Universidad de Córdoba

Departamento de Química Analítica

Tesis Doctoral

LA ELECTROFORESIS CAPILAR COMO HERRAMIENTA EN EL DESARROLLO DE PROCESOS ANALÍTICOS PARA LA EXTRACCIÓN DE INFORMACIÓN (BIO)QUÍMICA EN EL ÁMBITO AGROALIMENTARIO

Azahara Carpio Osuna

Córdoba, 2015

TITULO: *LA ELECTROFORESIS CAPILAR COMO HERRAMIENTA EN EL DESARROLLO DE PROCESOS ANALÍTICOS PARA LA EXTRACCIÓN DE INFORMACIÓN (BIO)QUÍMICA EN EL ÁMBITO AGROALIMENTARIO*

AUTOR: *Azahara Carpio Osuna*

Campus de Rabanales
Ctra. Nacional IV, Km. 396 A
14071 Córdoba

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LOS DIRECTORES,

Fdo. **Lourdes Arce Jiménez.** Profesora Titular del Departamento de Química Analítica de la Universidad de Córdoba

Fdo. **Miguel Valcárcel Cases**. Catedrático del Departamento de Química Analítica de la Universidad de Córdoba

Trabajo presentado para aspirar al

Grado de Doctor en Ciencias

EL DOCTORANDO,

Fdo. **Azahara Carpio Osuna**.

Licenciada en Química

Miguel Valcárcel Cases, Catedrático del Departamento de Química Analítica de la Universidad de Córdoba y **Lourdes Arce Jiménez**, Profesora Titular del Departamento de Química Analítica de la Universidad de Córdoba, en calidad de directores de la Tesis Doctoral presentada por la Licenciada en Química, Azahara Carpio Osuna,

CERTIFICAN: Que la Tesis Doctoral "LA ELECTROFORESIS CAPILAR COMO HERRAMIENTA EN EL DESARROLLO DE PROCESOS ANALÍTICOS PARA LA EXTRACCIÓN DE INFORMACIÓN (BIO)QUÍMICA EN EL ÁMBITO AGROALIMENTARIO" ha sido desarrollada en los laboratorios del Departamento de Química Analítica de la Universidad de Córdoba, y que, a nuestro juicio, reúne todos los requisitos exigidos a este tipo de trabajo.

Y para que conste y surta los efectos pertinentes, expiden el presente certificado en la ciudad de Córdoba, 27 de Noviembre de 2015.

Lourdes Arce Jiménez **Miguel Valcárcel Cases**

TÍTULO DE LA TESIS: La Electroforesis Capilar como herramienta en el desarrollo de procesos analíticos para la extracción de información (bio)química en el ámbito agroalimentario

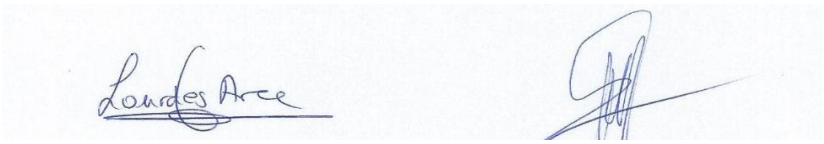
DOCTORANDO/A: Azahara Carpio Osuna

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

La Lcda. Carpio comenzó su trabajo en 2009 tras terminar su carrera de Química presentando con éxito su trabajo fin de Máster en 2010. En esta etapa se propuso el uso del ácido hipúrico como marcador para diferenciar entre muestras de leche de cabra alimentadas con pienso o con pastos usando la Electroforesis Capilar acoplada a un detector Ultravioleta-Visible (CE-UV/Vis) como técnica útil para autentificar el origen de un alimento. En 2012 comenzó a disfrutar de una beca FPI para poder seguir trabajando en su Tesis Doctoral, cuyo objetivo genérico ha sido el desarrollo y validación de metodologías analíticas para evaluar el potencial de la CE-UV/Vis en el ámbito agroalimentario. Para complementar el primer bloque de la Tesis se demostró como la CE puede determinar la concentración de una micotoxina producida por unos hongos cuyo potencial como bioinsecticida se está estudiando dentro del marco de un proyecto europeo en el que la doctoranda también ha colaborado. En este proyecto ha tenido la oportunidad de utilizar otras técnicas de separación complementarias a la CE complementando su formación analítica. En el segundo bloque se han usado las nanopartículas magnéticas recubiertas con grupos carboxílicos, una organosílice mesoporosa periódica con grupos fenilo y una estructura zeolítica de imidazolio 8 para la extracción y preconcentración de trazas de analitos (metales, pesticidas y bisfenoles) presentes en muestras de zumos o bebidas enlatadas. Todos estos materiales presentan un gran potencial como material sorbente en SPE para aumentar la sensibilidad de la CE-UV/Vis. Además se ha comprobado que la modalidad dispersiva de SPE es el mejor modo para obtener buenos valores de extracción y preconcentración de los analitos seleccionados. La producción científica de la doctoranda está abalada por los 5 trabajos de investigación ya publicados, un capítulo de una enciclopedia y los 2 trabajos enviados para su publicación en revistas de impacto internacional. Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 23 de noviembre de 2015

Firma del/de los director/es



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Agradezco al Ministerio de Economía y Competitividad la concesión de una beca de Formación de Personal Investigador (FPI) que ha hecho posible mi dedicación a este trabajo y el desarrollo de la presente Tesis Doctoral.

ÍNDICE

ACRÓNIMOS

Abreviaturas y acrónimos

AcN	*Acetonitrile*	**CM**	*Semi-synthetic Complete Medium*
ABA	*Abscisic Acid*	**CTAB**	*Cetyl Trimethyl Ammonium Bromide*
BADGE	*Bisphenol A diglycidyl ether*	**DAD**	*Diode Array Detection*
BADGE·2H$_2$O	*Bisphenol A (2,3-dihydroxypropyl) ether*	**dSPE**	*Dispersive Solid Phase Extraction*
BADGE·H$_2$O	*Bisphenol A (2,3-dihydroxypropyl) diglycidyl ether*	**Dtx**	*Destruxin*
BET	*Brunauer-Emmett-Teller method*	**EOF**	*Electroosmotic Flow*
BGE	*Background Electrolyte*	**FA**	*Formic Acid*
BJH	*Barret-Joyner-Halenda method*	**FBCA**	*Fungal Biocontrol Agent*
BPA	*Bisphenol A*	**GC**	*Gas Chromatography*
CE	*Capillary Electrophoresis*	**HA**	*Hippuric Acid*

HF	*Hollow Fiber*	**MM**	*Minimal Medium*
HLB	*Hydrophilic Lipophilic Balanced*	**MNP**	*Magnetic Nanoparticle*
HPLC	*High Performance Liquid Chromatography*	**MOF**	*Metal Organic Framework*
LLE	*Liquid Liquid Extraction*	**MRLs**	*Maximum Residue Limits*
LOD	*Limit of Detection*	**MS**	*Mass Spectrometry*
LOQ	*Limit of Quantification*	**MSPE**	*Magnetic Solid Phase Extraction*
MCM-41	*Mobil Cristallyne Material number 41*	**MWCO**	*Molecular Weight Cut Off*
MD	*Microdialysis*	**PCA**	*Principal Component Analysis*
MEKC	*Micellar Electrokinetic Chromatography*	**Ph-PMO**	*Phenylene-bridged Periodic Mesoporous Organosilica*
MeOH	*Methanol*	**PLE**	*Pressurized Liquid Extraction*
MIPs	*Molecular Imprinted Polymers*	**PMO**	*Periodic Mesoporous Organosilica*

RSD	*Relative Standard Deviation*
SBSE	*Stir Bar Sorptive Extraction*
SD	*Standard Deviation*
SDS	*Sodium Dodecyl Sulphate*
SEM	*Scanning Electron Microscopy*
SFE	*Supercritical Fluid Extraction*
SLM	*Supported Liquid Membranes*
SPE	*Solid Phase Extraction*
SPME	*Solid Phase Microextraction*
UHPLC	*Ultra High Performance Liquid Chromatography*
UV	*Ultraviolet*
Vis	*Visible*
XRD	*X-Ray Diffraction*
ZIF-8	*Zeolitic Imidazolate Framework 8*

OBJETO

El objetivo general de esta Tesis Doctoral será el desarrollo o aplicación y validación de metodologías analíticas para evaluar el potencial de la técnica Electroforesis Capilar acoplada a un detector Ultravioleta-Visible en el campo agroalimentario.

En concreto en esta Memoria se aportarán nuevos métodos analíticos que identifiquen marcadores químicos, que permitan comprobar si un alimento es o no ecológico, y determinar compuestos que puedan ser tóxicos tanto para la salud humana como el medio ambiente.

Los objetivos específicos de la investigación se han desarrollado en dos bloques, que se detallan a continuación.

1. Estudio de un marcador para caracterizar y/o diferenciar entre productos agroalimentarios, en concreto:

- Búsqueda de un marcador para diferenciar entre leche ecológica y convencional.
- Monitorización de la producción de un metabolito secundario (destruxina A), segregado por hongos entomopatógenos utilizados en agricultura como alternativa al uso de pesticidas químicos para el control de plagas.

2. Evaluación de nuevos materiales para aumentar la sensibilidad y/o selectividad de métodos electroforéticos, con el objetivo de determinar trazas de interés en muestras de alimentos. Los materiales estudiados han sido:

- Nanopartículas magnéticas para preconcentrar metales en zumos.

- Organosílices mesoporosas periódicas como sorbente para preconcentrar pesticidas en mosto.
- Estructuras organometálicas para preconcentrar bisfenol A, bisfenol diglicil éter y sus derivados hidrolizados en refrescos.

BLOQUE 1

INTRODUCCIÓN

El papel de la Electroforesis con detección Ultravioleta Visible dentro del campo agroalimentario

La Electroforesis Capilar (CE) es una técnica analítica de separación que se basa en la distinta velocidad de migración de los analitos en el interior de un capilar sometido a un alto campo eléctrico. Un equipo de CE está formado por dos disoluciones reguladoras (buffers) en los que se sumergen sendos electrodos conectados a una fuente de alto voltaje, que es lo que permite crear un campo eléctrico dentro del capilar cuyos extremos también están sumergidos en ambos viales. Este capilar tiene acoplado en una parte de los extremos el detector que monitoriza continuamente los analitos que circulan a su través. El movimiento de los analitos dentro del capilar está influenciado por el flujo electroosmótico (EOF) que se origina debido a la carga negativa de los grupos silanoles que recubren el interior del capilar. Este EOF va desde el ánodo hacia el cátodo, lo que posibilita que desde un punto de vista teórico los analitos pasen por el detector de forma secuencial: primero pasan los cationes, después los analitos neutros y finalmente los aniones. Las diferencias de movilidad entre los analitos se debe a las diferentes cargas y masas de los mismos: los más cargados se adelantarán más si son cationes o se retrasarán más si son aniones; y en cualquier caso, los de mayor masa siempre se retrasarían más. Los factores principales que influyen la separación de los analitos de interés son la composición, concentración y pH del buffer; y el voltaje aplicado. En la Figura 1 se muestra un esquema básico de un sistema de CE.

El nombre de CE engloba diferentes modalidades, como por ejemplo la electroforesis capilar de zona, isoelectroenfoque capilar, isotacoforesis capilar, cromatografía electrocinética, electroforesis capilar en gel o electrocromatografía.

Algunas de las ventajas de la CE como técnica analítica son las rápidas y eficientes separaciones que ofrece, con un mínimo consumo de muestra y reactivos, lo que conlleva que los análisis tengan un bajo coste e impacto ambiental; además esta técnica es fácilmente automatizable.

Estas ventajas encuadran a la CE dentro de la química analítica verde. El interés por este área ha crecido últimamente, y el término se utiliza para describir las estrategias analíticas que minimizan el consumo de reactivos y energía, y la generación de residuos peligrosos, que conllevan un incremento total del gasto por la introducción de la etapa de gestión de residuos. Por tanto, el desarrollo de métodos electroforéticos estaría conforme con los 12 principios de la química verde [1].

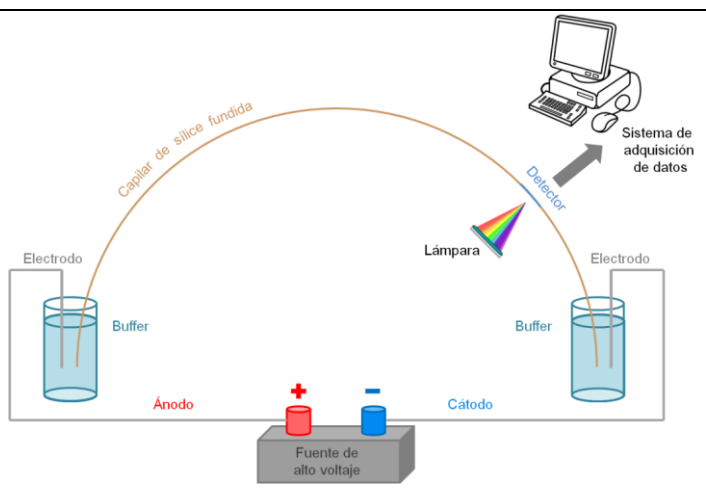

Figura 1. Esquema básico de un sistema de CE.

Hasta ahora la CE ha sido utilizada como técnica analítica en diferentes campos de aplicación, siendo en el ámbito clínico donde se han desarrollado la mayor parte de las investigaciones como puede observarse en la Figura 2. En este ámbito se engloba el análisis de ADN, drogas, enantiómeros, péptidos o proteínas [2], donde sólo pequeñas diferencias entre ellas son suficientes para su separación electroforética, ya que la CE separa en función del tamaño, carga y forma [3, 4]. Esta técnica es reconocida en farmacopeas y está bien establecida dentro de la industria biotecnológica para el control de calidad, el desarrollo de productos o formulación [5]. Esto se debe a la versatilidad de la técnica, ya que utilizando el mismo hardware se pueden usar los diferentes modos de separación antes señalados. Además es importante destacar los pocos nanolitros que hacen falta para realizar un análisis por CE. El uso de pequeños volúmenes de muestra es una gran ventaja en el ámbito clínico donde no es fácil disponer de grandes cantidades de muestra.

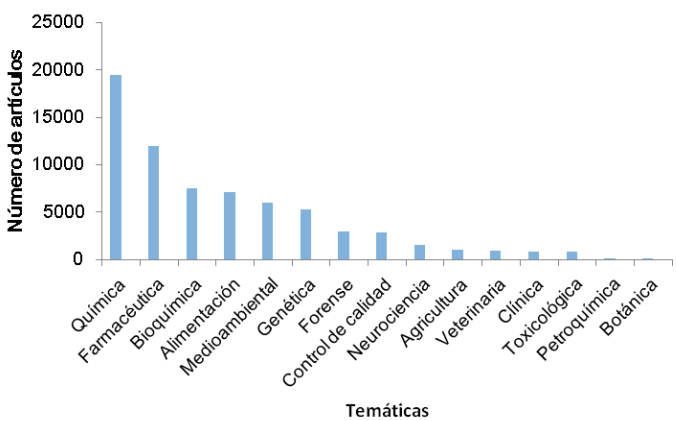

Figura 2. Número de artículos por área de investigación utilizando métodos de CE. Búsqueda realizada en "*ISI Web of Knowledge*" (hasta septiembre de 2015) combinando las palabras *capillary elect** y las diferentes temáticas que aparecen en la Figura.

Sin embargo, debido al potencial analítico de esta técnica también en los años 90 empezaron a aparecer diferentes métodos que usaban la CE en un ámbito muy distinto al clínico como es el agroalimentario. En la Figura 3 se puede observar la evolución de la técnica en el campo agroalimentario.

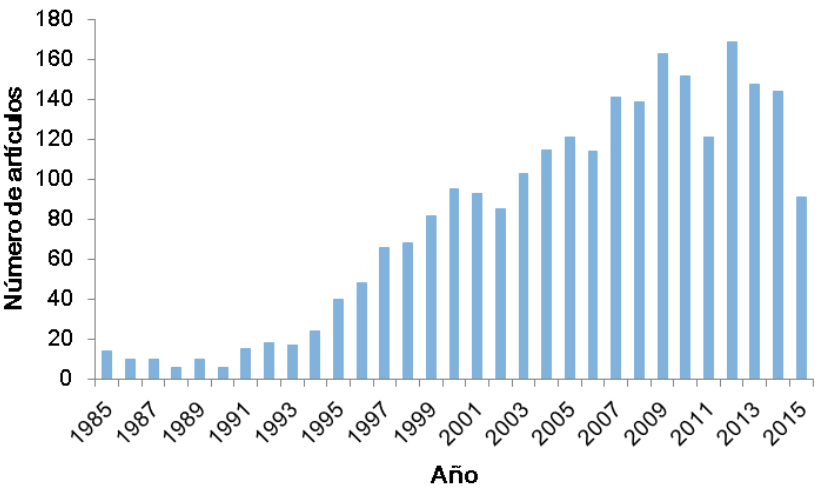

Figura 3. Número de artículos por año en el campo agroalimentario y usando la CE. Búsqueda realizada en "*ISI Web of Knowledge*" (hasta septiembre de 2015) combinando las palabras *food* y *capillary elect**.

Este desarrollo puede ser debido a la gran importancia de garantizar la seguridad, calidad y trazabilidad alimentaria; cumpliendo con las normativas vigentes. El análisis de productos agroalimentarios es una disciplina que engloba un amplio abanico de analitos diferentes, desde iones hasta proteínas, que pueden encontrarse a distintos niveles de concentración (desde trazas a macrocompuestos). Además como complicación añadida, las muestras en esta área suelen presentar matrices

complejas, lo que implica que en la mayoría de los métodos es necesario un tratamiento de muestra previo al análisis electroforético. Por todas estas razones su aceptación en la industria agroalimentaria es lenta, ya que además en esta área existen técnicas analíticas bien establecidas como la cromatografía líquida (HPLC). Aún así, existen investigaciones muy interesantes que destacan el papel que podría jugar la CE en el ámbito agroalimentario. A modo de ejemplo, en la Tabla 1 aparecen algunos artículos de revisión relacionados con el desarrollo de métodos electroforéticos en esta área durante los últimos cinco años.

Tabla 1. Artículos de revisión relacionados con métodos electroforéticos y el campo de investigación agroalimentario.

Título	Año	Ref.
CE-MS for metabolomics: developments and applications in the period 2012-2014	2015	[6]
Recent advances in the application of capillary electromigration methods for food analysis and Foodomics	2014	[7]
Characterization and study of transgenic cultivars by capillary and microchip electrophoresis	2014	[8]
Capillary electrophoresis and herbicide analysis: Present and future perspectives	2014	[9]
Metabolomics, peptidomics and proteomics applications of capillary electrophoresis-mass spectrometry in Foodomics: A review	2013	[10]
Analytical characterization of wine and its precursors by capillary electrophoresis	2012	[11]
Capillary electrophoresis of natural products: Highlights of the last five years (2006-2010)	2012	[12]
Thirty years of capillary electrophoresis in food analysis laboratories: Potential applications	2011	[2]
Chiral capillary electrophoresis in food analysis	2010	[13]
Capillary electrophoresis for the analysis of contaminants in emerging food safety issues and food traceability	2010	[14]

Como puede apreciarse en la Tabla 1, en los últimos han sido varios los objetos de atención en el uso de la CE, como el análisis de compuestos quirales, productos naturales, herbicidas e incluso su papel dentro de los laboratorios de rutina. Además se puede comprobar el gran avance en el desarrollo de aplicaciones relacionadas con las técnicas –ómicas (metabolómica, peptidómica, proteómica y foodómica), sobre todo utilizando CE acoplada a espectrometría de masas. Otra técnica cada vez más en auge es la CE miniaturizada o como ya se denomina "electroforesis en chip", destacando un método para diferenciar entre cultivos transgénicos de los que no lo son [8].

Hay que destacar que pese a que el número de aplicaciones de la CE en el ámbito agroalimentario haya aumentado, no existe un gran número de bibliografía acerca de la CE acoplada a la detección Ultravioleta Visible (UV/Vis) como puede apreciarse en la Figura 4.

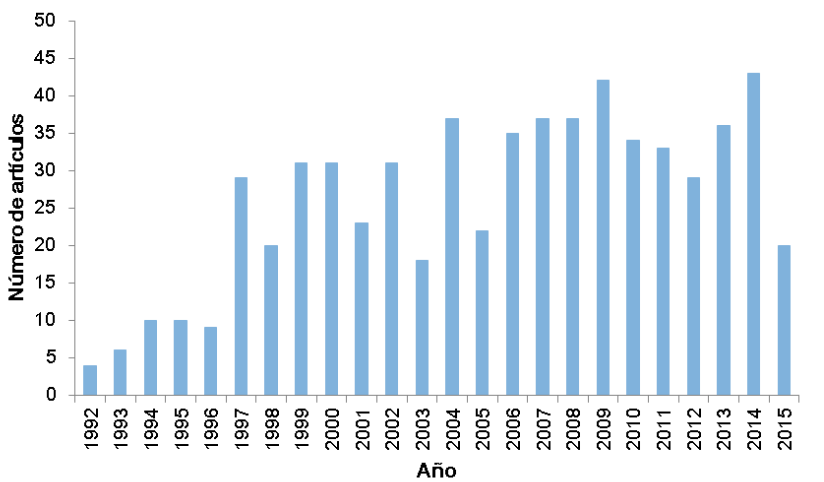

Figura 4. Número de artículos por año en el campo agroalimentario y utilizando CE-UV/Vis. Búsqueda realizada en "*ISI Web of Knowledge*" (hasta septiembre de 2015) combinando las palabras *food*, *capillary elect** y *UV/ultraviolet/Vis/visible*.

El bajo número de artículos publicados usando la CE-UV/Vis se podría atribuir a las desventajas del detector UV/Vis frente al uso de un espectrómetro de masas o un detector de fluorescencia. Uno de los principales inconvenientes del uso de la CE-UV/Vis comparado con HPLC-UV/Vis es el pequeño volumen de muestra inyectado (del orden de nanolitros) durante el análisis electroforético, que además de tener un impacto negativo en la precisión del método, implica una baja sensibilidad debido al pequeño paso óptico que se utiliza como ventana de detección.

Otra desventaja del uso de un detector UV/Vis es la baja selectividad que ofrece comparado con la que se puede obtener con otros detectores.

La baja sensibilidad y selectividad de una técnica son grandes inconvenientes cuando los analitos se encuentran a bajas concentraciones en muestras con matrices complejas. Una forma de evitar o minimizar estas desventajas es utilizar una etapa de preconcentración y/o limpieza de la muestra antes del análisis usando CE. Sin embargo es importante tener presente que en muchos casos, el problema objeto de estudio no requiere más sensibilidad o selectividad que la que puede ofrecer un detector UV/Vis, por lo tanto, en estos casos el uso de la CE-UV/Vis puede aportar ventajas o ser una alternativa a los métodos que actualmente usan HPLC-UV/Vis.

En esta Tesis Doctoral se han explotado las ventajas de utilizar la CE-UV/Vis en el ámbito agroalimentario por un lado en aplicaciones que no requieran el uso de una etapa previa de preconcentración; y por otro estudiando nuevos materiales para usarlos en la etapa de preconcentración, como herramienta para superar los problemas de sensibilidad y/o selectividad.

1. Uso de la Electroforesis con detección Ultravioleta Visible en el campo agroalimentario sin etapa de preconcentración en el tratamiento de muestra

El bajo número de publicaciones que existe utilizando CE-UV/Vis (Figura 4) comparado con otras técnicas, como HPLC, demuestra que esta técnica está todavía muy lejos de implantarse en los laboratorios agroalimentarios de rutina, aunque como se ha comentado anteriormente es una técnica común en la industria farmacéutica. Por este motivo hace falta todavía investigación dentro del campo agroalimentario para demostrar su potencial, y explotar sus ventajas en el desarrollo de nuevos métodos electroforéticos.

Indudablemente la HPLC es una de las técnicas de separación más extendidas tanto en los laboratorios de rutina como en los de investigación, pero no existen razones científicas suficientes para justificar el gran número de aplicaciones que se pueden encontrar usando HPLC en comparación con las aplicación que usan CE. Una justificación para explicar este hecho puede estar basada en el bajo conocimiento teórico sobre los principios fundamentales de la CE que todavía hoy existe entre el personal de los laboratorios. Además en la mayoría de los casos, el ámbito de aplicación de la CE-UV/Vis es casi el mismo que el de HPLC-UV/Vis, aunque entre ambas técnicas existen diferencias fundamentales como aparece en la Figura 5.

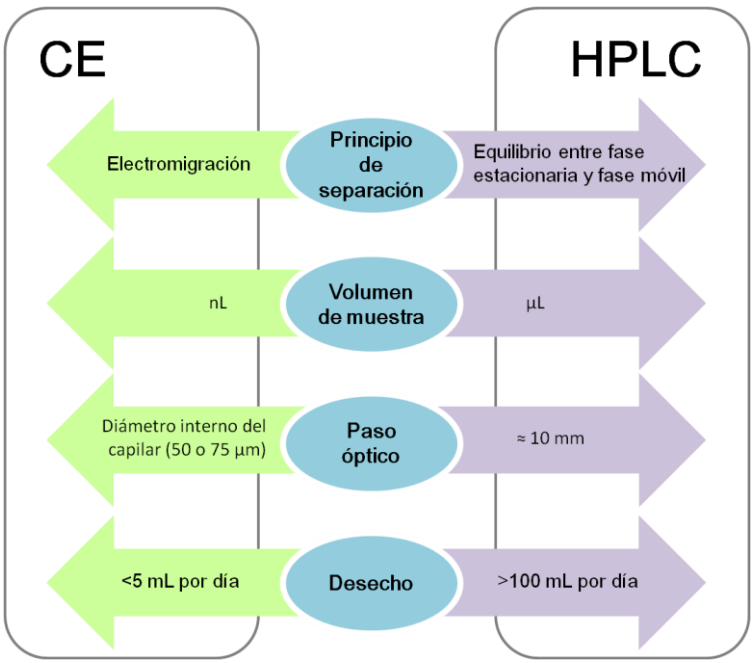

Figura 5. Comparación de algunas características de CE frente a HPLC.

En general los métodos de HPLC son más reproducibles, sin embargo exigen un gran consumo de disolventes orgánicos comparado con el bajo volumen que se necesita en la CE. Por otra parte, la CE es una técnica más versátil, genera menos residuos y el volumen de muestra que se necesita para un análisis es mucho menor. El principal inconveniente en algunos casos podría ser la menor sensibilidad que se obtienen en los métodos desarrollados debido al pequeño paso óptico. La nueva tendencia hacia el desarrollo de métodos englobados en la "química analítica verde", hace que el uso de la CE frente a HPLC pueda ser más apropiado en algunos casos. Por ejemplo recientemente Oliver y col. [15] extrajeron los monosacáridos de plantas, y determinaron su contenido tanto por HPLC-UV como por CE-UV. En este caso se demostró que la CE se comportaba de

igual forma frente a HPLC en términos de robustez y resolución, así como de recuperación. Además en comparación con HPLC, CE tuvo un coste de funcionamiento significativamente menor. Por otra parte Schaper, y col. [16] determinaron el contenido de cafeína presente en café descafeinado. En este caso la CE demostró comportarse como una buena alternativa frente al HPLC, proporcionando análisis más rápidos, además de reducir el consumo de disolvente, generando unos bajos niveles de residuos. Mu y col. [17] desarrollaron un método de CE-UV para la determinación de 4 purinas (adenina, guanina, hipoxantina y xantina) en leche de soja. Los resultados fueron comparables con los obtenidos mediante HPLC; mientras que el análisis por CE fue más corto, lo que implicó un menor coste de energía.

Con estos ejemplos se puede demostrar que la CE-UV/Vis presenta un gran potencial en los casos donde el detector UV/Vis ofrezca la suficiente sensibilidad y/o selectividad para resolver el problema analítico planteado. En estos casos no haría falta una etapa previa de preconcentración de la muestra, y sólo con extraer los analitos de interés en la muestra ya sería suficiente para obtener información analítica de calidad. La reducción de la etapa de tratamiento de muestra es una gran ventaja en un método analítico, ya que evita perder información de la muestra.

En la Tabla 2 se recogen algunos ejemplos interesantes de investigaciones donde se ha probado la validez de la CE-UV/Vis para resolver distintos problemas dentro del área agroalimentario. Esta lista no es exhaustiva y sólo se ha realizado para poner de manifiesto la variedad

de aplicaciones en diferentes muestras que existen utilizando la técnica objeto de estudio en esta Memoria, algunos de los ejemplos se comentarán a continuación.

El detector UV/Vis, aunque frecuentemente se encuentra subestimado, ha demostrado ser una herramienta económica capaz de ofrecer información que permite dar una respuesta rápida en relación a la composición de uno o varios analitos presentes en una muestra. En la mayoría de los ejemplos recogidos en la Tabla, se muestran artículos donde el objetivo fue encontrar un marcador o marcadores, y/o perfil electroforético característico de una determinada muestra, que se podría denominar “huella electroforética”.

Tabla 2. Ejemplos representativos de métodos de CE-UV/Vis en el ámbito agroalimentario sin etapa de preconcentración.

Objetivo	Analito/s marcador/es	Matriz	Año	Ref.
Uso de extractos de plantas medicinales para combatir plagas en olivos	Compuestos fenólicos en plantas	Plantas medicinales	2014	[18]
Origen de la fruta	Perfil de polifenoles	Productos basados en los arándanos	2014	[19]
Diferenciación del régimen de alimentación	Ácido hipúrico	Leche	2013	[20]
Caracterización del contenido a lo largo de un año de crecimiento de bayas	Catequina y epicatequina	Bayas	2013	[21]
Detección de cepas que producen ocratoxina A en alimentos	Ocratoxina A	Hongos que se pueden encontrar en alimentos	2013	[22]
Capacidad de producción de un metabolito	Ácido linoleico conjugado	Medios de cultivo	2012	[23]
Determinación del contenido de un analito en la carne	Hidroxiprolina como colágeno	Carne	2012	[24]
Caracterización de los polifenoles presentes y clasificación del origen del vino	Perfil de polifenoles	Vino	2012	[25]
Estudio de las alteraciones que influyen en la ternura de la carne	Proteínas sarcoplásmicas	Carne	2012	[26]
Estudio de las diferentes condiciones de almacenamiento de queso fresco de cabra	Perfil proteico	Queso fresco	2012	[27]
Seguimiento del contenido en ácidos grasos del queso durante su fabricación	Ácidos grasos tipo trans	Queso de untar	2012	[28]
Determinación del contenido de ácido lipoico en complementos dietéticos	Ácido lipoico S y R	Complementos dietéticos (cápsulas y tabletas)	2012	[29]
Detección de fraudes	Ácidos grasos omega 3	Huevos	2011	[30]
Identificación de un tipo de hierba procedente de China	Polifenoles	Hierba medicinal	2011	[31]
Diferenciación de productos transgénicos	Perfil proteico	Soja y maíz	2011	[32]

Tabla 2. Continuación.

Objetivo	Analito/s marcador/es	Matriz	Año	Ref.
Caracterización de diferentes productos de alimentación	Carbohidratos	Leche condensada, zumo de naranja, arroz, vino tinto, café y cereales	2011	[33]
Evaluación del contenido fenólico en uvas y vino, bajo diferentes tratamientos combinados (estrés hídrico y ácido abscísico exógeno)	Polifenoles	Uvas y vino	2011	[34]
Adulteración de aceite de oliva con aceite vegetal	Trigonelina	Aceite	2010	[35]
Diferenciación de variedades monovarietales de aceites de oliva	Perfil de proteínas	Aceite	2010	[36]
Determinación de melamina y derivados en diferentes productos alimenticios	Melamina, amelina, amelida y ácido cianúrico	Huevo, productos lácteos y piensos para mascotas	2010	[37]
Estudio de las denominaciones de origen protegidas de aceites españoles e italianos	Perfil de compuestos fenólicos	Aceite	2009	[38]
Clasificación geográfica del aceite de oliva virgen extra	Perfil de compuestos fenólicos	Aceite	2009	[39]
Estudio de la procedencia del vino blanco	Ácidos orgánicos	Vino blanco	2009	[40]
Caracterización de una planta medicinal	Sólo estudian los picos desconocidos del perfil	Hierba medicinal	2009	[41]
Caracterización de la cerveza	26 analitos, incluyendo alcoholes, iso-α-acidos, aminoácidos, flavonoides, isoflavonoides, una vitamina, bases de purina y pirimidina bases	Cerveza	2004	[42]
Conocer la edad de un vino	Perfil de antocianos (polifenoles)	Vino tinto	2004	[43]
Denominación de origen de queso portugués	Caseínas	Queso	2003	[44]

Tabla 2. Continuación.

Objetivo	Analito/s marcador/es	Matriz	Año	Ref.
Conocer la calidad de coñac y vino	Vanillina, siringaldehído, coniferaldehído y sinapaldehído	Coñac y vino	2002	[45]
Comparación zumos de naranja naturales y comerciales	Ácidos orgánicos (cítrico, isocítrico, tartárico y málico)	Zumo de naranja	2001	[46]
Diferencia entre zumos naturales y envasados	Ácidos orgánicos	Zumo de naranja	2000	[47]

Como puede observarse, la mayoría de los trabajos encontrados se basan en el uso de uno o varios marcadores para diferenciar muestras similares. Y en contraste con otros campos de estudio como el clínico, en el ámbito agroalimentario utilizando CE-UV/Vis, es más difícil encontrar trabajos donde se use toda la información del perfil electroforético. Esto se puede deber a que sería necesario el uso de herramientas quimiométricas, con el trabajo adicional que implica en el desarrollo del método. Estas herramientas son útiles para examinar pequeños cambios en los perfiles de las señales, que a simple vista son inapreciables. Además se podría eliminar el ruido de fondo o el desplazamiento de los picos (mediante tratamientos estadísticos se pueden alinear los picos, cuando existan cambios a lo largo del electroferograma). Este último problema es bastante pronunciado para la CE, ya que los tiempos de migración dependen del EOF dentro del capilar (que en el caso de la modalidad MEKC es más complejo, ya que entran en juego las micelas), de la muestra introducida, de las interacciones con la pared interna del capilar y los errores físicos (como pueden ser las variaciones en la irreproducibilidad en la inyección de la muestra o la temperatura).

De toda la Tabla 2, el único artículo donde estudian toda la información del perfil electroforético es el realizado por H. Franquet-Griell y col. [25]. Los autores haciendo uso del programa MatLab®, toman la información “bruta” del electroferograma para construir modelos de caracterización en una serie de vinos, y son capaces de corregir los tiempos de migración para alinear las señales. Posteriormente tienen en cuenta las señales de 15 picos (conocidos y desconocidos) para realizar un análisis de componentes principales (PCA), y así poder clasificar muestras

de vino por su origen. Además como paso previo al análisis, sólo realizan una filtración de la muestra, lo que conlleva que apenas existe pérdida de la información de la misma, y además implica un ahorro de tiempo y coste del análisis. El mismo método también lo usaron para cuantificar 20 polifenoles y así poder para caracterizar los vinos estudiados.

Las temáticas más relevantes que se han abordado utilizando la CE-UV/Vis (ver Figura 6), sin etapa de preconcentración previa, dentro del campo agroalimentario están relacionadas con la adulteración [24, 29, 30, 35, 46, 47], diferenciación [20, 32, 36, 46, 47] o el origen geográfico de un producto [19, 25, 38-40, 44]; seguidas del desarrollo de métodos para mejorar los controles de calidad que se aplican a un determinado producto [21, 26-28, 31, 33, 34, 41-43, 45], y/o determinar algún analito relevante [18, 22, 23, 37]. Las tres primeras temáticas mostradas en la Figura 6 se relacionan con la autenticidad del producto, que es algo que preocupa tanto a consumidores como a productores. Por una parte muchos consumidores están dispuestos a pagar un precio mayor por productos de mayor calidad, ya sea por su proceso de elaboración (p.ej. los productos ecológicos), o por su origen (p.ej. las denominaciones de origen). Y por otra, los productores necesitan que sus productos cumplan con la legislación vigente para poder ser vendidos. Con lo cual el desarrollo de este tipo de metodologías es necesario para satisfacer la demanda tanto de la industria como de los consumidores.

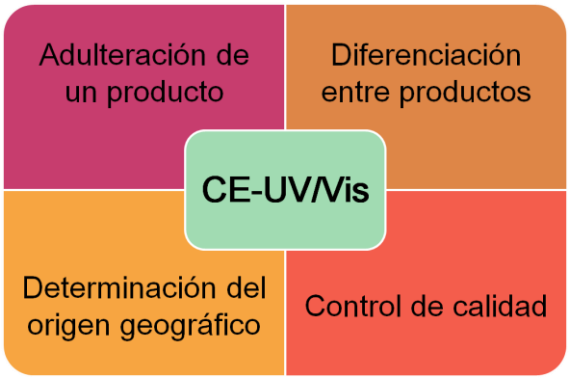

Figura 6. Temáticas más relevantes en el campo agroalimentario utilizando CE-UV/Vis.

Dentro del ámbito agroalimentario, se pueden destacar los trabajos relacionados con el aceite ya que ha sido una muestra estudiada tanto desde el punto de vista de la adulteración, como diferenciación y origen geográfico. Relacionado con el tema de adulteraciones, Sánchez-Hernández y col. [35], analizaron aceites de soja, girasol y oliva; y adulteraron el aceite de oliva con los de semilla. Mediante CE-UV, consiguieron diferenciar aceites de oliva adulterados con porcentajes desde el 10% de aceite de semilla, utilizando como marcador un alcaloide (trigonelina); el método presentó valores aceptables de precisión, detección y tiempo de análisis. Por otra parte Montealegre y col. [36] desarrollaron una metodología electroforética para la separación de proteínas procedentes del aceite de oliva, con el objetivo de diferenciar aceites monovarietales. Además el perfil de compuestos fenólicos que se puede obtener mediante CE-UV/Vis, ha demostrado ser de utilidad para clasificar geográficamente distintos tipos de aceite de oliva virgen extra [39]; incluso para certificar distintas denominaciones de origen protegidas (PDO) de

aceites tanto españoles como italianos [38]. En este último caso Carrasco-Pancorbo y col. [38], fueron capaces de clasificar 16 aceites con PDO utilizando herramientas estadísticas, mediante la identificación de 18 compuestos fenólicos, que pudieron ser resueltos en sólo 7 min. Además estudiaron la relación entre esos compuestos y las propiedades sensoriales encontradas en los aceites. En los tres ejemplos mencionados se observa que la CE-UV/Vis es capaz de dar respuesta a problemas de interés.

La CE es una técnica que sirve para analizar muestras tanto en estado sólido, como plantas o carnes, o muestras líquidas como vino o aceites. Dependiendo del tipo de muestra a analizar el tratamiento previo a la muestra es distinto, en el caso de muestras sólidas obligatoriamente se necesitará un paso de extracción, mientras que en muestras líquidas puede incluso que sólo sea necesaria una filtración. En cualquier caso el objetivo final de cualquier etapa de pretratamiento de muestra es buscar tratamientos de muestra simples con el menor número de operaciones, lo que implica una disminución del tiempo total de análisis y un ahorro de trabajo manual, además de minimizar los posibles errores, pero a la vez que reduzcan el máximo número de interferencias de la matriz, y cuando sea necesario que además se preconcentren los analitos objeto de análisis. Como cabe esperar, los tratamientos más sencillos corresponden a muestras líquidas (a excepción de la leche), como es el caso del vino, el coñac o el zumo, en los cuales los autores sólo han realizado una filtración y/o dilución. De hecho Panosyan y col. [45], no realizaron ningún tratamiento de muestra al coñac para estudiar su fracción fenólica, a través de la cual estudiaron la calidad de esta bebida. Por otra parte, en los demás

trabajos donde existen muestras sólidas o viscosas como el aceite, los tratamientos que aparecen son algo más complejos ya que en muchos casos lo que se necesita es extraer una fracción, lo que implica extraer y purificar. A modo de ejemplo se puede destacar la determinación de compuestos fenólicos en plantas utilizadas como alternativa a los fungicidas. En esta aplicación es necesario realizar distintos tratamientos de muestra como por ejemplo la extracción en fase sólida (SPE) como tratamiento para eliminar las interferencias o mayor parte de la matriz [19].

Por tanto los ejemplos recogidos en la Tabla 2 demuestran que en algunos casos utilizando un detector UV/Vis y sin necesidad de ninguna estrategia de preconcentración, el uso de las técnicas electroforéticas pueden ofrecer información analítica de calidad relacionada con analitos presentes en muestras complejas.

Como se ha comentado anteriormente, el uso de un detector económico como el UV/Vis está justificado en casos donde los analitos no se encuentren a muy bajas concentraciones, ya que la sensibilidad y/o selectividad está limitada cuando se usa este tipo de detección.

En la Tabla 2 se han recogido artículos de los analitos más estudiados mediante CE-UV/Vis. Además, para simplificar, en la Figura 7 se muestra una representación de los mismos.

Figura 7. Analitos más estudiados utilizando CE-UV/Vis.

Por ejemplo el contenido de polifenoles y ácido orgánicos es muy útil para caracterizar una muestra agroalimentaria. De hecho se han utilizado los perfiles de polifenoles en arándanos para clasificar las muestras según el origen geográfico a través del uso de PCAs; además los resultados se compararon con los obtenidos mediante HPLC [19]. Por otra parte Saavedra y col. [47] desarrollaron un método para determinar ácidos orgánicos y así poder comprobar si un zumo de naranja estaba recién exprimido o era envasado; en este caso, las muestras sólo se diluyeron y se filtraron. También mediante CE-UV se ha evaluado el contenido fenólico en uvas y vino bajo los efectos de diferentes tratamientos combinados, incluyendo estrés hídrico y ácido abscísico exógeno (ABA) [34]. Los resultados obtenidos mostraron que el suministro de ABA aumentó la

producción de catequina y malvidina, mientras que el estrés hídrico favoreció el contenido en resveratrol. Por otra parte la adición de ABA a las pieles de uva sometidas a estrés hídrico provocó la biosíntesis de antocianos. A través de estos factores se podría influir en el tipo y concentración de polifenoles en el vino. En este caso para el análisis mediante CE, el vino sólo se sometió a una filtración y la uva a una extracción.

De los analitos más estudiados mediante CE-UV/Vis en el ámbito clínico se pueden destacar las proteínas, por tanto esta técnica también es idónea para determinarlos en el área agroalimentaria. Por ejemplo, las alteraciones en las fracciones de proteínas sarcoplásmicas de carne sometida a tratamientos que influyen en su ternura (envejecimiento y el procesamiento por presión) por diferentes mecanismos, se estudiaron tanto por CE-UV como por HPLC-UV [26]. Masotti y col. [27] estudiaron el perfil proteolítico obtenido mediante CE-UV de queso fresco de leche de cabra, en diferentes condiciones de almacenamiento, obtenido tanto de supermercados (en diferentes tiempos, temperaturas, irradiación de luz) como de fábricas (en habitaciones frías bajo la oscuridad). En este caso la información obtenida mediante esta técnica junto con otras, sirvió para caracterizar el alimento. Por otro lado, se ha empleado la CE-UV para obtener el perfil de proteínas en extractos de variedades transgénicas y no transgénicas de maíz y soja [32]. Algunos de los cambios en este perfil se atribuyeron a la modificación genética; aunque los autores señalan que se necesitaría un número mayor de muestras para obtener resultados estadísticamente significativos.

Con respecto a los carbohidratos, por ejemplo, en un trabajo de Meinhart y col. [33], mediante la modalidad de CE-UV indirecta usando tensioactivos aniónicos, se consiguieron separar 13 carbohidratos en 6 matrices de alimentos diferentes (leche condensada, zumo de naranja, arroz, vino tinto, café y cereales).

También cabe destacar el uso de la CE-UV/Vis para controlar toxinas en alimentos, la modalidad micelar de CE-UV se utilizó para determinar el contenido de ocratoxina A producido por diferentes cepas. Esta técnica se utilizó para validar un nuevo protocolo de reacción en cadena de la polimerasa (PCR) con el objetivo de detectar hongos que producen esta micotoxina en alimentos [22].

Por otro lado, De Castro Barra y col. [28] optimizaron un método CE-UV con detección indirecta para el seguimiento de ácidos grasos tipo trans sin la necesidad de ninguna etapa previa de derivatización, durante el procesamiento industrial de quesos para untar. Los autores proponen este método como una alternativa al oficial de la Sociedad Americana de Químicos donde se usa cromatografía de gases para el análisis rutinario de este tipo de analitos.

La CE-UV/Vis también ha demostrado ser de gran utilidad en el análisis de compuestos quirales. Por ejemplo Kodama y col. [29] estudiaron el contenido en el antioxidante natural (R)-ácido lipoico en complementos alimenticios. Para desarrollar el método, los autores utilizaron un capilar sulfonado y el selector quiral trimetil-β-ciclodextrina. La presencia del ácido lipoico racémico puso de manifiesto la presencia de ácido lipoico de origen sintético en algunos suplementos dietéticos comerciales que aseguraban utilizar solamente ácido de origen natural.

En resumen, a pesar de que en los últimos años no exista mucha bibliografía en la que se usa la CE-UV/Vis en el campo agroalimentario, donde además no se incluya ninguna etapa de preconcentración, se han desarrollado investigaciones que ponen de manifiesto el potencial de esta técnica "verde" para poder competir con otras más establecidas como la HPLC. En esta Tesis Doctoral, se incluyen tres capítulos donde se demuestra el potencial de la CE-UV/Vis. En los dos primeros, a través de esta técnica se pudo diferenciar leche de cabras que habían sido sometidas a un régimen de alimentación diferente (pasto o pienso). Los resultados de este trabajo se validaron analizando parte del grupo de muestras por HPLC [20, 48]. En el tercer capítulo de este bloque, se evaluó la CE-UV/Vis como una alternativa a la HPLC para monitorizar un metabolito secundario segregado por un hongo que está siendo evaluado como alternativa a los insecticidas químicos.

2. Uso de la Electroforesis con detección Ultravioleta Visible en el campo agroalimentario con etapa de preconcentración en el tratamiento de muestra

A pesar de las ventajas que presenta la CE-UV/Vis, la sensibilidad que ofrece normalmente es menor frente a otras técnicas, lo cual ha limitado su aplicación en análisis de rutina. Como se ha comentado anteriormente, esta limitación deriva de los pequeños volúmenes de muestra que se inyecta en CE (del orden de nanolitros) y el pequeño paso óptico que se utiliza como ventana de detección. Sin embargo, se han desarrollado diferentes estrategias de preconcentración a realizar en la etapa de tratamiento de muestra para superar los inconvenientes de la técnica [49]. Este paso es crucial dentro del proceso analítico y debe ser cuidadosamente seleccionado en base a los analitos que se vayan a determinar y a la matriz de la muestra. De lo contrario es posible que compuestos de la matriz de la muestra puedan coeluir con los analitos de interés, e interferir en el análisis electroforético. Además esta etapa idealmente debería involucrar el menor número de operaciones como sea posible con el fin de minimizar los potenciales errores, y acortar los tiempos de análisis, que es fundamental cuando se tienen que analizar un gran número de muestras. Por otra parte existe una búsqueda creciente de enfoques más respetuosos con el medio ambiente, que usan un volumen pequeño de disolventes y de tamaño de las muestras.

Generalmente las estrategias de preconcentración se pueden dividir en *in-line*, *on-line*, *at-line* y *off-line*, las cuales han sido combinadas con la

CE para aumentar la sensibilidad de los métodos [50]. En la práctica la modalidad *off-line* es la más utilizada, ya que es la forma más sencilla de combinar una etapa de preconcentración con los equipos de CE. En la Figura 8 aparecen algunas de las más usadas antes del análisis por CE, donde puede observarse que los métodos de extracción clásicos continúan siendo los más utilizados, como es el caso de la extracción líquido-líquido (LLE) o SPE.

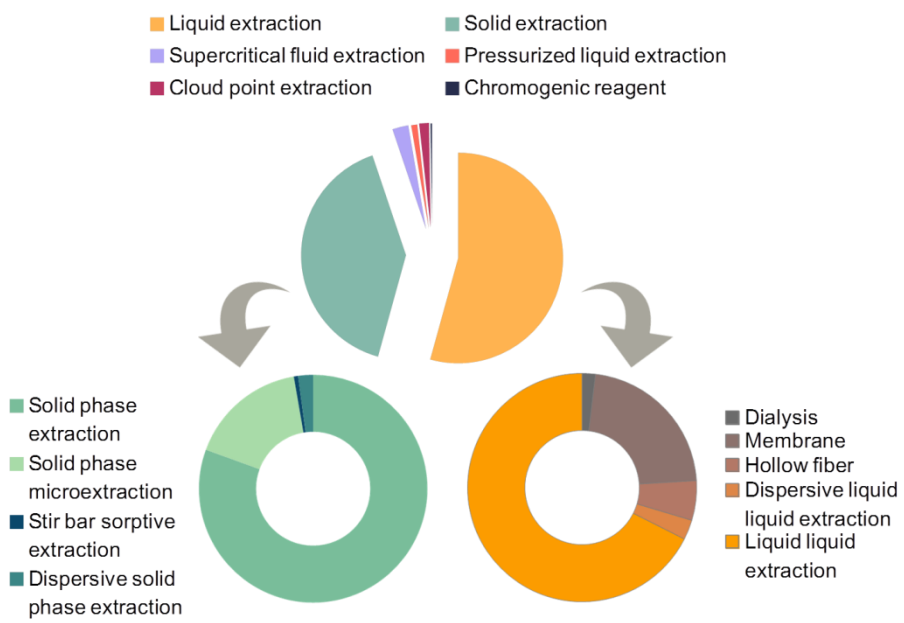

Figura 8. Relación del número de artículos relacionados con CE y diferentes estrategias de preconcentración. Búsqueda realizada en "*ISI Web of Knowledge*" (hasta septiembre de 2015) combinando las palabras *capillary electr** y las distintas técnicas que aparecen en la Figura.

Durante décadas la LLE ha sido una estrategia muy utilizada a pesar de sus desventajas debido al uso de grandes volúmenes de disolventes

orgánicos o su dificultad a la hora de ser automatizada. En contraposición, la SPE es una aproximación muy versátil gracias a los diferentes sorbentes que se pueden utilizar, como demostraron Almeda y col. [49], en una revisión exhaustiva que hicieron en 2010 sobre las estrategias de preconcentración más y menos comunes combinadas con la CE.

En esta Tesis Doctoral se ha seleccionado la SPE como sistema para mejorar la sensibilidad y selectividad de los métodos electroforéticos. Esta se desarrolló a partir de los años 70 [51]; y básicamente consiste en la puesta en contacto de una muestra líquida, que contiene los analitos de interés, junto con un material sorbente donde quedan retenidos los mismos; que después son eluidos generalmente con un disolvente orgánico.

La configuración más común para llevar a cabo la SPE es la de cartucho, el material se empaqueta en una columna entre dos fritas.

En esta modalidad clásica de SPE, las etapas más comúnmente usadas en el proceso de extracción se describen a continuación:

1. Etapa de acondicionamiento. Consiste en pasar uno o varios disolventes por la columna para eliminar interferencias y/o activar los grupos funcionales del material.
2. Etapa de carga de la muestra. Se pasa la muestra a través del sorbente, lo ideal es que se retengan los analitos de interés y se dejen pasar los componentes de la matriz que puedan interferir.
3. Etapa de lavado. El objetivo es pasar por la columna algún disolvente que pueda eliminar interferencias de la matriz, pero sin extraer los analitos.

4. Etapa de secado. Este paso ayuda a eliminar la muestra o el disolvente de lavado, circulando aire a través de la columna.
5. Etapa de elución de los analitos. Finalmente se usa un disolvente adecuado para eluir los analitos.

Por tanto, la técnica SPE permite preconcentrar, de forma relativamente sencilla, un volumen grande de muestra en una fase sólida, y eluir en un volumen pequeño para obtener una concentración detectable por CE-UV/Vis. Esta técnica puede utilizarse simultáneamente para eliminar interferencias de matriz a la vez que se aumenta la sensibilidad. Uno de las mayores desventajas del uso de la SPE en su modalidad clásica es que el paso del volumen de muestra por el cartucho suele ser un proceso lento, especialmente cuando se utilizan grandes volúmenes, ya que la difusión de la muestra está limitada dentro del cartucho. Además es bastante común que en muestras de alimentos puedan existir partículas en suspensión, que incluso pueden llegar a bloquear el cartucho.

Otras modalidades más recientes que han surgido como alternativa a la SPE son: la microextracción en fase sólida (SPME), la extracción por absorción en barras agitadoras (SBSE) o la extracción en fase sólida dispersiva (dSPE). De la misma forma que en la SPE clásica, los buenos resultados de estas estrategias finalmente se van a atribuir a la amplia variedad de sorbentes que existen en el mercado, además de los que día a día van apareciendo gracias a los trabajos recientemente desarrollados.

La SPME se basa en el uso de una fibra recubierta con una fase estacionaria que sirve para la extracción. Esta fibra se pone en contacto

con la muestra, y los analitos se transfieren hacia el recubrimiento. Después de alcanzar el equilibrio, la fibra se somete a una desorción líquida o térmica [50]; esta estrategia se explica con más detalle en el Anexo de este Bloque 1. A través de esta estrategia, por ejemplo Ravelo y col. desarrollaron dos métodos usando la SPME con fibras recubiertas de polidimetilsiloxano/divinilbenceno junto con CE-UV/Vis para determinar pesticidas en muestras de tomate [52] y vino [53].

Por otro lado, la SBSE se basa en los mismos principios que la SPME, sin embargo utilizan barras agitadoras en vez de fibras poliméricas recubiertas. Además, frente a la SPME necesita volúmenes de extractante mayores. Para la extracción se sitúa la muestra en un vial, donde posteriormente se añade la barra agitadora en condiciones químicas y físicas controladas. Después para la extracción se recoge la barra, se limpia y puede ser sometida a desorción líquida o térmica. Apenas existe bibliografía sobre el uso de esta estrategia junto con CE-UV/Vis en el ámbito agroalimentario; a modo de ejemplo se cita el trabajo de Juan-García y col. [54] donde la emplearon para preconcentrar pesticidas procedentes de frutas, sin embargo obtuvieron mejores resultados utilizando la SPE clásica.

Una de las estrategias de preconcentración alternativas a la SPE con más potencial para paliar las desventajas del detector UV/Vis es la dSPE, que fue propuesta por Anastassiades y col. [55], donde se usa el sorbente bajo condiciones de dispersión. En este caso se añade una pequeña cantidad de sorbente al extracto líquido de muestra, para retener los analitos de interés que después son eluidos. El material sorbente se separa de la muestra a través de filtración y/o centrifugación. Como en el caso de

la SPE clásica o en columna, la naturaleza del material que se utiliza como sorbente es un factor fundamental para optimizar el método.

Frente a la SPE clásica, la dSPE puede reducir el tiempo de preparación de la muestra, ya que el sorbente se dispersa de forma eficiente en toda la matriz de la muestra, en vez de pasar a través de la columna de extracción. La disminución del tiempo de análisis es algo esencial cuando se analizan un alto número de muestras, o cuando la caracterización de un producto implica el análisis por varias técnicas. Además se evita que partículas en suspensión que pueden aparecer en muestras de matrices complejas, puedan limitar el proceso de extracción (en el caso de la SPE clásica, pueden llegar a bloquear el cartucho).

En la actualidad ya se han desarrollado métodos electroforéticos donde hacen uso de la dSPE para aumentar la sensibilidad de los mismos. Por ejemplo, Chen y col. [56] sintetizaron un polímero de impresión molecular para mejorar la sensibilidad de CE-UV en la determinación de sulfometazinas en muestras de leche a través de la dSPE. El material mostró una rápida capacidad para alcanzar el equilibrio y se comportó de forma selectiva frente al analito. Obtuvieron señales 300 veces más intensas que de forma directa, con recuperaciones entre el 89 y el 110%. Herrera-Herrera y col. [57] utilizaron nanotubos de carbono multicapa como fase estacionaria para preconcentrar 7 quinolonas (residuos farmacéuticos) presentes en diferentes tipos de agua. Además se hace una preconcentración online. En este caso, se obtuvieron recuperaciones entre 62 y 116 %, con límites de detección en el nivel de los ng/L. Zeng y col. [58] también desarrollaron un método donde en la extracción en fase sólida dispersiva se utilizan nanotubos de carbono multicapa para la

preconcentración simultánea de hidroclorotiazida, clortalidona, indapamida, reserpina, nifedipina y valsartan. El método propuesto se aplicó a la determinación de estos 6 adulterantes en alimentos antihipertensivos (té, vino y medicamentos). En esto caso los resultados se compararon con los obtenidos por HPLC, con resultados similares. Cuando se usa la modalidad dispersiva se aprovecha una ventaja que aporta los nuevos materiales, como es su alta superficie específica.

Otra modalidad de la dSPE bastante interesante es la dispersión SPE magnética (MSPE), donde micro o nano partículas magnéticas se utilizan como sorbentes. Esta modalidad ha atraído en los últimos años la atención de los investigadores, ya que el sorbente con los analitos retenidos puede recogerse fácilmente de la disolución de la muestra aplicando un campo magnético externo después de la extracción, así se evitan las etapas de centrifugación o filtración. Los sorbentes más utilizados en esta técnica son los nanomateriales modificados basados en Fe_3O_4 [59, 60].

Por ejemplo, Ibarra y col. [61] preconcentraron 7 quinolonas mediante MSPE en muestras de leche. Bajo las condiciones óptimas, los LODs se encontraron en el intervalo de 9 a 12 μg/L, y los LOQs se encontraron por debajo de los límites máximos de residuos (LMRs) que marcan las regulaciones europeas. El método propuesto lo aplicaron a 20 muestras de leche, todas las muestras dieron resultados positivos. Los autores lo proponen como un método de rutina. Los mismos autores a través de la MSPE preconcentraron también de forma rápida tetraciclinas en matrices complejas como es la leche [62]. En este caso estudiaron 5 materiales diferentes basados en sílice fenilo. La metodología descrita es más rápida

que la modalidad clásica de preparación de la SPE, y además presenta unos buenos resultados en términos de sensibilidad y precisión. Los valores estuvieron por debajo de los MLRs. Por otra parte, Hu, y col. [63] preconcentraron 10 benzoimidazoles en diferentes tejidos de cerdo mediante Fe_3O_4/SiO_2/poly (MAA-*co*-EGDMA) para aumentar la sensibilidad del método, además se combinó con el uso de preconcentración online. Los límites de detección fueron del orden de ng/g, y la RSD fue menor del 13%. También se han utilizado nanopartículas magnéticas de Fe_3O_4/SiO_2-COOH como sorbente para preconcentrar metales, y luego determinar el contenido de metales tanto en zumos [64] como en orujo [65]. Después de la elución los metales se determinan por CE-UV mediante los complejos que forman con fenantrolina.

Como se ha comentado, en general los buenos resultados obtenidos con las distintas modalidades de SPE derivan de la idoneidad del sorbente utilizado para cada propósito, ya que finalmente va a ser lo que aporte sensibilidad y selectividad al método. Hasta la fecha, algunos de los sorbentes más utilizados se podrían clasificar en: fase reversa [66, 67], fase normal [68], poliméricos [69, 70], de intercambio iónico [71, 72] o incluso polímeros de impresión molecular (MIPs) [73] (en el Anexo de este Bloque 1 están descritos con detalle).

A pesar de los buenos resultados que ya se han obtenido con los materiales comerciales, el estudio de nuevos materiales abre la posibilidad de seguir mejorando las estrategias de preconcentración de analitos y limpieza de la muestra antes de su análisis por CE. A modo de ejemplo se

pueden destacar algunas aplicaciones recientemente publicadas en las que se usan nanopartículas [65], nanotubos de carbono [74], grafeno [75], etc.

En esta Tesis Doctoral se han usado las nanopartículas magnéticas, las organosílices mesoporosas periódicas (PMO, del inglés *periodic mesoporous organosilica*) y las estructuras organometálicas (MOFs, del inglés *metal organic framework*). A continuación se expondrá brevemente las características de estos materiales.

Materiales propuestos para aumentar la sensibilidad y/o selectividad de la Electroforesis con detección Ultravioleta Visible

Nanopartículas magnéticas

En general las partículas magnéticas consisten en elementos magnéticos como hierro, níquel, cobalto o sus óxidos y aleaciones con propiedades ferromagnéticas y superparamagnéticas. Las partículas magnéticas tienen un amplio intervalo de tamaños desde nano hasta micropartículas. Recientemente las nanopartículas magnéticas (MNPs) (1-100 nm) han atraído la atención de la comunidad científica debido a su naturaleza superparamagnética así como sus propiedades químicas y físicas como su gran dispersibilidad, gran área de superficie y la alta relación superficie-volumen, lo que implica a una gran capacidad de adsorción [76].

Entre todas las partículas magnéticas, los óxidos de hierro como la magnetita (Fe_3O_4) y la maghemita (γ-Fe_2O_3), han recibido una considerable

atención como nuevo sorbente de SPE, debido a su pequeño tamaño y su gran área superficial, dando lugar a mejores cinéticas y una mayor capacidad de extracción de los analitos [77].

Las partículas inorgánicas puras (Fe_3O_4 ó γ-Fe_2O_3) forman el núcleo del material magnético y son propensas a la formación de agregados dando lugar a cambios en sus propiedades magnéticas. Es más, la pérdida de selectividad los hace inadecuados para muestras con matrices complejas. Por tanto se lleva a cabo una modificación de la superficie de este núcleo magnético (en la capa/recubrimiento) con grupos activos específicos que normalmente ayudan a evitar esas limitaciones. Este recubrimiento de la superficie se consigue mediante la fijación de componentes inorgánicos (p.ej. silica, alúmina, zirconio o ferritas) o moléculas orgánicas (p.ej. un polímero o un surfactante), lo que hace aumentar su estabilidad química, evitando su oxidación, y dotando de nuevas funcionalidades como la selectividad o mejorar sus características para la sorción. Hay una gran variedad de recubrimientos y grupos funcionales que pueden anclarse a la superficie de las partículas [78].

La preparación de un material magnético implica 3 pasos incluyendo la síntesis de las partículas magnéticas (magnetita o maghemita), el recubrimiento del núcleo de la partícula y la modificación de la estructura final [79].

En esta Tesis Doctoral se va a hacer uso de MNPs funcionalizadas con grupos carboxílicos para estudiar el potencial de extracción de metales en muestras de zumos, previo al análisis por CE-UV/Vis [64]. En la Figura 9 aparece una representación de su estructura.

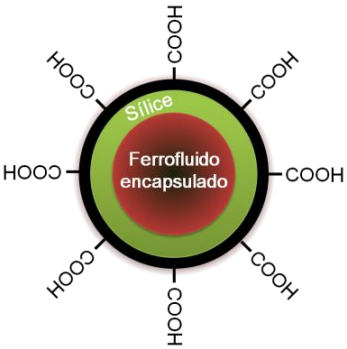

Figura 9. Representación de la estructura de las MNPs utilizadas en esta Tesis Doctoral.

Organosílices mesoporosas periódicas

Recientemente, los materiales mesoporosos están recibiendo atención en el campo de la investigación debido a algunas de sus características como son: el gran área superficial, gran volumen de poro, canales mesoporos modificables con una distribución de tamaño de poro bien definida, y controlable composición de su superficie.

De acuerdo con la nomenclatura de la IUPAC (*International Union of Pure and Applied Chemistry*), los materiales porosos se pueden clasificar en tres categorías: microporosos con tamaño de poro por debajo de 2 nm, macroporosos con tamaños por encima de 50 nm, y los mesoporosos con un tamaño entre 2 y 50 nm [80].

De forma general, las ventajas que presentan los materiales mesoporosos son:

1. Poseen una estructura mesoporosa altamente ordenada y con un tamaño controlado, que permite la adsorción selectiva por tamaño de pequeñas moléculas, pero a la vez excluye por tamaño moléculas

más grandes, permitiendo una preconcentración en base al peso molecular del compuesto.

2. Presentan una gran área superficial y un gran tamaño de poro que permite la capacidad de adsorción de analitos.
3. Poseen estabilidad térmica, química, y control de su composición, además permiten su pos-funcionalización permitiendo introducir grupos hidrófobos, hidrofílicos o polares.

En general se han explotado las ventajas de estos materiales en la extracción de iones metálicos [81], en la adsorción de compuestos orgánicos [82], como reactores enzimáticos para la digestión de proteínas [83] o para el enriquecimiento selectivo por tamaño de péptidos y proteínas [84].

Después de aparecer las primeras referencias en las que se mencionan los materiales mesoporosos, aparecieron otros trabajos en los que ya se referencian las sílices ordenadas mesoporosas. Estas estructuras se introdujeron a principios de los 90 [85] y a partir de ahí la síntesis de estos materiales ha experimentado grandes avances, provocando una diversificación de las áreas de investigación. Una de esas áreas es el desarrollo de materiales híbridos. En ese caso la funcionalidad de grupos orgánicos se combina con la estabilidad de matrices inorgánicas. Con el objetivo de introducir diferentes funcionalidades activas en la superficie mesoporosa se utilizan diferentes precursores organosilanos. Entre esos materiales híbridos se encuentran las organosílices mesoporosas periódicas (PMOs), que se desarrollaron en 1999 [86-89], y están atrayendo cada vez más el interés por su utilidad en diferentes áreas [90-96].

La síntesis de los PMO normalmente es un proceso de dos pasos, donde el primer paso implica la hidrólisis de un precursor organosilano en condiciones ácidas o básicas en presencia de un surfactante como plantilla, seguida de un tratamiento hidrotermal a altas temperaturas entre 24 y 48 horas. Después de esta etapa, la plantilla se elimina y se lleva a cabo la extracción del disolvente o la calcinación a altas temperaturas [97]. Finalmente la estructura básica de un PMO es $(R'O)_3Si\text{-}R\text{-}Si(OR')_3$, donde R´O es un grupo hidrolizable. Cambiando el puente orgánico que se utiliza como precursor para preparar el PMO, se puede promover la creación de diferentes características dependiendo del objetivo deseado. Hasta ahora se han incorporado con éxito etano, etileno, benceno, p-bifenilo o tiofeno como puente [86, 88, 89, 98, 99]. El uso potencial del PMO depende de su morfología y de la carga de sus grupos orgánicos accesibles dentro de su estructura. Esto da lugar a propiedades muy interesantes incluyendo una alta afinidad de adsorción para compuestos orgánicos, alta área superficial, y una distribución de tamaño de poro y diámetro de poro ajustable.

En esta Tesis Doctoral, se va a evaluar un PMO con grupos fenilo [91] (Figura 10) como material sorbente para aumentar la sensibilidad de la CE-UV/Vis en la determinación de pesticidas [100].

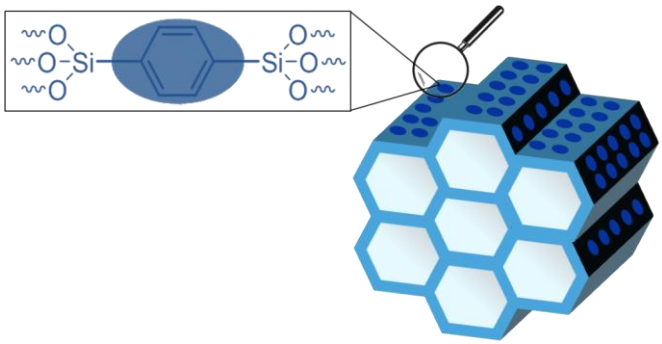

Figura 10. Representación de la estructura del Ph-PMO utilizado en esta Memoria.

Estructuras organometálicas

Las estructuras organometálicas (MOFs) son una clase de materiales cristalinos porosos, con una estructura altamente ordenada que se sintetiza a través de la combinación de ligandos orgánicos e iones metálicos [101]. Las partes inorgánicas de los MOFs se pueden seleccionar con varias dimensiones, creando cadenas lineales (1D), capas (2D) y estructuras (3D) [102].

Desde que en 1995 Yaghi y col. [103] introdujeron el término MOF, ha existido una intensa investigación en el tema. En comparación con las estructuras inorgánicas porosas convencionales como las zeolitas o los materiales basados en el carbón, la superficie activa de los MOFs es considerablemente mayor, pudiendo alcanzar casi los 7200 m^2/g [104]. Por otra parte las estructuras inorgánicas están limitadas a ciertos cationes como son Al, Si y calcógenos [105], mientras que en los MOFs existen casi todos los cationes de hasta 4 átomos tetravalentes [102]. La variedad con la que se pueden encontrar los MOFs, además depende de la elección de los

ligandos orgánicos (p.ej. carboxilatos o aminas), lo que ofrece un gran número de posibilidades a la hora de crear nuevos MOFs según los objetivos deseados. Por otra parte las estructuras de los MOFs tienen una flexibilidad sintética que les permite cambiar su tamaño sin cambiar la topología, lo que es llamado el principio isoreticular [101]. Este carácter isoreticular les permite a los MOFs albergar macromoléculas como vitaminas o proteínas, y aumentar el espacio de interacción entre los poros. La habilidad de añadir grupos funcionales en la estructura a través de la modificación después de la síntesis es otra ventaja.

La variedad de estructuras que se pueden diseñar de los MOFs les confieren unas propiedades químicas, térmicas y físicas únicas; que permiten su utilización en una gran variedad de aplicaciones [106, 107].

Dentro de la gran variedad de MOFs, se encuentran las estructuras zeolíticas de imidazolato (ZIF). Los ZIFs están formados por metales (M= Co, Cu, Zn, etc) unidos a través de los átomos de N de la estructura de imidazolato (Im) o de ligandos de imidazolato funcionalizados. En esta Tesis Doctoral se va a hacer uso del ZIF-8 (Figura 11), cuya estructura está compuesta por Zn unido al anillo Im. Al contrario de otros tipos de MOFs, el ZIF-8 presenta una estabilidad química y térmica excepcional en disolventes, agua y disoluciones acuosas alcalinas; por ello es uno de los ZIFs más estudiados [108-112].

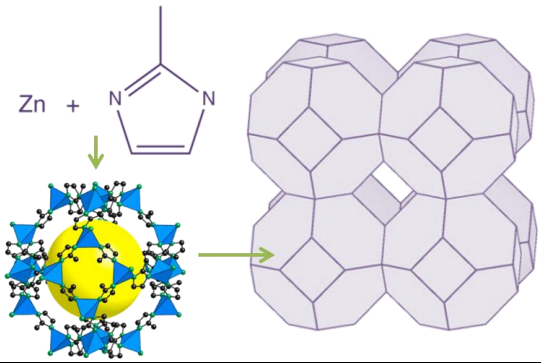

Figura 11. Representación de la estructura del ZIF-8 utilizado en esta Tesis Doctoral.

En concreto se estudiará el potencial del ZIF-8 como sorbente en dSPE para preconcentrar bisfenoles antes de la determinación de estos por CE-UV/Vis.

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ANEXO DEL BLOQUE 1

SAMPLE TREATMENT STRATEGIES FOR CAPILLARY ELECTROPHORESIS ANALYSIS

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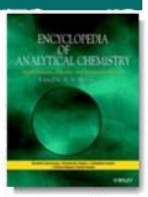

SAMPLE TREATMENT STRATEGIES FOR CAPILLARY ELECTROPHORESIS ANALYSIS

L.Arce, A. Carpio

Department of Analytical Chemistry, University of Córdoba, Annex C3 Building, Campus of Rabanales, E-14071 Córdoba, Spain

The determination of analytes included in different types of samples by Capillary Electrophoresis (CE) requires their prior extraction from the sample matrix. The extraction steps are the bottleneck of many analytical processes, which should ideally involve as few operations as possible in

order to minimize potential errors and shorten analysis times. In addition, there is a growing search for more environmental friendly approaches capable of using smaller amounts of solvents and sample sizes.

Sample treatment in CE is of great practical relevance by virtue of its inherent shortcomings, which include low sensitivity, small sample volume, and a marked influence of matrix components. The main types of coupling (on-line, in-line and at line) are systematically dealt with and compared to manually implemented approaches in order to derive practical conclusions with a view to facilitating the use of CE for the determination of analytes present in complex samples.

The more suitable approaches for sample treatment before CE analysis are presented in this chapter. Although solid phase extraction (SPE) continues to be the most widely used extraction technique, some other clean-up/concentration methodologies involving the use of membranes, microdialysis (MD), solid phase microextraction (SPME), hollow fibers (HF), supercritical fluid extraction (SFE), amongst others have proved effective alternatives for extraction, clean-up and sometimes preconcentration purposes.

Keywords: Capillary electrophoresis, sample treatment.

1. Introduction

In the first ten years of this century many books related with Capillary Electrophoresis (CE) have already been published, and in this Encyclopedia there are several chapters dealing with CE. All of these bibliography proofs the potential of CE in Analytical Chemistry nowadays. However, only in a small fraction of these books is reported the importance of sample treatment before CE analysis. In general, such essential step of chemical measurement processes (see Figure 1) is even more crucial in this separation technique owing to a number of factors, including:

1. The need to insert very small sample volumes (in the nanoliter region) which can detract from such important analytical features as precision and representativeness.
2. The typically low sensitivity of UV-vis detection systems (one of the detector more used in CE nowadays); and
3. The strong influence of the sample matrix components (e.g. salt content, macromolecules, etc.), which can disturb CE separations.

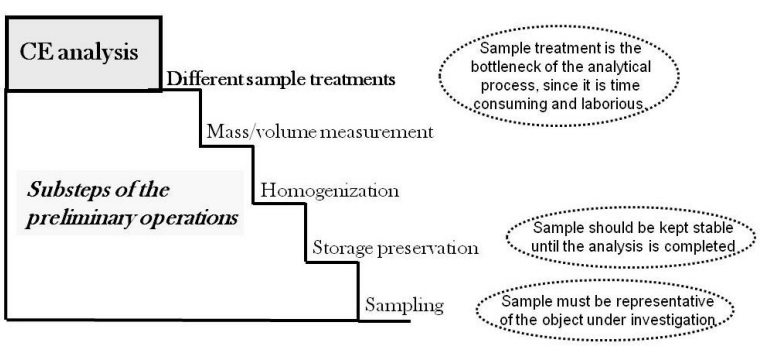

Figure 1. General features of a chemical measurement process in CE.

One way of avoiding or minimizing these inherent difficulties and their strong effects on the quality of analytical results is to consider sample preparation as a key part of electrophoretic processes. Unfortunately, no universal sample preparation processes for all types of sample exist. Sample processing and pre-treatment can take a number forms depending on the nature of analytes, sample matrix, and the final separation method. Typical processes may include sample homogenisation, filtration, centrifugation, distillation, extraction, fractionation, and concentration. The successful execution of these processes is required to ensure that the analytes are present in a compatible way with the electrophoretic analysis. Figure 2 depicts the more common interfaces between the initial gaseous, liquid or solid sample phase (which can be the original sample or the result of a previous preliminary step) and the final phase, which can be only liquid to be compatible with CE analysis.

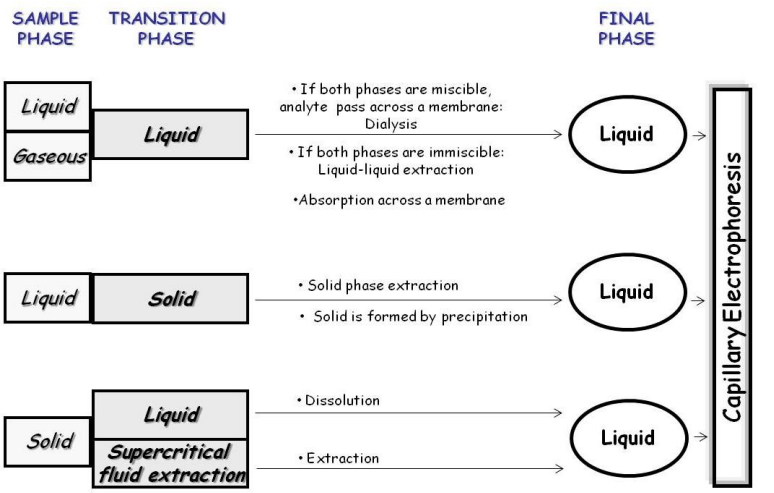

Figure 2. Main interfaces between the initial sample phase (gaseous, liquid or solid).

Mass transfer at the interface can occur through various well-known physico-chemical mechanisms, such as absorption, adsorption, selective transfer of species across a membrane, extraction, dissolution, chemical reaction and binary or ternary combination thereof (e.g. ion exchange). A second phase (transition phase) can be either added externally (e.g. active liquid and solid phase for absorption, adsorption or retention of the target substances dissolved in the initial liquid phase) or formed in situ (e.g. interfaces based on precipitation).

The characteristic of preliminary operations before CE are summarized in Figure 3.

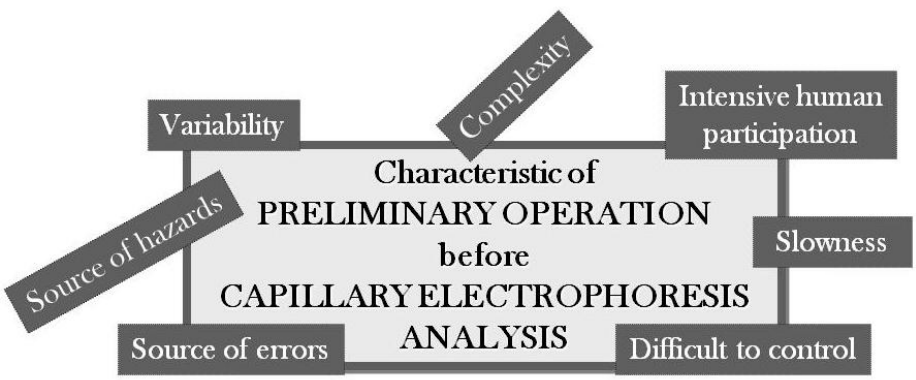

Figure 3. Characteristics of preliminary operations before CE.

Taking into account all of these features it can be confirmed that the whole analytical process may be jeopardized if an inappropriate way is chosen to prepare samples for analysis by CE. It is widely accepted that some sample preparation method is needed when complex samples are to be analysed by CE. The aims of the sample treatment can be:

(i) Suppressing potential interferences from the sample matrix (*e.g.*, by removing proteins which might be adsorbed on the capillary inner walls or solid particles which might clog the capillary).

(ii) Enhancing the analytical signal in order to improve the typically poor limits of detection when UV-vis detector is used.

The different sample preparation approaches can be performed off-line, at-line, on-line or in-line (see Figure 4).

Off-line sample pre-treatment includes manual sample preparation. At-line procedures are performed with a programmable arm or robotic station. In on-line or in-line sample preparation, the sample preparation is directly combined with the final separation step. Normally, on-line coupling are via a split-flow interface[1], which are used in combination with flow systems. Improved at-line, on-line, in-line and on-capillary sample preparation methods are in fact highly useful with a view to expanding the scope of CE methodology in as much as they afford automation, low sample consumption, quick analyses and a high throughput.

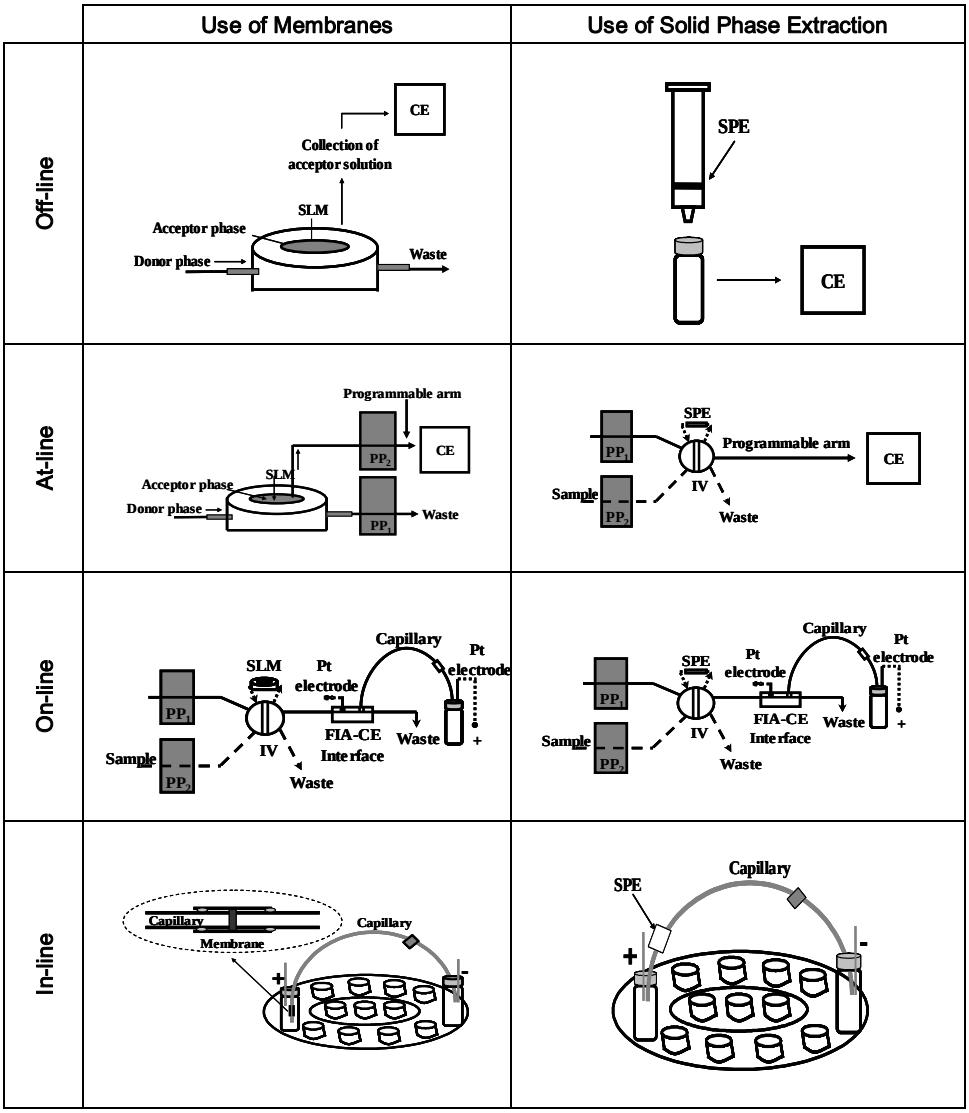

Figure 4. Different combinations of sample treatments with the CE (off-line, at-line, on-line and in-line). (Reproduced with permission from Ref. 47. Copyright 2010, Betham Science Publisher.)

A number of sample treatment systems intended to purify and/or concentrate analytes [1-4] have been used prior to CE analysis. These systems can be classified in two main categories:

- Liquid-liquid extraction (e.g. microdialysis (MD) or supported liquid membranes (SLM)).
- Solid-phase extraction (e.g. solid-phase extraction (SPE) or solid phase microextraction (SPME)).

Anyone starting to use CE for real sample analysis will be faced with a vast number of different approaches (see Figure 5) for extracting different analytes presented in real samples.

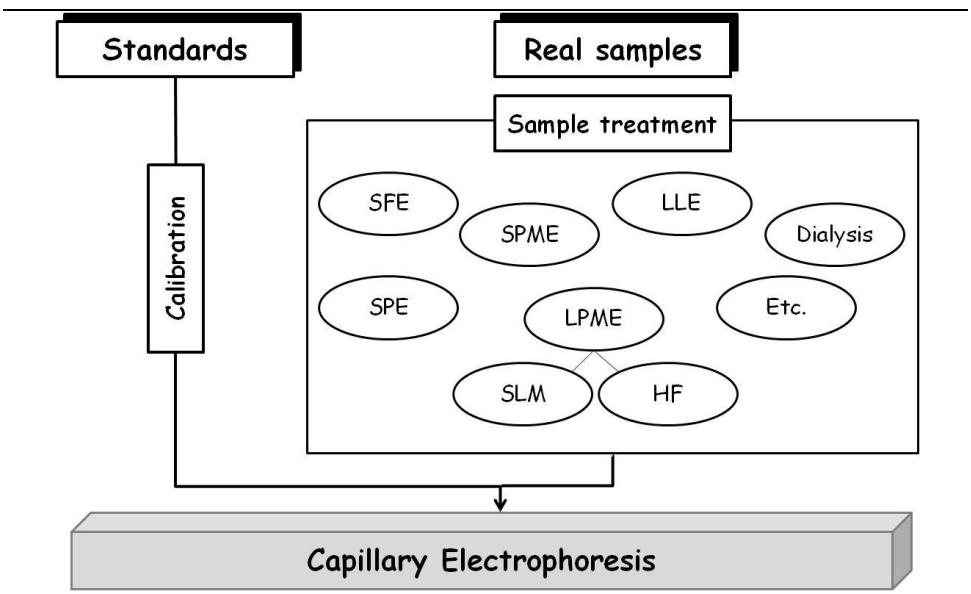

Figure 5. Different sample treatments strategies before CE analysis. (Reproduced with permission from Ref. 29. Copyright 2009. Elsevier.).

This chapter is intended to provide readers with the enough information to select the most convenience extraction technique for each different case in real situations. Finally, an overview of selected applications of these approaches is provided.

2. Sample treatment approaches

The sample preparation process involves isolation, and in many cases it is also required the preconcentration of the components of interest from several matrices making the analytes more suitable for separation and detection. Sample preparation gradually becomes a major part of analysis capable of taking up to 80% of the total time of a complete analysis process (sampling, extraction, clean-up, detection and data analysis). In this section the different extraction strategies more often used in CE are summarized.

2.1 Solid Phase (Micro)Extraction

2.1.1 Solid Phase Extraction

There is no doubt that SPE is today the most popular sample preparation method used before CE analysis (see Figure 6) and for this reason it will be the first approach to be covered in this chapter.

Although SPE was introduced in the mid-1970s [5], it was not until 1990 that it was first used before CE analysis by Meyer *et al.* [6]. Since then, it has been widely used in a variety of CE methods. SPE can be used to simultaneously enrich trace analytes and remove potentially interfering compounds in order to avoid undesirable effects on CE separation. If the samples have not significant amount of interferences, a large volume of a low-concentration sample can be loaded onto a solid phase and eluted in a small volume of eluent in order to obtain a CE-detectable concentration.

Further preconcentration can be obtained by evaporating the eluted sample to dryness and redissolving the residue in a smaller volume of solvent.

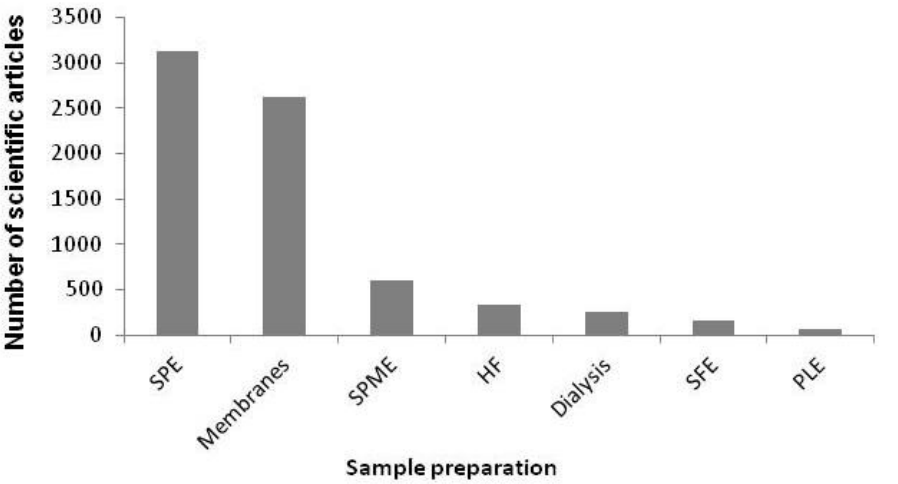

Figure 6. Number of scientific articles found using CE and the name of the different sample treatment strategies cover in this chapter, according to the database 'ISI Web of Knowledge' (until October 2013).

When reviewing the literature on SPE-CE method, we can still often observe that the part concerns with SPE are poorly developed with little consideration to the chemistry involved in the extraction step which is described as a largely empirical. To avoid these habits, CE methodologies should cover the following steps (to achieve a successful extraction of the target analytes to be analysed lately by CE):

1. Selection of the type and amount of sorbent.
2. Column conditioning.
3. Determination of the sample volume and pH which can be applied without loss in recovery.

4. The composition and volume of the washing or clean-up solution which can be applied without loss of analytes.
5. The composition and the volume of the elution or desorption solution.

When a procedure is being developed, a suitable adsorbent material and suitable washing and eluting solvents must be selected, in accordance with the characteristics of the analytes and the matrix, and the purpose of the analysis. The final extract should also be compatible with the CE methodology.

The good results obtained with this approach can be ascribed to the wide variety of SPE products now commercially available and also to its operational simplicity. A wide range of chemically modified adsorbent materials (silica gel or synthetic resins) enable precise group separation on the basis of different types of physicochemical interaction, i.e.:

A) Reversed-phase (e.g. C_2, C_8, C_{18}). SPE C_{18} silica cartridges provide high recoveries, not only for apolar analytes but also for polar ones. No special problem occurs for the extraction of moderately polar analytes. Retention depends on the number of C_{18} chains bonded at the surface of the silica. In order to have a maximum amount of octadecyl chains at the surface, a first requirement is to modify bare silica which is very porous. Starting from such porous silica, every manufacturer of SPE products now provides several types of modified silicas. There are also light loaded alkyl silicas with lower percentages of carbon which in general contain higher amounts of unmodified silanol groups. Residual silanols can play a significant

role in the extraction scheme, and modified silica using a monofunctional silane or with a lower amount of alkyl chains bonded at the surface have been designed for trapping polar analytes.

In order to obtain a better efficiency and a totally apolar material, the trends are to minimize the number of residual silanol groups of the original silica, and for this purpose, a trifunctional silane is used for bonding the n-alkyl chains and an endcaping is carried out with trimethylsilane after bonding [7-12].

Reverse-phase adsorbents are mainly used for extraction of apolar compounds from polar matrices, for example plasma, by use of hydrophobic interaction mechanisms. The compounds are eluted with a less polar organic solvent that disrupts the Van Der Waals forces. Apolar adsorbents, such as C_{18} are widely used in clinical and forensic toxicology because they are broad-range adsorbents which can be used to extract a wide range of compounds from a variety of biological samples [13].

B) Normal phase sorbents (e.g. bare silica, alumina, Florisil (synthetic magnesium silicate) and silica chemically are modified by polar groups such as amino, cyano or diol groups). These adsorbents can be used to extract polar compounds from an apolar matrix, as a result of hydrophilic interactions. Elution with a polar organic solvent is necessary to disrupt the hydrophilic interactions. In clinical applications these adsorbents are mostly used to purify apolar extracts (e.g. i hexane) of solid matrices such as body tissues.

Because silica and alumina adsorb water they should be kept dry before use and aqueous matrices should not be used. Water will

deactivate hydrogen-bonding sites, resulting in reduced retention of the analytes and variable recovery.

Adsorbent properties that increase retention are a larger surface area and a number and type of functional groups present. Hydrogen-bonding functional groups are strongly retained (e.g. sulfonic acid, carboxylic acid, phenol and hydroxyl) in the sorbents, those with a significant dipole character are retained to a lesser extent (e.g. nitro, ester and ketone) and polarisable functional groups (e.g. aromatic and alkene) are the least retained [14].

C) Cation- and anion-exchange. Sorbents that utilizes an ion-exchange interaction can be highly selective for compounds that can be ionized under either acidic or basic conditions. When this mode of extraction is used, pH control during the loading, washing, and elution steps is important.

The use of an exchange minicolumn containing Chelex-100 chelating resin to preconcentrate cations to be determined in CE is mentioned as an example of this type of sorbents [15]. The problem of using this material is the strong acid needed to elute the retained analytes. Normally, 2 M HNO_3 solution is selected given a wide peak in the electropherogram which can overlap the signal of some analytes of interest.

D) New polymeric adsorbents have recently been developed for the improved retention of polar organic compounds, which is a major limitation of the C_{18} adsorbent [16].

One limitation of both reversed-phase silica sorbents and many of the commercial polymeric sorbents is that they must be conditioned

with a wetting solvent and not allowed to dry before the loading of an aqueous solvent. Functionalized polymeric sorbents have been shown to provide a better wettability and to increase the extraction recoveries of polar compounds. The new generation of polymers (Oasis from Waters, Abselut from Varian) are designed to extract extensive spectrum of analytes, i.e., lipophilic, hydrophobic, acidic, basic and neutral with single cartridge [17].

E) New selective sorbents are emerging which are based upon molecular recognition.

E.1) A first approach uses antibodies which allow a high degree of molecular selectivity. Immunosorbents have long been used for sample pre-treatment in medicine, biology and food science, but more general applications, such as environmental samples, are relatively recent. In part, this is because of the difficulty of making antibodies selective to small molecules as well as a lack of familiarity among analytical chemists with the procedures used to make specific antibodies [14].

E.2) A second approach deals with molecular imprinted polymers (MIPs), which avoids the inherent instability of biological materials [17]. MIPs are sometimes referred to as plastic antibodies and are used in SPE as synthetic analogs of immunosorbents. Molecular imprinting is a technique used for preparing polymers with synthetic recognition sites having a predetermined selectivity for a specified analyte (or group of similar analytes). The imprint is obtained by the polymerization of functional and cross-linking monomers in the presence of a template molecule (the analyte). The resultant

imprints posses a steric (size and shape) and chemical (spatial arrangement of complementary functional groups) memory for the template molecule. Removal of the template from the polymer matrix creates vacant recognition sites that enable the polymer to selectively rebind the imprint molecule from a mixture of closely related compounds [14].

As an alternative to the use of a single SPE column, the use of combined or mixed-mode SPE columns was shown to be effective for the purification and isolation of different analytes. Mixed-mode sorbents containing both non-polar and strong ion (cation and/or anion) exchange functional groups have been targeted for the extraction of basic drugs before CE.

In summary, the choice of an appropriate chromatographic material allows SPE devices to be tailored to a variety of needs ranging from extremely selective (with immunoaffinity ligands) to completely general (with a C-18 or an ion-exchange phase).

SPE Formats

1. Syringe-barrel and/or cartridge types are still the most popular format with typically 40-60 □m d_p packing materials. Typical cartridges devices consist of short column (generally an open syringe barrel). Reservoirs have been adapted in order to increase the sample volume. The volume of the syringe barrel selected depends on sample volume, and the amount of

adsorbent determines the sample capacity, which is approximately 5% of the mass of the adsorbent [18].

To avoid previous filtration of the samples there are several cartridge which integrate filters in the SPE cartridges.

2. The second most popular format is the disk. SPE disk were developed to avoid the drawbacks of classical barrels, for example reduced retention of analytes because of channelling through the adsorbent bed. The disk allows higher flow-rates without channelling effects thanks to their large cross-sectional area and thin bed [19].

2.1.2 Solid Phase Microextraction

SPME was originally developed by Pawliszyn and coworkers in 1989, and became commercially available in 1993 [20]. The study of stationary phases for SPME assists the development of applications. Several coatings from non-polar to polar are commercially available, including polydimethylsiloxane, polyacrylate, divinylbenzene, Carboxen (a carbon molecular sieve) and Carbowak (polyethylene glycol) among others. Fibers are available in different film thicknesses with single coatings, combined coatings or co-polymers but remain very limited, which restricts the wide application of SPME.

SPME is done with a fiber coated with an extracting phase, whether liquid (a polymer) or solid (a sorbent)[21], that can be used to extract various types of analytes such as ephedrine derivates [22], amphetamine derivates [23], propranolol enantiomers [24] and pesticides [25] from various types of

matrices such as urine [22], water [22-24] and foods [25]. Its operational simplicity and convenience afford extraction of analytes from small volumes of sample. The coated fiber can be exposed in the headspace of a sample or to a sample solution in order to capture and accumulate analytes. After extraction, the SPME fiber is normally transferred to the injection port of a separating instrument (*e.g.* a gas chromatograph), where the analytes are desorbed and detected —this, however, is not the case with CE instruments. Although SPME was originally used to extract volatile and semi-volatile compounds by thermal desorption, it has subsequently also been used to determine weakly volatile and thermally labile compounds by chemical desorption with an organic solvent for direct analysis by CE or evaporation and reconstitution in an aqueous medium for injection into a CE instrument.

SPME has several important advantages compared to other extraction strategies:

- It is simple, solvent free and sensitive method for the extraction of analytes.
- It is a simple and effective adsorption/desorption technique.
- It provides linear results for wide concentration of analytes.
- It has small size, which is convenient for designing portable devices.
- It gives highly consistent, quantifiable results from very low concentration of analytes.

For all of these advantages, the use of SPME fibers gained popularity but this extraction strategy also presents important drawbacks such as:

- Their relatively low recommended operating temperature.
- Their instability and swelling in organic solvents.

- The breakage of the fiber.
- The stripping of coatings.
- Bending of the needle.
- Expensive cost.

These common problems that SPME users contend with are recognized by researchers and constitute areas of SPME improvement [21].

CE methodologies using SPME as extraction strategy should cover the following steps to achieve a successful extraction of the analytes from the sample:

1. Selection of the type of fiber.
2. Determination of the sample volume.
3. Sampling time and flow rate.
4. The composition and the volume of the extracting solution used to extract the analytes retained in the fiber.

The final extract should be compatible with the CE methodology.

SPME has been the subject of several comprehensive reviews in recent years [21,26]. Since its inception, SPME prior to CE analysis has steadily grown in popularity by virtue of its operational simplicity. In fact, the SPME–CE couple has so far been the subject of more than 200 papers. SPME can also be combined with other extraction and/or analytical procedures in order to further improve the sensitivity and selectivity of the overall method.

SPME should be considered as sample treatment strategies when others do not work well since the better character of this approach (direct extraction of the analytes and injection into the equipment) cannot be exploited with CE instruments.

2.2 Liquid Phase (Micro)Extraction

Liquid–liquid extraction (LLE) remains a classical choice in wide use for sample preparation. LLE allows the extraction of both trace analytes and macrocomponents. The selectivity and efficiency of the extraction process in LLE depends mainly on the election of the immiscible solvents, but other factors may also affect the distribution of the analytes into both phases like the pH, the addition of a complexation agent, the addition of salts (salting out effect), etc. Although the use of LLE alone provides goods results in terms of extraction efficiency and clean-up of the samples, it is often carried out in combination with other preconcentration procedures [27].

The LLE technique has traditionally required substantial amounts of organic solvents and is usually difficult to couple to analytical instruments, whether directly or via an interface. A number of LLE-flow injection approaches involve the mixing of aqueous and organic phases in a mixing coil and their subsequent separation elsewhere [28]. The analytes extracted by LLE are dissolved in a water-immiscible organic phase. Very often, this makes the procedure incompatible with, or unsuitable for, the direct analysis in aqueous electrophoretic buffers and requires using a non-aqueous medium or, alternatively, evaporating the organic solvent in a nitrogen stream and subsequently redissolving the analyte in an appropriate aqueous medium. Although the two previous approaches are the most widely used, there is also the choice of adding a modifier (*e.g.*, a surfactant) to the organic phase in order to make it compatible with the aqueous electrophoretic buffer. One alternative to avoid the addition of modifier is to

perform a non-aqueous capillary electrophoresis separation which is more compatible with the introduction of organic solvents [29].

Various membrane-based methods involving microdialysis, supported liquid membranes or hollow fibers have emerged as alternatives to classical liquid–liquid extraction and proved effective for determining trace analytes [30,31].

2.2.1 Dialysis

Dialysis is a separation technique that uses the mass flux through a semi-permeable inert membrane separating two chambers. One chamber contains an acceptor liquid and the other (the donor chamber) serves as a sample reservoir. Dialysis membranes of a particular molecular weight cut off (MWCO) provide an effective tool for molecular separation based on molecular hydrodynamic dimensions. Thus, molecules larger than the MWCO will be unable to cross over into the perfusing liquid, whereas species with a molecular weight less than the MWCO will diffuse across the membrane by effect of the concentration gradient between the two streams. The dialysis membrane can be non-porous or microporous; both types are permeable to small molecules, but impermeable to macromolecules such as proteins.

One of the most important advantages is that dialysates are protein-free solutions allowing direct injection into a CE system for separation and detection. Dialysis is primarily used to isolate drugs from macromolecular interferences in biological matrices and remove non-volatile ions from biopolymers such as DNA oligonucleotides and proteins.

As it was previously mentioned, dialysis systems provide an elegant solution for separating molecules differing significantly in molecular mass (size). Recently, intrinsic shortcomings such as poor recovery of analytes and high analyte dilution in the acceptor liquid [32] that restricted its use has been solved. For example, poor recoveries can be circumvented by using low sampling flow rates. Also, the use of capillary tubing and small volume dialysis systems has shown that dilution can be minimized. Other alternative, it is to compensate the loss of sensitivity due the dilution with an electrophoretic technique to preconcentrate the analyte in the CE capillary.

Dialysis equipment can be as simple as a static drop dialysis on a floating membrane or as complex as a fully automated system with continuous flowing liquids in the donor and acceptor channels [29].

CE methodologies using dialysis as extraction strategy should cover at least the following steps to achieve a successful extraction of the analytes from the sample:

1. Selection of the type of membrane (select MWCO).
2. Determination of the sample volume.
3. Composition and volume of acceptor phase.
4. Sampling time and flow rate.

In summary we can confirm that in general, dialysis is a relatively slow process and scarcely selective (especially when analytes and potential interferents have similar molecular hydrodynamic dimensions).

2.2.2. Membrane

One effective alternative to dialysis is membrane extraction, where the donor and acceptor phases are separated by a porous membrane. The pores of the membrane are filled with a water-immiscible organic solvent. The theory and uses of liquid membranes were described by Noble and Way in 1987 [33]. The use of membranes to enhance sensitivity and selectivity in CE has grown steadily since 1990.

Unlike dialysis, SLM affords precise control of selectivity in addition to high preconcentration factors for ionizable analytes [29]. By adjusting the pH of the two phases in a way that the analyte will be uncharged in the donor phase but charged in the acceptor phase, it is possible to selectively enrich the acceptor phase with the analytes. The acceptor solution can be directly analysed by CE or evaporated and reconstituted it in an aqueous medium for injection into the CE equipment. For practical purposes, SLMs should be stable for at least one working day. The stability of the liquid membrane is seemingly influenced by the nature of its support. Thus, the most commonly used PTFE membranes are slightly more stable than are polypropylene membranes. Membrane stability is also influenced by pore size.

A membrane can act as a selective filter, either just limiting diffusion between two solutions or as an active membrane —in which case its chemical structure dictates the selectivity of sample transfer [34].

In both dialysis and SLM, diffusion of the analyte through the interface is a rate-limiting step. This problem can be partially solved by continuously stirring the solution or by using an electric field (or voltage). In the latter case, the analyte flux is simultaneously driven through the membrane by a

concentration gradient and an electric potential. The electric potential causes charged analytes to electromigrate through the membrane towards the electrode with opposite charge. Electro-driven flux overcomes back-diffusion and enables nearly quantitative transfer, in general, higher than 70%, of the analyte to the acceptor chamber [35].

The most salient advantages of SLMs include the need for little organic solvent and their environmental friendliness, high preconcentration capabilities, low cost and high selectivity [36,37]. By contrast, SLMs have the disadvantage of typically long analysis times. Based on the foregoing, membrane systems provide an effective way of increasing the sensitivity and selectivity of CE and can be an attractive alternative to, or supplement for, SPE in this context.

CE methodologies using membrane or SLM as extraction strategy should cover at least the following steps to achieve a successful extraction of the analytes from the sample:

1. Selection of the type of membrane.
2. Selection of the organic phase to impregnate the membrane.
3. pH adjusting of the donor phase.
4. Determination of the sample volume.
5. Composition, pH and volume of acceptor phase.
6. Sampling time.

Finally, it is very important to study the compatibility of the acceptor solution with the electrophoretic buffer.

2.2.3. Hollow fiber

HF based liquid-phase microextraction has been a subject of research by several groups ever since it was first reported in 1999 [38-42]. In fact, about 300 references to the use of HF prior to CE separation have been published to date. Although the chemistry behind HF is similar to that of extraction with SLM, the two techniques differ significantly in terms of equipment and operation. A short piece of a porous HF can be used in a rod configuration with a closed bottom or an U-configuration (see selected examples in Figure 7).

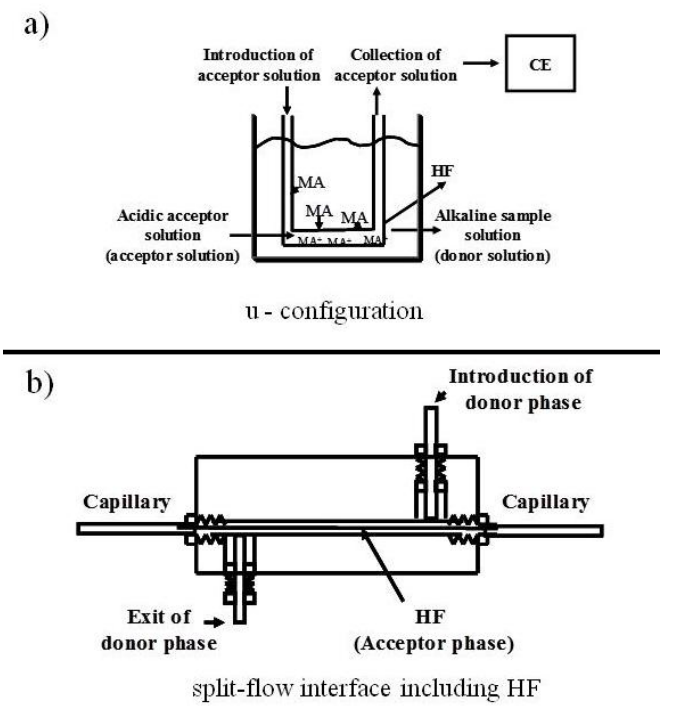

Figure 7. Different configurations of hollow fiber; (a) U-configuration and (b) split-flow interface including hollow fiber. (Reproduced with permission from Ref. 47. Copyright 2010, Betham Science Publisher.).

In any case, the target analytes are extracted from aqueous samples through a thin layer of organic solvent immobilized within the pores of a porous HF, and into an acceptor solution inside the lumen of the HF. Subsequently, the acceptor solution is directly subjected to final analysis with no further effort. HF of appropriate cut-off molecular weights can be used to concentrate diluted samples with an added purification effect. HF holds great promise for the analysis of drugs [41] and biological samples [40], and also for environmental monitoring [39]. Hollow fibers have a number of advantageous properties including a high extractive surface area-to-inner volume ratio and a low resistance to flow. All reported applications of HF in this context rely on the use of custom-built extraction units. By exception, Nozal *et al.* [41] developed an alternative involving the insertion of an HF based microextraction liquid unit into a commercial CE capillary for the selective enrichment of analytes (see Figure 8).

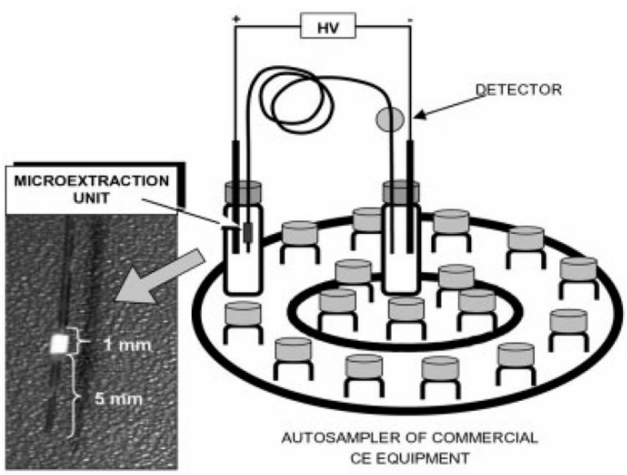

Figure 8. Hollow fiber into a commercial CE capillary. (Reproduced with permission from Ref. 41. Copyright 2007, John Wiley and Sons, Ltd.).

CE methodologies using HF as extraction strategy should cover at least the following steps to achieve a successful extraction of the analytes from the sample:

1. Selection of the type of HF (normally polypropylene).
2. Selection of the organic phase to impregnate the HF (like n-octanol, dihexyl ether or toluene).
3. pH adjusting of the donor phase.
4. Determination of the sample volume (mL range).
5. Composition, pH and volume of acceptor phase (μL range).
6. Sampling time.

Depending of the particular nature of the analytes, the extraction unit (HF) can be reused or must be replaced after each analysis.

2.3 Others

2.3.1 Supercritical Fluid Extraction

SFE emerged as a solution to some problems of SPE in the mid-1980s. Thus, among others, the use of supercritical carbon dioxide avoids the undesirable side effects of organic solvents. Non-polar supercritical CO_2 produces high extraction efficiency for compounds from non-polar to low polarity. Co-solvent systems combining CO_2 with one or more small amounts of modifiers extend the utility of CO_2 to polar and even ionic compounds.

Supercritical fluids, which constitute the intervening physical state between gases and liquids, possess unique properties. Thus, they are less

viscous than liquids, which facilitate their diffusion and results in more efficient extraction of the target substances. In fact, SFE can be made selective by adjusting the fluid properties via its pressure and temperature, and the addition of an appropriate modifier. The large number of SFE variables amenable to adjustment endows SFE with a high flexibility but make optimization of its operating conditions time-consuming. In any case, SFE has some major advantages such as:

(i) its relatively short extraction times.

(ii) its modest pressure and temperature requirements, which minimize the risks of activity losses in preserving the integrity of functional compounds in food and natural products [43] .

(iii) its ability to extract labile compounds from environmental samples [44] .

It was not until 1998, however, that SFE was used in conjunction with CE [43]; since then, around 150 references to the use of SFE prior to CE separation have been reported.

SFE works best with finely powdered solids of good permeability such as soils [45] and dried plant material [46]. This technique has proved effective for extracting organic pollutants such as phenolic compounds, herbicides, pesticides, among others, from soils and plants. Also, it has been used to extract bioactive compounds (metabolites) from botanicals, and medicinal plant materials. In addition, SFE can be used for extraction from wet or liquid samples and solutions, albeit with some difficulty.

CE methodologies using SFE as extraction strategy should cover at least the following steps to achieve a successful extraction of the analytes from the sample:

1. Determination of sample size (normally less than 1 g).
2. Selection of extractant (pure CO_2 or CO_2 with a percentage of modifier).
3. Time of sampling and mode (dynamic and static).
4. Selection of the type of trap to retain analytes.
5. Selection of the solvent (normally organic) to elute the analytes retained in the trap.

Finally, it is very important to study the compatibility of the organic solvent selected to extract the analytes with the electrophoretic buffer. If not, a good alternative could be to use an aqueous liquid trap to retain the analytes extracted from the sample.

Although SFE is inherently superior to many other sample treatment strategies, it is not as easily implemented as some, which has somehow limited broad expansion of its use in CE [47].

2.3.2 Pressurized Liquid Extraction

For rapid and efficient extraction of analytes from solid matrices, liquid extraction temperature is an important experimental factor, because elevated temperatures could lead to significant improvements in the capacity of extraction solvents to dissolve the analytes, in the rates of mass transport, and in the effectiveness of sample wetting and matrix penetration, all of which lead to overall improvement in the extraction and desorption of analytes from the surface and active sites of solid sample matrices. To achieve all these advantages, however, elevated pressure is needed to maintain the extraction solvents as liquids at high temperatures (usually

above their boiling points); this can be accomplished by use of a modern extraction and sampling technique known as pressurized-liquid extraction (PLE) or, more commonly, by its trade name (accelerated solvent extraction) [48] .

By adequately choosing the solvent, its temperature and pressure it is possible to control, among other factors, the dielectric constant of the extractant and with that the polarity of the compounds that can be extracted. Moreover, PLE works in an automatic way; it requires small amounts of solvents and low extraction times. Therefore, PLE can provide fast extractions and purifications allowing testing a high number of extraction conditions under controlled conditions.

As it can be seen in Figure 6, PLE is not a popular sample treatment strategy before CE analysis although for some specific application it may be advantageous over other alternatives (nowadays it is under study).

3. Strategies to couple sample treatment devices to CE equipments

Capillary electrophoresis offers highly efficient separations, short analysis times, simplicity, therefore is a flexible separation technique which has become a serious competitor for other separation techniques including chromatographies [49, 50]. However, sample requirements are more stringent in CE than they are in other separation techniques, to find an appropriate sample treatment is a critical step in the analytical process in order to obtain accurate and reproducible results; this entails, among other cautions, avoiding clogging of the capillary and adsorption of macromolecules on its walls.

Electroosmotic flow (EOF) is one of the driving forces in CE. This phenomenon appears due to the presence of surface charges on capillary walls. The result is a net flow of buffer solution in the direction of the negative electrode. Another important factor to consider is the small inner diameter of the capillary, which implies a very low consumption of samples and reagents [50, 52, 53].

The ability to couple different flow processing devices to CE equipment is limited by the following factors, all of which warrant careful consideration:

(*a*) Compatibility of hydrodynamic flow in the processing device with EOF in the capillary.

(*b*) Compatibility of the high flow-rates typically used in processing devices with the low rates of EOF in a CE system.

(*c*) Compatibility of the sample plug coming from the processing device with the small sample volume to be introduced in the CE capillary.

(*d*) Compatibility of the sample composition with the electrophoretic system.

(*e*) Decoupling of the high voltages and currents applied to the electrophoretic separation system and the flow processing device.

The following sections describe selected coupled systems and their interfaces [54].

3.1. Interfaces and types of coupling

Flow processing devices have been coupled at-, on- and in-line to CE equipment [3]. Overall, on-line coupled systems are the most commonplace. Interested readers can find a review of sample treatment devices used in combination with commercially available CE equipment elsewhere [2].

At-line system refers to a system that has two parts connected via flow line that serves to deliver the pretreated sample to the CE vial. One way of coupling a flow processing device at-line with CE equipment entails using a specifically designed robotic arm interface to place the sample coming from the former in an empty vial in the latter [50, 55].

An alternative to a programmable robotic arm is to couple at-line flow processing devices to a CE equipment by a replenishment system used to empty and fill the CE vial with fresh buffer solution. For instance, a Teflon tube can connect the replenishment bottles (one containing the buffer and the other used as a waste bottle) with the replenishment needle used to evacuate and fill the vial [56]. This entails disconnecting the replenishment needles from the Teflon tube coming from the replenishment bottles and replacing it with one coming from the flow processing device [2, 56].

The greatest advantage of at-line coupled systems is that the CE equipment is run in its normal operation mode; this implies samples to be introduced hydrodynamically or electrokinetically into the separation capillary. Also, it facilitates other CE operations such as capillary conditioning.

Coupling a flow processing device on-line to CE involves inserting the capillary end in a continuous stream of the former. In this case it is

necessary a split-flow interface to couple them, duet to the flow processing device and the electrophoretic system operate at a different flow-rate. The design of this interface is the most critical step. The split-flow interface was originally developed in a vertical configuration by Fang's group [57-59] and a horizontal configuration by Karlberg's [1, 60, 61]; they both consist in a flow-trough channel into an end of the separation capillary and an electrode are inserted. Figure 9 compares the two types of interface, which possess a low dead volume and are electrically grounded. The separation electrolyte and the sample are continuously replenished in the interface.

Samples and electrolyte solutions are introduced in the electrophoretic capillary by effect of electroosmotic flow and electrophoretic mobility, without interrupting the separation voltage or physically moving the capillary. Hydrodynamic flow or pressure generated flow should be avoided by ensuring that the liquid level in the reservoirs CE coincides (at the interface or capillary inlet and capillary outlet).

A split-flow interface can be readily constructed from dielectric materials such as teflon, methacrylate or plexiglas. Alternatively, a piece of tygon tubing can be used to insert the electrophoretic capillary [62].

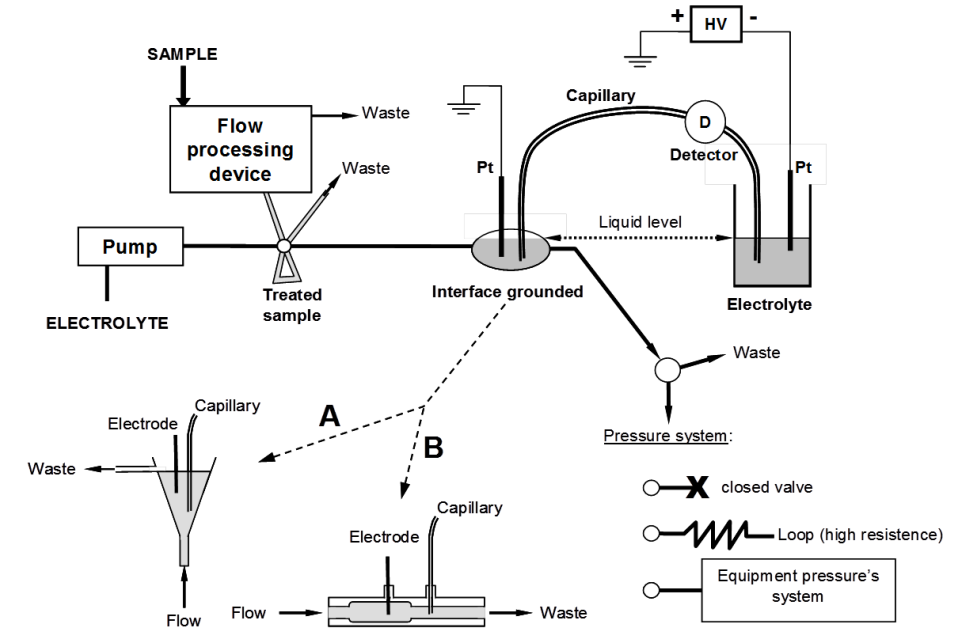

Figure 9. Schematic depiction of the on-line coupling of flow processing devices to capillary electrophoresis equipment. Detail of split flow interface A)- in vertical design and B)- in horizontal design. Description of systems to perform hydrodynamic injection mode for electrophoretic analysis. For more details see Ref. 54.

The flow processing device is made compatible with the electrophoretic system by grounding the interface, avoiding the voltage differences between the interface and flow system. Electrophoretic separation is accomplished by applying a voltage difference to the capillary end as shown in Figure 9. This configuration is highly recommended when an optical detector is used with the electrophoretic system, but not with other types of detectors such as spectrometers. According to Valcárcel's group, the split-flow interface must be subjected to a voltage difference in order to obtain one in the electrospray needle of the electrospray ionization interface of a mass spectrometer [63]. In Figure 10 can be seen two

configurations including a mass spectrometer with a grounded electrospray needle and electrospray chamber, respectively.

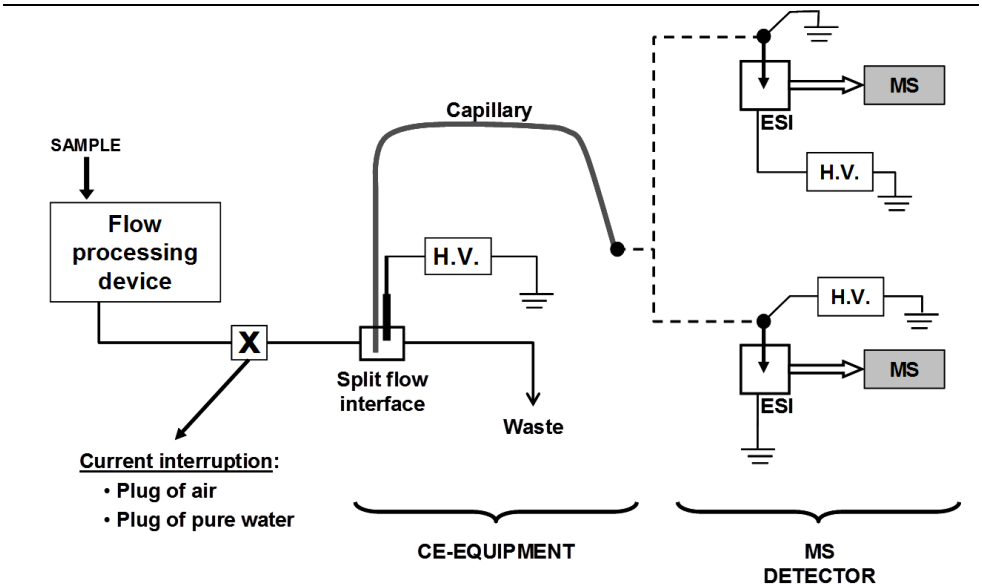

Figure 10. Schematic design of the on-line coupling of flow processing device to a capillary electrophoresis-mass spectrometry equipment by using a split flow interface. This configuration requires voltage isolation by using a plug of air or a large plug or pure water. For more details see Ref. 54.

The current obtained from the voltage difference between the grounded flow processing device and the split-flow interface –which is connected to a voltage source– must be interrupted in order to avoid arc discharges in valves and pumps in the flow system. Alternatively, the current can be interrupted by using a plug of a substance with a high electrical resistance such as pure water or air [64]. It is also advisable to insert a safety line consisting of a grounded electrode immediately in front of the pumps [2, 63].

In principle, the use of on-line coupled systems linked via a split-flow interface is limited to the electrokinetic introduction of samples. In this injection type the sample is induced into the capillary by a combination of the electrophoretic migration of the ions and the electroosmotic flow. However, this mode provides biassed results in some cases; due to the discrimination of analytes depending on their mobility. For this reason the development of an interface with hydrodynamic injection has promoted instead. Thus, Pu *et al.* [64] used a split-flow cell affording hydrodynamic injection of samples into the CE capillary by electroosmotic flow. In the interface, the capillary is fractured about 10 cm from the injection end and one Pt electrode is inserted in the vial together with the fractured portion of the separation capillary. Also, it included a Nafion joint to connect the CE capillary to the tube of the flow system (see Figure 11).

The high voltage cannot be constantly applied since it introduces the flow of sample matrix into the separation capillary, thus changing the magnitude of the EOF flow. Therefore, an electronic time relay was used to control the voltage and to switch to the EOF traction mode the period during which the injection takes place. Kuban *et al.* [61] inserted a valve at the end of a horizontal split-flow interface. Switching the valve off caused the sample to be forced into the capillary. The greatest limitations of this approach arise from the need to control the time during which the valve is on and off, and also the pressure generated in the system. Santos *et al.* [63] proposed controlling the pressure by using a valve loop capable of withstanding higher pressures. With commercial CE instruments, one can also connect the pressure system of the equipment [2, 63].

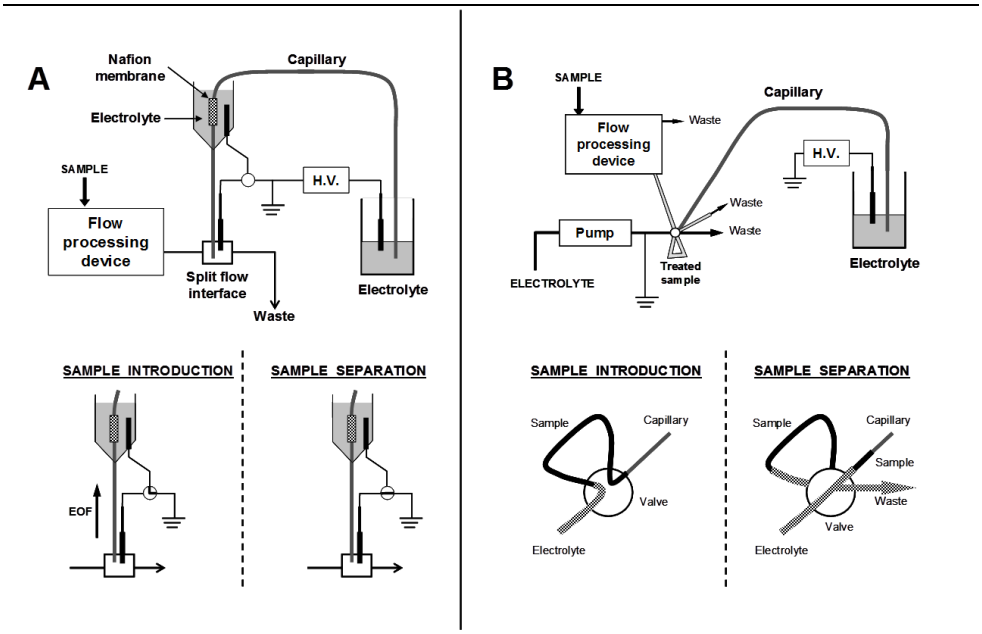

Figure 11. Alternatives to perform automatic hydrodynamic sample introduction for electrophoretic analysis in on-line flow system-capillary electrophoresis combinations. A)- use of Nafion membranes and B)- use of injection valve. For more details see Ref. 54.

The split-flow interface can be used for the on-line coupling of both flow injection and sequential injections systems. Based on a common approach, split-flow interfaces, microsequential injection systems or integrated Lab-on-a-valve systems [65, 66] have been coupled to CE. Sequential injection systems have also been on-line coupled via a microvalve allowing the insertion of a constant volume of sample into the capillary, and their suitability for repeated injections has been demonstrated [67, 68]. However, the loop volume is relatively higher than to those typically used in CE work and must be reduced by switching the valve or flushing the sample from the interface in order to facilitate electrokinetic insertion of a portion of sample (see Figure 11).

A modified version of the split-flow interface was used to accommodate a typical SPME fibre precisely at the capillary end. The modification consisted of an additional channel closed with a septum that permitted the introduction of the fiber in the interface, right at the inlet region of the separation capillary [69]. Although samples were treated on the fibre and the flow system was only used to couple SPME and CE, they can also be processed at the interface connecting the fibre to the flow system [70].

In-line coupling a flow processing device to CE involves carrying out the sample treatment in the capillary. This modality allows coupling both solid phase extraction and liquid phase extraction. SPE can be integrated in the capillary packing the sorbent in the inlet region, and using it such as cartridge [71]. Also it has been used hollow fibres to perform liquid-phase microextraction (LPME). As shown in Figure 12 the electrophoretic capillary can be inserted into the lumen of a hollow fibre or the extraction unit into the capillary [72].

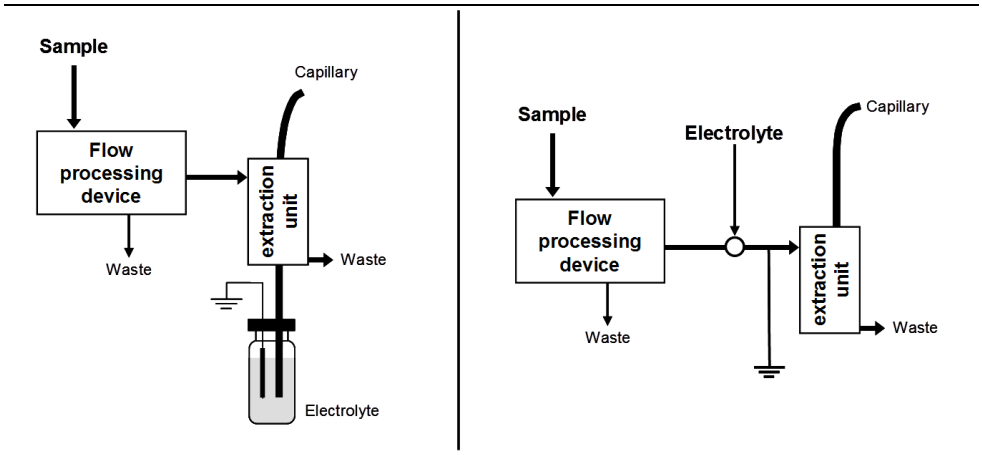

Figure 12. Schematic depiction of the in-line coupling of flow processing devices to capillary electrophoresis equipment. For more details see Ref. 54.

In the latter case, the fibre can be fitted to the capillary by heating [2]. The hollow fiber can be changed for a membrane where analytes migrate through it, and with this strategy is avoided the contact between the sample matrix and the internal capillary wall [41]. Alternatively, in-vial methodologies coupled to commercial CE equipment has been also developed for sample treatment, taking account the dimensions of the vial and the configuration of capillary and inlet electrode. In this case the vial containing the microextraction unit is located in the autosampler and operates to preconcentrate analytes [73, 74].

The use of CE on a chip has grown in recent years, due to the significantly shorter analysis times compared to concentional CE. Consequently flow systems have also been coupled to microchip electrophoretic systems [75], using a capillary as the interface between a sequential injection system and the microchip. For this purpose, one end of the capillary was attached to the microchip via a Teflon fitting. The principal shortcoming of this combination is the presence of residual hydrodynamic flow in the microchip separation channel [75, 76].

4. Analytical applications in biomedical, environmental and food analysis

The sample and target analytes dictate the most convenient sample treatments and conditions (e.g., solvent selection, sample and amount) to use before CE analysis, and, usually, the difficulty of determination is proportional to its complexity. In general, these procedures are performed sequentially, and a subsequent step cannot begin until the preceding one has been fully completed, implying that the slowest steps determine the rate

of the analytical process. For most of the users, the method should be cost-effective, consuming as minimum reagents and chemicals as possible and with as low expenses as possible on instrumentation and facility. A method is always preferred which is very easy to use, has the minimum steps and uses only simple devices or systems capable of full automation. It is highly significant to minimize sample preparation steps to reduce the sources of error. A sample preparation method can have more than one step, such as homogenization, extraction, cleanup, preconcentration and or derivatization. The more the number of steps are involved, the more will be uncertainty will be introduced into the assaying.

As it has been mentioned in other part of the chapter, sample treatments are of critical importance, since in general, real samples are complex matrices that include interferences. If the extraction is not selective, the extract will probably require a clean-up process in order to eliminate interferences that might compromise separation and identification of target compounds during instrumental analysis [77]. In general, it is of pivotal importance that the extraction method should provide an extract that reflects the real composition and the profile of the components of the original sample and is as free as possible of interferences, in order to allow accurate reproducible and high-quality results to be obtained during the analysis step.

Until now, CE has been used across a wide range of analytical chemistry and biochemistry applications, and in different fields (Figure 13).

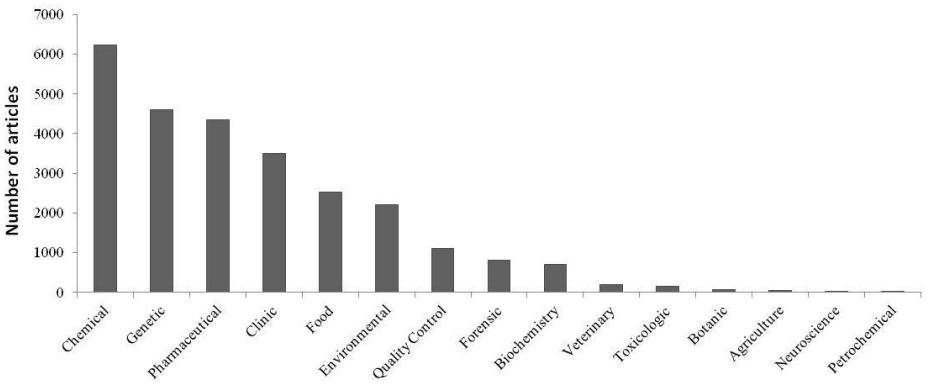

Figure 13. Bibliography concerning CE method categorized by the application field. According to the database 'ISI Web of Knowledge' (until October 2013).

More than 2000 articles were found in the database 'ISI Web of Knowledge' combining the keywords 'Capillary Electrophoresis' with the terms 'chemical', 'genetic', 'pharmaceutical', 'clinic', 'food' or 'environmental' up to October 2013. This bibliography demonstrates the versatility of the technique. Also, as Figure 14 shows, CE can be used for a variety of different separations, but the main application remains in the determination of proteins, DNA, drugs, aminoacids, peptides and enantiomers. However, there is a growing interest in the use of CE to determine other analytes such as enzymes, organic acids, metals or additives among others, where an increase in the published papers number can be observed each day.

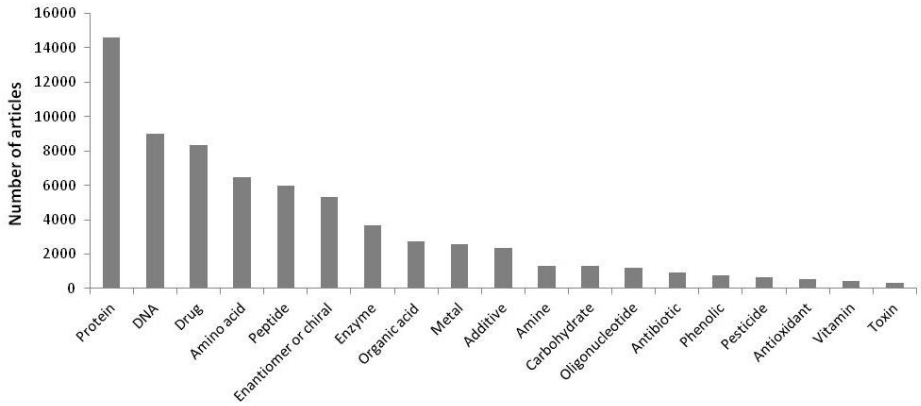

Figure 14. Bibliography concerning CE methods using the name of different groups of analytes. According to the database 'ISI Web of Knowledge' (until October 2013).

A summarize of some applications of CE methodologies using the sample treatments covered in this chapter are presented in Table 1. From the available literature, summarizes in Table 1, it can be concluded that SPE is the most popular sample treatment used nowadays before CE analysis. And there are several different combinatory possibilities that could be used to extract analytes from complex samples. Usually, when SPE is combined or hyphenated with another extraction technique, high selectivity and much cleaner samples can be obtained.

A study of the scientific literature shows that drug metabolites and protein are the analytes more extracted using SPE, although drug and metabolites have been extracted more times using LLE than SPE. Finally it can be highlighted that the use of SFE or PLE before CE is less popular than other approaches summarized in this chapter.

Table 1. Different sample treatments used for the determination of different analytes before CE analysis.

Analytes	Sample	Sample treatment	Ref.
Penicillin G	Urine, blood, amniotic fluid	Deproteinization by ACN, direct injection	78
Amoxicillin, Ampicillin, Cloxacillin, Dicloxacillin, Nafcillin, Oxacillin, Penicillin G	Milk	Extraction with ACN followed by SPE	79
Ciprofloxacin, enrofloxacin	Raw bovine milk	Deproteinization by HCl and SPE	80
Amoxicillin, Ampicillin, Penicillin G	Poultry muscles	Extraction with ACN, Methanol and EDTA followed by SPE	81
Cefoperazone, Ceftiofur	Cow plasma	Deproteinization by perchloric acid and on-line SPE	82, 83
Ciprofloxacin, Gatifloxacin, Moxifloxacin, Ofloxacin, Sparfloxacin	Tablets	Extraction with methanol	84
Ciprofloxacin, Norfloxacin, Ofloxacin	Urine	Centrifugation and direct injection	85
Ciprofloxacin, Danofloxacin, Difloxacin, Enrofloxacin, Flumequine, Marbofloxacin, Oxolinic acid, Sarafloxacin	Chicken	PLE with dichloromethane and in-line SPE, direct injection	86
Enoxacin, Fleroxacin, Norfloxacin, Ofloxacin, Pazufloxacin	Chicken	Extraction with TFA in ACN, followed with dichloromethane	87
Quinolone residue	Chicken, fish	Solvent extraction, SPE	88
Basic drugs	Whole blood	LLE (extraction solvent 1-chlorobutane, alkaline pH)	89
Amphetamine and Methamphetamine	Urine	In tube SPME	90
Carnitine, Acylcarnitines	Plasma, urine	LLE (ethyl acetate)	91
Oxprenolol	Human urine	LLE-SPE (C18)	92
Tetracyclines	Soil samples	Dilution, filtration, SPME (carbowax-divinylbenzene)	69

Table 1. Continuation.

Analytes	Sample	Sample treatment	Ref.
β-Lactam	Waste, well and river water	LLE-SPE (Oasis HLB)	93
Glycoproteins	Bovine proteins	Microcon filtration, enzymatic deglycosilation	94
Proteins	Spirulina platensis microalgae	Sonication, PLE, ultrafiltration, precipitation, dialysis, freeze drying	95
Proteins	Human serum	Dilution and Monolithic column with immobilized immunoglobulin G	96
Glycoprotein-derived Oligosaccharides	Bovine serum	Derivatization and LLE	97
Proteins	Olive samples	LLE and precipitation	98
Polypeptides and proteins	Urine	SPE, lyophilizacion	99
Peptides	Human plasma	On line SPE	100
Bioactive peptides	Infant milk formulas	SPE	101
Aminoacids	Juice	Derivatization and direct injection	102
N-phenylpropenoyl-l-amino acids	Cocoa samples	Soxhlet and centrifugation	103
Furosine	Flour, pasta and milk	Hydrolysis and filtration	104
Aminoacids	Serum, plasma, blood and urine	Electromenbrane extraction	105
Aminoacids	Standards	Derivatization and single drop microextraction (based in LLE)	106
Inorganic anions	River and tap water	Ion Exchange sorbent	107
Inorganic anions	Sea water	Monolithic column with cation exchange sites	108
Inorganic ions	Water	Ion exchange sorbent (Chelex-100)	15
Anionic arsenic compounds	Chicken litter and soils	C18 and anion exchange SPE	109
Inorganic anions and cations	environmental samples	Filtration and direct injection	110

Table 1. Continuation.

Analyte	Sample	Sample treatment	Ref.
Organic cations, inorganic anions and organic	Exhaled breath	Condensation and direct analysis	111
Selenium	Water	SPE	112
Metals	Juice	Mineralization and dispersive SPE	113
Sodium	Milk	Direct injection	114
Metals	Tap water and milk powder	Electromembrane extraction	115
Metal cations, anions, organic acids, carbohydrates	Red wines	Dilution and direct injection	116
Carboxylic acids	Aqueous extracts of atmospheric particles	Hollow fiber liquid-phase microextraction	117
Mono- and disaccharides	Drink and foodstuffs	Dilution and direct injection	118
Triclosan and bisphenol A	Water, beverage, urine samples	Liquid-liquid microextraction	119
Benzoyl peroxide	Wheat Flour	Derivatization	120
Heterocyclic amines	Urine	In capillary SPE	121
Heteroyciclic aromatic amines	Urine	Deproteinization SPE	122
Aromatic amines	Aqueous sample	Hollow fibre	123
Aromatic amines	Water	SLM	124
Phenolic compounds	Virgin olive oil	SPE	125
Flavonoids	Plant	Soxhlet, LLE, SPE	126
Flavonoids	Licorice	PLE	127
Antioxidants	*Rosmarinus officinalis L.*	PLE	128
Anthocyanins	Wine and wine musts	SPE	129

Table 1. Continuation.

Analyte	Sample	Sample treatment	Ref.
Phenolic compounds (bisphenol-A, beta-naphthol, a-naphthol, 2, 4-dichlorophenol)	Aqueous cosmetics	Ionic liquid based dispersive liquid-liquid microextraction	130
Phenols, lignans, phenolic acids, flavonoids	Virgin olive oil	SPE	131
Chlorophenols	Water	SLM	132
Chlorophenols	Water and urine	SFE	44
Pesticides	Water	SPE	133
Pesticides	Fruits and vegetables	LLE (water/acetone), filtration, evaporation, SPE	134
Pesticides	Peaches and nectarines	Solvent extraction, SPE	135
Pesticides	Fruit juices	SPME	136
Phenylureas	Green vegetables and rice	Dispersive SPE	137
Pesticides	Peaches, melon, watermelon, apricot	SPE and PLE	138

ABBREVIATIONS AND ACRONYMS

CE	Capillary Electrophoresis
EOF	Electroosmotic Flow
HF	Hollow Fiber
LLE	Liquid liquid Extraction
MD	Microdialysis
MIPs	Molecular Imprinted Polymers
MWCO	Molecular Weight Cut Off
SFE	Supercritical Fluid Extraction
SLM	Supported Liquid Membranes
SPE	Solid Phase Extraction
SPME	Solid Phase Microextraction
PLE	Pressurized Liquid Extraction

RELATED ARTICLES

Chemical Weapons Chemical Analysis (Volume 1)
Capillary Electrophoresis in Detection of Chemicals Related to the Chemical Weapons Convection

Liquid Chromatography (Volume 13)
Micellar Electrokinetic Chromatography

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BLOQUE 2

HERRAMIENTAS ANALÍTICAS

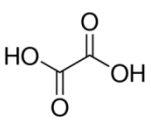
En el desarrollo experimental de la presente Tesis Doctoral se han empleado diferentes herramientas analíticas considerando como tales todos los elementos utilizados; incluyendo estándares, reactivos, disolventes, aparatos e instrumentación. En esta sección de la Memoria se enumeran dichas herramientas junto con sus características más relevantes.

2.1. Estándares, reactivos y disolventes

Todos los estándares, reactivos y disolventes utilizados a lo largo de esta Tesis Doctoral son de pureza analítica.

2.1.1. Estándares

- Ácidos orgánicos: ácido oxálico, ácido cítrico, ácido orótico, ácido benzoico y ácido hipúrico (Sigma Aldrich).



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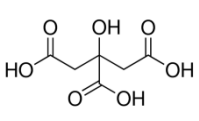

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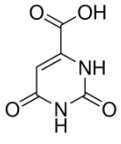

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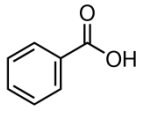
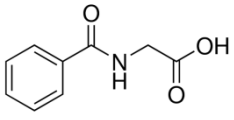
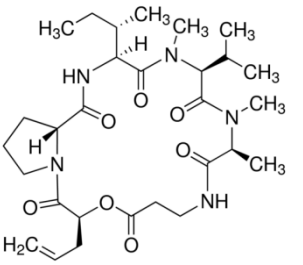


Ácido benzoico



Ácido hipúrico

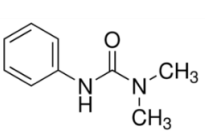
- Micotoxinas: destruxina A (Sigma Aldrich).



Destruxina A

- Sales inorgánicas: nitratos de Cd(II), Mg(II), Cu(II), Zn (II) y Ni(II) de Sigma Aldrich (patrones en medio ácido, pH 2).

- Pesticidas: fenuron, simazina, atrazina, carbaril y terbutrin (Sigma Aldrich).



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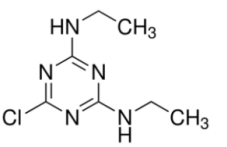

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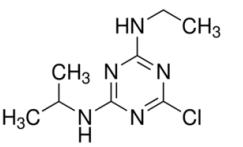
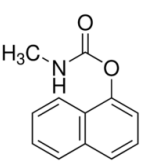
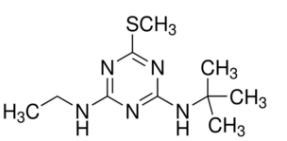


Atrazina

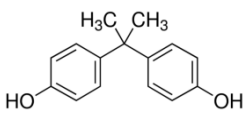


Carbaril

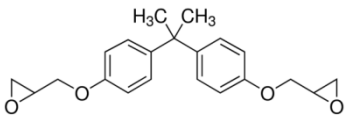


Terbutrin

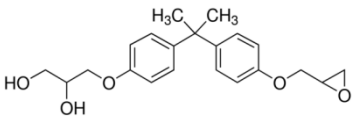
- Bisfenoles: bisfenol A, bisfenol A diglicidil éter, bisfenol A (2,3-hidroxipropil) diglicidil éter y bisfenol A (2,3-hidroxipropil) éter (Sigma Aldrich).



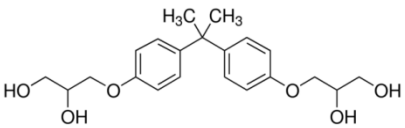
Bisfenol A



Bisfenol A diglicidil éter



Bisfenol A (2,3-dihidroxipropil) glicidil éter



Bisfenol A (2,3-dihidroxipropil) éter

2.1.2. Reactivos

- Reactivos utilizados para preparar los distintos bufferes de separación utilizados en la Electroforesis Capilar

Tipo	Compuesto	Casa comercial
Sales	Tetraborato de sodio	Sigma Aldrich
	Bromuro de hexadeciltrimetilamonio (CTAB)	Fluka
	Cloruro de hidroxilamina	Sigma Aldrich
	1,10-fenantrolina	Sigma Aldrich
	Urea	Sigma Aldrich
	Cloruro amónico	Sigma Aldrich
	Hidrógeno fosfato de sodio	Sigma Aldrich
	Dodecilsulfato sódico (SDS)	Sigma Aldrich
Bases y ácidos (ajuste pH)	Hidróxido sódico	Sigma Aldrich
	Ácido clorhídrico	Sigma Aldrich
	Ácido nítrico	Sigma Aldrich
Disolventes	Metanol	Sigma Aldrich
	Acetonitrilo	Sigma Aldrich
	Isopropanol	Sigma Aldrich

- Reactivos utilizados para preparar los medios de cultivo

Compuesto	Casa comercial
Dihidrógeno fosfato de potasio	Panreac
Hidrógeno fosfato dipotásico	Panreac
Hidrógeno fosfato disódico	Merck
Cloruro potásico	Panreac
Nitrato potásico	Merck
Sulfato magnésico hidratado	Panreac
Nitrato amónico	Merck
Glucosa	Panreac
Extracto de levadura	Oxoid
Triton X-100	Merck

- Reactivos utilizados para la extracción QuEChERS

Compuesto	Casa comercial
Fosfato monobásico de sodio	Sigma Aldrich
Sulfato magnésico	Sigma Aldrich
Cloruro sódico	Sigma Aldrich
Citrato sódico	Sigma Aldrich
Citrato disódico sesquihidratado	Sigma Aldrich
Ácido fórmico	Merck

- Reactivos utilizados para sintetizar la organosílice mesoporosa periódica con puentes fenileno

Compuesto	Casa comercial
1,4-bis(trietoxisilil)benceno	Sigma Aldrich
Surfactante Brij 76 (polioxietileno estearil alcohol)	Sigma Aldrich

2.1.3. Disolventes

Los disolventes básicos utilizados a lo largo de la parte experimental de esta Tesis Doctoral son: metanol de Panreac, acetonitrilo, etanol e isopropanol de Sigma Aldrich.

Estos se han empleado con tres finalidades; en la preparación de las disoluciones estándar de los analitos, la preparación de buffers de separación (que se ha indicado previamente), y como eluyentes en los procesos de extracción en fase sólida (tanto en la etapa de elución como de acondicionamiento).

2.2. Materiales sorbentes utilizados para preconcentrar

2.2.1. Materiales sorbentes clásicos

- Oasis HLB (Waters, Mildford, MA, USA). Es un sorbente polimérico en fase reversa que se usa para extraer un gran intervalo de compuestos tanto ácidos, básicos, o neutros.
- Bond Elut C18 (Varian, Harbor City, CA, USA) y Sep-pak C18 (Waters, Mildford, MA, USA). La C18 es una fase ligada a sílice que presenta características hidrofóbicas.

2.2.2. Nuevos materiales sorbentes

- Nanopartículas magnéticas (MNPs)

Nanopartículas magnéticas funcionalizadas con grupos carboxílicos se obtuvieron de MagmaMedics Diagnostic (Geleen, The Netherlands). Estas presentan un tamaño medio de 1,2 μm (±0.1 μm), un contenido magnético del 60% y un área superficial de 3,6 m^2/g.

Este material se utilizó en MSPE en el Capítulo 4.1. En la etapa de preconcentración se utilizó una combinación de tres imanes para separar las nanopartículas magnéticas de las disoluciones. Estos imanes tenían las siguientes características:

1. Recubiertos de Ni-Cu-Ni
2. Fuerza axial de 4.8 kg (axial pull force)
3. Diámetro interno de 6 mm y externo de 15 mm
4. Grosor de 5 ± 0.1 mm

- Organosílice mesoporosa periódica con puentes fenileno (Ph-PMO)

El Ph-PMO utilizado en esta Tesis Doctoral se sintetizó siguiendo procedimientos previamente descritos [1, 2].

El material se utilizó en el Capítulo 4.2 como sorbente para la modalidad clásica de SPE. Para ello, el material se empaquetó en cartuchos de SPE de un volumen de 3 mL (Sigma Aldrich), colocando una frita de polietileno (Sigma Aldrich) arriba y abajo del material.

- Estructura zeolítica de imidazolato 8 (ZIF-8)

El material se obtuvo de Sigma Aldrich bajo el nombre comercial Basolite Z1200. Este ZIF-8 presenta un tamaño de partícula sobre 4.9 μm y un área superficial entre 1300 y 1800 m^2/g.
El material se utilizó en el Capítulo 4.3 como sorbente tanto tanto en la estrategia de dSPE, como empaquetado en un cartucho de SPE clásico.

2.3. Otro material de laboratorio

- Filtros de nylon de 0.45 μm de Análisis vínicos.
- Jeringas de plástico de Terumo.
- Microfiltros Amicon-10 con un nivel de corte de 10 kDa (Millipore, Milford, MA, USA).

2.4. Muestras

- Muestras de leche cruda de cabra (Capítulo 3.1 y 3.2)

Se diseñó un plan de muestreo para poder obtener un conjunto de muestras representativo de los distintos pastos que se pueden encontrar en el sur de España. Este muestreo se realizó a lo largo de 4 años para estudiar cómo afecta el factor climatológico al pasto. En total se recogieron 198 muestras de leche de cabra procedentes de diferentes zonas de España (Tabla 1). Después se congelaron a -18 ºC hasta su análisis.

Tabla 1. Muestras de leche cruda de cabra.

Sistema de producción	Régimen de alimentación	Raza	Origen	Mes de muestreo	Número de muestras
Convencional	Pienso y pasto	Payoya	Cádiz	Febrero 2009	10
	Pienso y pasto	Payoya	Cádiz	Abril 2009	10
	Pienso	Florida	Córdoba	Diciembre 2010	11
	Pienso	Murciano-Granadina	Sevilla	Diciembre 2010	11
	Pienso y pasto	Serrana	Badajoz	Diciembre 2010	11
	Pienso	Florida	Badajoz	Febrero 2011	10
Ecológico	Pienso y pasto	Payoya	Cádiz	Febrero 2009	10
	Pienso y pasto	Payoya	Cádiz	Abril 2009	10
	Pasto	Payoya	Cádiz	Múltiple[1]	61
	Pienso	Murciano-Granadina	Murcia	Enero 2011	14
	Pasto	Serrana	Huelva	Junio y julio 2011	20
	Pienso	Serrana	Córdoba	Abril 2012	10
	Pasto	Serrana	Badajoz	Abril 2012	10

[1]Muestras recogidas en mayo, junio y julio 2010; enero 2011; y abril 2012.

- Muestras de leche comercial de cabra y vaca (Capítulo 3.2)

En total se reunieron 27 muestras de distintos orígenes tal y como se muestra en la Tabla 2.

Tabla 2. Muestras de leche comercial de cabra y vaca.

Sistema de producción	Animal	Raza	Origen	Número de muestras
Convencional	Vaca	Holstein-Friesian	España	11
	Vaca	British Friesian	Reino Unido	3
	Cabra	Murciano-Granadina	España	1
	Cabra	Saanen y Alpina	Reino Unido	2
Ecológico	Vaca	Holstein-Friesian	España	4
	Vaca	British Friesian	Reino Unido	5
	Cabra	Murciano-Granadina	España	1

- Muestras de medios de cultivo (Capítulo 3.3)

Cepas

Se evaluaron tres cepas procedentes del hongo *Metarhizium brunneum*, en concreto BIPESCO5, EAMA 01/58-Su y ART2825. La BIPESCO5 se aisló de *Cydia pomonella* y se obtuvo de la colección de cultivos de Innsbruk (Austria). EAMa 01/58-Su pertenece a la colección de cultivos de la Universidad de Córdoba, y originalmente se aisló de suelos de cultivos de trigo en Hinojosa del Duque (Córdoba, España). Finalmente, ART2825 se obtuvo de la colección de medios del EAER-Agroscope en Suiza, y se aisló del *Agriotes obscurus.*

Preparación de los medios y de las condiciones de cultivo

Las cepas BIPESCO5, EAMa 01/58-Su y ART2825 se cultivaron en dos medios diferentes llamados medio semisintético completo (CM) y medio mínimo (MM).

El medio CM se preparó mezclando 0.36 g KH_2PO_4, 1.7 g Na_2HPO_4, 1 g KCl, 0.29 g $MgSO_4(7H_2O)$, 0.7 g NH_4NO_3, 10 g glucosa, 5 g de extracto de levadura y 0.6 mL Triton X-100 en 1 L de agua. Mientras que el medio MM se preparó utilizando 1 g KH_2PO_4, 0.7 g Na_2HPO_4, 0.7 g KCl, 0.6 g $MgSO_4(7H_2O)$, 0.7 g NH_4NO_3, 0.5 g K_2HPO_4, 2.5 g KNO_3, y 20 g glucosa en 1 L de agua.

Para preparar el cultivo primario, se colocó 1 mL de suspensión de conidias (ajustada a una concentración de $1x10^7$ esporas/mL) en un erlenmeyer que contenía 25 mL del medio líquido. La mezcla se cultivó a 25 ºC en un agitador rotatorio (Ovan Multimix, Badalona, España) a 110 rpm durante 4 días.

Con el objetivo de inocular los cultivos secundarios que sirven posteriormente para incubar el hongo, 2 mL del cultivo primario se transfirieron a 125 mL del mismo medio. Este proceso se repitió seis veces, para obtener seis preparaciones independientes. Estos cultivos se incubaron a 25 ºC en el agitador a 110 rpm, y cada 3 días se fue retirando un cultivo para su posterior análisis, así hasta 18 días. Los micelios que se encontraron, se filtraron utilizando un filtro de papel Whatman nº 3 (Whatman, Kent, UK).

- Muestras de zumo de piña y melocotón (Capítulo 4.1), mosto de uva (Capítulo 4.2) y refresco de cola (Capítulo 4.3)

Todas las muestras se obtuvieron de supermercados locales.

2.5. Instrumentación

- Equipo de Electroforesis Capilar P/ACE MDQ Capillary Electrophoresis System procedente de Beckman (Palo Alto, CA, USA) equipado con un detector UV/Vis de diodos en fila, y utilizando un capilar también de Beckman.

- Cromatógrafo de Líquidos modelo Varian 920-LC equipado con un detector de diodos en fila, una columna de fase reversa C18 (5 μm; 250 x 4.6 mm) (Phenomenex, Torrance, CA).

- Los patrones de difracción de rayos X se obtuvieron utilizando radiación CuKα en un difractrómetro ARL X'TRA de Thermo Scientific (Waltham, MA, USA).

- Las isotermas de adsorción-desorción se obtuvieron en un analizador Micromeritics ASAP 2010 (Norcross, GA, USA) a -196 ºC. Antes las muestras se desgasaron a 120 ºC durante 24 h.

- Para las medidas del tamaño de partículas se utilizó un analizador de tamaño de partículas por difracción láser modelo Mastesizer S (Malvern Instruments, Worcestershire, UK).

- Las imagénes de microscopía electrónica de barrido se llevaron a cabo utilizando un microscopio modelo JEOL 6300 (Tokio, Japan) utlizando oro “sputtered” acelerado con un voltaje de 20 kV.

2.6. Aparatos y pequeña instrumentación

- Destilador de agua MilliQ de Millipore (Bedford, MA, USA).
- pHmetro modelo Micro-pH 2000 de Crison (Barcelona, España)
- Centrífuga modelo High-Speed Centrifuge procedente de J. P. Selecta (Abrera, Barcelona, España).
- Sistema de extracción en vacío modelo Visiprep DL de Supelco (Bellefonte, PA, USA). Constituida por una cámara con paredes de vidrio con una tapa de polietileno de 20 posiciones, dotada de una gradilla para situar los tubos en los que se recoge el eluido de cada cartucho. Este dispositivo tiene un sistema de control de presión mediante el cual se monitoriza el grado de vacío existente en la cámara de extracción.
- Agitador vortex (Heidolph, Merida, España).
- Agitador magnético (Velp Científica, Milán, Italia).
- Centrífuga Centronic BL-II (J.P. Selecta, Barcelona, España), con una velocidad máxima de 13500 rpm.

- Horno mufla Heron modelo HD-150, con una temperatura máxima de 1200 ºC.
- Balanza analítica OHAUS Explorer (OHAUS, Nänikon, Suiza) que puede realizar medidas de masas desde 0.01 a 110 mg, con un error de 0.0001 g.

Referencias

[1] M. C. Burleigh, S. Jayansundera, C. W. Thomas, M. S. Spector, M. A. Markowitz, B. P. Gaber, A versatile synthetic approach to periodic mesoporous organosilicas, Colloid Polym. Sci. 282 (2004) 728-733.

[2] D. Esquivel, C. Jiménez-Sanchidrián, F. J. Romero-Salguero, Thermal behaviour, sulfonation and catalytic activity of phenylene-bridged periodic mesoporous organosilicas, J. Mater. Chem. 21 (2011) 724-733.

BLOQUE 3

USO DE LA ELECTROFORESIS EN EL CAMPO AGROALIMENTARIO SIN ETAPA DE PRECONCENTRACIÓN EN EL TRATAMIENTO DE MUESTRA

En este Bloque se van a presentar los trabajos experimentales relacionados con dos campos de aplicación diferentes, pero ambos enmarcados dentro del ámbito agroalimentario, para demostrar la utilidad de la CE con detección UV/Vis sin necesidad de tratamientos de muestra complejos.

En primer lugar se ha utilizado la CE para diferenciar el régimen de alimentación suministrado a cabras. Generalmente los consumidores están dispuestos a pagar un precio mayor por productos ecológicos, porque los consideran más saludables o sostenibles con el medio ambiente; con lo cual es un sector del mercado que está creciendo rápidamente. Por ello para asegurar y certificar la leche ecológica, es necesario establecer parámetros o buscar analitos que puedan diferenciar ambos productos, y de esa forma identificar falsos etiquetados.

Por otra parte, el desarrollo de biopesticidas utilizando hongos entomopatógenos es un tema de creciente interés, ya que son una opción ambientalmente sostenible frente al uso de pesticidas químicos. Sin embargo producen metabolitos que pueden ser potencialmente tóxicos. En el tercer capítulo de este bloque, se muestra como la CE con detección UV puede ser una herramienta útil para los laboratorios con la que evaluar la producción de estas toxinas que en un futuro podrían considerarse como un problema de seguridad ambiental o alimentaria.

Todos los resultados presentados en este bloque se han comparado con los obtenidos por HPLC.

Capítulo 3.1

Differentiation of organic goat's milk based on its hippuric acid content as determined by Capillary Electrophoresis

ELECTROPHORESIS

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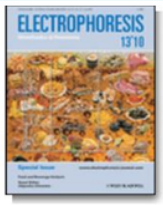

DIFFERENTIATION OF ORGANIC GOAT´S MILK BASED ON ITS HIPPURIC ACID CONTENT AS DETERMINED BY CAPILLARY ELECTROPHORESIS

A. Carpio[1], V. Rodríguez-Estévez[2], M. Sánchez-Rodríguez[2], L. Arce[1], M. Valcárcel[1*]

[1] Department of Analytical Chemistry, University of Córdoba, Annex C3 Building, Campus of Rabanales, E-14071 Córdoba, Spain, [*]qa1meobj@uco.es

[2] Department of Animal Production, University of Córdoba, Campus of Rabanales, E-14071 Córdoba, Spain

Organic foods have lately aroused interest by virtue of their quality and their essential, exclusive characteristics. In this work, we assessed the potential of capillary electrophoresis (CE) to detect a marker that is able to identify the particular food given to the goats and develop an effective method to determine directly the most abundant organic acid in goat's milk (*viz.* hippuric acid). Also, we examined the use of the hippuric acid content of goat's milk as a general marker for authenticating organic goat's milk. The feeding of these goats can be influenced by the time of the year. For this reason we have collected samples from 20 conventionally fed goats and

20 organically fed goats over a period of 2 months. A threshold value dependent on the hippuric acid content was thus established for the first time with a view to discriminating between conventional and organic goat's milk. Organic acids in goat's milk were separated in a running buffer consisting of 120 mM sodium tetraborate decahydrate and 0.5 mM cetyltrimethylammonium (CTAB) set to pH 8. The precision of the ensuing method is acceptable for hippuric acid; thus, the relative standard deviation for peak area and migration time was less than 10 % and 4 %, respectively. Also, calibration curves were linear throughout the studied concentration range.

Keywords: Capillary electrophoresis, classification, goat's milk, organic milk.

1. Introduction

The market for organic products is currently expanding thanks to the growing interest of consumers in their potential health benefits. Despite their wide popularity, however, the price of organic products usually surpasses that of similar, conventional products. In part, the difference in price is due to the increased production costs of organic products, which is a result of them being obtained with more environmentally friendly procedures. Surprisingly, the strong interest in organic products has not led to the development of effective, officially endorsed methods for distinguishing them from conventional substitutes in order to prevent fraudulent labelling. In this work, we sought to identify chemical markers allowing one to discriminate in a simple manner between milk from goats fed with organic products (fresh grass or silage) and milk from goats receiving commercial feed.

At present, organic and conventional cow milk could be distinguished by using gas (GC) or liquid chromatography (HPLC) to determine some specific compounds [1-4]. Thus, the carbon stable isotope and the linolenic acid content of milk fat were used to examine their usefulness as general markers for authenticating organic retail milk in Germany [1]. In other studies, organic and conventional milk samples were analyzed for fatty acids by GC [2-4] and the former found to contain a higher proportion of polyunsaturated fatty acids.

In recent years, capillary electrophoresis (CE) has found increasing use in agriculture and food laboratories. In this work, we assessed its potential for fulfilling the above-described objective. Specifically, we

investigated six different chemical compounds that are in an organic acid mixture as potential markers for distinguishing the two types of caprine milk.

Goat's milk is especially interesting by virtue of its peculiar composition, which has led to its appreciation as a high quality product for infants and the elderly, as well as for some population segments with special needs [5-8]. Some studies have shown that goat's milk protein is more digestible [5-9] and also more easily tolerated than cow's milk by humans [10-12]. Similarly, goat's milk fat is more digestible than cow milk [6, 13] and provides an excellent source of energy for use in various metabolic processes [5, 12] and even to combat metabolic diseases [14-16]. Based on mineral composition, goat's milk and its derivatives possess a higher nutritional quality than do cow milk and dairy products [5, 6, 17, 18]. Milk is a heterogeneous natural product consisting of major and minor components including minerals, vitamins, proteins, lipids and carbohydrates [19-21]. Its minor family components include organic acids, which can form by hydrolysis of milk fat. The organic acid content of milk depend on a variety of factors such as animal breed, diet and duration of the lactation period [22]. Orotic acid is especially important as it is a precursor of uracil and cytosine in the biosynthesis of pyrimidine nucleotides, which are in turn precursors of DNA and RNA [22]. Other organic acids are present in varying quantities and reflect the health condition of the animals yielding the milk. In mammals, organic acids are filtered directly from blood into milk. Capillary electrophoresis has only been used for the qualitative detection of minor organic species in whey from cow´s and human milk [22].

In this work, we assessed various organic acids (oxalic, citric, orotic, benzoic, uric and hippuric) as potential markers for distinguishing organic and conventional milk. Some organic acids (citric acid, formic acid, lactic acid, tartaric acid; amongst others) can be determined in dairy products (cow milk, cheese, liquid yogurt and fruit juice) and biological fluids (serum and urine), in a direct manner (*i.e.* without preconcentration) by CE [23-26]. However, in this case, only hippuric acid could be directly identified in the goat's milk samples studied. Other organic acids were also identified, using the standard addition method, but their spectral failed to match exactly those for the pure species in standards. Therefore, only hippuric acid was examined as a potential marker.

The primary purpose of this work was thus to confirm whether CE could be effective with a view to discriminating between organic and conventional goat's milk. The distinction was based on differences in hippuric acid content between milk from goats fed on fresh grass or silage and goats given any type of commercial feed.

2. Materials and methods

2.1. Reagents and milk samples

The organic acids used included oxalic, citric, orotic, benzoic, uric and hippuric were all supplied by Sigma (St. Louis, MO, USA). A stock standard solution containing a 1000 mg/L concentration of each acid was prepared in purified water from which working-strength standards were obtained by appropriate dilution with purified water. Sodium tetraborate decahydrate was

obtained from Sigma and CTAB from Fluka (Buchs, Switzerland). All water used was purified by passing it through a Milli-Q apparatus from Millipore (Bedford, MA, USA).

A total of 40 milk samples were obtained from goats of the *Payoya* breed grown in *Sierra de Cádiz (Andalusia, Spain)*. The samples were obtained from organically and conventionally fed goats in order to examine the effect of their diet. In each case, 10 milk samples were collected in February 2009 and another 10 samples were collected in April 2009. The fodder composition is shown in Table 1.

Table 1. Composition of the fodder given to the goats.

Goat type	Fodder type
Organic	0.5 kg/day organic mixture (ECORSEVILLA) 12:00–18:00 h: grazing in a fallow meadow Rest of day: grazing in bush
Conventional	1 kg/day corn, oats, beans 2–3 h grazing in an oat field Rest of day: grazing in a dehesa

Milk samples were collected in sterile 30 mL plastic screw-top containers and farm production figures were obtained by interviewing the farmers. The samples were placed on ice to transfer them to the laboratory and frozen at –18 ºC until analysis.

2.2. Apparatus and operating conditions

Following the measurement of their pH with a MicropH 2000 pH meter from Crison, the samples were centrifuged in a High Speed Centrifuge with Microprocessor Control from J.P. Selecta, S.A. (Abrera, Barcelona, Spain).

Analytes were separated and quantified on a P/ACE MDQ Capillary Electrophoresis System from Beckman (Palo Alto, CA, USA) equipped with a diode array detector and using a fused-silica capillary (Beckman Coulter) of 75 μm inner diameter, 60.2 cm total length and 50 cm effective separation length.

The CE operating conditions were similar to those previously used by Buiarelli *et al.* [22] but re-optimized for our particular purpose. The running buffer consisted of 120 mM sodium tetraborate decahydrate and 0.5 mM CTAB adjusted to pH 8 with hydrochloric acid. The applied voltage was 10 kV, the average current 150 μA and the working temperature 25 ºC. Samples were injected in the hydrodynamic mode at 0.5 psi for 5 s. Electropherograms were recorded at 200 nm, using reversed polarity. All buffer solutions and milk whey samples were filtered through a Nylon membrane of 0.45 μm pore size before analysis.

Prior to first use, the capillary was conditioned by rinsing with 1 M HCl for 5 min, 0.1 M NaOH for 10 min and water for 5 min using a pressure of 20 psi in all cases. The capillary was prepared for daily use by rinsing with 0.1 M NaOH for 5 min, water for 5 min and separation buffer for 15 min, with a pressure of 20 psi.

Between individual analyzes, the capillary was cleaned with water for 1 min, 0.1 M NaOH for 2 min, water for 1 min and separation buffer for 2 min prior to injection. An additional rinse cycle was included as a blank analysis after each series of 6 injections.

2.3. Sample preparation

The procedure followed to extract the mixture of organic acids that are in the goat's milk samples proposed by Buiarelli *et al.* [22] was re-optimized to achieve our objectives. A volume of 9 mL of goat's milk was deproteinated (*i.e.* its casein precipitated) by adding 2 M HCl to lower the pH to 3.4–3.6. The extract thus obtained was defatted by centrifugation at 6000 rpm for 20 min. The whey was then separated and additional centrifugation at 6000 rpm for 10 min used to remove residual caseins and fat. Finally, the whey was filtered with a 0.45 μm pore size filter to remove any particles that might block the capillary, and was ready for injection.

2.4. Statistical analysis

The statistics software package SPSS 14.0. (SPSS Inc. Chicago, Illinois) was used for statistical analysis. A Kolmogorov-Smirnov test was used to examine the normal distribution of variables and a one-way analysis of variance was used to analyze the effect of the feeding system and seasonal sampling on hippuric acid concentration. The comparison between the samples was carried out using Tukey's HSD multiple comparisons test for a confidence interval of 99 %.

Milk samples were classified by using principal component analysis (PCA) implemented in SPSS 14.0. PCA is a powerful visualization tool for data evaluation; it allows the graphic representation of relationships between samples and between variables, and provides a way to reduce dimensionality in the data.

3. Results and discussion

As noted earlier, the primary aim of this work was to assess the potential of CE as classification technique for discriminating organic and conventional goat's milk. The ability to distinguish goats by the diet (organic or conventional) that they have been given is very important, especially since organic products have become so popular. In this work, we sought to identify one or several potential markers enabling the discrimination of milk from organically and conventionally fed goats in a mixture of organic acids specifically chosen for this purpose. The acids in question were selected on the grounds of their amenability to direct determination by CE with ultraviolet UV detection. We should note that effective separation and identification of all organic acids that are in goat's milk is impossible unless appropriate steps are taken to preconcentrate the analytes and remove some interferences.

The experimental procedure used involved (*a*) adapting an existing CE method for separating organic acids to the target matrices (organic and conventional goat's milk); (*b*) optimizing a clean-up procedure for extracting organic acids from such matrices; (*c*) analysing all samples by CE; and (*d*) processing the CE data.

3.1. Optimization of instrumental and electrophoretic variables

The background electrolyte (BGE) used to determine the target organic acids by CE was selected from the literature [22]. The influence of the borate concentration used on the separation efficiency was studied over

the range 100–140 mM. Peak resolution was poor at 100 mM but improved for all organic acids above 120 mM, which was therefore adopted for further testing as it resulted in optimal peak separation. The effect of the CTAB concentration was studied at 0.25, 0.5 and 0.75 mM. At the lowest CTAB level, benzoic and uric acids comigrated and virtually all other acids were unaffected. Also, the results obtained at 0.5 and 0.75 mM were virtually identical. A CTAB concentration of 0.5 mM was therefore chosen. The effect of the buffer pH was examined from 6.5 to 9, in 0.5 increments. pH 8 ensured efficient separation of all acids.

The UV absorption spectra for organic acids typically exhibit several fairly strong, sharp absorption maxima (their maximum absorption wavelengths are: for oxalic acid 193 nm; for citric acid 190 nm; for orotic acid 207 nm and 278 nm; for uric acid 200 nm, 233 nm and 291 nm; for benzoic acid 193 nm and 222 nm; and for hippuric acid 196 nm and 225 nm). Measurements were made at 200 nm, where the organic acids were found to absorb maximally. The peaks for the six selected organic acids in the electropherograms for the goat's milk samples were identified by comparing their spectra and migration times with those for standard solutions of each compound.

3.2. Sample clean-up

A volume of 9 mL of milk was used to obtain a large enough amount of whey extract for CE analysis. Proteins (casein, mainly) present in the samples were removed by adding 2 M HCl to pH 3.4–3.6. Centrifugation allowed two phases to be separated and a clear extract obtained. After

centrifugation, the whey was ultrafiltered on an Amicon-10 Microfilter (Millipore, Milford, MA, USA) to cut off soluble proteins in order to avoid potential interferences with the determination of the target analytes (organic acids). As can be seen from Figure 1, however, using the microfilter did not improve identification; in fact, the microfilter failed to efficiently retain interferents that are in the goat's milk sample matrix and was hence useless to preconcentrate the organic acids to a significant extent.

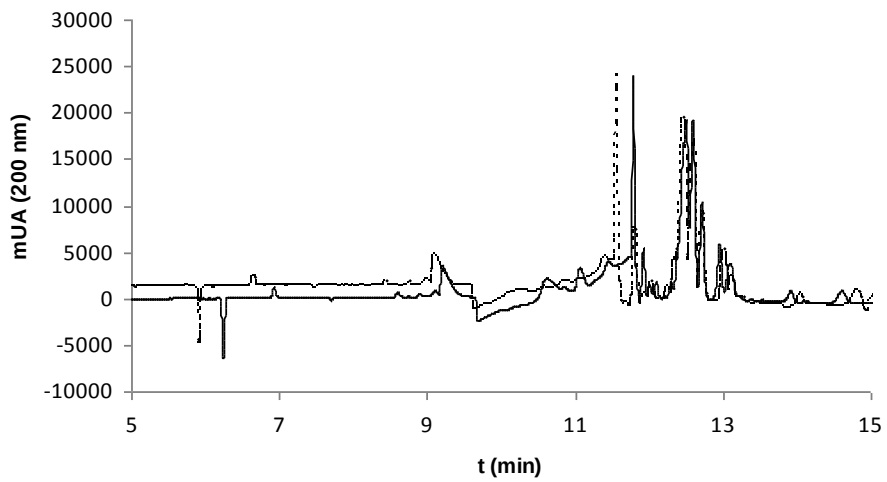

Figure 1. Electropherograms for whey before (solid line) and after ultrafiltration on an Amicon-10 microfilter (dashed line). Buffer consisting of 120 mM sodium tetraborate decahydrate, 0.05 mM CTAB, pH 8; voltage applied 10 kV; UV detection at 200 nm.

Therefore, the whey was instead passed through filters of 0.45-μm pore size to remove any particles that might clog the capillary. Additional clean-up procedures (solid phase extraction included) will be tested in future work with a view to removing interferences with the identification of other organic acids.

3.3. Analytical features of the CE system

The electrophoretic method was validated directly with standard solutions of the target organic acids. A standard calibration curve was constructed by injecting solutions containing the mixture of organic acids at variable concentrations up to 250 mg L^{-1} into the electrophoretic system. The detector response was linear over the concentration range 0.5–100 mg L^{-1} for all acids. Based on the corresponding regression equations and coefficients (see Table 2), oxalic acid, benzoic acid and hippuric acid exhibited a good linear response (R > 0.99) throughout their respective concentration ranges.

Table 2. Calibration curves and statistical figures of merit of the determination of a mixture of organic acids standard.

Organic acid	y= mx + b		Sy/x	r
	m	b		
Oxalic acid	400 ± 6	-135 ± 295	1081	0.9972
Citric acid	206 ± 10	555 ± 572	1477	0.9798
Orotic acid	2161 ± 52	-3703 ± 2904	7975	0.9946
Benzoic acid	6109 ± 154	-10912 ± 5988	21090	0.9931
Uric acid	3203 ± 104	-5015 ± 5160	18900	0.9871
Hippuric acid	5304 ± 102	-6625 ± 5043	18473	0.9954

The applicability of the proposed CE system was assessed in terms of precision as determined by using standard solutions of the analytes and goat's milk samples.

(a) *Standard solutions of the analytes.* Repeatability (as % relative standard deviation RSD) in peak area and in migration time was calculated by injecting organic acids standards at two different

concentration levels (n = 9). In almost all cases (see Table 3) the values of repeatability at 10 mg L^{-1} were worse than those at higher concentration (100 mg L^{-1}).

Table 3. Repeatability and reproducibility of the mixture of the six organic acids (n=9).

Organic acid	Repeatability[1] RSD (%)		Reproducibility[1] RSD (%)		Repeatability[2] RSD (%)		Reproducibility[2] RSD (%)	
	peak area	migration time	peak area	migration time	peak area	migration time	peak area	migration time
Oxalic acid	9.77	0.30	3.14	3.18	8.49	0.37	4.72	1.54
Citric acid	7.70	0.18	1.60	3.33	22.70	0.19	10.21	2.16
Orotic acid	9.07	0.20	5.49	3.93	10.80	0.21	19.24	2.62
Benzoic cid	11.14	0.20	4.51	4.08	15.74	0.21	2.97	2.64
Uric acid	5.04	0.27	9.38	3.74	9.31	0.24	3.55	2.56
Hippuric acid	6.64	0.20	2.86	4.13	9.27	0.21	2.28	1.19

[1]Results of a mixture of organic acids at 100 mg L^{-1}. [2]Results of a mixture of organic acids at 10 mg L^{-1}.

This may be attributed to the low signal obtained with this CE method at low concentrations and therefore the poor integration of the signals (especially the case of citric acid and benzoic acid). For hippuric acid, RSD for peak area was 9.3 % at 10 mg L^{-1} and slightly lower (6.6 %) at 100 mg L^{-1}; and less than 0.2 % for migration time at both concentration levels.

Reproducibility (as % RSD) in peak area and migration time was calculated by injecting organic acids standards during three consecutive days, preparing a different solution each day (n = 3). Overall reproducibility values were better than repeatability. For hippuric acid, RSD for peak area was lower than 3 % at two different concentration levels (10 mg L^{-1} and 100 mg L^{-1}).This fact could be

attributed to lack of effectiveness of the rinsing cycle of the capillary after a certain number of analysis. Special care was exercised with regards to cleaning the capillary. This led us to perform a blank analysis after each 6 standards or whey sample injections.

(b) *Goat's milk samples.* Repeatability was also assessed by injecting the extract obtained with clean-up of a goat's milk sample (n = 9). RSD was 5.3 % for hippuric acid peak area and less than 1.7 % for migration time. Therefore, the precision of the method is not affected by the presence of other compounds in goat's milk provided analyzes are conducted on whey.

Application of the whole extraction sequence to three different aliquots (n = 3) from the same goat's milk sample allowed the repeatability of the extraction process to be calculated. RSD was 4 % for hippuric acid peak area and less than 1.5 % for migration time. Additionally, these values testify the outstanding precision of the analytical procedure, which involves extraction of the analytes from the milk and their determination by CE.

In some cases, the capillary stopped working properly —migration times varied and measurements were irreproducible— after use with a large number of whey samples (approximately 170 analysis). This signalled the need to replace it with a new one.

3.4. Analysis of goat's milk samples

Because the dietary intake of organic goats is usually subject to seasonal variations, the present study was conducted on animals during

different seasons. A total of 40 animals from two different dairy farms were studied. One herd consisted of 20 goats given conventional feed and the other of 20 organically fed goats. Variability between animals was minimized by selecting two farms with goats of the same race, age, lactation regime and sexual development. Also, the farms were in similar locations, and comparable in size and mode of operation. Both were run by a co-operative which requires its farmers to follow identical procedures with regards to feeding, milk collection and veterinary controls, amongst others.

Qualitative analyzes were based on the diode array detector (DAD) spectra and also on the results for milk samples spiked with standards of the pure organic acids. Figure 2 shows the electropherograms obtained by applying the proposed extraction–CE method to a goat's milk sample before and after spiking it with a 50 mg L^{-1} concentration of a standard mixture of the acids.

In the absence of clean-up, the whey sample only allowed hippuric acid to be directly identified. In fact, the peak purity for this analyte exceeded 0.998, whereas those for the other organic acids were inadequate for direct detection in the whey. Full identification of the organic acids would therefore require additional sample treatment (*e.g.* solid phase extraction) and detract from the expeditiousness with which hippuric acid can be determined in order to discriminate between organic and conventional goat's milk. Therefore, the proposed analytical method is more economical and cost-effective than any other alternative method requiring the determination of additional organic acids as potential markers.

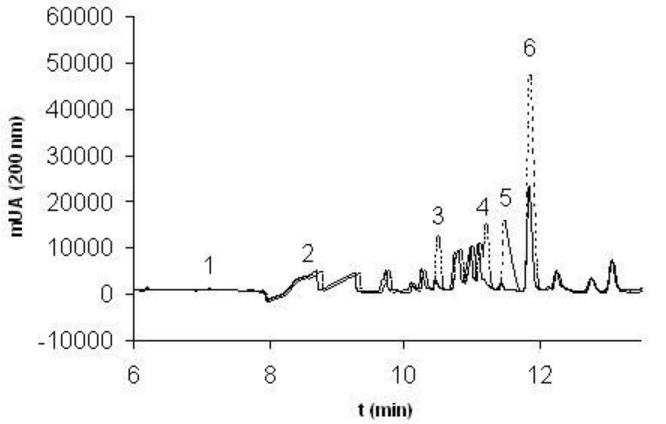

Figure 2. Electropherograms for whey before (solid line) and after spiking with a 50 mg L^{-1} concentration of a standard mixture of organic acids (1 oxalic, 2 citric, 3 orotic, 4 uric, 5 benzoic, 6 hippuric) (dashed line). Buffer consisting of 120 mM sodium tetraborate decahydrate, 0.05 mM CTAB, pH 8; voltage applied 10 kV; UV detection at 200 nm.

The proposed CE method was validated by using the standard addition method. The recoveries obtained from three different goat's milk samples spiked with 20, 40 and 60 mg L^{-1} concentrations of hippuric acid respectively ranged from 88, 78 and 72 %. Based on these results, the proposed extraction method is effective towards the determination of hippuric acid in such a complex matrix as goat's milk to achieve the objective of this work. Although a revision on the extraction method should be carried out if higher recoveries are needed.

The method was used to analyze 20 samples of organic goat's milk and another 20 of conventional goat's milk in terms of hippuric acid, the potential marker adopted to distinguish between the two types of milk. Based on the data in Table 4, the concentration of hippuric acid in organic milk exceeds that in conventional milk (*i.e.* milk from goats fed basically with commercial compound feed (corn, oats, beans etc.)).

Table 4. Hippuric acid concentrations found in 20 organic goat samples and 20 conventional organic samples.

Sample type	Sampling period	Range of hippuric acid concentration (mg L^{-1})	$\overline{X}$ ± SE (mg L^{-1})	
Organic	February 2009	72.59-167.82	128 ± 10^{a}	132 ± 7^{a}
	April 2009	93.35-188. 96	137 ± 10^{a}	
Conventional	February 2009	17.75-66.59	33 ± 5^{b}	59 ± 7^{b}
	April 2009	53.83-125.84	83 ± 7^{c}	

$\overline{X}$ ± SE: mean ± statistic error. Mean values with the same letters (a,b,c) indicate homogeneous subsets for α = 0.001 according to Tukey's HSD test.

Statistical differences were found between samples from organic and conventional feed (see values with different superscript in Table 4). Differences between conventional goat's milk from February and April could be explained by variations in the commercial feed composition, bearing in mind that this is usually formulated looking for a lower cost.

The hippuric acid concentration present in goat's milk may in fact depend on various factors including breed, season, geographic location, access to fresh pasture, silage type, cereal feeding and oil supplementation of feed. Goats (feeding under an organic diet) have browsing habits and their diet is based on grazing different fodder-groups (grass, shrubs, trees…) over the year, and their browsing depends on the vegetative status of the plants, preferring shoots [27]. Vegetative activity increases the DNA level [28]; as a result of this, we propose the following hypothesis: the fodder material that is in shoots increases the DNA content in an organic diet, producing a higher level of puric bases in rumen. Some of these bases might be absorbed and metabolized producing hippuric acid [29].

We collected forage samples as well as milk samples in order to consider the potential effect of the goats' dietary intake in interpreting the results, which are in the course of being processed.

In the first part of this project, seasonal variations of the hippuric acid concentration in organic milk samples were studied systematically over only two seasons (winter and spring). Some preliminary data for the summer were also obtained (results not shown) that followed the same trend (*i.e.* the content in hippuric acid was higher in organic milk than in conventional milk).

The present research was planned as a pilot study aimed at identifying in future work a characteristic fingerprint enabling the discrimination of organic and conventional goat's milk from Spain. A characteristic fingerprint may be more useful than an individual marker with a view to detecting the reformulation of feed to increase the amount of hippuric acid given to goats in order to have their produce pass as organic milk. Some steps have already been taken in this direction. Thus, the information contained in the electropherograms for the goat's milk samples was used to examine the influence of other, unidentified compounds on class membership. A comparison of the average electropherograms obtained for each group of samples (organic and conventional whey) revealed that the profiles were quite similar (see Figure 3).

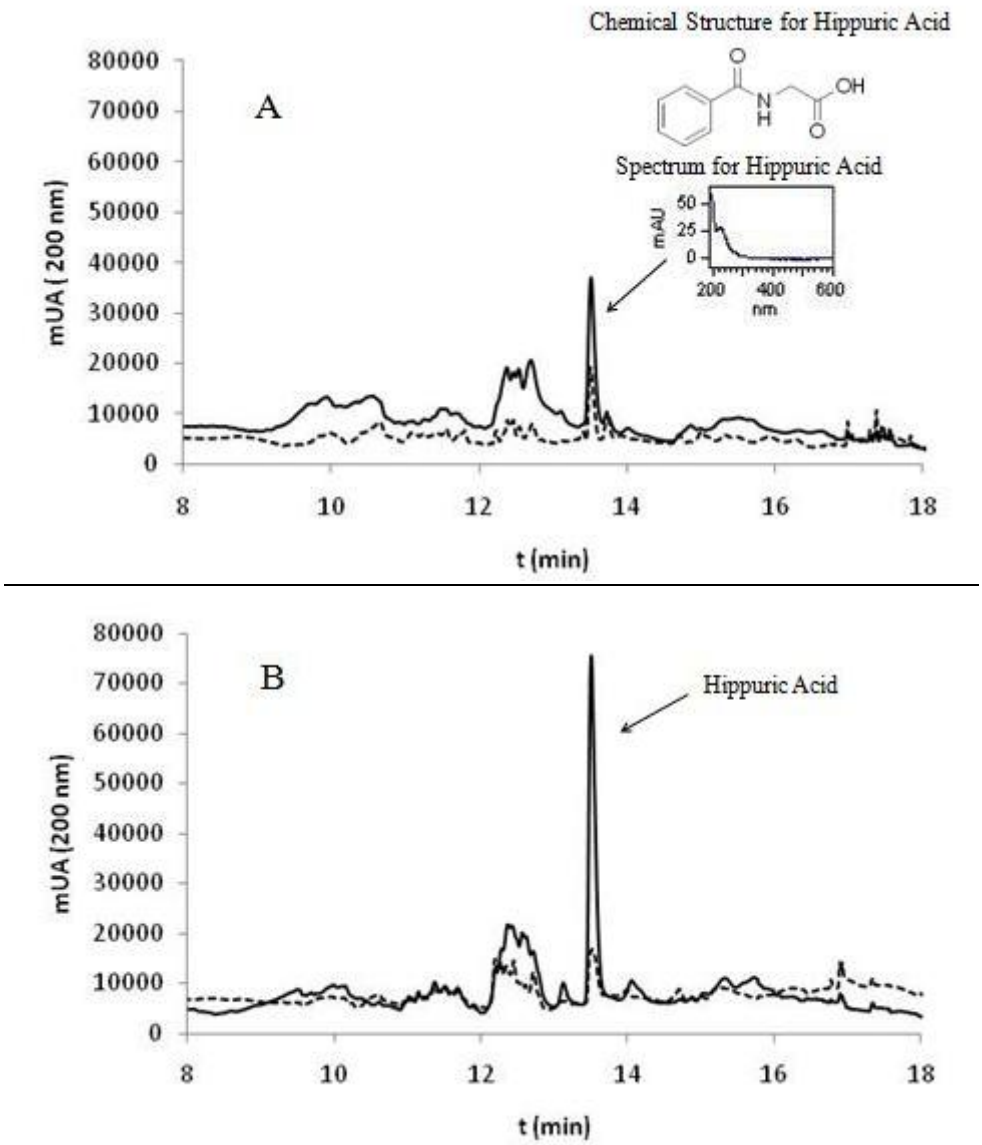

Figure 3. (A) Average electropherograms obtained from 20 individual recordings for conventional (A) and organic goat's milk samples (B). Buffer consisting of 120 mM sodium tetraborate decahydrate, 0.05 mM CTAB, pH 8; voltage applied 10 kV; UV detection at 200 nm. Solid line = average. Dashed line = standard deviation.

The greatest difference was only in the peak area for hippuric acid, which was higher in organic milk. As it has been mentioned before, this led us to select it as a potential marker for discriminating organic and conventional milk. Figure 3 also shows the spectrum and chemical structure for hippuric acid in milk, whereby this organic acid was identified.

A preliminary statistical study using principal component analysis was carried out with the information gathered over the elution time range used to identify the organic acids (viz. minutes 9 to 15). This can be seen from Figure 4, the two types of samples exhibit a slight separation.

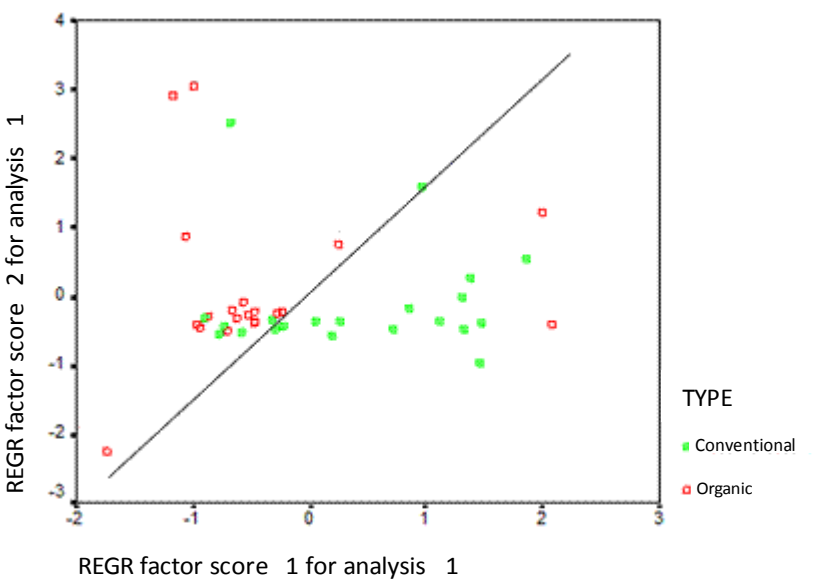

Figure 4. Principal Component Analysis (PCA) conducted by using the time interval between 9 and 15 min from the electropherograms from organic and conventional goat's milk samples.

Therefore, other compounds eluting in the same time range as the organic acids appear to contribute to the overall separation results. As can

also be seen from the Figure, however, 6 conventional goat's milk samples were classified in the organic group. We are planning to increase the number of samples from the same area to be studied and compare them with samples from goats of other races and origin. Also, electrophoretic results will be subject to a deeper statistical analysis. Finally, the potential of using hippuric acid as an effective marker for organic goat's milk and its combination with the information provided by the whole electropherogram will be examined.

4. Concluding remarks

The proposed electrophoretic method is able to detect and quantify the hippuric acid present in goat's milk. To our knowledge, this is the first time hippuric acid in such a complex matrix as goat's milk has been quantified with a rapid, simple electrophoretic method. The preliminary results shown in this manuscript led us to think that by using hippuric acid as marker, we could distinguish between milk from organically and conventionally fed goats. To support this hypothesis new experiments increasing the number of milk samples from different goats are being carried out.

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The authors have declared not conflict of interest.

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Capítulo 3.2

Evaluation of hippuric acid content in goat milk as a marker of feeding regime

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EVALUATION OF HIPPURIC ACID CONTENT IN GOAT MILK AS A MARKER OF FEEDING REGIMEN

A. Carpio,* D. Bonilla-Valverde,† C. Arce,‡ V. Rodríguez-Estévez,‡1 M. Sánchez-Rodríguez,‡ L. Arce,* and M. Valcárcel*

**Department of Analytical Chemistry, Institute of Fine Chemistry and Nanochemistry, University of Córdoba, Annex C3 Building, Campus of Rabanales, e-14071 Córdoba, Spain*
†Centro de Investigación y Calidad Agroalimentaria de Los Pedroches (CICAP) Research, Apdo. Correos 105, Ctra. de la Canaleja s/n, 14400 Pozoblanco, Córdoba, Spain
‡Department of Animal Production, University of Córdoba, Campus of Rabanales, e-14071 Córdoba, Spain

Organic producers, traders, and consumers must address 2 issues related to milk: authentication of the production system and nutritional differentiation. The presence of hippuric acid (HA) in goat milk samples has been proposed as a possible marker to differentiate the feeding regimen of goats. The objective of this work is to check the hypothesis that HA could be

a marker for the type of feeding regimen of goats by studying the influence of production system (conventional or organic) and feeding regimen (with or without grazing fodder). With this purpose, commercial cow and goat milk samples (n = 27) and raw goat milk samples (n =185; collected from different breeds, localizations, and dates) were analyzed. Samples were grouped according to breed, feeding regimen, production system, and origin to compare HA content by ANOVA and honestly significant difference Tukey test at a confidence level of ≥95 %. Hippuric acid content was obtained by analyzing milk samples with capillary electrophoresis. This method was validated by analyzing part of the samples with HPLC as a reference technique. Sixty-nine raw goat milk samples (of the total 158 samples analyzed in this work) were quantified by capillary electrophoresis. In these samples, the lowest average content for HA was 7 ± 3 mg/L. This value corresponds to a group of conventional raw milk samples from goats fed with compound feed. The highest value of this group was 28 ± 10 mg/L, corresponding to goats fed compound feed plus grass. Conversely, for organic raw goat milk samples, the highest concentration was 67 ± 14 mg/L, which corresponds to goats fed grass. By contrast, the lowest value of this organic group was 26 ± 10 mg/L, which belongs to goats fed organic compounds. Notice that the highest HA average content was found in samples from grazing animals corresponding to the organic group. This result suggests that HA is a good marker to determine the type of goats feeding regimen; a high content of HA represents a diet based mainly or exclusively on eating green grass (grazing), independently of the production system. Hence, this marker would not be useful for the actual organic policies to distinguish organic milk under the current regulations, because

organic dairy ruminants can be fed with organic compound feed and conserved fodder without grazing at all.

Keywords: Authentication, organic farming, Capillary Electrophoresis, High Performance Liquid Chromatography.

1. Introduction

In recent decades, debate has been growing about the ethical aspects of production and trade. The International Federation of Organic Agriculture Movements defines organic livestock production as a system based on the harmonious relationship between land, plants and livestock, respect to the physiological and behavioral needs of livestock, and using organically grown foodstuffs or natural resources as fodders (IFOAM, 2002). Consumers associate organic farming with grazing animals (fodder diet, freedom, and welfare); however, the standards for organic livestock farming detailed by the organic farming regulations of the European Community (Council of the European Union, 2007) allow an intensive open air production, without grazing and feeding animals with organic compound feeds adding green or conserved fodder (e.g., silage, hay, straw, and so on). Producers, traders, and consumers of organic food regularly use the concept of the natural (naturalness) to characterize organic farming and organic food, in contrast to the unnaturalness of conventional farming. Critics sometimes argue that such use lacks any rational (scientific) basis and only refers to sentiment (Verhoog et al., 2003).

On the basis of a systematic review from a total of 52,471 articles about nutritional quality of foods, Dangour et al. (2009) identified 162 studies (137 crop and 25 livestock products) dealing with organic food; only 55 were of satisfactory or higher quality. Those authors established that the analysis of the very limited database on livestock products found no evidence of a difference in nutrient content between organically and conventionally produced livestock products. Hence, organic producers,

retailers, and consumers must address 2 issues related to food: authentication of the production methods and nutritional differentiation.

In the case of organic milk, different techniques have been used for nutritional differentiation. Many authors have compared the composition and content of FA between organic and conventional milk (Ellis et al., 2006; Collomb et al., 2008; Molkentin, 2009, Molkentin and Giesemann, 2010). Ellis et al. (2006) found that organic milk had a higher proportion of PUFA and n-3 FA, and this effect of production system remained significant even after accounting for some potentially confounding management and nutritional factors in the analyses. Collomb et al. (2008) also found a higher content in PUFA, conjugated linoleic acids, and branched FA in organic milk with significantly higher levels of grasses and lower levels of concentrates in the fodder of organic farming.

Other authors have also carried out different comparisons in the composition and content of different analytes between organic and conventional milk samples to find suitable markers to certify the milk production system and avoid fraud. Molkentin (2009) studied the influence of the production system (organic or conventional) and the season by measuring the carbon-stable isotope ratio (δ13C) using isotope-ratio mass spectrometry, and the content of α-linoleic acid in milk using gas chromatography. These parameters were selected because both of them are measured in the easily accessible milk fat. Molkentin (2009) concluded that δ13C and α-linoleic acid are not good markers to discriminate between organic and conventional milk; one of the main reasons being the seasonal variation showed by both components. They also investigated their applicability as markers for authentication of organic milk in Germany, due

to its higher content in organic milk. However, they did not consider the feeding regimen of animals (only the production system).

Furthermore, Molkentin and Giesemann (2010) concluded that analyses involving the combination of threshold values for δ13C, δ15N, or C18:3n-3 content in milk components can improve the authentication of organic milk. Thus, multivariable analyses can increase robustness and reduce the number of exceptions in organic milk authentication.

After the determination of a mixture of organic acids (oxalic, citric, orotic, benzoic, uric, and hippuric acids) in milk samples, Carpio et al. (2010) found that only hippuric acid (HA) could be a marker to distinguish milk from goats fed on different production system. However, these authors concluded that it is necessary to check if the HA content comes from grazing fodder or from organic handling.

This initial hypothesis, highlighted by Carpio et al. (2010), is supported by the conclusions summarized by other authors. Some of the first articles that mentioned the natural presence of HA in cow milk were those presented by Karabinos and Dittiner (1943), Patton (1953), and Svensen (1974). According to Sieber et al. (1995), HA concentration of cow milk may be up to 50 mg/kg, although Patton (1953) found that HA concentration ranged from 31 to 64 mg/L in skim milk. Svensen (1974) observed a higher amount of HA in milk from grazing than from indoor forage feeding. Also, Besle et al. (2010) found a higher content of HA in milk from cows with a diet based on grazing grassland pasture in comparison to those with a diet based on different diets of concentrate and silage or hay forages. Besle et al. (2010) related milk HA to the presence of chlorogenic acid, neochlorogenic acid, or caffeoyl compounds in the diets based on

grassland pasture (per Gonthier et al., 2003). Forages contain large amounts of aromatic compounds both in the insoluble cell wall and in the cellular content in the form of water and ethanol-soluble polyphenols specific to each plant taxon. These aromatic compounds, including HA, are partially degraded in the rumen and partly absorbed in the rumen and intestinal mucosa, following an immediate conjugation before they are transformed in the liver and excreted in urine or milk (Gatley and Sherratt, 1977; Scheline, 1991). Hence, an important factor in the variation of content of aromatic compounds in milk samples could be the feeding regimen and the fodder quality that goats have consumed, which are seasonally influenced by climatic conditions of the area and the month of sample collection (Peinado-Lucena et al., 1992).

The aim of the present work was, first, to check the hypothesis proposed by Carpio et al. (2010), that the evaluation of HA in goat milk samples is a possible marker to differentiate the type of feeding regimen supplied to goats; second, to validate capillary electrophoresis (CE) to determine HA content by a reference technique, such as HPLC; and third, elucidate if the differences in HA content between organic and conventional milk are due to feeding regimen more than production system (organic or conventional).

2. Material and methods

2.1. Samples, chemicals and standards

Goat milk samples analyzed in this work are individual raw milk from farms and commercial milk from markets, with a total of 162 organic and conventional goat milk samples. Most of these were raw goat milk samples (n = 158). As only 4 different commercial goat milk brands were found in the Spanish and English markets (only 1 of them being organic), it was decided to include commercial cow milk samples to increase the size of this group. For this reason, 23 samples of commercial cow milk (organic and conventional) were also analyzed (see Table 1).

Table 1. Total commercial milk samples analysed.

Production system	Animal	Breed	Origin	Number of samples
Conventional	Cow	Holstein Frisian	Spain	11
	Cow	British Frisian	United Kingdom	3
	Goat	Murciano-Granadina	Spain	1
	Goat	Saanen & Alpine	United Kingdom	2
Organic	Cow	Holstein Frisian	Spain	4
	Cow	British Frisian	United Kingdom	5
	Goat	Murciano-Granadina	Spain	1

All commercial conventional cow milk samples were whole and UHT, and all commercial conventional goat milk samples were semiskimmed by UHT or pasteurization processes. To obtain the highest number of commercial organic milk samples, all the samples found in the market were

included in this study. Raw goat milk samples were grouped into 9 groups according to breed, feeding regimen, production system, and origin (zone); each group corresponds to a different farm (see Table 2).

Table 2. Total raw milk samples analysed.

Production system	Feeding regime	Animal	Breed	Origin	Sampling month	Number of samples	Group
Conventional	Feed	Goat	Florida	Córdoba (Spain)	December 2010	11	1
	Feed	Goat	Murciano-Granadina	Sevilla (Spain)	December 2010	11	2
	Feed+ Grazing	Goat	Serrana	Badajoz (Spain)	December 2010	11	3
	Feed	Goat	Florida	Badajoz (Spain)	February 2011	10	4
Organic	Grazing	Goat	Payoya*	Cádiz (Spain)	*	61	5
	Feed	Goat	Murciano-Granadina	Murcia (Spain)	January 2011	14	6
	Grazing	Goat	Serrana	Huelva (Spain)	June-July 2011	20	7
	Feed	Goat	Serrana	Córdoba (Spain)	April 2012	10	8
	Grazing	Goat	Serrana	Badajoz (Spain)	April 2012	10	9

*Samples were collected in May, June and July 2010, January 2011, and April 2012

Raw milk samples were collected in situ into sterile 30-mL plastic screw-top containers and farm production information (production system, feeding regimen, and diet) was obtained by an interview with the farmer. Note that each sample was from 1 individual goat, not from a tank. Samples were transported cold to the laboratory, where all samples were frozen at −18°C until their analysis. Commercial milk samples were analyzed immediately after opening.

Hippuric acid (CAS Number 495-69-2) with a purity of 98 % was supplied by Sigma (St. Louis, MO). A stock standard solution containing a

1,000 mg/L of HA was prepared in purified water. Working standard solutions were prepared by diluting the stock standard solution with purified water. Sodium tetraborate decahydrate was obtained from Sigma and cetyl trimethyl ammonium bromide from Fluka (Buchs, Switzerland). All water used was purified by passing through a Milli-Q apparatus from Millipore (Bedford, MA).

2.2. Sample preparation

Commercial milk samples were bought pasteurized. This treatment may produce losses of compounds that form part of the milk (including HA). To study the stability of HA in the pasteurization process, a cow raw milk sample and another from goat were taken from milk companies tanks. These milk samples came from different animals fed in conventional farms. The study was carried out analyzing 2 aliquots of each cow and goat milk samples before pasteurizing it, and 2 aliquots after the pasteurization process (3 replicates from each aliquot were analyzed).

The steps followed to extract a mixture of organic acids present in milk samples were a reoptimization of the process proposed by Buiarelli et al. (2003); these are shown in detail in a previous work (Carpio et al., 2010). In summary, a volume of 9 mL of milk was deproteinated by a simple addition of 2 M HCl to lower the pH to 3.4 to 3.6. Then, the extract was defatted and the residual proteins were eliminated by centrifugation. Finally, the separated whey was filtered through 0.45-μm filters (Terumo, Leuven, Belgium) to remove any particle that might block the capillary or the column,

and the sample was ready for injection. All the samples were analyzed 3 times to obtain an average value of each sample.

2.3. Apparatus and operating conditions

The pH of the samples was adjusted with a MicropH 2000 from Crison (Barcelona, Spain). Samples were centrifuged with a high-speed centrifuge with microprocessor control from J.P.Selecta S.A. (Abrera, Barcelona, Spain). Analytes were separated and quantified on a P/ ACE MDQ capillary electrophoresis system instrument from Beckman (Palo Alto, CA) equipped with a diode array detector and using a fused-silica capillary (Beckman Coulter) of 75 μm i.d., a total length of 60.2 cm, and an effective separation length of 50 cm.

Capillary electrophoresis operating conditions were similar to those previously used by Buiarelli et al. (2003), but reoptimized for this particular purpose (Carpio et al., 2010). The running buffer consisted of 120 mM sodium tetraborate decahydrate and 0.5 mM cetyl trimethyl ammonium bromide adjusted to pH 8 with hydrochloric acid. The applied voltage was 10 kV, the average current was 150 μA, and the working temperature was 25°C. Samples were injected in the hydrodynamic mode at 0.5 psi for 5 s. Electropherograms were recorded at 200 nm using reversed polarity. All buffer solutions and milk whey samples were filtered through a nylon membrane with a 0.45-μm pore size (Terumo) before analysis.

Prior to first use, the capillary was conditioned by rinsing with 1 M HCl for 5 min, 0.1 M NaOH for 10 min, and water for 5 min, using a pressure of 20 psi in all cases. The capillary was prepared for daily use by rinsing with

0.1 M NaOH for 5 min, water for 5 min, and separation buffer for 15 min, with a pressure of 20 psi. Between individual analyses, the capillary was conditioned with water for 5 min and separation buffer for 5 min before injection. After 6 injections, an extra rinse cycle was included as a blank analysis.

The HPLC analyses were carried out using a Varian 920-LC analytical chromatograph equipped with a photodiode array detector (Varian Inc., Mulgrave, Australia). A C18 reversed-phase column (5 μm; 250 × 4.6 mm; Phenomenex, Torrance, CA) was used. Hippuric acid was eluted at isocratic flow rate of 1 mL/min, with a mobile phase of acetonitrile-water-acetic acid (12.5:86.5:1). The UV detection wavelength was set at 229 nm. All analyses were performed at room temperature and the volume of solution injected into the column was 20 μL for each run.

2.4. Comparison between data obtained with CE and with HPLC

Results obtained for the milk samples analyzed by CE were validated using a separation technique widely employed in routine laboratories, such as HPLC. For this comparison study, 33 raw conventional goat milk samples, 36 raw organic goat milk samples, and 9 commercial milk samples (organic and conventional milk) were analyzed simultaneously by CE and HPLC.

Before analyzing the milk samples by HPLC, a calibration curve was calculated showing a linear response between 1 and 100 mg/L and a correlation coefficient of 0.9981. A study of precision was also carried out with standard solutions of HA at 2 levels of concentration, 10 and 100 mg/L.

Precision was assessed in terms of repeatability and reproducibility (as % relative standard deviation, RSD). Repeatability in the peak area was calculated by injecting HA (n = 9). Reproducibility in the peak area was calculated by injecting HA during 3 consecutive days, preparing a different solution for each day.

2.5. Analytical strategies followed to compare the results from analyzed milk samples

Different strategies were followed to compare data (peak area or concentration of HA) obtained from 2 different equipment (CE and HPLC).

Strategy 1. A standard addition method was used to determine the exact concentration of HA present in commercial milk samples. The standard addition method is a useful, but time-consuming tool to quantify compounds in complex samples (such as milk); with this methodology the matrix effect is avoided. In this work, the same set of commercial samples was analyzed with 2 different techniques (HPLC and CE).

Strategy 2. The area value of the HA peak was used to compare the content of HA from the different samples analyzed (raw milk samples) using the same equipment. This strategy was the simplest one used in this work, as the data from the electropherogram or chromatogram can be directly used to determine the related content of HA from a big set of samples avoiding the quantification step, which is time consuming.

Strategy 3. Finally, a direct quantification of the HA present in raw milk samples by using calibration curves was used to compare the results obtained for CE and HPLC. This strategy was used only when the number

of samples analyzed was high and we could not afford to run the standard addition method to calculate the exact concentration of each sample. In this case, the strategy 2 (peak area values) could not be used to compare results, as they were obtained with 2 different techniques (HPLC and CE). For this reason, calibration curves were used to obtain an approximated concentration value (as matrix effects were not considered).

2.6. Statistical analysis

The statistics software package SPSS 14.0 (SPSS, Chicago, IL) was used for statistical analysis. The values obtained for samples (conventional or organic and with or without grazing) were compared statistically by ANOVA and Tukey honestly significant difference (HSD) test at 3 confidence levels (95, 99, and 99.9 %). In addition, the statistical significance test of Student's t-test was applied to data of milk samples analyzed using both techniques (CE and HPLC) to confirm if the results were comparable at a confidence level of 95 %. The calibration curves for CE and HPLC method were obtained using Excel software (Microsoft Corp., Redmond, WA).

3. Results and discussion

3.1. Determination of HA in 2 types of commercial milk samples (organic and conventional)

Values of peak areas for HA in the electropherograms remained practically constant before and after the pasteurization process of raw milk. Therefore, HA concentration is not affected by the pasteurization treatment; consequently, it could be used as a potential marker for differentiating the feeding regimen of the animals analyzing any type of milk (raw and pasteurized).

Once the study of HA stability in the pasteurization process was carried out, the content of HA in commercial milk samples (conventional and organic) was calculated using the standard addition method (strategy 1). The average and standard deviation values are similar for conventional cow and goat milk samples (P = 0.01), as well as between these and organic cow milk samples (Table 3; note that all samples in this table were found in different supermarkets from Spain and United Kingdom). Unfortunately, only 1 brand of organic commercial goat milk was found in the market.

Table 3. Content of hippuric acid in commercial milk samples analysed by CE using the standard addition method.

Type of milk	Animal	Total number of brands	Concentration* (mg / L)
Conventional	Cow	14	14 ± 8[a]
	Goat	3	12 ± 3[a]
Organic	Cow	9	15 ± 8[a]
	Goat	1	35 ± 9[b]

*Average ± Standard deviation.
Mean values with the same letters (a, b) indicate homogeneous subsets for *P* = 0.01 according to Tukey's HSD test.

However, all its samples resulted in the highest HA content (with a mean value of 35 mg/L), significantly different from the other milk brands analyzed (P = 0.01). The last data confirm the initial hypothesis, that organic goat milk samples have higher values of HA than conventional goat milk samples (Carpio et al., 2010); but the same conclusion cannot be extrapolated to the organic cow samples. Because the standards for organic livestock farming (Council of the European Union, 2007) allow an intensive production (without grazing) of animals fed organic compound feeds with added green or conserved fodder. For this reason we developed a second hypothesis: Could HA be a marker to distinguish the feeding regimen of goats, but not the production system (organic or conventional)?

3.2. Determination of HA in 2 types of raw goat milk samples (organic and conventional)

Table 4 shows the average and standard deviation of the peak area values of HA for each group of samples corresponding to the same goat breed and localization. As the number of samples analyzed was large, area peak values (strategy 2) obtained by CE, and not concentration, were used in this study. Data shown in Table 4 are represented in Figure 1. As can be seen in Figure 1, the highest values of HA (398,925 and 363,886) correspond, respectively, to organic raw milk samples of Payoya breed goats (group 5) and Serrana breed goats from Badajoz (group 9), both of which graze.

Table 4. Average and standard deviation of peak area values of hippuric acid in each group of raw goat milk samples.

Type of milk	Group	Feeding regime	Goat breed*	Total number of samples	Peak area values
Conventional	1	Feed	F	11	51,317 ± 16,001 [ab]
	2	Feed	MG	11	31,968 ± 16,738 [a]
	3	Feed+ Grazing	S	11	139,714 ± 55,799 [ab]
Organic	4	Feed	F	10	146,866 ± 42,253 [bc]
	5	Grazing	P	61	398,925 ± 143,737 [d]
	6	Organic feed	MG	14	94,547 ± 23,617 [ab]
	7	Grazing	S	20	237,698 ± 53,014 [c]
	8	Organic feed	S	10	141,148 ± 47,816 [b]
	9	Grazing	S	10	363,886 ± 73,958 [d]

* F: Florida; MG: Murciano-Granadina; S: Serrana; P: Payoya
Mean values with the same letters (a, b, c or d) indicate homogeneous subsets for $P = 0.05$ according to Tukey's HSD test.

Conversely, the lowest and most homogeneous values (31,968) correspond to conventional raw milk samples of Murciano-Granadina breed goats (group 2), followed by Florida breed goats (group 1) values (51,317), which did not graze.

Figure 1 attempts to establish a peak area value to be used as the cut-off level between organic and conventional raw milk samples. Two groups of organic milk (groups 6 and 8) might be easily misclassified as conventional (see Figure 1), as they have a low-to-medium value of HA (94,547 and 141,148, respectively). Table 5 shows the peak area values of HA for different dates corresponding to a same organic flock with a diet based on grazing (group 5). Statistically significant differences exist between different months (P = 0.05), the summer months being those with the highest values.

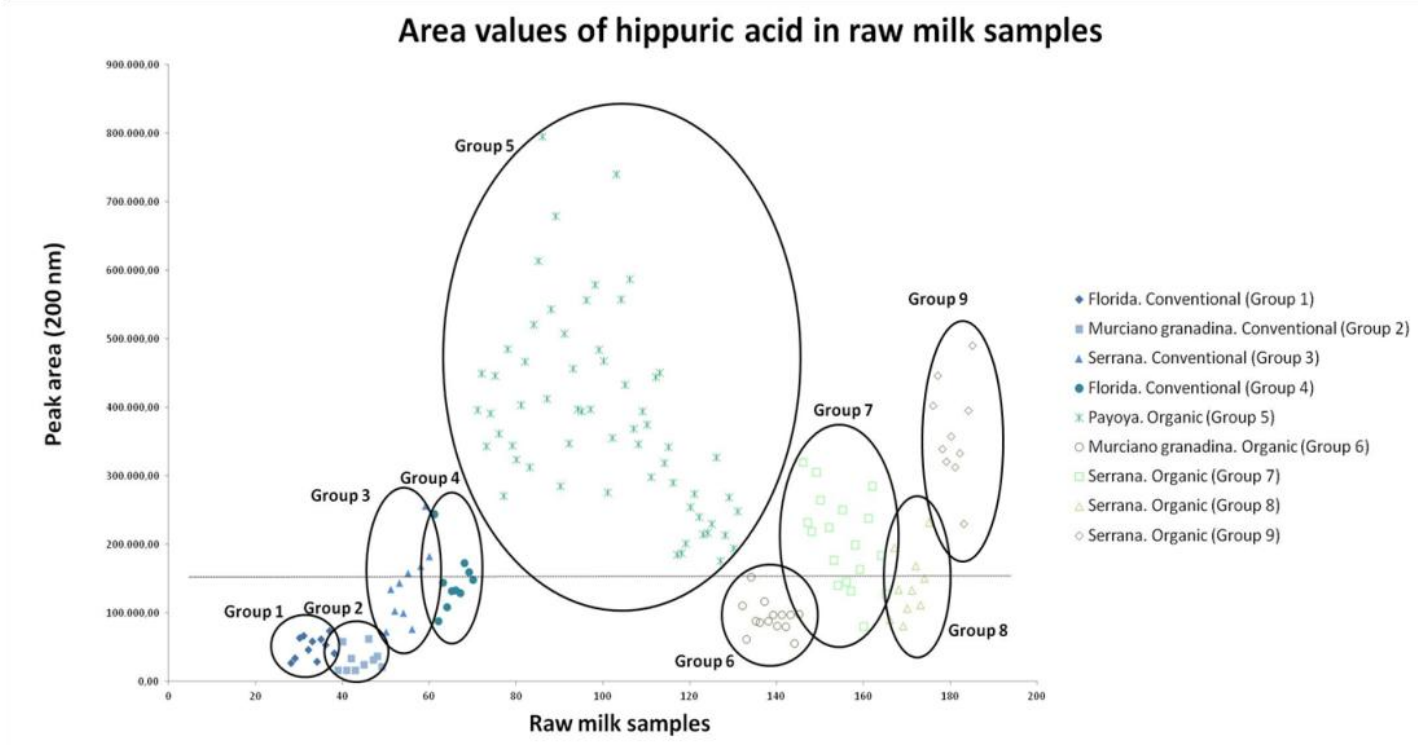

Figure 1. Peak area values of hippuric acid corresponding to the electropherograms at 200 nm of raw milk samples analyzed.

This fact can be explained taking into account that the feeding regimen and the fodder quality the goats have consumed are seasonally influenced by climatic conditions of the area and the month of sample collection (Peinado-Lucena et al., 1992). According to Peinado-Lucena et al. (1992), during summer, the grazing goat diet is more dependent of shrub and tree fodder; hence this change of the grazing date could explain the HA differences.

Conversely, milk samples of group 6 (see Table 2) and a subgroup of group 5 (see Table 5) was collected in the same month (January). However, the HA content is almost 4 times higher in raw milk from group 5 than in group 6 (398,925 vs. 94,547; P = 0.001; see Table 4). Both groups are organic, but the group 6 diet is based on organic compound feed, whereas group 5 is completely grazing dependent.

Table 5. Average and standard deviation of peak area values of hippuric acid in raw organic Payoya goat milk samples (group 5) from different dates.

Sampling months	Total number of samples	Peak area values*
May 2010	12	391,152 ± 64,258 [a]
June 2010	12	490,296 ± 153,144 [b]
July 2010	12	486,689 ± 125,940 [b]
January 2011	9	377,295 ± 52,030 [a]
April 2012	16	248,286 ± 42,326 [c]
Total samples	61	398,925 ± 143,737

Mean values with the same letters (a, b, c) indicate homogeneous subsets for $P = 0.05$ according to Tukey's HSD test.

Finally, we can conclude that, in group 5 (Payoya breed goats), a high content of HA exists because its diet is based on grazing fodder. These data confirm the experimental values of a previous work (Carpio et al., 2010) where the average and standard deviation of the peak areas of raw organic

milk samples from Payoya breed goats collected in February and April 2009 were 430,293 ± 100,283, similar to the high values obtained from samples analyzed in this study (398,925± 143,737).

Carrying on with this hypothesis, when comparing feeding regimen instead of production system (Table 6), the highest HA peak area values correspond to the grazing samples (all of them organic; 362,445 ± 178,620 vs. 94,025 ± 53,040; P = 0.001). Similarly, the difference between diets (grazing or feed) is higher than the one between the production regimens (organic or conventional; 312,053 vs. 86,458; P = 0.001) because the values of the samples from animals fed organic compound compound feed (with an average of 108,774) reduce the HA average of organic milk samples.

Table 6. Average and standard deviation of peak area values of hippuric acid in raw goat milk samples according to feeding and production regime.

Group	Total number of samples	Peak area values
Total organic samples	115	312,053 ± 190,101 [a]
Total conventional samples (all feed samples)	43	86,458 ± 57,295 [b]
Total grazing samples	91	362,445 ± 178,620 [a]
Total compound feed samples	67	94,025 ± 53,040 [b]
Total organic and feed samples	24	108,774 ± 40,055 [b]

Mean values with the same letters (a or b) indicate homogeneous subsets for *P* = 0.0001 according to Tukey's HSD test.

According to these data, HA could be a good marker of the type of feeding regimen of goats. A high content of HA would represent a diet based mainly or exclusively on eating green grass or fresh fodder (grazing).

However, this would not represent organic farming because the organic rules do not imply grazing. Hence, this marker would not be useful

for the actual organic policies under the current regulations, because organic dairy ruminants can be fed with organic compound feed and conserved fodder without grazing at all.

Differences between raw conventional goat milk could be explained by variations in the commercial feed composition, considering that this is usually formulated for the lowest cost. Furthermore, the high standard deviations of Payoya goat samples (in group 5 and previous studies) could be explained by individual variations of diet, which are proper for foraging animals (Peinado-Lucena et al., 1992; Rodríguez-Estévez et al., 2009). In this sense, Collomb et al. (2008) take into account the fodder composition to compare organic and conventional milk, and Besle et al. (2010) find differences in milk HA content related to the presence of caffeoylquinic compounds in forages. Hence, the high difference in HA content found in the samples analyzed could be due to climatic and soil factors affecting growth and botanical composition of fodder in each geographical zone.

3.3. Comparison between the data obtained with CE and with HPLC

The analytical methodology proposed by Carpio et al. (2010) was validated with a classical analytical technique found in agrifood laboratories such as HPLC. In both cases, the same samples were analyzed. Commercial milk samples were quantified using the standard addition method (strategy 1) by CE and its values were compared with the results obtained by HPLC. The results of both techniques (CE and HPLC) are 15 ± 3 and 13 ± 3 mg/L for conventional milk, respectively, and 12 ± 4 and 16 ± 8 mg/L for organic milk, respectively. The Student's test of the total average

values obtained for conventional milk analyzed with both techniques does not show significant differences at a confidence level of 95 %. Hence, the results obtained by HPLC and CE are comparable. Besides, the Student's test carried out to compare the total average values in organic milk leads to the same conclusion.

From these data, it can also be concluded that HA is not a good marker to distinguish if pasteurized milk is organic or not; probably because organic milk can be obtained by feeding goats or cows organic compound feed. These organic compounds are produced by companies, and can be bought and supplied to the animals.

Raw conventional and organic goat milk samples were also analyzed by HPLC and CE methods. The average concentration values and standard deviation obtained (see strategy 3) from raw milk samples by HPLC and CE are shown in Table 7.

Table 7. Average values of hippuric acid concentration obtained by HLPC and CE in conventional and organic raw goat milk samples (mean ± SD).

Type of milk	Goat breed	Total number of samples	HPLC (mg/L)	CE (mg/L)
Conventional	Florida (Group 1)	11	12 ± 3 ^{a}	11 ± 3 ^{a}
	Murciano granadina (Group 2)	11	5 ± 2 ^{a}	7 ± 3 ^{a}
	Serrana (Group 3)	11	26 ± 10 ^{b}	28 ± 10 ^{b}
			15 ± 11 $^{\beta}$	14 ± 10 $^{\beta}$
Organic	Payoya (Group 5)*	16	49 ± 13 ^{c}	43 ± 8 ^{c}
	Serrana (Group 8)	10	21 ± 6 ^{b}	26 ± 10 ^{b}
	Serrana (Group 9)	10	57 ± 13 ^{d}	67 ± 14 ^{d}
			47 ± 19 $^{\delta}$	45 ± 19 $^{\delta}$

*Only samples collected in April 2012

Mean values with the same letters (a, b, c or d) indicate homogeneous subsets for $P = 0.05$ according to Tukey's HSD test; and mean values with different letters (β or δ) indicate homogeneous subsets for $P = 0.001$.

It can be seen that similar results were obtained using both techniques, the total average in conventional and organic milk, respectively, are statistically comparable using the Student's test at a level of confidence of 95 %.

As mentioned before, the Murciano-Granadina breed of goats (group 2) is the group with lowest content of HA. This low value could be explained by the fact that the food provided to these goats was based on conventional compound feed. The low standard deviation also obtained confirms that all the goats had the same diet (compound feed). Hence, the individual variation proper of foraging animals was not expected. The complete opposite was found in the Serrana breed of goats (group 3). For those goats, the highest content of HA was found among the conventional milk group, as they had grazed despite their conventional farming system.

However, this was an extensive regimen with foraging and individual variations of grazing diet, highlighting the high standard deviation (±10) found in the samples analyzed by HPLC and CE, respectively. Conversely, the results of analyzing raw organic milk using both techniques also show values statically comparable according to the Student's test (P = 0.05). For these results, the deviation values are higher than for conventional milk samples, which indicate the variability of the grazing fodder ingested by the goats.

Finally, the average values of HA obtained by CE after analyzing conventional and organic raw goat milk are 14 ± 10 and 45 ± 19 mg/L, respectively. Hence, it can be concluded that the HA content in the organic raw milk analyzed is higher than in conventional milk, and it can be due to a

higher ingestion of green or conserved fodder following the organic regulations (Council of the European Union, 2007).

4. Conclusions

Hippuric acid content depends on the feeding regimen (with or without grazing) more than on the production system (organic or conventional). We confirmed that HA is not a suitable marker to certify and label goat milk as organic, as organic milk production has to only pass the minimum standard of feeding organic feed and fodder (green or conserved), but it does not imply grazing. The double analysis carried out with CE and HPLC served to validate the electrophoresis methodology to measure HA and reinforces the experimental data obtained. Finally, for a successful valuation program of dairy ruminant organic farming and the milk produced, it would be necessary to promote not only the general principles of the International Federation of Organic Agriculture Movements, but also specific feeding rules requiring grazing, which is closer to consumer perception of organic milk than the current minimum standards. Then, it would be necessary and useful to go into nutritional qualities and markers (e.g., HA) to distinguish organic milk in depth.

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Capítulo 3.3

Is Capillary Electrophoresis a promising technique to evaluate metabolites secreted by fungal biocontrol agents?

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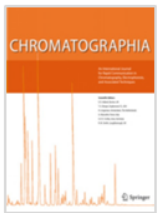

IS CAPILLARY ELECTROPHORESIS A PROMISING TECHNIQUE TO EVALUATE METABOLITES SECRETED BY FUNGAL BIOCONTROL AGENTS?

Azahara Carpio[1], Alex Ríos-Moreno[2], Inmaculada Garrido-Jurado[2], Enrique Quesada-Moraga[2], Lourdes Arce[1*]

[1] Department of Analytical Chemistry, University of Cordoba, Campus of Rabanales, Annex C3 Building, Cordoba, Spain,
[2] Department of Agricultural and Forestry Sciences, ETSIAM, University of Cordoba, Campus of Rabanales, C4 Building, Cordoba, Spain.
**Author to whom correspondence should be addressed. Email: Lourdes.arce@uco.es*

Capillary Electrophoresis (CE) with ultraviolet (UV) detection has been evaluated in this work as a valuable tool to use in the risk assessment of fungal biocontrol agents (FBCA). The CE-UV proposed method provided faster analyses, with low cost reagents, reduced solvent consumption and generated low levels of residues compared to HPLC-UV methods. The risk assessment of FBCA, used nowadays as an alternative to chemical insecticides, was carried out measuring the secretion of destruxin (dtx) A, produced by three strains of a fungus (*Metarhizium brunneum*) since it could

be a risk to humans and the environment and hence interest about its production has increased. Results from the monitoring of dtx A over 18 days in different conditions, show that in a complete medium the BIPESCO5 and EAMa 01/58-Su strains produced dtx A at levels that could be directly analyzed by CE (BIPESCO5 produced the highest dtx A concentration at day 9). However, for a minimal medium, a preconcentration step was necessary prior the determination of this metabolite by CE-UV. C18 and HLB cartridges were successfully used to improve the sensitivity of the CE method when necessary. In addition, dtx A was not detected in ART2825 strain in any culture medium. All these results were in agreement with the values found in bibliography using HPLC.

Keywords: Biological control agents, Capillary Electrophoresis, culture medium, entomopathogenic fungi, metabolites.

1. Introduction

Recently, the interest in green analytical chemistry has grown tremendously and some manuscripts on green analytical methods development can be found in literature [1]. This term is used to describe analytical approaches that minimize the consumption of reagents and energy, as well as the reduction in the generation of hazardous waste. For analytical method development, the focus is oriented toward reduction or elimination of toxic solvents and decreasing the analysis time [2]. High Performance Liquid chromatography (HPLC) remains today the technique of choice for non-volatile analytes present in liquid samples. Acetonitrile and methanol are mostly used as organic modifier in the mobile phase in HPLC due to the fact that it has a low viscosity, is miscible with water and possesses a low ultraviolet (UV) absorbance. From a green analytical chemistry point of view however it is less attractive as they are toxic solvents. Green environment friendly alternatives such as the use of Capillary Electrophoresis (CE) should be considered to determine analytes in routine analysis. This work will therefore focus on the study of the potential of CE to develop sensitive, selective, high-throughput methods for determination of analytes in complex samples. Its major advantages include high separation efficiency, ease of method optimization, the possibility to analyze small sample volumes, the use of low toxic organic solvents volume and inexpensive capillaries. Although, the sensitivity is lower compared to LC, tremendous improvements can be obtained using different preconcentration methods [3, 4].

To the best of our knowledge, the potential of CE has not fully described for the application in the risk assessment of fungal biocontrol agents (FBCA). They are an alternative to the use of chemical insecticides since it is well known the negative effects of them in living organisms and the environment, including the human health [11]. Therefore the use of FBCA (also called bioinsecticides) that are based on entomopathogenic fungi, are an alternative to the use of chemical agents. In fact, *Metarhizium spp* has been used as a biocontrol agent for a long time within integrated pest management programs due to its safety to human and animal health [1, 12].

The impact on the use of *Metarhizium spp* as a biocontrol agent is currently not totally known, as a consequence investigations are necessary to determine if the metabolites produced can enter to the food chain and therefore pose a risk to human and animal health [13, 14]. On the other hand, crude extracts produced by other entomopathogenic fungi genera have been shown to be allergens for humans [15].

Destruxins (dtxs) are major secondary metabolites produced by the genus *Metarhizium spp.* These metabolites belong to the cyclic hexadepsipeptides family and they are composed of an α-hydroxy acid and five amino acid residues [16, 17]. To date, 39 dtxs analogues have been reported and categorized into five groups (A-E) on the basis of their chemical structure [12, 18]. Individual dtxs differ on the hydroxyl acid, N-methylation and R group of the amino acid residues [17]. The dtxs are known to exhibit a wide variety of biological activities as well as a potential role as a virulence factor in fungi [17]. In fact, they have been associated with the insecticidal activity due to the acceleration of the damage and death

of infected insects [2, 6]. Dtxs are also able to suppress the insect immune response and have shown to be ATPase inhibitor and insect development inhibitor [19, 20]. Therefore, robust analytical methodologies are necessary to provide information about the amount of dtxs produced by the different *Metarhizium* strains used as bioinsecticides.

Until now, all the developed investigations have used HPLC or ultra high pressure liquid chromatography (UHPLC) coupled with an UV detector for the quantification of the different dtxs. In fact, 41 articles have been found using 'ISI Web of Knowledge' database combining the words destruxin and liquid chromatography (some representative examples are shown in Table 1). However, it is important to highlight that in spite of the strengths of CE to be used as environmental friendly technique; only 1 article has been found related with this topic. In that research, CE-UV was used to determine dtxs (A, B, E and desmethyl B) from *Metarhizium anisopliae* [10], however this method was not validated (no calibration curves, precision or accuracy is shown). As a consequence, in this paper, CE technique has been deeply studied and compared with HPLC.

In the examples summarized in Table 1, a wide range of dtx concentrations has been found, from low values near to 0.01 mg/L up to values around 100 mg/L. Considering these concentration levels, CE coupled to a UV detector can be presented as an alternative technique to HPLC or UHPLC to determine dtxs in the risk assessment of FBCA. In comparison with HPLC, CE is a versatile technique that presents some advantages, previously mentioned. Moreover, CE can be classified as an environmental friendly technique since it does not need the use of high volumes of organic phases (with less than 5 mL of organic solvent, the

device can be working for at least 8 hours) and also the generation of waste volume is minimum.

Therefore, the objective of this article has been to show that CE-UV has the potential to be an alternative to chromatographic techniques in the risk assessment of FBCA. For that purpose, the production of dtx A by three strains (BIPESCO5, EAMa 01/58-Su and ART2825) from *Metarhizium brunneum* with differences in their dtxs secretion profiles have been monitored, in two different culture media (semi-synthetic complete medium and minimal medium) for 18 days. This period of study was selected according with Amiri-Besheli et al. [2] for maximal production of dtxs by *Metarhizium* strains. Furthermore, different commercial solid phase extraction (SPE) cartridges, C18 and for first time hydrophilic-lipophilic balanced (HLB) cartridges have been evaluated in order to improve the sensitivity and/or selectivity of the methodology to determine dtx A presents in different culture media.

Table 1. Representative examples of methods developed to determine dtxs.

Objective	Strains	Samples	Study time	Quantification tecnhique	Studied dtxs	Concentration range of dtxs found	Ref.
Study the dtx production in insects, and its degradation factors	V245, V275	Insects, soil	10 days	HPLC-DAD	A, B, E	N.D. – 6.3±2.5 µg/insect	[1]
Identification of the dtx production in different strains and examine the possible correlation between dtx profiles and virulence/specificity	V220, V304, Ma23, Me1, V245, ARSEF297, ARSEF1092, ARSEF1946, ARSEF2081, DSM 1136, DSM1137	Culture media, insects	30 days	HPLC-UV	A, E, B	0.7 – 40 mg/L in culture media N.D. – 1 µg/insect	[2]
Investigation of dtx A and B production in different strains	80 *Metarhizum* strains	Culture media	10 days	HPLC-DAD	A, B	0.43 – 106.78 mg/L	[3]
Investigation of dtx production in different culture conditions	V245, V275	Culture media	30 days	HPLC-UV	A, B, E, E diol	N.D – 18.5 ± 0.7 mg/L	[4]
Quantification of dtxs in different culture conditions	F005, F007, F024, F061	Culture medium	10-12 days	HPLC-DAD a)	A, B, desmethyl B, E	0.22 ± 0.04 – 66.89 ± 2.57 mg/L	[5]
Study of the pathogenesis of M. anisoplae in three insects	39 *Metarhizium* strains	Insects	8-15 days	HPLC-UV	A, E	N.D. – 12.97± 0.86 µg/mg dry weight	[6]
Purification and quantification of dtx A and B	ARSEF925	Culture medium	14 days	HPLC-DAD a)	A, B	5.18 – 33.67 mg/L	[7]
Development of a HPLC method to determine dtxs	V275	Culture medium	8 days	HPLC-DAD a)	16 dtxs, only quantified A, B, E	Unknown – 289 ± 63 mg/L	[8]
Development of a UHPLC method to determine dtxs	V275, ART2825, EAMa 01/58-Su	Culture media	5 days	UHPLC-DAD a)	22 dtxs, only quantified A, B, E	N.D.- 146.7 ± 2.1 mg/L	[9]
Separation of dtxs by CE	A strain from *M.anisoplae*	Culture medium	Unknown data	CE-UV	A, B, desmethyl B, E	Not studied	[10]

a) Dtx identification was carried out with mass spectrometry. N.D.: non detected

2. Materials and methods

2.1. Standards and reagents

All chemicals and solvents were of analytical grade. Methanol and acetonitrile were obtained from Panreac (Barcelona, Spain). The only commercial dtx, dtx A (Fig. 1), was purchased from Sigma Aldrich (St. Louis, MO, USA). One stock solution (1000 mg/L) was prepared in acetonitrile, and working solutions were prepared by dilution in the property solvent (culture medium or water). All solutions were prepared under a vented hood. The reagents used to prepare the buffer solution (sodium tetraborate and sodium dodecyl sulphate (SDS) and sodium hydroxide) were also supplied by Sigma Aldrich (St. Louis, MO, USA).

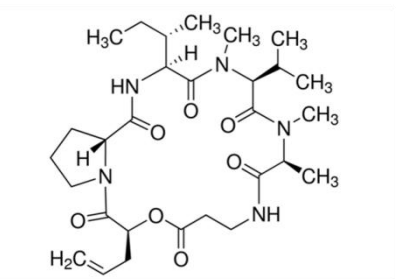

Figure 1. Destruxin A structure.

The reagents necessary to prepare all culture media were: dipotassium hydrogen phosphate anhydrous (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), potassium chloride (KCl), magnesium sulfate 7-hydrate ($MgSO_4(7\ H_2O)$) and glucose obtained from Panreac

(Barcelona, Spain); ammonium nitrate (NH_4NO_3), potassium nitrate (KNO_3), disodium hydrogen phosphate (Na_2HPO_4), and triton X-100 from Merck (Darmstadt, Germany); and yeast extract was supplied by Oxoid (Basingstoke, United Kingdom).

All water used was purified by passing it through a Milli-Q apparatus from Millipore (Bedford, MA, USA). Finally, the following commercial SPE cartridges were used: 30 mg of Supelco HLB (Sigma Aldrich, St. Louis, MO, USA) and 30 mg of Sep-Pak C18 (Waters, Milford, MA, USA).

2.2. Strains

In this study, three strains of *Metarhizium brunneum* were evaluated: BIPESCO5, EAMa 01/58-Su and ART2825. BIPESCO5 was isolated from *Cydia pomonella* and obtained from the Innsbruck onsite culture collection in Austria. EAMa 01/58-Su belongs to the culture collection of University of Cordoba and it was originally isolated from soil of wheat crop in Hinojosa del Duque (Cordoba, Spain). Finally, ART2825 was isolated from *Agriotes obscurus* and obtained from EAER–Agroscope from the Switzerland culture collection.

2.3. Culture media and cultivation conditions

The strains BIPESCO5, EAMa 01/58-Su and ART2825 were cultivated in semi-synthetic complete medium (CM) (CM was prepared using 0.36 g KH_2PO_4, 1.7 g Na_2HPO_4, 1 g KCl, 0.29 g $MgSO_4$(7 H_2O), 0.7 g NH_4NO_3, 5 g yeast extract, 10 g glucose, 0.6 mL Triton X-100 and 1 L

water) and in minimal medium (MM) (MM was prepared 1 g KH_2PO_4, 0.7 g Na_2HPO_4, 0.7 g KCl, 0.6 g $MgSO_4(7H_2O)$, 0.7 g NH_4NO_3, 0.5 g K_2HPO_4, 2.5 g KNO_3, y 20 g glucose and 1 L water).

To prepare a primary culture, 1 mL of conidial suspension (adjusted to 1×10^7 spores/mL) was inoculated into a 100 mL erlenmeyer flask containing 25 mL of liquid medium, and it was cultured at 25°C on a rotator shaker (OVAN Multimix, Badalona, Spain) at 110 rpm for 4 days. To inoculate secondary cultures for large-scale growth of the fungus, 2 mL of the primary culture were transferred into 125 mL of the same medium in a 500 mL erlenmeyer flask (six preparations in total). The six preparations were cultured at 25°C on the rotator at 110 rpm and every three days one was taken for analysis over 18 days, removing the mycelia by filtration through a Whatman no. 3 filter paper (Whatman, Kent, UK).

2.4. Apparatus

Dtx A was detected and quantified on a P/ACE MDQ Capillary Electrophoresis System from Beckman (Palo Alto, CA, USA) equipped with a diode array detector and using a fused-silica capillary (Beckman Coulter) of 75 μm inner diameter, a total length of 60.2 cm and an effective separation length of 50 cm. The applied voltage was 15 kV, the average current 72 μA and the working temperature 25°C. Samples were injected in the hydrodynamic mode at 0.5 psi for 5 s. Electropherograms were recorded at 205 nm, using normal polarity.

The running buffer consisted of 50 mM SDS, 25 mM sodium tetraborate at pH 9. Prior to first use, the capillary was conditioned by

rinsing with 1 M HCl for 5 min, 0.1 M NaOH for 10 min, and separation buffer for 10 min. The capillary was prepared for daily use by rinsing with 0.1 M NaOH for 5 min, milli-Q H_2O for 5 min and separation buffer for 10 min. Before each analysis, the capillary was flushed with milli-Q H_2O for 1 min, 0.1 M NaOH for 2 min, milli-Q H_2O for 1 min and separation buffer for 2 min prior to injection. All solutions were filtered through a nylon membrane of 0.45 μm pore size (Terumo, Leuven, Belgium) before analysis. The SPE procedure was performed on a Visiprep DL SPE Vacuum Manifold from Supelco (Bellefonte, PA, USA).

2.5. Solid phase extraction procedure

Different SPE cartridges were tested in order to improve the sensitivity and/or selectivity of the CE method, taking into account that the target analyte, dtx A, was added in a complex culture media (CM or MM). The chosen cartridges and their sorbent characteristics were Supelco HLB (hydrophilic modified styrene polymer, the reversed-phase interactions dominate the retention) and Sep-Pak C18 (hydrophobic, reverse-phase, it is a silica-based bonded phase that is used to adsorb analytes of even weak hydrophobicity from aqueous solution).

For the extraction procedure, before each use of the cartridge, the sorbent was conditioned by passing 3 mL of methanol and 3 mL of water in that sequence. For the dtx A preconcentration, a volume of 10 mL of standard or culture media was passed through the column. This was followed by elution with 1 mL of acetonitrile, evaporation to dryness of the

eluent under a nitrogen stream and reconstitution of the residue in 1 mL of water.

3. Results and discussion

This section discusses the potential of CE as an alternative to HPLC-UV fulfilling the requirements of a routine laboratory control will need (fast and cheap method) to use in the risk assessment of FBCA. For that purpose, the production of dtx A has been monitored by three strains from *Metarhizium brunneum* over time. This study is focus on dtx A because it is the only dtx commercially available. However, in bibliography is reported that dtx A is a major secondary metabolite produced by *Metarhizium spp* together with other major ones like dtx B or E [1-6], hence dtx A could be considered as a general indicator of the presence of dtxs.

3.1. Characterization of MEKC-UV method

In order to choose a suitable buffer to detect dtxs, different buffers previously used to separate other mycotoxins were taken into account [11-14]. The best conditions were achieved using micellar electrokinetic chromatography (MEKC) with a mixture of sodium tetraborate, SDS and a basic pH. Unlike the article published by Liu et al. [10] concerning the determination of dtxs by CE, the use of an organic solvent was discarded because the background noise increased in the electropherograms when it was added to the buffer. The best resolution was obtained with 25 mM

sodium tetraborate, 50 mM SDS and pH 9. Electropherograms were recorded at a wavelength of 205 nm.

As part of the characterization of the methodology proposed, calibration curves were carried out for dtx A in different media (water, MM and CM culture media), without any sample treatment before CE. Calibration curves were obtained by linear regression analysis of the peak area (y) against concentration (x) of each analytes with the equation y = mx + b. Each concentration level was injected in triplicate. The limit of detection (LOD) of each analyte was calculated through three times the standard deviation of the intercept divided by the slope, and the limit of quantification (LOQ) at ten times the standard deviation of the intercept divided by the slope.

The range studied to build the calibration curves was between 0.5 and 20 mg/L. This range of concentration was selected taking into account the level of dtx A expected in the fungal samples studied. The results obtained for the two culture broths are shown in Table 2.

Table 2. Calibration curves and statistical figures of merit for the determination of destruxin A in the different media.

	Calibration curve (y= mx + b)		Sy/x	R^2	LOD[a] (mg/L)	LOQ[a] (mg/L)	Using SPE	
	m	b					LOD[a] (mg/L)	LOQ[a] (mg/L)
Water	4743 ± 116	-1624 ± 626	1710	0.9910	0.39	1.32		
MM medium	2568 ± 54	-11 ± 645	1106	0.9989	0.75	2.51	0.08[b]	0.26[b]
CM medium	1928 ± 58	1868 ± 686	832	0.9991	1.06	3.55	0.20[c]	0.67[c]

Sy/x: Regression standard deviation; R^2: Correlation coefficient. a) Calculated using Sb; b) Using HLB cartridge; c) Using C18 cartridge.

The LODs and LOQs for dtx A in MM were 0.75 and 2.51 mg/L respectively and were slightly higher, 1.06 and 3.55 mg/L, when dtx A was added to CM. As can be expected, the values obtained in both cases were slightly higher than those obtained for dtx A in water (0.39 and 1.32 mg/L, for LOD and LOQ, respectively).

In addition, the precision of the method was also evaluated in terms of within-day and between-day precision (Table 3). Within-day precision (as %RSD, n=6) in peak area and migration time was evaluated injecting six times, from the same vial, dtx A in different media (a standard of dtx A, and spiked CM and MM culture media) at a level of 5 mg/L. Between-day precision (as %RSD, n=9) in peak area and migration time was calculated during three consecutives day preparing a different solution each day (a standard of dtx A, and spiked CM and MM culture media with 5 mg/L of dtx A), injecting them in triplicate. In all cases, the values obtained for dtx A were acceptable for the analytical purpose, and similar between them.

Table 3. Precision obtained for destruxin A in the different media.

	Within-day precision (% RSD, n=6)		Between-day precision (% RSD, n=9)	
	Peak Area	Migration Time	Peak Area	Migration Time
Water	7.1	0.3	11.3	2.5
MM medium	7.2	0.3	11.8	2.2
CM medium	7.0	0.8	12.7	2.5

As it was expected RSD values between days were slightly higher than within days but in all cases values were lower than 13 % for the dtx A peak.

3.2. Analysis of fungal culture media samples by CE-UV

As it was previously mentioned, the aim of this work is to present CE-UV as a useful technique in the field of risk assessment of FBCA. Nowadays, there is a lack of official guidelines about how to detect dtxs in production systems, final products and in the environment; hence, CE methodologies could be used as environmental friendly instrument to obtain and evaluate data from fungal production of bioinsecticides.

As a starting point, in this article for first time, CE-UV is used to evaluate the dtx A production of three strains in different conditions over a period of time. Specifically, dtx A production by three *Metarhizium brunneum* strains was monitored for 18 days. For that purpose, two culture media, CM and MM, were selected to force the strains to grow under opposite conditions, and therefore obtain different profiles of dtx A secretion. In particular, the CM medium represents the ideal growth conditions, whereas MM causes a nutritional stress.

Table 4 represents the dtx A production values belonging to the BIPESCO5 and EAMa 01/58-Su strain cultivated in the CM culture broth. As can be seen in Table 4, for BIPESCO5 the dtx A concentration reached the maximum level at day 9, with a value of 18.2 ± 0.2 mg/L. However, after this day the concentration decreased to 6.0 ± 0.1 mg/L and then it remained fairly constant up to day 18 where dtx was not detected. In the case of EAMa 01/58-Su strain, the highest level of dtx A was found in day 6 with a concentration of 7.6 ± 0.4 mg/L. The sample taken in day 9 showed a small decrease in the concentration (up to 6.3 ± 0.3 mg/L), but in the following days the decrease became more significant.

Table 4. Destruxin A concentration (mg/L) in CM culture medium inoculated with BIPESCO5 and EAMa 01/58-Su strains at different days of incubation.

Day	BIPESCO5	EAMa 01/58-Su
3	n.d.	n.d.
6	15 ± 1	7.6 ± 0.4
9	18.2 ± 0.2	6.3 ± 0.3
12	6.0 ± 0.1	3.5 ± 0.2
15	5.6 ± 0.6	4.4 ± 0.8
18	n.d.	3.5 ± 0.3

n.d.: non detected

The electropherograms corresponding to the production of dtx by A EAMa 01/58-Su strain over a period of 18 days are represented in Fig. 2. Finally dtx A was not detected in CM medium inoculated with the ART2825 strain.

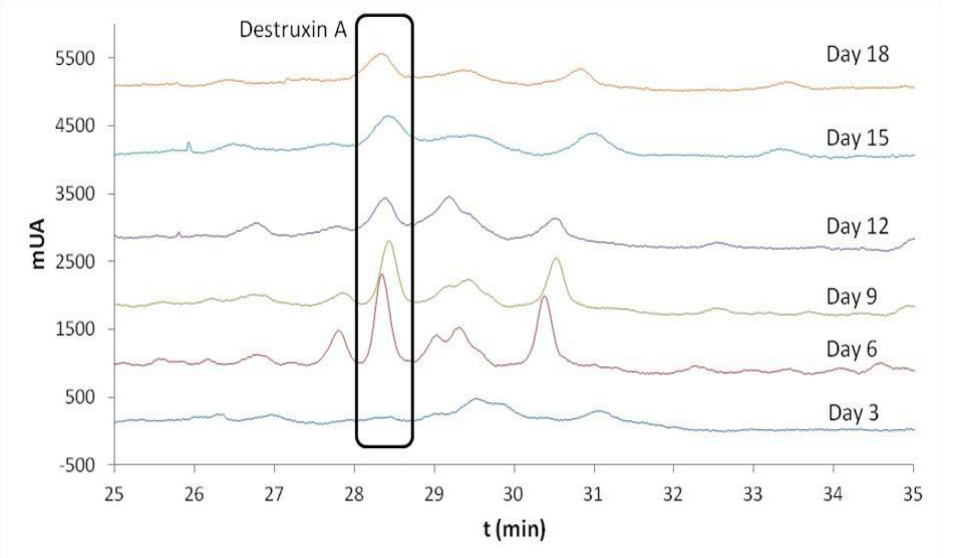

Figure 2. Electropherograms corresponding to the production of destruxin A by EAMa 01/58-Su strain in CM culture medium, over a period of 18 days. The buffer used consisted of 25 mM sodium tetraborate, 50 mM SDS at pH 9. Other conditions: hydrodynamic injection at 0.5 psi for 5 s; applied voltage 15 kV; UV detection wavelength 205 nm.

Regarding the strains cultivated in MM medium, any detectable amount of dtx A was found in a direct analysis by CE-UV. The nutritional stress that strains were submitted to when they were cultivated on MM could be the reason why any amount of dtx A was detected. This hypothesis agrees with other authors, Hu et al. [3]. They confirmed that dtxs production was not only attributable to the strain, but also to the culture media compounds and fermentation conditions. The results presented in this article are also in accordance with previous research, where other authors using HPLC showed that culture media influenced the production of dtx A secreted *in vitro* by *Metarhizium spp* [2-4, 6, 8]. Differences of dtx A production between BIPESCO5 and EAMa 01/58-Su strains was also previously studied, with BIPESCO5 being the strain that produced the largest amount of dtx A [9]. Furthermore, the same authors found that more enriched culture media yielded a higher concentration of dtx A, in agreement with the results presented here. On the other hand, data obtained of dtx A secreted by BIPESCO5 obtained by CE-UV were similar to those obtained by Wang et al. [4] using HPLC in similar fermentation period times.

Taking into account the fermentation time period, several authors have previously studied the differences of dtx production depending on this variable, in all cases HPLC was used [4, 6, 8]. In the present study, using CE-UV the maximum concentration of dtx A was obtained at the incubation days 6 and 9, for EAMa 01/58-Su and BIPESCO5 strains, respectively. These results were in agreement with the one obtained by Seger et al. [8], reporting the highest concentrations of dtxs (including dtx A) for BIPESCO5 strain on 7 day-old cultures.

In view of the results shown here, CE-UV could be considered a reliable analytical technique to use in routine controls in order to monitor the production of dtxs when *Metarhizium spp* will be used as bioinsecticide. Moreover, the potential of CE-UV could be considered as a cost effective alternative to HPLC or UHPLC coupled to UV to identify and quantify dtx A in fungal culture media.

3.3. Assessment of solid phase extraction procedure to increase sensitivity of CE-UV

Taking into account that the production of dtxs by the strains depends on the *Metarhizium* genus and the culture medium in which it is incubated, a wide range of concentrations can be found. Only for cases in which dtx A concentration is lower than 2.5 or 3.5 mg/L (in MM and CM culture media, respectively) a SPE step is necessary before CE-UV to quantify it. One of the main purposes of this work was to include the minimum sample treatment, in order to have an easy and cost effective method which will be able to analyze a high number of samples per day. Thus in this work, SPE was chosen due to its well-known advantages compared to other preconcentration strategies [15].

As it has been previously mentioned, each culture medium has a different composition that could interfere in the extraction of dtx A, consequently one type of SPE cartridge could be more or less suitable for use. Therefore, a study of two different SPE cartridges was carried out in order to improve the selectivity and/or sensitivity of the method to extract dtx A from the two culture media used in this research. Based on the

physicochemical characteristics of the target analyte (dtx A), HLB and C18 cartridges were chosen. C18 was previously evaluated with dtx A [9], whereas it is the first time that HLB has been used to preconcentrate this secondary metabolite.

Firstly, a standard SPE extraction procedure (following the steps described in section 2.4) was carried out with a dtx A standard at 0.5 mg/L in water and spiking the different culture media with dtx A at the same level, and submitting them to the SPE with the two sorbents chosen. Each experiment was done in triplicate, and the sample extracted from SPE was injected in triplicate into the CE-UV. The peak area obtained for dtx A in the culture media was compared with the peak area obtained for a standard of dtx A in water, in order to assess how the cartridges were able to eliminate interferences present in the culture media. The results about the interference study were expressed as a percentage using the following equation: [(sample signal)*100/standard signal]. The values obtained when dtx A was spiked to MM culture medium were 85 ± 1 and 90 ± 5 % for HLB and C18, respectively; whereas those obtained for CM were 94 ± 5 and 97 ± 1 % for HLB and C18. These values means that dtx A signals obtained in the culture media were quite similar to the standard; therefore the cartridges and the conditions used were highly selective for our purpose.

On a separate matter, in order to check the trueness of the SPE procedure, recovery experiments at a dtx A concentration level of 0.5 mg/L were carried out using the two different cartridges and considering the different culture broths. In this case, the signal of dtx A in the culture media spiked before and after the SPE procedure was compared. Each experiment was done in triplicate and the obtained extracts were injected

three times into the CE-UV system. The recoveries obtained for MM culture medium were 98 ± 1 % and 71 ± 3 % using HLB and C18, respectively; whereas in case of CM, the results showed a recovery value of 72 ± 3 % for HLB and 96 ± 4 % for C18. In summary, for the MM medium the highest recoveries were obtained with HLB and for the CM medium the highest recoveries were found using C18. Therefore, depending on the experimental conditions it is more suitable to use one type of cartridge, and the culture broth composition has to be considered in order to choose the type of sorbent to preconcentrate dtx A present in real fungal culture media samples.

Following the conclusions obtained in the previous SPE study, one calibration curve was obtained for dtx A in MM using the HLB sorbent, and another for CM using C18. Calibration curves were obtained by linear regression analysis of the peak area (y) against concentration (x) of each analytes with the equation $y = mx + b$. Each concentration level was injected in triplicate. LODs and LOQs were calculated as three times the standard deviation of the intercept divided by slope, and ten times the standard deviation of the intercept divided by slope respectively.

The LOD and LOQ obtained for dtx A in MM using HLB were 0.08 and 0.26 mg/L, and in CM using C18 were 0.20 and 0.67 mg/L, respectively. Comparing these values with the one obtained directly (without SPE, see Table 2), it can be shown how the sensitivity of the method was increased by just including the SPE step before CE-UV separation. If lower LODs are necessary the values obtained could be reduced just passing more sample volume through the SPE cartridges. In fact, using HLB column and passing 100 mL of MM medium spiked with dtx A allows obtaining better LOD (0.026

mg/L) than by passing 10 mL (always eluting with 1 mL). Unfortunately, the same conclusion can not be highlighted when dtx A presents in CM culture media was extracted with C18, in this case the use of higher volumes of sample (above 10 mL) allowed interferences to coelute with the dtx A and for this reason the identification of this secondary metabolite was not possible.

3.4. Analysis of fungal culture media samples by SPE-CE-UV

Under the conditions optimized for the SPE-CE-UV method, firstly, the fungal culture media samples from CM that couldn't be directly determined its content in dtx A using CE-UV (BIPESCO5 in days 3 and 18, and EAmA 01/58-Su in day 3) were tested including the SPE procedure. No positive result was found. One possible reason is that at earlier stages, in the fungal culture media there isn`t a detectable amount of dtx A, whereas in the final days the secondary metabolite can be degraded in other compounds.

Secondly, fungal samples cultivated in MM were submitted to SPE to determine the low dtx A level produced. In these conditions, dtx A produced by EAMa 01/58-Su and BIPESCO5 strains could be detected and quantified in some samples. Specifically, dtx A produced by BIPESCO5 was only quantified in days 12, 15 and 18 with concentration values of 0.88 ± 0.02, 0.78 ± 0.01 and 1.33 ± 0.03 mg/L, respectively; whereas in EAMa 01/58-Su strain, dtx A was quantified only in day 12 (0.266 ± 0.006 mg/L). In day 15, dtx A was detected but its quantification was not possible as its concentration was lower than the LOQ. In addition, it can be confirmed that ART2825 does not produce dtx A above the LOD of the method. Finally, as

it has been detailed in section 3.2, the variability in these results can be due to the different behavior of the strains in the cultivation conditions tested in this work.

Otherwise, SPE allowed for the successful enrichment of dtx A with the different culture media, but it is important to take into account the possible interferences from the culture medium during sorbent selection.

5. Conclusions

The results summarized in this study have shown the potential of CE-UV to be considered as a cost effective alternative to HPLC or UHPLC coupled to UV to identify and quantify dtx A in fungal culture media. Although HPLC analysis still remains the technique of choice for this kind of applications, in this paper has been demonstrated that CE is a fast, and sensitive technique which can handle low sample volumes compare with the method based on the use of HPLC-UV. CE has been used for first time to monitor the production of this secondary metabolite in different culture media and over a period of time and it can be concluded that CM favours the production of dtx A; whereas in case of MM, the use of SPE was necessary to preconcentrate in order to determine this metabolite by CE-UV. Additionally, among the three strains, BIPESCO5 produced a higher content of dtx A, and ART2825 did not produce any detectable amount of dtx A in any culture media.

Further research with more promising strains to be used as bioinsecticides should be conducted with other kind of media. Those media should more closely resemble plant composition. A final stage should be to

validate analytical methodologies to test the presence of dtxs in plants treated with biocontrol agent, since the secretion of these secondary metabolites will also be different according to the plant in which bioinsecticides were applied.

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Conflict of Interest

They authors have declared no conflict of interest.

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BLOQUE 4

USO DE LA ELECTROFORESIS EN EL CAMPO AGROALIMENTARIO CON ETAPA DE PRECONCENTRACIÓN EN EL TRATAMIENTO DE MUESTRA

En este Bloque se presentan los trabajos que han tenido como objetivo común estudiar las ventajas de tres materiales no muy usados para extraer analitos y preconcentrarlos por SPE antes de su determinación por CE-UV/Vis. Los materiales seleccionados han sido unas nanopartículas magnéticas carboxiladas, una organosílice mesoporosa periódica con grupos fenileno, y una estructura zeolítica de imidazolio. Dependiendo de las características físicas de cada uno de ellos se han usado diferentes modalidades de la SPE en modo convencional (empaquetando el material en un cartucho) o dispersivo (usando un imán o una centrífuga para separar las fases). El potencial de estos materiales se ha estudiado con diferentes grupos de analitos (metales, pesticidas y bisfenoles) en distintas muestras de interés agroalimentarios. En todos los casos se ha estudiado el efecto matriz. Todos los resultados obtenidos se han comparado con los que ofrecen los materiales (C18 y HLB) que se usan más comúnmente en SPE para extraer y preconcentrar los analitos objeto de estudio en esta memoria.

Capítulo 4.1

Use of carboxylic group functionalized magnetic nanoparticles for the preconcentration of metals in juice samples prior to the determination by Capillary Electrophoresis

ELECTROPHORESIS

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USE OF CARBOXYLIC GROUP FUNCTIONALIZED MAGNETIC NANOPARTICLES FOR THE PRECONCENTRATION OF METALS IN JUICE SAMPLES PRIOR TO THE DETERMINATION BY CAPILLARY ELECTROPHORESIS

A. Carpio[1], F. Mercader-Trejo[2], L. Arce[1], M. Valcárcel[1*]

[1] Department of Analytical Chemistry, Institute of Fine Chemistry and Nanochemistry (IQFN), University of Córdoba, Agrifood Campus of International Excellence (ceiA3), Annex C3 Building, Campus of Rabanales, E-14071 Córdoba, Spain.
[*]qa1meobj@uco.es

[2] Polytechnic University of Santa Rosa Jauregui Carr. Qro – SLP km 28.5 Parque Industrial Querétaro. Privada la Noria s/n Santa Rosa Jáuregui, Querétaro, México.

Nowadays food industry demands reliable and precise methods to resolve problems related to quality and security control. Food samples matrices are very complex and for this reason the pre-treatment steps prior to sample analysis are compulsory to extract the target analytes. In this work, we have studied for the first time, the potential of carboxylic group functionalized magnetic nanoparticles to preconcentrate metals from liquid

samples before CE analysis. For the extraction of metals, 10 mL of an aqueous sample which contains the metal mixture was added to 2 mg of carboxylic group functionalized magnetic nanoparticles. Metals retained in the nanoparticles were re- extracted with 200 μL of a solution consisting of 0.8 mM 1,10- phenanthroline and 0.04 % hydroxylamine chloride set to pH 2. The electrophoretic buffer used in this work to separate different metals (Co, Cu, Zn, Ni and Cd) consisting of 30 mM hydroxylamine chloride, 0.30 mM 1,10-phenanthroline, 80 mM urea, 15 mM ammonium chloride and 0.1% methanol set to pH 3.6. Finally, measurements were made at 270 nm. Under the optimized conditions, detection limits for Co, Zn, Cu, Ni and Cd were 0.004, 0.003, 0.004, 0.008 and 0.009 mg L^{-1}, respectively.

Keywords: Carboxylic group functionalized magnetic nanoparticles, 1,10-phenanthroline, metals, Capillary Electrophoresis.

1. Introduction

Recently, nanoparticles with novel optical, electrical, and magnetic properties have become more prevalent in diverse fields, such as biosensor [1], nanomedicine [2], and separation science [3] among others. Typical advantages of nanoparticles are that they can be used to improve selectivity, sensitivity, rapidity, miniaturization or portability of the analytical system. Consequently, nanoparticles can be used for purposes such as sample treatment, instrumental separation of analytes, or even detection.

Among various nanostructured materials, magnetic nanoparticles such as magnetite, magemite, nickel and cobalt, due to their phenomena such as super paramagnetism, high field irreversibility and high saturation field, have attracted growing interest in magnetic nanostructured materials for their unique properties and potential applications in various fields, especially in biomedicine and bioengineering such as magnetically assisted drug delivery [4], cell isolation [5], magnetic resonance imaging with contrast agents [6] and biomacromolecule purification [7].

Magnetic nanoparticles have three properties: (i) they have a large surface, (ii) their surface can also be functionalized with various chemical groups to increase their affinity towards analytes, and (iii) the magnetic nanoparticles with adsorbed analyte can be easily collected by using an external magnet, which makes sampling and collection easier and faster. Moreover, the magnetic nanoparticles can be reused o recycled. For these reasons, magnetic nanoparticles are attractive sorbents, compared with traditional solid-phase extraction. Among the wide variety of existing commercial magnetic nanoparticles, in this work we have selected one type which is

functionalized with carboxylic groups because of their affinity with cationic metals. The potential of these nanoparticles has already been demonstrated in the mRNA isolation and extraction of supercoiled plasmid DNA [8].

In Capillary Electrophoresis (CE), different magnetic nanoparticles have been used in the electrophoretic system [9-11], in the pre-treatment of the sample [12-14], and also CE has been used to separate magnetic nanoparticles [15]. As it is well known, CE suffers from poor concentration sensitivity for absorbance detection due to the low injected sample volumes and to the narrow inner capillary diameter being the optical path length. Preconcentration with magnetic nanoparticles in the sample treatment is a new alternative to improve the sensitivity of this technique.

In this project, the potential of carboxylic group functionalized magnetic nanoparticles in CE has been proved by concentration metals in liquid samples. The determination of metals is important for routine quality control of beverage products, among other fields, due to their essential or toxic action; there are studies that indicate that some metals act as catalysts in the oxidative reactions of biological macromolecules [16]. The U.S. Food and Nutrition Board of the Institute of Medicine have carried out risk assessments dealing with the toxicity by establishing the Tolerable Upper Intake Level for trace elements. The value calculated for Zn is 40 mg/day and for Co is 10 mg/day [17]. Although the trace metal content in food is important, in juices only the content in Pb and Sn is legislated by the European Union according to Directive 2001/112/EC, being the maximum permissible concentration (0.05 mg/Kg and 100 mg/Kg respectively).

One problem in the determination of metal ions by CE is that most of the transition metals have almost the same mobility due to their similar size

and identical charge. An alternative to resolve this problem is to use a chromogenic chelating reagent capable of strong complex formation and conversion of metal ions into positively charged chelates with different electrophoretic mobilities. Many chelating reagents such as porphyrins [18], 4-(2-pyridylazo)resorcinol [19], several aminopolycarboxylic acids [20], 1,10-phenanthroline [21], etc; have been employed for the direct absorbance-based detection of alkaline earth, transition and lanthanide metals ions. In this work, the potential of a new material to increase the sensitivity in CE with UV-VIS detection for the determination of Co, Cu, Zn, Mg, Ni and Cd has been studied. The results shown in this paper could be used to extend the application of these magnetic nanoparticles to preconcentrate other analytes. We combine the advantages of carboxylic group functionalized magnetic nanoparticles and the formation of complexes with 1,10-phenanthroline to carry out the extraction and preconcentration of metals present in juice samples prior to analysis by CE.

2. Materials and methods

2.1. Reagents

Nitrate salts of Cd(II), Mg(II), Cu(II), Co(II), Zn(II) and Ni(II) were obtained from Sigma (St. Louis, MO, USA). They were used for preparing stock solutions of 1000 mg L^{-1} at pH 2 adjusted with HCl. The working solutions of the metals were prepared by appropriately diluting the stock solutions with distilled water and adjusting the pH to 8 with NaOH to carry out the extraction and preconcentration of metals with the carboxylic group

functionalized magnetic nanoparticles. Reagents used for preparing the buffer solution and re-extraction solution (hydroxylamine chloride, 1,10-phenanthroline, urea, ammonium chloride) were supplied by Sigma (St. Louis, MO, USA), except methanol that was obtained from Panreac (Barcelona, Spain). All water used was purified by passing it through a Milli-Q apparatus from Millipore (Bedford, MA, USA). Carboxylic group functionalized magnetic nanoparticles were provided from MagnaMedics (Geleen, The Netherlands). Finally, juice samples were purchased from local commercial stores.

2.2. Apparatus and operating conditions

The measurements of pH solutions were realized in a MicropH 2000 pH meter from Crison. The magnetic field was applied with a combination of three magnets, being their characteristics: (i) magnets with a coating of Ni-Cu-Ni, (ii) 4.8 kg of axially pull force, (iii) inner diameter of 6 mm and external diameter of 15 mm, and (iiii) thickness of 5 mm ± 0.1 mm. Analytes were separated and quantified on a P/ACE MDQ Capillary Electrophoresis System from Beckman (Palo Alto, CA, USA) equipped with a diode array detector and using a fused silica capillary (Beckman Coulter) of 50 μm inner diameter, 50.2 cm total length and 40 cm effective separation length.

The running buffer was composed of 30 mM hydroxylamine chloride, 0.30 mM 1,10-phenanthroline, 80 mM urea, 15 mM ammonium chloride and 0.1 % methanol set to pH 3.6. The applied voltage was 20 kV and the working temperature 25 ºC. The samples were injected into the capillary by hydrodynamic injection during 30 s at 0.5 psi. Electropherograms were

recorded at a wavelength of 270 nm, using normal polarity. All buffer solutions were filtered through a Nylon membrane of 0.45 μm pore size before analysis.

Prior to first use, the capillary was conditioned by rinsing with 1 M HCl for 5 min, 0.1 M NaOH for 10 min and water for 5 min using a pressure of 20 psi in all cases. The capillary was prepared for daily use by rinsing with 0.1 M NaOH for 5 min, water for 5 min and separation buffer for 15 min, with a pressure of 20 psi. Between individual analysis, the capillary was conditioned with water for 5 min and separation buffer for 5 min prior to injection.

2.3. Sample treatment

2.3.1. Mineralization of juice samples

15 mL of juice sample was added in a crucible with 0.5 mL of concentrated nitric acid, and the mixture was taken to dryness in a sand bath, keeping the temperature less than 100 ºC. After that, the sample was carbonized with a Bunsen burner. Then the sample was incinerated at 400 ºC approximately for 16 hours in an oven until the ashes were almost white. In the case that ashes were not clear enough, an additional 5 drops of concentrated nitric acid must be added and both dryness and incineration processes must be repeated. Next, 5 mL of nitric acid 1 M were added to the final residue in the crucible, and it was taken to almost dryness in a sand bath. Finally, the residue was dissolved in 10 mL of water (if it is necessary this solution can be filtered) and the pH of this extract was adjusted to pH 8,

for preparing the mineralized juice sample to the extraction and preconcentration method with carboxylic group functionalized magnetic nanoparticles.

2.3.2. Extraction and preconcentration method using carboxylic group functionalized magnetic nanoparticles

The carboxylic group functionalized magnetic beads used in this work are composed of a ferrofluid encapsulated in a matrix of silica. This ferrofluid is composed of magnetic nanoparticles of diameter less than 10 nm containing iron oxides (magemita, γ-Fe_2O_3, and magnetite, Fe_3O_4). The mean size of the magnetic beads is 1.2 μm. By controlling the pH in the magnetic nanoparticles, the surface can be charged positively or negatively, and the nanoparticles can be applied to separate different molecules.

Prior to their first use, (see Figure 1) commercial magnetic nanoparticles must be pre-conditioned. For this purpose, in a vial, 2 mg of nanoparticles were shaken in a vortex for 5 min with 10 mL of water solution at pH 8. Then magnetic nanoparticles were aggregated by an external magnet, and the solution was removed using a pipette. After that, magnetic nanoparticles were re-suspended in 200 μL of re-extraction solution of metals (0.8 mM 1,10-phenanthroline and 0.04% hydroxylamine chloride at pH 2) and they were shaken in the vortex for 5 minutes. Following this, nanoparticles were separated from solution with an external magnet and the solution was removed. This cycle of washing must be repeated 4 times.

Once the magnetic nanoparticles are washed, 10 mL of aqueous sample which contains the metal mixture at pH 8 were added. The solution was shaken in the vortex for 5 minutes. Then nanoparticles with metals retained were separated from solution applying a magnet and removed the solution with a pipette. After, 200 µL of re-extraction solution (0.8 mM 1,10-phenanthroline and 0.04 % hydroxylamine chloride at pH 2) were added to the nanoparticles with metals retained. The solution was shaken in the vortex for 5 minutes. Finally, magnetic nanoparticles with metals retained were separated from solution with an external magnet, and the extract was analyzed by CE. Figure 1 shows the steps followed to extract and preconcentrate the metals from the aqueous samples.

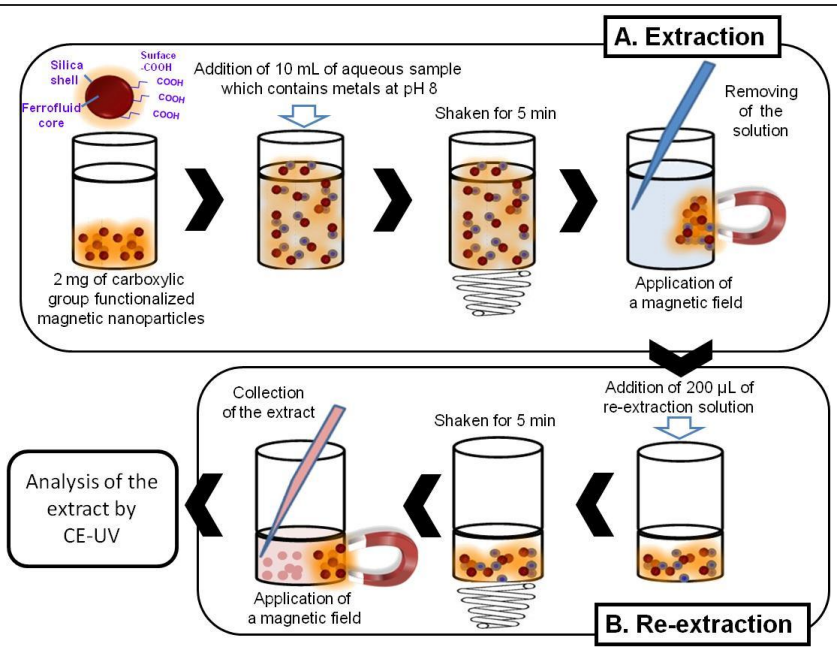

Figure 1. Scheme of the method to extract and preconcentrate metals from aqueous samples using carboxylic group functionalized magnetic nanoparticles.

Magnetic nanoparticles were reused after a cleaning process. After each extraction the nanoparticles were washed twice with 200 µL of re-extraction solution (0.8 mM 1,10-phenanthroline and 0.04 % hydroxylamine chloride at pH 2), and then with 5 mL of water solution at pH 8.

3. Results and discussions

The potential of carboxylic functionalized magnetic nanoparticles has been studied to preconcentrate metals in aqueous food samples (such as juices, tea, etc.) by using and electrophoretic buffer containing a strong complexing agent, 0.30 mM 1,10-phenanthroline for direct UV detection, 30 mM hydroxylamine chloride, 80 mM urea 15 mM ammonium chloride and 0.1% methanol set to pH 3.6. The urea acts as a weak complexing agent with its ion-pair of electrons on the nitrogen atom interacting with the complexes. This decreases the charge density of the complexes and its migration times are changed [22]. The metal chelates formed showed different electrophoretic mobilities and solved the comigration problem for CE separation of free metal ions. To obtain stable metal-phenanthroline chelates during the CE run, both pre-capillary and on-capillary complexation are required and threefold excess of phenanthroline over metal ions is added to the sample. Among the varied electrophoretic methods to determine metals in CE found in the literature, for this work a reoptimized electrophoretic method reported by previous authors [22, 23] has been used, in order to achieve better electrophoretic resolution between analytes.

3.1. Optimization of extraction and preconcentration method

In order to optimize the best method for extraction and preconcentration of metals from aqueous samples, the following variables were studied: the effect of extraction time, the effect of pH of the solution which contains the metal mixture; the effect of re-extraction time; and the effect of pH, concentration of 1,10-phenantroline, and concentration of hydroxylamine chloride in the re-extraction solution; and finally, the effect of the amount of magnetic nanoparticles in the extraction method was also studied.

A mixture solution containing 0.1 mg L^{-1} of each metal (Co, Cu, Zn, Mg, Ni and Cd) was used in the optimization experiments. The optimization process was done based on the peak area of the mixture of analytes.

3.1.1. Optimization of extraction conditions

The effect of extraction time and the effect of the pH of the aqueous solution which contains the mixture of analytes were studied for the extraction of metals (see Figure 1.A) from the aqueous solution into carboxylic group functionalized magnetic nanoparticles.

In order to determine the optimal extraction time required for quantitative adsorption, 10 mL of a metals mixture at 0.1 mg L^{-1} were mixed with 2 mg of magnetic nanoparticles, the extraction times studied were 1, 3, 5, 7, 10 and 20 minutes. The electrophoretic signal of metals was constant after 5 min, therefore this value was chosen as the optimum extraction

time. In these conditions, the sorption of metals onto magnetic nanoparticles reached the equilibrium.

The effect of the pH in the solution of metals was also checked. The studied range of pH was between 2 and 10. Improvement in signals for metals was obtained while increasing pH. pH 8 was selected as optimum value. At this pH the carboxylic groups in the surface of nanoparticles are negatively charged and they attract the positive charge of metals. When pH decreases, adsorption also decreases.

3.1.2. Optimization of re-extraction conditions

For the re-extraction of metals (see Figure 1.B) from the carboxylic group functionalized magnetic nanoparticles to re-extraction solution, the effect of re-extraction time has been studied; and the effect of pH of re-extraction solution, concentration of 1,10-phenantroline, and concentration of hydroxylamine chloride were also studied.

The re-extraction time was studied at 1, 3, 5, 7, 10 and 20 min. Signals practically constant were observed from 3 to 7 min. Therefore 5 min was selected as optimum re-extraction time.

The influence of pH of re-extraction solution was studied in the range of 2-9. The results are shown in Figure 2.

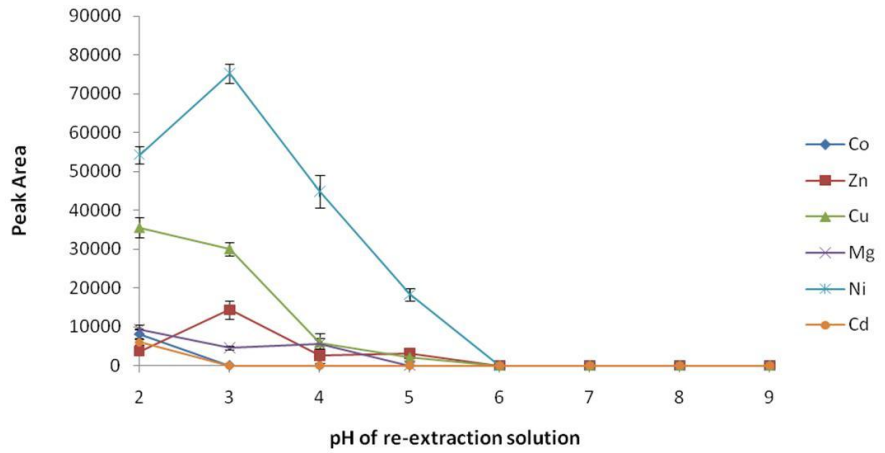

Figure 2. Effect of the pH of the re-extraction solution. Buffer consisting of 30 mM hydroxylamine chloride, 0.30 mM 1,10-phenanthroline, 80 mM urea, 15 mM ammonium chloride and 0.1 % methanol set to pH 3.6; hydrodynamic injection during 30 s at 0.5 psi; voltage applied, 20 kV; UV detection at 270 nm.

For this variable, pH 2 was selected as optimum value in order to obtain a signal in less sensitive elements such as Cd, Co, Mg, and Zn. However, the manufacturers of the magnetic nanoparticles recommend working at pH values between 3 and 13, in order to avoid Fe leaching at pH below 3. For this reason, after 4 cycles of use, it was observed that there was a formation of a layer of brown particles without magnetic properties. This was attributed to the deterioration of the silica matrix causing the leaching of the iron oxides into the solution. Taking into account these recommendations, pH 2 was selected since protonation of carboxylic acid groups is favored causing that metals are not attracted by the magnetic nanoparticles and with the presence of 1,10-phenanthroline in the acid medium it favours the formation of metal-1,10-phenanthroline complexes.

The 1,10-phenanthroline was used to extract and complex metals from magnetic nanoparticles. The concentration range studied was between 0 and 1 mM. The results are shown in the Figure 3.

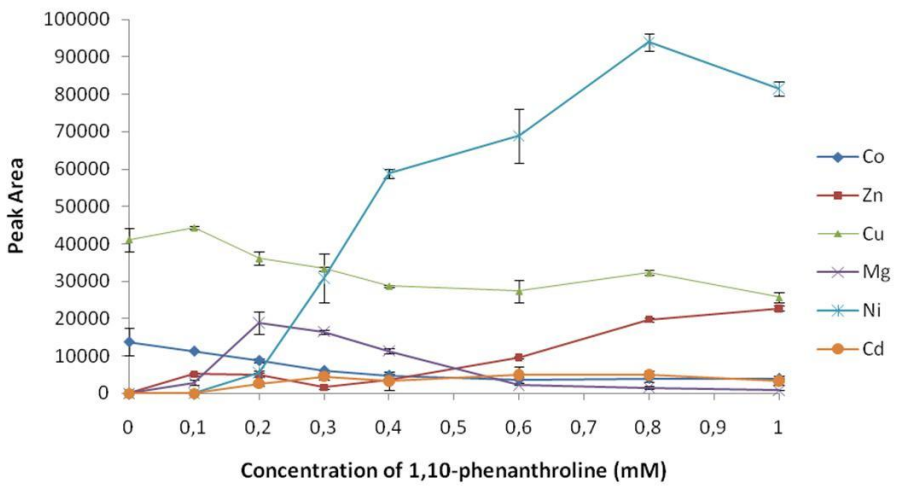

Figure 3. Effect of concentration of 1,10-phenanthroline (mM) in the re-extraction solution. Others conditions as in Figure 2.

From the data shown in this Figure, it can be seen that Co and Cu are able to be re-extracted even in the absence of 1,10-phenanthroline only by the change of pH. It was observed that the signal of these metals was decreasing with the increase of phenanthroline concentration, especially for Co. As we expected in the case of Zn, Ni and Cd the signal increases with the increase of the concentration of 1,10-phenanthroline, while the Mg has a maximum at 0.2 mM. In order to have an excess of phenanthroline for metal complexation, 0.8 mM was selected as optimum value.

Finally, hydroxylamine chloride was used to change the ionic strength and to improve the resolution of the analytes in the

electropherogram, and to avoid oxidation of metals with valence +2 to +3 (Co and Ni), and thus, to favour the complexation of metals with the 1,10-phenanthroline. In this study, the concentration of hydroxylamine hydrochloride varied from 0 to 0.07 %, while the 1,10-phenanthroline was kept at 0.8 mM. The best resolution was at 0.04 %, therefore it was chosen as optimum concentration.

3.1.3. Effect of amount of magnetic nanoparticles in the extraction method

The amount of magnetic nanoparticles was also evaluated, over the range of 1, 2 and 3 mg of magnetic nanoparticles.

Using 1 mg and 10 mL of 0.1 mg L^{-1} metals mixture solution (solution A), the extraction was not completed. This was checked analyzing the re-extraction solution once it was separated from magnetic nanoparticles in which metal were retained. Traces of signals of the metals were obtained in the electropherograms.

With 3 mg of magnetic nanoparticles a more difficult separation of the nanoparticles from the solution A using the magnets available was observed, causing this process to be slower. The disadvantage of using this amount of nanoparticles is that in each cycle of use a little amount of magnetic nanoparticles was lost, thus the extraction efficiency of the process is changing and therefore the process will not have repeatability. It is also possible that these suspended nanoparticles can enter into the capillary and clogging it. For all of these reasons, it was decided to use 2 mg of magnetic nanoparticles for the separation process.

Once the variables of the extraction and preconcentration method were optimized, a metals mixture at a concentration of 0.1 mg L^{-1} was analyzed. The electropherogram obtained was compared with that obtained only complexing the metal mixture with phenanthroline in the presence of hydroxylamine hydrochloride without using through the process of extraction and preconcentration with magnetic nanoparticles. These results are shown in Figure 4. In the metals mixture analyzed without preconcentration, the metals cannot be identified with any certainty. After performing the preconcentration process, there is a large increase in signal intensity, and metals can be easily identified. It is also observed that the complete separation of Mg ion peak with that corresponding to the excess 1,10-phenanthroline was not obtained, for this reason, we decided to eliminate the Mg from the group of metals to be determined with this methodology.

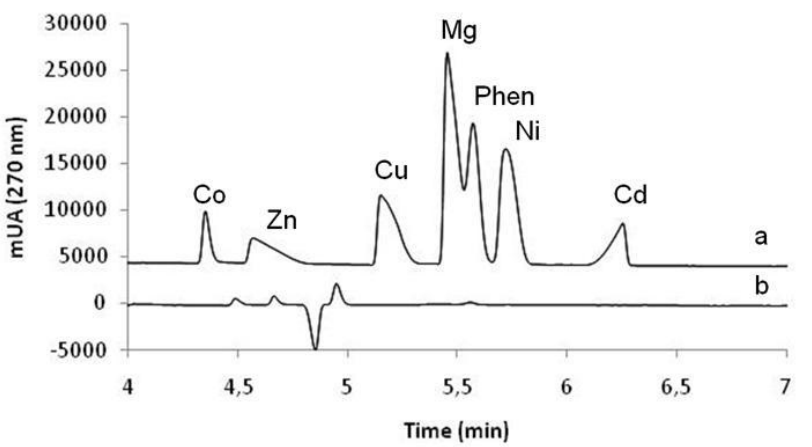

Figure 4. Electropherogram of a standard metal mixture at 0.1 mg L^{-1} preconcentrated with magnetic nanoparticles (a), and without preconcentration (b). Others conditions as in Figure 2.

3.2. Reusability

In order to investigate the recycling of magnetic nanoparticles, several extraction and preconcentration cycles with a metal mixture at 0.1 mg L^{-1} were carried out. After each cycle, the nanoparticles were washed according to the method described before.

An amount of 2 mg of nanoparticles were stable up to at least 4 cycles, because there was no obvious decrease or increase for the peak area of the analytes. These results are shown in the Figure 5.

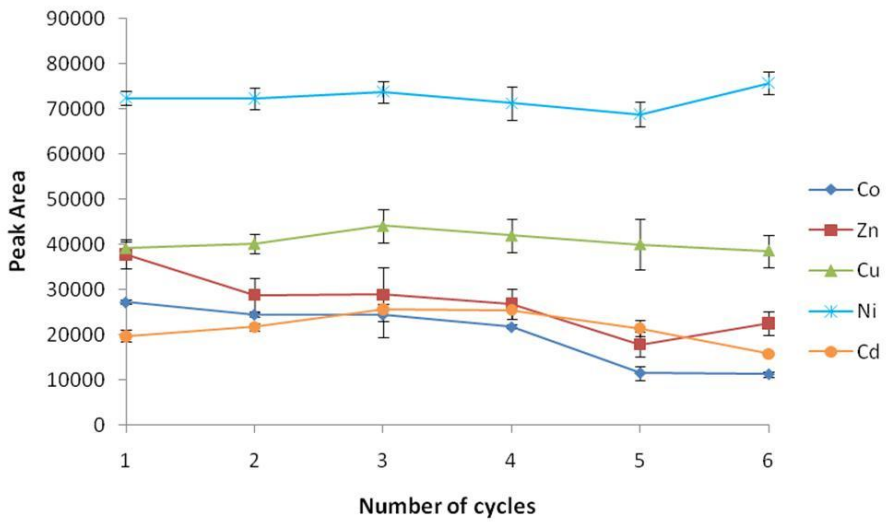

Figure 5. Number of cycles of use of 2 mg of carboxylic group functionalizated magnetic nanoparticles to extract and preconcentrate metals. Others conditions as in Figure 2.

Taking into account the amount of nanoparticles used and the price of the product (e.g. a commercial solution of 40 mg cost approximately 75 €;

and using portions of 2 mg, 80 analyzes can be performed with this amount of nanoparticles), it can be confirmed that this material is very adequate according with the market prices of this magnetic nanoparticles.

3.3. Validation

Under the optimal experimental conditions, the calibration curves for each metal were established in the range of 0.005–0.12 mg L^{-1} by plotting the peak area versus concentration, and three repetitions at each concentration were performed. This range was chosen because at higher concentrations it is possible to determine the metals by CE forming complexes with 1,10-phenanthroline without the need to use a preconcentration method. LOD of each metal determination was calculated through three times the standard deviation of the intercept of the plot divided by its slope, and LOQ at ten times the standard deviation of the intercept of the plot divided by its slope. The figures of merit are shown in Table 1. The proposed method is highly effective to determine Co, Zn and Cu at low levels.

The LODs achieved by using magnetic nanoparticles before CE-UV has been compared with the experimental LOD (0.3 mg L^{-1}) obtained without any preconcentration step before CE-UV analysis and it can be confirmed that, with this new methodology has been possible to reduce the LODs in two orders of magnitude.

Table 1. Calibration curves and statistical figures of merit for the determination of a metal mixture using carboxylic group functionalized magnetic nanoparticles before CE analysis.

Metal	Calibration curve y = mx + b		Sy/x	R	LOD (mg L^{-1})	LOQ (mg L^{-1})
	m	b				
Co	479415 ± 10904	-480 ± 628	1955	0.9939	0.004	0.013
Zn	475461 ± 11605	-244 ± 562	1709	0.9956	0.003	0.012
Cu	724855 ± 16115	4857 ± 891	2277	0.9971	0.004	0.012
Ni	1939666 ± 133231	970 ± 5403	5891	0.9839	0.008	0.028
Cd	468257 ± 21977	-3888 ± 1424	2394	0.9892	0.009	0.030

The proposed method to extract and preconcentrate metals has been compared with other methodologies used to preconcentrate and determine metals by CE. In the bibliography can be found different sorbent types such as chelex-100 [24] or silica gel [25] to carry out a solid phase extraction to preconcentrate heavy metals before CE analysis. Other alternatives to preconcentrate metals have been reported, e.g. the use of electromembranes and electrochemical preconcentration with CE-C^4D [26, 27], or the online combination of transient isotachophoresis and CE-UV [28]. In all cases, the new simple and cheap methodology proposes is able to reach similar or lower limits of detection. Taking to account the advantages of the use of magnetic nanoparticles, the methodology proposed in this work is an interesting alternative to improve the sensitivity of CE-UV technique.

The precision of the proposed method was also assessed in terms of reproducibility and repeatability expressed as relative standard deviation percent (% RSD). The values are shown in Table 2. Repeatability of injection was studied over a solution of metals at 0.1 mg L^{-1} which was previously extracted and preconcentrated by using 2 mg of magnetic nanoparticles. The re-extracted solution was injected 9 times in the CE system. Reproducibility was calculated applying the extraction and re-

extraction process to 3 independent aliquots of the metal mixture at 0.1 mg L^{-1} using 3 different portions of 2 mg of magnetic nanoparticles between three consecutive days. Every extract was injected 3 times in the CE system. The lack of repeatability and reproducibility for some analytes could be attributed to the small volume solution (200 μL) used to extract the metals from the magnetic nanoparticles. Values less than 8 % were obtained for Cu and Cd demonstrating the potential of this methodology for the determination of these metals.

Table 2. Repeatability and reproducibility of the metal mixture at 0.1 mg L^{-1} (n=9) by using the optimized methodology.

Metal	Injection Repeatability RSD (%)		Reproducibility RSD (%)	
	Peak area	Migration time	Peak area	Migration time
Co	9.35	1.99	12.91	2.51
Zn	9.76	3.22	11.86	2.81
Cu	7.06	1.03	6.86	1.27
Ni	14.65	3.10	16.63	1.66
Cd	5.99	1.71	8.58	1.69

Finally it was carried out a recovery study; a pineapple juice sample was spiked with 0.07 mg L^{-1} of a metal mixture and all the experimental steps described above were followed previous to the analysis. The recoveries obtained were 89.8, 87.8, 88.1, 83.1 and 95.9 % for Co, Zn, Cu, Ni and Cd, respectively.

3.4. Analysis of juice samples

The developed method was applied to quantify the concentration of metals in 4 commercial juices (2 of pineapple juice and 2 of peach juice).

The results are shown in Table 3. The concentrations of metals have been determined taking into account the calibration curves and the preconcentration factor of the volume in the mineralization of the sample. Any matrix effect was observed since juice samples were effectively mineralized and the final extract was an aqueous medium.

Table 3. Determination of metals in juice samples[(1)].

Number of sample	Type of juice	Analyte (mg L^{-1})				
		Co	Zn	Cu	Ni	Cd
1	Pineapple	-	0.015 ± 0.003	0.0209 ± 0.0008	*	*
2	Pineapple	-	0.012 ± 0.001	0.0098 ± 0.0006	-	-
3	Peach	-	0.0169 ± 0.0004	0.030 ± 0.001	*	-
4	Peach	-	0.041 ± 0.002	-	-	*

[(1)]The values shown in this Table take into account that 15 mL of juice samples were used to mineralize the sample and the final residue was dissolved in 10 mL of water. *Concentration found below LOQ.

The corresponding electropherogram of a juice sample (sample 1) is shown in Figure 6. Trace amounts of Zn and Cu were found in the pineapple juice. Ni and Cd were also detected, but not quantified.

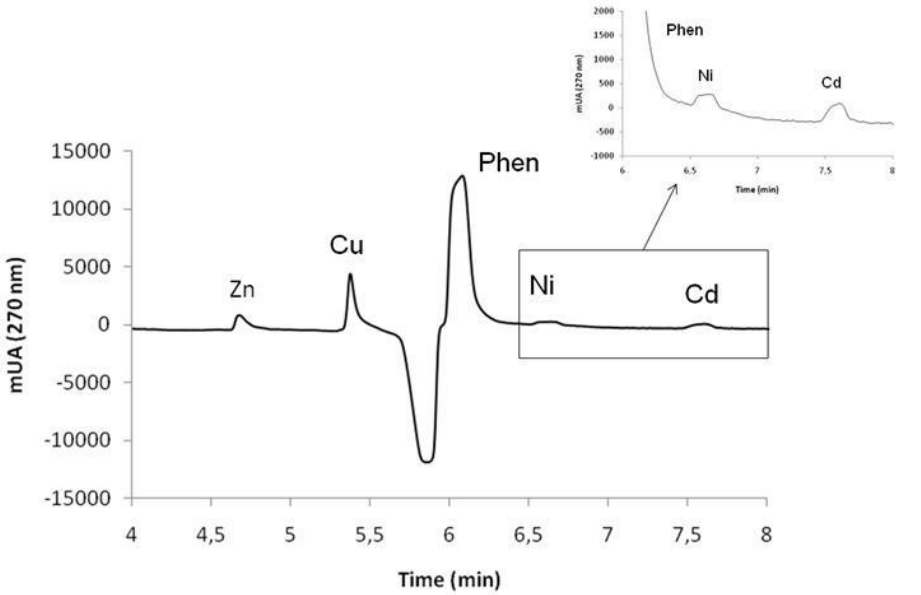

Figure 6. Electropherogram of pineapple juice sample applying the extraction and preconcentration steps using carboxylic group functionalized magnetic nanoparticles. Others conditions as in Figure 2.

These results have shown the potential of this carboxylic group functionalized magnetic nanoparticles to be used as preconcentrator of a group of metals before CE analysis. Further studies could be carried out with other food matrices by using the same optimized methodology.

4. Concluding remarks

The potential of the combination of carboxylic groups functionalized magnetic nanoparticles with the chelating reagent 1,10-phenanthroline to extract and preconcentrate metals (Cd, Co, Cu, Ni and Zn) has been demonstrated. Also, it was demonstrated that the sensibility improvement of

the CE with UV detection in addition with other capabilities, like being an easy and quick technique to separate and determinate metals in juice samples as an example of food matrices.

The main advantages of using carboxylic group magnetic nanoparticles as a sorbent to preconcentrate metals were (i) the low time consumption due to magnetically assisted separation; and (ii) their high surface areas; therefore, satisfactory results can be achieved by using less amounts of the sorbents. Consequently, if the sensitivity needs to be enhanced to determine a metal in a food sample by CE, the use of carboxylic group functionalized magnetic nanoparticles represents a very interesting alternative.

Acknowledgments

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Capítulo 4.2

Evaluation of phenylene-bridged periodic mesoporous organosilica as a stationary phase for solid phase extraction

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EVALUATION OF PHENYLENE-BRIDGED PERIODIC MESOPOROUS ORGANOSILICA AS A STATIONARY PHASE FOR SOLID PHASE EXTRACTION

A. Carpio[1], D. Esquivel[2], L. Arce[1], F.J. Romero-Salguero[3], P. Van Der Voort[2], C. Jiménez-Sanchidrián[3], M. Válcarcel[1*]

*[1]Department of Analytical Chemistry, University of Cordoba, Annex C3 Building, Campus of Rabanales, Cordoba, Spain. *qa1meobj@uco.es*

[2]Department of Inorganic and Physical Chemistry, Center for Ordered Materials, Organometallics and Catalysis (COMOC), Ghent University, Krijgslaan 281-S3, B-9000 Ghent, Belgium.

[3]Department of Organic Chemistry, Nanochemistry and Fine Chemistry Research Institute (IUIQFN), Campus of Rabanales, University of Cordoba, Campus de Excelencia Internacional Agroalimentario (CeiA3), Cordoba, Spain.

A periodic mesoporous organosilica, in particular, a phenylene-bridged material (Ph-PMO), was evaluated for the first time as a sorbent for retaining and eluting fenuron, simazine, atrazine, carbaryl and terbutryn in grape must by solid phase extraction (SPE) prior to their determination with capillary electrophoresis coupled with ultraviolet detection (CE-UV). The analytes were used as model compounds to demonstrate the potential of

Ph-PMO for increasing the sensitivity of CE. Under optimal conditions, the limits of detection for the analytes ranged from 0.6 to 4 μg/L, and their limits of quantitation from 2 to 10 μg/L. These values were comparable and, in some cases, even better than those obtained with C18 and HLB materials. Ph-PMO was characterized physicochemically by X-ray diffraction analysis, N_2 adsorption–desorption measurements and laser diffraction particle sizing. The sorbent afforded the extraction of atrazine, carbaryl and terbutryn from grape must with mean recoveries ranging from 86 to 105 %. Therefore, periodic mesoporous organosilicas possess a high potential as SPE materials.

Keywords: Pesticides, periodic mesoporous organosilicas, solid phase extraction, Capillary Electrophoresis.

1. Introduction

The synthesis and applications of mesoporous materials have grown rapidly ever since the discovery of “Mobil Crystalline Material number 41” (MCM-41) and related silica families by Mobil researchers in 1992 [1]. Although these materials were extensively studied, they required surface functionalization by “co-condensation” or “grafting” for some applications. In 1999, three research groups independently synthesized periodic mesoporous organosilicas (PMOs) [2-4]. These novel, interesting materials are generally synthesized by condensation of bridged organosilanes of the type $(R'O)_3Si\text{-}R\text{-}Si(OR')_3$ (with R’O being a hydrolyzable group such as methoxy or ethoxy) in the presence of a surfactant. As a result, the organic units are uniformly incorporated into the silica framework. The chemical and physical properties of PMOs can be modified by changing the bridging organic group in the organosilane precursor. Thus, a great variety of PMOs have been synthesized by using organosilanes of variable nature including methylene- ($–CH_2–$), ethylene- ($–C_2H_4–$), ethenylene- ($–C_2H_2–$) and phenylene-bridged ($–C_6H_4–$) materials [5]. Various complex bridges including heteroatomic and organometallic species have also been incorporated into PMOs [5]. In general, PMOs possess a high surface area, large pore volume, tunable mesoporous channels with a well-defined pore size distribution, controllable wall composition and easily modified surface properties. Moreover, they usually exhibit a higher hydrothermal and mechanical stability than their silica counterparts.

Mesoporous silicas have proved very useful as sorbents for diverse applications in analytical chemistry. In fact, different modified mesoporous materials have been successfully used to preconcentrate and separate

inorganic compounds including metal ions [6-8]; organic compounds such as VOCs [9, 10] or trinitrotoluene [11]; and chiral molecules such as L-valine [12]. For example, Barreto et al. [13] used the mesoporous silica MCM-41 to extract the pesticides trichlorfon, pyrimethanil, tetraconazole, thiabendazole, imazalil and tebuconazole from mango fruit for subsequent analysis by gas chromatography–mass spectrometry.

Unlike mesoporous silicas, PMOs are highly hydrophobic by virtue of the uniform distribution of organic moieties throughout their pore walls. In fact, PMOs have proved excellent adsorbents for organic molecules. Recently, the adsorption of toxic organic pollutants such as benzene and 4-nitrophenol [14], and the herbicide S-metolachor [15], in ethylene- and phenylene-bridged PMOs (Ph-PMO) was examined and Ph-PMOs were found to clearly surpass ethylene-bridged PMOs and related all-silica materials in this respect.

In any case, PMOs remain scarcely used owing to their unknown potential for extracting many chemical families (especially from complex matrices). To our knowledge, no application of Ph-PMOs (Figure 1a) to the extraction of pesticides from complex matrices or PMOs as solid phase extraction (SPE) sorbents has to date been reported.

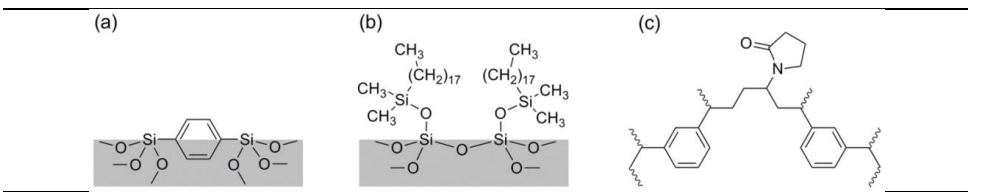


Figure 1. Surface structure of phenylene-bridged periodic mesoporous organosilica (Ph-PMO) (a) and C18 stationary phase (b), both organic–inorganic hybrid materials, and polymeric structure of Oasis HLB SPE sorbent (c).

The main objective of this study was therefore to assess the potential of Ph-PMO as a SPE sorbent for preconcentrating the pesticides fenuron, simazine, atrazine, terbutryn and carbaryl as model analytes from grape must prior to their determination by capillary electrophoresis with ultraviolet detection (CE-UV) (Figure 2). The results thus obtained were compared with those for commercial products such as C18 (Figure 1b) and Oasis hydrophilic–lipophilic balance (HLB) materials (Figure 1c) in terms of sensitivity and selectivity. In addition, the Ph-PMO material was characterized physicochemically and found to remain stable throughout the SPE process.

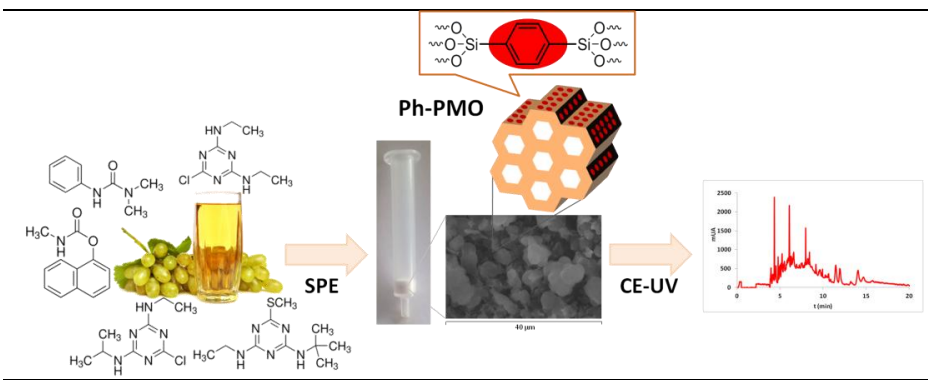


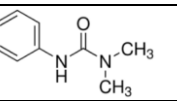
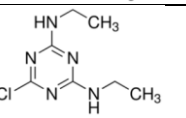
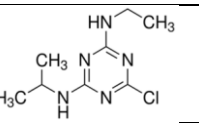
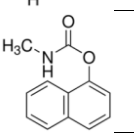
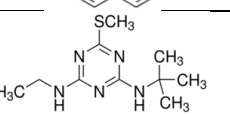
Figure 2. Representation of the SPE process showing the chemical structures of the studied pesticides, a picture of the prepared cartridges and a scanning electron micrograph of the Ph-PMO sorbent for extraction before CE analysis.

2. Experimental

2.1. Standards, reagents and grape must samples

Fenuron, simazine, atrazine, carbaryl and terbutryn were obtained from Sigma–Aldrich (St. Louis, MO, USA). Table 1 shows their chemical structures and selected properties.

Table 1. Chemical structure of the selected pesticides.

Peak number	Pesticide	Family	Molecular weight (g/mol)	Structure	Use
1	Fenuron	Ureas	327.59		Herbicide
2	Simazine	Triazines	201.66		Herbicide
3	Atrazine	Triazines	215.68		Herbicide
4	Carbaryl	Carbamates	201.22		Insecticide
5	Terbutryn	Triazines	241.36		Herbicide

The reagents were used to prepare stock solutions containing a 100 mg L^{-1} concentration in acetonitrile. Working solutions of the pesticides were prepared by appropriate dilution of the stock solutions with Milli-Q ultrapure water. Methanol, ethanol and the reagents used to prepare the buffer solution (sodium hydrogen phosphate, sodium dodecyl sulfate and acetonitrile) were also purchased from Sigma–Aldrich. The water used was purified by passage through a Milli-Q apparatus from Millipore (Bedford, MA, USA).

The SPE columns were manually prepared in 3 mL empty SPE cartridges and polypropylene frit purchased from Sigma–Aldrich.

The periodic mesoporous organosilica material used (Ph-PMO) was synthesized by following a previously reported procedure [16, 17]. The organosilica precursor, [1,4-bis(triethoxysilyl)benzene (BTEB)] and the surfactant Brij 76 [polyoxyethylene(10) stearyl alcohol] were also purchased from Sigma–Aldrich (St. Louis, MO, USA). Commercial SPE cartridges packed with Oasis HLB (Waters, Milford, MA, USA) and Bond Elut C18 (Varian, Harbor City, CA, USA) were also used. Grape must was purchased at local outlets.

2.2. Apparatus and operating conditions

The pH of the samples was adjusted with a MicropH 2000 meter from Crison (Barcelona, Spain). The analytes were separated and quantified on a P/ACE MDQ Capillary Electrophoresis System from Beckman (Palo Alto, CA, USA) equipped with a diode array detector and using a fused-silica capillary (Beckman Coulter) of 75 μm inner diameter, a total length of 60.2 cm and an effective separation length of 50 cm.

The CE operating conditions used to separate the pesticides were similar to those previously used by Hinsmann et al. [18]. The running buffer consisted of 10 mM sodium hydrogen phosphate, 60 mM sodium dodecyl sulfate and 8 % acetonitrile at pH 9.5. The applied voltage was 25 kV, the average current 72 μA and the working temperature 25 ºC. Samples were injected in the hydrodynamic mode at 0.5 psi for 5 s. Electropherograms

were recorded at 226 nm, using normal polarity. All buffer solutions were filtered through a Nylon membrane of 0.45 μm pore size (Terumo, Leuven, Belgium) before analysis.

The SPE procedure was performed on a Visiprep DL SPE Vacuum Manifold from Supelco (Bellefonte, PA, USA).

The Ph-PMO material was characterized by using various techniques. Powder X-ray diffraction (XRD) patterns were recorded by using CuKα radiation on an ARL X'TRA diffractometer from Thermo Scientific (Waltham, MA, USA). Nitrogen adsorption–desorption isotherms were obtained on a Micromeritics ASAP 2010 analyzer (Norcross, GA, USA) at –196 ºC. Prior to measurement, the samples were degassed at 120 ºC for 24 h. Specific surface areas were calculated by using the Brunauer–Emmett–Teller (BET) method and pore size distributions determined by analyzing the desorption branch of each isotherm with the Barrett–Joyner–Halenda (BJH) method. Particle sizes were measured with a Mastersizer S laser diffraction analyzer (Malvern Instruments, Worcestershire, UK), using water as dispersant. All samples were sonicated for 10 min before analysis. Scanning electron microscopy (SEM) was carried out using a JEOL 6300 microscope (Tokyo, Japan) with an Au-sputtered specimen at an accelerating voltage of 20 kV.

2.3. Solid phase extraction procedure

For SPE, an amount of 30 mg of sorbent material was packed into a 3 mL empty SPE cartridge capped with polypropylene frits at the bottom and

top. Before each use, the sorbent was conditioned by passing 5 mL of methanol and 5 mL of water in that sequence.

For preconcentration, a volume of 100 mL of standard or grape must samples was passed through the column. This was followed by elution with 1 mL of acetonitrile, evaporation to dryness of the eluate under a nitrogen stream and reconstitution of the residue in 200 μL of water.

3. Results and discussion

The potential of Ph-PMO as a SPE sorbent was assessed by using selected pesticides as model analytes which were extracted from grape must and subsequent determination by CE-UV. In addition, the PMO material was characterized in physicochemical terms simultaneously in order to confirm its stability during the SPE process.

3.1. Optimization of the SPE procedure

As stated in the Introduction, Ph-PMO is an excellent candidate as SPE sorbent in virtue of its favorable physicochemical properties, i.e. a high surface area, a uniform mesoporous size and a high pore volume in addition to its intrinsic hydrophobicity (Table 2).

Table 2. Physicochemical properties of phenylene-bridged PMO.

Material	d_{100} [a] (nm)	a_0 [b] (nm)	S_{BET} [c] ($m^2\ g^{-1}$)	V_p [d] ($cm^3\ g^{-1}$)	D_p [e] (nm)	Wall thickness [f] (nm)
Ph-PMO	5.4	6.2	790	0.57	3.2	3.0
Ph-PMO after use in SPE	5.3	6.1	658	0.47	3.2	2.9

[a] d_{100} spacing as determined from XRD patterns; [b] Unit cell dimension, calculated as $a_0 = 2d_{100}/\sqrt{3}$; [c] Specific surface area as determined with the BET method; [d] Pore volume; [e] Pore diameter as determined with the BJH method; [f] Estimated from ($a_0 - D_p$).

A scanning electron micrograph of Ph-PMO (Figure 2) revealed a morphology consisting of smooth, unevenly shaped particles with sizes between 3 and 12 μm. The physicochemical properties of these materials can be conveniently tailored by modifying the synthetic procedure, which makes the extraction of analytes of different natures possible. In this study, the molecular size of all pesticides was small enough to diffuse easily into the mesopores of Ph-PMO. Therefore, the potential of Ph-PMO as a sorbent was assessed by optimizing the following solid phase extraction variables: amount of PMO, volume and pH of sample and type of eluent.

The SPE process was optimized by using a mixture of the selected pesticides at a 100 μg/L concentration each. The influence of operational variables was assessed via the electrophoretic peak area.

3.1.1. Optimization of the amount of PMO

The effect of the amount of PMO packed in the SPE cartridge was assessed over the range 10–90 mg. A 100 mL of sample and 1 mL of solvent were used in all cases. As can be seen from Figure 3, the best results were obtained with 30 mg, which was thus selected for subsequent testing.

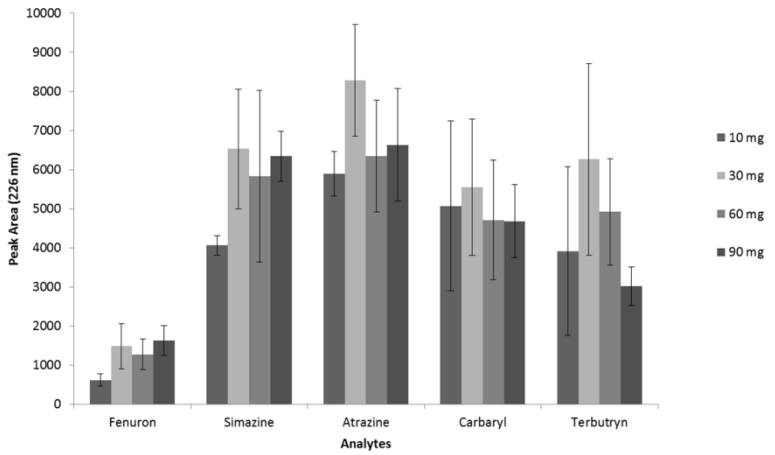


Figure 3. Influence of the amount of Ph-PMO used in the SPE procedure. The buffer used consisted of 10 mM sodium hydrogen phosphate, 60 mM sodium dodecyl sulfate and 8 % acetonitrile at pH 9.5. Other conditions: hydrodynamic injection at 0.5 psi for 5 s; applied voltage 25 kV; UV detection wavelength 226 nm.

Greater amounts decreased the analytical signals, probably because the solvent volume used to elute the analytes was inadequate to ensure complete extraction. If more than 30 mg of PMO had been used, the amount of eluent would have been increased above 1 mL to extract all analytes. No further optimization of the amount of PMO or eluent volume was done here, however, because one of the objectives was to use as little PMO material as possible in order to achieve the limits of detection (LODs) required to determine the selected pesticides in grape must.

3.1.2. Optimization of the sample volume

The volume of sample was optimized in order to be able to determine the selected pesticides in grape must with adequate sensitivity. The influence of the sample volume was studied by passing 40–200 mL of the standard mixture through Ph-PMO cartridges. Volumes below 100 mL led to poor electrophoretic signals, volumes from 100 to 130 mL gave virtually identical peak areas and higher volumes resulted in diminished signals — possibly as a result of the analytes being swept by too high a volume passing through the SPE column. A volume of 100 mL was therefore chosen as optimal for operational reasons.

3.1.3. Optimization of the sample pH

The influence of the sample pH on the SPE performance of the PMO material was initially studied by using a standard mixture of the pesticides at a variable pH from 2 to 9. Following passage of standards of variable pH through the SPE cartridges, the stationary phases were examined by XRD analysis. As can be seen from Figure 4, Ph-PMO exhibited identical XRD patterns whichever the pH; therefore, it retained its ordered hexagonal mesostructure (with a *P*6*mm* space group) throughout the sample pH range, which testifies to its high stability.

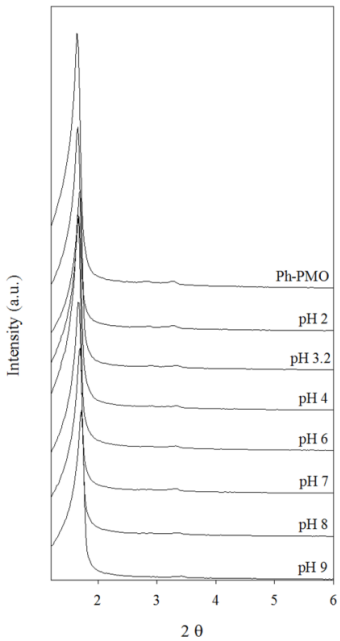


Figure 4. X-ray diffraction patterns for Ph-PMO after use as an SPE stationary phase at different pH values. The sample at pH 3.2 was real grape must.

The surface charge of Ph-PMO was expected to change with the pH of the sample. Also, hydrophobic and aromatic π–π interactions between aromatic rings in the pore walls of the Ph-PMO sorbent and the pesticides should be considered. At its isoelectric point (pH around 4 [15]), the surface of this material is essentially neutral, so hydrophobic and aromatic interactions prevail over electrostatic forces. The surface of Ph-PMO is positively charged below pH 4 and negatively charged above pH 4. Therefore, electrostatic interactions can also play a prominent role in retention of pesticides on Ph-PMO in this pH region.

The highest peak areas for the standard mixtures were obtained at pH 8 (the pH provided by the pesticides). Nevertheless, the pH of grape must is

around 3.2, therefore, the sample had to be initially adjusted to a pH of 8 prior to the SPE procedure. However, grape must contains a complex mixture of compounds of diverse nature and, when altering its natural pH, its color changes from yellow to brown. These conditions cause the formation of new compounds that compete with the pesticides for retention on Ph-PMO. Interferents and pesticides co-emigrated during electrophoretic separation, which precluded quantitation of the latter. This led us to use the natural pH of grape must in subsequent tests.

3.1.4. Optimization of the eluent

The nature of the elution solvent (particularly its polarity) is highly influential on SPE performance. Because pesticides are polar compounds, they are best extracted with polar solvents. In this work, we studied acetonitrile, ethanol and methanol as eluting solvents for extracting the pesticides from Ph-PMO. The highest signals were invariably obtained with the most polar eluent (acetonitrile), followed by ethanol and methanol —the respective polarity indices are 5.8, 5.2 and 5.1 (see Figure 5).

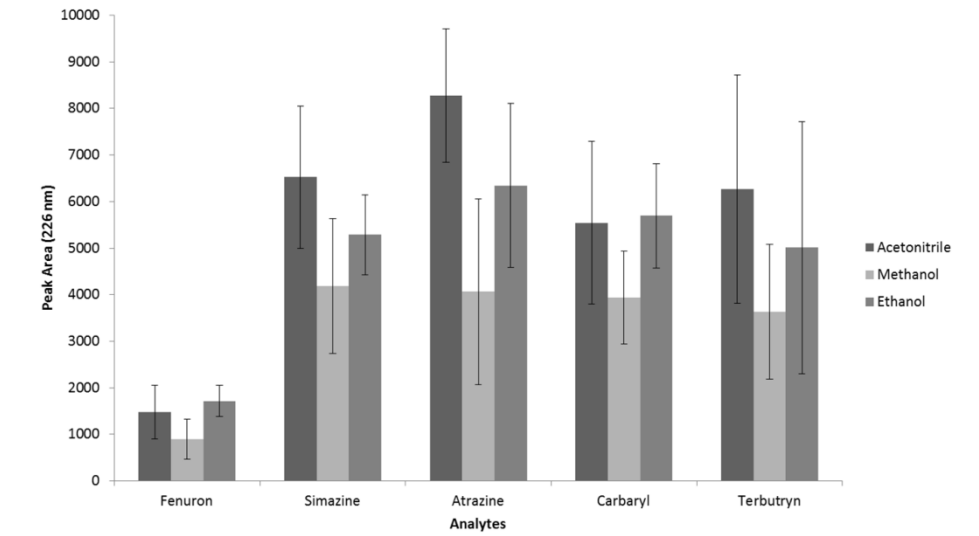


Figure 5. Influence of the type of eluent used in the SPE procedure. Other conditions identical as in Fig. 3.

The influence of the eluent pH was examined by using mixtures of acetonitrile and water at a variable ratio at pH 2. This choice of pH was dictated by the fact that the standard mixture had a naturally basic pH, so pH 2 would allow the influence of both polarity and pH on the elution efficiency to be assessed. As confirmed by XRD patterns, changing the solvent pH did not modify the mesostructure of Ph-PMO. The highest signal in virtually all instances was obtained with 100 % acetonitrile. Also, adding water at pH 2 to acetonitrile required subsequent time-consuming evaporation and slowed the SPE process as a result. Consequently, pure acetonitrile was chosen as the most suitable eluent to extract the mixture of pesticides from Ph-PMO.

3.2. Validation

The determination of the selected pesticides by CE-UV without preconcentration provided limits of quantification (LOQs) much higher than 10 μg/L for all analytes, which signaled the need for preconcentration. Table 3 shows the analytical figures of merit of the proposed method as applied to a standard mixture of pesticides containing 1–20 μg/L of fenuron and 0.5–20 μg/L concentration of the other pesticides. Calibration curves were obtained by plotting peak areas versus concentrations. Measurements were all done in triplicate. LODs and LOQs were calculated as three and ten times, respectively, the intercept deviation (Sb) divided by the slope.

With direct analysis by CE, LODs ranged from 0.2 to 0.8 mg/L and LOQs from 0.7 to 2.7 mg/L; with SPE, LODs were 0.6–4 μg/L and LOQs 2–10 μg/L. Therefore, SPE with Ph-PMO as sorbent prior to CE separation provided LODs well below the European Union's Maximum Residue Limits (MRLs) for the pesticides in wine grapes (viz., 0.01 mg/kg for fenuron, simazine, carbaryl and terbutryn; and 0.05 mg/kg for atrazine) in addition to acceptable LOQs.

Table 3. Parameters of the calibration curves and statistical figures of merit for the determination of a mixture of pesticides using Ph-PMO as a sorbent for SPE before CE analysis, and comparison with C18 and HLB.

	PMO						C18		HLB	
Analyte	Calibration curve ($y = mx + b$)		$S_{y/x}$	R^2	LOD* (μg/L)	LOQ* (μg/L)	LOD* (μg/L)	LOQ* (μg/L)	LOD* (μg/L)	*LOQ* (μg/L)*
	m	b								
Fenuron	255513 ± 2757	139 ± 31	54.8	0.9345	4	10	2	6	1	3
Simazine	258824 ± 6441	251 ± 66	171.7	0.9932	0.8	3	3	9	1	3
Atrazine	361032 ± 6711	99 ± 72	167.6	0.9966	0.6	2	1	4	0.8	3
Carbaryl	535695 ± 17782	118 ± 211	441.9	0.9923	1	4	1	4	3	10
Terbutryn	278283 ± 39473	2346 ± 289	441.6	0.9086	3	10	2	7	2	5

$S_{y/x}$ Regression standard deviation; R^2 Correlation coefficient; * Calculated from S_b

One important consideration in validating the overall analytical method was its usefulness for determining the pesticides in grape must, which is a complex matrix. Figure 6 shows the electropherogram for a grape must sample spiked with the pesticides and subjected to the SPE procedure using Ph-PMO, and analyzed by CE.

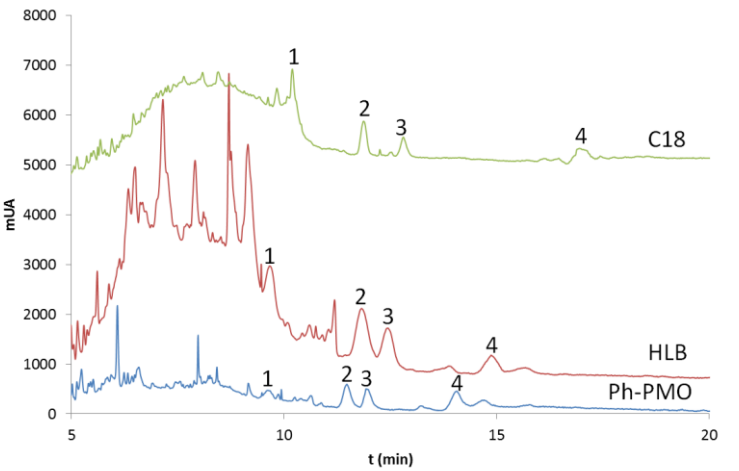


Figure 6. Electropherograms obtained after SPE of a grape must sample spiked with a 10 μg/L concentration of the standard mixture of pesticides by using C18, HLB and Ph-PMO as sorbents. Other conditions as in Fig. 3. 1, simazine; 2, atrazine; 3, carbaryl; 4, terbutryn.

As can be seen, different compounds present in grape must can co-emigrate with the analytes of interest, so they should be considered in analyzing real samples. Thus, fenuron could not be identified —not only because of its low absorbance relative to the other pesticides, but also because it co-eluted with other unknown compounds present in the must matrix. This analyte was therefore not measured in subsequent tests. Simazine was identified but, similarly to fenuron, it migrated with other interferences; therefore, the procedure was useless for determining

simazine in grape must. Under optimal SPE conditions, the Ph-PMO cartridges allowed the other pesticides (carbaryl, atrazine and terbutryn) to be successfully extracted from grape must.

The efficiency of Ph-PMO in retaining the selected pesticides was assessed by conducting recovery tests involving triplicate must samples spiked with the analytes at three different concentrations (10, 30 and 60 μg/L). The results are summarized in Table 4. The mean recoveries for atrazine, carbaryl and terbutryn ranged from 86 to 105 % and confirmed that Ph-PMO was an effective sorbent material for extracting these pesticides.

Table 4. Recoveries from grape must samples spiked with the analytes at different concentration levels as obtained by using Ph-PMO as SPE sorbent.

Analyte	Added (μg/L)	Recovery (%)
Atrazine	10	105 ± 7
	30	103 ± 4
	60	90 ± 10
Carbaryl	10	86 ± 3
	30	97 ± 6
	60	91 ± 5
Terbutryn	10	91 ± 7
	30	96 ± 12
	60	97 ± 9

The precision of the proposed method was assessed in terms of within-day and between-day precision, both expressed as % RSD, by repeatedly applying the whole SPE procedure. Standard mixtures of the pesticides and grape must spiked with them at a concentration of 100 μg/L were used for this purpose. Within-day precision was assessed by applying the procedure six times on the same day, and between-day precision by

analyzing three samples over three consecutive days. Each extract was injected three times into the CE system. As can be seen from Table 5, the results were acceptable in both cases.

Table 5. Precision in the analysis by SPE-CE of a standard mixture of the pesticides and grape must samples spiked with them.

Analyte	Within-day precision (%RSD, *n* = 6)		Between-day precision (%RSD, *n* = 9)	
	Standard	Sample	Standard	Sample
Fenuron	13	–	13	–
Simazine	10	–	13	–
Atrazine	12	13	14	15
Carbaryl	11	12	12	16
Terbutryn	12	13	13	14

3.3. Reusability

The reusability of a sorbent is important for process economy. That of Ph-PMO was assessed here in a series of extraction tests using an identical amount of sorbent.

Firstly, the sorbent was tested by passing a standard mixture of pesticides at a concentration of 100 μg/L through it. Using the same column a second time led to slightly decreased signals which, however, leveled off after a third use. The Ph-PMO columns performed similarly for all analytes except carbaryl, for which reusing the columns is not recommended. The reusability of Ph-PMO was also assessed with grape must, using a sample spiked with a mixture of 100 μg/L atrazine, carbaryl and terbutryn for SPE. The resulting signal was slightly smaller and the process slower in the second extraction. The decreased flow rate observed may have resulted

from some structural change in the material caused by the matrix components or by retention of some substance not eluted in the cleaning step between uses. Both the mesostructure and the particle size (Figure 7) of Ph-PMO were preserved after the first use of the SPE cartridge.

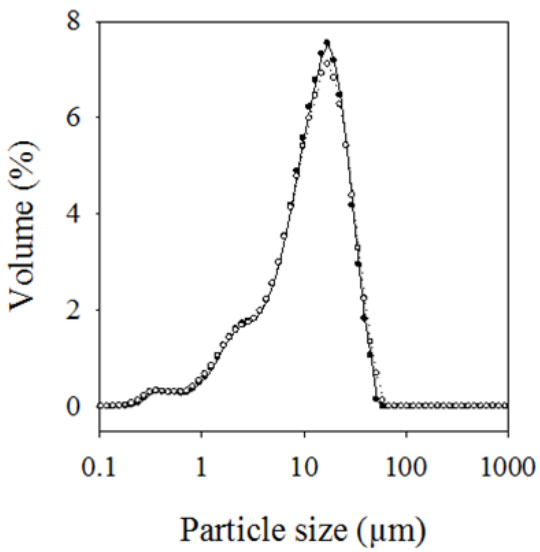


Figure 7. Particle size distribution curves for the parent Ph-PMO material (●) and Ph-PMO after use as an SPE sorbent (○).

In fact, the XRD pattern of the material used was identical to that shown in Figure 4. However, as can be seen from Table 2 and Figure 8, both the specific surface area and the pore volume decreased after use, even though the N_2 adsorption–desorption isotherm remained of the type IV, which is typical of mesoporous materials.

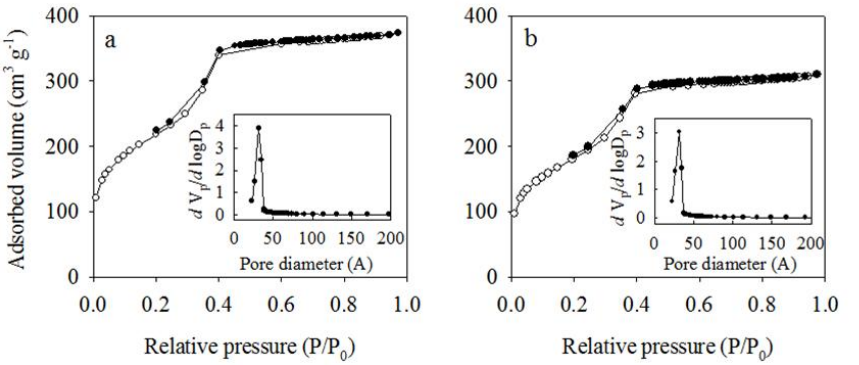


Figure 8. N_2 adsorption–desorption isotherms and pore size distribution curves for (a) the parent Ph-PMO material and (b) Ph-PMO after use as an SPE sorbent.

This can be ascribed to the presence of compounds from grape must that were retained by the sorbent but not removed by the eluent. Moreover, the third extraction left the material useless as it provided nonquantitative results. Consequently, Ph-PMO sorbent material can be reused twice for extracting pesticides from grape must without substantial changes in the analytical signals.

3.4. Comparison with commercial SPE cartridges

Following optimization for a mixture of pesticides, the proposed SPE procedure using Ph-PMO as sorbent was compared with others involving commercially available sorbents. Although C18 based materials are the most widely used for pesticides extraction, HLB sorbents have also proved effective for this purpose. In this study, 30 mg of both commercial cartridges were used for comparison with custom-made cartridges packed with Ph-PMO.

Firstly, the sorbents were compared in terms of sensitivity. Table 3 shows the LODs and LOQs in water obtained with C18, HLB and the Ph-PMO material. As can be seen, the Ph-PMO achieved the best limits for simazine and atrazine. The opposite situation was found for fenuron and terbutryn, although these limits were low enough to accomplish the official MRLs for pesticides in wine grapes. In the case of carbaryl, the results obtained were similar to those for C18, and better than those for HLB. These data demonstrated the potential of the Ph-PMO material as an alternative to commercial sorbents.

Secondly, a comparison of the selectivity between cartridges was carried out. In this case, the analyses consisted in passing a grape must sample spiked with 10 μg/L of a mixture of the pesticides through the different sorbents. As can be seen from Figure 6, Ph-PMO and C18 exhibited a similar ability not only to remove interferences but also to retain the selected analytes. However, HLB proved to be even more efficient in extracting the pesticides. Based on these results, it can be confirmed that Ph-PMO was as selective as C18 and HLB for the determination of the target pesticides in grape must, and hence equally useful for this purpose.

Finally, the reusability of C18 and HLB versus Ph-PMO was also assessed. With this objective, grape must samples spiked with 100 μg/L of pesticides were passed through the commercial sorbents in order to compare the results with those obtained with Ph-PMO material. In all cases it was observed that the more times the same cartridge was used, the darker the extracts were. The capacity of C18 to extract the pesticides in the first and second use was similar to that of Ph-PMO. However, in the third extraction, other compounds were dragged out of the material and some

interferences appeared in the electropherogram that made a reliable determination of the analytes impossible. On the other hand, the peak area of the analytes decreased for HLB in the second use and they were not well resolved in the third and fourth extractions. Consequently, in terms of number of extractions per cartridge of grape must samples, C18 and Ph-PMO could be used twice, whereas HLB could be employed only once due to the decrease in the intensity of the signals.

The results of this comparative test highlight the outstanding properties of this new type of material. Based on its properties, Ph-PMO could be assessed for clean-up and preconcentration steps of other types of analytes present in other samples in the future.

4. Conclusions

In this work, an alternative material consisting of a phenylene-bridged periodic mesoporous organosilica was successfully used as an SPE sorbent for the extraction of standard mixtures of pesticides prior to analysis by CE-UV. The new sorbent proved effective for extracting atrazine, carbaryl and terbutryn from grape must. The LODs and LOQs obtained were below the MRLs for these analytes in wine grapes.

Ph-PMO was similar in terms of selectivity and sensitivity to commercial C18 and HLB sorbents in extracting the target pesticides from grape must. Consequently, the Ph-PMO material studied in this work may be the starting point for developing a new family of PMO based stationary phases for SPE thanks to the ease with which their structure (symmetry,

pore size, particle size), nature of their organic bridges and even other functionalities can be broadly modified.

Acknowledgments

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Capítulo 4.3

Suitability of the zeolitic imidazolate framework 8 as sorbent in dispersive solid phase extraction of bisphenols

In Progress

SUITABILITY OF THE ZEOLITIC IMIDAZOLATE FRAMEWORK 8 AS SORBENT IN DISPERSIVE SOLID PHASE EXTRACTION OF BISPHENOLS BEFORE CAPILLARY ELECTROPHORESIS

A. Carpio, L. Arce, M. Valcárcel*.

*Department of Analytical Chemistry, University of Cordoba, Annex C3 Building, Campus of Rabanales, Cordoba, Spain. *qa1meobj@uco.es*

The metal-organic framework zeolitic imidazolate framewok 8 (ZIF-8) was evaluated as sorbent in dispersive solid phase extraction (dSPE), taking advantages of its high surface area and water stability, for preconcentrating for first time bisphenol A, bisphenol A diglycidyl ether and its hydrolyzed derivatives. Several parameters affecting the extraction of the target analytes by dSPE, including sample volume and sample pH, agitation time in retention and elution steps, and type of eluent were investigated. The optimized methodology was applied to the determination of these analytes in soft drinks by Capillary Electrophoresis (CE). Under those conditions, the limit of detection obtained ranging from 0.04 to 0.1 mg/L. The precision, expressed as relative standard deviation was between 7 and 14 %. And the recoveries were higher than 81%.

The suitability of ZIF-8 was compared with those obtained using C18 and HLB sorbents; and in dSPE mode, the signals obtained using ZIF-8 and

C18 were comparable. In addition, the best extraction efficiency was obtained when ZIF-8 was used under dSPE mode compared with the results obtained placing this material in a cartridge.

Keywords: Metal-organic framework, zeolitic imidazolate framework 8, dispersive solid phase extraction, bisphenols.

1. Introduction

Metal-organic frameworks (MOFs) are a new class of hybrid inorganic-organic microporous crystalline materials self assembly from metals ions with organic linkers via coordination bonds. A wide number of MOFs can be prepared depending on the combination of building blocks of metal ions and organic linkers, and this will involve diverse structures, topologies and porosities in the material [1-3]. They are resembling zeolites, able to use a minimum amount of metal ions and organic ligands to build maximum surface areas with predictable, controllable, tailorable and post-modifiable pores and cavities [4]. All these characteristics have made possible that MOFs have a great potential in diverse applications such as gas storage [5, 6], separation [7], catalysis [8] and drug delivery [9, 10].

Zeolitic imidazolate frameworks (ZIFs) are a subclass of MOFs, which have attracted considerable interest due to their remarkable properties such as high specific surface areas, high porosity, good thermal stability, and most importantly, water stability, a property that is rare among other porous MOFs [11]. ZIFs structures are made up of metal ions (Co, Cu, Zn,…) linked through N atoms of imidazolate or functionalized imidazolate ligands to provide four, six, eight, and twelve membered ring tetrahedral clusters [12]. Zeolitic imidazolate framework 8 (ZIF-8) is one of the most studies ZIF compounds, a prototypical ZIF material formed by 2-methylimidazole served as a bridging ligand between zinc central ions characterized by sodalite zeolite-type structure containing larger cavities (11.6 Å) and small apertures (3.4 Å) [13]. In addition, this MOF is characterized by its permanent porosity, hydrophobic properties and open metal sites, and especially it is very stable

in water samples [12]. Therefore, ZIF-8 has the potential to be used as a sorbent material for solid phase extraction (SPE) in sample preparation. Generally, the most often SPE configuration is the cartridge, in which the sorbent is packed in a column between two frits. The efficiency of SPE directly depends on the particle size and the surface area of the sorbent [14]. Consequently, to take advantages of the properties of new developed materials, different SPE configurations should be taken into account. Up to now, ZIF-8 and its derivatives have been employed to retain analytes from different chemical nature using various SPE modalities. For example, ZIF-8-derived nanoporous carbon was packed in a cartridge to preconcentrate carbamate pesticides [15]; polycyclic aromatic hydrocarbons [12, 16] and acidic drugs [17] were preconcentrated using membrane-protected-μ-SPE; online SPE was carried out to retain tetracyclines from water and milk [18]; also a derivatized magnetic ZIF-8 was used as a coating in solid phase microextraction (SPME) to determine estrogens in fish and pork [19].

With the objective of obtaining a high retention of target analytes, the contact between sample and sorbent should be favoured; therefore the use of dispersive SPE (dSPE) could be an attractive approach to achieve this objective. In this strategy a small amount of the sorbent is added to the sample, its dispersion is carried out by vortex or ultrasounds, and the phases are easily separated by centrifugation or filtration. Until now, only in two different papers ZIF-8 has been tested under dSPE; specifically a derivated magnetic ZIF-8 has been used as a sorbent in magnetic SPE to preconcentrate phthalate esters [20, 21]. In these papers the phases are separated via magnet. In addition, adsorption experiments were carried out with ZIF-8 to retain selectively theophylline over caffeine [13], or removal

arsenic [22] and p-arsanilic [20] from water samples. In these cases, the sorbent is dispersed in the sample; however, the authors study the concentration of analytes not retained to determine the capacity of this MOF to remove those analytes from the solution.

ZIF-8 was used for first time as a sorbent in dSPE to preconcentrate bisphenol A (BPA), bisphenol A diglycidyl ether (BADGE) and its hydrolyzed derivatives, bisphenol A (2,3-dydroxyprophyl) glycidyl ether (BADGE·H_2O) and bisphenol A bis (2,3-dihydroxyprophyl) ether (BADGE·$2H_2O$). These substances were selected due to endocrine disruptors such as BPA have been attracting the attention of the scientific community due to their possible negative effects on human health and the environment [23, 24]. BPA is mainly used as a monomer in the preparation of polycarbonate plastic and epoxy resins, and it is the substrate to produce BADGE, the main monomer used in the epoxy industry. Epoxy resins are the predominant protective coatings used for lining the interior of metal food cans, and it is documented that these compounds can migrate into the product. For all these reasons, there is a need of analytical methods to determine these type of compounds and the European Union has set specific migration limits (SMLs) for the sum of BADGE and its derivates in food, establishing s SML of 9 mg/Kg for the sum of BADGE and its hydrolyzed derivates [25], and 0.6 mg/Kg for BPA [26]. Therefore, these analytes can migrate from containers into a variety of foods and beverages and considered to be a potential toxic food contaminant.

To date, various MOFs have been used to extract BPA from different samples [27-29]; however, any article has reported the use of a MOF to preconcentrate BPA. The main objective of this article was to study the

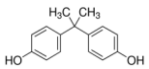
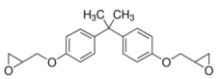
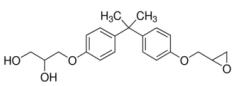
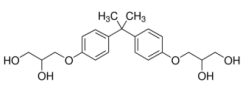
suitability of ZIF-8 as a SPE sorbent to preconcentrate the selected analytes prior to their determination by Capillary Electrophoresis with Ultraviolet detection (CE-UV) to increase the sensitivity of the analytical technique. The dSPE approach was compared with the classical SPE strategy using a cartridge to show the importance of selecting the appropriate mode for preconcentrating analytes according to the characteristics of the material selected in each case. In addition, the results obtained were compared with those for commercial products such as C18 and Oasis HLB.

2. Experimental

2.1. Standards, reagents and samples

All chemicals and solvents were of analytical grade. BPA, BADGE and BADGE·H_2O were obtained from Sigma–Aldrich (St. Louis, MO, USA), and BADGE·$2H_2O$ from Santa Cruz Biotechnology (Heidelberg, Germany). Table 1 shows their chemical structures and selected properties. The reagents were used to prepare stock solutions containing a concentration of 2500 mg L^{-1} in acetonitrile. Working solutions at 100 mg L^{-1} were prepared by appropriate dilution with acetonitrile from the stock solutions. Methanol, ethanol and the reagents used to prepare the buffer solution (sodium hydrogen phosphate, sodium dodecyl sulfate and isopropanol) were also purchased from Sigma–Aldrich. The water used was purified by passage through a Milli-Q apparatus from Millipore (Bedford, MA, USA).

Table 1. Chemical structure of target analytes.

Analyte	CAS number	Structure	Molecular Weight (g/mol)	Volume (Å^3)	pKas
Bisphenol A (BPA)	80-05-7		139.11	221.36	10.29 10.93
Bisphenol A diglycidyl ether (BADGE)	1675-54-3		340.41	319.26	-
Bisphenol A (2,3-dydroxyprophyl) glycidyl ether (BADGE•H_2O)	76002-91-0		358.42	339.02	13.53 15.02
Bisphenol A bis (2,3-dihydroxyprophyl) ether (BADGE•2H_2O)	5581-32-8		376.44	359.24	14.72 15.32

ZIF-8 was provided from Sigma Aldrich, under the commercial name Basolite Z1200. SPE cartridges packed with Oasis HLB (Waters, Milford, MA, USA) or Bond Elut C18 (Varian, Harbor City, CA, USA) were also used. Soft drinks were purchased at local supermarkets.

The SPE procedure using a cartridge was conducted with 3 mL empty SPE cartridges and polypropylene frit purchased from Sigma–Aldrich.

2.2. Apparatus and operating conditions

The pH was measured with a MicropH 2000 meter from Crison (Barcelona, Spain). An ultrasound bath from Pce Instruments (Tobarra, Spain) and a vortex from Heidolph (Schwabach) were used in the sample treatment step. Samples were centrifuged in a High Speed Centrifuge with Microprocessor Control from J.P. Selecta, S.A. (Abrera, Barcelona, Spain).

The analytes were separated and quantified on a P/ACE MDQ Capillary Electrophoresis System from Beckman (Palo Alto, CA, USA) equipped with a diode array detector and using a fused-silica capillary (Beckman Coulter) of 75 µm inner diameter, a total length of 40.2 cm and an effective separation length of 30 cm.

The CE operating conditions used to separate the BPA, BADGE and its hydrolyzed derivatives were similar to those previously used by [30]. The running buffer consisted of 25 mM sodium hydrogen phosphate, 200 mM sodium dodecyl sulfate and 35% isopropanol at pH 2.5. The applied voltage was 10 kV, the average current 85 µA and the working temperature 25 ºC. Samples were injected in the hydrodynamic mode at 0.5 psi for 15 s. Electropherograms were recorded at 194 nm, using reverse polarity.

Prior to first use, the capillary was conditioned by rinsing with 1 M HCl for 5 min, 0.1 M NaOH for 10 min, and separation buffer for 10 min. The capillary was prepared for daily use by rinsing with 0.1 M NaOH for 5 min, milli-Q H_2O for 5 min and separation buffer for 10 min. Before each analysis, the capillary was flushed with milli-Q H_2O for 1 min, 0.1 M NaOH for 2 min, milli-Q H_2O for 1 min and separation buffer for 2 min prior to injection.

All buffer solutions were filtered through a Nylon membrane of 0.45 µm pore size (Terumo, Leuven, Belgium) before analysis.

2.3. Dispersive solid phase extraction procedure

10 mg of ZIF-8 was transferred into a centrifuge tube. Prior each experiment ZIF-8 was preconditioned shaking it in a vortex for 1 min with 1

mL of methanol, and after discarded the supernatant. Then the same operation was repeated using milli-Q H_2O.

After that, 10 mL of sample was added to the tube, and it was placed in an ultrasound bath for 1 min for the dispersion of ZIF-8. In case of using soft drinks, they were previously degassed by ultrasounds. Then, the mixture was shaken in a vortex for 1 min and centrifuged for 3 min at 9000 rpm. The supernatant was discarded. Following this procedure, 1 mL of acetonitrile was added to the tube, and the same procedure of sonication and agitation was carried out. After centrifugation, the eluate were evaporated to dryness under a nitrogen stream, and dissolved in 200 μL of water. Finally, the extract was filtered with 0.45 μm nylon filter prior its analysis by CE-UV.

To compare the results obtained using dSPE, the same material was packing in a cartrigde to carry out the preconcentration of the analytes. Therefore, an amount of 10 mg of sorbent material was packed into a 3 mL empty SPE cartridge capped with polypropylene frit at the bottom and top. The same procedure describes above was also followed in this case.

3. Results and discussion

3.1. Optimization of the dispersive solid phase extraction procedure

As it has been aforementioned in the Introduction, BPA, BADGE and its derivatives can appear in plastic and canned products. Therefore previously to the optimization step, a study of the possible components

present in distillated water samples which can interfere with the analytes of interest was carried out, because of contamination derived from the labware could interfere in the CE analysis. None of the compounds selected as target analytes was found in the lab material used to perfom the dSPE-CE method.

To evaluate the use of ZIF-8 as sorbent in dSPE, four bisphenols were selected as model compounds; specifically BPA, BADGE, BADGE·H_2O and BADGE·$2H_2O$. The effects of different experimental parameters such as the amount of ZIF-8 and sample volume, the agitation time in the retention and elution steps, the pH of the sample solution and the type of eluent were investigated.

The dSPE procedure was optimized using a mixture of analytes at a concentration level of 0.5 mg/L. The influence of each variable was assessed via the electrophoretic peak area.

3.1.1. Optimization of the retention variables

3.1.1.1. Optimization of the amount of ZIF-8 and sample volume

The sample volume and the amount of ZIF-8 are related variables; hence a bivariant approach was selected to obtain an optimum value. Therefore, both variables were optimized together considering three different amounts of ZIF-8 (5, 10 and 15 mg) and three different sample volumes (2, 6 and 10 mL). The sample volume was limited by the volume of the centrifuge tube. Generally, when higher samples volumes are

processed, higher amount of sorbent should be used. The other initial conditions were 1 min for the dispersion of ZIF-8 with ultrasounds and 3 min with agitation via vortex; this procedure was followed in retention and elution steps.

The optimization of both variables was performed taking into account all the peaks from target analytes. However, as a similar behavior for all of them was observed, to simplify the data, the sum of the peak area of each compound was considered as a response. As a result (Figure 1), the highest signals were obtained using 10 or 15 mg of ZIF-8 and 10 mL of sample; therefore, with the objective of using the minimum amount of sorbent necessary, 10 mg and 10 mL were chosen as optimum values.

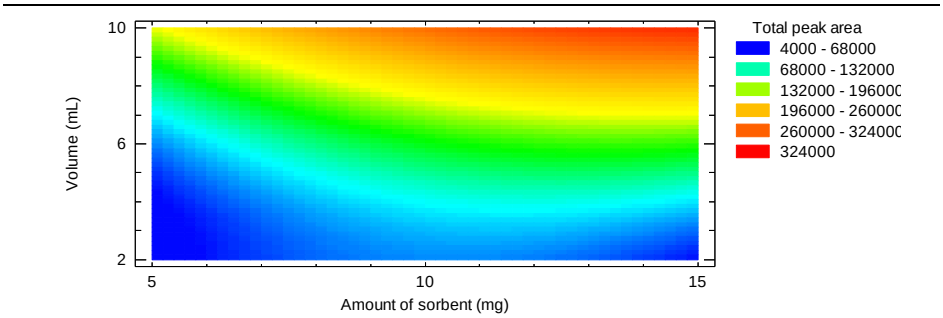


Figure 1. Bivariant effect of the sample volume and amount of ZIF-8 in the dSPE procedure. The buffer used consisted of 10 mM sodium hydrogen phosphate, 25 mM sodium sodium hydrogen phosphate, 200 mM sodium dodecyl sulfate and 35% isopropanol at pH 2.5. Other conditions: hydrodynamic injection at 0.5 psi for 5 s; applied voltage 10 kV; UV detection wavelength 194 nm.

3.1.1.2. Optimization of the agitation time in the retention step

In a preliminary experiment, the effect of ultrasounds and shaking methods on the extraction was investigated. The agitation via vortex was the procedure with mayor influence in the retention of the analytes, on the contrary the ultrasounds only increased slightly the peak areas. Hence, ultrasounds were only chosen to promote the dispersion of ZIF-8 into the sample solution, and the agitation time using vortex was optimized.

The effect of the retention time was studied in the range of 1-10 min. The results showed that there is no considerable change in the signals during the experiments (Figure 2). The adsorption process seemed to reach equilibrium after 1 min, at this time the electrophoretic signals stabilized.

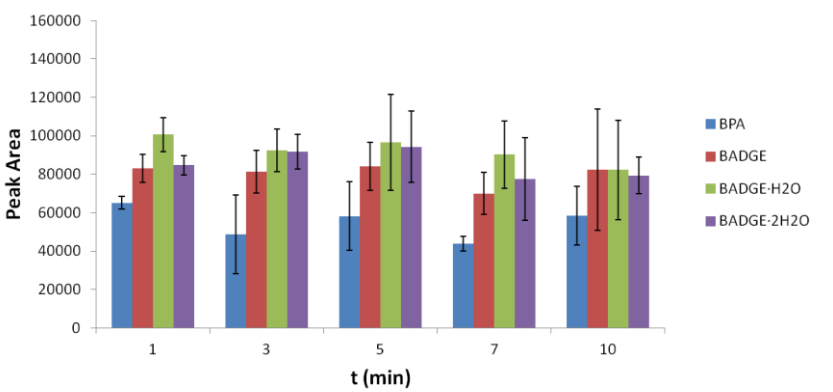


Figure 2. Influence of agitation time in the retention step of the dSPE procedure. Other conditions identical as in Figure 1.

Therefore this value was chosen as optimum in the extraction procedure. Visually, using this modality of SPE, a fast good dispersion of the material in the sample containing the analytes was observed. The high

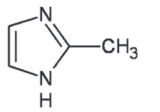
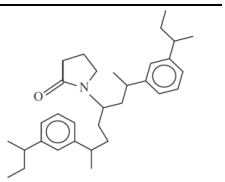
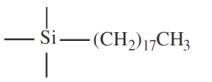
surface area that presents ZIF-8, and in general MOFs, helps to a quick contact between the sorbent and the target analytes. Obviously, in the desorption step this fact was also beneficial.

3.1.1.3. Optimization of the sample pH

In general the pH of sample plays an important role in the retention, it affects not only to the analytes, but also to the sorbent material. In this article, a range from 2 to 12 was studied. The electrophoretic peak areas remained practically constant at pH values from 4 to 10. Further, an increase in the pH values led to decrease electrophoretic signals. The pH of the standard mixture is ≈8, therefore in the following experiments the pH was not adjusted.

According to the previous reports on zeta potential of ZIF-8, ZIF-8 is positively charged over the pH range 2-10 [13]. Taking into account the characteristics of the analytes (BADGE is totally hydrophobic), electrostatic interactions cannot be responsible of the retention of them, but from the experimental data it can be confirmed that analytes are efficiently extracted in the ZIF-8. Therefore the mechanism that could be responsible of the adsorption of the analytes into the ZIF-8 is the π-π interactions between the imidazolium rings on the surface of ZIF-8 (Table 2) and the benzene ring of the analytes, as it has been stated previously in the retention of BPA with other MOFs [27-29].

Table 2. Physicochemical properties of sorbents.

Sorbent	Surface area (m^2/g)	Pore diameter (Å)	Pore volumen (mg/L)	Particle size (μm)	Functional group
ZIF-8	1300-1800	11.6	0.65	4.9	
HLB	823	82	1.34	30.3	
C18	463	73	0.85	53	

3.1.2. Optimization of the elution variables

3.1.2.2. Optimization of the agitation time in the elution step

As the same manner that in the retention step, firstly, 1 min in the ultrasounds bath were used to disperse the ZIF-8, in this case, through the solvent. The effect of agitation via vortex on the extraction of the target analytes in 1 mL of acetonitrile was explored by using times ranging from 1 to 10 min.

Electrophoretic peak areas did not change significantly when different agitation times were used. This indicates that extraction of analytes to the solvent was very fast since the contact surface area between the extraction solvent and ZIF-8 was very large, due to the high surface of the material. Therefore, 1 min was considered to be sufficient to the elution of the analytes from the sorbent.

3.1.2.1. Optimization of the eluent

In previous experiments the volume of organic solvent was tested, and 1 mL was chosen to assure the complete extraction of the target analytes. In a second step the type of eluent was studied, and acetonitrile, ethanol and methanol were used as potential eluting solvents. There were no significant differences between the solvents, but the highest peak areas were obtained using acetonitrile; therefore, this solvent was used in the subsequent experiments.

3.2. Characterization of the method

In order to validate the methodology proposed, a standard mixture of the analytes and cola soft drink were selected. As it has been stated in the Introduction, these compounds can migrate from the lining of canned drinks to the sample, so the validation was done using the analytes dissolved in bottle soft drink.

Calibration curves were obtained by plotting peak areas versus concentrations. All experiments were done by triplicate. Limits of detection (LODs) and quantification (LOQs) were obtained as three and ten times, respectively, the intercept deviation (S_b) divided by the slope. Table 3 shows the analytical figures of merit of the proposed dSPE using CE-UV for a standard mixture of the analytes in the range of 0.005-1.5 mg/L; and in same Table appears the values obtained diluting the analytes in soft drink to take into account the matrix effect. As can be seen, the LOQs were acceptable according to the European Commission [25, 26].

Table 3. Figures of merit for the determination of target analytes using ZIF-8 as a sorbent for dSPE before CE-UV analysis.

Analyte	Standard in water						Cola soft drink	
	Calibration curve (y = mx + b)		$S_{y/x}$	R^2	LOD[a] (mg/L)	LOQ[a] (mg/L)	LOD[a] (mg/L)	LOQ[a] (mg/L)
	m	b						
BPA	134604±1389	142±791	1874	0.9995	0.02	0.06	0.05	0.16
BADGE	136604±6517	1996±1279	2796	0.9887	0.03	0.09	0.06	0.19
BADGE·H_2O	183933±2173	-176±1620	3257	0.9993	0.03	0.09	0.04	0.15
BADGE·$2H_2O$	143088±1945	18429±1198	1668	0.9994	0.02	0.08	0.10	0.34

$S_{y/x}$. regression standard deviation; R^2. correlation coefficient. [a]Calculated from S_b.

A typical electropherogram corresponding to the extraction with ZIF-8 of spiked cola soft drink with 0.5 mg/L of the target analytes is represented in Figure 3. As it can be seen, the four analytes were perfectly separated and all peaks resulted with good shape.

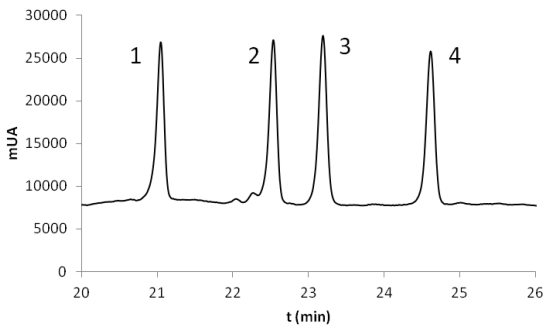


Figure 4. Electropherogram corresponding to the extraction with ZIF-8 of spiked cola soft drink with 0.5 mg/L of the target analytes. 1: BPA; 2: BADGE; 3: BADGE·H_2O; 4: BADGE·$2H_2O$.Other conditions identical as in Figure 1.

The precision of the method was evaluate in terms of within day and between day precision, both expressed as % RSD of peak areas. Standard mixtures of the analytes and soft drink samples spiked with them at 0.4 mg/L and 0.8 mg/L were used for this purpose. Within day precision was assessed by application the proposed method during six times on the same day. Between day precision was evaluated with a similar procedure and analyzing three samples over three consecutive days. Each sample was injected by triplicate. As can be seen in Table 4, the precision values were acceptable, ranging from 7% to 14%.

Table 4. Precision in the analysis by dispersive SPE-CE of a standard mixture of the target analytes and cola soft drink samples spiked with them (level 1: 0.4 mg/L; level 2: 0.8 mg/L).

Analyte	Within-day precision (% RSD. *n* = 6)				Between-day precision (% RSD. *n* = 9)			
	Standard		Cola soft drink		Standard		Cola soft drink	
	Level 1	Level 2	Level 1	Level 2	Level 1	Level 2	Level 1	Level 2
BPA	9	10	11	9	9	11	13	12
BADGE	7	9	10	10	9	9	13	12
BADGE·H_2O	8	7	9	7	9	10	14	12
BADGE·$2H_2O$	7	10	10	7	10	11	10	14

In addition, recovery experiments were carried out in order to check the trueness of the method. As a consequence, recovery tests involved triplicate cola soft drink samples spiked with the analytes at three different concentrations (0.4, 0.8 and 1 mg/L). The results are shown in Table 5, and recoveries between 81 and 89 % were obtained, that confirmed the suitability of ZIF-8 to extract the analytes.

Table 5. Recoveries from cola soft drink samples spiked with the analytes at different concentration levels as obtained by using ZIF-8 as sorbent.

Analyte	Added (mg/L)	Recovery (%)
BPA	0.4	88±7
	0.8	87±7
	1	85±3
BADGE	0.4	86±6
	0.8	81±4
	1	87±5
BADGE·H_2O	0.4	89±6
	0.8	87±3
	1	85±4
BADGE·$2H_2O$	0.4	84 ±6
	0.8	86±5
	1	88±9

3.3. Reusability

The potential commercial application of this method can be dependent on the reusability of the material in terms of to achieve a reduction in the cost procedure. In this study the reusability of the ZIF-8 was evaluated in a series of extraction tests using the same amount of sorbent (10 mg). The test was performed with a mixture of standards at 0.5 mg/L, and sample spiked with the mixture of analytes at the same level of concentration. In order to reuse the sorbent, any residual should be thoroughly desorbed prior to the next use; therefore, between uses of the ZIF-8 the material was washed with 1 mL methanol and 1 mL water. When the standards dissolved in water are used, as shown in Figure 4, the material can be used twice. The same conclusion was achieved for cola soft drink samples spiked with a mixture of analytes.

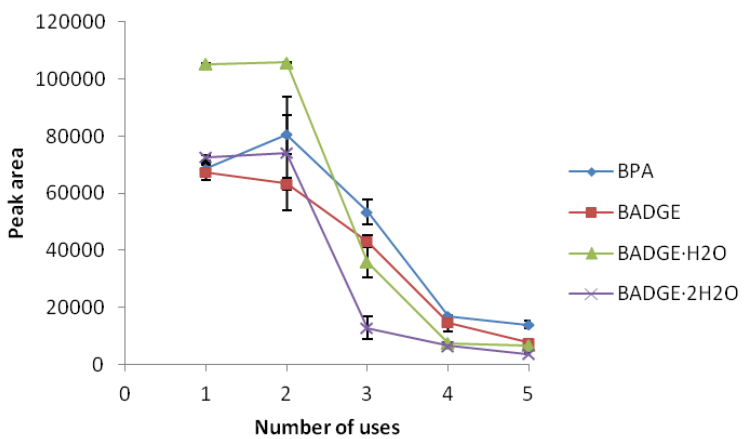


Figure 4. Number of cycles of use of 10 mg of ZIF-8 to extract and preconcentrate the target analytes. 1: BPA; 2: BADGE; 3: BADGE·H_2O; 4: BADGE·$2H_2O$. Other conditions identical as in Figure 1.

3.4. Comparison with other commercial sorbents

BPA has been the bisphenol most studied till now, and it was selected together with BADGE and its hydrolyzed derivatives as analytes test to demonstrate the potential of this new material. Nevertheless, HLB and C18 are the most common classical sorbents used to retain BPA [31, 32]. As a consequence, to evaluate the applicability of ZIF-8, its performance was compared with the results obtained with these classical sorbents. HLB is a divinylbenzene/N-vinylpyrrolidone copolymer and C18 is a hydrophobic silica-based bonded phase, therefore HLB and C18 provides reversed-phase retention. Chemically comparing with ZIF-8 (see Table 2), both materials have less surface area and higher pore diameter than the MOF selected in this study.

In order to carry out the comparison, 10 mg of HLB and C18 were used to preconcentrate a standard mixture of the target analytes at a level of 0.5 mg/L using the optimized dSPE procedure. As can be seen in Figure 5A, when a mixture of analytes in water was analyzed by dSPE-CE, the highest signals resulted when HLB was used, followed by ZIF-8 and C18. The high retention of the analytes on HLB versus ZIF-8 could be due to the presence of two benzene rings in the structure of the polymeric sorbent that could provokes stronger attractions with the benzene rings of the analytes. The signal between C18 and ZIF-8 were comparable, even it can be said, that the signals in ZIF-8 were slightly higher than those obtained with C18.

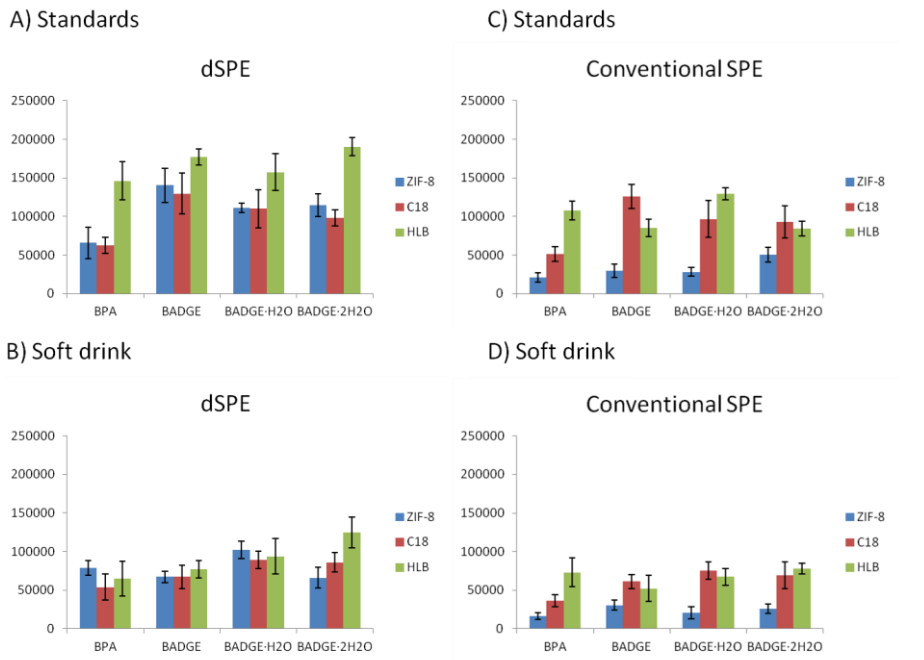


Figure 5. Comparison of peak areas obtained using dSPE and conventional SPE for ZIF-8, C18 and HLB; corresponding to a standard mixture of the target analytes at 0.5 mg/L, and spiked cola soft drink with 0.5 mg/L with the mixture of analytes. Other conditions identical as in Figure 1.

Although, ZIF-8 posses the highest surface area, it was not an advantage to retain the target analytes. The experimental values obtained can be explained due to the fact that analytes have a size larger than the pore diameter of ZIF-8 and therefore they couldn't enter to the pores, and the interaction only occurs on the outer surface of the MOF. The same experiments were also carried out spiking a cola soft drink with the mixture of analytes at a concentration of 0.5 mg/L (Figure 5B) and the same conclusion was achieved. However, the LOD and LOQ obtained for BPA, BADGE and its hydrolyzed derivatives using ZIF-8 is enough for the purpose of this work.

3.5. Comparison between SPE modalities (cartridge, dispersive)

Nowadays the most common approach to retain analytes is packing sorbent in a cartridge. This method is usually preferred to conventional liquid-liquid extractions because of SPE consumes minimal amount of organic solvents among other advantages. However, generally the sample loading step requires a relative long period of time due to the limited diffusion of the sample through the SPE cartridge, and even in case of use complex matrices and high sample volumes, the sorbent can suffer clogging. dSPE reduces the time required for this operation and overcome the second drawback exposed. In addition, this procedure enables the sorbent to interact equally rapidly with all the target analyte by means of ultrasounds, vortex or using an emulsifier. This approach permitted higher extraction efficiencies due to the increasing interactions that are established between the MOF sorbent and the analytes.

To check the suitability of ZIF-8 to be used as a sorbent in the dSPE modality, a comparison between both modalities (cartridge and dispersive) in the same conditions was carried out using a standard mixture of the analytes and spiked cola soft drink at a concentration of 0.5 mg/L. The peak areas obtained can be seen in Figure 5C and 5D. In addition the same experiment was performance using C18 and HLB sorbents to check also their behavior in both modalities. It is noticeable that in case of standards and real samples, the highest increase of signal using dSPE was found with ZIF-8 that is the material that presents the highest surface area. That confirms that the potential of ZIF-8 to retain the analytes is fulfilled using the dispersive modality; its large surface area and short diffusion route can

result in high extraction efficiency and rapid extraction dynamics. However, in case of C18 and HLB that structurally possesses less surface area, the increase was not as noticeable as when ZIF-8 was used as a sorbent in SPE.

4. Conclusions

In the present work a dSPE method based on the use of ZIF-8 to preconcentrate BPA, BADGE and its hydrolyzed derivatives has been successfully developed. This MOF was suitable for the extraction of the analytes from soft drinks samples prior to their determination by CE-UV, increasing the sensitivity of the methodology.

When materials with high surface area are used, it has been demonstrated that the sorbent dispersion leads to an improvement in the extraction. The potential of using dSPE before CE could be extended to others different applications fields. ZIF-8 in particular, and MOFs in general are a new family material with a characteristics properties which can be used in analytical chemistry field as a sorbent in dSPE.

Acknowledgements

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BLOQUE 5

RESULTADOS Y DISCUSIÓN

Este bloque de la Tesis Doctoral ofrece una visión general de los resultados más relevantes que se han obtenido a lo largo del desarrollo de la misma. Para ello se va a evaluar el potencial de la CE-UV/Vis para actuar como herramienta en el desarrollo de procesos analíticos para la extracción de información (bio)química en el ámbito agroalimentario. Por un lado se va a discutir el papel que puede representar a la hora de resolver problemas en esta área; y por otro cómo se pueden resolver las limitaciones que presenta la técnica. En este sentido, los resultados y discusión se ha estructurado en dos apartados correspondientes a los trabajos experimentales contenidos en los Bloques 3 y 4 de esta Memoria.

La Electroforesis Capilar en el ámbito agroalimentario sin etapa de preconcentración en el tratamiento de muestra

Dentro de este primer apartado se engloban las metodologías electroforéticas que se han desarrollado en el campo de la diferenciación de productos y seguimiento de metabolitos secundarios tóxicos producidos por hongos.

Con respecto a la diferenciación de productos, recientemente ha incrementado el interés de la población en la forma de producción de los alimentos, ya que es un aspecto que influye en las propiedades sensoriales del alimento, su valor nutricional y su seguridad. Este desarrollo ha impulsado el mercado de los productos ecológicos. Y debido a que su producción implica un mayor coste, como consecuencia su precio en el mercado es mayor; y esto lo convierte en un área susceptible al fraude.

Con lo cual se necesitan herramientas analíticas que puedan certificar los productos ecológicos, y así evitar cualquier tipo de adulteración. En esta Memoria se ha demostrado el potencial de la CE-UV/Vis para contribuir a la toma de decisiones dentro de esta área. En concreto a la diferenciación del régimen de alimentación suministrado a las cabras.

El éxito del proceso analítico comienza con el muestreo, y acaba con la interpretación de los datos obtenidos. El muestreo es la primera subetapa de las operaciones previas (Figura 1), y debe ser coherente con el problema analítico planteado. Además es la base de la representatividad, la propiedad analítica suprema que se atribuye a los resultados que se generan. Un resultado que sea exacto pero no representativo, es un resultado de mala calidad, ya que no proporciona la información requerida por la sociedad. Por tanto al ser el muestreo una de las mayores fuentes de error, se debe planificar cuidadosamente como parte del método analítico. En el caso de los trabajos relacionados con el análisis de la leche de cabra, el muestreo se hizo con ayuda de veterinarios y ganaderos expertos en el tema.

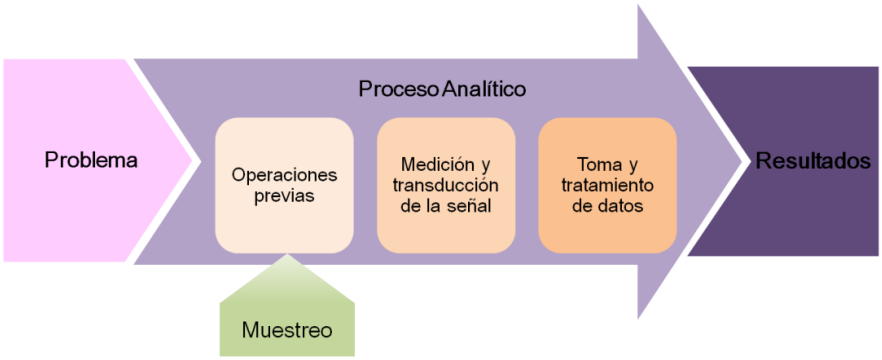


Figura 1. Etapas del proceso analítico en Química Analítica.

Para el estudio de la leche ecológica y convencional, se tomaron muestras de leche en diferentes condiciones. En total a lo largo de todo el estudio se analizaron 225 muestras de leche (27 de leche comercial de cabra y vaca, y 198 de leche cruda de cabra). En el caso de la leche cruda de cabra, esta procedió de animales sometidos a diferentes regímenes de alimentación. Se escogieron dos sistemas de producción de explotaciones ganaderas; el convencional, donde la alimentación básica del animal se basa en piensos; y el ecológico, en el cual la alimentación se basa en el pastoreo y/o en piensos ecológicos. Como el pastoreo disponible va a depender de las condiciones meteorológicas del momento, el muestreo se realizó durante 4 años en diferentes meses. En la Tabla 1 aparece un resumen de todas las muestras analizadas en el Capítulo 3.1 y 3.2.

Tabla 1. Número total de muestras de leche analizadas.

Tipo de leche	Animal	Sistema de producción	Nº total de muestras	Variables consideradas
Cruda	Cabra	Ecológico	135	- Recolección entre 2009 y 2012 - 4 variedades de cabra - Diferentes localizaciones
		Convencional	63	
Comercial	Vaca	Ecológico	9	Compradas en el mercado inglés y español
		Convencional	14	
	Cabra	Ecológico	3	
		Convencional	1	

Cuando existe un gran número de muestras para analizar, es conveniente realizar tratamientos de muestra sencillos. Por una parte para no perder información de la muestra, y por otro para disminuir el tiempo total del método de análisis, que es algo fundamental sobre todo en laboratorios de rutina. En el caso de las muestras de leche, se precipitó la proteína principal de la leche (caseína), y a continuación las muestras se

centrifugaron para separar el precipitado junto con la grasa de la leche del suero, que fue el que finalmente se analizó.

Entre los compuestos presentes en la leche, se escogieron los ácidos orgánicos para buscar diferencias entre muestras, en concreto el ácido oxálico, cítrico, orótico, benzoico, úrico e hipúrico, ya que debido a la composición de la leche se encuentran a unos niveles de concentración que se pueden determinar directamente con un detector UV/Vis.

En la literatura existen pocos ejemplos en los que se haya demostrado el potencial de la CE como técnica de rutina en el ámbito agroalimentario por lo que no hay muchos datos acerca de cómo puede afectar el análisis de un número elevado de muestras con un mismo capilar o cómo podrían cambiar los tiempos de migración a lo largo de los análisis. Por ello las conclusiones obtenidas en esta Memoria podrían ser de gran utilidad en caso de transferir el método electroforético optimizado a la industria. En este caso se observó que cada 6 análisis era necesario un blanco para limpiar el capilar y poder tener resultados repetitivos. Además en las condiciones óptimas de trabajo la vida media útil del capilar fue de 170 análisis.

Por otra parte, como aparece en la Figura 1 dentro del proceso analítico es fundamental la etapa de toma y tratamiento y datos. En una primera aproximación para encontrar diferencias entre la leche de cabras sometidas a un régimen convencional y ecológico, se intentó buscar una huella electroforética característica, entre los minutos 9 y 15 del

electroferograma. En este caso se usaron todos los picos obtenidos en este intervalo de tiempo, aunque no estuvieran identificados. Sin embargo la mayor diferencia se encontró teniendo en cuenta sólo la señal correspondiente al ácido hipúrico (Figura 2), por tanto a partir de ahí se comenzaron a estudiar las diferencias en su contenido, para utilizarlo como marcador químico para diferenciar si una muestra de leche es o no ecológica, o poder identificar el tipo de alimentación suministrado a un animal.

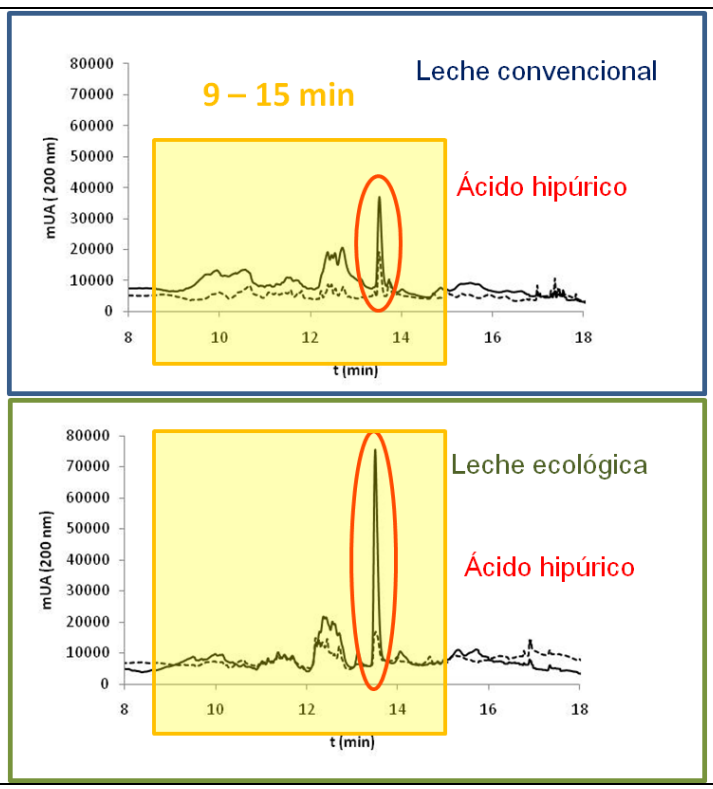


Figura 2. Comparación de los perfiles electroforéticos obtenidos para 20 muestras de leche cruda de cabras sometidas a un régimen convencional y otras 20 a un régimen ecológico. (Línea continua: valores medios; línea punteada: desviaciones estándar).

A partir de aquí se marcaron dos objetivos a través de la comparación del contenido en ácido hipúrico en:

1. Leche cruda de cabras de diferentes razas y criadas en diferentes zonas. En este caso debido al gran número de muestras se tomó como dato para comparar el área de pico del ácido hipúrico.
2. Leche comercial. Donde se incluyeron muestras de leche de vaca debido a la poca variedad de muestras de leche comercial de cabra que existía en el mercado.

Después del análisis por CE-UV de las 198 muestras de leche cruda de cabra (135 ecológicas y 63 convencionales) se llegó a la conclusión de que el ácido hipúrico es un marcador del régimen de alimentación, es decir indica si el animal ha consumido pasto o pienso; y no del sistema de explotación ganadera (ecológico o convencional). Los valores de hipúrico más alto se encontraron en muestras de leche cruda de cabra ecológica, recogidas durante los meses de verano (julio 2010). En contraste los valores más bajos de ácido hipúrico aparecieron en muestras de leche cruda convencional procedentes de cabras de la raza murciano granadina recogidas en diciembre de 2010, cuya alimentación fue exclusivamente a base de pienso.

En general hay que resaltar que los mayores valores de desviación estándar de los resultados se encontraron en muestras de leche cruda ecológicas, esto puede ser debido al consumo aleatorio de pasto de cada animal; mientras que para las cabras a las que se les suministró pienso, se obtuvieron unos valores de desviación estándar menores, lo que significa que todas las cabras llevaban un sistema de alimentación similar.

Por otra parte se estudiaron los resultados obtenidos al analizar las 27 muestras de leche comercial (Tabla 1). Como es bien sabido, para la comercialización de la leche, esta se somete a diferentes tratamientos. Con lo cual en primer lugar se realizó un estudio de la estabilidad del ácido hipúrico en muestras de leche tanto de cabra como de vaca durante el tratamiento de pasteurización, para ello se analizaron las muestras antes y después del mismo. Se encontró que el contenido de hipúrico es similar antes y después de este proceso, y por tanto se puede afirmar que el ácido hipúrico se puede usar como marcador químico porque no se pierde durante la pasteurización. Al comparar los datos obtenidos (Tabla 3 del Capítulo 3.2), sólo existieron diferencias significativas entre las muestras de leche comercial de cabra ecológica (35 ± 9 mg/L) y convencional (12 ± 3 mg/L). Sin embargo no se encontraron diferencias significativas entre las muestras de leche de vaca. Por ello el ácido hipúrico no es un buen marcador para diferenciar leche pasteurizada ecológica o convencional, porque normalmente las vacas productoras de leche ecológica a gran escala se alimentan con pienso ecológico y no con pasto.

Finalmente para validar la metodología desarrollada y reforzar la hipótesis planteada sobre la utilidad del ácido hipúrico como marcador del régimen de alimentación, parte de las muestras de leche (69 muestras de leche cruda, 33 de convencional y 36 de ecológica, 2 muestras de leche comercial de vaca ecológica y convencional) se analizaron también por HPLC-UV/Vis, que es una de las técnicas más explotadas tanto en laboratorios de investigación como de rutina. De hecho si se hace una breve búsqueda en la base de datos "*ISI Web of Knowledge*" con las

palabras "*organic acid and liquid chromatography"* y "*organic acid and capillary electrophoresis"*, el resultado que se obtiene para el caso de HPLC es de 16415 artículos frente a los 2523 que se obtienen para CE, un valor alrededor de 6 veces mayor.

Los resultados obtenidos por ambas técnicas se compararon a través de la t de Student, y como se observa en la Tabla 7 del Capítulo 3.2 no se encontraron diferencias significativas. Esto valida los resultados obtenidos mediante CE-UV/Vis, y nos ayuda a soportar una de las hipótesis de partida de esta tesis, ¿es la CE una herramienta fiable en un laboratorio agroalimentario? La respuesta a esta pregunta es sí, ya que como se ha demostrado en este trabajo, con una simple precipitación y centrifugación, se han obtenido resultados comparables de CE frente a HPLC. Además el uso de la CE ayuda a los laboratorios de rutina a trabajar con metodologías más respetuosas con el medio ambiente, ya que genera menos residuos, y el volumen de muestra que se necesita para un análisis es menor.

Por otra parte, la Química Analítica tiene un papel muy importante en la vida diaria, y la CE-UV/Vis puede ser también una herramienta útil para la resolución de problemas actuales. Últimamente, existe un abierto debate sobre el uso de pesticidas para el control de organismos nocivos a lo largo de la cadena alimenaria, ya que existe la preocupación de la toxicidad de estos productos sobre la salud humana y el medio ambiente. Actualmente las normativas tienden a restringir el uso de los pesticidas químicos, disminuyendo sus concentraciones permitidas o incluso prohibiéndolos. En este marco, recientemente se están regulando como alternativa los

biopesticidas basados en hongos entomopatógenos (Directiva 91/414/ECC). En esta Memoria se ha trabajado con un equipo multidisciplinar dentro del marco europeo "FP7-ENV.2011.3.1.9-1 ECO-INNOVATION INBIOSOIL" estudiando el potencial de la CE frente a la HPLC. Y se ha demostrado que la CE-UV/Vis se puede usar como una herramienta analítica que permite controlar la presencia de destruxina A en medios de cultivos de hongos.

De esta forma se está evaluando el hongo *Metarhizium* como agente de control biológico para el control de insectos; pero hay que tener en cuenta que al ser un organismo natural, también se debe estudiar la posible secreción de metabolitos secundarios que en algunos casos puedan presentar efectos tóxicos. En concreto uno de los grupos de metabolitos mayoritarios que produce *Metarhizium* son las destruxinas, un grupo de micotoxinas. Aunque las destruxinas actúan junto con otros factores patogénicos en la muerte de los insectos, hay que tener en cuenta que la cantidad y el tipo de destruxinas pueden jugar un papel importante en la virulencia y especificidad de las diferentes cepas de *Metarhizium*. Por otra parte la producción de destruxinas está influenciada por los nutrientes del medio donde se encuentra el hongo; además existen variaciones de producción de destruxinas entre hongos *Metarhizium*, e incluso entre las cepas de un mismo hongo (por ello hay cepas que atacan de forma más virulenta a ciertos insectos) [1].

Por tanto, el primer paso para desarrollar un nuevo biopesticida o agente de control biológico es encontrar las cepas adecuadas, y por tanto estudiar sus características microbiológicas [2]. Para ello se necesita primero estudiar las características de las cepas que se podrían utilizar con

este fin. Y en estudios futuros el efecto que tendrían sobre plantas, insectos, etc.; en definitiva en la cadena alimentaria.

Pese a la problemática presentada, en la actualidad no existe ninguna normativa vigente sobre cuáles son los métodos analíticos óptimos para la determinación de estas micotoxinas, y por tanto es un nuevo campo de investigación en el ámbito de la Química Analítica.

Hasta la fecha prácticamente sólo se ha utilizado HPLC-UV/Vis para cuantificar destruxinas, y la espectrometría de masas para identificarlas. En la Tabla 1 del Capítulo 3.3 aparecen algunos ejemplos representativos de estos métodos. A lo largo de esta Memoria se han ido comentando las ventajas que puede aportar la CE-UV/Vis (tales como la menor generación de residuos, y el menor volumen de muestra necesario) frente a HPLC-UV/Vis. Por ello en esta Tesis Doctoral, se propone la CE-UV/Vis como una herramienta económica y ambientalmente sostenible para evaluar los riesgos de los agentes de biocontrol de la producción de estos hongos. Para demostrar esta hipótesis se eligieron dos medios diferentes (CM y MM) con condiciones nutricionales opuestas para cultivar tres cepas diferentes (BIPESCO5, ART2825 y EAmA 01/58-Su) procedentes de *Metarhizium brunneum*, y se evaluó el contenido de destruxina A producido durante 18 días.

En bibliografía sólo existe un método en el que se muestra el potencial de la CE-UV/Vis para determinar destruxinas [3], sin embargo en este trabajo sólo se estudia la separación de una mezcla de 4 destruxinas. En este Memoria se reoptimizaron las condiciones de separación anteriormente propuestas, y el buffer que se usó fue una mezcla de 25 mM

de tetraborato de sodio, 50 mM de SDS a pH 9. El método se validó usando el estándar de destruxina A disuelto en agua como en los medios de cultivo CM y MM. Es importante resaltar que las muestras de los medios de cultivo a pesar de ser una matriz muy compleja, sólo se filtraron antes del análisis por CE-UV/Vis, lo que destaca la sencillez del método. De forma directa los LODs estuvieron entre 0.39 y 1.06 mg/L, y los LOQs entre 1.32 y 3.55 mg/L. El método tiene una sensibilidad limitada, aunque por estudios previos utilizando UHPLC-MS/MS [4], pudimos comprobar que podrían ser suficientes para obtener el día de máxima producción de destruxina A. Sin embargo en el caso de que el método electroforético propuesto se quisiera utilizar para determinar destruxina A presente en muestras agroalimentarias como frutas o verduras, o incluso insectos, sería obligatorio optimizar una etapa previa de preconcentración de extracción y preconcentración del analito, ya que las concentraciones presentes en estas muestras probablemente serían más bajas que las encontradas en los medios de cultivo.

En relación a la precisión, como se puede observar en la Tabla 3 del Capítulo 3.3, los valores de RSD para el área de pico estuvieron entre 7.1 y 12.7 %, mientras que para el tiempo de migración estuvieron comprendidos entre 0.3 y 2.5 %. Lo que demuestra que el método electroforético es bastante reproducible, lo cual es interesante pensando en esta técnica como una herramienta futura en los laboratorios de rutina.

Con respecto al estudio de las muestras de medios de cultivo, se comprobó que el medio CM favorece la producción de destruxina A frente a MM, ya que el medio MM se caracteriza por presentar unas condiciones de

estrés nutricional para el hongo. Además de entre las tres cepas, la que produce mayor concentración de este metabolito secundario es BIPESCO5 (su máxima producción se encuentra en el día 9 con un valor de 18.2 ± 0.2 mg/L), seguido de EAmA 01/58-Su (máxima producción el día 6 con 7.6 ± 0.4 mg/L) y finalmente se ha comprobó que ART2825 no produce ninguna concentración detectable.

La necesidad de disponer de métodos analíticos fiables para determinar destruxinas en distintas muestras agroalimentarias se encuentra recogido en el proyecto europeo INBIOSOIL "FP7-ENV.2011.3.1.9-1 ECO-INNOVATION, Grant Agreement No 282767", y para dar respuesta a esta necesidad, como trabajo adicional a esta Tesis, se ha colaborado en el desarrollo de otros métodos para determinar destruxinas en muestras de patata (Anexo de la Tesis Doctoral). En este caso se comenzó con un estudio preliminar utilizando la SPE como estrategia de preconcentración de la destruxina A en medios de cultivos, en los casos menos favorables de producción. Para ello se probaron dos sorbentes clásicos como son el C18 y el HLB. Y se estudió su capacidad para eliminar interferencias de la matriz, así como las recuperaciones obtenidas tanto en el medio MM como el CM. A partir de este estudio se comprobó que dependiendo del medio de cultivo donde se encuentra la destruxina es mejor utilizar un sorbente u otro, siendo el HLB el sorbente más idóneo para determinar destruxina A en el medio MM y el C18 para el CM. Como en el medio MM ninguna cepa produjo la cantidad suficiente de destruxina A para que pudiera ser determinada directamente por CE-UV/Vis, se preconcentraron los medios procedentes de las tres cepas. De igual forma que en el medio CM,

BIPESCO5 y EAmA 01/58-Su fueron las cepas que produjeron una mayor cantidad del metabolito secundario en el medio M. En este caso la destruxina A sólo se cuantificó en algunas muestras. Su máximos días de producción fueron el 18 (con 1.33 ± 0.03 mg/L) y el 12 (0.266 ± 0.006 mg/L), respectivamente. Sin embargo, la cepa ART2825 no produjo la cantidad suficiente de destruxina A para que pudiera ser detectada usando SPE-CE.

Uno de los primeros objetivos de este proyecto europeo (que fue la determinación de destruxinas secretadas por el hongo *Metarhizium* en medios de cultivo) se logró con éxito usando la CE-UV/Vis. Por lo tanto se puede proponer utilizarla en los estudios de evaluación de riesgos de los agentes de control biológico. Además los resultados obtenidos con CE-UV/Vis se compararon con los obtenidos mediante UHPLC-MS/MS, lo cual confirmó los datos obtenidos con la técnica propuesta en esta Tesis Doctoral. Aunque como era de esperar el poder de identificación de un detector de masas permitió la identificación de un mayor número de destruxinas.

El siguiente paso de este proyecto será aplicar el hongo en diferentes plantas, frutas, verduras, etc., a fin de combatir diferentes plagas. Sin embargo, hoy en día hay una falta de conocimiento sobre la posible presencia de destruxinas en muestras agroalimentarias. Por tanto, debido a la toxicidad de estos metabolitos, es necesario tener métodos analíticos validados para detectar la presencia de destruxinas en la cadena alimentaria, para proteger la salud pública.

Para poder determinar destruxinas en muestras agroalimentarias, por ejemplo de patata, es necesario utilizar una estrategia de extracción eficiente, ya que las interferencias que pueden presentar estas matrices pueden afectar a la determinación de estos metabolitos secundarios. Además el extracto obtenido debe ser compatible con el sistema de detección seleccionado. Actualmente existen pocos tratamientos de muestra para extraer estos metabolitos en plantas [5, 6]. Los que se encuentran en bibliografía se basan en las clásicas extracción sólido-líquido [6], aunque recientemente se ha publicado un método para determinar destruxinas en muestras de patata usando la metodología QuEChERS [5] (Anexo de la Tesis Doctoral). Este último procedimiento se escogió para estudiar la compatibilidad de la extracción QuEChERS con el método electroforético desarrollado. Para ello se siguió el procedimiento para extraer destruxinas en la planta de la patata, en concreto en el tubérculo y las hojas; y se analizó tanto un blanco de cada muestra como cada muestra fortificada con 100 mg/L de destruxina A. Como puede observarse en la Figura 3, en ambos casos no se obtuvieron muchas interferencias.

Sin embargo, a pesar de la alta concentración escogida para hacer las pruebas preliminares, el pico correspondiente a destruxina A no es muy intenso, esto se debe a que la metodología QuEChERS realiza una dilución de la muestra. Por lo que si se desea usar la CE-UV/Vis como técnica de separación, el uso de un detector más sensible que el detector UV es completamente necesario para alcanzar el objetivo propuesto.

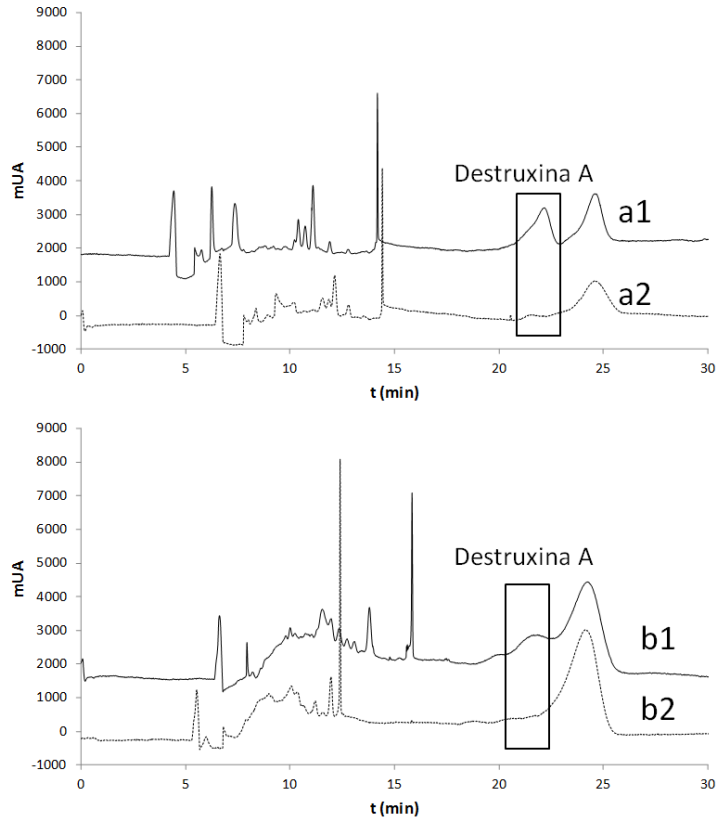


Figura 3. Electroferogramas correspondientes a la extracción de destruxina A de diferentes partes de la patata utilizando QuEChERS; a: tubérculo fortificado con 100 mg/L de destruxina A (a1), y sin fortificar (a2); b: hojas fortificadas con 100 mg/L de destruxina A (b1), y sin fortificar (b2).

En resumen, existe un nuevo campo de investigación donde desarrollar estrategias para determinar destruxinas en diferentes matrices, y la CE acoplada a un espectrómetro de masas puede desempeñar un papel interesante en la evaluación del riesgo de los agentes de biocontrol de hongos, además los resultados que se podrían comparar los resultados obtenidos con equipos más establecidos en los laboratorios como el UHPLC-MS.

Considerando las dos metodologías propuestas en el Bloque 3 de esta Tesis Doctoral; en el primer caso, se ha comprobado el potencial de la CE para diferenciar muestras de leche de cabras alimentadas con distintos regímenes de alimentación. En la segunda parte de este Bloque se ha estudiado la viabilidad de la técnica para monitorear la producción de metabolitos secundarios, donde se ha demostrado que se puede determinar la destruxina A en medios de cultivo, pero es necesario sustituir el detector de UV por un espectrómetro de masas si se quieren estudiar matrices más complejas como la patata.

En este Bloque se ha demostrado el potencial de la CE-UV/Vis como técnica útil para resolver problemas dentro del campo agroalimentario, aplicando tratamientos de muestra sencillos (precipitación, centrifugación o filtración). Además se ha demostrado que los resultados son comparables con los obtenidos por técnicas más aceptadas dentro del área de la agroalimentación, como es la HPLC. Además, teniendo en cuenta la nueva tendencia hacia el uso de métodos analíticos respetuosos con el medio ambiente, la CE se podría considerar una alternativa frente a la HPLC, ya que el volumen de muestra como los residuos generados son muchos menores.

La Electroforesis Capilar en el ámbito agroalimentario con etapa de preconcentración en el tratamiento de muestra

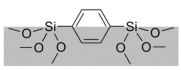
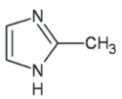
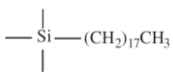
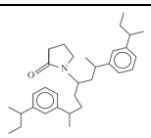
En esta Tesis Doctoral se ha hecho uso de tres materiales nuevos con características diferentes para paliar una de las mayores limitaciones de la CE-UV/Vis que es su sensibilidad, derivada del pequeño paso óptico que se utiliza como ventana de detección y de los pequeños volúmenes de muestra que se inyectan en el capilar. Todos estos materiales se han estudiado para comprobar el potencial de ellos, y las mejoras que podrían aportar con respecto a los materiales que se usan clásicamente para mejorar la sensibilidad de la CE.

Los materiales utilizados en esta Memoria han sido: nanopartículas magnéticas recubiertas de grupos carboxílicos (MNPs), una organosílice mesoporosa periódica con puentes de fenileno (Ph-PMO), y una estructura zeolítica de imidazolato 8 (ZIF-8). Todos ellos se han utilizado como sorbentes de SPE usando la modalidad convencional o dispersiva.

Como se puede observar en la Tabla 2, los tres materiales estudiados como fases estacionarias son de diferente naturaleza. Las nanopartículas magnéticas están funcionalizadas con grupos carboxílicos. Por otra parte el Ph-PMO y el ZIF-8 son materiales híbridos con una parte orgánica y otra inorgánica, en el caso del Ph-PMO la parte orgánica es el fenileno y la inorgánica la sílice; y para el ZIF-8, la parte orgánica son los anillos imidazolios mientras que la inorgánica son metales de Zn. El PMO se caracteriza por ser un material mesoporoso, mientras que el ZIF-8 es una estructura cristalina microporosa. El material con mayor tamaño de

partícula corresponde al ZIF-8, pero al ser microporoso, presenta la mayor área superficial, seguida del Ph-PMO y las MNPs.

Tabla 2. Características de los diferentes sorbentes utilizados como fases estacionarias en esta Tesis Doctoral.

		Propiedades		
		Unidad básica	Tamaño de partícula (μm)	Superficie (m^2/g)
Materiales nuevos propuestos	MNPs carboxiladas[1]	- COOH	1.2	3.6
	Ph-PMO[2]		3-12	790
	ZIF-8[3]		4.9	1300-1800
Materiales clásicos	C18[4]		55-105	325
	HLB[4]		30-60	160-420

Información obtenida de: [1]MagmaMedics, [2]experimentalmente, [3]Sigma Aldrich, [4]Waters.

En el caso del Ph-PMO y el ZIF-8 que en los Capítulos 4.2 y 4.3, respectivamente, se han comparado con el C18 y el HLB, presentan tamaños de partícula considerablemente menores frente a ellos, y por tanto mayores áreas superficiales. Como se comentó en la Introducción, la superficie de contacto disponible del sorbente para entrar en contacto con los analitos es algo fundamental. Por ello estos materiales presentan un gran potencial como nuevas fases estacionarias.

Estos tres materiales se evaluaron a través de tres estrategias de preconcentración diferentes. En el caso de las MNPs se han aprovechado sus propiedades magnéticas para realizar una extracción dispersiva asistida con un imán (MSPE), el Ph-PMO se ha utilizado como sorbente en la modalidad clásica de SPE (utilizando el formato de cartucho), y el ZIF-8 se ha utilizado como sorbente en fase dispersiva (dSPE).

A continuación se van a describir los resultados más relevantes obtenidos en cada caso, y en segundo lugar se van a comparar las estrategias entre sí.

- Uso de MNPs recubiertas con grupos carboxilícos para preconcentrar metales mediante MSPE

En este caso se ha demostrado el potencial de las MNPs funcionalizadas con grupos carboxilo junto con el agente complejante 1,10-fenantrolina para extraer y preconcentrar metales, en concreto Cd, Co, Cu, Ni y Zn, a través de una MSPE.

En la estrategia de dispersión, se añadió el material sorbente a la muestra, y se necesitaron 5 min mediante agitación en vortex para alcanzar el equilibrio de adsorción de los metales (0.1 mg/L en 10 mL) sobre la superficie de 2 mg de MNPs. Además el pH de la muestra juega un papel fundamental en la extracción; por ello se seleccionó un pH 8 donde los grupos carboxílicos están negativativamente cargados, y atraen la carga positiva de los metales.

Una vez retenidos los metales en la superficie, éstos se extrajeron poniendo en contacto durante 5 min las MNPs con 200 μL de una

disolución compuesta por 0.8 mM 1,10-fenantrolina, 0.04 % de clorhidrato de hidroxilamina a pH 2. A este pH están protonados los grupos carboxílicos de las MNPs, que junto con la presencia del agente complejante en el medio, se favorece la formación de los complejos metal-1,10-fenantrolina (que se añadió en exceso). Además la hidroxilamina se añadió para evitar la oxidación de los metales de Co y Ni de valencia +2 a +3 y así poder formar el complejo. Aunque a este pH tan ácido se deteriora la matriz de sílice provocando la lixiviación de los óxidos de hierro al medio, se eligió con el objetivo de obtener mayor señal en los metales que presentaban menos sensibilidad en el método (Cd, Co, Mg y Zn). Debido al uso de este pH tan ácido, se comprobó que las MNPs sólo se podían reutilizar cuatro veces.

También se observó que los metales de Co y Cu se podían extraer sólo cambiando el pH del medio, con lo cual para ser determinados formarían complejo con la fenantrolina presente en el buffer.

Finalmente se estudió la cantidad de MNPs que se podían utilizar durante el proceso de extracción. Con 1 mg la extracción de los metales no era suficiente, y con 3 mg la separación de las MNPs de la disolución ser hacía más complicada, quizás con unos imanes más potentes se podría haber conseguido. Con lo que en las condiciones de trabajo seleccionadas, se comprobó que 2 mg de material era la cantidad óptima para alcanzar los objetivos propuestos.

Teniendo en cuenta las condiciones electroforéticas utilizadas, hay que resaltar que a 270 nm se detectan los complejos de metal-1,10 fenantrolina, por lo que se favoreció la estabilidad del complejo añadiendo un exceso de complejante al buffer.

Como se puede observar en la Figura 4 del Capítulo 4.1 el aumento de intensidad en las señales electroforéticas de los metales Co, Zn, Cu, Ni y Cd, fue bastante significativo. Además la señal del Mg solapa con la de la fenantrolina, por ello este método no es óptimo para su identificación.

En las condiciones optimizadas los LODs se encontraron entre 3 y 9 μg/L, mientras que los LOQs estuvieron entre 1.2 y 30 μg/L. El método optimizado fue efectivo para determinar Co, Cu y Zn a niveles bajos de concentración. Por otra parte la precisión del método fue aceptable con valores entre 5.99 y 14.65 % en términos de repetitividad, y entre 6.86 y 16.63 % para la reproduciblidad, respectivamente. Por último, se demostró la utilidad del método en el ámbito agroalimentario determinando Zn, Cu, Ni y Cd en muestras de zumos.

El método desarrollado permite realizar una determinación multiparamétrica de 4 metales, que lo hace atractivo si se compara con el potencial de la absorción atómica, que no permite la detección simultánea de estos metales.

- Uso de Ph-PMO para preconcentrar pesticidas mediante SPE convencional

En este caso el Ph-PMO se ha evaluado como sorbente en la modalidad clásica de SPE, donde el material está empaquetado en un cartucho, para preconcentrar un grupo de pesticidas modelo (fenuron, simazina, atrazina, carbaril y terbutrin).

En las condiciones óptimas, para la extracción se empaquetaron 30 mg en una columna de SPE, y se paso 100 mL de muestra. Luego los analitos se eluyeron en 1 mL de acetonitrilo que se evaporó a sequedad y se reconstituyó en 200 μL de agua.

La optimización del pH de la muestra fue un punto importante. Según los análisis de difracción de rayos X, el material mostró ser estable desde pH 2 hasta pH 9. La mezcla del patrón a una concentración de 100 μg/L presentó un pH alrededor de 8. Cuando se ajustó el pH de la muestra de mosto a este valor, la apariencia física de la muestra cambió y aparecieron interferentes nuevos coemigrando con los analitos de interés; por tanto, se dejó la muestra a su pH natural (alrededor de 3.2), para evitar la interferencia de compuestos con los analitos de interés. A ese valor de pH el fundamento de la retención se basa en las interacciones π-π que existen entre los anillos aromáticos del PMO y los analitos. Con estos experimentos se demuestra la importancia de validar las metodologías con matrices reales ya que en muchos casos las condiciones óptimas encontradas para una mezcla de analitos en agua, no se pueden transferir directamente para identificar el mismo grupo de analitos en muestras reales, debido a que los componentes presentes en la matriz pueden afectar a las señales de los analitos de estudio.

En las condiciones optimizadas se obtuvieron unos valores de LODs y LOQs entre 0.8 y 4 μg/L, y 2 y 10 μg/L, respectivamente. Posteriormente se estudió la señal electroforética de la muestra de mosto fortificada con 10 μg/L (Figura 6 del Capítulo 4.2) con el método optimizado, y se comprobó que debido a la baja absorbancia del fenuron, éste no se pudo identificar

dentro de la muestra a esa concentración; además la simazina coemigraba también con otros compuestos presentes en la matriz. Por lo tanto el método desarrollado sólo es válido para determinar atrazina, carbaril y terbutrin en zumos; obteniéndose valores de recuperación entre 86 y 105 % para tres niveles de concentración diferentes (10, 20 y 30 μg/L). Además, se evaluó la precisión del método fortificando el mosto con 100 μg/L, obteniéndose valores entre 12 y 16 %. Los valores de recuperación y precisión demuestran la utilidad del método para determinar pesticidas en este tipo de muestras en un laboratorio agroalimentario.

Por otra parte se estudió la estabilidad del Ph-PMO para reconcentrar los pesticidas disueltos en agua, y se comprobó que el material se pudo reutilizar dos veces (donde en la segunda, las señales electroforéticas obtenidas ya fueron menores). Cuando los pesticidas están disueltos en mosto, se encontró que tanto la mesoestructura como el tamaño de partícula del Ph-PMO se mantuvieron después del primer uso, pero el área específica y el volumen de poro disminuyó, lo que indica que algunos compuestos del mosto se quedaron retenidos en el sorbente. Esto hizo que los valores de área de pico de las señales en la segunda extracción fueran algo menores, y en la tercera extracción el material se bloqueará al pasar la muestra.

Los resultados obtenidos al usar el Ph-PMO como sorbente para preconcentrar pesticidas, se compararon con los materiales sorbentes clásicos como C18 y HLB. En primer lugar se compararon los LODs y LOQs obtenidos con cada material para los estándares. En el caso de la

simazina y atrazina, los mejores límites se obtuvieron con el Ph-PMO; sin embargo se observó la situación contraria para el fenuron y el terbutrin. En el caso del carbaril se obtuvieron valores similares al C18 y mejores que para el HLB.

En segundo lugar se comparó la selectividad obtenida al extraer los pesticidas disueltos en el mosto. El PMO y el C18 se comportaron de forma similar, sin embargo para extraer los pesticidas en el mosto se comprobó que el HLB daba mejores resultados, debido a que se obtuvieron mayores áreas de pico correspondientes a los analitos de interés (Figura 6 del Capítulo 4.2).

Finalmente se estudió cuántas veces se podría reutilizar tanto el C18 como el HLB en la extracción de pesticidas en las muestras de mosto. Obteniéndose que el C18 se podía reutilizar dos veces (como el Ph-PMO), mientras que el HLB sólo una. Los resultados presentados en este trabajo demuestran que el Ph-PMO ofrece unas características similares a las del C18 para usarlo como sorbente de pesticidas en mostos.

- Uso del ZIF-8 para preconcentrar bisfenoles mediante dSPE

Se ha utilizado el ZIF-8 para aumentar la sensibilidad del método electroforético aplicado para preconcentrar bisfenoles (bisfenol A, diglicidil éter de bisfenol A y sus derivados hidrolizados) en muestras de refrescos.

En las condiciones óptimas del método, se añadieron 10 mg de sorbente a 10 mL de muestra conteniendo los analitos, y se dispersó ZIF-8 mediante 1 min de ultrasonidos. El tiempo de contacto entre ambas fases es importante, con lo cual en este caso se optimizó el tiempo de agitación

en vortex. Al presentar una gran área superficial (1300-1800 m^2/g), en el primer minuto se alcanzó el equilibrio. La retención de los bisfenoles en el material se debió a las interacciones π-π de los anillos imidazolio del ZIF-8 y los anillos benceno de los analitos.

Para la extracción de los analitos se utilizó 1 mL de acetonitrilo, y se siguió el mismo procedimiento de dispersión y agitación utilizando el vortex, y centrifugación. Luego el extracto se llevó a sequedad y se constituyó en 200 μL de agua.

En las condiciones optimizadas, disolviendo los estándares en agua se obtuvieron unos LODs y LOQs entre 0.02 y 0.03 mg/L, y 0.06 y 0.08 mg/L, respectivamente. Además el método se validó disolviendo los analitos en muestras de refresco de cola procedente de botellas, donde no se esperaba la presencia de los analitos (previamente se realizó un blanco de la muestra para asegurar que no se encontraban los analitos de estudio). En ese caso, se encontraron unos LODs y LOQs entre 0.04 y 0.1 mg/L, y 0.15 y 0.34 mg/L. Aunque los valores obtenidos no fueron muy bajos, fueron suficientes para el propósito del trabajo teniendo en cuenta los límites legales. Por otra parte la precisión del método estuvo entre 7 y 11 % para estándares, y 7 y 14 % para muestras reales. Además las recuperaciones a tres niveles de concentración (0.4, 0.8 y 1 mg/L) estuvieron entre 81 y 89 %. Estos datos confirman la eficacia de la estrategia de preconcentración propuesta para la determinación de estos analitos por CE-UV/Vis.

Por otra parte se estudió, las veces que se podría reutilizar el sorbente en las condiciones optimizadas, ya que un aspecto importante de

un método analítico es el coste del mismo. En este caso los 10 mg se pudieron reutilizar hasta 2 veces.

Además este material se comparó con el C18 y el HLB. A pesar de tener el ZIF-8 una mayor área superficial (Tabla 2 del Capítulo 4.3), en el caso de estándares las áreas de pico más altas se encontraron cuando se usó el HLB, esto puede ser debido a su estructura química, ya que los dos anillos de benceno que presenta podrían provocar una mayor interacción con los presentes en los bisfenoles. Sin embargo las señales obtenidas usando el C18 y el ZIF-8 fueron comparables. Teniendo en cuenta el efecto de la matriz de la muestra, se llevó a cabo el mismo experimento con muestras de refresco de cola fortificadas con los analitos, y se llegó a la misma conclusión. Aunque se podría esperar que debido al área superficial del ZIF-8 las mayores retenciones de los analitos se encontraran usando este material, aunque esto no ocurrió. Una posible hipótesis para explicar este hecho es que los analitos no puedan interaccionar con toda la superficie del mismo debido a su microporosidad y al tamaño de los analitos.

Finalmente, se realizó una comparación las modalidades de SPE dispersiva y en cartucho. El resultado más relevante para destacar es que cuando se usan materiales con gran área superficial como el ZIF-8, es mejor seleccionar la modalidad dispersiva, ya que se favorece la interacción entre la superficie y los analitos. De esa forma se incrementa notablemente las señales de área de pico en la dSPE frente a la otra modalidad. Hecho que no fue tan notable cuando se usan sorbentes tipo C18 y el HLB.

En resumen, en la Tabla 3 aparecen las características de los tres métodos propuestos para aumentar la sensibilidad de la CE-UV/Vis. En los tres casos se han obtenido valores aceptables de precisión y recuperación para alcanzar los objetivos inicialmente propuestos en cada trabajo; que en los tres casos ha sido proponer un método donde utilizando la CE con un detector UV/Vis se puedan detectar los límites de concentración máximos oficiales marcados para cada grupo de analitos.

Tabla 3. Resumen de las características de los métodos de SPE-CE mostrados en el Bloque 4 de esta Tesis Doctoral.

	MNPs	**Ph-PMO**	**ZIF-8**
Modalidad	MSPE	SPE en cartucho	dSPE
Analitos	Metales	Pesticidas	Bisfenoles
Muestra	Zumos	Mosto	Refresco
Cantidad de sorbente	2 mg	30 mg	10 mg
Volumen de muestra	10 mL	100 mL	10 mL
Extracto final	200 µL	200 µL	200 µL
LODs	3 - 9 µg/L	0.6 – 4 µg/L	40 – 100 µg/L
LOQs	12 - 30 µg/L	2 -10 µg/L	150 – 340 µg/L
Precisión	6 – 17 %	10-16 %	7- 14 %
Recuperaciones	83 – 96 %	86 – 105 %	81 – 89 %
Reutilización	4 veces	2 veces	2 veces

Como se observa en la Tabla 3, para cada material se ha utilizado una modalidad diferente de SPE. En el caso del Ph-PMO, se seleccionó la SPE en el formato de columna. En este caso la carga de la muestra se hace por gravedad o vacío, además la difusión de los analitos por el sorbente está limitada debido a la configuración del cartucho. Sin embargo, en la comparación de modalidades clásica y dSPE que se ha hecho en el trabajo del ZIF-8, se comprobó que en el caso de materiales con una alta área superficial, los mejores resultados se obtienen dispersando el sorbente sobre toda la disolución. Por tanto, en el método desarrollado con el PMO,

utilizando dSPE se podrían haber obtenido unos resultados similares disminuyendo el volumen de muestra o la cantidad de sorbente. Lo cual es importante para reducir los costes del método desarrollado, o en el caso de que la muestra esté limitada. Además, debido al procedimiento que se sigue en la modalidad dispersiva, normalmente reduce el tiempo de extracción frente a la SPE clásica, sobre todo si se trata de volúmenes grandes. También es más económica, ya que se evita el uso de cartuchos y fritas.

Por otra parte, cuando se utiliza un material con propiedades magnéticas en la modalidad dispersiva, como fue el caso de las MNPs, se elimina la etapa de centrifugación para separar el sorbente de la disolución, al usar imanes para separar ambas fases, lo cual simplifica el procedimiento.

En resumen, se ha demostrado que los tres materiales utilizados son eficaces para aumentar la sensibilidad de la CE-UV/Vis. Las características más relevantes que se pueden destacar para el Ph-PMO y el ZIF-8 son la gran relación superficie/volumen, siendo la estrategia de dSPE donde más se aprovecha su potencial, ya que aumenta el contacto entre el sorbente y la muestra. Con lo cual son ideales como sorbentes de SPE para aumentar la sensibilidad de las técnicas analíticas, y en algunos casos podrían mejorar los valores de sensibilidad y selectividad obtenidos con los materiales comúnmente utilizados.

Por otra parte, la gran ventaja de los materiales con propiedades magnéticas es que el proceso de extracción más sencillo que en los otros

casos. En un futuro se podría estudiar la posibilidad de magnetizar los nuevos materiales propuestos.

Teniendo en cuenta todos los resultados mostrados en esta Tesis Doctoral, se puede comprobar que la CE-UV/Vis es una técnica analítica apropiada para ofrecer información (bio)química relacionada con diferentes problemas dentro del área agroalimentaria. Además, como cualquier problema analítico [7], los trabajos se pueden englobar en las dos vertientes que pueden tener las investigaciones relacionadas con esta técnica actualmente (Figura 4).

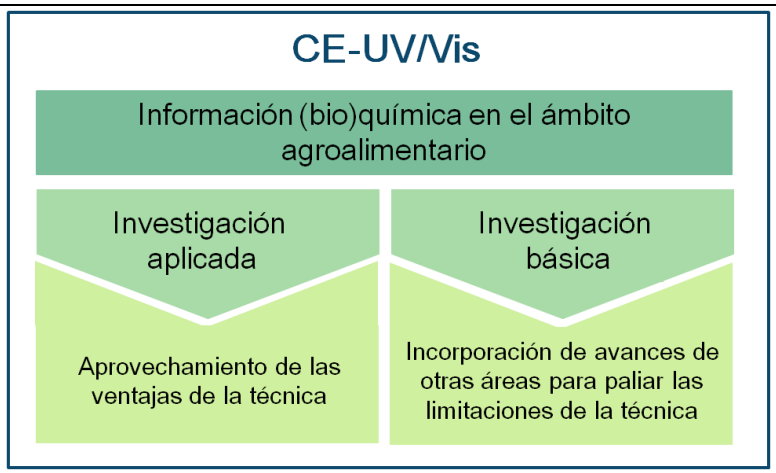


Figura 4. Problema analítico de la CE-UV/Vis en el ámbito agroalimentario.

En la primera parte, se ha realizado una investigación aplicada aprovechando las ventajas de la técnica. Esta investigación aplicada puede servir como prueba del papel que puede jugar la CE-UV/Vis en un laboratorio de rutina. En el caso de los trabajos relacionados con el análisis

de la leche, se ha comprobado que la técnica es lo suficientemente robusta como para analizar un gran volumen de muestras y obtener información de calidad. Además el método desarrollado para la determinación de destruxina A en muestras de medios de cultivo de hongos, es un buen ejemplo de cómo la técnica puede considerarse para resolver nuevos problemas que van surgiendo dentro del área agroalimentaria.

En el segundo bloque de esta Tesis, se ha seguido trabajando en el estudio del potencial de nuevos materiales para mejorar la sensibilidad y selectividad de la CE, en este caso se ha hecho uso de uso de avances de otras áreas para solventar estos problemas.

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under different stress conditions and their detection using UHPLC-MS/MS, (2015) *In press*.

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CONCLUSIONES

Las conclusiones más relevantes generadas a lo largo de esta Tesis Doctoral se han dividido en aquellas que tienen una repercusión química analítica, y las que inciden sobre el área agroalimentaria.

En relación con las aportaciones más relevantes en el ámbito químico analítico, cabe destacar:

- Se ha demostrado que la CE-UV/Vis es una herramienta válida para resolver problemas del ámbito agroalimentario. Además para los casos presentados en el Bloque 3, se puede considerar una alternativa más económica y respetuosa con el medio ambiente que la HPLC-UV/Vis, ya que en la CE el uso de disolventes orgánicos y/o residuos es mínimo.

- Se ha comprobado como en algunos casos, es posible la introducción de muestras en el equipo con un tratamiento de muestra mínimo, y sin necesidad de preconcentrar el analito, obteniendo datos exactos y precisos. Esto simplifica el proceso analítico, haciendo que los métodos desarrollados sean rápidos y de fácil uso; por lo que podrían ser implantados en un laboratorio de control lechero o agronómico.

- Se han empleado con éxito nuevos materiales sorbentes a través de diferentes modalidades de SPE, para aumentar la sensibilidad y/o selectividad de la CE-UV/Vis. En concreto se ha evaluado el potencial de:

- Nanopartículas magnéticas funcionalizadas con grupos carboxílicos para preconcentrar de forma sencilla y rápida metales mediante una SPE magnética dispersiva.

- Una organosílice mesoporosa con puentes de fenileno como sorbente en la modalidad clásica de SPE (utilizando un cartucho) para la extracción de pesticidas. Estos resultados fueron comparables los obtenidos con el C18 y el HLB.

- Una estructura zeolítica de imidazolio 8 como un sorbente idóneo en SPE dispersiva, frente a la modalidad de SPE en cartucho, para preconcentrar bisfenoles. Se ha demostrado que cuando se utilizan materiales de gran área superficial se obtienen mejores resultados cuando se usa la modalidad dispersiva de SPE, sin embargo la misma conclusión no se llega cuando se usan materiales como el C18 o el HLB.

En relación con las aportaciones más relevantes en la generación de información (bio)química utilizando la CE-UV/Vis en el ámbito agroalimentario, cabe destacar:

- A través del análisis del contenido en ácido hipúrico de 225 muestras de leche, se ha demostrado que la concentración de ácido hipúrico presente en la leche de cabra depende del régimen de alimentación al que se someten estos animales. Una mayor concentración de ácido hipúrico se relaciona con la alimentación

basada en pasto. También se ha demostrado que con la legislación actual el ácido hipúrico no se puede utilizar para clasificar una leche como ecológica, ya que también es posible obtener ésta alimentando cabras en un régimen intensivo con pienso ecológico.

- Se ha monitoreado la producción de destruxina A de varias cepas del hongo *Metarhizium brunneum* que actualmente se están evaluando como agente de control biológico como alternativa al uso de pesticidas químicos.

- Se ha llevado a cabo la preconcentración de los metales Cd, Co, Cu, Ni y Zn muestras de zumo. Los límites de detección (del nivel de los μg/L) que ofrece el método electroforético desarrollado permite confirmar el potencial de éste en otras aplicaciones agroalimentarias.

- Se han extraído los pesticidas atrazina, carbaril y terbutrin en muestras de mosto, permitiendo su detección hasta bajos niveles de concentración utilizando la CE-UV/Vis.

- Se ha preconcentrado bisfenol A, diglicidil éter de bisfenol A y sus derivados hidrolizados en muestras de refresco.

AUTOEVALUACIÓN CIENTÍFICA

La perspectiva que aporta el tiempo y la experiencia adquirida, permite analizar de forma crítica el trabajo desarrollado. Además de las aportaciones más relevantes que se han resumido en la sección anterior, también conviene mencionar aquellas limitaciones o dificultades que presentan las metodologías desarrolladas.

En el caso de los trabajos contenidos en los Capítulos 3.1 y 3.2 presentan al ácido hipúrico como un marcador para diferenciar el régimen de alimentación al que se someten las cabras. Aunque el uso de un marcador es beneficioso como parámetro para diferenciar productos, hay que tener en cuenta que se abrirían nuevas vías para aumentar el fraude. Con lo cual sería más adecuado el uso de un perfil o huella electroforética donde no todos los picos sean identificados con su respectivo compuesto químico. Por ello hubiera sido también interesante estudiar los perfiles electroforéticos obtenidos con herramientas estadísticas más potentes para evaluar toda la información que se obtiene en el análisis, siguiendo las estrategias que se usan en metabolómica, y en general en todas las técnicas -ómicas.

En el trabajo del Capítulo 3.3, se propone el uso de la CE-UV/Vis como una herramienta para controlar el contenido de destruxina A producido por distintas cepas de un hongo, en medios de cultivo de con diferentes composiciones y estrés nutricional. La gran debilidad de este trabajo se manifiesta cuando se necesita determinar concentraciones de destruxina A a niveles muy bajos. En esos casos, se necesitaría evaluar y

validar nuevas metodologías de preconcentración y/o utilizar un espectrómetro de masas acoplado a un equipo de CE, ya que la sensibilidad del UV/Vis está limitada. Además este detector no sólo limita por la sensibilidad, sino también la posibilidad de identificar otras destruxinas, ya que comercialmente sólo existe la destruxina A (hoy en día la producción de estándares a través de medios de cultivo es un desafío), con lo cual es la única que se puede determinar con seguridad. Sin embargo es una de las mayoritarias, y su presencia suele indicar que existen también la B o la E, con lo cual en este trabajo se plantea como un marcador de la presencia de este grupo de metabolitos secundarios. Aunque por otra parte, el uso de la espectrometría de masas permite determinar un mayor número de destruxinas al conocer sus fragmentaciones mediante bibliografía. Por tanto como futura línea de investigación sería interesante estudiar el potencial de la CE-MS en esta área.

Además aunque en el trabajo se confirma a través de la bibliografía que los resultados obtenidos son comparables con los de otros autores, hubiera sido interesante analizar las mismas muestras de forma simultánea por CE-UV/Vis y HPLC-UV/Vis. De esta forma se podrían comparar los resultados obtenidos mediante ambas técnicas, y comprobar si para esta aplicación la CE ofrece alguna ventaja, diferente de las comentadas en la Introducción, con respecto a HPLC.

Por otra parte, se van a comentar las nuevas metodologías de SPE desarrolladas con nuevos materiales propuestos en esta Memoria.

Se ha propuesto el uso de un nanomaterial con propiedades magnéticas para preconcentrar Cd, Co, Cu, Ni, Zn en muestras agroalimentarias complejas. El mecanismo de retención y elución de los analitos se basa en un cambio drástico de pH del medio de 8 a 2 (para desprotonar o protonar los grupos carboxílicos de las nanopartículas) y complejación con la 1,10-fenantrolina. Sin embargo el pH ácido extremo al que se someten las nanopartículas hace que se deterioren con cada uso, apareciendo interferencias correspondientes al hierro que contienen las nanopartículas usadas. Además el pico del exceso de fenantrolina de la disolución de reextracción es otra interferencia permanente, que afecta a la determinación de Mg, con lo cual el método no es válido para determinar este analito.

Por otra parte, este es un método con potencial para aplicarse en otras muestras agroalimanterias tanto líquidas (por ej. bebidas) como sólidas (por ej. galletas), en todos los casos eliminando la materia orgánica con la etapa de mineralización. El LOD de la metodología propuesta para determinar metales se podría mejorar si fuera necesario, simplemente usando más volumen de muestra.

También hubiera sido interesante estudiar el efecto que tendría sobre la retención de los analitos sobre las nanopartículas el uso de la agitación mediante vortex frente al de ultrasonidos.

Con respecto a las partículas de organosílice mesoporosa periódicas con puentes de fenileno, se ha comprobado su efectividad para preconcentrar fenuron, simazina, atrazina, carbaril y terbutrin. Cuando estos

analitos están disueltos en agua, el material es capaz de retener por su afinidad, los cinco pesticidas estudiados. Aunque cuando el método se usa para la determinación de estos analitos en muestras de mosto, algunas interferencias hacen que sólo sea eficiente para determinar la atrazina, carbaril y terbutrin. Esto pone de relevancia la importancia de la optimización de los métodos usando muestras reales.

Además los resultados obtenidos han sido comparables a los obtenidos con C18 y HLB.

Por otra parte la estructura del material es estable en el intervalo de pHs de 2 a 9, lo que aumenta su capacidad de aplicación a diferentes muestras del campo agroalimentario. Sin embargo en la metodología desarrollada el volumen que se necesita para alcanzar los límites es bastante alto, haciendo la extracción lenta y tediosa. Quizás con el uso de una optimización bivariante para estudiar la cantidad de material y volumen de muestra simultáneamente, se podría haber disminuido alguna de estas dos variables.

Por último se ha aplicado con éxito una estructura zeolítica de imidazolato-8 para preconcentrar bisfenol A, diglicidil éter de bisfenol A y sus derivados hidrolizados en muestras de refresco. Al igual que en el trabajo de las nanopartículas magnéticas, en caso de ser necesario mejorar el LOD y LOQ de la metodología, se debería utilizar más volumen de muestra.

Finalmente como análisis general de las posibilidades que tiene la CE-UV/Vis como herramienta analítica dentro del campo agroalimentario para obtener información (bio)química de calidad, se ha realizado un análisis DAFO (debilidades, amenazas, fortaleza y oportunidades), que está representado en la Figura 1.

Figura 1. Análisis DAFO de la CE-UV/Vis dentro del campo de la agroalimentación.

En primer lugar, las debilidades que presenta la técnica en este área son las propias ya asignadas a la técnica. Es decir, su limitada sensibilidad debido al detector UV/Vis y al pequeño paso óptico del capilar. Además como amenaza, se presenta el desafío de presentar la CE-UV/Vis en un ámbito donde otras técnicas como la HPLC están bien establecidas. Sin embargo una fortaleza de la CE es su versatilidad, que hace que utilizando el mismo instrumento se puedan analizar analitos de diferente naturaleza. A

modo de ejemplo en esta Tesis Doctoral se han determinado ácidos orgánicos, metales, pesticidas y moléculas orgánicas como el bisfenol A y derivados.

Para posicionar mejor la CE-UV/Vis las investigaciones deben aprovechar las nuevas oportunidades que hay actualmente con el desarrollo de nuevos materiales. Además las nuevas áreas de investigación, como el caso de la evaluación de biopesticidas, se presentan como nuevos desafíos donde la CE-UV/Vis puede jugar un papel interesante como técnica analítica.

PRODUCCIÓN CIENTÍFICA

A. PUBLICACIONES DERIVADAS DE LA TESIS DOCTORAL

B. CONTRIBUCIONES A CONGRESOS

C. PÓSTERES PRESENTADOS

A. Publicaciones derivadas de la Tesis Doctoral

1. Differentiation of organic goat's milk based on its hippuric acid content as determined by Capillary Electrophoresis.

A. Carpio, V. Rodríguez-Estévez, M. Sánchez-Rodríguez, L. Arce, M. Valcárcel.

Electrophoresis 31 (2010) 2211-2217.

2. Use of carboxylic group functionalized magnetic nanoparticles for the preconcentration of metals in juice samples prior to the determination by Capillary Electrophoresis.

A. Carpio, F. Mercader-Trejo, L. Arce, M. Valcárcel.

Electrophoresis 33 (2012) 2446-2453.

3. Evaluation of hippuric acid content in goat milk as a marker of feeding regimen.

A. Carpio, D. Bonilla-Valverde, C. Arce, V. Rodríguez-Estévez, M. Sánchez-Rodríguez, L. Arce, M. Valcárcel.

Journal of Dairy Science 96 (2013) 5426-5434.

4. Sample treatment strategies for Capillary Electrophoresis analysis.

L. Arce, A. Carpio.

Encyclopedia of Analytical Chemistry 2014.

DOI: 10.1002/9780470027318.a9178.

5. Evaluation of phenylene-bridged periodic mesoporous organosilica as a stationary phase for solid phase extraction.

A. Carpio, D. Esquivel, L. Arce, F.J. Romero-Salguero, P. Van der Voort, C. Jiménez-Sanchidrian, M. Valcárcel.

Journal of Chromatography A 1370 (2014) 25-32.

6. Is Capillary Electrophoresis a promising technique to evaluate metabolites secreted by fungal biocontrol agents?

A. Carpio, A. Ríos-Moreno, I.Garrido-Jurado, E. Quesada-Moraga, L. Arce.

Enviado a Chromatographia, 2015.

7. Suitability of the zeolitic imidazolate framework 8 as sorbent in dispersive solid phase extraction of bisphenols.

A. Carpio, L. Arce, M. Valcárcel.

In progress.

B. Contribuciones a congresos

1. XII Reunión del grupo regional andaluz de la sociedad española de química analítica.

Fecha y lugar: 10-11 junio, 2010. Córdoba (España).

Póster: Differentiation of organic goat's milk based on its hippuric acid content as determined by capillary electrophoresis.

Autores: A. Carpio, V. Rodríguez-Estévez, M. Sánchez-Rodríguez, L. Arce, M. Valcárcel.

2. Jornadas Doctorales Andaluzas

Fecha y lugar: 18-23 septiembre, 2011. Mengíbar, Jaén (España).

Comunicación oral y póster: Diferenciación de alimentos ecológicos y convencionales usando estrategias analíticas.

Autores: A. Carpio, L. Arce, M. Valcárcel.

3. V Workshop NyNA

Fecha y lugar: 21-23 septiembre, 2011. Toledo (España).

Póster: Use of carboxylic group functionalized magnetic nanoparticles for the preconcentration of metals in food samples prior to the determination by capillary electrophoresis.

Autores: A. Carpio, F. Mercader-Trejo, L. Arce, M. Valcárcel.

4. II Congreso Científico de Investigadores en Formación de la Universidad de Córdoba

Fecha y lugar: 8-9 mayo, 2012. Córdoba (España).

Comunicación oral: Uso de la electroforesis capilar para buscar marcadores que distingan una leche ecológica.

Autores: A. Carpio, L. Arce, M. Valcárcel.

5. ITP 2013-20th International Symposium on Electro- and Liquid Phase-Separation Techniques.

Fecha y lugar: 6-9 octubre, 2013. Tenerife, Islas Canarias (España).

Comunicación oral: Determination of majority versus minority compounds in agrifood samples using capillary electrophoresis with UV-VIS detection.

Autores: L. Arce, A. Carpio.

6. XIV Reunión del grupo regional andaluz de la sociedad española de química analítica.

Fecha y lugar: 26-27 junio, 2014. Baeza, Jaén (España).

Póster: Evaluation of phenylene-bridged periodic mesoporous organosilica as a stationary phase or solid phase extraction.

Autores: A. Carpio, D. Esquivel, L. Arce, F.J. Romero-Salguero, P. Van Der Voort, C. Jiménez-Sanchidrián, M. Válcarcel.

7. 47th Annual meeting of the society of invertebrate pathology and International congress on invertebrate pathology and microbial control.

Fecha y lugar: 3-7 agosto, 2014. Mainz (Alemania).

Póster: Evaluation of destruxin A production in four strains of *Metarhizium* by capillary electrophoresis.

Autores: A. Ríos-Moreno, A. Carpio, I. Garrido-Jurado, L. Arce, M. Valcárcel, E. Quesada-Moraga.

8. 15th Meeting of the International organisation for biological and integrated control (IOBC)-WPRS Working group. Microbial and nematode control of invertebrate pests.

Fecha y lugar: 7-11 junio, 2014. Riga, Latvia (Letonia).

Comunicación oral: Development of a QuEChERS-based extraction method for the determination of destruxins in potato plants by UHPLC-MS/MS.

Autores: A. Carpio, N. Arroyo-Manzanares, A. Ríos-Moreno, I. Garrido-Jurado, L. Gámiz-Gracia, A. García-Campaña, E. Quesada-Moraga, L. Arce.

C. Pósteres presentados

DIFFERENTIATION OF ORGANIC GOAT´S MILK BASED ON ITS HIPPURIC ACID CONTENT AS DETERMINED BY CAPILLARY ELECTROPHORESIS

A. Carpio,[1] V. Rodríguez-Estévez,[2] M. Sánchez-Rodríguez,[2] L. Arce,[1] M. Valcárcel[1*]

[1] Department of Analytical Chemistry, University of Córdoba, Annex C3 Building, Campus of Rabanales, E-14071 Córdoba, Spain, *qa1meobj@uco.es

[2] Department of Animal Production, University of Córdoba, Campus of Rabanales, E-14071 Córdoba, Spain

Is it possible to differentiate organic milk from conventional milk by using CE?

Goat type: Conventional
Composition of the fodder type:
1 kg/day corn, oats, beans
2–3 h grazing in an oat field
Rest of day: grazing in a *dehesa*

Goat type: Organic
Composition of the fodder type:
0.5 kg/day organic mixture (ECORSEVILLA)
12:00–18:00 h: grazing in a fallow meadow
Rest of day: grazing in bush

OBJECTIVE

To assess the potential of capillary electrophoresis to find a marker that is able to identify the particular food given to the goats

- Possible markers??
  - Organic acids
    - Oxalic acid, Benzoic acid, Citric acid, Uric acid, Orotic acid, Hippuric acid
- The feeding of these goats can be influenced by the time of the year
  - For this reason we have collected samples from 20 conventionally fed goats and 20 organically fed goats over a period of 2 months

EXPERIMENTAL

Sample preparation

9 mL milk → Addition of 2 M HCl to adjust sample pH to 3.4 – 3.6 → Centrifugation 6000 rpm, 20 min → Separation of the whey → Centrifugation 6000 rpm, 10 min → Filtration of the whey trough 0.45 µm filters → Sample ready for injection

Electrophoretic Conditions

Buffer: 120 mM sodium tetraborate decahydrate + 0.5 mM CTAB at pH 8
Voltage: 10 kV, reverse polarity
Hydrodynamic injection: 0.5 psi for 5 s
Wavelength: 200 nm
Capillary total length: 60.2cm (75 µm I.D.)

RESULTS

Method Validation

Calibration curves and statistical figures for merit of the determination of a mixture of organic acids standard.

Organic acid	y= mx + b		Sy/x	R
	m	b		
Oxalic acid	400 ± 6	-135 ± 295	1081	0.9972
Citric acid	206 ± 10	555 ± 572	1477	0.9798
Orotic acid	2161 ± 52	-3703 ± 2904	7975	0.9946
Benzoic acid	6109 ± 154	-10912 ± 5988	21090	0.9931
Uric acid	3203 ± 104	-5015 ± 5160	18900	0.9871
Hippuric acid	5304 ± 102	-6625 ± 5043	18473	0.9954

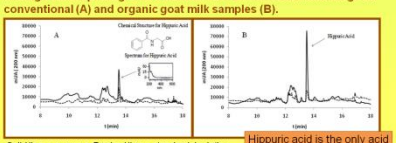
Repeatability and reproducibility of the mixture of the six organic acids (n=9).

Organic acid	Repeatability[1] RSD (%)		Reproducibility[1] RSD (%)		Repeatability[2] RSD (%)		Reproducibility[2] RSD (%)	
	peak area	migration time	peak area	migration time	peak area	migration time	peak area	migration time
Oxalic acid	9.77	0.30	3.14	3.18	8.49	0.37	4.72	1.54
Citric acid	7.70	0.18	1.60	3.33	22.70	0.19	10.21	2.16
Orotic acid	9.07	0.20	5.49	3.93	10.80	0.21	19.24	2.62
Benzoic cid	11.14	0.20	4.51	4.08	15.74	0.21	2.97	2.64
Uric acid	5.04	0.27	9.38	3.74	9.31	0.24	3.55	2.56
Hippuric acid	6.64	0.20	2.86	4.13	9.27	0.21	2.28	1.19

[1]Results of a mixture of organic acids at 100 mg L^{-1}. [2]Results of a mixture of organic acids at 10 mg L^{-1}.

Sample Analysis

Average electropherograms obtained from 20 individual recordings for conventional (A) and organic goat milk samples (B).



Solid line = average. Dashed line = standard deviation

Hippuric acid is the only acid directly identified

Hippuric acid concentrations found in 20 organic goat samples and 20 conventional organic samples.

Sample type	Sampling period	Range of hippuric acid concentration (mg L^{-1})	$\bar{X} \pm SE$ (mg L^{-1})	
Organic	February 2009	72.59-167. 82	128 ± 10[a]	132 ± 7[a]
	April 2009	93.35-188. 96	137 ± 10[a]	
Conventional	February 2009	17.75-66.59	33 ± 5[b]	59 ± 7[b]
	April 2009	53.83-125.84	83 ± 7[c]	

$\bar{X} \pm$ **SE:** mean ± statistic error. Mean values with the same letters (a,b,c) indicate homogeneous subsets for α = 0.001 according to Tukey´s HSD test.

CONCLUSIONS

- The proposed electrophoretic method is able to detect and quantify the hippuric acid present in goat's milk. To our knowledge, this is the first time hippuric acid in such a complex matrix as goat's milk has been quantified with a rapid, simple electrophoretic method.
- The preliminary results shown in this manuscript led us to think that by using hippuric acid as marker, we could distinguish between milk from organically and conventionally fed goats. To support this hypothesis new experiments increasing the number of milk samples from different goats are being carried out.

DIFERENCIACIÓN DE ALIMENTOS ECOLÓGICOS Y CONVENCIONALES USANDO DISTINTAS ESTRATEGIAS ANALÍTICAS

Azahara Carpio Osuna
Lourdes Arce Jiménez, Miguel Valcárcel Cases
Departamento de Química Analítica FQM-215, Campus de Rabanales, Universidad de Córdoba

OBJETIVO GENÉRICO

Asegurar la **calidad de los alimentos** (el valor nutricional, las propiedades funcionales de algunos alimentos, su composición exacta y si es o no ecológico, entre otros parámetros o índices de calidad) mediante técnicas analíticas como la **Electroforesis Capilar (CE)** o la **Espectrometría de movilidad Iónica (IMS)**.

OBJETIVOS ESPECÍFICOS

1. Evaluar el **potencial de la Electroforesis Capilar** para su implantación en laboratorios agroalimentarios.
2. Búsqueda de un **marcador o un perfil electroforético** característico para diferenciar entre **leche ecológica y convencional**, o el régimen de alimentación suministrado a cabras o vacas, y así evitar posibles fraudes comerciales.
3. Aplicación de la técnica **IMS** para caracterizar los compuestos volátiles presentes en leche.
4. Uso de **nanopartículas** magnéticas para la **preconcentración** de metales presentes en muestras de alimentos por Electroforesis Capilar.
5. Estudio de la "**Foodomics**" y su relación con técnicas electroforéticas.

EJEMPLO

DIFERENCIACIÓN DE LECHE ECOLÓGICA Y CONVENCIONAL DE CABRA MEDIANTE ELECTROFESIS CAPILAR CON DETECCIÓN ULTRAVIOLETA VISIBLE (CE-UV-VIS)

PROBLEMA

¿SERÁ REALMENTE LECHE ECOLÓGICA?

SOLUCIÓN

La **Electroforesis Capilar** es una técnica de separación basada en la velocidad de migración de partículas cargadas debido a la acción de un campo eléctrico. El soporte de la separación es un capilar de sílice fundida.

ETAPAS DEL PROCESO ANALÍTICO

MUESTREO

Recogida de leche ecológica y convencional de cabra

Leche convencional: cabra con régimen de alimentación basado en pienso

Leche ecológica: cabra con régimen de alimentación basado en pasto y pienso ecológico

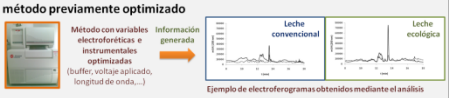
Envío al laboratorio

TRATAMIENTO DE MUESTRA

Extracción de ácidos orgánicos de cada muestra de leche

TÉCNICA ANALÍTICA

Análisis de los extractos de leche mediante CE-UV-VIS a través de un método previamente optimizado

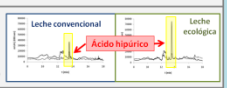


Ejemplo de electroferogramas obtenidos mediante el análisis

TRATAMIENTO DE DATOS

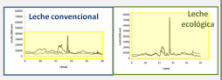
Estudio de uno o varios analitos

Búsqueda de marcadores que identifiquen un producto como ecológico



Estudio de toda la información analítica obtenida (con tratamientos estadísticos)

"Foodomics"



CONCLUSIONES

1. El método propuesto permite detectar y cuantificar el ácido hipúrico presente en la leche de cabra mediante un método electroforético simple y rápido
2. Los estudios preliminares indican que el ácido hipúrico podría ser un marcador para diferenciar leche ecológica de leche convencional

USE OF CARBOXYLIC GROUP FUNCTIONALIZED MAGNETIC NANOPARTICLES FOR THE PRECONCENTRATION OF METALS IN FOOD SAMPLES PRIOR TO THE DETERMINATION BY CAPILLARY ELECTROPHORESIS

A. Carpio[1], F. Mercader[2], L. Arce[1], M. Valcárcel[1*]
[1] Department of Analytical Chemistry, University of Córdoba, Annex C3 Building, Campus of Rabanales, E-14071 Córdoba, Spain,
[2] Metrología de Materiales, Centro Nacional de Metrología, km.4.5 Carretera a Los Cúes, El Marqués, Querétaro, México
*qa1meobj@uco.es

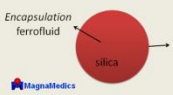
OBJECTIVE

Food industry → **It demands reliable and precise methods** to resolve problems related to quality and security control

Food samples → The food **matrices are very complex** and for this reason **pre-treatment steps prior to sample analysis** are compulsory to extract the target analytes

In this work, we took the advantages of **magnetic nanoparticles** using them to carry out the **preconcentration of metals** presents in food samples before the determination of the complex formed by **capillary electrophoresis with UV-VIS detection**

CARBOXILIC GROUP FUNCTIONALIZED MAGNETIC NANOPARTICLES



Technical Data	
Mean size	1.2 µm (±0.1 µm)
Material	Magnetic silica beads with high magnetic content. Optimized for nucleic acid isolation.

It's the first time that this type of magnetic nanoparticles has been used to preconcentrate metals before their determination by capillary electrophoresis

EXPERIMENTAL AND PRELIMINARY RESULTS

SAMPLE TREATMENT

EXTRACTION OF METALS IN FOOD SAMPLES

1 COMPLEXATION OF METALS (DIRECT METHOD)

Aqueous extract with metals
+
1,10-phenantroline 0.2%
+
hydroxylammonium chloride 10%

Analysis CE-UV-VIS

2 PRECONCENTRATION AND COMPLEXATION OF METALS WITH CARBOXYLIC GROUP FUNCIONALIZED MAGNETIC NANOPARTICLES

STEP 1 — **10 mL of aqueous sample** which contains the mixture of metals at pH 8 is added to a **2 mg** of carboxylic group functionalized magnetic **nanoparticles**

STEP 2 — The solution is **shaken** in the vortex for **5 minutes**

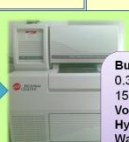
STEP 3 — The nanoparticles with metals retained are separated from solution with an **external magnet**

STEP 4 — **200 µL** of **re-extraction solution** (0.8 mM 1,10-phenantroline and 0.04% hydroxylammonium chloride at pH 2) is added to the nanoparticles with metals retained

STEP 5 — The solution is **shaken** in the vortex for **5 minutes**

STEP 6 — The nanoparticles with metals retained are separated from solution with an **external magnet,** and then the solution is analysed by CE-UV-VIS
Nanoparticles are **reused** after a cleaning process.

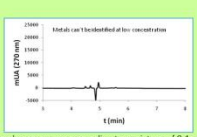
ANALYSIS



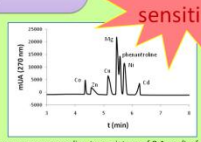
Electrophoretic conditions

Buffer: 30 mM hydroxylammonium chloride + 0.30 mM 1,10-phenantroline + 80 mM urea + 15 mM ammonium chloride + 0.1% methanol + pH 3.6
Voltage: 20 kV, normal polarity
Hydrodynamic injection: 0.5 psi for 30 s
Wavelength: 270 nm
Capillary total length: 50.2cm (50 µm I.D.)

Improved sensitivity



Electropherogram corresponding to a mixture of 0.1 mg/L of Co, Zn, Cu, Mg, Ni, Cd



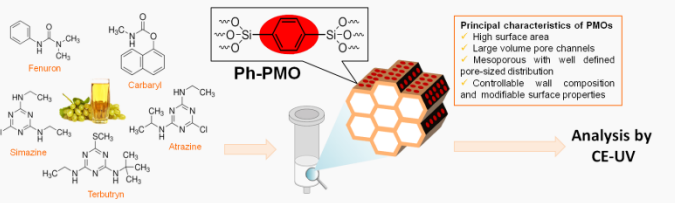
Electropherogram corresponding to a mixture of 0.1 mg/L of Co, Zn, Cu, Mg, Ni, Cd

Evaluation of Phenylene-Bridged Periodic Mesoporous Organosilica as a Stationary Phase for Solid Phase Extraction

A. Carpio[1], D. Esquivel[2], L. Arce[1], F.J. Romero-Salguero[2], P. Van Der Voort[2], C. Jiménez-Sanchidrián[2], M. Valcárcel[1]*
[1]Department of Analytical Chemistry, University of Cordoba, Annex C3 Building, Campus of Rabanales, Cordoba, Spain. *qa1mecbj@uco.es
[2]Department of Inorganic and Physical Chemistry, Center for Ordered Materials, Organometallics and Catalysis (COMOC), Ghent University, Krijgslaan 281-S3, B-9000 Ghent, Belgium.
[3]Department of Organic Chemistry, Nanochemistry and Fine Chemistry Research Institute (IUIQFN), Campus of Rabanales, University of Cordoba, Campus de Excelencia Internacional Agroalimentario (CeiA3), Cordoba, Spain

Objective

A periodic mesoporous organosilica consisting of phenylene-bridged material (Ph-PMO) was for the first time evaluated as a sorbent for retaining and eluting fenuron, simazine, atrazine, carbaryl and terbutryn in grape must by solid phase extraction (SPE) prior to their determination with a simple analytical method based on capillary electrophoresis with ultraviolet detection (CE-UV). The analytes were used as model compounds to demonstrate the potential of Ph-PMO for increasing the sensitivity of CE



Solid phase extraction procedure

Variable	Range studied	Optimal value
Amount of Ph-PMO	30 – 90 mg	30 mg
Sample volume	40 – 200 mL	100 mL
Sample pH	2 – 9	8 in standards mixture of pesticides natural pH in grape must (3.2)
Eluent	acetonitrile, ethanol and methanol	1 mL acetonitrile

Evaporation to dryness of the eluate under a nitrogen stream and reconstitution of the residue in 200 µL of water

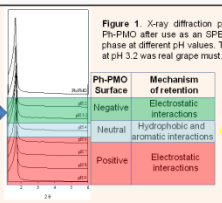


Figure 1. X-ray diffraction patterns for Ph-PMO after use as an SPE stationary phase at different pH values. The sample at pH 3.2 was real grape must.

Ph-PMO Surface	Mechanism of retention
Negative	Electrostatic interactions
Neutral	Hydrophobic and aromatic interactions
Positive	Electrostatic interactions

Identical XRD patterns testifies a high stability of Ph-PMO in a wide range of pH

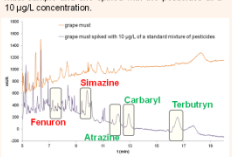
Electrophoretic conditions*

Buffer	10 mM Na_2HPO_4+ 60 mM SDS + 8 % AcN + pH 9.5
Capillary	Total lenght 60.2 cm Inner diameter 75 µm
Voltage	25 kV
Hydrodinamic Injection	0.5 psi for 5s
Temperature	25 ºC
λ	226 nm

*P.Hinsmann, L.Arce, A.Rios, M.Valcárcel, J. Chromatogr. A 866 (2000) 137

Validation

Figure 2. Electropherograms for an unspiked grape must sample and one spiked with the pesticides at a 10 µg/L concentration.



× Fenuron and simazine cannot be determined because they coemigrate with other unknown compounds

✓ Atrazine, carbaryl and terbutryn can be determined free of interferences

Table 1. LODs and LOQs for the determination of a mixture of pesticides using Ph-PMO as a sorbent of SPE before CE analysis

Analyte	LOD* (µg/L)	LOQ* (µg/L)	Official MRLs (European Union)
Fenuron	4	10	0.01 mg/kg
Simazine	0.8	3	0.01 mg/ kg
Atrazine	0.6	2	0.05 mg/kg
Carbaryl	1	4	0.01 mg/ kg
Terbutryn	3	10	0.01 mg/kg

Acceptable LOQs and LODs below official MRLS

Table 2. Recoveries from grape must samples spiked with the analytes at different concentration levels as obtained by using Ph-PMO as a sorbent in SPE

Analyte	Added (µg/L)	Recovery (%)
Atrazine	10	105 ± 7
	30	103 ± 4
	60	90 ± 10
Carbaryl	10	86 ± 3
	30	97 ± 6
	60	91 ± 5
Terbutryn	10	91 ± 7
	30	96 ± 12
	60	97 ± 9

Table 3. Precision corresponding to a standard mixture of pesticides and the same pesticides added to grape must samples submitted to the SPE procedure

Analyte	Intraday precision (%RSD, n=6)		Interday precision (%RSD, n=9)	
	Standard	Sample	Standard	Sample
Fenuron	13	-	13	-
Simazine	10	-	13	-
Atrazine	12	13	14	15
Carbaryl	11	12	12	16
Terbutryn	12	13	13	14

Ph-PMO was an effective sorbent material for extracting the selected pesticides

Comparison with commercial SPE cartridges

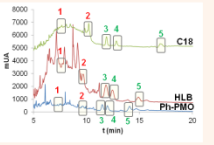


Figure 3. Electropherograms obtained after SPE of a grape must sample spiked with a 10 µg/L concentration of the standard mixture of pesticides by using C18, HLB and Ph-PMO as sorbents. 1, fenuron; 2, simazine; 3, atrazine; 4, carbaryl; 5, terbutryn.

Ph-PMO and C18 exhibited a similar ability not only to remove interferences, but also to retain the selected analytes

HLB proved even more efficient in extracting the pesticides

Also Ph-PMO was as selective as C18 and HLB for the determination of the target pesticides in grape must, and hence equally useful for this purpose

The results of this comparative test highlight the outstanding properties of this new type of material

Reusability

Table 4. Physicochemical properties of phenylene-bridged PMO

Material	d_{100} [a] (nm)	a_0 [b] (nm)	S_{BET} [c] ($m^2 g^{-1}$)	Vp [d] ($cm^3 g^{-1}$)	D_p [e] (nm)	Wall thickness [f] (nm)
Ph-PMO	5.4	6.2	790	0.57	3.2	3.0
Ph-PMO after use in SPE	5.3	6.1	658	0.47	3.2	2.9

[a] d_{100} spacing determined from XRD; [b] Unit cell dimension, calculated as $a_0 = 2d_{100}/\sqrt{3}$; [c] Specific surface area determined by the BET method; [d] Pore volume; [e] Pore diameter determined by the BJH method; [f] Estimated from (a_0-D_p).

Mesostructure and particle size were preserved after the first use

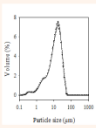
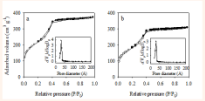


Figure 5. Particle size distribution curves for the parent Ph-PMO material (•) and Ph-PMO after used as an SPE sorbent (○).

Figure 4. N2 adsorption–desorption isotherms and pore size distribution curves for (a) the parent Ph-PMO material and (b) Ph-PMO after use as an SPE sorbent



Both the specific surface area and the pore volume decreased after use, even though the N_2 adsorption–desorption isotherm remained of the type IV, which is typical of mesoporous materials

Ph-PMO can be used twice as a sorbent

This can be ascribed to the presence of compounds from grape must that were retained by the sorbent but not removed by the eluent

Conclusions

- A phenylene-bridged periodic mesoporous organosilica (Ph-PMO) has been successfully applied as a sorbent in SPE of the pesticides selected.
- Ph-PMO has shown similar properties in terms of selectivity to extract pesticides in grape must compared with other commercial sorbents such as C18 and HLB.
- Ph-PMO is the starting point for the development of a new family of stationary phases for SPE applications based on PMOs.