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DEPARTAMENTO DE QUÍMICA ANALÍTICA

METODOLOGÍAS AVANZADAS PARA LA DETERMINACIÓN DE 5-NITROIMIDAZOLES EN MUESTRAS ALIMENTARIAS, AMBIENTALES Y CLÍNICAS

PROGRAMA OFICIAL DE
DOCTORADO EN QUÍMICA

Maykel Hernández Mesa

Granada, 2016

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METODOLOGÍAS AVANZADAS PARA LA DETERMINACIÓN DE 5-NITROIMIDAZOLES EN MUESTRAS ALIMENTARIAS, AMBIENTALES Y CLÍNICAS

(Advanced methodologies for 5-NDZ determination in food, environmental and clinical samples)

por

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DEPARTAMENTO DE QUÍMICA ANALÍTICA
UNIVERSIDAD DE GRANADA

MEMORIA presentada para aspirar al Grado de Doctor en Química (Mención Internacional)

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VISADA en Granada a 25 de Enero de 2016

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

CERTIFICA:

Que el trabajo que se presenta en esta TESIS DOCTORAL, con el título de "METODOLOGÍAS AVANZADAS PARA LA DETERMINACIÓN DE 5-NITROIMIDAZOLES EN MUESTRAS ALIMENTARIAS, AMBIENTALES Y CLÍNICAS", ha sido realizado en los laboratorios del citado grupo bajo mi dirección y la de la Profesora Carmen Cruces Blanco, en el Departamento de Química Analítica de la Facultad de Ciencias de la Universidad de Granada y reúne todos los requisitos para poder optar al Grado de Doctor, con mención de "Doctor Internacional", dentro del programa de Doctorado en Química.

Fdo.: Dra. Ana Mª García Campaña

En Granada a 25 de Enero de 2016

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A mi madre

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"An essential condition for any fruitful research is the possession of suitable methods. Any scientific progress is progress in the method."

Mikhail Semenovich Tswett

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Goals of this Thesis

The goals of this Thesis are within the scopes of several research projects carried out in the last years and included within the research lines of the group in which this Thesis has been performed.

The main goal has been the development of novel, sensitive and selective analytical methods for the determination of several drugs belonging to the antibiotic family called 5-nitroimidazoles (5-NDZs). These compounds are prescribed in human medicine but their use in veterinary medicine is forbidden in the European Union. However, their use is allowed in other countries, so the control of their residues in food is a requirement in order to guarantee that current legislation is enforced. For such a reason, in this Thesis, several methods have been developed for the determination of 5-NDZ residues, including their metabolites, and which are suitable for their application in drug monitoring in biological fluids, food safety and residue determination in environmental field. Miniaturized techniques such as capillary electrophoresis (CE), capillary electrochromatography (CEC), and capillary liquid chromatography (CLC), as well as a high efficiency technique such as ultra-high performance liquid chromatography (UHPLC), have been selected for accomplishing this purpose. These techniques have been coupled to different detection techniques such as ultraviolet-visible (UV-Vis) spectrophotometry and tandem mass spectrometry (MS/MS). Furthermore, both on-line preconcentration (in the case of CE) and off-line preconcentration methods have been proposed, allowing sensitivity enhancement. Moreover, they result in new, more efficiency and environmental-friendly sample treatments for the analysis of 5-NDZ residues, according to the current trends in Green Chemistry.

The following specific goals of this Thesis must be remarked:

- ◊ Determination of 5-NDZs by CE-UV in environmental water samples and food products of animal origin, considering both off-line and on-line preconcentration techniques for overcoming the lack of sensitivity attributed to CE-UV.

◊ Demonstration of the potential of CE-MS/MS hyphenation for the control of 5-NDZ residues in biological fluids, considering the identification capacity of the selected detection.

◊ Proposal of an efficiency procedure for packing capillaries for their further application in CEC and consequently, evaluation of the potential of this technique coupled to UV detection for the determination of 5-NDZs.

◊ Evaluation of CEC-MS hyphenation for the therapeutic monitoring of these drugs (research carried out in the group of Dr. Salvatore Fanali during a predoctoral stay in the *'Istituto di Metodologie Chimiche (IMC)* of *Consiglio Nazionale delle Ricerche di Montelibretti'*, Rome, Italy).

◊ Use of CLC for the determination of 5-NDZs in food products as alternative to traditional HPLC.

◊ Investigation of the advantages of UHPLC coupled to both UV and MS/MS detection, for the quick quantification of 5-NDZ residues in products of animal origin intended to human consumption.

◊ Proposal of new, simple, quick, selected and environmental-friendly simple treated for the determination of 5-NDZs, including their metabolites, as alternative to the methods that have been previously reported. Evaluation of these simple treatments in different matrices with the aim of demonstrating their suitability in terms of selectivity and sensitivity.

Objeto de la memoria

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

El objetivo principal ha sido la puesta a punto de nuevos métodos de análisis sensibles y selectivos para un grupo de fármacos pertenecientes a la familia de los 5-nitroimidazoles (5-NDZs), empleados en medicina humana aunque de uso prohibido en veterinaria en la Unión Europea. Su aplicación si está permitida como antibiótico de uso veterinario en otros países productores y exportadores de alimentos de origen animal, lo que hace necesario el control de sus residuos con objeto de garantizar el cumplimiento de la legislación. Por ello, en esta Tesis, se han propuesto diversos métodos para su determinación y la de sus metabolitos, de aplicación en monitorización terapéutica de fármacos en fluidos biológicos, en calidad y seguridad alimentaria y en control medioambiental. Se han seleccionado técnicas de separación miniaturizadas, como la electroforesis capilar (CE), la electrocromatografía capilar (CEC) y la cromatografía líquida capilar (CLC), y de ultra eficacia, como es la cromatografía líquida de ultra resolución (UHPLC), acopladas a diferentes técnicas de detección como son la espectrofotometría ultravioleta-visible (UV-Vis) y la espectrometría de masas en tándem (MS/MS). Asimismo, en esta Tesis se proponen métodos de preconcentración tanto on-line (en el caso de la CE) como off-line, con los que se consigue un aumento de sensibilidad, aportando además tratamientos de muestra alternativos para el control de los 5-NDZs y sus metabolitos en diversas matrices, más eficaces y respetuosos con el medioambiente, de acuerdo con las nuevas tendencias de la Química Verde.

Como objetivos concretos de esta Tesis Doctoral destacan los siguientes:

- ◊ Evaluar la CE-UV para la determinación de 5-NDZs en aguas naturales y alimentos de origen animal, empleando técnicas de preconcentración off-line y on-line que mejoren la sensibilidad inherente a esta técnica.

◊ Demostrar la potencialidad del acoplamiento CE-MS/MS para el control de estos residuos en fluidos biológicos, fundamentalmente considerando la capacidad de identificación de la detección.

◊ Establecer un procedimiento eficaz de relleno de capilares para CEC y evaluar el potencial de esta técnica acoplada a la detección UV para la determinación de 5-NDZs.

◊ Acoplar la CEC a la detección mediante MS con objeto de estudiar las posibilidades que ofrece dicho acoplamiento en la monitorización terapéutica de estos fármacos (estudio desarrollado en el grupo del Dr. Salvatore Fanali durante la realización de una estancia predoctoral en el *'Istituto di Metodologie Chimiche (IMC)* del *Consiglio Nazionale delle Ricerche di Montelibretti'*, Roma, Italia).

◊ Emplear la HPLC capilar para la determinación de 5-NDZs en alimentos, como alternativa a la HPLC convencional.

◊ Explorar las ventajas de la UHPLC, tanto acoplada a la detección UV como a MS/MS como potente herramienta para la cuantificación rápida de residuos de estos compuestos en alimentos de origen animal.

◊ Proponer nuevos tratamientos de muestra más simples, rápidos, selectivos y respetuosos con el medioambiente para la determinación de 5-NDZs y sus metabolitos, como alternativa a los ya existentes y evaluarlos en los diferentes tipos de muestras considerados, con objeto de demostrar su validez en cuanto a la selectividad y sensibilidad requerida en este tipo de análisis.

Summary

The discovery of antibiotics has involved several advantages to human health, being recognized as a contributing factor to increased life expectancy. Moreover, the use of antimicrobial agents in veterinary medicine has improved animal health and welfare, and besides it has allowed an obvious reduction of economic losses caused by contagious livestock diseases. However, the extended use of antibiotics has led to the appearance of resistant bacteria which constitutes a major public health problem.

5-nitroimidazoles (5-NDZs) are a wide-spectrum antibiotic class used for treating infections due to anaerobic protozoan and bacteria. Their importance in human medicine has been evidenced by the inclusion of metronidazole (MNZ), which is the most representative 5-NDZ compound, in the 'World Health Organization (WHO) Model List of Essential Medicines'. Nevertheless, carcinogenic, genotoxic and mutagenic properties have been attributed to these compounds, and as a consequence, their use in veterinary medicine has been restricted within European countries. Although 5-NDZ residues are not allowed in food of animal origin according to Regulation (EU) No 37/2010, alerts about their presence in animal products destined to human consumption are still notified by the Rapid System of Food and Feed (RASFF) portal. Consequently, analytical methods are required for 5-NDZ determination in order to ensure food safety.

On the other hand, 5-NDZs are polar molecules that present low biodegradability, and therefore, they are liable to bioaccumulate. Current wastewater treatments have demonstrated to be inefficient for the removal of 5-NDZ residues, so these compounds are released into the environment. In fact, the occurrence of 5-NDZ residues in wastewater, surface water and groundwater has already been reported, but their risk to human health is still unknown. As a result, there is no legislation dealing with the control of 5-NDZs or other antibiotics in environmental sources. However, it is certainly important to monitor the concentration levels of these substances in the environment in order to evaluate their potential risk.

Based on the foregoing, novel analytical methods are presented in this Thesis for 5-NDZ determination in food, environmental and clinical matrices. The proposed methods are based on miniaturized separation techniques namely capillary electrophoresis (CE), capillary electrochromatography (CEC) and capillary liquid chromatography (CLC) because they involve low solvent consumption as it is required by the new trends in analytical chemistry. Furthermore, the use of ultra-high performance liquid chromatography (UHPLC) has also been investigated because it offers lower solvent consumption than traditional LC methods, but also because this technique allows separations with high efficiency and in short analysis time. In addition to ultraviolet/visible (UV/Vis) detection, mass spectrometry (MS) has been considered through this Thesis due to its advantages such as the unequivocal identification of the analytes. It is important to emphasize that it is presented, for the first time, the CE-MS and CEC-MS hyphenation for the evaluation of 5-NDZ determination. Moreover, quick, simple and environmentally-friendly sample treatments have been proposed in order to achieve high extraction efficiency and sample throughput.

The present Thesis has been divided into four different parts. **Part I** presents the benefits and adverse effects of the use of antibiotics (**Chapter 1**), as well as an overview of the recent analytical methods developed for 5-NDZ determination (**Chapter 2**). The experimental studies of this Thesis have been included in the other three parts according to the separation technique considered for the development of each method. Each part includes an introduction chapter, where the most relevant features of the selected separation technique are discussed.

Part II consists of an introduction and three chapters including the CE-based methods proposed for 5-NDZ determination. **Chapter 3** introduces the CE technique and describes the developed strategies for improving the low sensitivity inherent to CE methods and presents also CE-MS hyphenation.

In **Chapter 4**, a micellar electrokinetic chromatography (MEKC)-UV method has been proposed for the determination of up to nine 5-NDZ compounds, including metabolites, in milk and environmental water samples. The influence of various parameters affecting

MEKC separation (buffer pH, concentration and nature, surfactant concentration, capillary temperature and applied voltage) was investigated. Finally, 5-NDZ separation was accomplished at 20°C and 25 kV using a background solution (BGS) consisted of 20 mM phosphate buffer (pH 6.5) containing 150 mM of sodium dodecyl sulphate (SDS). In order to enhance signal sensitivity, separation was performed in a 'bubble' capillary (61.5-64.5 cm total length × 50 μm inner diameter (i.d.), 150 μm of optical path length). Analytical signals were monitored at 320 nm. Moreover, on-line preconcentration by sweeping was attempted, so samples were injected in BGS without micelles for 15-25 s at 50 mbar. On the other hand, solid phase extraction (SPE) using Oasis®HLB cartridges and dispersive liquid-liquid microextraction (DLLME) were considered as sample treatments for environmental water samples. SPE using Oasis®MCX cartridges was evaluated for 5-NDZ extraction from milk samples.

In **Chapter 5**, a novel on-line preconcentration technique has been assayed for 5-NDZ determination. The considered approach combines field-enhanced sample injection (FESI) and sweeping, and it is named cation selective exhaustive injection (CSEI)-sweeping. The proposed method consisted of rinsing the CE capillary with a low conductivity buffer (LCB; 50 mM phosphate buffer, pH 2.5), followed by a plug of a high conductivity buffer (HCB; 100 mM phosphate buffer, pH 2.5, 50 mbar, ≈ 31.5% of capillary volume) and a plug of water (50 mbar, 2 s). Afterwards, samples with lower conductivity than BGS were electrokinetically injected at 9.8 kV for 632 s. Finally, separation was carried out at -30 kV and 20°C using a 44 mM phosphate buffer (pH 2.5) containing 8% (v/v) THF and 123 mM SDS as BGS. This method was applied to the analysis of 5-NDZ residues in different matrices, and as consequence, different sample treatments were evaluated. DLLME was applied to water samples and egg samples were submitted to SPE, whereas diluted human urine and serum samples were directly analyzed.

Finally, in **Chapter 6**, a novel CE-ion trap (IT)-MS approach has been developed for the simultaneous determination of eleven 5-NDZs. A comparison between MEKC and capillary zone electrophoresis (CZE) was performed, obtaining higher selectivity when 1 M formic acid (pH 1.8) was selected as BGS. Using this BGS, 5-NDZs were hydrodynamically injected in water (40 s, 50 mbar) and separated at 28 kV and 25°C in a

fused silica capillary (110 cm × 50 μm i.d.). Furthermore, a pressure of 50 mbar was applied to the inlet vial during runs in order to improve migration time reproducibility. Electrospray ionization (ESI) in positive mode was selected, establishing a nebulization pressure of 7.4 psi, a dry gas flow of 6 L/min and a dry gas temperature of 160°C. A coaxial sheath-flow interface was employed for CE-MS hyphenation. Sheath liquid consisted of a mixture of 2-propanol/water/acetic acid (60.0:38.8:0.2, v/v/v) and was supplied at a flow rate of 3.3 μL/min. In addition, MS parameters were also optimized for analyte identification through their MS^2 and MS^3 spectra. The proposed method was applied to the determination of 5-NDZs in urine samples using molecularly imprinted solid phase extraction (MISPE) as sample treatment.

Part III consists of an introduction and two chapters that present the developed CEC-based methods for the analysis of 5-NDZ residues. In **Chapter 7**, CEC technique is described, with particular emphasis on capillary packing procedures and frit formation. Additionally, CEC-MS hyphenation is also introduced.

In **Chapter 8**, a novel CEC-UV method for the analysis of eight 5-NDZ compounds using laboratory made packed columns is described. Capillaries were packed at high pressure using acetone as driving solvent and C18 silica uncapped particles (5 μm) as packing material. Column frits consisted of sintered particles and were made by heating the packed column for 20 s with a nichrome ribbon (80% Ni – 20% Cr, 28 cm × 2 mm × 0.2 mm, electric resistance 1.3 Ω) connected to a 7 V AC power supply. Laboratory made columns (40 cm × 50 μm i.d.) were employed for the determination of 5-NDZs in milk samples which were submitted to salt-assisted liquid-liquid extraction (SALLE) followed by SPE prior to their analysis. Samples were hydrodynamically injected into the column (120 s, 11.5 bar) and 5-NDZs were separated in isocratic mode at 27 kV and 30 °C. Mobile phase consisted of a mixture 60:40 (v/v) acetonitrile (MeCN)/ammonium acetate buffer (2.5 mM, pH 5). Separation was monitored at 320 nm and was accomplished in less than 15 min.

Chapter 9 presents an experimental work carried out in collaboration with the group of Dr. Salvatore Fanali during a pre-doctoral stay in the *'Istituto di Metodologie Chimiche (IMC)* of

Consiglio Nazionale delle Ricerche di Montelibretti' (Rome, Italy). The separation of eight 5-NDZ family was carried out by means of CEC coupled to MS. Different stationary phases were studied, namely Lichrospher C18 (5 μm), Cogent™ Bidentate C18 (4.2 μm), Pinnacle II™ Phenyl (3 μm), and Pinnacle II™ Cyano (3 μm). Finally, Cogent™ Bidentate C18 (4.2 μm) gave the best performance when CEC-UV assays were carried out. For CEC-MS coupling, a laboratory assembled liquid-junction-nano-spray interface was used. Sheath liquid consisted of a mixture of 2-propanol/water (50:50, v/v) containing formic acid (0.05%, v/v). Under optimized CEC-ESI-MS conditions, the separation was accomplished under 15 kV using a mixture of 45:10:45 (v/v/v) MeCN/methanol (MeOH)/water containing ammonium acetate (5 mM, pH 5) as mobile phase. Separation was achieved within 22 min using a column packed with a mixture 3:1 (w/w) of Bidentate C18 (4.2 μm)/Lichrospher Silica-60 (5 μm). Additionally, a simultaneous hydrodynamic and electrokinetic injection (96 s, 8 bar, 15 kV) was considered. The proposed method was applied to the determination of MNZ, secnidazole (SCZ), and ternidazole (TRZ) in spiked urine samples that were submitted to SPE prior to their analysis. Furthermore, MS^2 spectrum was obtained for the selected antibiotics, providing the unambiguous confirmation of these drugs in urine samples.

Part IV consists of an introduction and two chapters that include the LC-based methods that have been proposed for 5-NDZ determination. **Chapter 10** introduces the LC technique, with particular emphasis on miniaturized LC-methods, especially CLC, and UHPLC technology.

In **Chapter 11**, the optimization a CLC-UV method is discussed. Parameters affecting the separation such as mobile phase composition, mobile phase flow rate, separation temperature and gradient program as well as the type of chromatographic column, were evaluated. Finally, the separation of eleven 5-NDZs was carried out in a Zorbax XDB-C18 (150 × 0.5 mm, 5 μm) column, using a mobile phase consisting of water (eluent A) and MeCN (eluent B) at a flow rate of 7 μL/min and a column temperature of 20°C. Analytical signal was monitored at 320 nm. Furthermore, full loop injection mode (8 μL) was selected and water was considered as injection solvent. The application of the optimized method was limited to the determination of nine 5-NDZ compounds, including three metabolites,

in aquaculture products, namely crab, salmon, prawn and swimming velvet crab. A MISPE procedure was evaluated for sample clean-up.

Chapter 12 presents an exhaustive study of the parameters involved in a SALLE procedure for the extraction of eight 5-NDZ antibiotics from milk samples prior to their analysis by UHPLC-UV. Ethyl acetate was used as extraction solvent whereas Na_2SO_4 was considered as salt agent. Once sample treatment was performed, sample extract was reconstituted in a mixture of MeCN/water (6:94, v/v) containing formic acid (0.1%, v/v) and was further analyzed by a novel UHPLC-UV method. An injection volume of 20 μL (full loop injection mode) was considered and separation was accomplished in a C18 Zorbax Eclipse Plus (50 mm × 2.1 mm, 1.8 μm) column within 8 min. Mobile phase flow rate was established at 0.45 mL/min and it consisted of a 0.1% (v/v) formic acid aqueous solution (eluent A) and MeCN containing 0.1% (v/v) formic acid (eluent B). In addition, the column was thermostatically controlled at 45 °C during the analysis. Analytical signals were monitored at 320 nm.

Finally a novel UHPLC-MS/MS method has been developed in **Chapter 13** for the determination of twelve 5-NDZ drugs in fish roe samples. A quick, simple, cheap and environmental-friendly sample treatment was proposed for 5-NDZ extraction, because only 5 mL of MeCN per sample were required for achieving this purpose. Separation was performed in a C18 Zorbax Eclipse Plus (50 mm × 2.1 mm, 1.8 μm) column in less than 4 min. Mobile phase consisted of 0.025% (v/v) formic acid aqueous solution as eluent A and pure MeOH as eluent B, and was supplied at flow rate of 0.5 mL/min. During the separation, column temperature was maintained at 25°C. Furthermore, samples were injected in a mixture of 95:5 (v/v) formic acid solution (0.025%, v/v)/MeOH and an injection volume of 17.5 μL was considered.

Resumen

El descubrimiento de los antibióticos ha supuesto grandes mejoras en la salud humana, siendo reconocido, este hecho, como uno de los factores causantes del aumento de la expectativa de vida. Además, el uso de agentes antimicrobianos en la ganadería y en general en la medicina veterinaria, ha contribuido a mejorar la salud y el bienestar de los animales, así como a disminuir los costes económicos causados por enfermedades contagiosas entre el ganado. Sin embargo, el uso extendido de los antibióticos ha supuesto la aparición de bacterias resistentes, lo cual constituye un problema de salud pública.

Los 5-nitroimidazoles (5-NDZs) son una clase de antibióticos de amplio espectro que se usan en el tratamiento de infecciones debidas a bacterias y protozoos anaerobios. Su importancia en la medicina humana ha quedado evidenciada mediante la inclusión del metronidazol (MNZ), que es el 5-NDZ más representativo, en la 'Lista de Medicinas Esenciales' elaborada por la Organización Mundial de la Salud (WHO). Sin embargo, a estos compuestos se le atribuyen propiedades carcinogénicas, genotóxicas y mutagénicas y como consecuencia, su uso en medicina veterinaria ha sido restringido dentro de los países europeos.

Por otra parte, y a pesar de que la presencia de residuos de 5-NDZs en alimentos de origen animal está prohibida de acuerdo con la Regulación (EU) 37/2010, todavía se siguen registrando alertas sobre este hecho en el portal del 'Sistema de Alerta Rápida para Alimentos y Piensos' (RASFF). Por este motivo, se requieren métodos analíticos para la determinación de 5-NDZs con el objetivo de garantizar la seguridad alimentaria.

Los compuestos 5-NDZs son moléculas polares que presentan baja biodegradabilidad, y por lo tanto, son susceptibles de ser bioacumulados. Los tratamientos de aguas residuales que se llevan a cabo actualmente han resultado ser poco eficaces para la eliminación de residuos de 5-NDZs, y por lo tanto estos compuestos son emitidos al medioambiente.

De hecho, ha sido descrita la presencia de residuos de 5-NDZs en aguas residuales, superficiales y subterráneas, aunque el riesgo que suponen para la salud humana aún es desconocido y no existe ningún tipo de legislación que regule y controle su presencia. Sin embargo, es importante la monitorización de los niveles de concentración de estas sustancias en el medioambiente con el objetivo de regular su riesgo potencial.

En base a lo anterior, en esta Tesis Doctoral se describen nuevos métodos analíticos para la determinación de 5-NDZs en matrices alimentarias, ambientales y clínicas. Los métodos propuestos están basados en técnicas miniaturizadas de separación, concretamente en la electroforesis capilar (CE), la electrocromatografía capilar (CEC) y la cromatografía capilar de líquidos (CLC), debido a que éstas implican un bajo consumo de disolventes tal y como es requerido por las nuevas tendencias en la Química Analítica. Además, el uso de la cromatografía de líquidos de ultra-alta resolución (UHPLC) también se ha investigado puesto que ofrece un menor consumo de disolventes además de dar proporcionar una alta eficacia en un tiempo de análisis corto. Junto con la detección basada en la absorción ultravioleta/visible (UV/Vis), a lo largo de esta Tesis se ha considerado la espectrometría de masas (MS) debido a las ventajas que aporta como sistema de detección, tales como la identificación inequívoca de los analitos. Es importante señalar que en esta Tesis Doctoral se presentan, por primera vez, los sistemas CE-MS y CEC-MS como una herramienta para la determinación de 5-NDZs. Además, se ha propuesto el uso de tratamientos de muestra rápidos, simples y poco contaminantes con el objetivo de alcanzar una alta eficacia en la extracción y un alto rendimiento en el tratamiento de muestra.

La presente Tesis se ha dividido en cuatro partes. La **Parte I** presenta los beneficios y los efectos adversos del uso de antibióticos (**Capítulo 1**), y da una visión general de los métodos analíticos que se han desarrollado recientemente para la determinación de 5-NDZs (**Capítulo 2**). Los estudios experimentales llevados a cabo en esta Tesis se han incluidos en las otras tres partes de acuerdo con la técnica de separación considerada para el desarrollo de cada uno de los métodos propuestos. Cada una de estas tres partes incluye un capítulo introductorio en el que se discuten las características más relevantes de la técnica de separación seleccionada.

La **Parte II** consiste en una introducción y tres capítulos que incluyen los métodos basados en CE propuestos para la determinación de 5-NDZs. El **Capítulo 3** introduce la técnica CE y describe las estrategias desarrolladas para mejorar la baja sensibilidad inherente a estos métodos, así como presenta el acoplamiento CE-MS.

En el **Capítulo 4**, se propone un método de cromatografía electrocinética micelar (MEKC)-UV para la determinación de hasta nueve 5-NDZs, incluyendo alguno de sus metabolitos, en muestras de leche y aguas naturales. En dicho capítulo se evalúa la influencia de varios parámetros que afectan a la separación (pH, concentración y naturaleza del tampón, concentración del surfactante, temperatura del capilar y voltaje aplicado). Finalmente, la separación de los 5-NDZs se ha llevado a cabo a 20°C y 25 kV usando como medio de separación un tampón fosfato (20 mM, pH 6.5) conteniendo dodecil sulfato sódico (SDS) con una concentración de 150 mM. Con el objetivo de aumentar la sensibilidad, la separación se llevó a cabo en un capilar 'burbuja' (61.5-64.5 cm longitud total × 50 μm diámetro interno (i.d.), 150 μm longitud de paso óptico). Las señales analíticas se registraron a una longitud de onda de 320 nm. Además, se evaluó una preconcentración 'on-line' mediante 'sweeping', por lo que se consideró una inyección durante 15-25 s a 50 mbar de las muestras disueltas en tampón de separación pero en ausencia de micelas. Por otra parte, se consideró la extracción en fase sólida (SPE) con cartuchos Oasis®HLB y la microextracción líquido-líquido dispersiva (DLLME) como tratamiento para las muestras de aguas. Para la extracción de los 5-NDZs de las muestras de leche, se evaluó la SPE usando cartuchos Oasis®MCX.

En el **Capítulo 5**, se ensayó una nueva técnica de preconcentración 'on-line' para la determinación de 5-NDZs. La estrategia considerada combina la inyección de la muestra en un campo eléctrico ampliado (FESI) y la preconcentración mediante 'sweeping', la cual se denomina inyección exhaustiva-selectiva de los cationes-sweeping (CSEI)-sweeping. El método propuesto consiste en el llenado del capilar con un tampón de baja conductividad (LCB; 50 mM tampón fosfato, pH 2.5), seguido de la inyección de un bolo de tampón de alta conductividad (HCB; 100 mM tampón fosfato, pH 2.5, 50 mbar, ≈ 31.5% del volumen del capilar) y finalmente de la inyección de un bolo de agua (50 mbar, 2 s). A continuación, las muestras, disueltas en una disolución con menor conductividad que el medio de

separación, se inyectaron electrocinéticamente a 9.8 kV durante 632 s. Finalmente, la separación se llevó a cabo a -30 kV y 20°C usando como medio de separación un tampón fosfato (44 mM, pH 2.5) conteniendo 8% (v/v) THF y 123 mM SDS. El método propuesto se aplicó al análisis de residuos de 5-NDZs en diferentes matrices, y como consecuencia, se evaluaron diferentes tratamientos de muestras. Se aplicó un método de extracción DLLME a las muestras de agua, mientras que para las muestras de huevo, se aplicó un método de extracción SPE. Por otra parte, las muestras de orina y suero se diluyeron y analizaron directamente.

Finalmente, en el **Capítulo 6**, se llevó a cabo el desarrollo de un nuevo método de CE acoplado a MS con trampa de iones (CE-IT-MS) para la determinación simultánea de once 5-NDZs. Además, se llevó a cabo una comparación de dos modos de separación, MEKC y CZE, obteniendo una mayor selectividad cuando se empleó una disolución de ácido fórmico 1 M como medio de separación. Usando este medio de separación, los 5-NDZs se inyectaron hidrodinámicamente en agua (40 s, 50 mbar) y se separaron a 28 kV y 25°C en un capilar de sílice fundida (110 cm × 50 μm i.d.). Además, se aplicó al vial de entrada una presión de 50 mbar durante la separación con el objetivo de mejorar la reproducibilidad de los tiempos de migración. Por otra parte, se empleó la ionización por electrospray (ESI) en modo positivo, estableciendo una presión de nebulización de 7.4 psi, un flujo del gas de secado de 6 L/min y una temperatura del gas de secado de 160°C. Así mismo, se empleó una interfase coaxial de líquido adicional para el acoplamiento CE-MS. El líquido adicional consistía en una mezcla 2-propanol/agua/ácido acético (60.0:38.8:0.2, v/v/v) y se suministró con un flujo de 3.3 μL/min. Además, se llevó a cabo la optimización de los parámetros relativos al espectrómetro de masas con el objetivo de identificar los analitos a través de sus espectros de MS^2 y MS^3. El método propuesto se aplicó a la determinación de 5-NDZs en muestras de orina utilizando como tratamiento de muestra la extracción en fase sólida con polímeros molecularmente impresos (MISPE).

La **Parte III** consiste en una introducción y dos capítulos que presentan los métodos basados en la CEC y que se han desarrollado para el análisis de residuos de 5-NDZs. En el **Capítulo 7**, se describe la técnica CEC, incidiendo en los procedimientos de empaquetado

de los capilares y la formación de las fritas. Asimismo, se introduce el acoplamiento CEC-MS.

En el **Capítulo 8**, se describe un nuevo método CEC-UV para el análisis de ocho compuestos 5-NDZs usando columnas empaquetadas en el laboratorio. Los capilares se empaquetaron a alta presión utilizando acetona como disolvente transportador y partículas C18 no-protegidas (5 μm) como material a empaquetar. Por otra parte, se consideró la sinterización de las partículas de la fase estacionaria para la fabricación de las fritas. La sinterización se llevó a cabo mediante el calentamiento de la columna con una cinta de cromo-níquel (80% Ni – 20% Cr, 28 cm × 2 mm × 0.2 mm, resistencia eléctrica 1.3 Ω) conectada a una fuente de voltaje de 7 V AC durante 20 s. Las columnas preparadas en el laboratorio (40 cm × 50 μm i.d.) se emplearon para la determinación de 5-NDZs en muestras de leche que fueron tratadas antes de su análisis mediante una extracción líquido-líquido asistida por sales (SALLE) seguida de una extracción SPE. Las muestras se inyectaron hidrodinámicamente en la columna (120 s, 11.5 bar) y los 5-NDZs se separaron en modo isocrático a 27 kV y 30 °C. Se empleó una fase móvil consistente en una mezcla 60:40 (v/v) acetonitrilo (MeCN)/tampón acetato amónico (2.5 mM, pH 5). La separación se monitorizó a 320 nm y se llevó a cabo en menos de 15 minutos.

El **Capítulo 9** presenta el trabajo experimental llevado a cabo en colaboración con el grupo del Dr. Salvatore Fanali durante una estancia predoctoral desarrollada en el *'Istituto di Metodologie Chimiche (IMC)* del *Consiglio Nazionale delle Ricerche di Montelibretti'* (Roma, Italia). La separación de ocho compuestos pertenecientes a la familia de los 5-NDZs se abordó usando la CEC acoplada a MS. En este método se evaluaron diferentes fases estacionarias, concretamente Lichrospher C18 (5 μm), Cogent™ C18 Bidentada (4.2 μm), Pinnacle II™ Phenyl (3 μm), and Pinnacle II™ Cyano (3 μm). Finalmente, en los estudios llevados a cabo mediante CEC-UV, se obtuvo mejor separación cuando se usó la fase estacionaria Cogent™ C18 Bidentada (4.2 μm). Para el acoplamiento CEC-MS se empleó una interfase 'nano' de unión líquida fabricada en el laboratorio. Por otra parte, el líquido adicional consistió en una mezcla 2-propanol/agua (50:50, v/v) conteniendo ácido fórmico (0.05%, v/v). Considerando las condiciones optimizadas del método CEC-ESI-MS, la separación se llevó a cabo a 15 kV empleando como fase móvil una mezcla 45:10:45 (v/v/v)

MeCN/MeOH/agua conteniendo acetato amónico (5 mM, pH 5). Finalmente, la separación se llevó a cabo en 22 minutos en una columna empaquetada con una mezcla 3:1 (m/m) C18 Bidentada (4.2 μm)/Lichrospher Silica-60 (5 μm). Asimismo, la inyección de las muestras se realizó mediante la combinación simultánea de una inyección electrocinética y una inyección hidrodinámica (96 s, 8 bar, 15 kV). El método propuesto se aplicó a muestras de orina dopadas con MNZ, secnidazol (SCZ), y ternidazol (TRZ), las cuales fueron tratadas mediante una extracción SPE, antes de ser analizadas. Además, el espectro de MS^2 se obtuvo para cada uno de los antibióticos seleccionados, obteniendo la identificación inequívoca de estos compuestos en las muestras de orina.

La **Parte IV** consiste en una introducción y dos capítulos que incluyen los métodos basados en la LC y que se han propuesto para la determinación de 5-NDZs. El **Capítulo 10** introduce la técnica LC, incidiendo en la miniaturización, especialmente en la CLC, así como en la tecnología UHPLC.

En el **Capítulo 11** se discute la optimización de un método de CLC-UV. Se evaluaron los distintos parámetros que afectan a la separación tales como la composición de la fase móvil, el flujo de la fase móvil, la temperatura de separación, el programa de gradiente así como el tipo de columna cromatográfica empleada. Finalmente la separación de once 5-NDZs se llevó a cabo en una columna Zorbax XDB-C18 (150 × 0.5 mm, 5 μm) bajo una temperatura de separación de 20°C, usando una fase móvil constituida por agua como eluyente A y MeCN como eluyente B, y suministrada con un flujo de 7 μL/min. Las señales analíticas se registraron a una longitud de onda de 320 nm. Además, se consideró una inyección de bucle completo (8 μL), en tanto que el disolvente de inyección considerado era agua. El método optimizado se aplicó a la determinación de nueve compuestos 5-NDZs, incluyendo tres metabolitos, en productos de acuicultura, concretamente cangrejo, salmón, gamba y nécora. Se llevó a cabo la evaluación de un procedimiento MISPE, como tratamiento de muestra.

El **Capítulo 12** presenta un estudio exhaustivo de los parámetros involucrados en un proceso SALLE para la extracción de ocho antibióticos 5-NDZs en muestras de leche, aplicado antes de su análisis mediante UHPLC-UV. Se consideró el uso de acetato de etilo

como disolvente de extracción, en tanto que el Na_2SO_4 se empleó como agente salino. Después de la aplicación del tratamiento de muestra, el extracto obtenido se reconstituyó en una mezcla MeCN/agua (6:94, v/v) conteniendo ácido fórmico (0.1%, v/v), y posteriormente se analizó mediante un nuevo método UHPLC-UV. Se consideró un volumen de inyección de 20 μL (modo de inyección de bucle completo) y la separación se llevó a cabo en una columna C18 Zorbax Eclipse Plus (50 mm x 2.1 mm, 1.8 μm) en 8 minutos. El flujo de la fase móvil se estableció en 0.45 mL/min y consistía en una disolución acuosa 0.1% (v/v) de ácido fórmico como eluyente A, y MeCN conteniendo 0.1% (v/v) ácido fórmico como eluyente B. Además, la columna se termostatizó a 45 °C durante el análisis y las señales analíticas se monitorizaron a 320 nm.

Además se ha llevado a cabo la determinación de doce compuestos 5-NDZs en huevas de merluza mediante un nuevo método UHPLC-MS/MS desarrollado en el **Capítulo 13.** En dicho capítulo se propone un tratamiento de muestra rápido, simple, barato y respetuoso con el medio ambiente para la extracción de 5-NDZs, dado que se necesitaron 5 mL de MeCN por muestra para lograr dicho objetivo. La separación se llevó a cabo en una columna C18 Zorbax Eclipse Plus (50 mm × 2.1 mm, 1.8 μm) en menos de 4 minutos. La fase móvil consistía en una disolución acuosa al 0.025% (v/v) en ácido fórmico como eluyente A y MeOH como eluyente B, usando un flujo de 0.5 mL/min. Durante la separación, la temperatura de la columna se mantuvo a 25°C. Además, las muestras se inyectaron en una mezcla 95:5 (v/v) de disolución acuosa de ácido fórmico (0.025%, v/v)/MeOH, siendo el volumen de inyección de 17.5 μL.

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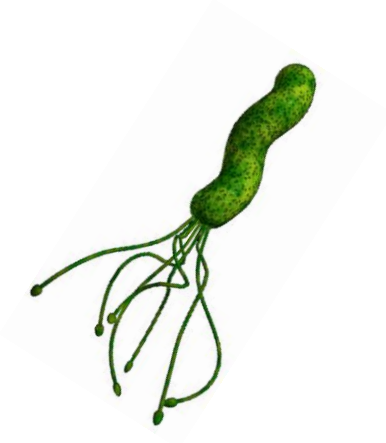
PART I

GENERAL INTRODUCTION



Chapter 1:

Analytical challenges on antibiotic determination

Chapter 2:

5-Nitroimidazoles

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1

Analytical challenges on antibiotic determination

1.1. Background

Since the beginning of history, the fight against microorganism infections has been one of the greatest challenges that humanity has faced. In Ancient Mesopotamia, China and Greece, the use of certain products such as medicine herbs or clays was quite common for the treatment of various diseases. Ancient Egyptian texts such as the 'Great Medical Papyrus' described the medical benefits of moldy wheaten preparations for healing pustular scalp infections and inflammations of the bladder and urinary tract [1]. However, it was not until the end of 19th century when specific microbial pathogens were identified as the causative agents of many diseases. The earliest known chemotherapeutic antibacterial discovery began in the 20th century by screening of compounds from the dye industry [2]. In 1910 the first antibacterial drug was released and it was known as Salvarsan, arsphenamine or sometimes just compound 606. It was discovered by Paul Ehrlich and it is an arsenic derivative of hydroxyl-aniline that made possible to treat syphilis [3].

Nevertheless, the 'Golden Age' of antibacterial discovery began in 1929 when Alexander Fleming published his observations about the effects of a mold identified as *Penicillium nonatum* over bacteria cultures. *Penicillium nonatum* produced a substance named penicillin, which was described as the active substance. Despite of his discovery, Fleming was unable to isolate and purify the penicillin. This achievement was accomplished by Ernst Chain and Howard Florey who extracted the first real sample of penicillin in 1941. Their extract was a million times more powerful than Fleming's original extract [1]. Fleming, Florey and Chain were awarded the Nobel Prize in Medicine in 1945.

On the other hand, in 1935, Gerhard Domagk from Bayer Laboratories discovered and developed the first sulfonamide, a synthetic red dye called by the trade name Prontosil. This fact supposed a great milestone that ushered the era of antibacterials. It was the first commercially available antibacterial, causing a true revolution in the treatment of bacterial infections. Several diseases such as streptococcal infections (including childbed fever and septicemia), pneumonia, meningitis, dysentery, gonorrhea, and urinary tract infections, were brought under a substantial measure of control by chemotherapy. As a result, Domagk's discovery saved many lives, including prominent figures such as Winston Churchill [4]. Domagk was awarded the Nobel Prize in Medicine in 1939.

Inspired by the introduction of penicillin and sulfa drugs, a wide number of antimicrobials were discovered. Although the isolation and development of the majority of antimicrobials was accomplished between the 1940s and the 1970s, further discoveries such as the introduction of ciprofloxacin in the mid-1980s changed the antibiotic market scene [5]. **Figure 1.1** summarizes the most relevant milestones on antibacterial agent discovery.

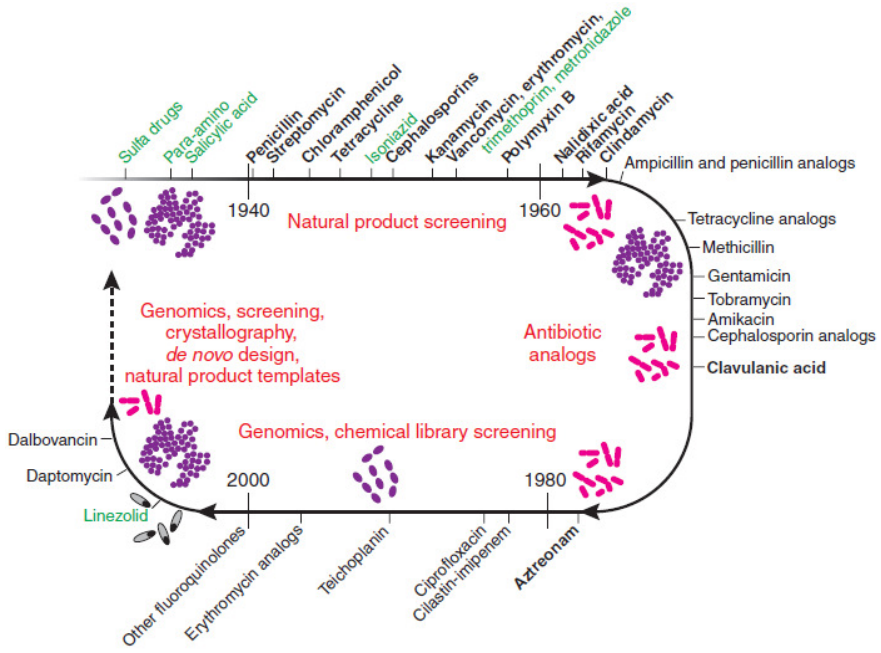

Figure 1.1. A timeline for antibiotic research. Major clinically used antibiotics and the type of resistant bacteria are selected for each decade. New antibiotic classes are shown in bold letters. Those of natural product origin are shown in black and those that are synthetic are in green. Technologies used in antibacterial discovery are shown in red letters. Gram-positive bacteria are in purple and Gram-negative bacteria are in pink. *Clostridium difficile* is shown in black. Reproduced from [5].

1.2. General features of antibiotic usage

The term 'antibiotic' derives from the Greek words ἀντί (anti, 'against') and βιωτικός (biōtikos, 'fit for life or lively'). At first, it referred to those substances of microbial origin that specifically inhibit the growth of other microorganisms. Nowadays, its usage has been extended to those compounds with low molecular-weight (Mw) that at low concentrations are able to kill or inhibit the growth of certain microorganisms. These compounds can be a microbial, a living organism's metabolite or a synthetic compound [1]. However, several authors limit the term antibiotic to those substances which have a microbial origin, so sulfonamides, quinolones and 5-nitroimidazoles (5-NDZs) cannot be included because they are synthetic molecules. Despite this, the extended definition of antibiotic has been accepted in this Thesis.

Antibiotic agents can inhibit the growth of bacteria (bacteriostatic), kill them without causing lysis or cell rupture (bactericidal), or induce killing by lysis (bacteriolystic). Bacteriolystic antibiotics can also inhibit cell synthesis and damage the cytoplasmic membrane. On the other hand, bacteriostatic antibiotics are frequently inhibitors of protein synthesis and act by binding to ribosomes. If the concentration of bacteriostatic antibiotics is lowered or if the antibiotic is removed, ribosomes are unblocked and bacterial cells resume growth. However, bactericidal antibiotics generally bind tightly to their cellular targets, and thus, bacteria are killed although antibiotic concentration is further decreased [1,6].

Most antibiotics usually attack a specific target that is peculiar to bacteria, interfering with the construction of the bacterial cell wall, the synthesis of protein, or the replication and transcription of deoxyribonucleic acid (DNA). Relatively few clinically useful drugs act at the level of the cell membrane or by interfering with specific metabolic processes of the bacterial cell [7]. In general, the most successful antibiotics attack mainly three targets or pathways: the ribosome (specifically the components of the ribosome known as 50S and 30S subunits), cell wall synthesis and DNA gyrase or DNA topoisomerase [8]. Therefore, antibiotics can be classified according to their target as shown in **Figure 1.2**. Furthermore,

those substances with similar molecular structure and/or identical mechanism of action have been grouped together under an antibiotic family name.

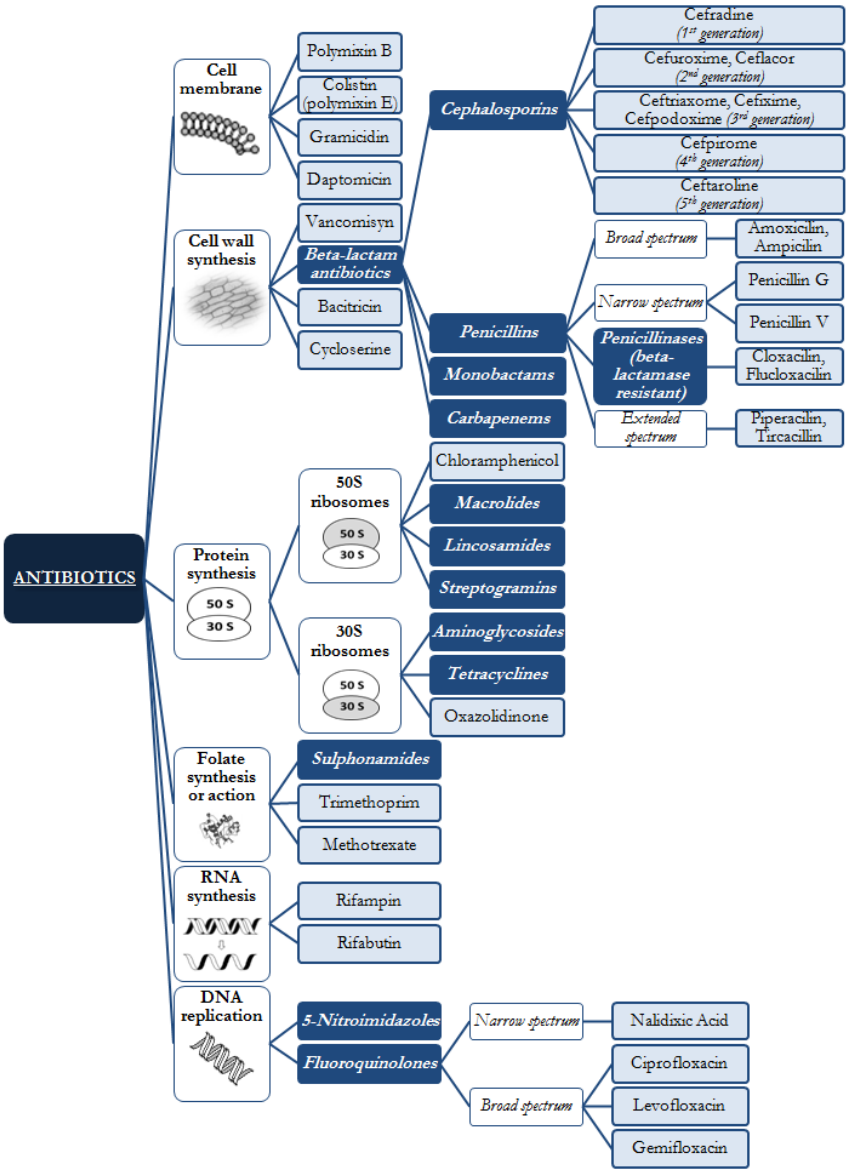

Figure 1.2. **Classification of antibiotic agents according to their site of action. White boxes indicate the target of the antibiotic on the bacteria, dark-blue boxes indicate antibiotic families, and light-blue boxes indicate a specific antibiotic compound.**

Without any doubt, antibiotics have become essential to human health. They have contributed to double the life expectancy from roughly 25 years to about 65 for men and 70 for women over the past two centuries [9]. Furthermore, the use of antibiotics has been

extended to veterinary medicine, improving not only animal health but also reducing economical losses due to contagious livestock diseases.

In 2010, a total of 73.6 billion antibiotic units, namely pills, capsules, and ampoules, were consumed in seventy-one countries, including the majority of European countries, United States of America (USA), Canada, Australia, Brazil, Russia, India, China and South Africa. Antibiotic consumption was increased about 36% in the first decade of the 21st century [10]. In order to express antibiotic consumption in units of mass, 500 mg can be considered as the average weight of a standard dose. Therefore, 36,800 tons of antibiotics were used by the humans in 2010. Considering that the majority of antibiotics are excreted in active form in the urine and/or feces, and with about half of the human population living in cities, about 15,000 tons of antibiotics are dumped yearly into the sewage [11]. As can be seen in **Figure 1.3**, penicillins, cephalosporins, macrolides, quinolones and tetracyclines, lead the list of antibiotic consumption in European Union (EU)/European Economic Area (EEA) countries.

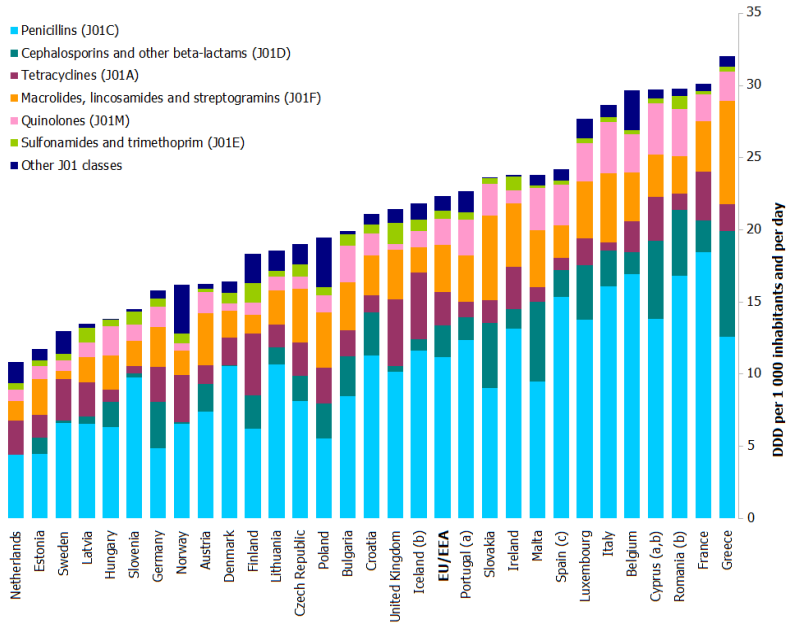

Figure 1.3. Consumption of antibiotics for systemic use by groups in 30 EU/EEA countries in 2013 (expressed in defined daily doses (DDD) per 1,000 inhabitants and per day). (a) For Cyprus and Portugal, data for 2012. (b) Cyprus, Iceland and Romania provided total care data (i.e. including the hospital sector). (c) Spain provided reimbursement data (i.e. not including consumption of antibiotics obtained without a prescription and other non-reimbursed courses). 5-NDZs are included in the group of others J01 classes. Reproduced from [12].

Furthermore, the fourth 'European Surveillance of Veterinary Antimicrobial Consumption' report has established overall sales of veterinary antimicrobial agents about 8,046.4 tons within twenty-six EU/EAA countries in 2012 [13]. This data includes drugs used in companion animals and food-producing animals, including horses. In food-producing animals, the most used antibiotics are tetracyclines, penicillins and sulfonamides, supposing up to 69% of the total consumption. Additionally, it reported the sold of substances such as chloramphenicol, metronidazole (MNZ), furazolidone, or furaltadone for veterinary medicine. They are prohibited substances in food-producing animals according to Regulation (EU) No 37/2010 [14], so it is important to control if their use has been restricted to veterinary medicine for companion animals or if they have been illegally employed for treating food-producing animals.

1.3. Adverse effects of antibiotic usage

In spite of the benefits of antibiotics, their extended use has led to the appearance of various adverse effects on human health. The main safety concern is the emergence of bacteria that are resistant to antibiotics, also named superbugs. The development of resistant mechanisms to antibiotics by bacteria has been widely explained [15,16]. Nowadays, there are not antibiotics for which resistance has eventually appeared and more than 70% of bacteria have shown to be insensitive to at least one antibiotic [17]. Initially the occurrence of resistant bacteria was a problem related to hospitals, but today it affects the world at large. As an example, some strains of disease-causing bacteria in USA may now be untreatable such as the vancomycin-resistant enterococcus, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, and *Acinetobacter baumanii* [18].

Alexander Fleming already warned about the dangers of rising levels of antibiotic resistance in his Novel Prize acceptance speech in 1945: "The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant" [19]. In fact, the emergence of resistant bacteria can be considered one of the most rapid and striking phenomena of biological evolution caused

by mankind [20]. Despite the bad health habits, there are other risky practices that have lead to the appearance of bacterial resistances.

Many antibiotics are applied in human medicine as well as they are used for treating bacterial infections in animals. They are usually applied at therapeutic levels for treating diseases in animals or in prophylactic treatments against bacterial infections in livestock. However, antimicrobials agents can also be used as growth promoters at subtherapeutic levels. Therefore it is clear that food-producing animals are exposed to antimicrobial agents, and consequently, it provides a great pathway for the emergence of resistant bacteria, especially if antibiotics are used as growth promoters. Resistance can emerge from the use of antibiotics in animals and the subsequent transfer of resistance genes and bacteria among animals and animal products [21]. As a result these resistant bacteria can infect the human population through the food chain and transfer their resistance genes to other bacteria belonging to the endogenous human flora [22]. For example, resistance related to foodborne pathogens such as *Salmonella* that is rarely transferred from person to person in industrialized countries, has emerged due to the use of antimicrobials in food-producing animals [23]. Additionally, several evidences have been recently reported about the presence of resistant bacteria in human coming from animals [24], and consequently, the occurrence of superbugs is considered an important human health concern.

Although the presence of certain antibiotics in food products of animal origin is allowed at certain levels (see **Section 1.4**), other compounds such as chloramphenicol, nitrofurans and 5-NDZs are strictly forbidden within EU countries [14]. Carcinogenic and genotoxic properties have been attributed to these pharmaceuticals. Therefore the use of these substances in food-producing animals has been banned with the aim of avoiding the exposure of people to them [25-27].

On the other hand, it is well known that antibiotics can cause hypersensitivity or allergic reactions. A large number of drug allergies are related to beta-lactam antibiotics that include penicillins and cephalosporins as shown in **Figure 1.2**. Up to 10% of patient population has been diagnosed with penicillin allergy [28]. Besides, allergic reactions such as urticaria, angioedema and anaphylaxis have been reported to fluoroquinolones,

macrolides, sulfonamides and tetracyclines [29]. All these antimicrobials are widely used in veterinary medicine as was previously mentioned (**Figure 1.3**), thus allergic reactions may also occur due to the presence of antibacterial agents in food products. However, these adverse effects are less common because if these substances are presented in food products of animal origin, it will be at low levels that do not show important allergenicity [30].

1.4. Legal framework of antibiotic residue control in food

The Food and Agriculture Organization of the United Nations (FAO) in the Codex Alimentarius Commission defines 'contaminant' as any substance not intentionally added to food, which is present in such food as a result of the production (including operations carried out in crop husbandry, animal husbandry, and veterinary medicine), manufacture, processing, preparation, treatment, packing, packaging, transport, or holding of such food or as a result of environmental contamination or from fraudulent practices [31]. Nevertheless, some substances are found in food as a result of their intentional use such as pesticides in food of plant and animal origin or veterinary medicines in food of animal origin. Therefore, these substances are referred to as 'residues'. It must be pointed that residues of veterinary drugs include the parent compounds and/or their metabolites in any edible portion of the animal product, and also include residues of associated impurities of the veterinary drug concerned [31].

As was stated in the above section, the presence of antibiotic residues in foodstuff of animal origin can suppose a risk to human health, and thus the sanitary authorities have launched several legislative actions in order to guarantee the food safety. Within EU countries, Regulation of European Commission (EC) No 178/2002 [32] can be regarded as the top in the hierarchy of EU food regulations and it establishes the framework of the EU food laws that allows the free movement of food and feed within the EU [33]. The objective of this regulation is to ensure a high level of protection for consumers whilst also taking into account the protection of animal health and welfare, plant health and the environment. It also establishes the European Food Safety Authority (EFSA) which is an independent scientific authority that provides advice and technical support for Community

legislation and policies in all fields which have a direct or indirect impact on food and feed safety. Moreover, it provides independent information on all matters within these fields and communicates on risks. Additionally, Regulation (EC) No 178/2002 establishes the Rapid Alert System for Food and Feed (RASFF) which notifies about any direct or indirect risk to public health derived from food or feed and involves EU national food safety authorities, EC, EFSA, European Space Agency (ESA), Norway, Liechtenstein, Iceland and Switzerland [34].

Moreover, it is required to ensure that food-producing animals are fed with quality in order to guarantee the level of animal health and consumer protection pursued by Regulation (EC) No 178/2002. Normally, additives are incorporated in the feedstuffs for enhancing their nutritive properties or improving the palatability of the diet, availability of ingredients, feed conversion and a healthy balance of the digestive tract's microflora, etc. Regulation (EC) No 1831/2003 [35] lays down on the procedure for approving the additives that can be used in feedstuffs. Furthermore, it established that antibiotics, other than coccidiostats and histomonostats, shall not be further authorized as feed additives, and besides, it has banned their used as growth promoters within EU countries since January 2006. Therefore, the use of antibiotics in food-producing animals within EU countries is only allowed at therapeutic levels for treating and preventing diseases. Nevertheless, the use of antibiotics as growth promoters has not been forbidden in countries such as USA, Japan, or Canada [36].

Additionally, Regulation (EC) No 726/2004 [37] lays down on Community procedures for the authorization, supervision and pharmacovigilance of medicinal products for human and veterinary use. Furthermore, the structure and the aims of the European Medicines Agency (EMA) have been established by this regulation. The main task of EMA is to provide scientific advice to the Community institutions and the member states in relation to the authorization and supervision of medicinal products for human and veterinary use [33]. Therefore, only those pharmacologically active substances that have received a favorable evaluation by EMA can be used in food-producing animals. Besides, EMA provides scientific advice on the use of antibiotics in food-producing animals in order to

minimize the occurrence of bacterial resistances and advices on the maximum residue limits (MRLs) of medicinal products which may be accepted in foodstuffs of animal origin.

The MRL is the maximum concentration of residue accepted by EU in a food product obtained from an animal that has received veterinary medicines or that has been exposed to biocide products for use in animal husbandry [38]. It is based on the type and amount of residue considered to be without any toxicological hazard for human health as expressed by the acceptable daily intake (ADI), or on the basis of a temporary ADI that utilizes an additional safety factor. It also takes into account other relevant public health risks as well as food technology aspects. Currently, Regulation (EC) No 470/2009 [39] lays down on the procedures for the establishment of MRLs for pharmacologically active substances intended for use in veterinary medicinal products.

On the other hand, the Directive 96/23/CE [40] has established the control measures and the alert plans to be applied for the detection of special substances and their residues, potentially toxic for the consumer, in alive animals or products of animal origin used in human feeding. The substances to be monitored in food and animal products have been classified in two groups according to the Annex I to Directive 96/23/CE. An overview of substances per category is presented in **Table 1.1**.

Table 1.1. Annex I to EU Directive 96/23/EC.

GROUP A **Substances having anabolic effect and unauthorized substances**	**GROUP B** **Veterinary drugs* and contaminants**
(1) Stilbenes, stilbene derivatives, and their salts and esters (2) Antithyroid agents (3) Steroids (4) Resorcylic acid lactones including zeranol (5) Beta-agonists (6) Compounds included in Annex IV to Council Regulation (ECC) No 2377/90 of June 1990 [41]	(1) Antibacterial substances, including sulfonamides, quinolones (2) Other veterinary drug *(a)* Anthelmintics *(b)* Anticoccidials, including nitroimidazoles *(c)* Carbamates and pyrethroids *(d)* Sedatives *(e)* Non-steroidal anti-inflammatory drugs (NSAIDs) *(f)* Other pharmacologically active substances (3) Other substances and environmental contaminants *(a)* Organochlorine compounds including PCBs *(b)* Organophosphorus compounds *(c)* Chemical elements *(d)* Mycotoxins *(e)* Dyes *(f)* Others

*Including unlicensed substances which could be used for veterinary purposes.

Furthermore, Directive 96/23/EC established the European laboratory infrastructure assigning, in addition to national reference laboratories, Community Reference Laboratories (CRLs). Nowadays, CRLs are called EU Reference Laboratories (EURLs) and they are responsible of the development and approval of validated methods and providing technical advice to national reference laboratories (NRLs). The tasks, duties and requirements for all the EURLs are indicated in Regulation (EC) No 882/2004 [42] that has been amended by Regulation (EC) No 776/2006 [43].

The list of MRLs for veterinary drugs in food, including antibiotics, has been recently revised by Regulation (EU) No 37/2010 [14]. It establishes the MRL allowed for each substance in different food products of animal origin such as in multiple animal tissues (e.g. muscle, liver, kidney and fat), milk, or eggs. All pharmacologically active substances that are allowed in food of animal origin are grouped on table 1 of this regulation. Furthermore, table 2 includes those substances that are strictly prohibited in foodstuffs of animal origin, and for which no maximum levels in food have been established. Table 2 of Regulation (EU) No 37/2010 replaces Annex IV of Regulation (ECC) No 2377/90.

In order to harmonize the analytical performance of analytical methods focus on banned substances, the concept of minimum required performance limit (MRPL) was introduced by Commission Decision 2002/657/EC [44]. It is defined as the minimum content of an analyte in a sample which at least has to be detected and confirmed. Official MRPLs were further established for chloramphenicol, nitrofuran residues and medroxyprogesterone acetate [45] and later for the sum of malachite green and its metabolite leuco-malachite green [46]. However, MRPLs have not been established for other banned antibiotics such as MNZ, ronidazole (RNZ) or dimetridazole (DMZ). Thus EURLs have made several recommendations about the concentration that should be detected for these and other compounds in certain food products of animal origin [47]. However, these recommendations are not legally binding. In any case, food inspectors in the EU have established to consider any foodstuff containing these substances as non-compliant within current EU legislation. This has been addressed as the policy of zero tolerance. The term itself does not appear in the current EU legislation [48].

Finally, technical guidelines and performance characteristics, such as detection level, selectivity, and specificity for residue control in the framework of Directive 96/23/EC are described in the Commission Decision 2002/657/EC [44], including additional requirements for confirmatory methods. It means the introduction of the concept of identification points in order to achieve the unambiguous identification of the legislated residues that are monitored. Three identification points are required for the identification of group B substances (see **Table 1.1**) whereas four identification points are claimed for the unequivocal determination of group A substances.

Mass spectrometry (MS) detection coupled to chromatographic techniques such as liquid chromatography (LC) or gas chromatography (GC) is the most adequate analytical tool for the determination and confirmation of any substance. Commission Decision 2002/657/EC assigns one identification point to the precursor ion and one and half identification points to the transition products when LC or GC coupled with low resolution MS is used. Therefore, in this case, one precursor ion and two transition products are required for the unequivocal identification of a group A substance such as 5-NDZs.

1.5. Importance of drug monitoring in environmental matrices

Antibiotics are released to the environment either directly, as a result of the disposal of unused medicines and their containers and from their use in aquaculture and the treatment of pasture animals, or indirectly during the land application of manure and slurry from livestock facilities [49]. However, in general, the majority of the antibiotics is discharged to the sewer systems together with the human urine and feces and enters the sewage treatment plants (STP). In spite of the sewage treatments stages carried out in them, they are insufficient for the complete removal of antibiotics that mainly occurs in secondary processes, such as biological filters and activated sludge [50]. Therefore, the effluents from urban STP are suspected to be among the main anthropogenic sources for antibiotics spread in the environment [51]. Recent studies have demonstrated the occurrence of antimicrobials agents in STP [50,52-54]. The main pathways followed by the antibiotics for entering into the environment are shown in **Figure 1.4**.

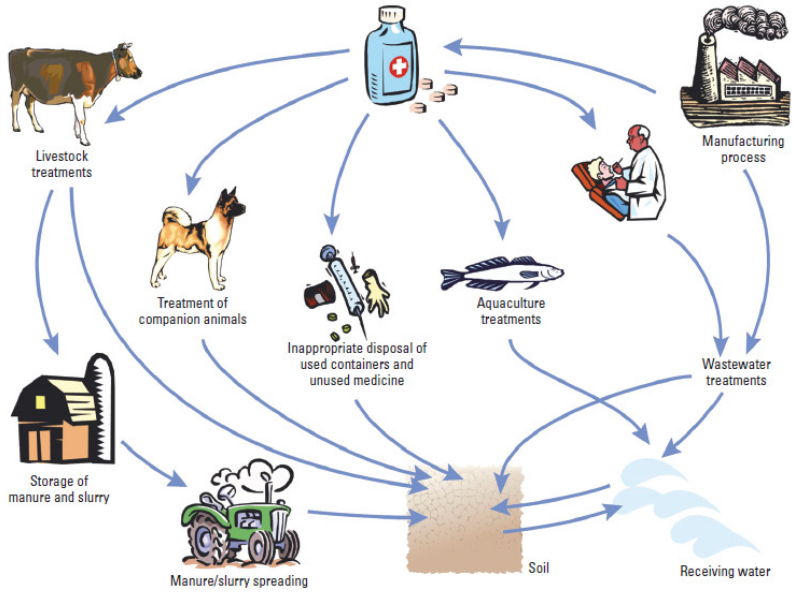

Figure 1.4. Major pathways of pharmaceutical release into the environment. Reproduced from [49].

Antimicrobial agents are present at low levels (typically < 100 ng/L) in the effluents of wastewater plants, and consequently, the risk of toxicity is low [55]. However, toxic effects due to long-term exposure to a combination of low concentrations of these compounds and other emerging pollutants are not totally understood. In this sense, the appearance of resistant bacteria should probably be considered the first concern with regard to the presence of antibiotics in STP and their subsequent release to the environment [56]. Additionally, the occurrence of antimicrobials agents in environmental waters and sediments has been already reported [57-59], and as a consequence, studies are required for the evaluation of their ecotoxicity and the possible risks to the human health due to their bioaccumulation. Concerning is the presence of antibiotics in groundwater [60,61], because it is the previous step toward their occurrence in drinking waters.

Drinking water should not contain any chemicals of anthropogenic origin, so the prudent use of antibiotics and the restriction of their input into the aquatic environment seem to be necessary [62]. It must be taken into account that Regulation (EC) No 178/2002 establishes that drinking water is included in the term 'food', and obviously it contributes to the overall exposure of a consumer to ingested substances, including chemical and microbiological contaminants, so a high protection for human health must be guaranteed.

Nowadays the quality of the water within EU countries is regulated by the Directive 2000/60/EC [63], also called 'Water Framework Directive'. It establishes the main strategies to follow against water pollution, outlining the steps to be taken. As a consequence, Directive 2455/2001/EC [64] has established a list of priority substances consisted of 33 substances or group of substances that must be monitored in water sources. The monitoring of these substances is required because they are toxic, persistent and liable to bio-accumulate or they belong to a group of substances which give rise to an equivalent level of concern. The list of priority substances has been recently extended by Directive 2008/105/CE [65], and there is a proposal for including other additional priority substances [66]. Nonetheless, antibiotics are not included in this list as priority contaminant substances because, as it was mentioned before, it is not well known the adverse effects of these compounds in the environment. Furthermore, Directive 98/83/EC [67] lays down the quality of consumption, but among the several parameters and chemicals regulated in this directive, none relates to pharmaceuticals.

1.6. Analytical methods for the determination of antibiotic residues

According to the facts already exposed, antibiotics have provided benefits to human health, but their extended use is also a risk that must be taken in consideration. Consequently, analytical methods for their determination are required in clinical field studies such as pharmacokinetic and metabolomic assays or for therapeutic drug monitoring. But moreover, they are also needed in food field for guarantying food safety, and therefore, the compliance of the current legislation. Additionally, the occurrence of antibiotics in environmental sources has been demonstrated, but their real risk level is still unknown. In order to evaluate their ecotoxicity, it is essential to understand the degradation pathways of antibiotics in the environment as well as quantifying the presence of antimicrobial agents in surface and groundwater.

Obviously, the determination of antibiotic substances in biological matrices such as urine or serum does not present the same problems as the determination of antibiotic residues in environmental or food matrices. Antibiotics are found at trace levels (between 1 and 100 μg/g) in biological samples whereas they are present in environmental and food

samples at low microtrace levels (between 1 and 1000 μg/kg) [68]. On the other hand, the complexity of water samples is different to the supposed complexity of milk and serum samples which possess high protein content. Therefore, specific solutions are required for each analytical problem.

1.6.1. Chromatographic and electromigration methods

Generally, the determination of traces of antibiotic substances is carried out by LC coupled to an ultraviolet/visible (UV/Vis) detector or using other more selective detection modes such as fluorescence or MS [69,70]. Moreover, GC has been also used as separation technique instead of LC, but LC is preferred to GC because many pharmaceuticals are thermolabile, such as tetracycline antibiotics [71], whereas other compounds require to be derivatized in order to be volatile substances. In addition, capillary electrophoresis (CE) has emerged as an alternative to LC and GC [72,73], mainly owing to the development of on-line preconcentration strategies for increasing the injected sample volume. They have allowed reaching lower detection limits, and as a result, overcoming the low sensitivity inherent to this technique that constitutes its main disadvantage for the application in trace analysis [74].

In this Thesis, CE methods (**Chapter 4**, **5** and **6**) and LC methods (**Chapter 11**, **12** and **13**) have been proposed for the determination of traces of 5-NDZ antibiotics in different matrices including food, environmental and biological samples. Furthermore, electrochromatographic methods are proposed in **Chapter 8** and **9** in order to evaluate the potential of capillary electrochromatography (CEC) as tool for the determination of residues in food and biological matrices. Because the most relevant features related to each separation technique are widely discussed in **Chapter 3** for CE, **Chapter 7** for CEC and **Chapter 10** for LC, they are not further addressed in this section.

1.6.2. Sample treatments

Typically, sample treatments are required prior to residue determination, especially in the residue analysis field because analyte preconcentration is required in addition to sample clean-up. Although the technology related to chromatographic separations and MS

techniques has experienced a great advance, sample treatment remains as a key challenge in the analytical process, being essential for the determination of compounds at trace levels in food, environmental and clinical samples [75-79]. The majority of residue analyses require clean-up procedures for avoiding matrix interferences, and analyte preconcentration is usually crucial for achieving low detection limits (LODs). Conventional extraction procedures have been mainly focused on the determination of a single class of analytes and techniques such as solid-liquid extraction (SLE), liquid-liquid extraction (LLE) and solid-phase extraction (SPE) have been widely used and are still in development. In food analysis, different solvents have been used for SLE according to the nature of analytes but, in general, ethyl acetate, acetonitrile (MeCN) and methanol (MeOH) are the most common solvents employed for performing analyte extraction [77,78].

During the last decade, a great effort has been made toward the development of more efficient extraction and clean-up approaches which includes system automation by coupling sample preparation and detection units, application of new sorbents and application of greener methods for reducing solvent consumption. Numerous novel extraction strategies have been developed and nowadays there is a vast spread of techniques for being successful on analyte extraction and sample clean-up [79-81]. However, in this Thesis, only common methods employed in routine laboratories such as SPE, dispersive-solid-phase extraction (d-SPE), or LLE assisted by salts are addressed. Additionally, dispersive liquid-liquid microextraction (DLLME) is discussed because it has shown to be simple and reproducible, and as a consequence, capable of being successfully implemented for routine analyses.

Solid phase extraction (SPE)

The most common analytical method reported for multiresidue determination in water and food samples is the use of SPE prior to LC or CE methods [82-85] because it offers some advantages such as robustness and high versatility. It is ease of operation and allows performing different analytical processes such as purification, trace enrichment, desalting, derivatization, solvent exchange, and class fractionation. SPE can be accomplished by two ways, off-line or on-line with the separation system. Off-line SPE is a very simple

technique that employs low-cost and simple equipment consisted of disposable extraction columns, microplates or cartridges with a wide range of reservoir volumes, formats, and sorbents. On the other hand, on-line SPE minimizes sample manipulation and provides both high preconcentration factors and good recoveries, improving sample throughput, but specific instrumentation is required for its performance.

Off-line SPE methodology is the most common mode and it is characterized by high recovery efficiency, a moderate solvent consumption and satisfactory reproducibility. Generally, analytes are extracted from a liquid phase using a solid stationary phase, which can be contained in cartridges, disks, SPE pipette tips, or 96-well SPE microliter plates. The most often applied design is the polypropylene cartridge containing the sorption phase [86]. SPE is achieved through the interaction of three components: the sorbent, who acts as stationary phase, the analyte and the solvent. Two strategies can be followed for performing SPE. First, matrix compounds are retained in the sorbent whereas analytes are eluted. On the other hand, analytes can be strongly retained in the sorbent more than matrix components, so analytes must be eluted in a final stage using a suitable solvent. Cartridge washing stages can be considered in order to remove other matrix components without eluting the analytes. For that reason, the selection of adequate washing solvents is crucial. The selection of the appropriate SPE mode and protocol is defined by the characteristics of the analyte and the sample.

Different sorbents have been evaluated for SPE, ranging from traditional reversed-phase sorbents (C18, C8), normal phase (silica, alumina), and ion exchange, to mixed-mode (ion exchange + reversed phase) and functionalized resins based on styrene-divinylbenzene polymers. Among them, silica and bonded silica sorbents, polymer-based sorbents, graphitized or porous carbon are the most frequently used. Besides, new extraction sorbents such as molecularly imprinted polymers (MIPs) and immunosorbents have been evaluated [86]. Traditionally C18 sorbents have been mainly considered for SPE in residue analyses [87-89]. However, polymeric-based sorbents are increasingly used because they are applicable to analytes within a wide range of polarity [90-93]. Polymer-based SPE involves macroporous polymeric media, offering a higher loading capacity than conventional functionalized silica-based sorbents. Normally, analytes are retained in these cartridges due

to dispersion forces (London) and π-π interactions such as in hydrophilic-lipophilic balance (HLB) cartridges. Moreover, electrostatic interactions can occur if mixed-mode cartridges are employed [86].

Nowadays, polymer-based SPE cartridges can be acquired from various manufacturers under different trademarks (™) or register marks ®, such as Strata® from Phenomenex (Torrence, CA, USA), Supel™ from Sigma Aldrich (St. Louis, MO, USA) or Oasis® from Waters (Milford, MA, USA). Because each manufacturer develops SPE cartridges of the same nature following different recipes, a vast number of sorption phases can be actually found in the market, especially cartridges of polymer-based sorbents. As a consequence, there are countless choices for selecting the adequate SPE cartridge to carry out a specific application. Polymer-based SPE cartridges from Waters were employed in the analytical methods discussed through this Thesis, so the chemical structures of the most used sorbents from this manufacturer are shown in **Figure 1.5.**

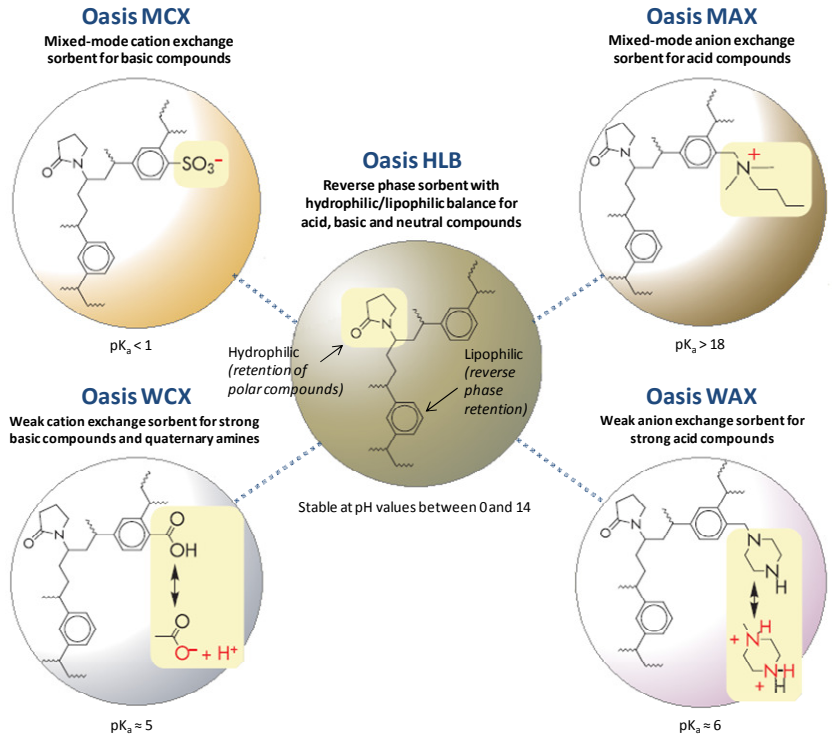

Figure 1.5. Polymer-based sorbents of SPE cartridges from Oasis®. Adapted from [94].

Molecular imprinted polymers-solid phase extraction (MISPE)

MIPs have also been used as sorbents in SPE, introducing the so-called MISPE methodology. MIPs are synthetic polymers produced in the presence of a molecule called template (**Figure 1.6**). Once the synthesis is performed, the template is removed and specific recognition sites are created. These sites are complementary in shape, size, and functional group, to the analyte of interest, providing high selectivity for retaining target molecules [95]. For this reason MISPE can be applied to the extraction and isolation of target analytes from complex mixtures, such as food, environmental and clinical matrices [96,97].

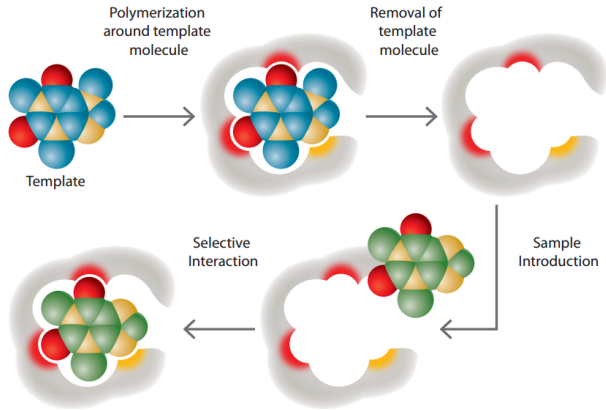

Figure 1.6. **A generalized diagram of MIP synthesis and selective analyte interaction. Reproduced from [98].**

Salt-assisted liquid-liquid extraction (SALLE)

LLE is one of the oldest extraction techniques and consists of the extraction of the analytes from aqueous samples using water-immiscible solvents such as dichloromethane. Normally, the sample is submitted to consecutive extraction stages with fresh portions of the solvent or with a series of solvents of increasing polarity. Consequently, various fractions of the solvent enriched with the analytes are obtained. This procedure is a time consuming method, involves a high consumption of toxic solvents and, besides, low extraction efficiency can be achieved for polar compounds. Furthermore, the risk of losing analytes or of sample contamination increases with the increasing number of operations performed on the same sample. In order to overcome these drawbacks, various

miniaturized extraction techniques have been developed in the last decades [99], but they usually require the use of water-immiscible solvents, and therefore, they are incompatible with the extraction of polar compounds.

However, performance characteristics of traditional LLE compounds can be improved for the extraction of polar compounds by using water-miscible solvents such as MeCN, MeOH, ethanol (EtOH), 2-propanol or acetone. The addition of suitable salts, normally NaCl, into aqueous sample solutions induces phase separation between the organic solvent and the aqueous sample because their miscibility is reduced [100]. The intermolecular forces (hydrogen links) between water and organic solvent molecules are easily interrupted due to the hydration process of electrolytes from the added salts.

This extraction method is named salt-assisted liquid-liquid extraction (SALLE), and although it is not a novel extraction strategy [101], it has now emerged as an important sample treatment [102], especially in bioanalysis field [103]. It is a simple and fast process that results in extracts that can be injected directly into a gas or liquid chromatograph and that is especially useful when selective detection such as MS is employed [104].

Dispersive solid phase extraction (d-SPE) – QuEChERS

In 2003, a generic method named QuEChERS (quick, easy, cheap, effective, rugged, and safe) was developed by Anastassiades *et al.* [105]. This technique involves liquid-liquid partitioning using MeCN and purifying the extract by d-SPE. The first stage consists of a SALLE or a salt-assisted solid-liquid extraction (SASLE) procedure. Analytes are extracted from the sample matrix using MeCN, whereas $MgSO_4$ with or without NaCl is added to the sample for producing phase partitioning. Then, all or an aliquot of the extraction solvent is submitted to a clean-up stage by d-SPE. This approach involves the addition of the extraction solvent to sorbent powders contained in a centrifuge tube and the subsequent manual or mechanical agitation. Generally, interfering matrix components are adsorbed by the selected sorbent whereas analytes remain in the extraction solvent. The most used d-SPE sorbents are primary-secondary amine (PSA) sorbent, HLB, C18 or graphitized carbon black (GCB). Recently, new sorbents have been successfully assayed,

namely ChloroFilr® for the removal of chlorophylls, Z-Sep$^+$ which is a zirconium-based sorbent for removing fat and pigments, and multi-walled carbon nanotubes [106].

In general, high recoveries are achieved for a wide polarity and volatility range of analytes when QuEChERS methods are performed. It is a low-cost technique that offers high sample throughput of about 30 samples per hour with high accuracy. Moreover, it involves low solvent consumption, and no chlorinated solvents are used. However, low sample preconcentration is achieved by QuEChERS or even sample dilution can occurs. Therefore, it is mandatory the use of chromatographic systems coupled with MS in order to get satisfactory results in terms of sensitivity. In the last years, this extraction technique has been increasingly used for the determination of residues in a wide range of applications [107-109].

Dispersive liquid-liquid microextraction (DLLME)

DLLME is a miniaturized LLE approach that was introduced by Rezaee *et al.* in 2006 [110]. It involves a ternary component solvent system which usually consists of an aqueous sample, an higher-density extraction solvent (a water immiscible organic solvent such as chlorobenzene, chloroform or CS_2) and a dispersive solvent that has to be miscible in both extractant and aqueous sample (such as MeOH, MeCN, or acetone) [111]. The general DLLME procedure is shown in **Figure 1.7**.

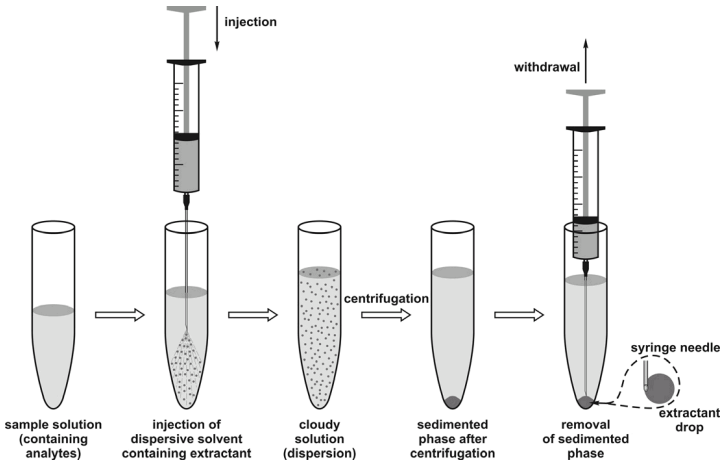

Figure 1.7. Scheme of DLLME procedure. Reproduced from [112].

First, the mixture of extractant and dispersant agent is rapidly injected into the sample, causing a high turbulence. This turbulent regimen gives rise to the formation of small droplets, which are dispersed throughout the aqueous sample. After the formation of the cloudy solution, the surface area between the extractant solvent and the aqueous sample becomes very large, so the equilibrium state is achieved quickly and, therefore, the extraction time is very short. After centrifuging the cloudy solution, a sedimented phase is settled in the bottom of the centrifugation tube. This phase is the extraction solvent containing the analytes, so it is collected and analyzed with the most appropriate analytical technique.

It is an environmental-friendly method because only a few microliters of the extraction solvent and low volume of dispersant agent are required for performing DLLME methods. Furthermore, DLLME is a simple and cheap extraction technique that requires short extraction times (a few minutes including centrifugation stage). Besides, it provides high recoveries and high enrichment factors. Due to these advantages, DLLME has been widely applied in the last years [112,113].

Although the traditional DLLME procedure has been mentioned, numerous modifications have been recently proposed for enhancing the potential of this technique. The use of ionic liquids [114] or low-density solvents [115] as extractant agents has also demonstrated to be suitable for DLLME. Furthermore, the dispersion of the solvents through the sample can be improved by ultrasound or vortex agitation as well as by the addition of surfactants to the extraction media [113]. Additionally, as it occurs in SALLE procedures, DLLME efficiency can be enhanced by adding salts to the sample prior the extraction.

DLLME has been generally used for the extraction of residues from aqueous samples such as environmental waters, however it has also been applied to the determination of several compounds at trace levels in more complex samples [116,117].

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2

5-Nitroimidazoles

2.1. Background

Although 5-NDZs are synthetic compounds, their origin is related to the discovery of a natural compound called azomycin. It is a 2-NDZ drug which is a naturally occurring alkaloid produced by bacteria (*Nocardia spp.* and *Streptomyces eurocidicus*) [1] and was discovered in 1953 by Umezawa *et al.* As azomycin was structurally similar to the trichomonicidal agents aminitrozole and 2-amino-5-nitrothiazole, researchers at Rhône Poulenc tested it and found it to have similar activity, but it turned out to be too toxic for clinical exploitation [2]. However, a series of analogues of azomycin were synthesized, leading to the discovery of MNZ in the late 1950s by Cosar and Julou. MNZ possessed appreciable antiprotozoal properties against *Trichomonas vaginalis*. Afterwards, its application was extended for treating infections due to other protozoans such as *Entamoeba histolytica* and *Giardia lamblia* [3].

Serendipitously, in 1962, MNZ was found to be active against ulcerative gingivitis, a bacterial infection of the gums, and as a result, this fact led to an evaluation of its broader antibacterial activity [4]. It has showed to posses antibacterial properties toward most Gram-negatives and many Gram-positive anaerobic bacteria, although its efficiency against aerobic bacteria is very limited. Nowadays, MNZ is usually prescribed as antibacterial, especially in the case of *Clostridium difficile* infections, because it is considered to be one of the few drugs with activity against these bacteria. MNZ is also used for treating anaerobic bacterial vaginosis caused by *Gardnerella vaginalis*, in combined therapy for the eradication of *Helycobacter pylori* or as treatment for Crohn disease. Additionally, it is the agent of choice for the treatment of bacterial endocarditis caused by *Bacteroides* and *Prevotella* species [3,5,6].

Due to its importance, MNZ has been included in the 'World Health Organization (WHO) Model List of Essential Medicines' where it has been listed as one of the two essential antiamoebic and antigiardiasis medicines [7]. However, although MNZ is the most representative compound of 5-NDZ group, other drugs belonging to the same antibacterial group as ornidazole (ORZ) and tinidazole (TNZ) are also used in human medicine, but they are less prescribed than MNZ. ORZ is used in the treatment and prophylaxis of susceptible anaerobic infections in gastric surgery, and it is a preferred antibiotic over MNZ for the treatment of severe hepatic and intestinal amoebiasis [8]. Moreover, TNZ has demonstrated its effectiveness in the treatment of trichomoniasis, giardiasis, intestinal amebiasis and amebic liver abscess [9]. Additionally, other 5-NDZs such as ternidazole (TRZ), carnidazole (CRZ) or secnidazole (SCZ) are also clinically useful antibacterial and antiprotozoal drugs [10]. On the contrary, the use of other 5-NDZs, namely DMZ, ipronidazole (IPZ) and RNZ, has almost been restricted to veterinary medicine. In Spain, only MNZ, ORZ and TNZ are commercialized for therapeutic treatments in human medicine [11]. **Table 2.1** shows the physical and chemical properties, such as Mw, acid dissociation constant (pKa) or partition coefficient (log P) of some of the mentioned 5-NDZs.

Table 2.1. Physical and chemical properties of 5-NDZs. Data obtained from SciFinder Scholar.

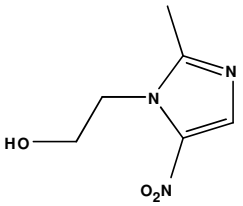
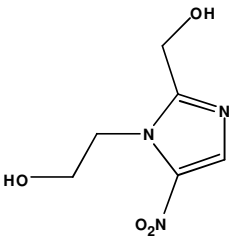
Compound identification	Molecular structure	Chemical and physical properties
Metronidazole (MNZ) **IUPAC name:** 1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole **Chemical formula:** $C_6H_9N_3O_3$ **CAS registry number:** 443-48-1		**Mw:** 171.15 g/mol **pKa:** 2.58±0.34/14.44±0.10 **log P:** -0.135±0.301 **Solubility:** 0.17 mol/L at pH 7 **Vapor pressure:** 2.67×10^{-7} Torr
Hydroxyl-metronidazole (MNZ-OH) **IUPAC name:** 1-(2-hydroxyethyl)-2-(hydroxymethyl)-5-nitroimidazole **Chemical formula:** $C_6H_9N_3O_4$ **CAS registry number:** 4812-40-2		**Mw:** 187.15 g/mol **pKa:** 1.98±0.34/13.28±0.10 **log P:** -0.298±0.424 **Solubility:** 0.18 mol/L at pH 7 **Vapor pressure:** 7.73×10^{-10} Torr

Table 2.1 (continued). **Physical and chemical properties of 5-NDZs. Data obtained from SciFinder Scholar.**

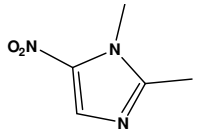
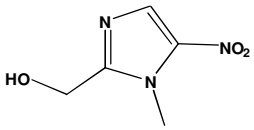
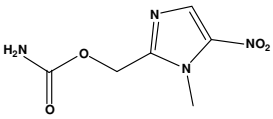
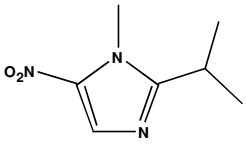
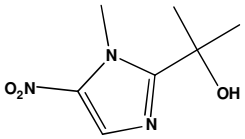
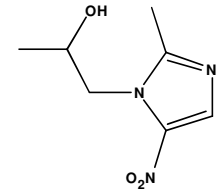
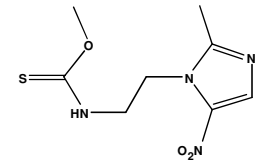
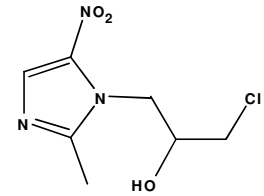
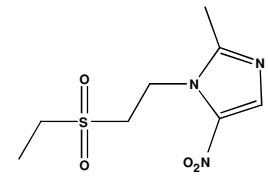
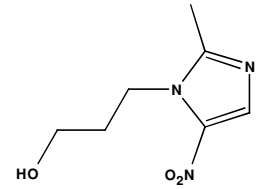
Compound identification	Molecular structure	Chemical and physical properties
Dimetridazole (DMZ) **IUPAC name:** 1,2-dimethyl-5-nitroimidazole **Chemical formula:** $C_5H_7N_3O_2$ **CAS registry number:** 551-92-8		**Mw:** 141.13 g/mol **pKa:** 2.81±0.25 **log P:** -0.135±0.301 **Solubility:** 0.08 mol/L at pH 7 **Vapor pressure:** 9.01×10^{-4} Torr
Hydroxyl-dimetridazole (HMMNI) **IUPAC name:** 1-methyl-2-hydroxymethyl-5-nitroimidazole **Chemical formula:** $C_5H_7N_3O_3$ **CAS registry number:** 936-05-0		**Mw:** 157.13 g/mol **pKa:** 2.21±0.25/13.31±0.10 **log P:** -0.068±0.350 **Solubility:** 0.26 mol/L at pH 7 **Vapor pressure:** 1.02×10^{-6} Torr
Ronidazole (RNZ) **IUPAC name:** 1-methyl-2-carbamoyloxymethyl-5-nitroimidazole **Chemical formula:** $C_6H_8N_4O_4$ **CAS registry number:** 7681-76-7		**Mw:** 200.15 g/mol **pKa:** 1.32±0.25/12.99±0.50 **log P:** -0.109±0.433 **Solubility:** 0.06 mol/L at pH 7 **Vapor pressure:** 3.21×10^{-10} Torr
Ipronidazole (IPZ) **IUPAC name:** 1-methyl-2-isopropyl-5-nitroimidazole **Chemical formula:** $C_7H_{11}N_3O_2$ **CAS registry number:** 14885-29-1		**Mw:** 169.18 g/mol **pKa:** 2.55±0.25 **log P:** 0.958±0.239 **Solubility:** 0.014 mol/L at pH 7 **Vapor pressure:** 1.18×10^{-3} Torr
Hydroxyl-ipronidazole (IPZ-OH) **IUPAC name:** 1-methyl-2-(2'-hydroxyisopropyl)-5-nitroimidazole **Chemical formula:** $C_7H_{11}N_3O_3$ **CAS registry number:** 35175-14-5		**Mw:** 185.18 g/mol **pKa:** 2.21±0.25/13.44±0.29 **log P:** -0.135±0.301 **Solubility:** 0.055 mol/L at pH 7 **Vapor pressure:** 4.69×10^{-6} Torr

Table 2.1 (continued). **Physical and chemical properties of 5-NDZs. Data obtained from SciFinder Scholar.**

Compound identification	Molecular structure	Chemical and physical properties
Secnidazole (SCZ) **IUPAC name:** 1-(2-methyl-5-nitroimidazol-1-yl)propan-2-ol **Chemical formula:** $C_7H_{11}N_3O_3$ **CAS registry number:** 3366-95-8		**Mw:** 185.18 g/mol **pKa:** 2.62±0.35/14.50±0.20 **log P:** 0.218±0.306 **Solubility:** 0.076 mol/L at pH 7 **Vapor pressure:** 5.49×10^{-7} Torr
Carnidazole (CRZ) **IUPAC name:** 1-(2-ethylcarbamothioic acid O-methyl ester)-2-methyl-5-nitroimidazole **Chemical formula:** $C_8H_{12}N_4O_3S$ **CAS registry number:** 42116-76-7		**Mw:** 171.15 g/mol **pKa:** 2.58±0.34/13.03±0.70 **log P:** 1.048±0.307 **Solubility:** 2.5×10^{-3} mol/L at pH 7 **Vapor pressure:** 2.35×10^{-7} Torr
Ornidazole (ORZ) **IUPAC name:** 1-(3-chloro-2-hydroxypropyl)-2-methyl-5-nitroimidazole **Chemical formula:** $C_7H_{10}ClN_3O_3$ **CAS registry number:** 16773-42-5		**Mw:** 219.63 g/mol **pKa:** 2.72±0.35/13.29±0.20 **log P:** 0.600±0.337 **Solubility:** 0.028 mol/L at pH 7 **Vapor pressure:** 1.23×10^{-8} Torr
Tinidazole (TNZ) **IUPAC name:** 1-(2-ethylsulfonylethyl)-2-methyl-5-nitroimidazole **Chemical formula:** $C_8H_{13}N_3O_4S$ **CAS registry number:** 19387-91-8		**Mw:** 247.27 g/mol **pKa:** 2.30±0.34 **log P:** -0.293±0.358 **Solubility:** 0.013 mol/L at pH 7 **Vapor pressure:** 1.00×10^{-10} Torr
Ternidazole (TRZ) **IUPAC name:** 1-(3-hydroxypropyl)-2-methyl-5-nitroimidazole **Chemical formula:** $C_7H_{11}N_3O_3$ **CAS registry number:** 1077-93-6		**Mw:** 185.18 g/mol **pKa:** 2.72±0.34/14.90±0.10 **log P:** -0.377±0.254 **Solubility:** 0.13 mol/L at pH 7 **Vapor pressure:** 8.40×10^{-8} Torr

Although the incidence of MNZ-resistant bacteria is still generally low [3,6], high rates of MNZ-resistance in *Helycobacter pylori* have already been reported [12]. Considering this fact, the development and use of MNZ-analogue substances has great relevance. For example, the efficiency of TNZ for treating *Trichomonas vaginalis* infections has been demonstrated in those cases in which resistances towards MNZ are observed [13].

2.2. Mechanism of action

As can be seen in **Table 2.1**, 5-NDZ compounds are characterized by an imidazole ring, containing a nitro (NO_2) group on the fifth position. There are four major stages involved in the mechanism of action of 5-NDZ compounds [14,15]. Firstly, 5-NDZ drug enters the cells by passive diffusion that is enhanced by the rate of intracellular reduction. Then, an electron is transferred to the nitro group and a short-lived nitrous free radical is produced (**Figure 2.1**). The formed intermediate compound is cytotoxic and can interact with cellular DNA.

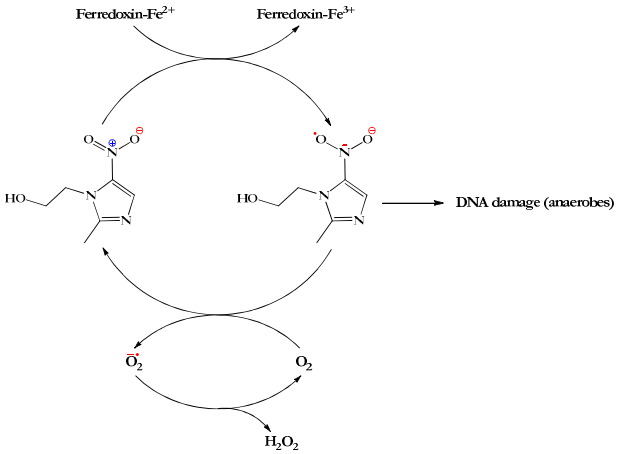


Figure 2.1. **Mechanism of activation of MNZ drug in anaerobic cells. Reproduced from [16].**

In anaerobic bacteria, 5-NDZ reduction is mainly accomplished by the pyruvate: ferredoxin oxidoreductase complex, while the nitro group of 5-NDZ molecules acts as an electron acceptor. On the other hand, 5-NDZ drugs are rarely activated in aerobic bacteria because they lack the necessary electron transport proteins with sufficient negative redox potential for achieving the reduction of the nitro group. Furthermore, even if reduction

occurs in the presence of oxygen, DNA damage is very limited or absent because aerobic cells are able to detoxify active 5-NDZ's intermediate through reaction with molecular oxygen. This is because oxygen rapidly removes the electron from the nitro radical anion, forming the original drug and a superoxide. This oxidation process is known as 'futile cycling'. The superoxide formed in the latter reaction is then scavenged by superoxide dismutase [16].

In the third stage, 5-NDZ's intermediate reacts with cellular DNA, inhibiting its synthesis and inducing its damage via oxidation. As a result, DNA strand breakage and helix destabilization occur. The degree of damage is related to base composition, and is greater with increased adenine and thymine content [17]. Thus, DNA degradation and cell death is induced by 5-NDZs. Finally, inactive end products of 5-NDZs are released.

2.3. Pharmacokinetic and pharmacodynamic properties

Administration and pharmacokinetic properties of MNZ have been widely described, whereas few reports have described the pharmacology behavior of other 5-NDZ drugs. According to the pathology, normal doses of MNZ for human therapeutic treatment range from 250 mg three times a day during 7 days to 750 mg three times a day during 10 days. Single doses of 2 g can also be prescribed. Daily dose of MNZ is usually restricted to a maximum of 2.5 g [18]. MNZ can be administered orally, intravenously, rectally or intravaginally as suppository or cream. Its bioavailability related to intravenous is complete after oral administration (close to 100%) whereas it is reduced to 80% when rectal administration is applied. Moreover, it is slowly and poorly absorbed after intravaginal administration [19]. TNZ, ORZ and SCZ have also shown high absorption for oral administration, reaching a bioavailability higher than 90% [20].

Depending on the oral dose, maximum plasma concentration level (C_{max}) after maximum absorption ranges between 6 and 40 mg/L while the time to reach C_{max} ranges between 0.25 to 4 h [18]. Similar C_{max} have been observed for TNZ and SCZ after oral administration of a single dose of 2 g. Values between 35.7 and 46.3 mg/L has been reported for SCZ, whereas C_{max} ranging from 40 to 58 mg/mL has been obtained for

TNZ. For ORZ, C_{max} between 23.6 and 31.5 mg/mL has been reported after oral administration of a single dose of 1.5 g [20]. Therefore, these concentrations are higher than the observed minimum bactericidal concentration of MNZ for causing bacteria death and inhibiting their growth which has been established between 0.25 and 4 mg/mL depending on the type of microorganism [3].

A high penetration and distribution of MNZ into cerebrospinal fluid has been reported, as well as into rectal muscle, fallopian tube, umbilical cord plasma and uterus, while it has shown to be lower in pancreatic tissues and colonic mucosa and poor in adipose tissues. Furthermore, MNZ protein binding is lower than 20%, whereas it is even lower for ORZ and SCZ (< 15%) [20].

MNZ undergoes hepatic metabolism and approximately 90% of it is metabolized to oxidative products, mainly to a hydroxyl-metabolite named hydroxyl-metronidazole (MNZ-OH) [21]. This metabolite is clinically significant with an antimicrobial activity of 30-65% in relation to that of MNZ. MNZ is also metabolized to an acetic acid product, but it has only minimal activity (5%) and is detectable only in patients with renal dysfunction. Other metabolites, such as glucuronide compounds have also been detected, but they represent a small proportion of MNZ metabolized [22]. The main chemical and physical properties of MNZ-OH are shown on **Table 2.1**.

MNZ dose is mainly eliminated via renal excretion, recovering in the urine up to 77%, whereas a 14% is excreted with the feces. Unchanged MNZ in the urine accounts for approximately 8-10% of the total dose [22-24]. Pharmacokinetic studies carried out over patients treated with 200 mg of MNZ each eight hours during seven days reported antibiotic levels in urine in a concentration above 10 mg/L the ninth day after the first administration day [25]. In other assay, patients were treated with 250 or 500 mg of MNZ and urine analyses were carried out. Samples were collected between 4 and 8 h after single dose administration and MNZ concentration ranging between 15.7 to 115.0 mg/L was observed.

Hydroxyl-metabolites have also been described for IPZ, DMZ and RNZ. IPZ is mainly metabolized to hydroxyl-ipronidazole (IPZ-OH) whereas DMZ and RNZ are metabolized

into the same hydroxyl-metabolite called hydroxyl-dimetridazole (HMMNI) (see **Table 2.1)**. However, DMZ and RNZ metabolisms follow different pathways. Metabolites for other compounds such as TNZ are present at low concentrations or are not detected either in serum or in urine [20,23]. In general, 5-NDZ parent drugs and their metabolites are mainly recovered in urine [20].

2.4. Importance of the determination of 5-NDZ residues in foodstuffs

In addition to human medicine, MNZ, DMZ, RNZ, TNZ and CRZ have been considered for treating diseases in animals. For example, they are the drugs of choice for the treatment of *trichomonas gallinae* in racing pigeons and wild pigeons [26], and RNZ has also been used for treating *trichomonas foetus* infections in felines [27]. Furthermore, they have been used in the prophylactic and therapeutic treatment of histominiasis and coccidiosis in poultry [28] and for combating hemorrhagic enteritis in pigs [29]. Moreover, DMZ, RNZ and IPZ were used as food additives in the past [30], however, the use of antibiotics as food additives is currently forbidden within EU (see **Section 1.4**).

On the other hand, as was described in **Chapter 1**, the presence of antibiotics in food products of animal origin can suppose an important risk to human health. This risk is especially relevant with regard to the presence of 5-NDZ residues because carcinogenic, genotoxic and mutagenic properties have been attributed to them [31-33]. In order to ensure the food safety, their use in veterinary medicine has been restricted to non-food producing animals. As a consequence, the presence of 5-NDZ residues in animal products intended to human consumption is forbidden within EU according to Regulation (EU) No 37/2010 [34]. Additionally, this ban has been extended to other countries and the use of 5-NDZs in food-producing animals is also forbidden in USA and China [35,36].

Nevertheless, in spite of being forbidden substances in food-producing animals, several alerts about the presence of 5-NDZs in some foods derived from animals have been notified by RASFF Portal in the last years [37]. These alerts are mainly related to the presence of MNZ residues in different food matrices such as honey, royal jelly, trout, fish products, meat and meat products, although RNZ residues have also been found in chilled

fresh turkey. Therefore, considering the globalization of food production, any alert about the illegal use of these antibiotics should be taken into account as international food safety concern.

According to current EU legislation, MRLs or MRPLs have not been established for 5-NDZ residues in food products. However, the EURLs for veterinary residues have established 'action limits' for the validation of analytical methods in relation to the control of unauthorized analytes in different matrixes [38]. In relation to 5-NDZs, a recommended concentration of 3 μg/kg has been set and it involves that detection capability (CCβ) for screening methods or decision limit (CCα) for confirmatory methods should be lower than this value. In the case that LOD is estimated, the value of this parameter should be lower than the mentioned limit. However, this reference point is just a recommendation and thus it does not have any legal force.

Additionally, depletion studies have shown that after a single oral dose of 75 mg of 5-NDZ drugs, namely IPZ, DMZ and RNZ, residues of IPZ-OH and HMMNI in a concentration above 10 μg/kg can be present in eggs for up to 5 days. Furthermore, residues of unchanged RNZ drug exceeding 10 μg/g have been found in eggs after 7 days [39]. IPZ and DMZ are extensively metabolized to their respective hydroxyl-metabolites, thus it is important to monitor these compounds when 5-NDZ determination analyses are performed. On the other hand, Mitrowska *et al.* carried out depletion assays in tissues samples from rainbow trouts which were treated with MNZ in feed at the average dose of 25 mg/kg body weight per day for 7 days [40]. MNZ was rapidly converted into MNZ-OH after oral administration of MNZ in feed and depletion studies showed that MNZ-OH residues were present in muscle up to 21 days post-administration. Moreover, unchanged MNZ was detected at a concentration of 0.20 μg/kg after 42 days post-administration.

Furthermore, cooking cannot be considered as a safeguard against the ingestion of 5-NDZ residues. Rose *et al.* demonstrated that DMZ and HMMNI residues are relatively stable to heat, while RNZ is metabolized to HMMNI at 100°C in water [41]. In addition, residue depletion was evaluated in prepared eggs and chicken meat. Residues of DMZ and HMMNI in eggs were only decomposed by about a quarter in an omelet cooked for

3 min. Furthermore, both residues in chicken meat were reduced between 26 and 45% during frying and microwaving. However, the reduction was attributed to the loss of residues with meat juices which were exuded during cooking. Additionally, Gadaj *et al.* evaluated the effect of different cooking procedures on the concentration of 5-NDZ residues, mainly MNZ, RNZ and DMZ, in shrimp tissue [42]. Residue concentration in the evaluated food products was significantly depleted by boiling and/or microwaving, but these residues showed to be resistant to conventional grilling or frying, because residue concentration only decreased between 7 and 25% after accomplishing one of both cooking procedures.

Considering that the use of 5-NDZ drugs in food-producing animals is forbidden by law, but alerts about the presence of 5-NDZ residues in foodstuffs of animal origin are still reported; simple, cheap and green analytical methods are required for the determination of 5-NDZ residues in food products. Furthermore, as described above, food safety is not ensured although food products are cooked. Indeed there is the potential risk that 5-NDZ residues may be converted to more toxic substances during cooking [42].

2.5. Presence of 5-NDZ residues in environmental sources

As was described in **Section 1.5**, the presence of pharmaceuticals in environmental sources is an emerging concern. Regarding 5-NDZ residues, the evaluation of their occurrence in the environment is important because they present high polarity and low biodegradability, and therefore, they are susceptible to be bioaccumulated [43,44].

The presence of 5-NDZ residues in hospital sewage water samples can be expected [45,46]; however, their presence in wastewater effluents has also been reported, and consequently, it can be supposed that 5-NDZs are getting into the environment [47]. Occurrence assays have normally been carried out for MNZ rather than for other 5-NDZ drugs. MNZ residues with an average concentration of 55 ng/L were found by Rosal *et al.* in wastewater samples collected from the effluent of a sewage treatment plant (STP) located in Alcalá de Henares (Madrid, Spain) [48]. On the other hand, Kasprzyk-Hordent *et al.* reported MNZ residue concentration up to 591 ng/L in samples collected from the

effluent of a STP located in South Wales (United Kingdom, UK). Furthermore, it was demonstrated that typical water treatments carried out in STP provide low efficiency for the removal of MNZ residues. In the same study, the presence of MNZ residues was also evaluated in River Taff (Wales, UK) downstream of the investigated SPT. MNZ concentrations up to 24 ng/L were found [49]. Moreover, a greater concentration of MNZ was detected in samples collected from the effluent of a STP located in Lausanne (Switzerland) [50]. In this study Morash *et al.* reported a concentration of 1.1 μg/L for MNZ that was only a 15% lower than the concentration measured in the influent, showing that low efficiency for MNZ removal is achieved in the studied STP.

In addition, MNZ in a concentration of 0.3 ng/L has been detected in surface water samples collected in the Rhône-Alpes region (France) [51], and the presence of DMZ and MNZ residues has been reported in groundwater samples collected from wells located in Taiwan [52]. A concentration of 1.8 ng/L was reported for a sample containing DMZ, whereas higher concentration was observed for MNZ, ranging between 4.9 and 35.6 ng/L according to the analyzed sample. In both cases, considering all analyzed samples, 5-NDZ residues were detected with a frequency lower than 5%. Nevertheless, their presence in these environmental sources is concerning because a medium environment risk has been attributed to these compounds [53].

2.6. Analytical methods for the determination of 5-NDZs

In view of all that has been set out above, analytical methods are required in order to determine 5-NDZ drugs in clinical, food and environmental fields. Normally, 5-NDZ analyses in clinical field are needed for carrying out pharmacokinetic studies and for the evaluation of certain therapeutic treatments. On the other hand the determination of 5-NDZ residues in food and environmental matrices is performed for ensuring the protection of human health.

Despite 5-NDZ determination has been mainly accomplished by LC-based methods using both UV and MS detection [54], other techniques such as GC [55-58] or CE [59-66] have also been considered for that purpose. Nevertheless, GC is not widely used for the

determination of antibiotic residues due to their low volatility, and therefore they have to be derivatized prior to their analysis. In the case of 5-NDZ compounds, derivatization is accomplished by thermal reaction with N,O-bis-(trimethylsilyl)acetamid (BSA) to produce trimethylsilyl- (TMS)-derivatives [56-58]. On the contrary, Wang has proposed a GC method using nitrogen-phosphorus detection without requiring any derivatization step [55]. On the other hand, electromigration techniques such as CE or CEC have been less used for residue determination than chromatographic techniques because low robustness and sensitivity are attributed to them. However, the applicability of CE in residue analysis field is increasingly, showing its suitability for the determination of compounds at trace levels [67,68].

CE has been applied mainly to pharmaceutical quality controls [59-61], although the determination of MNZ in diluted human urine by capillary zone electrophoresis (CZE) using amperometric detection has also been proposed [62]. Additionally, Lin *et al.* developed a CZE coupled with UV detection for the separation and determination of DMZ, MNZ, SCZ, benzoylmetronidazole and RNZ in porcine muscle tissue [66]. In order to improve the separation between two of the studied analytes, namely MNZ and SCZ, the addition of tetrabutylammonium bromide (TBAB) to the background solution (BGS) was proposed. Furthermore, Nozal *et al.* evaluated the coupling of a micro-membrane device to the separation capillary for carrying out an in-line sample extraction treatment of complex matrices prior the separation of the analytes. The developed method was successfully applied to the determination of MNZ, RNZ and DMZ in pig liver samples by micellar electrokinetic chromatography (MEKC) coupled to UV detection [63]. Furthermore, 5-NDZ separation was assayed in a CE two-dimension approach, but the developed method was not further applied to any type of sample [65].

In addition to separation-based techniques, other alternatives such as enzyme linked immuno-sorbent assays (ELISAs) [69,70] or immuno-biosensor assays [71,72] have been proposed as inexpensive and reliable techniques that can be useful for screening purposes. Furthermore, electrochemical [73,74] and spectrophotometric [75,76] methods as well as nuclear magnetic resonance spectroscopy [77] and Raman spectroscopy [78] techniques have also been assayed for 5-NDZ determination.

As mentioned above, LC has been the most employed technique for the analysis of 5-NDZ residues due to its performance characteristics. On the other hand, its applicability has been greater in food safety control, and few applications have been reported for their determination in environmental matrices. **Table 2.2** presents an overview of LC-based methods developed for 5-NDZ determination in food products since 1995, whereas environmental applications are included on **Table 2.3**. Both tables present the main operational characteristics of the selected LC methods and their respective applicability to food and environmental samples. In each case, the considered sample treatment has also been indicated. Additionally, clinical applications have been summarized on **Table 2.4** following the same criteria. In addition to the selected methods, several multiclass LC-based approaches have been reported for the determination of two or more veterinary drug groups, including 5-NDZs [79-82]. However, the evaluation of multiclass methods has not been the aim of this Thesis.

In general, LC-UV and LC-MS methods have been proposed for performing 5-NDZ determination. Despite MS advantages, mainly the unequivocal identification of the analyzed compounds, LC-UV methods are also useful because they are ease of operation, involve lower operating costs, allow the detection of an analyte or group of analytes at the first level of interest and furthermore provide quantitative results. Detection wavelength has usually been established between 312 and 350 nm when 5-NDZ determination has been carried out by LC-UV [83-93]. Therefore it supposes a great advantage compared to other LC-UV methods that use lower detection wavelengths involving lower selectivity.

On the other hand, triple quadrupole (QqQ) mass spectometer has been the most common detector for performing 5-NDZ determination by LC-MS [94-111], while only one study has evaluated the use of an ion trap (IT) mass spectrometer [87]. A single quadrupole (Q) detector [85,112-116] and a hybrid Q-ToF (time of flight) mass spectrometer [117] have also been considered as detection device. Although the majority of the proposed LC-MS methods employ electrospray ionization (ESI) as ionization mode for being the most universal [87-108,110,111,113,114,116,117], other ionization modes such as atmospheric pressure chemical ionization (APCI) [85,109,115] and thermospray ionization [112] have also been considered.

Table 2.2. Operational features of LC methods proposed for 5-NDZ determination in food samples.

Analytes	Matrix	Sample treatment	Column	Mobile phase	Analysis time*	Detection mode	Ref.
MNZ, DMZ, RNZ, IPZ, CRZ, ORZ, MNZ-OH, TRZ, HMMNI, IPZ-OH	Animal plasma (5 mL)	SALLE + clean-up with hexane	Luna C18 (100 × 2 mm; 3 µm; Phenomenex)	A: water with 0.1% (v/v) acetic acid B: MeCN with 0.1% (v/v) acetic acid Flow rate: 0.25 mL/min	12.6 min	ESI-QqQ-MS	[94]
MNZ, DMZ, TNZ, RNZ, HMMNI	Honey (5 g)	SALLE + SPE (Bakerbond® amine)	Symmetry C18 (250 × 4.6 mm, 5 µm; Waters)	Isocratic mode: 91:9 (v/v) 0.01 M anhydrous sodium acetic/MeCN Flow rate: 0.8 mL/min	≈ 23 min	UV (315 nm)	[83]
MNZ MNZ-OH (TNZ as internal standard)	Trout tissue (5 g)	SLE + clean-up with hexane + SPE (Bont Elute silica)	NovaPak C18 (300 × 3.9 mm, 4 µm; Waters)	A: 9:3:88 (v/v/v) MeCN/MeOH/H_3PO_4 solution B: 80:20 (v/v) MeCN/H_3PO_4 solution H_3PO_4 solution consisted of 0.12% (v/v) H_3PO_4 aqueous solution adjusted to pH 3 with diethyl amine Flow rate: 0.8 mL/min	≈ 16 min	UV (325 nm)	[84]
DMZ, RNZ, HMMNI	Meat tissue (5 g) Eggs**	SLE + SPE (Bont Elute strong cation exchange, SXC)	LC-UV: Genesis C18 (250 × 3.0 mm, 4 µm; Jonas Chromatography) LC-MS: Prodigy ODS3 (250 × 3.2 mm, 5 µm; Phenomenex)	Isocratic mode: 0.01 M potassium phosphate (pH 4) containing 10% (v/v) MeCN for LC-UV analyses. Flow rate: 0.5 mL/min Isocratic mode: 0.05 M ammonium acetate containing 13% (v/v) of MeCN for LC-MS analyses. Flow rate: 0.5 mL/min	≈ 14 min	UV (315 nm) APCI-Q-MS	[85]
MNZ, RNZ, DMZ, MNZ-OH, HMMNI	Plasma, milk, muscle, egg, feed, honey (5 g)	SLE or LLE + SPE (Strata-SDB)***	Kinetex XB-C18 (100 × 3 mm, 2.6 µm; Phenomenex)	Isocratic mode: 0.1% (v/v) formic acid (pH 2.6)/MeOH (88:12, v/v) Flow rate: 0.7 mL/min	2.3 min	ESI-QqQ-MS	[95]
MNZ, RNZ, DMNZ, TNZ, ORZ, SCZ, HMMNI	Poultry muscle (5 g) Pork muscle (5 g)	SLE + SPE (Bont Elute SXC)	Diamonsil™ C18 (250 × 4.6 mm, 5 µm; Dikma)	A: water B: MeCN C: MeOH Flow rate: 1 mL/min	≈ 18 min	UV (320 nm)	[86]

* Analysis time has been estimated considering the elution time of the last eluted analyte.
** The treated sample amount has not been specified.
*** Sample treatment was selected according to the sample.

Table 2.2 (continued). Operational features of LC methods proposed for 5-NDZ determination in food samples.

Analytes	Matrix	Sample treatment	Column	Mobile phase	Analysis time*	Detection mode	Ref.
DMZ, HMMNI, RNZ, ORZ, IPZ, IPZ-OH, TRZ, SCZ, TNZ, CRZ, MNZ, MNZ-OH	Serum (2.5 mL) Eggs (2.5 g) Muscle (5 g)	SALLE or SLE** + MISPE	Zorbax Eclipse XDB-C8 (150 × 4.6 mm, 5 µm; Agilent Technologies)	A: 90:10 (v/v) water/MeCN containing 0.1% (v/v) of formic acid B: 90:10 (v/v) MeCN/water containing 0.1% (v/v) of formic acid Flow rate: 0.6 mL/min	6.9 min	ESI-QqQ-MS	[96]
DMZ, MNZ, HMMNI, RNZ, MNZ-OH, IPZ-OH, IPZ	Eggs (2.5 g)	SALLE + MISPE	SymmetryShield RP18 (150 × 2.1 mm, 3.5 µm; Waters)	A: 0.1% (v/v) formic acid solution B: MeCN containing 0.1% (v/v) of formic acid Flow rate: 0.3 mL/min	6.4 min	ESI-QqQ-MS	[97]
MNZ, DMZ, RNZ, MNZ-OH, HMMNI, IPZ, IPZ-OH, CRZ, ORZ, TRZ, TNZ, chloramphenicol	Milk (1 mL) Honey (3 g)	SALLE + clean-up with hexane	Zorbax Eclipse Plus C18 (100 × 2 mm, 1.8 µm; Agilent Technologies)	A: 0.1% (v/v) acetic acid solution B: MeCN containing 0.1% (v/v) of acetic acid Flow rate: 0.5 mL/min	4.3 min	ESI-QqQ-MS	[98]
MNZ, DMZ, RNZ, MNZ-OH, HMMNI, IPZ, IPZ-OH, CRZ, ORZ, TNZ, SCZ	Muscle (3 g)	SLE assisted by ultrasound agitation	Kinetex XB-C18 (100 × 2.1 mm, 1.7 µm; Phenomenex)	A: 0.1% (v/v) formic acid solution B: MeCN containing 0.1% (v/v) of formic acid Flow rate: 0.3 mL/min	6.8 min	ESI-QqQ-MS	[99]
MNZ	Fish tissue (5 g)	SASLE + SPE (Supelco NH_2) or QuEChERS**	Gemini-NX C18 (150 × 4.6 mm, 5 µm; Phenomenex	A: 90:10 (v/v) 1 mM ammonium acetate/MeCN (pH 3.57) B: MeCN Flow rate: 1 mL/min	≈ 8.5 min	UV (315 nm) ESI-IT-MS	[87]
MNZ, RNZ, DMZ, HMMNI	Porcine liver (5 g)	SLE + clean-up with hexane + SPE (Oasis®MCX)	SunFire™ C8 (100 × 2.1 mm, 3.5 µm; Waters)	A: water B: MeCN Flow rate: 0.2 mL/min	8.0 min	ESI-QqQ-MS	[100]

* Analysis time has been estimated considering the elution time of the last eluted analyte.
** Sample treatment was selected according to the sample.

Table 2.2 (continued). Operational features of LC methods proposed for 5-NDZ determination in food samples.

Analytes	Matrix	Sample treatment	Column	Mobile phase	Analysis time*	Detection mode	Ref.
MNZ, DMZ, IPZ, MNZ-OH, HMMNI, ORZ, TNZ, TRZ, RNZ, IPZ-OH, CRZ	Eggs (3 g)	SALLE + clean-up with hexane	Luna C18 (100 × 2 mm; 3 µm; Phenomenex)	A: water with 0.1% (v/v) acetic acid B: MeCN with 0.1% (v/v) acetic acid Flow rate: 0.25 mL/min	12.7 min	ESI-QqQ-MS	[101]
DMZ, MNZ, RNZ, MNZ-OH, IPZ-OH, HMMNI	Lyophilized pork meat (1.5 g)	SLE	Genesis C18 (150 × 3 mm, 5 µm; Phenomenex)	A: 90:10 (v/v) 10 mM ammonium formate (pH 3.5)/MeCN B: 10:90 (v/v) 10 mM ammonium formate (pH 3.5)/MeCN Flow rate: 0.4 mL/min	15.1 min	ESI-QqQ-linear IT-MS	[102]
DMZ, MNZ, RNZ, SCZ, CRZ, ORZ, IPZ-OH, HMMNI, MNZ-OH, IPZ, TRZ, menidazole, TNZ, nimorazole	Honey (3 g)	MISPE	Kinetex XB-C18 (150 × 2.1 mm, 2.6 µm; Phenomenex)	A: 0.1% (v/v) formic acid solution B: MeCN containing 0.1% (v/v) of formic acid Flow rate: 0.25 mL/min	8.1 min	ESI-QqQ-linear IT-MS	[103]
DMZ, IPZ, MNZ, RNZ, MNZ-OH, TRZ, HMMNI, IPZ-OH	Eggs (5g)	LLE + SPE (Strata-X-C)	Acquity BEH C18 (100 × 2.1 mm, 1.7 µm; Waters)	A: 0.05% (v/v) formic acid solution B: MeCN containing 0.05% (v/v) of formic acid Flow rate: 0.6 mL/min	6.5 min	ESI-QqQ-MS	[104]
MNZ, MNZ-OH, DMZ, RNZ, HMMNI, IPZ, IPZ-OH	Chicken muscle Chicken plasma Eggs (5 g)	SALLE or SASLE** + SPE (SCX)	Gemini C18 (150 mm × 2 mm, 5 µm; Phenomenex)	A: 0.1% (v/v) formic acid solution B: MeCN containing 0.1% (v/v) of formic acid Flow rate: 0.2 mL/min	11.2 min	ESI-QqQ-MS	[105]
DMZ	Poultry liver Poultry muscle Eggs (4 g)	LLE or SLE** + SPE (Bakerbond® Silica)+ clean-up with hexane	Partisil 5 ODS 3 (250 × 4 mm, 5 µm; Whatman)	Isocratic mode: 50:50 (v/v) water/MeOH containing 0.05 M of ammonium acetate Flow rate: 1 mL/min	≈ 7 min	Thermospray-Q-MS	[112]

* Analysis time has been estimated considering the elution time of the last eluted analyte.
** Sample treatment was selected according to the sample.

Table 2.2 (continued). Operational features of LC methods proposed for 5-NDZ determination in food samples.

Analytes	Matrix	Sample treatment	Column	Mobile phase	Analysis time*	Detection mode	Ref.
MNZ, DMZ, RNZ, HMMNI	Poultry meat (4 g)	SLE + clean-up with a mixture of hexane and CCl_4	Symmetry C18 (150 × 3.9 mm, 5 μm; Waters)	Isocratic mode: 6:13:81 (v/v/v) MeCN/MeOH/formic acid solution (0.2%, v/v) Flow rate: 0.6 mL/min	6.2 min	ESI-Q-MS	[113]
MNZ, RNZ, DMZ, TNZ	Honey (2.5 g)	Stir bar sorptive extraction (SBSE)	Thermo LC-C18 (250 × 4.6 mm, 5 μm; Thermoscientific)	Isocratic mode: 85:15 (v/v) 10 mM sodium acetate buffer/MeCN Flow rate: 1 mL/min	9.2 min	UV (320 nm)	[88]
MNZ, DMZ, IPZ, RNZ, SCZ, TNZ	Feedstuff (0.5 g)	SLE + SPE (Oasis®HLB)	Symmetry C18 (150 × 2.1 mm, 5 μm; Waters)	A: 50 mM ammonium acetate buffer (pH 4.3) B: MeCN Flow rate: it has not been indicated	23.3 min	ESI-Q-MS	[114]
RNZ, MNZ, DMZ	Swine liver (10 g)	SLE + clean-up with hexane + SPE (Oasis®HLB)	Symmetry C18 (150 × 4.6 mm, 5 μm; Waters)	Isocratic mode: 0.01 M ammonium acetate containing 9% (v/v) of MeCN Flow rate: 1 mL/min	7.4 min	APCI-Q-MS	[115]
DMZ, HMMNI, IPZ, IPZ-OH, MNZ, MNZ-OH, RNZ	Eggs (5 g) Processed egg (2.5 g) Chicken meat (5 g)	SALLE + SPE (Oasis®HLB) or SLE + LLE + clean-up with hexane**	Symmetry Shield C18 (150 × 2.1 mm, 3.5 μm; Waters)	A: 0.1% (v/v) formic acid solution (pH 3) B: MeCN containing 0.1% (v/v) of formic acid Flow rate: 0.3 mL/min	5.9 min	ESI-QqQ-MS	[106]
DMZ, MNZ, ORZ, RNZ, MNZ-OH, IPZ, HMMNI, IPZ-OH	Prawn tissue (2 g) Finfish tissue (2 g)	SASLE + clean-up with hexane	Acquity UPLC®BEH C18 (100 × 2.1 mm; 1.7 μm, Waters)	A: 0.01% (v/v) formic acid solution B: MeOH containing 0.01% (v/v) of formic acid Flow rate: 0.45 mL/min	5.8 min	ESI-QqQ-MS	[107]
MNZ, RNZ, DMZ	Eggs (10 g)	LLE	Alltima C18 (150 × 2.1 mm, 5 μm)***	A: 0.1% (v/v) formic acid solution B: MeCN containing 0.1% (v/v) of formic acid Flow rate: 0.25 mL/min	< 9.8 min	ESI-QqQ-MS	[108]

* Analysis time has been estimated considering the elution time of the last eluted analyte.
** Sample treatment was selected according to the sample.
*** The manufacturer of the chromatographic column has not been indicated.

Table 2.2 (continued). Operational features of LC methods proposed for 5-NDZ determination in food samples.

Analytes	Matrix	Sample treatment	Column	Mobile phase	Analysis time*	Detection mode	Ref.
MNZ, DMZ	Poultry muscle Poultry liver Eggs (5 g) Poultry serum (2 mL)	SLE or LLE** + SPE (Bakerbond C18)	Bakerbond BDS-C18 (200 × 4.6 mm, 5 µm; Baker)	Isocratic mode: 70:30 (v/v) 0.05 M ammonium acetate (pH 4.3)/MeCN Flow rate: 1 mL/min	2.6 min	UV (350 nm)	[89]
MNZ, RNZ, DMZ, MNZ-OH, IPZ, HMMNI, IPZ-OH	Pig plasma (5 mL)	LLE + clean-up with hexane + SPE (Chromabond XTR®)	Genesis C18 (250 × 3 mm, 4 µm; Jones Chromatography)	A: 0.1% (v/v) acetic acid solution B: MeCN Flow rate: 0.6 mL/min	13.3 min	APCI-QqQ-MS	[109]
RNZ, DMZ, HMMNI, MNZ, MNZ-OH	Bovine muscle (10 g)	SASLE + clean-up with 0.1% (v/v) formic acid solution and petroleum ether	Eclipse C18 (150 × 4.6 mm, 3 µm; Agilent Technologies)	Isocratic mode: 82:18 (v/v) formic acid solution/MeCN Flow rate: 1 mL/min	3.9 min	ESI-QqQ-MS	[110]
RNZ, DMZ, MNZ, MNZ-OH, IPZ, IPZ-OH, HMMNI	Swine kidney (5 g)	SLE + SPE (Oasis®MCX)	Acquity UPLC®BEH C18 (50 × 2.1 mm; 1.7 µm, Waters)	A: water B: MeCN Flow rate: 0.3 mL/min	2.5 min	ESI-QqQ-MS	[111]

* Analysis time has been estimated considering the elution time of the last eluted analyte.
** Sample treatment was selected according to the sample.

Table 2.3. Operational features of LC methods proposed for 5-NDZ determination in environmental samples.

Analytes	Matrix	Sample treatment	Column	Mobile phase	Analysis time*	Detection mode	Ref.
MNZ	Sediment (0.5 g and 5 g) Water samples (500 mL)	SALLE or SLE + SPE (Strata XC)**	Gemini-NX C18 (150 × 4.6 mm, 5 µm; Phenomenex)	A: 90:10 (v/v) 1 mM ammonium acetate/MeCN (pH 3.57) B: MeCN Flow rate: 1 mL/min	≈ 8.5 min	UV (315 nm) ESI-IT-MS	[87]
MNZ, DMZ, IPZ, RNZ, HMMNI (TRZ as internal standard)	Water samples (5 mLand 50 mL)	SPE (Oasis® HLB)	C18*** (150 × 2.1 mm, 5 µm)	A: 5 mM ammonium acetate solution (pH 4.3) B: MeCN Flow rate: 0.3 mL/min	23.2 min	ESI-Q-MS	[116]

* Analysis time has been estimated considering the elution time of the last eluted analyte.
** Sample treatment was selected according to the sample.
*** The manufacturer of the chromatographic column has not been indicated.

Table 2.4. Operational features of LC methods proposed for 5-NDZ determination in clinical samples.

Analytes	Matrix	Sample treatment	Column	Mobile phase	Analysis time*	Detection mode	Ref.
MNZ, MNZ-OH, (TNZ as internal standard)**	Human plasma Gastric juice Saliva (0.5 mL)	LLE	Hypersil ODS (150 × 4.6 mm, 5 μm; Shandon HPLC)	Isocratic mode: 10:90 (v/v) MeCN/0.05 phosphate buffer (pH 7) containing 0.1% triethylamine (TEA) Flow rate: 1 mL/min	7 min (15 min with TNZ)	UV (317 nm)	[90]
MNZ (clenbuterol-d_9 as internal standard)	Equine plasma (500 μL)	LLE	ACE C18 (75 × 2.1 mm, 5 μm; MaC-Mod Analytical)	A: 5 mM ammonium formate (pH 3.5) B: MeCN Flow rate: 0.3 mL/min	3.7 min	ESI-Q-ToF-MS	[117]
MNZ, its metabolites including MNZ-OH and their conjugates	Urine	Sample dilution	LiChrosorb Si 60 (120 × 4.6 mm; 5 μm; Knauer)	Isocratic mode: 25:30:45 (v/v/v) MeOH/0.2 M potassium phosphate (pH 7)/water Flow rate: 0.5 mL/min and increased to 1 mL/min after 9 min	≈ 2.2 min	UV (312 nm)	[91]
MNZ, omeprazole	Human plasma Gastric fluid (0.3 mL)	SPE (C2 cartridges)	Omnipac Pax-500, anion exchange and reversed phase column (250 × 4 mm, 8.5 μm; Dionex)***	Isocratic mode: 60:20:20 (v/v/v) 0.1 M sodium phosphate buffer/MeOH/MeCN (pH 7) Flow rate: 0.7 mL/min	7.6 min	UV (254 nm)	[118]
MNZ, ranitidine	Human plasma (250 μL)		Shimpak C18 (300 × 4.6 mm, 5 μm; Phenomenex)	Isocratic mode: 90:10 (v/v) 10 mM potassium dihydrogen phosphate (pH 3.5)/MeCN Flow rate: 1 mL/min	≈ 6.5 min	UV (315 nm)	[119]
MNZ, (TNZ as internal standard)	Vaginal tissue (0.25 g)	SPE (C18 cartridges from J&W Scientific)	Zorbax SB-phenyl (150 × 4.6 mm, 5 μm; Mac-Mod Analytical)	Isocratic mode: 85:15 (v/v) 0.01 M potassium phosphate (pH 4)/MeOH Flow rate: 1 mL/min	23.2 min	UV (313 nm)	[92]
MNZ (TNZ as internal standard)	Human serum (1 mL)	LLE	LiChrospher 100 RP18 (125 × 4 mm, 5 μm; Merck)	Isocratic mode: 85/15 (v/v) 0.01 M phosphate solution (pH 4.7)/MeCN Flow rate: 1 mL/min	4.8 min	UV (318 nm)	[93]

* Analysis time has been estimated considering the elution time of the last eluted analyte.
** Internal standard was only added to gastric juice samples.
*** Resin composition: ethylvinylbenzene/divinylbenzene macroporous substrate (55% cross-linked) agglomerated with 60-nm MicroBead alkanolquaternary amine-functionalized latex (4% cross-linked).

Additionally, 5-NDZ separation has been normally performed in columns consisted of C18 stationary phase, so the use of other type of stationary phase has scarcely been investigated. However, some studies using C8 [96,100], phenyl [92], silica [91] and mixed anion exchange-reverse phase stationary phase [118] have also been reported. On the other hand, conventional [83,84,86-93,96,110,112,113,115,119] and microbore [85,94,95,97-109,111,114,116,117] columns have been evaluated for accomplishing 5-NDZ separation, but no micro- or capillary columns have been tested for that purpose (column characteristics are described on **Chapter 10**). Furthermore, most methods for 5-NDZ determination have been developed using as stationary phase particles with a size between 2.5 and 5 μm, whereas few methods using ultra-high performance liquid chromatography (UHPLC) technology have been proposed [98,99,104,107,111].

A wide variety of food-producing animals can be treated with 5-NDZ antibiotics, thus residues of them can be present in a wide variety of food products such as milk, eggs, honey, and animal tissues. The development of LC-methods for 5-NDZ determination in animal tissues [85,86,89,95,96,99,102,105-107,110-113] and eggs [89,95-97,101,104-106,108,112] has been widely investigated. Moreover, the analysis of 5-NDZ residues has also been evaluated in animal serum or plasma [89,94-96,105,109] as well as in honey [83,88,95,98,103] and milk [95,98]. However, few works have been reported for 5-NDZ determination in fish tissue [84,87,107] or crustaceans such as prawn [107], but no methods have been reported for their determination in fish roes according to the reviewed literature. Additionally, two methods have been found for the analysis of 5-NDZ compounds in animal feed [95,114].

Despite the wide range of food applications, few methods have been reported for 5-NDZ determination in environmental matrices [87,116]. Consequently, methods for that purpose are required, as previously suggested by Mahugo-Santana *et al.* [54]. On the other hand several LC-methods have been proposed for the analysis of 5-NDZ drugs in complex matrices in the clinical field, such as urine, serum, vaginal tissue and gastric juice (see **Table 2.4**). Nevertheless, these methods are mainly focused on the determination of one 5-NDZ substance and its metabolites, or on the analysis of two or more pharmaceuticals including one 5-NDZ antibiotic.

Regarding sample treatment, SLE and LLE has been usually accomplished as first sample treatment stage. MeCN [84,89,95,99,108] and ethyl acetate [86,100,102,113,115] have been mainly used for that purpose, but other extraction solvents such as acidified MeOH [89], toluene and dichloromethane [105], phosphate solution (pH 2) [114] and sodium acetate solution (pH 5) with trichloroacetic acid (TCA) solution (5%, w/v), have been also considered. Moreover, protein precipitation can also be achieved through this extraction stage. Furthermore, salts such as NaCl have been added to the sample in order to favor the extraction due to a salting-out effect, thus SASLE [87,107,110] and SALLE [83,87,94,96-98,101,105,106] have been carried out.

On the other hand, several sample clean-up procedures have been proposed after the extraction stage, or the whole sample treatment has mainly consisted of a clean-up stage. In addition to protein precipitation, fat removal is usually carried out for complex samples. It is usually accomplished by the addition of hexane to the sample and its subsequent removal [84,94,98,100,101,106,107,109,112,115], although the use of a mixture of hexane and CCl_4 [113] or a mixture consisted of 0.1% (v/v) formic acid solution and petroleum ether [110] has also been proposed. Moreover, SPE has been widely considered for sample clean-up. Cartridges with different sorbents have been evaluated for 5-NDZ extraction, namely cation exchange [85-87,100,104,105,111], silica [84,112], amine [83,87], HLB [106,114-116], C18 [89,92], C2 [118] and MIPs [96,97,103] cartridges. Other techniques such as QuEChERS [87] or SBSE [88] have been less employed.

In view of the above, LC methods have been widely proposed for 5-NDZ determination, but the use of miniaturized techniques such as CE, CEC, capillary liquid chromatography (CLC) or nano-LC has barely been tested. One of the main advantages of miniaturized separation techniques is the low solvent consumption. However, few methods using CE have been reported [59-66], and although CEC has been evaluated for 5-NDZ separation, the proposed methods have not been applied to any sample [120,121].

In addition to miniaturized techniques, a reduction of solvent consumption can also be achieved by the use of UHPLC technology, but it has not been widely explored for

5-NDZ determination. Moreover, when it has been used, only MS detection has been considered.

Furthermore, analytical methods are required for the analysis of 5-NDZ residues in environmental samples, but also for their determination in several food products that have been scarcely investigated. The control of 5-NDZ residues in milk, crustaceans, fish tissues or related products has been suggested by EURLs [38], but few methods have been reported for that purpose.

In view of all that has been set out above, there is a lot of work to do regarding to the development of analytical methods for the determination of 5-NDZ residues in environmental, food and other biological samples. With this aim, novel analytical methods based on CE, CEC, CLC and UHPLC techniques are proposed for the analysis of these compounds in different matrices. The use of UV and MS as detection mode is also evaluated. Additionally, simple, low-cost and environmental-friendly sample treatments are investigated for 5-NDZ extraction and sample clean-up prior to their analysis.

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PART II

5-NDZ DETERMINATION BY CE-BASED METHODS

Chapter 3:

Capillary electrophoresis

Chapter 4:

Determination of 5-NDZs in environmental water and milk samples by micellar electrokinetic chromatography

Chapter 5:

CSEI-sweeping-MEKC-UV approach for 5-NDZ residue analysis in water, urine, serum and egg samples

Chapter 6:

Simultaneous determination of 5-NDZs in urine samples by CE-ESI-MS/MS using molecular imprinted solid phase extraction

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3

Capillary electrophoresis

3.1. Background

Electrophoresis is the phenomenon whereby ions and solutes migrate under the influence of an electric field. The beginning of electrophoresis can be attributed to the studies of the migration of hydrogenium ions in a phenolphthalein gel carried out by Lodge in 1886, and the subsequent description of the migration of ions in saline solutions proposed by Kohlraush in 1897 [1]. However, electrophoresis was not employed as separation technique until 1937 when, based on his previous studies of moving-boundary electrophoresis, Tiselius demonstrated that a mixture of serum proteins could be separated in an electric field [2]. Because of this discovery, Tiselius was awarded the Nobel Prize in Chemistry in 1948.

With the aim of improving electrophoresis features and, especially, in order to reduce the generation of Joule heat, systems of different materials were evaluated for performing electrophoretic separations. In 1939, Coolidge assessed the electrophoretic separation of serum proteins in tubes of glass wool. Furthermore column electrophoresis using glass powder was developed by Haglund and Tiselius in 1950, whereas the use of cellulose powder was subsequently tested by Porath in 1956 [3].

An important breakthrough in CE development was achieved by Hjertén in 1967. For the first time, he carried out the 'free solution electrophoresis' using quartz glass tubes of 3 mm of inner diameter (i.d.) coated with methylcellulose, showing a reduction of electroosmotic flow (EOF) and convection phenomena [4]. Later on, in 1979, Mikkers *et al.* reported that the use of narrow-bore tubes in free solution electrophoresis reduced the dispersive effects, achieving high efficiency separations [5].

Although applications using free solution electrophoresis were further reported, it did not become popular until 1981. In this year, Jorgenson and Lukacs performed electrophoresis analyses in silica capillaries of 75 μm of i.d. and 100 cm of length considering a separation voltage of 30 kV. Under these conditions, they accomplished the determination of amino acids, peptides and urinary amines using a fluorescence detector [6]. This achievement supposed the beginning of CE, namely the beginning of CZE. In 1984, Terabe developed an alternative CE method called MEKC following Nakagawa's approaches about electrophoretic separation using ionic micelles [7]. With this methodology, neutral and charged compounds were simultaneously separated by the simple addition of a surfactant to the BGS [8].

In 1988, Applied Biosystems (Foster City, CA, USA) and Beckman Coulter (Fullerton, CA, USA) launched to the market the first CE instruments, and as a consequence, the applicability of CE was rapidly extended [9-15], and further challenges such as CE hyphenation with MS appeared.

CE may be regarded as the most important milestone in separation sciences during the latter part of the 20th century, because it can be applied to simple problems such as the assay of pharmaceutical products and to more complex problems such as the mapping of the human genome and proteome [16]. CE is a miniaturized separation technique which is characterized by the high efficiency achieved in the separations, involving short analysis times. Furthermore, it is a green analytical technique because it consumes a low volume of solvents and requires low sample volumes.

In general, CE can be considered as a collection of electrophoretic separation techniques, which have only in common that they are performed in a narrow capillary with a high-voltage source providing the energy required in the separation [17]. In order to clarify this aspect, different CE modes are described on **Table 3.1** according to definitions proposed by European Pharmacopeia and International Union of Pure and Applied Chemistry (IUPAC) [18,19].

Table 3.1. **Classification of main CE modes according to definitions from European Pharmacopeia and IUPAC [18,19].**

Separation mode	Separation mechanism
Capillary zone electrophoresis (CZE)	Analyte ions are separated in a BGS present in the capillary and in both electrode vials before the analysis. With this technique, separation takes place because the different components of the sample migrate as discrete bands with different velocities. The velocity of each band depends on the electrophoretic mobility of the solute and the EOF in the capillary. Using this CE mode, the analysis of both small analytes (relative mass, M_r, < 2000 Dalton) and large molecules (2000 < M_r < 10^5 Dalton) can be accomplished.
Micellar electrokinetic chromatography (MEKC)	Separation takes place in an electrolyte solution which contains a pseudo-stationary phase, normally a surfactant at a concentration above the critical micellar concentration (CMC). One of the most widely used surfactants in MEKC is sodium dodecyl sulfate (SDS). The solute molecules are distributed between the aqueous buffer and the pseudo-stationary phase composed of micelles, according to the partition coefficient of the solute. Separation of both neutral and charged solutes can be performed, maintaining the efficiency, speed and instrumental suitability of CE.
Capillary electrochromatography (CEC)	Separation technique in which the mobile phase movement through a capillary, filled, packed or coated with a stationary phase, is achieved by EOF (which may be assisted by pressure). The retention is due to a combination of electrophoretic migration and chromatographic retention. The advantage of CEC over pressure-driven chromatography is that there is no pressure limit on the size of the particles that can be used.
Capillary gel electrophoresis (CGE)	Separation takes place inside a capillary filled with a gel that acts as a molecular sieve. Molecules with similar charge-to-mass ratios are separated according to molecular size since smaller molecules move more freely through the network of the gel and therefore migrate faster than larger molecules. Different biological macromolecules (for example, proteins and DNA fragments), which often have similar charge-to-mass ratios, can thus be separated according to their molecular mass by capillary gel electrophoresis.
Capillary isoelectric focusing (CIF)	Separation of amphoteric analytes according to their isoelectric points is performed by the application of an electric field along a pH gradient formed in a capillary.
Capillary isotachophoresis (CITP)	Electrophoretic separation technique in a discontinuous buffer system in which the analytes migrate according to their electrophoretic mobility, forming a chain of adjacent zones moving with equal velocity between two solutions, leading and terminating electrolytes, bracketing the mobility range of the analytes.

In addition to CE modes indicated on **Table 3.1**, other less used electrophoretic modes such as capillary sieving electrophoresis (CSE), affinity capillary electrophoresis (ACE) and microemulsion electrokinetic chromatography (MEEKC) can be included as proper CE modes. Similarly, chiral capillary electrophoresis (CCE) and non-aqueous capillary electrophoresis (NACE) can also be regarded as other types of CE. However, some authors consider CCE as a CZE modification where additives such as cyclodextrins are employed for achieving a chiral separation [20]. NACE can also be considered as CZE modification in which an organic BGS is used instead of an aqueous running buffer [21].

Since CE has been integrated in analytical laboratories, it has found applications within many fields, especially pharmaceutical science and biochemistry, being an important tool in drug control assays and analyses related to proteins, peptides, etc. [22-24]. Nowadays the use of CE has been extended to environmental and food analysis fields [25-28] where CZE is the most common CE methodology and its applicability has been successfully demonstrated in residue analyses. It supposes an important milestone considering the lack of sensitivity that is generally attributed to CE methods, especially when UV detection is employed. As it will further discuss, different strategies have carried out in order to overcome this drawback.

Recent applications of CZE methods include the determination of veterinary drugs such as aminoglycosides in honey [29], fluoroquinolones in milk [30] or sulfonamides in meat samples [31], and pesticides such as sulfonylureas in water and grape samples [32] or amitrol and triazines in water samples [33]. Furthermore, the analysis of compound traces in other matrices apart from food and environmental samples have also been accomplished by CZE. The determination of pesticide residues in marijuana samples [34] or abuse drugs in urine and hair samples [35-37] are just a few examples of the applicability of CZE in residue analyses. Although to a lesser extent, MEKC and CEC modes have also been applied for the purpose of monitoring residues in food and environmental samples, as well as in other biological matrices [25,38,39].

In this Thesis, both CZE and MEKC modes have been considered for the determination of 5-NDZ residues. In **Chapter 4**, a novel MEKC methodology is proposed for the

determination of 5-NDZs, including some of their more relevant metabolites, in environmental and food matrices. It supposes the first time that 5-NDZ metabolites are analyzed by CE. Furthermore, a comparison between CZE and MEKC as separation techniques is carried out in **Chapter 6**. Besides, the hyphenation of both techniques with MS is addressed. Moreover, in **Part 2** of this Thesis, which includes **Chapter 8** and **Chapter 9**, CEC using packed capillaries and coupled to both UV and MS detection is evaluated for the first time as separation technique for 5-NDZ determination.

3.2. CE-UV/Vis

Separation by CE can be monitored with several devices as fluorescence, chemiluminescense or conductivity detectors, however, UV/Vis detection is the most commonly used. Nowadays, commercial CE instruments coupled with diode array detector (DAD) are available from Beckman Coulter (Fullerton, CA, USA) and Agilent Technologies (Waldbronn, Germany). The primary advantage of the DAD is the increased confidence with which purity and identity of a peak can be established [40]. Furthermore, the use of a DAD presents other important advantages such as UV/Vis spectra monitoring during the entire sample analysis, electropherogram collection at any wavelength by one single run and determination of UV maximum absorption of each compound.

However, CE-UV/Vis methods have a major drawback that is the lack of sensitivity attributed to them due to the low sample volume injected in each analysis and the short optical path length of the capillary detection window. This inconvenience is significantly relevant for residue determination methods that require high sensitivity in order to reach low detection limits. With the aim of overcoming this lack of sensitivity, several strategies can be followed when a CE-UV determination is performed. Sample preconcentration can be achieved through different sample treatments as was described in **Chapter 1**. This strategy has been widely used, and its suitability to CE-UV methods in the residue analysis field has been successfully demonstrated [41-43]. In addition to the preconcentration achieved by off-line sample treatment procedures, high sample preconcentration can be

reached by on-line and/or in-line strategies [44,45]. On-line preconcentration approaches will be widely discussed in **Section 3.3**.

UV absorption (A) is a function of the optical path length (l) according to Lambert-Beer's law (**Equation 3.1**), as well as compound molar absorptivity coefficient (ε), which is a specific characteristic of every species, and analyte concentration (c). I_0 and I are the intensity of the incident light and the transmitted light, respectively.

$$A = log\ \frac{I}{I_0} = \varepsilon \times c \times l \quad \textbf{Equation 3.1.}$$

Therefore, extension of the optical path length should lead to increase detection sensitivity. However, an increased capillary i.d. is inadvisable because increased Joule heating results, involving a loss of resolution from increased peak widths. For that reason, different approaches have been proposed for increasing the optical path length without increasing the i.d. of the capillary along its whole length. Among the proposed alternatives, the use of extended light path capillaries is the most frequent strategy although the use of high detection Z-shaped flow cells has also been widely evaluated (**Figure 3.1**). Less known approaches are the used of square or rectangular capillaries and multireflection flow cells [46].

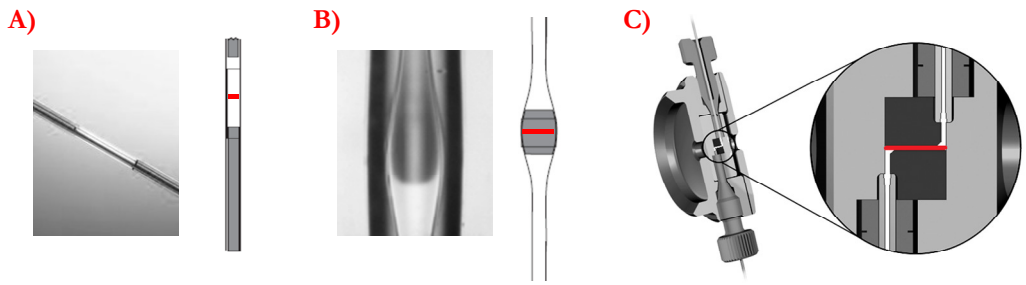

Figure 3.1. Scheme of the optical path of different CE capillaries: A) standard capillary, B) extended light path capillary or 'bubble' capillary, and C) capillary coupled to a Z-shaped flow cell. Considering capillaries of 75 µm i.d., optical path length can be increased from 75 µm, when a standard capillary is used, to 202.5 µm if a bubble capillary from Agilent Technologies is considered, and to 1200 µm if Z-shaped flow cell from Agilent Technologies is employed. Adapted from Agilent Technologies website.

Extended light path capillaries or 'bubble' capillaries are commercially available from Agilent Technologies, and they are made by forming an expanded region, a bubble, directly

on the capillary column. In the region of the bubble, the electrical resistance is reduced and thus the electric field is decreased. When the sample zone enters into the bubble region, its velocity decreases in a manner similar to field-amplified (FA) injection and the zone is compressed axially but spread radially, resulting in the same concentration in the compressed analyte zone [47]. Sample preconcentration factors about 3- to 5-fold can be achieved for CE methods using "bubble" capillaries [48,49], without any significant band broadening. On the other hand, two strategies have been established for sensitivity enhancement by Z-shaped flow cells. Capillary can be bent parallel to the light path at right angles to the capillary with the aim of providing a longer path length, or a special Z-shaped capillary cartridge interfaced with ends of capillary column can be employed [50]. Signal enhancement up to 14-fold in comparison with standard capillary sensitivity has been reported for the use of Z-shaped flow cells [51].

3.3. CE preconcentration modes

Due to small dimensions of CE capillaries, typically 25-150 μm of i.d. and 40-80 cm of capillary length, only a few nanoliters of sample can be loaded onto the column when a CE method is performed. In order to increase the amount of sample that is injected, different on-line and in-line preconcentration strategies have been proposed. On-line approaches, commonly known as stacking techniques, are performed in a completely integrated and automated manner in the CE system, whereas in-line strategies are usually carried out within the capillary [52]. Furthermore, on-line approaches involve changes in the electrophoretic velocity of the analytes, being the analyte velocity in the sample zone faster than the analyte velocity in the BGS, whereas other mechanisms such as interactions between a stationary phase and the analytes are involved in in-line preconcentration systems.

Although different strategies have been evaluated for residue preconcentration in CE methodologies such as in-line SPE [53,54] or the use of a supported liquid membrane device [55,56], in this Thesis only on-line preconcentration approaches are addressed. In **Figure 3.2**, stacking techniques have been classified according to the mechanism that causes the variation of analyte velocity, and as a consequence, analyte preconcentration.

Changes in analyte electrophoretic velocity during on-line preconcentration methods can be accomplished by the variation of electric field strength or composition between different capillary zones, including BGS and sample solvent. Furthermore, modification of analyte electrophoretic velocity can be physically achieved by ion-selective membranes and nano/microchannel interfaces [57,58]. Strategies based on field-strength or chemically induced modifications in velocity are discussed in detail for being one of the purposes of **Chapter 4** and **Chapter 5**.

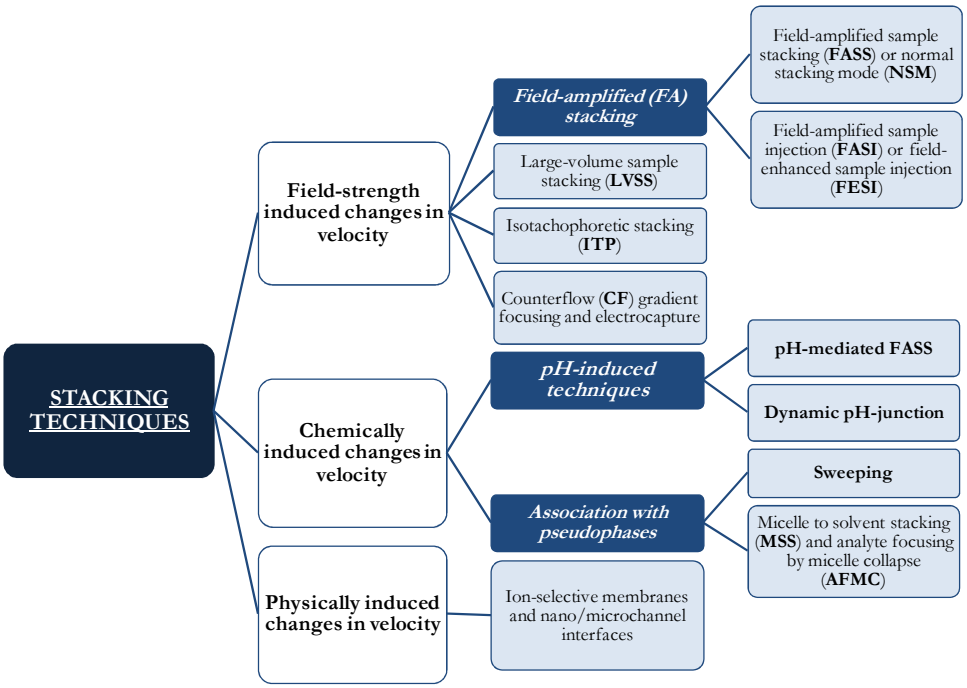

Figure 3.2. Classification of stacking techniques according to the phenomena that produces the variation on analyte velocity. Dark-blue boxes indicate a group of preconcentration techniques and light-blue boxes indicate a specific preconcentration technique. Information extracted from [52].

FA stacking is one of the most popular and simplest stacking techniques and it was firstly introduced by Mikkers *et al.* [59]. It is based on the conductivity difference between sample and BGS, being sample conductivity lower than BGS conductivity. Because samples have lower conductivity than BGS, the electric field in the sample zone is higher than that in the BGS. Thus analytes move faster in the sample zone than in the BGS. Once injection is carried out and separation voltage is established, analytes migrate faster through the sample zone, suffering a decrease of their velocity when they pass the boundary between sample

matrix and BGS. As a consequence, analytes are stacked in this boundary. FA can be easily accomplished by sample dilution with a pure solvent or by placing a section of pure solvent in front of the sample in the capillary [42]. FA stacking strategies can be divided in two groups, depending if sample injection is hydrodynamically or electrokinetically performed. When samples are hydrodynamically injected, field-amplified sample stacking (FASS) takes place, whereas field-amplified sample injection (FASI) occurs when samples are electrokinetically injected.

For FASS, also known as normal stacking mode (NSM), the sample must be at least ten times less conductive than BGS [60]. A short plug of low conductivity medium (for example a water plug) is sometimes injected before sample for achieving analyte preconcentration by this strategy. Although FASS has been proposed for the preconcentration of compounds at trace levels [61,62], other on-line preconcentration strategies have demonstrated to reach higher sensitivity enhancement factors (SEFs) [63,64]. The main limitation of FASS is that only short sample injections can be accomplished without any loss of peak resolution or separation efficiency [65]. In **Chapter 6**, a FASS approach is considered for the preconcentration of 5-NDZ residues and their subsequent determination by CZE-MS.

On the other hand, FASI also reported as field-enhanced sample injection (FESI) [52] allows injecting a greater amount of sample than in FASS without showing any loss of peak resolution. Furthermore, FESI offers another advantage over FASS due to its selectivity because only charged analytes can be successfully injected by this technique. Because samples are electrokinetically injected in FESI, the injected sample amount is not proportional to the injection time due to depletion of the analytes in sample solution during the injection [66]. Although an electrokinetic injection can be more irreproducible than a hydrodynamic one, higher reproducibility has been achieved when a water plug is injected prior to sample injection, and therefore it results in an adequate strategy for performing FESI [67]. It should be noted that FESI is probably one of the most employed on-line preconcentration strategies in CE and its effectiveness has been demonstrated in different fields such as food [68,69], environmental [70,71] and forensic [35,72] analyses.

Figure 3.3 shows a schematic diagram of FESI injection and separation of cationic compounds.

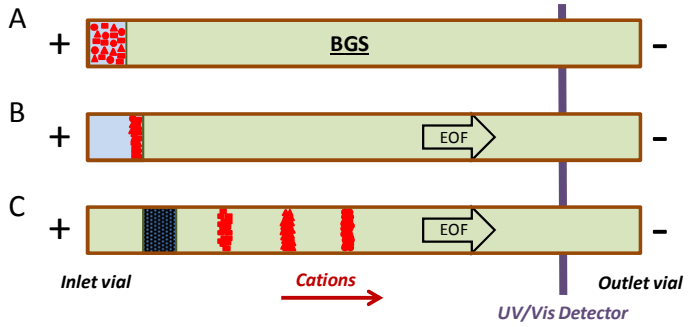

Figure 3.3. Scheme of FESI preconcentration for cationic analytes. A) Capillary is conditioned with BGS and the sample, dissolved in a lower conductivity solvent, is injected for a certain time under positive voltage. B) Separation voltage is applied (positive mode) and analytes are stacked in the boundary between the sample zone and BGS because of their mobility changes. C) Stacked analytes migrate and are separated by CZE. Matrix sample enters into the capillary due to the EOF, which is directed to the cathode under the considered conditions.

Large-volume sample stacking (LVSS) is an efficient alternative to FASS in which samples are hydrodynamically injected for longer times than in FASS without showing any loss of peak resolution. Separation efficiency is preserved by removing the sample matrix after performing the injection. LVSS with matrix removal can be carried out with or without polarity switching and a large sample volume (up to the entire capillary volume) of a low conductivity sample can be hydrodynamically injected.

In LVSS with polarity switching, the analytes are stacked in the boundary between the sample solution and the BGS because of the conductivity difference. Afterwards, sample matrix is removed using EOF, while the stacked zone gradually moves toward the inlet. Electrical polarity must be reversed for the separation before stacked analyte zone reaches the exit of the inlet. The timing for switching the polarity is established by the electrical current. Polarity is reversed when the current reaches 95% of the current related to a capillary filled with BGS [73]. In spite of the fact that LVSS with polarity switching have been successfully applied to the determination of residues such as β-lactam antibiotics in milk [74], pesticides in red wines [75], or cephalosporins in environmental water samples [76], an important disadvantage is attributed to this methodology. The main drawback of

LVSS with polarity switching is that it cannot be automated by the majority of commercial CE instruments, so the presence of an operator is required for switching the polarity of the system. In order to overcome this disadvantage, the suppression or direction-inversion of the EOF has been proposed by the addition of dynamic coating materials or other additives such as organic solvents to the BGS or the use of very acid BGSs [77-80].

Isotachophoresis was employed as on-line preconcentration method in early studies at the beginning of 1990s, introducing the term transient isotachophoresis (tITP) [81]. In tITP, larger sample volumes than in FASS are injected between a higher mobility co-ion (leading electrolyte) and a lower mobility terminating electrolyte, while the sample ions have an intermediate mobility. Because electric field strength is inversely proportional to ion mobility in that region, analytes are concentrated between the leading and termination ions when a voltage is applied to the system, and subsequently they are separated by normal CZE [82]. Although tITP is a strategy widely used in biological applications [52,83] other preconcentration strategies such as FESI or LVSS have been much exploited for residue preconcentration in food and environmental fields.

Finally, among on-line preconcentration strategies based on electric field strength variations, counter-flow (CF) gradient focusing should be at least mentioned, though its applicability has not been explored for residue preconcentration. CF gradient focusing techniques are defined as methods whereby a combination of electrophoresis and a bulk solution counter-flow is used to accumulate or focus analytes at stationary points along the separation column, being a powerful tool for sample concentration in lab-on-chip systems [84].

Stacking strategies due to compound velocity variations induced by chemical phenomena can be classified in pH-induced techniques and strategies associated to pseudo-stationary phases. Among pH-induced alternatives, dynamic pH-junction is the most common approach. It consists of injecting the analyte in a medium of different pH from BGS pH, ensuring that analyte mobility in the sample zone is higher than in the BGS. Because analyte ionization is modified when it reaches the boundary between sample zone and BGS, its mobility is reduced, and as a consequence it is focused in this region [85]. As

occurs with tITP or CF gradient focusing, the application of dynamic pH-junction strategies to the preconcentration of pesticides or pharmaceuticals in food or environmental matrices has not been extensively investigated. On the contrary, preconcentration strategies associated to pseudo-stationary phases, mainly sweeping, have been widely evaluated for pesticide determination in different matrices such as water samples [86], vegetables and fruits [87,88] and fruit juices [89].

Sweeping is an on-line preconcentration strategy related to MEKC separations and it involves both electrophoretic and chromatographic principles. Firstly introduced by Quirino and Terabe [90], sweeping phenomenon is based on the effect occurred when a sample is hydrodynamically injected in a solution devoid of micelles with lower, similar or higher conductivity than the BGS. When separation voltage is applied, charged micelles in BGS penetrate the sample zone and "sweep" the analytes. As a consequence, analytes are accumulated and preconcentrated in narrow bands due to their interaction with the pseudo-stationary phase (micelles). In **Chapter 4**, a sweeping approach is proposed as on-line preconcentration for 5-NDZ determination in water and milk samples. A scheme of sweeping procedure in a homogeneous electric field (matrix zone conductivity is similar to BGS conductivity) is shown in **Figure 3.4**.

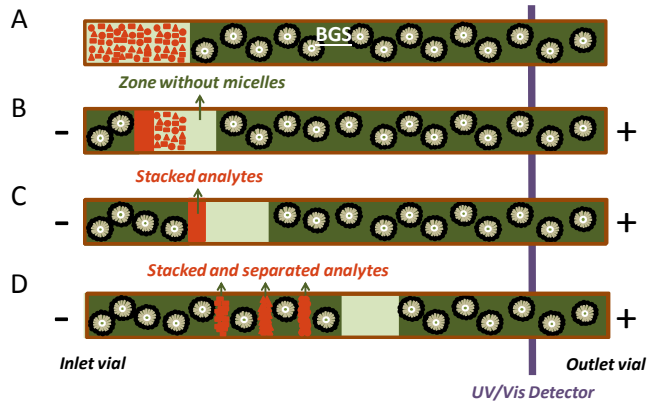

Figure 3.4. Sweeping and separation in a homogeneous electric field. Evolution of analyte zones MEKC using negatively charged micelles and the zero EOF condition. A) Injection of a large volume of sample devoid of micelles and prepared in a solution with similar electric conductivity to the BGS containing micelles. B) Application of voltage via the cathode at the inlet end while both ends of the capillary are immersed into two vials containing BGS. Micelles enter into the sample zone and sweep the analyte molecules. C) Formation of the final swept zone when micelles completely fill the sample zone. D) Separation of analytes by MEKC. Adapted from [91].

Since its introduction in 1998, sweeping has grown to be one of the most prominent and universal concentration systems in the field because of its applicability to both charged and neutral species and its tolerance of high ionic strength samples [52]. Thus, following sweeping principles, other preconcentration techniques such as analyte focusing by micelle collapse (AFMC) and micelle to solvent stacking (MSS) have been developed.

AFMC technique relies on the use of a micelle carrier phase (found in the sample) that collapse in a "micelle dilution zone" located between the sample zone and BGS devoid of micelles. Samples must be dissolved in solutions with higher conductivity than BGS and it has to contain micelles at a concentration above the CMC [92,93]. Because micelles migrate to the anode, neutral analytes are transported to micelle dilution zone. When micelle carrier phase reaches this zone, it is diluted below the CMC, causing the collapse of the micelles and releasing the previously bound analyte molecules. The continued transport and release causes the stacking of the analytes at the micelle dilution zone. The main drawback of AFMC is that neutral analytes cannot be separated after their concentration [94].

On the other hand, MSS can be considered a variation of AFMC where charged analytes are concentrated instead of neutral ones. For performing MSS samples must be prepared in solvents containing micelles. Furthermore, micelles have an opposite charge compared to analytes. The focusing effect relies on the reversal in the effective electrophoretic mobility at the boundary zone between the matrix containing micelles and the BGS modified with organic solvent [95]. At the beginning, when separation voltage is applied, analyte bound to the micelles migrates to the anode, reaching the boundary between the sample zone and the BGS. However, in this boundary, the effective electrophoretic mobility of the analyte is inversed, and as a consequence, it is stacked. The inversion of the electrophoretic mobility is due to the lower affinity of the analytes to the micelles in the boundary between sample zone and BGS because of the presence of an organic solvent [96]. Afterwards, analytes migrate to the cathode and are separated by CZE or NACE, according to the organic solvent content of the BGS.

APFC and MSS have been considered as on-line preconcentration strategies for environmental, clinical and food applications such as the determination of hypolipidemic drugs in wastewater samples [97], alkaloids in urine [98], 5-NDZs in rabbit plasma [99], or herbicides in milk samples [100].

As it has been mentioned, 5-NDZ on-line preconcentration have been proposed for DMZ, MNZ and SCZ determination in rabbit plasma by CZE-UV. According to the checked bibliography, this supposes the only study that has reported an on-line preconcentration approach for 5-NDZ determination by CE. 5-NDZ separation was accomplished at 28 kV and 25°C using a BGS consisted of 25 mM phosphate buffer (pH 1.5) containing 0.5% (v/v) of MeOH. 5-NDZ preconcentration was achieved by the combination of two on-line preconcentration techniques, namely sweeping and MSS, and it followed a strategy that was previously proposed by Quirino [101].

However, it is not the only time that the combination of two on-line preconcentration methods has been assayed in order to achieve a greater sample preconcentration. Thus, the combination of LVSS with sweeping [102-104], LVSS with pH-mediated techniques [105] and FESI with MSS [100,106] have also been tested. In addition, the combination up to three different strategies, namely LVSS, dynamic pH-junction and sweeping has been successfully evaluated for the determination of methotrexate and its eight metabolites in cerebrospinal fluid [107] and for the analysis of nucleosides in human urine [108].

Despite all the mentioned on-line preconcentration methods, their application to residue analyses, considering pharmaceuticals such as 5-NDZ compounds, has not been frequently explored. Nevertheless their efficiency has been largely demonstrated in biological applications and, as a consequence, they suppose a powerful tool for compound determination at trace levels. With the aim of testing their effective potential, the use of sweeping as preconcentration strategy has been evaluated in **Chapter 4**, whereas the combination of FESI and sweeping has been assessed in **Chapter 5**. In both cases, the application to the determination of 5-NDZ residues in environmental and food matrices has been considered. Furthermore, the direct analysis of urine and serum samples for the

determination of 5-NDZs at trace levels has also been assayed in **Chapter 5** by the developed FESI-sweeping method.

3.4. CE-MS hyphenation

Since CE-MS coupling was firstly accomplished by Olivares *et al.* [109], the importance of MS as detection and quantification tool in CE methods has significantly grown. MS has enhanced the utility of CE as analytical technique because it allows the unequivocal identification of the separated analytes, providing information about the chemical structure of the compounds, and adding a second dimension in separation selectivity for co-eluting molecules of different nominal masses. CE-MS has found its main applicability in "omics" approaches such as proteomics, metabolomics, genomics and foodmics [23,110-112], but its potential has also been evaluated in residue analysis field [25,113,114].

It is clear that CE-MS is already a mature analytical technique, but CE-MS hyphenation has required a lot of research in order to solve several difficulties. It must be taken into account that CE-MS interface has to convert the analytes from the aqueous CE eluate into a gas phase providing their ionization and, the most important issue, it must guarantee that the electric circuit of the electrophoresis system is closed [115]. Although different ionization interfaces have been proposed for CE-MS coupling, electrospray ionization (ESI) interfaces remain as the most commonly used in CE-MS methods [116], because they offers high ionization efficiency and the ionization process is soft.

In ESI, a spray of fine droplets is created under the influence of a strong electrical field. In CE-ESI-MS, the process for the electrospray formation is due to the application of a high voltage (± 1-5 kV) between the end of the electrophoretic capillary and the MS entrance orifice and occurs at atmospheric pressure. Initially, the electrical gradient provides a driving force for electrochemical reactions in the liquid phase coming from the electrophoretic system through the sprayer tip (Taylor cone), and as a consequence, an aerosol plume formed by charged droplets is obtained. After subsequent solvent evaporation, the electrostatic repulsion due to the excess charge of the droplets overcomes the surface tension (the Rayleigh limit is reached), and consequently, a cascade of coulomb

fissions occurs, resulting in the formation of a number of smaller offspring droplets. According to 'Charged Residue Model' proposed by Dole *et al.* [117], coulomb fissions continue to occur until nanodroplets containing only a single analyte molecule are formed. After further desolvation, the analyte retains the droplet's excess charge [116]. A scheme of the electrospray formation in an ESI process is shown in **Figure 3.5.**.

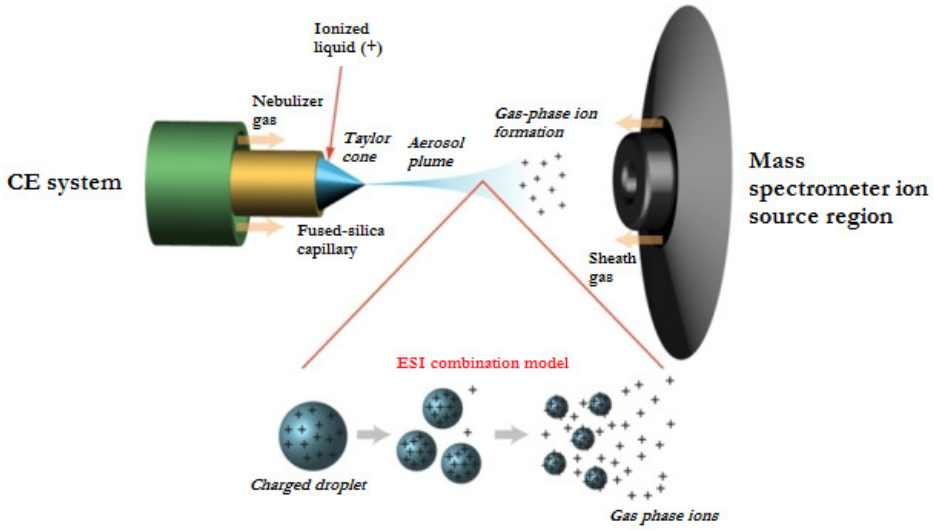

Figure 3.5. Schematic diagram of a typical ESI process in positive ion mode. Adapted from [118].

Furthermore, the compatibility of ESI with the commonly employed BGSs must be considered when a CE-MS method is developed. Indeed, it supposes an important disadvantage of CE-ESI-MS technique because the majority of the BGSs used in CE such as phosphate, citrate or tris(hydroxymethyl)aminomethane (Tris), are non-volatile buffers. Therefore signal suppression can be caused due to salt deposits on the source and a decrease of the analytical response due to the formation of ion-pairing with the analytes [119]. With the aim of overcoming this drawback, the use of formic acid, acetic acid, and their ammonium salts as BGS is recommended.

Moreover, the substitution of SDS for a more volatile pseudo-stationary has to be taken into account in MEKC separations using MS as detection tool [120] or, if not, a partial filling strategy must be considered [121]. In addition to the restricted number of BGSs that can be used due to their poor volatility, CE-ESI-MS coupling faces other problems

inherent to the hyphenation. ESI-MS is an end-capillary detection and as a consequence CE capillary must be removed from the outlet vial. Thus, it is necessary for the CE and ESI processes, which require a closed electric circuit, to occur in the absence of conductive buffer throughout their surroundings. In this case, ESI needle generally acts as a shared electrode, leading to certain undesirable electrochemical reactions that can affect the ionization and separation in terms of efficiency, sensitivity and stability [122].

In order to close CE-ESI electric system, three designs of ESI sources have been developed, namely coaxial sheath-flow, liquid-junction, and sheathless interface [123]. **Figure 3.6** shows a schematic representation of the three types of interface designs employed in CE-ESI-MS. Coaxial sheath-flow interface as well as liquid-junction interface requires an additional fluid to ensure system stability, whereas it is not needed if a sheathless interface is employed. This supplemental flow is mixed with the BGS coming from the electrophoretic system at the capillary end, and consequently, analyte dilution is observed. For this reason, higher signal sensitivity is achieved for sheathless interfaces.

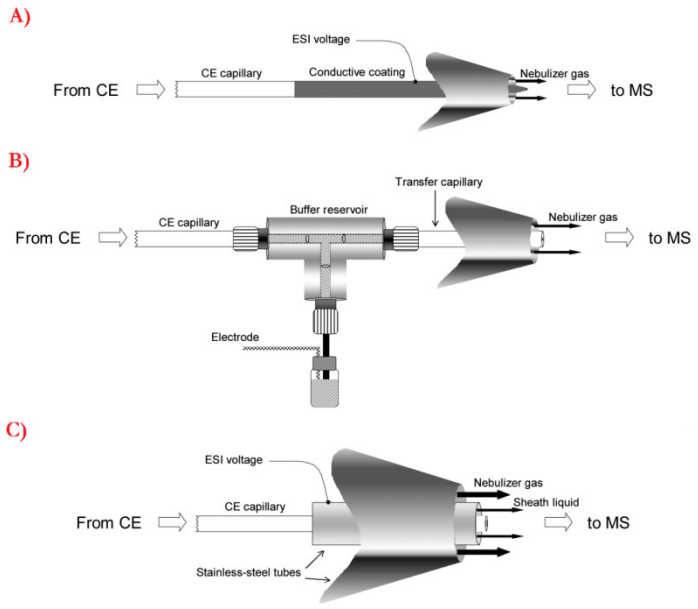

Figure 3.6. Scheme of CE-ESI-MS interfaces: A) sheathless, B) liquid-liquid junction and C) coaxial sheath-flow. Reproduced from [124].

In sheathless interfaces, the tip of the CE capillary itself is the spray point. This tip is sharpened or pulled to a fine point and a metallic deposition or a wire, typically made from silver or gold, is connected to it for closing the electric system [125]. However, this design presents an important disadvantage that it is the physical and electrical removal of the metallic deposition or the wire degradation. Additionally, the formation of bubbles as a result of electrochemical reactions also contributes to lose the electrical contact during the analyses [126]. Improvements in sheathless interfaces have been carried out for avoiding these inconveniences, resulting in the recent porous junction interfaces [127,128]. In spite of the improvements, sheathless interfaces are prototypes, so their implementation in laboratories is still limited. On the contrary, coaxial sheath-flow interface is commercially available, showing higher analysis precision than laboratory-manufactured sheathless interface [129]. Thus, the use of a coaxial sheath-flow interface is widely extended in CE-ESI-MS applications, despite of sample dilution associated to this interface.

First coaxial sheath-flow interface design was proposed by Smith *et al.* [130,131], and its commercial design from Agilent Technologies consists of three concentric capillaries, as shown in **Figure 3.7**(**A)**. In CE-ESI-MS hyphenation, CE capillary is inserted into the atmospheric region of the ESI source by means of a narrow stainless steel capillary *(d)*, which is placed concentrically around the capillary and delivers the so-called sheath liquid.

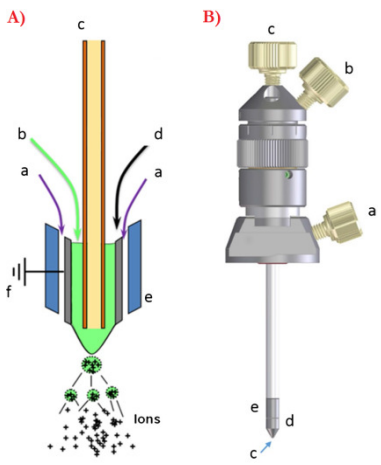

Figure 3.7. **A) Pictorial representation of Agilent coaxial sheath-liquid CE-MS interface. B) Engineering sketch of the coaxial sheath-liquid CE-MS interface. Interface parts: a, nebulizer gas; b, sheath liquid; c, CE-capillary with BGS; d, stainless steel spray needle (0.4 mm i.d., 0.5 mm outer diameter, (o.d.)); e, outer tube; f, ground connection. Reproduced from [132].**

CE-ESI electrical circuit is close due to the contact among CE separation solvent, the sheath liquid and the electrospray needle. A third concentric stainless steel tube delivers the nebulizer gas, generally nitrogen, which assists in spray formation, scavenges free electrons to prevent corona discharge and provides cooling for the CE capillary [125]. As can be seen in **Figure 3.7(B)**, sheath liquid is coaxially supplied to the interface compared to BGS flow direction.

CE-ESI-MS methods using a coaxial sheath-flow interface have proved to be robust, largely due to the high spray stability achieved by the constant flow rate of the sheath liquid that is supplied to the ESI interface [122]. Furthermore, sample dilution due to the sheath liquid can be compensated by the addition of chemicals to it, which will enhance analyte ionization and consequently will improve signal sensitivity. Sheath liquid usually consists of mixtures of water and an organic solvent such as MeCN or MeOH, containing a volatile acid or base for encouraging analyte ionization. On the other hand, the considered nebulizer gas in the coaxial ESI interface ensures electrospray stability, enhancing the analytical signal due to faster vaporization and formation of smaller droplets. However, the flow of nebulizer gas must be carefully optimized because high flows can causes an aspirating effect through the CE capillary, resulting in a parabolic flow profile and thus worse separation efficiency [133].

Liquid junction is the third ESI interface that has been considered for CE-MS coupling, being by far the least used interface. It was firstly developed by Lee *et al.* [134,135], and it uses a T-section reservoir that allows the electrical connection between the CE capillary and the ESI emitter via the electrolytes introduced through the buffer reservoir [123]. An advantage of this approach is that the separation and spray voltages can be controlled independently. However, in coaxial sheath-flow and sheathless interface, separation and spray voltage always share a common lead [17]. Because a liquid-junction interface has been considered for CEC-MS hyphenation in **Chapter 9**, it will be further described in **Chapter 7** where an introduction of CEC technique is addressed.

Regardless of the employed interface, CE methods can be coupled with a variety of mass spectrometers for detection such as Q, QqQ, fourier transform-ion cyclone resonance,

ToF and IT detectors. Therefore, the speed and sensitivity of the selected MS detector are crucial, especially when residue analyses are performed [112].

In the present Thesis, 5-NDZ separation and determination by CE-ESI-MS is proposed for the first time (**Chapter 6**). Because the detector employed in the optimized method was an IT, only this type of mass spectrometer will be described in this section.

Figure 3.8 shows a schematic representation of CE-ESI-IT-MS hyphenation used in this Thesis.

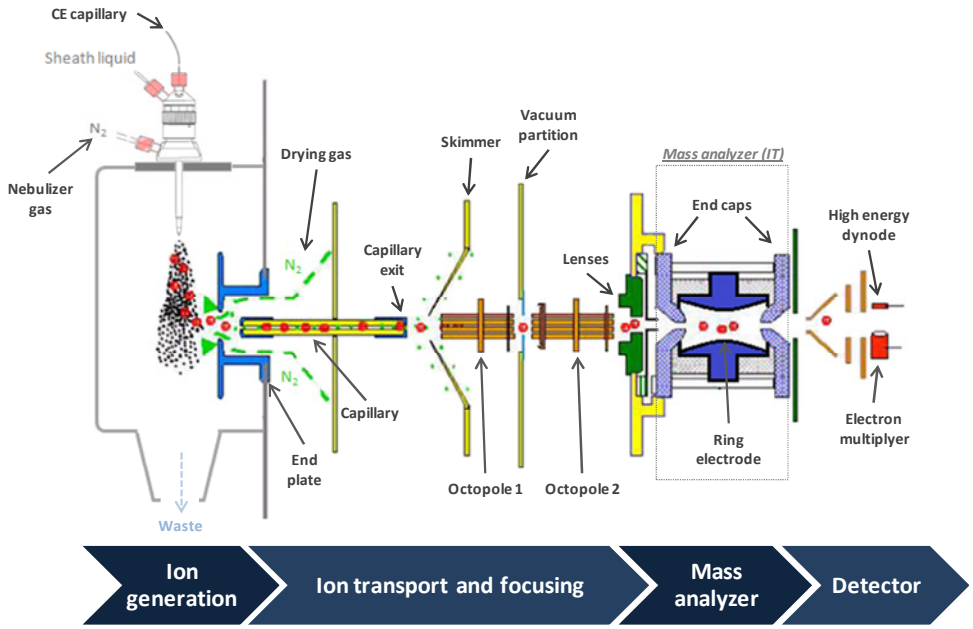

Figure 3.8. Design of the ion trap mass spectrometer with an ESI source coupled to a CE system.

As can be seen in **Figure 3.8**, IT analyzer consists of a doughnut-shaped central ring electrode that is flanked by two convex-shaped end-caps with entry and exit orifices in their centres [112]. Initially, a low high-frequency voltage is applied to the ring electrode of the trap and ions with the required mass/charge ratio (m/z) are introduced inside it. Subsequently, they are subjected to oscillating electric fields that are applied to the ring electrode by a radio-frequency voltage and are trapped via helium gas. In all cases, the ion trap is able to store either positively and negatively charged ions, or ions of one specific polarity [136]. In the created tridimensional electric field, ions move in complex trajectories

according to their m/z for a specific length of time. Furthermore, these trajectories are expanded due to the repulsive forces among the trapped ions and, as a result, a loss of some ions can occur. In order to control these interactions, the trap contains helium gas at low pressure which interacts with the ions, reducing their kinetic energy and forcing them to the centre of the IT. When ions are trapped, they are subjected to a gradual increase of the high-frequency voltage until these trajectories are axially destabilized, and as a consequence, they are ejected from the trap via the hole in the end-cap electrode [137]. Ions with lower m/z are firstly discharged whereas ions with higher m/z are trapped for longer times. An important advantage of IT over other MS analyzers is that it is able to trap specific ions, fragmenting them and detecting the resulting fragment ions. Besides, fragment ions can also be trapped and subjected to subsequent fragmentations, obtaining MS^n spectra. Consequently, IT is considered a mass spectrometer specialized for qualitative analysis, and a potential tool for molecular structure identification.

Figure 3.8 shows a CE system coupled to an IT detector by means of an ESI interface, namely a commercial coaxial sheath-flow interface. Initially, BGS coming from the CE system and containing the sample is mixed with the sheath liquid solution. Then the mixture is nebulized according to the previously described ESI process (**Figure 3.5**). Afterwards, charged ions are carried out into the vacuum region of the mass spectrometer through a glass capillary. With the aim of maintaining the high vacuum in the system, the skimmer prevents that gas molecules enters into the system. Ions cross the skimmer and they are guide to the lenses through two octopoles. Lenses are responsible for the final guidance of the ions to the ion trap. Finally, ions enter into the trap and they are subjected to the process previously described. Once ions are ejected from the trap, they are detected. The detection part of the IT system possesses a dynode which allows the detection of ions on both positive and negative mode. All the variables affecting compounds determination by ESI-IT-MS, including MS instrumental parameters, have been described in more detail by Kruve *et al.* [138].

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4

Determination of 5-NDZs in environmental water and milk samples by micellar electrokinetic chromatography

Abstract ► In this chapter, a MEKC method coupled with UV detection has been developed for the determination of up to nine 5-NDZs, including metabolites, in milk and environmental water samples. Separation was performed in an extended light path capillary (61.5-64.5 cm total length × 50 μm i.d., 53-56 cm effective length, 150 μm optical path length) using a BGS consisted of 20 mM sodium phosphate buffer (pH 6.5) and 150 mM SDS. During the run, capillary temperature was kept constant at 20°C and a voltage of 25 kV was applied (normal mode). Analytical signals were monitored at 320 nm. Due to the low sensitivity associated to UV detection, two preconcentration strategies were explored. On-line preconcentration was achieved by sweeping. Samples in BGS without micelles (20 mM sodium phosphate buffer, pH 6.5) were hydrodinamically injected at 50 mbar for 15-25 s. On the other hand, off-line preconcentration was accomplished through the application of different extraction procedures to the samples. SPE using Oasis®HLB cartridges and DLLME were considered as sample treatments for environmental water samples. SPE using Oasis®MCX cartridges was evaluated for 5-NDZ extraction from milk samples. In all cases, LODs at low μg/L levels were reached due to the combination of both preconcentration effects. The proposed method provides an efficient and economical alternative to the use of chromatographic methods for monitoring 5-NDZ residues. Moreover, it supplements the relatively few methods available for the analysis of these compounds in environmental samples.

4.1. Background

Since penicillin was discovered, the use of antibiotics against diseases caused by microorganisms has been a contributing factor to increased life expectancy. With the aim of improving human health, the utilization of these drugs has been extended to veterinary medicine reducing economic losses due to cattle illness and improving the quality of products of animal origin offered to the consumers. Moreover, in the race of reaching high quality products at low cost, antibiotics have been proposed as growth promoters. However, this practice causes higher risks than benefits, so it has recently been banned [1]. Additionally, within EU countries, the use of 5-NDZs is forbidden in animals intended to human consumption, even when they pretend to be used as antibiotics and not as growth promoters [2]. This ban is the consequence of several reports that have attributed genotoxic, carcinogenic and mutagenic properties to these compounds [3]. Despite the ban, alerts about the presence of 5-NDZs in foodstuffs of animal origin are still reported by RASFF [4]. The need to check the illegal use of 5-NDZs has stimulated the development of analytical methods for 5-NDZ determination in many matrices, especially in eggs [5-7], poultry meat [8,9] or swine tissues [10].

Furthermore, the presence of 5-NDZ residues in hospital sewage waters [11] as well as in fish farm and meat industry effluents owing to their illegal use supposes an additional negative consequence of the wide use of antibiotics. 5-NDZs present high polarity and low biodegradability that involves their bioaccumulation, creating a potential environmental problem. At the moment, only a reduced number of preliminary studies have been reported about 5-NDZ removal from wastewaters [12,13]. Taking into account the lack of reports about the effects of 5-NDZs as environmental contaminants, the presence of these drugs in water sources could be considered as a health risk. A few methods have been reported for 5-NDZ determination in waters [14] although the occurrence of pharmaceutical residues in environmental sources is recognized as an emerging issue. Therefore, additional contributions in this area are required, considering that 5-NDZs have been recently detected in natural waters [15].

Traditionally, LC has been the most employed technique for the determination of 5-NDZ residues, using both UV [16,17] and MS [5-10,18-24] detection. Moreover, GC [25-28], ELISA [29], and optical biosensors [30] have also been employed. A few methods have been reported for 5-NDZ determination by CE, but different CE modes have been considered such as CZE coupled with amperometric detection for MNZ determination in human urine [31], MEKC for the evaluation of an in-line micromembrane extraction unit and the subsequent determination of MNZ, DMZ and RNZ in pig liver tissue [32] and MEEKC, which was compared with a MEKC methodology for an antibiotic mixture separation, including MNZ, DMZ and RNZ [33]. Furthermore, a two-dimensional CE system for the determination of MNZ, DMZ, RNZ, and three tetracyclines has also been proposed [34]. Nevertheless, and apart from the methods proposed in this Thesis, the above-mentioned CE methods have been limited to the determination of a maximum of three 5-NDZs, while the analysis of their metabolites has not been considered.

CE has demonstrated to be a suitable tool for antibiotic residue analyses in environmental and food field [35], and moreover, it accomplishes with Green Chemistry basis that requires methods with low solvent consumption avoiding toxic reagents. Under this consideration, great efforts have been made for the development of environmental-friendly methods, including sample preparation procedures [36,37]. In this chapter, an alternative CE method is proposed for the analysis of 5-NDZ residues in environmental and food samples, together with the evaluation of different sample treatments. A novel SPE procedure has been optimized for 5-NDZ extraction from milk samples whereas other SPE method was assayed for 5-NDZ determination in water samples. SPE is a simple and robust technique with high implementation in routine analysis laboratories and which has been widely used as sample treatment prior to CE separations [38]. From the point of view of Green Chemistry, SPE was developed as environmental-friendly technique even before this concept was introduced [39]. Furthermore, a DLLME method has also been proposed for 5-NDZ extraction from water samples. DLLME is a miniaturized version of traditional LLE methods and involves lower solvent consumption, but high efficient extractions are also achieved [40,41]. Nowadays, its use has become popular and it has been successfully applied to both food and environmental analysis using CE as analytical tool [42-45].

4.2. Materials and methods

4.2.1. Materials and reagents

All reagents used through this work were analytical reagent grade and solvents were high performance liquid chromatography (HPLC) grade, unless otherwise specified. Dichloromethane, NaCl, NaOH, NaH_2PO_4, Na_2HPO_4, H_3PO_4 (85%, v/v), 2-propanol, 1,4-dioxane and ammonium hydroxide (30%, v/v) were obtained from Panreac-Química (Madrid, Spain). MeOH, chloroform, acetic acid, acetone, 2-butanol, dimethylformamide (DMF) and HCl (37%, v/v) were purchased from VWR International (West Chester, PA, USA) while MeCN, SDS, imidazole, 1,2-dichloroethane, dibromomethane, dibromoethane, and urea were supplied by Sigma-Aldrich (St. Louis, MO, USA). Citric acid monohydrate, EtOH, diethyl ether, tetrahydrofuran (THF), formic acid (98%, v/v), triethylamine (TEA) and TCA were acquired from Merck (Darmstadt, Germany) and CS_2 was supplied by Carlo Erba (Rodano, MI, Italy).

Ultrapure water (Milli-Q plus system, Millipore, Bedford, MA, USA) was used throughout the work. Sodium phosphate buffer (0.1 M, pH 2) was prepared from an H_3PO_4 aqueous solution by the subsequent pH adjustment with a 1 M NaOH solution.

Analytical standards of MNZ, DMZ, RNZ, ORZ, HMMNI, MNZ-OH, IPZ-OH, CRZ and TNZ were supplied by Sigma-Aldrich (St. Louis, MO, USA) while IPZ, SCZ and TRZ were purchased from Witega (Berlin, Germany). Stock standard solutions were obtained by dissolving the appropriate amount of each 5-NDZ drug in MeCN, reaching a final concentration of 1 mg/mL. Stock standard solutions were kept in the freezer at -20°C avoiding exposure to light. Intermediate standard solutions (50-100 μg/mL of each 5-NDZ) were obtained by mixing the stock standard solutions and subsequent dilution with MeCN. They were stored in dark at 4°C and equilibrated to room temperature before their use. Working standard solutions were freshly prepared by the dilution of an intermediate standard solution aliquot with water or injection solvent (20 mM phosphate buffer, pH 6.5) according to the desired concentration.

Acrodisc 13 mm nylon membrane filters (0.2 μm of pore size) from Pall Corp. (Ann Arbor, MI, USA) were used for the filtration of the samples prior to their injection into the electrophoretic system. A 0.2 μm nylon membrane filter (Supelco, Bellefonte, PA, USA) was used for the filtration of water samples prior to SPE. Milk samples were filtered through 0.2 μm nylon membrane filters from Pall Corp. prior to SPE, except goat raw milk samples that were filtered through 0.45 μm polyethersulfone membrane filters (VWR International, West Chester, PA, US).

Oasis®HLB cartridges (Waters, Milford, MA, USA) of different sorbent mass (60 mg, 3 mL; 200 mg, 6 mL; and 500 mg, 6 mL) were evaluated for 5-NDZ extraction from environmental water samples. Oasis®MCX cartridges (Waters, Milford, MA, USA) of different sorbent mass (60 mg, 3 mL; and 150 mg, 6 mL) were tested for 5-NDZ extraction from milk samples.

4.2.2. Instrumentation

CE experiments were carried out with an HP^{3D}CE instrument and an Agilent 7100 CE system (Agilent Technologies, Waldbronn, Germany) both equipped with a DAD. Data were collected by ChemStation (version B.02.01) software. Separations were performed in an uncoated fused-silica capillary (61.5-64.5 cm total length × 50 μm i.d. × 375 μm o.d., and 53-56 cm of effective length). CE capillaries were supplied by Polymicro Technologies (Phoenix, AZ, USA). Moreover, extended light-path capillaries (61.5-64.5 cm total length × 50 μm i.d., 53-56 cm of effective length, and 150 μm of optical-path diameter) were also employed and acquired from Agilent Technologies. Compound UV/Vis absorption spectra were obtained by an Agilent 8453 spectrophotometer (Agilent Technologies).

SPE procedures were carried out on a Visiprep™ DL vacuum manifold for 12 cartridges from Supelco (Bellefonte, PA, USA). A Universal 320R centrifuge (HettichZentrifugen, Tuttlingen, Germany), a nitrogen dryer EVA-EC System (VLM GmbH, Bielefeld, Germany) and a vortex-2 Genie (Scientific Industries, Bohemia, NY, USA) were also used through sample treatment. Solution pH was adjusted with a pH meter (Crison model pH 2000, Barcelona, Spain) with a resolution of ±0.01 pH unit.

4.2.3. Sample treatment procedures

SPE for environmental water samples

Water samples were collected from different rivers such as Genil River (Granada, Spain) located in an urban area, Riofrío River (Granada, Spain) where the sampling point was located in an area close to a fish farm, and Zújar River (Badajoz, Spain) where the sampling point was located in a cattle-farming area.

A sample treatment previously proposed [14] was evaluated and modified for 5-NDZ extraction and preconcentration. Sample volumes higher than 25 mL were filtered through a 0.2 μm nylon membrane in order to remove suspended matter, and subsequently sample pH was adjusted to 2 with a 1:4 (v/v) H_3PO_4 aqueous solution. Afterwards, an aliquot of the sample (25 mL) was fortified at the desired 5-NDZ concentration level and submitted to SPE using Oasis®HLB (500 mg, 6 mL) cartridges. Previously, the cartridge was sequentially conditioned with 2 mL of MeCN, 1 mL of MeOH and 1 mL of 0.1 M phosphate buffer (pH 2). Then, sample was passed through the cartridge at 1 mL/min by gravity. Thereafter, cartridge was washed by passing 1 mL of MeOH. Finally, the analytes were eluted with 1.5 mL of MeCN. The extract was evaporated to dryness under gentle nitrogen current and reconstituted in 0.5 mL of 20 mM sodium phosphate buffer (pH 6.5) by vortex agitation for 2 min.

DLLME for environmental water samples

Environmental water samples collected from different sources were selected. River water samples were collected in Zújar River (Badajoz, Spain), which is located in a cattle-farming area. Tap water samples (Granada, Spain) and bottled water samples were also analyzed.

Initially, 5 mL of a water sample were placed into a 15 mL centrifuge tube with a conical bottom and fortified at the desired 5-NDZ concentration level. An amount of 1.65 g of NaCl (33%, w/v) was added to the sample and it was agitated by vortex for a few minutes. Later, a mixture of dibromomethane (extractant solvent, 1600 μL) and 2-propanol (disperser agent, 2000 μL) was rapidly injected into the sample tube using a syringe coupled

to a needle with flat tip, and then, sample was subsequently centrifuged for 5 min at 5000 rpm. After that, it was observed that the aqueous matrix phase remained as the upper layer while the organic extraction phase was moved down as sediment. Extraction solvent was collected using a syringe coupled to a needle with flat tip and discharged in a glass vial. Finally it was evaporated to dryness under gentle nitrogen current and reconstituted in 200 μL of 20 mM sodium phosphate buffer (pH 6.5) by vortex agitation for 2 min.

SPE for milk samples

A wide variety of milk samples were analyzed. Samples were selected considering different compositions, nature and the thermal treatments that were applied to them in the milk industry before their reception in our laboratory. Whole pasteurized cow milk and semi-skimmed goat milk were acquired in a local supermarket (Granada, Spain); goat raw milk and ewe raw milk were gently supplied by a local farm from Extremadura (La Serena, Spain).

A novel sample treatment was developed for analyte extraction and preconcentration using Oasis®MCX cartridges. An aliquot of a milk sample (3.5 mL) was placed in a 15 mL polypropylene centrifuge tube and fortified at the desired 5-NDZ concentration level. Then 0.35 g of TCA was added to the sample and the mixture was subsequently shaken by vortex for a few seconds and centrifuged for 10 min at 9000 rpm. Afterwards, it was observed that milk fat remained at the top of the solution while protein precipitation occurred. Sample aqueous phase was collected using a syringe coupled to a needle and filtered through a 0.2 μm nylon membrane for removing the suspended matter. Filtered solution was discharged on a conditioned Oasis®MCX (150 mg) cartridge. SPE cartridge was sequentially conditioned with 1 mL of MeOH and 1 mL of 0.1 g/mL TCA aqueous solution. Following that, sample was passed through the column at 1 mL/min. Thereafter, a cartridge washing step was sequentially carried out with 1 mL of 2% (v/v) formic acid aqueous solution, 1 mL of MeOH and 1.5 mL of an aqueous solution containing 5% (v/v) of MeOH and 2% (v/v) of ammonium hydroxide. Cartridge was vacuum dried prior to sample elution. Sample elution was performed with 2 mL of MeOH solution containing 2% (v/v) of ammonium hydroxide. Finally, sample extract was evaporated to dryness

under gentle nitrogen current and reconstituted in 200 μL of 20 mM sodium phosphate buffer (pH 6.5) by vortex agitation for 2 min.

4.2.4. Capillary electrophoresis separation

CE experiments were carried out in a bare fused-silica capillaries (61.5 or 64.5 cm of total length, according to the application × 50 μm i.d. × 375 μm o.d.) and in extended light-path capillaries of the same dimensions and 150 μm of optical path. 5-NDZ separation was performed using a BGS consisted of 20 mM phosphate buffer (NaH_2PO_4/Na_2HPO_4, pH 6.5) containing 150 mM SDS. A voltage ramp from 0 to 25 kV was applied for 30 s at the beginning of the electrophoretic separation, and separation was accomplished at 25 kV (normal mode). Capillary was kept at 20°C during runs. Standard solutions and samples dissolved in BGS in absence of micelles were hydrodynamically injected at 50 mbar for 15-25 s. Analyses were monitored at 320 nm. A stable electrical current of 58 μA was observed during separations when capillaries of 64.5 cm of total length were used (61 μA for capillaries of 61.5 cm of total length).

Before the first use, capillaries were flushed with 1 M NaOH solution for 20 min at 1 bar and 60°C, followed by deionized water for 10 min at 1 bar and 25°C and BGS for 30 min at 1 bar and 25°C. At the end of the working day, the capillary was washed with deionized water for 5 min at 5 bar and 20°C and, afterwards, it was dried with compressed air for 5 min at 5 bar and 25°C.

Capillary conditioning at the beginning of each session and between runs was considered according to the application as follows.

Capillary conditioning for the analysis of water samples by SPE

At the beginning of each session, the capillary was subsequently rinsed with 0.1 M NaOH for 7 min, deionised water for 1 min and running buffer for 20 min at 1 bar and 25°C. Between runs, the capillary was subsequently conditioned with 0.1 M NaOH for 2 min, deionised water for 1 min and BGS for 5 min at 1 bar and 20°C.

Capillary conditioning for the analysis of water samples by DLLME and milk samples by SPE

At the beginning of each session, the capillary was rinsed at 1 bar and 20°C with the BGS for 15 min. Before each run, the capillary was flushed with running buffer at 1 bar and 20°C for 2 min.

4.3. Results and discussion

4.3.1. Electrophoretic separation

Due to the lack of CE methods proposed for 5-NDZ separation, preliminary studies were performed for the evaluation of their electrophoretic behavior. Furthermore, separation of eleven 5-NDZ compounds by CE was initially intended. However, and as will be discussed below, the optimized CE-UV method was only suitable for the determination of eight or nine compounds according to the application.

In order to select the UV detection wavelength, the UV spectrum of each 5-NDZ in water (2 μg/mL) was firstly registered. UV spectra of MNZ, IPZ-OH, RNZ and CRZ are shown in **Figure 4.1**. Maximum UV absorption for almost all 5-NDZs was observed between 311 and 322 nm, as indicated on **Table 4.1**. Finally, 320 nm was considered for monitoring all 5-NDZs.

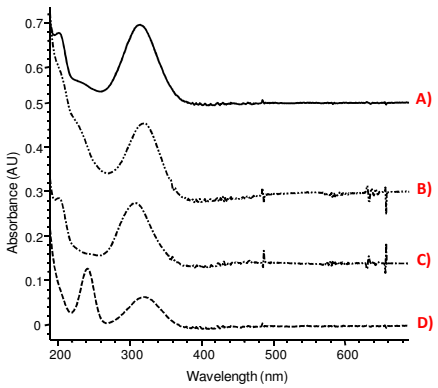

Figure 4.1. UV spectra for individual 5-NDZ standard solutions in water (2 μg/mL): A) IPZ-OH, B) MNZ, C) RNZ and D) CRZ. Arbitrary Unit, AU.

Table 4.1. Maximum UV absorption wavelength (nm) for each studied 5-NDZ compound.

Analyte	Maximum UV absorption wavelength (nm)	UV absorption (AU)	Analyte	Maximum UV absorption wavelength (nm)	UV absorption (AU)
SCZ	319	0.178	TRZ	320	0.113
DMZ	319	0.250	HMMNI	311	0.257
IPZ	322	0.204	TNZ	318	0.097
RNZ	202, 308*	0.213, 0.202	MNZ	321	0.288
ORZ	319	0.166	IPZ-OH	314	0.178
MNZ-OH	311	0.116	CRZ	242, 320*	0.123, 0.065

*Second maximum UV absorption wavelength is also given.

On the other hand, CE-UV method optimization was carried out considering peak resolution and signal sensitivity, in terms of peak height, as analytical response. Generated electrophoretic current was kept lower than 90 μA in each experiment. Initially, 5-NDZ separation was assayed in fused-silica capillaries (64.5 cm × 50 μm i.d., 56 cm of effective length). Sample injection in BGS was considered in those studies carried out prior to injection solvent optimization.

All studied 5-NDZs are neutral compounds across a wide pH range (as was indicated in **Table 2.1**), but they are positively charged at pH values about 2. Therefore, 5-NDZ separation was firstly attempted by CZE considering a pH range between 1.5 and 4.0. The employed BGSs consisted of 50 mM phosphate buffers, and low-pH BGSs were prepared from H_3PO_4 solutions and subsequent pH adjustment with 1 M NaOH solution while high-pH BGSs were prepared from NaH_2PO_4/Na_2HPO_4 mixtures. Baseline peak resolution was not reached for all 5-NDZs under any of the tested conditions (**Figure 4.2,I**), but 5-NDZ separation was improved when low pH BGSs were considered. Nevertheless, the lack of EOF significantly increased the analysis time when 5-NDZ separation was performed at low pH conditions. For this reason, CZE was discarded as the appropriate CE mode for achieving 5-NDZ separation at reasonable analysis time. As alternative, 5-NDZ separation by MEKC was proposed. The addition of a surfactant to the BGS, namely SDS, was considered, and as a consequence, interactions between the analytes and the micelles were also involved in the 5-NDZ electromigration behavior. At

first, 50 mM phosphate buffer containing 50 mM SDS was employed as running solution and BGS pH was evaluated between 6 and 9. Low-pH BGSs were prepared from NaH_2PO_4/Na_2HPO_4 mixtures while high-pH BGSs were prepared from H_3PO_4/Na_2HPO_4 mixtures. Higher peak resolution was obtained when low-pH BGSs were tested, but longer analysis times were obtained under these conditions. As a compromise between analysis time and peak resolution, pH 6.5 was established as optimum (**Figure 4.2,II**). Further experiments were carried out in order to increase peak resolution between peaks 2 and 3 while achieving separation among peaks 5, 6, 7 and 8 was the main objective.

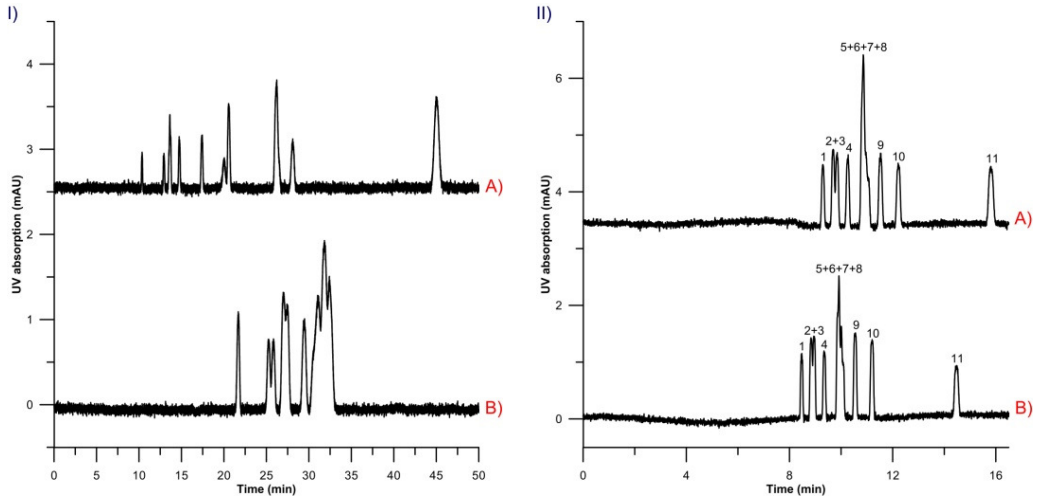

Figure 4.2. Evaluation of BGS pH. I) BGS consisted of 50 mM phosphate buffer: A) pH 2.0 and B) pH 4.0. II) BGS consisted of 50 mM phosphate buffer containing 50 mM SDS: A) pH 6.5 and B) pH 9.0. 5-NDZ separations were performed in a standard fused-silica capillary (64 cm × 50 µm i.d.) at 20 kV and 20°C. Separations were monitored at 320 nm. Standard solutions (5 µg/mL of each 5-NDZ) were hydrodynamically injected for 5 s at 50 mbar. Peaks are numbered by migration order.

Furthermore, the influence of BGS nature on the separation was also evaluated. Phosphate buffer (50 mM, pH 6.5), imidazole/HCl buffer (50 mM, pH 6.5) and McIlvaine buffer (50 mM, pH 6.5, prepared by the mixture of Na_2HPO_4 and citric acid according to [46]) were evaluated. Each tested BGS contained 50 mM SDS. **Figure 4.3** shows that no significant differences in terms of peak resolution were obtained when any of the mentioned buffers were employed as BGS. On the other hand, McIlvaine buffer increased

analysis time from 13 min to 20 min in comparison to imidazole and phosphate buffers. Finally, phosphate buffer (pH 6.5) was selected as BGS for further experiments.

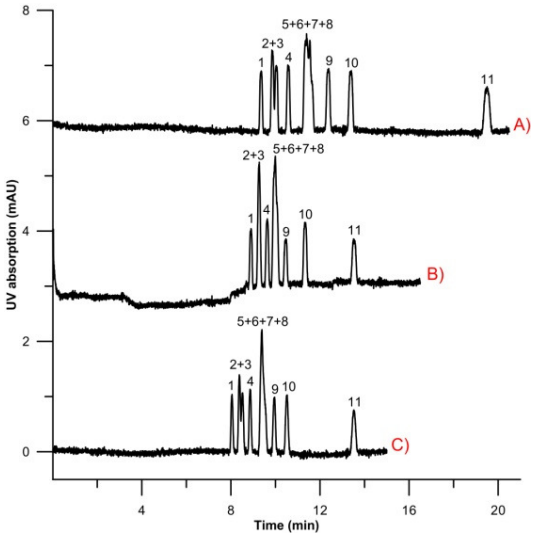

Figure 4.3. **Evaluation of BGS nature: A) 50 mM McIlvanine buffer, B) 50 mM imidazole/HCl buffer, and C) 50 mM phosphate buffer. All tested BGSs (pH 6.5) contained 50 mM SDS. 5-NDZ separations were performed in a standard fused-silica capillary (64 cm × 50 μm) at 20 kV and 20°C. Separations were monitored at 320 nm. Standard solutions (5 μg/mL of each 5-NDZ) were hydrodynamically injected (5 s, 50 mbar). Peaks are numbered by migration order.**

Furthermore, phosphate buffer concentration was evaluated between 10 and 70 mM using BGSs (pH 6.5) containing 50 mM SDS (**Figure 4.4,I**). It was observed that 20 mM phosphate buffer provided better results in terms of peak resolution. Peak resolution was even improved at lower BGS concentrations, but unstable electrophoretic current was observed. Obviously, concentrations higher than 20 mM resulted in lower peak resolution.

On the other hand, SDS concentration in the BGS was assessed between 30 and 200 mM, obtaining higher peak resolution at higher surfactant concentrations. Finally, 150 mM was established as optimum. Although higher concentrations improved the resolution between some peaks, analysis time was considerably increased (**Figure 4.4,II**). Additionally, the addition of a separation modifier, such as MeOH, EtOH, 2-propanol, MeCN, and urea to the running buffer was also considered. However, the addition of any of these separation modifiers did not improve peak resolution.

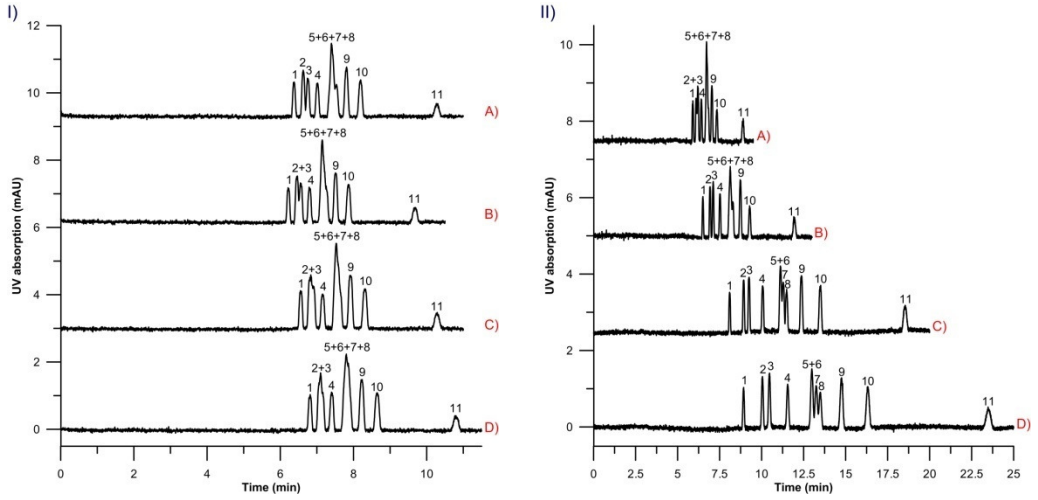

Figure 4.4. **I) Evaluation of phosphate buffer concentration: A) 10 mM, B) 20 mM, C) 40 mM and D) 70 Mm; BGS (pH 6.5) containing 50 mM SDS. II) Evaluation of SDS concentration: A) 50 mM, B) 100 mM, C) 150 mM and D) 200 mM; BGS consisted of 20 mM phosphate buffer (pH 6.5). 5-NDZ separations were performed in a standard fused-silica capillary (64 cm × 50 μm) at 20 kV and 20°C. Separation was monitored at 320 nm. Standard solutions (5 μg/mL of each 5-NDZ) were hydrodynamically injected (5 s, 50 mbar). Peaks are numbered by migration order.**

Once running buffer composition was optimized, separation voltage was studied. It was ranged from 19 to 30 kV, setting 25 kV as a compromise between peak resolution, analysis time, and an adequate separation current with the aim of avoiding capillary heating due to Joule effect. Moreover, capillary temperature during the separation was investigated between 15 and 30°C and it was finally set to 20 °C. A higher number of co-migrating peaks were observed when higher capillary temperatures were assayed while lower temperatures increased analysis time.

Method sensitivity evaluation and injection optimization

In order to improve method sensitivity, in terms of peak height, 5-NDZ separation was assayed in an extended light-path capillary (64.5 cm of total length × 50 μm i.d., 150 μm of optical path length). Obtained signals were compared to those observed in a standard capillary (64.5 cm total length × 50 μm, 50 μm of optical path length) with. As can be extracted from **Figure 4.5**, signal intensity was increased two-fold for all the analytes when an extended optical path capillary was employed.

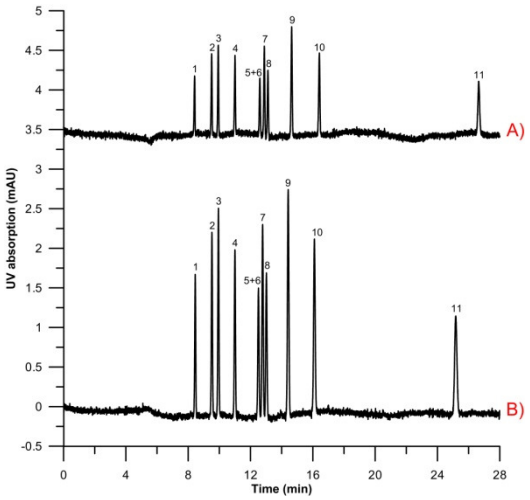

Figure 4.5. 5-NDZ separation performed in: A) a standard fused-silica capillary (64.5 cm × 50 µm, 56 cm of effective length, 50 µm of optical path diameter); B) an extended light-path capillary (64.5 cm × 50 µm, 56 cm of effective length, 150 µm of optical path capillary). 5-NDZ separations were carried out at 25 kV and 20°C using a BGS consisted of 20 mM phosphate buffer (pH 6.5) containing 150 mM SDS. Separation was monitored at 320 nm. Standard solutions (3 µg/mL of each 5-NDZ) were hydrodynamically injected for 5 s at 50 mbar. Peaks: 1, MNZ-OH; 2, HMMNI; 3, MNZ; 4, RNZ; 5, DMZ; 6, TNZ; 7, SCZ; 8, TRZ; 9, IPZ-OH; 10, ORZ; 11, IPZ.

At this point of the method optimization, TNZ was excluded from the proposed method because it co-migrated with DMZ. Furthermore, peak resolution lower than 1.5 (the minimum expected in a baseline separation) was obtained between DMZ and SCZ and SCZ and TRZ peaks depending on the run. As a consequence, SCZ was also excluded in further studies.

Finally, injection solvent and injection time were evaluated in order to enhance signals through an on-line preconcentration strategy. Different sample solvents such as BGS without SDS and deionized water were evaluated for achieving analyte preconcentration due to sweeping effect. Furthermore, sample injection in 100 mM NaCl solution was also assessed in order to evaluate a high conductivity solvent as injection media. Although similar signal enhancement was observed with both, BGS without micelles and 100 mM NaCl solution, BGS without micelles was chosen as injection solvent because the homogeneity of the system encouraged higher separation current stability. Signal intensity was increased from 1.2- to 1.9-fold (depending on the considered analyte) when this solution was employed instead of using BGS as injection solvent (**Figure 4.6**).

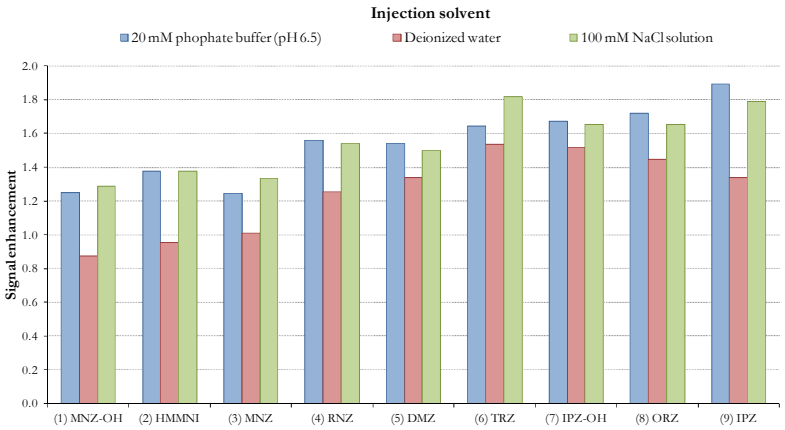

Figure 4.6. **Relative signal enhancement obtained when BGS (20 mM phosphate buffer containing 150 mM SDS, pH 6.5) was replaced by 20 mM phosphate buffer (pH 6.5), deionized water, or 100 mM NaCl solution as injection solvent.**

Finally, injection time was studied between 10 and 25 s. The considered range was limited by the low resolution observed between DMZ and TRZ when higher injection times were evaluated. Finally, 15 s at 50 mbar was chosen as optimum injection time, which supposed an injection volume of approximately 17.8 nL (≈ 1.4% of the total capillary volume for a capillary of 64.5 cm total length and 50 μm i.d.). **Figure 4.7** shows an electropherogram of a standard solution separation under the optimized conditions.

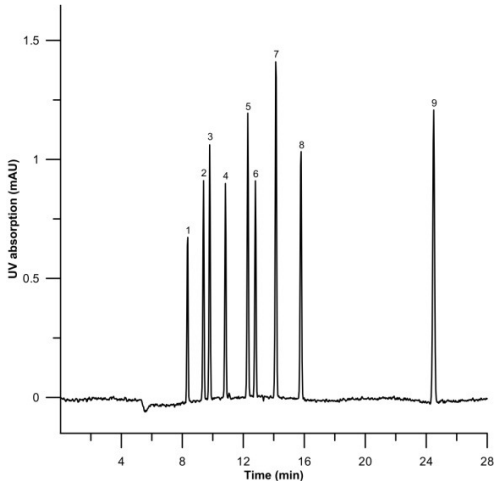

Figure 4.7. **5-NDZ separation performed in an extended optical path fused-silica capillary (64.5 cm × 50 μm, 56 cm of effective length, 150 μm of optical path diameter). 5-NDZ separation was carried out at 25 kV and 20°C using 20 mM phosphate buffer (pH 6.5) containing 150 mM SDS as BGS. Separation was monitored at 320 nm. Standard solutions (2 μg/mL of each 5-NDZ) in BGS without micelles were hydrodynamically injected for 15 s at 50 mbar. Peaks: 1, MNZ-OH; 2, HMMNI; 3, MNZ; 4, RNZ; 5, DMZ; 6, TRZ; 7, IPZ-OH; 8, ORZ; 9, IPZ.**

4.3.2. Optimization and application of SPE to the determination of 5-NDZs in environmental water samples

In order to determine 5-NDZ residues in environmental water samples by the optimized MEKC-UV method, a SPE method was proposed as a sample treatment. 5-NDZ determination was performed by the previously described MEKC procedure. The SPE procedure proposed by Capitán-Valley *et al.* [14] for 5-NDZ extraction from water samples was initially considered, although some modifications were subsequently carried out. Water sample volume was evaluated between 10 and 50 mL whereas Oasis®HLB cartridges with different sorbent mass were assayed (60 mg, 3 mL; 200 mg, 6 mL; 500 mg, 6 mL). The appropriate selection of both variables was crucial for achieving satisfactory extraction efficiencies and reaching high preconcentration factors, considering that 5-NDZ elution could occurred in the sample charging step. Higher recoveries were obtained when 500 mg Oasis®HLB cartridges were used and 25 mL of water sample were submitted to the SPE.

In the original SPE procedure dichloromethane was employed for cartridge conditioning, and its substitution by a less contaminant solvent such as MeCN was evaluated. Finally, MeCN was selected as cartridge conditioning solvent instead of dichloromethane, and as a consequence, extraction recoveries were improved between 5 and 10%. Therefore, cartridge was subsequently conditioned with 2 mL of MeCN, 1 mL of MeOH, and 1 mL of 0.1 M phosphate buffer (pH 2). Afterwards water sample was charged into the cartridge at a flow rate of 1 mL/min by gravity. Before elution, cartridge was vacuum dried for removing the water sample remaining in the cartridge. However, analytes were lost in this stage and low recoveries were obtained, so a cartridge washing step with 1 mL of MeOH was proposed for removing the water sample remaining in the column instead of drying it. Thereafter, analytes were eluted by passing 1.5 mL of MeCN through the cartridge. The eluate was dried under gentle nitrogen current and reconstituted in 0.5 mL of 20 mM phosphate buffer (pH 6.5). Consequently, an off-line analyte preconcentration of 50-fold was achieved by the application of the optimized SPE procedure. **Figure 4.8** shows a scheme of the proposed sample treatment.

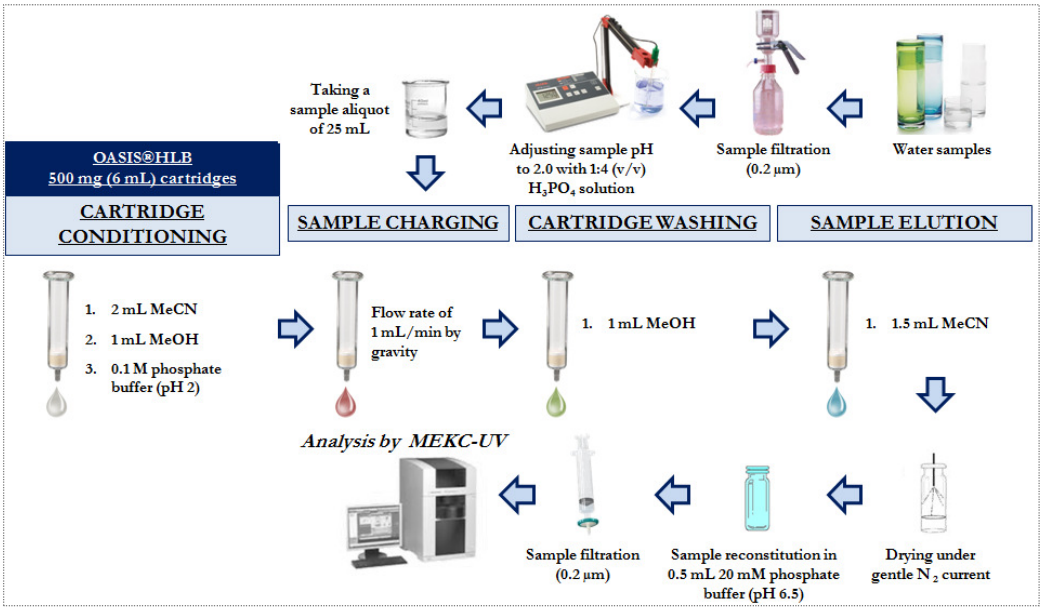

Figure 4.8. SPE procedure for 5-NDZ determination in water samples by MEKC-UV.

Calibration curves and performance characteristics

Calibration curves were established for the studied analytes in water samples which were previously treated according to the optimized SPE procedure. Urban river water samples from Genil River (Granada, Spain) were used for performing method characterization. Matrix-matched calibration curves were established in water samples fortified at different concentration levels (3, 20, 40, and 60 µg/L). Two samples per concentration level were processed following the developed sample treatment and injected in triplicate. Peak area was considered as a function of analyte concentration on the sample. A blank sample was also processed, and no peaks were detected at 5-NDZ migration times. Statistical parameters calculated by least-squares regression and performance characteristics of the proposed SPE-MECK-UV method for river water samples are shown in **Table 4.2**. LODs and limits of quantification (LOQs) were calculated as the minimum analyte concentration yielding a signal-to-noise ratio equal to three and ten, respectively. For all studied compounds, LODs at low µg/L levels were obtained after the application of the off-line and on-line preconcentration procedures.

Table 4.2. Statistical and performance characteristics of the SPE-MEKC-UV method for the analysis of nine 5-NDZs in river water samples.

Analyte	Linear range (µg/L)	R^2	Linear regression equation (y=m·x+a)		LOD (µg/L)	LOQ (µg/L)
			Slope	*Intercept*	$3\times S/N$	$10\times S/N$
MNZ-OH	3.0 – 60	0.985	0.147	0.528	0.9	3.0
HMMNI	2.7 – 60	0.993	0.194	0.386	0.8	2.7
MNZ	1.8 – 60	0.983	0.217	0.894	0.5	1.8
RNZ	2.3 – 60	0.993	0.186	0.649	0.7	2.3
DMZ	1.6 – 60	0.996	0.295	0.797	0.5	1.6
TRZ	3.7 – 60	0.990	0.208	0.480	1.1	3.7
IPZ-OH	1.6 – 60	0.995	0.329	0.933	0.5	1.6
ORZ	2.7 – 60	0.998	0.296	0.391	0.8	2.7
IPZ	3.5 – 60	0.992	0.380	1.176	1.0	3.5

Figure 4.9 shows electropherograms of a blank water sample and water samples collected from Genil River and fortified at different 5-NDZ concentration levels.

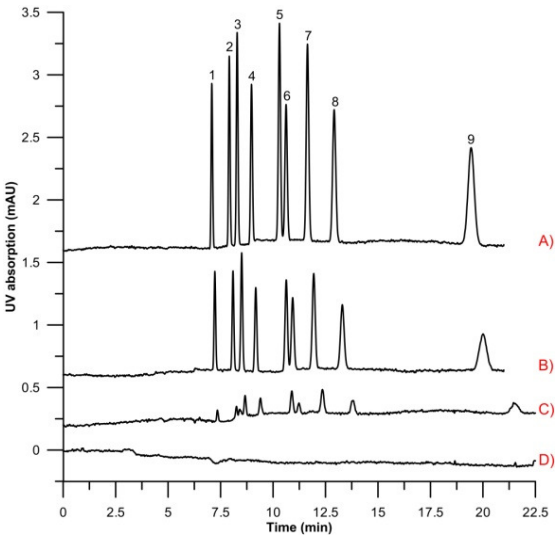

Figure 4.9. Electropherograms of Genil River water samples (Granada, Spain) treated and analyzed by the proposed SPE-MEKC-UV method. Samples were fortified with 5-NDZs at a concentration of: A) 40 µg/L; B) 20 µg/L; C) 3 µg/L; and D) blank sample. 5-NDZ separations were performed in an extended optical path capillary (64.5 cm × 50 µm, 56 cm of effective length, and 150 µm of optical path) at 25 kV and 20°C and using 20 mM phosphate buffer (pH 6.5) containing 150 mM SDS as BGS. Separation was monitored at 320 nm. Samples in BGS without micelles were hydrodynamically injected for 15 s at 50 mbar. Peaks: 1, MNZ-OH; 2, HMMNI; 3, MNZ; 4, RNZ; 5, DMZ; 6, TRZ; 7, IPZ-OH; 8, ORZ; 9, IPZ.

Precision assays

Method precision was evaluated in terms of repeatability (intra-day precision) and intermediate precision (inter-day precision) by applying the proposed SPE-MEKC-UV method to river water samples from Genil River (Granada, Spain) spiked at three different concentrations levels (3, 20, and 40 μg/L). Repeatability was evaluated by analyzing three samples fortified at the same concentration level (experimental replicates) which were injected in triplicate on the same day under the same conditions. Intermediate precision was assessed for five consecutive days by analyzing a sample fortified at each concentration level in triplicate using a similar procedure to that employed for repeatability studies. The results, expressed as relative standard deviation (RSD, %) of peak area are summarized in **Table 4.3**. In order to establish the RSD of the analyte migration time, migration time averages resulted from intermediate precision studies (45 runs) were considered.

Table 4.3. **Precision of the SPE-MEKC-UV method for the analysis of nine 5-NDZs in river water samples.**

Analyte	Repeatability (% RSD, n = 9)			Intermediate precision (% RSD, n = 15)			Migration time, min (% RSD, n = 45)
	3 μg/L	*20 μg/L*	*40 μg/L*	*3 μg/L*	*20 μg/L*	*40 μg/L*	
MNZ-OH	10.4	4.7	2.2	13.5	9.0	6.7	7.2 (5.7)
HMMNI	6.8	6.7	3.7	11.8	11.3	9.5	8.1 (6.4)
MNZ	7.3	5.4	2.6	12.7	8.9	7.3	8.4 (6.7)
RNZ	10.3	6.6	5.9	12.4	7.9	9.3	9.2 (7.2)
DMZ	13.7	12.0	10.1	12.7	10.0	11.5	10.5 (8.3)
TRZ	8.2	6.5	4.4	13.3	7.5	8.3	10.9 (8.5)
IPZ-OH	9.5	13.4	6.8	11.9	9.4	9.5	12.0 (9.3)
ORZ	9.3	10.3	7.9	11.7	9.8	9.6	13.4 (10.2)
IPZ	7.0	11.6	9.7	10.7	12.9	12.4	20.9 (14.9)

Trueness

In order to check the trueness of the proposed method, recovery experiments were carried out in water samples collected in different rivers and fortified at three different 5-NDZ concentration levels (3, 20, and 40 μg/L). River water samples collected from an urban

area (Genil River, Granada, Spain), from an area close to a fish farm (Riofrío River, Granada, Spain), and from a cattle farming area (Zújar River, Badajoz, Spain) were considered as representative matrices subjected to a possible environmental contamination. Three samples of each matrix were considered per fortification level. They were treated following the proposed SPE procedure and analyzed in triplicate. Moreover, a blank sample of each matrix was also analyzed, and no peaks were detected at 5-NDZ migration times above the estimated LODs. Satisfactory recoveries, ranging from 61.3 to 111.7%, were obtained for all considered analytes and samples (**Table 4.4**).

Table 4.4. Recovery studies in environmental water samples of different origins.

Analyte	River water samples from an urban river (%, n = 9)			River water samples from an area close to a fish farm (%, n = 9)			River water samples from a cattle farming area (%, n = 9)		
	3 µg/L	*20 µg/L*	*40 µg/L*	*3 µg/L*	*20 µg/L*	*40 µg/L*	*3 µg/L*	*20 µg/L*	*40 µg/L*
MNZ-OH	95.4	94.7	93.9	96.7	98.4	108.5	102.8	95.7	96.0
HMMNI	91.1	72.5	88.9	95.3	89.6	95.4	94.1	87.2	96.0
MNZ	97.0	83.3	91.2	90.0	92.3	92.1	98.0	95.2	99.2
RNZ	83.2	81.4	85.0	85.5	90.8	96.6	95.1	92.6	93.8
DMZ	84.5	70.2	78.6	76.9	74.6	93.9	73.5	82.2	82.8
TRZ	74.6	89.7	91.5	91.8	104.2	103.2	96.4	94.2	98.0
IPZ-OH	80.5	80.2	86.4	90.3	82.3	95.8	80.3	92.6	86.3
ORZ	98.6	111.7	82.5	86.1	81.8	95.4	81.2	88.0	92.8
IPZ	85.6	65.8	65.6	84.4	61.3	82.1	82.8	65.0	65.4

4.3.3. Optimization and application of DLLME to the determination of 5-NDZs in environmental water samples

A DLLME procedure was optimized for 5-NDZ determination in environmental water samples as an alternative to the previously described SPE sample treatment. This sample treatment was initially proposed for the determination of nine 5-NDZ compounds, namely MNZ, MNZ-OH, HMMNI, RNZ, TNZ, TRZ, IPZ-OH, IPZ and ORZ. However, low reproducibility was found for IPZ recoveries, so this analyte was discarded from this study. The drying step performed after DLLME was involved in the observed low reproducibility. The reason for this anomalous IPZ behavior was attributed to its high

vapor pressure in comparison with those of other 5-NDZs. Nevertheless, IPZ could be detected in the IPZ-OH form, so this metabolite was included in the present method.

5-NDZ separation was performed by the optimized MEKC method introducing slight modifications. First of all, a shorter extended light-path capillary (61.5 cm total length × 50 μm i.d., 56 cm of effective length, and 150 μm of optical path length) was employed. Furthermore, capillary conditioning at the beginning of the working day and between runs was studied in order to reach higher migration time reproducibility. It was noticed that the use of alkaline solutions (for daily conditioning or as conditioning solution prior to each analysis) had a slight negative effect on migration time reproducibility. In order to improve it, an alternative conditioning protocol was established. At the beginning of each working day and before each analysis, capillary was exclusively rinsed with BGS avoiding the use of NaOH solutions. As a result, higher migration time reproducibility was reached because RSDs were decreased from 5.7-10.2% to 2.0-4.5% for the analytes under study.

As higher migration time reproducibility was achieved, higher injection times than 15 s were allowed without involving any loss of peak resolution. As a consequence, injection time was re-evaluated between 15 and 35 s. In order to achieve a sweeping effect, standard solutions were prepared in BGS without micelles and were hydrodynamically injected at 50 mbar. Poor resolution between HMMNI and MNZ peaks was observed when injection times higher than 25 s were considered. Moreover, signal intensity was not significantly improved for most of the analytes when injection times higher than 25 s were assayed. For these reasons, injection was accomplished at 50 mbar for 25 s, and consequently, signals were enhanced between 1.3 and 2.3-fold in comparison with those achieved when sample was injected in BGS for 15 s at 50 mbar.

Optimization of DLLME parameters

Initially chloroform (600 μL) was established as extraction solvent, MeCN (1200 μL) was chosen as dispersive solvent and NaCl (10%, w/v) was considered as salting-out agent. DLLME procedure was applied to 5 mL of water sample. The following factors involved in the extraction procedure were optimized: sample pH, extraction and dispersive solvent

nature and volume, amount of salting-out agent, and timing of the shaking step performed after the extraction/dispersive solvent mixture was injected into the sample.

Sample pH was evaluated between 2 and 8. Buffered aqueous samples were used for these experiments. Low-pH buffers were prepared from NaH_2PO_4/H_3PO_4 mixtures. High-pH buffers were prepared from Na_2HPO_4/NaH_2PO_4 mixtures. Sample pH influence was not significant, and only low recoveries were observed when very acid samples (pH 2) were analyzed. At this pH value, 5-NDZs were positively charged, so its extraction with an apolar solvent such as chloroform was disadvantaged. Due to the fact that most of the water samples present a pH around 6.5, sample pH adjustment was not further considered because 5-NDZs are neutral molecules at this pH value.

When a DLLME procedure is performed, extractant is typically denser than water and it must be immiscible with it. For 5-NDZ extraction, six different organic solvents were tested: CS_2, dichloromethane, 1,2-dichloroethane, dibromomethane, dibromoethane and chloroform. On the other hand, dispersive solvent must be miscible either with aqueous phases or organic ones. Ten dispersive solvents were assayed in this study, namely MeOH, EtOH, 2-propanol, 2-butanol, acetone, MeCN, diethyl ether, 1,4-dioxane, DMF and THF. The selection of extraction and dispersive solvents was carried out for improving the extraction of polar analytes, namely MNZ-OH, HMMNI, MNZ and RNZ, due to their low recoveries.

Initially, each extraction solvent (600 µL) was evaluated using MeCN (1200 µL) and 10% (w/v) of NaCl. Higher signals, in terms of peak area, were obtained when dibromomethane was chosen as extraction solvent, whereas 5-NDZs were not recovered when CS_2 was employed (**Figure 4.10,I**). Then, each dispersive solvent (1200 µL) was studied employing dibromomethane (600 µL) as extractant and 10% (w/v) of NaCl as salting-out agent. Under these conditions, the use of 2-butanol as dispersive solvent gave the best results in terms of peak area, as can be seen in **Figure 4.10,II**, observing that MeOH was ineffective as dispersive agent.

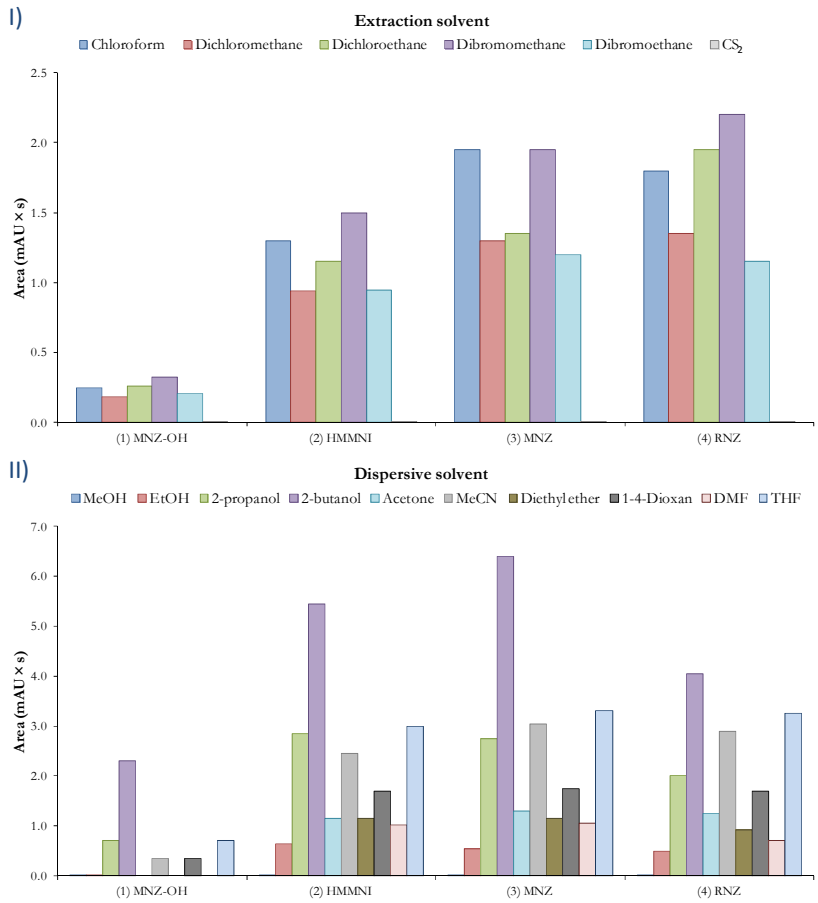

Figure 4.10. **Evaluation of DLLME solvents. I) Influence of extraction solvent nature on 5-NDZ extraction in terms of peak area. Extractant volume was set to 600 µL while MeCN (1200 µL) was used as dispersive solvent and 10% (w/v) of NaCl as salting-out agent. II) Influence of dispersive solvent nature on 5-NDZ extraction in terms of peak area. Dispersive solvent volume was set to 1200 µL while dibromomethane (600 µL) was used as extraction solvent and 10% (w/v) of NaCl as salting-out agent.**

Additionally, an experimental design (central composite blocked cube-star design, 16 runs) was carried out for the optimization of solvent volumes and the amount of NaCl added to the sample. Extraction and dispersive solvent volumes were continuously studied between 118 and 1882 µL while the amount of salting-out agent was ranged from 0.0 to 33.0% (w/v). 5-NDZ extraction was not achieved when lower solvent volumes were considered, while cloudy solution formation during DLLME was not favored when higher solvent volumes were tested. Moreover, NaCl was evaluated according to the solubility of this salt in water. In each experiment, 5 mL of a water sample fortified at 50 µg/L of each 5-NDZ were treated. The sum of the peak area of each compound was considered as response

variable. Lack-of-fit was not significant at a confidence level of 95.0% (p value > 0.05). The dispersive solvent volume appeared to be the only significant factor, while other factors and their interactions were not significant (p > 0.05). Resulted response surfaces are shown in **Figure 4.11**. The combination of factors which maximized peak area sum was: 1156 µL of dibromomethane, 1363 µL of 2-butanol and 16% (w/v) of NaCl.

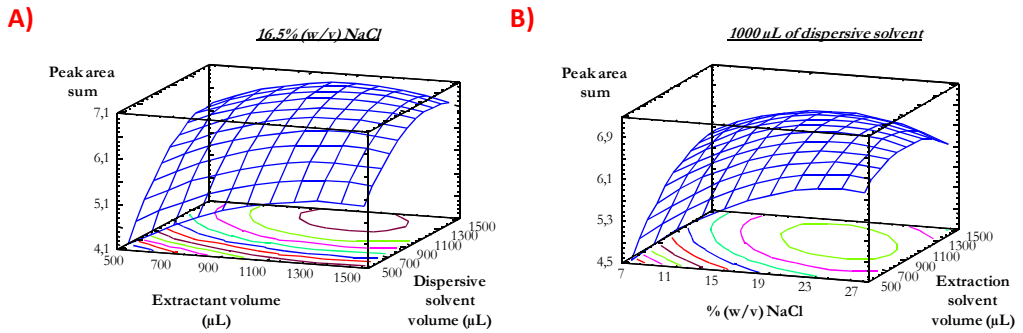

Figure 4.11. Experimental design results: A) estimated response surface for extraction solvent volume and dispersive solvent volume, and B) estimated response surface for extraction solvent volume and salting-out agent amount (%, w/v).

Under the optimized conditions, recoveries higher than 60% were obtained for all the analytes, except for MNZ-OH (recovery value ≈ 40%). Because of dispersive solvent volume was the only significant variable obtained from the previous experimental design, dispersive solvent nature and volume were again optimized in order to improve MNZ-OH extraction recovery. From these studies, MNZ-OH recovery was improved when 2-propanol was used as dispersive agent instead of 2-butanol at higher volumes than 1200 µL. As a consequence, a new experimental design, with the same characteristics as the one proposed before, was carried out for the optimization of dibromomethane and 2-propanol volumes and the amount of NaCl added to the sample. Extraction and dispersive solvent volumes were continuously studied between 98 and 1982 µL while the amount of salting-out agent was ranged from 0.0 to 33.0% (w/v). Peak area sum was considered as response variable. Although data fitted to the proposed model (p > 0.05), the obtained optimum parameters, which maximized the response function, were out of the studied variable ranges, except for extraction solvent volume. According to these results, dispersive solvent volume and salting-out agent amount higher than the previously considered values were required in order to maximize the response variable. For technical

reasons, dispersive agent volume was established to 2000 µL because higher volumes did not allow the formation of the cloudy solution during DLLME. Salting-out agent amount was limited by the insolubility of NaCl in water at levels higher than 33% (w/v), so this value was considered as optimum. Taking into account these parameter values, extractant volume was optimized in a univariate way between 1000 and 2000 µL. Maximum recoveries for most of 5-NDZs were obtained when 1600 µL of dibromomethane were used.

Improvements in extraction recoveries were observed when DLLME was performed under the last proposed conditions (1600 µL of dibromomethane, 2000 µL of 2-propanol, and 33% (w/v) of NaCl) instead of the initial DLLME conditions (1156 µL of dibromomethane, 1363 µL of 2-butanol and 16% (w/v) of NaCl). Recoveries were improved about 10 and 15% for most of the analytes. Therefore, 2-propanol was finally selected as dispersive agent in the present application.

Finally, the effect of a shaking step after the injection of the DLLME mixture (dibromomethane/2-propanol) in the sample was evaluated. The introduction of a manual shaking mode (1, 2, and 4 min) or vortex shaking mode (1 min) to the DLLME procedure was compared to the results reached by the DLLME methodology without any shaking stage. No significant differences in the recoveries were found, so no shaking was incorporated to the proposed DLLME procedure. A scheme of the DLLME procedure that was finally proposed is shown in **Figure 4.12**.

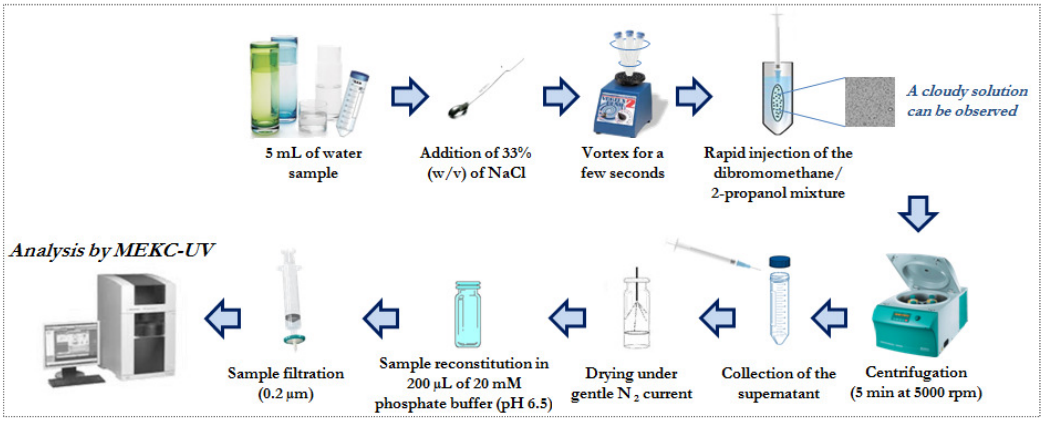

Figure 4.12. DLLME procedure for 5-NDZ determination in water samples by MEKC-UV.

Calibration curves and performance characteristics

In order to establish matrix-matched calibration curves for the studied analytes (MNZ, MNZ-OH, HMMNI, RNZ, TNZ, TRZ, IPZ-OH and ORZ), tap water was chosen as representative matrix for the characterization of the developed DLLME-MEKC-UV method. Tap water samples were spiked at five 5-NDZ concentration levels (5, 15, 30, 60, and 90 μg/L) and processed following the sample treatment described above. Three water samples were analyzed in triplicate per concentration level. Peak area was considered as a function of the analyte concentration on the sample. A blank sample was also analyzed according to the proposed DLLME-MEKC-UV methodology and no peaks were detected at 5-NDZ migration times.

Statistical parameters calculated by least-squares regression and method performance characteristics for the application of the DLLME–MEKC–UV method to water samples are shown in **Table 4.5**.

Table 4.5. **Statistical and performance characteristics of the proposed DLLME–MEKC–UV method for 5-NDZ determination in tap water samples.**

Analyte	Linear range (μg/L)	R^2	Linear regression equation (y=m·x+a)		LOD (μg/L)	LOQ (μg/L)
			Slope	*Intercept*	$3\times S/N$	$10\times S/N$
MNZ-OH	7.9 – 90	0.996	0.081	0.283	2.4	7.9
HMMNI	4.0 – 90	0.995	0.149	0.571	1.2	4.0
MNZ	3.7 – 90	0.995	0.169	0.596	1.1	3.7
RNZ	5.2 – 90	0.997	0.118	0.405	1.6	5.2
TNZ	3.8 – 90	0.996	0.159	0.544	1.1	3.8
TRZ	3.6 – 90	0.995	0.167	0.619	1.1	3.6
IPZ-OH	2.8 – 90	0.991	0.230	0.539	0.8	2.8
ORZ	3.0 – 90	0.994	0.213	0.713	0.9	3.0

LODs and LOQs were calculated as the minimum analyte concentration yielding a signal-to-noise ratio equal to three and ten, respectively. As can be seen, LODs at the low μg/L levels were obtained for all the studied compounds. These LODs are slightly higher than those obtained in the previous proposed application (see **Section 4.3.2**) using SPE as

sample treatment. In that application LODs ranging between 0.5 and 1.1 µg/L were obtained (**Table 4.2**) because a sample volume of 25 mL was considered and, as a consequence, a preconcentration factor of 50-fold was achieved. However, in the present application, DLLME was applied to 5 mL of sample and a preconcentration factor of 25-fold was reached. Electropherograms of a tap water sample fortified at 5 µg/L of each 5-NDZ and a blank sample are shown in **Figure 4.13**.

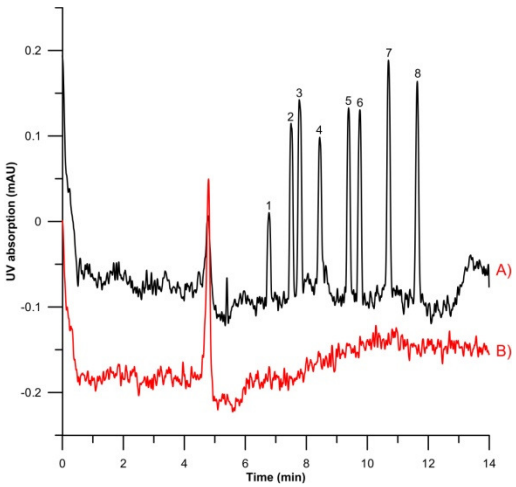

Figure 4.13. **Electropherograms of tap water samples analyzed by the proposed DLLME-MEKC-UV method. A) Fortified sample at 5 µg/L with each 5-NDZ; B) blank sample. 5-NDZ separations were performed in an extended light-path capillary (61.5 cm × 50 µm, 56 cm of effective length, and 150 µm of optical path diameter) at 25 kV and 20°C, using 20 mM phosphate buffer (pH 6.5) containing 150 mM SDS as BGS. Separation was monitored at 320 nm. Samples in BGS without micelles were hydrodynamically injected for 25 s at 50 mbar. Peaks: 1, MNZ-OH; 2, HMMNI; 3, MNZ; 4, RNZ; 5, TNZ; 6, TRZ; 7, IPZ-OH; 8, ORZ.**

Precision assays

The precision of the optimized DLLME–MEKC–UV method was evaluated in terms of repeatability (intra-day precision), intermediate precision (inter-day precision) and inter-worker precision by the application of the proposed method to tap water samples spiked at three different 5-NDZ concentration levels (5, 15, and 60 µg/L). Repeatability was assessed over three treated samples (experimental replicates) analyzed in triplicate on the same day under the same conditions. For intermediate precision assays, a sample fortified at each concentration level was processed and analyzed in triplicate for five consecutive days. For inter-worker precision, one sample per concentration level was treated by three

workers with different level of experience in performing DLLME methods. Each sample was injected in triplicate. Precision results expressed as peak area RSD (%) are summarized in **Table 4.6**. As can be observed, inter-worker precision was lower than intermediate precision, which indicates high robustness in terms of operator influence.

Table 4.6. Precision studies for the determination of 5-NDZs in tap water samples by the proposed DLLME–MEKC–UV method.

Analyte	Repeatability (% RSD, n = 9)			Intermediate precision (% RSD, n = 15)			Inter-worker precision (% RSD, n = 9)		
	5 µg/L	*15 µg/L*	*60 µg/L*	*5 µg/L*	*15 µg/L*	*60 µg/L*	*5 µg/L*	*15 µg/L*	*60 µg/L*
MNZ-OH	9.5	6.2	6.3	15.1	13.7	10.2	12.8	9.0	12.4
HMMNI	10.4	7.2	5.9	15.7	11.1	9.0	13.6	9.2	8.3
MNZ	10.6	6.1	6.8	16.4	12.0	9.8	14.6	8.2	8.7
RNZ	6.5	7.4	7.8	16.8	12.8	10.0	15.7	8.6	10.3
TNZ	7.8	8.6	6.7	16.5	13.1	10.7	11.2	6.6	8.4
TRZ	8.1	8.0	6.8	15.2	12.8	10.6	16.9	8.0	8.6
IPZ-OH	10.4	11.4	4.6	15.5	11.5	10.7	15.8	13.9	7.4
ORZ	10.5	10.4	8.1	16.1	13.4	10.7	16.0	9.1	7.4

Trueness

With the aim of checking the trueness of the proposed method for the analysis of real samples, recovery experiments were carried out in water samples of different origins, namely river water, tap water and bottled water. Three samples of each matrix per 5-NDZ concentration level (5, 15, and 60 µg/L) were treated according to the proposed DLLME procedure and analyzed in triplicate. A blank sample of each matrix was also processed and analyzed and no compounds co-migrating with the studied analytes were observed.

Recoveries higher than 70% were obtained for six of the eight studied compounds, namely HMMNI, MNZ, TNZ, TRZ, IPZ-OH, and ORZ, in all considered water samples and concentration levels. Recoveries higher than 60% were obtained for RNZ whereas recoveries ranging between 45 and 60% were observed for MNZ-OH. MNZ-OH is one of the most polar 5-NDZ compounds, and therefore, it justifies its low recovery in an apolar extractant such as dibromomethane. Results are shown on **Table 4.7**.

Table 4.7. Recovery studies in water samples with different origin.

Analyte	Tap water samples (%, n = 9)			Bottled water samples (%, n = 9)			River water samples (%, n = 9)		
	5 µg/L	*15 µg/L*	*60 µg/L*	*5 µg/L*	*15 µg/L*	*60 µg/L*	*5 µg/L*	*15 µg/L*	*60 µg/L*
MNZ-OH	50.2	51.9	52.2	45.5	53.9	50.7	53.3	58.4	61.5
HMMNI	70.5	70.9	69.2	76.0	71.7	71.2	70.8	74.9	81.0
MNZ	72.2	76.8	73.6	71.8	77.5	74.7	75.6	83.3	85.8
RNZ	67.7	67.0	66.3	60.1	70.3	66.7	69.0	79.8	77.8
TNZ	80.5	79.9	78.7	79.1	82.1	78.5	75.2	90.2	91.2
TRZ	86.5	80.6	77.8	72.4	83.1	79.5	78.6	87.7	92.3
IPZ-OH	81.8	81.6	77.0	78.7	82.5	83.2	73.5	88.9	89.9
ORZ	81.9	86.5	81.2	85.1	88.9	85.0	81.5	94.4	96.9

4.3.4. Optimization and application of SPE for the determination of 5-NDZs in milk samples

5-NDZ separation was carried out by the optimized MEKC-UV method considering the modifications already indicated in the **Section 4.3.3**. On the other hand, a two-step sample treatment was optimized for 5-NDZ determination in milk samples. The proposed method was applied to MNZ, MNZ-OH, DMZ, RNZ, HMMNI, TRZ, ORZ, IPZ, and IPZ-OH.

Fat removing and protein precipitation

Numerous methods have been proposed for fat removing and protein precipitation in milk samples [47-49]. In addition to these strategies, the simultaneous fat removing and protein precipitation is proposed in this section. Both processes were achieved by the addition of a protein precipitation agent to the sample and subsequent centrifugation. MeCN, TCA and acetic acid were assayed, being TCA the most adequate because less suspended matter was observed in the supernatant after sample centrifugation. Furthermore, the use of TCA allowed the decrease of sample pH and, as a consequence, 5-NDZ compounds were positively charged. Solid TCA was employed in order to avoid sample dilution. TCA concentration in the milk sample was studied between 0.10 and 0.18 g/mL. No improvement on analyte recoveries was observed when concentrations higher than

0.10 g/mL were used, so this value was chosen as optimum. Once the solid acid was added to the milk samples, they were shaken by vortex for a few seconds in order to guarantee sample homogenization. Afterwards, samples were centrifuged at 9000 rpm for 10 min and, as a consequence, protein precipitation occurred. Furthermore a fat layer was observed on the top of the acidic sample supernatant.

The loss of sample mass after the removal of fat and protein precipitation process was evaluated in four milk samples namely whole pasteurized cow milk, semi-skimmed goat milk, goat raw milk and ewe raw milk. A loss of sample mass about 25% was observed for raw goat milk, giving an idea about the complexity of this matrix. For whole pasteurized cow milk, weight difference was about 19%, while a lower loss of mass was observed for raw ewe milk and semi-skimmed goat milk (around 7%). As will be discussed later, raw goat milk sample complexity entailed low 5-NDZ extraction recoveries.

SPE optimization

Due to the fact that TCA was previously added to milk samples, the supernatant collected after the fat removing and protein precipitation stage was very acid (pH 0.6), and as a consequence, 5-NDZ molecules were positively charged in this medium. For this reason SPE using cation exchange cartridges was proposed for sample clean-up and analyte extraction. Previous assays were carried out using Oasis®MCX cartridges with 60 mg of sorbent mass (30 μm particle diameter).

The proposed SPE protocol consisted of a cartridge preconditioning step with 1 mL of MeOH and 1 mL of 0.1 g/mL of TCA aqueous solution. Then milk supernatant was filtered through a 0.2 μm nylon membrane to the SPE cartridge (except for goat raw milk for which 0.45 μm polyethersulfone filters were used). Filtrated sample was charged into the cartridge at 1 mL/min by gravity. Afterwards a washing step was carried out by passing 1 mL of 2% (v/v) formic acid solution (for removing salts and to block alkaline analytes) and 1 mL of MeOH (for removing neutral and acidic interferences) through the cartridge at 1 mL/min by gravity. Finally, analytes were eluted with 1.5 mL of MeOH containing 2% (v/v) of ammonium hydroxide.

Recoveries around 90% were obtained for MNZ-OH, HMMNI, MNZ, RNZ, DMZ, TRZ, IPZ-OH and ORZ. It was shown that an alkaline organic medium was needed for 5-NDZ elution and therefore no analyte was lost when pure methanol was used as cartridge washing agent. Strong retention of positive charged 5-NDZs was achieved in the Oasis®MCX cartridges because of the low working pH. Although satisfactory recoveries were achieved, these results were obtained when 1 mL of milk was treated. It was an inconvenience because expected LOD level was not enough considering the target value of 3 μg/L proposed by EURLs as the minimum determination level for analytical methods focused on 5-NDZ residue analysis.

In order to improve reached LODs, sample volumes between 1 and 5 mL were evaluated. However, MNZ-OH and RNZ analytical signals were drastically reduced when high sample volumes were treated. Furthermore, matrix interference compounds with 5-NDZ peaks were monitored when these samples were analyzed. With the aim of solving these drawbacks, an extra cartridge washing step and the use of cartridges with higher sorbent mass were considered.

In this sense, alkaline aqueous solutions (1 mL) containing a low concentration of MeOH were also tested as cartridge washing solvents. Assays were carried out for the treatment of 2 mL of milk samples. The use of these solvents allowed removing alkaline polar matrix compounds during cartridge washing step. So, aqueous solutions containing 0–15% (v/v) of MeOH in presence of 2% (v/v) of ammonium hydroxide or 2% (v/v) of TEA were evaluated. In terms of peak area, better results were obtained when ammonium hydroxide was employed instead of TEA. The consideration of these solvents in the cartridge washing step did not involve significant loss for TRZ, ORZ, DMZ, IPZ and IPZ-OH drugs. However, a decrease in the analytical signal was observed for MNZ-OH, RNZ, HMMNI and MNZ when MeOH concentration in the alkaline solution was higher than 5% (v/v) (**Figure 4.14,I**), so it was selected as optimum. As can be seen in **Figure 4.14,II**, a very clean extract was obtained when this extra washing stage was considered.

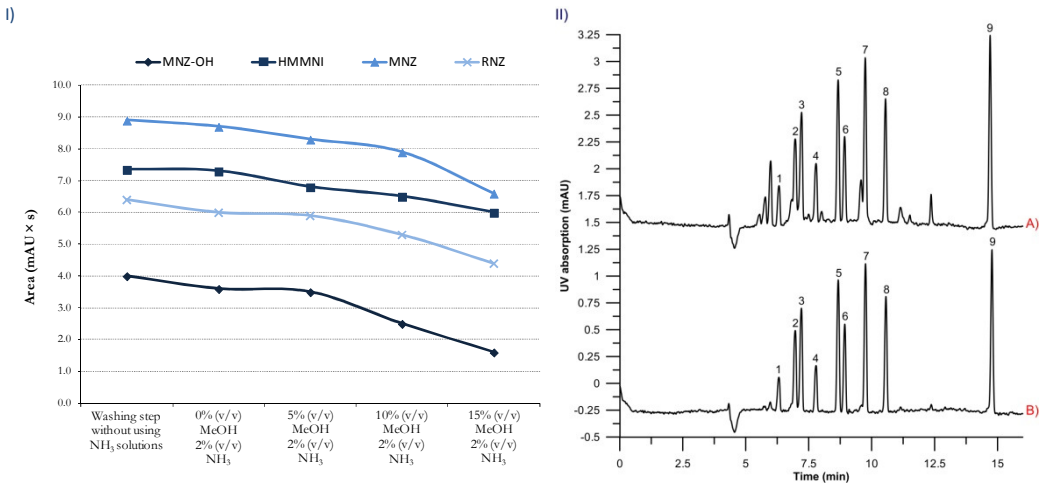

Figure 4.14. **I) Influence on 5-NDZ peak areas of the MeOH solution employed in the cartridge washing process in SPE. II) Electropherograms of whole pasteurized cow milk samples subjected to the proposed SPE method including a washing step consisted of: A) 1 mL of 2% (v/v) formic acid solution and 1 mL of MeOH, and B) 1 mL of 2% (v/v) formic acid solution, 1 mL of MeOH and 1 mL of 2% (v/v) ammonium hydroxide solution containing 5% (v/v) MeOH. Peaks: 1, MNZ-OH; 2, HMMNI; 3, MNZ; 4, RNZ; 5, DMZ; 6, TRZ; 7, IPZ-OH; 8, ORZ; 9, IPZ.**

On the other hand, Oasis®MCX cartridges with higher sorbent mass (150 mg instead of 60 mg) were tested in order to increase the treated sample volume, following the SPE procedure previously described. With this purpose, aliquots of a milk sample fortified at 100 μg/L of each 5-NDZ were processed and analyzed. Aliquot volumes of 2, 3 and 4 mL were considered, observing a decrease of about 20% on MNZ-OH recovery when 4 mL samples were evaluated. Nevertheless recoveries for the rest of analytes were not significantly affected by the assessed sample volumes. Finally, 3.5 mL were chosen as milk sample volume as a compromise between MNZ-OH recovery and sample preconcentration factor.

Because of cartridge sorbent mass was increased respect to initial assays, washing and elution solvent volumes were re-optimized. Washing step consisted of 1 mL of 2% (v/v) formic acid solution, 1 mL of pure MeOH and 1.5 mL of an aqueous solution containing 2% (v/v) ammonium hydroxide and 5% (v/v) MeOH. The effect of the volume for this last solution was investigated between 1 and 2.5 mL. In this range, a loss of analytes was not observed while the obtained extracts were clean enough when 1.5 mL of this cartridge washing solution was applied. On the other hand, elution solvent volume was increased

from 1.5 to 2 mL, being enough for achieving the complete elution of 5-NDZ compounds. Due to the high sorbent mass of the employed SPE cartridges, it was necessary to apply vacuum between washing and elution steps in order to remove the washing solution from the cartridge before analyte elution.

Finally, sample eluate was collected, dried under gentle N_2 current and reconstituted in 200 µL of 20 mM phosphate buffer (pH 6.5). An off-line preconcentration factor of 18-fold was achieved by the proposed SPE procedure. A summary of the final sample treatment is included in **Section 4.2.3 'SPE for milk samples'** and in **Figure 4.15**.

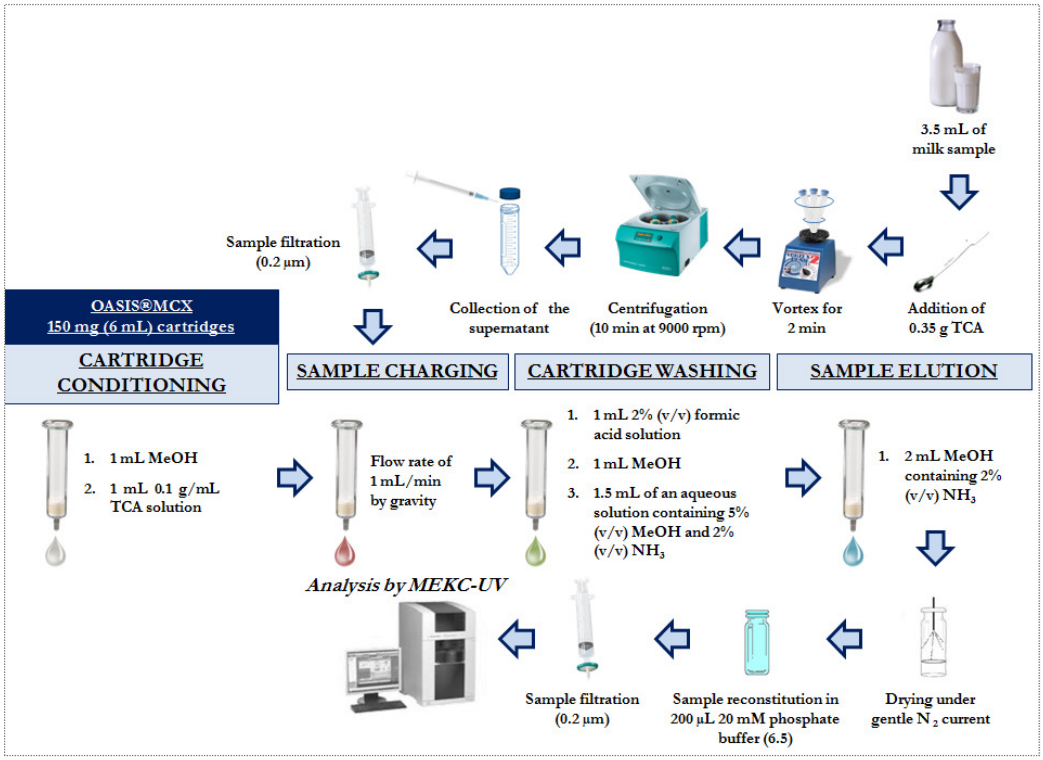

Figure 4.15. SPE procedure for 5-NDZ determination in milk samples by MEKC-UV.

Calibration curves and performance characteristics

Matrix-matched calibration curves were established for the studied analytes in milk samples fortified at different 5-NDZ concentration levels (5, 10, 25, 50 and 100 µg/L). Whole pasteurized cow milk was selected as representative matrix. Two samples per each

concentration level were processed following the developed SPE method and analyzed in triplicate. Peak area was considered as a function of analyte concentration on the sample. A blank sample was also processed, and no matrix interferences were detected at 5-NDZ migration times.

Statistical parameters, calculated by least-square regression, and performance characteristics of the proposed SPE-MEKC-UV method for milk samples are shown in **Table 4.8**. LODs and LOQs were calculated as the minimum analyte concentration yielding a signal-to-noise ratio equal to three and ten, respectively. As can be seen, LODs lower than 3 μg/L were achieved for all the studied compounds as a consequence of both off-line and on-line preconcentration strategies.

Table 4.8. **Statistical and performance characteristics of the developed SPE-MEKC-UV method for the analysis of nine 5-NDZs in whole pasteurized cow milk samples.**

Analyte	Linear range (μg/L)	R^2	Linear regression equation (y=m·x+a)		LOD (μg/L)	LOQ (μg/L)	Migration time (min, n = 45)
			Slope	*Intercept*	$3\times S/N$	$10\times S/N$	
MNZ-OH	6.0 – 100	0.996	0.062	0.606	1.8	6.0	6.7
HMMNI	5.2 – 100	0.994	0.099	0.281	1.6	5.2	7.4
MNZ	3.7 – 100	0.995	0.111	0.799	1.1	3.7	7.7
RNZ	5.7 – 100	0.995	0.079	0.631	1.7	5.7	8.3
DMZ	3.2 – 100	0.994	0.137	0.980	1.0	3.2	9.4
TRZ	4.0 – 100	0.996	0.119	0.710	1.2	4.0	9.7
IPZ-OH	3.1 – 100	0.995	0.139	1.196	0.9	3.1	10.7
ORZ	3.7 – 100	0.993	0.141	1.032	1.1	3.7	11.7
IPZ	4.3 – 100	0.990	0.219	1.716	1.3	4.3	17.3

Precision assays

Method precision was evaluated in terms of repeatability (intra-day precision) and intermediate precision (inter-day precision) by the application of the proposed SPE-MEKC-UV method to whole pasteurized cow milk samples fortified at three different 5-NDZ concentration levels (10, 50 and 100 μg/L). Repeatability was assessed over three treated samples (experimental replicates) which were analyzed in triplicate on

the same day under the same conditions. For intermediate precision assays, a fortified sample at each concentration level was processed and analyzed in triplicate for five consecutive days. The results, expressed as peak area RSD (%), are summarized in **Table 4.9**.

Table 4.9. Precision studies for the determination of 5-NDZs in whole pasteurized cow milk samples by the proposed SPE-MEKC-UV method.

Analyte	Repeatability (% RSD, n = 9)			Intermediate precision (% RSD, n = 15)		
	10 µg/L	***50 µg/L***	***100 µg/L***	***10 µg/L***	***50 µg/L***	***100 µg/L***
MNZ-OH	10.3	7.5	2.8	11.8	11.3	9.2
HMMNI	4.2	8.0	2.3	15.2	9.7	9.4
MNZ	5.7	4.4	2.7	12.1	9.0	9.3
RNZ	8.4	3.0	3.5	14.4	9.0	10.6
DMZ	8.2	3.8	4.2	13.8	11.1	13.2
TRZ	7.2	6.4	2.4	13.9	10.7	11.5
IPZ-OH	5.0	3.4	3.5	11.0	10.8	13.6
ORZ	5.7	4.1	3.8	16.1	11.8	13.1
IPZ	14.3	4.5	5.1	19.3	18.3	20.1

Trueness

In order to check the trueness of the proposed method for the analysis of real samples, recovery experiments were carried out in different types of milk samples spiked at three different 5-NDZ concentration levels (10, 50 and 100 µg/L) (**Table 4.10**). Commercial milk samples, namely whole pasteurized cow milk and semi-skimmed goat milk, and raw milk samples, namely goat and ewe milk were selected as representative milk products destined to human consumption. Three samples of each matrix were fortified at each concentration level, processed by the proposed SPE procedure and analyzed in triplicate. In all cases, a blank sample was analyzed in order to check the presence of matrix compounds co-migrating with 5-NDZ peaks. An unknown peak was detected at MNZ migration time in both goat milk samples. This peak was considered as an inherent compound of this type of milk. However, MS assays should be required in order to discard a positive result for MNZ in these samples.

Table 4.10. Recovery studies for each 5-NDZ at different spiked levels in different milk samples.

Analyte	Whole pasteurized cow milk samples (n = 9)			Semi-skimmed goat milk samples (n = 9)			Raw goat milk samples (n = 9)			Raw ewe milk samples (n = 9)		
	10 µg/kg	*50 µg/kg*	*100 µg/kg*	*10 µg/kg*	*50 µg/kg*	*100 µg/kg*	*10 µg/kg*	*50 µg/kg*	*100 µg/kg*	*10 µg/kg*	*50 µg/kg*	*100 µg/kg*
MNZ-OH	76.3	65.8	65.4	77.5	65.3	64.4	66.1	67.8	66.8	66.8	68.1	69.1
HMMNI	66.7	73.3	76.1	74.9	85.5	84.9	58.0	69.0	68.0	72.5	83.1	83.4
MNZ	76.2	77.4	76.8		N/A			N/A		97.7	84.1	84.6
RNZ	73.1	70.7	68.8	62.6	72.5	74.2	57.6	62.5	63.6	83.9	76.8	77.9
DMZ	63.5	65.6	68.4	79.3	78.1	78.1	39.3	51.4	54.5	79.4	71.8	70.2
TRZ	64.5	78.4	78.5	81.7	83.8	84.0	57.5	66.9	70.4	79.8	82.3	82.2
IPZ-OH	64.6	70.1	74.8	79.3	80.4	81.9	59.9	61.2	65.8	88.1	78.3	78.4
ORZ	66.7	70.0	70.4	85.7	73.9	76.4	54.7	58.3	59.5	83.0	73.0	73.4
IPZ	57.0	53.0	58.5	76.7	68.5	68.5	37.4	34.7	38.3	71.0	57.5	59.4

Not applicable (NA) due to the presence of a co-migrating matrix compound.

In spite of this interference, MNZ can be indirectly detected in real samples by the monitoring of MNZ metabolite (MNZ-OH). For other cases no compounds were co-migrating at the same time of the studied analytes. **Figure 4.16** shows the obtained electropherograms from the analysis of a blank and a spiked raw ewe milk sample (10 µg/L of each 5-NDZ).

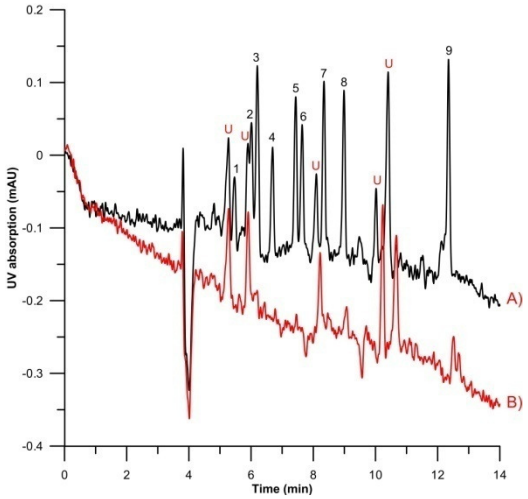

Figure 4.16. **Electropherograms of raw ewe milk samples treated and analyzed by the proposed SPE-MEKC-UV method. A) fortified sample at 10 µg/L of each 5-NDZ; B) blank sample. 5-NDZ separations were performed in an extended optical path capillary (61.5 cm × 50 µm, 56 cm of effective length and 150 µm of optical path diameter) at 25 kV and 20°C, using a BGS consisted of 20 mM phosphate buffer (pH 6.5) containing 150 mM SDS. Separation was monitored at 320 nm. Samples in BGS without micelles were hydrodynamically injected for 25 s at 50 mbar. Peaks: 1, MNZ-OH; 2, HMMNI; 3, MNZ; 4, RNZ; 5, DMZ; 6, TRZ; 7, IPZ-OH; 8, ORZ; 9, IPZ.**

In general, recoveries over 60% were obtained for all analytes in milk samples. Recoveries ranged between 50% and 70% for all analytes in raw goat milk samples, except for DMZ and IPZ that showed recoveries lower or equal to 50%. These results indicate the complexity of raw goat milk samples, as was commented before.

4.4. Conclusions

In this chapter, a MEKC-UV method was proposed for the simultaneous determination of 5-NDZ residues in different environmental water and milk samples. The optimized method represents the first time that a CE-based method has been employed to analyze samples containing a relatively high number of 5-NDZ drugs, including their

corresponding metabolites. In spite of using UV detection, high sensitivity was achieved by both off-line and on-line preconcentration strategies. Sweeping was considered as on-line preconcentration, reaching a signal enhancement up to 2.3-fold. On the other hand SPE and DLLME procedures were proposed as sample treatments, and moreover, as off-line preconcentration procedures.

The developed MEKC-UV method was successfully applied to the analysis of river water samples from different origins in which contamination by antibiotics such as 5-NDZs is possible, such as an urban place, a fish farm, and a cattle farming area. For this application a SPE procedure was successfully performed. It was demonstrated that this method provides a much-needed alternative technique for determining these antimicrobials in environmental samples, as the LODs attained with the method are at the low μg/L level. Therefore, the method developed here provides an efficient, sensitive, and simple alternative approach for monitoring these drug residues in environmental water samples at the low μg/L level.

Furthermore, in this chapter, a simple and efficient way for 5-NDZ extraction from aqueous samples was proposed. In general, the combination of MEKC with DLLME is a green alternative to the traditional LC methods combined with high organic solvent consumption sample treatments, such as LLE or the well-established SPE, and it is simpler, faster and cheaper, reducing the consumption of organic solvents and waste. The proposed method could be routinely used for water analysis in the control of 5-NDZ residues, considering the low detection limits obtained, even by using UV detection. By using DLLME, an off-line preconcentration factor of 25 was achieved, demonstrating the suitability of this sample treatment even in the extraction of polar compounds, such as 5-NDZ antibiotics, from water samples of different origins with satisfactory precision and trueness.

Additionally, a novel sample treatment combined with the proposed MEKC method as analytical separation technique was evaluated as quick, simple and low solvent consumption strategy for the simultaneous determination of nine 5-NDZ residues in different milk samples. Sample treatment consisted of two steps in which fat removing and

protein precipitation occur simultaneously, following by SPE with Oasis®MCX cartridges for sample clean-up and off-line preconcentration. On other hand, it was the first time that these group of analytes were determined in such a complex matrix by CE-UV, which constitutes a new green alternative for the simultaneous monitoring of a high number of 5-NDZs and their metabolites in foods, with very low detection limits, even lower that the recommended by EURLs.

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CSEI-sweeping-MEKC-UV approach for 5-NDZ residue analysis in water, urine, serum and egg samples

Abstract ► In this chapter, a novel cation-selective exhaustive injection-sweeping-MEKC-UV method is proposed for 5-NDZ residue determination in different matrices, such as water, urine, serum and egg samples. The proposed method consisted of rinsing the CE capillary with a low conductivity buffer (LCB; 50 mM phosphate buffer, pH 2.5), followed by a plug of a high conductivity buffer (HCB; 100 mM phosphate pH 2.5, 50 mbar, ≈ 31.5% of capillary volume) and a plug of water (50 mbar, 2 s). Afterwards, samples with lower conductivity than BGS were electrokinetically injected at 9.8 kV for 632 s in a bare fused-silica capillary (total length up to the application, 50 μm i.d.). Finally, separation was carried out at -30 kV and 20°C using a BGS consisted of 44 mM phosphate buffer (pH 2.5) containing 123 mM SDS and 8% (v/v) THF. The method was applied to environmental, clinical and food samples considering different sample treatment strategies for accomplishing with CSEI-sweeping requirements. DLLME was applied to water samples (5 mL), using dibromomethane (1156 μL) as extractant, 2-butanol (1363 μL) as dispersive solvent and NaCl (16%, w/v) as salting-out agent. On the other hand, untreated human urine and serum samples were analyzed by the simple injection of diluted samples (143 and 22-fold, respectively). Additionally, a SPE procedure, using Oasis®HLB (60 mg) cartridges, was considered for egg sample (2.0 g) analyses. As a result, LODs at low μg/L and μg/kg levels were achieved for water samples and egg samples, according to EURLs' recommendations. LODs at low μg/mL levels were reached for urine and serum samples, and as a consequence, this method allows the detection of these drugs at therapeutic levels in biological fluids.

5.1. Background

MNZ is an antiprotozoal drug that possesses appreciable properties against *Trichomonas vaginalis*, *Entamoeba histolytica*, and *Giardia lamblia* and its effectiveness has also been shown for treating *Clostridium difficile* and *Gardnerella vaginalis* infections [1]. According to the pathology, doses of MNZ can be prescribed from 250 to 750 mg three times per day during 7 or 10 days, restricting the daily dose to a maximum of 2.5 g. Depending on the dose, maximum plasma concentration levels (C_{max}) of 6 to 40 μg/mL are reached after maximum absorption within 0.25 to 4 h [2]. Furthermore, this drug undergoes hepatic metabolism and it is mainly eliminated via renal excretion, recovering in the urine up to 77% of the dose, of which 10% is MNZ as unchanged drug [3]. MNZ is the only 5-NDZ that has been included in the list of essential medicines proposed by WHO [4]. However, other 5-NDZs such as ORZ and TNZ have demonstrated to be an effective alternative to MNZ [5,6].

Although 5-NDZ drugs have also been tested in veterinary medicine [7-9] for the prophylactic and therapeutic treatment of histomoniasis and coccidiosis in poultry [10] and for combating hemorrhagic enteritis in pigs [11], their use in animals intended to human consumption has been banned in EU [12], USA [13] and China [14]. The presence of 5-NDZ residues in food of animal origin can supposes a serious risk to human health, not only because carcinogenic properties have been attributed to these compounds [15,16], but also because the emergence of resistant bacteria to these drugs have already been reported [17,18]. Despite the ban, several alerts about the presence of 5-NDZ residues in foodstuff have been notified by RASFF portal in the last years [19]. These alerts are mainly concerned with the presence of MNZ traces in different food matrices such as honey, royal jelly, trout, fish products, meat and meat products. Furthermore, RNZ residues have also been found in chilled fresh turkey.

On the other hand, the uncontrolled disposal of 5-NDZs among other pharmaceutical residues in wastewaters also supposes an environmental and health risk because urban wastewater treatment plants are among the main sources of antibiotic release to the environment [20]. As a consequence, natural environment has become an important via for

the emergence of resistant bacteria [21-23]. In the case of 5-NDZs, this problem is highly relevant because these compounds present high polarity and low biodegradability characteristics, which contribute to their persistence in water and, consequently, favors their bioaccumulation and ecotoxicity [24].

Therefore, analytical methods for 5-NDZ determination are required in environmental, clinical and food safety fields. Traditionally, LC coupled to both UV and MS detection has been the most popular choice for monitoring 5-NDZ residues in environmental [25], clinical [26-28] and food [29-33] samples. Nevertheless, other alternative methods to LC such as GC [34,35] or CE [36,37] have been proposed for 5-NDZ determination. CE represents a powerful tool for analytical separations due to its unique characteristics such as low solvent consumption or high efficiency. However its use is limited due mainly to its poor sensitivity, especially when it is coupled to UV detection. In order to overcome this disadvantage, different preconcentration strategies have been developed, including on-line (sample stacking techniques) [38], in-line methods which can also be considered as sample clean-up strategies [39] and off-line (through sample pretreatment) procedures [40]. The most common on-line preconcentration methodologies include: FASS and FESI [41], sweeping [42], dynamic pH junction [43] and tITP [44], although others less known strategies offer even higher SEFs.

A decade ago, Quirino *et al.* proposed a novel on-line preconcentracion technique based on CSEI-sweeping. It combines two on-line preconcentration techniques: FESI and sweeping. This combination allows achieving enhancement factors from a thousand- to almost a million-fold in relation to conventional CE [45]. CSEI-sweeping involves an electrokinetic injection (FESI) of a high amount of charged cationic compounds, creating long analyte zones in the capillary with higher concentration than in the original sample solution [46]. After sample injection, BGS vials containing micelles are placed at both ends of the capillary and a negative voltage is applied. Micelles focus the analytes in narrow bands by sweeping, and they are consequently separated by conventional MEKC. In order to achieve FESI, analytes must be dissolved in a low conductivity medium, which is not an easy task, especially for complex real samples showing high or moderate salinity. In such cases, a proper sample treatment is required for decreasing sample conductivity. Another

drawback inherent to CSEI-sweeping-MEKC is the run time since it requires longer analysis times compared to conventional CE methods, considering capillary conditioning, injection and separation time [45]. However, most commercial CE instruments allow the automation of capillary preconditioning, sample injection and CE separation. CSEI-sweeping-MEKC is a quite novel technique since it has not been very widely employed at the moment, but it has been successfully considered in different applications such as the determination of herbicides in water samples [47], drugs of abuse in urine [48,49], methadone in serum [50] and melamine and cyromazine in milk [51].

In this chapter, a novel CSEI-sweeping-MEKC-UV method has been developed for 5-NDZ determination in water, urine, serum and egg samples. Both water and egg samples required a sample treatment for decreasing sample conductivity. In this respect, SPE has shown to be a suitable option for sample clean-up prior to CSEI-sweeping-MEKC [52,53], and an alternative widely used for 5-NDZ extraction from eggs samples. In this sense, LLE with MeCN or MeOH has usually been carried out in order to induce sample deproteinization, followed by SPE with reverse phase [54,55] or cation exchange [56,57] cartridges. Thus, in this chapter, a SPE procedure has been employed for 5-NDZ preconcentration and extraction from eggs and egg-based products. On the other hand, DLLME has been proposed for water sample clean-up and 5-NDZ extraction. According to the checked bibliography, it supposes the first time that DLLME is coupled with a CSEI-sweeping-MEKC method. Furthermore, FESI selectivity was demonstrated by the direct injection of urine and serum sample. Consequently the determination of 5-NDZ antibiotics in untreated urine and serum samples has been directly carried out, avoiding tedious sample treatments.

5.2. Materials and methods

5.2.1. Materials and reagents

All reagents used through this work were analytical reagent grade and solvents were HPLC grade, unless otherwise specified. NaCl, NaOH, NaH_2PO_4 and H_3PO_4 (85%, v/v) were obtained from Panreac-Química (Madrid, Spain). MeOH and 2-butanol were purchased

from VWR International (West Chester, PA, USA) while MeCN, 2-propanol, dibromomethane, SDS and oxalic acid were supplied by Sigma-Aldrich (St. Louis, MO, USA). Formic acid (98–100%, v/v) and THF were acquired from Merck (Darmstadt, Germany). Ultrapure water (Milli-Q plus system, Millipore, Bedford, MA, USA) was used throughout the work.

Analytical standards of ORZ, MNZ, TNZ, and CRZ were supplied by Sigma-Aldrich (St. Louis, MO, USA) while IPZ, SCZ and TRZ were purchased from Witega (Berlin, Germany). Individual standard solutions were obtained by dissolving the appropriate amount of each 5-NDZ in MeCN, reaching a final concentration of 1 mg/mL. They were kept in the freezer at -20°C avoiding exposure to light. Intermediate standard solutions were obtained by mixing the appropriate amount of each individual standard solution and their subsequent dilution with MeCN. The concentration of the analytes in intermediate standard solutions ranged between 2 and 5 μg/mL. They were stored in dark at 4°C and equilibrated to room temperature before their use. Working standard solutions were prepared by the dilution of an intermediate standard solution with injection solvent (5 mM H_3PO_4 in water containing 5% (v/v) of MeOH) to the desired 5-NDZ concentration. Furthermore, a standard solution (15.0 μg/mL of ORZ and MNZ and 40.0 μg/mL of TNZ) was weekly prepared in injection solvent (5 mM H_3PO_4 in water containing 5% (v/v) of MeOH) from the individual standard solutions for the fortification of serum and urine samples. It was stored at 4°C avoiding exposure to direct light.

Oasis®HLB cartridges (60 mg, 3 mL, 30 μm of particle size) were supplied by Waters (Milford, MA, USA). Acrodisc 13 mm nylon membrane filters (0.2 μm pore size) were supplied by Pall Corp. (Ann Arbor, MI, USA).

5.2.2. Instrumentation

CE experiments were carried out with an Agilent 7100 CE System (Agilent Technologies, Waldbronn, Germany) equipped with a DAD. Data were collected using the software supplied with the HP ChemStation (Version B.02.01). Separations were performed in fused silica capillaries of different dimensions according to the application. Method optimization

was accomplished in a standard capillary (48.5 cm of total length × 50 μm i.d., 40 cm of effective length) whereas water sample analyses were carried out in an extended light-path capillary (57.2 cm of total length × 50 μm i.d., 48.7 cm of effective length, and 150 μm of optical path length) acquired in Agilent Technologies. Serum and urine analyses were performed in an extended light-path capillary (65 cm total length × 50 μm i.d., 56.5 cm of effective length, and 150 μm of optical path length) while egg and egg-based product analyses were carried out in a standard capillary (70 cm total length × 50 μm i.d., and 61.5 cm of effective length).

SPE treatment was carried out on a Visiprep™ DL vacuum manifold for 12 cartridges from Supelco (Bellefonte, PA, USA). A Universal 320R centrifuge (Hettich Zentrifugen, Tuttlingen, Germany), a nitrogen dryer EVA-EC System (VLM GmbH, Bielefeld, Germany) and a vortex-2 Genie (Scientific Industries, Bohemia, NY, USA) were also used. Solution pH was adjusted with a pH meter (Crison model pH 2000, Barcelona, Spain) with a resolution of ±0.01 pH unit.

5.2.3. Sample treatment procedures

DLLME for water samples

Natural water samples from different sources were considered in this study. River water samples were collected from Riofrío River (Riofrío, Granada, Spain), being the sampling point located after a fish farm drain, and from Genil River which is an urban river (Granada, Spain). Besides, water samples from a well placed in a cattle area (La Serena, Badajoz, Spain) were also analyzed. Water samples were kept at 4 °C and equilibrated to room temperature before their analysis.

Water samples were treated following the DLLME procedure optimized in **Chapter 4**. The proposed sample treatment was applied to aliquots (5 mL) of each water sample that were placed in 15 mL-centrifuge tubes with a conical bottom. Analyte extraction was assisted by a salting-out effect and achieved by the addition of 0.8 g of NaCl (16%, w/v) to each sample. Salt was dissolved in the water samples by vortex agitation before extraction. 5-NDZ extraction took place through the quick injection of an organic solvent mixture

into the water sample. The organic mixture consisted of 1156 µL of dibromomethane (extraction solvent) and 1363 µL of 2-butanol (dispersive solvent) and its injection into the sample was carried out with a syringe coupled to a needle with a flat point, causing the formation of a cloudy solution. Afterwards, the sample tube was agitated by vortex for 30 s and centrifuged for 5 min at 9000 rpm. Consequently, phase separation occurred, obtaining the extractant as the lower phase. It was carefully collected with a syringe coupled to a needle with a flat point, avoiding the collection of aqueous phase, and it was placed into a glass vial. Then the extractant was evaporated to dryness under gentle nitrogen current and it was re-dissolved with 1.2 mL of injection solvent (5 mM H_3PO_4 aqueous solution containing 5% (v/v) of MeOH), agitated by vortex for 2 min and filtered. Finally, 1 mL of the extract was analyzed. **Figure 5.1** shows a scheme of the proposed sample treatment.

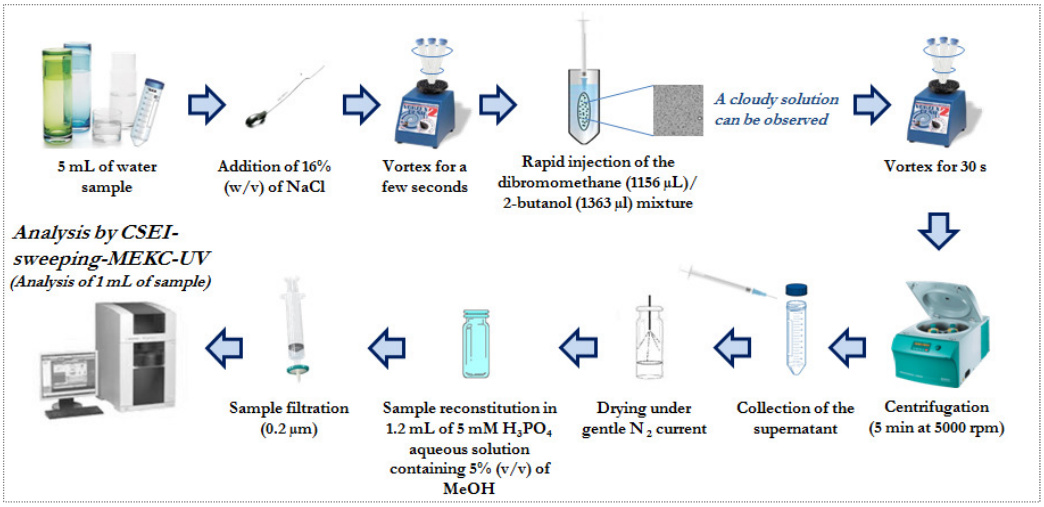

Figure 5.1. DLLME procedure for 5-NDZ determination in water samples.

Urine and serum sample dilution

Urine samples were daily obtained from healthy volunteers, disposed in flasks and spiked at the required 5-NDZ concentration. Afterwards, 7 µL of a spiked sample was placed in a CE vial and diluted with injection solution (5 mM H_3PO_4 aqueous solution containing 5% (v/v) of MeOH) to a final volume of 1 mL. Serum samples were stored at –20°C and brought to room temperature before use. Before analysis, 200 µL of serum sample were

placed in a 15 mL-conical tube and spiked with the required standard solution volume. Injection solution (5 mM H_3PO_4 aqueous solution containing 5% (v/v) of MeOH) was added up to a final volume of 300 μL. Afterward, 400 μL of MeCN were added. The mixture was agitated by vortex for a few seconds and centrifuged for 5 min at 9000 rpm in order to precipitate serum proteins. Finally, 46 μL of deproteinized serum was placed in a CE vial which was filled with injection solution up to 1 mL.

SPE for egg and egg-based product samples

Hen and quail eggs and a pasteurized egg white commercial product were purchased from a local supermarket. Eggs were cracked, mixed, and stirred until homogenization while egg white product was homogenized by stirring it. Protein precipitation was performed by adding 4 mL of MeCN to 2.0 g of stirred egg sample and their subsequent centrifugation for 10 min at 9000 rpm and 20ºC. The resulting supernatant was isolated and 0.8 g of NaCl were added to it. The mixture was agitated by vortex for 2 min and centrifuged at 9000 rpm and 20ºC for 5 min, obtaining the separation between MeCN and the matrix aqueous phase due to the salting-out effect. Three layers were perfectly distinguished in the tube: some salt in the bottom, a clear aqueous phase in the middle and a yellowish organic phase in the top. Then, 3.4 mL of the organic phase were isolated and transferred to a 4 mL-vial. The organic extract was evaporated under a gentle nitrogen stream at room temperature. The residue was re-dissolved in 2 mL of ultrapure water and loaded into an Oasis®HLB (60 mg, 3 mL) cartridge which was previously conditioned with 1 mL of MeOH and 2 mL of ultrapure water. After sample loading, cartridge was washed with 3.0 mL of a mixture 95:5 (v/v) water/MeOH. Then vacuum was applied for a few seconds and elution was accomplished with 2 mL of pure MeOH. The obtained extract was collected in a new 4 mL-vial and dried under a gentle nitrogen stream. Finally, it was re-dissolved in 850 μL of 5 mM H_3PO_4 aqueous solution containing 5% (v/v) of MeOH and filtered through a 0.2 μm nylon membrane filter. The resulting solution was analyzed by the developed CSEI-sweeping-MEKC-UV procedure. **Figure 5.2** shows a scheme of the proposed sample treatment.

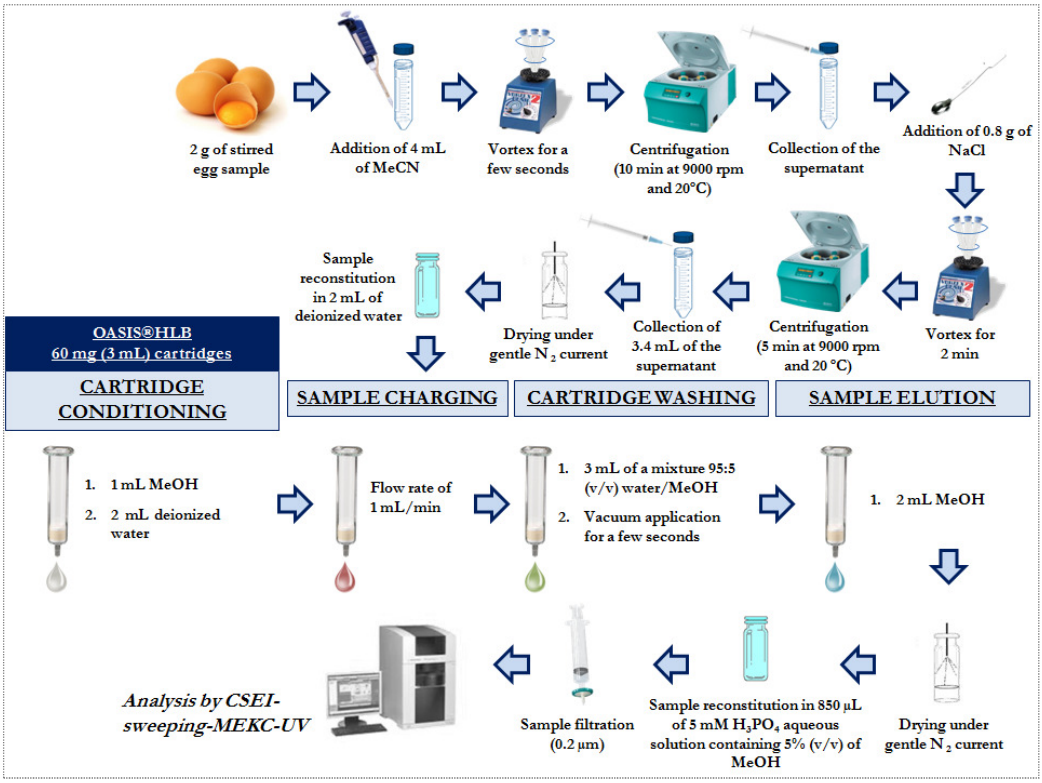

Figure 5.2. SALLE-SPE procedure for 5-NDZ determination in egg and egg-based product samples.

5.2.4. CSEI-sweeping-MEKC method

The proposed method was performed in an uncoated fused-silica capillaries whose dimensions have been previously indicated (**Section 5.2.2**). New capillaries were conditioned with 1 M NaOH solution for 15 min at 1 bar and 20°C. Afterwards and under the same conditions, capillary was rinsed with ultrapure water for 5 min and with LCB for 15 min. LCB consisted of 50 mM phosphate buffer (NaH_2PO_4/H_3PO_4, pH 2.5). At the beginning of each working day, capillary was flushed with LCB for 15 min at 1 bar and 20°C. At the end of each day, capillary was washed with deionized water for 5 min at 5 bar and dried with air for 5 min at 5 bar and 20°C. Between runs, capillary was subsequently rinsed with 0.1 M NaOH solution for 2 min at 3 bar, ultrapure water for 0.5 min at 3 bar and LCB for 3 min at 3 bar. Afterwards, a plug of HCB was flushed into the capillary at 50 mbar and 20°C, filling 31.5% of capillary volume. HCB consisted of 100 mM

phosphate buffer (NaH_2PO_4/H_3PO_4, pH 2.5). Afterwards, inlet electrode was submerged into a vial containing ultrapure water for 5 s in order to wash it. Finally, an ultrapure water plug was hydrodynamically injected into the capillary for 2 s at 50 mbar. After capillary conditioning, samples were electrokinetically injected at 9.8 kV (normal mode) for 632 s. Electrophoretic separation was performed under a voltage of −30 kV programming a voltage ramp from 0 to −30 kV for 0.5 min at the beginning of the run. Separation temperature was set to 20°C. BGS consisted of phosphate buffer (44.0 mM, pH 2.5) containing 123 mM SDS and 8% (v/v) THF. Analytical signals were monitored at 276 nm, except for CRZ peak (244 nm). A procedure scheme of the proposed CSEI-sweeping-MEKC method is shown in **Figure 5.3**.

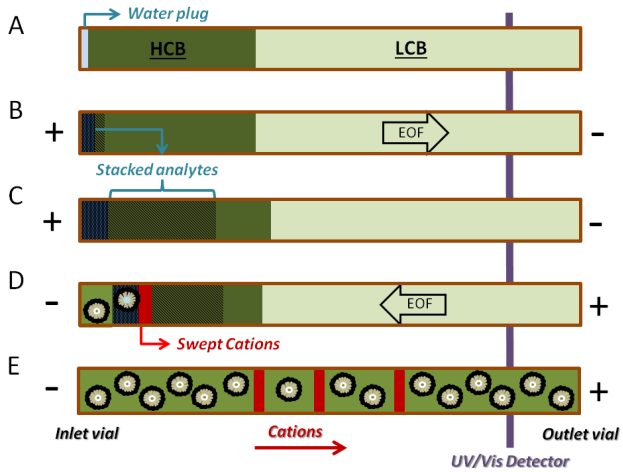

Figure 5.3. CSEI-Sweeping-MEKC procedure scheme. Procedure stages: A, capillary is rinsed with LCB, followed by a plug of a HCB and a water plug; B, electrokinetic injection at positive polarity is performed, being cationic analytes stacked at the interface between water and HCB zones; C, cationic analytes are stacked at HCB zone because of the long injection, but not at water or matrix zones; D, BGS is placed in both ends of the capillary and a negative voltage is applied, removing matrix sample from the capillary; E, ordinary MEKC separation takes place. Adapted from [45].

5.3. Results and discussion

5.3.1. Optimization of CSEI-sweeping-MEKC method

Separation optimization was initially performed in a standard capillary (48.5 cm × 50 µm i.d., 40 cm of effective length). It was rinsed for 3 min with LCB solution (25 mM phosphate buffer, pH 2.5, prepared from a mixture of NaH_2PO_4/H_3PO_4) at 3 bar and

20°C. Afterwards, a plug of HCB solution (150 mM phosphate buffer, pH 2.5, prepared from a mixture of NaH_2PO_4/H_3PO_4) was injected into the capillary for 150 s at 50 mbar and 20°C. Then inlet electrode was submerged in ultrapure water for 5 s in order to wash it. Prior to sample injection, a water plug was injected for 1 s at 50 mbar. Finally, sample dissolved in 3 mM H_3PO_4 aqueous solution was injected for 600 s at 10 kV. 5-NDZ separation was performed at −30 kV and 20°C, using a BGS consisted of 20 mM phosphate buffer (pH 2.5) containing 150 mM SDS. Analytical signals were monitored at 276 nm, except for CRZ which presents a maximum UV absorption at 244 nm, instead of considering a wavelength of 320 nm as was established in **Chapter 4**. As it has been previously reported [58], maximum UV absorption of imidazole derivatives is moved to lower wavelengths at low-pH solutions (UV spectra for some 5-NDZ compounds at pH 2.5 are shown in **Figure 5.4**).

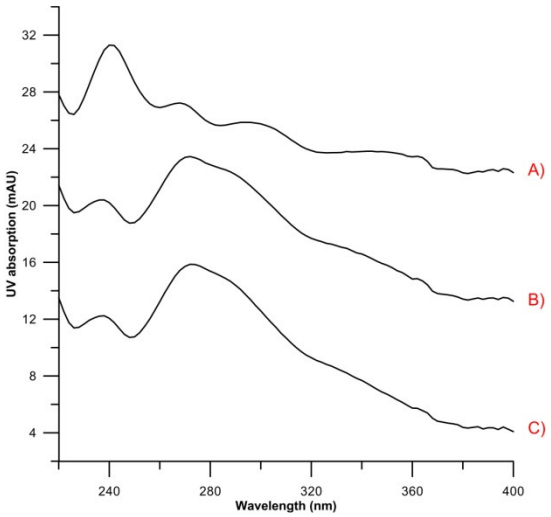

Figure 5.4. **UV/Vis spectrum signals of A) CRZ, B) TRZ and C) MNZ. UV spectrum measurement was done with the DAD from CE instrument at pH 2.5.**

Variables related to the CSEI-sweeping-MEKC method were divided in three groups for their optimization: parameters affecting 5-NDZ separation (buffer concentration, organic percentage in the buffer and surfactant concentration); chemical variables related to the capillary conditioning (LCB and HCB concentration, and capillary volume filled with HCB); and instrumental variables related to the injection (voltage and injection time). These groups of variables were optimized through experimental designs in order to

evaluate their interactions. Other parameters, such as separation pH, separation buffer nature, injection solvent, buffer concentration in the outlet vial during the sample injection, influence of a water plug before the sample injection and separation voltage and temperature were independently studied. Standard solutions of 70 μg/L of each 5-NDZ (IPZ, ORZ, SCZ, TRZ and MNZ), except for CRZ (210 μg/L), were used through optimization studies.

BGS nature and pH

Separation buffer pH was studied in a narrow range (between 2 and 3). At lower pH values, EOF was very low and consequently worse analyte stacking effect was observed. At higher pH values, a stronger EOF was produced, and as a consequence, EOF was higher than the electrophoretic velocity of the micelles. It resulted in analyte migration toward the cathode instead of from the inlet vial (cathode) to the outlet vial (anode). Considering the evaluated range, longer analysis times were observed at pH 3, while poorer stacking effect was observed at pH 2. Thus, a separation pH of 2.5 was established.

Different buffer natures as phosphate, oxalate and formate were studied. A concentration of 20 mM was considered for all tested buffers. Oxalate and formate buffers were prepared from their respective acid solutions and subsequent pH adjustment with 1 M NaOH solution, while phosphate buffer was prepared by mixing NaH_2PO_4 and H_3PO_4. Phosphate buffer was chosen as optimum because oxalate buffer showed poor separation reproducibility while lower sensitivity in terms of peak height was obtained when formate buffer was employed as BGS.

In order to improved peak resolution, different organic solvents were tested as BGS modifiers, namely MeCN, MeOH, 2-propanol and THF. All organic solvents were added to the BGS in a concentration of 10% (v/v). In all cases, similar peak resolution was shown without observing any improvement, although higher sensitivity in terms of peak height was obtained when an organic solvent was added to the BGS. Signal increase was more appreciable when THF was added to the separation buffer. Furthermore, negatively charged micelles are required in CSEI-sweeping methodologies. In this work, SDS was

selected considering that it has been the most employed surfactant in the application of this technique [47-51].

BGS composition

A central composite design was employed for the multivariate optimization of surfactant concentration, phosphate buffer concentration and percentage of THF contained in the BGS. Ranges for each parameter were established as follows: from 10.23 to 70.77 mM for phosphate buffer concentration, from 49.32 mM to 200.7 mM for SDS concentration and between 0 and 15.07% (v/v) for THF concentration. **Equation 5.1** represents the employed response function (R.F.). Parameters in the R.F. were normalized respect to the maximum value shown in the experimental set. The selected R.F. considered the most critical parameters for the proposed 5-NDZ separation. Lowest peak resolution was obtained between SCZ and TRZ peaks, so normalized peak resolution among both peaks was included in the R.F. Other terms such as normalized CRZ and MNZ theoretical plates ($Plates_{norm.}$) were also included in the R.F. because preliminary studies indicated that CRZ presented the lowest sensitivity while MNZ is the representative 5-NDZ drug due to its wide use.

$$R.F. = Plates_{norm.}^{CRZ} + Plates_{norm.}^{MNZ} + Peak\ resolution_{norm.}^{SCZ-TRZ} \quad \textbf{Equation 5.1.}$$

In the corresponding analysis of variance (ANOVA), a second-degree quadratic model was assumed. Lack-of-fit was no significant at a confidence level of 95.0% (p-value > 0.05). In this case, interaction between THF percentage and SDS concentration was significant (p-value = 0.0131) as well as the quadratic interactions (p-value < 0.05). The optimum values for the studied variables were: 44 mM for phosphate buffer concentration, 123 mM for SDS concentration and 8% (v/v) for THF concentration in the BGS. These values were considered for further experiments.

Separation voltage and temperature

Separation was carried out at negative polarity. Voltage values from −25 kV to −30 kV were evaluated. Values lower than −30 kV provided longer analysis times without

achieving any improvement on peak resolution. According to that, −30 kV was established as optimum. On the other hand, low separation temperature was desired in order to compensate the heat generated due to the high voltage that was used. Temperature was set at 20 °C in order to avoid capillary heating because of Joule effect.

Water plug prior to sample injection

Some reports attribute an improvement on method sensitivity to a water plug introduced into the capillary prior to sample injection. For such a reason, the injection of a water plug was assayed between 1 and 4 s at 50 mbar. In this case, the water plug did not produce any increase on sensitivity but it was decided to plug water for 2 s prior to sample injection because it produced higher injection reproducibility [59].

Chemical parameters of CSEI

Phosphate buffer was selected as LCB and HCB solutions. Concentration of both solutions, together with the capillary length filled with HCB solution, was evaluated by a central composite design. Studied experimental domains were ranged from 9.82 to 50.18 mM for LCB concentration and from 74.43 to 200.57 mM for HCB concentration, while HCB injection time was studied from 60.18 to 241.82 s considering an injection pressure of 50 mbar. The considered R.F is indicated in **Equation 5.2**.

$$R.F. = \frac{H^{MNZ}}{1+|1-Sym^{MNZ}|} \quad \textbf{Equation 5.2.}$$

In this R.F., only the symmetry (Sym^{MNZ}) and height (H^{MNZ}) of MNZ peak were included. According to that, analyte stacking into the capillary was evaluated considering MNZ peak as representative. A higher stacking effect involves higher sensitivity in terms of peak height, without showing any loss of peak symmetry. In the proposed experimental design, lack of fit was no significant ($p > 0.05$) at a confident level of 95.0%. The obtained optimum values from the surface response were 50 mM phosphate for LCB concentration, 100 mM phosphate for HCB concentration and 190 s for HCB injection time, which involved filling the capillary 31.5% of its total length. The influence of LCB and HCB

concentrations on R.F. was found to be significant at a confidence level of 95% (p-value = 0.0030 and p-value = 0.0163, respectively) as well as quadratic HCB concentration interaction (p-value = 0.0344). Estimated response surfaces for the proposed R.F. are shown in **Figure 5.5**.

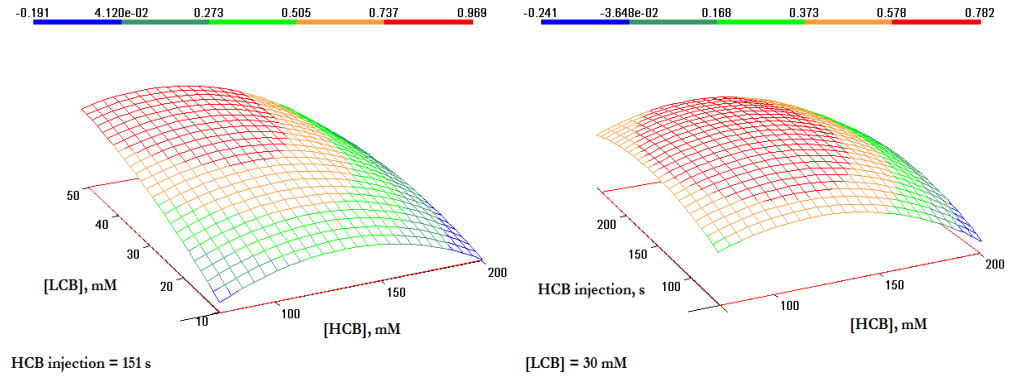

Figure 5.5. Estimated response surfaces for CSEI chemical parameters.

Instrumental parameters for FESI performance

A central composite design was also considered for the optimization of voltage and injection time. Variables were continuously studied between 5 and 15 kV for injection voltage and between 5 and 15 min for injection time. **Equation 5.3** was established as R.F. in order to improve CRZ and MNZ peak signals. For that reason, CRZ and MNZ normalized peak height ($H_{norm.}$) were proposed as R.F. terms. Because of shapeless peaks were shown under some of the employed injection conditions, a term that represents the number of symmetrical peaks ($n_{gaussians\ integrable\ peaks}$) was also included in the R.F. The number of analytes ($n_{analytes}$) studied in this experimental design was six.

$$R.F. = \lfloor H_{norm.}^{CRZ} + H_{norm.}^{MNZ} \rfloor \times \frac{n_{gaussians\ integrable\ peaks}}{n_{analytes}} \quad \textbf{Equation 5.3.}$$

Lack-of-fit was no significant at a confidence level of 95.0%. The maximum of the response surface corresponds to an injection voltage of 9.8 kV and an injection time of 10.53 min. The response surface for the described experimental design can be found in **Figure 5.6**.

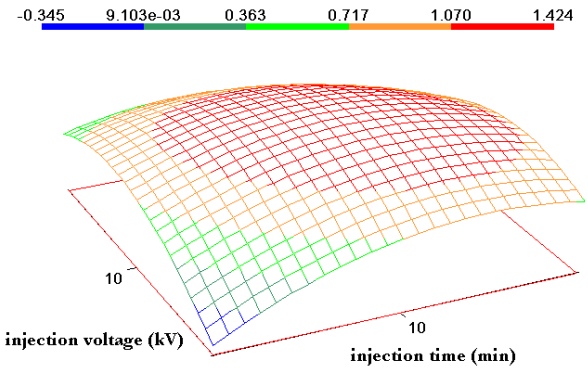

Figure 5.6. Estimated response surface for voltage and injection time associated to FESI.

Injection solvent evaluation

Low-conductivity samples benefit analyte injection into the capillary by FESI, resulting in an important signal enhancement. However, the presence of a certain amount of protons in sample matrices is needed for obtaining charged analytes and consequently achieving a satisfactory injection by FESI. The required proton concentration is determined by analyte pK_a values. Because of that, H_3PO_4 solution was proposed as injection media. An organic solvent was added to the injection solvent in order to decrease its conductivity. MeCN, MeOH and THF at concentrations of 10% (v/v) were evaluated. Although an improvement of peak signals was expected, results did not show this behavior. This could be due to the increase of injection solution viscosity when an organic solvent is added, involving lower analyte mobility and, consequently a lower amount of injected analytes, resulting in lower peak signals. Nevertheless, the addition of organic additives was considered in order to guarantee its low conductivity, and therefore, a satisfactory sample injection as well as better peak symmetries.

Better peak symmetries were observed when MeOH was employed (**Figure 5.7**), and besides, the use of MeCN or THF as organic modifiers was discarded due to the presence of unknown peaks in the obtained electropherograms. Finally, MeOH percentage in the injection solvent was evaluated in a range between 2 and 20% (v/v). Slight differences were observed in these experiments, so 5% (v/v) was considered as optimum in order to avoid a high solution viscosity.

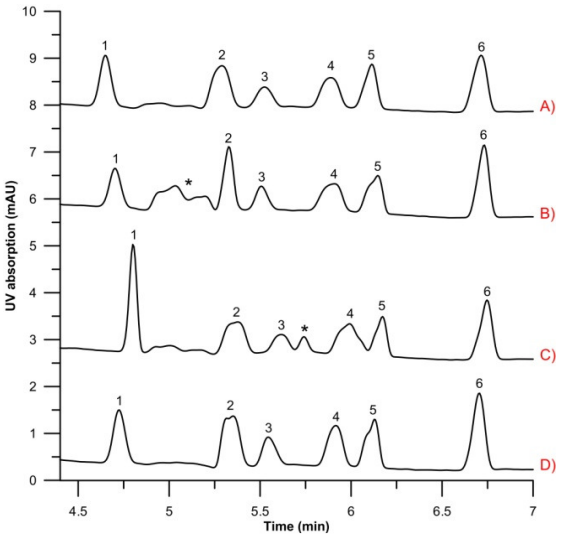

Figure 5.7. **Evaluation of injection solvent composition. A) 3 mM H_3PO_4 aqueous solution containing 10% (v/v) of MeOH; B) 3 mM H_3PO_4 aqueous solution containing 10% (v/v) of MeCN; C) 3 mM H_3PO_4 aqueous solution containing 10% (v/v) of THF; D) 3 mM H_3PO_4 aqueous solution. 5-NDZ separations were performed in a fused-silica capillary (48.5 cm × 50 μm i.d., 40 cm of effective length) at -30 kV and 20°C, using a BGS consisted of 44 mM of phosphate buffer (pH 2.5) containing 123 mM SDS and 8% (v/v) THF. Separations were monitored at 276 nm. Samples were electrokinetically injected following the proposed FESI injection. Peaks: 1, CRZ; 2, IPZ; 3, ORZ; 4, SCZ; 5, TRZ; 6, MNZ; *, Unknown peaks.**

Acid concentration in the injection solvent was studied in a range between 1 and 10 mM. Signals did not show significant differences when acid concentration was higher than 5 mM. At lower concentrations, peak signal depended on each analyte, observing poor staking effect for IPZ peak. Furthermore, low reproducibility was obtained when low concentration of H_3PO_4 was considered in the injection solvent. Finally, a concentration of 5 mM was chosen as optimum because higher concentrations could result in high conductivity samples.

In order to obtain good sample injection reproducibility, the nature of the solution placed in the outlet vial during sample injection was further evaluated. Phosphate buffers (pH 2.5) of different concentration were assayed. Better reproducibility was showed when HCB (100 mM phosphate buffer, pH 2.5) was used as the solution contained into the outlet vial during sample injection.

Figure 5.8 shows an electropherogram of standard samples analyzed under the proposed CSEI-sweeping-MEKC method considering the optimum conditions.

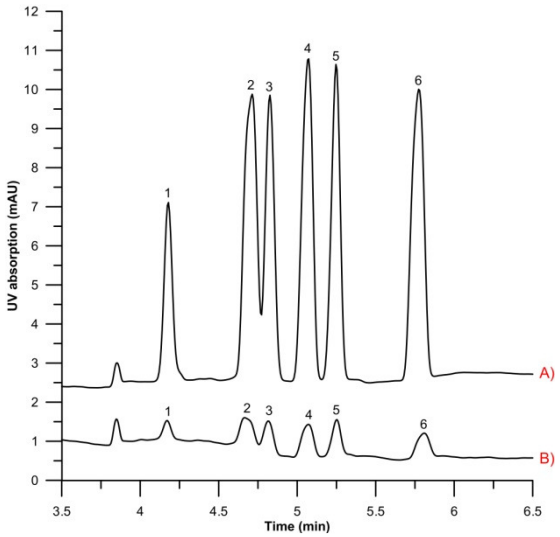

Figure 5.8. Electropherograms of standard solutions analyzed by the proposed CSEμ-sweeping-MEKC-UV method. A) 75 μg/L of each 5-NDZ; B) 10 μg/L of each 5-NDZ. 5-NDZ separations were performed in an extended light-path capillary (48.5 cm × 50 μm i.d., 40 cm of effective length, and 150 μm of optical path length) at -30 kV and 20°C, using a BGS consisted of 44 mM of phosphate buffer (pH 2.5) containing 123 mM SDS and 8% (v/v) THF. Separations were monitored at 276 nm. Samples were electrokinetically injected following the proposed CSEμ-sweeping conditions (**Section 5.2.4**). Peaks: 1, CRZ; 2, μPZ; 3, ORZ; 4, SCZ; 5, TRZ; 6, MNZ.

Furthermore, SEFs were estimated by the comparison of the peak height of each 5-NDZ achieved by the proposed CSEI-sweeping-MEKC-UV method with the peak height resulted from the analysis of a standard solution by the MEKC-UV method developed in **Chapter 4** considering a normal injection of 5 s, and multiplied by the dilution factor [60,61]. The SEF obtained for each evaluated 5-NDZ are indicated on **Table 5.1**.

Table 5.1. SEF for each 5-NDZ obtained by the developed CSEI-sweeping-MEKC-UV method.

Analyte	Sample concentration (μg/L) analyzed by the proposed CSEI-sweeping-MEKC method	Sample concentration (μg/L) analyzed by MEKC-UV considering a normal injection of 5 s	SEF*
CRZ	10	3000	262
IPZ	10	3000	178
ORZ	10	3000	189
SCZ	10	3000	102
TRZ	10	3000	215
MNZ	10	3000	73

$$*SEF = \frac{Peak\ height\ with\ CSEI-sweeping-MEKC}{Peak\ height\ with\ MEKC\ considering\ a\ normal\ injection} \times dilution\ factor$$ **Equation 5.4.**

5.3.2. Application of DLLME to the determination of 5-NDZs in natural water samples

For the first time DLLME is proposed as sample treatment coupled to CSEI-sweeping-MEKC. In this chapter, DLLME previously optimized in **Chapter 4,** was applied to water samples collected from different sources. As it is usual, separation method was optimized employing analyte standard solutions. However, during method characterization with fortified samples, slight variations were observed for 5-NDZ migration times. Lower peak resolution was shown among the analytes in water samples after DLLME treatment, so a longer capillary was used for achieving 5-NDZ baseline separation. A capillary of 57.2 cm of total length was finally employed for method characterization on real water samples. Considering that, capillary was filled with HCB solution for 264 s at 50 mbar during capillary conditioning stage in order to reach the same experimental conditions (31.5% of the total capillary length filled with HCB) that those proposed for this CSEI-sweeping-MEKC strategy in standard solutions.

ORZ determination in water samples was not possible due to the presence of matrix interference at the same migration time. However, the optimized injection solvent composition (3 mM H_3PO_4 aqueous solution containing 10% (v/v) of MeOH) compared to preliminary conditions (3 mM H_3PO_4 aqueous solution) allowed the determination of TNZ, that was not previously included in this study. TNZ presents the lowest pK_a of all studied 5-NDZ compounds, being injected under the actual injection conditions, although it was not injected under the initial established conditions.

Calibration curves and performance characteristics

Matrix-matched calibration curves for the studied analytes (CRZ, IPZ, SCZ, TRZ, MNZ and TNZ) were established using river water samples collected from Riofrío River (Granada, Spain). It was considered as the representative matrix to characterize the present method. River water samples were spiked at 2.0, 4.0, 8.0, 14.0 and 20.0 μg/L for all 5-NDZ compounds except for TNZ that was injected at 4.0, 8.0, 16.0, 28.1 and 40.1 μg/L). Five aqueous samples were spiked at the same concentration level. They were

processed following the previously described procedure, injected and analyzed according to the developed CSEI-sweeping-MEKC method. Peak area was considered as a function of analyte concentration on the sample. A blank sample was also treated and no interferences were co-migrating with any 5-NDZ peak.

Statistical parameters calculated by least-square regression and the performance characteristics of the DLLME-CSEI-sweeping-MEKC-UV method for water samples are shown on **Table 5.2**. LODs and LOQs were calculated as the minimum analyte concentration yielding a signal-to-noise ratio equal to three and ten, respectively, as well as LODs were also estimated by Long and Winefordner [62] and Clayton criteria [63]. LOQs were calculated as 10 × S/N. Despite that the off-line preconcentration factor due to DLLME was only of 4.2-fold, all 5-NDZ compounds were able to be quantified at the low μg/L levels using the proposed method, although poor sensitivity is attributed to CE-UV methods.

Precision assays

Method precision was evaluated in terms of repeatability (intra-day precision) and intermediate precision (inter-day precision) by the application of the proposed method to water samples collected from Riofrío River and spiked at three different concentration levels (4.0, 8.0 and 20.0 μg/L) of each analyte (CRZ, IPZ, SCZ, TRZ and MNZ) except for TNZ (8.0, 16.0 and 40.1 μg/L).

Repeatability was evaluated by analyzing seven samples (experimental replicates) per concentration level while intermediate precision was assessed by analyzing one sample per concentration each day for five consecutive days. Instrumental replicates of each sample were not considered because FESI involves an exhaustive injection and an important depletion of the analytes is produced in the vial as a consequence of a single injection [45]. The obtained results, expressed as RSD (%) of peak areas, are summarized in **Table 5.3**. Satisfactory results were obtained in terms of precision, being RSD (%) lower than 10% in almost all assayed 5-NDZ concentrations.

Table 5.2. Statistical and performance characteristics of the proposed CSEI-MEKC-sweeping-UV method for 5-NDZ determination in river water samples.

Analyte	Linear range (µg/L)	R^2	Linear regression equation (y=m·x+a)				LOD (µg/L)			LOQ (µg/L)
			Slope	*SD for the slope*	*Intercept*	*SD for the intercept*	*3 × S/N*	*Long*	*Clayton*	*10 × S/N*
CRZ	4.4 – 20.0	0.980	0.25	0.01	-0.26	0.09	1.3	1.0	2.2	4.4
IPZ	2.1 – 20.0	0.998	0.39	0.00	-0.10	0.05	0.6	0.2	0.6	2.1
SCZ	4.6 – 20.0	0.995	0.24	0.00	-0.21	0.04	1.4	0.4	1.0	4.6
TRZ	2.1 – 20.0	0.995	0.27	0.00	-0.04	0.05	0.6	0.4	1.1	2.1
MNZ	2.8 – 20.0	0.985	0.33	0.01	-0.12	0.10	0.8	0.7	1.7	2.8
TNZ	8.1 – 40.1	0.980	0.07	0.00	0.04	0.05	2.4	1.9	4.3	8.1

Table 5.3. Precision studies for the proposed method for 5-NDZ determination in river water samples.

Analyte	Repeatability (% RSD, n = 7)			Intermediate precision (% RSD, n = 5)		
	4.0 µg/L	***8.0 µg/L***	***20.0 µg/L***	***4.0 µg/L***	***8.0 µg/L***	***20.0 µg/L***
CRZ	7.6	8.1	6.4	23.6	8.1	9.9
IPZ	15.9	11.3	4.3	24.3	3.2	8.4
SCZ	15.5	6.5	7.6	11.4	10.9	7.9
TRZ	4.9	7.7	6.9	10.7	3.8	5.0
MNZ	4.3	7.1	7.3	6.9	6.5	4.9
	8.0 µg/L	***16.0 µg/L***	***40.1 µg/L***	***8.0 µg/L***	***16.0 µg/L***	***40.1 µg/L***
TNZ	3.9	7.0	9.8	4.1	3.8	5.6

Figure 5.9 shows an electropherogram resulted from the analysis of a river water sample spiked at 14.0 µg/L of each 5-NDZ compound except for TNZ (28.1 µg/L). Moreover, the observed electrophoretic current during 5-NDZ separation is also shown.

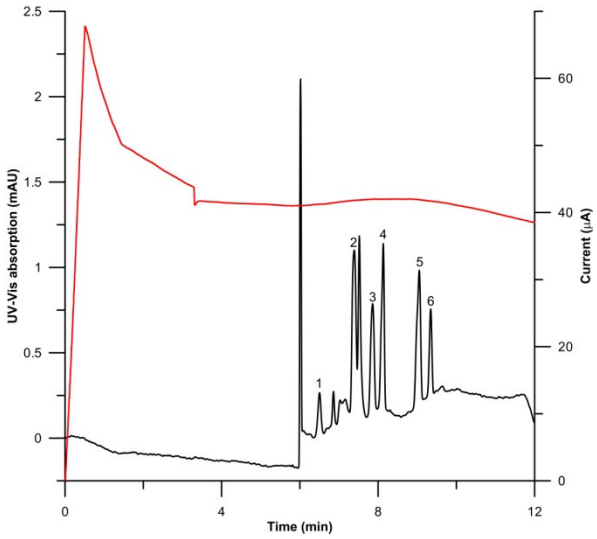

Figure 5.9. Electropherogram of a river water sample fortified at 14 µg/L of each compound, except for TNZ (28.1 µg/L), treated and analyzed by the proposed DLLME-CSEI-sweeping-MEKC-UV method. 5-NDZ separation was performed in an extended light-path capillary (57.2 cm × 50 µm i.d., 48.7 cm of effective length, and 150 µm of optical path length) under -30 kV and 20°C, using a BGS consisted of 44 mM of phosphate buffer (pH 2.5) containing 123 mM SDS and 8% (v/v) THF. Separation was monitored at 276 nm. Samples were electrokinetically injected following the proposed CESI-sweeping conditions (**Section 5.2.4**). Peaks: 1, CRZ; 2, IPZ; 3, SCZ; 4, TRZ; 5, MNZ; 6, TNZ. Separation current is also shown (red line).

Trueness

Trueness assays were carried out over different spiked water samples. Water samples from Riofrío River (Granada, Spain), Genil River (Granada, Spain) and well water samples (Badajoz, Spain) were evaluated. Water samples were spiked at three different concentration levels (4.0, 8.0 and 20.0 μg/L) of each analyte (CRZ, IPZ, SCZ, TRZ and MNZ) except for TNZ (8.0, 16.0 and 40.1 μg/L). Seven samples from Riofrío River were fortified at each concentration level, treated and analyzed following the proposed DLLME-CSEI-sweeping-MEKC-UV method. In the case of Genil River water and well water samples, five samples were assessed per each concentration level. A blank of each type of water was processed, and an interfering peak associated to the matrix was co-migrating with CRZ for Genil River water and well water samples. As a consequence, recovery values could not be established for CRZ in these matrices. For the rest of 5-NDZ drugs, the obtained results are shown on **Table 5.4**. In general, recoveries over 70% were achieved.

Table 5.4. **Recovery studies for each 5-NDZ compound in different water samples analyzed by the proposed DLLME-CSEI-sweeping-MEKC-UV method.**

Analyte	Riofrío River water samples (%, n = 7)			Genil River water samples (%, n = 5)			Well water samples (%, n = 5)		
	4.0 μg/L	***8.0 μg/L***	***20.0 μg/L***	***4.0 μg/L***	***8.0 μg/L***	***20.0 μg/L***	***4.0 μg/L***	***8.0 μg/L***	***20.0 μg/L***
CRZ	83.3	73.0	82.8		N/A			N/A	
IPZ	87.9	95.1	93.2	72.0	86.1	73.1	66.0	68.5	63.7
SCZ	68.6	73.6	97.2	78.9	91.3	82.0	84.9	83.1	74.0
TRZ	85.3	96.9	93.8	90.5	76.7	76.9	74.0	72.9	69.3
MNZ	76.9	83.8	72.0	80.3	81.5	74.6	68.7	60.9	67.3
	8.0 μg/L	***16.0 μg/L***	***40.1 μg/L***	***8.0 μg/L***	***16.0 μg/L***	***40.1 μg/L***	***8.0 μg/L***	***16.0 μg/L***	***40.1 μg/L***
TNZ	85.1	91.8	100.0	87.6	87.1	88.1	81.9	73.2	79.3

Not applicable (N/A) due to the presence of a co-migrating matrix interference

5.3.3. Determination of 5-NDZ compounds in diluted urine and serum samples

MNZ, ORZ and TNZ determination in untreated human urine and serum was also assayed by the developed CSEI-sweeping-MEKC-UV method. 5-NDZ preconcentration and separation were initially evaluated considering the optimized method characteristics. However, the presence of matrix compounds that co-migrated with 5-NDZ peaks was observed when the proposed procedure was applied to these complex matrices. Therefore, it was necessary to re-optimize some of the parameters involved in the separation. Preliminary studies showed that buffer composition and capillary length had influence on 5-NDZ separation in these new matrices. However, 5-NDZ separation was not influenced by the separation voltage because, when separation voltage was decreased from its established value (30 kV, negative polarity). Only an increase on analysis time was observed at lower voltages without achieving any improvement on peak resolution.

From optimized conditions, separation buffer composition (44 mM phosphate buffer, pH 2.5, 123 mM SDS and 8% (v/v) THF) was checked, but it was not modified. On the other hand, capillary length was increased from 48.5 cm to 65.0 cm, achieving baseline peak resolution among 5-NDZ drugs and matrix endogenous compounds. The rest of parameters involved in the separation were kept as before, and 5-NDZs were separated under a voltage of -30 kV and 20°C.

Due to the fact that capillary length was increased, capillary conditioning procedure between runs was readjusted. Finally, capillary was subsequently rinsed with 0.1 M NaOH solution for 2 min, ultrapure water for 0.5 min and LCB (50 mM phosphate buffer, pH 2.5) for 3 min at 3 bar and 20°C. Afterwards a plug of HCB solution (100 mM phosphate buffer, pH 2.5) was injected into the capillary at 50 mbar for 341 s instead of 190 s as it was previously proposed. Under these conditions, 31.5% of capillary volume was filled with HCB solution as was established during method optimization. The rest of CSEI-sweeping-MEKC stages were kept as before. As a consequence, a water plug was injected (2 s at 50 mbar) prior to sample injection in order to obtain better injection reproducibility. Then samples were injected in a low conductivity solvent (5 mM H_3PO_4

solution containing 5% (v/v) of MeOH) at 9.8 kV (positive mode) for 632 s. Finally, MEKC separation was performed.

Low-conductivity injection solvents benefit FESI injection, and for that reason some inconveniences were shown when direct injection of urine and serum samples was intended because these samples possess a high content of salt. In order to decrease sample conductivity, dilution of both samples with injection solvent was carefully evaluated. Dilution factors were established as a compromise between sensitivity in terms of peak height and a satisfactory FESI injection. A dilution factor of 143-fold was considered for urine samples while a dilution factor of 76-fold was applied to deproteinized serum samples. Both factors represented the minimum dilution required for performing a stable sample injection and achieving a satisfactory 5-NDZ separation (without current drops during the injection or the separation) and which let to obtain Gaussian peaks. Although FESI injection was successfully carried out, unshaped peaks were obtained if lower dilution factors were considered.

Finally, analytical method was characterized in terms of linearity, LODs and LOQs, precision, and trueness, considering that FESI does not allow analyzing a sample twice because as was mentioned before, it involves an exhaustive sample injection and an important depletion is produced in the vial after a single injection [45]. Therefore, samples were injected once and only experimental replicates were considered through the method characterization.

Calibration curves and performance characteristics

Matrix-matched calibration curves were established in urine and serum samples at six concentration levels that were selected according to the sensitivity of each 5-NDZ in terms of peak height. Four samples of each matrix were spiked at each concentration level, treated and analyzed according to the proposed CSEI-sweeping-MEKC-UV method. Peak area was considered as function of analyte concentration on the sample. Blank urine and serum samples were also evaluated and no matrix endogenous interfering peaks were detected at 5-NDZ migration times.

LODs and LOQs, calculated as the minimum analyte concentration yielding a signal-to-noise ratio equal to three and ten, respectively, are shown on **Table 5.5** where it is also shown the LODs estimated by Long and Winefordner [62] and Clayton criteria [63]. According to the results, ORZ, MNZ and TNZ can be quantified at low μg/mL levels in both biological fluids by the proposed method. Therapeutic concentrations of 5-NDZ in serum and urine are usually determined at these levels [2,3], so the proposed method accomplishes with the needs for monitoring 5-NDZ drugs in this kind of matrices. Furthermore, the optimized method is also valid for pharmacokinetic studies via elaboration of absorption/elimination curves for the considered drugs, because obtained LOQs are lower than the typical 5-NDZ concentration levels in these matrices.

In order to check the influence of the matrix on the calibration curves, slope comparison tests between matrix-matched and external calibration curves were applied. Statistical and performance characteristics of related external standard calibration curves are indicated in **Table 5.6**. For slope comparison tests, matrix-matched calibration curves shown on **Table 5.5** were scaled to external standard calibration curve units taking into account sample dilution factors. No significant difference between the slopes of matrix-matched and external standard calibration curves was observed, at a confident level of 95%, for ORZ in urine samples. However, significant differences were found for the rest of analytes in both, urine and serum samples.

Precision assays

Precision studies were carried out to evaluate for the evaluation of method repeatability and intermediate precision. Repeatability was tested over nine samples (experimental replicates), while intermediate precision was studied by analyzing one sample per day for five consecutive days. **Table 5.7** shows precision study results in terms of RSD (%) of peak areas, which were lower than 9.3 and 14.5% for repeatability and intermediate precision, respectively. Furthermore, electropherograms of spiked urine and serum samples, and their corresponding blanks, are shown in **Figure 5.10,I** and **Figure 5.10,II**, respectively.

Table 5.5. Statistical and performance characteristics of the proposed CSEI-sweeping-MEKC-UV method for 5-NDZ determination in urine and serum samples.

Analyte	Linear range (µg/mL)	R^2	Linear regression equation (y=m·x+a)				LOD (µg/mL)			LOQ (µg/mL)
			Slope	*SD for the slope*	*Intercept*	*SD for the intercept*	*3 × S/N*	*Long*	*Clayton*	*10 × S/N*
Urine										
ORZ	1.7 – 14.6	0.988	0.122	0.003	0.228	0.025	0.5	0.6	1.2	1.7
MNZ	1.5 – 11.5	0.998	0.235	0.003	0.060	0.015	0.5	0.2	0.4	1.5
TNZ	6.0 – 14.6	0.991	0.047	0.001	0.030	0.010	1.8	0.6	1.1	6.0
Serum										
ORZ	2.0 – 7.5	0.989	0.175	0.005	0.012	0.022	0.6	0.3	0.6	2.0
MNZ	0.8 – 7.5	0.996	0.418	0.007	-0.024	0.029	0.2	0.2	0.4	0.8
TNZ	5.1 – 20.0	0.997	0.065	0.001	0.002	0.010	1.5	0.4	0.8	5.1

Table 5.6. Statistical and performance characteristics of external standard calibration curves for the proposed CSEI-sweeping-MEKC-UV method.

Analyte	Linear range (µg/L)	R^2	Linear regression equation (y=m·x+a)				LOD (µg/L)			LOQ (µg/L)
			Slope	*SD for the slope*	*Intercept*	*SD for the intercept*	*3 × S/N*	*Long*	*Clayton*	*10 × S/N*
ORZ	15.2 – 100.0	0.987	0.019	0.001	0.050	0.035	4.6	3.7	7.9	15.2
MNZ	10.2 – 100.0	0.996	0.044	0.001	0.050	0.039	3.1	2.6	5.1	10.2
TNZ	35.4 – 100.0	0.997	0.008	0.000	0.021	0.010	10.6	2.5	4.6	35.4

Table 5.7. Precision studies of the proposed method for ORZ, MNZ, and TNZ determination in untreated urine and serum samples.

Analyte	Urine					
	Repeatability (% RSD, n = 9)			Intermediate precision (% RSD, n = 5)		
	3.1 µg/mL	***6.3 µg/mL***	***11.5 µg/mL***	***3.1 µg/mL***	***6.3 µg/mL***	***11.5 µg/mL***
ORZ	4.3	5.4	5.9	5.9	14.1	12.5
	1.5 µg/mL	***3.1 µg/mL***	***9.4 µg/mL***	***1.5 µg/mL***	***3.1 µg/mL***	***9.4 µg/mL***
MNZ	9.3	8.4	4.0	4.9	13.4	7.4
	6.3 µg/mL	***11.5 µg/mL***		***6.3 µg/mL***	***11.5 µg/mL***	
TNZ	9.0	2.5		10.6	12.0	
	Serum					
	Repeatability (% RSD, n = 9)			Intermediate precision (% RSD, n = 5)		
	3.0 µg/mL	***6.0 µg/mL***		***3.0 µg/mL***	***6.0 µg/mL***	
ORZ	8.0	7.5		14.5	14.3	
	1.5 µg/mL	***3.0 µg/mL***	***6.0 µg/mL***	***1.5 µg/mL***	***3.0 µg/mL***	***6.0 µg/mL***
MNZ	3.5	2.8	1.7	5.5	7.9	8.2
	8.0 µg/mL	***16.0 µg/mL***		***8.0 µg/mL***	***16.0 µg/mL***	
TNZ	4.2	3.8		7.4	7.7	

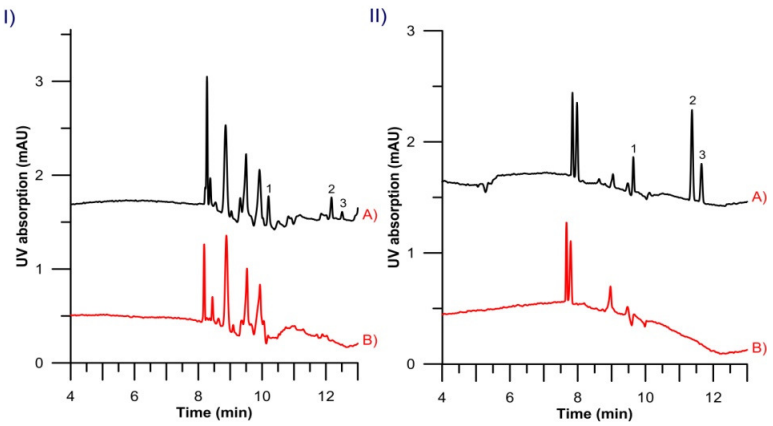

Figure 5.10. I) Electropherograms resulted from the analysis of urine samples: A) urine sample fortified at 6.3 µg/mL of ORZ and TNZ and at 3.1 µg/mL of MNZ; and B) a blank of urine sample. Urine samples were diluted 143-fold and analyzed according to the proposed CSEI-sweeping-MEKC-UV method. II) Electropherograms resulted from the analysis of serum samples: A) serum sample fortified at 6.0 µg/mL of ORZ and MNZ and at 16.0 µg/mL of TNZ. Serum samples were diluted 76-fold and analyzed according to the proposed CSEI-sweeping-MEKC-UV procedure. 5-NDZ separations were performed in an extended light-path capillary (65.0 cm × 50 µm i.d., 56.5 cm of effective length, and 150 µm of optical path length) at -30 kV and 20°C, using a BGS consisted of 44 mM of phosphate buffer (pH 2.5) containing 123 mM SDS and 8% (v/v) THF. Separation was monitored at 276 nm. Samples were electrokinetically injected following the proposed CSEI-sweeping conditions (**Section 5.2.4**). Peaks: 1, ORZ; 2, MNZ; 3, TNZ.

Trueness

Trueness was evaluated by means of recovery assays for each studied 5-NDZ at three different concentration levels in fortified human urine and serum samples (**Table 5.8**). For each concentration level, four different samples were analyzed. Found concentrations were estimated by the matrix-matched calibration curves and recovery values were calculated by comparison of the obtained value with the added concentration. In all cases satisfactory recoveries were obtained. Average recovery values for ORZ, MNZ and TNZ in urine were 101.8, 100.1 and 99.9%, respectively, whereas average recoveries in serum were 96.9, 101.7 and 101.1% for ORZ, MNZ and TNZ, respectively.

Table 5.8. **Recovery studies of the proposed method for ORZ, MNZ and TNZ determination in untreated urine and serum samples.**

Analyte	Urine		
	Concentration added (μg/mL)	**Concentration found (mean ± SD, μg/mL)**	**Recovery (%)**
ORZ	3.1	3.0 ± 0.5	96.8
	6.3	6.4 ± 0.6	101.9
	11.5	12.3 ± 0.4	106.7
MNZ	1.5	1.4 ± 0.1	97.0
	3.1	3.0 ± 0.2	97.2
	9.4	10.0 ± 0.5	106.2
TNZ	6.3	6.2 ± 0.4	99.3
	11.5	11.5 ± 0.5	99.6
	14.6	14.7 ± 0.4	100.9
	Serum		
	Concentration added (μg/mL)	**Concentration found (mean ± SD, μg/mL)**	**Recovery (%)**
ORZ	3.0	2.8 ± 0.1	94.2
	4.5	4.4 ± 0.3	98.5
	7.5	7.4 ± 0.5	98.1
MNZ	1.5	1.6 ± 0.0	105.1
	3.0	3.0 ± 0.1	99.6
	7.5	7.5 ± 0.2	100.4
TNZ	8.0	8.2 ± 0.5	102.0
	12.0	12.0 ± 0.6	99.7
	20.0	20.4 ± 0.8	101.7

5.3.4. Application of SPE to the determination of 5-NDZs in eggs and egg-based product samples

Additionally, the proposed CSEI-sweeping-MEKC-UV method was tested for the determination of IPZ, ORZ, SCZ, TRZ, MNZ and TNZ in eggs and egg-based products. Eggs and egg-based products are quite complex matrices and the determination of traces of organic analytes on them usually involves the application of clean-up procedures to the samples prior to their analysis. In the case of the proposed analytical method, a sample treatment was especially needed due to the complexity of the considered samples. As mentioned before, high conductivity samples can causes inconveniences when FESI injection is performed. Therefore a three-stage sample treatment was proposed for 5-NDZ determination in eggs and egg-based products.

5-NDZ extraction and sample clean-up procedure was initially evaluated using a pool of hen eggs samples, and the final characteristics of the proposed sample treatment are indicated in **Section 5.2.3**. In short, first stage of sample treatment consisted of protein precipitation that was caused by the addition of 4 mL of MeCN to 2.0 g of blended eggs, and subsequent sample centrifugation. Afterwards, a SALLE procedure was carried out by the addition of 0.8 g of NaCl to the supernatant collected after protein precipitation. As a consequence, MeCN was separated from the aqueous egg matrix and 5-NDZ compounds were extracted in the organic phase due to a salting-out effect. Finally, 3.4 mL of the organic phase was dried and re-dissolved in 2 mL of ultrapure water in order to submit the sample to a SPE procedure with Oasis®HLB (60 mg, 3 mL) cartridges.

In spite of the exhaustive clean-up procedure that was proposed, matrix inherent compounds co-migrating with 5-NDZ peaks were still observed when sample analyses were carried out. In order to solve this inconvenience, two strategies were followed. Initially, a capillary with longer effective length was considered as it was assessed for 5-NDZ determination in water, urine and serum samples. Finally, a capillary of 61.5 cm of effective length (70 cm total length × 50 cm i.d.) was selected, and therefore, capillary was rinsed with HCB solution for 395 s at 50 mbar during capillary conditioning prior to sample injection. Under these conditions, capillary was filled 31.5% of its volume as it was

established during method optimization. Although higher peak resolution was achieved among 5-NDZ peaks and interfering compounds, it was not enough, and the presence of an interfering compound from the egg matrix that co-migrated with ORZ peak was still observed. This unknown compound absorbed quite strongly at 276 nm, being the wavelength at which 5-NDZs present maximum UV absorption in acid media. On the other hand, the six considered 5-NDZs present quite strong absorption at 300 nm, while the absorption of the interference at 300 nm was almost negligible. Thus it was decided to perform the detection of all the 5-NDZ at 300 nm, avoiding the cited interference. **Figure 5.11** shows the electropherograms monitored at 276 nm and 300 nm for egg blank samples and samples spiked with the studied 5-NDZs. Slightly weaker signals were obtained for 5-NDZ when the detection wavelength was changed from 276 to 300 nm, but it was completely assumable due to the gain in selectivity, since the interfering compound was not detected anymore.

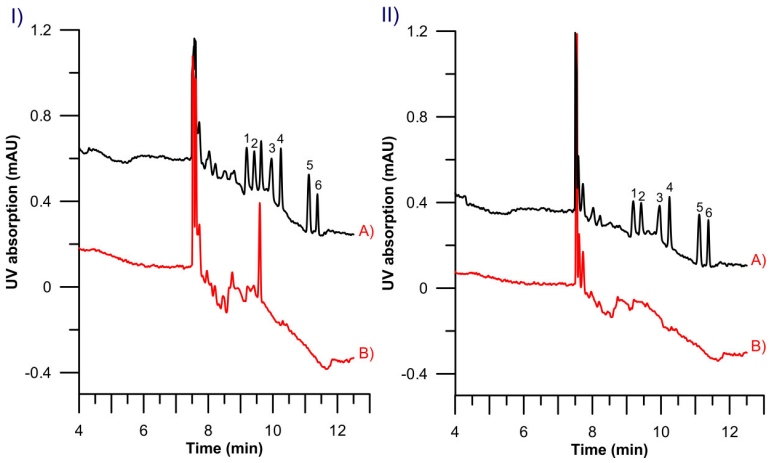

Figure 5.11. I) Electropherograms registered at 276 nm and obtained from the analysis of: A) a hen egg sample fortified at 20.0 µg/kg of each 5-NDZ, expect for TNZ (42.2 µg/kg); and B) a hen blank sample. II) Electropherograms registered at 300 nm and obtained from the analysis of: A) a hen egg sample fortified at 20.0 µg/kg of each 5-NDZ, expect for TNZ (42.2 µg/kg); and B) a hen blank sample. 5-NDZ separations were performed in a standard fused-silica capillary (70.0 cm × 50 µm i.d., 61.5 cm of effective length) at -30 kV and 20°C, using a BGS consisted of 44 mM of phosphate buffer (pH 2.5) containing 123 mM SDS and 8% (v/v) THF. Samples were electrokinetically injected following the proposed CSEI-sweeping conditions (**Section 5.2.4**). Peaks: 1, IPZ; 2, ORZ; 3, SCZ; 4, TRZ; 5, MNZ; 6, TNZ.

Calibration curves and performance characteristics

Matrix-matched calibration curves were carried out with samples from a pool of blended eggs spiked at five concentration levels that were submitted to the proposed SPE-CSEI-sweeping-MEKC-UV method. The obtained results, in terms of analytical figures of merit, are summarized in **Table 5.9**.

LODs and LOQs, calculated as the minimum analyte concentration yielding a signal-to-noise ratio equal to three and ten, respectively, are also shown on **Table 5.9**. LODs and LOQs ranged between 2.1 and 5.0 μg/kg and 7.4 and 16.8 μg/kg, respectively. TNZ presented the poorest sensitivity, which might be due to a less effective injection for this compound. However, reached LODs demonstrate the applicability of the developed method for the determination of 5-NDZs in egg samples. In **Table 5.9**, LODs estimated by Long and Winefordner [62] and Clayton criteria [63] are also included.

Precision assays

The precision of the method was evaluated in terms of repeatability (intra-day precision) and intermediate precision (inter-day precision) by applying the proposed method to hen eggs. Precision was checked at three different concentration levels for each analyte. The assayed levels were 10.0, 20.0 and 50.0 μg/kg for IPZ, ORZ, SCZ, TRZ and MNZ and 21.1, 42.2 and 106 μg/kg for TNZ.

Repeatability was evaluated by preparing nine experimental replicates at each concentration and analyzing them in the same day and under identical experimental conditions. Replicate injections were not performed due to the exhaustive character of the applied injection procedure. Intermediate precision was assessed during eight consecutive days at the same concentration levels as for the repeatability studies, by analyzing each day one sample of each concentration. The results, expressed as RSDs (%), are summarized in **Table 5.10**.

Table 5.9. Statistical and performance characteristics of the proposed CSEI-sweeping-MEKC-UV method for 5-NDZ determination in egg samples.

Analyte	Linear range (µg/kg)	R^2	Linear regression equation (y=m·x+a)				LOD (µg/kg)			LOQ (µg/kg)
			Slope	*SD for the slope*	*Intercept*	*SD for the intercept*	*3 × S/N*	*Long*	*Clayton*	*10 × S/N*
IPZ	14.7 – 50.0	0.986	-0.148	-0.050	0.050	0.002	4.4	2.0	3.9	14.7
ORZ	9.8 – 50.0	0.990	0.077	0.018	0.029	0.001	2.9	1.1	2.6	9.8
SCZ	11.4 – 50.0	0.994	0.044	0.019	0.046	0.001	3.4	0.8	1.9	11.4
TRZ	7.0 – 50.0	0.996	0.015	0.014	0.044	0.001	2.1	0.7	1.7	7.0
MNZ	7.4 – 50.0	0.994	0.006	0.029	0.062	0.001	2.2	1.3	2.9	7.4
TNZ	16.8 – 105.5	0.994	0.019	0.015	0.018	0.000	5.0	2.1	4.8	16.8

Table 5.10. Precision of the proposed method for the determination of 5-NDZs in hen egg samples.

Analyte	Repeatability (% RSD, n = 9)			Intermediate precision (% RSD, n = 8)		
	10.0 µg/kg	***20.0 µg/kg***	***50.0 µg/kg***	***10.0 µg/kg***	***20.0 µg/kg***	***50.0 µg/kg***
IPZ	-	17.4	9.1	-	13.7	17.9
ORZ	18.0	8.2	15.5	15.3	14.9	12.3
SCZ	10.7	9.5	9.2	15.8	8.2	14.2
TRZ	9.5	7.3	8.1	10.4	7.6	14.1
MNZ	8.8	7.2	6.0	9.5	9.1	11.3
	21.1 µg/kg	***42.2 µg/kg***	***106 µg/kg***	***21.1 µg/kg***	***42.2 µg/kg***	***106 µg/kg***
TNZ	8.0	5.0	6.6	6.3	6.9	9.9

Trueness

Trueness was checked by means of recovery assays. Recovery percentages of the studied 5-NDZs were firstly estimated in hen eggs. For this purpose, a calibration curve for each 5-NDZ at the same concentration ranges as detailed in **Table 5.9**, was established in treated aliquots of a pool of blended eggs by their fortification after they were treated according to the optimized sample treatment procedure. The recovery percentage for each analyte was calculated as the ratio between the slopes of the calibration curves obtained from samples spiked before and after the sample treatment, respectively. Average recoveries in hen eggs of 62.9, 101.0, 89.2, 95.1, 99.0 and 104.0% were calculated for IPZ, ORZ, SCZ, TRZ, MNZ and TNZ, respectively.

In order to complete and extend the trueness assay to other samples than hen eggs, recovery percentages were also calculated in quail eggs and a commercial pasteurized egg white product respectively. For these samples, recovery percentages were estimated at three different concentration levels, namely, 10.0, 20.0 and 50.0 µg/kg for TRZ and MNZ, 15.0, 30.0 and 45.0 µg/kg for IPZ, ORZ and SCZ, and 21.1, 42.2 and 106 µg/kg for TNZ. The obtained recoveries for each analyte in each matrix are summarized in **Table 5.11**. Low recoveries were obtained for IPZ, ranging between 51.9 and 70.2% and 25.8 and 31.1% in quail eggs and egg whites, respectively. For the rest of the assayed analytes, recovery percentages higher than 57% were achieved in both matrices.

Table 5.11. Recovery percentages for 5-NDZs in quail egg and commercial egg white samples.

Analyte	Recoveries (%), n = 4 (except for IPZ, n = 2)					
	Quail egg			Egg white-based product		
	15.0 µg/kg	***30.0 µg/kg***	***45.0 µg/kg***	***15.0 µg/kg***	***30.0 µg/kg***	***45.0 µg/kg***
IPZ	70.2	51.9	54.6	25.7	25.8	27.7
ORZ	112.0	104.8	106.1	71.9	57.9	75.1
SCZ	61.0	107.4	99.2	86.2	94.6	90.6
	10.0 µg/kg	***20.0 µg/kg***	***50.0 µg/kg***	***10.0 µg/kg***	***20.0 µg/kg***	***50.0 µg/kg***
TRZ	103.7	110.1	96.1	74.7	84.0	80.2
MNZ	88.5	101.6	96.5	91.9	86.2	82.1
	21.1 µg/kg	***42.2 µg/kg***	***106 µg/kg***	***21.1 µg/kg***	***42.2 µg/kg***	***106 µg/kg***
TNZ	98.4	105.6	108.3	107.0	93.5	90.6

Figure 5.12 shows the electropherograms corresponding to a blank sample of a commercial pasteurized egg white-based product and quail eggs, and the same samples spiked at 42.2 µg/kg of TNZ, 30 µg/kg of IPZ, SCZ and ORZ, and 20 µg/kg of MNZ and TRZ.

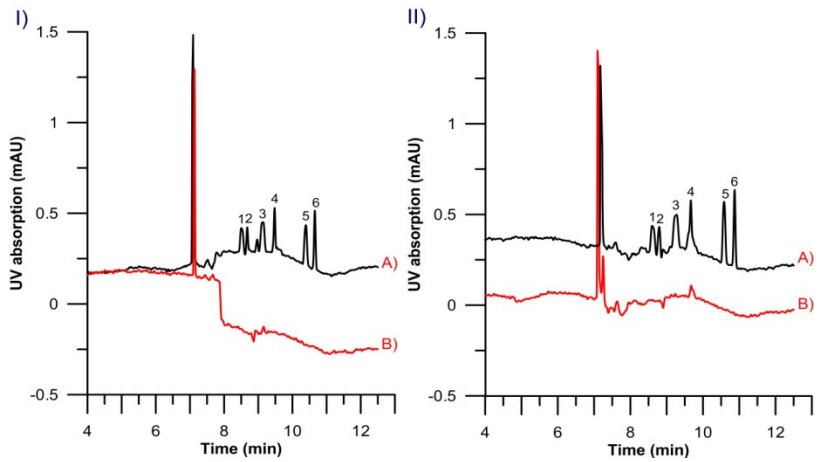

Figure 5.12. µ) Electropherograms resulted from the analysis of egg white-based product samples: A) white egg sample fortified at 20.0 µg/kg of MNZ and TNZ, 30.0 µg/kg of SCZ, µPZ and ORZ, and 42.2 µg/kg of TNZ; and B) a blank of white egg sample. µµ) Electropherograms resulted from the analysis of quail egg samples: A) quail egg sample fortified with 20.0 µg/kg of MNZ and TNZ, 30.0 µg/kg of SCZ, µPZ and ORZ, and 42.2 µg/kg of TNZ; and B) a blank of quail egg sample. 5-NDZ separations were performed in a standard fused-silica capillary (70.0 cm × 50 µm i.d., 61.5 cm of effective length) at -30 kV and 20°C, using a BGS consisting of 44 mM of phosphate buffer (pH 2.5) containing 123 mM of SDS and 8% (v/v) of THF. Samples were electrokinetically injected following the proposed CSEµ-sweeping conditions (**Section 5.2.4**). Peaks: 1, µPZ; 2, ORZ; 3, SCZ; 4, TRZ; 5, MNZ; 6, TNZ.

5.4. Conclusions

A novel CSEI-sweeping-MEKC was developed for 5-NDZ determination in environmental, clinical and food samples, demonstrating its suitability for the residue analysis in different fields. Several of the involved chemical and instrumental variables were optimized by chemometrics tools. SEFs between 73 and 262-fold were achieved for the proposed method in comparison with the MEKC method developed in **Chapter 4** considering a normal injection of 5 s.

For the first time, DLLME has been coupled to this CE-based method. DLLME is a miniaturized sample clean-up treatment with a low solvent consumption. Low conductivity samples were obtained after the DLLME procedure, accomplishing with the proposed CSEI-sweeping method requirements. Thus, DLLME showed to be adequate as sample treatment prior to CSEI-sweeping-MEKC analyses.

The developed DLLME-CSEI-sweeping-MEKC was successfully applied to the determination of 5-NDZ residues in river and well water samples. In spite of the low sensitivity attributed to CE-UV, the method results in a high on-line preconcentration factor, reaching detection limits lower than 2.44 μg/L for all the studied analytes. Satisfactory results were achieved for repeatability and intermediate precision studies, obtaining RSDs lower than 10% in most of the cases. The combination of DLLME with the evaluated CE technique supposes a cheap and green alternative for monitoring 5-NDZ residues in water samples.

On the other hand, the CSEI-sweeping-MEKC-UV method demonstrated to be suitable for 5-NDZ determination in diluted urine and serum samples. The selectivity and sensitivity of this automated technique allow 5-NDZ determination at therapeutic levels (μg/mL) in diluted urine and serum samples, avoiding tedious off-line sample treatments that also require the use of organic solvents. The satisfactory results obtained in terms of accuracy led to the conclusion that this is a good alternative for monitoring 5-NDZ drugs in biological fluids.

Finally, the optimized CSEI-sweeping-MEKC method was also applied to the determination of 5-NDZ residues in egg samples and egg-derived products, considering a SPE procedure as sample treatment prior to sample analyses. LODs at low μg/kg were achieved for all analytes due to the applied on-line preconcentration procedure, overcoming the lack of sensitivity attributed to CE-UV methods. Furthermore, a SPE protocol, using Oasis®HLB cartridges, was successfully implemented for sample clean-up, obtaining sample extracts which were compatible with the low conductivity samples required for the application of the CSEI-sweeping procedure.

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Simultaneous determination of 5-NDZs in urine samples by CE-ESI-MS/MS using molecular imprinted solid phase extraction

Abstract ► A novel CE-ESI-MS/MS approach has been proposed for the simultaneous determination of eleven 5-nitroimidazoles. A comparison between MEKC and CZE has been performed, obtaining higher selectivity when 1 M formic acid (pH 1.8) was selected as BGS instead of using a running solution containing a micellar pseudo-stationary phase (100 mM ammonium perfluorooctanoate (APFO), pH 9). Considering CZE as separation mode, 5-NDZs were hydrodynamically injected in water for 40 s at 50 mbar and separated at 28 kV and 25°C. In order to improve migration time reproducibility, a pressure of 50 mbar was applied to the inlet vial during the run without showing any loss of peak resolution. For sensitivity enhancement, ESI parameters were evaluated and established as follow: 6 L/min, dry gas flow rate; 7.4 psi, nebulization pressure; 160°C, dry gas temperature. CE-MS hyphenation was accomplished by means of a coaxial sheath-flow interface. Sheath liquid consisted of a mixture 2-propanol/water/acetic acid (60.0:38.8:0.2, v/v/v) and was supplied at a flow rate of 3.3 μL/min. Moreover, MS parameters were also optimized for analyte identification through their MS^2 and MS^3 spectra. The proposed method was applied to the determination of 5-NDZs in urine samples. For first time a MISPE protocol has been considered for 5-NDZ extraction from urine, resulting in recoveries higher than 79.2% while low matrix effect was observed. Obtained LODs ranged from 9.6 to 130.2 μg/L while precision assays resulted in RSDs for peak areas lower than 16.1%.

6.1. Background

The importance of 5-NDZ substances as human medicines has already been described in **Chapter 2**, and summarized in **Section 5.1**. As was mentioned, MNZ has been listed as one of the two essential antiamoebic and antigiardiasis medicines in the 'WHO Model List of Essential Medicines' [1]. MNZ undergoes hepatic metabolism, being mainly metabolized to MNZ-OH and an acetic acid compound. Metabolites are mostly excreted in the urine, while a small proportion of the unchanged drug is also excreted in the urine (about 10-15%) [2]. Similar metabolic and excretion characteristics have been reported for other 5-NDZ drugs, namely TNZ, ORZ and SCZ [3]. DMZ and RNZ are metabolized to the same metabolite, namely HMMNI, although both compounds follow different metabolism pathways [4]. Furthermore, pharmacokinetic studies carried out over patients treated with 200 mg of MNZ three times daily during seven days have shown drug levels in urine up to 1.6 μg/mL the twelfth day after the first administration day [5].

Traditionally, LC-MS has been the most employed technique for 5-NDZ analyses [6-9], although in less extension other techniques such as GC-MS [10,11] have also been considered. In spite of CE advantages, i.e. low solvent consumption, low sample volume requirement and high separation efficiency, this separation technique coupled to MS has not been explored for 5-NDZ identification and quantification yet. On the contrary, some approaches have been reported for 5-NDZ separation by CE, but always coupled with UV detection. 5-NDZs are neutral molecules in almost the entire pH range, being cationic species at low pH values. Therefore, they have to be separated by MEKC because CZE mode is only suitable if a very acid BGS is selected [12,13]. However, both CE modes present drawbacks when the use of MS detection is attempted as alternative to UV detection.

Long analysis times result from CZE separations performed at low pH because the EOF is minimized. Moreover, a slow EOF can also be problematic in CE-MS analysis because the transfer rate of analytes from the CE to the mass spectrometer depends on the magnitude of the EOF [14]. This inconvenience can be solved by the application of a pressure to the inlet vial when the analysis is running and even though peak resolution loss is associated to

it, this effect could not be so critical [15]. On the other hand, SDS as pseudo-stationary phase is a usual choice when MEKC separations are carried out. Nevertheless, this surfactant is not volatile and therefore micelles in the BGS solvent tend to soil the mass spectrometer and, as a consequence, lower sensitivity is obtained. In order to avoid this problem, partial filling strategies were firstly proposed [16,17], but in the last years the use of volatile surfactants has emerged as a robust alternative, allowing the direct MEKC-MS coupling [18,19]. Some recent applications involve the use of a volatile pseudo-stationary phase based on APFO [20-23].

Regarding sample treatment, the compatibility between the sample and the features of CE methods has been widely described as an important drawback for their implementation. Sample matrix inherent compounds can disturb CE separations through the action of saline constituents, macromolecules and other substances [24,25]. Moreover, low sensitivity is attributed to these methods due to the small sample volume that is injected in a CE analysis. In order to achieve sample clean-up and improve method sensitivity, SPE procedures have been proposed as suitable sample treatments prior to CE analyses. Several sorbents have been evaluated for SPE [26], being MIP an attractive alternative due to its selectivity. For 5-NDZ extraction, this approach has barely been evaluated and the proposed applications have been focused on food analysis [27,28].

In this chapter, a comparison between MEKC and CZE separation modes has been carried out in order to establish the best strategy for analyzing 5-NDZ drugs by CE-MS. MEKC separation was performed using APFO as pseudo-stationary phase while the application of pressure to the inlet separation vial was considered for CZE separation due to the benefits obtained for CZE-MS coupling. The optimization of both methods is widely discussed as well as the evaluation of ESI and MS parameters. According to the reviewed literature, it supposes the first proposal of a CE-MS method for 5-NDZ determination and quantification. Furthermore, the developed method was applied to the analysis of 5-NDZ antibiotics in urine samples. With the aim of avoiding matrix effects, a SPE procedure using molecular MIP cartridges was also assayed, being the first time that this procedure has been applied to a biological matrix such as urine.

6.2. Materials and methods

6.2.1. Materials and reagents

All reagents used through this work were analytical reagent grade and solvents were HPLC grade, unless otherwise specified. Ammonium hydroxide solution (30%, v/v), NaOH, toluene and heptane were obtained from Panreac-Química (Madrid, Spain). MeOH, MeCN, 2-propanol and hexane were purchased from VWR International (West Chester, PA, USA) while acetic acid (MS grade) and perfluorooctanoic acid (PFOA, 96%, v/v) were supplied by Sigma-Aldrich (St. Louis, MO, USA). Formic acid (98–100%, v/v) was acquired from Merck (Darmstadt, Germany). Ultrapure water (Milli-Q plus system, Millipore, Bedford, MA, USA) was used throughout this work. Ammonium acetate, ammonium formate and APFO solutions were prepared from acetic acid, formic acid and PFOA, respectively, adjusting the pH with a 5 M ammonium hydroxide solution.

Analytical standards of DMZ, RNZ, IPZ-OH, HMMNI, ORZ, MNZ, MNZ-OH and TNZ were supplied by Sigma-Aldrich (St. Louis, MO, USA) while IPZ, SCZ and TRZ were purchased from Witega (Berlin, Germany). Stock standard solutions were obtained by dissolving the appropriate amount of each 5-NDZ drug in MeCN, reaching a final concentration of 1 mg/mL. Stock standard solutions were kept in the freezer at -20°C avoiding exposure to light. Intermediate standard solutions were obtained by mixing the appropriate amount of each stock standard solution and their subsequent dilution with MeCN. The concentration of the analytes in intermediate standard solutions ranged between 50-100 μg/mL. They were stored in dark at 4°C and equilibrated to room temperature before their use. Working standard solutions were prepared by dilution of an intermediate standard solution with water to the desired 5-NDZ concentration.

SupelMIP®SPE-Nitroimidazole cartridges (50 mg, 3 mL) (Sigma Aldrich; St. Louis, MO, USA) were considered for the sample treatment procedure. Clearinert™ 13 mm syringe filters (0.22 μm pore size) were supplied by Bonna-Agela Technologies (Wilmington, DE, USA).

6.2.2. Instrumentation

CE experiments were carried out with an HP^{3D}CE instrument (Agilent Technologies, Waldbronn, Germany). An Agilent 1100 Series LC/MSD SL mass spectrometer equipped with an ion trap (IT) mass analyzer from Agilent Technologies (Waldbronn, Germany) was employed as detector. A coaxial sheath-flow interface (Agilent Technologies, Waldbronn, Germany) was selected for CE-MS hyphenation. A KD Scientific 100 series syringe pump (KD Scientific Inc., Holliston, MA, USA) was considered for supplying the sheath liquid. MS and MS/MS spectra and ion electropherograms were collected and processed by Esquire software 4.1 from Bruker Daltonics (Bremen, Germany)

MISPE treatment was carried out on a Visiprep™ DL vacuum manifold for 12 cartridges from Supelco (Bellefonte, PA, USA). A Universal 320R centrifuge (HettichZentrifugen, Tuttlingen, Germany), a nitrogen dryer EVA-EC System (VLM GmbH, Bielefeld, Germany), a mechanical shaker (model 384 from Vibromatic, Noblesville, USA) and a vortex-2 Genie (Scientific Industries, Bohemia, NY, USA) were also used. Solution pH was adjusted with a pH meter (Crison model pH 2000, Barcelona, Spain) with a resolution of ±0.01 pH unit.

6.2.3. Sample treatment procedure

Urine samples were supplied by a healthy volunteer. Aliquots of 5 mL were made taken and they were fortified at the desired 5-NDZ concentration. Prior to sample analyses, a sample clean-up procedure was performed using commercial MISPE cartridges. The extraction protocol recommended by the supplier [29], considering slight modifications, was followed through this work. MISPE cartridges were sequentially conditioned with 1 mL of toluene, 1 mL of MeCN and 1 mL of ammonium acetate buffer (10 mM, pH 5). Afterwards, 2 mL of spiked sample were passed through the extraction cartridge by gravity. Then, the column was washed considering four stages. First, 0.5 mL of deionized water were charged onto the cartridge, followed by loading twice 1 mL of hexane and 1 mL of a mixture 3:1 (v/v) heptane/toluene. Between washing steps, the cartridge was vacuum dried (-400 mbar) for 10 s. Finally, sample was eluted in two stages passing through the column

1 mL of 60:40 (v/v) MeCN/water solution containing acetic acid 0.5% (v/v) in each stage. Between elution stages, the cartridge was vacuum dried (-400 mbar) for 10 s. Both elution fractions were mixed and the eluted sample was dried under nitrogen current at 40°C. Sample was re-dissolved in 250 µL of deionized water using vortex agitation and filtered through a syringe filter to the vial for the CE-MS/MS analysis (**Figure 6.1**).

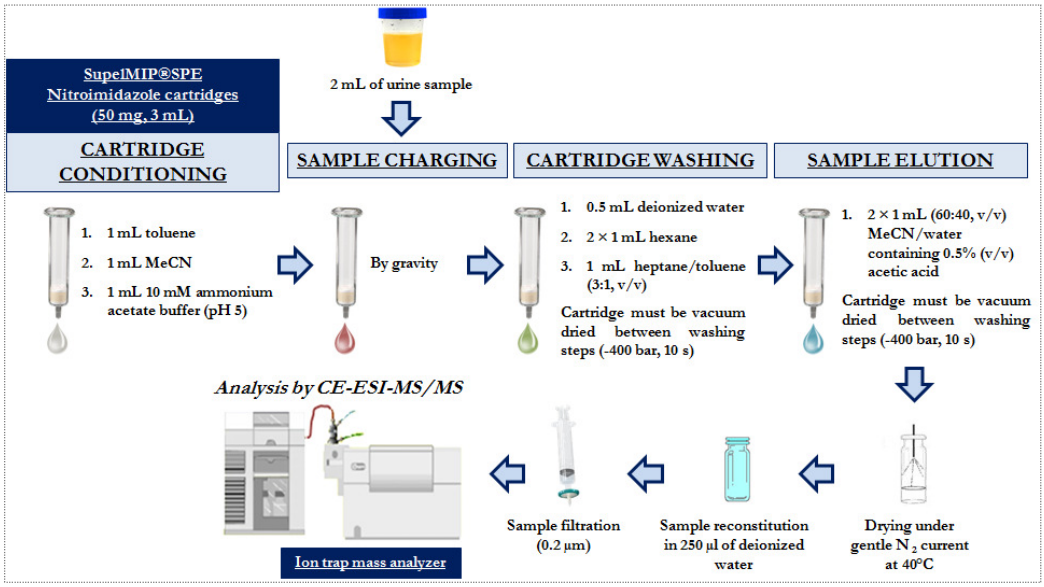

Figure 6.1. MISPE procedure for 5-NDZ determination in urine samples.

6.2.4. Capillary electrophoresis separation

CE experiments were carried out in a bare fused-silica capillary (110 cm of total length × 50 µm i.d. × 375 µm o.d.) from Polymicro Technologies (Phoenix, AZ, USA). APFO buffer (100 mM, pH 9) was employed as BGS for MEKC analyses and samples were hydrodynamically injected at 50 mbar for 10 s. Separation was performed at 25 kV and 25°C. On the other hand, 1 M formic acid solution (pH 1.8) was considered as BGS for CZE separation. A pressure of 50 mbar was applied to the inlet vial during the separation in order to improve migration time reproducibility. A voltage of 28 kV and a temperature of 25°C were applied for 5-NDZ separation. Samples were hydrodynamically injected at 50 mbar for 40 s when CZE analyses were carried out.

Before the first use, capillaries were flushed with 1 M NaOH solution for 10 min, followed by deionized water for 10 min and BGS for 20 min at 5 bar and 25°C. At the beginning of each session, capillary was subsequently rinsed with 5 M ammonium hydroxide solution for 5 min, deionized water for 5 min and running buffer for 10 min at 5 bar and 25°C. Between runs, capillary was conditioned with BGS for 2 min at 4 bar and 25°C. At the end of the working day, capillary was cleaned with deionized water for 5 min at 5 bar and 25°C and, afterwards, it was dried with compressed air for 5 min at 5 bar and 25°C.

6.2.5. Mass spectrometry and electrospray interface

Sheath liquid consisted of a mixture 60:38.8:0.2 (v/v/v) 2-propanol/water/acetic acid and was supplied at a flow rate of 3.3 μL/min. Compounds ionization was achieved in positive mode under ESI voltage of -4900 V. Other electrospray characteristics were established as follow: nebulizer pressure, 7.4 psi; dry gas flow rate, 6 L/min; and dry gas temperature, 160°C.

For MS experiments, IT parameters were evaluated using the ion charge control mode, setting a target of 100,000 ions, a maximum accumulation time of 300 ms and four averages per experiment. Scan range was established from 125.0 to 250.0 m/z. For MS/MS experiments, a target of 90,000 ions was selected while the maximum accumulation time was set to 100 ms and two averages per experiment were considered. Parent molecular ions ($[M+H]^+$) were fragmented by means of collision induced dissociation with the helium present in the trap for 40 ms in multiple reaction monitoring (MRM) mode. Product ions were scanned in the range of 35.0-252.0 m/z. MS/MS parameters are summarized on **Table 6.1**.

For method characterization, electrophoretic parameters such as retention time, peak height and peak area, and S/N, were acquired from the extracted ion chromatogram of each 5-NDZ.

Table 6.1. Mass spectrometer parameters according to the separation time range.

Segment time (min)	0.0 – 15.6	15.6 – 18.2	18.2 – 20.9	20.9 – 24.4	24.4 – 30.0
Detected analytes	DMZ	IPZ MNZ TRZ SCZ	ORZ HMMNI	IPZ-OH TNZ MNZ-OH	RNZ
Capillary (V)	-4900.0	-4695.5	-4900.0	-4740.4	-4900.0
Skimmer (V)	15.0	26.5	21.0	15.5	31.7
Cap Exit (V)	107.4	107.4	62.3	93.7	86.9
Oct 1DC (V)	7.4	8.8	8.4	7.3	8.5
Oct 2DC (V)	0.0	0.9	1.2	1.4	1.5
Trap Drive	33.3	32.3	32.6	32.4	31.2
Oct RF (Vpp)	91.0	69.5	60.9	50.0	82.8
Lens 1 (V)	-7.4	-4.7	-4.4	-3.3	-4.4
Lens 2 (V)	-100.0	-61.3	-51.3	-45.9	-48.4

6.3. Results and discussion

6.3.1. Electrophoretic separation

A comparison between MEKC and CZE was carried out in order to establish the best strategy for analyzing 5-NDZ drugs by CE-ESI-MS. MEKC separation was performed using APFO surfactant as pseudo-stationary phase while formic acid was selected as BGS for CZE separation. Both approaches were evaluated in a bare fused-silica capillary (110 cm × 50 μm i.d.), under a separation voltage of 25 kV and a separation temperature of 25°C. Additionally, samples were hydrodynamically injected for 10 s at 50 mbar.

First, MEKC separation was evaluated in terms of separation pH. Using a 75 mM APFO buffer as BGS, pH value was investigated between 8.5 and 10.0 and better peak resolution was observed when pH 9 was considered. Because 5-NDZs were not baseline resolved under these conditions, APFO concentration was further studied between 75 and 150 mM. Lower APFO concentrations were not considered because peak resolution is usually improved when micelle concentration is increased although it also tends to increase analysis time. As a compromise between peak resolution and analysis time, a concentration

of 100 mM was established as optimum (**Figure 6.2**). In order to improve 5-NDZ separation, the addition of 5% (v/v) of MeCN, MeOH and 2-propanol to the separation solution was assayed. However, no improvement in peak resolution was achieved when BGS modifiers were employed, so finally, a BGS consisted of 100 mM APFO buffer (pH 9) was established.

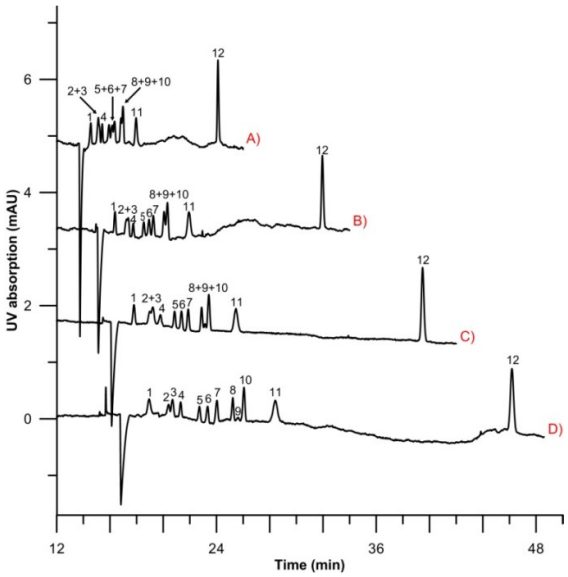

Figure 6.2. **Evaluation of APFO concentration in the BGS (pH 9): A) 75 mM; B) 100 mM; C) 125 mM; D) 150 mM. UV detection at 320 nm was used for this assay. MEKC-UV runs were performed in a fused-silica capillary (110 cm of effective length × 50 μm i.d.). Other experimental conditions: separation voltage, 25 kV; temperature, 25°C. Standard solutions (5 μg/mL of each 5-NDZ, except for IPZ, 10 μg/mL) were hydrodynamically injected for 10 s at 50 mbar. Peaks are numbered by elution order. Initially, CRZ was initially considered in the standard mixture but its determination was discarded in further assays.**

On the other hand, the evaluation of formic acid concentration in the BGS was firstly assessed for the CZE method. Formic acid concentration was studied between 0.1 and 2.0 M, but some 5-NDZ peaks were not baseline resolved under any of the tested conditions. Higher concentrations were not tested because a separation current of 40 μA was observed when 2.0 M formic acid was considered as BGS. Finally 1.0 M formic acid solution was established as BGS. Under this condition co-migrating peaks were not observed, but 5-NDZ separation was partially accomplished. At least two co-migrating peaks were observed under other separation conditions as shown in **Figure 6.3**.

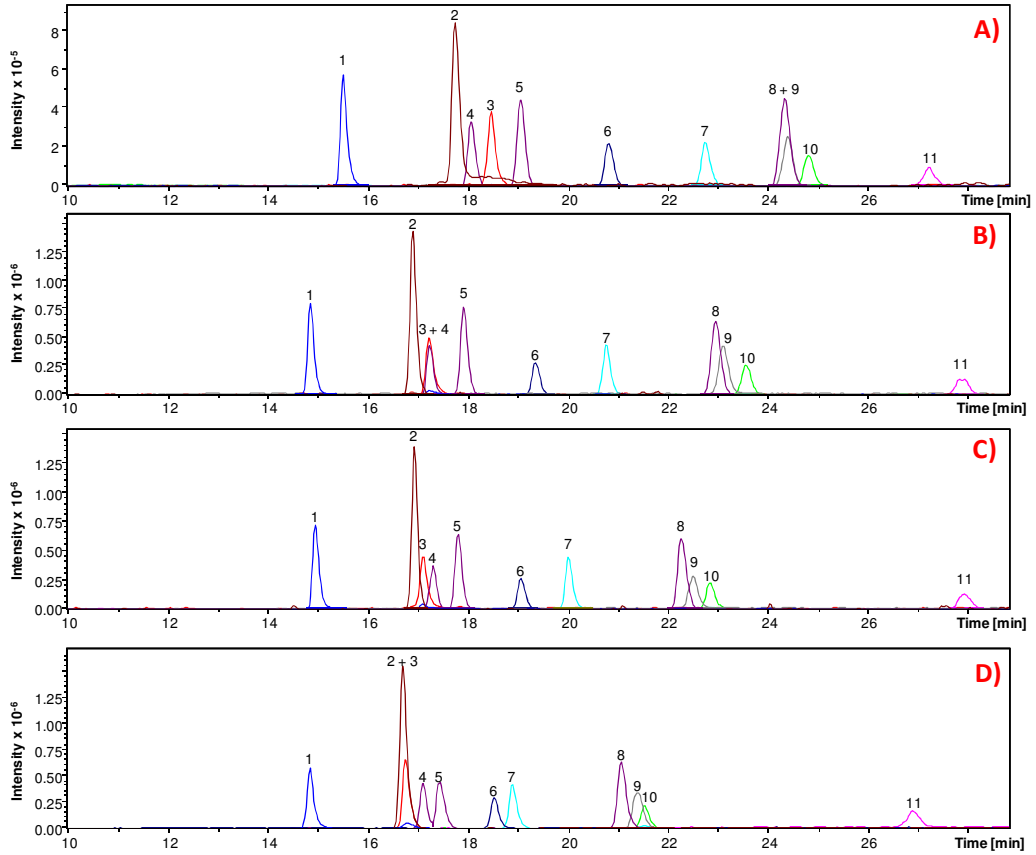

Figure 6.3. Evaluation of formic acid concentration in the BGS: A) 0.1 M; B) 0.5 M; C) 1 M; D) 2 M. CZE-MS runs were performed in a fused-silica capillary (110 cm × 50 μm i.d.). Other experimental conditions: separation voltage, 25 kV; temperature 25°C. Standard solutions (8 μg/mL of each 5-NDZ) were hydrodynamically injected for 10 s at 50 mbar. Peak identification: 1, DMZ; 2, IPZ; 3, MNZ; 4, TRZ; 5, SCZ; 6, ORZ; 7, HHMNI; 8, IPZ-OH; 9, TNZ; 10, MNZ-OH; 11, RNZ.

Additionally, separation pH was evaluated from 1.8 that corresponds to the 1.0 M formic acid solution pH, to 2.5. Solution pH was adjusted by the addition of 5 M ammonia solution to the 1.0 M formic acid solution. Higher pH values were not tested because separation current reached the limit of 50 μA when pH 2.5 was assessed. It was observed that an increase of the pH value caused an increase on the running time without achieving any improvement on peak resolution. As a consequence, 1.0 M formic acid solution was finally selected as BGS and no pH adjustment was considered. Because all analytes were not baseline resolved, the addition of an organic modifier to the separation media was proposed. A 10% of MeCN or MeOH was added to the BGS (1.0 M formic acid aqueous solution). As can be seen in **Figure 6.4**, peak resolution between IPZ, MNZ and TRZ was

improved when an organic modifier was added to the BGS but IPZ-OH, TNZ and MNZ-OH co-migrated under these conditions. Moreover, longer analysis times were obtained when an organic solvent was added to the BGS due to the increase of the separation solvent viscosity. Consequently the use of an organic modifier was discarded and 1.0 M formic acid (pH 1.8) was established as BGS for CZE separation.

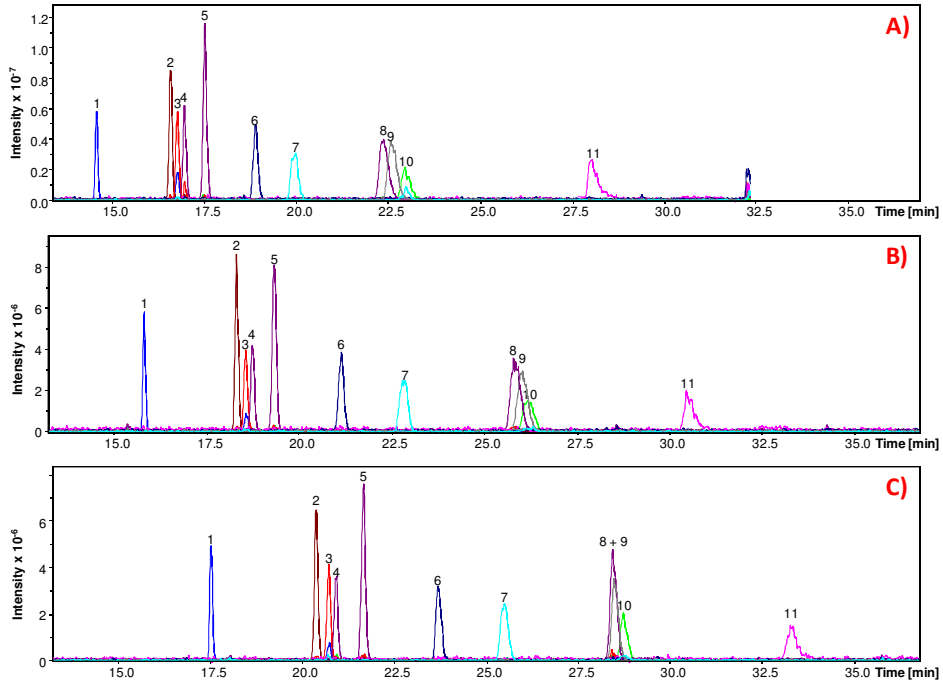

Figure 6.4. Evaluation of the addition of an organic modifier to the BGS (1.0 M formic acid, pH 1.8): A) without any modifier; B) 10% (v/v) MeCN; C) 10% (v/v) MeOH. CZE-MS runs were performed in a fused-silica capillary (110 cm × 50 µm i.d.). Other experimental conditions: separation voltage, 28 kV; temperature, 25°C. Standard solutions (2 µg/mL of DMZ and IPZ; 3 µg/mL of MNZ and TRZ; 5 µg/mL of SCZ, ORZ, HMMNI, IPZ-OH, TNZ, MNZ-OH and RNZ) were hydrodynamically injected for 40 s at 50 mbar. Peak identification: 1, DMZ; 2, IPZ; 3, MNZ; 4, TRZ; 5, SCZ; 6, ORZ; 7, HHMNI; 8, IPZ-OH; 9, TNZ; 10, MNZ-OH; 11, RNZ.

The application of pressure (50 mbar) to the inlet vial was considered during CZE separation in order to obtain better reproducibility in terms of migration times and for overcoming the lack of EOF under the established conditions. When pressure was applied during the separation, it was observed that migration time reproducibility, in terms of RSD (%), ranged from 0.5 to 1.2% when 54 runs were randomly performed during three

days. These values were doubled when separations were performed without applying any pressure to the inlet vial during the run.

Figure 6.5 shows the electropherograms resulted from the analysis of a 5-NDZ standard solution by MEKC and CZE. Considering peak resolution, higher selectivity was observed when CZE was considered as separation mode, although similar analysis time was achieved in both cases. Therefore, CZE was considered for further experiments.

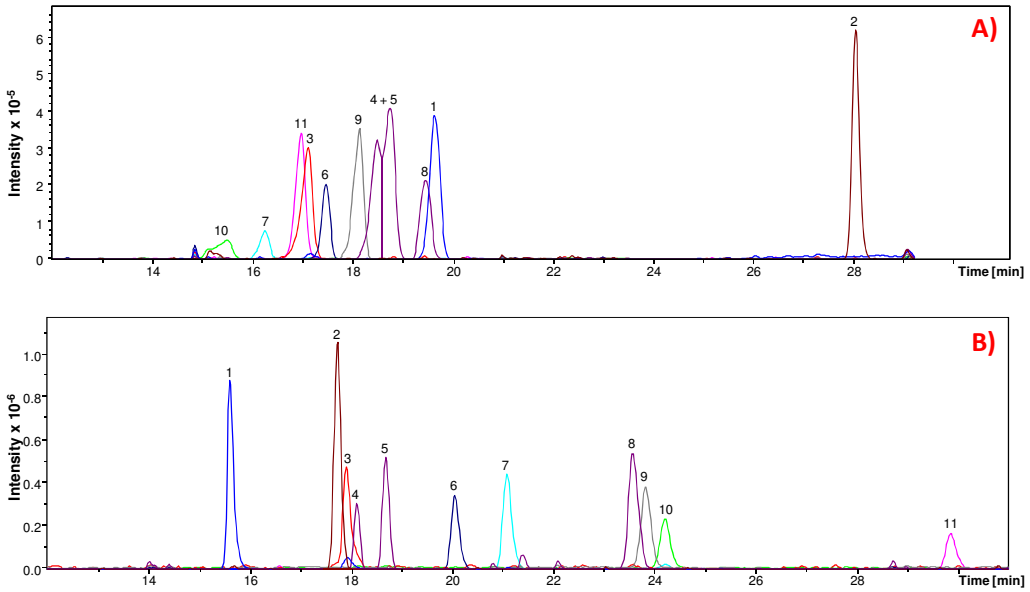

Figure 6.5. Comparison of 5-NDZ separation carried out by (A) MEKC-MS and (B) CZE-MS. BGS consisted of: A) 100 mM APFO (pH 9), B) 1 M formic acid (pH 1.8). Both separations were performed in a fused-silica capillary (110 cm × 50 µm i.d.). Other experimental conditions: separation voltage, 25 kV; temperature, 25°C. Standard solutions (8 µg/mL of each 5-NDZ) were hydrodynamically injected for 10 s at 50 mbar. Peak identification: 1, DMZ; 2, IPZ; 3, MNZ; 4, TRZ; 5, SCZ; 6, ORZ; 7, HHMNI; 8, IPZ-OH; 9, TNZ; 10, MNZ-OH; 11, RNZ.

Once separation mode was chosen, other parameters regarding electrophoretic separation were evaluated. Separation voltage was ranged between 20 and 30 kV, observing shorter analysis times at higher applied voltages, while peak resolution was not affected by this parameter. Because running time was not significantly decreased when separation voltage was increased from 28 to 30 kV, 28 kV was established as optimum. Moreover, capillary temperature was evaluated from 20 to 35°C, choosing 25°C as optimum. Higher analysis times were observed at lower temperatures while higher temperatures did not involve any

improvement on analysis time. Furthermore, peak resolution was not significantly affected by capillary temperature.

On the other hand, deionized water and BGS solution were evaluated as injection media considering a hydrodynamic sample injection for 10 s at 50 mbar. Standard solutions of 5 μg/mL of each 5-NDZ were prepared in each injection solvent and analyzed according to the proposed separation method. Similar results were obtained when both media were considered and no significant sensitivity differences, in terms of peak height, were observed. In this case, water was selected as injection medium. Additionally, injection time was assayed from 10 to 50 s. Maximum signal intensity was reached when 40 s was tested, so it was selected as optimum. Moreover, it should be remarked that higher injection times resulted in wider peaks, involving lower peak efficiencies.

6.3.2. CE-ESI-MS/MS optimization

Once separation conditions were selected, parameters related to CE-MS and CE-MS/MS were optimized. MS instrument was operated in ESI positive mode as it has been previously reported [30,31]. Other acquisition parameters such as scan range, accumulation time and ion accumulation target have been defined in **Section 6.2.5**. The selection of sheath-liquid parameters is also very important in CE-ESI-MS/MS methods. In order to achieve optimum signals, sheath liquid composition and flow rate, nebulizer pressure and dry gas flow rate and temperature were optimized by analyzing 5-NDZ standard solutions (5 μg/mL of HMMNI, IPZ-OH, ORZ, TNZ, MNZ-OH, RNZ and SCZ; 3 μg/mL of MNZ and TRZ; 2 μg/mL for IPZ and DMZ). S/N for each studied analyte was selected as response variable.

Sheath liquid composition and flow rate

Preliminary studies were carried out using a sheath liquid consisted of a mixture 70.0:29.9:0.1 (v/v/v) 2-propanol/water/formic acid that was supplied at a flow rate of 3.3 μL/min. Both parameters were evaluated considering a dry gas flow rate of 7.0 L/min, a nebulizer pressure of 8.0 psi and a dry gas temperature of 200°C. The use of MeCN and MeOH instead of 2-propanol was assayed. Higher signal sensitivity in terms of S/N was

obtained when 2-propanol was employed (**Figure 6.6,I**). Furthermore, the replacement of formic acid for acetic acid reported higher sensitivity in terms of S/N (**Figure 6.6,II**). Therefore, sheath liquid composition was also evaluated in terms of S/N by ranging 2-propanol percentage between 40.0 and 80.0% (v/v) and acetic acid percentage from 0.01 to 0.50% (v/v).

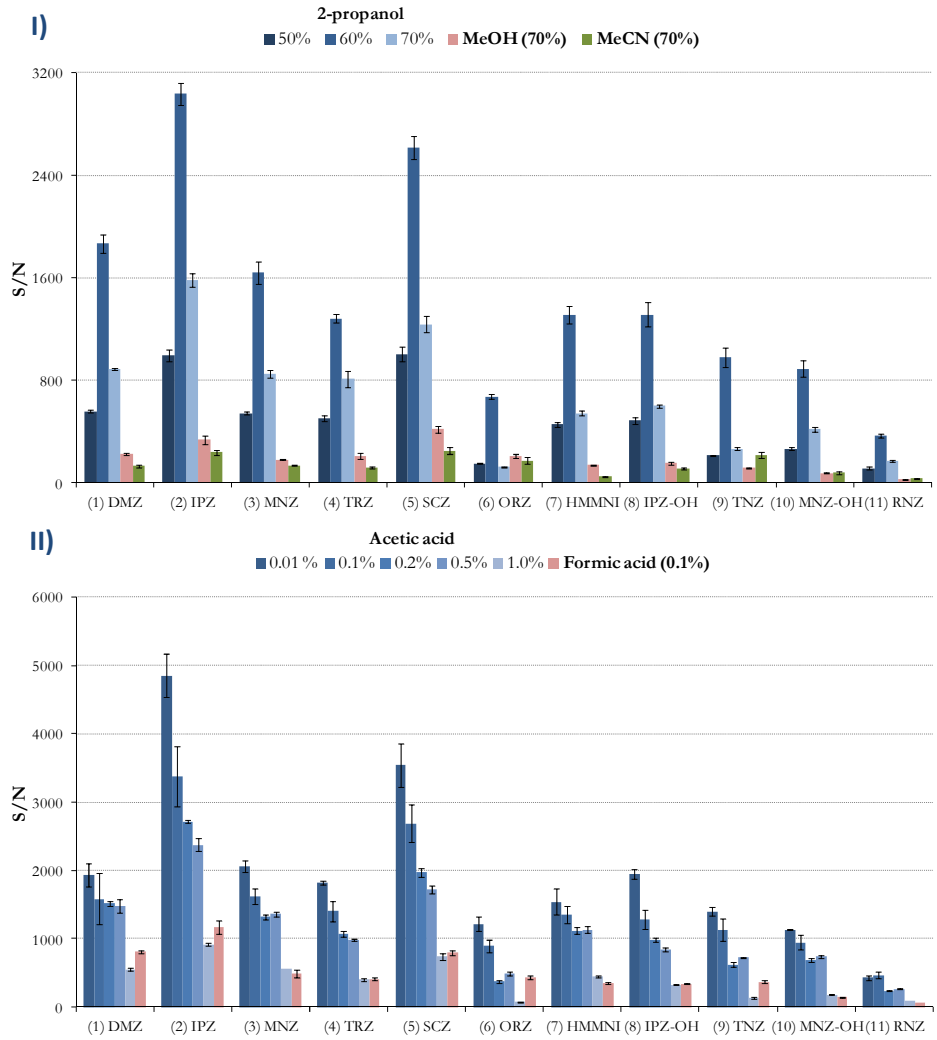

Figure 6.6. I) Optimization of the organic solvent nature and percentage (v/v) added to the sheath liquid. II) Optimization of the acid nature and percentage (v/v) added to the sheath liquid. (n = 3).

Low organic solvent content caused worse desolvation process while high content involved unstable electrospray currents and consequently no analytical signals were

obtained. This phenomena has been previously described [32] and it occurs because the capillary tip gets dry off and the electric circuit is not closed. As can be seen in **Figure 6.6,I**, maximum S/N was achieved when 60.0% (v/v) of 2-propanol was selected. Regarding acetic acid percentage, higher S/N were observed when low quantities of acid were added to the sheath liquid (**Figure 6.6,II**) because as it is known, organic acids increase background noise which deteriorates S/N [33]. However, it was observed that low acetic acid concentrations increased electrospray current instability resulting in higher RSDs for the considered analytical response. As a compromise between both effects, 0.20% (v/v) of acetic acid was chosen as optimum. Consequently, the selected sheath liquid consisted of a mixture 60.0:39.8:0.2 (v/v/v) 2-propanol/water/acetic acid, obtaining maximum signal sensitivity in terms of S/N under these conditions which guarantee separation and electrospray current stability. Finally, sheath liquid flow rate was assayed between 2.0 and 8.0 μL/min. High flow rates resulted in sample dilution which gave low S/N as it was expected. However, flow rates lower than 3.3 μL/min were discarded because in addition to parent ions, some of their fragmentation ions were also observed. Therefore, 3.3 μL/min was established as optimum flow rate (**Figure 6.7**).

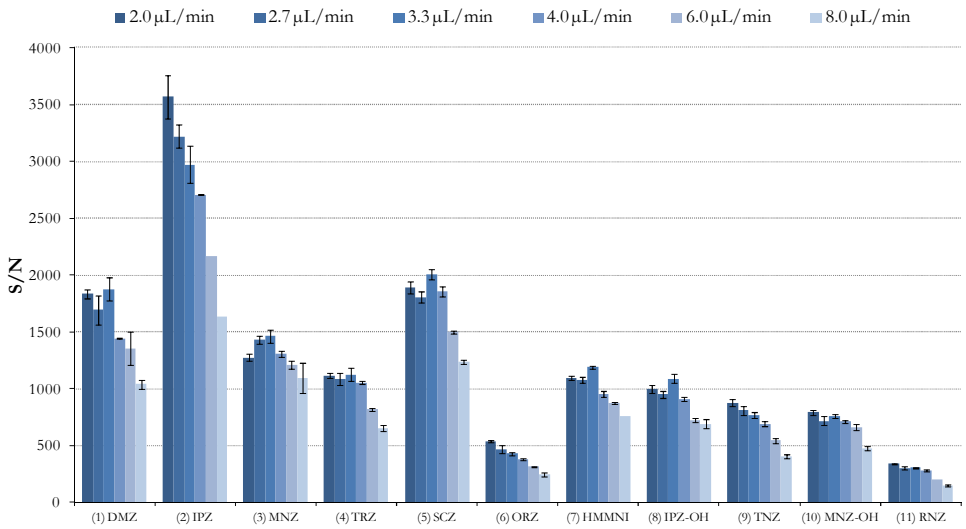

Figure 6.7. **Evaluation of sheath liquid flow rate (n = 3). Sheath liquid consisted of a mixture 60.0:38.8:0.2 (v/v/v) 2-propanol/water/acetic acid.**

Electrospray ionization interface parameters

Dry gas flow rate was assessed from 2 to 10 L/min, resulting in maximum peak sensitivity in terms of S/N as well as in terms of peak height when 6 L/min was selected. Nebulization pressure was studied between 2 and 10 psi. As S/N was improved when high nebulization pressures were tested, 10 psi was established as optimum. Higher pressures were not evaluated due to electrospray instability observed at high nebulization pressures. Regarding dry gas temperature, it was studied from 150 to 310°C. Although differences among analytes behavior were noticed, in general, maximum S/N was reached for almost all 5-NDZs at 190ºC.

These obtained optimum values did not agree with those observed during preliminary assays because initial studies showed higher S/N values at low nebulization pressures and high dry gas temperatures. Therefore, in order to clarify the influence of nebulization pressure and dry gas temperature on the considered analytical response (S/N) as well as their interaction, an experimental design involving both parameters was proposed. Dry gas flow rate was set to 6 L/min (univariate assay optimum) while a central composite blocked cube-star design (12 runs, 4 central points) was performed for the evaluation of nebulization pressure (4-8 psi) and dry gas temperature (180-270°C). Lack of fit P-value for the proposed model was 65% (confidence level of 95%) and determination coefficient (R^2) was 89.7%. Consequently, experimental data satisfactorily fitted to the predicted model. Estimated response surface and main effects plot are shown in **Figure 6.8**.

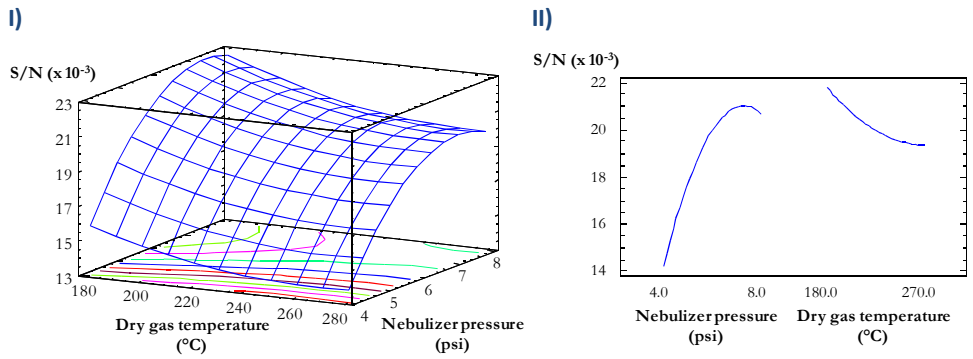

Figure 6.8. Experimental design results. A) Estimated response surface for dry gas temperature (°C) and nebulization pressure (psi). B) Main effect plot.

Optimum values (7.4 psi, nebulization pressure; 160°C, dry gas temperature) were close to those obtained from the univariate assays, selecting these values for further studies.

Mass spectrometer parameters

MS parameters (such as capillary voltage, skimmer, cap exit, Oct 1 DC, Oct 2 DC, Trap drive, Oct RF, Lens 1 and Lens 2) were evaluated by performing direct infusions of 5-NDZ solutions. Each analyte (5 μg/mL) was dissolved in BGS solution (1 M formic acid, pH 1.8) and each standard solution was flushed from the CE instrument to the MS instrument by applying a pressure of 1 bar to the inlet vial. Optimized sheath liquid characteristics (60.0:38.8:0.2, v/v/v, 2-propanol/water/acetic acid; 3.3 μL/min) and optimized nebulization parameters (6 L/min, dry gas flow rate; 7.4 psi, nebulization pressure; 160°C, dry gas temperature) were considered. For MS parameter optimization, the enhancement of ion signals was the target, considering that $[M+H]^+$ resulted in the most abundant ions. Because of some 5-NDZs showed similar migration times, MS detection windows were established, so optimum MS parameters were considered as an average of the MS parameters previously estimated for each compound (**Table 6.1**). **Figure 6.9** shows the total ion chromatogram, the base peak chromatogram and the extracted ion chromatogram for the separation and detection of eleven 5-NDZs under the optimized conditions.

Hereafter, MS/MS mode optimization was carried out. Considering that some peaks were not well-resolved, MRM mode was chosen. For fragmentation experiments, a cut-off of 27% of the precursor mass was set (i.e., the minimum m/z of the fragment ion able to be trapped by the analyzer). 5-NDZs were fragmented using the SmartFrag™ option that automatically ramps the fragmentation energy from 30 to 200% of the excitation amplitude. Fragmentation amplitude was manually varied and it was optimized considering that the maximum signal should be reached for parent ion and at least two fragmentation ions should be obtained. However, several 5-NDZ parent ions, namely MNZ, TRZ, SCZ, HMMNI, IPZ-OH, MNZ-OH and RNZ, only resulted in one fragmentation ion when MS/MS experiments were performed.

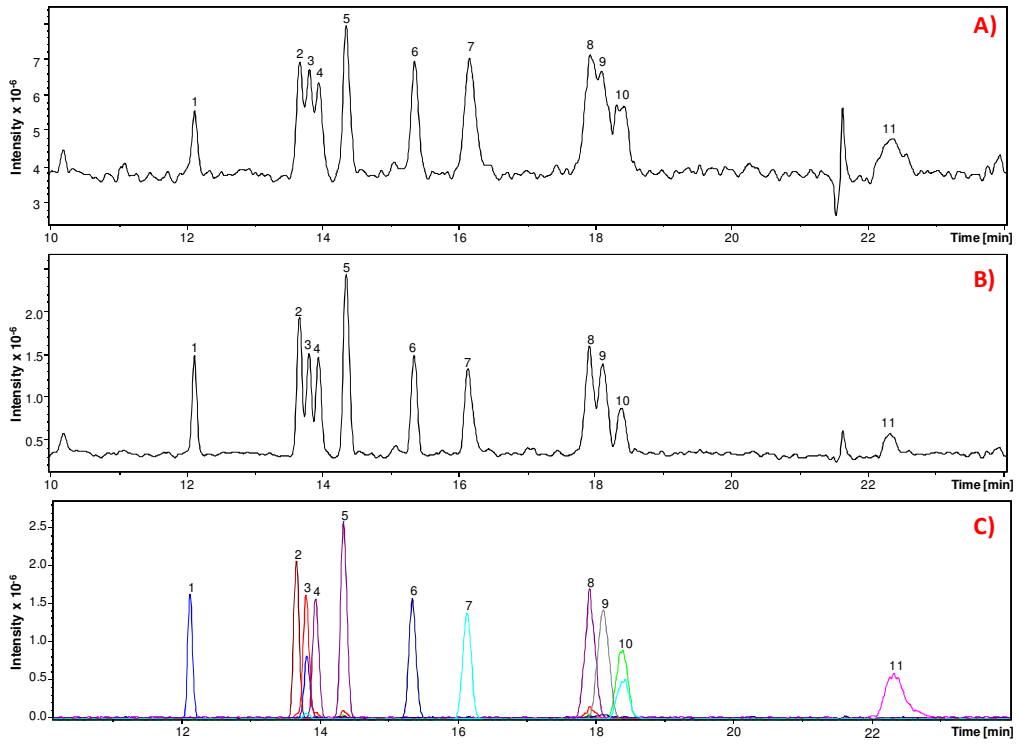

Figure 6.9. 5-NDZ separation by CZE-ESI-MS. (A) Total ion electropherogram, (B) base peak electropherogram, and (C) extracted ion electropherograms. Separation was performed in a fused-silica capillary (110 cm × 50 µm i.d.) using 1 M formic acid (pH 1.8) as BGS. Other experimental conditions: separation voltage, 28 kV; temperature, 25°C. Standard solutions (2 µg/mL of DMZ and IPZ; 3 µg/mL of MNZ and TRZ; 5 µg/mL of SCZ, ORZ, HMMNI, IPZ-OH, TNZ, MNZ-OH and RNZ) were hydrodynamically injected for 40 s at 50 mbar. Sheath liquid consisted of a mixture 60.0:38.8:0.2 (v/v/v) 2-propanol/water/acetic acid and was supplied at 3.3 µL/min. ESI and MS parameters are described on **Section 6.2.5**. Peak identification: 1, DMZ; 2, IPZ; 3, MNZ; 4, TRZ; 5, SCZ; 6, ORZ; 7, HHMNI; 8, IPZ-OH; 9, TNZ; 10, MNZ-OH; 11, RNZ.

In order to guarantee the unambiguous determination of these compounds, a second fragmentation ion was required. Therefore, MS^3 mode was selected for the detection of the previously mentioned analytes. For these compounds, first fragmentation amplitude value was established considering a maximum signal for the fragmentation ion. On the other hand, second fragmentation amplitude value was selected under the consideration of a fragmentation ion ratio equal to 1:10 (second fragmentation ion:first fragmentation ion). Final fragmentation data is appointed on **Table 6.2**. Observed fragmentation ions (m/z) were confirmed by previously reported data [31,34]. **Figure 6.10** shows MS^2 and MS^3 spectra of the studied 5-NDZs as well as the proposed molecule fragmentations according to the observed m/z signals.

Table 6.2. Main parameters of the CZE-MS/MS method.

	Segment time (min)																					
	0.0-15.6	**15.6-18.2**						**18.2-20.8**						**20.8-24.4**						**24.4-30.0**		
	DMZ	IPZ	MNZ		TRZ		SCZ			ORZ	HMMNI			IPZ-OH			TNZ	MNZ-OH	RNZ		
	MS²	*MS²*	*MS²*	*MS³*	*MS²*	*MS³*	*MS²*	*MS³*		*MS²*	*MS²*	*MS³*		*MS²*	*MS³*		*MS²*	*MS²*	*MS²*	*MS³*	
Width (m/z)	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0		2.0	2.0	4.0		2.0	2.0		2.0	2.0	4.0	2.0	
Cutoff (m/z)	38.0	46.0	46.0	35.0	50.0	35.0	50.0	35.0		59.0	43.0	38.0		50.0	45.0		67.0	51.0	54.0	38.0	
Amplitude (V)	0.23	0.23	0.23	0.20	0.25	0.20	0.25	0.25		0.26	0.24	0.16		0.25	0.18		0.20	0.18	0.28	0.14	
Precursor ion $[M+H]^+$	142.0	170.0	172.0	128.0	186.0	128.0	186.0	128.0		220.0	158.0	140.0		186.0	168.0		248.0	188.0	200.9	140.0	
IPZ Observed fragmentation ions	112.0 96.1	140.0 124.0 109.1	128.0	82.1	128.0	82.1	128.0	82.1		128.0 82.1	140.0	110.0 55.5		168.0	122.0		202.0 121.0 110.0	170.0 123.0	140.0	55.0	

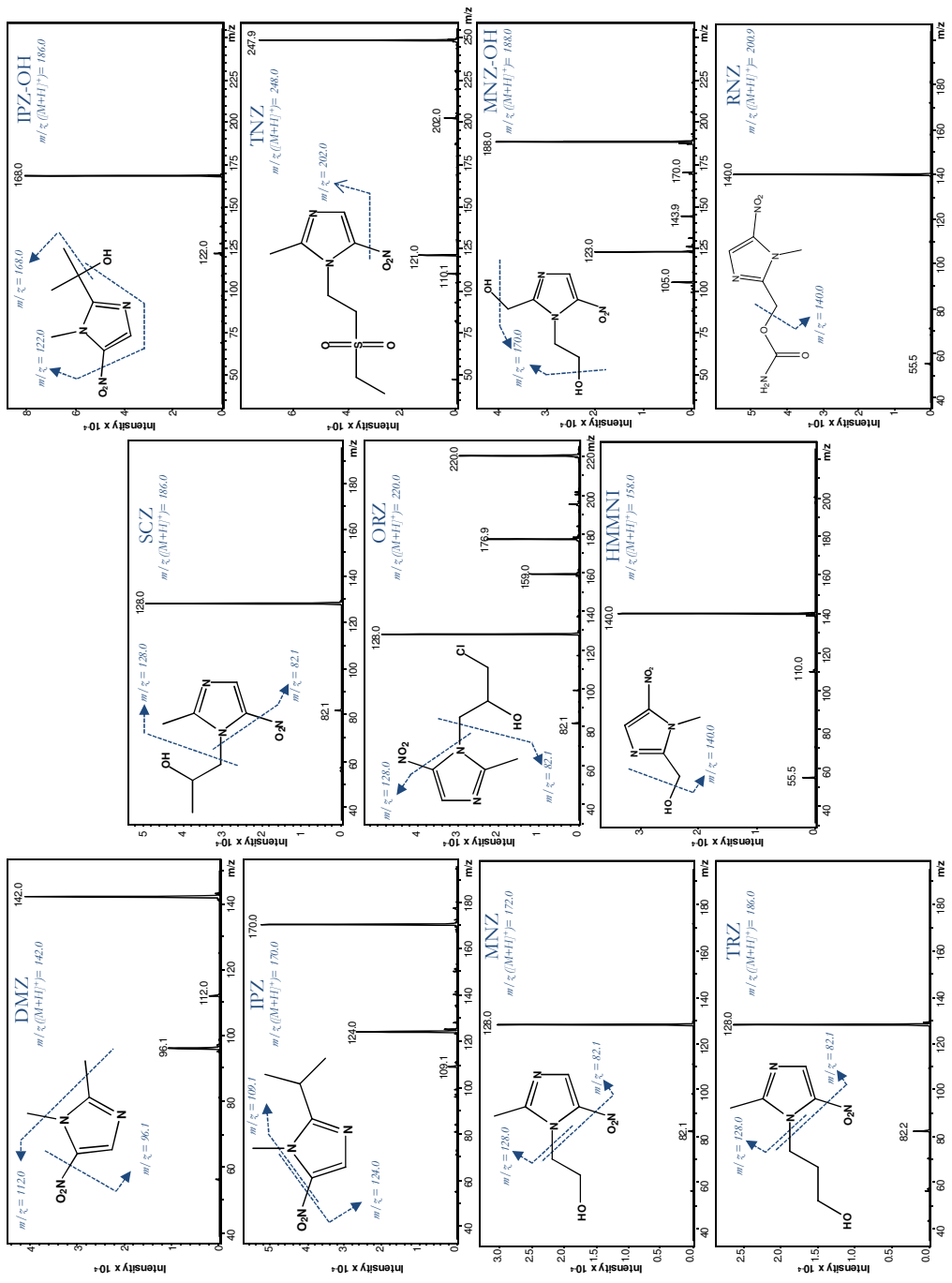

Figure 6.10. MS² and MS³ spectra for the studied 5-NDZs obtained from a standard solution (5 µg/mL of DMZ, MNZ, TRZ, SCZ, HMMNI and RNZ; 3 µg/mL of IPZ, ORZ, IPZ-OH, TNZ and MNZ-OH) under the optimized CE-MSⁿ conditions. Molecule fragmentations are proposed in order to justify the observed m/z signals.

6.3.3. Method characterization

Generally, 5-NDZ determination in urine samples has been carried out by LC-MS, however CE-MS is a good alternative because it is a more cost-effective and greener analytical tool. In order to test the usefulness and potential of the proposed method, urine samples were treated by the previously mentioned MISPE procedure (see **Section 6.2.3**) and subsequently analyzed by CZE-tandem MS. The optimized method was instrumentally evaluated in terms of linearity, LODs, LOQs, trueness, matrix effect (ME), process efficiency (PE) and peak area repeatability and intermediate precision.

Calibration curves and performance characteristics

Matrix-matched calibration curves were established in urine samples fortified at the following concentration levels: 37.5, 125, 250, 625 and 500 μg/L for DMZ, MNZ, TRZ, SCZ and ORZ; 22.5, 75, 150, 375 and 600 μg/L for IPZ, IPZ-OH, TNZ and MNZ-OH; 60, 195, 400, 1000 and 1600 μg/L for HMMNI and 156.3, 312.5, 781.3 and 1250 μg/L for RNZ. Characteristics of matrix-matched calibration curves are shown on **Table 6.3**.

Table 6.3. **Matrix-matched calibration curves and statistical and performance characteristics of the proposed MISPE-CE-MS method.**

Analyte	Linear range (μg/L)	R^2	Linear regression equation (y=m·x+a)				LOD (μg/L)	LOQ (μg/L)
			Slope $\times 10^{-3}$	*SD for the slope* $\times 10^{-2}$	*Intercept* $\times 10^{-5}$	*SD for the intercept* $\times 10^{-5}$	$3 \times S/N$	$10 \times S/N$
DMZ	22.8 - 1000	0.9955	3.05	1.18	2.28	0.64	6.9	22.8
IPZ	11.6 – 600	0.9972	9.05	2.78	0.63	0.91	3.5	11.6
MNZ	37.5 – 1000	0.9940	3.57	1.60	1.70	0.87	11.3	37.5
TRZ	15.4 – 1000	0.9949	2.64	1.10	1.53	0.60	4.6	15.4
SCZ	21.0 - 1000	0.9960	3.17	1.16	1.11	0.63	6.3	21.0
ORZ	19.9 - 1000	0.9981	4.11	1.02	0.08	0.55	6.0	19.9
HMMNI	51.3 - 1600	0.9969	4.33	1.40	0.33	1.21	15.4	51.3
IPZ-OH	9.6 - 600	0.9960	9.87	3.61	0.97	1.17	2.9	9.6
TNZ	19.1 - 600	0.9992	6.88	1.10	-0.20	0.36	5.7	19.1
MNZ-OH	21.4 - 600	0.9975	6.56	1.91	1.25	0.62	6.4	21.4
RNZ	130.2 - 1250	0.9983	2.16	0.52	-0.22	0.35	39.1	130.2

Two spiked urine samples per level were treated following the MISPE procedure. Afterwards, each sample was analyzed in duplicate according to the proposed CZE-tandem MS method. A urine blank sample was analyzed as it has been described and no matrix interferences were found at any analyte migration time. The sum of peak areas from all product ions was considered as function of analyte concentration on the sample. LODs and LOQs of the method were calculated as the minimum analyte concentration yielding a S/N equal to three and ten, respectively. In all cases, LODs were lower than 39.1 µg/L. Additionally, electropherograms obtained from the analysis of urine samples spiked at different 5-NDZ concentrations are shown in **Figure 6.11**. An electropherogram of a blank sample is also shown.

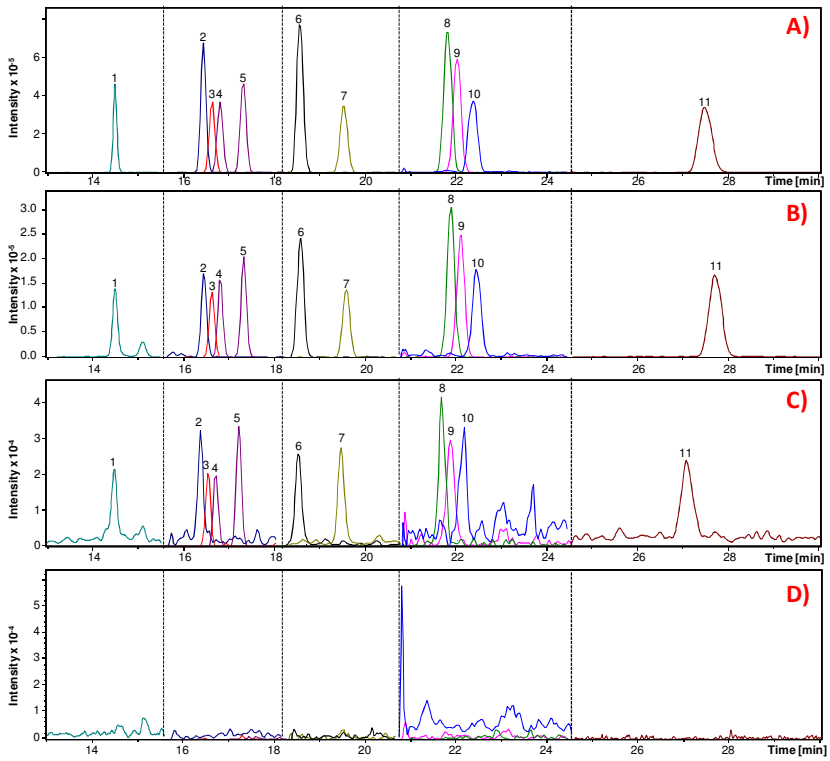

Figure 6.11. **Electropherograms obtained from the analysis of urine samples fortified at different 5-NDZ concentrations. MISPE-CZE-MS/MS conditions are described in Section 6.2. A) 600 µg/L for IPZ, IPZ-OH, TNZ and MNZ-OH; 1000 µg/L for DMZ, MNZ, TRZ, SCZ and ORZ; 1600 µg/L for HMMNI; and 1250 µg/L for RNZ. B) 240 µg/L for IPZ, IPZ-OH, TNZ and MNZ-OH; 400 µg/L for DMZ, MNZ, TRZ, SCZ and ORZ; 640 µg/L for HMMNI; and 500 µg/L for RNZ. C) 30 µg/L for IPZ, IPZ-OH, TNZ and MNZ-OH; 50 µg/L for DMZ, MNZ, TRZ, SCZ and ORZ; 80 µg/L for HMMNI; and 62.5 µg/L for RNZ. D) Blank urine sample.**

Precision assays

Precision studies were carried out in order to evaluate the repeatability (intra-day precision) and intermediate precision (inter-day precision) of the proposed MISPE-CE-MS/MS method. Repeatability was assayed at three concentration levels by analyzing three spiked samples per level in triplicate. Intermediate precision was assessed at three concentration levels by analyzing one spiked sample in triplicate per level and per day for five consecutive days. Results expressed as peak area RSDs (%) are shown on **Table 6.4**. In all cases RSDs were lower than 11.9% and 16.1% for repeatability and intermediate precision, respectively.

Table 6.4. **Precision studies in terms of RSDs (%) for spiked urine samples.**

Analyte	Repeatability (% RSD, n = 9)			Intermediate precision (% RSD, n = 15)		
	50 µg/L	***400 µg/L***	***1000 µg/L***	***50 µg/L***	***400 µg/L***	***1000 µg/L***
DMZ	11.7	3.2	3.3	13.1	8.9	8.4
MNZ	11.9	5.5	3.0	12.7	7.9	9.1
TRZ	11.6	3.3	5.1	16.1	6.9	8.5
SCZ	10.2	4.2	3.8	13.9	6.9	14.9
ORZ	7.6	3.2	9.2	7.0	15.1	13.7
	30 µg/L	***240 µg/L***	***600 µg/L***	***30 µg/L***	***240 µg/L***	***600 µg/L***
IPZ	5.8	3.1	4.2	12.2	8.9	8.4
IPZ-OH	8.3	4.3	2.8	11.7	7.5	8.3
TNZ	8.5	4.4	3.0	11.6	5.0	10.2
MNZ-OH	8.5	4.0	3.9	13.0	6.3	15.8
	80 µg/L	***640 µg/L***	***1600 µg/L***	***80 µg/L***	***640 µg/L***	***1600 µg/L***
HMMNI	4.3	4.6	4.8	10.9	10.1	14.4
	500 µg/L	***1250 µg/L***		***500 µg/L***	***1250 µg/L***	
RNZ	5.4	6.6		13.1	12.9	

Trueness

Recovery studies were performed over urine samples fortified at three different concentration levels. Three samples per concentration level were treated and analyzed in triplicate. Recoveries (%) were estimated by comparing the analytical responses obtained

from the application of the full MISPE-CZE-tandem MS to those obtained from the analysis of blank urine samples spiked after the application of the MISPE protocol and prior to the measurement (**Equation 6.1**).

$$R = \frac{Signal\ of\ a\ sample\ spiked\ before\ its\ extraction}{Signal\ of\ a\ sample\ spiked\ after\ its\ extraction}$$ **Equation 6.1.**

These studies evaluate the efficiency of the extraction process. As can be seen on **Table 6.5**, recoveries higher than 79.2% were obtained for all the compounds, proving the convenience of using MISPE for 5-NDZ determination in urine samples.

Table 6.5. Recovery studies for spiked urine samples.

Analyte	Recovery (%, n = 9)		
	50 µg/L	***400 µg/L***	***1000 µg/L***
DMZ	99.3	105.0	87.0
MNZ	99.3	95.6	100.8
TRZ	101.4	102.6	98.5
SCZ	97.1	99.9	100.3
ORZ	98.8	98.6	114.1
	30 µg/L	***240 µg/L***	***600 µg/L***
IPZ	86.5	90.1	80.0
IPZ-OH	88.4	98.5	91.7
TNZ	101.3	102.3	93.8
MNZ-OH	105.2	103.0	79.2
	80 µg/L	***640 µg/L***	***1600 µg/L***
HMMNI	96.2	96.6	93.9
	500 µg/L	***1250 µg/L***	
RNZ	100.0	97.7	

Process efficiency and matrix effect

ME is the result caused by other components of the sample except the specific compounds to be quantified on the analytical response. It can be attributed to many sources affecting the analyte ionization, resulting in ion suppression or signal enhancement when MS is employed as detection tool for quantification and/or identification [35]. In order to evaluate both phenomena, Matuszewski *et al.* introduced the term of absolute ME which is

directly related to R and PE [36]. Therefore, ME (%) can be estimated by comparing the analytical response obtained from a sample spiked post-extraction at any given analyte concentration to the response resulted from a standard solution of the same analyte concentration according to the **Equation 6.2**. If ME (%) > 100, matrix components produce a signal enhancement whereas ME (%) < 100 means that ion suppression is occurring. No significant ME involves a value close to 100%. Additionally, overall PE can be calculated by **Equation 6.3**, in which R as well as ME are considered.

$$ME = \frac{Signal\ of\ a\ sample\ spiked\ after\ its\ extraction}{Standard\ solution\ signal}$$ **Equation 6.2.**

$$PE = \frac{Signal\ of\ a\ sample\ spiked\ before\ its\ extraction}{Standard\ solution\ signal} = R \times ME$$ **Equation 6.3.**

ME and PE were calculated according to **Equation 6.2** and **Equation 6.3** at the same concentration levels for which R studies were performed. Average values obtained for each 5-NDZ are shown on **Table 6.6**. ME values range from 71.3 to 97.9%, suggesting a slight ion suppression which was more significant for MNZ, TRZ and SCZ. Satisfactory results were obtained for overall PE, ranging between 70.4 and 102.9% that correspond to MNZ and ORZ, respectively. From these results, it can be concluded that the proposed method is suitable for 5-NDZ determination in urine samples because it results in high R (%) and low ME (close to 100%) which supposes high PE for all studied analytes.

Table 6.6. Estimated process efficiency and matrix effect averages.

Analyte	Matrix effect (%)	Process efficiency (%)
DMZ	84.0	80.1
IPZ	90.1	76.2
MNZ	71.4	70.4
TRZ	74.3	74.7
SCZ	71.3	70.9
ORZ	97.9	102.9
HMMNI	92.6	88.4
IPZ-OH	91.4	84.8
TNZ	85.3	84.4
MNZ-OH	84.5	79.7
RNZ	83.6	82.7

6.4. Conclusions

In this chapter, a comparison between two separation modes, namely MEKC and CZE, was evaluated for 5-NDZ determination by CE-MS. Higher selectivity was achieved under CZE conditions, establishing a novel strategy for the analysis of eleven 5-NDZ drugs, including some of their metabolites, in urine. On the other hand, high sensitivity has been reached through nebulization and MS parameters optimization achieving LOQs between 9.6 and 130.2 μg/L which are lower than those previously reported in urine samples. This proposal represents a good alternative to traditional LC methods because it accomplishes with the basis of Green Chemistry, involving low consumption of solvents, reagents and samples. Furthermore, it has been showed the usefulness of MISPE as efficient sample treatment for 5-NDZ extraction from complex biological samples such as urine, avoiding complicated clean-up procedures.

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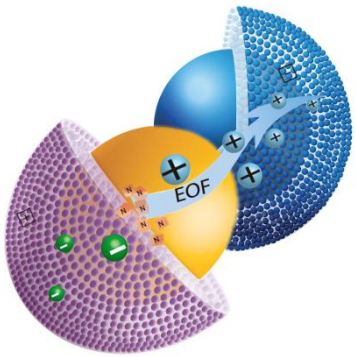
PART III

5-NDZ DETERMINATION BY CEC-BASED METHODS



Chapter 7:

Capillary electrochromatography

Chapter 8:

Determination of 5-NDZ residues in milk by capillary electrochromatography with packed C18 silica beds

Chapter 9:

Capillary electrochromatography - mass spectrometry for the determination of 5-NDZ antibiotics in urine samples

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7

Capillary electrochromatography

7.1. Background

CEC is a hybrid separation technique which combines the high efficiency of CE and the selectivity of LC chromatography. As was mentioned in **Chapter 3**, CEC is a CE mode where analyte separation is practically accomplished by LC mechanisms due to interactions between analytes and a stationary phase. Therefore, CEC can be defined as a LC separation in which the mobile phase flow is due to the EOF originated by the application of a voltage at the ends of the separation column. Furthermore, electrophoretic velocity of the compounds must be taken into account for charged molecules, and thus, separation can also occurs due to electrophoresis.

In 1939, Strain proposed for the first time the combination of chromatographic and electrophoretic methods. The separation of colored water-soluble compounds was performed on Tswett adsorption columns by applying a voltage between 175 to 200 V to the ends of the column [1]. Later on, in 1952, Mould and Synge demonstrated the potential of the EOF as a driving force in separation techniques by using the EOF in thin liquid chromatography (TLC) for the separation of polysaccharides through colloid membranes [2]. However, the beginning of CEC was not established until 1974 when Pretorious *et al.* studied the effects of propelling the solvents in TLC and in high speed liquid chromatography (HSLC) by electro-osmosis instead of using pressure [3]. They experimentally demonstrated that smaller plate heights could be obtained under EOF compared to those observed under pressure driven flow. Consequently, they concluded that a flat flow profile reduces band spreading arising from resistance to mass transfer in the mobile phase.

Indeed, flat flow profile of CEC compared to parabolic flow profile of LC (**Figure 7.1**) is the main advantage of CEC methods because, as a consequence, higher separation efficiencies can be achieved.

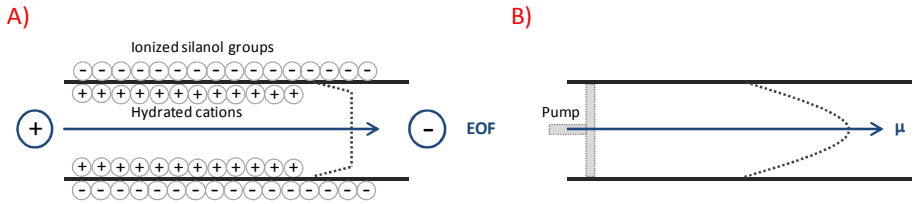

Figure 7.1. **A) 'Plug flow' profile in CEC separation. B) 'Parabolic flow' profile in HPLC. μ represents the lineal velocity of the flow.**

Although the beginning of CEC is connected to LC-based methods, the development of CEC is more related to CE advances than LC ones, or at least, it should be considered as the meeting point of both separation techniques. In 1981, Jorgensen and Lukacs proposed the first CEC methodology as it is developed today [4]. Packed capillaries were laboratory-made by filling a Pyrex glass tube (68 cm, 170 μm i.d.) with C18 particles (10 μm of particle size). The separation of 9-methylantracene and perylene was performed using MeCN as mobile phase and applying a separation voltage of 30 kV. Although the technique was crude and poorly packed columns were employed, an improvement of peak efficiency was achieved. In comparison with a reverse-phase chromatography using a pressure-driven flow, plate heights were reduced 1.9 and 2.5-fold, respectively. Nevertheless, and in spite of these achievements, Jorgensen and Lukacs also suggested which was going to be the main limitation for the application of CEC methods by concluding that "the performance of these (CEC) columns appears to offer a modest improvement over conventional (pressure driven) flow, but may not justify the increased difficulty in working with electroosmotic flow". During the last decades and in order to overcome this pessimistic conclusion, great efforts have been done for improving CEC performance.

An important influence on CEC research can be attributed to Knox and Grant who stimulated it when they proposed to group all separation methods carried out using a flow driven by EOF under the term of 'electrochromatography'. Their paper, published in 1987, supposed a well discussed theoretical study with regard to electrochromatography features [5]. Furthermore, in 1991, they experimentally demonstrated that greater efficiencies can be

achieved by CEC compared to pressure-driven systems when the same separation column is employed. Additionally, they confirmed that EOF velocity in CEC is unaffected by particle size at least down to 1.5 μm which is consistent with their conclusion derived from double layer theory [6].

The enthusiasm generated by CEC as separation tool was significant during the 1990s when several reviews and research papers about CEC were reported [7-10]. For example, Rathore and Horváth presented a review in 1997 in which they pretended to reveal the complexity of the electrokinetic phenomena underlying EOF in porous media, and as a consequence, to cause motivation for performing the experimental and theoretical research required for developing CEC as a powerful analytical tool [11].

Nowadays, CEC is a mature technique whose general principles have been already established. However, their application is still locked in research laboratories. Numerous efforts have been made for improving the reliability and robustness of this technique and an important instrumentation development has been carried out since Pretorious proposed, for the first time, the separation of molecules by this tool. Thus, it is the time for the application of CEC with the aim of solving analytical problems.

7.2. CEC and residue determination

In general, CEC methods can be classified in three groups according to the column type that is chosen for performing the separation. CEC columns can be open-tubular (OT), continuous bed, also known as monolithic columns, or packed columns (**Figure 7.2**).

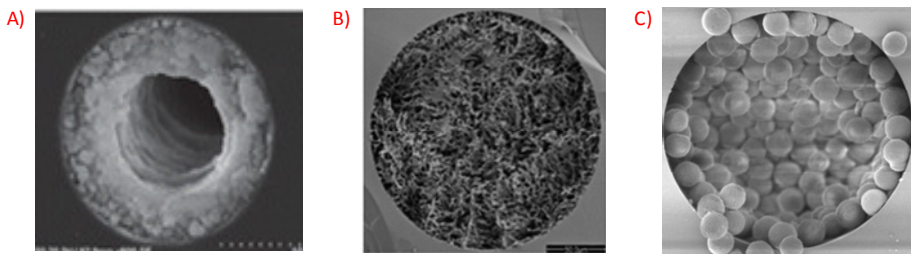

Figure 7.2. Scanning electron microscopy (SEM) images of: A) an OT column with a monolithic layer thickness of approximately 25 μm; B) monolithic cryopolymer based on poly(polyethylene glycoldiacrylate) containing 10% poly(divinylbenzene) nanoparticles; C) cross-sectional view on cut capillaries (50 μm i.d.) packed with 5 μm-sized Zorbax SB-C18 particles. Reproduced from [12], [13] and [14], respectively.

An OT column is a capillary bonded with a wall-supported stationary phase that can be a coated polymer, a bonded molecular monolayer, or a synthesized porous layer network. A monolithic column is a capillary containing a wall-supported porous continuous bed that is synthesized in-situ in the capillary. Finally, a packed column is a capillary filled with particles of the stationary phase that are retained by two end-frits [15].

In 1984, Tsuda *et al.* proposed for the first time the use of OT columns for carrying out an electrochromatographic separation. ODS was employed as stationary phase and it was chemically bonded to pretreated soda-lime glass capillaries [16]. Since then, different approaches have been proposed for achieving high performance in open-tubular CEC. The main drawback of OT columns compared to monolithic or packed columns is that they offer lower sample capacity, and therefore, worse signal sensitivity is achieved in OT-CEC methods. Many efforts have been made for increasing the area-to-volume ratio in order to overcome this disadvantage, and as a consequence, several procedures have been proposed for manufacturing OT columns [17]. Besides, research on OT-CEC has been mainly focused on developing new stationary phases which give an important selectivity to the column. The use of MIPs [18,19], gold nanoparticles [20,21], cyclodextrins [22,23] or molecular organic frameworks (MOFs) [24,25] are just a few examples of materials that have been employed for achieving high selective separations by OT-CEC.

Unlike OT-CEC columns, in monolithic columns the stationary phase occupies all the capillary volume and is manufactured inside the capillary. Monolithic capillaries are classified in four groups according to their manufacturing process: molded porous polymer, molded porous sol-gel, particle-fixed continuous-bed, and microfabricated monolith channels [15]. First monolithic columns for CEC were proposed by Hjertén [26] and Fujimoto [27] in 1995, and they consisted of a highly crosslinked acrylamide-based continuous bed. As it has occurred with OT-CEC, the research on monolithic CEC separations has been mainly focused on the development of new stationary phases [28]. Furthermore, due to their unique properties, the synthesis of monolithic materials for CEC columns has attracted great interest among researchers.

The production of monolithic columns is usually carried out in-situ following easy polymerization procedures, and moreover, there are a large number of readily available chemistries. Therefore, a wide variety of stationary phases are easily available for CEC using monolithic capillaries [29]. However, the use of monolithic columns presents several inconveniences that must be taken into account. Polymeric monolithic stationary phases can swell and shrink due to the organic solvents of the mobile phase, leading to a lack of stability. Furthermore, the preparation of polymeric monoliths usually leads to micropores, which negatively affects their efficiency and peak symmetry. Thus, it is difficult to reach high efficiency for small molecules. Additionally, monolithic columns present lower specific area compared to packed capillaries, and as a result, lower column capacity is observed [30]. Probably monolithic capillaries will be the future of CEC due to their simplicity; however packed capillaries may still be the most common choice for performing CEC separations.

Packed columns offer the highest specific area among all the electrochromatographic columns, and therefore, higher sample capacity can be obtained. As a consequence, higher sensitivity and reproducibility of the separation can be achieved. Besides, CEC packed columns offer the inherent selectivity and reproducibility of commercially available packing materials [15]. Despite of these advantages, the use of this type of capillaries has important limitations. First, the cost and the fragility of the commercially available columns have led routine users to prepare their own columns. As a result, many CEC users pack their own capillaries in the laboratory, which involves several inconveniences. The success in packing a CEC capillary mainly depends on researcher's skills and experience. Poor pack capillaries provide low separation efficiency and reproducibility. Besides, bubble formation during the separation is attributed to the two end-frits that have to retain the stationary phase inside the capillary. However, both problems can be overcome, and working with laboratory-packed capillaries should not be a real inconvenience for using this type of columns in CEC separations. With the aim of being successful in the manufacturing procedure, approaches with regard to capillary packing and frit formation are described in **Section 7.4**.

Regardless of the employed CEC column, the application of CEC methods to residue determination can be limited by the same drawbacks as CE. A lack of sensitivity can be attributed to them, especially when UV detection is used due to the low sample volume injected. With the aim of enhancing signal sensitivity, it has been proposed to inject the sample in a solvent with lower elution strength than mobile phase for both hydrodynamic [31] or electrokinetic [32,33] injection. Additionally, the simultaneous electrokinetic and hydrodynamic injection of the sample has shown to enhance signal sensitivity compared to a simple electrokinetic or hydrodynamic injection [34].

Considering residue analysis field, recent applications have been reported about the use of CEC for the determination of veterinary drugs and pesticide residues in food matrices. Pesek *et al.* developed an OT-CEC methodology for the analysis of five veterinary drugs in milk serum, including tetracyclines [35]. Moreover, the analysis of sulfonamide antibiotics in meat samples [33] or penicillin antibiotics in milk [36] has been proposed by CEC using methacrylate-based monolithic columns. Additionally, packed capillaries with C18 particles have been employed for the separation and determination of phenlyurea herbicides in vegetables and vegetable processed food, namely fresh potatoes and soya products [37] and pyrethroid pesticides in cabbage [38]. Furthermore, residue determination by CEC has been carried out in other matrices such as environmental or clinical samples. The determination of phenylureas in groundwater samples using a C18 packed capillary [39] or the enantioselective separation of mirtazapine and its metabolites in urine using a packed capillary with vancomycin-modified diol stationary phase [40] are just a few examples of CEC applications in this field.

5-NDZ determination has only been proposed by monolithic-CEC, although its applicability to complex samples has not been shown. The preparation of polymethacrylate-based MIP columns for the chiral recognition of ORZ in pharmaceutical tablets was evaluated [41], and further separation of MNZ, SCZ, RNZ, TNZ, DMZ, (R)-ORZ and (S)-ORZ standards was assessed [42]. In **Chapter 8**, a novel CEC-UV method using packed capillaries is developed and applied for the first time to the determination of traces of 5-NDZs in a complex food matrix such as milk. Additionally, the analysis of 5-NDZ residues in urine samples is proposed by CEC-MS in **Chapter 9**.

Furthermore, the nature of the selected stationary phase is also discussed and it represents the first CEC method used for the determination of 5-NDZs employing MS as detection technique.

7.3. General features of packed capillaries

As was previously mentioned, packed capillaries are the most employed columns for performing CEC separation. Packed columns present two sections: a packed section and an open section. Packed section is limited by stationary phase length. Stationary phase is retained by two frits which can be mechanically or chemically made. The open section follows the packed section and the detection window is located on it (**Figure 7.3**).

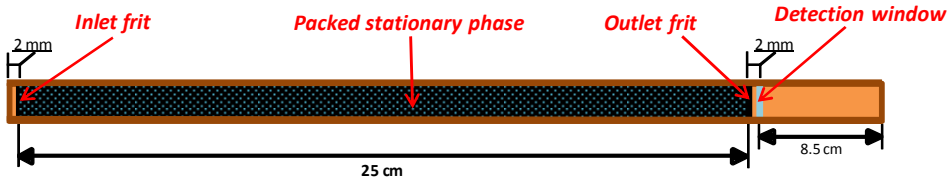

Figure 7.3. Scheme of a packed capillary. Represented dimensions are according to those of a packed capillary normally used in CEC.

Normally, fused-silica capillaries with i.d. lower than or equal to 100 μm are packed with materials based on silica microparticles (1.5-5 μm). A capillary with low i.d. minimizes the Joule effect during runs, but a low i.d. also increases the difficulty of packing the column. Furthermore, stationary phases based on hydrocarbon structures bonded to silica particles or reversed-phase packing, are usually employed. In 2000, Pursh and Sander established that about 70% of all the selected applications involving CEC separations (180 publications were revised) employed C18 stationary phases [43]. Although this percentage may have decreased due to the growing use of polymeric monolithic stationary phases, C18 stationary phases are still widely used.

It must be remarked the role of the stationary phase in a CEC separation because it has to guarantee selective interactions toward analytes and the presence of a quite strong and constant EOF. Therefore, the employed stationary phases have to possess charge or chargeable functional groups [44]. In the case of silica particles-based stationary phases, the

residual silanols promote the EOF, so end-capped phases may not be suitable for CEC [43]. In a packed column, the electrical double layer is formed on both the surface of the particles and column wall [45]. Additionally, Pesek *et al.* demonstrated that approximately 95% of the silanol groups on particle surface could be replaced by Si-H moieties without affecting the EOF. In this case, the generated EOF is due to the silanols below the particulate silica hybrid surface as well as the silanol groups on the fused silica capillary wall [46]. Following strategies like this, the properties of the stationary phases can be modified and, consequently, a different selectivity can be achieved. **Figure 7.4** shows different chemical groups that can be considered for the functionalization of silica particles further used as stationary phase in CEC.

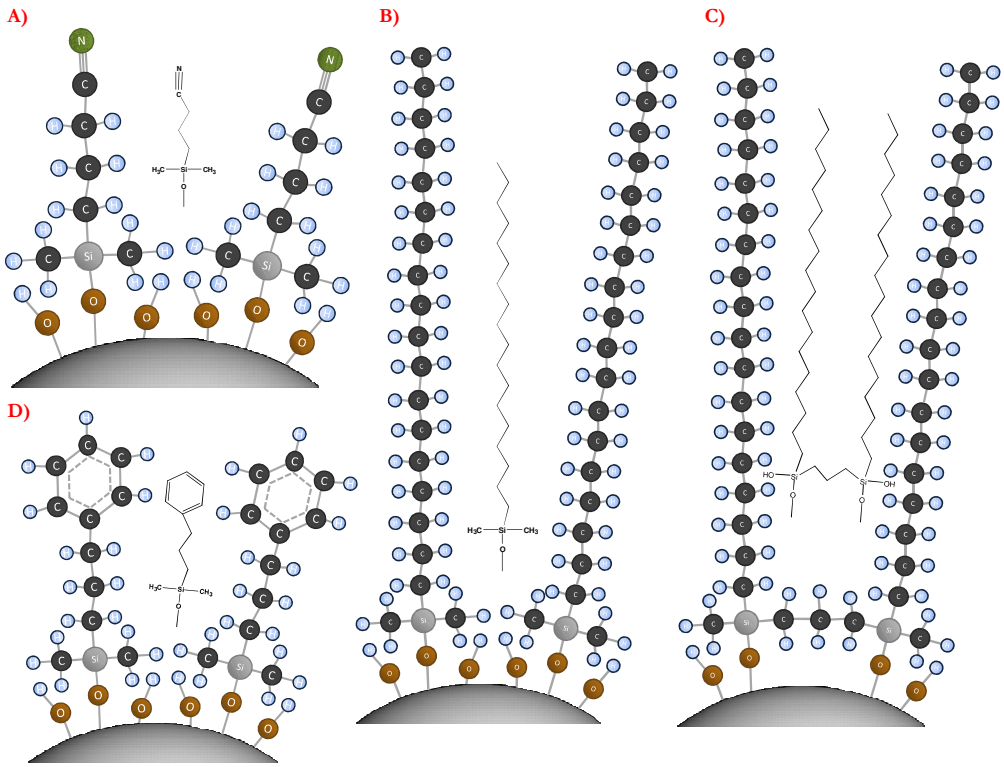

Figure 7.4. **Stationary phases consisted of silica-based particles with different moieties. A) cyano-based stationary phase; B) C18-based stationary phase; C) bidentate-C18-based stationary phase with reduced silanophilic activity; D) phenyl-based stationary phase.**

Despite of particulate C18 stationary phases are the most commonly used, other packing natures have been successfully tested, and as result, columns with different selectivity can

be employed in CEC. Aturki *et al.* showed that cyano-based stationary phase was more convenient than C18-based stationary phases for the separation of basic molecules which present hydrophilic and polar features [47]. Furthermore, a comparison between C18-bidentate stationary phase and cyano-based stationary phase was performed for the analysis of bovine cytochrome c tryptic digest by Fanali *et al.* [48]. Higher selectivity was achieved when cyano-based stationary phase was employed. On the other hand, the separation of eleven estrogenic compounds has been proposed by CEC using a phenyl-based stationary phase which showed higher selectivity for the referred molecules than C18-based ones [49].

In general, CEC offers a great advantage over LC. The mass amount of stationary phase required for packing electrochromatographic capillaries is lower than the amount needed for manufacturing a LC column. As a consequence, columns with different selectivity can be tested for CEC separation without involving a high cost.

7.4. Manufacturing procedures for packing CEC columns

7.4.1. Packing techniques

As was mentioned in **Section 7.2**, researchers use to manufacture their own capillaries for performing CEC separations instead of buying commercial columns. Different manufacturing procedures have been proposed for packing the capillaries; however, a universal procedure has not been established. Capillary packing by pressure using slurry of the stationary phase is the most popular technique. Nevertheless, other procedures such as electrokinetic packing, packing by centripetal forces or packing with slurry using supercritical CO_2 as carrier have shown to provide capillaries with higher efficiency [50].

When a capillary is packed by pressure using slurry, the capillary with a temporary frit is connected to a packing reservoir such as a short LC column or another suitable unit. **Figure 7.5** shows a set up for capillary packing by pressure with slurry. Temporary frit can be a mechanical retainer (**Figure 7.5,G**) or it can be chemically fabricated by dipping the capillary in a solution containing wet silica particles. When a small amount of the material is introduced in the capillary, it is heated and silica particles sinter forming a temporary frit

[51]. Nowadays, Western Fluids Engineering (Wildomar, CA, USA) commercializes a column packing unit where the capillary is directly connected to the reservoir vial (**Figure 7.5,C**). The reservoir vial containing the stationary phase slurry (approximately 50-100 mg/mL) is placed inside the packing unit [52]. The reservoir system is connected to a high-pressure solvent delivery pump (**Figure 7.5,A**) which propels the driving solvent to the packing reservoir, and as a result, capillary is packed. The packing material is transported into the capillary column by applying a pressure between 350 and 700 bar [50].

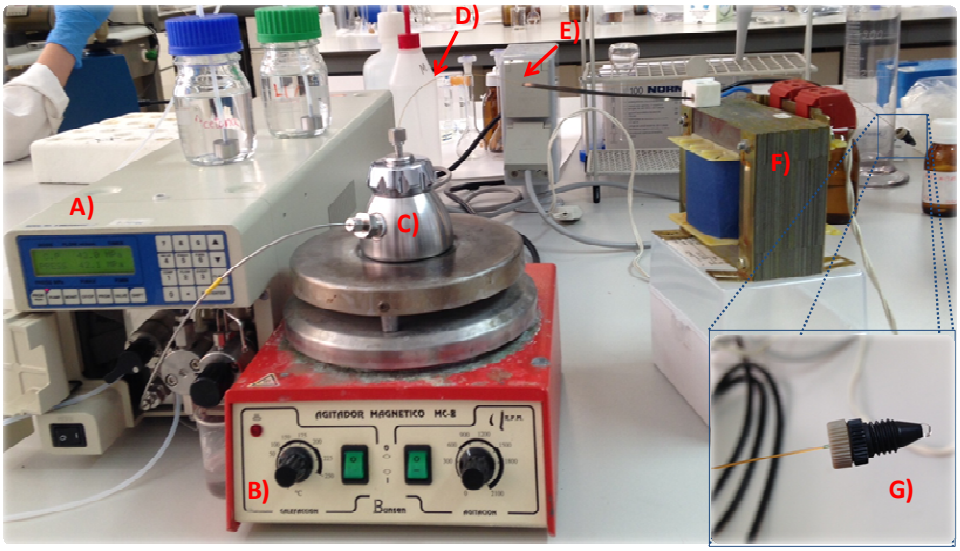

Figure 7.5. Set up for packing capillaries by pressure using slurry. A) High pressure pump which propels the driving solvent to the packing unit and, subsequently, through the capillary. B) Magnetic stirrer which keeps the stationary phase as a suspension in the slurry solvent. C) Packing unit where a vial containing the stationary phase is placed. D) Capillary to be packed. E) Interaction point between the capillary and the Nichrome ribbon where an end-frit is fabricated. F) Power supply for heating the Nichrome ribbon and, as a consequence, sintering the frit. G) Initial temporary frit.

Different solvents such as water, MeCN, acetone, hexane or mixtures of them have been evaluated as driving or packing solvents whereas water, hexane, THF, MeCN, 2-propanol, MeOH and their mixtures have been tested as slurry solvents. Indeed, the selection of the slurry and driving solvents is critical for obtaining a well packed stationary phase. If capillary packing is poor, particle rearrangement during CEC separation can occur and thus holes inside the capillary are observed and low separation efficiency is obtained. Van den Bosh *et al.* observed that the use of acetone instead of n-propanol and MeOH as slurry and

driving solvents, respectively, allowed higher flow-rate during the packing. Consequently, more dense packing was obtained and, as a result, more efficient and stable columns were manufactured [53].

Additionally, good slurry solvents are those which provide a stationary phase suspension without any aggregated particles. It is recommended to agitate the slurry by sonication prior to packing the capillary in order to avoid particle aggregates. Furthermore, magnetic agitation (**Figure 7.5,B**) or other agitation mechanisms favor the slurry during the packing procedure.

On the other hand, the way of applying the pressure during the packing process has created some contradictions [54]. Some authors have recommended applying directly a high pressure to the driving solvent because it provides higher velocity and kinetic energy to the particles, and therefore, a dense and stable bed structure is obtained. However, other authors have observed that more uniform and dense beds are obtained when the pressure of the system is slowly increased.

Capillary packing can also be accomplished using supercritical CO_2 as carrier instead of an organic driving solvent. The packing system is similar to the previously described for capillary packing by pressure with slurry, but important modifications can be appreciated. With the aim of maintaining the system above the critical temperature and pressure of CO_2, the capillary end, where the temporary frit is located, is connected to a pressure restrictor. Moreover, the capillary column must be immersed into an ultrasonic bath with hot water. Packing pressures between 200 and 300 bar have been reported whereas packing temperatures ranging from 50 to 70ºC have been considered [55-57]. Furthermore, the system and material must be dried before beginning the packing procedure.

An alternative to packing by pressure is to pack the capillaries by gravity or centripetal forces. Reynolds *et al.* proposed the use of a simple device for capillary packing by gravity [58]. A 1-mL syringe was attached to the column to be packed by means of a tube connection. Afterwards, the slurry was placed in the syringe using acetone as slurry solvent because it possess low viscosity and thus stationary phase could be maintained in suspension for longer time. A suspension of about 10 mg of the stationary phase per

milliliter of acetone (100 μL) was employed for capillary packing and it was replaced each four hours in order to maintain the suspension of the particles. This approach can be quite tedious because it could take between 10 and 12 h to pack a capillary.

On the other hand, Fermier and Colón have designed a laboratory-made device for capillary packing by centripetal forces [59]. The device consists of a central reservoir where between 10 and 50 mg of stationary phase per milliliter of solvent are placed. The use of low viscosity solvents, such as acetone, as slurry solvent is recommended. Two extending arms made of stainless steel tubing are connected to the central device. The columns to be packed must be placed inside of the extending arms and they are attached to the central reservoir. A temporary frit has to be previously made in the other end of the capillaries. Rotation of the device forces the stationary phase particles to move toward the inlet frit of both capillaries and, consequently, capillaries are packed. The main advantage of this last approach is that two capillaries can be packed at the same time. Furthermore, it is a fast packing procedure according to the reported results [59]. Capillaries of 25 to 30 cm length and 50 μm of i.d. were packed in less than 15 min under a centrifugation velocity of 2000 rpm.

Finally, electrokinetic packing approaches have proposed as other alternative strategy for capillary packing. The main advantage of this technique is that it can be performed at low cost and besides, multiple columns can be simultaneously packed [50]. Electrokinetic packing procedures consist of introducing the stationary phase particles into the capillary by the action of the EOF [60]. In general, the ends of the capillary to be packed are inserted into two vials containing a background electrolyte (BGE). Both vials are equipped with electrodes, and the inlet vial also contains the slurry. A temporary frit is previously made in the end of the capillary located in the outlet vial [56]. Furthermore, both vials can be mechanically shaken to aid the packing process. Once a voltage is applied between both electrodes, capillary is packed. High separation efficiency can be achieved using capillaries packed by electrokinetic techniques. Lim *et al.* obtained higher efficiency for the separation of six opiate compounds by CEC than that achieved by a MEKC method. CEC separation was performed in a C18 packed capillary (15 cm packed length × 75 μm i.d., 5 μm particle size) manufactured following an electrokinetic packing approach [61]. Moreover, Wiedmer

et al. evaluated both electrokinetic packing and packing by pressure with slurry for the fabrication of packed capillaries with polyethylenimine-modified silica particles (23 cm packed length × 100 μm i.d., 5 μm). They claim to have achieved similar performance characteristics for columns manufactured following both techniques. However, they also observed that electrokinetic packed capillaries could only be employed at very low electric field strength [62].

Additionally, a comparison among different packing procedures, namely packing by pressure with slurry, CO_2 and electrokinetic packing, and packing with centripetal forces, was performed by Maloney and Colón. They demonstrated that similar capillary performance can be achieved independently of the followed packing approach, but they also concluded that packing by pressure with slurry is easier to implement because no special instrumentation is required [56].

In this thesis, two procedures for capillary packing by pressure with slurry are described in **Chapter 8** and **9**. Microscope images of a capillary column before and after being packed are shown in **Figure 7.6**.

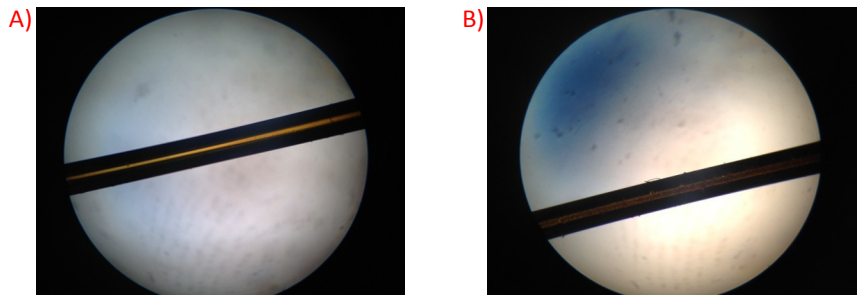

Figure 7.6. **Microscope images of: A) unpacked capillary (50 μm i.d.) and B) packed capillary (50 μm i.d.) with C18 particles (5 μm i.d.). Microscope magnification of 40-fold.**

7.4.2. Frit formation

Once capillary is packed, frits must be fabricated for retaining the stationary phase inside the column when packed capillaries are used. This stage of the capillary packing procedure has been declared as the "Achilles heel" of column packing [50]. In general, frits have to be mechanically strong to contain the stationary phase, and besides they have to withstand the

high pressure reached during the packing procedure or when the column is flushed for cleaning or conditioning. Nevertheless, porosity and permeability of the frits may be enough to ensure the solvent flow through them.

At the beginning of packed-column technology, CEC inlet frits were prepared by sintering silica beads. These particles were wetted with potassium or sodium silicate solutions, introduced into the capillary end and sintered by heating. This type of frit consists of beads connected by polysilicate network, purely silicate network and gaps between frit fragments. Consequently, these frits present high heterogeneity, while high irreproducibility is associated to the fabrication process. Moreover, capillary can be blocked during frit formation following this process, so capillary with poor performance characteristics are obtained.

Later on, it was shown that making the frits by sintering a section of the packed bed resulted in high homogenous frits [63]. As can be seen in **Figure 7.7.**, higher homogeneity is achieved when frits are prepared following this second approach.

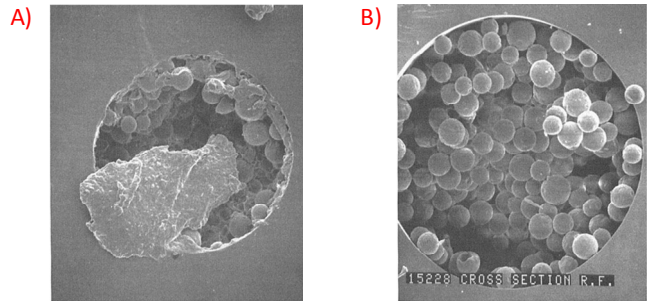

Figure 7.7. **SEM images of: A) cross-section of an end frit made in the usual way (from wet silica beads) and B) cross-section of a frit made from a portion of the packed stationary phase. Reproduced from [63].**

Normally, a located point of the capillary is heated, the stationary phase bed sinter at this point and, as a result, a retainer frit is made. The amount of heat required for sintering the particulate material depends on the column diameter and particle size as well as the employed heating element (i.e. splicer, thermal wire strippers, microtorch, burners, or assemblies with heating elements as it is shown in **Figure 7.5,F**) [50]. The main drawback

of this process is to control the heating temperature which ensures the resulting frit porosity and permeability.

When frits are prepared from sintering the stationary phase, the presence of free silanol groups is essential for frit formation by particle sintering. In absence of silanol groups, particles can sinter in presence of NaCl solutions (1-8 mM) [44]. Moreover, when capillaries are packed by pressure with slurry, slurry and driving solvents must be removed from the capillary prior to making the frits in order to guarantee the bed structure stabilization and a consistent frit fabrication. It is recommended forming the frits in presence of water. The presence of organic solvents during frit fabrication leads to the formation of carbonaceous residues, lowering the column performance [51]. Angus *et al.* have published an interesting paper including this and other advices for having success in packing the capillaries [54].

This approach is the most common technique for preparing the frits. Although it seems a simple procedure, it can lead to several problems if it is not properly carried out. These inconveniences have widely described by various authors [15,45,50,64]. First of all, heating the capillary for particles sintering involves the removal of the protective capillary coating, normally polyamide, and the column becomes fragile at frit positions. Furthermore, there is a difficulty in controlling the permeability of the frit and besides the fabrication of the frits is not always a reliable and reproducible process. Additionally, the EOF is different in each zone of the capillary (packed section, open section and frit zones) so pressure differences can be developed across the frits. Consequently, air bubbles can be generated at the boundary between the frit and the open section of the capillary. This leads to current instability during the analyses and therefore to low separation performance. Separation system is usually pressurized in order to avoid bubble formation [65]. An alternative strategy is to apply pressure only to the inlet vial. In this last case, the separation technique is often called pressure-assisted capillary electrochromatography (pCEC) because as it was suggested by Poppe: "when one sticks to pure electrodrive operation the pressure has to be applied to both ends of the tube" [66]. A loss of efficiency can be observed when pCEC is performed instead of traditional CEC. For that reason, the application of pressure to the inlet vial is limited to those applications where it is strictly required.

In a recent review, Cheong have summarized all techniques considered for frit formation since 1997 [67]. According to his conclusions, stationary phase sintering is the prevailing technique for frit formation because this fabrication procedure is the one which involves least frit-related band broadening. However, other alternatives have been proposed for overcoming the poor column-to-column reproducibility attributed to this methodology, and with the aim of manufacturing less fragile capillaries.

Monolithic frits have become an attractive alternative to sintered frits. Rocco and Fanali proposed the preparation of inlet frit through the in-situ thermal radical polymerization of vinylsulfonic acid using 1,4-bis(acryloyl)pipezarine as crosslinker [68]. No significant differences were observed on packed column performance compared to a column with both sintered frits. Furthermore, bubble formation was not observed and thus separations were performed without pressure assistance. An alternative to thermal polymerization for preparing monolithic frits is to consider photoinduced polymerization. This strategy was followed by D'Orazio and Fanali [69] for manufacturing C18 packed capillaries with both inlet and outlet frits prepared by the photo-polymerization of glycidyl methacrylate monomers using ethylene dimethacrylate as crosslinker. Although both methodologies shown good performance characteristics, monolithic frits tend to cause band broadening [67] and as consequence they could not be the best option for replacing sintering frits.

In addition to monolithic frits, the preparation of them from sol-gel solutions has been also investigated. Kato *et al.* reported that the preparation of monolithic sol-gel frits is easy and quick, and moreover, the length of sol-gel frits is easier to control compared to the fabrication of other photopolymerized or silicate frits [70]. Moreover, sol-gel solutions have been also employed as 'glue' for the preparation of frits with silica or ODS particles [71]. In this approach, silica or ODS particles are coated by sol-gel solution and when polymerization occurs, particles are bonded among them and to the column surface through polymer networks.

On the other hand, fritless packed capillaries techniques have been proposed with the aim of solving the bubble formation problem attributed to sintered frits. Mayer *et al.* proposed to retain the stationary phase in a capillary with tapered inlet end without considering any

outlet frit [72]. Inlet frit was made by a reduction of the i.d. of capillary inlet end from 100 μm to 10-15 μm. This approach is only possible if the stationary phase consists of charged particles electrophoretically attracted towards the anode and the electrophoretic velocity of the particles is larger than the EOF velocity. As the authors concluded, it seems to be a good alternative for those capillaries packed with particles which cannot be sintered. Furthermore, stationary phase of a packed column can be contained by physical retainers such as capillaries with lower i.d. connected by means of a polyetheretherketone (PEEK) tube to both packed capillary ends. Baltussen *et al.* demonstrated that capillaries of 100 μm of i.d. were able to be packed with ODS particles of 5 μm using retainers of 50 μm of i.d. As a result, electrical currents up to 50 μA were tolerated by the packed capillaries without observing bubble generation [73]. Because this type of fritless capillaries introduces a dead-volume to the column, band broadening can occur.

Other strategies have recently emerged as alternatives to traditional procedures for preparing frits. The use of single particles as frits or retaining the stationary phase by means of magnetic particles are two of the new proposals. Zhang *et al.* packed capillaries of 100 μm of i.d. with C18 particles (3 μm of particle size) using silica particles (110 μm in diameter and 0.9 μm of pore size) as frits [74]. Both inlet and outlet frits consisted of one of this particles introduced into the column with the aid of a capillary with lower o.d. than the i.d. of the column. On the other hand, micro-magnetic particles (10 μm) have been satisfactorily used for containing ODS particles (5 μm) in a column of 20 cm of packed length and 75 μm of i.d. [75]. Micro-magnetic particles were immobilized at frit positions because two magnets were directly and permanently glued onto the surface of the capillary column. Although several promising procedures are still under investigation, stationary phase sintering is the prevailing technique for frit preparation in packed columns for CEC [67].

7.5. CEC coupled to MS detection

In general, CEC-MS hyphenation is affected by the same drawbacks related to CE-MS coupling, but it also reports the same advantages (i.e. unequivocal identification of compounds, information about molecule structures). The absence of a column outlet

electrolyte reservoir for ensuring the electrical continuity of CEC system or the need of transferring the liquid phase coming through the CEC column to gas phase ions prior to mass analyzer, are common inconveniences whose solutions have been addressed in **Chapter 3**.

Verheij *et al.* were the first of carrying out CEC-MS coupling, and fast atom bombardment (FAB) was considered as ionization source [76]. However, other ionization sources such as APCI or ESI have been also assayed for CEC-MS hyphenation. Nowadays, ESI source is widely used due to its robustness, the effectiveness of ion production, the possibility of ensure the electrical contact without breakdown at the column outlet and the ability of working at low flow rates [77]. With regard to MS analyzers, CEC has been satisfactorily coupled to different MS instruments such as IT, Q, QqQ or ToF [77,78].

CEC-ESI-MS coupling have been satisfactorily accomplished by means of sheath-flow [79], sheathless [80,81] or liquid-junction interface [48,82]. Sheathless and coaxial sheath-flow interface for CE-MS hyphenation have been widely discussed in **Chapter 3**. In this section, only liquid-junction interface is described for being the type of interface employed in the CEC-MS method developed in **Chapter 9**.

The liquid-junction interface consists of a tee in which a cross flow of the make-up fluid is mixed with the CEC mobile phase, ensuring that the electrical circuit of the CEC system is closed. The exit of the CEC column and ESI emitter inlet are faced in the center of the tee, leaving a gap of 20 to 200 μm between them [83,84]. The fluid for the liquid-junction is delivered through the third branch of the tee piece and also acts as outlet buffer reservoir. The gap between both capillaries is known as liquid-junction, and the transference of the analytes coming through the CEC system to the ESI chamber occurs on it [85]. A benefit of liquid-junction interface compared to sheath-flow and sheathless interface is that CEC column is partially decoupled from the ESI emitter capillary [78].

In spite of the gap that separates both capillaries, peak broadening can be avoided if they are properly aligned and a reasonable liquid pressure is applied to the system [83]. If the mixture procedure between the sample from CEC and the fluid from liquid-junction is too turbulent or too slow, band broadening can be appreciated and consequently, the high

efficiency attributed to CEC will be lost. For this reason, the liquid pressure is a critical parameter to control.

Furthermore, sample is diluted when reaches the liquid-junction, so signal sensitivity will be decreased. With the aim of reducing this effect, nano-liquid-junction interfaces where sample dilution is minimized have been recently proposed [84,86-88]. A schematic representation of a nano-liquid-junction interface is shown in **Figure 7.8**. It consists of a polymethyl methacrylate (PMMA) block which is crossed by a hole where packed column and ESI emitter are placed. An additional orifice has been drilled on the block, so direct access is provided to the liquid-junction where packed column and ESI emitter are facing each other. This orifice is closed by a Teflon screw. A perpendicular channel to the capillary position has been done for supplying the fluid to the liquid junction. It is connected on the top with the sheath liquid reservoir, while the lower exit is connected to a Hamilton valve which has to be closed during CEC runs. It can be opened between analyses for replacing the fluid on the liquid-junction [87].

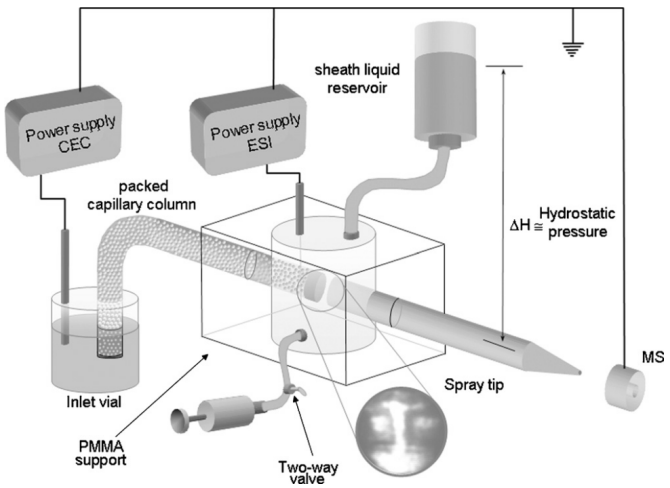

Figure 7.8. **Scheme of the CEC–MS instrument with a liquid-junction ESI interface. The distance between the packed capillary and the tip was about 100 μm. Reproduced from [87].**

In **Chapter 9**, a novel CEC-ESI-MS method using an IT analyzer is proposed for the determination of 5-NDZ residues in urine samples. The detection mechanism of an IT has been already described in **Chapter 3**. Furthermore, CEC-ESI-MS coupling was achieved by means of the nano-liquid-junction interface represented in **Figure 7.8**.

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Determination of 5-NDZ residues in milk by capillary electrochromatography with packed C18 silica beds

Abstract ► This chapter presents a novel method for analyzing 5-NDZ residues in milk samples by CEC using laboratory-made packed columns, produced by carrying out a high pressure packing procedure using acetone as driving solvent and C18 silica uncapped particles (5 μm particle size) as packing material. Column frits resulted from sintering the proper stationary phase by heating the packed material for 20 s with a nichrome ribbon (80% Ni – 20% Cr, 28 cm × 2 mm × 0.2 mm, electric resistance 1.3 Ω) connected to a 7 V AC power supply. Laboratory-made C18 silica packed capillaries (40 cm × 50 μm i.d.) were employed for the determination of 5-NDZ drugs. Milk samples were treated by a SALLE procedure followed by a SPE with Oasis®HLB cartridges prior to their injection. Samples were hydrodynamically injected into the column for 120 s at 11.5 bar. Afterwards eight 5-NDZ compounds were separated in isocratic mode under an applied voltage of 27 kV and a temperature of 30 °C. The selected mobile phase consisted of a mixture 60:40 (v/v) MeCN/ammonium acetate buffer (2.5 mM, pH 5). Separation was monitored at 320 nm and performed in less than 15 min. The method was characterized in terms of linearity ($R^2 \geq 0.993$) and precision (repeatability, RSD ≤ 12.2% and reproducibility, RSD ≤ 14.5%), obtaining detection limits lower than 29 μg/L for all the compounds under study.

8.1. Background

During the last years several contributions have been reported about analytical methods intended to determine 5-NDZ in food residues. LC with UV detection [1,2] or MS detection [3-6] is the most employed technique. Additionally, GC coupled to MS [7,8] and CE with UV detection [9,10] have also been proposed. The high solvent consumption and waste generation is the main drawback of LC methods, which could be avoided by employing greener techniques such as CE. However, the applicability of CE is limited due to the low sensitivity reached by this methodology as consequence of the short optical path length in the UV detection and injection of low sample volumes. In order to combine the advantages of both methodologies, one of the CE modes, named CEC, has been proposed as a hybrid separation technique which shows high efficiency and selectivity [11].

Three different CEC modalities can be considered depending on the stationary phase morphology: OT, monolithic and packed CEC. OT-capillaries offer poor sensitivity as a consequence of the narrow bore size of the capillary and low separation capability due to the low phase ratio caused by the limited surface area of the stationary phase [12]. On the other hand, monolithic capillaries can suffer from a lack of stability because of polymeric monolithic stationary phases tend to swell in organic solvents. Moreover, the preparation of polymeric monoliths usually leads to micropores which results in low efficiency and peak asymmetry. Monolithic capillaries also possess low column capacity attributed to their low specific surface area, providing some limited applications [13]. In contrast to the above mentioned types of columns, packed capillaries offer higher surface area which improves sample loading capacity. The success of these packed capillaries lies in this advantage, becoming the most commonly employed CEC capillaries. They are commercially available but the problems of bubble formation, column fragility and above all, their high price, retard their extensive use [14], so researchers often prepare their own columns and as a result, several protocols have been proposed for packing capillaries. However, some authors still consider these procedures too arduous, an art or even as 'black magic' methodologies because they require specific skills to achieve highly efficient and reproducible capillaries [15].

Since packed columns in CEC were introduced by Pretorius *et al.* in 1974 [16], different packing techniques have been tested in order to obtain the perfect packed column, even comparisons among them have been reported [17-19]. But generally, all packing methodologies have a common challenge that is to avoid poorly packed capillaries which can lead to low efficiency, poor resolution and asymmetric peaks. Slurry packing with pressure [20-22] is the most established methodology because it is simple to implement and it does not require long recipes for start-up. The main difficulty of column packing resides in the production of the frits because they have to retain the stationary phase in the capillary when a separation voltage is applied and, at the same time, to allow the mobile phase to penetrate freely through them. If frits are too thin, they will not retain the sorbent, and if they are too thick and non-porous, the mobile phase will not pass through them. In these packed columns, EOF is not homogeneous along the capillary, showing different zones (packed part, frit and open part) which produces pressure differences across the frit [23]. As a result, bubbles are formed in the boundary region between the frit and the unpacked part of the capillary, causing lost of efficiency and current disruptions. In order to solve these problems different frit types have been proposed and compared [24,25]. Therefore, the major challenge of the packing procedure is the satisfactory fabrication of retention frits. Excluding methodologies such as single particle [26] and internal taper approaches [27], sintering is the prevailing mode in frit fabrication because it leads to the least frit-related band broadening [14].

In this chapter an easy methodology is proposed for the fabrication of packed capillaries and their subsequent use for monitoring 5-NDZ residues in milk by CEC. According to the reviewed bibliography, there is just one published CEC method that involves 5-NDZ determination by CEC [28]. Nevertheless, this work is focused on monolithic MIP-based capillary development and 5-NDZ standard separation is proposed without any application to real matrixes. Moreover, it is worth to mention that most of the papers about CEC are focused on testing new sorbents or developing new procedures to make CEC columns. However applications of these developments to solve practical problems with real samples are not usually carried out. In this sense, CEC remains as a technique with a great potential to be explored.

8.2. Materials and methods

8.2.1. Materials and reagents

All reagents were analytical reagent grade, unless indicated otherwise, and solvents were HPLC grade. Ammonium hydroxide (30%, v/v), sulfuric acid (98%, v/v), NaCl and NaOH were obtained from Panreac-Química (Madrid, Spain). MeOH and acetone were purchased from VWR International (West Chester, PA, USA) while MeCN and acetic acid were supplied by Sigma-Aldrich (St. Louis, MO, USA). Formic acid (98–100%, v/v), Tris and HCl (37%, v/v) were acquired from Merck (Darmstadt, Germany). Ultrapure water (Milli-Q plus system, Millipore, Bedford, MA, USA) was used throughout the work.

Analytical standards of DMZ, RNZ, CRZ, ORZ, MNZ, TNZ were supplied by Sigma-Aldrich (St. Louis, MO, USA) while analytical standards of IPZ, SCZ and TRZ were purchased from Witega (Berlin, Germany). Individual standard solutions of each 5-NDZ were prepared at 1 mg/mL by dissolving each pure compound in MeCN. These solutions were stored in dark bottles at –20°C and equilibrated to room temperature before use. They were stable for at least six months. Additionally, an intermediate standard solution containing 100 μg/mL of each 5-NDZ except for CRZ (200 μg/mL) was obtained by mixing aliquots from the individual standard solutions and their subsequent dilution with MeCN. It was stored at 4°C avoiding exposure to direct light. It was stable for at least three months. Working solutions were prepared in water from the intermediate standard solution. Milk samples were also fortified from the intermediate standard solution according to desired concentrations.

Packed columns consisted of uncoated fused silica capillaries of 50 μm, 75 μm and 100 μm i.d. which were purchased from Polymicro Technologies (Phoenix, AZ, USA) and LiChrospher RP-C18 non-encapped particles (5 μm particle size) (Agilent Technologies, Waldbronn, Germany) which were recycled from a damage LC column.

Oasis®HLB cartridges (60 mg, 3 mL) (Waters, Milford, MA, USA) were considered for the sample treatment procedure. Clearinert™ 13 mm syringe filters with 0.22 μm nylon

membrane (Wilmington, DE, USA) were used for sample filtration prior to sample injection into the CEC system.

8.2.2. Instrumentation

A SP-400 Nanobaume™ column packing unit (Western Fluids Engineering, Wildomar, CA, USA) coupled to a PU-2080 high pressure pump (Jasco, Easton, MD, USA) was employed for capillary packing. Capillary packing process was assisted by a MC-8 magnetic stirrer (Bunsen, Madrid, Spain). Capillary frits were made using a nichrome ribbon (80% Ni – 20% Cr, 28 cm × 2 mm × 0.2 mm, electric resistance 1.3 Ω) connected to a 7 V AC power supply which was made by a local technician (**Figure 8.1**).

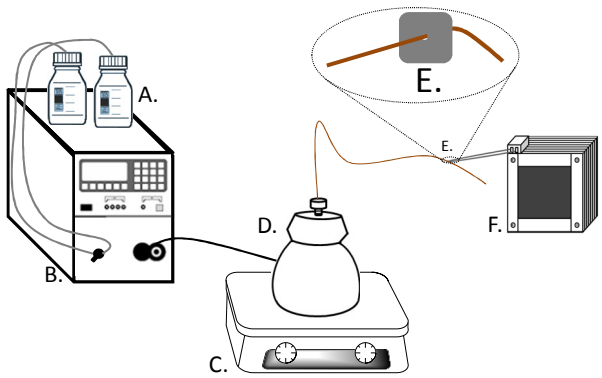

Figure 8.1. Packing capillary setup diagram: (A) solvent containers, (B) high pressure pump, (C) magnetic stirrer, (D) packing capillary unit, (E) capillary placed through the hole made on the nichrome ribbon; particles from the stationary phase are sintered at this capillary position and (F) 7 V AC power supply.

CEC experiments were carried out with an Agilent 7100 CE System (Agilent Technologies, Waldbronn, Germany) equipped with a DAD. Data were acquired using the supplied software with the CE system (HP ChemStation, Version B.02.01).

A Universal 320R centrifuge (Hettich Zentrifugen, Tuttlingen, Germany) and a vortex-2 Genie (Scientific Industries, Bohemia, NY, USA) were also used. Buffer pH was adjusted with a pH meter (Crison model pH 2000, Barcelona, Spain) with a resolution of ±0.01 pH unit.

8.2.3. C18 silica packed capillaries fabrication procedure

A fused silica capillary (50 μm i.d. and 20 cm longer than the desired packed capillary length) was rinsed with a 1 M NaOH solution for 10 min at 5 bar. Subsequently it was flushed with deionized water for 5 min at 5 bar, followed by acetone for 5 min at 5 bar. After conditioning, the capillary was mounted in the packing unit and a seven step protocol was carried out for capillary packing and frit formation (**Figure 8.2**).

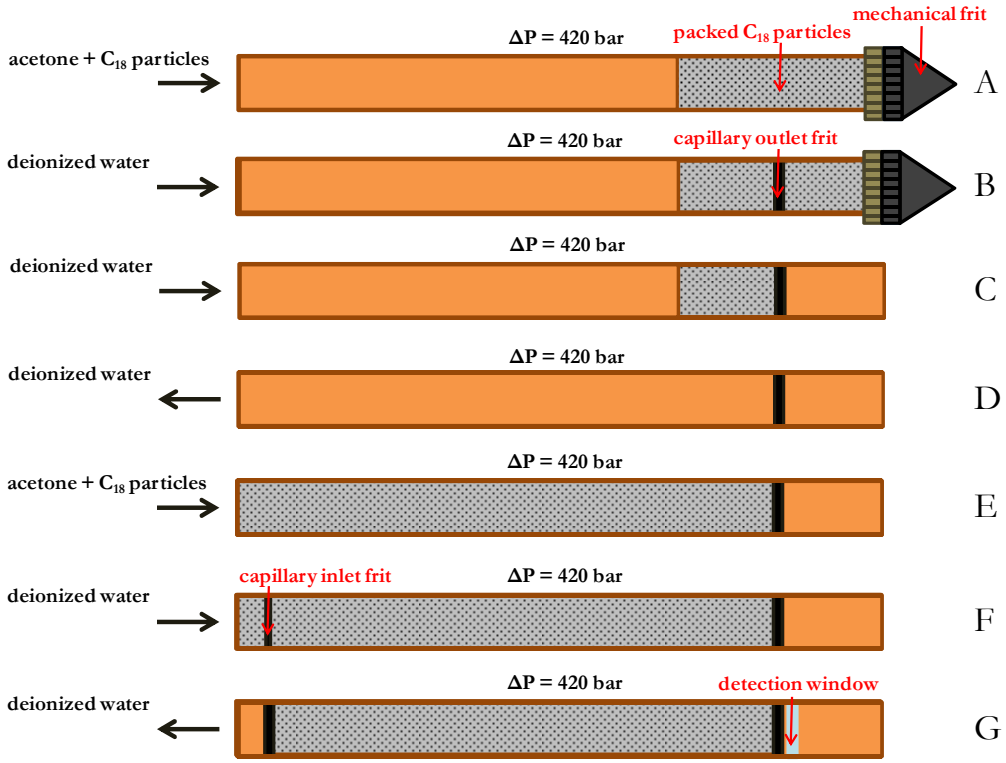

Figure 8.2. Capillary packing procedure scheme: (A) capillary is partially filled with C18 silica particles; (B) deionized water is passed through packed capillary for 1 h at 420 bar and outlet frit is made 10 cm far away from the mechanical retainer. C18 particles are sintered and consequently frit is made by heating a nichrome ribbon for 20 s. Deionized water is passed through the capillary for 1 h at 420 bar when frit formation is carried out; (C) mechanical retainer is removed. (D) Capillary is emptied. Outlet frit is able to support a pressure of 420 bar; (E) capillary is fully packed at high pressure; (F) deionized water is passed through packed capillary for 1 h at 420 bar. Afterwards, inlet frit is sintered considering the desired packed capillary length as outlet frit was made; (G) the excess of stationary phase at capillary inlet is removed. Detection window is done and capillary ends are cut according to the desired capillary dimensions.

Experimental set-up, which mainly consisted of a high pressure pump connected to a Nanobaume™ column packing unit, is shown in **Figure 8.1**. The driving solvent (acetone) is initially propelled by a high pressure pump to the packing unit where C18 silica particles (20 mg) are suspended in 1.5 mL of slurry solvent (acetone) by magnetic agitation (**Figure 8.2, step A**). At the same time that the system pressure increases from 0 to 420 bar, acetone carries the particles along the capillary. Particles are initially retained inside the capillary because of a mechanical frit made of a 0.5 μm membrane filter is placed at the end of it. As a consequence, the capillary is packed. Packing velocity is increased and much more homogeneous packing is obtained if a plastic screw is coupled to the mechanical frit and it is removed when the system pressure reaches 200 bar. When the capillary is partially filled with C18 silica particles, 12 cm far away from the mechanical frit, the high pressure pump is turned off. As result, system pressure dropped down and capillary packing is interrupted. Afterwards, capillary is rinsed with deionized water for 1 h at 420 bar to ensure a proper packing of this capillary portion and to ensure that acetone is totally drained from the capillary (**Figure 8.2, step B**). Then, outlet frit is made at 10 cm far away from the mechanical retainer. To make the frit, the capillary is introduced through a small hole (380–400 μm) that was previously made in the centre of the 28 cm nichrome ribbon. A 7 V AC power supply is connected to the nichrome ribbon for 20 s in order to heat the C18 silica particles so they are sintered. Only particles in contact with the ribbon surface and the closer ones are sintered. Frit formation is done by passing deionized water through the capillary at 420 bar. Once frit is made, mechanical frit is taken out and the stationary phase is removed from the capillary (**Figure 8.2, steps C** and **D**).

Then, the capillary is totally packed in one single step in order to obtain a compact packing (**Figure 8.2, step E**). The beads travel up the capillary using acetone as driving solvent at 420 bar. The packing procedure can be monitored by the observed light/dark transition in the capillary. After filling the capillary, deionized water is used to flush the column for 1 h in order to consolidate the packed bed (**Figure 8.2, step F**). As it was done before, inlet frit is fabricated at the desired length from outlet frit.

Finally, a detection window is made 2 mm from the outlet frit using hot sulfuric acid for removing capillary coating (**Figure 8.2, step G**). In addition, outlet capillary end is cut

8.5 cm away from the detection window and the inlet capillary end is cut 2 mm away from the inlet frit.

8.2.4. Sample treatment protocol

Whole cow's milk samples were purchased in a local supermarket. A sample of 3 mL was placed in a conical tube and it was centrifuged for 5 min at 9000 rpm for removing the majority of the fat content. Liquid phase was collected avoiding the upper fat layer. Sample deproteinization was carried out by adding 4 mL of MeCN to the liquid sample. Mixture was homogenized by vortex for a few seconds and it was centrifuged for 10 min at 9000 rpm, occurring protein precipitation. Supernatant was collected and 0.8 g of NaCl were dissolved in it by vortex for 2 min. Sample was centrifuged for 5 min at 9000 rpm and two separated phases were obtained based on SALLE procedure. Highly saline aqueous phase was discarded while 3.3 mL from the upper organic phase were dried under nitrogen current at 25°C. Sample was reconstituted in 1.5 mL of deionized water.

Afterwards, a sample clean-up was performed following a SPE protocol. An Oasis®HLB cartridge (60 mg, 3 mL) was conditioned with 1 mL of MeOH and 2 mL of deionized water. Then the sample was loaded onto the cartridge at 1 mL/min. Later the cartridge was washed up with 2 mL of deionized water at 1 mL/min and analytes were eluted with 2 mL of MeOH at 1 mL/min by applying vacuum. Sample was dried under a nitrogen current at 25°C and it was reconstituted in 200 μL of deionized water. Finally, it was filtered through a 0.22 μm nylon filter and it was analyzed by the proposed CEC method.

8.2.5. Capillary electrochromatography method

A C18 packed capillary (40 cm packed length × 50 μm i.d.) was used for 5-NDZ determination. New packed capillaries were initially rinsed with mobile phase in the CE instrument by pressure (11.5 bar) for one hour. Then, a voltage of 27 kV was applied between two vials containing mobile phase for 30 min. During voltage application, inlet and outlet vials were pressurized to 5 bar in order to suppress bubbles formation. After capillary conditioning, a stable baseline was observed. At the beginning of each working day, capillary conditioning consisted of discarding the first two runs of the day. Between

runs the capillary was flushed with mobile phase for 2 min at 11.5 bar of pressure. After a working day, the capillary was stored with mobile phase and capillary ends were placed in vials containing mobile phase.

Samples were hydrodynamically injected for 120 s at 11.5 bar. After sample injection, a plug of mobile phase was hydrodynamically injected for 20 s at 11.5 bar to ensure sample injection reproducibility. Analyses were performed under isocratic conditions using a mixture 60:40 (v/v) MeCN/ammonium acetate (2.5 mM, pH 5) as mobile phase. 5-NDZ separation was carried out at 30°C under an applied voltage of 27 kV. A voltage ramp from 0 to 27 kV for 0.5 min was programmed at the beginning of the run, obtaining a separation current of 0.4 μA. UV detection was employed and analytical signals were monitored at 320 nm (244 nm for CRZ detection).

8.3. Results and discussion

8.3.1. Capillary packing optimization

In the fabrication of packed capillaries C18 particles were selected as stationary phase because it has been demonstrated that this phase has high selectivity for 5-NDZ separation by LC methods [1,3,5].

Although MeCN is a quite common driving solvent employed for packing stationary phases and it was our first choice, after several experiences, an alternative solvent was needed. Capillaries that were packed using MeCN presented holes through the stationary phase after some CEC runs, leading to irreproducible analyses in terms of elution times. This effect has already been noticed by van den Bosch *et al.* [29] when MeOH was used as driving solvent as a consequence of particle rearrangement when voltage was applied. Finally, acetone was selected as driving solvent instead of MeCN, accomplishing more stable and very efficient capillaries. Packing was carried out at 420 bar that is very close to the pressure limit of the employed pump (500 bar). Lower pressures are not recommended because they produced poor and inefficient packed capillaries.

While the capillary packing procedure resulted relatively easy to develop, to find an adequate way for making the frits was a harder task. At the beginning, thermal polymerization of silica particles wetted with sodium silicate solution was tested as frit formation methodology [30]. Although different experimental parameters were changed, it always resulted in inconsistent or nonporous frits. Particle sintering was proposed as alternative to make the frits, considering that the selected C18 silica particles are non-endcapped, hence they can sinter.

Two parameters are involved on a sintering process: time and temperature. It is difficult to measure with precision the sintering temperature and, although most of the CEC papers refer to this parameter to explain frit fabrication, they do not indicate how to control it. Therefore frit fabrication can be more reproducible if instead of the temperature value, the parameters leading to that sintering temperature are given, i.e., dimensions and electric resistance of the wire or ribbon and applied voltage to the wire/ribbon for producing the frit, which are easier to measure. In any case, if the sintering temperature is very high, the sintering time has to be very short to obtain permeable frits and not to make the capillary fragile at this zone. Sintering times as short as just a couple of seconds can lead to irreproducible results.

For particles sintering process, nichrome ribbon (80% Ni – 20% Cr; cross section area of 2 mm × 0.2 mm; electric resistance 1.3 Ω) of different lengths were tested. Finally, a 28 cm metallic strip was chosen because higher sintering times could be applied avoiding the formation of nonporous frits. Sintering times from 5 to 25 s were considered, reaching an optimum at 20 s as a good compromise between frit permeability and robustness.

Moreover, frit fabrication must be carried out in absence of driving solvent. Acetone is rapidly expanded when heat is applied to the capillary and as a result, air bubbles can be formed and ineffective frits can be obtained. Therefore inlet and outlet frits were made under a water flow at high pressure for avoiding bubble formation.

8.3.2. Electrochromatographic separation of 5-NDZs

Effect of mobile phase composition

CEC separations are usually carried out under isocratic conditions. Therefore the selection of the proportion between the organic solvent and the buffer in the mobile phase is crucial for achieving satisfactory peak resolutions. Initially a mixture 65:35 (v/v) MeCN/ammonium acetate buffer (14 mM, pH 5) was considered as mobile phase, reaching an ammonium acetate concentration of 5 mM. Other buffers and pH values (14 mM ammonium formate, pH 3; 71 mM Tris–HCl, pH 8) were also evaluated. Ammonium formate buffer (pH 3) resulted in worse peak resolution as well as Tris–HCl buffer (pH 8) in comparison with the firstly used ammonium acetate buffer (pH=5). Moreover, Tris–HCl buffer was discharged because of high pH values can damage C18 silica particles according to manufacturer's specifications (recommended pH range between 2 and 7.5). Different mixtures of MeCN/ammonium acetate buffer containing percentages of the organic solvent from 55% to 70% were studied (**Figure 8.3**).

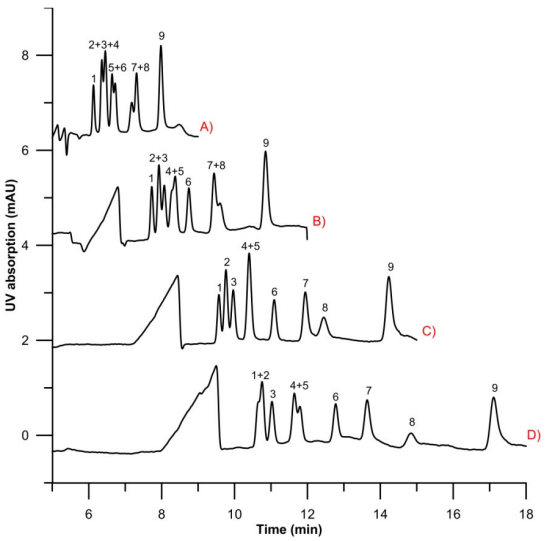

Figure 8.3. **Influence of MeCN content in the mobile phase. Electrochromatograms were obtained using different mixtures of MeCN/ammonium acetate buffer (pH 5) (in all cases, final ammonium acetate concentration in the mobile phase, 5 mM): A) 70:30 (v/v) MeCN/ammonium acetate buffer (17 mM, pH 5); B) 65:35 (v/v) MeCN/ammonium acetate buffer (14 mM, pH 5); C) 60:40 (v/v) MeCN/ammonium acetate buffer (13 mM, pH 5); D) 55:45 (v/v) MeCN/ammonium acetate buffer (11 mM, pH 5). Standard solutions containing 2 µg/mL of each compound were injected for 30 s at 11.5 bar. Separation conditions: 20 kV and 20°C. Packed capillary dimensions: 25 cm × 75 µm i.d. Signals were monitored at 320 nm. Peaks are numbered by elution order.**

As it was expected, longer retention times were observed when MeCN percentage in the mixture was decreased due to lower elution strength. In terms of peak resolution, a greater number of compounds were resolved when a mixture 60:40 (v/v) MeCN/ammonium acetate buffer (13 mM, pH 5) was considered as mobile phase. So it was selected as optimum.

Additionally, the use of MeOH instead of MeCN as organic solvent in the mobile phase was also explored. As it occurs in LC separations, longer retention times were obtained when MeOH was added to the mobile phase due to its lower elution strength. As a result, MeCN was selected because 5-NDZ separation using MeOH took more than twice the time required when MeCN was employed.

The pH value of the mobile phase buffer was deeply studied between 4 and 6 (**Figure 8.4,I**), but peak resolution improvement was not observed, so pH 5 was kept. The use of ammonium formate instead of ammonium acetate was evaluated as alternative mobile phase buffer (**Figure 8.4,II**). Solutions of each buffer (50 mM) were prepared from their respectively acids and adjusted to pH 5 with ammonium hydroxide solution (1:5, v/v). The required buffer volume was diluted in water and mixed with MeCN according to the selected proportion (v/v). This assay was carried out considering a buffer concentration of 13 mM. Better peak resolutions were obtained when ammonium acetate was employed instead of ammonium formate.

Finally, ammonium acetate concentration was studied from 2.5 mM to 25 mM. The main observed effect was the decrease of the analysis time when lower buffer concentrations were considered without any effect on the resolution, so a final concentration of 2.5 mM ammonium acetate (pH 5) was selected. Lower concentrations were not evaluated because low and unstable separation currents can be obtained. Under the selected conditions, 1 mM represents the ammonium acetate concentration in the mobile phase.

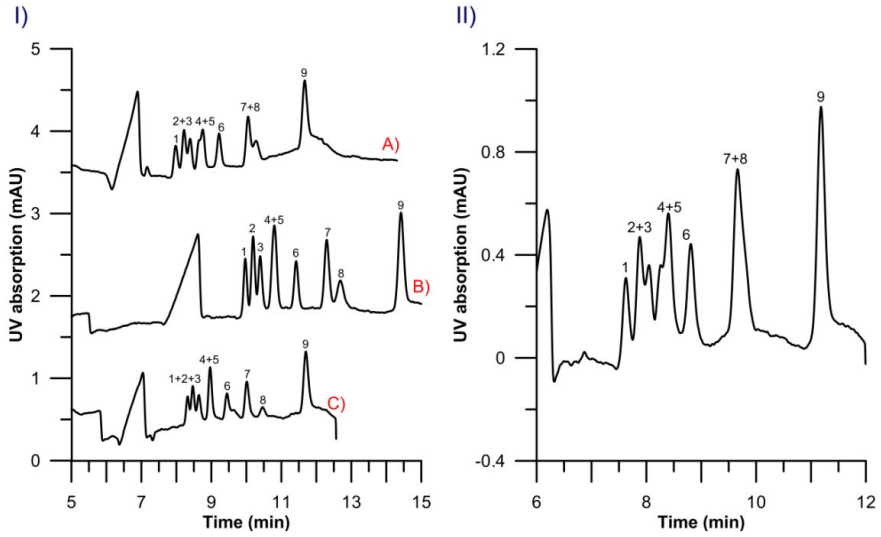

Figure 8.4. I) Influence of ammonium acetate buffer pH: A) 5.8; B) 5.0; C) 4.0. II) 5-NDZ separation by CEC-UV using ammonium formate buffer instead of ammonium acetate buffer. In all cases a mixture 60:40 (v/v) MeCN/buffer (13 mM, pH 5) was employed as mobile phase. Standard solutions containing 2 µg/mL of each compound were injected for 30 s at 11.5 bar. Other separation conditions: 20 kV and 20°C. Packed capillary dimensions: 25 cm × 75 µm i.d. Signals were monitored at 320 nm. Peaks are numbered by elution order.

Effect of capillary dimensions

It is more affordable to study the effect of both, the packed length and the i.d. with laboratory-made CEC columns than with the commercial ones due to their high price. In this work different packed capillary lengths have been evaluated (8, 25, 32 and 40 cm) using laboratory made packed capillaries of 75 µm i.d. As it was expected, higher separation time was observed when packed capillary length was increased, improving peak resolution. **Figure 8.5** shows how peaks 1, 2 and 3 are much better resolved when analyses were carried out in a 40 cm length packed capillary.

Besides, packed capillaries (40 cm) of different i.d. (50, 75 and 100 µm) were tested. A packed capillary of higher id provides higher sensitivity because a much greater amount of sample is injected under the same conditions (injection time and pressure). However, lower peak efficiency is reached when higher id columns are used, reducing peak resolution. Diffusion along the axis is reduced when a smaller packing diameter is selected and column efficiency is improved. Peak efficiency in terms of theoretical plates was improved between

1.3 and 1.7-fold when capillary i.d. was decreased from 75 μm to 50 μm (**Figure 8.6**). Furthermore, it was improved 1.6–2.3 times when a packed capillary of 50 μm i.d. was employed instead of a 100 μm i.d. capillary. Finally, a 50 μm i.d. column was chosen for 5-NDZ separation because peak resolution was crucial for the proposed method.

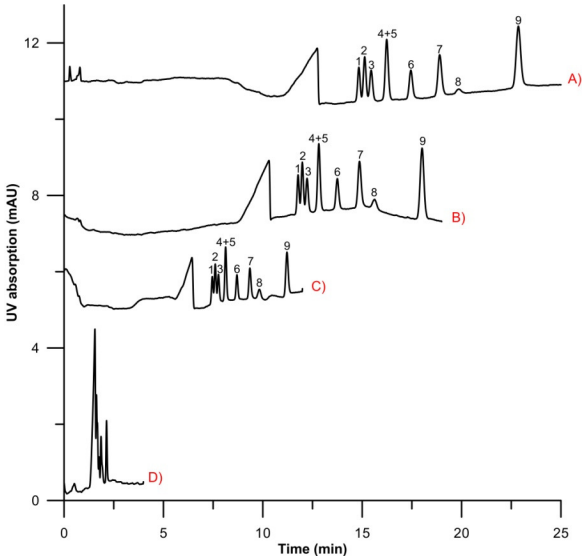

Figure 8.5. Influence of packed capillary length evaluated in capillaries of 75 μm i.d. Electrochromatograms were obtained using different packed capillary lengths: A) 40 cm; B) 32 cm; C) 25 cm; D) 8 cm. Standard solutions containing 2 μg/mL of each compound were injected for 60 s at 11.5 bar. Separation conditions: 20 kV and 20°C. Mobile phase: 60:40 (v/v) MeCN/ammonium acetate buffer (pH 5, 2.5 mM). Signals were monitored at 320 nm. Peaks are numbered by elution order.

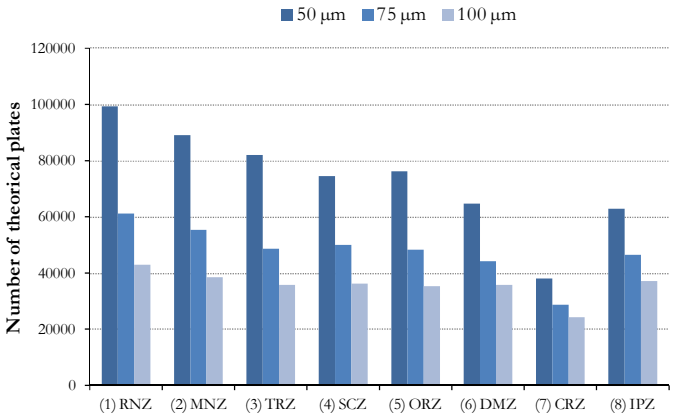

Figure 8.6. Peak efficiency, in terms of theoretical plates, for each compound as a function of the selected capillary i.d.

Effect of separation voltage and temperature

Separation voltage was evaluated between 20 and 30 kV, reducing the analysis time from 20 to 13 min in this voltage range. However, RNZ and MNZ peak resolution and MNZ and TRZ peak resolution decreased when separation voltage was increased. As a compromise between analysis time and peak resolution, a separation voltage of 27 kV was established.

Separation temperature was ranged from 17 to 35°C. An increase in the temperature improved CRZ peak efficiency; however, MNZ and TRZ peak resolution was drastically reduced. Considering both effects, a separation temperature of 30°C was selected.

Injection optimization

Standard solutions in water were hydrodynamically injected in the capillary at 11.5 bar. Injection time was studied from 60 to 180 s (**Figure 8.7**). Maximum sensitivity for all 5-NDZ compounds in terms of peak height was accomplished for an injection time ranging between 120 s and 150 s. Finally 120 s was set up as injection time because longer injection times resulted in band-broadening and peak resolution losses.

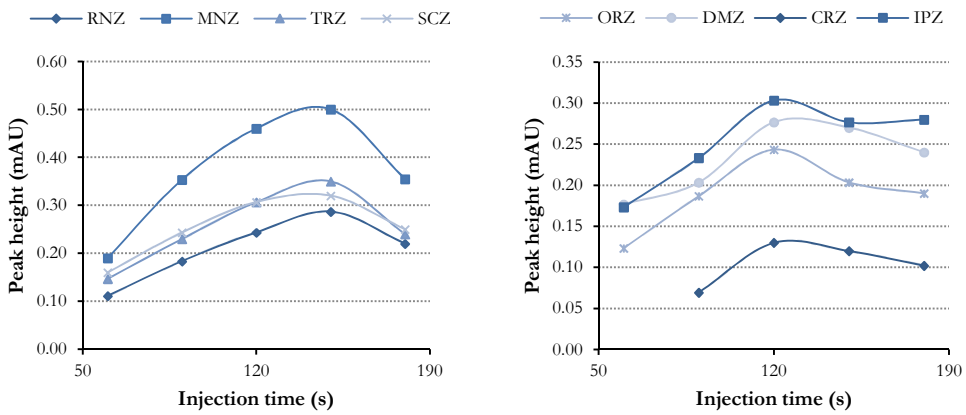

Figure 8.7. **Injection time influence on peak height. Standard solutions containing 2 µg/mL of each compound were injected at 11.5 bar.**

8.3.3. Method characterization in standard solutions

In order to check the suitability of the laboratory-made packed capillaries for 5-NDZ determination, the proposed analytical method was instrumentally evaluated in terms of linearity, LODs, LOQs, intra-day and inter-day precision. Besides, reproducibility among different laboratory-made packed capillaries was also studied. Method characterization was finally proposed for eight of the nine firstly considered 5-NDZs, excluding TNZ because it co-eluted with SCZ under the final separation conditions. All peak signals have been monitored at 320 nm, except for CRZ peak (244 nm).

Calibration curves and performance characteristics

Standard calibration curves were established considering 5-NDZ standard solutions at six different concentration levels. Two replicates of standard solutions of each concentration were analyzed in duplicate. Peak area was considered as function of analyte concentration. Instrumental LODs and LOQs were calculated as the minimum analyte concentration yielding a S/N equal to three and ten respectively (**Table 8.1**). According to the results, satisfactory instrumental LODs have been reached. All 5-NDZ drugs were detected at low μg/L levels, ranging from 38 to 67 μg/L (except for CRZ, 160 μg/L).

Table 8.1. **Statistical and instrumental performance characteristics of the proposed CEC-UV method for 5-NDZ determination.**

Analyte	Linear range (μg/L)	R^2	LOD (μg/L) $3 \times S/N$	LOQ (μg/L) $10 \times S/N$
RNZ	177 - 5000	0.996	53	177
MNZ	128 - 5000	0.996	38	128
TRZ	203 - 5000	0.996	61	203
SCZ	177 - 5000	0.997	53	177
ORZ	222 - 5000	0.996	67	222
DMZ	153 - 5000	0.997	46	153
IPZ	186 - 5000	0.996	56	186
CRZ	534 - 10000	0.996	160	534

An electrochromatogram showing the separation of eight 5-NDZ compounds under the optimized conditions is included in **Figure 8.8**.

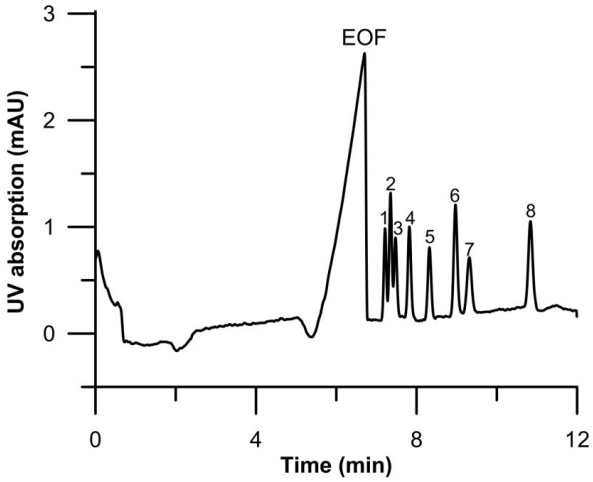

Figure 8.8. **Electrochromatogram of a 5-NDZ standard solution containing 1200 µg/L of each studied compound except for CRZ (2400 µg/L). A C18 packed capillary (40 cm × 50 µm i.d.) was employed for the separation according to the optimized CEC method. Separation conditions: 27 kV and 30°C. Mobile phase: 60:40 (v/v) MeCN/ammonium acetate buffer (2.5 mM, pH 5). Sample was injected for 120 s at 11.5 bar. Signals were monitored at 320 nm. Peaks: 1, RNZ; 2, MNZ; 3, TRZ; 4, SCZ; 5, ORZ; 6, DMZ; 7, CRZ; 8, IPZ.**

Precision assays

Precision studies were carried out in order to evaluate the repeatability (intra-day precision) and intermediate precision (inter-day precision) of the proposed CEC method for 5-NDZ determination. In the repeatability study, three standard solutions at three concentration levels were analyzed in triplicate. Intermediate precision (inter-day) was assayed at three different concentration levels by analyzing a standard solution in triplicate per day for five consecutive days. Results expressed as RSDs (%) of peak areas are shown in **Table 8.2**. In all cases RSDs were lower than 11.2%. In addition, four CEC columns were prepared following the same experimental conditions and they were tested in order to verify the reproducibility within columns. Standard solutions at three different concentrations were analyzed in triplicate in each capillary.

Table 8.2. Precision results for 5-NDZ standard analyses.

Analyte	Repeatability (intra-day) (% RSD, n = 9)			Intermediate precision (inter-day) (% RSD, n = 15)			Reproducibility among 4 different capillaries (inter-capillary) (% RSD, n = 12)			
	300 µg/L	***1500 µg/L***	***3000 µg/L***	***300 µg/L***	***1500 µg/L***	***3000 µg/L***	***300 µg/L***	***1500 µg/L***	***3000 µg/L***	**Elution time (min) (% RSD, n = 36)**
RNZ	6.9	2.6	8.2	10.5	7.1	5.3	6.4	5.3	4.7	7.7 (8.4)
MNZ	4.9	2.7	8.2	11.2	7.5	5.0	6.8	4.5	4.8	7.8 (8.4)
TRZ	8.1	4.0	7.9	8.6	7.7	5.0	7.7	5.5	5.7	8.0 (8.4)
SCZ	7.6	2.6	8.5	8.2	8.4	5.8	5.8	4.4	5.1	8.3 (8.5)
ORZ	5.4	3.1	8.8	10.0	9.3	6.7	7.9	4.6	4.0	8.9 (8.7)
DMZ	6.7	2.8	7.4	5.7	9.3	6.7	5.7	4.2	3.6	9.5 (8.6)
IPZ	6.9	3.2	7.5	7.2	9.7	9.4	7.4	4.0	3.3	11.5 (8.8)
	600 µg/L	***3000 µg/L***	***6000 µg/L***	***600 µg/L***	***3000 µg/L***	***6000 µg/L***	***600 µg/L***	***3000 µg/L***	***6000 µg/L***	
CRZ	6.8	3.5	7.9	8.7	6.4	8.4	4.4	3.1	3.5	9.9 (8.8)

In all cases the RSDs (%) of peak areas were, in general, lower than that observed in the intra-day and inter-day precision studies. It supposes that the change of the capillary during the method application did not involve a higher variability among the data and a low precision of the results. RSDs lower than 7.9% were achieved for reproducibility assays in four different packed capillaries.

In terms of elution times, RSDs ranged from 0.4% to 1.3% in repeatability assays for all studied compounds, while for intermediate precision, RSDs between 4.7% and 10.7% were obtained. High reproducibility was also obtained in terms of elution times (RSD ≤ 8.8%) when 5-NDZ separation in four different CEC columns was assessed. This fact supposes a great success considering the reported disadvantages about packing lab-made columns for CEC analyses [31]. Packed capillaries that were properly made showed a half-life of at least one hundred and fifty runs. After that, the use of a new lab-made packed column is recommended because the charged particles themselves tended to move in the electrical field during the CEC process and the column performance is no longer optimum.

8.3.4. Method characterization in whole milk samples

The proposed analytical method was applied to whole milk samples in order to test its usefulness and potential for complex samples. The analytical method was evaluated in terms of linearity, LODs and LOQs, intra-day and inter-day precision and trueness. All peak signals have been monitored at 320 nm although CRZ presents a UV absorption maximum at 244 nm. Elecrochromatogram baseline was unstable at 244 nm when milk samples were analyzed.

In preliminary studies, samples were treated by applying only the first step of the final proposed sample treatment (SALLE using MeCN in presence of NaCl). Sample extracts were reconstituted in water and analyzed by the proposed CEC method. Although peak interferences were not found during 5-NDZ separation, peak elution times were not reproducible after several runs. It was attributed to matrix constituents. In order to remove them, a second step was proposed. It was based on the application of SPE using Oasis®HLB cartridges. Treated blank milk samples were analyzed and no endogenous

interferences were detected at the same 5-NDZ elution times. Moreover, non unknown peaks were observed during all the analyses. The combination of the proposed sample treatment with the developed CEC method shows a high selectivity for 5-NDZ determination (**Figure 8.9**).

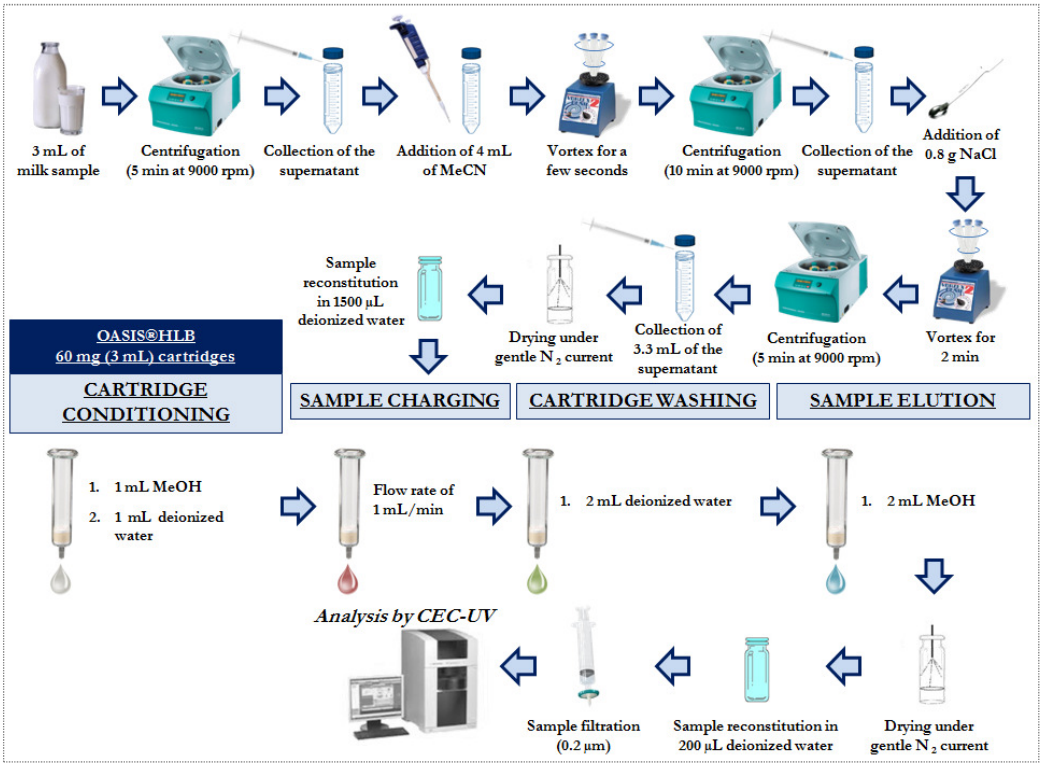

Figure 8.9. SALLE-SPE procedure for 5-NDZ determination in milk samples by CEC-UV.

Matrix-matched calibration curves were established in milk samples fortified at six different concentration levels: 25, 50, 100, 200, 350 and 500 µg/L for all considered 5-NDZs, except for CRZ (50, 100, 200, 400, 700 and 1000 µg/L). Two spiked milk samples per level were treated following the SALLE–SPE procedure previously described. Afterwards, each sample was analyzed in duplicate according to the proposed CEC–UV method. Peak area was considered as a function of analyte concentration on the sample. LODs and LOQs of the method were calculated as it was mentioned before (**Table 8.3**). In spite of the lack of sensitivity attributed to UV detection, LODs lower than 12 µg/L were accomplished for all studied 5-NDZ compounds except for CRZ (29 µg/L).

Table 8.3. **Statistical and performance characteristics of the proposed SALLE-SPE-CEC-UV method for the determination of 5-NDZ residues in whole milk samples.**

Analyte	Linear range (µg/L)	R^2	LOD (µg/L)	LOQ (µg/L)	Repeatability (% RSD, n = 9)		Intermediate precision (% RSD, n = 12)	
			$3 \times S/N$	$10 \times S/N$	***50 µg/L***	***350 µg/L***	***50 µg/L***	***350 µg/L***
RNZ	19 – 500	0.997	6	19	7.3	4.0	10.4	8.1
MNZ	11 - 500	0.997	3	11	6.5	6.3	11.4	9.3
TRZ	17 - 500	0.996	5	17	9.0	5.3	11.4	8.2
SCZ	14 - 500	0.995	4	14	9.7	7.1	10.7	9.3
ORZ	19 - 500	0.995	6	19	8.5	4.1	10.8	7.0
DMZ	24 - 500	0.993	7	24	9.7	4.1	11.1	14.5
IPZ	38 - 500	0.995	12	38	12.2	6.3	11.4	11.4
					100 µg/L	***700 µg/L***	***100 µg/L***	***700 µg/L***
CRZ	96 – 1000	0.995	29	96	10.8	5.8	10.1	7.3

The precision of the whole method, including the sample treatment, was evaluated by analyzing three spiked milk samples at two concentration levels in the same day (intra-day studies) and one spiked milk sample at two concentration levels for four different days (inter-day studies). Each sample was injected in triplicate. Results are shown in **Table 8.3**. Satisfactory results were obtained in terms of RSD (%), being lower than 12.2% and 14.5% for repeatability and intermediate precision, respectively.

Trueness assays were carried out over whole milk samples spiked at 50 and 350 µg/L with each 5-NDZ drug except for CRZ (100 and 700 µg/L). For each concentration level three samples were analyzed in triplicate. Obtained data were compared with those obtained by analyzing extracts of blank samples submitted to the sample treatment and spiked with 5-NDZ compounds just before the measurement. Recoveries over 68% were obtained for all 5-NDZ antibiotics, reaching RSDs lower than 12.2% for all cases (**Table 8.4**). All the presented results show that the developed method is suitable for the analysis of these compounds in milk samples.

Table 8.4. Recovery studies (%) in whole milk samples.

Analyte	Recovery (% RSD, n = 9)	
	50 µg/L	***350 µg/L***
RNZ	107 (7.3)	81 (4.0)
MNZ	94 (6.5)	85 (6.3)
TRZ	100 (9.0)	85 (5.3)
SCZ	107 (9.7)	93 (7.1)
ORZ	104 (8.5)	91 (4.1)
DMZ	79 (9.7)	70 (4.1)
IPZ	81 (12.2)	68 (6.3)
	100 µg/L	***700 µg/L***
CRZ	102 (10.8)	75 (5.8)

Additionally, **Figure 8.10** shows the electrochromatograms obtained from milk samples by applying the proposed SALLE–SPE procedure and analyzed considering the developed CEC method.

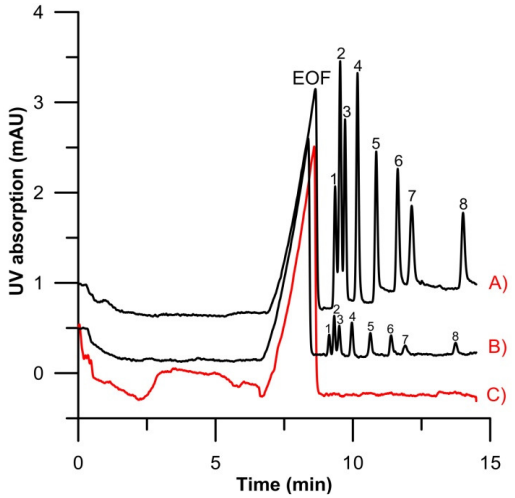

Figure 8.10. Electrochromatograms of A) milk sample spiked at 350 µg/L of each analyte except for CRZ (700 µg/L); B) milk sample spiked at 50 µg/L of each analyte except for CRZ (100 µg/L); C) blank milk sample analyzed by the proposed SALLE-SPE-CEC-UV method. Separation conditions: C18 packed capillary (40 cm × 50 µm i.d.), 27 kV and 30°C. Mobile phase: 60:40 (v/v) MeCN/ammonium acetate buffer (2.5 mM, pH 5). Samples were injected for 120 s at 11.5 bar. Signals were monitored at 320 nm. Peaks: 1, RNZ; 2, MNZ; 3, TRZ; 4, SCZ; 5, ORZ; 6, DMZ; 7, CRZ; 8, IPZ.

In comparison with other previously reported methods for the analysis of 5-NDZs using HPLC or CE with UV detection in food matrixes, the proposed CEC method involves a

shorter analysis time for a similar or higher number of analytes, showing also higher selectivity when the advantages of both chromatographic and electrophoretic separation modes are combined, reaching LODs at similar low μg/L levels [2,9,10].

8.4. Conclusions

A new method to determine eight 5-NDZs in whole milk samples by CEC using lab-made columns has been proposed and evaluated. A detailed study of the procedure proposed for packing capillaries and making the required frits has also been described. This procedure results in robust and reproducible CEC capillaries considerably cheaper than the commercial ones. The optimized CEC method is able to separate eight 5-NDZs in less than 15 min with good sensitivity and precision. The selectivity of a chromatographic separation was achieved together with the advantage of a lower reagent consumption and waste generation of an electrophoretic separation. A selective sample treatment based on the combination of SALLE and SPE was used to extract 5-NDZ residues from whole milk samples. The satisfactory results obtained with this complex sample demonstrate that CEC is a separation technique that could be used in routine analysis laboratories.

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Capillary electrochromatography-mass spectrometry for the determination of 5-NDZ antibiotics in urine samples

Abstract ► The separation of eight antibiotics belonging to 5-NDZ family was carried out by means of CEC coupled to MS. Preliminary experiments were carried out with UV detection in order to select the suitable stationary and mobile phase. Among the different stationary phases studied (namely Lichrospher C18, 5 μm; Cogent™ Bidentate C18, 4.2 μm; Pinnacle II™ Phenyl, 3 μm; Pinnacle II™ Cyano, 3 μm), Cogent™ Bidentate C18 (4.2 μm) gave the best performance. For CEC-MS coupling, a laboratory assembled liquid-junction-nano-spray interface was used. In order to achieve a good sensitivity, special attention was paid to the optimization of both sheath liquid composition and selection injection mode. Under optimized CEC-ESI-MS conditions, the separation was accomplished within 22 min by using a column packed with a mixture of Bidentate C18 (4.2 μm)/Lichrospher Silica-60 (5 μm) 3:1 (w/w), an applied inlet pressure of 11 bar, a voltage of 15 kV and a mobile phase composed by 45:10:45 (v/v/v) MeCN/MeOH/water containing ammonium acetate (5 mM, pH 5). A combined hydrodynamic and electrokinetic injection of 8 bar, 15 kV, and 96 s was adopted. The method was validated in terms of repeatability and intermediate precision of retention times and peak areas, linearity, and LODs and LOQs. RSDs values were < 2.9% for retention times and < 16.1% for peak areas in both intra-day and inter-day experiments. LOQ values were between 0.09 and 0.42 μg/mL for all compounds. Finally, the method was applied to the determination of MNZ, SCZ and TRZ in spiked urine samples subjected to a SPE procedure. Recovery values in the 67–103% range were obtained. Furthermore, for the selected antibiotics, CEC-MS^2 spectra were obtained providing the unambiguous confirmation of these drugs in urine samples.

9.1. Background

LC coupled to MS or UV detection has been the most employed technique for 5-NDZ determination [1], although other methodologies employing GC [2,3] or CE [4,5] have also been considered. In spite of LC advantages, the high amount of required solvents remains as its main drawback. Solvent consumption in LC has been reduced since UHPLC has been implemented; however, the generated residues are still high compared with those produced by greener miniaturized separation techniques as nano-LC or CEC. These methodologies present another advantage additionally related to cost savings. For instance, the amount of stationary phase required for making capillary columns is very low (only few mg). This fact lets to test different stationary phases for the separation of a mixture without a high investment. Concerning CEC, the combination of these advantages and its intrinsic properties as high efficiency, high resolution, and selectivity [6] makes it attractive as analytical tool for antibiotics determination [7,8].

Laboratory CEC columns preparation is a well-consolidated process, offering several strategies for packing [9]. In spite of different possibilities, slurry packing [10-12] remains as the most established methodology because it is easy to implement and it does not require long recipes for start-up. Concerning frit formation for retaining the stationary phase in the capillary, numerous alternatives have been reported [13-15], being silica-based particle sintering the prevalent mode [16]. To sum up, the manufacturing of CEC columns should not suppose a handicap at the time of developing CEC methodologies, but it should be considered as a useful way to test different column types for achieving a satisfactory separation.

On the other hand, for achieving the unequivocal identification of the analyzed compounds, the use of MS as detection tool has became essential. CEC-MS hyphenation has followed the CE-MS groundwork and, in spite of several ionization methods have been proposed, ESI interface has resulted as the most suitable for the majority of CEC-MS applications [17]. In principle, CEC-ESI-MS coupling could be considered a hard task because it presents several potentially challenging instrumental aspects which complicate their successful combination, such as the absence of a CEC column outlet electrolyte

reservoir or the need to achieve electrical continuity for the CEC system and also for the ESI ion source [18]. In order to solve these disadvantages three different ESI interfaces designs have been investigated namely CEC-MS sheathless, coaxial sheath flow and liquid junction interface. Successful results have been obtained using a new nano-liquid junction interface prototype [19,20].

In this chapter, a CEC-MS method for 5-NDZ determination in clinical samples has been developed. Different stationary phases were tested as column packing for obtaining the baseline separation of all studied compounds. Electrochromatographic parameters, such as mobile phase composition, were also evaluated. Likewise, special consideration was paid to the injection mode in order to improve method sensitivity. Furthermore, in order to have a certain identification of studied compounds by means of MS detection, exhaustive studies of parameters related to CEC-MS coupling were carried out for improving sensitivity. At the best of our knowledge, the developed methodology supposes the first approach for the determination of 5-NDZ drugs involving CEC coupled to MS. Finally, the novel optimized method was applied to the analysis of urine samples spiked with the most administrated 5-NDZ drugs, specifically MNZ, SCZ, and TRZ.

9.2. Materials and methods

9.2.1. Materials and reagents

All chemicals were of analytical reagent grade, unless indicated otherwise, and solvents were HPLC grade. Ammonium hydroxide solution (30%, v/v), MeOH, acetone, 2-propanol, ethanol, MeCN, and acetic acid were acquired from Carlo Erba (Milan, Italy). Formic acid (98–100%, v/v) was supplied by Merck (Darmstadt, Germany). Ultrapure water, obtained from a Milli-Q system (Millipore, Bedford, MA, USA), was used throughout the work. Ammonium acetate buffer was prepared from acetic acid solutions adjusting their pH with an ammonia solution. Mobile phases were prepared by mixing proper volumes of buffer, water and organic solvents.

Analytical standards of DMZ, RNZ, CRZ, ORZ, and MNZ were supplied by Sigma-Aldrich (St. Louis, MO, USA) while IPZ, SCZ and TRZ were purchased from Witega

(Berlin, Germany). Stock standard solutions were obtained by dissolving the appropriate amount of each 5-NDZ drug in MeCN, reaching a final concentration of 1 mg/mL. Working standard solutions were obtained by mixing the appropriate amount of stock standard solutions and their subsequent dilution with water. All standard solutions were stored in the freezer at –20°C avoiding exposure to direct light. Before their use, they were equilibrated to room temperature. They were stable for at least four months.

Oasis®HLB (30 mg, 1 mL) cartridges supplied by Waters (Milford, MA, USA) were considered for the sample treatment procedure.

9.2.2. Instrumentation

An Agilent Technologies CE system (Waldbronn, Germany), equipped with a UV diode array detector, an autosampler and an external high-pressure nitrogen source, was employed for CEC-UV experiments. Chemstation software (Rev. A.09.01, Agilent Technologies) was used for controlling the instrument and to handle the obtained data.

CEC-MS experiments were run by coupling the CEC system to a LCQ™ ion-trap mass spectrometry detector (Thermo Finnigan, San Jose, CA, USA) equipped with an ESI source and operating in positive ion mode. A laboratory-made liquid-junction-nano-spray-ESI interface, already described [20,21], was used for the hyphenation. It was implemented machining a block of polysulfone resin PSU 1000 (Plastotecnica Emiliana Srl, Bologna, Italy). A commercial x-y-z translation stage was used for setting up the liquid junction interface. Interface stainless steel electrode was connected to a high voltage power supply CZE 1000 R (Spellman High Voltage Electronics, NY, USA). The MS and MS/MS spectra and the ion electrochromatograms were collected and processed by using Xcalibur™ software, version 1.3 (Thermo Finnigan).

9.2.3. Capillary column preparation

Uncoated fused silica capillaries of 75 μm i.d. × 375 μm o.d., purchased from Polymicro Technologies (Phoenix, AZ, USA), were packed with the appropriate stationary phase (packed, effective, and total length of 25.0, 27.0, and 33.5 cm, respectively) and used for

CEC experiments. Five different stationary phases were considered, including: Lichrospher C18 (5 µm) from Merck (Darmstadt, Germany); Type-C Silica Cogent™ Bidentate C18 (4.2 µm) from MicroSolv Technology Corporation (Eatontown, NJ, USA); Pinnacle II™ Phenyl (3 µm), and Pinnacle II™ Cyano (3 µm) from Restek Corporation (Bellefonte, PA, USA); and a mixture of Type-C Silica™ Bidentate C18 (4.2 µm)/Lichrospher Silica-60 (5 µm, Merck) 3:1 (w/w).

Columns were laboratory made and prepared following a protocol previously described [22], which is based on a slurry packing procedure. In short, about 20 mg of stationary phase were suspended in 1 mL of acetone and sonicated in order to avoid particle agglomeration. The slurry was pumped into the capillary under a maximum pressure of 37 MPa. Particles were retained into the capillary by means of a mechanical frit, disposed at the end of the capillary. Once the capillary was packed, it was filled with deionized water for 30 min followed by a 5 mM NaCl solution for 30 min. Afterwards, frits were fabricated by sintering the stationary phase at the desired location for 6 s at about 700°C using a laboratory-made heated electrical wire. Finally, capillaries were rinsed with mobile phase at 7 MPa for 40 min for equilibration.

9.2.4. Capillary electrochromatography

Preliminary CEC-UV analyses were carried out under a voltage of 20 kV at 20°C. During voltage application, inlet and outlet vials were pressurized to 8 bar in order to avoid bubbles formation. Samples were injected by pressure at 8 bar for 24 s. Analyte separation was performed using a mixture 45:10:45 (v/v/v) MeCN/MeOH/water containing 5 mM ammonium acetate buffer (pH 5) as mobile phase. Analytical signals were monitored at 320 nm.

To carry out CEC-MS experiments, capillary column was placed in a modified CE cartridge and the CE instrument was positioned very close to the CEC-MS interface. CEC-MS analyses were performed using the previously described mobile phase, under a voltage of 15 kV and a pressure of 11 bar applied to the inlet vial. Column was totally packed for a length of 25.0 cm, while the stationary phase was composed by a mixture 3:1 (w/w)

Type-C Silica™ Bidentate C18/Lichrospher Silica 60. Samples were injected by mixed mode for 96 s at 8 bar and 15 kV. The temperature of the system was controlled by continuous room conditioning (20°C).

At the end of working day, the inlet reservoir was immersed in MeOH while, the MS interface was kept in aqueous-organic mixture 1:1 (v/v) MeOH/water.

9.2.5. MS and electrospray interface

Sheath liquid consisted of a mixture 50:50 (v/v) 2-propanol/water containing 0.05% (v/v) formic acid. Sheath liquid was supplied by gravity to the interface, being the level of the sheath liquid 52 cm upper than the liquid junction. As emitter tip a fused silica capillary (50 μm i.d. × 375 μm o.d. × 6 cm) was used. The tip was laboratory-made using a rotating disk supporting emery paper. MS parameters were selected as follow: spray voltage, 1.8 kV; capillary voltage, –7.5 V; capillary temperature, 200°C; tube lens voltage, –15 V; automatic gain control (AGC), 3×10^7; number of microscans, 3; max injection time, 70 ms and scan range m/z, 130.0–250.0.

The MS/MS experiments were performed by the fragmentation of the molecular ions $[M+H]^+$ which were selected as the precursor ion. Normalized collision energy (%) was adjusted in a range of 24–40% and product ions were analyzed in the range of 130–250 m/z.

For method validation, electrochromatographic variables such as retention time, peak height and peak area were acquired from the extracted ion chromatograms of each studied compound.

9.2.6. Sample treatment

Urine samples were supplied by a healthy male volunteer and aliquots of 5 mL from them were made in order to be fortified with the corresponding 5-NDZ concentration. Sample clean-up was carried out following a SPE protocol, reported in literature and generically recommended by Waters [23,24]. An Oasis®HLB (30 mg, 1 mL) cartridge was conditioned

with 1.0 mL of MeOH and 1.0 mL of deionized water. Then, 0.5 mL of the sample was loaded onto the cartridge at about 1 mL/min. The cartridge was washed up with 1 mL of a mixture 95:5 (v/v) deionized water/MeOH at 1 mL/min and analytes were eluted with 0.5 mL of MeOH at 1 mL/min. Later 0.2 mL of the eluted sample were dried under nitrogen stream at ambient temperature and it was reconstituted in 0.2 mL of ammonium acetate buffer (2.5 mM, pH 5)/MeCN (90:10, v/v). Finally, the extract was analyzed using the optimized CEC-MS method.

9.3. Results and discussion

9.3.1. Stationary phase selection

Preliminary experiments concerning the separation of selected 5-NDZs were performed by CEC-UV. Separations were carried out in capillaries packed with different silica-based stationary phases (modified with different groups, namely C18, phenyl or cyano) under a voltage of 20 kV and 20°C. At first, a mixture 60:40 (v/v) MeCN/water containing 1 mM ammonium acetate (pH 5) was employed as mobile phase, considering the compatibility of the buffer for coupling CEC with MS. The pH 5 was selected considering that the studied compounds are neutral at this value [25]. In such form, interactions with stationary phases based on reverse phase mechanism are enhanced and they move through the capillary column by means of EOF. Afterwards, mobile phase composition was slightly modified in order to get the best separation when each stationary phase was evaluated. MeCN content in the mobile phase was studied in a range from 60 to 45% (v/v) and MeOH content was considered up to 10% (v/v). Both organic solvents were added to the mobile phase depending on the tested column. Decreasing organic solvent percentage and/or considering MeOH instead of MeCN resulted in a mobile phase with lower elution strength and less strong EOF. As a consequence, analytes were more retained into the column and better peak separation was achieved, although longer analysis time was observed. Considering the influence of the buffer, ammonium acetate concentration was raised from 1 to 5 mM. Finally 5 mM was selected as optimum because higher concentration decreased EOF and increased analysis time. Under this value a stable current was observed. **Figure 9.1** shows the separation of the eight 5-NDZ compounds achieved

in capillary columns packed with different stationary phases, under the considered working parameters that gave the best separation.

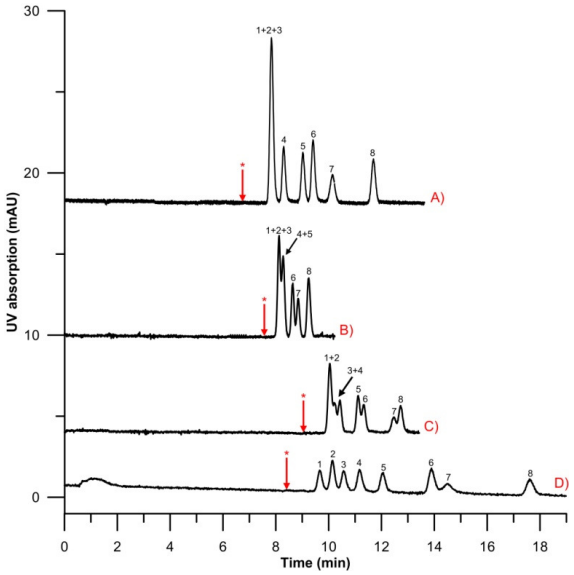

Figure 9.1. Selectivity of different stationary phases toward the studied compounds. Experimental conditions: capillary column: 75 μm i.d., 25.0 cm packed length, 27.0 cm effective length, 35.5 cm total length; A) Lichrospher C18, 5 μm; mobile phase: 60:40 (v/v) MeCN/water containing 5 mM ammonium acetate, pH 5; current, 2.2 μA; B) capillary column: Pinnacle II™ Cyano, 3 μm; mobile phase: 50:10:40 (v/v/v) MeCN/MeOH/water containing 5 mM ammonium acetate, pH 5; current, 2.3 μA; C) capillary column: Pinnacle II™ Phenyl, 3 μm; mobile phase: 50:10:40 (v/v/v) MeCN/MeOH/water containing 5 mM ammonium acetate, pH 5; current, 2.0 μA; D) Cogent Bidentate C18, 4.2 μm; mobile phase: 45:10:45 (v/v/v) MeCN/MeOH/water containing 5 mM ammonium acetate, pH 5; current 2.2 μA. Other experimental conditions: applied voltage, 20 kV; temperature 20°C; pressurized column at both ends with 8 bar; injection: 8 bar × 24 s; UV detection, 320 nm; standard concentration, 20 μg/mL for A), B) and C) and 12.5 μg/mL for D). Peak identification: 1, RNZ; 2, MNZ; 3, TRZ; 4, SCZ; 5, ORZ; 6, DMZ; 7, CRZ; 8, IPZ; *, t_{EOF}.

In the case of Cyano stationary phase, besides the mentioned mobile phase conditions, a mobile phase containing a buffer with pH lower than 5 was tested in order to improve the separation. Considering data from literature [26], buffer with pH 3 was used for benefitting electrostatic interactions between analytes and the stationary phase. At this pH value, 5-NDZ drugs are positively charged in certain grade according to their pKa values. However, no peak eluted in reasonable time until MeCN was increased up to 80%. At this percentage, the studied compounds eluted within 15 min, however they were barely resolved and not further experiments were carried out for this stationary phase.

Concerning the chromatographic separation by means of phenyl silica support, both the strong polar component and the alkyl moiety of 5-NDZ were supposed to prevent π–π interactions with the stationary phase, giving explanation for the poor separation observed.

Relating to the chromatographic separation by C18 phases, although Lichrospher has a higher carbon load respect to Cogent Bidentate, 21 and 16.5%, respectively [27], the presence of silicon-hydride groups (Si-H) enhances the hydrophobic properties of Cogent Bidentate, allowing the complete separation of the mixture.

All considered stationary phases were characterized by the presence of no chargeable or chargeable (Pinnacle CN) groups. However, EOF generation was mostly guaranteed by the presence of a silica support in each stationary phase. As can be seen in **Figure 9.1**, the EOF varied only partially among the studied stationary phases, and these variations can be due to differences of the used mobile phase composition and the properties of the silica used as support. While the composition of mobile phase was optimized considering the higher number of resolved analytes, the role of silica support in the EOF generation was not investigated.

Concluding, Type-C Silica™ Bidentate C18 showed the highest selectivity, and it was chosen as stationary phase for carrying out further studies.

9.3.2. CEC separation with MS detection

Once the stationary phase was selected, electrochromatographic separation of studied 5-NDZs was assayed using MS detection instead of UV detection. The capillary column used in CEC-UV experiments was closely cut to the outlet frit, inserted into the pressurized liquid-junction interface and aligned with the spray emitter. The optimized CEC-UV separation conditions were also used for CEC-MS analysis considering slight modifications. In particular, an inlet applied pressure up to 11 bar and a decreased separation voltage of 15 kV were necessary to avoid current instability during the run.

Despite the mentioned changes, no satisfactory repeatability was obtained. Specifically, retention times increased run after run because capillary was getting dried. This

phenomenon appeared even when a new column was prepared and, finally it was related to the nature of the stationary phase. Bidentate C18 stationary phase is characterized by the presence of silicon hydrides instead of silanols and as main consequence the adsorbed water layer is precluded, by virtue of other chromatographic benefits [28]. Considering this aspect, the use of a stationary phase consisting of Bidentate C18 and Lichrospher Silica-60 particles mixed in a ratio 3:1 (w/w) was considered. A new column was prepared and its performance was firstly evaluated by CEC-UV in the same experimental conditions described in **Section 9.2.4**. The introduction of silica did not substantially affect both the EOF and 5-NDZ separation (see **Figure 9.2**). It was not surprising since in literature it was already reported that switching from a mixed stationary phase composed by 3:1 (w/w) vancomycin-CSP (chiral stationary phase)/silica to a 'pure' vancomycin-CSP produced only a slight modification of the EOF [29].

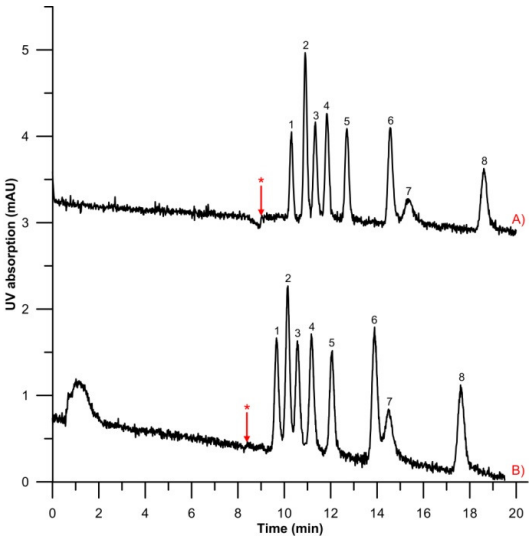

Figure 9.2. **Comparison between the column packed with A) a mixture of 3:1 (w/w) Cogent Bidentate C18 (4.2 μm)/Lichrospher Silica-60 (5 μm) and B) Cogent Bidentate C18 (4.2 μm). Experimental conditions: capillary column: 75 μm i.d., 25.0 cm packed length, 27.0 cm effective length, 35.5 cm total length; mobile phase, 45:10:45 (v/v/v) MeCN/MeOH/water containing 5 mM ammonium acetate, pH 5; current 2.2 μA; applied voltage, 20 kV; temperature 20°C; pressurized column at both ends with 8 bar; injection: 8 bar × 24 s; UV detection, 320 nm, standard concentration, 12.5 μg/mL. Peak identification: 1, RNZ; 2, MNZ; 3, TRZ; 4, SCZ; 5, ORZ; 6, DMZ; 7, CRZ; 8, IPZ; *, t_{EOF}.**

Successively, CEC-MS experiments were carried out and good repeatability in terms of retention times was obtained after several runs. Then, improved performance was related

to the presence of silica particles which were able to retain the water layer and thus avoiding column dryness observed in the course of earlier experiments. In any case, it was necessary to apply a pressure to the inlet vial in order to carry out experiments with good reproducibility, even if this stratagem could negatively affect the electrochromatographic performance [20].

9.3.3. Mass spectrometer evaluation

Mass spectrometer parameters

Initially, MS parameters were established by the direct infusion of 5 μg/mL of MNZ in MeOH. MNZ is a representative compound of 5-NDZ family and it possesses an intermediate m/z ratio if the m/z range for all studied compounds is considered. MS instrument was operated in the ESI positive mode as described in literature [30,31]. However, when a mixture of 5-NDZ standards (5 μg/mL) was analyzed by CEC-MS following the proposed method, signal sensitivity resulted too low. In order to increase signal sensitivity, S/N was optimized by means of tune MS mode. Therefore, MS parameters such as tube lens voltage, capillary voltage and capillary temperature were evaluated. Initial values of these parameters were: −5 V for tube lens voltage, 8 V for capillary voltage and 200°C for capillary temperature. Tube lens voltage was assayed from −20 to 10 V, resulting in a higher sensitivity (in terms of peak height) when a value of −15 V was set up. Capillary voltage was modified in the range −12.5–7.5 V, obtaining higher peak signal when it was set at −7.5 V. Finally, capillary temperature was fixed at 200°C because its variation did not produce any signal improvement.

Sheath liquid composition evaluation

The sheath liquid has a predominant influence on the ionization into the ESI interface and its composition has to be carefully investigated in order to obtain a stable electrospray and good sensitivity. The effect of the sheath liquid on the MS-signal for the studied compounds is described below.

At the beginning, a sheath liquid consisted of 50:50 (v/v) MeOH/water containing 0.1% (v/v) of acetic acid was used. Its composition was further investigated considering other organic solvents (2-propanol, EtOH) and other sheath liquid additives (ammonium hydroxide and formic acid). For most compounds, similar ionization was obtained when sheath liquid contained 2-propanol instead of MeOH whereas the use of EtOH resulted in a decrease of sensitivity (**Figure 9.3,I**). The addition of formic acid to the sheath liquid gave the highest signal, compared to the use of acetic acid and a sheath liquid without any additive. Moreover, the choice of using sheath liquid without any additive caused an unstable electrospray current. As it was expected, no MS signal was observed when 0.1% (v/v) ammonium hydroxide was used as additive in accordance with the selected positive detection mode. In order to further improve the sensitivity, formic acid percentage in the sheath liquid was studied in a range of 0.01–0.50% (v/v). In general, higher signal was obtained when a lower concentration of formic acid was used. However, an unstable electrospray was observed when formic acid percentage was lower than 0.05% (v/v). Therefore, 0.05% (v/v) formic acid was finally added to the sheath liquid in order to enhance compound ionization and guarantying a stable electrospray current.

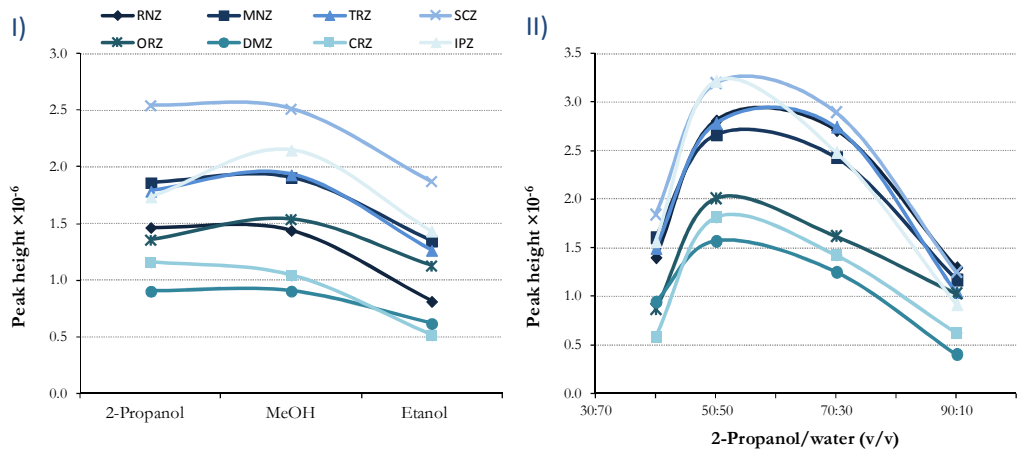

Figure 9.3. Influence of sheath-liquid composition on the MS signal. Sheath-liquid: I) 50:50 (v/v) organic solvent/water containing 0.1% (v/v) acetic acid; II) Different mixtures (v/v) 2-propanol/water containing 0.05% (v/v) of formic acid. Experimental conditions: capillary column, Cogent-Bidentate C18/Lichrospher Silica 60 (3:1, w/w), 25 cm packed length × 75 µm i.d.; inlet pressure, 11 bar; applied voltage, 15 kV; mobile phase, 45:10:45 (v/v/v) MeCN/MeOH/water containing 5 mM ammonium acetate, pH 5; hydrostatic pressure ≈ 4.2 kPa. MS parameters: spray voltage, 1.8 kV; capillary voltage, -7.5 V; capillary temperature, 200 °C; tube lens voltage, -15 V; automatic gain control (AGC), 3×10^7; number of microscans, 3; max. injection time, 70 ms; scan range (m/z), 130.0-250.0.

Lastly, the effect of different 2-propanol/water ratios in the sheath liquid was evaluated in order to enhance signal sensitivity. The content of 2-propanol in the sheath liquid was tested from 40 to 90% (v/v), always adding 0.05% (v/v) formic acid to the considered sheath liquid. A high aqueous content (> 50%, v/v) produced an increase in the surface tension that together with a lower volatility of the sheath liquid caused an unstable electrospray. As a consequence, a decrease of compound ionization and MS signal were observed. Likewise, a high content in 2-propanol (> 50%, v/v) caused an increase in the level of noise likely due to high ionization of the sheath liquid, supposing a decrease in analyte MS signal (**Figure 9.3,II**).

Finally, a mixture 50:50 (v/v) 2-propanol/water containing 0.05% (v/v) formic acid was selected as sheath liquid composition for further experiments.

Effect of outlet hydrostatic pressure

In the liquid junction interface used in the present chapter, the transport of the chromatographic bands from the outlet column to the MS detector via the emitter-tip is ensured by a sheath liquid flow resulting from the effect of a hydrostatic pressure [19,20]. Therefore, the applied outlet pressure may have effects on the chromatographic peak efficiency. For example, a too high sheath liquid flow can produce turbulence (liquid moving and stirring) within the interface, where the outlet of capillary column (from CEC instrument) and the emitter tip are placed and aligned at an optimal distance of about 100–150 μm [19,20], affecting negatively to the peak efficiency. Consequently, the optimization of the outlet pressure results in a compromise between the MS signal and the chromatographic efficiency.

For this purpose, the applied outlet pressure, which affects the sheath liquid flow rate into the tip emitter, was varied moving the height of the reservoir with respect to the interface. The height was moved in the 22–62 cm range, obtaining a pressure between 1.9 and 4.9 kPa. If pressure was rinsed, an increase of the flow rate into the emitter-tip was observed, with a generally improved S/N up to 4.2 kPa (except for the first eluted compound RNZ). At higher flow rate of the spray liquid, electrospray instability, dilution

of the sample and an increase of the noise level were noted. Consequently, 4.2 kPa was selected as optimum value of applied outlet hydrostatic pressure.

Influence of different sample injection methods on sensitivity

In order to increase the sensitivity (hydrodynamic injection for 24 s at 8 bar was initially employed), the combination of hydrodynamic and electrokinetic injection was evaluated and the results were compared with those obtained employing both injection modes separately. Keeping the injection time constant at 24 s, 8 bar and 15 kV were applied for hydrodynamic and electrokinetic injection, respectively. The combined mode was carried out at 8 bar and 15 kV. A comparison of efficiency, in terms of peak height and width at half height ratio (h/w_h), of both electrokinetic and combined injection was carried out. Higher values were obtained for some of the studied analytes (MNZ, TRZ, IPZ) when the combined mode was chosen while no significant differences were observed for SCZ, ORZ, DMZ and CRZ, while only for RNZ, the efficiency was slightly improved by employing the electrokinetic injection, as shown in **Figure 9.4**.

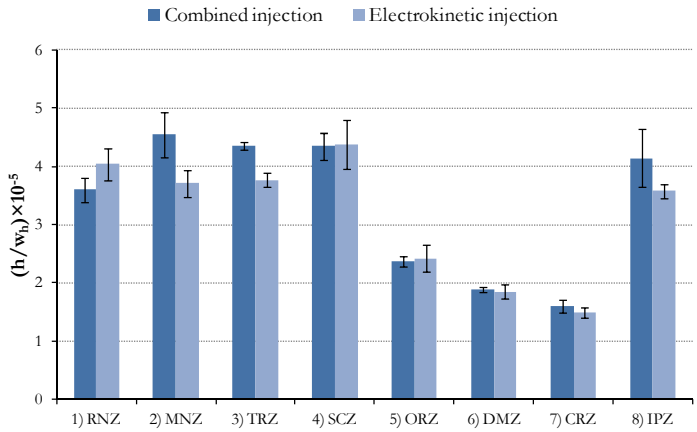

Figure 9.4. Influence of injection mode on peak efficiencies represented as ratio of peak height and peak width at half height (h/w_h) (n = 3). Injection time, 24 s. Combined injection, 15 kV at 8 bar; electrokinetic injection, 15 kV. Capillary column, Cogent-Bidentate C18/Lichrospher Silica 60 (3:1, w/w), 25 cm packed length × 75 µm i.d.; mobile phase, 45:10:45 (v/v/v) MeCN/MeOH/water containing 5 mM ammonium acetate, pH 5. Inlet pressure, 11 bar; applied voltage, 15 kV. Sheath liquid, 2-propanol/water (50:50, v/v) containing formic acid (0.05%, v/v). MS parameters: spray voltage, 1.8 kV; capillary voltage, −7.5 V; capillary temperature, 200°C; tube lens voltage, –15 V; automatic gain control (AGC), 3×10^7; number of microscans, 3; max. injection time, 70 ms; scan range m/z, 130.0–250.0.

Taking into account, separately, the influence of the injection mode on sensitivity (peak height) and efficiency (peak width at half height), peak height showed the same trend as h/w_h, while efficiency remained almost unchanged. As a consequence, sensitivity can be evaluated in terms of peak height and without taking into account peak efficiency. Regarding to hydrodynamic injection, the same assayed standard mixture (2.5 μg/mL) gave signal intensities lower than ten times the S/N (LOQ), consequently this injection mode was discarded and the results are not reported in the figure.

It was concluded that combined injection gave optimum results, so it was selected for further studies which involved the evaluation of the injection time and the sample solvent composition. This last factor plays a very important role especially when large sample volume is injected, promoting a sample stacking and/or a focusing effect without affecting the peak efficiency and/or resolution [26,32,33]. Among the different studied solvents, namely water, mobile phase (45:10:45 (v/v/v) MeCN/MeOH/water, containing 5 mM ammonium acetate, pH 5), mobile phase without buffer, 2.5 and 5 mM ammonium acetate buffers adjusted to pH 5, MeCN and MeOH; a sensitivity improvement, in terms of S/N and repeatability, was obtained for a solvent consisted of 90:10 (v/v) 2.5 mM ammonium acetate (pH 5)/MeCN, where the presence of 10% (v/v) MeCN came from the working standard solution. This result was not surprising, since the lower conductivity of the sample solvent compared to the mobile phase is responsible of an on-line preconcentration approach so-called FASI [34]. In addition to the stacking effect, high water content, as a non-eluting phase, focused and concentrated the analytes at the column inlet in a narrow injection plug. On the other hand, the presence of a small amount of organic solvent (as 10% MeCN) can improve analyte solubility working in on-column focusing conditions [33]. The presence of 10% MeCN in the sample solvent was considered even for additional dilutions of the analyte mixture, i.e. for the determination of LOD and LOQ values.

In order to further improve method sensitivity, the volume of sample introduced into the column was expanded, increasing the injection time. To find the maximum volume of sample that could be injected without compromising the chromatographic performance, the effect of the injection time on the ratio of peak height and peak width at half height

(h/w_h) was studied. The results reported in **Figure 9.5** clearly show that increasing the injection time from 24 to 96 s supposes an almost linear increase of the h/w_h ratio for the represented analytes. Concerning the other studied compounds, the influence of injection time was similar. Further increases in the injection time, up to 120 s, caused peak band broadening resulting in a constant value of the h/w_h ratio without any improvement of sensitivity. Moreover, it was observed bubble formation when large plugs of sample were injected. Accordingly, the optimum injection time to obtain the highest MS signal with the best chromatographic efficiency was found at 96 s.

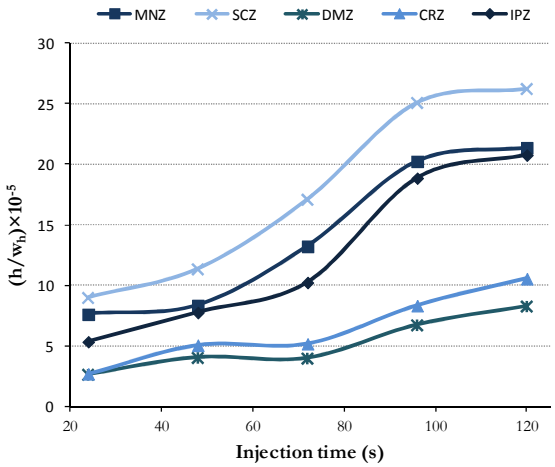

Figure 9.5. Influence of injection time on peak efficiencies represented as ratio of peak height and peak width at half height (h/w_h). Other conditions as reported in **Figure 9.4**.

9.3.4. Method characterization

The optimized method was instrumentally evaluated in terms of linearity, LODs, LOQs, repeatability, and intermediate precision of retention times and peak areas. Calibration curves were obtained by analyzing 5-NDZ standard solutions at six concentration levels: 0.25, 0.50, 1.00, 2.00, 3.20, and 5.00 μg/mL for all considered 5-NDZs, except for CRZ and ORZ (0.50, 1.00, 2.00, 3.20, and 5.00 μg/mL). Three replicates of each concentration level were assayed. Peak area was considered as function of analyte concentration. Instrumental LODs and LOQs were calculated as the minimum analyte concentration yielding a S/N equal to three and ten, respectively. The obtained regression equations, correlation factors, and LODs and LOQs are shown in **Table 9.1**.

Table 9.1. Statistical and performance characteristics of the proposed CEC-MS method.

Analyte	Linear range (μg/mL)	R^2	Linear regression equation (y=m·x+a)				LOD (μg/mL)	LOQ (μg/mL)
			Slope, ($\times 10^{-7}$)	*SD for the slope ($\times 10^{-5}$)*	*Intercept ($\times 10^{-6}$)*	*SD for the intercept ($\times 10^{-6}$)*	*3×S/N*	*10×S/N*
RNZ	0.12-5.00	0.997	1.06	2.84	-1.04	0.74	0.04	0.12
MNZ	0.09-5.00	0.997	1.21	3.53	0.86	0.92	0.03	0.09
TRZ	0.24-5.00	0.996	1.10	3.47	1.17	0.90	0.07	0.24
SCZ	0.26-5.00	0.994	1.66	6.37	-1.72	1.66	0.08	0.26
ORZ	0.28-5.00	0.995	1.04	5.28	-3.07	1.60	0.08	0.28
DMZ	0.19-5.00	0.994	0.78	2.95	-0.56	0.77	0.06	0.19
CRZ	0.42-5.00	0.989	1.46	8.82	-6.11	2.51	0.13	0.42
IPZ	0.11-5.00	0.996	1.81	6.08	-1.38	1.58	0.04	0.11

Repeatability study was carried out by analyzing a standard solution containing the eight considered 5-NDZs at a concentration of 1.00 μg/mL for seven consecutive times. Intermediate precision was assayed by analyzing a standard solution (1.00 μg/mL of each 5-NDZ compound) in triplicate per day for three consecutive days. Results expressed as RSDs (%) of retention times and peak areas are shown in **Table 9.2**. As can be seen good results were obtained with RSD values for intra-day analyses between 0.5 and 1.2% for retention time and below 10.4% for peak area, while for the inter-day they were in the range 1.4–2.9% and below 16.1%, in the same order.

Additionally, **Figure 9.6** shows the separation of the eight 5-NDZ drugs (2.50 μg/mL) under CEC-MS optimized conditions. In comparison with HPLC-MS [35], a shorter analysis time for a higher number of analytes and better peak efficiencies were obtained. Exactly, the proposed CEC-MS method has allowed a complete chromatographic separation of eight 5-NDZs (instead of six by HPLC-MS) in 20 min under isocratic conditions. Moreover HPLC analyses were performed in a gradient mode and required a total run time of 28 min for a standard mixture analysis, while it needed up to 35 min when real samples (feedstuff) were analyzed due to the required column conditioning between runs.

Table 9.2. Precision studies for the analysis of 5-NDZ compounds by the proposed CEC-MS method.

Analyte	Repeatability RSD (%, n = 7)		Intermediate precision RSD (%, n = 9)	
	t_R	*Area*	t_R	*Area*
RNZ	0.7	6.3	1.4	13.1
MNZ	0.7	8.9	1.6	14.6
TRZ	0.5	7.8	1.8	13.3
SCZ	0.7	10.4	1.8	15.2
ORZ	1.0	9.1	2.0	15.5
DMZ	1.0	9.3	2.1	16.1
CRZ	1.2	6.1	2.9	15.9
IPZ	1.1	6.0	2.9	13.4

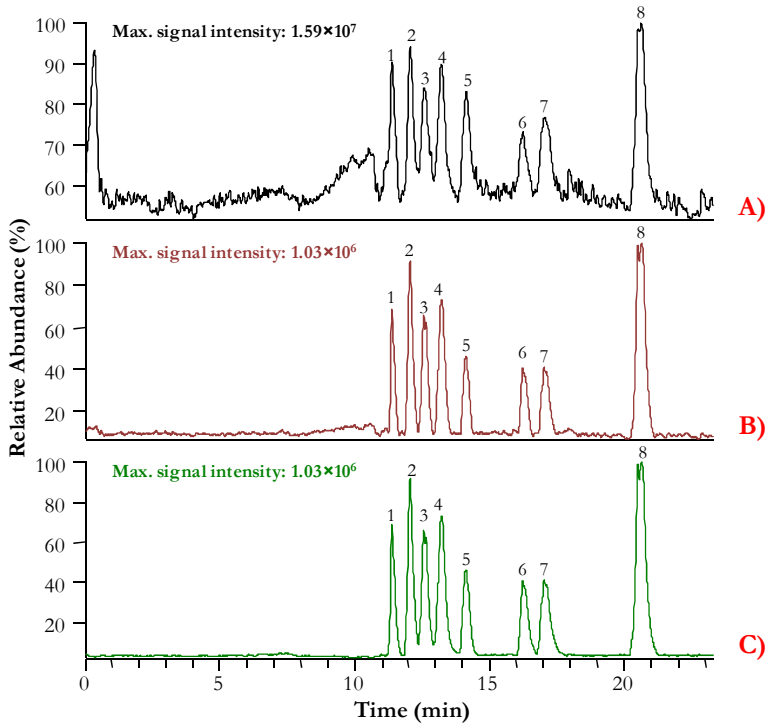

Figure 9.6. 5-NDZ determination by CEC-MS. A) Total ion electrochromatogram, B) base peak electrochromatogram, C) extracted ion electrochromatogram. Experimental conditions: capillary column, Cogent-Bidentate C18/Lichrospher Silica 60 (3:1, w/w), 25 cm packed length × 75 µm i.d.; inlet pressure, 11 bar; applied voltage, 15 kV; mobile phase, 45:10:45 (v/v/v) MeCN/MeOH/water containing 5 mM ammonium acetate, pH 5; sample, 2.5 µg/mL in 90:10 (v/v) ammonium acetate (2.5 mM, pH 5)/MeCN; injection parameters, 15 kV for 96 s at 8 bar; sheath liquid, 2-propanol/water (50:50, v/v) containing 0.05% (v/v) formic acid; hydrostatic pressure ≈ 4.2 kPa. Peak identification: 1, RNZ; 2, MNZ; 3, TRZ; 4, SCZ; 5, ORZ; 6, DMZ; 7, CRZ; 8, IPZ. For other experimental conditions see **Section 9.2.5**.

On the other hand, HPLC offered higher sensitivity than CEC with LOD and LOQ values ranging between 1.2–6.1 and 2.0–10.3 ng/mL, respectively. However both methodologies have been applied to different matrices (foodstuffs in the case of HPLC-MS and urine samples for CEC-MS) that claim for different detection limits. Also, comparing with the work mentioned before [35], reproducibility of HPLC method is referred only to recovery values, and is not exceeding 11% in terms of RSD. For the proposed CEC-MS method RSDs < 16.1% in terms of peak area were obtained and it was considered satisfactory. Finally, an attempt was made to demonstrate the applicability of the CEC-MS method.

9.3.5. Analysis of spiked urine samples

5-NDZs are commonly used singly as antimicrobial agents, being MNZ, SCZ, and ORZ the most commonly administrated. There are few research papers where the use of the combination of more than one of these 5-NDZ is proposed [36,37]. In order to verify the applicability of the proposed CEC-MS method, the determination of three 5-NDZs, namely MNZ, SCZ, and ORZ, in urine was considered. Urine samples were subjected to a standard SPE procedure (**Section 9.2.6**) and extracts were analyzed. As can be observed in **Figure 9.7**, no interfering peaks with the same ion mass co-eluted with the studied 5-NDZs.

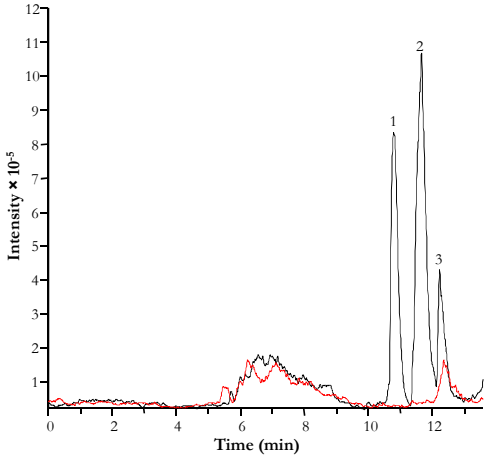

Figure 9.7. Extracted ion electrochromatogram of a blank urine sample (red line) and a urine sample spiked with a mixture of 1 µg/mL MNZ (1), SCZ (2) and ORZ (3) which were treated according to the proposed SPE procedure and subsequently analyzed by the optimized CEC-MS method (black line). Experimental conditions as reported in **Section 9.2.4** and **9.2.5**.

Trueness

Trueness assays were carried out over urine samples spiked at two concentration levels (1.00 and 2.50 μg/mL) with MNZ, SCZ, and TRZ. For each concentration level, three samples were analyzed in duplicate. The data were compared with those obtained by analyzing extracts of blank samples submitted to the sample treatment and spiked with 5-NDZs just before the measurement. Recoveries, in the 67–103% range, and precision data in terms of RSD are shown in **Table 9.3**. The obtained values were considered satisfactory for all the studied drugs. Even if recovery values are lower for the highest studied concentration level, it can be remarked that RSD is about 15%, which involved a wide confidence interval around the mean recovery value. In this sense, the values could belong to the same confidence interval and the differences could not be so significant from a statistical point of view. However, additional studies will be carried out regarding the applicability of the CEC-MS method and eventually improvements of SPE procedure will be also considered.

Table 9.3. Recovery results (%) for spiked urine samples.

Analyte	Level 1: 1.00 μg/mL (RSD, %; n = 6)	Level 2: 2.50 μg/mL (RSD, %; n = 6)
MNZ	97 (11.5)	67 (12.3)
SCZ	99 (15.6)	75 (15.5)
ORZ	103 (9.5)	67 (15.5)

9.3.6. MS/MS experiment for identification

In order to obtain the unambiguous confirmation of 5-NDZ drugs by the fragmentation spectra of each studied molecule, CEC-MS^2 experiments were performed. Normalized collision energy (%) for each compound was evaluated by the flow injection of 5 μg/mL of each 5-NDZ standard compound dissolved in MeCN. Normalized collision energy was set for a precursor/ion ratio of 100:10. Results are shown in **Table 9.4**.

Table 9.4. MS and MS/MS parameters of the studied 5-NDZ compounds.

Analyte	Precursor ion (m/z) $[M+H]^+$	Product ion (m/z)	Normalized collision energy (%)
RNZ	201	140	24
MNZ	172	128	30
TRZ	186	128	30
SCZ	186	128	28
ORZ	220	128	29
DMZ	142	96	34
CRZ	245	118	40
IPZ	170	124	37

MS/MS analyses were carried out for the separation of MNZ, SCZ, and ORZ (1.00 µg/mL) in urine samples. **Figure 9.8** shows 5-NDZ MS/MS spectra that agree with that obtained for the fragmentation of standard compounds. Moreover, MS/MS spectra were in accordance with those reported in literature [38,39].

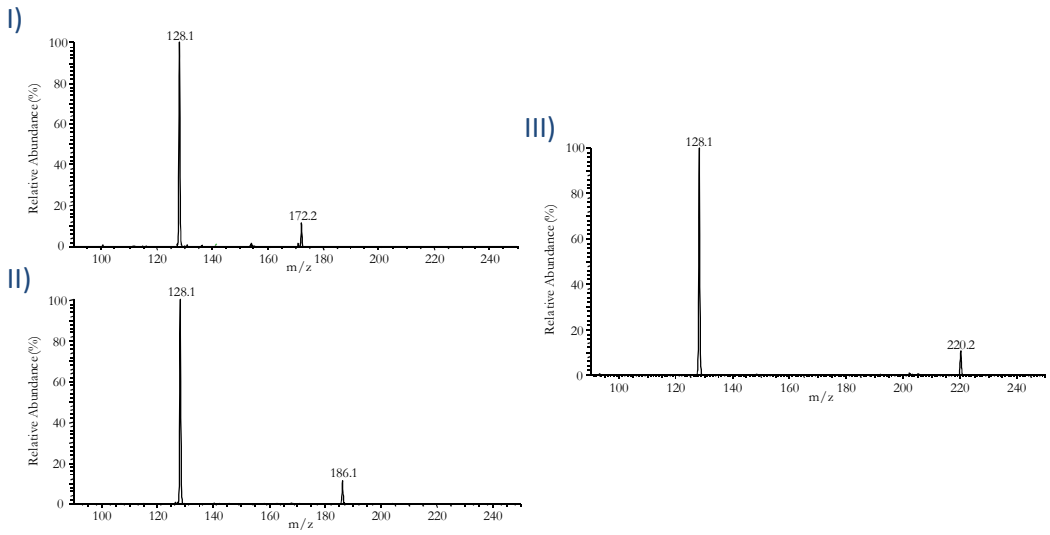

Figure 9.8. MS/MS spectra of I) MNZ, II) SCZ, and III) ORZ resulted from the analysis of a spiked urine sample (1 µg/mL of each 5-NDZ) following the proposed SPE-CEC-MS method. For MS parameters see **Section 9.2.5**. MS/MS experimental conditions: normalized collision energy: 24–40%; range scan: 130–250 m/z.

Additionally, a MS electropherogram of each analyte is also shown and it is compared with the electropherogram obtained for the analyses of a blank sample. The blank sample showed an interfering peak at 12.7 min (**Figure 9.9, II**). However, 5-NDZ peaks can be integrated from their extracted ion chromatogram without any inconvenience.

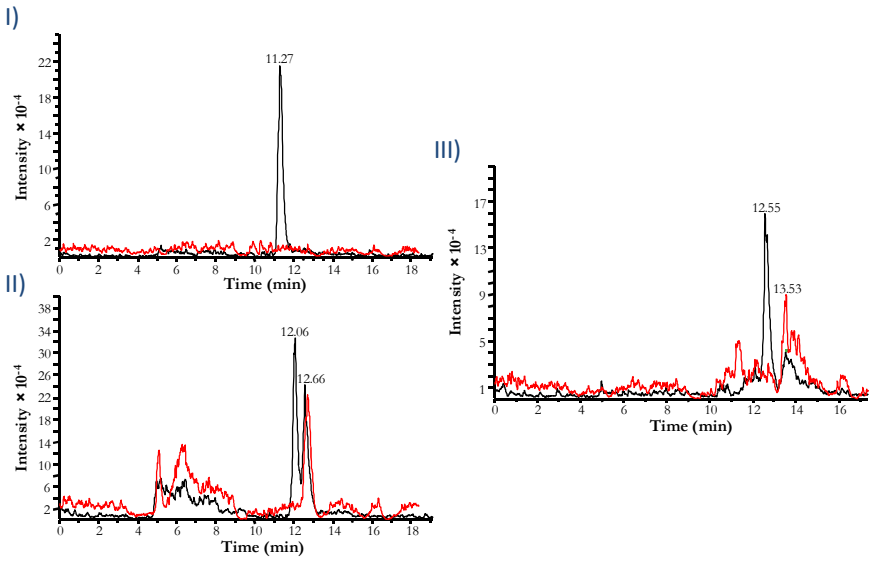

Figure 9.9. **Extracted ion electrochromatogram of a blank urine sample (red line) and a urine sample spiked with 1 µg/mL (black line) of I) MNZ; II) SCZ; III) ORZ. Experimental conditions are reported in Section 9.2.4 and 9.2.5.**

9.4. Conclusions

A novel CEC-MS method was developed and validated for the simultaneous determination of eight 5-NDZs. Good precision in terms of migration time and peak area, and satisfactory trueness from recovery studies were obtained. CEC-MS hyphenation was achieved by means of a laboratory-assembled liquid-junction interface and the identification of all studied analytes was achieved. The method was successfully applied to urine samples spiked with three of most administered 5-NDZs, namely MNZ, SCZ, and ORZ, which were also unambiguously confirmed by MS^2 experiments. The use of a miniaturized technique such as CEC permitted to perform analyses in reasonable time with a minimal consumption of reagents (e.g. solvents and stationary phase), and as a consequence, waste disposal is reduced and it results in higher effective cost if it is compared with HPLC methodologies.

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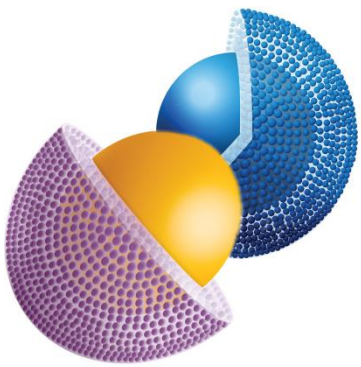
PART IV

5-NDZ DETERMINATION BY LC-BASED METHODS



Chapter 10:

Liquid chromatography

Chapter 11:

Determination of 5-NDZs in aquaculture products by capillary liquid chromatography-UV using MIPSE

Chapter 12:

Determination of 5-NDZs in milk samples by UHPLC-UV coupled to salt-assisted liquid-liquid extraction

Chapter 13:

UHPLC-MS/MS method for the determination of 5-nitroimidazole residues in fish roe samples

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Liquid chromatography

10.1. Background

The discovery of chromatography goes back to the beginning of 20th century, namely to 1903, when the Russian botanist Mikhail S. Tswett presented his results of the separation of colored pigments of plants by means of open-column liquid chromatography [1]. The term 'chromatography' comes from the Greek words 'chroma', meaning 'color', and 'graph', meaning 'writing', and it refers to the processes occurred in a flow system containing two phases, one mobile and the other stationary, in which the sample components are separated according to differences in their distribution between the two phases [2].

Since its discovery, innumerable contributions have been reported on chromatography, including LC. However, the aim of this Thesis is far away from numbering all the advances in chromatography and especially in LC, but it seems necessary to mention at least the most relevant milestones achieved in this field as it has been done with CE in **Chapter 3** and with CEC in **Chapter 7**. **Table 10.1** summarizes some of the advances in chromatography through the last century.

Table 10.1. Landmarks in chromatography. Information extracted from [2-6].

Authors	Milestone	Year
M. Tswett	Discovery of chromatography. Separation of chlorophylls.	1903
L.S. Palmer	Separation of carotenoids from butterfat. It represents the first application of chromatography after Tswett's discovery.	1922
E. Lederer	Preparative separation of α- and β-carotene from carrots.	1931
N.A. Izmailov and M.S. Shraiber	First publication regarding TLC.	1938

Table 10.1 (continued). Landmarks in chromatography. Information extracted from [2-6].

Authors	Milestone	Year
A.J.P. Martin and R.L.M. Synge	Development of liquid partition chromatography using water as liquid stationary phase fixed to silica gel as the support for it, and chloroform, containing 0.5% (v/v) alcohol, as mobile phase. Separation of monoamino monocarboxylic acids present in wool.	1941
A.J.P. Martin	Development of paper chromatography and its subsequent application to the separation of dicarboxylic and basic amino acids.	1944
J.E. Meinhard and N.F. Hall	For first time a starch binder was used for holding the adsorbent layer to the rigid support in TLC [7].	1949
F. Prior and E. Cremer	The first chromatogram was obtained for the separation of air and carbon dioxide by adsorption gas chromatography using a thermal-conductivity detector connected to a galvanometer [8].	1947
A.W. Tiselius	Development of many gel types for specific biochemical adsorption.	1948
F. H. Spedding	First papers on ion-exchange chromatography [9].	1949
J.G. Kirchner	The first real TLC separation was carried out using glass plates coated with silicic acid for the analysis of terpenes in essential oils.	1950
A.J.P. Martin and A.T.James	First gas-liquid partition chromatographic system.	1952
R.S. Alm, R.J.P. Williams and A.Tiselius	Introduction of gradient elution analysis [10].	1952
J.J. Van Deemter	Publication of the article describing the relation between plate height and the linear flow velocity in chromatography.	1956
J.E. Lovelock	First chromatography using mobile phases in supercritical state.	1958
J.O. Porath and P. Flodin	Development of size exclusion chromatography.	1959
J.C. Moore	Development of gel-permeation chromatography, enabling the determination of the molecular weight distribution of high-molecular-weight synthetic polymers [11].	1964
C.G. Horvath	Development of high pressure chromatography, using microcolumns of 1 mm i.d. [12].	1967
Waters Corporation	The first LC instrument was commercially available. It was named ALC100 and pumps only had a pressure capability of 35 bar.	1969
J. Kirkland at DuPont	Development of the corresponding 'bonded' stationary phases consisting of long-chain hydrocarbon or other moieties covalently bound on silica particles.	1970
	Development of UPLC chromatographic system and 1.7 μm columns.	2004

In general, during the last decades, the development of chromatography has been characterized by the advances in instrumentation, column technology, computing technology and software development. Regarding HPLC, it is under permanent development, and research is now focused on the introduction of advanced detectors and data evaluation systems with high performance characteristics, the development of new stationary phases including monolithic and hydrophilic interaction liquid chromatography (HILIC) columns and the evaluation of sub-2 μm and analogous particles as column packing materials and the subsequent proposal of UHPLC methods. Furthermore, miniaturization of the technique is also pursued with the aim of reducing solvent consumption and operation costs.

Nowadays, HPLC has matured to one of the most versatile techniques in the analytical and preparative fields [3]. In relation to residue analyses, it has demonstrated to be a powerful tool and is only substituted by GC in the case of the analysis of residues that present high volatility. On the other hand the analysis of residues of chemicals in food products of animal origin is a relatively novel discipline, because the first traceable documents within EU date from 1970s [13]. Initially, the determination of persistent halogenated pesticide residues in food and in environmental matrices was initially carried out with GC and an electron capture detector (ECD), however the advances on LC and MS have involved the development of more efficient methods for the determination of pesticide and pharmaceutical residues at lower detection levels [14]. **Figure 10.1** shows a timeline that represents the evolution of the analytical methods used in residue analysis field.

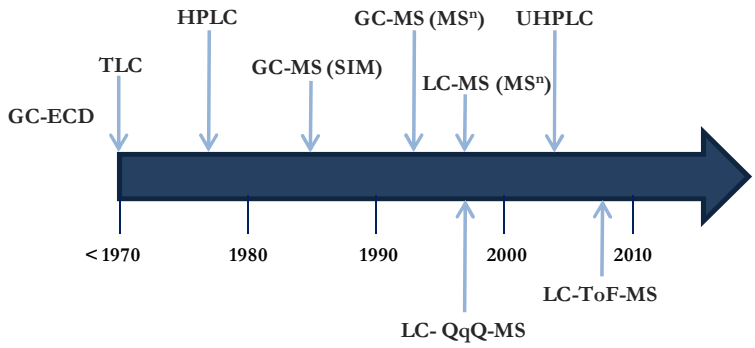

Figure 10.1. Evolution of methods used in residue analysis. SIM: Selected ion monitoring. Adapted from [13].

In this Thesis the use of LC-based methods is proposed for 5-NDZ determination in milk and aquaculture samples. Considering the recent trends in LC-techniques, 5-NDZ separation by CLC and UHPLC has been considered.

10.2. Miniaturized LC-methods

Miniaturization is a general trend common to science and technology, and therefore, LC has not been unaffected by this trend. In 1967, Horváth *et al.* introduced the use of LC microcolumns by performing the separation of ribonucleotides using stainless steel columns of 1 mm i.d. packed with glass beads coated with an anion exchanger containing benzyl-dimethylammonium groups [12]. In 1970's, Ishii *et al.* reported the use of packed Teflon microcolumns of 500 μm i.d. in a serial of publications [15-18], while afterwards Tsuda and Novotny reported the reduction of column i.d. further to 50 – 200 μm [19]. Other important contributions to the development of microcolumns have been attributed to Yang [20] and Scott and Kucera [21].

During the past decades, important advances in LC miniaturization have been accomplished, especially in terms of instrumentation and detection development [22-25]. Miniaturized HPLC systems based on the use of micro-, capillary and nanocolumns present several advantages in comparison with traditional or conventional LC-instrumentation involving columns with i.d. higher than 3.2 mm. In general, HPLC techniques are classified according to the i.d. of the chromatographic column that is used as shown on **Table 10.2**.

Table 10.2. Names and definitions for HPLC techniques. Adapted from [26].

Name	Column i.d.	Flow rate
Conventional HPLC	3.2 – 4.6 mm	0.5 – 2.0 mL/min
Microbore HPLC	1.5 – 3.2 mm	100 – 500 μL/min
Micro-LC	0.5 – 1.5 mm	10 – 100 μL/min
Capillary LC	150 – 500 μm	1 – 10 μL/min
Nano-LC	10 -150 μm	10 – 1000 nL/min

Miniaturized LC-methods are characterized by the high efficiency that can be achieved, as well as the low flow rate that is required for performing LC-separation, and consequently, the reduction of solvent consumption in comparison with traditional LC-based methods. Moreover, lower mass amount of stationary phase is required due to the smaller column dimensions. As a result, miniaturized LC-methods are greener and involve lower economic cost than traditional LC-methods. Considering this aspect, more expensive additives, stationary phases or exotic mobile phases can be used maintaining a low analysis cost and enabling new interaction mechanisms and an improvement in chromatography selectivity [25]. Additionally, an increase in sensitivity in terms of peak height of 235-fold has been theoretically estimated for the reduction in the i.d. of a column from 4.6 mm to 300 μm [23]. As a consequence, it seems to be the perfect technique for the analysis of samples in which a small amount of them are available.

Despite the advantages of HPLC using micro-, capillary or nanocolumns, methods for the determination of 5-NDZs have not been reported in the reviewed literature. However, miniaturized LC-based methods have been proposed for the analysis of compound residues at trace levels in food matrices [27,28]. For that reason, in **Chapter 11**, a novel CLC method has been proposed for 5-NDZ determination in aquaculture products.

10.3. UHPLC technology

In addition to miniaturized LC-columns, in the last years another important evolution in column technology has been the introduction of columns packed with sub-2-μm particles with a higher backpressure requirement ($\Delta P > 400$ bar), leading to the development of UHPLC. This technology was introduced by Jorgenson *et al.* who were the first to describe the use of nano-columns packed with non-porous 1.0–1.5-μm silica-based particles on a prototype system compatible with very high pressure [29]. Since then, UHPLC has been widely used in multiresidue analysis [30-32], particularly because of the ability to transfer existing HPLC conditions directly. Furthermore, because it is possible to work up to 1,000 bar and mass transfer is improved with small particles, a high mobile phase flow rate can be applied. As a consequence, UHPLC speeds up the analytical process without a loss in peak capacity or change in selectivity in comparison with HPLC, as well as solvent

consumption is reduced [33,34]. Indeed, UHPLC increases throughput by 3–10 fold in comparison to conventional HPLC [35], and according to the van Deemter equation, the use of smaller particle diameter of the column packing material results in higher efficiency, which is also predicted by van Deemter plots (**Figure 10.2**). Therefore, height equivalent theoretical plate (HETP) is reduced when smaller particle diameter of column packing material is considered at the same linear velocity.

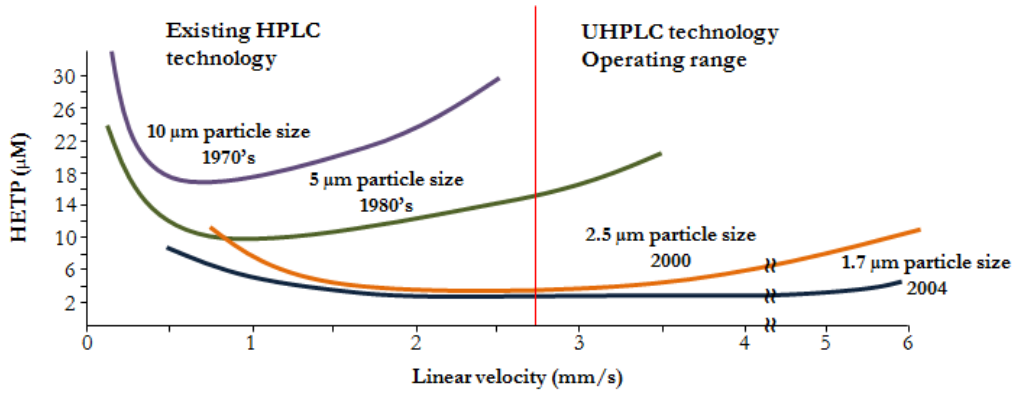

Figure 10.2. Van Deemter plots for several column particle sizes. Adapted from [36].

One of the drawbacks of UHPLC is associated with the frictional heating generated by the elevated backpressure, which can produce temperature gradients inside the column. This heating problem, which is particularly critical for 4.6-mm i.d. columns and/or when the pressure is close to or higher than 1,000 bar, can be resolved by reducing the column i.d. to 2.1 or 1 mm [33]. UHPLC technology provides very narrow peaks, requiring a small detection volume and fast acquisition rate of the detector in order to ensure high efficiency. The flow cell volume is usually much lower than that for conventional HPLC in order to minimize the extra-column volume, typically 0.5–2.0 µL.

On the other hand, most of the applications in the determination of residues in food and environmental samples are based on reversed-phase separations using Acquity UPLC BEH C18 columns of 1.7 µm particle size with different column lengths, but other C18 reversed-phase columns such as Zorbax Eclipse XDB-C18 (1.8 µm particle size) or Hypersil GOLD C18 have also been used [37]. Additionally, on the last years, few applications in the monitoring of residues in food and environmental samples have been

reported either using UV detection [38] or fluorescence detection [39,40], but MS has become the technique of choice in order to guarantee the unequivocal confirmation of target compounds [30,31].

Due to its robustness, speed of analysis and low solvent consumption, UHPLC is the perfect analytical tool for routine analyses. Regarding 5-NDZ determination, a few UHPLC-based methods have been proposed for that purpose [41-45]. However, only MS has been considered as detection tool, so it seems necessary to propose new applications involving UHPLC methods for the analysis of 5-NDZ residues. Furthermore, the proposal of new methods using UV detection as alternative to MS, will supplement and enriched the existing UHPLC-MS methods. With that aim, in **Chapter 12**, a UHPLC-UV method is proposed as low-cost approach for the determination of 5-NDZs compounds in milk samples.

10.4. LC-MS for residue determination

In the last years, LC-tandem MS (LC-MS/MS) has been the technique of choice for multiresidue analysis in food and environmental matrices [46-48], offering good sensitivity and selectivity, and using the IT and mainly the QqQ as analyzers. The possibility of detecting as many analytes as possible in a single analytical run has made the coupling of reverse phase (RP)-LC with MS^2 the most useful option, fulfilling with the requirements of regulations in term of MRLs and required identification points. IT allows multiple stages in fragmentation (MS^n) of target ions, which are selectively trapped and separated of the unwanted species. The limitations of this type of instruments, using tridimensional or linear ion trap (LIT), are the poor resolution and mass shift, the reduced dynamic range and the low number of ions that can be simultaneously isolated. The operation mode of IT was described in **Chapter 3**.

On the other hand, QqQ is a mass analyzer consisting of three quadrupoles arranged in series. Each quadrupole consisted of four circular rods placed in parallel to which an oscillating electric field is applied. Q1 and Q3 are responsible of filtering sample ions according to their m/z ratio. Between both quadrupoles is placed Q2 which acts as a non-

linear collision cell. Ions are selected or scanned in Q1 and Q3 based on the stability of their paths in the established electric field. Once they reach Q2, they are accelerated by the electric field and collided with a neutral gas such as N_2 or Ar to produce small fragments. Therefore, the ionized molecules can be selected in Q1, fragmented in Q2 and their fragmentation ions can be selected and leading in Q3 to the detector. **Figure 10.3** shows a scheme of a QqQ mass spectrometer.

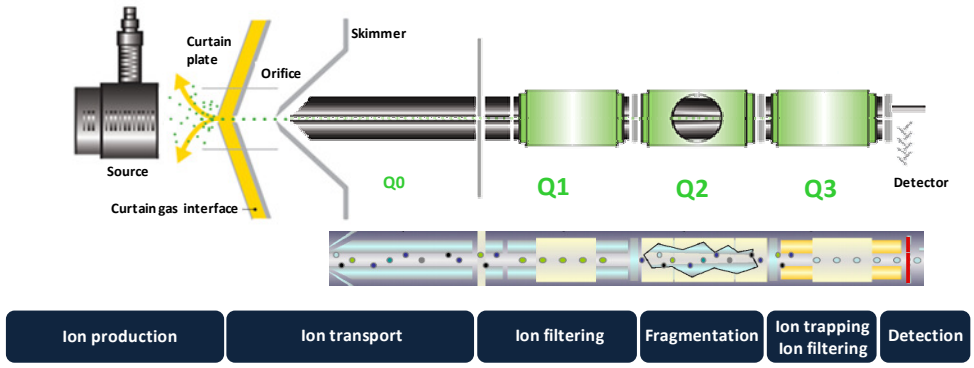

Figure 10.3. Scheme of a QqQ mass spectrometer. Adapted from AB SCIEX website.

QqQ can operate with three types of ion sources, ESI, APCI and atmospheric pressure photoionization (APPI). In practice ESI is the most widely used technique for multiresidue analysis because it does not require additional dopant agent (extra pump is required for APPI) and offers better sensitivity at low flow rates (compared with APCI). ESI procedure has already been described in **Chapter 3**. **Figure 10.4** shows the molecular weight range, and the polarity, in which each ionization mode is suitable for reaching the ionization of the compounds.

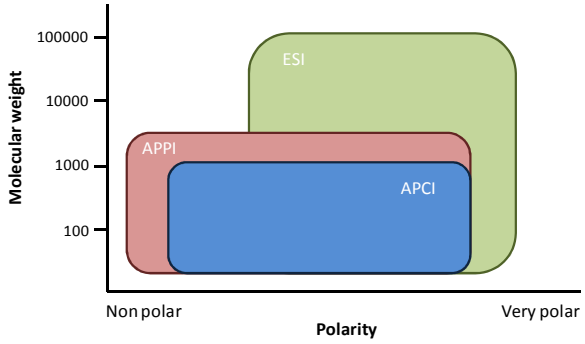

Figure 10.4. Application of the ionization techniques on the basis of the polarity and molecular weight of the compounds. Adapted from [49].

Moreover, MRM is the most common mode applied for target compounds or selected reaction monitoring (SRM), allowing the detection of target analytes preselected prior to their mass signal acquisition (pre-target screening). In this mode, the analytes are usually detected by monitoring the ionic signal of at least two mass transitions, in combination with their chromatographic retention time.

More recent couplings involve the replacing of HPLC by UHPLC and the use of MS instruments working at low dwell times and low inter-channel and interscan delays in order to obtain a sufficient amount of data points per peak for UHPLC applications. In this sense, with new analyzers such as new-generation QqQs, capable of full acquisition rates up to 10000 m/z per s, dwell times of 1 ms and polarity switching in 30 ms or less, are appeared [30]. This combination allows the detection of analytes at a lower LOQ due to the increased resolution of the system, offering shorter injection cycles times. By using UHPLC-MS/MS, different multiresidue methods have been established for the determination of single class of veterinary drugs (e.g. antibiotics such as quinolones, tetracyclines, β-lactams, sulfonamides, aminoglucosides, or antihelmintics, coccidiostats, etc.) as well as pesticides (carbamates, neonicotinoids, sulfonylureas, etc.). Furthermore, several multiclass-multianalyte methods have been developed in the last years for the determination of residues of pesticides and veterinary drugs in a great variety of food and environmental samples [50-53].

In this Thesis, UHPLC-ESI-QqQ-MS has been used for the development of an analytical method for 5-NDZ determination in fish roe samples. As was mentioned in **Chapter 2**, various methods using this technology have been reported for 5-NDZ analysis in food products of animal origin, but this supposes the first time that a method of these characteristics is applied to this type of matrix.

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Determination of 5-NDZs in aquaculture products by capillary liquid chromatography-UV using MISPE

Abstract ► In this chapter, the optimization of a CLC method coupled to UV detection is discussed. Parameters affecting the separation such as mobile phase composition, mobile phase flow rate, separation temperature and gradient program as well as the type of chromatographic column, have been evaluated. Finally, the separation of eleven 5-NDZ compounds has been carried out in a C18 (150 × 0.5 mm, 5 μm) column, using a mobile phase consisted of water (eluent A) and MeCN (eluent B) and supplied at a flow rate of 7 μL/min. Column was thermostated at 20°C during the analysis and 320 nm was established as detection wavelength. Furthermore, full loop injection mode (8 μL) was selected and water was considered as injection solvent. Finally, the optimized method has been applied to the analysis of 5-NDZ residues, including three metabolites, in aquaculture products, namely crab, salmon, prawn and swimming velvet crab. A MISPE procedure has been evaluated for sample clean-up, resulting in extraction recoveries higher than 67% for all considered compounds. The method was characterized in all the matrices in terms of linearity ($R^2 \geq 0.9964$), precision (repeatability, RSD ≤ 7.9% and reproducibility, RSD ≤ 11.1%) and trueness (recoveries ≥ 80.4% and ≤ 108.7%). Decision limits, CCα, ranging from 0.2 to 1.5 μg/L and detection capabilities, CCβ, from 0.2 to 1.8 μg/kg, were obtained.

11.1. Background

Nowadays consumer concerns about food safety are quite relevant and consequently many efforts have been done in the analytical chemistry field in order to ensure it. Occurrence of harmful compounds in foodstuffs can be due to their application during food production (residues such as pesticides or veterinary drugs) or they can be formed during production, storage or food processing (contaminants such as mycotoxins) [1]. Regarding the presence of veterinary drugs in food matrices, EU legislation has classified them in allowed substances for which MRLs have been established and in banned substances [2], while several analytical strategies have been proposed for their determination [3,4]. Carcinogenic, genotoxic and mutagenic properties have been attributed to 5-NDZ drugs [5,6], and as a consequence, their use in animals intended to human consumption has been forbidden. Therefore 5-NDZ residues should not be found in any foodstuff of animal origin. However, alerts about their presence in food matrices as meat and fish products are still reported by RASFF portal. As a consequence, sensitive analytical methods for 5-NDZ determination should be developed. Considering that, EURLs have recommended CCβ or CCα values of 3 μg/L for the developed methods in the case of screening and confirmatory methods, respectively [7]. This recommendation has only been made for the determination of MNZ, DMZ, RNZ and their hydroxyl-metabolites, namely MNZ-OH and HMMNI. However, it is convenient to extrapolate this recommendation to the detection of other substances belonging to 5-NDZ family such as IPZ and its hydroxyl-metabolite (IPZ-OH) or SCZ, TNZ, TRZ and ORZ, having in mind that no legislation has been established for them yet.

Laboratories dedicated to residue determination routinely face the analysis of a large number of samples. For this reason, cheap and green methods are required in order to reduce operating costs and waste generation. Regarding antibiotic monitoring, LC-MS is the most employed analytical technique [8], which could be due to its versatility, robustness and to the valuable data obtained from it. Although MS is a powerful tool, its use in routine laboratories can be limited to the unequivocal identification of the analyte(s) while other cheaper alternatives are performed for detecting the presence of an analyte or group of analytes at the first level of interest. In this sense, LC coupled to UV detection

constitutes a convenient option because it is ease of use, offers low operating costs and qualitative and quantitative information can be obtained from it. In spite of it, few methods based on LC coupled with UV have been proposed for 5-NDZ determination [9-11] and only the detection of a maximum of seven compounds, including some of their metabolites, has been reported [12]. In general these methods are able to determine from two to seven compounds while analysis time ranges from 14 to 23 min without considering column equilibration between runs. Their mobile phase flow rate has been established between 0.5 and 1 mL/min. As a consequence, all referenced methods involve high solvent consumption which could result in an inconvenience for their application in routine analyses.

From the analytical chemistry point of view and with the aim of complying with the basis of Green Chemistry, the reduction of organic solvent consumption, and consequently the decrease in the production of organic waste, is a must. A good strategy for achieving this goal is to modify the relevant column-related parameters as the i.d. of the column. Reduction of column i.d. involves a decrease on the mobile phase flow rate and, logically, on the solvent consumption [13]. With this aim, techniques such as CLC and nano-LC which are characterized for column i.d. of 0.1-0.5 mm and 0.01-0.1 mm, respectively [14], have been successfully implemented in the food analysis field [15]. For example, CLC has been considered for the determination of beta-lactam antibiotics in food of animal origin and waters [16,17], quinolones in milk [18] or pesticides in vegetable, fruits and waters [19,20], proving its potential as analytical tool for residue analysis in food and environmental matrices.

In this chapter a novel separation method based on CLC-UV has been developed for the analysis of 5-NDZ residues in aquaculture products such as crab, salmon, prawn and swimming velvet crab. According to the reviewed literature, the proposed method is the first that employs a miniaturized LC technique for 5-NDZ determination. In order to solve the lack of selectivity associated to UV detection, a specific sample treatment using MIP cartridges has been considered. The effectiveness of the employed extraction procedure has been previously reported [21-23], but it supposes the first time that it is considered for

the selected matrices. MISPE-CLC-UV method has been fully characterized for nine 5-NDZ compounds, including three of their metabolites.

11.2. Materials and methods

11.2.1. Materials and reagents

All reagents were analytical reagent grade, unless indicated otherwise, and solvents were HPLC grade. Ammonium hydroxide solution (30%, v/v), $MgSO_4$, toluene and heptane were obtained from Panreac-Química (Madrid, Spain). MeOH, MeCN and hexane were purchased from VWR International (West Chester, PA, USA) while acetic acid was supplied by Sigma-Aldrich (St. Louis, MO, USA). Formic acid (98–100%, v/v) was acquired from Merck (Darmstadt, Germany). Ultrapure water (Milli-Q plus system, Millipore, Bedford, MA, USA) was used throughout the work. Ammonium acetate solutions were prepared from acetic acid solutions by subsequent pH adjustment with 5 M ammonium hydroxide solution.

Analytical standards of DMZ, RNZ, IPZ-OH, HMMNI, ORZ, MNZ, MNZ-OH and TNZ were supplied by Sigma-Aldrich (St. Louis, MO, USA) while IPZ, SCZ and TRZ were purchased from Witega (Berlin, Germany). Stock standard solutions were obtained by dissolving the appropriate amount of each 5-NDZ drug in MeCN, reaching a final concentration of 1 mg/mL. Stock standard solutions were kept in the freezer at -20°C avoiding exposure to light. Intermediate standard solutions (50 μg/mL of each 5-NDZ) were obtained by mixing the appropriate amount of each stock standard solution and their subsequent dilution with MeCN. They were stored in dark at 4°C and equilibrated to room temperature before their use. Working standard solutions were freshly prepared by diluting intermediate standard solution aliquots with water in order to reach the desired 5-NDZ concentration level. Aquaculture samples were spiked at the required 5-NDZ concentration level using working standard solutions or the intermediate standard solution according to the fortification level.

SupelMIP®SPE-Nitroimidazole cartridges (50 mg, 3 mL) (Sigma Aldrich; St. Louis, MO, USA) were considered for the sample treatment procedure. Clearinert™ 13 mm syringe filters were supplied by Bonna-Agela Technologies (Wilmington, DE, USA).

11.2.2. Instrumentation

Analyses were carried out on a 1200 Series Capillary LC System with UV detection supplied by Agilent Technologies (Santa Clara, CA, USA). Data were collected with HP ChemStation (version A.09.01) software. Different chromatographic columns were tested for 5-NDZ separation, namely Luna C18 (150 × 0.3 mm, 5 μm particle size), Luna C18 (150 × 0.5 mm, 5 μm) and Luna C8 (150 × 0.3 mm, 3 μm), which were acquired from Phenomenex (Torrance, CA, USA), and Zorbax XDB-C18 (150 × 0.5 mm, 5 μm) column which was supplied by Agilent Technologies.

MISPE treatment was carried out on a Visiprep™ DL vacuum manifold for twelve cartridges from Supelco (Bellefonte, PA, USA). A Universal 320R centrifuge (Hettich Zentrifugen; Tuttlingen, Germany), a nitrogen dryer EVA-EC System (VLM GmbH; Bielefeld, Germany), a mechanical shaker (model 384 from Vibromatic; Noblesville, USA) and a vortex-2 Genie (Scientific Industries; Bohemia, NY, USA) were also used. Solution pH was adjusted with a pH meter (Crison model pH 2000; Barcelona, Spain) with a resolution of ±0.01 pH unit.

11.2.3. Chromatographic conditions

CLC separations were performed in a Zorbax XDB-C18 (150 × 0.5 mm, 5 μm) column, using a mobile phase consisted of water (eluent A) and MeCN (eluent B) and supplied at a flow rate of 7 μL/min.

The gradient program was established as follows: 0 min, 5% (v/v) of B; 14 min, 40% (v/v) of B; 17 min, 70% (v/v) of B; 19 min, 95% (v/v) of B. At 20 min, mobile phase composition was back to initial conditions in 4 min. In order to guarantee column equilibration, initial conditions were maintained for 11 min. Column temperature was set to 20°C and 8 μL (full loop injection) was selected as injection volume.

11.2.4. Sample treatment procedure

Crab, salmon, prawn and swimming velvet crab samples were bought from a local supermarket and the muscle tissue was considered as the relevant part of the different analyzed samples. Samples were crushed and homogenized and portions of 2 g were placed in 15-mL conical tubes. Afterwards, they were spiked at the desired 5-NDZ concentration level, 1 mL of deionized water was added to each sample, and the mixture was finally agitated by vortex until sample homogenization was achieved. Before extraction, samples were left to stand for 15 min.

Later on 5 mL of MeCN were added to each sample and they were vortexed for 30 s. Furthermore, 0.5 g of $MgSO_4$ were added and samples were mechanically agitated for 5 min. Then, samples were centrifuged for 10 min at 5000 rpm and 4 mL of each sample supernatant were collected and dried under nitrogen current at 40°C. Finally, samples were reconstituted in 2.5 mL of deionized water by vortex agitation for 2 min.

Hereafter, a clean-up procedure was applied to each sample using commercial MIP cartridges. Extraction protocol recommended by the seller [24], considering slight modifications, was followed in this work. MIP columns were sequentially conditioned with 1 mL of toluene, 1 mL of MeCN and 1 mL of ammonium acetate buffer (10 mM, pH 5). Afterwards, reconstituted samples (2.5 mL) were passed through extraction cartridges by gravity.

Then, columns were washed considering four stages. First, 0.5 mL of deionized water were charged onto the cartridge, followed by loading 1 mL of hexane twice and 1 mL of a mixture 3:1 (v/v) heptane/toluene. Between washing steps, cartridges were vacuum dried (-400 mbar) for 10 s. Finally, samples were eluted in two steps, passing through the column 1 mL of 60:40 (v/v) MeCN/water containing 0.5% (v/v) of acetic acid twice. Between elution steps, cartridges were vacuum dried (-400 mbar) for 10 s. Both elution fractions were mixed and dried under nitrogen current at 40°C. Extracts were re-dissolved in 200 µL of deionized water by vortex agitation and filtered through syringe filters to LC vials and then, they were submitted to CLC-UV analysis.

11.3. Results and discussion

11.3.1. Chromatographic separation optimization

Initially, 5-NDZ separation was evaluated in a Luna C18 (150 × 0.3 mm, 5 μm) column because C18 stationary phase columns have been mainly employed for this purpose [25,26]. Mobile phase was supplied at a flow rate of 10 μL/min and it consisted of 0.5% (v/v) formic acid aqueous solution (eluent A) and pure MeCN (eluent B). Separation was carried out using gradient conditions under a temperature of 30°C.

During 5-NDZ separation, mobile phase composition was kept at 5% (v/v) of eluent B from 0 to 6 min, increasing MeCN content to 20% (v/v) at 7.5 min and to 25% (v/v) at 8.5 min and reaching a maximum of 95% (v/v) at 9.5 min. Maximum MeCN concentration (95%, v/v) was kept for 1 min and initial conditions were reestablished by a 3.5 min linear gradient. In order to reach a proper column equilibration, initial conditions were maintained for 5 min. An injection volume of 1 μL was considered and water was selected as injection solvent. Standard solutions of 2 μg/mL were analyzed under these conditions.

The percentage of formic acid added to eluent A was evaluated from 0.0 to 1.0% (v/v). In **Figure 11.1,I**, it is shown that a higher number of resolved peaks can be distinguished when water without any formic acid is employed as eluent A. Moreover, an increase on formic acid percentage decreased almost all compound elution times and caused a loss of peak resolution. Consequently the addition of an acid to eluent A was discarded.

Furthermore, the use of MeOH as eluent B instead of MeCN was also considered. However, no improvement in terms of peak resolution was observed (**Figure 11.1,II**), hence MeCN was selected as mobile phase organic solvent.

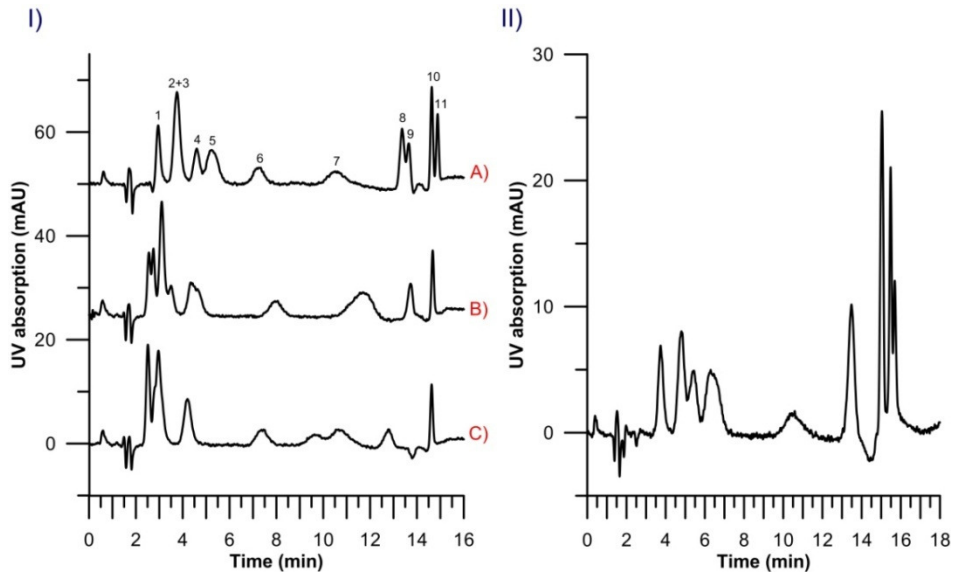

Figure 11.1. Evaluation of the mobile phase composition. I) Chromatograms obtained using pure MeCN as eluent B. Eluent A was selected as follows: A) water, B) 0.5% (v/v) formic acid aqueous solution and C) 1.0% (v/v) formic acid aqueous solution. II) Chromatogram obtained using pure MeOH as eluent B and water as eluent A. Separations were performed in a Luna C18 (150 × 0.3 mm, 5 μm) column under a separation temperature of 30°C. Injection volume was set to 1 μL and standard solutions in water (2 μg/mL) were injected. Peaks are numbered by elution order.

In order to better assess the influence of mobile phase flow rate, the gradient program was modified. Initially eluent B was set to 3% (v/v) and this condition was maintained for 5 min. Content of eluent B was linearly increased to 40% (v/v) in 5 min, then to 70% (v/v) in 4.5 min and finally it reached a 95% (v/v) at 19 min. At the time of 20 min the gradient elution was changed for reaching initial conditions at 24 min. They were stated for a period of 11 min for guarantying column equilibration and obtaining a stable and reproducible separation. Although slight modifications were subsequently assayed, the mentioned gradient program was used for the optimization of the rest of chromatographic parameters and it was finally selected as separation gradient program.

Taking all of these into account, mobile phase flow rate was studied from 6 to 12 μL/min. Although analysis time was slightly increased, a flow rate of 8 μL/min was established as optimum because higher flow rates involved lower peak resolution between peaks 3 and 4 and peaks 7 and 8. On the other hand poor peak resolution was accomplished between

peaks 2 and 3 at lower flow rates whereas peaks 5 and 6 were not resolved under these conditions (**Figure 11.2,I**).

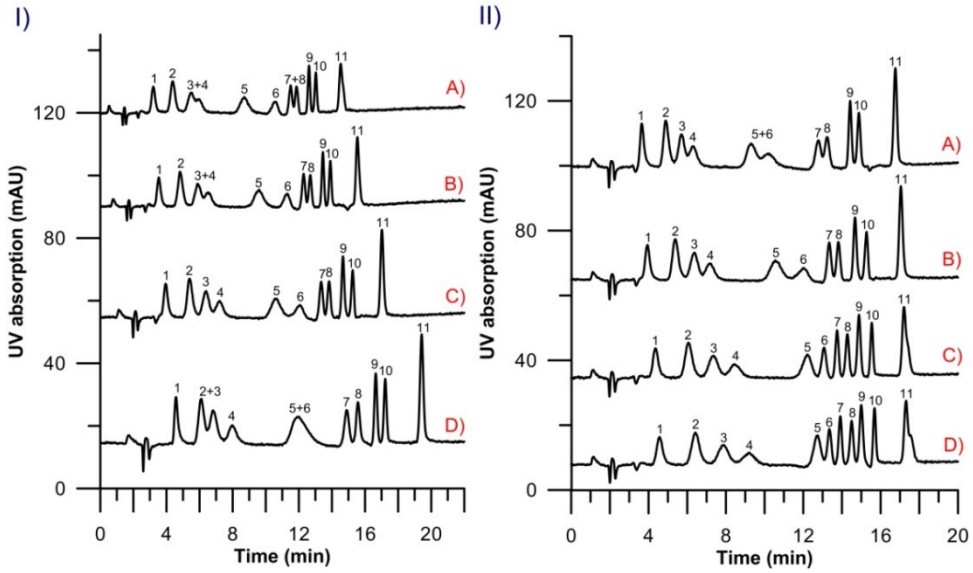

Figure 11.2. **I) Optimization of mobile phase flow rate: A) 12 µL/min, B) 10 µL/min, C) 8 µL/min and D) 6 µL/min. Runs were performed at 30°C. II) Optimization of separation temperature considering a mobile flow rate of 8 µL/min: A) 40°C, B) 30°C, C) 20°C and D) 15 °C. Separations were performed in a Luna C18 (150 × 0.3 mm, 5 µm) column and mobile phase consisted of water (eluent A) and MeCN (eluent B). Injection volume was set to 1 µL and standard solutions (2 µg/mL) in water were analyzed. Peaks are numbered by elution order.**

Furthermore, column temperature was evaluated from 15 to 40°C. Although similar analysis time was obtained under all tested conditions (around 17-18 min), migration times of more polar analytes were lower when higher column temperatures were used (**Figure 11.2,II**). Considering the highest number of peaks that were baseline resolved, 20°C was selected as optimum. Although peak efficiency is usually examined when separation temperature is evaluated, in this case it was not taken into account because different trends were observed depending on the selected analyte.

11.3.2. Comparison among chromatographic columns

All peaks were not baseline resolved under the optimized conditions, so other capillary LC columns were evaluated for 5-NDZ separation. A Luna C18 (150 × 0.5 mm, 5 µm) column and a Zorbax XDB-C18 (150 × 0.5 mm, 5 µm) column were assessed. Both

columns use a C18 stationary phase with similar length of that previously used (Luna C18, 150 × 0.3 mm, 5 μm), but their i.d. is 1.7 times larger. Moreover, a Luna C8 (150 × 0.3 mm, 3 μm) was also evaluated. **Figure 11.3** shows different chromatograms related to 5-NDZ separation performed in each CLC column.

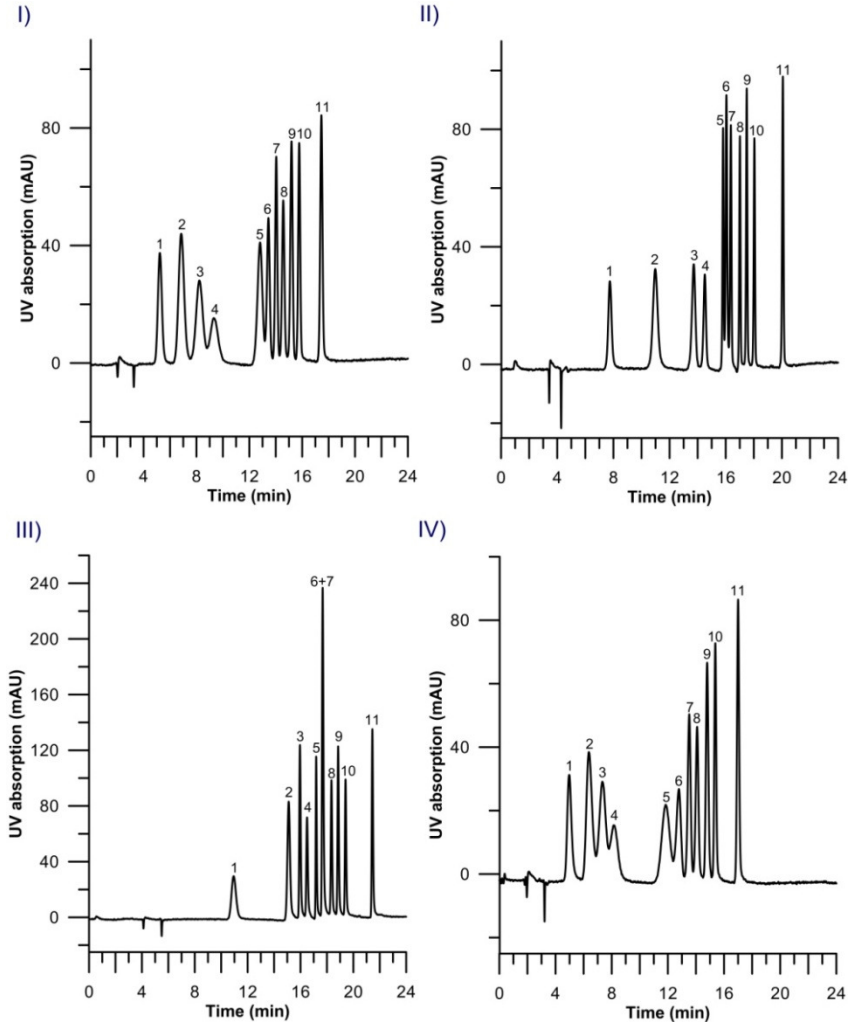

Figure 11.3. **Chromatograms obtained from the analysis of standard solutions (0.5 μg/mL of each 5-NDZ) using different CLC columns: I) Luna C18 (150 × 0.3 mm, 5 μm), II) Zorbax XDB-C18 (150 × 0.5 mm, 5 μm), III) Luna C18 (150 × 0.5 mm, 5 μm) and IV) Luna C8 (150 × 0.3 mm, 3 μm). Separations were performed at 20°C and a mobile phase consisted of water (eluent A) and MeCN (eluent B) was applied at flow rate of 8 μL/min. Gradient program was established as follows: 0 min: 3% (v/v) of B; 5 min: 3% (v/v) of B; 10 min: 40% (v/v) of B; 14.5 min: 70% (v/v) of B; 19 min: 95% (v/v) of B; 20 min: 95% (v/v) of B; 24 min: 3% (v/v) of B and 35 min: 3% (v/v) of B. Injection volume was set to 8 μL. Peaks are numbered by elution order.**

As it can be deduced from **Figure 11.3**, C8 and C18 stationary phases present similar selectivity for 5-NDZ separation and slightly lower retention times were observed (variations from 0.2 to 1.2 min) when C8 column was considered. On the other hand, peak efficiency was not increased when C8 column was employed in spite its smaller particle size, so C8 stationary phase column was discarded. However, promising results were obtained when C18 columns with larger i.d. were tested. Higher peak efficiency was reached when a Zorbax XDB-C18 (150 × 0.5 mm, 5 μm) and Luna C18 (150 × 0.5 mm, 5 μm) columns were employed instead of a Luna C18 (150 × 0.3 mm, 5 μm) column, although analysis time was slightly increased. Finally, a Zorbax XDB-C18 (150 × 0.5 mm, 5 μm) column was selected. According to previous assays, it was assumed that this column could have many more potential for achieving baseline resolved peaks.

11.3.3. Reoptimization of chromatographic conditions

Taking into account that a Zorbax XDB-C18 (150 × 0.5 mm, 5 μm) column was finally selected for the proposed method instead of the previous considered Luna C18 (150 × 0.3 mm, 5 μm) column, parameters affecting the separation such as mobile flow rate or gradient program were reevaluated. Finally, separation was carried out in a Zorbax SB-C18 (150 × 0.5 mm, 5 μm particle size) column at 20°C. Mobile phase consisted of water (eluent A) and MeCN (eluent B) was supplied at 7 μL/min. Initially eluent B was set a 5% (v/v) and was linearly increased to 40% (v/v) for 14 min. Afterwards, it reached a 70% (v/v) at 17 min and a 95% at 19 min. At the time of 20 min the gradient elution was changed and initial conditions were reached at 24 min. Finally they were stated for a period of 11 min in order to ensure a stable and reproducible separation.

11.3.4. Injection optimization

Standard solutions in water (0.5 μg/mL of each 5-NDZ) were analyzed and injection volumes from 1 μL to 8 μL (full loop injection) were studied. As it can be deduced from **Figure 11.4**, a linear dependence between injection volume and peak height for each analyte was established. As a consequence, full loop injection was set and no band-broadening or loss of peak resolution was observed under this condition.

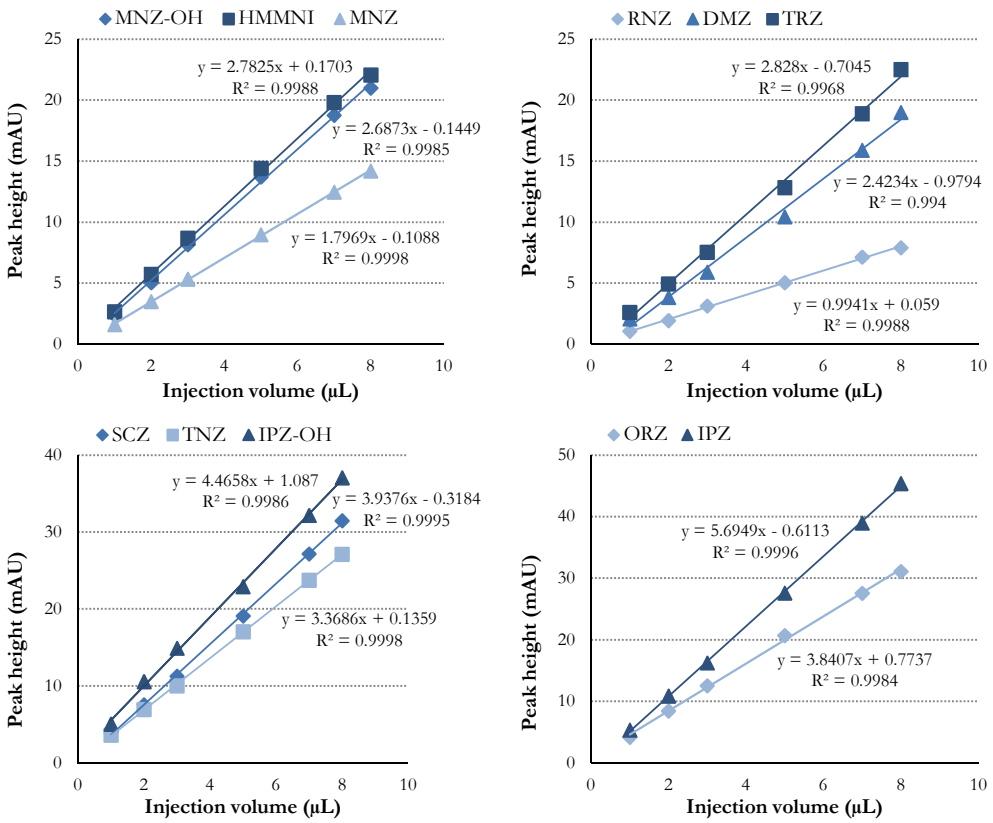

Figure 11.4. Variation of the peak height according to the injection volume. Standard solutions in water (0.5 µg/mL of each compound) were injected and analyzed.

11.3.5. Method characterization in aquaculture products

The proposed analytical method was applied in aquaculture products, namely crab, salmon, prawn and swimming velvet crab. Following EURLs' recommendations, muscles were considered as the relevant part of the different analyzed products. Prior to analysis, samples were submitted to a sample treatment based on MISPE procedure (**Section 11.2.4**). The analytical method was evaluated for all matrices in terms of linearity, LODs and LOQs, extraction recoveries, trueness, intra-day and inter-day precision. All peak signals were monitored at 320 nm. **Figure 11.5,I** shows a chromatogram resulting from the analysis of a standard solution (50 µg/L) under the optimized conditions. Moreover, **Figure 11.5,II** shows chromatograms related to the separation of eight 5-NDZs and three of their metabolites in crab samples fortified at different concentration

levels. It was observed that matrix inherent compounds did not co-eluted with the studied 5-NDZ analytes.

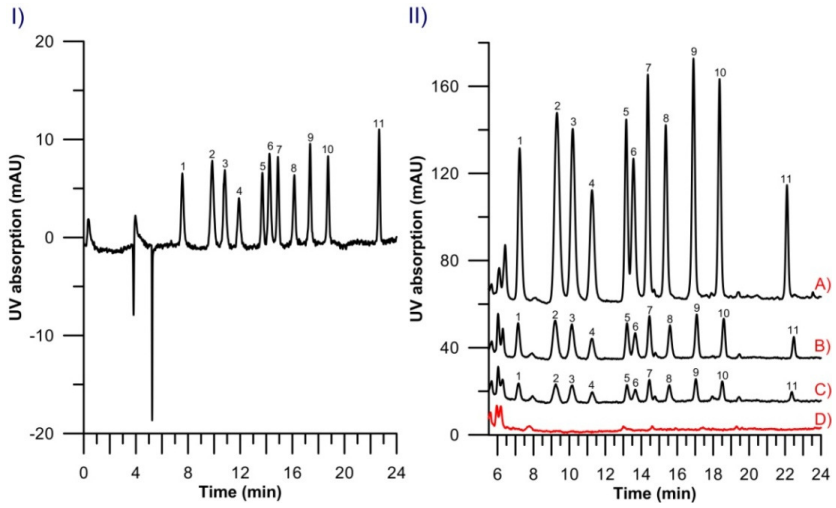

Figure 11.5. Chromatograms resulted from the analysis of I) 5-NDZ standard solution (50 µg/L) and II) Crab samples spiked with 5-NDZ compounds at different concentration levels (A, 100 µg/kg; B, 50 µg/kg; C, 10 µg/kg and D, blank sample). Separations were performed in a Zorbax SB-C18 (150 × 0.5 mm, 5 µm) column using a mobile phase consisted of water (eluent A) and MeCN (eluent B) and supplied at 7 µL/min. Injection volume was set to 8 µL. UV detection wavelength was established at 320 nm. Peaks: 1, MNZ-OH; 2, HMMNI; 3, MNZ; 4, RNZ; 5, TRZ; 6, DMZ; 7, SCZ; 8, TNZ; 9, IPZ-OH; 10, ORZ; 11, IPZ.

Calibration curves and performance characteristics

Firstly, treated blank samples were analyzed and no endogenous interferences were detected at the same 5-NDZ retention times, except for MNZ determination in swimming velvet crab samples (**Figure 11.6,III**). However, the fraudulent use of MNZ in this type of product can be still monitored by the proposed method through the detection of its metabolite (MNZ-OH). Due to the fact that the analyzed samples are complex matrices, the combination of the proposed sample treatment with the developed CLC-UV method shows a high selectivity for 5-NDZ determination. Matrix-matched calibration curves were performed for all aquaculture samples by fortifying them at the following concentration levels: 10, 20, 50, 100 and 150 µg/kg. Two spiked samples per level were treated following the MISPE procedure. Afterwards, each sample was analyzed in duplicate according to the proposed CLC-UV method. Peak height was considered as function of analyte

concentration on the sample. LODs and LOQs of the method were calculated as the minimum analyte concentration yielding a S/N equal to three and ten, respectively (**Table 11.1**).

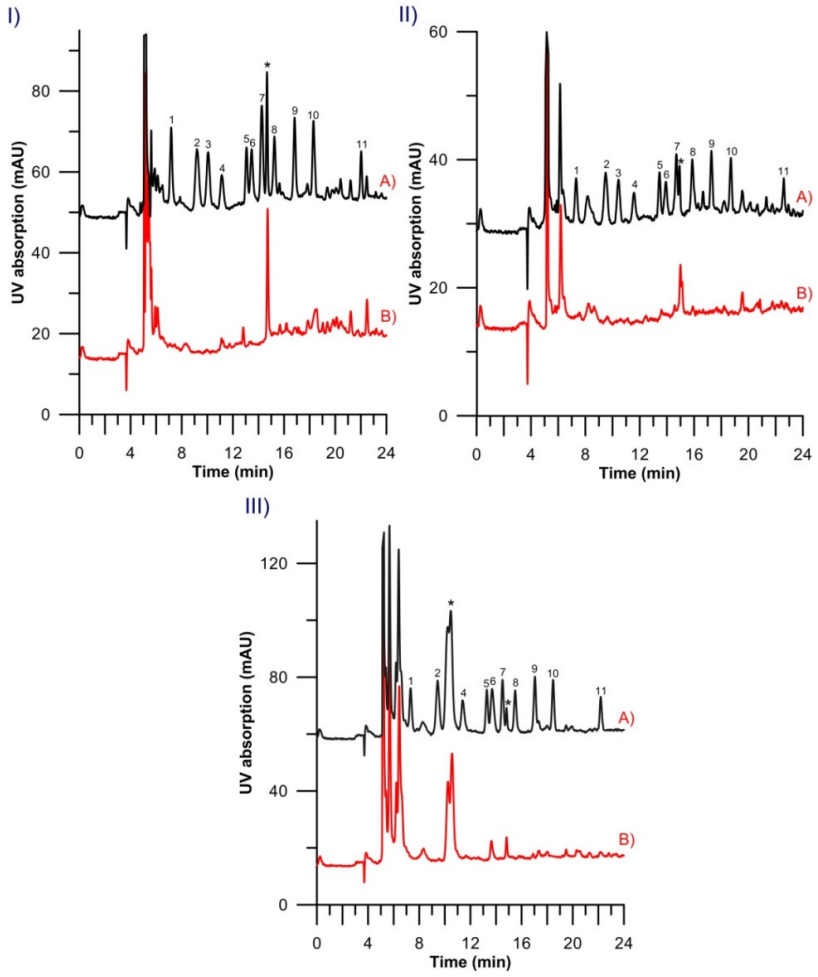

Figure 11.6. Chromatograms resulted from the analysis of I) salmon samples (A, spiked at 20 µg/kg of each 5-NDZ; B, blank sample), II) prawn samples (A, spiked at 20 µg/kg of each 5-NDZ; B, blank sample) and III) swimming velvet crab samples (A, spiked at 20 µg/kg of each 5-NDZ; B, blank sample). Separations were performed in a Zorbax SB-C18 (150 × 0.5 mm, 5 µm) column using a mobile phase consisted of water (eluent A) and MeCN (eluent B) and supplied at 7 µL/min. Injection volume was set to 8 µL. UV detection wavelength was established at 320 nm. Peaks: 1, MNZ-OH; 2, HMMNI; 3, MNZ; 4, RNZ; 5, TRZ; 6, DMZ; 7, SCZ; 8, TNZ; 9, IPZ-OH; 10, ORZ; 11, IPZ; *, unknown peaks.

Table 11.1. Statistical and performance characteristics of the proposed CLC-UV method for 5-NDZ determination.

Analyte	Linear range (µg/kg)	R^2	Linear regression equation (y=m·x+a)				LOD (µg/kg)	LOQ (µg/kg)	CCα (µg/kg)	CCβ (µg/kg)
			Slope	*SD for the slope*	*Intercept*	*SD for the intercept*	$3 \times S/N$	$10 \times S/N$		
					Crab					
MNZ-OH	10-150	0.9986	0.713	0.016	1.369	1.310	1.5	5.1	0.3	0.4
HMMNI	10-150	0.9999	0.851	0.005	0.506	0.423	1.2	4.1	0.3	0.3
MNZ	10-150	1.0000	0.764	0.002	0.011	0.130	1.4	4.8	0.3	0.4
RNZ	10-150	0.9999	0.484	0.002	0.267	0.183	2.1	6.9	0.4	0.5
TRZ	10-150	0.9999	0.788	0.004	0.639	0.371	1.4	4.7	0.3	0.3
DMZ	10-150	0.9992	0.499	0.010	1.385	0.817	1.7	5.8	0.4	0.8
SCZ	10-150	1.0000	0.957	0.001	0.098	0.079	1.1	3.8	0.2	0.2
TNZ	10-150	0.9999	0.746	0.004	-0.062	0.345	1.5	5.0	0.3	0.4
IPZ-OH	10-150	0.9998	1.008	0.008	-0.425	0.681	1.1	3.7	0.2	0.2
ORZ	10-150	0.9998	0.937	0.008	-0.938	0.669	1.2	4.0	0.2	0.3
IPZ	10-150	0.9993	0.367	0.007	1.559	0.529	2.2	7.4	0.6	1.2
					Salmon					
MNZ-OH	10-150	0.9972	0.620	0.019	1.041	1.599	1.9	6.5	0.9	1.1
HMMNI	10-150	0.9988	0.728	0.015	1.611	1.248	1.6	5.2	0.8	0.9
MNZ	10-150	0.9964	0.697	0.024	1.618	2.048	1.7	5.7	0.8	0.9
RNZ	10-150	0.9984	0.445	0.010	0.826	0.871	2.4	8.1	1.3	1.4
TRZ	10-150	0.9984	0.712	0.017	1.382	1.399	1.6	5.3	0.8	0.9
DMZ	10-150	0.9932	0.350	0.017	0.281	1.405	2.9	9.6	1.6	1.8
SCZ	10-150	0.9987	0.880	0.018	1.762	1.523	1.2	4.2	0.6	0.7
TNZ	10-150	0.9985	0.688	0.015	2.358	1.283	1.4	4.8	0.8	0.9
IPZ-OH	10-150	0.9984	0.944	0.022	-0.711	1.836	1.3	4.4	0.6	0.7
ORZ	10-150	0.9998	0.918	0.007	0.236	0.551	1.2	4.2	0.6	0.7
IPZ	10-150	0.9993	0.367	0.007	1.559	0.529	2.3	7.7	1.5	1.8

Table 11.1 (continued). Statistical and performance characteristics of the proposed capillary CLC-UV method for 5-NDZ determination.

Analyte	Linear range (µg/kg)	R^2	Linear regression equation (y=m·x+a)				LOD (µg/kg)	LOQ (µg/kg)	CCα (µg/kg)	CCβ (µg/kg)
			Slope	*SD for the slope*	*Intercept*	*SD for the intercept*	$3 \times S/N$	$10 \times S/N$		
					Prawn					
MNZ-OH	10-150	0.9991	0.772	0.014	0.439	1.145	2.1	7.1	0.4	0.5
HMMNI	10-150	0.9997	0.933	0.009	0.206	0.784	1.7	5.8	0.4	0.4
MNZ	10-150	0.9999	0.826	0.006	0.085	0.481	2.0	6.5	0.4	0.5
RNZ	10-150	0.9999	0.533	0.002	0.033	0.205	3.1	10.2	0.6	0.7
TRZ	10-150	0.9999	0.822	0.004	0.010	0.369	1.9	6.5	0.4	0.5
DMZ	10-150	0.9855	0.603	0.042	3.942	3.554	2.5	8.2	0.5	0.8
SCZ	10-150	1.0000	1.003	0.003	-0.098	0.288	1.6	5.4	0.3	0.4
TNZ	10-150	0.9998	0.777	0.006	0.451	0.537	2.0	6.8	0.4	0.5
IPZ-OH	10-150	1.0000	1.100	0.003	-0.108	0.251	1.5	5.0	0.3	0.3
ORZ	10-150	0.9997	0.993	0.010	0.382	0.807	1.6	5.5	0.3	0.4
IPZ	10-100	0.9954	0.693	0.047	-1.113	1.485	3.2	10.5	0.6	0.8
					Swimming velvet crab					
MNZ-OH	10-150	0.9994	0.694	0.009	2.260	0.797	1.2	3.9	0.5	0.6
HMMNI	10-100	0.9989	0.791	0.018	1.205	1.034	1.0	3.3	0.4	0.4
MNZ					N/A					
RNZ	10-150	0.9981	0.474	0.012	0.647	1.004	1.7	5.6	0.7	0.9
TRZ	10-150	0.9997	0.759	0.007	1.601	0.613	1.2	3.9	0.4	0.5
DMZ	10-150	0.9995	0.698	0.009	-0.672	0.781	1.5	5.0	0.5	0.6
SCZ	10-150	0.9992	0.920	0.015	1.338	1.302	0.9	3.1	0.4	0.4
TNZ	10-150	1.0000	0.736	0.002	0.267	0.205	1.3	4.2	0.4	0.5
IPZ-OH	10-150	0.9995	1.014	0.013	0.254	1.091	0.9	3.1	0.3	0.4
ORZ	10-150	0.9994	0.942	0.014	0.656	1.141	0.9	3.1	0.3	0.4
IPZ	10-150	0.9993	0.367	0.007	1.559	0.529	2.0	6.8	0.9	1.0

Not applicable (NA) due to the presence of a co-eluting matrix compound.

Despite the lack of sensitivity attributed to UV detection, LODs lower than 3.2 μg/kg were obtained for all the studied 5-NDZ compounds in all evaluated matrices. Furthermore, validation procedure was completed by the estimation of CCα and CCβ parameters [27]. This procedure allowed the determination of both parameters for non-permitted veterinary drug substances. As can be seen on **Table 11.1**, the obtained data for CCα and CCβ ranged between 0.2-1.5 and 0.2-1.8 μg/kg, respectively. Both parameters were lower than 3 μg/kg, accomplishing with EURLs' recommendations.

Evaluation of the proposed MISPE procedure

In order to evaluate the efficiency of the extraction process and the possible loss of analytes, recovery studies were performed over crab, salmon, prawn and swimming velvet crab samples fortified at three different concentration levels. For this purpose, calibration curves were established using samples which were spiked at the following fortification levels (10, 20, 50, 100 and 150 μg/kg) after they were treated by the proposed sample treatment procedure. The same levels were considered for the establishment of matrix-matched calibration curves with samples spiked just before the sample treatment. Recovery percentage for each analyte in each matrix was calculated as the ratio between slopes obtained from the calibration curves of samples spiked before and after the sample treatment, respectively. These studies evaluated the efficiency of the extraction process.

As can be seen on **Table 11.2**, extraction efficiency is more dependent on the considered analyte than the assayed matrix. Higher polar compounds, namely MNZ-OH, HMMNI, MNZ and RNZ showed high extraction efficiency (≥ 79.5%) while recoveries ranging between 67.0 and 82.6% were obtained for the rest of analytes, except for IPZ and DMZ. Although MISPE demonstrated that it is suitable for extracting the majority of 5-NDZ compounds from aquaculture products, recoveries lower than 52% showed that this procedure could be not convenience for IPZ and DMZ determination. However, the proposed extraction procedure is suitable for their metabolite extraction, IPZ-OH and HMMNI, respectively. As consequence, the illegal use of IPZ and/or DMZ could be assumed by the presence of their metabolites in the studied matrix.

Table 11.2. Recovery studies (%) in aquaculture products (crab, salmon, prawn and swimming velvet crab).

Analyte	Recovery (%)			
	Crab	Salmon	Prawn	Swimming velvet crab
MNZ-OH	94.8	92.7	94.3	95.0
HMMNI	99.9	99.0	100.5	79.5
MNZ	86.6	87.0	88.1	NA
RNZ	82.7	85.3	85.5	83.2
TRZ	72.4	82.6	72.5	80.4
DMZ	38.5	26.5	45.1	58.6
SCZ	70.3	70.9	70.5	74.0
TNZ	67.6	68.6	69.5	71.6
IPZ-OH	67.0	69.2	72.1	72.8
ORZ	74.4	75.2	78.5	78.3
IPZ	24.0	24.1	42.4	24.4

Not applicable (NA) because of the presence of a co-eluting matrix compound.

Precision assays

Precision studies were carried out in order to evaluate the repeatability (intra-day precision) and the intermediate precision (inter-day precision) of the proposed MISPE-CLC-UV method. Repeatability was assessed by the application of the whole procedure to three samples per matrix (experimental replicates) spiked at three 5-NDZ concentration levels (10, 20 and 100 μg/kg), and analyzed on the same day. Each sample was injected in triplicate (instrumental replicates). Intermediate precision was evaluated at three different concentration levels, namely 10, 20 and 100 μg/kg, by analyzing a spiked sample in triplicate per matrix and per day for five consecutive days. Results expressed as RSDs (%) of peak heights can be found on **Table 11.3.** In all cases RSDs were lower than 11.1%, excluding IPZ and DMZ.

Table 11.3. Statistical and performance characteristics of the proposed CLC-UV method for 5-NDZ determination.

Analyte	Crab						Salmon					
	Repeatability (% RSD, n = 9)			Intermediate precision (% RSD, n = 12)			Repeatability (% RSD, n = 9)			Intermediate precision (% RSD, n = 12)		
	10 µg/kg	*20 µg/kg*	*100 µg/kg*	*10 µg/kg*	*20 µg/kg*	*100 µg/kg*	*10 µg/kg*	*20 µg/kg*	*100 µg/kg*	*10 µg/kg*	*20 µg/kg*	*100 µg/kg*
MNZ-OH	7.1	6.4	6.7	4.5	5.0	3.9	7.1	1.1	0.9	7.6	4.2	0.9
HMMNI	3.1	3.2	5.8	2.9	4.1	2.9	4.1	2.4	0.4	3.8	5.4	0.3
MNZ	5.3	3.2	6.5	2.6	5.0	1.6	2.6	1.3	0.6	3.6	3.4	1.2
RNZ	3.4	3.2	6.7	6.8	3.9	2.2	3.6	3.7	1.8	7.7	3.0	4.3
TRZ	2.7	5.1	7.8	3.6	5.4	1.7	4.7	1.6	0.9	6.8	1.6	1.8
DMZ	13.3	>20%	>20%	9.6	3.3	12.8	4.1	2.0	1.1	18.4	6.3	5.9
SCZ	2.0	1.3	6.0	2.5	3.7	2.2	3.0	2.0	0.7	5.3	1.2	1.5
TNZ	5.1	2.1	5.1	4.1	2.7	2.8	4.9	2.3	0.7	2.2	2.6	7.7
IPZ-OH	2.4	1.8	5.2	4.2	1.9	3.5	5.6	2.8	0.8	2.4	2.1	2.9
ORZ	3.4	1.6	4.2	5.3	5.2	3.5	6.8	3.8	1.1	5.4	1.8	0.7
IPZ	16.9	>20%	>20%	13.3	5.7	18.9	6.4	5.1	1.7	8.4	6.4	>20%
	Prawn						**Swimming velvet crab**					
	Repeatability (% RSD, n = 9)			Intermediate precision (% RSD, n = 12)			Repeatability (% RSD, n = 9)			Intermediate precision (% RSD, n = 12)		
	10 µg/kg	*20 µg/kg*	*100 µg/kg*	*10 µg/kg*	*20 µg/kg*	*100 µg/kg*	*10 µg/kg*	*20 µg/kg*	*100 µg/kg*	*10 µg/kg*	*20 µg/kg*	*100 µg/kg*
MNZ-OH	3.3	6.7	3.5	7.3	5.4	6.4	5.0	3.7	0.4	6.8	5.7	0.9
HMMNI	4.9	2.8	2.3	7.0	6.5	4.6	2.3	2.5	0.6	5.0	4.2	3.8
MNZ	3.0	4.3	2.2	7.8	5.0	3.8			N/A			
RNZ	4.2	6.6	1.3	8.4	9.2	3.3	7.9	3.9	1.1	4.7	5.9	1.9
TRZ	2.5	4.0	2.1	7.5	5.1	2.0	5.3	2.0	1.4	2.2	5.1	1.3
DMZ	9.8	11.3	5.0	16.1	10.4	7.7	5.2	2.6	0.9	9.9	7.4	1.7
SCZ	6.0	3.8	1.9	7.6	11.1	2.5	4.1	3.7	1.7	5.7	2.7	0.9
TNZ	5.6	5.5	2.6	3.7	6.1	10.7	3.3	2.8	0.8	4.9	3.4	0.7
IPZ-OH	2.2	1.1	1.3	5.2	5.6	3.9	2.8	2.4	1.2	2.9	2.8	0.8
ORZ	1.7	2.2	1.8	6.9	6.6	3.9	2.9	3.5	0.9	3.6	3.2	0.9
IPZ	5.8	15.8	12.4	16.1	15.8	11.2	5.0	3.5	1.6	14.1	7.8	5.0

Not applicable (NA) because of the presence of a co-eluting matrix compound.

On the basis of these results, it can be definitively concluded that the proposed sample treatment could not be the most appropriate for IPZ and DMZ extraction from aquaculture products. However, the proposed MISPE-CLC-UV method is still valid for determination of nine 5-NDZs, including MNZ, DMZ, RNZ and IPZ metabolites, namely MNZ-OH, HMMNI and IPZ-OH, achieving their separation in less than 18.7 min. In comparison with previously reported LC-methods using UV detection [9-12], the proposed method involves a similar analysis time for the determination of a higher number of analytes. Moreover, LODs at similar μg/kg levels have been reached with the advantage of a lower solvent consumption.

Trueness

Trueness was assessed in terms of method recoveries. Samples of each matrix spiked at three different concentration levels (10, 20 and 100 μg/kg) were considered. They were treated following the MISPE procedure and finally they were analyzed in triplicate. Considering matrix-matched calibration curves shown on **Table 11.1**, the concentration found in the samples were estimated and compared with the added concentration. These studies were repeated for three days. As can be seen on **Table 11.4**, recoveries between 80.4 and 108.7% were obtained, resulting in a trueness measurement uncertainly ranging from -20 to +10%, accomplishing with the trueness range established for residue concentration ≥ 10 μg/kg by Regulation 2002/657/EC [28].

Table 11.4. Trueness assays of 5-NDZ determination in aquaculture products by the proposed MISPE-CLC-UV method.

Analyte	Crab			Salmon		
	Concentration added (µg/kg)	Concentration found (mean ± SD, µg/kg) (n = 9)	Recovery (%)	Concentration added (µg/kg)	Concentration found (mean ± SD, µg/kg) (n = 9)	Recovery (%)
MNZ-OH	10	8.2 ± 0.5	82.1	10	10.6 ± 0.3	105.9
	20	18.0 ± 0.9	90.0	20	19.2 ± 0.8	96.1
	100	97 ± 2	96.9	100	105 ± 1	104.5
HMMNI	10	9.6 ± 0.2	95.8	10	9.2 ± 0.5	92.2
	20	19.7 ± 0.9	98.6	20	20 ± 1	98.7
	100	99 ± 3	98.8	100	98.8 ± 0.8	98.8
MNZ	10	10.0 ± 0.3	100.3	10	8.1 ± 0.4	80.6
	20	20 ± 1	98.5	20	18.0 ± 0.8	90.0
	100	101 ± 2	100.8	100	103 ± 1	102.9
RNZ	10	10.1 ± 0.8	100.5	10	9.2 ± 0.6	92.0
	20	19.9 ± 0.8	99.6	20	17.9 ± 0.6	89.5
	100	101 ± 2	100.8	100	93 ± 2	93.1
TRZ	10	9.4 ± 0.2	93.6	10	8.4 ± 0.7	84.5
	20	19 ± 1	97.3	20	18.5 ± 0.4	92.7
	100	102 ± 2	101.7	100	100 ± 2	99.8
SCZ	10	10.3 ± 0.3	102.6	10	8.2 ± 0.3	82.2
	20	20.6 ± 0.8	103.0	20	18.2 ± 0.3	90.8
	100	102 ± 2	102.7	100	101 ± 1	100.5
TNZ	10	10.2 ± 0.4	101.9	10	9.1 ± 0.3	90.6
	20	20.4 ± 0.6	101.8	20	17.3 ± 0.4	86.4
	100	103 ± 2	103.6	100	92 ± 7	92.1
IPZ-OH	10	10.6 ± 0.5	106.3	10	10.7 ± 0.3	107.3
	20	20.9 ± 0.3	104.4	20	20.7 ± 0.5	103.7
	100	105 ± 2	105.8	100	101 ± 3	100.6
ORZ	10	10.8 ± 0.6	108.7	10	9.6 ± 0.5	96.5
	20	21 ± 1	105.6	20	19.6 ± 0.4	97.9
	100	103 ± 4	103.3	100	97.5 ± 0.5	97.5

Table 11.4 (continued). Trueness assays of 5-NDZ determination in aquaculture products by the proposed MISPE-CLC-UV method.

Analyte	Prawn			Velvet swimming crab		
	Concentration added (μg/kg)	Concentration found (mean ± SD, μg/kg) (n = 9)	Recovery (%)	Concentration added (μg/L)	Concentration found (mean ± SD, μg/kg) (n = 9)	Recovery (%)
MNZ-OH	10	9.2 ± 0.8	91.7	10	8.0 ± 0.8	80.4
	20	20 ± 1	98.9	20	18.3 ± 0.7	91.7
	100	93 ± 5	93.1	100	99.5 ± 0.9	99.5
HMMNI	10	9.0 ± 0.6	89.6	10	9.7 ± 0.3	97.2
	20	19 ± 1	95.5	20	19.0 ± 0.9	94.9
	100	93 ± 3	92.9	100	93 ± 3	93.2
MNZ	10	8.9 ± 0.7	88.8			
	20	18.7 ± 0.9	93.3		N/A	
	100	93 ± 2	93.3			
RNZ	10	9.5 ± 0.9	95.5	10	10.3 ± 0.6	102.6
	20	20 ± 2	98.3	20	21 ± 1	102.6
	100	93 ± 1	93.3	100	97 ± 2	96.9
TRZ	10	9.6 ± 0.8	95.9	10	8.1 ± 0.2	81.1
	20	19 ± 1	95.6	20	18.9 ± 0.8	94.5
	100	97 ± 2	97.3	100	102 ± 1	101.6
SCZ	10	11 ± 2	107.5	10	8.8 ± 0.3	88.0
	20	20 ± 3	101.7	20	18.8 ± 0.5	94.2
	100	98 ± 3	97.9	100	100.8 ± 0.8	100.8
TNZ	10	10.1 ± 0.4	101.1	10	9.9 ± 0.6	99.2
	20	21 ± 1	102.7	20	19.6 ± 0.8	97.9
	100	106 ± 12	105.8	100	101.2 ± 0.6	101.2
IPZ-OH	10	9.2 ± 0.5	92.5	10	9.7 ± 0.3	97.1
	20	19 ± 1	96.2	20	19.1 ± 0.5	95.5
	100	96 ± 4	95.5	100	100.4 ± 0.9	100.4
ORZ	10	8.9 ± 0.7	88.6	10	9.5 ± 0.3	94.9
	20	19 ± 1	94.9	20	18.9 ± 0.6	94.6
	100	96 ± 3	95.7	100	99.2 ± 0.5	99.2

Not applicable (NA) because of the presence of a co-eluting matrix compound.

11.4. Conclusions

A novel CLC-UV method has been developed for 5-NDZ residue determination in aquaculture products. A MISPE procedure has been evaluated as sample clean-up resulting in satisfactory extraction recoveries and achieving high selectivity and sensitivity in spite of using UV detection. Furthermore, the optimized method involves low solvent consumption which supposes a great advantage and accomplishes with the basis of Green Analytical Chemistry. The proposed method has been fully characterized for nine 5-NDZ determination, including three of their metabolites. All analytes were separated in less than 18.7 min. High sensitivity was also observed, being able to detect these veterinary drugs at low μg/kg levels. Linearity, precision and trueness showed that the proposed method can be applied to routine analysis laboratories, replacing traditional LC-UV methods which are less environmental friendly.

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Determination of 5-NDZs in milk samples by UHPLC-UV coupled to salt-assisted liquid-liquid extraction

Abstract ► In this chapter an exhaustive study of the parameters involved in a SALLE procedure has been carried out for the extraction of eight 5-NDZ antibiotics from milk samples prior to their analysis by UHPLC-UV. Extraction solvent nature and volume, the amount of the salt agent and its nature as well as centrifugation and vortex timing were the optimized parameters for SALLE procedure. The extract resulted from the SALLE procedure was filtered and analyzed by UHPLC-UV. An injection volume of 20 μL (full loop injection mode) was considered. Separation was accomplished in a C18 Zorbax Eclipse Plus (50 mm × 2.1 mm, 1.8 μm) column within 8 min. Gradient mode was considered and mobile phase consisted of 0.1% (v/v) formic acid aqueous solution (eluent A) and MeCN containing 0.1% (v/v) formic acid (eluent B). Mobile phase flow rate was established at 0.45 mL/min and the column was thermostatically controlled at 45 °C during the analysis. Analytical signals were monitored at 320 nm. Matrix-matched calibration curves showed satisfactory linearity ($R^2 \geq 0.996$). LODs ranging from 2 to 4 μg/L were achieved and precision studies showed RSDs, in terms of peak height, lower than 12.8%. Additionally, recoveries higher than 62.8% were obtained for all studied compounds in milk samples.

12.1. Background

Milk consumption is much extended around the world due to its nutritive components like saturated fat, proteins and calcium. Nevertheless, uncontrolled use of antibiotics in farms for treating veterinary diseases can turn this popular feed into a risk source for human health. Allergic reactions in hypersensitive individuals or appearance of drug-resistant microorganisms could be the consequences [1]. As was mentioned in **Chapter 1**, EU legislation has established MRLs for veterinary medicinal substances in food products derived from animals and intended for human consumption such as milk [2]. According to this regulation, pharmacologically active compounds have been grouped in two categories depending if the presence of their residues food products is allowed (these substances have an assigned MRL) or is banned. Consequently, residues of pharmaceuticals such as chloramphenicol, nitrofurans or 5-NDZ antibiotics are not admitted in food products with animal origin. EURLs for veterinary residues have established 'action limits' for the validation of analytical methods in relation to the control of unauthorized analytes in different matrixes. In relation to 5-NDZs, a recommended concentration of 3 μg/L has been set, and therefore, it involves that the proposed methods for 5-NDZ determination should reach decision limits lower than this value [3]. Although this recommendation has only been established for MNZ, RNZ, DMZ and their metabolites determination, it is usually applied to the analysis of other 5-NDZ residues.

Several analytical methods have been proposed for monitoring the presence of these compounds in a quite wide variety of matrices such as egg [4,5], poultry meat [6,7], swine tissue [8], honey [9] or fish tissue [10]. Moreover, few studies have been focused on the development of analytical methods for 5-NDZ determination in milk samples, and LC-MS has been mainly used for that purpose [11,12]. Additionally, the use of an optical biosensor has also been proposed as screening method [13], and in this Thesis a MEKC-UV method has been evaluated for the analysis of 5-NDZ residues in this type of matrix (see **Chapter 4**). In spite of the advantages offered by LC-MS, mainly the unequivocal identification of the target compounds, other methods are required for those cases in which MS is not available. In this sense, LC-UV methods suppose a cheaper and an easier option in terms of instrumentation costs and technical knowledge, respectively.

On the other hand, the development of methods involving lower solvent consumption and shorter analysis time is the trend in food analysis in the last years [14,15]. For this reason, the use of miniaturized techniques such as CLC or nano-LC, and UHPLC has exponentially grown in the last decade. However, UHPLC coupled with alternative detectors to MS has been scarcely evaluated for residue analyses [16]. For example, it has been proposed the analysis of quinolone residues in fish and milk samples by UHPLC-FL [17,18] and the determination of the coccidiostat toltrazuril and its metabolites in chicken and porcine tissues by UHPLC-UV [19]. However, according to the reviewed literature, UHPLC-UV has never been used for 5-NDZ determination in food matrices.

The use of UV detection presents a high drawback due to its lack of sensitivity and selectivity. Additionally, milk is a complex matrix that presents several inconveniences because some analytes can bind easily to proteins. Furthermore, milk contains significant amounts of divalent and trivalent cations that can form complexes with these compounds, increasing their retention in the matrix [20]. With the aim of accomplishing the extraction of the target compounds and removing analytical interferences from such complex matrix, some sample treatments have been proposed. SPE [7,21,22], including MIPSE mode [23,24], and LLE are the common choices for that purpose [25].

In LLE, water-immiscible organic solutions are relatively poor for the extraction of polar compounds such as 5-NDZs, due to their low dielectric constants. Although the use of more polar organic solvents such as EtOH, MeOH, acetone or MeCN, can provide the extraction of the mentioned compounds, they are water-miscible and, as a consequence, they cannot be adopted for conventional LLE methods. However, it has been demonstrated that the addition of salts can reduce their water-miscibility, leading to phase separation. Therefore, in presence of a salt agent, the polar analytes which are present in the aqueous sample phase can selectively move into the polar organic phase. This technique is called SALLE [26].

In this chapter, a new UHPLC-UV method is introduced for the rapid determination of eight 5-NDZs in milk samples. Moreover, a SALLE procedure has been optimized considering the type and volume of the extraction solvent as well as the type and amount

of the salt agent. Besides, other parameters as vortex agitation or centrifugation timing were evaluated. This optimization was carried out by using the experimental design methodology.

12.2. Materials and methods

12.2.1. Materials and reagents

All reagents used through this work were analytical reagent grade and solvents were HPLC grade, unless otherwise specified. Ammonium hydroxide solution (30%, v/v), $CaCl_2$, NaCl, $MgSO_4$ and Na_2SO_4 were acquired from Panreac-Química (Madrid, Spain). MeOH, MeCN, acetone, 2-propanol, $(NH_4)_2SO_4$ and ethyl acetate were purchased from VWR International (West Chester, PA, USA) while acetic acid and 2-butanone were supplied by Sigma-Aldrich (St. Louis, MO, USA). Formic acid (98–100%, v/v) and KCl were acquired from Merck (Darmstadt, Germany). Ultrapure water (Milli-Q plus system, Millipore, Bedford, MA, USA) was used throughout the work. Ammonium acetate and ammonium formate solutions were prepared from acetic acid and formic acid, respectively, adjusting the pH with 5 M ammonium hydroxide solution.

Analytical standards of DMZ, RNZ, ORZ, MNZ, TNZ and CRZ were supplied by Sigma-Aldrich (St. Louis, MO, USA) while IPZ, SCZ and TRZ were purchased from Witega (Berlin, Germany). Stock standard solutions were obtained by dissolving the appropriate amount of each 5-NDZ drug in MeCN, reaching a final concentration of 1 mg/mL. Stock standard solutions were kept in the freezer at -20°C avoiding their exposure to light. Intermediate standard solution (100 μg/mL of each 5-NDZ compound) was obtained by mixing the appropriate amount of each stock standard solution and its subsequent dilution with MeCN. It was stored in dark at 4°C and it was equilibrated to room temperature before its use. Working standard solutions were prepared by dilution of the intermediate standard solution with water or mobile phase (up to the experiment) to the desired 5-NDZ concentration.

Clearinert™ 13 mm syringe filters (0.22 μm pore size) were supplied by Bonna-Agela Technologies (Wilmington, DE, USA).

12.2.2. Instrumentation

Chromatographic separations were carried out on a UHPLC from Jasco that consisted of two extreme pressure pumps (X-LC™ 3185PU), a degasser module with four channels (X-LC™ 3080DG), a mixing unit (X-LC™ 3180MX), a column oven (X-LC™ 3067CO), an auto-sampler (X-LC™ 3059AS) equipped with an exchangeable loop (5 μL and 20 μL) and a UV detector (Model X-LC™ 3070). LC-Net II/ADC was used as hardware interface between the UHPLC system and the computer. A C18 Zorbax Eclipse Plus RRHD (50 × 2.1 mm, 1.8 μm) column from Agilent Technologies (Waldbronn, Germany) was used as chromatographic column.

A Universal 320R centrifuge (HettichZentrifugen, Tuttlingen, Germany), a nitrogen dryer EVA-EC System (VLM GmbH, Bielefeld, Germany) and a vortex-2 Genie (Scientific Industries, Bohemia, NY, USA) were used through the sample preparation procedure. Solution pH was adjusted with a pH meter (Crison model pH 2000, Barcelona, Spain) with a resolution of ±0.01 pH unit.

12.2.3. Sample treatment procedure

Whole pasteurized cow's milk and semi-skimmed goat's milk were acquired in a local supermarket (Granada, Spain) while ewe's raw milk was gently supplied by a local farm. Aliquots of 4 mL were made from each sample and they were fortified at the desired 5-NDZ concentration. 5-NDZ extraction was accomplished by the developed SALLE procedure. First, the sample disposed in a 15-mL conical tube was centrifuged for 10 min at 7500 rpm in order to remove the majority of milk fat. After centrifugation, a portion of the milk fat was observed at the top of the solution and it was discarded. Liquid phase was transferred to a new 15-mL centrifuge tube and 10 mL of ethyl acetate were added on it. Sample was agitated by vortex for a few seconds and centrifuged for 5 min at 7500 rpm, and as a consequence, protein precipitation occurred. In the same centrifugation tube and without removing precipitated proteins, 1.0 g of Na_2SO_4 was added to the sample. Subsequently, sample was once again centrifuged for 10 min at 7500 rpm and 6.3 mL were collected from the upper organic layer. Sample was dried under a nitrogen current at 40°C

and afterwards it was re-dissolved in 200 μL of mobile phase (6:94 (v/v) MeCN/water containing 0.1% (v/v) of formic acid) using vortex agitation for 2 min. Finally, sample was filtered through a syringe filter to a vial and was further submitted to the UHPLC-UV analysis.

12.2.4. Chromatographic conditions

UHPLC separations were performed in a C18 Zorbax Eclipse Plus RRHD (50 × 2.1 mm, 1.8 μm) column, using a mobile phase consisted of 0.1% (v/v) formic acid aqueous solution (eluent A) and MeCN containing 0.1% (v/v) of formic acid (eluent B) at a flow rate of 0.45 mL/min.

Gradient program was established as follows: 0 min, 6% (v/v) of B; 2 min, 6% (v/v) of B; 5 min, 15% (v/v) of B; 7 min, 25% (v/v) of B; 8 min, 95% (v/v) of B. At 8.5 min, mobile phase composition was back to initial conditions in 1.25 min. In order to guarantee column equilibration, initial conditions were maintained for 1.75 min. Column temperature was set to 40°C and 20 /L (full loop injection mode) was selected as injection volume. 5-NDZ separation was monitored at 320 nm.

12.3. Results and discussion

12.3.1. Optimization of chromatographic conditions

5-NDZ separation was evaluated in a C18 Zorbax Eclipse Plus RRHD (50 × 2.1 mm, 1.8 μm) column because C18 stationary phase columns have been mainly employed for this purpose [27,28]. Initially, mobile phase consisted of 0.025% (v/v) formic acid aqueous solution (eluent A) and pure MeCN (eluent B) and was supplied at 0.3 mL/min. Separation was carried out under gradient conditions and the column was thermostatized at 40°C. Gradient program was established as follows: mobile phase composition was kept at 5% (v/v) of eluent B from 0.0 to 2.0 min, increasing MeCN content to 30% (v/v) at 4.0 min, and reaching a maximum of 95% (v/v) at 5.0 min. Maximum MeCN concentration (95%, v/v) was kept for 0.5 min and initial conditions were re-established by a 2.5 min linear gradient. In order to reach a proper column equilibration, initial conditions were

maintained for 2 min. An injection volume of 5 μL (full loop) was considered and water was initially selected as injection solvent. Standard solutions (2.0 μg/mL of each 5-NDZ compound) were analyzed under these conditions and analytical signal was monitored at 320 nm. As can be seen in **Figure 12.1**, analytes numbered as peaks 8 and 9 co-eluted while peak resolution lower than 1.5 was obtained for peaks 2 and 3 and peaks 5 and 6 under these initial conditions.

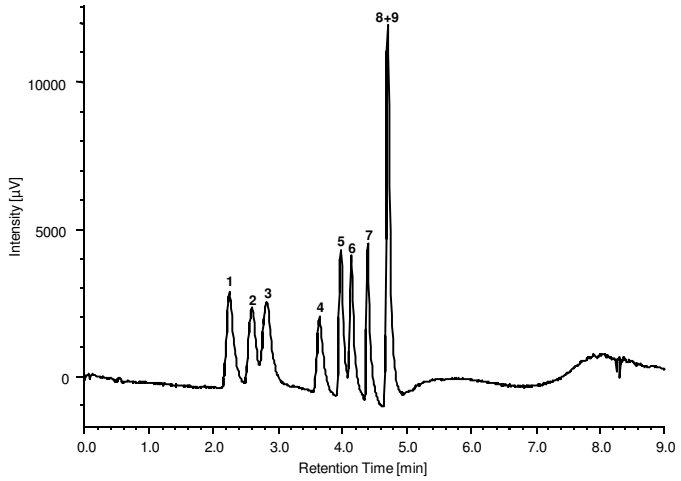

Figure 12.1. **Chromatogram obtained from the analysis of a standard solution (2.0 μg/mL of each 5-NDZ) using a C18 Zorbax Eclipse Plus RRHD (50 × 2.1 mm, 1.8 μm) column. Separation was performed at 40°C and mobile phase consisted of 0.025% (v/v) formic acid aqueous solution (eluent A) and pure MeCN (eluent B) and was supplied at flow rate of 0.3 mL/min. Gradient program was established as follows: 0 min, 5% (v/v) of B; 2 min, 5% (v/v) of B; 4 min, 70% (v/v) of B; 5 min, 95% (v/v) of B; 5.5 min, 95% (v/v) of B; 7 min, 5% (v/v) of B; and 9 min, 5% (v/v) of B. Injection volume was set to 5 μL. Analytical signal was monitored at 320 nm. Peaks are numbered by elution order.**

In order to improve 5-NDZ separation, parameters such as mobile phase composition, mobile phase flow rate, separation temperature and gradient program were evaluated in detail. Gradient program was also modified during the optimization of other separation parameters with the aim of achieving a better selection of optimum values.

First, different solutions such as 0.1% (v/v) formic acid solution (pH 2.5), 0.1% (v/v) acetic acid solution (pH 3.1), 0.1% (v/v) ammonium formate solution (pH 4.5), 0.1% (v/v) ammonium acetate solution (pH 5.5) and deionized water, were tested as eluent A. Better separation in terms of peak resolution was achieved when solvents with lower pH value were employed as eluent A, so formic acid solution was selected. Afterwards, formic acid

concentration was assessed from 0.025 to 0.5% (v/v). **Figure 12.2** shows that more baseline resolved peaks were obtained when 0.1% (v/v) formic acid solution was used as eluent A.

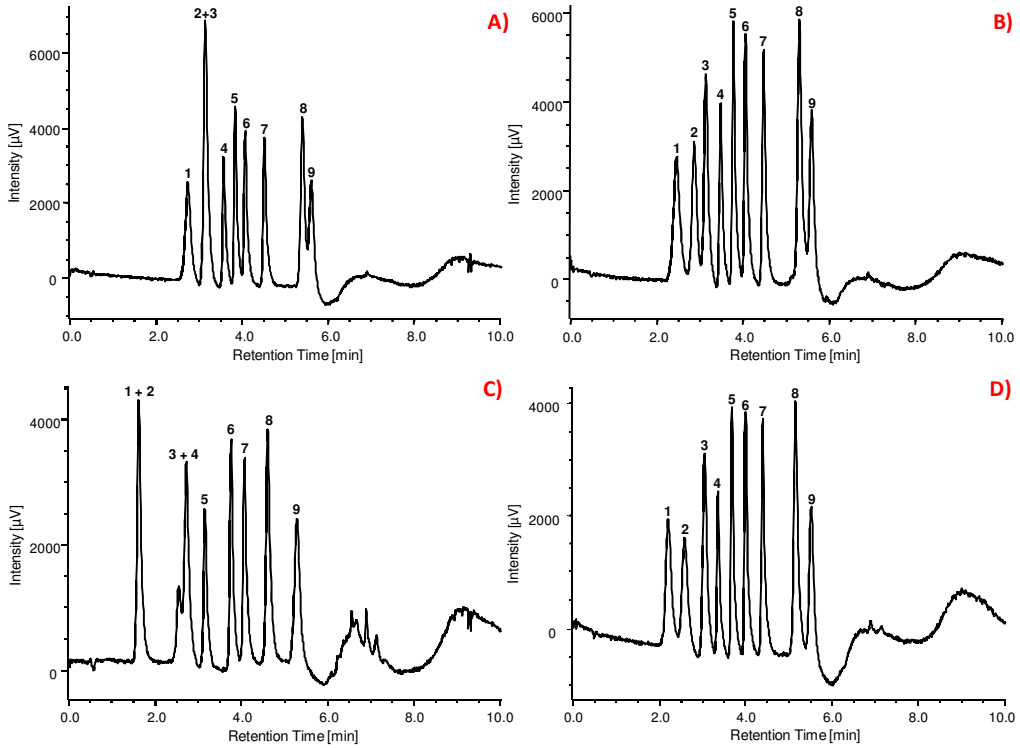

Figure 12.2. Evaluation of formic acid solution (eluent A) concentration: A) 0.05% (v/v), B) 0.1% (v/v) and C) 0.5% (v/v), using pure MeCN as eluent B; and D) evaluation of the use of MeCN containing 0.1% (v/v) of formic acid as eluent B, using 0.1% (v/v) formic acid aqueous solution as eluent A. Separations were performed in a C18 Zorbax Eclipse Plus RRHD (50 × 2.1 mm, 1.8 µm) column under a separation temperature of 40°C. Mobile phase flow rate: 0.3 mL/min. Gradient program was established as follows: 0 min, 3% (v/v) of B; 1.5 min, 3% (v/v) of B; 3 min, 25% (v/v) of B; 4.5 min, 25% (v/v) of B; 6 min, 95% (v/v) of B; 6.5 min, 95% (v/v) of B; 8 min, 3% (v/v) of B; and 10 min, 3% (v/v) of B. Injection volume was set to 5 µL and standard solutions (2 µg/mL of each compound) were analyzed. Analytical signals were monitored at 320 nm. Peaks are numbered by elution order.

The use of MeOH as eluent B instead of MeCN was also considered. Furthermore, a mixture 50:50 (v/v) MeCN/MeOH was also assayed as eluent B. However no improvement in terms of peak resolution was achieved and even co-eluted peaks were observed under both conditions. Therefore MeCN was selected as mobile phase organic solvent. Moreover, the addition of 0.1% (v/v) formic acid to the eluent B was also

considered. As a consequence, peak resolution between peaks 2 and 3 and peaks 8 and 9 was slightly improved (**Figure 12.2,B** and **Figure 12.2,D**).

On the other hand, mobile phase flow rate was ranged from 0.2 to 0.5 mL/min. A flow rate of 0.35 mL/min was established as optimum because higher flow rates involved a decrease on peak resolution between peaks 1 and 2, while co-eluting peaks were observed when lower flow rates were evaluated. Furthermore, column temperature was studied from 30 to 50°C. Although a shorter analysis time could be expected when a higher temperature is considered because mobile phase viscosity decreases, similar separation time (≈ 6 min) was observed for the assessed temperature range. Finally, 45°C was selected as optimum because all peaks were baseline resolved under this condition.

Considering the optimized separation parameters, mobile phase composition through the separation was varied according to the following gradient program. Initially eluent B was set to 3% (v/v) and this condition was maintain for 2 min. Content of B was linearly increased to 20% (v/v) in 1.5 min, then to 25% (v/v) in 1.5 min and finally mobile phase composition reached a 95% (v/v) of B at 7 min. At the time of 7.5 min gradient elution was changed for reaching initial conditions at 8.75 min and they were stated for a period of 1.75 min with the aim of guarantying column equilibration and obtaining a stable and reproducible separation.

Injection conditions

In addition to separation optimization, injection solvent was also investigated. It was observed that the use of mobile phase (3:97 (v/v) MeCN/water containing 0.1% (v/v) of formic acid) gave the same results, in terms of peak efficiency, peak resolution and retention times, as those obtained using water as injection solvent. As a consequence, mobile phase (3:97 (v/v) MeCN/water containing 0.1% (v/v) of formic acid) was selected as injection solvent, because it is highly recommended to inject the sample in a solution of the same characteristics that the initial composition of the mobile phase. Moreover, the addition of a 3% (v/v) of MeCN to the samples encourages its reconstitution after a sample treatment.

Finally, the initial injection loop (5 μL) was replaced by an injection loop of 20 μL in order to increase sample injection volume, enhancing method sensitivity. Signals, in terms of peak height, were enhanced for MNZ (2.6-fold) and RNZ (3.4-fold). Both signals were the lowest signals when a standard solution containing 2 μg/mL of each 5-NDZ was injected using the loop of 5 μL and analyzed following the optimized conditions (**Figure 12.3,I**). **Figure 12.3,II** shows that peak resolution is lost between peaks 1 and 2 and 2 and 3, because peak efficiency loss occurred when 20 μL of sample were injected instead of 5 μL.

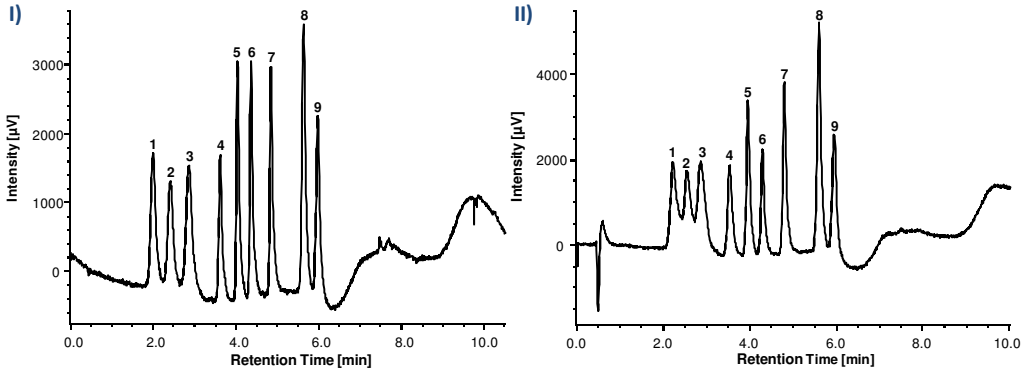

Figure 12.3. **Chromatograms resulted from the analysis of I) 5-NDZ standard solution (2 μg/mL) considering an injection volume of 5 μL and II) 5-NDZ standard solution (0.5 μg/mL) considering an injection volume of 20 μL. Separations were performed in a C18 Zorbax Eclipse Plus RRHD (50 × 2.1 mm, 1.8 μm) column under a separation temperature of 40°C. Mobile phase consisted of 0.1% (v/v) formic acid solution as eluent A and MeCN containing 0.1% (v/v) of formic acid as eluent B and was supplied at a flow rate of 0.35 mL/min. Gradient program was established as follows: 0 min, 3% (v/v) of B; 2 min, 3% (v/v) of B; 3.5 min, 20% (v/v) of B; 5 min, 25% (v/v) of B; 7 min, 95% (v/v) of B; 7.5 min, 95% (v/v) of B; 8.75 min, 3% (v/v) of B; and 10.5 min, 3% (v/v) of B. Samples were injected in 3:97 (v/v) MeCN/water containing 0.1% (v/v) of formic acid. Analytical signals were monitored at 320 nm. Peaks: 1, MNZ; 2, DMZ; 3, RNZ; 4, TRZ; 5, SCZ; 6, TNZ; 7, ORZ; 8, IPZ; 9, CRZ.**

Finally, the loop of 20 μL was selected in order to improve sensitivity. As a consequence, DMZ (peak 2) was excluded from the present study in order to achieve a baseline separation among the considered analytes. Prior to this decision, gradient program modifications were assessed for improving peak resolution among MNZ, DMZ and RNZ, but satisfactory results were not achieved.

12.3.2. Sample treatment optimization

The applicability of the proposed UHPLC-UV method was evaluated for the analysis of 5-NDZ residues in milk samples. SALLE was chosen as sample treatment for 5-NDZ extraction from milk samples because it is a cheap and easy process and, moreover, low solvent volumes are required, accomplishing with the aims of Green Chemistry. And above all it is suitable for being implemented in routine laboratories.

Initially, 4 mL of fortified milk were centrifuged for 10 min at 7500 rpm. The liquid phase was transferred to a 15-mL conical tube, discarding upper fat layer. Afterwards, 8 mL of organic solvent (MeCN) were added to the sample and it was agitated by vortex for a few seconds and centrifuged for 5 min at 7500 rpm. After centrifugation, a suspension of proteins was observed in the sample. Then, 1.6 g of the salt (NaCl) were added to the sample and it was agitated by vortex for 2 min and centrifuged for 10 min at 7500 rpm. Finally, 3 mL of the organic supernatant were collected and dried under nitrogen at 40°C. Sample was reconstituted in 200 μL of mobile phase (3:97 (v/v) MeCN/water containing 0.1% (v/v) of formic acid) and submitted to UHPLC-UV analysis. From the mentioned procedure, extraction solvent and salt nature, solvent volume, amount of salt (%, w/v) added to the sample and vortex agitation and centrifugation timing were studied through sample treatment optimization.

Extraction solvent and salt nature

Six different organic solvents were assessed as extraction solvents, namely, MeCN, MeOH, acetone, 2-propanol, butanone and ethyl acetate. MeOH and butanone were discarded because separation between sample aqueous phase and solvent organic phase was not observed. On the other hand, 3 mL from the organic phase were collected when MeCN, 2-propanol and acetone were employed as extraction solvents, but a lower solvent volume was able to be collected when ethyl acetate was used. Therefore, lower off-line preconcentration factor was achieved in this last case. However, ethyl acetate was finally selected as extraction solvent because no matrix compounds were co-eluting with 5-NDZ peaks. **Figure 12.4** shows that an interfering peak was co-eluted with ORZ when MeCN

was employed as extraction solvent. The same interfering compound was also observed when sample extraction was performed using 2-propanol and acetone as extractant agent. Regarding extraction recoveries, similar recoveries were obtained for each compound independently of the used extraction solvent. In all cases recoveries higher than 60% were achieved.

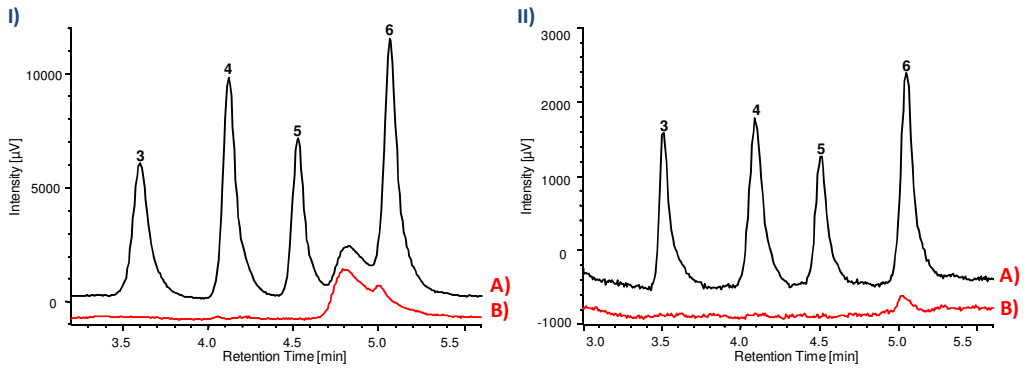

Figure 12.4. Extraction solvent evaluation. I) Chromatogram sections obtained from the application of the SALLE procedure to milk samples using MeCN as extraction solvent: A) fortified sample (0.5 µg/mL of each 5-NDZ) and B) blank sample. II) Chromatogram sections obtained from the application of the SALLE procedure to milk samples using ethyl acetate as extraction solvent: A) fortified sample (0.5 µg/mL of each 5-NDZ) and B) blank sample. Separations were performed in a C18 Zorbax Eclipse Plus RRHD (50 × 2.1 mm, 1.8 µm) column under a separation temperature of 40°C. Mobile phase consisted of 0.1% (v/v) formic acid solution as eluent A and MeCN containing 0.1% (v/v) of formic acid as eluent B and was supplied at a flow rate of 0.35 mL/min. Gradient program was established as follows: 0 min, 3% (v/v) of B; 2 min, 3% (v/v) of B; 3.5 min, 20% (v/v) of B; 5 min, 25% (v/v) of B; 7 min, 95% (v/v) of B; 7.5 min, 95% (v/v) of B; 8.75 min, 3% (v/v) of B; and 10.5 min, 3% (v/v) of B. Samples were injected in 3:97 (v/v) MeCN/water containing 0.1% (v/v) of formic acid. Analytical signals were monitored at 320 nm. Peaks: 3, TRZ; 4, SCZ; 5, TNZ; 6, ORZ.

On the other hand, three different chloride salts ($CaCl_2$, KCl, NaCl) and three different sulfate salts ($MgSO_4$, $(NH_4)_2SO_4$, Na_2SO_4) were evaluated as salting-out agents. The obtained recoveries for each 5-NDZ according to the employed salt agents are shown in **Figure 12.5**.

In general, satisfactory recoveries were achieved, being higher than 65% in all cases. Moreover, similar recoveries were obtained for each compound independently of the employed salt. **Figure 12.5** shows that a higher salting-out effect can be attributed to sulfate salts because slightly higher recoveries were observed for some analytes such as SCZ, ORZ and IPZ when these salts were used, so Na_2SO_4 was selected.

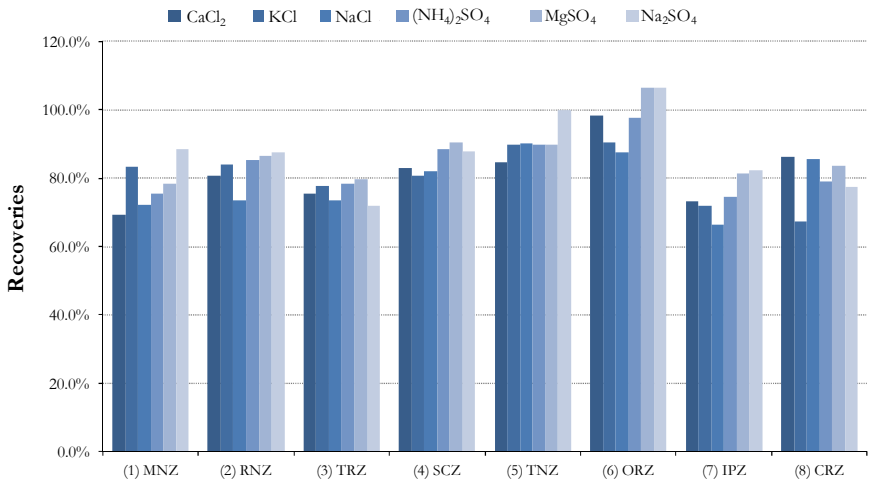

Figure 12.5. Salting-out agent evaluation for 5-NDZ extraction from milk samples by a SALLE procedure.

Experimental designs for the extraction procedure optimization

Taking into account the previously selected extraction solvent and salting-out agent, ethyl acetate and $MgSO_4$, respectively, and in order to detect the significant factors related to 5-NDZ extraction from milk samples in a SALLE procedure, a half fraction screening experimental design 2^{5-1} in one block (18 runs) was performed. The evaluated variables are indicated on **Table 12.1** as well as the studied range for each parameter.

Table 12.1. Variables evaluated in the proposed screening experimental design.

Variable	Studied range
A: centrifugation timing after the addition of the extraction solvent to the sample	0 – 5 min
B: extraction solvent volume	6 – 9 mL
C: vortex agitation timing after the addition of the salt agent to the mixture constituted by the sample and the organic solvent	0 – 2 min
D: timing of the centrifugation process performed after vortex agitation (variable C)	2 – 10 min
E: amount of salting-out agent (%, w/v)	0.5 – 1.5 g

From each run, the resulting organic phase from the SALLE procedure was collected. The selected response was the sum of peak heights because peak height defines method sensitivity. From the screening design, a Pareto chart was obtained (**Figure 12.6,I**), in which neither the timing of the first considered centrifugation step nor the amount of salt agent (%, w/v) were found significant in the studied range. For further experiments, samples were centrifuged for 5 min at 7500 rpm after the addition of the extraction solvent to them (variable A) and 1.0 g of Na_2SO_4 was finally considered as salt agent (variable E). Moreover, other conclusions resulted from the Pareto Chart should be mentioned. As was expected, extraction solvent volume (variable B) showed the highest influence on the analytical response. This influence was positive, meaning that higher sensitivity was achieved when higher volume was used, and as a consequence, higher amount of organic phase was collected after performing SALLE procedure. On the other hand, vortex agitation timing (variable C) showed a surprisingly negative effect on the analytical response while the second considered centrifugation step timing (variable D) had a positive influence on the analytical response.

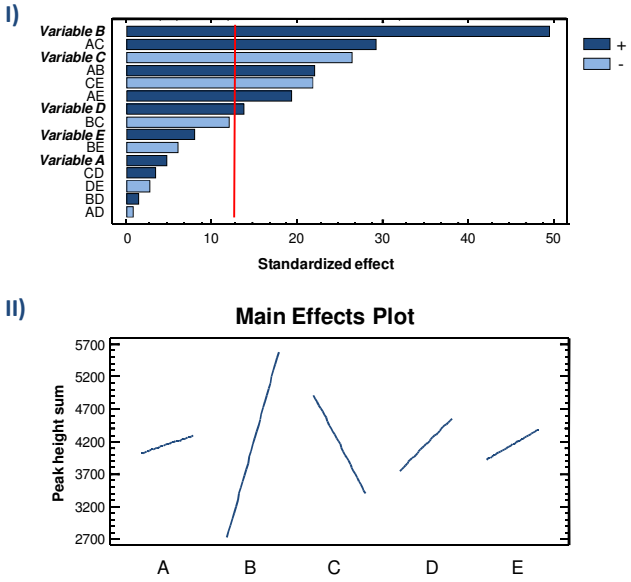

Figure 12.6. I) Pareto chart showing the effects on the peak height sum from the screening design in the study of important variables for SALLE procedure. (+) Positive effects on the response; (-) negative effects on the response. Red line shows the limit of decision to consider the significance of the factors (based on the standardized effect = estimated effect/standard error, p-value = 0.05 at 95% of confidence level). II) Main effects plot for all studied variables which are defined on **Table 12.1**.

In order to optimize the significant factors (variables B, C and D) and evaluate the interactions among them, a Doehlert experimental design [29] for three factors and two central points (14 runs) was proposed (**Table 12.2**).

Table 12.2. Doehlert experimental design matrix for the optimization of the significant variables on the SALLE procedure.

Experiment	Variable B (mL)	Variable C (min)	Variable D (min)
1	8	2	6
2	8	4	6
3	9.7	3	6
4	8.6	3	9.3
5	8	0	6
6	6.3	1	6
7	7.4	1	2.7
8	6.3	3	6
9	7.4	3	2.7
10	9.7	1	6
11	9.2	2	2.7
12	8.6	1	9.3
13	6.8	2	9.3
14	8	2	6

In this case, centrifugation step timing (D) was evaluated at three levels (between 2.0 and 10.0 min), vortex agitation timing (C) was investigated at five levels (between 0 to 4 min) and extraction solvent volume (B) was studied at seven levels (between 6 and 10 mL). The selected analytical response was the same as that employed in the previous screening design. The obtained lack of fit p-value for the proposed model was 0.06, which indicated that the model was adequate to describe the observed data at the 95.0% confidence level. Moreover, the determination coefficient (R^2) was 89.2%, indicating that the predicted model fitted well the experimental data. The response surfaces corresponding to the significant interactions are shown in **Figure 12.7**, resulting in an optimum value of 10 mL for extraction solvent volume. According to the screening design, the highest assayed extraction solvent volume maximized the analytical response as was expected. Under this condition, 6.3 mL of the upper organic phase were able to be collected after separation

between aqueous and organic phase occurred. On the other hand, from **Figure 12.7,I**, it can be concluded that vortex agitation and centrifugation steps carried out after salting-out agent addition to the sample presented opposite effects. Analytical response was maximized when low centrifugation timing and high vortex agitation timing were established, and vice versa. Neither of those two conditions led to achieve a clear maximum, so a decision had to be taken. Finally, vortex agitation step was discarded, and therefore, samples were only centrifuged for 10 min at 7500 rpm after salting-out agent addition.

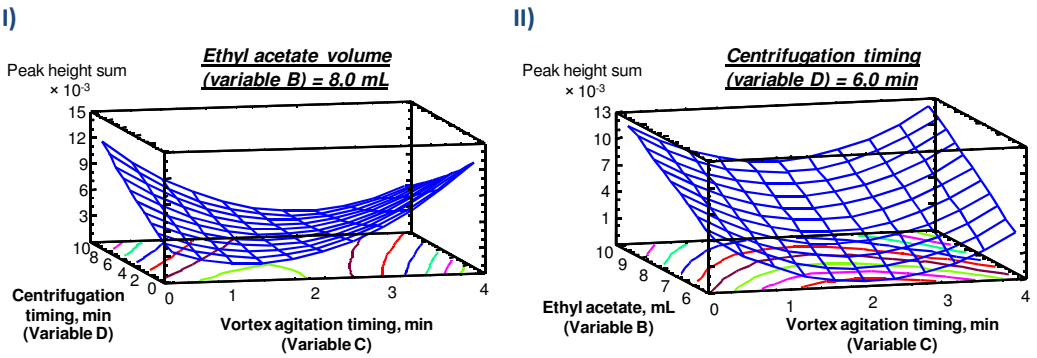

Figure 12.7. Estimated response surface for I) the interaction between variables C and D and II) the interaction between variables B and C.

Figure 12.8 shows a scheme of the optimized SALLE procedure.

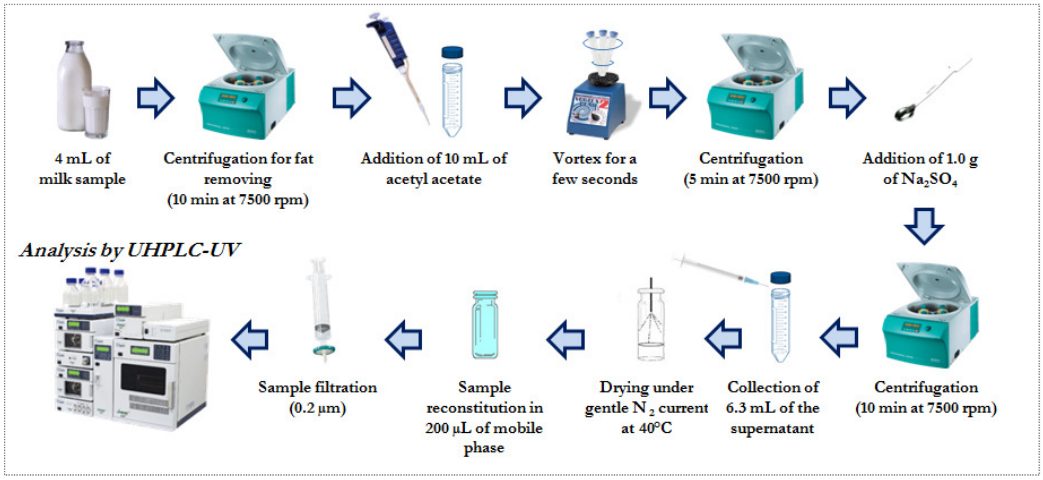

Figure 12.8. SALLE-UHPLC-UV procedure for 5-NDZ determination in milk samples.

Finally, two milk samples, a blank and a fortified sample at 25 μg/L of each 5-NDZ compound, were treated following the proposed SALLE procedure and analyzed by the optimized UHPLC-UV method. The chromatograms obtained for both samples are shown in **Figure 12.9**.

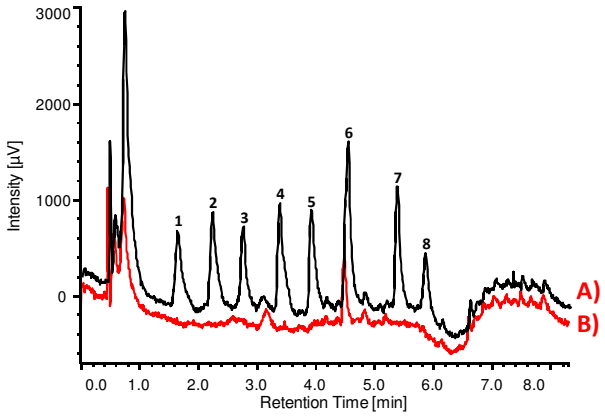

Figure 12.9. **Chromatograms obtained from the application of the proposed SALLE-UHPLC-UV procedure to A) a milk sample fortified at 25 μg/L of each 5-NDZ and B) a blank of milk sample. Analyses were performed in a C18 Zorbax Eclipse Plus RRHD (50 × 2.1 mm, 1.8 μm) column under a temperature of 40°C. Mobile phase consisted of 0.1% (v/v) formic acid as eluent A and MeCN containing 0.1% (v/v) of formic acid as eluent B and was supplied at a flow rate of 0.35 mL/min. Gradient program was established as follows: 0 min, 3% (v/v) of B; 2 min, 3% (v/v) of B; 3.5 min, 20% (v/v) of B; 5 min, 25% (v/v) of B; 7 min, 95% (v/v) of B; 7.5 min, 95% (v/v) of B; 8.75 min, 3% (v/v) of B; and 10.5 min, 3% (v/v) of B. Samples were injected in 3:97 (v/v) MeCN/water containing 0.1% (v/v) of formic acid. Analytical signals were monitored at 320 nm. Peaks: 1, MNZ; 2, RNZ; 3, TRZ; 4, SCZ; 5, TNZ; 6, ORZ; 7, IPZ; 8, CRZ.**

Gradient program reevaluation

As can be seen in **Figure 12.9,B**, the proposed sample treatment was very effective even if any clean-up step was applied, but the presence of a co-eluting peak with ORZ was still observed. In order to clarify if this interfering peak was exclusive from the evaluated milk sample or it was an inherent compound of any milk sample possessing the same retention time as ORZ, other milk samples from different product batches were investigated. Performed analyses exposed the common presence of matrix compounds co-eluting with ORZ, showing even higher intensity than the interfering peak intensity observed in **Figure 12.9**.

Because ORZ, and as a consequence, its interfering peak were well retained in the employed LC column (stationary phase consisted of C18 silica-based particles) under the considered chromatographic conditions, their separation was investigated by means of gradient program modifications. This strategy could result less effective in the case of more polar compounds which are less retained by reverse phase LC columns. When gradient program modifications were carried out, co-elution among 5-NDZ peaks and matrix compounds were avoided. Once new gradient program was established (**Table 12.3**), mobile phase flow rate was reevaluated with the aim of decreasing analysis time. Thus, the flow rate was increased from previously established 0.35 to 0.45 mL/min. As can be seen in **Figure 12.10**, separation time was decreased in 0.5 min after flow rate was increased in 0.1 mL/min.

Table 12.3. **Comparison between gradient program used during sample treatment optimization and the gradient program established for the separation of 5-NDZ peaks and matrix interfering peaks.**

Previous gradient program		New gradient program	
Time (min)	***% (v/v) of B***	***Time (min)***	***% (v/v) of B***
0.00	3	0.00	6
2.00	3	2.00	6
3.50	20	5.00	15
5.00	25	7.00	25
7.00	95	8.00	95
7.50	95	8.50	95
8.75	3	9.75	6
10.50	3	11.50	6

In summary, milk samples treated by the proposed SALLE procedure (**Figure 12.8**) were reconstituted in 6:94 (v/v) MeCN/water containing 0.1% (v/v) of formic acid and analyzed by UHPLC-UV. Separation using a gradient program (see 'New gradient program' from **Table 12.3**) was carried out in a C18 Zorbax Eclipse Plus RRHD (50 × 2.1 mm, 1.8 μm) column at 40°C under a mobile phase flow rate of 0.45 mL/min. Mobile phase consisted of 0.1% (v/v) formic acid aqueous solution as eluent A and MeCN containing 0.1% (v/v) of formic acid as eluent B. Analytical signals were monitored at

320 nm. **Figure 12.10,II** shows chromatograms of a spiked pasteurized cow milk sample (25 μg/L) and a blank sample, treated and analyzed according to the mentioned method.

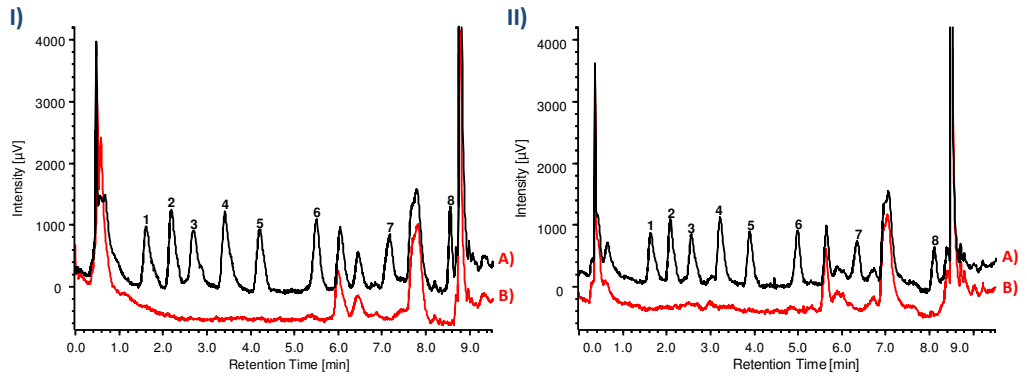

Figure 12.10. Chromatograms obtained from the analysis of milk samples by SALLE-UHPLC-UV. I) Mobile phase flow rate was established at 0.35 mL/min (A, fortified milk sample at 25 μg/L of each 5-NDZ and B, blank sample). II) Mobile phase flow rate was established at 0.45 mL/min (A, fortified milk sample at 25 μg/L of each 5-NDZ and B, blank sample). Mobile phase consisted of 0.1% (v/v) formic acid aqueous solution as eluent A and MeCN containing 0.1% (v/v) of formic acid as eluent B. Gradient program was established as is indicated on **Table 12.3** ('new gradient program'). Analytical signals were monitored at 320 nm. Peaks: 1, MNZ; 2, RNZ; 3, TRZ; 4, SCZ; 5, TNZ; 6, ORZ; 7, IPZ; 8, CRZ.

12.3.3. Method characterization

In order to test the usefulness and potential of the proposed method, milk samples were treated by the optimized SALLE procedure (see **Section 12.2.3**) and subsequently analyzed by UHPLC-UV. The optimized method was evaluated in terms of linearity, LODs, LOQs, trueness, and peak height repeatability and intermediate precision.

Calibration curves and performance characteristics

Matrix-matched calibration curves were established in milk samples fortified at the following concentration levels: 10, 20, 50, 100 and 200 μg/L for each studied 5-NDZs. Two spiked milk samples per level were treated following the SALLE procedure. Afterwards, each sample was analyzed in duplicate according to the proposed UHPLC-UV method. A blank milk sample was analyzed as described before and no matrix interferences were found at any analyte retention time. Peak height of each compound was considered as function of analyte concentration on the sample. LODs and LOQs of the method were

calculated as the minimum analyte concentration yielding a S/N equal to three and ten, respectively (**Table 12.4**).

Table 12.4. **Matrix-matched calibration curves and statistical and performance characteristics of the proposed SALLE-UHPLC-UV method.**

Analyte	Linear range (µg/L)	R^2	Linear regression equation (y=m·x+a)				LOD (µg/L)	LOQ (µg/L)
			Slope	*SD for the slope*	*Intercept*	*SD for the intercept*	*3×S/N*	*10×S/N*
MNZ	9.2 – 200	0.9999	33.6	0.2	-61.2	23.8	2.8	9.2
RNZ	7.3 – 200	0.9956	46.6	1.8	-282.9	184.4	2.2	7.3
TRZ	6.6 – 200	0.9994	44.6	0.6	-85.7	66.1	2.0	6.6
SCZ	11.6 – 200	0.9949	26.6	1.1	51.9	113.8	3.5	11.6
TNZ	9.4 – 200	0.9916	25.4	1.4	158.4	139.1	2.8	9.4
ORZ	8.2 – 200	0.9998	35.7	0.3	-34.8	30.2	2.5	8.2
IPZ	11.1 – 200	0.9978	30.3	0.8	-159.0	84.9	3.3	11.1
CRZ	13.4 – 200	0.9973	24.5	0.7	-124.2	75.4	4.0	13.4

Table 12.4 shows that LODs ranging between 2.0 and 4.0 µg/L were achieved for all 5-NDZ compounds. As a consequence, the proposed SALLE-UHPLC-UV method is suitable for its implementation in routine analysis laboratories that are focused on the determination of 5-NDZ residues in food matrices such as milk.

Precision assays

Precision studies were carried out in order to evaluate the repeatability (intra-day precision) and intermediate precision (inter-day precision) of the proposed SALLE-UHPLC-UV method. Repeatability was assayed at three concentration levels by analyzing three spiked samples per level in triplicate. Intermediate precision was assessed at three concentration levels by analyzing one spiked sample in triplicate per level and per day for three consecutive days. Results expressed as peak height RSDs (%) are shown on **Table 12.5**. In all cases RSDs were lower than 13.3% and 12.8% for repeatability and intermediate precision, respectively.

Table 12.5. Precision studies in terms of RSDs (%) for spiked pasteurized cow's milk samples.

Analyte	Repeatability (% RSD, n = 9)			Intermediate precision (% RSD, n = 9)		
	15 µg/L	*75 µg/L*	*150 µg/L*	*15 µg/L*	*75 µg/L*	*150 µg/L*
MNZ	13.3	8.9	8.8	5.3	8.4	9.1
RNZ	10.9	5.4	9.1	5.1	5.6	6.5
TRZ	8.8	8.8	12.1	11.6	7.6	9.0
SCZ	13.3	5.5	5.0	7.9	4.7	8.9
TNZ	12.0	6.0	7.6	10.0	6.7	9.1
ORZ	8.1	8.4	8.4	6.0	5.0	7.3
IPZ	10.9	8.5	10.6	11.1	10.9	5.5
CRZ	9.9	8.6	11.1	7.0	12.8	7.9

Trueness

In order to check the trueness of the proposed method, recovery experiments were performed in three kinds of milk, namely pasteurized cow's milk, skimmed goat's milk and raw ewe's milk. Three samples of each type of milk was fortified at three different concentration levels, treated following the procedure described in **Section 12.2.3**, and analyzed in duplicate by the proposed UHPLC-UV method. Moreover, a blank of each milk sample was analyzed and no matrix interferences were observed at 5-NDZ retention times (see **Figure 12.10,II** and **Figure 12.11**).

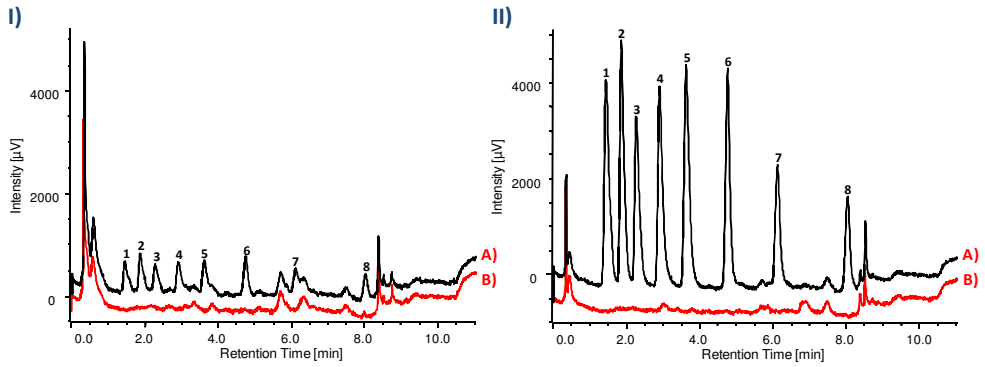

Figure 12.11. Chromatograms obtained from the analysis of I) skimmed goat's milk samples (A, sample fortified at 25 µg/L of each 5-NDZ and B, blank sample) and II) raw ewe's milk samples (A, sample fortified at 150 µg/L of each 5-NDZ and B, blank sample). Analysis conditions are indicated in **Section 12.2.4**. Analytical signals were monitored at 320 nm. Peaks: 1, MNZ; 2, RNZ; 3, TRZ; 4, SCZ; 5, TNZ; 6, ORZ; 7, IPZ; 8, CRZ.

Recoveries were estimated by the comparison of the obtained signal for each sample with the signal obtained for a blank sample spiked after the sample treatment and prior to its analysis. Recovery results obtained for each type of milk are shown in **Table 12.6**. As can be seen, recoveries higher than 62.8% were obtained for all compounds, demonstrating the convenience of using the proposed SALLE procedure as cheap and easy sample treatment for 5-NDZ extraction from different milk samples.

Table 12.6. **Recoveries (%) obtained for pasteurized cow's milk, skimmed goat's milk and raw ewe's milk samples.**

Analyte	Recoveries (%), n = 9								
	Pasteurized cow's milk			Skimmed goat's milk			Raw ewe's milk		
	15 µg/L	*75 µg/L*	*150 µg/L*	*15 µg/L*	*75 µg/L*	*150 µg/L*	*15 µg/L*	*75 µg/L*	*150 µg/L*
MNZ	85.9	76.5	106.4	75.4	85.8	99.8	81.5	76.9	107.5
RNZ	89.3	84.3	105.3	83.9	88.9	95.7	85.5	75.7	102.7
TRZ	86.2	62.8	95.8	80.5	87.4	96.6	83.7	66.4	102.4
SCZ	90.2	86.3	98.1	81.6	87.5	93.6	76.4	79.9	100.8
TNZ	94.8	89.3	99.0	90.0	90.1	93.3	82.0	86.4	102.0
ORZ	90.8	85.5	95.4	88.0	88.6	87.0	80.6	77.0	98.4
IPZ	71.2	71.3	83.2	63.4	67.7	66.0	72.9	71.8	109.2
CRZ	82.4	69.1	74.5	81.4	80.2	70.6	64.8	65.6	79.3

12.4. Conclusions

A rapid and simple UHPLC-UV method was developed for the determination of eight 5-NDZ drugs within 8 min, involving a total analysis time lower than 12 min. The optimized method was successfully applied to the analysis of 5-NDZ residues in milk samples of different origin, proposing a novel SALLE procedure as cheap, green and easy sample treatment. The variables involved in the SALLE procedure were optimized through a screening and Doehlert experimental design, achieving satisfactory analyte recoveries under the optimum obtained parameters. LODs at lower µg/L levels were reached, and therefore, the proposed SALLE-UHPLC-UV method supposes an environmentally friendly alternative to the determination of 5-NDZs in food matrices in these laboratories where analyses by LC-MS cannot be performed in the first instance.

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13

UHPLC-MS/MS method for the determination of 5-nitroimidazole residues in fish roe samples

Abstract ► In this chapter a new multiresidue method has been proposed for the determination of twelve 5-NDZs, including some of the most relevant metabolites, in fish roe samples by UHPLC-MS/MS. A SALLE procedure has been proposed for the extraction of the analytes whereas a sample treatment based on QuEChERS has also been evaluated in order to reduce the matrix effect. The variables that affected the separation such as the mobile phase composition and flow rate as well as the parameters involved in the ionization and in the MRM for MS detection were studied in detail. Finally, the separation of 5-NDZ compounds was accomplished in a C18 Zorbax Eclipse Plus (50 mm × 2.1 mm, 1.8 μm) column using a mobile phase consisted of 0.025% (v/v) formic acid aqueous solution as eluent A and pure MeOH as eluent B and supplied at 0.5 mL/min. Column temperature was maintained to 25°C during the analysis and an injection of 17.5 μL of sample in mobile phase (5:95 (v/v) MeOH/0.025% (v/v) formic acid aqueous solution was considered. The proposed method was characterized in terms of linearity ($R^2 \geq 0.9996$), extraction efficiency (≥ 71.4%), repeatability (≤ 9.8%), reproducibility (≤ 13.9%) and trueness (≥ 72.3%). CCα and CCβ values between 0.3-1.5 and 0.5-2.5 μg/kg, respectively, were obtained for all 5-NDZ compounds. The reached results accomplish with the requirements of Regulation (2002/657/EC) and EURLs.

13.1. Background

The concern that supposes the presence of 5-NDZ residues in food products as well as the current legislation that forbids them in food matrices of animal origin have been widely discussed through this Thesis, especially in **Chapter 2** in which the methods developed for the analysis of 5-NDZs were summarized. In general, LC-MS has been proposed for 5-NDZ determination [1], because it has demonstrated to be a robust technique and offers the unequivocal identification of the target compounds. Furthermore, the recent proposed methods have replaced the use of traditional HPLC for UHPLC which involves a significant decrease on analysis time and reagent consumption, and as a consequence, enhances the features of the methods [2-6]. On the other hand they have been focused on the determination of these antibiotics in several foods such as milk [2,7], honey [7,8], eggs [3,9] or poultry and swine tissues [5,10-12], but methods for 5-NDZ analysis in aquaculture products have been barely evaluated as mentioned in **Chapter 11**. According to the reviewed literature, few methods have been reported for 5-NDZ determination in fish tissue [13-15] or crustaceans such as prawn [15], but no methods have been reported for their determination in fish roe samples. However, EURLs have included these matrices, namely fish muscle, crustaceans and fish eggs as relevant matrices for monitoring 5-NDZs, so new methods are required for their determination in these products.

Regarding sample treatment, the extraction assisted by salts has recently reemerged as a simple and high efficiency sample treatment [16], which in combination with UHPLC-MS/MS constitutes the perfect tandem for multiresidue analysis. **Chapter 2** showed that this sample treatment has been widely employed for 5-NDZ extraction from various matrices [9,12,17,18], while a novel SALLE procedure was proposed in **Chapter 12** for 5-NDZ determination in milk samples prior UHPLC-UV analysis. However, this strategy presents a drawback when MS determination is carried out. Taking into account that sample clean-up is barely carried out when SALLE is performed, a high matrix effect could be observed and, as a result, analytical signal could decrease or even been inhibited. In order to solve this inconvenience, a clean-up step can be carried out after SALLE. QuEChERS methods are based on the combination of SALLE followed by d-SPE as clean-up, and they have demonstrated their suitability for multiresidue determination prior

to LC-MS analyses [19,20]. However, this type of sample treatment has been scarcely employed for 5-NDZ extraction as mentioned in **Chapter 2**.

In this chapter the determination of twelve 5-NDZ compounds, including the more relevant metabolites, was carried out by a novel UHPLC-MS/MS method. Additionally, the developed method was applied to the determination of 5-NDZ residues in hake roe samples using a SALLE procedure as sample treatment, although a QuEChERS procedure was also investigated for reducing the observed matrix effect. The proposed method was characterized following the current European legislation for analytical methods focused on residue analysis [21] as well as the recommendation of EURLs for 5-NDZ analyses in food products of animal origin [22].

13.2. Materials and methods

13.2.1. Materials and reagents

All reagents used through this work were analytical reagent grade and solvents were HPLC grade, unless otherwise specified. NaCl and $MgSO_4$ were acquired from Panreac-Química (Madrid, Spain). MeOH, MeCN, acetone, ethyl acetate were purchased from VWR International (West Chester, PA, USA) while EtOH were acquired from Merck (Darmstadt, Germany). Formic acid and acetic acid, both MS grade, were supplied by Sigma-Aldrich (St. Louis, MO, USA). Additionally, d-SPE sorbents namely C18 and PSA were acquired from Agilent Technologies (Waldbronn, Germany) and Z-Sep$^+$ sorbent was purchased from Supelco (Bellafonte, PA, USA). Ultrapure water (Milli-Q plus system, Millipore, Bedford, MA, USA) was used throughout the work.

Clearinert™ 13 mm syringe filters (0.22 μm pore size) were supplied by Bonna-Agela Technologies (Wilmington, DE, USA).

Analytical standards of DMZ, RNZ, HMMNI, ORZ, MNZ, MNZ-OH, IPZ-OH, TNZ and CRZ were supplied by Sigma-Aldrich (St. Louis, MO, USA) while IPZ, SCZ and TRZ were purchased from Witega (Berlin, Germany). Stock standard solutions were obtained by dissolving the appropriate amount of each 5-NDZ drug in MeCN, reaching a final

concentration of 1 mg/mL. Stock standard solutions were kept in the freezer at -20°C avoiding their exposure to light. Intermediate standard solution (5-NDZ concentration ranged from 1.25 to 3.75 μg/mL, depending of the compound) was obtained by mixing the appropriate amount of each stock standard solution and its subsequent dilution with water. It was stored in dark at 4°C and it was equilibrated to room temperature before its use. Working standard solutions were prepared by dilution of the intermediate standard solution with water or mobile phase (up to the experiment) to the desired 5-NDZ concentration.

13.2.2. Instrumentation

All experiments were performed on an Agilent 1290 Infinity LC (Agilent Technologies, Waldbronn, Germany) equipped with a binary pump, online degasser, autosampler (20 μL loop), and a column thermostat. MS measurements were carried out on a QqQ mass spectrometer API 3200 (AB SCIEX, Toronto, ON, Canada) with ESI interface. A C18 Zorbax Eclipse Plus RRHD (50 × 2.1 mm, 1.8 μm) column from Agilent Technologies (Waldbronn, Germany) was used as chromatographic column. Furthermore, a Phoroshell 120 EC-C18 (50 × 2.1 mm, 2.7 μm) column from Agilent Technologies was also tested. Data were collected by the Analyst® Software version 1.5 using the Scheduled MRM™ Algorithm (AB SCIEX).

A Polytron® PT 2500 E homogenizer (Kinematica AG; Luzern, Switzerland), a Universal 320R centrifuge (Hettich Zentrifugen; Tuttlingen, Germany), a nitrogen dryer EVA-EC System (VLM GmbH; Bielefeld, Germany), a mechanical shaker (model 384 from Vibromatic; Noblesville, USA) and a vortex-2 Genie (Scientific Industries; Bohemia, NY, USA) were used through the sample preparation procedure.

13.2.3. Sample treatment procedure

Fresh hake roes were purchased from a local supermarket (Granada, Spain). Before homogenization, hake roe was cut with a knife and the eggs were collected. Then the sample was placed in a 50-mL conical tube and was subsequently ground with a homogenizer for 10 min at 10000 rpm. Afterwards, 1.0 g of the sample was weighted in a

15-mL conical tube and was fortified at the desired 5-NDZ concentration. Furthermore, 1 mL of water was added to the sample and it was homogenized by vortex agitation for a few seconds. Before extraction, samples were left to stand for 15 min.

Later on, 5 mL of MeCN were added to the sample and it was agitated by vortex for 30 s. Moreover, 0.1 g of NaCl and 0.5 g of $MgSO_4$ were added and, subsequently, the sample was mechanically agitated for 10 min and centrifuged for 10 min at 5000 rpm and 25°C. Finally, 1 mL of the sample was dried under a nitrogen current at 40°C and afterwards it was re-dissolved in 200 µL of a mixture 5:95 (v/v) MeOH/formic acid aqueous solution (0.025%, v/v) using vortex agitation for 2 min. Sample was filtered through a syringe filter to a vial and was further analyzed by the proposed UHPLC-MS/MS method.

13.2.4. UHPLC-MS/MS analyses

UHPLC separations were accomplished in a C18 column using a mobile phase consisted of 0.025% (v/v) formic acid aqueous solution (eluent A) and MeOH (eluent B) and supplied at a flow rate of 0.5 mL/min. Gradient program was established as follows: 0.0 min, 5% of B; 1.5 min, 5% of B; 3.0 min, 30% of B; 4.0 min, 95% of B; 5.0 min, 95% of B; and, 6 min, 5% of B. In order to guarantee that initial conditions were properly reached, they were stated for 2 min. Column temperature was set to 25°C and 17.5 µL was selected as injection volume.

Additionally, ionization source parameters were established as follows: temperature of ion source (TEM), 600 ºC; curtain gas (CUR), nitrogen and 45 psi; collision gas (CAD), nitrogen and 10 psi; ion spray voltage (IS), 5250 V; and nebulizing gas (GAS 1) and drying gas (GAS 2), both of them were nitrogen and were set to 50 psi. In all cases a precursor ion and two product ions (the most abundant for quantification and the other one for confirmation) were studied. Therefore, four identification points were obtained as it is required by Regulation (EU) 2002/657/EC for forbidden substances in food products of animal origin [21]. On the other hand, mass spectrometer was working with ESI in positive mode under the MRM conditions shown in **Table 13.1**.

Table 13.1. Monitored ions of the target analytes and MRM parameters.

Analyte	Retention time (min)	Precursor ion (m/z)	Molecular ion	DP (V)	EP (V)	CEP (V)	Product ions	CE (V)	CXP (V)
MNZ-OH	1.28	188.1	$[M+H]^+$	26	4.5	10	126.1, Q_{ion} 123.2, I_{ion}	25 19	4
HMMNI	1.72	158.0	$[M+H]^+$	21	5	8	140.0, Q_{ion} 55.1, I_{ion}	17 29	4 2
MNZ	2.30	172.1	$[M+H]^+$	31	5	10	128.1, Q_{ion} 82.0, I_{ion}	21 33	4
RNZ	2.40	201.1	$[M+H]^+$	21	4	12	140.1, Q_{ion} 55.1, I_{ion}	17 33	4
DMZ	2.53	142.1	$[M+H]^+$	26	5	8	96.0, Q_{ion} 95.0, I_{ion}	23 33	4
TRZ	3.02	186.1	$[M+H]^+$	31	4.5	10	128.3, Q_{ion} 82.2, I_{ion}	21 37	4
TNZ	3.10	248.1	$[M+H]^+$	31	4.5	18	121.1, Q_{ion} 128.0, I_{ion}	23 29	4
SCZ	3.22	186.2	$[M+H]^+$	21	5.5	10	128.1, Q_{ion} 82.2, I_{ion}	19 35	4
ORZ	3.64	220.1	$[M+H]^+$	36	5	10	128.2, Q_{ion} 82.1, I_{ion}	25 39	4
IPZ-OH	3.65	186.2	$[M+H]^+$	31	4.5	10	168.1, Q_{ion} 121.0, I_{ion}	19 37	4
IPZ	3.84	170.1	$[M+H]^+$	41	5	12	109.0, Q_{ion} 123.0, I_{ion}	33 33	4
CRZ	3.91	245.1	$[M+H]^+$	16	3	14	118.0, Q_{ion} 75.0, I_{ion}	17 45	4

Declustering potential, DP; entrance potential, EP; collision entrance potential, CEP; collision energy, CE; collision cell exit potential, CXP; quantification ion, Q_{ion}; and identification ion, I_{ion}.

13.3. Results and discussion

13.3.1. Optimization of chromatographic conditions

5-NDZ separation was evaluated in a C18 Zorbax Eclipse Plus RRHD (50 × 2.1 mm, 1.8 μm) as in **Chapter 12**. Although the separation was also assayed in a Phoroshell 120 EC-C18 (50 × 2.1 mm, 2.7 μm) column, significant differences were not observed compared to the separation achieved in the C18 Zorbax column, and only the retention time of the most polar compounds was slightly decreased. Finally, the C18 Zorbax column was used because analysis time and peak resolution were similar in both cases.

Initially, mobile phase consisted of 0.1% (v/v) formic acid aqueous solution (eluent A) and pure MeOH (eluent B) and was supplied at 0.4 mL/min. Separation was carried out under gradient conditions and the gradient program was established as follows: mobile phase composition was kept at 5% (v/v) of eluent B from 0.0 to 1.0 min, increasing MeOH content to 95% (v/v) in 3.0 min. Maximum MeOH concentration (95%, v/v) was kept for 1.0 min and initial conditions were re-established by a 1.0 min linear gradient. In order to reach a proper column equilibration, initial conditions were maintained for 2.0 min. During separation, column temperature was established at 40°C. Moreover, an injection volume of 10 μL (full loop) was initially considered. Method conditions were optimized using standard solutions in water (100 μg/L of each 5-NDZ), except for the optimization of injection conditions in which standard solutions containing 50 μg/L of each 5-NDZ and dissolved in the evaluated solvents were analyzed.

The use of MeCN instead of MeOH as organic eluent was evaluated, but peak resolution was decreased. Analytes such as DMZ and RNZ co-eluted when MeCN was employed although they were separated when MeOH was used. On the other hand, the addition of acetic acid (0.1%, v/v) instead of formic acid (0.1%, v/v) to the eluent A was also studied. Better separation in terms of peak resolution was observed when formic acid was used, so it was established as the additive of eluent A. Furthermore, formic acid concentration in eluent A was evaluated between 0.01 and 0.1% (v/v). Finally, 0.025% (v/v) was considered as optimum because worse peak shape in terms of peak symmetry was observed at higher values whereas a loss of resolution was obtained at lower concentrations. Moreover, the addition of formic acid (0.025%, v/v) to the eluent B was investigated but no differences in the separation were observed, so pure MeOH was finally used as eluent B.

Mobile phase flow rate was investigated between 0.4 and 0.6 mL/min. As a compromise between peak resolution and analysis time, 0.5 mL/min was selected as optimum. Additionally, the effect of the column temperature on 5-NDZ was studied and it was ranged from 20°C to 45°C. However, no changes on any parameter related to the separation such as analysis time, efficiency or peak resolution were observed in the evaluated temperature range. Therefore, column was thermostatized to 25°C because it is a soft temperature and, as a consequence, column life could be extended.

Under the optimized separation conditions, IPZ-OH and ORZ co-eluted (**Figure 13.1**), but it was not a problem considering that both analytes possess different m/z and, as a result, their unequivocal identification by MS/MS was possible as shown in **Section 13.3.2**.

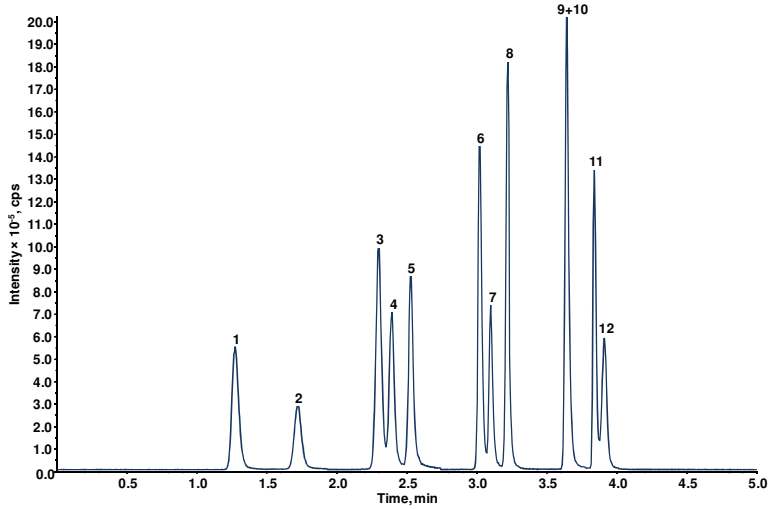

Figure 13.1. Total ion chromatogram obtained from the analysis of a standard solution (5 µg/L of TRZ, SCZ, ORZ, IPZ-OH, IPZ and CRZ; 10 µg/L of HMMNI, MNZ, RNZ, DMZ and TNZ; 15 µg/L of MNZ-OH) by the proposed UHPLC-MS/MS method. Separation was performed in a C18 Zorbax Eclipse Plus RRHD (50 × 2.1 mm, 1.8 µm) column under a temperature of 25°C. Mobile phase consisted of 0.025% (v/v) formic acid aqueous solution as eluent A and MeOH as eluent B and was supplied at a flow rate of 0.5 mL/min. Gradient program was established as follows: 0 min, 5% (v/v) of B; 1.5 min, 5% (v/v) of B; 3 min, 30% (v/v) of B; 4 min, 95% (v/v) of B; 5 min, 95% (v/v) of B; 6 min, 5% (v/v) of B; and 8 min, 5% (v/v) of B. Samples were injected in 5:95 (v/v) MeOH/formic acid aqueous solution (0.1%, v/v). Ionization and MRM parameters are indicated in **Section 13.2.4**. Peaks: 1, MNZ-OH; 2, HMMNI; 3, MNZ; 4, RNZ; 5, DMZ; 6, TRZ; 7, TNZ; 8, SCZ; 9, ORZ; 10, IPZ-OH; 11, IPZ; 12, CRZ.

Injection conditions

In addition to separation optimization, injection solvent was also investigated. It was observed that the use of mobile phase (formic acid solution (0.025%, v/v) containing 5% (v/v) of MeOH) gave the same results in terms of peak efficiency, peak resolution and retention times, as those obtained using water as injection solvent. As a consequence, the use of mobile phase was selected as injection solvent because it is highly recommended to inject the sample in a solution of the same characteristics that the composition of the mobile phase established by the initial conditions of the gradient program. Moreover, the addition of a 5% (v/v) of MeOH to the samples encouraged their reconstitution after

performing the sample treatment. Furthermore, injection volume was studied from 5 to 20 µL, being 20 µL the total volume of the employed injection loop. As can be seen in **Figure 13.2**, peak height linearly increased with the injection volume for all analytes. However, higher irreproducibility was observed for a sample injection volume of 20 µL in comparison to 17.5 µL. For such a reason, an injection volume of 17.5 µL was finally selected.

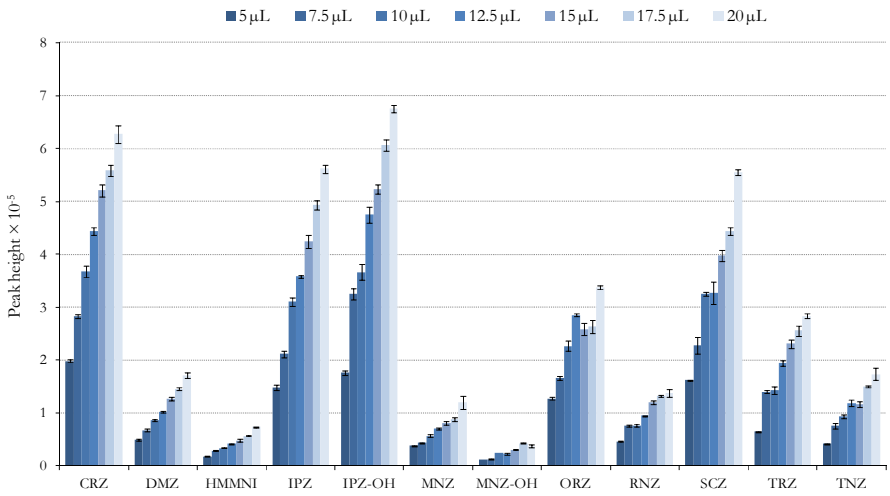

Figure 13.2. **Evaluation of injection time influence on peak height considering standard solutions which contained 50 µg/L of each 5-NDZ. Standard deviation (n = 4).**

13.3.2. Optimization of ionization and MRM parameters

In order to achieve the highest sensitivity, MRM parameters were optimized for each 5-NDZ. Standard solutions of 1.0 mg/L of each analyte dissolved in a mixture 50:50 (v/v) MeOH/formic acid aqueous solution (0.1%, v/v) were individually infused into the mass spectrometer. All the compounds were monitored considering ESI in positive mode as it was done in **Chapter 6** and **8**. The use of acid in the mobile phase favored the ionization step by ESI (+), and under this condition, precursor ions were protonated molecules ($[M+H]^+$). Furthermore, the following MRM parameters were optimized: DP, EP, CEP, CE and CXP, when standard solutions were infused. The optimum values of MRM parameters are shown in **Table 13.1**.

On the other hand, each analyte was characterized by its retention time and two precursor-product ion transitions. The most intense product ion (Q_{ion}) was used for quantification while the second one (I_{ion}) was considered as the confirmation point required for the unequivocal identification of each compound (see **Table 13.1**). During analysis, MRM detection window was established in 10 s and target scan time for each transition was set to 0.5 s.

Finally, under the optimized chromatographic conditions described in **Section 13.3.1**, the ionization source parameters were optimized. Source temperature was tested between 400 and 600°C in steps of 100 °C. This range was selected following the recommendations of the MS manufacturer (AB SCIEX) when a mobile flow rate from 0.3 to 1 mL/min is employed. Finally, 600°C was selected as optimum because higher signal sensitivity was achieved (**Figure 13.3,A**).

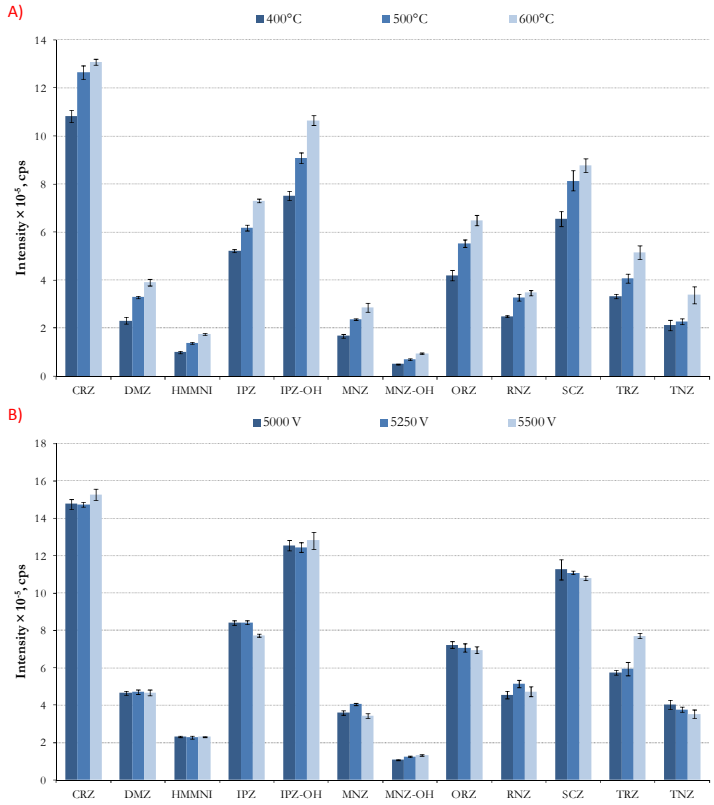

Figure 13.3. Evaluation of ionization parameters: A) temperature of ion source and B) ion spray voltage. Standard deviation (n = 4).

On the other hand, IS was evaluated from 5000 to 5500 V but no differences were observed on the signal in the studied range. As a consequence, an average value was considered for this parameter and 5250 V was established as IS (**Figure 13.3,B**). Moreover, both GS1 and GS2 parameters were established at 50 psi following the recommendations of the manufacturer, so they were not optimized. Additionally, CUR pressure was studied from 25 to 45 psi in steps of 5 psi. Considering that this gas prevents that solvent droplets enter into the MS system and contaminate the ions optics, it must be maintained as high as possible without damaging compound stability and signal sensitivity. It was observed that working a high CUR pressure such as 45 psi was possible without losing signal sensitivity (**Figure 13.4,A**), so it was selected as optimum.

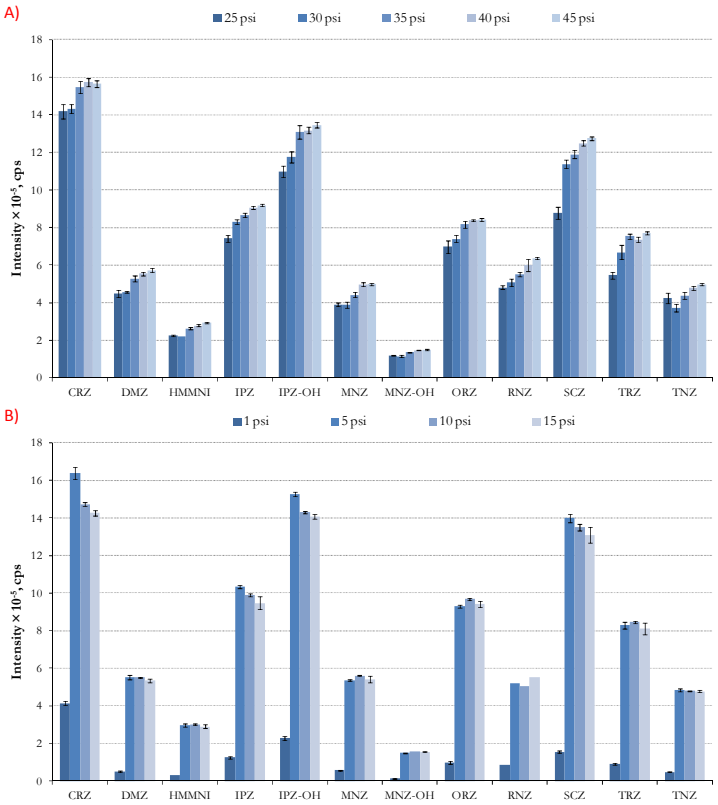

Figure 13.4. Evaluation of ionization parameters: A) curtain gas and B) collision gas. Standard deviation (n = 4).

Finally, CAD pressure was assayed from 1 to 10 psi (**Figure 13.4,B**). Although slightly higher signal sensitivity was reached at 5 psi, better reproducibility in terms of peak area

and height was obtained when 10 psi was considered. Therefore, 10 psi was finally selected as optimum.

Figure 13.5 shows the separation and determination of a standard solution containing the twelve studied 5-NDZ compounds.

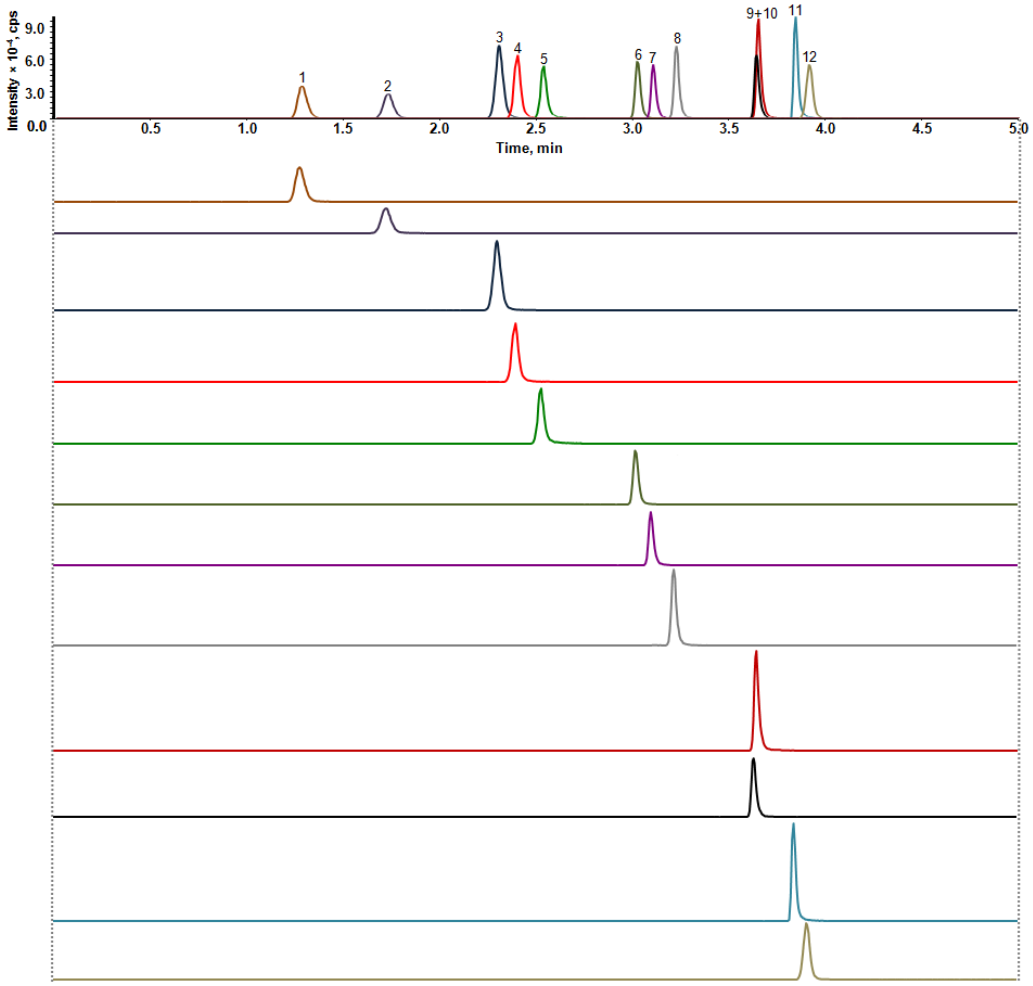

Figure 13.5. Extracted ion chromatograms obtained from the analysis of a standard solution (5 µg/L of TRZ, SCZ, ORZ, IPZ-OH, IPZ and CRZ; 10 µg/L of HMMNI, MNZ, RNZ, DMZ and TNZ; 15 µg/L of MNZ-OH) by the proposed UHPLC-MS/MS method. Separation was performed in a C18 Zorbax Eclipse Plus RRHD (50 × 2.1 mm, 1.8 µm) column under a temperature of 25°C. Mobile phase consisted of 0.025% (v/v) formic acid aqueous solution as eluent A and MeOH as eluent B and was supplied at a flow rate of 0.5 mL/min. Gradient program was established as follows: 0 min, 5% (v/v) of B; 1.5 min, 5% (v/v) of B; 3 min, 30% (v/v) of B; 4 min, 95% (v/v) of B; 5 min, 95% (v/v) of B; 6 min, 5% (v/v) of B; and 8 min, 5% (v/v) of B. Samples were injected in 5:95 (v/v) MeOH/formic acid aqueous solution (0.1%, v/v). Ionization and MRM parameters are indicated in **Section 13.2.4**. Peaks: 1, MNZ-OH; 2, HMMNI; 3, MNZ; 4, RNZ; 5, DMZ; 6, TRZ; 7, TNZ; 8, SCZ; 9, ORZ; 10, IPZ-OH; 11, IPZ; 12, CRZ.

Separation was achieved in less than 4.0 min, and all compounds were resolved except IPZ-OH and ORZ which co-eluted under the optimized conditions. However, from the extracted ion chromatograms it can be concluded that both analytes were satisfactorily identified without damaging signal sensitivity or peak shape. In both cases more than ten points per signal peak were observed.

13.3.3. Sample treatment optimization

Initially, a QuEChERS procedure was proposed as sample treatment and it was further evaluated and optimized. Sample (1.0 g) was spiked at the desired 5-NDZ concentration and 1 mL of water was added to it. Afterwards, sample was allowed to stand for 15 min. Then, 5 mL of MeCN (extraction solvent) were added to the sample and it was subsequently agitated by vortex for 30 s. A mixture of salts (0.1 g of NaCl and 0.5 g of $MgSO_4$) was later added to the sample and it was mechanically agitated for 5 min. Finally, sample was centrifuged for 10 min at 5000 rpm and 25°C. Two liquid phases were differenced, being separated by a solid interface. The upper organic phase was collected and transferred to other 15-mL conical tube. A mixture of the clean-up sorbent (25 mg) and $MgSO_4$ (150 mg) was added to the collected sample, and it was mechanically agitated (5 min) and centrifuged (10 min at 5000 rpm and 25°C). Finally, 2 mL of the sample were collected and dried under nitrogen current at 40°C. Sample was reconstituted in the mobile phase as was established in **Section 13.3.1** and analyzed by the proposed UHPLC-MS/MS method.

During the sample treatment optimization, extraction efficiency and matrix effect were considered as analytical parameters. Both parameters were estimated as it was proposed in **Chapter 6**. Extraction efficiency was calculated by the comparison between the analytical signal obtained for a sample fortified before the sample treatment and the analytical signal obtained for a sample fortified after the sample treatment. In the case of matrix effect, the analytical signal of a sample fortified before the sample treatment was compared with the signal of a standard solution with the same 5-NDZ concentration.

Initially, three clean-up sorbents, namely Z-Sep$^+$, C18 and PSA were assayed. Although similar matrix effect was obtained for all of them, slightly higher recoveries were obtained when Z-Sep$^+$ was used, so it was considered in further experiments (**Figure 13.6**). Furthermore, the amount of sorbent employed in the clean-up stage was also studied. It was assessed from 25 to 100 mg, but no differences were obtained for the evaluated parameters in the studied range. Therefore, 25 mg was considered in further experiments.

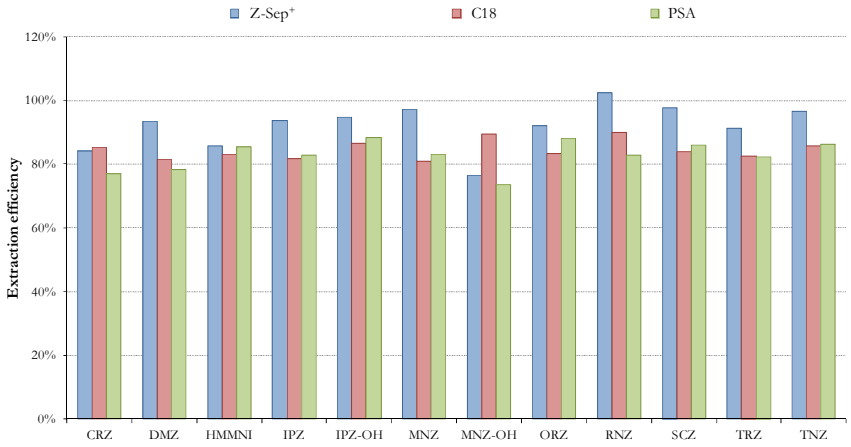

Figure 13.6. Extraction efficiency according to the employed clean-up sorbent.

On the other hand, the use of other extraction solvents as alternative to MeCN was also evaluated. Ethyl acetate, EtOH and MeOH were tested. **Figure 13.7** shows that the use of MeCN allowed achieving higher extraction efficiencies. Therefore it was finally selected as extraction solvent.

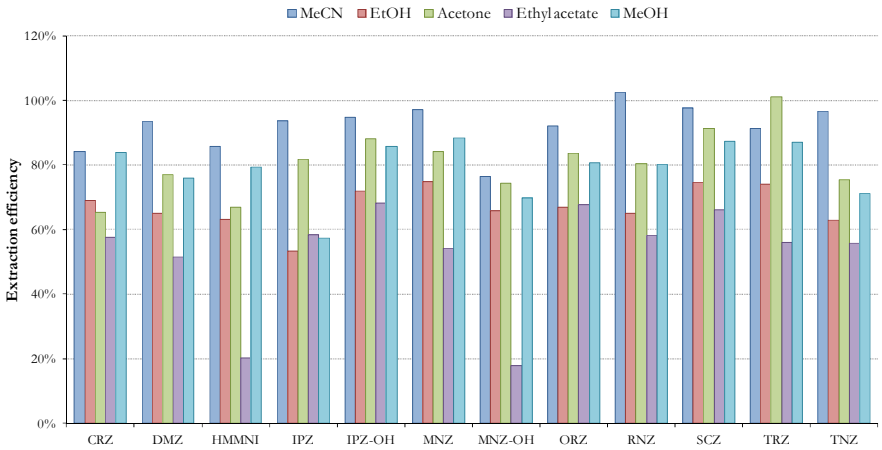

Figure 13.7. Evaluation of the extraction solvent.

In order to evaluate the matrix effect according to the sample volume that was collected after accomplishing the sample treatment, different samples were treated and volumes of them of 0.5, 1 and 2 mL were collected, dried, reconstituted and analyzed. Additionally, the same study was carried out for a sample treated avoiding the clean-up stage, so only the extraction assisted by salts was carried out. Therefore, the evaluation of a SALLE procedure was compared with the initial QuEChERS procedure.

In general, similar extraction recoveries were obtained for each analyte. Moreover, **Figure 13.8** shows that higher matrix effect was obtained when higher sample volume was collected after sample treatment. However, it was surprising that the performance of a clean-up stage did not reduce the matrix effect. The definition of matrix effect proposed by Matuszewski *et al.* [23], and which was discussed in **Chapter 6**, has been followed through this Thesis. Therefore, a value of 100% means that no matrix effect is observed whereas lower values involve a negative matrix effect. Considering the above, the clean-up stage of the sample treatment was discarded and, as a consequence, a SALLE procedure was simply carried out. Moreover it was decided to collect 1 mL of the sample after performing the sample treatment as a compromise between reducing the matrix effect and achieving a high preconcentration factor.

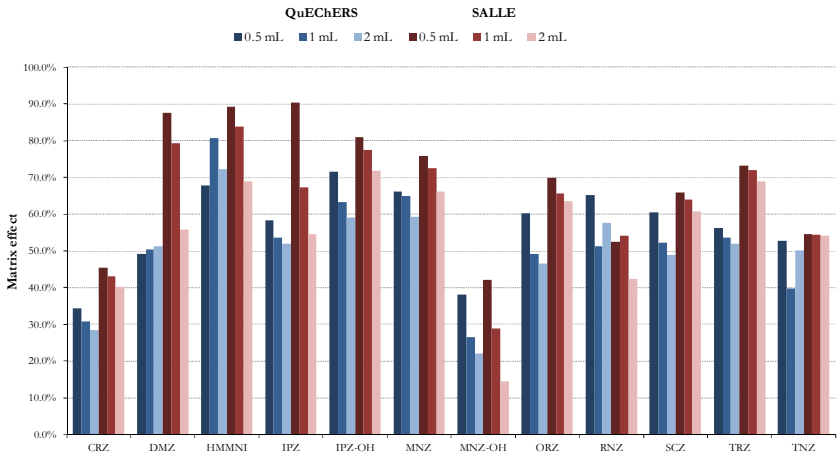

Figure 13.8. **Matrix effect (%) related to the proposed QuEChERS and SALLE procedures according to the collected sample volume after performing the sample treatment.**

The sample treatment procedure that was considered in the proposed method is schematized in **Figure 13.9**.

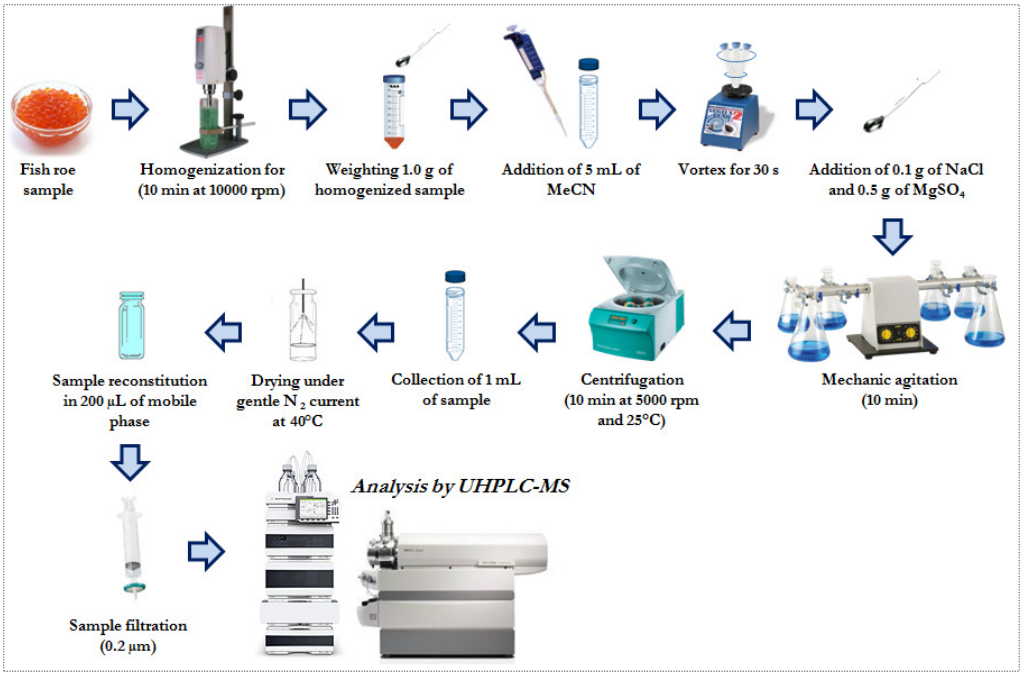

Figure 13.9. Scheme of the sample treatment proposed for 5-NDZ extraction from fish roe samples.

13.3.4. Method characterization

In order to test the usefulness and potential of the proposed method, fish roe samples were treated following the optimized SALLE procedure (see **Section 13.2.3**) and subsequently analyzed by UHPLC-MS/MS. The optimized method was instrumentally evaluated in terms of linearity, LODs, LOQs, extraction recoveries, ME, PE, peak height repeatability and intermediate precision, and trueness.

Calibration curves and performance characteristics

Matrix-matched calibration curves were established in fresh hake roe samples fortified at different concentration levels as follows: 0.5, 1.0, 5.0, 10.0, 30.0 and 64.0 µg/kg for TRZ, SCZ, ORZ, IPZ-OH, IPZ and CRZ; 1.0, 2.0, 10.0, 20.0, 60.0 and 128.0 µg/kg for HMMNI, MNZ, RNZ, DMZ and TNZ; 1.5, 3.0, 15.0, 30.0, and 90.0 µg/kg for MNZ-OH. Two samples per concentration level were analyzed in duplicate. Peak height was considered as function of analyte concentration on the sample. Characteristics of matrix-matched calibration curves are shown on **Table 13.2**.

Table 13.2. Statistical and performance characteristics of the proposed UHPLC-MS/MS method for 5-NDZ determination in hake roe samples.

Analyte	Linear range (µg/kg)	R^2	Linear regression equation (y=m·x+a)				LOD (µg/kg)	LOQ (µg/kg)	CCα (µg/kg)	CCβ (µg/kg)
			Slope $\times 10^{-4}$	*SD for the slope* $\times 10^{-2}$	*Intercept* $\times 10^{-3}$	*SD for the intercept* $\times 10^{-3}$	$3 \times S/N$	$10 \times S/N$		
MNZ-OH	2.9 – 192.0	0.9997	0.019	0.02	0.23	0.77	0.88	2.93	0.73	1.24
HMMNI	0.8 – 128.0	0.9996	0.156	0.15	4.09	0.90	0.24	0.79	1.47	2.50
MNZ	0.4 – 128.0	0.9998	0.385	0.28	2.76	1.62	0.11	0.36	0.83	1.41
RNZ	9.9 – 128.0	0.9996	0.217	0.22	1.50	1.29	2.96	9.85	1.13	1.93
DMZ	0.6 – 128.0	0.9998	0.271	0.19	1.21	1.11	0.17	0.56	0.78	1.33
TRZ	0.2 – 64.0	0.9999	0.569	0.26	1.39	0.76	0.07	0.23	0.26	0.45
TNZ	0.5 – 128.0	0.9998	0.273	0.18	1.68	1.05	0.15	0.51	0.75	1.33
SCZ	0.4 – 64.0	0.9999	0.821	0.48	2.62	1.40	0.11	0.36	0.34	0.58
ORZ	0.2 – 64.0	0.9998	0.797	0.50	2.45	1.46	0.07	0.24	0.36	0.62
IPZ-OH	0.1 – 64.0	0.9996	1.299	1.22	4.59	3.60	0.03	0.11	0.53	0.91
IPZ	0.1 – 64.0	0.9998	0.843	0.58	1.18	1.61	0.04	0.14	0.36	0.62
CRZ	0.7 – 64.0	0.9998	0.491	0.33	1.02	0.97	0.22	0.74	0.38	0.64

LODs and LOQs of the method were calculated as the minimum analyte concentration yielding a S/N equal to three and ten, respectively. Moreover, LODs lower than 0.9 μg/kg were obtained for all the studied 5-NDZ compounds, expect for RNZ (LOD equal to 3.0 μg/kg). Furthermore, validation procedure was completed by the estimation of CCα and CCβ parameters [24]. This procedure allows the determination of both parameters for non-permitted veterinary drug substances. As can be seen on **Table 13.2**, the obtained data for CCα and CCβ ranged between 0.3-1.5 and 0.5-2.5 μg/kg, respectively. Both parameters were lower than 3 μg/kg, accomplishing with EURLs' recommendations.

Recoveries studies, matrix effect and process efficiency

R, ME and PE studies were performed over fresh fish roe samples. R studies evaluate the efficiency of the extraction process whereas ME is the result caused by other components of the sample except the specific compounds to be quantified on the analytical response. As it was previously described, ME (%) > 100 involves that matrix components produce a signal enhancement whereas ME (%) < 100 means that ion suppression is occurring. A value around 100% involves that no ME is observed. Both phenomena are included in PE which evaluates the efficiency of the whole determination procedure.

These parameters were evaluated as proposed in **Chapter 6**, although in this case they were estimated as average value for the whole linear range instead of estimating a value at three different concentration levels. Therefore, matrix-matched calibration curves, external standard calibration curves and calibration curves of samples spiked after sample treatment with the same linear range were required for these studies. The estimation of RE, ME and PE was performed by comparison between calibration curve slopes as indicated in **Equation 13.1**, **Equation 13.2**, and **Equation 13.3**, respectively.

$$R = \frac{Slope\ of\ matrix{-}matched\ calibration\ curve}{Slope\ of\ calibration\ curve\ \ of\ samples\ spiked\ after\ sample\ treatment}$$ **Equation 13.1.**

$$ME = \frac{Slope\ of\ calibration\ curve\ \ of\ samples\ spiked\ after\ sample\ treatment}{Slope\ of\ external\ standard\ calibration\ curve}$$ **Equation 13.2.**

$$PE = \frac{Slope\ of\ matrix{-}matched\ calibration\ curve}{Slope\ of\ external\ standard\ calibration\ curve}$$ **Equation 13.3.**

Table 13.3 shows that high R values were obtained for all analytes (> 70%) which demonstrates the suitability of the proposed SALLE procedure for the extraction of 5-NDZ residues from fish roe samples. However, low PE was achieved for RNZ (45.5%) and very poor PE was observed for MNZ-OH (9.4%). These values are due to the high ME observed for both analytes, so a sample clean-up stage should be considered in order to improve the PE for MNZ-OH and RNZ by reducing the ME. However, lower ME was observed for the rest of the studied 5-NDZ compounds, so the proposed sample treatment is suitable for their determination in fresh hake roe samples.

Table 13.3. **Estimated extraction recovery, matrix effect and process efficiency for the determination of 5-NDZ residues in fresh hake roe samples by the proposed SALLE-UHPLC-MS/MS method.**

Analyte	R (%)	ME (%)	PE (%)
MNZ-OH	72.2	13.1	9.4
HMMNI	85.9	77.0	66.1
MNZ	83.9	82.0	68.9
RNZ	83.2	54.7	45.5
DMZ	71.4	79.4	56.7
TRZ	80.6	81.2	65.6
TNZ	83.7	85.2	71.3
SCZ	83.9	78.6	65.9
ORZ	91.0	80.2	73.0
IPZ-OH	93.6	84.1	78.6
IPZ	75.4	71.5	53.9
CRZ	80.0	67.8	54.2

Precision assays

Precision studies were carried out in order to evaluate the repeatability (intra-day precision) and intermediate precision (inter-day precision) of the proposed SALLE-UHPLC-MS/MS method. Repeatability was assayed at three concentration levels by analyzing four spiked samples per level in triplicate. Intermediate precision was assessed at three concentration levels by analyzing one spiked sample in triplicate per level and per day for five consecutive

days. Results expressed as peak height RSDs (%) are shown on **Table 13.4**. In all cases RSDs were lower than 9.8% and 13.9% for repeatability and intermediate precision, respectively.

Table 13.4. Precision studies in terms of RSDs (%) for hake roe samples.

Analyte	Repeatability (% RSD, n = 12)			Intermediate precision (% RSD, n = 15)		
	1 µg/kg	***5 µg/kg***	***30 µg/kg***	***1 µg/kg***	***5 µg/kg***	***30 µg/kg***
TRZ	3.4	3.6	1.2	8.5	5.8	3.7
SCZ	2.9	3.6	1.4	10.6	8.1	3.4
ORZ	2.7	1.9	1.7	9.1	8.1	4.6
IPZ-OH	3.1	2.3	1.9	8.4	7.7	3.5
IPZ	8.1	6.0	3.9	9.9	7.3	8.9
CRZ	5.3	3.8	5.6	9.1	7.2	8.7
	2 µg/kg	***10 µg/kg***	***60 µg/kg***	***2 µg/kg***	***10 µg/kg***	***60 µg/kg***
HMMNI	4.3	8.9	2.2	10.5	5.6	3.9
MNZ	3.8	5.9	1.8	13.9	7.4	3.4
RNZ	4.1	6.6	2.1	10.7	5.2	2.8
DMZ	8.5	7.1	8.0	7.3	6.8	4.1
TNZ	4.3	4.3	2.1	4.8	6.2	3.5
	3 µg/kg	***15 µg/kg***	***90 µg/kg***	***3 µg/kg***	***15 µg/kg***	***90 µg/kg***
MNZ-OH	8.8	9.8	5.9	10.8	5.5	6.9

Trueness

Trueness was assessed in terms of method recoveries considering hake roe samples spiked at three different concentration levels. Four samples per concentration level were treated following the SALLE procedure and analyzed in triplicate. Considering matrix-matched calibration curves shown on **Table 13.2**, the concentration found in the samples was estimated and compared with the added concentration.

As can be seen on **Table 13.5**, recoveries between 73.7 and 109.5% were obtained. In all cases results accomplish with Regulation 2002/657/EC [21] that establishes a trueness uncertainly range between -30 and +10% for a sample with a residue concentration between 1 and 10 µg/kg and a range from -20 to +10% for a residue concentration higher than 10 µg/kg.

Table 13.5. Trueness assays of 5-NDZ determination in hake roe samples by the proposed SALLE-UHPLC-MS/MS method.

Analyte	Concentration added (µg/kg)	Concentration found (mean ± SD, µg/kg) (n = 9)	Recovery (%)
MNZ-OH	3.0	2.6 ± 0.2	86.2
	15.0	14.2 ± 1.4	94.8
	90.0	87.3 ± 5.2	97.0
HMMNI	2.0	1.5 ± 0.1	73.7
	10.0	10.1 ± 1.0	101.2
	60.0	65.7 ± 0.9	109.6
MNZ	2.0	1.7 ± 0.1	85.6
	10.0	10.5 ± 0.6	104.6
	60.0	65.7 ± 1.2	109.5
RNZ	2.0	1.4 ± 0.1	72.3
	10.0	10.1 ± 0.7	101.4
	60.0	64.3 ± 1.4	107.2
DMZ	2.0	1.9 ± 0.2	93.0
	10.0	10.3 ± 0.8	103.1
	60.0	62.4 ± 5.0	104.0
TRZ	1.0	0.9 ± 0.0	92.0
	5.0	5.1 ± 0.2	102.8
	30.0	31.8 ± 0.4	106.0
TNZ	2.0	1.8 ± 0.1	89.4
	10.0	10.4 ± 0.5	104.4
	60.0	63.5 ± 1.3	105.8
SCZ	1.0	0.9 ± 0.0	91.1
	5.0	5.2 ± 0.2	104.3
	30.0	31.4 ± 0.5	104.8
ORZ	1.0	0.9 ± 0.0	88.8
	5.0	5.1 ± 0.1	102.6
	30.0	30.2 ± 0.5	100.7
IPZ-OH	1.0	0.8 ± 0.0	83.9
	5.0	5.1 ± 0.1	102.3
	30.0	30.0 ± 0.6	100.1
IPZ	1.0	1.0 ± 0.1	103.8
	5.0	5.2 ± 0.3	104.1
	30.0	24.5 ± 5.1	81.7
CRZ	1.0	0.9 ± 0.1	90.4
	5.0	5.1 ± 0.2	101.4
	30.0	28.3 ± 1.6	94.3

Selectivity

The confirmation of the identification of 5-NDZ compounds in hake roe samples was carried out according to Regulation 2002/657/EC [21], which establishes a tolerance level for the relative intensity between Q_{ion} and I_{ion} for the obtained MRM transitions in the analysis of real samples by LC-MS. The tolerance range depends on the Q_{ion}/I_{ion} ratio established from the analysis of a standard solution (see **Table 13.6**). **Table 13.6** shows the average of the Q_{ion}/I_{ion} ratios obtained from the samples analyzed in intermediate precision studies. In all cases Q_{ion}/I_{ion} ratios were within the tolerance range for relative ion intensities established by the current European legislation [21].

Table 13.6. **Q_{ion}/I_{ion} ratios obtained from the determination of 5-NDZs in standard solutions and in hake roe samples.**

Analyte	Q_{ion}/I_{ion} ratio in standards (%)	Ratio range	Tolerance range (%)	Q_{ion}/I_{ion} ratio in hake eggs (%)
MNZ-OH	81	> 50%	±20	82
HMMNI	26	> 20% to 50%	±25	25
MNZ	50	> 20% to 50%	±25	46
RNZ	20	> 10% to 20%	±30	21
DMZ	50	> 20% to 50%	±25	48
TRZ	47	> 20% to 50%	±25	46
TNZ	46	> 20% to 50%	±25	42
SCZ	33	> 20% to 50%	±25	32
ORZ	37	> 20% to 50%	±25	37
IPZ-OH	47	> 20% to 50%	±25	47
IPZ	90	> 50%	±20	93
CRZ	13	> 10% to 20%	±30	12

Additionally, the total ion chromatogram obtained from the analysis of hake roe sample spiked with 5-NDZ compounds, treated following the proposed SALLE procedure and analyzed by the developed UHPLC-ESI-MS/MS method is shown in **Figure 13.10,A**. Moreover, the total ion chromatogram of a blank sample is also shown. **Figure 13.10,B** also shows the extracted ion chromatogram of each 5-NDZ.

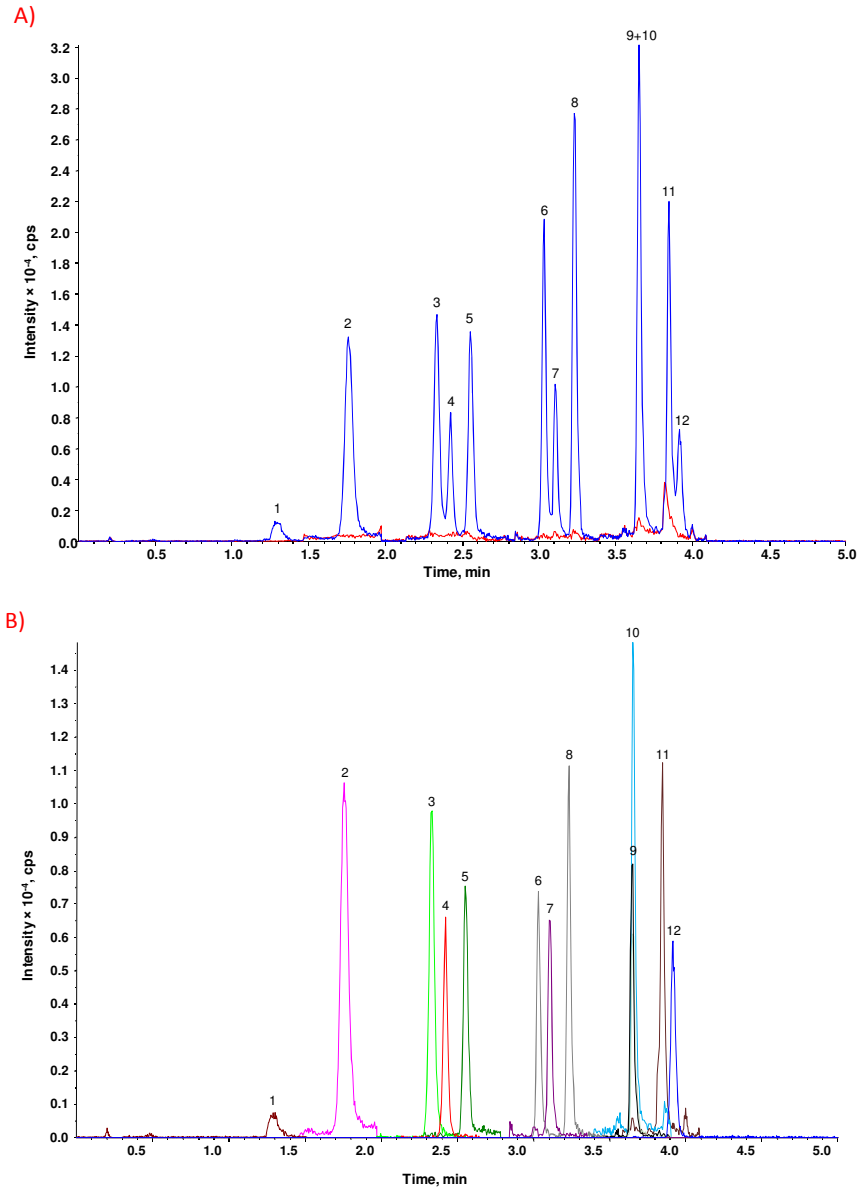

Figure 13.10. Chromatograms of a hake roe sample spiked at 1 µg/kg of TRZ, SCZ, ORZ, IPZ-OH, IPZ and CRZ, 2 µg/kg of HMMNI, DMZ, RNZ, MNZ and TNZ, and 3 µg/kg of MNZ-OH, treated and analyzed following the proposed SALLE-UHPLC-ESI-MS/MS method. A) Total ion chromatogram; red line represents the total ion chromatogram of a blank sample. B) Extracted ion chromatograms of the quantification ions. Separation was performed in a C18 Zorbax Eclipse Plus RRHD (50 × 2.1 mm, 1.8 µm) column under a temperature of 25°C. Mobile phase consisted of 0.025% (v/v) formic acid aqueous solution as eluent A and MeOH as eluent B and was supplied at a flow rate of 0.5 mL/min. Gradient program was established as follows: 0 min, 5% (v/v) of B; 1.5 min, 5% (v/v) of B; 3 min, 30% (v/v) of B; 4 min, 95% (v/v) of B; 5 min, 95% (v/v) of B; 6 min, 5% (v/v) of B; and 8 min, 5% (v/v) of B. Samples were injected in 5:95 (v/v) MeOH/formic acid aqueous solution (0.1%, v/v). Ionization and MRM parameters are indicated in **Section 13.2.4**. Peaks: 1, MNZ-OH; 2, HMMNI; 3, MNZ; 4, RNZ; 5, DMZ; 6, TRZ; 7, TNZ; 8, SCZ; 9, ORZ; 10, IPZ-OH; 11, IPZ; 12, CRZ.

13.4. Conclusions

A rapid and simple UHPLC-MS/MS method was developed for the determination of twelve 5-NDZ drugs in less than 4 min, which involves a total analysis time within 8 min, including column equilibration. The optimized method was successfully applied to the analysis of 5-NDZ residues in hake roe samples. According to the reviewed literature, it supposes the first application proposed for the determination of 5-NDZ antibiotics in this type of food matrix. Moreover, a cheap, fast, simple and environmental friendly SALLE procedure was considered as sample treatment. Satisfactory extraction efficiency was observed for all the studied compounds, but high matrix effect was obtained for RNZ and especially for MNZ-OH, which suggests that a clean-up sample stage should be required for the determination of both compounds. However, it was demonstrated that the performance of a d-SPE stage using C18, PSA or Z-Sep$^+$ sorbent was inefficiency for achieving a decrease of matrix effect. Additionally, the developed method was successfully characterized in terms of linearity, repeatability and reproducibility, trueness and selectivity.

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GENERAL CONCLUSIONS

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Conclusions

This Thesis contributes to increase the number of analytical methods available for the control of 5-NDZ residues in food and environmental samples and the monitoring of these substances in other biological matrices. Additionally, the developed methods have evaluated the potential of CE, CEC, CLC and UHPLC as well as of new alternative sample treatments, according to the trends of green analytical chemistry, for the determination of 5-NDZs at trace levels in several matrices. The following conclusions have been achieved:

- ◊ CE, CEC, CLC and UHPLC methods involve lower solvent consumption, especially organic solvents, in the case of CLC and UHPLC, in comparison with traditional LC-based methods. Among all considered techniques, CE is less polluting due to the use of buffers as BGS and the low waste generation.

- ◊ Although low sensitivity has been attributed to CE-UV methods, high sensitivity have been reached by the combination of on-line and off-line preconcentration strategies. In this Thesis, sweeping and CSEI-sweeping have been evaluated as on-line preconcentration approaches, whereas SPE and DLLME procedures has been assessed as sample treatments. The developed methods have shown that detection limits at low μg/L (or μg/kg) can be achieved for the analysis of 5-NDZ residues in food and environmental samples by MEKC-UV. Furthermore, CSEI-sweeping has demonstrated to be a powerful strategy for the direct analysis of 5-NDZs in urine and serum samples, at therapeutic levels (μg/mL), without involving tedious sample treatments.

- ◊ MEKC and CZE have been evaluated for 5-NDZ separation. A MEKC-UV method has been developed for the analysis of up to nine 5-NDZ compounds within 20 min, while a CZE-MS method has been proposed for the determination of eleven 5-NDZs within 28 min. This supposes the first contribution for the determination of relatively high number of 5-NDZ drugs by CE, including their metabolites, and the first CE-MS applied to 5-NDZ determination.

In comparison with the proposed CLC-UV method, which involves the separation of eleven 5-NDZs within 22 min, CE-based methods have demonstrated to be a suitable alternative to LC-methods.

◊ The suitability of CEC-UV for 5-NDZ determination in complex matrices, such as milk, has also been investigated. Despite of using laboratory-made capillaries, high reproducibility in terms of migration time was achieved, and the separation of eight 5-NDZ compounds was accomplished in less than 15 min. Although low detection limits were achieved, they are higher than those reported for 5-NDZ determination in milk by MEKC-UV. However, it supposes an example of the applicability of CEC.

◊ Furthermore, and for the first time, a CEC-MS method has been proposed for 5-NDZ determination. This method was developed in collaboration with the group of Dr. Salvatore Fanali during a pre-doctoral stay in the *'Istituto di Metodologie Chimiche (IMC), Consiglio Nazionale delle Ricerche di Montelibretti'* (Rome, Italy). CEC-MS hyphenation was carried out by means of a nano-liquid-junction interface, and the proposed method was successfully applied to urine samples. The use of a miniaturized technique such as CEC permitted to perform analyses in a reasonable time with minimum reagent consumption, and as a consequence, waste disposal is reduced and it results in a lower effective cost if it is compared with HPLC methods.

◊ SPE has shown to be a good alternative to sample clean-up prior to sample analysis by CE. HLB and mixed cation exchange-reverse phase cartridges have demonstrated to be suitable for 5-NDZ extraction from complex matrices such as milk and eggs, as well as from water samples. Moreover, its applicability prior to CEC analysis was also checked in milk and urine samples.

◊ MISPE has demonstrated its high selectivity for the extraction of 5-NDZs from urine and aquaculture products, namely crab, salmon, prawn and swimming velvet crab. High recoveries were obtained in the case of urine samples while lower

values were observed for some analytes in the case of aquaculture samples. However, analyte loss was attributed to the SALLE procedure performed before MISPE, a stage that was avoided for urine samples.

◊ A novel SALLE procedure was evaluated as cheap, green and easy sample treatment for the analysis of 5-NDZ antibiotics in milk samples. Moreover, this procedure has been proposed in combination with UHPLC-UV method that allowed the determination of eight 5-NDZs within 8 min. Therefore, this method supposes a great alternative for its implementation in routine analysis because it is a quick, cheap, easy and offers high sample throughput.

◊ Finally, a new UHPLC-MS/MS method has been developed for the determination of 5-NDZs in a fish roe samples. It supposes the first application reported for this purpose according to the reviewed literature. Furthermore, the whole analytical procedure, including sample treatment, is quick, simple and environmental friendly, considering that only an extraction with 5 mL of MeCN per sample is required and separation takes places in less than 4 min.

As a summary, the most significant analytical characteristics of the developed methods are shown in **Table C1**.

Table C1. Analytical characteristics of the proposed methods.

Analytes	Matrix	Sample treatment	Instrumental technique	Analysis time (min)	LODs
MNZ-OH, HMMNI, MNZ, RNZ, DMZ, TRZ, IPZ-OH, ORZ, IPZ	River water (25 mL)	SPE Oasis®HLB (500 mg) cartridges	**Sweeping-MEKC-UV** 'Bubble' capillary: 64.5 cm × 50 μm i.d. BGS: 20 mM phosphate buffer (pH 6.5) containing 150 mM SDS 25 kV, 20°C	20 min	0.5 – 1.1 μg/L
MNZ-OH, HMMNI, MNZ, RNZ, TNZ, TRZ, IPZ-OH, ORZ	Tap, river and bottled water (5 mL)	DLLME	**Sweeping-MEKC-UV** 'Bubble' capillary: 61.5 cm × 50 μm i.d. BGS: 20 mM phosphate buffer (pH 6.5) containing 150 mM SDS 25 kV, 20°C	12 min	0.9 – 2.4 μg/L
MNZ-OH, HMMNI, MNZ, RNZ, DMZ, TRZ, IPZ-OH, ORZ, IPZ	Milk (3.5 mL)	SPE Oasis®MCX (150 mg) cartridges	**Sweeping-MEKC-UV** 'Bubble' capillary: 61.5 cm × 50 μm i.d. BGS: 20 mM phosphate buffer (pH 6.5) containing 150 mM SDS 25 kV, 20°C	15 min	0.9 – 1.8 μg/L
CRZ, IPZ, SCZ, TRZ, MNZ, TNZ	Well and river water (5 mL)	DLLME	**CSEI-sweeping-MEKC-UV** 'Bubble' capillary: 57.2 cm × 50 μm i.d. BGS: 44 mM phosphate buffer (pH 2.5) containing 123 mM SDS and 8% (v/v) THF -30 kV, 20°C	10 min	0.6 – 2.4 μg/L
IPZ, ORZ, SCZ, TRZ, MNZ, TNZ	Eggs (2 g)	SALLE + SPE Oasis®HLB (60 mg) cartridges	**CSEI-sweeping-MEKC-UV** Standard capillary: 70.0 cm × 50 μm i.d. BGS: 44 mM phosphate buffer (pH 2.5) containing 123 mM SDS and 8% (v/v) THF -30 kV, 20°C	12 min	2.1 – 5.0 μg/kg
MNZ, ORZ, TNZ	Urine (7 μL) Serum (200 μL)	Urine dilution 143-fold Serum dilution 76-fold	**CSEI-sweeping-MEKC-UV** 'Bubble' capillary: 65.0 cm × 50 μm i.d. BGS: 44 mM phosphate buffer (pH 2.5) containing 123 mM SDS and 8% (v/v) THF -30 kV, 20°C	13 min	Urine: 0.5 – 1.8 μg/mL Serum: 0.2 – 1.5 μg/mL

Table C1 (continued). Analytical characteristics of the proposed methods.

Analytes	Matrix	Sample treatment	Instrumental technique	Analysis time (min)	LODs
MNZ-OH, TNZ, HMMNI, MNZ, RNZ, DMZ, TRZ, IPZ-OH, ORZ, IPZ, SCZ	Urine (2 mL)	MIPE SupelMIP®SPE (50 mg) cartridges	**CZE-ESI-IT-MS** Standard capillary: 110 cm × 50 μm i.d. BGS: 1 M formic acid solution (pH 1.8) 28 kV, 25°C	28 min	2.9 – 39.1 μg/L
MNZ, CRZ, RNZ, TRZ, SCZ, IPZ, DMZ, ORZ	Milk (3 mL)	SALLE + SPE Oasis®HLB (60 mg) cartridges	**CEC-UV** C18 (5 μm) packed capillary: 40 cm × 50 μm i.d. Mobile phase: 60:40 (v/v) MeCN/ammonium acetate buffer (2.5 mM, pH 5) 27 kV, 30°C	15 min	3 – 29 μg/L
MNZ, CRZ, RNZ, TRZ, SCZ, IPZ, DMZ, ORZ	Urine (0.5 mL)	SPE Oasis®HLB (30 mg) cartridges	**CEC-nano-liquid-junction-ESI-IT-MS** 3:1 (w/w) bidentate C18 (4.2 μm)/silica (5 μm) packed capillary: 25 cm × 75 μm i.d. Mobile phase: 60:40 (v/v) MeCN/ammonium acetate buffer (5 mM, pH 5) 15 kV, 11 bar of applied pressure to inlet vial	21 min	0.03 – 0.13 μg/mL
MNZ-OH, TNZ, HMMNI, MNZ, RNZ, DMZ, TRZ, IPZ-OH, ORZ, IPZ, SCZ	Crab, salmon, prawn, swimming velvet crab (2 g)	SALLE + MISPE SupelMIP®SPE (50 mg) cartridges	**CLC-UV** Zorbax SB-C18 (150 × 0.5 mm, 5 μm) Mobile phase: water (eluent A), MeCN (eluent B) Flow rate: 7 μL/min	22 min	0.9 – 3.2 μg/kg
MNZ, RNZ, TRZ, SCZ, TNZ, ORZ, IPZ, CRZ	Milk (4 mL)	SALLE	**UHPLC-UV** Zorbax Eclipse Pluss RRHD C18 (50 × 2.1 mm, 1.8 μm) Mobile phase: 0.1% (v/v) formic acid solution (eluent A), MeCN containing 0.1% (v/v) formic acid (eluent B) Flow rate: 0.45 mL/min	8 min	2.0 – 4.0 μg/L
MNZ-OH, HMMNI, MNZ, RNZ, DMZ, TRZ, TNZ, SCZ, ORZ, IPZ-OH, IPZ, CRZ	Hake roe (1 g)	SALLE	**UHPLC-ESI-QqQ-MS** Zorbax Eclipse Pluss RRHD C18 (50 × 2.1 mm, 1.8 μm) Mobile phase: 0.025% (v/v) formic acid solution (eluent A), pure MeOH (eluent B) Flow rate: 0.5 mL/min	4 min	0.03 – 2.96 μg/kg

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Conclusiones

En esta Tesis se ha llevado a cabo la propuesta de nuevos métodos de análisis de 5-NDZs, alternativos a los ya existentes, para el control de residuos de estos compuestos en muestras alimentarias y medioambientales, así como para la monitorización terapéutica de estas sustancias en muestras biológicas. Por otra parte, y considerando las tendencias actuales en la Química Analítica Verde, se ha evaluado el potencial de diversas técnicas de separación tales como la CE, CEC, CLC y UHPLC, además de nuevos tratamientos de muestras, para la determinación de 5-NDZs a niveles de trazas en diversas matrices.

Con estas premisas, las conclusiones de este estudio son las siguientes:

- ◊ Los métodos que utilizan CE, CEC, CLC y UHPLC como técnicas de separación implican un bajo consumo de disolventes, concretamente de disolventes orgánicos en el caso de la CLC y UHPLC, en comparación con los métodos de LC tradicionales. Entre las técnicas consideradas, la CE es la técnica menos contaminante debido al uso de disoluciones tampón como medio de separación, generando una menor cantidad de residuos.

- ◊ Aunque se atribuye una baja sensibilidad a los métodos CE-UV, ésta puede incrementarse utilizando estrategias de preconcentración 'on-line' y 'off-line'. En esta Tesis se ha llevado a cabo la evaluación de las técnicas de preconcentración 'on-line' denominadas 'sweeping' y 'CSEI-sweeping' con el objetivo de mejorar la sensibilidad, así como se han evaluado diferentes tratamientos de muestras basados en la SPE y DLLME para aumentar dicha sensibilidad a través de la preconcentración 'off-line'. En los métodos desarrollados se han alcanzado límites de detección muy bajos (niveles de μg/L o μg/kg), concretamente en los métodos basados en MEKC-UV, propuestos para el análisis de residuos de 5-NDZs en muestras alimentarias y medioambientales. Además, la preconcentración 'CSEI-sweeping' ha demostrado ser una estrategia potencialmente útil para la determinación directa de 5-NDZs en muestras biológicas como la orina y el suero.

En este último caso, el método propuesto permite la determinación de dichas sustancias a niveles de concentración terapéuticos (μg/mL).

◊ Así mismo, dos de los modos de trabajo de la CE, como son MEKC y CZE, se han empleado en la separación de diversos 5-NDZs. El método MEKC-UV desarrollado permite el análisis de hasta nueve 5-NDZs en 20 minutos. Por otra parte, mediante el método propuesto basado en CZE-MS ha sido posible analizar simultáneamente once compuestos en tan solo 28 minutos. Estos dos métodos han permitido, por primera vez, el análisis simultáneo de una cantidad tan elevada de antibióticos 5-NDZs, incluyendo sus metabolitos, mediante CE, además de plantear por primera vez el uso de CE-MS para tal fin.

En comparación con el método CLC-UV propuesto, el cual implica la separación de once 5-NDZs en 22 minutos, los métodos de CE desarrollados han demostrado ser una alternativa a los métodos de análisis basados en la LC.

◊ Se ha investigado la idoneidad de un método CEC-UV para la determinación de 5-NDZs en matrices complejas tales como la leche. A pesar de emplear capilares preparados en el laboratorio, se alcanzó una alta reproducibilidad en términos de tiempos de migración. Por otra parte, la separación de ocho 5-NDZs se llevó a cabo en menos de 15 minutos. Aunque se alcanzaron límites de detección bajos, éstos eran superiores a los alcanzados para la determinación de 5-NDZs en leche mediante el método MEKC-UV desarrollado. Sin embargo, este método constituye un ejemplo de la aplicabilidad de la CEC.

◊ Por primera vez se ha propuesto un método CEC-MS para la determinación de 5-NDZs. Este método se ha desarrollado en colaboración con el grupo del Dr. Salvatore Fanali durante una estancia predoctoral en el *'Istituto di Metodologie Chimiche (IMC)* del *Consiglio Nazionale delle Ricerche di Montelibretti'* (Roma, Italia). El acoplamiento CEC-MS se llevó a cabo mediante una interfase 'nano' de unión líquida, en tanto que el método propuesto se aplicó satisfactoriamente a muestras de orina. El uso de una técnica miniaturizada como la CEC permite obtener los

análisis en un tiempo razonable involucrando un consumo mínimo de reactivos, y como consecuencia, los residuos generados se reducen, constituyendo, a su vez, un ahorro económico comparado con los métodos de HPLC.

◊ La extracción SPE ha demostrado ser una buena alternativa como tratamiento de muestra antes de llevar a cabo un análisis mediante CE. El sorbente HLB y el sorbente mixto de intercambio catiónico y fase reversa han demostrado ser útiles para la extracción de 5-NDZs de muestras complejas tales como la leche y los huevos, así como para su extracción de muestras de aguas. Además, la aplicabilidad de esta técnica de extracción también se comprobó en las muestras de leche y orina que iban a ser analizadas posteriormente mediante CEC.

◊ Por otra parte, MISPE ha demostrado su alta selectividad para la extracción de 5-NDZs en muestras de orina y productos de acuicultura, concretamente cangrejo, gamba, salmón y nécora. Se obtuvieron altas recuperaciones, en el caso de las muestras de orina, aunque los valores fueron inferiores, para algunos analitos, en el caso de los productos de acuicultura. Sin embargo, estas recuperaciones más bajas fueron atribuidas a pérdidas de analito en el proceso SALLE, etapa que fue evitada en el caso de las muestras de orina, llevada a cabo antes del procedimiento MISPE.

◊ Se ha evaluado un nuevo procedimiento de extracción (SALLE) para el análisis de antibióticos 5-NDZs en muestras de leche, por ser un tratamiento de muestra barato, ecológico y simple. Además, este tratamiento de muestra se propuso en combinación con un método UHPLC-UV, el cual permite la determinación de ocho 5-NDZs en 8 minutos. Los resultados obtenidos demuestran que este método supone una alternativa eficaz para su implementación en los laboratorios de rutina debido a que es un método rápido, barato, verde y ofrece un alto rendimiento en el tratamiento de muestras.

◊ Finalmente, se ha desarrollado un nuevo método UHPLC-MS/MS para la determinación de 5-NDZs en muestras de huevas de merluza. Este método es la primera aplicación que se ha llevado a cabo para dicho fin de acuerdo con la

bibliografía consultada. Además, todo el proceso analítico, incluyendo el tratamiento de muestra, es rápido, simple y respetuoso con el medio ambiente, teniendo en cuenta que sólo son necesarios 5 mL de MeCN por muestra para llevar a cabo la extracción, en tanto que la separación se realiza en menos de 4 minutos.

Como resumen, las características más significativas de los métodos analíticos desarrollados se muestran en la **Tabla C2**.

Tabla C2. Características analíticas de los métodos desarrollados.

Analitos	Muestra	Tratamiento de muestra	Técnica instrumental	Tiempo de análisis (min)	LODs
MNZ-OH, HMMNI, MNZ, RNZ, DMZ, TRZ, IPZ-OH, ORZ, IPZ	Agua de río (25 mL)	SPE con cartuchos Oasis®HLB (500 mg)	**Sweeping-MEKC-UV** Capilar 'burbuja': 64.5 cm × 50 μm i.d. BGS: 20 mM tampón fosfato (pH 6.5) conteniendo 150 mM SDS 25 kV, 20°C	20 min	0.5 – 1.1 μg/L
MNZ-OH, HMMNI, MNZ, RNZ, TNZ, TRZ, IPZ-OH, ORZ	Agua del grifo, de río y embotellada (5 mL)	DLLME	**Sweeping-MEKC-UV** Capilar 'burbuja': 61.5 cm × 50 μm i.d. BGS: 20 mM tampón fosfato (pH 6.5) conteniendo 150 mM SDS 25 kV, 20°C	12 min	0.9 – 2.4 μg/L
MNZ-OH, HMMNI, MNZ, RNZ, DMZ, TRZ, IPZ-OH, ORZ, IPZ	Leche (3.5 mL)	SPE con cartuchos Oasis®MCX (150 mg)	**Sweeping-MEKC-UV** Capilar 'burbuja': 61.5 cm × 50 μm i.d. BGS: 20 mM tampón fosfato (pH 6.5) conteniendo 150 mM SDS 25 kV, 20°C	15 min	0.9 – 1.8 μg/L
CRZ, IPZ, SCZ, TRZ, MNZ, TNZ	Agua de pozo y de río (5 mL)	DLLME	**CSEI-sweeping-MEKC-UV** Capilar 'burbuja': 57.2 cm × 50 μm i.d. BGS: 44 mM tampón fosfato (pH 2.5) conteniendo 123 mM SDS y 8% (v/v) THF -30 kV, 20°C	10 min	0.6 – 2.4 μg/L
IPZ, ORZ, SCZ, TRZ, MNZ, TNZ	Huevos (2 g)	SALLE + SPE con cartuchos Oasis®HLB (60 mg)	**CSEI-sweeping-MEKC-UV** Capilar estándar: 70.0 cm × 50 μm i.d. BGS: 44 mM tampón fosfato (pH 2.5) conteniendo 123 mM SDS y 8% (v/v) THF -30 kV, 20°C	12 min	2.1 – 5.0 μg/kg
MNZ, ORZ, TNZ	Orina (7 μL) Suero (200 μL)	Dilución de la orina 143 veces Dilución del suero 76 veces	**CSEI-sweeping-MEKC-UV** Capilar 'burbuja': 65.0 cm × 50 μm i.d. BGS: 44 mM tampón fosfato (pH 2.5) conteniendo 123 mM SDS y 8% (v/v) THF -30 kV, 20°C	13 min	Orina: 0.5 – 1.8 μg/mL Suero: 0.2 – 1.5 μg/mL

Tabla C2 (continuación). Características analíticas de los métodos desarrollados.

Analitos	Muestra	Tratamiento de muestra	Técnica instrumental	Tiempo de análisis (min)	LODs
MNZ-OH, TNZ, HMMNI, MNZ, RNZ, DMZ, TRZ, IPZ-OH, ORZ, IPZ, SCZ	Orina (2 mL)	MISPE con cartuchos SupelMIP®SPE (50 mg)	**CZE-ESI-IT-MS** Capilar estándar: 110 cm × 50 µm i.d. BGS: 1 M ácido fórmico (pH 1.8) 28 kV, 25°C	28 min	2.9 – 39.1 µg/L
MNZ, CRZ, RNZ, TRZ, SCZ, IPZ, DMZ, ORZ	Leche (3 mL)	SALLE + SPE con cartuchos Oasis®HLB (60 mg)	**CEC-UV** Capilar empaquetado C18 (5 µm): 40 cm × 50 µm i.d. Fase móvil: 60:40 (v/v) MeCN/tampón acetato amónico (2.5 mM, pH 5) 27 kV, 30°C	15 min	3 – 29 µg/L
MNZ, CRZ, RNZ, TRZ, SCZ, IPZ, DMZ, ORZ	Orina (0.5 mL)	SPE con cartuchos Oasis®HLB (30 mg)	**CEC-nano-interfase unión líquida-ESI-IT-MS** Capilar empaquetado 3:1 (w/w) C18 bidentada (4.2 µm) /silica (5 µm): 25 cm × 75 µm i.d. Fase móvil: 60:40 (v/v) MeCN/tampón acetato amónico (5 mM, pH 5) 15 kV, presión aplicada al vial de entrada de 11 bares	21 min	0.03 – 0.13 µg/mL
MNZ-OH, TNZ, HMMNI, MNZ, RNZ, DMZ, TRZ, IPZ-OH, ORZ, IPZ, SCZ	Cangrejo, salmón, gamba, nécora (2 g)	SALLE + MIPSE con cartuchos SupelMIP®SPE (50 mg)	**CLC-UV** Zorbax SB-C18 (150 × 0.5 mm, 5 µm) Fase móvil: agua (eluyente A), MeCN (eluyente B) Flujo de la fase móvil: 7 µL/min	22 min	0.9 – 3.2 µg/kg
MNZ, RNZ, TRZ, SCZ, TNZ, ORZ, IPZ, CRZ	Leche (4 mL)	SALLE	**UHPLC-UV** Zorbax Eclipse Pluss RRHD C18 (50 × 2.1 mm, 1.8 µm) Fase móvil: 0.1% (v/v) ácido fórmico (eluyente A), MeCN conteniendo 0.1% (v/v) ácido fórmico (eluyente B) Flujo de la fase móvil: 0.45 mL/min	8 min	2.0 – 4.0 µg/L
MNZ-OH, HMMNI, MNZ, RNZ, DMZ, TRZ, TNZ, SCZ, ORZ, IPZ-OH, IPZ, CRZ	Hueva de merluza (1 g)	SALLE	**UHPLC-ESI-QqQ-MS** Zorbax Eclipse Pluss RRHD C18 (50 × 2.1 mm, 1.8 µm) Fase móvil: 0.025% (v/v) ácido fórmico (eluyente A), MeON (eluyente B) Flujo de la fase móvil: 0.5 mL/min	4 min	0.03 – 2.96 µg/kg

Annex I: List of publications

From the present Thesis, the following publications have been reported:

◊ M. Hernández-Mesa, C. Cruces-Blanco, A.M. García-Campaña, Determination of 5-nitroimidazoles and metabolites in environmental samples by micellar electrokinetic chromatography, Anal. Bioanal. Chem. 404 (2012) 297–305. (**Chapter 4**).

◊ M. Hernández-Mesa, C. Cruces-Blanco, A.M. García-Campaña, Green methodology based on dispersive liquid-liquid microextraction and micellar electrokinetic chromatography for 5-nitroimidazole analysis in water samples, J. Sep. Sci. 36 (2013) 3050–3058. (**Chapter 4**).

◊ M. Hernández-Mesa, A.M. García-Campaña, C. Cruces-Blanco, Novel solid phase extraction method for the analysis of 5-nitroimidazoles and metabolites in milk samples by capillary electrophoresis, Food Chem. 145 (2014) 161–167. (**Chapter 4**).

◊ M. Hernández-Mesa, D. Airado-Rodríguez, C. Cruces-Blanco, A.M. García-Campaña, Novel cation selective exhaustive injection-sweeping procedure for 5-nitroimidazole determination in waters by micellar electrokinetic chromatography using dispersive liquid-liquid microextraction, J. Chromatogr. A 1341 (2014) 65–72. (**Chapter 5**).

◊ M. Hernández-Mesa, D. Airado-Rodríguez, A.M. García-Campaña, C. Cruces-Blanco, Development of an ultrasensitive stacking technique for 5-nitroimidazole determination in untreated biological fluids by micellar electrokinetic chromatography, Electrophoresis 36 (2015) 2538–2541. (**Chapter 5**).

◊ D. Airado-Rodríguez, M. Hernández-Mesa, A.M. García-Campaña, C. Cruces-Blanco, Evaluation of the combination of micellar electrokinetic capillary chromatography with sweeping and exhaustive injection in the determination of 5-nitroimidazoles in egg samples, *(Submitted for publication)*. (**Chapter 5**).

◊ M. Hernández-Mesa, C. Cruces-Blanco, A.M. García-Campaña, Simultaneous determination of eleven 5-nitroimidazoles and metabolites in urine samples by capillary electrophoresis-tandem mass spectrometry using molecular imprinted solid phase extraction, *(Submitted for publication).* (**Chapter 6**).

◊ M. Hernández-Mesa, F.J. Lara, C. Cruces-Blanco, A.M. García-Campaña, Determination of 5-nitroimidazole residues in milk by capillary electrochromatography with packed C18 silica beds, Talanta 144 (2015) 542–550. (**Chapter 8**).

◊ M. Hernández-Mesa, G. D'Orazio, A. Rocco, A.M. García-Campaña, C. Cruces-Blanco, S. Fanali, Capillary electrochromatography-mass spectrometry for the determination of 5-nitroimidazole antibiotics in urine samples, Electrophoresis 36 (2015) 2606–2615. (**Chapter 9**).

◊ M. Hernández-Mesa, D. Moreno-González, C. Cruces-Blanco, A.M. García-Campaña, Selective approach for the determination of 5-nitroimidazoles in aquaculture products by capillary liquid chromatography-UV using molecular imprinted solid phase extraction, *(Submitted for publication).* (**Chapter 11**).

◊ M. Hernández-Mesa, A.M. García-Campaña, C. Cruces-Blanco, Novel approach for 5-nitroimidazole determination in milk samples by UHPLC-UV coupled to salt assisted liquid-liquid extraction as cheap and easy sample treatment, *(In process of drafting).* (**Chapter 12**).

◊ M. Hernández-Mesa, C. Cruces-Blanco, A.M. García-Campaña, High-throughput method for the analysis of 5-NDZ residues in fish roe samples by UHPLC-MS/MS and SALLE as sample treatment, *(In process of drafting).* (**Chapter 13**).

Annex II: List of abbreviations and acronyms

ACE	Affinity capillary electrophoresis
ADI	Acceptable daily intake
AFMC	Analyte focusing by micelle collapse
ANOVA	Analysis of variance
APCI	Atmospheric pressure chemical ionization
APFO	Ammonium perfluorooctanoate
APPI	Atmospheric pressure photoionization
AU	Arbitrary unit
BGE	Background electrolyte
BGS	Background solution
BSA	N,O-bis-(trimethylsilyl)acetamid
CAD	Collision gas
CCE	Chiral capillary electrophoresis
CCα	Decision limit
CCβ	Detection capability
CE	Capillary electrophoresis
CE	Collision energy
CEC	Capillary electrochromatography
CF	Counter-flow
CGE	Capillary gel electrophoresis
CIF	Capillary isoelectric focusing
CITP	Capillary isotachophoresis
CLC	Capillary liquid chromatography
C_{max}	Maximum plasma concentration level
CMC	Critical micellar concentration
CRL	Community Reference Laboratory
CRZ	Carnidazole
CSE	Capillary sieving electrophoresis
CSEI	Cation selective exhaustive injection

CSP	Chiral stationary phase
CXP	Collision cell exit potential
CZE	Capillary zone electrophoresis
DAD	Diode-array detector
DLLME	Dispersive liquid-liquid microextraction
DMF	Dimethylformamide
DMZ	Dimetridazole
DNA	Deoxyribonucleic acid
DP	Declustering potential
d-SPE	Dispersive solid-phase extraction
EC	European Commission
ECD	Electron capture detector
EEA	European Economic Area
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicine Agency
EOF	Electroosmotic flow
EP	Entrance potential
ESA	European Space Agency
ESI	Electrospray ionization
EU	European Union
EURL	European Union Reference Laboratory
FA	Field-amplified
FAB	Fast atom bombardment
FAO	Food and Agriculture Organization of the United Nations
FASI	Field-amplified sample injection
FASS	Field-amplified sample stacking
FESI	Field-enhanced sample injection
GC	Gas chromatography
GCB	Graphitized carbon black
GS1	Nebulizing gas
GS2	Drying gas

HCB	High conductivity buffer
HETP	Height equivalent theoretical plate
HILIC	Hydrophilic interaction liquid chromatography
HLB	Hydrophilic-lipophilic balance
HMMNI	Hydroxyl-dimetridazole
HPLC	High performance liquid chromatography
HSLC	High speed liquid chromatography
i.d.	Inner diameter
I_{ion}	Identification ion
IPZ	Ipronidazole
IPZ-OH	Hydroxyl-ipronidazole
IS	Ion spray voltage
IT	Ion trap
LC	Liquid chromatography
LCB	Low conductivity buffer
LIT	Linear ion trap
LLE	Liquid-liquid extraction
LOD	Limit of detection
Log P	Partition coefficient
LOQ	Limit of quantification
LVSS	Large-volume sample stacking
m/z	Mass/charge ratio
ME	Matrix effect
MEEKC	Microemulsion electrokinetic chromatography
MEKC	Micellar electrokinetic chromatography
MIP	Molecular imprinted polymer
MISPE	Molecular imprinted solid phase extraction
MNZ	Metronidazole
MNZ-OH	Hydroxyl-metronidazole
M_r	Relative mass
MRL	Maximum residue level
MRM	Multiple reaction monitoring

MRPL	Minimum required performance limit
MS	Mass spectrometry
MSS	Micelle to solvent stacking
Mw	Molecular-weight
NACE	Non-aqueous capillary electrophoresis
NDZ	Nitroimidazole
NSM	Normal stacking mode
o.d.	Outer diameter
ODS	Octadecylsilane
ORZ	Ornidazole
OT	Open-tubular
pCEC	Pressure-assisted capillary electrochromatography
PE	Overall process efficiency
PEEK	Polyetheretherketone
PFOA	Perfluorooctanoic acid
pKa	Acid dissociation constant
PMMA	Polymethyl methacrylate
PSA	Primary-secondary amine
Q	Single quadrupole
Q_{ion}	Quantification ion
QqQ	Triple quadrupole
QuEChERS	Quick, easy, cheap, effective, rugged, and safe
R	Recovery
R^2	Regression coefficient
RASFF	Rapid Alert System for Food and Feed
RF	Response function
RNZ	Ronidazole
RP	Reverse phase
rpm	Revolution per minute
RSD	Relative standard deviation
S/N	Signal to noise ratio
SALLE	Salt-assisted liquid-liquid extraction

SASLE	Salt-assisted solid-liquid extraction
SBSE	Stir bar sorptive extraction
SCZ	Secnidazole
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEF	Sensitivity enhancement factor
SEM	Scanning electron microscopy
SIM	Selected ion monitoring
SLE	Solid-liquid extraction
SPE	Solid phase extraction
SRM	Selected reaction monitoring
STP	Sewage treatment plant
TBAB	Tetrabutylammonium bromide
TCA	Trichloroacetic acid
TEA	Triethylamine
TEM	Temperature of ion source
THF	Tetrahydrofuran
tITP	Transient isotachophoresis
TLC	Thin layer chromatography
TMS	Trimethylsilyl
TNZ	Tinidazole
ToF	Time of flight
Tris	Tris(hydroxymethyl)aminomethane
TRZ	Ternidazole
UHPLC	Ultra-high performance liquid chromatography
UK	United Kingdom
USA	United States of America
UV/Vis	Ultraviolet/visible
WHO	World Health Organization

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