers in sediments [10-12]. The recent research carried out by Nakata and coworkers in Japan has also confirmed the presence of these compounds in sediments as well as their bioaccumulation in the marine food chain [13]. However, with the exception of the above studies, the analytical determination of benzotriazole UV stabilizers and the assessment of their occurrence and fate in the aquatic media have been scarcely investigated.

Solid-liquid extraction approaches followed by gas (GC), liquid (LC), and even supercritical chromatography with UV, mass spectrometry (MS), and less often infrared spectroscopy detection have been used for the determination of microgram per gram levels of benzotriazole derivatives in host materials [8,14-16]. GC-MS and LC-MS are also suitable for the determination of trace residues of the above compounds in environmental samples [10,13]. However, in this case, they need to be combined with effective sample

preparation approaches providing high enrichment factors. As regards water samples, solid-phase extraction (SPE) and solid-phase microextraction (SPME) fulfill the above requirement; they are available in most laboratories, and they present just a moderate, or even null, consumption of organic solvents. In addition, their selectivity can be somehow controlled by selecting an appropriate sorbent or SPME coating. Both approaches have been also applied to the determination of UV filters in water samples [17,18]; however, as far as we could trace, their performance for the extraction and concentration of benzotriazole-type UV stabilizers has never been reported.

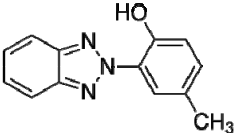
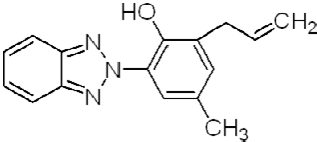
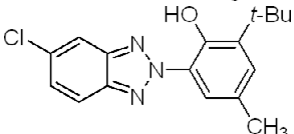
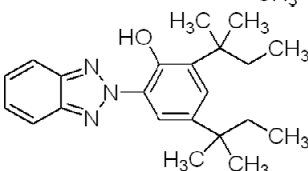
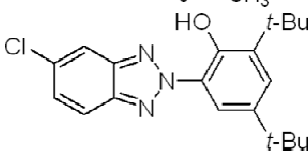
The basic aim of this research was to optimize and validate a procedure for the determination of five benzotriazole UV stabilizers in environmental water samples at the low nanogram per liter level. Headspace (HS) SPME was evaluated as sample concentration technique, and compounds were determined using GC in combination with tandem MS (MS/MS) detection. Parameters affecting the performance of SPME sample preparation (fiber coating, sample temperature, pH, ionic strength, stirring, sampling mode and time) are carefully evaluated together with determination steps, and preliminary data related to the presence of target compounds in sewage water are provided.

2. Experimental

2.1. Reagents, sorbents, and samples

HPLC-grade methanol, acetone, and ethyl acetate (trace analysis grade) were obtained from Merck (Darmstadt, Germany). Standards of 2-(2H-benzotriazol-2-yl)-4-methylphenol (Tinuvin P), 2-(2H-benzotriazol-2-yl)-4-methyl-6-(2-propen-1-yl)-phenol (Allyl-bzt), 2-(5-chloro-2H-benzotriazol-2-yl)-6-(1,1-dimethylethyl)-4-methyl-phenol (Tinuvin 326), 2-(2H-benzotriazol-2-yl)-4,6-bis(1,1-dimethylpropyl)-phenol (Tinuvin 328), and 2-(5-chloro-2H-benzotriazol-2-yl)-4,6-bis(1,1-dimethylethyl)-phenol (Tinuvin 327) were acquired from Aldrich (Milwaukee, WI, USA). Their chemical structures, molecular weights, pK_a values, octanol–water partition coefficients ($\log K_{ow}$), and water solubilities are summarized in **Table 1**. Individual stock solutions of each UV stabilizer (approximately $1000 \mu\text{g mL}^{-1}$) were made in acetone. Further dilutions and mixtures of target species

Table 1. Abbreviated names, CAS numbers, chemical structures and some relevant properties of selected UV-stabilizers.

Abbreviated name (CAS number)	Molecular weight (a.m.u.)	Structure	^a pK _a	^a Log K _{ow}	Mass solubility ^{a,b} (mg L ⁻¹)
Tinuvin P (2440-22-4)	225.25		8.2	4.3	18.0
Allyl-bzt (2170-39-0)	265.31		8.5	5.3	4.0
Tinuvin 326 (3896-11-5)	315.80		9.5	6.6	0.47
Tinuvin 328 (25973-55-1)	351.49		8.9	8.3	0.095
Tinuvin 327 (3864-99-1)	357.88		9.4	7.8	0.10

^a Values obtained from SciFinder Scholar Database, <http://www.cas.org/products/sfacad/>^b pH 7, 25°C

were prepared in the same solvent, when used to fortify water samples, and in ethyl acetate when employed to optimize GC–MS/MS detection conditions.

Oasis HLB (60 mg) SPE cartridges were acquired from Waters (Milford, MA, USA). A manual SPME holder and fibers coated with different polymers: poly(dimethylsiloxane) (PDMS, 100 µm film thickness), polyacrylate (PA, 85-µm film thickness), Carboxen-PDMS (CAR-PDMS, 75-µm film thickness), and PDMS-divinylbenzene (PDMS-DVB, 65-µm film thickness) were obtained from Supelco (Bellefonte, PA, USA). Before being used for first time, SPME fibers were thermally conditioned following conditions recommended by the supplier. Ultrapure (Milli-Q), river, and wastewater samples, obtained from the inlet and outlet streams of an urban sewage treatment plant, equipped with primary and activated sludge units, were employed throughout this study. Blind specimens of raw wastewater

were supplied by a local laboratory. River and wastewater samples were passed through a glass fiber filter and stored in the dark at 4 °C, for a maximum of 48 h, until analysis.

2.2. Sample preparation

Optimization of extraction conditions was carried out with spiked aliquots of ultrapure water. SPE assays were performed with 100 mL volume samples, containing variable percentages of methanol (up to 30%). SPME extractions were made in 22 mL glass vessels, sealed with a polytetrafluoroethylene (PTFE)-layered rubber septa, maintained at different temperatures, and containing variable amounts of sodium chloride (NaCl). Under final working conditions, SPME was selected as the extraction technique. A PDMS-DVB coated fiber was exposed to the HS of the vessel containing 15 mL of water, a 15% of NaCl (2.25 g), and a PTFE covered stirrer (10 mm × 3 mm). The sample vessel was equilibrated in a water bath at 100 °C for 5 min, and then the fiber was exposed to its HS for 30 min.

2.3. Determination

Analytes were selectively determined by GC-MS/MS, using a Varian (Walnut Creek, CA, USA) 450 GC instrument connected to an ion trap Varian 240 MS, furnished with an electron impact source in the external configuration mode. Separations were carried out in a Varian Factor Four, BP-5 type column (30 m × 0.25 mm i.d., d_f 0.25 μ m), operated at a constant helium flow of 1.2 mL min⁻¹. The GC oven was programmed as follows: 80 °C (held for 1 min), first rate 12 °C min⁻¹ to 245 °C, second rate 25 °C min⁻¹ to 285 °C (held for 10 min). The temperature of the injector was maintained at 270 °C, and standards, in ethyl acetate, were injected in the splitless mode, with the solenoid valve changing to the split position after 1 min. SPME fibers were desorbed at the same temperature, increasing the splitless as well as the initial oven temperature times from 1 to 3 min. Transfer line and electron impact ionization source and trap were set at 285, 180, and 120 °C, respectively. The helium dumping gas flow was fixed at 0.8 mL min⁻¹.

The mass spectrometer was operated in the electron impact ionization mode (70 eV). MS spectra were acquired in the m/z range from 70 to 400. The base peak in the spectra of each compound was isolated with a window of 3 a.m.u. and subjected to

collision-induced dissociation in order to obtain the corresponding MS/MS spectra. Under final conditions, compounds were detected in the MS/MS mode, applying an offset +200 V to the electron multiplier and a filament emission current of 50 μ A.

3. Results and discussion

3.1. GC-MS/MS parameters

Table 2 summarizes some relevant data related to GC-MS/MS detection conditions. The parent ion of each compound, appearing at m/z ratios of $[M]^+$ (Tinuvin P), $[M-15]^+$ (Allyl-BzT, Tinuvin 326, and Tinuvin 327), and $[M-29]^+$ (Tinuvin 328), was isolated in the trap and further fragmented using a resonant wavelength. Individual acquisition segments were defined for each species, except Tinuvin 328 and Tinuvin 327, whose chromatographic peaks were not baseline-resolved. Thus, their MS/MS transitions were recorded in the multiple reaction mode. Tinuvin 328 and 327 followed a similar MS/MS fragmentation pattern. It consisted of the replacement of one of the alkyl chains bonded to the phenolic ring by hydrogen to render the corresponding $[M-29-70]^+$ and $[M-15-56]^+$ product ions appearing at m/z ratios of 252 and 286, without any further noticeable transition. The other three analytes involved in this research showed a higher number of transitions and a more complex fragmentation pattern.

Table 2. Retention times, MS/MS detection parameters, linearity and instrumental LOQs of the GC-MS/MS system.

Compound	Ret. Time (min)	Parent ion (m/z)	Storage level (m/z)	Excitation voltage (V)	Product ions (m/z) ^a	Linearity (R^2)	LOQ (ng mL ⁻¹)
Tinuvin P	16.66	225	86	0.9	<i>196, 168</i>	0.9995	0.5
Allyl-bzt	18.30	250	95	1.1	<i>194, 132, 131</i>	0.9990	0.4
Tinuvin 326	19.47	300	114	0.95	<i>272, 154, 147</i>	0.9999	0.5
Tinuvin 328	20.09	322	123	1.1	252	0.9998	0.1
Tinuvin 327	20.15	342	130	1.05	286	0.9998	0.2

^a Italicized ions were used for quantification in the MS/MS detection mode.

Figure 1 presents the tentative structures assigned to the daughter ions in their spectra. Some of them can be only explained assuming the opening of the aromatic

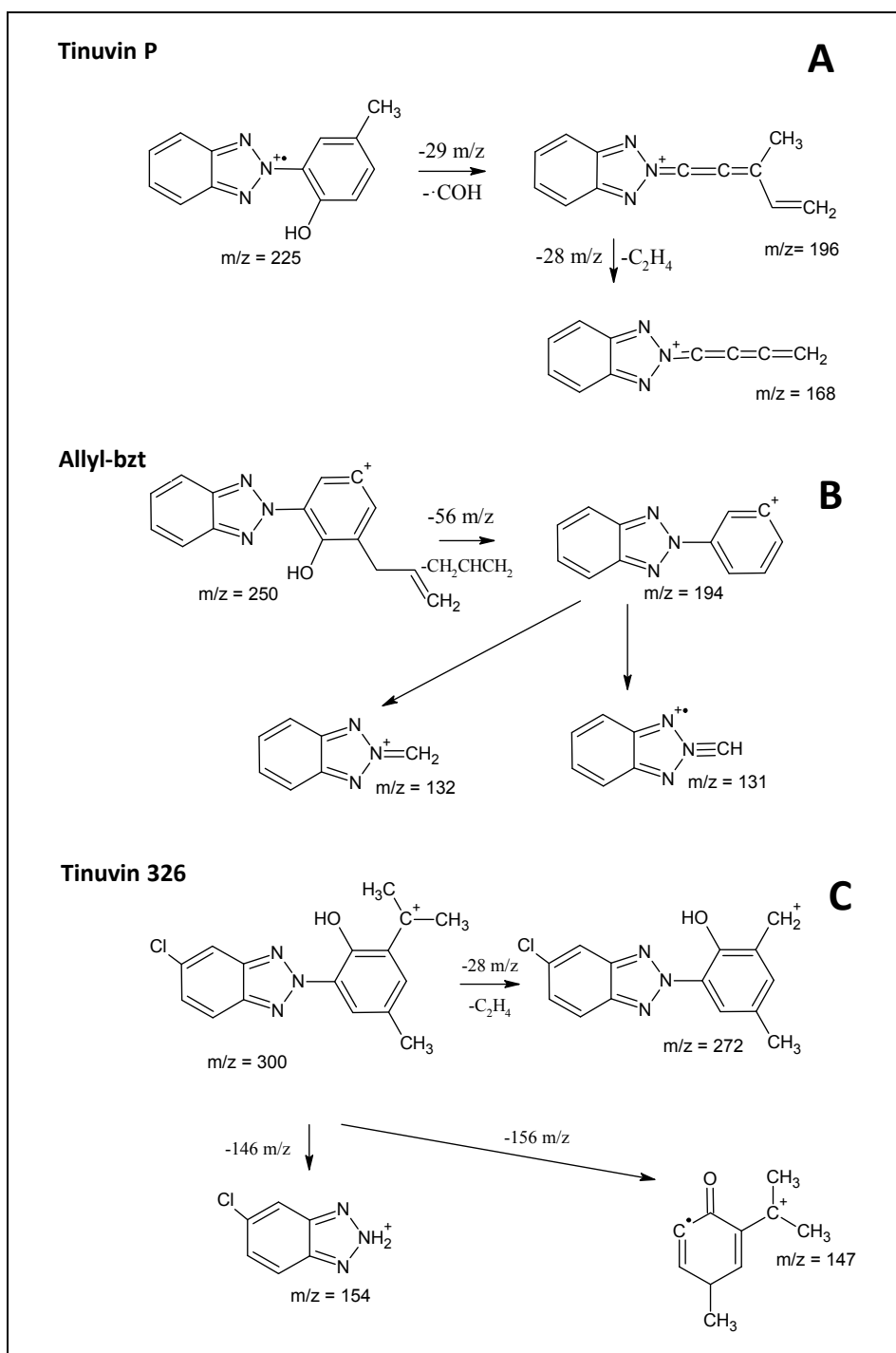


Figure 1. Proposed structures for product ions observed in the MS/MS spectra of Tinuvin P (A), Allyl-bzt (B) and Tinuvin 326 (C).

phenolic group. The linearity in the response of the GC-MS/MS system was evaluated with standards at six different concentrations in the range from 1 to 500 ng mL⁻¹. Within this interval, linear response plots (peak area versus concentration) were obtained for all compounds, with correlation coefficients (R^2) higher than 0.999. The instrumental limits of quantification (LOQs), defined as the concentration given a chromatographic peak with a signal ten times higher than the baseline noise, remained below 0.5 ng mL⁻¹, considering an injection volume of 2 µL, **Table 2**. The repeatability of the system for injections performed in the same day ranged from 2% to 4%, depending on the compound and the concentration level.

3.2. Sample preparation

3.2.1. Solid-phase extraction

SPE was initially chosen as concentration technique for the sensitive determination of target compounds in water samples. The OASIS HLB polymer, which has been previously tested for the concentration of different families of organic species with large differences of polarities [19], was used. Compounds were recovered from this sorbent with 2 mL of ethyl acetate. This extract was evaporated to 1 mL and injected in the GC-MS/MS system. Recovery values, corresponding to spiked (5 ng mL⁻¹) 100 mL aliquots of ultrapure water (pH 6), ranged from 70% for Tinuvin P and Allyl-bzt up to only 5% for the most hydrophobic Tinuvin 328 and 327. On the other hand, values above 90% were achieved when compounds were added directly to the sorbent, and then, the same volume of unspiked ultrapure water was passed through the cartridge. The above behavior suggests that the most lipophilic UV stabilizers are lost due to adsorption on glass beakers, containing the spiked samples, and PTFE connections with the SPE cartridge. In order to minimize this problem, increasing percentages of methanol were added to the spiked water samples. Even considering 30% of methanol, the recoveries obtained for Tinuvin 328 and 327 remained around 50% (data not shown); thus, the use of SPE as enrichment technique was discarded.

3.2.2. SPME optimization

Performance of SPME extractions is affected by a considerable number of variables, among them the coating of the SPME fiber, the temperature of the sample, and the sampling mode are some of the most important ones. Preliminary assays, using an exposure time of 30 min, showed that (1) the extraction efficiency of the CAR-PDMS sorbent was much lower than those provided by the other three fiber coatings considered in this study and that (2) increasing the temperature of the sample improved the yield of the extraction, particularly in the HS mode, data not shown.

In a second series of experiments, the effect of the above variables was simultaneously investigated using a mixed-mode $3^1 \times 2^2$ experimental factorial design. Extractions were carried out in 22-mL vessels, containing a magnetic stir bar and 15-mL aliquots of ultrapure water (pH 6) spiked with 1 ng mL^{-1} of compounds. The percentage of acetone in the SPME vessel was maintained below 1%. Again, the sampling time was limited to 30 min. After this period, fibers were desorbed at 270°C for 3 min. **Table 3** summarizes the domain of the design and the codified value for each factor. Responses (peak areas) obtained in the 12 experiments involved in the above design were processed using the StatGraphics Centurion XV software (Manugistics, Rockville, MD, USA) in order to obtain the main effects associated to each factor and also the two-factor interactions. The absolute value of the main effect for a given factor is proportional to the variation in the efficiency of the SPME process, when it changes from the low to the high level, within the domain of the design. A positive sign indicates an improvement in the yield of the extraction and a negative one the opposite effect.

Table 3. Experimental domain, standardized values for main effects and the interaction extraction mode-sample temperature.

Factor	Code	Level			Standardized value				
		-1	0	1	Tinuvin P	Allyl-bzt	Tinuvin 326	Tinuvin 328	Tinuvin 327
Fibre	A	PDMS	PDMS-DVB	PA	0.5	0.04	1.7	0.7	1.0
Mode	B	Direct	-	HS	-2.2	-2.4	14.6 ^a	12.0 ^a	19.4 ^a
Temperature (°C)	C	60	-	100	0.7	3.5 ^a	20.1 ^a	11.9 ^a	20.1 ^a
Samp. mode- temperature	BC	-	-	-	0.1	1.2	15.8 ^a	10.0 ^a	16.8*

^aStatistically significant factors and interactions at the 95% confidence level.

Numerical values of main effects and significant two-factor interactions are summarized in **Table 3**. The variable temperature (code C) played a positive effect on the efficiency of the SPME, being statistically significant (95% confidence level) for all species, except Tinuvin P. The extraction mode (code B) was also an important factor. Tinuvin 326, 328, and 327 were recovered in a significantly higher extension in the HS mode, whereas direct exposure of the fiber to the water sample was preferred for Tinuvin P and Allyl-bzt but without achieving the statistically significant boundary. Finally, the type of fiber (code A) was the least important of the three evaluated factors. As regards two-factor interactions, just the term BC (exposure mode-temperature) showed a significant importance for Tinuvin 326, 328, and 327. Main effects and interaction plots provided by the StatGraphics software for Tinuvin P and Tinuvin 328 are shown in **Figure 2**. Allyl-bzt followed the same trend as Tinuvin P, whereas Tinuvin 326 and 327 behaved similarly to Tinuvin 328. The curvature associated to the factor fiber (code A) in the main effect graph for Tinuvin P indicates that the highest response for this species corresponded to the

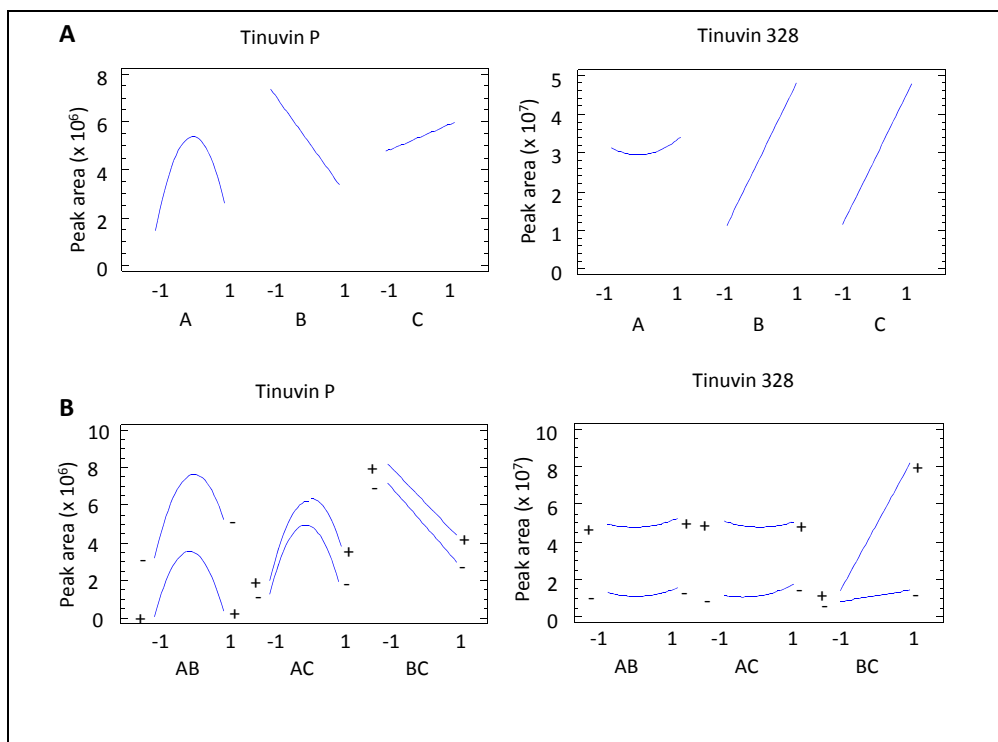


Figure 2. Main effects (A) and two-factor interactions (B) plots for Tinuvin P and Tinuvin 328. Values corresponding to -1, 0 and 1 levels for each factor are given in **Table 3**.

PDMS–DVB coating, whereas very little differences were noticed among the three fibers for Tinuvin 328, **Figure 2a**. The two-factor interaction plot for Tinuvin 328 shows that the effect of the temperature (C) depended on the sampling mode (B). When the fiber is dipped into the sample, little differences in the efficiency of the extraction were observed as function the temperature. On the other hand, in the HS mode, maintaining the sample at 100 °C was much more favorable than operating at 60 °C, **Figure 2b**.

On the basis of the above results, the PDMS-DVB fiber was chosen and extractions were carried out after equilibration of the samples at 100 °C. The effect of the sampling mode was further evaluated for samples without and with a 20% of NaCl. Increasing the ionic strength of water also increases the distribution coefficients of nonionic species between the coating of the SPME fiber and the sample, at the expense of slower extraction kinetics (particularly in the direct mode) due to a diminution in the diffusion coefficients of the analytes in the sample. The highest responses for all compounds were attained in the HS mode either without NaCl (Tinuvin 326, 327, and 328) or with 20% NaCl (Tinuvin P and Allyl-bzt). Taking into account this pattern, in addition to the higher selectivity of HS extractions, this mode was adopted for further experiments and the optimal concentration of NaCl was evaluated in detail. Obtained data are shown in **Figure 3**. For the large molecular weight and more lipophilic species (Tinuvin 328 and 327, log K_{ow} 8.3 and 7.8), the efficiency of the extraction underwent a steady decrease with the increase in the NaCl content in the sample. The effect observed for Tinuvin P (log K_{ow} 4.3) was just the opposite. Responses for Allyl-bzt and Tinuvin 326 increased until NaCl

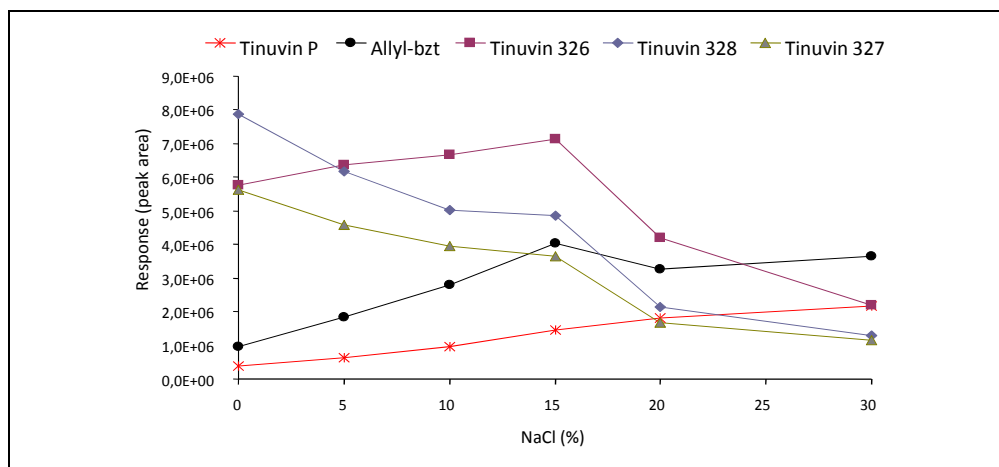


Figure 3. Effect of NaCl concentration (%) on the HS SPME responses of UV-stabilizers. Average values for duplicate extractions.

concentrations of 15% and then started to decrease. The depicted behavior matched with that previously reported for other families of compounds with variable polarities [20]. As a compromise solution, 15% of NaCl was added to water samples.

Stirring is another variable that potentially affects the yield of SPME extractions. Experimental data showed no significant differences between responses measured for Tinuvin P and Allyl-bzt in stirred (600 rpm) and nonstirred HS extractions of aliquots from the same sample. On the other hand, for Tinuvin 326, 328, and 327, higher extraction efficiencies were noticed for stirred samples, data not shown. Probably, stirring contributes to increase (1) the migration of these highly lipophilic species to the interface with the gas phase and (2) the surface of contact between the sample and the HS due to the formation of a vortex. The neat result was an improvement in the yield of the extraction for a fixed sampling time.

The presence of a phenol group in the structure of the benzotriazole UV stabilizers confers them a slightly acidic character; therefore, the performance of the HS SPME process might decrease in wastewater samples, showing pH values close to the pK_a of target species (between 8.3 and 9.5 units). Extractions carried out with samples adjusted at three different pH, 4, 6, and 9, showed no differences in the amount of compounds transferred to the fiber, data not shown. Consequently, the pH of real-life water samples was not modified, unless it falls outside of the above interval.

3.2.3. HS SPME kinetics

Figure 4 shows the time course plots of the HS SPME extraction. As observed, equilibrium conditions were not achieved for any compound after 2 h of sampling at 100 °C. For practical reasons, the sampling time was maintained at 30 min, assuming the corresponding loss of sensitivity at the expense of improving the productivity of the method. Obviously, automation of the microextraction step will make feasible the use of longer extraction steps, in order to reduce the overall LOQs of the method.

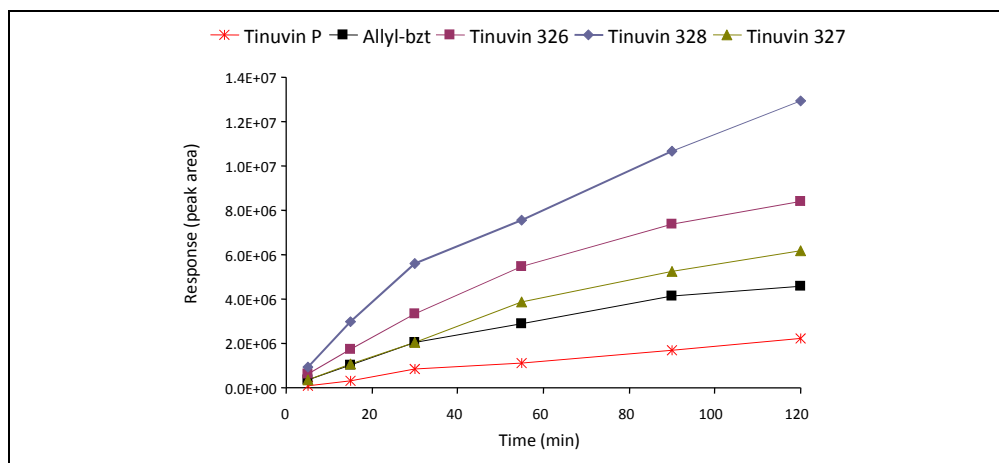


Figure 4. Time-course of the HS SPME at 100 °C.

3.2.4. Figures of merit of the HS SPME method

Data related to the performance of the optimized protocol are summarized in **Table 4**. Linearity was evaluated with samples spiked at seven concentration levels, from 2 to 1000 ng L⁻¹. The plot of peak area versus concentration fitted a linear response model with correlation coefficients (R^2) higher than 0.997. Repeatability of the HS SPME method ranged from 5% to 12%, for samples spiked at two different concentration levels (50 and 500 ng L⁻¹) and extracted within the same day ($n=4$ replicates). Reproducibility for extractions carried out in three consecutive days ($n=9$ replicates) remained between 9% and 11% for a concentration level of 100 ng L⁻¹. Overall LOQs, estimated considering the S/N ratios of chromatographic peaks in the lowest levels of the linearity study and the presence of trace amounts of some species in the procedural blanks (**Figure 5**), varied between 0.5 and 2 ng L⁻¹, depending on the compound. As commented in previous sections, target compounds are prone to remain sorbed on solid surfaces. The use of new vessels for each extraction is mandatory to avoid cross-contamination problems. Teflon-layered magnetic stir bars are another source of contamination between samples. Replacement of conventional stirrers by a disposable metallic screw (10 mm × 3 mm) wrapped with Teflon tape, minimizes cross-contamination problems. Finally, incomplete desorption of PDMS-DVB fibers may also be a source of carryover problems. Experimental data showed that between 0.6% and 1.5% of the compounds remained in the PDMS-DVB fiber after a desorption step of 3 min at 270 °C, with 1.2 mL min⁻¹ of helium flowing through the liner. Thus, before exposing the fiber to the next sample, it was kept at the

same temperature for 5 min under a helium flow rate of 20 mL min⁻¹. In addition to above considerations, procedural blanks must be processed systematically to avoid the report of false positives during application of the method to environmental water samples.

Table 4. Linearity (2-1000 ng L⁻¹), repeatability (n=4), reproducibility (n=9) and estimated limits of quantification (LOQs), defined for a signal to noise (S/N) of 10.

Compound	Correlation coefficient (R ²)	Repeatability (RSD, %)		Reproducibility (RSD, %)	LOQs (ng L ⁻¹)
		50 ng L ⁻¹	500 ng L ⁻¹	100 ng L ⁻¹	
Tinuvin P	0.9991	9.4	11.6	11.0	2
Allyl-bzt	0.9991	10.4	12.2	10.3	1
Tinuvin 326	0.9976	5.0	5.9	8.6	2
Tinuvin 328	0.9977	9.6	8.1	11.1	0.5
Tinuvin 327	0.9994	7.9	9.4	10.5	0.5

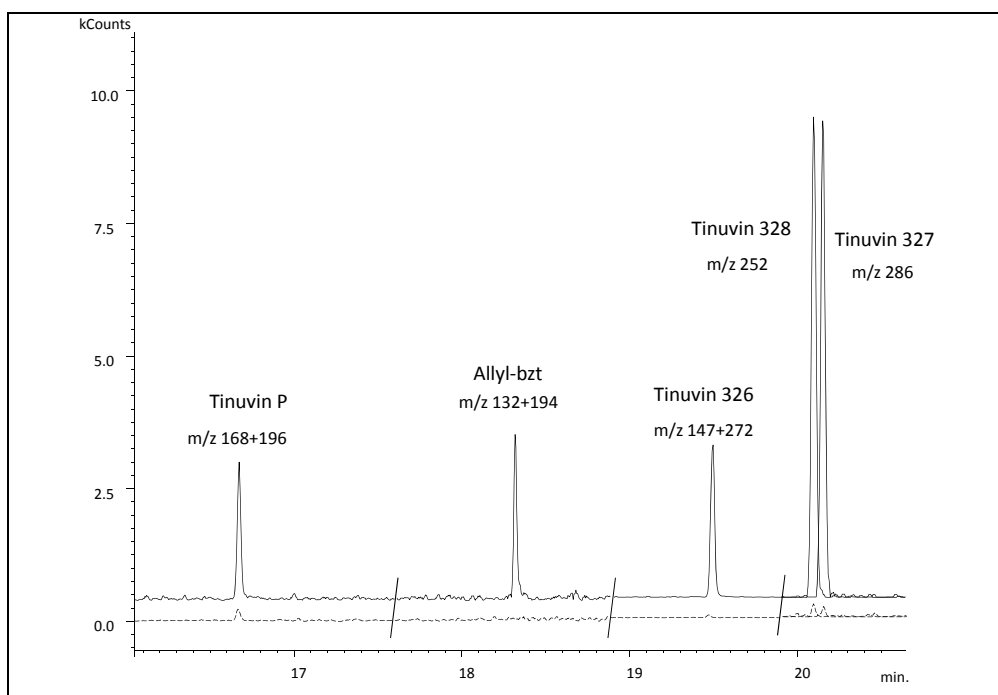


Figure 5. GC-MS/MS chromatograms corresponding to a procedural blank (dotted line) and ultrapure water spiked at 10 ng L⁻¹ per compound (solid line).

The effect of the sample matrix on the efficiency of the HS SPME process was evaluated with aliquots of river and urban wastewater. After filtration, each sample was divided in two aliquots. One was processed directly and the other spiked with selected compounds at 500 ng L⁻¹. Differences between responses attained for spiked and

nonspiked fractions of each sample were normalized to those obtained for ultrapure water. As shown in **Table 5**, similar efficiencies were noticed among ultrapure, river, and treated wastewater. On the other hand, significant enhancement and suppression effects were noticed for raw wastewater, depending on the considered UV stabilizer. Taking this behavior into account, external calibration against spiked aliquots of ultrapure water can be used as quantification technique to determine the presence of target compounds in river and treated wastewater; however, the standard addition methodology is required for their accurate quantification in raw wastewater.

Table 5. Effect of the type of water sample on the relative efficiency of the HS SPME method. Normalized responses to those achieved for ultrapure water. Addition level 500 ng L⁻¹, n=4 replicates.

Compound	Normalized values with standard deviations within parenthesis			
	Ultrapure water	River water	Treated wastewater	Raw wastewater
Tinuvin P	100 (8)	105 (7)	109 (5)	144 (14)
Allyl-bzt	100 (7)	108 (2)	107 (7)	92 (14)
Tinuvin 326	100 (6)	94 (9)	102 (8)	44 (2)
Tinuvin 328	100 (6)	89 (11)	91 (7)	30 (4)
Tinuvin 327	100 (3)	86 (12)	89 (6)	28 (1)

3.3. Real sample analysis

The developed methodology was used to investigate the presence of UV stabilizers in filtered aliquots of river water (n=3 samples), two samples taken in the inlet and outlet streams of an urban wastewater plant and four additional specimens of raw wastewater samples provided by a local laboratory. Compounds were not detected either in river or in treated wastewater. As regards raw wastewater samples, Allyl-bzt and Tinuvin 327 also remained under the LOQ of the method; however, the rest of species were noticed at concentrations up to 57 ng L⁻¹, **Table 6**. Chromatographic traces and MS/MS spectra for species quantified in sample code 2 (**Table 6**) are shown in **Figure 6**.

Table 6. Concentrations of UV-stabilizers in raw wastewater samples, n=3 replicates.

Code	Concentration (ng L ⁻¹) ± SD				
	Tinuvin P	Allyl-bzt	Tinuvin 326	Tinuvin 328	Tinuvin 327
1	16 ± 1	n.d.	39 ± 6	19 ± 2	n.d.
2	6 ± 1	n.d.	57 ± 9	16 ± 4	n.d.
3	16 ± 4	n.d.	19 ± 1	n.d.	n.d.
4	n.d.	n.d.	n.d.	1.0 ± 0.1	n.d.
5	5 ± 2	n.d.	3.5 ± 0.5	2.0 ± 0.1	n.d.

n.d. not detected

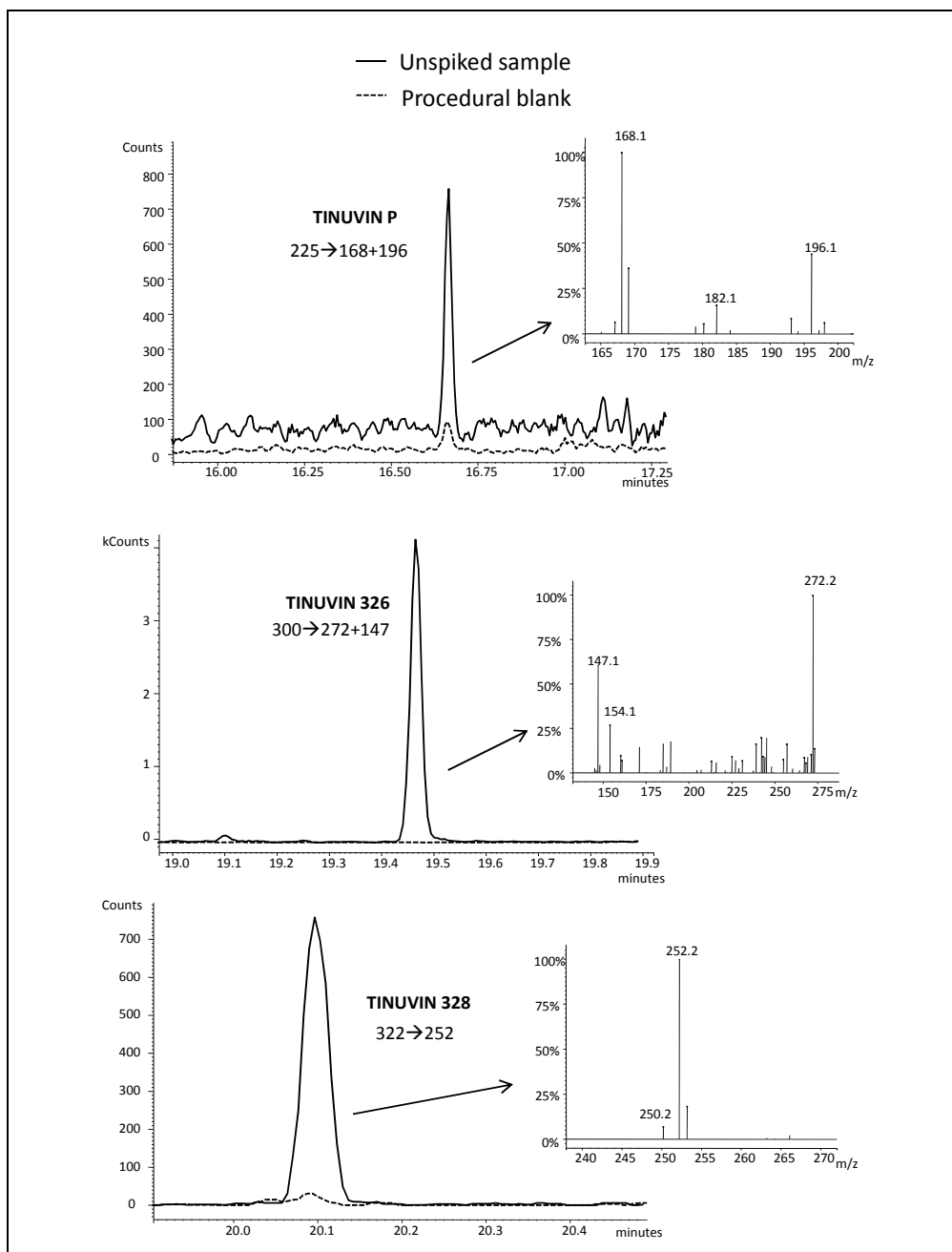


Figure 6. Chromatographic traces and MS/MS spectra of Tinuvin P, 326 and 328 in a non-spiked wastewater sample (code 2, Table 6).

4. Conclusions

GC-MS/MS and SPME have been combined for the first time for the sensitive and selective determination of five benzotriazole UV stabilizers in water samples. Performance of the sample preparation step is mainly controlled by the extraction temperature, mode, ionic strength, and sampling time. A suitable combination of the above factors, in addition to the use of a PDMS–DVB fiber, allows the effective extraction of selected compounds in the HS mode with the inherent advantages of higher selectivity and better stability of the SPME coating, in comparison to the direct exposure mode. Considering a sampling period of 30 min, similar to the duration of the GC separation step, LOQs in the low and even the sub-nanogram per liter level and acceptable precision were achieved. Trace amounts of some of the investigated species have been detected in filtered samples of raw wastewater samples. This fact confirms the contribution of sewage water to the introduction of UV stabilizers in the aquatic environment, where they are likely fixed to suspended particles and sediments. Further research is necessary to evaluate the suitability of the proposed HS SPME method for the analysis of unfiltered wastewater and even sludge, in order to obtain an estimation of the concentrations of benzotriazole UV stabilizers discharged through urban wastewater.

Acknowledgments

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Publicación III.1.2:

Matrix solid-phase dispersion followed by gas chromatography tandem mass spectrometry for the determination of benzotriazole UV absorbers in sediments

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Matrix solid-phase dispersion followed by gas chromatography tandem mass spectrometry for the determination of benzotriazole UV absorbers in sediments

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Abstract

A cost effective and low solvent consumption method, based on the matrix solid-phase dispersion (MSPD) technique, for the determination of six benzotriazole UV absorbers in sediments is presented. Sieved samples (0.5 g) were first mixed in a mortar with a solid sorbent and then transferred to a polypropylene syringe containing a layer of clean-up co-sorbent. Analytes were eluted with a suitable solvent and further determined by gas chromatography with tandem mass spectrometry (GC-MS/MS). Under final conditions, diatomaceous earth and silica, deactivated to 10%, were used as inert dispersant and clean-up co-sorbent, respectively. Analytes were recovered using just 5 mL of dichloromethane and this extract was concentrated and exchanged to 1 mL of isoctane. Further removal of co-extracted sulphur was achieved adding activated copper powder to final extracts, which were stored overnight, before injection in the GC-MS/MS system. The accuracy of the method was assessed with river and marine sediment samples showing different carbon contents and spiked at different concentrations in the range from 40 to 500 ng g⁻¹. Recoveries varied between 78% and 110% with associated standard deviations below 14%. The limits of quantification of the method stayed between 3 and 15 ng g⁻¹. Levels of target compounds in sediment samples ranged from not detected up to a maximum of 56 ng g⁻¹ for Tinuvin 328.

Keywords: Benzotriazole UV absorbers, sediment, matrix solid-phase dispersion, gas chromatography-mass spectrometry

1 Introduction

Tinuvin (2-hydroxyphenyl benzotriazole derivatives) are high volume production chemicals, which are employed as additives in paints, coatings, adhesives, polymeric surfaces, food packing films and construction materials [1-3]. Similar to the UV filters incorporated in personal care products and sunscreens, Tinuvin are sun-blocking agents, which prevent light-induced degradation reactions of host materials. Although some previous monitoring studies had confirmed the accumulation and persistence of these hydrophobic compounds in sediments collected in the vicinity of chemical manufacturing plants [4-5], the global fate of Tinuvin in the environment received scarce attention until the recent findings of Nakata's group. In a first work, Nakata and co-workers reported the presence of Tinuvin 320, 326, 327 and 328 in muscle and livers from different aquatic organisms as well as in sediments from several rivers in Japan, at concentrations ranging from the low ng g^{-1} to 300 ng g^{-1} [6]. In a further work, they also found Tinuvin 327 and 328 residues in blubber from marine mammals [7]. The estimated bio-concentration factors of both species were similar to those reported for well-known persistent chemicals, such as hexachlorocyclohexane [7]. After these early works, other authors have confirmed the presence of Tinuvin in fish from Manila Bay [8] sediments and sludge samples obtained in China and USA [9], and other geographic areas in Japan [10]. In Europe, to the best of our knowledge, the existence of Tinuvin residues in sediment and biota samples has not been reported, yet; however, their detection at trace levels (low ng L^{-1}) in sewage water [11] and dust from indoor environments [12] points out to the world wide use of these light stabilizers.

From the perspective of analytical chemistry, most Tinuvin are amenable to gas chromatography-mass spectrometry (GC-MS) and GC-tandem mass spectrometry (MS/MS) determination [6,12], after extraction and concentration from environmental matrices. In the case of sediments, only conventional solid-liquid extraction and Soxhlet, followed by additional time and solvent-consuming clean-up steps, based on gel permeation and/or column purification, have been proposed as sample preparation techniques [6,9-10]. Although effective in terms of attained recoveries, the above approaches generate a large amount of organic wastes and require dedicated operators. Thus, development and validation of alternative sample preparation strategies, showing reduced costs, higher sample throughput and improved sustainability, is a challenging issue previously to the design of survey campaigns aiming to study the spread of these compounds in the environment.

Among modern sample preparation techniques for organic compounds determination, matrix solid-phase dispersion (MSPD), first reported by Barker et al. [13], combines some unmatched properties such as low cost (dedicated instrumentation is not required), limited consumption of organic solvents, use of mild extraction conditions and possibility to integrate extraction and purification in the same step. The fundamentals and the most relevant applications of the technique have been revised recently [14-16]. In brief, in MSPD samples are first mixed and dispersed with a suitable sorbent and then packed in a disposable polypropylene cartridge, or syringe, which may contain a layer of the same, or different material as clean-up sorbent to retain co-extracted interfering species. Analytes are recovered with a small volume of an organic solvent, which ideally should be compatible with the determination technique. MSPD was initially designed to deal with semi-solid food and biological samples; nevertheless, during last years, a relevant number of successful applications have also been proposed for solid environmental matrices, such as soils, sediments and freeze-dried sludge [17-19].

In this research we investigate the suitability of the MSPD technique, followed by GC-MS/MS, for the sensitive determination of six Tinuvin UV absorbers in sediment samples. The effect of different experimental parameters, such as dispersant and clean-up sorbents and elution solvent in the efficiency of the extraction process was thoroughly evaluated using river and marine sediment samples with different carbon contents. After validation, the proposed method was applied to investigate the existence of Tinuvin residues in several sediment samples.

1. Experimental

1.1. Solvents, standards, sorbents and material

n-Hexane, dichloromethane, ethyl acetate and isooctane for trace analysis grade were obtained from Merck (Darmstadt, Germany). Standards of 2-(2H-benzotriazol-2-yl)-4-methyl-phenol (Tinuvin P), 2-(2H-benzotriazol-2-yl)-4-methyl-6-(2-propen-1-yl)-phenol (Allyl-bzt), 2-(5-chloro-2H-benzotriazol-2-yl)-6-(1,1-dimethylethyl)-4-methyl-phenol (Tinuvin 326), 2-(2H-benzotriazol-2-yl)-4,6-bis(1,1-dimethylpropyl)-phenol (Tinuvin 328) and 2-(5-chloro-2H-benzotriazol-2-yl)-4,6-bis(1,1-dimethylethyl)-phenol (Tinuvin 327) were acquired from Aldrich (Milwaukee, WI, USA). 2-(2H-benzotriazol-2-yl)-4,6-bis(1,1-

dimethylethyl)-phenol (Tinuvin 320) was provided by Dr. Ehrenstorfer GmbH (Augsburg, Germany).

Stock solutions of each compound were dissolved in acetone. Further dilutions and mixtures of target analytes were prepared in acetone and isooctane. Standards in acetone were used to fortify sediment samples employed during optimization of extraction conditions. Standards in isooctane were used to quantify the levels of target compounds in the extracts from sediment samples. All standard solutions were protected from light and stored at 4 °C.

Acid-washed calcined quartz sand and diatomaceous earth were bought from Riedel-de-Haën (Seelze, Germany) and Aldrich, respectively. C18 (70-230 mesh), Florisil (60-100 mesh), alumina (150 mesh) and silica (150 mesh) were provided by Aldrich and Merck (Darmstadt, Germany). Normal-phase materials were first activated at 130 °C, for 24 h, allowed to cool down in a desiccator and stored in closed, amber vials. When necessary, they were deactivated with a given percentage of ultrapure water before use. Activated copper powder was purchased from Aldrich.

Empty polypropylene solid-phase extraction (SPE) syringes (15 mL capacity) and 20 µm polyethylene frits were purchased from International Sorbent Technology (Mid Glamorgan, UK). Syringe filters (Millex GV, 13 mm, 0.22 µm) were obtained from Millipore (Billerica, MA, USA).

1.2. Samples and sample preparation

Extraction conditions were optimized using a pool of river and marine sediments with a total carbon (TC) content of 3%, fortified with selected analytes at 500 ng g⁻¹. The spiking procedure consisted of adding a standard solution in acetone to an accurately weighed mass of sieved sediment (fraction below 0.3 mm). The obtained slurry was homogenized thoroughly and left in the hood, at room temperature, for 2-3 days until complete evaporation of the solvent. Thereafter, fortified samples were stored in closed vessels at 4 °C, for 2 weeks, before extraction. The efficiency of the MSPD extraction was further evaluated with individual samples of river and marine sediments fortified at different concentration levels. In all cases, fresh sediment samples were air-dried in the

hood for several days and then sieved. The fraction with a particle size below 0.3 mm was considered in this study.

During optimization of sample preparation conditions, samples (0.5 g) were thoroughly homogenized in a mortar, using a pestle, with a given mass of sorbent and then poured in a polypropylene syringe containing the same, or a different, material as clean-up sorbent. Analytes were eluted with a suitable organic solvent and this extract was concentrated and exchanged to 1 mL of isooctane before injection in the GC-MS/MS system.

The influence of different experimental factors (amount and type of dispersant and clean-up sorbents as well as the composition of the elution solution) on the efficiency of the extraction was assessed using univariant and experimental factorial design strategies. In the latter case, the Statgraphics statistical package (Warrenton, VA, USA) was used to generate the matrix of experiments and to calculate the standardized main effects of each factor in the efficiency of the extraction.

Under final working conditions, samples (0.5 g) were mixed with the same amount of anhydrous sodium sulphate and dispersed with 1 g of diatomaceous earth for 5 min. Silica (0.5 g), deactivated to 10% with ultrapure water, was employed as clean-up sorbent and 5 mL of dichloromethane were used as extraction solvent. This extract was mixed with 1 mL of isooctane, concentrated to around 0.5 mL, using a gentle stream of nitrogen at room temperature, and finally adjusted to 1 mL with the same solvent.

Procedural blanks, employed to identify possible contamination problems, were performed repeating the whole sample preparation process, but without introducing the sediment sample in the MSPD cartridge.

1.3. Determination

A GC-MS/MS system, consisting of a Varian (Walnut Creek, CA, USA) 450 GC instrument connected to an ion-trap Varian 240 mass spectrometer (MS), furnished with an electron impact ionization source, in the external configuration mode, was used for the determination of Tinuvin light stabilizer compounds. Separations were carried out in a HP-5ms capillary column (30 m × 0.25 mm i.d., d_f : 0.25 μ m), acquired from Agilent

(Wilmington, DE, USA) and operated at a constant helium flow of 1.2 mL min^{-1} . The GC oven was programmed as follows: $80 \text{ }^{\circ}\text{C}$ (held for 1 min), first rate $12 \text{ }^{\circ}\text{C min}^{-1}$ to $245 \text{ }^{\circ}\text{C}$, second rate $25 \text{ }^{\circ}\text{C min}^{-1}$ to $285 \text{ }^{\circ}\text{C}$ (held for 10 min). The temperature of the injector was maintained at $280 \text{ }^{\circ}\text{C}$. Standards and sample extracts ($1\text{--}2 \text{ }\mu\text{L}$), both in isooctane, were injected in the pulsed splitless mode (35 psi for 1.1 min). After 1 min, the solenoid valve was switched to the split mode (split flow 50 mL min^{-1}). Retention times and MS/MS parameters for target species are summarized in **Table 1**. Further details about optimal MS/MS conditions are given elsewhere [11]. In addition to MS/MS detection, GC-MS, in scan mode (acquisition range m/z 80-400), was also used to investigate the efficiency of the clean-up process.

Table 1. CAS number, retention times and MS/MS determination conditions.

Compound	CAS number	Retention time (min)	Parent ion (m/z)	Excitation voltage (V)	Product ions (m/z) ^a
Tinuvin P	2440-22-4	14.53	225	0.90	<u>196</u> , <u>168</u>
Allyl-bzt	2170-39-0	16.33	250	1.10	<u>194</u> , 132, 131
Tinuvin 320	3846-71-7	16.95	308	1.00	<u>252</u>
Tinuvin 326	3896-11-5	17.37	300	0.95	<u>272</u> , <u>147</u> , 154
Tinuvin 328	25973-55-1	18.01	322	1.10	<u>252</u>
Tinuvin 327	3864-99	18.08	342	1.05	<u>286</u>

^aUnderlined ions were used for quantification purposes

Recoveries of the optimized method and levels of target compounds in non-spiked sediment samples were determined using external standard quantification and GC-MS/MS detection. Calibration curves, corresponding to the plots of peak area versus concentration, fitted a linear model in the range of concentrations comprised between 1 and 1000 ng mL^{-1} , with determination coefficient (R^2) values higher than 0.995 for all species.

2. Results and discussion

2.1. Preliminary experiments

C18 is the most popular sorbent employed in MSPD methods. Moreover, some authors have also reported the possibility of using inert materials, such as diatomaceous earth and even sand, as cost-effective dispersants in MSPD protocols [15,20]. Obviously, in

the latter case, the role of the dispersant is just increasing the surface of sample available to the extraction solvent. A first series of extractions was conducted in order to assess the effect of the above dispersants (C18, sand and diatomaceous earth, 1 g) in the efficiency of the extraction process. As on-line clean-up sorbent, 1 g of silica, deactivated to 5%, was placed in the bottom of the MSPD syringe [12]. Packed samples were sequentially eluted with 10 mL aliquots of *n*-hexane, *n*-hexane:dichloromethane (1:1), dichloromethane and ethyl acetate. The combined extracts from each sample were reduced from 40 mL to 1 mL (isooctane) and injected in the GC-MS/MS system. Average peak areas for target analytes (*n*=3 replicates) were scarcely affected by the type of dispersant. However, a slightly higher variability was observed when using quartz sand, data not shown. On the basis of its negligible cost in comparison with C18, diatomaceous earth was selected as sample dispersant.

Thereafter, the elution profile of target analytes was investigated collecting the consecutive 10 mL fractions of the above solvents in separated vessels. The first and the fourth fraction (corresponding to *n*-hexane and ethyl acetate, respectively) contained just traces or non-detectable amounts of target analytes, around 70-80% of their peak areas appeared in the second fraction (*n*-hexane: dichloromethane, 1:1) and the rest was noticed in the third one, corresponding to pure dichloromethane. Altogether, these findings point out to the possibility of recovering target compounds with moderate volumes of medium-polarity solvents.

Figure 1 compares the responses obtained in an additional series of extractions using 15 mL of three solutions: hexane: dichloromethane (1:1), dichloromethane and ethyl acetate. Ethyl acetate rendered the lowest peak areas and also the darkest extracts. The mixture of hexane: dichloromethane (1:1) provided colourless extracts, although its extraction efficiency was lower than that achieved with dichloromethane, which produced pale yellowish extracts.

In addition to dispersant sorbent and elution solvent, the performance (extraction efficiency and selectivity) of MSPD protocols is affected by the characteristics of the clean-up co-sorbent. Using normal-phase sorbents, deactivated to 5%, and considering 20 mL of elution solution (10 mL of 1:1 *n*-hexane:dichloromethane followed by 10 mL of dichloromethane), the lowest responses in the GC-MS/MS chromatograms corresponded to alumina, followed by Florisil and silica, **Figure 2**. As regards the selectivity of the extraction, little differences were noticed among the total ion current (TIC) GC-MS

chromatographic profiles corresponding to above extracts. Thus, silica was retained as clean-up material in the MSPD syringe.

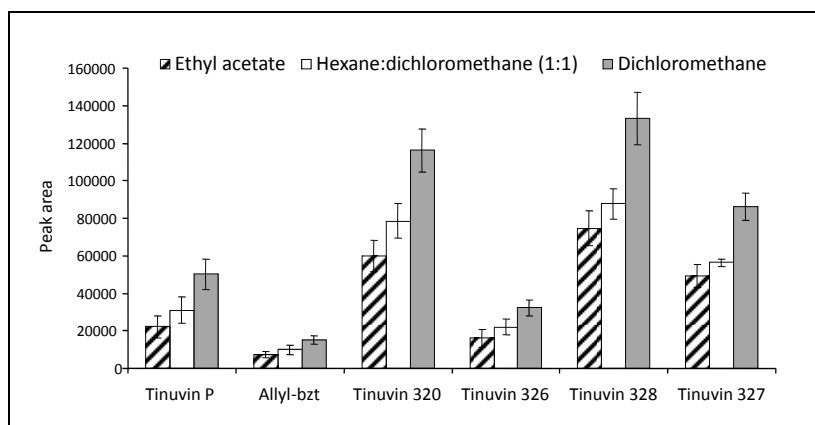


Figure 1. Influence of the extraction solvent (15 mL) on the responses obtained for a spiked ($0.5 \mu\text{g g}^{-1}$) pooled sediment sample, $n=3$ replicates.

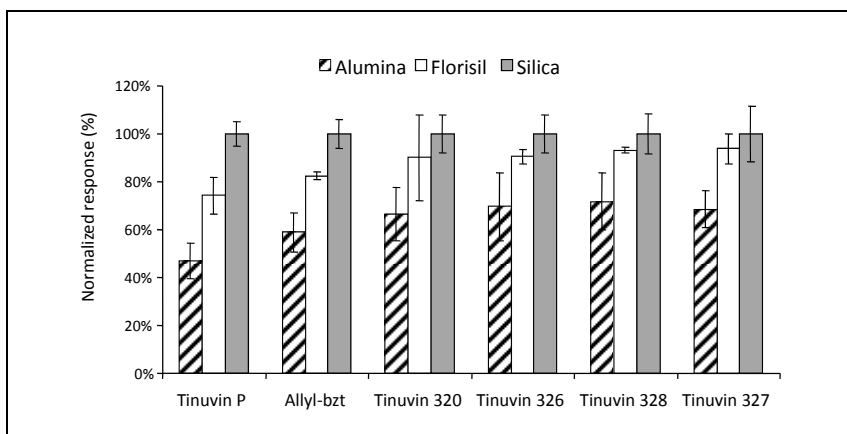


Figure 2. Effect of the clean-up sorbent (1 g, 5% deactivation) in the normalized peak areas of target species, $n=3$ replicates.

2.2. Experimental design optimization

After the preliminary extraction conditions were fixed, a Box-Behnken experimental factorial design was used to further investigate the effects of the amount and deactivation percentage of the clean-up sorbent (silica), as well as the percentage of *n*-hexane added to the extraction solvent (dichloromethane) in the yield of the extraction.

The domain of the design is summarized in **Table 2**. In all cases, 15 mL of extract were collected from the MSPD syringe. Peak areas for target compounds, measured by GC-MS/MS, were used as response variables in order to calculate the main effects associated with each experimental factor, their quadratic terms and the two-factor interactions. **Table 3** summarizes the numerical values of the standardized main effects and their quadratic terms. The absolute value of a main effect is proportional to the influence of the associated experimental variable on the efficiency of the extraction. A positive sign indicates an improvement in the efficiency of the MSPD process when the variable changes from the low to the high level, within the domain of the design. Obviously, a negative value points out to the opposite trend.

Table 2. Domain of the Box-Behnken experimental factorial design.

Variable	Units	Code	Level		
			Low	Medium	High
<i>n</i> -Hexane percentage	(%)	A	0	25	50
Silica deactivation grade	(%)	B	1	5.5	10
Silica amount	g	C	0.5	1.25	2

Table 3. Standardized values of main effects and quadratic terms in the Box-Behnken experimental factorial design.

Code	Standardized values					
	Tinuvin P	Allyl-bzt	Tinuvin 320	Tinuvin 326	Tinuvin 328	Tinuvin 327
A	-2.52	-3.81 ^a	-0.83	-1.06	-1.31	-0.93
B	5.90 ^a	7.33 ^a	3.49 ^a	4.05 ^a	3.82 ^a	4.04 ^a
C	-2.58 ^a	-1.84	-1.01	-0.91	-0.96	-1.41
AA	7.18 ^a	8.13 ^a	2.97 ^a	3.51 ^a	3.10 ^a	3.22 ^a
BB	-1.67	-1.09	-1.66	-1.68	-1.33	-1.31
CC	3.84 ^a	4.19 ^a	-0.05	0.01	0.21	0.07

^aStatistically significant factors at the 95% confidence level

Data compiled in **Table 3** indicate that the deactivation grade of the clean-up silica sorbent (code B) was the most important of the investigated variables, showing a positive and statistical significant effect (95% confidence level) in the extraction of all compounds. This behaviour shows that, despite the high octanol-water partition coefficients of Tinuvin species (log K_{ow} from 4.3 to 8.3), they are strongly retained by activated silica (probably through hydrogen-bond interactions) being difficult to elute at room temperature with aprotic solvents, such as the *n*-hexane:dichloromethane mixture used in this study. In agreement with the above discussion, the amount of clean-up sorbent (code C) affected negatively to the response of all compounds; however, it was

statistically significant just for Tinuvin P, the most polar of the tested species ($\log K_{ow}$ 4.3). Finally, the percentage of *n*-hexane included in the extraction solution (code A) also showed a negative influence on the efficiency of the MSPD extraction; however, the standardized value for the quadratic term of this factor (code AA) was higher than that observed for the main effect (code A). Such situation corresponded to a non-linear diminution in the responses of Tinuvin species with the increase in the percentage of *n*-hexane. The main effect graphs (figure not given) confirmed a minimum extraction yield for an intermediate percentage of *n*-hexane. In general, the other quadratic terms remained below the threshold of statistical significance, except that associated with the amount of silica (CC) for Tinuvin P and Allyl-bzt, **Table 3**. Finally, two-factor interactions did not play significant effects in the MSPD extraction, data not shown.

On the basis of the above comments, pure dichloromethane was selected as extraction solvent and 0.5 g of silica, previously deactivated to 10% with ultrapure water, used as clean-up co-sorbent at the bottom of the MSPD syringe. Although different masses of diatomaceous earth (0.5 to 2 g) were further assayed as sample dispersants, no differences were observed in the efficiency of the extraction, thus 1 g was kept as the optimum value for this parameter. Once these conditions were established, the minimum volume of dichloromethane required for the quantitative extraction of target compounds was evaluated by collecting successive 2 mL fractions from the MSPD syringe. As observed in **Figure 3**, about 95% of the obtained responses corresponded to the first and second fractions. Considering the availability of volumetric glass material, 5 mL of dichloromethane were adopted as working value for this variable.

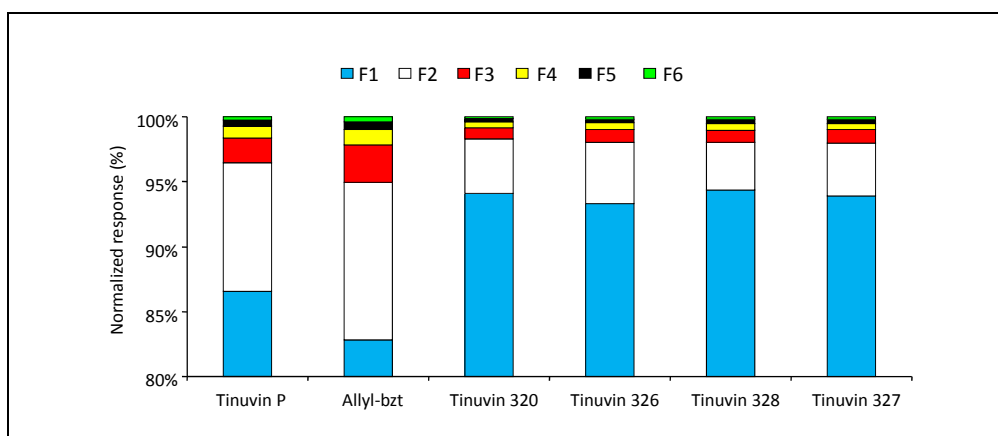


Figure 3. Cumulative normalized responses in the consecutive dichloromethane fractions (2 mL each) collected from the MSPD syringe. Average values for triplicate extractions.

2.3. Sulphur removal

Most of the sediment samples considered in this study contained significant amounts of sulphur (up to 0.8%), which was co-extracted together with target compounds and could not be effectively removed with any of the tested clean-up sorbents (silica, alumina and Florisil). Using GC-MS detection, it was verified that the chromatographic signal of S8 (a broad band in most cases) overlapped the peak of Tinuvin P. The MS spectra of S8 showed a cluster of ions with m/z ratios: 224 ($[^{32}\text{S}_7]^+$), 226 ($[^{32}\text{S}_6^{34}\text{S}]^+$) and 225 ($[^{32}\text{S}_6^{33}\text{S}]^+$); whereas, the base peak in the spectra of Tinuvin P appeared at m/z 225 (spectra not given). Therefore, GC combined with low resolution MS does not provide enough selectivity for the reliable determination of Tinuvin P in sediments displaying high levels of sulphur. Even in the MS/MS mode the performance of ion-trap type mass spectrometers might be affected by the existence of a large number of ions with the same, or very close, nominal m/z ratios as the parent ion.

With the aim of reducing the sulphur content in sediment extracts, activated copper (0.5-2 g) was combined with the MSPD sample preparation protocol; however, at difference with Soxhlet and PLE applications [21,22], significant removal of sulphur was not achieved. Likely, at room temperature, the kinetics of sulphur reduction with copper is too slow to be effectively implemented in the MSPD extraction process. As a compromise solution, sulphur was eliminated adding activated copper (ca. 400 mg) to the concentrated sediment extracts, which were kept overnight at room temperature before analysis [23].

2.4. Performance of the method

The recoveries of the method were investigated using spiked samples of river and marine sediments with different carbon contents. Peak areas for Tinuvin in the extracts corresponding to spiked fractions from each sample were corrected with responses attained for un-spiked fractions. Then, they were compared with calibration curves corresponding to pure standards prepared in isooctane. Recoveries were defined as the ratio between measured and spiked concentrations multiplied by 100. The obtained values varied from a minimum of 78% up to 110%, with standard deviations remaining below 14, **Table 4**.

Table 4. Recoveries of the proposed method (n=3 replicates) for spiked sediment samples.

Sample code	TC (%)	Added concentration. (ng g ⁻¹)	Recovery (%) ± standard deviation					
			Tinuvin P	Allyl-bzt	Tinuvin 320	Tinuvin 326	Tinuvin 328	Tinuvin 327
R. 1	2.8	100	101 ± 8	94 ± 8	88 ± 6	93 ± 7	100 ± 7	97 ± 8
R. 1	2.8	500	110 ± 13	83 ± 9	104 ± 14	90 ± 8	93 ± 12	93 ± 12
R. 2	1.0	500	78 ± 8	88 ± 6	95 ± 7	97 ± 7	99 ± 7	98 ± 7
R. 3	1.2	40	84 ± 2	93 ± 7	79 ± 2	92 ± 8	82 ± 4	82 ± 3
M. 1	15.0	100	96 ± 2	80 ± 8	87 ± 10	94 ± 5	99 ± 2	96 ± 8

R. river sediment, M. marine sediment, TC total carbon

The limits of quantification (LOQs) of the method were 3 ng g⁻¹ for Tinuvin 320, 326, 328 and 327 and 15 ng g⁻¹ for Tinuvin P and Allyl-bzt. Considering 0.5 g and 1 mL as sample intake and final extract volume respectively, the linear response range of the method extended up to 500 ng g⁻¹. **Figure 4** shows the MS/MS chromatographic traces corresponding to a procedural blank (**Figure 4A**) superposed with those of a low level calibration standard (**Figure 4B**). As appreciated, small signals appeared at the retention times of Tinuvin 320, 326, 328 and 327; thus, their LOQs were defined as the concentration of each species providing a response 10 higher than the standard deviation of procedural blanks (n= 5 replicates). In the case of Tinuvin P and Allyl-bzt, LOQs were defined as the concentration of each compound rendering a peak with a height 10 times higher than the average baseline noise in the corresponding MS/MS acquisition window. As shown in **Figure 4B**, both compounds tend to produce slightly tailing peaks; moreover, the selectivity of their MS/MS transitions was lower than those corresponding to the rest of Tinuvin, which explains the higher LOQs of Tinuvin P and Allyl-bzt versus those reported for the rest of analytes. Overall, the LOQs achieved in this work (3-15 ng g⁻¹) remain in the same order of values as LODs, estimated for a signal to noise (S/N) of 3, comprised between 0.3 and 10 ng g⁻¹, previously reported in the literature using conventional solid-liquid extraction followed by multistep clean-up strategies, which consumed up to 200 mL of different organic solvents per sample [9-10].

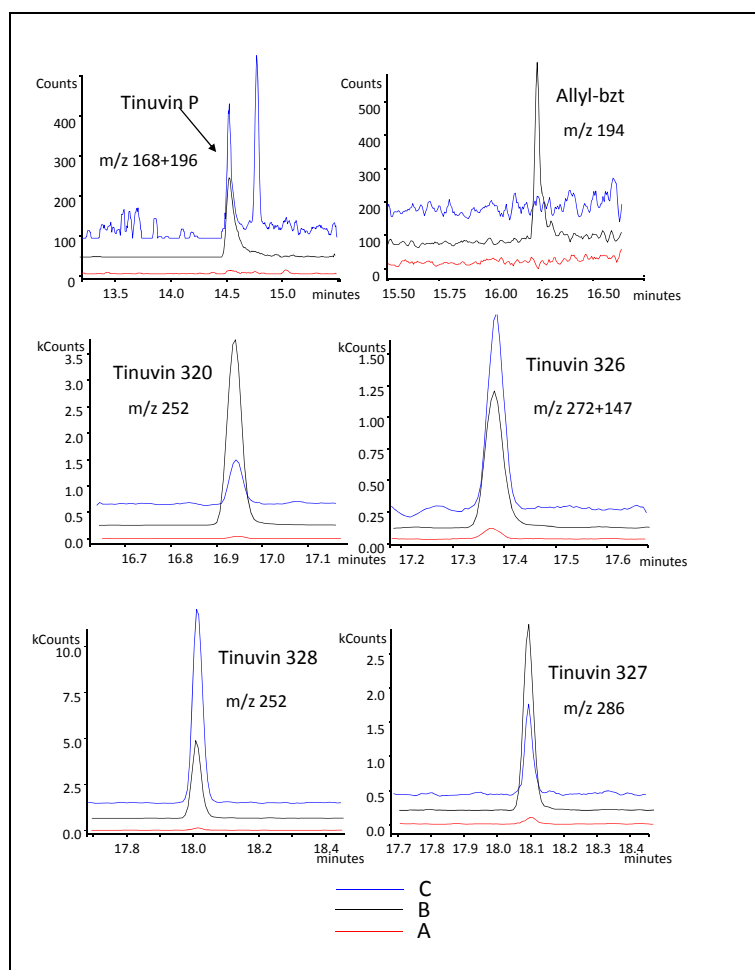


Figure 4. GC-MS/MS traces for a procedural blank (A), a 10 ng mL⁻¹ standard in isooctane (B), and a non-spiked sediment sample, code 1, **Table 5** (C).

2.5. Application to real samples

The optimized method was used to investigate the presence of target species in ten samples of river and estuarine sediments with different carbon contents. Six of the processed specimens contained detectable levels of at least one Tinuvin, with the highest concentration (56 ng g⁻¹) corresponding to Tinuvin 328, **Table 5**. On the other hand, Allyl-bzt remained undetected in all samples. The GC-MS/MS chromatogram for sample code 1 (see **Table 5**) has been also incorporated in **Figure 4** (trace C). Concentrations summarized in **Table 5** stay below the levels reported for heavily polluted river sediments in Japan

[6,10]; nevertheless, they point out to the ubiquitous spread of this family of light stabilizers in the aquatic environment.

Table 5. Concentrations (ng g^{-1}) of Tinuvin species measured in sediment samples, $n=3$ replicates.

Code	TC (%)	Concentration (ng g^{-1}) $\pm$ standard deviation				
		Tinuvin P	Tinuvin 320	Tinuvin 326	Tinuvin 328	Tinuvin 327
1	3.0	30 ± 4	5.6 ± 0.8	32 ± 4	56 ± 2	15 ± 2
2	3.9	n.d.	n.d.	n.d.	10 ± 1	10.3 ± 0.4
3	5.5	27 ± 3	n.d.	7.8 ± 0.8	8.3 ± 0.3	n.d.
4	4.6	n.d.	n.d.	n.d.	11.2 ± 0.8	9.5 ± 0.2
5	2.2	n.d.	n.d.	n.d.	7.9 ± 0.7	n.d.
6	8.0	n.d.	n.d.	15 ± 2	8 ± 1	n.d.

n.d., not detected, TC total carbon

3. Conclusions

The suitability of the MSPD technique for the extraction of Tinuvin species from sediment samples has been demonstrated for first time. The developed method renders quantitative recoveries and similar LOQs than previously developed methodologies, with the advantages of integrating extraction and clean-up (except sulphur removal) in the same step, and presenting reduced cost and organic solvent consumption. The GC-MS determination of Tinuvin P is interfered by the presence of large amounts of sulphur in sediment samples. Although sulphur removal could not be implemented in MSPD process, the above problem was overcome by storing overnight the concentrated extracts with activated copper powder and by using tandem MS/MS, instead of single MS, detection. Data obtained for non-spiked sediment samples confirmed the accumulation of Tinuvin UV absorbers in this matrix, with the highest occurrence frequency corresponding to Tinuvin 328.

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Publicación III.1.3:

Pressurized solvent extraction followed by gas chromatography tandem mass spectrometry for the determination of benzotriazole light stabilizers in indoor dust

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Pressurized solvent extraction followed by gas chromatography tandem mass spectrometry for the determination of benzotriazole light stabilizers in indoor dust

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Abstract

A novel and sensitive method for the determination of five benzotriazole compounds (commonly used as light stabilizers) in indoor dust is presented. Pressurized liquid extraction (PLE) and gas chromatography followed by tandem in time mass spectrometry (GC-MS/MS) were used as sample preparation and determination techniques, respectively. Extraction and clean-up were integrated on-line and, after an evaporative concentration step, the extract provided by the PLE instrument was injected directly in the GC-MS/MS system. Parameters affecting the performance of the sample preparation process were evaluated using experimental factorial designs. Under optimized conditions, analytes were recovered from 0.5 g samples in 3 static extraction cycles of 10 min, using a hexane: dichloromethane (7:3) mixture, at 90 °C. Silica (1 g) was placed in the bottom of the extraction cells as clean-up sorbent. The recoveries of the method varied from 82 to 122%, with standard deviations below 13. The inter-day precision ranged from 9 to 12%, and the limits of quantification (LOQs) remained below 10 ng g⁻¹ for all species. For first time, four of the five investigated species were found in dust from indoor environments. Their mean concentrations ranged from 71 to 780 ng g⁻¹.

Keywords: Dust, light stabilizers, tinuvin, pressurized liquid extraction, gas chromatography, mass spectrometry

1. Introduction

Chronic exposure to chemicals in confined atmospheres has become a matter of concern during recent years, particularly in the case of small children and older people, who spend up to 90% of their time indoors [1]. Floor coverings, electronic devices, furniture, paints and building materials incorporate a large number of additives to improve their physical properties, stability and to reduce the risk of ignition. These additives are slowly released from host materials. Then, they may remain in the vapour phase and/or be adsorbed on suspended particulate matter and settled dust, depending on their volatilities [2,3]. Well-known examples of concerning chemicals detected at relevant concentrations in dust from closed areas are brominated and organophosphorus flame retardants, phthalates and polycyclic aromatic hydrocarbons [4-6]. Compounds systematically included in the formulation of personal care products are also ubiquitous in indoor dust [7,8].

The use of more efficient insulation in private houses and administrative buildings, together with the low impact of light-induced degradation reactions in indoor areas, leads to low removal rates of compounds associated with dust particles [2,5]. As a result, dermal contact, inhalation and even oral intake constitute significant sources of exposure to the cocktail of chemicals adsorbed on settled dust and airborne particles in confined environments [2,9]. Some authors have suggested a potential relationship between chronic exposure and the increased incidence of allergies, respiratory diseases and other health-related problems included in the so-called sick building syndrome [10,11].

The derivatives of 2-hydroxyphenyl benzotriazole, normally referred to as Tinuvs, are incorporated in polymeric materials, paints and varnishes to prevent yellowing and light-induced degradation reactions [12,13]. Multi-residue surveys have confirmed the stability of these species in sediments under anaerobic conditions [14,15]. More recently, Nakata et al. have also demonstrated bio-accumulation of these compounds in aquatic organisms and birds [16]. This information points to the classification of Tinuvs as possible persistent pollutants. However, we did not find any information concerning their occurrence in confined environments. Nevertheless, on the basis of their moderate molecular weights and low vapour pressures, it may be expected that 2-hydroxyphenyl benzotriazole species would mainly remain associated with

suspended particulate matter and settled dust after being released from surfaces treated with the light stabilizers.

Toxicological aspects related to exposure to Tinuvin species are scarce. Acute effects of direct contact with Tinuvin treated surfaces, such as dermatitis and skin irritation problems have been reported [17,18], but we did not find any information about the effects of chronic exposure. However, bio-accumulation data reported for birds and aquatic organisms [16] suggests that the compounds might also be concentrated on the human body as the result of continuous exposure.

From an analytical perspective, the determination of organic pollutants in dust is a challenging issue. The high adsorption capacity of dust particles requires the use of exhaustive extraction strategies, which normally render highly complex extracts, with a large number of co-extracted interferences. Pressurized liquid extraction (PLE) presents some appealing features to address this problem. The technique shows considerable versatility as regards the type of extraction solvent, temperature and time, moderate consumption of organic solvents and high degree of automation. A further characteristic of PLE is the possibility of integrating a clean-up step in the extraction process, simply using an active sorbent, which can be placed at the bottom of the cell and/or mixed with the sample matrix [19,20]. Some of these advantages have already been exploited for the extraction of different groups of anthropogenic pollutants from dust samples [21-23]. However, as far as we could trace, PLE has never been applied to the extraction of Tinuvin light stabilizers from environmental samples.

The aim of this research was to evaluate the suitability of PLE, combined with gas chromatography (GC) followed by tandem in time mass spectrometry (MS/MS) detection, for the sensitive and selective determination of five benzotriazole type light stabilizers in dust samples from indoor environments. Parameters affecting the efficiency and selectivity of sample preparation are discussed. Moreover, the occurrence of target species in dust, collected in different confined areas, is reported for first time.

2. Experimental

2.1. Solvents, standards and sorbents

n-Hexane, dichloromethane, acetone, isooctane and ethyl acetate, trace analysis grade, were obtained from Merck (Darmstadt, Germany). Standards of 2-(2H-benzotriazol-2-yl)-4-methyl-phenol (Tinuvin P), 2-(2H-benzotriazol-2-yl)-4-methyl-6-(2-propen-1-yl)-phenol (Allyl-bzt), 2-(5-chloro-2H-benzotriazol-2-yl)-6-(1,1-dimethylethyl)-4-methyl-phenol (Tinuvin 326), 2-(2H-benzotriazol-2-yl)-4,6-bis(1,1-dimethylpropyl)-phenol (Tinuvin 328) and 2-(5-chloro-2H-benzotriazol-2-yl)-4,6-bis(1,1-dimethylethyl)-phenol (Tinuvin 327) were acquired from Aldrich (Milwaukee, WI, USA). The chemical structures and some relevant properties of the above compounds are shown in **Figure 1**. In general, these are moderately acidic species (the pK_a values varied between 8.15 and 9.49) with medium to high octanol water partition coefficients ($\log K_{ow}$ ranged from 4.30 for Tinuvin P to 8.28 for Tinuvin 328).

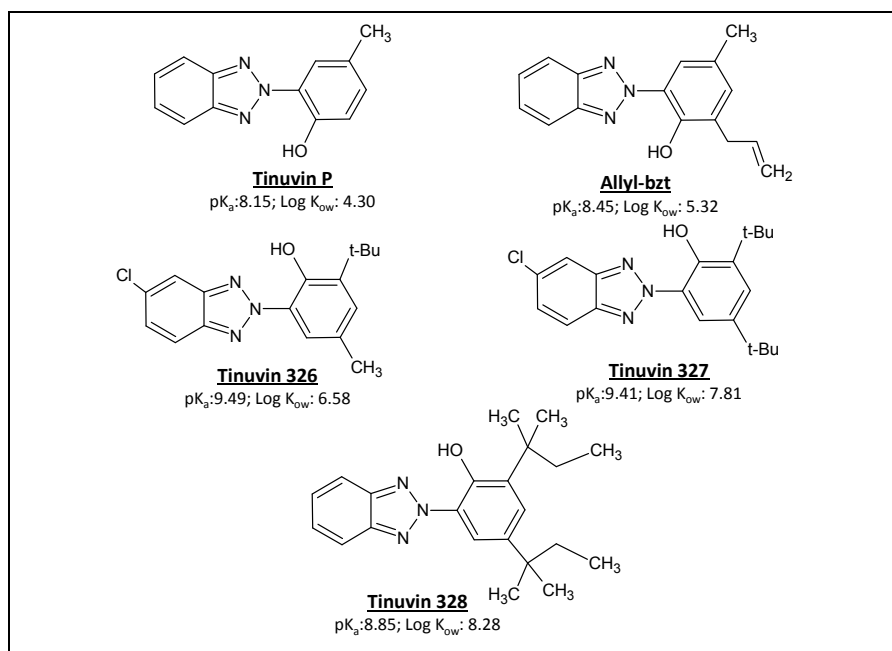


Figure 1. Structures, pK_a values and octanol water partition coefficients ($\log K_{ow}$) for target species.

Stock solutions of each species were made in acetone. Further dilutions and mixtures of target analytes were prepared in acetone and isooctane. Standards in acetone

were used to fortify the samples employed during optimization of extraction conditions. Standards in isooctane were used to quantify the levels of compounds in the extracts from dust samples. All standard solutions were protected from light and stored in the fridge at 4 °C.

Acid washed and calcined quartz sand was bought from Riedel-de-Haën (Seelze, Germany). Florisil (60-100 mesh), alumina (150 mesh) and silica (150 mesh) were provided by Aldrich and Merck (Darmstadt, Germany). Normal-phase materials were first activated at 130 °C, for 24 h, and then stored in a desiccator, at room temperature, before being used as clean-up sorbents in the PLE cell.

2.2. Samples

Dust was collected from several private houses, vehicle cabins and an administrative building by using domestic vacuum cleaners equipped with paper filter bags. The contents of the bags were sieved to improve the homogeneity of the material. Coarse particles, debris and lint were removed and the fraction below 60 µm was retained for this study. Sieved samples were stored in amber glass vessels at room temperature. Reference material SRM 2585, organic compounds in house dust, was acquired from the National Institute of Standards and Technology (NIST; Gaithersburg, MD, USA). This sample was used to confirm the ubiquity of Tinuvins in dust although no certified or indicative values of their levels in this reference material are available.

Optimization of PLE conditions was performed with a pooled dust matrix, prepared from individual samples, fortified at the 1 µg g⁻¹ level. The total carbon (TC) content of the matrix was 21.8%. The spiking procedure consisted of the addition of a measured volume of a standard in acetone to an accurately weighed fraction of the above described pooled sample. The slurry was protected from the light, homogenized periodically and kept in a hood until complete elimination of the acetone. The recoveries of the method were evaluated with fortified samples containing lower levels of the target analytes. All spiked samples were aged for a minimum of two weeks before extraction.

2.3. Sample preparation

Extractions were performed with a pressurized liquid extractor, ASE 200 Dionex (Sunnyvale, CA, USA), furnished with 11 mL capacity stainless-steel cells. A cellulose filter, followed by a glass fibre one, were placed on the bottom of each cell. Then, 1 g of a given normal-phase material and 0.5 g of dust, previously mixed with 1 g of quartz sand in a mortar, were loaded. The remaining volume of the cell was filled completely with quartz sand and another cellulose filter was placed on top. Extractions were carried out under different experimental conditions. In all cases, 1 mL of isooctane was added as a keeper to the extracts collected from the PLE cell. The extracts were then evaporated, c.a. 0.5-0.7 mL, under a gentle stream of dry nitrogen at room temperature. Finally, the concentrated extracts were passed through a nylon syringe filter (pore size, 0.45 μm) and made up to 2 mL with isooctane.

Under optimized conditions, analytes were extracted with a hexane:dichloromethane (7:3) mixture. Silica (1 g) was placed on the bottom of the PLE cell as clean-up sorbent, to retain polar interferences. The extraction was carried out at 90 $^{\circ}\text{C}$, with the cells pressurized at 1500 psi, using 3 consecutive static cycles of 10 min each. The flush volume and the purge time (using nitrogen) were 11 mL (equivalent to 100% of the cell capacity) and 1 min, respectively.

2.4. GC-MS/MS determination

A GC-MS/MS system, consisting of a Varian (Walnut Creek, CA, USA) 450 GC instrument connected to an ion-trap Varian 240 mass spectrometer (MS), furnished with an electron impact (EI) ionization source, in the external configuration mode, was used for the determination of light stabilizers. Separations were carried out in a Varian Factor Four, BP-5 type column (30 m $\times$ 0.25 mm i.d., d_f : 0.25 μm) operated at a constant helium flow of 1.2 mL min^{-1} . The GC oven was programmed as follows: 80 $^{\circ}\text{C}$ (held for 1 min), first rate 12 $^{\circ}\text{C min}^{-1}$ to 245 $^{\circ}\text{C}$, second rate 25 $^{\circ}\text{C min}^{-1}$ to 285 $^{\circ}\text{C}$ (held for 10 min). The temperature of the injector was maintained at 280 $^{\circ}\text{C}$. Standards and sample extracts (1-2 μL), both in isooctane, were injected in the pulsed splitless mode (35 psi for 1.1 min). After 1 min, the solenoid valve was switched to the split mode. Most intense ions in the MS

spectra of each compound and optimal MS/MS detection conditions have been reported elsewhere [24].

External calibration was used to evaluate the recoveries of the method and also the levels of target compounds in non-spiked samples. Calibration graphs, corresponding to the representation of peak areas versus the concentration of each compound, were constructed with standards prepared in isooctane at increasing concentrations in the range from 0.5 to 1000 ng mL⁻¹. Assessment of possible contamination and carry-over problems was made by repeating the whole sample preparation process without dust, filling the cells of the PLE system with quartz sand and 1 g of silica.

3. Results and discussion

3.1. Optimization of sample preparation conditions

3.3.1. Extraction solvent and clean-up sorbent

The choice of a suitable solvent is a key step in the optimization of any solid-liquid based extraction method. In the earlier steps of this research, 0.5 g fractions of a spiked (1 µg g⁻¹) pooled dust sample were mixed with sand and transferred to stainless steel PLE cells, containing 1 g of activated silica as clean-up sorbent. Temperature and pressure were fixed at 90 °C and 1500 psi, which are similar conditions to those used in the PLE extraction of other organic compounds from the same matrix [21]. Cells were extracted sequentially using first a hexane: dichloromethane (8:2) solution, followed by dichloromethane and finally ethyl acetate. For each solvent, a single extraction cycle of 5 min with a 100% flush volume, followed by a purging step of 5 min, was used. Extracts were evaporated, filtered and adjusted to 2 mL. The higher the polarity of the extraction solution, the more complex the visual appearance of the corresponding extracts was, with colours ranging from pale (hexane: dichloromethane) to intense brown (ethyl acetate), which prevented the injection of this latter fraction in the GC-MS/MS system. Levels of target compounds in the dichloromethane fraction represented less than 5% of those measured in the hexane: dichloromethane (8:2) one, data not shown. On the basis of these preliminary results, it was further assessed if n-hexane alone sufficed to recover target analytes from the spiked dust sample. Using the above described PLE conditions, n-

hexane rendered colourless, completely transparent extracts. However, it failed to extract Tinuvin P and Allyl-bzt, which are the most polar species involved in this study ($\log K_{ow}$ 4.3 and 5.3, respectively). Thus, n-hexane combined with a medium polarity organic modifier was adopted as the extraction mixture.

The clean-up sorbent also affects the efficiency and the selectivity of PLE extractions. Florisil and alumina (1 g each) were considered as alternatives to silica. Under the PLE conditions described above, with hexane: dichloromethane (80:20) as the extraction solution, very little differences were noticed between responses obtained for Florisil and silica, whereas alumina appeared to be a little more retentive, **Figure 2**. In terms of selectivity, all extracts showed a similar appearance with a very pale coloration. Thus, silica was maintained as clean-up sorbent. Increasing the amount of silica from 1 to 2 g led to a dramatic diminution in the responses measured for Tinuvin P and Allyl-bzt, **Figure 3**. Consequently, the amount of silica was limited to 1 g.

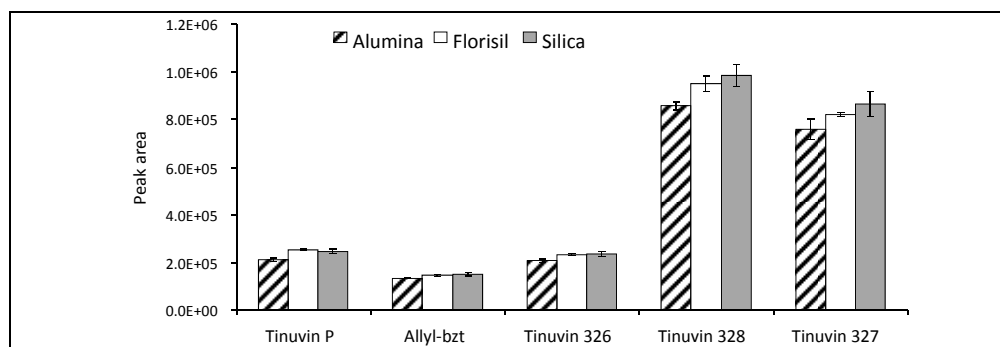


Figure 2. Influence of the clean-up sorbent (1 g) on the efficiency of the extraction process, $n = 3$ replicates. Extractions were performed with hexane: dichloromethane (8:2) at 90 °C and 1500 psi, for 10 min with a 100% flush volume.

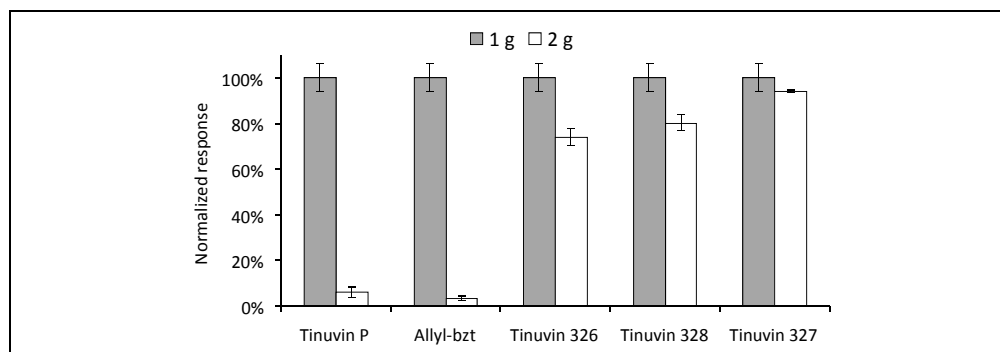


Figure 3. Normalized responses for 1 and 2 g of silica as clean-up sorbent, $n = 3$ replicates. Same extraction conditions as in **Figure 2**.

3.3.2. Multivariate optimization of PLE conditions

The yield of PLE extractions is affected by many instrumental variables, which are sometimes correlated. A multivariate strategy, based on the use of experimental factorial designs, was adopted in order to reduce the effort necessary to establish the optimal working conditions. A $3^1 \times 2^{3-1}$ type partial factorial design was used to obtain an initial overview of the effects of extraction temperature (70, 100 and 130 °C), time (1, 10 min), type (dichloromethane, acetone) and percentage of organic modifier (5 and 30%) on the efficiency of the extraction. Peak areas obtained in the 12 extractions involved in the above design were used as variable responses. Standardized values for the main effects corresponding to each factor were calculated with the Statgraphics Centurion XV software (Manugistics, Rockville, MD, USA). Their graphical representation is summarized in the plots grouped in **Figure 4**. The length of depicted lines is proportional to the variation in the response of the investigated species when a given factor changes from the low to the high level, within the domain of the design. A positive slope indicates an improvement in the efficiency of the extraction and a negative slope the opposite effect. For all compounds, the type of modifier was the least important of the considered factors. With the exception of Tinuvin P, slightly higher responses were noticed using acetone; however, the extracts for this modifier were more complex than those corresponding to dichloromethane. Thus, the latter solvent was used in further extractions. Time and percentage of modifier exerted positive effects on the extraction process. The first variable was fixed at 10 min and the percentage of dichloromethane was evaluated with more detail, between 15 and 30%, in a second experimental design. With regard to the extraction temperature, maximum responses were attained at intermediate or high values within the explored range.

Table 1 summarizes the experimental domain of the second design. In this case, a full factorial design, with four variables evaluated at two levels (2^4), and three replicates of the central point, was used. In addition to temperature and percentage of dichloromethane in the extraction solution, the number of extraction cycles and the flush volume were also considered as experimental factors. The standardized values for their main effects, calculated after carrying out the 19 extractions involved in the above design, are also shown in **Table 1**. The number of extraction cycles affected positively to the efficiency of the extraction for all compounds; moreover, its effect was statistically

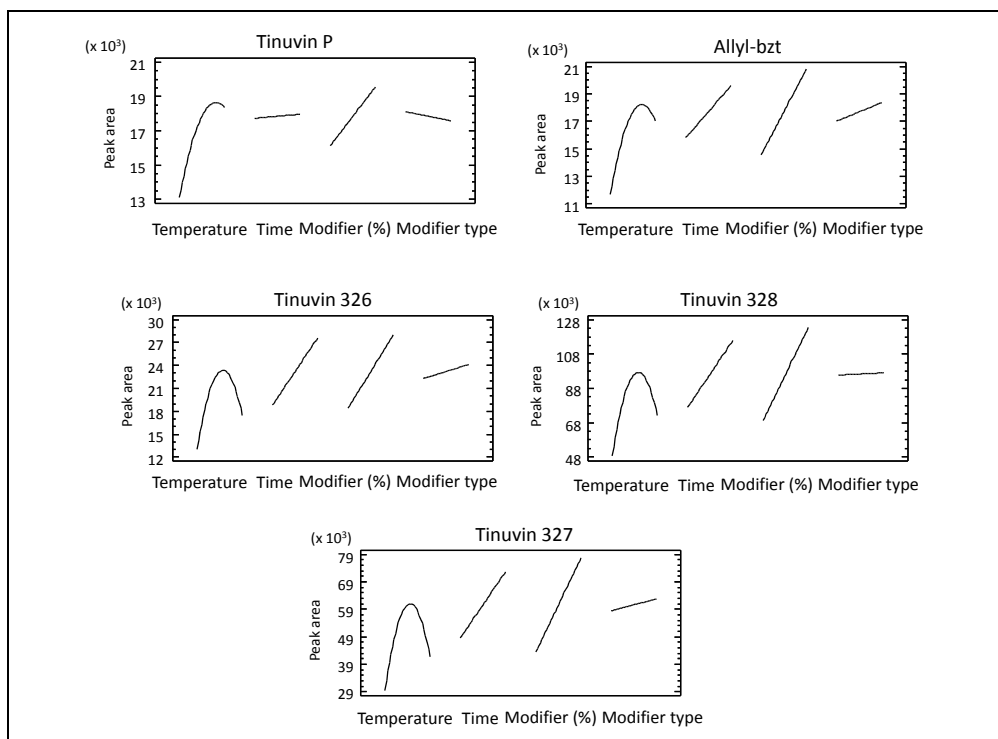


Figure 4. Main effect graphs corresponding to the partial ($3^1 \times 2^{3-1}$) factorial experimental design.

Table 1. Experimental domain and standardized main effects of factors considered in the 2^4 full experimental factorial design.

Factor	Level		Standardized value				
	Low	High	Tinuvin P	Allyl-bzt	Tinuvin 326	Tinuvin 328	Tinuvin 327
Temperature (°C)	90	110	0.42	0.46	-2.29	-1.75	-2.13
CH ₂ Cl ₂ percentage (%)	15	30	-1.06	-1.13	0.59	0.27	0.35
Cycles	1	3	1.12	0.80	2.32 ^a	2.31 ^a	2.44 ^a
Flush volume	60	100	0.24	-0.40	0.04	-0.39	-0.08

^a Statistically significant factors at the 95% confidence level

significant (95% confidence level) for three of the five analytes considered (**Table 1**). Main effects for the rest of variables remained below the threshold of statistical significance and their sign was compound dependant: Tinuvin 326, 327 and 328 were recovered in a higher extension using low temperatures and high percentages of dichloromethane, whereas the opposite combination improved the extraction of Tinuvin P and Allyl-bzt (**Table 1**). Two-factor interactions were also below the level of statistical significance; however, in the case of Tinuvin P and Allyl-bzt their standardized values were higher than

those corresponding to the main effects, data not shown. On the basis of these findings, a global desirability function was used to get the combination of experimental variables that provided the highest overall extraction yield for the considered compounds. First, the responses for each compound in the experiments of the above design were normalized between 0 and 1. The overall desirability function was then estimated as their geometric mean. **Figure 5** shows the plots of this function versus the experimental values of the considered factors. The best compromise values, which maximized the global desirability function (0.86 over a maximum value of 1), were 90 °C, 30 % of dichloromethane and a 100% of flush volume, distributed in 3 static extraction cycles.

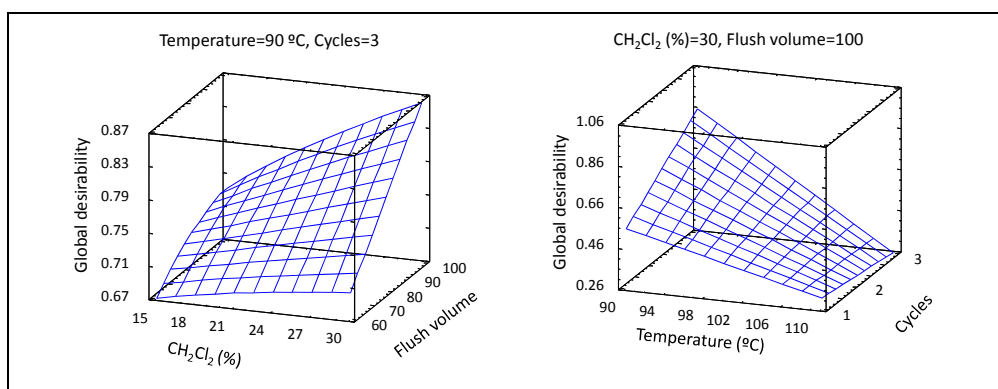


Figure 5. Plots of the global desirability function against extraction conditions.

Purging the extraction cells with nitrogen is necessary to recover completely the solvent, which impregnates the packed sample. Between 1 and 5 min no differences were observed in the yield of the extraction; thus, 1 min was adopted as working value for this variable.

3.2. Performance of the method

Table 2 summarizes some data related to the performance of the GC-MS/MS system, without considering the sample preparation step. The plots of peak area versus the concentration of each analyte fitted a linear model, with determination coefficients (R^2) higher than 0.999 for all species. The instrumental limits of quantification (LOQs), defined as the concentration of each compound that produced a chromatographic peak

with a signal 10 times higher than the baseline noise, were below 0.5 ng mL^{-1} for an injection volume of $2 \mu\text{L}$, whereas the repeatability of the injection remained below 5%.

Table 2. Retention times, quantification ions, linearity and limits of quantification (LOQs) of the GC-MS/MS system for standards in isooctane.

Compound	Ret. Time (min)	Quantification ions (m/z)	Linearity ^a (R^2)	Repeatability ^b (RSDs %)	LOQs (ng mL^{-1})
Tinuvin P	14.43	168+196	0.9994	4.4	0.4
Allyl-bzt	16.12	194+132	0.9992	5.3	0.4
Tinuvin 326	17.23	147+272	0.9991	2.6	0.3
Tinuvin 328	17.83	252	0.9999	2.3	0.2
Tinuvin 327	17.88	286	0.9996	2.4	0.3

^aCalibration range $0.5\text{--}1000 \text{ ng mL}^{-1}$, nine levels.

^bRelative standard deviations for a 10 ng mL^{-1} standard, $n = 5$ replicates.

The accuracy of the sample preparation method was assessed with a new pool of dust samples (TC 22.5%) spiked at two concentration levels, 100 and 300 ng g^{-1} . Peak areas, corresponding to extracts from spiked and non-spiked fractions of the above matrix, were introduced in the equations of calibration curves constructed for standards in isooctane. The differences of concentrations were then compared with the added level and multiplied by 100. Obtained recoveries ranged from 82 to 122 %, with associated standard deviations from 3 to 13 (**Table 3**). As expected, the best precision was attained for the sample fortified at the highest concentration.

Table 3. Recoveries of the method for spiked samples ($n = 4$ replicates), reproducibility, and estimated LOQ values.

Compound	Addition level 100 ng g^{-1}		Addition level 300 ng g^{-1}		^a Reproducibility RSD (%)	LOQ (ng g^{-1})
	Recovery (%)	SD	Recovery (%)	SD		
Tinuvin P	122	8	108	4	9	10
Allyl-bzt	105	13	82	10	12	10
Tinuvin 326	108	11	94	4	9	9
Tinuvin 328	97	4	89	7	10	4
Tinuvin 327	82	11	92	3	12	4

^aRSDs corresponding to 9 extractions on 3 different days.

The inter-day reproducibility of the method was evaluated with another spiked sample (ca. 150 ng g^{-1} per compound) extracted in triplicate during 3 consecutive days. Relative standard deviations between 9 and 12% were obtained (**Table 3**).

Procedural blanks showed small peaks at the retention times of some species (**Figure 6**). The source of this contamination may be related to the trend of target

compounds to be retained on solid surfaces [24]. Glass material, extraction cells and connections in the PLE system might contribute to the presence of benzotriazole light stabilizers in procedural blanks due to carry over problems. After being cleaned using a conventional detergent followed by rinsing with tap water, ultrapure water and acetone, PLE cells and any other container in contact with sample extracts were covered with aluminium foil to prevent the deposition of particulate matter, and thus, to minimize its contribution to contamination problems. Anyhow, procedural blanks must be performed periodically in order to (1) evaluate the magnitude of contamination problems and (2) avoid the report of false positives during analysis of real samples. The LOQs of the whole method were estimated on the basis of the sample intake (0.5 g), final extract volume (2 mL), the above commented problems related to blanks and the instrumental LOQs of the GC-MS/MS system (**Table 2**). Estimated values ranged from 4 to 10 ng g⁻¹, depending on the compound (**Table 3**). On the basis of the linear response range for the GC-MS/MS system reported in **Table 2**, the sample intake and the final extract volume (0.5 g and 2 mL, respectively), the proposed method can be applied to samples containing concentrations of up to 4 µg g⁻¹ of each compound.

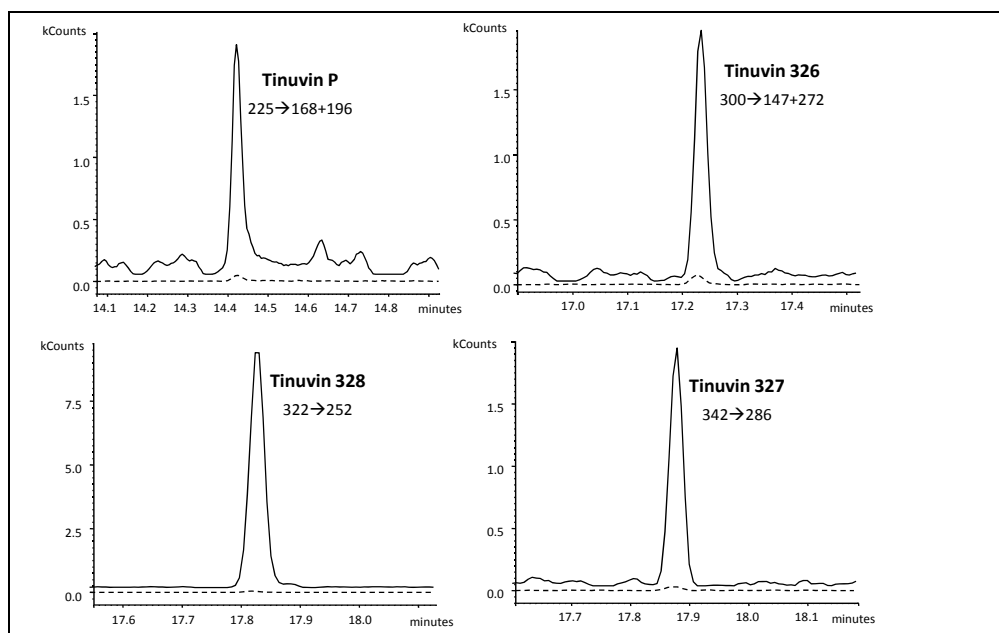


Figure 6. GC-MS/MS traces for a procedural blank (dotted line) and compounds found in sample code 4, **Table 4** (solid line).

3.3. Analysis of dust samples

The presence of benzotriazole light stabilizers in confined environments was evaluated with dust specimens obtained from private houses (5 samples), administrative buildings (1 sample) and cabins from vehicles (3 samples); moreover, an indoor dust reference material (SRM 2585) was also processed. GC-MS/MS traces corresponding to sample code 4 (**Table 4**) and a procedural blank are shown in **Figure 6**. Allyl-bzt always remained below the LOQ of the method, whereas the other compounds were ubiquitous in dust, with measured values from 22 to more than 600 ng g⁻¹ (**Table 4**). Moreover, Tinuvin 326 was found in one sample (code 7, **Table 4**) at a concentration of almost 5 µg g⁻¹. Except for this compound, the average levels for the rest of Tinuvin in the processed samples were lower than their concentrations in SRM 2585. This may be related to the different geographical origin of the SRM material.

Table 4. Levels of benzotriazole light stabilizers in non-spiked dust samples, n= 3 replicates.

Code	Source	Concentration (ng g ⁻¹) ± SD			
		Tinuvin P	Tinuvin 326	Tinuvin 328	Tinuvin 327
1	Private house	77 ± 5	42 ± 4	46 ± 3	86 ± 6
2	Private house	98 ± 14	58 ± 14	127 ± 15	101 ± 13
3	Private house	657 ± 27	333 ± 14	100 ± 5	29 ± 3
4	Private house	75 ± 6	73 ± 1	68 ± 5	22 ± 1
5	Private house	94 ± 2	269 ± 12	149 ± 4	52 ± 3
6	Public building	212 ± 17	676 ± 4	62 ± 4	131 ± 7
7	Car cabin	65 ± 6	4880 ± 150	88 ± 7	48 ± 2
8	Car cabin	71 ± 5	522 ± 7	124 ± 5	127 ± 5
9	Car cabin	93 ± 12	170 ± 11	52 ± 5	43 ± 3
10	SRM 2585	369 ± 8	121 ± 4	259 ± 5	322 ± 6
Min.		65	42	46	22
Max.		657	4883	149	127
Mean ^a		160	780	91	71

^aMean values without considering the SRM 2585 material

As far as we are aware, the data shown in **Table 4** constitutes the first report of the target compounds in indoor dust. Their average values are approximately 10 times lower than the mean levels of other UV-filters, used in sunscreens and other personal care products (e.g. octocrylene and 2-ethylhexyl-p-methoxycinnamate), measured in dust samples collected in the same geographical area and also in the NIST reference material SRM 2585 [8]. However, they are similar to, or even higher than those previously reported by Nakata and co-workers in sediments from heavily polluted estuaries in Japan [16].

Considering an average intake of 20 mg of indoor dust per day and person [25], 22 ng of the four Tinuvin species included in **Table 4** will enter the body of an adult person. In the same work, a 2-3 times higher intake of settled dust was estimated for crawling infants [25].

4. Conclusions

A novel, automated sample extraction method for the determination of five benzotriazole light stabilizers in dust samples has been proposed. Its major advantages are: (1) moderate consumption of organic solvents, (2) high extraction efficiency and (3) integration of extraction and clean-up in the same step, rendering a ready to inject extract, immediately after concentration to a lower volume. When combined with GC-MS/MS determination, the proposed method enables the sensitive determination of target compounds in dust from indoor environments. Four of the five species involved in this study were found in all the processed samples at significant concentrations. The results point out to the widespread use of these additives in plastics, upholstery, paints and building materials and thus to the chronic exposure of humans to Tinuvin in confined areas through inhalation and oral ingestion of dust.

Acknowledgements

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2. Determinación de Fungicidas



Los fungicidas son los fitosanitarios más empleados en viticultura para prevenir, y tratar, enfermedades provocadas por hongos que merman la producción y la calidad de la uva. Dependiendo de una combinación compleja de variables (atmosféricas, prácticas agrícolas, modo de acción y estructura química de los fitosanitarios empleados, así como operaciones de bodega), una fracción de los fungicidas aplicada en los viñedos, persiste en la uva en el momento de su recolección, pudiendo pasar al vino [1]. Con objeto de evaluar los niveles de fungicidas en vinos, y estimar la exposición de los consumidores, es preciso disponer de metodología analítica robusta para su determinación en esta matriz. En la bibliografía se describen diferentes técnicas de extracción, como LLE, SPE y varias modalidades de microextracción, seguidas de una etapa de determinación mediante GC-MS, GC-ECD, LC-UV o LC-MS. En ocasiones, sobre todo en el caso de vinos tintos, los extractos procedentes de SPE y LLE son demasiado complejos para su inyección en el sistema cromatográfico, por lo que se requieren etapas posteriores de limpieza que aumentan el coste y la complejidad de las determinaciones [2]. En esta memoria se ha desarrollado un procedimiento en el que se combina SPE con LC-MS/MS para la determinación de varios fungicidas en muestras de vino, incidiendo en mejorar la selectividad de la etapa de extracción, de manera que se minimicen los efectos de matriz durante el proceso de ionización en *electrospray* (ESI), garantizando la exactitud de los resultados obtenidos mediante calibración externa.

Como consecuencia de la fumigación de viñedos, y otros cultivos agrícolas, del lavado de los cultivos por el agua de lluvia, deposición directa, y otros fenómenos, los fungicidas contaminan los suelos agrícolas donde pueden permanecer un tiempo variable. Por lo tanto, pueden suponer un riesgo para el ecosistema del suelo [3], así como de filtración hacia las aguas subterráneas y superficiales, desembocando en un problema medioambiental [4]. Para la extracción de fungicidas de suelo se emplean tanto técnicas clásicas, como SLE [5], como otras más modernas, por ej. PLE [6]. En el primer caso, además del consumo de material y tiempo, la influencia del tipo de suelo en la eficacia de extracción es habitual [5], siendo necesario recurrir al método de adiciones estándar para obtener resultados cuantitativos. En el caso de PLE, aunque es una técnica con un alto grado de automatización, se necesita un equipamiento especial que incrementa el coste de las determinaciones. MSPD presenta un bajo coste y moderado consumo de disolventes orgánicos, aunque no ha sido considerada de forma sistemática para la extracción de fungicidas de muestras de suelo. Dentro de esta memoria, se ha investigado la aplicabilidad de MSPD a la extracción de 15 fungicidas, pertenecientes a 8 familias

químicas diferentes, de suelos agrícolas con diferentes porcentajes de materia orgánica, con especial atención a suelos de viñedo.

A continuación, se muestran los esquemas de las metodologías desarrolladas en esta Tesis para la determinación de fungicidas en vino (**Figura III.2.1**) y en suelo de viñedo (**Figura III.2.2**).

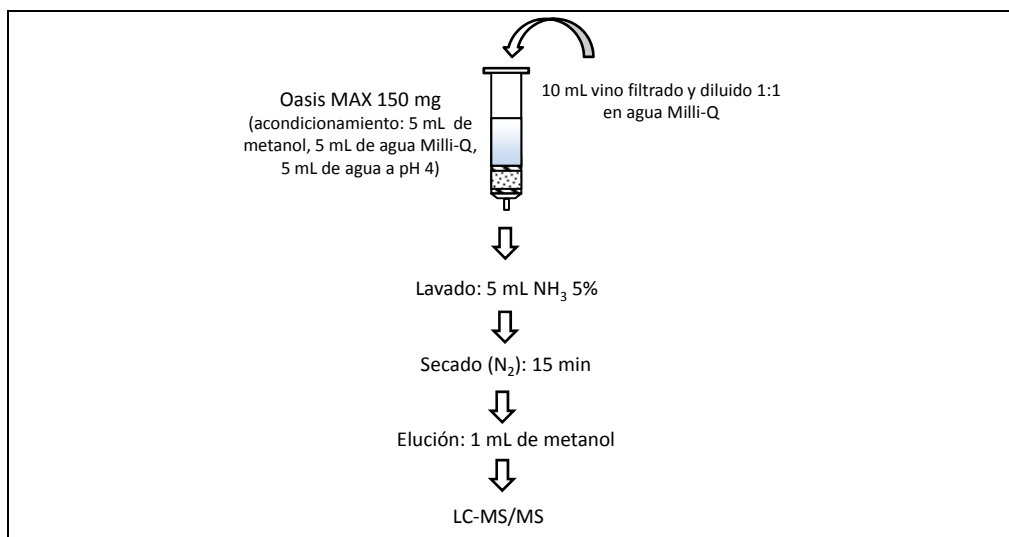


Figura III.2.1. Esquema seguido para la determinación de fungicidas en vino mediante SPE y LC-MS/MS.

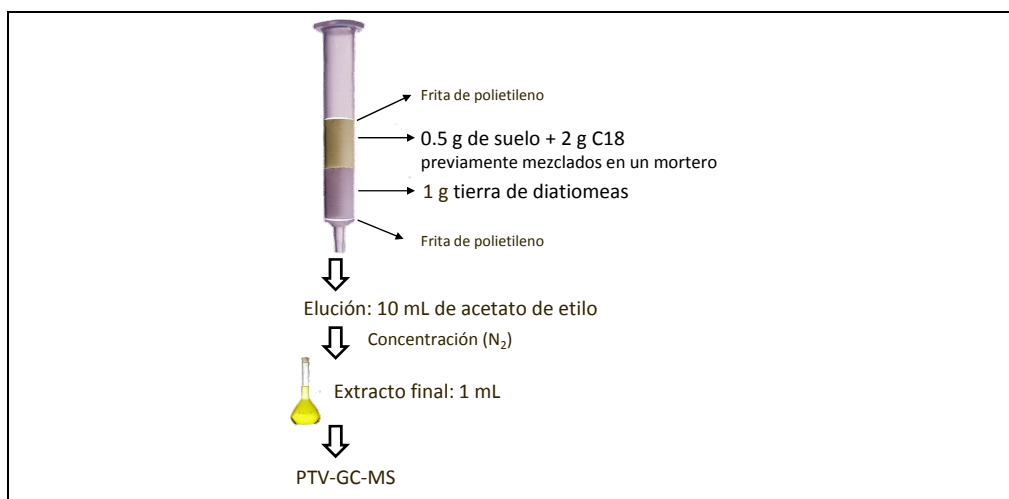


Figura III.2.2. Esquema de trabajo para la determinación de fungicidas en suelo mediante MSPD y GC-MS.

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Publicación III.2.1:

Determination of fungicides in wine by mixed-mode solid-phase extraction and liquid chromatography coupled to tandem mass spectrometry

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Determination of fungicides in wine by mixed-mode solid-phase extraction and liquid chromatography coupled to tandem mass spectrometry

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Abstract

A novel procedure for the determination of nine selected fungicides (metalaxyl-M, azoxystrobin, myclobutanil, flusilazole, penconazole, tebuconazole, propiconazole, diniconazole and difenoconazole) in wine samples is presented. Sample enrichment and purification is simultaneously performed using mixed-mode, anion exchange and reversed-phase, OASIS MAX solid-phase extraction (SPE) cartridges. Analytes were determined by liquid chromatography coupled to tandem mass spectrometry using atmospheric pressure electrospray ionization (LC–ESI–MS/MS). Parameters affecting the chromatographic determination and the extraction-purification processes were thoroughly investigated. Under optimized conditions, 10 mL of wine were firstly diluted 1:1 with ultrapure water and then passed through the mixed-mode SPE cartridge at a flow of ca. 5 mL min⁻¹. After a washing step with 5 mL of an aqueous NH₄OH solution (5%, w:v), analytes were recovered with just 1 mL of methanol and injected in the LC–MS/MS system without any additional purification. The selective extraction process avoided significant changes in the ionization efficiency for red and white wine extracts in comparison with pure standards in methanol. Performance of the method was good in terms of precision (RSDs < 11%) and accuracy (absolute recoveries > 72%, determined against pure standards in methanol) reporting method LOQs in the range of 0.01–0.8 ng mL⁻¹ for target compounds, which are far below the EU maxima residue levels (MRLs) for fungicides in vinification grapes and wine. Several commercial wines from different geographic areas in Spain were analyzed. In most samples, metalaxyl-M and azoxystrobin were found at concentrations up to several ng mL⁻¹.

Keywords: Fungicides, wine analysis, mixed-mode solid phase extraction, liquid chromatography coupled to tandem mass spectrometry, pesticides

1. Introduction

Pesticides are used all over the world to protect vines against diseases caused by insects, fungi, molds and other agents. Fungicides belong to this group of high volume production agrochemicals, being mainly used for treating grey rot, downy and powdery mildew and oidium [1,2]. It is well-known that fungicide residues existing in vinification grapes can be removed in a high degree by different dissipation mechanisms (sorption, degradation, etc.) during the wine production process; however, depending on factors such as their physico-chemical properties and wine-making operations, some of these agrochemicals can be also found at low concentration levels in the final commercial product [2–6]. The maxima residue limits (MRLs) allowed in the European Community for different fungicides in vinification grapes are well established and legislated [7]; however, despite of being a direct exposure source to consumers, MRLs for wine are still scarcely regulated [8]. Thus, in order to establish future legal measures, it is mandatory to perform both monitoring and toxicological studies to assess the background levels of these chemicals in different wines and to evaluate the chronic exposure of wine consumers to these contaminants, respectively.

Analysis of wine samples is a challenging issue due to the high complexity of the matrix. Depending on the fungicides nature, either gas chromatography (GC) [9-11] or liquid chromatography (LC) [12-14] coupled to different detectors are frequently employed as determination techniques. In terms of selectivity, mass spectrometry (MS) represents, at present, one of the most powerful detection tools for both chromatographic techniques. Particularly, LC-tandem MS appears increasingly in food applications related to this type of compounds [15-20] and it has been proven to provide better performance than GC-MS [21].

Traditionally, sample preparation is performed either by liquid-liquid extraction (LLE) using organic solvents [10,13] or solid-phase extraction (SPE), using reversed-phase materials [11,14,20]. LLE and commonly used reversed-phase SPE sorbents, such as C18 or Oasis HLB, usually render extracts containing too many interferences, which require further clean-up before analysis and an increase in time and cost of the sample

preparation. Purification approaches can be based on additional SPE steps using either normal-phase sorbents [12,22] or cartridges containing a combination of materials such as aminopropyl-MgSO₄ [11], or graphitized carbon black/primary secondary amine (GCB/PSA) [6,23]. Recently, the application of dispersive liquid-liquid microextraction (DLLME) has also been successfully reported for the purification of wine extracts obtained by SPE [24]. Ion-exchange based sorbents have been used by Schermerhorn et al. during fungicides determination for purification of fruit and water extracts [25]; however, to the best of our knowledge, no analytical methodology based on ion-exchange SPE has been optimized to perform simultaneous wine samples enrichment and purification.

More recently, miniaturized enrichment techniques such as solid-phase microextraction (SPME) have also been applied to the determination of fungicides in wine using polyacrylate (PA) [26], polydimethylsiloxane (PDMS) [9] or PDMS-divinylbenzene (DVB) fibers [27]. Despite SPME normally provides a higher selectivity than SPE, factors such as the ethanol content and the complexity of wine matrices reduce its applicability in terms of extraction efficiency compared to water samples [28,29]. These limitations are also common to stir-bar sorptive extraction (SBSE) [30], which, in addition, is only valid for the concentration of low polar fungicides.

In this work, a SPE methodology based on the use of a mixed-mode sorbent, that combines ionic exchange and reversed-phase mechanisms, for the extraction of nine selected fungicides (belonging to azolic, phenylamide and strobilurine classes) from wine samples is presented. The combination of the above retention mechanisms during sample preparation improved the selectivity of the process, avoiding further tedious and labour intensive cleanup steps. Different parameters affecting the extraction process (sorbent/eluent type and amount) have been optimized. Analysis was performed by LC-tandem MS after optimization of chromatographic conditions and MS parameters. Under final conditions, good performance was obtained for both red and white wines. Levels of fungicides in some commercial wines are also reported.

2. Experimental

2.1. Standards, solvents and material

Standards of azoxystrobin (99.9%), diniconazole (99.1%), difenoconazole (97%), flusilazole (99.8%), metalaxyl-M (99%), myclobutanil (99.4%), penconazole (99.1%), propiconazole (98.6%) and tebuconazole (99.6%), as well as ammonium acetate were purchased from Riedel de Haën (Seelze, Germany). The chemical structures of these compounds and some properties of relevance to optimize extraction (SPE) and LC separation processes are compiled in **Table 1**. Acetic and formic acid, acetone, ammonium hydroxide and HPLC-grade methanol were acquired from Merck (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q system (Millipore, Billerica, MA, USA). Stock solutions of each compound were prepared in methanol. Further dilutions and mixtures of them were made in the same solvent. Standards in methanol were stored in the dark, at 4 °C for a maximum of two months.

2.2. Samples and sample preparation

Red and white wine samples obtained from local supermarkets were used in this study. After opening the bottles, wine was filtered and processed within 48 h. Optimization of sample preparation conditions was performed using two spiked pooled samples of red (*Grenache* and *Cabernet Sauvignon*) and white (*Albariño* and *Palomino*) wines. Spiked samples were maintained at 4 °C and used for a maximum of 48 h. Unless otherwise stated, during optimization of extraction conditions samples were spiked at the 50 ng mL⁻¹ level.

SPE cartridges containing either OASIS HLB (60 mg) or OASIS MAX sorbents (60 and 150 mg) were provided by Waters (Milford, MA, USA).

Cellulose acetate membrane filters (0.45 µm pore size) were purchased from Millipore (Bedford, MA, USA).

Table1. Names, CAS numbers, structures, pK_a and octanol-water partition coefficients of selected fungicides.

Name (abbreviation), CAS number and properties	Structure	Name (abbreviation), CAS number, pK_a and Log K_{ow}	Structure
Azoxystrobin (AZO) CAS: 131860-33-8 pK_a : -0.67 log K_{ow} : 5.13		Metalaxyl-M (MET) CAS: 70630-17-0 pK_a : 1.41 log K_{ow} : 2.15	
Difenoconazole (DIF) CAS: 119446-68-3 pK_a : 3.06 log K_{ow} : 4.92		Penconazole (PEN) CAS: 66246-88-6 pK_a : 2.83 log K_{ow} : 3.67	
Diniconazole (DIN) CAS: 70217-36-6 pK_a : 12.89, 2.19 log K_{ow} : 4.23		Propiconazole (PRO) CAS: 60207-90-1 pK_a : 3.06 log K_{ow} : 3.88	
Fluxilazole (FLU) CAS: 85509-19-9 pK_a : 3.02 log K_{ow} : 3.84		Tebuconazole (TEB) CAS: 107534-96-3 pK_a : 13.70, 3.39 log K_{ow} : 3.58	
Myclobutanil (MYC) CAS: 88671-89-0 pK_a : 2.30 log K_{ow} : 2.82			

Spiked and non-spiked samples were diluted (1:1) with ultrapure water and passed through the SPE cartridges at a flow of approximately 5 mL min^{-1} . After a washing step, the sorbent was dried using a stream of nitrogen and analytes were eluted with methanol. Breakthrough studies were carried out passing the spiked samples through two serially connected cartridges. The minimum elution volume was established by collecting consecutive fractions of methanol (1 mL each) from SPE cartridges.

Under optimal conditions, 10 mL wine samples were diluted with the same volume of ultrapure water and concentrated using Oasis MAX cartridges (150 mg),

previously conditioned with methanol, ultrapure water (pH 6) and ultrapure water (pH 4) (5 mL each). Afterwards, cartridges were washed with 5 mL of a 5% (w:v) ammonium hydroxide solution, dried during 15 min with a gentle nitrogen stream and eluted with just 1 mL of methanol.

2.3. Equipment

Analytes were determined using a Varian (Walnut Creek, CA, USA) LC-MS/MS system. The LC instrument comprised two isocratic, high-pressure mixing pumps (Varian ProStar 210), an autosampler and a thermostated compartment for the column (Varian ProStar 410). The mass spectrometer was a triple quadrupole (Varian MS 1200L) furnished with an electrospray ionization (ESI) interface. A Varian ProStar 335 diode array detector (190-950 nm) connected in series after the column was occasionally used to test the complexity of the extracts obtained from red and white wines. The LC-MS/MS instrument was entirely controlled by the Varian MS Workstation Version 6.9 software. The same software was used to monitor the signal of the diode array detector.

Compounds were separated using a Kromasil C18 column (5 μ m; 2.1 mm $\times$ 100 mm) acquired from Sugelabor (Madrid, SPAIN) and connected to a C18 (2 mm $\times$ 4 mm) guard cartridge supplied by Phenomenex (Torrance, CA, USA). Ultrapure water (A) and methanol (B), containing different amounts of formic acid or ammonium acetate as modifiers, were employed as mobile phases. Under final conditions, both phases were modified with formic acid (0.01%) and compounds were separated using the following gradient: 0-2 min, 15% B; 6min, 40% B; 10 min, 70% B; 18 min, 75% B; 21-24 min, 100% B; 27-36 min, 15% B. The mobile phase flow was set at 0.25 mL min⁻¹ and the temperature of the column fixed at 35 °C. The injection volume for standards and sample extracts was 15 μ L.

Nitrogen (99.999%), used as nebulising (50 PSI) and drying gas (300 °C, 21 PSI) in the ESI source, was provided by a high purity generator (Domnick Hunter, Durham, UK). The ESI interface was operated in the positive mode and the voltage of the ESI needle fixed at 5000 V. The temperature of the ESI housing was set at 50 °C. Argon (99.999%) was employed as collision gas (2.2 mTorr) in the mass spectrometer. Fungicides were recorded in the multiple reaction monitoring (MRM) mode, using two transitions per compound and a dwell time of 0.18 s per transition. The most intense one was used to quantify the

response of each species in standards and SPE extracts from real wine samples. **Table 2** summarizes retention times, most intense MS/MS transitions, capillary voltages and collision energies for target species. Conversely to GC, the LC column was not able to separate the isomers of propiconazole and diniconazole [24].

Table 2. Retention times and optimized ESI(+)-MS/MS conditions for selected fungicides. CV (capillary voltage, V); CE (collision energy, eV).

Compound	Ret. time (min)	MRM1 (quantification)	CV/CE	MRM2 (confirmation)	CV/CE	MRM1:MRM2 Ratio
MET	12.27	280>220	72/8	280>192	72/11.5	64:36
AZO	12.93	404>372	39/10	404>329	39/25.5	82:18
MYC	14.54	289>70	70/12.5	289>125	70/24.5	72:28
FLU	16.42	316>165	63/18.5	316>247	63/13	51:49
PEN	17.22	284>159	71/21	284>70	71/11	55:45
TEB	17.62	308>70	65/12	308>125	65/27	83:17
PRO	17.92	342>159	56/20.5	342>69	56/13	94:6
DIN	19.79	326>70	60/14	326>159	60/23	80:20
DIF	20.19	406>251	39/18.5	406>337	39/12	82:18

2.4. Extraction efficiency, matrix effects and LOQ calculations

Absolute recoveries (as percentages) provided by the proposed method were calculated as: $\%R = [(C_s - C_b)/C_t] \times 100$, where C_s is the measured concentration in the extracts from spiked wine samples, C_b is the concentration measured in un-spiked aliquots of the same wine and C_t is the theoretical concentration, being C_s and C_b values established by external calibration. Matrix effects (%) during electrospray ionization were evaluated as recoveries using the difference of peak areas measured for spiked (A_{se}) and non-spiked extracts (A_{ne}) from 10 mL wine samples (addition was done after SPE) divided by the response obtained for a standard with the same concentration prepared in methanol (A_{ss}): $\%ME = ((A_{se} - A_{ne})/A_{ss}) \times 100$. Therefore, the ion suppression percentage corresponds to $100 - \%ME$. LOQs of the whole methodology ($LOQ_{SPE-LC-MS/MS}$) were calculated from instrumental $LOQ_{LC-MS/MS}$, taking into account mean absolute recoveries: $LOQ_{SPE-LC-MS/MS} = LOQ_{LC-MS/MS} \times (V_{ext} / V_s R)$, being V_{ext} and V_s the extract and sample volumes, respectively.

3. Results and discussion

3.1. Optimization of LC–MS/MS parameters

3.1.1. Ionization and fragmentation conditions

The optimization of ESI-MS/MS parameters was performed by direct infusion of $5\text{ }\mu\text{g mL}^{-1}$ individual standards in methanol using an isocratic flow of methanol–ultrapure water (1:1) ($50\text{ }\mu\text{L min}^{-1}$). Both, positive and negative ESI were investigated; however, compounds were only ionized in a significant extent in the positive mode, which agrees with their basic character. The capillary voltage was fixed at the maximum parent ion $[\text{M}+\text{H}]^+$ intensity, and afterwards, the collision energy for the most intense daughter ions was also optimized. Once main MS/MS transitions were identified for each compound, the fragmentation pattern was investigated. In the case of azoxystrobin, the main transition ($404\rightarrow 372\text{ m/z}$) corresponds to the methanol loss [17–21]. Additional losses of carbonyl and methyl groups produce 344 and 329 m/z ions, respectively (**Figure 1A**). Although, the $404\rightarrow 344\text{ m/z}$ transition has been used as quantifier by most authors [17–21], the m/z 372 showed higher intensity and was chosen in our case.

Methanol loss is also observed during the fragmentation of metalaxyl-M. This yields the minority m/z 248 fragment and it is followed by a CO elimination giving the quantification ion m/z 220 [17,19–21] (**Figure 1B**). Afterwards, the cleavage of the C–N bond takes place producing the m/z 192 species. Some authors have used the m/z 160 ion, corresponding to a further methanol loss from the m/z 192 fragment as primary product ion [21], but under our ionization conditions it presented lower intensity.

The cleavage of the C–N bond to release the triazolic ring is a common fragmentation pattern in the case of triazolic compounds [15]. However, there are some differences in fragmentation depending on fungicide structure. On one hand, PEN, DIN, MYC and TEB follow different rearrangements to produce the m/z 70 product ion [15] which is commonly used as quantification (MYC, TEB and DIN) or as confirmation (PEN) ion. The other positively charged fragment for the above compounds corresponded to a di- or monochlorinated tropylium ion (m/z 159 and 125, respectively) (**Table 2**). On the other hand, PRO, FLU and DIF undergo a C–N bond simple cleavage to release the much less intense m/z 69 product ion. The $[\text{M}-\text{C}_2\text{H}_3\text{N}_3]^+$ ions correspond to m/z 247 and m/z 337

for FLU and DIF, respectively. In case of PRO, a transposition of the methylene group, followed by a CO₂ loss, yields the m/z 159 ion [15,18], a similar mechanism yields the m/z 251 ion for DIF (see **Table 2**).

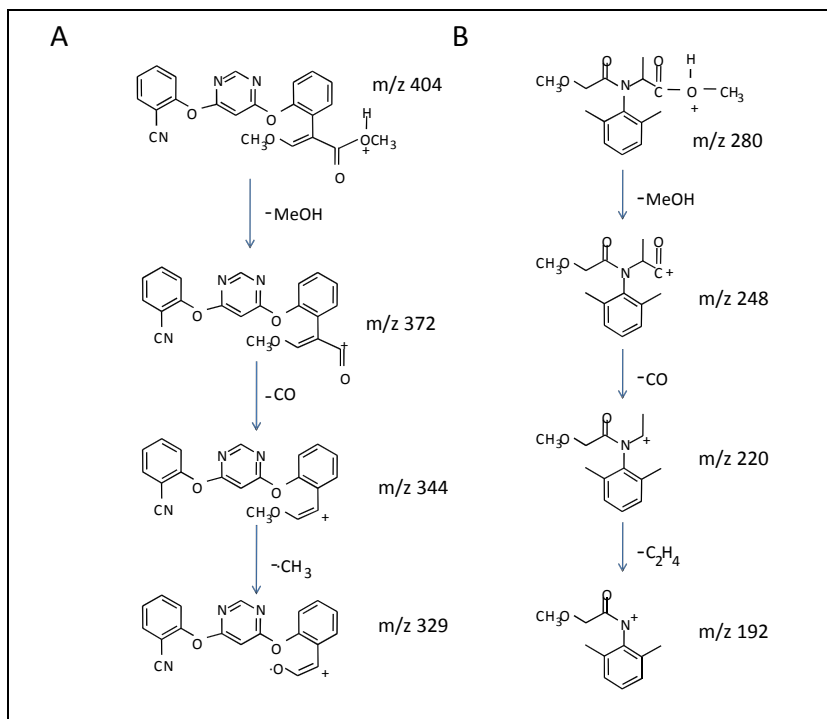


Figure 1. LC-ESI-MS/MS fragmentation pattern for A, azoxystrobin and B, metalaxyl-M.

3.1.2. Selection of modifier

The ionic strength and the pH of the mobile phase are parameters that can affect notoriously the ionization efficiency and the chromatographic separation. Two commonly used modifiers in LC-MS, one with acidic nature (formic acid) and another with saline character (ammonium acetate), were separately evaluated for signal enhancement. With this purpose, a standard of 250 ng mL⁻¹ was injected in the chromatographic system in consecutive runs increasing the amount of modifier in the mobile phase up to 0.5% of formic acid, or up to 3mM ammonium acetate. **Figure 2** shows the signal variation with the increase of formic acid content in the mobile phase. As it can be noticed, just a small percentage of acidic modifier (0.01%) is enough to provide a significant signal increase for the seven triazolic species, which show a higher basic character than AZO and MET. The

ionization is favored because a slightly acid medium improves the formation of the $[M+H]^+$ precursor ions, and consequently, the intensity of MRM transitions. On the other hand, higher percentages lead to a progressive decrease in the monitored responses, possibly due to ionic suppression phenomena.

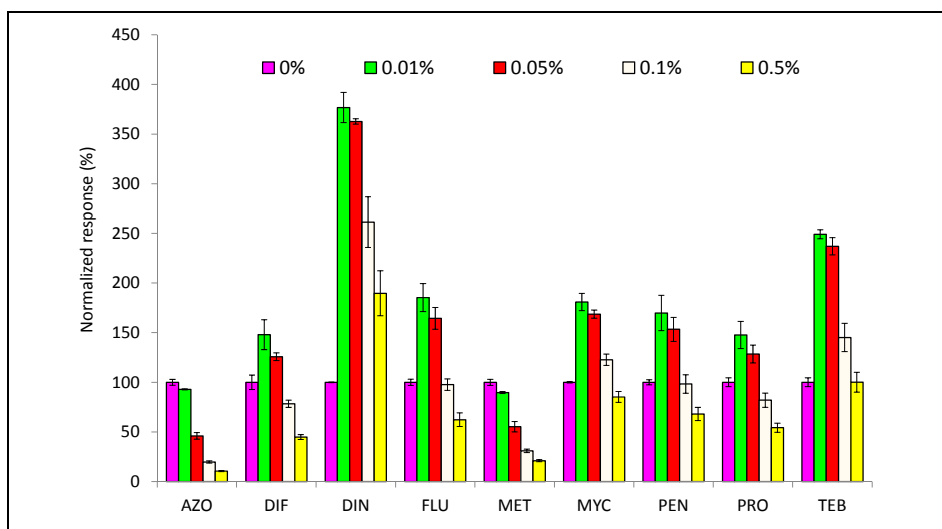


Figure 2. Influence of formic acid percentage on the ionization efficiency.

The addition of ammonium acetate did not produce a significant improvement in the response, and slightly reduced the obtained peak areas at concentrations higher than 0.05 mM, data not shown. No changes in the LC separation efficiency were observed for the evaluated modifiers concentration range. Thus, based on the above comments, a percentage of 0.01% formic acid was selected as mobile phase modifier.

3.1.3. LC–MS/MS performance

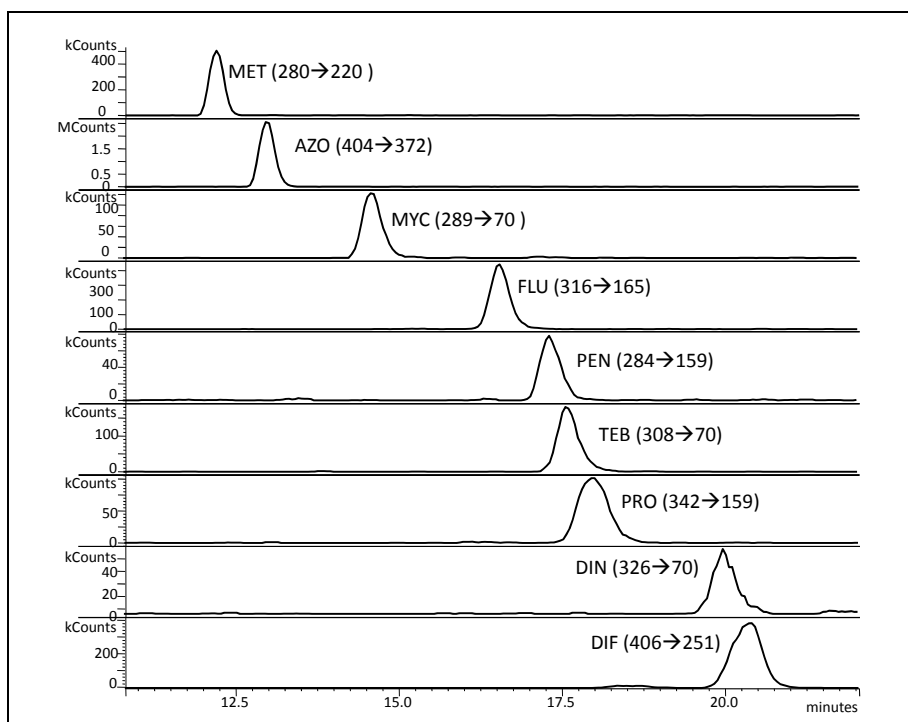
Instrumental performance is summarized in **Table 3**. Calibration standard solutions at six different levels, in the range LOQ–1000 ng mL⁻¹, were injected in the system, providing a linear relationship between peak area and concentration with determination coefficients (R^2) higher than 0.997 for all analytes.

Table 3. Linearity, instrumental limits of quantification (LOQs), repeatability and reproducibility of the LC-MS/MS system

Compound	Linearity, R^2 (LOQs-1000 ng mL ⁻¹)	LOQs (ng mL ⁻¹)	Repeatability (RSD, %) ^a		Reproducibility (RSD, %) ^b	
			20 ng mL ⁻¹	500 ng mL ⁻¹	20 ng mL ⁻¹	500 ng mL ⁻¹
MET	0.9987	1	7.1	1.1	8.8	5.2
AZO	0.9978	0.1	3.1	2	6.3	6.7
MYC	0.9996	2.5	1.4	0.5	6.9	4.6
FLU	0.9998	1	4.7	1.0	8.5	6.8
PEN	0.9992	2.2	3.5	1.4	8.9	4.3
TEB	0.9997	2	10.2	0.4	9.5	3.9
PRO	0.9998	2.5	7.8	0.5	8.3	5.4
DIN	0.9997	7	7.4	1.2	10.6	7.4
DIF	0.9999	1	9.0	1.9	7.3	5.4

^a n= 3 injections in the same day. ^b n= 9 injections in 3 consecutive days.

Limits of quantification determined by a signal to noise ratio of 10 ($S/N = 10$) were in the range 0.1 ng mL⁻¹ obtained for AZO till 7 ng mL⁻¹ for DIN. **Figure 3** shows a chromatogram obtained for a standard solution of target fungicides (20 ng mL⁻¹) monitoring the primary transitions. As it can be noticed, after optimization of the mobile phase gradient, fungicides were separated in a chromatographic run of about 20 min.

**Figure 3.** MRM chromatogram for a standard solution of target fungicides (20 ng mL⁻¹).

Instrumental response precision was investigated with standards at two concentration levels: 20 and 500 ng mL⁻¹. Relative standard deviations (RSD, %) for consecutive injections (n = 3 replicates) made in the same day ranged between 0.4 and 10.2%, and a maximum RSD of 10.6% was obtained for injections (n = 9 replicates) in three consecutive days.

3.2. Optimization of SPE conditions

3.2.1. Evaluation of sorbent selectivity

Two polymeric materials were evaluated as potential sorbents to concentrate wine samples. They were (1) the hydrophilic–lipophilic balance OASIS HLB sorbent, commonly found in environmental and food applications, and (2) the OASIS-MAX material which combines the same reversed-phase interactions as OASIS HLB (Van der Waals and π - π interactions) with an additional mechanism of anionic exchange, based on quaternary amine functionalized groups.

SPE experiments were performed with 20 mL aliquots of spiked, pooled red and white wine samples. Methanol was selected as elution solvent taking into account (1) its compatibility with reversed-phase LC separation process and (2) elution strength. In a first series of extractions, HLB and MAX cartridges (60 mg) were just washed with ultrapure water (5 mL). After being dried, analytes were recovered with 1 mL of methanol. The visual appearance of the corresponding extracts was related to the type of wine and the nature of the SPE sorbent. The most complex ones corresponded to the concentration of red wine using the HLB sorbent, which rendered intense red extracts. For the same matrix, light reddish extracts were noticed using the MAX cartridge, which kept a dark appearance after being eluted with methanol.

In a further series of extractions, the SPE cartridges were washed with 5 mL of an aqueous 5% (w:v) NH₄OH solution. Modification of the clean-up step did not change the color of the HLB extracts; however, it allowed obtaining colorless, completely transparent extracts from the MAX sorbent (**Figure 4**). Both types of extracts were injected in the LC column using UV detection at 254 nm. As depicted in **Figure 4** for red wine, a significantly

better baseline, showing less interferences between 6 and 15 min, was achieved with the mixed mode MAX cartridge.

Using any of both sorbents, phenols, polysaccharides and organic acids are retained together with target fungicides by the reversed-phase mechanism. After concentration of the sample, the basic washing step allows acids and phenols to become ionized and retained strongly by electrostatic interactions with the quaternary amine groups in MAX cartridges. When this sorbent is further eluted with 1 mL of MeOH, just neutral and weak bases, as our target analytes, are recovered. This selective extraction does not occur with the HLB sorbents and therefore is the responsible for cleaner extracts. Consequently, the MAX sorbent was selected to continue this research.

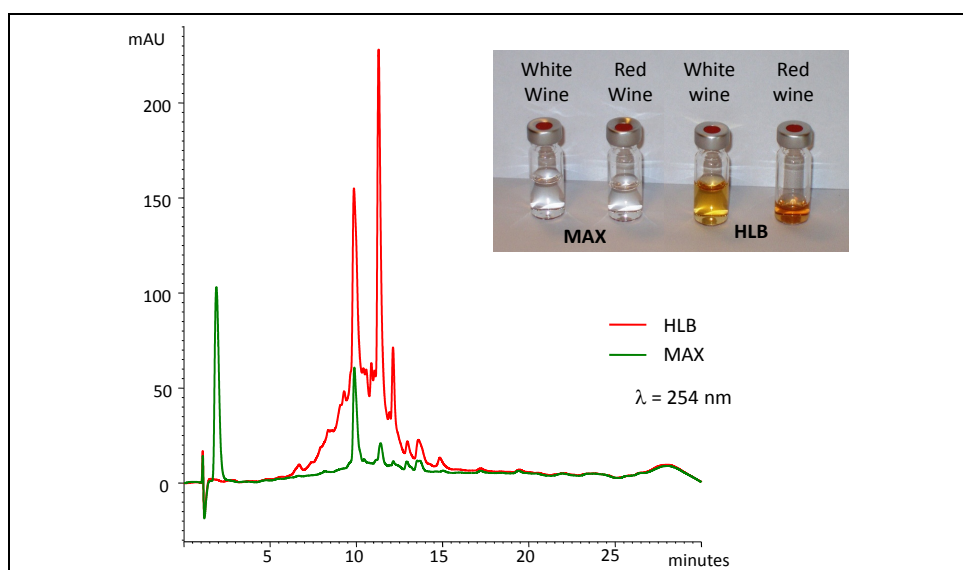


Figure 4. LC-UV chromatograms ($\lambda = 254$ nm) corresponding to red wine extracts obtained after SPE with OASIS HLB and MAX cartridges. Inserted picture shows different extracts appearance.

Breakthrough studies showed that the 60 mg MAX sorbent failed to quantitatively retain MET, the most polar of the selected compounds (**Table 1**), from 20 mL wine samples, with a significant percentage of the fungicide appearing in the second cartridge. Even when the sample intake was reduced to 10 mL, around 10% of the compound passed to the second cartridge. The 150 mg MAX cartridges allowed concentrating up to 10 mL of wine without noticeable breakthrough problems for any compounds. On the other hand, 1 mL of methanol suffices for the elution step.

3.3. Matrix effects

One of the main drawbacks of LC-MS atmospheric pressure ionization interfaces, particularly ESI, is the signal suppression caused by matrix co-eluting components competing with target analytes during the interface ionization process.

Figure 5 shows the normalized responses obtained for 10 mL spiked wine samples versus a reference standard in methanol. For most compounds, relative responses around 90% were noticed in the two types of wine. Responses for MET, the earliest eluting species from the LC column, corresponded to 80% of those obtained for this compound in the reference standard. Globally, data in **Figure 5** prove the existence of just weak (between 10 and 20%) ionic suppression effects for red and white wine samples, pointing to the feasibility of considering external calibration as a valid, straightforward quantification approach.

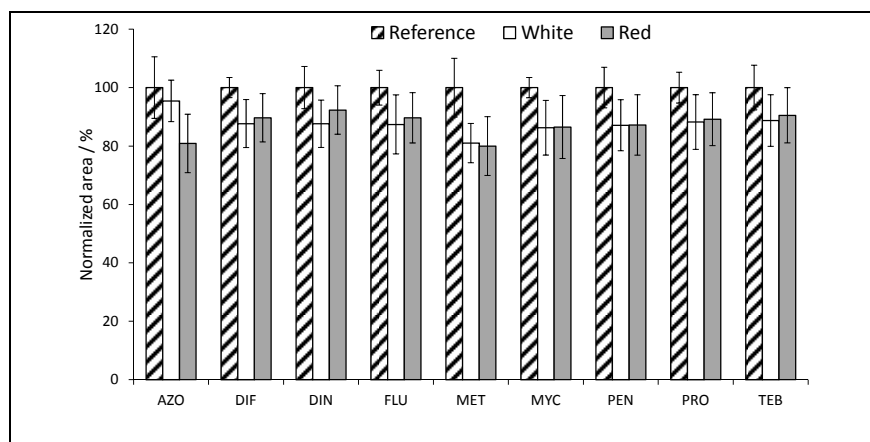


Figure 5. Fungicide recoveries affected by matrix effects for both white and red wines as normalized areas.

3.4. Validation of the analytical methodology

Figures of merit of the proposed methodology are presented in **Table 4**. Precision and accuracy were assessed with the analysis of non-spiked red/white wine samples and also with spiked samples at two levels of concentration: 2 ng mL⁻¹ and 20 ng mL⁻¹. The absolute recoveries of the whole procedure ranged from 72% to 97%. These values, combined with the previous evaluation of matrix effects confirmed that (1) compounds

are quantitatively recovered (retained and then eluted) in the sample preparation process and (2) external calibration is a valid quantification strategy. Acceptable repeatability was also obtained for both wine types, with SD lower than 9 for all fungicides.

Table 4. Accuracy, precision and quantification limits for the proposed methodology for red and white wine at two different concentration levels.

Compound	Absolute recoveries (%) (N= 3)				EU MRLs grape,wine (ng g ⁻¹)	LOQ _{SPE-LC-MS/MS} (ng mL ⁻¹)
	White wine		Red Wine			
	2 ng mL ⁻¹	20 ng mL ⁻¹	2 ng mL ⁻¹	20 ng mL ⁻¹		
MET	83±9	78±5	77±1	73±7	1000	0.1
AZO	78±8	88±2	83±3	77±8	2000	0.01
MYC	80±6	93±1	89±6	81±7	1000	0.3
FLU	72±4	76±2	72±2	74±5	200, 200 ^a	0.1
PEN	78±5	96±1	96±4	84±8	200	0.2
TEB	82±4	97±1	87±6	84±9	2000,2000 ^a	0.2
PRO	82±6	97±1	88±8	84±8	50	0.3
DIN	80±6	97±2	96±5	81±7	200	0.8
DIF	80±3	94±1	93±4	81±7	500	0.1

^aThese MRLs are for wine produced after 1 September 2008

Method LOQs, calculated for free of fungicides wines, were mainly controlled by the instrumental LOQs of the LC–MS/MS system and the enrichment factor of the sample preparation process. The calculated method LOQs ranged from 0.01 µg L⁻¹ for AZO to 0.8 µg L⁻¹ for DIN; thus, they remain far below the EU MRLs considered for this type of fungicides in grapes (0.05–2 µg g⁻¹) and wine (0.2 and 2 µg g⁻¹ for FLU and TEB, respectively) (**Table 4**). They would also fulfill the *Organisation Internationale de la Vigne* (OIV) recommendation which suggests taking for wine 1/10 of grapes MRLs [31]. Moreover, these LOQs are of the same range or better than those reported by other authors using SPE coupled to GC–MS (0.02–0.12 µg L⁻¹) [24], LC–MS (9–28 µg L⁻¹) [14] or LC–MS/MS (2–9 µg L⁻¹) [20] for wine analysis.

3.5. Application to real samples

The developed methodology was applied to the analysis of different commercial red and white wines mostly from northwestern Spain. **Table 5** summarizes the type of grape and geographic denomination of the different processed wines together with found fungicide concentrations. **Figure 6** shows the MRM chromatograms obtained for

compounds detected in the red wine sample code R2, a procedural blank and a 20 ng mL⁻¹ standard solution.

Table 5. Levels of different fungicides in un-spiked wine samples. Average concentrations (ng mL⁻¹) with their standard deviations, n= 3 replicates.

Wine code	Geographic denomination	Type of grape	AZO	MET	MYC	TEB
W1	--	<i>Albariño</i>	n.d.	n.d.	n.d.	n.d.
W2	--	<i>Palomino</i>	0.279±0.002	0.96±0.03	n.d.	n.d.
W3	Rías Baixas	<i>Albariño</i>	2.9±0.2	28.6±0.3	n.d.	n.d.
W4	Rías Baixas	<i>Albariño</i>	0.299±0.006	21.7±0.7	n.d.	n.d.
W5	Ribeiro	<i>Palomino</i>	0.3±0.1	20.5±1.3	n.d.	n.d.
W6	Ribeiro	<i>Dona Branca</i>	n.d.	30.2±0.5	n.d.	n.d.
W7	Bierzo	<i>Dona Branca and Palomino</i>	0.52±0.03	1.8±0.1	n.d.	n.d.
R1	Ribeiro	<i>Garnacha</i>	2.6±0.2	6.5±0.4	n.d.	n.d.
R2	Ribeira Sacra	<i>Mencia</i>	0.56±0.02	8.8±0.7	1.12±0.07	<LOQ
R3	Valdepeñas	<i>Tempranillo and Cabernet Sauvignon</i>	0.390±0.002	1.79±0.06	0.35±0.05	n.d.
R4	Penedés	<i>Tempranillo and Cabernet Sauvignon</i>	0.2±0.1	0.15±0.05	0.35±0.01	0.43±0.04

n.d. not detected; Codes W and R correspond to white and red wines respectively.

In most samples, AZO and MET were detected at concentrations in the range of 0.2-2.9 ng mL⁻¹ and 0.15-30.2 ng mL⁻¹, respectively. These findings are in agreement with the observation of Cabras et al. [1], who reported the incomplete removal of both agrochemicals during wine-making from grapes proceeding from treated vineyards. Detection of TEB also reveals its just partial elimination during the wine-making as postulated by González-Rodríguez et al. [6].

Although at present EU MRLs for MET and AZO have not yet been established in wine, an adequate analytical methodology and reliable occurrence data are powerful tools to develop future legislation. Thus, other European country, Switzerland, has already regulated these fungicides in wine at 0.6 and 0.5 µg g⁻¹, respectively [32].

It must be considered that most data in **Table 5** corresponds to wines from a region with wet weather, where fungi are a common problem in vineyards, and a high number of fungicide treatments are applied before harvest. Anyhow, mean values reported for these samples are in agreement with those measured by other authors. For instance, Trösken et al. [15] found TEB levels between 0.25 and 33 ng mL⁻¹ and MYC levels of 0.5-35 ng mL⁻¹ in red and white wines from different origins. TEB measured

concentration was far below the legal EU ($2 \mu\text{g g}^{-1}$) and Switzerland ($0.3 \mu\text{g g}^{-1}$) MRLs for wine.

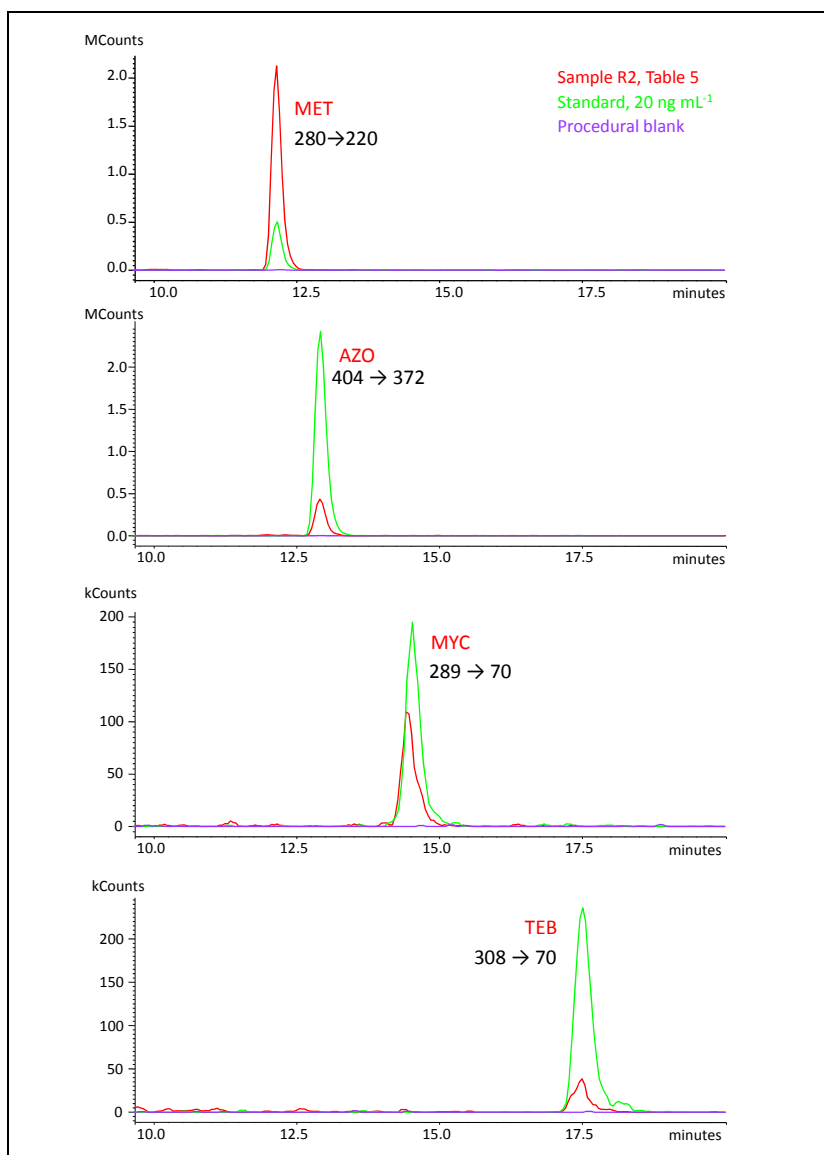


Figure 6. MRM chromatograms showing selected transitions for MET, AZO, MYC and TEB in a red wine sample (code R2, Table 5), a standard solution (20 ng mL^{-1}) and a procedural blank.

4. Conclusions

The analytical methodology for the determination of 9 fungicides in wine samples has been successfully developed. For sample preparation, SPE was optimized using divinylbenzene-N-vinylpyrrolidone functionalized with quaternary amine groups as sorbent and analysis was performed by LC-ESI-tandem MS. The developed method presents good linearity, accuracy and precision with LOQs in the range of 0.01-0.8 ng mL⁻¹ for target fungicides in wine, which are far below EU MRLs for vinification grapes and wine. The high selectivity of the sorbent material makes the SPE based on MAX cartridges a promising alternative to conventional extraction techniques for determination of fungicides in complex matrices such as wine. Its ionic exchange capacity allows the selective retention of matrix interferences and provides purified extracts that can be quantified by external calibration. AZO and particularly MET were found in most of the processed wine samples, with the latter compound being measured at concentrations as high as 30 ng mL⁻¹.

Acknowledgments

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**Optimization of matrix solid-phase dispersion conditions for organic
fungicides determination in soil samples**

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Optimization of matrix solid-phase dispersion conditions for organic fungicides determination in soil samples

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Abstract

A simplified sample preparation method, based on the matrix solid-phase dispersion technique, is proposed for the sensitive determination of 15 organic fungicides in vineyard soils by gas chromatography-mass spectrometry (GC-MS). Under final working conditions, sieved samples (0.5 g) were blended and dispersed with 2 g of C18 and transferred to a polypropylene syringe containing 1 g of diatomaceous earth. Analytes were recovered using 10 mL of ethyl acetate, this extract was concentrated to 1 mL and fungicides determined by GC-MS, without additional clean-up. The method provided recoveries in the range from 74% to 122% for soils with total carbon contents up to 5.5% and it allowed the use of external standard as quantification technique. Inter-day precision, given as relative standard deviations, stayed between 3% and 13%, and the limits of quantification were comprised between 0.6 ng g⁻¹ and 15 ng g⁻¹. Several fungicides were found in the top layer of vineyard soils with the highest detection frequency and maximum concentration corresponding to iprovalicarb. Some real samples were also submitted to pressurized liquid extraction. Measured concentrations were in excellent agreement with those obtained by matrix solid-phase dispersion, which reinforces the accuracy of the latter methodology.

Keywords: Fungicides, gas chromatography-mass spectrometry, matrix solid-phase dispersion, vineyard soil

1. Introduction

Organic fungicides are one of the main groups of pesticides employed in agriculture. Their use is particularly intense in viticulture to prevent, and/or to stop, fungal infections (e.g. downy and powdery mildew, oidium and botrytis) which ruin grapes production [1,2]. During application, in spring and summer, a fraction of these agrochemicals is directly deposited on vineyard soils; thereafter, in autumn, fallen leaves contribute to their indirect introduction in this compartment. On the other hand, fungicides are removed from soil through chemical and biological degradation, volatilization, photo-decomposition, water run-off and plants uptake [3-9]. Considering that some fungicides show soil half-lives of several months [10,11] and that vineyards are repeatedly fumigated (up to 3-4 applications of the same fungicide per campaign) during decades, the global balance of the above referred processes might be a slow, although steady, increase in their soil residues. A continuous exposure to sub-lethal concentrations of fungicides might lead to the apparition of resistant fungal strains, as it has already been reported for certain azolic compounds [12,13]. Assessing the background levels of fungicides in vineyard soils and evaluating their temporal variation is thus advisable to detect and to prevent the above described scenario, as well as to understand the risk of migration to other environmental compartments. In practice, the feasibility of such monitoring studies depends on the availability of multi-analyte procedures, displaying suitable characteristics in terms of analytical performance, cost and also wastes generation.

Gas chromatography (GC) and liquid chromatography (LC), combined with mass spectrometry (MS), provide suitable characteristics for fungicides determination in the extracts from food, agriculture and environmental matrices [1,14,15]. In the particular case of soils, sample preparation remains as the critical step, which controls the sample throughput and wastes generation of the analytical method, contributing also significantly to its cost. Classic approaches rely on solid-liquid extraction of soils with organic solvents, under manual shaking [16] or sonication [17-19]. The use of aqueous extraction solutions followed by liquid-liquid back extraction of fungicides to an organic solvent has also been reported [4,20]. Although effective, the above strategies consume large amounts of organic solvents, normally involve further clean-up steps and thus, they render low sample throughputs [16,18]. Modern sample preparation techniques, such as pressurized liquid extraction (PLE) [21] and microwave assisted extraction (MAE) [22], overcome some

of the above limitations and present a high automation grade; on the other hand, they precise acquiring and maintaining expensive instrumentation.

Matrix solid-phase dispersion (MSPD) extraction combines some interesting features such as moderate consumption of organic solvents, very low cost, use of mild extraction conditions and possibility to integrate extraction and clean-up in the same step [23-25]. Although MSPD was initially intended to be used with biological samples, further studies have demonstrated its usefulness for the extraction of organic compounds from solid matrices such as freeze-dried sludge and dust [26-28]. Despite these recent works, MSPD applications to the extraction of fungicides from soils are still scarce [6] and, as far as we could trace, only three azolic compounds have been considered in previous studies [29].

Thus, the goal of this research was to evaluate the performance of MSPD, followed by GC-MS, for the determination of a group of 15 organic fungicides, belonging to different chemical classes, in vineyard soils. Parameters affecting the efficiency of the sample preparation step have been carefully investigated and optimized, with particular attention focused on dispersant and clean-up sorbents as well as extraction solvents. The effect of soils carbon content in the accuracy of the method is also discussed. Thereafter, its applicability was demonstrated with samples collected from vines in the Northwest of Spain.

2. Experimental

2.1. Solvents, standards and sorbents

n-Hexane, dichloromethane and ethyl acetate, trace analysis grade, were obtained from Merck (Darmstadt, Germany). HPLC grade acetonitrile was also purchased from Merck. Standards of target analytes were acquired from Riedel de Haën (Seelze, Germany) and Aldrich (Milwaukee, WI, USA). Their abbreviated names, CAS numbers, family class and octanol-water (K_{ow}) partition coefficients are summarized in **Table S1**. CYP, PROC, FLUD and FEN are used to treat botrytis infections in vines, whereas the rest of fungicides is employed against oidium and mildew fungus. Most of the selected

Table S1. Abbreviations, CAS numbers, log K_{ow} values, GC-MS determination conditions and instrumental performance for standards of selected compounds.

Analyte	Abbreviation	CAS number	Chemical class	^a log K_{ow}	Retention time (min)	Quantification ions (m/z)	Other ions (m/z)	Linear range (ng mL ⁻¹)	Determination coefficient (R ²)	LOQ (ng mL ⁻¹)
Metaxyl-M	MET	70630-17-0	Phenylamide	1.76	17.83	160	234,206,132	5-500	0.9992	2
Cyprodinil	CYP	121552-61-2	Anilinopyrimidine	4.00	19.49	224	225	1-500	0.9993	0.4
Penconazole	PEN	66246-88-6	Triazole	4.64	19.71	248	250, 159	1-500	0.9992	1
Procymidone	PROC	32809-16-8	Dicarboximide	2.93	20.17	283	285, 255	5-500	0.9966	2
Fludioxonil	FLUD	131341-86-1	Phenylpirrole	3.59	21.49	248	182,154,127	1-500	0.9996	1
Iprovalicarb	IPRO	140923-17-7	Carbamate	3.56	21.58, 21.87	116+134	119, 98	1-500	0.9982	1
Myclobutanil	MYC	88671-89-0	Triazole	3.07	21.69	179	152,154	1-500	0.9992	1
Flusilazole	FLU	85509-19-9	Triazole	3.70	21.78	233	315, 206	1-500	0.9995	1
Diniconazole	DIN	83657-24-3	Triazole	4.34	22.64	268	270, 232	1-500	0.9986	1
Benalaxyl	BEN	71626-11-4	Phenylamide	3.88	23.58	148	206,176,132	1-500	0.9998	0.3
Propiconazole	PRO	60207-90-1	Triazole	3.65	23.75, 23.95	173	259, 193	2-500	0.9997	2
Fenhexamid	FEN	126833-17-8	Hidroxyanilide	3.86	23.86	177	179, 97	1-500	0.9986	1
Tebuconazole	TEB	107534-96-3	Triazole	3.58	24.32	250	252, 125	5-500	0.9990	2
Difenoconazole	DIF	119446-68-3	Triazole	4.90	30.75, 30.88	323	325, 265	10-500	0.9996	3
Azoxystrobin	AZO	131860-33-8	Strobilurin	5.13	31.98	344	388	1-500	0.9994	1

^aValues compiled from SciFinder Scholar Database.

compounds have been previously reported in grapes and wine samples [1,14,15,30], which is indicative of their often application in vines. Individual solutions of each compound were dissolved in methanol, further dilutions and mixtures of them were prepared in the same solvent, when used to fortify soil samples, and in ethyl acetate for optimization of GC-MS determination conditions and quantification purposes. All standard solutions were stored at 4 °C, using light protected glass vessels. Diluted standards and mixture solutions were employed within one month after preparation.

Diatomaceous earth and acid-washed quartz sand were bought from Aldrich. Florisil (60-100 mesh), alumina (150 mesh) and silica (150 mesh) were provided by Aldrich and Merck. C18 (70-230 mesh) was purchased from Agilent (Wilmington, DE, USA). Normal-phase materials were first activated at 130 °C, for 24 h, allowed to cool down in a desiccator and stored in closed, amber vials. Thereafter, they were used directly or after deactivation with a 5% of ultrapure water, depending on the investigated sample preparation conditions.

Empty polypropylene solid-phase extraction (SPE) syringes (15 mL capacity) and 20 µm polyethylene frits were purchased from International Sorbent Technology (Mid Glamorgan, UK).

2.2. Samples and sample preparation

Vineyard soil samples were employed throughout this study; moreover, in some experiments, other agriculture soils, with higher organic matter contents, were also considered. After reception, samples were air-dried in the hood for 2-3 days, sieved and stored at -20 °C. The fraction with a particle size below 0.3 mm was considered in this research. Sample preparation conditions were optimized with a pool of vineyard soils, with a total carbon (TC) content of 1.8%, spiked with selected pesticides at different levels in the range from 0.6 to 4 µg g⁻¹. The spiking procedure consisted of adding a known volume of a methanolic standard to accurately weighed fractions of soil (ca. 0.5 g) in amber glass vessels. Vessels were left open in the hood for 2 hours for solvent evaporation and then closed and stored in the freezer at -20 °C for at least one week before extraction. The accuracy of the optimized method was assessed with soil samples, showing different TC values and spiked at several levels from 0.04 to 0.6 µg g⁻¹.

MSPD extractions were carried out considering different combinations of dispersant sorbents, clean-up materials and extraction solvents. Under final working conditions, 0.5 g samples were mixed and dispersed with 2 g of C18 in mortar, ca. 1 min. Thereafter, the blend was transferred to a polypropylene syringe containing (bottom to top) a polyethylene frit and 1 g of diatomaceous earth. Another frit was placed on top and analytes were recovered with 10 mL of ethyl acetate. The extract was concentrated to ca. 0.5 mL, using a gentle stream of nitrogen at room temperature, and finally adjusted to 1 mL with the required volume of ethyl acetate.

In addition to the above described MSPD protocol, fractions from some vineyard soils were extracted using a pressurized liquid extractor (PLE), model ASE 200, acquired from Dionex (Sunnyvale, CA, USA) and furnished with 11 mL volume stainless-steel cells. Extractions were performed with ethyl acetate under conditions reported elsewhere [21].

2.3. Determination conditions

Analytes were determined by GC-MS, using a Varian (Walnut Creek, CA, USA) 450 GC instrument connected to an ion-trap Varian 240 mass spectrometer (MS). The GC-MS system was equipped with an electron impact (EI) ionization source and a programmable temperature vaporization injector (PTV). Separations were carried out in an Agilent (Wilmington, DE, USA) HP-5MS capillary column (30 m $\times$ 0.25 mm i.d., d_f: 0.25 μ m), operated at a constant helium flow of 1.2 mL min⁻¹. The GC oven was programmed as follows: 55 °C (held for 1 min), first rate at 10 °C min⁻¹ to 200 °C, second rate at 5 °C min⁻¹ to 250 °C, last rate at 30 °C min⁻¹ to 285 °C (held for 10 min). The injector was furnished with a Siltek fritted liner and operated in the solvent venting mode. Its temperature was maintained at 50 °C, for 0.3 min and then increased to 280 °C with a rate of 200 °C min⁻¹. During the first 0.2 min, the solenoid valve was in the split position (split flow 20 mL min⁻¹) to remove the excess of solvent and then, it was switched to the splitless mode. After 4 min, it was turned again to the split position and a flow rate (helium) of 80 mL min⁻¹ used to sweep the liner. Unless otherwise is stated, the injection volume was 10 μ L.

Transfer line, ion source and trap temperatures were set at 285, 200 and 150 °C, respectively. The helium damping gas flow in the mass analyzer was set at 2.5 mL min⁻¹. MS spectra were acquired in the m/z range between 80 and 450 a.m.u., with the

electromultiplier voltage set at 1900 V. Retention times and m/z values for the most intense ions in the spectrum of each fungicide are listed in **Table S1**.

Recoveries of the sample preparation method and levels of target analytes in vineyard soils were determined using external calibration as quantification technique. Calibration curves were built in the range from 1 to 500 ng mL⁻¹, with standards prepared in ethyl acetate. Within this interval, using an injection volume of 10 μ L, the plots of peak area versus concentration fitted a linear model with determination coefficients (R^2) above 0.997 for all species (**Table S1**). For IPRO, PRO and DIF, the sum of peak areas corresponding to both diastereoisomers was considered as response variable.

3. Results and discussion

3.1. Preliminary experiments

Polarity of elution solvent and MSPD sorbents (dispersant and clean-up) exert a major influence on the yield of the extraction. In a first series of extractions, spiked fractions (0.5 g, addition level 4 μ g g⁻¹) of a pooled vineyard soil were dispersed with 1 g of five different sorbents (C18, diatomaceous earth, Florisil, alumina or silica) and loaded in a polypropylene syringe containing a clean-up layer (1 g) of the same material. Normal-phase sorbents were previously activated as described in the Section 2. C18 and diatomaceous earth were employed as received. In all cases, ethyl acetate (15 mL) was used as elution solvent. **Figure 1** compares the normalized peak areas obtained in the above experiments. As observed, the highest responses corresponded to C18 followed by diatomaceous earth. Alumina, silica and, in a lesser extent, Florisil rendered lower signals for most species. Independently of the selected sorbent, none of the compounds was found in a 2nd ethyl acetate fraction (10 mL) passed through the MSPD syringe. This behaviour points out to strong interactions between the activated, normal-phase sorbents and target analytes, which cannot be broken with ethyl acetate. Such interactions were more significant for the triazole type fungicides, particularly in the case of TEB and DIN with an aliphatic hydroxyl group, and also for FEN, which contains a phenolic group in its structure. In fact, these three hydroxylated species could not be recovered, in a detectable amount, from alumina containing in MSPD syringes.

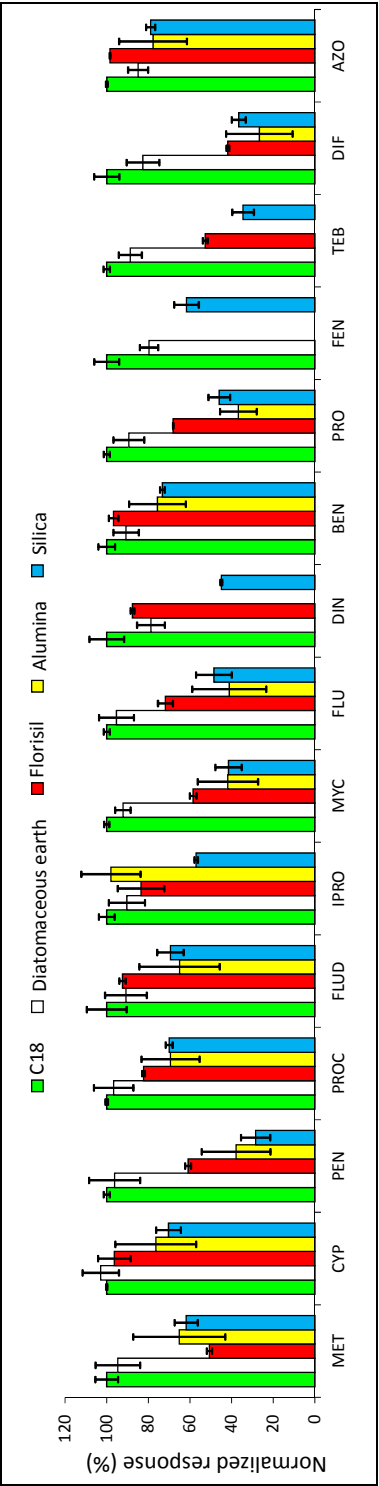


Figure 1. Normalized responses as function of MSPD sorbents (1 g of dispersant and clean-up co-sorbent) using ethyl acetate (15 mL) as extractant, n=3 replicates.

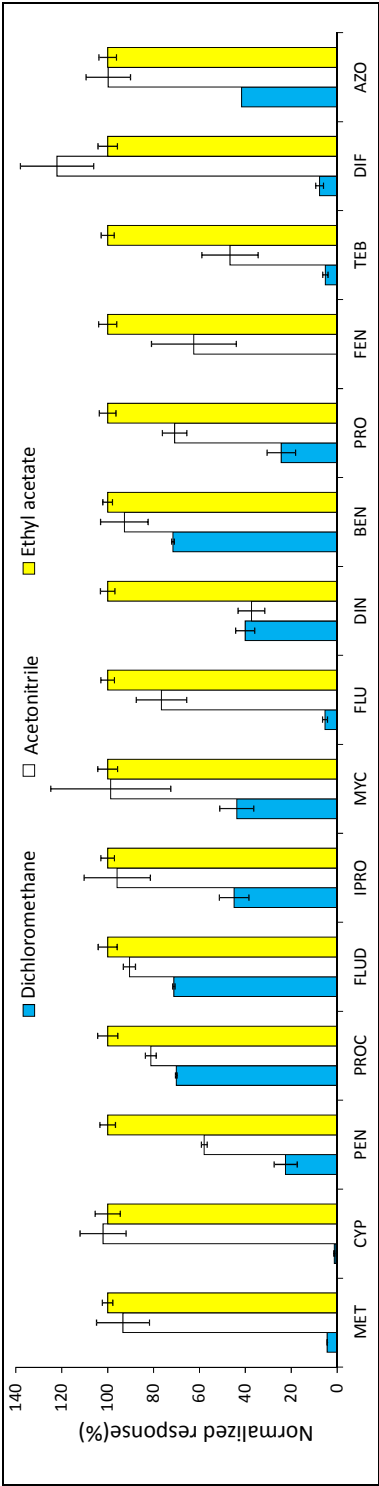


Figure 2. Influence of the elution solvent on the responses of target compounds, n=3 replicates.

In addition to ethyl acetate, n-hexane, dichloromethane and acetonitrile (20 mL aliquots) were also tested as possible extraction solvents. In this case, only C18 was considered as dispersant and clean-up sorbent. **Figure 2** compares the responses for dichloromethane and acetonitrile normalized versus those achieved with ethyl acetate. As observed, the former solvent completely failed (relative recoveries below 10%) to extract five of the considered species (MET, CYP, FLU, TEB and DIF), whereas for the rest it provided significantly lower responses than ethyl acetate. Acetonitrile showed a similar efficiency to ethyl acetate for nine analytes and slightly lower for the rest. Finally, for all compounds, the responses obtained with n-hexane remained below 5% of those achieved with ethyl acetate, data not shown. Hence, ethyl acetate was retained as elution solvent.

3.2. Type and amount of dispersant

The effects of the type and amount of dispersant, as well as the volume of ethyl acetate, in the yield of MSPD extractions were simultaneously investigated using a 2^3 full experimental factorial design, with 4 replicates of the central point. Low and high levels for each variable are summarized in **Table 1**. Whatever the selected dispersant (C18 or diatomaceous earth), 1 g of the former material (C18) was used as clean-up co-sorbent. Ethyl acetate extracts, obtained under those conditions defined in each experiment of the design, were concentrated to 1 mL before GC-MS analysis. Peak areas measured for target compounds were used as response variables to calculate the main effects associated to each experimental factor, their quadratic terms and the two-factor interactions.

Table 1 lists the numerical values of standardized main effects. The absolute value of a main effect is proportional to the influence of the associated factor on the extraction yield. A positive sign indicates an improvement in the efficiency of the MSPD process when the factor varies from the low to the high level, within the domain of the design. Obviously, a negative sign points out to the opposite trend. The most important of the three considered variables was the type of dispersant, with a positive, statistically significant effect (95% confidence level) in the efficiency of the extraction for most species. The only exceptions were IPRO and TEB, with positive effects below the statistical significance threshold and FEN, which showed a negative effect. These data clearly indicate that, globally, soil dispersion with C18 favours the efficiency of the extraction in comparison to diatomaceous earth. The amount of dispersant also played a positive

influence on the extraction, being statistically significant for half of the considered compounds. The volume of ethyl acetate remained as the least important of the investigated variables, **Table 1**. First order interactions and quadratic terms did not affect significantly the extraction yield for any species, data not shown. Taking into account the above comments, C18 (2 g) was selected as dispersant, whereas the volume of ethyl acetate was initially maintained in the upper level of the design (15 mL).

Table 1. Experimental domain and standardized values of main effects obtained from the experimental factorial design.

Factor	Dispersant type	Dispersant mass (g)	Ethyl acetate volume (mL)
Low level	Diatomaceous earth	0.5	10
High level	C18	2	15
MET	3.50 ^a	1.88	0.28
CYP	3.76 ^a	2.51	0.6
PEN	7.73 ^a	3.29 ^a	1.22
PROC	4.75 ^a	3.06 ^a	0.62
FLUD	4.07 ^a	2.70	1.07
IPRO	1.56	2.56	0.34
MYC	5.83 ^a	3.03 ^a	0.91
FLU	5.98 ^a	2.96 ^a	1.04
DIN	5.36 ^a	2.94 ^a	0.92
BEN	2.64 ^a	2.25	0.48
PRO	4.15 ^a	2.81 ^a	1.58
FEN	-3.18 ^a	0.04	0.22
TEB	2.25	2.28	1.14
DIF	9.62 ^a	3.82 ^a	2.47
AZO	2.63 ^a	2.21	1.11

^aStatistically significant factors at the 95% confidence level

3.3. Clean-up co-sorbent and elution solvent volume

Once type and amount of dispersant were selected, the effect of the clean-up sorbent was investigated. To this end, extractions were carried out introducing 1 g of Florisil (previously deactivated with ultrapure water to 5%), diatomaceous earth or C18 in the bottom of the MSPD syringe. Alumina and silica were not considered because of their strong interaction with several fungicides, **Figure 1**. Overall, the clean-up sorbent did not affect significantly to the responses of target compounds, see **Figure S1**. Also, similar GC-MS chromatographic profiles were noticed in all cases, figure not shown. Thus, to reduce the cost of sample preparation, diatomaceous earth was load in the bottom of the MSPD

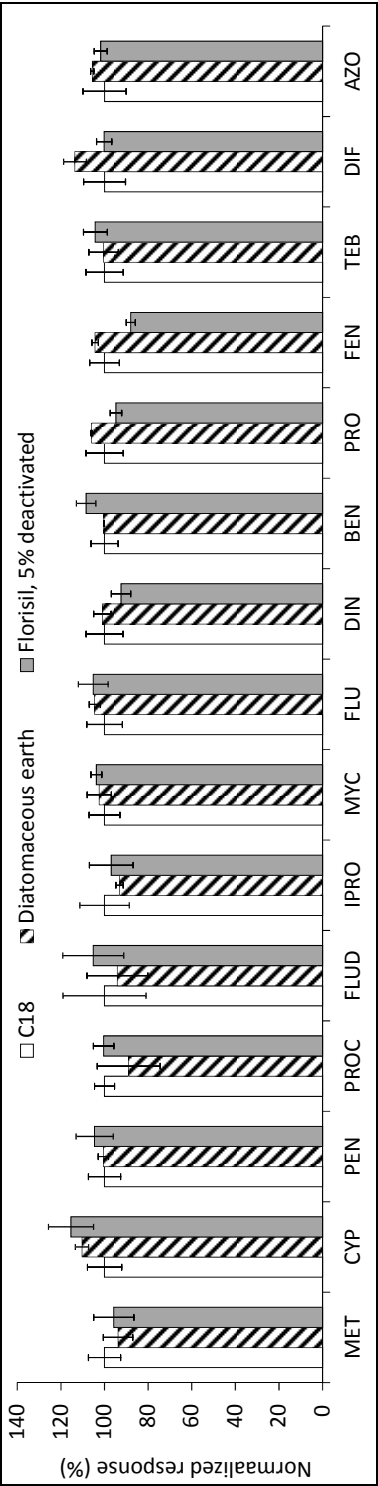


Fig. S1. Normalized responses as function of the clean-up co-sorbent (1 g), n= 3 replicates.

syringe. This layer of inert material served as a filter, avoiding that fine soil particles passed through the pores of the polyethylene frit.

The minimum volume of ethyl acetate required for the quantitative elution of the analytes was investigated collecting consecutive fractions (5 mL each) from the MSPD syringe. Above 80% of the responses measured for all compounds corresponded to the 1st fraction, the 2nd fraction contained between 6% and 21%, and the maximum percentage noticed in the 3rd fraction was 2%. Thus, the volume of ethyl acetate was limited to 10 mL.

Dryness evaporation of the above extract led to losses between 10 and 20%, depending on the compound. On the other hand, significant losses were not noticed when extracts were concentrated to ca. 0.5 mL. Thereafter, they were made up to 1 mL with the required volume of ethyl acetate.

3.4. Performance of the method

Table 2 summarizes the recoveries of the optimized method for three agriculture soils. Differences between responses obtained for spiked and non-spiked fractions of each sample were compared with calibration curves corresponding to standards prepared in ethyl acetate. Recoveries were defined as the ratio between measured and spiked concentrations multiplied by 100. Sample A corresponds to a vineyard soil with a moderate TC value (1.8%). The attained recoveries ranged from 83% to 122% with standard deviations below 17% in all cases (**Table 2**). As expected, the highest variability corresponded to the fraction spiked at the lowest level (40 ng g⁻¹). In order to investigate whether higher carbon contents would affect the extraction efficiency, two different agriculture soils, with TC values of 4.2% (vineyard soil, sample B) and 5.5% (corn field soil, sample C), were also fortified with target compounds at 160 ng g⁻¹ and submitted to MSPD extraction. In this case, recoveries ranged from 74% to 118% with similar standard deviations to those reported for the fraction of sample A (TC 1.8%) fortified at the same level (**Table 2**). The overall mean recoveries obtained in the above experiments varied between 84% for CYP and 111% for IPRO and DIN (**Table 2**). These data point out to the quantitateness of the MSPD extraction, as well as to the suitability of external calibration as quantification technique.

Table 2. Recoveries obtained for spiked fractions of different agricultural soils. Percentages of recovery (%) with standard deviations (SDs), n=3 replicates.

Sample identification	Sample A, TC 1.8%		Sample A, TC 1.8%		Sample A, TC 1.8%		Sample B, TC 4.2%		Sample C, TC 5.5%		
	600 ng g ⁻¹		160 ng g ⁻¹		40 ng g ⁻¹		160 ng g ⁻¹		160 ng g ⁻¹		
Added conc. (ng g ⁻¹)	Recovery (%)	SD	Recovery (%)	SD	Recovery (%)	SD	Recovery (%)	SD	Recovery (%)	SD	Average recoveries (%)
Analyte	Recovery (%)	SD	Recovery (%)	SD	Recovery (%)	SD	Recovery (%)	SD	Recovery (%)	SD	
MET	98	6	114	3	94	9	104	5	100	8	102
CYP	90	7	85	6	83	11	77	5	86	10	84
PEN	90	2	97	4	103	6	74	2	90	9	91
PROC	100	4	113	13	95	8	95	5	94	9	99
FLUD	95	7	98	3	111	15	106	2	94	10	101
IPRO	122	9	110	8	99	17	110	6	115	9	111
MYC	100	9	116	5	117	11	102	7	103	3	108
FLU	97	10	89	4	88	13	75	4	89	10	88
DIN	117	8	115	9	116	17	93	6	114	9	111
BEN	105	7	109	3	116	15	98	2	101	9	106
PRO	96	16	117	6	104	4	95	6	96	6	102
FEN	120	11	83	7	114	13	118	7	108	7	109
TEB	108	12	96	9	100	15	81	11	104	13	98
DIF	95	12	90	6	108	13	82	8	105	12	96
AZO	101	14	101	9	110	15	92	5	91	14	99

The inter-day precision and accuracy was assessed with another vineyard soil (TC 2.0%) spiked at the 80 ng g⁻¹ level and extracted in three consecutive days (n= 9 replicates). Precision, expressed as standard deviation, ranged from 3% to 13%, and recoveries varied between 85% and 122%, which can be considered as acceptable values, considering that internal standards were not used (**Table 3**). Again, minimum and maximum values corresponded to CYP and IPRO, respectively. Recoveries compiled in **Tables 2** and **3** are similar to those reported in the earlier application of MSPD to the determination of three azolic fungicides (uniconazole, DIN and PRO) in a soil sample with lower organic matter content (TC 0.72%) [29]. Also, they remain in the same range of values as those achieved using sonication-assisted solid-liquid extraction with ethyl acetate [19] and aqueous buffered solutions [20]; however, in the latter case, recoveries varied depending on soil samples characteristics.

Table 3. Inter-day accuracy and precision of the method for a spiked vineyard solid (TC, 2.0%; addition level 80 ng g⁻¹), n= 9 replicates. The limits of quantification (LOQs) have been calculated for a signal to noise (S/N) ratio of 10.

Analyte	Recovery (%) $\pm$ standard deviation	LOQs (ng g ⁻¹)
MET	112 $\pm$ 5	5
CYP	85 $\pm$ 3	1
PEN	97 $\pm$ 7	4
PROC	101 $\pm$ 5	10
FLUD	106 $\pm$ 7	4
IPRO	122 $\pm$ 12	5
MYC	112 $\pm$ 5	8
FLU	97 $\pm$ 4	2
DIN	91 $\pm$ 13	4
BEN	111 $\pm$ 3	3
PRO	97 $\pm$ 7	10
FEN	110 $\pm$ 7	15
TEB	97 $\pm$ 9	8
DIF	101 $\pm$ 7	8
AZO	96 $\pm$ 12	3

Procedural blanks demonstrated the absence of contamination problems; thus, the limits of quantification of the method (LOQs), defined as the concentration of each compound rendering a peak with a signal ten times higher than the average baseline noise (S/N=10), were estimated from peaks obtained for the low level (40 ng g⁻¹) spiked soil sample. The extracted ion chromatograms for a procedural blank and a low level spiked soil are provided in **Figure S2**. The achieved LOQs varied from 1 ng g⁻¹ to 15 ng g⁻¹ (**Table 3**). Previously published methods, dealing with GC-MS determination of fungicides in

agriculture soils, reached LOQs of 30-50 ng g⁻¹ [20], 6-30 ng g⁻¹ [19], 0.2-6 ng g⁻¹ [17] and 3-20 ng g⁻¹ [21]. Generally, these methods consume larger volumes of organic solvents and involved more sample handling than the approach optimized in this work. Considering an injection volume of 10 µL, the proposed method provides linear responses for samples containing up to 1 µg g⁻¹ of each analyte. This value extended up to 5 µg g⁻¹ when the injection volume was reduced to 2 µL. On the other hand, the width of GC peaks increased significantly for injection volumes above 10 µL, with a negligible reduction of LOQs reported in **Table 3**.

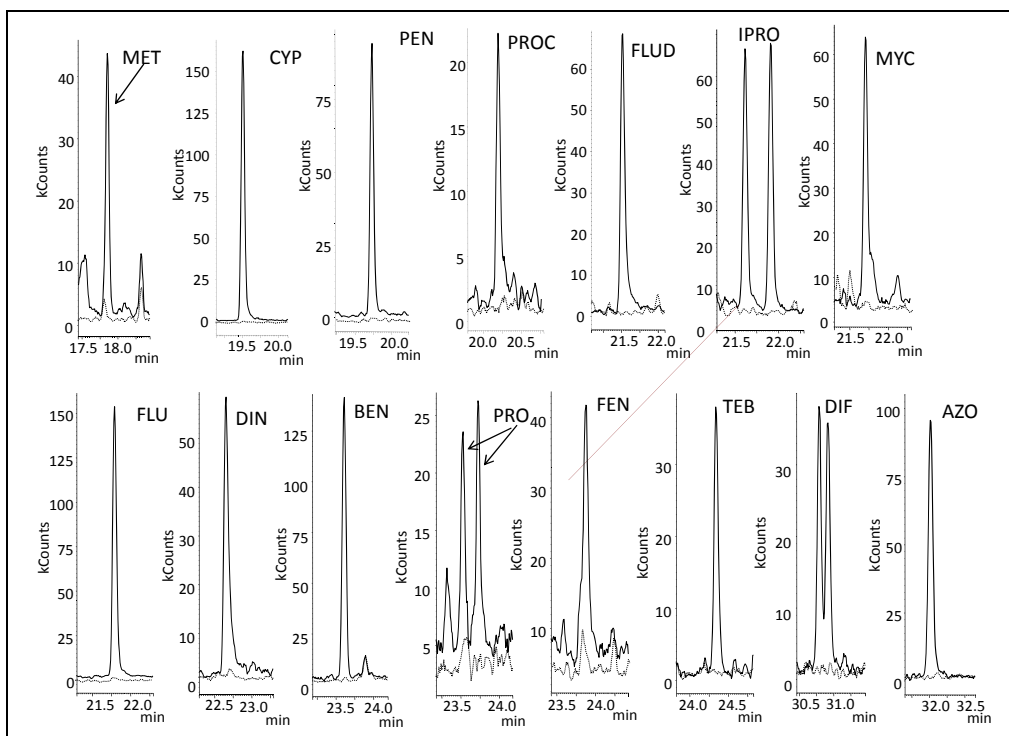


Figure S2. Selected ion GC-MS chromatograms for a procedural blank (dotted line) and a vineyard soil spiked with 40 ng g⁻¹ of each compound (solid line).

3.5. Real samples analysis

The optimized method was used to analyze a total of 19 samples, collected from the top 5 cm soil layer in different vineyards located in the Northwest of Spain, between the end of May and the beginning of September. During these months, vines are intensively fumigated with different cocktails of agrochemicals; thus, processed samples

provide information about the highest expected concentrations of fungicides in this compartment. Ten of the fifteen species involved in this research were found in the samples (**Table 4**). Among fungicides used against mildew and oidium infections, IPRO, MET and PEN were those displaying the highest occurrence frequencies, with a maximum residue level close to $3 \mu\text{g g}^{-1}$ for IPRO (**Table 4**). The prevalence of IPRO and MET in vineyard soils is in agreement with their often detection in wines elaborated in the same geographic area [30]. The anti-botrytis agrochemicals CYP, FLUD and PROC showed occurrence frequencies of 16%, with the highest concentration corresponding to the latter species ($1.5 \mu\text{g g}^{-1}$). Also, it is significant the detection of CYP and FLUD in samples collected in May and June (codes 4, 10 and 11). Under good agriculture practices, none of both fungicides should be sprayed on vineyards before grapes ripening, during the first half of august; thus, they might stay in soils since the previous year. Although confirming this assumption requires a detailed field study, controlling different atmospheric and agriculture variables, Rial-Otero and co-workers [20] have also suggested the persistence of FLUD for longer than 9 months in vineyard soils from the same geographic area.

In addition to the optimized MSPD method, samples codes 2, 3 and 4 were submitted to PLE extraction under optimum conditions previously reported for MET and MYC [21]. Obtained values were in excellent agreement with those provided by MSPD.

4. Concluding remarks

The MSPD sample preparation method optimized in this study provides suitable figures of merits (accuracy, precision and LOQs) for the extraction of 15 fungicides, belonging to different chemical families, in vineyard soils, with the advantages versus previously developed methodologies of lower cost, reduced consumption of organic solvents and less sample handling, as well as the possibility of using external calibration as quantification technique. Thus, MSPD constitutes an appealing alternative to monitor the temporal evolution of fungicide residues in vineyard, and in general in agriculture, soils, as well as to evaluate their possible depth migration. To the best of our knowledge, MSPD had been only previously proposed for 2 of the species considered in this work; moreover, the former method required 3 times higher solvent volumes than that used in this study.

Table 4. Concentrations (ng g^{-1}) of fungicides measured in vineyard soil samples, $n=3$ replicates.

Code	Sampling month	Concentration (ng g^{-1}) $\pm$ standard deviation									
		MET	CYP	PEN	PROC	FLUD	IPRO	MYC	FLU	BEN	TEB
1	May	n.d.	n.d.	n.d.	n.d.	n.d.	732 $\pm$ 50	n.d.	n.d.	n.d.	n.d.
2	May	n.d.	n.d.	n.d.	n.d.	n.d.	273 $\pm$ 30	n.d.	n.d.	n.d.	n.d.
2 ^a	May	n.d.	n.d.	n.d.	n.d.	n.d.	289 $\pm$ 21	n.d.	n.d.	n.d.	n.d.
3	May	1374 $\pm$ 25	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3 ^a	May	1436 $\pm$ 35	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4	May	n.d.	34.3 $\pm$ 0.1	1845 $\pm$	n.d.	59 $\pm$ 1	n.d.	n.d.	n.d.	n.d.	n.d.
4 ^a	May	n.d.	34.8 $\pm$ 0.3	1701 $\pm$	n.d.	53 $\pm$ 5	n.d.	n.d.	n.d.	n.d.	n.d.
5	May	912 $\pm$ 179	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
6	May	n.d.	n.d.	n.d.	n.d.	n.d.	81 $\pm$ 3	n.d.	n.d.	n.d.	62 $\pm$ 1
7	June	501 $\pm$ 22	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
9	June	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	808 $\pm$ 19
10	June	n.d.	42 $\pm$ 2	n.d.	n.d.	n.d.	2855 $\pm$	n.d.	79 $\pm$ 7	n.d.	n.d.
11	June	243 $\pm$ 6	n.d.	51 $\pm$ 2	n.d.	36 $\pm$ 5	n.d.	333 $\pm$ 10	n.d.	62 $\pm$ 5	n.d.
12	July	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	109 $\pm$ 10	n.d.	n.d.	n.d.
13	August	n.d.	n.d.	n.d.	n.d.	n.d.	176 $\pm$ 31	n.d.	n.d.	n.d.	n.d.
14	August	733 $\pm$ 49	415 $\pm$ 11	n.d.	n.d.	636 $\pm$	n.d.	n.d.	n.d.	n.d.	n.d.
15	August	n.d.	n.d.	156 $\pm$ 8	1379 $\pm$ 21	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
16	September	n.d.	n.d.	n.d.	n.d.	n.d.	173 $\pm$ 7	n.d.	n.d.	n.d.	n.d.
17	September	n.d.	n.d.	15 $\pm$ 1	118 $\pm$ 8	n.d.	63 $\pm$ 4	n.d.	31 $\pm$ 3	446 $\pm$	n.d.
18	September	n.d.	n.d.	n.d.	1499 $\pm$ 66	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
19	September	n.d.	n.d.	82 $\pm$ 6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Detection frequency (%)		26%	16%	26%	16%	16%	37%	11%	11%	11%	11%
Maximum concentration (ng g^{-1})		1374	415	1845	1499	636	2855	333	79	446	808

^aValues obtained using PLE extraction; n.d. below detection limit

The analysis of top vineyard soils revealed the existence of residues for 10 fungicides comprised between the LOQs of the method and $3 \mu\text{g g}^{-1}$. Those values can be considered as relatively low considering that samples were collected during months that vineyards are fumigated with these agrochemicals.

Acknowledgements

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3. Determinación de Etilfenoles



Las características organolépticas del vino afectan a la calidad del mismo. Los etilfenoles pueden contribuir de forma positiva, a bajos niveles de concentración, al aroma del vino; mientras que, por encima de un determinado umbral, provocan rechazo por parte de algunos consumidores. Los etilfenoles más importantes desde el punto de vista de su contribución aromática son EP, EG y, en menor medida, EC. Después de una etapa de derivatización, los tres compuestos anteriores son determinables mediante GC-MS. Además, el proceso de preparación de muestra empleado debe proporcionar un cierto factor de concentración, necesario para obtener LOQs al menos un orden de magnitud inferiores a sus umbrales sensoriales, y una elevada selectividad. Entre otras, en la bibliografía se recogen metodologías basadas en ELL [1], SPME [2] y SPE [3]. Con objeto de superar algunas limitaciones intrínsecas a las técnicas anteriores y disminuir el coste y/o el tiempo de extracción, se han desarrollado dos técnicas de microextracción alternativas, optimizando las variables que controlan la eficacia de las mismas.

El primer trabajo que se presenta emplea la microextracción en fase sólida sobre discos de silicona para realizar la extracción de estas especies en vino. Al igual que Carrillo y col. [2] cuando empleaban la SPME, la extracción y la derivatización tiene lugar en un solo paso. La ventaja del uso de este material radica en su bajo coste (<0.05€) lo que permite su empleo como absorbente desechable, de un solo uso. Así, se evitan algunos de los problemas asociados a la SPME como la contaminación cruzada entre muestras, elevado coste o su relativa fragilidad. Aunque al compararla con otras metodologías descritas en la bibliografía requiere un mayor tiempo de extracción, el uso de discos de silicona permite la extracción de varias muestras de manera simultánea. Además, se ha evaluado el uso de este material como medio de almacenamiento de etilfenoles, con objeto de hacer posible la preparación de la muestra *in-situ*, seguida del transporte y desorción de los discos en el laboratorio.

Para mejorar los LOQs obtenidos en el anterior trabajo ($5\text{--}15\text{ ng mL}^{-1}$) y en SPME ($3\text{--}30\text{ ng mL}^{-1}$) [2], se profundiza en el empleo de DLLME como técnica de extracción aplicada a la determinación de etilfenoles en vino. A priori, DLLME parece una técnica adecuada ya que permite obtener altos factores de concentración sin necesidad de introducir una etapa de evaporación (que podría ocasionar pérdidas de analitos volátiles, como ocurre con las especies consideradas). Además, se evalúa la posibilidad de realizar la derivatización en el propio tubo donde transcurre DLLME, de forma similar a la metodología de extracción/acetilación simultánea propuesta por Rezaee y col. [4] para la extracción de clorofenoles en agua mediante esta técnica, aumentando la simplicidad del

método. Con objeto de mejorar la selectividad de la extracción, y evitar problemas relacionados con la separación de fases, en este trabajo se propone realizar una etapa de SPE previa a la DLLME. Así, se ha conseguido reducir los LOQs hasta valores inferiores a 1 ng mL^{-1} .

A continuación se muestran los esquemas de trabajo correspondientes a las metodologías desarrolladas para la determinación conjunta de EP, EG y EC (**Figura III.3.1** y **Figura III.3.2**).

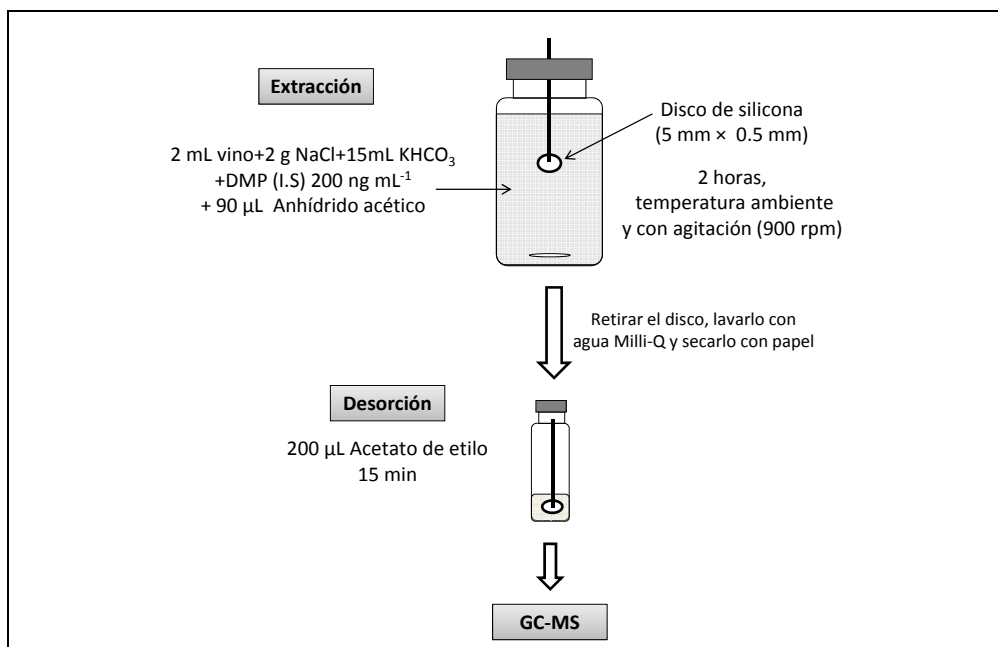


Figura III.3.1. Esquema de trabajo para la determinación de EP, EG y EC en vino mediante extracción con absorbentes de silicona, en formato disco, y GC-MS.

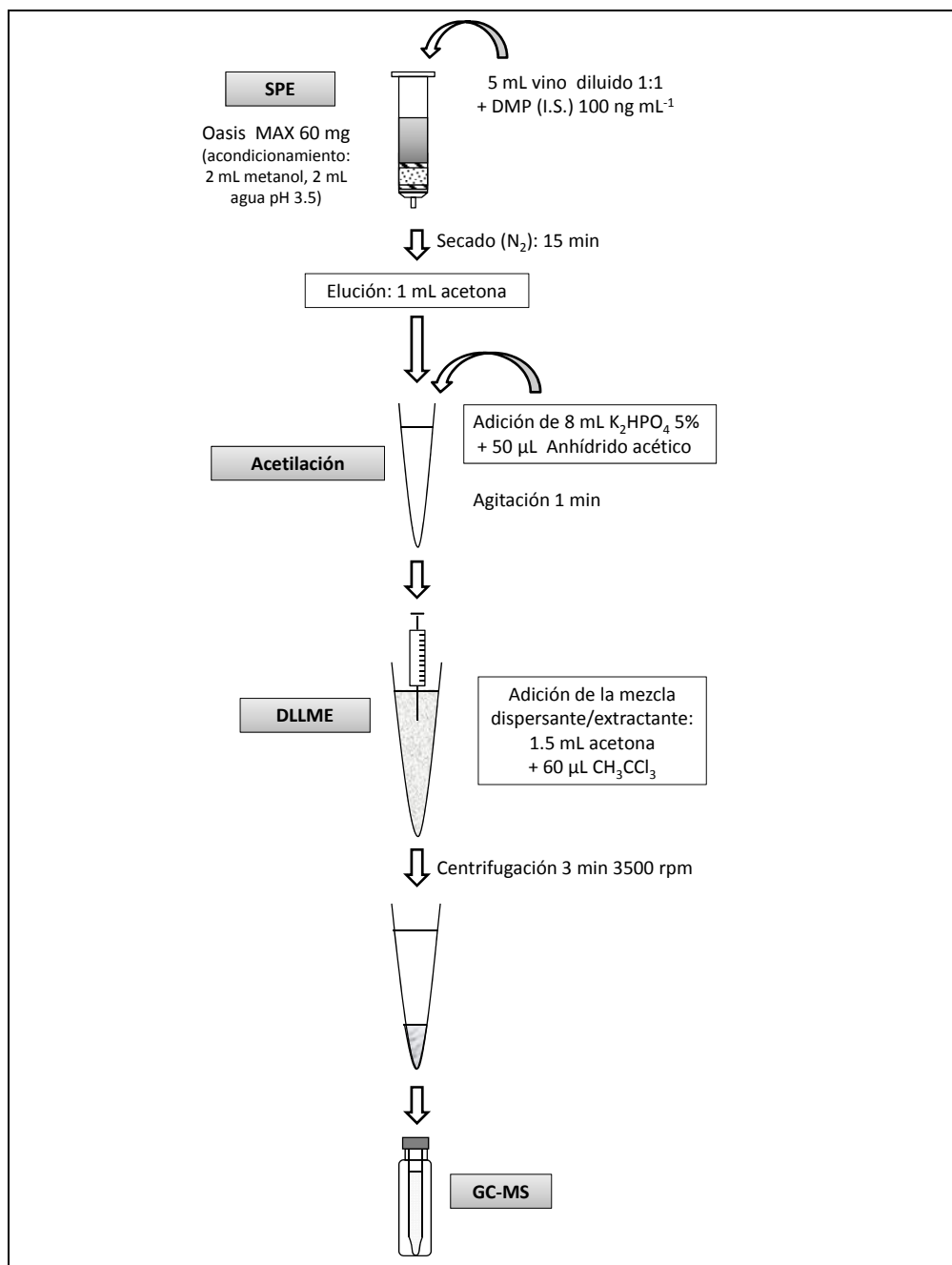


Figura III.3.2. Esquema de trabajo para la determinación de EP, EG y EC en vino mediante SPE-DLLME y GC-MS.

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Publicación III.3.1:

**Sorptive extraction with in-sample acetylation for gas chromatography
mass spectrometry determination of ethylphenol species in wine
samples**

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Sorptive extraction with in-sample acetylation for gas chromatography mass spectrometry determination of ethylphenol species in wine samples

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Abstract

An inexpensive and effective sample preparation procedure for the determination of three ethylphenolic off-flavours (4-ethylphenol, 4-ethylguaicol and 4-ethylcatechol) in wine samples is presented. Analytes were in-situ acetylated and concentrated using a disposable silicone sorbent (DSS) exposed to the diluted sample. After that, the analytes were recovered with ethyl acetate and determined by gas chromatography with mass spectrometry. The influence of different parameters (volume of acetic anhydride, basic catalyst, ionic strength, sorbent format, sampling mode and extraction time) on the efficiency of derivatization and extraction steps is discussed. Under optimized conditions, 2 mL of wine were diluted with 15 mL of an aqueous solution of potassium bicarbonate (5%, w/v) in a 22 mL vessel, containing 2 g of sodium chloride. The volume of acetic anhydride and the extraction time were set at 90 μ L and 2 hours, and the extraction was carried out at room temperature (20 ± 2 °C). Analytes were concentrated using a silicone disc (5 mm diameter $\times$ 0.5 mm thickness) and further desorbed with 0.2 mL of ethyl acetate. The achieved limits of quantification (LOQs), defined as the concentration of each compound providing a signal 10 times higher than the baseline noise, stayed between 5 and 15 ng mL⁻¹. The method provided a linear response range of up to 5000 ng mL⁻¹ and relative recoveries from 91 to 116%. The 4-ethylphenol off-flavour was detected in most red wine samples at concentrations of up to 2700 ng mL⁻¹.

Keywords: Ethylphenolic off-flavours, wine, acetylation, sorptive extraction, gas chromatography, mass spectrometry

1. Introduction

Organoleptic parameters greatly affect the quality of wine and on the acceptance of this product by consumers. Ethylphenol species, e.g. 4-ethylphenol (EP) and 4-ethylguaiacol (EG), contribute to the complexity of wine aroma; however, when their concentrations exceed a given threshold, which depends on the type of wine and the perception skills of the individual consumers, they provide undesirable off-flavours to wine, described as horse sweat or medicinal, and normally referred as *Brett* character [1,2]. The above species and 4-ethylcatechol (EC) are generated from the enzymatic decarboxylation and the further reduction of hydroxycinnamic acids contained in grapes must. These reactions are enhanced by yeasts of the *Brettanomyces* genus and occur during the aging of wine in wood barrels [3–4] and, to a lesser extent, in bottles [5]. Thus, monitoring the levels of the above ethylphenols is recommended to understand and to control those variables affecting their formation during winemaking and maturation steps.

Recently, many efforts have been focussed on the optimization of improved and/or simplified sample preparation methodologies for the determination of volatile phenols in wine. Thus, solid-phase microextraction (SPME) [6–10] and stir bar sorptive extraction (SBSE), using polydimethylsiloxane (PDMS) coated magnetic stirrers [11–13], have been proposed as appealing alternatives to conventional liquid-liquid extraction (LLE) [14] and solid-phase extraction (SPE) [15]. In most applications, SPME and SBSE are combined with gas chromatography-mass spectrometry (GC-MS) for the direct determination of EP and EG, using medium-polarity capillary columns. The higher polarity of EC limits its extraction yield and also impairs the determination of this species by GC-based methods. To overcome this drawback, in-sample acetylation has been combined with SPME for the simultaneous determination of EP, EG and EC in wine by GC-MS [10]. SPME and SBSE avoid completely the use of organic solvents and provide limits of quantification (LOQs) below the commonly accepted thresholds for ethylphenol species (from 33 ng mL⁻¹ for EG to 440 ng mL⁻¹ for EP) [10,13]. On the other hand, they also show some inherent limitations, such as the fragility of SPME coatings and fibres, the risk of cross-contamination problems between samples and the relatively high cost of fibres and PDMS coated stir bars.

Sorptive extraction, using disposable pieces of silicone polymers produced on an industrial scale, offers interesting features to overcome the above reported limitations. Popp and co-workers [16,17] first proposed the use of silicone rods for the extraction of

environmental pollutants from water samples, demonstrating that partition of the analytes between the matrix and the bulk sorbent was governed by the same equations as those used to predict the yield of SPME and SBSE processes. Following these early studies, different applications have been reported for direct and headspace extraction of organic species from aqueous matrices, liquid foodstuffs and air [18–23]. Bulk silicone sorbents are available in different formats (cord, hollow tube and sheets), they are suitable for thermal and organic solvents desorption, their dimensions (length and thickness) can be adapted for each particular application and they are inexpensive [24]. An immediate advantage of the latter feature is that they are considered as single-use sorbents (similar to SPE cartridges), which avoids carry-over and cross-contamination problems. In addition, some analytes show an excellent stability once they have been taken up into the silicone sorbent [25], thus samples can be processed and concentrated in-situ. Thereafter, only the pieces of sorbent are transported to the laboratory for further desorption and analysis.

The aim of this study was to evaluate the suitability of sorptive extraction, using disposable silicone sorbents (DSS), for the determination of EP, EG and EC in wine samples, at concentrations below their sensorial thresholds. In-sample acetylation was considered in order to increase the affinity of the analytes for the sorbent and to improve the performance of their further GC-MS determination. Parameters affecting the efficiency of the sample preparation process are discussed and the performance of the method is compared with that reported for other microextraction approaches followed by GC-MS determination.

2. Experimental

2.1. Solvents, standards and sorbents

HPLC-grade methanol, trace analysis ethanol, acetone and ethyl acetate, sodium chloride, sodium hydroxide, potassium carbonate, potassium bicarbonate, tartaric acid and acetic anhydride were supplied by Merck (Darmstadt, Germany). Standards of EP, EG, EC and 3,4-dimethylphenol (DMP), used as internal standard (I.S.) [10,11] in the extraction process, were purchased from Aldrich (Milwaukee, WI, USA) and TCI Europe (Zwijndrecht, Belgium). Individual solutions of each compound (ca. 1000 $\mu\text{g mL}^{-1}$) were prepared in methanol, further dilutions and mixtures of the three ethylphenolic off-flavours were

made in the same solvent. Diluted solutions of DMP were also prepared in methanol. Aqueous solutions of potassium carbonate, potassium bicarbonate and sodium hydroxide were made in ultrapure (Milli-Q) water.

Silicone sorbents were acquired from Goodfellow (Bad Nauheim, Germany) in two different formats: cord with a diameter of 1 mm and sheets with a 0.5 mm thickness. Rods (12 mm length $\times$ 1 mm diameter) were cut using a sharp blade; discs (5 mm diameter $\times$ 0.5 mm thickness) were obtained by pressing the silicone sheet with a sharp hollow punch (internal diameter 5 mm). The obtained pieces of silicone were weighed and those differing by more than 2% in weight were discarded. Those fulfilling the above requirement were soaked twice with a methanol: acetone (1:1) solution, for 15 min, and then conditioned overnight at 250 °C, before use.

Red and white wine samples were acquired from local markets. Synthetic wine was obtained by addition of tartaric acid (3.5 g L⁻¹) to 12% ethanol solutions in ultrapure water, followed by pH adjustment to 3.5 with NaOH (1 M).

2.2. Sample preparation

Extraction experiments were carried out in 22 mL volume, cylindrical glass vessels furnished with PTFE-lined septa and aluminium caps. An aliquot of wine was poured in each vessel, together with an aqueous solution of potassium carbonate or bicarbonate, a PTFE-covered magnetic stirrer (6 mm $\times$ 3 mm) and a certain volume of acetic anhydride. The silicone sorbent (rod or disc) was fixed to the septum of the vessel with a stainless steel pin (i.d. 0.4 mm) and immersed into the liquid sample or suspended in the headspace (HS) of the vessel, depending on the selected conditions. Normally, extractions were performed at room temperature (20 $\pm$ 2 °C); however, some assays were carried out at higher values, using a water bath to control the temperature of sample vessels. After finishing the extraction step, the pieces of silicone were rinsed with ultrapure water, dried with a lint free tissue and analytes were recovered with a small volume of ethyl acetate. Discs were desorbed in a GC autosampler vial (2 mL). Rods were first introduced into a glass insert (0.3 mL) and then desorbed with the same solvent.

Under optimised conditions, 2 mL of wine were transferred to extraction vessels containing 2 g of sodium chloride, 15 mL of potassium bicarbonate (5%, w/v) and a

magnetic stirrer. After homogenization of the mixture, 90 μL of acetic anhydride were added. Analytes were in-situ acetylated and extracted with a silicone disc exposed directly to the stirred sample, for 2 hours at room temperature. Desorption was also performed at room temperature, for 15 min with 0.2 mL of ethyl acetate. After this period, the silicone disc, still attached to a piece of stainless steel, was removed with tweezers, and the ethyl acetate extract injected directly in the GC-MS system. Further details describing the handling of silicone sorbents have been reported in a previous work [18].

2.3. GC-MS determination

Analytes were determined by GC-MS, using a Varian (Walnut Creek, CA, USA) 450 GC instrument connected to an ion-trap Varian 240 mass spectrometer (MS), furnished with an electron impact (EI) ionization source in the external configuration mode. Separations were carried out in an Agilent (Wilmington, DE, USA) HP-5ms type capillary column (30 m $\times$ 0.25 mm i.d., d_f : 0.25 μm) operated at a constant helium flow of 1.2 mL min^{-1} . The GC oven was programmed as follows: 60 $^{\circ}\text{C}$ (held for 1 min), first rate at 8 $^{\circ}\text{C min}^{-1}$ to 245 $^{\circ}\text{C}$, second rate at 25 $^{\circ}\text{C min}^{-1}$ to 285 $^{\circ}\text{C}$ (held for 15 min). The temperature of the injector was maintained at 270 $^{\circ}\text{C}$. Injections (2 μL) were done in the pulsed splitless mode (25 psi, 1.1 min), with the solenoid valve switched to the split position after 1 min. Transfer line, ion source and trap temperatures were set at 290, 200 and 150 $^{\circ}\text{C}$, respectively. The helium damping gas flow was set at 2.5 mL min^{-1} .

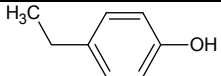
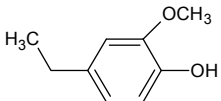
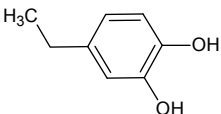
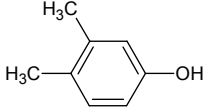
The mass spectrometer was operated in the electron impact ionization mode (70 eV) with a filament emission current of 50 μA . MS spectra were acquired in the m/z range between 80 and 400 a.m.u. The electromultiplier voltage was set at 1700 V. The sum of responses for the two most intense ions in the spectra of acetylated compounds (m/z 107 + 122, EP and DMP; m/z 137+152, EG; and m/z 123+138, EC) was used for quantification purposes.

3. Results and discussion

3.1. Preliminary extractions

The analytes involved in this research (EP, EG and EC) were of high to medium polarity (log K_{ow} values from 1.87 to 2.47 units) and thus had a limited affinity for silicone. In addition, the ethanol content of wine increases their solubility reducing even more their partitioning between the non-polar sorbent and the sample. Moreover, phenols are difficult to determine using GC columns coated with non-polar stationary phases. This problem is particularly relevant for EC, which contains two hydroxyl moieties in its structure, **Table 1**. Therefore, in-situ acetylation was used in order to improve the performance of extraction and determination steps. In practise, this means that the parameters affecting the yield of both steps have to be considered simultaneously.

Table 1. Structures, CAS numbers and relevant properties of selected compounds.

Abbreviation	Structure	CAS	Log K_{ow}	pK_a
EP		123-07-9	2.47	10.2
EG		2785-89-9	2.18	10.3
EC		1124-39-6	1.87	9.7
DMP (I.S.)		95-65-8	2.40	10.4

Preliminary extractions were carried out in order to obtain an initial overview of the effects of the sampling mode (direct vs. HS) and the base used as catalyst of the acetylation reaction (potassium carbonate vs. bicarbonate, both 5% w/v) on the efficiency of the sample preparation process. Assays were accomplished with 1 mL aliquots of a red wine sample previously spiked with target compounds (500 ng mL⁻¹) and diluted with 8 mL of the selected base aqueous solution (5% w/v). The volume of acetic anhydride and the sampling time were 100 μ L and 1 hour, respectively. A silicone disc was used as extraction

device and it was subsequently desorbed with ethyl acetate. EC was detected in the sorbent extracts only when potassium bicarbonate was used as catalyst of the acetylation reaction, but not in the presence of potassium carbonate. On the other hand, responses obtained in the HS mode, at room temperature and 60 °C, remained far below of those measured by dipping the silicone disc into the diluted wine sample. Consequently, direct exposure was adopted as the sampling mode; moreover, extractions were carried out at room temperature (1) to simplify the setup of the extraction process, and (2) to prevent changes in the composition of wine during extraction. In further experiments, in order to reduce the HS volume in the extraction vessel, wine and potassium bicarbonate volumes were up-scaled to 2 and 15 mL, respectively.

The effects of the volume of ethyl acetate used for desorption of the analytes and the duration of this step were further evaluated. The first variable was investigated between 15 and 60 min. Within this range, the desorption time did not affect the response of target analytes and, therefore, it was set to 15 min. Silicone pieces were desorbed twice with 0.2 mL or 0.1 mL of ethyl acetate. For the larger volume of solvent, the relative responses in the second desorption represented less than 3% of those measured in the first one; whereas, values around 10% were obtained with 0.1 mL of ethyl acetate. Thus, 0.2 mL of ethyl acetate were adopted as working value for the elution solvent volume. Under the above conditions desorption was not quantitative; however, as silicone discs were considered as single-use sorbents, there was not risk of cross-contamination problems.

Figure 1 compares the responses (peak areas, without internal standard correction) obtained using silicone sorbents in two different formats (discs and rods), exposed to spiked aliquots of red wine for 2 hours. Both formats have similar volumes and surfaces (ca. 10 μL and 39 mm^2 , respectively); however, under the conditions used, discs rendered higher responses than rods. This suggests that not only the total volume and surface, but also the format of the sorbent affects the performance of the extraction process. Obviously, discs were therefore used as extraction devices.

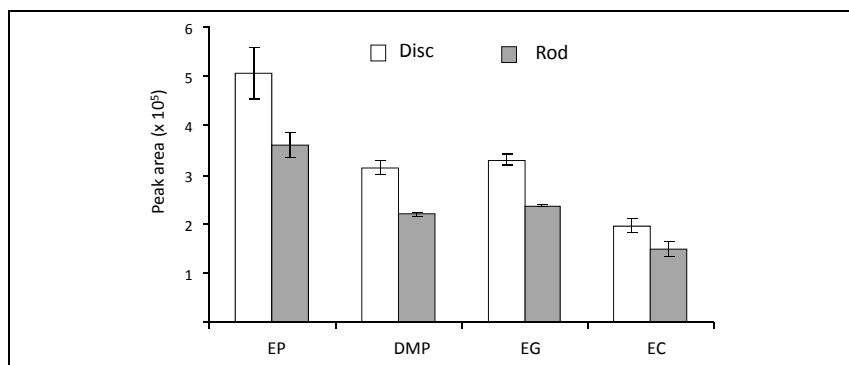


Figure 1. Responses (peak areas) provided by silicone rods and discs for 2 mL aliquots of spiked (500 ng mL^{-1}) red wine. Direct extraction at room temperature for 2 hours, $n=3$ replicates. EP, 4-ethylphenol; DMP, 3,4-dimethylphenol; EG, 4-ethylguaiaicol; EC, 4-ethylcatechol.

3.2. Ionic strength, acetic anhydride volume and potassium bicarbonate concentration

The influence of these parameters on the efficiency of the sample preparation process was simultaneously investigated using a Box-Behnken type, experimental factorial design with each variable considered at three levels (0, 1 and 2 g for the amount of NaCl; 50, 100 and 150 μL as acetic anhydride volume; 2, 5 and 8% as the concentration of KHCO_3). The above design involved a total of 15 experiments, including a triplicate of the central point, and it allows estimating the main effects associated with each experimental factor, their quadratic terms, and the two-factor interactions. The experimental domain of the design was selected on the basis of the previous results reported by Carrillo and Tena [10], using SPME as the extraction technique. Graphs showing the standardized main effects for target compounds, and the internal standard, are shown in **Figure 2**. The length of depicted lines is proportional to the variation in the response obtained for each compound when the corresponding factor varied within the domain of the design. As observed, increasing the ionic strength (mass of NaCl in the extraction vessel) favoured the efficiency of the extraction for all species. Analysis of variance (ANOVA) of responses obtained in the experiments involved in the Box-Behnken design demonstrated that the amount of NaCl was the only variable with a statistical significant effect (95% confidence level) on the extraction efficiency; whereas, the concentration of KHCO_3 and the volume of acetic anhydride did not achieve the threshold of statistical significance, data not given. As shown in **Figure 2**, most compounds were better extracted when the above variables were set at the medium level of the design. The exception was EC, which preferred low KHCO_3

concentrations and high acetic anhydride volumes. Two-factor interactions remained below the level of statistical significance at the 95% confidence level. The best compromise conditions, which maximized the efficiency of the extraction for all compounds, were calculated with a global desirability (D) function. This function is defined as the geometric mean of the normalized (between 0 for the minimum and 1 for the maximum) predicted responses (d_i) for each compound, by the Box-Behnken design. The maximum value of D (0.70) was obtained for 2 g of NaCl, 90 μL of acetic anhydride and 5% (w/v) of KHCO_3 , **Figure 3**.

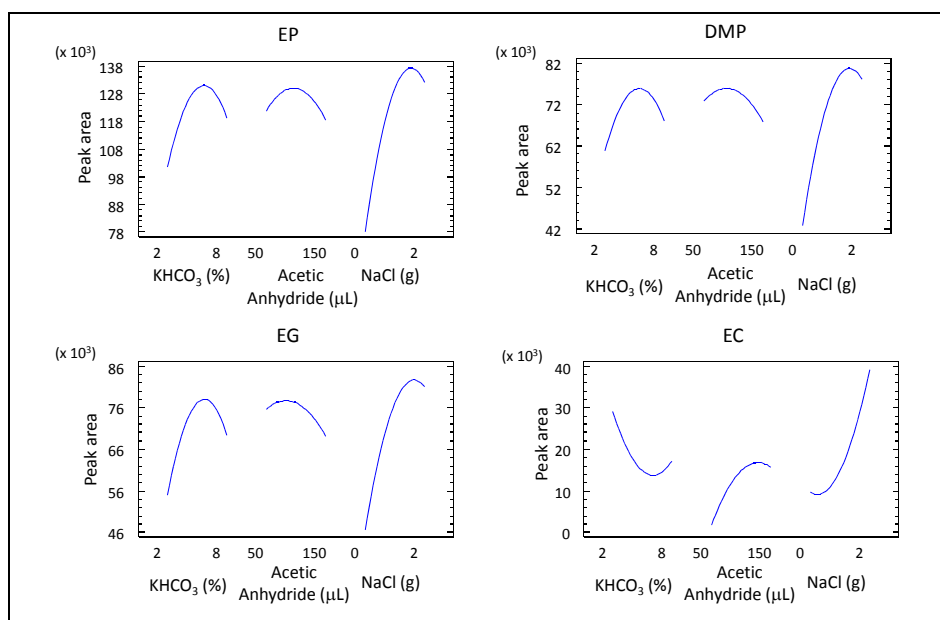


Figure 2. Main effect graphs corresponding to the Box-Behnken experimental factorial design.

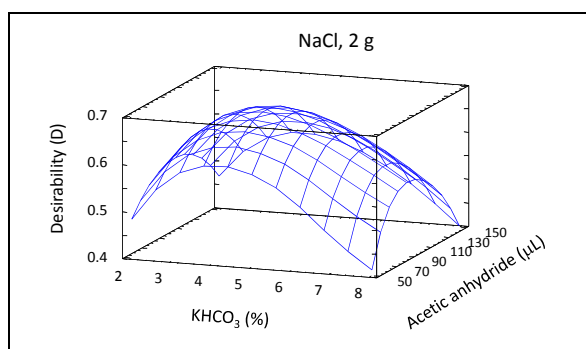


Figure 3. Plot of the global desirability function.

3.3. Stirring and extraction kinetics

Stirring favours migration of the analytes from the sample in the extraction vessel to the interface with the silicone disc; thus, it is expected to improve the efficiency of the extraction under non-equilibrium conditions. Considering a sampling time of 2 hours, 10-fold higher responses were measured for stirred samples versus non-stirred ones; however, no differences were noticed working at three different stirring speeds: 300, 900 and 1500 rpm. The same behaviour has been reported for PDMS coated stir bars [13]. A working value of 900 rpm was adopted for this variable.

The time-course of the sorptive extraction at room temperature was investigated with spiked aliquots (2 mL) of red wine, considering sampling times between 10 min and 8 hours. As normally occurs with sorptive techniques, the extraction process showed a slow kinetics [20]. In the case of EC, two hours of sampling were enough to obtain equilibrium conditions, whereas the other two analytes and the I.S. required four hours, **Figure 4**. It seems that, even after in-situ acetylation, EC remains as the most polar specie and therefore migrates more rapidly from the sample to the silicone disc. In this study, the sampling time was limited to 2 hours in order to increase the sample throughput, assuming the corresponding loss of efficiency for EP and EG.

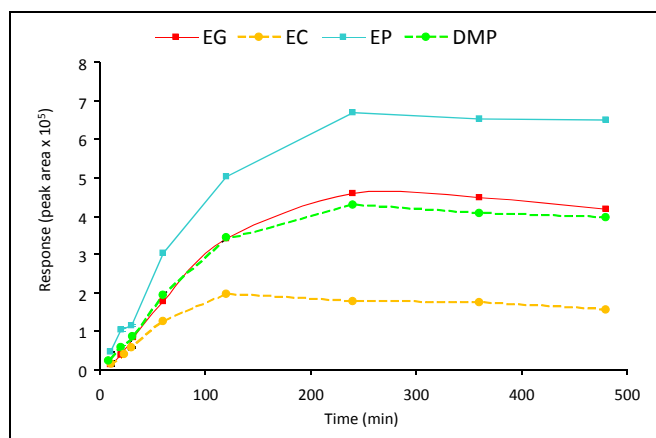


Figure 4. Kinetics of sorptive extraction. Direct sampling, at room temperature.

3.4. Performance of the method

Table 2 summarizes some data related to the linearity and the precision of the proposed method. The linearity was evaluated with a young red wine (*Mencia* variety), which did not contain detectable levels of target analytes. Aliquots of this matrix were spiked at eight different levels, prepared in duplicate, between LOQs and 5000 ng mL⁻¹. This interval covers the range of concentrations reported for target analytes in aged samples of red wines [10]. The concentration of the I.S. was maintained at 200 ng mL⁻¹. The plots of the ratios between the peak areas measured for each compound and the I.S. versus their concentrations in the wine sample fitted a linear model, with determination coefficients (R^2) higher than 0.997, **Table 2**. Precision was investigated with spiked samples of red and white wines processed on the same day (repeatability) and on different days (reproducibility). The relative standard deviations (RSDs) of the corrected responses (peak area divided by the I.S. peak area) ranged from 2% to 9%, **Table 2**. On the other hand, RSDs up to 16% were obtained before I.S. correction, data not shown.

Table 2. Linearity, precision and LOQs of the proposed method.

Analyte	Linearity (LOQs-5000 ng mL ⁻¹)			LOQs (ng mL ⁻¹)		
	Slope (SD)	Intercept (SD)	R ²			
EP	0.0046 (4×10 ⁻⁵)	0.01 (0.04)	0.998	5		
EG	0.0032 (4×10 ⁻⁵)	-0.01 (0.05)	0.998	5		
EC	0.0019 (9×10 ⁻⁶)	0.026 (0.009)	0.997	15		
Precision (RSDs, %)						
Analyte	Intra-day (n=4 replicates)				Inter-day (n=9 replicates, 3 days)	
	Red wine		White wine		Red wine	White wine
	^a 100 ng mL ⁻¹	^a 500 ng mL ⁻¹	^a 100 ng mL ⁻¹	^a 500 ng mL ⁻¹	^a 200 ng mL ⁻¹	^a 200 ng mL ⁻¹
EP	3.8	6.0	8.9	4.5	2.4	1.9
EG	4.2	5.3	6.0	6.9	4.5	8.6
EC	6.6	7.5	6.8	5.0	6.2	6.5

^aAdded concentration, SD, standard deviation

Procedural blanks, corresponding to synthetic wine solution, demonstrated the absence of contamination problems; thus, the limits of quantification (LOQs) of the method, defined for a S/N ratio of 10, were controlled by the efficiency of the sample preparation process and the sensitivity of the GC-MS instrument. Achieved LOQs, estimated for S/N ratios of chromatographic peaks in the lower levels of the linearity study, ranged from 5 to 15 ng mL⁻¹, depending on the particular analyte, **Table 2**. These values are similar to or lower than those obtained for EP and EG with SBSE followed by

thermal desorption and GC-MS determination [11–13], HS-SPME with in-situ derivatization [10] and dispersive liquid-liquid microextraction (DLLME) [26], **Table 3**. Thermal desorption of PDMS coated stir bars allows transferring the whole entire extracted amount of each compound to the GC column; thus, the achieved LOQs are expected to be much lower than those attained in this work using solvent desorption followed by injection of a fraction of the extract (1%, considering injection and desorption volumes of 2 and 200 μL , respectively) in the GC-MS system. However, on the other hand, the affinity of the polar ethylphenol species for the sorbent increases significantly after in-situ acetylation, which contributes significantly to reduce the LOQs of the proposed method. Most authors have reported sensorial thresholds for ethylphenol species of between 33 ng mL^{-1} for EG and 440 ng mL^{-1} for EP [10,13]. These values are higher than the LOQs of the methodology optimised in this study.

Table 3. Summary of sample preparation conditions and LOQs reported for ethylphenolic off-flavours in wine samples using different microextraction techniques followed by GC-MS determination.

Technique	Wine vol. (mL)	Extraction time and temperature	In-situ acetylation	LOQs (ng mL^{-1})			Ref.
				EP	EG	EC	
SBSE	4	60 min, room temperature	No	21	529	n.a.	[11]
SPME	4	70 min, 70 °C	Yes	30	3	6	[10]
SBSE	10	60 min, room temperature	No	1.1	0.9	n.a.	[13]
SBSE	25	90 min, room temperature	No	21	0.1	n.a.	[12]
DLLME	5	5 min, room temperature	No	147	95	n.a.	[26]
Sorptive extraction	2	120 min, room temperature	Yes	5	5	15	This work

n.a. not available

The effect of the type of sample on the efficiency of the extraction was first evaluated by comparing the corrected responses (peak areas after subtraction of the signals obtained for non-spiked aliquots of each matrix) measured for spiked aliquots of red and white wine samples, fortified at two different concentrations. Despite considerable dilution of samples (2 mL of wine plus 15 mL of a 5% potassium bicarbonate solution), the yield of the extraction for target analytes decreased around 35% for red wines versus white ones. This may be explained by the higher complexity of the former matrix, and the significant increase in the viscosity of red wines when adjusted at basic pHs in comparison with white wines. The signal measured for DMP was reduced to a similar extent as those corresponding to the rest of species. Thus, after I.S. normalization, the use of matrix matched standards, instead of the standard addition methodology, appears as a feasible quantification approach. In order to confirm the accuracy of the

latter methodology, samples of white (*Palomino*) and red (*Grenache*) wines were spiked at different levels and quantified against spiked aliquots of another red wine (*Mencia*). The obtained relative recoveries varied between 91% and 116% (**Table 4**), confirming the usefulness of DMP to compensate for changes in the efficiency of the sorptive extraction, as well as the possibility of using matrix-matched standards for quantification purposes.

Table 4. Relative recoveries obtained for spiked wine samples, n= 4 replicates.

Compound	Recovery (%) $\pm$ SD			
	Added concentration			
	^a 150 ng mL ⁻¹	^b 100 ng mL ⁻¹	^b 300 ng mL ⁻¹	^b 1000 ng mL ⁻¹
EP	105 $\pm$ 2	103 $\pm$ 4	105 $\pm$ 9	108 $\pm$ 5
EG	101 $\pm$ 10	92 $\pm$ 9	116 $\pm$ 12	100 $\pm$ 4
EC	91 $\pm$ 4	103 $\pm$ 5	99 $\pm$ 9	112 $\pm$ 9

^awhite wine, ^bred wine

The combination of inexpensive extraction devices with simplified sample preparation conditions opens the possibility for in-situ sample preparation, avoiding potential changes in the concentration of ethylphenol species during transport and/or storage of wine samples in the laboratory. In practise, the feasibility of this alternative is determined by the stability of the acetylated species in the extraction discs. **Figure 5** shows the normalized responses corresponding to the extracts from a spiked (300 ng mL⁻¹) wine sample. Three series of extractions (n=4 replicates, each) were carried out by desorbing the silicone discs immediately after finishing the sample preparation step, after 5 and 10 days of storage in 2 mL amber vessels, at 4 °C. As observed in **Fig. 5**, no differences were noticed among the obtained responses.

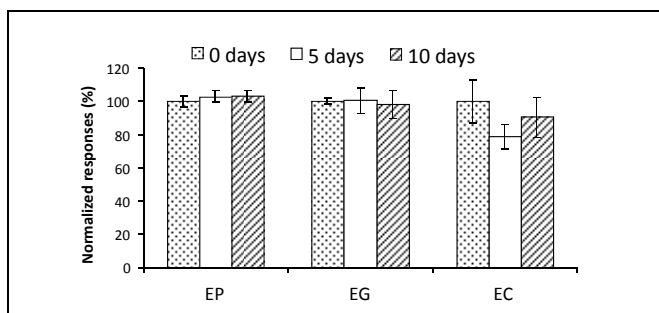


Figure 5. Stability of acetylated species in the silicone discs stored at 4 °C. Normalised values to those measured for silicone discs desorbed immediately after the extraction step, n=4 replicates.

3.5. Application to commercial wines

The proposed method was applied to samples of red and white wines, acquired in local supermarkets, from different geographic areas in Spain. None of the compounds was detected in white wines of the Albariño, Ribeiro, Rueda and Valdepeñas geographic regions. The levels measured in red wines are summarized in **Table 5**. The maximum concentration in all the processed samples corresponded to EP, with a level between 10 and 20 times higher than that of EG. The latter species was found at similar concentrations to EC. Overall, the above ratios matched with those reported for Rioja and other red wines from different countries aged in wood containers [10,13]. **Figure 6** shows the GC-MS chromatograms corresponding to a procedural blank, a red wine sample (code 3, **Table 5**) and same sample fortified with target species at 300 ng mL⁻¹.

It is worth noting that EP was not only detected in wood-aged wines (codes 2–5, **Table 5**), but also in most of the young wine samples (codes 6–10, **Table 5**) at levels up to 310 ng mL⁻¹. On the other hand, EG and EC were only found at relevant concentrations in the first type of wines.

Table 5. Summary of concentrations measured in red wine samples, n=3 replicates.

Code	Wood-aged	Year	Concentration (ng mL ⁻¹) ± SD		
			EP	EG	EC
1	n.a.	2008	2414 ± 53	114 ± 11	292 ± 25
2	Yes	2008	1892 ± 139	171 ± 22	162 ± 18
3	Yes	2007	359 ± 13	34 ± 2	62 ± 6
4	Yes	2002	2708 ± 115	204 ± 6	344 ± 39
5	Yes	2002	212 ± 18	n.d.	n.d.
6	No	2009	n.d.	n.d.	n.d.
7	No	2008	34 ± 4	n.d.	n.d.
8	No	2009	132 ± 10	n.d.	n.d.
9	No	2009	311 ± 24	9 ± 1	n.d.
10	No	2009	191 ± 7	6 ± 1	n.d.
<i>Sensorial threshold (ng mL⁻¹)</i>			440	33	n.a.

n.a., not available information

n.d., below detection limits

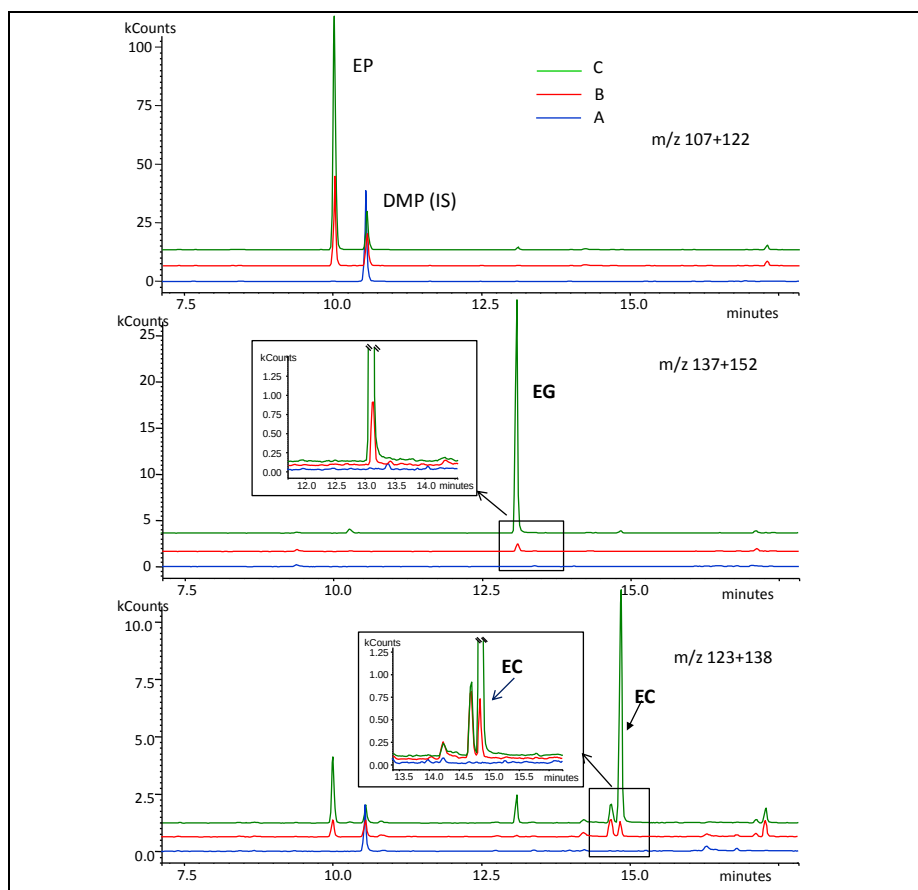


Fig. 6. Selected ion GC-MS chromatograms for a procedural blank of synthetic wine (A), sample code 3 in Table 5 (B), and same sample spiked at 300 ng mL⁻¹ per compound (C). The I.S. was added to all samples at 200 ng mL⁻¹.

4. Conclusions

In-sample acetylation followed by sorptive extraction with inexpensive silicone discs was found to be an effective, low cost method for extraction of ethyl phenolic off-flavours from wine samples. When combined with GC-MS determination, the developed method provides LOQs below the sensorial thresholds of target phenols, it presents a limited consumption of organic solvents, it is free of cross-contamination problems and sample preparation can be in-situ performed. Moreover, the use of DMP as internal standard allows compensating for changes in the extraction efficiency among different wine samples; thus, calibration with matrix-matched standards, instead of the standard

addition methodology, can be used for samples quantification. The 4-ethylphenol compound was found in most of the analyzed red wine samples, even when they had not been aged in wood barrels. On the other hand, none of the ethylphenol off-flavours was detected in white wines. Finally, EG and EC were mainly present in wood-aged wines.

Acknowledgements

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Publicación III.3.2:

Mixed-mode solid-phase extraction followed by dispersive liquid-liquid microextraction for the sensitive determination of ethylphenols in red wines

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Abstract

Selectivity of mixed-mode solid-phase extraction (SPE) was combined with the concentration power of dispersive liquid-liquid microextraction (DLLME) to obtain a sensitive, low solvent consumption method for gas chromatography-mass spectrometry determination of ethylphenol off-flavours (4-ethylphenol, EP; 4-ethylguaiacol, EG and 4-ethylcatechol, EC) in complex red wine samples. Under optimized conditions, limits of quantification (LOQs) between 0.3 and 0.8 ng mL⁻¹ were obtained using just 5 mL of wine and 0.06 mL of 1,1,1-trichloroethane (TCE) as extractant in the DLLME step. Analytes were acetylated after SPE and previously to DLLME concentration to enhance the performance of their GC-MS determination. The overall extraction efficiency of the method was unaffected by the particular characteristics of each wine; thus, accurate results (relative recoveries from 89 to 109% for samples spiked at concentrations from 20 to 1000 ng mL⁻¹) were obtained using matrix-matched standards, without requiring the use of the time consuming standard addition quantification methodology. The applicability of the method was demonstrated with the analysis of different red wines. Analytes concentrations varied from 6 to 2265 ng mL⁻¹ (EP), 0.8 to 251 ng mL⁻¹ (EG) and non-detected to 158 ng mL⁻¹ (EC).

Keywords: Ethylphenol off-flavours, wine, dispersive liquid-liquid microextraction, mixed-mode solid-phase extraction

1. Introduction

The organoleptic characteristics of wine are the result of a complex balance of volatile and semi-volatile compounds, displaying different concentration ranges and contributions to wine aroma [1]. Among them, ethylphenol species are considered as responsible for organoleptic defects when present at levels above a given threshold, which varies from a few ng mL^{-1} for 4-ethylguaiacol (EG) up to several hundreds of ng mL^{-1} for 4-ethylphenol (EP) [2-3]. In addition to EP and EG, the presence of significant amounts of 4-ethylcatechol (EC) has also been correlated with negative aromatic notes (spicy or medicinal) in wine [4]. On the other hand, at lower concentrations, ethylphenols positively contribute to the aroma of wine [2,5]. Ethylphenol species are the result of hydroxycinnamic acids (coumaric, caffeic and ferulic) decarboxylation, followed by reduction of the intermediate vinylphenols [6]. The above reactions are enhanced by certain yeasts and take place during wine elaboration and maturation, particularly when ageing in wood barrels [7]; thus, organoleptic defects related to ethylphenols are usually more significant in red wines than in white ones. Understanding the exact mechanisms controlling the formation of these off-flavours, and monitoring their evolution during wine maturation, requires sensitive analytical methods, able to provide reliable data at concentrations further below their sensorial thresholds.

Sample preparation plays several relevant roles in the determination of ethylphenol off-flavours, aiming (1) to reduce the complexity of wine matrices, (2) to concentrate target species and, in some cases, (3) to improve the performance of their further determination. Solid-phase extraction (SPE) [8-10] and several solid-phase type microextraction techniques [11-14] have been proposed to achieve the first two goals; whereas, analytes derivatization, usually with acetic anhydride, is advisable when considering gas chromatography-mass spectrometry (GC-MS) determination [12]. Overall, microextraction techniques, such as solid-phase microextraction (SPME) and stir bar sorptive extraction (SBSE), render similar limits of quantification (LOQs) to, or even lower than, those achieved by SPE, with the advantage of requiring lower wine volumes. On the other hand, SPME and SBSE show a low sample throughput due to (1) the limited kinetics of mass transference processes from the sample to the surface of the sorbent and (2) the use of standard addition as quantification technique, which is justified since the yield of the microextraction might change depending on the characteristics of each wine sample and/or the state of the polymeric coating.

The dispersive liquid-liquid microextraction technique (DLLME), first reported by Rezaee and co-workers [15], overcomes the problem associated with the slow kinetics of SPME and SBSE. However, on the other hand, the complexity of wine samples, particularly red wines, makes difficult obtaining a neat interface between the sample and the small drop of organic extractant. The first application of DLLME to the determination of ethylphenols in wine was proposed by Fariña et al. [16]. Using GC-MS, they achieved a LOQ for EG (95 ng mL^{-1}), which remains above the sensorial threshold of 33 ng mL^{-1} , reported for this compound in the bibliography [12]. Thereafter, EP and EG were included in a DLLME method dealing with GC tandem mass spectrometry (MS/MS) for the determination of several off-flavours in wine [5]. This method was further implemented replacing the dispersant solvent by ultrasound energy to achieve an emulsion of extractant droplets in the sample [17]. Unfortunately, the above methods are not applicable to EC, since they do not consider a derivatization step, required to improve the GC detectability of this dihydroxylated species. Also, as further explained, some doubts arise about their performance when transferred from hydro-alcoholic solutions, considered during optimization of DLLME, to red wines displaying a much higher complexity.

The aim of this research was to develop a sensitive and selective sample preparation method for the determination of three ethylphenol species in red wine samples, covering the range of concentrations from the sub ng mL^{-1} level (below their sensorial thresholds) up to the $\mu\text{g mL}^{-1}$ range. The selectivity of SPE was combined with the concentration capability of DLLME to attain the above aims, minimizing sample and organic solvent consumption, as well as time requirements to obtain quantitative data. Moreover, acetylation of target species was also considered to improve the performance of their GC-MS determination. Parameters affecting the efficiency of extraction, derivatization and concentration steps were thoroughly investigated and their effects in the overall performance of the method discussed. Finally, the method was used to determine the levels of target species in commercial red wines.

2. Experimental

2.1. Standards, solvents and sorbents

Standards of EP, EG, EC and 3,4-dimethylphenol (DMP), used as internal surrogate (I.S.) in the sample preparation process, were purchased from Aldrich (Milwaukee, WI, USA) and TCI Europe (Zwijndrecht, Belgium). Individual solutions of each compound (ca. 1000 $\mu\text{g mL}^{-1}$) were prepared in ethanol, further dilutions and mixtures of the three ethylphenol off-flavours were made in the same solvent. Diluted solutions of DMP were also prepared in ethanol.

Ethanol, acetonitrile, methanol and acetone (chromatographic analysis grade), as well as isooctane, chlorobenzene (ClBz), carbon tetrachloride (CCl_4) and 1,1,1-trichloroethane (TCE), all trace analysis grade, were supplied by Merck (Darmstadt, Germany). Potassium hydrogen carbonate (KHCO_3), potassium carbonate (K_2CO_3) and dipotassium hydrogen phosphate (K_2HPO_4) were also purchased from Merck. The above salts were dissolved in ultrapure water to prepare 5% (w/v) solutions.

SPE cartridges, containing 60 mg of the mixed-mode (reversed-phase and anionic exchanger) OASIS MAX sorbent, were acquired from Waters (Milford, MA, USA).

Samples of red wine, elaborated at different geographic areas in Spain, were acquired from local markets. Bottles were protected from light and stored at room temperature until analysis.

2.2. Sample preparation

The sample preparation method developed in this study involves three consecutive steps. First, wine samples were passed through SPE cartridges in order to extract and separate target analytes from other matrix components. The extract from the cartridge was diluted with an aqueous alkaline solution and analytes were derivatized using acetic anhydride. Finally, acetylated species were concentrated by DLLME.

Under optimized conditions, 5 mL of wine were diluted with the same volume of ultrapure water and passed through the SPE cartridge, previously conditioned with

methanol and water adjusted at pH 3.5 (2 mL each). After drying the sorbent with a gentle stream of nitrogen, analytes were recovered with 1 mL of acetone, which was collected in a conical bottom glass tube (12 mL volume). This extract was diluted with 8 mL of a 5% (w/v) K_2HPO_4 aqueous solution, 0.05 mL of acetic anhydride were added and tubes were shaken for 1 min. DLLME was carried out adding a binary mixture consisting of 0.06 mL of TCE in 1.5 mL of acetone. After centrifugation (3 min at 3000 rpm), the settled drop of TCE was transferred to an insert and 1-2 μ L injected in the GC-MS system.

2.3. Determination

Analytes were determined by GC-MS, using a Varian (Walnut Creek, CA, USA) 3800 GC instrument connected to an ion-trap Varian 2000 mass spectrometer (MS), furnished with an electron impact (EI) ionization source. Separations were carried out in an Agilent (Wilmington, DE, USA) HP-5ms type capillary column (30 m $\times$ 0.25 mm i.d., d_f : 0.25 μ m) operated at a constant helium flow of 1.2 mL min⁻¹. The GC oven was programmed as follows: 60 °C (held for 1 min), first rate at 8 °C min⁻¹ to 245 °C, second rate at 25 °C min⁻¹ to 285 °C (held for 15 min). The temperature of the injector was maintained at 270 °C. Injections (2 μ L) were done in the splitless mode, with the solenoid valve switched to the split position after 1 min (split flow 50 mL min⁻¹). Transfer line, manifold and trap temperatures were set at 290, 50 and 220 °C, respectively. MS spectra were acquired in the m/z range between 80 and 400 a.m.u. The sum of responses for the two most intense ions in the spectra of acetylated compounds (m/z 107 + 122, EP and DMP; m/z 137+152, EG; and m/z 123+138, EC) was used for quantification purposes.

Levels of target species in wine samples were determined with matrix-matched standards, corresponding to aliquots of red wine (*Mencia* variety) spiked with increased concentrations of target species, from 1 to 5000 ng mL⁻¹ (100 ng mL⁻¹ for the I.S.), and submitted to the global sample preparation method. The ratios between the responses (peak areas) measured for each compound and the I.S. were plotted against the added concentration and fitted to a linear model.

3. Results and discussion

3.1. Sample preparation strategy

Previous studies from our research group have shown that chlorinated solvents used as extractants in DLLME render viscous extracts for undiluted wine samples, particularly for red wines, unsuitable for direct injection in the GC column [18,19]. Increasing the pH of wine, which is required for analytes acetylation, worsened the aforementioned problem. Replacing chlorinated extractants, typically employed in DLLME, by room temperature melting point, low density solvents (such as 1-undecanol and hexadecane) provides cleaner extracts [19]; however, these solvents might interfere with chromatographic peaks corresponding to volatile compounds, as it is the case of ethylphenol off-flavours. Thus, SPE and DLLME were combined to improve the selectivity of the overall method by removing, during the SPE step, those matrix compounds which difficult phases separation in DLLME [18,20].

3.2. Solid-phase extraction

Optimization of the SPE step was performed with spiked (20 ng mL^{-1}) aliquots of red wine, diluted with the same volume of ultrapure water, passed through SPE cartridges previously conditioned with methanol and ultrapure water adjusted at pH 3.5. The mixed-mode OASIS MAX sorbent was preferred to reversed-phase polymers since the former has demonstrated a very high affinity for red wine matrix components, e.g. tannins, which remain retained in the sorbent after analytes elution [21]. As a result, colourless, transparent extracts are obtained using polar, water soluble solvents compatible with further steps of the analytical method. After passing a dry stream of nitrogen through the sorbent bed (ca. 10 min), analytes were recovered with methanol, acetone or acetonitrile. This extract was diluted with 8 mL of a 5% K_2HPO_4 aqueous solution and 50 μL of acetic anhydride were added, followed by manual shaking (5 min) and extraction of acetylated species with 2 mL of isooctane [22]. Recoveries of the SPE step were determined comparing the difference between responses measured for spiked and non-spiked fractions of the same wine versus standards, prepared in the same solvent used to elute MAX cartridges and derivatized under above reported conditions.

Breakthrough studies, performed passing the spiked samples through two cartridges connected in series, showed losses around 10% for EP considering 10 mL of wine, whereas, the rest of species were not detected in the extract from the second cartridge. Using 5 mL of wine, all compounds were quantitatively retained in the first SPE cartridge. As regards the elution solvent, acetone and acetonitrile rendered completely transparent extracts versus reddish ones obtained with methanol, which was discarded for further experiments. **Table 1** summarized the recoveries of the SPE step as function of the elution solvent (1 mL). Acetone provided absolute recoveries between 103% and 108%, with standard deviations below 12%. In the case of acetonitrile, recoveries ranged from 59% for EC to 102% for DMP, with standard deviations below 12%, **Table 1**. Moreover, EC was still detected in the 2nd fractions (1 mL) of acetonitrile eluted from SPE cartridges. Thus, 1 mL of acetone was selected as extraction solvent.

Table 1. Absolute recoveries of the SPE step as function of the elution solvent (1 mL), n=4 replicates. Data corresponding to 20 ng mL⁻¹ spiked red wine samples.

Compound	Recovery (%) $\pm$ SD	
	Acetone	Acetonitrile
EP	108 $\pm$ 6	98 $\pm$ 7
EG	103 $\pm$ 6	99 $\pm$ 7
EC	107 $\pm$ 2	59 $\pm$ 12
DMP (I.S.)	103 $\pm$ 12	102 $\pm$ 10

3.3. Acetylation and DLLME

Departure derivatization and liquid-liquid microextraction conditions were adopted from a previous work reporting the determination of chlorophenols in water samples after SPE and DLLME [23]. In brief, the acetone extract from the SPE cartridge was collected in a conical bottom glass tube, mixed with 0.1 mL of a chlorinated extractant and a few microliters (20-100 μ L) of acetic anhydride. Then, 8 mL of an alkaline aqueous solution (KHCO₃ or K₂HPO₄, both 5% w/v) were rapidly injected in the tube to obtain an emulsion of dispersed droplets of extractant. Under these conditions, it was expected that analytes were simultaneously acetylated and concentrated in the chlorinated extractant, which settled at the bottom of the conical tube after centrifugation [23]. Experimental findings showed that, whatever the volume of acetic anhydride, extractant type and alkaline catalyzer, extracts contained mixtures of derivatized and underivatized forms of EP, DMP and EG. Taking into account these unsatisfactory results, it was decided to

perform first the acetylation reaction, in the aqueous phase, and then to concentrate the acetylated derivatives by DLLME.

3.3.1. Optimization of acetylation conditions

Derivatization conditions were optimized using a pool of SPE extracts obtained from unspiked aliquots (5 mL) of a red wine sample (*Tempranillo* variety) containing detectable amounts of target compounds. Fractions (1 mL volume) of this extract were diluted with 8 mL of an alkaline 5% aqueous solution, a given volume of acetic anhydride was added and then the mixture was manually shaken before DLLME extraction using, in all experiments, a mixture consisting of 0.9 mL of acetone and 0.1 mL of TCE. The effects of acetic anhydride volume (50-200 μ L), type of alkaline catalizer (KHCO_3 versus K_2HPO_4) and derivatization time (1-10 min) in the responses (peak areas) obtained for each analyte and the I.S. were simultaneously investigated using a 2^3 type experimental factorial design, with four replicates of the central point. The Statgraphics statistical package was used to estimate the main effects associated with each variable and two-factor interactions. **Figure 1** shows the Pareto charts with standardized values of above mentioned parameters. The length of depicted bars is proportional to the effect of the considered factor (or two-factor interaction) in the response of acetylated species. A positive sign points out to an improvement in the efficiency of the reaction when the factor changes from the low to the high level, within the domain of the design and, a negative one indicates the opposite behaviour. The vertical dotted line in the graphs represents the statistical significance bound, established for a 95% confidence level.

As appreciated in **Figure 1**, the derivatization time was the less important variable, thus it was fixed at 1 min. The type of catalyzer played a positive influence on the acetylation process. Although the associated main effect did not reach the significance threshold for any compound, it is evident that the most favourable situation corresponded to the use of K_2HPO_4 (5%, w/v). This salt provides a higher pH (9.2 units) than KHCO_3 (pH 8.3) and thus, it shifts the acid-base equilibrium of ethylphenols towards phenolate forms, which are supposed to be more reactive than neutral species. Finally, the volume of acetic anhydride was the most relevant factor, with a significant negative influence on the performance of the derivatization step, **Figure 1**. Likely, an excess of acetic anhydride reduces the pH of the aqueous solutions, which negatively affects the yield of acetylation.

Thus, 50 μL was adopted as the working value for this variable. Some additional experiments were carried out using K_2CO_3 instead of K_2HPO_4 ; however, in this case EC could not be acetylated, thus K_2HPO_4 was retained as catalyzer in the derivatization reaction.

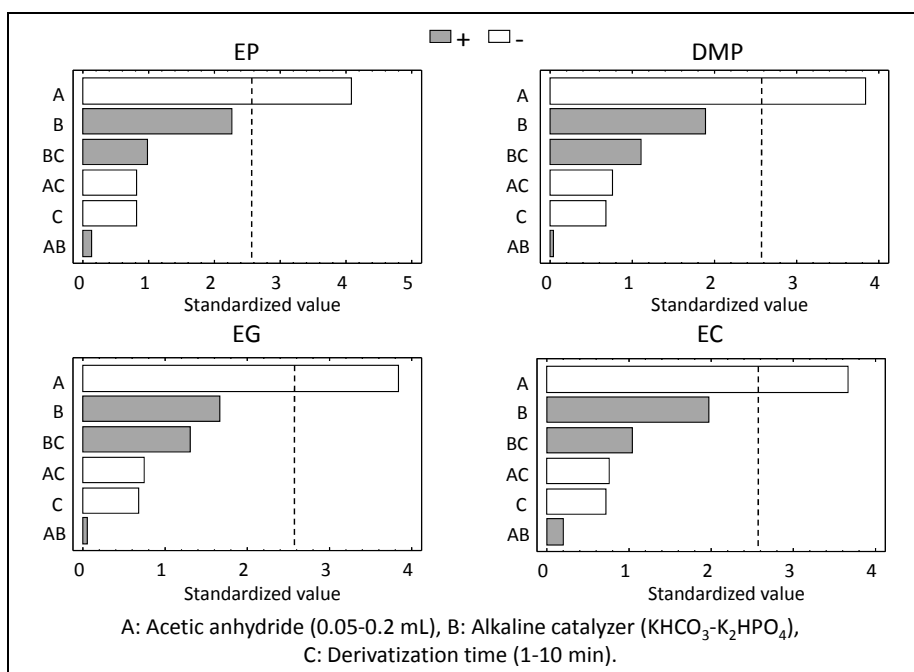


Figure 1. Standardized Pareto charts corresponding to optimization of acetylation conditions.

Figure 2 compares the extracted GC-MS chromatograms obtained using above optimized acetylation conditions, followed by DLLME with 1 mL of a binary mixture of acetone-TCE (9:1), and considering simultaneous DLLME and acetylation, as proposed by Fattahi and co-workers for chlorophenols [23]. As observed, the two-step method, optimized in this work, provided higher peak areas for EP, DMP and EG without traces of underivatized species for any of the considered phenols.

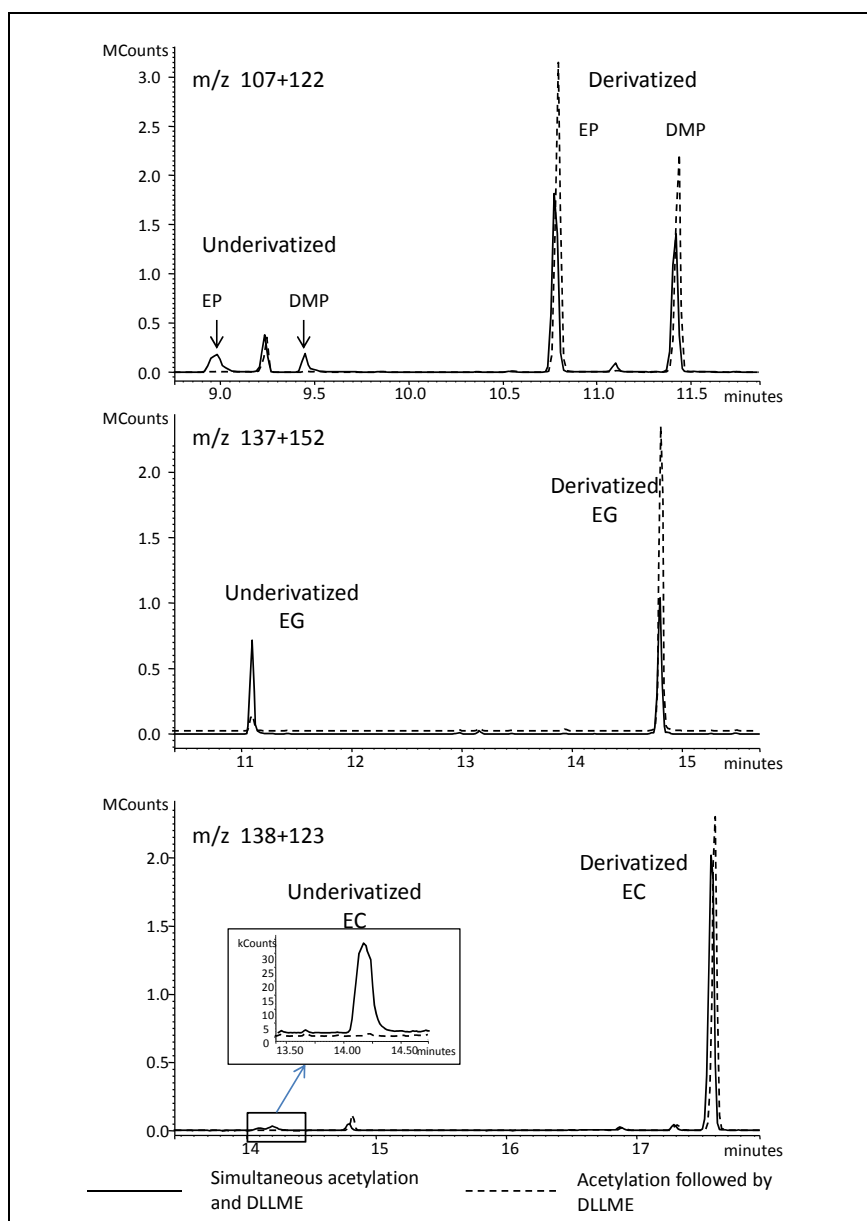


Figure 2. GC-MS chromatograms corresponding to a spiked red wine (addition level 200 ng mL^{-1}) under optimized acetylation and DLLME conditions (dotted line) and performing simultaneously acetylation and DLLME (solid line).

3.3.2. DLLME parameters

3.3.2.1. Dispersant and extractant selection

Performance of DLLME methods is mainly affected by the composition of the binary extraction solution. The effects of both solvents (extractant and dispersant) in the responses of target compounds were again investigated using the combined acetone extracts obtained after SPE of red wine. Comparison of responses obtained using methanol, acetone and acetonitrile (1 mL each) as dispersants, reflected non-significant differences between acetone and methanol, whereas around 30% lower peak areas were noticed for acetonitrile, figure not shown. Considering that acetone was also used for elution of SPE cartridges, it was selected as dispersant in the DLLME step.

Figure 3 shows the peak areas measured using three different extractants (0.1 mL). Responses for ClBz and CCl_4 stayed below those obtained with TCE, which was maintained as extractant.

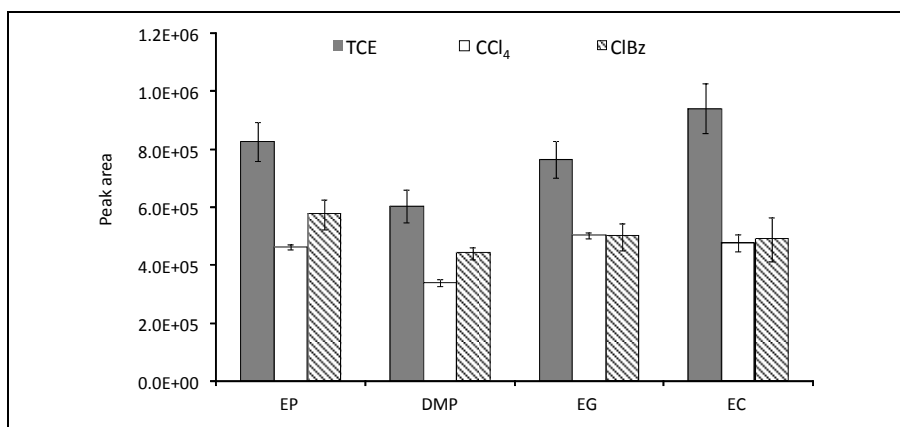


Figure 3. Comparison of responses as function of the extractant used in the DLLME step, n=4 replicates.

3.3.2.2. Dispersant and extractant volume

In DLLME, the volume of dispersant must be high enough to provide an emulsion of extractant droplets in the aqueous phase containing the acetylated ethylphenols.

Larger volumes result in lower extraction yields due to an increase in the solubility of the analytes.

Figure 4 shows the peak areas obtained for four different volumes of acetone (0.5, 1.0, 1.5 and 2.0 mL) combined with either 0.06 mL or 0.1 mL of TCE. The highest responses for all compounds were attained for the combination of 1.5 mL of acetone with 0.06 mL of TCE. Under these conditions, around 45 μL of TCE were recovered from the bottom of conical shaped derivatization-extraction tubes. This volume is large enough to be handled by the autosampler of the GC-MS system.

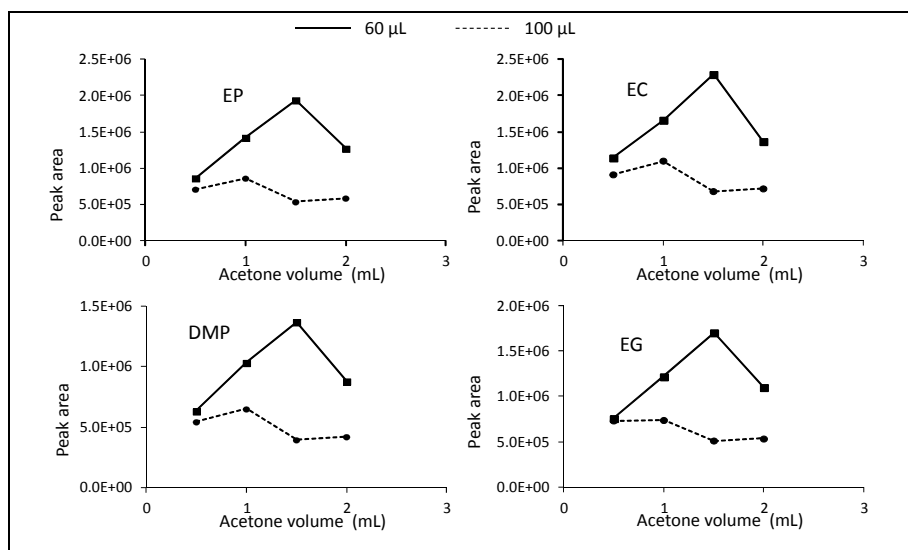


Figure 4. Effect of dispersant (acetone) and extractant (TCE) volumes in the responses of target compounds. Average values for duplicate experiments.

3.3.2.3. Extraction and centrifugation time

The extraction time is defined as the period comprised between addition of the extraction mixture to the aqueous sample, containing the acetylated compounds, and centrifugation. During this step DLLME tubes were manually shaken to maintain the emulsion of dispersed TCE droplets. Experimental data did not reveal any difference considering extraction times of 1, 5 and 10 min, which is in agreement with the extremely fast kinetics of the DLLME technique [20]. As regards the centrifugation time, values of 3, 10 and 15 min were tested. Again, this factor exerted a negligible effect in the responses of target

compounds. Thus, extraction and centrifugation times were set at 1 and 3 min, respectively.

3.4. Performance of the method

The extraction efficiencies (EEs, %) of the optimized DLLME step were assessed comparing the differences between responses obtained for spiked (100 ng mL^{-1} , added to the extract of the SPE cartridge) and non-spiked extracts from the same red wine sample, submitted to acetylation and DLLME under optimized conditions, with those corresponding to pure standards prepared in acetone, acetylated and further extracted by conventional liquid-liquid extraction (LLE) using 3 mL of TCE. Concentrations found in DLLME extracts were divided by the theoretic expected one (2222 ng mL^{-1} , assuming a drop volume of $45 \text{ }\mu\text{L}$) and multiplied by 100 in order to obtain the EEs reported in **Table 2**. Considering the whole analytical procedure (SPE-acetylation-DLLME), enrichment factors (EFs) obtained for 5 mL wine samples varied between 57 and 78 times.

Table 2. Extraction efficiencies (EEs, %) of the DLLME step and enrichment factors (EFs) of the whole procedure for 5 mL wine samples, $n=3$ replicates.

Compound	EEs (%)	RSD (%)	EFs	SD
EP	70	11	78	8
EG	60	10	67	6
EC	60	11	67	7
DMP (I.S.)	51	5	57	5

The overall performance of the developed method was evaluated using spiked and non-spiked samples. In all cases, DMP (I.S.) was added to wine at a constant concentration of 100 ng mL^{-1} . Precision was investigated with three red wines, corresponding to different geographic denominations and presenting low, medium and high ethylphenol contents. The intra-day precision ($n=4$ replicates) varied between 2 and 8%, whereas under reproducibility conditions ($n=12$ replicates, 3 days) relative standard deviations (RSDs) ranged from 3 to 10%, **Table 3**.

Table 3. Precision of the proposed method for non-spiked red wine samples with different ethylphenol contents, linearity (1–5000 ng mL⁻¹) and limits of quantification (LOQs) defined for a signal to noise (S/N) of 10.

Compound	Intra-day precision (RSDs %, n=4 replicates)		Inter-day precision (RSDs %, n=12 replicates)	Linearity (R ² , 1–5000 ng mL ⁻¹)	LOQs (ng mL ⁻¹)
	Low level sample	High level sample	Medium level sample		
EP	2.2	4.1	2.9	0.997	0.4
EG	7.8	3.7	9.6	0.998	0.3
EC	2.0	6.8	8.0	0.998	0.8

Linearity was assessed using a red wine (*Mencía*) sample, displaying low concentrations of ethylphenol species. Aliquots of this sample were spiked at nine concentration levels from 1 ng mL⁻¹ to 5000 ng mL⁻¹. **Figure 5** shows the chromatograms for the non-spiked wine and a fraction of the same matrix fortified with 5 ng mL⁻¹ per compound (the I.S. was maintained at 100 ng mL⁻¹). Within the above interval, a good linearity was observed with determination coefficients (R²) above 0.997 (**Table 3**). Procedural blanks (corresponding to the extraction of 5 mL aliquots of synthetic wine) did not show signals at retention times of ethylphenol species. Thus, method LOQs were estimated from the signal to noise (S/N) values corresponding to chromatographic peaks in the lower levels of the linearity study. Values obtained for S/N=10 stayed between 0.3 ng mL⁻¹ for EG and 0.8 ng mL⁻¹ for EC (**Table 3**). These LOQs remain two orders of magnitude below those achieved by Fariña and co-workers [16] applying the DLLME technique directly to wine samples followed by GC-MS determination and are similar to those attained by GC-MS/MS [5,17]. It is worth noting that, none of the above methods was tested for EC. LOQs reported in **Table 3** are also significantly lower than those published using SPME (LOQs, 3–30 ng mL⁻¹) and sorptive microextraction with polydimethylsiloxane type sorbents (LOQs, 5–15 ng mL⁻¹) after analytes acetylation [12,24]. On the other hand, SPE based methods require the concentration of wine volumes between 50 and 100 mL to achieve LOQs in the sub ng mL⁻¹ range [8,9].

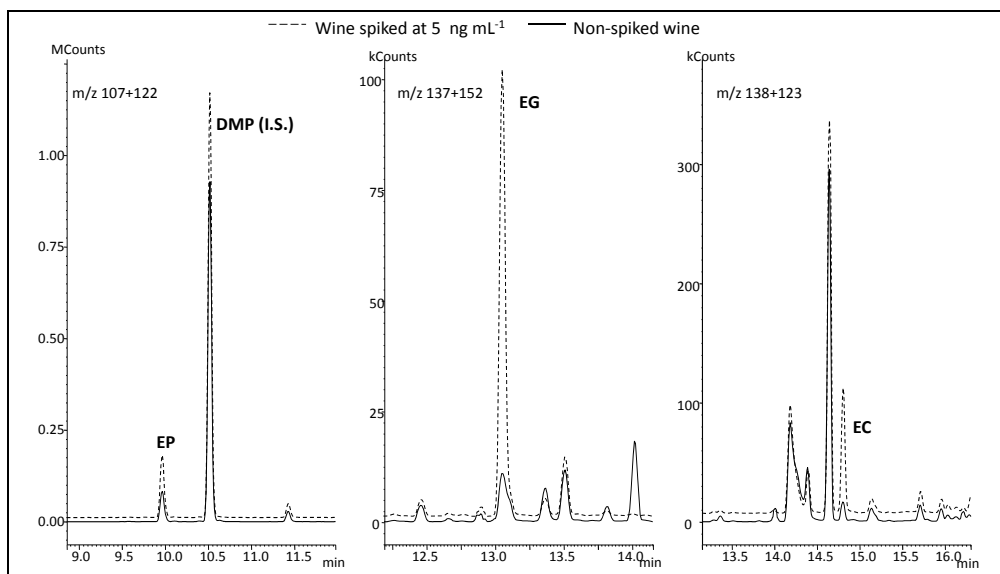


Figure 5. Extracted ion chromatograms corresponding to a wine sample before and after addition of ethylphenols (5 ng mL^{-1}).

As shown in **Table 2**, the EEs provided by the DLLME step are considerably high for a microextraction technique; however, the liquid-liquid microextraction process cannot be considered as exhaustive. Thus, quantification of ethylphenol levels in wine samples must rely either on the time-consuming standard addition method, or on matrix-matched standards. The accuracy of the later approach depends on whether the yield of the sample preparation process varies among wine samples, or if it remains basically unaltered. In order to answer this question, samples of young red wines, from three different geographic denominations, were spiked with increased concentrations of target compounds. After I.S. normalization, the maximum differences among the slopes of calibration curves accounted for a 15% for EP and EC, whereas, they remained below 10% for EG, see **Figure S1**. On the basis of these moderate sensitivity differences, the matrix-matched quantification technique was considered to assess the accuracy of the method. **Table 4** shows the relative recoveries for a red wine (*Grenache* variety) spiked at three different levels (20, 100 and 1000 ng mL^{-1}), quantified using a calibration curve corresponding to spiked aliquots of a different wine (*Mencía* variety). Relative recoveries between 89% and 108%, with associated standard deviations below 10, were achieved.

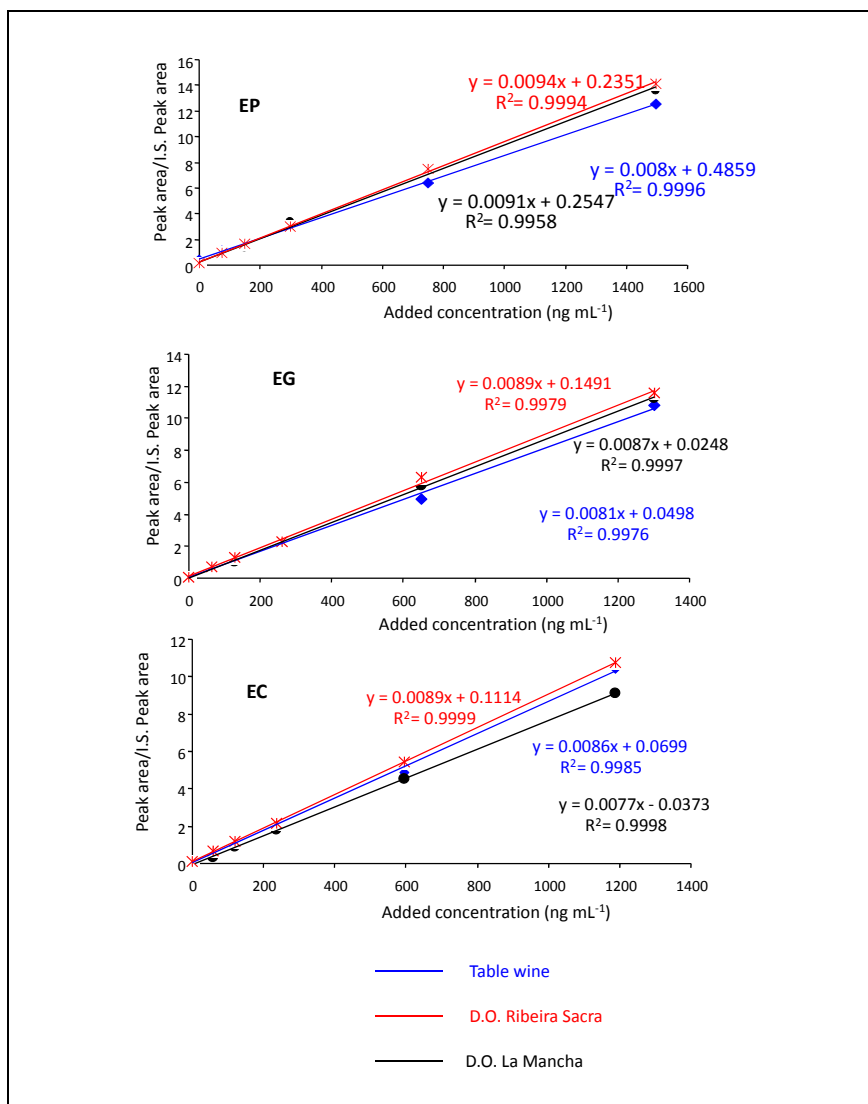


Figure S1. Calibration curves obtained for three different young red wines.

Table 4. Relative recoveries of the method (accuracy) for spiked aliquots of red wine.

Compound	Relative recovery (%) $\pm$ SD		
	^a 20 ng mL ⁻¹	^a 100 ng mL ⁻¹	^a 1000 ng mL ⁻¹
EP	108 $\pm$ 5	97 $\pm$ 2	92 $\pm$ 3
EG	106 $\pm$ 10	95 $\pm$ 2	89 $\pm$ 4
EC	89 $\pm$ 3	98 $\pm$ 2	89 $\pm$ 4

^aAdded concentration

3.5. Application to real samples

The proposed method was applied to a total of 10 red wine samples. Six samples were young wines (codes 1–5, 8), two were table wine (codes 6 and 7), without geographic denomination and distributed in Tetra Pack type packages; finally, codes 9 and 10 correspond to red wines aged in wood barrels. EC was quantified in 8 of the 10 processed samples, whilst the rest of species overpass the LOQs of the method in all of them (**Table 5**). Globally, EP levels stayed one order of magnitude above those of EG and EC, which were measured at similar concentrations in most samples. As expected, the highest concentrations corresponded to wood aged wines (codes 9 and 10); however, the presence of target analytes in young and table wines, which were never in contact with wood, points out to ethylphenols as intrinsic components of the aromatic profile of red wines.

Table 5. Concentrations (average values in ng mL^{-1} with their standard deviations) in measured in red wine samples, $n = 3$ replicates.

Code	Wooden aged	Mean $\pm$ SD		
		EP	EG	EC
1	No	5.9 ± 0.1	0.81 ± 0.08	n.d.
2	No	151 ± 6	16.5 ± 0.7	19 ± 1
3	No	7.2 ± 0.7	1.2 ± 0.2	n.d.
4	No	150 ± 9	22 ± 2	22.9 ± 0.5
5	No	38 ± 2	6.1 ± 0.4	5.0 ± 0.6
6	No	65 ± 1	8.6 ± 0.5	7.0 ± 0.4
7	No	11.42 ± 0.02	1.1 ± 0.1	1.85 ± 0.04
8	No	81 ± 2	5.8 ± 0.1	9.6 ± 0.2
9	Yes	1177 ± 50	73 ± 2	43 ± 3
10	Yes	2265 ± 44	251 ± 9	158 ± 11

4. Conclusions

The combination of mixed-mode SPE and DLLME provides an un-matched performance for the sensitive and accurate determination of ethylphenol compounds in red wines, overcoming the limited selectivity and capability of DLLME to deal with complex samples, as well as the reduced enrichment factors of SPE when considered individually. Despite acetylation and DLLME have to be performed sequentially, the method shows an acceptable sample throughput (both steps require about 5 min and many samples can be simultaneously submitted to SPE); moreover, it provides accurate

recoveries without using the time-consuming standard addition quantification methodology. Also, it permits the determination of target compounds in red wines at levels further below their sensorial thresholds, being suitable to investigate their evolution during wine maturation. Advantages of the proposed sample preparation approach (SPE followed by DLLME) are expected to be also useful for the determination of other volatile species, related to the organoleptic quality of wine.

Acknowledgements

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CAPÍTULO IV

Conclusiones/*Conclusions*

CONCLUSIONES

En esta memoria de Tesis se han presentado los desarrollos analíticos para la determinación de un grupo de contaminantes emergentes, utilizados como estabilizantes UV en productos industriales; varios fungicidas, empleados de forma frecuente en la producción de uva; y tres etilfenoles, relacionados con defectos sensoriales, en matrices medioambientales y en vino. Las actividades realizadas se han centrado, sobre todo, en la etapa de preparación de muestra, aportando procedimientos que puedan ser ventajosos desde el punto de vista de generación de residuos, coste y rapidez, manteniendo o mejorando las prestaciones analíticas (límites de cuantificación, precisión y exactitud) frente a metodologías ya disponibles en la bibliografía. La evaluación de la exactitud ha merecido un especial interés sobre todo a la hora de plantear técnicas de microextracción, tanto en fase sólida como líquida, aplicadas a muestras de agua y vino. Como es sabido, la eficacia de estas técnicas puede variar en función de las características concretas de cada muestra, lo que dificulta la obtención de datos cuantitativos con un nivel de esfuerzo experimental asumible. Globalmente, los resultados obtenidos han permitido proponer alternativas frente a métodos analíticos ya descritos en la bibliografía, así como profundizar en el comportamiento y distribución de las tres familias de compuestos en matrices con diferentes características.

A continuación se presentan las principales conclusiones que pueden extrapolarse de los trabajos realizados:

1. Determinación de Tinuvines en matrices medioambientales

Se han desarrollado tres metodologías para la determinación de Tinuvines en agua, en sedimentos y en polvo, empleando en todos los casos GC-MS/MS como técnica de determinación y aportando nuevos métodos de preparación de muestra, capaces de proporcionar factores de concentración y selectividad adecuados para evaluar la distribución de estos filtros UV en matrices reales.

En el caso de muestras de agua, el uso de SPME, en la modalidad de HS a temperatura elevada, ha conseguido minimizar los problemas de pérdidas por adsorción, particularmente relevantes en el caso de Tinuvin 327 y 328, detectados para metodologías

de SPE, a la vez que limita el consumo de disolventes orgánicos. Por otro lado, el muestreo en modo HS permite mejorar la selectividad de la extracción. La eficacia de extracción no se vio alterada de manera significativa entre muestras de agua ultrapura, superficial y residual tratada; sin embargo, si se observaron cambios relevantes en el caso de agua residual sin tratar. Por tanto, para esta última matriz la cuantificación debe de realizarse mediante el método de adiciones estándar. En combinación con GC-MS/MS y utilizando sólo 15 mL de agua, HS SPME permitió alcanzar LOQs entre 0.5 y 2 ng L⁻¹, inferiores a los descritos previamente en la bibliografía.

En lo referente a muestras de sedimento y polvo, se desarrollaron métodos de preparación de muestra, que combinaron extracción y *clean-up* en la misma etapa, basados en MSPD y PLE, respectivamente. En el caso de sedimentos, la presencia de azufre representa una importante dificultad para la cuantificación de Tinuvin P, incluso operando en modo MS/MS. Además, no fue posible integrar los tratamientos clásicos con Cu en polvo en el cartucho de MSPD. Así pues, los extractos deben almacenarse en contacto con Cu para reducir el azufre antes de su inyección en el sistema cromatográfico. Dejando a un lado esta dificultad, tanto MSPD como PLE proporcionaron recuperaciones cuantitativas para muestras de sedimentos y polvo, respectivamente, con consumos de disolventes orgánicos inferiores a 20 mL por muestra. El método de MSPD presenta a su favor un menor consumo de disolventes orgánicos y un coste muy inferior. Por su parte, PLE muestra un mayor grado de automatización.

Las mayores dificultades para la determinación de niveles traza de Tinuvines se deben por un lado a problemas de contaminación y, por otro, a su fuerte interacción con adsorbentes en fase normal. En el primer caso, la presencia de Tinuvines asociados a materia particulada y su fuerte carácter lipofílico favorece su deposición y/o adsorción sobre el material empleado en los procesos de preparación de muestra. Así pues, se recomienda reducir al mínimo la manipulación de las muestras, usar material desechable siempre que sea posible (por ej. métodos de MSPD) y cubrir el material de vidrio y las celdas de PLE, para evitar posibles contaminaciones por deposición de materia particulada en suspensión. Por otro lado, pese a su elevado carácter lipofílico, los Tinuvines han mostrado una elevada afinidad por adsorbentes en fase normal, probablemente debido a la formación de enlaces tipo puente de hidrógeno entre el grupo fenólico y/o el triazólico con estos materiales. Así pues, el grado de activación y la cantidad de estos adsorbentes empleados en el proceso de *clean-up* ejercen una influencia considerable en la eficacia

global del proceso de preparación de muestra, sobre todo en el caso de MSPD al emplear condiciones menos enérgicas que PLE.

Los datos obtenidos para muestras reales han confirmado el papel de las aguas residuales urbanas como vía de introducción de estos contaminantes emergentes en el medio acuático y la existencia de niveles traza de algunos de ellos en sedimentos. Esta información es coherente con los datos publicados en estudios realizados en Japón y otras regiones del Sudeste Asiático. Además, por primera vez, se ha demostrado la presencia de niveles significativos de Tinuvines en muestras de polvo de ambientes confinados.

2. Determinación de fungicidas en vino y suelos agrícolas

Se han desarrollado dos metodologías para la determinación de fungicidas en vino y en suelos agrícolas empleando SPE LC-MS/MS y MSPD GC-MS, respectivamente. El uso de MS o MS/MS, combinadas con una técnica cromatográfica, permite la identificación inequívoca de los analitos estudiados en las dos matrices consideradas.

Los estudios realizados con vino han puesto de manifiesto la eficacia de SPE como técnica de extracción y concentración. El uso de adsorbentes de modo-mixto (cartuchos Oasis MAX), ha permitido la obtención de extractos con un menor contenido en interferentes, hecho que se ha traducido en eficacias de ionización en electrospray (ESI) prácticamente constantes para patrones y extractos de muestras reales. Además, se han conseguido recuperaciones cuantitativas en la etapa de concentración. Como resultado, se ha obtenido una excelente exactitud para muestras de vinos blancos y tintos, usando calibración externa para la cuantificación. Los LOQs obtenidos ($0.01\text{--}0.8\text{ ng mL}^{-1}$) son al menos 2 órdenes de magnitud inferiores a los MRLs de estos fungicidas en uva.

Para la determinación de fungicidas en suelo, se ha optimizado un método de MSPD que permite la extracción de un grupo de 15 analitos, pertenecientes a 8 clases químicas. A diferencia de protocolos convencionales de extracción sólido-líquido encontrados en la bibliografía, MSPD no requiere etapas adicionales de limpieza y las recuperaciones no se vieron afectadas por el contenido de materia orgánica de las muestras, dentro del rango de valores esperables en suelos de viñedo. El procedimiento desarrollado ha proporcionado recuperaciones cuantitativas para muestras con adición y, concentraciones equivalentes a las obtenidas mediante PLE para suelos contaminados de

forma natural, durante la fumigación de viñedos. En conjunto, los resultados obtenidos para fungicidas y Tinuvines confirman la aplicabilidad de MSPD para la extracción de compuestos orgánicos de muestras sólidas de origen medioambiental.

La existencia de residuos de ciertos fungicidas en vino apunta a la necesidad de desarrollar una legislación específica para esta matriz, con objeto de erradicar el uso de aquellos fitosanitarios que presenten los mayores factores de transferencia de uva a vino. Además, la mayor o menor persistencia de estos compuestos en suelos agrícolas debería tomarse en cuenta a la hora de autorizar su uso. De esta forma es posible predecir problemas de resistencia y/o migración a otros compartimentos medioambientales.

3. Determinación de etilfenoles en vino

Se han desarrollado dos metodologías de microextracción para tres etilfenoles relacionados con defectos organolépticos en vino. Después de una etapa de acetilación, los analitos se han determinado mediante GC-MS alcanzando límites de cuantificación inferiores a sus umbrales sensoriales y factores de respuesta (una vez corregidas con el patrón interno) independientes de las características de cada vino. Por tanto, se ha utilizado una estrategia de pseudo-calibración externa para la obtención de resultados cuantitativos.

El procedimiento de microextracción en fase sólida, sobre discos de silicona, integra las etapas de derivatización y concentración, presenta un bajo consumo de disolventes orgánicos (0.2 mL por muestra) y rebaja los costes de determinación frente a metodologías de microextracción en fase sólida comerciales, como SBSE y SPME. Considerando la baja manipulación de muestra que requiere este método, y la estabilidad de los analitos una vez concentrados en el absorbente, la preparación de muestra podría realizarse *in-situ*, es decir, en las propias bodegas.

El procedimiento basado en la técnica de microextracción líquido-líquido dispersiva (DLLME) ha reflejado las dificultades para su aplicación directa a muestras de vino tinto sin diluir, como resultado de problemas con la separación de fases. Este inconveniente se ha solucionado combinando SPE, en modo mixto, con DLLME. El potencial de esta combinación para abordar la determinación de niveles traza de compuestos orgánicos en muestras de vino ha quedado patente en esta Tesis. En el caso

de los etilfenoles, aunque el procedimiento resultante consta de tres etapas (SPE, acetilación y DLLME), presenta una elevada productividad y los límites de cuantificación alcanzados ($0.3\text{--}0.8\text{ ng mL}^{-1}$) son un orden de magnitud inferiores a los proporcionados por la microextracción sobre discos de silicona ($5\text{--}15\text{ ng mL}^{-1}$).

CONCLUSIONS

This memory of Thesis summarizes the analytical developments applied to the determination of a group of emerging pollutants, used as UV stabilizers of industrial goods; several fungicides, often applied to vines; and three ethylphenols, related to sensorial defects, in environment samples and wine. Performed activities have been mainly focused on the sample preparation step, leading to advantageous procedures as regards wastes generation, cost and quickness. These methods maintain, or even improve, the analytical features (limits of quantification, precision and accuracy) of previously proposed methodologies. Accuracy assessment has become a major issue throughout this Thesis, particularly in the case of solid and liquid-based microextraction techniques applied to water and wine samples. As it is known, the efficiency of such techniques may change depending on the characteristics of each sample, a fact which makes difficult obtaining quantitative results with a level of acceptable experimental effort. Overall, obtained results have permitted to propose alternatives to methods already described in bibliography, as well as to obtain a deeper knowledge of the behaviour and distribution of the three studied families of compounds in matrices with different characteristics.

Next, the main conclusions that came out from developed activities are outlined:

1. Determination of Tinuvin in environmental samples

Three methodologies have been developed for the determination of Tinuvin in water, sediments and dust, using GC-MS/MS in all cases as determination technique, and providing new sample preparation methods rendering suitable enrichment factors and enough selectivity to evaluate the distribution of these UV filters in real samples.

In the particular case of water samples, the use of SPME, in HS mode at high temperature, has overcome adsorption losses, which were particularly important for Tinuvin 327 and 328, detected for SPE-based protocols; furthermore, SPME avoided organic solvents during sample preparation. In addition, the use of HS sampling improved the selectivity of the extraction. The efficiency of the microextraction did not change significantly among ultrapure, surface and treated wastewater; however, relevant variations were noticed for raw wastewater. Thus, the standard addition method was

required to quantify their levels in the latter matrix. In combination with GC-MS/MS, and using only 15 mL of water, HS-SPME reached LOQs between 0.5 and 2 ng L⁻¹, which are lower than those previously reported in the literature.

As regards sediment and dust samples, sample preparation methods based on MSPD and PLE, which combined analytes extraction and clean-up in the same step, have been developed. For sediments, co-extraction of elemental sulphur represented an important drawback for the quantification of Tinuvin P, even operating the GC-MS system in MS/MS mode. Moreover, attempts to integrate a reductive treatment, using copper powder, in the MSPD cartridge were unsuccessful. Thus, extracts had to be overnight stored, in contact with copper powder, before their injection in the chromatographic system. Leaving aside this difficulty, both MSPD and PLE provide quantitative recoveries for sediment and dust samples, respectively, with organic solvent consumptions below 20 mL per sample. The MSPD protocol presents some advantages like less (above half) organic solvent consumption than PLE and very little cost. On the other hand, PLE extractions are performed in an automated, un-attended mode.

Contamination problems and strong interactions with normal phase sorbents were the major problems for trace determination of Tinuvin in environmental samples. In the first case, occurrence of Tinuvin associated with particulate matter and their high lipophilic character favor their deposition and/or adsorption on material used in the sample preparation step. Thus, reducing sample handling, using disposable material whenever possible (e.g. MSPD methods), and covering glass material and PLE cells with aluminium foil, to prevent contamination arising from deposition of particulate matter, is recommended. On the other hand, and despite their high lipophilic character, Tinuvin display a high affinity for normal phase adsorbents, likely due to the formation of hydrogen bonds between phenolic and/or triazolic groups and these sorbents. Thus, the activation grade and the amount of these materials, used in the clean-up process, exerted a significant influence on the global efficiency of the sample preparation process, particularly in MSPD, that uses milder conditions than PLE.

Data obtained for real samples have confirmed the role of urban wastewater as a source of these emerging pollutants in the aquatic environment and the existence of trace levels of some of them in sediment samples. These results are in agreement with data reported for studies carried out in Japan and other regions from Southeast Asian. Moreover, for first time, the presence of significant levels of Tinuvin in dust from indoor environment has been reported.

2. Determination de fungicides in wine and agricultural soil

Two methodologies have been developed for the determination of fungicides in wine and agricultural soils using SPE LC-MS/MS and MSPD GC-MS, respectively. The use of MS or MS/MS, combined with a chromatographic technique, allows the unequivocal identification of target compounds in the two considered matrices.

The studies performed with wine have demonstrated the efficiency of SPE as extraction and concentration technique. The use of mixed-mode adsorbents (Oasis MAX cartridges) has rendered more selective extractions than reversed-phase ones, a fact that led to ESI efficiencies, which remained unaltered between standards and wine extracts. Furthermore, quantitative recoveries were achieved in the SPE step. As a result, accurate results were obtained using the straightforward external calibration technique for red and white wines. The achieved LOQs ($0.01\text{--}0.8\text{ ng mL}^{-1}$) remained, at least, two orders of magnitude below fungicides MRLs in grapes.

For the determination of fungicides in soil, a MSPD method, allowing the extraction of 15 compounds belonging to 8 different chemical classes, has been optimized. Unlike conventional protocols based on solid-liquid extraction, MSPD does not need additional clean-up steps and recoveries were not affected by the organic matter content of soil, within the range of values expected for vineyard soils. The developed procedure provided not only quantitative recoveries for spiked samples, but also equivalent concentrations to the ones obtained by PLE for soils becoming contaminated during fumigation of vineyards. Altogether, results obtained for fungicides and Tinuvins confirm the applicability of MSPD for the extraction of organic compounds from solid environmental samples.

The existence of detectable residues of certain fungicide in wine points out to the convenience of developing an specific legislation for this foodstuff, with the aim of eradicate the use of those agrochemicals displaying the highest transference factors from grapes to wine. Moreover, their persistence in agricultural soils should be another variable to consider before approving their use. In this manner, it is possible to predict the apparition of resistant organisms, as well as to prevent migration and contamination of other environmental compartments.

3. Determination of ethylphenols in wine

Two microextraction methodologies have been developed for the determination of three ethylphenols, related to sensorial defects, in wine. After a previous acetylation step, analytes were determined by GC-MS achieving limits of quantification far below their sensorial thresholds, and response factors (after internal standard correction) independent of the characteristics of each wine. Therefore, the matched-matrix standard approach could be used for quantification purposes.

The solid-phase microextraction procedure with silicone discs integrates derivatization and concentration steps, shows a low organic solvent consumption (0.2 mL per sample) and reduces analysis costs in comparison with commercial solid-phase microextraction techniques, such as SBSE and SPME. Considering the low sample handling requirements of this method, and the stability of the analytes once they have been incorporated in the silicone discs, sample preparation could be carried out *in-situ*, that is, in wineries.

Attempts to directly apply the DLLME technique to undiluted wine samples, particularly red wines, failed due to phase separation problems. This drawback was overcome combining SPE, in the mixed-mode, with DLLME. The potential of this combination to address the determination of trace level organic compounds in wine samples has been demonstrated in this study. In the particular case of ethylphenols, although the resulting procedure requires three sequential steps (SPE, acetylation and DLLME), it shows a high sample throughput. Moreover, the achieved limits of quantification ($0.3\text{--}0.8\text{ ng mL}^{-1}$) are one order of magnitude lower than those achieved using silicone discs ($5\text{--}15\text{ ng mL}^{-1}$).



ANEXO

1. Abreviaturas y acrónimos/

Abbreviations and acronyms

ACN	Acetonitrilo	Acetonitrile
%R	Recuperación (%)	% Recovery
AcOEt	Acetato de etilo	Ethyl acetate
AED	Detector de emisión atómica	Atomic emission detector
ASE	Extracción acelerada con disolvente	Accelerated solvent extraction
AZO	Azoxistrobina	Azoxystrobin
BEN	Benalaxil	Benalaxyl
C18	Octadecilsilano	Octadecylsilane
C8	Octilsilano	Octylsilane
CAR	Carboxen	Carboxen
CEE	Comunidad Económica Europea	
CYP	Ciprodinil	Cyprodinil
CW	Carbowax	Carbowax
D.O.	Denominación de origen	
DAD	Detección con array de diodos	Diode array detection
DCM	Diclorometano	Dichloromethane
DI	Inmersión directa	Direct Immersion
DIF	Difenoconazol	Difenoconazol
DIN	Diniconazol	Diniconazol
DLLME	Microextracción líquido-líquido dispersiva	Dispersive liquid-liquid microextraction
dSPE	Extracción dispersiva en fase sólida	Dispersive solid-phase extraction
DSS	Adsorbente de silicona desechable	Disposable silicone sorbent
DVB	Divinilbenceno	Divinylbenzene
EC	4-Etilcatecol	4-Ethylcatechol
ECD	Detector de captura electrónica	Electron-capture detector
EDAR	Estaciones depuradoras de agua residual	Wastewater treatment plant
EF	Factor de enriquecimiento	Enrichment factor
EG	4-Etilguayacol	4-Ethylguaiacol
EI	Ionización por impacto electrónico	Electronic impact ionization
EP	4-Etilfenol	4-Ethylphenol
EPA	Agencia de Protección Medioambiental	Environmental Protection Agency
ESI	Ionización por electrospray	Electrospray ionization

FAO	Organización de las Naciones Unidas para la Alimentación y Agricultura	Food and Agriculture Organization of United Nations
FID	Detector de ionización de llama	Flame ionization detector
FEN	Fenhexamida	Fenhexamide
FLU	Flusilazol	Flusilazole
FLUD	Fludioxonil	Fludioxonil
GC	Cromatografía de gases	Gas chromatography
HF	Fibra hueca	Hollow fiber
HPLC	Cromatografía líquida de alta resolución	High performance liquid chromatography
HS	Espacio de cabeza	Headspace
IL	Líquido iónico	Ionic liquid
IPRO	Iprovalicarb	Iprovalicarb
IS	Patrón interno	Internal standard
K _{ow}	Constante de distribución octanol-agua	Octanol-water partition constant
LC	Cromatografía líquida	Liquid chromatography
LDPE	Polietileno de baja densidad	Low density polyethylene
LLE	Extracción líquido-líquido	Liquid-liquid extraction
LOD	Límite de detección	Limit of detection
LOQ	Límite de cuantificación	Limit of quantification
LPME	Microextracción en fase líquida	Liquid-phase microextraction
LVI	Inyección de grandes volúmenes	Large volumen injection
m/z	Relación masa/carga	Mass/charge ratio
MAE	Extracción asistida por microondas	Microwave assisted extraction
MASE	Extracción con disolventes asistida por membrana	Membrane assisted solvent extraction
MeOH	Metanol	Methanol
MET	Metalaxilo-M	Metalaxyl-M
MIP	Polímeros de impresión molecular	Molecular imprinted polymer
MMLE	Extracción líquido-líquido con membrana microporosa	Microporous membrane liquid-liquid extraction
MRL	Límite máximo de residuos	Maximum residue limit
MRM	Monitorización de múltiples iones	Multiple reaction monitoring
MS	Espectrometría de masas	Mass spectrometry

MS/MS	Espectrometría de masas en tandem	Tandem mass spectrometry
MYC	Miclobutanilo	Myclobutanil
NOAELs	Máxima concentración que no causa efectos adversos observables	No-observed-adverse-effect-level
NPD	Detector de nitrógeno-fósforo	Nitrogen-phosphorus detector
p/p	Peso/peso	Weight/weight (w/w)
PA	Poliacrilato	Polyacrilate
PAH	Hidrocarburo aromático policíclico	Polycyclic Aromatic Hydrocarbon
PBDE	Difeniléter polibromado	Polybrominated diphenyl ether
PCB	Bifenilo policlorado	Polychlorinated biphenyl
PDMS	Polidimetilsiloxano	Polydimethylsiloxane
PEG	Polietilenglicol	Polyethylenglycol
PEN	Penconazol	Penconazole
PLE	Extracción con fluidos presurizados	Pressurized liquid extraction
PES	Polietersulfona	Polyethersulfone
PP	Polipropileno	Polypropylene
PRO	Propiconazol	Propiconazole
PROC	Procimidona	Procymidone
PS	Poliestireno	Polystirene
PSA	Amina primaria y secundaria	Primary and Secondary Amine
PTV	Vaporización con programa de temperatura	Programmable temperature vaporization
p/v	Peso/volumen	Weight/volumen (w/v)
Q	Cuadrupolo	Quadrupole
RSD	Desviación estándar relativa	Relative standard deviation
SBSE	Extracción con barra agitadora	Stir-bar sorptive extraction
SD	Desviación estándar	Standard deviation
SDME	Microextracción con gota suspendida	Single-drop microextraction
SFE	Extracción con fluidos supercríticos	Supercritical fluid extraction
SIM	Monitorización de único ion	Single ion monitorization
SLE	Extracción sólido-líquido	Solid-liquid extraction
SPE	Extracción en fase sólida	Solid-phase extraction
SPME	Microextracción en fase sólida	Solid-phase microextraction
t _{1/2}	Tiempo de vida media	Half-life time
TEB	Tebuconazol	Tebuconazole

TOF	Tiempo de vuelo	Time of flight
UPLC	Cromatografía líquida de ultra eficacia	Ultra performance liquid chromatography
USAEME	Microextracción-emulsificación asistida por ultrasonidos	Ultrasound assisted emulsification- microextraction
UV	Ultravioleta	Ultraviolet
VP	N-Vinilpirrolidona	N-Vinylpyrrolidone
v/v	Volumen/volumen	Volume/volume
WHO	Organización Mundial de la Salud	World Health Organization

2. Other publications



Headspace solid-phase microextraction of halogenated toluenes in environmental aqueous samples with polypropylene microporous membranes

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ABSTRACT

The optimization of the polypropylene microporous membrane based solid-phase microextraction (MMSPE) of several halogenated (Cl^- and Br^-) toluenes was carried out. The influence of several factors such as sampling mode, sample volume, stirring rate, ionic strength, exposure time, etc. on the performance of the microextraction process was thoroughly investigated. Under optimized conditions, analytes were concentrated onto a 2-cm long membrane exposed to the headspace (HS) of the sample vial containing 80 mL of water with a 30% of sodium chloride. Equilibrium was achieved after a sampling period of 1 h, at room temperature and vigorous magnetic stirring. After analytes desorption, with just 250 μL of *n*-hexane, they were determined by gas chromatography with micro-electron capture detection (GC- μ -ECD). The developed methodology was characterized and validated also by gas chromatography–mass spectrometry (GC–MS). The proposed approach presented good precision (RSDs of 6–15% and 2–8% under reproducibility and repeatability conditions, respectively) and linear responses (R^2 : 0.990–0.999) over more than two order of magnitude concentration ranges for di- to penta-substituted species either with GC–MS or GC- μ -ECD detection. The absolute extraction efficiency was directly related with the lipophilic nature of the studied species and their volatile character, ranging between 27% and 47% for di- to pentahalotoluenes. Despite being an adsorption based methodology, no matrix effects were observed for complex environmental water matrices such as river water or treated wastewater. The proposed approach provided a very simple and low-cost microextraction alternative rendering adequate limits of quantification, in the low/sub ng L^{-1} level, for environmental sample analysis of poly-halogenated toluenes.

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1. Introduction

Contamination of surface waters with chlorobenzenes has been commonly reported during last decades due mainly to their extent use as solvents and dye carriers in industrial applications [1]. Nowadays, increasing levels of halogenated toluenes have been detected in surface waters located in the nearby of dense industrialized areas. The substitution of the typical trichlorobenzene mixtures in the textile industry by mainly dichlorinated isomers of toluene has been pointed out as a possible explanation [2]. Although chlorinated toluenes are commonly used also as solvents and dye carriers [3], very few data are available regarding their analysis in water samples. Typical sample preparation methodologies for VOCs analysis (headspace [4,5], purge and trap extraction [6–8] or SPME

methods [9]) have been applied to the determination of just the most volatile congeners (monochlorinated isomers) in water samples. Particularly, Martí et al. [2] reported concentrations for mono- to trichlorinated toluenes in rivers receiving wastewater discharges from a highly industrialized area. For most samples in this study, the dichlorinated isomers, not considered in VOCs analysis, presented two order of magnitude higher values than mono- or trihalogenated species, exceeding in some samples the $10 \mu\text{g L}^{-1}$ level. This is in concordance with the pattern observed in wastewater treatment plants and textile industry of the same area where dichlorinated toluenes were clearly the main discharged isomers. In some textile industry wastewater effluents, values higher than $1000 \mu\text{g L}^{-1}$ were measured [2].

Solid-phase microextraction (SPME) techniques, either fibre-coated based SPME [10–12] or stir bar sorptive extraction (SBSE) [13], have experienced an important development during the last two decades for the enrichment of VOCs and semivolatile compounds. More recently, liquid-phase microextraction (LPME)

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[14–16], under different configurations, has emerged as an appealing alternative to conventional SPME methodologies, due to the lower cost of the set-up, the elimination of carry-over problems and the wide range of extractants to be used in comparison with the limited commercially available SPME coatings. The most robust configuration for LPME, introduced by Pedersen-Bjergaard and Rasmussen [17,18] utilizes a microporous membrane (MM) to support the acceptor phase during the extraction. Typically, polypropylene membranes with a wall thickness of 200 μm and an internal diameter of 600 μm , also referred as hollow fibres (HF), are employed. They have a porosity around 70% and a pore size of 0.2 μm . The acceptor solvent penetrates the pores of the hollow fibre and it remains bound, by capillary forces, to the polypropylene network resulting in a thin film [19]. Moreover, the lumen of the fibre may also be filled with the same or with a different solvent. The principles of the hollow fibre LPME considers therefore, the MM just as an inert material acting as support either for a liquid phase (LPME) or for a polymeric coating (polymer coating hollow fibre microextraction: PC-HFME) where analytes are extracted and concentrated [20,21]. However, Müller et al. [22] highlighted that, in addition to liquid–liquid partition, sorption of the compounds onto the network of the microporous membrane might also contribute to their extraction from water samples. They found that, in the case of analytes with very high K_{ow} values, such as *t*-nonylphenols or tonalide, adsorption onto the hydrophobic polypropylene membrane was relevant. Based on their observations, Montes et al. [23] described, for the first time, the application of dry porous polypropylene membranes for the concentration of off-flavour anisoles in aqueous matrices.

To the best of our knowledge, except in case of mono-halogenated congeners, microextraction techniques have not been yet applied to the concentration of poly-halogenated toluenes in water samples. In this work, a simple and low-cost methodology to quantify the presence of halogenated toluenes, at the low ng L^{-1} level, in environmental water samples is presented. Inexpensive polypropylene microporous hollow fibres were used, as disposable sorbents, for the extraction and concentration of these contaminants from aqueous matrices (ultrapure, tap, surface water and wastewater). Optimization of several factors potentially affecting the microextraction process such as sampling mode, exposure time, fibre length, desorption solvent, etc. was performed. Analytes were recovered from the polypropylene fibres with a few μL of an appropriate solvent and determined by gas chromatography (GC) either with micro-electron capture detection ($\mu\text{-ECD}$) or mass spectrometry (MS).

2. Experimental

2.1. Chemicals and material

Acetone, *n*-hexane, toluene, methanol, isooctane (trace analysis solvents) and ascorbic acid were acquired from Merck (Darmstadt, Germany). Sodium chloride was purchased from Fluka (Büchs, Switzerland). Standards of 2-chlorotoluene (2CT), 4-chlorotoluene (4CT), 2,4-dichlorotoluene (24DCT), 2,6-dichlorotoluene (26DCT), 4-bromo-2-chlorotoluene (4Br2CT) and 1,2,4-trichlorobenzene (124TCB) (internal standard) were obtained from Sigma–Aldrich Chemie (Steinheim, Germany). Pentachlorotoluene (PCT) and 2,3,6-trichlorotoluene (236TCT) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany), and 3,4-dichlorotoluene (34DCT) and 2,4,5-trichlorotoluene (245TCT) were from Fluka and Supelco (Bellefonte, PA, USA), respectively. Individual solutions of each analyte were prepared in methanol and further dilutions, in the same solvent, were used to spike the water samples. Calibration stan-

dards were prepared also by dilution of individual stock solutions prepared in isooctane.

Accurel polypropylene Q3/2 membranes in a HF configuration (600 μm I.D., 200 μm wall thickness, 0.2 μm pore size and 0.6 m length) were supplied by Membrana (Wuppertal, Germany). A sharp blade was used to cut each fibre in segments with different length (0.5–2 cm). Sampling with 4-cm individual fibres was not possible due to the limited HS volume. For that approach, 2 identical 2-cm fibres were used, desorbed individually afterwards and responses for each compound combined. Under optimal conditions, 2-cm long fibres were employed as single use sorbents. They were soaked with acetone, allowed to dry, wrapped with aluminium foil and stored in a closed vessel previously to their use.

2.2. Sample preparation

Optimization of the microextraction process was performed using samples of ultrapure (Milli Q) water spiked with 40 ng mL^{-1} of DCTs, 20 ng mL^{-1} of 4Br2CT, 10 ng mL^{-1} of 245TCT and 4 ng mL^{-1} of PCT. Since, during optimization of extraction conditions, GC- $\mu\text{-ECD}$ was used as determination technique, mono-chlorotoluenes were not considered. Optimized methodology was validated for tap, river and wastewater (influent and effluent). Grab wastewater samples were collected from inlet and outlet streams from an urban sewage treatment plant with primary and secondary (activated sludge) treatments. Tap, river and wastewater were filtered using cellulose acetate membranes (0.45 μm) (Millipore, Billerica, MA, USA). Influent was also previously filtered using glass fibre filters. Ascorbic acid was added to tap water to eliminate the free chlorine before filtration.

Microextractions were accomplished in glass vessels furnished with PTFE-lined septa and aluminium caps (Sugelabor, Madrid, Spain). A stainless steel wire (0.6 mm diameter), previously rinsed with methanol and dried, was inserted in the lumen of the hollow fibres, allowing just the outer surface and the pores of the fibre to be available for sorption. Three different sizes of stir bar were considered for agitation (3 $\times$ 8 mm, 5 $\times$ 20 mm and 7 $\times$ 60 mm).

In MMSPE experiments, fibres were either immersed in the sample (direct sampling) or suspended 0.5 cm over the surface of the water sample (headspace (HS) mode). In the LPME approach, toluene was used as extraction solvent due to its structural similarity with the target compounds and its compatibility with the MM material [14–16]. The fibres were soaked during 2 min with toluene previously to the extraction in order to immobilise the solvent in the pores of the membrane. Sampling was carried out either at room temperature ($20 \pm 2^\circ\text{C}$) or heating the water sample in a water bath at 100°C .

Once the sampling period was finished, the hollow fibre, with the stainless steel wire still in its lumen, was desorbed with 250 μL of solvent in a 0.3 mL insert located in a 1.5 mL GC vial. The stainless steel wire was cut using pliers to allow the vials to be closed. Three different solvents: isooctane, *n*-hexane and pentane were evaluated for desorption of the fibres. Traces of water on the surface of membranes exposed directly to the sample were removed before analytes desorption with a lint free tissue.

After desorption, 50 μL of a solution with an appropriate concentration of the internal standard in isooctane were added to the extract and the volume reduced to ca. 50 μL by evaporation under a gentle stream of nitrogen.

Under optimal conditions, MMSPE were carried out in the HS mode, using 2-cm long dry fibres. Extractions were performed for 1 h, at room temperature ($20 \pm 2^\circ\text{C}$) in 120 mL glass vessels containing 80 mL of water with a 30% of sodium chloride and samples were stirred vigorously with a 7 $\times$ 60 mm magnetic bar. Desorption time was fixed in 30 min using *n*-hexane as extractant.

2.3. Instrumentation

Determination of target analytes was performed with an Agilent 6890 gas chromatograph (GC) (Wilmington, DE, USA) equipped with a split/splitless injection port, autosampler and a micro-electron capture detector (μ -ECD). Compounds were separated in an Agilent HP-35 capillary column (30 m $\times$ 0.25 mm I.D., d_f 0.25 μ m) using helium (99.999%) as carrier gas at a constant flow of 1.4 mL min⁻¹. Injector and detector temperatures were set at 260 and 300 °C, respectively. The extract (2 μ L) was injected in the splitless mode (splitless time 1 min) with the following oven temperature program: 80 °C (1 min), 8 °C min⁻¹ till 180 °C, 12 °C min⁻¹ till 250 °C (held for 5 min). The makeup nitrogen flow in the μ -ECD system was fixed at 60 mL min⁻¹.

Due to the limited sensitivity of the μ -ECD for toluenes with a low degree of halogenation, and the risk of compounds misidentification in complex water samples, validation of the proposed analytical methodology was assessed also using a Varian (Walnut Creek, CA, USA) CP 3900 GC connected to an ion-trap mass spectrometer (MS) (Varian Saturn 2100). Separations were carried out with the same Agilent HP-35 capillary column, using helium (99.999%) as carrier gas at a constant flow of 1 mL min⁻¹. The GC oven was programmed as follows: 50 °C (1 min), 5 °C min⁻¹ till 180 °C, 12 °C min⁻¹ till 250 °C (held for 5 min). Injections (2 μ L of extract) were made in the splitless mode (splitless time 1 min) with the injector port at 250 °C. The GC–MS interface and the ion-trap temperatures were set at 280 and 220 °C, respectively. The mass spectrometer was operated in the electron impact ionisation mode (70 eV). MS spectra were recorded in the range from 75 to 400 m/z units at 1 scan s⁻¹. For quantification, the sum of the signals for the 2 most intense ions of each analyte was selected: 91 + 126 m/z for monochlorinated toluenes, 125 + 160 for dichlorinated toluenes, 159 + 196 for trichlorinated toluenes, 135 + 206 for the bromo-chlorinated isomer, 229 + 264 for pentachlorotoluene and 180 + 182 for the internal standard (124TCB).

3. Results and discussion

3.1. Extraction mechanism and sampling mode

Up to now, polypropylene MMs had been considered simply as an inert material to be used either as support of a liquid phase (LPME) or as holder for a polymeric coating sorbing phase (PC-HFME). Very recently, Montes et al. [23] reported for the first time the use of these HF-MMs to carry out the direct microextraction of organic compounds (halogenated anisoles) by adsorption mechanisms.

To further evaluate the possibilities of such technique, its applicability to the microextraction of halogenated toluenes in water samples was investigated. The first step was to compare, in a simple and single approach, the efficiencies of MMSPE and LPME using toluene as extraction solvent. Obtained results are shown in Fig. 1. Both techniques provided very similar responses for the dichlorinated species, whereas a slightly better response was obtained by MMSPE for more lipophilic isomers. Therefore, the MMSPE technique was found a good potential alternative to LPME for these contaminants, avoiding completely the use of organic solvents

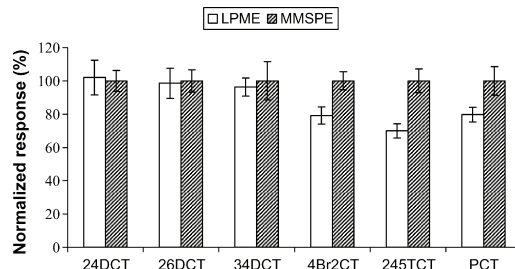


Fig. 1. Normalized responses for LPME and MMSPE of spiked water samples during 30 min in the HS mode. $N = 3$ replicates.

Table 2
Domain for the experimental factorial design.

Factor	Low level	Medium level	High level
NaCl	0%	15%	30%
Stirring (300 rpm)	No	–	Yes
Sample volume	30 mL	–	80 mL

during the extraction step, and factors affecting the extraction efficiency were optimized as follows.

The extraction efficiency of the MMSPE process by membrane immersion in the spiked water was compared to that achieved in the HS mode. As shown in Table 1, efficiencies of extraction using the HS mode were clearly superior for all halogenated toluenes, especially for the most lipophilic ones. Apart from differences between kinetics of direct and HS extraction modes, during direct sampling, analytes can interact only with the outlet surface of the membrane, since its lipophilic nature prevents the direct contact between the water sample and the internal surface of the micropores in the membrane. This limitation does not exist when analytes have previously migrated to HS of the sampling vessel.

An increase in temperature will improve the diffusion kinetics of analytes from the liquid to the vapour phase in the HS of the extraction vessel, however, at the same time, heating will also alter the distribution between polypropylene and the HS, favouring the desorption of the most volatile analytes from the warmed fibre. As reflected in Table 1, responses attained by HS sampling at 100 °C were significantly lower than those obtained at room temperature, being the less halogenated species more negatively affected by this factor.

3.2. Optimization of extraction conditions

3.2.1. Ionic strength, stirring and sample volume

A mixed level 3×2^2 experimental factorial design, involving a total of 12 experiments, was performed in order to simultaneously evaluate the influence of salt addition, stirring and sample volume on the efficiency of the extraction process in the HS mode. Table 2 summarizes the different levels chosen for the three selected factors. The effect of each factor on the response is represented in Fig. 2. The slope and the length of the depicted curves represent the variation in the response measured for each compound when the

Table 1
Normalized responses for MMSPE of spiked water samples using different sampling modes and temperatures, $n = 3$ replicates.

Normalized response (%)	24DCT	26DCT	34DCT	4Br2CT	245TCT	PCT
Direct sampling	33 $\pm$ 6	31 $\pm$ 5	38 $\pm$ 5	23 $\pm$ 4	16 $\pm$ 4	7 $\pm$ 8
HS, room temperature	100 $\pm$ 6	100 $\pm$ 6	100 $\pm$ 4	100 $\pm$ 5	100 $\pm$ 6	100 $\pm$ 10
HS, 100 °C	7 $\pm$ 9	6 $\pm$ 9	8 $\pm$ 11	10 $\pm$ 8	15 $\pm$ 10	58 $\pm$ 11

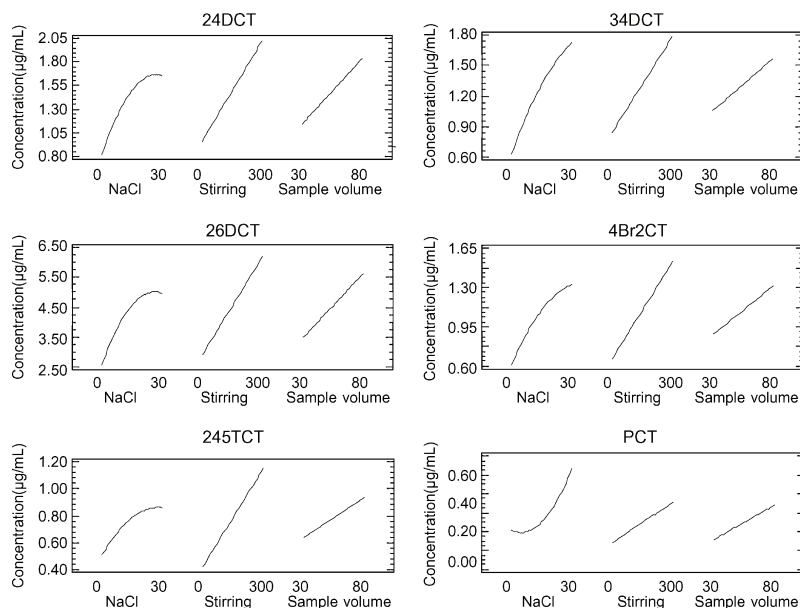


Fig. 2. Main effects graphs obtained from the experimental factorial design.

associated factor changes from the low to the high level considered in the domain of the design. The sign (+ or –) of the slope is related to the positive or negative exerted influence. Main effects and interactions were evaluated also by ANOVA. The *p*-values showed that, at a 95% confidence level, the main effects associated with the

three investigated factors: salt addition, stirring and sample volume were statistically significant ($p < 0.05$) for all species. Stirring was the most important factor for 5 of the 6 studied compounds. It is believed that, the deep vortex produced in the vials increased the surface of contact between the sample and the HS, speeding

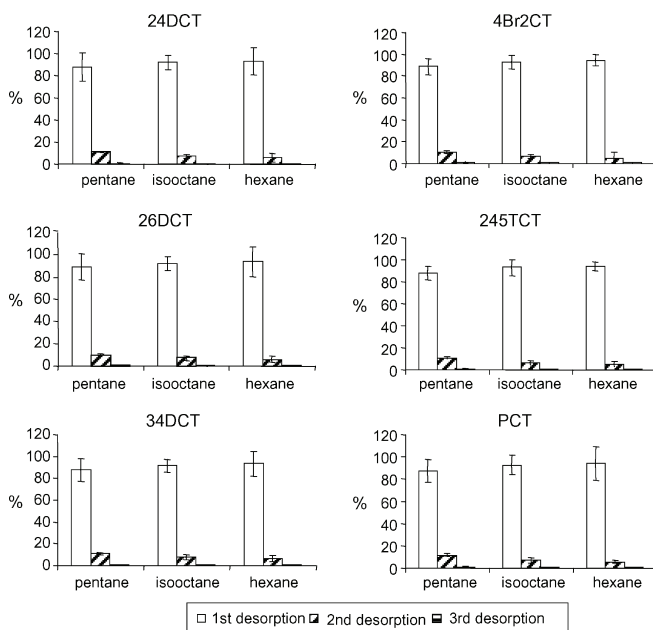


Fig. 3. Desorption profile of the hollow-fibres using different solvents. Normalized responses as percentages, $n = 3$ replicates.

the evaporation of the compounds and thus their incorporation to the polypropylene. In order to confirm this assumption, stir bars with different sizes were considered. The highest responses were observed for the larger bars, data not shown.

An increase in the ionic strength of the water sample altered the water–HS partitioning of dissolved species and improved significantly the response for all halotoluenes. In general, the addition of salt rises the amount of polar or semipolar substances transferred to the HS, and therefore to the fibre whilst this effect is minimal for apolar compounds. Sample volume is proportional to the analyte mass present in the water sample, and therefore a higher mass transference to the reduced HS was allowed. In general, the quadratic term associated with salt concentration, and interactions between factors, played a minor effect on the efficiency of the extraction. The mathematical model derived from the experimental design explained at least 94% of the variability in the responses for all compounds. On the basis of the above results, extraction conditions were fixed at the highest level for all factors: NaCl addition (30%), 80 mL water sample and stirring (300 rpm using 7 × 60 mm stir bars).

3.2.2. Fibre length and desorption conditions

The amount of sorbent material and sorbing sites is a relevant factor in microextraction processes. The HF configuration of the MMs allows the user to select the most appropriate length for every application. Different fibre lengths were used to assess the influence of their size on the efficiency of the extraction. An increasing response was observed till a 2 cm length for PCT and 245TCT and till 4 cm for the most volatile compounds (data not shown). However the signal rise from 2 cm to 4 cm was no significant, and the 2 cm fibre was chosen as the best compromise between the efficiency of the extraction and the easiness of the desorption process with a minimal consumption of organic solvents.

Three different solvents: isooctane, *n*-hexane and pentane were evaluated for the desorption of the fibres and three consecutive desorptions of each fibre were performed with each solvent. Results in Fig. 3 show that high percentual recoveries of all compounds were obtained in the first desorption with all solvents. Less than 10% and 1% of the total recovered amount were present in the second and third fractions, respectively. As extracts are evaporated to a final volume of 50 µL, isooctane was ruled out due to its lower volatility. Hexane showed the best recoveries in the first fraction and facilitates the evaporation process; therefore, it was selected as desorption solvent. Further experiments were carried out using shorter desorption times (5 and 15 min) and the achieved recoveries com-

Table 3

Normalized responses as function of desorption time. Data for 2-cm long MMs using *n*-hexane, *n* = 3 replicates.

Desorption time (min)	24DCT	26DCT	34DCT	4Br2CT	245TCT	PCT
5	70 ± 4	70 ± 2	70 ± 5	68 ± 3	68 ± 2	58 ± 12
15	78 ± 8	77 ± 9	77 ± 7	77 ± 4	77 ± 2	76 ± 9
30	100 ± 9	100 ± 8	100 ± 8	100 ± 7	100 ± 8	100 ± 10

pared with those for 30 min (Table 3). However, significant lower responses were obtained at shorter times, and therefore 30 min was set as desorption time.

3.3. Extraction kinetics

Extraction kinetics was evaluated under optimized conditions at different times (5–480 min). Fig. 4 plots the time profile of the extraction process. The response increased with the sampling time for all halogenated toluenes until 60 min, after this time, a steady signal was reached and no further improvement was noticed. Therefore, 1 h was fixed as the sampling time.

3.4. Performance evaluation

Performance of the optimized MMSPE method was characterized by GC–MS and an additional compound (236TCT) included in the list of target species. Precision was evaluated at different concentrations for all halogenated toluenes. Repeatability of HS–MMSPE, as relative standard deviations (RSDs) for samples extracted the same day, remained below 8% for di- to pentachlorinated toluene and in the range of 6–15% for different days, Table 4. Extraction efficiencies (%) were determined as the ratio between the analytes amount in the final extract and that spiked in the water sample (addition level 200 ng L⁻¹). Extraction efficiencies for di- to penta-chlorotoluenes ranged between 27 and 47% (Table 4). Enrichment factors were calculated using the ratio between sample (80 mL) and final extract volumes (0.05 mL) and corrected with the corresponding extraction yield. Obtained values ranged between 432 and 746 times for di- to penta-halogenated species depending on the compound, Table 4.

Using extraction conditions optimized for di- to penta-halogenated toluenes, extraction efficiencies for 2CT and 4CT were also assessed. Their extraction yields remained around 10 times lower than those reported in Table 4 for di-chlorotoluenes (DCTs). Their larger polarity (log *K*_{ow}: 3.3 vs 3.8–3.9 for DCTs) and

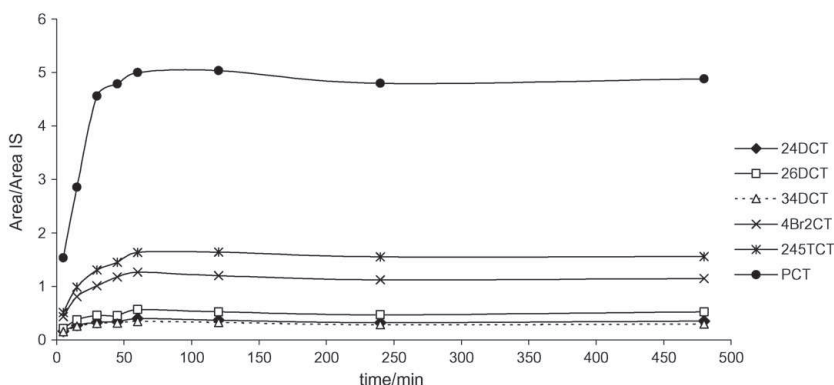


Fig. 4. Extraction kinetics of the HS-MMSPE process.

Table 4

Intra-day ($n=3$ replicates) and inter-day ($n=9$ replicates in three different days) precision at different concentration levels (relative standard deviations, RSDs, given as percentages), extraction efficiencies and enrichment factors for studied compounds.

Added concentration (ng L ⁻¹)	Intra-day				Inter-day	Extraction efficiency (%)	Enrichment factor
	50	200	1000	4000			
24DCT	3.3	4.6	7.8	7.6	14.6	31.7 ± 1.6	507
26DCT	5.1	6.9	6.8	7.2	13.6	32.5 ± 2.5	520
34DCT	7.9	7.1	7.2	6.0	14.3	31.4 ± 2.4	502
4Br2CT	4.4	5.1	6.8	6.2	13.7	41.0 ± 2.4	656
236TCT	8.1	1.6	7.3	7.8	5.6	27.3 ± 1.6	432
245TCT	3.8	5.7	2.8	7.7	14.5	43.4 ± 2.5	694
PCT	4.8	6.1	5.3	8.0	10.6	46.6 ± 3.5	746

Table 5

Linearity and limits of quantification ($S/N=10$) of the HS-MMSPE method.

Analyte	GC- μ ECD			GC-MS		
	Concentration range (ng L ⁻¹)	Correlation coefficient (R^2)	LOQ (ng L ⁻¹)	Concentration range (ng L ⁻¹)	Correlation coefficient (R^2)	LOQ (ng L ⁻¹)
24DCT	50–5000	0.998	10	20–2000	0.999	10
26DCT	50–5000	0.998	10	20–2000	0.999	10
34DCT	50–5000	0.997	10	20–2000	0.999	10
4Br2CT	40–6800	0.998	1.2	20–2000	0.999	12.5
236TCT	–	–	–	20–2000	0.998	12
245TCT	20–3800	0.998	0.8	20–2000	0.999	7
PCT	2–750	0.990	0.08	20–2000	0.994	12.5

volatility (vapour pressure: 70 Pa vs 500 Pa for DCTs) [3] probably led to a higher affinity for the water sample and the HS phase rather than for the lipophilic porous sorbent. Moreover, RSDs in the 20% range were associated with the GC-MS peaks of 2CT and 4CT. Consequently, the proposed method is deemed as unsuitable for these two compounds.

Linearity was investigated with water samples spiked at increasing concentration levels and considering GC-MS and GC- μ -ECD as detection techniques. Correlation coefficients obtained from the calibration curves, using 124TCB as internal standard, ranged from 0.990 to 0.999 depending on the considered compound and technique (Table 5).

Limits of quantification lower than 12.5 ng L⁻¹ were achieved for di- to penta-chlorinated toluenes by GC-MS and GC- μ -ECD, Table 5. For dichlorinated species, both techniques showed very similar LOQs, while GC- μ -ECD attained lower values than GC-MS for the highly chlorinated species. In any case, LOQs achieved are

suitable for the environmental monitoring of these compounds at the low/sub ng L⁻¹ level and equivalent to those reported by other authors for di- and tri-chlorinated toluenes determined by closed loop stripping analysis [2]. The proposed approach also provides better LOQs than average values reported by EPA purge and trap (P&T) based methods for volatile species [24,25], which require dedicated and expensive instrumentation; and similar values to those EPA methods for semivolatile compounds based on LLE or SPE and GC-MS [26,27], which require the use of a higher amount of organic solvents and present lower selectivity than HS microextraction techniques.

3.5. Real samples analysis and matrix effects

The optimized MMSPE methodology was applied to different matrices: tap water, river water close to a textile industry, raw and treated wastewater from a municipal sewage treatment plant, and

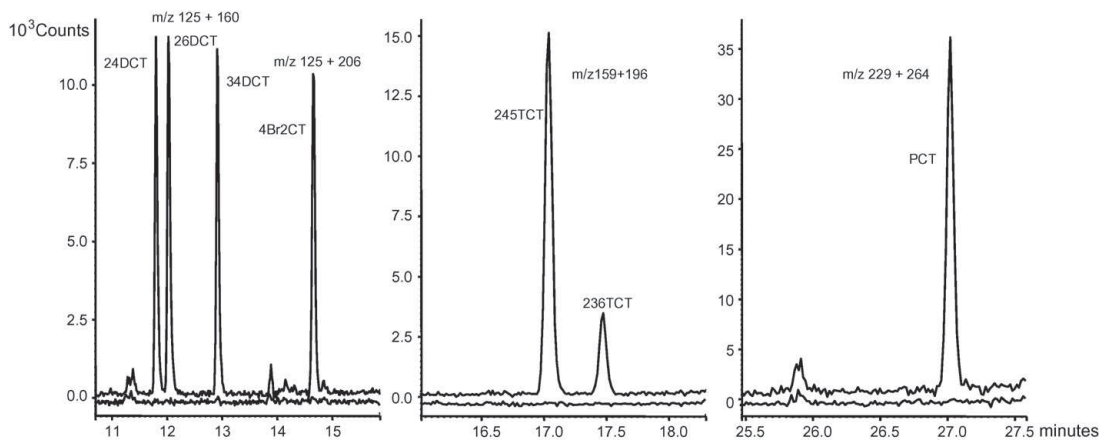


Fig. 5. Overlaid GC-MS chromatograms for an effluent blank and an effluent sample with addition of the analytes at the 50 ng L⁻¹ level.

Table 6Relative recoveries, with their standard deviations ($n=3$ replicates), for different water samples spiked at the (50 ng L^{-1}) level.

Analytes	Milli Q water	Tap water	River water	Treated wastewater	Raw wastewater
24DCT	100 ± 1.3	100.5 ± 3.5	101.5 ± 5.0	99.8 ± 4.0	70.1 ± 4.4
26DCT	100 ± 1.7	99.7 ± 2.3	102.3 ± 4.2	99.4 ± 3.8	69.3 ± 6.4
34DCT	100 ± 1.3	97.8 ± 2.9	101.3 ± 1.5	99.6 ± 2.8	72.5 ± 5.6
4Br2CT	100 ± 5.4	99.3 ± 2.8	104.6 ± 8.2	98.8 ± 2.1	60.6 ± 7.4
245TCT	100 ± 9.3	96.1 ± 2.7	100.9 ± 8.6	94.5 ± 2.1	43.3 ± 8.3
PCT	100 ± 9.5	106.9 ± 7.3	103.2 ± 8.0	107.8 ± 5.9	68.8 ± 8.9

obtained responses compared to those measured for Milli Q water spiked at the same level. For the evaluation of the matrix effects, samples were divided in two aliquots after filtration: one was used as a blank and the other was spiked with the chlorinated toluenes (50 ng L^{-1}). Fig. 5 shows the chromatograms corresponding to a spiked sample of treated wastewater and its blank. None of the target analytes was detected in the blanks. Normalized responses are presented in Table 6. No significant differences were found in the responses obtained for tap, river, treated wastewater and ultrapure water, spiked at the same level. However, a decrease in the signal in the range of 30–55% was observed for raw wastewater. The less volatile species were more affected by the matrix effects, due probably to the presence of organic matter in the matrix which affected the distribution coefficient between the sample and the hollow fibre by increasing their solubility. Therefore, external calibration is suitable for quantification of tap, river or treated wastewater, whereas for raw wastewater the standard addition method is recommended.

4. Conclusions

Determination of different halogenated toluenes in diverse water matrices (tap, river, wastewater) has been performed by microextraction using MMs as sorbent material. The technique provided limits of quantification in the low/sub ng L^{-1} , and a good precision and linearity for di- to pentahalogenated toluenes.

The simplicity and reduced cost of the extraction sorbent make it a valuable alternative to other extraction techniques for the determination of semi-volatile halogenated toluenes. For the highly volatile mono-chlorotoluenes, conventional approaches such as P&T or HS methods are preferred. It requires a very low consumption of organic solvents, is almost free of matrix effects and avoids completely carry-over problems. To the best of our knowledge, this is the first application of a microextraction technique to the determination of poly-halogenated toluenes in water samples.

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Simultaneous determination of benzotriazole and benzothiazole derivatives in aqueous matrices by mixed-mode solid-phase extraction followed by liquid chromatography–tandem mass spectrometry

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Abstract An improved selectivity method for the simultaneous determination of four benzotriazoles (benzotriazole, 4-methylbenzotriazole, 5-methylbenzotriazole, and 5,6-dimethyl-1*H*-benzotriazole) and six benzothiazoles (benzothiazole, 2-hydroxybenzothiazole, 2-benzothiazolamine, mercaptobenzothiazole, 2-methylbenzothiazole, and 2-methylthiobenzothiazole) in aqueous matrices has been developed. Under optimal conditions, analytes are concentrated using a MAX solid-phase extraction (SPE) cartridge, based on divinylbenzene-*N*-vinylpyrrolidone functionalized with quaternary amine groups, which allows reversed-phase interactions in combination with ionic exchange. Selected compounds are recovered with methanol–acetone 7:3 (v/v) whereas acidic interferences remained attached to the sorbent, and as determined by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), LOQs for surface, urban and industrial wastewater are in the range of 0.002–0.29 ng/mL. Figures of merit of the method revealed good precision (RSD% <12%), linearity ($R^2 > 0.99$) and accuracy (%R=80–100%) for surface waters and effluents allowing direct external standard quantification. For more complex samples, such as urban and industrial raw wastewater, either the standard addition method or pseudo-external standard calibration using matrix matched standards are recommended. Analysis of different real samples,

surface, urban wastewater and, for the first time, metal industry wastewater, reflected concentrations up to 310 ng/mL. The methylbenzotriazole isomers ratio was also determined.

Keywords Benzotriazoles · Benzothiazoles · Water analysis · Mixed-mode solid-phase extraction · Liquid chromatography coupled to tandem mass spectrometry · Emerging contaminants

Introduction

Benzotriazole and benzothiazole derivatives are considered as emerging pollutants, widely distributed in the aquatic environment [1, 2]. Benzotriazoles are extensively employed as corrosion inhibitors and also in deicing liquids and dishwashing agents [3, 4]. Benzothiazoles are also used as corrosion inhibitors and as vulcanization accelerators and biocides [5]. As a result of the above applications, both families of compounds have been detected in surface water [4, 6–8], raw and treated urban wastewater [5, 9–11], and even in ground water [3, 12].

Regarding emission sources, some authors have described the incomplete removal of these compounds during sewage treatment, highlighting sewage treatment plants as the main continuous source of these emerging pollutants to the aquatic environment [13, 14]. They have also reported different removal efficiencies for isomers of the same compound (tolylbenzotriazole) [3]. Due to their use as deicing fluids, airport run-offs are also described as release media [15]. The paper industry has been also reported as a potential source of contamination [16]; however, despite their main use as corrosion inhibitors, there is a lack of studies dealing with the

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contribution of the metal industry to their emission to surface waters. Metal pieces are treated with benzotriazole and benzothiazole containing fluids to avoid corrosion. During the industrial process, they suffer dewaxing, oxidation, and reducing processes which generate wastewater which can contain these compounds. If they are not efficiently removed, they could be still present in industrial effluents.

Benzotriazoles are rather polar compounds, thus, liquid chromatography (LC) is the preferred determination technique. Most authors use the combination of LC with mass spectrometry (MS) and either electrospray ionization (ESI) [17] or atmospheric pressure chemical ionization [18, 19] considering different types of analyzers [20, 21]. Depending on the nature of each analyte, ionization has been carried out by some authors separately either in positive or negative mode, in two different chromatographic runs [5].

Despite strong matrix effects having been described during ionization in their LC-MS analysis [5, 22], sample preparation is limited to direct injection of water samples or solid-phase extraction with typical reversed-phase adsorbents, such as OASIS HLB [5, 16] or Strata X [23, 24] which requires the standard addition methodology for quantification. Regarding solid-phase microextraction, Fries et al. [25] have recently described an extraction method for just benzothiazole from raw wastewater using stir bar sorptive extraction which again can be considered as a non-selective technique.

Although mixed-mode solid-phase extraction has been successfully applied for the determination of different polar compounds in biological [26], food [27], and environmental matrices [28], it has not been tested for benzotriazoles and benzothiazoles.

The aim of this work was the development of a SPE methodology based on the use of a mixed-mode sorbent, that combines ionic exchange and reversed-phase mechanisms, for the simultaneous (positive and negative mode) determination by LC-tandem MS of four benzotriazolic compounds and six benzothiazole derivatives. The use of the above-mentioned sorbent improves the selectivity of the extraction allowing a simple external standard mode quantification of these compounds in surface and sewage waters. Parameters affecting the extraction process such as sorbent/eluent type and amount were thoroughly investigated. Finally, the applicability of the method was evaluated in urban and industrial wastewater. Information about the tolyl isomers ratios before and after wastewater treatment is also given.

Experimental section

Standards, solvents and material

Standards of 1*H*-benzotriazole (BTri; 98%), 5,6-dimethyl-1*H*-benzotriazole (XTri; 99%), benzothiazole (BT;

96%), 2-benzothiazolamine (ABT; 97%), mercaptobenzothiazole (MBT; 97%), 2-methylbenzothiazole (MeBT; 99%), 2-methylthio-benzothiazole (MTBT; 97%), 2-hydroxybenzothiazole (OHBT; 98%), 5-methyl-1*H*-benzotriazole (5-TTri; 98%) and reduced L-glutathione were purchased from Sigma-Aldrich (Milwaukee, WI, USA). The technical mixture of 4- and 5-methyl-1*H*-benzotriazoles (TTri; 99.5%) was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Formic acid, acetone, ammonium hydroxide, hexane (GC grade) and HPLC-grade methanol and acetonitrile were acquired from Merck (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q system (Millipore, Billerica, MA, USA). Stock solutions of each compound were prepared in methanol. Further dilutions and mixtures of them were made in the same solvent. Standards in methanol were stored in the dark, at -20°C for a maximum of 2 weeks.

SPE cartridges containing either OASIS HLB (60 mg) or OASIS MAX sorbents (150 mg) were provided by Waters (Milford, MA, USA). Cellulose acetate membrane filters (0.45 μm pore size) were purchased from Millipore (Bedford, MA, USA).

Samples and sample preparation

River water was collected in a severely polluted stream (Sar River) in the Northwest of Spain. Raw and treated urban wastewaters (influent and effluent) were collected as grab samples in the sewage treatment plant of a town with 90,000 inhabitants. Industrial raw and treated wastewater was received from two different factories belonging to the metal industrial sector.

Under optimal conditions, Oasis MAX cartridges (150 mg) were conditioned with methanol, ultrapure water (5 mL each) and 50–500 mL of filtered water samples, with 5 mL of L-glutathione 0.1 M as reductor agent, passed through the sorbent at a flow rate of 5 mL/min. Afterwards, cartridges were dried during 15 min with a gentle nitrogen stream and eluted with just 5 mL of a MeOH/acetone (7:3, v/v) mixture. The sample extract was concentrated to 0.5 mL and made up to 1 mL with Milli-Q water for injection in the LC-MS system.

Equipment

Analytes were determined using a Varian (Walnut Creek, CA, USA) LC-MS/MS system. The LC instrument comprised two isocratic, high-pressure mixing pumps (Varian ProStar 210), an autosampler and a thermostated compartment for the column (Varian ProStar 410). The mass spectrometer was a U-shaped triple quadrupole (Varian MS 1200 L) furnished with an ESI interface. The LC-MS/MS instrument was entirely controlled by the Varian MS Workstation Version 6.9 software.

Compounds were separated using a Zorbax SB-phenyl (3.5 μm ; 2.1 mm $\times$ 100 mm) acquired from Agilent (Wilmington, DE, USA) and connected to a C18 (2 mm $\times$ 4 mm) guard cartridge supplied by Phenomenex (Torrance, CA, USA). Ultrapure water (A) and methanol (B), containing different amounts of formic acid or ammonium acetate as modifiers, were employed as mobile phases. Under final conditions, both phases were modified with ammonium acetate (1 mM) and compounds separated using the following gradient: 0–2 min, 15% B; 6 min, 40% B; 12 min, 70% B; 17–20 min, 100% B; 23–30 min, 15% B. The mobile phase flow was set at 0.15 mL min⁻¹ and the temperature of the column fixed at 35 °C. Standards and sample extracts injection volume was fixed at 15 μL .

Nitrogen (99.999%), used as nebulizing (50 psi) and drying gas (200 °C, 19 psi) in the ESI source, was provided by a high purity generator (Domnick Hunter, Durham, UK). The ESI interface was operated simultaneously in the positive and negative modes and the voltage of the ESI needle fixed at 5,000 V. The temperature of the ESI housing was set at 50 °C. Argon (99.999%) was employed as collision gas (2.2 mTorr) in the mass spectrometer. Compounds were recorded in the multiple reaction monitoring (MRM) mode, using two transitions per compound and a dwell time of 0.115 s per transition. The most intense one was used to quantify the response of each species in standards and SPE extracts from water samples, Table 1.

Extraction efficiency, matrix effects, and LOQ calculations

Percentages of absolute recoveries were calculated as: $\%R = [(C_s - C_b)/C_t] \times 100$, where C_s is the measured concentration in the extract from a spiked sample, C_b is the concentration measured in a non-spiked fraction of the same sample and C_t is the theoretical added concentration,

being C_s and C_b values established by external calibration. Recoveries affected by matrix effects were evaluated as percentage of the quotient between the difference in the compound area in a spiked SPE methanol extract (A_{se}) and the extract of the same sample without spike (A_{be}), and the area of an equal concentration standard solution (A_{ss}): $\%ME = (A_{se} - A_{be}/A_{ss}) \cdot 100$. Therefore, the ion suppression percentage corresponds to $100 - \%ME$.

Results and discussion

Optimization of LC-MS/MS conditions

Ionization and fragmentation parameters

The optimization of LC-MS/MS determination was carried out by injecting with direct infusion individual standards of each compound prepared in methanol/water (1:1) with a concentration of 5 $\mu\text{g/mL}$. As mobile phase, methanol/water (1:1) with a flow of 0.05 mL/min was employed. N₂ as drying gas was applied at 200 °C with a pressure of 19 psi.

Electrospray ionization (ESI) was evaluated in positive mode (compounds ionized by adding a proton) and negative mode (losing a proton). Significant ionization in positive mode was observed for most compounds with the exception of OHBT and MBT, which were only ionized in the negative mode. MeBT, BT, and TTri rendered also negative ionization but with lower intensity. Thereafter, capillary voltages and collision energies were optimized in order to maximize the intensity for the parent ion for each species, and to identify a minimum of two transitions in the MS/MS spectra. Optimal detection conditions are summarized in Table 1. As shown in the case of BTri, just one MS/MS transition was found; whereas, OHBT was quantified in the SIM mode, since it did not undergo significant MS/MS fragmentation.

Table 1 Optimized ESI-MS/MS conditions for selected compounds, repeatability, reproducibility and instrumental limits of quantification (LOQ) and detection (LOD) for the LC-MS/MS method

Analyte	Precursor ion (m/z)	Ionization mode	MRM1 (quantification)	CV/CE	MRM2 (confirmation)	CV/CE	Repeatability/%RSD		Reproducibility/% RSD		LOD ng/mL	LOQ ng/mL
							50 ng/mL	200 ng/mL	50 ng/mL	200 ng/mL		
BTri	120	Positive	120>65	60/15	—	—	4.2	1.9	9.3	9.7	0.75	2.5
TTri	134	Positive	134>77	52/20	134>79	52/13	2.4	2.9	10.9	8.5	0.75	2.5
OHBT	150	Negative	150	64/—	—	—	5.6	3.0	6.9	10.0	3	10
ABT	151	Positive	151>109	56/19	151>79	56/13	2.4	2.8	8.1	8.5	5.6	3.0
XTri	148	Positive	148>77	52/21	148>93	52/12	2.1	2.2	5.3	9.8	0.3	1
MBT	166	Negative	166>134	68/19	166>58	68/12	7.9	4.3	7.8	9.9	6	20
BT	136	Positive	136>109	64/17	136>65	64/26	3.4	4.2	10.8	7.7	0.3	1
MeBT	150	Positive	150>109	64/16	150>65	64/14	5.6	5.0	10.6	9.7	3	10
MTBT	182	Positive	182>167	60/16	182>109	60/26	9.8	5.4	9.7	9.9	0.75	2.5

CV capillary voltage, in voltage; CE collision energy, in electronvolts

Selection of modifier

Ionic strength and mobile phase pH can affect notoriously the ionization efficiency. In this study, formic acid (0–0.1%) and ammonium acetate (0–10 mM) were selected as mobile phase modifiers. The signal for OHBT and MBT (which ionize in negative mode) is very low or even null when formic acid is added to the mobile phase. For benzotriazoles, the highest intensity corresponds to the addition of 0.05% formic acid. In the case of salt addition, the higher the amount the lower the signal, and therefore the best response is obtained with 1 mM ammonium acetate which was selected as modifier for the rest of the study.

LC-MS/MS performance

Once the modifier was selected, the optimization of the elution gradient was carried out with methanol. Figure 1 shows a chromatogram (MRM) obtained for a 50 ng/mL standard under optimized conditions. The interference of the

ABT transition 151→109 can be observed in the MeBT window (150→109) due to the mass spectrometer limited resolution. As both compounds elute at very different times, this does not constitute a problem. It must be also taken into account that tolyl isomer separation was not possible under these conditions, because they coelute and present identical fragmentation pattern. An isocratic run with a 15% ACN and ammonium acetate as modifier (1 mM) is necessary to separate and quantify both isomers, Fig. 1. Unless otherwise stated, in this study, both isomers were quantified together.

Repeatability (precision) and reproducibility of the instrument were also investigated under optimized chromatographic conditions. Two different concentrations were tested, 50 and 200 ng/mL in four consecutive injections (Table 1). To test reproducibility, the protocol was repeated in three consecutive days. As it can be noticed, the relative standard deviation (%RSD) was acceptable (<11%) for all analytes at both concentrations. In order to test the response linearity, calibration curves were built in the following ranges, 1 to 1000 ng/mL for XTri and MBT; 10 to

Fig. 1 MRM chromatogram for a standard solution of target analytes (50 ng/mL) and tolyl isomers chromatogram (20 ng/mL)

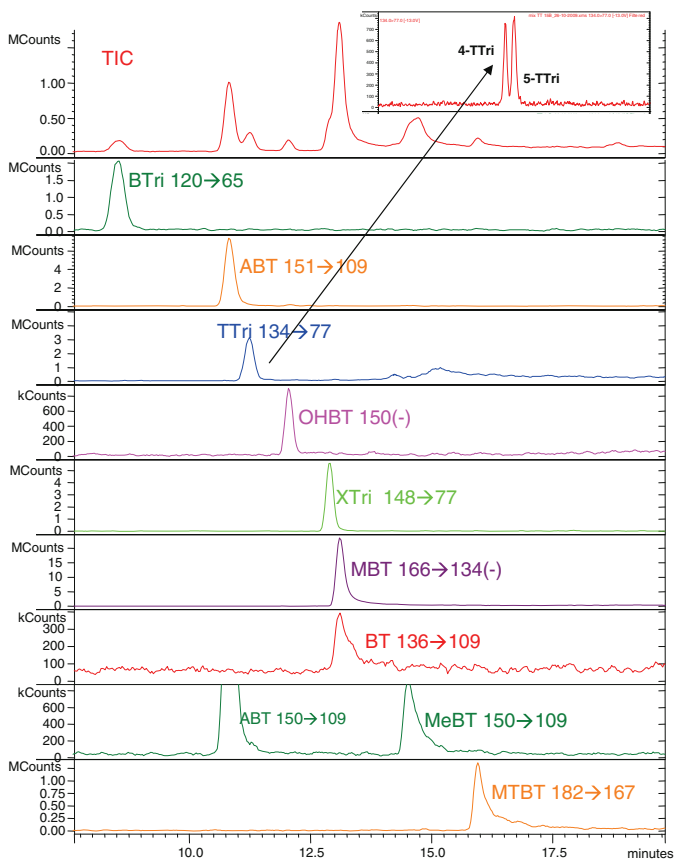
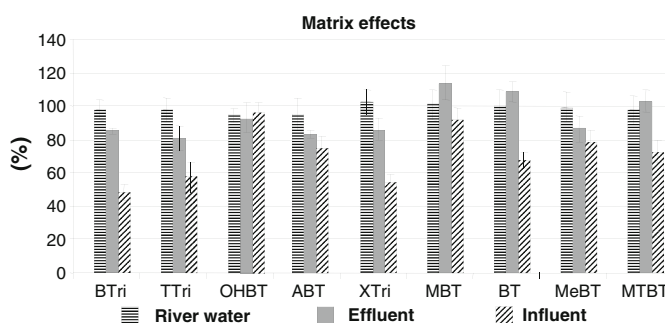


Fig. 2 Recoveries affected by matrix effects using the optimized methodology. Sample volume of 500 mL for river water (spiked concentration in extract 250 ng/mL) and 100 mL for raw and treated wastewater (spiked concentration in extract of 400 and 200 ng/mL respectively)



1000 ng/mL for MeBT e OHBT; 20 to 1000 ng/mL for BT; and 2.5 to 1000 ng/mL for the rest of compounds. Good linearity with determination coefficients higher than 0.995 for all compounds were obtained (data not shown).

Instrumental LOQs defined for a signal to noise ratio of 10 ($S/N=10$), with the noise integrated 1 min before and after the retention time of each compound, ranged between 1 and 20 ng/mL and LODs corresponding to a S/N equal to 3, stayed between 0.3 and 6 ng/mL, Table 1.

Optimization of SPE conditions

An alternative extraction protocol using mixed-mode solid-phase extraction (MAX) is proposed. This type of sorbent retains basic and neutral species by a reversed-phase mechanism and improves the acidic compounds retention by strong anionic exchange, with protonated amino moieties existing in the structure of the sorbent. Considering the same elution mixture as for OASIS HLB, MAX cartridges provided clear extracts, with a lower complexity.

The recovery percentage corresponding to the extraction step ($\%R_{SPE}$) was assessed by comparing the responses obtained for samples spiked before and after the SPE steps. River water (500 mL) was spiked at the 0.5 ng/mL level and effluent (treated urban wastewater) and influent (raw urban wastewater; 100 mL) with 2 and 4 ng/mL respectively. Results for the MAX cartridge are compiled in Electronic Supplementary Material Table S1. A second cartridge connected in series checked the sample breakthrough. No signals were observed in the extract from the second sorbent.

Recoveries between 80% and 118% were obtained for effluent, influent and river water. MBT presented too low recoveries for the wastewater matrices, (just 27% for influent) probably due to the reactive thiol group of the compound which can undergo oxidation processes. Some authors have added some reducing agent to the solution to avoid this problem [5, 7]. As described in the literature, L-glutathione was added in different concentrations as reducing agent to avoid this reaction. Thus, 1, 5, and 10 mL of

Table 2 Absolute recoveries (evaluated with external calibration), precision, and quantification limits for the proposed methodology for the three different water samples

	River				LOQ (ng/mL)
	Recovery 0.5 ng/mL	RSD%	Recovery 1 ng/mL	RSD%	
BTri	98	4	99	5	0.005
TTri	96	3	87	11	0.005
OHBT	97	4	88	5	0.020
ABT	96	1	99	8	0.005
XTri	102	3	89	9	0.002
MBT	101	5	97	11	0.002
BT	100	8	95	10	0.040
MeBT	99	10	90	11	0.020
MTBT	99	8	94	10	0.005
	Effluent				LOQ (ng/mL)
	Recovery 2 ng/mL	RSD%	Recovery 10 ng/mL	RSD%	
BTri	86	2	83	9	0.028
TTri	82	11	81	12	0.028
OHBT	92	11	87	11	0.111
ABT	83	4	82	11	0.028
XTri	86	9	91	11	0.011
MBT	110	10	99	11	0.011
BT	110	5	80	10	0.222
MeBT	90	4	87	11	0.111
MTBT	104	7	106	12	0.028
	Influent				LOQ (ng/mL)
	Recovery 4 ng/mL	RSD%	Recovery 20 ng/mL	RSD%	
BTri	50	10	64	9	0.050
TTri	55	10	65	9	0.042
OHBT	95	7	89	7	0.111
ABT	75	9	84	6	0.036
XTri	55	10	62	10	0.020
MBT	90	6	82	12	0.013
BT	68	10	71	3	0.286
MeBT	78	7	91	10	0.125
MTBT	72	10	70	7	0.036

0.1 M L-glutathione solution were added to 100 mL of influent water. At least 5 mL of this solution were necessary to prevent the oxidation of MBT achieving recoveries of 95%. Benzothiazole recovery for river water was also a bit low (70%) probably due to some loss during the extract concentration. Therefore in following experiments, very gentle N_2 stream was used for concentration.

Matrix effects

Matrix effects ($\%R_{ME}$) were calculated by comparing spiked sample extracts of 1 mL (after the SPE extraction) with standard solutions with the same concentration. River water samples, effluent and influent were assessed with spiked extracts concentrations of 250 ng/mL for river water (500 mL enriched) and 200 and 400 ng/mL for effluent and influent respectively (100 mL enriched). Results are shown in Fig. 2.

No matrix effects are reported for river water as shown in the picture. In the case of effluent, just a small decrease is observed, with quantitative recoveries for all studied compounds ($>80\%$). Therefore, the developed extraction methodology is highly selective and it can be applied for the determination of target analytes in matrices such as surface water and urban treated wastewater using simply direct external standard calibration. Thus, it seems a very good

alternative to the conventional reversed-phase materials which require standard addition method.

However, in the case of influent, which is an aqueous matrix with maximum complexity, the ionization suppression can be noticed with variable recoveries depending on the compound. Thus, in the case of OHBT, recoveries around 100% are obtained, whilst for benzotriazolic compounds (BTri, TTri, and XTri) they remained below 60%.

Industrial waters also produced strong matrix effects due to the incomplete removal of interferant analytes during the extraction, data not shown. For these type of very complex matrices, either the standard addition or the matrix matched standards methodologies are recommended.

Validation of the analytical methodology

Precision of the overall method (SPE followed by LC-tandem MS) was studied at two different addition levels for river, effluent and influent samples. RSD% ranging from 1% to 12% are reported in Table 2. Absolute recoveries were evaluated at the same addition levels to test accuracy. Table 2 shows quantitative recoveries ($>80\%$) for river and effluent samples, while influent water suffering ionic suppression during the determination provides recoveries of just 50% for some compounds. These recovery values confirmed the need of using standard addition as quantification technique.

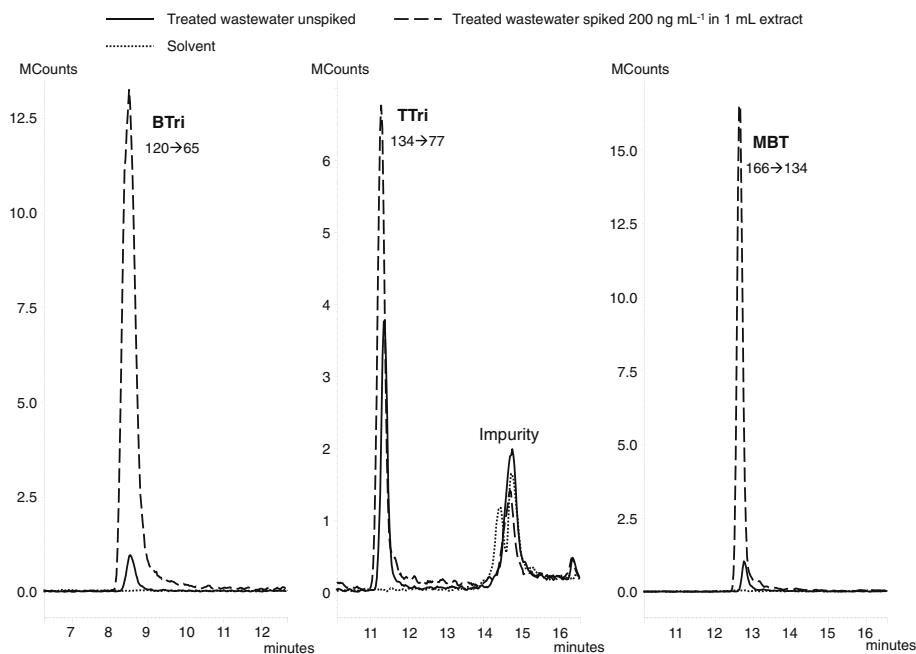


Fig. 3 MRMs chromatogram showing selected transitions for BTri, TTri, and BT in an urban wastewater sample

Obviously, the accuracy of such methodology depends on the linearity of the procedure. Experiments performed with aliquots (100 mL) of raw wastewater spiked at seven increasing levels from the LOQ of each compound up to 10 ng/mL demonstrated an excellent linearity for all species with determination coefficients between 0.991 and 0.999. The ratio between slopes of standard addition curves for wastewater (raw) and ultrapure stayed between 0.60 for BTri and 0.91 for OHBT which is in agreement with signal suppression effects depicted in Fig. 2 and Table 2. Limits of quantification of the whole method ($LOQ_{SPE-LC-MS/MS}$) were calculated from the instrumental limits of quantification ($LOQ_{LC-MS/MS}$) with the following equation: $LOQ_{SPE-LC-MS/MS} = LOQ_{LC-MS/MS} \cdot V_{\text{extract}} / (V_{\text{sample}} \cdot R)$, where V_{extract} and V_{sample} are the volume of the extract and sample respectively, and R represents the whole absolute recovery of the compound ($R=1$ for river water, $R=0.9$ for effluent and $R=0.5-0.9$ for influent). Obtained data provides LOQs of 0.002–0.04 ng/mL for river water, 0.11–0.222 ng/mL for effluent and 0.0125–0.286 ng/mL for influent. These values are similar or even better than those found in literature. Kloepper et al. reported LOQs in the range 0.04–0.62 ng/mL [29] and between 0.02 and 0.2 ng/mL [5] for wastewater. Weiss et al. [3] reached LOQs for wastewater in the 0.025 ng/mL level and in surface water around 0.01 ng/mL.

Application to real samples

The optimized methodology was applied to the determination of benzotriazoles and benzothiazoles in river, raw and treated urban wastewater. No signals for ABT, XTri or MeBT were observed in any tested matrix. Maximum concentrations measured for the rest of the compounds are compiled in Table 3. As it can be observed, the highest levels corresponded to BTri, TTri, and BT in influent samples with concentrations up to 10 ng/mL for BTri. This is probably a consequence of their high production and use. OHBT, MBT, and MTBT were also found in several matrices in concentrations up to 1.4 ng/mL of

Table 4 Tolyt isomers calculated ratios

Sample		4-TTri (ng/mL)	5-TTri (ng/mL)	Isomers Ratio
1	Effluent	0.31	0.45	0.68
	Influent	0.28	0.38	0.75
2	Effluent	0.37	1.14	0.32
	Influent	0.32	0.95	0.33

OHBT. These values are in agreement with concentrations reported by other authors [5, 29]. In surface waters just BTri and TTri were detected. River Sar found concentrations (up to 0.21 ng/mL for TTri) are rather lower than those reported by Walter et al. [4] for Glatt river (0.1–6.3 ng/mL), Loos et al. for Danube river (0.13–0.38 ng/mL) [30] or Leerdam et al. [20] in rivers from the Netherlands (0.1–1 ng/mL). Figure 3 shows the chromatograms corresponding to an effluent sample before and after being spiked with a standard solution. Analytes were also determined for the first time in metal industry wastewater samples. As it is shown in Table 3, concentrations of these compounds in the industrial raw wastewater were much higher than those found in urban wastewater (up to 310 ng/mL) and most compounds were also found in the industrial effluents. Therefore, the metal industry is confirmed as a relevant source of these contaminants.

Finally, the 4- and 5-tolyttriazole ratio was investigated in the urban wastewater grab samples of two different sampling dates (1 and 2). Table 4 shows similar ratios in influent and effluent samples collecting in the same day. This is not in agreement with those results observed by Weiss et al., where 4-TTri showed a higher resistance to be degraded in the wastewater treatment plants. On the other hand, the observed ratio seems to change with the sampling date.

Conclusions

A novel analytical methodology has been developed for the determination of four benzotriazole and six benzothiazoles

Table 3 Detected levels in different aqueous matrices; n = number of samples

Samples	n	Concentration (ng/mL)	BTri	TTri	OHBT	MBT	BT	MTBT
River	4	Average	0.01	0.20	n.d.	n.d.	n.d.	n.d.
		Maximum	0.04	0.21				
Urban influent	6	Average	2.47	2.1	0.15	n.d.	0.83	n.d.
		Maximum	10.02	2.3	0.41		1.81	
Urban effluent	6	Average	0.11	0.88	0.44	0.26	0.74	0.06
		Maximum	0.27	2.01	1.35	0.28	1.02	0.22
Industrial raw wastewater	3	Average	210	91	10.9	0.58	0.17	n.d.
		Maximum	310	122	14.3	0.89	0.35	
Industrial effluent	3	Average	n.d.	0.37	0.10	0.34	n.d.	n.d.
		Maximum		0.56	0.17	0.57		

in aqueous samples. The proposed sample preparation method provides an enhanced selectivity versus conventional reversed-phase SPE, allowing the determination of target analytes in surface and effluent samples using simply external standard calibration. For industrial wastewater or urban raw wastewater, either standard addition or pseudo-external calibration with matrix matched standards is recommended. Analytes were found in surface, urban effluent and influent samples and also for first time in metal industry wastewaters at concentrations up to 310 ng/mL. These results confirm the ubiquitous presence of these compounds in the water cycle and their potential environmental risk.

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Research Article

Combining stir-bar sorptive extraction and large volume injection-gas chromatography-mass spectrometry for the determination of benzotriazole UV stabilizers in wastewater matrices

Stir-bar sorptive extraction and liquid desorption followed by large volume injection-gas chromatography coupled to mass spectrometry under selected ion monitoring mode acquisition (SBSE-LD/LVI-GC-MS(SIM)) was applied for the determination of six benzotriazole UV stabilizers (Tinuvin P, Allyl-bzt, Tinuvin 320, Tinuvin 326, Tinuvin 327 and Tinuvin 328) in wastewater matrices. Parameters affecting the performance of extraction and desorption steps were thoroughly evaluated using uni- and multivariate optimization strategies, based on the use of experimental factorial designs. Assays performed with stir bars, coated with 24 μL of polydimethylsiloxane, on 25 mL of ultra-pure water samples spiked at the 0.5 ng/mL level, yielded recoveries ranging from $47.9 \pm 1.4\%$ (Tinuvin P) to $103.1 \pm 3.6\%$ (Tinuvin 326), under optimized experimental conditions. When applied to complex matrices (e.g. wastewater), the methodology showed also excellent linear dynamic ranges (0.02–10.00 ng/mL) for the six benzotriazole UV stabilizers studied with correlation coefficients higher than 0.9970, limits of quantification in between 0.004 and 0.015 ng/mL, suitable repeatability ($\text{RSD} < 12.7\%$) and reproducibility ($\text{RSD} < 4.5\%$). The application of the proposed methodology to urban sewage waters from Spain and Portugal wastewater plants revealed the presence of low contents of some benzotriazole UV stabilizers.

Keywords: Benzotriazoles / Large volume injection-gas chromatography-mass spectrometry (LVI-GC-MS) / Stir-bar sorptive extraction (SBSE) / UV stabilizers / Wastewater matrices
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1 Introduction

Fate, occurrence and potential adverse effects of organic compounds designed to absorb the ultraviolet (UV) wavelengths of solar radiation are receiving an increased attention [1]. Organic UV filters, which are included in the formulation of sun screens and personal care products, are considered emerging pollutants often detected in surface and sewage water, sediments, sludge and, less often, in fish and other aquatic organisms [2–5]. UV absorbing species are also added to building materials, automobile components, furniture, paints, food packages and polymeric materials to improve their stability and to prevent light-induced degradation reactions and yellowing [6–7]. This latter group of compounds is referred under the generic name of UV stabilizers. The 2-hydroxyphenyl derivatives of benzotriazole

(Tinuvin) constitute one of the most important families of UV stabilizers, providing protection against UV radiations in the range from 280 to 400 nm. Tinuvin are stable and hydrophobic compounds with higher octanol–water partition coefficients (K_{OW}) than most of the UV filters included in the formulation of personal care products. For instance, the use and production of Tinuvin 320 has been banned in Japan due to its persistence and bio-accumulation potential [8]. However, conversely to UV filters, the determination of Tinuvin has received a limited attention from the analytical and environmental point of view.

One of the first references describing the presence of Tinuvin in the environment was presented by López-Avila and Hites [9], who reported high levels (up to 1 mg/g) of these species in marine and freshwater sediments collected in the vicinity of a chemical manufacturing plant in USA. Further sampling campaigns, performed in the same geographic area, confirmed that Tinuvin persisted in the sediment core at least during 20 years [10, 11]. However, the evidence of their ubiquity in a large geographic area has been only recently obtained. Thus, Nakata and co-workers highlighted the bio-accumulation of Tinuvin 326 and 328 in mussel samples collected in different countries along the southeast coast of

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Asia [12], as well as their presence in sediments from heavily polluted areas in Japan [8]. Tinuins 327 and 328 were measured in blubber from marine mammals [8] and livers from other top predators in the marine food chain, such as sharks and birds [13]. Apart from direct discharges from chemical manufacturing plants, Nakata's group has demonstrated the contribution of urban wastewater to the indirect release of Tinuins 326, 327 and 328 in the aquatic environment [14]. This conclusion also has been supported with data obtained for a sewage treatment plant in Spain [15]. Further studies have revealed the existence of Tinuvin residues in sludge from urban sewage plants [16].

Assessing the contribution of wastewater to the spread of Tinuins in the environment requires effective sampling preparation methodologies providing high enrichment factors, but also simplified set-up without using toxic organic solvents. The novel emerging sorbent-based techniques (e.g. solid-phase microextraction, SPME) fulfil some of the above requirements, which are attractive alternatives to the conventional liquid–liquid extraction (LLE) approaches, as those reported by several authors for the pre-concentration of Tinuins from water matrices [14, 15]. Another potential sorbent-based technique is stir-bar sorptive extraction (SBSE), which was introduced by Baltussen et al. in 1999 [17]. In SBSE, the volume of the coated phase ranging from 24 to 126 μL , which is much higher when compared with the 0.5 μL incorporated into the SPME fibers [18], resulting in greater concentration factors. Moreover, the non-polar polydimethylsiloxane (PDMS) polymeric phase, the only commercially available coating for SBSE, presents a large affinity for low polar species, like Tinuins. Additional advantages of SBSE are the remarkable robustness of the microextraction device (*Twister*) and its lower cost in comparison with SPME fibers. Moreover, the analytes back-extraction can be further performed by thermal desorption for maximum sensitivity or liquid desorption (LD), using a small volume of a convenient organic solvent, where several other analytical advantages have been demonstrated recently [19–26].

The aim of the present contribution was to evaluate the suitability of the combination of SBSE with large volume injection-gas chromatography-mass spectrometry (LVI-GC-MS) for the determination of six benzotriazole type UV stabilizers in water matrices. Parameters affecting the efficiency of the sample preparation process were discussed in detail and the performance of the methodology was compared with other analytical alternatives. Finally, the optimized method was applied to investigate the presence of these priority pollutants in wastewater samples from Spain and Portugal.

2 Materials and methods

2.1 Solvents, materials and samples

HPLC-grade methanol (MeOH, 99%), acetonitrile (ACN, 99.99%) and ethyl acetate (AcOEt, trace analysis grade) were purchased from Merck (Germany). Trace analysis acetone

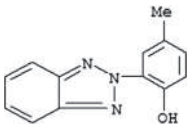
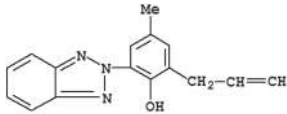
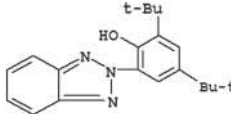
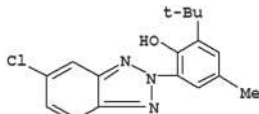
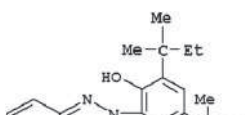
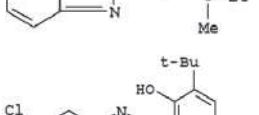
(99.5%), diethyl ether (99.5%) and *n*-pentane (99%) were obtained from Riedel-de-Haën (Germany). Hydrochloric acid was also supplied by Riedel-de-Haën and sodium hydroxide (NaOH, 98%) was acquired from AnalaR BDH Chemicals (UK). Ultra-pure water was obtained from a MilliQ water purification system (Millipore, USA). Standards of phenol, 2-(2H-benzotriazol-2-yl)-4-methyl- (Tinuvin P); phenol, 2-(2H-benzotriazol-2-yl)-4-methyl-6-(2-propen-1-yl)- (Allyl-bzt); phenol, 2-(2H-benzotriazol-2-yl)-4,6-bis(1,1-dimethylethyl)- (Tinuvin 320); phenol, 2-(5-chloro-2H-benzotriazol-2-yl)-6-(1,1-dimethylethyl)-4-methyl- (Tinuvin 326); phenol, 2-(2H-benzotriazol-2-yl)-4,6-bis(1,1-dimethylpropyl)- (Tinuvin 328) and phenol, 2-(5-chloro-2H-benzotriazol-2-yl)-4,6-bis(1,1-dimethylethyl)- (Tinuvin 327) were acquired from Aldrich (Milwaukee, WI, USA). Their chemical structures, molecular weights, pKa and log K_{OW} values can be consulted in Table 1. Individual stock solutions of each UV stabilizer were prepared in acetone. Further dilutions and mixtures of target analytes were prepared in MeOH when used to spike water samples, and in AcOEt when employed to optimize the LVI-GC-MS instrumental conditions. Stir bars (*Twister*TM, 10 mm length $\times$ 0.5 mm thickness, PDMS, 24 μL) were obtained from Gerstel (Müllheim a/d Ruhr, Germany). Prior to use, they were cleaned with ACN for 15 min.

Grab samples of wastewater were obtained from inlet and outlet streams of two urban sewage plants, equipped with primary and activated sludge treatment units, located in the region of Lisbon (Portugal) and in the Northwest of Spain. Samples were collected in 1 L glass vessels previously rinsed with acetone and ultra-pure water. After reception at the laboratory, they were passed through glass fibre filters, adjusted to pH 3 and stored in the refrigerator at 4°C for a maximum of 24 h before being used.

2.2 SBSE-LD assays

In a typical assay, 25 mL of ultra-pure water spiked at the 0.5 ng/mL level was introduced into a glass vial (Macherey-Nagel, Düren, Germany), a stir bar was immersed and the vial was closed with a seal using a hand crimper. For SBSE optimization studies, assays were performed in triplicate using a 15-positions agitation plate (Variomag H+P Labor-technik AG Multipoint 15, Oberschleibheim, Germany) at room temperature ($20 \pm 2^\circ\text{C}$). MeOH was added to water matrices up to 40% v/v and the extraction time was varied in between 60 and 960 min. Parameters such as agitation speed (700, 1000 and 1300 rpm), ionic strength (NaCl: 0, 10 and 20%, w/v) and pH (3, 6 and 9) were also optimized using the Box-Behnken-type experimental factorial design. For back-extraction optimization studies, the stir bars were removed from the sampling vessel using a magnetic rod, rinsed with ultra-pure water, dried with a lint-free tissue and soaked with 1 mL of several solvents (MeOH, ACN, AcOEt, *n*-pentane and diethyl ether) in 2 mL glass vials. For this purpose, other parameters were evaluated such as soak time (5, 10 and 15 min) and number of consecutive desorption steps under ultrasonic treatment (Branson 3510, Branson Ultrasonic

Table 1. Abbreviated names, CAS numbers, molecular weight, chemical structures, pKa values and octanol–water partition coefficients (log $K_{O/W}$) of the selected UV-stabilizers

Abbreviated name	CAS number	Molecular weight (a.m.u.)	Chemical structure	pKa ^{a)}	Log $K_{O/W}$ ^{a)}
Tinuvin P	2440-22-4	225.25		8.2	4.3
Allyl-bzt	2170-39-0	265.31		8.5	5.3
Tinuvin 320	3846-71-7	323.43		9.4	7.2
Tinuvin 326	3896-11-5	315.80		9.5	6.6
Tinuvin 328	25973-55-1	351.49		8.9	8.3
Tinuvin 327	3864-99-1	357.88		9.4	7.8

a) Values obtained from SciFinder Scholar Database (<http://www.cas.org/products/sfacad/>).

Corporation, Danbury, USA) at constant temperature (25°C). Subsequently, the organic extracts were evaporated to dryness under a gentle stream of nitrogen, and after reconstituted with 0.2 mL of AcOEt, the vials were closed and placed in the automatic liquid sampler tray for LVI-GC-MS(SIM) analysis. The standard addition methodology (SAM) was used to quantify and compensate for matrix effects on real matrices. Blank assays were also performed using the same procedure as above, employing water samples without spiking.

2.3 Instrumental set-up

Analytes were determined by GC-MS, using an Agilent 6890 Series gas chromatograph equipped with an Agilent 7683

automatic liquid sampler interfaced to an Agilent 5973 quadrupole mass spectrometer (Agilent Technologies, Little Falls, DE, USA). A programmed temperature vaporization (PTV) injector, configured with a septum-less head (Gerstel, Müllheim an der Ruhr, Germany) and equipped with an empty baffled liner, was used for large volume injection (LVI). The injector was operated in the solvent vent mode, using nitrogen to cool down the liner to the initial operating temperature. The temperature of the PTV injector was varied as follows: 40°C (0.35 min) to 300°C (3 min) at a rate of 600°C/min and subsequently, decreased to 200°C at a rate of 50°C/min. The solenoid valve was maintained in the split position until 0.30 min, with a split flow of 10 mL/min; then, it was switched to the splitless mode for 2 min and again returned to the split position, sweeping the liner with

Table 2. Retention times, target and qualifying ions, linearity, instrumental LODs and LOQs and repeatability obtained by LVI-GC-MS(SIM), under optimised instrumental conditions

Compound	RT (min)	SIM ions ^{a)} (<i>m/z</i>)	Linearity (<i>r</i> ²)	LODs (ng/mL)	LOQs (ng/mL)	Repeatability (RSDs, %)
Tinuvin P	13.2	225, 168, 192	0.9953	0.12	0.4	2.8
Allyl-bzt	15.6	<u>250</u> , 265, 281	0.9941	0.15	0.5	3.9
Tinuvin 320	16	<u>308</u> , 323	0.9956	0.05	0.2	1.3
Tinuvin 326	16.4	<u>300</u> , 315, 272	0.9981	0.05	0.4	2.9
Tinuvin 328	16.9	<u>322</u> , 351	0.9964	0.08	0.4	3.2
Tinuvin 327	17	<u>342</u> , 357	0.9984	0.11	0.4	1.9

a) Underlined ions were used for quantification purposes.

a helium flow of 60 mL/min. Injection volume and speed were set at 20 μ L and 100 μ L/min, respectively. Analytes were separated on an HP-5MS (30 m $\times$ 0.25 mm id, 0.25 μ m film thickness, Agilent Technologies) capillary column, using helium as the carrier gas at a constant column head pressure of 21 psi. The GC oven was programmed as follows: 80°C (held for 1 min), first rate 12°C/min to 245°C, second rate 25°C/min to 285°C (held for 10 min). Transfer line, ion source and quadrupole temperatures were set at 280, 230 and 150°C, respectively. The solvent delay time was set at 9 min. The MS system was operated in the electronic ionization mode (70 eV) using an ionization current of 35 μ A. Full-scan MS spectra were recorded in the range from 35 to 500 *m/z* units. Quantification was performed in the selected ion monitoring (SIM) mode, using ions compiled in Table 2. Quantification ions corresponded to [M]⁺ species for Tinuvin P, [M–29]⁺ for Tinuvin 328 and [M–15]⁺ for the rest of UV absorbers. Analytes were included in different temporal segments, except in the case of Tinuvin 328 and 327, which could not be baseline separated. In all cases, a dwell time of 100 ms per ion was used. Data recording and instrument control were performed by the MSD ChemStation software (G1701CA; version C.00.00; Agilent Technologies).

2.4 Statistics data

The Statgraphics Centurion XV software (Manugistics, Rockville, MD, USA) was used to implement the Box-Behnken-type experimental factorial design, in order to estimate the main effect of each experimental factor, their quadratic terms and two-factor interactions in the Tinuvin species response.

3 Results and discussion

3.1 LVI-GC-MS(SIM) conditions

Six benzotriazole UV stabilizers (Tinuvin P, Allyl-bzt, Tinuvin 320, Tinuvin 326, Tinuvin 327 and Tinuvin 328) were selected as model compounds for the present study (Table 1). In a first approach, a standard mixture (10 μ g/mL)

was analyzed to evaluate the fragmentation pattern of each compound, through GC-MS operating in the full-scan mode acquisition. From the data obtained, good resolution and sensitivity was noticed in an analytical time lower than 18 min. For trace-level quantification, the GC-MS data were acquired in SIM mode using the target and qualifier ions listed in Table 2. The PTV was used in the solvent venting mode for LVI (20 μ L), where the best compromise between peak height, symmetry and peak width was achieved, since larger volumes led to an increment in the solvent background, which negatively affected the symmetry, and thus the signal-to-noise ratios (*S/N*) of the chromatographic peaks. This observation matches with the behavior reported for other compounds using LVI as the inlet system [19–26]. Under SIM mode acquisition, the instrumental linearity was evaluated with standards at six different levels in the range of 0.5–500 ng/mL, where good linear dynamic responses were obtained for all compounds (*r*² > 0.994). Table 2 gives the figures of merit of the LVI-GC-MS(SIM) method under optimized conditions. Relative standard deviations (RSDs) on the peak areas (*n* = 5) at the 50 ng/mL level gave values ranging from 1.3 to 3.9%. Furthermore, the instrumental limits of detection (LODs) and quantification (LOQs), defined as the concentration of each specie providing a chromatographic peak with *S/N* of 3 and 10, remained below 0.15 and 0.50 ng/mL, respectively. It must be emphasized that the LOQs achieved are two orders of magnitude lower than those reported for other systems such as liquid chromatography coupled to mass spectrometry (LC-MS) using different ionization sources [7], and in the same range of values as those attained by tandem systems (GC-MS/MS) [27]. Therefore, the LVI-GC-MS(SIM) system seems to be one of the most convenient instruments for the trace-level determination of benzotriazole UV stabilizers under study.

3.2 SBSE-LD conditions

3.2.1 Back-extraction parameters

Parameters that affect the yield of analytes back-extraction from the stir bars are the stripping solvent type and desorption time. During the optimization evaluation, five

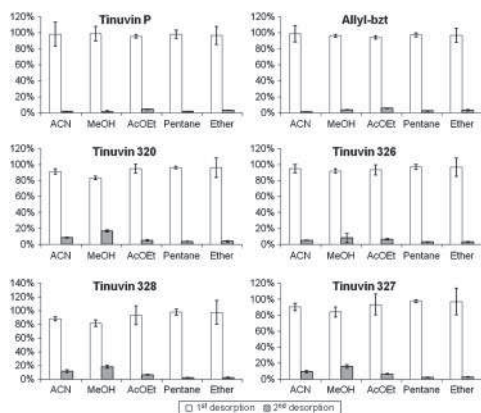


Figure 1. Desorption profile of the UV stabilizers from the stir-bar PDMS coating using different solvents. Normalized responses as percentages ($n = 3$).

water samples were spiked with the target analytes solutions in MeOH to give a final concentration of 0.5 ng/mL. SBSE assays were performed during 1.5 h (20°C, 1000 rpm), in which after the enrichment process were transferred into 2 mL vials and extracted twice with 1 mL of the desorption solvent for 15 min, to speed up the release of trapped compounds [18, 19–26]. Two consecutive back-extractions fractions having 1 mL of the considered solvent were obtained, where cumulative recoveries, as percentage, were obtained with MeOH, ACN, AcOEt, *n*-pentane and diethyl ether (Fig. 1). The lowest yields (around 80%) corresponded to Tinuvin 327 and 328 (the most lipophilic analytes) using MeOH (1 mL) as the desorption solvent. On the other hand, around 98% of all benzotriazoles were recovered with the first fraction (1 mL) of *n*-pentane and diethyl ether, where the former was preferred as the desorption solvent due to the lower variability observed (Fig. 1). In order to improve the enrichment factor provided by SBSE, the *n*-pentane extract was concentrated to dryness and the target analytes were further re-dissolved with 0.2 mL of AcOEt. In the subsequent experiments, it was verified that no differences were attained considering 1 mL of *n*-pentane and three different soaking times (5, 10 and 15 min). Therefore, 5 min was adopted as the working value for this parameter.

3.2.2 Extraction parameters

The SBSE efficiency depends on several variables, such as pH, ionic strength and polarity characteristics of the sample media, as well as, agitation speed and equilibrium time. In the first approach, organic modifiers are useful to control the polarity characteristics of the sample media in order to prevent the adsorption of non-polar compounds on the walls of the glass vessels [19, 28]. This phenomenon competes with the extraction equilibrium, reducing the amount of analytes incorporated into the PDMS coating. Therefore,

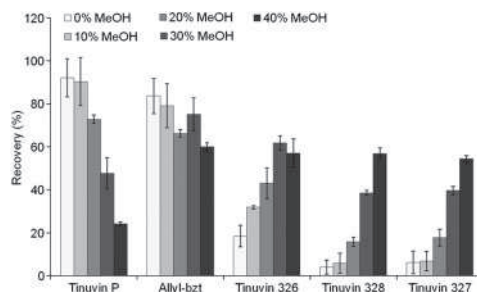


Figure 2. Effect of MeOH in the extraction efficiency of the UV stabilizers in ultra-pure water matrices obtained by SBSE-LD/LVI-GC-MS(SIM), under standard experimental conditions.

series of extractions were carried out with fortified aliquots of ultra-pure water containing different percentages of MeOH up to 40%. Under the above conditions, the yields of the assays were assessed by comparing the responses (peak areas) obtained for each analyte in the final extracts from the stir bars with those corresponding to calibration standards dissolved in AcOEt. MeOH addition increased the recoveries and improved the precision of the method for the less polar analytes (Tinuvin 320, 326, 327 and 328), as shown in Fig. 2. On the other hand, the efficiency yields for Tinuvin P underwent a significant diminution with the MeOH content in sampling vessels. Finally, the efficiency obtained for Allyl-bzt was scarcely affected by this factor. As a compromise solution, a 30% of MeOH was added to water matrices (7.5 mL of MeOH + 15 mL of water sample) in the extraction vial.

The ionic strength, sample pH and agitation speed were also optimized to maximize the efficiency of the extraction step. For this purpose, these factors were simultaneously evaluated, at three different levels, using a Box-Behnken-type experimental factorial design, involving a total of 15 assays, and considering directly the peak areas of target analytes as variable response. For three factors, the Box-Behnken design has demonstrated to be more efficient than other surface response designs, such as central composite or full factorial design [29, 30]. Table 3 summarizes the levels considered for each factor in the above design. As regards sample pH, the selected range covers the interval expected in sewage water samples. In all the experiments, an equilibrium time of 4 h was considered. Numerical values of the standardized main effects are also summarized in Table 3. Their absolute values are proportional to the change in the response measured for a given compound when the associated factor varies from the low to the high level, within the domain of the design. A positive sign indicates an improvement in the extraction yield and, a negative one the opposite trend. The concentration of NaCl (code A) was statistically significant at the 95% confidence level, with a negative influence on the responses obtained for Tinuvin 320, 326, 327 and 328, whereas Tinuvin P showed the opposite behavior. Probably, the ionic strength increment of

Table 3. Experimental domain and standardized values for the main effects in the Box-Behnken design

Factor	Code	Level			Standardized value					
		Low	Medium	High	Tinuvin P	Allyl-bzt	Tinuvin 320	Tinuvin 326	Tinuvin 328	Tinuvin 327
NaCl	A	0	10	20	12.86 ^{a)}	−2.05	−14.18 ^{a)}	−12.85 ^{a)}	−7.88 ^{a)}	−8.60 ^{a)}
Agitation speed	B	700	1000	1300	−1.29	1.69	−1.26	1.04	−2.45	−2.21
pH	C	3	6	9	−2.93 ^{a)}	−2.29	1.84	1.44	0.83	0.76

a) Statistically significant factors at the 95% confidence level.

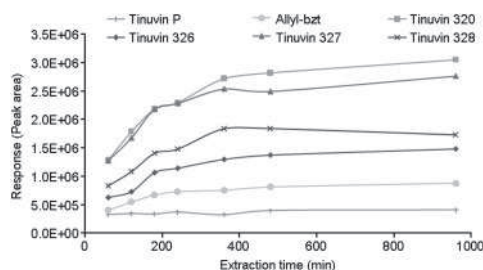
Table 4. Linear dynamic range, repeatability ($n = 4$), reproducibility ($n = 9$), accuracy and estimated limits of quantification (LOQs) for the proposed method (SBSE-LD/LVI-GC-MS(SIM)) obtained for water samples, under optimised experimental conditions

	Linearity (0.02–10.00 ng/mL)			Repeatability (RSD, %)		Reproducibility (RSD, %)	Accuracy (%) (SD)		LOQs (ng/mL)
	Slope (SD) ^{a)}	Intercept (SD)	Correlation coefficient (r^2)	0.1 ng/mL	1 ng/mL	0.5 ng/mL	0.1 ng/mL	1 ng/mL	
Tinuvin P	9.9E+5 (2E+4)	−1.2E+5 (9E+4)	0.9970	12.7	3.7	2.9	114 (3)	101 (7)	0.015
Allyl-bzt	1.94E+6 (9E+3)	−5.8E+4 (4E+4)	0.9999	5.8	1.8	4.5	118 (5)	107 (2)	0.013
Tinuvin 320	5.79E+6 (4E+4)	−3.8E+4 (1.7E+5)	0.9997	3.7	2.8	2.6	120 (4)	92.8 (0.5)	0.01
Tinuvin 326	2.85E+6 (3E+4)	9.7E+4 (1.2E+5)	0.9994	3.7	2.3	3.5	89.3 (1.1)	97 (3)	0.007
Tinuvin 328	4.98E+6 (1E+5)	3.5E+5 (4E+5)	0.9976	6.0	6.2	3.8	103 (10)	100 (2)	0.005
Tinuvin 327	3.19E+6 (7E+4)	2.6E+5 (3E+5)	0.9975	2.2	5.6	2.9	98 (4)	99.1 (1.2)	0.004

a) SD – standard deviation within parenthesis; RSD – relative standard deviation.

the sample favours losses of less polar analytes due to competitive sorption on the surface of glass vessels; furthermore, an increase in the ionic strength leads to more viscous samples, which turns in slower migration rates from the bulk of the water sample to the interface with the PDMS coater stir bar. On the other hand, in the case of the more polar Tinuvin P, the salting out phenomena is responsible for the improvement of the extraction yield. Sample pH (code C) had a negative, statistical significant effect only in the response of Tinuvin P, the most acidic and polar of the target analytes. Finally, the effect of agitation (code B) did not achieve the level of statistical significance for any compound (Table 3). In general, quadratic terms and two-factor interactions exerted non-significant effects in the responses of Tinuvin (data not shown). As a compromise conditions, the pH of water samples was adjusted to 3.0 units, the stirring was maintained at the lowest level (700 rpm) and no salt was added to water samples, assuming the diminution in the efficiency of the SBSE for Tinuvin P.

The kinetics of the SBSE process was assessed considering equilibrium times from 60 to 960 min, at room temperature ($20 \pm 2^\circ\text{C}$). The obtained profiles are shown in Fig. 3. In the case of Tinuvin P, the equilibrium was already achieved after one hour of sampling, whereas six hours were required for the remaining compounds. In further experiments, an equilibrium time of six hours was adopted. Using a 15-positions magnetic stirrer, the sample throughput of the proposed method remained around 30 min.

**Figure 3.** Average kinetic plots of the SBSE process ($n = 2$) for the UV stabilizers in ultra-pure water matrices obtained by SBSE-LD/LVI-GC-MS(SIM), under standard experimental conditions.

3.3 Figures of merit

Table 4 summarizes the data related to the performance of the optimized SBSE-LD/LVI-GC-MS(SIM) methodology. The linearity was evaluated with samples spiked at eight concentration levels, between 0.02 and 10.00 ng/mL. The plots of peak areas measured for each compound against the concentration in the water sample, fitted a linear model, with correlation coefficients (r^2) higher than 0.997. The precisions were investigated with spiked samples processed in the same day (intra-day – repeatability) and in three different days (inter-day – reproducibility). The RSDs

Table 5. Theoretical and experimental recoveries of the proposed method (SBSE-LD/LVI-GC-MS(SIM)) for different water matrices, under optimised experimental conditions

	Recovery, % (SD)					
	Tinuvin P	Allyl-bzt	Tinuvin 320	Tinuvin 326	Tinuvin 328	Tinuvin 327
Theoretical	95	100	100	100	100	100
Ultra-pure water	47.9 (1.4)	96.9 (4.4)	98.6 (2.6)	103.1 (3.6)	87.0 (3.3)	92.5 (2.7)
Treated wastewater	24.6 (0.5)	76.8 (1.6)	80.1 (2.4)	83.7 (1.9)	64.9 (2.2)	64.7 (1.8)
Raw wastewater	29.2 (1.0)	76.2 (2.7)	90.7 (2.9)	61.6 (1.8)	24.9 (2.2)	29.3 (1.5)

SD – standard deviation within parenthesis.

obtained ranged from 2 to 6%, with the exception of Tinuvin P in the sample spiked at the lowest level (12.7%). The LOQs of the proposed method, estimated considering the S/N ratios of 10 in the lowest levels of the linear dynamic range, and the presence of trace amounts of some species in the procedural blanks (Fig. 4), varied between 0.004 and 0.015 ng/mL. Using LLE, Nakata et al. [14] achieved LODs (defined for an $S/N = 3$) in between 0.002 and 0.009 ng/mL. Nevertheless, their procedure consumed 200 mL of organic solvent per sample and it required additional clean-up of the concentrated extract using a column of deactivated silica. On the other hand, SPME followed by GC-MS/MS [15] achieved slightly lower LOQs (from 0.0005 to 0.0020 ng/mL) than those reported in Table 4.

The control of procedural blanks and cross-contamination problems is a major requirement to achieve the LOQs reported in Table 4. For this purpose, new vials have to be used for each extraction. Moreover, additional soaking of PDMS-coated stir bars, after each extraction–desorption cycle, using 5 mL of ACN for 15 min, is mandatory. It is also highly recommended to protect glass material from deposition of particulate matter due to the existence of significant concentrations of Tinuvin associated with dust particles [27].

An often limitation of microextraction techniques is the influence of the sample matrix on the recovery efficiency. Table 5 shows the theoretical and experimental extraction efficiencies (absolute values) for target analytes in different water samples spiked at the 0.5 ng/mL level. Theoretical recoveries were calculated as reported elsewhere [17, 18], using the $K_{O/W}$ values and considering 25 and 0.024 mL as the volume of sample and PDMS coating, respectively. In the case of ultra-pure water, the experimental extraction yields were almost quantitative and they are in acceptable agreement with theoretical values predicted from $K_{O/W}$ partitioning coefficients. The exception to the above behavior corresponded to Tinuvin P, the most polar of the considered species. Likely, its log $K_{O/W}$ value (4.3) undergoes a dramatic reduction when MeOH (30%) was added to the water sample. This fact explains the difference between the theoretical and the experimental extraction efficiencies obtained for this compound in ultra-pure water. Although $K_{O/W}$ values for the rest of analytes are also reduced due to the presence of MeOH, they remained high enough to guar-

antee their quantitative recovery from the sampling vial. The data presented in Table 5 also prove that the extraction yield depends on the matrix characteristics. As observed, it significantly decreased for raw and even treated wastewater in comparison with ultra-pure water. The reduction was more significant for the less polar species (Tinuvin 328 and 327) in raw wastewater, probably due to competitive equilibria between the PDMS polymeric phase and the dissolved organic matter for Tinuvin species. The same trend has been also reported for sorptive extraction of other lipophilic species, such as the UV filter octocrylene, using silicone-type sorbents [30]. Providing that isotopic labeled analogues of target compounds are not commercially available, the SAM is definitely required guaranteeing the accuracy of the methodology when applied to sewage water matrices.

3.4 Application to wastewater matrices

The proposed method was applied to samples of treated and raw wastewater from two urban wastewater plants, located near Lisbon (Portugal) and in the Northwest of Spain. Levels of Tinuvin in the above samples were quantified through the SAM, processing three aliquots of each un-spiked sample plus four addition levels (from 0.02 to 0.50 ng/mL). Moreover, procedural blanks were processed to identify any possible contamination problem. Figure 4 shows fragmentograms obtained by SBSE-LD/LVI-GC-MS(SIM) corresponding to a procedural blank and a non-spiked aliquot of raw sewage water matrices (code 1, Table 6). From the data obtained, summarized in Table 6, the SAM plots provided a linear relationship between peak areas against concentrations with correlation coefficients (r^2) higher than 0.991 for all targets in the six processed samples. Allyl-bzt remained undetected in all samples, whereas Tinuvin 326 and 328 were measured in all raw sewage water specimens. In the case of treated samples, analytes remained below the LODs achieved for the methodology (sample codes 4 and 6), ranging from 0.021 to 0.031 ng/mL (sample code 2). The contents achieved are in the same order of magnitude as those previously reported in the literature [14, 15]. Furthermore, they point out to the apparent removal of Tinuvin UV stabilizers in the wastewater treatment plants.

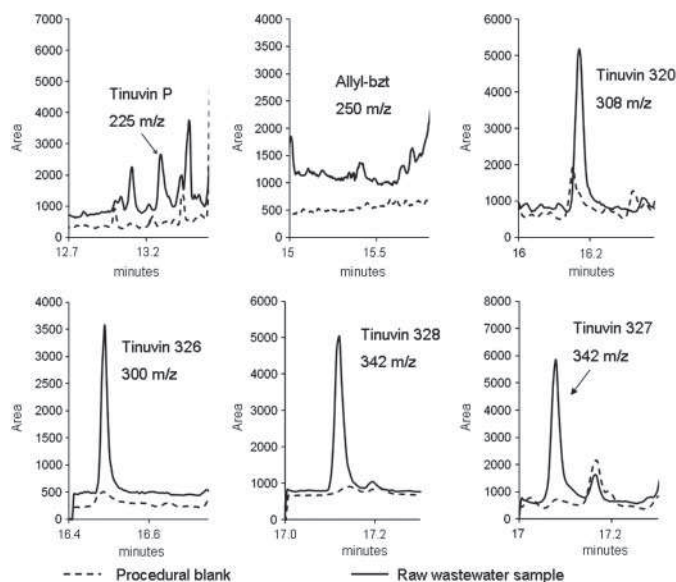


Figure 4. Fragmentograms of the UV stabilizers obtained by SBSE-LD/LVI-GC-MS(SIM) in a procedural blank and a non-spiked raw wastewater sample (code 1, Table 6), under optimized experimental conditions.

Table 6. Contents of Tinuvin found in several wastewater matrices from Portugal and Spain, obtained by SBSE-LD/LVI-GC-MS(SIM) using the SAM, under optimised experimental conditions

Code	Type	Sampling date	Place	Average concentrations, ng/mL (SD)				
				Tinuvin P	Tinuvin 320	Tinuvin 326	Tinuvin 328	Tinuvin 327
1	RW ^{a)}	Nov-10	Portugal	0.046 (0.004)	0.024 (0.007)	0.026 (0.005)	0.076 (0.014)	0.085 (0.019)
2	TW ^{b)}	Nov-10	Portugal	n.d.	n.d.	n.d.	0.021 (0.006)	0.031 (0.009)
3	RW ^{a)}	Jan-11	Spain	n.d.	n.d.	0.040 (0.006)	0.053 (0.007)	n.d.
4	TW ^{b)}	Jan-11	Spain	n.d.	n.d.	n.d.	n.d.	n.d.
5	RW ^{a)}	Feb-11	Spain	0.020 (0.005)	n.d.	0.034 (0.003)	0.065 (0.006)	0.022 (0.003)
6	TW ^{b)}	Feb-11	Spain	n.d.	n.d.	n.d.	n.d.	n.d.

SD – standard deviations within parenthesis, $n = 3$ replicates; n.d. – not detected.

a) Raw wastewater samples.

b) Treated wastewater samples.

4 Concluding remarks

The present methodology (SBSE-LD/LVI-GC-MS(SIM)) was successfully applied to determine benzotriazole UV stabilizers in wastewater matrices, demonstrating very high selectivity and sensitivity for trace-level analysis. Assays performed under optimized experimental conditions, yielded recoveries ranging from $47.9 \pm 1.4\%$ (Tinuvin P) to $103.1 \pm 3.6\%$ (Tinuvin 326). The methodology showed also excellent linear dynamic ranges (0.02–10.00 ng/mL) for the six benzotriazole UV stabilizers studied with correlation coefficients higher than 0.9970, limits of quantification in between 4 and 15 ng/L, suitable repeatability (RSD < 12.7%)

and reproducibility (RSD < 4.5%). A further advantage of the developed methodology is the low sample requirement. On the other hand, the efficiency of the extraction is sample dependent; therefore, the SAM method is required for the accurate quantification of Tinuvin UV filters in sewage water matrices. Although Tinuvin appeared to be conveniently removed from the water phase in sewage treatment plants, further studies are required to address their potential concentration in sludge.

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