UNIVERSIDAD DE GRANADA

FACULTAD DE CIENCIAS

DEPARTAMENTO DE QUÍMICA ANALÍTICA

DESARROLLO DE METODOLOGÍAS ANALÍTICAS PARA LA DETERMINACIÓN DE RESIDUOS DE CARBAMATOS EN ALIMENTOS Y AGUAS

TESIS DOCTORAL

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DESARROLLO DE METODOLOGÍAS ANALÍTICAS PARA LA DETERMINACIÓN DE RESIDUOS DE CARBAMATOS EN ALIMENTOS Y AGUAS

(DEVELOPMENT OF ANALYTICAL METHODOLOGIES FOR THE DETERMINATION OF RESIDUES OF CARBAMATES IN FOODS AND WATERS)

Por

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DEPARTAMENTO DE QUÍMICA ANALÍTICA
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MEMORIA presentada para aspirar al Grado de Doctor en Ciencias,
Sección Químicas

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D[a]. Ana María García Campaña, Catedrática del Departamento de Química Analítica de la Facultad de Ciencias de la Universidad de Granada y responsable del grupo de investigación "Calidad en Química Analítica, Alimentaria, Ambiental y Clínica" (FQM-302).

CERTIFICA:

Que el trabajo que se presenta en esta TESIS DOCTORAL, con el título de *"DESARROLLO DE METODOLOGÍAS ANALÍTICAS PARA LA DETERMINACIÓN DE RESIDUOS DE CARBAMATOS EN ALIMENTOS Y AGUAS",* ha sido realizado en los laboratorios del citado grupo bajo mi dirección y la de los profesores D[a]. Laura Gámiz Gracia y D. Juan Manuel Bosque Sendra, en el Departamento de Química Analítica de la Facultad de Ciencias de la Universidad de Granada y reúne todos los requisitos para poder optar al Grado de Doctor.

Granada a 2 de Abril de 2013

Esta Tesis Doctoral ha sido realizada gracias a la financiación obtenida en los siguientes proyectos de investigación:

- Grupo de Investigación *"Calidad en Química Analítica Alimentaria Ambiental y Clínica", FQM-302.*
- *"Nuevas estrategias basadas en técnicas miniaturizadas acopladas con detección por fluorescencia y espectrometría de masas para el control de plaguicidas y fármacos en muestras ambientales y biológicas"* (Ref.: CTM2006-06363, Ministerio de Educación y Ciencia)
- *"Determinación de micotoxinas y plaguicidas en bebidas y derivados de cereales mediante técnicas miniaturizadas con detecciones UV-VIS, espectrometría de masas y fluorescencia inducida por láser"* (Ref.: P07-AGR-03178, Proyectos de Excelencia. Junta de Andalucía)
- Subvención para estancias de movilidad de estudiantes en programas de doctorado con Mención hacia la Excelencia (Ministerio de Educación, Cultura y Deporte)

El doctorando David Moreno González y los directores de la tesis D[a]. Ana M[·] García Campaña, D[a]. Laura Gámiz Gracia y D. Juan Manuel Bosque Sendra garantizamos, al firmar esta tesis doctoral, que el trabajo ha sido realizado por el doctorando bajo la dirección de los directores de la tesis y hasta donde nuestro conocimiento alcanza, en la realización del trabajo, se han respetado los derechos de otros autores a ser citados, cuando se han utilizado sus resultados o publicaciones.

Granada a 2 de Abril de 2013

Directores de la Tesis

Doctorando

Fdo.: Ana María García Campaña

Fdo.: David Moreno González

Fdo.: Laura Gámiz Gracia

Fdo.: Juan Manuel Bosque Sendra

ÍNDICE

OBJETO DE LA MEMORIA

Los objetivos de esta Tesis Doctoral se enmarcan dentro de diversos Proyectos de Investigación desarrollados a lo largo del transcurso de este trabajo y que forman parte de las líneas de investigación del grupo en el que se ha realizado.

El objetivo principal ha sido la puesta a punto de nuevos métodos de análisis sensibles y selectivos para un grupo de plaguicidas pertenecientes a la familia de los carbamatos, empleando técnicas de separación miniaturizadas, como la electroforesis capilar (CE) y la cromatografía líquida capilar (HPLC capilar), y de ultra eficacia, como es la cromatografía líquida de ultra resolución (UHPLC), acopladas a diferentes técnicas de detección como son la espectrofotometría ultravioleta-visible (UV-Vis) y la espectrometría de masas en tándem (MS/MS). Asimismo, en esta Tesis se proponen métodos alternativos a los ya existentes para el tratamiento de muestras de diversos alimentos y aguas de distinta procedencia, más eficaces y respetuosos con el medioambiente, de acuerdo con las nuevas tendencias de la Química Verde.

Como objetivos concretos de esta Tesis Doctoral destacan los siguientes:

- Emplear la HPLC capilar para la determinación de carbamatos, como alternativa a la HPLC convencional.
- Evaluar la CE-UV para la determinación de carbamatos empleando técnicas de preconcentración *off-line* y *on-line* que mejoren la sensibilidad inherente a esta técnica.
- Demostrar la potencialidad de los diferentes acoplamientos CE-MS existentes en la determinación de carbamatos, empleando surfactantes volátiles como medio electroforético y comparando los resultados obtenidos con objeto de evaluar las dos modalidades empleadas.
- Explorar las ventajas de la UHPLC-MS/MS como potente herramienta para la identificación inequívoca y cuantificación rápida de un elevado número de residuos de carbamatos.
- Proponer nuevos tratamientos de muestra más simples, rápidos y respetuosos con el medioambiente para la determinación de carbamatos, como alternativa a los ya existentes y evaluarlos en diferentes tipos de muestras alimentarias y de aguas con objeto de demostrar su validez en cuanto a la selectividad y sensibilidad requeridas en este tipo de análisis.

RESUMEN

En esta Tesis se han desarrollado diferentes métodos para la determinación de residuos de carbamatos en alimentos y muestras medioambientales. En el caso de los alimentos, la legislación vigente establece para los carbamatos y otros contaminantes unos Límites Máximos de Residuos (MRLs) que no deben ser superados con objeto de garantizar la calidad del producto y permitir su distribución y consumo. En el caso de las aguas, el interés de la determinación de carbamatos es un hecho contrastado, ya que el uso de plaguicidas, incluidos los carbamatos, está muy extendido, y si se utilizan mal o en exceso, los plaguicidas pueden contaminar el agua, la atmósfera y el suelo y de este modo pueden entrar en la cadena alimentaria y provocar efectos desfavorables en la salud humana.
Considerando las recientes e importantes mejoras de las técnicas separativas en cuanto a miniaturización y aumento de la eficacia, y con objeto de explorar sus indudables ventajas (bajo consumo de disolventes, alta sensibilidad, elevada resolución, tiempos de análisis reducidos y baja cantidad de muestra), en esta Tesis Doctoral se proponen nuevos métodos analíticos para la determinación de residuos de carbamatos empleando técnicas de separación miniaturizadas, como la electroforesis capilar (CE) y la cromatografía líquida capilar (HPLC capilar), y de alta eficacia (como la cromatografía líquida de ultra resolución, UHPLC) con objeto de explorar las ventajas mencionadas. Estas técnicas, acopladas a sistemas de detección como la espectrofometría ultravioleta visible (UV-Vis) y la espectrometría de masas en tándem (MS/MS) permiten la cuantificación e identificación inequívoca de estos compuestos a las bajas concentraciones esperadas en estas matrices.

A continuación se describe brevemente el trabajo desarrollado:

- En el primer capítulo se describe el método desarrollado para la determinación de seis carbamatos en muestras vegetales (pepino) y aguas de diversa procedencia mediante HPLC capilar-UV como técnica instrumental y extracción en fase sólida (SPE) como técnica de tratamiento de muestra, con diferentes tipos de sorbentes dependiendo de la matriz.
- En el segundo capítulo se propone la determinación de doce carbamatos en diferentes zumos de frutas empleando la CE-UV. Para aumentar la sensibilidad del método se aplicaron técnicas de preconcentración dentro del capilar (*on-line)* como el denominado *barrido (sweeping)*, consiguiéndose también la

preconcentración previa (*off-line)* de los analitos mediante la microextracción líquido-líquido dispersiva (DLLME).

- En el tercer capítulo se propuso el acoplamiento CE-MS empleando una interfase de flujo coaxial o *sheath-flow* y un surfactante volátil como electrolito de fondo, para la determinación de diecisiete carbamatos en aguas de diversa procedencia y la DLLME como tratamiento de muestra.
- En siguiente capítulo se recoge el trabajo desarrollado durante la estancia predoctoral en la Universidad de Utrecht (Holanda), que consistió en la evaluación de un prototipo de interfase sin flujo adicional (*sheathless*) para el acoplamiento CE-MS. El método desarrollado se aplicó a los mismos analitos considerados en el capítulo anterior y se validó para aguas destinadas al consumo humano.
- El quinto capítulo describe un método de análisis para la determinación de veinticinco carbamatos en muestras de vino. Para ello se desarrolló un método UHPLC-MS/MS que permitió la separación cromatográfica de estos plaguicidas en menos de cinco minutos con eficacias muy elevadas. Además se propuso un nuevo tratamiento de muestra llamado microextracción asistida por ultrasonidos con emulsificación mejorada con surfactante (UASEME), escasamente explorado en el análisis de alimentos.
- Finalmente, en el último capítulo se evaluó la metodología QuEChERS para el análisis de treinta y tres carbamatos en productos botánicos empleando de nuevo UHPLC-MS/MS.

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SUMMARY

In this Thesis, different methods for the determination of carbamate residues in foodstuff and environmental samples have been developed. In the case of foods, in order to ensure their quality and consumer health, maximum residue limits (MRLs) have been established by legislation for carbamates and other residues. In the case of waters, the indiscriminate or inappropriate use of pesticides could pollute air, water sources and/or soils, and finally these pesticides could enter into the feed chain, causing a threat to human health.

Considering these problems and taking into account the last technical advances in terms of efficiency and miniaturization, different miniaturized separation techniques (capillary electrophoresis (CE) and capillary liquid chromatography (capillary HPLC)) as well as ultra-high performance liquid chromatography (UHPLC) have been assessed, coupled to detection techniques such as ultraviolet-visible spectrophotometry (UV-Vis) and tandem mass spectrometry (MS/MS). In addition, alternative sample treatments have been proposed, making possible an increased efficiency and sample throughput.

As a summary, the Thesis compiles the following works:

- In the first chapter, a capillary HPLC-UV has been developed for the analysis of six carbamates in vegetable (cucumber) and enviromental water samples. The proposed sample treatment was solid phase extraction (SPE) using different sorbents.
- In the second chapter, the determination of twelve carbamates in fruit juice samples was carried out by CE-UV. In order to increase the sensitivity of the method, different preconcentration techniques were proposed, such as sweeping (on-line preconcentration) and a sample treatment such as dispersive liquid-liquid microextraction (DLLME), which also allows an off-line preconcentration.
- For the third chapter, a sheath-flow interface was proposed to the CE-MS coupling, using a volatile surfactant as background electrolite. The determination of seventeen carbamates was carried out in environmental waters using DLLME.
- In the fourth chapter the work carried out in Utrecht University (The Netherland) during the predoctoral stay is described. It consisted of the evaluation of a sheathless interface prototype for the CE-MS coupling. The proposed method was

applied to the same analytes considered in the previous chapter and was validated for waters intended for human consumption.

- In the fifth chapter, the determination of twenty five carbamates in wine samples was carried out. For that purpose, a new UHPLC-MS/MS method was optimized; with this methodology the analysis time was lower than six minutes with very high efficiencies. In addition, a new sample treatment named ultrasound-assisted surfactant-enhanced emulsification microextraction (UASEME) was developed for extraction and clean-up.
- Finally, in the last chapter, QuEChERS methodology was evaluated for the analysis of thirty three carbamates in herbal products using UHPLC-MS/MS.

INTRODUCCIÓN

1. PLAGUICIDAS: Generalidades y clasificación.

Edad Antigua

Egipto

"Papiro de Eber" donde se describe la preparación de varias sustancias que se emplean para repeler insectos.

Grecia

Homero menciona la utilidad del azufre quemado como fumigante (La Odisea, Capítulo XXII).

Hasta finales del siglo XIX

Compuestos derivados del arsénico, tabaco molido, cianuro de hidrógeno, compuestos de mercurio, zinc y plomo, etc, son empleados para luchar contra las plagas.

1ª GENERACIÓN

Principios del siglo XX hasta la actualidad

2ª GENERACIÓN

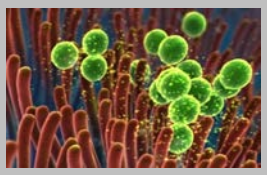
Los avances de la ciencia y de la industria química hicieron posible la aparición de plaguicidas más eficaces. Estos plaguicidas se pueden clasificar según su estructura química. Las tres familias más importantes son los organofosforados, los carbamatos y los organoclorados, incluido el DDT.

En 1939 el químico suizo Müller descubrió las propiedades del DDT como veneno para los insectos. Recibe el Nobel en 1948. Se calcula que el DDT salvó a 5 millones de personas en sus primeros años de uso ya que se evitó la pérdida de las cosechas y la consiguiente hambruna.

En 1970 el uso del DDT se prohibe debido a sus efectos secundarios para el medioambiente y la salud, lo que puso de manifiesto que el uso de plaguicidas debía ser regulado.

3ª,4ª y 5ª GENERACIÓN

Empleo de la ingeniería genética



Su uso todavía no está muy extendido

La organización mundial de la salud (WHO) define "plaguicida" como cualquier material fabricado o formulado con el fin de destruir alguna plaga [1]. Los herbicidas, fungicidas, insecticidas y acaricidas son, por tanto, plaguicidas. El uso de los plaguicidas está mundialmente extendido y su utilización ha contribuido a la mejora en la producción de las cosechas y a la disminución de la hambruna. Sin embargo, y paralelo a su uso, se han comprobado los numerosos efectos adversos sobre la salud de los consumidores y, sobre todo, de los trabajadores que los aplican, producen o manipulan. La industria química ha ido buscando siempre mejorar la relación efectividad-toxicidad; sin embargo, en la actualidad no existe todavía el plaguicida ideal.

Los plaguicidas se pueden clasifican en función de algunas de sus características principales, como son la toxicidad aguda, la estructura química y su uso [2]. Así, en 1978, la WHO estableció una clasificación basada en su peligrosidad o grado de toxicidad aguda, definida ésta como la capacidad del plaguicida de producir un daño agudo a la salud a través de una o múltiples exposiciones, en un período de tiempo relativamente corto. La toxicidad se mide a través de la dosis letal media (DL_{50}) que es la estimación estadística de la cantidad de una sustancia tóxica (mg kg^{-1}) por peso corporal, necesaria para matar al 50% de animales de experimentación en los que se ensaya el efecto letal de la sustancia, administrándola por vía oral o dérmica, durante un período de tiempo determinado y seguimiento en un período postexposición. Esta clasificación (Tabla 1) es periódicamente evaluada siendo la última versión la de 2009 [3]. En ella se clasifica a los plaguicidas en cuatro clases atendiendo a su grado de toxicidad.

Tabla 1. Clasificación de los plaguicidas según su toxicidad, expresada en DL_{50} (mg kg^{-1}).

Clase	Toxicidad	DL_{50}
Clase IA	Extremadamente peligrosos	0-5 mg kg^{-1}
Clase IB	Altamente peligrosos	5-50 mg kg^{-1}
Clase II	Moderadamente peligrosos	50-500 mg kg^{-1}
Clase III	Ligeramente peligrosos	mayor de 500 mg kg^{-1}

[1] http://www.fao.org/waicent/faostat/Pest-Residue/pest-s.htm#E10E3

[2] J.A. Ramírez, M. Lacasaña, *Arch. Prev. Riesgos Labor.* 4 (2001) 67.

[3] "The WHO recommended classification of pesticides by hazard and guidelines to classification" from WHO Press, World Health Organization 2009. ISBN 978 92 4 154796 3

Los plaguicidas se pueden clasificar además teniendo en cuenta el tipo de plaga a la que se dirige principalmente su acción [4].

Tabla 2. Clasificación de los plaguicidas en función del tipo de plaga que controlan.

Tipo	Acción principal
Insecticidas	Control de insectos
Fungicidas	Control de hongos causantes de enfermedades
Herbicidas	Luchan contra las malas hierbas, ya sea de un modo general o selectivo, es decir, dejando indemne el cultivo y destruyendo todas o buena parte de las hierbas adventicias (malas hierbas)
Acaricidas	Combaten la araña roja y los ácaros
Nematicidas	Control de nemátodos
Molusquicidas	Control de babosas y caracoles
Rodenticidas	Control de roedores (ratas, ratones, topillos, etc)
Desinfectantes del suelo	Su acción se extiende a nemátodos, insectos, hongos y malas hierbas que se encuentran en los suelos destinados a cultivo
Antibióticos de uso agrícola	Luchan contra las bacteriosis propias de los cultivos
Reguladores fisiológicos	Aceleran o retardan el crecimiento, estimulan la floración o fructificación o cambian en alguna forma el comportamiento normal de las plantas
Repelentes	Usados para ahuyentar las plagas
Atrayentes	Usados para atraer las plagas (generalmente a trampas)
Defoliantes	Provocan la caída de las hojas sin matar las plantas

Por otra parte, de acuerdo a su estructura química los plaguicidas se clasifican en diversas familias, que incluyen los compuestos organoclorados, organofosforados, carbamatos, etc. En la siguiente tabla se recogen las familias de compuestos más importantes y su acción principal [5].

Tabla 3. Clasificación de los plaguicidas según su estructura química.

Tipo	Principal uso
Organoclorados	Insecticida
Organofosforados	Insecticida
Carbamatos	Insecticida
Derivados del ácido carboxílico	Herbicida
Triazinas	Herbicida
Ureas sustituidas	Herbicida
Piretroides	Insecticida
Organometálicos	Funguicida
Tiocianatos	Insecticida
Fenoles	Insecticida

[4] U.S. Environmental Protection Agency. http://www.epa.gov/pesticides/about/types.htm

[5] H.S. Rathore, en "Handbook of pesticides, Methods of pesticide residues analysis", 1ª Ed. CRC Press 2010, p. 8.

Dado que los métodos de análisis desarrollados en esta Tesis Doctoral se han centrado en la determinación de plaguicidas de la familia de los carbamatos (CRBs), en el siguiente apartado se comentarán algunos aspectos relevantes de estos compuestos.

2. CARBAMATOS

La actividad biológica de los CRBs se descubrió en 1923, cuando se describió por primera vez la estructura del alcaloide eserina (o fisostigmina) contenido en la nuez de Eseré [6]. En 1929 se sintetizaron análogos de fisostigmina, y pronto se pudo disponer de derivados del ácido ditiocarbámico tales como tiram y ziram. El estudio de los compuestos carbámicos comenzó el mismo año, y ahora se conocen más de 1000 derivados del ácido carbámico. De ellos, más de 50 se utilizan como plaguicidas (herbicidas, fungicidas y nematocidas). En 1947 se sintetizaron los primeros productos de esta clase dotados de propiedades insecticidas. Algunos CRBs han demostrado ser eficaces como aceleradores de la vulcanización, y se han utilizado derivados en el tratamiento de tumores malignos, hipoxia, neuropatías, heridas por radiación y otras enfermedades. También se utilizan como plaguicidas aril ésteres del ácido alquilcarbámico y alquil ésteres del ácido arilcarbámico [7].

[6] D. Osindky, J.M. Stellman en "Enciclopedia de Salud y Seguridad en el trabajo", 3ª Ed., Ministerio de Trabajo y Asuntos Sociales (1998).
[7] N.E.l S. de Nudelman, en "Química sustentable", 1ª Ed., Universidad Nac. del Litoral (2004).

2.1. ESTRUCTURA Y PROPIEDADES FÍSICO-QUÍMICAS DE LOS CARBAMATOS

Los CRBs tienen la estructura común mostrada en la Figura 1.

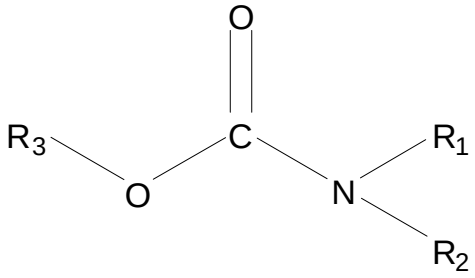


Figura 1. Estructura general de los CRBs.

En función de la naturaleza de los sustituyentes, los CRBs presentan diversas acciones, dando lugar a la clasificación mostrada en la Tabla 4. Además, la sustitución de uno o los dos oxígens por azufre, hace que tengan la actividad insecticida más baja [8].

Tabla 4. Clasificación de los CRBs.

Tipo	Sustituyentes	Acción
Metilcarbamatos	R_1 grupo metilo, R_2 Hidrógeno y R_3 grupo aromático o alifático	Insecticida
Carbamatos	R_1 grupo arómatico, R_2 Hidrógeno y R_3 grupo aromático o alifático	Fungicida
Ditiocarbamatos	R_1 grupo arómatico, R_2 Hidrógeno y R_3 grupo aromático o alifático; los dos oxígenos se sustituyen por azufre	Fungicida
Tiocarbamatos	R_1 grupo aromático o alifático, R_2 Hidrógeno y R_3 grupo benzimidazol; uno de los dos oxígenos se puede sustituir por azufre	Herbicida
Fenilcarbamatos	R_1 grupo aromático o alifático, R_2 Hidrógeno y R_3 grupo benzimidazol	Herbicida

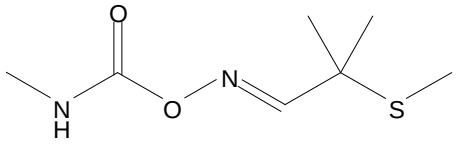
En general, los ésteres simples o derivados N-sustituidos del ácido carbámico, son compuestos inestables, especialmente bajo condiciones alcalinas. Las sales y ésteres del

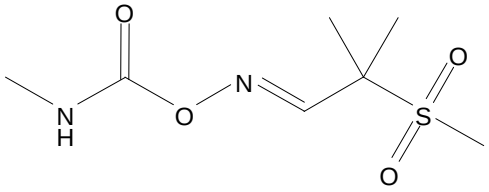
[8] R.J. Flanagan,R.A. Braithwaite, S.S. Brown, B. Widdop, F.A. de Wolff, "Basic Analytical Toxicology", World Health Organization, Geneva (1995).
http://www.who.int/ipcs/publications/training_poisons/basic_analytical_tox/en/index8.html

ácido carbámico son más estables que el ácido. Este incremento de la estabilidad es la base de la síntesis de muchos derivados que actúan como plaguicidas.

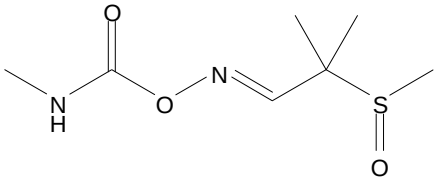
En cuanto a su solubilidad, los ésteres de carbamato son sólidos cristalinos de baja presión de vapor, con solubilidades variables, pero normalmente bajas en disoluciones acuosas. Son moderadamente solubles en disolventes como benceno, tolueno, xileno, cloroformo, diclorometano y 1,2-dicloroetano. En general, son escasamente solubles en disolventes no polares, pero muy solubles en disolventes polares orgánicos como metanol, etanol, acetona, dimetilformamida, etc.

A continuación se muestran las propiedades físico-químicas de los CRBs que han sido estudiados en la presente Tesis Doctoral. Los datos han sido obtenidos de diversas fuentes como Scifinder o Chemfinder.

Aldicarb (ALD)	
	
Nombre químico:	2-metil-2-(metiltio)propionaldehído-O-metilcarbamoiloxima
pKa:	-1.1 y 13.8
Peso molecular:	190.26

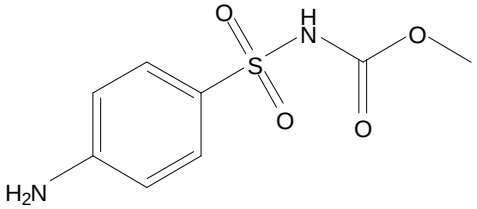
Aldicarb sulfona (ALDSLF)	
	
Nombre químico:	2-metil-2-(metilsulfonil)-O (metilamina)carboniloxima
pKa:	-1.2 y 13.4
Peso molecular:	222.26

Aldicarb sulfóxido (ALDSFX)



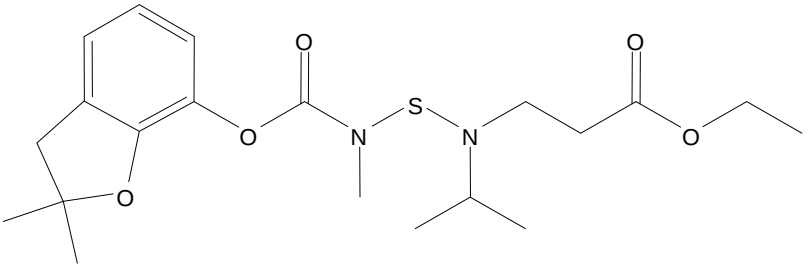
Nombre químico:	2-metil-2-(metilsulfonil)propionaldehído-O-metilcarbamoiloxima
pKa:	-1.2 y 13.5
Peso molecular:	206.26

Asulam (ASL)



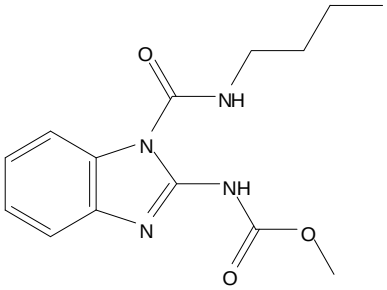
Nombre químico:	metil-N-(4-aminofenilsulfonilo)carbamato
pKa:	1.4 y 4.6
Peso molecular:	230.24

Benfuracarb (BF)



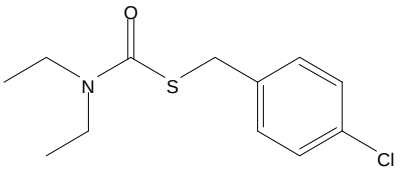
Nombre químico:	N-2,3-dihidro-2,2-dimetilaminotiol-N-isopropil-β-alanilato de metilo
pKa:	2.1
Peso molecular:	410.53

Benomil (BY)



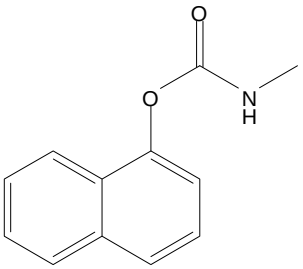
Nombre químico:	metil-1-(butilcarbamoil)bencimidazol-2-il carbamato
pKa:	3.5 y 11.7
Peso molecular:	290.32

Bentiocarb (BTH)



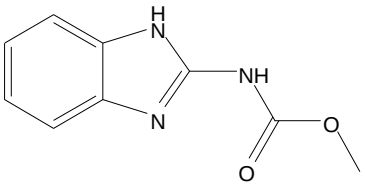
Nombre químico:	S-4-clorobencil dietil(tiocarbamato)
pKa:	-1.3
Peso molecular:	257.78

Carbaril (CAR)



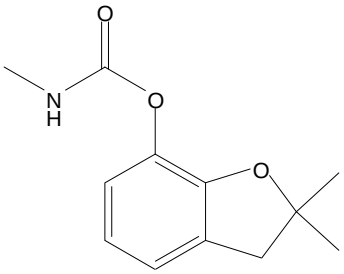
Nombre químico:	1-naftil metilcarbamato
pKa:	-1.5 y 12.0
Peso molecular:	201.22

Carbendazima (CBZ)



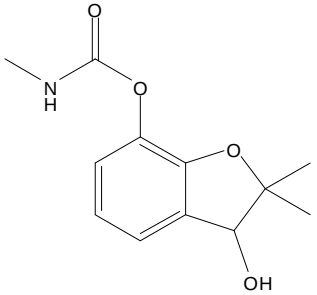
Nombre químico:	Metil benzimidazol-2-ilcarbamato
pKa:	5.6 y 11.6
Peso molecular:	191.19

Carbofurano (CF)



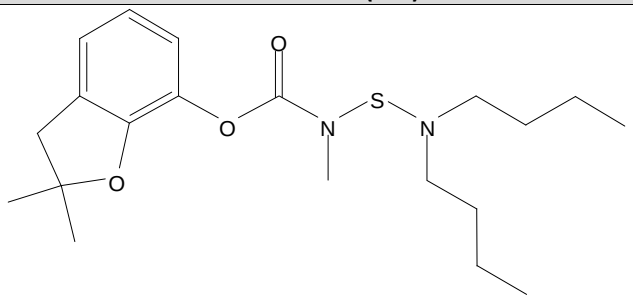
Nombre químico:	2,3-dihidro-2,2-dimetil-7-benzofuranil metilcarbamato
pKa:	-1.5 y 12.3
Peso molecular:	221.25

3-hidroxi-Carbofurano (CFH)



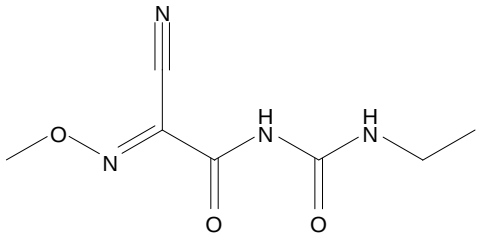
Nombre químico:	2,3-dihidro-3-hidroxi-2,2-dimetil-7-benzofuranil metilcarbamato
pKa:	-1.5 y 12.3
Peso molecular:	237.25

Carbosulfano (CSF)



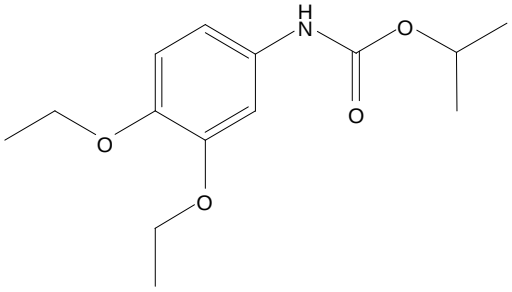
Nombre químico:	2,3-dihidro-2,2-dimetilbenzofuran-7-il (dibutilaminotio)metilcarbamato
pKa:	3.15
Peso molecular:	380.54

Cimoxanilo (CY)



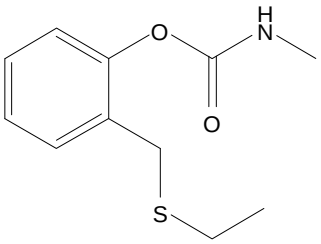
Nombre químico:	1-(2-ciano-2-metroximinoacetil)-3-etilurea
pKa:	-1.7 y 7.4
Peso molecular:	198.18

Dietofencarb (DETH)



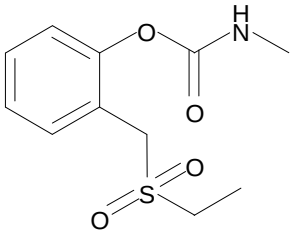
Nombre químico:	3,4-dietoxifenilcarbamato de isopropilo
pKa:	0.9 y 12.8
Peso molecular:	267.32

Etiofencarb (ETH)



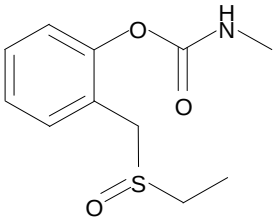
Nombre químico:	(2-etiltiometil-fenil)-n-metilcarbamato
pKa:	-1.5 y 12.1
Peso molecular:	225.31

Etiofencarb sulfona (ETHSL)



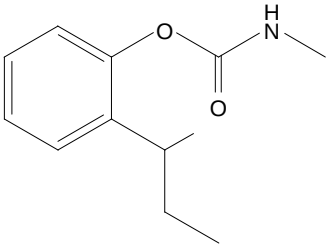
Nombre químico:	2-((etillsulfonil)metil)-fenol metilcarbamato
pKa:	-1.6 y 11.9
Peso molecular:	257.31

Etiofencarb sulfóxido (ETHSLX)



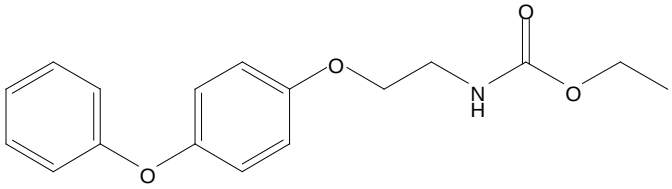
Nombre químico:	2-((etisulfinil)metil)-fenol metilcarbamato
pKa:	-1.6 y 12.0
Peso molecular:	241.31

Fenobucarb (FEN)



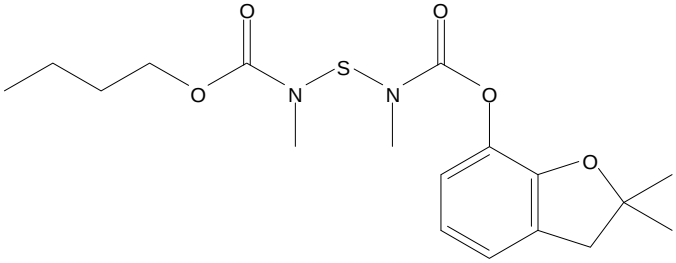
Nombre químico:	2-(1-metilpropil)fenil metilcarbamato
pKa:	-1.5 y 12.2
Peso molecular:	207.27

Fenoxicarb (FNX)



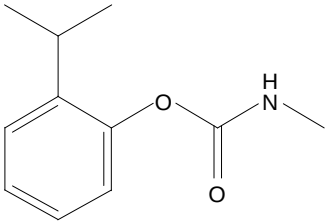
Nombre químico:	[2-(4-fenoxifenoxi)etil] carbamato de etilo
pKa:	-1.5 y 12.5
Peso molecular:	301.34

Furatiocarb (FR)



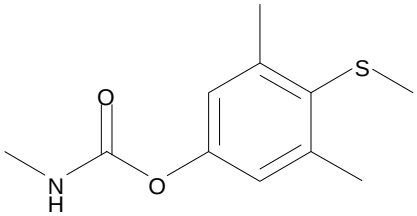
Nombre químico:	2,4-dimetil-6-oxa-5-oxo-3-tia-2,4-diazadecanoato de 2,3 dihidro-2,2-dimetil-7-benzofurilo
pKa:	-2.1
Peso molecular:	382.47

Isoprocarb (ISP)



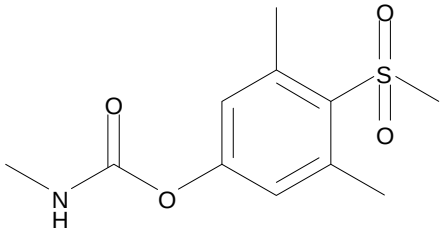
Nombre químico: 2-(1-metiletil)-fenol metilcarbamato
pKa: -1.5 y 12.2
Peso molecular: 193.24

Metiocarb (MTH)



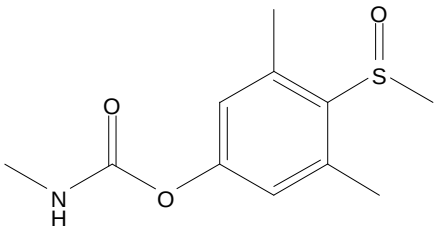
Nombre químico: 3,5-dimeti -4-(metiltio)-fenol metilcarbamato
pKa: -1.6 y 12.3
Peso molecular: 225.31

Metiocarb sulfona (MTHSL)



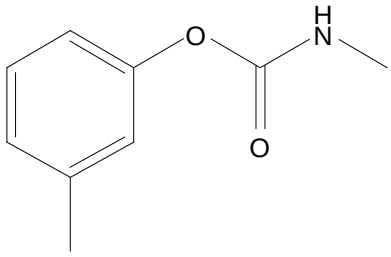
Nombre químico: 3,5-dimetil-4-(metilsulfonil)-fenol metilcarbamato
pKa: -1.5 y 11.9
Peso molecular: 257.31

Metiocarb sulfóxido (MTHSFX)



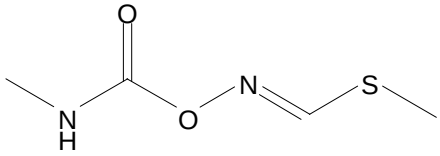
Nombre químico:	3,5-dimetil-4-(metilsulfinil)-fenol metilcarbamato
pKa:	-1.6 y 12.0
Peso molecular:	241.31

Metolcarb (MTL)



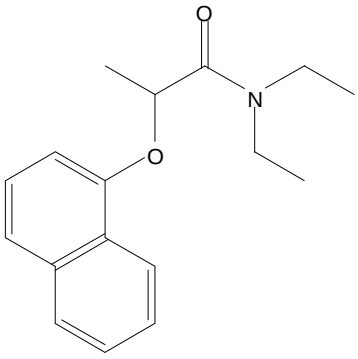
Nombre químico:	3-metilfenil metilcarbamato
pKa:	-1.5 y 12.4
Peso molecular:	165.19

Metomilo (MTY)



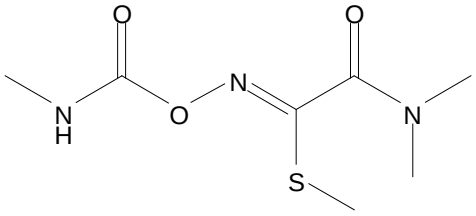
Nombre químico:	metil N-(metilcarbamoiloxi)tioacetimidato
pKa:	-1.3 y 13.3
Peso molecular:	162.21

Napropamida (NP)



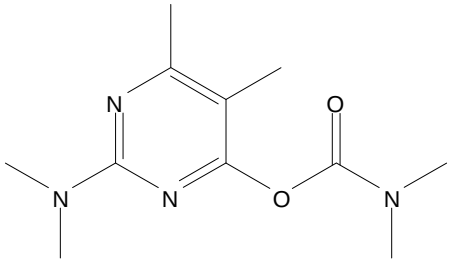
Nombre químico:	N,N-dietil-2-(1-naftiloxi)propionamida
pKa:	-0.8
Peso molecular:	271.35

Oxamil (OX)



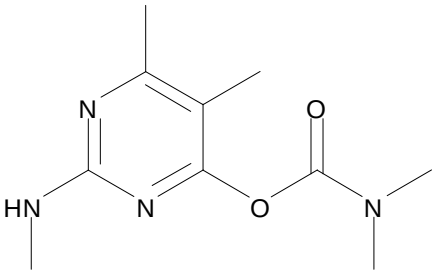
Nombre químico:	N,N-dimetil-2-metilcarbamoiloxiimino-2-(metiltio)acetamida
pKa:	-1.7 y 10.4
Peso molecular:	219.26

Pirimicarb (PRC)



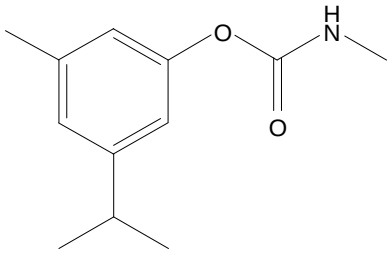
Nombre químico:	2-dimetilamino-5,6-dimetilpirimidin-4-il dimetilcarbamato
pKa:	5.6 y 9.0
Peso molecular:	238.29

Pirimicarb dismetil (PIRD)



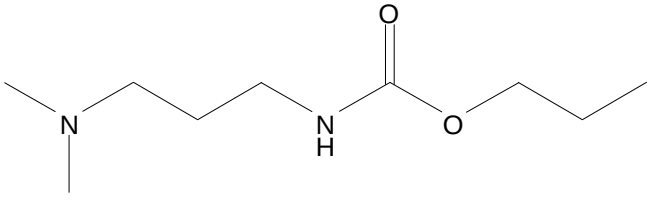
Nombre químico:	2-metilamino--5,6-dimetilpirimidin-4-il dimetilcarbamato
pKa:	4.4 y 8.7
Peso molecular:	224.29

Promecarb (PR)

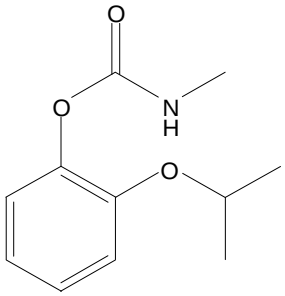


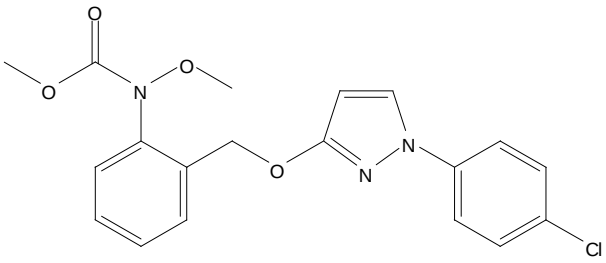
Nombre químico:	3-isopropil-5-metilfenil metilcarbamato
pKa:	-1.5 y 12.4
Peso molecular:	207.27

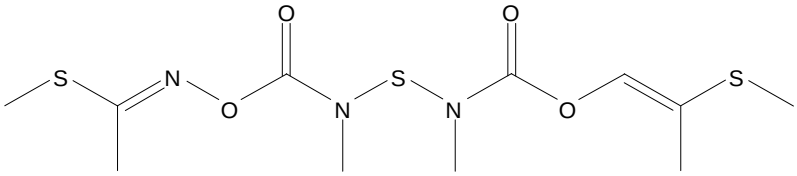
Propamocarb (PRP)



Nombre químico:	propil-3-(dimetilamino)propil carbamato
pKa:	9.5 y 12.7
Peso molecular:	188.27

Propoxur (PX)	
	
Nombre químico:	2-isopropoxifenil metilcarbamato
pKa:	-1.5 y 12.3
Peso molecular:	209.24

Pyraclostrobin (PYC)	
	
Nombre químico:	metil{2-[1-(4-clorofenil)pirazol-3-iloximetil]fenil} (metoxi)carbamato
pKa:	-0.3
Peso molecular:	387.82

Tiodicarb (TH)	
	
Nombre químico:	3,7,9,13-tetrametil-5,11-dioxa- 2,8,14-tritia-4,7,9,12-tetra-azapentadeca-3,12-dien-6,10-diona
pKa:	-1.8
Peso molecular:	354.47

Como se puede observar, la mayoría de los CRBs presentan unos valores de pKa muy extremos, por lo que se pueden considerar compuestos neutros en un amplio rango de pH.

2.2. ACTIVIDAD DE LOS CARBAMATOS

Los CRBs se emplean comúnmente como insecticidas, debido a su efecto neurotóxico como inhibidores reversibles de la enzima colinesterasa, siendo mortales a dosis elevadas. Sus características principales son alta toxicidad, estabilidad baja y nula acumulación en tejidos finos orgánicos. Afectan a los mamíferos a través de la piel, vía respiratoria y vía digestiva.

Según su modo de acción, los CRBs se pueden clasificar en dos grupos principales [9-11]:

a) Inhibidores de la enzima colinesterasa, entre ellos metilcarbamatos y dimetilcarbamatos, que se emplean fundamentalmente como insecticidas (tanto de contacto como sistémicos), pero también como miticidas, rodenticidas y nematocidas.

b) Inhibidores de la enzima colinesterasa por carbamilación del sitio esteárico de la enzima, e inhibidores de la acetilcolinesterasa ya que impiden su desesterificación, como los metilcarbamatos y dimetilcarbamatos. Entre ellos, algunos inhiben selectivamente a la acetilcolinesterasa (que se encuentra en las células rojas sanguíneas y tejido nervioso), a la butirilcolinesterasa (que se encuentra en el plasma) o a ambas.

Además de los efectos sobre la acetilcolinesterasa, los CRBs también pueden provocar disfunción del sistema reproductivo y endocrino, al afectar a las concentraciones en sangre de las hormonas implicadas en el metabolismo y en la reproducción [12,13]. En general, los síntomas producidos por la exposición del organismo a la acción de los CRBs incluyen fatiga, dolor en las articulaciones y muscular, efectos sobre el sueño, dolor de cabeza, afecciones cutáneas, efectos cognitivos y neurológicos.

---

[9] A. Tunek, L.Å. Svensson, *Drug Metab. Dispos.* 16 (1988) 759.
[10] W. Luo, Q.S. Yu, S.S. Kulkarni, D.A. Parrish, H.W. Holloway, D. Tweedie, A. Shafferman, D.K. Lahiri, A. Brossi, N.H. Greig, *J. Med. Chem.* 49 (2006) 2174.
[11] S. Darvesh, K.V. Darvesh, R.S. McDonald, D. Mataija, R. Walsh, S. Montana, O. Lockridge, E. Martin, *J. Med. Chem*. 51 (2008) 4200.
[12] D. J. Ecobichon, " Carbamate Insecticides" en "Handbook of Pesticide Toxicology", 2a Ed., Academic Press (2001). P 1087.
[13] N.C. Rawlings, S.J. Cook, D.N.C Waldbillig, *J. Toxicol. Environ. Health* 54 (1998) 21.

2.3. DISTRIBUCIÓN Y TRANSPORTE EN EL MEDIOAMBIENTE

La distribución y el tranporte de los CRBs desde el lugar donde se han empleado hasta llegar a afectar a diversos ecosistemas dependen de sus propiedades físico-químicas, pero también del lugar donde se han empleado. En la Figura 2 se muestran las principales vías de contaminación por plaguicidas, asi como los procesos de transporte y de transformación que pueden sufrir los mismos.

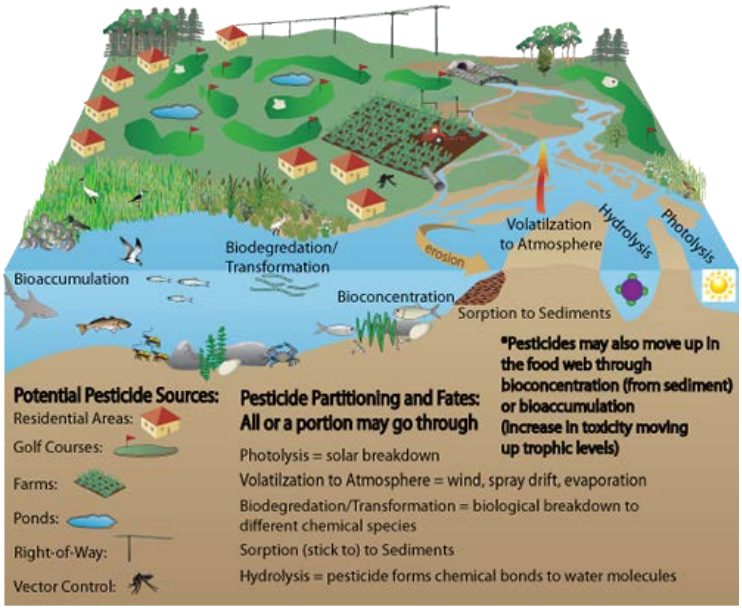


Figura 2. Contaminación por residuos de plaguicidas (fuentes y ecosistemas afectados) [14].

En general, la presión de vapor de los CRBs es baja. Sin embargo, pueden evaporarse o sublimar lentamente a temperatura ambiente, lo que puede dar lugar a la volatilización de los CRBs contenidos en el agua o el suelo. En cualquier caso, la distribución por vía aérea es minoritaria, mientras que el agua es una importante ruta de distribución para los CRBs muy solubles en ésta. La absorción de luz característica de los CRBs contribuye a su rápida

[14] http://sccoastalpesticides.org/knowledgebase/pesticide_movement_in_the_environment.php

descomposición (por fotodegradación o fotodescomposición) en medio acuoso. Por tanto, el riesgo de contaminación a largo plazo por parte de los CRBs es pequeño [15].

Los CRBs con propiedades insecticidas se emplean fundamentalmente en tratamientos agrícolas, por lo que pueden alcanzar el suelo, mientras que los nematocidas y herbicidas se aplican directamente sobre éste. Hay varios factores que pueden influenciar la biodegradación de los CRBs en el suelo, como su volatilidad, el tipo de suelo, la humedad de éste, adsorción, pH, temperatura y fotodescomposición. Ya que los distintos CRBs presentan a su vez propiedades diferentes, cada uno de ellos deberá ser evaluado individualmente, sin poder extrapolar los resultados de unos a otros. Así, mientras algunos CRBs se descomponen con facilidad, otros pueden ser fuertemente adsorbidos en el suelo o pasar fácilmente del suelo al agua, y en este proceso el tipo de suelo y la solubilidad del CRB juegan un papel muy importante. Más aún, hay que tener en cuenta que todo lo anterior no sólo es aplicable al CRB, sino también a sus productos de degradación o metabolitos, ya que debido a los procesos de biotransformación, se generan en muchos casos metabolitos tóxicos con capacidad de acumulación similar a la del compuesto original [15-17].

Las condiciones medioambientales que favorecen el crecimiento de microorganismos también favorecen la degradación de los CRBs. El primer paso en la degradación metabólica de los CRBs en suelo es su hidrólisis, y los productos de esta hidrólisis serán posteriormente metabolizados en el sistema suelo-planta por la acción de microorganismos, plantas y animales o por ruptura en el agua o suelo. Así, los microorganismos del suelo son capaces de metabolizar (hidrolizar) CRBs y pueden adaptarse fácilmente a la metabolización de diversos tipos de CRBs. Sin embargo, los CRBs y sus metabolitos pueden, a dosis elevadas, afectar a la microflora y causar cambios que pueden afectar a la productividad del suelo [15].

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[15] Environmental Health Criteria 64, Carbamate Pesticides: a General Introduction, World Health Organization, Geneva, International Programme on Chemical Safety, (1986), http://www.inchem.org/documents/ehc/ehc/ehc64.htm#subsectionnumber:1.1.7.

[16] S. Morais, E. Dias, M.L. Pereira "Carbamates: Human Exposure and Health Effects" en "The Impact of Pesticides", 1a Ed., AcademyPublish.org (2012) p.21.

[17] J.L. Martínez Vidal, P. Plaza-Bolaños, R. Romero-González, A. Garrido Frenich, *J. Chromatogr. A* 1216 (2009) 6767.

Aunque los CRBs no son muy estables en medios acuosos, y no persisten durante mucho tiempo en el ambiente, pueden producirse efectos bioacumulativos en peces, debido a que el metabolismo en estos animales es muy lento. Así, algunos CRBs se metabolizan rápidamente sin efectos acumulativos, mientras que otros son altamente tóxicos para invertebrados y peces [15].

3. INTERÉS Y LEGISLACIÓN SOBRE EL CONTROL DE RESIDUOS DE PLAGUICIDAS EN ALIMENTOS

La utilización de los plaguicidas en las cosechas puede conllevar la presencia de residuos, (esto es, restos de la utilización de un producto fitosanitario, incluidos sus metabolitos y los productos resultantes de su degradación o reacción) en los alimentos derivados. Los plaguicidas son indudablemente uno de los grupos de compuestos químicos mejor regulados y controlados en la sanidad alimentaria. Así, la Agencia Española de Seguridad Alimentaria y Nutrición (AESAN) incluye a los plaguicidas en los factores de peligro para la seguridad alimentaria, siendo factor de peligro todo agente biológico, químico o físico presente en un alimento o un pienso o toda condición biológica, química o física de un alimento o de un pienso que pueda causar un efecto perjudicial para la salud [18].

No obstante, a pesar de los estrictos controles ejercidos por las diversas autoridades, surgen permanentemente nuevas situaciones de alarma alimentaria y social que tienen importantes implicaciones en el comercio internacional. En la Unión Europea (EU), una herramienta clave para reaccionar con rapidez ante los incidentes registrados en el ámbito de los alimentos y los piensos es el RASFF (sistema de alerta rápida para alimentos y piensos), que permite que las diferentes organizaciones intercambien información de forma rápida y eficiente en caso de detectarse un riesgo para la salud y evitar así que perjudique a los consumidores. Como se ve en la Figura 3, el número de alertas relacionadas con plaguicidas ha ido aumentando en los últimos años, lo que pone de manifiesto la problemática que presentan estos compuestos en la actualidad.

[18] http://www.aesan.msc.es/

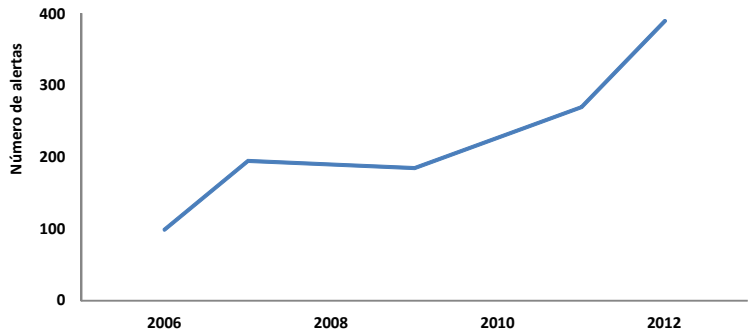

Figura 3. Número de alertas relacionadas con plaguicidas en los ultimos años [19].

A la vista de este problema, en 1979 se introdujeron por primera vez en la EU actos legislativos y medidas vigentes en relación con los plaguicidas, y desde entonces han ido evolucionando considerablemente, hasta culminar en 1991 con la adopción de una directiva sobre la comercialización de productos fitosanitarios [20].

Asimismo, con el fin de garantizar que la utilización de estas sustancias químicas es segura para los consumidores, se han establecido límites máximos de residuos (MRLs) para diversos plaguicidas en diferentes alimentos, definidos como la concentración máxima de residuos de un plaguicida (expresada en mg kg^{-1}) [21], recomendada por la Comisión del Codex Alimentarius, para que se permita legalmente su uso en la superficie o la parte interna de productos alimenticios para consumo humano y de piensos.

Los MRLs no son límites toxicológicos, sino que son límites toxicológicamente aceptables, basados en una buena práctica agrícola y que representan la cantidad máxima de un

[19] http://ec.europa.eu/food/food/rapidalert/docs/rasff_annual_report_2011_en.pdf.
[20] Directiva 91/414/CEE del Consejo, de 15 de julio de 1991, relativa a la comercialización de productos fitosanitarios Diario Oficial n° L 230 de 19/08/1991 p. 0001 – 0032.
[21] Reglamento (CE) n° 396/2005 del Parlamento Europeo y del Consejo, de 23 de febrero de 2005 relativo a los límites máximos de residuos de plaguicidas en alimentos y piensos de origen vegetal y animal y que modifica la Directiva 91/414/CEE del Consejo Diario Oficial n° L 070 de 16/03/2005 p. 0001 – 0016.

residuo que es posible encontrar en un producto alimentario de origen vegetal como consecuencia del uso legal y racional de ese plaguicida evaluado. Según la AESAN [22]:

- Los MRLs no son límites toxicológicos porque no representan la cantidad máxima de esa sustancia activa que puede ser perjudicial para la salud de los consumidores. Es decir, la superación de un MRL no implica necesariamente la existencia de un riesgo para la salud.
- Los MRL son toxicológicamente aceptables porque su cumplimiento asegura que no producen efectos tóxicos en los individuos, ni a corto ni a largo plazo.

La EU, a través de un reglamento comunitario de 2005 fijó los MRLs de sustancias activas (incluidos plaguicidas) que pueden aparecer en piensos y alimentos [21]; de forma periódica este reglamento ha sido sufriendo modificaciones en forma de anexos incluyendo nuevas sustancias que han de ser controladas en diferentes productos [23]. Como consecuencia de esta armonización de los MRLs en todo el territorio de la EU, se ha logrado paralelamente la eliminación de las barreras comerciales de los productos vegetales.

Además, como consecuencia de esta legislación podemos decir que en la EU no pueden autorizarse sustancias activas de productos fitosanitarios a menos que se haya probado científicamente antes que:

- No produzcan efectos perjudiciales en los consumidores, los agricultores ni terceros, y
- No provoquen efectos inaceptables en el medio ambiente, y
- Sean suficientemente eficaces.

[22]http://www.aesan.msssi.gob.es/AESAN/web/cadena_alimentaria/subdetalle/que_son_LMR.shml
[23] http://www.aesan.msc.es/AESAN/web/legislacion/subdetalle/residuos_plaguicidas.shtml

4. INTERÉS Y LEGISLACIÓN SOBRE EL CONTROL DE RESIDUOS DE PLAGUICIDAS EN EL MEDIOAMBIENTE

Hasta mediados del siglo XX el problema de la contaminación del agua para consumo humano se reducía a la presencia de microorganismos causantes de enfermedades infecciosas. En la actualidad y debido a las actividades agroindustriales, este problema se ha visto claramente incrementado debido a la contaminación por plaguicidas. Los agricultores usan los plaguicidas para mantener o mejorar el rendimiento y la calidad de las cosechas de un cultivo determinado. Las autoridades públicas los utilizan, por ejemplo, para eliminar las malas hierbas a lo largo de las autopistas o las vías del ferrocarril. También se utilizan en el sector privado, por ejemplo, en instalaciones recreativas, en casas y jardines. Si se utilizan mal o en exceso, los plaguicidas pueden contaminar el agua, la atmósfera y el suelo. Pueden ser nocivos para organismos distintos del que se pretende atacar y perjudicar así a la naturaleza, contribuyendo a la pérdida de biodiversidad. Además, la contaminación por sustancias químicas puede originar efectos adversos en la salud humana después de periodos prolongados de exposición, siendo de particular importancia los tóxicos que son acumulativos y los carcinógenos. Por otra parte, pueden entrar en la cadena alimentaria y provocar efectos desfavorables en la salud humana [16,24].

A este respecto, en la UE la Directiva marco del agua 2000/60/ CE [25] nace como respuesta a este problema, y en ella se establecen las bases de las políticas en materia de gestión de aguas aplicadas en todo el marco europeo. Los objetivos de esta directiva son:

- La prevención del deterioro adicional y la protección y mejora de los ecosistemas acuáticos, así como de los ecosistemas terrestres dependientes.
- La promoción de los usos sostenibles del agua.
- La protección y mejora del medio acuático.

[24] M. Amanullah, B.Y. Hari, *Indian J. Cancer* 48 (2011) 74.

[25] Directiva 2000/60/CE del Parlamento Europeo y del Consejo, de 23 de octubre de 2000, por la que se establece un marco comunitario de actuación en el ámbito de la política de aguas. *Diario Oficial* n° L 327 de 22/12/2000 p. 0001 – 0073.

- La reducción de la contaminación de las aguas subterráneas.
- La paliación de los efectos de inundaciones y sequías.

En este sentido la propia Directiva establece un propósito ambiental claro: para el año 2015 debe de haberse alcanzado el buen estado de los ecosistemas acuáticos de todos los países de la Unión, para lo cual será preciso desarrollar y aplicar planes de gestión que garanticen este objetivo.

También se deben destacar la Directiva 98/83/CE relativa a relativa a la calidad de las aguas destinadas al consumo humano [26], y la Directiva 2006/118/CE relativa a la protección de las aguas subterráneas contra la contaminación y el deterioro [27], donde se establece que la concentración de un plaguicida individual no puede superar los 0.1 μg l^{-1} mientras que la suma de plaguicidas totales no puede exceder de 0.5 μg l^{-1}.

5. MÉTODOS ANALÍTICOS PARA LA DETERMINACIÓN DE CARBAMATOS EN ALIMENTOS Y MUESTRAS MEDIOAMBIENTALES

A la vista de la problemática expuesta, se hace patente la necesidad de desarrollar métodos analíticos sensibles y selectivos para la determinación de plaguicidas (incluidos los CRBs) en muestras tanto alimentarias como medioambientales.

Existen diversos métodos recomendados para el análisis de CRBs en diversas muestras, como la determinación de ditiocarbamatos en alimentos mediante cromatografía de gases (GC) acoplada a un detector de captura de electrones [28] o el propuesto por diversas organizaciones incluida la EPA, para la determinación de metilcarbamatos en muestras de agua de diversa procedencia, que emplea cromatografía líquida (HPLC) acoplada con

[26]Directiva 98/83/CE del Consejo de 3 de noviembre de 1998 relativa a la calidad de las aguas destinadas al consumo humano Diario Oficial n° L 330 de 05/12/1998 p. 0032 - 0054

[27]Directiva 2006/118/CE del Parlamento Europeo y del Consejo, de 12 de diciembre de 2006 , relativa a la protección de las aguas subterráneas contra la contaminación y el deterioro Diario Oficial n° L 372 de 27/12/2006 p. 0019 - 0031

[28] http://www.crl-pesticides.eu/library/docs/srm/meth_DithiocarbamatesCs2_EurlSrm.PDF

detección fluorescente [29], basado en el método desarrollado por de Kok y col. [30]en el que se requiere derivatización post-columna. No obstante, en cuanto a los métodos de análisis para la determinación de residuos de plaguicidas en alimentos, la Decisión 2002/657/CE [31] y los criterios técnicos relativos a la validación de los métodos de ensayo y al aseguramiento de la calidad de los resultados reflejados en la guía SANCO/12495/2011 que entró en vigor en enero de 2012 [32] no establecen obligación de usar métodos normalizados en dicho control. Sin embargo, se establecen distintos criterios de funcionamiento y requisitos que deben cumplir los métodos analíticos, en cuanto a manipulación de muestras, veracidad (siempre que se pueda se utilizarán materiales de referencia certificados y cuando no se disponga de ellos se valorará mediante recuperación de adiciones de cantidades conocidas de analitos a una matriz en blanco, estableciéndose valores mínimos de veracidad), especificidad (se analizarán muestras en blanco para detectar posibles interferencias y valorar sus efectos) o criterios y requisitos de confirmación de los residuos y contaminantes de los métodos de cuantificación y cribado (puntos de identificación mediante espectrometría de masas (MS) u otros requisitos en los casos de otros sistemas de detección como UV-Vis, infrarrojos, etc.). Así, en el caso de requerir confirmación inequívoca de los residuos encontrados, se indica que la detección por MS es la adecuada tras la separación cromatográfica, empleándose un sistema de puntos de identificación para interpretar los datos, basado en el uso de la MS mediante análisis de las masas moleculares (en baja y alta resolución) de cada compuesto y de los fragmentos obtenidos en la fragmentación molecular producida en el detector cuando se trabaja con MS/MS.

HPLC es la técnica más ampliamente usada para la determinación de CRBs en alimentos y muestras medioambiantales empleando como sistemas de detección la espectrofotometría

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[29] EPA Method 531.1, Measurement of N-methylcarbamoyloximes and N-methylcarbamates in water by direct aqueous injection HPLC with post column derivatisation, Revision 3.1, edited 1995.
[30] A. de Kok, M. Hiemstra, C.P. Vreeker, *Chromatographia* 24 (1987) 469.
[31] 2002/657/CE: Decisión de la Comisión, de 12 de agosto de 2002, por la que se aplica la Directiva 96/23/CE del Consejo en cuanto al funcionamiento de los métodos analíticos y la interpretación de los resultados, Diario Oficial n° L 221 de 17/08/2002 p. 0008 - 0036
[32] Method validation and quality control procedures for pesticide residues analysis in food and feed. European Commission 2011, SANCO/12495/2011.

UV-Vis [33-36], fluorescencia [37-39], MS [40-42] y en menor medida la detección quimiolumiscente [43,44]. Algunos artículos de revisión recopilan los diferentes métodos propuestos para la determinación de CRBs en muestras medioambientales [45,46] y en alimentos [47].

En la presente Tesis Doctoral se han propuesto nuevos métodos de análisis que emplean técnicas separativas alternativas a la HPLC convencional, como son las técnicas miniaturizadas y de ultra resolución, que se comentan a continuación.

5.1. APLICACIÓN DE TÉCNICAS MINIATURIZADAS EN LA DETERMINACIÓN DE CARBAMATOS EN ALIMENTOS Y MUESTRAS MEDIOAMBIENTALES

Las técnicas separativas miniaturizadas han cobrado gran relevancia en los últimos tiempos debido a las ventajas que presentan, tales como el incremento en la resolución o una mejora en la sensibilidad y notable reducción del consumo de disolventes. Igualmente es de destacar los pequeños volúmenes de muestras requeridos, lo que constituye una gran ventaja, fundamentalmente en el ámbito biomédico [48]. En general, dentro de los sistemas miniaturizados se engloban actualmente los que utilizan columnas de pequeño diámetro interno o capilares de sílice fundida y aquellos en los que la separación se realiza en microchips. En la presente Tesis Doctoral se han utilizado dos técnicas pertenecientes al

[33] Z. Liu, W. Liu, H. Rao, T. Feng, C. Li, C. Wang, Z. Wang, *Int. J Environ. Anal. Chem*. 92 (2012) 571.
[34] X. Liu, W. Feng, C. Bao, Q. Jia, *Anal. Lett.* 45 (2012) 2663.
[35] G. Zhao, C. Wang, Q. Wu, Z. Wang, *Anal. Methods* 3 (2011) 1410.
[36] K. Farhadi, M.A. Farajzdeh, A.A. Matin, *J. Sep. Sci.* 32 (2009) 2442.
[37] M. Asensio-Ramos, J. Hernández-Borges, G. González-Hernández, M.A. Rodríguez-Delgado, *Electrophoresis* 33 (2012) 2184.
[38] F. Koc, Y. Yogit, Y.K. Das, Y. Gurel, C. Yarali, *J. Food Drug Anal*. 16 (2008) 39.
[39] C. Sanchez-Brunete, B. Albero, J.L. Tadeo, *J. Food Protection* 67 (2004) 2565.
[40] Y. Picó, D. Barceló, *TrAC Trends Anal. Chem*. 28 (2008) 40.
[41] F. Boujelbane, F. Oueslati, N. Ben-Hamida, *Desalination* 250 (2010) 473.
[42] J.M.F. Nogueira, T. Sandra, P. Sandra, *J. Chromatogr. A* 996 (2003) 133.
[43] E. Orejuela, M. Silva, *J. Chromatog. A* 1007 (2003) 197.
[44] J.F. Huertas-Pérez, A.M. García-Campaña, *Anal. Chim. Acta* 194 (2008) 630.
[45] J.M. Soriano, B. Jiménez, G. Font, J.C. Molto, *Crit. Rev. Anal. Chem.* 31 (2001) 19.
[46] Y. Santaladchaiyakit, S. Srijaranai, R. Burakham, *J.Sep. Sci.* 35 (2012) 2373.
[47] C. Goranka, S. Wolfgang, *TrAC Trends Anal. Chem.* 28 (2008) 40.
[48] M. Szumski, B. Buszewski, *Crit. Rev. Anal. Chem*. 32 (2002) 1.

primer grupo: la electroforesis capilar (CE) y la HPLC capilar, que pasan a comentarse brevemente.

5.1.1. ELECTROFORESIS CAPILAR

Son numerosas las referencias bibliográficas relacionadas con la CE, donde se pueden consultar sus fundamentos, instrumentación, diferentes modalidades y aplicaciones [49-54]. En esta introducción, se dará sólo una vision general de la CE, así como de las diferentes vias para mejorar la sensibilidad de esta técnica miniaturizada.

La CE presenta como principales ventajas una elevada eficacia, tiempos de separación cortos, y volúmenes de muestras y disolventes muy pequeños ya que se emplean capilares muy estrechos (25-150 µm de diametro interno), por lo que el coste y la contaminación ambiental disminuyen considerablemente en comparación con la HPLC convencional (flujos de ml min^{-1}).

Existen diversos modos de separación en CE, entre los que se encuentran la electroforesis capilar en zona (*capillary zone electrophoresis*, CZE), la cromatografía capilar electrocinética micelar (*micellar electrokinetic chromatography*, MEKC), electroforesis capilar en gel (*capillary gel electrophoresis,* CGE), isotacoforesis capilar (*capillary isotacophoresis,* CITP), enfoque isoeléctrico capilar, (*capillary isoelectrofocusing*, CIEF), y electrocromatografía capilar (*capillary electrochromatography*, CEC). En la Figura 4 se muestra un esquema de las diferentes modalidades en CE.

[49] C. Cruces Blanco, en "Electroforesis Capilar", 1ª Ed., Universidad de Almería (1998).
[50] W. Th. Kok, *Chromatographia Supplement* 51 (2000) 1.
[51] H.H. Lauer, G.P. Rozing, en "High Performance Capillary Electrophoresis", 2ª Ed, Agilent Tecnhologies (2010).
[52] J.L. Felhofer, L. Blanes, C. D. García, *Electrophoresis* 31 (2010) 2469.
[53] P. Camilleri, en "Capillary Electrophoresis: Theory and Practice", 2ª Ed., CRC PressINC (1998).
[54] K.D. Altria en "Capillary Electrophoresis Guidebook: Principles, Operation, and Applications", 2ª Ed. Humana Press (2010).

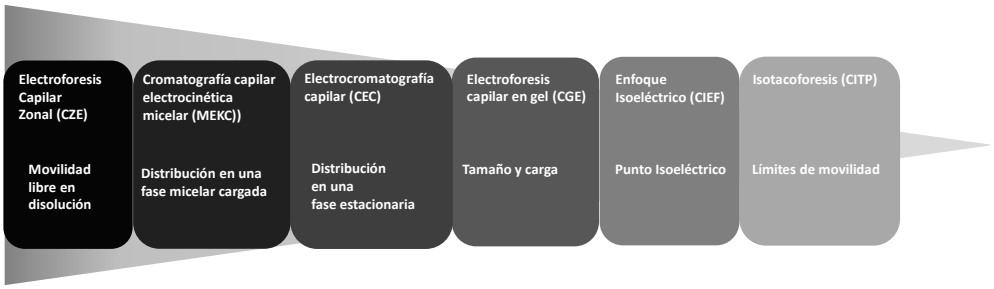

Figura 4. Modos de operación en CE.

En esta Tesis Doctoral, la modalidad utilizada ha sido la MEKC. En ella, el uso de disoluciones micelares de surfactantes iónicos a concentraciones superiores a la concentración micelar crítica (CMC) [55] permite la separación de moléculas tanto cargadas como neutras como es el caso de los CRBs, que como se comentó anteriormente, son compuestos neutros en un amplio rango de pHs. En MEKC, existen dos fases, la acuosa y la micelar (fase pseudoestacionaria), ambas en movimiento; la separación se basa en las diferencias en la interacción de cada uno de los analitos con las micelas presentes en la disolución tampón. Se trata de un modo de separación que combina el mecanismo de separación de la cromatografía (las micelas actúan como fase pseudoestacionaria) con los movimientos electroforéticos y electroosmótiocos de los solutos y las disoluciones.

Esta modalidad de MEKC se ha empleado en esta Tesis Doctoral acoplada con dos sistemas de detección diferentes: UV-Vis y MS, por lo que a continuación se comentarán las características principales de estos acoplamientos.

5.1.1.1. CE-UV-VIS

Este es el modo de detección más comúnmente empleado en CE y que presentan la mayoría de los equipos comerciales. La utilización de un detector de diodos en fila (DAD) en lugar de la detección por única o múltiple longitud de onda supone muchas ventajas, como son: visualización del espectro UV-Vis en todo momento del análisis, obtención del electroferograma a cualquier longitud de onda en una sola inyección, determinación del

[55] M. Silva, Electrophoresis 34 (2013) 141.

máximo de absorbancia para todos los analitos, identificación de compuestos y determinación de la pureza de pico.

Sin embargo, el principal inconveniente que presenta la aplicación de la CE-UV-Vis en la determinación de residuos de contaminantes radica en la necesidad de una elevada sensibilidad, lo cual contrasta con la limitación de esta técnica, debido a los bajos volúmenes de inyección introducidos y a la baja sensibilidad de esta detección al usar el capilar como celda de detección. Para solventar este inconveniente se han propuesto distintas aproximaciones para aumentar la sensibilidad, siendo las más comunes:

- Uso de tratamientos de muestra para preconcentrar la muestra.
- Mejora del camino óptico.
- Técnicas de preconcentración *on-line*.

La primera de ellas supone el desarrollo de métodos de extracción y limpieza que permitan aumentar la concentración de analito en el extracto final. Estos métodos se comentarán en un apartado posterior.

En cuanto a la segunda aproximación, la dependencia de la absorbancia con el camino óptico, según la ley de Lambert-Beer, hace que la sensibilidad esté limitada como consecuencia del pequeño camino óptico que el capilar ofrece. Esto hace que el detector tenga que ser cuidadosamente diseñado para enfocar la máxima luz en el capilar y para minimizar la luz dispersa que llega al monocromador.

Existen varias alternativas que permiten mejorar la sensibilidad mediante el aumento del camino óptico (Figura 5), consistentes en el uso de iluminación axial en lugar de perpendicular, utilizando una celda de flujo de alta detección o el uso de capilares con camino óptico extendido (capilares de burbuja), en los que el diámetro interno está aumentado de 3 a 5 veces en el punto de detección, sin que esto implique un aumento en la difusión del analito. En los capilares burbuja se crea una región expandida en el interior del capilar, de manera que es el diámetro interno el que se modifica localmente pero no el externo. Dado que la burbuja se localiza solamente en la región de detección, no se produce incremento alguno de la corriente. Sin embargo, en dicha zona se produce una disminución local de la velocidad de flujo y de la resistencia eléctrica y, por tanto, del

campo eléctrico, disminuyendo así la velocidad de migración de los analitos. En la celda de flujo de alta detección, por su parte, lo que se hace es cambiar el sentido de la radiación sobre el capilar, deformándolo en la región de detección formando una zeta y haciendo incidir la luz longitudinalmente; en este caso, el volumen interno del capilar no se modifica. El uso de capilares burbuja esta bastantante extendido ya que para su empleo, no se precisa ningún cambio en las condiciones electroforéticas (siempre y cuando la resolución entre los analitos sea aceptable). Por el contrario el uso de celdas de flujo de alta detección no se ha generalizado, dado que su manejo a veces no es sencillo (ya que consiste en una unión entre varios capilares que requiere que el operador tenga bastante experiencia para conseguir que la corriente sea estable) y, aunque el incremento de sensibilidad obtenido es apreciable, la resolución y la eficacia de los picos pueden verse afectadas de manera importante.

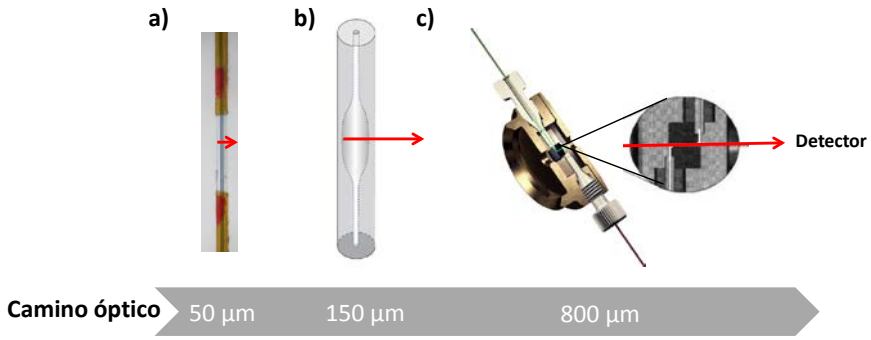

Figura 5. (a) Ventana de detección típica en CE, **(b)** capilar burbuja y **(c)** celda de flujo de alta detección.

Como se ha comentado anteriormente, la tercera aproximación para aumentar la sensibilidad es con técnicas que, al introducir la muestra de un modo determinado, producen un efecto de concentración *"on-column"*, permitiendo además el uso de volúmenes mayores de muestra y mejorando la relación señal/ruido. Dentro de estas técnicas, la más simple y popular es la conocida como apilamiento o *"stacking"* [56-58]

[56] A. Šlampová, Z. Malá, P. Pantůčková, P. Gebauer, P. Boček, *Electrophoresis* 34 (2013) 3.
[57] H.Y. Xie, Y.Z. He, W.E. Gan, G.N. Fu, L. Li, F. Han, Y. Gao, *J. Chromatogr. A* 1216 (2009) 3353.

cuya modalidad más usual consiste en disolver la muestra en agua pura, tampón diluido o disolvente orgánico y colocarla entre dos porciones del tampón empleado en la separación. Esta zona de muestra tendrá una fuerza iónica notablemente inferior al resto del capilar. Cuando se aplica un voltaje elevado a lo largo del capilar, la fuerza del campo eléctrico es considerablemente más alta en la zona de la muestra. Como consecuencia, los iones de la muestra se mueven rápidamente hasta que llegan al tampón donde su velocidad cae debido a la diferencia de fortaleza del campo eléctrico. Por tanto, la muestra es focalizada eléctricamente o apilada ("*stacked*"), y la concentración en esa zona del capilar aumenta.

Una variación de *stacking* que ha sido empleada en esta Tesis, es la denominada "*barrido"* o *"sweeping*", que se realiza en modo MEKC [59-61]. Dicho proceso consiste en disolver la muestra en un tampón de la misma composición que el tampón electroforético de fondo (BGE) pero exento de micelas. Con esta metodología se consigue focalizar los analitos en una banda muy estrecha dentro del capilar, aumentando así el volumen de muestra que se puede inyectar sin ninguna pérdida de eficacia. Este fenómeno implica la acumulación de los analitos cargados y neutros en la fase pseudoestacionaria que penetra en la zona de muestra (BGE libre de micelas), produciendo un efecto de enfoque de los analitos. El esquema básico de este tipo de preconcentración *on-line* se muestra en la Figura 6.

[58] M.C. Breadmore, A.I. Shallan, H.R. Rabanes, D. Gstoettenmayr, A.S. Abdul Keyon, A. Gaspar, M. Dawod, J.P. Quirino, *Electrophoresis* 34 (2013) 29.
[59] J.P. Quirino, S. Terabe, *Science* 282 (1998) 465.
[60] M. Silva, *Electrophoresis* 30 (2009) 50.
[61] A.T. Aranas, M. Guidote, J.P. Quirino, *Anal. Bioana.l Chem.* 394 (2009) 175.

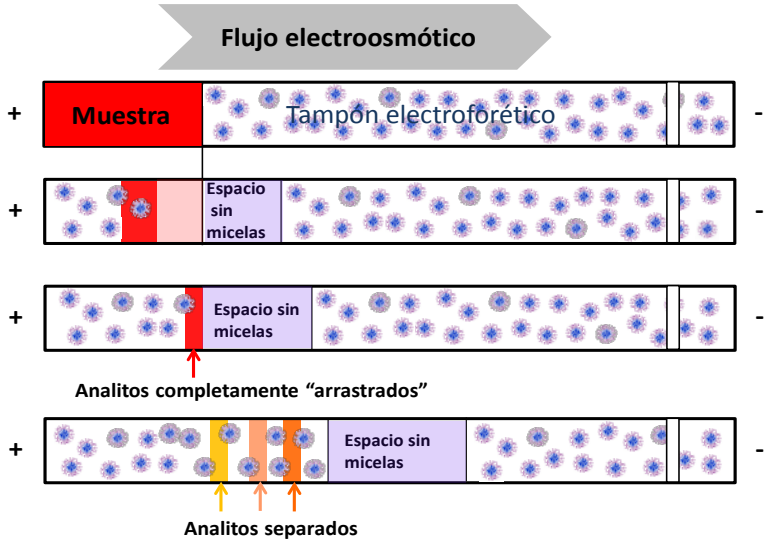

Figure 6. Principio básico de la preconcentración *on-line* mediante *sweeping*.

En cuanto a las aplicaciones desarrolladas para la determinación de CRBs, se han propuesto diversos métodos empleando CZE con detección amperometrica [62] y UV-Vis [57,63], aunque la MEKC con detección UV-Vis ha sido la más empleada debido a su capacidad para separar CRBs neutros y cargados [64- 69]. También se han planteado diversas aproximaciones de preconcentración *on-line*, como la determinación de CRBs en zanahorias y aguas empleando diferentes técnicas de *stacking* [66], en manzana

[62] X. Cheng, Q. Wang, S. Zhang, W. Zhang, P. He, Y. Fang, *Talanta* 71 (2007) 1083.
[63] E. Rodríguez-Gonzalo, J. Domínguez-Álvarez, L. Ruano-Miguel, R. Carabias-Martínez, *Electrophoresis* 29 (2008) 4066.
[64] A. Segura-Carretero, C. Cruces-Blanco, S. Cortacero-Ramírez, A. Carrasco-Pancorbo, A. Fernández-Gutiérrez, *J. Agric. Food Chem*. 52 (2004) 5791.
[65] L.M. Ravelo-Pérez, J. Hernández-Borges, T.M. Borges-Miquel, M.A. Rodríguez-Delgado, *J. Chromatogr. A* 1185 (2008) 151.
[66] C.L. da Silva, E.C. de Lima, M.F.M. Tavares, *J. Chromatogr. A* 1014 (2003) 109.
[67] S. Zhang, C. Li, S. Song, T. Feng, C. Wang, Z. Wang, *Anal. Methods* 400 (2010) 54.
[68] A. Juan-García, G. Font, Y. Picó, *J. Chromatogr. A* 1153 (2007) 104.
[69] M. Molina, D. Perez-Bendito, M. Silva, *Electrophoresis* 20 (1999) 3439.

empleando *sweeping* [67], en diferentes frutas y vegetales empleando diferentes técnicas de *stacking* incluida el *sweeping* [68] y en zumos empleando *sweeping* [70].

5.1.1.2. CE-MS

La MS ha cobrado una importancia cada vez mayor como método de detección y cuantificación en CE. El acoplamiento de un detector tan selectivo con una técnica tan versátil y de eficacias tan elevadas, hace que la CE-MS sea una potente herramienta que presenta importantes perspectivas en su aplicación en calidad y seguridad alimentaria [71-73] proporcionando una segunda dimensión en la separación, ya que los analitos además de separarse por su relación carga/tamaño son detectados por su relación masa/carga. La CE-MS se ha afianzado como técnica analítica gracias al desarrollo de instrumentación que ha solventado el principal inconveniente de este acoplamiento: la compatibilidad entre la CE, que trabaja con muestras líquidas, con la MS que trabaja con muestras en estado gaseoso. Además, dicho acoplamiento debe solucionar una segunda dificultad como es el cierre del circuito eléctrico en el que trabaja todo equipo de CE.

Así, se han desarrollado interfases adecuadas que trabajan de forma satisfactoria para este acoplamiento, como son el plasma de acoplamiento inductivo (se emplea fundamentalmente para átomos metálicos y en menor medida para no metálicos), bombardeo de átomos rápidos de flujo continuo, ionización química a presión atmosférica (APCI), fotoionización a presión atmósferica (APPI), electrospray asistido neumáticamente e ionización por electrospray o electronebulización (ESI), siendo ésta última una de las más usadas [74].

La ESI ha revolucionado el campo de los acoplamientos entre técnicas de separación que trabajan en fase líquida y MS para el análisis de biomoléculas polares, no volátiles y térmicamente lábiles, en parte por su capacidad de formar iones mono- o multicargados

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[70] D. Moreno-González, L. Gámiz-Gracia, A.M. García-Campaña, J.M. Bosque-Sendra, *Anal. Bioanal. Chem.* 400 (2011) 1329.

[71] C. Simó, C. Barbas, A. Cifuentes, *Electrophoresis* 26 (2005) 1306.

[72] L. Ravelo-Pérez, M. Asensio-Ramos, J. Hernández-Borges, M.A. Rodríguez-Delgado, *Electrophoresis* 30 (2009) 1624.

[73] M. Castro-Puyana, V. Garcia-Canas, C. Simó, A. Cifuentes, *Electrophoresis* 33 (2012) 147.

[74] J. Hernández-Borges, C. Simó, A. Cifuentes, *Cromatografía y Técnicas Afines* 24 (2003) 45.

[75,76]. El proceso de formación del electrospray puede describirse de forma sencilla: Se establece una diferencia de potencial entre el final del capilar electroforético y la entrada del MS (±1-5 kV). La formación del electrospray se lleva a cabo a presión atmosférica, intervieniendo diversos mecanismos al mismo tiempo (Figura 7). El primer paso en la creación de iones aislados es la formación de un cono de líquido ("cono de Taylor") en la punta de la aguja de la interfase, desde donde salen microgotas cargadas; es entonces cuando la muestra, procedente del capilar, se carga y se dispersa simultáneamente. El disolvente se va evaporando de las microgotas, aumentando su densidad de carga eléctrica, de modo que los iones de la misma polaridad son atraídos hacia la superficie de la misma debido a la diferencia de potencial existente. Cuando las fuerzas de repulsión electrostática de los iones son mayores que la tensión superficial que mantiene unidas las gotas en forma esférica ("límite de Rayleight") , las microgotas se van dividiendo en otras aún más pequeñas ("explosiones de Coulomb"), que seguirán sufriendo procesos de evaporación y explosión sucesivos hasta que finalmente se forman iones cargados que son transferidos al sistema óptico del MS, zona en que los iones serán tranportados y dirigidos hacia el analizador de masas y posteriormente al detector.

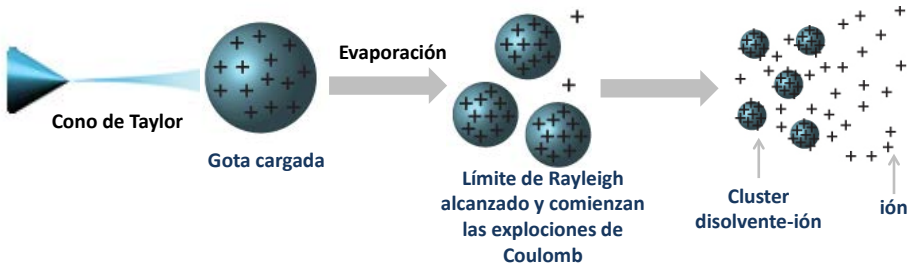

Figura 7. Esquema del proceso de formación del electrospray.

Hasta ahora, se han comentado las caractericticas de la ESI que pueden ser aplicadas tanto para LC como para CE. Pero para un acoplamiento CE-MS satisfactorio se tiene que tener en cuenta el método utilizado para establecer el cierre del circuito eléctrico a la salida del capilar. Para ello es necesario modificar el modelo convencional de CE en el cual los

[75] C. Simó, A. Cifuentes, en "Electroforesis capilar: aproximación según la técnica de detección", 1ª Ed., Universidad de Granada (2005).
[76] S. Banerjee, S. Mazumdar, *Int. J. Anal. Chem.* doi:10.1155/2012/282574.

extremos del capilar y los electrodos se encuentran sumergidos en una disolución conductora, normalmente la misma que se encuentra en el interior del capilar. Otro inconveniente que aparece es la incompatibilidad del espectrómetro de masas con algunos de los electrolitos usados en los diferentes modos de CE, ya que con su escasa volatilidad contribuyen a incrementar el ruido de fondo en la detección por MS, disminuyendo la sensibilidad, y llegando a obstruir y/o contaminar el sistema de detección en algunos casos [77].

El primer acoplamiento CE-MS a través de una interfase ESI fue desarrollado por Olivares y col. en 1987 [78]. En este acoplamiento, la conexión eléctrica se realizó mediante un recubrimiento de la punta del capilar con un metal conductor, siendo necesaria la presencia de flujo electroosmótico (EOF) para completar el circuito eléctrico. Era necesario por un lado un alto porcentaje de medio acuoso y por otro, una fuerza iónica adecuada en el tampón de separación. Aunque esta interfase es compatible con el pequeño volumen de líquido que fluye del capilar (0.1 -1 µl min^{-1}), el mantenimiento de un electrospray estable con un flujo inferior a 0.5 µl min^{-1} resultaba muy complicado, además de tener como desventaja que el tiempo de vida del recubrimiento métalico para cerrar el circuito era muy corto, con un máximo de 20 inyecciones [74].

En estos ultimos años se han desarrollado diferentes tipos de interfases para poder solventar este problema, que han dado lugar en la actualidad a tres modelos de interfase ESI para CE-MS:

- Interfase de unión líquida (*liquid-junction interface*)
- Interfase coaxial con flujo adicional (*coaxial liquid sheath-flow interface*)
- Interfase que no emplea flujo adicional (*sheathless interface*)

Las dos últimas interfases han sido evaluadas en la presente Tesis Doctoral, siendo sin duda la interfase coaxial con flujo adicional o *sheath-flow* la más empleada hasta la fecha para llevar a cabo el acoplamiento CE-MS. Esta interfase fue desarrollada por Smith y col. [79,80] y proporciona una mayor adaptación que el diseño comentado anteriormente, ya

[77] G.W. Somsen, R. Mol, G.J. de Jong, *J. Chromatogr. A* 1217 (2010) 3978.
[78] J.A. Olivares, N.T. Nguyen, C.R. Yonker, R.D. Smith, *Anal. Chem.* 59 (1987) 1230.
[79] R.D. Smith, C.J. Barinaga, H.R. Udseth, *Anal. Chem.* 60 (1988) 1948.
[80] C.G Edmonds, J.A. Loo, C.J. Barinaga, H.R. Udesh, R.D. Smith, *J. Chromatogr. A* 474 (1989) 21.

que al emplear un líquido adicional, los flujos de trabajos son superiores a 1 µl min^{-1} permitiendo la formación de un spray estable. Además, este flujo adicional permite el cierre del circuito eléctrico ya que la disolución se pone en contacto con un tubo metálico (conectado a tierra) que rodea el extremo final del capilar de separación con el electrolito de separación que proviene de la CE y que está fluyendo del interior del capilar. Esta interfase esta formada por tres tubos concéntricos (Figura 8): el primero de ellos es el propio capilar de separación que se encuentra rodeado por un tubo de acero inoxidable por el que se hace fluir el flujo adicional, y por un tercer tubo por el cual se introduce un gas de nebulización (geralmente N_2) que favorece la formación del electrospray. Estos tres tubos están situados en una cámara de nebulización, la cual se encuentra a una temperatura de entre 50 y 350 ^oC.

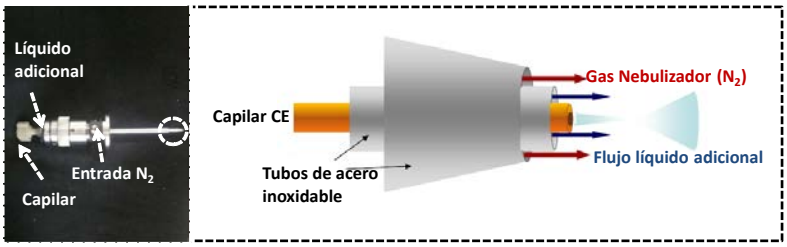

Figura 8. Esquema una interfase ESI con líquido adicional *(sheath-flow).*

Las principales limitaciones de este acoplamiento son la disminución de la sensibilidad debido a la dilución que provoca el empleo del líquido adicional. Además, hay que considerar multitud de parámetros que se han de tener en cuenta a la hora de optimizar un método CE-MS, como son la composición de dicho líquido, situación del capilar con respecto al tubo concéntrico que lo rodea, presión del gas de nebulización, etc., ya que van a influir tanto en la intensidad de la señal MS como en la calidad de la separación [81].

La segunda interfase que ha sido evaluada en esta Tesis Doctoral no requiere este líquido adicional (*sheathless*). Esta interfase fue desarrollada por Olivares y col. y fue la primera que se empleó para llevar a cabo el acoplamiento CE-MS en 1987 [78]; no obstante, como

[81] F.J. Lara, A.M. García-Campaña, F. Alés-Barrero, J.M. Bosque-Sendra, L.E. García-Ayuso, *Anal. Chem.* 78 (2006) 7665.

se ha comentado anteriormente, el principal problema de esta interfase era la degradación del recubrimiento metálico de la punta del capilar [82]. Con el fin de mejorar la estabilidad de spray y del recubrimiento metálico, se desarrollaron diferentes alternativas, como el empleo de recubrimientos de oro [83,84], plata [85], cobre [86], níquel [87] o grafito [88]. Otros dispositivos permitían el cierre del circuito eléctrico introduciendo un electrodo a la salida del capilar [89,90]. En otros acoplamientos la fuente de alto voltaje proporcionaba el campo eléctrico tanto para la separación en CE como para el electrospray, de modo que la posición del extremo final del capilar se ajustaba cerca del electrodo de entrada al espectrómetro de masas (1 mm) y el contacto eléctrico entre ambos se establecía a través del aire [91].

No obstante, todas estas alternativas no consiguieron solventar el problema del cierre del circuito eléctrico de forma eficiente. En estas interfases se producen procesos electroquímicos entre el electrodo y el electrolito de separación, que conducen a la formación de burbujas debido a las reacciones redox del agua en contacto con el electrodo, en oposición con lo que ocurre en las interfases de flujo coaxial en las que estas burbujas son expulsadas, no teniendo un impacto en la estabilidad eléctrica [51].

Finalmente, Moini publicó un nuevo enfoque para la interfase *sheathless* que evitaba los problemas relacionados con los procesos electroquímicos [92]. En esta interfase el recubrimiento de poliimida del capilar de separación de sílice fundida se retira y es atacado con fluoruro de hidrógeno hasta conseguir un diámetro externo de 80-90 micras. Gracias a

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[82] E.J. Maxwell, D.D.Y. Chen, *Anal. Chim. Acta* 627 (2008) 25.
[83] S. Nilsson, O. Klett, M. Svedberg, A. Amirkhani, L. Nyholm, *Rapid Commun. Mass Spectrom*. 17 (2003) 1535.
[84] Z. Kele, G. Ferenc, T. Klement, G.K. Toth, T. Janaky, *Rapid Commun. Mass Spectrom*. 19 (2005) 881.
[85] Y.R. Chen, G.R. Her, *Rapid Commun. Mass Spectrom*. 17 (2003) 437.
[86] A.D. Zamfir, N. Dinca, E. Sisu, J. Peter-Katalinic, *J. Sep. Sci.* 29 (2006) 414.
[87] L. Bendahl, S.H. Hansen, J. Olsen, *Rapid Commun. Mass Spectrom*. 16 (2002) 2333.
[88] Y.Z. Chang, G.R. Her, *Anal. Chem*. 72 (2000) 626.
[89] P. Cao, M. Moini, *J. Am. Soc. Mass Spectrom*. 8 (1997) 561.
[90] L. Fan, R. Zang, E.R. Williams, R.N. Zare, *Anal. Chem.* 66 (1994) 3696.
[91] M. Mazereeuw, J.P. Hofte, U.R. Tjaden, J. van der Greef, *Rapid Commun. Mass Spectrom*. 11 (1997) 981.
[92] M. Moini, Anal. Chem. 79 (2007) 4241.

este tratamiento con ácido, el extremo del capilar de separación se vuelve poroso, permitiendo la conexión entre el electrolito de separación con un líquido estancado que está dentro de un tubo concéntrico conectado a tierra. Por lo tanto, los procesos redox se producen fuera del capilar de separación y se evitan los problemas anteriormente citados.

Basándose en esta idea, Beckman Coulter (Brea, California, E.E.U.U.) ha desarrollado un prototipo llamado interfase *sheathless* de elevada sensibilidad con spray de punta porosa HSPS (*high-sensitivity porous sprayer*). Esta interfase HSPS ha sido aplicada de forma satisfactoria para el análisis de mezclas de péptidos [93,94], proteínas [95,96] y herbicidas [97], poniendo de manifiesto el gran potencial que presentaría para el acoplamiento CE-MS cuando sea comercializado, debido a las ventajas que ofrece como son la no dilución de la muestra y su fácil manejo. Un esquema de esta interfase se muestra en la Figura 9.

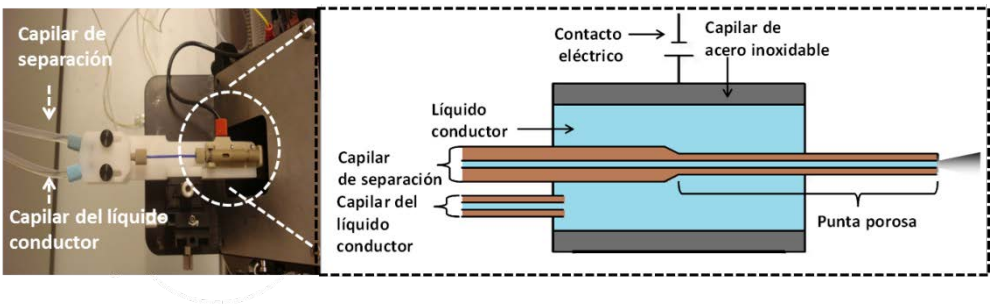

Figura 9. Esquema de una interfase ESI *sheathless*.

Sea cual sea la interfase empleada, una vez llevado a cabo el acoplamiento CE-MS donde se ha producido la transferencia de los iones desde la fase líquida procedente del capilar a la fase gaseosa, los iones son dirigidos hacia el analizador de masas. Estos analizadores permiten la caracterización con un grado de sensibilidad y selectividad muy elevado, ya que proporcionan información sobre la masa molecular de los analitos.

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[93] J.M. Busnel, B. Schoenmaker, R. Ramautar, A. Carrasco-Pancorbo, C. Ratnayake, J.S. Feitelson, J.D. Chapman, A.M. Deelder, O.A. Mayboroda, *Anal. Chem.* 82 (2010) 9476.
[94] K. Faserl, B. Sarg, L. Kremser, H. Lindner, *Anal. Chem.* 83 (2011) 7297.
[95] R. Haselberg, C.K. Ratnayake, G.J. de Jong, G.W. Somsen, *J. Chromatogr. A* 1217 (2010) 7605.
[96] R. Haselberg, G.J. de Jong, G.W. Somsen, *Anal. Chem.* 85 (2013) 2289.
[97] M. Kawai, Y. Iwamuro, R. Iio-Ishimaru, S. Chinaka, N. Takayama, K. Hayakawa, *Anal. Sci.* 27 (2011) 857.

Existe una variada gama de equipos de MS que pueden ser utilizados en los acoplamientos CE-MS: los cuadrupolos (Q), las trampas de iones (IT), los sectores magnéticos (EBE), los analizadores de tiempo de vuelo (TOF), así como sistemas híbridos como el Q-TOF. De todos ellos los más usados debido a que presentan menores dificultades técnicas son los Q y la IT, siendo esté último el que permite llevar a cabo fragmentaciones sucesivas de los iones seleccionados dando lugar a los espectros MS^n, muy importantes para la confirmación en el análisis de plaguicidas.

El analizador IT (empleado en esta Tesis) consiste fundamentalmente en un electrodo anular y dos electrodos laterales de geometría hiperbólica, que poseen una perforación que permite la entrada y la salida de los iones. Cuando los iones están dentro, se aplican diferentes voltajes generando un campo eléctrico tridimensional en la cavidad de la trampa. Este campo atrapa y concentra los iones dada su trayectoria de oscilación estable. La naturaleza de la trayectoria depende del potencial y de la relación m/z de los iones. Para llevar a cabo la determinación de las especies que entran o se forman en la trampa, los potenciales de los electrodos se alteran, sometiendo a los iones confinados a una rampa lineal de radiofrecuencia, de manera que son expulsados progresivamente en la dirección axial en función de su relación m/z, como resultado de desestabilizaciones de las trayectorias que mantienen dentro de la trampa. Una vez que estos iones llegan al detector, la señal se procesa y da lugar al espectro de masas.

En la Figura 10 puede verse el esquema del equipo empleado para llevar a cabo parte del desarrollo experimental de la presente memoria con sus distintas partes [98]. El equipo está constituido por la interfase (ESI en este caso, en sus dos variantes *sheath-flow* o *sheathless*), la zona de "transporte y convergencia" de iones formada por skimmers, octopolos y lentes, el analizador (IT) y el detector.

[98] Agilent Technologies en "Agilent 6300 Series Ion Trap Systems Concepts Guide", 3ª Ed., (2006) p.24.

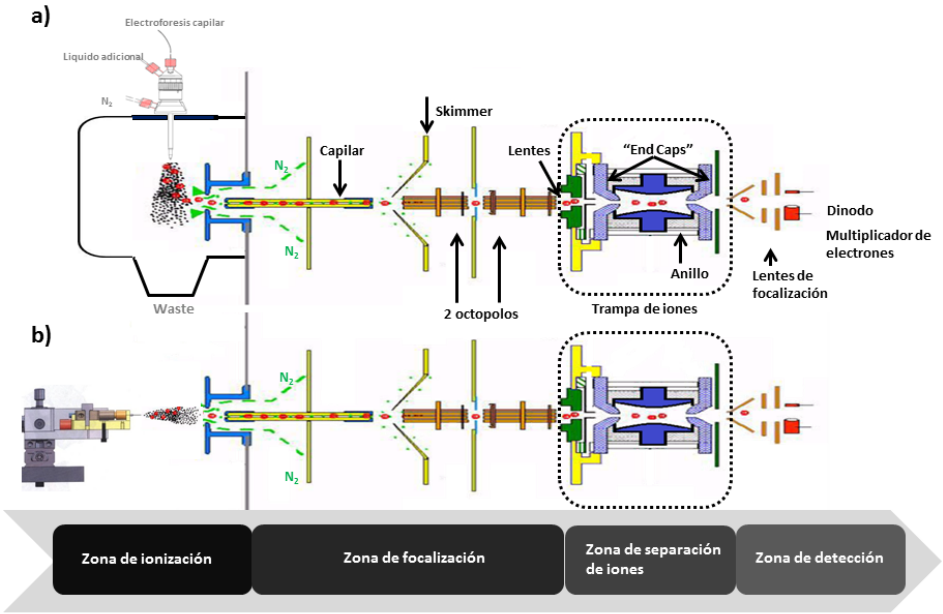

Figura 10. Esquema de un espectrómetro de masas ESI-IT: **(a)** interfase *sheath-flow* e **(b)** interfase *sheathless*.

En la primera zona se forma el spray (interfase) donde, como ya se comentó anteriormente, se nebuliza la disolución de la muestra y se ioniza a través de un proceso de desolvatación. La zona de transporte y focalización de iones posee cuatro zonas a alto vacío, originado por una serie de bombas que posee el sistema. Los iones pasan a esta zona a través de un capilar de vidrio: el skimmer elimina el volumen del gas de secado; a continuación pasan por los octopolos que los transportan y guían desde justo detrás del skimmer hasta el detector, atravesando una serie de lentes. Por último los iones entran en el analizador de IT, que los colecta y libera en función de su relación m/z. Después del analizador, los iones pasan a la zona de detección, que tiene también una serie de lentes que dirigen los iones hasta del propio detector, y un dínodo, que permite cambiar el modo de detección de positivo a negativo.

Cuando los iones se encuentran dentro de la trampa puede llevarse a cabo tanto el análisis de sus masas (espectro de MS) como el aislamiento de uno o varios iones precursores y su posterior fragmentación (dando lugar a lo que se denomina espectros de MS/MS).

En cuanto al empleo de la CE-MS para la determinación de CRBs, los tampones de separación volátiles frecuentemente empleados en MS (como acetato amónico o carbonato amónico) no son adecuados, ya que el modo CZE requiere que los analitos presenten carga y, como se indicó anteriormente, la mayoría de los CRBs son neutros en un amplio rango de pHs. Por ello son escasas las aplicaciones de CZE-MS en el análisis de CRBs (determinación de algunos plaguicidas, incluidos CRBs en zumos [99] y leche [100,101]). MEKC es la modalidad más apropiada para su separación, sin embargo los surfactantes que normalmente se emplean como por ejemplo el dodecil sulfato sódico (SDS) no son volátiles, por lo que la introducción directa de las micelas en espectrómetro de masas puede conducir a la contaminación y aumento de la supresión iónica. Para solventar este problema se han desarrollado diferentes alternativas [77]. Centrándonos en la determinación de CRBs, se ha propuesto emplear en lugar del ESI la APPI empleando rellenos monolíticos para el análisis de leche [100], y el uso de técnicas de llenado parcial del capilar en modo MEKC con SDS [102] que por la aplicación de cambios de polaridad en el voltaje de separación consigue que el surfactante no llegue al MS, aunque esta técnica presenta poca reproducibilidad y escasa sensibilidad. Para solventar estos problemas, en la presente Tesis Doctoral se ha propuesto el uso de surfactantes volátiles como son el perfluorooctanoato de amonio (APFO) o el sulfonato de perfluorooctano (PFOSA) [103,104] reduciendo la complejidad del sistema, ya que el surfactante se evapora antes de llegar al espectrómetro de masas. Estos surfactantes poseen una cadena fluorocarbonada, que les confiere una baja energía superficial, lo que está relacionado con bajas interacciones moleculares [105] y una elevada volatilidad [106], haciéndolos compatibles con el acoplamiento CE-MS. Esta estrategia ha sido empleada para la

[99] J. Hernández-Borges, M.A. Rodríguez-Delgado, F.J. García-Montelongo, A. Cifuentes, *Electrophoresis* 25 (2004) 2065.
[100] C. Gu, S.A. Shamsi, *Electrophoresis* 31 (2010) 1162.
[101] C. Blasco, Y. Picó, V. Andreu, *Electrophoresis* 30 (2009) 1698.
[102] M. Molina, S.K. Wiedmer, M. Jussila, M. Silva, M.L. Riekkola, *J. Chromatogr. A* 927 (2001) 191.
[103] Y. Ishihama, H. Katayama, N. Asakawa, *Anal. Biochem.* 287 (2000) 45.
[104] P. Petersson, M. Jörntén-Karlsson, M. Stålebro, *Electrophoresis* 24 (2003) 999.
[105] C.A. Barton, M.A. Botelho, M.A. Kaiser, *J. Chem. Eng. Data* 54 (2009) 752.
[106] C.A. Barton, M.A. Kaiser, M.H. Rusell, *J. Environ. Monitor* 9 (2007) 839.

determinación de CRBs en agua de grifo [107], y para otro tipo de aguas [108] ampliando el campo de aplicación del acoplamiento CE-MS tanto a moléculas cargadas como neutras.

5.1.2. CROMATOGRAFÍA LÍQUIDA CAPILAR

La HPLC es una técnica fundamental en la mayoría de los laboratorios de ensayo. Su continuo desarrollo ha conseguido una mejora en los métodos de análisis establecidos con anterioridad. Así, en los últimos años, para incrementar la eficacia de la separación, la sensibilidad de la detección y disminuir los tiempos de análisis de las columnas convencionales de HPLC, se ha reducido el diámetro interno de las columnas así como el tamaño de partícula del material de empaquetamiento. Los importantes avances que han tenido lugar en las tecnologías de fabricación de columnas, han permitido el desarrollo de columnas micro y capilares que respecto a las convencionales de diámetro interno 4.6 mm ofrecen diversas ventajas [48,109,110]. Así, debido al diámetro interno reducido, los caudales de fase móvil utilizados con micro-columnas son muy pequeños, lo que produce una disminución considerable del consumo de reactivos y disolventes, y por ello de los desechos generados y del impacto medioambiental. Por otra parte, el pequeño volumen en el que se eluyen los analitos proporciona mayor sensibilidad y mejoras de hasta 20 órdenes de magnitud en la respuesta del detector [111]. En comparación con los sistemas convencionales de HPLC, este tipo de técnicas (Tabla 5) mejoran la detección y permiten obtener eficacias de separación elevadas. Por ello, las micro-columnas han sido muy utilizadas para aumentar la sensibilidad de la detección y la resolución cuando el volumen de muestra disponible para la inyección es limitado o los compuestos a determinar se encuentran presentes a bajas concentraciones en las distintas muestras.

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[107] G. Van-Biesen, C.S. Bottaro, *Electrophoresis* 27 (2006) 4456.

[108] D. Moreno-González, L. Gámiz-Gracia, J.M. Bosque-Sendra, A.M. García-Campaña, *J. Chromatogr. A* 1247 (2012) 26.

[109] J. Hernández-Borges, Z. Aturki, A. Rocco, S. Fanali, *J. Sep. Sci*. 30 (2007) 1589.

[110] P. Molander, T.E. Gundersen, C. Haas, T. Greibrokk, R. Blomhoff, E. Lundanes *J. Chromatogr. A* 847 (1999) 59.

[111] A. Braithwaite, F.J. Smith en "Chromatographic Methods", 5ª Ed., Kluwer Academic Publishers (1996) p. 1.

Tabla 5. Terminología empleada en técnicas de cromatografía liquida [48]

Nombre	Diámetro interno de la columna	Velocidad de flujo	Tamaño de partícula (µm)	Longitud (cm)
HPLC analítico	3.2-4.6 mm	0.5-2.0 ml min^{-1}	3-10	3-25
HPLC microbore	1.5-3.2 mm	100-500 µl min^{-1}	3-8	15-25
Micro-LC	0.5-1.5 mm	10-100 µl min^{-1}	3-5	5-15
HPLC-capilar	150-500 µm	1-10 µl min^{-1}	3-5	5-15
Nano-HPLC	10-150 µm	10-1000 nl min^{-1}	3-5	5-15

La HPLC capilar ha sido ampliamente aplicada en el campo de la proteómica [112,113], pero el número de trabajos en el análisis de contaminantes es reducido. En concreto para la determinación de CRBs solo se encuentra el método propuesto en la presente Tesis Doctoral para su determinación en muestras de pepino y medioambientales [114] y otros dos trabajos para su determinación en aguas [115,116], empleándose en todos los casos la detección UV-Vis.

[112] S. Funke, D. Azimi, D. Wolters, F. Grus, N. Pfeiffer, *J. Proteom*. 75 (2012) 3177.

[113] X. Guan, G. Yan, M. Gao, G. Hong, C. Deng, X. Zhang, *J. Chromatogr. A* 1217 (2010) 6875.

[114] D. Moreno-González, J.F. Huertas-Pérez, L. Gámiz-Gracia, A.M. García-Campaña, *Intern. J. Environ. Anal. Chem.* 91 (2011) 1329.

[115] Y. Gou, J. Pawliszyn, *Anal. Chem.* 72 (2000) 2774.

[116] N. Rosales-Conrado, M.E. León-González, L.V. Pérez-Arribas, L.M. Polo-Díez, *J. Chromatogr. A* 1081 (2005) 114.

5.2. APLICACIÓN DE LA CROMATOGRAFÍA LÍQUIDA DE ULTRA RESOLUCIÓN PARA LA DETERMINACIÓN DE CARBAMATOS EN ALIMENTOS Y MUESTRAS MEDIOAMBIENTALES

Además de la miniaturización, otro paso importante dentro del avance de la HPLC ha sido el desarrollo de fases estacionarias de partícula híbrida, con un componente inorgánico (sílice) y un componente orgánico (organosiloxanos) [117,118] y con un tamaño de partícula inferior a los 2 µm [119], originando lo que se ha denominado "Cromatografía Líquida de Ultra Resolución (UHPLC)" [117]. En la Figura 11 se representa una gráfica de Van Deemter en función de los tamaños de partícula desarrollados desde 1970 hasta la fecha. Se puede observar que incluso la eficacia de las partículas de 2.5 µm comienza a disminuir a caudales muy altos debido a la contrapresión del sistema. Con columnas de tamaño de partícula inferiores a 2 µm la eficiencia se mantiene incluso a velocidades lineales altas, por lo que se puede aprovechar el uso de columnas más cortas y mayor velocidad de flujo para obtener mejores separaciones cromatográficas con mayor resolución y sensibilidad [120].

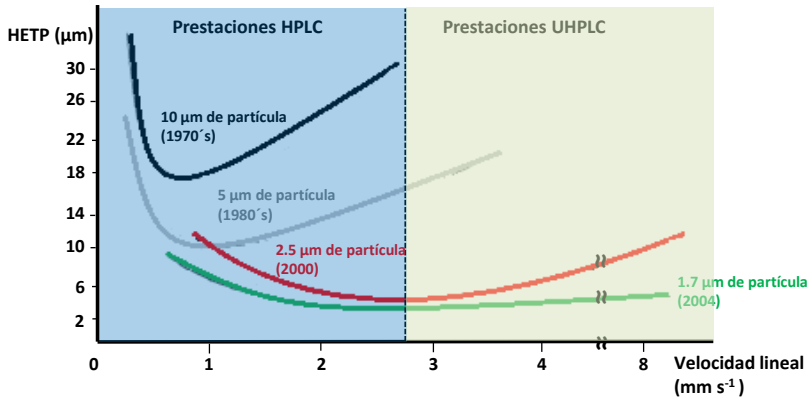

Figura 11. Gráfica de Van Deemter ilustrando la evolución del tamaño de partícula en las últimas décadas [120].

[117] M.E. Swartz, *J. Liq. Chromatogr. Rel. Tech.* 28 (2005) 1253.
[118] E.S. Grumbach, T.E. Wheat, M. Kele, J.R. Mazzeo*, LC-GC N. Am.* 23 (2005) 40.
[119] D.T.T. Nguyen, D. Guillarme, S. Rudaz, J.L. Veuthey, *J. Sep. Sci.* 29 (2006) 1836.
[120] Waters Corporation en "UPLC: New Boundaries for the Chromatography Laboratory", Hoja técnica (2004).

En cuanto a los sistemas de detección, la mayoría de las aplicaciones de UHPLC desarrolladas hasta ahora han implicado la utilización de MS como sistema de detección con interfase ESI [119]. En este acoplamiento, al contrario de lo que ocurre en CE, el principal obstáculo es la enorme cantidad de disolvente que acompaña al analito, con flujos de hasta 2-3 ml min^{-1} en algunos casos. Los espectrómetros de masas son aparatos de alto vacío que no pueden recibir fácilmente caudales de disolvente tan elevados, ya que se ven muy afectados por la humedad que entra al sistema dando lugar a grandes desviaciones en la exactitud de masa. Normalmente las interfases ESI empleadas para HPLC aceptan flujos comprendidos entre 0.001-1 ml min^{-1}, aunque el flujo recomendado a la entrada del MS está entre 0.2-0.5 ml min^{-1}, dependiendo del analizador usado. Cuando se utilizan flujos elevados, es recomendable el uso de divisores de flujo entre la salida de la columna cromatográfica y la entrada de la interfase, aunque esto suponga una disminución de la sensibilidad al eliminarse parte de la muestra. En el caso de la tecnología UHPLC este problema esta solventado ya que el flujo normal de trabajo esta comprendido entre 0.1 a 0.5 ml min^{-1}.

En el campo del análisis de plaguicidas se han empleado diferentes analizadores de masas acoplados a sistemas de HPLC convencional como UHPLC, como son IT, TOF, Q, Q-TOF [121-123] y analizadores de triple cuadrupolo (QqQ) [124,125]. En la presente Tesis Doctoral se ha propuesto la utilización de un analizador de QqQ para la determinación de CRBs en muestras de alimentos. El analizador QqQ (Figura 12), consiste en tres cuadrupolos conectados en serie. El cuadrupolo 1 (Q1) y el cuadrupolo 3 (Q3) funcionan como dos analizadores de tipo cuadrupolo conectados en serie. El cuadrupolo 2 (Q2), o celda de colisión, se sitúa entre el Q1 y Q3. En este cuadrupolo se aplica la energía de colisión, que permite fragmentar los iones obtenidos en la fuente de ionización. Esta energía puede

[121] C. Ferrer, O. Malato, A. Agüera, A.R. Fernández-Alba, *Compr. Anal.Chem*. 58 (2012) 1.

[122] Y. Picó, G. Font, M. J Ruiz, M. Fernández, *Mass Spectom. Rev*. 25 (2006) 917.

[123] M.L. Gómez-Péreza, P.Plaza-Bolaños, R. Romero-González, J.L. Martínez-Vidal, A. Garrido-Frenich, *J. Chromatogr. A* 1248 (2012) 130.

[124] L. Alder, "Targeted pesticide residue analysis using triple quad LC-MS/MS" en "Methods in Molecular Biology" (New York, NY, United States) (2011).

[125] R. Cazorla-Reyesa, J.L. Fernández-Moreno, R. Romero-Gonzáleza, A. Garrido Frenich, J.L. Martínez Vidal, *Talanta* 85 (2011) 183.

tomar diferentes valores, lo que permite obtener espectros de masas en los que aparecen diferentes fragmentos y/o diferentes relaciones de intensidad entre ellos.

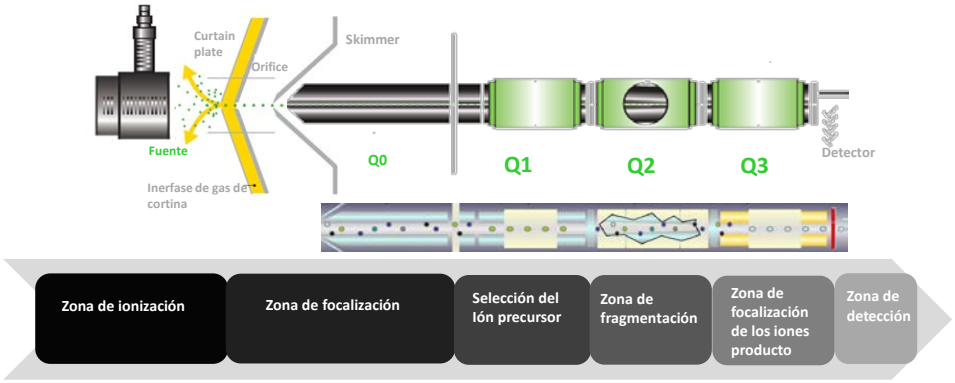

Figura 12. Esquema de un espectrómetro de masas de ESI-QqQ.

El modo de adquisición más utilizado en los QqQ es el MRM (Multiple Reaction Monitoring). Este modo permite obtener espectros de masas altamente selectivos, con gran sensibilidad. Es muy útil en los procesos en los que se requiere analizar unas sustancias muy concretas, de las que se conoce el peso molecular y el patrón de fragmentación. Asimismo, gracias a la selectividad se mejora considerablemente la sensibilidad, ya que se obtiene una mejor relación señal ruido (S/N). Además, la información obtenida por los analizadores MS/MS permite identificar los distintos compuestos, cumpliendo con la legislación vigente referente a puntos de identificación, para la confirmación inequívoca de residuos de plaguicidas en alimentos [31,32].

Actualmente, la metodología más empleada para la determinación de CRBs es la HPLC convencional acoplada a la MS, existiendo numerosos artículos donde se han determinado CRBs en diferentes matrices como vinos [126-128], frutas y vegetales [129-131], y muestras medioambientales [130]. No obstante, la UHPLC es una alternativa prometedora

[126] A. Economou, H. Botitsi, S .Antoniou, D. Tsipi, *J. Chromatogr. A* 1216 (2009) 5856.
[127] M. Moeder, C. Bauer, P. Popp, M. van Pinxteren, *Anal. Bioanal. Chem.* 403 (2012) 1731.
[128] T. Goto, Y. Ito, H. Oka, I. Saito, H. Matsumoto, H, Sugiyama, C. Ohkubo, H, Nakazawa, H. Nasage, *Anal. Chim. Acta* 531 (2005) 79.
[129] S. W.C. Chung, B.T.P. Chan, *J. Chromatogr. A* 1217 (2010) 4815.
[130] I. Ferrer, E.M. Thurman, *J. Chromatogr. A* 1175 (2007) 24.
[131] M.S. Filigenzi, N. Ehrke, L.S. Aston, Linda R.H. Poppenga, *Food Addit. Contam.*28 (2011) 1324.

para la determinación de CRBs ya que permite aumentar la eficiencia de la separación reduciendo notablemente los tiempos de análisis. Esto ha provocado que en los últimos años esta técnica esté emergiendo como alternativa a la HPLC. Así, su uso en combinación con la MS/MS ha proporcionado resultados satisfactorios en la determinación de CRBs en muestras como miel [123], arroz [132], frutos secos [133], bebidas alcoholicas [134] y cereales [135].

6. TRATAMIENTOS DE MUESTRA PARA LA DETERMINACIÓN DE CARBAMATOS

El proceso analítico es el conjunto de etapas u operaciones comprendidas entre la toma de muestra y la obtención e interpretación de los resultados obtenidos, siendo una de las etapas más importantes el tratamiento de muestra, ya que existen multitud de compuestos que pueden interferir en la medida analítica, siendo muy pocas las muestras que actualmente pueden ser analizadas directamente.

La etapa de tratamiento de la muestra tiene dos objetivos fundamentales:

- La extracción del analito de la propia matriz de la muestra para que pueda ser cuantificado con fiabilidad.
- La preconcentración del analito, sobre todo si se encuentra a concentraciones muy bajas.

Entre las principales técnicas de tratamiento de muestra actuales empleadas en la determinación de CRBs, destacan la extracción líquido-líquido (LLE) [64,136] y la extracción

[132] J. Wang, W. Schnute, Quantitative Determination of Ultratrace Level N-Methyl Carbamates in Rice Samples by Accelerated Solvent Extraction (ASE) and Ultrahigh Performance Liquid Chromatography Tandem Mass Spectrometry (UHPLC-MS/MS). Technical Sheet from Thermo Fisher Scientific (2011).

[133] Q.B. LIN, Y.Y. Xue, H. Song, *J. Chromatogr. Sci.* 48 (2010) 7.

[134] P. Plaza Bolaños, R. Romero-González, A. Garrido Frenich, J. L. Martínez Vidal, *J. Chromatogr. A* 1208 (2008) 16.

[135] J.M. Zhang, Y.L. Wu, Y.B. Lu, *J. Chromatogr. B* 915 (2013) 13.

[136] S.M. Goulart, R.D. Alves, A.A. Neves, J.H. de Queiroz, T.C. de Assis, M.E.L.R. de Queiroz, *Anal. Chim. Acta* 671 (2010) 41.

sólido-liquído (SPE) [137], aunque en los últimos años se han producido importantes avances en el desarrollo de técnicas de extracción, dirigidos principalmente al desarrollo de métodos más rápidos y con los que se consiga una mayor preconcentración [138,139].
En la presente Tesis Doctoral, se han evaluado diferentes metodologías considerando las tendencias actuales que persiguen una reducción del tamaño de muestra, reducción de disolventes orgánicos y procedimientos génericos multirresiduo. Estas metodologías se comentan a continuación.

6.1. EXTRACCIÓN EN FASE SÓLIDA (SPE)

La SPE mediante el uso de adsorbentes es idónea para la extracción de analitos presentes en muestras líquidas y para la purificación de los extractos obtenidos a partir de muestras sólidas mediante distintas técnicas de extracción. En esta metodología el disolvente compite con los analitos (y/o compuestos interferentes) por los sitios activos de adsorción del sorbente. Es decir, la elución se describe como un desplazamiento del soluto por la acción del disolvente. De este modo, cuanto más polar es el disolvente, mayor poder de elución presenta. Así, mediante una adecuada selección de disolventes empleados es posible la eliminación de compuestos interferentes del sorbente para después, utilizando un disolvente más polar, eluir los analitos (y/o compuestos interferentes). Existen disponibles diferentes tipos de cartuchos con distintos adsorbentes, siendo posible el empleo de adsorbentes polares, no polares y de intercambio iónico, cuya elección depende del tipo de matriz, analitos de interés e interferentes.

Los rellenos de C_{18} y Alumina han sido muy utilizados para la SPE de un elevado número de compuestos de diferente polaridad [125,140]. Sin embargo, cada vez están siendo más utilizados los rellenos poliméricos con balance hidrofílico-lipofílico [44,141,142], de fase reversa, humectable en agua, conocido como HLB. Su composición consiste en una

[137] L.M. Ravelo-Pérez, J. Hernández-Borges, M.A. Rodríguez-Delgado, *J. Sep. Sci.* 29 (2006) 2557.
[138] L. Ramos, *J. Chromatogr. A* 1221 (2012) 84.
[139] L. Zhang, S. Liu, X. Cui, C. Pan, A. Zhang, F. Chen, *Cent. Eur. J. Chem.* 10 (2012) 900.
[140] G.S. Nunes, M.L. Ribeiro, L. Polese, D. Barcelo, *J.Chromatogr. A* 795 (1998) 43.
[141] S.S. Petropoulou, A. Tsarbopoulos, P.A. Siskos, *Anal Bioanal Chem*. 385 (2006) 1444.
[142] A. Belmonte-Vega, A. Garrido-Frenich, J.L. Martinez-Vidal, *Anal. Chi. Acta* 538 (2005) 117.

proporción equilibrada de dos polímeros: N-vinilpirrolidona (hidrofílica) y divinilbenceno (lipofílica). El uso de este tipo de sorbente esta ampliante extendido, debido o a sus excelentes características para la extracción de compuestos de un amplio rango de polaridad. Con el empleo de HLB, los analitos de interés quedan retenidos en el cartucho, por lo que deberán ser eluidos por medio de un pequeño volumen de disolvente.

Diversos artículos de revisión han tratado esta metodología aplicada a la separación de componentes en alimentos, y en la actualidad existen descritas multitud de aplicaciones [143-145], entre las que se encuentran aplicaciones para la determinación de CRBs tanto en muestras medioambientales [140,143] como en alimentos [44,144,145].

6.2. EXTRACCIÓN EN FASE SÓLIDA DISPERSIVA (dSPE)-QuEChERS

La SPE dispersiva (dSPE) es un método rápido y sencillo de limpieza de muestras, actualmente aplicado fundamentalmente en el análisis multirresiduo de plaguicidas en frutas y vegetales. Basado en este tipo de extracción Anastassiades y col. [146] desarrollaron el procedimiento comúnmente conocido por su acrónimo en ingles "QuEChERS" -**Qu**ick (rápido), **E**asy (sencillo), **Ch**eap (barato), **E**ffective (eficaz), **R**ugged (robusto), **S**afe (seguro)- que implica un primer paso inicial de extracción simple con acetonitrilo, seguida de un fenómeno de reparto líquido-líquido por adición de $MgSO_4$ anhidro y NaCl (ver Figura 13). El sulfato magnésico, en el proceso de extracción, ayuda a mejorar las recuperaciones al favorecer el reparto de los plaguicidas en la fase orgánica. La cantidad utilizada debe superar la concentración de saturación. El NaCl ayuda a controlar la polaridad, aunque demasiada sal podría impedir el reparto. El acetato sódico y otras sales actúan controlando el pH. El segundo paso (dSPE) implica la eliminación de agua residual y limpieza (*clean-up*) mediante la adición de una mezcla basada en $MgSO_4$ (con objeto de eliminar el agua y favorecer el reparto) y un sorbente, como una amina primaria y secundaria (PSA), C_{18} (para matrices lipofílicas) o mezclas de ambos, que proporciona una elevada capacidad de eliminación de los componentes de la matriz (azúcares, ácidos

[143] S. Zhou, B. Wu, C. Ma, Y. Ye, H. Chen, *J. Liq. Chromatogr. R.T.* 35 (2012) 2860.
[144] S. Zhou, H. Chen, B. Wu, C. Ma, Y. Ye, *Microchim. Acta* 176 (2012) 419.
[145] M.C. Bruzzoniti, C. Sarzanini, G Costantino, M. Fungi, *Anal. Chim. Acta* 578 (2006) 241.
[146] M. Anastassiades, S.J. Lehotay, D. Stajbaher, F.J. Schenck, *J. AOAC Int.* 86 (2003) 412.

grasos, ácidos orgánicos, pigmentos polares, etc.). Después de este proceso de limpieza, se lleva a cabo una centrifugación y el extracto está listo para ser directamente analizado o sometido a evaporación y recomposición en el disolvente apropiado para su análisis.

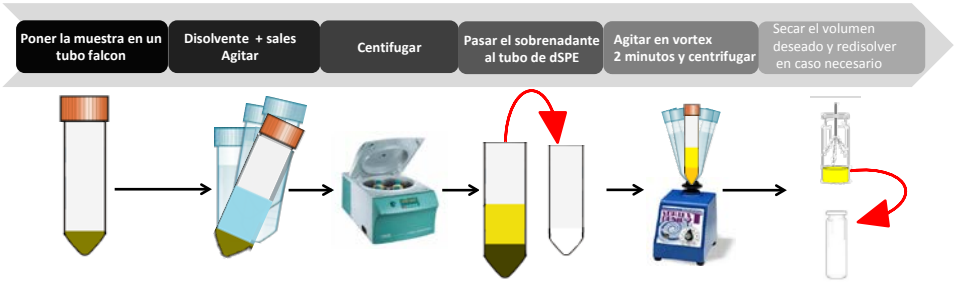

Figura 13. Esquema del método QuEChERS.

Existen multitud de aplicaciones de la metodología QuEChERS para la determinación de CRBs en muestras como miel [123], cereales [135], frutas y vegetales [131,147-150], productos botánicos [151-153], leche [154], vino [155], etc. Actualmente existen kits comercializados por diferentes empresas para desarrollar este tratamiento de muestra

[147] S.J. Lehotay, K.A. Sonb, H. Kwonb, U. Koesukwiwata, W. Fud, K. Mastovskaa, E.Hoha, N. Leepipatpiboon, *J. Chromatogr. A* 1217 (2010) 2548.
[148] M. Arienzo, D. Cataldo, L. Ferrara, *Food Control* 31 (2013) 108.
[149] U.Koesukwiwata, S. J. Lehotaya, S. Miaoc, N. Leepipatpiboon, *J. Chromatogr. A* 1217 (2010) 6692.
[150] S.C. Cunha, J.O. Fernandes, A. Alves, M.B.P.P. Oliveira, *J. Chromatogr. A* 1216 (2009) 119.
[151] Z. Huang, Y. Li, B. Chen, S. Yao, *J. Chromatogr. B* 853 (2007) 154.
[152] J. Wang, W. Chow, D. Leung, *J. AOAC Int.* 94 (2011) 1685.
[153] P.Zhao, L.Wang, Y.Jiang, F. Zhang, C.Pan, *J. Agric. Food Chem.* 60 (2012) 4026.
[154] J. Keegan, M. Whelan, M. Danaher, S. Crooks, R. Sayers, A. Anastasio, C. Elliott, D. Brandon, A. Furey, R. O'Kennedy, *Anal. Chim Acta* 654 (2009) 111.
[155] Y. Jiang, X. Li, J. Xu, C. Pan, J. Zhang, W. Niu, *Food Addit. Contam.* 26 (2009) 859.

[156,157], que ha sido establecido como método normalizado europeo para plaguicidas [158].

6.3. MICROEXTRACCIÓN LÍQUIDO-LÍQUIDO DISPERSIVA (DLLME)

En los últimos años existe un creciente interés en la simplificación y miniaturización de los sistemas de tratamiento de muestra, introduciendo disolventes menos contaminantes y disminuyendo considerablemente las cantidades de disolventes orgánicos utilizados, en consonancia con los principios de la llamada Química Verde [159], o Química beneficiosa para el medioambiente, que se ocupa del diseño de productos y procesos químicos que reducen o eliminan el uso y producción de sustancias peligrosas, implicando una mayor seguridad y un menor coste en relación a los procesos convencionales.

La microextracción en fase líquida (LPME) es una técnica simple y económica en la que se requieren sólo unos microlitros de disolvente para concentrar a los analitos a partir de las muestras, en lugar de los elevados volúmenes requeridos en la LLE convencional. Además, es compatible con GC, HPLC y CE. En LPME la extracción normalmente tiene lugar en una pequeña cantidad de disolvente miscible con el agua (fase aceptora) a partir de una muestra acuosa que contiene los analitos (fase donora). Dentro de este tipo de extracción, podemos distinguir las siguientes modalidades:

- Microextracción de gota simple (*single drop microextraction*, SDME)
- Microextracción de fibra hueca (*hollow-fiber microextraction*, HF-LPME)
- Microextracción líquido-líquido dispersiva (*dispersive liquid-liquid microextraction*, DLLME)

[156] http://www.chem.agilent.com/en-US/products-services/Columns-Sample-Preparation/Sample-Preparation/QuEChERS/Pages/default.aspx

[157] http://www.sigmaaldrich.com/analytical-chromatography/sample-preparation/spe/dispersive-spe-tubes.html

[158] Norm EN 15662:2008. "Foods of plant origin — Determination of pesticide residues using GC-MS and/or LC-MS/MS following acetonitrile extraction/partitioning and clean-up by dispersive SPE - QuEChERS-method", 01-02-2009.

[159] A.S. Matlack, "Introduction to Green Chemistry", 2 Ed., Marcel Dekker (2010).

En esta Tesis Doctoral se ha empleado la DLLME, introducida por Rezaee y col. en 2006 [160]; hasta la fecha este artículo ha sido referenciado en más de 700 ocasiones [161] lo que pone de manifiesto el impacto que ha tenido en el campo de la Química Analítica. Esta técnica ha sido ampliamente empleada en la determinación de CRBs debido a su simplicidad de operación, rapidez, bajo coste, altas recuperaciones, elevados factores de enriquecimiento y alta compatibilidad con el medioambiente, debido al reducido volumen de disolventes orgánicos requerido. Sus aplicaciones han sido recogidas en diversos artículos de revisión [162- 165]. La DLLME está basada en la rápida inyección de una mezcla, en proporciones adecuadas, de unos pocos microlitros de disolvente extractante y un disolvente dispersivo en el seno de una muestra acuosa que contiene los analitos de interés. Esta rápida inyección produce una gran turbulencia, originándose la formación de finas gotas de extractante que se dispersan a través de la muestra acuosa. Tras centrifugación, se obtiene la separación de las fases acuosa y orgánica, siendo posible el análisis de esta última fase para la determinación de los analitos de interés. Un esquema general de la DLLME se muestra en la Figura 14.

La DLLME ha sido empleada para la determinación de CRBs mayoritariamente en muestras medioambientales [36,166- 169], pero también se encuentran aplicaciones en alimentos, como en melón y tomate [33], manzana [67] o zumos [170].

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[160] M. Rezaee, Y. Assadi, M.R. Milani Hosseini, E. Aghaee, F. Ahmadi, S. Berijani, *J. Chromatogr. A* 1116 (2006) 1.

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[163] M. Rezaee, Y. Yamini, M. Faraji, *J. Chromatogr. A* 1217 (2010) 2342.

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[165] A. Zgoła-Grzeskowiak, T. Grzeskowiak, *TrAC Trends Anal. Chem.* 30 (2011) 1382.

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[167] H. Lijun, W. Chunjian, S. Yinjuan, X. Luo, J. Zhang, K. Lu, *Int. J. Environ. Anal. Chem.* 89 (2009) 439.

[168] Z.M. Liu, X.H. Zang, W.H. Liu, C. Wang, Z. Wang, *Chin. Chem. Lett.* 20 (2009) 213.

[169] H. Chen, R. Chen, S. Li, *J. Chromatogr. A* 1217 (2010) 1244.

[170] L. Fu, X. Liu, J. Hu, X. Zhao, H. Wang, X. Wang, *Anal. Chim. Acta* 632 (2009) 289.

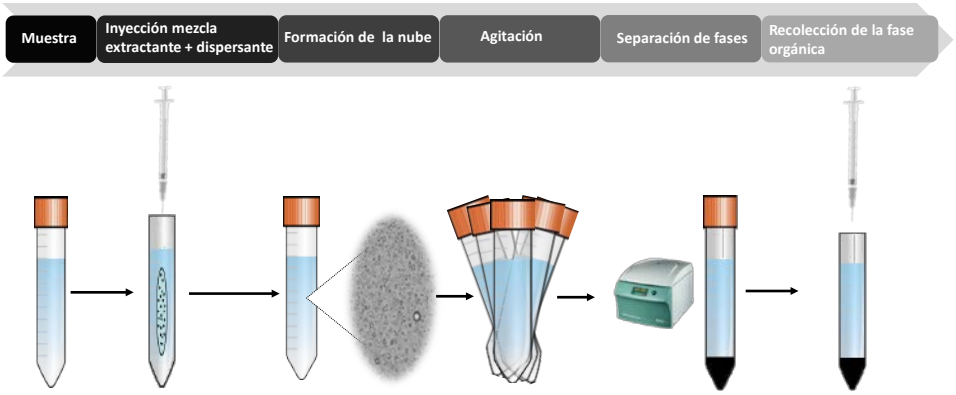

Figura 14. Esquema del procedimiento de DLLME.

6.4. MICROEXTRACCIÓN ASISTIDA POR ULTRASONIDOS CON EMULSIFICACIÓN MEJORADA CON SURFACTANTE (UASEME)

La microextracción asistida por ultrasonidos con emulsificación mejorada con surfactante (*Ultrasound-assisted surfactant-enhanced emulsification microextraction*, UASEME) se puede considerar una técnica relacionada con la DLLME, que ha ido evolucionando desde que Regueiro y col. propusieran la microextracción asistida por ultrasonidos (UME) para el análisis de plaguicidas en muestras medioambientales [171,172]. En la UME, un pequeño volumen de disolvente extractante inmiscible con el agua es dispersado en una fase acuosa gracias a la acción del ultrasonidos, sin el uso de ningún agente dispersante; esta microextracción suele llevarse a cabo a 25 ^{o}C y en un tiempo que varía de 10 a 20 minutos. Una vez producida la extracción de los analitos, y tras un paso de centrifucación, se obtiene la separación de fases acuosa y orgánica, siendo posible el análisis de esta última fase para la determinación de los analitos de interés. El principal inconveniente de esta técnica es el tiempo de extracción requerido para que se produzca la transferencia entre la muestra y el disolvente extractante, que es muy largo comparado con la DLLME, pudiendo ocurrir la degradación de los analitos por la acción prolongada del ultasonidos [173]. Recientemente, Wu y col. [174] y Vichapong y

[171] J. Regueiro, M. Llompart, C. García-Jares, J.C. García-Monteagudo, R. Cela, *J. Chromatogr. A* 1190 (2008) 27.

[172] M.M. Delgado-Povedano, M.D. Luque de Castro, *TrAC Trends Anal. Chem.* 45 (2013) 1.

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col. [175] han demostrado que el uso de surfactantes por debajo de la CMC, puede mejorar la dispersión fase acuosa-fase inmiscible, ya que estos surfactantes actuarían como una emulsión, reduciendo el tiempo de extracción prolongado en el ultrasonidos y dando lugar a la UASEME. Al ser un tratamiento relativamente nuevo, solo existe una aplicación para la determinación de CRBs [174] en muestras de aguas. El proceso global puede verse en la Figura 15.

Figura 15. Esquema del procedimiento UASEME.

En esta Introducción se ha pretendido abordar algunos principios y antecedentes de las técnicas y procedimientos de tratamientos de muestra que se proponen en los siguientes capítulos, así como la problemática del análisis de residuos de CRBs en muestras de alimentos y medioambientales. Como complemento a esta Introducción, en cada capítulo se incluye una introducción específica en función de la problemática abordada, método de análisis y detección, matriz y procedimiento de tratamiento de muestra, incluyendo algunos antecedentes bibliográficos, con objeto de facilitar la comprensión independiente de los mismos. El conjunto de los capítulos pone de manifiesto que el control de la contaminación alimentaria y ambiental se puede llevar a cabo por metodologías analíticas alternativas que en algunos casos implican mayor selectividad, menor coste e impacto ambiental y alta capacidad de identificación.

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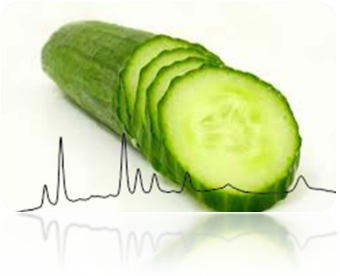
[174] Q. Wu, Q. Chang, C. Wu, H. Rao, X. Zeng, C. Wang, Z. Wang, *J. Chromatogr. A* 1217 (2010) 1773.
[175] J. Vichapong, R. Burakham, *Anal. Methods* 4 (2012) 2101.

CAPÍTULO 1

Determinación de carbamatos a niveles traza en aguas y pepino por cromatografía líquida capilar

CHAPTER 1

Determination of carbamates at trace levels in waters and cucumber by capillary liquid chromatography



RESUMEN

En este primer capítulo se ha desarrollado y validado un método analítico para la determinación de trazas de seis plaguicidas de la familia de los carbamatos en pepino y muestras medioambientales de aguas de diversa procedencia, usando HPLC capilar con detección UV mediante diodos en fila. Los analitos incluidos en el estudio (Carbofuran, Carbaril, Metiocarb, Promecarb, Bentiocarb y Fenoxicarb) presentan niveles máximos de residuos regulados por la Directiva 2006/118/CE del Parlamento Europeo y del Consejo, relativa a la protección del aguas subterráneas contra la contaminación y el deterioro y por el Reglamento (CE) n º 396/2005 relativo a los límites máximos de residuos de plaguicidas en alimentos. Para la preconcentración y limpieza se seleccionó la extracción en fase sólida *off-line.* La separación cromatográfica, realizada en modo gradiente, se llevó a cabo utilizando una columna C_{18} (150 mm x 0.5 mm ID, tamaño de partícula 5 µm) y una fase móvil compuesta por acetonitrilo:agua, con un caudal de 10 µl min^{-1}. El método presenta las ventajas de la cromatografía líquida capilar, con un bajo consumo de disolventes y muestra, además de proporcionar bajos límites de detección para los compuestos estudiados: entre 10.0-29.6 ng l^{-1} para las muestras de agua y entre 1.8-5.6 mg kg^{-1} para las muestras de pepino. Además, se llevaron a cabo estudios de recuperación en muestras dopadas a diferentes niveles de concentración, obteniéndose recuperaciones entre 70.0-111.1%, con desviaciones estándar relativas inferiores a 10.6%.

SUMMARY

In this chapter, an analytical method for trace determination of six carbamates in cucumber and environmental water samples using capillary HPLC with UV-diode array detection has been developed and validated. The selected analytes (Carbofuran, Carbaryl, Methiocarb, Promecarb, Benthiocarb and Fenoxycarb) present maximum residue levels regulated by the EU Council Directive 2006/118/EC on the protection of groundwater against the pollution and by the Regulation (EC) No. 396/2005 on pesticides in food. A previous off-line solid phase extraction procedure was required for preconcentration and sample clean-up. The separation was achieved using a C_{18} column (150 mm x 0.5 mm ID, 5 µm particle size) and a mobile phase consisting of acetonitrile: water using gradient mode, with a flow rate of 10 µl min^{-1}. Taking advantages of the characteristics of capillary HPLC, low volume of sample and solvents were required, achieving limits of detection for the studied compounds ranged from 10.0-29.6 ng l^{-1} for water samples and 1.8-5.6 mg kg^{-1} for cucumber. Recoveries studies for fortified samples at three different concentration levels were carried out, obtaining recoveries ranging from 70.0 to 111.1%, with relative standard deviations lower than 10.6%.

1. INTRODUCTION

In the European Union (EU), the Regulation (EC) No. 396/2005 on maximum residue levels of pesticides in products of plant and animal origin brings together and harmonizes in a single act all the limits applicable to the various types of food and feed. It also establishes maximum residue limits (MRL) for contaminants, including carbamates (CRBs), in different commodities [1]; for instance, in the case of cucumber samples, the MRLs are between 10 to 200 μg kg^{-1}. Moreover, the MRL for individual CRBs allowed by the European Directive on the quality of water intended for human consumption [2] and in the European Directive on the protection of groundwater against the pollution [3] is 100 ng l^{-1}; consequently, very sensitive analytical methods to detect trace levels of CRBs in these matrices are needed.

Chromatographic methods have been usually applied for the determination of CRBs in food and waters, using mainly fluorescence [4-6] and mass spectrometry (MS) detection [7-9]. Some reviews concerning the analysis of these compounds in water [10], foods [11,12] or including methodological aspects related to sample preparation prior to their analysis [13] can be found in the literature. Post-column hydrolysis of CRBs to methylamine (MA) and subsequent derivatization with o-phtalaldehide (OPA) is the basis of the HPLC-fluorescence method which has been accepted as a standard protocol by several official organizations,

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[1] Regulation (EC) No. 396/2005 of the European Parliament and of the Council on maximum residue levels of pesticides in or on food and feed of plant and animal origin and amending Council Directive 91/414/EEC. *Official Journal of the European Communities*, 16 March 2005, L70/1.

[2] Council Directive 98/83/EC on the quality of water intended for human consumption. *Official Journal of the European Communities*, 3 November 1998, L330/32.

[3] Council Directive 2006/118/EC on the protection of groundwater and deterioration. *Official Journal of the European Communities*, 27 December 2006, L372/19.

[4] M. Asensio-Ramos, J. Hernández-Borges, G. González-Hernández, M.A. Rodríguez-Delgado, *Electrophoresis* 33 (2012) 2184.

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[8] F. Boujelbane, F. Oueslati, N. Ben-Hamida, *Desalination* 250 (2010) 473.

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[10] J.M. Soriano, B. Jiménez, G. Font, J.C. Molto, *Crit. Rev. Anal. Chem.* 31 (2001) 19.

[11] C. Goranka, S. Wolfgang, *TrAC Trends Anal. Chem.* 28 (2008) 40.

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[13] Y. Santaladchaiyakit, S. Srijaranai, R. Burakham, *J.Sep. Sci.* 35 (2012) 2373.

including the Environmental Protection Agency (EPA) [14]. Chemiluminescence, a less usual detection technique in HPLC, has also been proposed [15,16].

During the last years, capillary HPLC has emerged as an alternative miniaturized technique to conventional HPLC and capillary electrophoresis (CE) [17]. In this type of chromatography, columns of ID of typically 500 mm and flow rates up to 20 µl min^{-1} are used. Capillary HPLC shows several advantages compared to analytical HPLC, such as better resolution, lower detection limits and lower solvent consumption, being more environmentally friendly than conventional HPLC [18]. It is recommended when sample volume is limited, and especially to gain sensitivity. In this sense, a relatively low sensitive detector such as UV-Vis could be used for detecting trace of contaminants, thanks to the higher sensitivity provided by capillary HPLC, as has been shown for different analytes and matrices [19,20]. Thus, the aim of this chapter is to show the applicability of this technique for the analysis of trace amounts of CRBs, as an alternative to analytical HPLC.

With this purpose, we have proposed the separation and sensitive determination of six CRBs in environmental waters from different origins and cucumber samples by means of capillary HPLC with diode array detection (DAD). In order to reach the very low limits of detection (LOD) and quantification (LOQ) required for the analysis of these matrices, a previous solid phase extraction (SPE) procedure was necessary for preconcentration and clean-up. A rigorous optimization of the significant variables involved in the extraction and chromatographic separation has been carried out. As far as we know, this is the first time that this miniaturized technique has been used for the quantitative analysis of this family of pesticides.

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[14] EPA Method 531.1, Measurement of N-methylcarbamoyloximes and N-methylcarbamates in water by direct aqueous injection HPLC with post column derivatisation, Revision 3.1, edited 1995.
[15] E. Orejuela, M. Silva, *J. Chromatogr. A* 1007 (2003) 197.
[16] J.F. Huertas-Pérez, A.M. García-Campaña, *Anal. Chim. Acta* 194 (2008) 630.
[17] M. Asensio-Ramos, J. Hernández-Borges, A. Rocco, S. Fanali, *J. Sep.Sci.* 32 (2009) 3764.
[18] J.RC. Vissers, H.A. Claessens, C.A. Cramers, *J. Chromatog. A* 779 (1997) 1.
[19] C. Cháfer-Pericás, R. Herráez-Hernández, P. Campíns-Falcó, *J. Chromatog. A* 1125 (2006) 159.
[20] C. Carrillo-Carrión, B.M. Simonet, M. Valcárcel, B. Lendl, *J. Chromatog A* 1225 (2012) 55.

2. EXPERIMENTAL

2.1. CHEMICALS

All the reagents were of analytical reagent grade and solvents were of HPLC grade. Organic solvents as methanol (MeOH), acetone (ACO), acetonitrile (ACN) and ethyl acetate (EtOAc), hydrochloric acid and anhydrous sodium sulfate were supplied by Panreac-Química (Madrid, Spain). Ultrapure water (18.2 $M\Omega cm^{-1}$, Milli-Q plus system, Millipore, Bedford, MA, USA) was used throughout the work. All solvents used as mobile phase and water samples were filtered under vacuum through a 0.45 mm nylon filter (Supelco, Bellefonte, PA, USA).

Analytical standards of carbofuran (CF), carbaryl (CAR), methiocarb (MTH), fenoxycarb (FNX), promecarb (PR) and benthiocarb (BTH) were supplied by ChemService Inc. (West Chester, USA) and FNX was supplied by Riedel-de Haën (Seelze, Germany). Individual stock standard solutions containing 500 mg l^{-1} of each compound were prepared by dissolving accurately weighed amounts in MeOH and stored in the dark at 4 ^{o}C. They were stable for at least four months. Working standard solutions were freshly prepared by dilution to the desired concentration with MeOH.

Extraction cartridges containing a hydrophilic–lipophilic balance (Oasis HLB, 200 mg, 6 ml; Waters, Milford, MA, USA) and Alumina N from Supelco in laboratory-prepared cartridges (1.25 g, 3 ml) were used in the SPE step. Acrodisc 13 mm syringe filters with 0.2 mm nylon membrane (Pall Corp., MI, USA) were used for filtration of cucumber extracts prior to the injection in the chromatographic system.

2.2. INSTRUMENTATION

Chromatographic analyses were performed with an Agilent HP-1200 series capillary HPLC (Agilent Technologies, Waldbronn, Germany) equipped with a binary pump (20 µl min^{-1} maximum flow-rate), on-line degasser, autosampler (8 µl loop), column thermostat and DAD. ChemStation software (A.10.20 [1757] version) was used for data acquisition and

processing. The chromatographic separation was performed on a Luna C_{18} column (150 mm x 0.5 mm ID, 5 µm particle size) from Phenomenex (supplied by Micrón, Madrid, Spain).

A Preppy vacuum manifold for 12 cartridges (Supelco), a rotavapor (Büchi RE 121, Flawil, Switzerland), and a domestic blender from Taurus (Lerida, Spain) were used for sample preparation. For pH measurements, a pH-meter (Crison model pH 2000, Barcelona, Spain) with a resolution of ±0.1 pH unit was used.

2.3. CHROMATOGRAPHIC CONDITIONS

The reverse phase separation was developed in a C_{18} column with a mobile phase consisting of ACN:H_2O, using the following gradient elution: 30% ACN (1 min) to 70% ACN (5 min), maintaining these conditions (12 min). The flow rate was 10 µl min^{-1}, the column temperature was kept at 25 ^oC and the injection volume was 0.5 µl. Based on literature the DAD wavelength was set at 210 nm [21,22].

2.4. SAMPLE PREPARATION

2.4.1. Water sample preparation

This procedure was based on a previously reported work, with some modifications [16]. Freshly collected water samples from growing areas susceptible of contamination by CRBs (river water samples from Dílar and ground water samples from Santa Fe, both in Granada) were spiked with proper concentrations of CRBs, filtered to remove suspended particulate matter and pH adjusted to 3.0 with 1 M HCl to prevent the potential degradation of the analytes. The samples were stored in the dark at 4 ^oC until analysis. Extraction and preconcentration of the analytes were achieved by SPE using Oasis HLB cartridges (200 mg, 6 ml) preconditioned with 6 ml of EtOAc, 6 ml of MeOH and 6 ml of ultrapure water, at a flow rate of 3 ml min^{-1}. Then, 500 ml of water sample were loaded on the SPE cartridge at a flow rate of 10 ml min^{-1}. After sample loading, the cartridges were washed with 5 ml of water at a flow rate of 3 ml min^{-1} and air-dried for 15 min. The analytes were eluted with 6

[21] S. Peng, J. Xiao, J. Chen, M. Zhang, X. Li, M. Cheng, *Microchim. Acta* 179 (2012) 193.
[22] A.I. Valenzuela, R. Lorenzini, M.J. Redondo, G. Font, *J. Chromatogr. A* 839 (1999)101.

ml of ACO at 3 ml min^{-1}. The eluate was dried under a gentle nitrogen stream at 30 ^{o}C. The dry residue was dissolved in 2 ml of MeOH, thoroughly vortexed and filtered before analysis. Sample throughput was about 12 samples per day.

The whole procedure is described in Figure I.1.

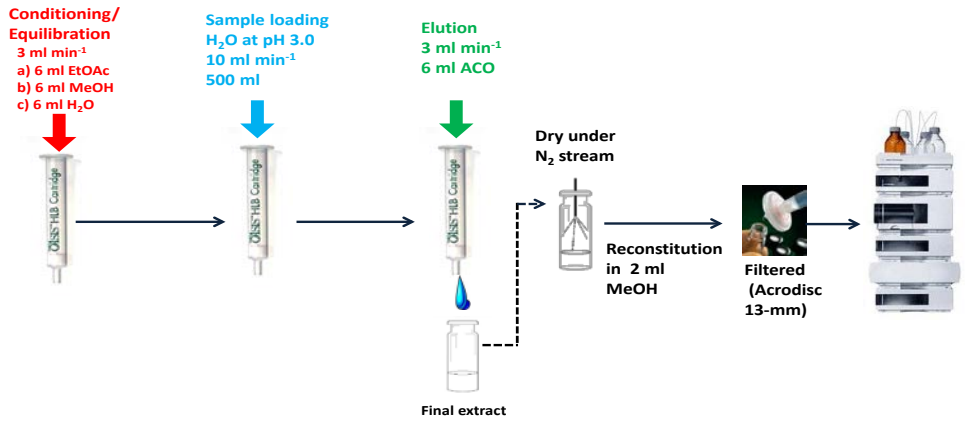

Figure I.1. Diagram of the SPE procedure for environmental samples using Oasis HLB cartridges.

2.4.2. Cucumber sample preparation

Portions of 20 g of cucumber collected from an ecological farm (Motril, Granada), were spiked with proper concentrations of CRBs. After equilibration for 1 hour at room temperature, samples were chopped and blended for 5 min with 60 ml of EtOAc and 60 g of anhydrous sodium sulfate. The mixture was filtered through a paper filter with vacuum and transferred to a round-bottom flask. Subsequently, 15 ml of EtOAc was used to clean both the filter and the filtering flask and transferred to the round-bottom flask. The obtained solution was concentrated in a rotavapor to ca. 2 ml, using a bath temperature of 30 ^{o}C. Then, the solution was made up to 5 ml with EtOAc and this solution was cleaned-up through an alumina cartridge at a flow rate of 0.5 ml min^{-1} (1.25 g of alumina previously conditioned with 5 ml of water, 5 ml of MeOH and 5 ml of EtOAc, at a flow rate of 3 ml min^{-1}); the eluate was collected in a glass vial. The EtOAc was then evaporated at 30 ^{o}C under nitrogen stream until dryness. The final residue was re-dissolved with 1 ml of MeOH, thoroughly vortexed and injected in the chromatographic system. Sample throughput was about 12 samples per day. Figure I.2 describes this procedure.

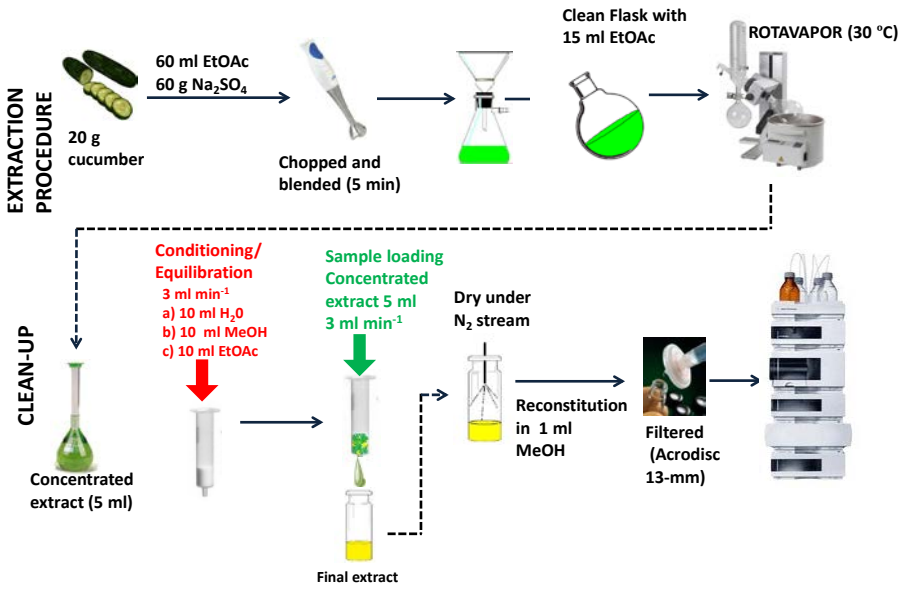

Figure I.2. Diagram of the SPE procedure for cucumber using Alumina cartridges.

3. RESULTS AND DISCUSSION

3.1. OPTIMIZATION OF THE CHROMATOGRAPHIC SEPARATION

The optimization of all the parameters involved in the chromatographic separation (such as gradient profile, column temperature and injection volume) was carried out. Mixtures of MeOH or ACN with water have been widely used for the separation of CRBs on C_{18} and C_8 columns [23] so we tested them. The use of MeOH as organic mobile phase was not adequate because the baseline presented a considerable drift, and the resolution was not appropriate. Thus, ACN:water was selected as the mobile phase since it produces smoother baseline and better peak shapes (see Figure I.3). The optimized elution program was as follows: 30% ACN for 1 min, a linear increase to 70% ACN (5 min) and then maintaining these conditions (12 min), at a flow rate of 10 µl min^{-1}.

[23] S. Khodadoust, M. Hadjmohammadi, *Anal. Chim. Acta* 699 (2011) 113.

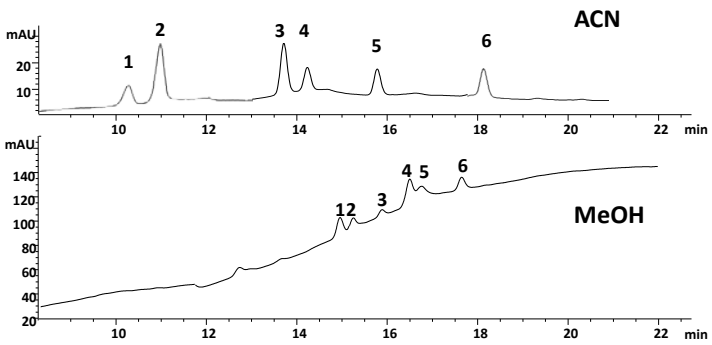

Figure I.3. Chromatograms obtained using different organic solvents in the aqueous mobile phase. Peaks: (1) CF, (2) CAR, (3) MTH, (4) PR, (5) FNX, and (6) BTH.

Column temperature was studied between 15 and 35 ^{o}C. No significant influence was observed, as can be seen in Figure I.4, and 25 ^{o}C was selected as optimum.

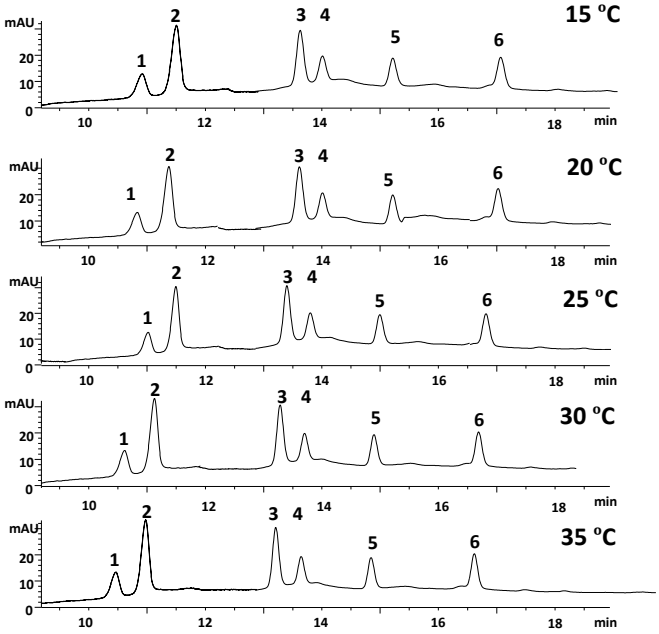

Figure I.4. Influence of the column temperature. Peaks: (1) CF, (2) CAR, (3) MTH, (4) PR, (5) FNX, and (6) BTH.

The injected volume was also tested from 0.10 to 2.00 µl in order to increase sensitivity without losing of resolution. When injection volumes higher than 0.50 µl were tested, the peak efficiency of CF was not satisfactory (see Figure I.5). For this reason, a volume of 0.50 µl was selected.

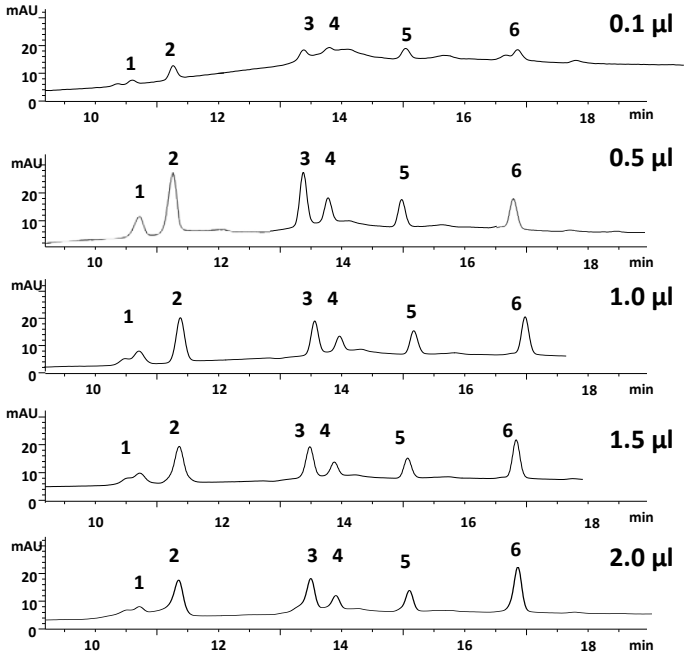

Figure I.5. Influence of the injection volume. Peaks: (1) CF, (2) CAR, (3) MTH, (4) PR, (5) FNX, and (6) BTH.

As a summary, all the optimum values for the capillary HPLC-DAD separation are included in Table I.1.

Table I.1. Optimum values for the variables involved in the capillary HPLC separation.

Variable	Optimum value
Column	Luna C_{18} (150 x 0.5 mm ID, 5 µm particle size)
Mobile phase composition	Solvent A: Water Solvent B: ACN
Gradient program	A:B (70:30) for 1 min 30% to 70% B in 5 min A:B (30:70) in 12 min
Injection volume	0.50 µl
Mobile phase flow rate	10 µl min^{-1}
Wavelength	210 nm
Column temperature	25 ^{o}C

3.2. OPTIMIZATION OF SOLID PHASE EXTRACTION FOR ENVIRONMENTAL WATER SAMPLES

As commented before, the MRLs of individual CRB pesticides in environmental and water samples allowed by the European Directory is 100.0 ng l^{-1} [3]; consequently a preconcentration step prior to HPLC determination is required to reach LODs below this value, and also to quantify these pesticides in environmental waters, where they are suspected to be at trace levels.

Preliminary studies were carried out in order to avoid losses of the analytes due to thermal degradation. As can be seen in Figure I.6, a degradation of CRBs was observed at temperatures higher than 30 ^{o}C. Thus, the drying temperature of the final extract was set at 30 ^{o}C as maximum.

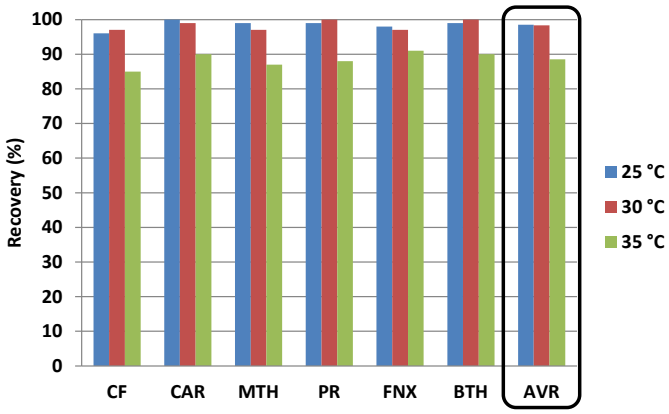

Figure I.6. Effect of the drying temperature on the extraction recovery of the CRBs (AVR: average recovery).

Several methodologies have been proposed for preconcentrating these analytes [10]. We have selected off-line SPE for its operational flexibility and simplicity. Oasis HLB sorbent has been presented as the best option for preconcentrating and cleaning-up CRBs in environmental samples [24,25]. Thus, those cartridges were selected for water samples.

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[24] X. Yang, H. Zhang, Y. Liu, J. Wang, Y. C. Zhang, A. J. Dong, H. T. Zhao, C. H. Sun, J. Cui, *Food Chem.* 127 (2011) 855.

[25] J.J. Soto-Chinchilla, A.M. García-Campaña, L. Gámiz-Gracia, L. Cuadros-Rodríguez, J.L. Martínez Vidal, *Anal. Chim. Acta* 524 (2004) 235.

Different amounts of sorbent (60 mg and 200 mg) were tested. As can be seen in Figure I.7, cartridges containing 200 mg were selected since they allowed to process a higher amount of sample and provided the highest retention percentage for BTH, the CRB with the lowest recovery.

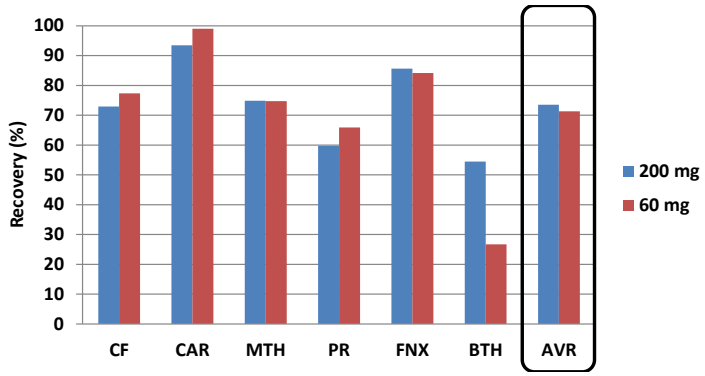

Figure I.7. Effect of amount of sorbent on the extraction recovery of the CRBs (AVR: average recovery).

Taking into account the recommendations of the cartridge supplier [26], a sample volume of 500 ml was selected. With this volume, a high preconcentration factor for CRBs could be achieved, allowing their determination below the established MRL for these samples.

Subsequently, different elution solvents were studied (6 ml of ACO-diethyl ether (90:10, v/v), ACN, MeOH and ACO). The optimization of this parameter showed that the most appropriate elution solvent was ACO (Figure I.8).

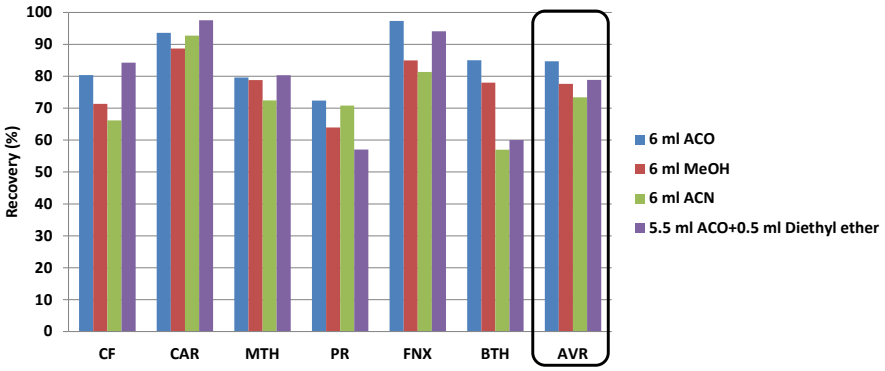

Figure I.8. Effect of different extraction solvents on the extraction recovery of the CRBs (AVR: average recovery).

[26] Oasis sample preparation. Application notebook. Waters Corporation (2008) 18.

Then, different volumes of ACO as elution solvent were tested. Although the differences were not significant for most of the analytes, and even for the average recovery, we decided to use 6 ml as the best value, which mainly improved recoveries for FNX and BTH (Figure I.9).

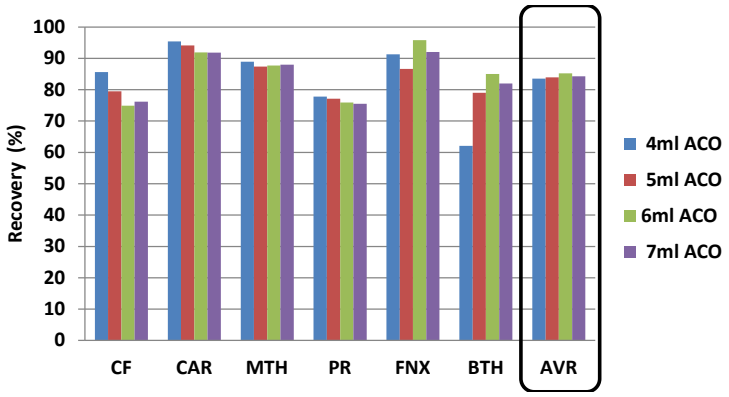

Figure I.9. Effect of different extraction solvents on the extraction recovery of the CRBs (AVR: average recovery).

Finally, different volumes of MeOH (0.5, 0.75, 1 and 2 ml) were tested for the reconstitution of the dried extract (Figure I.10). A volume of 2 ml gave the best result in terms of recovery for most of the analytes, except for MTH, and it was selected as optimum. Thus, considering that sample volumes of 500 ml were used, a preconcentration factor of 250 was achieved.

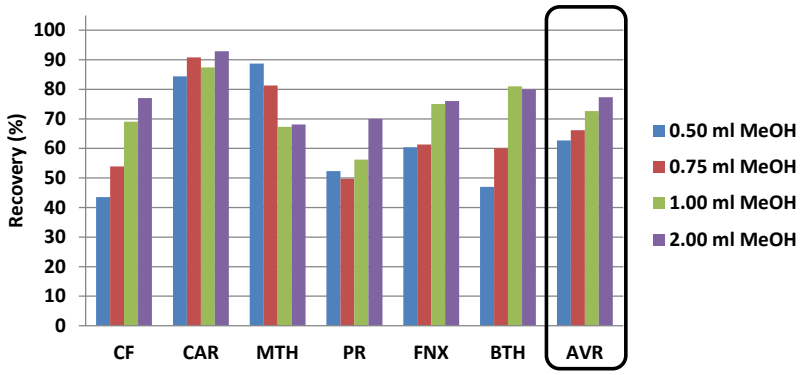

Figure I.10. Effect of different extraction solvents on the extraction recovery of the CRBs (AVR: average recovery).

A diagram of the water sample treatment is shown in Figure I.1.

3.3. OPTIMIZATION OF SOLID PHASE EXTRACTION FOR CUCUMBER

Extraction and clean-up procedure for the analysis of CRBs in cucumber samples was based in a previously reported work [16], but it should be noted that, in our case, the number of CRBs analyzed was higher. The procedure implied a previous solid-liquid extraction with EtOAc by means of a blender. Then, the extract was concentrated to ca. 2 ml by means of a rotavapor. In order to avoid losses of the analytes due to thermal degradation the temperature of the bath was set at 30 ^{o}C [27].

A subsequent clean-up step by SPE was necessary in order to remove certain interferences from the matrix. For this purpose, Alumina was selected as sorbent. This strategy is usually chosen when the sample component to be retained is present at a high concentration [28], allowing to adsorb matrix interferences while components of interest pass through the cartridge unretained.

Different quantities of Alumina were tested (from 0.50 to 1.50 g), and the best results in terms of selectivity (removal of interferences) and recoveries were obtained for 1.25 g (Figure I.11).

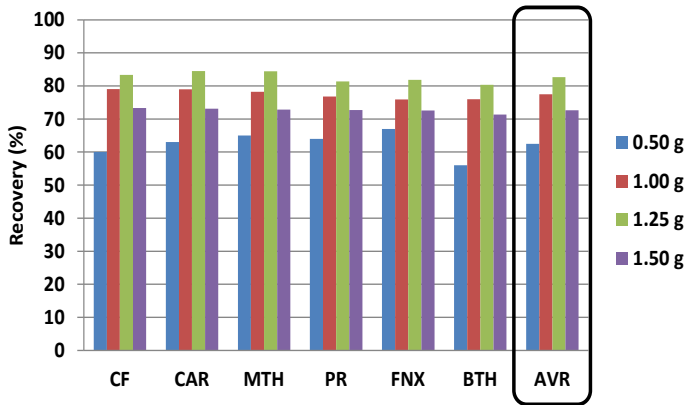

Figure I.11. Effect of different extraction solvents on the extraction recovery of the CRBs (AVR: average recovery).

Finally, the flow rate of extract through the cartridge was studied from 0.5 to 2 ml min^{-1} as it can be observed in Figure I.12; when a flow higher than 0.5 ml min^{-1} was used, the final

[27] G.S. Nunes, M.L. Ribeiro, L. Polese, D. Barceló, *J. Chromatogr. A* 795 (1998) 43.
[28] http://www.waters.com/waters/nav.htm?cid=10083883.

extract was not so clean, obtaining interferences which co-eluted with MTH. Therefore a flow rate of 0.5 ml min^{-1} was selected as optimum.

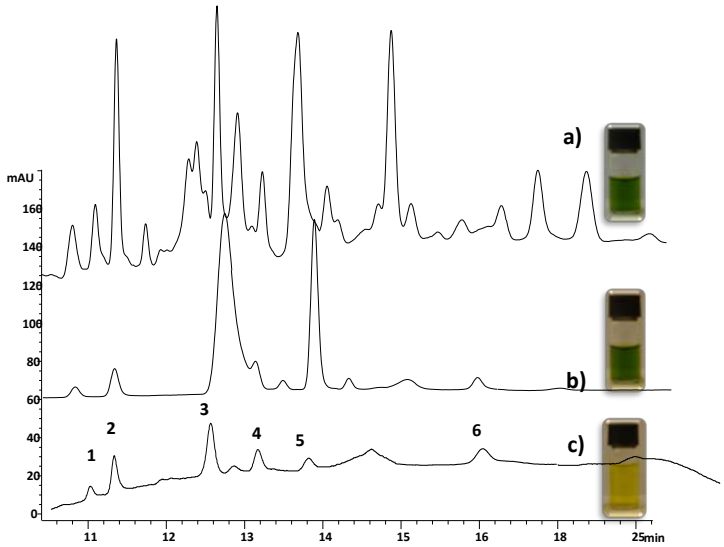

Figure I.12. Effect of flow rate extract through the cartridge. **(a)** 2 ml min^{-1} **(b)** 1 ml min^{-1}, **(c)** 0.5 ml min^{-1}. Peaks: (1) CF, (2) CAR, (3) MTH, (4) PR, (5) FNX, and (6) BTH.

The final sample treatment is shown in Figure I.2.

3.4. METHOD VALIDATION

The whole method was validated by means of matrix calibration curves, establishment of the performance characteristics of the method (such as LODs, LOQs, linearity and linear dynamic ranges), precision study (intraday and interday study) and trueness assessment (recovery study).

3.4.1. Calibration curves and analytical performance characteristics of the method

Matrix calibration curves were established using samples free of analytes, previously spiked with different analyte concentrations, processed following the SPE procedures and considering peak areas as a function of the analyte concentration in the sample. In the case of water samples, matrix calibration curves were obtained using river water spiked with

400, 1000, 2000, 3000 and 4000 ng l^{-1} of each analyte. For cucumber samples, matrix calibration curves were established from samples spiked with 12.5, 50, 100, 150 and 200 µg kg^{-1} of CF, CAR, MTH, FNX and BTH, and 5, 25, 50, 200 and 250 µg kg^{-1} of PR. All the samples were injected in triplicate. LODs and LOQs were evaluated as the concentration giving a signal-to-noise (S/N) ratio equal to 3 and 10, respectively. Performance characteristics of the method for the analysis of CRBs in water and cucumber samples are shown in Table I.2 and Table I.3, respectively.

Table I.2. Performance characteristics of the capillary HPLC-DAD method for the analysis of CRBs in waters (n=3 for each level).

Analyte	Linear dynamic range (ng l^{-1})	R^2 (%)	LOD (ng l^{-1})	LOQ (ng l^{-1})
CF	98.7-4000	99.8	29.6	98.7
CAR	33.3-4000	99.9	10.0	33.3
MTH	40.0-4000	99.9	12.0	40.0
PR	98.7-4000	99.6	29.6	98.7
FNX	50.0-4000	98.1	15.0	50.0
BTH	61.5-4000	99.8	18.5	61.5

100 ng l^{-1} MRL for individual CRBs allowed by the European Directive [2,3]

Table I.3. Performance characteristics of the capillary HPLC-DAD method for the analysis of CRBs in cucumber samples (n=3 for each level).

Analyte	Linear dynamic range (µg kg^{-1})	R^2 (%)	LOD (µg kg^{-1})	LOQ (µg kg^{-1})	MRL [1] (µg kg^{-1})
CF	18.7-200	99.6	5.6	18.7	20.0
CAR	4.6-150	99.7	1.9	4.6	50.0
MTH	7.2-250	99.6	2.2	7.2	200.0
PR	6.0-250	99.9	1.8	6.0	10.0
FNX	6.7-200	99.4	2.0	6.7	20.0
BTH	8.5-200	99.7	2.6	8.5	50.0

As can be seen, very good LOQs were obtained for all the analytes, being lower than the corresponding MRLs in cucumber samples and allowing their determination at trace level in environmental water samples.

3.4.2. Precision study

The precision of the method was evaluated in terms of repeatability (intraday precision) and intermediate precision (interday precision). Repeatability was evaluated by application of the proposed SPE and the chromatographic method to two different samples (experimental replicates) spiked at two different concentration levels: 1000 and 4000 ng l^{-1} in the case of water samples, and 50 and 150 µg kg^{-1} (except for PR, 25 and 200 µg kg^{-1}), in the case of cucumber samples. The samples were injected in triplicate (instrumental replicates) on the same day, with the same instrument and the same operator. Intermediate precision was evaluated with a similar procedure, but the samples were analyzed in three consecutive days. The results, expressed as relative standard deviation (%RSD) of the peak areas, are summarized in Table I.4 and Table I.5 for water and cucumber samples, respectively. The results showed good precision, less than 11% in all cases.

Table I.4. Precision of the method for spiked river water samples.

	CF	CAR	MTH	PR	FNX	BTH
Repeatability RSD (%) (n=6)						
Level 1[a]	2.5	1.9	5.8	6.4	9.3	7.5
Level 2[b]	3.1	3.0	3.1	2.7	4.6	4.6
Intermediate precision RSD (%) (n=9)						
Level 1[a]	6.8	2.1	8.9	7.1	10.6	8.0
Level 2[b]	2.7	3.6	4.2	4.5	6.5	5.5

[a] **Level 1:** 1000 ng l^{-1}
[b] **Level 2:** 4000 ng l^{-1}

Table I.5. Precision of the method for spiked cucumber samples.

	CF	CAR	MTH	PR	FNX	BTH
Repeatability RSD (%) (n=6)						
Level 1[a]	1.6	3.6	1.8	1.8	5.8	1.5
Level 2[b]	2.4	2.1	1.6	3.3	6.0	5.9
Intermediate precision RSD (%) (n=9)						
Level 1[a]	4.4	4.9	4.0	2.3	5.6	9.2
Level 2[b]	4.4	1.9	1.9	3.6	6.4	6.4

[a] **Level 1:** 50 μg kg^{-1}, except for PR (25 μg kg^{-1})
[b] **Level 2:** 150 μg kg^{-1}, except for PR (200 μg kg^{-1})

3.4.3. Trueness assessment

In order to check the trueness of the proposed methodology, recovery experiments were carried out in different types of spiked samples. In all cases, a sample free of analytes was analyzed to check the presence of CRBs, and none of them gave a positive result above the LOD of the proposed method. In water samples, recovery was estimated for well water and river water samples spiked at two different concentration levels (1000 and 4000 ng l^{-1}). The results are shown in Table I.6.

Table I.6. Recovery study in spiked water samples (n=6).

			CF	CAR	MTH	PR	FNX	BTH
River water	Level 1[a]	R (%)	93.6	92.7	93.5	83.3	71.6	67.9
		(RSD%)	(2.5)	(1.9)	(6.3)	(4.7)	(12.8)	(8.9)
	Level 2[b]	R (%)	103.3	100.6	95.6	88.5	72.5	91.8
		(RSD%)	(2.6)	(1.9)	(6.3)	(4.8)	(15.0)	(12.1)
Well water	Level 1[a]	R (%)	83.8	94.9	104.2	95.5	104.8	71.9
		(RSD%)	(4.6)	(2.0)	(10.6)	(13.9)	(12.0)	(10.7)
	Level 2[b]	R (%)	93.1	102.9	106.2	101.1	111.1	97.2
		(RSD%)	(4.8)	(2.0)	(10.5)	(14.3)	(14.0)	(14.5)

[a] **Level 1:** 1000 ng l^{-1}
[b] **Level 2:** 4000 ng l^{-1}

A chromatogram corresponding to a separation of the analytes in a river water sample under developed conditions is shown in Figure I.13.

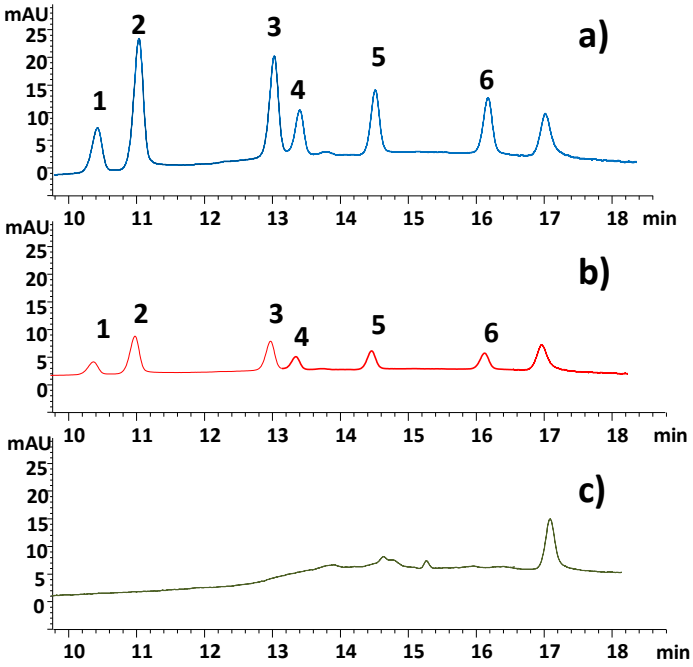

Figure I.13. Chromatograms of river water samples analyzed by capillary HPLC-DAD: **(a)** sample spiked with 4000 ng l^{-1}, **(b)** sample spiked with 1000 ng l^{-1}, **(c)** blank sample. Peaks: (1) CF, (2) CAR, (3) MTH, (4) PR, (5) FNX, and (6) BTH.

For cucumber samples, recoveries were checked at three different concentration levels, which varied depending on the CRB (see Table I.7). Taking into account that in pesticide residue analysis, the acceptable range for recovery is usually between 70 and 120% for routine analysis [29], the results obtained with the proposed method can be considered in agreement with current demands [30].

[29] D.J. Hamilton, *Pure Appl. Chem.* 75, 1123 (2003).

[30] Method validation and quality control procedures for pesticide residues analysis in food and feed. European Commission 2011, SANCO/12495/2011.

Table I.7. Recovery study in spiked cucumber samples (n=6).

		CF	CAR	MTH	PR	FNX	BTH
Level 1[a]	R (%)	75.9	70.0	70.5	76.3	86.1	90.4
	(RSD %)	(9.1)	(10.9)	(5.7)	(8.0)	(6.3)	(10.6)
Level 2[b]	R (%)	70.0	71.2	71.8	71	71.0	80.0
	(RSD %)	(1.5)	(1.1)	(2.2)	(3.4)	(5.5)	(8.9)
Level 3[c]	R (%)	80.3	80.4	84.3	90.2	86.9	84.2
	(RSD %)	(11.3)	(3.7)	(1.2)	(2.0)	(5.0)	(3.4)

[a] **Level 1:** 5 µg kg^{-1} for PR, 10 µg kg^{-1} for CAR and MTH, 12.5 µg kg^{-1} for FNX and BTH, 20 µg kg^{-1} for CF.
[b] **Level 2:** 50 µg kg^{-1} , except CF (100 µg kg^{-1}).
[c] **Level 3:** 150 µg kg^{-1}, except PR (200 µg kg^{-1}).

A typical chromatogram corresponding to a separation of the analytes in cucumber sample under developed conditions is shown in Figure I.14.

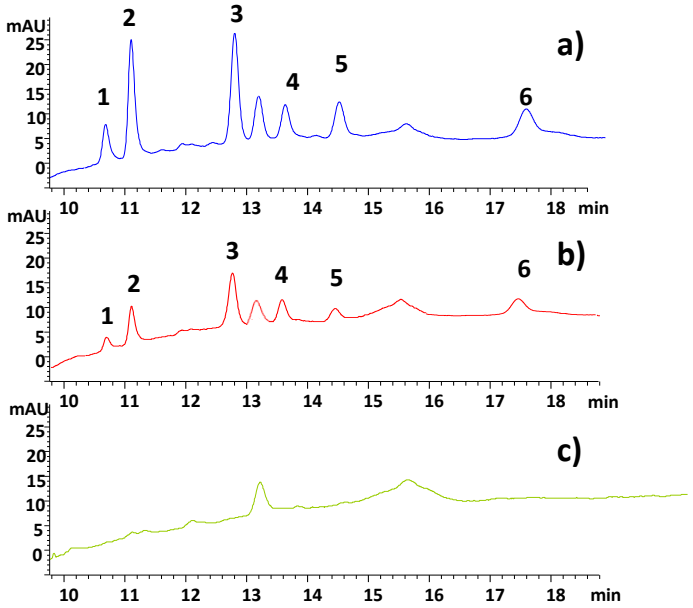

Figure I.14. Chromatograms of cucumber samples analyzed by capillary HPLC-DAD: **(a)** sample spiked with 150 µg kg^{-1} for all CRBs, except PR (200 µg kg^{-1}), **(b)** sample spiked with 50 µg kg^{-1} for all CRBs, except PR (25 µg kg^{-1}), **(c)** blank sample. Peaks: (1) CF, (2) CAR, (3) MTH, (4) PR, (5) FNX, and (6) BTH.

4. CONCLUSIONS

A sensitive capillary HPLC-DAD method has been proposed for the determination of six CRBs below the established MRLs in environmental waters and cucumber. The method has demonstrated its applicability for the analysis of these samples, showing very low LODs, with the advantage of lower solvent consumption, being in agreement with the Green Chemistry concept. The applied SPE procedure for sample preparation was very adequate for complex matrices such as cucumber; in the case of natural waters, it also allows a preconcentration step which can enhance the sensitivity for the analysis of the low level of residues expected in these samples. The developed capillary HPLC-DAD method could be satisfactorily applied as a routine procedure to determine CRBs in laboratories of food quality and safety control and also for the monitoring these residues in environmental waters, due to its robustness and feasibility, demonstrating the possibilities of capillary HPLC-DAD in this field.

This work was published as:

"Determination of carbamates at trace levels in water and cucumber by capillary liquid chromatography". David Moreno-González, José F. Huertas-Pérez, Laura Gámiz-Gracia, Ana M. García-Campaña, *Intern. J. Environ. Anal. Chem.* 91 (2011) 1329.

CAPÍTULO 2

Uso de la microextracción líquido-líquido dispersiva para la determinación de carbamatos en muestras de zumo mediante *c*romatografía capilar electrocinética micelar.

CHAPTER 2

Use of dispersive liquid–liquid microextraction for the determination of carbamates in juice samples by micellar electrokinetic chromatography

RESUMEN

En este capítulo se ha propuesto la microextracción líquido-líquido dispersiva (DLLME) para la extracción y preconcentración de 12 carbamatos en muestras de zumo, seguido de su determinación por cromatografía capilar electrocinética micelar con detección por fila de diodos. Con el fin de mejorar la sensibilidad, se ha desarrollado un método de preconcentración *on-line* en el capilar basado en el barrido de analitos (*sweeping)*, además de realizar la separación electroforética en un capilar burbuja de sílice fundida. El tampón de separación consistió en borato 100 mM y SDS 50 mM (pH 9.0) con 5% de acetonitrilo. Las muestras se introdujeron mediante inyección hidrodinámica disueltas en el tampón de separación, pero libre de micelas. Se realizó una optimización de los parámetros implicados en el procedimiento DLLME: tipo y volumen de los disolventes de extracción y de dispersión, pH, adición de sal, y tiempo de extracción. Las recuperaciones obtenidas para muestras de zumo (plátano, piña y tomate) fortificadas a tres niveles de concentración diferentes, estuvieron comprendidas entre 78% y el 105%, con desviaciones estándar relativas por debajo del 9%. Los límites de detección variaron de 1 a 7 $\mu g\ l^{-1}$. Además, el método es rápido, sencillo y respetuoso con el medioambiente.

SUMMARY

In this chapter, DLLME has been proposed for the extraction and preconcentration of 12 carbamate pesticides in juice samples, followed by their determination by micellar electrokinetic chromatography with diode array detection. To improve sensitivity, an on-capillary sample concentration method based on sweeping has been developed. Also, separations were performed in an extended light path fused-silica capillary; the separation buffer consisted of 100 mM borate and 50 mM SDS (pH 9.0) with 5% acetonitrile. Samples were introduced by hydrodynamic injection, dissolved in the separation buffer, but free of micelles. Several parameters of the DLLME procedure (such as type and volume of extraction and dispersive solvents, pH, salt addition, and extraction time) were optimized. Recoveries obtained for spiked juice samples (banana, pineapple, and tomato) at three different concentration levels, ranged from 78% to 105%, with relative standard deviations lower than 9%. The limits of detection ranged from 1 to 7 μg l^{-1}. Moreover, the method is fast, simple and environmentally friendly.

1. INTRODUCTION

Some of the CRBs, including CAR, carbendazim (CBZ), aldicarb (ALD), methomyl (MTY), oxamyl (OX), PR, CF, and MTH have been included in the final list of compounds to be considered for periodic re-evaluations by the Joint FAO/WHO Meeting on Pesticide Residues [1-3]. In the European Union (EU), the Regulation (EC) No. 396/2005 on MRLs of pesticides in products of plant and animal origin, and the

Regulation (EC) N. 149/2008 (amending Regulation (EC) No 396/2005), bring together and harmonizes in a single act all the limits applicable to the various types of food and feed and establishes a MRL of 10 μg kg^{-1} as a default value [4,5]. Currently, EU and USA are preparing recommendations on principles and practices for the establishment of MRLs for fruit juices [6].

Chromatographic methods have been usually used for the determination of CRBs in food, using mainly UV-Vis [7-10], fluorescence [11-15], and mass spectrometry (MS) detection

[1] Summary report. Acceptable daily intakes, acute reference doses, short-term and long-term dietary intakes, recommended maximum residue limits and supervised trials median residue values recorded by the 2010 meeting. 2010 Joint FAO/WHO Meeting on Pesticide Residues.

[2] Alinorm 01/24A Appendix IX. Priority list of compounds scheduled for evaluation or reevaluation by JMPR. 2001 Joint FAO/WHO Meeting on Pesticide Residues.

[3] Alinorm 07/30/24 Appendix VIII. Priority list of compounds scheduled for evaluation or reevaluation by JMPR. 2007 Joint FAO/WHO Food Standards Programme.

[4] Regulation (EC) No. 396/2005 of the European Parliament and of the Council on maximum residue levels of pesticides in or on food and feed of plant and animal origin and amending Council Directive 91/414/EEC. *Official Journal of the European Union* L 70, 16 March 2005, pp. 1–16.

[5] Regulation (EC) No 149/2008 amending Regulation (EC) No 396/ 2005 of the European Parliament and of the Council by establishing Annexes II, III and IV setting maximum residue levels for products covered by Annex I thereto. *Official Journal of the European Union* L 58, 2008, pp. 1–398.

[6] http://www.crlpesticides.eu/library/docs/fv/Beijing2009.pdf.

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[16- 22]. Capillary zone electrophoresis (CZE) with different detection systems [23,24] , and micellar electrokinetic chromatography (MEKC) with UV-Vis or diode array detection (DAD) has also been proposed for the determination of some CRBs in cucumber [25], tomato [26], carrots [27] and lettuce sample [28]. CE has been also coupled with MS for determination of CRBs in apple juices [29] and grape and orange juices [30].

Concerning sample treatment, several methods have been developed for the determination of CRBs in foodstuff [31], mainly SPE [8,29,32- 35], and liquid–liquid extraction [18,22,25]. Most of these methods are often complicated and their sample throughput is too low to achieve the challenges of food analysis. In recent years, different strategies have been proposed for simplifying sample treatment and preconcentration in pesticide residue analysis, which include QuEChERS (quick, easy, cheap, effective, rugged and safe) methodology [16,21], cloud-point extraction [7], floated organic drop

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microextraction [36], solid-phase microextraction (SPME) [9] and dispersive liquid–liquid microextraction (DLLME). This last method was introduced by Rezaee et al. in 2006 [37] and shows simplicity of operation, low cost and high recovery.

DLLME is based on a ternary component solvent system: the sample in an aqueous phase, an appropriate extraction solvent (i.e., a few microliters of an organic solvent such as chlorobenzene, chloroform or carbon disulfide) with high density, and a disperser solvent (such as MeOH, ACN, or ACO) with high miscibility in both extractant and aqueous phases. When the mixture of extractant phase and disperser is rapidly injected into the sample, a high turbulence is produced. This turbulent regimen gives rise to the formation of small droplets, which are dispersed throughout the aqueous sample. After the formation of the cloudy solution, the surface area between the extractant solvent and the aqueous sample becomes very large, so the equilibrium state is achieved quickly and, therefore, the extraction time is very short. In fact, this is the principal advantage of DLLME. After centrifugation of the cloudy solution, a sedimented phase is settled in the bottom of a conical tube and analyzed with the most appropriate analytical technique. The general aspects of DLLME are compiled in some recent reviews [38-41]. The applications of DLLME include the determination of CRBs in water samples [42-44] and the determination of CAR in fruit juices [12].

In order to overcome the lack of sensitivity of CE with UV detection, an on-line sample concentration method based on sweeping has been developed. This approach is designed to focus the analytes into a narrow band within the capillary, thereby increasing the sample volume that can be injected without any loss of efficiency. It uses the interactions between

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an additive (i.e., a pseudostationary phase or micellar media) in the separation buffer and the sample in a solvent similar to the separation buffer but free of additive. It involves the accumulation of charged and neutral analytes in the pseudostationary phase that penetrates the sample zone and "sweeps" the analytes, producing a focusing effect [45-47].

In the present chapter, sweeping-MEKC-DAD is proposed for the determination of twelve CRBs (OX, MTY, benomyl (BY), CBZ, asulam (ASL), propoxur (PX), CAR, ALD, CF, PR, MTH and napropamid(NP)) in fruit juice samples. Moreover, DLLME is proposed as extraction and preconcentration technique for a high number of analytes in a matrix no commonly explored with this methodology, being the main aim to show the applicability of both, the CE as separation technique and DLLME, as suitable alternatives for the determination of CRBs in juice samples.

2. EXPERIMENTAL

2.1. CHEMICALS

All reagents were of analytical reagent grade, unless indicated otherwise, and solvents were HPLC grade. Ultrapure water (Milli-Q plus system, Millipore, Bedford, MA, USA) was used throughout the work. Sodium hydroxide and sodium chloride were obtained from Panreac-Química (Madrid, Spain); tris(hydroxymethyl)aminomethane (Tris) was obtained from Merck (Darmstadt, Germany), hydrochloric acid from Scharlab (Barcelona, Spain); and boric acid, sodium dodecyl sulfate (SDS), sodium tetraborate decahydrate and imidazol from Sigma (St. Louis, MO, USA).

ACN, acetone (ACO), MeOH, EtOH, tetrahydrofuran (THF), and EtACO, were obtained from Merck; chloroform ($CHCl_3$), and dichloromethane (CH_2Cl_2), were obtained from VWR (West Chester, PA, USA); carbon disulfide (CS_2, analysis grade) was supplied by Carlo Erba

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(Rodano, Mi, Italy) and chlorobenzene (synthesis grade) by Alfa Aesar (Karlsruhe, Germany).

Analytical standards of CF, CAR, MTH, PR, OX, ALD, MTH, PX, and ASL were supplied by ChemService Inc (West Chester, USA), while BY, NP, BTH, FNX, and CBZ were supplied by Riedel-de Haën (Seelze, Germany). Individual stock standard solutions containing 3 g l^{-1} of each compound were prepared by dissolving accurately weighed amounts in MeOH and stored in the dark at 4 °C. They were stable for at least 4 months. Standard solutions containing all the CRBs were freshly prepared by proper dilution of the stock standard solutions with MeOH.

Acrodisc 13-mm syringe filters with 0.2-µm nylon membrane (Pall Corp., MI, USA) were used for filtration of sample extracts prior to the injection in the electrophoretic system.

2.2. INSTRUMENTATION

CE experiments were carried out with a $HP^{3D}CE$ instrument (Agilent Technologies, Waldbron, Germany) equipped with DAD. Data were collected using the software supplied with the HP ChemStation (Version A.09.01). Separations were performed in an uncoated fused-silica capillary (64.5 cm×50 µm ID, 56 cm effective length) with an optical path length of 200 µm (bubble cell capillary from Agilent Technologies).

A pH meter (Crison model pH 2000, Barcelona, Spain) with a resolution of ±0.01 pH unit, a centrifuge (Universal 320 model from Hettich, Leipzig, Germany), a mechanical shaker (model 384 from Vibromatic, Noblesville, USA), an evaporator with nitrogen (System EVA-EC from VLM GmbH, Bielefeld, Germany) and a vortex (Genie 2 model from Scientific Industries, Bohemia, USA) were also used.

2.3. ELECTROPHORESIS PROCEDURE

Before the first use, the new capillary was rinsed with 1 M NaOH at 60°C for 10 min, then with deionized water for 10 min, and finally with the background electrolyte (BGE) for 20 min. At the beginning of each session, the capillary was cleaned with deionized water for 3 min, then with 0.1 M NaOH for 7 min, deionized water for 1 min and finally with the BGE

for 20 min. Before each run, the capillary was pre-washed with 0.1 M NaOH for 2.5 min, deionized water for 1 min and finally with the BGE for 5 min. In all instances, a pressure of 7 bar was applied.

The BGE consisted of an aqueous solution of 100 mM boric acid and 50 mM SDS, pH 9.0 adjusted with 1 M NaOH and containing 5% ACN, v/v. A voltage of 27 kV (normal mode) was applied for the electrophoretic separation using a ramp of 0–27 kV in 0.5 min. The temperature of the capillary was kept constant at 27.5 ^{o}C. Standard solutions and samples prepared in a buffer similar to the BGE but without micellar medium, were hydrodynamically injected at 35 mbar for 25 s. The signal was monitored at a wavelength of 210 nm.

2.4. SAMPLE PREPARATION

DLLME was performed as follows: 50 ml of fruit juice (pH 7.5 adjusted with 1 M NaOH) was placed in a 50-ml falcon tube and centrifuged for 10 min at 9000 rpm. A 5-ml aliquot of the upper layer was placed into a 15-ml falcon tube with conical bottom. A mixture of MeOH (disperser solvent, 1500 µl) and chloroform (extraction solvent, 800 µl) was rapidly injected into the sample tube. The tube was closed and mechanically shaken for 5 min. After that, it was centrifuged for 2 min at 5000 rpm; the sedimented phase was removed using a syringe and collected in a glass vial. The chloroform phase was then evaporated at 30°C under nitrogen stream until dryness. The final residue was re-dissolved with 500 µl of 100 mM boric acid pH 9.0, 5% ACN (v/v), vortexed (2 min), filtered (Acrodisc 13-mm) and injected into the electrophoretic system. A schematic diagram of the sample preparation procedure is shown in Figure II.1. Following this treatment, sample throughput was approximately 12 samples per hour, obtaining a preconcentration factor of 10.

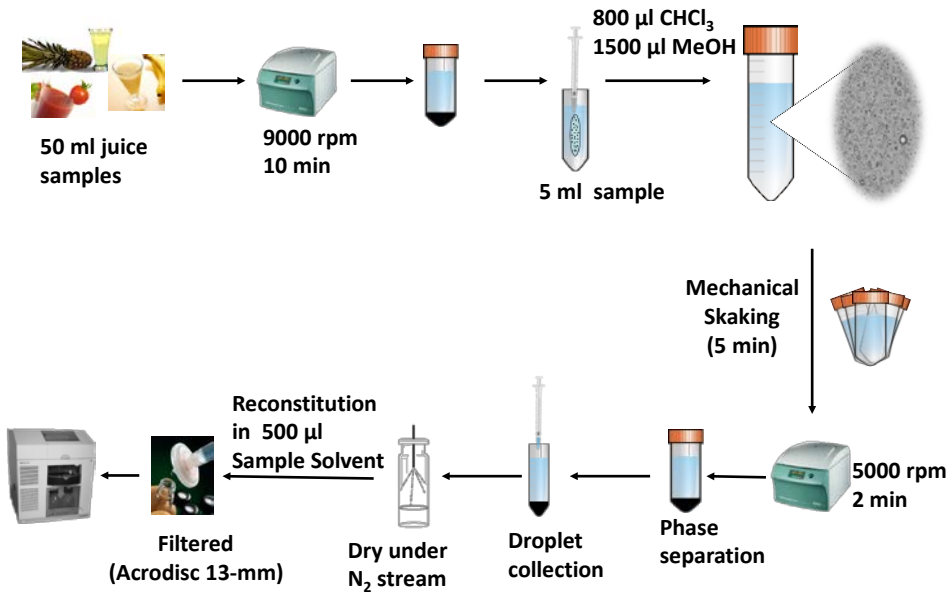

Figure II.1. Diagram of the DLLME procedure.

3. RESULTS AND DISCUSSION

3.1. OPTIMIZATION OF THE SWEEPING-MEKC EXPERIMENTAL CONDITIONS

Most of the CRBs studied are neutral compounds in a wide range of pH (see pKs in Introduction section), therefore using CZE, they cannot be separated. However, when a surfactant as SDS is used, different migration behaviors can be achieved, due to the different interactions between the CRBs and the micelles. Thus, MEKC was the separation mode of choice. The optimization of the main variables affecting the separation and simultaneous quantification of the CRBs by MEKC-DAD was carried out considering the resolution and the sensitivity (peak area) as response variables, keeping the generated current at optimum values.

First of all, the effect of pH between 7.5 and 9.5 was investigated using 100 mM boric acid with 50 mM SDS as BGE, adjusting the pH with NaOH. The effect of pH on the separation of fourteen CRBs is shown in Figure II.2. The best resolution was obtained at pH 9.0, especially in the case of BY and CBZ (peaks 3 and 4).

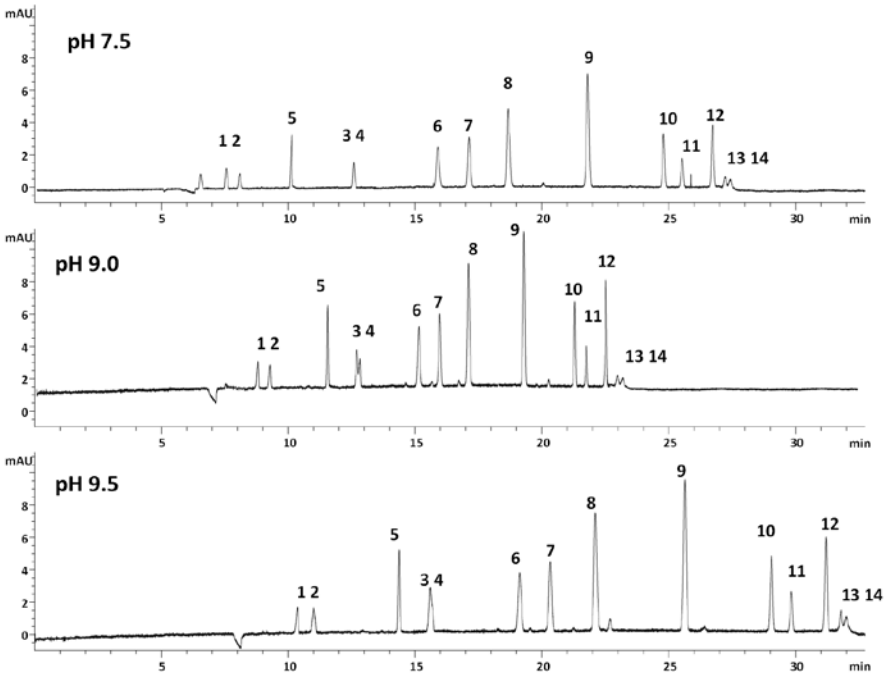

Figure II.2. Influence of the BGE pH (100 mM boric acid, 50 mM SDS). Peaks: (1) OX, (2) MTY, (3) BY, (4) CBZ, (5) ASL, (6) PX, (7) CF, (8) ALD, (9) CAR, (10) PR, (11) MTH, (12) NP, (13) BTH, and (14) FNX.

Then, different buffer solutions were tested ($Na_2B_4O_7$/HCl; H_3BO_3/NaOH; imidazol/HCl and Tris/HCl), all of them at a concentration of 100 mM, pH 9.0 and 50 mM SDS. As it can be observed in Figure II.3, the best results in terms of resolution and analysis time were obtained using H_3BO_3/NaOH, pH 9.0.

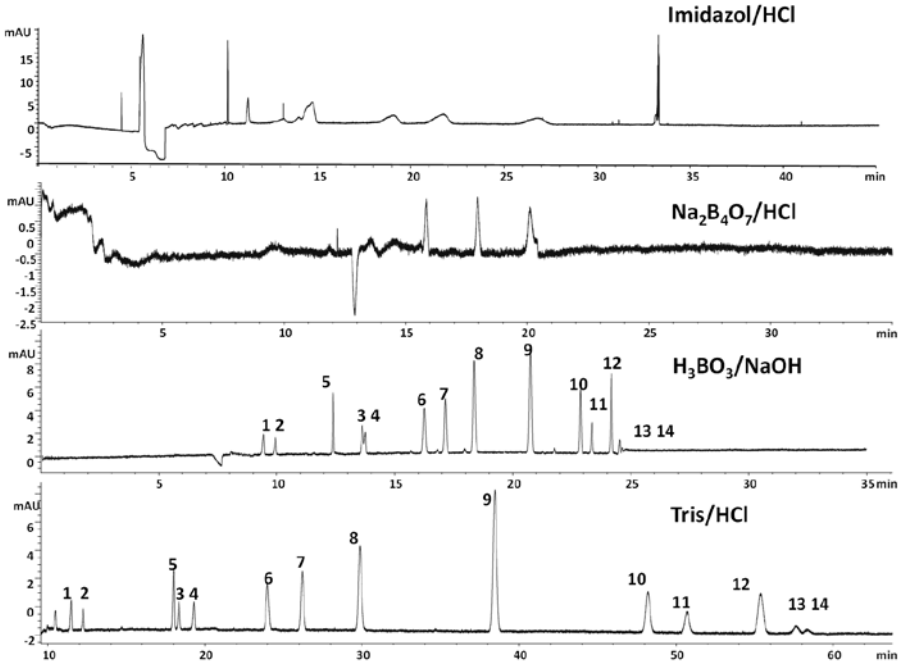

Figure II.3. Influence of the buffer BGE nature (buffer concentration 100 mM, pH 9.0, 50 mM SDS). Peaks: (1) OX, (2) MTY, (3) BY, (4) CBZ, (5) ASL, (6) PX, (7) CF, (8) ALD, (9) CAR, (10) PR, (11) MTH, (12) NP, (13) BTH, and (14) FNX.

Subsequently, boric acid concentration was modified between 50 and 150 mM, keeping the concentration of SDS at 50 mM and the pH at 9.0. The best results were obtained using a concentration of 100 mM, obtaining also a very low electric current (≈20 μA). The influence of the buffer concentration is shown in Figure II.4.

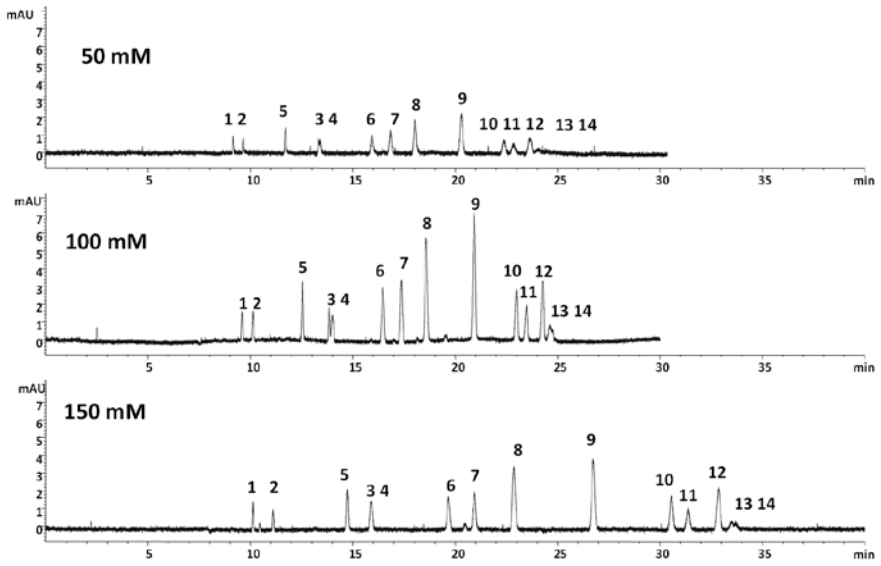

Figure II.4. Influence of the boric acid concentration in the BGE (pH 9.0, 50 mM SDS). Peaks: (1) OX, (2) MTY, (3) BY, (4) CBZ, (5) ASL, (6) PX, (7) CF, (8) ALD, (9) CAR, (10) PR, (11) MTH, (12) NP, (13) BTH, and (14) FNX.

Then, the SDS concentration was modified between 25 and 75 mM. According to the Figure II.5, the use of CZE (0 mM of SDS) resulted in an inefficient separation. As a result, it is clear that the CRBs present a great interaction with the micelles, showing an alteration in the migration times with the increase in the SDS concentration. The best signals were obtained using a concentration of 50 mM, as a compromise between resolution and analysis time.

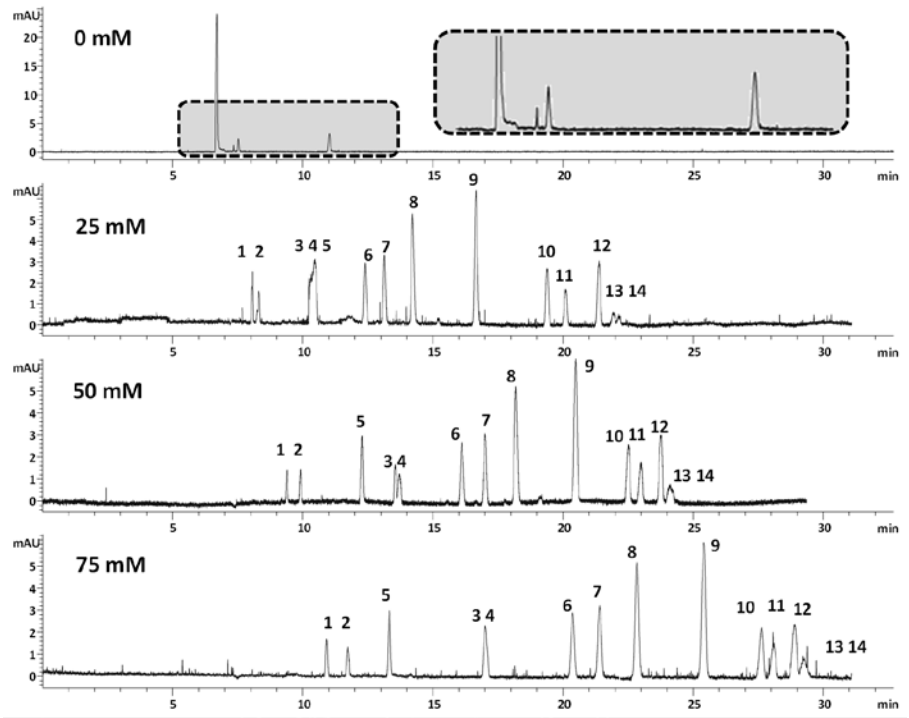

Figure II.5. Influence of the SDS concentration in the BGE (100 mM boric acid, pH 9.0). Peaks: (1) OX, (2) MTY, (3) BY, (4) CBZ, (5) ASL, (6) PX, (7) CF, (8) ALD, (9) CAR, (10) PR, (11) MTH, (12) NP, (13) BTH, and (14) FNX.

The addition of different organic modifiers to the running buffer, such as MeOH, EtOH, and ACN, was also considered. A significant improvement in resolution was obtained in the presence of ACN, and percentages of 2.5%, 5.0%, and 10% (v/v) were tested. An optimum of 5% ACN was selected, as it provided the best results in terms of resolution (R_S) between BY and CBZ and of analysis time. Also, it was observed that these analytes and ASL changed their migration sequence when ACN was added. These effects can be seen in Figure II.6.

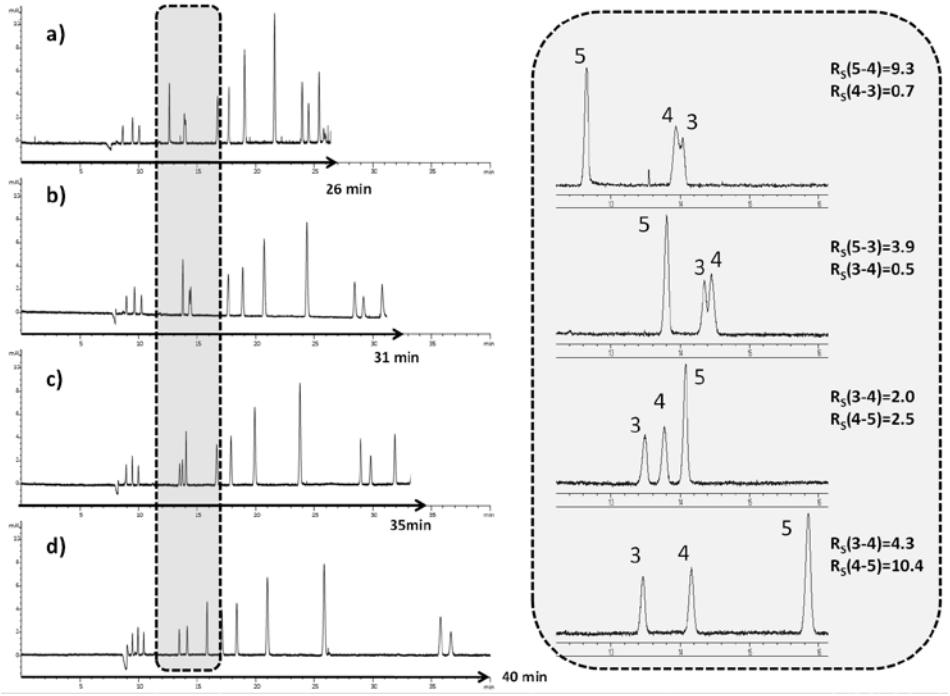

Figure II.6. Influence of organic modifier (ACN) in the BGE: **a)** without organic modifier; **b)** 2.5% ACN; **c)** 5% ACN; **d)** 10% ACN. Peaks: (3) BY, (4) CBZ and (5) ASL.

Once the running buffer had been optimized, the separation voltage was modified between 15 and 28 kV. A voltage of 27 kV was selected as optimum as a compromise between running time, resolution, and electric current (Figure II.7).

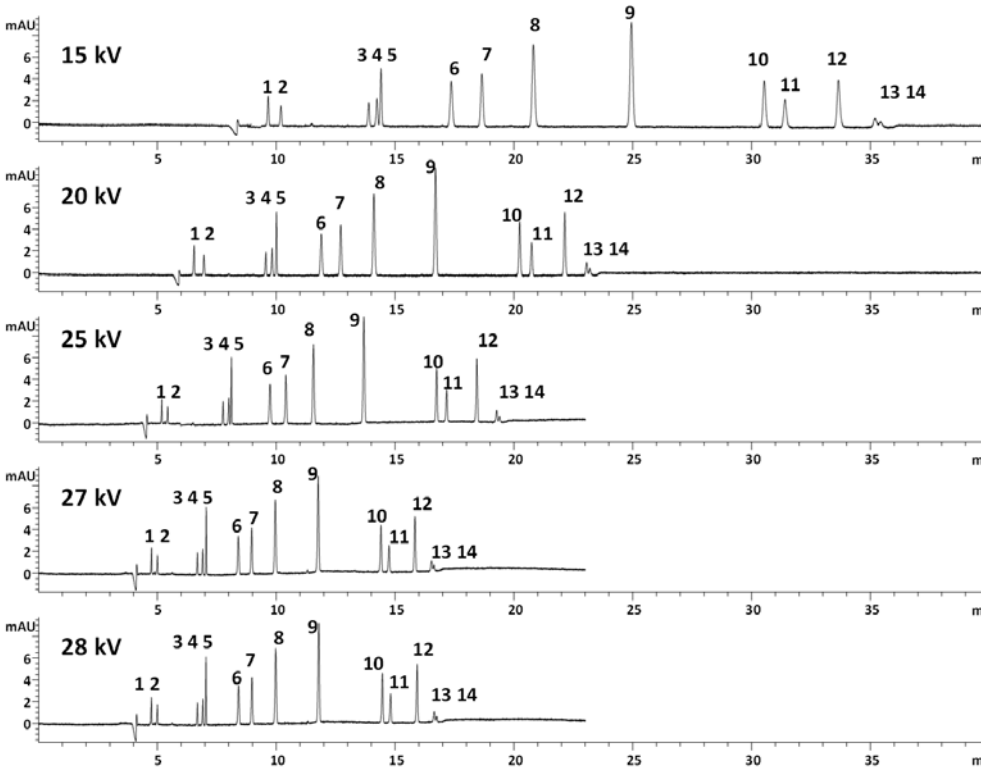

Figure II.7. Influence of the voltage. Peaks: (1) OX, (2) MTY, (3) BY, (4) CBZ, (5) ASL, (6) PX, (7) CF, (8) ALD, (9) CAR, (10) PR, (11) MTH, (12) NP, (13) BTH, and (14) FNX.

The effect of the temperature on the separation was also studied in the range of 20–30°C (Figure II.8). A decrease in the temperature resulted in a decrease in the generated current and EOF, and an increase in the migration times (due to lower electrolyte viscosity) and resolution. According to these effects, 27 °C was selected as suitable.

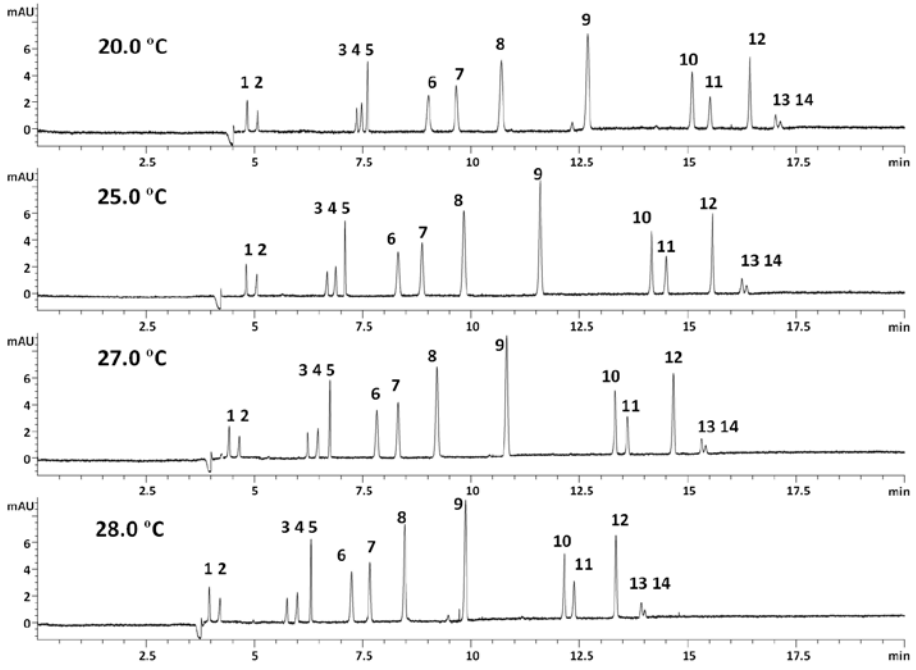

Figure II.8. Influence of the capillary temperature. Peaks: (1) OX, (2) MTY, (3) BY, (4) CBZ, (5) ASL, (6) PX, (7) CF, (8) ALD, (9) CAR, (10) PR, (11) MTH, (12) NP, (13) BTH, and (14) FNX.

In order to obtain the maximum sensitivity, different on-line preconcentration modes such as stacking by injection in water as low conductivity buffer and sweeping were evaluated (Figure II.9); in most of the cases, a slight improvement in sensitivity was obtained when sweeping strategy was used, so sweeping preconcentration was selected. As previously described in bibliography [45], the adequate sample solvent for an optimum collection and accumulation of analyte molecules by the pseudostationary phase was the running buffer but without micellar medium (SDS).

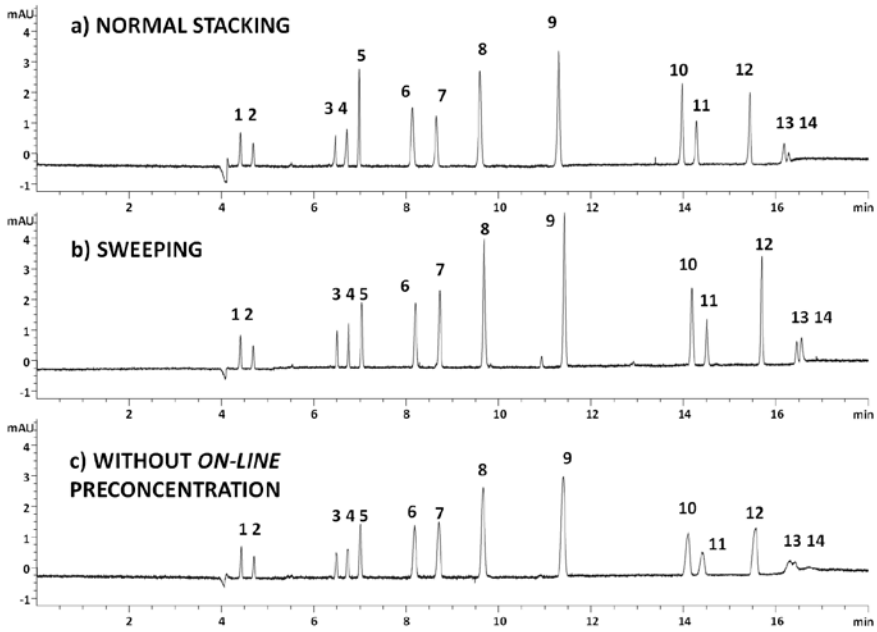

Figure II.9. Electropherograms corresponding to an injection of 35 mbar x 10 sec: **(a)** Solvent Sample: water; **(b)** Solvent Sample: Borate 100 mM (pH 9.0), 5 % ACN (V/V); **(c)** Solvent Sample: Borate 100 mM (pH 9.0), SDS 50 mM, 5 % ACN (V/V). Peaks: (1) OX, (2) MTY, (3) BY, (4) CBZ, (5) ASL, (6) PX, (7) CF, (8) ALD, (9) CAR, (10) PR, (11) MTH, (12) NP, (13) BTH, and (14) FNX.

Finally, the hydrodynamic injection time was studied from 10 to 75 s; 25 s at 35 mbar was used as optimum, equivalent to a volume of approximately 21 nl (≈2% of the total volume of the capillary). As a result (Figure II.10), an increment in the sensitivity up to four times was obtained, depending on the CRB, compared with the same injection volume without sweeping.

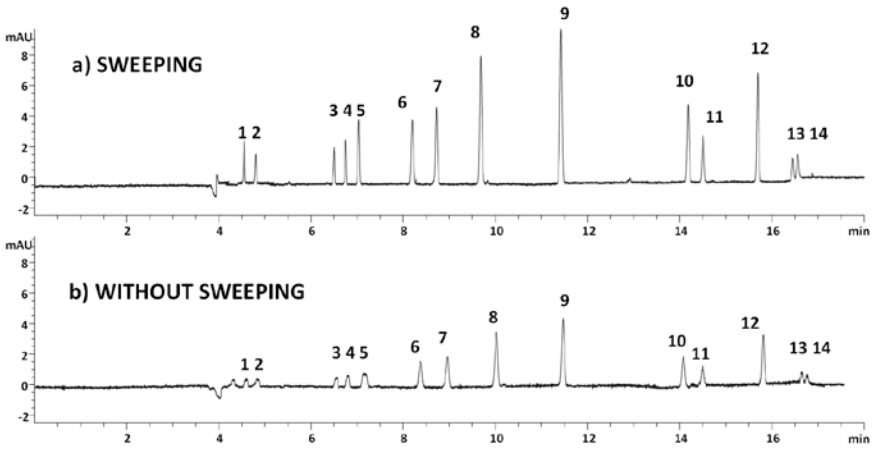

Figure II.10. Electropherograms corresponding to an injection of 35 mbar x 25 sec: **(a)** Solvent Sample: Borate 100 mM (pH 9.0), 5 % ACN (V/V); **(b)** Solvent Sample: Borate 100 mM (pH 9.0), SDS 50 mM, 5 % ACN (V/V). Peaks: (1) OX, (2) MTY, (3) BY, (4) CBZ, (5) ASL, (6) PX, (7) CF, (8) ALD, (9) CAR, (10) PR, (11) MTH, (12) NP, (13) BTH, and (14) FNX.

Once all the experimental variables were optimized, in order to improve the sensitivity, a capillary with an optical path length of 200 µm (bubble cell capillary) was tested. The increase in sensitivity was approximately 3 times.

As a summary, all the optimum values for the sweeping-MEKC-DAD separation are included in Table II.1.

Table II.1. Optimum values for the variables involved in the CE separation.

Variable	Optimum value
Capillary	64.5 cm x 50 µm ID, 56 cm effective length, 200 µm optical path length
BGE	Borate 100 mM (pH 9.0), SDS 50 mM, 5 % ACN (V/V)
Sample solvent	Borate 100 mM (pH 9.0), 5 % ACN (V/V)
Voltage	27 kV
Temperature	27 ^{o}C
Hydrodynamic injection	35 mbar x 25 sec
Wavelength	210 nm

3.2. OPTIMIZATION OF DLLME

There are some factors affecting the DLLME process, namely: extraction solvent, disperser solvent, volumes of extraction and disperser solvent, salt addition, pH, and extraction time. The initial optimization of these parameters was carried out using 5 ml of an aqueous mixture standard solution with a concentration of 150 µg l^{-1} of each CRB. The recovery (for each analyte and also the average recovery) was used to evaluate the extraction efficiency.

3.2.1. Type and volume of extraction solvent

The selection of an appropriate extraction solvent is very important for the DLLME process. The extraction solvent must meet three requirements, namely: a higher density than water; be immiscible with water; and provide the highest extraction efficiency for all the analytes. Among the solvents with a higher density than water, CH_2Cl_2 (1.32 g ml^{-1}), $CHCl_3$ (1.47 g ml^{-1}), chlorobenzene (1.11 g ml^{-1}), and CS_2 (1.26 g ml^{-1}) were tested, using a volume of 100 µl of extraction solvent with 1.0 ml of ACO as disperser solvent. As can be seen in Figure II.11, the best results were obtained using $CHCl_3$ as extraction solvent.

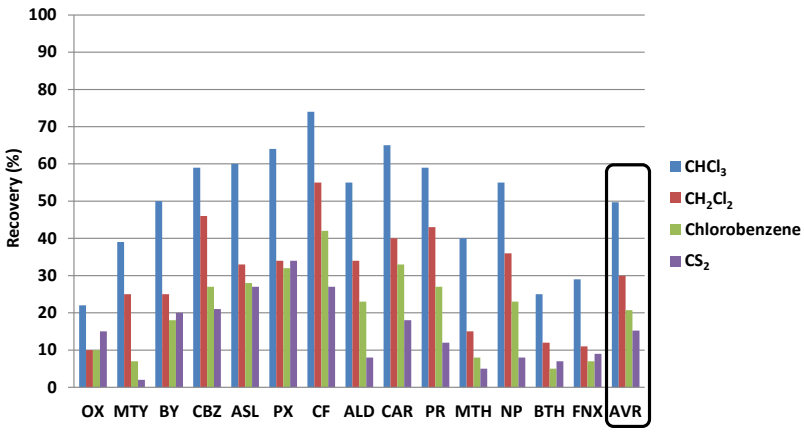

Figure II.11. Effect of different extraction solvents on the extraction recovery of the CRBs (AVR: average recovery) using 1 ml of ACO as disperser solvent.

Then, in order to study the effect of the extraction solvent volume on the extraction efficiency, different volumes of chloroform (between 50 and 700 µl) were tested, using 1.0 ml of ACO as disperser solvent (Figure II.12). The recovery increased with volumes of $CHCl_3$

up to 600 µl, and after that, the recoveries slightly decreased or did not suffer a significant variation. Therefore, 600 µl was selected as optimum.

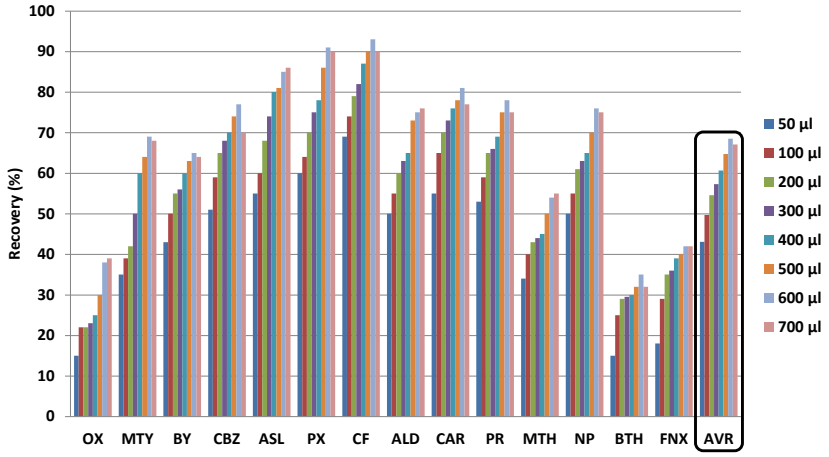

Figure II.12. Effect of the extraction solvent ($CHCl_3$) volume on the extraction recovery (AVR: average recovery) using 1 ml of ACO as disperser solvent.

3.2.2. Type and volume of disperser solvent

The disperser solvent must be miscible with the sample solution and the extraction solvent. Therefore, MeOH, EtOH, ACO, EtOAc, and THF were tested as disperser solvents, using 1 ml of each one containing 600 µl $CHCl_3$. According to Figure II.13, the best recoveries were obtained with MeOH.

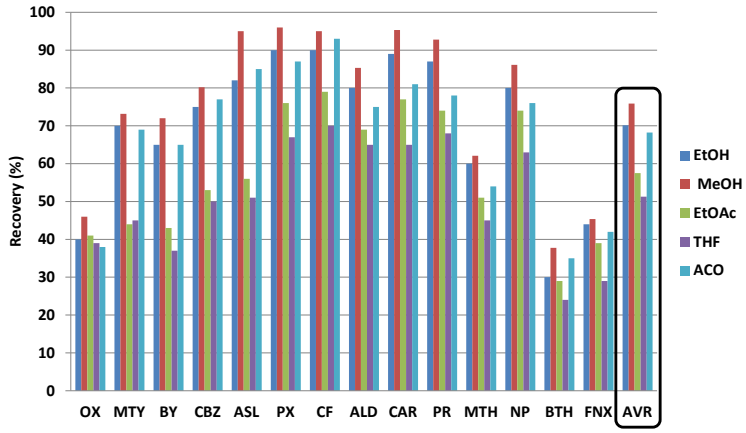

Figure II.13. Effect of different disperser solvents on the extraction recovery (AVR: average recovery) using 600 µl of $CHCl_3$ as extraction solvent**.**

Then, volumes of MeOH between 700 and 2200 µl, using 600 µl of $CHCl_3$ were tested (Figure II.14). A value of 1500 µl was selected, as it provided the best recoveries in most of the CRBs, including those with the lowest recoveries.

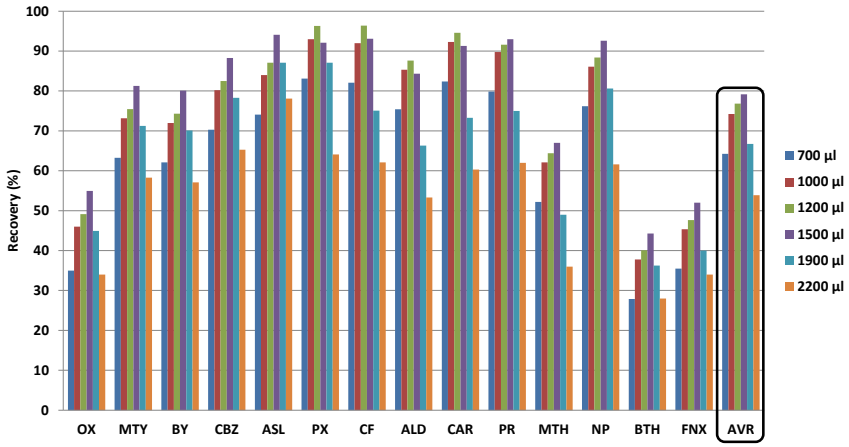

Figure II.14. Effect of the disperser solvent (MeOH) volume on the extraction recovery of the CRBs (AVR: average recovery) using 600 µl of $CHCl_3$ as extraction solvent.

3.2.3. Sample pH

In order to evaluate the effect of the sample pH in DLLME, experiments were carried out with buffered aqueous samples with pHs from 2.5 to 7.5. This pH range was selected according to the pKs of the CRBs, keeping most of them neutral. The results (Figure II.15) showed that the sample pH had a significant effect on the recoveries, being optimum at a pH of 7.5.

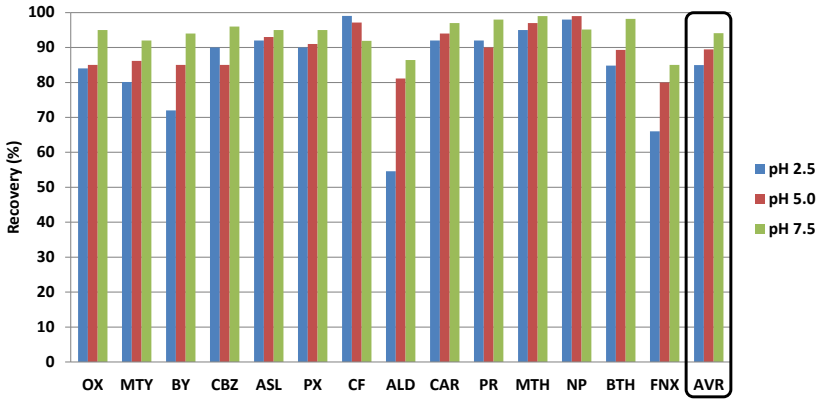

Figure II.15. Effect of different pHs on the extraction recovery (AVR: average recovery).

3.2.4. Salt addition

The influence of ionic strength on the performance of DLLME was investigated by adding different amounts of NaCl (0–5%, w/v) under the previous optimum conditions. However, as shown in Figure II.16, a decrease on the recoveries of all CRBs (except MTY) was observed when the ionic strength was increased. As a consequence, DLLME was carried out without any addition of salt.

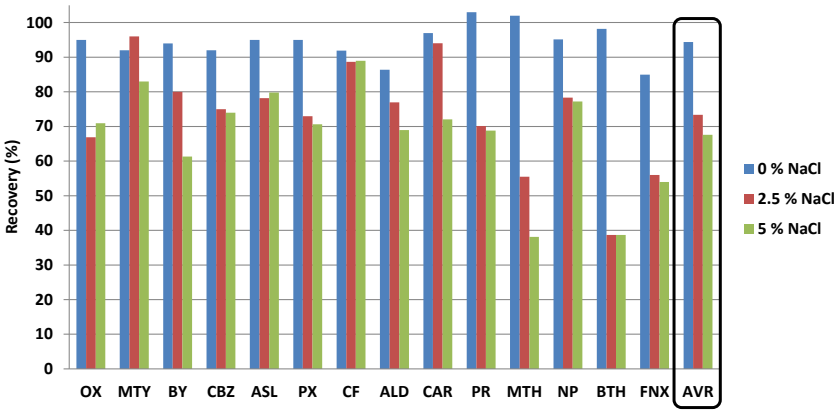

Figure II.16. Effect of addition of NaCl on the extraction recovery (AVR: average recovery).

3.2.5. Extraction time

In DLLME, extraction time is defined as the interval between the injection of the mixture of disperser and extraction solvents and the centrifugation step. In order to evaluate this parameter, different extraction times (from 0–10 min) were evaluated. The results showed that recoveries increased with extraction times up to 5 min, without a significant effect (or even with a slight decrease) for longer extraction times (Figure II.17). Therefore, 5 min was selected as extraction time.

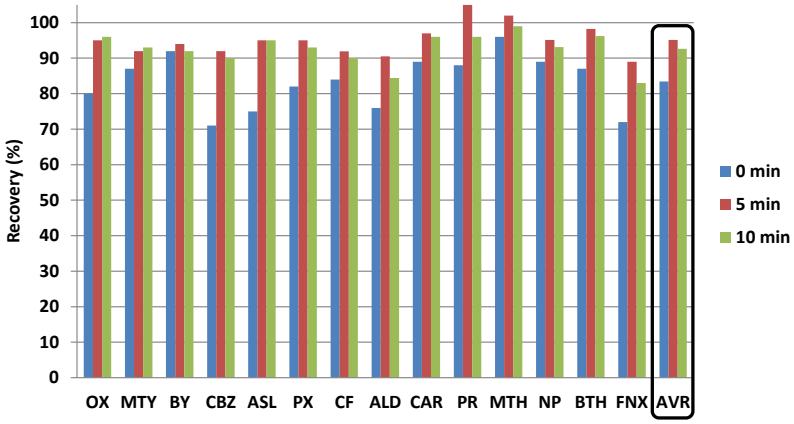

Figure II.17. Effect of the extraction time on the extraction recovery (AVR: average recovery).

3.2.6. Shaking mode

Finally, different shaking modes were compared: vortex, manual shaking, and mechanical shaking. Although no significant differences on the recoveries were observed, more reproducible results were obtained with the mechanical shaker (see Figure II.18), which was selected for the extraction.

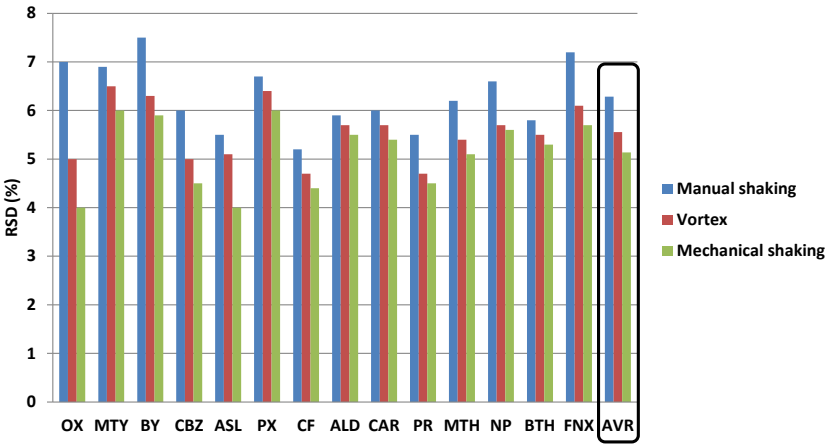

Figure II.18. RSD (%) of the results obtained with different shaking modes (n=6, AVR: average recovery).

The recoveries under these optimum conditions varied from 89% to 105% for the studied CRBs.

3.2.7. Optimization of DLLME for juice samples

Once the DLLME had been optimized with aqueous standard solutions, it was tested with commercially available banana juice samples. With this purpose, 50 ml of sample was spiked with 50 µg l^{-1} of each CRB and placed in a 50-ml falcon tube. This sample was centrifuged for 10 min at 9000 rpm and a 5-ml aliquot of the upper layer was placed into a 15-ml falcon tube with conical bottom. As the recoveries obtained were slightly lower than those obtained with aqueous standard solutions, a re-optimization of the volumes of extraction and disperser solvents was carried out; the optimum value for the extraction solvent was finally 800 µl of $CHCl_3$ (Figure II.19), while the optimum volume of dispersive solvent did not change. The rest of the variables remained constant.

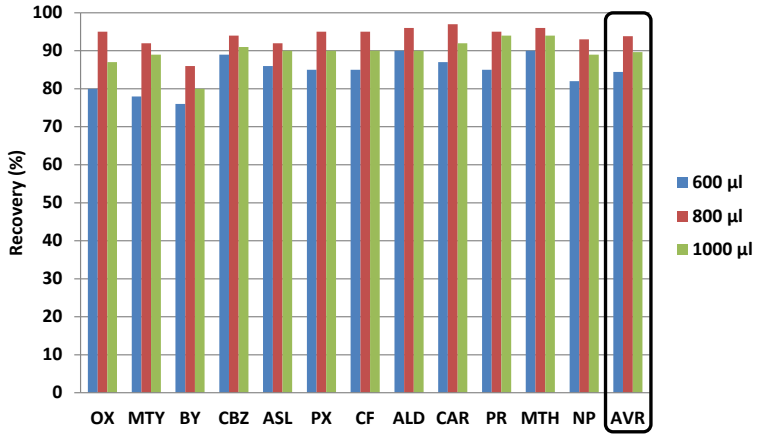

Figure II.19. Effect of the extraction solvent ($CHCl_3$) volume on the extraction recovery of the CRBs in banana juice (AVR: average recovery).

As can be observed in Figure II.20, migration times for the majority of CRBs changed from those obtained with aqueous standards. On the other hand, some interfering substances were found co-migrating with analytes 13 and 14 (corresponding to BTH and FNX respectively); attending this result, these analytes could not be determined in this matrix, and were not included in the validation of the method.

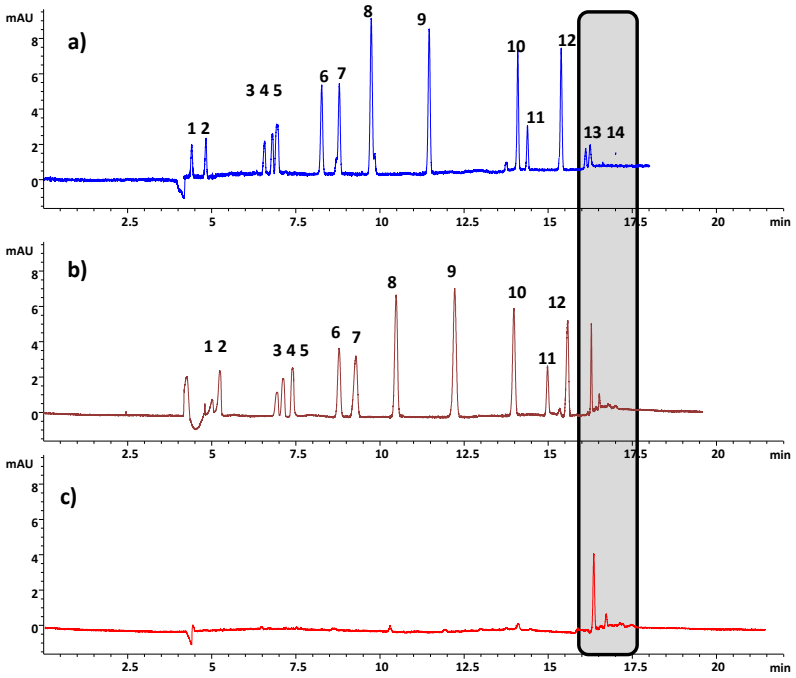

Figure II.20. Electropherograms corresponding to: **(a)** standard solution of 500 µg l^{-1} for all CRBs; **(b)** banana juice sample spiked with 500 µg l^{-1} of each compound after sample treatment; **(c)** blank sample. Peaks: (1) OX, (2) MTY, (3) BY, (4) CBZ, (5) ASL, (6) PX, (7) CF, (8) ALD, (9) CAR, (10) PR, (11) MTH, (12) NP, (13) BTH, and (14) FNX.

3.3. METHOD VALIDATION

3.3.1. Calibration curves and analytical performance characteristics of the method

Matrix-matched calibration curves were established using banana juice samples free of analytes as representative matrix, spiked with different analyte concentrations: 10, 20, 50, 100 and 200 µg l^{-1} for CAR and NP; 5, 10, 20, 50 and 100 µg l^{-1} for OX, MTY, CBZ, ASL and ALD; and 25, 50, 100, 200 and 500 µg l^{-1} for BY, CF, PR, MTH and PX. Each concentration level was processed following the DLLME method and injected in triplicate. Peak area was considered as a function of analyte concentration in the sample. A blank sample was also processed, and none of the CRBs was detected.

LODs and LOQs were calculated as 3×S/N and 10×S/N, respectively. Statistics and performance characteristics of the method in banana juice samples are shown in Table II.2. MRLs for these types of matrices have not been established; so MRLs for natural fruit were selected as reference value. As can be seen, good LOQs were obtained for all the analytes, being lower than the corresponding MRLs, except for OX. Compared to other reported methods for the determination of CRBS in juice samples, the proposed CE method provides similar sensitivity, allowing the determination of a higher number of compounds [8,9].

Table II.2. Statistic and performance characteristics of the DLLME-sweeping-MEKC-DAD method in banana juice samples.

Analyte	Linear dynamic range ($\mu g\ l^{-1}$)	R^2 (%)	LOD ($\mu g\ l^{-1}$)	LOQ ($\mu g\ l^{-1}$)	MRL ($\mu g\ Kg^{-1}$)[a]
OX	9.7-1000	99.6	4.3	9.7	10
MTY	10.2-1000	99.7	3.1	10.2	20
BY	24.3-500	99.5	7.2	24.3	100
CBZ	10.4-1000	99.3	3.2	10.4	100
ASL	16.3-1000	99.6	4.9	16.3	500
PX	8.7-500	99.5	2.9	8.7	50
CF	13.6-500	99.8	4.1	13.6	20
ALD	13.4-1000	99.7	4.2	13.4	20
CAR	4.2-200	99.4	1.1	4.2	50
PR	7.9-500	99.1	3.2	7.9	20
MTH	8.0-500	99.7	2.1	8.0	100
NP	6.3-200	99.6	1.7	6.3	50

[a] Pesticide EU-MRLs for banana fruit. Directive 91/414/EEC Regulation (EC) No 396/2005 (MRLs updated on 11/10/2010) [4].

3.3.2. Trueness assessment

In order to check the trueness of the proposed methodology for the analysis of juice samples, recovery experiments were carried out in different commercial juices (banana, tomato and pineapple). Trueness was evaluated over five samples spiked at three different concentrations of CRBs and injected in triplicate (see Table II.3). In all the cases, a blank sample was analyzed to check the presence of CRBs, and none of them gave a result above the LODs of the method. Taking into account that in pesticide residue analysis the acceptable range for recovery has been established between 70% and 120%, the results obtained with the proposed method (ranging between 78% and 105%) can be considered in agreement with the current demand [48].

A typical electropherogram corresponding to a banana juice sample spiked with increasing concentrations of CRBs, analyzed by the proposed DLLME-sweeping-MEKC-DAD method is shown in Figure II.21.

[48] Method validation and quality control procedures for pesticide residues analysis in food and feed. European Commission 2011, SANCO/12495/2011.

Table II.3. Recovery study of NMCs in spiked juice samples (n=15).

			OX	MTY	BY	CBZ	ASL	BPX	CF	ALD	CAR	PR	MTH	NP
Banana juice	Level 1[a]	R (%)	80.1	85.5	78.9	95.5	89.3	91.2	92.7	95.0	98.1	97.7	96.2	97.6
		(RSD)	(4.6)	(4.2)	(4.9)	(5.6)	(4.5)	(6.9)	(4.0)	(6.7)	(5.3)	(4.5)	(5.3)	(5.5)
	Level 2[b]	R (%)	81.2	89.3	82.2	93.2	80.1	93.9	94.3	92.1	95.5	95.0	94.7	93.1
		(RSD)	(4.9)	(5.4)	(5.8)	(4.7)	(4.2)	(6.0)	(4.5)	(5.9)	(5.4)	(4.5)	(5.1)	(5.7)
	Level 3[c]	R (%)	92.1	91.1	93.7	92.1	91.1	92.7	92.0	93.0	93.7	92.7	91.6	92.0
		(RSD)	(5.3)	(4.9)	(5.2)	(4.1	(3.9)	(5.2)	(4.9)	(4.9)	(5.1)	(3.7)	(4.7)	(5.1)
Pineapple juice	Level 1[a]	R (%)	89.0	88.8	94.6	93.0	92.3	93.5	94.1	96.7	93.4	95.0	92.2	94.3
		(RSD)	(4.9)	(4.7)	(5.1)	(5.4	(4.5)	(6.0)	(4.6)	(5.9)	(5.4)	(4.7)	(5.5)	(5.9)
	Level 2[b]	R (%)	91.1	92.3	94.7	97.0	94.6	97.6	92.9	95.6	98.9	93.6	99.1	105.6
		(RSD)	(4.8)	(4.5)	(5.0)	(5.7)	(4.0)	(5.8)	(4.7)	(5.5)	(5.2)	(4.6)	(5.0)	(5.7)
	Level 3[c]	R (%)	93.8	93.6	96.0	95.9	96.7	98.9	94.7	95.6	99.1	98.1	97.8	102.3
		(RSD)	(4.5)	(4.0)	(4.5)	(5.5)	(3.9)	(5.3)	(4.8)	(5.1)	(4.7)	(4.2)	(5.1)	(5.5)
Tomato juice	Level 1[a]	R (%)	81.3	87.7	81.1	94.2	91.1	93.0	95.3	94.2	96.2	95.5	93.2	92.2
		(RSD)	(5.1)	(4.1)	(5.6)	(5.5)	(4.9)	(5.7)	(5.0)	(5.3)	(5.0)	(5.3)	(4.9)	(5.6)
	Level 2[b]	R (%)	83.7	85.3	83.4	95.5	92.2	94.5	95.1	93.3	95.0	92.2	9.6	94.5
		(RSD)	(5.0)	(3.7)	(5.4)	(5.0)	(4.2)	(5.4)	(4.8)	(4.9)	(5.2)	(4.5)	(4.4)	(4.9)
	Level 3[c]	R (%)	83.3	84.9	86.7	96.2	93.1	95.2	96.0	94.0	101.7	95.9	99.4	97.8
		(RSD)	(4.5)	(3.5)	(4.9)	(5.1)	(4.3)	(5.0)	(4.3)	(5.0)	(4.4)	(4.7)	(4.2)	(4.1)

[a]**Level 1:** 10 µg l^{-1} for CAR, NP, PR and MTH, 25 µg l^{-1} for OX, MTY, CBZ, ASL, ALD, BY, PX and CF.
[b]**Level 2:** 100 µg l^{-1} for CAR and NP, 200 µg l^{-1} for OX, MTY, CBZ, ASL, ALD, BY, PX, CF, PR and MTH.
[c]**Level 3:** 200 µg l^{-1} for CAR and NP, 500 µg l^{-1} for BY, PX, CF, PR and MTH, 1000 µg l^{-1} for OX, MTY, CBZ, ASL and ALD.

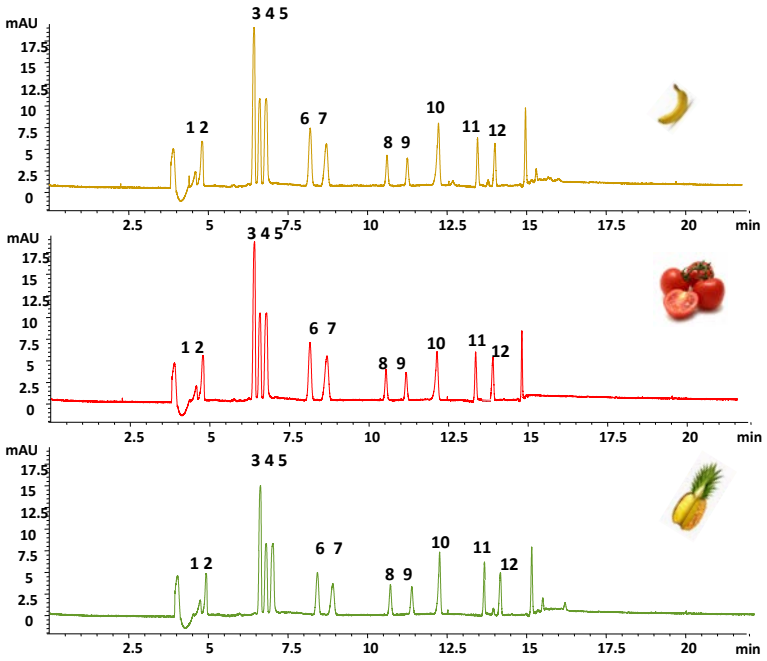

Figure II.21. Electropherograms of a banana, tomato and pineapple juice samples analyzed by the DLLME-MEKC-sweeping-DAD method: spiked with 200 µg l^{-1} for CAR and NP, 500 µg l^{-1} for BY, PX, CF, PR and MTH, 1000 µg l^{-1} for OX, MTY, CBZ, ASL and ALD (level 3). Peaks: (1) OX, (2) MTY, (3) BY, (4) CBZ, (5) ASL, (6) PX, (7) CF, (8) ALD, (9) CAR, (10) PR, (11) MTH, and (12) NP.

3.3.3. Precision study

The precision of the method was evaluated in terms of repeatability (intraday precision) and intermediate precision (interday precision) by application of the proposed DLLME-sweeping-MEKC-DAD method to samples of banana, pineapple and tomato juices spiked at three different concentrations of CRBs. Repeatability was evaluated over five samples prepared and injected in triplicate on the same day, under the same conditions. Intermediate precision was evaluated with a similar procedure, but the samples were analyzed in five consecutive days. The results, expressed as RSD (%) of the peak areas, are summarized in Table II.4. Good precision, lower than 8.9%, was obtained in all cases.

Table II.4. Precision of the method for spiked juice samples.

	OX	MTY	BY	CBZ	ASL	PX	CF	ALD	CAR	PR	MTH	NP
Banana juice												
Repeatability RSD (%) (n=15)												
Level 1[a]	4.6	4.2	4.9	5.6	4.5	6.9	4.0	6.7	5.3	4.5	5.3	5.5
Level 2[b]	4.9	5.4	5.8	4.7	4.2	6.0	4.5	5.9	5.4	4.5	5.1	5.7
Level 3[c]	5.3	4.9	5.2	4.1	3.9	5.2	4.9	4.9	5.1	3.7	4.7	5.1
Intermediate precision RSD (%) (n=15)												
Level 1[a]	6.1	7.1	7.8	5.5	7.7	7.6	5.7	7.7	5.8	6.4	7.5	6.3
Level 2[b]	6.9	6.7	7.1	5.7	7.5	7.5	5.5	7.8	5.5	6.3	7.3	6.0
Level 3[c]	5.7	6.5	6.9	5.3	7.1	7.1	6.1	7.5	5.8	6.0	7.1	5.9
Pineapple juice												
Repeatability RSD (%) (n=15)												
Level 1[a]	4.9	4.7	5.1	5.4	4.5	6.0	4.6	5.9	5.4	4.7	5.5	5.9
Level 2[b]	4.8	4.5	5.0	5.7	4.0	5.8	4.7	5.5	5.2	4.6	5.0	5.7
Level 3[c]	4.5	4.0	4.5	5.5	3.9	5.3	4.8	5.1	4.7	4.2	5.1	5.5
Intermediate precision RSD (%) (n=15)												
Level 1[a]	6.2	7.0	6.9	6.3	8.2	8.9	6.3	6.5	6.7	6.5	7.2	8.7
Level 2[b]	6.1	6.3	5.7	5.9	7.4	7.2	6.2	6.0	6.3	6.4	7.3	7.5
Level 3[c]	5.8	6.2	5.9	6.2	7.0	7.3	6.0	6.2	6.3	6.6	6.9	7.9
Tomato juice												
Repeatability RSD (%) (n=15)												
Level 1[a]	5.1	4.1	5.6	5.5	4.9	5.7	5.0	5.3	5.0	5.3	4.9	5.6
Level 2[b]	5.0	3.7	5.4	5.0	4.2	5.4	4.8	4.9	5.2	4.5	4.4	4.9
Level 3[c]	4.5	3.5	4.9	5.1	4.3	5.0	4.3	5.0	4.4	4.7	4.2	4.1
Intermediate precision RSD (%) (n=15)												
Level 1[a]	7.2	7.0	7.9	7.3	6.7	6.5	5.9	6.2	7.2	6.9	7.2	8.7
Level 2[b]	7.0	6.6	7.6	7.1	6.1	6.6	6.2	6.3	6.7	6.6	7.0	8.5
Level 3[c]	6.8	6.4	7.0	6.7	5.8	6.9	5.7	6.9	6.5	6.7	8.3	8.5

[a]**Level 1:** 10 μg l^{-1} for CAR, NP, PR and MTH, 25 μg l^{-1} for OX, MTY, CBZ, ASL, ALD, BY, PX and CF.
[b]**Level 2:** 100 μg l^{-1} for CAR and NP, 200 μg l^{-1} for OX, MTY, CBZ, ASL, ALD, BY, PX, CF, PR and MTH.
[c]**Level 3:** 200 μg l^{-1} for CAR and NP, 500 μg l^{-1} for BY, PX, CF, PR and MTH, 1000 μg l^{-1} for OX, MTY, CBZ, ASL and ALD.

4. CONCLUSIONS

In this chapter, a method for the simultaneous determination of 12 CRBs in fruit juice samples by MEKC-DAD with sweeping on-line preconcentration has been developed. The analytes can be separated and detected in less than 18 min. Moreover, DLLME has been proposed for sample preparation as an attractive methodology for extraction and off-line preconcentration, being an easy, inexpensive and fast method for aqueous matrices such as different fruit juices, allowing high extraction efficiency. Both off-line and on-line preconcentration steps increased the sensitivity of the analysis, allowing the determination of the pesticides at low concentrations, even using DAD detection. Compared to other reported methods for the determination of CRBs in juice samples, the proposed MEKC method provides similar sensitivity, allowing the determination of a higher number of compounds, with higher recoveries and with the advantage of a cleaner, simpler, and quicker sample treatment. As a conclusion, the proposed method could be satisfactorily applied as a routine procedure to quantify CRBs in laboratories of food quality and safety, demonstrating the possibilities of DLLME and CE in the analysis of foodstuff.

This work was published as:

"Use of dispersive liquid-liquid microextraction for the determination of carbamates in juice samples by sweeping-micellar electrokinetic chromatography". David Moreno-González, Laura Gámiz-Gracia, Ana M. García-Campaña and Juan M. Bosque-Sendra. *Analytical and Bioanalytical Chemistry* 400 (2011) 1329.

CAPÍTULO 3

Determinación de carbamatos en aguas por cromatografía capilar electrocinética micelar-espectrometría de masas empleando la microextracción líquido-líquido dispersiva

CHAPTER 3

Determination of carbamates in waters by micellar electrokinetic chromatography-mass spectrometry using dispersive liquid–liquid microextraction

RESUMEN

Se ha optimizado un nuevo método analítico basado en la cromatografía capilar electrocinética micelar acoplada a espectrometría de masas en tándem empleando un surfactante volátil (perfluorooctanoato de amonio). El método se ha validado para la identificación y cuantificación simultánea de diecisiete carbamatos en diferentes tipos de agua, tanto medioambientales como de consumo. El detector de masas empleado fue una trampa de iones en modo de reacción múltiple. Los diferentes parámetros, tanto de la separación electroforética como de la detección, han sido optimizados con el fin de obtener una adecuada sensibilidad. Por otra parte, para el tratamiento de muestra se ha propuesto la microextracción líquido-líquido dispersiva, empleando un disolvente de extracción de baja densidad, obteniéndose un factor de preconcentración de 10. En condiciones óptimas, la recuperación de las muestras fortificadas varió entre el 83% al 101%, con desviaciones estándar relativas inferiores al 8%. Se obtuvieron límites de detección desde 1 hasta 144 ng l^{-1}, lo que demuestra la sensibilidad y la aplicabilidad del método para la determinación de estos analitos en aguas de diversa procedencia, siendo además rápido, simple y respetuoso con el medioambiente.

SUMMARY

A new analytical method based on micellar electrokinetic chromatography tandem mass spectrometry employing a mass spectrometry friendly surfactant (ammonium perfluorooctanoate) has been optimized and validated for the identification and simultaneous quantification of seventeen carbamate pesticides in environmental and drinking water samples. Mass spectrometry detection using an ion trap as analyzer operating in the multiple reaction monitoring mode was used. Different parameters were optimized in order to obtain an adequate separation combined with the highest sensitivity. Dispersive liquid–liquid microextraction using a low-density extraction solvent has been proposed for extraction, obtaining a preconcentration factor of 10. Under optimum conditions, recoveries for fortified samples ranged from 83% to 101%, with relative standard deviations lower than 8%. The limits of detection ranged from 1 to 144 ng l^{-1}, showing the sensitivity and applicability of this fast, simple, and environmentally friendly method.

1. INTRODUCTION

As stated in Chapter 1, MRL of individual CRBs allowed by the European Directive on the protection of groundwater against the pollution is 100 ng l^{-1} [1]. In the case of drinking water, the pesticide control is also highly relevant, because it has been shown that the presence of these pesticides could produce adverse effects. For example, their presence may be playing a role in the increasing prevalence of food allergies [2]. For this reason, EU has established an individual MRL of 100 ng l^{-1} for each CRB in this type of samples [3]; consequently very sensitive analytical methods to detect trace levels of these compounds in environmental and drinking waters are needed.

As commented in the previous Chapter, when using CE for the determination of CRBs, MEKC is the mode of choice, as most of these pesticides are neutral compounds in a wide range of pH and different migration behaviors can be achieved, due to the different interactions between the CRBs and the surfactant micelles [4- 9]. However, MEKC with UV detection has inadequate sensitivity for direct detection of pesticide residues at the trace level of these compounds in water samples and important preconcentration factors must be achieved by using different sample pretreatment, mainly SPE [10]. Moreover, when coupling CE to MS detection, there is an additional drawback, as commonly used surfactants (as SDS) are non-volatile and can cause analyte signal suppression and contamination of the MS. A number of different approaches have been suggested to

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circumvent the problem [11]; specifically in the analysis of CRBs, the application of atmospheric pressure photoionization (APPI) mode instead of electrospray ionization (ESI) for CEC-MS using an approach based on surfactant-bound monolithic column [12] or partial-filling MEKC [13], introduced by Ozaki and Terabe [14], have been proposed, but the results showed low resolution and poor reproducibility. Nevertheless, direct coupling of MEKC to MS is possible using ammonium perfluorooctanoate (APFO) as volatile surfactant [15,16]. This surfactant has a fluorocarbon chain, giving it a low surface energy, which is related to low inter-molecular interactions [17] and a high volatility [18], being compatible with the ESI-MS systems. This strategy was firstly applied for the analysis of ten CRBs in tap water using MS as detection mode [19].

In this chapter we propose the combination of DLLME with a "home-made" extraction vessel using a low-density extraction solvent, with a powerful technique, such as sheath flow MEKC–ESI–MS/MS employing APFO as surfactant, for simultaneous quantification and identification of seventeen CRBs in different types of waters. The designed vessel consisted of a pipette tip appropriately covered which can be directly used in the centrifuge to separate both phases more quickly. To the best of our knowledge, the use of APFO as surfactant in a sheath-flow MEKC–ESI–MS/MS method had not yet been reported to determine such a high number of CRBs simultaneously; moreover, this is the first time that this strategy is combined with a renovated and efficient green method for extraction and preconcentration such as DLLME, obtaining satisfactory recoveries and a higher sensitivity compared with other MEKC–MS methods previously reported.

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2. EXPERIMENTAL

2.1. CHEMICALS

Water, Isopropanol (IPA), MeOH, EtOH and ACN (LC–MS Chromasolv grade) were supplied from Fluka Analytical (Steinheim, Germany). APFO 75 mM pH 9.0 solution was prepared with perfluorooctanoic acid 96% (Sigma–Aldrich; St. Louis, MO, USA) and ammonium hydroxide (Panreac-Química; Barcelona, Spain). Formic acid was obtained from Sigma–Aldrich. Acetic acid and citric acid were supplied from Merck (Darmstadt, Germany).

Analytical grade ACN, ACO, and MeOH for sample treatment were obtained from Merck, while toluene, cyclohexane and n-hexane were obtained from VWR (West Chester, PA, USA).

Pestanal grade analytical standards of CF, CAR, MTH, PR, OX, ALD, MTY, PX, ASL, BY, NP, CBZ, ethiofencarb (ETH), aldicarb sulfoxide (ALDSFX), carbosulfan (CSF), BTH and FNX were supplied by Fluka. Individual stock standard solutions containing 3 g l^{-1} of each compound were prepared by dissolving accurately weighed amounts in MeOH and stored in the dark at 4 ^{o}C. They were stable for at least 4 months. Working standard solutions containing all the CRBs were freshly prepared by proper dilution of the stock standard solutions with MeOH.

Pipette tips (5 ml) were supplied by Brand GmbH (Wertheim, Germany). Acrodisc 13-mm syringe filters with 0.2 μm nylon membrane (Pall Corp., MI, USA) were used for filtration of sample extracts prior to the injection in the electrophoretic system.

2.2. INSTRUMENTATION

CE experiments were carried out with an HP^{3D} CE instrument (Agilent Technologies, Waldbron, Germany) equipped with a UV-Vis detector working at 210 nm with a bandwidth of 16 nm. UV-Vis electropherograms were acquired using the software provided by the HP ChemStation version A.09.01. The coaxial sheath liquid sprayer was supplied by Agilent Technologies. MS was performed using an Agilent 1100 Series LC/MSD SL mass

spectrometer equipped with an ion trap (IT) analyzer. MS was controlled by a PC running the Esquire software 4.1 from Bruker Daltonics (Bremen, Germany).

The StatGraphics Plus Software 5.1 (Statistical Graphics Rockville; MD, USA) was used to generate the experimental designs and data processing. Thermogravimetric Analysis (TGA,TA instruments, Delaware, USA) was also used.

A pH-meter (Crison model pH 2000; Barcelona, Spain) with a resolution of ±0.01 pH unit, a centrifuge (Universal 320 model from Hettich; Leipzig, Germany), a nitrogen evaporator (System EVA-EC from VLM GmbH, Bielefeld, Germany) and a vortex (Genie 2 model from Scientific Industries; Bohemia, NY, USA) were also used.

2.3. ELECTROPHORETIC PROCEDURE

Separation was carried out in a bare fused-silica capillary (70.5 cm total length, 50 µm ID, 375 µm OD) from Polymicro Technologies (Phoenix, AZ, USA). The electrophoretic separation was achieved using a voltage of 23 kV (normal mode) with an initial ramp of 0.3 min. The BGE was an aqueous solution of 75 mM perfluorooctanic acid adjusted to pH 9.0 with 14.2 M ammonium hydroxide. The temperature of the capillary was kept constant at 24 ^{o}C. Before the first use, the capillary was conditioned by flushing with 1 M NaOH for 10 min at 60 ^{o}C, then with water for 10 min, and finally with the BGE for 20 min. At the beginning of each session, the capillary was prewashed with water (3 min), 5 M ammonium hydroxide (3 min), water again (3 min) and BGE (20 min), always applying a N_2 pressure of 1 bar. In order to obtain a satisfactory repeatability of run-to-run injections, before each run the capillary was pre-washed with 5 M ammonium hydroxide for 2.5 min, water for 1 min and finally with the BGE for 5 min, applying a pressure of 1 bar. At the end of the analysis, the capillary was washed with water during 5 min, and then it was left empty with air for 5 min, applying a N_2 pressure of 1 bar.

2.4. ESI INTERFACE

A coaxial sheath liquid sprayer was used for MEKC–MS coupling (Agilent Technologies). The fused-silica capillary was mounted in such a way that the tip just protruded from the surrounding steel needle −1/2 of the capillary OD. The sheath liquid consisted of IPA: formic acid (99.9:0.1, v/v) and was delivered at a flow rate of 1.66 µl min^{-1} by a KD Scientific 100 series syringe pump (KD Scientific Inc.; Holliston, MA, USA). The ESI voltage was set to −4800 V. Other electrospray parameters at optimum conditions were: nebulizer pressure, 12 psi; dry gas flow rate, 8 l min^{-1}; and dry gas temperature, 180 ^{o}C.

2.5. MS AND MS/MS CONDITIONS

The mass spectrometer was operating in the positive ion mode and scanned from 85 to 385 m/z at 13000 m/z s^{-1}. In the MS experiments, the IT parameters were selected in ion charge control mode using a target of 30000, maximum accumulation time of 200 ms, and 5 averages per experiment. In MS/MS experiments, the maximum accumulation time was set at 50 ms with 2 averages per experiment, using a target of 50000. Fragmentation was carried out by means of collision induced dissociation with the helium present in the trap for 40 ms in multiple reaction monitoring (MRM) mode. MS/MS spectra and their corresponding structures are shown in Figure III.1, while MS/MS parameters are summarized in Table III.1.

Table III.1. Main parameters of the MS/MS method.

Segment time (min)																
	0-6	6-7				7-9.5					9.5-12			12-15.5		15.5-17
	CBZ+BY	MTY	ALDSFX	ASL	CAR	ALD	CF	MTH	OX	PX	PR	ETH	BTH	FNX	NP	CSF
Width (m/z)	1.0	1.0	3.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.0
Cutoff (m/z)	52	44	56	62	55	56	60	61	60	58	56	61	70	82	73	103
Amplitude (V)	0.82	0.50	0.55	0.60	0.60	0.50	0.65	0.55	0.5	0.65	0.65	0.55	0.80	0.80	0.80	0.75
Precursor ion	192[a]	163[a]	207[a]	231[a]	202[a]	116[b]	222[a]	226[a]	237[c]	210[a]	208[a]	226[a]	258[a]	302[a]	272[a]	381[a]
Product ions	160	122 106 88 65	132 89.1 76.2	188 156	145 127	89 70 68	165 163	169 121	90 72	168 153 111	151 109	169 164 107	125 116 100	256 116 88	199 171 128	222 195 160 128 118

[a] $[M+H]^+$; [b] $[M+H-75]^+$; [c] $[M+NH_4]^+$

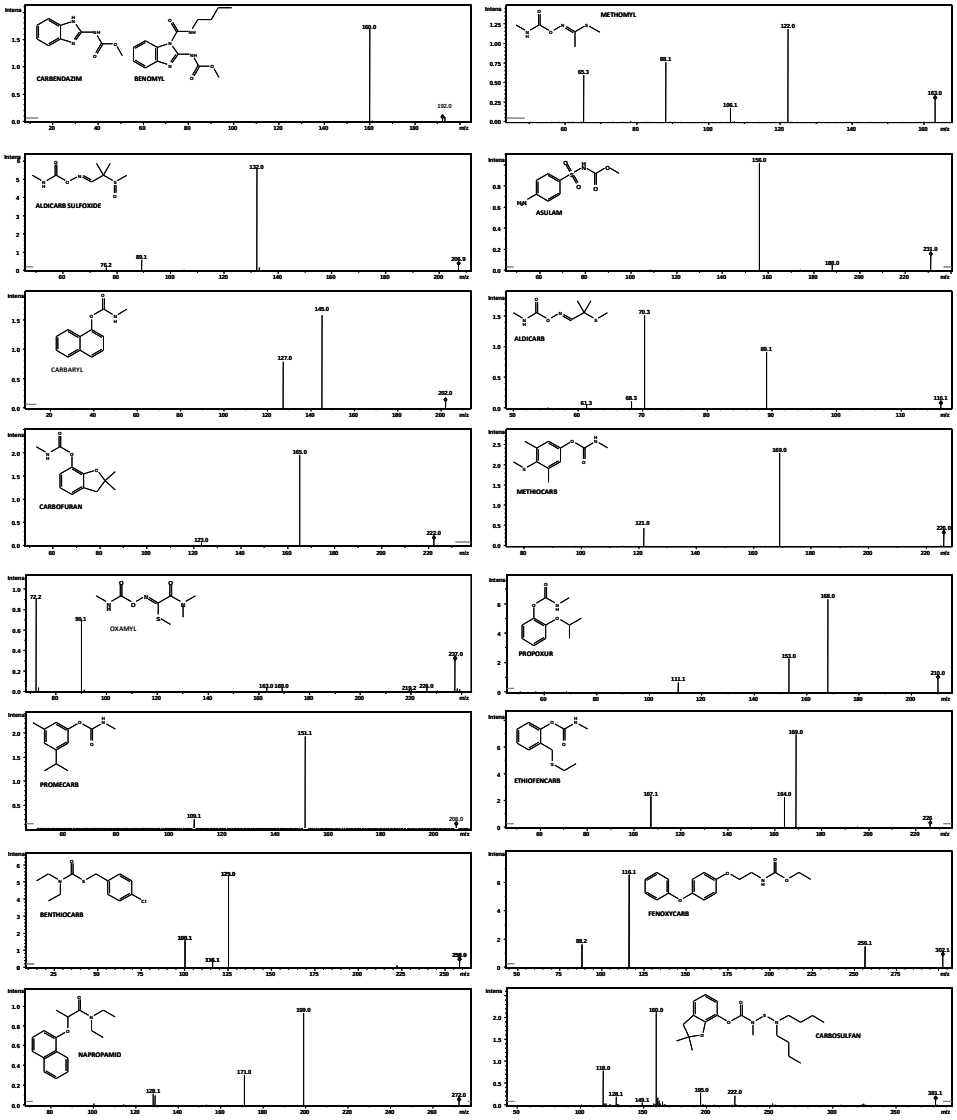

Figure III.1. MS/MS spectra of the studied CRBs and their corresponding structures.

2.6. SAMPLE PREPARATION

DLLME was performed as follows: 4.5 ml of water sample (pH 2.0, adjusted with 50 mM citric acid) was placed in a 5 ml pipette tip (extraction vessel). A mixture of ACN (disperser solvent, 940 µl) and toluene (extraction solvent, 636 µl) was rapidly injected into the sample tube. The extraction vessel was closed and shaken for 5 min by vortex. Then, it was centrifuged for 5 min at 2000 rpm; the upper phase was removed using a syringe and collected in a glass vial. The toluene was evaporated under nitrogen stream until dryness. The final residue was re-dissolved with 450 µl of 57 mM APFO pH 9.0, shaken by vortex (2 min), filtered (Acrodisc 13-mm) and injected into the electrophoretic system. A schematic diagram of the sample preparation procedure is shown in Figure III.2. Following this treatment, sample throughput was approximately 12 samples per hour, obtaining a preconcentration factor of 10.

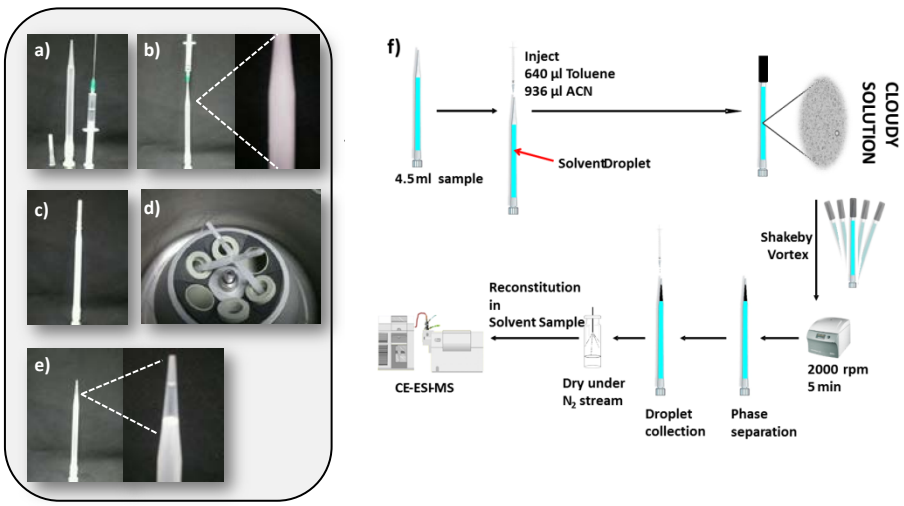

Figure III.2. Diagram of the DLLME procedure: **(a)** before injection; **(b)** injection and formation of a cloudy solution; **(c)** covered extraction vessel; **(d)** centrifugation; **(e)** after centrifugation; **(f)** total process.

3. RESULTS AND DISCUSSION

3.1. OPTIMIZATION OF THE MEKC EXPERIMENTAL CONDITIONS

As commented in the Introduction, most of the CRBs studied are neutral compounds in a wide range of pH (see Introduction). Thus, MEKC was the selected separation mode. Before coupling to MS, the electrophoretic separation of the selected compounds was optimized using a UV-Vis detector, considering the resolution and the sensitivity (peak area) as response variables. In order to consider the compatibility of CE with MS, electric current was kept below 50 µA, as upper values could produce plugging of the dielectric capillary between the spray chamber and the MS, originating an unstable spray.

First of all, the effect of pH was investigated between 8.0 and 9.5 using 50 mM APFO as BGE, and adjusting pH with ammonium hydroxide. As can be observed in Figure III.3, the best resolution was obtained at pH 9.0.

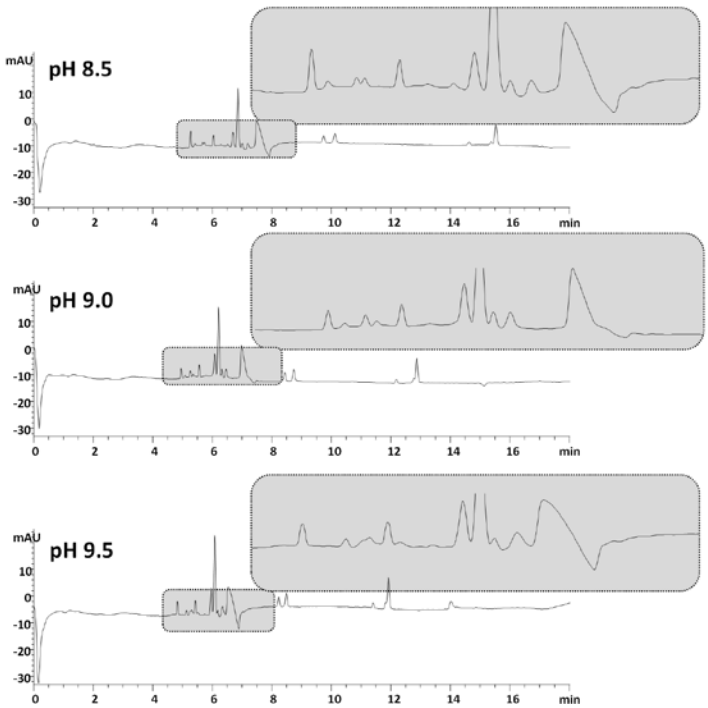

Figure III.3. Obtained electropherograms for the studied CRBs at different pHs.

Thus, APFO concentration was modified between 25 and 100 mM, keeping the pH at 9.0, being this variable one of the most critical to obtain a satisfactory separation. As shown in Figure III.4, the best results were obtained using a concentration of 75 mM, obtaining also a very low electric current (IQ≈31 µA). It can be observed that low concentrations of APFO did not allow adequate resolution of the peaks, while with values higher than 75 mM, a longer analysis time was obtained; thus, 75 mM was selected.

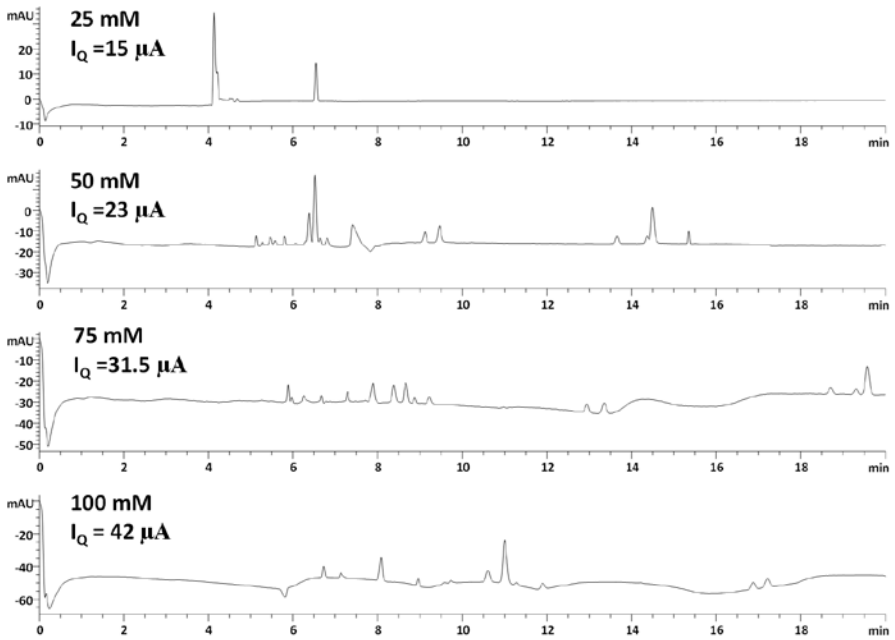

Figure III.4. Influence of the APFO concentration on the migration times of the CRBs. I_Q=current intensity.

The addition of different organic modifiers to the BGE (such as MeOH and ACN) was also considered (Figure III.5). However, a negative effect in separation efficiency was observed, because organic modifiers changed partitioning mechanism and, as consequence, most of CRBs overlapped. For this reason, organic modifiers were not considered for the rest of the study.

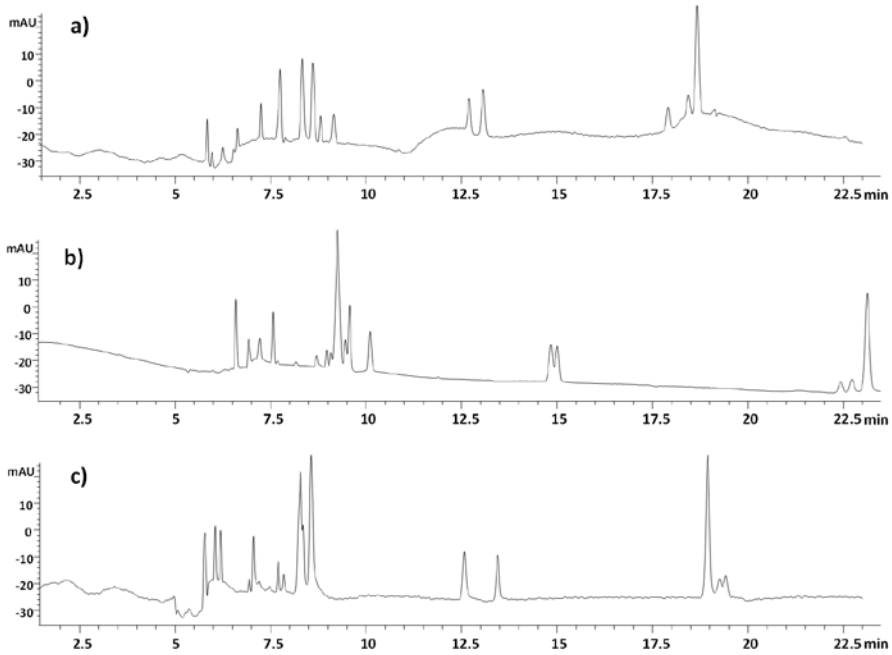

Figure III.5. Influence of organic modifiers in the BGE: **(a)** without organic modifier **(b)** 5% MeOH **(c)** 5% ACN.

Subsequently, the separation voltage was modified between 15 and 28 kV. A voltage of 23 kV was selected as optimum as a compromise between running time, resolution, and electric current. Finally, the effect of the temperature on the CE separation was studied in the range of 20–30 ^{o}C, and a capillary temperature of 24 ^{o}C was selected as optimum.

As a summary, all the optimum values for the CE separation are included in Table III.2.

Table III.2. Optimum values for the variables involved in the CE separation.

Variable	Optimum value
Capillary	70.5 cm x 50 µm ID
BGE	75 mM APFO, pH 9.0
Voltage	23 kV
Temperature	24 ^{o}C

3.3. THERMOGRAVIMETRIC ANALYSIS OF APFO

TGA was used in order to know the thermal stability of APFO in order to consider its compatibility with MS detection. The TGA analysis of APFO (Figure III.6) showed that it decomposed practically in 100 % at approximately 150 °C. Thus, it can be concluded that APFO can be used as volatile surfactant as it has a decomposition point compatible with MS.

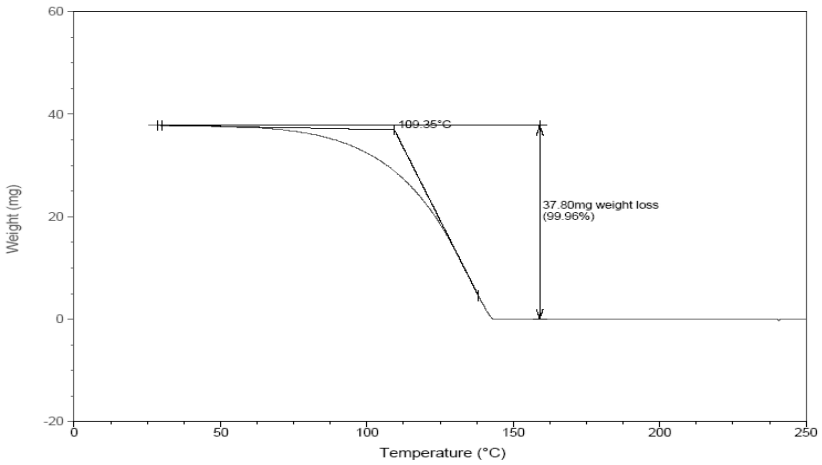

Figure III.6. Thermogravimetric curve of APFO.

3.4. MEKC–ESI–MS/MS OPTIMIZATION

Once the optimum values for separation had been selected, the optimization of the parameters affecting the CE–MS and CE–MS/MS was carried out. In order to select the best operation mode (ESI+ or ESI−), 1% formic acid or 1% ammonium hydroxide were added to the sheath liquid, which consisted of a mixture of IPA: water (90:10). The best results were obtained when the positive mode was applied. When negative mode was used, analytes were not detected because no ionization was observed in presence of APFO (see Figure III.7).

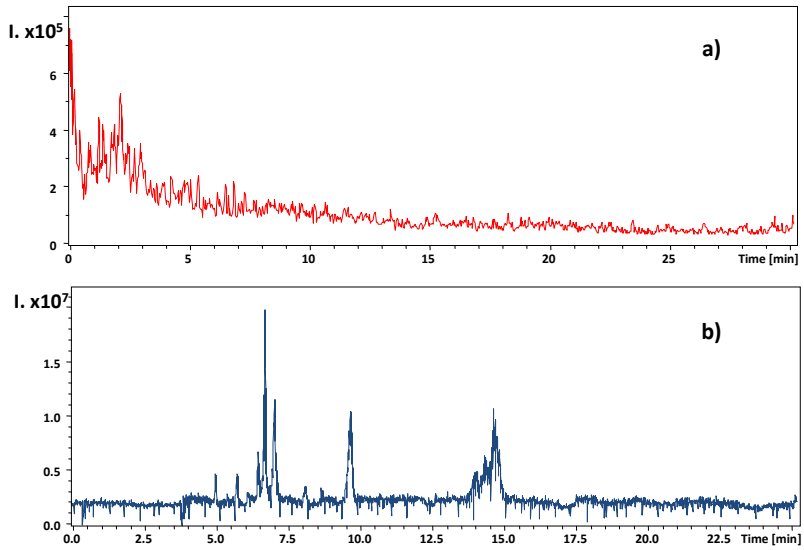

Figure III.7. Total Ion Chromatogram of the 17 CRBs: **a)** ESI-negative mode; **b)** ESI-positive mode.

Then, in order to know what type of acid is more adequate for an optimum ionization of the CRBs, 1% acetic and formic acid were evaluated (Figure III.8), obtaining the best results when formic acid was included in the sheath liquid.

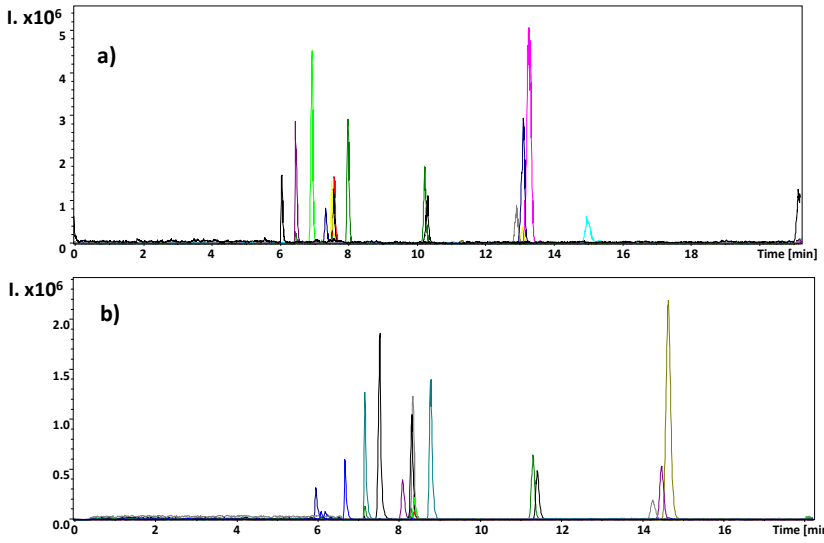

Figure III.8. Influence of the type of acid in the sheath liquid IPA: water (90:10): **(a)** 1% formic acid; **(b)** 1% acetic acid.

In addition, it was necessary to select the organic solvent of the sheath liquid. Mixtures of ACN, MeOH, EtOH and IPA with water (1% formic acid) were tested, all at a ratio of 90:10. The best results in terms of MS signal intensity for the studied CRBs were obtained using the mixture IPA: water. The influence of the organic solvent is shown in Figure III.9.

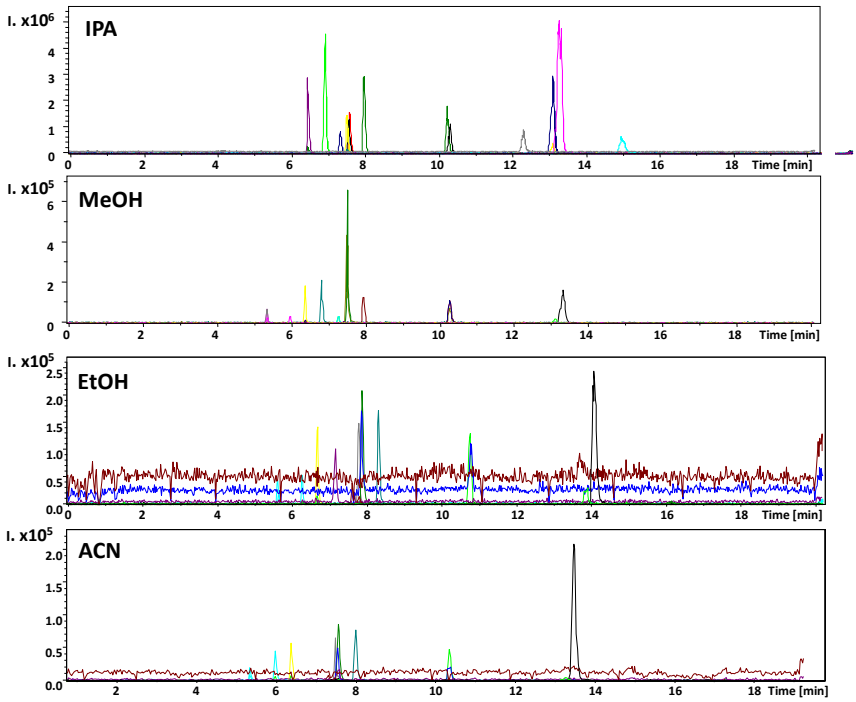

Figure III.9. Influence of the kind of organic solvent in the sheath liquid.

Then, the percentages of IPA (from 80 to 100%) and formic acid (from 0.1 to 1.5%) were evaluated; the best sheath liquid composition in terms of signal intensity and stable current was IPA: formic acid (99.9:0.1%).

After optimizing the sheath liquid, parameters affecting the sensitivity of MS detection (such as capillary voltage, skimmer, cap exit, Oct 1 DC, Oct 2 DC, Trap drive, Oct RF, Lens 1 and Lens 2) were optimized by direct infusion, filling the capillary with each analyte dissolved in the BGE and applying the separation voltage, using the selected sheath liquid in the syringe infusion pump. This implies a continuous supply of each analyte in order to optimize satisfactorily these parameters. In most of the ESI-MS reported methods, neither MS parameters nor the optimization procedure are mentioned. If low detection limits are

not required, the default MS parameters could be applied, and an extra-optimization is not critical. However, this procedure is crucial for obtaining the highest S/N ratio and the lowest detection limits. As can see in Figure III.10, after this optimization, intensity signal was up to one order of magnitude higher.

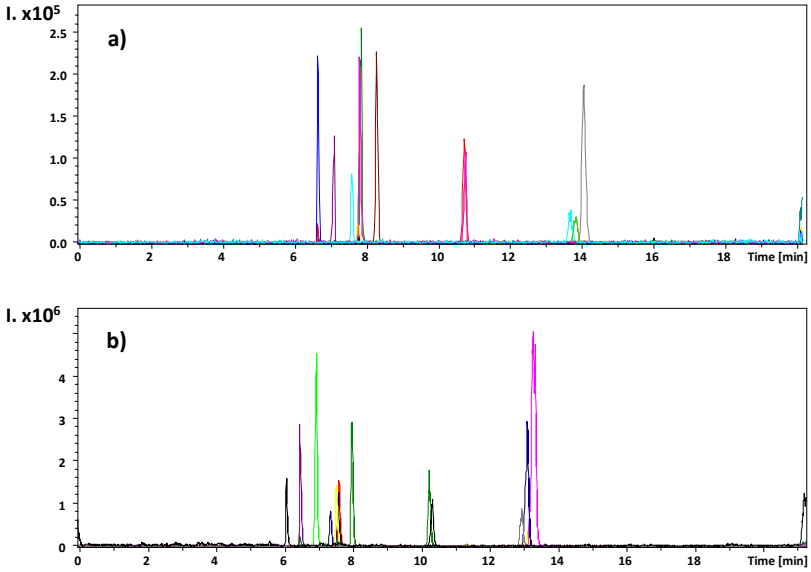

Figure III.10. Electropherograms for the CRBs obtained: **(a)** before optimization of parameters affecting the sensitivity of MS detection, **(b)** after optimization.

It has to be highlighted that the improved sensitivity could be due to the presence of $[M+H]^+$ as more abundant fragment. Thus, as it can be observed in Figure III.11, a higher number of fragments were obtained before the optimization, being $[M+H]^+$ the less intense of all. However, when all parameters were optimized, $[M+H]^+$ showed a good intensity for most CRBs. To illustrate this effect, Figure III.12 shows the improvement on the sensitivity for PR after lens optimization, including the fragmentation process.

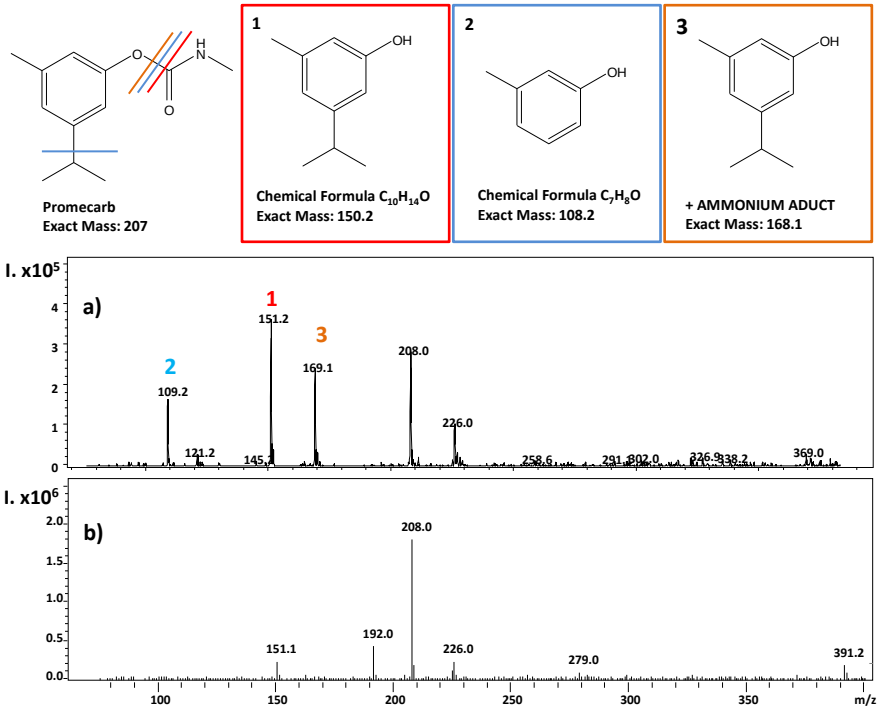

Figure III.11. Improvement on the sensitivity due to the lens optimization in the case of PR. Mass spectrum **a)** before the optimization, **b)** after the optimization.

However, in the case of OX the highest peak corresponded to the ammonium adduct $[M+NH_4]^+$, which presented a very low signal, showing lower sensitivity than the rest of CRBs. With respect to ALD, the $[M+H]^+$ ion was not detected, but the $[M+H-75]^+$ ion presents a strong intensity, in agreement to literature [20]. CBZ and BY were identified together, as it is known that BY in alkaline medium is easily degraded to CBZ and butyl isocyanate [21].

As a summary, the final optimum values for MS/MS detection are shown in Table III.3.

[20] T. Goto, H. Oka, I. Saito, H. Matsumoto, H. Sugiyama, C. Ohkubo, H. Nakazawa, H. Nagase, *Anal. Chim. Acta* 531 (2005) 79.
[21] E. Mallar, D. Barceló, R. Tauler, *Chromatographia* 46 (1997) 342.

Table III. 3. Optimized parameters of MS/MS for the detection of CRBs.

	Segment time (min)															
	0-6	6-7					7-9.5					9.5-12			12-15.5	15.5-17
	CBZ+BY	MTY	ALDSFX	ASL	CAR	ALD	CF	MTH	OX	PX	PR	ETH	BTH	FNX	NP	CSF
Capillary (V)	-4800	-4800					-4800				-4800			-4800		-4800
Skimmer (V)	27.1	21.5					28.5				30.6			29.9		33.6
Cap Exit (V)	70.6	50.8					40.7				65.4			63.9		100
Oct 1DC (V)	15.4	12.8					11.1				11.7			16.0		14.5
Oct 2DC (V)	1.1	1.5					0.6				0.6			0.4		0
Trap Drive (V)	39.6	33.2					34.1				35.7			45.2		49.9
Oct RF (V)	111.5	115.6					72.1				79.9			81.6		218.3
Lens 1 (V)	-6.6	-5.3					-5.9				-6.3			-8.9		-7.4
Lens 2 (V)	-100	-100					-68.4				-79.8			-100		-100

Under these conditions, the rest of parameters of the electrospray were studied in order to obtain the best S/N for all the analytes, with the highest sensitivity, using experimental design. Thus, nebulizer pressure, dry gas flow rate, sheath liquid flow rate and dry gas temperature were optimized by means of a Doehlert design plus three central points (23 runs) in order to select the optimum values that provide the maximum response, considering the possible interactions between the different factors [22]. Taking into account the influence of each factor on the response, dry gas flow rate (in the range 2–10 l min^{-1}) and sheath liquid flow rate (in the range 1–9 µl min^{-1}) were studied at seven levels, the nebulizer pressure (in the range 4–12 psi) at five levels, and the dry gas temperature (in the range 150–350 ^{o}C) at three levels. The lack-of-fit P-value for the model was 30.04% and the determination coefficient (R^2) was 85.34, showing a satisfactory fit of the experimental data to the predicted model. Estimated response surfaces are shown in Figure III.12.

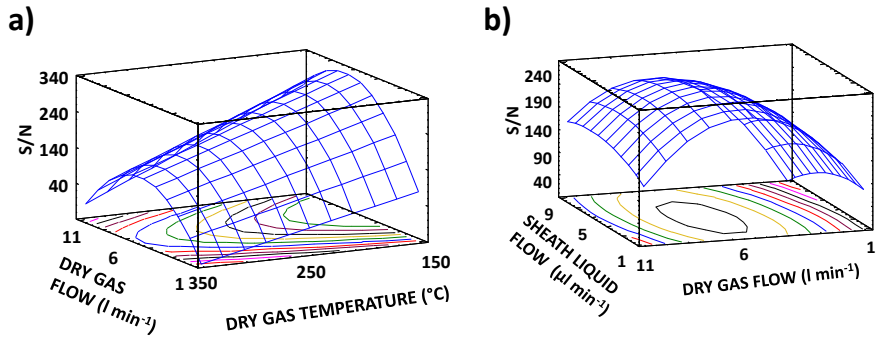

Figure III.12. Estimated response surfaces obtained in the multioptimization procedure for variables affecting the spray using a Doehlert design. **(a)** dry gas flow rate vs dry gas temperature; **(b)** dry gas flow rate vs sheath liquid flow rate.

Figure III.13 (a) shows the Pareto chart, including the effects of the variables affecting ESI conditions on the CRBs S/N ratio of the CRBs. The black line shows the limit of decision to consider the significance of the factors (based on the standardized effect estimated effect/standard error, P-value: 0.05 at 95% of confidence level). These effects can also be observed in Figure III.13 (b), which shows the main effects plots for S/N. All the variables were significant except the sheath liquid flow rate but also its interaction with the dry gas temperature.

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[22] S.L.C. Ferreira, W.N.L. dos Santos, C.M. Quintella, B.B. Neto, J.M. Bosque-Sendra, *Talanta* 63 (2004) 1061.

Finally, the obtained optimum values were: dry gas flow rate, 8 l min^{-1}; sheath liquid flow rate, 1.66 µl min^{-1}; nebulizer pressure, 12 psi; and dry gas temperature, 150 °C. Although the design recommended decreasing the dry gas temperature below 150 °C, it was kept at 180 °C (APFO boiling point) in order to prevent the contamination of MS by APFO.

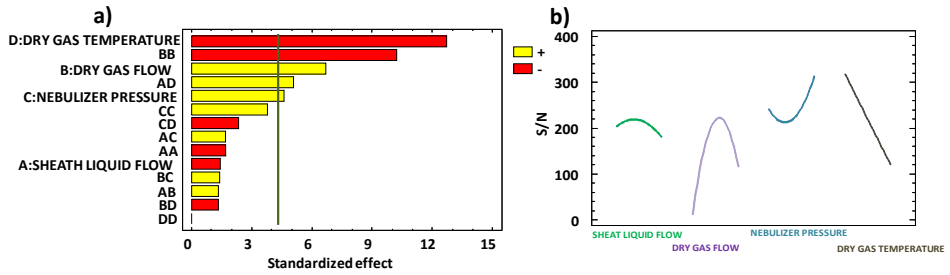


Figure III.13. **a)** Standarized Pareto chart for S/N (yellow: positive effects; red: negative effects), **b)** main effects plots for S/N.

Once the ESI conditions were established using MS mode, an optimization of the MS/MS detection was carried out. Considering that some peaks could not be well-resolved, the MRM mode was chosen. For fragmentation experiments, a cutoff of 27% of the precursor mass was set (i.e., the minimum m/z of the fragment ion able to be trapped by the analyzer). CRBs were fragmented using the SmartFragTM option that automatically ramps the fragmentation energy from 30 to 200% of the excitation amplitude. The fragmentation amplitude was manually varied and optimized by visualizing the intensities of the fragment ions with the aim to obtain a maximum which allows detecting the precursor ion.

Finally, in order to obtain the maximum sensitivity, the sample solvent and the sample injection volume were studied. The adequate sample solvent for an optimum collection and accumulation of analyte molecules by the pseudostationary phase was 57 mM APFO (pH 9.0), obtaining a small stacking effect [23,24]. The injection time was studied from 20 to 40 s. Finally, 30 s at 50 mbar was chosen as optimum, equivalent to a volume of approximately of 32.6 nl (≈2% of the capillary volume).

[23] A.T. Aranas, A.M. Guidote Jr., J.P. Quirino, *Anal. Bioanal. Chem.* 394 (2009) 175.

[24] M.C. Breadmore, A.I. Shallan, H.R. Rabanes, D., Gstoettenmayr, A.S.A. Keyon, A. Gaspar M. Dawod, J.P. Quirino, *Electrophoresis* 34 (2013) 29.

3.5. OPTIMIZATION OF DLLME

There are some factors affecting the extraction process, namely: kind of extraction and disperser solvents and their volumes, salt addition and pH. The optimization of these parameters was carried out using 4.5 ml of an aqueous standard solution containing a concentration of 500 ng l^{-1} of each CRB. The recovery was used to evaluate the extraction efficiency.

At the beginning, 15 ml falcon tubes were use as extraction vessel, but hydrocarbon solvents were difficult to collect, due to the meniscus formed at the top of the conical tube. Thus, very low precisions and recoveries were obtained. For this reason, a new extraction vessel was proposed, consisting of a 5 ml pipette tip properly covered, which allowed good reproducibility in the collection of the extraction solvent (see Figure III.2).

The selection of an appropriate extraction solvent is very important in the DLLME process. It must be immiscible with water, providing the highest extraction efficiency for all the analytes. In this study, 800 µl of extraction solvent with lower density than water were chosen (toluene, n-hexane and cyclohexane) using a volume of 1.0 ml of ACN as disperser solvent. According to the results (see Figure III.14) the best recoveries were obtained with toluene.

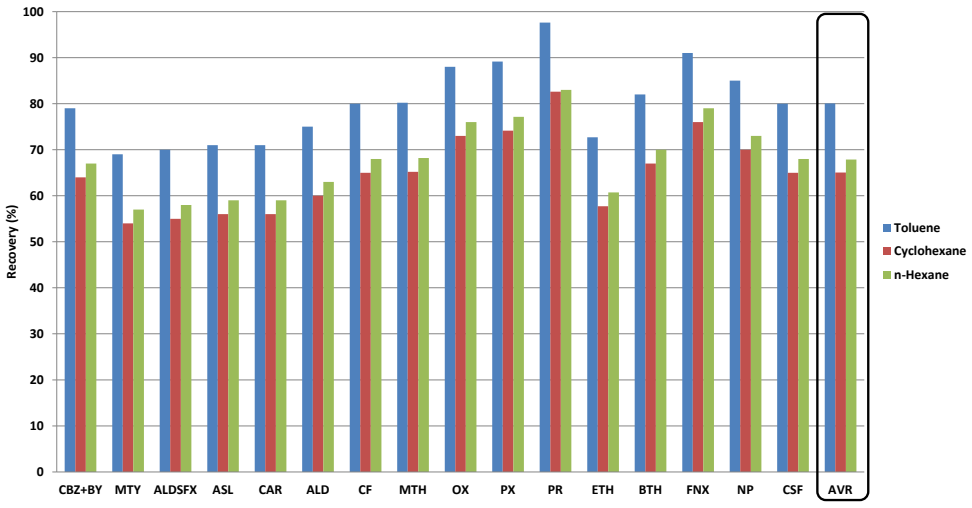


Figure III.14. Effect of different extraction solvents on the extraction efficiency of the CRBs(AVR: average recovery) using 1.0 ml of ACN as disperser solvent.

The disperser solvent must be miscible with both sample solution and extraction solvent. Therefore, 1 ml of ACN, ACO and MeOH were tested as disperser solvents, each one containing 800 µl of toluene. As can be seen in Figure III.15, the best recoveries were obtained with ACN as disperser solvent.

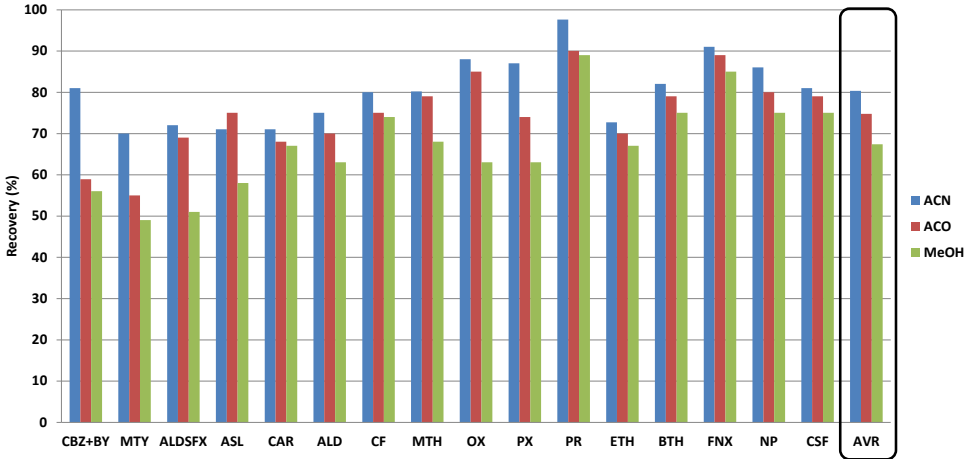


Figure III.15. Effect of different disperser solvents on the extraction recovery (AVR: average recovery) using 800 µl of toluene as extraction solvent**.**

Subsequently, extraction and disperser solvent volumes were optimized by means of a 3^2 full factorial design with 3 replicates of the central point. Toluene volume was studied in the range 400-800 µl and ACN volume in the range 750-1250 µl. The experiments were run randomly in order to minimize the effect of uncontrolled variables. The average recovery for all CRBs was considered as experimental response. The lack-of-fit P-value for the model was 20.2 % and the determination coefficient (R^2) was 91.57, showing a satisfactory fit of the experimental data to the predicted model. The surface response (see Figure III.16 (a)) shows that the optimum volumes were 940 µl for ACN and 636 µl for toluene. Figure III.16 (b) shows the Pareto chart, including the effects of both volumes on the recoveries.

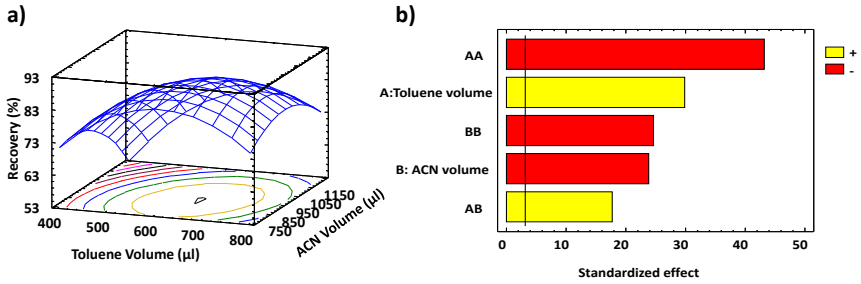


Figure III.16. **a)** Estimated response surfaces obtained in the optimization of extraction and disperser solvent using a 3^2 full factorial design, **b)** Standarized Pareto chart for Recovery (%) (yellow: positive effects; red: negative effects).

In order to evaluate the effect of the sample pH in DLLME, experiments were carried out with buffered (50 mM citric acid) aqueous samples with pHs from 2.0 to 7.0. This pH range was selected according to the pKs of the CRBs, keeping most of them neutral. The results (Figure III.17) showed that the sample pH had a significant effect on the recoveries for CF, FNX and CSF, being optimum at pH 2.0.

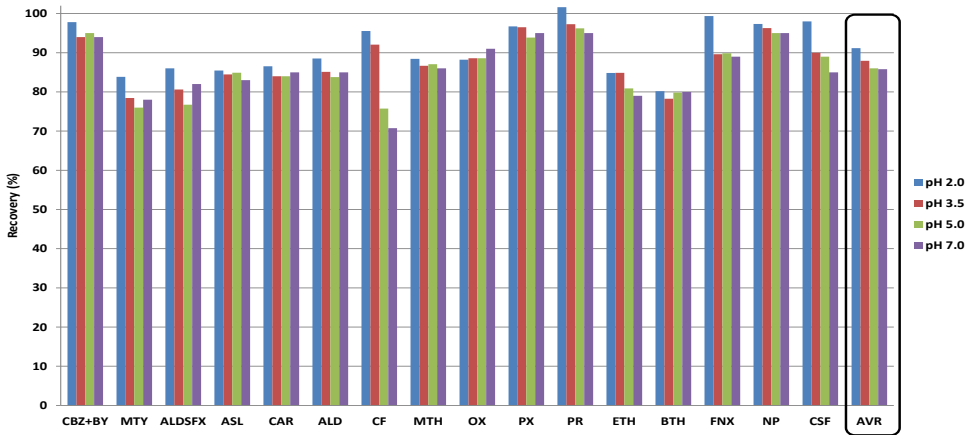


Figure III.17. Effect of pH on the extraction efficiency (AVR: average recovery).

The influence of the ionic strength on the performance of DLLME was investigated by adding different amounts of NaCl (0–10%, w/v) under the previous optimum conditions. However, as shown in Figure III.18, a decrease on recoveries was observed when the ionic

strength was increased, according to an effect previously described [25,26]. As a consequence, DLLME was carried out without addition of salt.

The whole procedure is summarized in Figure III.2.

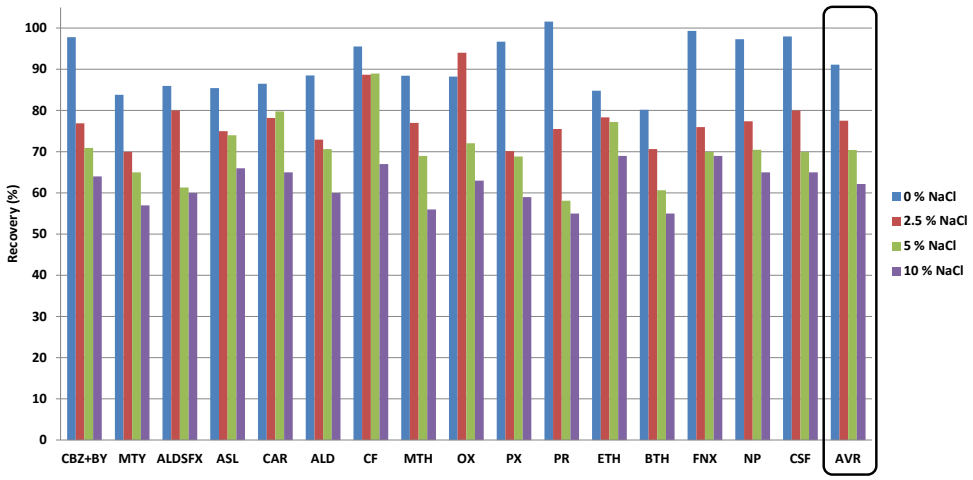


Figure III.18. Effect of addition of NaCl on the extraction efficiency (AVR: average recovery).

3.6. METHOD VALIDATION

In order to check the suitability of the method for the determination of CRBs in water samples, it was characterized in terms of linear dynamic ranges, LODs and LOQs, precision and trueness.

3.6.1. Calibration curves and analytical performance characteristics of the method

Matrix-matched calibration curves were established using river water (Genil river, Granada, Spain) as representative matrix, spiked with different analyte concentrations (500, 1000, 2500, 5000 and 10000 ng l^{-1} for OX; and 50, 100, 250, 500, 2500, 5000 and 10000 ng l^{-1} for the rest). Each concentration level was processed following the DLLME method and analyzed in triplicate.

[25] X. Lin, X. Chen, X. Huo, Z. Yu, K. Bi, Q. Li, *J. Sep. Sci.* 34 *(*2011) 202.
[26] H. Lijun, W. Chunjian, S. Yinjuan, X. Luo, J. Zhang, K. Lu, *Int. J. Environ. Anal. Chem*. 89 (2009) 439.

In order to achieve unambiguous identification of the CRBs, it was necessary to obtain the minimum number of product ions usually established by legislation for the quantification of pesticides [27]. In the case of MS/MS analysis, 2 or more product ions are required. As can be observed in Table III.1, between 2 and 5 product ions were obtained for all CRBs, except to BY+CBZ.

The sum of the peak areas of all the product ions were considered as a function of analyte concentration in the sample. A blank sample was also processed, and none of the CRBs were detected.

LODs and LOQs were calculated as 3 × S/N and 10 × S/N, respectively. All the LOQs were below the MRL established in the European Directives for water (100 ng l^{-1}) [3] except for OX.

Statistics and performance characteristics of the method are shown in Table III.4.

Table III.4. Statistics and performance characteristics of the DLLME-MEKC-MS/MS method for the analysis of CRBs in waters.

Analyte	Linear dynamic range (ng l^{-1})	R^2 (%)	LOD (ng l^{-1})	LOQ (ng l^{-1})
CBZ+BY	47.0-10000	99.8	21.3	47.0
MTY	46.0-10000	99.8	17.5	46.0
ALDSFX	48.5-10000	99.8	14.5	48.5
ASL	4.7-10000	99.7	1.4	4.7
CAR	49.1-10000	99.7	16.5	49.1
ALD	24.3-10000	99.6	7.3	24.3
CF	12.3-10000	99.5	3.7	12.3
MTH	36.9-10000	99.7	11.1	36.9
OX	478.9-10000	99.7	143.7	478.9
PX	42.4-10000	99.6	12.7	42.4
PR	15.3-10000	99.7	4.6	15.3
ETH	33.1-10000	99.4	9.4	33.1
BTH	33.1-10000	99.9	10.8	33.1
FNX	41.3-10000	99.8	12.4	41.3
NP	46.6-10000	99.7	14.0	46.6
CSF	38.8-10000	99.6	11.6	38.8

Comparing the instrumental LODs obtained in the proposed sheath flow MEKC–MS/MS method with those obtained from previous papers using sheath flow MEKC–MS for the

[27] Method validation and quality control procedures for pesticide residues analysis in food and feed. European Commission 2011, SANCO/12495/2011.

analysis of CRBs using SDS with different techniques (partial filling techniques, reverse migrating micelles and reverse migrating micelles with coated capillaries [13]) or APFO [19], a substantial decrease in the LODs with a higher number of analyzed compounds was observed. The same conclusion was obtained when the proposed method was compared with other methods using HPLC–MS for the determination of CRBs in water samples (see Table III.5).

Table III.5. Instrumental detection limits (ng l^{-1}) for CRBs using HPLC-MS, MEKC-MS and MEKC-MS/MS (this chapter).

Analyte	HPLC-MS [28]	HPLC-MS [29]	HPLC-MS [30]	MEKC-MS with SDS [13]	MEKC-MS with APFO [19]	MEKC-MS/MS Present chapter
CBZ+BY	na	na	300	na	na	260
MTY	na	500	1000	1.1×10^{6}	0.08×10^{6}	282
ALDSFX	na	na	na	na	na	170
ASL	na	na	na	na	na	20
CAR	na	100	2000	0.04×10^{6}	0.02×10^{6}	168
ALD	na	100	3000	0.4×10^{6}	0.01×10^{6}	82
CF	na	100	400	0.07×10^{6}	0.02×10^{6}	40
MTH	5000	na	na	na	na	120
OX	na	100	4000	0.9×10^{6}	na	1400
PX	na	na	na	0.2×10^{6}	0.03×10^{6}	140
PR	na	na	na	na	0.05×10^{6}	55
ETH	na	na	200	na	na	100
BTH	5000	na	na	na	na	120
FNX	na	na	na	na	na	130
NP	na	na	na	na	na	150
CSF	na	na	30	na	na	131

na: not analyzed.

3.6.2. Precision study

The precision of the method was evaluated in terms of repeatability (intraday precision) and intermediate precision (interday precision) by application of the proposed DLLME–MEKC–ESI–MS/MS method to river water samples spiked at three different concentration levels of CRBs. Repeatability was evaluated over five samples prepared and injected in triplicate on the same day, under the same conditions. Intermediate precision was evaluated with a similar procedure, but the samples were analyzed in five consecutive

[28] F. Boujelbane, F. Oueslati, N. Ben-Hamida, *Desalination* 250 (2010) 473.
[29] J.M.F. Nogueira, T. Sandra, P. Sandra, *J. Chromatogr. A* 996 (2003) 133.
[30] N. Makaihata, T. Kawamoto, K. Teranishi, *Anal. Sci.* 19 (2003) 543.

days. The results, expressed as %RSD of the peak areas, are summarized in Table III.6. Good precision, lower than 8%, was obtained in all cases.

Table III.6. Precision of the method for spiked water samples.

	CBZ+BY	MTY	ALDSFX	ASL	CAR	ALD	CF	MTH	OX	PX	PR	ETH	BTH	FNX	NP	CSF
Repeatability RSD (%) (n=15)																
Level 1[a]	4.7	5.7	5.3	4.9	5.3	4.5	5.2	6.1	5.7	4.5	5.3	4.9	5.0	4.5	4.9	5.2
Level 2[b]	4.5	5.5	5.3	5.1	4.6	4.3	4.9	5.5	4.9	3.6	4.8	5.1	4.9	4.6	3.8	4.9
Level 3[c]	4.3	5.2	4.3	4.0	5.0	3.9	4.2	5.3	4.7	3.7	4.4	4.6	5.1	4.1	3.6	4.2
Intermediate precision RSD (%) (n=15)																
Level 1[a]	6.3	6.8	7.0	5.5	6.5	5.9	7.1	7.5	8.0	6.5	6.0	6.5	7.0	6.9	6.4	6.7
Level 2[b]	6.9	7.1	6.8	5.6	7.1	6.0	6.5	7.6	6.7	6.4	6.3	7.0	7.2	6.5	6.5	6.3
Level 3[c]	5.9	6.3	6.1	5.3	6.3	5.9	6.0	7.1	6.9	6.0	5.9	6.9	6.7	6.1	5.9	6.3

[a] **Level 1:** 500 ng l^{-1} for OX, 50 ng l^{-1} for the rest.
[b] **Level 2:** 2500 ng l^{-1} for OX, 100 ng l^{-1} for the rest.
[c] **Level 3**: 10000 ng l^{-1} for OX, 500 ng l^{-1} for the rest.

3.6.3. Trueness assessment

In order to check the trueness of the proposed methodology, recovery experiments were carried out in different types of water samples (river, tap and mineral water) spiked at three different concentration levels of CRBs. In all the cases, a sample free of analytes was analyzed to check the presence of CRBs; none of them gave a result above the LODs of the method. Low recoveries were obtained for tap water, as ion suppression effect was observed due to the presence of chlorine; thus, this type of water was stored in an open vessel during one day previously to the analysis, in order to decrease chlorine presence.

Finally, recoveries higher than 81% were obtained in all cases, with satisfactory precision (see Table III.7).

Table III.7. Recoveries obtained for each CRB at different spiked levels in river, tap and mineral water samples (n=15).

			CBZ+BY	MTY	ALDSFX	ASL	CAR	ALD	CF	MTH	OX	PX	PR	ETH	BTH	FNX	NP	CSF
River water	**Level 1**[a]	**R (%)**	97.3	82.8	84.3	87.4	85.7	87.8	96.6	89.3	86.7	96.7	97.8	83.3	81.2	98.3	96.3	99.5
		(RSD)	(4.7)	(5.7)	(5.3)	(4.9)	(5.3)	(4.5)	(5.2)	(6.1)	(5.7)	(4.5)	(5.3)	(4.9)	(5.0)	(4.5)	(4.9)	(5.2)
	Level 2[b]	**R (%)**	93.4	83.3	85.2	83.4	83.4	89.5	95.9	88.7	84.2	95.9	98.1	85.3	82.3	99.5	97.9	97.5
		(RSD)	(4.5)	(5.5)	(5.3)	(5.1)	(4.6)	(4.3)	(4.9)	(5.5)	(4.9)	(3.6)	(4.8)	(5.1)	(4.9)	(4.6)	(3.8)	(4.9)
	Level 3[c]	**R (%)**	97.5	83.8	86.0	85.4	86.7	88.5	95.5	88.3	88.2	96.7	101.1	82.3	80.1	99.3	97.3	95.5
		(RSD)	(4.3)	(5.2)	(4.3)	(4.0)	(5.0)	(3.9)	(4.2)	(5.3)	(4.7)	(3.7)	(4.4)	(4.6)	(5.1)	(4.1)	(3.6)	(4.2)
Tap water	**Level 1**[a]	**R (%)**	96.4	83.9	85.3	89.3	87.7	88.8	95.9	92.4	85.7	95.7	98.7	84.2	82.2	99.4	95.3	99.1
		(RSD)	(4.6)	(6.0)	(5.2)	(5.1)	(5.7)	(4.3)	(6.0)	(5.8)	(5.7)	(5.0)	(5.6)	(5.8)	(5.2)	(4.6)	(4.7)	(5.6)
	Level 2[b]	**R (%)**	93.5	85.7	89.5	83.3	81.4	90.5	96.9	90.4	84.1	96.0	98.1	87.3	83.9	99.6	97.5	96.5
		(RSD)	(4.7)	(5.3)	(5.9)	(5.5)	(4.7)	(4.5)	(5.5)	(5.6)	(4.8)	(4.5)	(4.9)	(5.7)	(4.7)	(4.8)	(4.0)	(4.7)
	Level 3[c]	**R (%)**	97.9	87.7	87.7	85.5	81.1	87.5	98.5	84.4	88.3	96.1	99.8	83.5	79.1	99.4	98.4	95.4
		(RSD)	(4.5)	(5.5)	(4.4)	(4.5)	(5.5)	(4.3)	(4.5)	(5.0)	(4.5)	(4.0)	(4.6)	(4.9)	(5.0)	(4.7)	(3.9)	(4.0)
Mineral water	**Level 1**[a]	**R (%)**	98.3	82.7	84.5	86.5	83.7	85.7	95.7	89.7	86.5	97.7	97.9	83.3	81.4	98.3	96.3	98.4
		(RSD)	(4.5)	(5.6)	(5.5)	(4.8)	(5.9)	(4.7)	(5.2)	(6.0)	(5.7)	(4.9)	(5.4)	(5.5)	(5.4)	(4.7)	(4.9)	(5.0)
	Level 2[b]	**R (%)**	94.4	85.3	83.3	85.5	87.1	89.5	95.7	88.5	87.3	98.7	98.1	88.9	81.3	97.5	97.9	95.5
		(RSD)	(3.5)	(5.6)	(5.6)	(5.0)	(4.6)	(4.2)	(5.1)	(5.4)	(4.9)	(4.0)	(5.1)	(5.2)	(4.5)	(4.5)	(3.8)	(5.1)
	Level 3[c]	**R (%)**	96.3	86.8	86.1	85.4	85.7	82.5	96.7	88.7	87.2	97.6	102.3	83.3	81.1	98.6	97.5	98.5
		(RSD)	(4.4)	(5.1)	(4.6)	(4.5)	(4.9)	(3.8)	(4.8)	(5.4)	(5.0)	(4.7)	(4.8)	(4.7)	(5.0)	(3.9)	(3.8)	(4.6)

[a] **Level 1:** 500 ng l^{-1} for OX, 50 ng l^{-1} for the rest.
[b] **Level 2**: 2500 ng l^{-1} for OX, 100 ng l^{-1} for the rest.
[c] **Level 3:** 10000 ng l^{-1} for OX, 500 ng l^{-1} for the rest.

A typical electropherogram corresponding to river water spiked with 500 ng l^{-1} for OX and 50 ng l^{-1} for the rest, and analyzed by the proposed DLLME–MEKC–ESI–MS/MS method is shown in Figure III.19.

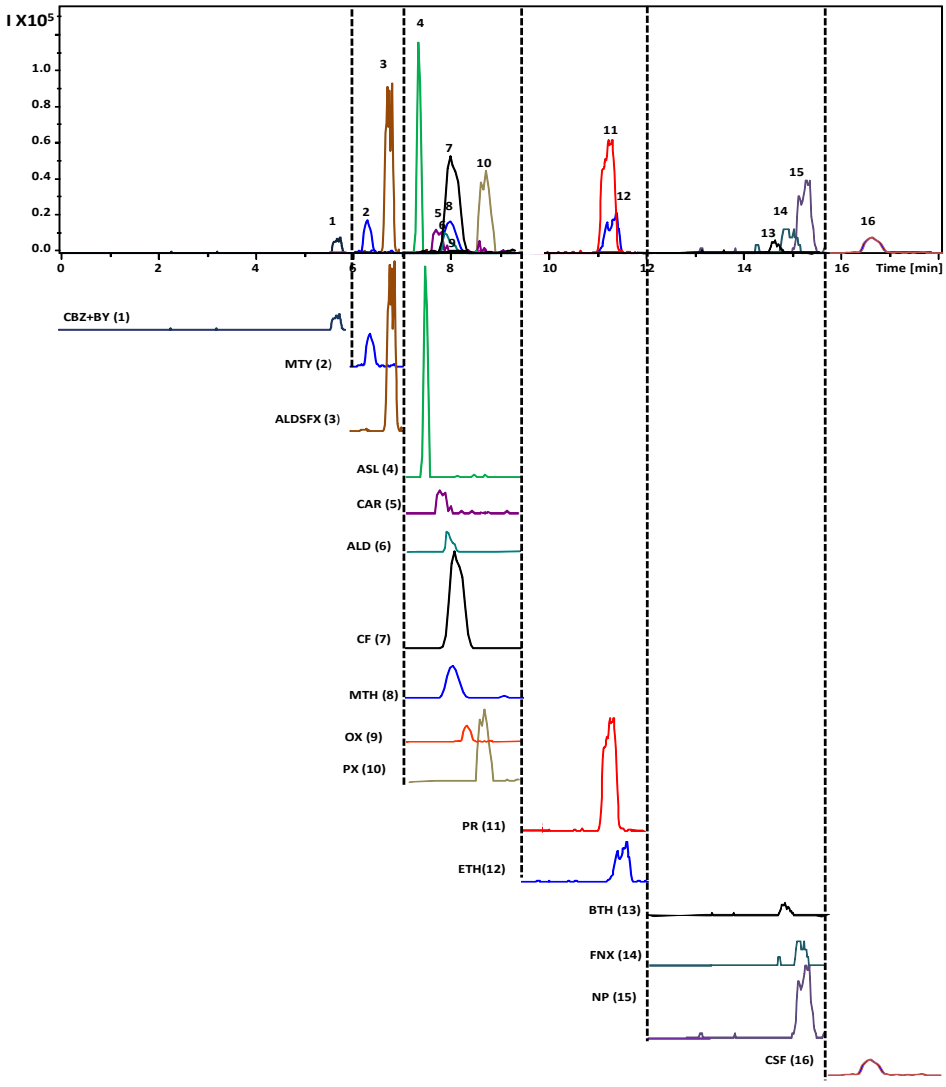


Figure III.19. Electropherogram of a spiked river water sample applying the proposed DLLME–MEKC–ESI-MS/MS method: 500 ng l^{-1} for OX, and 50 ng l^{-1} for the rest; peaks: (1)CBZ + BY, (2) MTY, (3) ALDSFX, (4) ASL, (5) CAR, (6) ALD, (7) CF, (8) MTH, (9) OX, (10) PX (11) PR, (12) ETH, (13) BTH, (14) FNX, (15) NP and (16) CSF.

4. CONCLUSIONS

In the present study, DLLME using an extraction solvent with a lower density than water combined with a new sheath-flow MEKC–ESI–MS/MS method has been proposed as a green alternative for the determination of seventeen CRBs in environmental and drinking water samples, with LOQs below the MRL established in the European Directives except for OX.

A new extraction vessel has been proposed in order to obtain good reproducibility and an easy mode to operate in the sample treatment. Moreover, APFO has been used as a volatile surfactant compatible with MS, increasing the possibilities of this coupling for non-polar analytes. In the optimization of DLLME and ESI parameters, experimental designs have been used in order to take into account possible interactions between the variables. This new method combines the advantages of DLLME and MEKC in terms of miniaturized techniques with low consumption of organic solvents, being a very environmentally friendly strategy for the monitoring of a high number of CRBs in water samples at trace levels, considering also the potential of MS detection in terms of identification and quantification.

This work was published as:

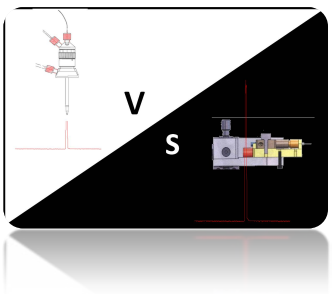
"Dispersive liquid–liquid microextraction using a low density extraction solvent for the determination of 17 N-methylcarbamates by micellar electrokinetic chromatography–electrospray–mass spectrometry employing a volatile surfactant". David Moreno-González, Laura Gámiz-Gracia, Juan M. Bosque-Sendra, Ana M. García-Campaña*. J. Chromatogr. A* 1247 (2012) 26.

CAPÍTULO 4

Cromatografía capilar electrocinética micelar acoplada a espectrometría de masas empleando una interfase sin líquido adicional de punta porosa como método altamente sensible para el control de carbamatos en aguas

CHAPTER 4

Highly sensitive micellar electrokinetic chromatography-mass spectrometry method employing a sheathless porous tip interface for the control of carbamates in waters



RESUMEN

Para la determinación de dieciséis carbamatos en aguas de consumo, se ha utilizado el acoplamiento de la espectrometría de masas usando una nueva interfase sin líquido adicional (*sheathless)* y la cromatografía capilar electrocinética micelar, empleando como surfactante volátil el perfluorooctanoato de amonio. Con el objetivo de conseguir una sensibilidad adecuada y una pulverización estable se han optimizado los parámetros de la interfase (distancia entre la punta del emisor y la entrada al espectrómetro de masas, el voltaje de electronebulización, la temperatura del gas de secado y el flujo del gas de secado). Los límites de detección obtenidos estuvieron comprendidos entre 0.5 y 14.6 ng l^{-1}. Para todos los plaguidas, la linealidad obtenida fue satisfactoria (R^2 > 99.1%). Asimismo, se obtuvo una buena reproducibilidad, tanto para los valores de área de pico con desviaciones estándar relativas entre 4.0 y 11.3 % como para los tiempos de migración, con valores alrededor de 0.9%. La utilización de la interfase sin líquido adicional (*sheathless*) ha permitido obtener menores límites de detección (al menos 10 veces menores y en algunos casos 100), que los obtenidos con la interfase con líquido adicional (*sheath-flow)* empleada en el capítulo anterior o los que se obtienen en cromatografía líquida utilizando el mismo espectrómetro de masas. Esto representa una mejora considerable sobre la interfase sin líquido adicional, permitiendo la cuantificación de carbamatos por debajo de los límites máximos de residuos establecidos por la legislación europea para el agua potable (100 ng l^{-1}).

SUMMARY

A sheathless interface coupled with micellar electrokinetic chromatography employing the semi-volatile surfactant ammonium perfluorooctanoate has been evaluated for the analysis of sixteen CRB pesticides in water. The parameters of the interface (as the distance between the emitter tip and MS inlet, electrospray voltage, dry gas temperature and dry nitrogen flow) have been optimized, in order to attain satisfactory sensitivity and stable spray. Limits of detection ranging from 0.5 to 14.6 ng l^{-1} were achieved, and good linearity (R^2 > 99.1%) and satisfactory reproducibility were obtained, with relative standard deviations for peak area between 4.0 and 11.3 % and for migration times around 0.9%. The detection limits using this sheathless interface, were at least 10 times lower (in some cases 100 times) than those obtained with a traditional sheath-flow interface for capillary electrophoresis, and with liquid chromatography using the same mass spectrometer. That represents a considerable improvement over sheath-flow interfaces, allowing the quantification of these pesticides below the maximum residue limits established by European legislation for drinking water (100 ng l^{-1}).

1. INTRODUCTION

In Chapter 3 we proposed a MEKC-ESI-MS/MS method for the determination of 17 CRBs in water samples using APFO as surfactant [1], obtaining very low LOQs (ranging from 50 to 5123 ng l^{-1}), similar to those obtained with HPLC-MS methods [2-4]. However, a sample treatment including a preconcentration step was required in order to reach the established MRLs in water for human consumption [5].

A sheathless interface is another alternative to increase sensitivity in CE-MS/MS. ESI sprayer in CE-MS needs a sheath liquid to establish electrical contact between the end of the capillary and to maintain a stable electrospray; this entails dilution of the analyte by sheath liquid with the subsequent decrease of sensitivity, being the main disadvantage of this configuration [6]. The first ESI interface, introduced by Olivares et al. in 1987, was in fact a sheathless interface [7]. However, that design was quickly abandoned in favour of a coaxial sheath-flow interface in order to increase the stability and compatibility of the hyphenated techniques [8].

Lately, a porous sheathless interface CE–MS was introduced by Moini [9]. The novelty in this interface was that it provided electrical contact without the need for microelectrodes or liquid junctions. In this approach, hydrofluoric acid was used to etch the last 3-4 cm of the bare fused-silica capillary, producing a ~5 µm thick porous wall, which is conductive when it is in contact with an electrolyte. CE and ESI keep the electrical contact by letting the porous capillary outlet protrude from a stainless steel ESI needle filled with static conductive liquid, allowing electrospray formation at the capillary tip. Any bubble

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[3] F. Boujelbane, F. Oueslati, N. Ben-Hamida, *Desalination* 250 (2010) 473.
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[5] Council Directive 98/83/EC on the quality of water intended for human consumption. Official *Journal of the European Communities*, 3 November 1998, L330/32.
[6] M. Moini, *Anal. Bioanal. Chem.* 373 (2002) 466.
[7] J.A. Olivares, N.T. Nguyen, C.R. Yonker, R.D. Smith, *Anal. Chem*. 59(1987) 1230.
[8] E.J. Maxwell, D.D.Y. Chen, *Anal. Chim. Acta* 627 (2008) 25.
[9] M. Moini, *Anal. Chem.* 79 (2007) 4241.

formation at the electrode occurs outside the separation capillary, and no dilution of the CE effluent takes place. The capillary inner diameter remains unchanged (i.e. no tapering) so that chances of clogging are minimized. The potential usefulness of the porous tip sprayer was demonstrated by the analysis of amino acids, peptides, protein digests and protein–metal complexes [9,10].

Based on this concept, a high-sensitivity porous sprayer (HSPS) sheathless interface prototype for CE-MS was developed by Beckman Coulter (Brea, CA). This HSPS has been used for the analysis of peptide mixtures [11,12], intact proteins [13,14], and phosphorus-containing amino acid-type herbicides [15].

Taking into account the advantages of volatile surfactants and sheathless ESI, a MEKC-ESI-MS/MS method using the prototype HSPS sheathless interface has been evaluated. APFO has been used as a volatile surfactant compatible with MS and sixteen CRBs have been chosen as neutral model compounds, allowing the comparison of the proposed method with earlier reported systems. Thus, the method has been applied to the analysis of mineral water, and it has been compared in terms of sensitivity, precision and resolution with the conventional sheath-flow MEKC-ESI-MS/MS method previously reported in Chapter 3 [1]. To our knowledge, this is the first time that MEKC has been coupled with a sheathless MS interface.

[10] A. Nguyen, M. Moini, *Anal. Chem.* 80 (2008) 7169.
[11] J.M. Busnel, B. Schoenmaker, R. Ramautar, A. Carrasco-Pancorbo, C. Ratnayake, J.S. Feitelson, J.D. Chapman, A.M. Deelder, O.A. Mayboroda, *Anal. Chem.* 82 (2010) 9476.
[12] K. Faserl, B. Sarg, L. Kremser, H. Lindner, *Anal. Chem.* 83 (2011) 7297.
[13] R. Haselberg, C.K. Ratnayake, G.J. de Jong, G.W. Somsen, *J. Chromatogr. A* 1217 (2010) 7605.
[14] R. Haselberg, G. J. de Jong, G. W. Somsen, *Anal. Chem.* DOI: 10.1021/ac303158f.
[15] M. Kawai, Y. Iwamuro, R. Iio-Ishimaru, S. Chinaka, N. Takayama, K. Hayakawa, *Anal. Sci.* 27 (2011) 857.

2. EXPERIMENTAL

2.1. CHEMICALS

A 75 mM APFO solution (pH 9.0) was prepared with perfluorooctanoic acid 96% (Sigma Aldrich; St. Louis, MO, USA) and ammonium hydroxide (VWR, West Chester, PA, USA). Water was deionized and purified with a Milli-Q purification system (Millipore, Belford, USA). ACO and MeOH (LC-MS Chromasolv grade) were supplied by Sigma.

Pestanal grade analytical standards of CF, CAR, MTH, PR, OX, ALD, PX, ASL, BY, NP, CBZ, ETH, ALDSFX, CSF, BTH and FNX were supplied by Fluka (Steinheim, Germany). Individual stock standard solutions containing 3 g l^{-1} of each compound were prepared by dissolving accurately weighed amounts in MeOH and stored in the dark at 4 °C. They were stable for at least 4 months. Standard solutions containing all the CRBs were freshly prepared by proper dilution of the stock standard solutions with MeOH.

2.2. INSTRUMENTATION

CE experiments were carried out with a ProteomeLabTM PA 800 plus Protein Characterization System (Beckman Coulter, Brea, CA, USA) equipped with a temperature controlled autosampler and a power supply able to deliver up to 30 kV. Separations were performed in a prototype bare fused-silica capillary with porous tip (Beckman Coulter) with an ID of 30 µm, an OD of 150 µm, and a total length of 90 cm. The HSPS tip prototype is under development by Beckman Coulter and is not yet available for commercial use. The conventional ESI source was replaced by a nano-ESI source (Bruker Daltonics), which features a gas diverter. The nano-ESI source is especially designed to handle the very low CE flow rates (in the nl min^{-1} range). The porous tip capillary was placed in a grounded stainless steel needle that could be positioned by an XYZ-stage fitting the ion trap instrument (Figure IV.1). A nanospray end plate and a gas diverter were installed to allow using nano-ESI.

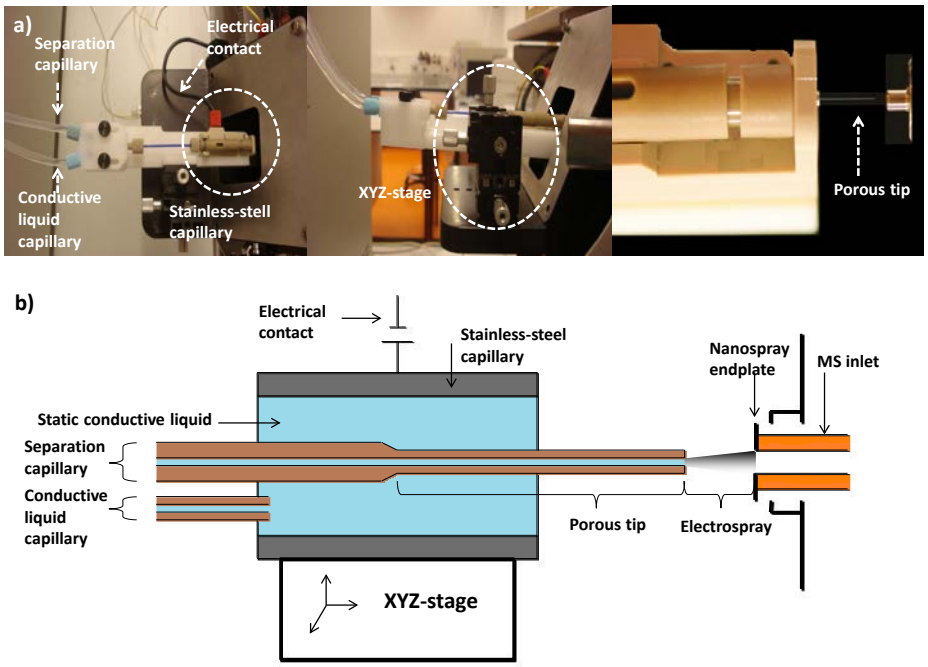

Figure IV.1. The high-sensitivity porous sprayer interface **(a)** photographs and **(b)** scheme of the prototype interface.

MS was performed using an Agilent 6330 Series XCT mass spectrometer equipped with an IT analyzer. MS spectrometer was controlled by a PC running the Esquire software 4.1 from Bruker Daltonics (Bremen, Germany).

A pH-meter (Metrohm model 744; Herisau, Switzerland), a nitrogen evaporator (Reacti-Therm from Pierce Chemical Co., Rockford, Illinois, USA) and a shaker (Heidolph Reax 2000 model from Heidolph Instruments GmbH & Co.; Kelheim, Germany) were also used.

2.3. ELECTROPHORESIS PROCEDURE

The separation capillary (fused-silica capillary, 30 µm ID, 150 µm OD, 90 cm length) has a porous segment at its outlet side. The porous segment was surrounded by a stainless steel needle that was filled with a conductive liquid, which made possible the electrical contact between the electrolyte and the conductive liquid. The separation conditions were taken from the previous work from Chapter 3 [1]: the BGE was 75 mM APFO adjusted to pH 9.0 with 15 M ammonium hydroxide. The conductive liquid was the same as BGE in this study.

The separation voltage was 24 kV and the capillary temperature was 23 ^{o}C. The sample was hydrodynamically injected for 15 s at 5 psi (equal to 2% of the capillary volume). In order to improve the sensitivity for some CRBs, the sample solvent was 57 mM APFO at pH 9.0, less concentrated than the BGE. With this sample solvent a small stacking effect was obtained, allowing on-line preconcentration [1,16].

At the beginning of each session, the separation capillary was rinsed with 1 M ammonium hydroxide by applying a pressure of 50 psi for 3 min; the conductive liquid capillary was rinsed for 0.5 min at 50 psi. Then, the separation capillary was rinsed with BGE by applying a pressure of 50 psi for 3 min, and the conductive liquid capillary was rinsed for 0.5 min at 50 psi with the same BGE. Between each run, the same process was carried out. The introduction of ammonium hydroxide prevented the solidification of APFO on the tip of the sprayer. At the end of the session, the capillaries were washed with water during 5 min.

For CE-UV experiments, a capillary of 30 μm ID, 90 cm length was prepared. The separation conditions were: voltage, 24 kV; temperature, 23 ^{o}C; BGE, 75 mM APFO pH 9.0; wavelength, 210 nm.

2.4. MS AND MS/MS CONDITIONS

The mass spectrometer was operating in the positive ion mode and scanned from 85–385 m/z at 13000 m/z s^{-1}. In the MS experiments, the IT parameters were selected in ion charge control mode using a target of 30000, maximum accumulation time of 200 ms, and 5 averages per experiment. In MS/MS experiments, the maximum accumulation time was set at 50 ms with 2 averages per experiment, using a target of 50000. Fragmentation was carried out by means of collision induced dissociation with the helium present in the trap for 40 ms in MRM mode. MS/MS parameters are summarized in Table IV.1.

The optimal spray conditions were as follows; dry gas temperature, 180 ^{o}C; dry gas nitrogen flow, 4.0 l min^{-1}. Electrospray in positive ionization mode was achieved using an ESI voltage of -1.3 kV. The porous tip protruded the grounded needle approximately 1.8 mm and the needle was filled with BGE to establish the electrical contact.

[16] A.T. Aranas, A.M. Guidote Jr., J.P. Quirino, *Anal. Bioanal. Chem.* 394 (2009) 175.

Table IV.1. Main parameters of the MS/MS method.

	Segment time (min)														
	0-10.5	**10.5-12.0**			**12.0-16.0**					**16.0-23.0**			**23.0-28.0**		**28.0-33.0**
	CBZ+BY	**ALDSFX**	**ASL**	**CAR**	**ALD**	**CF**	**MTH**	**OX**	**PX**	**PR**	**ETH**	**BTH**	**FNX**	**NP**	**CSF**
Width (m/z)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Cutoff (m/z)	52	56	62	55	56	60	61	60	58	56	61	70	82	73	103
Amplitude (V)	0.82	0.55	0.60	0.60	0.50	0.65	0.55	0.5	0.65	0.65	0.55	0.80	0.80	0.80	0.75
Precursor ion	192[a]	207[a]	231[a]	202[a]	116[b]	222[a]	226[a]	220[a]	210[a]	208[a]	226[a]	258[a]	302[a]	272[a]	381[a]
Product ions	160	132 89 76	188 156	145 127	89 70 68	165 123	169 121	90 72	168 153 111	151 109	169 164 107	125 116 100	256 116 88	199 171 128	222 195 160 128 118

[a] $[M+H]^+$; [b] $[M+H-75]^+$

3. RESULTS AND DISCUSSION

3.1. OPTIMIZATION OF SHEATHLESS PARAMETERS

One of the aims of this work was the evaluation of the sheathless CE-ESI-MS interface for MEKC applications using as model system the CRBs, target analytes in this Thesis and previously characterized using sheath liquid CE-MS [1], enabling good comparisons in terms of analytical performance. As preliminary study, in order to know the lineal velocity into the electrophoretic medium, CE-UV experiments were carried out (see conditions in experimental section). For that purpose ACO was used as EOF marker [17], dissolved in APFO 57 mM pH 9.0 (sample solvent). The migration time corresponding to ACO was 6 min, and taking into account the dimensions of the capillary, the calculated flow rate was 106 nl min^{-1} and the pressure to do infusion experiments in CE-MS should be 11 psi. Also, it is remarkable that in sheathless CE-MS employing non-coated capillaries, it is critical to get a significant EOF, which is necessary for successful coupling. As an alternative, Busnel et al. [11] showed that applying pressure and with flow rates ranging from 10 nl min^{-1} to 340 nl min^{-1}, approximately, this interface could generate a stable spray. However, EOF generated by APFO is high enough for the separation, without using additional pressure in the electrophoretic separation.

To optimize the sheathless interfacing conditions, CF (200 ng l^{-1}) was selected as a representative CRB, and it was infused by pressure of 11 psi through the capillary. In order to obtain good sensitivity and high spray stability, the influence of spray voltages and distance between the capillary tip and the MS inlet were studied together. For that purpose, the distance between the capillary tip and the MS inlet was studied between 0 and 5 mm (0.3 mm steps); at each position the voltage was varied between -1000 to -1700 V (50 V steps). It is important to note that the study was aborted when ESI current was higher than 100 nA in order to prevent breakdown capillary. The results are shown in Figure IV.2. As it can be observed, when the distance between the capillary tip and the MS inlet was very short, a high signal was obtained due to suction and very efficient spray sampling, but did not allow a stable ESI signal (Figure IV.2B). Moreover, when the distance

[17] P. Jandera, J. Fischer, J. Jebavá, H. Effenberger, *J. Chromatogr. A* 914 (2001) 233.

was increased, the ESI signal get more stable; however the voltage required to be increased as well to obtain higher signals. The ESI signal was stable over the entire range from 1 to 5 mm, obtaining a maximum signal in the region 1.8 mm -1300 V (ESI current 55 nA). This is consistent with Faserl et al. [12], whose studies showed that with a shorter distance between MS inlet and the capillary tip, the probability of arcing increases, whereas reduced spray voltage destabilizes the spray. Moreover when this distance increased, it was necessary to increase the ESI voltage in order to produce a stable electrospray. For these reasons, a combination of values (1.8 mm distance to MS inlet and -1300 V ESI voltage) were selected as they provided a compromise between spray stability and good intensity signal. Moreover, considering these results, it can be concluded that apparently, APFO causes a minimal ionization suppression of CRB signals.

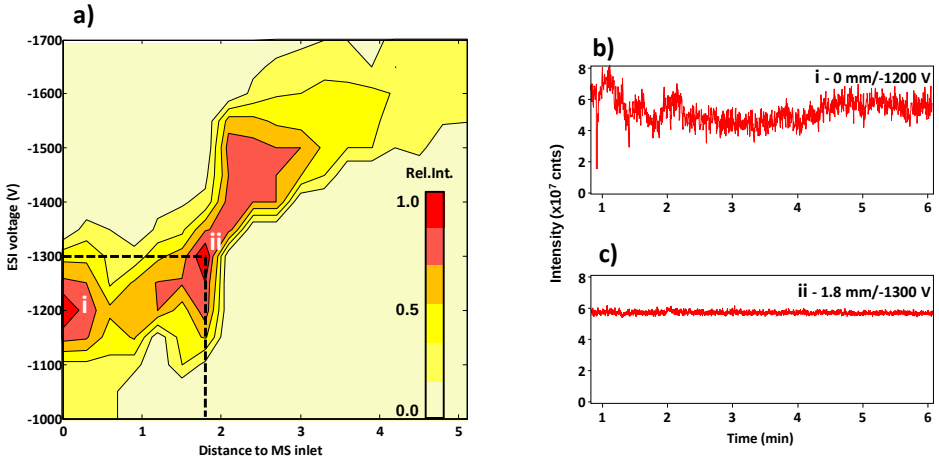

Figure IV.2. **(a)** Signal intensity of CF (200 ngl^{-1}) represented in contour plot obtained in the optimization of the distance to MS inlet versus ESI voltage and **(b and C)** baseline signal generated for the two maximum signal.

Subsequently, other parameters related to the sprayer stability and signal, such as dry gas temperature and dry gas nitrogen flow, were monitored by infusion experiments. The results of this optimization are shown in Figure IV.3. The effect of dry gas temperature was investigated between 180 ^{o}C and 220 ^{o}C. Although the spray was stable in the whole range (50-75 nA of ESI current), a decrease in the signal was observed when a dry gas temperature higher than 180 ^{o}C was used, probably due to the easy degradation of CRBs at higher temperatures. On the other hand, in order to prevent the MS ion source

contamination by APFO, dry gas temperatures below 180 °C (APFO boiling point) were not considered. Finally, dry gas nitrogen flow rate was investigated between 3.0 l min^{-1} and 5.0 l min^{-1}, the variations on signal and noise were not critical, so a dry gas nitrogen flow rate of 4 l min^{-1} was selected as optimum.

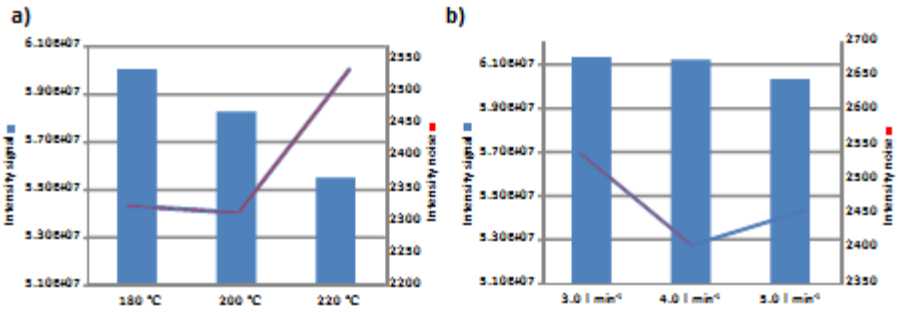

Figure IV.3. Signal intensity of CF (200 ng l^{-1}) and noise intensity obtained in the optimization of **(a)** dry gas temperature and **(b)** dry gas flow rate.

3.2. MS/MS OPTIMIZATION

Parameters affecting the sensitivity of MS detection such as capillary voltage, skimmer, cap exit, Oct 1 DC, Oct 2 DC, Trap drive, Oct RF, Lens 1 and Lens 2 were optimized by direct infusion, filling the capillary with each analyte dissolved in the BGE and applying a pressure of 11 psi with sheathless ESI parameters already optimized. Using this methodology it was observed that most of the CRBs gave clearly the $[M+H]^+$ ion when these parameters were optimized, except ALD, where $[M+H]^+$ ion was not observed, while $[M+H-75]^+$ ion had strong intensity, as previously described [18]. CBZ and BY were identified together as permitted by European legislation, as it is known that BY in alkaline medium is easily degraded to CBZ and butyl isocyanate [19]. The final optimum values are shown in Table IV.2.

[18] E. Mallar, D. Barceló, R. Tauler, *Chromatographia* 46 (1997) 342.
[19] N. Makaihata, T. Kawamoto, K. Teranishi, *Anal. Sci.* 19 (2003) 543.

Table IV.2. Optimized parameters of MS/MS.

	Segment time (min)														
	0-10.5	**10.5-12.0**				**12.0-16.0**					**16.0-23.0**		**23.0-28.0**		**28.0-33.0**
	CBZ+BY	**ALDSFX**	**ASL**	**CAR**	**ALD**	**CF**	**MTH**	**OX**	**PX**	**PR**	**ETH**	**BTH**	**FNX**	**NP**	**CSF**
Capillary (V)	-1300	-1300				-1300					-1300		-1300		-1300
Skimmer (V)	40	42.5				48.7					39.1		37.3		33.6
Cap Exit (V)	105.4	106.5				104.0					107.3		111.8		119.6
Oct 1DC (V)	12.23	12.96				13.6					14.98		14.12		12.0
Oct 2DC (V)	1.70	1.65				1.94					1.70		1.89		1.63
Trap Drive	32.3	33.3				42.6					34..3		38.1		45.3
Oct RF (V)	192.2	132.8				130.5					135.2		148.2		167.7
Lens 1 (V)	-5.3	-4.9				-6.1					-5.0		-5.3		-5.4
Lens 2 (V)	-60	-60				-60					-60		-60		-60

3.3. METHOD VALIDATION

In order to check the suitability of the method for the determination of CRBs in water samples, it was characterized in terms of linear dynamic ranges, limits of detection (LODs) and quantification (LOQs), precision and trueness.

3.3.1. Calibration curves and analytical performance characteristics of the method

Calibration curves were established using standard solutions of different concentrations (50, 75, 100, 150 and 200 ng l^{-1} for all CRBs). Each concentration level was injected in triplicate. The sum of the peak areas of all the product ions were considered as a function of the analyte concentration in the sample.

System variability was investigated with repetitive injections of the CRB solution (100 ng l^{-1}) in the same day (intraday precision, n=3) and in different days (interday precision, n=6). A satisfactory precision, lower than 8.4 and 11.3% for intraday and interday conditions, respectively were obtained for all CRBs.

LODs and LOQs were calculated as 3×S/N and 10×S/N, respectively. The results ranged from 0.5 to 14.6 ng l^{-1} for LODs and from 1.6 to 48.6 ng l^{-1} for LOQs, and, as can be observed, the method allows the determination of CRBs at the very low MRL established by legislation in drinking waters [5]. A summary of the performance characteristics of the method is listed in Table IV.3.

Table IV.3. Statistics and performance characteristics of the sheathless-MEKC-MS/MS method for the analysis of CRBs.

Analyte	Linear dynamic range (ng l^{-1})	R^2 (%)	LOD (ng l^{-1})	LOQ (ng l^{-1})	RSD(%) n=3 100 ng l^{-1} INTRADAY	RSD(%) n=6 100 ng l^{-1} INTERDAY
CBZ+BY	11.0-200.0	99.3	3.0	11.0	5.2	5.6
ALDSFX	43.6-200.0	99.9	13.1	43.6	6.4	7.1
ASL	5.6-200.0	99.6	1.7	5.6	8.1	9.0
CAR	38.9-200.0	99.6	11.8	38.9	4.1	5.5
ALD	42.7-200.0	99.9	12.8	42.7	8.4	11.3
CF	1.6-200.0	99.8	0.5	1.6	3.6	4.0
MTH	31.9-200.0	99.5	9.6	31.9	4.1	5.8
OX	48.7-200.0	99.7	14.6	48.7	4.1	8.8
PX	28.7-200.0	99.2	8.6	28.7	4.1	4.9
PR	18.2-200.0	99.7	5.5	18.2	4.2	7.0
ETH	20.4-200.0	99.3	6.1	20.4	4.8	5.2
BTH	20.4-200.0	99.8	6.1	20.4	6.5	6.7
FNX	21.8-200.0	99.4	6.6	21.8	5.1	5.4
NP	19.7-200.0	99.3	5.9	19.7	5.7	6.0
CSF	48.1-200.0	99.7	14.4	48.1	7.2	8.9

3.3.2. Trueness assessment

In order to check the trueness of the proposed methodology for the analysis of drinking water, recovery experiments were carried out in a commercial sample of bottled mineral water with weak mineralization, spiked at 100 ng l^{-1} of CRBs. In order to obtain the same stacking effect applied in the previous chapter, the final concentration of APFO in samples must be 57 mM, for this reason spiked water samples were diluted (1:1) with 114 mM APFO solution. Previously, a blank sample was analysed to check the presence of CRBs, and none of them were detected. Recoveries higher than 96% were obtained for all CRBs, with satisfactory precision (RSD from 4.1 to 8.2 %, n=3). An extracted ion electropherogram corresponding to spiked mineral water (100 ng l^{-1} for all CRBs) and analysed by sheathless MEKC-ESI-MS/MS method is shown in Figure IV.4.

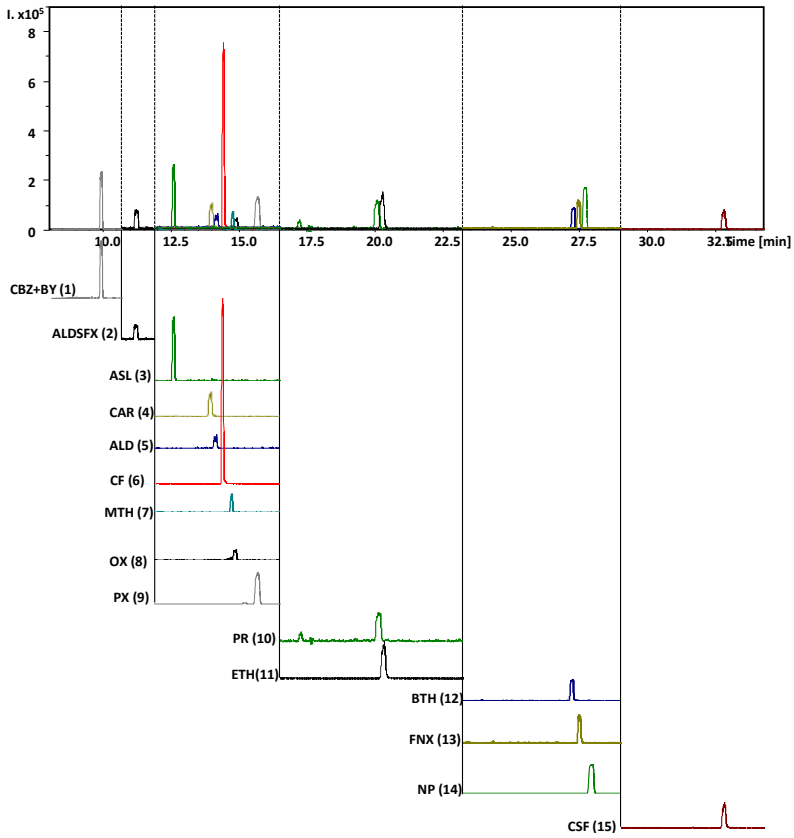

Figure IV.4. Extracted ion electropherogram corresponding to a standard solution of 50 ng l^{-1} for each CRB. Peaks: (1) CBZ +BY, (2) ALDSFX, 3) ASL, (4) CAR, (5) ALD, (6) CF, (7) MTH, (8) OX, (9) PX, (10) PR,(11) ETH, (12) BTH, (13) FNX, (14) NP and (15) CSF.

3.3.3. Comparison between sheathless and sheath-flow MEKC-ESI-MS/MS methods

Instrumental performance characteristics of sheathless MEKC-ESI-MS/MS were compared with the results obtained in the previous work using sheath-flow MEKC-ESI-MS/MS [1]. In both cases the same IT mass spectrometer and similar CE conditions were used. The results are summarised in Table IV.4.

Table IV.4. Comparison of sheath-flow and sheathless MEKC-ESI–MS/MS methods in terms of instrumental performance characteristics.

	Sheath-flow MEKC-ESI-MS/MS	**Sheathless MEKC-ESI-MS/MS**
Working range (ng l^{-1})	Up to 100000	Up to 200
R^2 (%)	99.2-99.8	99.3-99.9
RSD (%) intraday precision (n=3)	3.8-7.8 (500 ng l^{-1})	3.6-10.2 (100 ng l^{-1})
LOD (ng l^{-1})	20-1400	0.5-14.6
LOQ (ng l^{-1})	50-5123	1.5-48.7
Analysis time	18 min	33 min

As can be seen, linearity (R^2 above 99%) and precision (estimated in terms of intraday precision, n=3) were similar in both methods. The working range established in the case of sheathless method was lower than in sheath-flow method. The reason was that evaluation of higher concentrations was not necessary with the sheathless interface, because LODs obtained were low enough to achieve MRL established by EU in this type of samples (100 ng l^{-1}) [5].

Instrumental LOQs (without considering the preconcentration obtained in the sheath-flow method) were at least 10 times lower in the sheathless method for most CRBs. Moreover, LOQs for CF, CBZ+BY and OX, improved more than 80 times compared with conventional sheath-flow ESI method (see table IV.5). In the case of CF, this noteworthy improvement may be due to the optimization of sheathless ESI parameters, which was carried out with CF as model.

It must be highlighted that, due to the very low LOQs achieved, additional sample treatment for preconcentration was not necessary in the case of the sheathless method, while the sheath-flow required a previous DLLME achieving preconcentration of analytes. This supposed a simplification of the method, reducing the sample manipulation and increasing throughput.

Table IV.5. Instrumental detection and quantification limits for CRBs using sheath-flow and sheathless MEKC-ESI–MS/MS methods.

	SHEATH-FLOW-MS/MS		SHEATHLESS-MS/MS	
Analyte	**LOD (ng l^{-1})**	**LOQ (ng l^{-1})**	**LOD (ng l^{-1})**	**LOQ (ng l^{-1})**
CBZ+BY	260	436	3.0	11.0
ALDSFX	170	490	13.1	43.6
ASL	20	50	1.7	5.6
CAR	168	454	11.7	38.9
ALD	82	250	12.8	42.7
CF	40	147	0.5	1.6
MTH	120	369	9.6	31.9
OX	1400	5123	14.6	48.7
PX	140	475	8.6	28.7
PR	55	169	5.5	18.2
ETH	100	345	6.1	20.4
BTH	120	333	6.1	20.4
FNX	130	445	6.6	21.8
NP	150	501	6.0	19.7
CSF	131	360	14.4	48.1

Regarding peak efficiencies, they were higher in the sheathless mode than in the sheath-flow method (N>200000 and N>70000, respectively). Taking into account this improvement in efficiency, the resolution between peaks was better in the sheathless method, and the majority of CRBs were not overlapped. On the other hand, due to the longer capillary used in the sheathless system the sheath-flow method presented a lower analysis time (see Figure IV.5).

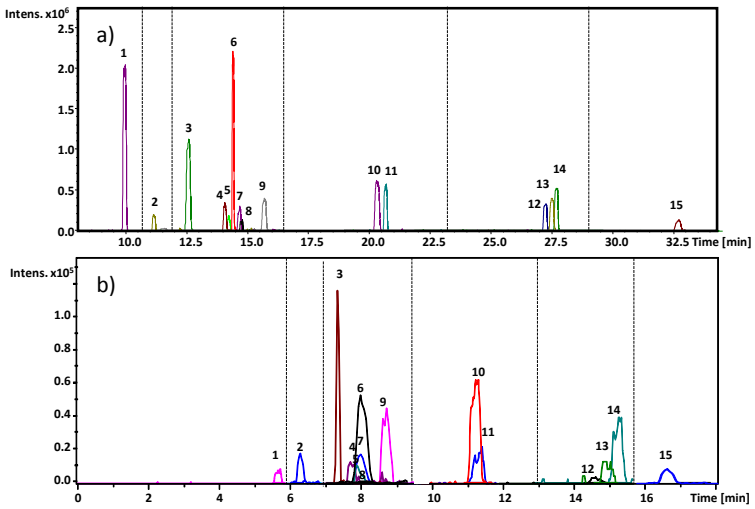

Figure IV.5. Electropherogram corresponding to a standard solution: **(a)** sheathless system (50 ng l^{-1}). **(b)** sheath-flow system (500 ng l^{-1}); peaks: (1) CBZ +BY, (2) ALDSFX, 3) ASL, (4) CAR, (5) ALD, (6) CF, (7) MTH, (8) OX, (9) PX, (10) PR,(11) ETH, (12) BTH, (13) FNX, (14) NP and (15) CSF.

4. CONCLUSIONS

In the present study, a new sheathless MEKC-ESI-MS/MS method using APFO as a volatile surfactant compatible with MS and a prototype HSPS has been evaluated. The determination of sixteen CRBs in water has been optimised to show the performance of the system. Stable CE and ESI conditions were established by placing the HSPS capillaries in a stainless steel needle filled with a static conductive liquid. Optimization of the sprayer tip position versus ESI voltage allowed obtaining conclusive results, in order to get good spray stability and satisfactory intensity signal for CRBs. The instrumental LOQs obtained in the proposed sheathless method were lower than those obtained from previous studies using sheath-flow MEKC-ESI-MS/MS for the determination of CRBs using SDS with different techniques (partial filling, reverse migrating micelles and reverse migrating micelles with coated capillaries) or APFO. Moreover, compared to previously reported HPLC-MS methods for the determination of CRBs, similar LOQs were obtained. These low LOQs allowed the determination of CRBs in drinking water with a minimum sample treatment (i.e. 1:1 mixing with BGE) with satisfactory recoveries. Thus, neutral compounds, which cannot be separated by CZE, are nicely separated by MEKC, and effectively ionized and detected by porous tip sheathless ESI with an improved sensitivity.

Looking to its potential, this methodology could be applied for the analysis of other neutral compounds where the use of MEKC and the advantages of MS detection are required.

To our knowledge, this MEKC-MS coupling with sheathless ESI using volatile surfactants have not been proposed before.

This work was carried out during the predoctoral stay, in collaboration with the gropu of Prof. G.J. de Jong, from Departament Biomolecular Analysis in Utrecht University (The Netherland) and is currently in preparation:

“Highly sensitive micellar electrokinetic chromatography-mass spectrometry employing a volatile surfactant and a sheathless porous tip interface”.

CAPÍTULO 5

Microextracción asistida por ultrasonidos con emulsificación mejorada con surfactante para la determinación de carbamatos en vinos por UHPLC-MS/MS

CHAPTER 5

Ultrasound-assisted surfactant-enhanced emulsification microextraction for the determination of carbamates in wines by UHPLC-MS/MS

RESUMEN

En este capítulo se ha desarrollado un nuevo método multirresiduo para la confirmación y cuantificación de veinticinco carbamatos en muestras de vino mediante cromatografía líquida de ultra resolución utilizando como sistema de detección la espectrometría de masas en tándem. Utilizando una columna Zorbax Eclipse plus C_{18} (50 mm x 2.1 mm, 1.8 µm) y una fase móvil compuesta por agua y metanol, ambos con un 0.01% de ácido fórmico, la separación se completó en 5.5 min. La detección se llevó a cabo mediante ionización positiva empleando el modo de monitorización de reacciones múltiples. Para obtener una mayor sensibilidad se optimizaron todos los parámetros de la fragmentación. Asimismo, para la extracción de los carbamatos y limpieza de los extractos de diferentes tipos de vino se propone la microextracción asistida por ultrasonidos con emulsificación mejorada con surfactantes, empleando un disolvente de baja densidad. Para conocer el efecto de la supresión iónica se evaluó el efecto matriz, pudiéndose comprobar que con el procedimiento propuesto se consiguen extractos muy limpios. En condiciones óptimas, las recuperaciones de las muestras enriquecidas variaron del 74 al 102%, con desviaciones estándar relativas inferiores al 5%. Además, los límites de cuantificación estuvieron comprendidos entre 0.15 y 0.92 µg l^{-1}, demostrando que el método propuesto proporciona una alta sensibilidad, siendo además rápido y simple.

SUMMARY

In this chapter a new sensitive multiresidue ultra-high performance liquid chromatography-tandem mass spectrometry method has been developed for the detection, confirmation and quantification of twenty five carbamates in wine samples. The separation was achieved in 5.5 min, using a Zorbax Eclipse plus C_{18} column (50 mm × 2.1 mm, 1.8 µm), with a mobile phase of water and methanol, both of them with 0.01% formic acid. The analytes were detected in positive mode with multiple reaction monitoring mode and MS/MS conditions were optimized in order to obtain a high sensitivity. Ultrasound-assisted surfactant-enhanced emulsification microextraction using a low-density extraction solvent has been optimized for satisfactory extraction of carbamates and clean-up of extracts from different types of wine. The matrix effect was evaluated in order to know the ion suppression effect, showing that this procedure provides very clean extracts. Under optimum conditions, recoveries for fortified samples ranged from 74 to 102%, with relative standard deviations lower than 5 %. The limits of quantification ranged from 0.15 to 0.92 µg l^{-1}, showing the high sensitivity and applicability of this fast and simple method.

1. INTRODUCTION

CRB pesticides are known to be used on a broad spectrum of crops, including vineyards [1]. As already discussed in Chapter 2, the content in pesticides in different foods and feeds (including wine grapes) is controlled by EU, which has established a MRL of 10 μg kg^{-1} as a default value for those matrices not included in the regulation (as wine) [2,3]. Moreover, since 2003 the EC's Rapid Alert System for Food and Feed (RASFF) has regularly alerted member States to the problems with pesticide residues in grapes. In response to these problems the Secretariat publishes results for grape monitoring as part of its continuous reporting ever years. Since then, RASFF has informed a remarkable number of cases of pesticide residue contamination in grapes, including CRBs as MTY, CBZ, and OX among others [4].

Taking into account these data, it could be possible that part of these pesticides may be transferred into the wine produced from these grapes. According to the international Organization of Vine and Wine, 241.9 mega hectoliters of wine are consumed annually all over the world [5]. Therefore, the control of pesticide contents in this kind of samples is a matter of concern. Thus, although MRLs are generally set for raw commodities, when MRLs are established the assessment of dietary intakes takes into account the potential for residues to remain in processed foods produced from the raw agricultural commodities. As an example, MRLs have been set for processed infant foods, and in future may be extended to other processed food products, including wine.

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[2] Regulation (EC) No. 396/2005 of the European Parliament and of the Council on maximum residue levels of pesticides in or on food and feed of plant and animal origin and amending Council Directive 91/414/EEC. *Official Journal of the European Union* L 70, 16 March 2005, pp. 1–16.

[3] Regulation (EC) No 149/2008 amending Regulation (EC) No 396/ 2005 of the European Parliament and of the Council by establishing Annexes II, III and IV setting maximum residue levels for products covered by Annex I thereto. *Official Journal of the European Union* L 58, 2008, pp. 1–398.

[4] http://www.crl-pesticides.eu/docs/public/tmplt_article.asp?CntID=661&LabID=100&Lang=EN. RASFF-Notifications.

[5] http://www.oiv.int/oiv/info/ennoteconjoncture2012.

Several methods have been published for the determination of pesticides, including CRBs in wine, using mainly GC-MS [6-8] or LC-MS [9-11]. UHPLC is a good alternative to conventional HPLC, due to its advantages associated with the use of columns of less than 2.0 µm porous stationary phase able to with stand very high pressures, which allows an increased efficiency with a shortened analysis time. UHPLC provides higher peak capacity, greater resolution, increased sensitivity and a higher speed of analysis and it is recommended especially to reduce analysis time and sample preparation [12,13] in combination with MS/MS.

Concerning sample treatment, several methods have been developed for the determination of CRBs in wines, mainly SPE [9,14], and LLE [14]. In recent years, different strategies have been proposed for simplifying sample treatment and preconcentration in pesticide residue analysis, including QuEChERS [6,8], cloud point extraction [15],membrane-assisted solvent extraction [10], SPME [16] and DLLME [17].

Another microextraction technique related with DLLME, named ultrasound-assisted emulsification microextraction (USAEME) was developed by Regueiro *et al*. [18,19]. In this

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[15] A. Santalad, R. Burakham, S. Srijaranai, R.L. Deming, Richard, J. Chromatogr. Sci. 50 (2012) 523.
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methodology, a microvolume of extraction solvent is dispersed into an aqueous sample solution without any disperser under ultrasound irradiation. However, it was observed that the extraction time in USAEME was quite long, because the mass-transfer efficiency between sample and extraction organic solvent is longer than DLLME. Moreover, the long ultrasound time required could accelerate the degradation of the analytes [20]. Lately, Wu *et al.* [21] and Vichapong and Burakham [22] showed that the use of surfactants above their CMC as emulsifier could enhance the dispersion of water-immiscible phase into aqueous phase. This methodology has been named ultrasound-assisted surfactant-enhanced emulsification microextraction (UASEME). In the UASEME sample treatment, the combination of ultrasound and surfactant allows shorter extraction time than USAEME.

In the present Chapter, the combination of UHPLC-MS/MS as separation and detection technique, and UASEME as sample treatment, has been proposed for the simultaneous determination of twenty five CRBs in different wine varieties. Taking into account the study of the European Food Safety Authority (EFSA) showing the risk associated with the presence of metabolite pesticide residues in the diet [23], some of these scarcely studied metabolites have been included in this work. UHPLC-MS/MS parameters were optimized in order to obtain a good separation and good sensitivity in the shortest possible time, while UASEME was optimized by means of an experimental design. The method was applied to different types of wine samples achieving LOQs below the default MRL (stablished for samples which are not regulated) and MRLs specified for wine grapes.

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2. EXPERIMENTAL

2.1. CHEMICALS

Water, MeOH, and ACN (LC-MS Chromasolv grade) were supplied from Fluka Analytical (Steinheim, Germany). Formic acid and 2-butanone were obtained from Sigma-Aldrich (ST. Louis, MO, USA) and acetic acid was supplied from Merck (Darmstadt, Germany).

For sample treatment, tween 80, triton X-114, NaCl and $MgSO_4$ were obtained from Panreac-Química (Barcelona, Spain). Toluene, tetrahydrofuran (THF) and $(NH_4)_2SO_4$ were supplied by VWR (West Chester, PA, USA). Triton X-100 was obtained from Riedel-de-Haën (Hanover, Germany).

Pestanal grade analytical standards of ASL, ALDSFX, aldicarb-sulfone (ALDSFN), OX, MTY, ethiofencarb-sulfone (ETHSFN), pirimicarb-desmethyl (PIRDES), ethiofencarb-sulfoxide (ETHSFX), methiocarb sulfoxide (MTHSFX), carbofuran-3-hidroxy (3-CF), cymoxanil (CY), ALD, metolcarb (METOL), PX, CF, CAR, ETH, thiodicarb (TH), isoprocarb (ISO), fenobucarb (FEN), diethofencarb (DETH), MTH, PR, NP and BTH, were supplied by Fluka. Individual stock standard solutions containing 3 g l^{-1} of each compound were prepared by dissolving accurately weighed amounts in MeOH and stored in the dark at 4 ^{o}C. They were stable for at least 4 months. Working standard solutions containing all the CRBs were freshly prepared by proper dilution of the stock standard solutions with MeOH.

Pipette tips (5 ml) were supplied by Brand GmbH (Wertheim, Germany). Acrodisc 13-mm syringe filters with 0.2 μm nylon membrane (Pall Corp., MI, USA) were used for filtration of sample extracts prior to the injection in the UHPLC system.

2.2. INSTRUMENTATION

Separation was performed on an Agilent 1290 Infinity LC from Agilent Technologies (Waldbron, Germany). The mass-spectrometer measurements were performed on a triple quadrupole mass spectrometer API 3200 (Applied Biosystems, Darmstadt, Germany) with electrospray ionization (ESI). The instrument data were collected using the Analyst®

Software version 1.5 with Schedule MRMTM Algorithm (ABSCIEX). A C_{18} column (Zorbax Eclipse Plus RRHT 50 mm × 2.1 mm, 1.8 µm) was supplied by Agilent Technologies.

The StatGraphics Plus Software 5.1 (Statistical Graphics Rockville; MD, USA) was used to generate the experimental designs and data processing.

An ultrasonic bath (USS-300 model; VWR) was used for UASEME sample treatment. A pH-meter (Crison model pH 2000; Barcelona, Spain) with a resolution of ±0.01 pH unit, a centrifuge (Universal 320 model from Hettich; Leipzig, Germany), a nitrogen evaporator (System EVA-EC from VLM GmbH, Bielefeld, Germany) and a vortex (Genie 2 model from Scientific Industries; Bohemia, NY, USA) were also used.

2.3. CHROMATOGRAPHIC PROCEDURE

UHPLC separations were performed in a C_{18} column using a mobile phase consisting of 0.01% aqueous formic acid solution (solvent A) and MeOH with the same percentage of acid (solvent B) at a flow rate of 0.5 ml min^{-1}. The gradient profile was 5% B at the beginning; 20% B from 0.7 to 1.2 min; then 50% B from 2.5 to 3 min; 95% B from 6.5 to 7.0 min; and finally in order to come back to the initial conditions, 5% B at 7.5 min, equilibrating for 3 min. The run time for each injection was 9.5 min in total, the temperature of the column was 25 ^{o}C and the injection volume was 10 µl. Under optimum conditions, all analytes were eluted in 6 min. The mass-spectrometer was working with ESI in positive mode under the multiple reaction monitoring (MRM) conditions shown in Table V.1. The ionization source parameters were: source temperature 400 ^{o}C; curtain gas (nitrogen) 30 psi, ion spray voltage -5000 V; and GAS 1 and GAS 2 (both of them nitrogen) were set at 50 psi.

Table V.1. Optimized MS parameters.

	Precursor ion	Rt	DP	EP	CEP	Product ions*	CEN	CXP
ASL	231.0	1.2	36	8	12	155.9 (Q)	15	4
						92.1 (I)	33	4
ALDSFX	207.1	1.3	21	8.5	16	132.1 (Q)	11	4
						89.1 (I)	17	2
ALDSFN	223.1	1.5	36	6	12	85.9 (Q)	23	4
						76.2 (I)	13	0
OX	237.1	1.5	16	6.5	12	71.9 (Q)	27	4
						90.2 (I)	13	2
MTY	163.1	1.7	26	7.5	10	88.2 (Q)	13	2
						106 (I)	15	2
ETHSFN	258.1	2.2	41	7	16	107 (Q)	21	4
						201 (I)	13	8
PIRDES	225.1	2.3	26	9	14	72.1 (Q)	27	4
						168.1 (I)	19	6
ETHSFX	242.1	2.3	21	6	20	107.1 (Q)	27	4
						185.2 (I)	13	4
MTHSFX	242.1	2.5	26	6.5	14	185.2(Q)	19	6
						170.0 (I)	29	4
3- CF	238.1	2.5	36	9	12	163.1 (Q)	19	4
						220 (I)	11	4
MTHSFN	258.1	2.7	41	6	12	122 (Q)	33	6
						201 (I)	13	6
ALD	116.1	3	31	9.5	12	89.1 (Q)	13	0
						70.3 (I)	13	0
METOL	166.0	3.1	31	6.5	14	109 (Q)	17	4
						94 (I)	43	4
PIR	239.1	3.2	36	7.5	16	72.1 (Q)	29	4
						182.2 (I)	21	4
PX	210.2	3.3	21	7.5	15	111.1 (Q)	21	4
						168.2 (I)	13	4
CF	222.9	3.4	41	6.5	15	165.1 (Q)	17	4
						123.1 (I)	29	4
CAR	202.1	3.6	26	9	15	145(Q)	17	4
						127.2 (I)	39	4
ETH	226.1	3.8	26	8	15	107.1 (Q)	23	4
						164.4 (I)	13	2
TH	355.2	3.9	21	7	19	88.1 (Q)	33	4
						108.1 (I)	19	2
ISO	194.2	4	26	8.5	15	95.1 (Q)	21	2
						137.2 (I)	13	4
FEN	208.2	4.5	36	7	15	95.2 (Q)	23	2
						151.9 (I)	13	4
DETH	268.2	4.6	31	8.5	16	226 (Q)	15	6
						124.2 (I)	41	4
MTH	226.1	4.6	21	8.5	15	169.1 (Q)	15	4
						121.1 (I)	25	4
PR	208.2	4.7	26	8.5	15	151.1 (Q)	13	4
						109 (I)	25	4
NP	272.1	5	36	11	17	129.2 (Q)	23	4
						171.3 (I)	25	6
BTH	258.1	5.5	46	7.5	16	125 (Q)	33	4
						89.2 (I)	71	4

*(Q) Transition used for quantification, (I) Transition employed to complete the identification.
Rt: Retention Time. DP: Declustering Potential. EP: Entrance Potential. CEP: Collision Cell Entrance potential. CEN: Collision Energy. CXP: Collision Exit Potential.

2.4. SAMPLE PREPARATION

Aliquots of 1 ml of wine were diluted with 3 ml of $MgSO_4$ solution (30% w/v). Then, the obtained solution was placed in a 5 ml pipette tip (extraction vessel). A mixture of 1150 µl of 2-butanone as extraction solvent and 333 µl of 1.8 mM triton X-114 as emulsifier (giving a final concentration of triton X-114 in sample solution of 0.15 mM) was rapidly injected into the sample tube. The extraction vessel was closed and it was immersed into an ultrasonic water bath for extraction (50 kHz of ultrasound frequency and 200 W for 5 min at 25 ^{o}C, to prevent the degradation of CRBs). Then, it was centrifuged for 5 min at 2000 rpm; the upper phase was removed using a syringe and collected in a glass vial. The 2-butanone was evaporated under nitrogen stream until dryness. The final residue was re-dissolved with 500 µl of sample solvent (H_2O:MeOH, 80:20), shaken by vortex (2 min), filtered (Acrodisc 13-mm) and injected into the UHPLC-MS/MS system. A schematic diagram of the sample preparation procedure is shown in Figure V.2. Following this treatment, sample throughput was approximately 16 samples per hour, obtaining a preconcentration factor of 2.

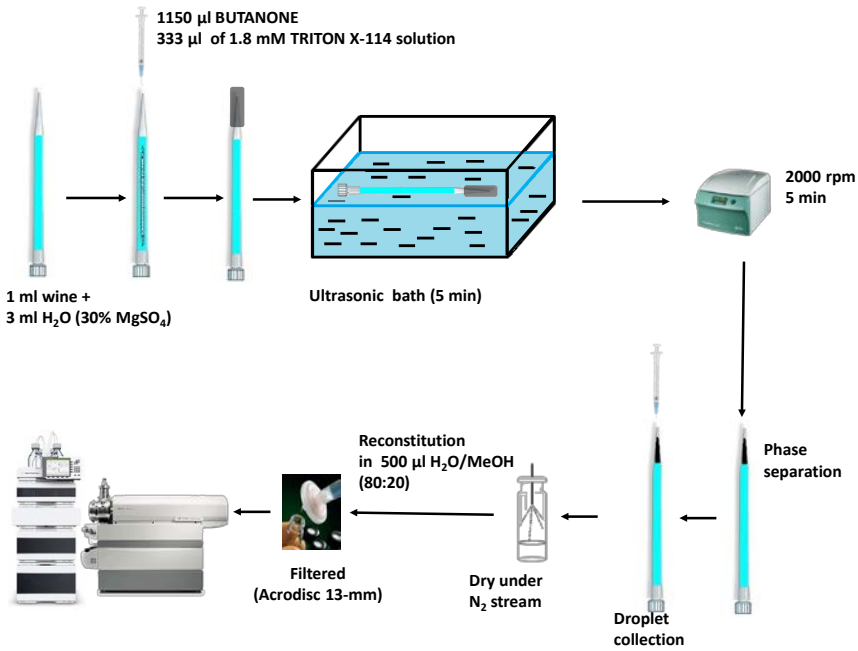

Figure V.1. Diagram of the proposed UASEME procedure.

3. RESULTS AND DISCUSSION

3.1. OPTIMIZATION OF MS/MS DETECTION AND CHROMATOGRAPHIC SEPARATION

For each individual CRB, the mass spectrometer parameters were optimized to provide the best responses for quantification. In order to get high sensitivity, a standard solution of 1 mg l^{-1} in 0.1 % aqueous formic acid solution:MeOH (50:50, v:v) of each analyte was individually infused directly into the mass spectrometer. All compounds were tested using ESI positive/negative mode. As it was expected from previous data [9,10,12,13] ESI operating in positive mode showed the best results in term of sensitivity. During the infusion, the DP, EP, CEP, CEN and CXP were optimized for each compound in order to obtain the maximum sensitivity (see results in Table V.1). Each compound was characterized by its retention time and by two precursor ion-product transitions. The most intense product ion (Q) was used for quantification (see Figure V.2) whereas the second one (I) was used to complete the identification (see selected ions in Table V.1). The target scan time established for each transition was 0.2 s. Under the experimental conditions selected, protonated molecules $[M+H]^+$, were observed for all the compounds except OX and ALD. In the case of OX, ammonium adduct was obtained as majority precursor ion. Moreover, in the case of ALD $[M+H]^+$ ion was not observed, while $[M+H-75]^+$ ion had a strong intensity, as previously described [24].

Concerning the chromatographic conditions, aqueous standard solutions of CRBs were used during the optimization of chromatographic separation. According to previous papers [9,10] the mobile phase consisted of 0.01% aqueous formic acid solution (solvent A) and MeOH (solvent B). The use of ACN as organic mobile phase was also considered, nevertheless a negative effect in the ionization was observed, obtaining lower signal intensity than those obtained when MeOH was used. For this reason MeOH was finally chosen. Subsequently, the gradient was studied to get the best separation, peak shape and sensitivity in the shortest time. The MeOH percentage was increased until 95% in order to

[24] E. Mallar, D. Barceló, R. Tauler, *Chromatographia* 46 (1997) 342.

elute the most retained analytes and other possible components included in the final sample extract.

The use of acid in the mobile phase is required to improve the ionization step in ESI+. Therefore, different acids (formic and acetic acid) were tested. Formic acid provided better results than acetic acid and it was selected for the rest of the experimental work, evaluating different percentages (0-0.1%). Finally, a 0.01% formic acid, in both the aqueous and organic solvents, gave the higher signals and the best peak shapes.

The flow rate was studied from 300 to 600 µl min^{-1} and finally 500 µl min^{-1} was selected as a compromise between signal, peak shape and run time. The temperature of the column was studied between 20 °C and 30 °C and 25 °C was selected, as it provided the highest peak height with the best resolution and good analysis time. The injection volume was evaluated from 5 to 15 µl, and 10 µl was selected as optimum because with a higher injection volume the efficiency of the peaks decreased.

After the chromatographic optimization, a study of the ionization source was carried out considering the following parameters:

- Dry Gas temperature (TEM)

The TEM parameter controls the temperature of the turbo gas in the TurboIonSpray™ source or the temperature of the probe in the heated nebulizer (or APCI) source. It helps to evaporate the solvent to produce gas phase sample ions.

TEM was studied in the range from 300 to 600 °C in steps of 100 °C. The range was selected according to recommendations by the MS manufacturer (AB SCIEX) when a 300 to 1000 µl min^{-1} flow is employed. The study showed a slight improvement on the signal in the case of BTH and NP when the TEM was increased up 500 ^{o}C, while for the rest of CRBs the signal remained almost constant. For this reason a TEM of 500 ^{o}C was selected as optimum.

- Curtain Gas^{TM} (CUR)

The CUR parameter controls the gas which flows between the curtain plate and the orifice. CUR prevents solvent droplets from entering into the MS system and contaminating the ion optics. CUR should be maintained as high as possible without losing sensitivity and compound stability. In this way the entrance of small droplets of mobile phase is avoided

as far as possible. CUR was optimized between 25 and 40 psi in steps of 5. Attending to the results, 30 psi was selected, as higher values produced a slightly decrease in the signals.

- Ion spray voltage (IS)

The IS parameter controls the voltage applied to the needle that ionizes the sample in the ion source. It depends on the polarity, and affects the stability of the spray and the sensitivity. This parameter was studied between -5000 and -5500 V, as recommended by the MS manufacturer, in steps of 250 V. Similar results in terms of intensity signal were obtained for all analytes. For this reason, a IS of -5000 V was considered enough to obtain satisfactory signals.

- Gas 1 (GS1) and Gas 2 (GS2).

The GS1 parameter controls the nebulizer gas, which helps to generate small droplets of chromatographic eluent flow and affects spray stability and sensitivity. The GS2 parameter controls the auxiliary gas (also called turbo gas). It helps to evaporate the spray droplets and prevent solvent from entering the instrument. This parameter was not optimized and considering the recommendations of the manufacturer, values of 50 psi for both GS1 and GS2 were set.

As can be observed in Figure V.2, using the above specified values for the considered variables, all CRBs could be separated and detected in less than 6.0 minutes.

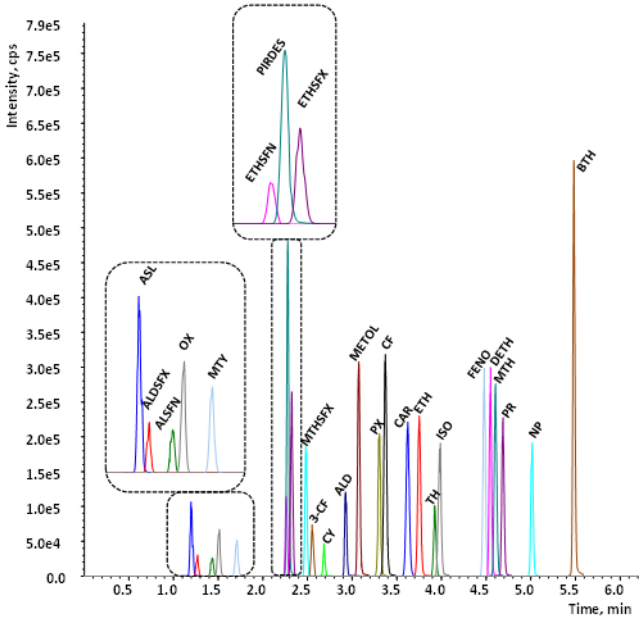

Figure V.2. UHPLC-MS/MS extracted ion chromatogram under optimum conditions (standard solution of 100 μg l^{-1} for each CRB).

As a summary, all the optimum values for the UHPLC separation are included in Table V.2.

Table.V.2. Optimum values for the variables involved in the UHPLC separation.

Variable	Optimum value
Column	C_{18} column (Zorbax Eclipse Plus RRHT 50 mm × 2.1 mm, 1.8 μm)
Mobile phase composition	Solvent A: Water (0.01 % Formic acid) Solvent B: MeOH (0.01 % Formic acid)
Gradient program	0 min (95:5) 0.7 min (80:20) 1.2 min (80:20) 2.5 min (50:50) 3 min (50:50) 6.5 min (5:95)
Sample solvent	Water: MeOH (80:20)
Injection volume	10.0 μl
Mobile phase flow rate	500 μl min^{-1}
Column temperature	25 ºC

3.2. OPTIMIZATION OF UASEME

There are some factors affecting the extraction process, namely: kind and volume of extraction solvent, kind and concentration of emulsifier, salt addition and pH. The optimization of these parameters was carried out using 4.0 ml of an aqueous standard solution with a concentration of 12.5 μg l^{-1} of each CRB. In order to induce phase separation between water and organic solvents, it was necessary to add $MgSO_4$ (30% w/v). The recovery was used to evaluate the extraction efficiency.

The selection of an appropriate extraction solvent is very important in UASEME. It must be immiscible with water, providing the highest extraction efficiency for all the analytes. In this study, extraction solvents with lower density than water (800 μl of toluene, 2-butanone and THF) were chosen using triton X-100 (0.15 mM) as emulsifier surfactant. According to the results (see Figure V.3), in most of the cases the best recoveries were obtained with 2-butanone, giving the highest average recovery.

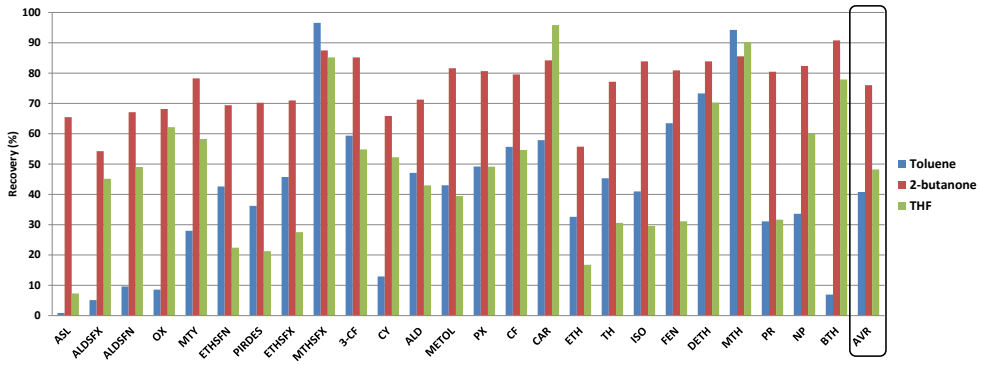

Figure V.3. Effect of different extraction solvents on the extraction efficiency of the CRBs using triton X-100 (0.15 mM) as emulsifier. (AVR: average recovery)

The nature of surfactant is also of great importance for obtaining a satisfactory extraction. Surfactants, which act as emulsifier, could accelerate the emulsification of the water-immiscible extraction solvent into the aqueous solution under ultrasound radiation. After emulsification, the extraction solvent is dispersed as droplets in the sample solution, which are favorable for the mass transfer of the analytes from the aqueous phase to the organic phase. The effect of different emulsifier non-ionic surfactants (0.15 mM triton X-100, triton X-114 and tween 80) on the extraction recovery is shown in Figure V.4. As could be

observed, triton X-114 gave the best results in terms of recovery for the majority of CRBs, especially for those with lower recoveries.

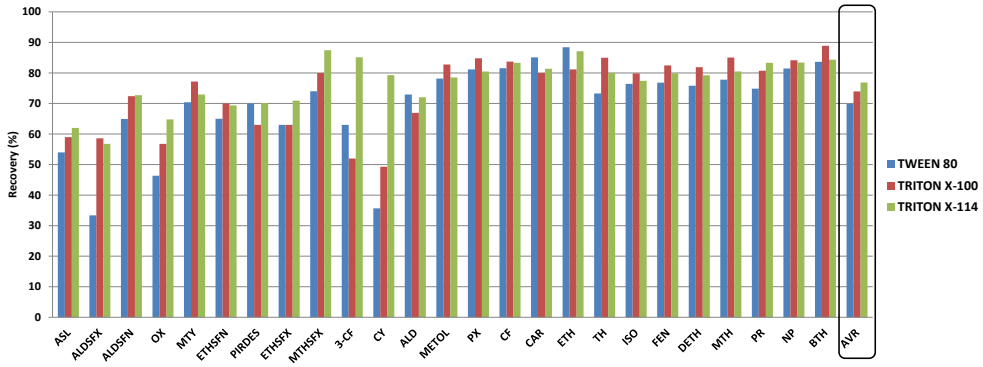

Figure V.4. Effect of different emulsifier surfactants on the extraction recovery using 800 µl of 2-butanone as extraction solvent. (AVR: average recovery)

As discussed earlier in this chapter, the influence of ionic strength on the performance of this type of UASEME was a key point. A salting-out effect is required for separation between organic solvent and water and, as expected, it was observed that with a low salt percentage, the separation was not possible. Moreover, the nature of the salt is also significant in the separation; thus, in order to evaluate this factor, different salts (30 % w/v of NaCl, $(NH_4)_2SO_4$, and $MgSO_4$) were tested. Figure V.5 clearly shows that the best recoveries were obtained when $MgSO_4$ was used.

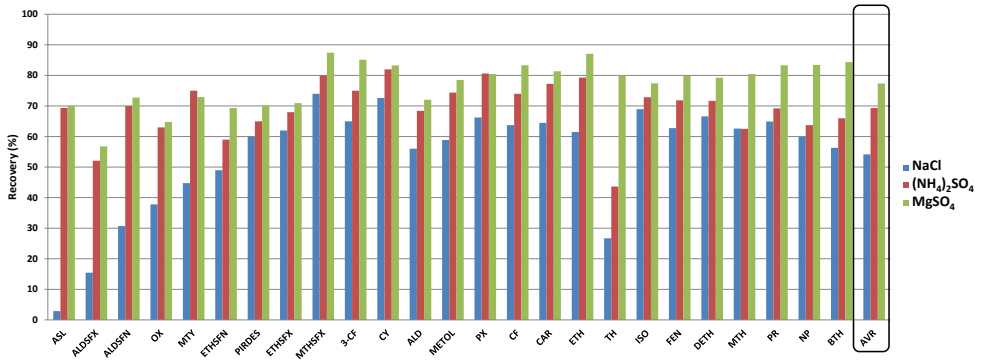

Figure V.5. Effect of different salt natures (30% w/v) on the extraction recovery using 800 µl of 2-butanone as extraction solvent and triton X-114 (0.15 mM) as emulsifier surfactant. (AVR: average recovery)

Subsequently, under these conditions, a quarter fraction factorial screening design 2^{5-2} plus three central points (11 runs) was carried out to establish the significant variables involved in the UASEME and to select their final optimum values, taking into account their interactions. The ranges for the different factors studied in this design were as follows: 2-butanone volume (700-1200 µl), concentration of triton X-114 (0.15-0.25 mM), percentage of $MgSO_4$ (20-30 %), pH (3.0-8.0), and sonication time (5-15 min). The average recovery for all CRBs was considered as experimental response. The lack-of-fit P-value for the model was 17.2 % and the determination coefficient (R^2) was 85.48%, showing a satisfactory fit of the experimental data to the predicted model.

From the screening design, a Pareto Chart (Figure V.6) was obtained, showing that neither the percentage of $MgSO_4$ nor the sonication time were significant for the extraction recovery in the studied range. A possible explanation of the non-significant effect of the percentage of $MgSO_4$ could be that in the studied range (20-30 %), the salting out effect was enough to obtain the maximum insolubility between water and 2-butanone, although a positive effect was observed in the Pareto chart, showing that the highest percentage of salt (30%) was recommended. Regarding sonication time, it has been reported in literature that 5 min was enough to obtain satisfactory recoveries, as the recoveries remained almost constant with longer sonication times [21,22]. The negative effect observed in the Pareto chart would be in accordance with literature. So, 5 min of sonication time was considered as optimum. The effect of sample pH resulted also non-significant for the majority of CRBs; only for CBZ+BY, the recovery was slightly better at pH 3 than without pH adjustment, so that the UASEME was carried out without pH adjustment. The other factors had an expected effect; for example, the volume of extraction solvent was significant, because it is the main factor to obtain a satisfactory recovery. On the other hand, the effect of the triton X-114 concentration was also remarkable, as values above the CMC (0.20 mM for triton X-114) induced a decrease in the extraction efficiency. A possible explanation of this decrease is that a fraction of the CRBs could be incorporated into the already formed micelles, increasing their solubility in the aqueous phase.

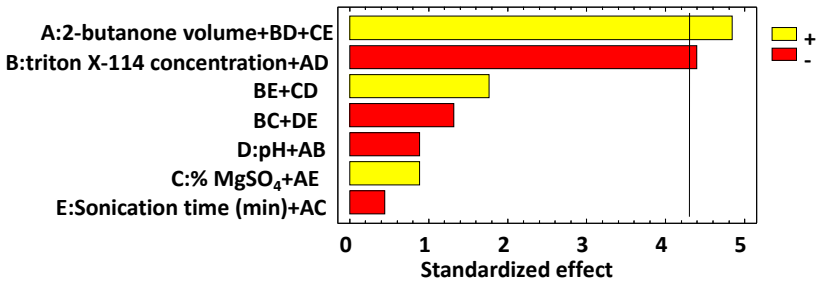

Figure V.6. Pareto chart showing the effects of important variables of UASEME on the average recovery (%) from the screening design (yellow: positive effects; red: negative effects).

Once identified, the next step was the optimization of the significant factors. Thus, extraction solvent volume and emulsifier surfactant concentration were optimized by means of a 3^2 full factorial design with 3 replicates of the central point. The volume of 2-butanone was studied in the range 700-1300 µl and triton X-114 concentration in the range 0.10-0.20 mM (below the CMC of triton X-114, 0.20 mM). The experiments were run randomly in order to minimize the effect of uncontrolled variables and the average recovery for all CRBs was considered as experimental response. The lack-of-fit P-value for the model was 20.2 % and the determination coefficient (R^2) was 91.57%, showing a satisfactory fit of the experimental data to the predicted model. The surface response (see Figure V.7 (a)) showed that the optimum conditions were 1150 µl for 2-butanone and 0.15 mM for triton X-114, a concentration below its CMC.

Figure V.7 (b) shows the Pareto chart, including the effects of both 2- butanone volume and triton X-114 concentration on the average recovery. As can be observed, the effect of triton X-114 concentration was not significant; this can be explained by the fact that the studied range (0.10-0.20 mM) did not exceed the CMC of triton X-114; as a consequence, it was as an emulsion into the aqueous system in the whole range, preventing the solubilization of the CRBs in the aqueous phase.

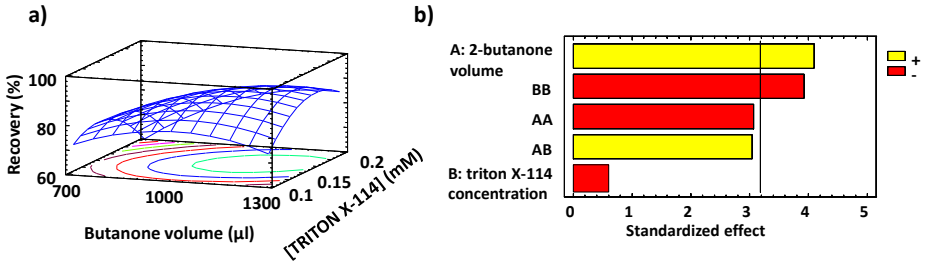

Figure V.7. **a)** Estimated response surface obtained in the optimization of the extraction solvent volume and surfactant concentration using a 3^2 full factorial design; **b)** Standardized Pareto chart for average Recovery (%) (yellow: positive effects; red: negative effects).

3.2.1. Optimization of UASEME for wine samples

Once the UASEME had been optimized with aqueous standard solutions, it was tested with commercially available wine samples. It was observed that with 4 ml of wine (the volume of aqueous standard solution used for the previous optimization) the recoveries were very low. This fact could be explained by the alcohol content of the wine which impaired the effectiveness of the extraction. Thus, in order to reduce this effect, it was decided to dilute the sample in the UASEME procedure; thus, different extractions were carried out with 1, 2, 3 and 4 ml of wine, making up to 4 ml with water. As can be observed in Figure V.8, the higher the dilution, the better the recoveries for all CRBs. Therefore, 1 ml of wine was diluted with 3 ml of water (30% $MgSO_4$, w/v), following the UASEME sample treatment previously described.

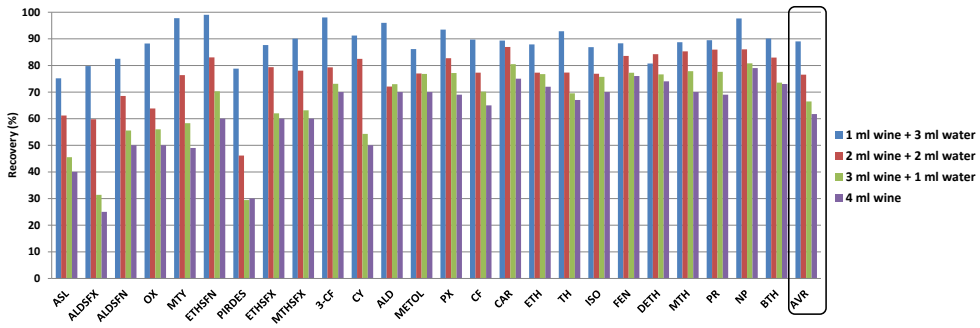

Figure V.8. Effect of wine sample volume on the extraction recovery using 1150 µl of 2-butanone as extraction solvent and triton X-114 (0.15 mM) as emulsifier surfactant. (AVR: average recovery)

3.3. METHOD VALIDATION

In order to check the suitability of the method for the determination of CRBs in wine samples, it was characterized in terms of linear dynamic ranges, LODs and LOQs, matrix effect evaluation, precision, trueness, and selectivity using white wine ("Chardonnay" variety) as representative matrix.

3.3.1. Calibration curves and analytical performance characteristics of the method

Matrix-matched calibration curves were established using white wine spiked with different analyte concentrations (1, 5, 10, 20, 50 and 100 μg l^{-1} for each CRB). Each compound was analyzed in MRM mode, selecting the two highest precursor ion/product ion transitions, which, with retention times, were used to ensure adequate analyte identification, according to SANCO guideline [25]. Each concentration level was processed following the UASEME method and analyzed in triplicate.

LODs and LOQs were provided by the software Analyst, as 3×S/N and 10×S/N, respectively. Statistics and performance characteristics of the method in white wine are shown in Table V.3. As already commented in the introduction, MRLs for these types of matrices have not been established; so in the Table the MRLs for wine grape and the default MRL value established when the matrix is not legislated are summarized, as reference values. As can be seen, good LOQs were obtained for all the analytes, being lower than the default MRL. Therefore, the proposed method is adequate for the determination of very low levels of these compounds in the selected matrix. Compared to other reported methods for the determination of CRBs in wine samples [9,10], the proposed UHPLC method provides similar LOQs, also allowing the determination of a higher number of compounds.

[25] Method validation and quality control procedures for pesticide residues analysis in food and feed. European Commission 2011, SANCO/12495/2011.

Table V.3. Statistics and performance characteristics of the UASEME-UHPLC-MS/MS method for the analysis of CRBs in white wine.

	Linear dynamic range (µg l^{-1})	R^2 (%)	LOD (µg l^{-1})	LOQ (µg l^{-1})	MRL[a] (µg l^{-1})	MRL[b] (µg kg^{-1})
ASL	0.92-200.00	99.5	0.31	0.92	10	500
ALDSFX	0.61-200.00	99.6	0.21	0.61	10	20
ALDSFN	0.51-200.00	99.7	0.15	0.51	10	20
OX	0.42-200.00	99.8	0.12	0.42	10	10
MTY	0.31-200.00	99.8	0.11	0.31	10	500
ETHSFN	0.21-200.00	99.9	0.06	0.21	10	Not established
PIRDES	0.15-200.00	99.8	0.04	0.15	10	1000
ETHSFX	0.24-200.00	99.7	0.07	0.24	10	Not established
MTHSFX	0.55-200.00	99.9	0.16	0.55	10	300
3-CF	0.23-200.00	99.8	0.07	0.23	10	20
CY	0.41-200.00	99.9	0.12	0.41	10	200
ALD	0.33-200.00	99.8	0.10	0.33	10	20
METOL	0.33-200.00	99.6	0.10	0.33	10	Not established
PX	0.37-200.00	99.8	0.11	0.37	10	50
CF	0.40-200.00	99.7	0.08	0.28	10	20
CAR	0.38-200.00	99.7	0.11	0.38	10	50
ETH	0.41-200.00	99.8	0.12	0.41	10	Not established
TH	0.50-200.00	99.7	0.15	0.50	10	500
ISO	0.40-200.00	99.5	0.12	0.40	10	Not established
FEN	0.21-200.00	99.6	0.06	0.21	10	1000
DETH	0.26-200.00	99.4	0.08	0.26	10	1000
MTH	0.20-200.00	99.6	0.06	0.20	10	300
PR	0.19-200.00	99.5	0.06	0.19	10	50
NP	0.26-200.00	99.7	0.08	0.26	10	100
BTH	0.16-200.00	99.8	0.05	0.16	10	Not established

[a] Default MRL value.
[b] MRL for wine grapes.

3.3.2. Quantitative matrix effect evaluation

Matrix effects (MEs) were evaluated by comparison of the slopes of matrix-matched calibration curves with the slopes of standard solution calibration curves at the same final concentration, using the following equation:

$$\mathrm{ME}\ (\%) = \left(\left(\frac{\text{Slope of matrix} - \text{matched curve}}{\text{Slope of standard solution curve}}\right) - 1\right) \mathrm{X}\ 100$$

Figure V.9 shows the matrix effect values for all CRBs analyzed by UASEME-UHPLC-MS/MS method. Matrix effects for all analytes were very soft, with values from -0.5% for ETHSFX to -17.4% in the case of NP.

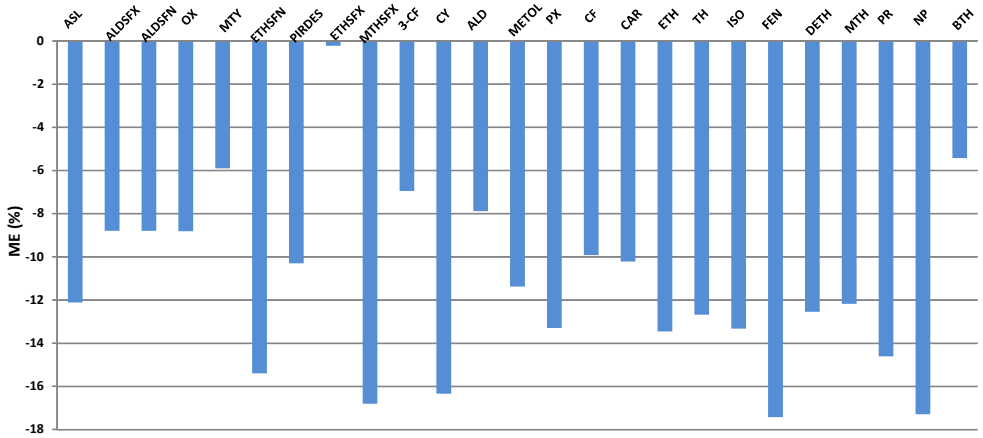

Figure V.9. Matrix effect (%) for CRBs in white wine.

3.3.3. Precision study

The precision of the method was evaluated in terms of repeatability (intraday precision) and intermediate precision (interday precision) by application of the proposed UASEME–UHPLC-MS/MS method in white wine samples spiked at three different concentration levels of CRBs. Repeatability was evaluated over five samples prepared and injected in triplicate on the same day, under the same conditions. Intermediate precision was evaluated with a similar procedure, but the samples were analyzed in five consecutive days. The results, expressed as % RSD of the peak areas, are summarized in Table V.4. Good precision, lower than 6%, was obtained in all cases. Thus, the results obtained with the proposed method could be considered in agreement with the current demand [25,26].

[26] European Union Commission Decision 2002/657/EC of 12 of August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Official Journal of the European Union* L 221, 2002, pp. 8–36.

Table V.4. Intraday and interday precision expressed as RSD (%) for white wine sample (n=15).

	Intraday precision [a]			Interday precision [a]		
	Level 1	**Level 2**	**Level 3**	**Level 1**	**Level 2**	**Level 3**
ASL	3.8	2.4	2.6	4.2	3.2	3.0
ALDSFX	2.4	2.0	1.9	3.0	2.3	2.7
ALDSFN	3.6	5.5	3.5	5.5	6.1	5.1
OX	3.9	3.1	2.5	4.4	3.5	2.8
MTY	2.8	3.1	2.7	3.6	3.6	3.2
ETHSFN	3.0	1.9	2.8	3.5	2.3	2.9
PIRDES	2.8	2.1	2.2	3.3	2.6	2.6
ETHSFX	2.0	1.2	1.0	2.5	1.7	1.9
MTHSFX	2.9	1.5	2.3	3.5	2.0	2.8
3-CF	2.6	2.5	2.9	3.0	3.3	3.2
CY	2.6	2.4	2.4	2.9	2.9	2.8
ALD	2.4	1.9	2.6	3.0	2.3	3.0
METOL	3.2	1.1	2.5	3.8	1.4	3.3
PX	2.7	2.2	1.9	3.6	2.7	2.4
CF	3.2	2.0	2.5	3.5	2.3	3.0
CAR	3.5	2.4	3.8	4.3	2.9	4.6
ETH	3.6	2.4	1.8	4.2	2.8	2.1
TH	3.2	2.1	2.1	3.7	2.4	2.7
ISO	4.1	2.2	2.0	4.7	2.8	2.4
FEN	3.7	2.1	2.3	4.1	2.6	3.1
DETH	4.1	2.4	2.2	4.5	2.9	2.6
MTH	4.2	2.5	2.2	4.8	3.1	2.8
PR	1.8	2.3	3.4	2.1	2.9	4.2
NP	2.8	2.7	5.2	3.4	3.5	5.7
BTH	2.7	3.1	2.8	3.2	3.4	3.2

[a] level 1=5 μg l^{-1}, level 2=10 μg l^{-1} and level 3=50 μg l^{-1}.

3.3.4. Trueness assessment

In order to check the trueness of the proposed methodology, recovery experiments were carried out in different types of wine samples obtained from local markets ("Chardonnay" and "Ribeiro" white wines, "Tempranillo" sparkling rose wine, and "Lambrusco" rose wine) spiked at three different concentration levels of each CRBs. Each sample was analyzed in triplicate and injected five times. In all the cases, a blank sample was analyzed to check the presence of CRBs; none of them gave a result above the LODs of the method.

Finally, recoveries between 75 and 103% were obtained, with satisfactory precision (see Table V.5, Table V.6, Table V.7 and Table V.8), fulfilling the requirements of current legislation about analytical performance, which establishes that the recovery must be between 70 to 120 % [25,26].

Table V.5. Recovery study and RSD for "Chardonnay" white wine (n=15).

	Level 1[a]		Level 2[a]		Level 3[a]	
	R (%)	RSD (%)	R (%)	RSD (%)	R (%)	RSD (%)
ASL	75.1	3.8	79.9	2.4	79.4	2.6
ALDSFX	79.8	2.4	76.0	2.0	80.8	1.9
ALDSFN	82.5	3.6	84.1	5.5	92.7	3.5
OX	88.2	4.0	80.5	3.1	80.3	2.5
MTY	97.8	2.8	83.1	3.1	82.8	2.7
ETHSFN	102.8	3.0	93.7	2.0	101.1	2.8
PIRDES	78.9	2.8	84.7	2.1	89.5	2.2
ETHSFX	87.6	2.0	95.4	1.2	85.6	1.1
MTHSFX	90.0	2.9	87.9	1.5	83.1	2.3
3-CF	103.1	2.6	89.0	2.5	93.0	2.9
CY	91.2	2.6	92.4	2.4	98.5	2.4
ALD	96.0	2.5	87.4	1.9	91.2	2.6
METOL	86.1	3.2	93.1	1.1	83.1	2.5
PX	93.4	2.8	96.7	2.2	93.9	2.0
CF	89.7	3.2	97.8	2.0	91.1	2.5
CAR	89.3	3.5	90.0	2.3	92.2	3.8
ETH	87.9	3.6	89.0	2.2	95.4	1.8
TH	92.8	3.2	91.4	2.0	85.6	2.1
ISO	86.9	4.1	94.4	2.2	93.5	2.0
FEN	88.3	3.7	90.2	2.1	83.9	2.2
DETH	80.7	4.0	84.9	2.4	81.9	2.3
MTH	88.7	4.2	94.3	2.5	93.4	2.2
PR	89.5	1.8	90.2	2.3	82.8	3.4
NP	97.6	2.8	85.1	2.7	92.8	5.2
BTH	90.0	2.7	84.6	3.1	89.4	2.8

[a] level 1=5 μg l^{-1}, level 2=10 μg l^{-1} and level 3=50 μg l^{-1}.

Table V.6. Recovery study and RSD for "Tempranillo" sparkling rose wine (n=15).

	Level 1[a]		Level 2[a]		Level 3[a]	
	R (%)	RSD (%)	R (%)	RSD (%)	R (%)	RSD (%)
ASL	79.1	3.1	81.1	3.0	87.1	3.1
ALDSFX	86.4	2.4	84.9	2.3	83.3	2.5
ALDSFN	82.0	2.0	83.5	1.9	87.9	1.1
OX	83.5	2.2	85.4	1.9	83.1	1.8
MTY	89.6	3.8	92.7	3.4	95.0	2.5
ETHSFN	95.1	2.2	97.5	1.8	95.6	1.7
PIRDES	98.2	2.9	99.4	2.6	101.5	2.0
ETHSFX	88.3	1.6	85.2	2.1	88.4	3.5
MTHSFX	94.4	2.8	97.0	2.0	93.4	2.1
3-CF	95.5	3.8	98.4	3.0	97.3	2.5
CY	82.4	3.4	84.9	2.5	85.2	2.1
ALD	87.5	3.1	86.2	2.7	84.1	2.4
METOL	87.2	3.1	89.7	2.8	95.7	2.1
PX	92.1	2.9	89.8	2.8	93.2	2.6
CF	88.3	3.2	86.2	2.5	89.5	1.5
CAR	85.8	3.5	89.5	2.8	88.1	2.1
ETH	89.1	2.7	91.2	2.1	93.0	2.3
TH	94.7	2.5	91.2	3.0	92.0	3.4
ISO	93.5	2.7	95.3	1.9	92.3	1.8
FEN	94.7	2.5	92.8	2.6	94.9	2.4
DETH	91.1	2.3	88.3	2.2	92.8	2.0
MTH	91.6	3.2	87.9	2.9	92.1	2.5
PR	91.5	2.7	95.3	2.8	96.4	3.6
NP	97.7	3.7	96.8	2.7	94.1	3.0
BTH	93.2	3.0	93.9	2.7	92.1	3.1

[a] level 1=5 μg l^{-1}, level 2=10 μg l^{-1} and level 3=50 μg l^{-1}.

Table V.7. Recovery study and RSD for "Lambrusco" rose wine (n=15).

	Level 1[a]		Level 2[a]		Level 3[a]	
	R (%)	RSD (%)	R (%)	RSD (%)	R (%)	RSD (%)
ASL	74.8	3.2	77.1	3.0	80.3	2.4
ALDSFX	75.5	3.0	77.8	2.9	81.6	2.2
ALDSFN	84.2	3.2	84.2	2.5	87.4	2.7
OX	87.5	3.7	89.4	3.6	91.8	2.1
MTY	95.7	3.0	94.1	2.5	94.1	2.7
ETHSFN	97.3	3.5	96.5	3.9	95.2	2.8
PIRDES	78.6	2.9	79.6	2.7	81.5	2.1
ETHSFX	77.1	2.5	82.7	2.4	83.4	2.2
MTHSFX	90.6	3.0	91.1	2.1	91.9	2.0
3-CF	91.2	3.9	95.4	2.6	93.0	3.0
CY	88.5	3.2	91.7	2.9	91.4	2.2
ALD	93.2	2.9	94.3	2.2	93.1	1.9
METOL	88.8	3.1	92.0	2.6	90.7	2.4
PX	94.5	3.5	99.0	2.7	97.1	2.6
CF	91.1	3.1	93.8	2.5	92.9	2.4
CAR	89.9	3.5	91.3	2.9	93.4	2.5
ETH	91.9	4.0	92.2	2.7	93.5	2.1
TH	90.5	3.0	91.9	2.2	92.1	2.2
ISO	87.3	2.8	91.9	2.7	92.7	2.3
FEN	90.3	2.7	91.7	2.3	92.9	2.6
DETH	85.3	2.3	83.9	2.5	85.1	2.1
MTH	88.3	3.4	91.0	2.4	89.9	1.7
PR	89.3	2.6	88.7	2.3	91.3	1.8
NP	96.2	3.4	94.7	2.2	96.3	1.8
BTH	94.3	2.9	96.6	2.6	96.2	2.4

[a] level 1=5 μg l^{-1}, level 2=10 μg l^{-1} and level 3=50 μg l^{-1}.

Table V.8. Recovery study and RSD for "Ribeiro" white wine (n=15).

	Level 1[a]		Level 2[a]		Level 3[a]	
	R (%)	RSD (%)	R (%)	RSD (%)	R (%)	RSD (%)
ASL	77.4	2.9	82.6	2.8	81.2	2.1
ALDSFX	79.0	2.7	77.5	2.3	81.1	1.7
ALDSFN	85.1	3.0	87.2	1.9	87.0	1.2
OX	89.6	2.9	90.8	2.0	93.4	2.4
MTY	94.0	2.7	92.0	2.1	96.3	2.4
ETHSFN	95.9	3.2	96.3	2.2	93.1	2.1
PIRDES	82.4	2.5	87.2	2.6	84.4	2.2
ETHSFX	91.4	2.5	94.3	2.1	93.1	2.2
MTHSFX	89.8	2.6	91.0	2.4	90.2	2.5
3-CF	93.3	2.3	94.5	2.3	95.9	2.4
CY	89.0	2.6	89.8	2.0	93.0	1.9
ALD	94.2	2.5	93.5	2.3	96.8	2.4
METOL	85.8	3.1	86.4	2.4	84.3	2.5
PX	94.6	2.9	93.4	2.5	95.5	2.2
CF	91.1	3.3	93.2	2.9	94.5	2.3
CAR	89.2	2.6	94.0	2.5	93.9	2.6
ETH	91.9	3.2	92.1	2.4	93.1	1.8
TH	90.7	3.1	92.4	2.4	93.5	2.1
ISO	85.9	2.9	87.8	2.6	88.7	2.1
FEN	90.9	3.0	92.2	2.5	92.1	2.4
DETH	81.3	3.5	86.0	2.2	87.2	2.7
MTH	85.9	3.1	86.8	2.7	89.7	3.2
PR	91.3	2.9	92.4	2.6	93.9	2.6
NP	92.5	2.9	96.9	2.9	95.0	2.6
BTH	89.8	3.6	91.8	2.7	92.1	2.7

[a] level 1=5 μg l^{-1}, level 2=10 μg l^{-1} and level 3=50 μg l^{-1}.

A typical extracted ion chromatogram corresponding to a "Chardonnay" white wine sample spiked with 1 µg l^{-1} for each CRB, and analyzed by the proposed UASEME–UHPLC–MS/MS method is shown in Figure V. 10.

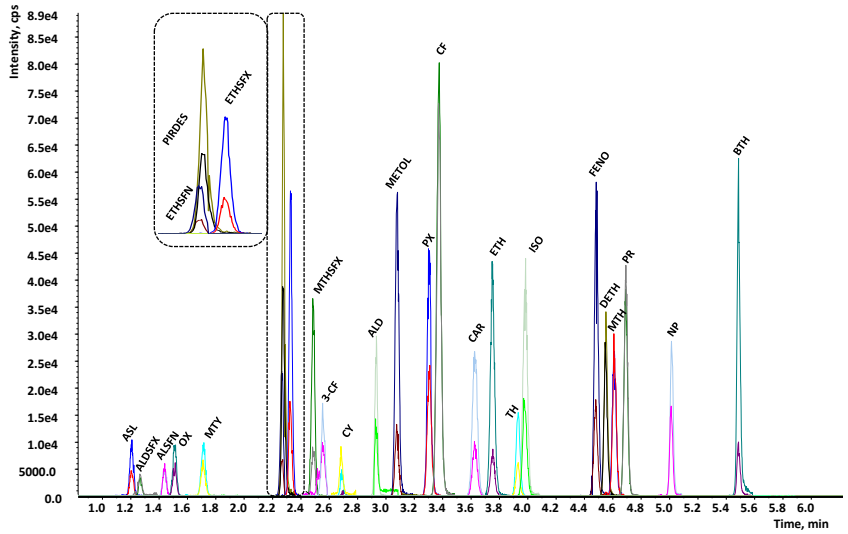

Figure V.10. Extracted ion chromatogram of a spiked "Chardonnay" white wine sample applying the proposed UASEME–UHPLC-MS/MS method (1 µg l^{-1} for each CRB).

3.3.5. Selectivity

The confirmation of CRBs was carried out according to European guidelines for determination of pesticides residues [25,26], which establishes a tolerance level for the relative intensity between Q and I MRM transitions in real samples. This tolerance value depends on the ratio Q/I value obtained from a standard solution (see Table V.1). Table V.9 shows the Q/I ratios obtained from a spiked wine sample (5 µg l^{-1} for each CRB), and from a standard solution (10 µg l^{-1}); as can be seen in all cases Q/I ratios in sample were within the tolerance range for relative ion intensities indicated in the current European guidelines [25,26]. The same conclusions were obtained for the other wines included in this study. Thus, considering these results along with values obtained in the matrix effect evaluation, it can be concluded that no significant interferences from the wine matrices tested affected the analytical response.

Table V.9. Ratio between the quantifier (Q) and qualifier (I) ions in standard and sample solutions.

	Relative ratio from standards		Relative ratio in white wine	
	Q/I ratio (%)	**Range**	**Tolerance range**	**Q/I ratio (%)**
ASL	48.7	>20% to 50%	±25%	50.4
ALDSFX	90.2	>50%	±20%	93.8
ALDSFN	98.9	>50%	±20%	87.3
OX	44.8	>20% to 50%	±25%	49.8
MTY	60.1	>50%	±20%	66.2
ETHSFN	28.7	>20% to 50%	±25%	30.3
PIRDES	50.3	>50%	±20%	55.6
ETHSFX	26.5	>20% to 50%	±25%	33.9
MTHSFX	23.5	>20% to 50%	±25%	26.0
3-CF	68.7	>50%	±20%	65.6
CY	71.6	>50%	±20%	85.9
ALD	43.9	>20% to 50%	±25%	49.0
METOL	22.6	>20% to 50%	±25%	21.3
PX	51.2	>50%	±20%	53.3
CF	89.0	>50%	±20%	87.5
CAR	35.0	>20% to 50%	±25%	34.9
ETH	20.7	>20% to 50%	±25%	20.1
TH	29.3	>20% to 50%	±25%	37.2
ISO	37.6	>20% to 50%	±25%	40.5
FEN	28.3	>20% to 50%	±25%	30.5
DETH	79.7	>50%	±20%	78.2
MTH	91.9	>50%	±20%	98.5
PR	87.1	>50%	±20%	84.5
NP	59.5	>50%	±20%	60.2
BTH	19.1	>10% to 20%	±30%	18.7

4. CONCLUSIONS

A new multiresidue UHPLC-MS/MS method has been developed for the simultaneous determination of twenty five CRBs in wine samples. UASEME using an extraction solvent with a lower density than water has been optimized in order to obtain satisfactory recoveries for all analytes. The results in terms of analyses time, sensitivity, selectivity, precision, cleanliness of extracts and matrix effects showed the suitability of this procedure for the monitoring of the CRB residues in different kinds of wines in a single run. To our knowledge, this is the first UASEME-UHPLC-MS/MS method described for CRBs using this type of organic solvent, and analyzing simultaneously such a high number of compounds. Applying this method, and considering the short analysis time achieved in UHPLC, a high sample throughput can be achieved (less than 20 min, including sample preparation and determination), shorter compared to traditional methods, such as HPLC-MS or GC-MS and very useful in pesticide monitoring programs with a large number of samples to analyze. Moreover, taking into account the lower consumption of solvents required in UHPLC, and the absence of organic disperser solvents in the UASEME procedure, the proposed method could be considered as an environmentally friendly strategy for the monitoring of a high number of CRBs, including some metabolites which have been scarcely studied in wines samples.

This work has been submitted for publication to Journal of Chromatography A as:

"Ultrasound-assisted surfactant-enhanced emulsification microextraction for the determination of carbamates in wines by UHPLC-MS/MS".

CAPÍTULO 6

Análisis simultáneo de carbamatos en productos herbales mediante UHPLC-MS/MS empleando la metodología QuEChERS

CHAPTER 6

Simultaneous analysis of thirty-three carbamates in herbal products by UHPLC-MS/MS using QuEChERS methodology

RESUMEN

En este capítulo, se ha desarrollado un nuevo método multirresiduo para la determinación de treinta y tres carbamatos (incluyendo algunos metabolitos) en tés, manzanilla y otros productos herbales empleados en la preparación de infusiones mediante cromatografía líquida de ultra resolución acoplada a espectrometría de masas en tándem, proponiendo el método QuEChERS para el tratamiento de la muestra. La optimización de la etapa de limpieza (extracción sólido-líquido dispersiva) ha permitido minimizar el efecto matriz en este tipo de muestras, reduciéndose alrededor del 30% para la mayoría de los plaguicidas, comparándolo con una extracción sin limpieza adicional. Para los tres niveles de concentración estudiados (5, 20 y 50 μg kg^{-1}), el método propuesto proporcionó recuperaciones entre el 74 y el 101%, con desviaciones estándar relativas inferiores al 7%. Los límites de cuantificación estuvieron comprendidos entre 1.9 y 4.0 μg kg^{-1}, siempre por debajo de los límites máximos establecidos por la legislación para este tipo de muestras.

SUMMARY

In this chapter, a new multiresidue method for the determination of thirty-three carbamates (including some metabolites) in teas, chamomile, and other herbal products for infusions by ultra-high performance liquid chromatography tandem mass spectrometry was developed. The QuEChERS method was evaluated as sample treatment and, by optimizing the clean-up step (dispersive solid phase extraction), matrix effects were reduced around 30% for most of the pesticides, compared with an extraction without additional cleaning. The method allowed recoveries between 74 and 101%, with relative standard deviations lower than 7% at three concentration levels (5, 20 and 50 μg kg^{-1}). Limits of quantification ranged from 1.9 to 4.0 μg kg^{-1}, below maximum residue limits established for this type of samples.

1. INTRODUCTION

Many types of plants are consumed throughout the world as infusions for both pleasure and therapeutic purposes (health benefits, to prevent neurodegenerative diseases or for their anticancer effects) [1-3]. Chamomile and tea are the most popular herbal infusions in the world; for example, according to the Food and Agriculture Organization of the United Nations (FAO), in 2010 the worldwide production of tea was 4.1 million tonnes [4], and the tea and other herbal infusions consumption in some countries can reach 3 kg/year per inhabitant [5]. As in other agricultural products which have been commented in previous chapters, pesticides used for growing these herbals must be subject to control, in order to minimize possible risks to human health, because the intake of such water-based drinks could represent a contribution of total human exposure to pesticides. Moreover, EFSA annual report in 2009, indicated that 5% of tea samples and 15% of other herbal products analyzed were positives for pesticides above MRLs established by EU [6]. Those MRLs for the CRBs considered in the present study are between 5 and 200 μg l^{-1} for chamomile, tea and other plants consumed as infusions [7]. So, it is important to develop versatile and trustworthy methods to analyze and control these samples.

In the field of instrumental analysis for pesticide residue determination in herbal products, methods based on the use of MS are frequently reported, coupled to both GC [8-10] or

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[6] *EFSA Journal* (2011) 2430.

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HPLC [11,12]. Recently, UHPLC with columns packed with small particles and high linear velocities have proved to give superior chromatographic resolution, reduced analysis time, less solvent consumption and increased sensitivity. Taking advantages of these features, some UHPLC-MS methods have been proposed for the analysis of this type of samples [13,14].

These commodities are complex matrixes as they may contain different components, including pigments, caffeine, alkaloids, polyphenols, flavonoids and essential oils [15,16,17]. This fact highlights the importance of establishing a good sample treatment, in order to minimize the amount of co-extractives compounds in the final extract, which could prevent the correct quantification of the analytes studied. Recently, extraction and clean-up methods have been published on the determination of pesticide residues in these commodities using SPE [14] or gel permeation chromatography [10]. Nevertheless, these methods usually require a high consumption of organic solvents and several clean-up procedures, which may be tedious and costly. Since the emerging of QuEChERS method, introduced by Anastassiades *et al.* in 2003 [18,19], this methodology has been increasingly used and has been adopted as recommended method for determination of pesticide residues [20]. This sample treatment is fast and inexpensive, because it presents a high extraction efficiency, smaller volume of organic solvent, simplicity of operation, low cost per sample and effectiveness for cleaning-up complex samples such as vegetables [21,22],

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fruits [22,23] and herbal products [9-14]. It involves two steps: the first one is an extraction step based on partitioning via salting-out involving the equilibrium between an aqueous and an organic layer, and the second one is a dispersive SPE (dSPE) step that involves further clean-up using combinations of different sorbents (such as $MgSO_4$, C_{18}, graphitized carbon or primary-secondary amines (PSA)), to remove interfering substances.

The purpose of this chapter has been to develop a fast, simple, selective and efficient UHPLC-MS/MS method for the simultaneous determination of thirty-three CRBs in commonly consumed herbal products. Taking into account the study of the EFSA showing the risk that suppose the presence of metabolite pesticide residues in the diet [24], some of these metabolites have been included in this work. QuEChERS methodology has been evaluated for the sample treatment, showing good recovery for all CRBs with satisfactory reproducibility. The method was applied to different types of herbal products (such as chamomile, green tea, red tea, valerian, thyme and linden) achieving LOQs below MRLs specified for this type of samples.

2. EXPERIMENTAL

2.1. CHEMICALS

Water, MeOH, and ACN (LC-MS Chromasolv grade) were supplied from Fluka Analytical (Steinheim, Germany). Formic acid and 2-butanone were obtained from Sigma-Aldrich (ST. Louis, MO, USA) and acetic acid was supplied from Merck (Darmstadt, Germany).

Pestanal grade analytical standards of propamocarb (PRM), ASL, ALDSFX, ALDSFN, OX, MTY, CBZ+BY, ETHSFN, PIRDES, ETHSFX, MTHSFX, 3-CF, methiocarb sulfone (MTHSFN), CY, ALD, METOL, pirimicarb (PIR), PX, CF, CAR, ETH, TH, ISO, FEN, DETH, MTH, PR, NP, FNX, pyraclostrobin (PY), BTH, benfurocarb (BFU) and furathiocarb (FURA) were supplied by Fluka. Individual stock standard solutions containing 3 g l^{-1} of each compound were prepared by dissolving accurately weighed amounts in MeOH and were stored in the dark

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at 4 ^{o}C. They were stable for at least 4 months. Working standard solutions containing all the CRBs were freshly prepared by proper dilution of the stock standard solutions with MeOH.

SampliQ QuEChERS (Agilent Technologies, Waldbron, Germany) consisted on 50 ml tube and QuEChERS extraction kit (buffered: 4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate and 0.5 g disodium citrate sesquihydrate; No buffered: 4 g $MgSO_4$ and 1 g NaCl), while dSPE tubes were prepared in the lab from C_{18}, PSA and $MgSO_4$, supplied by Agilent.

Acrodisc 13-mm syringe filters with 0.2 µm nylon membrane (Pall Corp., MI, USA) were used for filtration of sample extracts prior to the injection in the UHPLC system.

2.2. INSTRUMENTATION

Separation was performed on an Agilent 1290 Infinity LC using a C_{18} column (Zorbax Eclipse plus RRHT 50 mm × 2.1 mm, 1.8 µm) supplied by Agilent Technologies. The mass-spectrometer measurements were performed on a triple quadrupole mass spectrometer API 3200 (Applied Biosystems, Darmstadt, Germany) with ESI. The instrumental data were collected using the Analyst® Software version 1.5 with Schedule MRMTM Algorithm (ABSCIEX).

A pH-meter (Crison model pH 2000; Barcelona, Spain) with a resolution of ±0.01 pH unit, a centrifuge (Universal 320 model from Hettich; Leipzig, Germany), a mechanical shaker (model 384 from Vibromatic, Noblesville, USA), a nitrogen evaporator (System EVA-EC from VLM GmbH, Bielefeld, Germany) and a vortex (Genie 2 model from Scientific Industries; Bohemia, NY, USA) were also used.

2.3. CHROMATOGRAPHIC PROCEDURE

UHPLC separations were performed in a C_{18} column using a mobile phase consisting of 0.01% aqueous formic acid solution (solvent A), and MeOH with the same percentage of acid (solvent B) at a flow rate of 0.5 ml min^{-1}. The gradient profile was 0% B at the beginning; 20% B from 0.7 to 1.2 min; 50% B from 2.5 to 3 min; 95% B from 6.5 to 7.0 min; and finally in order to come back to the initial conditions, 0% B at 7.5 min, equilibrating for

3 min. Under optimum conditions, all analytes were eluted in 6 min, while the run time for each injection was 9.5 min. The temperature of the column was 25 ^{o}C and the injection volume was 10 µl. The mass-spectrometer was working with ESI in positive mode under the MRM conditions shown in Table VI.1. The ionization source parameters were: source temperature 400 ^{o}C; curtain gas (nitrogen) 30 psi, ion spray voltage -5000 V; and GAS 1 and GAS 2 (both of them nitrogen) were set to 50 psi.

Table VI.1. Optimized MS parameters.

	Precursor ion	Rt	DP	EP	CEP	Product ion*	CEN	CXP
PRM	189.2	1.0	41	6.5	22	102.1 (Q)	25	4
						73.8 (I)	37	4
ASL	231.1	1.2	36	8	12	155.9 (Q)	15	4
						92.1 (I)	33	4
ALDSFX	207.2	1.3	21	8.5	16	132.1 (Q)	11	4
						89.1 (I)	17	2
ALDSFN	223.1	1.5	36	6	12	85.9 (Q)	23	4
						76.2 (I)	13	0
OX	237.2	1.5	16	6.5	12	71.9 (Q)	27	4
						90.2 (I)	13	2
MTY	163.2	1.7	26	7.5	10	88.2 (Q)	13	2
						106 (I)	15	2
CBZ+BY	192.2	1.9	36	9	18	160 (Q)	41	4
						132.1 (I)	25	4
ETHSFN	258.1	2.2	41	7	16	107 (Q)	21	4
						201 (I)	13	8
PIRDES	225.1	2.3	26	9	14	72.1 (Q)	27	4
						168.1 (I)	19	6
ETHSFX	242.1	2.3	21	6	20	107.1 (Q)	27	4
						185.2 (I)	13	4
MTHSFX	242.1	2.5	26	6.5	14	185.21 (Q)	19	6
						170 (I)	29	4
3- CF	238.1	2.5	36	9	12	163.1 (Q)	19	4
						220 (I)	11	4
MTHSFN	258.1	2.7	41	6	12	122 (Q)	33	6
						201 (I)	13	6
CY	199.2	2.7	21	7	15	128 (Q)	13	4
						111.2 (I)	25	4
ALD	116.1	3	31	9.5	12	89.1 (Q)	13	0
						70.3 (I)	13	0
METOL	166.0	3.1	31	6.5	14	109 (Q)	17	4
						94 (I)	43	4

*(Q) Transition used for quantification, (I) Transition employed to complete the identification.
Rt: Retention Time. DP: Declustering Potential. EP: Entrance Potential. CEP: Collision Cell Entrance potential. CEN: Collision Energy. CXP: Collision Exit Potential.

Table VI.1. Continued.

	Precursor ion	Rt	DP	EP	CEP	Product ions*	CEN	CXP
PIR	239.2	3.2	36	7.5	16	72.1 (Q)	29	4
						182.2 (I)	21	4
PX	210.3	3.3	21	7.5	15	111.1 (Q)	21	4
						168.2 (I)	13	4
CF	222.2	3.4	41	6.5	15	165.1 (Q)	17	4
						123.1 (I)	29	4
CAR	202.2	3.6	26	9	15	145(Q)	17	4
						127.2 (I)	39	4
ETH	226.1	3.8	26	8	15	107.1 (Q)	23	4
						164.4 (I)	13	2
TH	355.2	3.9	21	7	19	88.1 (Q)	33	4
						108.1 (I)	19	2
ISO	194.2	4	26	8.5	15	95.1 (Q)	21	2
						137.2 (I)	13	4
FEN	208.2	4.5	36	7	15	95.2 (Q)	23	2
						151.9 (I)	13	4
DETH	268.3	4.6	31	8.5	16	226 (Q)	15	6
						124.2 (I)	41	4
MTH	226.1	4.6	21	8.5	15	169.1 (Q)	15	4
						121.1 (I)	25	4
PR	208.3	4.7	26	8.5	15	151.1 (Q)	13	4
						109 (I)	25	4
NP	272.2	5	36	11	17	129.2 (Q)	23	4
						171.3 (I)	25	6
FNX	302.1	5.2	36	5.5	17	87.9 (Q)	33	4
						116.1 (I)	17	4
PY	388.2	5.4	26	7	20	163.1 (Q)	21	4
						194.1 (I)	19	4
BTH	258.2	5.5	46	7.5	16	125 (Q)	33	4
						89.2 (I)	71	4
BFU	411.3	5.8	46	6.5	20	195.2 (Q)	31	4
						252.1 (I)	21	4
FURA	383.1	5.8	56	8	8	195.2 (Q)	23	4
						252 (I)	19	6

*(Q) Transition used for quantification, (I) Transition employed to complete the identification.
Rt: Retention Time. DP: Declustering Potential. EP: Entrance Potential. CEP: Collision Cell Entrance potential. CEN: Collision Energy. CXP: Collision Exit Potential.

2.4. SAMPLE PREPARATION

Chamomile, green tea, red tea, valerian, thyme and linden were purchased in local markets from Granada (Spain). The QuEChERS procedure was as follows: 1 g of sample previously homogenized was placed into a 50 ml falcon tube. Five ml of Milli-Q water was added to hydrate the samples; it was shaken in a vortex for 2 min, and then left for 30 min. Subsequently, 10 ml of ACN was added to the tube, and was mechanically shaken for 10 min. Agilent SampliQ QuEChERS extraction kit (4 g $MgSO_4$, 1 g NaCl,) was added and the tube was shaken for 10 min. After that, the sample was centrifuged at 9000 rpm for 5 min.

Then, 3 ml of the supernatant were transferred to the QuEChERS dispersive tube containing 150 mg of PSA and 150 mg of C_{18}, stirred in vortex for 2 min and centrifuged (9000 rpm for 2 min). An aliquot of 2 ml of supernatant was transferred to a vial, dried under N_2 stream and the final residue was re-dissolved with 500 µl of sample solvent (H_2O:MeOH, 80:20), shaken by vortex (2 min), filtered and injected into the UHPLC-MS/MS system. A schematic diagram of the sample preparation procedure is shown in Figure VI.1.

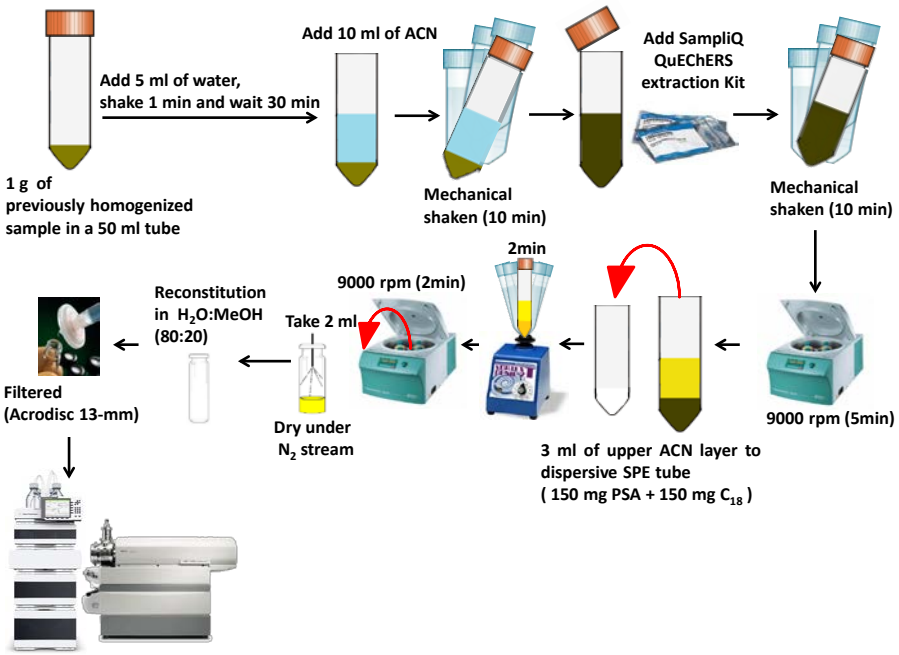

Figure VI.1. Diagram of the QuEChERS procedure.

For method validation, samples were spiked with the standard solution at appropriate levels. However, chamomile, teas and other herbal products are dehydrated samples; thus, in order to achieve a homogeneous distribution of the CRBs, after spiking the sample (1 g), it was continuously stirring, while at the same time the solvent of the CRB solution (MeOH) was evaporated under N_2 stream during 2 min. This method (shown in Figure VI.2) allowed an appropriate homogenization of the samples.

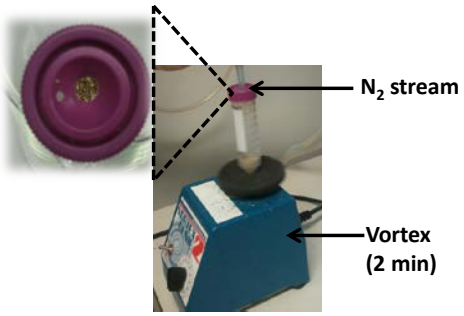

Figure VI.2. Spiking procedure.

3. RESULTS AND DISCUSSION

3.1. OPTIMIZATION OF MS/MS DETECTION AND CHROMATOGRAPHIC SEPARATION

For each individual CRB, the mass spectrometer was optimized to provide the best responses for quantification. In order to get high sensitivity, each analyte was individually infused as a standard solution of 1 mg l^{-1} in 0.1% aqueous formic acid solution:MeOH (50:50, v:v), directly into the mass spectrometer. All compounds were tested using ESI positive/negative mode. As it was expected from previous data [25-27] ESI operating in positive mode showed the best results in terms of sensitivity. During the infusion the variables DP, EP, CEP, CEN and CXP were optimized for each compound in order to obtain the maximum sensitivity (see results in Table VI.1). Each compound was characterized by its retention time and by two precursor ion-product ion transitions. The most intense product ion (Q) was used for quantification whereas the second one (I) was used to complete the identification (see selected ions in Table V.1). The target scan time established for each transition was 0.2 s. Under the experimental conditions, protonated molecules $[M+H]^+$ were observed for all the compounds except OX and ALD. In the case of

[25] A. Economou, H. Botitsi, S .Antoniou, D. Tsipi, *J. Chromatogr. A* 1216 (2009) 5856.
[26] M. Moeder, C. Bauer, P. Popp, M. van Pinxteren, *Anal. Bioanal. Chem.* 403 (2012) 1731.
[27] T.Goto, Y. Ito, H. Oka, I. Saito, H. Matsumoto, H, Sugiyama, C. Ohkubo, H, Nakazawa, H. Nasage, *Anal. Chim. Acta* 531 (2005) 79.

OX, ammonium adduct was obtained as majority precursor ion. Moreover, in the case of ALD, $[M+H]^+$was not observed, while $[M+H-75]^+$ ion had a strong intensity, as previously described [28].

Chromatographic conditions were the same of Chapter 5, except for the initial conditions; thus, in this case the gradient program started with 100% of aqueous phase, in order to improve the shape of PRM, the first compound to elute in the chromatographic analysis, keeping constant the rest of conditions.

As a summary, all the optimum values for the UHPLC separation are included in Table VI.2.

Table.VI.2. Optimum values for the variables involved in the UHPLC separation.

Variable	Optimum value
Column	C_{18} column (Zorbax Eclipse Plus RRHT 50 mm × 2.1 mm, 1.8 µm)
Mobile phase composition	Solvent A: Water (0.01 % Formic acid) Solvent B: MeOH (0.01 % Formic acid)
Gradient program	0 min (100:0) 0.7 min (80:20) 1.2 min (80:20) 2.5 min (50:50) 3 min (50:50) 6.5 min (5:95)
Sample solvent	Water: MeOH (80:20)
Injection volume	10.0 µl
Mobile phase flow rate	500 µl min^{-1}
Column temperature	25 ^oC

As can be observed in Figure VI.3, all the analytes could be separated in less than 6 min.

[28] E. Mallar, D. Barceló, R. Tauler, *Chromatographia* 46 (1997) 342.

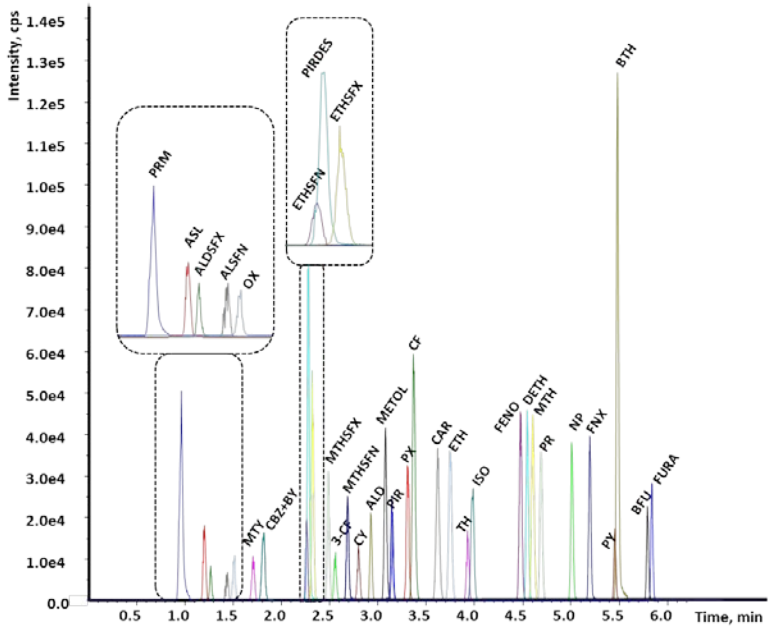

Figure VI.3. UHPLC-MS/MS extracted ion chromatogram of a standard solution at 1 μg l^{-1} for each CRB under optimal conditions.

3.2. OPTIMIZATION OF SAMPLE TREATMENT

QuEChERS methodology was selected in order to achieve a quick and effective extraction method. The optimization of QuEChERS was carried out using 1 g of chamomile (as representative matrix of herbal products) spiked at 20 μg kg^{-1} of each CRB. The recovery was used to evaluate the extraction efficiency.

First, different extraction kits were tested: buffered (4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate and 0.5 g disodium citrate sesquihydrate), and non-buffered (1 g $MgSO_4$, 1 g NaCl). As can be seen in Figure VI.4, similar recoveries were obtained with both options, observing that some compounds were slightly beneficed by buffered and some by non-buffered extraction media. So, a non-buffered kit was selected for the rest of the work.

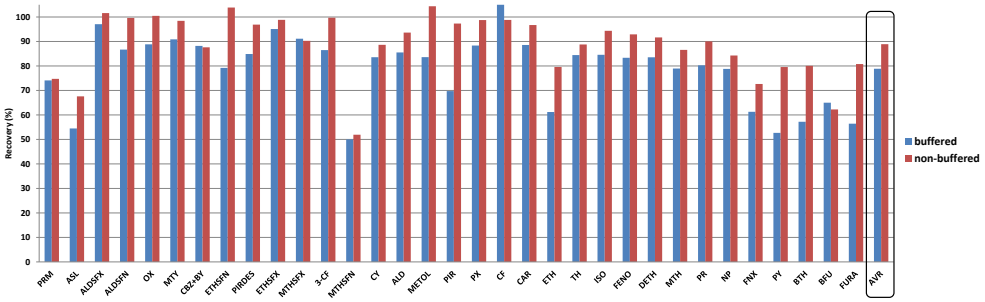

Figure VI.4. Selection of QuEChERS extraction kit: Blue: buffered; Red: non-buffered. (AVR: average recovery).

As CRBs are susceptible to be affected by matrix effect [18,29] and in order to avoid a negative impact on the separation performance of the UHPLC column by the complex herbal matrices, a dSPE clean-up step was necessary to obtain a cleaner extract. Three types of combination of sorbents were tested: (a) 150 mg PSA, (b) 150 mg PSA + 150 C_{18} + 150 $MgSO_4$ and (c) 150 mg PSA +150 mg C_{18}. The results of this study are shown in Figure VI.4 (a), while the final extracts obtained are shown in Figure VI.5 (b).

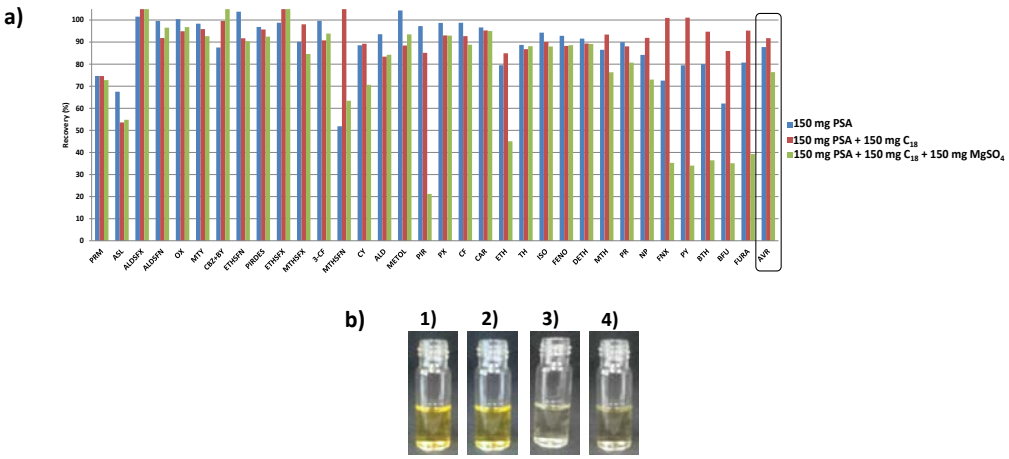

Figure VI.5. **(a)** Optimization of dSPE clean-up step: Blue: 150 mg PSA; Red: 150 mg PSA +150 mg C_{18}; Green: 150 mg PSA + 150 C_{18} + 150 $MgSO_4$. (AVR: average recovery). **(b)** Chamomile extracts: (1) without any clean-up, (2) 150 mg PSA, (3) 150 mg PSA +150 mg C_{18} + 150 mg $MgSO_4$, (4) 150 mg PSA +150 mg C_{18}.

[29] Y. Wang, H.Y. Jin, S.C. Ma, J. Lu, R.C. Lin, *J. Chromatogr. A* 1218 (2011) 334.

As can be observed, the best average recoveries were obtained when 150 mg PSA + 150 mg C_{18} were used, as PSA is useful to remove fatty acids, organic acids and some pigments, while C_{18} can remove additional lipids and sterols [30]. It is important to note that when $MgSO_4$ was included in this step, the recovery of some CRBs (PIR, FNX, PY, BFU and FURA) strongly decreased. This fact could be due to the thermal decomposition of these compounds during the generated exothermic reaction. Thus, as the only role of $MgSO_4$ in this step is to remove water from organic phase, it was decided to avoid its use.

3.3. METHOD VALIDATION

In order to check the suitability of the method for the determination of CRBs in herbal product samples, it was characterized in terms of linear dynamic ranges, LODs and LOQs, matrix effects, precision, trueness and selectivity.

3.3.1. Calibration curves and analytical performance characteristics of the method

Matrix-matched calibration curves were established using chamomile as representative matrix, spiked with different analyte concentrations (4, 10, 20, 50 and 100 μg kg^{-1} for each CRB). Each concentration level was processed following the proposed QuEChERS method and analyzed in triplicate in MRM mode, selecting the two highest precursor ion/product ion transitions, which, together with retention times, were used to ensure adequate analyte identification according to SANCO guideline [31].

LODs and LOQs were provided by the software Analyst, as 3×S/N and 10×S/N, respectively. Statistics and performance characteristics of the method are shown in Table VI.3. As can be seen, good LOQs were obtained for all the analytes, being lower than the current MRLs. Therefore, the proposed method is adequate for the determination of very low levels of these compounds in the selected matrix. Compared to other reported methods for the determination of pesticides in similar herbal products [8,11,14], the proposed UHPLC

[30] QuEChERS Informational Booklet, http://www.unitedchem.com/.

[31] Method validation and quality control procedures for pesticide residues analysis in food and feed. European Commission 2011, SANCO/12495/2011.

method provides similar LOQs, also allowing the determination of a higher number of CRBs.

Table VI.3. Statistics and performance characteristics of the QuEChERS-UHPLC-MS/MS method for the analysis of CRBs in chamomile.

	Linear dynamic range(μg kg^{-1})	R^2 (%)	LOD (μg kg^{-1})	LOQ (μg kg^{-1})	MRL (μg Kg^{-1}) [7]
PRM	2.5-200.0	99.7	0.7	2.5	200
ASL	3.5-200.0	99.8	1.1	3.5	50
ALDSFX	3.8-200.0	99.6	1.1	3.8	50
ALDSFN	3.4-200.0	99.5	1.0	3.4	50
OX	4.0-200.0	99.8	1.2	4.0	20
MTY	2.9-200.0	99.4	0.9	2.9	100
CBZ+BY	2.4-200.0	99.3	0.7	2.4	100
ETHSFN	2.9-200.0	99.6	0.9	2.9	Not established
PIRDES	2.2-200.0	99.7	0.6	2.2	5
ETHSFX	2.3-200.0	99.4	0.7	2.3	Not established
MTHSFX	3.9-200.0	99.6	1.2	3.9	100
3-CF	3.8-200.0	99.8	1.1	3.8	50
MTHSFN	2.8-200.0	99.4	0.8	2.8	100
CY	3.9-200.0	99.7	1.2	3.9	50
ALD	3.8-200.0	99.4	1.1	3.8	50
METOL	2.9-200.0	99.9	0.9	2.9	Not established
PIR	3.7-200.0	99.7	1.1	3.7	5
PX	2.7-200.0	99.9	0.8	2.7	100
CF	2.5-200.0	99.5	0.8	2.5	50
CAR	2.8-200.0	99.6	0.8	2.8	100
ETH	3.7-200.0	99.3	1.1	3.7	Not established
TH	3.3-200.0	99.7	1.0	3.3	100
ISO	2.6-200.0	99.4	0.8	2.6	Not established
FENO	2.5-200.0	99.8	0.7	2.5	Not established
DETH	2.9-200.0	99.7	0.9	2.9	50
MTH	2.9-200.0	99.8	0.9	2.9	100
PR	3.0-200.0	99.6	0.9	3.0	Not established
NP	2.3-200.0	99.7	0.7	2.3	50
FNX	3.0-200.0	99.8	0.9	3.0	50
PY	2.3-200.0	99.9	0.7	2.3	50
BTH	2.0-200.0	99.7	0.6	2.0	Not established
BFU	3.6-200.0	99.6	1.1	3.6	100
FURA	2.6-200.0	99.7	0.8	2.6	100

3.3.2. Quantitative matrix effect evaluation

ME was evaluated for each compound by comparison of the slopes of matrix-matched calibration curves with the slopes of standard solution calibration curves at the same final concentration (considering the proposed QuEChERS method and also the extraction procedure excluding the dSPE clean-up step) using the following equation:

$$\text{ME}\ (\%) = \left(\left(\frac{\text{Slope of matrix} - \text{matched}}{\text{Slope of standard solution}} \right) - 1 \right) \text{X}\ 100$$

As can be observed in Figure VI.6, dSPE step allowed the reduction of ME in about 30% for most of the CRBs, being mandatory for their sensitive determination.

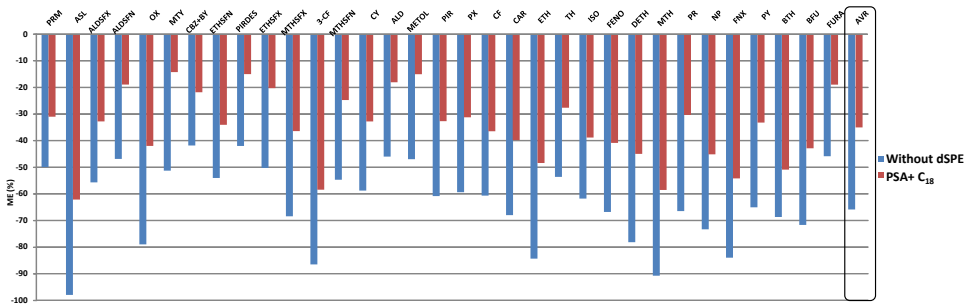

Figure VI.6. Matrix effects (%) for CRBs in chamomile excluding the dSPE step (blue) and using the whole QuEChERS procedure (red).

3.3.3. Precision study

The precision of the method was evaluated in terms of repeatability (intraday precision) and intermediate precision (interday precision) by application of the proposed QuEChERS–UHPLC-MS/MS method to chamomile samples spiked at three different concentration levels of CRBs. Repeatability was evaluated over three samples prepared and injected in triplicate on the same day, under the same conditions. Intermediate precision was evaluated with a similar procedure, but the samples were analyzed in five consecutive days. The results, expressed as % RSD of the peak areas, are summarized in Table VI.4. Good precision, lower than 7%, was obtained in all cases. These results can be considered in agreement with the current demand [25,32].

[32] European Union Commission Decision 2002/657/EC of 12 of August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Official Journal of the European Union* L 221, 2002, pp. 8–36.

Table VI.4. Intraday (n=9) and interday precision (n=15) expressed as RSD(%) for chamomile samples.

	Intraday precision [a]			Interday precision [a]		
	Level 1	**Level 2**	**Level 3**	**Level 1**	**Level 2**	**Level 3**
PRM	2.2	2.8	2.7	4.5	4.3	3.9
ASL	2.5	4.1	1.1	5.0	4.2	4.9
ALDSFX	3.4	2.5	2.3	5.1	4.1	3.7
ALDSFN	1.5	1.7	1.3	3.1	2.2	3.0
OX	2.0	1.6	1.7	4.1	3.3	3.5
MTY	2.9	2.5	2.2	2.8	4.6	2.4
CBZ+BY	4.5	3.6	3.3	6.0	4.1	4.9
ETHSFN	2.9	2.1	2.2	4.6	3.6	4.3
PIRDES	3.2	1.2	2.1	5.0	1.7	4.2
ETHSFX	3.3	2.2	2.4	4.8	2.9	3.2
MTHSFX	3.9	3.1	3.9	5.7	4.5	5.5
3-CF	2.7	2.2	1.7	4.8	4.0	2.4
MTHSFN	4.1	4.9	3.4	5.9	6.6	3.9
CY	2.5	2.0	1.9	3.0	3.6	3.3
ALD	3.3	2.2	2.2	5.4	4.3	4.3
METOL	3.3	1.5	2.2	3.8	2.3	4.0
PIR	1.7	1.6	1.7	3.4	3.7	3.4
PX	3.1	2.6	2.0	4.9	3.6	2.6
CF	2.8	1.5	1.4	4.5	3.0	3.5
CAR	2.4	1.7	2.0	4.2	3.8	3.8
ETH	2.3	2.0	2.4	4.4	3.7	4.1
TH	2.0	3.3	3.2	3.5	5.1	3.7
ISO	1.7	1.9	1.1	3.5	3.4	2.6
FENO	3.5	3.0	1.7	5.2	5.1	3.5
DETH	1.4	1.6	1.3	2.9	3.4	3.0
MTH	2.0	1.8	1.7	3.8	3.2	3.5
PR	3.4	2.7	2.9	5.5	4.4	3.4
NP	3.7	2.2	2.0	5.5	3.8	3.7
FNX	2.2	1.7	1.5	3.8	3.2	2.9
PY	1.1	1.8	1.7	2.6	3.5	3.8
BTH	1.9	1.4	1.7	3.6	2.9	3.3
BFU	1.9	1.6	1.5	2.4	3.5	3.6
FURA	2.1	1.8	1.8	3.3	3.9	2.3

[a] level 1=5 μg kg^{-1}, level 2=20 μg kg^{-1} and level 3=50 μg kg^{-1}.

3.3.4. Trueness assessment

In order to check the trueness of the proposed methodology, recovery experiments were carried out in different types of herbal samples (chamomile, green tea, red tea, valerian, thyme and linden) spiked at three different concentration levels of CRBs. In all the cases, a sample free of analytes was analyzed to check the presence of these compounds; none of them gave a positive result above the LODs of the method.

Finally, recoveries between 74.0 and 101.1% were obtained in all cases, with satisfactory precisions, fulfilling current legislation [31,32] (see Table VI.5, Table VI.6, Table VI.7, Table VI.8, Table VI.9 and Table VI.10).

Table VI.5. Recovery study and RSD for chamomile (n=9).

	Level 1[a]		Level 2[a]		Level 3[a]	
	R (%)	RSD (%)	R (%)	RSD (%)	R (%)	RSD (%)
PRM	77.3	2.2	76.4	2.8	74.7	2.7
ASL	87.1	2.5	89.8	4.1	83.7	1.1
ALDSFX	94.7	3.4	92.2	2.5	99.6	2.3
ALDSFN	90.7	1.5	97.8	1.7	91.9	1.3
OX	93.0	2.0	96.1	1.6	95.0	1.7
MTY	91.3	2.9	94.5	2.5	96.0	2.2
CBZ+BY	92.4	4.5	95.2	3.6	91.6	3.3
ETHSFN	92.3	2.9	94.4	2.1	91.8	2.2
PIRDES	99.3	3.2	92.7	1.2	95.8	2.1
ETHSFX	93.0	3.3	95.5	2.2	97.7	2.4
MTHSFX	84.5	3.9	97.4	3.1	98.1	3.9
3-CF	93.4	2.7	95.1	2.2	90.8	1.7
MTHSFN	91.4	4.1	95.3	4.9	96.9	3.4
CY	95.9	2.5	85.8	2.0	89.3	1.9
ALD	85.0	3.3	83.3	2.2	83.5	2.2
METOL	85.9	3.3	83.1	1.5	88.5	2.2
PIR	78.7	1.7	84.9	1.6	81.2	1.7
PX	89.2	3.1	98.4	2.6	97.1	2.0
CF	91.0	2.8	93.0	1.5	92.8	1.4
CAR	90.1	2.4	92.9	1.7	95.3	2.0
ETH	84.4	2.3	83.4	2.0	84.9	2.4
TH	89.7	2.0	86.3	3.3	86.9	3.2
ISO	90.1	1.7	91.0	1.9	90.1	1.1
FENO	89.0	3.5	86.3	3.0	88.3	1.7
DETH	88.2	1.4	87.1	1.6	89.4	1.3
MTH	92.8	2.0	91.9	1.8	93.5	1.7
PR	87.4	3.4	82.0	2.7	88.1	2.9
NP	91.8	3.7	97.7	2.2	92.4	2.0
FNX	97.4	2.2	91.1	1.7	99.0	1.5
PY	95.3	1.1	98.2	1.8	101.1	1.7
BTH	96.8	1.9	96.1	1.4	94.8	1.7
BFU	89.3	1.9	83.0	1.6	86.0	1.5
FURA	97.1	2.1	96.0	1.8	95.3	1.8

[a] level 1=5 µg kg^{-1}, level 2=20 µg kg^{-1} and level 3=50 µg kg^{-1}.

Table VI.6. Recovery study and RSD for green tea (n=9).

	Level 1[a]		Level 2[a]		Level 3[a]	
	R (%)	RSD (%)	R (%)	RSD (%)	R (%)	RSD (%)
PRM	77.0	2.2	78.3	2.3	74.5	2.3
ASL	88.8	2.6	93.7	2.2	90.5	2.1
ALDSFX	95.2	2.8	96.3	2.5	97.7	1.7
ALDSFN	93.9	1.7	95.1	1.6	95.8	1.4
OX	94.6	2.5	93.0	2.3	96.0	2.0
MTY	91.4	1.9	92.4	1.4	95.5	1.4
CBZ+BY	94.9	3.5	93.1	2.9	96.2	2.6
ETHSFN	95.3	2.7	95.2	2.1	94.8	2.3
PIRDES	96.6	2.9	95.7	2.6	96.8	2.2
ETHSFX	96.2	2.9	98.9	2.1	97.5	2.4
MTHSFX	90.1	2.8	94.3	2.3	95.8	2.1
3-CF	94.3	2.3	96.0	1.8	97.6	1.4
MTHSFN	97.9	2.9	98.2	2.7	98.7	2.5
CY	96.9	2.5	96.6	2.2	97.8	2.4
ALD	90.5	3.1	91.3	2.8	94.5	2.4
METOL	88.8	2.8	87.7	2.0	90.8	1.9
PIR	87.5	2.4	91.5	2.3	92.4	2.3
PX	91.8	2.1	93.9	2.3	92.5	1.8
CF	89.3	2.3	89.1	1.7	92.5	1.6
CAR	93.4	2.6	94.8	2.4	92.3	2.1
ETH	89.2	3.2	91.2	2.7	92.7	2.4
TH	87.3	2.9	91.2	2.0	92.6	1.8
ISO	90.4	2.5	93.5	2.1	92.8	1.8
FENO	92.7	2.6	94.7	2.1	95.3	2.0
DETH	90.4	2.0	89.7	1.9	92.1	1.6
MTH	94.2	2.3	91.9	2.1	95.2	2.1
PR	90.5	2.3	92.6	2.4	91.7	2.1
NP	91.2	3.3	90.8	2.7	92.9	2.6
FNX	95.4	2.4	98.2	2.3	97.6	1.8
PY	97.0	2.2	96.9	1.7	97.5	1.4
BTH	95.7	2.1	94.7	2.3	95.8	2.2
BFU	91.7	3.5	93.5	3.0	94.9	2.3
FURA	98.9	2.0	98.4	1.9	97.7	1.8

[a] level 1=5 μg kg^{-1}, level 2=20 μg kg^{-1} and level 3=50 μg kg^{-1}.

Table VI.7. Recovery study and RSD for red tea (n=9).

	Level 1[a]		Level 2[a]		Level 3[a]	
	R (%)	RSD (%)	R (%)	RSD (%)	R (%)	RSD (%)
PRM	78.1	2.8	82.9	2.5	84.1	2.2
ASL	86.9	2.5	91.7	2.0	92.4	1.8
ALDSFX	93.1	2.8	94.3	2.5	95.2	2.0
ALDSFN	93.8	2.8	91.8	2.6	95.4	2.3
OX	95.1	3.0	95.1	2.8	93.6	2.0
MTY	87.8	2.8	88.4	2.6	89.3	2.7
CBZ+BY	91.4	2.6	93.9	2.7	93.0	2.5
ETHSFN	94.4	2.7	95.8	2.3	95.8	2.0
PIRDES	94.2	2.8	95.8	1.9	96.5	2.6
ETHSFX	87.8	2.7	91.8	2.4	93.1	2.8
MTHSFX	85.3	3.0	87.9	2.3	90.5	2.0
3-CF	93.5	2.6	93.6	2.3	94.1	1.7
MTHSFN	89.5	3.2	92.8	3.0	93.4	2.2
CY	93.4	2.7	93.8	2.3	97.3	2.2
ALD	89.1	2.8	91.2	2.1	92.1	2.1
METOL	93.6	2.7	93.8	2.2	95.8	2.3
PIR	82.8	3.0	83.9	2.7	88.0	2.2
PX	91.4	2.5	88.6	2.3	93.5	2.6
CF	94.4	2.6	95.4	3.1	93.1	2.3
CAR	90.3	2.7	94.3	1.4	93.8	1.3
ETH	89.7	3.1	93.0	2.7	91.5	2.7
TH	88.9	2.8	87.6	2.2	90.1	2.3
ISO	90.0	3.0	93.1	2.6	93.9	2.2
FENO	86.7	2.6	93.5	2.4	94.7	2.7
DETH	88.1	1.8	89.3	1.6	87.0	1.0
MTH	94.8	2.5	96.2	2.4	95.7	2.1
PR	87.7	2.7	86.2	1.7	93.0	2.1
NP	90.4	2.8	94.1	2.7	93.9	2.9
FNX	96.9	2.5	99.7	2.2	98.2	2.7
PY	92.2	2.8	95.2	2.1	93.6	2.9
BTH	94.1	3.2	94.2	2.4	96.2	2.0
BFU	93.3	2.8	94.5	2.0	94.8	2.4
FURA	95.1	2.7	94.0	2.6	96.8	2.0

[a] level 1=5 μg kg^{-1}, level 2=20 μg kg^{-1} and level 3=50 μg kg^{-1}.

Table VI.8. Recovery study and RSD for valerian (n=9).

	Level 1[a]		Level 2[a]		Level 3[a]	
	R (%)	RSD (%)	R (%)	RSD (%)	R (%)	RSD (%)
PRM	79.9	2.8	80.9	2.7	77.2	2.3
ASL	90.5	2.1	88.7	2.2	90.2	2.1
ALDSFX	96.7	2.7	94.7	2.2	95.6	2.2
ALDSFN	92.0	2.8	92.6	2.2	94.7	1.9
OX	93.2	3.3	94.9	2.8	96.5	2.4
MTY	92.4	2.6	92.0	2.4	91.6	2.3
CBZ+BY	95.6	3.4	96.7	3.1	97.1	3.0
ETHSFN	92.0	2.5	90.7	2.0	90.9	1.8
PIRDES	95.3	2.7	97.5	2.9	98.0	2.5
ETHSFX	94.1	3.0	95.1	2.8	96.7	2.5
MTHSFX	95.5	2.3	93.6	2.3	97.8	2.2
3-CF	92.2	2.6	98.0	2.2	98.3	1.8
MTHSFN	96.3	2.3	94.5	2.3	95.2	2.0
CY	91.4	2.4	94.2	2.4	95.1	1.8
ALD	85.0	2.3	88.6	2.3	87.6	2.1
METOL	90.0	2.7	88.4	2.0	88.5	1.8
PIR	85.6	3.6	87.0	2.9	88.3	2.6
PX	91.8	2.7	91.2	2.6	90.3	2.3
CF	94.2	2.4	95.0	2.0	93.9	1.8
CAR	96.1	2.6	96.3	2.7	97.6	2.3
ETH	88.3	2.9	89.5	2.5	92.2	2.1
TH	86.4	3.2	88.6	3.1	90.0	2.8
ISO	92.4	3.0	93.0	2.3	96.1	1.8
FENO	89.6	2.7	87.8	2.4	90.2	2.2
DETH	90.2	2.8	89.6	2.8	91.5	2.2
MTH	93.3	2.6	92.0	2.0	93.0	1.8
PR	84.1	2.7	85.0	2.4	87.1	2.2
NP	92.0	2.0	93.8	2.3	92.3	1.9
FNX	98.6	2.7	99.1	2.7	98.2	2.3
PY	97.9	2.1	95.0	1.8	98.3	1.6
BTH	96.3	2.7	97.6	2.0	98.1	1.8
BFU	87.4	2.3	90.8	2.4	94.9	2.1
FURA	95.8	2.5	94.5	2.2	95.3	1.8

[a] level 1=5 μg kg^{-1}, level 2=20 μg kg^{-1} and level 3=50 μg kg^{-1}.

Table VI.9. Recovery study and RSD for thyme (n=9).

	Level 1[a]		Level 2[a]		Level 3[a]	
	R (%)	RSD (%)	R (%)	RSD (%)	R (%)	RSD (%)
PRM	74.6	3.0	77.1	2.5	78.3	2.3
ASL	86.8	2.6	87.4	2.3	88.4	2.2
ALDSFX	95.1	3.5	94.3	3.2	95.5	2.6
ALDSFN	91.8	3.2	95.3	2.8	91.9	2.2
OX	94.6	2.6	95.3	2.8	94.1	2.1
MTY	90.8	2.9	91.5	2.3	93.7	1.9
CBZ+BY	93.7	3.0	95.4	2.8	92.4	2.7
ETHSFN	91.9	2.5	91.0	2.2	93.4	1.9
PIRDES	95.9	3.0	97.0	2.6	97.4	2.2
ETHSFX	94.3	2.8	93.4	2.5	95.1	2.0
MTHSFX	95.6	2.0	96.1	1.9	97.8	1.8
3-CF	91.6	2.1	93.5	2.6	95.3	2.2
MTHSFN	96.3	3.4	98.4	2.8	97.8	2.4
CY	90.1	2.2	92.2	2.6	94.2	1.8
ALD	87.9	3.3	88.1	2.9	89.8	2.6
METOL	84.0	3.1	87.5	2.8	91.2	2.3
PIR	88.3	2.8	90.9	2.6	92.6	2.4
PX	94.8	2.6	96.0	2.0	97.7	1.9
CF	93.7	2.6	96.0	2.8	97.6	2.5
CAR	89.7	2.9	94.5	2.7	91.3	2.3
ETH	87.4	3.5	88.8	2.9	91.9	2.9
TH	88.0	2.3	88.6	2.6	90.2	2.1
ISO	93.9	3.0	95.9	2.3	93.3	2.3
FENO	87.6	2.6	82.6	2.0	85.6	2.0
DETH	90.2	3.0	92.9	2.1	93.4	1.7
MTH	93.7	2.1	92.1	1.8	95.5	1.9
PR	85.1	1.8	86.5	1.7	89.8	1.7
NP	93.3	3.3	95.9	2.7	93.6	2.5
FNX	95.2	3.1	98.9	2.7	97.1	2.6
PY	97.1	3.5	97.9	2.9	96.4	2.7
BTH	95.9	2.8	94.3	2.1	93.9	2.1
BFU	87.1	2.8	85.9	2.4	84.8	2.2
FURA	98.7	2.6	97.5	2.8	95.3	2.8

[a] level 1=5 μg kg^{-1}, level 2=20 μg kg^{-1} and level 3=50 μg kg^{-1}.

Table VI.10. Recovery study and RSD for linden (n=9).

	Level 1[a]		Level 2[a]		Level 3[a]	
	R (%)	**RSD (%)**	**R (%)**	**RSD (%)**	**R (%)**	**RSD (%)**
PRM	80.0	2.2	83.3	1.8	83.5	1.8
ASL	89.4	2.6	91.7	2.2	93.5	1.8
ALDSFX	92.2	2.3	93.3	2.0	95.7	1.7
ALDSFN	93.9	2.0	94.1	1.6	95.8	1.5
OX	95.6	2.4	93.0	1.5	96.0	1.3
MTY	91.4	1.9	92.4	1.7	96.5	1.4
CBZ+BY	94.9	2.9	96.1	2.6	96.2	2.3
ETHSFN	97.3	2.7	98.2	2.1	96.8	1.9
PIRDES	93.6	1.9	91.7	1.6	92.8	1.6
ETHSFX	92.2	2.4	95.0	2.1	94.1	1.8
MTHSFX	90.1	3.2	95.3	1.3	92.8	2.1
3-CF	90.3	2.3	89.0	1.8	93.6	1.8
MTHSFN	93.9	2.0	89.9	2.0	94.6	1.5
CY	95.9	3.5	97.6	3.2	98.8	2.8
ALD	92.5	2.7	90.3	3.1	93.5	2.4
METOL	85.8	2.8	87.7	3.0	84.8	2.3
PIR	87.5	2.4	91.5	2.3	92.4	2.1
PX	91.8	2.1	91.9	2.6	90.5	1.8
CF	89.3	2.3	89.1	1.5	92.5	1.1
CAR	94.4	2.6	96.8	2.2	95.3	2.2
ETH	89.2	2.2	91.2	2.0	92.7	1.9
TH	92.3	2.6	91.2	2.0	93.6	1.8
ISO	90.4	1.5	93.5	1.7	94.8	1.5
FENO	92.7	2.9	93.7	2.1	95.3	2.0
DETH	90.4	2.0	88.7	1.4	92.1	1.6
MTH	93.2	3.0	89.9	2.4	91.2	2.4
PR	90.5	2.3	94.6	2.7	91.7	1.9
NP	91.2	3.2	90.8	2.3	94.9	2.7
FNX	94.4	2.4	95.2	2.4	93.6	1.8
PY	93.0	2.2	94.9	1.7	94.5	1.4
BTH	95.7	2.1	96.7	2.6	96.8	1.9
BFU	89.7	2.3	92.5	2.0	93.9	2.0
FURA	94.9	2.0	96.4	1.7	94.7	1.5

[a] level 1=5 μg kg^{-1}, level 2=20 μg kg^{-1} and level 3=50 μg kg^{-1}.

A typical UHPLC chromatogram corresponding to green tea spiked with 5 µg kg^{-1} for each CRB, and analyzed by the proposed QuEChERS–UHPLC–MS/MS method is shown in Figure VI.7.

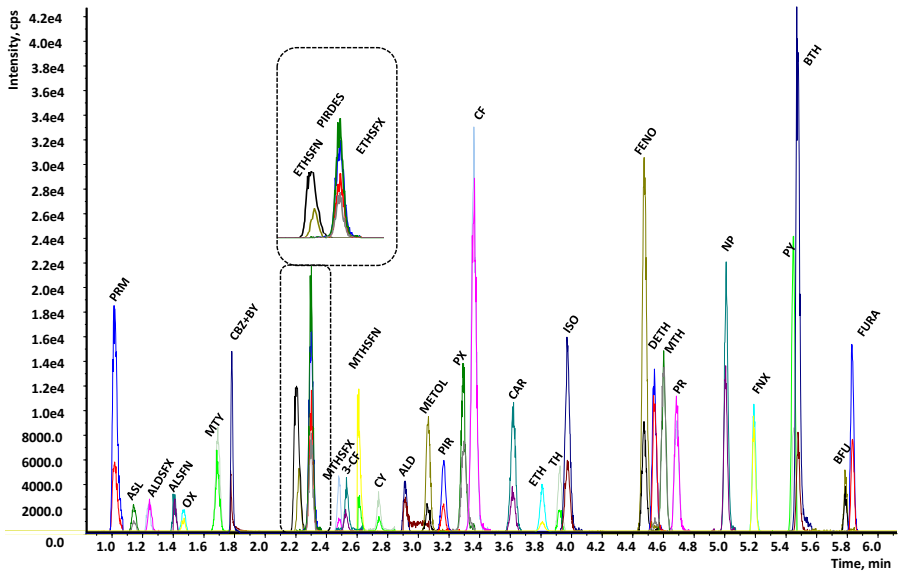

Figure VI.7. Chromatogram of a spiked green tea sample applying the proposed QuEChERS–UHPLC-MS/MS method: 5 µg kg^{-1} for each CRB.

3.3.5. Selectivity

The confirmation of the identification of CRBs was carried out according to European guidelines for determination of pesticides residues [31,32], which establishes a tolerance level for the relative intensity between Q and I MRM transitions in real samples. This tolerance value depends on the Q/I value obtained from a standard solution (see Table V.1). Table VI.11 shows the Q/I ratios obtained from a spiked chamomile sample (5 µg l^{-1} for each CRB), and from a standard solution (12.5 µg kg^{-1}); as can be seen in all cases Q/I ratios in sample were within the tolerance range for relative ion intensities indicated in the current European guidelines [31,32]. The same conclusions were obtained for the other herbals included in this study. Thus, considering these results along with the values obtained in the matrix effect evaluation, it can be concluded that no significant interferences from the studied matrices affected the analytical response.

Table VI.11. Ratio between the quantifier (Q) and qualifier (I) ions in standard solutions and analyzed chamomile samples.

	Relative ratio from standards		Relative ratio in chamomile	
	Q/I ratio (%)	Range	Tolerance range	Q/I ratio (%)
PRM	28.9	>20% to 50%	±25%	31.0
ASL	49.4	>20% to 50%	±25%	45.9
ALDSFX	95.0	>50%	±20%	91.6
ALDSFN	90.3	>50%	±20%	91.4
OX	44.2	>20% to 50%	±25%	50.8
MTY	63.9	>50%	±20%	62.1
CBZ+BY	17.3	>10% to 20%	±30%	14.6
ETHSFN	28.2	>20% to 50%	±25%	26.4
PIRDES	57.0	>50%	±20%	52.4
ETHSFX	32.6	>20% to 50%	±25%	34.6
MTHSFX	23.4	>20% to 50%	±25%	26.5
3-CF	69.6	>50%	±20%	65.5
MTHSFN	65.0	>50%	±20%	70.5
CY	76.3	>50%	±20%	73.7
ALD	49.5	>20% to 50%	±25%	55.7
METOL	17.6	>10% to 20%	±30%	23.7
PIR	45.3	>20% to 50%	±25%	48.2
PX	56.1	>50%	±20%	54.5
CF	90.4	>50%	±20%	86.8
CAR	35.2	>20% to 50%	±25%	39.9
ETH	22.6	>20% to 50%	±25%	28.6
TH	29.1	>20% to 50%	±25%	32.9
ISO	39.5	>20% to 50%	±25%	36.7
FENO	28.7	>20% to 50%	±25%	32.1
DETH	81.9	>50%	±20%	83.6
MTH	95.1	>50%	±20%	96.5
PR	90.4	>50%	±20%	93.3
NP	60.8	>50%	±20%	58.8
FNX	85.0	>50%	±20%	82.2
PY	30.7	>20% to 50%	±25%	36.0
BTH	16.9	>10% to 20%	±30%	18.3
BFU	77.7	>50%	±20%	74.1
FURA	47.6	>20% to 50%	±25%	50.3

4. CONCLUSIONS

A new method, based on a QuEChERS extraction procedure and UHPLC-MS/MS, has been developed for the simultaneous determination of thirty-three CRBs in herbal products. The sample treatment is simple, fast, and allows an effective clean-up of the extracts. Good linearity, sensitivity and selectivity, LOQs, satisfactory trueness and precision, as well as good selectivity were obtained, fulfilling the current legislation for pesticide residue analysis. Moreover, as a consequence of the UHPLC features, the separation of the pesticides was achieved in only six minutes. The results show the suitability of this procedure for the monitoring of CRBs in herbal products in a single run.

This work has been submitted for publication to Journal of Agricultural and Food Chemistry as:

“Simultaneous analysis of thirty-three carbamates in herbal products by UHPLC-MS/MS using QuEChERS methodology”

Conclusiones finales

En esta Tesis se han desarrollado diferentes métodos para la determinación de residuos de CRBs en alimentos y muestras medioambientales. Para ello se han evaluado diferentes técnicas instrumentales separativas, tanto miniaturizadas (CE y HPLC capilar) como de ultra resolución (UHPLC) acopladas a diferentes sistemas de detección (DAD y MS/MS). Con ello se ha pretendido contribuir al aumento y la mejora de los métodos de control de calidad de alimentos y aguas, en lo relativo a residuos contaminantes. Además se han estudiado y propuesto metodologías de tratamientos de muestra alternativas a las ya existentes que permiten aumentar la eficacia y el rendimiento de los análisis.

El estudio de las diferentes técnicas separativas empleadas acopladas a diferentes sistemas de detección, ha permitido demostrar su potencialidad para la determinación de residuos de CRBs en diversas matrices, obteniendo las siguientes conclusiones:

- Los métodos de CE y HPLC capilar implicaron un menor consumo de reactivos y disolventes, especialmente disolventes orgánicos en el caso de HPLC capilar, que los métodos cromatográficos convencionales. Además los consumibles y reactivos empleados en la CE son menos contaminantes y supusieron un coste inferior a los de LC.
- Los métodos propuestos proporcionaron adecuada resolución y sensibilidad con tiempos de análisis relativamente cortos. Cuando se utilizó HPLC capilar-DAD se emplearon 16 minutos para determinar 6 CRBs y cuando se utilizó la UHPLC se determinaron treinta y tres CRBs en menos de 6 minutos. En el caso de CE se emplearon menos de 18 minutos para determinar 17 CRBs en CE-MS y menos de 14 para determinar 12 CRBs en CE-DAD. Sólo en el caso de emplear la interfase *sheathless* en CE-MS el tiempo de análisis fue de 32.5 minutos debido a que la longitud del capilar empleado fue mayor.
- El empleo de surfactantes vólatiles como el APFO ha permitido la determinación de CRBs mediante CE-MS, evitando los problemas inherentes al uso de surfactantes no volátiles o los inconvenientes de otras propuestas anteriores, menos reproducibles.
- En CE-MS se utilizó la interfase de flujo coaxial (*sheath-flow)* obteniendo adecuados LODs para los compuestos en estudio aunque la dilución de la muestra supone una limitación en caunto a sensibilidad. Durante la estancia en la

Universidad de Utrecht se evaluó la interfase sin flujo adicional (*sheathless*) obteniéndose LODs para la mayoría de los CRBs 10 veces mejores, llegando en algunos casos a mejorarse hasta 100 veces.

- En cuanto a los métodos de detección, a pesar de las limitaciones de la espectrofotometría UV-Vis, gracias a las técnicas de preconcentración empleadas (tanto *on-line* como *off-line*) se llegaron a LOQs por debajo de los MRLs establecidos para la muestras analizadas. Además, la MS ha permitido diferenciar e identificar compuestos con similar tiempo de retención con alto grado de sensibilidad.

Otro de los objetivos planteados fue el estudio de tratamientos de muestra alternativos para evaluar la selectividad y sensibilidad de los mismos en el análisis de CRBs. Por ello se han evaluado tanto métodos novedosos de microextracción (DLLME o UASEME) como métodos más convencionales como la SPE o propuestas recientes para extracciones genéricas como los QuEChERS. Las conclusiones a este respecto se resumen a continuación:

- Cuando se empleó la SPE para el análisis de muestras de agua y pepino mediante HPLC- capilar se alcanzaron LOQs muy bajos ya que se pudo conseguir un factor de preconcentración muy elevado. En cambio, el uso de métodos basados en la metodología QuEChERS no permitió tan elevada preconcentración ya que el tratamiento implica la dilución de la muestra. Los métodos basados en la DLLME presentaron factores de preconcentración intermedios, siendo muy adecuados cuando la sensibilidad de la técnica instrumental es elevada. En cambio, el UASEME fue óptimo para la limpieza de los extractos de vino aunque el nivel de preconcentración no fuese muy elevado.
- Los tratamientos basados en QuEChERS resultan muy adecuados cuando se utilizan métodos de detección muy selectivos y sensibles como MS/MS, especialmente cuando se quiere estudiar un gran número de compuestos de naturaleza muy diferente, como es el caso del análisis multirresiduo.
- Las metodologías basadas en la microextracción liquid-líquido, ya sea DLLME o UASEME, son técnicas que presentaron como ventajas su bajo consumo de

reactivos, fácil manejo, rapidez y elevadas recuperaciones, estando además en sintonía con la nuevas tendencias de la quimica verde.

En la Tabla C1, y para acompañar las conclusiones anteriores, se pueden observar las características más notables de cada uno de los métodos desarrollados en esta Tesis.

Tabla C1. Resumen de los métodos desarrollados en esta tesis.

Analitos	Matriz	Tratamiento de muestra	Técnica instrumental	Tiempo de análisis (min)	LODs
CF, CAR, MTH, FNX, PR y BTH	Agua de pozo, agua de río y pepino	SPE (HLB para aguas y Alúmina para pepino)	HPLC-Capilar-DAD Columna C_{18} (Luna, 150 mm x 0.5 mm, 5 µm) Fase móvil: A:H_2O B: ACN	16	10.0-29.6 ng l^{-1} para aguas 1.8-5.6 mg kg^{-1} para pepino
OX, MTY, BY, CBZ, ASL, PX, CF, ALD, CAR, PR, MTH y NP	Zumo de plátano, tomate y piña	DLLME	*Sweeping*-MEKC-DAD BGE: Borato 100 mM (pH 9.0), SDS 50 mM, 5 % ACN (V/V)	14	1.1-7.2 µg l^{-1} para zumo de plátano
CF, CAR, MTH, PR, OX, ALD, MTY, PX, ASL, BY, NP, CBZ, ETH, ALDSFX, CSF, BTH y FNX	Agua de río, grifo y mineral	DLLME	*Stacking*-MEKC-MS/MS con interfase *sheath-flow* BGE: APFO 75 mM (pH 9.0)	17	1.4-143.7 ng l^{-1} para agua de río
CF, CAR, MTH, PR, OX, ALD, PX, ASL, BY, NP, CBZ, ETH, ALDSFX, CSF, BTH y FNX	Agua mineral	Dilución (1:1) con el BGE	*Stacking*-MEKC-MS/MS con interfase *sheathless* BGE: APFO 75 mM (pH 9.0)	16	0.5-14.6 ng l^{-1}
ASL, ALDSFX, ALDSFN, OX, MTY, ETHSFN, PIRDES, ETHSFX, MTHSFX, 3-CF, CY, ALD, METOL, PX, CF, CAR, ETH, TH, ISO, FEN, DETH, MTH, PR, NP y BTH	Vino blanco "Chardonnay" y "Ribeiro" Vino Rosado "Lambrusco" y "Tempranillo"	UASEME	UHPLC-MS/MS Columna C_{18} (Zorbax Eclipse Plus RRHT 50 mm × 2.1 mm, 1.8 µm) Fase móvil: A:H_2O (0.01 % Ácido fórmico) B: MeOH (0.01 % Ácido fórmico)	5.5	40-310 ng l^{-1} para vino blanco "Chardonnay"
PRM, ASL, ALDSFX, ALDSFN, OX, MTY, CBZ+BY, ETHSFN, PIRDES, ETHSFX, MTHSFX, 3-CF, MTHSFN, CY, ALD, METOL, PIR, PX, CF, CAR, ETH, TH, ISO, FEN, DETH, MTH, PR, NP, FNX, PY, BTH, BFU y FURA	Manzanilla, té verde, té rojo, valeriana, tomillo y tila	QuEChERS	UHPLC-MS/MS Columna C_{18} (Zorbax Eclipse Plus RRHT 50 mm × 2.1 mm, 1.8 µm) Fase móvil: A:H_2O (0.01 % Ácido fórmico) B: MeOH (0.01 % Ácido fórmico)	6.0	585-1158 ng l^{-1} para manzanilla

Final conclusions

In this Thesis, different methods for the determination of CRB residues in food and environmental water samples have been developed. Different analytical techniques have been evaluated, CE and capillary HPLC as miniaturized techniques and UHPLC as an advanced alternative, coupled to different detection systems (DAD and MS/MS). The aim has been to increase the number and to improve the quality control methods for foods and waters regarding contaminant residues. In addition, in order to increase efficiency and sample throughput, different sample treatments have been assessed as an alternative to those previously reported.

The study of these separation techniques coupled to different detection systems has allowed us to show their potential for the determination of CRBs in several matrices, achieving the following conclusions:

- CE and capillary HPLC methods involve lower solvent and reagent consumption, especially organic solvents in the case of capillary HPLC, compared with conventional chromatographic methods. In adittion, the solvent and reagent consumption employed for CE was less polluting than that for LC and materials and reagents have a lower cost.
- The proposed methods present an adequate resolution and sensitivity with relatively short times. Analysis time to determinate six CRBs by capillary HPLC-DAD was 16 min, when UHPC was used the time to determinate thirty three CRBs was less than 6 min. In the case of CE methods, less than 18 min were employed to determine seventeen CRBs by CE-MS and less than 14 min to determine twelve CRBs by CE-DAD. Only when sheathless interfase was used, analysis time was higher (32.5 min), this was due to the higher length of the capillary.
- The use of volatile surfactants as APFO has allowed the determination of CRBs using CE-MS. Thus, the problems related to the use of non-volatile surfactants were avoided, as well as the inconveniences for using other less reproducible techniques.
- For CE-MS, when a sheath-flow interface was evaluated, adequate LODs were obtained, althought this interface has the limitation of the dilution of the sample. On the other hand, the sheathless interfase evaluated during the predoctoral stay

at the University of Utrecht did not need sheath liquid, so the obtained LODs were at least 10 times lower (in some cases up to 100 times) than those obtained with a traditional CE sheath-flow interface.

- Regarding detection methods, do to the constraints of the spectophotometric UV-Vis detection, preconcentration techniques (both on-line and off-line) were carried out in order to obtain LODs below the established MRLs for the analyzed samples. However, the use of MS/MS allowed the unequivocal identification of compounds.

Another aim of this Thesis was the study of alternative sample treatments for CRBs in order to increase selectivity and sensitivity. So, novel sample treatments, as DLLME and UASEME, and generic sample treatments, as SPE or QuEChERS, have been checked. The main conclusions about this aspect are summarized as follows:

- When SPE was used to analyse water and cucumber samples by capillary HPLC-UV-Vis, a high preconcentration factor made possible reaching very low LOQs. On the other hand, the use of QuEChERS did not allow such a high preconcentration factor, as this treatment involved a dilution of the sample. The DLLME methods allowed intermediate preconcentration factors, being very suitable when the sensitivity of the instrumental technique was high. Instead, the UASEME was optimum for cleaning wine extracts although the preconcentration level was not very high.
- QuEChERS-based methodologies have shown to be ideal when a very sensitivity and selective detection technique such as MS/MS was used, especially when it was necessary to determine a high number of analytes, as in multiresidue methods.
- Liquid-liquid microextraction methodologies, including DLLME or UASEME, showed as advantages a very low solvent consumption, easy handling, high recoveries and quick sample treatment, being in agreement with the new trends in green chemistry.

As a summary, the most significant analytical characteristics of the developed methods are shown in Table C1.

Table C1. Summary of the methods developed in this Thesis.

Analyte	Matrix	Sample treatment	Instrumental technique	Analysis time (min)	LODs
CF, CAR, MTH, FNX, PR y BTH	Well water, river water and cucumber	SPE (HLB for water and alumina for cucumber)	Capillary-HPLC-DAD C_{18} column (Luna 150 mm x 0.5 mm, 5 µm) Mobil phase: A:H_2O B: ACN	16	10.0-29.6 ng l^{-1} for waters 1.8-5.6 mg kg^{-1} for cucumber
OX, MTY, BY, CBZ, ASL, PX, CF, ALD, CAR, PR, MTH and NP	Banana, tomato and pineapple juice	DLLME	Sweeping-MEKC-DAD Borate 100 mM (pH 9.0), SDS 50 mM, 5 % ACN (V/V)	14	1.1-7.2 µg l^{-1} for banana juice
CF, CAR, MTH, PR, OX, ALD, MTY, PX, ASL, BY, NP, CBZ, ETH, ALDSFX, CSF, BTH and FNX	River, tap and mineral water	DLLME	Stacking-MEKC-MS/MS with sheath-flow interface APFO 75 mM (pH 9.0)	17	1.4-143.7 ng l^{-1} for river water
CF, CAR, MTH, PR, OX, ALD, PX, ASL, BY, NP, CBZ, ETH, ALDSFX, CSF, BTH and FNX	Mineral water	Dilution (1:1) with the BGE	Stacking-MEKC-MS/MS with sheathless interface APFO 75 mM (pH 9.0)	32.5	0.5-14.6 ng l^{-1}
ASL, ALDSFX, ALDSFN, OX, MTY, ETHSFN, PIRDES, ETHSFX, MTHSFX, 3-CF, CY, ALD, METOL, PX, CF, CAR, ETH, TH, ISO, FEN, DETH, MTH, PR, NP and BTH	"Chardonnay" and "Ribeiro" white wine "Lambrusco" and "Tempranillo" rose wine	UASEME	UHPLC-MS/MS C_{18} column (Zorbax Eclipse Plus RRHT 50 mm × 2.1 mm, 1.8 µm) Mobil phase: A:H_2O (0.01 % Formic acid) B: MeOH (0.01 % Formic acid)	5.5	40-310 ng l^{-1} for "Chardonnay" white wine
PRM, ASL, ALDSFX, ALDSFN, OX, MTY, CBZ+BY, ETHSFN, PIRDES, ETHSFX, MTHSFX, 3-CF, MTHSFN, CY, ALD, METOL, PIR, PX, CF, CAR, ETH, TH, ISO, FEN, DETH, MTH, PR, NP, FNX, PY, BTH, BFU and FURA	Chamomile, green tea, red tea, valerian, thyme and linden	QuEChERS	UHPLC-MS/MS C_{18} column (Zorbax Eclipse Plus RRHT 50 mm × 2.1 mm, 1.8 µm) Mobil phase: A:H_2O (0.01 % Formic acid) B: MeOH (0.01 % Formic acid)	6.0	585-1158 ng l^{-1} for chamomile

Abreviaturas y acrónimos

Abbreviations and acronyms

En la mayoría de los casos se ha empleado la abreviatura o acrónimo anglosajón debido a su empleo más generalizado.

In most cases, the anglo-saxon abbreviation or acronym have been employed because their use is more widespread.

3-CF: carbofuran-3-hidroxy
AESAN: Agencia Española de Seguridad Alimentaria y Nutrición
ACN: acetonitrile
ACO: acetone
ALD: aldicarb
ALDSFN: aldicarb sulfone
ALDSFX: aldicarb sulfoxide
APCI: atmospheric pressure chemical ionization
APFO: ammonium perfluorooctanoate
APPI: atmospheric pressure photoionization
ASL: asulam
BFU: benfurocarb
BTH: benthiocarb
BGE: background electrolyte
BY: benomyl
CAR: carbaryl
CBZ: carbendazim
CF: carbofuran
CE: capillary electrophoresis
CEC: capillary electrochromatography
CEN: collision energy
CEP: collision cell entrance potential
CGE: capillary gel electrophoresis
CIEF: capillary isoelectrofocusing
CITP: capillary isotacophoresis
CMC: critical micellar concentration
CRB: carbamate

CSF: carbosulfan

CUR: curtain gas

CXP: collision cell exit potential

CY: cymoxanil

CZE: capillary zone electrophoresis

DAD: diode array detection

DETH: diethofencarb

DLLME: dispersive liquid-liquid microextraction

DP: declustering potential

dSPE: dispersive solid phase extraction

ETH: ethiofencarb

ETHSFN: ethiofencarb sulfone

ETHSFX: ethiofencarb sulfoxide

EFSA: European Food Safety Authority

EP: entrance potential

EPA: Environmental Protection Agency

EtOAc: ethyl acetate

EtOH: ethanol

EOF: electro-osmotic flow

ESI: electrospray ionization

EU: European Union

FAO: Food and Agriculture Organization of the United Nations

FEN: fenobucarb

FNX: fenoxycarb

FURA: furathiocarb

GC: gas chromatopraphy

GS1/GS2: dry gas

HETP: height equivalent to the theoretical plate

HLB: hydrophilic lipophilic balance

HPLC: high performance liquid chromatography

HSPS: high-sensitivity porous sprayer

ID: internal diameter

IPA: isopropanol

IS: ion spray voltage

ISO: isoprocarb

IT: ion trap

LC: liquid chromatography

LLE: liquid-liquid extraction

LLME: liquid-liquid microextraction

LOD: limit of detection

LOQ: limit of quantification

MA: methylamine

MAE: microwave assisted extraction

ME: matrix effect

MEKC: micellar electrokinetic chromatography

MeOH: methanol

MTH: methiocarb

MTHSFN: methiocarb sulfone

MTHSFX: methiocarb sulfoxide

METOL: metolcarb

MTY: methomyl

MRL: maximum residue limit

MRM: multiple reaction monitoring

MS: mass spectrometry

MS/MS: tandem mass spectrometry

NP: napropamid

OD: external diameter

OPA: o-phtalaldehide

OX: oxamyl

PIR: pirimicarb

PIRDES: pirimicarb desmethyl

PR: promecarb

PRM: propamocarb

PSA: primary secondary amine

PX: propoxur

PY: pyraclostrobin

Q: single quadrupole

QuEChERS: quick, easy, cheap, effective, rugged and safe

QqQ: triple quadrupole

R (%): percentage of recovery

rpm: revolution per minute

RSD: relative standard deviation

Rt: retention time

S/N: signal to noise ratio

SDS: sodium dodecyl sulphate

SPE: solid phase extraction

SPME: solid phase microextraction

TEM: dry gas temperature

TH: thiodicarb

THF: tetrahydrofuran

TOF: time of flight

Tris: tris(hydroxymetil)aminomethan

UASEME: ultrasound-assisted surfactant-enhanced emulsification microextraction

USAEME: ultrasound-assisted emulsification microextraction

UHPLC: ultra-high performance liquid chromatography

UV-Vis: ultraviolet-visible

WHO: World Health Organization