DEPARTAMENT DE MEDICINA PREVENTIVA I SALUT PÚBLICA, CIÈNCIES DE L'ALIMENTACIÓ, TOXICOLOGIA I MEDICINA LEGAL

ANÁLISIS DE RESIDUOS DE CONTAMINANTES ORGÁNICOS EN ALIMENTOS POR TÉCNICAS CROMÁTOGRÁFICAS

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ANÁLISIS DE RESIDUOS DE CONTAMINANTES ORGÁNICOS EN ALIMENTOS POR TÉCNICAS CROMATOGRÁFICAS

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Ana Juan García

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CERTIFIQUEN que:

Ana Juan García, Llicenciada en Farmacia per la Universitat de València, ha estat treballant sota la nostra direcció per a l´elaboració de la Tesi "Análisis de residuos de contaminantes orgánicos en alimentos por técnicas cromatográficas", i que presenta per a optar a Títol de Doctora en Farmacia per la Universitat de València.

Per a que així conste als efectes oportuns, signem el present document en València, a 18 de Gener de 2008

Guillermina Font Pérez Yolanda Picó García

This thesis is presented as European PhD and it is included in the

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Ana Juan García agraeix al Ministeri d´Educació i Ciència per la beca predoctoral de Formació de Personal Investigador BES-2004-4780 (2004-2008).

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ESTRUCTURA DE LA TESIS – *Thesis structure*

La memoria de la tesis que se presenta se ha estructurado en cuatro apartados: Introducción, Material y Reactivos, Resultados y Discusión, y Conclusiones.

En el primer apartado se hace una introducción general sobre los compuestos estudiados, los plaguicidas y los medicamentos de uso veterinario. Se abarcan aspectos tales como su importancia agrícola, ganadera, sanitaria, ecológica, económica, social y las Directivas Europeas por las que se regulan estos compuestos; así como la incidencia, toxicidad y las técnicas analíticas utilizadas para su extracción, separación, preconcentración y para su detección.

En el segundo apartado se detalla de manera íntegra los reactivos y el material utilizados para el desarrollo del trabajo experimental.

El tercer apartado se ha estructurado en seis capítulos, en los que se expone el trabajo experimental realizado a lo largo de la tesis doctoral. En general, estos capítulos se inician con un resumen, una optimización de la técnica de extracción, de separación y de detección, y su aplicación a muestras reales, así como una discusión de los resultados que se obtienen.

En el capítulo I, se establece la metodología analítica por cromatografía líquida con espectrometría de masas para el análisis de residuos de plaguicidas en uva. Se evalúan las interfases de presión atmosférica y de electrospray en los dos modos de ionización, positivo y negativo, dos procesos de extracción, extracción en fase sólida y extracción sobre barras magnéticas, y validación del método.

En el capítulo II se desarrolla la metodología analítica basada en la electroforesis capilar electrocinética micelar para el análisis de ocho plaguicidas y se comparan dos métodos de extracción, extracción en fase sólida y extracción sobre barras magnéticas, para muestras de lechuga, tomate, uva y fresas.

En el capítulo III se describe un método basado en el acoplamiento de la electroforesis capilar a la espectrometría de masas con interfase de electrospray con dos analizadores de masas, simple cuadrupolo y trampa de iones, para identificar y cuantificar seis plaguicidas en melocotones y nectarinas.

En el capítulo IV se ensayan métodos de preconcentración en línea de electroforesis capilar con detección ultravioleta: migración reversa de micelas con tapón de agua, técnica de focalización y de acumulación con migración reversa de micelas sin tapón de agua. El trabajo se desarrolla para cinco plaguicidas en muestras de uva y lechuga.

En el capítulo V se desarrolla una metodología analítica basada en electroforesis capilar-espectrometría de masas y se realiza un estudio comparativo de la fragmentación de cinco quinolonas utilizando dos analizadores de masas: trampa de iones y simple cuadrupolo. El método se aplica a muestras de carne de pollo y pescado.

En el capítulo VI se estudia la técnica de extracción presurizada con disolventes y se desarrolla un método analítico basado en electroforesis capilar-espectrometría de masas en tándem para la identificación y cuantificación de doce sulfonamidas en carne de cerdo.

En el apartado de resultados y discusión se incluye un resumen de los resultados obtenidos para la extracción, técnicas de preconcentración, separación, determinación y aplicación a muestras reales con los métodos desarrollados.

En el último apartado se recogen las conclusiones extraídas de los resultados obtenidos.

THESIS STRUCTURE

The reported thesis has been structured in four sections: Introduction, Material and Reagents, Results and Discussion and Conclusions.

The first section deals with general introduction about the compounds studied, pesticides and veterinary drugs. It takes in different aspects of importance as agricultural, livestock, sanitary, ecological, economy and social and European Guidelines which control these compounds; it includes also incidence, toxicity and analytical techniques used for its extraction, separation, preconcentration and detection.

In the second section exhaustively details material and reagents used for the experimental work.

The third section, Results and Discussion, has been structured in six chapters, that describes and analyzes the experimental work developed through the thesis. In general, these chapters start with a summary, followed by an optimization of the extraction, separation and detection techniques, and application to real samples, as well as a discussion of the obtained results.

Chapter I establishes the analytical methodology by liquid chromatography-mass spectrometry for analyzing pesticide residues in grape. Atmospheric pressure chemical ionization and electrospray interfaces in both ionization modes, positive and negative, two extraction procedures based in solid phase extraction and stir bar sorptive extraction, are developed and validated.

Chapter II describes the analytical methodology based on micellar electrokinetic capillary electrophoresis for analyzing eight pesticides. Two extraction methods, solid phase extraction and stir bar sorptive extraction are also compared and applied to lettuce, tomato, grapes and strawberry samples.

In Chapter III, a method based in coupling capillary electrophoresis to mass spectrometry using electrospray interface with two mass analyzers, single quadrupole and ion trap, to identify and quantify six pesticides in peaches and nectarines is developed.

Chapter IV describes the use of capillary electrophoresis on-line preconcentration strategies with diode array detector. This work is developed for five pesticides in grapes and lettuce samples.

Chapter V develops an analytical methodology based in capillary electrophoresis-mass spectrometry and a comparative study of five quinolone fragmentation by using two mass analyzers: ion trap and simple quadrupole. The method is applied in chicken and fish muscle.

In Chapter VI, pressurized liquid extraction method is studied and an analytical method based in capillary electrophoresis-tandem mass spectrometry is developed to identify and quantify twelve sulphonamides in pork meat.

Results and Discussions, also includes a summary of the obtained results for extraction and separation processes, preconcentration and determination techniques and application to real samples for the developed methods.

The last section collects the conclusions, extracted of the obtained results.

Introducción
Introduction

1.-Introducción

La contaminación de los alimentos por sustancias químicas es un problema de salud pública de gran preocupación a escala mundial. El motivo puede ser consecuencia de la contaminación ambiental que puede alcanzar a las cadenas alimentarias a través del aire, del agua y del suelo, como ocurre en el caso de metales, bifenilos policlorados (PCBs), dioxinas, etc. Contribuye también el uso intencionado de diversos productos químicos, tales como pesticidas, fármacos o sustancias administradas a los animales, y de otros productos agroquímicos (abonos, etc). Algunos aditivos utilizados en alimentos así como la fabricación y el procesamiento de los mismos también pueden aportar contaminantes.

Por tanto son muchos los contaminantes orgánicos que pueden estar presentes en alimentos, como micotoxinas, plaguicidas, y otros compuestos asociados a la contaminación ambiental o bien sustancias que por el procesado, conservación y/o envasado pueden encontrarse en los alimentos. Para vigilar la seguridad alimentaria, diversas agencias medioambientales y de calidad se encargan de que la legislación se cumpla de forma rigurosa, mediante la aplicación de métodos analíticos adecuados para la detección de contaminantes en alimentos de forma rápida y eficaz [1,2], utilizando metodologías multirresiduo y realizando muestreos y controles sobre los alimentos en fase de comercialización.

A lo largo del último siglo, los avances logrados en las condiciones sanitarias de las producciones vegetales y animales junto con el establecimiento de los sistemas veterinarios de higiene e inspección de los alimentos, han conducido a una situación de garantías sanitarias excelentes. A pesar de ello, el interés de la población por la "seguridad alimentaria" se ha convertido en una

exigencia por parte de los ciudadanos en tanto que son consumidores responsables.

Los hábitos alimentarios han evolucionado a lo largo de los años y se ha pasado de las necesidades basadas en la supervivencia del hombre primitivo, a las demandas que en las sociedades desarrolladas los ciudadanos plantean en lo relacionado con los alimentos, como son la calidad nutricional, sensorial y la garantía de los mismos. De igual modo la industria agroalimentaria también ha variado, desde la producción agrícola y ganadera y de una forma muy llamativa, mediante el desarrollo de nuevas tecnologías en el procesado y conservación de los alimentos, lo que se traduce en una mayor producción de alimentos, y como resultado el desarrollo de la agricultura intensiva con utilización de plaguicidas o alimentos modificados genéticamente entre otros aspectos.

Para desarrollar la ganadería, se han controlado los sistemas de producción y la salud de los animales, mediante la actuación de profesionales y la utilización de productos que contribuyen a la obtención de animales sanos y que proporcionan alimentos saludables y de alta calidad.

Los productos destinados al tratamiento o la prevención de enfermedades en los animales, deben ser sometidos a una evaluación científica y a estrictos controles orientados a que se cumplan las diferentes normativas tanto españolas como europeas que existen en este campo, para lo que se recurre a las autoridades competentes, nacional o europea, según el lugar donde se vaya a comercializar el producto. La nacional corresponde a la Agencia Española de Medicamentos y Productos Sanitarios y la europea a la Agencia Europea de Medicamentos. De este modo se satisface la evaluación, desde el punto de vista de la *calidad* del medicamento, de su s*eguridad* para los propios animales, el medio ambiente y los consumidores, y de su *eficacia* frente a las dolencias a las que está orientado.

Todo esto ha dado lugar a una gran sensibilización de la población a la contaminación de los alimentos por productos químicos y una necesidad de aumentar los niveles de control de los contaminantes en alimentos llevada a cabo por la Administración Sanitaria. Se han implantado sistemas de control de calidad que se basan en abordar la situación desde una perspectiva global, del conjunto de la cadena alimentaria y desde la producción al consumidor final también conocido como "trazabilidad" de los productos [1-3].

La Organización Mundial de la Salud para la Agricultura y la Alimentación (FAO) define Seguridad Alimentaria como: "El acceso físico y económico del consumidor en todo momento a suficientes alimentos inocuos y nutritivos para satisfacer sus necesidades alimentarías y sus preferencias en cuanto a los alimentos a fin de llevar una vida activa y sana".

En esta tesis la repercusión del uso plaguicidas y medicamentos veterinarios puede considerarse desde varias vertientes: agrícola y ganadera, sanitaria, ecológica o ambiental, social y económica.

1.1. Importancia Agrícola y Ganadera

La producción agrícola tiene una importancia considerable en la alimentación, pero su rendimiento se ve constantemente afectado por organismos y plantas dañinos de cuyos efectos es necesario proteger a las plantas, los productos vegetales y no solamente para evitar una reducción de la producción sino también para aumentar la productividad agrícola. Para este fin la utilización de plaguicidas químicos es uno de los métodos más importantes tanto en plantas y productos vegetales así como para el ganado para la protección de los efectos de organismos nocivos.

Los plaguicidas, por su propia naturaleza, no tienen únicamente un efecto favorable en la producción vegetal y animal, ya que generalmente se trata de sustancias o preparados con efectos tóxicos peligrosos, destinados a prevenir o controlar toda especie indeseable de plantas y animales que se utilice como defoliante, desecante o reguladora del crecimiento vegetal (según la Organización Mundial de la Salud-OMS) [4].

Los plaguicidas actúan negativamente alterando procesos vitales, y por lo tanto su uso supone un riesgo para los seres vivos. Por otra parte, el riesgo de toxicidad para las personas merece una mención especial ya que afecta tanto a las personas que los aplican, las cuales estan directamente expuestos durante mucho tiempo a los plaguicidas, como para los que consumen los productos tratados, por los residuos que puedieran contener los vegetales (frutas, hortalizas, cereales) en el momento de la recolección. La exposición a los plaguicidas es distinta según el grupo de población, ya que es más elevada en personas directamente expuestas (fabricantes, aplicadores) que en población en general, aunque esta es más numerosa [5].

Es de interés señalar que un gran número de plaguicidas y de sus productos de metabolización o de degradación, pueden tener efectos nocivos para los consumidores de productos vegetales y animales, así mismo, dichos plaguicidas y los contaminantes que puedan llevar incorporados, pueden presentar riesgos para el medio ambiente y afectar indirectamente al hombre a través de productos de origen animal.

El riesgo que suponen los plaguicidas para el medio ambiente implica la destrucción de determinados seres vivos, alterando las cadenas tróficas y provocando desequilibrios biológicos, que en algunos casos suponen aparición de nuevas plagas o intensificación de las existentes por eliminación de sus enemigos naturales, lo que supondría nuevas aplicaciones químicas para

controlar las nuevas plagas. Producen también contaminación química del medio (aire, suelo, agua) con sustancias potencialemtne peligrosas.

Por esta razón, existe una regulación en la distribución y utilización de plaguicidas en la que se especifica que la cantidad de aplicación del plaguicida debe ser lo más baja posible y el intervalo entre la aplicación y el consumo del producto debe ser lo más amplio posible de forma que el residuo se reduzca al mínimo, es lo que se denomina tiempo de espera.

En el caso de los medicamentos, es a través de las autoridades competentes nacionales o europeas, las encargadas de establecer el periodo que debe pasar desde el último tratamiento y la fecha de sacrificio del animal u obtención de otros productos como leche, huevos, etc. [5].

Con esta regularización se garantiza que los alimentos lleguen a los consumidores libres de residuos o al menos dentro de los límites permitidos.

1.2. Importancia Sanitaria y Toxicológica

Los productos químicos en general, y más en particular los de estudio en esta tesis, plaguicidas y medicamentos veterinarios, están relacionados con el desarrollo de la sociedad moderna y los avances en la tecnología alimentaria. La exposición a estos productos y la ingestión a través de alimentos que los contienen conlleva riesgos para la salud, siendo de especial relevancia el aumento de cánceres, alergias, enfermedades crónicas inexplicables como efectos sobre la fertilidad, defectos del nacimiento, deterioro del sistema inmunológico y lesiones cerebrales.

La Seguridad Alimentaria abarca actuaciones preventivas realizadas por la administración sanitaria así como la limitación del uso o prohibición de

ciertos compuestos químicos, mediante la publicación de normas legales y la realización de actuaciones de vigilancia y control, comprobándose el establecimiento por parte de la Industria Alimentaria de sistemas de autocontrol [3-6].

La toxicidad de los contaminantes se clasifica en tres categorías toxicológicas: nocivos, tóxicos y muy tóxicos. En algunos casos pueden tener otros efectos peligrosos (corrosivos, inflamables, comburentes, explosivos, ...). . Estas clasificaciones son útiles para una adecuada tipificación y conocimiento de la peligrosidad de un determinado compuesto, aunque en realidad debido a los diversos mecanismos de actuación y acciones secundarias de cada contaminante, existe más variedad [5].

En definitiva los contaminantes presentes en los alimentos tienen una dimensión toxicológica compleja e incluso más que los productos aislados, por las posibles interacciones con los propios nutrientes u otros constituyentes, siendo fundamental no sólo que estos contaminantes se identifiquen, sino que se establezcan las propiedades toxicológicas de cada uno de ellos y de la mezcla de todos, pues en la mayoría de los casos existen fenómenos de sinergia aditiva, potenciación y/o antagonismo.

1.3. Importancia Ecológica

En España de los 500 millones de kilos de plaguicida utilizados anualmente, sólo el 1 % de los productos alcanza a los organismos nocivos a los que van destinados. El 99 % restante permanece en los ecosistemas, se transfieren a la atmósfera por volatilización, al suelo y a los acuíferos. Los residuos que permanecen en los productos agrícolas, se ingieren por los animales y el hombre, si no se respetan los plazos de seguridad.

La utilización de plaguicidas y medicamentos veterinarios ha originado la aparición de especies resistentes a determinados compuestos, produciéndose una disminución de la eficacia de dichos productos. El desarrollo de resistencias ocasiona la necesidad de aumentar las dosis necesarias para conseguir el mismo efecto con el consiguiente aumento de los efectos indeseables.

Entre los efectos indeseables sobre el medio ambiente por la aplicación de plaguicidas se encuentra la desaparición de polinizadores, comprometiendo con ello el éxito reproductivo de gran variedad de plantas que necesitan de su consumo para reproducirse. Asimismo, la destrucción o desaparición de los enemigos naturales de distintas plagas, puede conducir a la proliferación de especies antagónicas de aquellas extinguidas y producir nuevas plagas.

El mecanismo de acción de estos compuestos no es selectivo, y afecta no sólo a las plagas que se pretenden controlar sino también a vectores relacionados y otras especies, provocando desequilibrios de los ecosistemas sobre los que se aplican.

A medida que se asciende en la cadena alimentaria se producen fenómenos de bioacumulación, bioconcentración y biomagnificación que contribuyen a que aquellas especies que se sitúan en la parte superior de la pirámide se vean afectadas. Por ejemplo, en algunas comunidades de aves se ha incrementado el porcentaje de esterilidad, entre otros efectos.

Todos estos fenómenos han originado, en los últimos años, un cambio de estrategia hacia lo que se denomina “lucha integrada” y “buenas prácticas agrícolas” que pretende optimizar la aplicación de los plaguicidas y el uso de medicamentos veterinarios.

Por residuos de plaguicidas o medicamentos veterinarios se entiende cualquier sustancia presente en alimentos productos agrícolas o alimentos para

animales, como consecuencia del uso de estos. El término incluye cualquier derivado de un plaguicida o medicamento, como producto de conversión, metabolitos y productos de reacción, y las impurezas consideradas de importancia toxicológica.

La "lucha integrada" se define como el método de control de plagas y enfermedades que aplica un conjunto de medidas satisfactorias desde el punto de vista económico, ecológico y toxicológico, dando prioridad a la utilización de elementos naturales de regulación, para mantener la población afectada por debajo del umbral de producción de daños [1].

Por "buenas prácticas" se entiende a los usos inocuos autorizados a nivel nacional, en las condiciones existentes, de los plaguicidas o medicamentos veterinarios necesarios para un control eficaz y fiable de las plagas y enfermedades. Comprende una gama de niveles y aplicaciones de plaguicidas o medicamentos hasta la concentración de uso autorizado más elevada, de forma que quede la concentración mínima posible del residuo. Los usos inocuos autorizados se determinan a nivel nacional y prevén usos registrados o recomendados en el país teniendo en cuenta las consideraciones de salud pública y profesional, y la seguridad del medio ambiente. Las condiciones existentes comprenden cualquier fase de la producción, almacenamiento, transporte, distribución y elaboración de alimentos para el consumo humano y piensos [1].

1.4. Importancia Económica

El empleo de plaguicidas y medicamentos veterinarios han aportado indiscutibles beneficios económicos, y ha resultado fundamental para elevar de manera constante la producción de los alimentos y cubrir las necesidades

producidas por el incremento demográfico de las últimas décadas, así como para colaborar en una indiscutible mejora de la calidad de vida de la población.

En España existe una gran variedad en la producción agrícola y debido a la diversidad climática y edáfica de las distintas comunidades autónomas que implica agriculturas notablemente diferentes, con especializaciones productivas muy señaladas, se cultivan desde especies propias del clima templado, hasta especies tropicales, pasando por cultivos mediterráneos. Concretamente en Andalucía, Región de Murcia, Canarias, Baleares y Comunidad Valenciana, la actividad agraria tiene una clara y fuerte predominancia agrícola basada, sobretodo, en la hortofruticultura.

En la Comunidad Valenciana las tierras de cultivo ocupan 734.627 Ha, lo que supone un 31,6 % de la superficie total de toda la Comunidad, la agricultura y la ganadería suponen el 6,9 % del total nacional del sector, inferior a la de otros sectores: industria (10,9 %) y servicios (9,5 %) [7].

En la Comunidad Valenciana predomina la contribución de la producción final agrícola sobre la ganadera. Esto no se debe a la escasa actividad ganadera, pues la ganadería sin tierra, es notable en Castellón y Valencia, sino al enorme peso relativo de la producción agrícola de regadío en general, y de los cítricos en particular.

La agricultura en la comunidad Valencia se basa fundamentalmente en el regadío, ya que el 44 % de la superficie cultivada está regada. La mayor parte de producción agraria se debe a cítricos y almendros y en menor proporción a hortalizas y verduras.

En cuanto a ganadería las cabañas más importantes son las de aves, con un gran número de centros, y la de porcino, con un gran desarrollo de cebaderos

y cría. Entre las dos suman el 85 % del total de la producción cárnica regional. No obstante no es un subsector fundamental pero tampoco marginal [4].

1.5. Importancia Social y Directivas Europeas

Con el fin de que los ciudadanos tengan confianza plena en los alimentos que consumen, dispongan de información adecuada para tener capacidad de elección, y hacer compatible la protección de la Salud Pública con la defensa sanitaria de los cultivos contra las plagas y enfermedades de los animales favoreciendo la producción, se han dictado una serie de normas legales que limitan o establecen los Límites Máximos de Residuos (LMRs). Se definen como "la concentración máxima de residuos (expresada en mg Kg^{-1}) de peso fresco para que se permita legalmente su uso en la superficie o la parte interna de los productos alimenticios para consumo humano y de piensos" [8].

Los LMRs se basan en los datos de Buena Práctica Agrícola (BPA) y en criterio toxicológicos, y tienen por objeto lograr que los alimentos derivados de los productos básicos que se ajustan a los respectivos LMRs sean toxicológicamente aceptables (Codex Alimentarius). Se establecen en función de la ingesta diaria admisible (IDA) y de la dieta alimentaria, y se regulan en la legislación de las distintas naciones. Dependiendo de las costumbres alimentarias de un país, los LMRs pueden variar. Para poder cumplir estos LMRs es muy importante que se respete el plazo de seguridad de los productos, conocido como tiempo de espera, ya mencionado.

Con el establecimiento de los LMRs se pretende evitar que un alimento contaminado, no llegue al consumo humano, entendiendo como alimento contaminado aquél que contiene gérmenes patógenos, sustancias químicas o

radiactivas, toxinas o parásitos, capaces de transmitir enfermedades al hombre o a los animales.

Las IDAs representan la cantidad de residuo de un contaminante que, ingerido diariamente por un ser humano, durante todo el periodo vital no muestra riesgos apreciables para la salud del consumidor (mg/kg de peso corporal/día). Se establece sobre el estudio de toda la información disponible, incluyendo datos de las propiedades bioquímicas, metabólicas, toxicocinéticas y toxicológicas del contaminante.

La IDA es un parámetro básico, tanto para la determinación de los LMRs como para la evaluación del rieso toxicológico de un determinado contenido en residuos de un alimento, ya que, teniendo encuenta la participación en la dieta de los alimentos que puedan contener residuos del plaguicida encuestión, la ingesta de los mismos no debe superar la IDA [5].

Debido a la posibilidad que ofrecen los productos químicos en contaminar la cadena trófica alimentaria, es necesario conocer la cantidad que contienen los alimentos y cuales son los niveles aceptables que se pueden ingerir, la dosis más alta de una sustancia que no causa efectos adversos detectables en animales de experimentación, (dosis/nivel sin efecto NOAEL mg/kg/día aplicando un factor de seguridad de acuerdo con la FAO y la OMS, para extrapolar a humanos).

En el ámbito de la Unión Europea existe una renovación continua de las Directivas del Consejo, que regulan los límites máximos de estos residuos tanto de origen vegetal como animal. Todo ello se traduce en una actualización constante de las legislaciones de los países miembros de la Unión Europea.

En España existe un Programa Nacional de Vigilancia de Residuos de Plaguicidas de origen vegetal y animal.

En esta tesis se lleva a cabo el estudio de sustancias orgánicas, concretamente plaguicidas y medicamentos de uso veterinario, a continuación se detallan algunos aspectos importantes de estos contaminantes:

Sustancias orgánicas

a) Contaminantes agrícolas

Los plaguicidas son sustancias que sirven para combatir los parásitos de los cultivos, del ganado, de los animales domésticos y del hombre y su ambiente. Desde el punto de vista toxicológico se clasifican según su:

-Acción específica sobre la plaga o la enfermedad que controlen (herbicida, fungicida, insecticida, nematocida, rodenticida, bactericidas, etc.)

-Toxicidad atendiendo a los efectos agudos por la DL_{50} (oral o dérmica para rata) o la CL_{50} (vía respiratoria para rata) siendo nocivos, tóxicos o muy tóxicos.

-Naturaleza química: organoclorados, organofosforados, carbamatos, piretroides, bipiridilos, ciclofenoxiácidos, cloro y nitrofenoles y organomercuriales, etc.

El control de productos plaguicidas se lleva a cabo mediante un sistema de registro. Se estudian los plaguicidas para determinar su toxicidad y sus posibles efectos sobre el medio ambiente, específico para el producto (dependiente de la dirección General de la Producción Agraria) debiendo figurar en el etiquetado del producto el número del registro que se le ha asignado [3, 9].

El uso de plaguicidas es una práctica cotidiana no sólo del sector agrícola, que permite controlar diferentes plagas y puede dar lugar a la presencia de Residuos, definido por el Código Alimentario como cualquier sustancia que se halle en los alimentos junto a otros productos derivados de su degradación, metabolitos y otro grupo de contaminantes importante desde el punto de vista toxicológico resultantes del uso de un producto fitosanitario.

Hay que tener en cuenta que algunos plaguicidas tienen tendencia a acumularse, aumentando el riesgo que suponen sus residuos, por lo tanto, la mayor importancia en relación con los residuos de plaguicidas en alimentos se centra en la toxicidad crónica, ya que las cantidades de residuos de plaguicidas, si están presentes siempre, son extremadamente pequeñas. No se conoce que estas concentraciones produzcan efectos adversos a corto plazo, estos efectos se producen normalmente por accidentes ocasionales o mal uso de los plaguicidas. Los efectos adversos que pueden provocar, dependiendo del tipo de producto son: efectos neurológicos, reproductivos, inmunológicos y cancerígenos.

b) Contaminación por Tratamientos Ganaderos

En la producción animal se utilizan sustancias con el fin de aliviar, curar, diagnosticar o evitar enfermedades como antibióticos (penicilinas, tetraciclinas, sulfonamidas y algunos aminoglucósidos), sulfamidas y quimioterapéuticos.

Es muy importante que cuando éstos se utilicen, se realice un uso controlado de los mismos y que se respete el periodo de supresión, considerado como el tiempo que tardan estos compuestos en desaparecer del organismo animal, previo a su sacrificio [3, 9].

Los residuos de sustancias prohibidas de uso ganadero y otros tipos de contaminantes se controlan a través del Plan de Vigilancia de determinadas sustancias y sus residuos en los animales vivos y sus productos.

En esto se ven implicados:

Sustancias con efecto anabolizante y sustancias no autorizadas: estilbenos, derivados y sus sales, agentes antitiroidianos, esteroides, β-agonistas, etc.

Medicamentos Veterinarios y contaminantes: Los residuos de medicamentos veterinarios son sustancias o combinación de sustancias con actividad farmacológica, inmunológica o metabólica, principios activos, excipientes o productos de degradación y sus metabolitos que presentan propiedades curativas o preventivas con respecto a las enfermedades animales, o que puede administrarse al animal con el fin de restablecer corregir o modificar las funciones fisiológicas del animal, que permanecen en los productos alimenticios obtenidos a partir de animales a los que se les ha administrado el medicamento veterinario (Reglamento 2377/90/CEE) [10].

Sustancias antibacterianas, incluidas las sulfamidas y las quinolonas

Otros medicamentos veterinarios: antihelmínticos, anticoccidianos (nitrommidazoles), carbamatos y piretroides, tranquilizantes, antiinflamtorios no esteroideos (AINES), otras sustancias que ejerzan una actividad farmacológica

Otras sustancias y contaminantes medioambientales. Compuestos organoclorados (PCB), compuestos organosfosforados, elementos químicos, micotoxinas, colorantes, otros.

Estos compuestos pueden llegar en forma de residuo al consumidor y producir efectos tóxicos o secundarios como: alergias (desde dermatitis por

contacto y otras reacciones en la piel hasta shock anafiláctico), resistencias bacterianas, afecciones tiroideas, metabólicas, nerviosas y cardiovasculares, carcinogénesis y teratogénesis.

2. INCIDENCIA DE RESIDUOS DE PLAGUICIDAS EN ALIMENTOS. RIESGO TOXICOLÓGICO

Los LMRs establecidos en las distintas legislaciones intentan hacer compatible la protección de la salud del consumidor y la defensa sanitaria de los cultivos. Con objeto de vigilar el cumplimiento de la legislación vigente se efectúan muestreos y controles sobre productos alimenticios en fase de comercialización para asegurar que no se superan los LMRs. De los distintos estudios realizados en laboratorios oficiales se deduce que si bien la mayoría de las muestras cumplen la legislación y no contienen residuos de plaguicidas detectables o los contienen en niveles inferiores a los LMRs, siempre hay una pequeña proporción que supera estos LMRs que oscila entre 2 y el 10 % de las muestras analizadas. Esta proporción no representa un riesgo real para la salud del consumidor pero pone de manifiesto la necesidad y la importancia de estos programas de monitorización de residuos para garantizar la seguridad alimentaria (Programa de control de LMRs 1999) [3, 11, 12].

El grado de exposición de la población a los residuos de plaguicidas en los alimentos, depende del tratamiento que se efectúe y, de la composición de la dieta media en la zona geográfica considerada. Para ello es necesario conocer la proporción de los alimentos y los residuos que cada uno de ellos puede contener. Estos datos permiten estimar la ingestión diaria media de los distintos plaguicidas por la población y garantizar que sea inferior a la ingesta diaria admisible (IDA) establecida por la FAO/OMS.

En España se han efectuado estudios teniendo encuentra la "composición de la cesta de la compra" y los LMRs establecidos en la normativa vigente. En éstos, la ingesta diaria estimada (IDE) teórica y la comparación con las ingestas diarias admisibles (IDAs) permite determinar que el riesgo para la población es mínimo cuando no se superan los LMRs.

2.1. Situación de España

Las mayores presiones sobre el medio natural en España provienen de los sectores con un mayor peso en la economía.

Los terrenos dedicados a la agricultura han ido cambiando en la última década. Las tierras más fértiles han sido urbanizadas. El desplazamiento obligado hasta otros emplazamientos menos fértiles ha llevado consigo en ocasiones un mayor uso de fertilizantes, una contaminación de suelos y en algunos casos, de acuíferos.

La superficie agrícola en España se cifra en aproximadamente 25 millones de *ha* dedicadas a cultivos y pastos. España es el segundo país de la UE, después de Francia, en superficie cultivada, y el cuarto –detrás de Francia, Italia y Alemania- por su aportación a la producción final agrícola de la UE.

Si bien, es cierto que el desarrollo está acompañado de presiones sobre el medio ambiente, puesto de manifiesto por indicadores ambientales, que ofrecen una imagen completa de la situación ambiental, existen problemas específicos, asociados a los fertilizantes sintéticos y plaguicidas [1,2].

La importancia de los indicadores ambientales radica en la posibilidad de poderse convertir en hilo conductor de un problema, en presentar una continuidad en el tiempo, mostrar tendencias y señalar las metas que es deseable alcanzar y los objetivos que es obligado conseguir. A la vez evalúan objetivamente el medio ambiente.

En agricultura se presentan indicadores que se detallan en la Tabla 1. De especial relevancia es el consumo o utilización de plaguicidas, estrechamente relacionado con el desarrollo del medio rural y con la orientación de la agricultura a corto y medio plazo.

Tabla 1. Indicadores en agricultura

Indicador	Meta	Tendencia
Consumo de fertilizantes	Disminución del consumo de fertilizantes	En 2005 se observa una disminución del consumo del 15%
Consumo de productos fitosanitarios	Disminución del consumo de productos fitosanitarios	Tras el fuerte incremento de 2004, se constata una fuerte siminución del consumo de fitosanitarios
Agricultura ecológica	Aumentar la superficie de agricultura ecológica respecto a la superficie agraria total	Sigue aumentando la superficie, pero disminuye el ritmo de crecimiento
Superficie de regadío	Introducción de sistemas de regadío más eficientes	Se detecta un incremento a nivel nacional, aumenta en alguna CCAA y se reduce en otras
Ecoeficiencia en la agricultura	Aumentar el valor económico de la producción agrícola disminuyendo las presiones sobre el medio	En el periodo 2000-2004 el VAB del sector (a precios constantes) disminuyó un 3.2 %.

CCAA: Comunidades Autónomas VAB: Valor Añadido Bruto

www.mma.es/portal/secciones/calidad_contaminacion/indicadores_ambientales/perfil_ambiental_2006/index.htm

2.2. Consumo de plaguicidas

El gráfico 1 muestra el consumo de plaguicidas (insecticidas, fungicidas, herbicidas y otros plaguicidas (acaricidas, nematocidas, fitorreguladores y molusquicidas)), en relación con la superficie del cultivo.

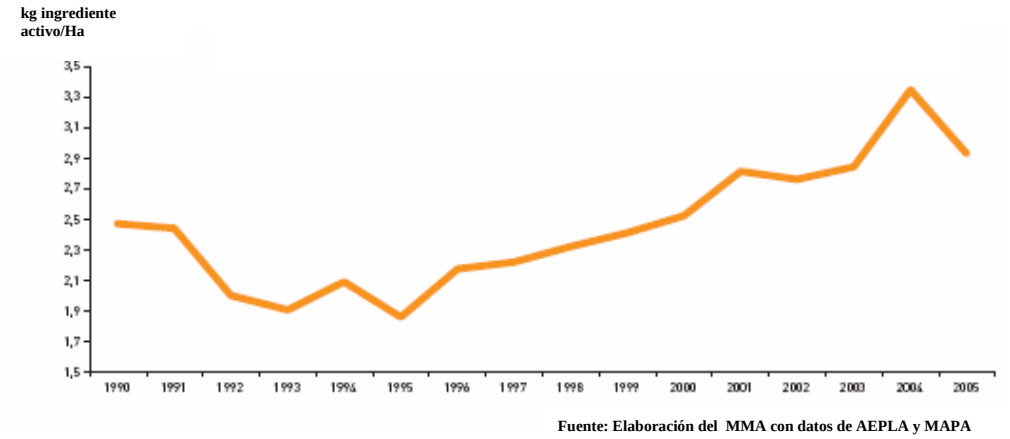


Gráfico 1 Consumo de productos fitosanitarios

En España se observa que, en lo que se refiere al indicador de consumo de plaguicidas con relación a la producción hortícola; se ha incrementado un 30 % por Ha en 2004 con respecto a 1997, alcanzándose en 2004 un 7.79 Kg/ha, pero hay que señalar que este incremento se había producido sobretodo en los años anteriores (gráfico 1). Entre el 2001 y el 2002 se frena el incremento en el uso de plaguicidas, y se produce incluso un ligero descenso. Los procesos de intensificación agraria y sobre todo el aumento de su especificidad y eficacia, están agravando el problema de aumentar su consumo y su toxicidad.

En la actualidad, España ocupa el noveno puesto de consumo de Europa. El consumo de plaguicidas presentaba un índice de crecimiento en la última década, pero en los tres últimos años se ha producido un estancamiento del consumo tendiendo a reducirse, debido a dos causas principalmente; la primera, el abandono de tierras de labor por el descenso de rentabilidad al no poder competir en precios con los países en vías de desarrollo y la segunda, la concienciación tanto por parte de los consumidores como de los agricultores, de que el uso de estas sustancias químicas contribuye a la contaminación difusa de los ecosistemas y afecta de un modo especial a los alimentos, las aguas y la biodiversidad.

La intensificación desigual de la agricultura en las Comunidades Autónomas se refleja también en el uso de plaguicidas, siendo mucho mayor en Canarias y en las regiones hortícolas, sobresaliendo la Región de Murcia (30 Kg/ha), la Comunidad Valenciana y La Rioja (20 Kg/ha). Por compuestos, los fungicidas ocuparían el primer lugar, seguido de herbicidas e insecticidas respectivamente. El gráfico 2 muestra el consumo de estos compuestos, distribuido por comunidades autónomas.

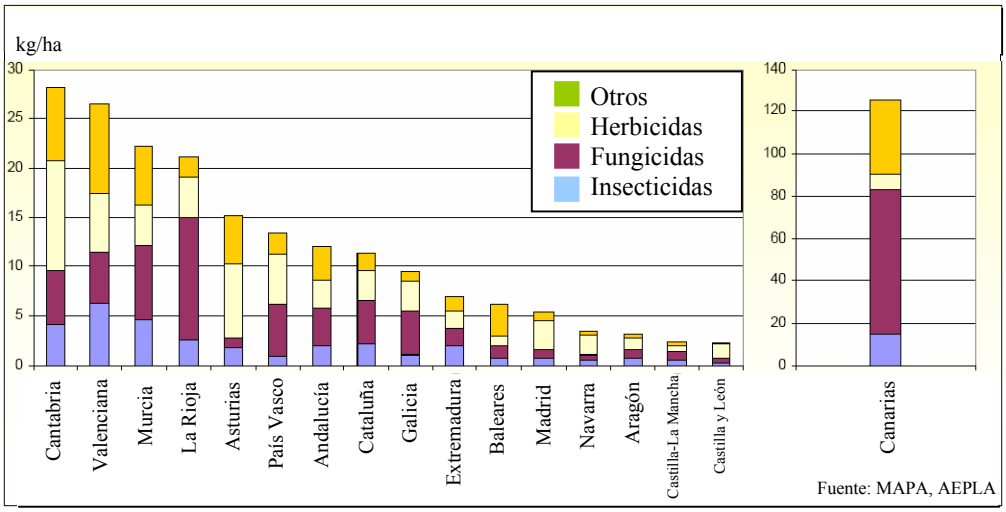


Gráfico 2 Consumo de plaguicidas por comunidades y productos (Ministerio de Medio Ambiente, www.mma.es)

Al no poder disponer de cifras desagregadas para los diferentes usos, debe tenerse en cuenta que este indicador (kg de ingrediente activo por ha) es una aproximación al uso de los fitosanitarios en el sector agrario.

El uso de dichos productos también viene condicionado por una combinación de factores agroclimáticos y estructurales, entre los que se pueden señalar las condiciones meteorológicas (como olas de frío y calor que reducen las superficies de siembra y plantación), las reformas en el marco de la Política Agraria Común (PAC) que no potencian determinados cultivos y el aumento de

costes de carburantes y energía. A ello se pueden unir condiciones del mercado en relación con los precios que inciden también en la utilización de insumos y, por tanto, en la disminución de la superficie agrícola tratada.

En el entorno europeo, la Organización para la Cooperación y el Desarrollo Económico (OCDE) (Organisation for economic co-operation and developement (OECD)), en su informe "OECD Enviromental *Indicators, 2005*" facilita los siguientes datos relativos a países europeos y referidos al año 2000 (Tabla 2):

Tabla 2. kg de ingrediente activo/ha de los países europeos

País	*kg de ingrediente activo/ha*	*País*	*kg de ingrediente activo/ha*
Bélgica	*6,75*	***España***	*1,30*
Italia	*5,79*	*Grecia*	*1,34*
Países Bajos	*4,38*	*Dinamarca*	*1,11*
Portugal	*4,00*	*Austria*	*1,00*
Luxemburgo	*3,10*	*Suecia*	*0,54*
Francia	*2,81*	*Finlandia*	*0,52*
Alemania	*1,72*	*Polonia*	*0,51*
ReinoUnido	*1,97*	*Irlanda*	*0,48*

3. RESIDUOS DE MEDICAMENTOS DE USO VETERINARIO

Los medicamentos veterinarios legalmente reconocidos como tales, son sustancias, o combinaciones de las mismas, destinadas a ser utilizadas en los animales, que se presentan dotadas de propiedades curativas o preventivas con respecto a las enfermedades de los mismos, o que pueden administrarse al animal con el fin de restablecer, corregir o modificar las funciones fisiológicas del mismo, ejerciendo una acción farmacológica, inmunológica o metabólica, o de establecer un diagnóstico.

Aunque los medicamentos veterinarios han contribuido decisivamente a la mejora de la calidad de los productos, en ocasiones plantean problemas de efectividad y de seguridad bien conocidos.

El medicamento de uso animal ha de ser seguro, eficaz, de calidad, correctamente identificado y debe ir acompañado por la información precisa, con el fin de garantizar su utilización racional [2, 13, 14].

Uno de los grupos farmacológicos que más indiscriminadamente se ha utilizado por su baja toxicidad y elevadas ventajas terapéuticas, ha sido el de los antibacterianos. Este uso inadecuado a dado lugar a un elevado número de problemas que van desde el fracaso terapéutico, por una elección inadecuada o una posología mal instaurada, a la presentación de resistencias, cada vez más extendidas o a la presentación de reacciones adversas iatrogénicas.

En todos los casos se requiere una dosificación adecuada y diversificada de acuerdo con los pesos, medidas y características fisiológicas de cada especie, por tanto, en todo momento se debe dotar al medicamento veterinario de la forma farmacéutica y posología necesarias, teniendo en cuenta su posterior metabolismo.

Los veterinarios deben considerar las implicaciones del uso con el fin de proteger la salud y bienestar de los animales, ya que los alimentos de origen animal destinados al consumo humano no deben contener residuos de medicamentos que excedan los niveles legales de concentración. Por otra parte, la presencia de residuos en los animales tiene una implicación potencial para la seguridad medioambiental.

La utilización de antibióticos en animales para algunas patologías y la presencia de residuos en alimentos destinados al consumo humano, trae como consecuencia la aparición de cepas de bacterias patógenas resistentes a estos antibióticos.

3.1. Directivas europeas, legislación estatal y regulaciones autonómicas

La seguridad de la alimentación humana y animal está siendo desde hace unos años foco de interés especial a nivel europeo y nacional, de forma que la producción animal no puede disociarse de la calidad y la seguridad de los alimentos que produce. Las crisis alimentarias de los últimos años han impulsado a la Comisión Europea y a los estados miembros de la UE a un replanteamiento fundamental acerca de la integridad de la cadena alimentaria y cómo debería ser regulada.

Desde 1999 la UE se planteó ejercer un control más estricto en la alimentación animal y en el 2000 la Comisión de las Comunidades Europeas publicó el *Libro Blanco sobre la Seguridad Alimentaria* [7] en el que se planteaban 84 acciones en favor de la seguridad alimentaria. Entre las más importantes fue la de la creación de un organismo alimentario europeo independiente para garantizar un nivel elevado de seguridad alimentaria, la Autoridad Europea para la Seguridad Alimentaria (EFSA); el resultado fue la

creación de Agencias de Seguridad Alimentaria en muchos estados miembros de la UE y en España la creación de la Agencia Española de Seguridad Alimentaria (AESA) con objeto de replantear la política de seguridad alimentaria y disponer de instrumentos para la gestión integral de la seguridad alimentaria en toda la cadena de producción, elaboración, distribución y consumo, y también creando agencias en ciertas comunidades autónomas.

Las diversas Directivas Europeas inciden en la legislación española, en especial la Directiva 2004/28/CE, del Parlamento Europeo, por la que se establece un código comunitario sobre medicamentos veterinarios, que modifica la Directiva 2001/82/CE. Actualmente a los medicamentos veterinarios les son de aplicación todos los criterios y exigencias generales que la Ley del Medicamento establece o que reglamentariamente se establezcan y que serán ejercidas por el Ministerio de Agricultura, Pesca y Alimentación de acuerdo con el Ministerio de Sanidad y Consumo, sin perjuicio de las competencias de las Comunidades Autónomas. En esta línea se publicó el Real Decreto 109/1995 sobre medicamentos veterinarios, y también se promulgó la Ley de Sanidad Animal (2003), que en conjunto regulan la fabricación, comercialización, información y publicidad, importación, exportación, almacenamiento, prescripción, dispensación, evaluación, autorización y registro de los medicamentos de uso animal y de las substancias, excipientes y materiales utilizados para su fabricación, preparación y envasado [15].

Actualmente la gran mayoría de las Comunidades Autónomas han aprobado disposiciones reguladoras específicas en relación con los medicamentos veterinarios. La legislación Española y la de las Comunidades Autónomas, se va adecuando a las Directivas de la Unión Europea, y en estos momentos está en pleno proceso de adaptación a la realidad de la sanidad animal y la salud pública en la vertiente de la seguridad alimentaria.

3.2. Grupos terapéuticos de medicamentos veterinarios

Los medicamentos veterinarios e pueden clasificar según los grupos terapéuticos siguientes (Tabla 3):

Tabla 3. Grupos terapéuticos de medicamentos veterinarios

-Sistema Cardiovascular	-Sistema muscular	-Antifúngicos
-Vacunas e inmunoterápicos	-Sistema urinario	-Antihelmínticos
-Antisépticos y desinfectantes	-Sistema digestivo	-Antibacterianos
-Sistema epitelial/dérmico	-Sistema nervioso	-Antivirales
-Promotores del crecimiento	-Antiinflamatorios	-Ectoparásitos
-Sistema respiratorio	-Antineoplásicos	-Ojos
-Sistema reproductor		

http://www.merckvetmanual.com [13]

En la presente tesis se han estudiado dos clases de medicamentos veterinarios: las quinolonas y las sulfonamidas, pertenecientes al grupo de antibacterianos.

3.3. Investigación, producción, distribución y dispensación en España

La realidad es que la investigación, producción, y distribución de los medicamentos veterinarios es similar en los países de nuestro entorno, siendo España el séptimo productor mundial (un 15% se destina a la exportación). Las grandes diferencias se dan en la prescripción mediante receta veterinaria, la dispensación y la aplicación práctica de los mismos.

La Subdirección General de Evaluación de Medicamentos de Uso Veterinario de la Agencia Española del Medicamento y Productos Sanitarios es la encargada de desarrollar las funciones relativas a la evaluación, autorización y

registro de las especialidades farmacéuticas y demás medicamentos especiales de uso animal, la evaluación y autorización de los productos en fase de investigación clínica y de los estudios y ensayos complementarios, así como la revisión y adecuación de los medicamentos de uso animal ya comercializados y la planificación estratégica y evaluación de la farmacovigilancia veterinaria.

Para facilitar la distribución de los medicamentos de uso animal desde los laboratorios fabricantes y entidades importadoras a las entidades legalmente autorizadas para la dispensación, en general se utiliza la mediación de los almacenes mayoristas que en todos los casos deben disponer de un director/a técnico/a farmacéutico/a.

En la mayoría de los casos para la dispensación en España se requiere la prescripción de una receta extendida por el veterinario legalmente capacitado.

La dispensación de los medicamentos de uso animal en España está reglamentada en general por las Comunidades Autónomas, que esencialmente establecen que se realizará por las oficinas de farmacia legalmente establecidas que además serán las únicas autorizadas para la elaboración de fórmulas magistrales y preparados oficinales, las entidades o agrupaciones ganaderas autorizadas para el uso exclusivo de sus miembros, y los establecimientos comerciales detallistas autorizados. Por razones de urgencia y lejanía de las oficinas de farmacia en determinados casos se utilizan botiquines de medicamentos de uso animal en las condiciones que se determinen. También en casos reglamentados el veterinario en ejercicio clínico está autorizado para la adquisición y cesión de medicamentos, siempre que tales actividades no impliquen actividad comercial con destino a los animales bajo su cuidado directo, en casos de urgencia, lejanía de los centros de dispensación o, cuando por imposición legal, la aplicación tenga que ser efectuada personalmente por el facultativo o bajo su directa dirección y control [16].

3.4. Consumo de medicamentos veterinarios- Datos mundiales

Los datos facilitados por la Federación Internacional de la Sanidad Animal (IFAH), indican que el mercado de la industria de la sanidad y nutrición animal a nivel mundial alcanzó en 2006 los 16.065 millones de dólares.

América del norte se situó en el primer puesto con el 34,9%, una décima por delante de Europa que alcanzó el 34,8%, seguidos a distancia por Asia con el 15,9%, Iberoamérica con el 11,6% y el resto del mundo con el 2,8% (gráfico 3).

En cuanto a los productos, los antiparasitarios supusieron el 28,8%, seguidos por los biológicos (vacunas, antígenos, alergenos, hormonas y antibióticos) con el 22,8%, otros fármacos con el 20,2%, antiinfecciosos con el 15,8% y los aditivos medicamentosos con el 12,4%.

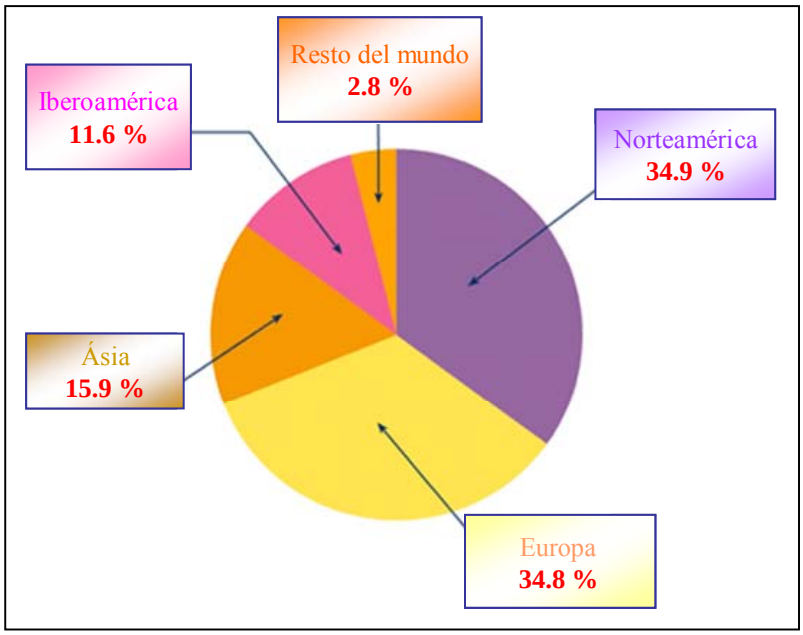
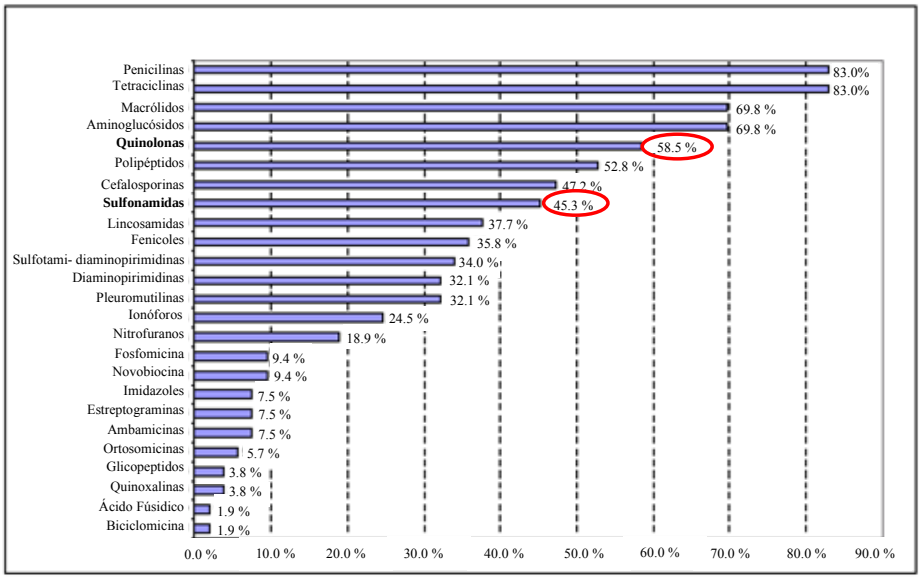


Gráfico 3 Distribución del mercado mundial www.veterindustria.com Veterindustria: Asociación Empresarial Española de la Industria de Sanidad y Nutrición Animal.

Tras el análisis de un cuestionario llevado a cabo por la Organización Mundial de la Salud (OMS) y la Organización Mundial de Sanidad Animal (World Organization for Animal Health (OIE)) en 2006, con el fin de definir y designar cuales eran los antimicrobianos veterinarios de importancia crítica (con las siglas en inglés VCIA por "*Veterinary Critically Important Antimicrobials*") se concluyó que los VCIA son: los antimicrobianos utilizados para el tratamiento, prevención y control de infecciones graves en animales que pueden tener consecuencias relevantes en la salud y el bienestar de los animales, la salud pública o consecuencias económicas importantes, y donde las alternativas son muy pocas o inexistentes. Estos antimicrobianos estarán disponibles en cantidades y formas farmacéuticas adecuadas, de calidad y económicamente accesibles.

Se designaron como VCIA a 122 antimicrobianos de 25 familias detallados en la tabla de a continuación (Tabla 4)

Tabla 4. Antimicrobianos designados como VCIA



VCIA: *Veterinary Critically Important Antimicrobials* (antimicrobianos veterinarios de importancia crítica)

Los porcentajes hacen relación a la importancia crítica de cada familia de antibióticos. Es de especial interés para esta tesis los valores de quinolonas y sulfonamidas, que ocupan el noveno y quinto lugar con un 58.5 % y 45.3 % respectivamente.

En la tabla 5 siguiente se detallan las quinolonas y sulfonamidas veterinarias de importancia y las especies de animales sobre las que se aplican.

Tabla 5. Lista de quinolonas y sulfonamidas de importancia crítica y especies de animales

Familia	Subfamilia	Antimicrobiano	Especies animales
Quinolonas	Quinolonas 1g	Flumequina	AV, BOV, CAP, EQU, CON, OVI, PES, POR
		Miloxacina	PES
		Acido nalidíxico	BOV
		Acido oxolínico	AV, BOV, CON, PES, POR
	Quinolonas 2 g (fluoroquinolonas)	Ciprofloxacino	AV, BOV, CAN, FEL, POR
		Danofloxacina	AVI, BOV, CAP, CON, OVI, POR
Quinolonas	Quinolonas 2 g (fluoroquinolonas)	Difloxacina	AV, BOV, CAN, CON, POR
		Enrofloxacina	AV, BOV, CAN, CAP, EQU, FEL, CON, OVI, PES, POR
		Marbofloxacino	AV, BOV, CAN, EQU, CON, POR
		Norfloxacina	AV, BOV, CAN, CAP, CON, OVI, POR
		Ofloxacina	AV, POR
		Orbifloxacina	BOV, CAN, POR
Quinoxalinas	Quinoxalinas	Carbadox	POR
Sulfonamidas	Sulfonamidas	Sulfacloropiridazina	AV, POR
		Sulfadiazina	BOV, CAP, OVI, POR
		Sulfadimerazina	AV, BOV, CON
		Sulfadimethoxina	AV, BOV, CAP, EQU, FEL, CON, OVI, PIS, POR

Tabla 5. Continuación

Familia	Subfamilia	Antimicrobiano	Especies animales
cont. Sulfonamidas	Sulfonamidas	Sulfadimidina	AVI, BOV, CAP, EQU, CON, OVI, POR
		Sulfadoxina	EQU, POR
		sulfafurazol	PIS
		Sulfaguanidina	CAP, OVI
		Sulfametazina	SUI
		sulfametoxazol	AV, BOV, POR
		Sulfametoxina	AV, PES, POR
		Sulfamonometoxina	AV, PIS, POR
		Sulfanilamida	BOV, CAP, OVI
		Sulfaquinoxalina	AV, BOV, CAP, CON, OVI
Sulfonamidas + diaminopirimi-dinas	Sulfonamidas + diaminopirimidinas	Sulfametoxipiridazina	AV, BOV, EQU
		Trimetoprimsulfona-mida	AV, BOV, CAN, CAP, EQU, FEL, CON, OVI, PES, POR

AV: aves, API: apicultura, BOV: bovino, CAN: canino, CAP: caprino, CAM: camélidos, EQU: equino, FEL: felino, CON: conejo, OVI: ovino, PES: pescado, POR: porcino.

3.5. Situación actual y consumo de medicamentos veterinarios en España

La industria de Sanidad y Nutrición animal española facturó unos 710 millones de euros en el año 2005, de los cuales unos 312 millones pertenecen a los medicamentos farmacológicos (45%), seguidos de los complementos nutricionales con una facturación de unos 138 millones (20%).

De todos es sabido que la administración de antibióticos y otros medicamentos a animales de producción sin control, puede tener posteriormente graves consecuencias para la salud de las personas, aunque comporta una ganancia económica a los infractores. Con estas prácticas en exceso e incontroladas, las resistencias de las bacterias y otros microorganismos, que afectan a los humanos, están provocando que ciertas enfermedades cada día sean más difíciles de sanar, además de provocar algunas alergias innecesarias. Por tanto se debe seguir reclamando la exigencia de un adecuado control sanitario de

los medicamentos veterinarios, mediante la receta veterinaria, y la dispensación farmacéutica a través de los establecimientos autorizados, y con el asesoramiento profesional que determina la legislación vigente [16].

3.5.1. Datos del Sector:

El sector español de la industria de Sanidad y Nutrición animal creció en total en el año 2006 un 9,60%, lo que traducido a euros supuso 919,15 millones. En este periodo el mercado interior aumentó un 6.12% con respecto al ejercicio anterior, 754,9 millones de euros.

Respecto a las exportaciones del sector, estas representan ya el 28.92% de las ventas totales de la industria, alcanzando la cifra en 2006 de 165,06 millones de euros.

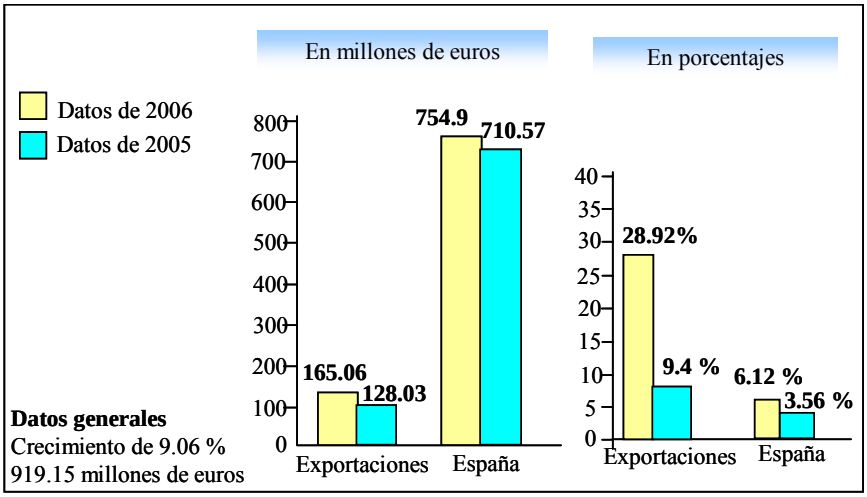


Gráfico 4 El mercado de la Sanidad y Nutrición Animal en España www.veterindustria.com

3.5.2. Grupo de Productos

Las cifras referidas al mercado de productos, sitúan a los Farmacológicos a la cabeza de las ventas. Los que más crecieron en 2006 fueron los biológicos

(vacunas, antígenos, alergenos, hormonas y antibióticos) con un aumento del 11.26% sobre el año anterior.

Las ventas en millones de euros, las encabezan los Farmacológicos con 329.35 millones de euros y el 43,68%;

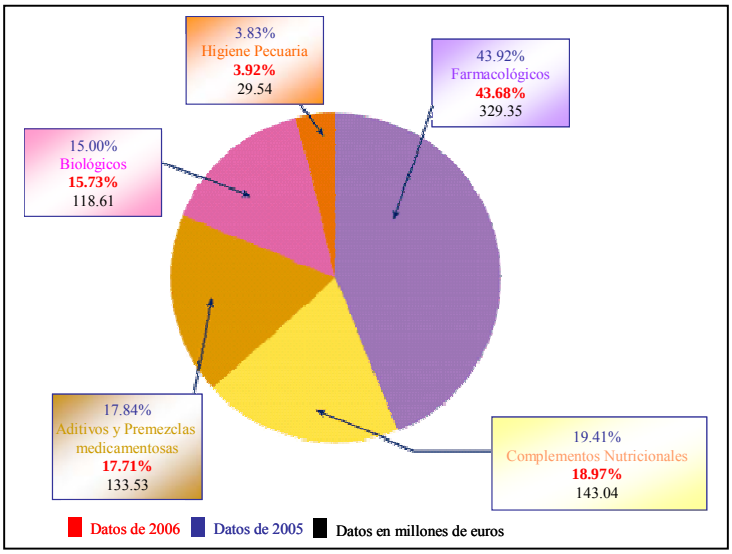


Gráfico 5 Distribución porcentual del mercado por grupos de productos www.veterindustria.com

3.5.3. Comunidades Autónomas

El mercado interior español está encabezado por la Comunidad Autónoma de Cataluña, con significativa diferencia respecto al resto, especialmente las más cercanas como Castilla y León o Andalucía.

Así, Cataluña con 189.97 millones de euros encabeza la lista nacional, y la Comunidad Valenciana con 32,58 ocupando el noveno lugar, por detrás de Castilla y León, Andalucía, Galicia, Aragón, Castilla–La Mancha, Comunidad de Madrid, y Región de Murcia.

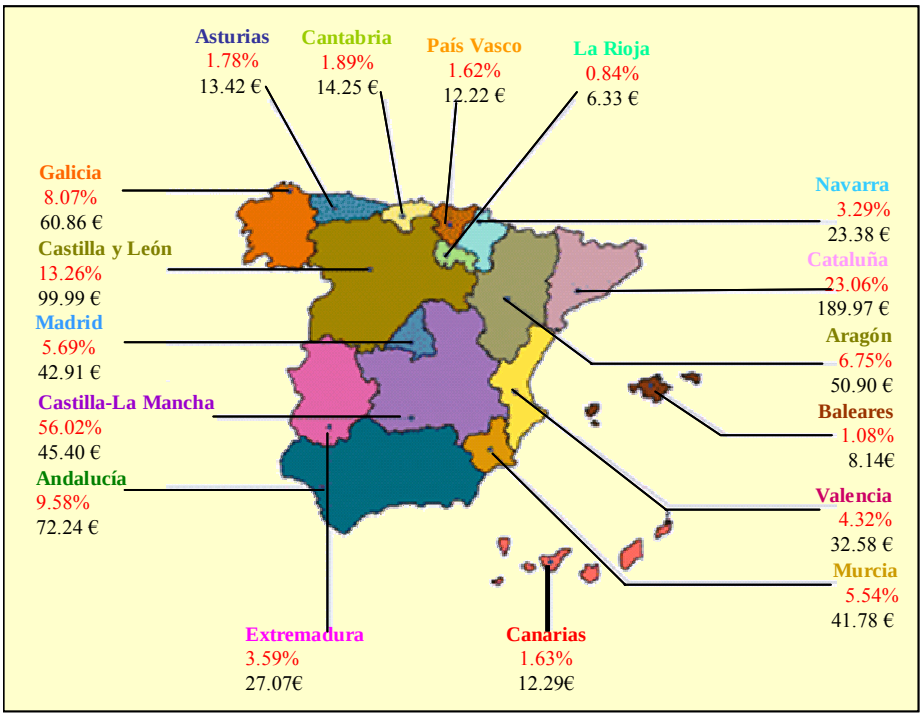


Gráfico 6 Distribución del mercado por comunidades autónomas www.veterindustria.com

4.-TÉCNICAS DE ANÁLISIS

El análisis de contaminantes orgánicos en alimentos es una tarea ardua que engloba muchas disciplinas incluidas la química, bioquímica y la microbiología. No es una tarea fácil, ya que estos contaminantes, de los que se pueden enumerar miles, están presentes a unas concentraciones extremadamente bajas, en mezclas complejas de compuestos de los alimentos naturales. Sin embargo, cualquier técnica que se utilice en el análisis de alimentos debe alcanzar o ser capaz de separar los compuestos de la matriz y la identificación de los contaminantes a niveles traza [17, 18].

La electroforesis capilar (EC) es una técnica analítica que se ha aplicado para el análisis de alimentos, forense, farmacéutico, ambiental, muestras biológicas, etc. [19-24]. Es una técnica muy estudiada para aquellos compuestos que no se pueden separar ni por cromatografía gaseosa (CG) ni por cromatografía líquida (CL). Son muchas las características inherentes a la EC, como: la velocidad del análisis, eficacia elevada, separación selectiva, capacidad de analizar tamaños de partícula pequeña y bajo consumo de reactivos. Con el fin de mejorar los límites de detección, se ha intentado optimizar la técnica de EC tratándose de establecer como una técnica viable y alternativa a la CL.

Una restricción importante de la EC es la baja sensibilidad, debido al diámetro tan estrecho del capilar, lo que limita los volúmenes de inyección hasta nanolitros y estar acoplado a un detector óptico en línea, que proporciona rangos de detección óptica reducida en comparación con la CL. Estos inconvenientes se han superado recientemente con alternativas como optimización de técnicas de inyección (preconcentración en línea, focalización (sweeping) y acumulación (stacking)), detectores de fluorescencia inducida por láser, una buena opción cuando el analito lo permite, y acoplamiento de EC a la espectrometría de masas (EM). Los métodos de concentración en línea para EC han sido revisados por

Osburn y col [25], Beckers y Bocek [26], y Quirino y col [27], así como los de EC-EM por Schmitt-Kopplin y Frommberger [28], y Choudhary y col [29].

En los últimos años se han demostrado separaciones por EC sorprendentes, incluidas la separación de contaminantes. El interés creciente de la EC, en el campo del análisis de contaminantes orgánicos, resalta como se pone de manifiesto en revisiones para la determinación cromatográfica de contaminantes orgánicos en alimentos u otras matrices ambientales, poniendo de manifiesto que la EC está establecida como una opción más desde sus inicios en el desarrollo de un método nuevo analítico [30-35].

No obstante, la aplicación de la EC para el análisis de alimentos está todavía limitada en comparación con otros campos de estudio [36]. Sin embargo, se han realizado progresos significativos para ampliar su aplicabilidad y en el campo de los alimentos y así como en proteómica, identificación microbiana, reconocimientos de especies o detección de alimentos transgénicos [36-38].

La tabla 6 recoge las aplicaciones estudiadas para el análisis de alimentos, y pone de manifiesto que la principal aplicación está en el campo de análisis de residuos de plaguicidas y antibióticos. Hay algunas aplicaciones sobre toxinas, principalmente micotoxinas, pero sobretodo toxinas bacterianas y algunas sobre patógenos en alimentación infantil.

Tabla 6. Aplicaciones de la EC para la determinación de contaminantes orgánicos en alimentos

Contaminantes orgánicos	Tipo	Alimento	Ref
Agentes antibacterianos	Quinolonas	Pollo, cerdo, pescado	[39-45]
	Tetraciclinas	Catfish	[46]
	Sulfonamidas	Leche, carne	[47-49]
	Mezclas	Alimentos	[50]
Pesticidas	Ditiocarbamatos	Trigo	[51-53]
	Fungicidas	Naranjas, limones, zumos de fruta, uva, tomates, lechugas	[54-60]
	Polares que contienen P (glifosato, glufosinato)	Trigo	[61, 62]
	Organofosforados	Hojas	[63]
	Derivados de la Urea	Tomates, naranjas	[64]
	Triazinas	Zumos	[65]
	Herbicidas Acídicos	Manzanas, uva, naranjas, tomates	[66]
	Diversos Pesticidas	Pepinos, zanahorias, zumos	[20, 45, 67-69]
Reguladores del crecimiento de plantas	Hidracina málica	Patatas, cebollas	[70]
Toxinas Biológicas			
Micotoxinas	Ocratoxina A	Café tostado, maíz, sorgo, pienso	[71, 72]
	Patulina	Sidra de manzana	[73]
	Aflatoxinas	Maíz, pienso	[72, 74, 75]
Bacteriotoxinas	Colero toxina A	Maíz, cacahuetes	[74]
Toxinas marinas	ASP *(toxina amnésica de los moluscos*) PSP *(toxina paralizante de los moluscos)*	Marisco (navajas, almejas, mejillones)	[76]
Patógenos de los alimentos	Especies de S. aureus, L. monocytogenes, Sal-monella	Carne	[77]
	Verotoxinas de Escherichia coli (VT1, VT2)	–	[78]

Existe una relación entre los contaminantes buscados y los alimentos seleccionados para su análisis, sin embargo, los antibacterianos veterinarios y las bacterias se han determinado en alimentos de origen animal, principalmente carne, pescado, y leche, micotoxinas en cereales, y plaguicidas en frutas y verduras. Los compuestos químicos y su contenido en estos alimentos es muy diferente. Así, por ejemplo, la carne y la leche son alimentos ricos en proteínas y

lípidos, los cereales tienen baja humedad y elevada proporción de polisacáridos complejos, frutas y verduras tienen un elevado contenido en agua (entre un 80-90 %) y una elevada proporción de azúcares (azúcares complejos como almidón en el caso de los vegetales y los simples como fructosa, glucosa y sacarosa en frutas).

En los últimos años se ha incrementado la utilización de compuestos más inestables y polares, caracterizados por ser poco persistentes. La mayoría de estos plaguicidas no se pueden analizar directamente por CG o requieren de procesos de derivatización previos al análisis cromatográfico.

Las primeras aplicaciones de la CL estaban enfocadas a la determinación de sustancias no analizables por CG; no obstante, recientemente la CL ha experimentado un importante auge, ampliando el numero de plaguicidas analizados por esta técnica y extendiendo su empleo para métodos multirresiduos en frutas y hortalizas [79].

La CL se ha utilizado para el analisis de muchos y muy diversos compuestos este hecho pone de manifiesto su gran importancia y versatilidad.

Se ha desarrollado detectores basados en principios muy diversos entre ellos. La EM se caracteriza por su buena sensibilidad y selectividad, y ha permitido revolucionar el campo del análisis químico.

5. PROCESOS DE EXTRACCIÓN Y CONCENTRACIÓN DE CONTAMINANTES ORGÁNICOS DE ALIMENTOS

El aislamiento y la preconcentración de contaminantes orgánicos son etapas críticas y complicadas en los procesos de EC para su determinación en alimentos. Los procedimientos utilizados se pueden clasificar de acuerdo al tipo de contaminante orgánico, químico o biológico. Para el primer tipo, se han utilizado diferentes aproximaciones en la extracción-principalmente extracción con disolventes clásica seguida de extracción en fase sólida (EFS) o microextracción en fase sólida (MEFS)- para la preparación de la muestra. Se puede conseguir una mayor concentración por evaporación de un disolvente orgánico y reconstitución de la muestra en un pequeño volumen. Para el segundo tipo la utilización de técnicas moleculares, principalmente basada en reacciones de la cadena de polimerasa (PCR), se ha convertido en una aproximación.

Aunque el método de pre-tratamiento de la muestra depende principalmente de la matriz, el procedimiento general es emplear un disolvente orgánico y llevar a cabo una purificación mediante un cartucho de EFS, independientemente de cual haya sido el procesado de la muestra y las diferencias químicas previamente descritas. Sin embargo cuanto más compleja es la muestra, más difícil es eliminar los compuestos interferentes de la matriz, por lo que se requiere de una homogeneización previa para la extracción de los residuos que se encuentran a concentraciones muy bajas en los alimentos y una purificación para eliminar las sustancias interferentes y facilitar la determinación.

En la tabla 7, se recoge la aplicación de algunas de estas técnicas para el análisis de contaminantes orgánicos en alimentos incluyendo algunos

comentarios de la viabilidad de los procedimientos y los límites de detección (LODs) obtenidos cuando se realiza una inyección con detección DAD o UV.

Otras aproximaciones, como la preconcentración en línea o la utilización de detectores selectivos se detallan más adelante.

Tabla 7. Métodos de extracción, aislamiento y concentración descritos en la literatura para la determinación de contaminantes orgánicos

Contaminante orgánico	Extracción	Aislamiento/Con-centración	Comentario	LDs[a] (Veces conc.)[b]	Ref.
Quinolonas	Phosphate buffer pH 7	EFS/tampón fosfato (pH 9)/CH_3OH	Ajuste del extracto a pH 3 previa a la EFS	0.08 µg/mL (1)	[40]
	HCl 0.1 N	EFS C_{18}/CH_3OH	–	120 µg/kg (0.5)	[42]
	Tampón fosfato pH 7/DCM	EFS (Bond Elut C_{18})/ ácido trifluoroacético/ H_2O/ACN	Neutralizar con tampón fosfato y desengrasar con hexano previo a la EFS	26 µg/kg (50)	[41]
	Tampón fosfato pH 7/DCM/NaOH	EFS (C_{18})/ ácido trifluoroacético/ H_2O/ACN	Neutralizar con tampón fosfato y desengrasar con hexano previo a la EFS	10–25 µg/kg (50)	[43]
	DCM/ NaOH	EFS (Zorbax C18, Bond Elut C18, Isolute ENV+, Oasis HLB, y Oasis MAX)/ ácido trifluoroacético/ H_2O/ACN	Desengrasar con hexano previo a la EFS. Ajustar el pH de los extractos cuando se utilicen los cartuchos Bond Elut y Oasis MAX.	10–15 µg/kg (50)	[39, 44, 45]
Tetraciclinas	Ácido tricloroacético, HCl, EDTA	EFS (Sep-Pak C_{18})/CH_3OH	–	a 0.1 mg/kg (10)	[46]
Sulfonamidas	ACN	EFS (Alumina N)/EFS con propanol (Oasis HLB)/CH_3OH	El ACN extrae muchas sustancias endógenas. Se utilizan dos cartuchos de EFS para purificar, uno polar y el otro es de un copolímero con funciones hidrofílicas y lipofílicas.	5– 10 µg/kg (50)	[48]
	Sin Extracción (muestras de leche)	Evapoar a sequedad, redisolver con CH_3OH, evaporar a sequedad, redisolver con agua	Precipitación ácida de proteinas y eliminación de lípidos con hexano. Una extracción y purificación más eficaz puede mejorar los LODs	EC-EM (–)	[47]

Tabla 7. Continuación

Contaminante orgánico	Extracción	Aislamiento/Con-centración	Comentario	LDs[a] (Veces conc.)[b]	Ref.
	Sin Extracción (muestras de leche)	EFS C_{18} (minicolumnas)/ CH_3OH	Precipitación ácida de las proteinas previo a la EFS	EC-EM (2 mL de leche en línea)	[49]
Mezclas	Quinolonas (ACN-nitrilo) Otros residuos (acetato de etilo/ NaOH)	–	Se realizaron modificaciones en la ELL con el fin de reducir los efectos de fondo	a 20 µg/kg (10)	[50]
Fungicidas	ACN/NaOH	EFS Sep-Pak PS-2 /ACN	Extracción bajo condiciones básicas	– (30)	[54]
	Acetato de etilo/NH_3	Particiones ácido-base	No hay interferencias en la EC de compuestos endógenos	0.6 mg/kg (6)	[60]
	5 mM NaCl/Ciclohexano/ acetato de etilo/acetona	–	Se precisa de enriquecimiento de la muestra (por EFS) para asegurar la sensibilidad adecuada	Acumulación (*Stacking*)	[55]
	H_2O/CH_3OH	EFS C_8/DCM	Se produce alguna degradación durante el proceso de evaporación y redisolución	0.1–1 mg/kg (10)	[56–58]
Polares que contienen P (glifosato, etc.)	H_2O/acetona	–	Pérdida de sensibilidad si no se usa EM	EC-EM EC-DFLL	[61, 62]
Organofos-forados	Acetona	ACN/ H_2O	Pérdida de sensibilidad	–	[63]
Derivados de la urea	CH_3OH/ H_2O	EFS C_{18}/DCM/ H_2O	Pérdida de sensibilidad	≈0.05 mg/kg (10)	[64]
Triazinas	0.5 M H_2SO_4/ CH_3OH	MSL-EFS	Problemas con interferencias de la matriz, sin embargo la MSL-EFS proporciona extractos más limpios, y LOD más bajos con sólo con EFS	10 µg/L (100)	[65]

Tabla 7. Continuación

Contaminante orgánico	Extracción	Aislamiento/Con-centración	Comentario	LDs[a] (Veces conc.)[b]	Ref.
Herbicidas ácidos	Acetona- H_2O (5:1)	MEFS CW/ fibras de TPR / CH_3OH	La acetona se deberia de evaporar previo a la MEFS con el fin de obtener buenas recuperaciones. La MEFS requiere de una cuidadosa optimización.	EC-EM	[66]
Multipesticidas	Diluciíon con H_2O	MEFS PDMS/ fibras de DVB / CH_3OH	La MEFS requiere de una cuidadosa optimización . Utilizacion de quimiométricos para optimizar	EC-EM	[69]
	Acetona/eter de petróleo/DCM	EFS NH_2/ DCM/ CH_3OH	Los extractos se concentran mediante 3 técnicas de preconcentración en línea	Acumulación (Stacking)	[68]
	DCM/ éter de petroleo	–	–	0.22–1.13 µg/L (75)	[67]
Hidracina málica	CH_3OH / H_2O	EFS C_{18}/ H_2O	Patatas con elevada cantidad de almidón	2.0 mg/kg	[70]
Ochratoxin A	DCM/ tampón fosfato	EFS (columnas de silice y de inmunoafinidad)	Las columnas de sílice purifican los extractos de la muestra permitiendo e paso de grndes cantidades de muestra a traves de las columnas de inmunoafinidad	EC-LIF	[71]
Aflatoxinas	ACN/ H_2O	EFS C_{18}/CH_3OH	Barrido y técnica de confirmación para procesar muchas muestras en poco tiempo	0.02–0.06 mg/kg (10)	[72]
Aflatoxina B1	Celita/ H_2O /cloroformo	EFS (columnas de silice y de inmunoafinidad)	Es posible aumentar la sensibilidad reconstituyendo el extracto seco conmenos tampón. Las columnas de inmunoafinidad disminuyen la cantidad de maiz que puede cargarse	CE-LIF	[75]
Patulina	Acetato de etilo	–	Método rápido	3.8 µg/L (20)	[73]

Tabla 7. Continuación

Contaminante orgánico	Extracción	Aislamiento/Concentración	Comentario	LDs[a] (Veces conc.)[b]	Ref.
Ácido domoico y toxinas PSP	CH_3OH: H_2O	El ácido domoico se extrajo por EFS en cartuchos de SAX y SCX. Las toxinas PSP fueron por EFS en C_{18}	Efecto fuerte de las condiciones ácidas y de la temperatura. Baja resolución de las muestras reales asociadas a la complejidad de la matriz (elevado contenido en sal, sustancias interferentes)	0.05–0.06 µg/mL (10)	[76]
Spp de S. aureus L. monocytogenes Salmonella.	Tampón de lisis de ADN	DNeasy Tissue Kit Multiplex PCR reaction	Detección simultánea de tres patógenos aislados. El procesamiento de la muestra se adaptó para la detección simultánea de tres especies incluendo lisozima y lisostafina en un simple paso de incubación. Los primers esepcíficos se seleccionaron teniendo en cuenta que debian tener la misma tempatura de fusión y longitud de ADN para evitar diferentes campos en los productos de amplificación de bandas.	ECG-LIF	[77]
Escherichia coli Vero toxinas (VT1, VT2)	Extracción del ADN por lisozimas SDS	PCR alelo específica SSCP CFLP	Los métodos proporcionaron resultados excelentes con respecto a especificidad y rapidez Cabe la posibilidad de detectar los genes VT1 y VT2 a la vez. Identificación del hongo tipo O157.	ECG-LIF	[78]

a) LODs sólo cuando se detecta con DAD

b) (Conc. Fold): Número de veces la concentración

Muchos antibacterianos presentan propiedades anfóteras porque tienen un lugar de unión de protones, como grupos amino que proporcionan especies protonadas, y grupos ácidos que se disocian en especies aniónicas. Sus propiedades anfóteras son a la vez una ayuda y un impedimento en la extracción y en la purificación, ya que hay una variación de pH dependiente de sus coeficientes de reparto entre disolventes acuosos y orgánicos en el rango de pH 7-9. Los plaguicidas y las micotoxinas son compuestos neutros que requieren del uso de mezclas alcohólicas para favorecer la recuperación de los compuestos desde la matriz sólida.

5.1 Extracción en fase sólida (EFS)

La EFS se realiza forzando el paso de los analitos disueltos en una matriz líquida a través de un soporte sólido de naturaleza extractante, donde los analitos quedan retenidos para posteriormente ser eluidos con una mínima cantidad de disolvente orgánico. En este proceso se ven implicados mecanismos de absorción, reparto y desorción.

La elección de la fase sólida depende de la polaridad de los plaguicidas y del tipo de matriz utilizada y aunque hoy en día están disponibles muchos tipos de materiales como fases, lo que puede originar diferente selectividad cuando se aplica a la extracción de contaminantes orgánicos, las más empleadas son C_{18} y C_8. El principal inconveniente es que con muestras sólidas hay que realizar una extracción previa de los residuos de plaguicidas con algún disolvente miscible con el agua [82].

Jiménez-Lozano y col. [44] compararon diferentes fases sólidas comerciales (Zorbax C_{18}, Bond Elut C_{18}, Isolute ENV+, Oasis HLN, Oasis MAX, SDS-RPS y MPC-SD) para la extracción en serie de quinolonas reguladas por la UE por EFS, en muestras de tejidos de pollo, y establecer el método de determinación por EC-DAD. Las fases sólidas probadas tenían diferentes características; C_{18} no polar (Bond Elut y Zorbax), fases sólidas poliméricas (ENV+ y HLB), y mezcla de fases como C_8 y de intercambio catiónico (MPC-SD), polímeros con grupos sulfónicos ácidos (SDS-RPS) y polímeros con intercambio aniónico fuerte (MAX). Se obtuvieron recuperaciones elevadas con los cartuchos de HLB, SDS-RPS y MAX. Con los cartuchos de HLB se observó un ensanchamiento de los picos más acusado que con los cartuchos de SDS-RPS y MAX en los que los picos fueron aceptables. El proceso de extracción fue más rápido cuando se utilizaron cartuchos de MAX que de SDS-RPS. Los límites de detección fueron más bajos utilizando

cartuchos MAX que SDS-RPS, excepto para ciprofloxacino, que fue similar con ambas fases.

5.2 Microextracción en fase sólida (MEFS)

La MEFS es una técnica de preparación de la muestra alternativa a la EFS, basada en la adsorción y/o reparto de los analitos sobre una microfibra, recubierta con una fase estacionaria (sílice fundida). Los analitos difunden hacia la fibra hasta que transcurrido un tiempo, se alcanza el equilibrio para cada compuesto entre el agua y la fibra. Posteriormente los analitos se desorben en el inyector de un cromatógrafo de gases o de líquidos con ayuda de una jeringa [82].

Solamente en dos trabajos se aplica la MEFS en cambinación con EC para la determinación de plaguicidas en alimentos. Rodríguez y col [66] determinaron cinco plaguicidas ácidos, o-fenilfenol, ioxonil, haloxifop, acifluorfen y picloram en frutas y obtuvieron recuperaciones muy similares a las que se obtiene con EFS. Por otra parte, Hernández-Borges y col [69] estudiaron otros plaguicidas diferentes, pirimetanil, pirifenox, ciprodinil, ciromazina, y pirimicarb en muestras de zumo. Ambos llegaron a la misma conclusión: la MEFS presenta la ventaja de ser más rápida y simple y promete buenas posibilidades de automatización. El inconveniente principal es la cantidad de parámetros que se deben considerar y optimizar antes de alcanzar una buena recuperación para algunos compuestos. De cualquier modo, la MEFS es una técnica útil para el análisis de alimentos, que alcanza recuperaciones, precisión y límites de cuantificación (LDC o LOQs) aceptables.

5.3 Extracción sobre barras magnéticas (ESBM)

La ESBM consiste en introducir una barra magnética recubierta de polidimetilsiloxano (PDMS) en una solución acuosa, a la cual se adsorben los analitos. Se basa en los mismos principios de la MEFS, sin embargo, la principal diferencia con la MEFS es que la cantidad de fase extractiva en las barras magnéticas es mayor que en las fibras, y por lo tanto la sensibilidad es también mayor. Presenta algunas limitaciones como es el recubrimiento de las barras, para las que sólo existen de PDMS, en cambio para las fibras de la MEFS se comercializan distintos tipos de recubrimientos.

El número de artículos que presentan aplicaciones en alimentos es limitado y el más frecuente y principal problema, durante el proceso de extracción, es la presencia de interferencias de matriz. Generalmente, este inconveniente se reduce por dilución de la muestra, lo que provoca un aumento del límite de detección (LOD), o mediante el uso de detectores más selectivos.

5.4 Extracción presurizada con disolventes (EPD o PLE o ASE)

La EPD permite la extracción de una gran variedad de compuestos tanto de muestras sólidas como semisólidas. Se utilizan disolventes a alta presión y temperatura sin alcanzar su punto crítico; estos dos parámetros determinaran la eficiencia de la extracción.

La extracción sigue tres etapas; el analito difunde del corazón de la matriz a la superficie, después se transfiere al solvente de extracción y por último el analito es eluído de la celda de extracción. El coeficiente de difusión viene determinado por la estructura del analito, la temperatura de extracción y el disolvente utilizado, lo que permite utilizar una gran variedad de disolventes.

La muestra se introduce dentro de una celda de extracción de acero inoxidable se cierra y se llena de disolvente. Bajo condiciones estáticas, el

disolvente y la muestra están en contacto a temperatura y presión constantes durante un tiempo (5-10 min). Este periodo de tiempo se denomina ciclo o tiempo estático. El ciclo se puede repetir varias veces con el fin de obtener mejores recuperaciones. A continuación se hace pasar disolvente a través de la celda seguido de nitrógeno con el fin de empujar el disolvente que pueda quedar atrapado en la matriz hacia un vial colector. Los parámetros experimentales a optimizar en el proceso serian la elección del disolvente, temperatura, presión, tiempo estático y tamaño de celda de extracción.

La EPD se ha aplicado para la determinación de ocratoxina A en arroz, productos elaborados a partir de arroz y pan [80-82], zearalenona en cereales y derivados [83, 84] y fumonisina B1, zearalenona y deoxinivalenol en maíz [85], en antibióticos [86,87] entre otros.

6. TÉCNICAS DE SEPARACIÓN CROMATOGRÁFICAS PARA CONTAMINANTES ORGÁNICOS

La separación de los solutos en EC se lleva a cabo en el interior de un capilar bajo la influencia de un campo eléctrico. La característica más interesante de la EC es su versatilidad y que es una técnica de aplicación muy amplia debido a la gran variedad de modalidades de separación.

6.1 Electroforesis capilar de zona (ECZ)

La ECZ es una técnica de separación llevada a cabo solamente con las diferencias de la movilidad electroforética de las especies cargadas, bien sean soluciones acuosas o no, del tampón de separación (o *background electrolite*, BGE). Estos pueden contener aditivos (como ciclodextrinas o polímeros) que pueden interaccionar con los analitos y alterar su movilidad electroforética.

El BGE normalmente se disuelve en medio acuoso ya que el agua es un buen disolvente químico ácido-base, es compatible con muchos modelos de detección y puede disolver un buen número de BGE (véase Tabla 8). El tampón óptimo, se obtiene seleccionando su concentración y composición para el que las diferencias entre las movilidades de las sustancias estudiadas sean grandes, proporcionando la mejor separación. La separación se puede mejorar fácilmente ajustando el pH del tampón, alterando la fuerza iónica del tampón, y añadiendo disolventes orgánicos. Los antibacterianos muestran una gran variedad de estructuras químicas, y por lo tanto una gran diversidad de propiedades químicas, físicas y biológicas. Muchos de ellos tienen grupos funcionales con características ácidas, básicas o anfóteras que pueden ser ionizadas de forma eficiente de acuerdo con el pH del tampón de separación. Esta propiedad los hace especialmente útiles para su estudio por EC. Las quinolonas son derivados del ácido nalidíxico y tienen un grupo de ácido carboxílico en posición 4; por

eso se separan bien en valores de pH entre 8.0 y 9.5 (ver tabla 8). Por el contrario, las sulfonamidas que tienen un grupo de carácter básico (grupo *para*-NH_2) se separan a pH ácido. Algunos antibacterianos tienen tiempos de migración similares y por tanto no es posible separarlos todos a la vez. Cuando las separaciones físicas de todos los componentes no son del todo completas, la resolución de los picos es baja y es posible utilizando sustancias químicas. A modo de ejemplo, hay dos quinolonas, ciprofloxacin y sarafloxacin, que coeluyen en EC y tienen una fuerte superposición de espectros. Mediante un procedimiento de calibración multivariante (regresión parcial de los cuadrados) que se puede aplicar a los espectros, obtenidos en el máximo de los picos de electroforesis [45], permite la cuantificación de ambos compuestos.

Tabla 8. Modos de separación utilizados en el análisis de contaminantes orgánicos en alimentos

Tipo	Compuestos	Modo de separación	Comentarios	Ref.
Quinolonas	Acid oxonílico, fluomequina, difloxacina, sarafloxacina, enrofloxacina, ciprofloxacino, danofloxacina, marbofloxacino, ácido piromídico.	EC 10 – 50 mM tampón fosfato ajustado a pH 8 – 9.0 con o sin CH_3OH	Compuestos anfotéricos con grupos ácidos y básicos. Control de esepcies iónicas cuando el pH del tampón está próximo al pKa	[39-41, 43-45]
		NACE con EOF reverso 20 mM acetato de amonio, 0.004 % HDB y 4 % ácido acético con una mezcla de CH_3OH/CH_3CN (50:50 v/v)		[42]
Tetraciclinas	Oxitetraciclina	EC con tampón fosfato (0.2 M, pH 2)	Grupos amfóteros	[46]
Sulfonamidas	Sulfametazina, sulfamerazina, sulfadiazina, sulfadimetoxina, sulfamonometoxina, sulfafenazol, sulfaquinoxalina, sulfisoxazol, sulfabenzamida, sulfacetamida, sulfametosazol, sulfameter, sulfacloropiridazina, sulfametizol, sulfametoxi-piridazina, sulfamoxol, sulfatiazol, sulfapiridina, sulfanilamida, sulfaguanidina, trimetoprim, omethoprim, sulfisomidina	EC con tampón fosfato (35 mM, pH 6.5)	Grupos amfóteros Buena resolución de todas las sulfonamidas	[48]
		EC con ácido fórmico (100 – 10000 mM) CH_3OH /H_2O ácido fórmico 1.5 %	Adsorción de los analitos catiónicos, un revestimiento catiónico del capilar elimina la adsorción de los analitos	[47, 49]

Tabla 8. Continuación

Tipo	Compuestos	Modo de separación	Comentarios	Ref.
Mezclas	β-Lactamas, aminoglicósidos, tretraciclinas, quinolonas, fenicoles	EC 5 – 25 mM de tampones fosfato y borato	Algunos de los medicamentos tienen tiempos de migración muy parecidos	[50]
Ditiocarbamatos	Ferbam (dimetilditiocarbamato férrico) Ziram (dimetilditiocarbamato de zinc) Zineb (zinc etilenebisditiocarbamato) Metham (sodio metilditiocarbamato) Maneb (manganeso etilenebisditiocarbamato)	EC 20 – 25 mM tampón de tetraborato de sodio (pH 9)	Los compuestos cargados negativamente del Ferbam se determinan tras una descomposición acídica y formación de complejos con EDTA como Fe-EDTA$^-$	[51–53]
Fungicidas	Amitrol, carbendazima, 2-aminobenzimidazol, tiabendazol, 1,2-diaminobenceno, procimidona, procloraz, imazalil, metalaxil, captan, folpet, captafol, vinclozolin, iprodiona	EC solución de fosfato 4 mM (pH 3.5) 12 mM de formiato amónico -20 mM ácido fórmico pH 3.5, 2% CH_3OH 50 mM ácido fórmico – 50 mM de formiato amónico	Separación de los fungicidas amitrol y benzimidazol que tienen grupos imidazol protonados. Tampones volátiles para acoplarse al EM	[57–60]
		5 mM amonio dihidrógenofosfato – 50 mM tampón fosfato (pH 3) – 4 mM 2-hidroxipropil-b-CD	Resolución quiral de los dos enantiómeros de imazalil	[65]
		MEKC 4 mM de borato pH 9.2, 75 mM colato sódico 30 mM NH_4Cl/NH_3 (pH 9), 15 mM SDS	Interacciones electrostáticas entre cargas negativas de las micelas de SDS y los analitos cargados positivamente.	[55, 56]
Polares que contienen P	Glifosato, glufosinato, fosetil-Al, acefato	ECG en capilar recubierto de poliacrilamida lineal 1 mM acetato de amonio (pH 4 – 9) – CH_3OH	El recubrimiento del capilar es para estabilizar la interfase con el EM.	[61]
		40 mM de acetato de amonio (pH 9)	Compuestos cargados negativamente en solución	[62]
Organofosforados	Clorpirifos	10 mM Na_2HPO_4/ 6 mM $Na_2B_4O_7$, 25 % ACN con 50 mM de SDS o 50 mM deoxicolato sódico	Compuestos neutros	[63]
Derivados de la urea	Triasulfuron, clorsulfuron, monuron, fluometuron, metobromuron, clorotoluron, isoproturon, diuron, meta-benzitiazuron, flufenuxuron	MEKC 4 mM de borato (pH 9.2), 35 mM SDS	Compuestos neutros al pH de trabajo	[64]
Herbicidas ácidos	o-Fenilfenol, ioxinil, haloxifop, acifluorfen, picloram	EC 32 mM de formiato amónico/ fórmico (pH 3.1)	Pesticidas con grupos ácidos cargados negativamente en solución	[66]

Table 8. Continuación

Tipo	Compuestos	Modo de separación	Comentarios	Ref.
Multipesticidas	Pirimetanil, pirifenox, ciprodinil, ciromazina, pirimicarb, carbendazima, simazina, atrazina, propazina, ametrin, diuron, linuron, carbaril, propoxur, naftaleno, acetamida, tiabendazol, 1-naftol, acrinatrin, bitertanol, ciproconazol, fludioxonil, flutriafol, miclobutanil, piriproxifen, tebuconazol	CE 0.3 M acetato de amonio/ácido acético (pH 4) con y sin adición de MeOH	Plaguicidas con grupos ácidos y básicos ionizables	[69]
		MEKC 30 mM NH_4Cl/NH_3 (pH 9) 15 mM SDS 20 mM de tampón fosfato (pH 2.5) 25 mM SDS, 10% CH_3OH	Compuestos neutros	[67, 68]
Ocratoxina A		MEKC Fosfato-borato (pH 8.02), SDS, γ-CD, CH_3CN	Compuestos neutros	[72]
		EC-LIF 20 mM tampón fsofato (pH 7)	Separa únicamente la ocratoxina de interferencias de la matriz	[71]
Patulina		MEKC-UV 25 mM tetraborato sódico (pH 9), 50 mM SDS	La paulina es químicamente lábil en soluciones alcalinas	[73]
Aflatoxinas	B1,B2,G1,G2	MEKC-UV Fosfato borato (pH 8.02), SDS, γ-CD, CH_3CN Fosfato borato (pH 9.1), deoxicolato sódico	Separación aceptable de cuatro aflatoxinas	[72, 75]
	B1,B2,G1	MEKC-MPE fluorescencia 20 mM Tris, 10 mM carboximetil-β-CD	Mejora la fluorescencia de las aflatoxinas	[74]
Colero	Toxina A	MEKC-MPE fluorescencia 20 mM Tris, 10 mM carboximetil-β-CD	Mejora la fluorescencia de las aflatoxinas	[74]
ASP	Ácido domoico	EC 10 – 50 mM tampón de borato sódico (pH 9.3)	Análisis bajo condicones básicas	[76]
PSP	STX, dcSTX, dcneoSTX GTX1, GTX2, GTX3, GTX4, GTX5,	CE-UV 50 mM tampón de mofolina (pH 5) 10% CH_3OH o CH_3CN	Buena capacidad para analizar muchas PSP	[88]

Los plaguicidas incluyen un gran número de compuestos químicos que compromete diferentes familias químicas. A pesar de ello, hay muchos compuestos como los ditiocarbamatos que existen como aniones en solución [51, 52]. La capacidad de la EC para separar los ditiocarbamatos como ziram, zineb, metam y maneb, ha sido estudiado por Malik y Faubel [51,52]. Por debajo de pH 7.0 no se observan picos diferentes, mientras que con pH 9.0 con borato se obtienen picos bien definidos. El comportamiento de la migración de estos compuestos en el capilar puede explicarse basándose en el impedimento estérico causado por los grupos alquilo. Como el dietilditiocarbamato tiene dos grupos alquilo, esta menos hidratado que los otros compuestos estudiados, y aparece antes que los del etil xantano que tiene un grupo alquilo. El metilditiocarbamato tiene el grupo alquilo más pequeño, sin embargo aparece más tarde, pero el maneb con dos grupos ditiocarbamato aparece mucho más tarde. Las especies neutras interferentes y los cationes se discriminan por la aplicación de un campo eléctrico de polaridad reversa. Los mismos autores determinaron el ferbam por un método de EC simple y selectivo convirtiendo el Fe(III) del ferbam en un complejo de Fe(III)-$EDTA^-$ [53]. Los plaguicidas polares que contiene P como glifosato, su metabolito el ácido aminometilfosfórico, acefato, fosetil-Al y etefon así como o-fenilfenol, ioxinil, haloxyfop, acifluorfen y picloran, se separaron de igual modo [62,66]. Muchos fungicidas tienen grupos imidazol, que se protonan a pH ácido; su movilidad electroforética está relacionada con los valores de pK_a, y varían considerablemente con el pH. Takeda y col [59] utilizaron la EC para llevar a cabo la separación simultánea de cinco plaguicidas: amitrol, carbendazima, 2-aminobenzoimidazol, tiabendazol y 1,2-diaminobenceno. Se separan completamente a pH 4.0 como resultado del cambio de pH, utilizando el tampón de ácido fórmico-formiato amónico. Rodríguez y col [57] demostraron la

separación simultánea de tiabendazol, procloraz y procimidona en uva con disolución de fosfato 4 mM a pH 3.5.

Los disolventes orgánicos son una alternativa al medio acuoso en EC; sin embargo su potencial como disolvente de BGE ha empezado a centrar la atención. La corriente baja en electroforesis capilar no acuosa (non-aqueous capillary electrophoresis, NACE) significa que se pueden utilizar elevadas concentraciones de sal y campos eléctricos estrechos y también que la muestra una vez cargada puede aumentar la escala utilizando un capilar con un diámetro interno grande. Solamente se ha propuesto un método para la determinación de siete quinolonas por NACE utilizando flujo electroosmótico reverso [42]. Esto proporciona buena selectividad y tiempos de análisis cortos. Una consecuencia práctica importante para utilizar medios de separación por NACE es que las fases orgánicas, resultado de la extracción de los analitos en las matrices de las muestras o de los eluyentes de la EFS, pueden ser inyectados directamente en el sistema de EC.

La disponibilidad de muchos selectores quirales hace a la EC una herramienta importante para el análisis quiral como ha sido previamente revisado por algunos autores [57, 89]. De estos selectores, las ciclodextrinas (CDs) y sus derivados han sido los más aplicados en la EC para la separación de enantiómeros de muchos compuestos. Kodama y col [54] desarrollaron un método para la separación quiral de imazalil, utilizando EC con CDs y determinar la enantioselectividad de (+)- y (-) imazalil en residuos de naranjas. El grupo de imidazoles de imazalil con un pK_b de 6.53 está protonado a pH 3.0. Sin embargo los analitos migran electroforéticamente hacia el cátodo. Cuando un analito cargado se introduce en la cavidad de la CD, el complejo formado tiene una carga idéntica a la del analito libre pero se incrementa la masa y además la movilidad electroforética es más baja que la del analito libre. Sin embargo el principio de separación de la EC con CD para la separación

enantiomérica es por las diferencias de formación constante de complejos entre un par de enatiómeros y las CDs. Cuanto más fuerte es la inclusión del enantiómero más baja es su movilidad. El imazalil se separó enantioméricamente por la adición de cada CD, excepto α-CD, porque el (+) isómero se mueve más despacio que el isómero (-). Esto indica que el isómero (+) forma complejos diasteroisoméricos más fuertes con cada CD que el isómero (-).

6.2 Electroforesis capilar de gel (ECG)

La ECG es un caso especial de EC donde el capilar está recubierto de una película de gel. Goodwin y col [61] determinaron dos herbicidas (glifosato y glufosinato) y sus metabolitos (ácido aminometilfosfórico y ácido metilfosfínico-propiónico) como especies naturales, utilizando una interfase simple de microelectrospray. La química de las paredes del interior del capilar tiene especial importancia en la agudeza de los picos, y una elección adecuada es esencial para una operación beneficiosa de la interfase porque el EOF (flujo electrosomótico o *electrosmotic flow*) negativo operaria en dirección opuesta para la presión necesaria aplicada para alcanzar flujos envolventes en la interfase de microelectrospray estable. Una solución posible es utilizar capilares con EOF positivo o cero. Los capilares con carga positiva o neutra en el interior de las paredes se prepararon por la derivatización de los capilares de sílice vacíos. Un capilar recubierto de poliacrilamida, sin flujo electroosmótico, da mejor reproducibilidad, con precisión en tiempo de migración y área de pico.

6.3 Cromatografía electrocinética capilar (ECC): electroforesis capilar electrocinética micelar (MEKC)

La ECC es una técnica de separación basada en la combinación de electroforesis e interacción de los analitos con aditivos (ejemplo tensioactivos) los cuales forman una fase dispersa móvil de diferente velocidad. Con el fin de alcanzar la separación tanto de los analitos como de la fase secundaria deben estar cargados. La misma técnica se llama también cromatografía electrocinética (EKC).

Una variante de la ECC, en la que la segunda fase es una fase micelar dispersa en el capilar, es la cromatografía electrocinética micelar. La misma técnica se conoce también como cromatografía electrocinética micelar capilar (MEKC). En MEKC, se añade un tensioactivo iónico al electrolito de separación en una concentración superior a la micelar crítica (CMC). El tensioactivo más común es este modo de operación es el sodio dodecil sulfato ya que tiene una CMC bastante baja (8 mM L^{-1}) a un nivel al que la conductividad del electrolito se puede mantener baja. Estos modos electroforéticos han alcanzado la separación de muchos fungicidas [55, 67, 68, 90], organofosforados [63], derivados de la urea [64, 68, 91-93], triazinas [65, 68, 94] y carbamatos [67, 68].

La MEKC es un modo de EC que proporciona una muy buena separación de analitos neutros (sin carga), y esto se utiliza frecuentemente con tampones fosfato y borato a valores de pH que maximizan su capacidad tamponadora (de 7.0 a 9.2 respectivamente) [55]. Molina y Silva [55] intentaron la separación por MEKC con un pH proporcionado por el tampón fosfato a partir de la estructura química de ocho fungicidas: carbendazima, emtalaxyl, captan, procimidona, folpet, captafol, vinclozolin e iprodiona. Sin embargo carbendazima no se comporto como se esperaba ya que un átomo de nitrógeno en su grupo benzimidazol puede estar protonado a este pH, favoreciendo una

consecuente interacción electrostática con carga negativa de las micelas de SDS. Un comportamiento similar observaron Rodríguez y col [56] en otros fungicidas benzimidazoles como el metil tiofanato, tiabendazol y procimidona. Los fungicidas se separaron muy bien con colato sódico como tensioactivo porque las micelas no están muy cargadas.

Otras técnicas de ECC como la cromatografía de microemulsión electrocinética capilar (MECC o MEEKC) se ha utilizado para separar EDCs [95], pero no hay ninguna aplicación en el análisis de alimentos

6.4 Electrocromatografía capilar (CEC)

Una alternativa a la ECC es la CEC, que es un caso especial de cromatografía liquida capilar, donde el movimiento de la fase móvil a través del capilar, relleno, recubierto de una fase estacionaria, se lleva a cabo por el flujo electroosmótico (que puede ser asistido por presión). La retención se debe a la combinación de la migración electroforética y a la retención cromatográfica. La CEC tiene muchas posibilidades para ser aprovechada en la determinación de contaminantes orgánicos en alimentos. Se ha empleado sólo par la separación de algunos plaguicidas [96]. Sin embargo la aplicación a alimentos todavía no se ha utilizado.

7. AVANCES PARA MEJORAR LA SENSIBILIDAD, SELECTIVIDAD O AMBAS EN ELECTROFORESIS CAPILAR

Una de las limitaciones que la EC presenta es la sensibilidad inadecuada para el análisis de contaminantes orgánicos a niveles traza en alimentos, debido al pequeño volumen de muestra inyectado (ca. 1-10 nl). Este inconveniente se ha recogido en algunos trabajos y ha sido subsanado por diseños de enriquecimiento a niveles traza en columna y por mejoras en la sensibilidad del detector. En la tabla 9 se recogen una lista de los contaminantes orgánicos, los LOD obtenidos, así como las características importantes y las referencias relativas a estas mejoras.

Tabla 9 Propuestas descritas en la literatura para mejorar la sensibilidad y/o selectividad para la determinación de contaminantes orgánicos en alimentos

Tipo	Inyección	Detección	LDs	Comentarios	Ref.
Sulfonamidas	Hidrodinámica (35 mbar, 10 s)	EC-NanoES Quasi-EM/EM/EM (triple cuadrupolo)	5 ppb	Espectrometría de masas en tándem (EM/EM) incluyendo baridos del ion precursos y MRM. EM/EM convencional produce iones no isómero específicos. Una mejora de la técnica en línea y una mayor eficacia en los procesos de extracción y purificación podrían mejorar los LDs.	[47]
	Hidrodinámica (50 mbar, 10s)	EC-ESI-EM (simple cuadrupolo)	2–6 ppb	El método de barrido permite monitorizar el ion de fragmentación caracteristifco m/z 156 para todas las sulfonamidas. Los métodos de confirmación permiten monitorizar iones característicos de cada sulfonamida. El acoplamiento de CFS a la EC juega un papel importante.	[49]
Fungicidas benzimidazólicos	"Acumulación simple" 50 mM ácido fórmico (30 s, 5 kPa)	EC-ESI-EM (simple cuadrupolo) "líquido envolvente"	1.4–4.7 ppb	Extracto de la muestra recontituido en agua mili-Q o en la matriz de la muestra en vez de en 50 mM de ácido fórmico da lugar a una pérdida en la eficacia de la separación. La sensibilidad se mejoró entre 1-10 veces con respecto a la deteción de UV	[59]

Tabla 9. Continuación

Tipo	Inyección	Detección	LDs	Comentarios	Ref.
Tiabendazol y procimidona	"Acumulación con supresión de la muestra" (15 s, 0.5 psi) voltaje aplicado +30 kV, 0.15 min)	EC-ESI-EM (simple cuadrupolo) "líquido envolvente de la interfase"	1–10 ppb	A pesar del proceso de acumulación (stacking) y la longitud del capilar no termostatizada no se observaron ensanchamientos significativos. Los LDs fueron 10 veces superiores a los LMRs. Los EM se caracterizan por las moléculas protonadas. Fue 100 veces más sensible que la EC-UV	[58]
Benzimidazoles ,triazinas, ureas, y carbamatos	Focalización (96 s, 2.5 kPa) SRMM (96 s, 2.5 kPa) voltaje aplicado + 20 kV, 2 min SRW (96s, 2.5 kPa)	MEKC-DAD	2–46 ppb	Comparación de las 3 esrategias en línea. Factores de mejora de 3 a 18. Los efectos de matriz son relevantes. Solamente algunos plaguicidas pueden ser claramente identificados sin interferencias de los constituyentes de la matriz.	[68]
Fungicidas	Acumulación con elevada cantidad de sal (0.5 psi, 100 s)	MEKC-DAD	0.7–10.4 µg/L	El extracto de la muestra se reconstituye en 50 mM de NaCl. Se observaron mejores resultados comparados con la baja conductividad de la estrategia de acumulación (extracto de la muestra en H_2O mili-Q) y acumlación de la muestra en micelas (extracto de la muestra en SDS 5 mM). Factor de mejora 7.4 – 21	[97]
Polares que contienen P	Hidrodinámica (3 psi, 5 s)	EC-ESI-EM Simple cuadrupolo Interfase envolvente	2.5 lM	Se detectan herbicidas sin derivatizar al 10% los LMRs en trigo. Permite la dilución de la muestra asociada a la utlización del líquido de la interfase.	[61]
	Electrocinética	EC-FPD (30 kV)	1000 ppb	Una característica de la EC es el reducido tiempo de análisis con respecto a la µCL. Sin embargo, EC-DFLL es menos sensible y los compuestos como el acefato no pueden concentrarse.	[62]
Herbicidas ácidos	Hidrodinámica (0.5 psi, 20 s)	EC-ESI-EM Simple cuadrupolo Sheath liquid inter-face	20–5000 ppb	Comparado con la detección Uv covencional, los LDs fueron 2 – 50 veces más bajos. Pérdidas de resolución, eficacia y reproducibilidad comparado UV.	[66]
Multi pesticidas	Hidrodinámica (0.5 psi, 18 s)	EC-ESI-EM Trampa de iones. Interfase del líquido envolvente	60 ppb	La EC-EM proporciona LDs un orden de magnitude major que EC-UV. Pérdidas de resolución, eficacia y reproducibilidad comparado UV.	[69]
Ocratoxina A	Hidrodinámica (0.5 psi, 5 s)	EC-LIF (He/Cd laser)	0.2–10 ppb	LDs muy bajos, comparados con los de CL. Fluorescencia natural de la micotoxina por lo que no necesita derivatización.	[71]

Tabla 9. Continuación

Tipo	Injección	Detección	LDs	Comentarios	Ref.
Aflatoxina B1	Hidrodinámica (0.5 psi, 5 s)	CE-LIF (He/Cd laser)	0.5 ppb	Fluorescencia natural de la micotoxina por lo que no necesita derivatización. Posibilidad de aumentar la sensibilidad del método reconstituyendo el extracto seco con menos tampón.	[75]

7.1 Técnicas de preconcentración

La IUPAC define el proceso de preconcentración como el proceso que tiene como resultado que el rango de la concentración o la cantidad de microcomponentes (elementos traza) y macrocomponentes (matriz) que constituyen una muestra se incrementen, obteniéndose una muestra sólida y pequeña ideal para el análisis [98].

Los procesos de preconcentración se han aplicado siempre que se analizan trazas de compuestos en muestras reales. En función del modo de utilización de los métodos de preconcentración se diferencia entre:

- preconcentración sin línea/ en discontínuo (off-line): cuando la preconcentración y manipulación de la muestra se realiza en una etapa externa previa a la inyección y separación electroforética de la muestra en la EC
- preconcentración en línea/contínuo (on-line): cuando la muestra se inyecta y la preconcentración y separación electroforética se producen de manera consecutiva en la misma etapa

En la EC, se ha estudiado una gran variedad de técnicas de preconcentración en línea, basadas en acumular (stacking) y focalizar (sweeping) los analitos, aprovechando las diferentes propiedades fisicoquímicas (viscosidad, fuerza iónica, conductividad, pH, etc) que presentan la matriz de la muestra y el tampón electroforético.

Estos métodos ayudan a la disminución de los LOD mediante la introducción de un volumen elevado de muestra en el capilar, sin comprometer la eficacia y la resolución del pico, aunque una de las modalidades implica el cambio de la movilidad electroforética del analito.

Acumulación simple:

La acumulación simple se lleva a cabo por la disolución de la muestra en un tampón de baja conductividad y por la inyección hidrodinámica de la disolución. Tras la inyección hidrodinámica de la muestra y al aplicar una diferencia de potencial en el capilar los iones presentes en dicha muestra migran hacia los electrodos correspondientes siendo frenados al llegar a la zona del tampón (donde tienen una movilidad menor) lo que produce la compactación y, por tanto, acumulación de los mismos en la interfase de separación muestra-tampón. Con este método se puede aumentar el volumen de muestra introducido en el sistema electroforético sin producirse pérdida de eficacia.

Takeda y col [59] estudiaron diferentes técnicas para llevar a cabo esta acumulación simple para cinco plaguicidas: amitrol, carbendazima, 2-aminobencimidazol, tiabendazol y 2-diaminobenceno, utilizando agua destilada, tampón de migración o disolución ácida para redisolver los extractos. Cuando se utilizó el tampón de migración o agua para disolver el extracto de la muestra, la agudeza de todos los picos se vio afectada con el aumento del tiempo de inyección. La adición de ácido fórmico a la matriz de la muestra dió lugar a picos más agudos. Esta mejora puede ser debida al ligero efecto isocatoforético, el cual ocurre estrictamente cuando se inyecta un elevado volumen de muestra entre el frente del electrolito y el final. Los analitos están en la muestra apelmazados en bandas estrechas uno tras otro de acuerdo a su movilidad. Para estos cinco plaguicidas, el ion amonio en el tampón de migración correspondería al ión mayoritario y el hidrogeno en la matriz correspondería al ión terminal o

final de la isocatoforesis. Los factores de concentración obtenidos oscilaron en un rango de 7.6-27 veces.

Acumulación con traslado de muestra:

Esta técnica de acumulación es con traslado de la muestra; el capilar se llena de un volumen elevado, y se mantiene una resolución elevada, sólo si el tampón de la muestras se elimina después de la acumulación para reducir la distribución no uniforme de las fuerzas iónicas y el flujo electroosmótico [99]. Esta técnica se comprobó con concentración en línea de tiabendazol y procimidona en extractos de fruta y verdura por Rodríguez y col [58]. La cantidad de analitos en la muestra acumulada fue tres veces mayor a las inyecciones estándar. El proceso de acumulación no mejoró la sensibilidad pero sí se obtuvieron mejores LODs y eficacia en los picos.

Preconcentración en electroforésis capilar electrocinética micelar:

Este grupo de técnicas de preconcentración, estudia la habilidad para separar los analitos en una fase pseudo-estacionaria en MEKC; los analitos no se acumulan (stacked) por el proceso normal hasta que no se aceleran por el campo eléctrico que se alcanza a lo largo de la zona de inyección. En este grupo son representativas las técnicas de focalización (sweeping) y acumulación (stacking) con migración reversa de micelas sin (SRMM) y con (SRW) tapón de agua antes de la inyección.

La focalización (SW) está basada en la captura y recolección de los analitos por las micelas que penetran en la zona de la muestra durante la aplicación de un voltaje. Las muestras se inyectan en la columna en una disolución tampón con una conductividad similar a la del tampón de separación (BGE), pero en ausencia de una fase pseudo-estacionaria. El capilar se introduce entonces en un tampón BGE micelar aniónico para migrar electroforéticamente

hacia el detector. El BGE se mantiene a pH bajo para eliminar el flujo electroosmótico (EOF), permitiendo a las micelas aniónicas la migración electroforética hacia el detector. Puesto que las micelas migran hacia el detector, serán ellas mismas las que "barreran" los analitos neutros. Este efecto es dependiente de un campo eléctrico uniforme y de la ausencia de micelas en la solución de la muestra. No se observa una acumulación micelar, y la velocidad de los analitos en la zona de la muestra con micelas es la misma que la de los analitos en el tampón de separación [25, 100].

El objetivo del mecanismo de la SRMM, se basa en el cambio desigual de la velocidad electroforética efectiva de los analitos en la zona de acumulación. La zona de acumulación separa las regiones de campo eléctrico alto y bajo. El fenómeno de focalización es en parte responsable del efecto en SRMM.

El SRW se lleva a cabo por la preparación de una muestra en una matriz que tenga una conductancia menor que la del tampón de separación. Sin embargo se añade un tensioactivo a una concentración ligeramente superior a la del tampón de separación. Primero se inyecta un tapón de agua en el capilar antes de la inyección de la muestra. El objetivo del mecanismo se basa en el cambio desigual de la velocidad electroforética efectiva de los analitos en la zona de acumulación.

Da Silva y col [68] compararon focalización y SRMM, con y sin inyección de un tapón de agua antes de la inyección de la muestra, para nueve plaguicidas de diferentes clases, carbendazim (benzimidazol), simazina, atrazina, propazina y ametrin (triazina), diuron y linuron (ureas), carbaril y propoxur (carbamato). Se introdujo una nueva versión de SRMM, basada en la aplicación momentánea de un voltaje positivo en la parte de inyección de la muestra (inlet) justo después de inyectarse la muestra. Compararon la efectividad de las técnicas de preconcentración, recogiendo los LODs y los

factores sobresalientes. A la vista de los resultados se dedujo que los tres procedimientos proporcionaban aproximadamente una sensibilidad entre 3-18 veces mejor, dependiendo del plaguicida. A pesar de que estos factores no son tan sorprendentes como los encontrados en la literatura [25, 26], se debe tener en cuenta que los plaguicidas estudiados comprenden un rango amplio de hidrofobicidad y polaridad; sin embargo, sus coeficientes de reparto difieren considerablemente. Las metodologías propuestas se aplicaron al análisis de plaguicidas en extractos de zanahorias obteniéndose LODs de 2.5 $\mu g\ L^{-1}$.

Las aproximaciones más recientes para la acumulación de volúmenes elevados de muestra, sin dispersión de la misma, implican la utilización de concentraciones de sal más elevadas que las del tampón de separación. Tras la aplicación del voltaje de separación, las micelas con carga negativa migran hacia la zona de la muestra, desde el detector hasta la interfase de separación de la muestra y el tampón. El campo eléctrico en la zona de la muestra de conductividad elevada, es más bajo que en la zona del tampón de separación, en consecuencia, la velocidad de migración de las micelas decrece. El efecto neto es que en las micelas acumuladas cerca del detector en la interfase, zona de separación muestra-tampón es mayor la relación de la fase de las micelas en la interfase de muestra-tampón. En consecuencia, los analitos más hidrofóbicos se concentran en esta zona. De acuerdo con Palmer y Landers [101] se necesita de dos requisitos decisivos para poder hacer acumulación en MEKC con concentración elevada de sal. Primero, la conductividad de la matriz de la muestra debe ser superior a la de la conductividad de la muestra, por eso el campo eléctrico disminuye significativamente en la zona de la muestra. En segundo lugar, la matriz de la muestra debe incluir un co-ion, con frecuencia el cloruro, con una movilidad electroforética intrínseca superior a la del tensioactivo, lo que garantiza la formación de una banda pseudo estacionaria entre la micela y el componente co-ion en la matriz. En contraste con el método

de focalización descrito anteriormente, la acumulación de alta concentración de sal utiliza un sistema de tampón discontinuo y no es necesaria la muestra sin fase pseudo-estacionaria [100]. Este mismo procedimiento lo llevaron a cabo Molina y Silva [55] y mejoraron la sensibilidad para la determinación de ocho fungicidas, carbendazima, metalaxilo, captan, procimidona, folpet, captafol, vinclozolina y iprodiona, en zumos de frutas, sin producirse ensanchamiento de los picos. Se demostró que los métodos de acumulación con concentración elevada de sal es la mejor condición para obtener los efectos sobresalientes en MEKC.

8. MÉTODOS DE DETECCIÓN

El sistema de detección es de especial relevancia para mejorar los límites de detección.

El detector UV-Vis ha sido utilizado en el análisis de plaguicidas por CL porque la mayoría de los plaguicidas absorben energía en dicha zona de espectro [83, 102, 103], al igual que muchas sustancias interferentes procedentes de la matriz estudiada e impurezas de los disolventes utilizados en los procesos de extracción. Este hecho contribuye a que este detector sea poco selectivo y poco sensible. Por otra parte el detector de fila de diodos (DFD o DAD) aunque es capaz de medir simultáneamente todas las longitudes de onda, continua presentando el inconveniente de poca sensibilidad [104-106].

La utilización de la espectrometría de masas (EM) acoplada a la CL permite la identificación y confirmación de las sustancias desconocidas presentes en las matrices de análisis, y aunque al principio este acoplamiento presentó incompatibilidades, debido a la cantidad de fase móvil (ml min^{-1}) que pasaba a vapor al llegar al EM, se superó con el diseño de interfases de ionización a presión atmosférica (IPA), las cuales poseen gran sensibilidad, son aptas para un gran número de compuestos y ofrecen una valiosa información estructural mediante la colisión inducida por disociación (CID). Las técnicas IPA son la ionización química a presión atmosférica (IQPA) y la ionización electrospray (ESI).

En cuanto a la EC, aunque es más fácil de conectar con métodos de detección ópticos basados en UV, el análisis de extractos complejos de alimentos utilizando este detector tiene posibilidades limitadas. La EC-UV no es lo suficientemente sensible para la detección de contaminantes orgánicos a niveles traza. Se han desarrollado algunas soluciones en los instrumentos para la

mejora de los LODs con detección UV, como detección con celdas con forma de z o de burbuja, las cuales son celdas de detección muy sensible y con más anchura que las convencionales. Desafortunadamente esta aproximación no se ha aplicado para el análisis de contaminantes en alimentos [43]. Además la identificación de los analitos diana es incierta porque el espectro de UV de diferentes compuestos incluidos en una clase de contaminantes son muy similares, y la co-migración de los analitos y de los compuestos de la matriz puede hacer una confirmación ambigua de estos residuos. En consecuencia se precisa de detecciones más selectivas y sensibles.

La fluorescencia inducida por láser (LIF) es una técnica de detección más sensible y selectiva que la UV, pero su uso esta restringido a moléculas con propiedades fluorescentes.

En la actualidad, el acoplamiento de la EC a la EM y la espectrometría de masas en tándem (EM/EM) son las técnicas de elección para la identificación

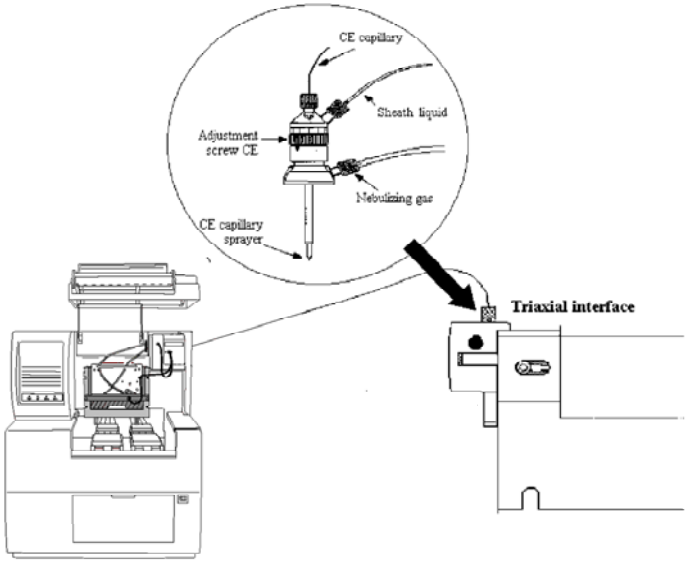


Figura 1 Acoplamiento de la Electroforesis Capilar a la Espectrometría de Masas

de residuos, pues la adquisición de información de masas facilita la identificación y caracterización de los analitos y suprime una gran cantidad de sustancias interferentes. La técnica de ionización más frecuente es la EC-EM con ionización de electrospray (ESI), que ha demostrado ser sensible, versátil y relativamente fácil de utilizar en combinación con la EC. El acoplamiento de la EC a la EM utilizando triple cuadrupolo (QqQ), o trampa de iones (QIT) ha sido revisada en la literatura [29, 74], mostrando que hay una gran variedad de interfases EC-ESI-EM, divididas en dos categorías: interfases de flujo envolvente e interfases sin envolvente. La primera es la más utilizada para EC-EM y proporciona características muy buenas como fácil fabricación, fiable, y de fácil aplicación. La EC acoplada a la EM se ha utilizado para la determinación de algunos antibacterianos en carne y leche y algunos residuos de plaguicidas en frutas y verduras.

Santos y col propusieron un método para la detección de residuos de seis sulfonamidas en leche por EC-EM utilizando un ion característico común a todas las sulfonamidas [67]. Los mismos autores utilizaron un método de EC-EM más específico para confirmar aquellas muestras que contenían los residuos a una concentración superior a los límites legales.

Takeda y col [59] probaron la determinación con EC-EM para cinco plaguicidas. Rodríguez y col [41] desarrollaron un método basado en la EC-EM para la determinación de procimidona y tiabendazol en frutas y verduras. Estas muestras son matrices complejas que contienen cantidades significativas de sal y varios compuestos endógenos. Estudios comparativos entre EC-UV y EC-EM se han llevado a cado para diferentes plaguicidas utilizando modos de ionización positivos [52] y negativos [49]. Los dos llegan a las mismas conclusiones. Se obtiene una buena separación de los compuestos en ambas técnicas utilizando tampones acuosos volátiles, pero la separación es más pobre por EC-EM, atribuible a la pequeña proporción del capilar que está termostatizado ya que la

mayor parte de él (entre EC y EM) se encuentra a temperatura ambiente. En el área de los picos se observa una reproducibilidad más baja para EC-EM que para EC-UV. Sin embargo, la EC-EM proporciona unos LODs al final de un orden de magnitud mejor que por EC-UV.

Goodwing y col [44] demostraron que la EC-EM sin interfase envolvente es apropiada para el análisis de glifosato, glufosinato, ácido aminometilfosfónico y ácido metilfosfinicopropiónico, utilizando la combinación de impulso eléctrico y de presión. La restricción de este mecanismo se establece en la necesidad de evitar descargas entre la punta del capilar y la introducción en el EM, ya que en el modo operativo el límite superior de la corriente es de 2 μA. La concentración adecuada de amonio fue de 1 mM, considerada como muy baja con respecto a los tampones (BGEs) típicos utilizados en EC. Esto limita el rango de muestras aceptables para el análisis, ya que la conductividad de la matriz debe ser inferior o igual a la del BGE para poder obtener picos aceptables. Los sistemas de interfase tienen ventajas sobre los líquidos envolventes típicos, porque no hay dilución de los analitos por el líquido envolvente.

La espectrometría de masas en tándem EM/EM o EM^n utiliza estadios múltiples del análisis de masas lo que permite la selección de un ion y el análisis inducido de fragmentos obtenidos por colisión con un gas inerte. Recientemente, EC-ESI-EM/EM es una técnica emergente utilizada para la caracterización de contaminantes orgánicos en alimentos, y esta tecnología ha sido aplicada satisfactoriamente para el análisis de sulfonamidas en leche y plaguicidas en frutas y verduras.

La aplicación de la combinación de EC/EM/EM con cribado (screening), cuantificación y confirmación de residuos de sulfonamidas en muestras de leche a niveles, de alrededor de 5 ppb ha sido estudiada por Bateman y col [30]. Se estudiaron tres pares de isómeros de sulfonamidas, los cuales difieren sólo en la

posición de un átomo de átomo de nitrógeno y uno de oxigeno en el anillo aromático heterocíclico de las moléculas. En el análisis convencional con EM/EM se produjeron iones no isómero específicos, no obstante, la EM/EM/EM se aplicó para suplir estas limitaciones. Específicamente, en una primera etapa con fuente de CID se utilizó como etapa de casi EM/EM para generar iones correspondientes a las aminas heteroatómicas. En una segunda etapa de EM/EM, estos iones se aislaron y se hicieron pasar por la CID en el cuadrupolo de colisión para producir iones isómero-específicos.

Una vía fiable de obtener información estructural es realizar experimentos con EM en tándem de iones de interés específicos. Los EM^2 y EM^3 a menudo producen fragmentos de iones formados por la fragmentación de cadenas laterales en la estructura molecular o por la apertura de los anillos heterocíclicos.

9. OBJETIVOS-Objectives

El objetivo principal de esta tesis es la determinación de residuos de diferentes contaminantes orgánicos por diferentes técnicas cromatográficas a fin de obtener resultados que permitan poner de manifiesto el grado de seguridad alimentaria

Para ello, se abordan diversos objetivos parciales:

1.-Extracción de residuos de plaguicidas y medicamentos veterinarios por procedimientos clásicos de extracción en fase sólida, y otros más innovadores, como extracción sobre barras magnéticas y extracción presurizada con disolventes.

2.- Análisis de plaguicidas por cromatografía líquida-espectrometría de masas previa utilización de los métodos de extracción en fase sólida y extracción sobre barras magnéticas.

3.-Comparación de las técnicas de preconcentración en línea, para el análisis de plaguicidas a fin de alcanzar unos límites de detección adecuados para su determinación en frutas y verduras por electroforesis capilar.

4.-Estudiar el empleo de la espectrometría de masas como técnica de determinación acoplada a la electroforesis capilar en sus variantes electroforesis capilar-simple cuadrupolo y electroforesis capilar-trampa de iones para llevar a cabo la detección de residuos de plaguicidas y medicamentos veterinarios.

5.-Aplicación de los procedimientos de análisis seleccionados a diversos alimentos de origen vegetal como, uva, fresa, tomate, lechuga, melocotones, y nectarinas, y alimentos de origen animal como pescado y carne de pollo y cerdo.

OBJECTIVES

The objective of this thesis is the determination of residues of different organic contaminats by using different chromatographic techniques to evaluate the degree of food safety.

For this, partial objectives have to be approached:

1.-Pesticide and veterinary drugs residues extraction by using classical extraction procedures, as solid phase extraction and other more innovatives, as stir bar soptive extraction and pressurized solvent extraction.

2.-Pesticide analysis by liquid cromatography-mass spectrometry previous the use of the extraction methods like solid phase extraction and stir bar soptive extraction.

3.-Comparison of on-line preconcentration techniques, for the pesticide analysis in order to reach adequated limits of detection for its determination in fruits and vegetables by capillary electrophoresis.

4.-Study the use of mass spectrometry as a determination technique coupled to capillary electrophoresis using different mass analyzers, capillary electrophoresis-single quadrupole and capillary electrophoresis-ion trap to carry out these techniques in pesticide and veterinary drugs residue detection.

5.-Apply the selected analytical procedures to different vegetable products as grapes, strawberry, tomato, lettuce, peaches and nectarines, as well as to animal food such as fish, chicken and pork.

Material y Reactivos
Material and Chemicals

1. Reactivos

Los plaguicidas carboxin, flutriafol, tebuconazol, bitertanol, fludioxonil, ciproconazol, acrinatrin, dinoseb, pirimicarb, procimidona, pirifenox, tiabendazol, y carbendazima (pureza > 99.3%) se obtuvieron de Riedel-de Haën (Seelze, Alemania), miclobutanil, piriproxifen (pureza > 99.3%), pirimetanil y triadimefon por Dr. Ehrenstorfer GmbH (Augsburg, Alemania).

Las quinolonas danofloxacina, flumequina, ofloxacina, y ácido pipemídico se obtuvieron de Riedel de-Haën (Seelze, Alemania), y enrofloxacina fue proporcionada amablemente por Cenavis (Reus España).

Las sulfonamidas (SAs): sulfanilamida sulfatiazol, sulfapiridina sulfadiazina, sulfametoxipiridazina, sulfaguanidina, sulfadimetoxina, sulfacloropiridazina, sulfisoxazol, sulfasalazina, sulfabenzamida y sulfadimidina, se adquirieron de Riedel de-Haën (Seelze, Alemania).

Las estructuras de los compuestos estudiados se muestran en la figura 1.

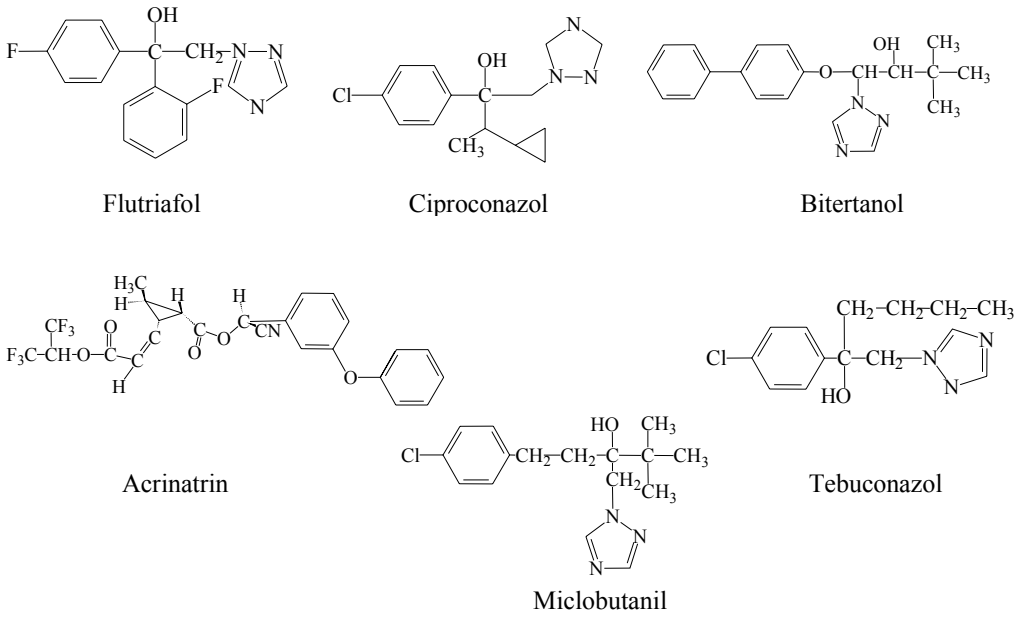
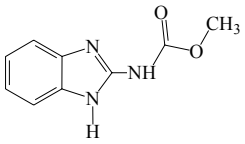
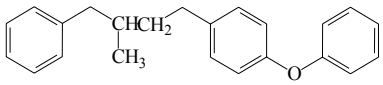


Figura 2. Estructura de los compuestos estudiados

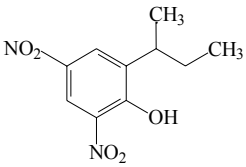
Figura 2. Continuación



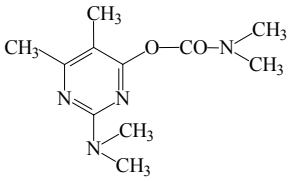
Carbendazima



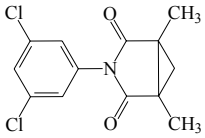
Piriproxifen



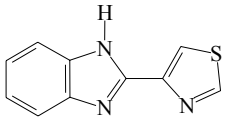
Dinoseb



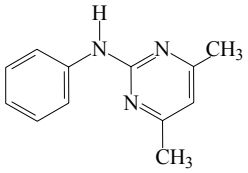
Pirimicarb



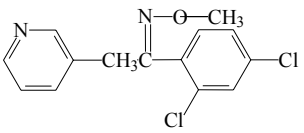
Procimidona



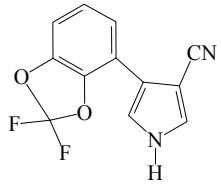
Tiabendazol



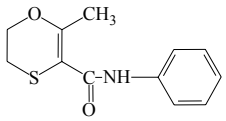
Pirimetanil



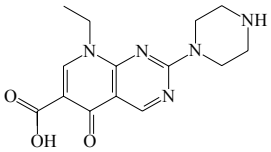
Pirifenoz isómeros D y Z



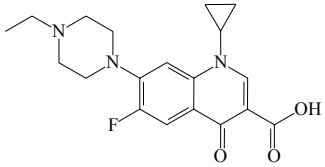
Fludioxonil



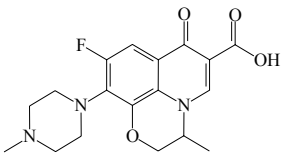
Triadimefon



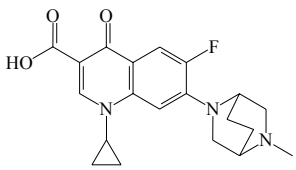
Ácido pipemídico



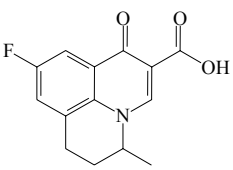
Enrofloxacina



Ofloxacina

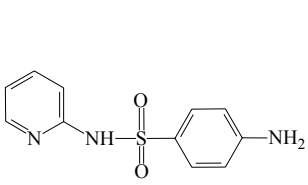


Danofloxacina

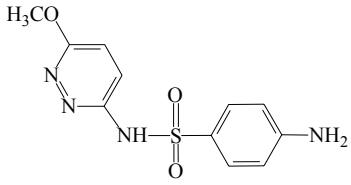


Flumequina

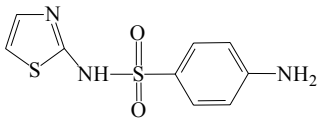
Figura 2. Continuación



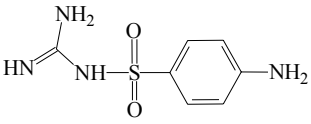
Sulfapiridina



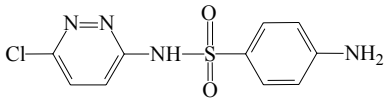
Sulfametoxipiridazina



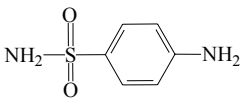
Sulfatiazol



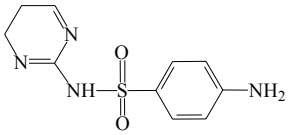
Sulfaguanidina



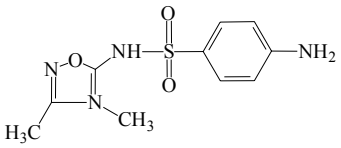
Sulfacloropiridazina



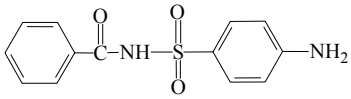
Sulfanilamida



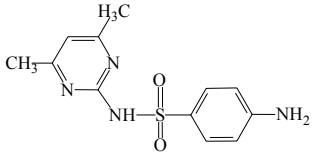
Sulfadiazina



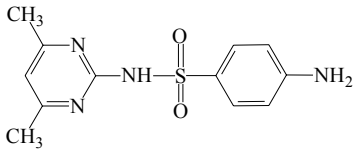
Sulfisoxazol



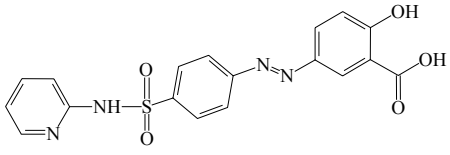
Sulfabenzamida



Sulfadimetoxina



Sulfadimidina



Sulfasalazina

Las disoluciones patrón de 1mg ml^{-1}se prepararon pesando la cantidad necesaria de cada plaguicida y disolviendo posteriormente con metanol. Se procedió de este modo para todos los compuestos que se estudian en la presente tesis excepto para la disolución patrón de carbendazima, que se preparó con 10 ml l^{-1} HCl a la misma concentración. Las disoluciones patrón se guardaron en viales de cristal a 4ºC y permanecieron estables en estas condiciones durante más de 3 meses.

Las disoluciones de trabajo se prepararon diariamente a varias concentraciones por disolución de pequeñas alícuotas de disoluciones patrón en metanol y en el tampón de separación. Todas las disoluciones y mezclas de compuestos, se hicieron pasar a través de filtros de celulosa de 0.45 µm de Scharlau (Barcelona, España).

Las disoluciones utilizadas para adicionar las muestras se prepararon en metanol, mezclando las disoluciones de trabajo hasta obtener concentraciones de 5 mg mL^{-1} de cada compuesto.

Todos los reactivos y disolventes utilizados fueron de grado analítico o calidad HPLC. El metanol y el diclorometano (DCM) se adquirieron de Merck (Darmstadt, Alemania); la acetona de Mallinckrodt (ChromARHPLC, KY, EEUU) y de Promochem (Wesel, Alemania); el ácido fórmico y ácido acético se obtuvieron de PanReac (Barcelona, España); el agua desionizada (resistividad <8 cmMΩ) de Milli-Q SP Reagent Water System (Millipore, Bedford, MA, EEUU) y fue utilizada para la preparación de todos los tampones y las disoluciones de las muestras y de compuestos. Todos los disolventes, antes de ser utilizados, se hicieron pasar a través de filtros de 0.45µm de celulosa Scharlau (Barcelona, España), o bien por filtros de Nylon de 0.45 mm de Análisis Vínicos (Tomelloso, España).

Se trabajó con ácido fosfórico de Panreac y de Scharlau (ambas de Barcelona, España).

El sodio dihigrógeno fosfato se obtuvo de PanReac (Barcelona, España). El sodio dodecil sulfato y el sodio tetraborato decahidratado se adquirieron de Aldrich (Madrid, España); el cloruro sódico, ácido ortofosfórico, disodio hidrogenofosfato dihidratado, potasio dihidrogenofosfato, disodio hidrogeno fosfato decahidratado, fosfato trisodio dodecahidratado, hidróxido sódico, hexano y acetonitrilo (ACN) se obtuvieron de Scharlau (Barcelona, España).

El carbonato amónico, acetato amónico y ácido trifluoroacético (TFA) se adquirieron de Fluka (Madrid, España); la sal de colato sódico se consiguió de Fluka (Buchs, Suiza) y la tierra de diatomeas (tamaño de la partícula 13 μm) se adquirió de Sigma (Steinheim, Alemania).

Los cartuchos de Oasis HLB [poli(divinilbenceno-co-N-pirrolidona), 200 mg fase sólida, cartuchos de 6 ml], se obtuvieron de Waters ; las columnas de C_{18} (15 cm×0.4 cm i.d., 5μm) fueron de Phenomenex (Madrid, España); la fase sólida ODS (MFE-Pak C18) y la MFE C_{18} (diámetro de la partícula entre 45–55μm y diámetro del poro de 60 Å) fueron de Análisis Vínicos (Tomelloso, España). Se utilizaron columnas de vidrio 100 mm × 9 mm i.d. con tapón (No. 3) de dimensiones (14/23) y un cono estándard (29/32), que se pudieron conectar a material de vidrio de laboratorio como embudos, kitasatos o adaptadores de vacío hechos a medida por Marcelo Glassware (Valencia, España).

Las barras magnéticas (Twister), de una longitud de 10 mm y recubiertas de una capa fina de 1mm de PDMS, lo que corresponde a 55 μl de PDMS, se adquirieron de Gerstel (Mülheim, Alemania).

2. Equipos

La evaporación se llevó a cabo con un rotavapor R200 (Büchi, Flawill, Suiza) o bien un TurboVapLR Concentration Work Station de Zymark (Hopkinton, MA, EEUU) en función del volumen a evaporar.

El pH de las disoluciones electrolíticas se midió con un pH-ímetro modelo Model DM-21 (Digined, Sao Paulo, Brasil).

La centrifugación realizó con un Macrotonic Selecta (J. P. Selecta SA, Abrera, España).

La extracción presurizada con disolventes (EPD o PLE o ASE) se llevó a cabo con un ASE 200 de Dionex (Sunyvale, CA, EEUU). Las celdas de extracción utilizadas, fueron de acero y selladas en los extremos con unos filtros de fibra de vidrio de 1.98 cm de diámetro (Dionex Co.).

Las separaciones electroforéticas se llevaron a cabo con un equipo de Beckman P/ACE System MDQ (Fullerton, CA, EEUU) equipado con un detector de difracción de diodos (DAD) y un software de System Gold versión 2.2 para registro de datos.

Los ensayos realizados con detección UV se llevaron a cabo en un cartucho convencional P/ACE System MDQ provisto de un capilar de sílice fundida de Beckman o bien de Supelco (Madrid, España) con las siguientes dimensiones: 57 cm de longitud total, 50 cm longitud efectiva (desde la inyección de la muestra hasta el final del capilar), 75 mm diámetro interno (ID), y 375 mm diámetro externo (OD). Sin embargo, cuando se llevó acabo la EC-EM se utilizó un capilar de una longitud total de 75 cm, 50 cm termostatizados y 25 cm a temperatura ambiente, las dimensiones de OD e ID fueron las mismas que las mencionadas anteriormente. A los capilares utilizados para EC-EM se les eliminó el recubrimiento del final del capilar (ca. 5 mm) y sobresalió 0.1 mm del spray para mantener estable el electrospray.

Se utilizaron dos espectrómetros de masas: un simple cuadrupolo Hewlett-Packard (HP) 1100 (Agilent Technologies Palo Alto, CA, EEUU), equipado de un HP Chem Station version A.06.03 y un cuadrupolo trampa de iones (QIT-MS) Esquire 3000 Ion Trap LC/$MS^{(n)}$ (Bruker Daltonik, Bremen, Alemania) y un procesador de datos Daltonic Esquire Control Software System 3.0. y Esquire Control versión 5.1 y Data Analysis versión 3.1 (Bruker)

El acoplamiento de la EC a la EM una vez conectado, se sincronizó mediante una señal de pulso externa, programada desde el equipo de electroforesis. Estas conexiones se llevaron a cabo con un G1607 Agilent CE-MS sprayer kit, una bomba binaria HP1100 y adaptador de detección externo (EDA) y cartuchos (Beckman). El objetivo de este sistema de acoplamiento fue permitir la conexión desde la salida del capilar del sistema P/ACE MDQ al espectrómetro de masas.

El kit del spray incluye un montaje de flujo triaxial para la interfase de electrospray (ESI), el cual permite la mezcla del eluyente del capilar con un líquido envolvente adecuado (para establecer el contacto eléctrico en la punta del capilar y el nebulizador) y ambos con un flujo de N_2.

El líquido envolvente proporcionado por una bomba de Hewlett-Packard 1100 Series, se conectó a la salida del flujo en finas gotículas en una relación 1:100 (también incorporado en el equipo de Agilent CE-MS).

Con el fin de optimizar las lentes de QIT-MS, los voltajes y las condiciones de fragmentación se utilizó una bomba de infusión simple (Cole-Parmer, Vernon Hills, IL, EEUU) dispuesta de una jeringa Hamilton de 240 a 500 mL (Hamilton, Bonaduz, Suiza).

**** Cromatografía líquida-espectrometría de masas (CL-EM)***

Se utilizó un HP-1100 CL-EM (Palo Alto, CA, USA) equipado de una bomba de disolventes binarios y un autoinyector de 5 µl, y un detector selectivo de masas con ionización química a presión atmosférica (APCI) acoplada a un

HP Chem Work Station. Las condiciones de trabajo para la interfase de APCI en modo positivo fueron: temperatura del vaporizador 325ºC, presión del gas (nitrógeno) nebulizador 60 psi (1 psi = 6894.76 Pa), flujo de 4 l min^{-1}, temperatura del gas de secado 250 ºC, voltaje del capilar 4000 V y corriente de la corona 10 µA.

La separación cromatográfica se llevo a cabo en una columna columna de C_{18} (15 cm×0.4 cm i.d., 5µm) de Phenomenex (Madrid, España), la fase móvil fue agua-metanol con un gradiente inicial de 65 % de metanol que se aumentó hasta un 80 % en 15 min a un flujo de 0.8 ml min^{-1}.

Los cromatogramas de CL-EM en modo de barrido completo, se obtuvieron desde m/z 80 hasta 340 en un tiempo de 0.687 s.

3. Tampón de separación y acondicionamiento del capilar

El tampón de separación (o BGE) seleccionado para el análisis de los compuestos, se realizó tras el estudio de parámetros clave, como pH y concentración del electrolito. Así mismo fue necesario considerar la importancia de la presencia de un modificador orgánico y la utilización de diferentes tensioactivos a diferentes concentraciones.

Para el análisis de plaguicidas por MEKC de fresas, uvas, lechugas y tomates el rango de pH estudiado fue de 4 a 11 y la concentración del electrolito de 3 a 30 mM. Se estudió la adición de metanol como modificador orgánico y sal de colato sódico y sodio dodecil sulfato como tensioactivos. El tampón seleccionado fue 6 mM de tetraborato sódico decahidratado con 75 mM de colato sódico preparado por disolución de las cantidades adecuadas en agua desionizada con ayuda de un baño de ultrasonidos. El pH del tampón borato se ajustó al valor deseado mezclando varias proporciones de solución de 6 mM de tetraborato sódico con la misma concentración de hidróxido sódico en ácido bórico.

Para el análisis de quinolonas el BGE elegido fue 60 mM $(NH_4)_2CO_3$ a pH 9.2 y para las sulfonamidas (SAs) 50 mM de acetato amónico a pH 4.16.

Cuando se llevaron a cabo técnicas de preconcentración en línea para el análisis de plaguicidas, el tampón seleccionado fue un tampón fosfato entre 4-25 mmol l^{-1} de pH 2.3 con o sin metanol (de 0 a 10%) y 10-25 mmol l^{-1} de dodecilsulfato sódico (SDS).

Antes de realizar las inyecciones por EC, el capilar, se acondicionó todos los días con 0.1 M de hidróxido sódico durante 30 min y entre cada inyección el capilar se lavó durante 2 min con agua desionizada, 2 min con 0.1 M de NaOH, 2 min de agua desionizada, y 2 min de tampón de separación aplicando una sobrepresión de 20 psi (1 psi = 6894.76 Pa).

4. Inyección de la muestra

Las muestras se inyectaron aplicando una presión de 1-0.25 psi durante 10-5 s.

Cuando se estudió la acumulación (stacking) para muestras de carne de cerdo la muestra se inyecto hidrodinámicamente a 50 mbar (1 mbar = 100 Pa) durante 5 s, lo que corresponde a un 1 % del volumen del capilar y se aplicó voltaje negativo (−20 kV) lo que proporcionó una corriente de 34 μA.

La separación se llevó a cabo utilizando viales de 2 ml para lavado, inyección y tamponar y viales de 500 μL de polipropileno para las muestras. Las muestras se inyectaron en capilares de sílice fundida con las características anteriormente descritas. Se aplicó un voltaje de 120-23 kV, y la temperatura del capilar se mantuvo a 20 ºC. La longitud de onda de detección fue de 214-230 nm para EC-DAD. Para las separaciones de pEC, se aplicó un exceso de presión al final del inyector (30 mbar) durante la inyección electroforética.

5. Técnicas de preconcentración en línea

Cuando se estudiaron las técnicas de preconcentración las inyecciones de la muestra fueron hidrodinámicas, a 50 mbar, y se estudió el efecto de cantidad de muestra inyectada con la eficacia en la resolución de los picos. Se estudiaron tiempos de inyección de 10 a 100 ms, a intervalos de 10 s, y se observó que el tiempo óptimo era de 60 s.

Para SW, los analitos (bien sean soluciones patrón de plaguicidas o extractos) se disolvieron en tampón fosfato 25 mmol l^{-1} pH 2.3 con un 10 % de metanol, obteniéndose un tampón no micelar con una conductividad de 2.1mS cm^{-1}. Una vez inyectada la muestra se aplicó un voltaje de -20kV.

Para SRMM, la muestra se diluyó en agua. Tras la inyección se aplicó un voltaje positivo (+20 V) durante 12 s en el vial del lado derecho (inlet-de entrada). En este tiempo la corriente aumentó progresivamente hasta alcanzar el 80% del valor inicial de corriente medido por el tampón de separación, llegado a este punto se invirtió el voltaje a modo negativo (−20 kV).

Para SRW, se inyectó un volumen de agua antes de la inyección de la muestra durante un tiempo establecido (se optimizó de 5 a 60 s a 0.4 psi obteniendo un valor óptimo a 10 s), y a continuación la muestra diluida en un tampón fosfato de 4 mmol l^{-1} pH 2.3 con 10 mmol l^{-1} SDS. Tras la inyección se aplicó un voltaje de −20 kV.

6. EC-ESI-EM y EC-ESI-EM/EM

Los parámetros instrumentales y experimentales de EC-UV, EC-EM y EC-EM/EM se detallan en la tabla 10.

Tabla 10. Parámetros instrumentales y experimentales para EC

	EC-UV	EC-EM	EC-EM/EM	Cap.
Acondicionamiento del capilar				
Al principio de cada día	0.1 M NaOH durante 30 min aplicando una sobrepresión de 20 psi (1 psi = 6894.76 Pa)			III, V, VI
Antes de cada inyección	Prelavado durante 2 min con agua desionizada, 2 min con 0.1 M NaOH, 2 min con agua desionizada, y 2 min con tampón de separación aplicando una sobrepresión de 20 psi			III, V, VI
Condicicones del equipo				
Tampón	0.3 M acetato de amonio - ácido acético, pH 4, en 10% MeOH			III
	60 mM $(NH_4)_2CO_3$ a pH 9.2			V
	50 mM acetato de amonio pH 4.16			VI
Temperatura		25ºC		III
		20 ºC		V, VI
Voltaje (media de la corriente)		30 kV (<50 µA)		III
		120 kV		V
		-20 kV (34 µA)		VI
		23 kV		
Inyección		0.5 psi durante 5 s		III
		1 psi durante 10 s		VI
Recogida de datos	Amplitud de barrido desde 190 a 280 nm		Barrido completo	III, V, VI
	Longitud de onda única 214 nm	SIM	MRM	III, V, VI
Interfase EC-ESI-EM				
Composición de líquido envolvente		0.3 M acetato de amonio-ácido acético, pH 4, en 10% MeOH		III
		MeOH-H_2O-ácido fórmico (49.5:49.5:1 v:v:v)		VI
Flujo del líquido envolvente *			5 mL/min	III
		0.01 mL/min		V, VI
Espray de la cámara de ESI				
Polaridad			Positivo/negativo	III
			Positivo	V, VI
Flujo del gas de secado			7 L/min	III
			10 L/min	VI
			7L/min	V
Temperatura del gas de secado			250ºC	III
			150 ºC	VI
			250 ºC	V
Presión del gas nebulizador			60 psi	III
			10 psi	V, VI
Voltaje			4000 V	III
				V, VI
Rango m/z de barrido		150-350 con un tiempo de barrido de 0.75 s		III
		100-500 y sumatorio cada 4 barridos		VI
		100-500 con un tiempo de barrido de 0.75 s		V
Control de carga de iones en la trampa		100000 durante 50 ms		III
		20000 durante 50 ms		V, VI
Voltaje del fragmentador		70 V		V

* valor del flujo en el extremo del capilar, teniendo en cuenta el split ratio de 1:100

6.1 Análisis de plaguicidas

Los espectros de masas de los plaguicidas estudiados por EC-EM, se recogieron en modo positivo y en modo negativo, en modo de barrido completo desde m/z 150 a 350 con un tiempo de barrido de 0.75 s, o bien por monitorización selectiva de iones (SIM). Las moléculas $[M+H]^+$ se monitorizaron de m/z 202 para tiabendazol, m/z 239 para pirimicarb, m/z 295 para pirifenox, m/z 200 para pirimetanil, m/z 284 para procimidona, y m/z 240 para dinoseb con una ganancia de 3 y un tiempo de espera de 400 ms.

El EM-QIT se ajustó para cada compuesto, y se optimizó el voltaje de las lentes en el ExpertTune con un software Daltonic Esquire Control mediante inyección por infusión de una disolución patrón (1 mg mL^{-1}) preparada en el tampón de separación a flujo de 0.005 mL min^{-1}. Con el EM se trabajó en modo de barrido completo y en modo monitorización de reacciones múltiples (MRM).

Los parámetros de la trampa se seleccionaron en control de carga de iones utilizando una diana de 100000, el tiempo máximo de acumulación fue de 50 ms y una media de agitación establecida en 2.

La colisión inducida por disociación (CID) se llevó a cabo en el ion de interés por colisión con un gas (helio) presente en la trampa durante 40 ms. Los plaguicidas protonados o desprotonados se sometieron a CID dando lugar a un primer conjunto de iones EM/EM o EM^2; uno de estos iones fragmentados se aislaron y refragmentaron para dar lugar al siguiente conjunto de iones de EM^3 y así sucesivamente. Las etapas de fragmentación de cada compuesto se optimizaron observando los cambios en intensidad de los iones fragmentados, mientras que la fragmentación de rotura (cutoff) y el ancho de fragmentación se modificaron de forma manual. Los parámetros optimizados se detallan en la tabla 11.

Tabla 11. Tiempo de ventana y transiciones utilizadas para la cuantificación por EM^n

	Definición de las ventanas del tiempo (min)					
	0–20			20–24	24–26	26–35
	Tiabendazol	Pirifenox	Pirimicarb	Pirimetanil	Procimidona	Dinoseb
Transiciones MS^2 (m/z)	202 → 175	295 → 264	239 → 182	200 → 183	284 → 255	240 → 194
Width (m/z)	1.0	4.0	1.0	1.0	4.0	1.0
Cut-off (m/z)	100	100	100	100	100	100
Amplitud (V)	2.0	1.5	1.6	2.4	1.3	2.0
Transiciones MS^3 (m/z)	175 → 132	264 → 230	182 → 132	183 → 168	255 → 213	194 → 164
Width (m/z)	1.0	4.0	1.0	1.0	4.0	1.0
Cut-off (m/z)	100	100	100	100	100	100
Amplitud (V)	2.0	2.3	2.0	2.4	1.8	2.0

6.2 Análisis de quinolonas

Para el análisis de quinolonas se trabajó en modo positivo con fuente de electrospray (ESI), los espectros de masas se adquirieron en un HP-MSD en modo de barrido completo (de m/z 100 a 500 con un tiempo de barrido (scan time) de 0.75 s) o en modo SIM (moléculas de $[M+H]^+$ a m/z 358 para danofloxacina, m/z 362 para ofloxacina, m/z 360 para enrofloxacina, m/z 304 para ácido pipemídico y m/z 262 para flumequina) con ganancia de 3, voltaje del fragmentador de 70 V y tiempo de espera de 63 ms.

Los parámetros de la fuente de ESI para la trampa de iones (QIT) fueron los mismos que los de simple cuadrupolo, véase tabla 10. El detector y los voltajes block fueron: 1600 V, voltage dynodo, 7.0 kV; skimmer block 51.2 V; lentes bloque 1 2200.0 V, amplidtud del octopolo de RF 50 Vpp; block de partición 4.6 V, lentes del bloque 2 0V, salida del bloque del capilar 195 V. La parámetros optimizados se recogen en la tabla 12.

Tabla 12. Transiciones utilizadas para la cuantificación por EM^n. Las pérdidas neutras están indicadas entre paréntesis

	Quinolonas				
	Danofloxacina	Ofloxacina	Enrofloxacina	Ácido pipemídico	Flumequina
Transiciones EM^2 (m/z)	358 → 314 (CO_2)	362 → 318 (CO_2)	360 → 316 (CO_2)	304 → 216 (CO_2,C_2H_5N)	262 → 244 (HO_2)
Width (*m/z*)	4.0	4.0	4.0	4.0	4.0
Cut-off (*m/z*)	150	100	100	100	100
Amplitud (V)	4.0	2.0	1.5	1.0	2.0
Transiciones EM^3 (m/z)	314 → 294 (80%) (HF) 314 → 283 (100%) (H_2NCH)	318 → 261 (C_3H_7N)	316 → 245 ($C_2H_5NC_2H_5$)	216 → 189 (CO)	244 → 202 (C_3H_6)
Width (*m/z*)		4.0	4.0	4.0	4.0
Cut-off (*m/z*)		100	100	100	100
Amplitud (V)		1.2	1.3	1.8	1.3
Transiciones EM^4 (m/z)		261 → 218 (NC_2H_5)	245 → 205 (C_3H_4)		
Width (*m/z*)		4.0	4.0		
Cut-off (*m/z*)		100	100		
Amplitud (V)		2.0	1.8		
Transiciones EM^5 (m/z)			205 → 189 (CH_4)		
Width (*m/z*)			4.0		
Cut-off (*m/z*)			100		
Amplitud (V)			2.0		

6.3 Análisis de sulfonamidas (SAs)

Los parámetros de interés para el análisis de SAs por EC-EM, fueron los voltajes de las lentes y de los bloques, que se fijaron utilizando las mismas condiciones de la página de tune. El espectrómetro de masas trabajó en los modos de barrido completo y en monitorización de reacciones múltiples (MRM). En la tabla 13 se recoge el ion precursor y los iones producto para cada SAs estudiada.

Tabla 13. Iones producto e iones precursores obtenidos para las SAs estudiadas

Sulfonamida	Ion precursor seleccionado $[M+H]^+$ (m/z)	Iones del espectro de masas del ion producto (m/z)
Sulfasalazina	398	317
		156
		108
Sulfabenzamida	276	156
		108
Sulfadimetoxina	311	245
		218
		156
		108
Sulfacloropiridazina	285	285
		156
		108
Sulfametoxipiridazina	281	265
		247
		215
		188
		156
		126
		108
Sulfadiazina	251	251
		174
		156
		108
Sulfisoxazol	268	156
		113
		108
Sulfatiazol	256	256
		156
		108
Sulfadimidina	279	279
		156
		124
		108
Sulfapiridina	250	250
		184
		156
		108
Sulfaguanidina	215	215
		156
		108
Sulfanilamida	173	173
		156
		108

Validación y calibración para el análisis de SAs

La validación del método se llevo a cabo de acuerdo a las reglamentaciones establecidas por la UE [131]. Los valores de los límites de detección y de cuantificación para cada SAs se consideraron como la concentración para una señal de ruido de fondo de 3 a 10 respectivamente. Para la determinación de la linealidad y la precisión del método se adicionó a las muestras de carne triturada unas disoluciones de fortificación en metanol que contenía las 12 SAs a concentraciones de: 50, 75, 100, 200, 300 y 500 μg kg^{-1}.

Cada muestra se analizó por triplicado y las curvas de calibrado se obtuvieron representando área del pico frente a la concentración. La variación entre días (la desviación estándar relativa, DSR) se calculó con la pendiente de la recta de calibrado.

La precisión del método se determinó con las recuperaciones a niveles de MRL/2, MRL y 2 MRL. Estas recuperaciones se calcularon comparando el área de los picos obtenidos en las muestras de carne adicionadas antes y después de la preparación de las muestras. La precisión en los ensayos se determinó analizando por quintuplicado las muestras adicionadas a los tres niveles en un mismo día (con precisión o repetibilidad en el día) o en días diferentes (entre días precisos).

El límite de decisión (CCα) y la capacidad de detección (CCβ) se determinó por el proceso de la curva de calibrado [131]. Para determinar la concentración a niveles de LMRs (MRLs) CCα y CCβ se calcularon con una certeza estadística de 1-α (α = 0.05) y 1-β (β=0.05) respectivamente.

El criterio de confirmación utilizado para la identificación de los compuestos, fue el tiempo de retención de los picos del cromatograma para cada compuesto, las transiciones seleccionadas (una etapa específica de aislamiento y de fragmentación, con dos iones productos (al final) en el espectro de masas) y la abundancia entre los iones diferentes. La cuantificación se llevó a cabo por procedimientos estándar externos.

7. Procesos de extracción y de purificación

7.1. Extracción en fase sólida (EFS/SPE)

Consistió en hacer pasar la disolución de la matriz que contiene los analitos, a través de una columna que contiene 0.5 g de fase sólida C_{18} o una columna de C_{18} a un flujo de 2 ml min^{-1}. El filtrado se desecha y los analitos retenidos en la fase sólida se eluyen con un disolvente capaz de resorber los analitos retenidos. El eluyente se recoge en un tubo cónico graduado (20 ml) y se concentra llevándolo a sequedad a 50 ºC con una corriente de nitrógeno. Posteriormente se redisuelve en 0.5 ml de metanol o en el tampón de separación.

7.2. Extracción sobre barras magnéticas (ESBM/SBSE)

Una vez se ha filtrado el extracto de la muestra, se introduce en un vaso de precipitados de vidrio de 50 ml y se agita con una barra magnética recubierta de PDMS (polidimetilsiloxano), durante 2-4 h a 900 rpm. Antes de utilizar las barras, se acondicionan en un vial con 15 ml de de metanol, y se lleva a un baño de ultrasonidos durante 5 min. El disolvente se descarta y el procedimiento se repite tres veces. Las barras magnéticas se extraen de la muestra de la fase acuosa con la ayuda de una barra imantada.

A continuación los analitos se desorben en el interior de un vial de 2 ml que contiene 1 ml de metanol. La desorción de los plaguicidas se lleva acabo en un baño de ultrasonidos (sonicando) durante 15 min. La concentración de la muestra se realiza llevándola a sequedad con una corriente de nitrógeno. Posteriormente se redisuelve con 0.5 ml de tampón de separación.

7.3. Extracción presurizada con disolventes (EPD o PLE o ASE)

Las muestras trituradas se introducen en celdas de acero de 22 ml, se sellan en una de las partes finales con un filtro de microfibra de vidrio de 1.98 cm de diámetro con el fin de asegurar que las celdas quedan bien cerradas. Cualquier espacio libre en la celda una vez llena, se completa con tierra de diatomeas y se sella con filtros de microfibra de vidrio.

Las celdas se cargan en el equipo de extracción ASE 200 y se estudian los parámetros con los que el se realizará la extracción: tiempo de calentamiento de la celda, tiempo de contacto del disolvente con la muestra (conocido como static time), presión, temperatura, tiempo de purga con nitrógeno, (expulsar el resto de disolvente de la celda), el volumen de agua pasando respecto al tamaño de la celda en porcentaje, y número de ciclos. El volumen final se recoge y se concentra llevándolo a sequedad con una corriente de nitrógeno, redisolviendo posteriormente con 0.5 ml de metanol o BGE.

8. Procesamiento de las muestras

Las muestras de fresa, uva, lechuga y tomate se adquirieron de diferentes supermercados de la ciudad de Valencia (España). Las muestras de melocotones y nectarinas se cogieron de una cooperativa agrícola local.

En cuanto a las muestras de carne y pescado, fueron todas compradas en supermercados, pescaderías y carnicerías de Valencia

Tanto para muestras de frutas y verduras como de carne y pescado, se coge una cantidad representativa, se trituran en un procesador de alimentos y 100 g se guardan en contendores especiales en el congelador.

8.1 Fresa, uva, melocotón, nectarina, lechuga y tomates por EFS

La extracción de plaguicidas de fresa, uva, melocotón, nectarina, lechuga y tomates se llevó a cabo por EFS: una porción de muestra (5-15 g) adicionada o no con plaguicidas, se introdujo en un vaso de precipitados de 250 mL, Posteriormente se añadieron 10 mL de agua-acetona (50:50 v/v) y se mezclaron y homogeneizaron durante 15 min en un baño de ultrasonidos. La suspensión resultante se filtró a través de un embudo Büchner con un filtro de Albet 40 (Barcelona, Spain) y la capa filtrada se lavó dos veces con 20 mL de agua-acetona. El extracto se llevó a evaporación en un rotavapor para eliminar la acetona. Una vez acondicionada la fase sólida de C_{18}, con 10 mL de metanol y

10 mL de agua destilada, la fase acuosa resultante se hizo pasar a través de una columna de C_{18} a un flujo de 2 ml min^{-1}. Tras la extracción, los plaguicidas retenidos se eluyeron con 10 mL de diclorometano. Cuando se estudiaron los plaguicidas: carboxin, flutriafol, tebuconazol, bitertanol, pirimetanil y triadimefon en uva, se utilizó una mezcla de diclorometano-metanol (50:50 v/v).

El eluyente, en ambos casos, se recogió en un tubo cónico graduado (20 ml) y se concentró llevándolo a sequedad con nitrógeno. Tras esto se redisolvió con 0.5 ml del tampón de separación.

8.2 Uva por ESBM

La extracción de los plaguicidas en uva por ESBM, se llevó a cabo por agitación durante 2 h a 900 rpm, con una barra magnética de PDMS, en una disolución filtrada de uva. Se estudió la linealidad, porcentaje de recuperación y repetibilidad (precisión dentro de un día) por la adición de 50 µl de la disolución de trabajo en 5 g de muestra. La muestra adicionada estuvo en contacto durante 1 h antes de la extracción para permitir la distribución de los plaguicidas en la matriz (uva).

8.3. Carne de pollo y pescado

5 gramos de carne de pollo o pescado triturados, se pusieron en un tubo de centrífuga de 20 mL.

Las muestras de carne fortificadas se prepararon por adición del volumen correspondiente de una disolución patrón de quinolonas (de 10 a 100 mL). Las muestras adicionadas se dejaron durante 15 min a temperatura ambiente para asegurar la distribución adecuada de las quinolonas en la matriz.

A continuación, la muestra, se mezcló con 5 mL de NaH_2PO_4 (pH 7.0), se agitó durante 10 min y se extrajo dos veces consecutivas con 20 y 10 ml de diclorometano por agitación durante 5 min. Las fases orgánica y acuosa se separaron por centrifugación durante 5 min a 2500 rpm.

La fase orgánica se recogió en un tubo de centrífuga de 40 mL y se extrajo dos veces con 5 mL de 0.5 M de NaOH.

La fase acuosa, ya separada del diclorometano se transfirió a un vial y fue ajustado a un pH 7 con 200 mM H_3PO_4 y desengrasado por extracción con 10 mL de hexano. La fase acuosa se pasó a través de una columna de C_{18} previamente acondicionada con 10 mL de metanol y 10 ml de agua destilada, a un flujo de 2 mL min^{-1}. La elución de las quinolonas se llevó a cabo con 2 mL de 4% de TFA en agua-ACN (25:75 v/v), seguido de 1mL de ACN.

El eluyente se recogió en un tubo cónico gradual (10 mL) y concentrado hasta sequedad con una corriente de aire y posteriormente redisuelto con 0.5 mL de la disolución tampón de trabajo.

8.4. Carne de cerdo por EPD

Con el fin de validar el desarrollo de este método, se analizaron muestras de carne de cerdo, medicados con sulfadimidina. Las muestras positivas se obtuvieron de animales tratados con 300 mg de compuesto, alimentados durante 5 días consecutivos, con pienso contenía 100 g. Estas muestras se consiguieron de animales que se sacrificaron tras la medicación.

La extracción de agentes antibacterianos de carne se llevó a cabo mediante PLE. Para estudios de recuperación, se pesaron porciones de 10 g de carne de cerdo triturado en cápsulas de porcelana y se adicionaron con volúmenes variables de una disolución patrón de SAs (de 10 a 100 µl).

Las muestras adicionadas, se dejaron durante 15 min a temperatura ambiente con el fin de asegura la distribución adecuada de las SAs en la matriz. A continuación se mezcló con 10 g de tierra de diatomeas con la ayuda de una maza durante 5 min, hasta que se obtuvo una mezcla homogénea. Esta mezcla se introdujo en una celda de extracción de acero del equipo de ASE y se selló con

un microfiltro. Cualquier espacio que quedo vacío en la celda, se rellenó con tierra de diatomeas.

Los parámetros de extracción en el equipo fueron los siguientes: 8 min de calentamiento de la celda, 5 min de contacto del disolvente con la muestra, 1500 psi de presión, temperatura 160ºC, 80 s de purga con nitrógeno, el volumen de agua pasando respecto al tamaño de la celda en porcentaje fue de 100 %, y un único ciclo. El volumen final recogido al final del proceso de extracción fue de 40 mL.

Los extractos obtenidos en el PLE se hicieron pasar a través de la columna Oasis HLB a un flujo de 2 ml min^{-1} aproximadamente. Las columnas previamente se lavaron con 2 mL de agua y 2 mL de metanol. Las SAs se eluyeron de la fase sólida con 2 mL de metanol, se evaporó a sequedad en una corriente de nitrógeno a 55ºC y posteriormente se redisolvió con 1 ml de tampón de separación. El extracto se guardó a 4ºC hasta su inyección, a temperatura ambiente, en la EC-EM para el análisis.

Resultados y Discusión
Results and Discussion

I.- EVALUATION OF SOLID-PHASE EXTRACTION AND STIR-BAR SORPTIVE EXTRACTION FOR THE DETERMINATION OF FUNGICIDE RESIDUES AT LOW-µg kg^{-1} LEVELS IN GRAPES BY LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

Evaluación de la extracción en fase sólida y extracción sobre barras magnéticas para la determinación de residuos de fungicidas en uva, a niveles por debajo de µg kg^{-1} por cromatografía líquida-espectrometría de masas

I.1.-Summary method developed

A liquid chromatography–mass spectrometry method has been developed for determining bitertanol, carboxin, flutriafol, pyrimethanil, tebuconazole and triadimefon. The evaluation of both atmospheric pressure interfaces (API), atmospheric pressure chemical ionization (APCI) and electrospray (ESI) using positive and negative ionization modes was carried out. Two procedures based on solid-phase extraction (SPE) and stir-bar sorptive extraction (SBSE) have been assessed for extracting these compounds in grape.

LC-MS conditions, extraction and clean-up procedure has been described previously in materials and method section.

Time scheduled selected-ion monitoring (SIM) of the most abundant ions of each compound was performed as is reported in Table I.1 using the high resolution setting.

Table I.1 Time scheduled SIM conditions for monitoring pesticides

	Group	Time (min)	SIM ion	Gain	Fragmentor (V)	Dwell time (ms)
Carboxin	1	0–8	236	1	100	132
			143			132
Flutriafol	2		302	1	100	132
Pyrimethanil	3	8–14	200	1	100	132
Triadimefon	4		294	1	100	132
			197			132
Tebuconazole	5	14–30	308	1	60	98
Bitertanol	6		269	1	60	98
			338			98

I.2.- Mass spectrometry remarks

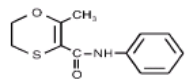
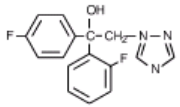
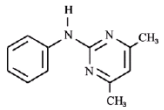
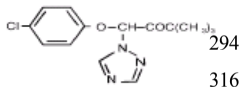
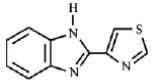
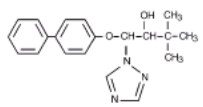
Although there are a lot of methods described now in the literature, established procedures for choosing the most sensitive interface or the best ionization mode do not exist.

Table I.2 summarizes the chemical structures, molecular weights, base peaks and the most abundant ions (with their relative abundance) of the mass spectra of the six studied fungicides using APCI and electrospray (ESI) interfaces in positive ionization (PI) and negative ionization (NI) modes. The studied compounds gave response in positive and negative mode by both interfaces, except that bitertanol did not give a signal in ESI

The ESI interface in PI mode provided mainly the protonated molecules and strong signal for sodium adducts. Only carboxin presents a fragment ion corresponding to the neutral loss of aniline. The sodium adduct is the main ion for flutriafol, triadimefon and tebuconazole whereas pyrimethanil did not form sodium adducts, confirming the theory that the sodium adduct formation requires a group that can donate a lone pair of electrons. Carboxin, flutriafol, triadimefon and tebuconazole contain carboxyl and hydroxyl groups that are absent in the pyrimethanil molecule.

The ESI interface in NI mode provided the deprotonated molecules as main ion for triadimefon and pyrimethanil. The main ion in the spectrum of flutriafol is the fragment obtained by the neutral loss of fluorobenzene, in the spectrum of carboxin the fragment obtained by the loss of ethene, and the only ion in the spectrum of tebuconazole is the fragment resulting from the loss of methyltriazole moiety.

Table I.2 Molecular and fragment ions and their relative abundance both API interface in NI and PI modes at voltage fragmentor 100V

Compound (M_W)	ES				APCI			
	Positive (PI)		Negative (NI)		Positive (PI)		Negative (NI)	
	m/z tentative ion	*R*(%)	*m/z tentative ion*	*R*(%)	*m/z tentative ion*	*R*(%)	*m/z tentative ion*	*R*(%)
Carboxina (235)	143 $[M + H - C_6H_6NH_2]^+$	25	206 $[M - H - CH_2CH_2]^-$	100	236 $[M + H]^+$	100	234 $[M - H]^-$	100
	236 $[M + H]^+$	100	234 $[M - H]^-$	50	143 $[M + H - C_6H_6NH_2]^+$	80	206 $[M - H - CH_2CH_2]^-$	20
	258 $[M + Na]^+$	25						
Flutriafol (301)								
	302 $[M + H]^+$	20	300 $[M - H]^-$	10	302 $[M + H]^+$	100	300 $[M - H]^-$	10
	324 $[M + Na]^+$	100	204 $[M - H - FC_6H_5]^-$	100	233 $[M + H - C_2HN_3]^+$	15	204 $[M - H - FC_6H_5]^-$	100
Pyrimethanil (199)								
	200 $[M + H]^+$	100	198 $[M - H]^-$	100	200 $[M + H]^+$	100	198 $[M - H]^-$	100
Triadimefon (293)								
	294 $[M + H]^+$	10	292 $[M - H]^-$	100	294 $[M + H]^+$	100	292 $[M - H]^-$	100
	316 $[M + Na]^+$	100			225 $[M + H - C_2HN_3]^+$	50	127 $[M - H - ClC_6H_4O]^-$	10
Tebuconazole (307)					197 $[M + H - C_2HN_3C_2H_4]^+$	50		
	308 $[M + H]^+$	25	223 $[M - H - C_3N_3H_5]^-$	100	308 $[M + H]^+$	100	306 $[M - H]^-$	25
Bitertanol (337)	330 $[M + Na]^+$	100					223 $[M - H - C_3N_3H_5]^-$	100
	–		–		338 $[M + H]^+$	20	169 $[C_{12}H_9O]^-$	100

The studied fungicides have similar mass spectra in APCI to those provided in ESI. However, in PI mode, there were no sodium adducts and some fragment ions can be obtained. Carboxin provides the fragment by neutral loss of aniline in higher proportion. Flutriafol, triadimefon and bitertanol commonly suffered the neutral loss of 69 u of the molecule that corresponds to the triazole ring. APCI in NI mode provided similar mass spectra for carboxin, flutriafol and tebuconazole to those obtained by ESI. Triadimefon provided a characteristic fragment ion at m/z 127 corresponding to the loss of p-chlorophenol, and bitertanol gave a fragment at m/z 169 that correspond to the p-phenylphenol. Fig. I.1 illustrates some examples of the mass fragmentation observed.

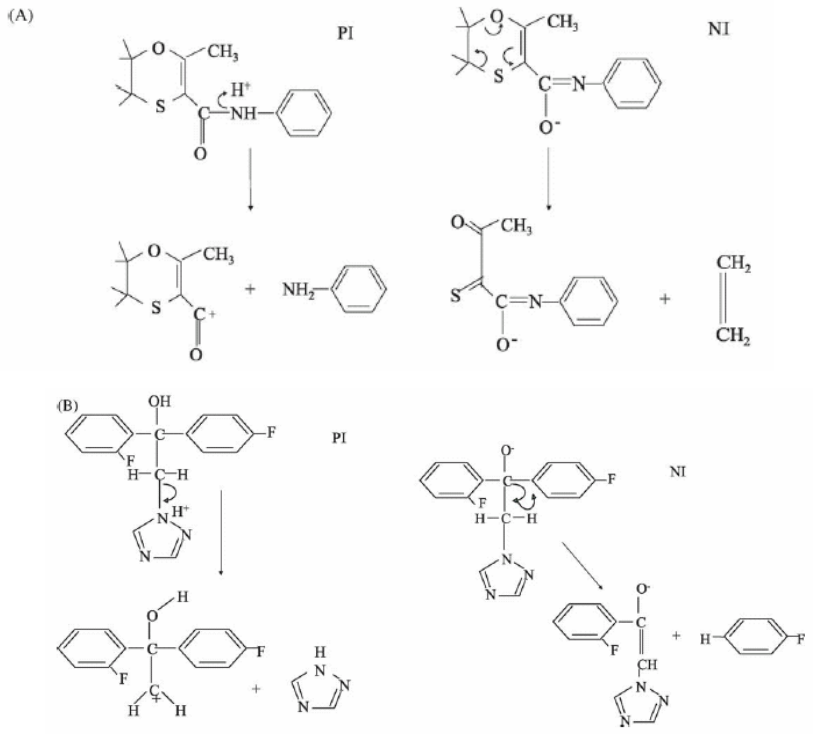


Fig. I.1. Mass fragmentation patterns observed in PI and NI modes for (A) carboxin and (B) flutriafol.

A summary of the results in terms of limits of detection (LODs) obtained using full scan mode is shown in Table I.3. The response varied from 250 pg for the six compounds in the APCI in PI mode, which are the most sensitive interface and mode, to no response in ESI for bitertanol. ESI was

Table I.3 Detection limits (ng injected) obtained using full-scan mode with both interfaces

	APCI		ESI	
	PI	NI	PI	NI
Carboxin	0.25	1	10	25
Flutriafol	0.25	0.25	10	25
Pyrimethanil	0.25	0.25	5	25
Triadimefon	0.25	0.25	10	125
Tebuconazole	0.25	0.25	5	25
Bitertanol	0.25	125	–	–

between 25 and 100 times less sensitive for the studied compounds than APCI. Taking into account these data, APCI in PI mode was chosen for further experiments. The system sensitivity was fully optimized using SIM. The time-schedule of SIM was performed following the procedure reported in Table I.1.

I.3.- Optimization of the extraction procedures

SPE and SBSE are significantly influenced by the aqueous volume selected, the amount of sample processed and the ionic strength of the medium. A set of experiments to determine the effect of these parameters in the recoveries of the studied compounds was designed. The elution step has already been widely studied in the literature for both techniques, and it is state that the best eluent for SPE is a mixture of dichloromethane–methanol since it provides highest recoveries and cleanest extracts, and that the best desorption of the compounds from the stir bar is accomplished sonicating 15 min with 0.5 ml of either methanol or acetonitrile [107-112].

Extraction efficiencies for a wide variety of compounds (depending on the polarity) can be improved increasing ionic strength since high ionic strength

reduces their water solubility. As SBSE provided recoveries below 90% for most of the studied fungicides, this effect was tested adding 10, 20 and 30% (w/w) of sodium chloride (NaCl). The recovery of all fungicides was increased in proportion to the amount of sodium chloride, and double recovery percentages are achieved using the highest amount of 30%, which was added to the samples processed by SBSE. The recoveries obtained by SPE (see the data presented below) do not require the salt addition.

Different water volumes (10–100 ml) were tested as it is shown in Table I.4. The recoveries obtained using SPE for these range of volumes are around 100% and almost independent of the aqueous volume passed through it, except for carboxin, the recovery of which is reduced to the half for volumes up to 50 ml, and for tebuconazole that is recovered on a 40% less when the sample volume is less than 25 ml. For SBSE, the results are quite different. The lower the sample volume is, the higher the recovery obtained. SBSE recoveries ranged from 42 to 98% using 10 ml of water, and are maintained for 25 ml of water, suffering an important decrease for higher volumes that lead to recoveries from 20 to 50% for 50 ml and from 5 to 25% for 100 ml. A water volume of 25 ml was selected for further experiments as a compromise to obtain appropriate sensitivity with a water volume that achieved the dissolution of an appropriate quantity of grape.

Table I.4 Influence of water volume on the extraction efficiency

Pesticide	SPE volume (ml)				SBSE volume (ml)			
	10	25	50	100	10	25	50	100
Carboxin	88	105	95	52	55	58	20	28
Flutriafol	87	100	95	102	65	63	30	11
Pyrimethanil	95	102	97	101	57	50	25	17
Triadimefon	96	99	93	114	97	97	50	29
Tebuconazole	60	111	102	111	60	53	26	11
Bitertanol	103	112	110	105	44	44	26	18

The influence of grape matrix on the extraction efficiency of SPE and SBSE was checked diluting different amounts of grape in 25 ml of water. Table I.5 illustrates the results in terms of recovery for SPE and SBSE. The matrix reduces the recovery obtained by SPE for carboxin, pyrimethanil and triadimefon whereas that obtained for flutriafol, tebuconazole and bitertanol are scarcely affected. Although the amount of grape tested (between 2 and 10 g) shows negligible effect on recovery, the variability of the results is greater with amounts up to 5 g.

Table I.5 Influence of grape amount on the extraction efficiency

Pesticide	SPE amount of matrix (g)				SBSE amount of matrix (g)			
	2	5	7	10	2	5	7	10
Carboxin	13	33	10	35	3	15	14	13
Flutriafol	95	100	33	93	80	45	9	14
Pyrimethanil	14	60	28	57	69	65	13	12
Triadimefon	79	72	65	72	100	105	26	9
Tebuconazole	68	98	99	95	92	51	31	13
Bitertanol	96	97	100	79	27	21	13	10

The effect of the grape matrix in the recoveries attained by SBSE presented a strong relation with the grape amount. Grape amounts of 2 g only reduced the recovery of carboxin and bitertanol respect to those obtained in pure water, grape amounts of 5 g also decreased the recovery of flutriafol, and higher grape amounts (from 7 to 10 g) were negative for all fungicides. The amount of 5 g of grape was used for the following experiments, since it provided acceptable recoveries and good sensitivity for all studied fungicides by both extraction techniques.

The pH of the spiked samples oscillated from 4.1 to 4.3. The pH of the unspiked grape matrix and that of the spiked grape matrix were controlled to ensure that are equivalent. Optimization of sample pH was not carried out because all the analytes are protonated at low pH because they contain basic

nitrogens, which enhanced their water solubility. These analytes are stables in aqueous solutions at slightly acid pH. The sample pH lower 4 can negatively affected the solid phase stability. Because of this, the sample pH was considered appropriate since it provides acceptable recoveries, which are comparable for flutriafol, tebuconazole and bitertanol to those obtained in the experiments performed with distilled water.

In addition, SBSE is an adsorption equilibrium and it is very influenced by the extraction time and temperature. Different extraction times were studied to obtain the sorption time profiles, which are presented in Fig. I.2. A 120 min extraction time was selected for SBSE to avoid unreasonable analysis time. Equilibrium was not reached for any of the studied pesticides. However, quantitative analysis can be carried out because the samples are extracted exactly the same time and analytical sensitivity is rather satisfactory.

In quantitative analysis one of the major problems is the suppression/enhancement of the analyte signal in presence of matrix components, which has been reported by many authors [113-115].

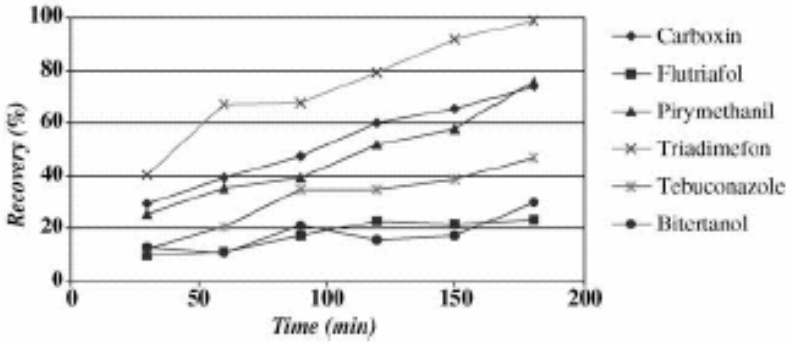


Fig. I.2 Effect of stirring time with the PDMS stir-bar on the recovery of the studied pesticides. Amount of each pesticide in solution: 50 ng.

Response suppression caused by sample matrix components using the ESI interface has been widely discussed in the literature [114, 115]. However, the information about the effects of this class of interferences on APCI interface is more conflicting.

This interference can be established comparing the signal intensity obtained in a standard solution (methanol) with those obtained in matrix matched standards. This was carried out for both procedures. Using SPE a slight enhancement of the response (ranging from 0 to 15%) depending on the compound was noted whereas using SBSE the response of the standard prepared in methanol and the standard prepared in matrix extract was the same. The absence of matrix effect using SBSE is an interesting characteristic of this technique that has already been reported in the literature [111].

The use of matrix-matched calibration standards was not necessary to compensate for signal enhancement of target analytes in matrix solution compared to their response in pure solvent since the enhancement is really low.

I.4.- Validation

Table I.6 shows the mean recovery and precision obtained by SPE from samples spiked at the limit of quantification (LOQ) levels and at around 10 times the LOQ levels. LOQs were calculated according to the European Union Guidelines as the lower concentration that provides repeatabilities lower than 20 %. Fungicide recoveries were between 60 and 100 % at the lowest concentration, and between 91 and 107 at the highest one. The relative standard deviations (RSDs) were from 7 % (bitertanol) to 17 % (carboxin) and from 4% (bitertanol) to 12 % (carboxin) for the lowest and highest concentration. The LOQ obtained, considering it as the lowest concentration for which the recovery and repeatability were acceptable, ranged from 3 to 10 μg kg^{-1}.

Table I.6 Recovery and RSD.s of the studied fungicides in grape samples spiked at LOQ and at 0.1 mg kg^{-1} (ca. LOQ × 10) obtained by SPE

Compound	Concentration (mg kg^{-1})	Recovery (%) ± RSD, n = 5	Concentration (mg kg^{-1})	Recovery (%) ± RSD, n =5
Carboxin	0.003	67 ± 17	0.1	91 ± 12
Flutriafol	0.005	100 ± 8	0.1	99 ± 6
Pyrimethanil	0.008	60 ± 10	0.1	107 ± 9
Triadimefon	0.01	71 ± 9	0.1	102 ± 5
Tebuconazole	0.005	98 ± 8	0.1	98 ± 5

Recovery only depends on concentration for three compounds carboxin, pyrimethanil and tebuconazole. The difference in recovery was only applied to the determination of the analyte concentration in the real samples when its concentration is close to the LOQ. Chromatograms of the SBSE–LC–MS analysis of an unspiked grape sample and grape sample spiked at 0.01 mg kg^{-1} of each compound are illustrated in Fig. I.3A and B.

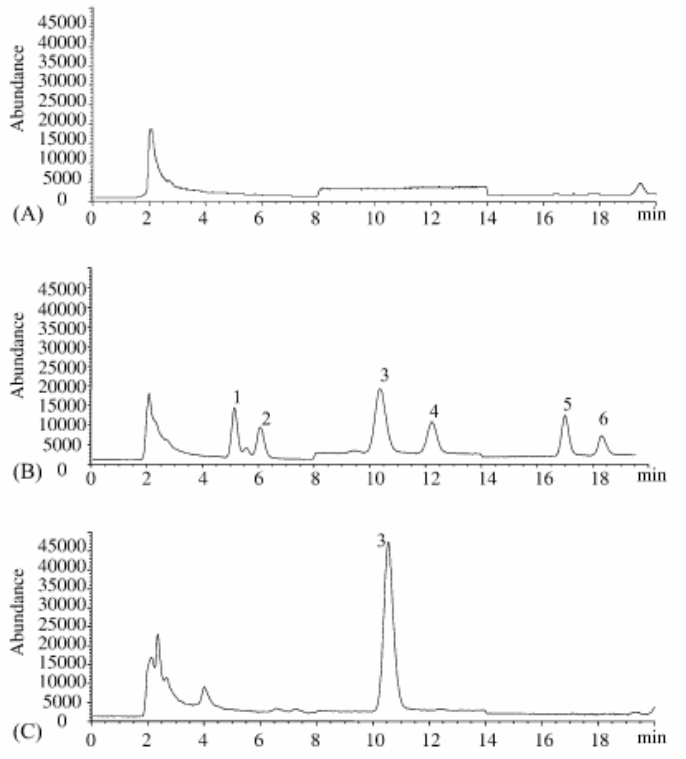


Fig. I.3 LC–MS chromatograms in SIM mode obtained after SPE of (A) untreated grape sample, (B) untreated grape sample spiked at 0.01 mg kg^{-1} of each compound and (C) grape sample that contains 0.05 mg kg^{-1} of pyrimethanil. Peak identification: (1) carboxin, (2) flutriafol, (3) pyrimethanil, (4) triadimefon, (5) tebuconazole and (6) bitertanol.

Table I.7 reports the same data but corresponding to the SBSE. The recoveries ranged from 15 % for carboxin to 100 %. for triadimefon and the RSDs from 10 % for triadimefon to 19 % for carboxin and bitertanol at the lowest concentration whereas at the higher one, the recoveries were between 17 % for carboxin to 101 %for triadimefon with RSDs < 17 %. The LOQs were 10 μg kg^{-1} for all studied fungicides.

Table I.7 Recovery and RSDs of the studied fungicides in grape samples spiked at LOQ and 10 times LOQ obtained by SBSE

Compound	Concentration (mg kg^{-1})	Recovery (%) ± RSD, *n* = 5	Concentration (mg kg^{-1})	Recovery (%) ± RSD, *n* =5
Carboxin	0.01	15 ± 19	0.1	17 ± 17
Flutriafol	0.01	45 ± 17	0.1	59 ± 16
Pyrimethanil	0.01	65 ± 16	0.1	73 ± 14
Triadimefon	0.01	100 ± 10	0.1	101 ± 8

Characteristic examples of LC–MS chromatograms of grapes spiked at LOQ level and non-spiked grapes samples are shown in Fig. I.4. It is remarkable the lack of interfering peaks and the low background noise compared with the chromatogram obtained by SPE. The comparison of both chromatograms also pointed out the higher sensitivity of SPE.

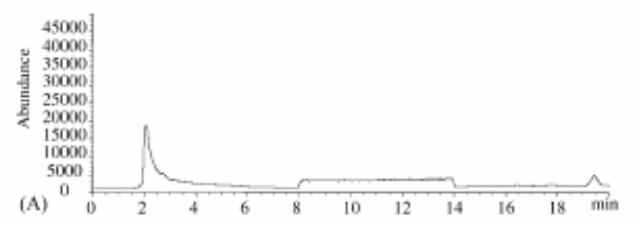


Fig. I.4 LC–MS chromatograms in SIM mode obtained after SBSE of (A) untreated grape sample

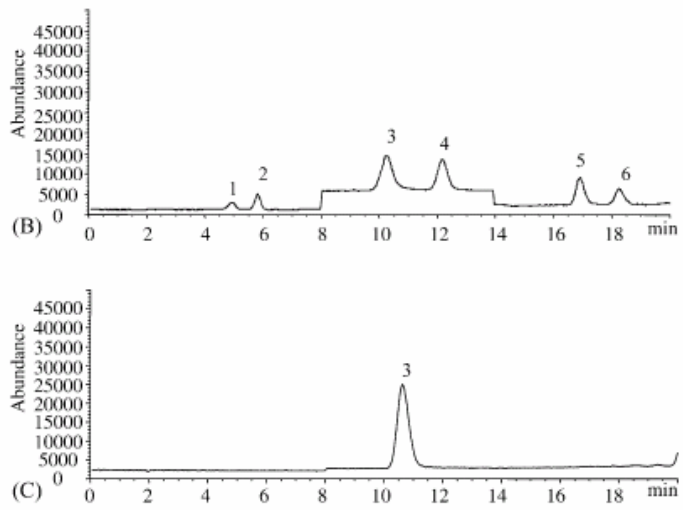


Fig. I.4 continued--LC–MS chromatograms in SIM mode obtained after SBSE of (B) untreated grape sample spiked at 0.01 mg kg^{-1} of each compound and (C) grape sample that contains 0.05 mg kg^{-1} of pyrimethanil.
Peak identification as in Fig. I.3.

Table I.8 compares the parameters indicative of the analytical performance of the two methodologies described. SPE provided LOQs slightly lower than those obtained by SBSE (three times as much), recoveries higher and RSDs lower than those obtained by SBSE. The low recoveries, higher RSDs and worse LOQs obtained by SBSE compared to those from the SPE can be explained because the SBSE is based on reaching adsorption equilibrium whereas SPE is a non-equilibrium process, based on partitioning between the aqueous extract of the sample and the solid-phase.

The linearity was evaluated at five concentrations, from the LOQ to 100 times the LOQ, showing correlation coefficients higher than 0.995 for SPE and 0.994 for SBSE. These coefficients (0.99) are relatively poor compared to conventional calibration techniques (0.999) because the extraction is included as it has been previously reported [112].

Other advantage of SPE is that is more rapid to perform that SBSE since it is not dependent on the sample equilibrium time. However, SBSE presents some advantages with respect to SPE as it reduces the organic solvent required,

Table I.8 Comparison of both methods features

	SPE	SBSE
Spiking concentration (mg kg^{-1})	0.01–1	0.01–1
Accuracy (% recovery)	67–106	15–100
Repeatibility (R.S.D., %)	4–17	8–19
Linearity (r^2)	>0.995	>0.994
Sensitivity (LOQ)	0.003–0.01	0.01
Organic solvent used to desorb analytes (ml)	10	0.5
Time required to process a sample (min)	90	150

provides cleanest chromatogram and less matrix interference effect (in spite that this effect can be considered negligible in both techniques).

LOQs obtained by both procedures were always lower than MRLs established by the EU [11], Codex Alimentarius Commission of FAO/WHO [8], Food and Drug Administration (FDA) from the USA [116] and Spanish legislation [117], which are in the interval of 0.05–2 mg kg^{-1} for bitertanol, 0.2 mg kg^{-1} for carboxin, 0.01 mg kg^{-1} for flutriafol, 5mg kg^{-1} for pyrimethanil, 2mg kg^{-1} for tebuconazole and 2mg kg^{-1} for triadimefon.

I.5.- Application

SPE and SBSE procedures were applied for determining these fungicides in 15 commercial grape samples from different markets (these samples include red and white grapes of different varieties). Only pyrimethanil was detected in one Muscat grape. This sample was extracted by triplicate and each replicate was injected twice. The mean concentration value and the standard deviation were 0.05 ± 0.002 mg kg^{-1} by SPE and 0.05 ± 0.003 mg kg^{-1} by SBSE. Fig. I.3C shows the chromatogram of the sample extracted by SPE and Fig. I.4C displays the chromatogram of the sample obtained by SBSE. Excellent conformity is obtained by both procedures.

II.- CAPILLARY ELECTROPHORESIS FOR ANALYZING PESTICIDES IN FRUITS AND VEGETABLES USING SOLID-PHASE EXTRACTION AND STIR-BAR SORPTIVE EXTRACTION

Análisis de plaguicidas en frutas y verduras utilizando extracción en fase sólida y extracción sobre barras magnéticas por electroforesis capilar

II.1.- Summary method developed

Two procedures based on solid-phase extraction (SPE) and stir-bar sorptive extraction (SBSE) in combination with micellar electrokinetic chromatography (MEKC)–diode array detection (DAD), conditions previously described, were compared for the simultaneous extraction of acrinathrin, bitertanol, cyproconazole, fludioxonil, flutriafol, myclobutanil, pyriproxyfen, and tebuconazole in lettuce, tomato, grape, and strawberry. Selectivity and resolution of the MEKC procedure were studied changing the pH and the molarity of the buffer, the type and the concentration of surfactant, and the methanol content in the mobile phase. A buffer consisting of 6 mM sodium tetraborate decahydrate with 75 mM of cholic acid sodium solution (pH 9.2) gave the best results. Advantages and disadvantages of SPE and SBSE procedures are also discussed.

II.2.- Optimization of the electrophoretic conditions

The optimization of the electrophoretic conditions is shown in Fig. II.1. The pH of the separation buffer was varied between 4 and 10. All buffers were 6 mM borate and 75 mM sodium cholate. The migration times decreased with pH (Fig. II.1A). The lower analysis time with appropriate resolution between analytes was at pH 10. Although the analysis time was higher, the pH 9.2 was selected as optimal because life-time of the capillary since higher pH degraded the silica inner wall of the capillary too quickly.

The influence of sodium tetraborate molarity on the pesticide separation was examined in the range 6–30 mM using a buffer 75 mM sodium cholate at pH 9.2 (Fig. II.1B). For all the analytes, the migration times were almost the same until 10 mM, then increased until 20mM maintaining again until 30 mM. Because of these results a sodium tetraborate concentration of 6 mM was selected.

Sodium dodecyl sulphate and sodium cholate were tested as surfactants. Peak resolution was much better using sodium cholate, which was selected for further experiments, this last surfactant achieved the differentiation of two isomers of cyproconazole, even through no chiral buffer is used. Different behavior was observed when the concentration of cholate was varied in the range 25–125 mM. Although the migration order of pesticide compounds with cholate concentration remains the same, the resolution varies considerably as cholate concentration increased from 25 to 75 mM. In addition, it can be see in Fig. II.1C that the migration time increased, when cholate concentration varied from 75 to 125 mM. This behaviour can be explained by both the increase of the ionic strength of the separation buffer and the greater interaction between analytes and micelles caused by the use of highest cholate concentrations. Therefore, the best separation was obtained with intermediate 75 mM cholate concentration in the buffer.

In order to find the best compromise between resolution and analysis speed, the effect of methanol concentration as organic modified was investigated, varying from 0 to 25 % (see Fig. II.1D). It was observed that increasing the percentage of methanol, the analysis time was increased and does not lead to real improvements in the resolution of the analytes. So, the use of organic modifier was avoided.

Table II.1 outlines some analytical parameters of the proposed method. The complete separation of the eight studied pesticides was obtained with resolution values >1. The repeatability and reproducibility of migration times were fairly high; the RSD values did not exceed 2.0 and 3.0 %, respectively. The repeatability and reproducibility of the peak area were worst; the RSD values for different analytes ranged from 2.1–5.3 and 2.9–5.8 %, respectively. The limits of detection (LODs), estimated as three times signal-to-noise ratio were 0.1 μgml^{-1} for bitertanol, fludioxonil, and pyriproxyfen, and 0.5 μgml^{-1} for the rest.

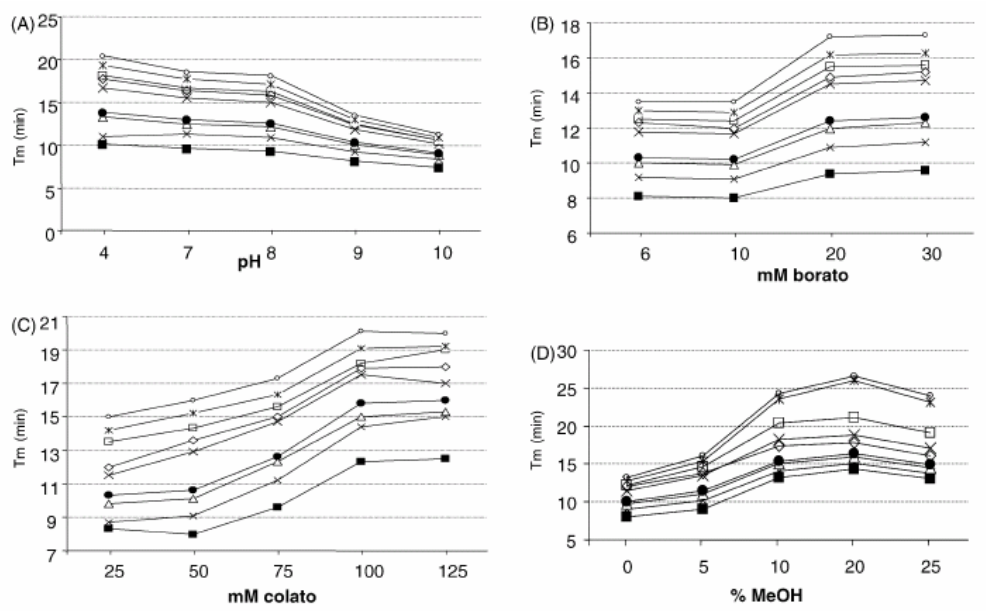


Fig. II.1. Variations of the migration time of pesticides as a function of (A) pH, (B) electrolyte concentration, (C) sodium cholate concentration, and (D) percentage of methanol. Capillary: 57 cm×50 µm i.d.; sample concentration: 10 µgml^{-1}; detection wavelength 214 nm; other operating conditions: 20 kV, 25 ºC. Curve identification: (■) flutriafol; (x) cyproconazole I; (Δ) cyproconazole II; (●) myclobutanil; (×) tebuconazole; (♦) acrinathrin; (□) bitertanol; (∗) fluodioxonil; (○) pyriproxyfen.

Table II.1 Analytical parameters of MEKC separation (R.S.D., %, n=5)

Pesticides	Resolution[a]	Average t_m (min)	Run-to-run R.S.D. (%)	Day-to-day R.S.D. (%)	Average area	Run-to-run R.S.D. (%)	Day-to-day R.S.D. (%)
Flutriafol	0	7.89	1.9	2.3	9,272	2.3	3.8
Cyproconazole I	5.3	9.01	1.6	2.1	3,382	3.4	2.9
Cyproconazole II	4.0	9.82	2.0	2.3	3,597	2.2	3.5
Myclobutanil	1.6	10.14	2.0	2.5	6,351	5.0	5.1
Tebuconazole	6.5	11.67	1.6	2.6	7,016	2.1	2.8
Acrinathrin	1.7	12.08	1.7	2.6	2,174	4.3	4.7
Bitertanol	1.1	12.37	1.5	2.6	20,857	4.6	5.0
Fludioxonil	1.7	12.92	1.6	2.9	25,626	3.3	4.3
Pyriproxifen	1.6	13.46	1.6	3.0	14,478	5.3	5.8

[a] Resolution was calculated using the equation: $R_s = 2(t_1 - t_2)/(w_1 + w_2)$, where t_1 and t_2 are the migration times and w_1 and w_2 are the widths of the peak at base line.

II.3.- SPE procedure

Fruit and vegetable samples are matrices that do not allow direct SPE of pesticides. They must be extracted with polar solvents to have the pesticides in an aqueous extract. Acetone was selected as extraction solvent because it is easy of evaporating and avoids losses of pesticides.

The influence of the amount of sample was also examined to obtain the smallest LOQs. Different sample sizes were tested from 5 to 15 g. Fig. II.2 shows the effect of the sample amount on pesticide recoveries for the four matrices tested. Two different behaviors were observed. The amount of lettuce and tomato samples could be increase to 15 g without important variations in recovery values and with RSDs within the guidelines of the EU (<20 %). On the contrary, the amount of grape and strawberry samples presented important influence on the recoveries because of the high viscosity of the extract. The maximum amount of these matrices that could be processed was 5 g to can recover all the analytes with RSDs within the guidelines of the EU (see Fig. II.2C and D). When the sample amount was increased the recoveries for most compounds diminished and RSDs increased to unacceptable values

In this study, LOQs were determined according to the guidelines of EU [11], as the lowest concentration for which acceptable recoveries (>50 %) and repeatabilities (<20 %) are obtained. Table II.2 presents LOQs lower than 0.5 mg kg^{-1}, satisfying the EU [11], Codex Alimentarius [8], US Food and Drug Administration [116], and Spanish [117] MRLs. For lettuce and tomato samples, LOQs can be diminished until 0.2 mg kg^{-1} working with 15 g of samples instead of 5 g. Considering these results, the method is adequate to determine the studied pesticides in grapes, lettuce, strawberries, and tomatoes. Table II.2 shows also the recoveries and precision obtained from spiked samples at LOQ levels. Average recoveries were between 40 and 106 % and RSD.s ranged from 10 to 19 %. Recoveries were similar in any of the matrix studied. Higher

concentrations were also tested (data not shown) providing results in the same interval.

Table II.2 Accuracy and precision at LOQ (amount of sample processed 5 g) SPE and MRLs established by the Spanish legislation [1]

Pesticide	Concentration (mg kg^{-1})	Recovery, % (R.S.D., %, n =5)				*MRL*
		Lettuce	Tomato	Grape	Strawberry	
Flutriafol	0.5	62 (17)	60 (14)	57 (8)	54 (17)	0.5–2
Cyproconazole I	0.5	58 (11)	53 (13)	55 (12)	58 (14)	0.05–1
Cyproconazole II	0.5	60 (16)	55 (12)	57 (12)	31 (18)	0.05–1
Myclobutanil	0.5	56 (13)	52 (14)	51 (17)	53 (15)	0.02–1
Tebuconazole	0.5	60 (17)	63 (13)	66 (14)	59 (15)	1–5
Acrinathrin	0.5	66 (12)	62 (17)	63 (14)	63 (19)	0.2–1
Bitertanol	0.5	49 (15)	40 (13)	44 (16)	47 (13)	2–3
Fludioxonil	0.5	50 (10)	46 (17)	57 (13)	47 (19)	1–2
Pyriproxyfen	0.5	94 (15)	97 (17)	106 (9)	95 (18)	0.05–1

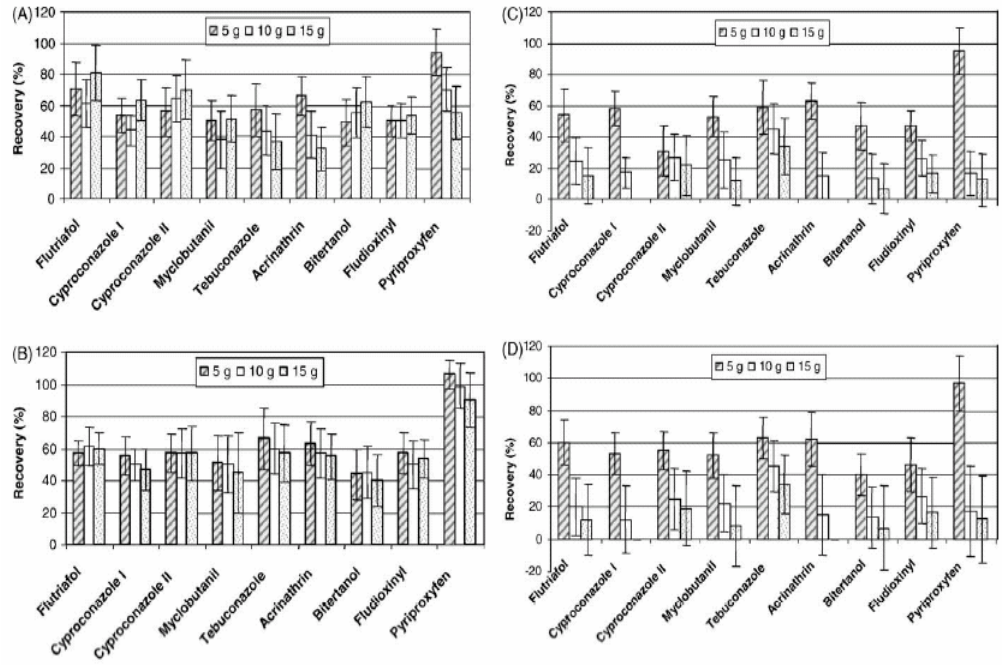


Fig. II.2. Effect of sample amount on pesticide recoveries and R.S.D.s by SPE in (A) lettuce, (B) tomato, (C) strawberry, and (D) grape.

The good performance of the electropherograms obtained from SPE extracts using 15 g of sample is illustrated in Fig. II.3. Unspiked samples do not

show peaks from the matrix that can interfered with the studied compounds. The lettuce sample presents pyriproxyfen (Fig. II.3B) as it was confirmed by liquid chromatography–mass spectrometry (LC–MS) using a HP 1100 system equipped with a APCI interface, using the standard source conditions, in positive ionization (PI) mode. The analysis was carried out in selected ion monitoring mode (SIM) selecting three characteristic ions of pyriproxyfen at m/z 322, 227, and 185 and using a fragmentor voltage of 100V, according to a method previously reported [111]. The identification and further confirmation of this pesticide showed the suitability of the method to determine these residues in real samples and the difficulty to find control samples without residues.

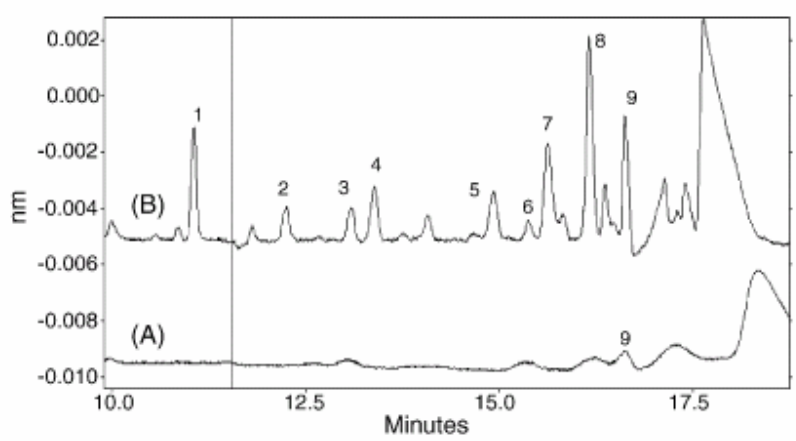


Fig. II.3 Electropherograms of SPE extracts from 15 g sample of (A) lettuce that contains pyriproxyfen at 0.2 mg kg^{-1} sample, and (B) lettuce sample spiked with the pesticides at 0.5 mg kg^{-1} levels. Peak identification: 1, flutriafol; 2, cyproconazole I; 3, cyproconazole II; 4, myclobutanil; 5, tebuconazole; 6, acrinathrin; 7, bitertanol; 8, fludioxonil; 9, pyriproxyfen.

II.4.- SBSE procedure

The optimum SBSE desorption conditions were obtained by exposure of the stir bar to 1ml of methanol for 15 min using a 1.5 ml volume glass vial; this volume enables the stir-bar to be completely immersed in the solvent. Those parameters were selected not only due to the higher quantitative desorption

obtained, but also because of the minimum carryover observed under this experimental conditions.

The matrix pH effect on the extraction efficiency of the pesticides was tested using pH values between 4 and 9 adjusted with potassium phosphate buffers. This range of pH values does not have influence in the extraction efficiency, and all subsequent analyses were performed without pH modification.

As expected, the addition of NaCl to the samples had a significant influence on the amount of pesticides adsorbed on the stir-bar. The addition of salt to the matrix altering the ionic strength and consequently decreasing the solubility of the pesticides in solution, can favour adsorption onto the stir-bar. The effect of NaCl concentration was investigated in the range 10–40 %. The saturated solution (40 % of NaCl) provided the best recoveries.

Fig. II.4 shows the time profile of the extraction for the pesticides. The extraction equilibrium time was reached at 4 h for all studied pesticides. So far, it has been reported that the shorter extraction, which could be of the order of minutes, is one of the advantages of the SBSE [113, 118]. However, for the studied pesticides, extraction times of over 240 min were required, enlarging the analysis more than practical convenience.

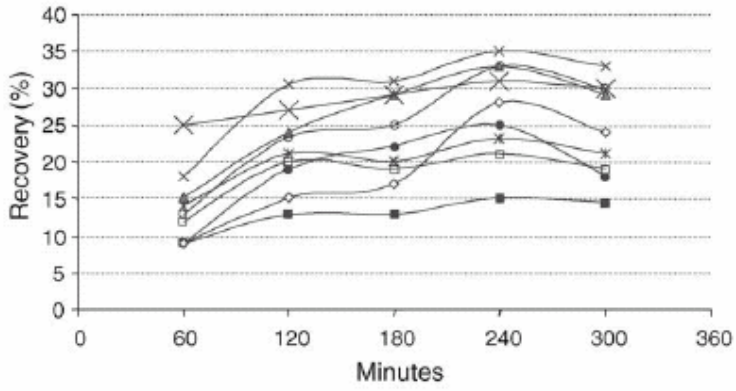


Fig. II.4 Effect of the extraction time on the recovery (each solution was added of 40% NaCl).Curve identification as in Fig. II.2.

The influence of the matrix on the extraction efficiency of the SBSE was negative for all compounds. A sample size of 5 g provided acceptable recoveries whereas higher amounts avoid the extraction of the flutriafol and cyproconazole. The quantity of matrix that can be used (no more than 5 g) is one limitation of this procedure.

Specificity of the method is demonstrated by representative electropherograms of tomato sample in Fig. II.5. Blank tomato sample showed no significant interference at the retention times of the analytes.

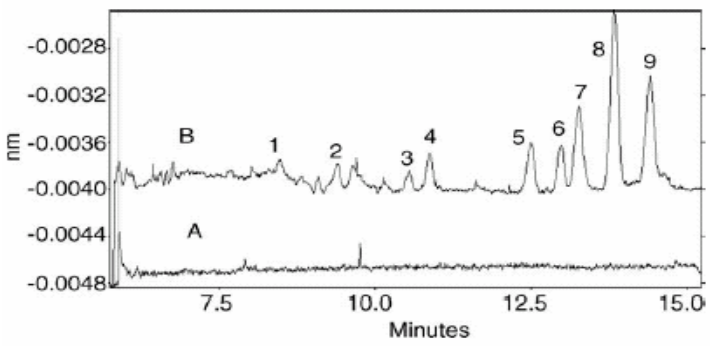


Fig. II.5. Electropherograms of SBSE extracts of (A) tomato blank sample, and (B) tomato sample spiked at 1mg kg^{-1} of each pesticide. Peak identification as in Fig. 4

The linearity was determined using spiked samples between 1 and 100 mg kg^{-1}. The evaluated interval was linear with correlation coefficients higher than 0.996. The precision and the recovery of the studied pesticides at LOQ levels for all the matrices tested are outlined in Table II.3. The recoveries obtained were between 12 and 47 % and the RSDs ranged from 3 to 17 %. The LOQ was 1 mg kg^{-1} for all the studied pesticides.

Table II.3 Accuracy and precision at LOQ (amount of sample processed 5 g) by SBSE

Pesticide	Concentration (mg kg^{-1})	Recovery, % (RSD, %, *n* =5)			
		Lettuce	Tomato	Grape	Strawberry
Flutriafol	1	12 (14)	13.4 (8)	17 (7)	14.6 (16)
Cyproconazole I	1	25 (9)	30.5 (3)	28 (5)	24 (17)
Cyproconazole II	1	29 (10)	33 (3)	34 (15)	31 (3)
Myclobutanil	1	20 (8)	24 (16)	26 (9)	19 (13)
Tebuconazole	1	32 (5)	35 (11)	25 (4)	31 (8)
Acrinathrin	1	47 (4)	45 (3)	43 (6)	41 (9)
Bitertanol	1	26 (13)	22 (4)	24 (4)	21 (4)
Fludioxonil	1	22 (15)	24 (12)	27 (12)	23 (11)
Pyriproxyfen	1	24 (9)	23 (6)	32 (8)	33 (12)

II.5.- Comparison of methods

Analytical parameters of both procedures are compared in Table II.4. Although SBSE extraction is less reliable than SPE to carry out the routine analyses, this procedure also presents some advantages over SPE such as better precision and cleaner electropherograms (as can be deduced from comparing Table II.4 and Figs. II.3 and II.5).

Table II.4 Methods performance comparison

	SPE	SBSE
Spiking concetrations (mg kg^{-1})	0.2–50	1–50
Accuracy (% recovery)	40–106	12–47
Repeatability (RSD, %)	8–19	3–17
Linearity (r^2)	>0.994	>0.996
Sensitivity (LOQ)	0.3–0.5	1
Analysis time (h)	1.5	5

SPE recoveries are, at least, twice higher than those obtained by SBSE. In addition, SPE showed better LOQs (twice) that can be improved for some matrices processing larger amounts of samples. The LOQs obtained mark the difference between both procedures because SPE combined with MEKC–UV achieves the detection of the studied pesticides at levels lower than MRLs established by the Spanish and EU legislations. Unfortunately, LOQs obtained by SBSE are too high to meet the MRLs. Because of this, the procedure is only useful in some cases. Other advantage of SPE, that consolidates it as that of

choice, is speediness (the complete analysis of each sample to obtained results are ca. 1.30 h in front of 5 h required by SBSE). Disadvantages described in the literature for the SPE such as breakthrough and plugging [56, 65] have not been observed in the present study, after optimization of the conditions.

III.- QUANTITATIVE ANALYSIS OF SIX PESTICIDES IN FRUITS BY CAPILLARY ELECTROPHORESIS-ELECTROSPRAY-MASS SPECTROMETRY

Análisis cuantitativo de seis plaguicidas en frutas por electroforesis capilar-electrospray-espectrometría de masas

III.1.- Summary method developed

A method to identify and quantify six pesticide residues – dinoseb, pirimicarb, procymidone, pyrifenox, pyrimethanil and thiabendazole – in peaches and nectarines using capillary electrophoresis-electrospray ionization-quadrupole ion trap-tandem mass spectrometry (CE-ESI-MS/MS) is described. Separation was carried out using a buffer of 0.3 M ammonium acetate at pH 4 with 10% methanol. Pesticide residues present in peach and nectarine samples were preconcentrated by solid-phase extraction using C_{18}, eluted with DCM, concentrated to dryness, and redissolved in buffer to obtain lower detection limits.

III.2.- CE-ESI-MS and CE-ESI-MS/MS optimization

The pH and ionic strength of the background electrolyte, the addition of organic modifiers, and the capillary temperature were evaluated using CE-UV to obtain the best separation of the six pesticides. A volatile buffer of low conductivity is required to avoid plugging of the dielectric capillary between the spray chamber and the mass spectrometer, as well as to obtain a stable electrospray. Ammonium acetate/acetic acid buffer was tested at four different pHs, 3, 4, 5, and 7. As CE is mainly governed by the charge/mass ratio of each analyte, the increase in pH decreases the migration time. The best arrangement between peak resolution and analysis time was obtained using pH 4.

The influence of buffer concentration on the migration time and resolution of the six compounds was studied with ammonium acetate-acetic acid buffers from 0.01 to 0.6 M at pH 4. An increment in the ionic strength of the carried electrolyte by increasing the buffer concentration reduced the adsorption of the compounds on the wall of the capillary and improved the peak shapes. The studied pesticides were resolved only with buffer of high molarity (> 0.2 M). The molarity of 0.3 M was selected to achieve a good compromise between compound separation and electrical conductivity of the buffer. The capillary current obtained using CE-UV was 180 mA (300 μA is the maximum current value suitable for the instrument) and coupling CE-MS was 87 μA.

The effect of adding an organic modifier to the background electrolyte on pesticide separation was checked using different concentrations of methanol and acetonitrile, in the range of 5–30 %. Organic solvents decrease the EOF prolonging the migration time, and can produce solvation effects, alteration of pKa, and improvement in the solubility of hydrophobic analytes. Methanol provided better separations than acetonitrile. The increment of methanol percentage increased the resolution between peaks from 5 to 15 % although the analysis times also increased. Higher percentage of methanol did not change the

resolution but enlarged the analysis time because longer migration times result in wide peaks. 10 % methanol improved the resolution between peaks without prolonging unreasonably the analysis time.

The migration time of the analytes decreased with an increase of the capillary temperature (15ºC–35ºC) because of the decrease in the distribution coefficient as well as of the buffer viscosity. The resolution between the peaks did not improve with low temperatures (< 25ºC) because the absolute rise of the temperature of the buffer is not detrimental to a separation if the Joule heating that provides wider peaks is not excessive. Temperatures of 30ºC–35ºC reduced resolution (especially between the last two peaks) because the Joule effect is higher, causes viscosity differences in the buffer, and gives rise to zone deformation. A capillary temperature of 25ºC was selected.

The ES-MS instrumental parameters, such as sheath liquid composition, sheath liquid flow-rate, drying gas flow-rate, and length of the CE capillary protruding from the sheath liquid tube, were optimized to obtain the higher response. The composition of the sheath liquid is critical to the fulfilment of the CE-MS system since it closes the electrical circuit between the CE and the electrospray source. The effect of the methanol percentage and of the buffer concentration was tested. The best results were obtained when the composition of the sheath liquid was the same as that of the running buffer. These results are in agreement with those already reported in the literature for similar connections [58, 66]. The sheath liquid flow-rate was varied from 3 to 15 mL min^{-1}. A flow-rate of 3 mL min^{-1} affected negatively the spray stability resulting in a low signal intensity, whereas the higher flow-rates tested did not provide higher peak areas, and the peak shape of procymidone was worst (peak-broadening is observed). A flow-rate of 5 mL min^{-1} provided the best results. In addition, a maximum response of the analytes was obtained when 7 L min^{-1} drying gas was used. At flow-rates lower or higher, the response decreased because of the

electrospray instability. The distance that the CE capillary protrudes from the electrospray needle was also optimized because it may seriously affect the performance of the system. This distance was varied between 0.05 and 1.5 mm. Maximum response was achieved when the CE capillary only protrudes 0.1 mm from the sheath liquid tube.

Figure III.1 shows the UV and MS electropherograms for the six pesticides under the best conditions. Successful coupling of CE with ESI-MS was accomplished for the separation of the six pesticides, and provided very similar separations compared to those achieved by CE-UV. The data reported in the published literature are quite polemic; some authors stated that separation efficiencies obtained by CE-ESI-MS are clearly lower than those achieved by CE-UV [57, 58, 66, 69] whereas others established that the separation efficiencies are not necessarily inferior compared with those obtained using CE-UV [119].

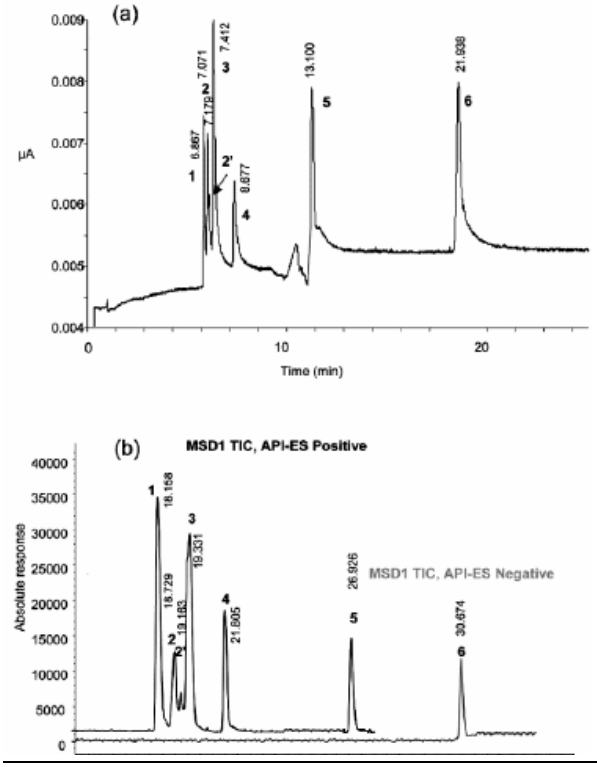


Fig. III.1. Electropherograms of a pesticide standard mixture by (A) CE-UV and (B) CE-ESI-MS using a single quadrupole. Conditions: capillary, fused silica (50 cm thermostated length, 75 mm ID); electrolyte, 0.3 M ammonium acetate/acetic acid with 10% methanol; voltage, 30 kV; injection, 5 s at 50 mbar; temperature, 25ºC; MS detection, in SIM mode. Concentrations: 25 and 5 mg mL^{-1} of each for CE-UV and CE-MS, respectively; other conditions see in Section 2. Peak identification: (1) thiabendazole; (2,2') pyrifenox; (3) pirimicarb; (4) pyrimethanil; (5) procymidone; (6) dinoseb.

The explanation of this contradiction could be that the coolant tube connector where the capillary is installed in the EDA cartridge, which has only 15 cm, has been changed in the present study by one of the same longitude than that used in the conventional cartridge. This confirms the general justification that the loss of resolution in the separation when an MS detector is used is caused by the small portion of the capillary thermostated. The tailing of the peaks observed using a UV detector is mainly because the capillary is older than that used with the MS detector, which originates in stronger adsorption on the wall silica capillary, producing tailing peaks.

Differences in migration times are because of the distinct interfacing between CE-UV and CE-MS. The capillary used for CE-UV has only 57 cm (50 cm effective length) whereas using an external mass spectrometer detector commonly requires considerably longer capillaries with an overall length of 75 cm (even through only 50 cm are thermostated). As the mobility of an ion is proportional to the applied field strength, the latter is inversely proportional to the capillary length, which means that the migration time will roughly double if the capillary is double.

However, there are several other factors that may interfere and alter the separation efficiency and migration times as well, such as moving ionic boundaries, buffer depletion (towards the end of the CE capillary in a hot ESI source), penetration of the sheath liquid by diffusion into the CE capillary, electrode reaction at the ESI needle which may result in pH modification, suction effect induced by the sheath gas flow used in addition to the sheath liquid flow, and alteration of electrosmotic flow (EOF) by the radial electric field which penetrates across the fused-silica capillary wall. Additionally, the separation efficiency may also be compromised by the data acquisition speed, the response time of the mass spectrometer, the liquid sheath flow rate, and the relative positioning of the CE capillary and the ESI needle [120].

The backpressure effect (siphoning of the sample) is caused by the pressure difference between the inlet and the outlet (commonly, the outlet can present a slight overpressure caused by the parameters of the vent system, e.g., drying gas) [120]. The overpressure is detected by a delay in retention time and analyte signal loss. The solution is kept at both capillary ends at approximately the same height, that is ± 0.5 cm around the sprayer's levels using a heath adjustable table placed under the CE instrument. However, typically this position was reach placing both instruments in the same bench without more adjustment. The appropriate values of RSDs obtained for retention time and peaks areas are a clear evaluation of the absence of backpressure (Table III.1). The coupling of CE to the QIT-MS was performed in the same way because the electrospray sprayer of the Agilent system has the same design than that of the Bruker Daltonik system without any important difference in the mechanism of electrical potential application.

Table III.1. Reproducibility, linearity, and sensitivity of pesticide detection by CE-UV and CE-MS using the single quadrupole.

	CE-UV				CE-MS			
	RSD (n = 10) (%)		Linearity correlation	LOD (mg/mL)	RSD (n = 10) (%)		Linearity correlation	LOD (mg/mL)
	Migration time	Peak area			Migration Time	Peak area		
Thiabendazole	1.2	3.9	0.998	2	1.5	5.0	0.998	0.05
Pyrifenox	1.3	5.0	0.996	2	1.4	6.2	0.992	0.5
Pirimicarb	1.3	4.5	0.994	1	1.7	8.7	0.992	0.05
Pyrimethanil	1.4	4.7	0.990	2	1.6	7.9	0.995	0.2
Procymidone	1.6	5.2	0.995	1	2.3	6.5	0.993	0.2
Dinoseb	2.7	5.8	0.992	1	3.0	8.9	0.990	0.5

III.3.- MS and MS/MS spectrum of pesticides

The only ion observed was the protonated $[M+H]^+$ or deprotonated $[M-H]^-$ molecules in full-scan MS mode, which is consistent with the "soft" nature of the ESI process. The voltages of the lenses were adjusted to obtain the

maximum response for the protonated or deprotonated molecule of each studied compound, which were chosen as precursor ions in further MS^n experiments. In addition, a reliable way of obtaining structural information is to perform tandem MS experiments on specific ions of interest. CID of $[M-H]^-$ or $[M+H]^+$ ions (MS^2) and CID of the main fragment ion (MS^3) often yield fragments ions formed by fragmentation of the lateral chains in the molecular structure or by the opening of the heterocyclic rings.

A three-stage mass analysis of the protonated thiabendazole at m/z 202 illustrates a dominant fragmentation pathway: m/z 202 → m/z 175 → m/z 131. MS^2 of thiabendazole is characterized by the loss of HCN from the thiazolic ring. It results in an abundant product ion at m/z 175, and a further loss of CS from the fragmentation of thiazolic ring leads to the little abundant ion of m/z 131.Further fragmentation of m/z 175 produces an abundant m/z 131 ion that confirms this fragmentation pathway. This fragmentation is almost the same as reported for LC-MS using the ion trap [121]. The MS^2 spectrum of $[M+H]^+$ for pyrifenox evidences the cleavage of the oxime bond, resulting in an intense signal at m/z 265 that corresponds to $[M-H+CH_3OH]^+$. Further fragmentation of this ion was verified by MS^3, showing an intense signal at m/z 230 formed by the loss of HCl molecule. Pirimicarb is a pesticide representative of the class of carbamates. Its MS^2 spectrum presents two main product ions at m/z 182 and 195, derived from the neutral loss of the CH_3NCO and $HN(CH_3)_2$ groups, respectively. In the further step (MS^3 of the ion at m/z 182), one product ion is formed at m/z 137 resulting from the loss of the group $HN(CH_3)_2$ located in the m-position at the carbamic group. The fragmentation of the protonated molecule of pyrimethanil exhibits major ions at m/z 184, formed through the loss of methane. MS^3 of m/z 184 leads to the formation of m/z 167 via the loss of the other methane group.

Figure III.2 illustrates the MS, MS^2, and MS^3 spectra for procymidone and dinoseb standards, obtained by CE-ESI-MS in full scan and CE-ESI-MS^n with the product ion scanning. These require the most complicated interpretation. Procymidone gave in the MS^2 a mass spectrum with a specific ion at m/z 256 by the neutral loss of CO. Its MS^3 analysis provides seven main fragment ions at m/z 162, 178, 186, 198, 212, 228, and 240 that are tentatively identified in

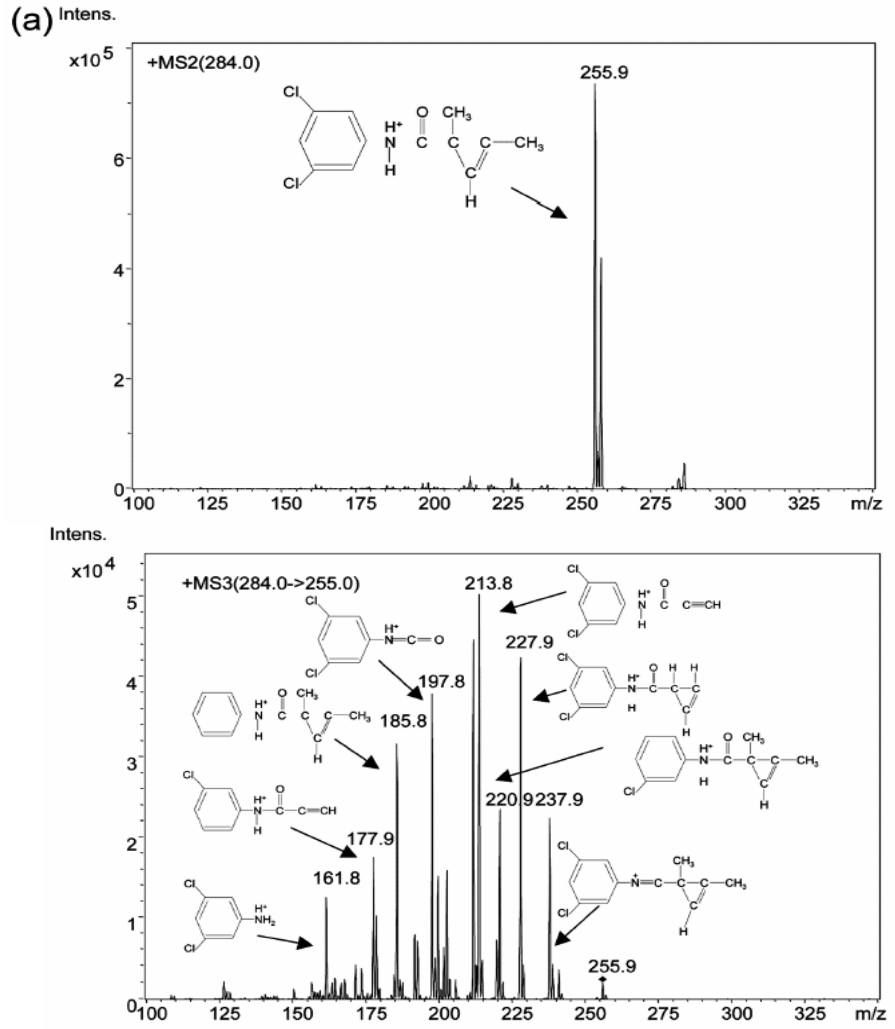


Fig. III.2. MS^2 and MS^3 mass spectra of (A) procymidone and (B, next page) dinoseb. The proposed fragmentation is displayed.

Fig, III.2. Continued.

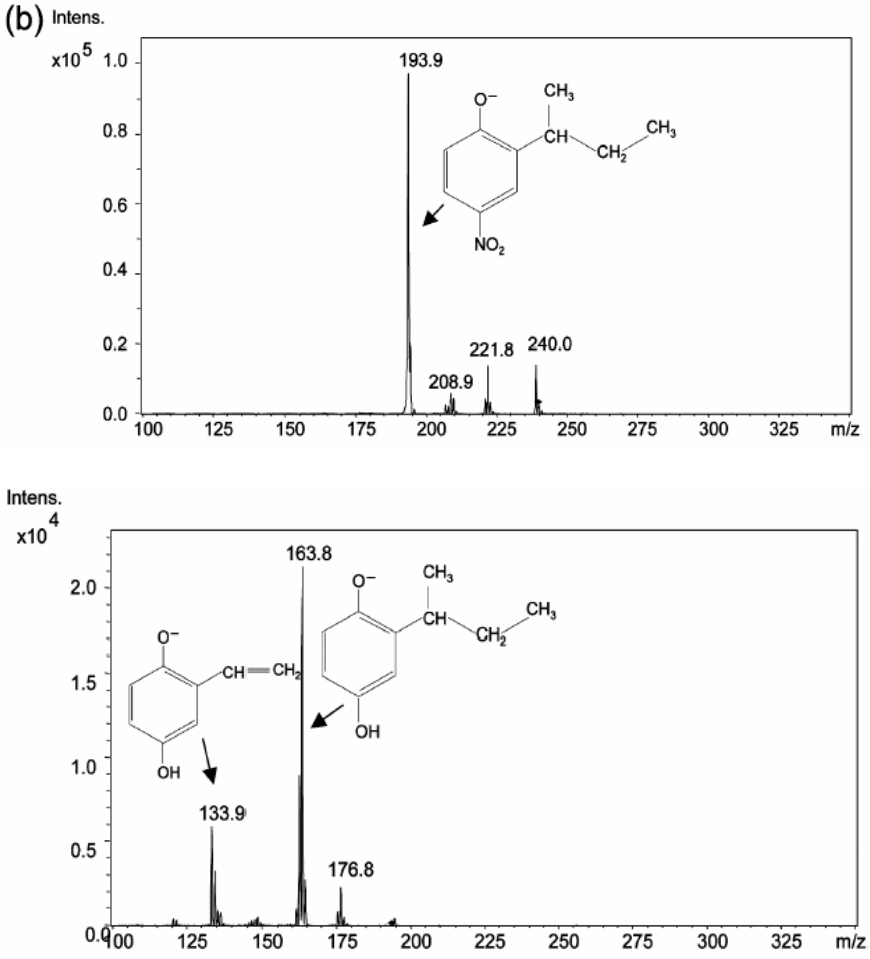


Fig. III.1. This mass fragmentation is in agreement with that already reported for GC-MS [122]. On the contrary, dinoseb is a phenol that formed mainly $[M-H]^-$ ions at pH 4 even though according to its pKa (4.62) hydroxyl groups should be predominantly nonionic. This phenomenon, called "wrong-way-around" results, has previously been reported for LC-ESI-MS [123] and for CE-ESI-MS [66]. The MS^2 spectrum evidences an intense signal at m/z 194 that corresponds to $[M+H-NO_2]^2$. Further fragmentation of this ion by MS^3 showed an intense signal at m/z 164 that corresponds to the neutral loss of NO and an additional fragment at m/z 134 formed by the loss of an ethane molecule. This course is

also in agreement with that reported in other studies [69]. From the observed fragmentation pathways the dominant dissociation was chosen (Table 11). The cut-off and the amplitude were set to a value that resulted in the maximum intensity of the product ions.

III.4.- Pesticide analysis by CE-UV, CE-ESI-MS, and CE-ESI-MS^n

The reproducibility, linearity, and sensitivity of the different detection systems tested in this study were evaluated. The results are listed in Tables III.1 and III.2. Satisfactory reproducibilities, calculated at concentrations five times higher than the LODs, were obtained with an RSD percentage (n = 10) for migration times better than 1.2 and for peak areas between 3.9 and 8.9. The reproducibility for the migration times was almost equal by all the detection systems tested, whereas that for the peak areas was slightly better by CE-UV (ranging from 3.9 to 5.8) than by the other detection systems (ranging from 4.3 to 8.9).

Among the different CE-ESI-MS approaches tested, there were no apparent differences in the RSDs. These results suggest that reproducible fragmentation was carried out for each pesticide. The calibration curves were linear in the range from 8 to 80 mg mL^{-1}, from 1.5 to 150 mg mL^{-1}, and from 0.05 to 50 mg mL^{-1} for CE-UV, CE-MS single quadrupole, and CE-QIT-MS with the different MS stages tested, respectively. The correlation coefficients were between 0.990 and 0.998. Calibration graphs showed good correlations for the six pesticides for any detection system.

Table III.2. Reproducibility, linearity, and sensitivity of pesticide detection by CE-MS with multiple stages of mass spectrum using the QIT.

	CE-MS				$CE-MS^2$				$CE-MS^3$			
	RSD (n = 10) (%)		Linearity correlation	LOD (μg/mL)	RSD (n = 10) (%)		Linearity correlation	LOD (μg/mL)	RSD (n = 10) (%)		Linearity correlation	LOD (μg/mL)
	Migration time	Peak area			Migration time	Peak area			Migration time	Peak area		
Thiabendazole	1.4	4.3	0.998	0.005	1.6	5.0	0.997	0.006	1.5	5.6	0.996	0.009
Pyrifenox	1.5	5.5	0.996	0.009	1.3	5.6	0.997	0.2	1.7	5.2	0.998	1.4
Pirimicarb	1.3	6.7	0.994	0.001	1.2	6.2	0.995	0.005	1.9	6.0	0.995	0.1
Pyrimethanil	1.7	5.8	0.993	0.01	1.7	5.5	0.994	0.1	2.0	6.4	0.997	0.3
Procymidone	2.0	5.9	0.992	0.015	2.3	6.3	0.990	0.02	2.5	7.1	0.996	0.5
Dinoseb	2.7	4.8	0.994	0.01	2.7	7.2	0.992	0.04	3.2	7.6	0.998	0.4

Using UV detection, the LODs, calculated as 3 times the signal/noise ratio, were 1 mg mL^{-1} for dinoseb, pirimicarb, and procymidone and 2 mg mL^{-1} for pyrifenox, pyrimethanil, and thiabendazole. Although the chemical structures of the studied pesticides are very different from each other, the LODs are quite similar because the molar absorptivities or extinction coefficients of the different analytes are similar as has already been reported [69]. These values would not be sufficient to detect thiabendazole, pyrifenox, pyrimethanil, and dinoseb, even when using an SPE preconcentration procedure, at concentrations lower than the maximum residue limits (MRLs) (see Table III.3). These LODs are in agreement with those described in the literature [57, 69]. LODs obtained by CE-MS with a single quadrupole were between 0.05 and 0.5 mg mL^{-1}. As previously, these values were not appropriate to determine pyrimethanil and dinoseb. Determination of thiabendazole and procymide in fruits using CE-ESI-MS with a single quadrupole has already been reported [58]. LODs reported in that work were five times lower than those obtained in the present study. A probable reason is that in the other study a stacking procedure was used, and that the running buffer and sheath liquid had different compositions. LODs ranged from 0.001 to 0.015 mg mL^{-1}, from 0.005 to 0.2 mg mL^{-1}, and from 0.009 to 1.4 mg mL^{-1} using MS, MS^2, and MS^3 with QIT-MS.

Table III.3. Results for pesticide analysis in spiked peach samples (basal pesticide levels , LODs) by CE-ESI-MS^2

Pesticide	Amount (mg/kg)	RSD (%, *n* =5)	Recovery (%)	MRLs (mg/kg)
Thiabendazole	0.005	15	58	0.05
	0.05	12	60	
Pyrifenox	0.06	18	91	0.2
	0.2	9	99	
Pirimicarb	0.001	19	69	0.5
	0.5	10	78	
Pyrimethanil	0.02	14	89	0.02
	0.2	11	92	
Procymidone	0.01	15	78	2
	2	9	82	
Dinoseb	0.02	16	60	0.05
	0.05	11	69	

The LODs for pyrifenox, pirimicarb, and pyrimethanil, obtained in the present study by CE-QIT-MS, were one order of magnitude better than those reported previously [69]. The explanation could be, as in the earlier case, the differences in the buffer and sheath liquid composition. According to the LODs obtained it can be deduced that MS and MS^2 resulted in a several fold improvement in sensitivity compared to CE coupled to UV or to single quadrupole MS in SIM mode. Both procedures seem to be appropriate to determine the studied pesticides at levels lower than the MRLs. On the contrary, LODs obtained by CE-ESI-QIT-MS^3 are higher than MRLs for pyrifenox, pyrimethanil, and dinoseb.

Since fruits contain a large number of organic compounds and some other contaminants that can cause isobaric interferences, highly selective methods are necessary to quantify pesticides precisely without interference from other compounds. MS/MS gives the highest degree of certainty in analyte identification and, therefore, CE-ESI-MS/MS was selected for further experiments.

III.5.- Identification and quantification of pesticides in fruits

The suitability of the CE-ESI-MS/MS was tested by applying it for the analysis of the selected pesticides in peaches. Peaches and nectarines are essentially the same, differing only in genes for surface fuzz. Pesticides were extracted using water-acetone as well as isolated and concentrated by SPE eluting with dichloromethane. Dichloromethane, a halogenated solvent, was selected because it provides higher analyte recoveries and coextracted lesser matrix components as has already been reported [57, 58]. The MRM mode of the mass spectrometers, which can detect mass spectra of fragmentation peaks of target precursor ions, provides sufficient selectivity and enables quantitative analysis. An example of a typical electropherogram obtained by CE-ESI-MS/MS from a spiked peach sample is shown in Fig. III.3. Electrophoretic resolution and peak performance were satisfactory. Although both mass spectrometers, single quadrupole and quadrupole ion trap, have almost the same design, different migration times compared with Fig. III.1b were observed because of small differences between both equipments, especially the distances in the mass spectrometer.

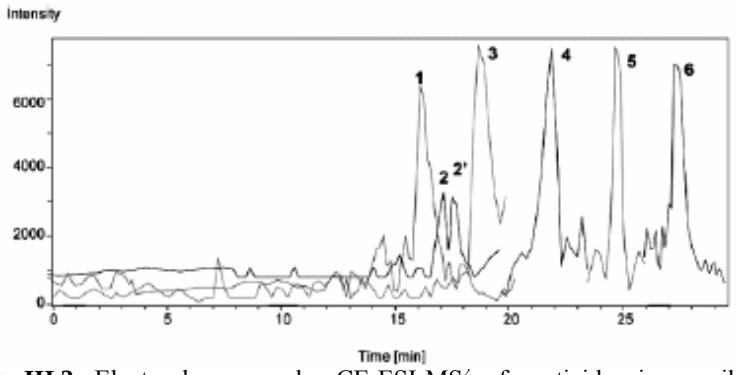


Fig. III.3. Electropherogram by CE-ESI-MS^2 of pesticides in a spiked peach sample at LOQ level (see Table III.3). The peaches (5 g) were extracted with water-acetone and the extracts were cleaned up and concentrated by SPE. The selected precursor ion→product ion in MRM used to quantify each analyte are indicated in Table 11.Same conditions as in Fig. III.1.

A matrix interference study was carried out comparing the calibration graph obtained from standards prepared in buffer and in fruit extracts. The equations of the least-square linear regressions obtained were similar, and the covariance analysis showed that the calculated F-values were lower than the tabulated ones, indicating that both straight lines were parallel ($P < 0.05$). These results proved that matrix interferences were negligible. Other important possible effects on the CE separation generated by matrix components, such as migration time variation or unresolved peaks, were not observed because the role of sample composition is more vital when on-line preconcentration techniques are applied instead of conventional hydrodynamic injection (5 s, 0.5 psi, ca. 5 nL injected) and the SPE helps to eliminate salts and inorganic components from the matrix that have the greatest influence on the ionic strength.

According to the guidance document on residue analytical methods established by the EU [11], Table III.3 reports recovery and precision for LOQs and MRLs (set or proposed) or 10 times LOQ when MRLs are similar to the LOQ fortification levels. Samples were analyzed prior spiking with pesticides to determine the basal levels or "blank values". There were no peaks at the retention times of the pesticides, indicating that basal levels were lower than the LODs. LOQs (lowest concentration outlined in Table III.3), calculated according to the European Guideliness as the lowest levels that provide acceptable recoveries and repeatabilities (< 20 %), were in the range of 0.001–0.2 mg kg^{-1}. Compared with the established MRLs, LOQs were equal for pyrimethanil and lower for thiabendazole, pyrifenox, pirimicarb, procymidone, and dinoseb a number of times of 10, 3, 500, 200, and 2.5, respectively. The MRLs reported in Table III.3 are those established by the Spanish legislation [124], which were in agreement with the MRLs instituted by the European Union (EU) [11] for dinoseb, procymidone, and thiabendazole, and with those

recommended by the Codex Alimentarius [15] for pirimicarb. There were no MRLs set by the US Food and Drug Administration [116] for these pesticides in peaches or nectarines. Recoveries and RSDs obtained for peaches and nectarines spiked at two concentrations are summarized in Table III.3. Mean recoveries ranged from 58 and 99% with RSDs from 9 to 19%. Recoveries seem to be higher at the higher spiked level but differences were within the range of the RSDs.

The method was applied to the determination of the six pesticides in ten samples (five of peaches and five of nectarines) obtained from an agricultural cooperative over different days. Three samples contained pesticide residues. One nectarine sample contained 0.03 mg kg^{-1} thiabendazole, and two peach samples 0.2 and 1.0 mg kg^{-1} procymidone. Electropherograms of the peach sample containing thiabenzole and using different CE-ESI-MS systems are shown in Fig. III.4. The impressive selectivity of multiple-stage MS can be observed.

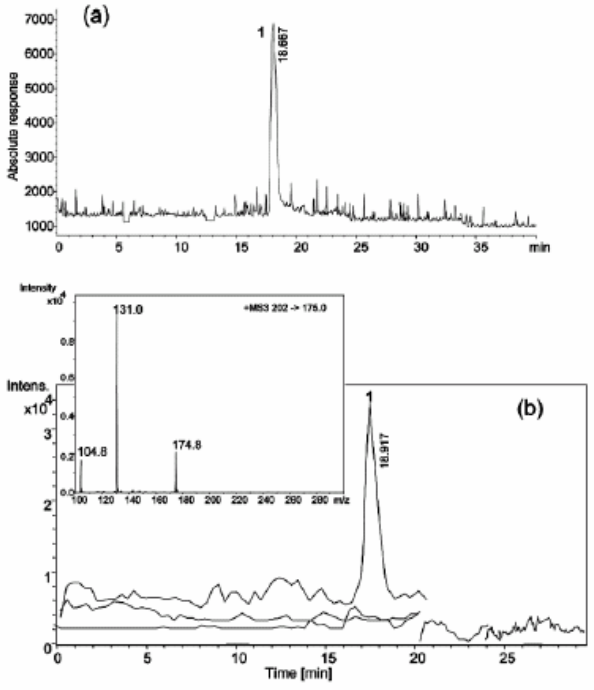


Fig. III.4. Electropherograms of sample containing thiabendazole using (A) single quadrupole in SIM monitoring the ion at m/z 202 and (B) QIT MS^3 (the superposed figure corresponds to the product ion spectrum).

IV.- ON-LINE PRECONCENTRATION STRATEGIES FOR ANALYZING PESTICIDES IN FRUITS AND VEGETABLES BY MICELLAR ELECTROKINETIC CHROMATOGRAPHY

Técnicas de preconcentración en línea para el análisis de plaguicidas en frutas y verduras por cromatografía electrocinética micelar

IV.1.- Summary method developed

Five pesticides (fludioxonil, procymidone, pyriproxyfen, dinoseb and carbendazim) were separated in reversed migration micellar electrokinetic chromatography (RM-MEKC) using 20 mmol l^{-1} phosphate buffer at pH 2.3, containing 25 mmol l^{-1} sodium dodecylsulfate and 10% methanol. Three on-line concentration strategies, sweeping (SW), normal stacking with reversed migration and a water plug (SRW) and stacking with reverse migration and removal of sample matrix using polarity switching (SRMM), were compared. A solid-phase extraction (SPE) procedure, for previous isolation and concentration of the analytes, was used in combination with any of the proposed on-line preconcentration strategies.

IV.2.- Optimum separation conditions

The preconcentration techniques tested in this study required separation of the compounds in RM-MECK, in which the EOF direction is reversed to attain matrix removal and preconcentration. EOF can be reversed mainly by two procedures: (i) at acidic pH by reducing the elect osmotic flow so far that it cannot overwhelm the electophoretic mobility of the anionic micelles and (ii) by the addition of a cationic surfactant that is adsorbed in the capillary walls charging the positively [27, 125, 126].

The separation of five pesticides in RM-MECK was optimized testing different running buffers consisted of mixtures of phosphate solutions (10–100 mmol l^{-1}) and SDS (15–150 mmol l^{-1}) at different pH (2–5) by direct hydrodynamic injection (5 s at 0.5 psi) of a 5 $\mu g ml^{-1}$ standard solution of the five pesticides prepared in the running buffer. According to the pKa for several compounds: carbendazim (pKa 4.48) and dinoseb (pKa 4.62), pH values around 5 should be used because at lower pH, these compounds are positively charged.

Experimental results are contradictory with this theory: the more acidic, the pH is, the best the separation is. Similar results have been reported by da Silva et al. [127], which separates nine pesticides of different classes including carbendazim. Among the different running buffers tested, the mixture containing 20 mmol l^{-1} phosphate and 25 mmol l^{-1} SDS at pH 2.3 provided the best results, although pyriproxyfen and carbendazim still overlap partially.

The separation between both compounds was attained adding a 10% of methanol to the previous buffer. This conclusion was achieved checking different solvents (methanol, acetonitrile, ethanol and isopropanol) at percentages between 2 and 15%.

Finally, the effect of the applied voltage and the temperature was study to shorten as much as possible the analysis time. Temperature was varied between 20 and 30 ◦C and the applied voltage between 16 and 25 kV. The

optimum results were obtaining applying a voltage of 20 kV at 20 °C. Fig. IV.1 presents the separation of 5 μgml^{-1} standard solution of the studied pesticides in the optimized running buffer.

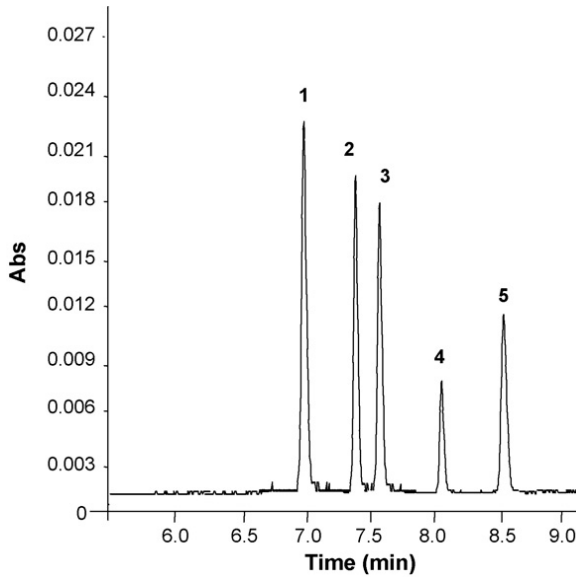


Fig. IV.1. Separation of the five pesticides standards at a concentration of 5 μgml^{-1} of each by RM-MECK in 20 mmol l^{-1} phosphate buffer at pH 2.3 containing 25mmmol l^{-1} SDS and 10% of methanol. Other conditions: hydrodynamic injection of 5 s at 0.5 psi pressure, applied voltage −20 kV at 20 ºC.
Peak identification:
1 fludioxonil,
2 pyriproxyfen,
3 carbendazim,
4 dinoseb
5 procymidone.

IV.3.- On-line sample preconcentration strategies

Fig. IV.2 illustrates the effects of the injection time on peak area for each of the three on-line preconcentration strategies tested. The peak area increases in proportion to the injection time. However, the dependency of the peak area is not linear in all cases. For example, procymidone and pyriproxyfen seem to present an exponential dependency and for dinoseb there is a maximum in the case of SRW. The 60 s injection was the most suitable in terms of peak shapes. However, as can be observed in Fig. IV.3, the application of the stacking techniques provoked certain loss of resolution (compared with Fig. IV.1), especially between pyriproxyfen and carbendazim, and some peak width increment, especially in SRW and SRMM. The lack of linearity can be explained because for longer injection time (up to 60 s), peaks showed

broadening and asymmetric shapes. The changes observed in the area give the impression to be dependent on the solute, which could be related to the pKa or log P values of the studied compounds. However, there are not available pKa for all the studied compounds but only for carbendazim and dinoseb and no relation can be establish according to the log P, which was 1.38 for carbendazim, 3.08 for procymidone, 3.56 for dinoseb, 4.12 for fludioxonil and 5.37 for fludioxonil. This lack of linearity or apparent exponentiality of the peak area with the injection time has already been reported by other authors [68, 126], which suggest that the injection length of the sample zone was limited by the dispersive effect brought about by the local electrososmotic velocity mismatch between the low- and high-conductivity zones.

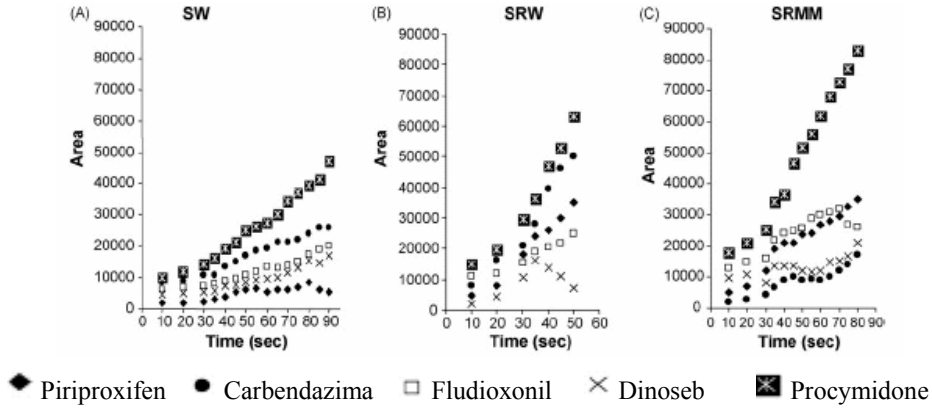


Fig. IV.2. Effects on injection time on peak area for (A) SW, (B) SRW and (C) SRMM.

Fig. IV.3 shows the electropherograms obtained by SW, SRW and SRMM RM-MECK-DAD analysis of a standard mixture containing 1 μg ml^{-1} of each analyte. A usual injection is included for comparison. About 10-, 30- and 50-fold increases in detection sensitivity, compared with standard hydrodynamic injection, were observed for SW, SRW and SRMM, respectively. Among the on-line concentration techniques, SRMM gave the highest improvement in detector response (>50-fold compared to usual injection). The

stacking techniques (SRMM and SRW) rendered better results compared to SW because the analytes were moderately polar. However, the peak shape obtained by SW was the most appropriate and its separation ability even better. The three on-line preconcentration procedures provided results applicable to the determination of pesticides at low concentration levels. The retention times obtained are slightly different upon the concentration method because the solution in which the standards are dissolved is different as has already been reported [127].

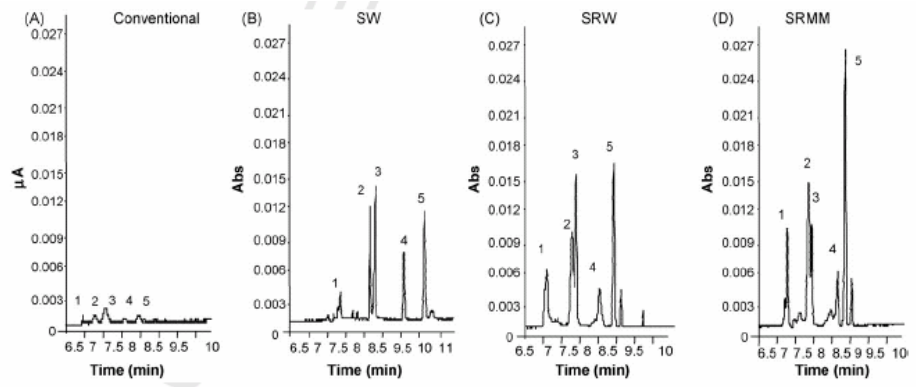


Fig. IV.3. Electropherograms of a standard mixture of containing 1μgml^{-1} of each pesticide obtained by (A) conventional RM-MEKC with hydrodynamic injection (5 s at 0.5 psi), (B) by the SW RM-MECK, (C) by SRW RM-MECK and (D) SRMM RM-MECK conditions. SW: hydrodynamic injection 60 s at 50 mbar, SRW: injection of water plug 10 s at 0.4 psi, then sample injection 60 s at 50 mbar and RMM: hydrodynamic injection 60 s at 50 mbar, application of positive voltage 1.2 s. Separation, other conditions and peak identification as in Fig. IV.1.

Table IV.1 summarizes and compares the mean retention times, linearity, limit of detection (LOD) values, relative standards deviations (RSD, %) of peak area and migration times for normal injection 0.5 s at 5 psi, SW, SRW and SRMM, respectively. The linearity of these methods was tested using five different concentrations within the range of 0.2–50 mg ml^{-1}, executing at least three replication injections. Pesticide responses were found linear over the concentration range explored with correlation coefficients (r) > 0.991 in all the cases. Data on the regression equations are listed in Table IV.1. The limits of

Table IV.1. LOD values, RSDs (n = 8) value and linearity (five points, injections in triplicate) for the studied pesticides with RM-MEKC-DAD using conventional injection, SW, SRW and SRMM.

Pesticides	Fludioxonil	Pyriproxyfen	Carbendazim
Conventional injection			
Mean migration time (min)	6.809	7.376	7.652
Migration time(RSDs, %)	1.58	2.27	2.64
Peak heigh(RSDs, %)	4.45	5.44	7.21
Peak area(RSDs, %)	10.01	6.95	9.82
Concentration range (mg ml^{-1})	4–80	4–80	2–80
Equation	y = (400 ± 29)x + (1487 ± 895)	y = (125 ± 10)x + (784 ± 124)	y = (405 ± 32)x + (111 ± 78)
Correlation coefficient (r)	0.993	0.997	0.995
LODs (µgml−1)	0.3	0.3	0.1
Sweeping			
Mean migration time (min)	7.799	8.608	8.841
Migration time (RSDs, %)	2.38	2.35	2.51
Peak heigh (RSDs, %)	3.71	9.09	5.91
Peak area (RSDs, %)	6.82	11.61	9.28
Concentration range (mg ml^{-1})	0.2–50	0.4–50	0.2–50
Equation	y = (1208 ± 95)x + (1051 ± 512)	y = (2319 ± 210)x + (5612 ± 609)	y = (5719 ± 450)x + (7371 ± 999)
Correlation coefficient (r)	0.994	0998	0.993
LODs (µgml^{-1})	0.02	0.04	0.02
SRW			
Mean migration time (min)	7.118	7.662	7.861
Migration time (RSDs, %)	3.65	2.47	3.63
Peak heigh (RSDs, %)	9.22	6.46	5.84
Peak area (RSDs, %)	2.23	4.92	3.04
Concentration range (mg ml^{-1})	0.2–50	0.2–50	0.2–50
Equation	y = (1202 ± 130)x + (18388 ± 1234)		y = (1202 ± 96)x + (2386 ± 1245)
Correlation coefficient (r)	0.998	0.993	0.992
LODs (µgml^{-1})	0.01	0.01	0.01
SRMM			
Mean migration time (min)	7.321	7.609	7.798
Migration time (RSDs, %)	1.30	1.54	1.33
Peak heigh (RSDs, %)	9.47	6.53	3.95
Peak area (RSDs, %)	6.85	5.01	2.86
Concentration range (mg ml^{-1})	0.2–50	0.2–50	0.2–50
Mean migration time (min)	7.321	7.609	7.798
Equation	y = (3069 ± 356)x + (2667 ± 503)	y = (4004 ± 395)x + (6047 ± 926)	y = (5449 ± 519)x + (7857 ± 698)
Correlation coefficient (r)	0.993	0.991	0.996
LODs (µgml^{-1})	0.01	0.01	0.01

Table IV.1. Continued

Pesticides	Dinoseb	Procymidone
Conventional injection		
Mean migration time (min)	8.098	8.538
Migration time (RSDs, %)	3.68	4.58
Peak heigh (RSDs, %)	11.79	12.06
Peak area (RSDs, %)	9.21	9.02
Concentration range (mg ml^{-1})	6–100	2–80
Equation	y = (578 ± 38)x + (517 ± 106)	y = (834 ± 59)x + (1089 ± 346)
Correlation coefficient (r)	0.993	0.992
LODs (μgml^{-1})	0.5	0.1
Sweeping		
Mean migration time (min)	9.617	10.206
Migration time (RSDs, %)	2.64	2.70
Peak heigh (RSDs, %)	9.73	6.71
Peak area (RSDs, %)	9.84	9.16
Concentration range (mg ml^{-1})	0.5–50	0.5–50
Equation	y = (2407 ± 202)x + (2640 ± 397)	y = (2203 ± 187)x + (7833 ± 451)
Correlation coefficient (r)	0.991	0.998
LODs (μgml^{-1})	0.02	0.02
SRW		
Mean migration time (min)	8.647	8.974
Migration time (RSDs, %)	3.76	3.24
Peak heigh (RSDs, %)	3.25	2.04
Peak area (RSDs, %)	2.58	0.22
Concentration range (mg ml^{-1})	1.2–50	0.4–50
Equation	y = (1003 ± 126)x + (1664 ± 925)	y = (1294 ± 113)x + (3604 ± 891)
Correlation coefficient (r)	0.995	0.997
LODs (μgml^{-1})	0.03	0.01
SRMM		
Mean migration time (min)	8.646	9.001
Migration time (RSDs, %)	1.36	1.45
Peak heigh (RSDs, %)	5.11	4.38
Peak area (RSDs, %)	6.24	2.95
Concentration range (mg ml^{-1})	1.2–50	0.2–50
Equation	y = (2541 ± 259)x + (3041 ± 735)	y = (7805 ± 707)x + (2637 ± 986)
Correlation coefficient (r)	0.997	0.992
LODs (μgml^{-1})	0.02	0.002

detection (S/N = 3) of the five compounds were 0.01–0.04 μgml^{-1}, (SW), 0.01–0.3 μgml^{-1} (SWR) and 0.0002–0.01 μgml^{-1}. The reproducibility (RSDs) of the proposed methods in terms of peak-area for eight replicate injections, were 6.82–11.61% for SW, between 3.95 and 9.47% for SRMM and 2.22–4.94% for SRW. In terms of migration time, between 2.35 and 2.70% for SW, between 1.30 and 1.54 % for SRMM and 2.47 and 3.76 % for SRW.

The selected pesticides have been determined using MECK or CZE in our laboratory. MECK using a running buffer consisted of 6 mmol l^{-1} sodium

tetraborate decahydrate and 75 mmol l^{-1} cholic acid at pH 9 separated eight pesticides, which fludioxonil and pyriproxyfen [128]. Conventional hydrodynamic injection (5 s, 0.5 psi) provided a LOD of 0.1 $\mu g ml^{-1}$. CZE using 0.3 M ammonium acetate-acetic acid at pH 4 with a 10 % of methanol provided resolution of procymidone and dinoseb among other pesticides. LODs obtained using UV detector and conventional injection were 10 times higher than those achieved in the present study [129]. However, using MS and MS/MS detectors LODs were improved, at least, by a factor of ten with the substantial increment in selectivity and specificity. However, any of the on-line preconcentration procedures assayed in this study allows to improve those previous LODs by a factor of 10.

IV.4.- Determination of pesticide residues in grape and lettuces

When applying this method to fruits, sample preconcentration via SPE was required (with 10-fold preconcentration achieved). A solvent extraction and SPE preconcentration and cleanup previously developed in our laboratory [128, 129] was used as off-line preconcentration step prior CE analysis.

Samples of grapes and lettuces unspiked and spiked at 0.01 mg kg^{-1} and at MRLs were analyzed by SPE, RM-MECK-DAD, using the three preconcentration procedures, under the optimized conditions described above. The electropherograms obtained for lettuce extracts are shown in Fig. IV.4. The electropherogram with the line in black of those figures shows the electrophrogram/chromatogram of the lettuce or grape extract without any of the studied pesticides, and the electropherogram with the line in grey the same extract spiked with the pesticides at 0.01 mg kg^{-1}, demonstrating that the analyte peaks of all matrix extracts were free from interferences and that there are no significant variations in the shape and the retention time of the peaks and, therefore, that the methods are comparable with complex food matrices. Grape

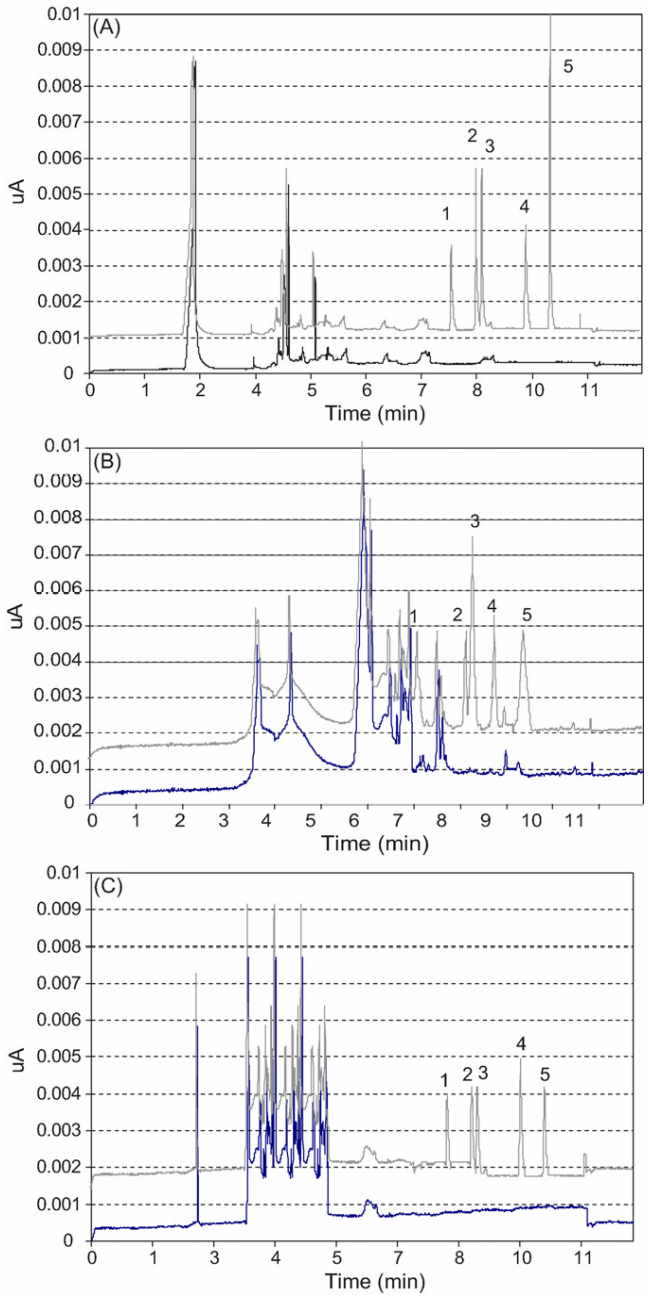


Fig. IV.4. Electropherograms from a unspiked (black line) and from a spiked (grey line) lettuce at 0.01 mg kg^{-1} of each pesticide obtained after solvent extraction and SPE clean-up by (A) SW, (B) SRW and (C) SRMM. Stacking conditions as in Fig. IV.2, and separation conditions and peak identification as in Fig. IV.1.

samples provided extracts a little cleaner than that of the lettuce maybe because the absence of chlorophylles and other colored or not compounds presents in the extracts. Fig. IV.5 shows worst case with corresponds to the electropherograms obtained for a grape sample using SRW. A much cleaner chromatogram with

less baseline deviation and less peaks is observed if compared with that corresponding to the lettuce sample.

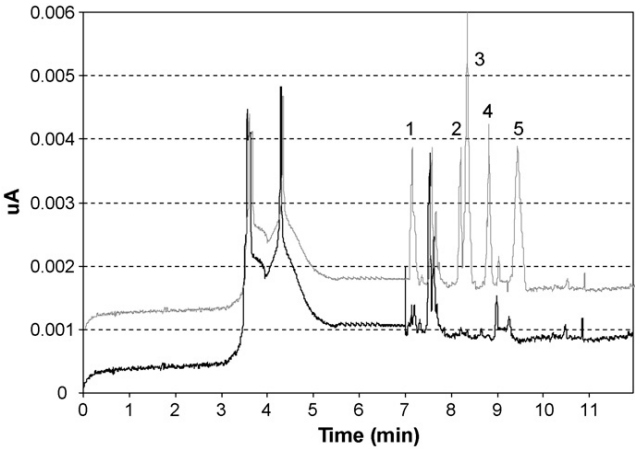


Fig. IV.5. Electropherograms from a unspiked (black line) and from a spiked (grey line) sample at 0.01 mg kg^{-1} of each pesticide obtained after solvent extraction and SPE clean-up by SRW. Stacking conditions as in Fig. IV.2, separation conditions and peak identification as in Fig. IV.1.

Recoveries and precision are summarized in Tables IV.2 and IV.3 as percentage and RSDs. Recoveries >70% for all pesticides were achieved being a little higher for fludioxonil and pyriproxyfen in grape than in lettuce. These values were between 72 and 98%, with RSDs from 13 to 19% by SW, between 75 and 97%, with RSDs from 11 to 19% by SRW and between 70 and 100%, with RSDs from 10 to18 % by SRMM. Although recovery was similar by all the methods and precision was within the range of the EU guidelines [130].

Table IV.2. Accuracy and precision at 0.01 mg kg^{-1} (amount of sample processed 5 g) after SPE and on-line preconcentration of lettuce samples using RM-MEKC with the three on-line preconcentration strategies.

Pesticide	Concentration (mg kg^{-1})	Recovery, % (RSD, *n* =5) RM-MEKC-DAD		
		SW	SRW	SRMM
Fludioxonil	0.01	72 (17)	75 (16)	73 (16)
	1	87 (15)	77 (14)	70 (10)
Pyriproxyfen	0.01	74 (19)	80 (19)	81 (16)
	5	77 (16)	82 (15)	80 (12)
Carbendazim	0.01	73 (18)	77 (18)	75 (17)
	2	75 (13)	81 (15)	76 (15)
Procymidone	0.01	78 (18)	88 (19)	82 (18)
	5	79 (15)	91 (11)	87 (14)
Dinoseb	0.01	70 (19)	75 (17)	73 (18)

Table IV.3. Accuracy and precision at 0.01 mg kg^{-1} (amount of sample processed 5 g) after SPE and on-line preconcentration of grape samples using RM-MEKC with the three on-line preconcentration strategies and conventional LC-DAD.

Pesticide	Concentration (mg kg^{-1})	Recovery, % (RSD, *n* =5) RM-MEKC-DAD		
		SW	SRW	SRMM
Fludioxonil	0.01	88 (16)	85 (19)	93 (18)
	2	92 (16)	88 (17)	90 (12)
Pyriproxyfen	0.01	94 (18)	90 (19)	98 (16)
	5	98 (15)	97 (15)	100 (12)
Carbendazim	0.01	70 (17)	77 (18)	78 (16)
	2	79 (14)	81 (15)	79 (13)
Procymidone	0.01	78 (16)	88 (19)	87 (17)
	5	79 (13)	91 (11)	87 (12)
Dinoseb	0.01	73 (18)	77 (16)	75 (16)

Table IV.4 shows the LOQs and the maximum residue limits (MRLs) established by different legislations [131-133]. LOQs were calculated according to the European Union (EU) guidelines as the lowest level that provides acceptable recoveries (>70 %) and reproducibilities (<20 %) [130]. The LOQs by RM-MECK-DAD of these pesticides were 0.008-0.01 mg kg^{-1} for SW,

0.004–0.01 mg kg^{-1} for SRW and 0.001–0.01 mg kg^{-1} for the SRMM method. Thus, SRMM yields the lowest LOQ, followed by SRW and finally SW. The lowest detection method yielded by the SRMM preconcentration can be explained by its ability to introduce the greatest quantity of sample to the capillary. Compared with the previous works carried out in our laboratory with some of the studied pesticides, LOQs are improved more than 10 times, which allows to reach the lowest MRLs established by the legislations [128, 129].

Table IV.4. LOQs obtained after SPE and on-line preconcentration of lettuce and grape samples using RM-MEKC with the three on-line preconcentration strategies and MRLs established by different legislation.

Pesticide	LOQs (mg kg^{-1})			MRLs (mg kg^{-1})	
	RM-MEKC-DAD			Grape	Lettuce
	SW	SRW	SRMM		
Fludioxonil	0.01	0.008	0.006	2[a],1[b]	1[b]
Pyriproxyfen	0.008	0.006	0.007	5[a]	5[a]
Carbendazim	0.008	0.004	0.008	5[c]	2[c]
Procymidone	0.008	0.010	0.010	0.05[c],5[a,b]	0.05[c],5[a ,b]
Dinoseb	0.008	0.005	0.001	5[c]	5[c]

[a] Codex Alimentarius [128]
[b] USA [129]
[c] EU MRLs [7]

IV.5.- Application to real samples

Table IV.5 reports the concentrations calculated for the selected pesticides in a total of 60 samples, 30 of grapes and 30 of lettuces, taken from different local markets, measured after SPE by RM-MECK using the three on-line preconcentration procedures. The reported concentrations are corrected by the individual recovery values of the compounds. The concentrations are in the μg kg^{-1} range. Owing to the increase in sensitivity provided by on-line preconcentration, pesticides can be detected by RM-MECK-DAD.

Pesticide residues were detected in 15 samples (25%) of the 60 analyzed. None exceeded MRLs. Dinoseb was the only pesticide that was not found in any

of the samples. Pyriproxyfen was only found in one sample of lettuce in quite low concentration. Carbendazim was found in nine samples (15 %), three lettuces and six grapes, at levels ranging from 0.01 to 1.54 mg kg^{-1}. Procymidone was also found in nine samples (15 %), two lettuces and seven grapes, at levels ranging from 0.19 to 2.03 mg kg^{-1}. Fludioxonil was found in five samples (8 %), one lettuce and four grapes, at concentrations between 0.02 and 3.02 mg kg^{-1}.

Of the contaminated samples, nine samples (60%) – seven grapes and two lettuces – contained residues of two pesticides and six samples (40 %) – three grapes and three lettuces – contained a single pesticide. This finding corroborates the presence and the co-occurrence of residues of pesticides in a significant number of samples.

Table IV.5. Concentrations of pesticides found in grapes and lettuces by RM-MECK-DAD using the three on-line preconcentration procedures and LC-DAD

Sample nº.	Pesticides found	Concentration (mg kg^{-1})[a]		
		SW-RM-MECK[b]	SRW-RM-MECK[c]	SRMM-RM-MECK[d]
Grape 1	Carbendazim	0.03	0.02	0.01
	Procymidone	1.23	1.53	1.05
Grape 4	Carbendazim	0.25	0.32	0.20
	Procymidone	1.64	1.54	1.65
Grape 5	Carbendazim	0.15	0.10	0.17
	Procymidone	1.92	2.03	1.54
Grape 10	Carbendazim	0.75	0.65	0.55
	Procymidone	1.85	1.80	1.95
Grape 11	Fludioxinil	0.04	0.05	0.03
Grape 12	Carbendazim	0.73	0.81	0.79
	Procymidone	0.21	0.19	0.26
Grape 15	Fludioxinil	0.44	0.33	0.35
Grape 22	Carbendazim	1.09	1.27	0.99
	Procymidone	0.23	0.26	0.27
Grape 24	Fludioxinil	0.09	0.07	0.08
Grape 28	Fludioxinil	0.05	0.03	0.04
	Procymidone	0.19	0.24	0.22
Lettuce 5	Pyriproxyfen	0.05	0.03	0.05
Lettuce 12	Carbendazim	0.15	0.21	0.19
Lettuce 25	Procymidone	0.34	0.24	0.45
Lettuce 28	Carbendazim	0.25	0.20	0.23
	Procymidone	0.53	0.53	0.61
Lettuce 30	Carbendazim	1.25	1.54	1.05
	Fludioxinil	3.02	2.05	2.95

a Triplicate measurements.
b RSDs were ranged between 19 and 25%.
c RSDs were ranged between 17 and 24%.
d RSDs were ranged between 13 and 22%.

V.- DETERMINATION OF QUINOLONE RESIDUES IN CHICKEN AND FISH BY CAPILLARY ELECTROPHORESIS- MASS SPECTROMETRY

Determinación de residuos de quinolonas en pollo y pescado por electroforesis capilar-espectrometría de masas

V.1.- Summary method developed

A specific pressure-assisted CE-MS method is described for the analysis of five quinolone residues (danofloxacin, enrofloxacin, flumequine, ofloxacin, and pipemidic acid). MS using a single quadrupole is compared with multiple-stage MS using a quadrupole IT (QIT-MS^n). The procedure involves a common sample preparation by SPE on disposable cartridges. The most suitable electrolyte is 60 mM $(NH_4)_2CO_3$ at pH 9.2. Single quadrupole does not provide enough fragmentation to confirm identities according to the current legislation. However, QIT-MS^n achieves selective fragmentation. Confirmation of the quinolones' identity is achieved using QIT-MS^3.

V.2.- Optimization of CE-MS conditions

The coupling between CE and MS requires the use of a volatile CE running buffer such as buffers containing formic acid, acetic acid, ammonium carbonate, and ammonium acetate. Four of the studied quinolones have a carboxylic group and a piperazinyl moiety including additional amino groups, which can be cationic, zwitterionic, and anionic depending on the pH. Because of this, the compounds can be analyzed by CE over a certain pH range with different separation selectivity. However, flumequine has only a carboxylic acid group that is negatively charged at basic pH. As it has already been reported, basic pH (between 8 and 9.50) provides better separation of quinolones than acidic or stronger alkaline pHs [41, 134, 135]. Using 60 mM ammonium carbonate buffer (pH 9.2) at 25 kV and an excess pressure of 30 mbar during the run, the separation of the five quinolones was achieved in 20 min. Carbonate buffers have previously been used in quinolone separation by CE-MS [136].

The sheath liquid flow is the dominant flow for CE-MS analysis, and its pH affects ESI-MS sensitivity. The effect of sheath liquid pH on ESI-MS sensitivity was tested by using three solutions: 0.5% acetic acid in water (pH 3.0), 60 mM ammonium acetate (pH 7.0), and 60 mM ammonium carbonate in water (pH 9.2). The MS signal and stability were better using 60 mM ammonium carbonate. The use of basic solutions (pH 9.2) had no significant decrease in the sensitivity compared with the lower pH sheath liquid solutions. The addition of different organic solvents to the sheath liquid (methanol, ACN, and isopropanol) was tested but they do not improve either MS signal or spray stability. Thus, further experiments were conducted with 60 mM ammonium carbonate. This empirical observation was in agreement with the general experience that the running buffer is the best sheath liquid [66, 129, 136].

Another important parameter, the effect of the sheath liquid flow rate on the ESI sensitivity, was also investigated in the range of 2–15 mL min^{-1}. The

addition of pressure to the separation capillary decreased analysis times and stabilized the ESI spray [137]. The application of 30 mbar increased the flow rate by 0.2 mL min^{-1}. The sheath liquid flow rate has only very little effect on the sensitivity, under the examined conditions. At higher flow rates (15 mL min^{-1}), the sensitivity decreased probably due to a dilution effect with the sheath liquid. At lower flow rates (< 6 mL min^{-1}), the sensitivity was also somewhat decreased, probably because those flows were too low to be able to stabilize the source spray. According to Frommberger et al. [138], a stable electrospray could not be achieved with flow rates less than 4 mL min^{-1}. In the present study, high stable and sensitive analysis is achieved at flow rates between 8 and 12 mL min^{-1}. Therefore, the following experiments were performed at 10 mL min^{-1}. This flow rate is relatively high compared with many papers that used pure CE [66, 129, 138]. However, the reason could be other than the use of pCE, for example the capillary temperature of the electrospray. This temperature is also higher than inmost of those studies because it must lead to solvent removal, and water has a high boiling temperature compared with organic solvents [138].

Table V.1 depicts the chemical structure and molecular weight of the studied quinolones to highlight their fragmentation patterns. The mass spectra obtained with the single quadrupole showed, as main ion, the peak corresponding to the protonated molecule $[M+H]^+$. According to the 657/2002/EC Commission Decision [139], the protonated molecule is equivalent to one IP, which is not enough for confirmation. Although an increase in the fragmentor voltage accelerates the collision between the ions inducing fragmentation, this in-source fragmentation is difficult, requires high fragmentor voltages, and can lead to a decrease in sensitivity.

Quinolone	Molecular Weight
Pipemidic Acid	303
Enrofloxacin	359
Ofloxacin	361
Danofloxacin	357
Flumequine	261

Table. V.1. Molecular weight of the quinolone studied.

Fragmentation patterns of quinolones using QIT were also studied to facilitate the identification. Table V.2 shows the precursor-product ions obtained using QIT and multiplestage MS. The first-order MS spectrum shows the peak corresponding to the protonated molecule of each quinolone, which was subjected to CID to produce a first set of fragment ions MS/MS or MS^2. One of these fragment ions was isolated and refragmented to give the next set of ions MS^3. CID was carried out, on the ion of interest, by collisions with the helium background gas present in the trap for 40 ms. Product ion mass spectra (MS^3) of quinolones showed that predominant fragment ions were m/z 283 for danofloxacin, m/z 245 for enrofloxacin, m/z 261 for ofloxacin, m/z 189 for

Table V.2. Transitions used for quantification by MS^n operation. Neutral loss is indicated between brackets

		Quinolones			
	Danofloxacin	Ofloxacin	Enrofloxacin	Pipemidic acid	Flumequine
MS^2 transitions (m/z)	358 → 314 (CO_2)	362 → 318 (CO_2)	360 → 316 (CO_2)	304 → 216 (CO_2,C_2H_5N)	262 → 244 (HO_2)
Width (*m/z*)	4.0	4.0	4.0	4.0	4.0
Cut-off (*m/z*)	150	100	100	100	100
Amplitude (V)	4.0	2.0	1.5	1.0	2.0
MS^3 transitions (m/z)	314 → 294 (80%) (HF) 314 → 283 (100%) (H_2NCH)	318 → 261 (C_3H_7N)	316 → 245 ($C_2H_5NC_2H_5$)	216 → 189 (CO)	244 → 202 (C_3H_6)
Width (*m/z*)		4.0	4.0	4.0	4.0
Cut-off (*m/z*)		100	100	100	100
Amplitude (V)		1.2	1.3	1.8	1.3
MS^4 transitions (m/z)		261 → 218 (NC_2H_5)	245 → 205 (C_3H_4)		
Width (*m/z*)		4.0	4.0		
Cut-off (*m/z*)		100	100		
Amplitude (V)		2.0	1.8		
MS^5 transitions (m/z)			205 → 189 (CH_4)		
Width (*m/z*)			4.0		
Cut-off (*m/z*)			100		
Amplitude (V)			2.0		

pipemidic acid, and m/z 202 for flumequine. These results are in agreement with those reported in other studies [140–144]. According to the EC Decision [139],

using QIT, each precursor-product ion transition earns 1.5 IPs. The monitoring of two precursor-product ion transitions achieves unequivocal confirmation. An example of the multiple stage spectra and the proposed interpretation of the ions is shown in Fig. V.1 for enrofloxacin, which is the quinolone that can be more repeatedly fragmented.

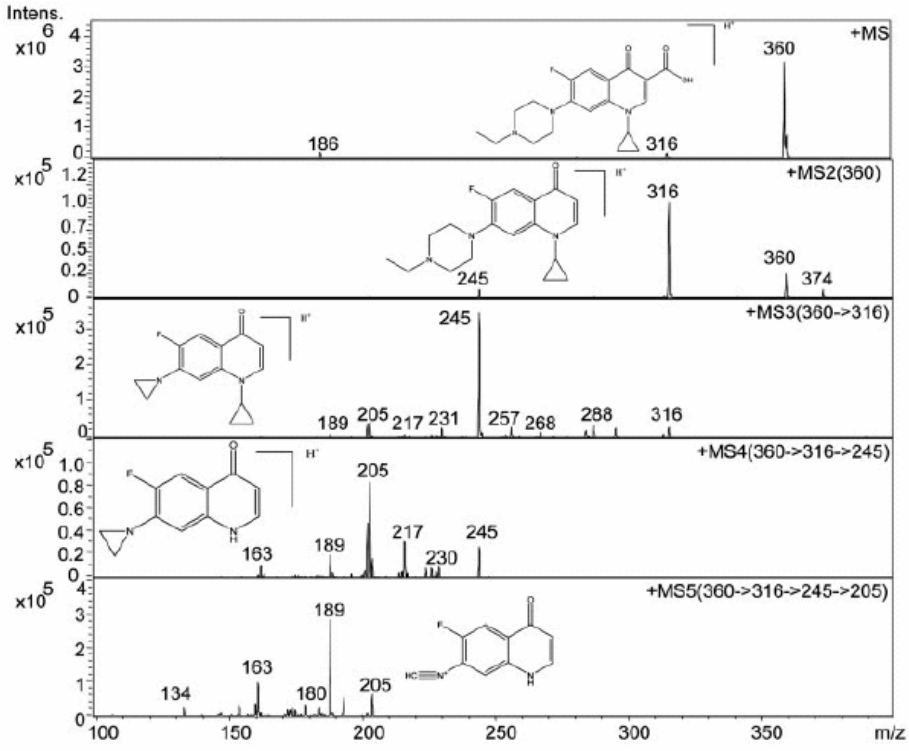


Fig. V.1. Multiple-stage mass spectra of enrofloxacin and proposed interpretation of the product ions.

V.3.- Method validation

In order to improve the detection sensitivity, an extraction procedure that achieves a ten-fold concentration was applied. The method was validated using both chicken and fish muscle tissue samples fortified with several levels of standard quinolone mixture, and subjected to the entire extraction procedure. LODs and LOQs were defined, respectively, as the signal corresponding to three and ten times the noise SD.

The developed method was validated in terms of repeatability and reproducibility of migration time and peak area, LOD, and linearity. The results obtained for chicken samples are summarized in Table V.3. The repeatability was calculated from six consecutive analyses (intraday) and the reproducibility from four consecutive days (day-to-day or interday) of the same extract. The RSDs of

Table V.3. Repeatability (intraday) and reproducibility (interday) of relative migration time, peak area, correlation coefficient (r), and LOD obtained by analyzing quinolone spiked chicken muscle with the proposed procedure by CE-MS using the single quadrupole (quantification was carried out in SIM mode) and by CE-MS^3 using QIT (quantification was carried out obtaining the EIC of the most intense product ion), respectively.

	CE-Q-MS						CE-QIT-MS^3					
	RSD (%)				LOD (ng/g)	$r^{c)}$	RSD (%)				LOD (ng/g)	$r^{c)}$
	Migration time		Peak area				Migration time		Peak area			
	Intraday[a)]	Interday[b)]	Intraday[a)]	Interday[b)]			Intraday[a)]	Interday[b)]	Intraday[a)]	Interday[b)]		
Danofloxacin	1.3	4.5	4.2	6.2	5	0.9992	2.2	6.6	6.2	9.0	20	0.9908
Oxofloxacin	1.7	3.8	4.6	6.8	10	0.9996	2.8	6.9	6.3	10.0	20	0.9922
Enrofloxacin	2.0	4.5	4.8	7.2	18	0.9996	2.6	5.6	6.0	10.4	20	0.9918
Pipemidic acid	2.0	5.8	4.8	7.0	20	0.9998	2.8	4.9	6.2	10.2	20	0.9934
Flumequine	2.3	3.8	4.4	8.8	20	0.9997	3.0	7.2	6.8	12.3	20	0.9902

a) Consecutive injections (n = 6) of an extract obtained of a chicken sample spiked at 100 ng/g.
b) Injections carried out in four consecutive days (n = 4) of an extract obtained of a chicken sample spiked at 100 ng/g.
c) Mean of three injections of six different levels in the concentration range of 0.05–5 mg/g.

the migration times for the studied quinolones were less than 2.6 intraday and 5.1 interday. Single quadrupole and QIT did not show appreciable differences. Although for the peak area acceptable results were also obtained, the reproducibility was lower for QIT. The LODs were calculated from the SIM electropherograms in the single quadrupole and from the EIC electropherograms in the QIT of spiked samples at 50 ng g^{-1}. LODs using single quadrupole were from 5 to 20 ng g^{-1} and using QIT were 20 ng g^{-1} for the five quinolones. Using single quadrupole, excellent linearity was observed in the concentration range of 50–5000 ng g^{-1}, with a correlation coefficient better than 0.9992. On the

contrary, for the QIT instrument, the linearity calculated in the same conditions was worst with a correlation coefficient better than 0.9903. The limited dynamic range of the QIT has been widely discussed in the literature; it is an intrinsic characteristic of this mass analyzer. Although the narrow range for which the response of QIT is linear, quinolones can be correctly quantified at low concentrations. Results obtained for fish samples were very similar (data not shown).

Table V.4 shows the accuracy and precision obtained for quinolones extracted from chicken and fish spiked samples at 50 ng g^{-1} using QIT as mass analyzer. These parameters for samples spiked at higher concentrations of quinolones (500 ng g^{-1}) provided similar values (data not shown). Good recoveries were generally obtained, in both chicken and fish tissues, for four of the five quinolones tested. Flumequine gave lower recovery ≥45 % because it is thermolabile and there are some losses in the evaporation step. All other recoveries were in the range of 60–93 %. The RSDs (intraday), summarized in Table V.3, showed satisfactory levels.

Table V.4. Results for quinolones analysis in spiked chicken and fish samples (basal quinolones levels ,LODs) by CE-ESI-MS^3.

Quinolones	Chicken				Fish			
	Amount (ng/g)	RSD (%, *n* =5)	Recovery (%)	MRLs (ng/g)	Amount (ng/g)	RSD (%, *n* =5)	Recovery (%)	MRLs (ng/g)
Danofloxacin	50	12	75	200	50	12	78	100
Ofloxacin	50	10	99	200	50	9	90	n.e
Enrofloxacin	50	11	65	100	50	10	68	100
Pipemidic acid	50	12	81	n.e	50	13	84	n.e
Flumequine	50	15	45	400	50	16	52	200

The proposed method is sufficiently sensitive to analyse these quinolones in fish and chicken because the LOQs obtained were below the MRL (100–200 ng g^{-1}) established for these drugs in the Council Regulation 2377/90 of EU [10]. Figures V.2, V.3 show the chromatograms of a standard solution of

the five quinolones at the LOQ level, an extract of chicken muscle spiked at the LOQ level, and an extract of an incurred chicken sample that contains enrofloxacin obtained using single quadrupole and QIT, respectively. As can be seen, no interfering peaks from endogenous tissue constituents or baseline aberrations were observed. Although the results obtained by QIT are more selective, single quadrupole also provide clean chromatograms without interfering peaks.

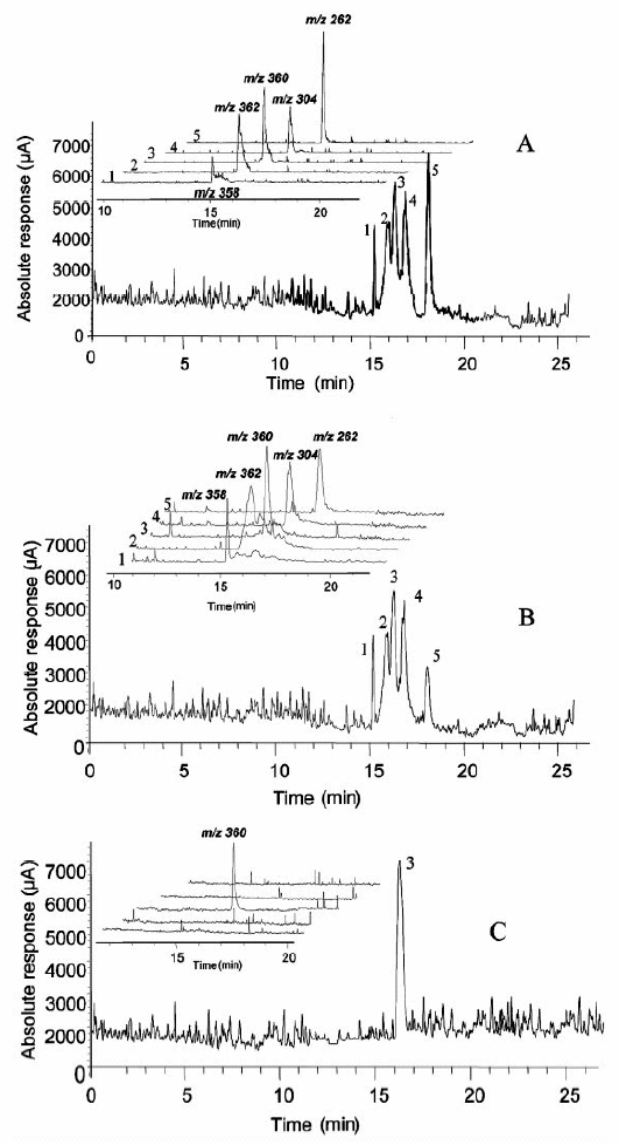


Fig. V.2. CE-ESI-MS electropherograms of a standard mixture of five quinolones at 0.5 mg mL^{-1} (A) of an extract of a chicken muscle spiked at 50 ng g^{-1} equivalent to a 0.5 mg mL^{-1} concentration in the injected extract (B), and incurred sample that contains 80 ng g^{-1} (C).

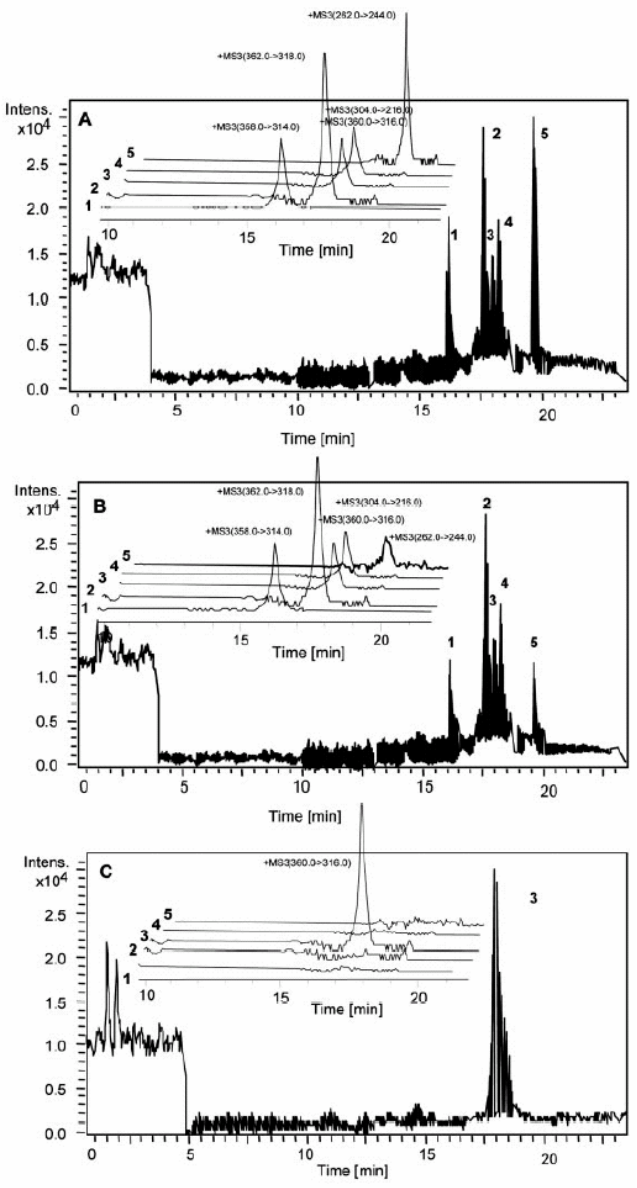


Fig. V.3. CE-ESI-MS^3 electropherograms of a standard mixture of five quinolones at 0.5 mg mL^{-1} (A) of an extract of a chicken muscle spiked at 50 ng g^{-1} (B), and incurred sample that contains 80 ng g^{-1} (C).

V.4.- Application to different samples

Several chicken and fish samples were taken from local markets of the Valencia city. These samples comprised 25 of chicken and 15 of fish (5 trout, 5

golden fish, 5 see bass). All the fish samples were from fish farm. None of the studied quinolones was detected in those samples.

The measurement of enrofloxacin in muscle samples of chicken, after oral administration (enrofloxacin 5 %) at a dose of 5 mg g^{-1}, confirmed the utility of the proposed analytical methodology. Figures V.2C, V.3C show CE-MS and CE-MS^3 electropherograms of treated chicken muscle extracts. The concentration found in the samples obtained 2 days after the treatment was ca. 80 ng g^{-1}.

VI.- PRESSURIZED LIQUID EXTRACTION COMBINED WITH CAPILLARY ELECTROPHORESIS-MASS SPECTROMETRY AS AN IMPROVED METHODOLOGY FOR THE DETERMINATION OF SULFONAMIDE RESIDUES IN MEAT

Extracción presurizada con disolvente, combinado con electroforésis capilar como metodología de mejora para la determinación de residuos de sulfonamidas en carne

VI.1.- Summary method developed

A new analytical method, based on capillary electrophoresis and tandem mass spectrometry (CE-MS^2), is proposed and validated for the identification and simultaneous quantification of 12 sulfonamides (SAs) in pork meat. The studied SAs include sulfathiazole, sulfadiazine, sulfamethoxypyridazine, sulfaguanidine, sulfanilamide, sulfadimethoxyne, sulfapyridine, sulfachloropyridazine, sulfisoxazole, sulfasalazine, sulfabenzamide and sulfadimidine. Different parameters (i.e. separation buffer, sheath liquid, electrospray conditions) were optimized to obtain an adequate CE separation and high MS sensitivity. MS^2 experiments using an ion trap as analyzer, operating in the selected reaction monitoring (SRM) mode, were carried out to achieve the required number of identification points according to the 2002/657/EC European Decision. For the quantification in pork tissue samples, a pressurized liquid extraction (PLE) procedure, using hot water as extractant followed by an Oasis HLB cleanup, was developed.

VI.2.- Optimization of SAs separation

Previous to the coupling with the mass spectrometer, an optimization of the electrophoretic separation was carried out using a DAD detector. The requirement for the subsequent connexion of CE to MS, of using a volatile buffer of low conductivity (i.e. electric current below 50 μA) to obtain a stable electrospray and to avoid plugging of the dielectric capillary between the spray chamber and the mass spectrometer was taken into account. Only ammonium acetate, ammonium formiate and ammonium carbonate buffers were studied, selecting the first one because better peak shapes were obtained. The pH and ionic strength of the background electrolyte and the addition of organic modifiers were considered to obtain the best selectivity, resolution and separation of the twelve SAs.

SAs are amphoteric compounds, and thus, behave both as acids and as bases depending on the pH. At high pH, the sulfonyl group, which is not directly attached to the ring, loses its proton to create a negative charge on the molecule. At low pH, the amine group is protonated and, therefore, a positive charge to the molecule is formed. The effect of pH (1.5–9.0) on the CE separation of SAs was evaluated. The results denoted that the examined pH range affected migrations of sulfixosazole, sulfadiazine and sulfanilamide in a greater extension than that of the other SAs. The migration time of each SA increased as pH rose from 6.0 to 9.0. In addition, the resolution among sulfasalazine, sulfabenzamide, sulfixosazole and sulfadimethoxyne decreased as the pH increased. Most of SAs could be baseline separated, with the exception of sufathiazole/sulfamethoxypyridine and sulfaguanidine/sulfanilamide, when pH is below 7.0. The 12 SAs had better separations in a pH 4.5 buffer, and the total separation was completed in less than 16 min.

The influence of the ammonium acetate concentration (15-60 mM) was also studied. The obtained results revealed that concentration of 50 mM provide a stable capillary current and good resolution. The consequence of adding an organic modifier to the background electrolyte on SAs separation was checked using different concentrations of methanol and acetonitrile, in the range of 5–30%. Imperceptible results were observed, reason why organic modifiers were not used. The effects of separation temperature and applied voltage on migration rate were studied, and 25 kV and 25 °C were utilized to obtain best separation. After optimization of the instrumental parameters, best separation of these SAs was obtained using 50 mM ammonium acetate/formic acid pH 4.26.

VI.3.- Optimization of CE-MS^n conditions

As it is mentioned above, coupling CE and MS implies the use of a sheath liquid, which permits a stable electrospray and the performance of the CE separation circuit. Different mixtures of volatile solvents, which provide electrical contact between CE and MS and a conductive modifier in terms of electrical contact, were tested. Mixtures of acetonitrile, methanol, ethanol, and 2-propanol with water were tested, all of them at 1: 1 v/v and containing 1 % formic acid. The presence of formic acid improved the signal of the SAs in the mass spectrometer because they are slightly basic compounds and the acidic pH favours their protonation. The highest intensity of the MS signal for the studied SAs maintaining, at the same time, the separation performance previously obtained by CE-DAD was achieved using methanol-water (1:1 v/v) with 1 % formic acid. The influence of the methanol and formic acid percentages in the sheath liquid were studied. The percentage of formic acid had no remarkable effect on the SA signals, probably, because at very low percentages of formic acid they already are totally in their cationic forms. A different methanol percentage, in any of both senses, lower or higher, produced a negative effect on

the response. Different parameters of the ESI source, such as nebulizer pressure, drying gas flow and drying gas temperature, were also tested. Drying gas temperature between 50-200 ºC has not effect in the signal of the analytes. Higher drying gas temperatures provided a decreasing in the signal, probably because those extreme temperatures caused some degradation of the compounds. Nebulizer pressure (2-10 psi) and drying gas flow rate (2-10 ml min^{-1}) had a positive effect on the response. The flow rate of the sheath liquid was also studied. The sensitivity increased when decreasing the flow rate of the sheath liquid, but at the same time, the instability of the system is increased, until the point that it became unavailable to keep going because the fall of the electric current. The flow rate studied ranged from 1 to 20 μl min^{-1}, and it was established at 4 μL min^{-1} as the best compromise between a good sensitivity and system stability.

Once the spray conditions were established using MS mode, the optimization of the MS^2 mode was carried out. Taking into account the confirmation requirements established in the European Commission Decision [139], and that some peaks are not well-resolved, the multiple reaction monitoring (MRM) mode of the instrument was the preferred option. Fragmentation was carried by means of collision-induced dissociation (CID) of the $[M+H]^+$ ions with the helium present in trap for 40 ms. The fragmentation steps for each compound were optimized visualizing the changes in the intensities of fragments ions, whereas the fragmentation cut-off and the fragmentation amplitude were manually varied. MS^2 characteristics for confirmation are shown in Table VI.1. As already reported [49, 129, 145-147], two major fragmentation pathways were observed in the MS^2 experiments by the bond cleavage between the sulfur and the nitrogen atom: the charge retention on the (4-aminophenyl) sulfonyl moiety to give generic ions at m/z 156 and 108,

and the charge retention on the heteroaromatic anime moiety to give compound-specific ions.

Table VI.1. Main parameters of the MS/MS method, precursor and product ions obtained, ratios of the product ions (with regards to the higher intensity one) and earned identification points.

SA	Precursor ion selected $[M+H]^+$ (*m*/*z*)	Product ion mass spectra (*m*/*z*)	Ions intensity (%)	Identification points (IPs)
Sulfasalazine	398	317	100	5.5
		156	80	
		108	20	
Sulfabenzamide	276	156	100	4
		108	20	
Sulfisoxazole	268	156	100	5.5
		113	20	
		108	20	
Sulfadimethoxyne	311	245	40	7
		218	28	
		156	100	
		108	15	
Sulfachloropyridazine	285	285	64	4
		156	100	
		108	20	
Sulfadiazine	251	251	10	5.5
		174	30	
		156	100	
		108	30	
Sulfamethoxypyridazine	281	265	20	11.5
		247	20	
		215	20	
		188	25	
		156	100	
		126	40	
		108	30	
Sulfathiazole	256	256	30	4
		156	100	
		108	20	
Sulfadimidine	279	279	20	5.5
		156	30	
		124	100	
		108	10	

Table VI.1. Continued

SA	Precursor ion selected [*M* +H]+ (*m*/z)	Product ion mass spectra (*m*/z)	Ions intensity (%)	Identification points (IPs)
Sulfapyridine	250	250	10	5.5
		184	70	
		156	100	
		108	20	
Sulfaguanidine	215	215	10	4
		156	100	
		108	15	
Sulfanilamide	173	173	10	4
		156	100	
		108	20	

VI.4.- Study of sample treatment

The utilization of PLE with hot water as extractant has proven to be suitable for the determination of SAs in meat, fish, milk, eggs and infant foods [148-151]. Among the solvents available for SAs extraction from pork tissues, water was chosen as extractant because of its low affinity towards fats and the polar character of the analytes. Additionally, water is cheap and environmental friendly. Temperature, extraction pressure, and static phase time were checked. Temperature (50-160 ºC) was the most important parameter in the extraction. The improvement of the recoveries was directly proportional to the temperature, with optimum values at 160 ºC.

The utilization of solid phase cartridges permits a sensitivity increase, preconcentrating the sample and eliminating the sample interferences. This alternative has already been applied for the clean-up of SAs extracts from eggs and meat [150-154]. In this work a polymeric phase cartridge (HLB) has been tested. The optimum retention was obtained at pH 7 observing elution of SAs at acidic and basic pH. Considering that the pH of the pork meat is between 6 and 7, it would be possible to apply the aqueous extract of the meat directly into the cartridge without the need of controlling pH. This procedure showed good

results in terms of recoveries and peak shapes, and a preconcentration factor of 10 was achieved.

VI.5.- Validation of the method

Representative CE-MS^2 electropherogram of pork muscle sample, fortified with a mixture containing 100 μg kg^{-1} of each SA, is presented in Fig. VI.1. The product ions (m/z 156 and 108) are the same for all the SAs, which can

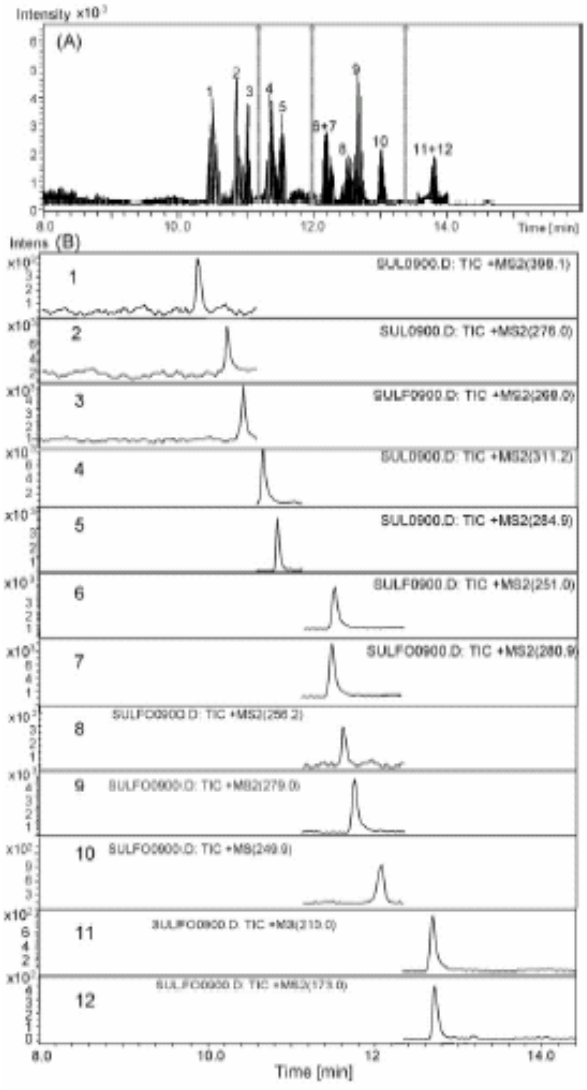


Fig. VI.1. CE–MS^2 electropherograms at optimum conditions obtained after PLE extraction and Oasis HLB clean-up and preconcentration of pork tissue samples spiked at 100 μg kg^{-1} of each SA (A) total ion electropherogram, and (B) extracted ion electropherograms.
Peak identification:
(1) sulfasalazine,
(2) sulfabenzamide,
(3) sulfisoxazole,
(4) sulfadimethoxyne,
(5) sulfachloropyridazine,
(6) sulfadiazine,
(7) sulfamethoxypyridazine,
(8) sulfathiazole,
(9) sulfadimidine,
(10) sulfapyridine,
(11) sulfaguanidine,
(12) sulfanilamide.
The dashed lines indicated each time segment programmed.

lead to cross-talk interferences with the SAs that coelute. The absence of these interferences were checked analyzing pork tissues spiked with each SA individually at 100 μg kg^{-1}. The resulting CE-MS^2 electropherograms, which only showed response for the investigated SA, gave almost identical response.

The statistic parameters, calculated by least squares regression and the performance characteristics are presented in Table VI.2. The reproducibility study was carried out at three levels of concentration (50, 100 and 200 μg kg^{-1}) using spiked pork tissues before the extraction procedure, only the values corresponding to the MRLs are showed in Table VI.2. RSD values obtained were lower than 2.4 % for migration times and lower than 3.8 % for the peaks areas within the same day (i.e. repeatability), while day-to-day precision RSD values were lower than 3.8 % for migration times and lower than 4.6 % for peak areas, showing that the procedure is reproducible. The linearity of the response was established from six calibration levels, between 50 and 500 μg kg^{-1}, injecting each level in triplicate and intending to establish the MRLs in the middle of the linear calibration range. Although previous calibration curves using standards prepared in methanol showed that MS response was linear at least two orders of magnitude, the studied working range was considered appropriate since real pork tissue samples with higher SAs content only occurs rarely. Table VI.2 also shows the calibration parameters as, for instance, calibration curve, correlation coefficient (r), Sy/x (standard deviation of residuals), LODs and LOQs. As it can be seen, a good linearity (r) higher than 0.996 was observed.

Table VI.2. Results of the intra-day and day-to-day precision study (both expressed as RSD%) (data given for 0.1μgkg^{-1}), calibration data (six points, three replicated of each), LOD (S/N = 3) and LOQ (S/N = 10) obtained by analyzing spiked pork muscle.

SA	t_m (min)	Intra-day precision (RSD %, n =5)		Day-to-day precision (RSD %, n =5)		Calibration equation	Sy/x	r^2	LOD (μgkg^{-1})	LOQ (μgkg^{-1})
		*t*m	Area	*t*m	Area					
Sulfasalazine	10.48	1.2	2.6	1.8	3.9	$y = (5{,}535 \pm 340)x - (56{,}571 \pm 2{,}495)$	0.032	0.997	6.25	21.3
Sulfabenzamide	10.95	1.3	2.8	2.0	3.6	$y = (11{,}001 \pm 1{,}125)x - (6{,}647 \pm 1{,}025)$	0.024	0.996	3.12	18.3
Sulfisoxazole	11.19	1.3	2.7	2.0	3.5	$y = (18{,}750 \pm 1{,}423)x - (34{,}845 \pm 1{,}678)$	0.039	0.997	1.56	14.0
Sulfadimethoxyn	11.42	1.2	2.9	1.8	3.7	$y = (42{,}473 \pm 3{,}628)x + (11{,}046 \pm 985)$	0.023	0.996	1.56	14.4
Sulfachloropyridazine	11.55	1.4	2.9	2.4	3.2	$y = (7{,}299 \pm 639)x + (12{,}974 \pm 1{,}340)$	0.045	0.996	3.12	18.7
Sulfadiazine	12.29	1.8	3.3	2.3	3.6	$y = (11{,}732 \pm 1{,}092)x + (2{,}783 \pm 283)$	0.021	0.996	1.56	14.6
Sulfamethoxypyridazine	12.31	1.7	3.5	2.7	3.9	$y = (20{,}091 \pm 1{,}762)x + (33{,}117 \pm 592)$	0.038	0.997	1.56	13.8
Sulfathiazole	12.63	1.9	3.2	2.6	4.2	$y = (12{,}438 \pm 1{,}098)x + (221 \pm 102)$	0.027	0.997	12.5	42.3
Sulfadimidine	12.84	1.6	3.0	2.8	4.3	$y = (18{,}158 \pm 1{,}535)x + (2{,}422 \pm 523)$	0.029	0.996	1.56	13.9
Sulfapyridine	13.04	2.0	3.8	2.8	4.6	$y = (29{,}524 \pm 2084)x - (6{,}110 \pm 610)$	0.036	0.996	5.56	21.3
Sulfaguanidine	13.82	2.2	3.8	3.8	4.6	$y = (13{,}674 \pm 1{,}032)x + (5{,}349 \pm 1{,}007)$	0.040	0.996	6.25	21.7
Sulphanilamide	13.84	2.4	3.6	3.4	4.6	$y = (12{,}857 \pm 1{,}054)x + (8{,}007 \pm 1{,}029$	0.042	0.996	6.25	22.0

In order to test the efficiency of the sample treatment, recovery studies at three concentration levels (50, 100 and 200 μg kg^{-1}) were carried out. Five replicates at each concentration were prepared, each one was injected in triplicate. Absolute recoveries were calculated by comparing peak areas of the pork tissue samples spiked before the PLE procedure with peak areas of pork tissue samples spiked after the PLE procedure. Recoveries higher than 76 % were obtained in all cases with acceptable RSDs. The results are shown in Table VI.3.

Table VI.3. Mean recoveries at half MRL, MRL and twice MRL levels for the selected SAs in pork muscle

SA	Concentration added (μgkg^{-1})	Mean recovery, % (RSDs, %)
Sulfasalazine	50	79 (9)
	100	80 (6)
	200	82 (4)
Sulfabenzamide	50	76 (11)
	100	81 (8)
	200	83 (7)
Sulfisoxazole	50	82 (9)
	100	87 (5)
	200	87 (4)
Sulfadimethoxyne	50	90 (12)
	100	86 (9)
	200	84 (6)
Sulfachloropyridazine	50	90 (12)
	100	95 (8)
	200	93 (6)
Sulfadiazine	50	91 (10)
	100	93 (8)
	200	96 (6)
Sulfamethoxypyridazine	50	92 (15)
	100	93 (13)
	200	91 (9)
Sulfathiazole	50	100 (12)
	100	99 (8)
Sulfadimidine	50	82 (10)
	100	85 (7)
	200	87 (6)

Table VI.3. Continued

SA	Concentration added (μgkg^{-1})	Mean recovery, % (RSDs, %)
Sulfapyridine	50	97 (10)
	100	98 (10)
	200	100 (7)
Sulfaguanidine	50	79 (14)
	100	83 (9)
	200	85 (5)
Sulfanilamide	50	92 (12)
	100	93 (9)
	200	91 (8)

Table VI.4 shows the CCα values with an error of 5% (probability of false non compliant ≤5%), considering the experimental standard deviation of within-laboratory reproducibility at the adequate contamination level and the CCβ values, calculated as the concentration at which the method is able to detect, identified and/or quantified MRL concentrations with a statistical certainty of (1 − β), considering the standard deviation of the within-laboratory reproducibility of the mean measured content at the decision limits, with an error β = 5% (probability of false compliant samples ≤5%). Validation was according to the draft EU criteria.

Table VI.4. CCα and CCβ values for studied SAs in pork muscle (MRL = $100\mu gkg^{-1}$)

SA	CCα (μgkg^{-1})	CCβ (μgkg^{-1})
Sulfasalazine	103	104
Sulfabenzamide	103	106
Sulfisoxazole	103	105
Sulfadimethoxyne	102	105
Sulfachloropyridazine	104	106
Sulfadiazine	103	104
Sulfamethoxypyridazine	104	107
Sulfathiazole	104	106
Sulfadimidine	104	108
Sulfapyridine	104	107
Sulfaguanidine	104	108
Sulfanilamide	104	109

VI.6.- Real sample analysis

The proposed method has been applied to determinate sulfadimidin in pork muscle samples from animals treated with this SA. Fig. VI.2A shows the electropherograms obtained from the extracts of the samples, from animals treated with SAs for 5 days and then slaughtered. The amount of sulfadimidin quantified was 400 μg kg^{-1}. As expected, in samples from animals immediately slaughtered, values of sulfadimidin are higher than MRL established by European Union.

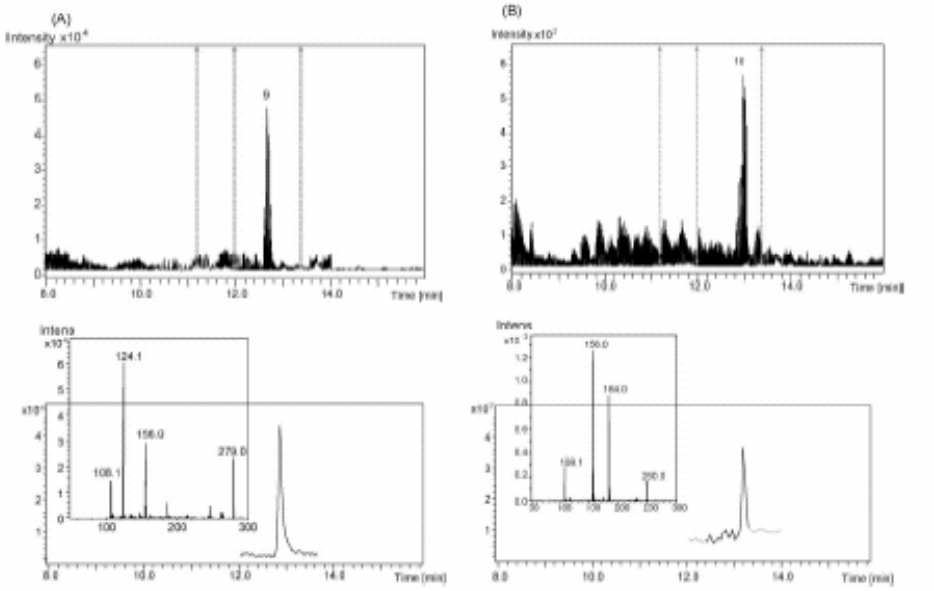


Fig. VI.2. CE–MS^2 electropherograms obtained after PLE extraction and Oasis HLB clean-up and preconcentration of pork tissue samples (A) treated with sulfadimidine (calculated concentration 400 μg kg^{-1}) and (B) from a butchery that contains sulfapyridine at 42 μg kg^{-1}. The upper electropherogram correspond to the total ion electropherogram. The lower to the extract ion electropherogram. The insert is the mass spectrum of each peak. Peak identification as in Fig. VI.1.

In addition, fifty swine muscle samples, randomly collected from the markets of Valencia city, were analyzed by the PLE-CE-MS^2 method. Two samples were contaminated with SAs. One contained 42 μg kg^{-1} of sulfapyridine

and the other 34 μg kg^{-1} of sulfadiazine. Fig. VI.2B shows the electropherogram corresponding to the sample that contains sulfapyridine. The product ion mass spectrum clearly confirms the identity of the compound.

Resumen

Summary

En la tabla 14 se detallan los plaguicidas y los medicamentos veterinarios que han sido objeto de estudio en esta tesis, las matrices en las que se han realizado los análisis y los LMRs para cada compuesto en cada matriz.

Tabla 14. Compuestos y LMRs (mg/kg) para las matrices estudiadas

	Matriz y LMRs (mg kg^{-1})				
Plaguicida	*Uva*	*Lechuga*	*Tomate*	*Fresa*	*Melocotón y nectarina*
Carboxin	0.2				
Flutriafol	0.01	0.01	0.01	0.01	
Tebuconazol	2	5	1	0.05	
Bitertanol	0.05	0.05	3	0.05	
Pirimetanil	5				
Triadimefon	2				
Fludioxonil	1	2	0.5	1	
Ciproconazol	0.1	0.01	0.05	0.01	
Tiabendazol					0.05
Acrinatrin	0.05	1	0.1	0.2	
Miclobutanil	1	0.02	0.3	1	
Piriproxifen	0.05	0.05	1	0.05	
Pirimicarb					0.5
Dinoseb	0.05	0.05			0.05
Procimidona	5	5			2
Pirifenox					0.2
Carbendazima	2	5			
Medicamentos veterinarios	*Carne de pollo*		*Pescado*		*Carne de cerdo*
Quinolonas					
Danofloxacina	$0.2\ 10^{-3}$		$0.1\ 10^{-3}$		
Ofloxacina	$0.2\ 10^{-3}$		n.e		
Enrofloxacina	$0.1\ 10^{-3}$		$0.1\ 10^{-3}$		
Ácido pipemídico	n.e.		n.e.		
Flumequina	$0.4\ 10^{-3}$		$0.2\ 10^{-3}$		
Sulfonamidas: *sulfasalazina, sulfabenzamida, sulfadimetoxina, sulfanilamida sulfadiazina, sulfisoxazol, sulfatiazol, sulfadimidina, sulfapiridina, sulfaguanidina, sulfacloropiridazina, sulfametoxipiridazina,*					0.1

n.e: no establecido

Aunque los niveles de plaguicidas y medicamentos veterinarios encontrados en la mayoría de las muestras son más bajos que los establecidos por la UE, es necesario realizar controles periódicos de los residuos contenidos en frutas, verduras y alimentos de origen animal. La presencia de residuos de estos no es un problema de salud publica, sino que también presenta repercusión económica debido a que la mayor parte de la producción se destina a la comercialización en países donde se realizan controles periódicos sobre los alimentos importados, que pueden rechazarse en caso de resultar positivas, con la pérdida de los mercados que ello supone.

En la definición de residuo se incluyen no sólo los productos de partida sino los productos de degradación tóxicas, y que pueden ser en algunos casos más tóxicos y persistentes que los compuestos que los originaron. Por tanto los métodos que se desarrollen deben ser capaces, no sólo de detectar los residuos de los plaguicidas que se aplicaron a lo largo del periodo de cultivo o en postcosecha o de los medicamentos veterinarios que se aplicaron durante las fases de desarrollo de los animales, sino también debe ser capaz de detectar los productos resultantes del metabolismo de los mismos.

La selectividad de la CL-EM y la EC-EM permite analizar extractos concentrados obtenidos de alimentos a partir de procedimientos de extracción multirresiduos rápidos y simples, en los que es posible eliminar etapas de purificación largas y tediosas que constituyen un foco de pérdidas de los plaguicidas y medicamentos veterinarios presentes en las muestras y en consecuencia de una disminución de la sensibilidad analítica.

Desde el siglo pasado las separaciones analíticas se llevaban a cabo por métodos clásicos como son la precipitación, destilación y extracción. Los crecientes esfuerzos en el campo de la investigación han obligado a buscar técnicas más precisas. Para elegir una técnica de separación, hay que atender a

dos tipos de consideraciones: las propiedades físicas y estructurales de las moléculas que se pretende separar, o las características de la matriz en que se encuentran. El método de selección incluye los pasos necesarios para la obtención, preparación y posible fraccionamiento de la muestra, la aplicación de la técnica analítica adecuada y el tratamiento de los datos obtenidos.

Cualquiera que sea de las técnicas de separación que se utilice, bien EC o CL y posterior determinación, se precisa de técnicas de extracción previas y debidamente optimizadas para las matrices y analitos que se vayan a estudiar.

Los tres métodos de extracción estudiados en esta tesis han sido:

Extracción en fase sólida (EFS)

Extracción sobre barras magnéticas (ESBM)

Extracción presurizada con disolventes (EPD)

Los métodos de extracción se validaron de acuerdo a las normas de la Unión Europea realizando los siguientes ensayos:

Límite de cuantificación (LDC o LOQ): es la mínima concentración adicionada a la muestra que permite obtener una recuperación aceptable (> 70 %) con una desviación estándar relativa inferior al 20 %. Para técnicas especiales, el valor de recuperación de 70 % puede ser menor.

Linealidad: se inyectan por triplicado un mínimo de 5 concentraciones diferentes que incluyan el LOQ y que cubra dos órdenes de magnitud, y se calcula la regresión lineal y coeficiente de correlación (r).

Exactitud y precisión: se calcula el porcentaje de recuperación medio y la desviación estándar relativa media en muestras adicionadas de plaguicidas y antibióticos al límite de cuantificación y aun nivel diez veces superior.

Efecto de matriz: comparan las regiones lineales, como mínimo de 5 concentraciones diferentes de los compuestos preparados en metanol y en extractos de muestras a la misma concentración.

1.- Extracción

Para la extracción en fase sólida (EFS) y extracción sobre barras magnéticas (ESBM) los parámetros de relevancia fueron: volumen de agua, cantidad de muestra que se procese y fuerza iónica del medio. Estos parámetros se estudian viendo su efecto en las recuperaciones de los compuestos. La elección de los disolventes adecuados para conseguir la extracción de los analitos es uno de los factores más importantes y más estudiado en la literatura ya que una de las causas de error en la determinación es la extracción incompleta a partir de la matriz.

En cambio para la extracción presurizada con disolventes (EPD/PLE) los parámetros importantes en esta técnica fueron: temperatura, presión de extracción y tiempo estático (tiempo de contacto del disolvente con la matriz y analitos), siendo la temperatura uno de los parámetros claves en este proceso de extracción.

1.1 Extracción de carboxin, flutriafol, pirimetanil, triadimefon, tebuconazol y bitertanol en uva.

La mezcla de disolventes idónea para llevar a cabo la EFS en este estudio fue la formada por diclorometano (DCM)-metanol. Esta mezcla proporcionó las mejores recuperaciones y los extractos más limpios. En cambio, la desorción de los compuestos de la barras magnéticas para la ESBM fue

utilizando 0.5 ml de metanol o bien de acetonitrilo y agitando a continuación durante 15 minutos [107-112].

Modificaciones de la fuerza iónica, mediante la adición de NaCl, mejoró las recuperaciones en la ESBM. Se estudiaron diferentes proporciones de NaCl: 10, 20 y 30 %. Los resultados óptimos se obtuvieron cuando se utilizó un 30 % de NaCl, alcanzándose recuperaciones de hasta casi el doble que sin la adición de NaCl.

Se probaron diferentes volúmenes de agua (10-100 ml) cuyos resultados se recogen en la Tabla I.4. Las recuperaciones obtenidas en EFS para estos volúmenes fue de aproximadamente el 100 % para la mayoría de compuestos independientemente del volumen utilizado, excepto para carboxin, la recuperación del cual se redujo a la mitad al utilizar volúmenes superiores a 50 ml, y para tebuconazol que fue inferior al 40 % cuando se trabajó con un volumen inferior a 25 ml. Para la ESBM, los resultados son bastante dispares, por lo que se seleccionaron 25 ml que proporcionó buena sensibilidad y permitió una adecuada disolución de la muestra.

Otro de los parámetros que se estudió fue la influencia en la eficacia de las técnicas de extracción (ESBM y la EFS), por interferencias de la matriz (uva). La Tabla I.5 recoge los resultados de las recuperaciones que se obtienen en las dos técnicas de extracción para el rango de cantidades de 2 a 10 g. Se eligieron 5 g de muestra, ya que proporcionó las mejores recuperaciones y la mejor sensibilidad.

La ESBM está basada en un equilibrio de adsorción, por lo que requiere de un tiempo adicional para realizar la extracción y una temperatura adecuada. En la figura I.2 se recogen los resultados obtenidos. El tiempo de extracción

seleccionado fue de 120 min, el cual proporcionó un tiempo de análisis razonable.

Las interferencias de la matriz son, para el análisis cuantitativo, uno de los problemas de mayor interés que se hace evidente al comparar la intensidad de la señal obtenida en una disolución patrón (en metanol). La ausencia de estos efectos de matriz mediante la ESBM es un factor de especial relevancia recogido en la literatura [111] mientras que en la EFS los efectos de matriz estan presentes en ocasiones tras realizarse procesos de purificación.

1.2 Extracción de flutriafol, ciproconazol (isómeros R y S), miclobutanil, tebuconazol, acrinatrin, bitertanol, fludioxonil y piriproxifen en lechuga, tomates, uva y fresas.

La EFS llevada a cabo en este estudio, se realizó utilizando acetona como disolvente eluyente, de fácil evaporación y que evita la pérdida o extracción incompleta de plaguicidas.

Con el fin de obtener los límites de cuantificación (LDC) bajos se estudiaron las influencias de la cantidad de muestra. La figura II.2 recoge cómo influyó este parámetro. Se observa que no existen variaciones significativas en las recuperaciones hasta 15 g para las matrices de lechuga y tomate. Sin embargo no ocurre lo mismo para las matrices de uva y fresa debido a la elevada viscosidad que se obtiene en el extracto. La máxima cantidad de muestra con la que se puede trabajar con estas matrices fue de 5 g, ya que para cantidades mayores las recuperaciones disminuyeron, para la mayoría de los compuestos. En la figura II.3B se muestra un electroferograma obtenido de un extracto de lechuga tras EFS de 15 g de muestra, en el que se observa la ausencia de picos interferentes en el análisis de los compuestos.

En la tabla II.2 se presentan los LDC, inferiores a 0.5 mg kg^{-1}, de acuerdo a la Unión Europea (UE) [11], el Codex Alimentarius [8] y el U.S. Food and Drug Administration [116]. Se recoge también la precisión y las recuperaciones para muestras adicionadas al LDC.

Durante el proceso de ESBM, las condiciones óptimas de desorción se obtuvieron por exposición de las barras magnéticas a 1 ml de metanol durante 15 minutos en un vial de vidrio. Este volumen permitió que la barra magnética estuviese completamente sumergida en el disolvente. Con estos parámetros se obtuvieron buenas recuperaciones y pocas interferencias.

Se estudió la alteración de la fuerza iónica mediante la adición de NaCl a la muestra. Influyó de forma significativa en la adsorción de los plaguicidas a las barras magnéticas y en consecuencia también en las recuperaciones. Tras estudiar varias concentraciones de NaCl, los mejores resultados se obtuvieron con disoluciones saturadas de NaCl (40 %).

Otro de los parámetros que afectan a la eficacia de la ESBM es el tiempo de extracción. En la figura II.4 se recoge el perfil para cada plaguicida a los diferentes tiempos de extracción estudiados. Para este estudio se necesitaron 240 minutos.

La cantidad de muestra para el análisis de los compuestos es una de las limitaciones de la ESBM por las posibles interferencias de matriz. Se obtuvieron recuperaciones aceptables para el análisis de 5 g de muestra, mientras que para cantidades mayores se impedía la extracción de ciproconazol y flutriafol.

La especificidad del método se representa en la figura II.5 en el electroferograma de una muestra de tomate sin adicionar (blanco, en la figura II.5A) en la que se muestra la ausencia de interferencias de la matriz con los picos de los plaguicidas estudiados.

En la tabla II.3 se recoge la linealidad, coeficientes de correlación, recuperaciones y DSR.

Comparando ambos procesos por parámetros analíticos recogidos en la tabla II.4, se observa que aunque la ESBM es menos práctica que la EFS, al menos como técnica de análisis de rutina, presenta algunas ventajas frente a la EFS como son: electroferogramas más nítidos y mejor precisión.

Los LDC obtenidos marcan la diferencia entre ambos procesos de extracción, debido a que la EFS combinada con la electroforesis capilar electrocinética micelar con detector de UV permite la detección de los plaguicidas estudiados a unos niveles inferiores a los Límites Máximos de Residuos (LMRs). Sin embargo, los LDC obtenidos por la ESBM son demasiado altos como para alcanzar los LMRs, lo que justifica su uso tan reducido como técnica analítica. La EFS es una técnica rápida, y aunque presente algunas desventajas, no se observaron en el desarrollo de este estudio, una vez optimizadas las condiciones.

1.3 Extracción de tiabendazol, pirifenox, pirimicarb, pirimetanil, procimidona y dinoseb en melocotones y nectarinas.

Los plaguicidas estudiados para las matrices de melocotones y nectarinas se extrajeron utilizando agua-acetona, y se concentraron con EFS, utilizando como eluyente DCM. El DCM, es un disolvente halogenado, que proporciona recuperaciones elevadas del analito y casi no extrae interferentes de la matriz. En la figura III.3 se muestra un electroferograma obtenido por EC-ESI-EM/EM de una muestra de melocotones adicionada.

Se llevó a cabo un estudio de interferencia de la matriz comparado con el gráfico de calibración obtenido con un patrón preparado en tampón y en

extractos de fruta y se observó que las interferencias de matriz eran prácticamente despreciables.

En la tabla III.3 se recogen las recuperaciones, DSR y precisiones para los LDC y los LMRs establecidos por la legislación española [1], los cuales coinciden con los establecidos por la (UE) [11] para dinoseb, procimidona, y tiabendazol, y para pirimicarb con los recomendados por el Codex Alimentarius [8].

1.4 Extracción de danofloxacina, ofloxacina, enrofloxacina, ácido pipemídico y flumequina en carne y pescado.

El método de extracción se basó en una modificación de un método publicado [155]. El método permitió concentrar el analito hasta 10 veces con el fin de mejorar la sensibilidad.

Se homogeneizaron 5 g de muestra (carne o pescado) en un tubo de centrifuga de 20 mL. Las muestras de carne fortificadas se prepararon añadiendo un volumen adecuado de las disoluciones estándar de quinolonas (de 10 a 100 μL). Las muestras adicionadas se dejaron durante 15 min a temperatura ambiente con el fin de asegurar la correcta distribución de las quinolonas en la matriz. Posteriormente la muestra se mezcló con 5 mL de 5 mM sodio dihidrógeno fosfato (pH 7) en agitación durante 10 min, y se extrajo dos veces consecutivas con 20 y 10 mL de DCM mediante agitación durante 5 min. Las fases orgánica y acuosa se separaron por centrifugación durante 5 min a 2500 rpm. La fase orgánica se recogió en un tubo de centrifuga de 40 mL y se extrajo dos veces con 5 mL de NaOH 0.5 M. La fase acuosa se separó del DCM centrifugando 5 min a 2500 rpm, y posteriormente se transfirió a un vial para ajustar a pH 7 con fosfórico 200 mM y se desengrasó con 10 mL de hexano. El

extracto acuoso se hizo pasar a través de una columna de C18, previamente activada con 10 mL de metanol y 10 mL de agua, a un flujo de 2 mL min^{-1}. La elución de las quinolonas se llevó acabo con 2 mL de TFA en agua al 4 % - ACN (25: 75 v/v), seguido de 1 mL de ACN.

El eluato se recogió en un tubo cónico y se concentró llevándolo a sequedad con nitrógeno y redisolviendo con 0.5 mL de disolución tampón.

El método se validó aplicándolo a muestras de tanto de carne de pollo como de pescado, adicionadas con diferentes niveles de mezcla de patrón de quinolonas.

1.5 Extracción de las sulfonamidas (SAs): sulfasalazina, sulfabenzamida, sulfisoxazol, sulfadimetoxina, sulfacloropiridazina, sulfadiazina, sulfametoxipiridazina, sulfatiazol, sulfadimidina, sulfapiridina, sulfaguanidina, sulfanilamida en carne.

La utilización de agua caliente para la EPD ha demostrado ser eficaz para la determinación de sulfonamidas (SAs) en carne, pescado, leche, huevos y alimentos infantiles [148-151]. Hay una gran variedad de disolventes para conseguir la extracción de SAs. Para la extracción de carne de cerdo, se eligió agua por su baja afinidad con la grasa y el carácter polar de los analitos. Por otra parte el agua es un disolvente económico y no perjudica el medio ambiente, características en la actualidad, de especial relevancia.

Se estudió la temperatura en el proceso de extracción (50-160ºC) y se observó una relación directa entre las recuperaciones y la temperatura. Los mejores resultados se consiguieron a 160 ºC.

La utilización de cartuchos de fase sólida permite un incremento de la sensibilidad, preconcentración de la muestra y eliminación de algunos compuestos interferentes de matriz. Esta alternativa, aplicada recientemente

para la purificación de extractos de SAs de huevos y carne [150-154], se llevó a cabo en este trabajo, con cartuchos de fase sólida polimérica (HLB). La retención fue óptima a pH 7 y elución de las SAs a pH básico y ácido. Teniendo en cuenta que el pH de la carne de cerdo oscila entre 6 y 7, es posible aplicar los extractos acuosos de carne directamente en los cartuchos sin la necesidad de controlar el pH. Este procedimiento proporcionó resultados buenos de recuperaciones, agudeza de picos, y se alcanzó un factor de precocentración del orden de hasta 10 veces.

2.- Técnicas de preconcentración

2.1 Técnicas de preconcentración en línea por electroforesis capilar electrocinética micelar para el análisis de dinoseb, procimidona, fludioxonil, carbendazima y piriproxifen en uva y lechuga.

Los métodos o técnicas de preconcentración más interesantes y que incrementan la sensibilidad de la técnica, son aquellas que evitan una manipulación de la muestra, es decir, los métodos en –línea.

Cuando se aplicó este método para la determinación de residuos de plaguicidas en frutas, fue necesario realizar una EFS como técnica de preconcentración previa (off-line) de los plaguicidas de estudio para las matrices: uva y lechuga.

En la figura IV.2 se representa el efecto de los tiempos de inyección en las áreas de los picos por los tres métodos de preconcentración en línea estudiados. Se observa que el área de los picos aumenta proporcionalmente con el tiempo de inyección. Sin embargo la dependencia del área del pico no es lineal en ninguno de los casos. Por ejemplo procimidona y piriproxifen parecen presentar una dependencia exponencial y para dinoseb hay un máximo en el

caso de SRW. El tiempo de inyección de 60 segundos, fue el más adecuado reflejado en la agudeza de los picos. Sin embargo, como se puede observar en la figura IV.3, la aplicación de las técnicas de acumulación provocan ciertas pérdidas de resolución (comparado con la figura IV.1), especialmente entre piriproxifen y carbendazima, y algunos picos aumentan su ensanchamiento, especialmente en SRW y SRMM. La falta de linealidad puede explicarse porque tras un tiempo de inyección largo, (superior a 60 seg), los picos muestran un ensanchamiento y una agudeza asimétrica. Los cambios que se observan en el área de los picos da la impresión de ser dependiente del soluto, que puede relacionarse con los valores de pKa o log P de los compuestos estudiados. Sin embargo no hay valores de pKa para todos los compuestos estudiados, solamente para carbendazima y dinoseb, y no hay ninguna relación con el log P, que es de 1.38 para carbendazima, 3.08 para procimidona, 3.56 para dinoseb, 4.12 para piriproxifen y 5.37 para fludioxonil. Esta falta de linealidad o aparente exponencialidad del área del pico con el tiempo de inyección ha sido estudiada por otros autores [126, 127], quienes sugieren que la longitud de inyección de la zona de la muestra esta limitada por el efecto de dispersión como consecuencia de una falta de velocidad electroosmótica entre las zonas de alta y baja conductividad.

El la figura IV.3 se muestra el electroferograma obtenido por análisis SW, SRW y SRMM RM-MECK-DAD de una mezcla estándar que contenía 1 $\mu g ml^{-1}$ de cada analito. Se incluye además una inyección convencional para comparar. Se observó un aumento en la sensibilidad de alrededor de 10-, 30- y 50- veces de detección para SW, SRW y SRMM respectivamente, comparado con inyecciones hidrodinámicas estándar. De entre las técnicas de concentración en línea estudiadas, SRMM proporcionó las respuestas más altas en los detectores (>50-veces comparado con una inyección convencional). Las técnicas de acumulación (SRMM y SRW) alcanzaron los mejores resultados

comparado con SW porque estos compuestos son moderadamente polares. Sin embargo, la agudeza de los picos obtenido por SW fue más eficaz y de mejor capacidad de separación. Los tres procesos de preconcentración en línea proporcionaron resultados aplicables para la determinación de plaguicidas a niveles de concentración bajos. Los tiempos de retención obtenidos son ligeramente diferentes a las concentraciones del método porque la solución en el cual los estándar se disuelven son diferentes, coincidiendo con otros autores, tal y como se recoge en la literatura [68].

En la tabla IV.1 se resume y comparan los valores de media de los tiempos de retención, linealidad, límites de detección (LOD), las desviaciones estándar relativas (RSD, %) del área de los picos y los tiempos de migración para inyecciones de 0.5 s a 5 psi, para SW, SRW y SRMM, respectivamente.

La linealidad de estos métodos se probó utilizando cinco concentraciones diferentes dentro del rango de 0.2–50 mg ml^{-1} y realizándose tres replicas. Las repuestas de los plaguicidas fue lineal para los rangos de concentración estudiados con coeficientes de correlación (r) > 0.991 para todos los casos. Los resultados de las ecuaciones de regresión se registran en la tabla IV.1. Los límites de detección (S/N = 3) de los cinco compuestos fueron 0.01–0.04 $\mu g ml^{-1}$, (SW), 0.01–0.3 $\mu g ml^{-1}$ (SRW) y 0.0002–0.01 $\mu g ml^{-1}$ (SRMM). La reproducibilidad (RSDs) de los métodos propuestos en términos de áreas de picos para réplicas de ocho inyecciones fueron entre 6.82–11.61 % para SW, entre 3.95 y 9.47 % para SRMM y 2.22–4.94 % para SRW. Los tiempos de migración fueron entre 2.35 y 2.70 % SW, entre 1.30 y 1.54 % SRMM y 2.47 y 3.76 %.

Los plaguicidas estudiados se determinaron por MECK o CZE. Con MECK y utilizando como tampón de separación 6 mmol l^{-1} de sodio tetraborato decahidratado y 75 mmol l^{-1} ácido cólico a pH 9, se separaron ocho plaguicidas [128]. Inyecciones hidrodinámicas convencionales (5 s, 0.5 psi) proporcionaron

LOD de 0.1 μgml^{-1}. Utilizando el tampón de 0.3 M de acetato de amonio-ácido acético pH 4 con 10 % de metanol para CZE se separaron procimidona y dinoseb entre otros plaguicidas. Los LDC obtenidos utilizando detector de UV e inyecciones convencionales fueron diez veces superiores a los que se alcanzan con este trabajo [129]. Sin embargo utilizando detectores de MS y MS/MS los LDC mejoran, al final, en un factor de diez con el incremento importante en selectividad y especificidad. Sin embargo, con ninguno de los procesos de preconcentración en líneas ensayadas en este estudio permitió mejorar los LODs mencionados anteriormente en un factor de 10.

3.- Separación

3.1 Separación electroforética.

3.1.1 *Separación de flutriafol, ciproconazol (isómeros R y S), miclobutanil, tebuconazol, acrinatrin, bitertanol, fludioxonil y piriproxifen*.

La optimización del tampón de separación en electroforesis capilar electrocinética micelar (MEKC), se llevó a cabo estudiando varios parámetros como: pH, concentración del tampón de separación, utilización de metanol como modificador orgánico y utilización de diferentes tensioactivos a diferentes concentraciones. La figura II.1 recoge las condiciones electroforéticas optimizadas para la separación de los compuestos de este estudio.

El pH del tampón de separación se estudió entre 4 y 10 para los tampones de borato 6 mM y colato sódico 75 mM. Los tiempos de migración disminuyeron con el pH (Figura II.1A) y el tiempo de análisi más corto con buena resolución entre los analitos se alcanzó con pH 10. No obstante, se trabajó con pH de 9.2, el cual aunque incrementó el tiempo de análisis, permitió

alargar el tiempo de vida media de los capilares, ya que a valores de pH elevado la pared interna del capilar se deteriora más rápidamente.

La influencia de la molaridad del tetraborato sódico en la separación de los plaguicidas se estudió en un rango de 6-30 mM utilizando un tampón de 75 mM de colato sódico pH 9.2 (Fig II.1B). Para todos los analitos el tiempo de migración se mantuvo hasta concentraciones de 10 mM, después aumentó hasta 20 mM y posteriormente se mantuvo de nuevo hasta 30 mM. Tras los resultados obtenidos se eligió como óptimo el tampón de tetraborato sódico en concentración de 6 mM.

Se estudió la adición de los tensioactivos: colato sódico y sodio dodecil sulfato (SDS). La resolución de los picos fue mejor con colato sódico, por lo que fue de elección, y permitió, además, la separación entre los dos isómeros de ciproconazol. Para concentraciones entre 25 y 125 mM de colato sódico, se obtuvo un comportamiento diferente. El orden de elución de lo plaguicidas fue prácticamente el mismo, sin variaciones importantes, pero sí lo fueron en cuanto a la resolución, siendo de especial relevancia el incremento de 25 a 75 mM, y más acusado en el incremento de 75 a 125 mM (figura II.1 C). Esto puede explicarse por el aumento de la fuerza iónica en el tampón de separación, y a la interacción entre los analitos y las micelas por las concentraciones elevadas de colato. La mejor separación se obtuvo con una concentración intermedia, de 75 mM de colato.

Se estudió el efecto de la adición de metanol como modificador orgánico entre un 0 y un 25 %, para mejorar la resolución y la velocidad del análisis (Figura II.1D). Se observó que un aumento en el porcentaje de metanol, el tiempo de análisis se incrementa, pero no se observa mejoras en la resolución de los analitos, por lo que se obvió la utilización de modificador orgánico.

La tabla II.1 recoge algunos de los parámetros analíticos como repetibilidad, reproducibilidad, DSR y límites de detección para el método propuesto. La separación completa de los ocho plaguicidas estudiados se obtuvo con resoluciones > 1. La repetibilidad y reproducibilidad de los tiempos de migración fue bastante elevada y las DSR que se obtuvieron no fueron superiores al 2 y 3 % respectivamente. En cambio, la repetibilidad y reproducibilidad del área de los picos no fue tan buena; las DSR para los diferentes analitos fueron del rango de 2.1-5.3 % y 2.9-5.8 % respectivamente. Los LODs estimados fueron tres veces la señal de ruido fondo, 0.1 μg ml^{-1} para bitertanol, fludioxonil y piriproxifen, y 0.5 μg ml^{-1} para el resto de plaguicidas.

Los resultados manifiestan que la separación electroforética por electroforesis capilar electrocinética micelar (MEKC) es un sistema útil, simple, y rápido para separar, identificar y determinar ocho plaguicidas con sensibilidad suficiente, para el análisis en frutas y verduras.

Se concluyo que con MEKC, previa extracción por fase sólida, es posible analizar un buen número de muestras de frutas y vegetales en un periodo corto de tiempo, utilizando un método rápido, de bajo coste y de rutina para la monitorización de residuos de plaguicidas.

3.1.2 ***Separación de fludioxonil, procimidona, dinoseb, carbendacima y piriproxifen.***

Los compuestos mencionados se estudiaron por técnicas de pre-concentración mediante la separación por MECK reversa (RM-MECK), en la que la dirección del flujo electroosmótico (EOF) se invierte para conseguir la supresión de la muestra y preconcentración. El EOF puede invertirse principalmente por dos procedimientos (i) a pH ácido por reducción del EOF sin que se suprima la movilidad electroforética de las micelas aniónicas y (ii) por la

adición de aditivos que se absorben en las paredes del capilar cargándolas positivamente [27, 125, 126].

La separación de los 5 plaguicidas por RM-MEKC se optimizó probando diferentes tampones de separación que consistían en mezclas de soluciones fosfato (10–100 mmol l^{-1}) y SDS (15–150 mmol l^{-1}) a diferente pH (2–5) por inyección hidrodinámica directa (5 s a 0.5 psi) de una disolución estándar de 5 $\mu g ml^{-1}$ de cada compuesto preparado en el tampón de separación. De acuerdo con los valores de pKa de los diferentes compuestos carbendazima (pKa 4.48) y dinoseb (pKa 4.62) se deberían utilizar valores de pH alrededor de 5 ya que a valores más bajos de pH, estos compuestos están cargados positivamente.

Los resultados experimentales fueron contradictorios a la teoría: cuanto más ácido es el pH, mejores separaciones se consiguen. Se obtuvieron resultados similares a los publicados por da Silva et al. [68], que separaron nueve plaguicidas de clases diferentes, incluida la carbendazima. De entre los diferentes tampones de separación probados, la mezcla que contenía 20 mmol l^{-1} de fosfato y 25 mmol l^{-1} de SDS a pH 2.3 proporcionaron los mejores resultados aunque piriproxifen y carbendazima fue se superpusieron parcialmente.

La separación entre ambos compuestos se consiguió añadiendo un 10 % de metanol al tampón anterior, conclusión que se alcanzó tras probar diferentes disolventes (metanol, acetonitrilo, etanol e isopropanol) en diferentes proporciones 2 y 15 %.

Por último, se estudió el efecto del voltaje aplicado y la temperatura para así acortar el máximo posible el tiempo de análisis. La temperatura varió entre 20 y 30 ºC y el voltaje entre 16 y 25 kV. Se obtuvieron resultados óptimos cuando se aplicó un voltaje de 20 kV a 20 ºC. En la figura IV.1 se muestra la separación de una disolución estándar de 5 $\mu g ml^{-1}$ de los plaguicidas estudiados en el tampón de separación óptimo.

3.2 Separación electroforética con acoplamiento posterior a espectrómetro de masas.

El acoplamiento de la EC a la EM implica optimizar la separación de los compuestos mediante la utilización de tampones volátiles (ácido fórmico, ácido acético, carbonato amónico o acetato de amonio) de baja conductividad (por ejemplo de corriente eléctrica por debajo de 50 μA) con el fin de obtener un electrospray estable y evitar posibles obturaciones entre el capilar de la cámara del espray y el EM.

El pH, la fuerza iónica del tampón y la adición de modificadores orgánicos también son de especial relevancia con el fin de obtener la mejor selectividad, resolución y separación entre los compuestos que se estudien

3.2.1 *Separación de dinoseb, pirimicarb, procimidona, pirifenox pirimetanil y tiabendazol.*

Para estos compuestos se estudió el tampón ácido acetato de amonio/ácido acético a pH 3, 4, 5, y 7. Los mejores resultados tanto en resolución como en tiempo de análisis fueron a pH 4.

Se estudió la relevancia de la concentración del tampón de separación en el tiempo de migración y en la resolución de los picos para los seis plaguicidas con el tampón acetato de amonio/ácido acético desde 0.01 a 0.6 mM a pH 4. Un incremento de la fuerza iónica de tampón de separación por aumento de su concentración, redujo la adsorción de los analitos a la pared del capilar y mejoró la agudeza de los picos. Los plaguicidas estudiados se resolvieron con tampones de molaridad elevada (> 0.2 M). Se escogió la molaridad de 0.3 M que proporcionó una buena relación entre la separación de los compuestos y la conductividad eléctrica del tampón. La molaridad influyó en la corriente del

capilar que en EC-UV fue de 180 µA (siendo 300 µA el máximo que puede alcanzar el equipo) y cuando se acopló a EC-EM fue de 87 µA.

El efecto de la adición de modificadores orgánicos en el tampón de separación se estudió con metanol y acetonitrilo en un rango entre 5 y 3 %. Cuanto mayor fue la proporción de metanol, mayor fue la resolución entre los picos, (de 5 a 15 %), y los tiempos de análisis disminuyeron. Para adiciones superiores al 30 % de metanol, la resolución no mejoró y el tiempo de análisis se incrementó, lo que provocó un ensanchamiento de los picos. Con un 10 % de metanol se mejoró la resolución entre los picos sin alargar de forma exagerada el tiempo de análisis.

Estudios de la temperatura del capilar demostraron que un aumento de la misma (15ºC - 35º C) disminuye el tiempo de migración de los analitos debido a una disminución de los coeficientes de distribución y de la viscosidad del tampón. A bajas temperaturas (< 25ºC) no se observaron mejoras en la resolución de los picos ya que el valor absoluto de temperaturas no interfiere en la separación, si el efecto Joule, (responsable del ensanchamiento de los picos), no es excesivo. Se observó que a temperaturas de 30-35 ºC la resolución disminuía (sobretodo entre los dos últimos picos) debido a que en este caso el efecto Joule era elevado, aparecían diferencias de viscosidad en el tampón, y aumentaba la zona de deformación del frente de los analitos. La temperatura óptima de elección fue de 25ºC.

3.2.2 ***Separación de danofloxacina, ofloxacina, enrofloxacina, ácido pipemídico y flumequina***.

Las quinolonas que se estudian, cuatro tienen un grupo carboxílico y un enlace piperazinilo incluyendo grupos amino (figura 2), que pueden ser catiónicos, anfóteros o aniónicos, dependiendo del pH. Esto explica el porque la

importancia del análisis de quinolonas por EC alrededor de un cierto rango de pH con separación selectiva muy variada. Sin embargo, la flumequina tiene solamente un grupo carboxílico cargado negativamente a pH básico. Algunos estudios han demostrado que a pH básico (entre 8 y 9.50) se obtiene una separación mejor de las quinolonas que a pH ácido o fuertemente alcalino [39, 66, 134]. Para obtener una separación óptima se utilizó como tampón de separación carbonato amónico 60 mM, pH 9.2, 25 kV y presión de 30 mbar durante la inyección, la separación de las 5 quinolonas se consiguió en 20 min.

3.2.3 ***Separación de las sulfonamidas (SAs): sulfapiridina, sulfametoxipiridazina, sulfatiazol, sulfaguanidina, sulfacloropiridazina, sulfanilamida, sulfadiazina, sulfisoxazol, sulfabenzamida, sulfadimetoxina sulfasalazina y sulfanilamida.***

La separación óptima de las SAs mencionadas se realizó mediante el estudio de los tampones de acetato de amonio, formiato amónico y carbonato amónico. El tampón de elección fue el primero ya que proporcionó la mejor resolución de los picos.

Las SAs son compuestos anfóteros, por lo que poseen comportamiento tanto ácido como básico, dependiendo del pH. A pH elevado, el grupo sulfonilo, que no está unido directamente con el anillo, pierde su protón para crear una molécula de carga negativa. A pH bajo, el grupo amino está protonado a pesar de la carga positiva de la molécula que se forma.

Se evaluó el efecto de pH (1.5–9.0) en la separación por EC de las SAs. Los resultados revelaron que en este rango de pH la migración de sulfisoxazol, sulfadiazina y sulfanilamida cambiaba con respecto a las otras SAs. El tiempo de migración de cada SAs aumentó con el pH desde 6.0 a 9.0, disminuyó la resolución entre sulfasalazina, sulfabenzamida, sulfixosazol y sulfadimetoxina

cuando el pH aumentó. Muchas de las SAs pueden separarse de la línea base, a excepción de sulfatiazol/sulfametoxipiridacina y sulfaguanidina/sulfanilamida, cuando el pH estuvo por debajo de 7.0. La mejor separación de las 12 SAs fue a pH 4.5 y en 16 min se consiguió la separación completa.

Se estudió también la influencia de la concentración de acetato de amonio (15-60 mM). De acuerdo con los resultados obtenidos, cuando la concentración del tampón fue de 50 mM se consiguió una corriente de capilar estable y buena resolución. Se estudiaron diferentes concentraciones de metanol y acetonitrilo, como modificadores orgánicos en el tampón de separación para la separación de las 12 SAs, en el rango de 5-30 %. No se obtuvieron resultados perceptibles, por lo que no se usaron modificadores orgánicos en la separación. Tras el estudio del efecto de la temperatura y del voltaje aplicado durante la separación los mejores resultados se obtuvieron con 25 kV y 25 ºC. Tras la optimización de los parámetros instrumentales la mejor separación de las SAs se obtuvo con el tampón de 50 mM de acetato de amonio/ácido fórmico pH 4.26.

4.- Determinación

4.1 *Determinación de dinoseb, procimidona, fludioxonil, carbendazima y piriproxifen en frutas y verduras por electroforesis capilar electrocinética micelar y técnicas de preconcentración en línea.*

Se analizaron muestras de lechuga y uva fortificadas y sin fortificar a 0.01 mg kg^{-1} y a los LMRs por EFS, RM-MECK-DAD utilizando los tres métodos de preconcentración en línea con las condiciones optimizadas previamente descritas (SW, SRW y SRMM). Los electroferogramas obtenidos de los extractos de lechuga se muestran en la figura IV.4. Se observa que no existen interferencias de la matriz así como tampoco variaciones en la agudeza

de los picos y en los tiempos de retención por lo tanto el método propuesto es viable para estas matrices. Los extractos de uva fueron un poco más limpios que los de lechuga, posiblemente debido a la ausencia de clorofila y otros compuestos coloreados. En la figura IV.5 se muestra un electroferograma de muy poca resolución de un extracto de uva utilizando SRW, no obstante es mucho más limpio y claro que el que se obtiene con el de lechuga.

Las recuperaciones y precisiones se recogen en la tabla IV.2 y IV.3 así como el porcentaje de las DSR. Se alcanzaron recuperaciones >70% para todos los plaguicidas siendo un poco superiores para fludioxonil y piriproxifen tanto en uva como en lechuga. Estos valores estuvieron entre el 72 y el 98 %, con DSR desde 13 a 19 % para SW, entre 75 y 97 % con DSRs desde 11 a 19 % para SRW y entre 70 y 100 % para SRMM. No obstante las recuperaciones fueron similares para todos los métodos y la precisión estuvo dentro de los límites establecidos por la legislación de la Unión Europea (EU) [11].

En la tabla IV.4 se muestran los límites de cuantificación y los LMRs establecido en diferentes legislaciones [8, 11, 133]. Los LOQ se calcularon de acuerdo a la UE como los niveles más bajos que proporcionan recuperaciones (>70%) y reproducibilidad (<20%) aceptables [130]. Los LOQ para RM-MECK-DAD para estos plaguicidas fue 0.008-0.01 mg kg^{-1} para SW, 0.004–0.01 mg kg^{-1} para SRW y 0.001–0.01 mg kg^{-1} para SRMM. Los LOQ más bajos se alcanzaron con SRMM, después SRW y finalmente con SW. La detección más baja la proporcionó el método de preconcentración SRMM probablemente debido a habilitadla posibilidad de introducir grandes cantidades de muestra en el capilar. Comparado con otros métodos explicados en los apartados anteriores para alguno de los plaguicidas estudiados, los LOQ se mejoraron más de 10 veces lo que permitió alcanzar los LMRs más bajos establecidos en la legislación [128, 129].

4.2. Determinación por espectrometría de masas.

En la determinación por EM, los compuestos se ionizan en modo positivo (IP) o en modo negativo (IN) dependiendo de las propiedades ácido-base del compuesto. Los fenoles y los ácidos se ionizan con mayor facilidad en modo negativo mientras que las bases lo hacen en modo positivo. Sin embargo muchos plaguicidas y antibióticos, independientemente de su carácter ácido o básico, contienen heteroátomos en la molécula que permiten la ionización en ambos modos.

4.2.1 ***Determinación de carboxin, flutriafol, pirimetanil, triadimefon, tebuconazol y bitertanol.***

La determinación de estos compuestos se realizó utilizando CL acoplada a la EM con interfases de ionización química a presión atmosférica (IQPA o APCI) y de electrospray (ESI) en modo positivo y negativo. La tabla I.2 resume la estructura química, peso molecular, base de los picos y los iones abundantes (con la abundancia relativa). Todos los fungicidas estudiados dieron respuesta en modo positivo y negativo con las dos interfases (APCI y ESI), excepto el bitertanol que no dio respuesta con electrospray.

La interfase de ESI en modo positivo proporcionó mayoritariamente moléculas protonadas y los aductos de sodio con mayor intensidad de señal. Solamente carboxin presentó un ion correspondiente al fragmento neutro que se genera tras la pérdida de anilina. El aducto de sodio es el ion mayoritario para flutriafol, triadimefon y tebuconazol, mientras que pirimetanil no crea aductos de sodio, confirmando la teoría de que para la formación de aductos de sodio se necesita de un grupo dador de un par de electrones. Carboxin, flutriafol, triadimefon y tebuconazol contienen grupos carboxilo e hidroxilo que no existen en la molécula de pirimetanil.

La interfase de ESI en modo negativo da lugar a moléculas desprotonadas como ion mayoritario en triadimefon y en pirimetanil. El ion mayoritario en el espectro del flutriafol es el fragmento obtenido por la pérdida neutra de fluorobenceno, en el espectro de carboxin el fragmento obtenido por la pérdida de etano, y el único ion obtenido en el espectro de tebuconazol es el fragmento resultado de la pérdida del enlace metiltriazol.

Todos los fungicidas estudiados tienen espectro de masas similares con la fuente de APCI, pero no ocurre así con la fuente de ESI. Por otra parte en modo positivo, no se obtienen aductos de sodio pero sí algunos fragmentos de iones. El carboxin proporciona el ion de mayor proporción originado por la pérdida neutra de anilina. Flutriafol, triadimefon y bitertanol sufren la pérdida neutra de 69 u de la molécula, que corresponde al anillo triazóilico. La fuente de APCI en modo negativo proporciona espectros similares a ESI para carboxin, flutriafol y tebuconazol. Triadimefon proporcionó el ion característico de m/z 127 correspondiente a la pérdida de *p*-clorofenol, y el bitertanol dio lugar al fragmento m/z 269 correspondiente a p-fenilfenol. En la figura I.1 se representan algunos espectros de la fragmentación de masas obtenida.

En la tabla I.3 se recogen los datos de los límites de cuantificación obtenidos en modo scan. La respuesta estaría entre 250 pg para los seis fungicidas con interfase de APCI en modo positivo, de mayor sensibilidad, frente a la ausencia de respuesta de bitertanol con interfase de ESI. La interfase de ESI osciló, entre 25 y 100 veces menos de sensibilidad, que con APCI. Teniendo en cuenta estos resultados, la APCI en modo positivo fue de elección para llevar a cabo el resto de experimentos. La sensibilidad del sistema se optimizó utilizando el modo SIM, de acuerdo con el procedimiento detallado en la tabla I.1.

A modo de resumen se puede decir que los compuestos estudiados fueron bien caracterizados con las interfases a presión atmosférica (API) tanto en modo positivo como en modo negativo.

4.3. Determinación simultánea por EC-EM.

Para la determinación simultánea EC-EM se han de considerar diferentes parámetros del EM como son: composición y flujo del líquido envolvente, flujo del gas de secado, y longitud del capilar sobresaliente del tubo del líquido envolvente.

Uno de los parámetros críticos para el sistema EC-EM es la composición del líquido envolvente, ya que será el encargado de cerrar el circuito eléctrico entre la EC y la fuente de electrospray, y la distancia entre lo que sobresale del capilar y la aguja de electospray, esta distancia puede variar entre 0.05 y 1.5 mm.

Otros factores que pueden interferir en la eficacia de la separación y en los tiempos de migración como son: la movilidad de los enlaces iónicos, la depleción del tampón (hacia el final del capilar de la EC en una fuente caliente de ESI), la entrada del líquido envolvente por difusión hacia el capilar de la EC, posibles reacciones en la aguja de la fuente de ESI que puede dar lugar a cambios de pH, efectos de ventosa producidos por flujo del gas envolvente, alteración del flujo electroosmótico por el campo eléctrico que se introduce a través de la pared del capilar de sílice, también se han de tener en cuenta.

Otro factor importante a considerar es el efecto presión invertida ("backpressure") que ocurre como consecuencia de la diferencia de presión entre el vial de inyección y el vial de salida [129]. La sobrepresión se detecta por un retraso en los tiempos de retención y en la pérdida de señal del analito

que se soluciona manteniendo los dos extremos del capilar a la misma altura, con una diferencia aproximadamente de 0.5 cm de los niveles del espray, que se puede conseguir poniendo ambos equipos en la mima bancada de trabajo, sin ningún otro ajuste en particular.

4.3.1 ***Determinación de tiabendazol, pirifenox, pirimicarb, pirimetanil, procimidona y dinoseb.***

Se estudio la composición del líquido envolvente mediante el efecto de la adición de metanol y la concentración del tampón de separación. Los mejores resultados se obtuvieron cuando la composición del líquido envolvente fue la misma que la del tampón de separación. El flujo del líquido envolvente se estudió entre 3 y 15 μL min^{-1}. Se observó que a flujo de 3 μL min^{-1} afectó negativamente a la estabilidad del spray disminuyendo la intensidad de los picos, mientras que a flujos elevados no se obtuvieron picos con más áreas y la agudeza del pico de la procimidona empeoró. Con un flujo de 5 μL min^{-1} se obtuvieron los mejores resultados. Se obtuvo la máxima respuesta de los analitos cuando se utilizo un flujo de gas de secado de 7 μL min^{-1}, ya que para flujos más elevados o más bajos la respuesta disminuyó debido a la inestabilidad del electrospray.

La distancia óptima entre el capilar y la aguja de la fuente de ESI que proporcionó la máxima resolución fue de 0.1 mm desde el tubo del líquido envolvente

En la figura III.1 se muestran los electroferogramas obtenidos con UV y EM en las mejores condiciones. El acoplamiento de la EC a la ESI-EM proporcionó buenos resultados para la separación de los seis plaguicidas, comparables, y con una separación de los plaguicidas muy similares a las obtenidas con la EC-UV. Los resultados que recoge la literatura son un poco

controvertidos: algunos autores apuntan que la eficacia en la separación obtenida por la EC-ESI-EM son claramente inferiores a las obtenidos por EC-UV [57, 58, 66, 69], mientras que otros han demostrado que la eficacia en la separación no son necesariamente inferiores a las obtenidas por EC-UV [119]. La explicación de esta controversia seria que el tubo del líquido refrigerante del adaptador externo del detector (EDA), de una longitud de 15 cm, para este estudio se cambió por uno de la misma longitud que el utilizado en un cartucho convencional. Esto justifica la pérdida de resolución cuando la EC se acopla a un detector de EM debido a la pequeña proporción de capilar que está termostatizado. La "cola" o ensanchamiento que se observa en algunos picos cuando se utiliza un detector UV se debe principalmente a que por el desgaste del capilar las adsorciones son más "fuertes" en la pared del capilar.

Las diferencias en los tiempos de migración se deben a las distintas conexiones entre EC-UV y EC-EM. El capilar utilizado en la EC-UV es de 57 cm (de los que sólo 50 cm son efectivos), mientras que utilizando un detector de EM externo se necesitan capilares de 75 cm de longitud (de los que sólo 50 cm están termostatizados). Debido a que la movilidad de los iones es proporcional a la longitud del capilar. Cuanto más largo sea el capilar, mayor será el tiempo de migración de los compuestos.

Se estudió el efecto de presión invertida ("backpressure") tras observarse un retraso en los tiempos de retención y pérdida de señal del analito. La solución es mantener los dos extremos del capilar a la misma altura, lo que supone ± 0.5 cm de los niveles del spray. Esto se alcanzó disponiendo una tabla ajustable por debajo del equipo de EC. Con frecuencia esta posición se ha alcanzado situando ambos equipos en la misma bancada de trabajo, sin ningún otro ajuste en particular. Los valores adecuados de DSR para tiempos de retención y áreas de picos es una evaluación clara de la ausencia del efecto de

presión invertida. (tabla III.2). El acoplamiento de EC con el EM trampa de iones, se llevo a cabo por el mismo procedimiento ya que el diseño de Agilent System y de Bruker Daltonik System es el mismo, y no presentan ninguna diferencia en el mecanismo de aplicación del potencial eléctrico (se ven los detalles más adelante (pag 229-230).

4.3.2 *Determinación de quinolonas: danofloxacina, ofloxacina, enrofloxacina, ácido pipémidico y flumequina.*

El efecto del pH en el líquido envolvente se probó utilizando tres soluciones: 0.5 % de ácido acético en agua (pH 3), 60 mM acetato de amonio (pH 7) y 60 mM de carbonato amónico en agua (pH 9.2) El EM proporcionó mejores señales cuando se utilizó carbonato amónico 60 mM. La utilización de soluciones básicas (pH 9.2) no implicó una disminución de la sensibilidad comparado con el pH bajo del líquido envolvente.

La adición de disolventes orgánicos en el líquido envolvente (como metanol, acetonitrilo e isopropanol) se estudió, pero ni la señal en EM ni la estabilidad de la interfase de electrospray mejoraron; de modo que las pruebas se llevaron a cabo con tampón corbonato amónico 60 mM.

El efecto del flujo del líquido envolvente en la sensibilidad de ESI se estudió para el rango de 2-15 µl min^{-1}. La aplicación de presión en el capilar para la separación, se estudió y se vió que estabilizaba el espray pero disminuía el tiempo de análisis [137]. La aplicación de 30 mbar aumentó el flujo en 0.2 µl min^{-1}. Tras realizar estudios del efecto de flujo en la sensibilidad se concluyó que a flujos altos (15 µl min^{-1}), la sensibilidad disminuye bastante, probablemente debido al efecto de dilución con el mismo líquido envolvente. A flujos bajos (< 6 µl min^{-1}), la sensibilidad disminuye muy poco, probablemente debido a que estos flujos tan bajos pueden estabilizar la fuente del espray. En

este estudio, con flujos entre 8-12 µl min^{-1} se conseguía la mayor estabilidad y sensibilidad. Los experimentos se llevaron a cabo con un flujo de 10 µl min^{-1}, un valor relativamente alto comparado con los propuestos por otros autores que utilizan EC [66, 129, 138]. La razón puede ser la temperatura del capilar del electrospray la cual es mucho mayor que en estos estudios ya que debe trasladar el disolvente, y el agua tiene un elevado punto de ebullición comparado con los disolventes orgánicos [138].

4.3.3 ***Determinación de las sulfonamidas (SAs): sulfapiridina, sulfametoxipiridazina, sulfatiazol, sulfaguanidina, sulfacloropiridazina, sulfanilamida, sulfadiazina, sulfisoxazol, sulfabenzamida, sulfadimetoxina sulfasalazina y sulfanilamida.***

El estudio de líquido envolvente se realizó con mezclas de acetonitrilo, metanol, etanol y 2-propanol con agua, todos en proporción 1:1 v/v con un 1 % de ácido fórmico. La presencia de ácido fórmico mejoró la señal de las SAs en el EM ya que son ligeramente compuestos básicos y el pH ácido favorece su forma protonada. La separación se llevó a cabo con las condiciones óptimas obtenida por EC-DAD, metanol-agua (1:1 v/v) con un 1 % de ácido fórmico. Se estudio el porcentaje de la influencia del metanol y del ácido fórmico. El porcentaje de ácido fórmico no tuvo efectos notables en la señal de las SAs, probablemente debido a que a bajos porcentajes de ácido fórmico están totalmente en su forma catiónica. Un porcentaje de metanol, alto o bajo, produjo una respuesta con efecto negativo.

Se estudiaron diferentes parámetros de la fuente de ESI: presión del nebulizador, flujo del gas de secado y temperatura del gas de secado. La temperatura del gas de secado (50-200 ºC) no produjo ningún efecto en la señal en los analitos, a temperaturas elevadas la señal disminuyó, probablemente debido a que en estos extremos de temperatura se produce degradación de los

compuestos. La presión del gas nebulizador (2-10 psi) y el flujo del gas de secado (2-10 ml min^{-1}) produjeron un efecto positivo en la respuesta de los analitos. Cuando se estudió el flujo del líquido envolvente la sensibilidad aumentó cuando este disminuyó, pero a la vez, la inestabilidad del sistema aumentó, hasta el punto de que hacerlo inviable por la caída de corriente. El rango de flujo estudiado fue entre 1 y 20 µl min^{-1} y se estableció como óptimo 4 µL min^{-1} ya que proporcionó buena sensibilidad y estabilidad del sistema.

Una vez se establecieron las condiciones del espray (modo ESI), se procedió a la optimización de EM^2. Teniendo en cuenta los requisitos de confirmación establecidos por la Comisión Europea [11], y que algunos picos no se resolvían bien, el modo de monitorización de reacción múltiple (MRM) fue la opción preferida a desarrollar. La fragmentación se llevó a cabo por medias de la colisión inducida por disociación (CID) de los iones $[M+H]^+$ con el helio presente en la trampa durante 40 ms. Los eslabones de fragmentación para cada compuesto se optimizaron visualizando los cambios en la intensidad de los iones fragmentados, mientras que la fragmentación cut-off y la amplitud de la fragmentación se cambió manualmente. Las características de EM^2 para su confirmación se muestran en la tabla VI.1. Como se ha recogido en algunas publicaciones [49, 129, 145-147], las dos vías alternativas de fragmentación mayoritariamente observadas en experimentos de EM^2 son la fragmentación por el enlace de unión entre el átomo de sulfuro y el átomo de nitrógeno; la retención de carga en la molécula de (4 aminofenil) sulfonilo da lugar a iones genéricos de m/z 156 y 108, y la retención de la carga de la amina heteroaromática da lugar a iones específicos de los compuestos.

4.4. *Determinación por espectrometría de masas en tándem (EM/EM).*

Las ventajas del empleo de la EC y/o CL acoplada a la EM son la reducción de los pasos correspondientes a la preparación de la muestra, y a la alta sensibilidad y selectividad de la técnica que permite el análisis de compuestos tóxicos a niveles traza. Sin embargo, una de las mayores desventajas es que empleando el simple cuadrupolo, no siempre se alcanzan los criterios de identificación establecidas por la Unión Europea en el documento Nº SANCO/10476/2003, por lo que otra técnica debe ser aplicada para la confirmación del análisis.

4.4.1 *Determinación de tiabendazol, pirifenox, pirimicarb, pirimetanil, procimidona y dinoseb.*

Los únicos iones observados en modo scan, fueron los de la molécula protonada $[M+H]^+$ o desprotonada $[M-H]^-$. Los voltajes de las lentes se ajustaron hasta obtener la máxima respuesta para moléculas protonadas y desprotonadas de los analitos, que fueron elegidas como iones precursores en los siguientes experimentos de EM^n. La fragmentación de los iones $[M+H]^+$ o $[M-H]^-$ (EM^2) y colisión inducida por disociación del ión de fragmentación mayoritario (EM^3) normalmente proporcionan fragmentos originados por la fragmentación de cadenas laterales o por la apertura de anillos heterocíclicos.

Se alcanzó el análisis de masas hasta un tercer estadío con el tiabendazol (m/z 202) con una secuencia de fragmentación: m/z 202→ m/z 175→ m/z 131. El EM^2 de tiabendazol se caracterizó por la pérdida de HCN (ácido nítrico) del anillo tiazólico. Esta pérdida da lugar a un ion abundante de m/z 175, y la pérdida posterior de CS por la rotura del anillo tiazólico da lugar al ion m/z 131. Sucesivas fragmentaciones del ion m/z 175 da lugar a m/z 131 lo que confirma la ruta de fragmentación.

El espectro de masas EM^2 de $[M+H]^+$ para pirifenox pone en evidencia la apertura del enlace de oxima dando una señal de gran intensidad a m/z 265 que corresponde al ion $[M+H-CH_3OH]^+$. Si se prosigue en la fragmentación de este ion, da una señal de m/z 230 ocasionado por la pérdida de una molécula de HCl que se verificó con EM^3.

El pirimicarb es un plaguicida representativo de los carbamatos, en su espectro de masas EM^2 se observaron dos iones mayoritarios m/z 182 y m/z 195, que derivan de la pérdida de los grupos neutros de CH_3NCO y $HN(CH_3)_2$ respectivamente. En el siguiente espectro, (EM^3 del ion m/z 182) se forma el ion m/z 137 como resultado de la pérdida del grupo $HN(CH_3)_2$ localizado en la posición meta del grupo carbámico. La fragmentación de la molécula protonada de pirimetanil de lugar a más iones de m/z 184, por la pérdida de metano. El EM^3 de m/z 184 da lugar al ion m/z 167 por la pérdida de otro grupo de metano.

La figura III.2 muestra los espectros de masas: EM, EM^2 y EM^3, para patrones estándar de procimidona y dinoseb obtenidos en EC-ESI-EM en modo de barrido y EC-ESI-EM^n con el ion producto escaneado. La procimidona en su espectro de masas EM^2 da lugar al ion específico m/z 256 por la pérdida neutra de CO; su EM^3 da lugar a fragmentos de m/z 162, 178, 186, 198, 212, 228 y 240 que se intentan identificar en la figura 3. Por el contrario, dinoseb es un fenol que mayoritariamente forma el ion $[M-H]^-$ a pH 4 aunque de acuerdo a su pK_a (4.82) los grupos hidroxilo deberían ser predominantemente no iónicos. Este fenómeno, conocido como "camino erróneo" ("wrong-way-around") se ha descrito para CL-ESI-EM [123] y para EC-ESI-EM [66]. El EM^2 pone en evidencia una señal intensa a m/z 194 que corresponde con $[M-H-NO_2]^-$, la fragmentación de este ion da lugar a un EM^3 con un ion mayoritario que es el m/z 164 que corresponde con la pérdida neutra de NO y un fragmento adicional a m/z 134 por la pérdida de una molécula de etano. De las diferentes rutas de

fragmentación se eligió aquella que fue la más dominante (table III.1). El cut-off y la amplitud se fijaron de acuerdo a la máxima intensidad obtenida del ion producido.

4.4.2 *Determinación de quinolonas: danofloxacina, enrofloxacina, ofloxacina, ácido pipemídico y flumequina por EC-EM trampa de iones.*

En la figura 2 y V.1 se muestra la estructura química y el peso molecular (PM) de las quinolonas estudiadas. El espectro de masas que se obtuvo con EM simple cuadrupolo mostró como ion principal, el correspondiente al de la molécula protonada $[M+H]^+$ siendo para danofloxacina m/z = 358, m/z = 360 para enrofloxacina, m/z = 362 para ofloxacina, m/z =304 = para ácido pipemídico y m/z = 262 para flumequina. De acuerdo con la Directiva 657/2002/EC [139] las moléculas protonadas equivalen a un punto de identificación (IP), insuficiente para la confirmación.

Se estudiaron las vías de fragmentación de las quinolonas mediante EM-trampa de iones para facilitar la identificación de las mismas. En la tabla V.2 se muestran los productos de los iones precursores utilizando trampa de iones y estadíos múltiples. El primer orden del espectro de masas muestra el pico correspondiente a la molécula protonada de cada quinolona, que fue sujeto a la detección por colisión inducida (CID) para producir fragmentos de iones EM^2. Uno de estos fragmentos se aisló y refragmentó para dar lugar a EM^3. La detección inducida por colisión (CID) se llevo a cabo sobre el ion de interés, por colisión con el gas de helio presente en la trampa, durante 40 ms.

Los iones producto del EM^3 de las quinolonas muestran que los iones mayoritarios fueron m/z 283 para danofloxacina, m/z 245 para enrofloxacina, m/z 261 par ofloxacina, m/z 189 para ácido pipemídico y m/z 202 para flumequina. De acuerdo con la Comisión Europea [11], utilizando la trampa de

iones, cada producto del ion precursor "gana" 1.5 puntos IP. La monitorización de dos de las transiciones de los iones precursores, proporciona una confirmación inequívoca. Un ejemplo de los espectros de los estadíos múltiples y una hipotética interpretación de los iones se muestran en la figura V.2 para enrofloxacina, que es la quinolona para la que más niveles se alcanzan.

4.5. *Comparación entre los analizadores de masas.*

4.5.1 *Simple cuadrupolo y trampa de iones para la determinación de tiabendazol, pirifenox, pirimicarb, pirimetanil, procimidona y dinoseb en nectarinas y melocotones.*

Los resultados de estudios de reproducibilidad, linealidad y sensibilidad de los sistemas de detección se muestran en las tablas III.2 y III.3. La reproducibilidad fue óptima y se obtuvieron DSR para los tiempos de migración (n=10) superiores a 1.2 y para las áreas de los picos del orden de 3.9 y 8.9. La reproducibilidad en los tiempos de migración fue muy similar para todos los sistemas de detección, mientras que para las áreas de los picos fueron mejor por EC-UV (entre 3.9 y 5.8) que por los otros sistemas de detección (4.3-8.9). A pesar de las diferencias en los sistemas de EC-ESI-EM, no se observaron diferencias en las DSRs lo que pone de manifiesto que una fragmentación reproducible es posible cuando se trabaja con cada plaguicida individualmente. Las rectas de calibrado fueron lineales para EC-UV a concentraciones entre 8 y 80 $\mu g\ ml^{-1}$, para EC-EM simple cuadruplolo entre 1.5 y 150 $\mu g\ ml^{-1}$ y para EC-trampa de iones con los diferentes estadios de masa estudiados entre 0.05 y 50 $\mu g\ ml^{-1}$. Los coeficientes de correlación oscilaron entre 0.990 y 0.998. Los gráficos de calibración mostraron buena correlación entre los 6 plaguicidas estudiados por cualquiera de los sistemas de detección.

Mediante detección UV, los límites de detección, (calculados como tres veces la relación señal- ruido) fue, 1 μg ml^{-1} para dinoseb, pirimicarb y procimidona y 2 μg ml^{-1} para pirifenox pirimetanil y tiobendazol. Aunque las estructuras químicas de los plaguicidas estudiados son muy diferentes entre sí figura 2, los límites de detección fueron muy similares posiblemente debido a la aproximación de los coeficientes de adsorción o extinción molar de los analitos. Los límites de detección obtenidos con EC-EM simple cuadrupolo fueron entre 0.05 y 0.5 μg ml^{-1}. Los límites de detección obtenidos con EM trampa de iones oscilaron entre 0.001 y 0.015 μg ml^{-1} para EM, de 0.005 a 2 μg ml^{-1} para EM^2 y de 0.009 a 1.4 μg ml^{-1} para EM^3. De acuerdo con los límites de detección obtenidos se puede deducir que la EM y la EM^2 son buenos comparado con la EC acoplado a UV o a EM simple cuadrupolo en modo SIM. Ambos métodos, EC-EM simpla cuadrupolo y EC-UV, parecen ser adecuados para la determinación de los plaguicidas estudiados a niveles inferiores a los LMRs. Por el contrario los límites de detección obtenidos por EC-ESI-trampa de iones-EM^3 fueron superiores a los LMRs para pirifenox, pirimetanil y dinoseb.

Debido a la cantidad de compuestos orgánicos y otros contaminantes presentes en las frutas, hay interferencias, por lo que se precisan de métodos muy selectivos para poder cuantificar los plaguicidas sin la interferencia de otros compuestos. La EM/EM identifica los compuestos y por ello la EC-ESI-EM/EM se utilizó para experimentos posteriores.

El método descrito permite, de forma simultánea, directa y sensible la determinación de residuos de plaguicidas en frutas. Comparado con otras técnicas, la electroforesis capilar acoplada a la espectrometría de masas (ESI-MS) presenta las ventajas significativas siguientes: el espectro de masas (MS^n) proporciona información estructural, y la seguridad de identificación es considerablemente alta. La sensibilidad, reproducibilidad y linealidad son

buenas, los plaguicidas se pueden detectar selectivamente sin interferencias de otros compuestos y la preparación de la muestra es mínima. Estos resultados indican que esta aproximación puede ser aplicable en muchos campos como el análisis de plaguicidas en alimentos.

4.5.2 *Simple cuadrupolo y trampa de iones para la determinación de quinolonas: danofloxacina, enrofloxacina, ofloxacina, ácido pipemídico y flumequina*

El método se validó usando muestras tanto de carne de pollo como de pescado, adicionadas con diferentes cantidades de mezcla de patrón de quinolonas y sujetos al proceso de extracción descrito anteriormente. Los límites de detección y de cuantificación se definieron respectivamente con la señal correspondiente a 3 y 10 veces la desviación estandard. El método propuesto fue validado en términos de repetitividad y reproducibilidad de tiempo de migración y de área del pico, límite de detección y linealidad. Los resultados obtenidos para las muestras de pollo están resumidos en la tabla V.3. La repetitividad se calculó a partir de seis análisis cuantitativos (en un mismo día) y la reproducibilidad a partir de 4 días consecutivos para el mismo extracto (en días diferentes).

Las DSRs de los tiempos de migración para las quinolonas estudiadas fueron inferiores a 2.6 en un mismo día y 5.1 entre días diferentes. No se observaron diferencias apreciables entre el análisis con simple cuadrupolo y trampa de iones. Aunque para el área del pico se obtuvieron resultados aceptables, la reproducibilidad fue más baja para la trampa de iones. Los límites de detección se calcularon con los electroferogramas en modo SIM en el EM y de los electroferogramas del cromatograma del ion extraído en la trampa de iones de muestras adicionadas a 50 ng g^{-1}. Los LODs con simple cuadrupolo fueron entre 5 y 20 ng g^{-1} y con trampa de iones fueron de 20 ng g^{-1} para las 5

quinolonas estudiadas. Se observó una excelente linealidad en el rango de concentración de 50-5000 ng g^{-1}, con un coeficiente de correlación mejor que 0.9992. Por el contrario la linealidad calculada en las mismas condiciones con la trampa de iones, fue peor, con un coeficiente de correlación mejor que 0.9903. El rango del límite para la trampa de iones se ha discutido mucho en la literatura y está considerado como una característica intrínseca a este analizador de masas. A pesar del rango tan estrecho para el que la respuesta con la trampa de iones es lineal, las quinolonas se pueden cuantificar correctamente a concentraciones muy bajas. Los resultados obtenidos para las muestras de pescado fueron muy parecidas.

La tabla V.4 muestra la exactitud y precisión obtenida en los extractos de quinolonas de las muestras de pollo y pescado a 50 ng g^{-1} utilizando EC-QIT como analizador de masas. Estos parámetros para muestras adicionadas a concentraciones elevadas de quinolonas (500 ng g^{-1}) proporcionan valores similares (no mostrados).

En general se obtuvieron buenas recuperaciones, tanto en carne de pollo como en pescado, para 4 de las 5 quinolonas estudiadas. Flumequina proporcionó recuperaciones inferiores al 45 %, debido a su termolabilidad y a posibles pérdidas durante el proceso de evaporación. Para el resto de quinolonas, las recuperaciones estuvieron entre 60-93 %. La DSR (en un mismo día) fueron adecuadas tal y como se muestra en la tabla V.4.

El método propuesto es suficientemente sensible para analizar estas quinolonas en pescado y carne, ya que los límites de cuantificación obtenidos están por debajo de los LMRs (100- a 200 ng g^{-1}) establecidos en la Directiva 2377/90 de la Unión europea [10]. Las figuras V.3 y V.4 muestran el cromatograma de una disolución estándar de las 5 quinolonas a los límites de cuantificación, un extracto de carne de pollo adicionado a los límites de

cuantificación y un extracto de muestra de pollo que contenía enrofloxacina, analizado por EC-EM y EC-QIT. Como se puede observar no existen picos endógenos de los tejidos que interfieran, ni distorsiones en la línea base. Aunque los resultados obtenidos con la trampa de iones son más selectivos, el EM simple cuadrupolo proporciona cromatogramas mucho más limpios y sin picos que interfieran en el análisis.

5.- Aplicación a muestras reales

Se determinó ***carboxin, flutriafol, pirimetanil, triadimefon, tebuconazol y bitertanol*** en 15 muestras de uva comercial de diferentes supermercados (uva blanca y negra de diferentes variedades), mediante los procesos de EFS y ESBM. Una muestra de Uva Moscatel fue positiva para pirimetanil, se extrajo por triplicado y cada extracto se inyecto dos veces. La media de la concentración encontrada y la desviación estándar fueron 0.05 ± 0.002 mg kg^{-1} para EFS (figura I.3C) y 0.05±0.003 mg kg^{-1} para ESBM (figura I.4C). La confirmación fue excelente por los dos procedimientos.

Se determinó ***flutriafol, ciproconazol (isómeros R y S), miclobutanil, tebuconazol, acrinatrin, bitertanol, fludioxonil y piriproxifen*** en muestras de tomate, lechuga, uva y fresas. En la figura II.3A se presenta una muestra de lechuga que fue positiva para piriproxifen, y se confirmo por CL-EM. La identificación y posterior confirmación de este plaguicida pone de manifiesto la idoneidad del método explicado anteriormente para determinar estos residuos en muestras reales y la dificultad de encontrar muestras exentas de residuos de plaguicidas.

Se determinó ***tiabendazol, pirifenox, pirimicarb, pirimetanil, procimidona y dinoseb*** en diez muestras (cinco de melocotones y cinco de

nectarinas), obtenidas de una cooperativa agrícola días diferentes. Tres de las muestras analizadas contenían residuos de plaguicidas. Una muestra de nectarina contenía 0.03 mg kg^{-1} de tiabendazol, y dos muestras de melocotones contenían 0.2 y 1.0 mg kg^{-1} de procimidona. En la figura III.4 se muestran los elctroferogramas de la muestra de melocotón positiva de tiabendazol utilizando diferentes métodos de EC-ESI-EM.

La determinación mediante técnicas de preconcentración para ***dinoseb, procimidona, fludioxonil, carbendazima y piriproxifen*** se aplicaron a un total de 60 muestras, 30 de uva y 30 de lechuga, obtenidas de supermercados locales. Se extrajeron por EFS y RM-MEKC. La tabla IV.5 recoge los resultados obtenidos

Las concentraciones recogidas fueron del rango µg kg^{-1}. Debido a la elevada sensibilidad proporcionada por las técnicas de preconcentración en línea, los plaguicidas se pudieron detectar por RM-MECK-DAD.

Se detectaron residuos de plaguicidas en 15 muestras (25%) de las 60 analizadas. Ninguna excedió los LMRs. Dinoseb fue el único plaguicida que no se encontró en ninguna muestra. Piriproxifen se encontró sólo en una muestra de lechuga a una concentración baja. Carbendazima se encontró en nueve muestras (15%), en tres de lechugas y en seis de uva, a niveles del rango 0.01 a 1.54 mg kg^{-1}. Procimidona se encontró en nueve muestras (15%) dos de lechugas y 7 de uva, a niveles entre 0.19 to 2.03 mg kg^{-1}. Fludioxonil se encontró en 5 muestras (8%), una en lechuga y 4 en uva, a concentraciones de 0.02 y 3.02 mg kg^{-1}.

De las muestras contaminadas, nueve muestras (60%)-siete de uva y dos de lechuga- contenían residuos de dos de los plaguicidas estudiados y seis muestras (40 %)- tres de uva y tres de lechuga- contuvieron un único plaguicida. La presencia de estos plaguicidas pone de manifiesto la existencia y frecuencia de residuos de plaguicidas en un número de muestras significativas.

La determinación de quinolonas (***danofloxacina, enrofloxacina, ofloxacina, ácido pipemídico y flumequina)*** se realizó para varias muestras de tejido de pollo y pescado de la ciudad de Valencia. De las muestras recogidas, 25 eran de pollo y 15 de pescado (5 truchas, 5 doradas y 5 lubinas). Ninguna de las quinolonas estudiadas se detecto en estas muestras.

La medida de enrofloxacina en muestras de carne de pollo, tras administración oral (enrofloxacina 5%) a una dosis de 5 mg g^{-1}, confirma la utilidad del método analítico propuesto. Las figuras V.3C y V.4C muestran el electroferograma por EC-EM y EC-EM^3 de extracción de carne de pollo tratados. Las concentraciones encontradas en las muestras dos días después de ser tratadas fueron de 80 ng g^{-1}.

El método propuesto para la determinación de sulfonamidas (***sulfapiridina, sulfametoxipiridazina, sulfatiazol, sulfaguanidina, sulfacloropiridazina, sulfanilamida, sulfadiazina, sulfisoxazol, sulfabenzamida, sulfadimetoxina sulfasalazina, sulfanilamida)*** se aplicó para determinar sulfadimidina en muestras de carne de cerdo de animales tratados con esta SA. La figura VI.2A muestra el electroferograma obtenido de los extractos de la muestras, de animales tratados con SAs durante 5 días y posteriormente sacrificados. La cantidad de sulfadimidina cuantificada fue de 400 μg kg^{-1}. Como fue de esperar de muestras de animales sacrificados inmediatamente, los valores de sulfadimidina fueron superiores a los LMRs establecidos por la UE.

Cincuenta muestras de carne de cerdo recogidas al azar de diversos supermercados de la ciudad de Valencia se analizaron por el método PLE-CE-MS^2. Dos muestra estaban contaminadas con SAs, una contenía 42 μg kg^{-1} de sulfapiridina y otra 34 μg kg^{-1} de sulfadiazina. En la figura VI.2B se muestra el

electroferograma correspondiente a la muestra que contenía sulfapiridina. El EM del ion producto confirmó claramente la identidad del compuesto.

Conclusiones
Conclusions

Primera

La extracción en fase sólida proporciona límites de cuantificación y desviaciones estándar ligeramente más bajos y recuperaciones más altas que las obtenidas por extracción sobre barras magnéticas, y es una técnica más rápida, como se demuestra para carboxin, flutriafol, pirimetanil, triadimefon, tebuconazol y bitertanol, extraídos desde uva y analizados por cromatografía líquida-espectrometría de masas.

Segunda

Los resultados obtenidos al extraer flutriafol, ciproconazol, miclobutanil, tebuconazol, acrinatrin, bitertanol, fludioxonil y piriproxifen desde muestras de lechuga, tomate, uva y fresa por extracción en fase sólida y sobre barras magnéticas, muestran que la extracción en fase sólida es la mejor técnica para el análisis por electroforesis capilar electrocinética micelar con detección de diodos, ya que se obtienen límites de cuantificación por debajo de los límites máximos de residuos establecidos y tiempos de análisis más cortos, mientras que con la técnica de extracción sobre barras magnéticas los límites de cuantificación fueron superiores a los límites máximos de residuos establecidos y el tiempo de análisis fue tres veces más, que el requerido por extracción en fase sólida.

Tercera

El análisis de tiabendazol, pirifenox, pirimicarb, pirimetanil, procimidona y dinoseb en melocotones y nectarinas por electroforesis capilar con detección de diodos, y por espectrometría de masas con simple cuadrupolo y trampa de iones, previa extracción en fase sólida, pone de manifiesto que la

espectrometría de masas, con simple cuadrupolo, y la espectrometría de masas en tándem, con trampa de iones, permiten obtener resultados adecuados para todos los compuestos a niveles por debajo de los límites máximos de residuos.

Cuarta

Las técnicas electroforéticas de preconcentración de analitos en línea basadas en fenómenos de acumulación y focalización permiten reducir los límites de detección para los plaguicidas fludioxonil, piriproxifen, carbendazima, procimidona y dinoseb, en muestras de lechuga y uva, siendo mejores que los obtenidos con la inyección hidrodinámica convencional, llegando a valores por debajo de los límites máximos de residuos utilizando detector de diodos.

Quinta

La preconcentración en línea por acumulación con migración reversa de micelas y tapón de agua, proporciona los mejores resultados en términos de sensibilidad, eficacia y reproducibilidad para los plaguicidas fludioxonil, piriproxifen, carbendazima, procimidona y dinoseb, en muestras de lechuga y uva, comparada con las técnicas de focalización del analito y de acumulación del mismo con migración reversa de micelas sin tapón de agua, utilizando detector de diodos.

Sexta

La extracción de cinco quinolonas: danofloxacina, ofloxacina, enrofloxacina, ácido pipemídico y flumequina, en carne de pollo y pescado mediante extracción líquida con diclorometano y purificación con extracción en

fase sólida, seguida de la separación por electroforesis capilar, permite su identificación y cuantificación por electrospray-espectrometría de masas por trampa de iones por debajo de los límites máximos de residuos.

Séptima

El método de extracción presurizada con disolventes empleando agua, desarrollado para la determinación de doce sulfonamidas en carne de cerdo, previa a la etapa de concentración, mediante extracción en fase sólida, permite la determinación por electroforesis capilar-espectrometría de masas por trampa de iones, por debajo de los límites máximos de residuos legislados.

Octava

La determinación simultánea y cuantitativa en muestras de alimentos procedentes de la Comunidad Valenciana, mediante la utilización de extracción en fase sólida, extracción sobre barras magnéticas o extracción presurizada con disolventes y electroforesis capilar con detector de filas de diodos o espectrometría de masas, pone de manifiesto la utilidad de la electroforesis capilar en la determinación de residuos de plaguicidas en matrices complejas hortofrutícolas como uva, lechuga, tomate, fresa, melocotón y nectarinas y residuos de medicamentos veterinarios en alimentos de origen animal como pescado y carne de pollo y cerdo.

Novena

La presencia de residuos de contaminantes orgánicos en alimentos, aunque en un pequeño porcentaje de muestras y generalmente a bajas concentraciones, hace necesario el desarrollo y la optimización de métodos para que su correcta determinación permita una adecuada seguridad alimentaria.

CONCLUSIONS

First

The solid phase extraction provides limits of quantification and standard deviations lower, and recoveries higher than the obtained by stir-bar sportive extraction and it is a faster technique, as it is showed for carboxin, flutriafol, pyrimethanil, triadimefon, tebuconazole and bitertanol, extracted from grape and analyzed by liquid chromatography-mass spectrometry.

Second

The extraction results obtained for flutriafol, cyproconazole, myclobutanil, tebuconazole, acrinathrin, bitertanol, fludioxonil and piriproxifen from lettuce, tomato, grape and strawberry samples by solid phase extraction and stir-bar sorptive extraction, showed that solid phase extraction is the best technique for analyzing by micellar electrokinetic chromatography with diode array detector, because the limits of quantification obtained, were lower than the maximum residue limits established, and shorter analysis time was employed, while with stir-bar sorptive extraction limits of quantification were higher than established maximum residue limits and the analysis time was three times longer than the required by solid phase extraction.

Third

The analysis of thiabendazole, pyrifenox, pirimicarb, pyrimethanil, procymidone and dinoseb in peaches and nectarines by capillary electrophoresis with diode array detector and by mass spectrometry with simple quadrupole and ion trap, previous solid phase extraction, reveals that mass spectrometry with

simple quadrupole and ion trap tandem mass spectrometry, allows to obtain appropriate results for all the analyzed compounds at levels lower than the maximum residue limits.

Fourth

The on-line preconcentration electrophoretic techniques based in sweeping and stacking phenomenons allows to reduce the limits of detection for pesticides fludioxonil, piriproxifen, carbendazim, dinoseb and procymidone in lettuce and grape samples, being better than the obtained by conventional hydrondinamic injection, and reaching values lower than the maximum residue limits using diode array detector.

Fifth

The preconcentration technique, stacking with reverse migration of micelles with water plug, proportionates the best results in terms of sensibility, efficiency and reproducibility for pesticides fludioxonil, pyriproxifen, carbendazim, dinoseb and procymidone in lettuce and grape samples, compared with both sweeping and stacking with reverse migration of micelles without water plug preconcentration techniques using diode array detector.

Sixth

The extraction of five quinolone danofloxacin, ofloxacin, enrofloxacin, pipemidic acid and fluemquine, in chicken and fish muscle by dichloromethane liquid extraction and clean-up by solid phase extraction, followed by capillary

electrophoresis, allowed to identify and quantify levels lower than the maximum residue limits by electrospray- ion trap mass spectrometry.

Seventh

The pressurized liquid extraction method with water, developed for the determination of twelve sulphonamides in pork meat, previous solid phase extraction step concentration, permits to determine levels lower than the maximum residue limits, by capillary electrophoresis-ion trap mass spectrometry.

Eighth

The quantitative and simultaneous determination in food samples from Valencian Community, through solid phase extraction, sitr-bar sorptive extraction or pressurized liquid extraction and by capillary electrophoresis with diode array or mass spectrometry detection, make known the utility of capillary electrophoresis for pesticide residues determination in complex fruit and vegetable matrices as grape, lettuce, tomato, strawberry, peach and nectarines and veterinary drug residues in animal origin food as fish and chicken and pork meat.

Nineth

The presence of organic contaminants residues in food, although in few samples percentage and often at a low concentrations, makes necessary to

develope and optimize methods in order to ensure that its correct determination allows an appropriate food safety.

Bibliografía
References

[1] Ministerio de Agricultura, Pesca y Alimentación, disponible en www.mapa.es, 2007

[2] Ministerio de Medio Ambiente, disponible en www.mma.es, 2007

[3] Font G., Fernández M., Ruiz M.J., Picó Y., *Residuos de plaguicidas en alimentos* pags. 349-372 en Toxicología Alimentaria, Cameán A.M., Repetto M., Ed. Díaz de Santos, 2006

[4] Organización Mundial de la Salud, disponible en http://www.who.int/es / , 2007

[5] Coscollà R., Coscollà C., *Cómo disminuir o eliminar los residuos de plaguicidas en frutas, hortalizas y alimentos transformados*, 71-111, Ed. Phytoma, 2006

[6] Ministerio de Sanidad y Consumo, disponible en http://www.msc.es, 2007

[7] Libro Blanco de la agricultura y el desarrollo rural disponible en www.libroblancoagricultura.com 2007

[8] Codex Alimentarius disponible en http://www.fao.org, 2007

[9] Rimblas M.E., Marsilla B., *Los compuestos químicos de los alimentos desde la perspectiva de la Seguridad Alimentaria*, Dirección general de Seguridad Alimentaria. 2004

[10] Council Regulation (EEC) No 2377/90 of 26 June 1990 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuff of animal origen, Off. J. Eur. Commun. 1990, L224, 1–20.

[11] European Community.http://europa.eu.int

[12] Asociación Empresarial para la Protección de las Plantas http://aepla.es

[13] The Merck Veterinary Manual http://www.merckvetmanual.com

[14] Portal de los Colegios Oficiales de Farmacia http://portalfarma.com

[15] Food and Agricultural Organization. http://www.fao.org

[16] Asociación Empresarial Española de la Industria de Sanidad y Nutrición Animal www.veterindustria.com

[17] Nollet L.M.L., Handbook of Food Analysis. Vol 2. Residues and Other Food Components, Marcel Dekker, New York, 2004.

[18] Watson D., Pesticides, Veterinary and Other Residues in Food, Woodhead, Cambridge, 2004.

[19] Bowser M., en: Encyclopedia of Analytical Science, P.J. Worsfold, A. Townshead, C.E. Poole (Eds.), Elsevier, Oxford 2005, 334-343.

[20] Scriba G.K.E., en: Encyclopedia of Analytical Science, P.J. Worsfold, A. Townshead, C.E. Poole (Eds.), Elsevier, Oxford 2005, 343-354.

[21] Pacakova V., Stulik K., en: Encyclopedia of Analytical Science, P.J. Worsfold, A. Townshead, C.E. Poole (Eds.), Elsevier, Oxford 2005, 354-361.

[22] Picó Y., en: Encyclopedia of Analytical Science, P.J. Worsfold, A. Townshead, C.E. Poole (Eds.), Elsevier, Oxford 2005, 362-374.

[23] Frazier R.A., en: Encyclopedia of Analytical Science, P.J. Worsfold, A. Townshead, C.E. Poole (Eds.), Elsevier, Oxford 2005, 374-381.

[24] Shihabi Z.K., en: Encyclopedia of Analytical Science, P.J. Worsfold, A. Townshead, C.E. Poole (Eds.), Elsevier, Oxford 2005, 381-392.

[25] Osbourn D.M., Weiss D.J., Lunte C.E., *On-line preconcentration methods for capillary electrophoresis*. Electrophoresis 21, (2000), 2768.

[26] Beckers J.L., Bocek P., *Sample stacking in capillary zone electrophoresis: Principles, advantages and limitations*. Electrophoresis 21, (2000), 2747.

[27] Quirino J.P., Kim J.B., Terabe S., *Sweeping: concentration mechanism and applications to high-sensitivity analysis in capillary electrophoresis*. J. Chromatogr. A 965, (2002), 357.

[28] Schmitt-Kopplin P., Frommberger M., *Capillary electrophoresis - mass spectrometry: 15 years of developments and applications*. Electrophoresis 24, (2003), 3837.

[29] Choudhary G., Apffel A., Yin H.F., Hancock W., *Use of on-line mass spectrometric detection in capillary electrochromatography*. J. Chromatogr. A 887, (2000), 85.

[30] Ahmed F.E., *Analysis of polychlorinated biphenyls in food products*. Trends Anal. Chem. 22, (2003), 170.

[31] Oka H., Ito Y., Matsumoto H., *Chromatographic analysis of tetracycline antibiotics in foods*. J. Chromatogr. A 882, (2000), 109.

[32] Sin D.W.M., Wong Y.C., *Analytical methodologies for identifying a polypeptide antibiotic*. Trends Anal. Chem. 22, (2003), 799.

[33] Hernández-Borges J., García-Montelongo F.J., Cifuentes A., Rodríguez-Delgado MA. *Analysis of triazolopyrimidine herbicides in soils using field-enhanced sample injection-coelectroosmotic capillary electrophoresis combined with solid-phase extraction*. J Chromatogr A 1100, (2005), 236.

[34] Hernández-Borges J, Garcia-Montelongo FJ, Cifuentes A, Rodriguez-Delgado MA. *Determination of herbicides in mineral and stagnant waters at ng L^{-1} levels using capillary electrophoresis and UV detection combined with solid-phase extraction and sample stacking*. J Chromatogr A 1070, (2005), 171.

[35] Stead D.A., *Current methodologies for the analysis of aminoglycosides*. J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 747, (2000), 69.

[36] Frazier R.A., *Recent advances in capillary electrophoresis methods for food analysis*. Electrophoresis 22, (2001), 4197.

[37] García-Canas V., González R., Cifuentes A, *The combined use of molecular techniques and capillary electrophoresis in food analysis*. Trends Anal. Chem. 23, (2004), 637.

[38] Sadecka J., Polonsky J., *Electrophoretic methods in the analysis of beverages*. J. Chromatogr. A 880, (2000), 243.

[39] Barrón D., Jiménez-Lozano E., Bailac S., Barbosa J., *Simultaneous determination of flumequine and oxolinic acid in chicken tissues by solid phase extraction and capillary electrophoresis*. Anal. Chim. Acta 477, (2003), 21.

[40] Saad B., Mohamad R., Mohamed N., Lawrence G.D., Jab M.S., Saleh M.I, *Determination of oxolinic acid in feeds and cultured fish using capillary electrophoresis.* Food Chem. 78, (2002), 383.

[41] Barrón D., Jiménez-Lozano E., Cano J., Barbosa J., *Determination of residues of enrofloxacin and its metabolite ciprofloxacin in biological materials by capillary electrophoresis*. J. Chromatogr. B 759, (2001), 73.

[42] Hernández M., Borrull F., Calull M., *Using nonaqueous capillary electrophoresis to analyze several quinolones in pig kidney samples*. Electrophoresis 23, (2002), 506.

[43] Barrón D., Jiménez-Lozano E., Bailac S., Barbosa J., *Determination of difloxacin and sarafloxacin in chicken muscle using solid-phase extraction and capillary electrophoresis*. J. Chromatogr. B 767, (2002), 313.

[44] Jiménez-Lozano E., Roy D., Barrón D., Barbosa J., *Effective sorbents for solid-phase extraction in the analysis of quinolones in animal tissues by capillary electrophoresis*. Electrophoresis 25, (2004), 65.

[45] Beltrán J.L., Jiménez-Lozano E., Barrón D., Barbosa J., *Determination of quinolone antimicrobial agents in strongly overlapped peaks from capillary electrophoresis using multivariate calibration methods*. Anal. Chim. Acta 501, (2004), 137.

[46] Huang T.S., Du W.X.,. Marshall M.R, Wei C.I., *Determination of oxytetracycline in raw and cooked channel catfish by capillary electrophoresis*. J. Agric. Food Chem. 45, (1997), 2602.

[47] Bateman K.P., Locke S.J., Volmer D.A., *Antibiotic resistance in Streptomyces lividans: Fluorescence assay for streptogramin B lyase*. J. Mass Spectrom. 32, (1997), 297.

[48] Fuh M.R.S., Chu S.Y., *Quantitative determination of sulfonamide in meat by solid-phase extraction and capillary electrophoresis*. Anal. Chim. Acta 499, (2003), 215.

[49] Santos B., Lista A., Simonet B., Rios A., Valcárcel M., *Screening and analytical confirmation of sulfonamide residues in milk by capillary electrophoresis-mass spectrometry*. Electrophoresis 26, (2005), 1567.

[50] Kowalski P., Oledzka I., Lamparczyk H., *Capillary electrophoresis in analysis of veterinary drugs*. J. Pharm. Biomed. Anal. 32, (2003), 937.

[51] Malik A.K., Faubel W., *Capillary electrophoretic determination of dithiocarbamates and ethyl xanthate.* Fresenius J. Anal. Chem. 367, (2000), 211.

[52] Malik A.K., Faube W., *Capillary electrophoretic determination of zinc dimethyldithiocarbamate (Ziram) and zinc ethylenebisdithiocarbamate (Zineb).* Talanta 52, (2000), 341.

[53] Malik A.K., Seidel B.S., Faubel W., *Capillary electrophoretic determination of ferric dimethyldithiocarbamate as iron(III) chelate of EDTA*. J. Chromatogr. A 857, (1999), 365.

[54] Kodama S., Yamamoto A., Ohura T., Matsunaga A., Kanbe T., *Enantioseparation of imazalil residue in orange by capillary electrophoresis with 2-hydroxypropyl-beta-cyclodextrin as a chiral selector*. J. Agric. Food Chem. 51, (2003), 6128.

[55] Molina M., Silva M., *Rapid determination of fungicides in fruit juices by micellar electrokinetic chromatography: Use of organic modifiers to enhance selectivity and on-column high-salt stacking to improve sensitivity.* Electrophoresis 21, (2000), 3625.

[56] Rodríguez R., Picó Y., Font G., Mañes J., *Analysis of post-harvest fungicides by micellar electrokinetic chromatography*. J. Chromatogr. A 924, (2001), 387.

[57] Rodríguez R., Boyer I., Font G., Picó Y., *Capillary zone electrophoresis for the determination of thiabendazole, prochloraz and procymidone in grapes*. Analyst 126, (2001), 2134.

[58] Rodríguez R., Picó Y., Font G., Mañes J., *Analysis of thiabendazole and procymidone in fruits and vegetables by capillary electrophoresis-electrospray mass spectrometry*. J. Chromatogr. A 949, (2002), 359.

[59] Takeda S., Fukushi K., Chayama K., Nakayama Y., Tanaka Y., Wakida S., *Simultaneous separation and on-line concentration of amitrole and benzimidazole pesticides by capillary electrophoresis with a volatile migration buffer applicable to mass spectrometric detection*. J. Chromatogr. A 1051, (2004), 297.

[60] Kaltsonoudis C.K., Lamari F.N., Prousalis K.P., Karamanos N.K., Tsegenidis T., *Analysis of carbendazim and thiabendazole in lemons by CE-DAD.* Chromatographia 57, (2003), 181.

[61] Goodwin L., Startin J.R., Keely B.J, Goodall D.M., *Analysis of glyphosate and glufosinate by capillary electrophoresis - mass spectrometry utilising a sheathless microelectrospray interface*. J. Chromatogr. A 1004, (2003), 107.

[62] Hooijschuur E.W.J., Kientz C.E., Dijksman J., Brinkman U.A.T., *Potential of microcolumn liquid chromatography and capillary electrophoresis with flame photometric detection for determination of polar phosphorus-containing pesticides*. Chromatographia 54, (2001), 295.

[63] Guardino X., Obiols J., Rosell M., Farran A., Serra C., *Determination of chlorpyrifos in air, leaves and soil from a greenhouse by gas-chromatography with nitrogen-phosphorus detection, high-performance liquid chromatography and capillary electrophoresis*. J. Chromatogr. A 823, (1998), 91.

[64] Rodríguez R., Picó Y., Font G., Mañes J., *Determination of urea-derived pesticides in fruits and vegetables by solid-phase preconcentration and capillary electrophoresis*. Electrophoresis 22, (2001), 2010.

[65] Khrolenko M., Dzygiel P., Wieczorek P., *Combination of supported liquid membrane and solid-phase extraction for sample pretreatment of triazine herbicides in juice prior to capillary electrophoresis determination.* J. Chromatogr. A 975, (2002), 219.

[66] Rodríguez R., Mañes J., Picó Y., *Off-line solid-phase microextraction and capillary electrophoresis mass spectrometry to determine acidic pesticides in fruits*. Anal. Chem. 75, (2003), 452.

[67] Carretero A.S., Cruces-Blanco C., Ramírez S.C., Pancorbo A.C., Gutierrez A.F., *Application of micellar electrokinetic capillary chromatography to the analysis of uncharged pesticides of environmental impact.* J. Agric. Food Chem. 52, (2004), 5791.

[68] da Silva C.L., de Lima E.C., Tavares M.F.M., *Investigation of preconcentration strategies for the trace analysis of multi-residue pesticides in real samples by capillary electrophoresis*. J. Chromatogr. A 1014, (2003), 109.

[69] Hernández-Borges J., Rodríguez-Delgado M.A., García-Montelongo F.J., Cifuentes A., *Highly sensitive analysis of multiple pesticides in foods combining solid-phase microextraction, capillary electrophoresis-mass spectrometry and chemometrics*. Electrophoresis 25, (2004), 2065.

[70] Kubilius D.T., Bushway R.J., *Determination of maleic hydrazide in potatoes and onions by capillary electrophoresis*. J. Agric. Food Chem. 46, (1998), 4224.

[71] Corneli S., Maragos C.M., *Capillary electrophoresis with laser-induced fluorescence: Method for the mycotoxin ochratoxin A.* J. Agric. Food Chem. 46, (1998), 3162.

[72] Peña R., Alcaraz M.C., Arce L., Rios A., Valcarcel M., *Screening of aflatoxins in feed samples using a flow system coupled to capillary electrophoresis*. J. Chromatogr. A 967, (2002), 303.

[73] Tsao R., Zhou T., *Micellar electrokinetic capillary electrophoresis for rapid analysis of patulin in apple cider*. J. Agric. Food Chem. 48, (2000), 5231.

[74] Wei J., Okerberg E., Dunlap J., Ly C., Shear J.B., *Determination of biological toxins using capillary electrokinetic chromatography with multiphoton excited fluorescence*. Anal. Chem. 72, (2000), 1360.

[75] Maragos C.M., Greer J.I., *Analysis of aflatoxin B-1 in corn using capillary electrophoresis with laser-induced fluorescence detection*. J. Agric. Food Chem. 45, (1997), 4337.

[76] Piñeiro N., Leao J.M., Martínez A.G., Vázquez J.A.R., *Capillary electrophoresis with diode array detection as an alternative analytical method for paralytic and amnesic shellfish toxins*. J. Chromatogr. A 847, (1999), 223.

[77] Alarcón B., García-Canas V., Cifuentes A., González R., Aznar R., *Simultaneous and sensitive detection of three foodborne pathogens by multiplex PCR, capillary gel electrophoresis, and laser-induced fluorescence*. J. Agric. Food Chem. 52, (2004), 7180.

[78] Arakawa H., Watanabe K., Kashiwazaki H., Maeda M., *Detection of two variant vero toxin genes in Escherichia coli by capillary electrophoresis.* Biomed. Chromatogr. 16, 2002, 41.

[79] Pang G.F., Cao Y.Z., Fan C.L., Zhang J.J., Li X.M. *Multiresidue gas chromatographic method for determining synthetic pyrethroid pesticides in agricultural products: Collaborative study*. J.AOAC Int. 2 (1999) 186.

[80] Tester P.A., Turner J.T., Shea D., *Vectorial transport of toxins from the dinoflagellate Gymnodinium breve through copepods to fish*. J. Plankton Res. 22, 2000, 47.

[81] Simó C., Barbas C., Cifuentes A., *Chiral electromigration methods in food analysis*. Electrophoresis 24, (2003), 2431.

[82] Picó Y., Fernández M., Ruiz M.J., Font G., *Current trends in solid-phase-based extraction techniques for the determination of pesticides in food and environment*. J. Biochem. Bioph. Meth. 2007, 70, 117.

[83] Shakulashvili N., Revia R., Steiner F., Engelhardt H., *Simultaneous determination of various classes of pesticides using micellar electrokinetic capillary chromatography in combination with solid-phase extraction.* Cromatografia 2004, 60, 145.

[84] Lambropoulou D.A., Albanis T.A., *Liquid-phase micro-extraction techniques in pesticide residue analysis*. J. Biochem. Bioph. Meth. 2007, 70, 195.

[85] Chicharro M., Bermejo E., Sánchez A., Zapardiel A., Fernández-Gutierrez A., Arraez D., *Multiresidue analysis of phenylurea herbicides in environmental waters by capillary electrophoresis using electrochemical detection*. Anal. Bioanal. Chem. 2005, 382, 519.

[86] Herranz S., Moreno-Bondi M.C., Marazuela M.D., *Developement of a new sample treatment procedure based on pressurized liquid extraction for the determination of fluoroquinolone residue in table eggs*. J. of Chromat A 2007, 1140, 63.

[87] StoobK., Singer H.P., Stettler S., *Exhaustive extraction of sulfonamide antibiotics from aged agricultural soils using pressurized solvent extraction* J. of Chromat A 2006, 1128, 1.

[88] Ravelo-Pérez LM., Hernández-Borges J., Rodríguez-Delgado M.A., *Pesticides analysis by liquid chromatography and capillary electrophoresis*. J. Sep. Sci. 2006, 29, 2557.

[89] Fogarty B., Dempsey E., Regan F., *Potential of microemulsion electrokinetic chromatography for the separation of priority endocrine disrupting compounds*. J. Chromatogr. A 2003, 1014, 129–139.

[90] Barceló-Barrachina E., Moyano E., Galceran M.T., *State-of-the-art of the hyphenation of capillary electrochromatography with mass spectrometry*. Electrophoresis 2004, 25, 1927–1948

[91] González L., Juan C., Soriano J.M., Moltó J.C., Mañes J., *Occurrence and daily intake of ochratoxin A of organic and non-organic rice and rice products*. Int J Food Microbiol. 107, (2006), 223.

[92] Juan, C., González L., Soriano J.M., Moltó J.C., Mañes J., *Accelerated solvent extraction of ochratoxin A from rice samples*. J Agric Food Chem. 53, (2005), 9348.

[93] González-Osnaya L., Soriano J.M., Moltó J.C., Mañes J., *Extraction and analysis of ochratoxin A in bread using pressurised liquid extraction and liquid chromatography*. J Chromatogr. A. 1113, (2006), 32.

[94] Pallaroni L., von Holst C., *Comparison of alternative and conventional extraction techniques for the determination of zearalenone in corn*. Anal Bioanal Chem. 376, (2003), 908.

[95] Urraca J.L., Benito-Pena E., Pérez-Conde C., Moreno-Bondi M.C., Pestka J.J., *Analysis of zearalenone in cereal and swine feed samples using an automated flow-through immunosensor*. J Agric Food Chem. 53, (2005), 3338.

[96] Royer D., Humpf H.U., Guy P.A. *Quantitative analysis of Fusarium mycotoxins in maize using accelerated solvent extraction before liquid chromatography atmospheric pressure chemical ionization tandem mass spectrometry*. Food Addit Contam. 21, (2004), 678.

[97] Kavran G., Erim F.B., *Separation of polycyclic aromatic hydrocarbons with sodium dodecylbenzenesulfonate in electrokinetic chromatography*. J. Chromatogr. A 949, (2002), 301.

[98] IUPAC Compendium of Chemical Terminology, disponible en http://www.iupac.org/goldbook/P04803.pdf .

[99] Picó Y., Rodríguez R., Mañes J., *Capillary electrophoresis for the determination of pesticide residues*. Trends Anal. Chem. 22, (2003), 133.

[100] Palmer J.E., *High-salt stacking principles and sweeping: comments and contrasts on mechanisms for high-sensitivity analysis in capillary electrophoresis*. J. Chromatogr. A 1036, (2004), 95.

[101] Palmer J., Landers J.P., *Stacking neutral analytes in capillary electrokinetic chromatography with high-salt sample matrixes*. Anal. Chem. 72, (2000), 1941.

[102] Valenzuela A.I., Redondo M.J., Picó Y., Font G., *Determination of abamectin in citrus fruits by liquid chromatography-electrospray ionization mass spectrometry*. J. Chromatogr. A 871, (2000) 57.

[103] Hernández-Borges J., Rodríguez-Delgado M.A., García-Montelogo F.J., Cifuentes A., *Analysis of pesticides in soy milk combining solid-phase extraction and capillary electrophoresis-mass spectrometry*. J. Sep. Sci. 28, (2005), 948.

[104] Jeannot R., Sabik H., Sauvard E., Genin E., *Application of liquid chromatography with mass spectrometry combined with photodiode array detection and tandem mass spectrometry for monitoring pesticides in surface waters*. J. Chromatogr. A 879, (2000), 51.

[105] Vandecasteele K., Gaus I., Debreuck W., Walraevens K., *Identification and quantification of 77 pesticides in groundwater using solid phase coupled to liquid-liquid microextraction and reversed phase liquid chromatography*. Anal. Chem. 72, (2000), 3093.

[106] Hiemstra M., Toonen A., DeKok A., *Determination of benzoylphenylurea insecticides in pome fruit and fruiting vegetables by liquid chromatography with diode array detection and residue data obtained in the dutch national monitoring program*. J. AOAC Int. 821, (1999), 198.

[107] Colume A., Cárdenas S., Gallego M., Valcárcel M., *Simplified method for the determination of chlorinated fungicides and insecticides in fruits by gas chromatography*. J. Chromatogr. A 882, (2000), 193.

[108] Otero R.R., Grande B.C., Gándara J.S., *Multiresidue method for fourteen fungicides in white grapes by liquid-liquid and solid-phase extraction followed by liquid chromatography-diode array detection*. J. Chromatogr. A 992, (2003), 121.

[109] Young M.S., Early M.F., Mallet C.R., Krol J., *Application of a mixed-mode solid-phase extraction and cleanup procedure for LC/MS determination of thiabendazole and carbendazim in apple juice*. J. AOAC Int. 84, (2001), 1608.

[110] Sandra P., Tienpont B., Vercammen J., Tredoux A., Sandra T., David F., *Stir bar sorptive extraction applied to the determination of dicarboximide fungicides in wine*. J. Chromatogr. A 928, (2001), 117.

[111] Blasco C., Font G., Picó Y., *Comparison of microextraction procedures to determine pesticides in oranges by liquid chromatography-mass spectrometry*. J. Chromatogr. A 970, (2002), 201.

[112] Blasco C., Fernández M., Picó Y., Font G., *Comparison of microextraction procedures to determine pesticides in oranges by liquid chromatography-mass spectrometry*. J. Chromatogr. A 1030, (2004), 77.

[113] González C.F., Otero R.R., Grande B.C., Gándara J.S., *Determination of fungicide residues in white grapes for winemaking by gas chromatography with mass spectrometric detection and assessment of matrix effects*. J. AOAC Int. 85, (2003), 1008.

[114] Zrostlikova J., Hajslova J., Kovalczuk T., Stefan R., Poustka J., *Determination of seventeen polar/thermolabile pesticides in apples and apricots by liquid chromatography/mass spectrometry*. J. AOAC Int. 86, (2003), 612.

[115] Ito Y., Goto T., Oka H., Matsumoto H., Miyazaki Y., N. Takahashi, H. Nakazawa, *Simple and rapid determination of thiabendazole, imazalil, and o-phenyl phenol in citrus fruit using flow-injection electrospray ionization tandem mass spectrometry*. J. Agric. Food Chem. 51, (2003), 861.

[116] Food and Drug Administration of the United States, http://www.cfsan.fda.gov, 2003.

[117] Ministerio de Agricultura, Pesca y Alimentación, http://www.mcx.plaguicidas.plagas.htm Official Maximum Residue Limits (MRLs), 2003.

[118] Sandra P., Tienpont B., David F., *Multi-residue screening of pesticides in vegetables, fruits and baby food by stir bar sorptive extraction-thermal desorption-capillary gas chromatography-mass spectrometry*. J. Chromatogr. A 1000, (2003), 299.

[119] Boughtflower R.J., Paterson C.J., Knox J.H., *Control of dispersion in capillary electrochromatography coupled to UV and mass spectrometric detection*. J. Chromatogr. A 887, (2000), 409.

[120] Lazar I.M., Lee M.L., *Effect of electrospray needle voltage on electroosmotic flow in capillary electrophoresis-mass spectrometry*. J. Am. Soc. Mass Spectrom. 10, (1999), 261.

[121] Blasco C., Font G., Picó Y., *Multiple-stage mass spectrometric analysis of six pesticides in oranges by liquid chromatography-atmospheric pressure chemical ionization-ion trap mass spectrometry*. J. Chromatogr. A 1043, (2004), 231.

[122] Jiménez J.J., Bernal J.L., Nozal M.J., Arias E., Bernal, J., *Determination of impurities in pesticides and their degradation products formed during the wine-making process by solid-phase extraction and gas chromatography with detection by electron impact mass spectrometry. I. Vinclozoline, procymidone and fenitrothion*. Rapid Commun. Mass Spectrom. 18, (2004), 657.

[123] Thurman E.M., Ferrer I., Barceló, D., *Choosing between atmospheric pressure chemical ionization and electrospray ionization interfaces for the HPLC/MS analysis of pesticides*. Anal. Chem. (2001), 73 5441.

[124] Spanish state secretary of trade and tourism. http://www.mcx.es/plaguicidas/espanol.asp

[125] Shihabi Z.K., *Stacking in capillary zone electrophoresis*. J. Chromatogr. A 902, (2000), 107.

[126] Kim J.B., Quirino J.P., Otsuka K., Terabe S., *On-line sample concentration in micellar electrokinetic chromatography using cationic surfactants*. J. Chromatogr. A 916 (2001), 123.

[127] Chen M.C., Chou S.H., Lin C.H., *Determination of corticosterone and 17-hydroxycorticosterone in plasma and urine samples by sweeping techniques using micellar electrokinetic chromatography*. J. Chromatogr. B 801, (2004), 347.

[128] Juan-García A., Picó Y., Font G., *Capillary electrophoresis for analyzing pesticides in fruits and vegetables using solid-phase extraction and stir-bar sorptive extraction*. J. Chromatogr. A 1073, (2005), 229.

[129] Juan-García, A., Font, G., Picó, Y., *Quantitative analysis of six pesticides in fruits by capillary electrophoresis-electrospray-mass spectrometry*. Electrophoresis 26, (2005), 1550.

[130] European Commission, Directorate General Health and Consumer Protection, Residue analytical methods for post-registration control and monitoring, SANCO/825/00 (rev. 7) 17.

[131] EU MRLs sorted by pesticide (disponible en http://europa.eu/food/plant/protection/pesticides/index.en.htm.

[132] Pesticide Residues in Food, Codex Alimentarius (disponible en http://www.codexalimentarius.net/mrls/pesticides/jsp/pest q-e.jsp).

[133] International Maximum Residue Limits Database (disponible en http://mrldatabase.com/query.cfm?CFID=1717249&CFTOKEN=).

[134] Hernández M., Borrull F., Calull M., *Determination of quinolones in plasma samples by capillary electrophoresis using solid-phase extraction*. J. Chromatogr. B 742, (2000), 255.

[135] Schmitt-Kopplin P., Burhenne J., Freitag D., Spiteller M., Kettrup A., *Development of capillary electrophoresis methods for the analysis of fluoroquinolones and application to the study of the influence of humic substances on their photodegradation in aqueous phase*. J. Chromatogr. A 837, (1999), 253.

[136] McCourt J., Bordin G., Rodríguez A. R., *Development of a capillary zone electrophoresis-electrospray ionisation tandem mass spectrometry method for the analysis of fluoroquinolone antibiotics.* J. Chromatogr. A 990, (2003), 259.

[137] Fernández F.M., Vadillo J. M., Kimmel J. R.,Wetterhall M., Markides N., Rodríguez K., Zare N.R., *Hadamard transform time-of-flight mass spectrometry: A high-speed detector for capillary-format separations*. Anal. Chem. 74, (2002), 1611.

[138] Frommberger M., Hertkorn N., Englmann M., Jakoby S., Hartmann a., Kettrup A., Schmitt-Kopplin P., *Analysis of N-acylhomoserine lactones after alkaline hydrolysis and anion-exchange solid-phase extraction by capillary zone electrophoresis-mass spectrometry*. Electrophoresis 26, (2005), 1513.

[139] Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning to the performance of analytical methods and the implementation of the results (Text with EEA relevance), Off. J. Eur. Commun. 2002, L221, 8–62.

[140] Nakata H., Kannan K., Jones P. D., Giesy J. P., *Determination of fluoroquinolone antibiotics in wastewater effluents by liquid chromatography-mass spectrometry and fluorescence detection*. Chemosphere 58, (2005), 759.

[141] Volmer D. A., Mansoori B., Locke S. J., *Study of 4-quinolone antibiotics in biological samples by short-column liquid chromatography coupled with electrospray ionization tandem mass spectrometry*. Anal. Chem. 69, (1997), 4143.

[142] Schilling J. B., Cepa S. P., Menacherry S. D., Bavda L. T., Heard B.M., Stockwell B.L., *Liquid chromatography combined with tandem mass spectrometry for the confirmation of sarafloxacin in catfish tissue*. Anal. Chem. 68, (1996), 1905.

[143] Toussaint B., Bordin G., Janosi A., Rodríguez A. R., *Validation of a liquid chromatography-tandem mass spectrometry method for the simultaneous quantification of 11 (fluoro)quinolone antibiotics in swine kidney*. J. Chromatogr. A 976, (2002), 195.

[144] van Vyncht G., Janosi A., Bordin G., Toussaint B., Maghuin-Rogister G., De Pauw E., Rodríguez A.R., *Multiresidue determination of (fluoro)quinolone antibiotics in swine kidney using liquid chromatography-tandem mass spectrometry*. J. Chromatogr. A 952, (2002), 121.

[145] Stoev G., Michailova A., *Quantitative determination of sulfonamide residues in foods of animal origin by high-performance liquid chromatography with fluorescence detection*. J. Chromatogr. A 871, (2000), 37.

[146] Van Eeckhout N., Pérez J.C., Van Peteghem C., *Determination of eight sulfonamides in bovine kidney by liquid chromatography/tandem mass spectrometry with on-line extraction and sample clean-up*. Rapid Commun. Mass Spectrom. 14, (2000), 2331.

[147] Furusawa N., *Rapid high-performance liquid chromatographic determining technique of sulfamonomethoxine, sulfadimethoxine, and sulfaquinoxaline in eggs without use of organic solvents*. Anal. Chim. Acta 481, (2003), 255.

[148] Bogialli S., Curini R., Di Corcia A., Nazzari M., Polci M.L., *Rapid confirmatory assay for determining 12 sulfonamide antimicrobials in milk and eggs by matrix solid-phase dispersion and liquid chromatography-mass spectrometry*. J. Agric. Food Chem. 51, (2003), 4225.

[149] Bogialli S., Curini R., Di Corcia A., Nazzari M., Samperi R., *A liquid chromatography-mass spectrometry assay for analyzing sulfonamide antibacterials in cattle and fish muscle tissues*. Anal. Chem. 75, (2003), 1798.

[150] Bogialli S., Curini R., Di Corcia A., Nazzari M., Sergi M., *Confirmatory analysis of sulfonamide antibacterials in bovine liver and kidney: extraction with hot water and liquid chromatography coupled to a single- or triple-quadrupole mass spectrometer*. Rapid Commun. Mass Spectrom. 17, (2003), 1146.

[151] Gentili A., Perret D., Marchese S., Sergi M., Olmi C., Curini R., *Accelerated solvent extraction and confirmatory analysis of sulfonamide residues in raw meat and infant foods by liquid chromatography electrospray tandem mass spectrometry*. J. Agric. Food Chem. 52, (2004), 4614.

[152] Berardi C., Bogialli S., Curini R., Di Corcia A., Lagana A., *A simple and rapid assay for analyzing residues of carbamate insecticides in vegetables and fruits: Hot water extraction followed by liquid chromatography-mass spectrometry*. J. Agric. Food Chem. 52, (2004), 4614.

[153] Stoob K., Singer H.P., Stettier S., Hartmann N., Mueller S.R., Stamm C.H., *Exhaustive extraction of sulfonamide antibiotics from aged agricultural soils using pressurized liquid extraction*. J. Chromatogr. A 1128, (2006), 1.

[154] Verzegnassi L., Savoy-Perroud M.C., Stadler R.H., *Application of liquid chromatography-electrospray ionization tandem mass spectrometry to the detection of 10 sulfonamides in honey*. J. Chromatogr. A 977, (2002), 77.

[155] Horstkotter C., Jiménez-Lozano E., Barron D., Barbosa J., Blaschke G., *Determination of residues of enrofloxacin and its metabolite ciprofloxacin in chicken muscle by capillary electrophoresis using laser-induced fluorescence detection* Electrophoresis 23, (2002), 3078.

Anexos
Annexes

Anexo I TABLAS -*TABLES*

Anexo II GRÁFICOS - *GRAFICS*

Anexo III FIGURAS - *FIGURES*

Anexo IV ABREVIATURAS Y ACRÓNIMOS
ABREVIATIONS AND ACRONYMS

AINES: antiinflamatorios no esteroideos

AEPLA: Asociación Española para la Agricultura/ Asociación Empresarial para la protección de plantas

APPCC: análisis de peligros y puntos críticos de control

AESA: Agencia Española de Seguridad Alimentaria

BPAs: Buenas prácticas agrícolas

BGE: "*background electrolite"* tampón de separación

CL/LC: cromatografía líquida

CG: cromatografía de gases

COP/COP´s: compuestos orgánicos persistentes

CL_{50}: concentración letal 50

CEE: Comunidad Económica Europea

CEC: Electrocromatografía capilar

CGP: cromatografía de gel permeación

CFS: extracción en fluidos supercríticos

CDs: ciclodextrinas

DAD: detector de fila de diodos

DL_{50}: dosis letal 50

DCM: Diclorometano

DCE: detector de captura de electrones

DFLL/FPD: detector de fotometría de llama

DNF: detector de nitrógeno fósforo

DMFS/MSFD: dispersión de matriz en fase sólida

DFD/DAD: detector de fila de diodos

dc: corriente directa

EC: electroforesis capilar

ECEM/MEKC: electroforesis capilar electrocinética micelar

ECG/CGE: electroforesis capilar de gel

ECZ/CZE: electroforesis capilar de zona

EFS/SPE: extracción en fase sólida

EIC/CIEF: enfoque isoeléctrico capilar

ELL: extracción líquido-líquido

EM/MS: espectrometría de masas

EM/EM o MS/MS: espectrometría de masas en tándem

EOF: flujo electroosmótico

EPD: extracción presurizada con disolventes

ESBM/SBSE: extracción sobre barras magnéticas

FAO: Organización Mundial para la Agricultura y la Alimentación

FAO/OMS de Expertos en Aditivos Alimentarios

ICP/CITP: isocatoforesis capilar

IDA: ingesta diaria admisible

IDE: ingesta diaria estimada

IFAH: Federación Internacional de la Sanidad Animal

IUPAC: "*International Union of Pure and Applied Chemistry*" Unión Internacional de Química Pura y Aplicada.

JECFA: *Joint FAO/WHO Expert Committeeon Food Additives*, Comité Mixto

LD/LOD: límites de detección

LIF: fluorescencia inducida por láser

LMR/MRL: límite máximo de residuos

LDC/LOQ: límites de cuantificación

MAPA: Ministerio de Agricultura Pesca y Alimentación

MEFS/SPME: microextracción en fase sólida

NOAEL: dosis sin efecto

OMS: Organización Mundial de la Salud

OECD/OCDE: Organización para la Cooperación y el Desarrollo Económico (OCDE) (Organisation for economic co-operation and developement (OECD)),

PABA: para-aminobenzoico

PAC: Política Agraria Común

PCBs: bifenilos policlorados

PCDD: dibenzo-p-dioxinas policloradas

PCDF: dibenzofuranos policlorados

PI: punto isoeléctrico

PLE: *Pressurized Solvent Extraction,* extracción presurizada con disolventes

PM/MW: peso molecular

POF: plaguicidas organofosforados

QIT: trampa de iones

QqQ: triple cuadrupolo

SDS: dodecilsulfato sódico

SIM/SIR: monitorización simple de iones

SLM: "*Supported liquid membrane*" membrana de soporte líquido

SRMM: acumulación por migración reversa de micelas con tapón de agua

SW: *sweeping*, focalización

SRW: acumulación por migración reversa de micelas sin tapón de agua

TFA/ATF: ácido trifluoroacético

UE: Unión Europea

UV: ultravioleta

VCIA: antimicrobianos veterinarios de importancia crítica

Anexo V DIFUSIÓN DE LOS RESULTADOS - *RESULTS SPREAD*

▪ *CONGRESOS-Congresses*

AUTORES: A. Juan, Y. Picó, R. Marín, G. Font
TÍTULO: Determinación de acefato en uva por electroforesis capilar de zona espectrometría de masas.
CONGRESO XV Congreso Español de Toxicología (AETOX)
PUBLICACIÓN Abstract Book
LUGAR DE REALIZACIÓN: Valencia (España)
AÑO: 2003

AUTORES: G. Font, A. Juan-García y Y. Picó
TÍTULO: A rapid screening method to estimate acephate, fosetyl aluminium and methamidophos in grape by capillary electrophoresis-electrospray mass spectrometry.
CONGRESO 27th Symposium on High Performance Liquid Phase Separations and Related Techniques. HPLC 2003
PUBLICACIÓN Abstract Book
LUGAR DE REALIZACIÓN: Niza (Francia)
AÑO: 2003

AUTORES: A. Juan-García, Y. Picó, J. Mañes, G. Font
TÍTULO: Potential of SPE and SBSE approaches for the extraction of pesticides in grapes
CONGRESO 3rd Scientific Meeting of the SECyTA and 3rd Waste Water Cluster European Workshop
PUBLICACIÓN Abstract Book
LUGAR DE REALIZACIÓN: Almeria (España)
AÑO: 2003

AUTORES: A. Juan-García, Y. Picó, S. Loran, G. Font
TÍTULO: SPE vs. SBSE as preconcentration techniques to determine pesticide residues inn lettuce by capillary electrophoresis
CONGRESO 28th international Symposium and Exhibit on high Performance Liquid Phase Separation and Related Techniques
PUBLICACIÓN Abstract Book
LUGAR DE REALIZACIÓN: Philadelphia (USA)
AÑO: 2004

AUTORES: A. Juan-García, Y. Picó, G. Font
TÍTULO: Determination of pesticides in grapes and strawberries by capillary electrophoresis
CONGRESO 25th International Symposium of Chromatography
PUBLICACIÓN Abstract Book
LUGAR DE REALIZACIÓN: Paris (Francia)
AÑO: 2004

AUTORES Y. Picó, G. Font;A. Juan-García
TÍTULO: CE-MS to determine pesticides in vegetables (Conference Paper)
CONGRESO: II Reunión de Espectrometría de Masas-SEEM
PUBLICACIÓN Abstract Book
LUGAR DE REALIZACIÓN: Barcelona (España)
AÑO: 2004

AUTORES A. Juan-Garcia,C. Juan, G. Sarais, P. Cabras, P. Caboni
TÍTULO: Separation and identification of main limonoid compounds in neem seeds extracts.
CONGRESO: INTRAFOOD 2005, Innovations Traditional Foods, by the European Federation of Food Science and Technology/ institute of Food Engineering for Development (UPV) Institute of Agrochemistry and Food Technology (CSIC)
PUBLICACIÓN: Abstract and Proceedeing Book
LUGAR DE REALIZACIÓN: Politecnic University of València. València (España)
AÑO 2005

AUTORES S. De Melo Abreu, A. Juan-García, P. Caboni, P. Cabras, A. Alves, V. L. Garau
TÍTULO: Study of disappearance mechanism of fenamidone with model systems
CONGRESO: 4th MGPR International Symposium of Pesticides in Food and the Environment in Mediterranean Countries and MGPR Annual Meeting 2005
PUBLICACIÓN Abstract Book
LUGAR DE REALIZACIÓN: Kuzadasi-Aydin (Turquía)
AÑO 2005

AUTORES A. Juan-García, Y. Picó, G. Font
TÍTULO: Determinación de residuos de quinolonas en alimentos de origen animal mediante electroforesis capilar espectrometría de masas (Comunication Paper)
CONGRESO: XVI Congreso de la Asociación Española de Toxicología (AETOX)
PUBLICACIÓN Abstract Book
LUGAR DE REALIZACIÓN: Cáceres (España)
AÑO 2005

AUTORES A. Juan-García, G. Font, Y. Picó
TÍTULO: Capillary electrophoresis-mass spectrometry for quinolone residue determination in fish and chicken.
CONGRESO 2nd International Symposium on Recent Advances in Food Analysis-IEAEC International
PUBLICACIÓN: Abstract Book
LUGAR DE REALIZACIÓN: Praga (República Checa)
AÑO 2005

AUTORES A. Juan-García, Y. Picó, G. Font
TÍTULO: Determination of quinolone residues in food of animal origin by capillary electrophoresis-mass spectrometry
CONGRESO 11as Jornadas de Análisis Instrumental (JAI-2005)
PUBLICACIÓN: Abstract Book
LUGAR DE REALIZACIÓN: Barcelona (España)
AÑO 2005

AUTORES A. Juan-García, Y. Picó, G. Font
TÍTULO: β-casomorphins implied in attention deficit disorder. Studies in derived milk products by liquid chromatography – multiple tandem mass spectrometry methods.
CONGRESO International Conference on Food Contaminants and Neurodevelopmental Disorders- Cátedra Santiago Grisolía
PUBLICACIÓN: Abstract Book
LUGAR DE REALIZACIÓN: Valencia (España)
AÑO 2006

AUTORES: A. Juan-García, K.J. James, A. Furey, Y. Picó, G. Font
TÍTULO Liquid chromatography – multiple tandem mass spectrometry methods to study β-casomorphins in derived milk products
CONGRESO VI Scientific Meeting of the Spanish Society of Chromatography and related Techniques
PUBLICACIÓN: Abstract Book
LUGAR DE REALIZACIÓN: Vigo (España)
AÑO 2006

AUTORES: A. Juan-García, Y. Picó, G. Font
TÍTULO: Determinación de residuos de sulfonamidas en carne por EC-EM/EM y extracción presurizada con disolventes
CONGRESO XVII Congreso Español de la Asociación Española de Toxicología
PUBLICACIÓN: Abstract Book
LUGAR DE REALIZACIÓN: Santiago de Compostela (España)
AÑO 2007

AUTORES: A. Juan-García, G. Font , Y. Picó
TÍTULO: Analysis and method validation of veterinary antibiotics residue in meat by capillary electrophoresis-mass spectrometry
CONGRESO: SEEM- Sociedad Española de Espectrometría de Masas
PUBLICACIÓN: Abstract Book
LUGAR DE REALIZACIÓN:Granada (España)
AÑO 2007

AUTORES: A. Juan-García, G. Font , Y. Picó
TÍTULO: Determination of pesticide residues in mediterranean summer fruits by capillary electrophoresis and pressurized liquid extraction
CONGRESO (SECyTA- Sociedad Española de Cromatorgrafía y Técnicas Afines
PUBLICACIÓN: Abstract Book
LUGAR DE REALIZACIÓN: Granada (España)
AÑO 2007

AUTORES: A. Juan-García, Y. Picó, G. Font
TÍTULO: Analysis of polar pesticide residues in fruits with high water content by hot water extraction and capillary electrophoresis-mass spectrometry
CONGRESO 3rd International Symposium on Recent Advances in Food Analysis
PUBLICACIÓN: Abstract Book
LUGAR DE REALIZACIÓN: Praga (República Checa)
AÑO 2007

AUTORES: C. Blasco, A. Juan-García, Y. Picó
TÍTULO: Validation of an analytical method to determine four tetracyclines in meat by pressuried liquid extraction and liquid chromatography-tandem mass spectrometry
CONGRESO 3 rd International Symposium on Recent Advances in Food Analysis
PUBLICACIÓN: Abstract Book
LUGAR DE REALIZACIÓN: Praga (República Checa)
AÑO 2007

▪ *PUBLICACIONES-Papers published*

A. Juan-García, J. Mañes, G. Font, Y. Picó, *Evaluation of solid-phase extraction and stir-bar sorptive extraction for the determination of fungicide residues at low-μg kg^{-1} levels in grapes by liquid chromatography–mass spectrometry. Journal of Chromatography A, 1050 (2004) 119–127*
TIMES CITED: 12 IMPACT FACTOR: 3.359

A. Juan-García, Y. Picó, G. Font, *Capillary electrophoresis for analyzing pesticides in fruits and vegetables using solid-phase extraction and stir-bar sorptive extraction. Journal of Chromatography A, 1073 (2005) 229–236*
TIMES CITED: 15 IMPACT FACTOR: 3.096

A. Juan-García, G. Font, Y. Picó, *Determination of organic contaminants in food by capillary electrophoresis. Journal of Separation Science 28 (2005) 793-812*
TIMES CITED: 13 IMPACT FACTOR: 1.829

A. Juan-García, G. Font, Y. Picó, *Quantitative analysis of six pesticides in fruits by capillary electrophoresis-electrospray-mass spectrometry. Electrophoresis 26 (2005) 1550-1561*
TIMES CITED: 11 IMPACT FACTOR: 3.850

A. Juan-García, G. Font, Y. Picó, *Determination of quinolone residues in chicken and fish by capillary electrophoresis-mass spectrometry. Electrophoresis 27 (2006) 2240-2249*
TIMES CITED: 13 IMPACT FACTOR: 4.101

A. Juan-García, G. Font, Y. Picó, *On-line preconcentration strategies for analyzing pesticides in fruits and vegetables by micellar electrokinetic chromatography. Journal of Chromatography A, 1153 (2007) 104–113*
TIMES CITED: 0

G. Font, A. Juan-García, Y. Picó, *Pressurized liquid extraction combined with capillary electrophoresis–mass spectrometry as an improved methodology for the determination of sulfonamide residues in meat. Journal of Chromatography A, 1159 (2007) 233–241*
TIMES CITED: 2

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Determination of organic contaminants in food by capillary electrophoresis

This review addresses recent advances in the analysis of organic contaminants, such as antibiotics, pesticides, biological toxins, and food-borne pathogens, in foods by capillary electrophoresis (CE). Special attention is paid to those aspects that increase sensitivity and/or selectivity, such as sample extraction and concentration, on-line preconcentration techniques (stacking), affinity capillaries or/and specific detectors (laser induced fluorescence (LIF), mass spectrometry (MS)). The various CE modes used to separate the compounds and the quantification strategies are also examined. As a result, this work presents an updated overview on the principal applications of CE, together with a discussion of their main advantages and drawbacks, and an outline of future trends in the analysis of organic contaminants in food.

Key Words: Capillary electrophoresis; Food contaminants; Antibacterials; Pesticides; Toxins

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1 Introduction

There are many contaminants of an organic nature that can occur in food, including mycotoxins, pesticides, pathogenic organisms, and impurities associated with environmental pollution or with contact migration from food packaging. Food safety is ensured through legislation that relies heavily on appropriate analytical methods to detect contamination of food, both rapidly and reliably [1, 2].

The analysis of organic contaminants in food is an important issue that encompasses many disciplines including chemistry, biochemistry, and microbiology. It is extremely intricate because these pollutants, which can be numbered in the thousands, are present at extremely low concentrations in a very complex mixture of natural food components. Thus, any instrumental technique that is to be used successfully in food analysis should achieve the separation of the matrix compounds and the identification of the contaminants at trace levels [1, 2].

Capillary electrophoresis (CE) has emerged over the last decade as a powerful analytical technique that has been applied to the analysis of food, forensic, pharmaceutical, environmental, e.g. clinical, molecular biological samples, etc. [3–8], because it is a very well suited technique for the analytes that are neither determinable by gas chromatography (GC) nor separable by liquid chromatography (LC). Commercial CE instruments are automated in a similar way to LC. Inherent characteristics of CE, such as speed of analysis, high efficiency, separation selectivity, small sample size capability, and low reagent consumption, have been recognized within the scientific community. A technical challenge to deal with in order to establish CE as a viable alternative to LC is to further improve the attained limits of detection (LODs). Sensitivity is an important restriction, as a consequence of the narrow bore of the CE capillary that limits injection volumes to nanoliters and, when interfaced with an on-line optical detector, gives shorter optical detection path-lengths in comparison with LC. This drawback has been already overcome by approaches such as optimised sample injection techniques (on-line preconcentration, stacking or sweeping), laser-induced fluorescence (LIF) detectors (a good option when the analyte is amenable), and hyphenation of CE with mass spectrometry (MS) (already a reality). Aspects of on-line preconcentration methods for CE have been reviewed by Osbourn et al. [9], Beckers and Bocek [10], and Quirino et al. [11], as well as those of CE-MS by

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Abbreviations: ASP, amnesic shellfish poisoning; CFLP, cleavase fragment length polymorphism; CFS, continuous flow system; CGE, capillary gel electrophoresis; CID, collision induced dissociation; EDCs, endocrine disrupting compounds; EDTA, ethylenediaminetetracetic acid; EU, European Union; GTX, gonyautoxin; HDB, hexadimethrine bromide; HEC, 2-hydroxyethyl cellulose; MPE, multiphoton-excited; PCDDs, polychlorinated dibenzo-*p*-dioxins; PSP, paralytic shellfish poisoning; SBSE, stir-bar sorptive extraction; SLM, supported liquid membrane; SRMM, stacking with reverse migration micelles; SRW, stacking with reverse migration micelles and a water plug; SSCP, single-strand conformation polymorphism; STX, saxitoxin; VT, verotoxin; YOPRO1, monomeric cyanine nucleic acid stain;

Schmitt-Kopplin and Frommberger [12], and Choudhary et al. [13].

Many impressive CE separations, including the separation of pollutants, have been demonstrated in the last few years. The growing importance of CE, in the field of organic contaminants analysis, is emphasized by the ubiquity of the technique in reviews of a more general nature on chromatographic determination of organic contaminants in food or other environmental matrices, showing that CE is established as an option from the beginning of a new method development [14–17].

The application of CE to food analysis is still limited in comparison with other fields of study [18]. However, significant progress has been made to widen its applicability and to address important issues relating to food quality and safety such as proteomics, food contamination, microbial identification, species recognition, or detection of transgenic food [18–20].

The aim of this work is to describe the role of CE in the analysis of organic contaminants in food, with special emphasis on work published in the last five years. This description focuses on how CE is used in those food applications (sample treatment, on-column preconcentration procedures, CE operation modes, and detectors). Finally, possible future trends and developments of CE in this area are briefly discussed.

2 Classification of organic contaminants

Food contaminants are defined in European Union (EU) legislation as any substance not intentionally added to food, which is present in food as result of the production, manufacture, processing, preparation, treatment, packaging, transport, or holding of such food, or as a result of environmental contamination. Among those, organic contaminants can be chemicals or biologicals [21].

Microorganisms are ubiquitous in the environment, and they may infect fresh food or stored products and produce toxic metabolites. Biological toxins, which are secondary metabolites of fungi, bacteria, and algae, can contaminate food. Under the right temperature and moisture conditions, stored food products contaminated by various microorganisms may become toxic. Certain types of microorganism may also proliferate on entering in gastrointestinal tract, where the conditions contribute to their development [22, 23].

The products of a fast-growing modern technology constitute a major source of food contamination. The increasing world population, as well as mass migration into the cities, demands a substantial and rapid increase of the food supply. Improved agricultural productivity requires constant large-scale application of fertilizers, insecticides, herbicides, fungicides, and other pesticides, growth stimulants, and antibiotics. Residues, sometimes at toxic levels, from most of these products, end up in the food supply. Recent reviews have detailed the application of CE to the trace analysis of pesticides [24–28] and veterinary antibiotic residues [29, 30], showing that this technique constitutes a viable alternative to other chromatographic resources.

Environmental contaminants also come from other origins besides agriculture. Transformation of the population's lifestyles has also caused the proliferation of synthetic chemicals for use in all aspects of modern life. Some organic contaminants, such as polychlorinated biphenyls (PCBs) [14, 31], polychlorinated dibenzo-*p*-dioxins (PCDDs) [32], endocrine disrupting compounds (EDCs) [33, 34], and polycyclic aromatic hydrocarbons (PAHs) [35–38], which enter food from the environment, have been separated and detected by CE, but without any application to foods.

However, the aim of this review is not to cover demonstrations of high resolution separations of specific compounds but to refer to their determination in real food samples. **Table 1** summarizes the applications developed in food analysis, showing that the main applications are in the field of pesticide residue analysis and antibiotics; some of them cover biological toxins, mainly mycotoxins but also some bacterial toxins, and a few examples treat foodborne pathogens and toxin-producing microorganisms.

There is a relationship between the contaminants sought and the foods selected for their analysis. Thus, veterinary antibacterials and bacteria have been determined in foods of animal origin, mainly meat, fish, and milk, mycotoxins in cereals, and pesticides in fruits and vegetables. The chemical components and their content in these foods are very different. For instance, meat and milk are rich in proteins and lipids, cereals have low moisture and high proportion of complex polysaccharides, and fruits and vegetables have a high water content (between 80–90%) and a certain proportion of sugars (complex sugars such as starch in the case of vegetables and simple ones as fructose, glucose, and saccharose in fruits).

3 Procedures for extracting and concentrating organic contaminants from food

Adequate isolation and preconcentration of the organic contaminants are mandatory and critical steps in the CE procedures for their determination in food. The procedures used can be classified according to the type of organic contaminant, chemical or biological. For the first type, several extraction approaches – mainly classical solvent extraction followed by either solid phase extraction (SPE) or solid phase microextraction (SPME) – have been employed for sample preparation. A further concentration is achieved by evaporating the organic solvent and

Table 1. Applications of CE to determine organic contaminants in food.

Organic contaminants	Type	Food	Ref.
Antibacterial agents	Quinolones	Chicken, Pig, Fish	[39–45]
	Tetracyclines	Catfish	[46]
	Sulfonamides	Milk, meat	[47–49]
	Miscellaneous	Food	[50]
Pesticides	Dithiocarbamates	Wheat	[51–53]
	Fungicides	Oranges, lemons, fruit juices, grapes, tomatoes, lettuces	[54–60]
	Polar P-containing (glyphosate, glufosinate)	Wheat	[61, 62]
	Organophosphorus	Leaves	[63]
	Urea-derived	Tomatoes, oranges	[64]
	Triazines	Juices	[65]
	Acidic herbicides	Apples, grapes, oranges, tomatoes	[66]
	Multiple pesticides	Cucumbers, carrots, juices	[4, 45, 67–69]
Plant growth regulator	Maleic Hydrazide	Potatoes, onions	[70]
Biological toxins			
Mycotoxins	Ochratoxin A	Roasted coffee, corn, sorghum, feed	[22, 71]
	Patulin	Apple cider	[72]
	Aflatoxins	Corn, feed	[71, 73, 74]
Bacterial toxins	Cholera toxin A	Corn, peanuts	[73]
Shellfish toxins	ASP (amnesic shellfish poisoning) PSP (paralytic shellfish poisoning)	Seafood products (razor clams, mussels)	[75]
Foodborne pathogens	*S. aureus, L. monocytogenes, Salmonella spp.*	Meat	[76]
	Escherichia coli Vero toxins (VT1, VT2)	–	[77]

reconstituting the sample in a small volume. For the second type, the use of molecular techniques, mostly PCR-based procedures, has become the preferred approach.

Although the particular sample pre-treatment method depends mainly on the matrix, the most general procedure is to employ an organic solvent and to perform clean-up through SPE cartridges, independently of the sample processed and the chemical differences described previously. However, the more complex the sample is, the more difficult it is to remove interfering sample components. In **Table 2**, the applications of some of these techniques for analyzing organic contaminants in food are summarized, including some comments on the procedures' viability, and the LODs obtained when conventional injection and DAD or UV detection are used. Other approaches, such as on-line preconcentration or selective detectors, are discussed in the next section.

Most antibacterials exhibit amphoteric properties because they have proton-binding sites, such as amino groups that provide protonated species, and acidic groups that are dissociated to the anionic species. Their amphoteric properties are both a help and an impediment in extraction and clean-up procedures because there is a marked pH-dependent variation of their partition coefficients between aqueous and organic solvents in the pH range 7–9. Pesticides and mycotoxins are neutral compounds that require the use of hydro-alcoholic mixtures to favour the recovery of the compounds from the solid matrix.

There is a general consensus that extraction with alkyl-bonded SPE sorbents, particularly C_8 and C_{18} sorbents, is the most common type of SPE performed. However, many types of sorbent materials are currently available, which may lead to different selectivities when applied to organic contaminants extraction

Jiménez-Lozano et al. [44] compared several commercial sorbents (Zorbax C_{18}, Bond Elut C_{18}, Isolute ENV+, Oasis HLN, Oasis MAX, SDS-RPS, and MPC-SD) for the SPE of series of quinolones regulated by the EU, in chicken tissues, to establish a method for determining them by CE-DAD. The sorbents tested were of different characteris-

Table 2. Extraction, isolation, and concentration methods described in the literature pertaining to the determination of organic contaminants in food.

Organic contaminant	Extraction	Isolation/concentration	Comment	LODs[a] (Conc. fold)[b]	Ref.
Quinolones	Phosphate buffer pH 7	SPE/Phosphate buffer (pH 9)/CH_3OH	Adjust the extract to pH 3 prior SPE	0.08 μg/mL (1)	[40]
	0.1 N HCl	SPE C_{18}/CH_3OH	–	120 μg/kg (0.5)	[42]
	Phosphate buffer pH 7/dichloromethane	SPE (Bond Elut C_{18})/trifluoroacetic acid/water/acetonitrile	Neutralize with a phosphate buffer and defat with hexane prior to SPE	26 μg/kg (50)	[41]
	Phosphate buffer pH 7/dichloromethane/NaOH	SPE (C_{18})/trifluoroacetic acid/water/acetonitrile	Neutralize with a phosphate buffer and defat with hexane prior to SPE	10–25 μg/kg (50)	[43]
	Dichloromethane/NaOH	SPE (Zorbax C_{18}, Bond Elut C_{18}, Isolute ENV+, Oasis HLB, and Oasis MAX)/trifluoroacetic acid/acetonitrile/water	Defat with hexane prior to SPE. Adjust the extracts' pH when Bond Elut and Oasis MAX cartridges are used	10–15 μg/kg (50)	[39, 44, 45]
Tetracyclines	Trichloroacetic acid, HCl, EDTA	SPE (Sep-Pak C_{18})/CH_3OH	–	< 0.1 mg/kg (10)	[46]
Sulfonamides	Acetonitrile	SPE (Alumina N)/propanol SPE (Oasis HLB)/CH_3OH	Acetonitrile extract a lot of endogenous substances Two SPE cartridges are used for sample clean-up, one polar and the other is a copolymer with hydrophilic and lipophilic functions	5–10 μg/kg (50)	[48]
	Without extraction (milk samples)	Evaporate to dryness, redissolve in CH_3OH, evaporate to dryness, redissolve in water	Acidic precipitation of proteins and elimination of lipids with hexane More efficient extraction and clean-up could further improve LODs	CE-MS (–)	[47]
	Without extraction (milk samples)	SPE C_{18} (minicolumn)/CH_3OH	Acidic precipitation of proteins prior to SPE	CE-MS (2 mL milk on-line)	[49]
Miscellaneous	Quinolones (acetonitrile) Other residues (ethyl acetate/NaOH)	–	Modification in LLE was introduced to reduce background effects	< 20 μg/kg (10)	[50]
Dithiocarbamates	NaOH solution	–	Insufficient sensitivity to reach MRLs	0.5–1 mg/kg (0.2)	[51, 52]
	Chloroform	–	Derivatization of ferbam to iron (III)	0.7 mg/kg (0.2)	[53]
Fungicides	Acetonitrile/NaOH	SPE Sep-Pak PS-2 /acetonitrile	Extraction under basic conditions	– (30)	[54]
	Ethyl acetate/NH_3	Acid-base partitions	No interferences in the CE from endogenous compounds	0.6 mg/kg (6)	[60]
	5 mM NaCl/Cyclohexane/ethyl acetate/acetone	–	Further sample enrichment (by SPE) may be required to ensure the required sensitivity	Stacking	[55]
	Water/CH_3OH	SPE C_9/Dichloromethane	Some degradation during evaporation and redissolution	0.1–1 mg/kg (10)	[56–58]
Polar-P containing (glyphosate, etc.)	Water/acetone	–	Lack of sensitivity if MS is not used	CE-MS CE-FPD	[61, 62]
Organophosphorus	Acetone	Acetonitrile/water	Lack of sensitivity	–	[63]
Urea-derived	CH_3OH/water	SPE C_{18}/dichloromethane/water	Lack of sensitivity	≈0.05 mg/kg (10)	[64]
Triazines	0.5 M H_2SO_4/CH_3OH	SLM-SPE	Problems with matrix interferences, however, SLM-SPE provides cleaner extracts and lower LODs than only SPE	10 μg/L (100)	[65]
Acidic herbicides	Acetone-water (5:1)	SPME CW/TPR fibers/CH_3OH	Acetone should be evaporate prior to SPME to obtain appropriate recoveries SPME requires careful optimization	CE-MS	[66]

Table 2. Continued.

Organic contaminant	Extraction	Isolation/concentration	Comment	LODs[a] (Conc. fold)[b]	Ref.
Multipesticides	Dilution with water	SPME PDMS/DVB fiber/ CH_3OH	SPME requires careful optimization Use of chemometrics to optimize	CE-MS	[69]
	Acetone/petroleum ether/dichloro-methane	SPE NH_2/Dichloromethane/ CH_3OH	Extracts are concentrated by 3 on-line preconcentration strategies	Stacking	[68]
	Dichloromethane/ petroleum ether	–	–	0.22 – 1.13 μg/L (75)	[67]
	Acetone/water	SPE C_{18}/dichloromethane SBSE/CH_3OH	Comparison of SPE and SBSE. SPE is more robust, rapid, and sensitive than SBSE	<0.5 mg/kg (10)	[78, 79]
Maleic hydrazide	CH_3OH /water	SPE C_{18}/water	Potatoes contain high amount of starch	2.0 mg/kg	[70]
Ochratoxin A	Dichloromethane/ phosphate buffer	SPE (silica and immunoaffinity column)	The silica column cleans up the sample extract allowing the passage of a larger sample amount through the immunoaffinity column	CE-LIF	[22]
Aflatoxins	Acetonitrile/water	SPE C_{18}/CH_3OH	Screening and confirmatory technique to process many samples in little time	0.02 – 0.06 mg/kg (10)	[71]
Aflatoxin B_1	Celite/water/chloroform	SPE (silica or immunoaffinity column)	It is possible to increase sensitivity by reconstituting the dried extract with less buffer. Immunoaffinity columns reduce the amount of corn that can be loaded	CE-LIF	[74]
Patulin	Ethyl acetate	–	Rapid method	3.8 μg/L (20)	[72]
Domoic acid and PSP toxins	CH_3OH:water	Domoic acid was SPE on SAX and SCX cartridges PSP toxins were SPE C_{18}	Strong effect of the acidic conditions and temperature. Low resolution of real samples associated to the complexity of the matrix (high salt content, interfering substances)	0.05 – 0.06 μg/mL (10)	[75]
S. aureus *L. monocytogenes* *Salmonella spp*	DNA lysis buffer	DNeasy Tissue Kit Multiplex PCR reaction	Simultaneous detection of three foodborne pathogens. The sample processing was adapted for the co-detection of three species by including both lysozyme and lysostapin in a single incubation step. Specific primers were selected taking into account that they should have similar melting temperature and length of DNA to prevent differential yields in band amplification products	CGE-LIF	[76]
Escherichia coli Vero toxins (VT1, VT2)	DNA extracted by SDS-lysozyme	Allele specific PCR SSCP CFLP	These methods provided excellent results with respect to specificity and speed. Possibility to detect VT1 and VT2 genes simultaneously. Identification of O157 fungal type	CGE-LIF	[77]

a) LODs only when typical injection and DAD is used.
b) (Conc. fold): Concentration fold.

tics: non-polar C_{18} (Bond Elut and Zorbax), polymeric sorbents (ENV+ and HLB), and mixed phases as C_8 and cationic exchange (MPC-SD), polymeric with sulfonic acid group (SDB-RPS) and polymeric with strong anion exchange (MAX). High recoveries were obtained with HLB, SDB-RPS, and MAX cartridges. An increase in the peak width was produced using HLB cartridge, while acceptable peaks were obtained with SDS-RPS and MAX cartridges, the sample extraction procedure being shorter in MAX than on using SDB-RPS. The LODs and LOQs were lower on using MAX than on using SDB-RPS, except for ciprofloxacin, which was similar in both.

SPME is an alternative sample preparation technique that uses a fused-silica fiber coated with a non-selective sorbent to extract samples and desorb analytes into a sol-

vent. Only two papers discuss the use of SPME combined with CE to determine pesticides in food. Rodríguez et al. [66] determined five acidic pesticides, *o*-phenylphenol, ioxynil, haloxyfop, acyfluoren, and pichloran, in fruit and reported recoveries almost equal to those reported by SPE. Hernández-Borges et al. [69] studied other different pesticides, pyrimethanil, pyrifenox, cyprodinil, cyromazine, and pirimicarb, in juices. Both arrived at the same conclusion: SPME has advantages as it is fast and simple and promises good possibilities of automation, but the main drawbacks are the many different parameters that have to be optimized before adequate analytical conditions are achieved, and the lack of recovery for some compounds. The former inconvenience can be palliated by the use of chemometrics. In any case, SPME is an innovative tactic for sample handling in food analysis, which achieves acceptable recoveries, precision, and LOQs.

Another option for SPE is stir-bar sorptive extraction (SBSE), which used a magnetic rod coated with polydimethylsiloxane (PDMS). Juan-García et al. [78] compares SPE and SBSE in combination with MEKC-DAD for the simultaneous extraction of acrinathrin, bitertanol, cyproconazole, fluodioxonil, flutriafol, myclobutanil, pyriproxyfen, and tebuconazole in lettuce, tomato, grape, and strawberry. SPE offers clear advantages that consolidate this procedure as the method of choice, such as higher recoveries (at least twice the recovery obtained by SBSE) and speed (complete analysis of each sample takes ca. 1.30 h instead of the 5 h required by SBSE). Although SBSE is less reliable than SPE for routine analysis, this procedure also presents some advantages over SPE such as better precision and less matrix compounds coextraction.

The number of published reports really concerned with food matrices is limited because a major problem appears during the extraction process: the presence of matrix interferences. Generally, this inconvenience is reduced by dilution of samples, leading to increased LODs, or by the use of more selective detectors, which will be discussed in next section. Another possibility, related to the extraction methods, is the application of immunoaffinity-based SPE or SPE with molecularly imprinted polymers. However, these methods require specially prepared sorbents that are significantly more expensive. In fact, the latter approach has never been applied to determine organic contaminants in food by CE. Immunoaffinity columns have been used to determine ochratoxin A and aflatoxins [22, 74]. Aflatoxins were isolated from corn using either silica columns or affinity columns. The affinity columns used are commercially available but have the limitation that less corn can be loaded onto the column than in the case of silica columns. This low load capacity effectively reduces the sensitivity 10-fold when affinity columns are used [74]. In this context, a procedure developed by Corneli and Maragos [22] for ochratoxin A combines a silica column followed by an ochatoxin-specific affinity column. The first clean-up step was not sufficient for a quantification of ochratoxin because of the large number of interfering peaks present in the electropherogram. Nevertheless, the silica column cleans up the extract enough to increase the amount of sample that passes through the commercial affinity column, which results in an improvement of the method sensitivity.

Khrolenko et al. [65] explored, as an alternative to the selective phases, the possibility of combining supported liquid membranes (SLM) and SPE for determining atrazine at microgram levels in different types of fruit juices. SLM extraction encompasses three simultaneous processes: extraction of the analyte into an organic phase, membrane transport, and reextraction. **Figure 1** shows the electropherograms obtained with only SPE as clean-up and enrichment method, and those obtained on using SLM-SPE for the enrichment of atrazine from four different juices. The results show that a single application of SPE is insufficient for the clean-up of juice samples. In the case of apple juice, even an approximate quantitative estimation was impossible (Figure 1.A). On using SLM-SPE, a significant improvement in the clean-up and enrichment was achieved. As can be seen in Figure 1.B, the extract was cleaner for all juices but interfering substances from the juices were still present and a significant switch in migration time of atrazine was evidenced, which underscored the pressing need for more selective extraction and/or detection methods. The switch in migration time can be caused by the different salt concentrations in the samples of juices that provided extracts with different ionic strengths.

A sample screening system can be used to minimise the need for permanent use of instruments with high purchase and maintenance cost. The sample screening systems can be described as systems that filter samples to select those with analyte contents similar to or higher than a previously established threshold.

A method for the extraction and determination of mycotoxins in food samples, incorporating a screening system to obtain a rapid response regarding the presence or not of these analytes, has been proposed by Peña et al. [71]. This system is based on the fluorescence of the oxidized aflatoxins, and consists of a continuous flow system (CFS) unit and a fluorimeter providing a yes/no binary response. An extraction step followed by a purification stage was carried out to remove interfering substances prior to the analysis. A C_{18} column was chosen to concentrate the mycotoxins, and the analytes were eluted from C_{18} with methanol. The confirmatory system uses a separation technique such as MEKC-DAD, and is justified if there is an interest in identifying the different mycotoxins.

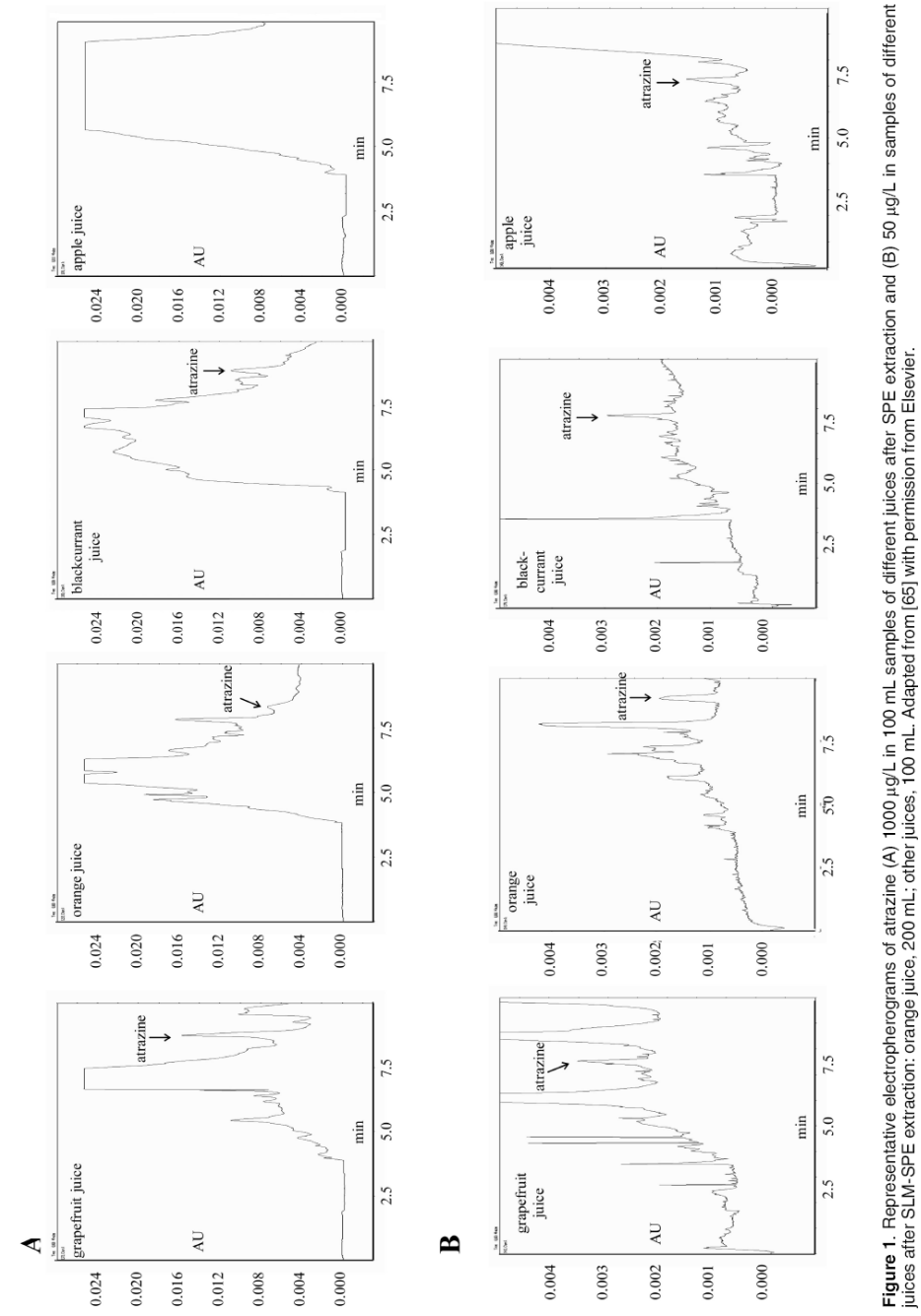

Figure 1. Representative electropherograms of atrazine (A) 1000 µg/L in 100 mL samples of different juices after SPE extraction and (B) 50 µg/L in samples of different juices after SLM-SPE extraction: orange juice, 200 mL; other juices, 100 mL. Adapted from [65] with permission from Elsevier.

Santos et al. [49] proposed a similar methodology for determining sulfonamides in milk but samples were analyzed by CE-MS. The screening method involves on-line treatment of the sample using a simple continuous flow system and rapid analysis with a CE-MS instrument, in which a common characteristic ion of all sulfonamides was monitored with the MS detector by flushing the sample through the capillary. The confirmatory method is based on the purification and preconcentration of sulfonamides in a CFS unit and subsequent analysis by CE-MS.

Bacterial pathogens may coexist, at different concentrations, in the same food sample, but they generally occur at low levels. Their detection is usually preceded by an enrichment step to increase cell numbers to the detection level. However, this type of procedure still relies on the use of selective media, biochemical reactions, and other parameters for bacterial identification, and requires several days to obtain results. The simultaneous detection of the three major foodborne pathogens considered as the most frequent causes of food poisoning (i.e. *Salmonella* spp., *S. aureus*, and *L. monocytogenes*) has been approached by Alarcon et al. [76]. The combination of PCR and CGE with LIF detection significantly improves the LODs of the three pathogens tested.

Arakawa et al. [77] developed a DNA diagnosis technique for the verotoxin $(VT)_1$ and VT_2 genes of *Escherichia coli* (O157) consisting of three methods, because DNA diagnosis using PCR sometimes exhibits false negatives as a consequence of the appearance of bacterial mutants and inhibition of PCR by unknown material in sample. These three methods are based on the use of different PCR primers and analytical approaches, i.e. analysis of base size, single-strand conformation polymorphism (SSCP), and cleavase fragment length polymorphism (CFLP) methodology. Therefore, the combination of these analytical approaches will lead to a more definitive diagnosis.

4 Approaches for enhancing sensitivity, selectivity, or both in CE

The originally reported CE limitation of inadequate sensitivity for trace analysis of organic contaminants in food, as a result of the small sample volume typically injected (ca. 1–10 nL), has been overcome by on-column trace enrichment schemes and improvements in detector sensitivity. Both approaches have been separately or simultaneously applied to determine some of the reported organic contaminants. A list of the class of organic contaminants, the approaches used, the LODs obtained, the most important characteristics, and the references is presented in **Table 3**.

Preconcentration procedures have always been implemented when trace analysis of compounds in real matrices is contemplated. In CE, a variety of on-line preconcentration strategies have been reported, aiming at decreasing LODs by the insertion a large volume of sample in the capillary, without compromising peak efficiency and resolution. In general, pre-concentration strategies can be distinguished in two categories, according to the physical phenomena related to the analyte concentration. One category involves the change of electrophoretic mobility of the analyte. The simplest stacking is performed by dissolving the sample in a low conductivity buffer and by injecting the resulted solution hydrodynamically. Takeda et al. [59] studied several strategies to carry out this "simple" stacking for five pesticides: amitrole, carbendazim, 2-aminobenzimidazole, thiabendazole, and 1,2-diaminobenzene, using distilled water, migration buffer, or acidic solution to redissolve the extracts. When migration buffer or water were used to redissolve the sample extract, the shapes of all peaks were distorted along with the increase of injection time. Addition of formic acid to the sample matrix gave better peak shapes. That improvement can be attributed to a transient isotachophoretic effect, which strictly occurs when a large volume of sample is placed between a leading and a terminating electrolytes. Analytes in the sample stack into narrow bands one after another according to their mobility. For these five pesticides, ammonium in the migration buffer would correspond to the leading ion and hydrogen in the sample matrix could match the terminating ion in transient isotachophoresis . The concentration factors obtained were in the range of 7.6–27-fold.

Another technique is stacking with sample removal, in which the capillary can be filled with an extremely large sample volume, while retaining high resolution, if the sample buffer is eliminated after stacking to reduce the non-uniform distribution of both the field strength and the electro-osmotic velocity [25]. This technique has been checked with on-line concentrated thiabendazole and procymidone from fruit and vegetable extracts by Rodriguez et al. [58]. The quantities of analytes in the stacked sample were 3 times higher than those of the standard injections. The stacked procedure did not cause a dramatic improvement in sensitivity but better LODs and high peak efficiencies were obtained for both analytes.

The other group of preconcentration strategies explores the ability of the analyte to partition into a pseudo-stationary phase because, in MEKC, compounds are not concentrated in a normal stacking procedure since they are not accelerated by the high electric field that is developed across the injection zone. Sweeping and stacking with reverse migration micelles (SRMM), with (SRW) and without the insertion of a plug of water before the injection, are representative of this group.

Sweeping is based on the capture and accumulation of the analyte by the micelles that penetrate into the sample

Table 3. Approaches to enhancement of sensitivity and/or selectivity described in the literature pertaining to the determination of organic contaminants in food.

Type	Injection	Detection	LODs	Comment	Ref.
Sulfonamides	Hydrodynamic (35 mbar, 10 s)	CE-NanoES Quasi-MS/MS/MS (triple quadrupole)	5 ppb	Tandem mass spectrometry (MS/MS) including precursor ion scans and MRM. Conventional MS/MS yields no isomer-specific ions. On-line enrichment technique and more efficient extraction and clean-up procedures could further improve LODs	[47]
	Hydrodynamic (50 mbar, 10s)	CE-ESI-MS (single quadrupole)	2–6 ppb	Screening method monitoring the characteristic fragment ion at *m/z* 156 for all the sulfonamides. Confirmatory methods monitoring a characteristic ion for each sulfonamide. Coupling of CFS to CE play an important role	[49]
Benzimidazole fungicides	"Simple stacking" 50 mM formic acid (30 s, 5 kPa)	CE-ESI-MS (single quadrupole) "sheath liquid homemade interface"	1.4–4.7 ppb	Sample extract reconstituted in milli-Q water or in sample matrix instead of 50 mM formic acid causes lost of separation efficiencies. Sensitivity was about one-tenth that with UV detection	[59]
Thiabendazole and procymidone	"Stacking with matrix removal" (15 s, 0.5 psi) applied voltage +30 kV, 0.15 min)	CE-ESI-MS (single quadrupole) "sheath liquid interface"	1–10 ppb	In spite of the stacking procedure and the large non-thermostated part of the capillary no significant band broadening was observed. LODs were 10 times lower than MRLs. Mass spectra characterized by protonated molecule. One hundred times more sensitive than CE-UV	[58]
Benzimidazoles, triazines, ureas, and carbamates	Sweeping (96 s, 2.5 kPa) SRMM (96 s, 2.5 kPa) applied voltage + 20 kV, 2 min SRW (96s, 2.5 kPa)	MEKC-DAD	2–46 ppb	Comparison of 3 on-line strategies. Enrichment factors 3–18. Matrix effects are relevant. Only some pesticides can be clearly identified in the sample with no interference from matrix constituents	[68]
Fungicides	High-salt stacking (0.5 psi, 100 s)	MEKC-DAD	0.7–10.4 μg/L	The sample extract is reconstitute in 50 mM NaCl. Best results compared with low conductivity stacking (sample extract in milli-Q water) and micellar sample stacking (sample extract in 5 mM SDS). Enrichment factor 7.4–21	[37]
Polar P-containing	Hydrodynamic (3 psi, 5 s)	CE-ESI-MS Single quadrupole Sheathless interface	2.5 μM	Underivatized herbicides are detected at 10% MRL in wheat. Avoid sample dilution associated with the use of sheath liquid interface	[61]
	Electrokinetic	CE-FPD (30 kV)	1000 ppb	CE features are shorter analysis times than μLC. However, CE-FPD is less sensitive and compounds such as acephate cannot be concentrated	[62]
Acidic herbicides	Hydrodynamic (0.5 psi, 20 s)	CE-ESI-MS Single quadrupole Sheath liquid interface	20–5000 ppb	Compared with conventional UV detection, LODs were 2–50 times lower. Losses of resolution, efficiency and reproducibility compared with UV	[66]
Multiple pesticides	Hydrodynamic (0.5 psi, 18 s)	CE-ESI-MS Ion trap Sheath liquid interface	60 ppb	CE-MS provides LODs one order of magnitude better than CE-UV. Losses of resolution, efficiency and reproducibility compared with UV	[69]
	Hydrodynamic (0.5 psi, 5 s)	CE-ESI-MS/MS Ion trap Sheath liquid interface	10–50 ppb	The MS^n spectra provide structural information and the identification confidence is considerably high	[79]
Ochratoxin A	Hydrodynamic (0.5 psi, 5 s)	CE-LIF (He/Cd laser)	0.2–10 ppb	Very low LODs, comparable to those of the LC. Natural fluorescent mycotoxin that does not require derivatization	[22]
Aflatoxin B_1	Hydrodynamic (0.5 psi, 5 s)	CE-LIF (He/Cd laser)	0.5 ppb	Natural fluorescent mycotoxin that does not require derivatization. Possible to further increase the sensitivity of the method by reconstituting the dried extract with less buffer	[74]

Table 3. Continued.

Type	Injection	Detection	LODs	Comment	Ref.
Aflatoxins B_1, B_2, G_1 Cholera toxin A	Electrokinetic	CE-MEF (Ti:S laser 730–770 nm)	0.2–0.4 nM	Use a wavelength tunable mode locked to produce MPE fluorescence of aflatoxins. Substantial emission at all tested wavelengths Improvement of $>10^4$ in mass detectability	[73]
Foodborne pathogens *S. aureus* *L. monocytogenes* *Salmonella spp*	Hydrodynamic (1 psi, 12 s)	CGE-LIF (Argon laser)	2.6 10^3 cfu mL^{-1}	Electrophoretic buffer containing a fluorescence reagent for DNA. LIF improves the LODs of the three pathogens tested allowing their simultaneous detection	[76]
Escherichia coli Vero toxins (VT1, VT2)	Electrokinetic (7.4 kV, 2–10 s) Hydrodynamic (40 s)	CGE-LIF (Argon laser)	—	Electrophoretic buffer containing a fluorescence reagent for DNA	[77]

zone during the application of a voltage. Samples are injected onto the column in a buffer solution with a conductivity similar to that of the background electrolyte (BGE), but in the absence of a pseudostationary phase. The capillary is then placed in an anionic micellar BGE solution and the separation is performed in reverse polarity mode. The BGE is kept at low pH to suppress the electrosmotic flow (EOF), allowing the anionic micelles to electrophoretically migrate towards the detector. As the micelles migrate towards the detector they "sweep" the neutral analytes along. This effect is dependent on a uniform electric field and the absence of micelles in the sample solution; no micellar stacking is observed, and the velocity of analytes in the sample zone in the presence of micelles is the same as the velocity of the analytes in the separation buffer [9, 80]. The SRMM focussing mechanism is based on the abrupt change of the analyte effective electrophoretic velocities at the stacking boundary. The stacking boundary separates regions of high and low electric fields. The sweeping phenomenon is also partly responsible for the focussing effect in SRMM. SRW is performed by preparing the sample in a matrix having a conductance lower than that of the separation buffer. However, a surfactant is added at a concentration slightly higher than the critical micelle concentration and lower than that of the separation buffer. A water plug is first injected into the capillary before injection of the sample. The focusing mechanism is primarily based on the abrupt change in analyte effective electrophoretic velocities at the stacking boundary. Da Silva et al. [68] contrasted sweeping and SRMM, with and without the insertion of a plug of water before sample injection, for nine pesticides from different classes, carbendazim (benzimidazole), simazine, atrazine, propazine, and ametryn (triazine), diuron and linuron (urea), carbaryl and propoxur (carbamate). A new version of SRMM was introduced consisting of momentarily applying a positive voltage at the inlet vial just after sample injection. The authors compare the effectiveness of the preconcentration strategies, computing LODs and enrichment factors. The results show that the three procedures provide a roughly 3–18 fold sensitivity increase, depending on the pesticide. Even through these factors were not as impressive as those found in the literature [9, 10] it must be considered that the covered pesticides comprise a wide range of hydrophobicity and polarity; therefore, their partition coefficients differ considerably. The proposed methodologies were applied to the analysis of pesticides in carrot extracts obtaining LODs of 2.5 μg/L.

The most recent and universal approach to the stacking of large sample volumes without removal of the sample matrix utilizes higher salt concentrations in the sample matrix than in the separation buffer. After the separation voltage is applied, the negatively-charged micelles move into the sample zone from the detector side of the sample zone–separation buffer interface. In the higher-conductivity sample zone the electric field strength is lower than in the separation buffer. As a result the micelle migration velocity decreases. The net effect is that micelles accumulate near the detector side of the sample zone–separation buffer interface resulting in a higher phase ratio of micellar phase at the sample–buffer interface. Consequently, the hydrophobic analyte is concentrated in this zone. According to Palmer and Landers [81], two requirements are decisive for successful high-salt stacking in MEKC. First, the conductivity of sample matrix must exceed that of the separation buffer so that the electric field decreases significantly inside the sample zone. Secondly, the sample matrix must include a co-ion (commonly chloride) with a higher intrinsic electrophoretic mobility than the surfactant, which guarantees the formation of a pseudo-steady-state boundary between the micelle and co-ion component in the sample matrix. In contrast to the sweeping method described above, the high-salt stacking uses a discontinuous buffer system and does not require a sample free of pseudostationary phase [80]. This high salt stacking approach was used by Molina and Silva [55] to enhance the sensitivity to determine eight fungicides (carbendazim, metalaxyl, captan, procymidone, folpet, captafol, vinclozoline, and iprodione) in fruit juices.

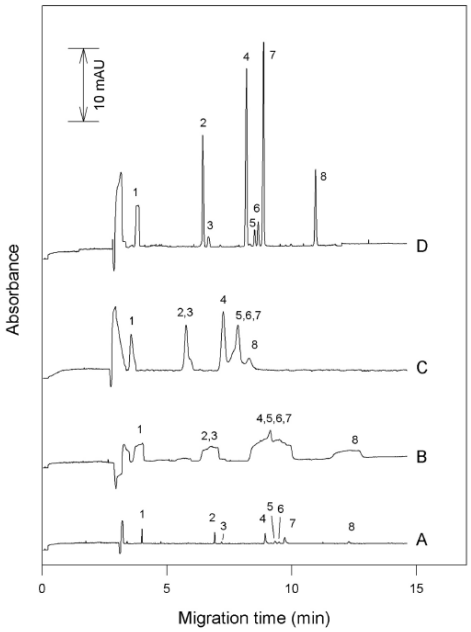

Figure 2. Electropherograms obtained by using various hydrodynamic injection procedures. (A), (B) without stacking; sample in running buffer; hydrodynamic injection, 5 and 100 s, respectively; (C) low-conductivity matrix stacking; sample in milli-Q water; hydrodynamic injection, 100 s; and (D) high-salt stacking; sample in 50 mM NaCl; hydrodynamic injection, 100 s. Reprinted from [55] with permission from Wiley.

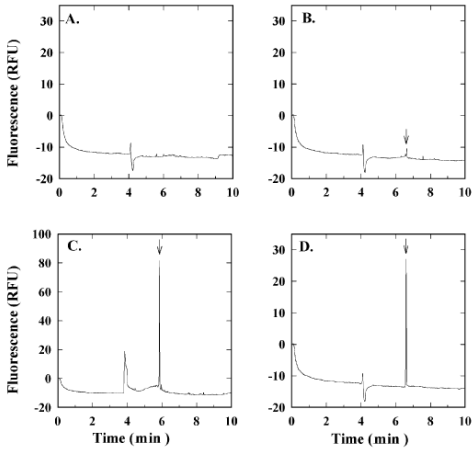

Figure 3. Detection of ochratoxin A in coffee by CE-LIF: (A) control, roasted coffee with no detectable ochratoxin A; (B) roasted coffee spiked with ochratoxins at the limit of detection of the method, 0.2 ppb; (C) 10 ppb spiked coffee; (D) ochratoxin standard, 0.05 ng/μL. Reprinted from [22] with permission from American Chemical Society.

Figure 2 shows an electropherogram obtained using high-salt concentration stacking, illustrating how the sensitivity is improved without peak broadening. Two other sample stacking approaches (viz. low-conductivity stacking and micellar-sample stacking) were also tested. Both proved ineffective compared to the high-salt stacking mode: the former was restricted by the conductivity of the running buffer and the latter by the small differences between the critical micelle concentration (CMC) (10 mM) and the surfactant concentration in the running buffer (30 mM). These results show that the high salt stacking method is the best way of obtaining high enrichment factors in MEKC.

The detection system is the other cornerstone for improving the concentration limits of detection. Although CE is more easily interfaced with optical detection methods based on UV, the analysis of complex food extracts using this detector has very limited possibilities. First, CE-UV is usually not sufficiently sensitive to detect organic contaminants at trace levels. Some instrumental solutions for the improvement of LODs for UV have recently been developed, such as z-shape or bubble detection cell, which are highly sensitive detection cells with longer path-lengths than conventional ones. Unfortunately, this approach has not been applied to the analysis of contaminants in foods [25]. In addition, identification of target analytes is uncertain because the UV spectra of different compounds included in one class of contaminants are very similar, and co-migrating analytes and matrix components can make the confirmation of these residues ambiguous. Consequently, more selective and sensitive detection is necessary.

Laser induced fluorescence (LIF) constitutes a more sensitive and selective detection than UV, but its use is restricted to molecules with fluorescent properties. The LIF detector is also commercially available for CE instruments. However, only a few different laser sources are available.

CE with LIF detection has been applied successfully to mycotoxin determination, reaching very low levels [22, 74]. Most mycotoxins show native fluorescence; for instance, ochratoxin and aflatoxin B_1 were exposed to light from an ultraviolet He/Cd laser, and were detected with a very high selectivity and sensitivity. **Figure 3** shows very good LODs and the lack of interference in the determination of ochratoxin A in three very different commodities: roasted coffee, corn, and sorghum.

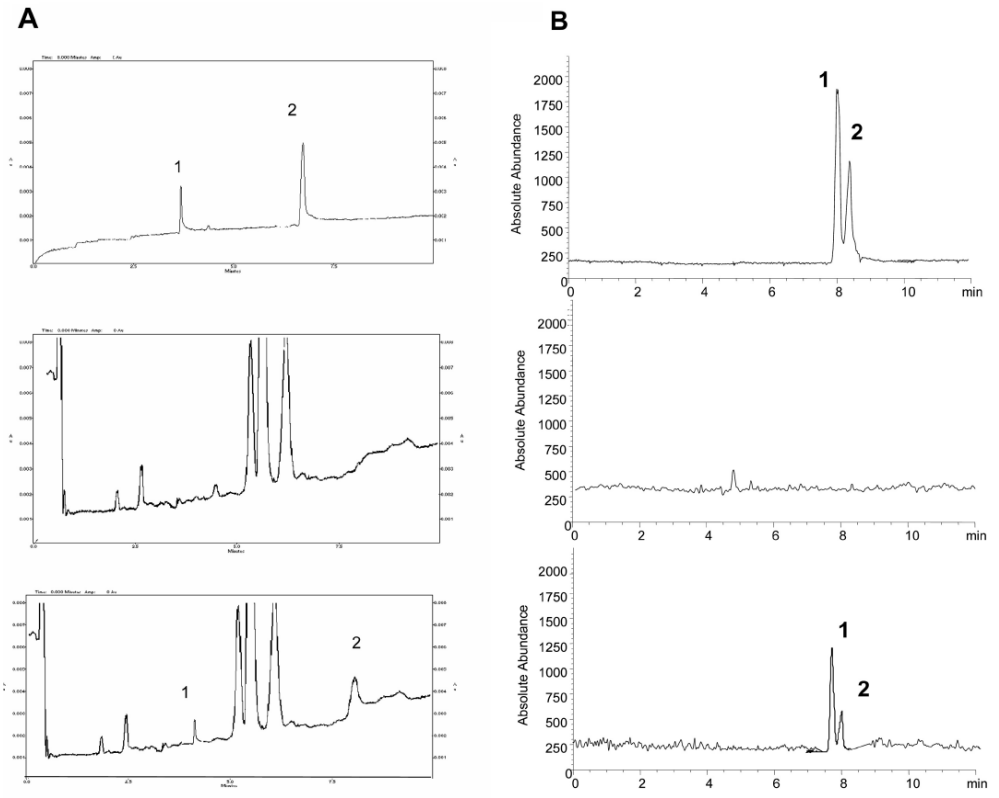

Figure 4. Electropherograms obtained (A) CE-DAD, and (B) CE-ESI-MS electropherograms in the SIM mode of thiabendazole and procymidone standard (10 mg kg^{-1} for the UV and 0.1 and 1 mg/kg, respectively, by CE-MS), untreated control orange sample, and control orange sample spiked (at 1 mg/kg of each compound for the UV, and at 0.01 and 0.1 mg/kg of thiabendazole and procymidone, respectively, by MS. Adapted from [58] with permission from Elsevier.

The use of multiphoton-excited (MPE) fluorescence has been explored as an alternative to conventional fluorescence for probing small quantities of toxins fractionated with electrokinetic capillary chromatography (aflatoxins and the cholerae toxin A). The research demonstrated that MPE fluorescence has the versatility to probe dissimilar toxins through different spectroscopic mechanisms, and can offer extremely low mass detection limits for compounds separated in micrometer-diameter channels [73].

For DNA, LIF detection has proved to be a powerful alternative to meet the growing need for species detection tools when contamination is suspected in food [19, 77]. The simultaneous LIF detection of *Staphylococcus aureus*, *Listeria monocytogenes*, and *Salmonella spp.* after a new multiplex PCR-based procedure has been studied by Alarcón et al. [76]. As compared to slab gel electrophoresis, the use of CGE-LIF improves the sensitivity of the multiplex PCR analysis from 10 to 1000-fold. The diagnosis of two variant vero toxin genes in *Escheria coli* by CE-LIF has been also accomplished after complex DNA replication procedures [77].

Presently, the coupling of CE to MS and tandem mass spectrometry (MS/MS) are the techniques of choice for identification of residues because the acquisition of mass information facilitates the identification and characterization of the analytes and overcomes a large number of interfering substances. The ionization technique most successfully used for CE-MS is electrospray ionization (ESI), which has proved to be sensitive, versatile, and relatively easy to use in combination with CE. The coupl-

ing of CE to MS using quadrupole, triple quadrupole, or ion trap instruments has been reviewed [13, 56], showing that there are a great variety of CE-ESI-MS interfaces, which are divided into two general categories: sheath-flow interfaces and sheathless interfaces. The first ones are the most widely used interfaces for routine CE/MS because they offer several valuable features such as simple fabrication, reliability, and ease of implementation. CE coupled to MS has been used to determine some antibacterials in meat and milk and some pesticide residues in fruits and vegetables.

A "screening" method to detect six sulfonamide residues in milk by CE-MS, using a common characteristic ion for all sulfonamides, has been reported by Santos et al. [49]. The same authors used a more specific CE-MS method to confirm those samples that contained the residues in a concentration exceeding the legal limits. Methods of this type of are less expensive and faster, allowing the analysis of more than 30 samples per hour.

Takeda et al. [59] tested CE-MS determination for five pesticides, applying a simple sample stacking. CE was coupled to MS using a laboratory-made ESI interface; sensitivity was about one-tenth that with UV detection, and RSDs of peak area exceeded 10% because of the high noise level originating from the instability of the electrospray at the laboratory-made interface. Although the obtained results do not allow strictly quantitative comparison, it was clear that on-line concentration by addition of formic acid was as successful as in the case of UV detection.

Rodríguez et al. [58] developed a method based on CE-MS to determine procymidone and thiabendazole in fruits and vegetables. These samples are complex matrices containing significant amounts of salt and endogenous compounds. **Figure 4** illustrates examples of representative electropherograms of orange samples obtained by CE-ESI-MS along with those obtained by CE-UV. The former show a stable baseline, good peak shapes, and no interferences by endogenous compounds, which clearly demonstrated better sensitivity (one hundred times) and selectivity compared to those obtained by CE-UV, and allows determination of procymidone and thiabendazole at levels 10-times lower than the MRLs established. Comparative studies between CE-UV and CE-MS have been carried out for different pesticides using positive [69] and negative [66] ionization modes. Both arrive at the same conclusion. A good separation of the compounds is achieved by both techniques using volatile aqueous buffers, but separation is slightly poorer by CE-MS, which is attributable to the small portion of the capillary that is thermostated and the large part of it (between the CE and the MS) that is at room temperature. Peak areas show poorer reproducibility for CE-MS than for CE-UV. However, CE-MS provides LODs at least one order or magnitude better than CE-UV.

Goodwing et al. [61] demonstrates that a sheathless interface is suitable for the CE-MS analysis of glyphosate, glufosinate, aminomethylphosphonic acid, and methylphosphinicopropionic acid, using a combination of electrical and pressure drive. The limitation of this device is set by the need to avoid discharges between the capillary tip and the MS inlet, and operationally the upper limit to the current is $\approx 2\ \mu A$. An appropriate ammonium concentration is 1 mM, considerably lower than is the norm for typical BGEs in CE. This restricts the range of acceptable sample matrices, since the conductivity of the matrix should be less than or equal to that of the BGE in order to provide acceptable peak shapes. The interface has advantages over typical sheath liquid interface systems, in that there is no analyte dilution by the sheath liquid.

Tandem mass spectrometry MS/MS or MS^n uses multiple stages of mass analysis that allow preselection of an ion and analysis the induced fragments obtained by collision with an inert gas. Recently, CE-ESI-MS/MS has emerged as a powerful tool for the characterization of food contaminants, and this technology has been successfully applied to the analysis of sulfonamides in milk and pesticides in fruits and vegetables.

The application of combined CE/MS/MS for screening, quantification, and confirmation of sulfonamide residues in milk samples at levels around 5 ppb has been reported by Bateman et al. [47]. Three pairs of isomeric sulfonamides, which differ only in the position of the nitrogen and oxygen atom in the heterocyclic aromatic ring of the molecules, were targeted. Conventional MS/MS analysis yielded no isomer-specific ions. Therefore, a quasi-MS/MS/MS was applied to overcome these limitations. Specifically, in a first step in-source collision induced dissociation (CID) was used as a quasi MS/MS stage to generate ions corresponding to the heteroatomic amine moiety. In a second MS/MS step, these ions were isolated and made to undergo CID in the collision quadrupole to yield isomer-specific ions. The results of these experiments are illustrated in **Figure 5**. As can be observed, all compounds exhibited at least one isomer-specific ion.

Juan-García et al. [79] described a method to identify and quantify six pesticide residues – dinoseb, pirmicarb, procymidone, pyrifenox, pyrimethanil, and thiabendazole – in peaches and nectarines using CE-quadrupole ion-trap (QIT)-MS/MS. A reliable way to obtain structural information is to perform tandem MS experiments on specific ions of interest. MS^2 and MS^3 often yield fragment ions formed by fragmentation of the lateral chains in the molecular structure or by the opening of the heterocyclic rings. However, according to the LODs obtained, MS and MS^2 resulted in a several-fold improvement in sensitivity com-

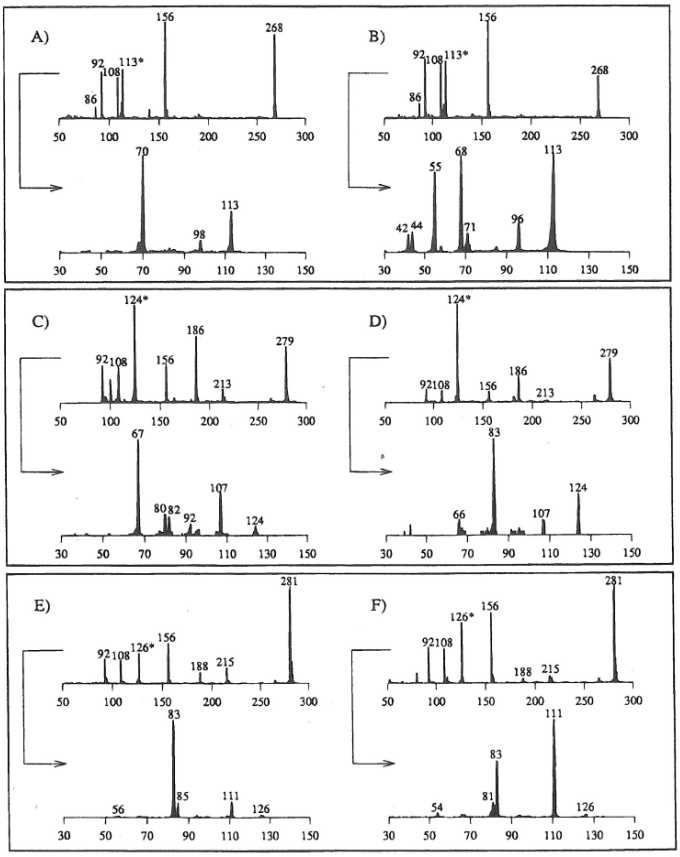

Figure 5. CZE/quasi-MS/MS/MS of three pairs of isomeric sulfonamides. The top mass spectrum of each isomer shows the quasi-tandem mass spectrum (in-source CID). The mass spectrum at the bottom is the tandem mass spectrum of the BH^+ ions isolated from the first quasi-MS/MS step (indicated by an asterisk in the top spectrum). (A) Sulfamoxole; (B) sulfisoxazole; (C) sulfamethazine; (D) sulfisomidine; (E) sulfameter; (F) sulfamethoxypyridazine. Reprinted from [47] with permission from Wiley.

pared with CE-UV, and are appropriate for determining pesticides at levels lower than MRLs. On the contrary, LODs obtained by CE-ESI-QIT-MS^3 are of the same order of magnitude that those obtained by CE-UV.

Among other possible detectors, flame photometric detection (FPD) in the P-selective mode has been studied for the determination of polar P-containing pesticides [62]. FPD shows certain promising features including high sensitivity and good selectivity, but this approach has not yet been accepted since it is not available commercially.

5 Capillary electromigration techniques to separate organic contaminants

CE performs the separation of solutes within a capillary under the influence of an electric field. The versatility of CE is its most attractive feature, and it is a widely applicable technique because of its range of separation modes.

5.1 Capillary Zone Electrophoresis (CZE)

CZE is a separation technique carried out in capillaries based only on the differences in the electrophoretic mobilities of charged species, either in aqueous or in non-aqueous BGE solutions. These can contain additives (such as cyclodextrins or polymers) that can interact with the analytes and alter their electrophoretic mobility.

The BGE is commonly dissolved in aqueous medium because water has a well-know acid-base chemistry, is compatible with several detection schemes, and can dissolve numerous BGE (see **Table 4**). The optimal buffer, at which the differences between the mobilities of the studied substances are greatest, and hence, at which these

Table 4. Separation modes used in the analysis of organic contaminant in food.

Type	Compounds	Separation mode	Comments	Ref.
Quinolones	Oxonilic acid, fluomequine, difloxacin, sarafloxacin, enrofloxacin, ciprofloxacin, danofloxacin, marbofloxacin, piromidic acid	CE 10–50 mM phosphate buffer adjusted to pH 8 – 9.0 with or without CH_3OH	Amphoteric compounds with acidic and basic groups Control of the ionic species when the buffer pH is close to pK_a	[39–41, 43–45]
		NACE with reversal EOF 20 mM ammonium acetate, 0.004% HDB and 4% acetic acid with a mixture CH_3OH/CH_3CN (50:50 *v/v*)		[42]
Tetracyclines	Oxitetracycline	CE Phosphate buffer (0.2 M, pH 2)	Amphoteric groups	[46]
Sulfonamides	Sulfamethazine, sulfamerazine, sulfadiazine, sulfadimethoxine, sulfamonomethoxine, sulfaphenazole, sulfaquinoxaline, sulfisoxazole, sulfabenzamide, sulfacetamide, sulfamethosazole, sulfameter, sulfachloropyridazine, sulfamethizole, sulfamethoxypyridazine, sulfamoxole, sulfathiazole, sulfapyridine, sulfanylamide, sulfaguanidine, trimethoprim, omethoprim, sulfisomidine	CE Phosphate buffer (35 mM, pH 6.5)	Amphoteric groups Good resolution of all sulfonamides	[48]
		CE Formic acid (100–10000 mM) CH_3OH /water 1.5% formic acid	Adsorption of cationic analytes, cationic coating of the capillary eliminates analyte adsorption	[47] [49]
Miscellaneous	β-Lactam, aminoglycoside, tretracycline, quinolone, phenicol	CE 5–25 mM phosphate and borate buffers	Some of the drugs have similar migration times	[50]
Dithiocarbamates	Ferbam (ferric dimethyldithiocarbamate) Ziram (zinc dimethyldiaminocarbamate) Zineb (zinc ethylenebisdithiocarbamate) Metham (sodium methyldithiocarbamate) Maneb (manganese ethylenebisdithiocarbamate)	CE 20 –25 mM Sodium tetraborate buffer (pH 9)	Negative charged compounds Ferbam is determined after its acidic decomposition and complexation with EDTA as Fe-$EDTA^-$	[51–53]
Fungicides	Amitrole, carbendazim, 2-aminobenzimidazole, thiabendazole, 1,2-diaminobenzene, procymidone, prochloraz, imazalil, metalaxyl, captan, folpet, captafol, vinclozolin, iprodione	CE 4 mM phosphate solution (pH 3.5) 12 mM ammonium formate- 20 mM formic acid pH 3.5, 2% CH_3OH 50 mM formic acid–50 mM ammonium formate	Separation of amitrole and benzimidazole fungicides, which have protonated imidazole groups Volatile buffers to combine with MS	[57–60]
		5 mM ammonium dihydrogenphosphate – 50 mM phosphate buffer (pH 3) – 4 mM 2-hydroxypropyl-β-CD	Chiral resolution of both enantiomers of imazalil	[95]
		MEKC 4 mM borate pH 9.2, 75 mM sodium cholate 30 mM NH_4Cl/NH_3 (pH 9), 15 mM SDS	Electrostatic interactions between negative charge of SDS micelles and positively charged analytes	[55, 56]
Polar P-containing	Glyphosate, glufosinate, fosetyl-aluminium, acephate	CGE Linear polyacrylamida coated capillary 1 mM ammonium acetate (pH 4–9) – CH_3OH	The coated capillary is to stabilize the sheathless interface to MS	[61]
		40 mM ammonium acetate (pH 9)	Compounds negatively charged in solution	[62]
Organophosphorus	Chlorpyriphos	10 mM Na_2HPO_4/6 mM $Na_2B_4O_7$, 25% acetonitrile containing 50 SDS or 50 mM sodium deoxycholate	Neutral compounds	[63]
Urea derived	Triasulfuron, chlorsulfuron, monuron, fluometuron, metobromuron, chlorotoluron, isoproturon, diuron, methabenzthiazuron, flufenuxuron	MEKC 4 mM borate (pH 9.2), 35 mM SDS	Neutral compounds at working pH	[64]

Table 4. Continued.

Type	Compounds	Separation mode	Comments	Ref.
Triazines	Atrazine	MEKC 10 mM phosphate buffer, 60 mM SDS, 20% CH_3OH	Neutral compounds	[65, 68]
Acidic herbicides	*o*-Phenylphenol, ioxynil, haloxyfop, acifluorfen, picloram	CE 32 mM ammonium formate/formic acid (pH 3.1)	Pesticides with acidic groups negatively charged in solution	[66]
Multiple pesticides	Pyrimethanil, pyrifenox, cyprodinil, cyromazine, pirimicarb, carbendazim, simazine, atrazine, propazine, ametryn, diuron, linuron, carbaryl, propoxur, naphthalene, acetamide, thiabendazole, 1-naphthol, acrinathrin, bitertanol, cyproconazole, fludioxonil, flutriafol, myclobutanil, pyriproxifen, tebuconazole	CE 0.3 M ammonium acetate/acetic acid (pH 4) with or without addition of methanol	Pesticides with ionizable acidic or basic groups	[69, 79]
		MEKC 30 mM NH_4Cl/NH_3 (pH 9) 15 mM SDS 20 mM phosphate buffer (pH 2.5) 25 mM SDS, 10% CH_3OH	Neutral compounds	[67, 68, 78]
Ochratoxin A		MEKC Phosphate-borate (pH 8.02), SDS, γ-CD, CH_3CN	Neutral compounds	[71]
		CE-LIF 20 mM phosphate buffer (pH 7)	Separate only ochratoxin from matrix interferences	[22]
Patulin		MEKC-UV 25 mM sodium tetraborate (pH 9), 50 mM SDS	Patulin is chemically labile in alkaline solutions	[72]
Aflatoxins	B_1, B_2, G_1, G_2	MEKC-UV Phosphate borate (pH 8.02), SDS, γ-CD, CH_3CN Phosphate borate (pH 9.1), sodium deoxycholate	Adequate separation of four aflatoxins	[71, 74]
	B_1, B_2, G_1	MEKC-MPE fluorescence 20 mM Tris, 10 mM carboxymethyl-β-CD	Improves the fluorescence of aflatoxins	[73]
Cholera	Toxin A	MEKC-MPE fluorescence 20 mM Tris, 10 mM carboxymethyl-β-CD	Improves also the fluoresce of aflatoxins	[73]
ASP	Domoic acid	CE 10–50 mM sodium borate buffer (pH 9.3)	Analysis under basic conditions	[75]
PSP	STX, dcSTX, dcneoSTX GTX1, GTX2, GTX3, GTX4, GTX5,	CE-UV 50 mM morpholine buffer (pH 5) 10% CH_3OH or CH_3CN	Good potential for the analysis of most PSP	[82]
S. aureus, L. monocytogenes, Salmonella spp.	S. typhi CECT 409, *S. typhimurium* CECT 443, *S. paratyphi* CECT 554, *S. typhi* CECT 725, CECT 4031^T, CECT 4032, CECT 940, CECT86^T CECT 976, CECT 4013, CECT 515, CECT 4456	CGE-LIF 20 mM Tris, 10 mM phosphoric acid, 2 mM EDTA, 1.5 M urea, 500 nM YOPRO1	Faster and more sensitive separation than agarose gel separation	[76]
Escherichia coli Vero toxins	VT_1, VT_2, O157	CGE-LIF Tris-borate (pH 7.8), 3% acrylamide, orange fluorescent reagent	Suitable for detection and identification of the pathogens	[77]

substances migrate with the best separation, is obtained by carefully selecting its concentration and composition. Separation is easily improved by adjusting the buffer pH, altering the buffer ionic strength, and adding organic solvents. Antibacterials exhibit a variety of chemical structures and therefore a diversity of chemical, physical, and biological properties. Most of them have functional groups with acidic, basic, or amphoteric characteristics that can be effectively ionized according to the running buffer pH. This property makes them especially suited for CE. Quinolones are derived from nalidix acid and they have a carboxylic acid group in position 4; because of this they are well-separated at pH values between 8.0 and 9.5 (see Table 4). On the contrary, sulfonamides that posses a group of basic character (*para*-NH_2 group) are separated at acidic pH. Some antibacterials have similar migration times and therefore it is not possible to validate all of them simultaneously. When physical separation of sample components is not fully accomplished, quantification of poorly resolved peaks is still possible mathematically using chemometrics. As an example, there are two quinolones, ciprofloxacin and sarafloxacin, which coelute in CE

and have strongly overlapping spectra. A multivariation calibration procedure (partial least square regression) can be applied to the spectra obtained at the maximum of the electrophoresis peaks [45], allowing the quantification of both compounds.

Pesticides include a large number of chemicals that comprise many different chemical families. Among them, there are some compounds such as dithiocarbamates that exist as anions in solution [51, 52]. The potential of CE for separating dithiocarbamates, such as ziram, zineb, metham, and maneb, has been studied by Malik and Faubel [51, 52]. Below pH 7.0 no distinct peaks were observed, whereas well defined peaks were obtained in borate at pH 9.0. The migration behaviour of these compounds in the capillary can be explained on the basis of the steric hindrance caused by alkyl groups. As diethyldithiocarbamate has two alkyl groups, it is less hydrated than the other compounds investigated, and its peak appears earlier than that of ethyl xanthate which has one alkyl group. Methyldithiocarbamate has the smaller alkyl group, therefore its peak appears later, but the peak of maneb with two dithiocarbamate groups appears last. Interfering neutral species and cations are discriminated by reversing the polarity of the electrical field applied. The same authors determined Ferbam by a simple and selective CE method by converting Fe(III) present in Ferbam into Fe(III)-EDTA$^-$ complex [53]. Polar P-containing pesticides such as glyphosate, its metabolite aminomethylphosphonic acid, acephate, fosetyl-aluminium and ethefon as well as *o*-phenylphenol, ioxynil, haloxyfop, acifluorfen, and pichloran, were also separated in the same way [62, 66]. Most fungicides have imidazole groups, which are protonated at acidic pH; their electrophoretic mobilities are related to the pK_a values, and vary considerable with the pH. Takeda et al. [59] used CE to carry out the simultaneous separation of five pesticides: amitrole, carbendazim, 2-aminobenzoimidazole, thiabendazole, and 1,2-diaminobenzene. They were separated completely at pH 4.0 as a result of changing pH, using formic acid-ammonium formate buffer. Rodriguez et al. [57] demonstrated the simultaneous separation of thiabendazole, prochloraz, and procymidone in grapes in 4 mM phosphate solution at pH 3.5.

Ochratoxin is a neutral molecule. However, the use of a 20 mM sodium phosphate buffer as electrolyte resulted in a good resolution of ochratoxin from the matrix component with a relative short migration time [22]. However, using these simple buffers, the different mycotoxins cannot be separated because they are neutral substances.

Piñeiro et al. [75] showed an example of the application of the CE technique to the analysis of amnesic shellfish poisoning (ASP) and paralytic shellfish poisoning (PSP) toxins. The structure of domoic acid, with three carboxyl groups and one amino group, can undergo protonation. Because of the distinct charge states, the proportions of these toxins in solutions are determined by the pK_a values and also by the pH; for this reason different modes of operation could be applied using either acidic or basic conditions. Based on the structure of PSP toxins with a global charge of +2, +1, 0, depending on the pH of the medium, these toxic compounds can migrate in an electric field, being separated by CE, with the exception of the group of neutral toxins.

Organic solvents are an alternative to aqueous media in CE. However, their potential as BGE solvent has only recently begun to attract close attention. The low current in non-aqueous capillary electrophoresis (NACE) means that higher electrolyte salt concentrations and electric field strengths can be used, and also that the sample load can be scaled up by using a capillary with a larger inner diameter. Only one method has been proposed for determining seven quinolones by NACE using reversal of EOF [42]. This provides good selectivity and short analysis times. An important practical consequence of using NACE separation medium is that the organic phases, resulting from the extraction of the analytes of the matrix samples or from the eluents of the SPE, can be directly injected into the CE system.

The availability of many chiral selectors makes CE an important tool for chiral analysis as previously reviewed [57, 83]. Of these selectors, cyclodextrins (CDs) and their derivatives have been most widely applied in CE for the separation of enantiomers of many compounds. Kodama et al. [54] developed a method for the chiral separation of imazalil, using CE with CDs and determined the enantioselectivity of (+)- and (−)-imazalil residues in orange. The imidazole group of imazalil with a pK_b of 6.53 is protonated at pH 3.0. Thus, the analytes migrate electrophoretically to the cathode. When a charged analyte is included in the CD cavity, the inclusion complex formed has a charge identical with the free analyte but an increased molecular mass and, hence, a lower electrophoretic mobility than free analyte. In an enantiomeric separation, free enantiomers have identical electrophoretic mobilities. Therefore, the separation principle of CE with CD for enantiomeric separation is the difference in inclusion complex formation constants between a pair of enantiomers and CD. The more strongly included enantiomer has a lower mobility. Imazalil was enantioseparated by the addition of each CD, except α-CD, because the (+) isomer moved more slowly than the (−) isomer. This indicates that the (+) isomer formed stronger diastereomer complexes with each CD than the (−) isomer.

5.2 Capillary Gel Electrophoresis (CGE)

CGE is a special case of CE where the capillary is filled with a cross-linked chemical gel. Goodwin et al. [61]

demonstrated the potential of CE-MS for simultaneous determination of two herbicides (glyphosate and glufosinate) and their metabolites (aminomethylphosphonic acid and methylphosphinicopropionic acid) as native species, using a simple microelectrospray interface. The chemistry of the inner walls of the capillary has a marked influence on peak shape, and an appropriate choice is essential for successful operation of the interface because the negative EOF should operate in the opposite direction to the applied pressure necessary to achieve a stable flow in the sheathless microelectrospray interface. A possible solution is to use capillaries with positive or zero EOF. Capillaries with neutral and positively charged inner walls were prepared by derivatization of the bare silica capillary. A linear-polyacrylamide-coated capillary, which has no electroosmotic flow, gave best reproducibility, with precision of migration time and peak area.

Application of CE to detect multiple DNA products should lead to analyses with higher sensitivity, separation efficiency (typically, several million theoretical plates/meter for DNA fragments 80–1000 bp), and resolution. CGE provides much faster separation than the traditional agarose gel procedure. A CGE separation method using dynamically coated fused silica capillaries, together with a replaceable buffer with a fluorescent intercalating dye and LIF detection, was used to analyse DNA samples. This method, developed by Alarcón et al. [76], allowed the detection of the three amplicons in 25 min. Allele-specific PCR, SSCP, and CFLP with DNA separation and detection by LIF-CE were developed by Arakawa et al. [77]. The increase in sensitivity and selectivity obtained applying LIF-CE is also remarkable.

5.3 Electrokinetic Capillary Chromatography (ECC)

ECC is a separation technique based on a combination of electrophoresis and interactions of the analytes with additives (e.g. surfactants), which form a dispersed phase moving at a different velocity. In order to achieve separation either the analytes or this secondary phase should be charged. The same technique is also called Electrokinetic Chromatography (EKC)

A special widely used variant of ECC, in which the secondary phase is a micellar dispersed phase in the capillary, is micellar electrokinetic chromatography. The same technique is also called Micellar Electrokinetic Capillary Chromatography (MECC). In MEKC, an ionic surfactant is added to the background electrolyte in a concentration higher than the so-called critical micelle concentration (CMC). The most commonly used surfactant, in this operation mode, is sodium dodecyl sulfate (SDS) since it has a fairly low CMC (8 mM/L), at which level the conductivity of the electrolyte can be kept low. These electrophoretic modes have achieved the separation of several fungicides [55, 67, 68], organophosphorus [63], urea-derived [64, 68], triazines [65, 68], and carbamates [67, 68].

MEKC is a CE mode that provides powerful separation of neutral (uncharged) analytes, and thus is often used with phosphate and borate buffers at the pH values that maximize their buffering capacity (viz. 7.0 and 9.2, respectively) [55]. Given the chemical structure of eight fungicides, carbendazim, metalaxyl, captan, procymidone, folpet, captafol, vinclozolin, and iprodione, Molina and Silva [55] attempted their MEKC separation at the pH provided by a phosphate buffer. However, carbendazim failed to behave as expected since one nitrogen atom in its benzimidazole group could be protonated at this pH, thus favouring a subsequent electrostatic interaction with the negative charge of SDS micelles. Similar behaviour has also been observed by Rodríguez et al. [56] in other benzimidazole fungicides such as methyl thiophanate, thiabendazole, and procymidone. Fungicides were best separated using sodium cholate as surfactant because the micelles are not so strongly charged.

Aflatoxins are uncharged over a wide pH range making electrophoretic separation impracticable unless a pseudostationary phase is established in the separation buffer. The four aflatoxins (B_1, B_2, G_1, and F_2), which are neutral molecules, will be separated according to the degree to which they interact with the micelles in solution (sodium deoxycholate). The separation of four aflatoxins was adequate and was accomplished within 10 min. The order of migration of the aflatoxins in MEKC was the same as that which has been commonly reported with reverse-phase LC [74]. The MEKC mode was used for detection and quantification of patulin in apple ciders [72].

When the anionic surfactant SDS is used to form micelles, different migration behaviour can be obtained due to the different interactions between the mycotoxin and the micelle. The SDS micelle, being slightly polar, results in stronger associations with the neutral hydrophobic aflatoxins. Hydrophobic compounds tend to completely associate with the micelles and probably are not resolved. However, in such situations increasing the organic content of the mobile phase will improve the resolution.

CDs are cylinder-shaped with a void apolar axial cavity and an outer hydrophilic surface, which allows certain molecules, of appropriate size and spatial configuration, to form CD inclusion complexes. The structure of the mycotoxins studied appears to meet these criteria for γ-CD, and could be inserted into the cavity of γ-CD. They are then carried along at the same rate as the electroosmotic flow, which implies a decrease in the retention times.

Because the micelle solutions formed from deoxycholic acid or SDS generate a prohibitive fluorescence background when exposed to tightly focused light, Wei et al. [73] evaluated anionic β-cyclodextrin (β-CD) deriv-

atives as differential complexation agents for aflatoxins. Baseline resolution of AFB_1, AFB_2, and AFG_1, and the cholera toxin A was readily achieved using Tris buffers, modified with either carboxymethyl-β-CD or sulphated β-CD that can form host-guest inclusion complexes with hydrophobic (or amphipathic) compounds. The use of an anionic β-CD as a differential complexation agent in the separation medium yields aflatoxin-β-CD complexes that have a net negative charge, making it possible to separate components through a chromatographic mechanism.

Other ECC techniques such as microemulsion electrokinetic capillary chromatography (MEECC or MEEKC) have been reported to separate EDCs [34], but there is no application to food analysis.

5.4 Capillary electrochromatography (CEC)

An alternative to EEC is CEC, which is a special case of capillary liquid chromatography, where the movement of the mobile phase through a capillary, filled, packed, or coated with a stationary phase, is achieved by electroosmotic flow (which may be assisted by pressure). The retention is due to a combination of electrophoretic migration and chromatographic retention. CEC has many latent possibilities to be exploited in organic contaminant determination in food. It has only been reported to separate some pesticides [84]. However, no food application has been reported yet.

6 Future perspectives

As can be discerned from the number of applications reviewed in this paper, CE has, undoubtedly, become an attractive alternative to other chromatographic techniques for determining organic contaminants in food. It offers high separation efficiency, fast analysis, low consumable expenses, and easy operation.

An exciting avenue of future research will be the development of multi-capillary arrays and chip based separations. Separations based on the principles of CE have been the basis for much of the recent explosion in chip-based analytical devices, and it is reasonable to imagine that many current CE applications will be transferable to a chip-based format. The potential advantages in terms of cost, throughput, and device portability are enormous.

Another promising research direction will be the application of CE as a bioanalytical tool to enable the determination of in vivo interactions involving food components such as proteins and organic contaminants.

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References

[1] L.M.L. Nollet, *Handbook of Food Analysis. Vol 2. Residues and Other Food Components*, Marcel Dekker, New York, 2004.

[2] D. Watson, *Pesticides, Veterinary and Other Residues in Food*, Woodhead, Cambridge, 2004.

[3] M. Bowser, in: *Encyclopedia of Analytical Science,* P.J. Worsfold, A. Townshead, C.E. Poole (Eds.), Elsevier, Oxford 2005.

[4] G.K.E. Scriba, in: *Encyclopedia of Analytical Science,* P.J. Worsfold, A. Townshead, C.E. Poole (Eds.), Elsevier, Oxford 2005.

[5] V. Pakacova, K. Stulik, in: *Encyclopedia of Analytical Science,* P.J. Worsfold, A. Townshead, C.E. Poole (Eds.), Elsevier, Oxford 2005.

[6] Y. Picó, in: *Encyclopedia of Analytical Science,* P.J. Worsfold, A. Townshead, C.E. Poole (Eds.), Elsevier, Oxford 2005.

[7] R.A. Frazier, in: *Encyclopedia of Analytical Science,* P.J. Worsfold, A. Townshead, C.E. Poole (Eds.), Elsevier, Oxford 2005.

[8] Z.K. Shihabi, in: *Encyclopedia of Analytical Science,* P.J. Worsfold, A. Townshead, C.E. Poole (Eds.), Elsevier, Oxford 2005.

[9] D.M. Osbourn, D.J. Weiss, C.E. Lunte, *Electrophoresis* **2000**, *21*, 2768–2779.

[10] J.L. Beckers, P. Bocek, *Electrophoresis* **2000**, *21*, 2747–2767.

[11] J.P. Quirino, J.B. Kim, S. Terabe, *J. Chromatogr. A* **2002**, *965*, 357–373.

[12] P. Schmitt-Kopplin, M. Frommberger, *Electrophoresis* **2003**, *24*, 3837–3867.

[13] G. Choudhary, A. Apffel, H.F. Yin, W. Hancock, *J. Chromatogr. A* **2000**, *887*, 85–101.

[14] F.E. Ahmed, *Trends Anal. Chem.* **2003**, *22*, 170–185.

[15] H. Oka, Y. Ito, H. Matsumoto, *J. Chromatogr. A* **2000**, *882*, 109–133.

[16] D.W.M. Sin, Y.C. Wong, *Trends Anal. Chem.* **2003**, *22*, 799–809.

[17] D.A. Stead, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2000**, *747*, 69–93.

[18] R.A. Frazier, *Electrophoresis* **2001**, *22*, 4197–4206.

[19] V. Garcia-Canas, R. Gonzalez, A. Cifuentes, *Trends Anal. Chem.* **2004**, *23*, 637–643.

[20] J. Sadecka, J. Polonsky, *J. Chromatogr. A* **2000**, *880*, 243–279.

[21] European Union, *Council Directive 92/59/EEC of 29 De June 1992 on General Product Safety*, Official Journal L228, 11/08/1992, P.0024–0032, Brussels, 2005.

[22] S. Corneli, C.M. Maragos, *J. Agric. Food Chem.* **1998**, *46*, 3162–3165.

[23] P. Kowalski, I. Oledzka, P. Okoniewski, M. Switala, H. Lamparczyk, *Chromatographia* **1999**, *50*, 101–104.

[24] J. Hernandez-Borges, S. Frias-Garcia, A. Cifuentes, M.A. Rodriguez-Delgado, *J. Sep. Sci.* **2004**, *27*, 947–963.

[25] Y. Picó, R. Rodríguez, J. Mañes, *Trends Anal. Chem.* **2003**, *22*, 133–151.

[26] D.T. Eash, R.J. Bushway, *J. Chromatogr. A* **2000**, *880*, 281–294.

[27] F. Menzinger, P. Schmitt-Kopplin, D. Freitag, A. Kettrup, *J. Chromatogr. A* **2000**, *891*, 45–67.
[28] A.K. Malik, W. Faubel, *Crit. Rev. Anal. Chem.* **2001**, *31*, 223–279.
[29] C.L. Flurer, *Electrophoresis* **2003**, *24*, 4116–4127.
[30] M. Hernandez, F. Borrull, M. Calull, *Trends Anal. Chem.* **2003**, *22*, 416–427.
[31] C. Garcia-Ruiz, Y. Martin-Biosca, A.L. Crego, M.L. Marina, *J. Chromatogr. A* **2001**, *910*, 157–164.
[32] K. Otsuka, H. Hayashibara, S. Yamauchi, J.P. Quirino, S. Terabe, *J. Chromatogr. A* **1999**, *853*, 413–420.
[33] F. Regan, A. Moran, B. Fogarty, E. Dempsey, *J. Chromatogr. A* **2003**, *1014*, 141–152.
[34] B. Fogarty, E. Dempsey, F. Regan, *J. Chromatogr. A* **2003**, *1014*, 129–139.
[35] J. Kuijt, C. Garcia-Ruiz, G.J. Stroomberg, M.L. Marina, F. Ariese, U.A.T. Brinkman, C. Gooijer, *J. Chromatogr. A* **2001**, *907*, 291–299.
[36] M.M. Hsieh, Y.C. Kuo, P.L. Tsai, H.T. Chang, *J. Chromatogr. A* **2001**, *924*, 397–405.
[37] G. Kavran, F.B. Erim, *J. Chromatogr. A* **2002**, *949*, 301–305.
[38] G.K. Belin, E.B. Erim, F. Gulacar, *Polycycl. Aromatic Comp.* **2004**, *24*, 343–352.
[39] D. Barron, E. Jimenez-Lozano, S. Bailac, J. Barbosa, *Anal. Chim. Acta* **2003**, *477*, 21–27.
[40] B. Saad, R. Mohamad, N. Mohamed, G.D. Lawrence, M.S. Jab, M.I. Saleh, *Food Chem.* **2002**, *78*, 383–388.
[41] D. Barron, E. Jimenez-Lozano, J. Cano, J. Barbosa, *J. Chromatogr. B* **2001**, *759*, 73–79.
[42] M. Hernández, F. Borrull, M. Calull, *Electrophoresis* **2002**, *23*, 506–511.
[43] D. Barron, E. Jiménez-Lozano, S. Bailac, J. Barbosa, *J. Chromatogr. B* **2002**, *767*, 313–319.
[44] E. Jiménez-Lozano, D. Roy, D. Barron, J. Barbosa, *Electrophoresis* **2004**, *25*, 65–73.
[45] J.L. Beltran, E. Jimenez-Lozano, D. Barron, J. Barbosa, *Anal. Chim. Acta* **2004**, *501*, 137–141.
[46] T.S. Huang, W.X. Du, M.R. Marshall, C.I. Wei, *J. Agric. Food Chem.* **1997**, *45*, 2602–2605.
[47] K.P. Bateman, S.J. Locke, D.A. Volmer, *J. Mass Spectrom.* **1997**, *32*, 297–304.
[48] M.R.S. Fuh, S.Y. Chu, *Anal. Chim. Acta* **2003**, *499*, 215–221.
[49] B. Santos, A. Lista, B. Simonet, A. Rios, M. Valcarcel, *Electrophoresis* **2005**, *26*, 1567–1575.
[50] P. Kowalski, I. Oledzka, H. Lamparczyk, *J. Pharm. Biomed. Anal.* **2003**, *32*, 937–947.
[51] A.K. Malik, W. Faubel, *Fresenius J. Anal. Chem.* **2000**, *367*, 211–214.
[52] A.K. Malik, W. Faubel, *Talanta* **2000**, *52*, 341–346.
[53] A.K. Malik, B.S. Seidel, W. Faubel, *J. Chromatogr. A* **1999**, *857*, 365–368.
[54] S. Kodama, A. Yamamoto, T. Ohura, A. Matsunaga, T. Kanbe, *J. Agric. Food Chem.* **2003**, *51*, 6128–6131.
[55] M. Molina, M. Silva, *Electrophoresis* **2000**, *21*, 3625–3633.
[56] R. Rodríguez, Y. Picó, G. Font, J. Mañes, *J. Chromatogr. A* **2001**, *924*, 387–396.
[57] R. Rodríguez, I. Boyer, G. Font, Y. Picó, *Analyst* **2001**, *126*, 2134–2138.
[58] R. Rodríguez, Y. Picó, G. Font, J. Mañes, *J. Chromatogr. A* **2002**, *949*, 359–366.
[59] S. Takeda, K. Fukushi, K. Chayama, Y. Nakayama, Y. Tanaka, S. Wakida, *J. Chromatogr. A* **2004**, *1051*, 297–301.
[60] C.K. Kaltsonoudis, F.N. Lamari, K.P. Prousalis, N.K. Karamanos, T. Tsegenidis, *Chromatographia* **2003**, *57*, 181–184.
[61] L. Goodwin, J.R. Startin, B.J. Keely, D.M. Goodall, *J. Chromatogr. A* **2003**, *1004*, 107–119.
[62] E.W.J. Hooijschuur, C.E. Kientz, J. Dijksman, U.A.T. Brinkman, *Chromatographia* **2001**, *54*, 295–301.
[63] X. Guardino, J. Obiols, M. Rosell, A. Farran, C. Serra, *J. Chromatogr. A* **1998**, *823*, 91–96.
[64] R. Rodríguez, Y. Picó, G. Font, J. Mañes, *Electrophoresis* **2001**, *22*, 2010–2016.
[65] M. Khrolenko, P. Dzygiel, P. Wieczorek, *J. Chromatogr. A* **2002**, *975*, 219–227.
[66] R. Rodriguez, J. Manes, Y. Pico, *Anal. Chem.* **2003**, *75*, 452–459.
[67] A. Segura-Carretero, C. Cruces-Blanco, S. Cortacero-Ramirez, A. Carrasco-Pancorbo, A. Fernández-Gutierrez, *J. Agric. Food Chem.* **2004**, *52*, 5791–5795.
[68] C.L. da Silva, E.C. de Lima, M.F.M. Tavares, *J. Chromatogr. A* **2003**, *1014*, 109–116.
[69] J. Hernandez-Borges, M.A. Rodriguez-Delgado, F.J. Garcia-Montelongo, A. Cifuentes, *Electrophoresis* **2004**, *25*, 2065–2076.
[70] D.T. Kubilius, R.J. Bushway, *J. Agric. Food Chem.* **1998**, *46*, 4224–4227.
[71] R. Peña, M.C. Alcaraz, L. Arce, A. Rios, M. Valcarcel, *J. Chromatogr. A* **2002**, *967*, 303–314.
[72] R. Tsao, T. Zhou, *J. Agric. Food Chem.* **2000**, *48*, 5231–5235.
[73] J. Wei, E. Okerberg, J. Dunlap, C. Ly, J.B. Shear, *Anal. Chem.* **2000**, *72*, 1360–1363.
[74] C.M. Maragos, J.I. Greer, *J. Agric. Food Chem.* **1997**, *45*, 4337–4341.
[75] N. Piñeiro, J.M. Leao, A.G. Martinez, J.A.R. Vazquez, *J. Chromatogr. A* **1999**, *847*, 223–232.
[76] B. Alarcon, V. Garcia-Canas, A. Cifuentes, R. Gonzalez, R. Aznar, *J. Agric. Food Chem.* **2004**, *52*, 7180–7186.
[77] H. Arakawa, K. Watanabe, H. Kashiwazaki, M. Maeda, *Biomed. Chromatogr.* **2002**, *16*, 41–46.
[78] A. Juan-García, Y. Picó, G. Font, *J. Chromatogr. A* **2005**, *1073*, 229–236.
[79] A. Juan-García, G. Font, Y. Picó, *Electrophoresis* **2005**, *26*, 1550–1561.
[80] J.E. Palmer, *J. Chromatogr. A* **2004**, *1036*, 95–100.
[81] J. Palmer, J.P. Landers, *Anal. Chem.* **2000**, *72*, 1941–1943.
[82] P.A. Tester, J.T. Turner, D. Shea, *J. Plankton Res.* **2000**, *22*, 47–61.
[83] C. Simo, C. Barbas, A. Cifuentes, *Electrophoresis* **2003**, *24*, 2431–2441.
[84] E. Barcelo-Barrachina, E. Moyano, M.T. Galceran, *Electrophoresis* **2004**, *25*, 1927–1948.

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Evaluation of solid-phase extraction and stir-bar sorptive extraction for the determination of fungicide residues at low-μg kg^{-1} levels in grapes by liquid chromatography–mass spectrometry☆

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Abstract

A liquid chromatography–mass spectrometry method has been developed for determining bitertanol, carboxin, flutriafol, pyrimethanil, tebuconazole and triadimefon. The evaluation of both atmospheric pressure interfaces (API), atmospheric pressure chemical ionization (APCI) and electrospray (ESI) using positive and negative ionization modes, clearly shows that the studied pesticides are more sensitive using APCI in positive mode. Two procedures based on solid-phase extraction (SPE) and stir-bar sorptive extraction (SBSE) have been assessed for extracting these compounds in grape. The recoveries obtained by SPE in samples spiked at the limit of quantification (LOQ) level ranged from 60 to 100% with relative standard deviation (R.S.D.s) from 7 to 17%. With the SBSE the recoveries obtained from samples spiked at LOQ level were between 15 and 100% and the R.S.D.s between 10 and 19%. The LOQs of most compounds are better by SPE (0.003–0.01 mg kg^{-1}) than by SBSE (0.01 mg kg^{-1} for all fungicides). Although SPE provided higher recoveries, lower R.S.D.s, best LOQs and is more rapid to carry out compared with SBSE, this last one has some advantages such as lower organic solvent consumption, and cleaner extracts. Results obtained applying both techniques to real samples are analogous.

Keywords: Food analysis; Solid-phase extraction; Stir-bar sorptive extraction; Fungicides; Pesticides

1. Introduction

Fungicides as bitertanol, flutriafol, triadimefon and tebuconazole (triazoles), carboxin (anilide) and pyrimethanil (pyridine) are intensively applied to grapes at various stages of cultivation and during post-harvest storage to provide protection against rotting [1,2]. Triazines, anilines and pyridines are important classes of fungicides with a wide range of useful activities. Many are systemic and they are highly active with as little as 60 g ha^{-1} being required (compared to the 250 g ha^{-1} for other fungicides as dithiocarbamates). They act by interfering with the synthesis of sterols, which are essential for the construction of normal cell membrane [3–5].

Although all these compounds have low mammalian toxicity, fungicide residue levels in foodstuffs are generally legislated to minimise the exposure of consumers to the harmful or unnecessary intake of pesticides; to control their correct use in terms of the authorisations or registrations granted (application rates and pre-harvest intervals); and to permit the free circulation of products treated with them as long as they comply with the maximum residue limits (MRLs) fixed [4–6]. MRLs are not toxicological limits but are toxicologically acceptables. Exceeded MRLs are strong indicators of violations of good agricultural practices. If MRLs are exceeded, comparison of the exposure with admissible daily intake (ADIs) will indicate whether or not there are possible chronic or

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acute health risks, respectively. Because of the reasons indicated above, monitoring residues of these fungicides in fruits and vegetables is still required [6].

The analysis of fungicides has been widely described in the recent literature and usually utilises the established multiresidue methods (MRM) of analysis [7,8]. These methods involve solvent extraction and partitioning followed by solid-phase or gel permeation cleanup to achieve removal of co-extractives present in the sample extract. Most analytical methods developed in the literature are modification and variations that can improve these extraction and cleanup methods through changes in technologies to reduce the analysis time because sample preparation is still the bottleneck in the analytical laboratory, occupying more than 60% of the analyst's time [8].

Advances could make by simplifying clean-up [9–12], improving extraction and miniaturization [9,12], increasing the use of liquid chromatography (LC) [11,13–18], intensifying automation [9], and introducing mass spectrometry (MS) detection [14–22]. A valid alternative is the enrichment on solid-phases cartridges, glass columns or disks packed with C_{18} [9,13,14], mixed cation exchange [10,11], hydrophilic/lipophilic balance phases [10] or polymeric resins [22]. Detection limits attained ranged from 0.1 to 180 $\mu g\,kg^{-1}$ depending on the compound and the determination technique used.

Another very elegant enrichment technique for aqueous extraction is the recently developed stir-bar sorptive extraction (SBSE). In SBSE, analytes are adsorbed into a magnetic rod coated with polydimethylsiloxane (PDMS) by stirring for a given time. After that, the stir-bar is either thermally desorbed on-line with capillary GC–MS or by organic solvents. SBSE has been already verified for analysing dicarboximide fungicides in wine [23], organophosphorus and carbamates in oranges [24], and organophosphorus pesticides in honey [25].

In the study, SPE and SBSE were evaluated to analyze bitertanol, carboxin, flutriafol, pyrimethanil, tebuconazole and triadimefon in grapes in combination with liquid chromatography–mass spectrometry (LC–MS). Several parameters controlling the recovery efficiency of the analytes from the samples are optimized. Both procedures were compared to establish the most suitable technique for quantifying these pesticides. The methods were applied to measure the levels of fungicides in grape samples taken from the market.

2. Experimental

2.1. Chemicals

Fungicides carboxin, flutriafol, tebuconazole and bitertanol were purchased from Riedel-de Haën (Seelze, Germany), pyrimethanil and triadimefon from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Stock solutions of 1 $mg\,ml^{-1}$ were prepared by weighting and dissolving each pesticide in methanol and stored in glass-stopper bottles at 4 °C. The stock solutions were stable in the stored conditions for more than 3 months. Standard working solutions at various concentrations were daily prepared by appropriate dilution of aliquots of the stock solutions in methanol.

HPLC-grade methanol and organic trace analysis grade dichloromethane were purchased from Merck (Darmstadt, Germany). Deionized water (<8 cm MΩ resistivity) was obtained from the Milli-Q SP Reagent Water System (Millipore, Bedford, MA, USA). All the solvents were passed through a 0.45 μm cellulose filter from Scharlau (Barcelona, Spain) before use.

MFE C_{18} solid phase sorbent (particle diameter in the range of 45–55 μm and pore diameter 60 Å) was acquired from Análisis Vínicos (Tomelloso, Spain). The solid-phase (500 mg amount, 900 μl volume) was placed into a 100 mm × 9 mm i.d. glass column fitted with a coarse frit (No. 3). The column was preconditioned by passing through it 10 ml of methanol and 10 ml of deionized water.

The stir bars (Twister) were from Gerstel (Mülheim, Germany) with a length of 10 mm and coated with a 1 mm PDMS layer, that correspond to an amount of 55 μl of PDMS. Prior to use, stir bars were conditioned into a vial containing 15 ml of methanol, and treated for 5 min by sonication, then the solvent was rejected and the procedure was repeated three times.

2.2. Extraction and clean-up procedures

2.2.1. Extraction of grapes

A representative portion of the sample (ca. 200 g of grapes) was chopped and homogenized in a food chopper. Then, a 5 g portion was placed in 250 ml glass beaker and homogenized with 25 ml of water by sonication over 15 min. The resulting suspension was filtered through Albet 40 μm folded filters (Barcelona, Spain).

Linearity, percentage of recovery and the repeatability (within-day precision) were determined by adding 50 μl of the appropriate working mixture to the 5 g portion placed in a jar. The spiked sample was allowed to stand for 1 h before extraction to attain the pesticide distribution in grapes.

2.2.2. Solid-phase extraction (SPE)

The solution was passed under vacuum through a column containing 0.5 g of solid-phase C_{18}. The filtrate was discarded and the pesticides retained in the solid phase were eluted with 10 ml of dichloromethane–methanol (50:50, v/v). The eluent was collected in a graduated conical tube (20 ml) and concentrated at 50 °C, under a stream of nitrogen, to dryness. After that it was redissolved with 0.5 ml of methanol.

2.2.3. Stir-bar sorptive extraction (SBSE)

The filtrate was place into a 50 ml glass beaker and stirred with the stir bar, coated with PDMS, for 2 h at 900 rpm.

After the extraction, the stir bar was removed from the aqueous sample with a magnetic stirring bar and tweezers.

Table 1
Time scheduled SIM conditions for monitoring pesticides

	Group	Time (min)	SIM ion	Gain	Fragmentor (V)	Dwell time (ms)
Carboxin	1	0–8	236	1	100	132
			143			132
Flutriafol	2		302	1	100	132
Pyrimethanil	3	8–14	200	1	100	132
Triadimefon	4		294	1	100	132
			197			132
Tebuconazole	5	14–30	308	1	60	98
Bitertanol	6		269	1	60	98
			338			98

Then, the analytes were desorbed into 2 ml vial filled with 1 ml of methanol. Desorption of the pesticides was performed by sonication for 15 min.

2.3. Liquid chromatography–mass spectrometry

A Hewlett Packard (Palo Alto, CA, USA) HP-1100 series LC–MS system equipped with a binary solvent pump, an autosampler with the volume injection set to 5 μl, and a mass-selective detector (MSD) with atmospheric pressure chemical ionization (APCI) coupled with an HPChem work station was used. Operating conditions of the APCI interface in positive ion mode were vaporizer temperature 325 °C; nebulizer gas (nitrogen) pressure of 60 psi (1 psi = 6894.76 Pa); drying gas (nitrogen) flow rate 4 l min^{-1}; drying gas temperature 250 °C; capillary voltage 4000 V; and corona current 10 μA.

Chromatographic separation was performed on a C_{18} column (15 cm × 0.4 cm i.d., 5 μm) from Phenomenex (Madrid, Spain) with a methanol–water gradient that started with 65% of methanol increasing linearly during 15 min until 80% of methanol. The flow rate was 0.8 ml min^{-1}.

Full-scan LC–MS chromatograms were obtained by scanning from *m/z* 80–340; with a scan time of 0.68 s. Time scheduled selected-ion monitoring (SIM) of the most abundant ions of each compound was performed as is reported in Table 1 using the high resolution setting.

3. Results and discussion

3.1. Mass spectrometry remarks

Although there are a lot of methods described now in the literature, established procedures for choosing the most sensitive interface or the best ionization mode do not exist.

Table 2 summarizes the chemical structures, molecular weights, base peaks and the most abundant ions (with their relative abundance) of the mass spectra of the six studied fungicides using APCI and electrospray (ESI) interfaces in positive ionization (PI) and negative ionization (NI) modes. The studied compounds gave response in positive and negative mode by both interfaces, except that bitertanol did not give a signal in ESI.

The ESI interface in PI mode provided mainly the protonated molecules and strong signal for sodium adducts. Only carboxin presents a fragment ion corresponding to the neutral loss of aniline. The sodium adduct is the main ion for flutriafol, triadimefon and tebuconazole whereas pyrimethanil did not form sodium adducts, confirming the theory that the sodium adduct formation requires a group that can donate a lone pair of electrons. Carboxin, flutriafol, triadimefon and tebuconazole contain carboxyl and hydroxyl groups that are absent in the pyrimethanil molecule.

The ESI interface in NI mode provided the deprotonated molecules as main ion for triadimefon and pyrimethanil. The main ion in the spectrum of flutriafol is the fragment obtained by the neutral loss of fluorobenzene, in the spectrum of carboxin the fragment obtained by the loss of ethene, and the only ion in the spectrum of tebuconazole is the fragment resulting from the loss of methyltriazole moiety.

The studied fungicides have similar mass spectra in APCI to those provided in ESI. However, in PI mode, there were no sodium adducts and some fragment ions can be obtained. Carboxin provides the fragment by neutral loss of aniline in higher proportion. Flutriafol, triadimefon and bitertanol commonly suffered the neutral loss of 69 u of the molecule that corresponds to the triazole ring. APCI in NI mode provided similar mass spectra for carboxin, flutriafol and tebuconazole to those obtained by ESI. Triadimefon provided a characteristic fragment ion at *m/z* 127 corresponding to the loss of *p*-chlorophenol, and bitertanol gave a fragment at *m/z* 169 that correspond to the *p*-phenylphenol. Fig. 1 illustrates some examples of the mass fragmentation observed.

A summary of the results in terms of limits of detection (LODs) obtained using full scan mode is shown in Table 3. The response varied from 250 pg for the six compounds in the APCI in PI mode, which are the most sensitive interface and mode, to no response in ESI for bitertanol. ESI was between 25 and 100 times less sensitive for the studied compounds than APCI. Taking into account these data, APCI in PI mode was chosen for further experiments. The system sensitivity was fully optimized using SIM. The time-schedule of SIM was performed following the procedure reported in Table 1.

Table I.2 Molecular and fragment ions and their relative abundance both API interface in NI and PI modes at voltage fragmentor 100V

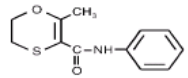
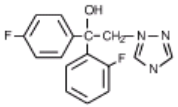
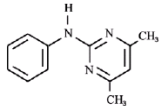
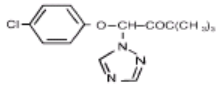
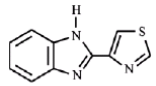
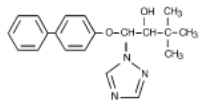
Compound (Mw)	ES				APCI			
	Positive (PI)		Negative (NI)		Positive (PI)		Negative (NI)	
	m/z tentative ion	*R*(%)	*m/z tentative ion*	*R*(%)	*m/z tentative ion*	*R*(%)	*m/z tentative ion*	*R*(%)
Carboxina (235)	143 [M + H − $C_6H_6NH_2$]+	25	206 [M − H − CH_2CH_2]-	100	236 [M + H]+	100	234 [M − H]-	100
	236 [M + H]+	100	234 [M − H]-	50	143 [M + H − $C_6H_6NH_2$]+	80	206 [M − H − CH_2CH_2]-	20
	258 [M + Na]+	25						
Flutriafol (301)								
	302 [M + H]+	20	300 [M − H]-	10	302 [M + H]+	100	300 [M − H]- 10	
	324 [M + Na]+	100	204 [M − H − FC_6H_5]-	100	233 [M + H − C_2HN_3]+	15	204 [M − H − FC_6H_5]-	100
Pyrimethanil (199)								
	200 [M + H]+	100	198 [M − H]-	100	200 [M + H]+	100	198 [M − H]- 100	
Triadimefon (293)								
	4 [M + H]+	10	292 [M − H]-	100	294 [M + H]+	100	292 [M − H]- 100	
	6 [M + Na]+	100			225 [M + H − C_2HN_3]+	50	127 [M − H − ClC_6H_4O]-	10
Tebuconazole (307)					197 [M + H − $C_2HN_3C_2H_4$]+	50		
	308 [M + H]+	25	223 [M − H − $C_3N_3H_5$]-	100	308 [M + H]+	100	306 [M − H]- 25	
Bitertanol (337)	0 [M + Na]+	100					223 [M − H − $C_3N_3H_5$]-	100
	–		–		338 [M + H]+	20	169 [$C_{12}H_9O$-]-	100
					269 [M + H − C_2HN_3]+	100		
					99 [$CH_2COHC(CH_3)_3$]+	100		

Table 3
Detection limits (ng injected) obtained using full-scan mode with both interfaces APCI and ESI in PI and NI modes at fragmentor voltage of 100 V

	APCI		ESI	
	PI	NI	PI	NI
Carboxin	0.25	1	10	25
Flutriafol	0.25	0.25	10	25
Pyrimethanil	0.25	0.25	5	25
Triadimefon	0.25	0.25	10	125
Tebuconazole	0.25	0.25	5	25
Bitertanol	0.25	125	–	–

Table 4
Influence of water volume on the extraction efficiency

Pesticide	SPE volume (ml)				SBSE volume (ml)			
	10	25	50	100	10	25	50	100
Carboxin	88	105	95	52	55	58	20	28
Flutriafol	87	100	95	102	65	63	30	11
Pyrimethanil	95	102	97	101	57	50	25	17
Triadimefon	96	99	93	114	97	97	50	29
Tebuconazole	60	111	102	111	60	53	26	11
Bitertanol	103	112	110	105	44	44	26	18

3.2. Optimization of the extraction procedures

SPE and SBSE are significantly influenced by the aqueous volume selected, the amount of sample processed and the ionic strength of the medium. A set of experiments to determine the effect of these parameters in the recoveries of the studied compounds was designed. The elution step has already been widely studied in the literature for both techniques, and it is state that the best eluent for SPE is a mixture of dichloromethane–methanol since it provides highest recoveries and cleanest extracts, and that the best desorption of the compounds from the stir bar is accomplished sonicating 15 min with 0.5 ml of either methanol or acetonitrile [9,13,14,23–25].

Extraction efficiencies for a wide variety of compounds (depending on the polarity) can be improved increasing ionic strength since high ionic strength reduces their water solubility. As SBSE provided recoveries below 90% for most of the studied fungicides, this effect was tested adding 10, 20 and 30% (w/w) of sodium chloride (NaCl). The recovery of all fungicides was increased in proportion to the amount of sodium chloride, and double recovery percentages are achieved using the highest amount of 30%, which was added to the samples processed by SBSE. The recoveries obtained

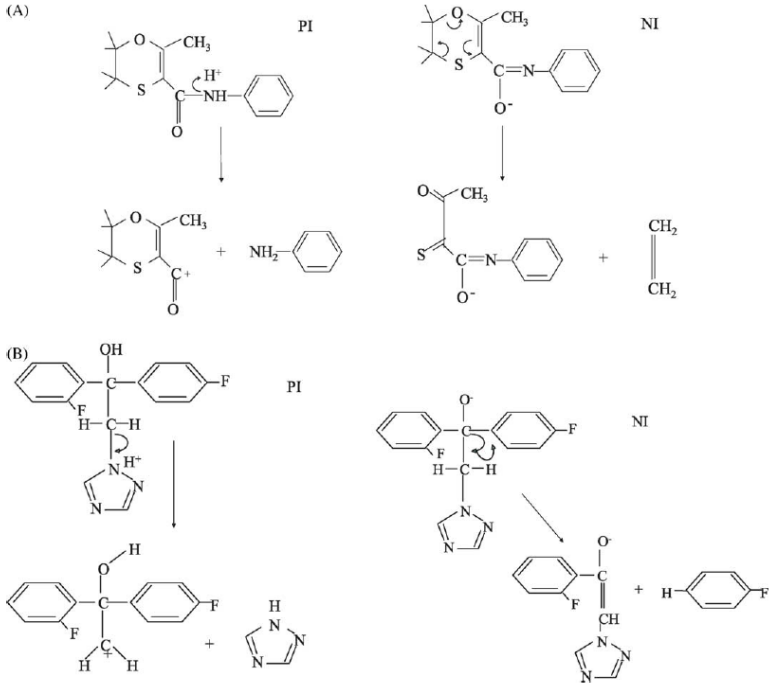


Fig. 1. Mass fragmentation patterns observed in PI and NI modes for (A) carboxin and (B) flutriafol.

Table 5
Influence of grape amount on the extraction efficiency

Pesticide	SPE amount of matrix (g)				SBSE amount of matrix (g)			
	2	5	7	10	2	5	7	10
Carboxin	13	33	10	35	3	15	14	13
Flutriafol	95	100	33	93	80	45	9	14
Pyrimethanil	14	60	28	57	69	65	13	12
Triadimefon	79	72	65	72	100	105	26	9
Tebuconazole	68	98	99	95	92	51	31	13
Bitertanol	96	97	100	79	27	21	13	10

by SPE (see the data presented below) do not require the salt addition.

Different water volumes (10–100 ml) were tested as it is shown in Table 4. The recoveries obtained using SPE for these range of volumes are around 100% and almost independent of the aqueous volume passed through it, except for carboxin, the recovery of which is reduced to the half for volumes up to 50 ml, and for tebuconazole that is recovered on a 40% less when the sample volume is less than 25 ml. For SBSE, the results are quite different. The lower the sample volume is, the higher the recovery obtained. SBSE recoveries ranged from 42 to 98% using 10 ml of water, and are maintained for 25 ml of water, suffering an important decrease for higher volumes that lead to recoveries from 20 to 50% for 50 ml and from 5 to 25% for 100 ml. A water volume of 25 ml was selected for further experiments as a compromise to obtain appropriate sensitivity with a water volume that achieved the dissolution of an appropriate quantity of grape.

The influence of grape matrix on the extraction efficiency of SPE and SBSE was checked diluting different amounts of grape in 25 ml of water. Table 5 illustrates the results in terms of recovery for SPE and SBSE. The matrix reduces the recovery obtained by SPE for carboxin, pyrimethanil and triadimefon whereas that obtained for flutriafol, tebuconazole and bitertanol are scarcely affected. Although the amount of grape tested (between 2 and 10 g) shows negligible effect on recovery, the variability of the results is greater with amounts up to 5 g. The effect of the grape matrix in the recoveries attained by SBSE presented a strong relation with the grape amount. Grape amounts of 2 g only reduced the recovery of carboxin and bitertanol respect to those obtained in pure water, grape amounts of 5 g also decreased the recovery

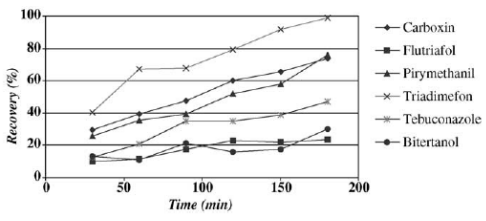


Fig. 2. Effect of stirring time with the PDMS stir-bar on the recovery of the studied pesticides. Amount of each pesticide in solution: 50 ng.

of flutriafol, and higher grape amounts (from 7 to 10 g) were negative for all fungicides. The amount of 5 g of grape was used for the following experiments, since it provided acceptable recoveries and good sensitivity for all studied fungicides by both extraction techniques.

The pH of the spiked samples oscillated from 4.1 to 4.3. The pH of the unspiked grape matrix and that of the spiked grape matrix were controlled to ensure that are equivalent. Optimization of sample pH was not carried out because all the analytes are protonated at low pH because they contain basic nitrogens, which enhanced their water solubility. These analytes are stables in aqueous solutions at slightly acid pH. The sample pH lower 4 can negatively affected the solid phase stability. Because of this, the sample pH was considered appropriate since it provides acceptable recoveries, which are comparable for flutriafol, tebuconazole and bitertanol to those obtained in the experiments performed with distilled water.

In addition, SBSE is an adsorption equilibrium and it is very influenced by the extraction time and temperature. Different extraction times were studied to obtain the sorption time profiles, which are presented in Fig. 2. A 120 min extraction time was selected for SBSE to avoid unreasonable analysis time. Equilibrium was not reached for any of the studied pesticides. However, quantitative analysis can be carried out because the samples are extracted exactly the same time and analytical sensitivity is rather satisfactory.

In quantitative analysis one of the major problems is the suppression/enhancement of the analyte signal in presence of matrix components, which has been reported by many authors [19–21]. Response suppression caused by sample matrix components using the ESI interface has been widely discussed in the literature [20–21]. However, the information about the effects of this class of interferences on APCI interface is more conflicting.

This interference can be established comparing the signal intensity obtained in a standard solution (methanol) with those obtained in matrix matched standards. This was carried out for both procedures. Using SPE a slight enhancement of the response (ranging from 0 to 15%) depending on the compound was noted whereas using SBSE the response of the standard prepared in methanol and the standard prepared in matrix extract was the same. The absence of matrix effect using SBSE is an interesting characteristic of this technique that has already been reported in the literature [24].

The use of matrix-matched calibration standards was not necessary to compensate for signal enhancement of target analytes in matrix solution compared to their response in pure solvent since the enhancement is really low.

3.3. Validation

Table 6 shows the mean recovery and precision obtained by SPE from samples spiked at the limit of quantification (LOQ) levels and at around 10 times the LOQ levels. LOQs were calculated according to the European Union Guidelines as the lower concentration that provides repeatabilities lower

Table 6
Recovery and R.S.D.s of the studied fungicides in grape samples spiked at LOQ and at 0.1 mg kg^{-1} (ca. LOQ × 10) obtained by SPE

Compound	Concentration (mg kg^{-1})	Recovery (%) ± R.S.D., $n = 5$	Concentration (mg kg^{-1})	Recovery (%) ± R.S.D., $n = 5$
Carboxin	0.003	67 ± 17	0.1	91 ± 12
Flutriafol	0.005	100 ± 8	0.1	99 ± 6
Pyrimethanil	0.008	60 ± 10	0.1	107 ± 9
Triadimefon	0.01	71 ± 9	0.1	102 ± 5
Tebuconazole	0.005	98 ± 8	0.1	98 ± 5
Bitertanol	0.003	96 ± 7	0.1	107 ± 4

than 20%. Fungicide recoveries were between 60 and 100% at the lowest concentration, and between 91 and 107 at the highest one. The relative standard deviations (R.S.D.s) were from 7% (bitertanol) to 17% (carboxin) and from 4% (bitertanol) to 12% (carboxin) for the lowest and highest concentration. The LOQ obtained, considering it as the lowest concentration for which the recovery and repeatability were acceptable, ranged from 3 to 10 μg kg^{-1}. Recovery only depends on concentration for three compounds carboxin, pyrimethanil and tebuconazole. The difference in recovery was only applied to the determination of the analyte concentration in the real samples when its concentration is close to the LOQ. Chromatograms of the SBSE–LC–MS analysis of an unspiked grape sample and grape sample spiked at 0.01 mg kg^{-1} of each compound are illustrated in Fig. 3A and B.

Table 7 reports the same data but corresponding to the SBSE. The recoveries ranged from 15% for carboxin to 100% for triadimefon and the R.S.D.s from 10% for triadimefon to 19% for carboxin and bitertanol at the lowest concentration whereas at the higher one, the recoveries were between 17% for carboxin to 101% for triadimefon with R.S.D.s < 17%. The LOQs were 10 μg kg^{-1} for all studied fungicides. Characteristic examples of LC–MS chromatograms of grapes spiked at LOQ level and non-spiked grapes samples are shown in Fig. 4. It is remarkable the lack of interfering peaks and the low background noise compared with the chromatogram obtained by SPE. The comparison of both chromatograms also pointed out the higher sensitivity of SPE.

Table 8 compares the parameters indicative of the analytical performance of the two methodologies described. SPE provided LOQs slightly lower than those obtained by SBSE (three times as much), recoveries higher and R.S.D.s lower than those obtained by SBSE. The low recoveries, higher R.S.D.s and worse LOQs obtained by SBSE compared to those from the SPE can be explained because the SBSE is based on reaching adsorption equilibrium whereas SPE is a

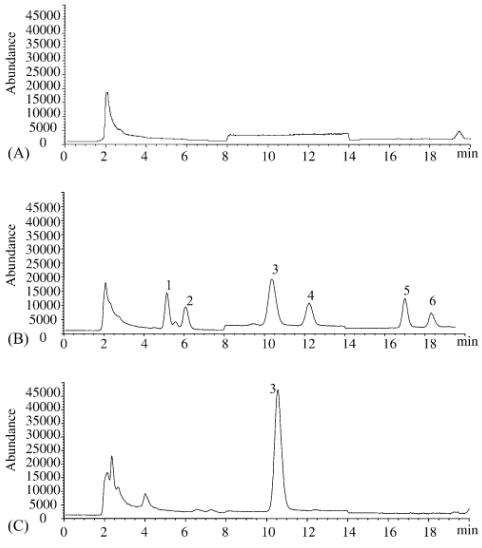


Fig. 3. LC–MS chromatograms in SIM mode obtained after SPE of (A) untreated grape sample, (B) untreated grape sample spiked at 0.01 mg kg^{-1} of each compound and (C) grape sample that contains 0.05 mg kg^{-1} of pyrimethanil. Peak identification: (1) carboxin, (2) flutriafol, (3) pyrimethanil, (4) triadimefon, (5) tebuconazole and (6) bitertanol.

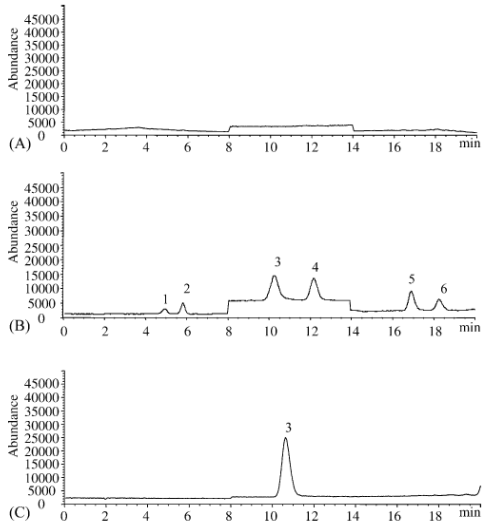


Fig. 4. LC–MS chromatograms in SIM mode obtained after SBSE of (A) untreated grape sample, (B) untreated grape sample spiked at 0.01 mg kg^{-1} of each compound and (C) grape sample that contains 0.05 mg kg^{-1} of pyrimethanil. Peak identification as in Fig. 3.

Table 7
Recovery and R.S.D.s of the studied fungicides in grape samples spiked at LOQ and 10 times LOQ obtained by SBSE

Compound	Concentration (mg kg^{-1})	Recovery (%) ± R.S.D., $n = 5$	Concentration (mg kg^{-1})	Recovery (%) ± R.S.D., $n = 5$
Carboxin	0.01	15 ± 19	0.1	17 ± 17
Flutriafol	0.01	45 ± 17	0.1	59 ± 16
Pyrimethanil	0.01	65 ± 16	0.1	73 ± 14
Triadimefon	0.01	100 ± 10	0.1	101 ± 8
Tebuconazole	0.01	55 ± 12	0.1	57 ± 15
Bitertanol	0.01	20 ± 19	0.1	25 ± 16

non-equilibrium process, based on partitioning between the aqueous extract of the sample and the solid-phase.

The linearity was evaluated at five concentrations, from the LOQ to 100 times the LOQ, showing correlation coefficients higher than 0.995 for SPE and 0.994 for SBSE. These coefficients (0.99) are relatively poor compared to conventional calibration techniques (0.999) because the extraction is included as it has been previously reported [25].

Other advantage of SPE is that is more rapid to perform that SBSE since it is not dependent on the sample equilibrium time. However, SBSE presents some advantages with respect to SPE as it reduces the organic solvent required, provides cleanest chromatogram and less matrix interference effect (in spite that this effect can be considered negligible in both techniques).

LOQs obtained by both procedures were always lower than MRLs established by the EU [6], Codex Alimentarius Commission of FAO/WHO [26], Food and Drug Administration (FDA) from the USA [27] and Spanish legislation [28], which are in the interval of 0.05–2 mg kg^{-1} for bitertanol, 0.2 mg kg^{-1} for carboxin, 0.01 mg kg^{-1} for flutriafol, 5 mg kg^{-1} for pyrimethanil, 2 mg kg^{-1} for tebuconazole and 2 mg kg^{-1} for triadimefon.

3.4. Application

SPE and SBSE procedures were applied for determining these fungicides in 15 commercial grape samples from different markets (these samples include red and white grapes of different varieties). Only pyrimethanil was detected in one Muscat grape. This sample was extracted by triplicate and each replicate was injected twice. The mean concentration value and the standard deviation were 0.05 ± 0.002 mg kg^{-1} by SPE and 0.05 ± 0.003 mg kg^{-1} by SBSE. Fig. 3C shows the chromatogram of the sample extracted by SPE and Fig. 4C displays the chromatogram of the sample obtained by SBSE. Excellent conformity is obtained by both procedures.

Table 8
Comparison of both methods features

	SPE	SBSE
Spiking concentration (mg kg^{-1})	0.01–1	0.01–1
Accuracy (% recovery)	67–106	15–100
Repeatibility (R.S.D., %)	4–17	8–19
Linearity (r^2)	>0.995	>0.994
Sensitivity (LOQ)	0.003–0.01	0.01
Organic solvent used to desorb analytes (ml)	10	0.5
Time required to process a sample (min)	90	150

4. Conclusion

The studied compounds have been well characterized by all the atmospheric pressure interfaces (API) sources and in PI and NI mode. The analysis of the six studied fungicides by SPE and SBSE has demonstrated to be an interesting alternative to more conventional methods that are usually more time consuming. SPE is more effective than SBSE to extract triazole, anilides and pyridines from grapes because it provides higher recoveries, lower R.S.D.s and best detection limits. In addition, the SPE procedure described is relatively simple and rapid. However, both procedures can be applied to determine bitertanol, carboxin, flutriafol, pyrimethanil, tebucanozole and triadimefon in real grape samples with comparable results.

Acknowledgements

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References

[1] R. Ryley, S. Bhuiyan, D. Herde, B. Gordan, Aust. Plant Pathol. 32 (2003) 329.
[2] A.D. Wilson, L.B. Forse, Mycologia 89 (1997) 468.
[3] N. Kurihara, J. Miyamoto, G.D. Paulson, B. Zeeh, M.W. Skidmore, R.M. Hollingworth, H.A. Kuiper, Pure Appl. Chem. 69 (1997) 1335.
[4] K.M. Crofton, Toxicol. Lett. 84 (1996) 155.
[5] G.L. Bateman, D. Hornby, R.W. Payne, P.H. Nicholls, Ann. Appl. Biol. 124 (1994) 241.
[6] http://europa.en.int/, The European Union "on-line", 2003.
[7] W.G. Fong, H.A. Moye, J.N. Seiber, J.P. Toth, Pesticides Residue in Food, Methods Techniques and Regulations, Wiley, New York, 1999.
[8] N.A. Botsoglou, D.J. Fletouris, in: L.M.L. Nollet (Ed.), Handbook of Food Analysis, Marcel Dekker, New York, 1996, p. 1189.

[9] A. Colume, S. Cárdenas, M. Gallego, M. Valcárcel, J. Chromatogr. A 882 (2000) 193.
[10] S. Navarro, A. Barba, G. Navarro, G. Vela, J. Oliva, J. Chromatogr. A 882 (2000) 221.
[11] Y. Yamazaki, T. Ninomiya, J. AOAC Int. 82 (1999) 1474.
[12] M. Navarro, Y. Picó, R. Marín, J. Mañes, J. Chromatogr. A 968 (2002) 201.
[13] R.R. Ottero, B.C. Grande, J.S. Gándara, J. Chromatogr. A 992 (2003) 121.
[14] M.S. Young, M.F. Early, C.R. Mallet, J. Krol, J. AOAC Int. 84 (2001) 1608.
[15] C. Blasco, Y. Picó, J. Mañes, G. Font, J. Chromatogr. A 947 (2002) 227.
[16] C. Blasco, M. Fernández, Y. Picó, G. Font, J. Mañes, Anal. Chim. Acta 461 (2002) 109.
[17] C. Blasco, Y. Picó, G. Font, J. AOAC Int. 85 (2002) 704.
[18] C. Blasco, G. Font, J. Mañes, Y. Picó, Anal. Chem. 75 (2003) 3606.
[19] C.F. González, R.R. Otero, B.C. Grande, J.S. Gándara, J. AOAC Int. 85 (2003) 1008.
[20] J. Zrostlikova, J. Hajslova, T. Kovalczuk, R. Stefan, J. Poustka, J. AOAC Int. 86 (2003) 612.
[21] Y. Ito, T. Goto, H. Oka, H. Matsumoto, Y. Miyazaki, N. Takahashi, H. Nakazawa, J. Agric. Food Chem. 51 (2003) 861.
[22] J.W. Wong, M.G. Webster, C.A. Halverson, M.J. Hengel, K.K. Ngim, S.E. Ebeler, J. Agric. Food Chem. 51 (2003) 1148.
[23] P. Sandra, B. Tienpont, J. Vercammen, A. Tredoux, T. Sandra, F. David, J. Chromatogr. A 928 (2001) 117.
[24] C. Blasco, G. Font, Y. Picó, J. Chromatogr. A 970 (2002) 201.
[25] C. Blasco, M. Fernández, Y. Picó, G. Font, J. Chromatogr. A 1030 (2004) 77.
[26] Codex Alimentarius, http://www.fao.org, 2003.
[27] Food and Drug Administration of the United States, http://www.cfsan.fda.gov, 2003.
[28] Ministerio de Agricultura, Pesca y Alimentación, http://www.mcx.plaguicidas.plagas, htm Official Maximum Residue Limits (MRLs), 2003.

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Capillary electrophoresis for analyzing pesticides in fruits and vegetables using solid-phase extraction and stir-bar sorptive extraction

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Abstract

Two procedures based on solid-phase extraction (SPE) and stir-bar sorptive extraction (SBSE) in combination with micellar electrokinetic chromatography (MEKC)–diode array detection (DAD) were compared for the simultaneous extraction of acrinathrin, bitertanol, cyproconazole, fludioxonil, flutriafol, myclobutanil, pyriproxyfen, and tebuconazole in lettuce, tomato, grape, and strawberry. Selectivity and resolution of the MEKC procedure were studied changing the pH and the molarity of the buffer, the type and the concentration of surfactant, and the methanol content in the mobile phase. A buffer consisting of 6 mM sodium tetraborate decahydrate with 75 mM of cholic acid sodium solution (pH 9.2) gave the best results. Linearity, extraction efficiencies and limits of quantitation (LOQs) of both extraction methods were compared. The recoveries obtained by SPE ranged from 40 to 106% with relative standard deviations (R.S.D.s) from 10 to 19% whereas by the SBSE method, the recoveries were 12–47% and the R.S.D.s 3–17%. The LOQs were much better by SPE (0.2–0.5 mg kg^{-1} depending on the processed sample amount) than those obtained by SBSE (1 mg kg^{-1} for each compound). Advantages and disadvantages of both procedures are also discussed. As SPE is more robust, rapid, and sensitive than SBSE, its application in combination with MEKC is recommended because provided LOQs below the MRLs established, which is not always attained by SBSE.

Keywords: Food analysis; Solid-phase extraction; Stir-bar sorptive extraction; Pesticides; Micellar electrokinetic chromatography

1. Introduction

The social concern about the levels of pesticides in food and the constant trend observed in the current legislations to reduce the maximum residue levels (MRLs) allowed in a variety of fruits and vegetables is increasing the number of samples to be analyzed as well as the need for their accurate determination reducing the analysis' costs [1].

Sample preparation is often the most time-consuming and laborious part of the analytical process. An ideal procedure should be simple, inexpensive, efficient, able to extract the largest number possible of pesticides, and compatible with various determination techniques [2,3]. That is the reason why these methods strive towards the simplification and miniaturization. Solid-phase extraction (SPE) and stir-bar sorptive extraction (SBSE) are well-established preconcentration techniques in food analytical applications because they offer significant advantages such as economy in terms of time and solvent needs [4–12].

Side by side with the classical techniques for detecting pesticide residues—gas chromatography (GC) or liquid chromatography (LC)—an increasing number of methods involve the use of capillary electrophoresis (CE) [13–15]. CE joints separation of compounds with GC-like resolution, and the capability of LC to determine thermally labile or non-volatile compounds. CE shows great potential in the analysis of contaminants in food because of its features, such as higher separation efficiency, shorter analysis time, simplicity with regards to instrumentation, and very less consumption of expensive reagents and solvents [13,14]. In the beginning, CE presented the disadvantage of inappropriate sensitivity for contaminant analysis, as a result of the small sample volumes typically injected (ca. 1–10 nl), but it has already been solved by off- and on-column trace enrichment schemes [15–18]. This is often performed by SPE that can be used directly as

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an extraction technique for liquid matrices, or as a clean-up method for solvents extracts [13,15]. SPE using C_{18} has already been applied for determining urea and fungicides in fruits and vegetables followed by CE [17,19–22]. However, the high matrix load required to achieve an appropriate pesticide concentration can cause the partial co-extraction of interfering substances, breakdown of the analytes, or clogging of the SPE material. SBSE, a relatively new extraction technique, uses as a magnetic rod encapsulate in a glass jacket and coated with polydimethylsiloxane (PDMS). Analytes are sorbed from aqueous samples based on partitioning between aqueous extract and PDMS. Because its unique characteristics, SBSE has the potential to overcome the problems associated with SPE of pesticides from fruits and vegetable extracts [8–12]. For example, SBSE, as an equilibrium technique, does not experience breakthrough and plugging, and can co-extract less water soluble co-extracts. To date, the application of SBSE to pesticide residues has focused mainly in GC determination [9–11]. Although there are recent reports on the application of SBSE to LC [8,12], no studies on the SBSE combined with CE determination have been reported.

The present study compares SPE and SBSE as enrichment schemes for determining eight pesticides in strawberries, grapes, lettuces, and tomatoes by MEKC and diode array detection (DAD). As target analytes (see Fig. 1), four conazole fungicides (flutriafol, cyproconazole, tebuconazole, and myclobutanyl), one azole fungicide (bitertanol), one pyrrole fungicide (fludioxonil), one pyrethroid insecticide (acrinathrin), and one juvenile hormone mimic insecticide (pyriproxyfen) were selected based on their application in fruits and vegetables and on the legislation requirements of the EU [23].

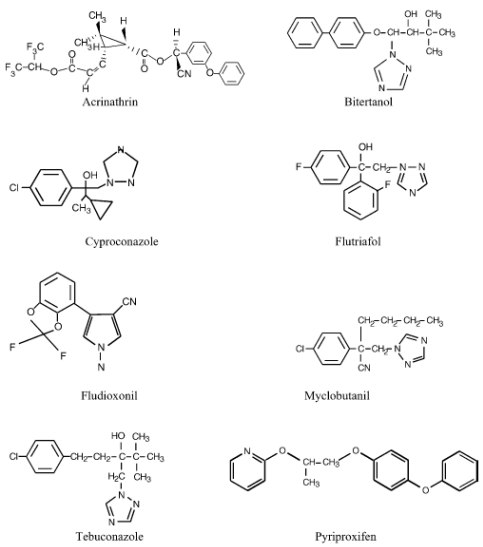

Fig. 1. Structures of the studied pesticides.

2. Experimental

2.1. Chemicals and reagents

Fludioxonil, cyproconazole, tebuconazole, bitertanol, acrinathrin, and flutriafol were purchased from Riedel-de-Haën (Seelze, Germany). Myclobutanil and pyriproxyfen were from Dr. Ehrenstorfer GmbH. Individual stock solutions were prepared at concentration of 1 mg ml^{-1} in methanol and stored in glass-stopper bottles at 4 °C. Working solutions, at different concentrations, were prepared by appropriate combination and dilution of the standard solutions with the running buffer. Mixtures were passed through a 0.45 μm cellulose filter from Scharlau (Barcelona, Spain).

HPLC-grade methanol and organic trace analysis grade dichloromethane were obtained from Merck (Darmstadt, Germany) and acetone from Mallinckrodt (ChromAR-HPLC, KY, USA). Deionized water (<18 MΩ cm resistivity) was used from the Milli-Q SP Reagent Water System (Millipore, Bedford, MA, USA).

Cholic acid sodium salt was purchased from Fluka (Buchs, Switzerland), sodium tetraborate decahydrate, and sodium dodecyl sulphate from Aldrich (Madrid, Spain), and sodium chloride, ortophosphoric acid, disodium hydrogenphosphate dihydrate, potassium dihydrogenophosphate, and trisodium phosphate dodecahydrate were of analytical grade from Scharlau (Barcelona, Spain).

MFE C_{18} solid phase (particle diameter in the range of 45–55 μm and pore diameter 60 Å) was acquired from Análisis Vínicos (Tomelloso, Spain). The solid-phase was placed into 100 mm × 9 mm i.d. glass column fitted with a coarse frit (No. 3). Prior to use, the column was activated by washing successively with methanol (10 ml) and deionized water (10 ml).

The stir bars (Twister) were from Gerstel (Mülheim, Germany) with a length of 10 μm and coated with a 1 mm PDMS layer. The stir bars were preconditioned by sonication 5 min into a vial containing 15 ml of methanol. The procedure was repeated three times.

2.2. Apparatus

All capillary electrophoresis separations were performed on a Beckman P/ACE System MDQ (Fullerton, CA, USA) equipped with a diode array detector and System Gold software for data acquisition. Uncoated fused-silica capillaries purchased from Beckman were used. The dimensions of the capillary were 57 cm × 75 μm i.d. The effective length of the capillary was 50 cm from the injection end of the capillary.

The electrolyte pH was measured by a pH meter (Model DM-21, Digined, Sao Paulo, Brazil).

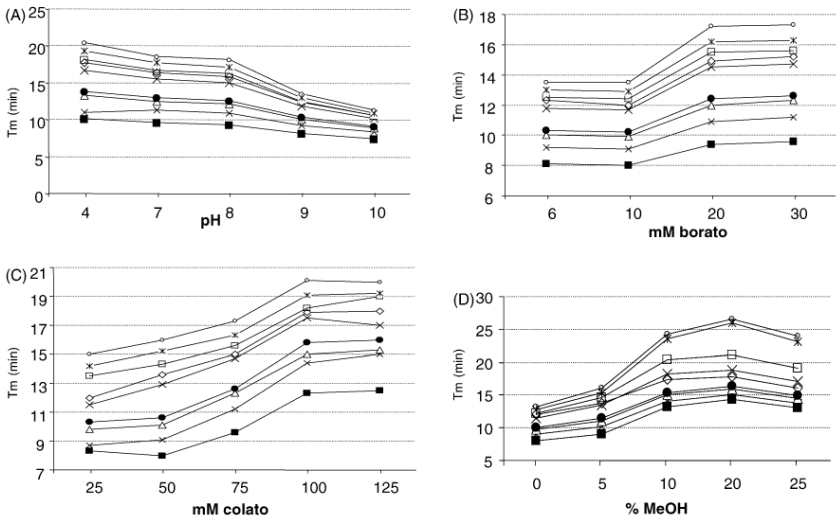

Fig. 2. Variations of the migration time of pesticides as a function of (A) pH, (B) electrolyte concentration, (C) sodium cholate concentration, and (D) percentage of methanol. Capillary: 57 cm × 50 μm i.d.; sample concentration: 10 μg ml^{-1}; detection wavelength 214 nm; other operating conditions: 20 kV, 25 °C. Curve identification: (■) flutriafol; (x) cyproconazole I; (Δ) cyproconazole II; (●) myclobutanil; (×) tebuconazole; (◊) acrinathrin; (□) bitertanol; (✱) fluodioxonyl; (◯) pyriproxyfen.

2.3. Preparation of running buffer and sample injection

Several parameters were studied during the optimization of the separation buffer, such as pH and concentration of the background electrolyte (BGE) ranging from 4 to 11 and from 3 to 30 mM, respectively with small increments. The presence of methanol as organic modifier, and the employment of different surfactants (cholic acid sodium salt and sodium dodecyl sulphate) at different concentrations were also tested. The buffer selected in MEKC was 6 mM sodium tetraborate decahydrate 75 mM cholic acid sodium salt prepared by diluting appropriate amounts in deionized water; complete dissolution was achieved by use of an ultrasonic bath.

The pH of a borate buffer was adjusted to the desired pH value by mixing various proportions of 6 mM sodium tetraborate solution with the same concentration of boric acid sodium hydroxide.

At the beginning of each day, the capillary was conditioned with 0.1 M sodium hydroxide for 30 min. Before each injection, the capillary was pre-washed for 2 min with deionized water, 2 min with 0.1 M NaOH, 2 min with deionized water, and 2 min with running buffer applying an overpressure of 20 psi (1 psi = 6894.76 Pa). Sample injections were carried out in the hydrodynamic mode over 5 s of 0.5 psi. A voltage of 30 kV was applied to keep the total current less than 80 μA at 25 °C. The detection wavelength was set at 214 nm.

2.4. Extraction procedure

Strawberry, grape, lettuce, and tomato samples were taken at some local supermarkets. A representative portion of the sample was chopped in a food chopper and 100 g portions were stored in closed containers in a freezer.

A portion of sample (between 5 and 15 g) spiked or not with pesticides was placed in a 250 ml glass beaker and mixed and homogenized in an ultrasonic bath for 15 min, therefore 10 ml of water–acetone (50:50, v/v) was added and homogenized 15 min more. The resulting suspension was filtered through a Buchner funnel and the cake filter was washed twice with 20 ml of acetone. The extract was evaporated by rotavapour to eliminate the acetone.

2.4.1. Solid-phase extraction

The aqueous sample extract was passed through the C_{18} column at a flow rate about 2 ml min^{-1}. Retained pesticides were eluted with 10 ml of dichloromethane. The eluent was collected in a graduated conical tube (20 ml) and concentrated, under stream of nitrogen, to dryness. After that, it was redissolved with 0.5 ml of buffer solution.

2.4.2. Stir-bar sorptive extraction

The filtrate was placed into a 50 ml glass beaker and stirring with the stir bar, for 4 h at 900 rpm. After the extraction, the stir bar was removed from the aqueous sample with a magnetic stirring bar. The analytes were desorbed into 2 ml vial

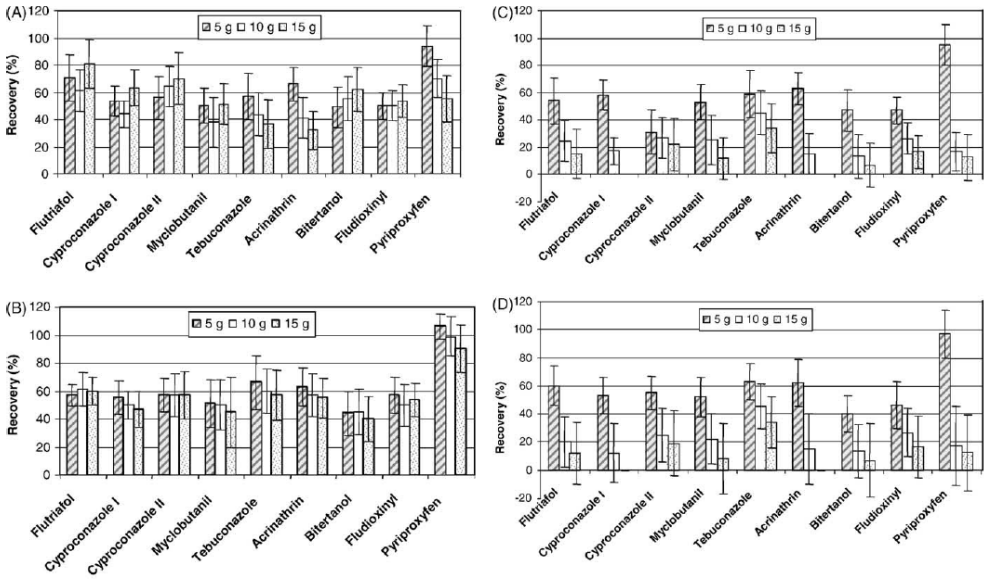

Fig. 3. Effect of sample amount on pesticide recoveries and R.S.D.s by SPE in (A) lettuce, (B) tomato, (C) strawberry, and (D) grape.

filled with 1 ml of methanol, concentrated to dryness under a stream of nitrogen, and redissolved with 0.5 ml of buffer.

3. Results and discussion

3.1. Optimization of the electrophoretic conditions

The optimization of the electrophoretic conditions is shown in Fig. 2. The pH of the separation buffer was varied between 4 and 10. All buffers were 6 mM borate and 75 mM sodium cholate. The migration times decreased with pH (Fig. 2A). The lower analysis time with appropriate resolution between analytes was at pH 10. Although the analysis time was higher, the pH 9.2 was selected as optimal because life-time of the capillary since higher pH degraded the silica inner wall of the capillary too quickly.

The influence of sodium tetraborate molarity on the pesticide separation was examined in the range 6–30 mM using a buffer 75 mM sodium cholate at pH 9.2 (Fig. 2B). For all the analytes, the migration times were almost the same until 10 mM, then increased until 20 mM maintaining again until 30 mM. Because of these results a sodium tetraborate concentration of 6 mM was selected.

Sodium dodecyl sulphate and sodium cholate were tested as surfactants. Peak resolution was much better using sodium cholate, which was selected for further experiments, this last surfactant achieved the differentiation of two isomers of cyproconazole, even through no chiral buffer is used. Different behavior was observed when the concentration of cholate was varied in the range 25–125 mM. Although the migration order of pesticide compounds with cholate concentration remains the same, the resolution varies considerably as cholate concentration increased from 25 to 75 mM. In addition, it can be see in Fig. 2C that the migration time increased, when cholate concentration varied from 75 to 125 mM. This behavior can be explained by both the increase of the ionic strength of the separation buffer and the greater interaction between analytes and micelles caused by the use of highest cholate concentrations. Therefore, the best separation was obtained with intermediate 75 mM cholate concentration in the buffer.

In order to find the best compromise between resolution and analysis speed, the effect of methanol concentration as organic modified was investigated, varying from 0 to 25% (see Fig. 2D). It was observed that increasing the percentage of methanol, the analysis time was increased and does not lead to real improvements in the resolution of the analytes. So, the use of organic modifier was avoided.

Table 1 outlines some analytical parameters of the proposed method. The complete separation of the eight studied pesticides was obtained with resolution values >1. The repeatability and reproducibility of migration times were fairly high; the R.S.D. values did not exceed 2.0 and 3.0%, respectively. The repeatability and reproducibility of the peak area were worst; the R.S.D. values for different analytes ranged

Table 1
Analytical parameters of MEKC separation (R.S.D., %, $n = 5$)

Pesticides	Resolution[a]	Average t_m (min)	Run-to-run R.S.D. (%)	Day-to-day R.S.D. (%)	Average area	Run-to-run R.S.D. (%)	Day-to-day R.S.D. (%)
Flutriafol	0	7.89	1.9	2.3	9,272	2.3	3.8
Cyproconazole I	5.3	9.01	1.6	2.1	3,382	3.4	2.9
Cyproconazole II	4.0	9.82	2.0	2.3	3,597	2.2	3.5
Myclobutanil	1.6	10.14	2.0	2.5	6,351	5.0	5.1
Tebuconazole	6.5	11.67	1.6	2.6	7,016	2.1	2.8
Acrinathrin	1.7	12.08	1.7	2.6	2,174	4.3	4.7
Bitertanol	1.1	12.37	1.5	2.6	20,857	4.6	5.0
Fludioximil	1.7	12.92	1.6	2.9	25,626	3.3	4.3
Pyriproxifen	1.6	13.46	1.6	3.0	14,478	5.3	5.8

[a] Resolution was calculated using the equation: $Rs = 2(t_1 - t_2)/(w_1 + w_2)$, where t_1 and t_2 are the migration times and w_1 and w_2 are the widths of the peak at base line.

from 2.1–5.3 and 2.9–5.8%, respectively. The limits of detection (LODs), estimated as three times signal-to-noise ratio were 0.1 μg ml^{-1} for bitertanol, fludioxonyl, and pyriproxyfen, and 0.5 μg ml^{-1} for the rest.

3.2. SPE procedure

Fruit and vegetable samples are matrices that do not allow direct SPE of pesticides. They must be extracted with polar solvents to have the pesticides in an aqueous extract. Acetone was selected as extraction solvent because it is easy of evaporating and avoids losses of pesticides.

The influence of the amount of sample was also examined to obtain the smallest LOQs. Different sample sizes were tested from 5 to 15 g. Fig. 3 shows the effect of the sample amount on pesticide recoveries for the four matrices tested. Two different behaviors were observed. The amount of lettuce and tomato samples could be increase to 15 g without important variations in recovery values and with R.S.D.s within the guidelines of the EU (<20%). On the contrary, the amount of grape and strawberry samples presented important influence on the recoveries because of the high viscosity of the extract. The maximum amount of these matrices that could be processed was 5 g to can recover all the analytes with R.S.D.s within the guidelines of the EU (see Fig. 3C and D). When the sample amount was increased the recoveries for most compounds diminished and R.S.D.s increased to unacceptable values.

In this study, LOQs were determined according to the guidelines of EU [23], as the lowest concentration for which acceptable recoveries (>50%) and repeatabilities (<20%) are obtained. Table 2 presents LOQs lower than 0.5 mg kg^{-1}, satisfying the EU [23], Codex Alimentarius [24], US Food and Drug Administration [25], and Spanish [26] MRLs. For lettuce and tomato samples, LOQs can be diminished until 0.2 mg kg^{-1} working with 15 g of samples instead of 5 g. Considering these results, the method is adequate to determine the studied pesticides in grapes, lettuce, strawberries, and tomatoes. Table 2 shows also the recoveries and precision obtained from spiked samples at LOQ levels. Average recoveries were between 40 and 106% and R.S.D.s ranged from 10 to 19%. Recoveries were similar in any of the matrix studied. Higher concentrations were also tested (data not shown) providing results in the same interval.

The good performance of the electropherograms obtained from SPE extracts using 15 g of sample is illustrated in Fig. 4. Unspiked samples do not show peaks from the matrix that can interfered with the studied compounds. The lettuce sample presents pyriproxyfen (Fig. 4B) as it was confirmed by liquid chromatography–mass spectrometry (LC–MS) using a HP 1100 system equipped with a APCI interface, using the standard source conditions, in positive ionization (PI) mode. The analysis was carried out in selected ion monitoring mode (SIM) selecting three characteristic ions of pyriproxyfen at m/z 322, 227, and 185 and using a fragmentor voltage of 100 V, according to a method previously reported

Table 2
Accuracy and precision at LOQ (amount of sample processed 5 g) SPE and MRLs established by the Spanish legislation [26]

Pesticide	Concentration (mg kg^{-1})	Recovery, % (R.S.D., %, $n = 5$)				*MRL*
		Lettuce	Tomato	Grape	Strawberry	
Flutriafol	0.5	62 (17)	60 (14)	57 (8)	54 (17)	0.5–2
Cyproconazole I	0.5	58 (11)	53 (13)	55 (12)	58 (14)	0.05–1
Cyproconazole II	0.5	60 (16)	55 (12)	57 (12)	31 (18)	0.05–1
Myclobutanil	0.5	56 (13)	52 (14)	51 (17)	53 (15)	0.02–1
Tebuconazole	0.5	60 (17)	63 (13)	66 (14)	59 (15)	1–5
Acrinathrin	0.5	66 (12)	62 (17)	63 (14)	63 (19)	0.2–1
Bitertanol	0.5	49 (15)	40 (13)	44 (16)	47 (13)	2–3
Fludioxinyl	0.5	50 (10)	46 (17)	57 (13)	47 (19)	1–2
Pyriproxyfen	0.5	94 (15)	97 (17)	106 (9)	95 (18)	0.05–1

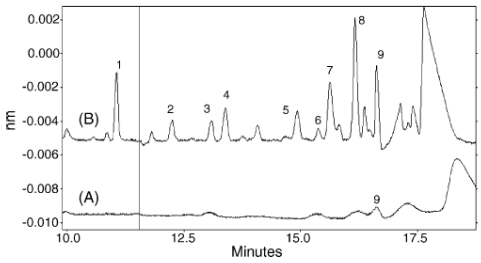

Fig. 4. Electropherograms of SPE extracts from 15 g sample of (A) lettuce that contains pyriproxyfen at 0.2 mg kg^{-1} sample, and (B) lettuce sample spiked with the pesticides at 0.5 mg kg^{-1} levels. Peak identification: 1, flutriafol; 2, cyproconazole I; 3, cyproconazole II; 4, myclobutanil; 5, tebuconazole; 6, acrinathrin; 7, bitertanol; 8, fludioxonil; 9, pyriproxyfen.

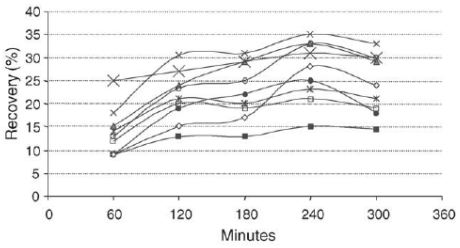

Fig. 5. Effect of the extraction time on the recovery (each solution was added of 40% NaCl). Curve identification as in Fig. 2.

[12]. The identification and further confirmation of this pesticide showed the suitability of the method to determine these residues in real samples and the difficulty to find control samples without residues.

3.3. SBSE procedure

The optimum SBSE desorption conditions were obtained by exposure of the stir bar to 1 ml of methanol for 15 min using a 1.5 ml volume glass vial; this volume enables the stir-bar to be completely immersed in the solvent. Those parameters were selected not only due to the higher quantitative desorption obtained, but also because of the minimum carryover observed under this experimental conditions.

The matrix pH effect on the extraction efficiency of the pesticides was tested using pH values between 4 and 9 adjusted with potassium phosphate buffers. This range of pH values does not have influence in the extraction efficiency, and all subsequent analyses were performed without pH modification.

As expected, the addition of NaCl to the samples had a significant influence on the amount of pesticides adsorbed on the stir-bar. The addition of salt to the matrix altering the ionic strength and consequently decreasing the solubility of the pesticides in solution, can favor adsorption onto the stir-bar. The effect of NaCl concentration was investigated in the range 10–40%. The saturated solution (40% of NaCl) provided the best recoveries.

Fig. 5 shows the time profile of the extraction for the pesticides. The extraction equilibrium time was reached at 4 h for all studied pesticides. So far, it has been reported that the shorter extraction, which could be of the order of minutes, is one of the advantages of the SBSE [9,11]. However, for the studied pesticides, extraction times of over 240 min were required, enlarging the analysis more than practical convenience.

The influence of the matrix on the extraction efficiency of the SBSE was negative for all compounds. A sample size of 5 g provided acceptable recoveries whereas higher amounts avoid the extraction of the flutriafol and cyproconazole. The quantity of matrix that can be used (no more than 5 g) is one limitation of this procedure.

Specificity of the method is demonstrated by representative electropherograms of tomato sample in Fig. 6. Blank tomato sample showed no significant interference at the retention times of the analytes.

The linearity was determined using spiked samples between 1 and 100 mg kg^{-1}. The evaluated interval was linear with correlation coefficients higher than 0.996. The precision and the recovery of the studied pesticides at LOQ levels for all the matrices tested are outlined in Table 3. The recoveries

Table 3
Accuracy and precision at LOQ (amount of sample processed 5 g) by SBSE

Pesticide	Concentration (mg kg^{-1})	Recovery, % (R.S.D., %, $n=5$)			
		Lettuce	Tomato	Grape	Strawberry
Flutriafol	1	12 (14)	13.4 (8)	17 (7)	14.6 (16)
Cyproconazole I	1	25 (9)	30.5 (3)	28 (5)	24 (17)
Cyproconazole II	1	29 (10)	33 (3)	34 (15)	31 (3)
Myclobutanil	1	20 (8)	24 (16)	26 (9)	19 (13)
Tebuconazole	1	32 (5)	35 (11)	25 (4)	31 (8)
Acrinathrin	1	47 (4)	45 (3)	43 (6)	41 (9)
Bitertanol	1	26 (13)	22 (4)	24 (4)	21 (4)
Fludioxinyl	1	22 (15)	24 (12)	27 (12)	23 (11)
Pyriproxyfen	1	24 (9)	23 (6)	32 (8)	33 (12)

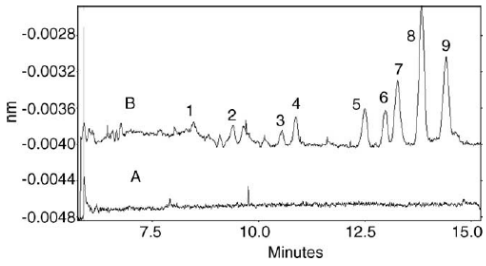

Fig. 6. Electropherograms of SBSE extracts of (A) tomato blank sample, and (B) tomato sample spiked at 1 mg kg^{-1} of each pesticide. Peak identification as in Fig. 4.

Table 4
Methods performance comparison

	SPE	SBSE
Spiking concetrations (mg kg^{-1})	0.2–50	1–50
Accuracy (% recovery)	40–106	12–47
Repeatability (R.S.D., %)	8–19	3–17
Linearity (r^2)	>0.994	>0.996
Sensitivity (LOQ)	0.3–0.5	1
Analysis time (h)	1.5	5

obtained were between 12 and 47% and the R.S.D.s ranged from 3 to 17%. The LOQ was 1 mg kg^{-1} for all the studied pesticides.

3.4. Comparison of methods

Analytical parameters of both procedures are compared in Table 4. Although SBSE extraction is less reliable than SPE to carry out the routine analyses, this procedure also presents some advantages over SPE such as better precision and cleaner electropherograms (as can be deduced from comparing Table 4 and Figs. 4 and 6).

SPE recoveries are, at least, twice higher than those obtained by SBSE. In addition, SPE showed better LOQs (twice) that can be improved for some matrices processing larger amounts of samples. The LOQs obtained mark the difference between both procedures because SPE combined with MEKC–UV achieves the detection of the studied pesticides at levels lower than MRLs established by the Spanish and EU legislations. Unfortunately, LOQs obtained by SBSE are too high to met the MRLs. Because of this, the procedure is only useful in some cases. Other advantage of SPE, that consolidates it as that of choice, is speediness (the complete analysis of each sample to obtained results are ca. 1.30 h in front of 5 h required by SBSE). Disadvantages described in the literature for the SPE such as breakthrough and plugging [17,20] have not been observed in the present study, after optimization of the conditions.

4. Conclusions

Comparing the SPE with SBSE, the first one is more sensitive, robust, and rapid than SBSE as well as it provides higher extraction efficiencies and sample throughput. The results of this work demonstrate that MEKC is useful, simple, and rapid for separating, identifying, and determining eight pesticides with sufficient sensitivity in fruits and vegetables. The LOQs show that the developed method can be used to detect the pesticides at concentrations below the MRLs established by the European Union, Spanish legislations, and other recommendations. SPE combined with MEKC achieves the analysis of a large number of fruit and vegetable samples in a short period of time attaining a fast and inexpensive method for routine pesticide residue monitoring in laboratories.

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References

[1] L. Nasreddine, D. Parent-Massin, Toxicol. Lett. 127 (2002) 29.
[2] F.E. Ahmed, Trends Anal. Chem. 20 (2001) 649.
[3] W.G. Fong, H.A. Moye, J.N. Seiber, J.P. Toth, Pesticide Residues in Food, Wiley–Interscience, New York, 1999, p. 1335.
[4] D. Stajnbaher, L. Zupancic-Kralj, J. Chromatogr. A 1015 (2003) 185.
[5] F.J. Schenck, S.J. Lehotay, V. Vega, J. Sep. Sci. 25 (2002) 883.
[6] M. Michel, B. Buszewski, J. Liq. Chromatogr. Relat. Technol. 25 (2002) 2293.
[7] V.J. Barwick, S.L.R. Ellison, S.J. Lacey, C.R. Mussell, C.L. Lucking, J. Sci. Food Agric. 79 (1999) 1196.
[8] C. Blasco, M. Fernández, Y. Picó, G. Font, J. Chromatogr. A 1030 (2004) 77.
[9] P. Sandra, B. Tienpont, F. David, J. Chromatogr. A 1000 (2003) 299.
[10] C. Bicchi, C. Cordero, P. Rubiolo, P. Sandra, Eur. Food Res. Technol. 216 (2003) 449.
[11] P. Sandra, B. Tienpont, J. Vercammen, A. Tredoux, T. Sandra, F. David, J. Chromatogr. A 928 (2001) 117.
[12] C. Blasco, G. Font, Y. Picó, J. Chromatogr. A 970 (2002) 201.
[13] Y. Picó, R. Rodríguez, J. Mañes, Trends Anal. Chem. 22 (2003) 133.
[14] F. Menzinger, Ph. Schmitt-kopplin, D. Freitag, A. Kettrup, J. Chromatogr. A 891 (2000) 45.
[15] Z. El Rassi, Electrophoresis 20 (1999) 3280.
[16] R. Rodríguez, J. Mañes, Y. Picó, Anal. Chem. 75 (2003) 452.
[17] M. Khrolenko, P. Dzygiel, P. Wieczorek, J. Chromatogr. A 975 (2002) 219.
[18] E.W.J. Hooijschuur, C.E. Kientz, J. Dijksman, U.A.Th. Brinkman, Chromatographia 54 (2001) 295.
[19] R. Rodríguez, Y. Picó, G. Font, J. Mañes, J. Chromatogr. A 949 (2002) 359.
[20] R. Rodríguez, Y. Picó, G. Font, J. Mañes, J. Chromatogr. A 924 (2001) 387.

[21] M. Molina, M. Silvia, Electrophoresis 21 (2000) 3625.
[22] J. Hau, S. Riediker, N. Varga, R.H. Stadler, J. Chromatogr. A 878 (2000) 77.
[23] The European Union "on-line" http://europa.en.int/, 2004.
[24] Codex Alimentarius, http://www.fao.org/, 2004.
[25] Food and Drug Administration of the United States, http://www.cfsan.fda.gov, 2004.
[26] Ministerio de Agricultura, Pesca y Alimentación, http://www.mcx.plaguicidas.plagas/, Límites Máximos de Residuos (MRLs) Oficiales, 2004.

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Quantitative analysis of six pesticides in fruits by capillary electrophoresis-electrospray-mass spectrometry

A method to identify and quantify six pesticide residues – dinoseb, pirimicarb, procymidone, pyrifenox, pyrimethanil, and thiabendazole – in peaches and nectarines using capillary electrophoresis-electrospray ionization-quadrupole ion trap-tandem mass spectrometry (CE-ESI-MS/MS) is described. Separation was carried out using a buffer of 0.3 M ammonium acetate at pH 4 with 10% methanol. Pesticide residues present in peach and nectarine samples were preconcentrated by solid-phase extraction using C_{18}, eluted with CH_2Cl_2, concentrated to dryness, and redissolved in buffer to obtain lower detection limits. The recoveries of the analytes ranged from 58 to 99% and the relative standard deviations were 9 to 19%. Under optimized CE-MS/MS conditions the minimum detectable levels for the six pesticides in spiked peach samples were between 0.01 mg/kg for pirimicarb and 0.05 mg/kg for procymidone with pressure injection of 50 mbar for 5 s (5 nL) at a signal-to-noise ratio of 3, which constitutes a severalfold increase in sensitivity compared to CE-MS, using a single quadrupole, and to conventional CE-UV. The potential of the method was demonstrated by analyzing different samples taken from regional agricultural cooperatives. The pesticides most often detected were thiabendazole and procymidone.

Keywords: Capillary electrophoresis / Fruits / Mass spectrometry / Pesticides / Quadrupole ion-trap mass spectrometer
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1 Introduction

Pests and diseases destroy up to one-third of the crops during growth, harvest, and storage, therefore pesticides are invaluable for increased agricultural production. However, the inherent toxicity and ubiquity of pesticides have spawned a research and development effort on part of government, industry, and non-profit institutions to develop analytical methods for detecting pesticide residues [1, 2]. The challenge is to balance a reliable, high-quality food supply with the need to protect the consumer from unnecessary exposure to toxic chemicals [3]. The need for fast and efficient analytical procedures within the food safety sector is growing rapidly [4]. To guarantee the high analytical quality required by the regulatory agencies, all methods need to be fully validated, which puts stringent demands on their stability and ruggedness [4, 5]. In combination with the introduction of new tools, *e.g.*, high-throughput screening in residue-monitoring programs, a constant search for new analytical techniques is anticipated [6]. One of these new techniques concerns capillary electrophoresis (CE), which shows high efficiency in separating pesticides [7, 8]. The practical application of CE for pesticide residue analysis in food is, however, still fairly limited because some of the CE shortcomings, such as the low sensitivity achieved or the lack of specificity of the UV detector, have prevented the speedy introduction in a routine analytical environment [9, 10]. The main reported solutions are the CE combination with different off- and on-column trace-enrichment schemes (stacking, isotachophoresis, solid-phase extraction (SPE), and solid-phase microextraction (SPME) as well as their coupling to selective detection systems, such as mass spectrometry) [7–11].

The use of CE coupled with electrospray ionization-mass spectrometry (CE-ESI-MS) has evolved as a useful tool to analyze polar pesticides in fruit and vegetable samples [12]. While CE confers high resolving power and separating speed to the analysis, MS provided high sensitivity and selectivity. The major advantages to combine CE-MS are an extremely high resolution and that almost any molecule can be infused into MS. Previous works demonstrated that CE-MS techniques were useful for the

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analysis of pesticides [13–16] but the definitive identification of certain pesticide residues by CE-MS has been somewhat limited by known and unknown compounds that provided isobaric interferences or by multiple-component spectra, which are definitively useless [12]. Tandem mass spectrometry (MS/MS or MS^n) uses multiple stages of mass analysis that allow to preselect an ion and analyze the induced fragments obtained by collision with an inert gas. Recently, CE-ESI-MS/MS has emerged as a powerful alternative for characterizing food toxicants successfully applied to the analysis of tetracycline antibiotics and heterocyclic amines in food [17, 18], and quaternary ammonium herbicides in water [19]. To date, no report describing the application of CE-ESI-MS/MS for analyzing pesticide residues in fruits has been published.

In this study, a CE-ESI-MS/MS method for determining six pesticides – dinoseb, pirimicarb, procymidone, pyrifenox, pyrimethanil, and thiabendazole – is described. The proposed approach was applied to the analysis of pesticide residues in peaches and nectarines. The detected peaks were selectively identified based on their MS^n spectra and their amounts were readily quantified by isolating the main ion. The performance of the method is compared with those obtained using CE-UV and CE-ESI-MS with the single quadrupole.

2 Materials and methods

2.1 Instrumentation

A P/ACE MDQ capillary electrophoresis system (Beckman Instruments, Fullerton, CA, USA), equipped with a diode array detector (DAD) and a System Gold software for data acquisition, was employed. Experiments with UV detection were performed using the conventional P/ACE System MDQ cartridge and uncoated fused-silica capillaries, purchased from Beckman with 57 cm total length, 50 cm effective length, 75 μm ID, and 375 μm OD. Two different mass spectrometers were handled: an HP1100 single quadrupole mass spectrometer (Palo Alto, CA, USA), equipped with an HP Chem Station, and a quadrupole ion-trap (QIT-MS), consisting of an Esquire3000 Ion Trap LC/MS(n) system (Bruker Daltonik, Bremen, Germany) and a data acquisition/processing Daltonic Esquire Control Software System 3.0. The coupling of CE to MS was carried out using a G1607 Agilent CE-MS sprayer kit and a special external detector adapter (EDA) cartridge (Beckman). The sprayer kit includes a triaxial flow assembly for the electrospray interface (ESI) that mixes the capillary effluent with a suitable sheath liquid to establish electrical contact at the probe tip and nebulizes both by N_2 stream. The coaxial sheath liquid was delivered by a Hewlett-Packard 1100 Series pump, which was connected to the outlet to a sheath flow splitter in the ratio 1:100 (also incorporated in the Agilent CE-MS sprayer kit). The EDA cartridge is designed to allow the capillary to exit the P/ACE MDQ system instrument for connection to the mass spectrometers. The accommodated fused-silica capillary (75 cm total length, 50 cm thermostated length, 25 cm room temperature length, 75 μm ID, 375 μm OD) was from Supelco (Madrid, Spain). The polyamide coating was removed at the end of the capillary (*ca.* 5 mm) and the capillary was protruded 0.1 mm of the sprayer to maintain a stable electrospray. A single-syringe infusion pump (Cole-Parmer, Vernon Hills, IL, USA) equipped with 240 or 500 μL Hamilton gastight syringes (Hamilton, Bonaduz, Switzerland) were used to optimize the QIT-MS lenses and block voltages and the ion fragmentation conditions. The solid phase was placed into a 100 × 9 mm ID glass column fitted with a coarse frit (No. 3) with a standardized hollow key (14/23) and a standard ground cone (29/32) that can be combined with several laboratory glassware, such as funnels, kitasato flasks, or vacuum adapters made-to-measure by Marcelo Glassware (Valencia, Spain). Evaporation of acetone was accomplished with a model R200 Rotavapor (Büchi, Flawill, Switzerland). Evaporation of dichloromethane was performed on a TurboVap^{LR} Concentration Work Station from Zymark (Hopkinton, MA, USA).

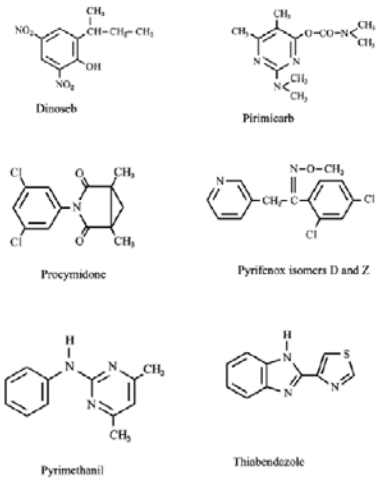


Figure 1. Chemical structure of the studied compounds.

2.2 Chemicals

Dinoseb, pirimicarb, procymidone, pyrimethanil, pyrifenox, and thiabendazole were purchased from Riedel de-Haën (Seelze, Germany). Their structures are shown in Fig. 1. Individual stock solutions of pesticides at a concentration of 1 mg/mL were prepared in methanol and stored in glass-stopper bottles at 4°C. The working mixture was prepared by diluting stock solutions with the running buffer, just prior to injection. All the solutions were passed through a 0.45 μm cellulose filter from Scharlau (Barcelona, Spain). HPLC-grade methanol and organic trace analysis-grade dichloromethane were obtained from Merck (Darmstadt, Germany) and acetone from Promochem (Wesel, Germany). Deionized water (< 18 MΩ cm resistivity) was used from the Milli-Q SP Reagent Water System (Millipore, Bedford, MA, USA). All other reagents were obtained from Fluka (Madrid, Spain) and were of analytical or reagent-grade. MFE C_{18} solid phase (particle diameter in the range of 45–55 μm and pore diameter 60 Å) was acquired from Análisis Vínicos (Tomelloso, Spain).

2.3 CE separation conditions

Details of the experimental and instrumental parameters of CE-UV, CE-MS, and CE-MS/MS are listed in Table 1. Several conditions were studied for the optimization of the separation buffer, such as pH and concentration of the background electrolyte (BGE) ranging from 4 to 11 and from 3 to 300 mM, respectively, with small increments. The presence of methanol and acetonitrile as organic modifiers was also tested.

2.4 CE-ESI-MS and CE-ESI-MS/MS conditions

The sheath liquid was delivered at a flow rate of 0.5 mL/min (taken into account that a split ratio of 1:100 means that the flow at the probe tip was 5 μL/min). The mass spectra in the HP1100 were recorded either in positive or negative ion modes using full-scan (from *m/z* 150 to 350 with a scan time of 0.75 s) or selected ion monitoring (SIM). $[M+H]^+$ molecules at *m/z* 202 for thiabendazole, *m/z* 239 for pirimicarb, *m/z* 295 for pyrifenox, *m/z* 200 for pyrimethanil, and *m/z* 284 for procymidone with a gain of 3 and dwell time of 114 ms or $[M-H]^-$ molecule at *m/z* 240 for dinoseb with a gain of 3 and a dwell time of 400 were monitored. The QIT-MS was tuned for each compound optimizing the voltages of the lenses in the ExpertTune mode of the Daltonic Esquire Control software whilst infusing a standard solution prepared in the running buffer (1 μg/mL) by a syringe pump at a flow rate of 0.005 mL/min. The mass spectrometer was operated in full-scan and multiple reaction monitoring (MRM) modes.

Table 1. Experimental and instrumental parameters for CE

	CE-UV	CE-MS	CE-MS/MS
Capillary conditioning			
At the beginning of each day	0.1 M NaOH for 30 min applying and overpressure of 20 psi (1 psi = 6894.76 Pa)		
Before each injection	Prewashed for 2 min with deionized water, 2 min with 0.1 M NaOH, 2 min with deionized water, and 2 min with running buffer applying an overpressure of 20 psi		
Instrument conditions			
Buffer	0.3 M ammonium acetate-acetic acid, pH 4, in 10% methanol		
Temperature		25°C	
Voltage (average current)		30 kV (< 50 μA)	
Injection		0.5 psi for 5 s	
Data recorder	Scan wavelength from 190 to 280 nm		Full scan
	Single wavelength 214 nm	SIM	MRM
CE-ESI-MS interface			
Sheath liquid composition		0.3 M ammonium acetate-acetic acid, pH 4, in 10% methanol	
Sheath liquid flow-rate			5 μL/min
ESI spray chamber			
Polarity			Positive/negative
Drying gas flow			7 L/min
Drying gas temperature			250°C
Nebulizer gas pressure			60 psi
Voltage			4000 V
Fragmentor voltage		70 V	

Table 2. Defined time windows and transitions used for quantification by MS^n operation

	Time windows definition (min)					
	0–20			20–24	24–26	26–35
	Thiabendazole	Pyrifenox	Pirimicarb	Pyrimethanil	Procymidone	Dinoseb
MS^2 transitions (*m/z*)	202 → 175	295 → 264	239 → 182	200 → 183	284 → 255	240 → 194
Width (*m/z*)	1.0	4.0	1.0	1.0	4.0	1.0
Cut-off (*m/z*)	100	100	100	100	100	100
Amplitude (V)	2.0	1.5	1.6	2.4	1.3	2.0
MS^3 transitions (*m/z*)	175 → 132	264 → 230	182 → 132	183 → 168	255 → 213	194 → 164
Width (*m/z*)	1.0	4.0	1.0	1.0	4.0	1.0
Cut-off (*m/z*)	100	100	100	100	100	100
Amplitude (V)	2.0	2.3	2.0	2.4	1.8	2.0

The trap parameters were selected in ion charge control mode using a target of 100 000, maximum accumulation time of 50 ms, and rolling average set at 2. Collision-induced dissociation (CID) was carried out on the ion of interest by collisions with the helium background gas present in the trap for 40 ms. In these experiments, the protonated or deprotonated pesticide was subjected to CID to produce a first set of fragment ions MS/MS or MS^2, one of these fragment ions was isolated and refragmented to give the next set of ions MS^3, and so on. The fragmentation steps for each compound were optimized by visualizing the changes in the intensities of the fragments ions, whereas the fragmentation cutoff and the fragmentation amplitude were manually varied. The optimized parameters are listed in Table 2.

2.5 Extraction procedure

Peach and nectarine samples were taken at local agricultural cooperatives. A representative portion of the sample was chopped in a food chopper and 100 g portions were stored in closed containers in a freezer. A portion of sample (5–15 g) spiked or not with pesticides was placed into a 250 mL glass beaker, mixed, and homogenized in an ultrasonic bath for 15 min. Then 10 mL water-acetone (50:50 v/v) was added and homogenized for 15 min. The resulting suspension was filtered through a Büchner funnel and the cake filter was washed twice with 20 mL water-acetone. The extract was evaporated by a rotavapour to eliminate the acetone. The C_{18} solid-phase was conditioned with 10 mL methanol and 10 mL distilled water. The aqueous sample extract was passed through the C_{18} column at a flow-rate of about 2 mL/min. Retained pesticides were eluted with 10 mL dichloromethane. The eluent was collected in a graduated conical tube (20 mL) and concentrated, under stream of nitrogen, to dryness. After that, it was redissolved with 0.5 mL buffer solution.

3 Results and discussion

3.1 CE-ESI-MS and CE-ESI-MS/MS optimization

The pH and ionic strength of the background electrolyte, the addition of organic modifiers, and the capillary temperature were evaluated using CE-UV to obtain the best separation of the six pesticides. A volatile buffer of low conductivity is required to avoid plugging of the dielectric capillary between the spray chamber and the mass spectrometer, as well as to obtain a stable electrospray. Ammonium acetate/acetic acid buffer was tested at four different pHs, 3, 4, 5, and 7. As CE is mainly governed by the charge/mass ratio of each analyte, the increase in pH decreases the migration time. The best arrangement between peak resolution and analysis time was obtained using pH 4.

The influence of buffer concentration on the migration time and resolution of the six compounds was studied with ammonium acetate-acetic acid buffers from 0.01 to 0.6 M at pH 4. An increment in the ionic strength of the carried electrolyte by increasing the buffer concentration reduced the adsorption of the compounds on the wall of the capillary and improved the peak shapes. The studied pesticides were resolved only with buffer of high molarity (> 0.2 M). The molarity of 0.3 M was selected to achieve a good compromise between compound separation and electrical conductivity of the buffer. The capillary current

obtained using CE-UV was 180 μA (300 μA is the maximum current value suitable for the instrument) and coupling CE-MS was 87 μA.

The effect of adding an organic modifier to the background electrolyte on pesticide separation was checked using different concentrations of methanol and acetonitrile, in the range of 5–30%. Organic solvents decrease the EOF prolonging the migration time, and can produce solvation effects, alteration of p*K*a, and improvement in the solubility of hydrophobic analytes. Methanol provided better separations than acetonitrile. The increment of methanol percentage increased the resolution between peaks from 5 to 15% although the analysis times also increased. Higher percentage of methanol did not change the resolution but enlarged the analysis time because longer migration times result in wide peaks. 10% methanol improved the resolution between peaks without prolonging unreasonably the analysis time.

The migration time of the analytes decreased with an increase of the capillary temperature (15°C–35°C) because of the decrease in the distribution coefficient as well as of the buffer viscosity. The resolution between the peaks did not improve with low temperatures (< 25°C) because the absolute rise of the temperature of the buffer is not detrimental to a separation if the Joule heating that provides wider peaks is not excessive. Temperatures of 30°C–35°C reduced resolution (especially between the last two peaks) because the Joule effect is higher, causes viscosity differences in the buffer, and gives rise to zone deformation. A capillary temperature of 25°C was selected.

The ES-MS instrumental parameters, such as sheath liquid composition, sheath liquid flow-rate, drying gas flow-rate, and length of the CE capillary protruding from the sheath liquid tube, were optimized to obtain the higher response. The composition of the sheath liquid is critical to the fulfilment of the CE-MS system since it closes the electrical circuit between the CE and the electrospray source. The effect of the methanol percentage and of the buffer concentration was tested. The best results were obtained when the composition of the sheath liquid was the same as that of the running buffer. These results are in agreement with those already reported in the literature for similar connections [14, 15]. The sheath liquid flow-rate was varied from 3 to 15 μL/min. A flow-rate of 3 μL/min affected negatively the spray stability resulting in a low signal intensity, whereas the higher flow-rates tested did not provide higher peak areas, and the peak shape of procymidone was worst (peak-broadening is observed). A flow-rate of 5 μL/min provided the best results. In addition, a maximum response of the analytes was obtained when 7 L/min drying gas was used. At flow-rates lower or higher, the response decreased because of the electrospray instability. The distance that the CE capillary protrudes from the electrospray needle was also optimized because it may seriously affect the performance of the system. This distance was varied between 0.05 and 1.5 mm. Maximum response was achieved when the CE capillary only protrudes 0.1 mm from the sheath liquid tube.

Figure 2 shows the UV and MS electropherograms for the six pesticides under the best conditions. Successful coupling of CE with ESI-MS was accomplished for the separation of the six pesticides, and provided very similar separations compared to those achieved by CE-UV. The data reported in the published literature are quite polemic;

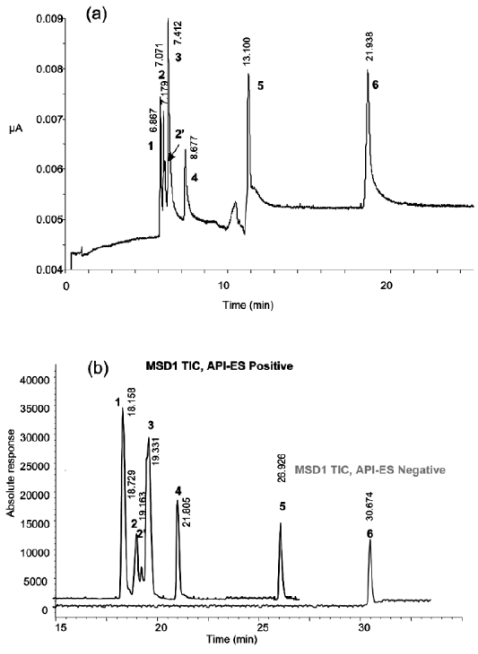


Figure 2. Electropherograms of a pesticide standard mixture by (A) CE-UV and (B) CE-ESI-MS using a single quadrupole. Conditions: capillary, fused silica (50 cm thermostated length, 75 μm ID); electrolyte, 0.3 M ammonium acetate/acetic acid with 10% methanol; voltage, 30 kV; injection, 5 s at 50 mbar; temperature, 25°C; MS detection, in SIM mode. Concentrations: 25 and 5 μg/mL of each for CE-UV and CE-MS, respectively; other conditions see in Section 2. Peak identification: (1) thiabendazole; (2,2') pyrifenox; (3) pirimicarb; (4) pyrimethanil; (5) procymidone; (6) dinoseb.

Table 3. Reproducibility, linearity, and sensitivity of pesticide detection by CE-UV and CE-MS using the single quadrupole

	CE-UV				CE-MS			
	RSD (n = 10) (%)		Linearity correlation	LOD (μg/mL)	RSD (n = 10) (%)		Linearity correlation	LOD (μg/mL)
	Migration time	Peak area			Migration time	Peak area		
Thiabendazole	1.2	3.9	0.998	2	1.5	5.0	0.998	0.05
Pyrifenox	1.3	5.0	0.996	2	1.4	6.2	0.992	0.5
Pirimicarb	1.3	4.5	0.994	1	1.7	8.7	0.992	0.05
Pyrimethanil	1.4	4.7	0.990	2	1.6	7.9	0.995	0.2
Procymidone	1.6	5.2	0.995	1	2.3	6.5	0.993	0.2
Dinoseb	2.7	5.8	0.992	1	3.0	8.9	0.990	0.5

some authors stated that separation efficiencies obtained by CE-ESI-MS are clearly lower than those achieved by CE-UV [13–15, 20] whereas others established that the separation efficiencies are not necessarily inferior compared with those obtained using CE-UV [21]. The explanation of this contradiction could be that the coolant tube connector where the capillary is installed in the EDA cartridge, which has only 15 cm, has been changed in the present study by one of the same longitude than that used in the conventional cartridge. This confirms the general justification that the loss of resolution in the separation when an MS detector is used is caused by the small portion of the capillary thermostated. The tailing of the peaks observed using a UV detector is mainly because the capillary is older than that used with the MS detector, which originates in stronger adsorption on the wall silica capillary, producing tailing peaks.

Differences in migration times are because of the distinct interfacing between CE-UV and CE-MS. The capillary used for CE-UV has only 57 cm (50 cm effective legth) whereas using an external mass spectrometer detector commonly requires considerably longer capillaries with an overall length of 75 cm (even through only 50 cm are thermostated). As the mobility of an ion is proportional to the applied field strength, the latter is inversely proportional to the capillary length, which means that the migration time will roughly double if the capillary is double.

However, there are several other factors that may interfere and alter the separation efficiency and migration times as well, such as moving ionic boundaries, buffer depletion (towards the end of the CE capillary in a hot ESI source), penetration of the sheath liquid by diffusion into the CE capillary, electrode reaction at the ESI needle which may result in pH modification, suction effect induced by the sheath gas flow used in addition to the sheath liquid flow, and alteration of electrosmotic flow (EOF) by the radial electric field which penetrates across the fused-silica capillary wall. Additionally, the separation efficiency may also be compromised by the data acquisition speed, the response time of the mass spectrometer, the liquid sheath flow rate, and the relative positioning of the CE capillary and the ESI needle [22].

The backpressure effect (siphoning of the sample) is caused by the pressure difference between the inlet and the outlet (commonly, the outlet can present a slight overpressure caused by the parameters of the vent system, *e.g.*, drying gas) [22]. The overpressure is detected by a delay in retention time and analyte signal loss. The solution is kept at both capillary ends at approximately the same height, that is ±0.5 cm around the sprayer's levels using a heath adjustable table placed under the CE instrument. However, typically this position was reach placing both instruments in the same bench without more adjustment. The appropriate values of RSDs obtained for retention time and peaks areas are a clear evaluation of the absence of backpressure (Table 3). The coupling of CE to the QIT-MS was performed in the same way because the electrospray sprayer of the Agilent system has the same design than that of the Bruker Daltonik system without any important difference in the mechanism of electrical potential application.

3.2 MS and MS/MS spectrum of pesticides

The only ion observed was the protonated $[M+H]^+$ or deprotonated $[M-H]^-$ molecules in full-scan MS mode, which is consistent with the "soft" nature of the ESI process. The voltages of the lenses were adjusted to obtain the maximum response for the protonated or deprotonated molecule of each studied compound, which were chosen as precursor ions in further MS^n experiments. In

addition, a reliable way of obtaining structural information is to perform tandem MS experiments on specific ions of interest. CID of $[M+H]^+$ or $[M-H]^-$ ions (MS^2) and CID of the main fragment ion (MS^3) often yield fragments ions formed by fragmentation of the lateral chains in the molecular structure or by the opening of the heterocyclic rings.

A three-stage mass analysis of the protonated thiabendazole at *m/z* 202 illustrates a dominant fragmentation pathway: *m/z* 202 → *m/z* 175 → *m/z* 131. MS^2 of thiabendazole is characterized by the loss of HCN from the thiazolic ring. It results in an abundant product ion at *m/z* 175, and a further loss of CS from the fragmentation of thiazolic ring leads to the little abundant ion of *m/z* 131. Further fragmentation of *m/z* 175 produces an abundant *m/z* 131 ion that confirms this fragmentation pathway. This fragmentation is almost the same as reported for LC-MS using the ion trap [23]. The MS^2 spectrum of $[M+H]^+$ for pyrifenox evidences the cleavage of the oxime bond, resulting in an intense signal at *m/z* 265 that corresponds to $[M+H-CH_3OH]^+$. Further fragmentation of this ion was verified by MS^3, showing an intense signal at *m/z* 230 formed by the loss of HCl molecule. Pirimicarb is a pesticide representative of the class of carbamates. Its MS^2 spectrum presents two main product ions at *m/z* 182 and 195, derived from the neutral loss of the CH_3NCO and $HN(CH_3)_2$ groups, respectively. In the further step (MS^3 of the ion at *m/z* 182), one product ion is formed at *m/z* 137 resulting from the loss of the group $HN(CH_3)_2$ located in the *m*-position at the carbamic group. The fragmentation of the protonated molecule of pyrimethanil exhibits major ions at *m/z* 184, formed through the loss of methane. MS^3 of *m/z* 184 leads to the formation of *m/z* 167 *via* the loss of the other methane group.

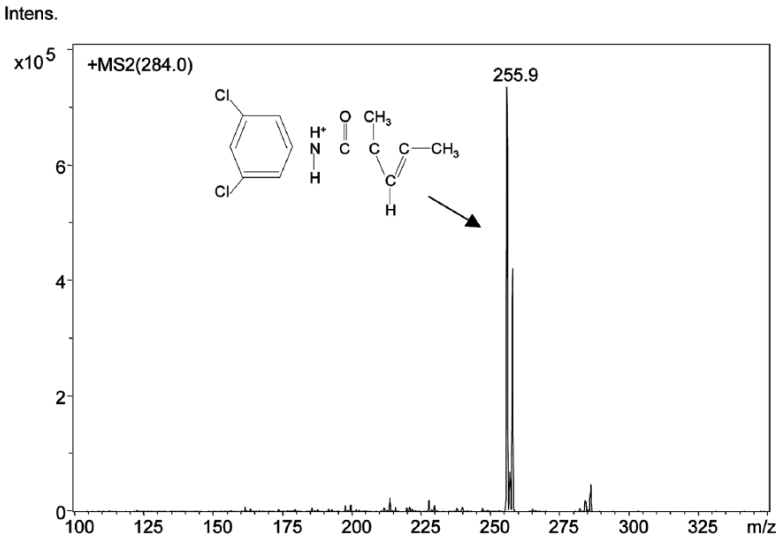
Figure 3 illustrates the MS, MS^2, and MS^3 spectra for procymidone and dinoseb standards, obtained by CE-ESI-MS in full scan and CE-ESI-MS^n with the product ion scanning. These require the most complicated interpretation. Procymidone gave in the MS^2 a mass spectrum with a specific ion at *m/z* 256 by the neutral loss of CO. Its MS^3 analysis provides seven main fragment ions at *m/z* 162, 178, 186, 198, 212, 228, and 240 that are tentatively identified in Fig. 3. This mass fragmentation is in agreement with that already reported for GC-MS [24]. On the contrary, dinoseb is a phenol that formed mainly $[M-H]^-$ ions at pH 4 even though according to its pK_a (4.62) hydroxyl groups should be predominantly nonionic. This phenomenon, called "wrong-way-around" results, has previously been reported for LC-ESI-MS [25] and for CE-ESI-MS [15]. The MS^2 spectrum evidences an intense signal at *m/z* 194 that corresponds to $[M-H\text{-}NO_2]^-$. Further fragmentation of this ion by MS^3 showed an intense signal at *m/z* 164 that corresponds to the neutral loss of NO and an additional fragment at *m/z* 134 formed by the loss of an ethane molecule. This course is also in agreement with that reported in other studies [26]. From the observed fragmentation pathways the dominant dissociation was chosen (Table 2). The cut-off and the amplitude were set to a value that resulted in the maximum intensity of the product ions.

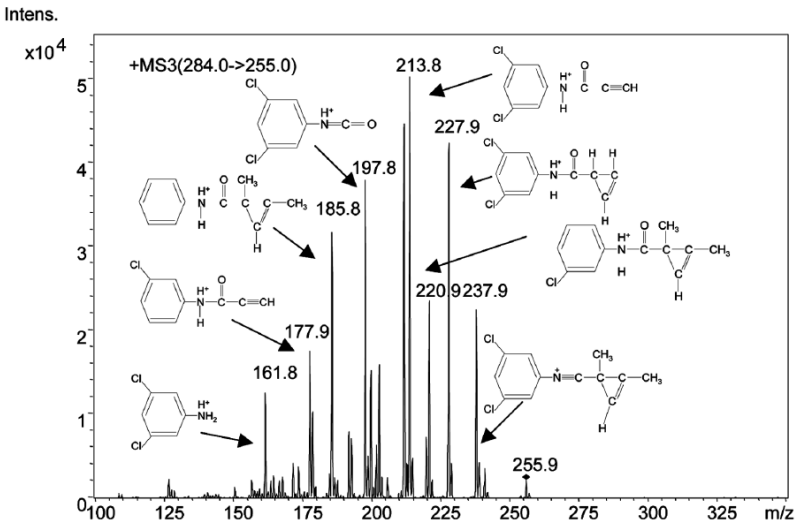
3.3 Pesticide analysis by CE-UV, CE-ESI-MS, and CE-ESI-MS^n

The reproducibility, linearity, and sensitivity of the different detection systems tested in this study were evaluated. The results are listed in Tables 3 and 4. Satisfactory reproducibilities, calculated at concentrations five times higher than the LODs, were obtained with an RSD percentage (n = 10) for migration times better than 1.2 and for peak areas between 3.9 and 8.9. The reproducibility for the migration times was almost equal by all the detection systems tested, whereas that for the peak areas was slightly better by CE-UV (ranging from 3.9 to 5.8) than by the other detection systems (ranging from 4.3 to 8.9). Among the different CE-ESI-MS approaches tested, there were no apparent differences in the RSDs. These results suggest that reproducible fragmentation was carried out for each pesticide. The calibration curves were linear in the range from 8 to 80 μg/mL, from 1.5 to 150 μg/mL, and from 0.05 to 50 μg/mL for CE-UV, CE-MS single quadrupole, and CE-QIT-MS with the different MS stages tested, respectively. The correlation coefficients were between 0.990 and 0.998. Calibration graphs showed good correlations for the six pesticides for any detection system.

Using UV detection, the LODs, calculated as 3 times the signal/noise ratio, were 1 μg/mL for dinoseb, pirimicarb, and procymidone and 2 μg/mL for pyrifenox, pyrimethanil, and thiabendazole. Although the chemical structures of the studied pesticides are very different from each other, the LODs are quite similar because the molar absorptivities or extinction coefficients of the different analytes are similar as has already been reported [13]. These values would not be sufficient to detect thiabendazole, pyrifenox, pyrimethanil, and dinoseb, even when using an SPE preconcentration procedure, at concentrations lower than the maximum residue limits (MRLs) (see Table 5). These LODs are in agreement with those described in the literature [13, 20]. LODs obtained by CE-MS with a single quadrupole were between 0.05 and 0.5 μg/mL. As previously, these values were not appropriate to determine pyrimethanil and dinoseb. Determination of thiabendazole and procymide in fruits using CE-ESI-MS with a single quadrupole has already been report-

(a)





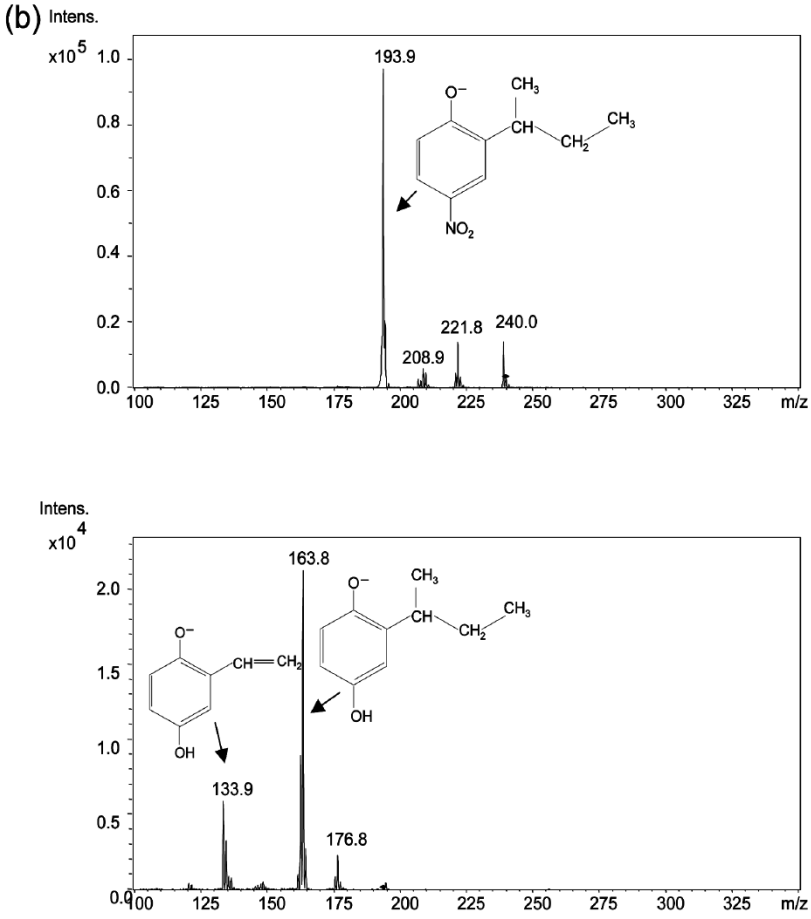


Figure 3. MS^2 and MS^3 mass spectra of (A) procymidone and (B) dinoseb. The proposed fragmentation is displayed.

ed [14]. LODs reported in that work were five times lower than those obtained in the present study. A probable reason is that in the other study a stacking procedure was used, and that the running buffer and sheath liquid had different compositions. LODs ranged from 0.001 to 0.015 μg/mL, from 0.005 to 0.2 μg/mL, and from 0.009 to 1.4 mg/mL using MS, MS^2, and MS^3 with QIT-MS. The LODs for pyrifenox, pirimicarb, and pyrimethanil, obtained in the present study by CE-QIT-MS, were one order of magnitude better than those reported previously [13]. The explanation could be, as in the earlier case, the differences in the buffer and sheath liquid composition. According to the LODs obtained it can be deduced that MS and MS^2 resulted in a severalfold improvement in sensitivity compared to CE coupled to UV or to single quadrupole MS in SIM mode. Both procedures seem to be appropriate to determine the studied pesticides at levels lower than the MRLs. On the contrary, LODs obtained by CE-ESI-QIT-MS^3 are higher than MRLs for pyrifenox, pyrimethanil, and dinoseb.

Table 4. Reproducibility, linearity, and sensitivity of pesticide detection by CE-MS with multiple stages of mass spectrum using the QIT

	CE-MS				$CE-MS^2$				$CE-MS^3$			
	RSD (n = 10) (%)		Linearity correlation	LOD (μg/mL)	RSD (n = 10) (%)		Linearity correlation	LOD (μg/mL)	RSD (n = 10) (%)		Linearity correlation	LOD (μg/mL)
	Migration time	Peak area			Migration time	Peak area			Migration time	Peak area		
Thiabendazole	1.4	4.3	0.998	0.005	1.6	5.0	0.997	0.006	1.5	5.6	0.996	0.009
Pyrifenox	1.5	5.5	0.996	0.009	1.3	5.6	0.997	0.2	1.7	5.2	0.998	1.4
Pirimicarb	1.3	6.7	0.994	0.001	1.2	6.2	0.995	0.005	1.9	6.0	0.995	0.1
Pyrimethanil	1.7	5.8	0.993	0.01	1.7	5.5	0.994	0.1	2.0	6.4	0.997	0.3
Procymidone	2.0	5.9	0.992	0.015	2.3	6.3	0.990	0.02	2.5	7.1	0.996	0.5
Dinoseb	2.7	4.8	0.994	0.01	2.7	7.2	0.992	0.04	3.2	7.6	0.998	0.4

Since fruits contain a large number of organic compounds and some other contaminants that can cause isobaric interferences, highly selective methods are necessary to quantify pesticides precisely without interference from other compounds. MS/MS gives the highest degree of certainty in analyte identification and, therefore, CE-ESI-MS/MS was selected for further experiments.

3.4 Identification and quantification of pesticides in fruits

The suitability of the CE-ESI-MS/MS was tested by applying it for the analysis of the selected pesticides in peaches. Peaches and nectarines are essentially the same, differing only in genes for surface fuzz. Pesticides were extracted using water-acetone as well as isolated and concentrated by SPE eluting with dichloromethane. Dichloromethane, a halogenated solvent, was selected because it provides higher analyte recoveries and co-extracted lesser matrix components as has already been reported [14, 20]. The MRM mode of the mass spectrometers, which can detect mass spectra of fragmentation peaks of target precursor ions, provides sufficient selectivity and enables quantitative analysis. An example of a typical electropherogram obtained by CE-ESI-MS/MS from a spiked peach sample is shown in Fig. 4. Electrophoretic resolution and peak performance were satisfactory. Although both mass spectrometers, single quadrupole and quadrupole ion trap, have almost the same design, different migration times compared with Fig. 2b were observed because of small differences between both equipments, especially the distances in the mass spectrometer.

A matrix interference study was carried out comparing the calibration graph obtained from standards prepared in buffer and in fruit extracts. The equations of the least-square linear regressions obtained were similar, and the covariance analysis showed that the calculated F-values were lower than the tabulated ones, indicating that both straight lines were parallel ($P < 0.05$). These results proved that matrix interferences were negligible. Other important possible effects on the CE separation generated by matrix components, such as migration time variation or unresolved peaks, were not observed because the role of sample composition is more vital when on-line preconcentration techniques are applied instead of conventional hydrodynamic injection (5 s, 0.5 psi, *ca.* 5 nL injected) and the SPE helps to eliminate salts and inorganic components from the matrix that have the greatest influence on the ionic strength.

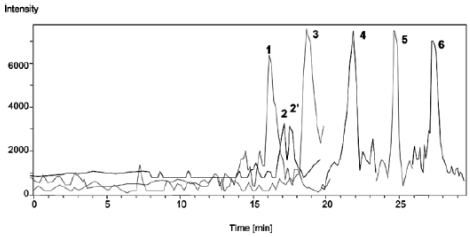


Figure 4. Electropherogram by CE-ESI-MS^2 of pesticides in a spiked peach sample at LOQ level (see Table 5). The peaches (5 g) were extracted with water-acetone and the extracts were cleaned up and concentrated by SPE. The selected precursor ion → product ion in MRM used to quantify each analyte are indicated in Table 1. Other conditions as in Fig. 2.

According to the guidance document on residue analytical methods established by the EU [27], Table 5 reports recovery and precision for LOQs and MRLs (set or proposed) or 10 times LOQ when MRLs are similar to the

Table 5. Results for pesticide analysis in spiked peach samples (basal pesticide levels < LODs) by CE-ESI-MS^2

Pesticide	Amount (mg/kg)	RSD (%, $n = 5$)	Recovery (%)	MRLs (μg/kg)
Thiabendazole	0.005	15	58	0.05
	0.05	12	60	
Pyrifenox	0.06	18	91	0.2
	0.2	9	99	
Pirimicarb	0.001	19	69	0.5
	0.5	10	78	
Pyrimethanil	0.02	14	89	0.02
	0.2	11	92	
Procymidone	0.01	15	78	2
	2	9	82	
Dinoseb	0.02	16	60	0.05
	0.05	11	69	

LOQ fortification levels. Samples were analyzed prior spiking with pesticides to determine the basal levels or "blank values". There were no peaks at the retention times of the pesticides, indicating that basal levels were lower than the LODs. LOQs (lowest concentration outlined in Table 5), calculated according to the European Guideliness as the lowest levels that provide acceptable recoveries and repeatabilities (< 20%), were in the range of 0.001–0.2 mg/kg. Compared with the established MRLs, LOQs were equal for pyrimethanil and lower for thiabendazole, pyrifenox, pirimicarb, procymidone, and dinoseb a number of times of 10, 3, 500, 200, and 2.5, respectively. The MRLs reported in Table 5 are those established by the Spanish legislation [28], which were in agreement with the MRLs instituted by the European Union (EU) [27] for dinoseb, procymidone, and thiabendazole, and with those recommended by the Codex Alimentarius [29] for pirimicarb. There were no MRLs set by the US Food and Drug Administration [30] for these pesticides in peaches or nectarines. Recoveries and RSDs obtained for peaches and nectarines spiked at two concentrations are summarized in Table 5. Mean recoveries ranged from 58 and 99% with RSDs from 9 to 19%. Recoveries seem to be higher at the higher spiked level but differences were within the range of the RSDs.

The method was applied to the determination of the six pesticides in ten samples (five of peaches and five of nectarines) obtained from an agricultural cooperative over different days. Three samples contained pesticide residues. One nectarine sample contained 0.03 mg/kg thiabendazole, and two peach samples 0.2 and 1.0 mg/kg procymidone. Electropherograms of the peach sample containing thiabenzole and using different CE-ESI-MS systems are shown in Fig. 5. The impressive selectivity of multiple-stage MS can be observed.

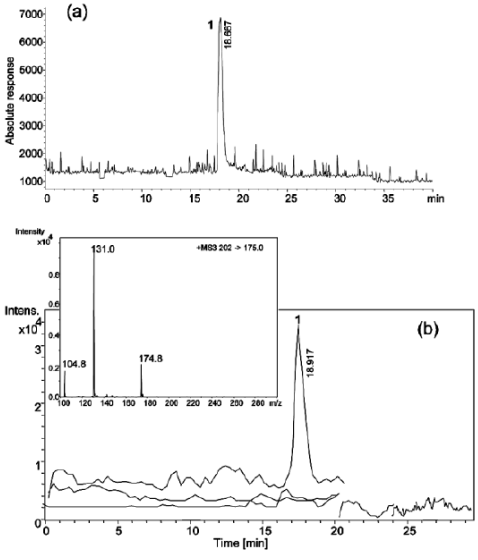


Figure 5. Electropherograms of sample containing thiabendazole using (A) single quadrupole in SIM monitoring the ion at *m/z* 202 and (B) QIT MS^3 (the superposed figure corresponds to the product ion spectrum).

4 Concluding remarks

The reported method enables the simultaneous, direct, and sensitive analysis of pesticide residues in fruits. Compared with other techniques, CE-ESI-MS/MS has significant advantages: (i) the MS^n spectra provided structural information, and the identification confidence is

considerably high, (ii) sensitivity, reproducibility, and linearity are good, (iii) pesticides can be selectively determined without interferences of other compounds, and (iv) sample preparation is minimal. Its utility was demonstrated by analyzing pesticide residues in fruits. These results indicate that this approach should be applicable in a wide range of fields, such as the analysis of pesticides in foods.

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5 References

[1] Diehl, J. F., *Acta Aliment.* 2002, *31*, 355–369.
[2] Peshin, S. S., Lall, S. B., Gupta, S. K., *Acta Pharmacol. Sin.* 2002, *23*, 193–202.
[3] Nasreddine, L., Parent-Massin, D., *Toxicol. Lett.* 2002, *127*, 29–41.
[4] Abbott, P., Baines, J., Fox, P., Graf, L., Kelly, L., *et al.*, *Food Control* 2003, *14*, 383–389.
[5] Lauwaars, M., Anklam, E., *Accredit. Qual. Assur.* 2004, *9*, 253–258.
[6] Ahmed, F. E., *Trends Anal. Chem.* 2001, *20*, 649–661.
[7] Mezinger, F., Schmitt-Kopplin, Ph., Freitag, D., Kettrup, A., *J. Chromatogr. A* 2000, *891*, 45–61.
[8] Picó, Y., Rodríguez, R., Mañes, J., *Trends Anal. Chem.* 2002, *22*, 133–151.
[9] Martínez, D., Cugat, M. J., Borrull, F., Calull, M., *J. Chromatogr. A* 2000, *902*, 65–89.
[10] Malik, A. K., Fabel, W., *Crit. Rev. Anal. Chem.* 2001, *31*, 223–279.
[11] da Silva, C. L., de Lima, C. E., Tavares, F. M. M., *J. Chromatogr. A* 2003, *1014*, 109–116.
[12] Schmitt-Kopplin, P., Frommberger, M., *Electrophoresis* 2003, *24*, 3837–3867.
[13] Hernandez-Borges, J., Rodríguez-Delgado, M. A., Garcia-Montelogo, F. J., Cifuentes, A., *Electrophoresis* 2004, *25*, 2065–2076.
[14] Rodríguez, R., Picó, Y., Mañes, J., *J. Chromatogr. A* 2002, *949*, 359–366.
[15] Rodríguez, R., Mañes, J., Picó, Y., *Anal. Chem.* 2003, *75*, 452–459.
[16] Goodwin, L., Startin, J. R., Keely, B. J., Goodall, D. M., *J. Chromatogr. A* 2003, *1004*, 107–119.
[17] Toribio, F., Moyano, E., Puignou, L., Galceran, M. T., *J. Chromatogr. A* 2002, *948*, 267–281.
[18] Oka, H., Ito, Y., Matsumoto, H., *J. Chromatogr. A* 2000, *882*, 109–113.
[19] Núñez, O., Moyano, E., Galceran, M. T., *J. Chromatogr. A* 2002, *974*, 243–255.
[20] Rodríguez, R., Boyer, I., Font, G., Picó, Y., *J. Chromatogr. A* 2001, *126*, 2134–2138.
[21] Boughtflower, R. J., Paterson, C. J., Knox, J. H., *J. Chromatogr. A* 2000, *887*, 409–420.
[22] Lazar, J. M., Lee, M. L., *J. Am. Soc. Mass Spectrom.* 1999, *10*, 261–264.
[23] Blasco, C., Font, G., Picó, Y., *J. Chromatogr. A* 2004, *1043*, 231–238.
[24] Jiménez, J. J., Bernal, J. L., Nozal, M. J., Arias, E., Bernal, J., *Rapid Commun. Mass Spectrom.* 2004, *18*, 657–663.
[25] Thurman, E. M., Ferrer, I., Barceló, D., *Anal. Chem.* 2001, *73*, 5441–5449.
[26] Budde, W. L., *Mass Spectrom. Rev.* 2004, *23*, 1–24.
[27] European Community. http://europa.eu.int
[28] Spanish state secretary of trade and tourism. http://www.mcx.es/plaguicidas/espanol.asp
[29] Food and Agriculture Organization. http://www.fao.org
[30] US Food and Drug Administration. http://wm.cfsan.fda.gov

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On-line preconcentration strategies for analyzing pesticides in fruits and vegetables by micellar electrokinetic chromatography

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Abstract

Five pesticides (fludioxonil, procymidone, pyriproxyfen, dinoseb and carbendazim) were separated in reversed migration micellar electrokinetic chromatography (RM-MEKC) using 20 mmol l^{-1} phosphate buffer at pH 2.3, containing 25 mmol l^{-1} sodium dodecylsulfate and 10% methanol. Three on-line concentration strategies, sweeping (SW), normal stacking with reversed migration and a water plug (SRW) and stacking with reverse migration and removal of sample matrix using polarity switching (SRMM), were compared. About 10-, 30- and 50-fold increases in detection sensitivity, compared with standard hydrodynamic injection (5 s at 0.5 psi), were observed with SW, SRW and SRMM, respectively. Limits of detection (LODs) ranged from 0.002 to 0.03 μg ml^{-1} using only the on-line preconcentration procedures without any off-line concentration of the extract. A solid-phase extraction (SPE) procedure, for previous isolation and concentration of the analytes, was used in combination with any of the proposed on-line preconcentration strategies, which achieves the determination of pesticides at limits of quantification (LOQs) lower than 0.01 mg kg^{-1}. The recoveries obtained by SPE in samples spiked at 0.01 mg kg^{-1} were between 70 and 100%, with RSDs between 10 and 18% using SRMM. Samples of fruits and vegetables were taken from the market, extracted by the proposed procedure and analyzed with RM-MEKC with the on-line strategies.

Keywords: Food analysis; SW; SRW; SRMM; Reversed migration micellar electrokinetic chromatography; Pesticides; Solid-phase extraction

1. Introduction

Over the years, research in the field of pesticide residues analysis in food has experienced a continuous expansion of the number of techniques available for determining their content in different fruits and vegetables [1,2]. The state-of-the-art has progressed to the point that most sophisticated techniques, such as liquid chromatography–mass spectrometry (LC–MS), special immunological assays, automatic clean-up/determination analyzers, biosensors, are in use to solve the more difficult analytical problems [3]. Many of these methods either are not generally available or have a little analytical utility at the present, but they are expected to become necessary for optimal care in a not too distant future [1–3].

Capillary electrophoresis (CE), the latest addition to analytical instrumentation, will undoubtedly have a great impact in pesticide residue analysis, because of its broad potential to separate a wide spectrum of small molecules [4–8]. A variety of CE modes are suitable to separate inorganic and organic substances, depending on the particular problem to be solved [4,5]. Among them, micellar electrokinetic chromatography (MEKC) [6,8] has become prevalent as a powerful separation technique for both, neutral and ionic compounds. Principle of MEKC separation is based on analyte partitioning between the micelle and the aqueous phase. Reversed MECK (RM-MEKC) is characterized by anionic micelles moving faster than the electroosmotic flow (EOF); thus, positive potential is applied to the detector end in order to detect the analytes. Electroosmotic flow is reduced using low pH buffers or cationic surfactants [6–8].

The disadvantage of MEKC, normal or reverse, to determine trace-analytes is its low sensitivity because of short path length in on-column UV detection and the low injection volumes (ca. 10–50 nl). That problem has prevented CE to be more widely used and to achieve the status of routine technique, as for example LC, which is now a fairly well, established one. Different on-column trace enrichment schemes, with the advantages of simplicity and economy, have been developed to overcome this

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limitation. Most popular ones are sweeping (SW) and stacking with reverse migration of micelles with (SRMM) or without (SRW) the insertion of a water plug prior to the sample and with or without polarity switching [8,9,11].

SW is based on the capture and accumulation of the analyte by the micelles that penetrate in the sample zone during the application of a voltage [11]. Samples must be free of micelles [7,8,11]. The SRMM focussing mechanism is based on the abrupt change of the analyte effective electrophoretic velocities at the stacking boundary [9,10]. The stacking boundary separates regions of high and low electric fields. The SW phenomenon is also partly responsible for the focussing effect in SRMM. SRW is performed by dissolving the sample in a buffer with lower conductance than the separation buffer, and with a surfactant at a concentration slightly higher than the critical micelle concentration and lower than that of the separation buffer. A water plug is first injected into the capillary before the injection of the sample [9]

Enhancement factors of the order of 3 up to 1000-folds, depending on the nature of sample analyte and the sample, have been reported. Those factors accomplish viable trace analysis of pesticides and metabolites in fruits and vegetables and result comparable to those obtained by LC [7]. Recently, the separation and concentration of triazines [12,13], urea-derived pesticides [13,14], carbamates [13] and ammonium quaternary herbicides [15] in water samples by CE using these preconcentration strategies have been reported to achieve ppb-level detection limits. Although the application of these techniques to food matrices has been scarcer described, few representative reports showed very promising results [13,16–18]. da Silva et al. [13] compared these three most popular on-line preconcentration techniques for determining nine pesticides in carrot extracts, without reporting data on recovery or precision. Hernández-Borges et al. [16,17] tested SMMR with other on-line preconcentration modes such as normal stacking mode (NSM) and field enhanced sample injection (FESI) for the analysis of a selected group of pesticides in juices and checked the NSM for determining pesticides in soy milk by CE-MS. Molina-Mayo et al. [18] applied SRMM to determine pesticides in wine obtaining LODs between 17.6 and 32.3 $\mu g\,l^{-1}$.

The objective of this study is to evaluate solid-phase extraction (SPE) [15], as an off-line preconcentration step, and on-capillary concentration and separation to analyze five pesticides (fludioxonil, procymidone, pyriproxyfen, dinoseb and carbendazim) in fruits and vegetables and to study the presence of these compounds in grapes and lettuces. Fig. 1 shows the structures of the studied compounds. As target analytes, three fungicides (fludioxonil, procymidine and carbendazim), one insecticide (pyriproxyfen) and one herbicide (dinoseb) were selected based on their wide use in the selected crops and the legislation requirements of the EU and Spain. Three on-capillary

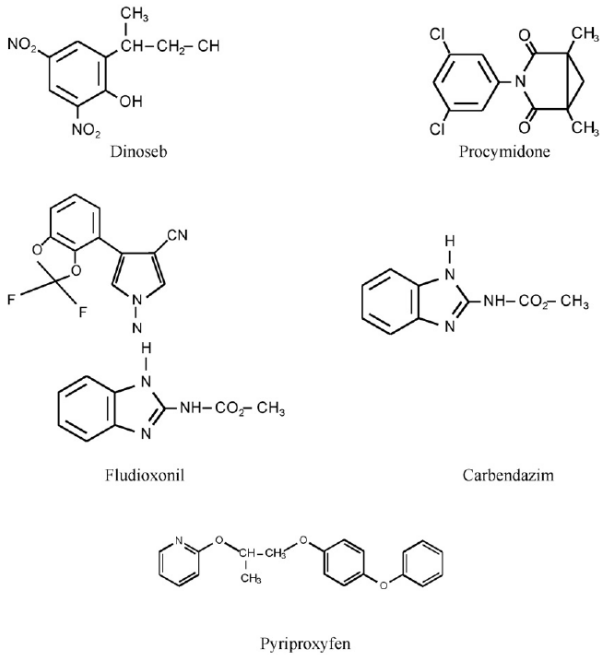

Fig. 1. Names and structures of the tested pesticides.

on-line concentration techniques, SW, SRW and SRMM, were optimized and compared.

2. Material and methods

2.1. Chemicals, materials and working solutions

Fludioxonil, procymidone, dinoseb and carbendazim (purity > 99.3%) were purchased from Riedel-de-Haen (Swelze, Germany). Pyriproxyfen (purity > 99.3%) was from Dr. Ehrenstorfer GmbH (CIUDAD, PAIS). HPLC-grade methanol and organic trace analysis grade dichloromethane were from Merck (Darmstadt, Germany) and acetone from Mallinckrodt (Chromar-HPLC, KY). Deionized water (<18 MΩ cm resistivity) from a Milli-Q SP Reagent Water System (Millipore, Bedford, MA, USA) was used to prepare all buffers and sample solutions. Sodium dodecylsulfate was purchased from Aldrich (Madrid, Spain), phosphoric acid and disodium hydrogenphosphate dihydrate were of analytical grade from Scharlau (Barcelona, Spain). MFE C_{18} solid phase (particle diameter in the range of 45–55 μm and pore diameter 60 Å) was acquired from Análisis Vinicos (Tomelloso, Spain).

The solid-phase (500 mg) was placed into a 100 mm × 9 mm I.D. glass column fitted with a coarse frit (No. 3). Prior to use, the column was activated by washing successively with methanol (10 ml) and deionized water (10 ml). All other reagents were obtained from Fluka (Madrid, Spain), and were of analytical or reagent grade.

Grapes and lettuces were purchased from different local markets in Valencia city (Valencia, Spain). Method validation experiments were performed with biologically farmed grapes and lettuces, obtained from an ecological farming cooperative (Valencia, Spain), which showed no detectable pesticide concentrations.

Individual stock solutions of fludioxonil, procymidone, dinoseb and pyriproxyfen were prepared at concentration of 1 mg ml^{-1} in methanol and that of carbendazim in 10 mol l^{-1} HCl at the same concentrations. They were stored in glass-stopper bottles at 4 °C. The stock solutions were stable in the stored conditions for more than 3 months. Standard working solutions at various concentrations were daily prepared by appropriate dilution of aliquots of the stock solutions in the suitable buffer for the on-line preconcentration procedure tested. Mixtures were passed through a 0.45 μm cellulose filter from Scharlau (Barcelona, Spain).

2.2. Sample preparation

A representative portion of the sample (ca. 500 g) was chopped in a food chopper and 100 g portions were stored, in closed containers, in a freezer. A portion of sample (5 g) spiked or not with pesticides was placed in a 250 ml glass beaker and mixed and homogenized by ultrasonic vibration for 15 min; therefore 10 ml of water/acetone (50:50, v/v) was added and homogenized 15 min more. The resulting suspension was filtered through a Buchner funnel and the cake filter was washed twice with 20 ml of acetone/water (50:50, v/v). The extract was evaporated by rotavapor to eliminate the acetone. The resulting aqueous sample solution was passed through the C_{18} column at flow-rate about 2 ml min^{-1}. After extraction, the solid-phase was dried under vacuum for approximately 10 min. Retained pesticides were eluted with 10 ml of dichloromethane. The eluent was collected in a graduated conical tube (20 ml) and concentrated, under stream of nitrogen, to dryness. After that, it was redissolved with 0.5 ml of the appropriate buffer according to the on-line preconcentration technique used.

2.3. Capillary electrophoresis/DAD with on-line preconcentration

Experiments were carried out on a P/ACE MDQ CE system (Beckman Instruments, Fullerton, CA, USA) equipped with a diode array detector (DAD) and System Gold software for data acquisition. The capillaries were untreated fused-silica capillaries (Beckman) 60 cm total length, 50 cm effective length, 75 μm I.D. and 375 μm O.D. The capillary was treated with 0.1 mol l^{-1} sodium hydroxide (pressure flow for 30 min), then Milli-Q water (5 min), followed by the carrier electrolyte (5 min) at the start of each working day. Prior to each analysis, the capillary was prewashed for 2 min with deionized water, 2 min with 0.1 mol l^{-1} NaOH, 2 min with deionized water again and 2 min with running buffer applying an overpressure of 20 psi (1 psi = 6894.76 Pa). Pesticides were monitored at 230 nm.

The CE running buffer consisted of a pH 2.3 phosphate buffer solution at 20 mmol l^{-1} concentration, containing 25 mmol l^{-1} SDS and 10% methanol (conductivity, 2.23 mS cm^{-1}). The sample was hydrodynamically injected at 50 mbar (1 mbar = 100 Pa) for 5 s, corresponding to a ∼1% of the capillary volume. Negative voltage (−20 kV) was applied, giving an average current of 34 μA.

For SW, the analytes (pesticide standard solutions or grape or lettuce extracts) were dissolved in a 25 mmol l^{-1} phosphate buffer at pH 2.3 and 10% of methanol, resulting in a nonmicelle buffer with 2.1 mS cm^{-1} conductivity. Hydrodynamic injections were performed at 50 mbar for 10–100 s, at intervals of 10 s, to study the effect of sample plug length on separation efficiency or resolution (optimum injection time was 60 s). After injection, a voltage of −20 kV was applied.

For SRMM, sample was diluted in plain water. Hydrodynamic injections were performed at 50 mbar for 10–100 s at intervals of 10 s as in the previous procedure (optimum injection time 60 s). A positive voltage was applied for 12 s at the inlet vial right (+20 V). In this period, current progressively increased until it reaches 80% of the value initially measure for the background electrolyte. Then, the voltage was switched to the negative (−20 kV).

For SRW, a water plug was injected before the sample injection during an established time (that was optimized from 5 to 60 s at 0.4 psi obtaining the optimum value at 10 s). The sample was diluted in 4 mmol l^{-1} phosphate buffer at pH 2.3, containing 10 mmol l^{-1} SDS. Injection time was also optimized for 10–100 s at intervals of 10 s, as in the previous procedure (optimum value was 60 s). After injection, a voltage of −20 kV was applied.

3. Results and discussion

3.1. Optimum separation conditions

The preconcentration techniques tested in this study required separation of the compounds in RM-MEKC, in which the EOF direction is reversed to attain matrix removal and preconcentration. EOF can be reversed mainly by two procedures: (i) at acidic pH by reducing the EOF so far that it cannot overwhelm the electophoretic mobility of the anionic micelles and (ii) by the addition of a cationic surfactant that is adsorbed in the capillary walls charging the positively [9–11].

The separation of five pesticides in RM-MEKC was optimized testing different running buffers consisted of mixtures of phosphate solutions (10–100 mmol l^{-1}) and SDS (15–150 mmol l^{-1}) at different pH (2–5) by direct hydrodynamic injection (5 s at 0.5 psi) of a 5 μg ml^{-1} standard solution of the five pesticides prepared in the running buffer. According to the pK_a for several compounds: carbendazim (pK_a 4.48) and dinoseb (pK_a 4.62), pH values around 5 should be used because at lower pH, these compounds are positively charged.

Experimental results are contradictory with this theory: the more acidic, the pH is, the best the separation is. Similar results have been reported by da Silva et al. [13], which separates nine pesticides of different classes including carbendazim. Among the different running buffers tested, the mixture containing 20 mmol l^{-1} phosphate and 25 mmol l^{-1} SDS at pH 2.3 provided the best results, although pyriproxyfen and carbendazim still overlap partially.

The separation between both compounds was attained adding a 10% of methanol to the previous buffer. This conclusion was achieved checking different solvents (methanol, acetonitrile, ethanol and isopropanol) at percentages between 2 and 15%.

Finally, the effect of the applied voltage and the temperature was study to shorten as much as possible the analysis time. Temperature was varied between 20 and 30 °C and the applied voltage between 16 and 25 kV. The optimum results were obtaining applying a voltage of 20 kV at 20 °C. Fig. 2 presents the separation of 5 μg ml^{-1} standard solution of the studied pesticides in the optimized running buffer.

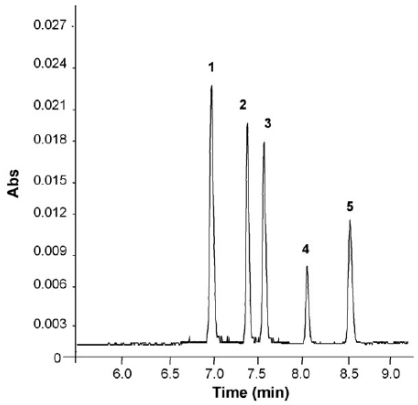

Fig. 2. Separation of the five pesticides standards at a concentration of 5 μg ml^{-1} of each by RM-MEKC in 20 mmol l^{-1} phosphate buffer at pH 2.3 containing 25 m mmol l^{-1} SDS and 10% of methanol. Other conditions: hydrodynamic injection of 5 s at 0.5 psi pressure, applied voltage −20 kV at 20 °C. Peak identification: 1 = fludioxinil, 2 = pyriproxyfen, 3 = carbendazim, 4 = dinoseb and 5 = procymidone.

3.2. On-line sample preconcentration strategies

Fig. 3 illustrates the effects of the injection time on peak area for each of the three on-line preconcentration strategies tested. The peak area increases in proportion to the injection time. However, the dependency of the peak area is not linear in all cases. For example, procymidone and pyriproxyfen seem to present an exponential dependency and for dinoseb there is a maximum in the case of SRW. The 60 s injection was the most suitable in terms of peak shapes. However, as can be observed in Fig. 4, the application of the stacking techniques provoked certain loss of resolution (compared with Fig. 2), especially between pyriproxyfen and carbendazim, and some peak width increment, particularly in SRW and SRMM. The lack of linearity can be explained because for longer injection time (up to 60 s), peaks showed broadening and asymmetric shapes. The changes observed in the area give the impression to be dependent on the solute, which could be related to the pK_a or log P values of the studied compounds. However, there are not available pK_a for all the studied compounds but only for carbendazim and dinoseb and no relation can be establish according to the log P, which was 1.38 for carbendazim, 3.08 for procymidone, 3.56 for dinoseb, 4.12 for fludioxinil and 5.37 for fludioxinil. This lack of linearity or apparent exponentiality of the peak area with the injection time has already been reported by other authors [10,19], which suggest that the injection length of the sample zone was limited by the dispersive effect brought about by the local electrososmotic velocity mismatch between the low- and high-conductivity zones.

Fig. 4 shows the electropherograms obtained by SW, SRW and SRMM RM-MEKC-DAD analysis of a standard mixture containing 1 μg ml^{-1} of each analyte. A usual injection is included for comparison. About 10-, 30- and 50-fold increases in detection sensitivity, compared with standard hydrodynamic injection, were observed for SW, SRW and SRMM, respectively. Among the on-line concentration techniques, SRMM gave the highest improvement in detector response (>50-fold compared to usual injection). The stacking techniques (SRMM and SRW) rendered better results compared to SW because the analytes were moderately polar. However, the peak shape obtained by SW was the most appropriate and its separation ability even better. The three on-line preconcentration procedures provided results applicable to the determination of pesticides at low concentration levels. The retention times obtained are slightly different upon the concentration method because the solution in which the standards are dissolved is different as has already been reported [13].

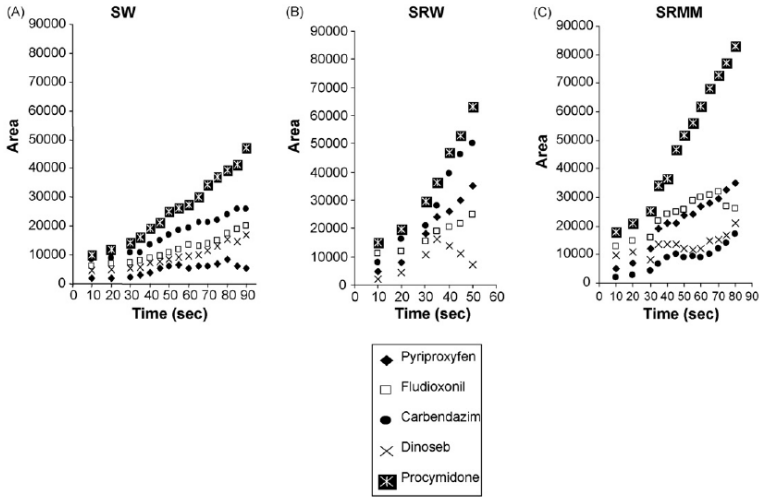

Fig. 3. Effects on injection time on peak area for (A) SW, (B) SRW and (C) SRMM.

Table 1 summarizes and compares the mean retention times, linearity, limit of detection (LOD) values, relative standards deviations (RSD, %) of peak area and migration times for normal injection 0.5 s a 5 psi, SW, SRW and SRMM, respectively. The linearity of these methods was tested using five different concentrations within the range of 0.2–50 mg ml^{-1}, executing at least three replication injections. Pesticide responses were found linear over the concentration range explored with correlation coefficients (r) > 0.991 in all the cases. Data on the regression equations are listed in Table 1. The limits of detection (S/N = 3) of the five compounds were 0.02–0.04 μg ml^{-1} (SW), 0.01–0.3 μg ml^{-1} (SRW) and 0.002–0.01 μg ml^{-1} (SRMM). The reproducibility (RSDs) of the proposed methods in terms of peak-area for eight replicate injections, were 6.82–11.61% for SW, between 3.95 and 9.47% for SRMM and 2.22–4.94% for SRW. In terms of migration time, between 2.35 and 2.70% SW, between 1.30 and 1.54% SRMM and 2.47 and 3.76%.

The selected pesticides have been determined using MEKC or CZE in our laboratory. MEKC using a running buffer consisted of 6 mmol l^{-1} sodium tetraborate decahydrate and 75 mmol l^{-1}

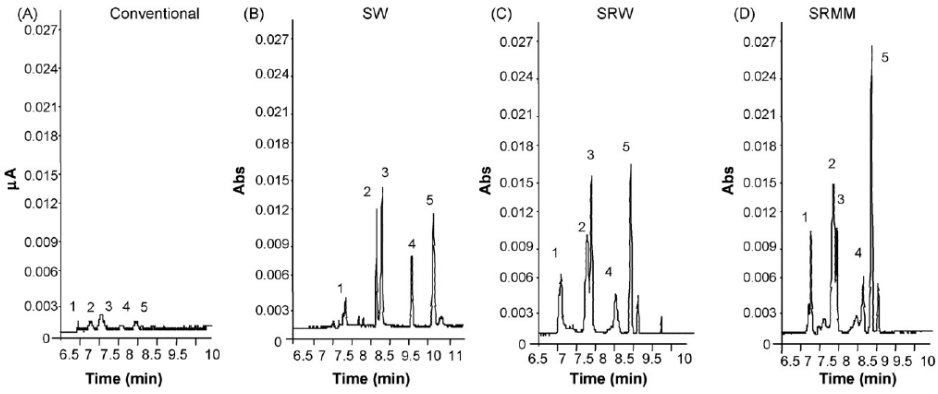

Fig. 4. Electropherograms of a standard mixture of containing 1 μg ml^{-1} of each pesticide obtained by (A) conventional RM-MEKC with hydrodynamic injection (5 s at 0.5 psi), (B) by the SW RM-MEKC, (C) by SRW RM-MEKC and (D) SRMM RM-MEKC conditions. SW: hydrodynamic injection 60 s at 50 mbar, SRW: injection of water plug 10 s at 0.4 psi, then sample injection 60 s at 50 mbar and SRMM: hydrodynamic injection 60 s at 50 mbar, application of positive voltage 1.2 s. Separation, other conditions and peak identification as in Fig. 2.

Table 1
LOD values, RSDs ($n = 8$) value and linearity (five points, injections in triplicate) for the studied pesticides with RM-MEKC-DAD using conventional injection, SW, SRW, SRMM

	Pesticides				
	Fludioxonil	Pyriproxyfen	Carbendazim	Dinoseb	Procymidone
Conventional injection					
Mean migration time (min)	6.809	7.376	7.652	8.098	8.538
Migration time (RSDs, %)	1.58	2.27	2.64	3.68	4.58
Peak heigh (RSDs, %)	4.45	5.44	7.21	11.79	12.06
Peak area (RSDs, %)	10.01	6.95	9.82	9.21	9.02
Concentration range (mg ml^{-1})	4–80	4–80	2–80	6–100	2–80
Equation	$y = (400 \pm 29)x + (1487 \pm 895)$	$y = (125 \pm 10)x + (784 \pm 124)$	$y = (405 \pm 32)x + (111 \pm 78)$	$y = (578 \pm 38)x + (517 \pm 106)$	$y = (834 \pm 59)x + (1089 \pm 346)$
Correlation coefficient (r)	0.993	0.997	0.995	0.993	0.992
LODs (μg ml^{-1})	0.3	0.3	0.1	0.5	0.1
Sweeping					
Mean migration time (min)	7.799	8.608	8.841	9.617	10.206
Migration time (RSDs, %)	2.38	2.35	2.51	2.64	2.70
Peak heigh (RSDs, %)	3.71	9.09	5.91	9.73	6.71
Peak area (RSDs, %)	6.82	11.61	9.28	9.84	9.16
Concentration range (mg ml^{-1})	0.2–50	0.4–50	0.2–50	0.5–50	0.5–50
Equation	$y = (1208 \pm 95)x + (1051 \pm 512)$	$y = (2319 \pm 210)x + (5612 \pm 609)$	$y = (5719 \pm 450)x + (7371 \pm 999)$	$y = (2407 \pm 202)x + (2640 \pm 397)$	$y = (2203 \pm 187)x + (7833 \pm 451)$
Correlation coefficient (r)	0.994	0998	0.993	0.991	0.998
LODs (μg ml^{-1})	0.02	0.04	0.02	0.02	0.02
SRW					
Mean migration time (min)	7.118	7.662	7.861	8.647	8.974
Migration time (RSDs, %)	3.65	2.47	3.63	3.76	3.24
Peak heigh (RSDs, %)	9.22	6.46	5.84	3.25	2.04
Peak area (RSDs, %)	2.23	4.92	3.04	2.58	0.22
Concentration range (mg ml^{-1})	0.2–50	0.2–50	0.2–50	1.2–50	0.4–50
Equation	$y = (1202 \pm 130)x + (18388 \pm 1234)$	$y = (1202 \pm 96)x + (2386 \pm 1245)$	$y = (2002 \pm 340)x + (2673 \pm 792)$	$y = (1003 \pm 126)x + (1664 \pm 925)$	$y = (1294 \pm 113)x + (3604 \pm 891)$
Correlation coefficient (r)	0.998	0.993	0.992	0.995	0.997
LODs (μg ml^{-1})	0.01	0.01	0.01	0.03	0.01
SRMM					
Mean migration time (min)	7.321	7.609	7.798	8.646	9.001
Migration time (RSDs, %)	1.30	1.54	1.33	1.36	1.45
Peak heigh (RSDs, %)	9.47	6.53	3.95	5.11	4.38
Peak area (RSDs, %)	6.85	5.01	2.86	6.24	2.95
Concentration range (mg ml^{-1})	0.2–50	0.2–50	0.2–50	1.2–50	0.2–50
Mean migration time (min)	7.321	7.609	7.798	8.646	9.001
Equation	$y = (3069 \pm 356)x + (2667 \pm 503)$	$y = (4004 \pm 395)x + (6047 \pm 926)$	$y = (5449 \pm 519)x + (7857 \pm 698)$	$y = (2541 \pm 259)x + (3041 \pm 735)$	$y = (7805 \pm 707)x + (2637 \pm 986)$
Correlation coefficient (r)	0.993	0.991	0.996	0.997	0.992
LODs (μg ml^{-1})	0.01	0.01	0.01	0.02	0.002

cholic acid at pH 9 separated eight pesticides, which fludioxinil and pyriproxyfen [20]. Conventional hydrodynamic injection (5 s, 0.5 psi) provided a LOD of 0.1 μg ml^{-1}. CZE using 0.3 M ammonium acetate-acetic acid at pH 4 with a 10% of methanol provided resolution of procymidone and dinoseb among other pesticides. LODs obtained using UV detector and conventional injection were 10 times higher than those achieved in the present study [21]. However, using MS and MS/MS detectors LODs were improved, at least, by a factor of ten with the substantial increment in selectivity and specificity. However, any of the on-line preconcentration procedures assayed in this study allows to improve those previous LODs by a factor of 10.

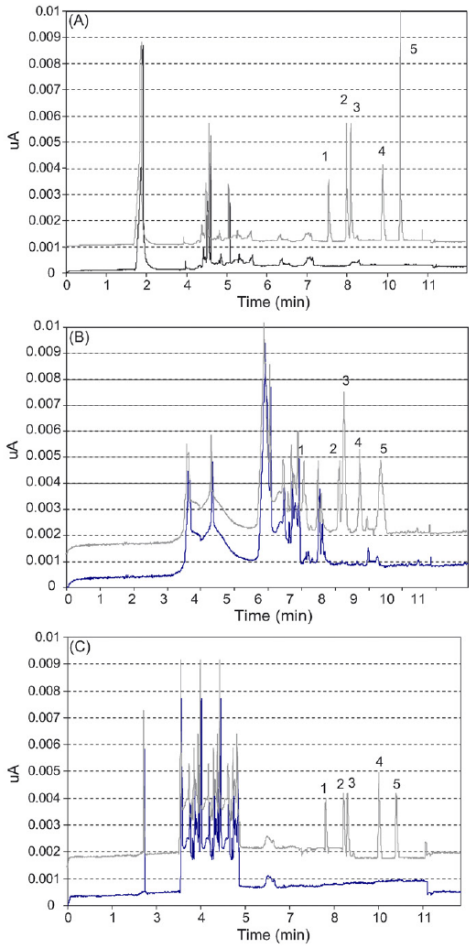

Fig. 5. Electropherograms from a unspiked (black line) and from a spiked (grey line) lettuce at 0.01 mg kg^{-1} of each pesticide obtained after solvent extraction and SPE clean-up by (A) SW, (B) SRW and (C) SRMM. Stacking conditions as in Fig. 3, and separation conditions and peak identification as in Fig. 2.

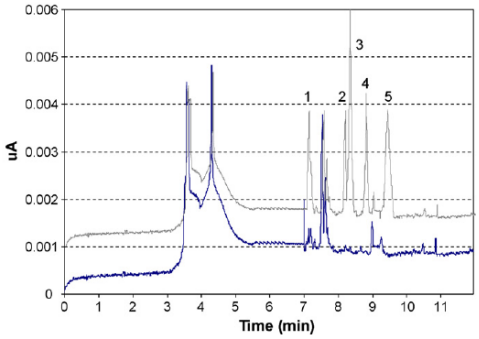

Fig. 6. Electropherograms from a unspiked (black line) and from a spiked (grey line) sample at 0.01 mg kg^{-1} of each pesticide obtained after solvent extraction and SPE clean-up by SRW. Stacking conditions as in Fig. 3, and separation conditions and peak identification as in Fig. 2.

3.3. Determination of pesticide residues in grape and lettuces

When applying this method to fruits, sample preconcentration via SPE was required (with 10-fold preconcentration achieved). A solvent extraction and SPE preconcentration and cleanup previously developed in our laboratory [20,21] was used as off-line preconcentration step prior CE analysis.

Samples of grapes and lettuces unspiked and spiked at 0.01 mg kg^{-1} and at MRLs were analyzed by SPE, RM-MEKC-DAD, using the three preconcentration procedures, under the optimized conditions described above. The electropherograms obtained for lettuce extracts are shown in Fig. 5. The electropherogram with the line in black of those figures shows the

Table 2
Accuracy and precision at 0.01 mg kg^{-1} (amount of sample processed 5 g) after SPE and on-line preconcentration of lettuce samples using RM-MEKC with the three on-line preconcentration strategies

Pesticide	Concentration (mg kg^{-1})	Recovery, % (RSD, $n=5$) RM-MEKC-DAD		
		SW	SRW	SRMM
Fludioxinil	0.01	72 (17)	75 (16)	73 (16)
	1	87 (15)	77 (14)	70 (10)
Pyriproxyfen	0.01	74 (19)	80 (19)	81 (16)
	5	77 (16)	82 (15)	80 (12)
Carbendazim	0.01	73 (18)	77 (18)	75 (17)
	2	75 (13)	81 (15)	76 (15)
Procymidone	0.01	78 (18)	88 (19)	82 (18)
	5	79 (15)	91 (11)	87 (14)
Dinoseb	0.01	70 (19)	75 (17)	73 (18)
	5	76 (16)	77 (12)	78 (13)

Table 3
Accuracy and precision at 0.01 mg kg^{-1} (amount of sample processed 5 g) after SPE and on-line preconcentration of grape samples using RM-MEKC with the three on-line preconcentration strategies and conventional LC-DAD

Pesticide	Concentration (mg kg^{-1})	Recovery, % (RSD, $n=5$) RM-MEKC-DAD		
		SW	SRW	SRMM
Fludioxinyl	0.01	88 (16)	85 (19)	93 (18)
	2	92 (16)	88 (17)	90 (12)
Pyriproxyfen	0.01	94 (18)	90 (19)	98 (16)
	5	98 (15)	97 (15)	100 (12)
Carbendazim	0.01	70 (17)	77 (18)	78 (16)
	2	79 (14)	81 (15)	79 (13)
Procymidone	0.01	78 (16)	88 (19)	87 (17)
	5	79 (13)	91 (11)	87 (12)
Dinoseb	0.01	73 (18)	77 (16)	75 (16)
	5	76 (13)	79 (14)	77 (12)

Table 4
LOQs obtained after SPE and on-line preconcentration of lettuce and grape samples using RM-MEKC with the three on-line preconcentration strategies and MRLs established by different legislation

Pesticide	LOQs (mg kg^{-1}) RM-MEKC-DAD			MRLs (mg kg^{-1})	
	SW	SRW	SRMM	Grape	Lettuce
Fludioxinil	0.01	0.008	0.006	2[a], 1[b]	1[b]
Pyriproxyfen	0.008	0.006	0.007	5[a]	5[a]
Carbendazim	0.008	0.004	0.008	5[c]	2[c]
Procymidone	0.008	0.010	0.010	0.05[c], 5[a,b]	0.05[c], 5[a,b]
Dinoseb	0.008	0.005	0.001	5[c]	5[c]

[a] Codex Alimentarius [23].
[b] USA [24].
[c] EU MRLs [22].

Table 5
Concentrations of pesticides found in grapes and lettuces by RM-MEKC-DAD using the three on-line preconcentration procedures and LC-DAD

Sample no.	Pesticides found	Concentration (mg kg^{-1})[a]		
		SW-RM-MEKC[b]	SRW-RM-MEKC[c]	SRMM-RM-MEKC[d]
Grape 1	Carbendazim	0.03	0.02	0.01
	Procymidone	1.23	1.53	1.05
Grape 4	Carbendazim	0.25	0.32	0.20
	Procymidone	1.64	1.54	1.65
Grape 5	Carbendazim	0.15	0.10	0.17
	Procymidone	1.92	2.03	1.54
Grape 10	Carbendazim	0.75	0.65	0.55
	Procymidone	1.85	1.80	1.95
Grape 11	Fludioxinil	0.04	0.05	0.03
Grape 12	Carbendazim	0.73	0.81	0.79
	Procymidone	0.21	0.19	0.26
Grape 15	Fludioxinil	0.44	0.33	0.35
Grape 22	Carbendazim	1.09	1.27	0.99
	Procymidone	0.23	0.26	0.27
Grape 24	Fludioxinil	0.09	0.07	0.08
Grape 28	Fludioxinil	0.05	0.03	0.04
	Procymidone	0.19	0.24	0.22
Lettuce 5	Pyriproxyfen	0.05	0.03	0.05
Lettuce 12	Carbendazim	0.15	0.21	0.19
Lettuce 25	Procymidone	0.34	0.24	0.45
Lettuce 28	Carbendazim	0.25	0.20	0.23
	Procymidone	0.53	0.53	0.61
Lettuce 30	Carbendazim	1.25	1.54	1.05
	Fludioxinil	3.02	2.05	2.95

[a] Triplicate measurements.
[b] RSDs were ranged between 19 and 25%.
[c] RSDs were ranged between 17 and 24%.
[d] RSDs were ranged between 13 and 22%.

electrophrogram of the lettuce or grape extract without any of the studied pesticides, and the electropherogram with the line in grey the same extract spiked with the pesticides at 0.01 mg kg^{-1}, demonstrating that the analyte peaks of all matrix extracts were free from interferences and that there are no significant variations in the shape and the retention time of the peaks and, therefore, that the methods are comparable with complex food matrices. Grape samples provided extracts a little cleaner than that of the lettuce maybe because the absence of chlorophylles and other colored or not compounds presents in the extracts. Fig. 6 shows worst case with corresponds to the electropherograms obtained for a grape sample using SRW. A much cleaner electropherogram with less baseline deviation and less peaks is observed if compared with that corresponding to the lettuce sample.

Recoveries and precision are summarized in Tables 2 and 3 as percentage and RSDs. Recoveries >70% for all pesticides were achieved being a little higher for fludioxynil and pyriproxyfen in grape than in lettuce. These values were between 72 and 98%, with RSDs from 13 to 19% by SW, between 75 and 97%, with RSDs from 11 to 19% by SRW and between 70 and 100%, with RSDs from 10 to 18% by SRMM. Although recovery was similar by all the methods and precision was within the range of the EU guidelines [22].

Table 4 shows the limits of quantification (LOQs) and the maximum residue limits (MRLs) established by different legislations [23–25]. LOQs were calculated according to the European Union (EU) guidelines as the lowest level that provides acceptable recoveries (>70%) and reproducibilities (<20%) [22]. The LOQs by RM-MEKC-DAD of these pesticides were 0.008-0.01 mg kg^{-1} for SW, 0.004–0.01 mg kg^{-1} for SRW and 0.001–0.01 mg kg^{-1} for the SRMM method. Thus, SRMM yields the lowest LOQ, followed by SRW and finally SW. The lowest detection method yielded by the SRMM preconcentration can be explained by its ability to introduce the greatest quantity of sample to the capillary. Compared with the previous works carried out in our laboratory with some of the studied pesticides, LOQs are improved more than 10 times, which allows to reach the lowest MRLs established by the legislations [20,21].

3.4. Application to real samples

Table 5 reports the concentrations calculated for the selected pesticides in a total of 60 samples, 30 of grapes and 30 of lettuces, taken from different local markets, measured after SPE by RM-MEKC using the three on-line preconcentration procedures. The reported concentrations are corrected by the individual recovery values of the compounds. The concentrations are in the μg kg^{-1} range. Owing to the increase in sensitivity provided by on-line preconcentration, pesticides can be detected by RM-MEKC-DAD.

Pesticide residues were detected in 15 samples (25%) of the 60 analyzed. None exceeded MRLs. Dinoseb was the only pesticide that was not found in any of the samples. Pyriproxyfen was only found in one sample of lettuce in quite low concentration. Carbendazim was found in nine samples (15%), three lettuces and six grapes, at levels ranging from 0.01 to 1.54 mg kg^{-1}. Procymidone was also found in nine samples (15%), two lettuces and seven grapes, at levels ranging from 0.19 to 2.03 mg kg^{-1}. Fludioxinil was found in five samples (8%), one lettuce and four grapes, at concentrations between 0.02 and 3.02 mg kg^{-1}.

Of the contaminated samples, nine samples (60%) – seven grapes and two lettuces – contained residues of two pesticides and six samples (40%) – three grapes and three lettuces – contained a single pesticide. This finding corroborates the presence and the co-occurrence of residues of pesticides in a significant number of samples.

4. Conclusions

This work demonstrated that fludioxonil, procymidone, pyriproxyfen, dinoseb and carbendazim, in lettuce and grape, are readily distinguishable at the MRLs through the combined use of off-line SPE using C_{18} bonded silica as solid sorbent, and any of the proposed on-line preconcentration strategies. The LOQs obtained in the determination of these compounds by CE are much better, than those obtained by conventional hydrodynamic injection, which pointed out that CE is a perfectly eligible technique for pesticide residue analysis in complex matrices such as fruits and vegetables.

The comparative study among the three different on-line preconcentration protocols carried out in this work has shown that reverse polarity-stacking with matrix removal (SRMM-RM) provides the best results in terms of sensitivity, efficiency and reproducibility. The other two procedures SW and SWR also provide good results, applicable to the determination of pesticides at low concentration levels.

The usefulness of this protocol has been further corroborated through their simultaneous and quantitative determination in real samples (lettuce and grape samples taken from different markets in the Valencian Community) below their MRL values.

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References

[1] C.M. Torres, Y. Picó, J. Mañes, J. Chromatogr. A 754 (1996) 301.
[2] F.E. Ahmed, Trends Anal. Chem. 20 (2001) 649.
[3] W.G. Fong, H.A. Moye, J.N. Seiber, J.P. Toth, Pesticide Residues in Foods, Wiley-Interscience, NY, 1999, p. 1335.
[4] A. Karcher, Z. El Rassi, Electrophoresis 20 (1999) 3280.
[5] Y. Picó, R. Rodríguez, J. Mañes, J. Trends Anal. Chem. 22 (2002) 133.
[6] D. Martinez, F. Borrull, M. Calull, Trends Anal. Chem. 18 (1999) 282.
[7] A.K. Malik, W. Faubel, Crit. Rev. Anal. Chem. 31 (2001) 223.
[8] J. Hernández-Borges, S. Frías-García, A. Cifuentes, M.A. Rodríguez-Delgado, J. Sep. Sci. 27 (2004) 947.
[9] Z.K. Shihabi, J. Chromatogr. A 902 (2000) 107.
[10] J.B. Kim, J.P. Quirino, K. Otsuka, S. Terabe, J. Chromatogr. A 916 (2001) 123.
[11] J.P. Quirino, J.B. Kim, S. Terabe, J. Chromatogr. A 965 (2002) 357.

[12] R. Carabias-Martínez, E. Rodríguez-Gónzalo, J. Domínguez-Álvarez, J. Hernández-Méndez, Electrophoresis 23 (2002) 494.
[13] C.L. da Silva, E.C. de Lima, M.F.M. Tavares, J. Chromatogr. A 1014 (2003) 109.
[14] J.P. Quirino, N. Inoue, S. Terabe, J. Chromatogr. A 892 (2000) 187.
[15] O. Núñez, E. Moyano, L. Puignou, M.T. Galceran, J. Chromatogr. A 912 (2001) 353.
[16] J. Hernández-Borges, A. Cifuentes, F.J. García-Montelogo, M.A. Rodríguez-Delgado, Electrophoresis 26 (2005) 980.
[17] J. Hernández-Borges, M.A. Rodríguez-Delgado, F.J. García-Montelongo, A. Cifuentes, J. Sep. Sci. 28 (2005) 948.
[18] C. Molina-Mayo, J. Hernández-Borges, T.M. Borges-Miquel, M.A. Rodríguez-Delgado, J. Chromatogr. A 1150 (2007) 348.
[19] M.C. Chen, S.H. Chou, C.H. Lin, J. Chromatogr. B 801 (2004) 347.
[20] A. Juan-García, Y. Picó, G. Font, J. Chromatogr. A 1073 (2005) 229.
[21] A. Juan-García, G. Font, Y. Picó, Electrophoresis 26 (2005) 1550.
[22] European Commission, Directorate General Health and Consumer Protection, Residue analytical methods for post-registration control and monitoring, Sanco/825/00 (rev. 7) 17.
[23] EU MRLs sorted by pesticide (available at http://europa.eu/food/plant/protection/pesticides/index_en.htm).
[24] Pesticide Residues in Food, Codex Alimentarius (available at http://www.codexalimentarius.net/mrls/pesticides/jsp/pest_q-e.jsp).
[25] International Maximum Residue Limits Database (available at http://mrldatabase.com/query.cfm?CFID=1717249&CFTOKEN=).

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Research Article

Determination of quinolone residues in chicken and fish by capillary electrophoresis-mass spectrometry

A specific pressure-assisted CE-MS method is described for the analysis of five quinolone residues. MS using a single quadrupole is compared with multiple-stage MS using a quadrupole IT (QIT-MS^n). The procedure involves a common sample preparation by SPE on disposable cartridges. The most suitable electrolyte is 60 mM $(NH_4)_2CO_3$ at pH 9.2. Single quadrupole does not provide enough fragmentation to confirm identities according to the current legislation. However, QIT-MS^n achieves selective fragmentation. Using this method, danofloxacin, enrofloxacin, flumequine, ofloxacin, and pipemidic acid are analyzed in fortified samples of chicken and fish. Recoveries at levels of 50 ng/g were 62–99%, except for flumequine, which gives recoveries $\geq$45%. RSDs are from 9 to 16% and the LOD is equal (20 ng/g) for the five analytes. Confirmation of the quinolones' identity is achieved using QIT-MS^3. Forty samples of chicken and fish taken from different local markets are analyzed. Enrofloxacin is also determined in incurred chicken muscle using this method.

Keywords: Antibiotics / Capillary electrophoresis / Chicken / Fish / Mass spectrometry / Quadrupole ion trap
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1 Introduction

Quinolones are a group of synthetic antimicrobial agents, widely used in human and veterinary medicine, with the common skeleton of 4-oxo-1,4-dihydroquinoline. These compounds are active against a broad spectrum of Gram-positive and Gram-negative bacteria. Their extensive administration to animals, destined for human consumption, has become a serious problem because their residues can persist in edible animal tissues. Quinolones may be directly toxic or be the source of resistant human pathogens representing a possible risk to human health. They can produce allergic hypersensitivity reactions or toxic effects on the articular cartilages causing arthralgia or juvenile arthropaties [1, 2].

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Abbreviations: EIC, extracted ion chromatogram; **EU**, European Union; **IP**, identifications point; **MRL**, maximum residue limit; **pCE**, pressure-assisted CE; **QIT**, quadrupole IT

In the European Union (EU), the use of veterinary drugs is regulated through 2377/90/EC Council Regulation [3], which describes the procedure for the establishment of maximum residue limits (MRLs) for veterinary medicinal products in foodstuffs of animal origin. Technical guidelines and performance criteria for residue control, in the framework of 96/23/EC Directive [4], are explained in the 657/2002/EC Commission Decision [5], concerning the performance of analytical methods for the determination of organic residues and contaminants in live animal and animal products. According to these regulations, there is no obligation to use standardized methods in the residue control of food-producing animals. Instead, a *criteria approach* is applied, which lays down performance characteristics, limits, and conditions that must be met by the analytical methods. Confirmation of quinolone residues requires a combination of MS with chromatographic separation that reaches a minimum of three identifications points (IPs). The IPs are a system to establish the number of ions necessary in the mass spectrum to confirm the identity of an analyte depending on the nature of the MS information, which is related to the MS technique applied. Any MS technique or combination of techniques may be employed to attain the

number of IPs needed for the identification of a compound. A significant advantage of this approach is its high degree of versatility [2, 6].

There are different reviews describing the determination of quinolones in biological fluids, or in edible animals [7–9]. The most common method for quinolone residue analysis involves extraction with an appropriate solvent followed by one or more clean-up processes, and determination by LC-MS. As direct consequence of the criteria approach, intensified MS specificity is pursued by the use of MS/MS, which becomes increasingly available to routine laboratories. This technique allows the multiresidue determination of quinolones in different matrices, with the possibility to confirm the presence of these compounds by means of fragment abundance ratios at rather low concentration levels, achieving the required number of IPs [6].

Capillary electrophoretic methods also seem to have potential for the analysis of antibacterials, but more research is still needed to make the technique widely known and expand their use in the field of the analysis of drug residues in food [10, 11]. In some studies, quinolones are determined in edible animal tissues by CE, usually by CE-UV [12–18], and to a lesser extent LIF [19]. However, in those cases, the EU guideline requires a confirmatory analysis. Commonly, CE has been hyphenated by ESI to MS detectors, such as single quadrupole, triple quadrupole (QqQ), quadrupole IT (QIT), magnetic sector MS, TOF, and quadrupole TOF (QqTOF) [20–22]. However, very limited works have been reported on the application of CE-MS to the determination of quinolones. The optimization of experimental parameters for the CE-MS determination of nine quinolones was reported using a single quadrupole, but its application for real samples has not been attempted [23].

In this work, the development of an analytical method for the separation and quantification of five quinolones – enrofloxacin, danofloxacin, pipemidic acid, flumequine, and ofloxacin – in fish and chicken muscles, using CE-MS, is reported. The pressure-assisted CE (pCE) system was optimized with regards to important operating parameters such as the type of buffer, its pH, and concentration. Two different mass spectrometers, single quadrupole and QIT, were compared to establish the best selectivity. Finally, the developed method was applied to analyze these drugs in chicken and fish samples taken from local markets as well as in muscles of treated chickens. To the best of our knowledge, the determination of quinolones using the pressure-assisted CE multiple-stages MS (pCE-QIT-MS^n) approach in fish and chicken muscle has not been reported yet.

2 Materials and methods

2.1 Chemicals

Danofloxacin, flumequine, ofloxacin, and pipemidic acid were obtained from Riedel de-Haën (Seelze, Germany), and enrofloxacin was kindly donated by Cenavis (Reus Spain). Individual standard stock solutions of 1000 mg/mL were prepared in methanol and stored in stained glass-stopper bottles under refrigeration at 4°C. Standard working mixtures at various concentrations were daily prepared by appropriate dilution of aliquots of the stock solutions in the running buffer. A working standard mixture containing 5 μg/mL of each compound was prepared in methanol, for using it as spiking solution.

All chemicals and solvents used were of analytical grade or HPLC quality. Ammonium carbonate and TFA were purchased from Fluka (Madrid, Spain). Sodium hydroxide, hexane, ACN were obtained from Scharlau (Barcelona, Spain). Phosphoric acid and sodium dihydrogen phosphate were from Panreac (Barcelona, Spain). Methanol and dichloromethane were from Merck (Darmstadt, Germany). Deionized ultrapure water (<18.2 MΩcm resistivity) was obtained from the Milli-Q SP Reagent Water System (Millipore, Bedford, MA, USA). Nylon filters (0.45 μm) were purchased from Análisis Vínicos (Tomelloso, Spain).

ODS (MFE-Pak C_{18}) solid phase (particle diameter in the range of 45–55 μm and pore diameter 60 Å) was also acquired from Análisis Vínicos. The solid phase (500 mg) was placed into 100 mm × 9 mm id glass column fitted with a coarse frit (No. 3) with a standardized hollow key (14/23) and a standard ground cone (29/32) that can be combined with several laboratory glassware. These columns were made-to-measure by Marcelo Glassware (Valencia, Spain).

2.2 Instrumentation

Experiments were carried out using a P/ACE MDQ CE system (Beckman Instruments, Fullerton, CA, USA), with an Agilent 1100 series MSD mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) or with an Esquire3000 IT LC/MS(n) system (Bruker Daltonik, Bremen, Germany). The coupling of CE to MS was carried out using a G1607 Agilent CE-MS sprayer kit, an HP1100 binary pump, and a special external detector adapter (EDA) cartridge (Beckman) as has been described elsewhere [24, 25].

Samples were injected by applying a pressure of 0.5 psi for 5 s. Separations were carried out in an uncoated fused-silica capillary (Supelco, Madrid, Spain) of 75 μm id, 375 μm od, and 75 cm total length, 50 cm of which were thermostated and 25 cm were at room temperature.

The polyamide coating was removed from the end of the capillary (*ca.* 5 mm) to maintain a stable electrospray. The applied voltage was +20 kV, and the capillary temperature was maintained at 20°C. The electrolyte was 60 mM $(NH_4)_2CO_3$ at pH 9.2. For the pCE separation, excess pressure at the injector end (30 mbar) was applied during the electrophoretic run. At the beginning of each run, the capillary was conditioned with 0.1 M NaOH for 10 min and with the electrolyte for another 10 min.

The coaxial sheath liquid was the same electrolyte, delivered at 10 μL/min by the pump equipped with a 1:100 splitter. ESI-MS was conducted in the positive ion mode, and the capillary voltage was set at 4.0 kV. Dry nitrogen gas was heated to 250°C and delivered at a flow rate of 7 L/min. The pressure of nebulizing nitrogen gas was set at 10 psi.

The mass spectra were acquired with the HP-MSD using full scan (from *m/z* 100 to 500 with a scan time of 0.75 s) or SIM ($[M + H]^+$ molecules at *m/z* 358 for danofloxacin, *m/z* 362 for ofloxacin, *m/z* 360 for enrofloxacin, *m/z* 304 for pipemidic acid, and *m/z* 262 for flumequine) with a gain of 3, fragmentor voltage of 70 V, and dwell time of 63 ms.

QIT source parameters were the same as single quadrupole. Detector and block voltages were: multiplier voltage, 1600 V; dynode voltage, 7.0 kV; skimmer block, 51.2 V; lens 1 block, −200.0 V; octopole RF amplitude block, 50 Vpp; partition block, 4.6 V; lens 2 block, 0 V; capillary exit block, 195 V. The trap was programmed in ion charge control mode to accumulate 20 000 ions, for a maximum accumulation time of 50 ms. The *m/z* range scanned was from 100 to 500, and every four scans were summed. The mass spectrometer was operated in full scan and multiple reaction monitoring (MRM) modes. Extracted ion chromatograms (EICs) of the predominant transitions were used for quantification.

Instrumental electrophoretic conditions were run by a System Gold software version 2.2, mass spectrometric parameters, and data acquisition for the single quadrupole were managed by an HP Chem Station version A.06.03, and instrumental operations and data analyses for the QIT were controlled under Esquire Control version 5.1 and the Data Analysis version 3.1 (Bruker). The CE and MS instruments were connected and synchronized by an external pulse signal, programmed by the electrophoresis instrument.

2.3 Sample preparation

The extraction procedure used in the present study is based on an already published one that has been modified in some details [19]. Five grams of chopped and minced chicken or fish muscle tissue was accurately weighed and placed in a 20 mL centrifuge tube. Fortified muscle samples were prepared by adding an adequate volume of stock solutions of quinolones (from 10 to 100 μL). The spiked sample was left for 15 min at room temperature to ensure the appropriate distribution of quinolones in the matrix. Then, the sample was mixed with 5 mL of 50 mM NaH_2PO_4 (pH 7.0) by a rotary shaker for 10 min and extracted twice consecutively with 20 and 10 mL of dichloromethane, by a rotary shaker for 5 min. Organic and aqueous phases were separated by centrifuging in a Macrotonic Selecta (J. P. Selecta SA, Abrera, Spain) for 5 min at 2500 rpm. The organic layers were collected into a 40 mL centrifuge tube and extracted twice with 5 mL of 0.5 M NaOH. The aqueous phase was separated from the dichloromethane by centrifuging for 5 min at 2500 rpm, and then was transferred to a clean vial and adjusted to pH 7 with 200 mM H_3PO_4 and defatted by extraction with 10 mL of hexane. The aqueous sample extract was passed through the C_{18} column, previously conditioned with 10 mL of methanol and 10 mL of distilled water, at a flow rate of about 2 mL/min. The elution of the quinolones was carried out with 2 mL of 4% TFA in water–ACN (25:75 v/v), followed by 1 mL ACN.

The eluent was collected in a graduated conical tube (10 mL) and concentrated under a stream of nitrogen on a TurboVap^{LR} Concentration Work Station from Zymark (Hopkinton, MA, USA) almost to dryness, and redissolved with 0.5 mL of buffer solution.

3 Results and discussion

3.1 Optimization of CE-MS conditions

The coupling between CE and MS requires the use of a volatile CE running buffer such as buffers containing formic acid, acetic acid, ammonium carbonate, and ammonium acetate. Four of the studied quinolones have a carboxylic group and a piperazinyl moiety including additional amino groups, which can be cationic, zwitterionic, and anionic depending on the pH. Because of this, the compounds can be analyzed by CE over a certain pH range with different separation selectivity. However, flumequine has only a carboxylic acid group that is negatively charged at basic pH. As it has already been reported, basic pH (between 8 and 9.50) provides better separation of quinolones than acidic or stronger alkaline pHs [17, 26, 27]. Using 60 mM ammonium carbonate buffer (pH 9.2) at 25 kV and an excess pressure of 30 mbar during the run, the separation of the five quinolones was achieved in 20 min. Carbonate buffers have previously been used in quinolone separation by CE-MS [23].

The sheath liquid flow is the dominant flow for CE-MS analysis, and its pH affects ESI-MS sensitivity. The effect of sheath liquid pH on ESI-MS sensitivity was tested by using three solutions: 0.5% acetic acid in water (pH 3.0), 60 mM ammonium acetate (pH 7.0), and 60 mM ammonium carbonate in water (pH 9.2). The MS signal and stability were better using 60 mM ammonium carbonate. The use of basic solutions (pH 9.2) had no significant decrease in the sensitivity compared with the lower pH sheath liquid solutions. The addition of different organic solvents to the sheath liquid (methanol, ACN, and isopropanol) was tested but they do not improve either MS signal or spray stability. Thus, further experiments were conducted with 60 mM ammonium carbonate. This empirical observation was in agreement with the general experience that the running buffer is the best sheath liquid [23–25].

Another important parameter, the effect of the sheath liquid flow rate on the ESI sensitivity, was also investigated in the range of 2–15 μL/min. The addition of pressure to the separation capillary decreased analysis times and stabilized the ESI spray [28]. The application of 30 mbar increased the flow rate by 0.2 μL/min. The sheath liquid flow rate has only very little effect on the sensitivity, under the examined conditions. At higher flow rates (15 μL/min), the sensitivity decreased probably due to a dilution effect with the sheath liquid. At lower flow rates (<6 μL/min), the sensitivity was also somewhat decreased, probably because those flow were too low to be able to stabilize the source spray. According to Frommberger *et al.* [29], a stable electrospray could not be achieved with flow rates less than 4 μL/min. In the present study, high stable and sensitive analysis is achieved at flow rates between 8 and12 μL/min. Therefore, the following experiments were performed at 10 μL/min. This flow rate is relatively high compared with many papers that used pure CE [24, 25, 29]. However, the reason could be other than the use of pCE, for example the capillary temperature of the electrospray. This temperature is also higher than in most of those studies because it must lead to solvent removal, and water has a high boiling temperature compared with organic solvents [29].

Figure 1 depicts the chemical structure and molecular weight of the studied quinolones to highlight their fragmentation patterns. The mass spectra obtained with the single quadrupole showed, as main ion, the peak corresponding to the protonated molecule $[M + H]^+$. According to the 657/2002/EC Commission Decision [5], the protonated molecule is equivalent to one IP, which is not enough for confirmation. Although an increase in the fragmentor voltage accelerates the collision between the ions inducing fragmentation, this in-source fragmentation is difficult, requires high fragmentor voltages, and can lead to a decrease in sensitivity.

Fragmentation patterns of quinolones using QIT were also studied to facilitate the identification. Table 1 shows the precursor-product ions obtained using QIT and multiple-stage MS. The first-order MS spectrum shows the peak corresponding to the protonated molecule of each quinolone, which was subjected to CID to produce a first set of fragment ions MS/MS or MS^2. One of these fragment ions was isolated and refragmented to give the next set of ions MS^3. CID was carried out, on the ion of interest, by collisions with the helium background gas present in the trap for 40 ms. Product ion mass spectra (MS^3) of quinolones showed that predominant fragment ions were *m/z* 283 for danofloxacin, *m/z* 245 for enrofloxacin, *m/z* 261 for ofloxacin, *m/z* 189 for pipemidic acid, and *m/z* 202 for flumequine. These results are in agreement with those reported in other studies [30–34]. According to the EC Decision [5], using QIT, each precursor-product ion transition earns 1.5 IPs. The monitoring of two precursor-product ion transitions achieves unequivocal confirmation. An example of the multiple stage spectra and the proposed interpretation of the ions is shown in Fig. 2 for enrofloxacin, which is the quinolone that can be more repeatedly fragmented.

3.2 Method validation

In order to improve the detection sensitivity, an extraction procedure that achieves a ten-fold concentration was applied. The method was validated using both chicken and fish muscle tissue samples fortified with several levels of standard quinolone mixture, and subjected to the entire extraction procedure. LODs and LOQs were defined, respectively, as the signal corresponding to three and ten times the noise SD.

The developed method was validated in terms of repeatability and reproducibility of migration time and peak area, LOD, and linearity. The results obtained for chicken samples are summarized in Table 2. The repeatability was calculated from six consecutive analyses (intraday) and the reproducibility from four consecutive days (day-to-day or interday) of the same extract. The RSDs of the migration times for the studied quinolones were less than 2.6 intraday and 5.1 interday. Single quadrupole and QIT did not show appreciable differences. Although for the peak area acceptable results were also obtained, the reproducibility was lower for QIT. The LODs were calculated from the SIM electropherograms in the single quadrupole and from the EIC electropherograms in the QIT of spiked samples at 50 ng/g. LODs using single quadrupole were from 5 to 20 ng/g and using QIT were 20 ng/g for the five quinolones. Using single quadrupole, excellent linearity was observed in the concentration range of 50–5000 ng/g, with a corre-

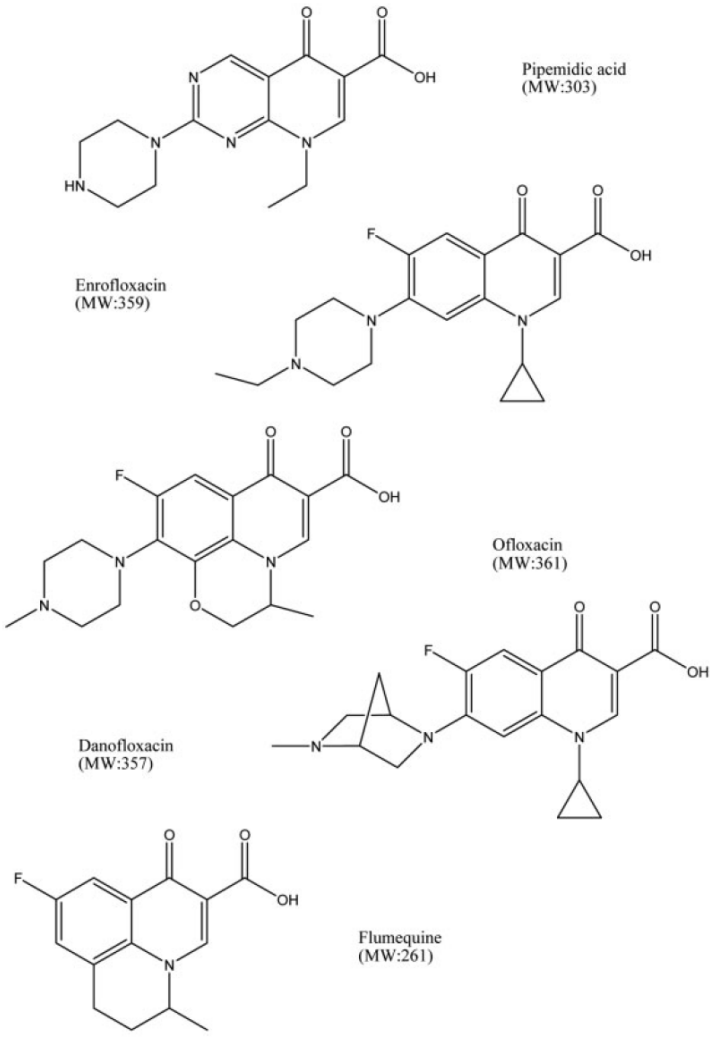

Figure 1. Chemical structure and molecular weight of the studied quinolones.

lation coefficient better than 0.9992. On the contrary, for the QIT instrument, the linearity calculated in the same conditions was worst with a correlation coefficient better than 0.9903. The limited dynamic range of the QIT has been widely discussed in the literature; it is an intrinsic characteristic of this mass analyzer. Although the narrow range for which the response of QIT is linear, quinolones can be correctly quantified at low concentrations. Results obtained for fish samples were very similar (data not shown).

Table 3 shows the accuracy and precision obtained for quinolones extracted from chicken and fish spiked samples at 50 ng/g using QIT as mass analyzer. These parameters for samples spiked at higher concentrations of quinolones (500 ng/g) provided similar values (data not shown). Good recoveries were generally obtained, in both chicken and fish tissues, for four of the five quinolones tested. Flumequine gave lower recovery $\geq$45% because it is thermolabile and there are some losses in the evaporation step. All other

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Table 1. Transitions used for quantification by MS^n operation. Neutral loss is indicated between brackets

	Quinolones				
	Danofloxacin	Ofloxacin	Enrofloxacin	Pipemidic acid	Flumequine
MS^2 transitions (*m/z*)	358 → 314 (CO_2)	362 → 318 (CO_2)	360 → 316 (CO_2)	304 → 216 (CO_2, C_2H_5N)	262 → 244 (HO_2)
Width (*m/z*)	4.0	4.0	4.0	4.0	4.0
Cut-off (*m/z*)	150	100	100	100	100
Amplitude (V)	4.0	2.0	1.5	1.0	2.0
MS^3 transitions (*m/z*)	314 → 294 (80%) (HF) 314 → 283 (100%) (H_2NCH)	318 → 261 (C_3H_7N)	316 → 245 ($C_2H_5NC_2H_5$)	216 → 189 (CO)	244 → 202 (C_3H_6)
Width (*m/z*)		4.0	4.0	4.0	4.0
Cut-off (*m/z*)		100	100	100	100
Amplitude (V)		1.2	1.3	1.8	1.3
MS^4 transitions (*m/z*)		261 → 218 (NC_2H_5)	245 → 205 (C_3H_4)		
Width (*m/z*)		4.0	4.0		
Cut-off (*m/z*)		100	100		
Amplitude (V)		2.0	1.8		
MS^5 transitions (*m/z*)			205 → 189 (CH_4)		
Width (*m/z*)			4.0		
Cut-off (*m/z*)			100		
Amplitude (V)			2.0		

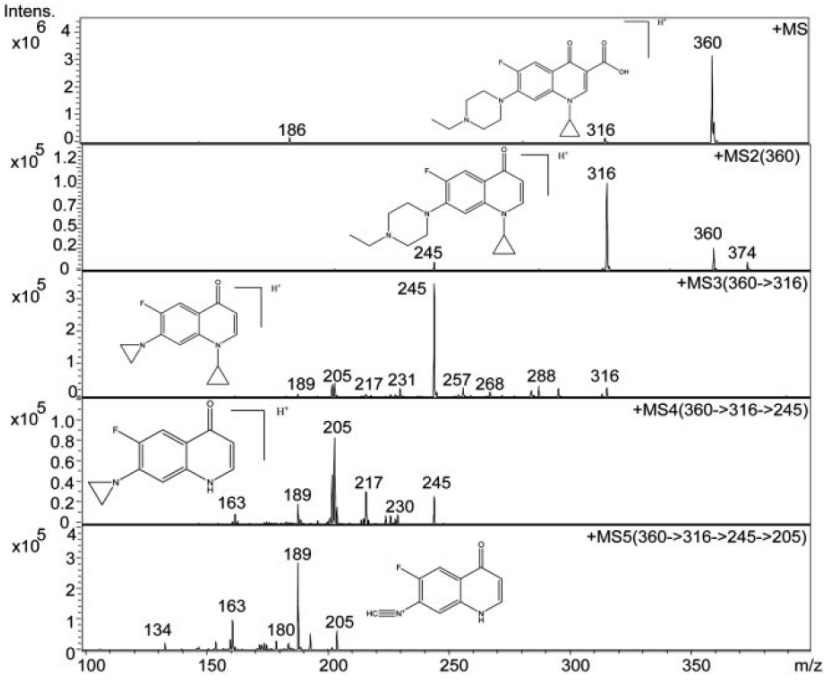

Figure 2. Multiple-stage mass spectra of enrofloxacin and proposed interpretation of the product ions.

Table 2. Repeatability (intraday) and reproducibility (interday) of relative migration time, peak area, correlation coefficient (*r*), and LOD obtained by analyzing quinolone spiked chicken muscle with the proposed procedure by CE-MS using the single quadrupole (quantification was carried out in SIM mode) and by CE-MS^3 using QIT (quantification was carried out obtaining the EIC of the most intense product ion), respectively

	CE-Q-MS						CE-QIT-MS^3					
	RSD (%)				LOD (ng/g)	$r^{c)}$	RSD (%)				LOD (ng/g)	$r^{c)}$
	Migration time		Peak area				Migration time		Peak area			
	Intraday[a)]	Interday[b)]	Intraday[a)]	Interday[b)]			Intraday[a)]	Interday[b)]	Intraday[a)]	Interday[b)]		
Danofloxacin	1.3	4.5	4.2	6.2	5	0.9992	2.2	6.6	6.2	9.0	20	0.9908
Oxofloxacin	1.7	3.8	4.6	6.8	10	0.9996	2.8	6.9	6.3	10.0	20	0.9922
Enrofloxacin	2.0	4.5	4.8	7.2	18	0.9996	2.6	5.6	6.0	10.4	20	0.9918
Pipemidic acid	2.0	5.8	4.8	7.0	20	0.9998	2.8	4.9	6.2	10.2	20	0.9934
Flumequine	2.3	3.8	4.4	8.8	20	0.9997	3.0	7.2	6.8	12.3	20	0.9902

a) Consecutive injections ($n = 6$) of an extract obtained of a chicken sample spiked at 100 ng/g.
b) Injections carried out in four consecutive days ($n = 4$) of an extract obtained of a chicken sample spiked at 100 ng/g.
c) Mean of three injection of six different levels in the concentration range of 0.05–5 μg/g.

Table 3. Results for quinolones analysis in spiked chicken and fish samples (basal quinolones levels <LODs) by CE-ESI-MS^3

Quinolones	Chicken				Fish			
	Amount (ng/g)	RSD (%, $n = 5$)	Recovery (%)	MRLs (ng/g)	Amount (ng/g)	RSD (%, $n = 5$)	Recovery (%)	MRLs (ng/g)
Danofloxacin	50	12	75	200	50	12	78	100
Ofloxacin	50	10	99	200	50	9	90	n.e
Enrofloxacin	50	11	65	100	50	10	68	100
Pipemidic acid	50	12	81	n.e	50	13	84	n.e
Flumequine	50	15	45	400	50	16	52	200

n.e: not established.

recoveries were in the range of 60–93%. The RSDs (intraday), summarized in Table 3, showed satisfactory levels.

The proposed method is sufficiently sensitive to analyse these quinolones in fish and chicken because the LOQs obtained were below the MRL (100–200 ng/g) established for these drugs in the Council Regulation 2377/90 of EU [3]. Figures 3, 4 show the chromatograms of a standard solution of the five quinolones at the LOQ level, an extract of chicken muscle spiked at the LOQ level, and an extract of an incurred chicken sample that contains enrofloxacin obtained using single quadrupole and QIT, respectively. As can be seen, no interfering peaks from endogenous tissue constituents or baseline aberrations were observed. Although the results obtained by QIT are more selective, single quadrupole also provide clean chromatograms without interfering peaks.

3.3 Application to different samples

Several chicken and fish samples were taken from local markets of the Valencia city. These samples comprised 25 of chicken and 15 of fish (5 trout, 5 golden fish, 5 see bass). All the fish samples were from fish farm. None of the studied quinolones was detected in those samples.

The measurement of enrofloxacin in muscle samples of chicken, after oral administration (enrofloxacin 5%) at a dose of 5 mg/g, confirmed the utility of the proposed analytical methodology. Figures 3C, 4C show CE-MS and CE-MS^3 electropherograms of treated chicken muscle extracts. The concentration found in the samples obtained 2 days after the treatment was *ca.* 80 ng/g.

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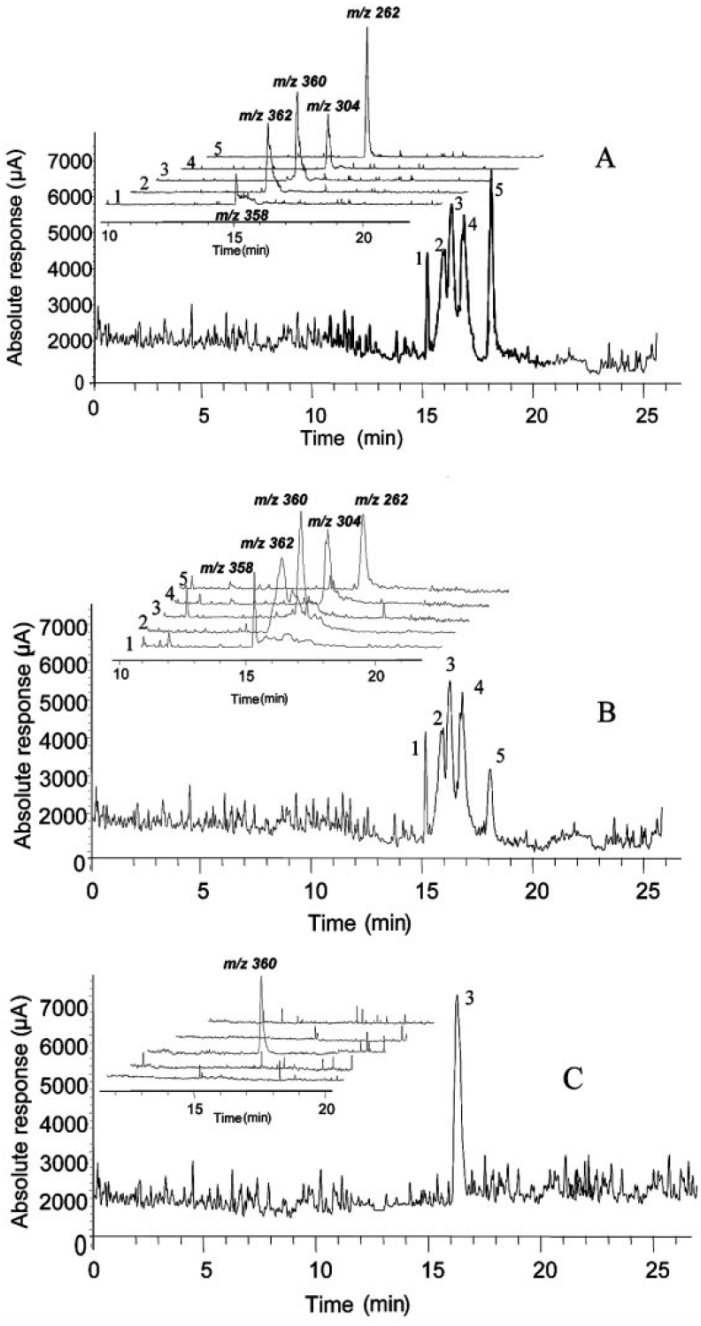

Figure 3. CE-ESI-MS electropherograms of a standard mixture of five quinolones at 0.5 μg/mL (A) of an extract of a chicken muscle spiked at 50 ng/g equivalent to a 0.5 μg/mL concentration in the injected extract (B), and incurred sample that contains 80 ng/g (C).

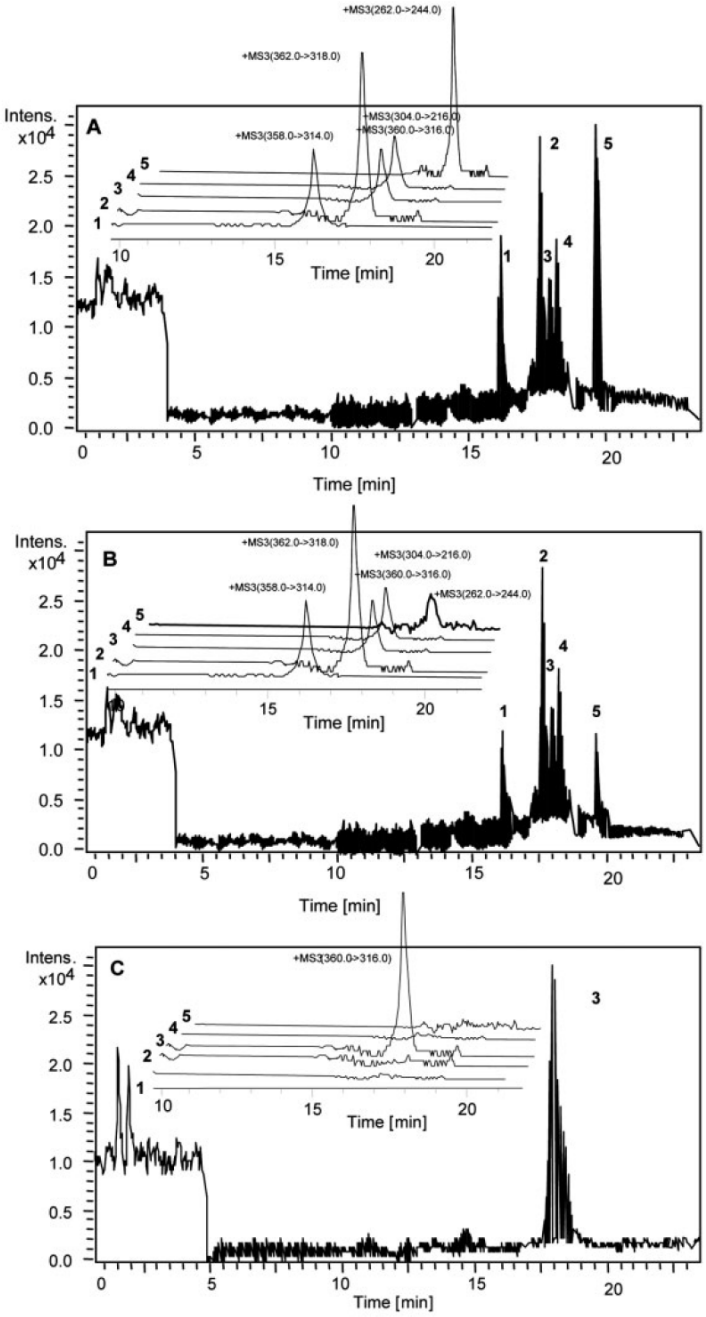

Figure 4. CE-ESI-MS^3 electropherograms of a standard mixture of five quinolones at 0.5 μg/mL (A) of an extract of a chicken muscle spiked at 50 ng/g (B), and incurred sample that contains 80 ng/g (C).

4 Concluding remarks

A simple, robust, and cost-effective universal CE-MS procedure for the analysis of quinolones has been developed using a common uncoated fused-silica capillary and a simple composition of alkaline volatile running buffer. The method presented in this paper, involving solvent extraction of quinolones in dichloromethane and SPE clean-up, followed by separation and detection of the quinolones by CE with on-line ESI-MS^3 detection is simple and does not require long sample preparation. Identification and quantification of five quinolones in chicken and fish muscle tissues were successfully achieved using CE-MS^3. The method was validated in fish and chicken muscles according to the Commission Decision No. 2002/657/CE. Satisfactory results were obtained with regard to selectivity, linearity, accuracy, and precision. LOQs lower than the MRLs set by the European Commission were attained. In addition, this method provides unequivocal detection and confirmation of different compound structures by isolating the corresponding ions and fragmented them, by collision with helium, to obtain characteristic product ions.

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5 References

[1] Woodward, K. N., in: Watson, D. H. (Ed.), *Pesticide, Veterinary and Other Residues in Food*, CRC Press, Boca Raton, Fl 2004, pp. 157–174.

[2] Botsoglou, N. A., Fletouris, D. J., in: Nollet, L. M. L. (Ed.), *Handbook of Food Analysis, Residues and Other Food Component Analysis*, Marcel Dekker, Ghent 2004, pp. 931–1037.

[3] Council Regulation (EEC) No 2377/90 of 26 June 1990 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuff of animal origen, *Off. J. Eur. Commun.* 1990, *L224*, 1–20.

[4] Council Directive 96/23/EC of April 1996 on measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EEC and 86/469/EEC and Decisions 89/18/EEC and 91/664/EEC, *Off. J. Eur. Commun.* 1996, *L125*, 10–18.

[5] Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning to the performance of analytical methods and the implementation of the results (Text with EEA relevance), *Off. J. Eur. Commun.* 2002, *L221*, 8–62.

[6] Stolker, A. A. M., Brinkman, U. A. Th., *J. Chromatogr. A* 2005, *1067*, 15–53.

[7] Belal, F., Al-Majed, A. A., Al-Obaid, A. M., *Talanta* 1999, *50*, 765–786.

[8] Hernandez-Arteseros, J. A., Barbosa, J., Compano, R., Prat, M. D., *J. Chromatogr. A* 2002, *945*, 1–24.

[9] Carlucci, G., *J. Chromatogr. A* 1998, *812*, 343–367.

[10] Simó, C., Barbas, C., Cifuentes, A., *Electrophoresis* 2005, *26*, 1306–1318.

[11] Juan-Garcia, A., Font, G., Picó, Y., *J. Sep. Sci.* 2005, *28*, 793–812.

[12] Beltran, J. L., Jimenez-Lozano, E., Barron, D., Barbosa, J., *Anal. Chim. Acta* 2004, *501*, 137–141.

[13] Saad, B., Mohamad, R., Mohamed, N., Lawrence, G. D. *et al.*, *Food Chem.* 2002, *78*, 383–388.

[14] Jimenez-Lozano, E., Roy, D., Barron, D., Barbosa, J., *Electrophoresis* 2004, *25*, 65–73.

[15] Barron, D., Jimenez-Lozano, E., Bailac, S., Barbosa, J., *J. Chromatogr. B* 2002, *767*, 313–319.

[16] Barron, D., Jimenez-Lozano, E., Bailac, S., Barbosa, J., *Anal. Chim. Acta* 2003, *477*, 21–27.

[17] Barron, D., Jimenez-Lozano, E., Cano, J., Barbosa, J., *J. Chromatogr. B* 2001, *759*, 73–79.

[18] Hernandez, M., Borrull, F., Calull, M., *Electrophoresis* 2002, *23*, 506–511.

[19] Horstkotter, C., Jimenez-Lozano, E., Barron, D., Barbosa, J., Blaschke, G., *Electrophoresis* 2002, *23*, 3078–3083.

[20] Schmitt-Kopplin, P., Englmann, M., *Electrophoresis* 2005, *26*, 1209–1220.

[21] Smyth, W. F., *Electrophoresis* 2005, *26*, 1334–1357.

[22] Schmitt-Kopplin, P., Frommberger, M., *Electrophoresis* 2003, *24*, 3837–3867.

[23] McCourt, J., Bordin, G., Rodriguez, A. R., *J. Chromatogr. A* 2003, *990*, 259–269.

[24] Juan-Garcia, A., Font, G., Picó, Y., *Electrophoresis* 2005, *26*, 1550–1561.

[25] Rodriguez, R., Manes, J., Picó, Y., *Anal. Chem.* 2003, *75*, 452–459.

[26] Hernandez, M., Borrull, F., Calull, M., *J. Chromatogr. B* 2000, *742*, 255–265.

[27] Schmitt-Kopplin, P., Burhenne, J., Freitag, D., Spiteller, M., Kettrup, A., *J. Chromatogr. A* 1999, *837*, 253–265.

[28] Fernandez, F. M., Vadillo, J. M., Kimmel, J. R., Wetterhall, M. *et al.*, *Anal. Chem.* 2002, *74*, 1611–1617.

[29] Frommberger, M., Hertkorn, N., Englmann, M., Jakoby, S. *et al.*, *Electrophoresis* 2005, *26*, 1513–1522.

[30] Nakata, H., Kannan, K., Jones, P. D., Giesy, J. P., *Chemosphere* 2005, *58*, 759–766.

[31] Volmer, D. A., Mansoori, B., Locke, S. J., *Anal. Chem.* 1997, *69*, 4143–4155.

[32] Schilling, J. B., Cepa, S. P., Menacherry, S. D., Bavda, L. T. *et al.*, *Anal. Chem.* 1996, *68*, 1905–1909.

[33] Toussaint, B., Bordin, G., Janosi, A., Rodriguez, A. R., *J. Chromatogr. A* 2002, *976*, 195–206.

[34] van Vyncht, G., Janosi, A., Bordin, G., Toussaint, B. *et al.*, *J. Chromatogr. A* 2002, *952*, 121–129.

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Pressurized liquid extraction combined with capillary electrophoresis–mass spectrometry as an improved methodology for the determination of sulfonamide residues in meat

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Abstract

A new analytical method, based on capillary electrophoresis and tandem mass spectrometry (CE–MS^2), is proposed and validated for the identification and simultaneous quantification of 12 sulfonamides (SAs) in pork meat. The studied SAs include sulfathiazole, sulfadiazine, sulfamethoxypyridazine, sulfaguanidine, sulfanilamide, sulfadimethoxyne, sulfapyridine, sulfachloropyridazine, sulfisoxazole, sulfasalazine, sulfabenzamide and sulfadimidine. Different parameters (i.e. separation buffer, sheath liquid, electrospray conditions) were optimized to obtain an adequate CE separation and high MS sensitivity. MS^2 experiments using an ion trap as analyzer, operating in the selected reaction monitoring (SRM) mode, were carried out to achieve the required number of identification points according to the 2002/657/EC European Decision. For the quantification in pork tissue samples, a pressurized liquid extraction (PLE) procedure, using hot water as extractant followed by an Oasis HLB cleanup, was developed. Linearity (r between 0.996 and 0.997), precision (RSD < 14 %) and recoveries (from 76 to 98%) were satisfactory. The limits of detection and quantification (below 12.5 and 46.5 $\mu g\,kg^{-1}$, respectively) were in all cases lower than the maximum residue limits (MRLs), indicating the potential of CE–MS^2 for the analysis of SAs, in the food quality and safety control areas.

Keywords: Antibacterials; Sulfonamides; Capillary electrophoresis; Mass spectrometry; Food; Animal tissues

1. Introduction

Sulfonamides (SAs) are analogues of the *para*-aminobenzoic acid (PABA) that act inhibiting the folic acid synthesis in susceptible micro-organisms. These compounds are not only employed to avoid bacterial proliferation and infections in food-producing animals, but also in the livestock industry to promote growth. SAs are often overused because they are inexpensive and readily available. In fact, several monitoring programmes have shown the presence of these compounds at trace levels in animal food products, which implies a human health risk [1,2]. Nowadays, SAs are among the priority pollutants to be monitored in animal derived food products, as well as in a wide variety of matrices, because their undesirable residues can remain and be incorporated into waters, soils, crops, animal tissues, and bio-fluids (milk and plasma) [3,4]. In Europe and the USA, an acceptable maximum residue limit (MRL) of 0.1 mg kg^{-1} total SA has been set in the target tissues of muscle, fat, liver and kidney [5,6].

For detecting SAs in food, microbial inhibition tests, biosensors and immunoassays are widely used as screening methods [7–9]. The use of these tests removes the need for time consuming extraction procedures and provides simplicity and economy, but they provide only semiquantitive measurements of detected residues and sometimes give rise to false positives [7]. Nevertheless, these methods need to be supported by highly selective and sufficiently sensitive analytical techniques before samples are condemned for containing levels of SA exceeding the MRLs [9].

Their analytical determination generally requires the use of lengthy extraction procedures prior to analysis [2,3]. Recently, the application of pressurized liquid extraction (PLE) for the extraction of SA residues has been described [10–15]. Bogialli et al. [10–12] and Gentili et al. [13] illustrated the extraction of SAs by rapid and simple PLE procedures with heated water as extractant in milk, eggs, fish and meat. In the same way, Berardi et al.

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[14] and Stoot et al. [15] outlined the use of PLE with mixtures of methanol–water for SAs concentration in cheese, soil and sediments. All groups remarked the benefits of the technology in offering rapid and reliable analysis.

The most widely applied analytical technique to analyze these compounds is liquid chromatography (LC) with fluorescence or mass spectrometry (MS) [16–20]. The former have a low limit of detection but the technique certainly requires derivatization to improve the fluorescence properties for detection. In addition, some SAs cannot be separated and the detection is non-specific [17]. Instead, LC–MS using electrospray (ESI) and tandem mass spectrometry (MS/MS) has been successfully applied to the determination of some SAs in milk, chicken liver, swine muscle tissue and shrimps [18–20]. This technique, contrarily to the previous one, is enough sensitive and selective not only to quantify but also to confirm the identity of the residues according to the European Commission Decision 2002/657/EC [21].

In this context, capillary electrophoresis (CE) techniques are attractive in terms of resolution and analysis time, and present some advantages over the LC methods, such as great separation power [22,23]. In fact, some papers have been published using CE–UV to separate SAs in different matrices, and two of them report successful applications to determine their residues in meat at levels below the MRLs. Fuh and Chu [24] described a conventional CE–UV with solid-phase extraction (SPE) method for the quantitative determination of eight SAs in meat. Soto-Chinchilla et al. [25] proposed CE with UV-detection, using large volume sample stacking with polarity switching, to determine nine SAs in meat. This procedure combined with a solvent extraction/SPE method, for off-line preconcentration and clean-up, provides a significant improvement in the LODs. CE can also be, as LC, coupled with MS achieving similar sensitivity, selectivity and specificity [26]. Bateman et al. [27] characterized isomeric SAs using CE coupled with nano-ESI quasi-MS/MS/MS in milk extracts. However, the approach is only superficially tested. Recently, Santos et al. [28] published a methodology for the screening and analytical confirmation of seven SA residues in milk using CE–MS. The method involves the use of a continuous flow system in order to automate and simplify the sample treatment step.

In this paper, a method based on pressurized liquid extraction (PLE) with CE–MS^2 detection has been developed and used for the determination of 12 SAs (Fig. 1) in pork meat. This combines the simple, fast and automatic extraction by pressurized liquids with the great efficiency and expeditiousness of CE, and the high sensitivity and selectivity of MS/MS detection using an ion trap (QIT). To our knowledge, this combination of techniques and the ability of CE-QIT–MS^2 to meet the confirmation requirements of the European Commission Decision [21] have not been tested yet.

2. Materials and methods

2.1. Chemicals

Sulfathiazole, sulfadiazine, sulfamethoxypyridazine, sulfaguanidine, sulfanilamide, sulfadimethoxyne, sulfapyridine,

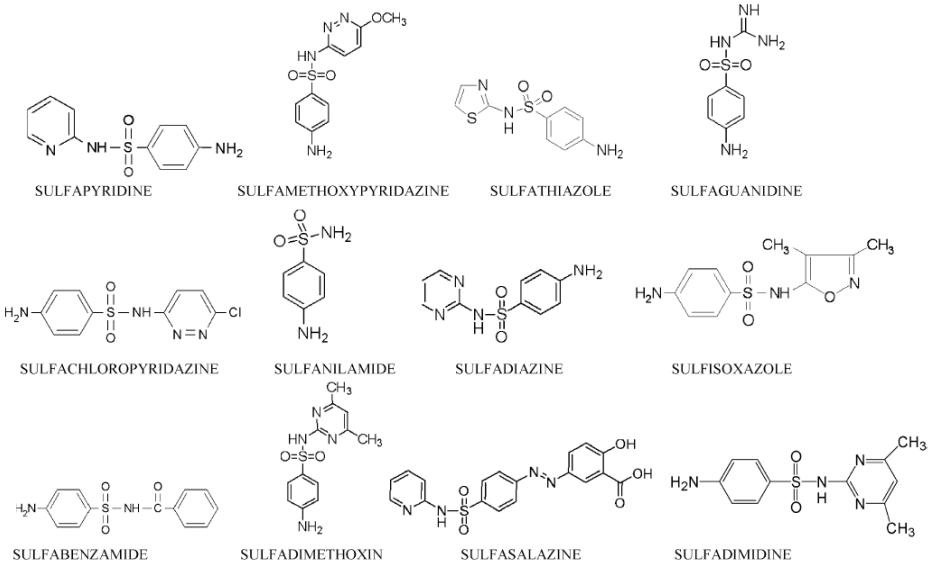

Fig. 1. Chemical structure of the SAs.

sulfachloropyridazine, sulfisoxazole, sulfasalazine, sulfabenzamide and sulfadimidine, were obtained from Riedel de-Haën (Seelze, Germany). Individual standard stock solutions of 1000 mg ml^{-1} were prepared in methanol and stored in stained glass-stopper bottles under refrigeration at 4 °C. Standard working mixtures at various concentrations were daily prepared by appropriate dilution of aliquots of the stock solutions in the running buffer.

All chemicals and solvents used in CE–MS and during the extraction process were of analytical grade or HPLC quality. Ammonium carbonate and ammonium acetate were purchased from Fluka (Madrid, Spain). Diatomaceous earth (particle size 13 μm) was from Sigma (Steinheim, Germany). Formic acid and acetic acid were from Panreac (Barcelona, Spain). Methanol was from Merck (Darmstadt, Germany). Deionised ultra pure water (<18.2 MΩ cm resistivity) was obtained from the Milli-Q SP Reagent Water System (Millipore, Bedford, MA, USA). Oasis HLB cartridges [poly(divinylbenzene-*co*-*N*-pyrrolidone), 200 mg sorbent, 6 ml cartridges] was also purchased from Waters. Nylon filters (0.45 μm) were purchased from Análisis Vínicos (Tomelloso, Spain).

2.2. Instrumentation

Experiments were carried out using an P/ACE MDQ capillary electrophoresis system (Beckman Instruments, Fullerton, CA, USA) with an Esquire 3000 Ion Trap LC/MS(n) system (Bruker Daltonik GmbH, Bremen, Germany). The coupling of CE to MS was carried out using a G1607 Agilent CE–MS sprayer kit, an HP1100 binary pump, and a special external detector adapter (EDA) cartridge (Beckman), as has been described elsewhere [29,30].

All separations were conducted using 2 ml vials for rinsing, washing, and buffering, and 500 μl polypropylene vials as sample vials. Samples were injected by applying a pressure of 1 psi for 10 s. Separations were carried out in an uncoated fused-silica capillary (Supelco, Madrid, Spain) with 75 cm total length, 50 cm thermostated, 25 cm at room temperature, 75 μm I.D., and 375 μm O.D. The polyamide coating was removed from the end of the capillary (ca. 5 mm) to maintain a stable electrospray. The applied voltage was +23 kV, and the capillary temperature was maintained at 20 °C. The electrolyte was 50 mM ammonium acetate at pH 4.16. At the beginning of each run, the capillary was conditioned by flushing for 10 min with 0.1 M NaOH, and 10 min with the electrolyte.

The coaxial sheath liquid was methanol–water–formic acid with 49.5:49.5:1 (v/v/v), portions delivered at 10 μl min^{-1} by the pump equipped with a 1:100 splitter. Electrospray ionization/MS was conducted in the positive ion mode, and the capillary voltage was set at 4.0 kV. Dry nitrogen gas was heated to 150 °C and delivered at a flow rate of 10 l min^{-1}. The pressure of nebulizing nitrogen gas was set at 10 psi. The lenses and block voltages were fixed using the smart option of the tune page. The mass spectra, in the Esquire 3000, were recorded programming the trap in ion charge control mode to accumulate 20,000 ions, for a maximum accumulation time of 50 ms. The m/z range scanned was from 100 to 500, and every 4 scans were summed. The mass spectrometer was operated in full scan and multiple reaction monitoring (MRM) modes.

Instrumental electrophoretic conditions were run by a System Gold software ver. 2.2, mass spectrometric parameters and data acquisition were managed by the Data Analysis ver. 3.1. package delivered by Bruker. The CE and MS instruments were connected and synchronized by an external pulse signal programmed from the electrophoresis.

2.3. Real sample analysis

Fifty porcine raw meat portions were brought from Valencian supermarkets and butchers' shops. In addition, to test the reliability of the developed method, samples of pork medicated with sulfadimidine were analyzed. Positive samples were obtained from animals treated with feed that contains 300 mg of the compound 100 g of feed for 5 consecutive days. Samples were from animals slaughtered just after medication.

2.4. Sample preparation

The extraction of antibacterial agents from meat was performed by PLE, using an ASE 200 system from Dionex (Sunyvale, CA, USA). For recovery studies, 10 g portion of chopped and minced pork muscle tissue was accurately weighed in a porcelain capsule and spiked with variable volume of stock solutions of SAs (from 10 to 100 μl), taken care to uniformly spread them on the sample. The spiked sample was left for 15 min at room temperature to ensure the appropriate distribution of SAs in the matrix. Then, the sample was blended with 10 g of diatomaceous earth using a pestle for ∼5 min, until a homogeneous material was obtained. This mixture was packed into a 22 ml pressure resistant stainsteel extraction cell of the ASE, which was sealed at one end with circular glass microfiber filters of 1.98 cm diameter (Dionex Co.). The packing of the extraction cell was carried out pouring the material into the tube in four aliquots. Each time, the cell was firmly tapped around its outer surfaces to ensure the homogeneous packing. Any void space, remaining after packing the solid material, was filled with diatomaceous earth and the cell was sealed with a glass microfiber filter.

The extraction cell was put in the ASE 200 instrument. Conditions used in the extraction for SAs compounds were as follows: time heating cell 8 min, time of solvent in contact with the sample 5 min (static time), pressure 1500 psi, temperature 160 °C, time purging with nitrogen to expulse rest of solvent in the cell 80 s, water volume flushing respect the cell size in percentage 100%, and one times cycled. At the end of each extraction a total extract volume of ca. 40 ml was obtained.

Clean-up and pre-concentration were carried out using Oasis HLB cartridge [poly(divinylbenzene-*co*-*N*-pyrrolidone), 200 mg sorbent, 6 ml cartridges] purchased from Waters, Milford, MA, USA. Column was conditioned first with 2 ml of methanol and then 2 ml of water. PLE extracts were passed through the Oasis column at approximately 2 ml min^{-1}, then, the column was washed with 2 ml of water and, finally, the SAs were eluted from the HLB-sorbent with 2 ml of methanol, evaporated

to dryness under a stream of nitrogen at 55 °C and re-dissolved in 1 ml running buffer. The extract was stored at 4 °C until injection at room temperature on the CE–MS for analysis.

2.5. Calibration and validation

The validation of the method was performed according with the EU guidelines [21]. The LODs and LOQ values of each SA were considered as the concentration giving a signal to noise ration of 3 and 10, respectively. For the determination of the linearity and accuracy of the method, choped and minced meat was spiked with the 12 SAs in the following concentrations 50, 75, 100, 200, 300, 500 $\mu g\,kg^{-1}$. Each sample was analyzed in triplicate and the calibration curves were generated by plotting the peak area versus the concentration. Day-to-day variation (relative standard deviation, RSD) on the slope of the calibration curves was calculated.

The accuracy of the method was determined as recovery at MRL/2, MRL and 2MRL levels. These recoveries were calculated by comparing the peak areas obtained from meat samples spiked before and after the sample preparation. The precision of the assay was determined by analyzing in quintuplicate the spiked samples at the three levels in 1 day (within-day precision or repeatability) or on different days (between days precision).

The decision limit (CCα) and the detection capability (CCβ) were determined by the calibration curve procedure [21]. CCα was calculated with a statistical certainty of $1-\alpha$ ($\alpha=0.05$), and CCβ was calculated with a statistical certainty of $1-\beta$ ($\beta=0.05$) to detect the concentration at the MRL level.

The confirmation criteria used for sulfonamide identification was the retention time of the chromatographic peak of each compound, the selected transitions (one specific isolation/fragmentation step, with at least two product ions in full scan mass spectrum), and the abundance ratio between the different ions. Quantification was carried out by conventional external standard procedure.

3. Results and discussion

3.1. Optimization of SAs separation

Previous to the coupling with the mass spectrometer, an optimization of the electrophoretic separation was carried out using a DAD detector. The requirement for the subsequent connexion of CE–MS, of using a volatile buffer of low conductivity (i.e. electric current below 50 μA) to obtain a stable electrospray and to avoid plugging of the dielectric capillary between the spray chamber and the mass spectrometer was taken into account. Only ammonium acetate, ammonium formate and ammonium carbonate buffers were studied, selecting the first one because better peak shapes were obtained. The pH and ionic strength of the background electrolyte and the addition of organic modifiers were considered to obtain the best selectivity, resolution and separation of the twelve SAs.

SAs are amphoteric compounds, and thus, behave both as acids and as bases depending on the pH. At high pH, the sulfonyl group, which is not directly attached to the ring, loses its proton to create a negative charge on the molecule. At low pH, the amine group is protonated and, therefore, a positive charge to the molecule is formed. The effect of pH (1.5–9.0) on the CE separation of SAs was evaluated. The results denoted that the examined pH range affected migrations of sulfixosazole, sulfadiazine and sulfanilamide in a greater extension than that of the other SAs. The migration time of each SA increased as pH rose from 6.0 to 9.0. In addition, the resolution among sulfasalazine, sulfabenzamide, sulfixosazole and sulfadimethoxyne decreased as the pH increased. Most of SAs could be baseline separated, with the exception of sufathiazole/sulfamethoxypyridine and sulfaguanidine/sulfanilamide, when pH is below 7.0. The 12 SAs had better separations in a pH 4.5 buffer, and the total separation was completed in less than 16 min.

The influence of the ammonium acetate concentration (15–60 mM) was also studied. The obtained results revealed that concentration of 50 mM provide a stable capillary current and good resolution. The consequence of adding an organic modifier to the background electrolyte on SAs separation was checked using different concentrations of methanol and acetonitrile, in the range of 5–30%. Imperceptible results were observed, reason why organic modifiers were not used. The effects of separation temperature and applied voltage on migration rate were studied, and 25 kV and 25 °C were utilized to obtain best separation. After optimization of the instrumental parameters, best separation of these SAs was obtained using 50 mM ammonium acetate/formic acid pH 4.5.

3.2. Optimization of CE–MS^n conditions

As it is mentioned above, coupling CE and MS implies the use of a sheath liquid, which permits a stable electrospray and the performance of the CE separation circuit. Different mixtures of volatile solvents, which provide electrical contact between CE and MS and a conductive modifier in terms of electrical contact, were tested. Mixtures of acetonitrile, methanol, ethanol, and 2-propanol with water were tested, all of them at 1:1, v/v and containing 1% formic acid. The presence of formic acid improved the signal of the SAs in the mass spectrometer because they are slightly basic compounds and the acidic pH favours their protonation. The highest intensity of the MS signal for the studied SAs maintaining, at the same time, the separation performance previously obtained by CE-DAD was achieved using methanol–water (1:1 v/v) with 1% formic acid. The influence of the methanol and formic acid percentages in the sheath liquid were studied. The percentage of formic acid had no remarkable effect on the SA signals, probably, because at very low percentages of formic acid they already are totally in their cationic forms. A different methanol percentage, in any of both senses, lower or higher, produced a negative effect on the response. Different parameters of the ESI source, such as nebulizer pressure, drying gas flow and drying gas temperature, were also tested. Drying gas temperature between 50 and 200 °C has not effect in the signal of the analytes. Higher drying gas temperatures provided a decreasing in the signal, probably because those extreme temperatures caused some degradation of the compounds. Nebulizer pressure (2–10 psi) and drying gas flow rate (2–10 $ml\,min^{-1}$) had a pos-

itive effect on the response. The flow rate of the sheath liquid was also studied. The sensitivity increased when decreasing the flow rate of the sheath liquid, but at the same time, the instability of the system is increased, until the point that it became unavailable to keep going because the fall of the electric current. The flow rate studied ranged from 1 to 20 μl min^{-1}, and it was established at 4 μL min^{-1} as the best compromise between a good sensitivity and system stability.

Once the spray conditions were established using MS mode, the optimization of the MS^2 mode was carried out. Taking into account the confirmation requirements established in the European Commission Decision [21], and that some peaks are not well-resolved, the multiple reaction monitoring (MRM) mode of the instrument was the preferred option. Fragmentation was carried by means of collision-induced dissociation (CID) of the $[M+H]^+$ ions with the helium present in trap for 40 ms. The fragmentation steps for each compound were optimized visualizing the changes in the intensities of fragments ions, whereas the fragmentation cut-off and the fragmentation amplitude were manually varied. MS^2 characteristics for confirmation are shown

Table 1
Main parameters of the MS/MS method, precursor and product ions obtained, ratios of the product ions (with regards to the higher intensity one) and earned identification points

SA	Precursor ion selected $[M+H]^+$ (*m/z*)	Product ion mass spectra (*m/z*)	Ions intensity (%)	Identification points (IPs)
Sulfasalazine	398	317	100	5.5
		156	80	
		108	20	
Sulfabenzamide	276	156	100	4
		108	20	
Sulfisoxazole	268	156	100	5.5
		113	20	
		108	20	
Sulfadimethoxyne	311	245	40	7
		218	28	
		156	100	
		108	15	
Sulfachloropyridazine	285	285	64	4
		156	100	
		108	20	
Sulfadiazine	251	251	10	5.5
		174	30	
		156	100	
		108	30	
Sulfamethoxypyridazine	281	265	20	11.5
		247	20	
		215	20	
		188	25	
		156	100	
		126	40	
		108	30	
Sulfathiazole	256	256	30	4
		156	100	
		108	20	
Sulfadimidine	279	279	20	5.5
		156	30	
		124	100	
		108	10	
Sulfapyridine	250	250	10	5.5
		184	70	
		156	100	
		108	20	
Sulfaguanidine	215	215	10	4
		156	100	
		108	15	
Sulfanilamide	173	173	10	4
		156	100	
		108	20	

in Table 1. As already reported [17–19,28,29], two major fragmentation pathways were observed in the MS^2 experiments by the bond cleavage between the sulfur and the nitrogen atom: the charge retention on the (4-aminophenyl) sulfonyl moiety to give generic ions at *m/z* 156 and 108, and the charge retention on the heteroaromatic anime moiety to give compound-specific ions.

3.3. Study of sample treatment

The utilization of PLE with hot water as extractant has proven to be suitable for the determination of SAs in meat, fish, milk, eggs and infant foods [10–13]. Among the solvents available for SAs extraction from pork tissues, water was chosen as extractant because of its low affinity towards fats and the polar character of the analytes. Additionally, water is cheap and environmental friendly. Temperature, extraction pressure, and static phase time were checked. Temperature (50–160 °C) was the most important parameter in the extraction. The improvement of the recoveries was directly proportional to the temperature, with optimum values at 160 °C.

The utilization of solid phase cartridges permits a sensitivity increase, preconcentrating the sample and eliminating some interferring compounds. This alternative has already been applied for the clean-up of SAs extracts from eggs and meat [12–16]. In this work, a polymeric phase cartridge (HLB) has been tested. The optimum retention was obtained at pH 7 observing elution of SAs at acidic and basic pH. Considering that the pH of the pork meat is between 6 and 7, it would be possible to apply the aqueous extract of the meat directly into the cartridge without the need of controlling pH. This procedure showed good results in terms of recoveries and peak shapes, and a preconcentration factor of 10 was achieved.

3.4. Validation of the method

Representative CE-MS^2 electropherogram of pork muscle sample, fortified with a mixture containing 100 μg kg^{-1} of each SA, is presented in Fig. 2. The product ions (*m/z* 156 and 108) are the same for all the SAs, which can lead to cross-talk interferences with the SAs that coelute. The absence of these interferences were checked analyzing pork tissues spiked with each SA individually at 100 μg kg^{-1}. The resulting CE–MS^2 electropherograms, which only showed response for the investigated SA, gave almost identical response.

The statistic parameters, calculated by least squares regression and the performance characteristics are presented in Table 2. The reproducibility study was carried out at three levels of concentration (50, 100 and 200 μg kg^{-1}) using spiked pork tissues before the extraction procedure, only the values corresponding to the MRLs are showed in Table 2. RSD values obtained were lower than 2.4% for migration times and lower than 3.8% for the peaks areas within the same day (i.e. repeatability), while day-to-day precision RSD values were lower than 3.8% for migration times and lower than 4.6% for peak areas, showing that the procedure is reproducible. The linearity of the response was established from six calibration levels, between 50 and 500 μg kg^{-1}, injecting each level in triplicate and intending to establish the MRLs in the middle of the linear calibration range. Although previous calibration curves using standards prepared in methanol showed that MS response was linear at least two orders of magnitude, the studied working range was considered appropriate since real pork tissue samples with higher SAs content only occurs rarely. Table 2 also shows the calibration parameters as, for instance, calibration curve, correlation coefficient (r), $S_{y/x}$ (standard deviation of residuals), LODs and LOQs. As it can be seen, a good linearity (r) higher than 0.996 was observed.

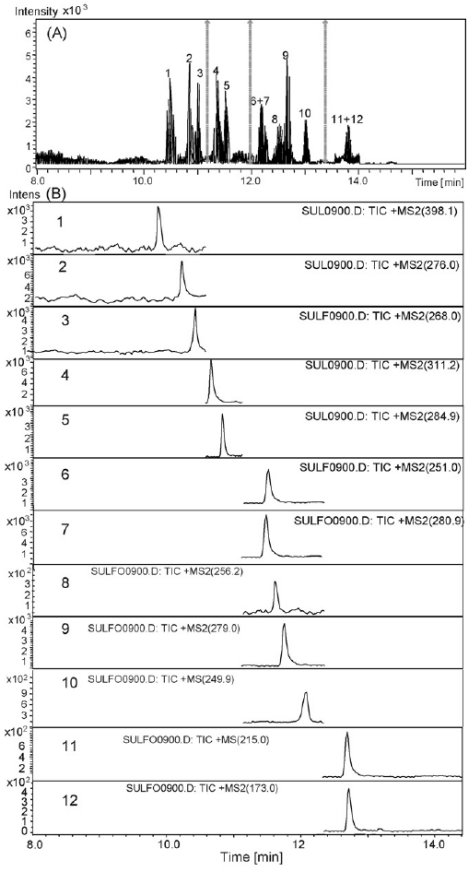

Fig. 2. CE–MS^2 electropherograms at optimum conditions obtained after PLE extraction and Oasis HLB clean-up and preconcentration of pork tissue samples spiked at 100 μg kg^{-1} of each SA (A) total ion electropherogram, and (B) extracted ion electropherograms. Peak identification: (1) sulfasalazine, (2) sulfabenzamide, (3) sulfisoxazole, (4) sulfadimethoxyne, (5) sulfachloropyridazine, (6) sulfadiazine, (7) sulfamethoxypyridazine, (8) sulfathiazole, (9) sulfadimidine, (10) sulfapyridine, (11) sulfaguanidine and (12) sulfanilamide. The dashed lines indicated each time segment programmed.

Table 2
Results of the intra-day and day-to-day precision study (both expressed as RSD%) (data given for 0.1 μg kg^{-1}), calibration data (six points, three replicated of each), LOD (S/N = 3) and LOQ (S/N = 10) obtained by analyzing spiked pork muscle

SA	t_m (min)	Intra-day precision (RSD %, n = 5)		Day-to-day precision (RSD %, n = 5)		Calibration equation	$S_{y/x}$	r^2	LOD (μg kg^{-1})	LOQ (μg kg^{-1})
		t_m	Area	t_m	Area					
Sulfasalazine	10.48	1.2	2.6	1.8	3.9	y = (5,535 ± 340)x − (56,571 ± 2,495)	0.032	0.997	6.25	21.3
Sulfabenzamide	10.95	1.3	2.8	2.0	3.6	y = (11,001 ± 1,125)x − (6,647 ± 1,025)	0.024	0.996	3.12	18.3
Sulfisoxazole	11.19	1.3	2.7	2.0	3.5	y = (18,750 ± 1,423)x − (34,845 ± 1,678)	0.039	0.997	1.56	14.0
Sulfadimethoxyn	11.42	1.2	2.9	1.8	3.7	y = (42,473 ± 3,628)x + (11,046 ± 985)	0.023	0.996	1.56	14.4
Sulfachloropyridazine	11.55	1.4	2.9	2.4	3.2	y = (7,299 ± 639)x + (12,974 ± 1,340)	0.045	0.996	3.12	18.7
Sulfadiazine	12.29	1.8	3.3	2.3	3.6	y = (11,732 ± 1,092)x + (2,783 ± 283)	0.021	0.996	1.56	14.6
Sulfamethoxypyridazine	12.31	1.7	3.5	2.7	3.9	y = (20,091 ± 1,762)x + (33,117 ± 592)	0.038	0.997	1.56	13.8
Sulfathiazole	12.63	1.9	3.2	2.6	4.2	y = (12,438 ± 1,098)x + (221 ± 102)	0.027	0.997	12.5	42.3
Sulfadimidine	12.84	1.6	3.0	2.8	4.3	y = (18,158 ± 1,535)x + (2,422 ± 523)	0.029	0.996	1.56	13.9
Sulfapyridine	13.04	2.0	3.8	2.8	4.6	y = (29,524 ± 2084)x − (6,110 ± 610)	0.036	0.996	5.56	21.3
Sulfaguanidine	13.82	2.2	3.8	3.8	4.6	y = (13,674 ± 1,032)x + (5,349 ± 1,007)	0.040	0.996	6.25	21.7
Sulphanilamide	13.84	2.4	3.6	3.4	4.6	y = (12,857 ± 1,054)x + (8,007 ± 1,029	0.042	0.996	6.25	22.0

Table 3
Mean recoveries at half MRL, MRL and twice MRL levels for the selected SAs in pork muscle

SA	Concentración added (μg kg^{-1})	Mean recovery, % (RSDs, %)
Sulfasalazine	50	79 (9)
	100	80 (6)
	200	82 (4)
Sulfabenzamide	50	76 (11)
	100	81 (8)
	200	83 (7)
Sulfisoxazole	50	82 (9)
	100	87 (5)
	200	87 (4)
Sulfadimethoxyne	50	90 (12)
	100	86 (9)
	200	84 (6)
Sulfachloropyridazine	50	90 (12)
	100	95 (8)
	200	93 (6)
Sulfadiazine	50	91 (10)
	100	93 (8)
	200	96 (6)
Sulfamethoxypyridazine	50	92 (15)
	100	93 (13)
	200	91 (9)
Sulfathiazole	50	100 (12)
	100	99 (8)
	200	98 (5)
Sulfadimidine	50	82 (10)
	100	85 (7)
	200	87 (6)
Sulfapyridine	50	97 (10)
	100	98 (10)
	200	100 (7)
Sulfaguanidine	50	79 (14)
	100	83 (9)
	200	85 (5)
Sulfanilamide	50	92 (12)
	100	93 (9)
	200	91 (8)

In order to test the efficiency of the sample treatment, recovery studies at three concentration levels (50, 100 and 200 μg kg^{-1}) were carried out. Five replicates at each concentration were prepared, each one was injected in triplicate. Absolute recoveries were calculated by comparing peak areas of the pork tissue samples spiked before the PLE procedure with peak areas of pork tissue samples spiked after the PLE procedure. Recoveries higher than 76% were obtained in all cases with acceptable RSDs. The results are shown in Table 3.

Table 4 shows the CCα values with an error of 5% (probability of false non-compliant ≤5%), considering the experimental standard deviation of within-laboratory reproducibility at the adequate contamination level and the CCβ values, calculated as the concentration at which the method is able to detect, identified and/or quantified MRL concentrations with a statistical

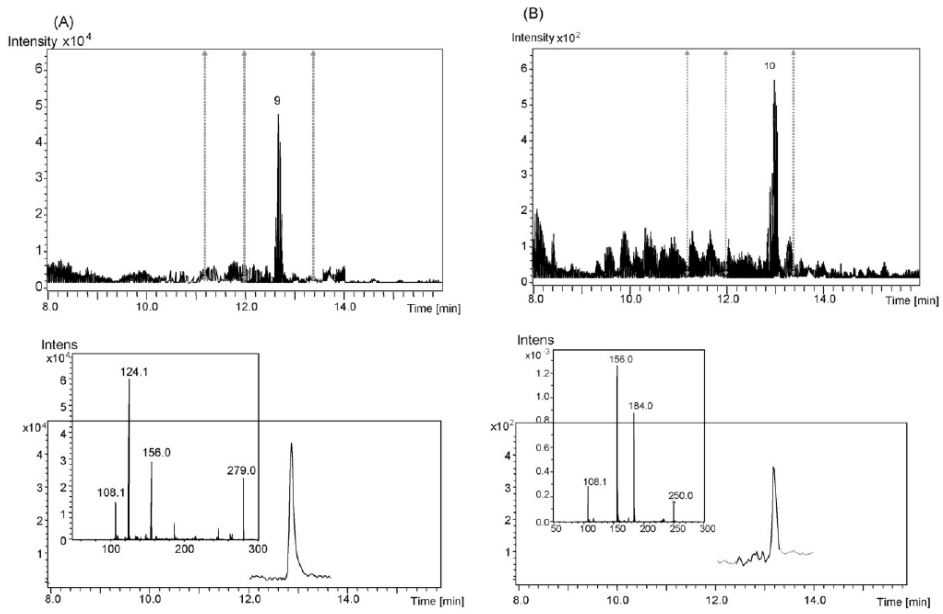

Fig. 3. CE–MS^2 electropherograms obtained after PLE extraction and Oasis HLB clean-up and preconcentration of pork tissue samples (A) treated with sulfadimidine (calculated concentration 400 $\mu g\,kg^{-1}$) and (B) from a butchery that contains sulfapyridine at 42 $\mu g\,kg^{-1}$. The upper electropherogram correspond to the total ion electropherogram. The lower to the extract ion electropherogram. The insert is the mass spectrum of each peak. Peak identification as in Fig. 2.

certainty of $(1-\beta)$, considering the standard deviation of the within-laboratory reproducibility of the mean measured content at the decision limits, with an error $\beta = 5\%$ (probability of false compliant samples $\leq 5\%$). Validation was according to the draft EU criteria.

3.5. Real sample analysis

The proposed method has been applied to determinate sulfadimidine in pork muscle samples from animals treated with this SA. Fig. 3A shows the electropherograms obtained from the extracts of the samples, from animals treated with SAs for 5 days and then slaughtered. The amount of sulfadimidine quantified was 400 $\mu g\,kg^{-1}$. As expected, in samples from animals immediately slaughtered, values of sulfadimidine are higher than MRL established by European Union.

In addition, 50 swine muscle samples, randomly collected from the markets of Valencia city, were analyzed by the PLE–CE–MS^2 method. Two samples were contaminated with SAs. One contained 42 $\mu g\,kg^{-1}$ of sulfapyridine and the other 34 $\mu g\,kg^{-1}$ of sulfadiazine. Fig. 3B shows the electropherogram corresponding to the sample that contains sulfapyridine. The product ion mass spectrum clearly confirms the identity of the compound.

Table 4
CCα and CCβ values for studied SAs in pork muscle (MRL = 100 $\mu g\,kg^{-1}$)

SA	CCα ($\mu g\,kg^{-1}$)	CCβ ($\mu g\,kg^{-1}$)
Sulfasalazine	103	104
Sulfabenzamide	103	106
Sulfisoxazole	103	105
Sulfadimethoxyne	102	105
Sulfachloropyridazine	104	106
Sulfadiazine	103	104
Sulfamethoxypyridazine	104	107
Sulfathiazole	104	106
Sulfadimidine	104	108
Sulfapyridine	104	107
Sulfaguanidine	104	108
Sulfanilamide	104	109

4. Conclusions

A sensitive and specific CE–MS^2 method is described for the determination of 12 SAs for veterinary use in pork meat samples below the MRLs as legislated by the EU. The proposed method eliminates the need to separate quantification and confirmation procedures, as required by most published methods for SAs. This method presents the advantages of application of CE as separation technique, such as simplicity and low cost combined with the characteristics inherent to MS detection. The utilization of PLE with hot water as extractant in combination with SPE to concentrate the samples without any doubt proves to be a

valuable alternative to long and tedious liquid–liquid extraction procedures. This sample treatment avoids the use of organic solvents or chlorinated acids harmful for the environment. This technique was comparable to the more conventional LC–MS/MS in terms of time consumption, sample preparation, selectivity and sensitivity. It gave shorter overall analysis time, provided good chromatographic resolution for the 12 SAs and adequate linearity. The developed method could be satisfactorily applied as routine procedure to identify and quantify SAs in laboratories of food quality and safety control, because of its robustness and feasibility, demonstrating the possibilities of CE–MS^2, scarcely used so far for quantification purposes.

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References

[1] K.N. Woodward, Pesticide, Veterinary and Other Residues in Food, Woodhead Publishing, Cambridge, 2000, p. 175.
[2] N.A. Botsoglou, D.J. Fletouris, Drug Residues in Foods, Marcel Dekker, New York, 2001, p. 85.
[3] A. Gentili, D. Perret, S. Marchese, Trends Anal. Chem. 24 (2005) 704.
[4] M. Gros, M. Petrovic, D. Barceló, Anal. Bioanal. Chem. 386 (2006) 941.
[5] Council Regulation (EEC) No. 2377/90 of 26 June 1990 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin, Off. J. Eur. Commun. L 224, 18.8.1990, p. 1.
[6] Food and Drug Administration, New animal drug for use in animal feed; Code of Federal Regulations, FDA, Washington, DC, 1988 (Title 21, Part 558).
[7] M. Farré, D. Barceló, Food Toxicants Analysis, Elsevier, The Netherlands, 2007, p. 599.
[8] W. Haasnoot, M. Bienenmann-Ploum, U. Lamminmäki, M. Swanenbutg, H. Van Rhijn, Anal. Chim. Acta 552 (2005) 87.
[9] S.Y. Ding, J.X. Chen, H.Y. Jiang, J.H. He, W.M. Shi, W.S. Zao, J.Z. Shen, J. Agric. Food Chem. 54 (2006) 6139.
[10] S. Bogialli, R. Curini, A. Di Corcia, M. Nazzari, M.L. Polci, J. Agric. Food Chem. 51 (2003) 4225.
[11] S. Bogialli, R. Curini, A. Di Corcia, M. Nazzari, R. Samperi, Anal. Chem. 75 (2003) 1798.
[12] S. Bogialli, R. Curini, A. Di Corcia, M. Nazzari, M. Sergi, Rapid Commun. Mass Spectrom. 17 (2003) 1146.
[13] A. Gentili, D. Perret, S. Marchese, M. Sergi, C. Olmi, R. Curini, J. Agric. Food Chem. 52 (2004) 4614.
[14] C. Berardi, S. Bogialli, R. Curini, A. Di Corcia, A. Lagana, J. Agric. Food Chem. 52 (2004) 4614.
[15] K. Stoot, H.P. Singer, S. Stettier, N. Hartmann, S.R. Mueller, C.H. Stamm, J. Chromatogr. A 1128 (2006) 1.
[16] L. Verzegnassi, M.C. Savoy-Perroud, R.H. Stadler, J. Chromatogr. A 977 (2002) 77.
[17] G. Stoev, A. Michailova, J. Chromatogr. A 871 (2000) 37.
[18] N. Van Eeckhout, J.C. Perez, C. Van Peteghem, Rapid Commun. Mass Spectrom. 14 (2000) 2331.
[19] N. Furusawa, Anal. Chim. Acta 481 (2003) 255.
[20] S.W. Yang, J.M. Cha, K. Carlson, J. Chromatogr. A 1097 (2005) 40.
[21] Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning to the performance of analytical methods and the implementation of the results (Text with EEA relevance), Off. J. Eur. Commun. L221, 2002, 8.
[22] A. Juan-Garcia, G. Font, Y. Pico, J. Sep. Sci. 28 (2005) 793.
[23] A. Cifuentes, Electrophoresis 27 (2006) 283.
[24] M.R.S. Fuh, S.Y. Chu, Anal. Chim. Acta 499 (2003) 215.
[25] J.J. Soto-Chinchilla, A.M. García-Campaña, L. Gámiz-Campaña, C. Cruces-Blanco, Electrophoresis 27 (2006) 4060.
[26] P. Schmitt-Kopplin, M. Englmann, Electrophoresis 26 (2005) 1209.
[27] K.P. Bateman, S.J. Locke, D.A. Volmer, J. Mass Spectrom. 32 (1997) 297.
[28] B. Santos, A. Lista, B.M. Simonet, A. Rios, M. Valcarcel, Electrophoresis 26 (2005) 1567.
[29] A. Juan-García, G. Font, Y. Picó, Electrophoresis 423 (26) (2005) 1550.
[30] A. Juan-García, G. Font, Y. Picó, Electrophoresis 27 (2006) 2240.