UNIVERSIDAD DE GRANADA

FACULTAD DE CIENCIAS

DEPARTAMENTO DE QUÍMICA ANALÍTICA

AVANCES EN EL ANÁLISIS DE BENZIMIDAZOLES EN MUESTRAS ALIMENTARIAS, MEDIOAMBIENTALES Y CLÍNICAS

TESIS DOCTORAL

CARMEN TEJADA CASADO

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AVANCES EN EL ANÁLISIS DE BENZIMIDAZOLES

EN MUESTRAS ALIMENTARIAS, MEDIOAMBIENTALES Y CLÍNICAS

ADVANCES IN THE ANALYSIS OF BENZIMIDAZOLES IN FOOD, ENVIRONMENTAL AND CLINICAL SAMPLES

Por

Carmen Tejada Casado

DEPARTAMENTO DE QUÍMICA ANALÍTICA

UNIVERSIDAD DE GRANADA

MEMORIA presentada para aspirar al Grado de Doctor en Química

(Mención internacional)

Fdo.: Carmen Tejada Casado

Los directores de la Tesis,

Fdo.: Dra. Monsalud del Olmo Iruela

Profesora Titular

Dpto. Química Analítica

Universidad de Granada

Fdo.: Dr. Francisco Jesús Lara Vargas

Investigador Posdoctoral

Dpto. Química Analítica

Universidad de Granada

VISADA en Granada el 30 de Octubre de 2018

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Dña. Ana María García campaña, Catedrática del Departamento de Química Analítica de la Facultad de Ciencias de la Universidad de Granada y responsable del grupo de investigación "Calidad en Química Analítica, Alimentaria, Ambiental y Clínica" (FQM302)

CERTIFICA:

Que el trabajo que se presenta en esta TESIS DOCTORAL, con el título de **"AVANCES EN EL ANÁLISIS DE BENZIMIDAZOLES EN MUESTRAS ALIMENTRIAS, MEDIOAMBIENTALES Y CLÍNICAS"**, ha sido realizado en los laboratorios del citado grupo bajo la dirección de la Profesora Monsalud del Olmo Iruela y el Investigador Posdoctoral Francisco Jesús Lara Vargas, 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 "Doctorado Internacional", dentro del programa de Doctorado en Química.

Fdo.: Dra. Ana Mª García Campaña

En Granada a 30 de Octubre de 2018

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Directores de la Tesis

Doctoranda

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Fdo.: Francisco Jesús Lara Vargas

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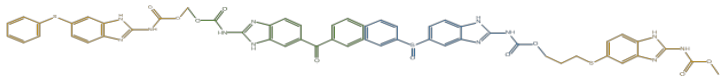
AGRADECIMIENTOS



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Agradecimientos

Hace ya más de 5 años que comencé a trabajar en el grupo FQM 302 con una beca de colaboración. En esos momentos ni me planteaba que algún día podría llegar a ser Doctora en Química Analítica. Pues bien, hoy es el día tan ansiado de la defensa de la Tesis Doctoral y es por ello que quiero mostrar mis agradecimientos a todas las personas que han estado acompañándome durante este camino.

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A mi madre, mi padre, mi hermana y Pablo

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"Conserva tus sueños, nunca sabes cuándo te harán falta"

"Keep your dreams, you never know when you will need"

(*La sombra del viento*)

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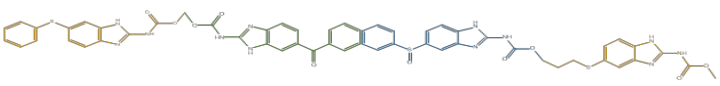

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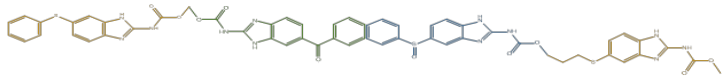
OBJETO DE LA MEMORIA



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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 veterinarios pertenecientes a la familia de los benzimidazoles (BZs) en matrices alimentarias, ambientales y clínicas. Para ello, en esta Tesis, se han empleado técnicas de separación miniaturizadas, como la electroforesis capilar (CE), electrocromatografía capilar (CEC) y la cromatografía líquida capilar (CLC), y de ultra alta eficacia, como es la cromatografía líquida de ultra resolución (UHPLC), así como la espectrometría de movilidad iónica (IMS). Todas ellas se han acoplado a diferentes técnicas de detección como son la espectrofotometría ultravioleta-visible (UV-Vis), la espectroscopia de fluorescencia (FL) y la espectrometría de masas (MS).

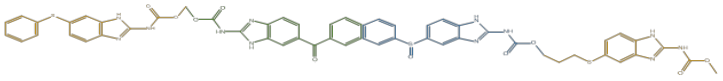
Así mismo, en esta Tesis se proponen métodos alternativos a los ya existentes para el tratamiento de muestras de diversos alimentos, aguas de distinta procedencia y fluidos biológicos de origen animal, más eficaces y respetuosos con el medio ambiente, de acuerdo con las nuevas tendencias de la Química verde.

Como objetivos concretos de esta Tesis Doctoral destacan los siguientes:

- Emplear la CLC para la determinación de BZs y sus metabolitos en alimentos, como alternativa a la cromatografía líquida de alta eficacia (HPLC) convencional.
- Explorar las ventajas de la UHPLC-FL, como una herramienta muy selectiva para la identificación de estos residuos en muestras alimentarias.
- Demostrar la potencialidad del acoplamiento CE-MS para el control de estos residuos en muestras alimentarias y en fluidos biológicos de origen animal respectivamente, 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 BZs y sus metabolitos en aguas naturales empleando técnicas de preconcentración off-line y on-line que mejoren la sensibilidad inherente a esta técnica.
- Conocer y emplear la IMS acoplada a MS para la caracterización de un amplio rango de fármacos de uso humano y veterinario, empleando la sección transversal de colisión (CCS) como parámetro de identificación junto con la masa exacta.
- Proponer nuevos tratamientos de muestra más simples, rápidos y respetuosos con el medio ambiente para la determinación de BZs y sus metabolitos, como alternativa a los ya existentes y evaluarlos en diferentes tipos de muestras alimentarias, aguas naturales y fluidos biológicos de origen animal, con objeto de demostrar su validez en cuanto a la selectividad y sensibilidad requeridas en este tipo de análisis.

GOALS OF THIS THESIS



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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 family of BZs in food, environmental and clinical samples. For this purpose, in this Thesis, miniaturized separation techniques have been used, such as capillary electrophoresis (CE), capillary electrochromatography (CEC) and capillary liquid chromatography (CLC). Moreover, a high efficiency technique such as ultra-high performance liquid chromatography (UHPLC), as well as ion mobility spectrometry (IMS). These techniques have been coupled to different detection techniques such as ultraviolet-visible (UV-Vis) spectrophotometry, fluorescence spectroscopy (FL) and mass spectrometry (MS).

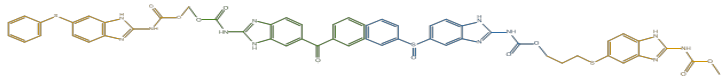
Likewise, this Thesis proposes alternative, more efficient and environmental-friendly sample treatments for the analysis of BZs residues in different foods, waters and animal body fluids according to the current trends in Green Chemistry.

The following specific goals of this Thesis must be remarked:

- To apply CLC for the determination of BZs and their metabolites in foods, as an alternative to conventional high-performance liquid chromatography (HPLC).
- To explore the advantages of UHPLC-FL, as a very selective tool for the identification of these residues in food samples.
- To study the potential of CE-MS hyphenation for the control of these residues in food samples and in animal body fluids respectively, considering the identification capacity of the selected detection.
- Proposal of an efficiency procedure for packing capillaries and their further application in CEC. This goal includes the evaluation of the potential of this technique coupled to UV detection for the determination of BZs and their metabolites in natural waters using off-line and on-line preconcentration techniques that improve the inherent sensitivity to this technique

- To know and use IMS coupled to MS for the characterization of a wide range of human and veterinary drugs, using the collision cross section (CCS) as an identification parameter together with the exact mass.
- Proposal of new, simple, quick and environmental-friendly sample treatments for the determination of BZs, including their metabolites, as alternative to the previously reported methods. This includes the evaluation of these sample treatments in different matrices with the aim of demonstrating their suitability in terms of selectivity and sensitivity.

RESUMEN



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En esta Tesis se han desarrollado diferentes métodos para la determinación de residuos de BZs y otros fármacos de origen veterinario y humano en matrices alimentarias, ambientales y clínicas. En el caso de los alimentos, la legislación vigente establece para estos compuestos unos Límites Máximos de Residuos (MRLs) que no deben ser superados con objeto de garantizar la calidad del producto y permitir su distribución y consumo. Por otro lado, la incorporación constante en el medioambiente de estos residuos sin el debido control está provocando serios problemas en los ecosistemas receptores que obligan al desarrollo de metodologías para su cuantificación y posterior evaluación de riesgos. Además, con el fin de seleccionar la dosis apropiada de un fármaco que evite riesgos innecesarios en la salud del animal, también es necesario el uso de métodos analíticos para el control de los principios activos de estos fármacos en fluidos biológicos.

Considerando las recientes e importantes mejoras de las técnicas separativas en cuanto a la miniaturización, aumento de la eficacia, alta sensibilidad, elevada resolución y bajo consumo de disolventes y muestra, en esta Tesis Doctoral se propone la utilización de la CE y la cromatografía líquida (LC), en sus diversas modalidades, además de la IMS, con objeto de explorar las ventajas mencionadas. Junto con la detección UV/Vis y la detección FL, a lo largo de esta Tesis se han considerado la MS debido a las ventajas que aporta como sistema de detección, tales como la identificación inequívoca de los analitos. Además, se ha utilizado IMS acoplada a MS ya que proporciona información complementaria, como es la sección transversal de colisión (CCS). Es importante señalar que en esta Tesis Doctoral se presentan, por primera vez los sistemas CEC-UV y CE-MS/MS como poderosas herramientas para la determinación de BZs y sus metabolitos. Además, es la primera vez que muchos de estos residuos y otros fármacos de uso humano y veterinario han sido caracterizados en términos de CCS haciendo uso de la IMS-MS. Además, se han propuesto 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.

A continuación se describe brevemente el trabajo desarrollado:

- En el **Capítulo 1**, se describe un método simple para la determinación de dieciséis BZs en diferentes muestras de leche mediante CLC-UV como técnica separativa y extracción líquido-líquido asistida por sales (SALLE) como técnica de tratamiento de muestra.
- En el **Capítulo 2**, se propone un nuevo método para la determinación de trece BZs en muestras de pescado. Para este propósito se empleó UHPLC-FL como técnica separativa y SALLE para el tratamiento de muestra.
- En el **Capítulo 3**, se llevó a cabo el desarrollo de un nuevo método para la determinación de doce BZs, empleando la electroforesis capilar zonal acoplado a la espectrometría de masas en tándem (CZE-MS/MS). Con el fin de mejorar la sensibilidad del método se llevó a cabo una preconcentración dentro del capilar (on-line). El método fue aplicado a diferentes tipos de carne haciendo uso de la micro-extracción líquido-líquido dispersiva (DLLME).
- En el **Capítulo 4**, se propuso un nuevo método para la determinación de trece BZs, haciendo uso de la cromatografía electrocinética micelar acoplada a la espectrometría en masas en tándem (MEKC-MS/MS) y empleando un surfactante volátil como electrolito de fondo. Para aumentar la sensibilidad del método se aplicó como técnica de preconcentración dentro del capilar (on-line) el denominado sweeping. Este método fue aplicado al análisis de muestras de orina animal haciendo una simple dilución de la muestra.
- En el **Capítulo 5**, se describe un nuevo método para la determinación de siete BZs en muestras de agua de diferente procedencia mediante CEC-UV, utilizando capilares de sílice fundida empaquetados en el laboratorio. Para aumentar la sensibilidad del método se aplicó una preconcentración dentro del capilar (on-

line) inyectando la muestra en un disolvente con menor fuerza de elución que la fase móvil, aplicando también una preconcentración previa (off-line) de los analitos mediante la DLLME.

- Finalmente, en el **Capítulo 6**, se recoge el trabajo desarrollado durante la estancia predoctoral en el "Laboratorire d'etude des residus et contaminants dans les aliments" (LABERCA, Francia), que consistió en la creación de una base de datos con los valores de CCS obtenidos mediante IMS-MS de 92 fármacos humanos y veterinarios. Con el fin de evaluar la aplicabilidad de esta base de datos, once fármacos veterinarios fueron analizados en orina de oveja.

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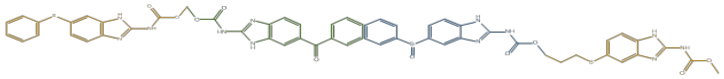
SUMMARY



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In this Thesis, different methods for the determination of BZs residues and other human and veterinary drugs in food, environmental and clinical matrices have been developed. In the case of food, current legislation establishes maximum residues limits (MRLs) for these compounds, which must not be exceeded in order to guarantee the quality of the product and allow its distribution and consumption. On the other hand, the constant incorporation of these residues into the environment without the proper control it is causing serious problems in the receiving ecosystems. Thus, it is required the development of methodologies for their quantification and subsequent risk assessment. In addition, in order to select the appropriate drug dose that avoids unnecessary risks in the animal health, it is also necessary the use of analytical methods for proper therapeutic drug monitoring.

Considering these problems and taking into account the last technical advances in terms of miniaturization, increased efficiency, high sensitivity, high resolution and low consumption of solvents and sample, different miniaturized separation techniques such as capillary electrophoresis (CE), capillary electrochromatography (CEC) and capillary liquid chromatography (CLC) as well as ultra-high performance liquid chromatography (UHPLC) and ion mobility spectrometry (IMS) have been assessed. In addition to ultraviolet-visible detection (UV-Vis) and fluorescence detection (FL), mass spectrometry (MS) has been considered through this Thesis due to its advantages such as the unequivocal identification of the analytes. Moreover, IMS coupled to MS has also been used as it provides complementary information to MS, the so-called collision cross section (CCS). It is important to note that this Doctoral Thesis presents for the first time CEC-UV and CE-MS/MS systems as a powerful tool for the determination of BZs and their metabolites. In addition, it is also the first time that most BZs and many human and veterinary drugs have been characterized in terms of CCS, using IMS-MS. Moreover, alternative sample treatments have been proposed, making possible an increased efficiency and sample throughput.

As a summary, the Thesis compiles the following works:

- In **Chapter 1**, a simple method for the determination of sixteen BZs in different milk samples by CLC-UV as separation technique and salting-out assisted liquid-liquid extraction (SALLE) as sample treatment technique was proposed.
- In **Chapter 2**, a new method for the determination of thirteen BZs in fish samples was carried out. For this purpose, UHPLC-FL was used as separation technique and SALLE as sample treatment.
- In **Chapter 3**, the development of a new method for the determination of twelve BZs using capillary zone electrophoresis coupled to tandem mass spectrometry (CZE-MS/MS) was proposed. In order to improve the sensitivity of the method, an on-line preconcentration technique based on stacking was carried out. Moreover, dispersive liquid-liquid microextraction (DLLME) was also considered as a sample treatment for the analysis of different types of meat, which also allowed an off-line preconcentration.
- In **Chapter 4**, a new method for the determination of thirteen BZs using micellar electrokinetic chromatography coupled to tandem mass spectrometry (MEKC-MS/MS) and using a volatile surfactant as background electrolyte was proposed. In order to increase the sensitivity of the method, the so-called sweeping was applied as an on-line preconcentration technique. This method was applied to the analysis of animal urine samples using “dilute and shoot” as a sample treatment.
- In **Chapter 5**, a new method for the determination of seven BZs in water samples using CEC-UV was described. The fused silica capillaries were packed in the laboratory. In order to increase the sensitivity of the method, different preconcentration techniques were proposed, such as an on-line preconcentration, in which the sample is injected in a solvent with lower elution strength than the mobile phase, and an off-line preconcentration by a sample treatment based on DLLME.

- Finally in **Chapter 6**, the work carried out in the "Laboratorire d'etude des residus et contaminants dans les aliments" (LABERCA, France) during the predoctoral stay is described. It consisted in the creation of a database of the CCS as a complementary parameter to characterize ninety-two human and veterinary drugs, using IMS-MS. In order to evaluate the applicability of this database, eleven veterinary drugs were analyzed in sheep urine.

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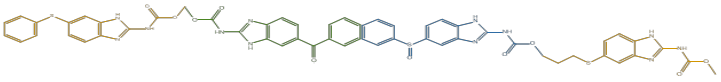
INTRODUCTION



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1. Residues of pharmaceutical compounds in food and environmental samples.

Human health is related to the environment and the quality and nature of the food among other things [1]. Quality of food from animal products is widely concerning public health agencies around the world since pharmaceutical compounds, mainly veterinary medicines have played an important role in the field of animal husbandry. Food-producing animals may be treated with veterinary drugs to prevent or cure disease. **Figure 1** shows the proportion of the total sales for food-producing species, expressed as mg sold per population correction unit (mg/PCU) of different veterinary medicines, in the 30 European countries, in 2015 [2]. As can be observed in **Figure 1** for all 30 countries, the sales of tetracyclines, penicillins and sulfonamides, in mg/PCU, accounted for 69.6 % of the total sales in 2015.

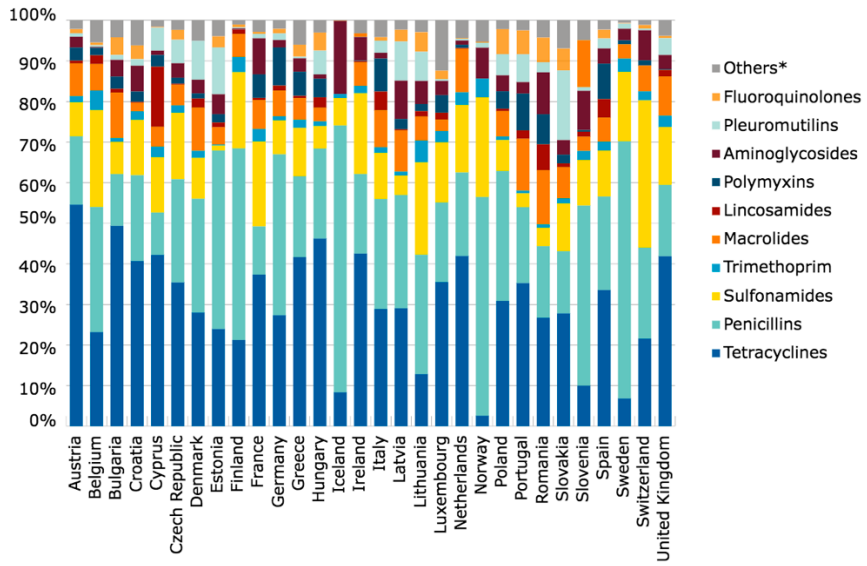


Figure 1. Proportion of the total sales for food-producing species, in mg/PCU, of different veterinary medicines, in the 30 European countries, in 2015. *Amphenicols, cephalosporins, other quinolones and other antibacterials [2].

[1] EPA research program overview, Science to protect public health and the environment, (2019).
[2] EMA, Sales of veterinary antimicrobial agents in 19 EU/EEA countries in 2010: Second ESVAC report, Ema. (2014) 74.

However, these substances may leave residues including parent compounds, their metabolites and/or transformation products in the food from treated animals. There are many factors influencing the occurrence of residues in animal products such as drug's properties and their pharmacokinetic characteristics, physicochemical or biological processes of animals and their products. The most likely reason for drug residues could be due to improper drug usage and failure to keep the withdrawal period [3].

In the last few years, the public concern about the utilization of pharmaceuticals in food-producing animals and their misuse in humans has increased due to the transfer of antimicrobial or anthelmintic resistance to man or hypersensitivity reaction, carcinogenicity, mutagenicity, teratogenicity, and disruption of intestinal normal flora among others [3,4]. Therefore, like other contaminants and residues, their determination in complex matrices (meat, egg, milk, honey, etc.) at trace levels is also of extreme importance and poses a health hazard to the consumer. In this sense, the control of pharmaceutical residues in edible animal tissues is of vital importance.

On the other hand, residues of pharmaceuticals, including veterinary drugs, pesticides, human residues, and personal care products, have experienced a fast-growing interest as emerging pollutants. Recent studies have shown that a multitude of drugs are present in aquatic systems, incorporated from a variety of sources, including discharges from domestic wastewater treatment plants and pharmaceutical companies, run-off from animal feeding operations, infiltration from aquaculture activities, or from compost made of animal manure containing

[3] T. Beyene, Veterinary drug residues in food-animal products: its risk factors and potential effects on public health, J. Vet. Sci. Technol. 07 (2015) 1–7.
[4] D. Moreno-González, F.J. Lara, M. del Olmo-Iruela, A.M. García-Campaña, Trends in multiresidue analysis, 2015.

drugs [5,6]. Moreover, some of their metabolites may have high stability and mobility in the environment. Therefore, it is very important to assess their fate in order to assess the environmental risks associated with the release of pharmaceuticals [7]. The main pathways followed by the pharmaceutical residues to enter the environment are shown in **Figure 2**.

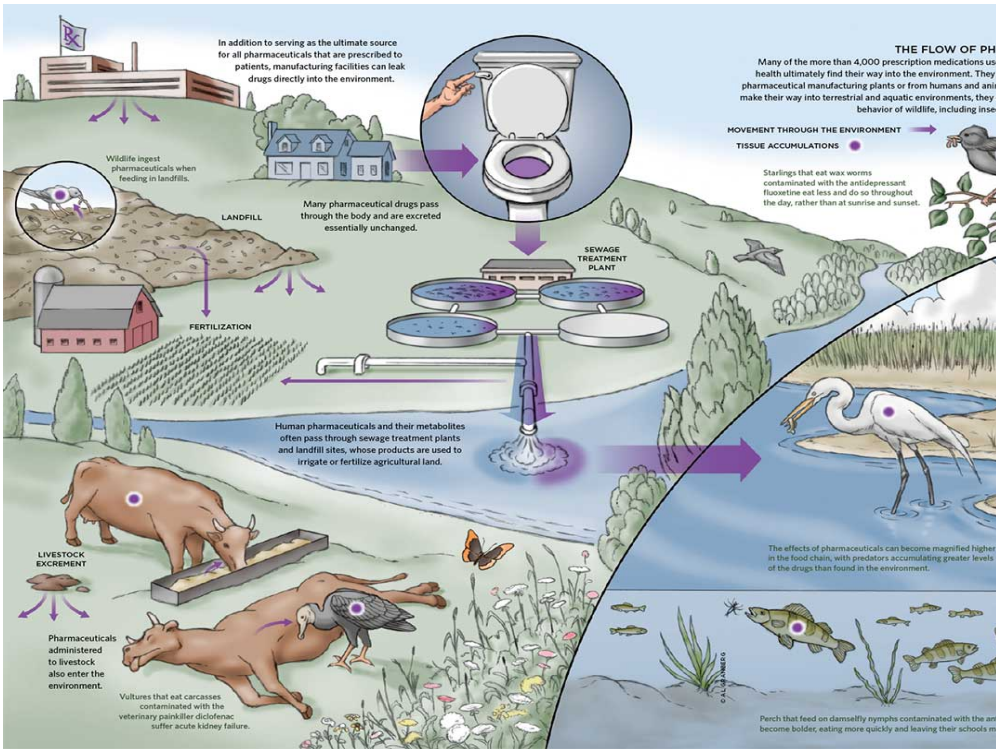


Figure 2. Mayor pathways of pharmacoceutical realease into the environment. Reproduced from [8].

[5] Y. Yang, Y.S. Ok, K.H. Kim, E.E. Kwon, Y.F. Tsang, Occurrences and removal of pharmaceuticals and personal care products (PPCPs) in drinking water and water/sewage treatment plants: A review, Sci. Total Environ. 596–597 (2017) 303–320.

[6] F. Kaczala, S.E. Blum, the occurrence of veterinary pharmaceuticals in the environment: a review, Curr. Anal. Chem. 12 (2016) 169–182.

[7] M. Gros, S. Rodríguez-Mozaz, D. Barceló, Fast and comprehensive multi-residue analysis of a broad range of human and veterinary pharmaceuticals and some of their metabolites in surface and treated waters by ultra-high-performance liquid chromatography coupled to quadrupole-linear ion trap tandem mass spectrometry, J. Chromatogr. A. 1248 (2012) 104–121.

[8] Website of AL GRANBERG: http://www.algranberg.com/index.html/ Accessed on 5 June 2018.

1.1 Legal framework

The global concern about food safety and environment quality has produced the development of a legal framework to control residues of organic compounds (e.g. pesticides and veterinary and human drugs). In this context, 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 [9]. Nonetheless, some substances are found in food as a result of intentional use. This definition refers to residues of pesticides in food of plant and animal origin and veterinary drugs in food of animal origin. It must be remarked that residues of veterinary drugs include the parent compounds and/or their metabolites in animal edible food, and also include residues of associated impurities of the veterinary drug concerned [9].

As was stated in the above section, the presence of pharmaceutical compounds in foodstuff of animal origin can involve a risk to human health. For this reason, the sanitary authorities have launched many legislative actions in order to guarantee the food safety. Within European Union (EU) countries, Regulation of European Commission (EC) No178/2002 [10] 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

[9] Codex Alimentarius Commission. Procedural manual. Joint FAO/WHO food standard program, 21 th edition (2013).
[10] Regulation (EC) No 178/2002 of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety, Off. J. Eur. Communities L31 (2002) 1-24.

the free movement of food and feed within the EU [11]. The main objective of this regulation is to ensure a high level of protection for consumers while also considering the protection and wellness of animal, plant heath and the environment. It also establishes the European Food Safety Authority (EFSA) which is an independent scientific authority that gives advice and technical support for Community legislation in all fields having a direct or indirect impact on food and feed safety. Also, it provides independent information on these matters and communicates on risks. Moreover, Regulation (EC) No 178/2002 establishes the Rapid Alert System for Food and Feed (RASFF). It notifies about any direct or indirect risk to public health due to food or feed and involves EU national food safety authorities, EC, EFSA, European Free Trade Association (EFTA) Surveillance Authority, Norway, Liechtenstein, Switzerland and Iceland [12].

Additionally, Regulation (EC) No 726/2004 [13] establishes the Community procedures for authorization, pharmacovigilance and supervision of medical products for human and veterinary use. Moreover, the structure and the goals of the European Medicines Agency (EMA) have been laid down by this regulation. EMA is responsible for the protection of public and animal health through the scientific evaluation and supervision of medical products for human and veterinary use [11]. Thus, only those pharmacologically active substances that have received a favorable evaluation by EMA can be used in food-producing animals. Furthermore, EMA provides scientific advice on the use of veterinary drugs in food-producing animals in order to minimize the occurrence of this residues. It also advices on the MRLs of

[11] R. Companyo, M. Granados, J. Guiteras, M.D. Prat, Antibiotics in food: Legislation and validation of analytical methodologies, Anal. Bioanal. Chem. 395 (2009) 877–891.
[12] Website source: http://ec.europa.eu/food/safety/rasff/index_en.htm. Accessed on 5 June 2018.
[13] Regulation (EC) No 726/2004 of 31 March 2004 of 31 March 2004 laying down Community procedures for the authorization and supervision of medical products for human and veterinary use and establishing a European Medicines Agency, Off. J. Eur. Union L136 (2004) 1-33.

medicinal products which may be accepted in foodstuffs of animal origin. Thus, MRLs for food of animal origin can be defined as 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 [14].

On the other hand, the EU Directive 96/23/CE [15] 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 of Directive 96/23/CE. A summary of substances per category is presented in **Table 1.**

Table 1. Annex I. 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 [16]	(1) Antibacterial substances, including sulfonamides, quinolones, β-Lactams, macrolides, quinolones and tetracyclines. (2) Other veterinary drug (a) Anthelmintic including BZs (b) Anticoccidials (c) Carbamates and pyrethroids (d) Sedatives (e) Non-steroidal anti-inflammatory drugs (NSAIDs) (f) Other pharmacologically active substances

[14] Website of European Medicines Agency: http://www.ema.europa.eu/ema/. Accessed on 5 June 2018.

[15] Council Directive 96/23/EC of 29 April 1996 on measures to monitor certain substances and residues thereof in live animals and animal products and repealing. Directives 85/358/EEC and 86/469/EEC and 91/664/EEC, Off. J. Eur. Communities (1996) 10-32.

[16] Council Regulation (EEC) No 2377/90 of 26 June 1990 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin, Off. J. Eur. Communities L224 (1990) 1–8.

GROUP A Substances having anabolic effect and unauthorized substances	Group B Veterinary drugs * and contaminants
	(3) Other substances and environmental contaminants
	(a) Organochlorine compounds including PCSs
	(b) Organophosphorus compounds
	(c) Chemical elements
	(d) Mycotoxins
	(e) Dyes
	(f) Others

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

In addition, MRLs for veterinary drugs in food has been recently revised by Regulation (EU) No 37/2010 [17]. 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.

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 [18], including additional requirements for confirmatory methods by introducing the concept of identification points (IPs) in order to achieve unambiguous identification of the monitored legislated residues. Three identification points are required for the identification of group B substances whereas four identification points are claimed for the unequivocal determination of group A substances (see **Table 1**).

[17] Commission regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits

[18] Commission Decision (2002/657/EC) of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, Off. J. Eur. Communities L221 (2002) 8–36.

In this regard, MS detection coupled to LC, gas chromatography (GC) or CE is the most adequate analytical tool for the unequivocal confirmation of these residues. This Commission Decision 2002/657/EC assigns one identification point to the precursor ion and one and half identification points to the transition products when low resolution MS is used. Therefore, in this case, one precursor ion and two transition products or only two transition products are required for the unequivocal identification of group B substances such as BZs. In this commission 2002/657/EC, FL as detection technique can be also used for unequivocal confirmation of group B substances. This approach can be achieved by the use of selective sample treatment which can remove most of the interferences from the sample. Moreover, this practice in combination with an adequate chromatographic separation coupling with FL detection could be a good option to fulfill these objectives.

On the other hand, in relation to quality standard for surface and groundwater, regulations and legislations have been proposed through the EU Water Framework Directive [19] and the Ground Water Directive [20], which involve water monitoring programs for the control of pollutant residues, including pesticides and new target compounds such as some veterinary medicines [21].

Therefore, legal requirements concerning the quality control of foods and environmental waters in relation to the determination of residues, require the development of satisfactory analytical performances of the proposed methods in terms of sensitivity, selectivity, resolution, identification, high number of simultaneously monitored analytes, analysis time, sample throughput, and

[19] European Commission, 'Commission Decision (EEC) 2000/60/EC', Off. J. Eur. Commun., L327, 1 (2000).
[20] European Commission, 'Directive (EEC) 2006/118/EC', Off. J. Eur. Commun., L372, (2006) 19
[21] R. Loos,G.Locoro, S. Comero, S.Contini, D. Schwesig, F. Werres, P. Balsaa, O. Gans, S.Weiss, L. Blaha,M. Bolchi, B.M. Gawlik, 'Pan-European survey on the ocurrence of selected polar organic persistent pollutants in ground water', Water Res., 44, (2010) 4115–4126.

applicability in different matrices such as high water content foods, fatty foods, dry food, wastewater, groundwater, among others [4].

2. Benzimidazoles

Benzimidazoles (BZs) are anthelmintic agents widely used in the prevention and treatment of parasitic infections in agriculture and aquaculture [22]. Also, some BZs are used as fungicidal agents for control of a wide range of fungi affecting field crops, stored fruit and vegetables [23]. BZs were introduced at the beginning of the 60s. Thiabendazole (TBZ) was the first BZ to be commercialized over 50 years ago and it has been used broadly for control of lungworms, gastrointestinal nematodes and as fungicidal agent [24]. After its introduction, other BZs with similar activity than TBZ reached the market, such as mebendazole (MBZ) [25], oxibendazole (OXI) [26], cambendazole (CAM) [27] and parbendazole (PAR) [28]. Later, at the end of the 70s, new BZs with sulphide and sulphoside functional groups, providing a broad-

[22] A. Loyacano, J. Williams, J. Gurie, A. DeRosa, Effect of gastrointestinal nematode and liver fluke infections on weight gain and reproductive performance of beef heifers, Vet. Parasitol. 107 (2002) 227–234.
[23] M. Danaher, H. De Ruyck, S.R.H. Crooks, G. Dowling, M. O'Keeffe, Review of methodology for the determination of benzimidazole residues in biological matrices, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 845 (2007) 1-37.
[24] H.D. Brown, A.R. Matzuk, I.R. Ilves, L.H. Peterson, S.A. Harris, L.H. Sarett, J.R. Egerton, J.J. Yakstis, W.C. Campbell, A.C. Cuckler, Antiparasitic drugs. IV. 2-(4'-thiazolyl)-benzimidazole, a new anthelmintic, J. Am. Chem. Soc. 83 (1961) 1764–1765.
[25] J.P. Brugmans, D.C. Thienpont, I. Van Wijngaarden, O.F. Vanparijs, V.L. Schuermans, H.L. Lauwers, Mebendazole in enterobiasis radiochemical and pilot clinical study in 1,278 subjects, JAMA J. Am. Med. Assoc. 217 (1971) 313–316.
[26] V.J. Theodorides, J. Chang, C.J. DiCuollo, G.M. Grass, R.C. Parish, G.C. Scott, Oxibendazole, a new broad spectrum anthelmintic effective against gastrointestinal nematodes of domestic animals, Br. Vet. J. 129 (1973) 97.
[27] D.R. Hoff, M.H. Fisher, R.J. Bochis, A new broad-spectrum anthelmintic: 2-(4-Thiazolyl)-5-isopropoxycarbonylamino-benzimidazole, Experienta 26 (1970) 550–551.
[28] P. Actor, E.L. Anderson, C.J. DiCuollo, R.J. Ferlauto, J.R.E. Hoover, J.F. Pagano, L.R. Ravin, S.F. Scheidy, R.J. Stedman, V.J. Theodorides, New broad spectrum anthelmintic, methyl 5(6)-butyl-2-benzimidazolecarbamate, Nature, 215 (1967) 321.

spectrum were introduced. Albendazole (ABZ) [29], fenbendazole (FBZ) [30] and fenbendazole-sulfoxide (FBZ-SO) [31] were the first BZs used against all growth stages of gastrointestinal nematodes. They could also be used in the treatment of tapeworms, lungworms and adult stages of liver fluke. Then, triclabendazole (TCB) was introduced as an anthelmintic agent for combating all stages of liver fluke, nonetheless it is an ineffective agent against nematodes [32]. However, the low solubility of BZs sulfosides and sulfides leads to their low absorption into the gut, resulting in low bioavailability [33]. Other BZs are known as pro-benzimidazoles because, shortly after being administered and already in the body of the host, they are transformed (especially in the liver) into a BZ which provides the anthelmintic efficacy. Thus, BZs such as netobimin (NETO) [34] and febantel (FEB) [35] which are pro-drugs of ABZ and FBZ, respectively, possess higher solubility in water resulting in increased absorption and enhanced bioavailability. There are also other similar

[29] V.J. Theodorides, R.J Gyurik, W.D. Kingsbury, R.C. Parish, Anthelmintic activity of albendazole against liver flukes, tapeworms, lung and gastrointestinal roundworms, Experienta 32 (1976) 702-703.

[30] C. Baeder,H. Bahr,O. Crist, D. Duwel, H.Kellner, R. Kirsch, H. Loewe, E. Schultes, E. Schutz, H.Westen, Fenbendazole: A new, highly effective anthelmintic ,Experienta 30 (1974) 753-754.

[31] E.A. Averkin, C.C. Beard, C.A. Dvorak, J.A. Edwards, J.H. Fried, J.G. Kilian, R.A. Schiltz, T.P. Kistner, J.H. Drudge, E.T. Lyons, M.L. Sharp, R.M. Corwin, Methyl 5(6)-Phenylsulfinyl-2-benzimidazolecarbamate, a New, Potent Anthelmintic, J. Med. Chem. 18 (1975) 1164–1166.

[32] J.C. Boray, P.D. Crowfoot, M.B. Strong, J.R. Allison, M. Von Schel- lenaum, M. Orelli, G. Sarasin, Treatment of immature and mature Fasciola hepatica infections in sheep with triclabendazole, Vet. Rec. 112 (1983) 315.

[33] C.E. Lanusse, R.K. Prichard, Relationship between pharmacological properties and clinical efficacy of ruminant anthelmintics, Vet. Parasitol. 49 (1993) 123–158.

[34] P. Delatour, M.C. Cure, E. Benoit, F. Garnier, Netobimin (Totabin-SCH): preliminary investigations on metabolism and pharmacology, (1986) 230–234.

[35] P. Delatour, J. Euzeby, Communautes structurale, metabolique et anthelminthique entre febantel, fenbendazole et oxfendazole, Le Point Vétér, 15 (1983) 63.

pro-benzimidazoles widely used as fungicidal agents, such as thiophanate-methyl (TM) and benomyl (BEN), which are precursors of carbendazim (CBZ) [36].

2.1. Structure and physicochemical properties of Benzimidazoles

BZs have the common structure showed in **Figure 3.**

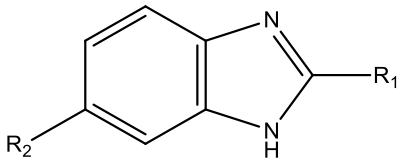


Figure 3. General structure of BZs

They can be classified as TBZ analogues and BZs carbamates. Substitution of side-chains on the parent BZ nucleus (**Figure 3**) produces the individual members. Newer BZ carbamates are characterized by novel substituents on the BZ nucleus and the replacement of the thiazole ring by methylcarbamate. It has led to a new generation of BZs with higher potencies, slower rates of elimination and broader activity spectra [23].

BZs are composed of a benzene ring fused to an imidazole ring at the positions 4 and 5. They are slightly basic, due to the imidazole nitrogen being able to accept a proton. However, when compared to imidazole, BZs are slightly weaker bases [37]. The benzene ring can help to delocalize the electrons of the imidazole nitrogen through a variety of resonance conformations. Conjugation increases stability of the

[36] M. Nakamura, Y. Furumi, F. Watanabe, K. Mizukoshi, M. Taniguchi, S. Nemoto, Determination of carbendazim, thiophanate, thiophanate-methyl and benomyl residues in agricultural products by liquid chromatography-tandem mass spectrometry, Shokuhin Eiseigaku Zasshi. 52 (2011) 148–155.

[37] G. Jerez, G. Kaufman, M. Prystai, S. Schenkeveld, K.K. Donkor, Determination of thermodynamic pKa values of benzimidazole and benzimidazole derivatives by capillary electrophoresis, J. Sep. Sci. 32 (2009) 1087–1095.

BZs and decreases the proton affinity of the imidazole nitrogen [38]. It is well known that the molecule could be protonated (pKa~3-6) or deprotonated (pKa~10-12) because the imidazole ring contains both acidic and basic nitrogen atoms, as can be seen in **Figure 4**.

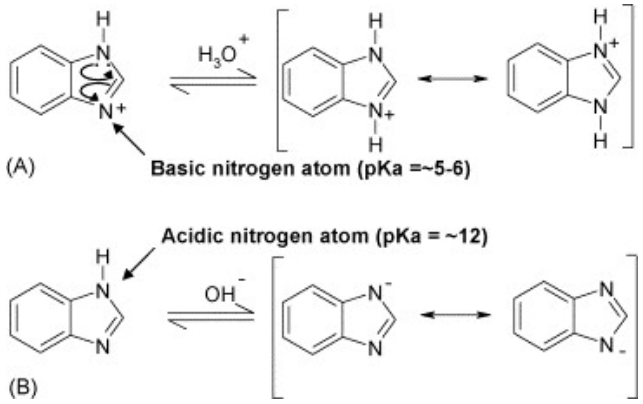
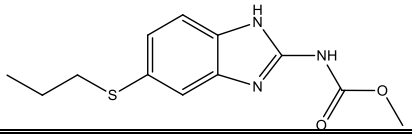


Figure 4. Ionization of benzimidazole molecule under acidic (A) and basic (B) conditions [23]

The physical and chemical properties of the BZs studied during the Doctoral Thesis, such as molecular weight (Mw), acid dissociation constant (pKa) or partition coefficient (log P) are shown below (Data obtained from SciFinder Scholar).

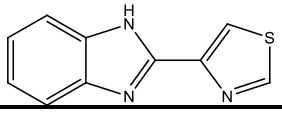
[38] P.N. Preston, Chemistry of heterocyclic compounds, Benzimidazoles, 40 (1981).

Albendazole (ABZ)



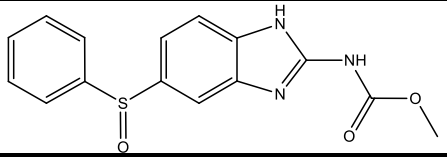
Compound identification	Chemical and physical properties
IUPAC name: Methyl 5-(propylthio)-2-benzimidazolecarbamate	**Mw:** 265.31 g/mol
	pKa1: 5.54 **pKa2:** 13.11
Chemical formula: $C_{12}H_{15}N_3O_2S$	**log P:** 3.0
CAS registry number: 54965-21-8	**Solubility:** $7.2x10^{-3}$ g/L at pH 7

Thiabendazole (TBZ)



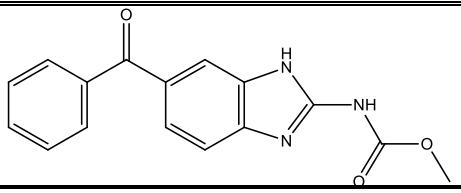
Compound identification	Chemical and physical properties
IUPAC name: 2-(4 Thiazolyl)benzimidazole	**Mw:** 201.25 g/mol
Chemical formula: $C_{10}H_7N_3S$	**pKa1:** 5.22 **pKa2:** 12.83
CAS registry number: 148-79-8	**log P:** 2.5
	Solubility: 0.060 g/L at pH 7

Fenbendazole-sulfoxide (FBZ-SO)



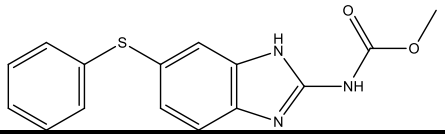
Compound identification	Chemical and physical properties
IUPAC name: Methyl 5-(phenylsulfinyl)-benzimidazol-2-carbamate	**Mw:** 315.35 g/mol
	pKa1: 4.13 **pKa2:** 11.79
Chemical formula: $C_{15}H_{13}N_3O_3S$	**log P:** 2.16
CAS registry number: 53716-50-0	**Solubility:** 0.027 g/L at pH 7

Mebendazole (MBZ)



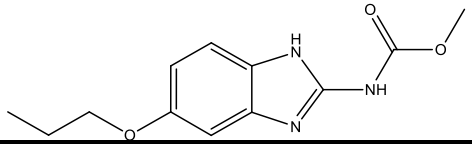
Compound identification	Chemical and physical properties
IUPAC name: 5-Benzoyl-2-benzimidazolecarbamic acid methyl ester	**Mw:** 295.30 g/mol **pKa1:** 4.13 **pKa2:** 1.79
Chemical formula: $C_{16}H_{13}N_3O_3$	**log P:** 2.83
CAS registry number: 31431-39-7	**Solubility:** $4.1x10^{-3}$ g/L at pH 7

Fenbendazole (FBZ)



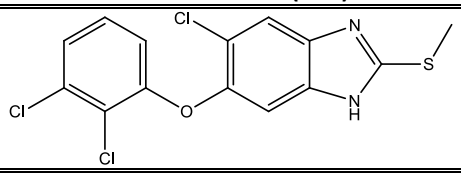
Compound identification	Chemical and physical properties
IUPAC name: Methyl 5-(phenylthio)-2-benzimidazolecarbamate	**Mw:** 299.35 g/mol **pKa1:** 5.12 **pKa2:** 12.72
Chemical formula: $C_{15}H_{13}N_3O_2S$	**log P**: 2.4
CAS registry number: 43210-67-9	**Solubility:** $9.0x10^{-3}$ g/L at pH 7

Oxibendazole (OXI)



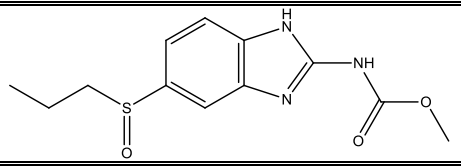
Compound identification	Chemical and physical properties
IUPAC name: Methyl (5-propoxy-1*H*-benzimidazol-2-yl) carbamate	**Mw:** 249.27 g/mol **pKa1:** 6.26 **pKa2:** 13.78
Chemical formula: $C_{12}H_{15}N_3O_3$	**log P**: 2.5
CAS registry number: 20559-55-1	**Solubility:** 0.027 g/L at pH 7

Triclabendazole (TCB)



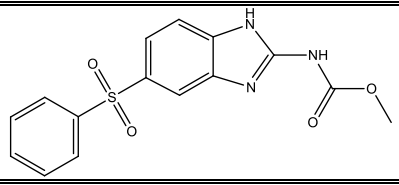
Compound identification	Chemical and physical properties
IUPAC name: 5-Chloro-6-(2,3-dichlorophenoxy)-2-(methylthio)-1*H*-benzimidazole	**Mw:** 359.65 g/mol **pKa1:** 5.31 **pKa2:** 12.91 **log P**: 5.4
Chemical formula: $C_{14}H_9Cl_3N_2OS$	**Solubility:** $2.3x10^{-4}$ g/L at pH 7
CAS registry number: 68786-66-3	

Albendazole-sulfoxide (ABZ-SO)



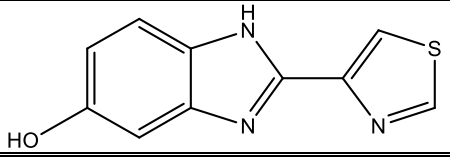
Compound identification	Chemical and physical properties
IUPAC name: Methyl [5-(propane-1-sulfinyl)-1*H*-benzoimidazol-2-yl]-carbamate	**Mw:** 281.33 g/mol **pKa1:** 5.69 **pKa2:** 13.25 **log P**: 0.7
Chemical formula: $C_{12}H_{15}N_3O_3S$	**Solubility:** 0.12 g/L at pH 7
CAS registry number: 54029-12-8	

Fenbendazole sulfone (FBZ-SO_2)



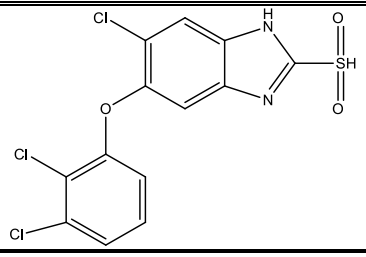
Compound identification	Chemical and physical properties
IUPAC name: 5-Benzenesulfonyl-1*H*-benzoimidazol-2-yl) carbamic acid methyl ester	**Mw:** 331.35 g/mol **pKa1:** 3.41 **pKa2:** 11.12 **log P**: 1.6
Chemical formula: $C_{15}H_{13}N_3O_4S$	**Solubility:** $4.3x10^{-3}$ g/L at pH 7
CAS registry number: 54029-20-8	

5-Hydroxy-Thiabendazole (5-OH-TBZ)



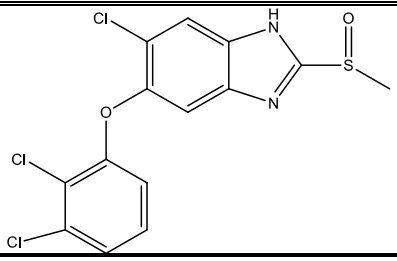
Compound identification	Chemical and physical properties
IUPAC name: 2-(4-Thiazolyl)-5-benzimidazolol	**Mw:** 217.25 g/mol **pKa1:** 3.78 **pKa2:** 8.76
Chemical formula: $C_{10}H_7N_3OS$	**log P**: 1.8
CAS registry number: 948-71-0	**Solubility:** 0.13 g/L at pH 7

Triclabendazole sulfone (TCB-SO_2)



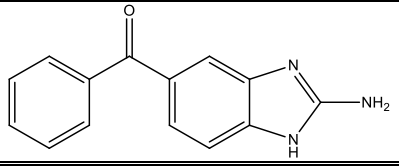
Compound identification	Chemical and physical properties
IUPAC name: 5-Chloro-6-(2,3-dichlorophenoxy)-2-(methylsulfonyl)-1*H*-benzimidazole	**Mw:** 391.66 g/mol **pKa1:** 1.86 **pKa2:** 9.99
Chemical formula: $C_{14}H_9Cl_3N_2O_3S$	**log P**: 5.3
CAS registry number: 106791-37-1	**Solubility:** $8.2x10^{-4}$ g/L at pH 7

Triclabendazole sulfoxide (TCB-SO)



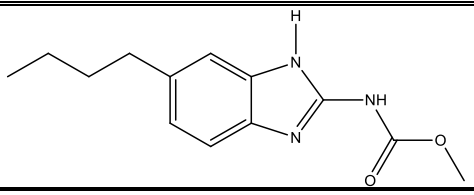
Compound identification	Chemical and physical properties
IUPAC name: 5-Chloro-6-(2,3-dichlorophenoxy)-2-(methylsulfinyl)-1*H*-benzimidazole	**Mw:** 375.66 g/mol **pKa1:** 3.00 **pKa2:** 10.95
Chemical formula: $C_{14}H_9Cl_3N_2O_2S$	**log P**: 4.3
CAS registry number: 100648-13-3	**Solubility:** $1.1x10^{-3}$ g/L at pH 7

Mebendazole-amine (MBZ-NH_2)



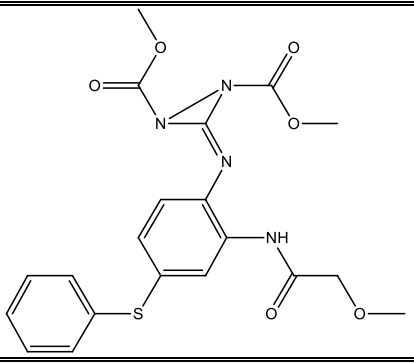
Compound identification	Chemical and physical properties
IUPAC name: 2-Amino-5-benzoyl-1*H*-benzimidazole	**Mw:** 2378.26 g/mol **pKa1:** 6.61 **pKa2:** 13.89
Chemical formula: $C_{14}H_{11}N_3O$	**log P**: 2.7
CAS registry number: 52329-60-9	**Solubility:** 0.047 g/L at pH 7

Parbendazole (PBZ)



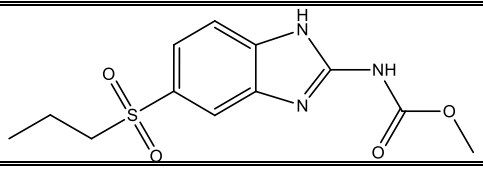
Compound identification	Chemical and physical properties
IUPAC name: Methyl (5-butyl-1*H*-benzoimidazol-2-yl)carbamate	**Mw:** 247.29 g/mol **pKa1:** 5.99 **pKa2:** 13.53
Chemical formula: $C_{13}H_{17}N_3O_2$	**log P**: 3.6
CAS registry number: 14255-87-9	**Solubility:** $7.4x10^{-3}$ g/L at pH 7

Febantel (FBT)



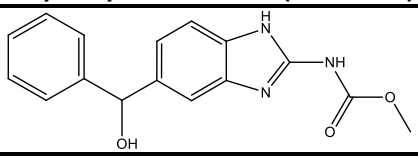
Compound identification	Chemical and physical properties
IUPAC name: *N*-{2-[2,3-Bis-(methoxycarbonyl)-guanido]-5-(phenylthio)-phenyl}-2-methoxyacetamide	**Mw:** 446.48 g/mol **pKa1:** 1.67 **pKa2:** 7.60
Chemical formula: $C_{20}H_{22}N_4O_6S$	**log P**: 1.8
CAS registry number: 58306-30-2	**Solubility:** 0.035 g/L at pH 7

Albendazole sulfone (ABZ-SO_2)



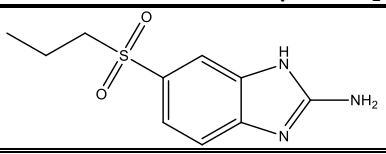
Compound identification	Chemical and physical properties
IUPAC name: *N*-{2-[2,3-Bis-(methoxycarbonyl)-guanido]-5-(phenylthio)-phenyl}-2-methoxyacetamide **Chemical formula:** $C_{20}H_{22}N_4O_6S$ **CAS registry number:** 58306-30-2	**Mw:** 446.48 g/mol **pKa1:** 3.5 **pKa2:**11.20 **log P**: 1.8 **Solubility:** 0.035 g/L at pH 7

5-Hydroxy-Mebendazole (5-OH-MBZ)

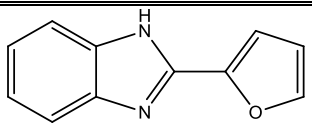


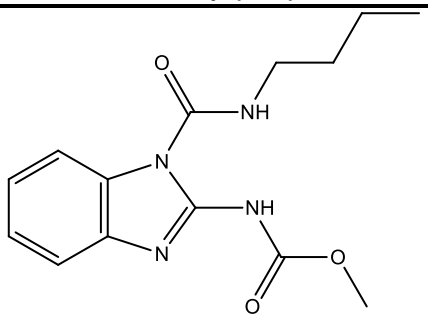
Compound identification	Chemical and physical properties
IUPAC name: Methyl 5-[(RS)-α-hydroxybenzyl]-1H-benzimidazole-2-carbamate **Chemical formula:** C16H15N3O3 **CAS registry number:** 60254-95-7	**Mw:** 297.31 g/mol **pKa1:** 5.60 **pKa2:** 13.16 **log P**: 1.8 **Solubility:** 0.029 g/L at pH 7

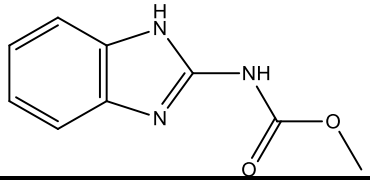
Albendazole-2-amine sulfone (ABZ-NH_2-SO_2)



Compound identification	Chemical and physical properties
IUPAC name: 2-Amino-5-propylsulfonylbenzimidazole **Chemical formula:** $C_{10}H_{13}N_3O_2S$ **CAS registry number:** 80983-34-2	**Mw:** 239.29 g/mol **pKa1:** 5.98 **pKa2:** 13.30 **log P**: 0.9 **Solubility:** 0.31 g/L at pH 7

Fuberidazole (FUB)	
	
Compound identification	**Chemical and physical properties**
IUPAC name: 2-(2-Furanyl)-1*H*-benzimidazole	**Mw:** 184.19 g/mol **pKa1:** 5.00 **pKa2:** 11.20
Chemical formula: $C_{11}H_8N_2O$	**log P**: 1.29
CAS registry number: 3878-19-1	**Solubility:** 0.77 g/L at pH 7

Benomyl (BEN)	
	
Compound identification	**Chemical and physical properties**
IUPAC name: Methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate	**Mw:** 290.32 g/mol **pKa1:** 3.48 **pKa2:** 11.78
Chemical formula: $C_{14}H_{18}N_4O_3$	**log P**: 2.12
CAS registry number: 17804-35-2	**Solubility:** 0.12 g/L at pH 7

Carbendazim (CBZ)	
	
Compound identification	**Chemical and physical properties**
IUPAC name: Methyl 2-benzimidazolecarbamate	**Mw:** 191.19 g/mol **pKa:** 5.52 **pKa2:** 13.09
Chemical formula: $C_9H_9N_3O_2$	**log P**: 1.52
CAS registry number: 10605-21-7	**Solubility:** 0.17 g/L at pH 7

2.2. Mode of action, biological activity and toxicity

Different modes of action have been described for BZs drugs in bibliography. Inhibition of microtube formation has been identified as the primary mode of action. BZs are attached to tubulin, a structural protein of cellular microtubes which are essential organelles in all types of organism (**Figure 5**). Microtubes act on the secretion of most digestive enzymes and BZs inhibit their normal functioning. As a consequence, the digestive process of worms is perturbed, which end up dying due to poor intake [39,40].

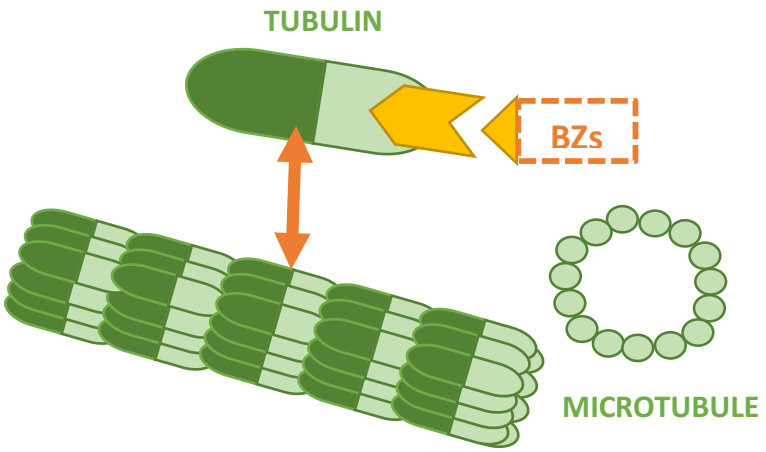


Figure 5. Union of BZs to tubulin protein

Other modes of action have also been investigated. ABZ has been shown to block glucose uptake in the larval and adult stages of susceptible parasites by depleting their glycogen reserves and in this way decreasing ATP formation. Blocking of glucose uptake was also proven to be a mechanism of action for MBZ and TBZ

[39] E. Lacey, The role of the cytoskeletal protein, tubulin, in the mode of action and mechanism of drug resistance to benzimidazoles, Int. J. Parasitol. 18 (1988) 885–936.
[40] E. Lacey, Mode of action of benzimidazoles, Parasitol. Today. 6 (1990) 112–115.

[41,42]. Likewise, it has been shown that BZs inhibit the enzyme fumaric acid reductase in the parasite, thus blocking the formation of succinic acid [43]. Consequently, this disturbs the normal operation of the dicarboxylic acid cycle, which replaces the Krebs cycle in anaerobic parasites [44].

With regards to biological activity, a summary of different BZs in the major food-producing animals is shown in **Table 2** [23]**.**

Table 2. Biological activity of BZs in food producing animals

BZs	Parasite	Animal
ABZ	Gastro intestinal (G.I) roundworms, lungworms, tapeworms and adult stage of fluke	Sheep
FBZ	G.I roundworms, lungworms and tapeworms	Sheep and goats
	G.I roundworms and G.I roundworms larva	Horses
	Lungworm	Pigs
FEB	G.I roundworms, lungworms and tapeworms	Sheep
	G.I roundworms	Pigs
MBZ	Lungworms, tapeworms	Sheep
	G.I roundworms, lungworms and tapeworms	Goats
	Gapeworms and G.I roundworms	Poultry
OXI	G.I roundworms	Sheep, horses and Pigs
TBZ	G.I roundworms and lungworms	Sheep
	G.I roundworms and P. equorum	Horses

[41] E. Lacey, J.H. Gill, Biochemistry of benzimidazole resistance, Acta Trop. 56 (1994) 245–262.
[42] P. Köhler, R. Bachmann, Intestinal tubulin as possible target for the chemotherapeutic action of mebendazole in parasitic nematodes, Mol. Biochem. Parasitol. 4 (1981) 325–336.
[43] A. Health, the Fumarate Reductase Reaction of Haemonchus Contortus and the Mode of Action of Some Anthelmintics, 3 (1973) 1–2.
[44] C. Bryant, E. Bennet, The vitally important role of the fumarate reductase system in helminths , and the fact that there is no parallel system in the mammalian host , have long suggested that it may be a particularly vulnerable site for chemotherapeutic attack . The broad-spec, 7 (1983) 281–292.

BZs	Parasite	Animal
TCB	Fluke	Sheep

Regarding toxicity of BZs in food producing animal, they have much more affinity for tubulin in helminth cells than for mammalian cells [45]. This allows, at the therapeutic dose, to kill the worms without harming the host [46]. Moreover, these compounds do not bind to the tissues of the host and are excreted rapidly, between 1 and 4 weeks, depending on the compound and the formulation.

BZs are regarded as safe up to 20 to 30 times the recommended dose. Acute toxicity is difficult to induce with these drugs and LD50 values are almost impossible to define for drugs such as TBZ and FBZ. Reports of acute toxicity of BZs in animals are very limited. The main toxic effect of the BZ compounds involves their teratogenic effect, which was first reported for PAR [47]. Thus, congenital malformations have been observed as a result of administration of FBZ-SO and ABZ during gestation in sheeps [48,49]. Nevertheless, FEB, FBZ, MBZ and OXI do not present teratogenic effect in ewes when they are administered in early gestation [50]. Moreover, OXI has been restricted to use in weaning piglets due to polyploidy was observed in

[45] P. Dustin, Microtubules, second ed., Springer, Berlin, 1984.
[46] A. Desai, T.J. Mitchison, Microtubule polymerization dynamics, Annu. Rev. Cell Dev. Biol. 13 (1997) 83–117.
[47] M. Lapras, J.P. Deschanel, P. Delatour, J. Gastellu, H. Lombard, Accidents teratologiques chez le mouton apres administration de parbendazole, Bull. Soc. Sci. Vet. Med. Comp. 75 (1973) 53.
[48] P. Delatour, J. Debroye, G. Lorgue, D. Courtot, Embryotoxicite experimentale de l'oxfendazole chez le rat et le mouton, Rec. Med.Vet. 153 (1977) 639.
[49] J.A. Bogan, S.E. Marriner, Pharmacodynamic and toxicological aspects of albendazole in man and animals, in: M. Fock (Ed.), Albendazole in helminthi- asis. Royal Society of Medicine International Congress and symposium series no. 61. London, Royal society of medicine, (1984) 13-21.
[50] H. Van Den Bossche, F. Rochet, C. Horig, Mebendazole and related anthelmintics, Adv. Pharmacol. Chemother. 19 (1982) 67.

hamster ovary cells [51]. It was also observed anemia in dogs after prolonged treatment with TBZ [52].

It must be remark that some BZ metabolites are more toxic than the parent drug. This happen in the case of MBZ-OH which has been found to be more embryotoxic than MBZ in rat. FBZ-SO is as well more teratogenic than FBZ in rat [53,54].

2.3. Benzimidazoles resistance

Anthelmintic resistance is defined as a hereditable reduction in the sensitivity of parasite population to the action of a drug [55]. The first reports of anthelmintic resistance were made on farms attached to parasitological research establishments where anthelmintics were often used intensively. Since the early 1980s resistance has been detected among the gastro-intestinal nematode parasites of sheep and goats throughout the world, and large scale surveys have shown that the situation is critical in many Latin American countries, South Africa, Australia and New Zealand [56]. A word map of anthelmintic resistance it is shown in **Figure 6.**

[51] European Agency for the Evaluation of Medicinal Products, EMEA/MRL/196/97-FINAL, 1997

[52] J.P. Seiler, Toxicology and genetic effects of benzimidazole compounds, Mutat. Res. Genet. Toxicol. 32 (1975) 151–167.

[53] D.W. Gottschall, V.J. Theodorides, R. Wang, The metabolism of benzimidazole anthelmintics, Parasitol. Today. 6 (1990) 115–124.

[54] P. Delatour, Some aspects of the teratogenicity of veterinary drugs, Vet. Res. Commun. 7 (1983) 125–131.

[55] S. Geerts, B. Gryseels, Drug resistance in human helminths: Current situation and lessons from livestock, Clin. Microbiol. Rev. 13 (2000) 207–222.

[56] FAO, Module 2. Helminths: Anthelmintic resistance: Diagnosis, management and prevention, Prevention. (2000) 78–118.

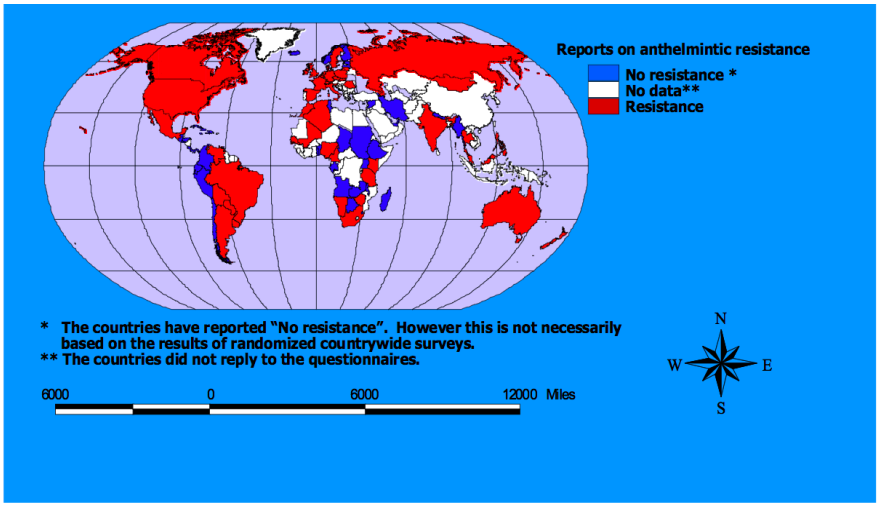


Figure 6. Anthelmintic resistance based on data from Survey of Word Organization Animal Health (OIE) member countries, FAO questionnaires (1998) and literature search (1999) [56].

Table 3 shows resistance of BZs parasites in sheep, goat and horses [55,56].

Table 3. BZs resistance in cattle according

HOST	Parasite
Sheep and goat	*Haemonchus contortus*
	Ostertagia spp
	Trichonstrongylus spp.
	Nematodirus spp.
	Fasciola hepática
Horses	*Cyathostomes*

3. Interest and legislation on the control of benzimidazoles in foodstuffs and presence of these compounds in environmental and clinical samples

As stated above, the use of BZs drugs can lead to the presence of their residues, including their metabolites and degradation products in foodstuff. Nowadays, there are some health alarms which are largely due to the irresponsible use of these

compounds. The presence of these residues in food products may have adverse effects on health, such as congenic malformations, teratogenicity, diarrhea, anemia, pulmonary edemas [57] and anthelmintic resistance among others [55]. Thus, the EU has set MRLs for BZs and their metabolites in animal products. This information is set out in the Commission regulation 37/2010 [17]. **Table 4** shows the MRLs for the different BZs legislated in different animal tissues.

Table 4 MRLs for BZs legislated in different animal tissues according to ref [17].

BZ	Marker residue	Animal Species	MRL	Target Tissues
ABZ	Sum of albendazole sulfoxide, albendazole sulfone, and albendazole 2-amino sulphone, expressed as albendazole	All ruminants	100 µg/kg 100 µg/kg 1000 µg/kg 500 µg/kg 100 µg/kg	Muscle Fat Liver Kidney Milk
FBZ	Sum of extractable residues which maybe oxidized to oxfendazole sulphone	All ruminants, porcine, *Equidae*	50 µg/kg 50 µg/kg 500 µg/kg 50 µg/kg	Muscle Fat Liver Kidney
		All ruminants	10 µg/kg	Milk
FEB	Sum of extractable residues which maybe oxidised to oxfendazole sulphone	All ruminants, porcine, *Equidae*	50 µg/kg 50 µg/kg 500 µg/kg 50 µg/kg	Muscle Fat Liver Kidney
		All ruminants	10 µg/kg	Milk
FBZ-SO	Sum of extractable residues which maybe oxidised to oxfendazole sulphone	All ruminants, porcine, *Equidae*	50 µg/kg 50 µg/kg 500 µg/kg 50 µg/kg	Muscle Fat Liver Kidney
		All ruminants	10 µg/kg	Milk

[57] B. Kinsella, S.J. Lehotay, K. Mastovska, A.R. Lightfield, A. Furey, M. Danaher, New method for the analysis of flukicide and other anthelmintic residues in bovine milk and liver using liquid chromatography-tandem mass spectrometry, Anal. Chim. Acta. 637 (2009) 196–207.

BZ	Marker residue	Animal Species	MRL	Target Tissues
MBZ	Sum of mebendazolemethyl (5-(1-hydroxy, 1-phenyl)methyl-1H-benzimidazol-2-yl) carbamate and (2-amino-1H-benzimidazol-5-yl) phenylmethanone, expressed as mebendazole equivalents	Ovine, caprine, *Equidae*	60 µg/kg 60 µg/kg 400 µg/kg 60 µg/kg	Muscle Fat Liver Kidney
OXI	Oxibendazole	Porcine	100 µg/kg 500 µg/kg 200 µg/kg 100 µg/kg	Muscle Skin and fat Liver Kidney
TBZ	Sum of thiabendazole and 5-hydroxy-thiabendazole	Bovine, caprine	100 µg/kg 100 µg/kg 100 µg/kg 100 µg/kg 100 µg/kg	Muscle Fat Liver Kidney Milk
TCB	Sum of extractable residues which maybe oxidised to ketotriclabendazole	All ruminants	225 µg/kg 100 µg/kg 250 µg/kg 150 µg/kg	Muscle Fat Liver Kidney

It must be remarked that in relation to fish samples, the EU has not set MRLs for BZs yet. However, most of the aquaculture systems in the world are based on crash cultivation methods, which are characterized by high stock density and volume, and the use of formulated feeds containing antibiotic, anthelmintic, antifungal, and pesticides among other substances which can cause serious health problems in consumers. Thus, in the absence of real data, several studies have demonstrated the presence of some BZs and their metabolites in different fish samples after an oral dose. Sørensen et al. [58] detected FBZ, FBZ-SO and FBZ-SO_2 shortly after administration, in muscle and skin tissues of trout given feed containing FBZ in an

[58] L.K. Sørensen, H. Hansen, Determination of fenbendazole and its metabolites in trout by a high-performance liquid chromatographic method, Analyst. 123 (1998) 2559–62.

aquaculture pilot plant. Shaikh et al. [59] determined ABZ and its three major metabolites in muscle tissue of three fish species sampled in the first day after the administration period. Thus, the presence of BZ residues in this type of samples is one of the key issues for food safety and raises much public concern.

On the other hand, BZ residues can be released into the environment through their presence in manure or their disposal into wastewater systems [60]. Once they reach the environment, these pollutants are transported and distributed into water, sediment, soil, and biota. They are subjected to processes (e.g., biodegradation, and chemical and photochemical degradation) that contribute to their elimination or they react with other compounds in the environment [61]. Furthermore, several studies have reported their presence in wastewaters and natural waters [62,63,64], however, how BZs can be removed from wastewater, aquatic and soil ecosystems surrounding pastures is still scarcely studied [65].

[59] B. Shaikh, N. Rummel, R. Reimschuessel, Determination of albendazole and its major metabolites in the muscle tissues of Atlantic salmon, tilapia, and rainbow trout by high performance liquid chromatography with fluorometric detection, J. Agric. Food Chem. 51 (2003) 3254–3259.

[60] A.B.A. Boxall, D.W. Kolpin, B. Halling-Sørensen, J. Tolls, Are veterinary medicines causing environmental risks?, Environ. Sci. Technol. 37 (2003) 286A–294A.

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[65] R. Podlipná, L. Skálová, H. Seidlová, B. Szotáková, V. Kubíček, L. Stuchlíková, R. Jirásko, T. Vaněk, I, Vokřál, Biotransformation of benzimidazole anthelmintics in reed (Phragmites australis) as a potential tool for their detoxification in environment. Bioresour. Technol. 144 (2013) 216–224.

Finally, therapeutic drug monitoring in veterinary medicine is a useful tool to assess when a treatment has attained therapeutic concentrations of a particular drug depending on the administered dose [66]. Subtherapeutic concentrations of these compounds are not going to be effective and concentration above a certain level can be toxic and cause some negative effects on the animal health [57]. In order to select the proper drug dose that avoids unnecessary risks in the animal health, the use of analytical methods that help to apply the right therapeutic concentrations of these compounds by means of the monitoring of their residues in biological fluids such as urine are needed [67,68].

4. Analytical techniques used for the determination of benzimidazoles in foodstuffs, environmental and clinical samples.

In view of the problem explained above, the development of sensitive, efficient, miniaturized and green analytical methods is required for the determination of BZ drugs in foodstuffs, environmental sources and biological fluids.

4.1. Liquid Chromatography

Liquid chromatography (LC) is the main technique used in routine laboratories. Therefore, during the last years, different analytical methods have been reported for the determination of BZs and their metabolites, using LC coupled to MS

[66] A. Loyacano, J. Williams, J. Gurie, A. DeRosa, Effect of gastrointestinal nematode and liver fluke infections on weight gain and reproductive performance of beef heifers, Vet. Parasitol. 107 (2002) 227–234.

[67] T.A.M. Msagati, M.M. Nindi, P. Bag, Determination of benzimidazole anthelmintic compounds by supported liquid membrane extraction and liquid chromatography, J.Sep.Sci. 24 (2001) 606–614.

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[57,69,70,71,72,73,74], UV detection [75,76,77,78,79] and FL detection [80, 81,82,83]. Although MS is a useful tool, which provides high selectivity, sensitivity, versatility and robustness, its use in routine laboratories could be limited due to its

[69] P. Jedziniak, T. Szprengier-Juszkiewicz, M. Olejnik, Determination of benzimidazoles and levamisole residues in milk by liquid chromatography-mass spectrometry: Screening method development and validation, J. Chromatogr. A. 1216 (2009) 8165–8172.

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[72] H. Sun, Q.W. Yu, H.B. He, Q. Lu, Z.G. Shi, Y.Q. Feng, Nickel oxide nanoparticle-deposited silica composite solid-phase extraction for benzimidazole residue analysis in milk and eggs by liquid chromatography-mass spectrometry, J. Agric. Food Chem. 64 (2016) 356–363.

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[79] D. Chen, Y. Tao, Z. Liu, Z. Liu, L. Huang, Y. Wang, Y. Pan, D. Peng, M. Dai, Z. Yuan, Development of a high-performance liquid chromatography method to monitor the residues of benzimidazoles in bovine milk, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 878 (2010) 2928–2932.

high cost. In addition, the analytical performances of these methods could be affected by matrix effect when electrospray ionization is used [84]. So, other less expensive alternatives such as LC coupled to UV detection are usually implemented for monitoring the presence of BZs and other contaminant residues. UV detection presents some advantages including low operating cost, simplicity and provides qualitative and quantitative information. Although BZs are LC-UV amenable, most published papers deal with a relatively low number of compounds [23] or they require long analysis time (80 min) [79]. Besides, downsizing the flow rates in LC may provide significant benefits in terms of sensitivity and lower solvent consumption [85], which is in agreement with green chemistry recommendations [86]. In this scenario, CLC, which is characterized for columns with internal diameters (i.d.) of 0.1–0.5 mm and flow rates up to 20 µL/min, has been successfully implemented in

[80] R. Halko, C.P. Sanz, Z.S. Ferrera, J.J.S. Rodriguez, Determination of benzimidazole fungicides by HPLC with fluorescence detection after micellar extraction, Chromatographia 60 (2004) 151–156.

[81] N. Rummel, I. Chung, B. Shaikh, Determination of albendazole, febendazole, and their metabolites in mouse plasma by high performance liquid chromatography using fluorescence and ultraviolet detection, J. Liq. Chromatogr. Relat. Technol. 6076 (2011) 2211-2223.

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[85] A. Uclés Moreno, S. Herrera López, B. Reichert, A. Lozano Fernández, M.D. Hernando Guil, A.R. Fernández-Alba, Microflow liquid chromatography coupled to mass spectrometry-an approach to significantly increase sensitivity, decrease matrix effects, and reduce organic solvent usage in pesticide residue analysis, Anal. Chem. 87 (2015) 1018–1025.

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food analysis [87,88,89] being possible to use a relatively low sensitive detector as UV/Vis to determine these compounds. Thus, in **Chapter 1**, it has been developed a green and simple multiresidue method using CLC with UV-diode array detection (DAD) for the determination of sixteen BZs and its metabolites in milk samples [90].

Another option for the determination of BZs after a LC separation is the use of FL detection, which can be highly selective and much more sensitive than UV and less expensive than LC-MS. Thus, BZs have a common structure containing a bicyclic ring formed by the fusion of benzene and imidazole and two amino groups which can be protonated or deprotonated under different conditions. Some BZs, such as ABZ and its metabolites, TBZ and FUB, present native fluorescence [23]. There are few studies dealing with the determination of BZ compounds by HPLC with FL detection, mainly applied in clinical samples [81,82,91] and environmental water [83,80,92]. To the best of our knowledge only one application of LC-FL has been found for the monitoring of BZ residues in food derived from animals, related to the determination of ABZ and its metabolites in muscle tissues of different fishes [82]. However, the Achilles heel of these methods is the low number of compounds analyzed (4) and the use of two isocratic modes of analysis, one for parent ABZ and

[87] Y. Saito, K. Jinno, T. Greibrokk, Capillary columns in liquid chromatography: Between conventional columns and microchips, J. Sep. Sci. 27 (2004) 1379–1390.
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the other for its metabolites, as they could not be determined in a single run. Consequently, the total analysis time increased to almost 40 minutes. These limitations could be overcome by the use of UHPLC, due to its advantages associated with the use of stationary phase particles smaller than 2.0 µm, allowing an increased efficiency with a shortened analysis time [93], and it is more environmentally friendly than a HPLC system. Therefore, in **Chapter 2**, it has been developed for the first time a method based on UHPLC-FL to determine thirteen BZs in farmed fish samples [94].

4.2. Capillary electrophoresis

CE is an interesting alternative to determine BZs. CE has several advantages in comparison with LC, such as low solvent consumption, low sample volume, high separation efficiency and rapid analysis [95,96, 97,98].

[93] A. Garrido. Frenich, R. Romero-González, M. Aguilera-Luiz, Comprehensive analysis of toxics (pesticides, veterinary drugs and mycotoxins) in food by UHPLC-MS, TrAC - Trends Anal. Chem. 63 (2014) 158–169.
[94] C. Tejada-casado, F.J. Lara, A.M. García-campa, M. Olmo-iruela, Ultra-high performance liquid chromatography with fluorescence detection following salting-out assisted liquid – liquid extraction for the analysis of benzimidazole residues in farm fish samples, 1543 (2018) 58–66.
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[96] M. Castro-Puyana, V. García-Cañas, C. Simó, A. Cifuentes, Recent advances in the application of capillary electromigration methods for food analysis and foodomics, Electrophoresis 33 (2012) 147–167.
[97] A.M. García-Campaña, L. Gámiz-Gracia, F.J. Lara, M. del Olmo-Iruela, C. Cruces-Blanco, Applications of capillary electrophoresis to the determination of antibiotics in food and environmental samples, Anal. Bioanal. Chem. 395 (2009) 967–986.
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For the determination of BZs, UV/Vis is the most common detection system coupled to CE in its different modalities, such as CZE [99,100] and MEKC [101]. The use of MS/MS as a detection system is an alternative to improve sensitivity and selectivity in CE, allowing the unequivocal identification of veterinary drug residues and therefore fulfilling EU regulation requirements [10]. To the best of our knowledge, only two studies about CZE-MS can be found in literature for the determination of these compounds [102,103]. However, these methods cannot allow the unequivocal identification of BZ compounds because no fragmentation experiments (MS/MS) were carried out.

During the last years, several strategies for on-line preconcentration have been developed [104,105] in order to increase the sensitivity in CE. Among them, the simplest one, named normal stacking mode (NSM), has hardly been tested for the determination of BZs [105]. For NSM the sample must be at least ten times less conductive than BGS. A short plug of low conductivity medium (for example water,

[99] X.Z. Hu, M.L. Chen, Q. Gao, Q.W. Yu, Y.Q. Feng, Determination of benzimidazole residues in animal tissue samples by combination of magnetic solid-phase extraction with capillary zone electrophoresis, Talanta 89 (2012) 335–341.
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[102] J. Domínguez-Álvarez, M. Mateos-Vivas, D. García-Gómez, E. Rodríguez-Gonzalo, R. Carabias-Martínez, Capillary electrophoresis coupled to mass spectrometry for the determination of anthelmintic benzimidazoles in eggs using a QuEChERS with preconcentration as sample treatment, J. Chromatogr. A 1278 (2013) 166–174
[103] S. Takeda, K. Fukushi, K. Chayama, Y. Nakayama, Y. Tanaka, S.I. Wakida, Simultaneous separation and on-line concentration of amitrole and benzimidazole pesticides by capillary electrophoresis with a volatile migration buffer applicable to mass spectrometric detection, J. Chromatogr. A 1051 (2004) 297–301.
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[105] Z. Malá, A. Slampova, L. Pantuckova, P. Gebauer, P. Bocek, Contemporary sample stacking in analytical electrophoresis, Electrophoresis 36 (2015) 15–35.

diluted buffer or organic solvent) is sometimes injected before the sample for achieving analyte preconcentration by this strategy [106]. In **chapter 3**, this on-line preconcentration approach has been carried out in a novel method based on CZE-MS/MS for the identification and simultaneous quantification of twelve BZs in meat samples [107].

However, the main drawback of these CZE-MS methods [102,103,107] is the lack of EOF (electroosmotic flow) due to the use of an acid background electrolyte (BGE) which is partially responsible of long analysis time (≈35 min) to obtain the full separation of at least 10 BZs. To improve the analysis time, the use of basic conditions is an alternative as the analytes can be separated under a stronger EOF. However, BZs are neutral in basic conditions [23], making the use of MEKC compulsory. Therefore, another option for determination BZs using CE coupled with MS/MS could be the use of this CE modality. The most commonly anionic surfactant employed in MEKC mode is sodium dodecyl sulfate (SDS) at basic conditions. However, this surfactant is not volatile and can cause ion-source contamination and serious suppression of analyte signals. To overcome this problem, several works have revealed the applicability of ammonium perfluorooctanoate (APFO) as volatile

[106] C.E. Sänger van de Griend, M.C. Breadmore, In-Capillary sample concentration in CE – "This is my analyte, how do I stack?," LGGC North Am. 3 (2014) 174–186.
[107] C. Tejada-Casado, D. Moreno-González, F.J. Lara, A.M. García-Campaña, M. del Olmo-Iruela, Determination of benzimidazoles in meat samples by capillary zone electrophoresis tandem mass spectrometry following dispersive liquid–liquid microextraction, J. Chromatogr. A. 1490 (2017) 212–219.

surfactant [108,109,110,111], producing a micellar medium of separation for neutral analytes, similar to SDS but with excellent results in relation to its high compatibility with MS [112]. On the other hand, in order to improve the sensitivity of the MEKC methods, an on-line preconcentration procedure based on sweeping could be applied which uses as sample solvent a BGE without micelles [113,114]. In **Chapter 4**, a new method based on MEKC-MS/MS has been performed for the identification and simultaneous quantification of thirteen BZs in animal urine using sweeping as on-line preconcentration to improve the sensitivity [115].

Among the CE modes, CEC can also be considered as a powerful separation technique because it combines the high efficiency of CE and the selectivity of LC. In CEC, separation occurs due to interactions between the stationary phase and the

[108] K. Brensinger, C. Rollman, C. Copper, A. Genzman, J. Rine, I. Lurie, M. Moini, Novel CE-MS technique for detection of high explosives using perfluorooctanoic acid as a MEKC and mass spectrometric complexation reagent, Forensic Sci. Int. 258 (2016) 74–79.
[109] S. Akamatsu, T.Mitsuhashi, MEKC–MS/MS method using a volatile surfactant for the simultaneous determination of 12 synthetic cannabinoids, J.Sep.Sci. 37 (2014) 304–307.
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analytes where the mobile phase flows through the stationary phase because of an electric field rather than an applied pressure [116,117]. CEC columns can be open-tubular (OT), continuous bed, also known as monolithic columns, or packed columns. In general, packed columns using C18 particles are the most employed [118], because they offer the highest specific area among all the electrochromatographic columns and therefore, higher sample capacity can be obtained. As a consequence, higher sensitivity and separation reproducibility can be achieved. Besides, CEC packed columns offer the inherent selectivity and reproducibility of commercially available packing materials [119]. The latest achievements related to stationary phases, columns, as well as method optimization have been recently summarized [120]. CEC is an environmentally friendly separation technique as other CE modes, involving a solvent consumption even lower than UHPLC, which reduces the generated waste in comparison with classical LC methods. Besides, lower amount of stationary phase is required for making a CEC column, so operational costs are reduced and more expensive packing materials can be tested. However, the application of CEC methods to residue determination can be limited by the same drawbacks as CE. A lack of concentration sensitivity can be attributed to them, especially when UV detection is used due to the low sample volume injected. Nevertheless, signal sensitivity can be enhanced by injecting the sample in a solvent with lower elution strength than the mobile phase for both,

[116] G.H. Leach, AP-8508A: Capillary Electrochromatography (1999) 1–52.
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hydrodynamic [121] or electrokinetic [122, 123] injection. Although, CEC has demonstrated its applicability for residue determination in food [124] and water samples [125, 126, 127, 128], it has not been evaluated for BZs determination. Therefore, a novel method for the analysis of BZs residues in water samples by CEC-UV, using laboratory-made packed columns was published **(Chapter 5)** [129].

4.3. Ion mobility spectrometry

As mentioned above, analytical techniques play an important role for the characterization of human and veterinary drugs and also for the elucidation of their metabolism behavior.

[121] D.A. Stead, R.G. Reid, R.B. Taylor, Capillary electrochromatography of steroids increased sensitivity by on-line concentration and comparison with high-performance liquid chromatography, J. Chromatogr. A 798 (1998) 259–67.

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[124] G. D'Orazio, M. Asensio-Ramos, C. Fanali, J. Hernández-Borges, S. Fanali, Capillary electrochromatography in food analysis, TrAC Trends Anal. Chem. 82 (2016) 250–267.

[125] A. De Rossi, C. Desiderio, Application of reversed phase short end-capillary electrochromatography to herbicides residues analysis, Chromatographia 61 (2005) 271–275.

[126] F. Ye, Z. Xie, X. Wu, X. Lin, Determination of pyrethroid pesticide residues in vegetables by pressurized capillary electrochromatography, Talanta 69 (2006) 97–102.

[127] C.M. Polcaro, A. Berti, A. De Rossi, C. Desiderio, Analysis of phenylurea herbicides in groundwater by reverse phase capillary electrochromatography, Chromatographia 57 (2003) 623–628.

[128] G. D'Orazio, J. Hernández-Borges, M. Asensio-Ramos, M.Á. Rodríguez-Delgado, S. Fanali, Capillary electrochromatography and nano-liquid chromatography coupled to nano-ESI interface for the separation and identification of estrogenic compounds, Electrophoresis 37 (2016) 356–362.

[129] C. Tejada-Casado, M. Hernández-Mesa, M. del Olmo-Iruela, A.M. García-Campaña, Capillary electrochromatography coupled with dispersive liquid-liquid microextraction for the analysis of benzimidazole residues in water samples, Talanta. 161 (2016) 8–14.

In general LC-MS is the most employed technique to characterize these substances [130, 131, 132, 133]. Nevertheless, such approaches provide incomplete structural information of molecules. Further, the differentiation of isobaric and isomeric compounds may be a challenge when they are not previously resolved by LC [134]. In this context, IMS–MS coupling may be an option to gather additional and complementary structural information of the investigated drugs.

IMS is a rapid gas phase separation technique in which ionized compounds are separated based on their shape, mass and charge in a neutral buffer gas (e.g. He, N_2, CO_2) at atmospheric pressure or reduced atmospheric pressure [135]. In TWIMS and drift tube ion mobility spectrometry (DTIMS), ions travel through the drift gas under the influence of a low electric field according to their rotationally averaged cross-sectional area, also referred as CCS. In this sense, compact ions reach the detector before elongated ions which exhibit greater CCS [136]. The main difference between TWIMS and DTIMS is that, whereas a uniform and continuous electric field is applied in DTIMS,

[130] L. Geis-Asteggiante, A. Nuñez, S.J. Lehotay, A.R. Lightfield, Structural characterization of product ions by electrospray ionization and quadrupole time-of-flight mass spectrometry to support regulatory analysis of veterinary drug residues in foods, Rapid Commun. Mass Spectrom. 28 (2014) 1061–1081.

[131] A. Nuñez, S.J. Lehotay, L. Geis-Asteggiante, Structural characterization of product ions by electrospray ionization and quadrupole time-of-flight mass spectrometry to support regulatory analysis of veterinary drug residues in foods. Part 2: Benzimidazoles, nitromidazoles, phenothiazines, and mectins, Rapid Commun. Mass Spectrom. 29 (2015) 719–729.

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a continuous sequence of voltage pulses is applied in TWIMS creating a traveling electric field that propels the ions towards the exit of the cell [137]. Despite TWIMS and DTIMS are the most widely used IMS forms, other technologies such as differential-mobility spectrometry (DMS) and field-asymmetric waveform ion mobility spectrometry (FAIMS) are also commercially available and their use is growing. In DMS and FAIMS, separation depends on the relation existing between the mobility of ions in high and low electric fields [138]. Unlike in TWIMS and DTIMS, the mobility of ions cannot be related to their CCS when using DMS and FAIMS.

CCS represents the effective area, expressed in $Å^2$, of the ionized molecule that interacts with the neutral buffer gas in the drift cell. In TWIMS and DTIMS instruments, CCS can be related with the mobility of ions by Mason-Schamp equation [139]. In the case of TWIMS, the instrument must be calibrated using compounds with known CCS values before performing CCS measurements [140], while CCS values can be directly measured when using DTIMS. Therefore, when combined with chromatography and MS, IMS provides a third separation dimension related to ion shape, and can allow measuring the CCS of each ion [137]. In this scenario, the hyphenation of IMS with high resolution mass spectrometry (HRMS) has become a relevant technique primarily oriented for the

[137] R. Cumeras, E. Figueras, C.E. Davis, J.I. Baumbach, I. Gràcia, Review on Ion Mobility Spectrometry. Part 1: current instrumentation, Analyst 140 (2015) 1376–1390.
[138] B.M. Kolakowski, Z. Mester, Review of applications of high-field asymmetric waveform ion mobility spectrometry (FAIMS) and differential mobility spectrometry (DMS), Analyst 132 (2007) 842-864.
[139] E.A. Mason, H.W. Schamp, Mobility of gaseous ions in weak electric fields, Ann. Phys. 4 (1958) 233–270
[140] K.M. Hines, J.C. May, J.A. McLean, L. Xu, Evaluation of collision cross section calibrants for structural analysis of lipids by traveling wave ion mobility-mass spectrometry, Anal. Chem. 88 (2016) 7329–7336.

separation and structural characterization of proteins, peptides, oligosaccharides and other biomolecules [141,142,143].

In addition, CCS is an intrinsic physicochemical property of each molecule and may be considered a particular molecular descriptor. Therefore, it could give additional information to retention time and *m/z*, and contribute to the unequivocal identification of the substances analyzed [144]. Direct correlation generally exists between *m/z* and CCS for compounds belonging to the same chemical family or presenting similar chemical structures because both are related parameters [145]. Nevertheless, recent findings have reported CCS databases showing more compact or elongated molecules than could be expected from their *m/z* in comparison to other molecular analogues such as observed for steroids [146]. In such cases, CCS cannot be directly predicted from *m/z*, so the measurement of CCS is expected to provide a complementary useful piece of information for the identification of molecules. Within this framework, CCS could be used for identification proposes in LC-HRMS workflows together with other well established identification criteria such as precursor ion, accurate mass, fragment ion, isotopic pattern and retention time. However, the main limitations for the implementation of CCS as potential identification parameter are the lack of CCS databases in which rely on, especially for contaminants and residues, and the lack of

[141]D.E. Clemmer, R.R. Hudgins, M.F. Jarrold, Naked protein conformations: Cytochrome c in the gas phase, J. Am. Chem. Soc. 117 (1995) 10141–10142.

[142] C. Wu, W.F. Siems, J. Klasmeier, H.H. Hill, Separation of isomeric peptides using electrospray ionization/high- resolution ion mobility spectrometry, Anal. Chem. 72 (2000) 391–395.

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[146] M. Hernández-Mesa, B. Le Bizec, F. Monteau, A.M. García-Campaña, G. Dervilly-Pinel, Collision Cross Section (CCS) database: an additional measure to characterize steroids, Anal. Chem. 90 (2018) 4616-4625.

inter-laboratory studies that would allow validating and accepting the reported CCS values. Only a few CCS databases related to contaminants and residues are currently published and available [134,145,146,147,148,149]. On the other hand, the need of inter-laboratory studies for the integration of IMS and, consequently, the CCS in current LC-MS workflows has recently been highlighted [150].

In this context, in **Chapter 6**, it has been proposed a database of human and veterinary drugs based on TWIMS-derived CCS measurements. Inter-laboratory reproducibility was also checked comparing the CCSs of the other 55 molecules with the values previously reported by CCS database of drug and drug-like compounds [134]. Additionally, the main fragment ions for most compounds have also been characterized for the first time in terms of CCS together with their structure elucidation. Moreover, in order to evaluate the applicability of the proposed database, bovine urine samples fortified with 11 veterinary drugs were analyzed by LC-TWIMS-MS.

5. Sample treatments to determine benzimidazoles in foodstuff, environmental and clinical samples.

Sample treatments are required prior to determination, especially in the residue analysis field because analyte preconcentration is required in addition to sample clean-up. Regarding the sample treatment to be applied for the control of BZs and other pharmaceuticals in different samples, the most relevant points, which have to

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[150] S.M. Stow, T.J. Causon, X. Zheng, R.T. Kurulugama, T. Mairinger, J.C. May, E.E. Rennie, E.S. Baker, R.D. Smith, J.A. McLean, S. Hann, J.C. Fjeldsted, An interlaboratory evaluation of drift tube ion mobility-mass spectrometry collision cross section measurements, Anal. Chem. 89 (2017) 9048–9055.

be taken into account, are the analyte extraction efficiency and the sample throughput.

5.1. Solid phase-extraction

Off-line solid-phase extraction (SPE) is the most used approach for the determination of BZs in foodstuff, environmental and clinical samples. Hereunder, a summary of some SPE methodologies to determine BZs in different samples are shown. Two different types of mixed mode SPE cartridges such as Oasis MCX and Isolute HCX were evaluated for the analysis of 5 BZs in milk and urine [68]. Other off-line SPE procedures using MCX [151] and C18 cartridges [152] have been developed for the extraction of 13 BZs and 6 BZs, respectively, from milk samples. In addition, online SPE employing different alkyl-diol-silica (ADS) cartridges with stationary phases made of alkyl chains of different lengths were evaluated, obtaining the best results with C_4 cartridges for the determination of 11 BZs in milk [153]. Moreover, nickel oxide nanoparticle-deposited silica (SiO_2@NiO) cartridges have also been developed for the determination of 9 BZs in milk and egg [72]. Regarding fish samples, 3 BZs were determine using C18 and CN cartridges in trout [58]. Moreover, SPE Oasis HLB cartridges were used to determine 10 BZs and other veterinary drugs in muscle, liver and kidney [154]. This analytical method offers some advantages

[151] X. Xia, Y. Dong, P. Luo, X. Wang, X. Li, S. Ding, J. Shen, Determination of benzimidazole residues in bovine milk by ultra-high performance liquid chromatography-tandem mass spectrometry, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 878 (2010) 3174–3180.

[152] L. Moreno, F. Imperiale, L. Mottier, L. Alvarez, C. Lanusse, Comparison of milk residue profiles after oral and subcutaneous administration of benzimidazole anthelmintics to dairy cows, Anal. Chim. Acta 536 (2005) 91–99.

[153] D. García-Gómez, M. García-Hernández, E, Rodríguez-Gonzalo & R. Carabias-Martínez (2012). A fast and reliable method for the quantitative determination of benzimidazoles and metabolites in milk by LC-MS/MS with on-line sample treatment. Anal. Bioanal. Chem. 404, 2909–2914.

[154] A. Kaufmann, P. Butcher, K. Maden, M. Widmer, Quantitative multiresidue method for about 100 veterinary drugs in different meat matrices by sub 2-µm particulate high-performance liquid chromatography coupled to time of flight mass spectrometry, J. Chromatogr. A 1194 (2008) 66–79.

such as robustness and high versatility. Nevertheless, they also are relatively tedious, involving several steps to remove the coextracted material from the matrix, with a high solvent consumption. Therefore, during the development of this thesis, other cheaper and quicker alternatives have been carried out such as SALLE, DLLLME or "dilute and shoot".

5.2. Salting out liquid –liquid extraction

Liquid-liquid extraction 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. LLE presents some advantages, including low cost, simplicity, high extraction efficiency and low time consumption. For example, LLE has been applied to determine BZs in different foodstuff such as milk [69], fish [59], and eggs [71] among others. However, it main drawback is the environmental impact of the organic solvent employed. LLE can be modified to extract more efficiently polar organic compounds like BZs with a water-miscible organic solvent (acetonitrile (MeCN), methanol (MeOH) or isopropanol (IPA)) upon addition of an electrolyte such as sodium chloride (NaCl), ammonium sulphate ($(NH_4)_2SO_4$) or magnesium sulfate ($MgSO_4$) to the mixture of solvents, producing a salting-out effect. This so-called salting-out assisted liquid/liquid extraction (SALLE) is a cost-effective, time-efficient, easy-to-use sample preparation method and in agreement with the aim of green analytical chemistry [155]. The general SALLE procedure is shown in **Figure 7.**

[155] R.E. Majors, Salting-out Liquid-Liquid Extraction (SALLE), LCGC North America 27 (2009) 526–533.

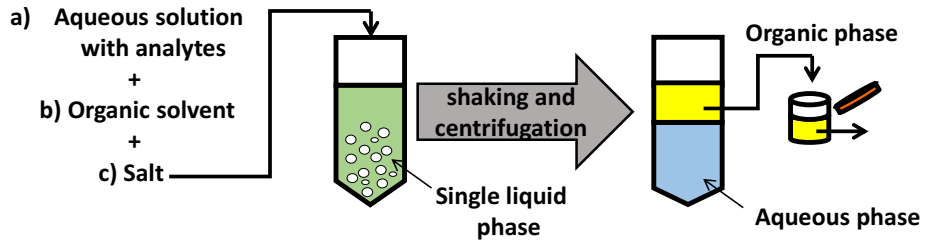

Figure 7. Scheme of SALLE procedure

It must be remarked that, to the best of our knowledge, just one method has been reported for the determination of 4 BZ fungicides in salinity samples employing a SALLE procedure [78]. Thus, during this thesis SALLE has been employed for the determination of 16 BZs in milk samples by CLC-UV (**Chapter 1**) [90] and 13 BZs in fish samples by UHPL-FL (**Chapter 2**) [94].

5.3. Dispersive liquid-liquid microextraction (DLLME)

DLLME is a miniaturized LLE approach that was introduced by Rezaee et al. in 2006 [156]. It involves a ternary component solvent system which usually consists of an aqueous sample, a higher-density extraction solvent (a water immiscible organic solvent such as chlorobenzene, chloroform or dichloromethane) and a dispersive solvent that has to be miscible in both extractant and aqueous sample (such as MeOH, MeCN, or acetone (ACO)) [157]. It is an environmental-friendly method because only a few microliters of the extraction solvent and a low volume of dispersive agent are required. Furthermore, DLLME is a simple and cheap technique that requires short extraction times (a few minutes, including centrifugation stages).

[156] M. Rezaee, Y. Assadi, H. Milani Mohammad-Reza, E. Aghaee, F. Ahmadi, S. Berijani, Determination of organic compounds in water using dispersive liquid-liquid microextraction, J. Chromatogr. A 1116 (2006) 1–9.
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Besides, it provides high recoveries and high enrichment factors. The general DLLME procedure is shown in **Figure 8.**

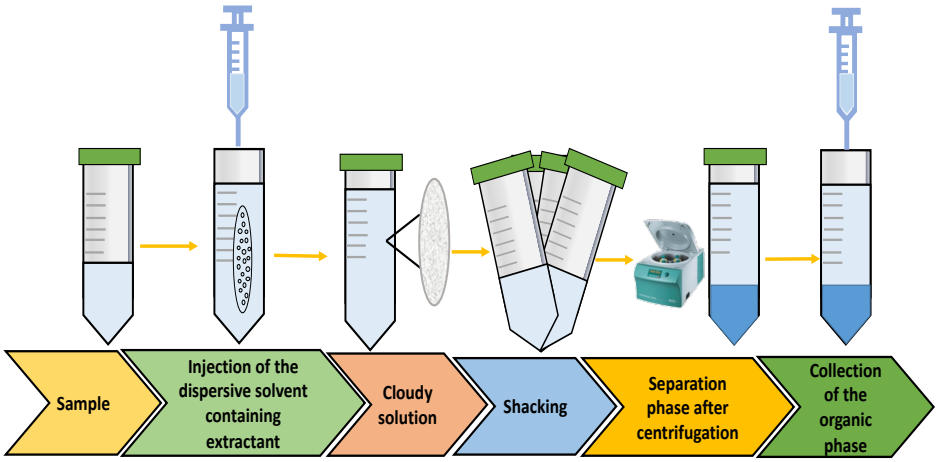

Figure 8. Scheme of DLLME procedure

Due to these advantages, DLLME has been widely applied in the last years for the determination of BZs mainly in water samples [83,158,159] or food with high water content such as tomato [160]. However, these applications include only a few number of these compounds. Therefore, during the development of this thesis DLLME has been employed for the determination of seven BZs in water samples using a novel CEC method (**Chapter 5**) [129]. In addition, DLLME as sample clean up

[158] Y. Santaladchaiyakit, S. Srijaranai, Alternative solvent-based methyl benzoate vortex-assisted dispersive liquid-liquid microextraction for the high-performance liquid chromatographic determination of benzimidazole fungicides in environmental water samples, J. Sep. Sci. 37 (2014) 3354–3361.
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[160] D. Han, B. Tang, M. Tian, K.H. Row, Solid-phase extraction combined with dispersive liquid-liquid microextraction for the determination of three benzimidazole pesticides (carbendazim, thiabendazole, and thiophanate-methyl) in tomatoes, Anal. Lett. 46 (2013) 557–568.

step after solvent extraction with MeCN has been evaluated for the determination of twelve BZs in meat samples by CZE-MS/MS (**Chapter 3**) [107].

5.4. Dilute and shoot

An attractive sample treatment called "dilute and shoot" is a current trend in the field of trace analysis. This procedure consists in a simple sample dilution with a proper solvent in order to reduce sample interferences, avoiding sample clean-up, providing a faster procedure and reducing the cost of labor, reagents and consumables [161]. However, the application of "dilute and shoot" can be limited by the sensitivity of the instrumental technique. For this reason, this sample treatment has been scarcely used in CE [162,163]. However, the use of MS detection and/or the application of stacking procedures could overcome this disadvantage [164,165 ,166,167]. Therefore, in **Chapter 4**, a MEKC-MS/MS method has been developed to determine 13 BZs in urine samples from sheep, cow and goat using

[161] K. Deventer, O.J. Pozo, A.G. Verstraete, P. Van Eenoo, Dilute-and-shoot-liquid chromatography-mass spectrometry for urine analysis in doping control and analytical toxicology, TrAC - Trends Anal. Chem. 55 (2014) 1-13.

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[164] F. Kitagawa, K. Otsuka, Recent applications of on-line sample preconcentration techniques in capillary electrophoresis, J. Chromatogr. A 1335 (2014) 43–60.

[165] M.C. Breadmore, R.M. Tubaon, A.I. Shallan, S.C. Phung, A.S. Abdul Keyon, D. Gstoettenmayr, P. Prapatpong, A.A. Alhusban, L. Ranjbar, H.H. See, M. Dawod, J.P. Quirino, Recent advances in enhancing the sensitivity of electrophoresis and electrochromatography in capillaries and microchips (2012-2014), Electrophoresis 36 (2015) 36–61.

[166] A. Slampova, Z. Mala, P. Gebauer, and P. Bocek. Recent progress of sample stacking in capillary electrophoresis (2014-2016). Electrophoresis 38 (2017) 20-32.

[167] M. C. Breadmore, A. Wuethrich, F. Li, S. C. Phung, U. Kalsoom, J. M. Cabot, M. Tehranirokh, A. I. Shallan, A. S. A. Keyon, H. H. See, M. Dawod, and J. P. Quirino. Recent advances in enhancing the sensitivity of electrophoresis and electrochromatography in capillaries and microchips (2014-2016). Electrophoresis 38 (2017) 33-59.

"dilute and shoot" as sample treatment [115]. In addition, in **Chapter 6**, in order to evaluate the applicability of the proposed CCS database, bovine urine samples with 11 veterinary drugs including 3 BZs were analyzed by LC-TWIMS-MS using as sample treatment "dilute and shoot".

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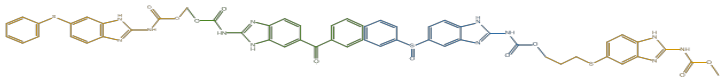
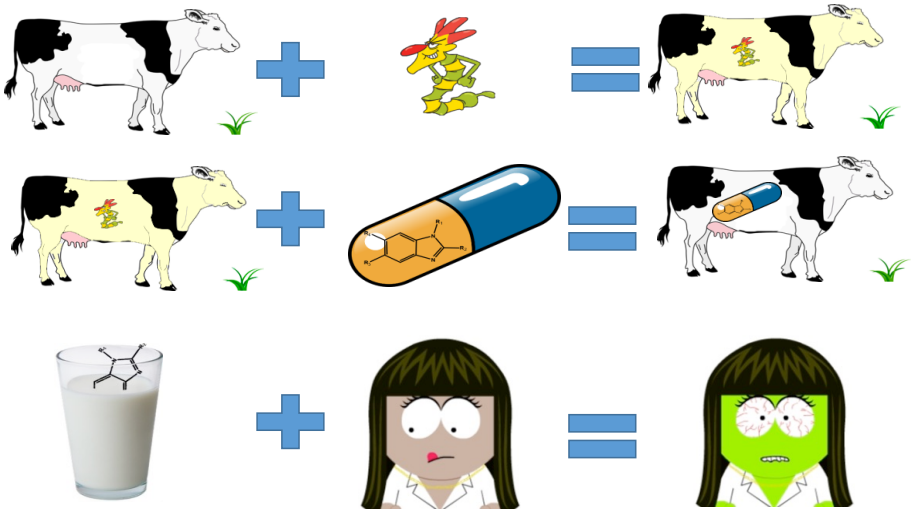
CHAPTER 1



GREEN AND SIMPLE ANALYTICAL METHOD TO DETERMINE BENZIMIDAZOLES IN MILK SAMPLES BY USING SALTING-OUT ASSISTED LIQUID-LIQUID EXTRACTION AND CAPILLARY LIQUID CHROMATOGRAPHY



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1. Summary

A green and simple multiresidue method using CLC with UV-diode array detection (DAD) has been developed for the determination of sixteen BZs and its metabolites in milk samples. The separation was achieved in less than 32 min, using a Zorbax XDB-C18 column (150 mm×0.5 mm i.d, 5 µm), with a mobile phase consisting of 50 mM ammonium acetate (solvent A) and a mixture of MeCN/MeOHl (1:1 v/v) (solvent B), at a flow rate of 9 µL/min. The temperature of the column was 20°C and 6 µL of sample were injected. In spite of the complexity of milk samples, an effective, simple and fast sample preparation method called SALLE was developed for the analysis of these compounds in cow milk samples. To obtain satisfactory extraction efficiencies for the studied analytes, several parameters affecting the SALLE procedure were optimized including the amount of sample, type and volume of the extraction solvent, and the nature and amount of the salt. Good linearity was obtained (R^2> 0.9985 for all BZs) with limits of detection (LOD) between 1.0-2.8 µg kg^{-1}. Relative standard deviations (RSDs) of repeatability and intermediate precision were below 1.6 and 14.2%, respectively. Satisfactory recoveries between 79.1 and 99.6% were also obtained for three types of milk samples (cow, sheep and goat). The advantages of a miniaturized technique such as CLC in terms of better efficiencies and reduced solvent consumption, combined with the simplicity of the SALLE procedure, make this method a useful alternative for the monitoring of these residues at trace level.

2. Materials and methods

2.1 Materials and reagents

Analytical standards of ABZ (99.0%), ABZ-SO_2 (99.3%), ABZ-SO (98.0%), BEN (99.0%), CBZ (99.0%), FBT (99.8%), FBZ (99.5%), FBZ-SO_2 (99.7%), FBZ-SO (99.6%), FUB (99.5%), MBZ (99.6%), MBZ-OH (99.5%), MBZ-NH_2 (99.7%), OXI (99.9%), PBZ (99.6%), TBZ (99.8%), 5-OH-TBZ (99.9%), TCB (99.7%), TCB-SO_2 (99.4%) and TCB-SO (99.8%) were supplied by Sigma-Aldrich (St. Louis, MO, USA). ABZ-NH_2-SO_2 (99.0%) was supplied by Dr. Ehrenstorfer (Augsburg, Germany).

Stock solutions of 1000 µg/mL (FUB, FBT, ABZ-NH_2-SO_2, ABZ-SO, FBZ-SO, MBZ-NH_2, TBZ, 5-OH-TBZ, TCB-SO_2, TCB-SO), 500 µg/mL (TCB, ABZ, BEN, 5-OH-MBZ, PBZ, ABZ-SO_2) and 250 µg/mL (MBZ, FBZ, OXI, CBZ, FBZ-SO_2) were dissolved in MeOH. These stock solutions were stored in amber-colored glass bottles at -20 °C for no less than six months. Intermediate solutions of 10 mg L^{-1} were prepared by diluting stock solutions with water. They were stored in amber-colored bottles at 4°C.

All reagents used in this study were of analytical grade and the solvents were of HPLC grade. MeOH, MeCN, ethyl acetate, IPA, and ACO (LC-MS grade) were obtained from VWR (Center Valley, PA, USA). Ethanol (EtOH) and ammonium acetate (99.9 %) were supplied from Merck (Darmstadt, Germany). Ultrapure water (Milli-Q plus system, Millipore, Bedford, MA, USA) was used throughout the work.

Salts used in the SALLE procedure for aqueous and organic-phase partitions were acquired from different providers: ammonium sulfate ($(NH_4)_2SO_4$) (99.5%) was obtained from VWR (Center Valley, PA, USA); and sodium chloride (NaCl) (> 99%) and magnesium sulfate anhydrous ($MgSO_4$) (96%) were obtained from Panreac (Barcelona, Spain).

Fiberglass syringe filters (0.72 µm x 13 mm) and teflon (PTFE) syringe filters (0.2 µm x 13 mm) (VWR, Center Valley, PA, USA) were used to filter the sample extracts before injection into the LC system.

2.2 Instrumentation

Analysis were performed in a 1200 Series Capillary LC System Agilent Technologies (Waldbronn, Germany) containing an online degasser, capillary pump (maximum flow rate 20 µL min^{-1}), autosampler (8 µL full loop), column thermostat and diode array detector (DAD). Data acquisition and processing were collected with HP ChemStation (version A.09.01) software.

A nitrogen evaporator, System EVA-EC from VLM GmbH (Bielefeld, Germany), a centrifuge Universal 320 R model from Hettich (Leipzig, Germany), and a multi-tube vortexer BenchMixer^{TM} XL model from Edison (New Jersey, USA) were also used.

2.3 Chromatography conditions

BZs separation was carried out in a Zorbax XDB-C18 column (150 mm×0.5 mm i.d, 5 µm) from Agilent Technologies (Waldbronn, Germany), employing a mobile phase consisted of ammonium acetate (solvent A) and a mixture of MeCN/MeOH (50:50 v/v) (solvent B) at a flow rate of 9 µL min^{-1}. Gradient mode was used for the separation of BZs as follows: 30% B (0 min), 40% B (14 min), 70% B (17 min), 95% B (19 min) to 95% B (29 min). Afterwards, mobile phase composition was back to initial conditions in 10 min. In order to guarantee column equilibration and achieve a reproducible and stable separation the initial conditions were maintained for 3 min. The temperature of the column was 20 °C and the DAD monitoring wavelength was set at 290 nm.

2.4 SALLE procedure

Different types of pasteurized and full fat milk (cow, goat and sheep) were purchased from a local supermarket located in Granada (Spain).

SALLE was selected as sample treatment for BZs extraction from milk samples due to its ability to extract polar organic compounds from aqueous matrices following a simple and cheap methodology. Initially, 4 g of milk were placed in a 15-mL conical tube. Two milliliters of MeCN were added and the conical tube was vortexed for 2 min. Subsequently, in the same tube 1.6 g of $(NH_4)_2SO_4$ were added to the sample in order to improve the separation between both, organic and aqueous phase. Then, the tube was shaken by vortex for 2 min and hereunder sample was centrifuged for 10 min at 9000 rpm. After that, 1.5 mL of the upper organic layer were transferred into a 4-mL vial and sample was dried under a nitrogen current at 35 °C. Afterwards, it was re-dissolved in 750 µL of a mixture MeCN/MeOH/H_2O (10:10:80 v/v/v) using vortex agitation for 2 min. The solution was then filtered with a 0.7 µm fiberglass syringe filter (Puradisc, Whatman, USA). Finally, 8 µL of the sample were injected into the CLC system. Taking into account all the steps involved in this procedure, 15 samples could be prepared per hour. The SALLE procedure it is showed in **Figure 1**.

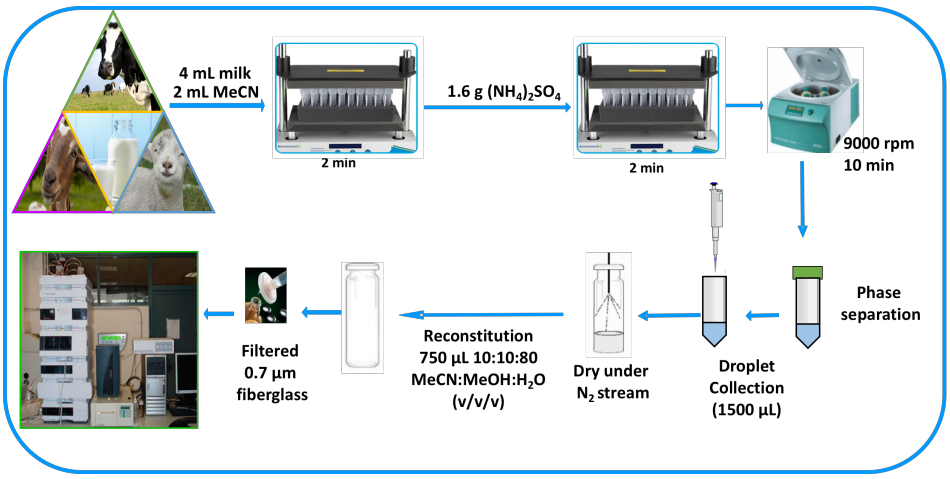


Figure 1. SALLE procedure

3. Results and discussion

3.1 Optimization of chromatographic separation

In order to achieve an optimum separation, parameters such as mobile phase composition, gradient program, mobile phase flow rate and separation temperature were studied. Initially, mobile phase consisting of water (eluent A) and MeCN (eluent B) was supplied at 7 µL min^{-1}. Separation was carried out under gradient conditions as follows: 5% B (0 min), 40% B (14 min), 70 % B (17 min), 95% B (19 min) and 95% B (20 min). Afterwards, mobile phase composition was back to the initial conditions in 14 min and stated for 11 min for column equilibration. Column temperature was set at 20°C and 3 µL was selected as injection volume using water as injection solvent. Under these conditions 14 BZs were separated from a total of 21 analytes included in the initial mixed standard solution. Further modifications of this gradient did not improve resolution. Thus, other chromatographic variables were tested in order to improve BZs separation. First of all, the effect on the separation of different organic solvents in the mobile phase (B), such as MeOH, EtOH or a mixture MeCN/MeOH (1:1 v/v) were evaluated (**Figure 2**). An increase was observed in the resolution between peaks when MeOH was employed. However, the elution of BZs was considerably delayed, increasing the analysis time, so it was discarded. EtOH was also evaluated since it has similar characteristics to MeOH but lower environmental impact. However, as can be observed in **Figure 2**, the resolution of several peaks was not satisfactory (R_{S3-4}, R_{S12-13}, R_{S14-15}), achieving Rs values lower than 0.7. When a mixture of MeCN/MeOH (1:1 v/v) was tested, the resolution between peaks improved, so a mixture of MeCN/ MeOH (1:1 v/v) was selected as organic solvent in the mobile phase (B).

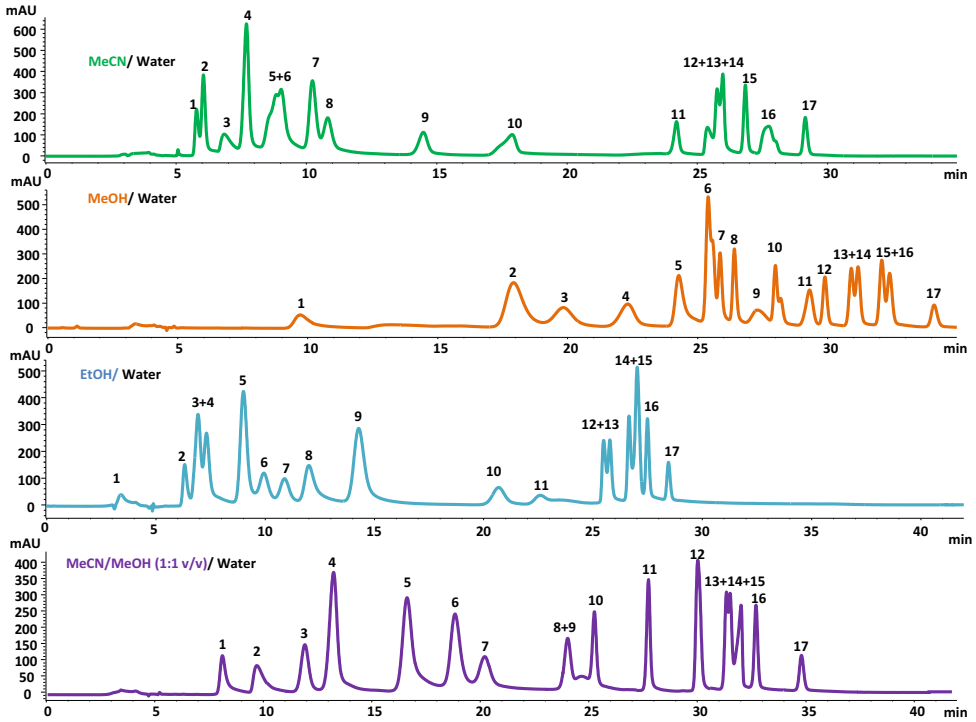


Figure 2. Optimization of the composition of the organic solvent of the mobile phase (B). Conditions: 1 mg L^{-1} of each benzimidazole; column temperature 20°C, flow rate 7 µL min^{-1} and 3 µL of injection volume using water as injection solvent.

Afterwards, different aqueous phase compositions (A) were checked such as water, water with 0.1 % of formic acid and 50 mM ammonium acetate (**Figure 3**). As can be observed in **Figure 3**, when a water solution containing 0.1% of formic acid was tested the resolution of the most polar compounds such as 5-OH-TBZ, ABZ-NH_2-SO_2, ABZ-SO, CBZ+BEN and TBZ was not adequate. The protonation of the analytes at this pH led to their fast elution and poor resolution. When 50 mM ammonium acetate was employed as eluent (A), the separation considerably improved, obtaining satisfactory peak efficiencies for all BZs in comparison with water (**Figure 3**). So, the final composition consisted of 50 mM ammonium acetate (eluent A) and a mixture of MeCN/MeOH (1:1 v/v) (eluent B). Under these conditions, 16 BZs were baseline resolved.

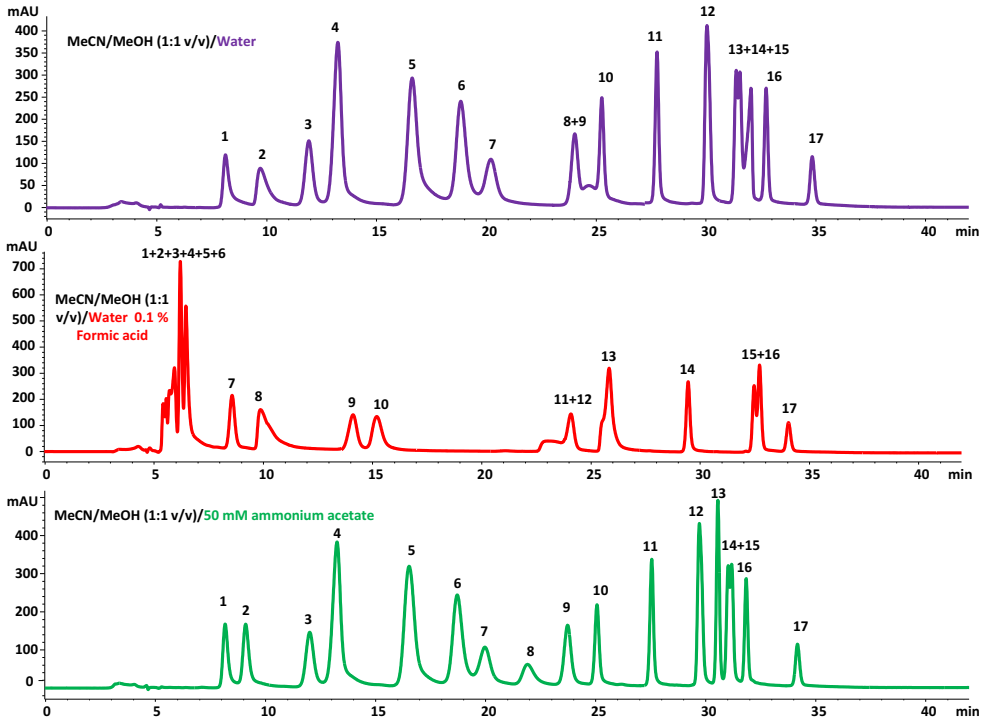


Figure 3. Optimization of the composition of the aqueous solvent of the mobile phase (A). Conditions: 1 mg L^{-1} of each BZ; column temperature 20°C, flow rate 7 µL min^{-1} and 3 µL of injection volume using water as injection solvent.

Then, the effect of mobile phase flow rate on both resolution and analysis time was evaluated from 7 to 17 µL min^{-1}. The responses selected to choose the optimum flow rate were resolution and analysis time. Thus, a flow rate of 9 µL min^{-1} was the best trade-off between them. On the other hand, the column temperature was assessed between 20 and 40 °C. It was observed that temperatures higher than 20 °C reduced the analysis time but the resolution between peaks got worse. A temperature of 20 °C was selected since all peaks were baseline resolved. Finally, the injection volume was studied from 3 to 8 µL (full loop). An injection volume of 6 µL was chosen as a compromise between resolution, peaks efficiency and sensitivity. In conclusion, under optimum conditions 16 BZs (5-OH-TBZ, NH_2-ABZ-SO_2, ABZ-SO, CBZ+BEN, TBZ, FBZ-SO, NH_2-MBZ, 5-OH-MBZ, FBZ-SO_2, MBZ, ABZ, TCB-SO_2, FBZ, TCB-

SO and TCB) were fully separated in less than 32 min, (42 min including column re-equilibration). It must be remarked that in the case of BEN and CBZ, they were quantified as a sum of both analytes because BEN is rapidly transformed into CBZ when it is dissolved in water [1].

3.2 Sample treatment

3.2.1 Extraction procedure

The amount of sample, type and volume of the extraction solvent, and type and amount of salt were studied. Recoveries of the extraction procedure, calculated as the peak area ratio of the analytes in samples spiked before and after the application of the sample procedure, were used to optimize each variable. All the experiments were carried out in triplicate (n=3) employing whole milk spiked at 1 mg L^{-1} of each BZ.

To select the best organic extraction solvent, MeCN, MeOH, EtOH, ethyl acetate, IPA and acetone were tested. The experiments were carried out employing 4 g of milk, and 1.6 g of $(NH_4)_2SO_4$. When EtOH and MeOH were employed, phase separation was not achieved, obtaining a slurry mixture. It was also observed that interference compounds co-eluted together with some BZs when acetone was used. Thus, they were discarded. As can be observed in **Figure 4**, when IPA, ethyl acetate and MeCN were used the average recoveries were 66%, 71% and 92% respectively, therefore, MeCN was selected as extraction solvent for salting-out liquid-liquid extraction (SALLE). The type of salt plays an important role in SALLE. Three different types of salts such as $MgSO_4$, $(NH_4)_2SO_4$ and NaCl were evaluated as salting-out agents using 1.6 g of each one. It was observed that in all cases the phase separation was produced. However, in the case of NaCl and $MgSO_4$, the recoveries for TCB and their metabolites were lower than 51% and 47% respectively. The best results in terms of

[1] R. Halko, C.P. Sanz, Z.S. Ferrera, J.J.S. Rodriguez, Determination of benzimidazole fungicides by HPLC with fluorescence detection after micellar extraction, Chromatographia 60 (2004) 151–156.

reproducibility and recoveries were obtained using $(NH_4)_2SO_4$. Therefore, $(NH_4)_2SO_4$ was used for the rest of optimization. Subsequently, the amount of salt was optimized, using 0.8, 1.2, 1.6 and 2 g of $(NH_4)_2SO_4$ (**Figure 4**). The recoveries for most BZs increased up to 1.6 g (higher than 80%). Thus, 1.6 g was selected. The volume of extraction solvent was also studied in the range of 1 to 4 mL. The average recovery was lower than 67% for all BZs when 1 mL was tested. Also, poor reproducibility was obtained due to the difficulty in collecting the organic upper layer. As it is shown in **Figure 4**, the best average recovery was achieved when 2 mL were used; being the extraction efficiency reduced above this value for all BZs. Finally, the amount of sample was studied (2–6 g). The recoveries of analytes increased up to 4 g of sample (recoveries in the range of 79-98%), remaining constant thereafter. Therefore, 4 g was selected as the optimum volume.

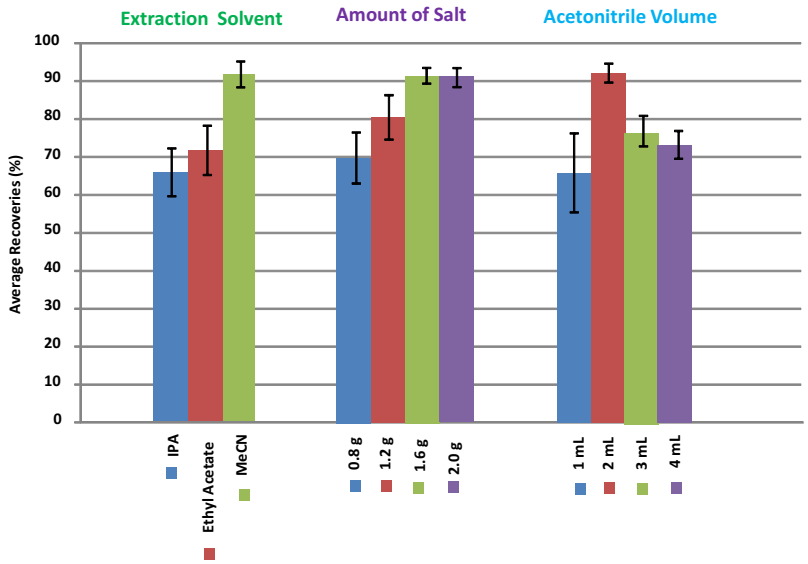


Figure 4. Average recoveries for the optimization of: extraction solvent; amount of salt and acetonitrile volume. Error bars represent the standard error (n = 3).

3.2.2 Filter nature and solvent selection

Preliminary studies showed that less polar analytes such as TCB and their metabolites (TCB-SO_2 and TCB-SO) were totally retained when nylon filters were employed before injection of the samples into the CLC system. Other types of filters were tested, including Teflon (0.2 µm x 13 mm) and fiberglass (0.7 µm x 13 mm) syringe filters. In the case of Teflon filters, these analytes were retained too. When fiberglass filters were employed, the analytes were still partially retained (≈38%). Thus, fiberglass filters were chosen although the optimization of the solvent to redissolve the sample before filtering was required in order to avoid partial retention of TCB and their metabolites onto fiberglass. Different proportions of MeCN/MeOH/H_2O (5:5:90; 10:10:80 and 15:15:70 v/v/v) were evaluated as sample solvent. With a mixture of MeCN/MeOH/H_2O (15:15:70 v/v/v) peak efficiency was not satisfactory for most compounds, so a mixture of MeCN/MeOH/H_2O (10:10:80 v/v/v) MeCN/H_2O was selected as injection solvent as all analytes were completely extracted from the fiber glass filter without any lost in the peak efficiency.

3.3 Method characterization

The applicability of the method for the determination of 16 BZs in cow milk samples was evaluated. With this purpose, parameters such as linearity, LODs, limits of quantification (LOQs), repeatability, intermediate precision and recoveries were studied.

3.3.1 Calibration curves and analytical performance characteristics

Matrix-matched calibration curves were performed employing as representative matrix cow milk samples free of analytes. Samples spiked with six different analyte concentrations were considered (10, 20, 50, 100, 150 and 200 µg L^{-1}) and peak area was used as analytical response. Each concentration level consisted of two experimental replicates and two instrumental replicates (n=4). A blank sample was analyzed and none of the BZs were detected. LODs and LOQs were calculated as the

minimum analyte concentration corresponding with a signal-to-noise ratio equal to three and ten, respectively.

Figures of merit of the proposed method in cow milk samples are collected in **Table 1**. LOQs ranged between 3.2 and 9.5 μg kg^{-1} were obtained. Therefore, the suggested method allows to determine these compounds in cow milk at levels below MRLs established by the European legislation.

Table 1. Statistic and performance characteristics of the proposed SALLE-CLC-UV methodology in cow milk samples.

Analyte	LOD (μg kg^{-1})	LOQ (μg kg^{-1})	Linear dynamic range (μg kg^{-1})	MRL (μg kg^{-1})	R^2
5-OH- TBZ	2.0	6.6	6.6-200	100	0.9990
ABZ-NH_2-SO_2	2.2	7.2	7.2-200	100	0.9994
ABZ-SO	2.8	9.5	9.5-200	100	0.9997
CBZ+BEN	2.7	8.9	8.9-200	Non established	0.9991
TBZ	2.3	7.8	7.8-200	100	0.9994
FBZ-SO	2.7	8.9	8.9-200	10	0.9998
MBZ-NH_2	2.8	9.1	9.1-200	Non established	0.9994
5-OH-MBZ	2.6	8.7	8.7-200	Non established	0.9995
FBZ-SO_2	2.8	9.3	9.3-200	10	0.9993
MBZ	2.0	6.6	6.6-200	Non established	0.9996
ABZ	1.0	3.2	3.2-200	100	0.9997
TCB-SO_2	1.9	6.2	6.2-200	10	0.9995
FBZ	1.2	4.0	4.0-200	10	0.9985

Analyte	LOD ($\mu g\ kg^{-1}$)	LOQ ($\mu g\ kg^{-1}$)	Linear dynamic range ($\mu g\ kg^{-1}$)	MRL ($\mu g\ kg^{-1}$)	R^2
TCB-SO	2.3	7.7	7.7-200	10	0.9995
TCB	2.4	7.8	7.8-200	10	0.9994

3.3.2 Precision study

The precision of the method was evaluated in terms of repeatability (intraday precision) and intermediate precision (interday precision) by application of the proposed SALLE-CLC-UV method to cow milk samples spiked at two different concentration levels of BZs (50 and 150 $\mu g\ Kg^{-1}$). Repeatability was evaluated over three samples injected by duplicate on the same day, under the same conditions. Intermediate precision was evaluated over two samples injected by duplicate during three consecutive days. The results, expressed as %RSD of the peak areas, are shown in **Table 2**. Good precision, with RSDs lower than 14.2% were obtained in all cases.

3.3.3 Recovery studies

In order to check the trueness of the proposed methodology for the analysis of real samples, recovery experiments were carried out in three different types of milk (cow, goat and sheep) spiked at two different BZ concentration levels (50 and 150 $\mu g\ Kg^{-1}$). Absolute recoveries were calculated by comparing peak areas of milk samples spiked before the sample treatment with peak areas of milk samples spiked after the sample treatment. In all cases, a sample free of analytes was analyzed to check the absence of BZs, and none of them gave signals corresponding to concentrations above the LODs. Recoveries higher than 79.1 % were obtained, with RSDs lower than 8.9 % (**Table 3**). These results suggest that the proposed method could be satisfactorily applied not just to cow milk but also to goat and sheep milk. Chromatograms corresponding to milk samples from cow, sheep and goat spiked

with each BZ at 150 μg kg^{-1} and blank samples of each type of milk are shown in **Figure 5**.

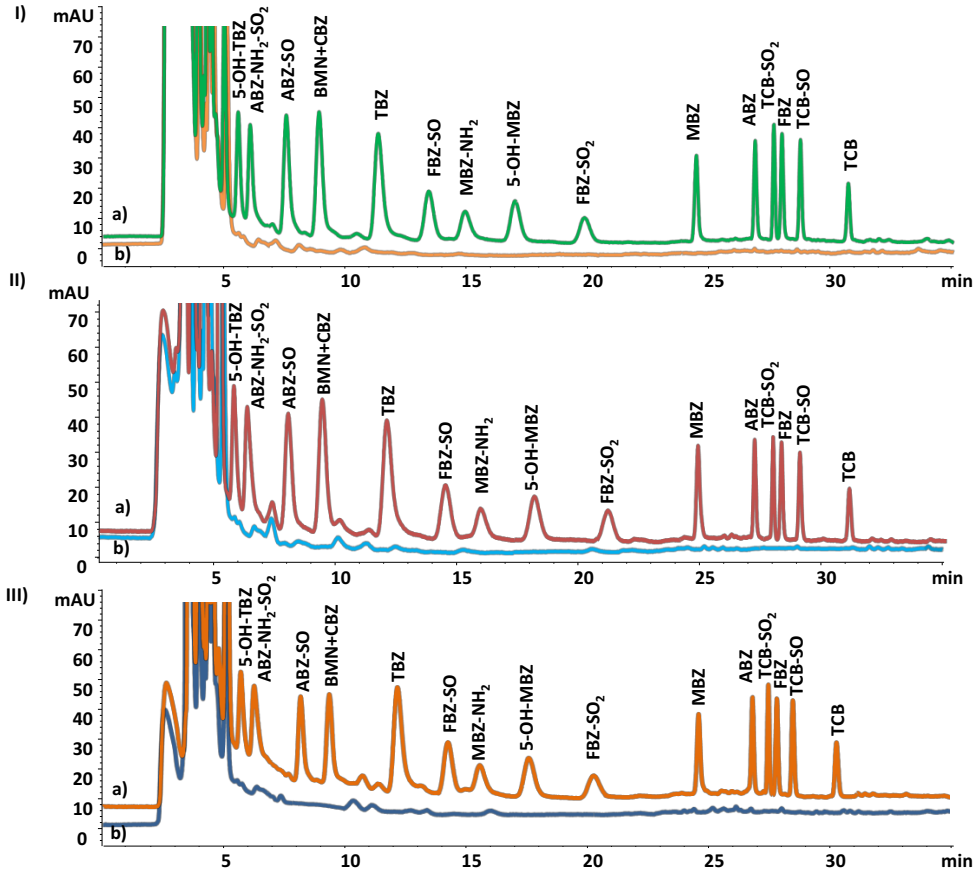


Figure 5. Chromatograms corresponding to: I) (a) a cow milk sample spiked with 150 μg kg^{-1} of each BZ and (b) a blank of cow milk sample; II) (a) a sheep milk sample spiked with 150 μg kg^{-1} of each BZ and (b) a blank of sheep milk sample; III) (a) a goat milk sample spiked with 150 μg kg^{-1} of each BZ and (b) a blank of goat milk sample.

Table 2. Precision of the method for spiked cow milk samples

	5-OH- TBZ	ABZ-NH_2-SO_2	ABZ-SO	CBZ+BEN	TBZ	FBZ-SO	MBZ-NH_2	5-OH-MBZ	FBZ-SO_2	MBZ	ABZ	TCB-SO_2	FBZ	TCB-SO	TCB
	Repeatability RSD (%) (n=6)														
Level 1	3.9	3.2	7.8	5.7	6.4	3.2	6.3	6.2	7.1	5.3	5.7	5.9	7.7	7.2	9.6
Level 2	1.6	2.1	3.8	2.6	2.2	0.5	3.0	2.6	3.7	2.9	3.7	2.9	3.1	2.3	2.1
	Intermediate precision RSD (%) (n=12)														
Level 1	5.1	8.0	10.6	7.0	10.8	4.5	9.1	6.5	8.4	7.8	7.3	9.1	8.2	10.8	14.2
Level 2	4.2	4.9	3.9	4.2	4.7	3.1	8.4	3.5	6.9	6.5	4.6	8.5	6.1	6.2	11.1

Level 1: 50 µg kg^{-1} and **Level 2:** 150 µg kg^{-1}

Table 3. Recoveries and RSD (n=4) for the proposed SALLE-CLC-UV methodology in cow milk, sheep milk and goat milk.

			5-OH-TBZ	ABZ-NH_2-SO_2	ABZ-SO	CBZ+BEN	TBZ	FBZ-SO	MBZ-NH_2	5-OH-MBZ	FBZ-SO_2	MBZ	ABZ	TCB-SO_2	FBZ	TCB-SO	TCB
Cow milk	**Level 1**	Recoveries (%)	92.1	91.4	87.8	79.1	84.8	88.3	80.7	84.9	99.6	88.7	89.6	92.7	90.7	94.9	92.8
		(RSD, %)	6.2	7.4	4.7	7.8	7.6	8.4	5.7	8.3	5.9	5.3	7.0	5.9	5.4	7.5	6.3
	Level 2	Recoveries (%)	82.2	94.2	91.1	98.8	96.2	97.7	87.0	97.9	96.0	95.1	90.2	94.2	92.4	88.8	93.1
		(RSD, %)	3.4	3.8	2.8	2.3	3.0	2.1	3.8	2.7	1.2	2.0	2.0	3.7	3.8	3.5	3.6
Sheep milk	**Level 1**	Recoveries (%)	94.7	90.9	85.0	81.7	81.8	86.2	81.1	85.3	96.8	99.7	87.5	84.4	92.6	83.1	82.6
		(RSD, %)	5.3	5.2	4.0	5.7	8.0	8.2	5.5	5.6	6.6	8.0	8.6	6.7	4.2	7.8	8.5
	Level 2	Recoveries (%)	81.1	79.4	82.2	83.5	87.7	87.0	81.6	95.3	96.9	97.8	95.3	97.0	94.1	97.2	94.9
		(RSD, %)	2.0	2.9	1.7	3.7	3.6	1.4	3.0	1.5	1.8	1.4	1.9	2.5	2.8	1.5	3.2
Goat milk	**Level 1**	Recoveries (%)	94.1	84.9	88.3	81.4	94.4	95.0	88.6	95.3	94.0	94.2	89.8	90.8	91.3	94.5	86.9
		(RSD, %)	6.4	5.3	7.3	8.9	5.8	7.3	5.5	5.9	7.2	4.9	7.1	5.7	4.9	5.4	6.0
	Level 2	Recoveries (%)	95.0	86.0	92.1	97.9	97.0	92.6	79.4	98.0	95.6	96.7	99.7	94.6	90.6	94.2	97.6
		(RSD, %)	3.6	1.4	4.8	5.1	1.3	1.9	3.0	2.6	1.4	3.1	2.9	3.9	2.0	3.6	3.0

Level 1: 50 µg kg^{-1} and **Level 2**: 150 µg kg^{-1}

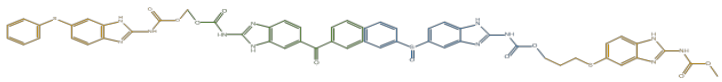
4. Conclusions

In this chapter, a capillary LC-UV method has been proposed for the first time to determine 16 BZs residues in milk samples, showing several advantages compared to analytical HPLC, such as lower limits of detection, better resolution, and lower solvent consumption. It is also friendlier with the environment. The so-called SALLE has been applied as sample treatment. SALLE is effective, fast, simple and allows an efficient clean-up of extracts. The combination of this sample treatment with a miniaturized technique such as CLC provided a useful method for the monitoring of BZ residues in milk samples. All analytes were separated in less than 32 min with detection limits in the range of low μg kg^{-1}. Good linearity, sensitivity and selectivity, satisfactory LOQs, trueness, precision, and selectivity were obtained with the proposed SALLE-CLC-UV method, fulfilling the current legislation for BZs residue analysis. The results show the suitability of this procedure for the monitoring of 16 BZs in milk samples in a single run.

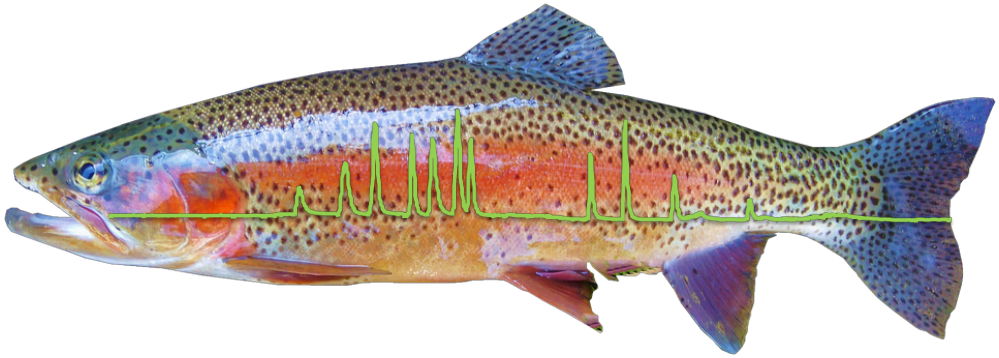
This work was published as:

"Green and simple analytical method to determine benzimidazoles in milk samples by using salting-out assisted liquid-liquid extraction and capillary liquid chromatography". **C. Tejada-casado, M. Olmo-iruela, A.M. García-campaña, F.J. Lara, J. Chromatogr. B. 1091 (2018) 46–52.**

CHAPTER 2



ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION FOLLOWING SALTING-OUT ASSISTED LIQUID–LIQUID EXTRACTION FOR THE ANALYSIS OF BENZIMIDAZOLE RESIDUES IN FARM FISH SAMPLES



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1. Summary

UHPLC coupled with FL has been proposed for the first time to determine thirteen BZs in farmed fish samples. In order to optimize the chromatographic separation, parameters such as mobile phase composition and flow rate were carefully studied, establishing a gradient mode with a mobile phase consisted of water (solvent A) and MeCN (solvent B) at a flow rate of 0.4 mL/min. The separation was performed on a Zorbax Eclipse Plus RRHD C_{18} column (50×2.1 mm, 1.8 µm), involving a total analysis time lower than 12 min. SALLE was applied as sample treatment to different types of farmed fish (trout, sea bream and sea bass). To obtain satisfactory extraction efficiencies for the studied analytes, several parameters affecting the SALLE procedure were optimized including the amount of sample, type and volume of the extraction solvent, and the nature and amount of the salt used. Characterization of the method in terms of performance characteristics was carried out, obtaining satisfactory results for the linearity (R^2≥0.997), repeatability (RSD≤6.1%), reproducibility (RSD≤10.8%) and recoveries (R≥79%; RSD≤7.8%). LODs between 0.04 to 29.9 µg kg^{-1} were obtained, demonstrating the applicability of this fast, simple and environmentally friendly method.

2. Materials and methods

2.1 Reagents and materials

MeCN (99.9%), MeOH (99.9%), IPA (99.9%), tetrahydrofuran (THF) (≥99.9%), and ACO (100%), (LC-MS HiPerSolv grade) were supplied from VWR (Radnor, PA, USA). Ammonium acetate and EtOH (99.9%), were supplied from Merck (Darmstadt, Germany). Ultrapure water (Milli-Q plus system, Millipore, Bedford, MA, USA) was used throughout the work.

Salts used for aqueous and organic-phase partitioning were purchased from different suppliers: ammonium sulfate ($(NH_4)_2SO_4$) (99.5%) was obtained from VWR (Radnor, PA, USA); magnesium sulfate anhydrous ($MgSO_4$) (96%) and sodium chloride (NaCl) (> 99%) were obtained from Panreac (Barcelona, Spain).

Analytical standards of ABZ (99.0%), ABZ-SO_2 (99.3%), ABZ-SO (98.0%), BEN (99.0%), CBZ (99.0%), FUB (99.5%), OXI (99.9%), TBZ (99.8%), 5-OH-TBZ (99.9%), TCB (99.7%), TCB-SO_2 (99.4%) and TCB-SO (99.8%) were supplied by Sigma-Aldrich (St. Louis, MO, USA). ABZ-NH_2-SO_2 (99.0%) was supplied by Dr. Ehrenstorfer (Augsburg, Germany).

Individual stock standard solutions of 1000 mg L^{-1} (ABZ-SO, ABZ-NH_2-SO_2, FUB, TBZ, 5-OH-TBZ, TCB-SO_2, TCB-SO), 500 mg L^{-1} (ABZ, ABZ-SO_2, BEN, TCB) and 250 mg L^{-1} (OXI, CBZ) were prepared by dissolving accurately weighed amounts of each compound in MeOH. They were stored in dark glass bottles at -20 °C, being stable for at least 6 months. Intermediate stock standard solutions for UV detection containing 10 mg L^{-1} of each BZ were obtained by mixing individual stock standard solutions and subsequent dilution with water. Intermediate stock standard solutions for FL detection containing 10 mg L^{-1} (5-OH-TBZ, TCB, OXI), 5 mg L^{-1} (TCB-SO, TCB-SO_2), 2.5 mg L^{-1} (ABZ-SO), 1.25 mg L^{-1} (CBZ, BEN, ABZ, TBZ), 625 µg L^{-1} (ABZ-NH_2-SO_2), 150 µg L^{-1} (ABZ-SO_2) and 40 µg L^{-1} (FUB) were obtained by mixing individual stock standard solutions and subsequent dilution with water. It must be noted that

different concentrations of BZs have been used due to the different fluorescence quantum yield of these compounds.

They were also stored in dark glass bottles at 4°C. Standard working solutions containing all the BZs were prepared by the proper dilution of the intermediate stock standard solutions with water at the required concentration.

Teflon (PTFE) syringe filters (0.2 µm x 13 mm) and fiberglass syringe filters (0.72 µm x 13 mm) from (VWR, Radnor, PA, USA) and nylon syringe filters, 0.2 µm x 13 mm (Bonna-Agela Technologies Inc, Wilmington, USA) were used for filtration of sample extracts prior to their injection into the UHPLC system.

2.2 Instrumentation

Chromatographic experiments were carried out with a UHPLC instrument from Jasco (Tokio, Japan) containing two extreme pressure pumps (X-LC-3185PU), a mixing unit (X-LC-3180MX), a degasser module with four channels (X-LC-3080DG), an auto-sampler (X-LC-3059AS) fitted with a 20-µL loop, a column oven (X-LC-3067CO), a fluorescence detector (Model X-LC3120FP) and a UV-Vis detector (Model X-LC-3070UV). LC-Net II/ADC was used as hardware interface between the UHPLC system and the computer. UV-Vis and fluorescence chromatograms were acquired using ChromNav version 1.18.03. A Zorbax Eclipse Plus RRHD C_{18} (50 × 2.1 mm, 1.8 µm) from Agilent Technologies (Waldbronn, Germany) was used as chromatographic column.

A centrifuge (Universal 320R model from Hettich, Leipzig, Germany), an evaporator with nitrogen (System EVA-EC from VLM GmbH, Bielefeld, Germany) and a multi-tube vortexer (BenchMixer™ XL model from Benchmark Scientific, New Jersey, USA) were also used.

2.3 UHPLC-UV and FL determination

The mobile phase consisted of water (solvent A) and acetonitrile (solvent B) at a flow rate of 0.4 mL/min. Gradient profile was as follows: 10% B (0 min), 10% B (1 min), 23 % B (4 min), 23% B (5 min), 50% B (6 min), 60% B (8.5 min) and 95% B (9.5 min). Afterwards, the mobile phase composition was back to initial conditions in 1 min. In order to guarantee column equilibration and obtaining a reproducible and stable separation, mobile phase composition was maintained at initial conditions for 2 min. The temperature of the column was fixed at 40 °C and the injection volume was 20 µL using a mixture of MeCN:water (20:80 v/v) as injection solvent. The UV detector was set at 290 nm. Fluorescence detection was performed at the maximum excitation/emission wavelength of each compound using five different windows: 316/400 nm for 5-OH-TBZ (0-3.5 min); 290/325 nm for ABZ-NH_2-SO_2 (3.5-4 min); 280/320 nm for CBZ and BEN (4-4.5 min); 290/325 nm for ABZ-SO, TBZ, FUB and ABZ-SO_2 (4.5-7 min); 290/340 nm for OXI, ABZ, TCB-SO_2, TCB-SO and TCB (7-12.5 min). Gain detector was set to 100.

2.4 Sample treatment procedure

Trout, sea bream and sea brass were purchased in local supermarkets from Granada (Spain). Before the analysis the fish were skinned and deboned in order to obtain the muscle and stored at −20 °C until they were used for BZs extraction, applying the developed SALLE procedure. First, portions of 0.5 g of muscle sample were weighed into 15 mL centrifuge tubes and spiked with different volumes of a standard solution containing 10 mg L^{-1} (5-OH-TBZ, TCB, OXI), 5 mg L^{-1} (TCB-SO, TCB-SO_2), 2.5 mg L^{-1} (ABZ-SO), 1.25 mg L^{-1} (CBZ, BEN, ABZ, TBZ), 625 µg L^{-1} (ABZ-NH_2-SO_2), 150 µg L^{-1} (ABZ-SO_2) and 40 µg L^{-1} (FUB). All spiked samples were allowed to stand for 10–15 min before proceeding. Then, 4.0 mL of deionized water was added to the sample, and the mixture was agitated by vortex for 2 min until a proper homogenization was achieved. Subsequently, 2 mL of MeCN was added and the

tube was vortexed again for 2 min. In the same conical tube, 1.6 g of $(NH_4)_2SO_4$ was added to the sample to induce the separation between the organic and aqueous phase. Then, the tube was shaken by vortex (2 min). After that, the sample was centrifuged at 9000 rpm for 10 min and 1.5 mL of the upper organic layer was transferred to a 4-mL vial. Then, the organic extract was dried under a nitrogen stream at 35 °C and afterwards it was re-dissolved in 500 µL of a mixture MeCN:water (20:80 v/v) by vortex. The sample was filtered with teflon (PTFE) syringe filters (0.2 µm x 13 mm) and transferred to a 200 µL vial insert for injection into the UHPLC-FL system.

Taking into account all steps involved in this procedure, the sample throughput was approximately 15 samples per hour. The sample procedure it is shown in **Figure 1**.

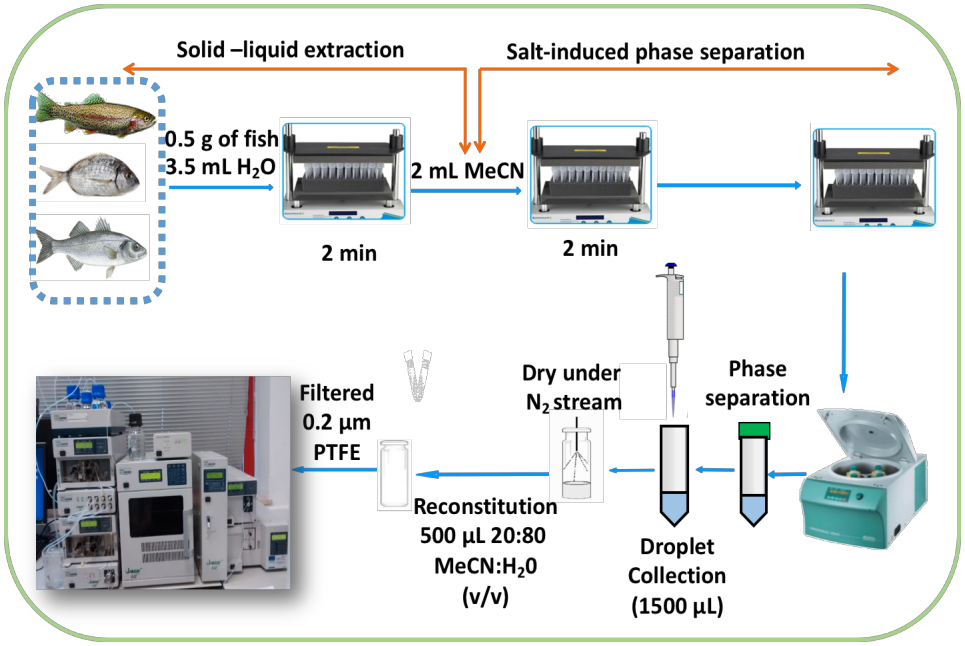


Figure 1. Extraction procedure

3. Results and discussion

3.1 Optimization of chromatographic separation

In order to improve BZs separation, parameters such as mobile phase composition, mobile phase flow rate, gradient program, separation temperature, gain of detector and data acquisition mode were evaluated. Initially, mobile phase consisted of ammonium acetate 50 mM (eluent A) and MeOH (eluent B) and it was supplied at 0.5 mL min^{-1}. Column temperature was set to 40°C and 5 µL was selected as injection volume using water as injection solvent. Separation was carried out under gradient condition described in Section 2.3. Unfortunately, several analytes co-eluted including ABZ-SO and TBZ, FUB and ABZ-SO_2 and TCB-SO and TCB-SO_2. CBZ and BEN were detected as a single chromatographic peak in any case because BEN is easily transformed into CBZ as soon as it dissolves in water [1,2]. In order to improve resolution, the composition of mobile phase in relation to the eluent B was evaluated, considering a mixture of 1:1 MeCN/MeOH (v/v) and MeCN instead of MeOH. It must be noted that the mobile phase composition study was carried out by monitoring UV-absortion, as all compounds can be easily detected using a single wavelength (290 nm). As can be observed in **Figure 2.b**, the use of a MeCN:MeOH mixture (50:50 v/v) allowed the separation of all analytes except for FUB and ABZ-SO_2, and TCB-SO_2 and TCB-SO (resolution < 1). The best separation in terms of peak resolution for all analytes was achieved when MeCN was employed as organic solvent in the mobile phase (**Figure 2.c**).

[1] R. Halko, C. Padrón Sanz, Z. Sosa Ferrera, J.J. Santana Rodríguez, Determination of benzimidazole fungicides by HPLC with fluorescence detection after micellar extraction, Chromatographia. 60 (2004) 151–156.
[2] E. Mallat, D.Barceló, R.Tauler, Degradation study of benomyl and carbendazim in water by liquid chromatography and multivariate curve resolution methods, Chromatographia 46 (1997) 342–350.

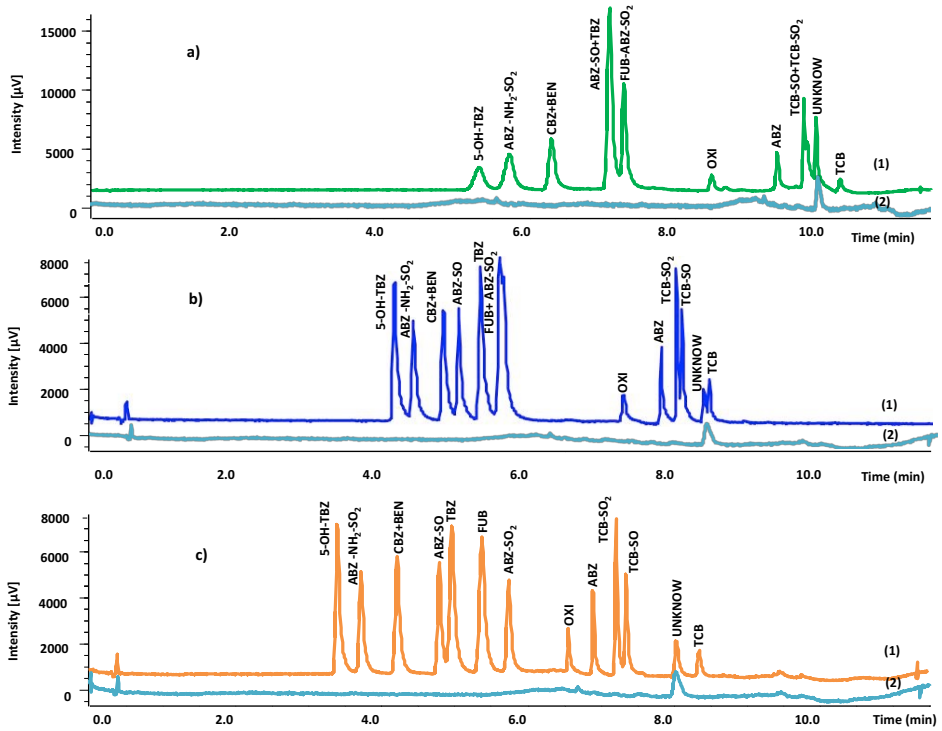


Figure 2. Optimization of the composition of the mobile phase. Eluent A: 50 mM ammonium acetate; Eluent B: (a) MeOH, (b) MeCN:MeOH (1:1, v/v), (c) MeCN. Conditions: 2 mg L^{-1} of each BZ; column temperature 40°C, flow rate 0.5 mL min^{-1} and 5 µL of injection volume using water as injection solvent. (1) Standard chromatogram (2) Blank chromatogram. (1) Standard chromatogram (2) Blank chromatogram

Water was studied as eluent (A) instead of ammonium acetate 50 mM (**Figure 3.b**). It was observed that the separation between TCB-SO and TCB-SO_2 was not satisfactory. However, the column pressure increased dramatically after a few analysis when ammonium acetate was used. This fact could be due to the precipitation of salts inside the column during the gradient program. In order to prevent the breakdown of the column, the use of ammonium acetate 50 mM was discarded sacrificing the separation of TCB-SO and TCB-SO_2 as they were detected as a single chromatography peak. So, the final mobile phase composition consisted of water (eluent A) and MeCN (eluent B) (**Figure 3.a**).

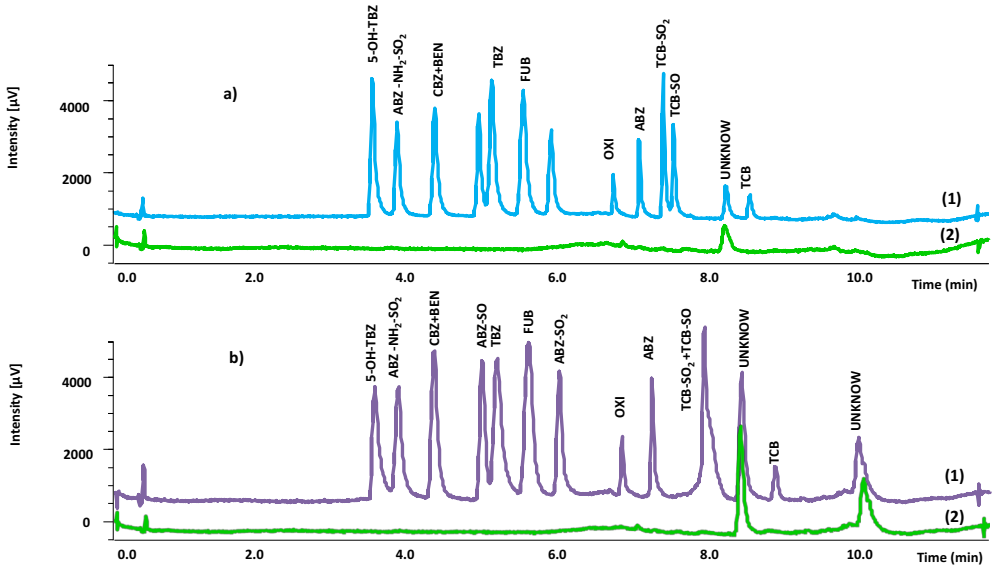


Figure 3. Optimization of the composition of the mobile phase. Eluent A: (a) 50 mM ammonium acetate; (b) water; Eluent B: MeCN. Conditions: 2 mg L^{-1} of each BZ; column temperature 40°C, flow rate 0.5 mL min^{-1} and 5 µL of injection volume using water as injection solvent. (1) Standard chromatogram (2) Blank chromatogram.

Consequently, mobile phase flow rate was studied at 0.4, 0.5 and 0.6 mL min^{-1}. A flow rate of 0.4 mL min^{-1} was selected as optimum because higher flow rates involved a decrease of resolution between ABZ-SO and TBZ (resolution< 1.46) (**Figure 4**). Then, the temperature of the column was tested for 30 °C, 40 °C and 50 °C. As can be observed in **Figure 5.a** with a temperature of 30 °C the resolution between FUB and ABZ-SO_2 was lower than 1.3. Temperatures higher than 40 °C decreased the resolution between ABZ-SO and TBZ (resolution < 1.42) (**Figure 5.c**). So, a temperature of 40 °C was selected since all peaks were baseline resolved under these conditions (**Figure 5.b**).

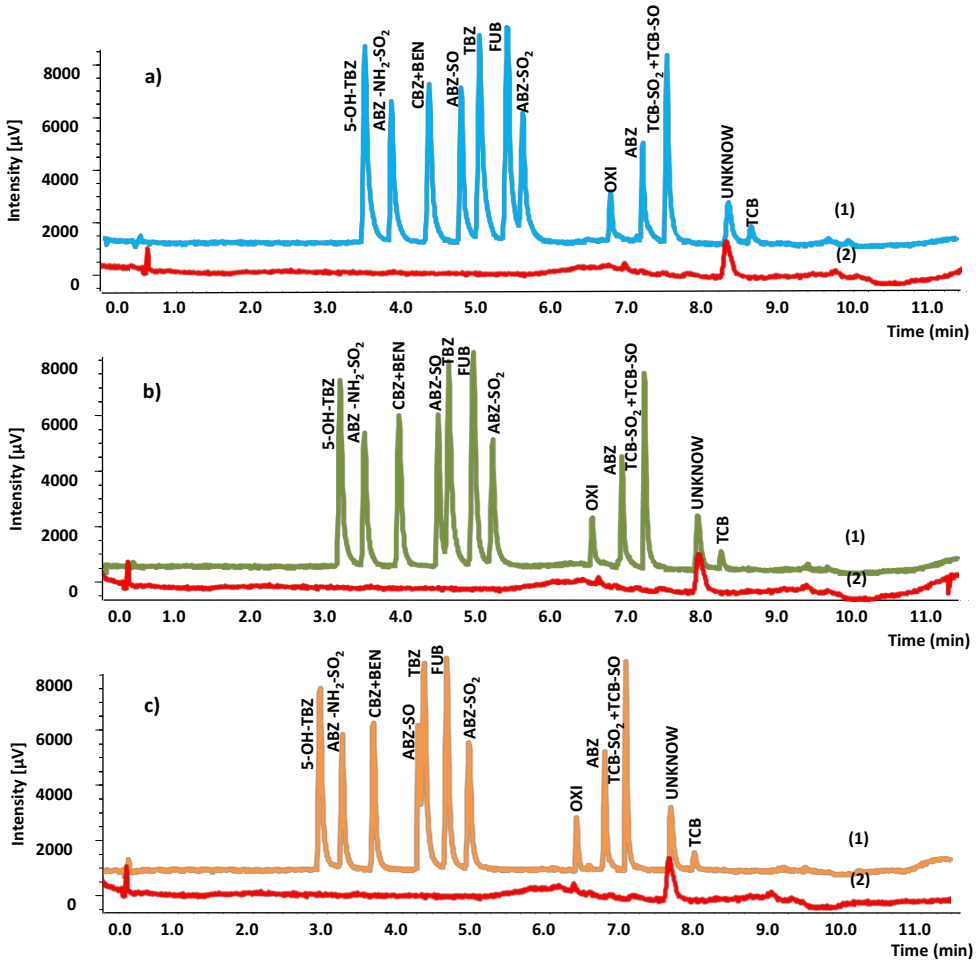


Figure 4. Optimization of the flow rate. (a) 0.4 ml min^{-1}, (b) 0.5 ml min^{-1}, (c) 0.6 ml min^{-1}. Conditions: 2 mg L^{-1} of each BZ in UV; column temperature 40°C and 5 µL of injection volume using water as injection solvent. (1) Standard chromatogram (2) Blank chromatogram.

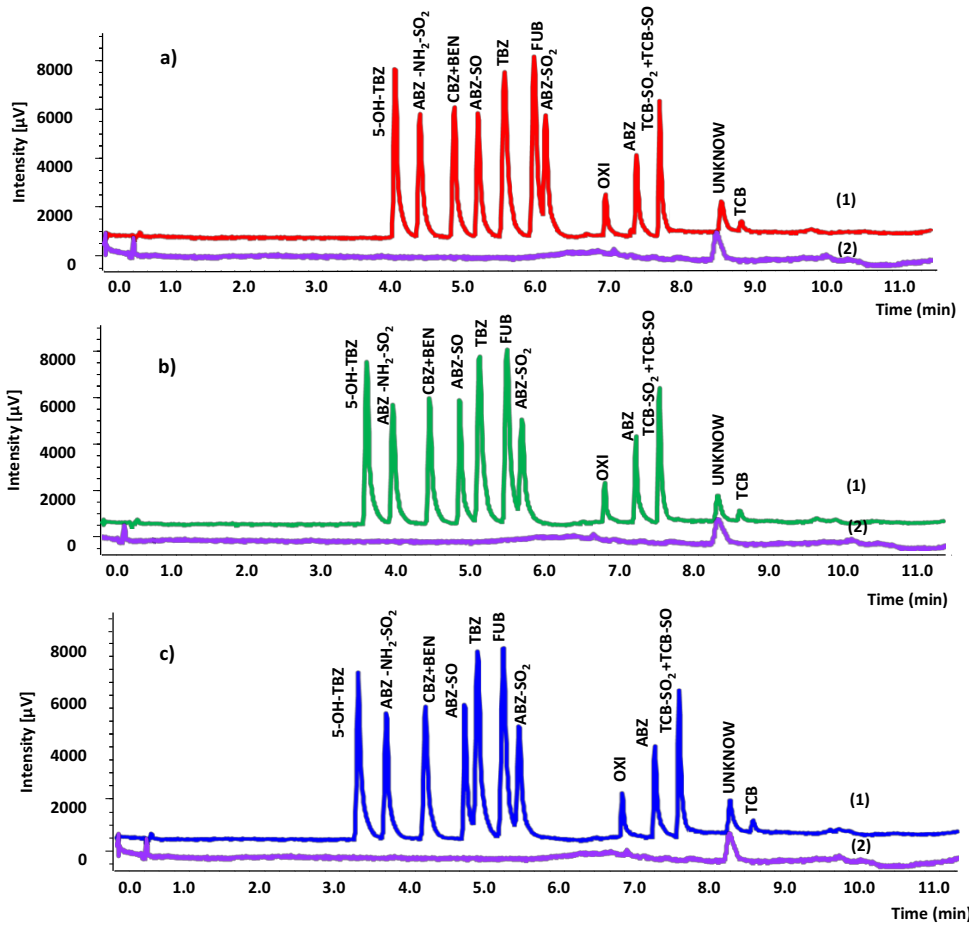


Figure 5. Optimization of the column temperature. (a) 30˚C, (b) 40˚C, (c) 50˚C. Conditions: 2 mg L^{-1} of each BZ in UV; flow rate 0.4 mL min^{-1} and 5 µL of injection volume using water as injection solvent. (1) Standard chromatogram (2) Blank chromatogram.

Subsequently, detector gain was investigated from 10 to 1000, selecting 100 as a compromise between sensitivity and selectivity. A "slow" mode for data acquisition (fifty points per second) in fluorescence was chosen because the S/N ratio enhanced (≈ 2.3 fold for all compounds) in contrast with a "standard" data acquisition (**Figure 6.a**). The separation of the BZs was carried out within 12 min.

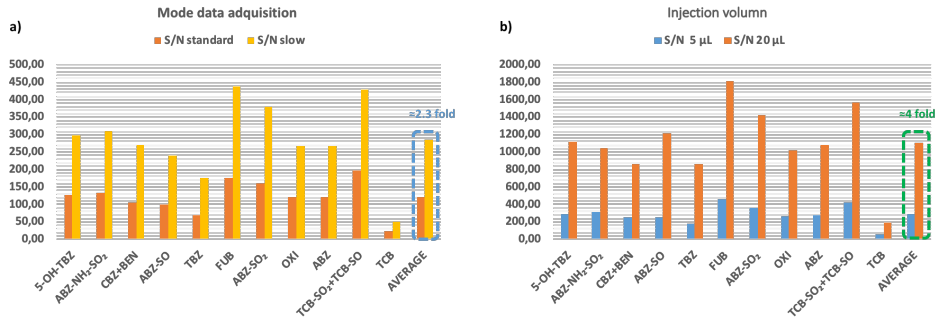


Figure 6. (a) Mode data acquisition; (b) Injection volume. BZs concentrations in FL: 1.0 mg kg^{-1} (5-OH-TBZ, TCB, OXI), 0.5 mg kg^{-1} (TCB-SO, TCB-SO_2), 250 µg kg^{-1} (ABZ-SO), 125 µg kg^{-1} (CBZ, BEN, ABZ, TBZ), 62.5 µg kg^{-1} (ABZ-NH_2-SO_2), 15 µg kg^{-1} (ABZ-SO_2) and 4 µg kg^{-1} (FUB).

Finally, two loops with injection volumes of 5 and 20 µL were checked with the option "full loop" (**Figure 6.b**). An injection volume of 20 µL was selected as optimum in order to improve sensitivity (≈ 4 fold for all compounds) without losing any resolution between peaks.

Figure 7 shows standard chromatograms with final separation conditions using UV and FL detection respectively. Taking into account the concentrations used for each detector, we can conclude that 5-OH-TBZ and the sum of the two metabolites of TCB present higher signal when UV detection is used. For TCB the signal is the same in both cases. The nine remaining compounds exhibit higher sensitivity for FL detection especially in the case of FUB, ABZ-SO_2 or ABZ-NH2-SO_2.

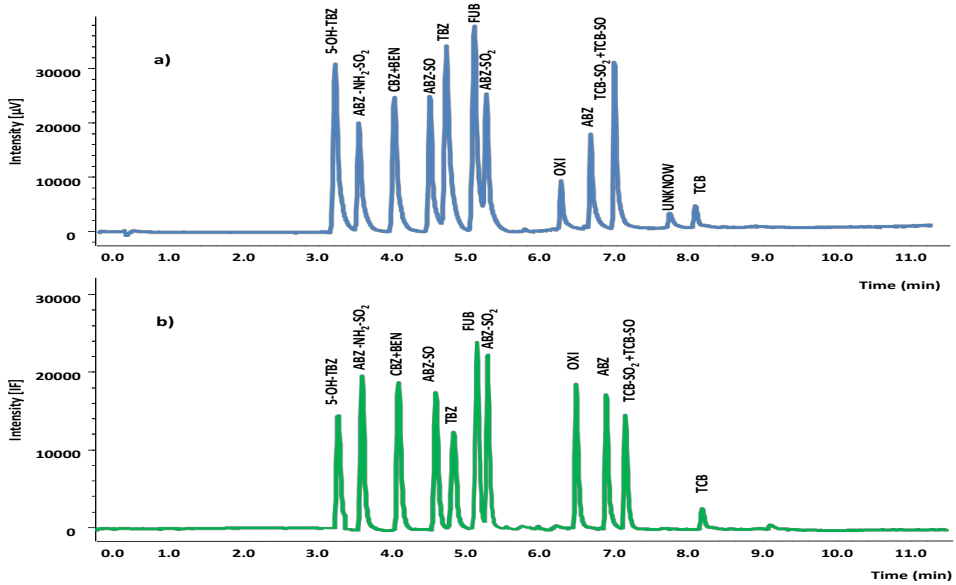


Figure 7. Standard chromatograms with final separation conditions: a) With UV detection using 2 mg L^{-1} of each BZ; b) With FL detection using 2.0 mg L^{-1} (5-OH-TBZ, TCB, OXI), 1 mg L^{-1} (TCB-SO, TCB-SO_2), 500 µg L^{-1} (ABZ-SO), 250 µg L^{-1} (CBZ, BEN, ABZ, TBZ), 125 µg L^{-1} (ABZ-NH_2-SO_2), 30 µg L^{-1} (ABZ-SO_2) and 8 µg L^{-1} (FUB). Conditions: column temperature 40°C; flow rate 0.4 mL min^{-1}; injection volume 20 µL; mobile phase composition: water as eluent (A) and MeCN as eluent (B).

3.2 Optimization of SALLE sample treatment

All the parameters affecting the extraction efficiency in the SALLE procedure, such as the amount of sample, type and volume of the extraction solvent, and type and amount of salt, were evaluated using standard analyte solutions. Efficiency of the extraction process was studied by means of a recovery study, considering the ratio between peak area for each analyte obtained by spiking the sample before the application of the sample treatment and that obtained by spiking the extract just before the instrumental measurement. All the experiments for the optimization procedure were carried out in triplicate (n=3) using 4 mL of aqueous sample containing 1.0 mg L^{-1} (5-OH-TBZ, TCB, OXI), 0.5 mg L^{-1} (TCB-SO, TCB-SO_2), 250 µg L^{-1} (ABZ-SO), 125 µg L^{-1} (CBZ, BEN, ABZ, TBZ), 62.5 µg L^{-1} (ABZ-NH_2-SO_2), 15 µg L^{-1} (ABZ-SO_2) and 4 µg L^{-1} (FUB).

This salt-induced phase separation study was made for six different water-miscible organic solvents namely MeOH, EtOH, MeCN, IPA, THF and ACO. These experiments were performed using 4 mL of aqueous sample, 2 mL of organic solvent and 1.6 g of $(NH_4)_2SO_4$. When MeOH and EtOH were employed, phase separation was not observed. When ACO was used, some interferents were co-eluting with BZs, so it was discarded. The results for the rest of solvents are shown in **Figure 8.a.** The best recoveries were achieved for most compounds when MeCN was employed as extraction solvent. So, MeCN was used throughout the study.

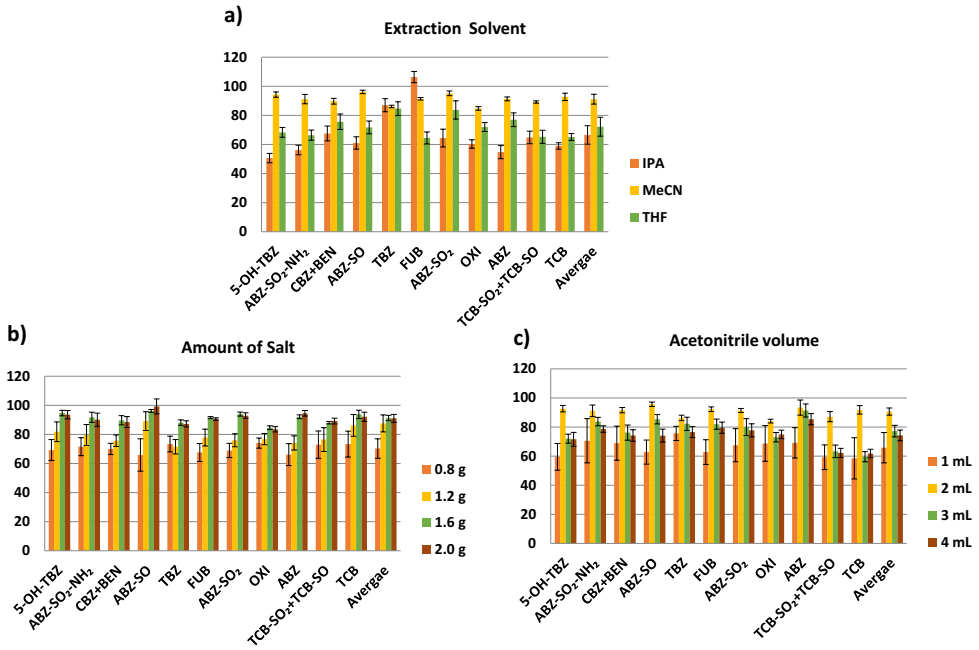


Figure 8. Study of the variables affecting the SALLE procedure. (a) Effect of extraction solvent type. Extraction conditions: 4 mL of aqueous sample, 2 mL of organic solvent and 1.6 g of $(NH_4)_2SO_4$; (b) Effect of the MeCN volume. Extraction conditions: 4 mL of aqueous sample, different volumes of MeCN and 1.6 g of $(NH_4)_2SO_4$; (c) Effect of the amount of $(NH_4)_2SO_4$. Extraction conditions: 4 mL of aqueous sample, 2 mL of MeCN and different amounts of $(NH_4)_2SO_4$. Error bars represent the standard error (n = 3).

Then, three different types of salts, such as $MgSO_4$, $(NH_4)_2SO_4$ and NaCl, were evaluated as salting-out agents using 1.6 g of each one. It was observed that in all cases the phase separation was induced. Nevertheless, in the case of NaCl, the recoveries for TCB and their metabolites were lower than 48% and in the case of $MgSO_4$, the recoveries for 5-OH-TBZ, OXI, TCB and their metabolites were lower than 46%, so both were discarded. The best result, in terms of efficiency recoveries (79 to 96 %) and reproducibility (RSD between 0.8 and 3.5 %), was obtained when $(NH_4)_2SO_4$ was used as salting-out agent.

Subsequently, different amounts of $(NH_4)_2SO_4$ (0.8, 1.2, 1.6 and 2 g) were investigated in the aqueous sample solution. The recoveries for most of the BZs studied increased up to 1.6 g (**Figure 8.b**). Thus, 1.6 g was selected as optimum with recoveries higher than 80% in all cases.

The volume of extraction solvent is also an important parameter to take into account in the SALLE procedure. Thus, different volumes of MeCN were tested from 1 to 4 mL. As can be observed in **Figure 8.c**, the recoveries for all BZs increased with the volume of MeCN up to 2 mL. When the volume was lower than 2 mL, it was very complicated to collect the organic layer because the interface between the acetonitrile and the aqueous phase was not clear, thus the recoveries for all compounds were lower than 75 %. On the other hand, when MeCN volume was higher than 2 mL, the recoveries decreased for most compounds except for ABZ. Moreover, in the case of TCB and their metabolites, the recoveries were lower than 63 % when 3 and 4 mL of MeCN were employed. On the basis of the results, 2 mL of MeCN was chosen as a compromise for all subsequent studies.

Then, the volume of BZs aqueous standard solution used for the optimization of the extraction procedure was studied (2–6 mL). The recoveries of analytes increased up

to 4 mL of solution (recoveries in the range of 79-96%), remaining constant thereafter. Therefore, 4 mL was selected as the optimum volume.

Once the SALLE procedure was optimized with aqueous standard solutions, it was tested with fish samples using trout muscle tissue samples. Thus, different extractions were carried out for 0.5, 1.0, 1.5 and 2.0 g of fish. Each amount of sample was spiked with 50, 100, 150 and 200 µL respectively with the standard solution previously described in Section 2.2. The obtained concentrations for each BZ were 1.0 mg kg^{-1} (5-OH-TBZ, TCB, OXI), 0.5 mg kg^{-1} (TCB-SO, TCB-SO_2), 250 µg kg^{-1} (ABZ-SO), 125 µg kg^{-1} (CBZ, BEN, ABZ, TBZ), 62.5 µg kg^{-1} (ABZ-NH_2-SO_2), 15 µg kg^{-1} (ABZ-SO_2) and 4 µg kg^{-1} (FUB). Subsequently, 4 mL of deionized water was added following the sample treatment procedure described in section 2. It was observed that 0.5 g of fish tissue gave the best recoveries and cleaner extracts for all BZs.

3.3 Filter and sample solvent selection.

Preliminary studies showed that TCB and their metabolites (TCB-SO_2 and TCB-SO) were partially retained when nylon filters were employed just before the sample injection in the UHPLC with recoveries ≈38% (Figure SD3). Teflon (0.2 µm x 13 mm) and fiberglass syringe filters (0.7 µm x 13 mm) were also evaluated. No retention was observed with fiberglass filters (**Figure 9**) but, unfortunately, they were not able to provide clean extracts due to their high pore size (0.7 µm). When teflon filters were employed, these analytes were retained to a lesser extent (recoveries ≈84%) compared to nylon filters, so teflon filters were selected. To prevent this partial retention of TCB and their metabolites in the selected filters, different proportions of MeCN/H_2O (10:90; 20:80 and 30:70) were evaluated as sample solvent in order to redissolve the extract before filtering. With a mixture of MeCN/H_2O 30:70 (v/v) peak area was not satisfactory for most compounds, so MeCN/H_2O 20:80 (v/v) was

selected as optimum injection solvent because all analytes passed through the teflon filter without any loss in the analytical signal.

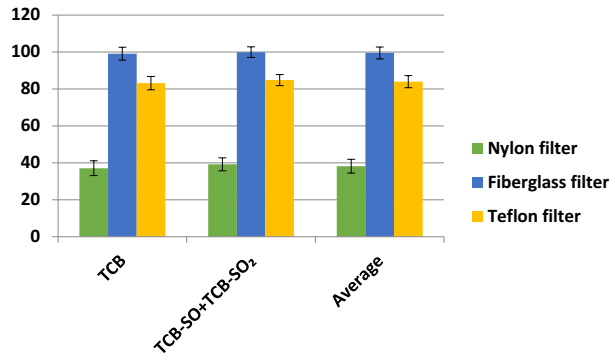


Figure 9. Optimization of the filter nature. Conditions: column temperature 40°C; flow rate 0.4 mL min^{-1}; injection volume 20 µL; mobile phase composition: water as eluent (A) and MeCN as eluent (B). BZs concentrations in FL: 1.0 mg kg^{-1} (5-OH-TBZ, TCB, OXI), 0.5 mg kg^{-1} (TCB-SO, TCB-SO_2), 250 µg kg^{-1} (ABZ-SO), 125 µg kg^{-1} (CBZ, BEN, ABZ, TBZ), 62.5 µg kg^{-1} (ABZ-NH_2-SO_2), 15 µg kg^{-1} (ABZ-SO_2) and 4 µg kg^{-1} (FUB). Error bars represent the standard error (n = 3).

3.4 Method characterization

The optimized methodology for the determination of 13 BZs in farmed fish muscle (trout, sea bream and sea bass) was characterized in terms of linearity, LODs, LOQs, intra-day and inter-day precision and trueness was finally evaluated with recovery experiments.

3.4.1 Calibration curves and analytical performance characteristics

Matrix-matched calibration curves were established using trout muscle samples free of analytes as representative matrix. Samples were spiked at six different analyte concentrations, including different levels for the different analytes, considering the differences in fluorescence intensity: 125, 250, 500, 750, 1000 and 1500 µg kg^{-1} for 5-OH-TBZ, TCB and TCB-SO+TCB-SO_2; 50, 125, 250, 500, 750 and 1000 µg kg^{-1} for OXI; 12.5, 31.3, 62.6, 125.3, 188 and 250 µg kg^{-1} for CBZ+BEN and ABZ-SO; 6.2, 15.7, 31.3, 62.6, 94 and 125 µg kg^{-1} for TBZ and ABZ; 7.8, 15.6, 31.3, 47, 62.5 and 94 µg

kg^{-1} for ABZ-NH_2-SO_2; 0.75, 1.9, 3.8, 7.5, 11.3 and 15 μg kg^{-1} for ABZ-SO_2; 0.2, 0.5, 1.0, 2.0, 3.0 and 4.0 μg kg^{-1} for FUB. Three samples of each concentration level were processed following the SALLE method and they were injected three times. Peak area was considered as a function of the analyte concentration in the samples. A blank sample was also processed, and none of the BZs were detected.

LODs and LOQs were calculated as 3×S/N and 10×S/N, respectively. Performance characteristics of the proposed method in trout muscle samples are shown in **Table 1**.

Table 1. Performance characteristics of the proposed SALLE-UHPLC-FL method in trout muscle samples.

Analyte	LOD (μg kg^{-1})	LOQ (μg kg^{-1})	Linear dynamic range (μg kg^{-1})	R^2
5-OH-TBZ	28.0	92.0	92-1500	0.9992
ABZ -NH_2-SO_2	1.7	5.6	5.6-94	0.9978
CBZ+BEN	2.1	7.1	7.1-250	0.9980
ABZ-SO	2.7	8.8	8.8-250	0.9991
TBZ	1.0	3.5	3.5-125	0.9979
FUB	0.05	0.15	0.15-4	0.9983
ABZ-SO_2	0.08	0.28	0.28-15	0.9993
OXI	11.0	36.0	36-1000	0.9980
ABZ	1.7	5.7	5.7-125	0.9983
TCB-SO_2 +TCB-SO	29.0	98.0	98-1500	0.9968
TCB	30.0	100.0	100-1500	0.9970

As can be seen, LOQs values ranged from 0.15 μg kg^{-1} for FUB to 100 μg kg^{-1} for TCB were obtained. A chromatogram corresponding to the BZs separation in an analyte-

free trout muscle sample spiked with each BZ at 750 µg kg^{-1} for 5-OH-TBZ, OXI, TCB and TCB-SO+TCB-SO_2; 188 µg kg^{-1} for CBZ+BEN and ABZ-SO; 94 µg kg^{-1} for TBZ and ABZ; 47 µg kg^{-1} for ABZ-NH_2-SO_2; 11.2 µg kg^{-1} for ABZ-SO_2; 3 for FUB µg kg^{-1} and a blank sample are shown in **Figure 10**.

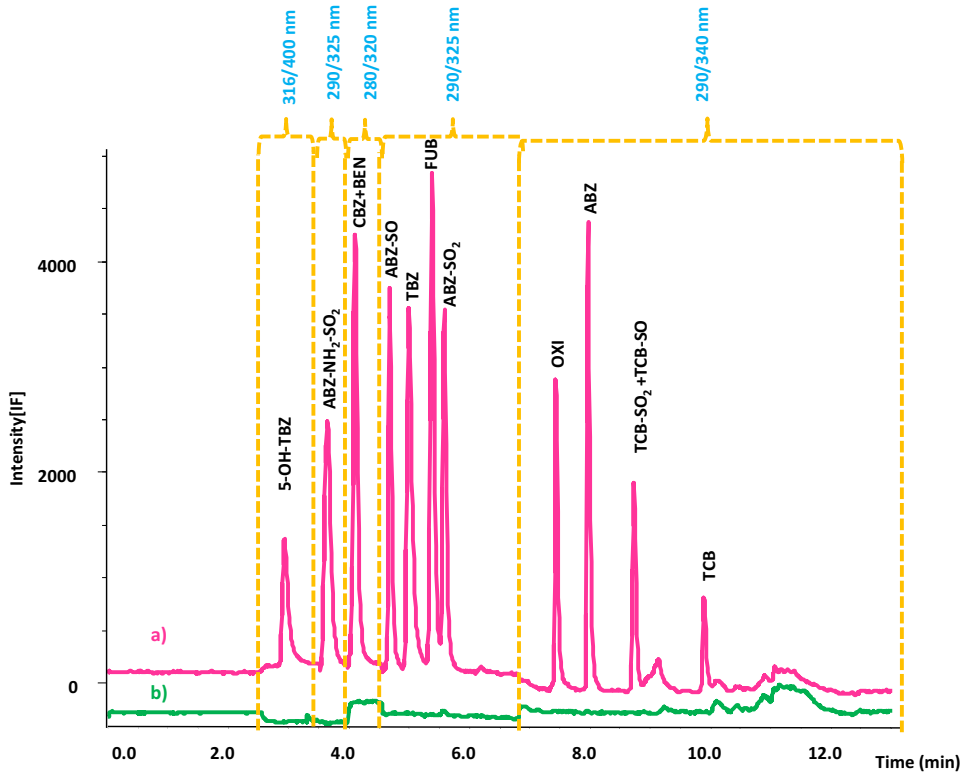


Figure 10. Chromatograms corresponding to (a) trout muscle tissue sample spiked with 750 µg kg^{-1} for 5-OH-TBZ, OXI, TCB and TCB-SO+TCB-SO_2; 188 µg kg^{-1} for CBZ+BEN and ABZ-SO; 94 µg kg^{-1} for TBZ and ABZ; 47 µg kg^{-1} for ABZ-NH_2-SO_2; 11.2 µg kg^{-1} for ABZ-SO_2; 3.0 for FUB µg kg^{-1} and (b) a blank of trout muscle sample.

3.4.2 Precision study

The precision of the method was evaluated in terms of repeatability (intraday precision n=6) and intermediate precision (interday precision n=12) by application of the proposed SALLE-UHPLC-FL method to trout muscle samples spiked at two different concentration levels of BZs (250 and 1000 µg kg^{-1} for 5-OH-TBZ, TCB and

TCB-SO+TCB-SO_2; 125 and 750 μg kg^{-1} for OXI; 31.3 and 188 μg kg^{-1} for CBZ+BEN and ABZ-SO; 15.7 and 94 μg kg^{-1} for TBZ and ABZ; 15.6 and 62.5 μg kg^{-1} for ABZ-NH_2-SO_2; 1.9 and 11.3 μg kg^{-1} for ABZ-SO_2 and 0.5 and 3.0 μg kg^{-1} for FUB). Repeatability was evaluated over three samples prepared for each concentration level and injected by duplicate on the same day and, under the same experimental and instrumental conditions. Intermediate precision was evaluated over two samples injected by duplicate during three consecutive days. The results expressed as %RSD of the peak areas, are shown in **Table 2.** Precision values in accordance with the guideline 2002/657/EC were obtained, with RSD lower than 10.8% in all cases.

3.4.3 Recovery studies

Recovery experiments were accomplished for three different types of farmed fish muscle samples (trout, sea bream and sea bass) spiked at two different BZ concentration levels (the same of the precision study). Relative recoveries were calculated by comparing peak areas of fish muscle samples spiked before the sample treatment with peak areas of extracts from fish muscle samples, spiked after the sample treatment, just before the measurement. In all cases, a fish muscle sample was analyzed prior to our deliberate spiking to check for the presence of BZs, and none of them gave signals corresponding to concentrations above the LODs. Recoveries higher than 79 % were obtained for all BZs with RSDs for n=4 lower than 7.8 % in all cases (**Table 3**).

Table 2. Repeatability and Intermediate precision study for the proposed SALLE-UHPLC-FL method

	[a]5-OH- TBZ	[b] ABZ - NH_2-SO_2	[c]CBZ+BEN	[c]ABZ-SO	[d]TBZ	[f]FUB	[g]ABZ-SO_2	[e]OXI	[d]ABZ	[a]TCB-SO_2 +TCB-SO	[a]TCB
Repeatability RSD (%) (n=6)											
Level 1	3.6	4.2	4.6	3.9	4.6	3.9	5.9	5.6	4.8	6.3	6.1
Level 2	1.5	3.0	2.1	2.1	1.9	2.7	2.4	3.8	2.7	4.2	5.5
Intermediate precision RSD (%) (n=12)											
Level 1	6.0	7.5	9.0	6.2	6.3	4.7	8.8	6.5	6.9	8.1	10.8
Level 2	2.8	4.7	3.1	4.9	2.5	3.2	4.0	5.4	5.3	7.3	8.9

[a]**Level 1:** 250 µg Kg^{-1} and **Level 2:** 1000 µg Kg^{-1}; [b]**Level 1:** 15.6 µg Kg^{-1} and **Level 2:** 62.5 µg Kg^{-1}; [c]**Level 1:** 31.3 µg Kg^{-1} and **Level 2:** 188 µg Kg^{1}; [d]**Level 1:** 15.7 µg Kg^{-1} and **Level 2:** 94 µg Kg^{-1}; [e]**Level 1:** 125 µg Kg^{-1} and **Level 2:** 750 µg Kg^{-1}; [f]**Level 1:** 0.5 µg Kg^{-1} and **Level 2:** 3 µg Kg^{-1}; [g]**Level 1:** 1.9 µg Kg^{-1} and **Level 2:** 11.3 µg Kg^{-1}

Table 3. Recoveries and RSD (n=4) for the proposed SALLE-UHPLC-FL method in trout muscle, sea bream muscle and sea bass muscle

			[a]5-OH-TBZ	[b]ABZ -NH_2-SO_2	[c]CBZ+BEN	[c]ABZ -SO	[d]TBZ	[f]FUB	[g]ABZ -SO_2	[e]OXI	[d]ABZ	[a]TCB-SO_2 +TCB-SO	[a]TCB
Trout muscle	Level 1	Recoveries (%)	93.9	90.8	86.4	85.8	92.0	81.2	82.3	89.8	84.5	91.4	86.8
		(RSD, %)	5.0	4.5	2.9	5.0	3.3	5.3	6.6	4.7	5.4	5.7	5.4
	Level 2	Recoveries (%)	96.1	88.3	95.6	86.9	90.7	94.9	93.5	85.7	92.4	91.6	96.8
		(RSD, %)	2.5	3.3	2.6	2.9	1.8	2.7	3.2	2.7	3.7	3.5	2.5
Sea bream muscle	Level 1	Recoveries (%)	88.9	93.0	86.0	94.3	87.7	84.7	94.2	80.9	85.8	81.4	90.6
		(RSD, %)	3.9	3.4	5.8	4.2	4.6	4.3	5.0	7.4	4.2	5.0	3.9
	Level 2	Recoveries (%)	86.7	91.6	90.3	85.8	87.5	95.9	98.3	97.3	86.5	84.9	90.8
		(RSD, %)	2.6	2.6	1.8	1.9	2.1	2.9	3.6	3.8	1.8	3.6	2.6
Sea bass muscle	Level 1	Recoveries (%)	90.9	91.1	90.8	83.1	90.8	91.4	91.6	85.7	87.7	79.0	85.0
		(RSD, %)	4.5	3.4	4.5	4.0	3.8	3.9	4.3	5.4	4.4	5.0	7.8
	Level 2	Recoveries (%)	90.7	86.0	92.4	88.8	93.7	90.5	97.6	81.6	80.1	86.1	94.6
		(RSD, %)	1.5	1.8	2.8	2.5	2.5	1.5	2.8	1.2	3.4	2.5	3.6

[a]**Level 1:** 250 μg Kg^{-1} and **Level 2:** 1000 μg Kg^{-1}; [b]**Level 1:** 15.6 μg Kg^{-1} and **Level 2:** 62.5 μg Kg^{-1}; [c]**Level 1:** 31.3 μg Kg^{-1} and **Level 2:** 188 μg Kg^{-1}; [d]**Level 1**: 15.7 μg Kg^{-1} and **Level 2:** 94 μg Kg^{-1}; [e]**Level 1:** 125 μg Kg^{-1} and **Level 2:** 750 μg Kg^{-1}; [f]**Level 1:** 0.5 μg Kg^{-1} and **Level 2:** 3 μg Kg^{-1}; [g]**Level 1:** 1.9 μg Kg^{-1} and **Level 2:** 11.3 μg Kg^{-1}

4. Conclusions

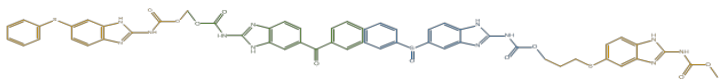
In this chapter, an UHPLC-FL methodology has been used for the first time to determine thirteen BZ drugs in farmed fishes in 10 min, involving a total analysis time lower than 12 min. SALLE has been proposed as sample treatment, offering a very efficient, easy and fast sample treatment procedure that allows the extraction and clean-up of these residues in complex and less commonly explored matrices to be performed simultaneously. Thus, the time required and the amount of solvent used are drastically reduced.

Good linearity, satisfactory trueness and precision and high sensitivity for most of the BZs, with LOQs in the range of low µg L^{-1} were obtained. The whole SALLE-UHPLC-FL method allowed recoveries higher than 79 % for all the studied analytes. Detection limits between 0.04-29.9 µg kg^{-1} were obtained in all cases, demonstrating the applicability of this quick, selective and simple method for the monitoring of BZ residues in laboratories of food quality and safety control.

This work was published as:

***"Ultra-high performance liquid chromatography with fluorescence detection following salting-out assisted liquid – liquid extraction for the analysis of benzimidazole residues in farm fish samples"*, C. Tejada-casado, F.J. Lara, A.M. García-campa, M. Olmo-iruela, J.chromatogr.A. 1543 (2018) 58–66.**

CHAPTER 3



DETERMINATION OF BENZIMIDAZOLES IN MEAT SAMPLES BY CAPILLARY ZONE ELECTROPHORESIS TANDEM MASS SPECTROMETRY FOLLOWING DISPERSIVE LIQUID-LIQUID MICROEXTRACTION

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1. Summary

A novel method based on CZE-MS/MS has been proposed and validated for the identification and simultaneous quantification of twelve BZs in meat samples. Electrophoretic separation was carried out using 500 mM formic acid (pH 2.2) as background electrolyte and applying a voltage of 25 kV at 25˚C. In order to improve the sensitivity, stacking mode injection was applied, using as injection solvent a mixture of 30:70 acetonitrile/water at 50 mbar for 75 s. Sensitivity enhancement factors from 74 to 317 were obtained under these conditions. Detection using an ion trap as analyzer, operating in multiple reactions monitoring (MRM) mode was employed. The main MS/MS parameters as well as the composition of the sheath liquid and other electrospray variables were optimized in order to obtain the highest sensitivity and precision in conjunction with an unequivocal identification. The method was applied to poultry and pork muscle samples. The deproteinization of samples and extraction of BZs was carried out with acetonitrile. $MgSO_4$ and NaCl were added as salting-out agents. Subsequently, dispersive liquid-liquid microextraction (DLLME) was applied as clean up procedure. The organic layer (MeCN, used as dispersant) containing the BZs was mixed with the extractant (chloroform) and both were injected in water, producing a cloudy solution. Recoveries for fortified samples were higher than 70 %, with RSDs lower than 16% were obtained in all cases. The LODs were below 3 μg Kg^{-1}, demonstrating the applicability of this fast, simple, and environmentally friendly method.

2. Materials and methods

2.1 Materials and reagents

All reagents used in this study were of analytical grade, unless otherwise indicated, and the solvents were of HPLC grade. Ultrapure water (Milli-Q plus system, Millipore, Bedford, MA, USA) was used throughout the work. MeCN, MeOH and IPA (LC-MS HiPerSolv grade) were supplied from VWR (Radnor, PA, USA). Acetic acid and formic acid were obtained from Sigma Aldrich (St. Louis, MO, USA). Ammonia solution (NH_3) (30%) and sodium hydroxide (NaOH) were obtained from Panreac-Química (Madrid, Spain). Chloroform ($CHCl_3$) was purchased from VWR International (West Chester, PA, USA). EtOH was purchased from Merck (Darmstadt, Germany).

Analytical standards of ABZ**,** ABZ-SO_2, ABZ-SO, CBZ, BEN, FBZ, FBZ-SO_2, FBZ-SO, MBZ, OXI, TBZ and MBZ-OH were supplied by Sigma-Aldrich (St. Louis, MO, USA).

Individual stock standard solutions of 1000 mg L^{-1} (ABZ-SO, FBZ-SO, MBZ-NH_2, TBZ), 500 mg L^{-1} (ABZ, ABZ-SO_2, BEN, MBZ-OH) and 250 mg L^{-1} (FBZ, FBZ-SO_2, MBZ, OXI, CBZ) were prepared by dissolving accurately weighed amounts of each compound in MeOH. They were stored in the dark at -20 °C, being stable for at least 6 months. Intermediate stock standard solutions containing 100 mg L^{-1} of each BZ were obtained by mixing individual stock standard solutions and subsequent dilution with 30:70 MeCN /water (v/v). They were also stored in dark bottles at 4°C. Working standard solutions containing all the BZs were freshly prepared by the proper dilution of the intermediate stock standard solutions at the required concentration.

Nylon syringe filters, 0.2 µm x 13 mm (Bonna-Agela Technologies Inc, Wilmington, USA) were used for filtration of sample extracts prior to the injection into the CE system.

2.2 Instrumentation

CE experiments were carried out with an HP3DCE instrument (Agilent Technologies, Waldbronn, Germany). The coaxial sheath liquid sprayer was supplied by Agilent Technologies. Mass spectra were performed using an Agilent 1100 Series LC/MSD SL mass spectrometer equipped with an ion trap (IT) mass analyzer. The mass spectrometer was controlled by a PC running the Esquire software 4.1 from Bruker Daltonics (Bremen, Germany). Separations were performed in an uncoated fused-silica capillary (100 cm total length, 50 µm i.d., 375 µm o.d.) from Polymicro Technologies (Phoenix, AZ, USA). A KD Scientific 100 series syringe pump (KD Scientific Inc., Holliston, MA, USA) was used for sheath liquid supplying.

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

2.3 CE conditions

Before the first use, the new capillary was rinsed with 1M NaOH for 10 min, followed by deionized water for 10 min, and then with the running buffer for 20 min. At the beginning of each day, the capillary was preconditioned with 5 M NH_3 for 3 min, deionized water for 3 min and finally with the running buffer for 20 min. In order to obtain an adequate repeatability in each analysis, before each run the capillary was prewashed with 1 M NH_3 for 2.5 min, deionized water for 1 min and at the end with the BGE for 5 min. At the end of each day, the capillary was washed with water during 5 min, and dried with air for another 5 min. In all instances, a N_2 pressure of 1 bar was applied. A voltage of 25 kV was applied for the electrophoretic separation. The temperature of the capillary was kept constant at 25°C. The BGE for CZE

separation was an aqueous solution of 500 mM formic acid at pH 2.2, obtaining an electric current of 21 µA. The sample solvent was a mixture 30:70 MeCN/water (v/v). The sample was hydrodynamically injected for 75 s at 50 mbar.

2.4 MS and MS/MS conditions

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 optimized. The direct infusion of the analytes to optimize ion optics in MS is an usual way to do it. However, a more realistic approach, which involves performing the study under CE separation conditions, was employed. This was carried out with BGE containing the BZs (1 mg L^{-1} for each compound) and applying 25 kV in order to get a constant concentration of them in the electrospray. The final optimum values for MS detection are shown in **Table 1**.

Table 1. MS ion optic parameters

	Time segments (min)	
	0-22.5 min	22.5-32 min
	TBZ, CBZ and BEN	OXI, ABZ, FBZ, MBZ-OH, MBZ, ABZ-SO, FBZ-SO, ABZ-SO_2 and FBZ-SO_2
Capillary (V)	-4500	-4500
Skimmer (V)	44.2	46.5
Cap Exit (V)	144.2	147.9
Oct 1DC	11.3	13.1
Oct 2DC	1.3	1.8
Trap Drive	29.8	41.1
OCT RF	110.1	140.6
Lens 1 (V)	-4.1	-4.4
Lens 2 (V)	-60.6	-66.7

The mass spectrometer was operated in the positive mode with an electrospray ionization (ESI) voltage of -4500 V. Sheath liquid, consisting of a mixture of 50:49.5:0.5 (v/v/v) EtOH/water/formic acid, was delivered at a flow rate of 0.1 mL h^{-1}. Other electrospray parameters optimized were dry gas temperature: 250 ^{o}C; nebulizer pressure: 6 psi and dry gas flow rate: 8 L/min. Scan range was established from 180.0 to 350.0 m/z. In all cases an isolation width of 1 m/z was selected. In the MS experiments, IT parameters were studied using the ion charge control mode (ICC), setting a target of 100000 ions, maximum accumulation time of 250 ms and an average of 4 scans per spectra. In MS/MS experiments a maximum accumulation time of 100 ms was selected with a target of 50000 ions and an average of 2 scans per spectra were established. Fragmentation was carried out by means of collision induced dissociation with the helium present in the trap for 40 ms in MRM mode. Product ions were monitorized in the range of 50.0-300.0 m/z. MS/MS parameters are summarized in **Table 2**.

Table 2. Main parameters for MS/MS detection.

	Time segments										
	0-22.5 min		23-32 min								
	TBZ	**CBZ+BEN**	**OXI**	**ABZ**	**FBZ**	**MBZ-OH**	**MBZ**	**ABZ-SO**	**FBZ-SO**	**ABZ-SO_2**	**FBZ-SO_2**
Amplitude fragmentation (V)	0.5	0.35	0.4	0.5	0.6	0.4	0.7	0.45	0.33	0.5	0.6
Precursor ion $[M+H]^+$	202	192	250	266	300	298	296	282	316	298	332
Product ions	175	160	218	234	268	266	264	240 222	284	266	300
	159		176	192	190	160	105	191	191	224	159

2.5 Sample treatment procedure

Poltry and porcine muscle samples were collected in a local store (Granada, Spain). The sample treatment was performed as follows: samples were crushed and homogenized. Portions of 1 g were placed in 15 mL conical tubes. Then, 1 mL of deionized water was added to the sample, and the mixture was agitated by vortex until a proper homogenization was achieved. Later on 2 mL of MeCN were added and mechanically agitated for 30 s. To induce the separation between the organic and aqueous phases 0.5 g of $MgSO_4$ and 0.1 g of NaCl were added and samples were mechanically agitated for 5 min. Then the mixture was centrifuged at 9000 rpm for 10 min. Subsequently, 1700 µL of the upper phase was transferred into a 4-mL vial. A mixture of this organic phase containing the extracted analytes (disperser solvent) and 950 µL of $CHCl_3$ (extractant) was injected in 5 mL of deionized water for DLLME. Then, the ternary system was vigorously shaken by hand for 60 s and a stable cloudy solution was formed. The mixture was centrifuged for 2 min at 5000 rpm for phase separation. The $CHCl_3$ phase was collected and dried under a gentle nitrogen stream. Finally, the residue was reconstituted with 250 µL of 30:70 MeCN/water (v/v) and filtered before injection into the electrophoretic system. Taking into account all steps involved in this procedure, the sample throughput was approximately 12 samples per hour. The sample procedure it is shown in **Figure 1.**

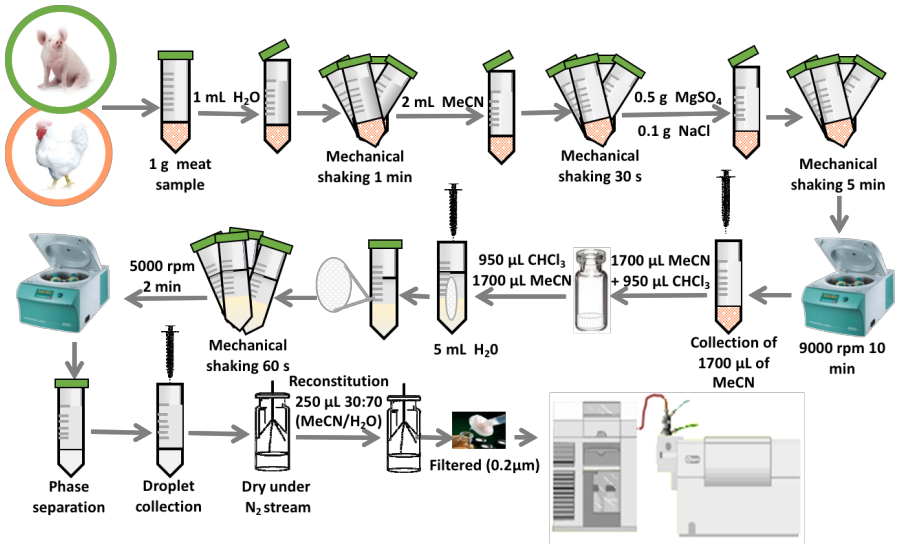

Figure 1. Sample treatment procedure

3 Results and discussion

3.1 Optimization of electrophoretic separation

The main variables affecting the electrophoretic separation of BZs by CZE were optimized. The use of volatile running buffers in CE-MS is recommended to prevent contamination of the MS and, therefore, analyte signal suppression effects. First of all, the effect of pH was studied. BZs have two p*K*a values, p*K*a$_1$ ~3-6 and p*K*a$_2$ ~10-12 corresponding to two amino groups which can be protonated or deprotonated under different conditions [1,2]. The higher differences in the electrophoretic mobilities can be achieved when the pH is slightly below p*K*a$_1$ or slightly above p*K*a$_2$. From an experimental point of view,

[1] M. Danaher, H. De Ruyck, S.R.H. Crooks, G. Dowling, M. O'Keeffe, Review of methodology for the determination of benzimidazole residues in biological matrices, J. Chromatogr. B 845 (2007) 1-37.

[2] A. Martínez-Villalba, E. Moyano, M.T. Galcerán, Ultra-high performance liquid chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry for the analysis of benzimidazole compounds in milk samples, J. Chromatogr. A 1313 (2013) 119–131.

the use of acidic conditions is more compatible with positive ionization and, the required basic conditions might be too strong for a volatile buffer such as ammonia. Thus, the pH effect was studied between 2.0 and 4.0 and formic acid (p*K*a = 3.75) was selected for this study. As can be observed in **Figure 2** when the pH was higher than 3 the peak shape and resolution were inadequate for some compounds such as ABZ-SO_2 and FBZ-SO_2.

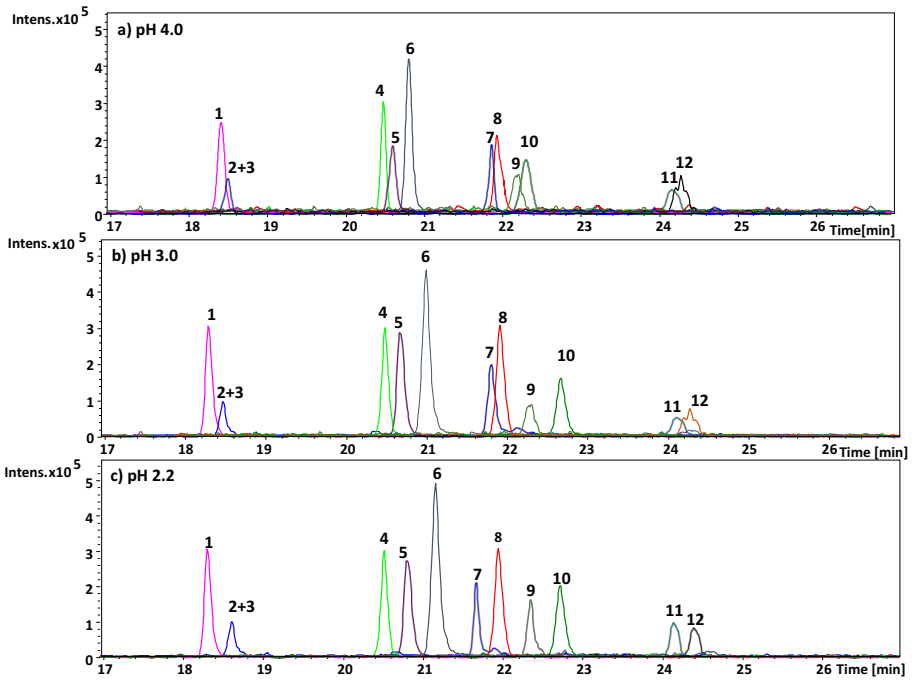

Figure 2. Optimization of the buffer pH. a) pH 4.0; b) pH 3.0; c) pH 2.2. Conditions: BGE, 500 mM formic acid; BZs concentration 500 µg L^{-1}; capillary temperature, 25˚C; separation voltage, 25 kV; sample injection 50 mbar for 75 s; sample solvent: water. Peak identification: 1, TBZ; 2+3, CBZ+BEN; 4, OXI; 5, ABZ; 6, FBZ; 7, MBZ-OH; 8, MBZ; 9, ABZ-SO; 10, FBZ-SO; 11, ABZ-SO_2; 12, FBZ-SO_2.

This might be due to the fact that BZs become neutral. The best results in terms of separation efficiency and analysis time were obtained using a pH of 2.2 (**Figure 2.c**).

Then, formic acid concentration was studied between 300 and 1000 mM at pH 2.2. It was observed (**Figure 3.a**) that with buffer concentrations lower than 500 mM the separation was not satisfactory.

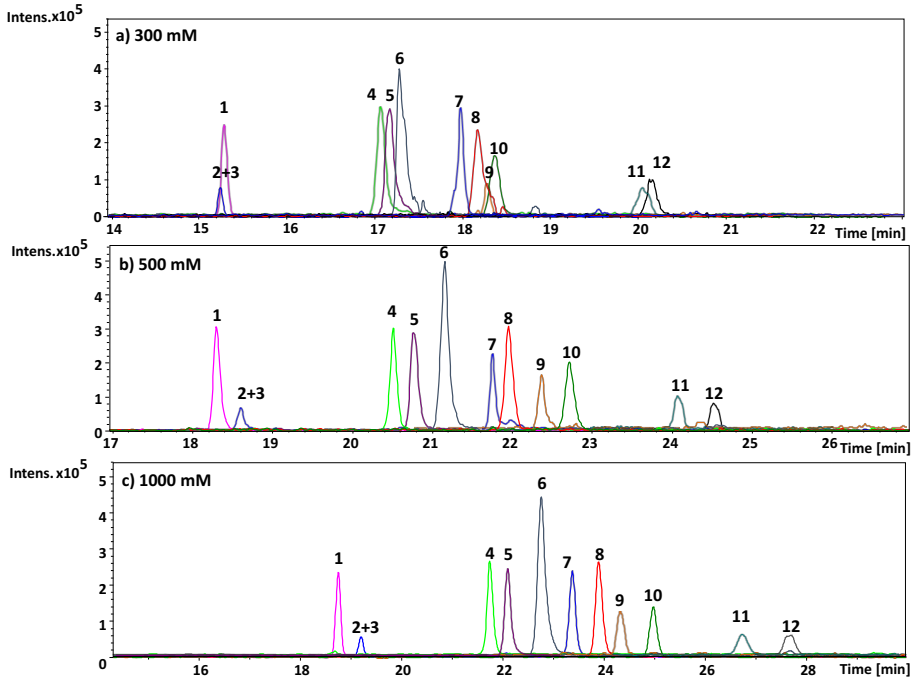

Figure 3. Optimization of the buffer concentration. a) 300 mM; b) 500 mM; c) 1000 mM. Conditions: BGE, formic acid pH 2.2; BZs concentration 500 µg L^{-1}; capillary temperature, 25 °C; separation voltage, 25 kV; sample injection 50 mbar for 75 s; sample solvent: water. Peak identification: 1, TBZ; 2+3, CBZ+BEN; 4, OXI; 5, ABZ; 6, FBZ; 7, MBZ-OH; 8, MBZ; 9, ABZ-SO; 10, FBZ-SO; 11, ABZ-SO_2; 12, FBZ-SO_2.

A formic acid buffer with a concentration of 500 mM was enough to achieve the full separation of all BZs (**Figure 3.b**). With concentrations higher than 500 mM peak shapes were not improved and the analysis time was increased (**Figure 3.c**). It must be remarked that CBZ and BEN cannot be separated with this method. This is due to the fact that BEN is easily transformed into CBZ as

soon as it dissolves in water [3]. For this reason, both compounds were quantified in a single peak.

Then, the separation voltage and the effect of the temperature were optimized. 25 kV was selected as a compromise between running time, resolution, and electric current and a capillary temperature of 25 ^{o}C.

In order to improve the sensitivity of the method, stacking mode injection was considered. For this purpose, different injection solvents such as water and several MeCN/water combinations (15/85; 20/80; 30/70 and 40/60) were evaluated. As shown in **Figure 4**, the mixture 30:70 (v/v) MeCN/water showed higher sensitivity in terms of signal to noise ratio (S/N).

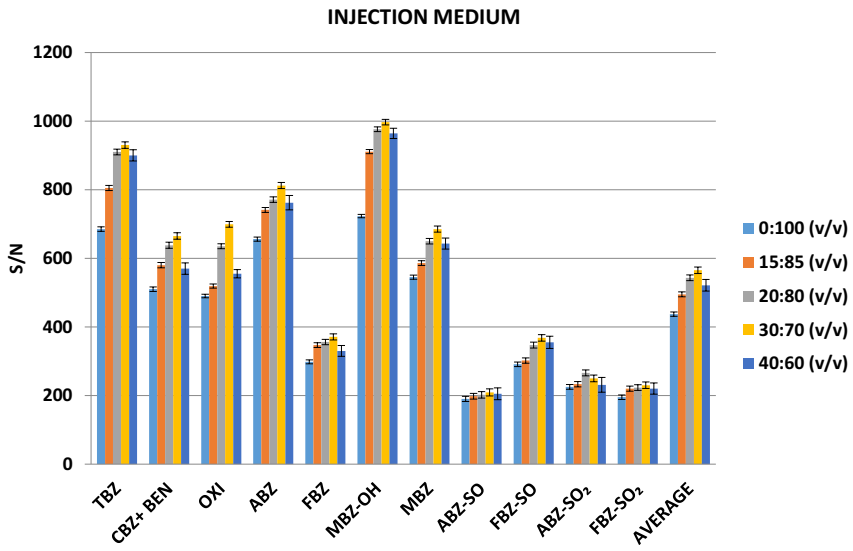

Figure 4. Optimization of the injection medium in terms of S/N ratio using different proportions of MeCN/water (v/v). Conditions: BGE, 500 mM formic acid (pH 2.2); BZs concentration 500 µg L^{-1}; temperature capillary, 25 °C; separation voltage, 25 kV and sample injection 50 mbar for 75 s. Error bars represent the standard error (n = 3).

[3] E. Mallat, D.Barceló, R.Tauler, Degradation study of benomyl and carbendazim in water by liquid chromatography and multivariate curve resolution methods, Chromatographia 46 (1997) 342–350.

Then, the hydrodynamic injection time was studied from 10s to 100s at 50 mbar. Maximum sensitivity was achieved using an injection of 75s at 50 mbar. Injection times higher than 75s produced peak broadening. This injection time corresponds to an approximate volume of 76.7 nL (4 % of the total volume of the capillary). Sensitivity enhancement factors based on peak heights (SEF_{height}) were estimated.

$$*SEF_{height} = \frac{\text{Peak height under NSM injection/Analyte concentration in NSM injection}}{\text{Peak height under hydrodynamic injection /Analyte concentration in hydrodynamic injection}}$$

*NSM injection: 50 mbar for 75s and 30:70 MeCN/H_2O as sample solvent.
Hydrodynamic injection: 50 mbar for 5 s and BGE as sample solvent.

The values of SEF_{height} obtained for all studied analytes and the injection conditions are included in **Table 3**. The use of this type of on-line preconcentration allowed the obtention of enhancement factors ranging from 75 to 317.

Table 3. Sensitivity enhancement factors based on peak heights (SEF height) and RSD values in percentages for studied BZs

Analyte	SEF height*	RSD (%) (n=3)
TBZ	141.3	1.3
CBZ+BEN	99.7	1.5
OXI	100.2	3.3
ABZ	204.7	3.4
FBZ	284.5	3.2
MBZ-OH	137.6	3.8
MBZ	217.9	5.9
ABZ-SO	74.8	4.2
FBZ-SO	317.3	3.9
ABZ-SO_2	217.8	3.7
FBZ-SO_2	122.1	4.1

3.2 Optimization of CZE-ESI-MS/MS procedure

Selection of the best sheath-liquid parameters in CZE-ESI-MS/MS separation is a key step to improve the sensitivity of the method. Thus, sheath-liquid composition, dry gas temperature and flow rate, nebulizer pressure and dry gas flow were optimized using a standard solution of 500 μg L^{-1} of each BZ. The signal-to-noise-ratio (S/N) was selected as response variable.

First of all, the sheath-liquid composition was evaluated considering a flow rate of 0.2 mL h^{-1}, a dry gas temperature of 250°C, a nebulizer pressure of 8.0 psi and a dry gas flow rate of 8.0 L min^{-1}. The influence of the nature and percentage of the organic solvent (MeOH, IPA, EtOH) in the sheath-liquid was studied. As it is shown in **Figure 5** (**a**,**b**), the response was similar for MeOH and EtOH, particularly for the analytes with smaller S/N ratio. Taking into account that EtOH is more environmentally friendly, it was selected as the organic solvent at a percentage of 50 %. Acetic acid or formic acid are very appropriate sheath-liquid additives as they ensure a proper analyte ionization and, therefore, good reproducibility. As can be observed in **Figure 5.c**, the best results in terms of S/N and signal reproducibility were reached with formic acid. Sequentially different percentages of formic acid were evaluated selecting 0.5% as optimum (**Figure 5.d**). In summary, the optimum sheath-liquid composition for the detection of BZs in CZE-MS/MS was a mixture of EtOH:water:formic acid (50:49.5:0.5).

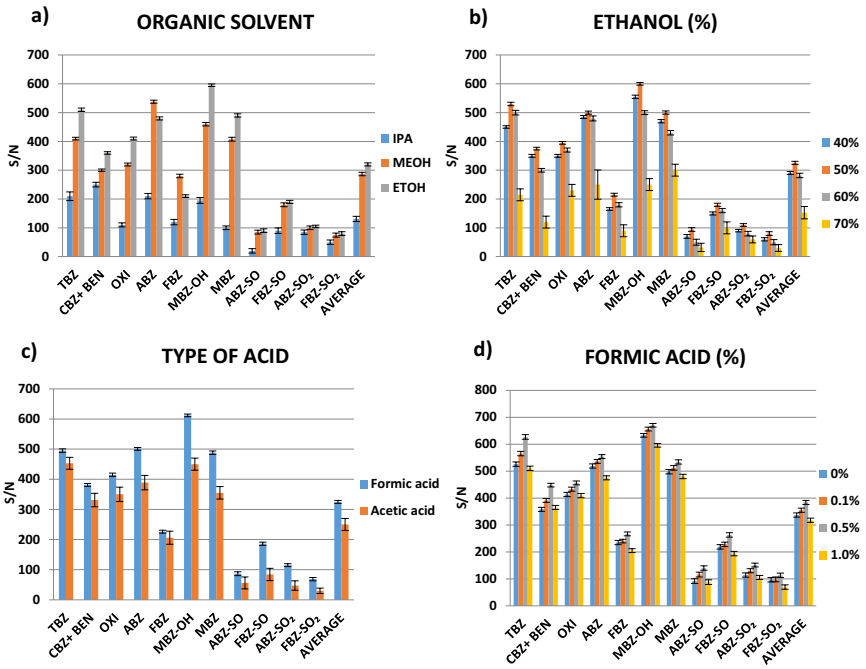

Figure 5. Effect of sheath liquid composition on S/N ratio. a) Type of organic solvent; b) Percentage of ethanol; c) Type of acid and d) Percentage of formic acid. Conditions: BGE, 500 mM formic acid (pH 2.2); BZs concentration 500 µg L^{-1}; capillary temperature, 25 °C; separation voltage, 25 kV; sample injection 50 mbar for 75 s; sample solvent, water. Error bars represent the standard error (n = 3).

The flow rate of the sheath-liquid was studied from 0.05 to 0.2 mL h^{-1}. As can be observed in **Figure 6.a**, increasing the flow rate showed a reduction in the S/N ratio. However, at a sheath-liquid flow of 0.05 mL h^{-1}, the spray stability decreased. Thus, an optimum value of 0.1 mL h^{-1} was selected. The dry gas temperature was fixed at 250°C. Then, nebulization pressure was studied between 4 and 10 psi. As can be observed in **Figure 6.b**, an increment of the nebulizer pressure from 4 to 6 psi had a positive effect. This is due to the fact that higher nebulizer pressures produce smaller spray droplets, enhancing analyte desolvation, and thus promoting ESI. Nevertheless, spray stability decreased at a nebulizer pressure of 8 psi. For this reason, a nebulizer pressure

of 6 psi was selected as optimum. The dry gas flow rate selected was 8 L min^{-1}.

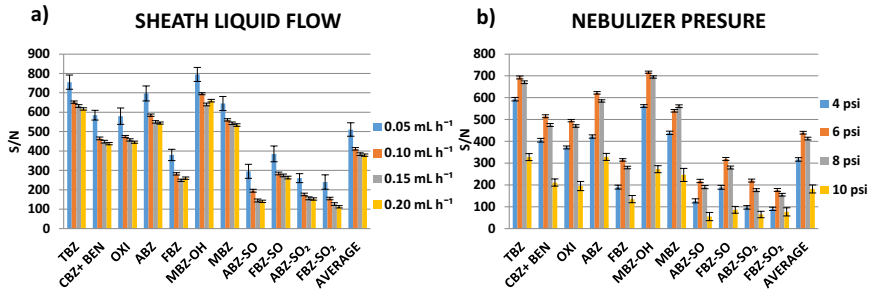

Figure 6. Effect of ESI conditions on S/N ratio. a) Sheath liquid flow; b) Nebulizer pressure. Conditions: BGE, 500 mM formic acid (pH 2.2); BZs concentration 500 µg L^{-1}; capillary temperature, 25 °C; separation voltage, 25 kV; sample injection 50 mbar for 75 s; sample solvent, water. Sheath liquid composition: ethanol/water/formic acid (50:49.5:0.5, v/v/v). Error bars represent the standard error (n = 3).

Finally, the optimization of the MS/MS parameters was carried out. Considering that some peaks were partially overlapped, the MRM mode was chosen. For fragmentation experiments, a cut-off of 27% of the precursor mass was fixed (i.e. the minimum m/z of the fragment ion able to be trapped by the analyzer). BZs were fragmented using the SmartFrag™ option that automatically ramps the fragmentation energy from 30 to 200% of the excitation amplitude. The fragmentation amplitude was manually varied and it was optimized considering that parent ion should be detected as it is recommended by the current legislation [4]. As can be observed in **Table 2**, it was enough to use MS^2 to achieve the minimum number of product ions for most of the studied BZs. Only in the case of CBZ+BEN, a single product ion was

[4] European Commission, European Commission decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, Commission Decision 2002/657/EEC, Off. J. Eur. Commun. L221 (2002) 8–36.

observed. Further attempts to get a second fragment by MS^3 were not successful.

3.3 Optimization of sample treatment

As stated in the Introduction section in this work DLLME, a miniaturized, fast and environment friendly procedure, has been used as clean up step after the extraction of the analytes from meat samples.

The starting DLLME conditions were obtained from a previous work, which has been developed in our group for the determination of 7 BZs (CBZ, MBZ, OXI, ABZ, FBZ, febantel (FBT) and parbendazol (PBZ)) in environmental water samples [5]. The extraction efficiency of DLLME for this new mixture of BZs showed values of recovery higher than 80 % for all the compounds. These values are similar to those obtained in the previous work so, it was not required to optimize the procedure again.

On the other hand, it was necessary to evaluate the extraction step previous to the DLLME sample treatment. The optimization was carried out using 1 g of poultry muscle (as representative matrix) free of BZs and spiked with 200 µg kg^{-1} of each BZ. The extraction of BZs was carried out using MeCN, which also acts as disperser solvent in the followed DLLME process. The recovery percentage was used to evaluate the extraction efficiency. A volume of 1 mL of water was added previously to get a better homogenization. As the addition of salt to the system significantly improved the extraction efficiency by salting-out effect, the influence of the ionic strength was investigated by adding different amounts of NaCl (0.05, 0.1 and 0.2 g) and $MgSO_4$ (0.1, 0.5 and 1.0). As can be observed in **Figure 7**, a mixture of 0.1 g of NaCl and 0.5 g of $MgSO_4$

[5] C. Tejada-Casado, M. Hernández-Mesa, M. del Olmo-Iruela, A.M. García-Campaña, Capillary electrochromatography coupled with dispersive liquid-liquid microextraction for the analysis of benzimidazole residues in water samples, Talanta 161 (2016) 8-14.

was enough to obtain a well-defined phase separation, obtaining also the highest recoveries. Thus, it was selected as optimum.

Finally, the effect of the volume of the reconstitution solvent was studied in the range of 250 to 1000 µL. A volume of 250 µL of 30:70 MeCN/water (v/v) was enough to dissolve the analytes and allowed the obtention of the highest enrichment factors for BZs.

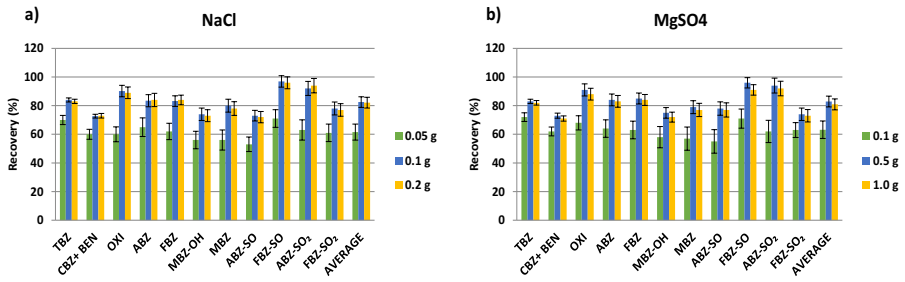

Figure 7. Extraction procedure optimization. Effect of the amount of a) NaCl and b) $MgSO_4$ on recoveries. Error bars represent the standard error (n = 3).

3.4 Method characterization

In order to check the applicability of the method for the determination of BZs in meat samples, linear dynamic ranges, LODs and LOQs, matrix effect, intra-day and inter-day precision and trueness were assessed.

3.4.1 Calibration curves and analytical performance characteristics

Matrix-matched calibration curves were established by the optimized DLLME-CZE-MS/MS method using poultry muscle free of analytes as representative matrix. Samples were spiked at seven different concentration levels: 5, 10, 25, 50, 75, 100, 125 µg L^{-1} for TBZ and CBZ+BEN; 10, 20, 50, 100, 150, 200, 250 µg L^{-1} for OXI, ABZ, FBZ, MBZ, FBZ-SO and ABZ-SO_2 and 20, 40, 100, 200, 300, 400, 500 µg L^{-1} for ABZ-SO, MBZ-OH and FBZ-SO_2. Two samples of each concentration level were processed following the DLLME method and they

were injected in duplicate. A blank sample was also processed, and no peaks were detected at BZs migration times. The sum of the peak areas of all the product ions and precursor ion (see **Table 2**) was considered the analytical signal.

LODs and LOQs of the method were calculated as the minimum analyte concentration yielding a signal-to-noise ratio equal to three and ten, respectively. Performance and statistic characteristics of the proposed method in poultry muscle are shown in **Table 4**. As can be seen, LOQs lower than 16 µg Kg^{-1} were obtained for all the analytes, being considerably lower than their corresponding MRLs. Therefore, the proposed method is adequate for the determination of very low concentration levels of these compounds in the selected matrix.

Table 4. Statistics, performance characteristics and matrix effect of the DLLME-CZE-MS/MS method for the analysis of BZs in poultry muscle.

Analyte	MRL (µg kg^{-1})	LOD (µg kg^{-1})	LOQ (µg kg^{-1})	Linear dynamic range (µg kg^{-1})	R^2	ME (%)
TBZ	100	1	4	4-125	0.9987	-12
CBZ+BEN	-	1	4	4-125	0.9971	-14
OXI	100	2	6	6-250	0.9982	-16
ABZ	100	2	8	8-250	0.9917	-27
FBZ	50	2	6	6-250	0.9941	-28
MBZ-OH	60	2	5	5-500	0.9963	-23
MBZ	60	1	4	4-250	0.996	-33
ABZ-SO	100	3	9	9-500	0.9914	-1
FBZ-SO	50	2	6	6-250	0.9928	-27
ABZ-SO_2	100	5	16	16-250	0.9959	-10
FBZ-SO_2	50	4	11	11-500	0.9981	-29

An electropherogram corresponding to BZs separation in poultry muscle samples spiked at different BZ concentrations is shown in **Figure 8**.

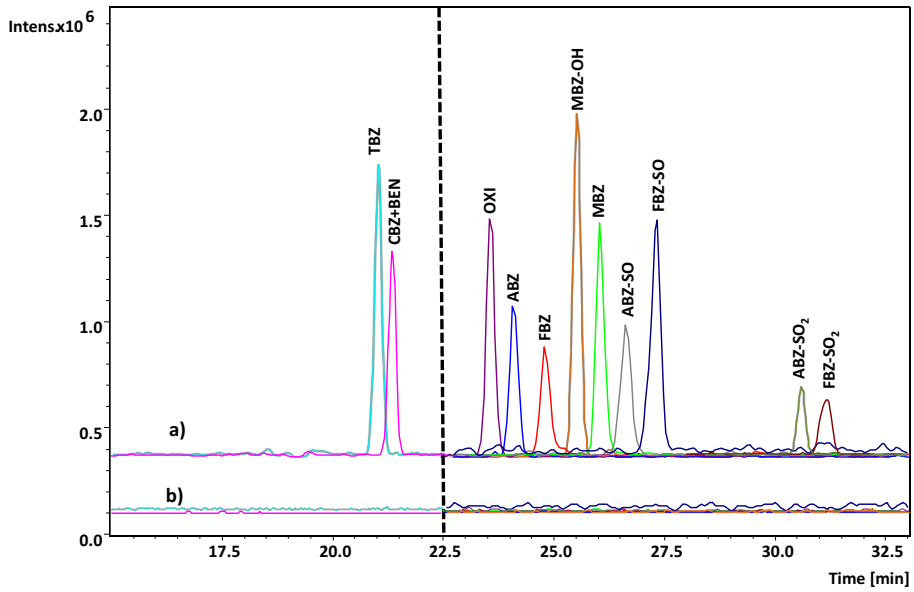

Figure 8. a) Extracted ion electropherogram obtained by DLLME-CZE-MS/MS of a poultry sample spiked at 25 µg kg^{-1} (TBZ, CBZ+BEN), 50 µg kg^{-1} (OXI, ABZ, FBZ, MBZ, FBZ-SO, ABZ- SO_2) and 100 µg kg^{-1} (MBZ-OH, ABZ-SO, FBZ-SO_2). b) Blank sample.

3.4.2 Matrix effect

The matrix effect (ME) is the result produced on the analytical response by the components of the sample other than the analytes. When MS is employed as detector, it can be attributed to many sources affecting analyte ionization and it can either enhance the analytical response or decrease it [6,7]. ME was assessed for each BZ in poultry muscle as representative matrix. It was

[6] Furey, M. Moriarty, V. Bane, B. Kinsella, M. Lehane, Ion suppression; A critical review on causes, evaluation, prevention and applications, Talanta 115 (2013) 104–122.

[7] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS, Anal. Chem. 75 (2003) 3019–3030.

estimated by comparing the slopes of calibration curves obtained from blank samples spiked post-extraction with BZs with the slopes of external standard calibration curves at the same concentration levels. The ME value for each BZ is showed in **Table 4**. Results obtained were lower than -33 % in all cases.

3.4.3 Precision study

Precision was evaluated in terms of repeatability (intraday precision) and intermediate precision (interday precision) by the application of the proposed DLLME-CZE-MS/MS methodology to poultry muscle samples spiked at two different BZ concentration levels: 10 μg L^{-1} and 25 μg L^{-1} for TBZ and CBZ+BEN; 20 μg L^{-1} and 50 μg L^{-1} for OXI, ABZ, FBZ, MBZ, FBZ-SO, MBZ-OH and ABZ-SO_2 and 40 μg L^{-1} and 100 μg L^{-1} for ABZ-SO and FBZ-SO_2. Repeatability was evaluated over two samples prepared and injected by duplicate on the same day, under the same conditions. Intermediate precision was assessed at two concentration levels by analysing one spiked sample in duplicate per level and per day, for three consecutive days. The results expressed as %RSD of peak areas are shown in **Table 5**. Satisfactory precision with RSD lower than 16.1 % was obtained in all cases.

Table 5. Precision of the DLLME-CZE-MS/MS method for spiked poultry muscle samples.

	TBZ[a]	CBZ+BEN[a]	OXI[b]	ABZ[b]	FBZ[b]	MBZ-OH[c]	MBZ[b]	ABZ-SO[c]	FBZ-SO[b]	ABZ- SO_2[b]	FBZ- SO_2[c]
Repeatability RSD (%) (n=4)											
Level 1	6.2	10.8	9.1	9.3	9.7	13.2	12.3	10.0	10.9	11.1	8.7
Level 2	2.8	2.8	6.6	7.1	6.4	6.5	11.4	7.7	8.8	8.5	7.8
Intermediate precision RSD (%) (n=6)											
Level 1	12.2	15.0	10.6	13.5	12.2	15.5	15.6	16.1	11.0	15.9	14.1
Level 2	11.3	8.4	8.8	9.4	9.3	14.1	13.5	13.6	10.2	12.5	10.3

[a]**Level 1**: 10 µg kg^{-1} and **Level 2**: 25 µg kg^{-1}; [b]**Level 1**: 20 µg kg^{-1} and **Level 2**: 50 µg kg^{-1}; [c]**Level 1:** 40 µg kg^{-1} and **Level 2**: 100 µg kg^{-1}

3.4.4 Trueness

In order to check the trueness of the proposed methodology for the analysis of real samples, recovery experiments were carried out in two different types of meat samples not used previously (poultry muscle and porcine muscle) spiked at two different BZ concentration levels. Recovery values (%) were estimated by substitution of the analytical responses obtained from the application of the DLLME-CZE-MS/MS method to spiked meat samples in the corresponding matrix-matched calibration curve. In all cases, a sample free of analytes was analyzed to check the absence of BZs, and no interferences were found. Recoveries higher than 70.1 % were obtained for all BZs, reaching RSDs lower than 13.6 % (**Table 6**).

4 Conclusions

In this chapter, a novel, selective and sensitive method for the simultaneous determination of 12 BZs including some of their metabolites in different meat samples (poultry and porcine) has been developed. DLLME has been proposed for sample treatment after an extraction step with MeCN. The proposed method is easy, fast and inexpensive for BZs determination in meat samples, allowing recoveries higher than 70%. In addition, the use of CZE-MS/MS with an online stacking preconcentration has proven to be very useful to improve the sensitivity of CZE-MS/MS method. Linearity and precision were satisfactory with values ranging from 0.9914-0.9987 and RSD ranging from 3-16 % for all studied analytes respectively. LOQs were lower than their corresponding MRLs, complying with the current legislation. ME values were also adequate, showing the efficiency of the clean-up procedure. Therefore, a new DLLME-CZE-MS/MS method is proposed as a powerful alternative to liquid chromatography for the monitoring of BZs and their metabolites in meat samples.

Table 6. Trueness assessment, expressed as percent recovery (R), for the proposed DLLME-CZE-MS/MS method.

			TBZ[a]	CBZ+BEN[a]	OXI[b]	ABZ[b]	FBZ[b]	MBZ-OH[c]	MBZ[b]	ABZ-SO[c]	FBZ-SO[b]	ABZ- SO_2[b]	FBZ-SO_2[c]
Poultry muscle	Level 1	R (%)	87.7	70.4	70.6	83.3	71.1	72.6	79.3	76.0	76.7	83.4	84.0
		RSD (%)	6.2	10.8	9.1	9.3	9.7	13.2	12.3	10.0	10.9	11.1	8.7
	Level 2	R (%)	84.0	72.7	90.2	83.5	83.8	74.5	80.5	73.3	97.3	92.1	78.0
		RSD (%)	2.8	2.8	6.6	7.1	6.4	6.5	11.4	7.7	8.8	8.5	7.8
Porcine Muscle	Level 1	R (%)	76.7	70.8	71.8	77.0	77.0	72.6	80.2	70.1	78.3	87.1	73.4
		RSD(%)	8.5	8.2	12.1	7.8	13.6	10.2	13.0	8.4	12.1	12.5	12.7
	Level 2	R (%)	80.8	73.3	87.1	78.1	79.4	84.1	93.0	72.9	81.6	95.5	90.5
		RSD(%)	5.7	2.7	6.0	4.3	12.0	4.8	11.7	6.7	10.2	4.8	8.4

[a]**Level 1**: 10 µg kg^{-1} and **Level 2**: 25 µg kg^{-1}; [b]**Level 1**: 20 µg kg^{-1} and **Level 2**: 50 µg kg^{-1}; [c]**Level 1**: 40 µg kg^{-1} and **Level 2**: 100 µg kg^{-1}

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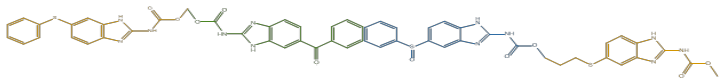
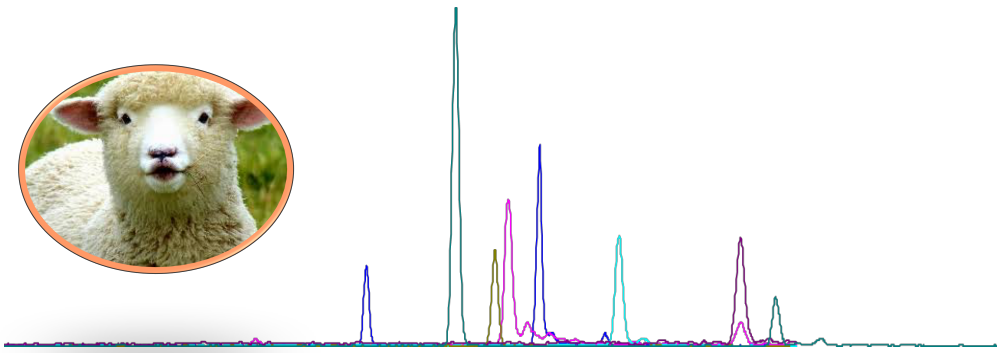
CHAPTER 4



COUPLING SWEEPING-MICELLAR ELECTROKINETIC CHROMATOGRAPHY WITH TANDEM MASS SPECTROMETRY FOR THE THERAPEUTIC MONITORING OF BENZIMIDAZOLES IN ANIMAL URINE BY DILUTE AND SHOOT



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1. Summary

A new method based on MEKC-MS/MS has been developed for the identification and simultaneous quantification of thirteen BZs in animal urine. In order to obtain an appropriate separation with the highest sensitivity, different electrophoretic parameters were evaluated. Under optimum conditions, the separation was performed using APFO as volatile surfactant and electrophoretic buffer (50 mM, pH 9). To increase the sensitivity, a stacking mode named sweeping was applied, using water as injection solvent at 50 mbar for 75 s, obtaining sensitivity enhancement factors from 50 to 181. The method was applied to different animal urine samples, including sheep, cow and goat. The sample treatment consisted of a 1:10 (v/v) dilution with water. Calibration using sheep urine samples can be used for both goat and cow urine samples with a relative bias below 25% and RSDs lower than 8 %. The LODs were below 70 μg L^{-1}. As a result, the applicability of this rapid, simple, sensitive, and environmentally friendly method for therapeutic drug monitoring of BZs based on the analysis of animal urine has been demonstrated.

2. Materials and methods

2.1 Materials and reagents

All reagents used in this study were of analytical grade and the solvents were of HPLC grade. MeCN, MeOH and IPA (LC-MS HiPerSolv grade) were supplied from VWR (Radnor, PA, USA). Formic acid, acetic acid and perfluorooctanoic acid (PFOA) 96% m/m, were obtained from Sigma Aldrich (St. Louis, MO, USA). Ammonia solution (NH_4OH) (30% m/m) and sodium hydroxide (NaOH) were purchased from Panreac-Química (Madrid, Spain). EtOH was obtained from Merck (Darmstadt, Germany). Ultrapure water (Milli-Q plus system, Millipore, Bedford, MA, USA) was employed throughout the work. A solution of APFO 50 mM at pH 9.0 was prepared with PFOA, dissolving the necessary amount of this acid in water and adjusting the pH with a 5 M NH_4OH.

Analytical standards of ABZ, ABZ-SO_2, ABZ-SO, CBZ, BEN, FBZ, FBZ-SO_2, FBZ-SO, MBZ-NH_2, MBZ, OXI, TBZ, MBZ-OH were obtained from Sigma-Aldrich.

Individual stock standard solutions of 1000 mg L^{-1} (ABZ-SO, FBZ-SO, MBZ-NH_2, TBZ), 500 mg L^{-1} (ABZ, ABZ-SO_2, BEN, MBZ-OH) and 250 mg L^{-1} (FBZ, FBZ-SO_2, MBZ, OXI, CBZ) were prepared by dissolving exactly weighed amounts of each compound in MeOH. They were stored in dark at -20 °C, being stable at least for 5 months. Intermediate stock standard solutions containing 25 mg L^{-1} of CBZ, BEN and MBZ-OH, 50 mg L^{-1} of OXI, ABZ, ABZ-SO, FBZ, MBZ, FBZ-SO and 100 mg L^{-1} ABZ-SO_2, MBZ-NH_2, FBZ-SO were obtained by mixing individual stock standard solutions and subsequent dilution with water. They were also stored in the dark at 4°C. Working standard solutions containing all the BZs were everyday prepared by dilution of the intermediate stock standard solution with water at the required concentration.

Nylon syringe filters, 0.2 µm x 13 mm (Bonna-Agela Technologies Inc, Wilmington, USA) were employed for filtration of sample extracts prior to the injection into the CE system.

2.2 Instrumentation

CE experiments were performed with an HP^{3D}CE instrument (Agilent Technologies, Waldbronn, Germany). Separations were carried out in uncoated fused-silica capillaries (100 cm total length, 50 µm i.d., 375 µm o.d.) from Polymicro Technologies (Phoenix, AZ, USA). The coaxial sheath liquid sprayer was provided by Agilent Technologies. MS was performed using an Agilent 1100 Series LC/MSD SL mass spectrometer fitted with an IT mass analyzer. A KD Scientific 100 series syringe pump (KD Scientific Inc., Holliston, MA, USA) was selected for sheath liquid supplying. MS data were collected and processed by Esquire software 4.1 from Bruker Daltonics (Bremen, Germany).

A pH meter (Crison model pH 2000, Barcelona, Spain) and a vortex (Genie 2 model from Scientific Industries, Bohemia, USA) were also employed.

2.3 MEKC conditions

Before the first use, new capillaries were rinsed with 1M NaOH for 10 min, followed by deionized water for 10 min, and running buffer for 20 min. The BGE was an aqueous solution of 50 mM APFO at pH 9, which gave an electric current of 17 µA. At the beginning of each day, the capillary was preconditioned with 5 M NH_3 solution for 3 min, deionized water for 3 min and finally with the running buffer for 20 min. To ensure an appropriate repeatability in each analysis, before each run the capillary was prewashed with 1 M NH_3 solution for 2.5 min, deionized water for 1 min and with BGE for 5 min. Finally, the capillary was washed with water for 5 min, and dried with air for 5 min at the end of each day. All time a N_2 pressure of 1 bar was applied.

The capillary was kept at 25 °C and 25 kV was used as separation voltage. The sample was hydrodynamically injected for 75 s at 50 mbar using water as sample solvent.

2.4 MS and MS/MS conditions

Compounds ionization was obtained in positive mode under an ESI voltage of -4500 V. Sheath liquid consisted of a mixture 50:49.5:0.5 (v/v/v) EtOH/water/formic acid and it was provided at a flow rate of 0.1 mL h^{-1}. Other electrospray parameters were as follows: dry gas flow rate, 8 L min^{-1}; nebulizer pressure, 6 psi (41.4 kPa) and dry gas temperature, 250 °C. MS experiments were carried out with the ICC target set to 100,000, 4 scans per spectra and a maximum accumulation time of 250 ms. Scan range was set from 180.0 to 350.0 m/z. In MS/MS experiments, a maximum accumulation time of 100 ms with an ICC target of 50,000 and 2 scans per spectra were set. Product ions were registered from 50 to 350 m/z. Fragmentation was performed by means of collision-induced dissociation with helium present in the trap for 40 ms in MRM mode. **Table 1** shows MS/MS parameters.

Table 1. Main parameters of the MEKC-MS/MS method for the determination of BZs.

	Time segment (min)												
	0-11 min		11-13 min	13-17.5 min									17.5-20 min
	CBZ+BEN	**TBZ**	**MBZ-OH**	**OXI**	**ABZ**	**ABZ-SO**	**FBZ**	**ABZ-SO_2**	**MBZ-NH_2**	**MBZ**	**FBZ-SO**	**FBZ-SO_2**
Width (m/z)	2	2	1	1	1	1	1	1	1	1	1	1
Cutoff (m/z)	52	52	80	68	72	76	81	81	64	80	85	90
Amplitude (V)	0.2	0.2	0.5	0.4	0.5	0.45	0.6	0.4	0.5	0.7	0.33	0.6
Precursor ion $[M+H]^+$	192	202	298	250	266	282	300	298	238	296	316	332
Fragmentations	160	175 159	266 160	218 176	234 192	240 222 191	268 190	266 224	105 77	264 105	284 191	300 159

2.5 Sample treatment procedure

Fresh animal urine samples of cow, goat and sheep were collected from a local farm (Jaén, Spain). As stated in the introduction, "dilute and shoot" sample treatment was employed. The procedure consisted in a 10 fold sample dilution with deionized water to decrease the effect of the ionic strength of the sample matrix and get both good separation and detection. Then, the diluted samples were filtered through 0.2 µm x 13 mm nylon syringe filters before the analysis by MEKC-MS/MS. The sample procedure it is shown in **Figure 1**.

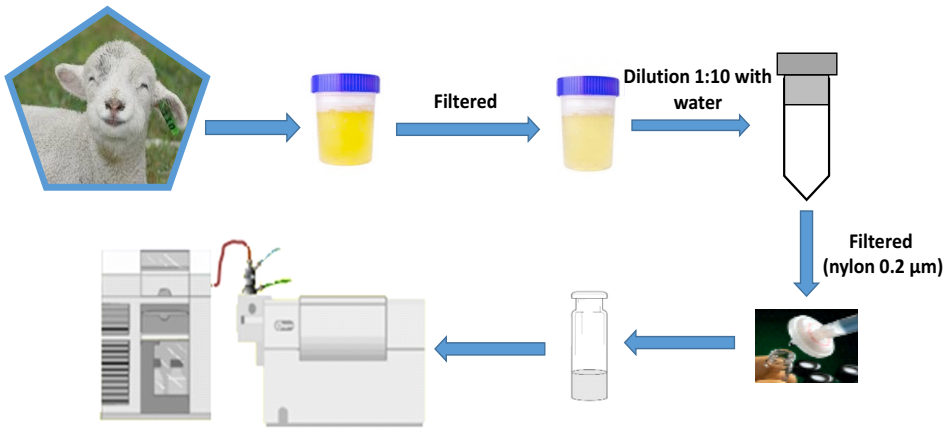

Figure 1. Sample treatment procedure

3. Results and discussion

3.1 Optimization of MEKC experimental conditions

Optimization of the main variables affecting the separation of BZs by MEKC were carried out considering different electrophoretic parameters such as peak height, peak area, S/N ratio, retention time and resolution for all analytes studied. MEKC mode has been evaluated to improve the separation efficiency of BZs. In MEKC mode, analytes show different electrophoretic mobilities due to their different interactions with the micelles. As stated in the introduction, the use of SDS is not recommended when MS detection is applied. Therefore, the separation performance using APFO as BGE was studied. First of all, the effect of pH was studied. To achieve an adequate MEKC separation using an anionic pseudo-stationary phase, pH values higher than 7 were considered to induce a strong EOF. In addition, PFOA at these pH values is fully deprotonated (PFOA pKa= 2.8 [1]). The pH effect was studied in the range between 8 and 10, in which all BZs are neutral compounds (pKa_1 ~3-6 and pKa_2 ~10-13 [2]) and they can be resolved by MEKC. As can be observed in **Figure 2,** when the pH was higher than 9, peak shapes and separation efficiencies were both poor for most of the analytes. With pH values lower than 9 peak shapes were slightly worse in the case of MBZ-OH and FBZ (**Figure 2**). Thus, the best results in terms of peak resolution and separation efficiency were obtained using pH 9.

[1] K.U. Goss, The pKa values of PFOA and other highly fluorinated carboxylic ccids, Environ. Sci. Technol. 42 (2008) 456–458.

[2]M. Danaher, H. De Ruyck, S.R.H. Crooks, G. Dowling, M. O'Keeffe, Review of methodology for the determination of benzimidazole residues in biological matrices, J. Chromatogr. B, 845 (2007) 1–37.

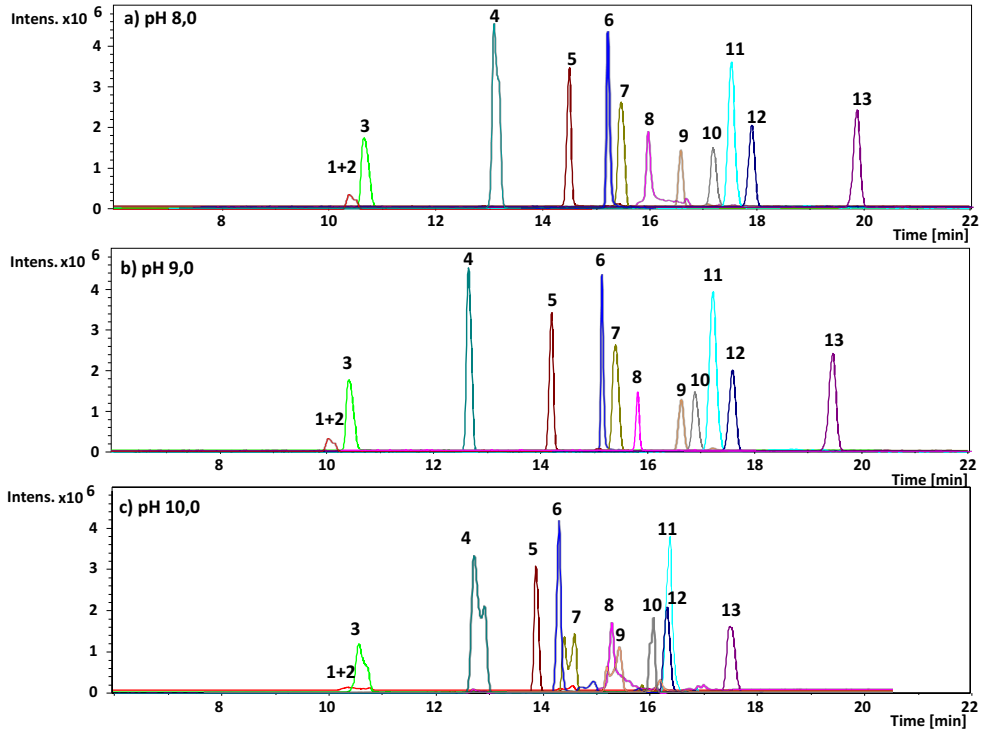

Figure 2. Optimization of buffer pH. Conditions: BGE, 50 mM APFO; BZ concentrations: 500 μg L^{-1} of (CBZ+ BEN, TBZ and MBZ-OH), 1000 μg L^{-1} of (OXI, ABZ, ABZ-SO, FBZ, MBZ, FBZ-SO) and 2000 μg L^{-1} (ABZ-SO_2, MBZ-NH_2, FBZ-SO); capillary temperature, 25 °C; separation voltage, 25 kV and sample injection, 50 mbar for 75 s. Peak identification: 1+2, CBZ+BEN; 3, TBZ; 4, MBZ-OH; 5, OXI; 6, ABZ; 7, ABZ-SO; 8, FBZ; 9, ABZ-SO_2; 10, MBZ-NH_2; 11, MBZ; 12, FBZ-SO; 13, FBZ-SO_2.

Subsequently different concentrations of APFO ranging from 25 to 100 mM at pH 9.0 were studied. As can be observed in **Figure 3,** most of the compounds were overlapped when a concentration of 25 mM was employed. APFO concentration was very close to the critical micelle concentration (≈25 mM) [3]) precluding a satisfactory separation. However, an APFO concentration of 50 mM was enough to achieve the full separation of BZs. For concentrations higher than 50 mM, peak resolutions were not improved and the analysis time was increased. In this regard,

[3] C. Wang, P. Yan, H. Xing, C. Jin, J. Xiao, Thermodynamics of aggregation of ammonium/tetraalkylammonium perfluorooctanoates: Effect of counterions, Engineering. 55 (2010) 1994–1999

a concentration of 50 mM was selected as optimum. It must be highlighted that CBZ and BEN were not resolved with this method. Probably because BEN is easily transformed into CBZ as soon as it dissolves in water [4]. For this reason, both compounds were quantified in a single peak.

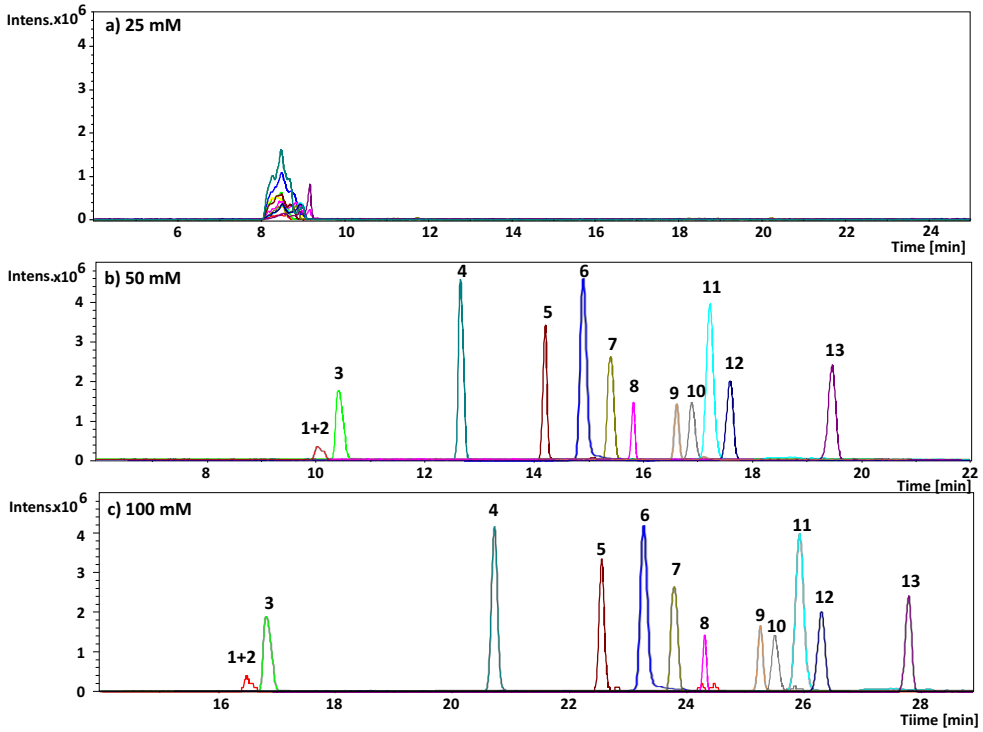

Figure 3. Optimization of buffer concentration. Conditions: BGE, APFO (pH 9). Other conditions as indicated in Figure 2.

In the next step, the separation voltage was studied in the range of 20-30 kV. The resolution of BZs did not improve significantly when this parameter was modified, so 25 kV was selected. The effect of the temperature on the MEKC separation was checked between 20 and 30 °C. It was observed that the analysis time decreased

[4] E. Mallat, D.Barceló, R.Tauler, Degradation study of benomyl and carbendazim in water by liquid chromatography and multivariate curve resolution methods, Chromatographia 46 (1997) 342–350.

with increasing the temperature. However, a temperature of 25°C was selected as optimum in order to avoid excessive Joule heating.

In order to improve signal sensitivity, a stacking procedure to preconcentrate the analytes was considered. First of all, different injection solvents including water and some MeCN/water combinations (15:85; 20:80; 30:70 and 40:60 v/v) were tested. The presence of organic solvent affected negatively the analyte peak shapes, probably due to the inadequate formation of micelles when organic solvent is present in the injection solvent. Therefore, water was chosen as optimum sample solvent. In this scenario, BZs were in their neutral form allowing their stacking when they were swept by the electrophoretic buffer containing APFO micelles. Finally, the effect of the hydrodynamic injection time was studied in the range from 25 to 100 s at 50 mbar. Injection times higher than 75 s produced band broadening; therefore, an injection time of 75 s was set. This injection time corresponds to an approximate volume of 57.5 nL (3 % of the total volume of the capillary). SEF_{height} were estimated with the following equation:

$$SEF_{height} = \frac{\text{Peak height under NSM injection/Analyte concentration in NSM injection}}{\text{Peak height under hydrodynamic injection /Analyte concentration in hydrodynamic injection}}$$

Table 2 shows the values of SEF_{height} achieved for all BZs studied. Water as sample solvent (sweeping effect) using a constant pressure of 50 mbar for 75 s as injection conditions was compared with the use of the BGE as sample solvent (conventional hydrodynamic injection) at constant pressure of 50 mbar for 5 s. Enhancement factors ranging from 50 to 181 were obtained for the studied analytes with this type of on-line preconcentration, improving sensitivity without any loss of peak efficiency.

Table 2. Sensitivity enhancement factors (SEF) for the studied BZs using sweeping.

Analyte	SEF height	RSD (%) (n=3)
CBZ and BEN	50.3	1.4
TBZ	67.8	1.6
MBZ-OH	102.9	3.9
OXI	113.5	4.1
ABZ	101.1	4.0
ABZ-SO	181.6	4.5
FBZ	52.4	1.8
ABZ-SO_2	72.6	1.9
MBZ-NH_2	57.6	1.8
MBZ	97.8	4.9
FBZ-SO	86.8	4.2
FBZ-SO_2	97.3	3.9

Electropherograms with and without sweeping using the same injection conditions (50 mbar for 75s) are shown in **Figure 4**. The peak efficiencies with sweeping (plate number for MBZ-OH: 28224; TBZ: 60526 and ABZ: 40656) were better than an injection with the BGE as sample solvent (plate number for MBZ-OH: 4096; TBZ: 4900 and ABZ: 4356), showing the good performance of this type of online preconcentration.

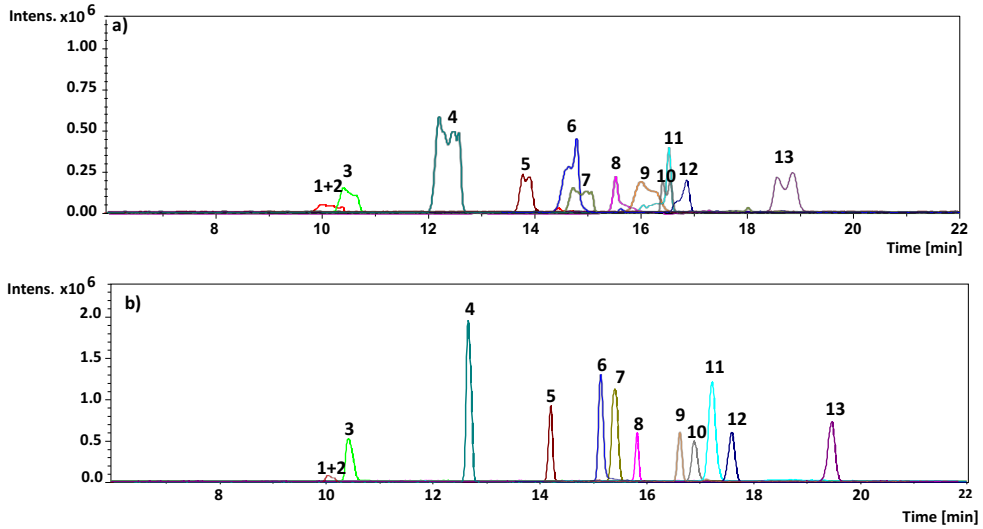

Figure 4. a) Injection of 50 mbar for 75 s using as injection solvent APFO 50 mM pH 9 (without sweeping) and b) Injection of 50 mbar for 75 s using as injection solvent water (with sweeping). BZ concentrations: 125 µg L^{-1} (CBZ+ BEN, TBZ and MBZ-OH), 250 µg L^{-1} of (OXI, ABZ, ABZ-SO, FBZ, MBZ, FBZ-SO) and 500 µg L^{-1} (ABZ-SO_2, MBZ-NH_2, FBZ-SO) 250 µg L^{-1} of each BZ; capillary temperature, 25 °C and separation voltage, 25 kV.

3.2 MEKC-ESI-MS/MS optimization

The electrospray parameters play an important role in a MEKC-ESI-MS/MS method because they should provide a stable spray with the highest sensitivity. Thus, the composition of the sheath-liquid and its flow rate, dry gas flow and nebulizer pressure and dry gas temperature were optimized employing a standard solution of 125 µg L^{-1} for CBZ+ BEN, TBZ and MBZ-OH, 250 µg L^{-1} for OXI, ABZ, ABZ-SO, FBZ, MBZ, FBZ-SO and 500 µg L^{-1} for ABZ-SO_2, MBZ-NH_2, FBZ-SO. The S/N was selected as response variable.

The sheath liquid composition was studied using as initial conditions a sheath liquid flow rate of 0.2 mL h^{-1}, a dry gas flow rate of 8.0 L min^{-1}, a nebulizer pressure of 8.0 psi (55.2 kPa), a dry gas temperature of 250°C and electrospray voltage of 4500 V. The influence of the nature of the organic solvent (MeCN, IPA, EtOH, MeOH) was optimized employing a mixture 50:49:1 (v/v/v) organic solvent/water/formic acid.

As can be observed in **Figure 5.a**, the use of MeCN and IPA decreased considerably the S/N ratio, so these solvents were discarded.

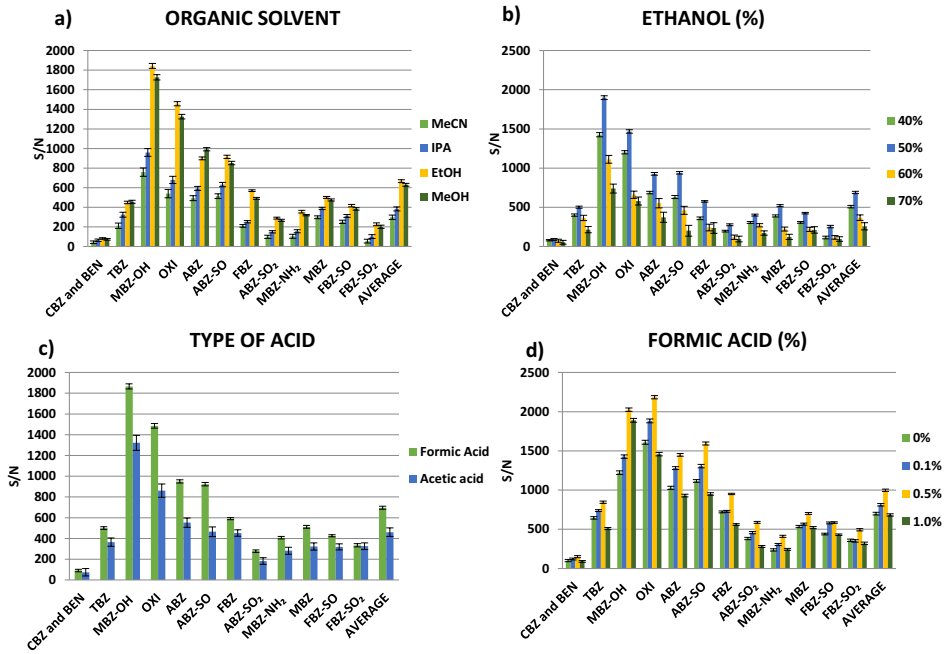

Figure 5. Effect of the sheath liquid composition. a) Organic solvent; b) Ethanol (%); c) Type of acid, d) Formic acid (%). Conditions: BGE, 50 mM APFO (pH 9); BZ concentrations: 125 µg L^{-1} (CBZ+ BEN, TBZ and MBZ-OH), 250 µg L^{-1} of (OXI, ABZ, ABZ-SO, FBZ, MBZ, FBZ-SO) and 500 µg L^{-1} (ABZ-SO_2, MBZ-NH_2, FBZ-SO) 250 µg L^{-1} of each BZ; capillary temperature, 25 °C; separation voltage, 25 kV; sample injection 50 mbar for 75 s; sample solvent, water. Error bars represent the standard error (n = 3).

Considering that EtOH is more environmentally friendly, it was selected as the organic solvent for the sheath liquid. Then, the percentage of EtOH was studied from 40 to 70 %. As can be observed in **Figure 5.b**, when the percentage of EtOH was below 50%, the S/N ratio for all BZs studied increased along with EtOH concentration. For higher values, an unstable spray was produced, so 50% was chosen as optimum. To ensure an adequate analyte ionization and reproducibility, the nature of different additives in the sheath-liquid was evaluated. Thus, formic acid and acetic acid were tested using a mixture of 50:49:1 (v/v/v) organic

solvent/water/acid. Using acetic acid an unstable electrospray current was observed, decreasing the S/N ratio for all BZs (**Figure 5.c**). As a consequence, acetic acid was discarded. Sequentially, the percentage of formic acid to ensure the positive ionization was checked from 0 to 1 %. It was observed (**Figure 5.d**) that formic acid percentages higher than 0.5 % did not improve the S/N ratio, selecting this value as optimum. In conclusion, the optimum sheath-liquid composition for the detection on these compounds in MEKC-MS/MS was a mixture of EtOH/water/formic acid (50:49.5:0.5).

The sheath liquid flow rate was assessed from 0.05 to 0.2 mL h^{-1}. As it is shown in **Figure 6.a**, a reduction in the S/N ratio was observed when the flow rate increased. This response may be originated for the dilution of the CE effluent causing lower analyte intensities. With 0.05 mL h^{-1} no stable electrospray current was achieved. Consequently 0.1 mL h^{-1} was selected as optimum. Then, the nebulizer pressure was evaluated between 4 and 10 psi (27.6 and 68.9 kPa). As can be observed in **Figure 6.b,** the best sensitivity in terms of S/N ratio was obtained with a pressure of 6 psi (41.4 kPa). Higher nebulizer pressures decreased the spray stability and led to lower peak resolutions for most of the compounds. The dry gas temperature was studied from 150 to 300 °C, but no significant differences were noticed for the majority of BZs, choosing 250 °C. Dry gas flow rate was studied from 4 to 10 L min^{-1}, obtaining the best S/N ratio when 8 L min^{-1} was employed.

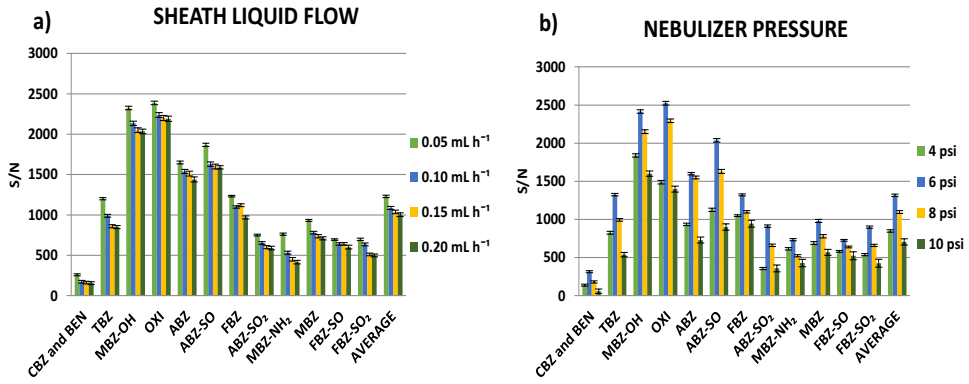

Figure 6. Effect of ESI conditions on S/N ratio. a) Sheath liquid flow and b) Nebulizer pressure. Other conditions as indicated in Figure 5.

To improve the sensitivity of the method, the main 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 optimized. Direct infusion of analytes diluted in the BGE can be a good option to optimize ion optics in MS. This was accomplished with BGE containing BZs (1 mg L^{-1} for each compound) and applying 25 kV in order to obtain a constant concentration of all compounds in the electrospray. The optimum values for the studied variables in MS detection are reported in **Table 3**.

Table 3. Optimum ion trap parameters.

	Segment time (min)			
	0-11 min	11-13 min	13-17.5 min	17.5-20 min
	CBZ,BEN,TBZ and MBZ-OH	**OXI**	**ABZ, ABZ-SO, FBZ, MBZ-NH2, ABZ-SO2, MBZ and FBZ-SO**	**FBZ-SO_2**
Capillary (V)	-4500	-4500	-4500	-4500
Skimmer (V)	46.94	44.3	39.2	52.6
Cap Exit (V)	157.9	156.6	138.9	168.8
Oct 1DC	12.0	13.1	13.4	13.1
Oct 2DC	1.6	1.9	2.0	1.9
Trap Drive	33.3	41.7	40.6	41.7
OCT RF	90.9	119.7	143.1	226.2
Lens 1 (V)	-3.93	-3.9	-4.4	-4.2
Lens 2 (V)	60.2	-54.3	-56.3	-67.5

As remarkable, in all cases the most abundant ion was $[M+H]^+$. In order to get optimum selectivity, the main MS/MS parameters were studied using MRM mode. For fragmentation experiments, a cut-off of 27% of the precursor mass was fixed (i.e. the minimum m/z of the fragment ion able to be trapped by the analyzer). BZs were fragmented using the SmartFrag™ option that automatically ramps the fragmentation energy from 30 to 200% of the excitation amplitude. The fragmentation amplitude was optimized considering that parent ion should be detected. As it is shown in **Table 1**, using MS^2 the number of product ions for the studied BZs was enough for a reliable identification.

3.3. Optimization of sample treatment

A very simple sample treatment based on “dilute and shoot” was used. First of all, different dilutions factors were tested for an urine:water mixture: 1:20, 1:15, 1:10 and 1:50 (v/v). A dilution factor of 1:10 (v/v) was selected as optimum, providing the best results in terms of sensitivity and CE current stability. It was necessary to filter

the sample before the injection to remove any particle which could block the capillary or induce an unstable electrophoretic current.

3.4 Method characterization

In order to test the suitability of the method for the determination of BZs in urine samples, it was validated in terms of linear dynamic ranges, LODs and LOQs, matrix effect, intra-day and inter-day precision and trueness.

3.4.1 Calibration curves and analytical performance characteristics

Procedural calibration curves were established using sheep urine free of analytes as representative matrix. Samples were spiked at five different concentration levels (62.5, 125, 250, 500 and 1000 μg L^{-1} for TBZ, MBZ-OH and CBZ+BEN; 125, 250, 500, 1000 and 2000 μg L^{-1} for OXI, ABZ, ABZ-SO, FBZ, MBZ and FBZ-SO; 250, 500, 1000, 2000 and 4000 μg L^{-1} for ABZ-SO_2, MBZ-NH_2 and FBZ-SO_2) and they were summited to the whole proposed analytical method. Therefore, two samples of each concentration level were processed following the sample treatment and they were injected in duplicate. Peak area was considered as a function of the analyte concentration in the samples. A blank sample was also analyzed, and none of BZs were detected.

LODs and LOQs were calculated as the minimum analyte concentration yielding a S/N equal to three and ten, respectively. The statistics and performance characteristics of the proposed method in sheep urine are shown in **Table 4**. As can be observed, LOQs lower than 231 μg L^{-1} were obtained for all compounds, estimated in urine samples before dilution. Thus, the method is adequate for the determination these analytes in the selected matrix.

Table 4. Statistics, performance characteristics and matrix effect of the sweeping-MEKC-MS/MS method for the analysis of BZs in sheep urine.

Analyte	LOD ($\mu g\ L^{-1}$)	LOQ ($\mu g\ L^{-1}$)	Linear dynamic range ($\mu g\ L^{-1}$)	R^2	ME (%)
CBZ + BEN	14.9	49.8	49.8-1000	0.9973	-2.4
TBZ	13.9	46.2	46.2-1000	0.9987	2.0
MBZ-OH	24.8	82.2	82.2-2000	0.9971	-2.2
OXI	29.7	99.1	99.1-2000	0.9970	-3.9
ABZ	25.3	84.3	84.3-2000	0.9997	-4.9
ABZ-SO	25.8	86.2	86.2-2000	0.9988	-7.6
FBZ	26.9	89.7	89.7-2000	0.9996	1.2
ABZ-SO_2	61.2	204.0	204.0-4000	0.9936	1.9
MBZ-NH_2	69.3	231.2	231.2-4000	0.9991	-3.9
MBZ	31.2	104.2	104.2-2000	0.9995	-0.3
FBZ-SO	31.7	105.8	105.8-2000	0.9979	6.8
FBZ-SO_2	58.7	195.8	195.8-4000	0.9930	-5.7

An electropherogram corresponding to BZs separation in sheep urine spiked at different BZ concentrations are shown in **Figure 7**.

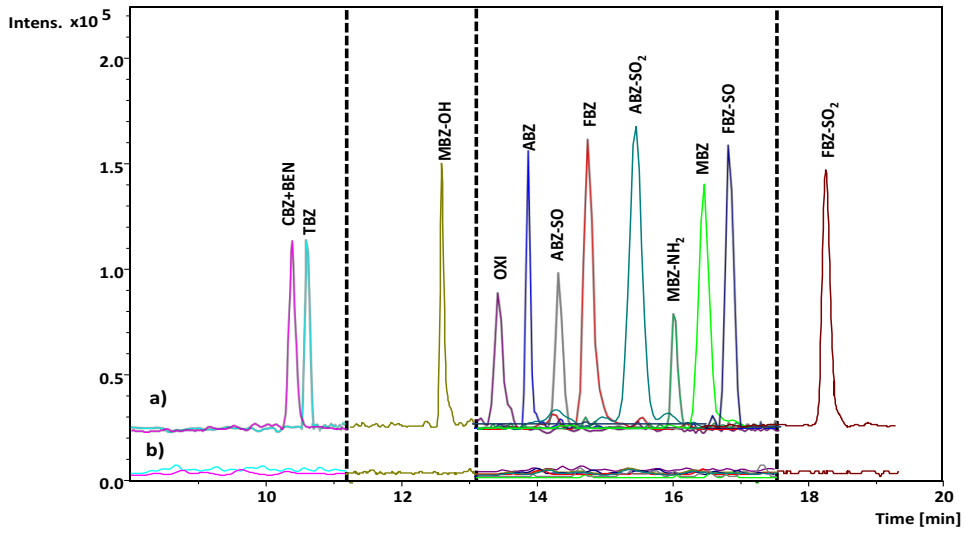

Figure 7. a) Extracted ion electropherogram of a sheep urine sample spiked with 125 $\mu g\ L^{-1}$ (CBZ+BEN, TBZ and MBZ-OH), 250 $\mu g\ L^{-1}$ (OXI, ABZ, ABZ- SO, FBZ, MBZ, FBZ-SO) and 500 $\mu g\ L^{-1}$ (MBZ-NH_2, ABZ-SO_2, FBZ-SO_2), obtained by sweeping-MEKC-MS/MS. b) Blank sample.

3.4.2 Matrix effect

ME is the outcome produced on the analytical response by the components of the sample different than the analytes. The analytical response can be enhanced or decreased when MS is used as detector. It may be caused by many factors affecting analyte ionization [5,6]. ME was obtained for each BZ considering sheep urine as a representative matrix. For that purpose, the slopes of matrix-matched calibration curves prepared from blank sample extracts spiked with BZs after the sample treatment and before the analysis were compared with the slopes of external calibration curves at the same concentration levels (see the following equation). As can be observed in **Table 4,** ME were lower than 7.6 % in all cases, showing an adequate selectivity of the proposed method. This fact can be partially attributed to the benefits of the "dilute and shoot" sample preparation method.

$$\text{ME}\ (\%) = \left(\left(\frac{\text{slope of matrix} - \text{matched calibration curve}}{\text{slope of external calibration curve}} \right) - 1 \right) \text{x}100$$

3.4.3 Precision study

Precision was evaluated in terms of repeatability (intraday precision) and intermediate precision (interday precision) by the application of the proposed sweeping-MEKC-MS/MS methodology to sheep urine samples spiked at two different BZ concentration levels: 125 μg L^{-1} and 500 μg L^{-1} for TBZ, MBZ-OH and CBZ+BEN; 250 μg L^{-1} and 1000 μg L^{-1} for OXI, ABZ, ABZ-SO, FBZ, MBZ and FBZ-SO and 500 μg L^{-1} and 2000 μg L^{-1} for ABZ-SO_2, MBZ-NH_2 and FBZ-SO_2.. Repeatability was evaluated over two samples prepared and injected by duplicate on the same day,

[5] Furey, M. Moriarty, V. Bane, B. Kinsella, M. Lehane, Ion suppression; A critical review on causes, evaluation, prevention and applications, Talanta 115 (2013) 104–122.
[6] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS, Anal. Chem. 75 (2003) 3019–3030.

under the same conditions. Intermediate precision was studied for three consecutive days with a slightly modified procedure: in this case just one sample was prepared per concentration level but it was injected by duplicate as well. **Table 5** shows the results expressed as %RSD of peak area. As can be observed, excellent precision was obtained, achieving RSDs lower than 7.6 %.

3.4.4 Trueness study

The trueness of the proposed methodology for the analysis of real samples was indicated by the percentage of the measured value from the nominal value. The samples were previously analyzed and no analytes were detected above the LODs, interferences were not found either. Therefore, samples were spiked at two concentration levels (similar to the precision study). Trueness was calculated for each BZ by interpolating the peak areas of a set of sheep, cow and goat urine samples in the corresponding procedural calibration curves previously obtained with a set of sheep urine samples. The dilution factor of 10 was applied in the samples before the analysis. Values for trueness between 75.2 % and 99.8% were obtained for all BZs with RSDs lower than 8.2 % (**Table 6**).

Table 5. Precision of the method for spiked sheep urine samples.

	CBZ+BEN[a]	**TBZ**[a]	**MBZ-OH**[a]	**OXI**[b]	**ABZ**[b]	**ABZ-SO**[b]	**FBZ**[b]	**ABZ- SO_2**[c]	**MBZ-NH_2**[c]	**MBZ**[b]	**FBZ-SO**[b]	**FBZ-SO_2**[c]
Repeatability RSD (%) (n=4)												
Level 1	2.1	4.0	3.4	3.3	2.4	2.5	4.2	5.9	4.1	3.2	2.5	3.8
Level 2	1.5	3.2	2.6	2.5	2.0	2.3	3.5	2.8	2.2	2.1	2.0	2.2
Intermediate precision RSD (%) (n=6)												
Level 1	7.6	5.9	4.6	4.4	5.4	4.5	6.8	7.3	7.1	5.2	6.9	7.4
Level 2	4.1	3.4	3.7	4.0	4.9	3.6	4.5	4.4	4.8	3.7	4.8	4.2

[a] **Level 1**: 125 µg L^{-1} and **Level 2**: 500 µg L^{-1}, [b] **Level 1**: 250 µg L^{-1} and **Level 2**: 1000 µg L^{-1}, [c] **Level 1**: 500 µg L^{-1} and **Level 2**: 2000 µg L^{-1}

Table 6. Trueness and RSD (n=4) for the proposed sweeping-MEKC-MS/MS methodology in sheep, cow and goat urine

			CBZ[a]	TBZ[a]	MBZ-OH[a]	OXI[b]	ABZ[b]	ABZ-SO[b]	FBZ[b]	ABZ-SO_2[c]	MBZ-NH_2[c]	MBZ[b]	FBZ-SO[b]	FBZ-SO_2[c]
Sheep urine	**Level 1**	Trueness (%)	97.6	91.3	99.3	94.6	94.6	95.9	93.5	96.9	97.6	95.4	93.2	87.6
		(RSD, %)	2.4	7.8	3.8	5.4	6.8	4.1	6.8	6.4	7.3	4.6	2.9	4.6
	Level 2	Trueness (%)	95.6	96.7	93.5	94.7	92.1	99.8	92.3	91.3	97.7	93.2	90.9	90.9
		(RSD, %)	1.6	3.9	2.6	2.9	2.1	2.7	4.0	2.5	3.0	2.4	2.0	2.3
Cow urine	**Level 1**	Trueness (%)	89.1	85.6	94.2	94.1	98.8	97.0	85.0	96.4	87.0	88.4	95.6	75.2
		(RSD, %)	4.8	8.1	4.3	7.1	7.5	6.6	7.5	8.2	8.2	8.0	5.6	5.0
	Level 2	Trueness (%)	89.6	89.9	95.4	93.0	87.5	99.8	84.6	86.9	97.4	83.7	87.4	85.9
		(RSD, %)	3.2	4.7	3.9	5.5	2.3	3.9	4.0	1.9	3.3	3.5	2.3	4.5
Goat urine	**Level 1**	Trueness (%)	93.5	97.3	95.8	92.6	98.1	90.7	84.1	97.1	90.0	87,4	93.4	83.8
		(RSD, %)	4.8	7.3	4.6	5.8	3.3	4.6	7.5	7.2	6.8	6.7	7.8	3.2
	Level 2	Trueness (%)	94.2	95.9	96.8	88.2	95.5	98.9	91.98	93.5	94.5	87.9	89.1	97.9
		(RSD, %)	3.7	2.9	2.5	3.5	2.2	2.4	3.3	2.1	2.2	3.3	3.5	4.0

[a] **Level 1**: 125 μg L^{-1} and **Level 2**: 500 μg L^{-1}; [b] **Level 1**: 250 μg L^{-1} and **Level 2**: 1000 μg L^{-1}; [c] **Level 1**: 500 μg L^{-1} and **Level 2**: 2000 μg L^{-1}

4. Conclusions

In this chapter a simple, fast, selective and sensitive method for the simultaneous determination of 13 BZs, including some of their metabolites, in animal urine samples (sheep, cow, goat) has been developed. To the best of our knowledge, this is the first time that MEKC coupled with MS/MS is applied to the monitoring of BZs using a volatile surfactant, APFO. This surfactant acts simultaneously as BGE and micellar medium and is highly compatible with MS. Moreover, it is able to efficiently separate BZs in a shorter time than CZE. The use of sweeping as online preconcentration step in the proposed MEKC-MS/MS method has proved to be very useful to improve sensitivity, with LOQs in the range of low μg L^{-1}. The simplicity of the "dilute-and-shoot" sample treatment, scarcely used in CE, and the rapidity to achieve a very high sample throughput with low cost and reduced waste are some of the main advantages of this green approach. This method can help veterinarians to customize the administered dose of BZs for each individual case and it can be a powerful alternative to LC methods for the monitoring of BZs and their metabolites in animal urine samples.

This work was published as:

***"Coupling sweeping-micellar electrokinetic chromatography with tandem mass spectrometry for the therapeutic monitoring of benzimidazoles in animal urine by dilute and shoot"*, C. Tejada-Casado, D. Moreno-González, M. del Olmo-Iruela, A.M. García-Campaña, F.J. Lara, Talanta. 175 (2017) 542–549.**

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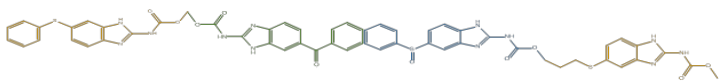
CHAPTER 5



CAPILLARY ELECTROCHROMATOGRAPHY COUPLED WITH DISPERSIVE LIQUID-LIQUID MICROEXTRACTION FOR THE ANALYSIS OF BENZIMIDAZOLE RESIDUES IN WATER SAMPLES

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1. Summary

A novel method for the analysis of BZ residues in water samples by CEC-UV detection (290 nm), using laboratory-made packed columns is presented. Capillaries (25 cm packed length × 75 µm inner diameter, 34 cm total length, 25.5 cm effective capillary length) were packed with C18 particles (5 µm, non-encapped) following a high pressure packing procedure and using a compact steel unit designed for packing capillary columns. Acetone was employed as solvent to carry the particles through the capillary and pack it under a pressure of 42 MPa. Outlet and inlet frits were made by sintering the particles of the stationary phase by heating the packed material with a nichrome ribbon connected to a 7 V AC power supply. With the aim of achieving a good analytical performance, the variables that affected the separation were studied, using a mobile phase composition of 60:40 (v/v) MeCN/water containing ammonium acetate (1 mM, pH 6.5), a separation voltage of 25 kV and a temperature of 25°C. In addition, a combined hydrodynamic-electrokinetic injection mode was considered and samples were injected for 75 s under a voltage of 12.5 kV and a pressure of 11.5 bar. Finally, the determination of BZs in water samples was accomplished by CEC using DLLME as sample treatment. Variables affecting the extraction efficiency were optimized, using chloroform and ethanol as extraction and disperser solvents, respectively.

This method was applied to different water samples, obtaining satisfactory results in terms of linearity ($R^2 \geq 0.990$), repeatability (RSD≤1.2%), reproducibility (RSD≤2.2%) and trueness (R≥87.7%). LODs and LOQs were lower than 2.8 µg L^{-1} and 9.3 µg L^{-1}, respectively.

2. Materials and methods

2.1 Materials and reagents

All reagents used in this study were of analytical grade, unless otherwise indicated, and the solvents were of HPLC grade. Ultrapure water (Milli-Q plus system, Millipore, Bedford, MA, USA) was used throughout the work. Ammonium hydroxide (NH_3) (30%), sulphuric acid (98%), and sodium hydroxide (NaOH) were obtained from Panreac-Química (Madrid, Spain). MeOH, ammonium bicarbonate, EtOH, IPA, isobutanol (ISOB), THF, chloroform, dichloromethane and ACO were purchased from VWR International (West Chester, PA, USA) whereas ammonium acetate, sodium tetraborate decahydrate and MeCN were supplied by Sigma-Aldrich (St. Louis, MO, USA). Tris(hydroxymethyl)aminomethane (TRIS) and hydrochloric acid 37% were acquired from Merck (Darmstadt, Germany).

Analytical standards of ABZ, CBZ, BEN, FBT, FBZ, MBZ, OXI and PBZ were supplied by Sigma-Aldrich (St. Louis, MO, USA). Individual stock standard solutions of 1000 mg L^{-1} (FBT), 500 mg L^{-1} (ABZ, BEN, PBZ) and 250 mg L^{-1} (FBZ, OXI, CBZ, MBZ) were prepared by dissolving accurately weighted amounts of each compound in MeOH. They were stored in dark at -20 °C, being stable for at least 6 months. Intermediate stock standard solutions containing 100 mg L^{-1} of each BZ were obtained by mixing individual stock standard solutions and subsequent dilution with MeOH. They were stored in dark at 4°C. Working standard solutions containing all the BZs were freshly prepared by the proper dilution of the intermediate stock standard solutions with MeOH at the required concentration.

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

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

2.2 Instrumentation

A SP-400 Nanobaume^{TM} 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). Packed capillary was heated with a metallic strip (80%Ni-20%Cr, 28 cm×2 mm×0.2 mm, electric resistance 1.3 Ω) connected to a 7 V AC power supply for frit fabrication was made by a local technician (**Figure 1**).

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

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

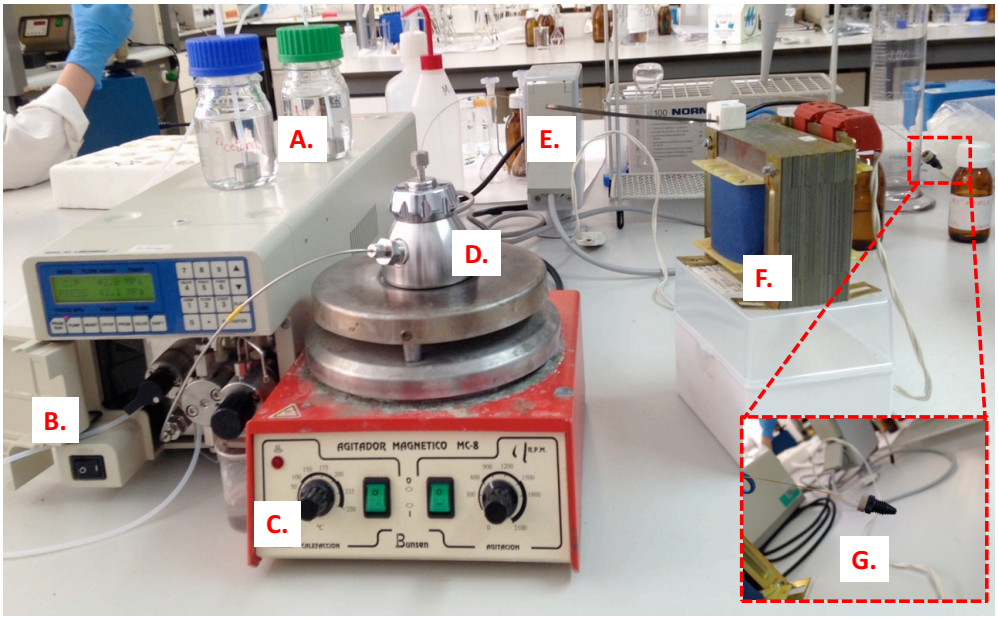

Figure 1. Packing capillary setup diagram: (A) solvent containers, (B) high pressure pump, (C) magnetic stirrer, (D) packing capillary unit, (E) capillary placed through the hole made on the nichrome ribbon; particles from the stacionary phase are sintered at this capillary position and (F) 7 V AC power supply, (G) Initial temporary frit.

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

2.3 Fabrication of packed capillary columns with C_{18} silica particles

A simple and cheap method previously reported was followed for producing C18 packed capillaries [1]. First of all, a new fused silica capillary (25 cm effective length and 75 µm i.d) was conditioned. It was conditioned by flushing with 1 M NaOH solution for 10 min at 5 bar. Afterwards it was rinsed with deionized water for 10 min at 5 bar, followed by acetone for 10 min at 5 bar. Finally, the conditioned capillary was placed in the packing unit which was connected to a high pressure pump in order to propel a particle transporting solvent through the capillary. After

[1] 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 C 18 silica beds, Talanta 144 (2015) 542–550.

conditioning, the capillary was mounted in the packing unit and a seven step protocol was carried out for capillary packing and frit formation (**Figure 2**).

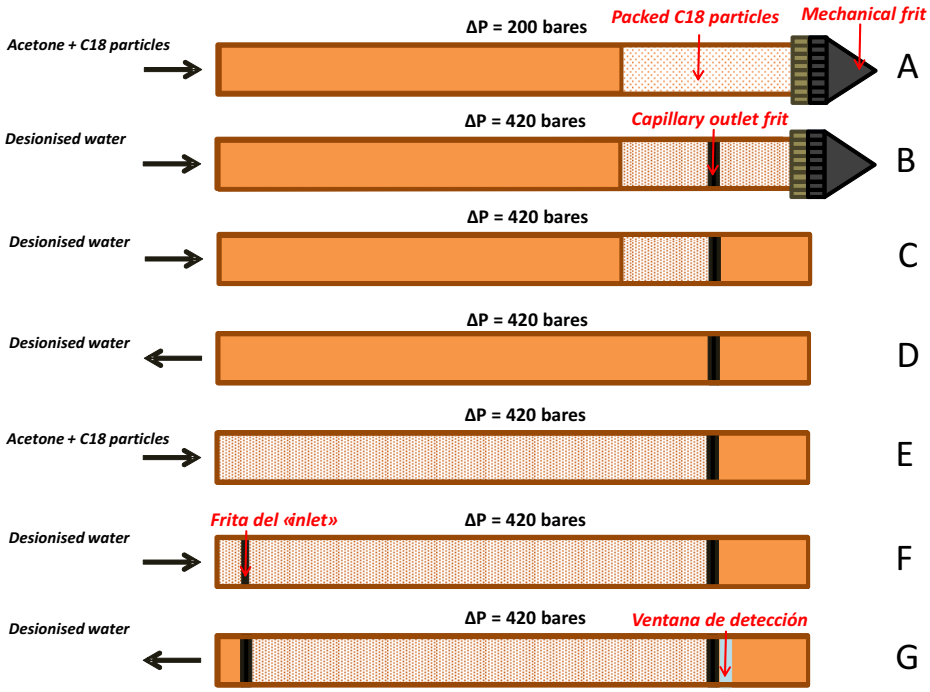

Figure 2. Capillary packing procedure scheme: (**A**) capillary is partially filled with C18 silica particles; (**B**) deionized water is passed through packed capillary for 1 h at 420 bar and outlet frit is made 10 cm far away from the mechanical retainer. C18 particles are sintered and consequently 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 [1].

The experimental set-up, which mainly consisted of a high pressure pump connected to a Nanobaume^{TM} column packing unit, is shown in **Figure 1**.

Firstly, the driving solvent (acetone) with the particles suspended by magnetic agitation was propelled by a pressure pump to the packing unit and consequently it

dragged the C_{18} silica particles (20 mg) into the capillary (**Figure 2, step A**). In order to obtain a good packing, the pressure of the system was slowly increased from 0 to 200 bar. At the beginning of the packing procedure, a mechanical frit was placed at the end of the capillary for retaining the particles inside the capillary. Additionally, a plastic screw initially sealed the mechanical frit, so driving solvent could not pass through it, and as a consequence system pressure was rapidly increased. The plastic screw was removed when a system pressure of 200 bar was reached. Consequently, the packing velocity was increased and capillary was homogeneously packed for 12 cm at 420 bar. Subsequently, the high pressure pump was turned off, the pressure decreased and capillary packing was stopped.

The packed capillary was then flushed 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 2, step A)**. Outlet frit was made at 10 cm far away from the mechanical retainer. Previously, the capillary was introduced through a small hole of 380-400 µm made on the center of the nichrome ribbon. The outlet frit was made by heating the capillary for 20 s with a 7 V AC power supplier which was connected to the nichrome ribbon. The mechanical frit was removed and the nichrome ribbon was taken away when outlet frit was made. C_{18} particles located between the sintered particles and the mechanical frit were dragged out from the capillary by passing deionised water through the capillary at 420 bar (**Figure 2, step C** and **D)**.

Then, the capillary was totally packed using acetone as driving solvent at 420 bar (**Figure 2, step E)**. After capillary packing, deionized water was passed through it for 1.5 hour at 420 bar in order to achieve a better packed stationary phase (**Figure 2, step F)**. Finally, inlet frit was made at 25 cm from outlet frit following the same procedure employed for the fabrication of the outlet frit.

A detection window was made at 2 mm from the outlet frit using sulphuric acid in order to remove capillary coating (**Figure 2, step G)**. Furthermore, the inlet capillary

end was cut 2 mm from the inlet frit and the outlet capillary end was cut at 8.5 cm from the detection window. Microscope images of capillary column before and after being packed are shown in **Figure 3**.

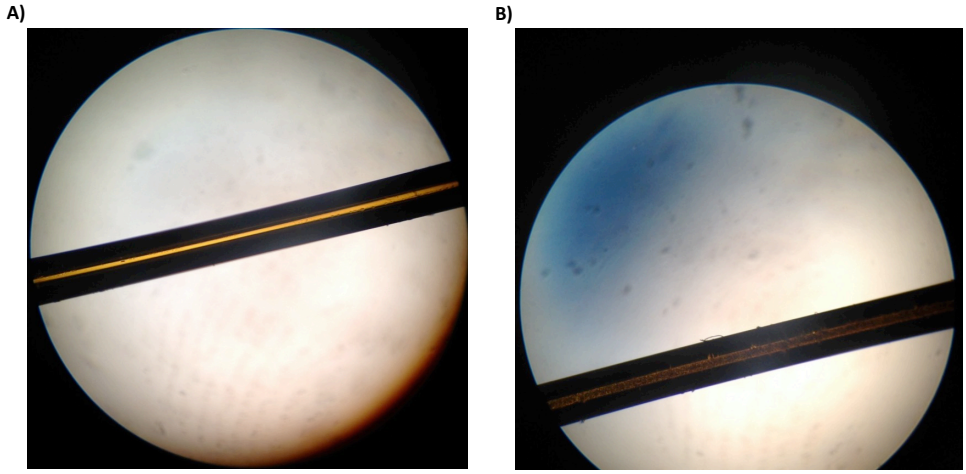

Figure 3. Microscope images of: A) unpacked capillary (50 µm i.d.) and B) packed capillary (50 µm i.d.) with C18 particles (5 µm i.d.). Microscope magnification of 40-fold.

2.4 Capillary electrochromatography procedure

A C18 packed capillary (75 µm i.d. x 25 cm packed length) was used for BZs determination. A simple and cheap method previously reported was followed for producing C18 packed capillaries [1] (see section 2.3). New packed capillaries were conditioned with mobile phase consisted of 60:40 (v/v) MeCN/water containing ammonium acetate (1 mM, pH 6.5) for 1 h by applying a pressure of 11.5 bar to the capillary inlet in the CE instrument. A voltage of 30 kV was applied between two vials containing mobile phase for 30 min. Both vials were pressurized at 7 bar during voltage application for avoiding any bubbles formation. After capillary conditioning, a stable baseline was observed. Analytes were injected by a combined hydrodynamic-electrokinetic injection for 75 s and 12.5 kV at 11.5 bar and were separated in isocratic mode. In order to achieve good injection reproducibility, a mobile phase plug was injected for 60 s at 11.5 bar after sample injection. A voltage of 25 kV and a temperature of 25°C were used for BZs separation. A separation

current of 1.8 µA was observed during the analysis. UV detection was employed and analytical signals were monitored at 290 nm.

2.5 DLLME procedure

Three different types of water samples were analyzed: spring water from Valle de Lecrín (Granada, Spain), well water from Valderrubio (Granada, Spain) and water samples collected from a fish farm located in Huéscar (Granada, Spain). The samples were collected directly in PVC bottles and they were stored at 4°C in the dark until the analysis, without any further sample pretreatment.

An aliquot of filtered water sample (5 mL) was placed into a 15-mL falcon tube with conical bottom. A mixture of EtOH (disperser solvent, 1700 µL) and $CHCl_3$ (extraction solvent, 950 µL) was rapidly injected into the sample tube. The tube was closed and it was shaken for 1 min. After that, it was centrifuged for 2 min at 3000 rpm. The sedimented phase was removed using a syringe, placed in a 4-mL vial and evaporated under nitrogen stream until dryness. The final residue was re-dissolved with 300 µL of 30:70 (v/v) MeCN/water containing ammonium acetate (1 mM, pH 6.5), vortexed (2 min), filtered (0.2 µm) and injected into the electrocromatographic system. Taking into account all the steps of this treatment, sample throughput was approximately of 12 samples per hour, obtaining a preconcentration factor of 16.7. The sample procedure it is shown in **Figure 4**.

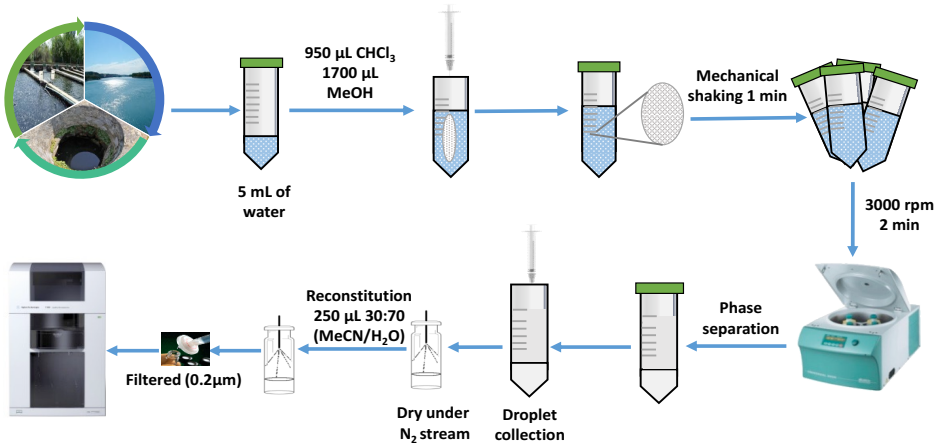

Figure 4. Sample treatment procedure

3 Results and discussion

3.1 Optimization of electrochromatographic experimental conditions

Optimization of the main variables affecting the separation was carried out considering that a satisfactory peak resolution and high signals in terms of peak height must be achieved. It was ensured that the electric current remained at stable values.

3.1.1 Selection of packed column length

Electrochromatographic conditions were initially established as follows: mobile phase composition 60:40 (v/v) MeCN/water containing ammonium acetate (5 mM, pH 5); separation voltage, 25 kV; temperature, 25°C. Samples were injected for 150 s applying a pressure of 11.5 bar to the inlet vial.

Under these conditions, different packed capillary lengths ranging from 25 to 40 cm were evaluated using lab-made packed capillaries of 75 µm i.d. It was observed that lengths higher than 25 cm caused an increase of separation time without supposing higher peak resolution. Thus, 25 cm packed capillary was selected as optimum because it provided a good separation in shorter time.

Additionally, reproducibility of the packing procedure was evaluated considering the analysis of a BZ standard solution (500 μg L^{-1} of each analyte) in three different packed capillaries. Ten runs were made in each capillary, obtaining total RSDs of peak area for each analyte lower than 8.0%, which confirm that no significant differences exist when different capillaries were used.

3.1.2 Mobile phase optimization

Initially a mixture of 60:40 (v/v) MeCN/water containing ammonium acetate (5 mM, pH 5) was selected as mobile phase. Under these conditions any of the peaks were not baseline resolved. For this reason, buffer pH was assayed between 5.0 and 7.5. The pH of ammonium acetate solutions (5 mM) was adjusted at the desired value with 1:20 (v/v) NH_4OH solution. According to particle manufacturer, the employed C18 particles are not stable at pH values higher than 7.5, therefore higher pH values were not studied. On the other hand, as it is known, higher pH values cause higher EOF, resulting in a higher flow of mobile phase. In this case, it was observed that working at pH 7.5 faster analysis was achieved without any loss in peak resolution. However at this pH a high baseline drift and fluctuations were obtained so, pH 6.5 was finally selected as a compromise between analysis time and stable baseline.

In order to check the influence of the BGE on the analysis time and peak efficiency in terms of theoretical plates, BGE nature and its concentration in the mobile phase were evaluated. The following BGE natures were tested: ammonium bicarbonate, TRIS, ammonium acetate and sodium tetraborate at a concentration of 5 mM. Band broadening and, as a consequence, a loss of peak efficiency was observed when mixtures of 60:40 (v/v) MeCN/water containing ammonium bicarbonate or sodium tetraborate (5 mM, pH 6.5) were employed. Therefore both of them were discarded as BGE. On the other hand, peak efficiency was satisfactory when mixtures of 60:40 (v/v) MeCN/water containing TRIS or ammonium acetate (5 mM, pH 6.5) were tested. However TRIS buffer produced unstable electric currents, so ammonium acetate was selected as optimum BGE. Afterwards, buffer concentration in the mobile phase was studied from 0.5 mM to 10 mM. It was observed that an increase of buffer concentration caused longer analysis times and band broadening.

Buffer concentration of 0.5 mM was discarded because of electric current was very low and unstable, so a concentration of 1 mM was selected as optimum (**Figure 5**).

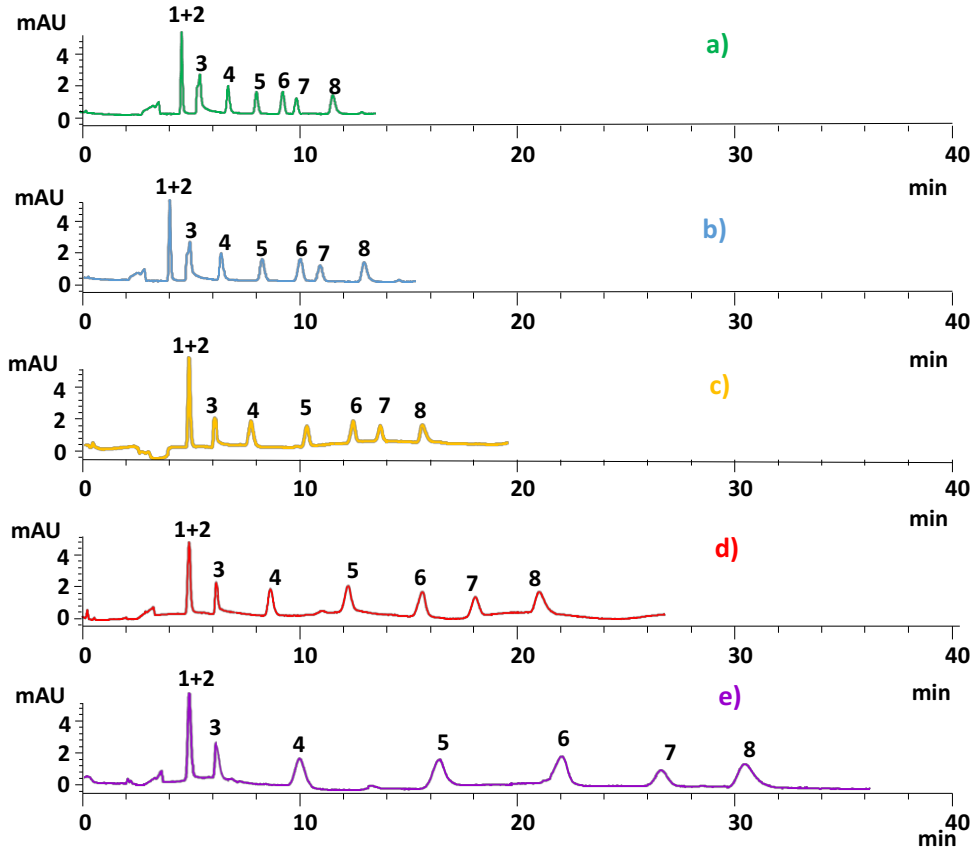

Figure 5. Optimization of the concentration of ammonium acetate (pH 6.5). a) 0.5 mM; b) 1 mM; c) 2.5 mM; d) 5 mM; e) 10 mM. Peak number: 1, CBZ; 2,BEN; 3,MBZ; 4,OXI; 5,ABZ; 6,FBZ; 7,FBT; 8,PBZ.

Then, different proportions of the mixture MeCN/water containing ammonium acetate (1 mM, pH 6.5) were evaluated. Proportions were ranged from 70:30 (v/v) to 50:50 (v/v). It was observed the co-elution of some analytes when higher percentages of organic solvents were used. In turn, lower percentages of organic solvents resulted in higher retention times, increasing analysis time. **Figure 6** shows the separation of BZ compounds when different proportions of MeCN/water containing ammonium acetate (1 mM, pH 6.5) were employed. Considering a

proportion of 70:30 (v/v) the compounds listed as 6 and 7 co-eluted. Something similar was observed when a mixture of 50:50 (v/v) was tested because analytes listed as 7 and 8 co-eluted, and additionally analysis time was increased. Finally a mixture of 60:40 MeCN/water (v/v) containing ammonium acetate (pH 6.5 and 1 mM) was selected as optimum due to all analytes, except 1 and 2, were resolved in an adequate analysis time. It must be remarked that peaks 1 and 2 are referred to CBZ and BEN, which cannot be resolved by traditional LC using C18 columns due to CBZ is the main degradation compound of BEN [2].

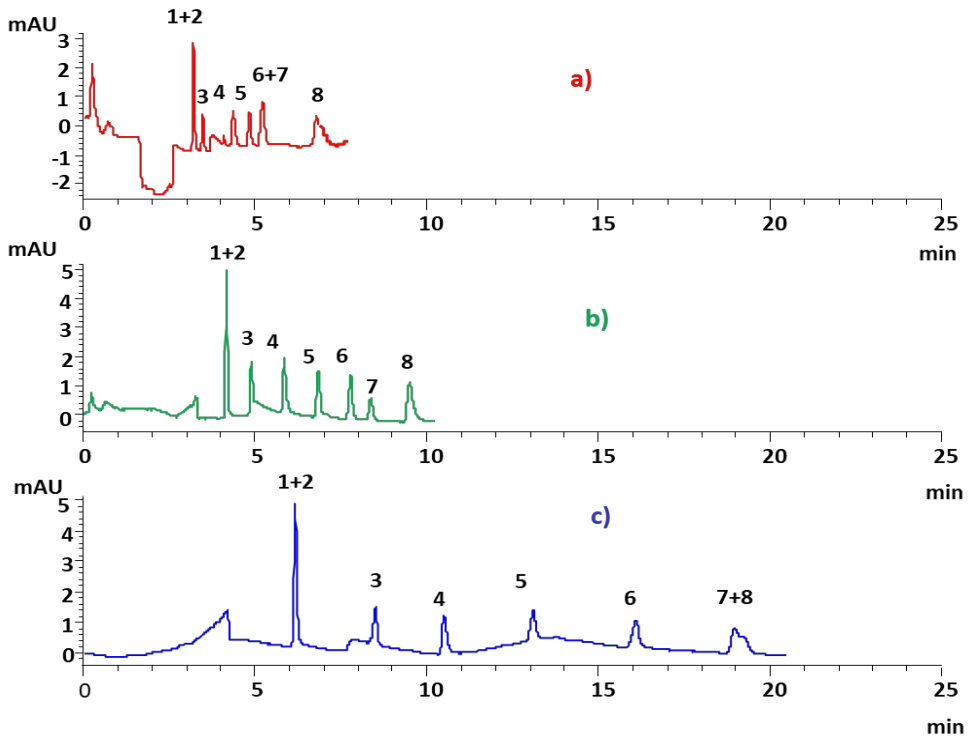

Figure 6. Optimization of the percentages of mobile phase composition (MeCN/water (v/v) containing ammonium acetate, 1 mM, pH 6.5). A) 70:30; B) 60:40; C) 50:50. Peak number: 1, CBZ; 2,BEN; 3,MBZ; 4,OXI; 5,ABZ; 6,FBZ; 7,FBT; 8,PBZ.

[2] E. Mallat, D. Barceló, R. Tauler, Degradation study of benomyl and carbendazim in water by liquid chromatography and multivariate curve resolution methods. Chromatographia 46 (1997) 342-350.

3.1.3 Sample injection solvent

From the beginning, water was employed as sample injection solvent. In order to improve method efficiency in terms of number of theoretical plates, on-column focusing was investigated. For this aim different injection solvents were evaluated. Instead of water as injection medium, different proportions of a mixture of MeCN/water containing ammonium acetate (pH 6.5, 1 mM) were studied, ranging between 20:80 (v/v) and 40:60 (v/v). Finally a mixture of 30:70 (v/v) MeCN/water containing ammonium acetate (pH 6.5, 1 mM) was selected as injection medium because higher efficiency was reached (**Figure 7**).

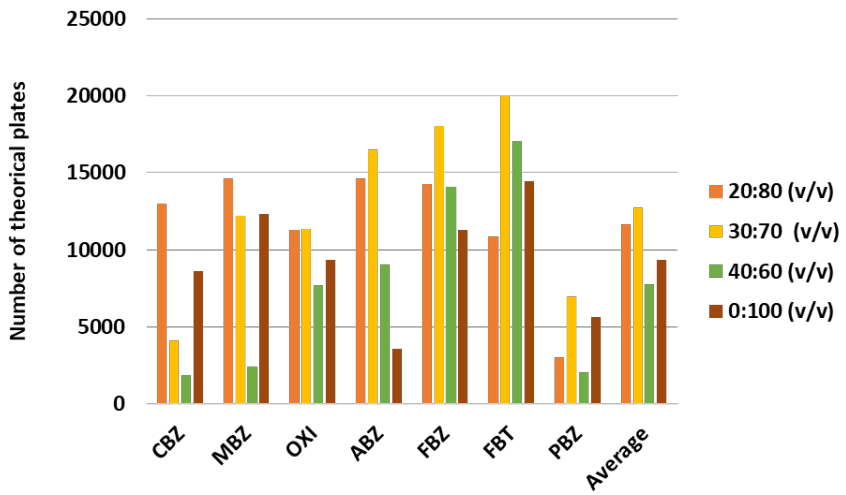

Figure 7. Optimization of the injection solvent composition (v/v) containing ammonium acetate (1 mM, pH 6.5).

3.1.4 Injection mode evaluation

In order to improve method efficiency different injection modes were assessed. Hydrodynamic injection mode considering an injection time of 50 s and a pressure of 11.5 bar, electrokinetic injection mode selecting an applied voltage of 10 kV for 50 s, and a combination of both injection modes (a voltage of 10 kV was applied for 50 s under a pressure of 11.5 bar) were considered as injection options. The best

peak efficiency in terms of number of theoretical plates was observed when the combined injection mode (hydrodynamic-electrokinetic mode) was considered (**Figure 8**).

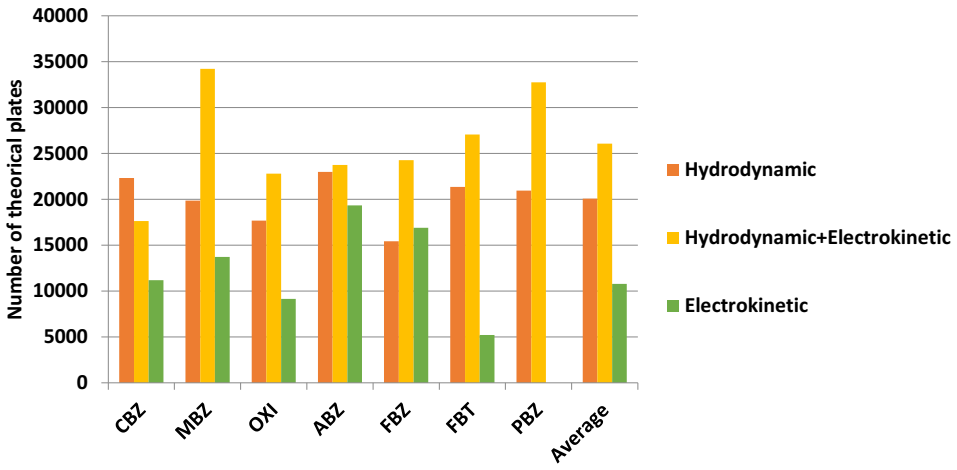

Figure 8. Evaluation of the sample injection mode. Hydrodynamic injection was performed at 11.5 bar for 50 s, combined hydrodynamic-electrokinetic injection was assayed for 50 s at 11.5 bar and 10 kV and electrokinetic injection was carried out at 10 kV for 50 s.

The effect of the sample injection volume was studied by varying the injection time in a range from 50 to 100 s at a constant pressure of 11.5 bar and an applied voltage of 10 kV. Injection times longer than 100 s produced extra-band broadening of the BZ peaks, and as a result an injection time of 75 s was chosen as optimum. Injection voltage was evaluated from 5 to 15 kV. Finally a voltage of 12.5 kV was selected as optimum because efficiency was reduced when higher voltages were tested.

3.1.5 Optimization of separation voltage and temperature

Separation voltage was investigated between 20 and 30 kV in positive mode. Baseline separation and a shorter analysis time was achieved for all analytes when a voltage between 28 and 30 kV was used. A voltage of 28 kV was selected as optimum because analysis time was not significantly reduced at higher voltages.

The effect of temperature during the separation by CEC was evaluated in the range of 20-35 ºC. Temperature of 35 ºC was discarded because of Joule effect. Lower temperatures originated longer analysis time. Finally a capillary temperature of 25 ºC was selected as a compromise between resolution and analysis time.

3.2 Optimization of DLLME

The main factors affecting the DLLME procedure are: extraction and disperser solvent nature, and solvent volumes. The optimization of these parameters was carried out considering an aqueous mixture standard solution (5 mL) containing a concentration of 60 µg L^{-1} of each BZ. The influence of the experimental parameters on the extraction performance was evaluated utilizing the absolute extraction recovery (R). It was calculated as the ratio between the peak area of the sample spiked with the analytes before the application of the DLLME procedure and that of the corresponding sample fortified after the application of the DLLME procedure just before the analysis.

Considering 850 µL of extraction solvent and 1500 µL of MeOH as disperser solvent, CH_2Cl_2, and $CHCl_3$ were evaluated as extraction solvent. The highest recovery for the eight considered BZs was obtained using $CHCl_3$. PBZ was not recovered when CH_2Cl_2 was used, and consequently $CHCl_3$ was selected (**Figure 9.a**).

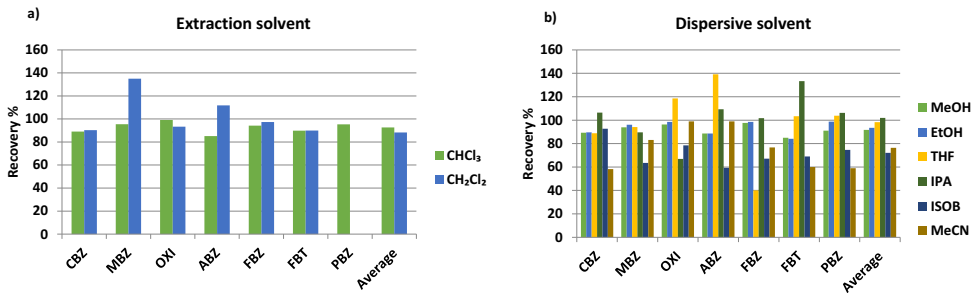

Figure 9. a) Optimization of extraction solvent; b) Optimization of dispersive solvent.

Solvents such as MeCN, IPA, ISOB, EtOH, THF and MeOH were tested as disperser solvents because they are miscible with the extraction solvent and the aqueous

sample phase. Considering a disperser volume of 1500 µL and a volume of 850 µL of the extractant solvent ($CHCl_3$), each solvent was tested. As can be observed in **Figure 9.b** when THF and IPA were used higher recoveries than 100% were obtained for several compounds (CBZ, OXI, ABZ, FBT and PBZ), so they were discarded. Finally, EtOH was selected because higher recoveries were obtained for most of the analytes.

Therefore, extraction and disperser solvent volumes were optimized by means of a 3^2 full factorial design with 3 replicates of the central point. $CHCl_3$ volume was studied between 700 and 1300 µL and EtOH volume was range between 1200 and 2200 µL. The experiments were randomly run in order to minimize the effect of uncontrolled variables. Recovery average for all BZs was considered as experimental response. The lack-of-fit P-value for the model was 9.1% and the determination coefficient (R^2) was 98.1%, showing a satisfactory fit of the experimental data to the predicted model. From the response surface shown in **Figure 10**, optimum values of 1700 µL and 950 µL were established for EtOH and $CHCl_3$ volumes, respectively.

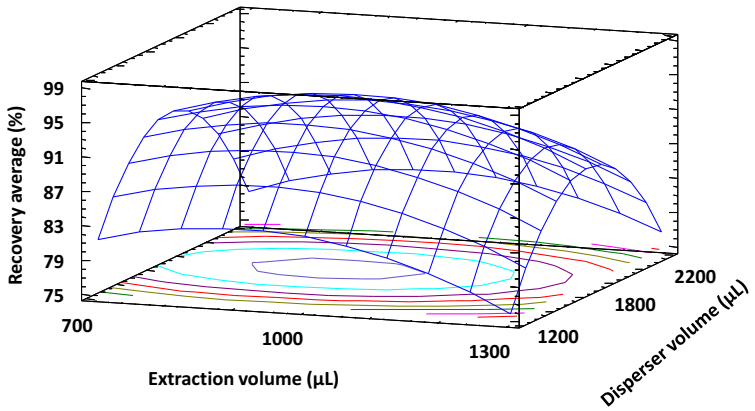

Figure 10. Estimated response surface obtained for the optimization of extraction and disperser solvent volumes using a full factorial design (3^2).

3.3 Method characterization

The suitability of the method for the determination of BZs in water samples, namely fish farm water, spring water and well water, was evaluated. With this aim parameters such as linearity, LODs, LOQs, intra-day and inter-day precision and trueness were assessed. Method characterization was developed for seven of the eight firstly considered BZ, excluding BEN because it co-eluted with CBZ under the final separation conditions (peaks 1 and 2 in **Figure 5**).

3.3.1 Calibration curves and analytical performance characteristics

Matrix-matched calibration curves were established using fish farm water samples free of analytes as representative matrix. Samples spiked at five different concentration levels (10, 20, 30, 50, 75 and 100 μg L^{-1}) were considered. Two samples of each concentration level were processed following the DLLME method and they were injected in duplicate. A blank sample was also processed, and no peaks were detected at BZs retention times. Peak area was considered as a function of the analyte concentration in the samples.

LODs and LOQs of the method were calculated as the minimum analyte concentration yielding a signal-to-noise ratio equal to three and ten, respectively. Performance and statistics characteristics of the proposed method in fish farm water samples are shown in **Table 1**.

Table 1. Statistic and performance characteristics of the proposed DLLME-CEC- UV methodology in fish farm water samples.

Analyte	LOD (μg L^{-1})	LOQ (μg L^{-1})	Linear dynamic range (μg L^{-1})	R^2
CBZ	2.8	9.2	9.2-100	0.997
MBZ	1.7	5.7	5.7-100	0.996
OXI	2.4	8.1	8.1-100	0.996
ABZ	2.5	8.4	8.4-100	0.990
FBZ	2.4	7.9	7.9-100	0.993

Analyte	LOD (µg L^{-1})	LOQ (µg L^{-1})	Linear dynamic range (µg L^{-1})	R^2
FBT	2.5	8.2	8.2-100	0.990
PBZ	2.8	9.3	9.3-100	0.990

As can be seen, LOQs lower than 9.3 µg L^{-1} were obtained for all the analytes. As a consequence, the proposed method is adequate for the determination of very low levels of these compounds in the selected matrix. Electrochromatograms corresponding to BZ separation in fish farm water samples spiked with each BZ at 20 µg L^{-1} and 50 µg L^{-1} and a blank sample are shown in **Figure 11**.

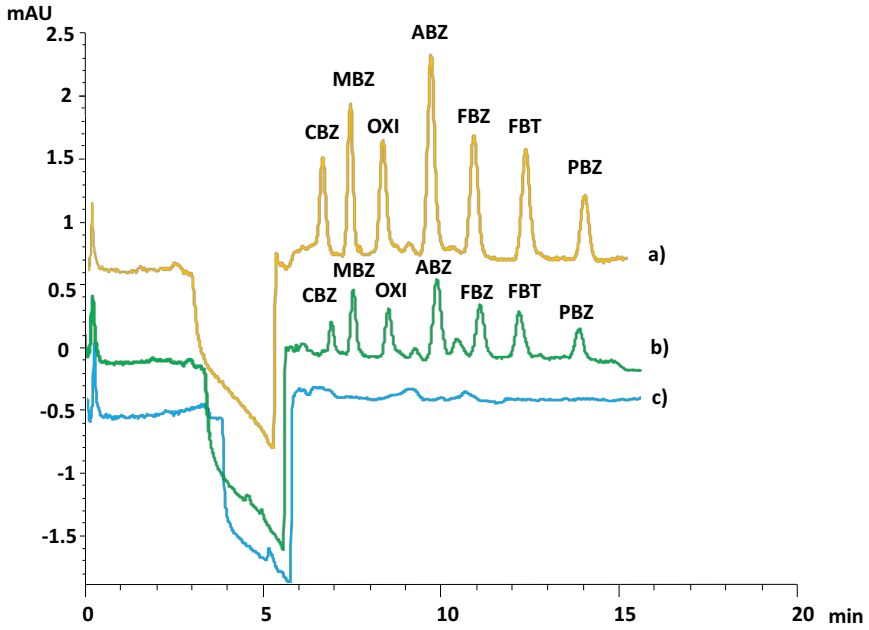

Figure 11. Electrochromatograms obtained by the proposed DLLME-CEC-UV methodology for a) a fish farm water sample spiked with seven BZs at 50 µg L^{-1}, b) a fish farm water sample spiked with seven BZs at 20 µg L^{-1} and c) a blank fish water sample.

3.3.2 Precision study

Precision was evaluated in terms of repeatability (intraday precision) and intermediate precision (interday precision) by the application of the proposed DLLME-CEC method to fish farm water samples spiked at two different BZ concentration levels (20 and 50 μg L^{-1}). Repeatability was evaluated over two samples prepared and injected by duplicate on the same day, under the same conditions. Intermediate precision was evaluated with a similar procedure, but the samples were analyzed in three consecutive days. The results expressed as %RSD of peak areas are summarized in **Table 2**. Satisfactory precision, with RSD lower than 4.8%, was obtained in all cases.

Table 2. Repeatability and Intermediate precision study for the proposed DLLME-CEC-UV methodology

	CBZ	MBZ	OXI	ABZ	FBZ	FBT	PBZ
Repeatability RSD (%) (n=4)							
Level 1	4.1	1.5	3.2	1.2	3.2	2.9	1.9
Level 2	3.3	1.7	2.2	1.8	2.0	1.9	1.8
Intermediate precision RSD (%) (n=6)							
Level 1	4.8	2.6	2.7	2.7	2.4	3.9	3.4
Level 2	4.0	2.5	3.2	2.2	2.5	3.4	3.0

Level 1: 20 μg L^{-1} and **Level 2**: 50 μg L^{-1}

3.3.3 Trueness assessment

Recovery experiments were carried out in three different types of water samples (fish farm water, spring water and well water) spiked at two different BZ concentration levels (20 and 50 μg L^{-1}) in order to check the trueness of the proposed methodology for the analysis of real samples. In all cases, a sample free of analytes was analyzed to check the presence of BZs, and none of them gave signals corresponding to concentrations higher than the LODs. Recoveries over 87.7 % were obtained for all BZs, reaching RSDs lower than 5.8 % in all cases (**Table 3**).

Table 3. Trueness assessment for the proposed DLLME-CEC-UV methodology

			CBZ	MBZ	OXI	ABZ	FBZ	FBT	PBZ
Fish farm water	**Level 1**	R (%)	91.3	91.7	94.5	92.3	96.0	92.3	94.4
		(RSD, %)	4.1	1.5	3.2	1.2	3.2	2.9	1.9
	Level 2	R (%)	92.0	98.3	96.4	96.8	94.5	96.6	97.2
		(RSD)	3.3	1.7	2.2	1.8	2.0	1.9	1.8
Spring water	**Level 1**	R (%)	91.4	93.1	94.3	92.4	93.9	97.4	90.4
		(RSD, %)	5.7	3.9	2.1	2.4	1.9	3.9	3.4
	Level 2	R (%)	93.6	91.3	94.5	96.0	95.6	96.1	95.8
		(RSD, %)	4.1	2.71	2.0	1.6	3.1	3.1	1.98
Well water	**Level 1**	R (%)	87.7	92.8	90.6	90.5	91.4	92.08	88.8
		(RSD)	5.1	4.8	5.4	2.4	3.0	4.1	3.6
	Level 2	R (%)	88.6	92.7	96.4	97.4	95.8	97.0	91.6
		(RSD)	5.8	3.0	2.6	2.3	1.1	1.7	2.1

Level 1: 20 μg L^{-1} and **Level 2**: 50 μg L^{-1}

4 Conclusions

In this chapter, a new method for the simultaneous determination of BZs in water samples by CEC-UV using lab-made columns has been developed. The optimized CEC method is able to separate seven BZs in less than 15 min. Good linearity, satisfactory trueness and precision and high sensitivity have been obtained, with LODs even lower that a previous method using CE and MS detection [3], even with a DAD, usually available in routine laboratoires. Moreover, DLLME has been proposed as sample treatment, being an attractive methodology for BZs extraction and off-line preconcentration. In comparison with previous LC based methods for the monitoring of these residues using also DLLME [4], this proposal is able to extract a

[3] J. Domínguez-Álvarez, M. Mateos-Vivas, D. García-Gómez, E. Rodríguez-Gonzalo, R. Carabias-Martínez, Capillary electrophoresis coupled to mass spectrometry for the determination of anthelmintic benzimidazoles in eggs using a QuEChERS with preconcentration as sample treatment, J. Chromatogr. A. 1278 (2013) 166–174.

[4] Y. Santaladchaiyakit, S. Srijaranai, Alternative solvent-based methyl benzoate vortex-assisted dispersive liquid-liquid microextraction for the high-performance liquid chromatographic determination of benzimidazole fungicides in environmental water samples, J. Sep. Sci. 37 (2014) 3354–3361.

higher number of compounds with satisfactory efficiency, combining this extraction technique with the advantages of CEC in terms of low environmental impact and cost. The proposal supposes a green and inexpensive method for BZs determination in environmental waters of different origins.

This work was published as:

***"Capillary electrochromatography coupled with dispersive liquid-liquid microextraction for the analysis of benzimidazole residues in water samples"*. C. Tejada-Casado, M. Hernández-Mesa, M. del Olmo-Iruela, A.M. García-Campaña, Talanta. 161 (2016) 8–14.**

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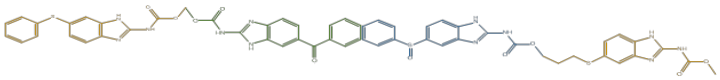
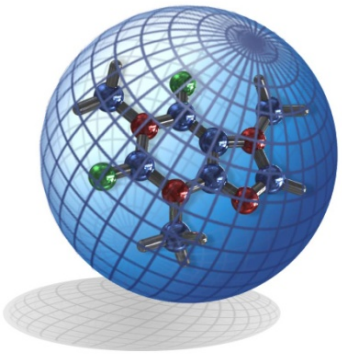
CHAPTER 6



COLLISION CROSS SECTION (CCS) AS A COMPLEMENTARY PARAMETER TO CHARACTERIZE HUMAN AND VETERINARY DRUGS: CREATION OF A DATABASE



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1. Summary

In the context of human and veterinary drugs identification, ion mobility spectrometry (IMS) in combination with mass spectrometry (MS) may provide a relevant complementary piece of information to mass-to-charge ratio (*m/z*), the so-called collision-cross-section (CCS). Up to now, however, the application of CCS as identification parameter has not been fully investigated due to the reduced number of these drugs that have being characterized in terms of CCS. This work proposes a CCS database for 92 human and veterinary drugs, including eighteen benzimidazoles, eleven 5-nitroimidazoles, eleven aminoglycosides, nineteen quinolones, eighteen β-lactams, ten sulfonamides and five tetracyclines. Among them, 37 drugs have been characterized in terms of CCS for the first time. The CCS values of the other 55 compounds have been compared with those from a recently published database in order to evaluate inter-laboratory reproducibility, which is crucial for the implementation of the CCS as identification parameter. CCS values were measured by traveling wave ion mobility spectrometry (TWIMS) under positive ionization conditions. Nitrogen was used as drift gas in the ion mobility cell. The proposed database covers 173 ions including $[M+H]^+$ and $[M+Na]^+$ species. High correlation between *m/z* and CCS has been observed for $[M+H]^+$ (R^2 = 0.9518, n = 91) and $[M+Na]^+$ (R^2 = 0.9135, n = 82) ions. As expected, CCS values for sodium adducts are generally greater than for protonated molecules because they exhibit higher molecular weight. However, sodium adducts of aminoglycosides, β-lactams, and of several quinolones and benzimidazoles, were characterized as more compact ions than their related protonated molecule. In addition, this work describes the fragmentation pattern observed for the studied molecules. For the first time, the main fragment ions for most of the compounds have also been characterized in terms of CCS, involving a total of 238 ions. As proof of concept, for the application of this database to biological matrices, eleven veterinary drugs in bovine urine

samples were characterized in terms of CCS, showing that this parameter was not influenced by the matrix.

2. Materials and methods

2.1 Materials and reagents

Acetonitrile, propan-2-ol and methanol (LC-MS Chromasolv® grade) were provided by Sigma-Aldrich (St. Louis, Mo, USA) and formic acid (eluent additive for LC-MS) was acquired from LGC Standards GmbH (Wesel, Germany). Water (HiPerSolv Chromanorm® for HPLC) was supplied by VWR International (West Chester, PA, USA).

Mass calibration was performed using a solution of sodium formate (0.5 mM in 90/10 (%, v/v) isopropanol/water). This solution was prepared from sodium hydroxide (1 M, Fisher ChemicalTM) and formic acid (Promochem®) which were purchased from Fisher Scientific (Loughborough, UK) and LGC Standards (Wesel, Germany), respectively. The Major Mix IMS/ToF Calibration Kit (Ref.186008113) acquired from Waters (Manchester, UK) was used for the calibration of the instrument in terms of CCS. This calibration solution contains small molecules such as sulfadimethoxine (molecular weight, 310.328 g/mol), which makes it ideal for the characterization of small molecules such as human and veterinary drugs. A solution of leucine-Enkephalin purchased from Waters was used as a lock mass standard (2 ng/L in 50/50 (%, v/v) water/acetonitrile solution containing 0.2% (v/v) of formic acid).

Standards of **benzimidazoles** (albendazole, thiabendazole, oxfendazole, mebendazole, fenbendazole, oxibendazole, triclabendazole, albendazole-sulfoxide, fenbendazole-sulfone, 5-hydroxythiabendaole, triclabendazole-sulfone, triclabendazole-sulfoxide, mebendazole-amine, parbendazole, febantel, albendazole-sulfone, 5-hydroxymebendazole, albendazol-2-aminosulfone); **5-nitroimidazoles** (ronidazole, secnidazole, ipronidazole, ternidazole hydrochride, metronidazole, dimetridazole, tinidazole, ornidazole, hydroxydimetridazol,

hydroxymetronidazole, hydroxyipronidazole); **aminoglycosides** (amikacin sulfate salt, dihydrostreptomycin sesquisulfate, streptomycin sulfate salt, apramycin sulfate salt, paromomycin sulfate salt, spectinomycin dihydrochloride pentahydrate, kanamycin disulfate salt, neomycin disulfate salt hydrate, gentamicin sulfate which includes gentamicin C1a, gentamicin C1 and gentamicin C2,); **quinolones** (pipemidic acid, marbofloxacin, oxolonic acid, ciprofloxacin, enrofloxacin, difloxacin hydrochloride, norfloxacin, cinoxacin, ofloxacin, enoxacin, flumequine, lomefloxacin hydrochloride, danofloxacin, levofloxacin, pefloxacin mesylate hydrochloride, orbifloxacin, nalidixic acid); **β-lactams** (oxacillin sodium salt, cloxacillin sodium salt, nafcillin sodium salt, amoxicillin, ampicillin sodium salt, piperacillin sodium salt, penicillin V potassium salt, dicloxacillin sodium salt hydrate, cephalexin, ceftiofur, cefaloim hydrate, cefazolin sodium salt, cefoperazone sodium salt, cefquinone sulfate, cefapirin sodium, cefadroxil, cefamandole sodium salt); **sulfonamides** (sulfadiazine, sulfadimetohoxine, sulfadoxine, sulfhamethizole, sulfapyridine, sulfapethoxazole, sulfamerazine, sulfameter, sulfamethazine, sulfachloropyridazine); and **tetracyclines** (oxytetracycline hydrochloride, methacycline hydrochloride, tetracycline hydrochloride, chlortetracycline hydrochloride, doxycycline hydrochloride) were provided by Sigma Aldrich (St. Louis, MO, USA), Dr. Ehrenstorfer (Augsburg, Germany), Honeywell Riedel-de HaënTM (Seelze, Germany) and Witega (Berlin, Germany). Individual stock standard solutions of each analyte were prepared in methanol (i.e. benzimidazoles, β-lactams, sulfonamides and tetracyclines), water (i.e. aminoglycosides), acetonitrile (i.e. 5-nitroimidazoles) and 50/50 (%, v/v) acetonitrile/water solution containing 0.02% (v/v) of formic acid (i.e. quinolones). They were stored in dark glass bottles at -20 ^{o}C. Standard concentrations ranged between 250 and 1000 µg/mL depending on the analyte. Working standard solutions of each analyte (2 µg/mL in 70/30 (%, v/v) acetonitrile/water), except for aminoglycosides, were prepared from individual

stock standard solutions for CCS characterization. Working standard solutions of aminoglycosides were prepared in water (6 µg/mL).

2.2 Analysis conditions

CCS characterization of human and veterinary drugs was accomplished by flow injection analysis (FIA) using an ACQUITY UHPLC separation system (Waters, Manchester, UK). A stainless steel flexible capillary restrictor was employed instead of a LC column. The restrictor (2 m x 0.12 mm and 1/16 in female connector at both ends) was acquired from Agilent Technologies (Waldbronn, Germany). Mobile phase composition consisted of 0.1% (v/v) aqueous formic acid (solvent A) and acetonitrile containing 0.1 % (v/v) of formic acid (solvent B). Analyses were carried out under isocratic conditions (70/30 (%, v/v) A/B) at a mobile phase flow rate of 0.175 mL/min. Injection volume was 5 µL and total analysis time of each run was 2 min.

Bovine urine samples were analyzed by LC-IMS-MS, and separations were carried out in a Hypersil Gold C18 column (100 x 2.1 mm, 1.9 µm). Mobile phase consisted of 0.1 % (v/v) aqueous formic acid (solvent A) and acetonitrile containing 0.1 % (v/v) of formic acid (solvent B), and was supplied at a flow rate of 0.6 mL min^{-1}. The following gradient program was established: 0 min, 5% (v/v) B; 0.3 min, 5% (v/v) B; 9.6 min, 43% (v/v) B; 10.5 min, 100% (v/v) B; 12.5 min, 100% (v/v) B. Afterwards, mobile phase composition was back to initial conditions in 0.5 min. In order to guarantee column equilibration and achieve reproducible separations, initial conditions were maintained for 3.5 min. Column temperature was set at 50 °C and 5 µL of sample were injected.

2.3 Instrument conditions

IMS-MS analyses were carried out on a hybrid quadrupole-TWIMS-orthogonal acceleration time-of-flight (ToF) mass spectrometer (Synapt G2-S HDMS from Waters) equipped with an electrospray ionization (ESI) interface (**Figure 1**).

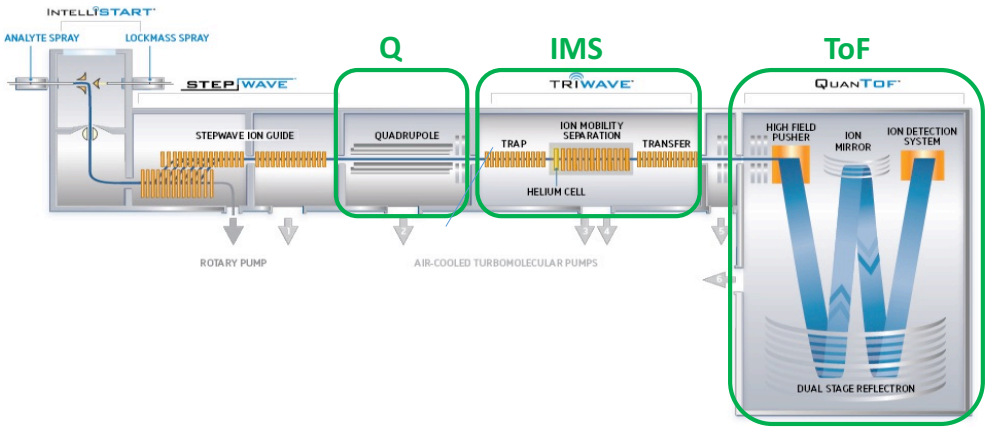

Figure 1. Synapt G2-Si High Definition Mass Spectrometry

ESI parameters were fixed as follows: capillary voltage (positive mode), 3 KV; cone voltage, 40 V; source temperature, 150 °C; desolvation temperature and gas flow, 450 °C and 800 L/h, respectively; nebulizer pressure, 6.0 bar; and cone gas flow, 100 L/h. Gas flows of ion mobility instrument were established as follows: trap gas, 2 mL/min; IMS gas, 80 mL/min; and helium cell gas, 180 mL/min. IMS DC bias and trap DC bias were set at 3 and 45 V, respectively. IMS wave velocity and height were fixed at 850 m/s and 40 V, respectively. Step wave velocity and height were adjusted to 300 m/s and 5 V, respectively. In addition, trap wave velocity and height were set at 311 m/s and 4 V, respectively, while transfer wave velocity and height were fixed at 160 m/s and 4 V, respectively.

2.4 CCS measurements

The procedure for TWIMS calibration has been previously reported [1]. It is known that CCS does not only depend on the characteristics of the related molecule, but also on the drift gas employed for CCS measurements [2,3]. In this work, CCS values were measured using nitrogen as drift gas. CCS measurements were carried out in positive ion mode. Major Mix CCS calibration solution was used in order to obtain the CCS of ions by the application of CCS calibration curves. The proposed calibration curves covered a CCS between 138 and 306 $Å^2$, and a *m/z* range from 195 to 1013. CCS was calculated by extrapolation in the case of *m/z* lower than 195. It must be remarked that CCS calibration was performed taking into account singly charged ions, so CCS measurements were only suitable to singly charged ions. Experiments were carried out in triplicate and each analyte was individually injected. All ions detected for each compound were identified with a deviation lower than 5 ppm in relation to their accurate mass.

2.5 Sample treatment procedure

Bovine urine samples were treated following a "dilute-and-shoot" procedure [4]. In short, urine samples were filtered using centrifugal filters (PES (polyethersulfone) membrane, molecular weight cut-off of 10 kDa) from VWR International (Fontenay-sous-Bois, France). Samples were centrifuged for 10 min at 9000 rpm and 15° C. Afterwards, they were spiked with a mixture of drugs (i.e. albendazole, albendazole-sulfone, albendazole-sulfoxide, ciprofloxacin, ornidazol, tinidazole, metronidazole,

[1] G. Paglia, G. Astarita, Metabolomics and lipidomics using traveling-wave ion mobility mass spectrometry, Nat. Protoc. 12 (2017) 797-813.
[2] K.L. Davidson, M.F. Bush, Effects of drift gas selection on the ambient-temperature, ion mobility mass spectrometry analysis of amino acids, Anal. Chem. 89 (2017) 2017–2023.
[3] R.T. Kurulugama, E. Darland, F. Kuhlmann, G. Stafford, J. Fjeldsted, Evaluation of drift gas selection in complex sample analyses using a high performance drift tube ion mobility-QTOF mass spectrometer, Analyst. 140 (2015) 6834–6844.
[4] K. Deventer, O.J. Pozo, A.G. Verstraete, P. Van Eenoo, Dilute-and-shoot-liquid chromatography-mass spectrometry for urine analysis in doping control and analytical toxicology, TrAC - Trends Anal. Chem. 55 (2014) 1–13.

hydroxymetronidazol, sulfadimethoxine, tetracycline hydrochloride, penicillin G sodium salt) at two concentration levels (i.e. 250 and 2500 µg L^{-1}). Finally, samples were diluted 10-fold with 0.1 % (v/v) aqueous formic acid and injected into the LC-IMS-MS system. On the other hand, the abovementioned mixture of drugs was selected in order to cover a wide range of CCS and *m/z* with representative compounds of the different drug families studied. Aminoglycosides and β-lactams were not included in this experiment because they were not detected under the reverse phase (RP)-LC conditions that were applied. The sample procedure it is shown in **Figure 2.**

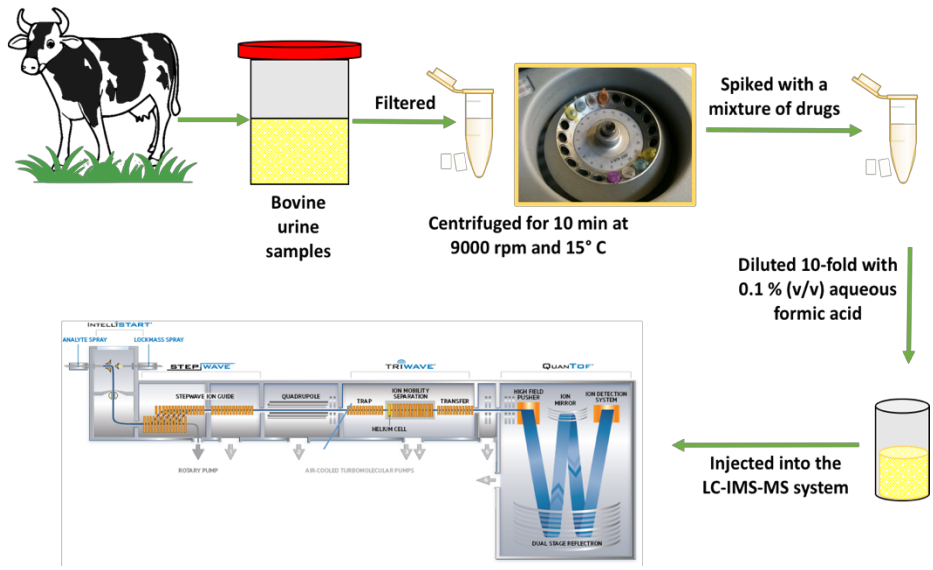

Figure 2. Scheme of the sample treatment procedure

2.6 Software and data analysis

Mass and mobility spectra were analyzed employing MassLynx software (version 4.2) which incorporates Driftscope (version 2.8) to obtain CCS data. Structure elucidation of each fragment was carried out by Chem Draw Ultra 12.0 software.

3 Results and discussion

3.1 CCS characterization of protonated molecules and sodium adducts

In this work, seven families of human and veterinary drugs have been characterized in terms of *m/z* and CCS, including: eighteen benzimidazoles, eleven 5-nitroimidazoles, eleven aminoglycosides, nineteen quinolones, eighteen β-lactams, ten sulfonamides and five tetracyclines. From a total number of 92 compounds, 37 of them have been characterized for the first time in terms of CCS. All compounds were analyzed applying positive ionization conditions because it is the most used mode for their detection [5,6]. The protonated molecule of all compounds was detected except for cefamandole, and these ions covered a *m/z* range from 142 to 646 and a CCS range from 133 to 239 $Å^2$. In addition, a wide number of $[M+Na]^+$ ions were also identified and characterized in terms of *m/z* and CCS. Sodium adducts were identified for 82 compounds and covered *m/z* and CCS ranges from 208 to 668 and from 150 to 244 $Å^2$, respectively. **Table 1** shows the $^{TW}CCS_{N_2}$ of all $[M+H]^+$ and $[M+Na]^+$ species that were detected. CCS measurements are reported following formalized nomenclature [7]. CCS measurements were carried out in triplicate, and relative standard deviations (RSDs) lower than 0.1% were generally obtained.

[5] L. Geis-Asteggiante, A. Nuñez, S.J. Lehotay, A.R. Lightfield, Structural characterization of product ions by electrospray ionization and quadrupole time-of-flight mass spectrometry to support regulatory analysis of veterinary drug residues in foods, Rapid Commun. Mass Spectrom. 28 (2014) 1061–1081.
[6] A. Nuñez, S.J. Lehotay, L. Geis-Asteggiante, Structural characterization of product ions by electrospray ionization and quadrupole time-of-flight mass spectrometry to support regulatory analysis of veterinary drug residues in foods. Part 2: Benzimidazoles, nitromidazoles, phenothiazines, and mectins, Rapid Commun. Mass Spectrom. 29 (2015) 719–729.
[7] J.C. May, C.B. Morris, J.A. McLean, Ion mobility collision cross section compendium, Anal. Chem. 89 (2017) 1032-1044.

Table 1. $^{TW}CCS_{N_2}$ database for human and veterinary drugs. *Compounds that have been characterized for the first time in terms of CCS.

Compound	CAS number	Chemical formula	Molecular weight (g/mol)	$[M+H]^+$		$[M+Na]^+$	
				m/z	$^{TW}CCSN_2$ (Å^2)	*m/z*	$^{TW}CCSN_2$ (Å^2)
Benzimidazoles							
ABZ	54965-21-8	$C_{12}H_{15}N_3O_2S$	265.331	266.0958	166.6	288.0777	178.8
TBZ	148-79-8	$C_{10}H_7N_3S$	201.247	202.0433	141.7	224.0253	152.4
FBZ-SO	53716-50-0	$C_{15}H_{13}N_3O_3S$	315.347	316.0750	177.4	338.0570	195.4
MBZ	31431-39-7	$C_{16}H_{13}N_3O_3$	295.298	296.1030	174.4	318.0849	187.8
FBZ	43210-67-9	$C_{15}H_{13}N_3O_2S$	299.348	300.0801	174.5	322.0621	186.5
OXI	20559-55-1	$C_{12}H_{15}N_3O_3$	249.270	250.1186	161.4	272.1006	173.5
TCB	31431-39-7	$C_{14}H_9Cl_3N_2O_S$	359.649	358.9574	179.3		
ABZ-SO*	54029-12-8	$C_{12}H_{15}N_3O_3S$	281.330	282.0907	169.7	304.0726	187.7
FBZ-SO_2*	54029-20-8	$C_{15}H_{13}N_3O_4S$	331.346	332.0700	181.3	354.0519	196.9
5-OH-TBZ *	948-71-0	$C_{10}H_7N_3OS$	217.246	218.0383	145.0	240.0202	156.7
TCB-SO_2*	106791-37-1	$C_{14}H_9Cl_3N_2O_3S$	319.647	390.9472	188.7	412.9292	202.9
TCB-SO*	100648-13-3	$C_{14}H_9Cl_3N_2O_2S$	375.648	374.9523	186.1	296.9345	199.5
MBZ-NH_2*	52329-60-9	$C_{14}H_{11}N_3O$	237.262	238.0975	161.9	260.0794	175.5
PBZ*	14255-87-9	$C_{13}H_{17}N_3O_2$	247.298	248.1394	164.3	270.1213	175.7
FBT*	58306-30-2	$C_{20}H_{22}N_4O_6S$	446.478	447.1333	221.5	469.1152	208.4
ABZ-SO_2*	75184-71-3	$C_{12}H_{15}N_3O_4S$	297.329	298.0856	173.0	320.0675	188.2
5-OH-MBZ*	60254-95-7	$C_{16}H_{15}N_3O_3$	297.314	298.1186	175.6	320.1006	187.8
ABZ-NH_2-SO_2*	80983-34-2	$C_{10}H_{13}N_3O_2S$	239.293	240.0801	160.7	262.0621	171.6
5-nitroimidazoles							
Ronidazole	7681-76-7	$C_6H_8N_4O_4$	200.154	201.0618	140.3	223.0438	150.0
Secnidazole	227622-73-3	$C_7H_{11}N_3O_3$	185.183	186.0873	138.6		
Ipronidazole*	14885-29-1	$C_7H_{11}N_3O_2$	169.184	170.0924	138.1		

Compound	CAS number	Chemical formula	Molecular weight (g/mol)	$[M+H]^+$		$[M+Na]^+$	
				m/z	$^{TW}CCSN_2$ (Å^2)	*m/z*	$^{TW}CCSN_2$ (Å^2)
Ternidazole*	70028-95-4	$C_7H_{11}N_3O_3$	185.183	186.0873	138.6	208.0693	152.9
Metronidazole	443-48-1	$C_6H_9N_3O_3$	171.156	172.0717	133.3		
Dimetridazole*	551-92-8	$C_5H_7N_3O_2$	141.130	142.0611	133.3		
Tinidazole*	19387-91-8	$C_8H_{13}N_3O_4S$	247.269	248.0700	149.7	270.0519	161.2
Ornidazole	16773-42-5	$C_7H_{10}ClN_3O_3$	219.625	220.0483	144.6		
Hydroxydimetridazol*	936-05-0	$C_5H_7N_3O_3$	157.129	158.0560	133.5		
Hydroxymetronidazole*	4812-40-2	$C_6H_9N_3O_4$	187.155	188.0666	136.9		
Hydroxyipronidazole*	35175-14-5	$C_7H_{11}N_3O_3$	185.183	186.0873	139.9		
Quinolones							
Pipemidic acid	51940-44-4	$C_{14}H_{17}N_5O_3$	303.322	304.1404	174.3	326.1224	185.6
Marbofloxacin*	115550-35-1	$C_{17}H_{19}FN_4O_4$	362.361	363.1463	190.6	385.1283	198.5
Oxolinic acid	14698-29-4	$C_{13}H_{11}NO_5$	261.233	262.0710	152.5	284.0529	167.2
Ciprofloxacin	85721-33-1	$C_{17}H_{18}FN_3O_3$	331.347	332.1405	188.1	354.1224	191.7
Enrofloxacin	93106-60-6	$C_{19}H_{22}FN_3O_3$	359.401	360.1718	194.9	382.1537	204.7
Difloxacin*	91296-86-5	$C_{21}H_{19}F_2N_3O_3$	399.398	400.1467	205.3	422.1287	210.4
Norfloxacin	70458-96-7	$C_{16}H_{18}FN_3O_3$	319.336	320.1405	186.9	342.1224	188.8
Cinoxacin	28657-80-9	$C_{12}H_{10}N_2O_5$	262.221	263.0662	152.1	285.0482	165.2
Ofloxacin	82419-36-1	$C_{18}H_{20}FN_3O_4$	361.373	362.1511	191.5	384.1330	200.1
Enoxacin	74011-58-8	$C_{15}H_{17}FN_4O_3$	320.324	321.1357	170.8	343.1177	185.0
Flumequine	42835-25-6	$C_{14}H_{12}FNO_3$	261.252	262.0874	153.0	284.0693	166.8
Lomefloxacin	98079-52-8	$C_{17}H_{19}F_2N_3O_3$	351.354	352.1467	191.3	374.1287	196.0
Danofloxacin*	112398-08-0	$C_{19}H_{20}FN_3O_3$	357.385	358.1561	193.3	380.1380	199.3
Levofloxacin	138199-71-0	$C_{18}H_{20}FN_3O_4$	361.373	362.1511	191.4	384.1330	199.9
Pefloxacin	149676-40-4	$C_{17}H_{20}FN_3O_3$	333.363	334.1561	188.8	356.1381	194.2
Fleroxacin*	79660-72-3	$C_{17}H_{18}F_3N_3O_3$	369.344	370.1373	189.8	392.1193	198.8

Compound	CAS number	Chemical formula	Molecular weight (g/mol)	$[M+H]^+$		$[M+Na]^+$	
				m/z	$^{TW}CCSN_2$ (Å^2)	*m/z*	$^{TW}CCSN_2$ (Å^2)
Moxifloxacin	186826-86-8	$C_{21}H_{24}FN_3O_4$	401.438	402.1824	198.5	424.1643	210.7
Orbifloxacin	113617-63-3	$C_{19}H_{20}F_3N_3O_3$	395.382	396.1530	199.5	418.1349	206.1
Nalidixic acid	389-08-2	$C_{12}H_{12}N_2O_3$	232.239	233.0921	148.0	255.0740	161.6
Tetracyclines							
Oxytetracycline*	2058-46-0	$C_{22}H_{24}N_2O_9$	460.439	461.1555	198.1	483.1374	207.3
Methacycline	3963-95-9	$C_{22}H_{22}N_2O_8$	442.424	443.1449	197.8	465.1268	207.4
Tetracycline	64-75-5	$C_{22}H_{24}N_2O_8$	444.440	445.1611	196.9	467.1425	207.1
Chlortetracycline	64-72-2	$C_{22}H_{23}ClN_2O_8$	478.882	479.1216	199.0	501.1035	210.5
Doxycycline	10592-13-9	$C_{22}H_{24}N_2O_8$	444.440	445.1605	196.8	467.1425	205.7
Sulfonamides							
Sulfadiazine	68-35-9	$C_{10}H_{10}N_4O_2S$	250.053	251.0597	153.9	273.0417	163.0
Sulfadimethoxine	122-11-2	$C_{12}H_{14}N_4O_4S$	310.328	311.0809	170.5	333.0628	178.4
Sulfadoxin	24447-57-6	$C_{12}H_{14}N_4O_4S$	310.328	311.0809	169.1	333.0628	177.8
Sulfamethizole	144-82-1	$C_9H_{10}N_4O_2S_2$	270.325	271.0318	157.8	293.0137	171.2
Sulfapyridine	14483-2	$C_{11}H_{11}N_3O_2S$	249.288	250.0645	154.6	272.0464	163.9
Sulfamethoxazole	723-46-6	$C_{10}H_{11}N_3O_3S$	253.276	254.0595	154.5	276.0413	164.8
Sulfamerazine	127-79-7	$C_{11}H_{12}N_4O_2S$	264.303	265.0754	159.0	287.0573	168.3
Sulfameter	651-06-9	$C_{11}H_{12}N_4O_3S$	280.302	281.0703	161.6	303.0522	171.3
Sulfamethazine	57-68-1	$C_{12}H_{14}N_4O_2S$	278.330	279.0910	164.4	301.0730	172.8
Sulfachloropyridazine	80-32-0	$C_{10}H_9ClN_4O_2S$	284.718	285.0208	160.9	307.0027	171.7
Aminoglycosides							
Gentamicin C1a*	1405-41-0	$C_{19}H_{39}N_5O_7$	449.549	450.2922	212.1	472.2742	212.3
Gentamicin C1*	1405-41-0	$C_{21}H_{43}N_5O_7$	477.603	478.3235	214.5	500.3055	222.8
Gentamicin C2*	1405-41-0	$C_{20}H_{41}N_5O_7$	463.576	464.3079	215.8	486.2898	217.5
Amikacin*	149022-22-0	$C_{22}H_{43}N_5O_{13}$	585.608	586.2930	235.1	608.2750	233.7
Dihydrostreptomycin*	5490-27-7	$C_{21}H_{41}N_7O_{12}$	583.596	584.2886	232.1	606.2705	230.0
Streptomycin*	3810-74-9	$C_{21}H_{39}N_7O_{12}$	581.580	582.2729	231.0	604.2549	228.5
Apramycin*	65710-07-8	$C_{21}H_{41}N_5O_{11}$	539.580	540.2875	232.9	562.2695	222.4
Paromomycin*	1263-89-4	$C_{23}H_{45}N_5O_{14}$	615.634	616.3036	239.1	638.2855	237.7

Compound	CAS number	Chemical formula	Molecular weight (g/mol)	$[M+H]^+$		$[M+Na]^+$	
				m/z	$^{TW}CCSN_2$ (Å^2)	*m/z*	$^{TW}CCSN_2$ (Å^2)
Spectinomycin*	22189-32-8	$C_{14}H_{24}N_2O_7$	332.353	333.1656	176.2	355.1476	183.5
Kanamycin*	64013-70-3	$C_{18}H_{36}N_4O_{11}$	484.503	485.2453	206.6	507.2273	210.7
Neomycin*	1405-10-3	$C_{23}H_{46}N_6O_{13}$	614.650	615.3196	238.4	637.3015	238.6
B-Lactams							
Oxacillin	1173-88-2	$C_{19}H_{19}N_3O_5S$	401.437	402.118	191.5	424.0938	195.4
Cloxacillin	642-78-4	$C_{19}H_{18}ClN_3O_5S$	435.879	436.0728	193.8	458.0548	200.4
Nafcillin	985-16-0	$C_{21}H_{22}N_2O_5S$	414.476	415.1322	196.1	437.1142	202.0
Penicillin G	69-57-8	$C_{16}H_{18}N_2O_4S$	334.0987	335.1060	176.6	357.0879	182.9
Amoxicillin	26787-78-0	$C_{16}H_{19}N_3O_5S$	365.404	366.1118	192.2	388.0938	189.3
Ampicillin	69-52-3	$C_{16}H_{19}N_3O_4S$	349.405	332.1063	187.5	372.0988	183.2
Piperacillin	59703-84-3	$C_{23}H_{27}N_5O_7S$	517.1631	518.1704	227.2	540.1523	226.4
Penicillin V	132-98-9	$C_{16}H_{18}N_2O_5S$	350.389	351.1009	182.6	373.0829	186.7
Dicloxacillin	13412-64-1	$C_{19}H_{17}Cl_2N_3O_5S$	470.321	470.0339	197.0	492.0158	205.9
Cefalexin	15686-71-2	$C_{16}H_{17}N_3O_4S$	347.389	348.1013	186.9	370.0832	183.1
Ceftiofur*	80370-57-6	$C_{19}H_{17}N_5O_7S_3$	523.553	524.0363	215.0	546.0182	211.2
Cefalonium*	5575-21-3	$C_{20}H_{18}N_4O_5S_2$	458.507	459.0791	203.5		
Cefazolin	27164-46-1	$C_{14}H_{14}N_8O_4S_3$	454.498	455.0373	186.2	477.0192	192.7
Cefoperazone	62893-20-3	$C_{25}H_{27}N_9O_8S_2$	645.666	646.1497	239.1	668.1316	244.1
Cefquinone*	118443-89-3	$C_{23}H_{24}N_6O_5S_2$	528.602	529.1322	223.2	551.1142	226.8
Cefapirin	24356-60-3	$C_{17}H_{17}N_3O_6S_2$	423.458	424.0632	183.5	446.0451	191.2
Cefadroxil	50370-12-2	$C_{16}H_{17}N_3O_5S$	363.388	364.0962	188.8	386.0781	187.7
Cefamandole	42540-40-9	$C_{18}H_{18}N_6O_5S_2$	462.499			485.0672	197.9

CCS is a molecular characteristic closely related to *m/z*. Therefore, correlation between both parameters was expected for a group of compounds belonging to the

same chemical family with similar structural composition [8,9]. **Figure 3** shows the correlation existing between CCS and *m/z* for both $[M+H]^+$ and $[M+Na]^+$ species of all studied compounds. In the present work, we observed that the majority of ions were located within a narrow interval from correlation curve as previously reported by other studies [10,11] (i.e. ±4 and ±10%, respectively). Consequently, it was considered as the interval of CCS predictability.

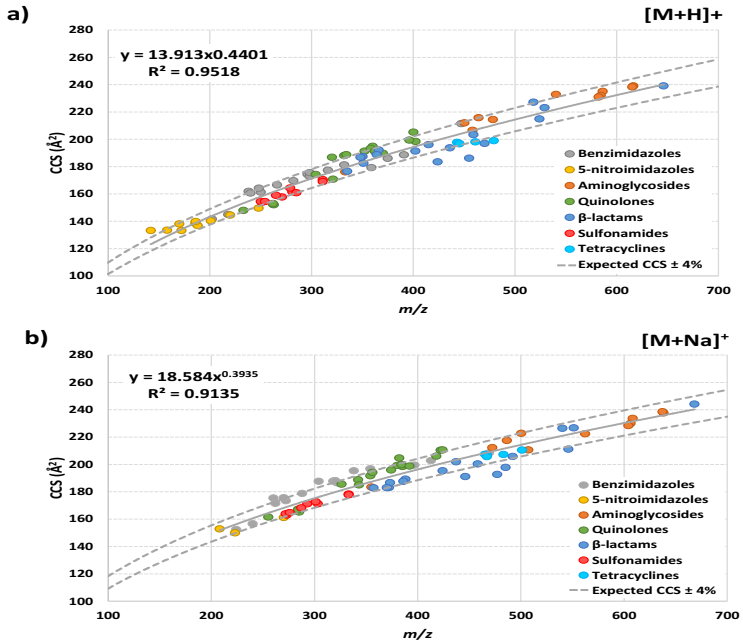

Figure 3. Representation of CCS vs *m/z* for all singly charged ions: a) $[M+H]^+$ ions and b) $[M+Na]^+$ adducts

[8] H. Borsdorf, G.A. Eiceman, Ion mobility spectrometry: Principles and applications, Appl. Spectrosc. Rev. 41 (2006) 323–375.
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In **Figure 3**, dashed lines represent approximately ±4% from the center of the data (solid line) according to the regression model proposed (power regression model). According to these results, one single regression model can describe the correlation existing between *m/z* and CCS for the seven different families of human and veterinary drugs studied, assuming a similar basic structure for all the considered compounds. On the contrary, in certain cases, several regression models are required to explain the correlation between both parameters, as previously observed for different families of steroids [11] and lipids [12]. Therefore, the study of the correlation existing between these molecular characteristics seems to be interesting, especially in untargeted analysis, because it could allow the assignment of a putative chemical nature to unidentified compounds. In general, high correlation between *m/z* and CCS was observed for $[M+H]^+$ (R^2 = 0.9518; n = 91) and $[M+Na]^+$ ions (R^2 = 0.9135; n = 82). However, the CCS values proposed by the correlation model for several compounds presented a deviation higher than 4% from the experimental CCS value. In the case of $[M+H]^+$ ions, a total number of 10 compounds presented a CCS smaller than the one predicted by the regression model (**Figure 3.a**). In particular, this was observed for four β-lactams (e.g. cefapirin, *m/z* 424.0632, $^{TW}CCS_{N_2}$ = 183.5 Å^2 or cefazolin, *m/z* 455.0373, $^{TW}CCS_{N_2}$ = 186.2 Å^2), two tetracyclines (i.e. oxytetracycline, *m/z* 461.1555, $^{TW}CCS_{N_2}$ = 198.1 Å^2 and chlortetracycline, *m/z* 479.1216, $^{TW}CCS_{N_2}$ = 199.0 Å^2), three quinolones (e.g. cinoxacin, *m/z* 263.0662, $^{TW}CCS_{N_2}$ = 152.1 Å^2 and flumequine, *m/z* 262.0874, $^{TW}CCS_{N_2}$ = 153.0 Å^2) and one 5-nitroimidazole (i.e. tinidazole, *m/z* 248.0700, $^{TW}CCS_{N_2}$ = 149.7 Å^2). In addition, CCS values were slightly higher than expected for eleven drugs, such as one 5-nitroimidazole (i.e dimetridazole, *m/z* 142.0611, $^{TW}CCS_{N_2}$ = 133.3 Å^2), two benzimidazoles (i.e. amino mebendazole, *m/z* 238.0975, $^{TW}CCS_{N_2}$ = 161.9 Å^2 and

[12] G. Paglia, P. Angel, J.P. Williams, K. Richardson, H.J. Olivos, J.W. Thompson, L. Menikarachchi, S. Lai, C. Walsh, A. Moseley, R.S. Plumb, D.F. Grant, B.O. Palsson, J. Langridge, S. Geromanos, G. Astarita, Ion mobility-derived collision cross section as an additional measure for lipid fingerprinting and identification, Anal. Chem. 87 (2015) 1137-1144.

parbendazole, *m/z* 248.1394, $^{TW}CCS_{N_2}$ = 164.3 Å^2), six quinolones (e.g. difloxacin, *m/z* 400.1467, $^{TW}CCS_{N_2}$ = 205.3 Å^2 or difloxacin, *m/z* 400.1467, CCS = 205.3 Å^2), one beta-lactamn (i.e. piperacillin, *m/z* 518.1704, $^{TW}CCS_{N_2}$ = 227.2 Å^2) and one aminoglycoside (i.e. apramycin, *m/z* 540.2875, $^{TW}CCS_{N_2}$ = 232.9 Å^2). In the case of $[M+Na]^+$ adducts, there were some analytes with a CCS that significantly differed from the value provided by the correlation curve (ΔCCS > 4%) (**Figure 3.b**). Thus, five compounds including one 5-nitroimidazole (i.e. tinidazole, *m/z* 270.0519, $^{TW}CCS_{N_2}$ = 161.2 Å^2) and four beta-lactams (e.g. cefapirin, *m/z* 446.0451, $^{TW}CCS_{N_2}$ = 191.2 Å^2 or cefazolin, *m/z* 477.0192, $^{TW}CCS_{N_2}$ = 192.7 Å^2) exhibited a CCS smaller than expected from the proposed correlation model. Moreover, there were eleven compounds including seven benzimidazoles (e.g. albendazole-sulfoxide, *m/z* 304.0726, $^{TW}CCS_{N_2}$ = 187.7 Å^2 or oxfendazole, *m/z* 304.0726, $^{TW}CCS_{N_2}$ = 187.7 Å^2), three quinolones (e.g. enrofloxacin, *m/z* 382.1537, $^{TW}CCS_{N_2}$ = 204.7 Å^2 or moxifloxacin, *m/z* 424.1643, $^{TW}CCS_{N_2}$ = 210.7 Å^2) and one aminoglycoside (i.e. gentamicin C1, *m/z* 500.3055, $^{TW}CCS_{N_2}$ = 222.8 Å^2) whose CCSs were greater than the CCSs predicted from the correlation curve.

In short, several drugs resulted in more compact or elongated molecules than predicted by the power correlation model. Consequently, their CCS provides additional structural information to *m/z* that may be useful for the identification of these compounds. Molecular modeling studies are undoubtedly required to evaluate, for example, intramolecular interactions and to further explain why certain protonated molecules and sodium adducts result in CCSs that are smaller or greater than could be expected from other set of ions presenting structural similarities. The general charge distribution in the molecule and its rigidity can be the main factors affecting its CCS as well as the charge localization resulting from the ionization (i.e. H^+, Na^+, K^+, etc.). In this sense, molecules possessing several electronegative atoms which are closely located can present a concentration of negative charge in their structure. Consequently, they could be more prone to

accommodate the positive charge, minimizing charge repulsion and leading to more compact structures than expected. This effect can also be more relevant for flexible molecules which can easily embrace the proton or the sodium atom. For example, cefapirin and cefazolin possess several electronegative atoms such as oxygen, sulfur and nitrogen (i.e. charge polarization) and are large molecules that present high flexibility, so they can easily fold onto themselves when catching a proton or a sodium atom.

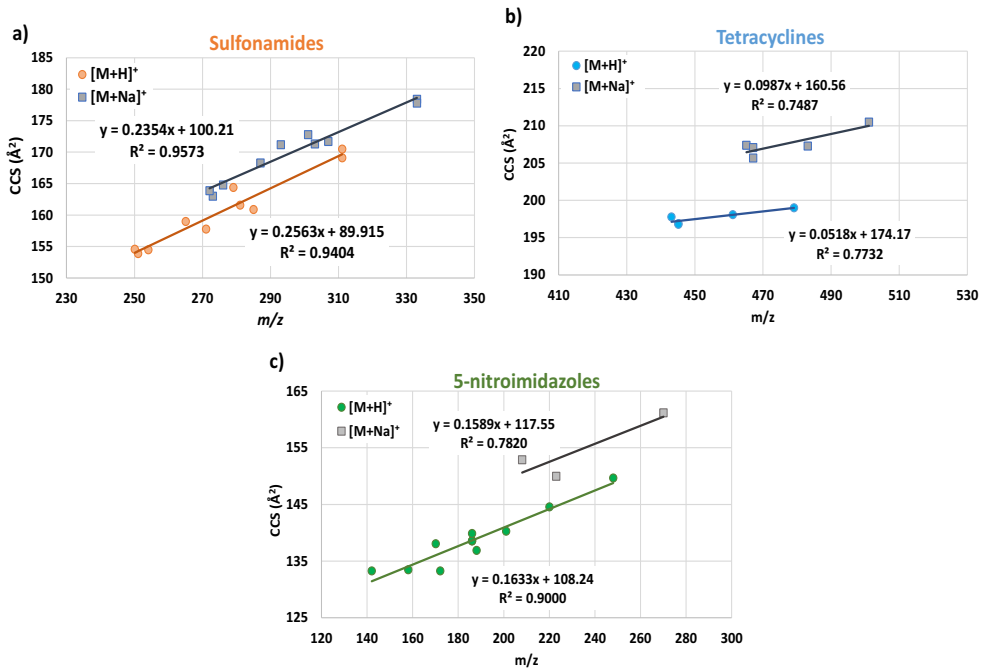


Figure 4. Representation of CCS vs *m/z* for $[M+H]^+$ and $[M+Na]^+$ species: A) sulfonamides, B) tetracyclines, and C) 5-nitroimidazoles.

In order to study in greater depth the differences between the CCS of $[M+H]^+$ and $[M+Na]^+$ ions, each family of human and veterinary drugs has been individually examined (**Figure 4** and **Figure 5**). Despite CCS and *m/z* are non-linearly correlated [13,14], as seen in **Figure 3**, we have also observed that data were better adjusted to a lineal model when a narrow *m/z* range was considered. Therefore, this has been the criteria followed through this work.

Sulfonamides showed good linearity between CCS and *m/z* for both $[M+H]^+$ and $[M+Na]^+$ ions (r = 0.9697 and r = 0.9784, respectively; n = 10), and data fitted well to the linear regression model (**Figure 4.a**). For tetracyclines, the linearity was also high (r = 0.8793 for $[M+H]^+$ and r = 0.8653 for $[M+Na]^+$; n = 5) and data deviation from correlation curves was not observed (**Figure 4.b**). However, higher difference was observed between the slopes of correlation curves for both $[M+H]^+$ and $[M+Na]^+$ species in comparison with the trends showed by other families of drugs (**Figure 4** and **Figure 5**). In the case of 5-nitroimidazoles, it must be remarked that only three sodium ions were detected from a total of eleven compounds. For protonated molecules and sodium adducts, high linearity between CCS and *m/z* was obtained (r = 0.9487; n = 11, for $[M+H]^+$, and r = 0.8843; n = 3, for $[M+Na]^+$; **Figure 4.c**). High linearity between CCS and *m/z* was also shown by benzimidazoles (r = 0.9633 for $[M+H]^+$ and r = 0.9316 for $[M+Na]^+$; n = 18; **Figure 5.a**). Regarding aminoglycosides, high linearity between CCS and *m/z* was also observed for protonated molecules and sodium adducts (r = 0.9723 for $[M+H]^+$ and r= 0.9855 for $[M+Na]^+$; n = 11; **Figure 5.b**). In relation to β-lactams, high linearity between CCS and *m/z* was also shown by $[M+H]^+$ and $[M+Na]^+$ species (r = 0.9101 and r = 0.9475, respectively; n = 17) (**Figure 5.c**). Finally, as can be observed in **Figure 5.d**, quinolones also presented high

[13] G. Paglia, M. Kliman, E. Claude, S. Geromanos, G. Astarita, Applications of ion-mobility mass spectrometry for lipid analysis, Anal. Bioanal. Chem. 407 (2015) 4995–5007
[14] J.A. McLean, W.B. Ridenour, R.M. Caprioli, Profiling and imaging of tissues by imaging ion mobility-mass spectrometry, J. Mass Spectrom. 42 (2007) 1099–1105.

linearity between CCS and *m/z* for protonated molecules and sodium adducts (r = 0.9628 and r = 0.9855, respectively; n = 19).

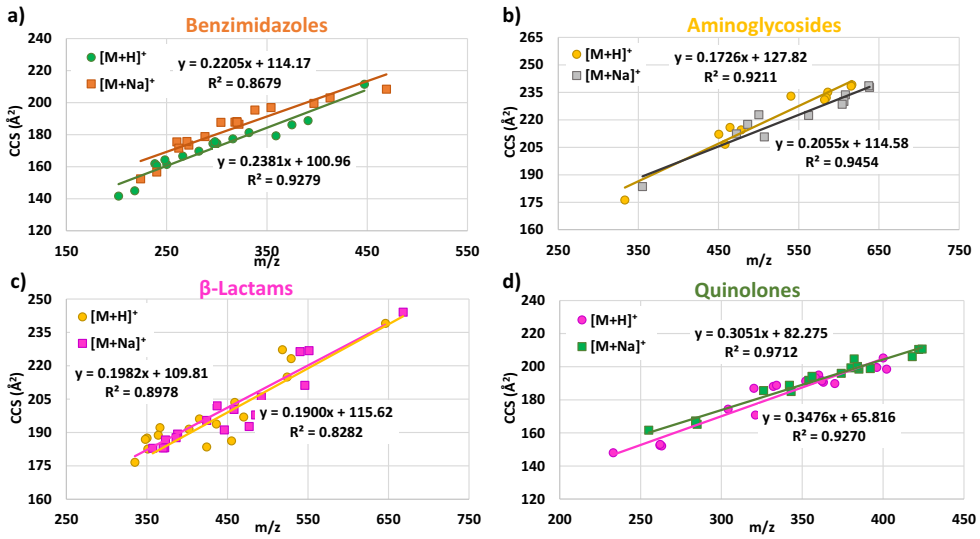


Figure 5. Representation of CCS vs *m/z* for $[M+H]^+$ and $[M+Na]^+$ species: A) benzimidazales, B) aminoglycosides, C) β-lactams, and D) quinolones.

On the other hand, the CCS of sodium adducts should be greater than the CCS of protonated adducts since $[M+Na]^+$ species are theoretically larger molecules. Consequently, they should migrate slower in the drift cell than $[M+H]^+$ ions. Under this context, it was expected that higher *m/z* values involved greater CCSs as shown by sulfonamides, tetracyclines and 5-nitroimidazoles (**Figure 4**). In these cases, the difference between the CCS of sodium adducts and protonated molecules was found similar for all the compounds belonging to these families of drugs (i.e. sulfonamides, ΔCCS = 9.8 ± 1.6 $Å^2$, tetracyclines ΔCCS = 9.9 ± 1.0 $Å^2$, and 5-nitroimidazoles, ΔCCS = 11.8 ± 2.3 $Å^2$) (**Table 2**). In general, for benzimidazoles, the CCS of sodium adducts was greater than the CCS of protonated adducts except for febantel (ΔCCS = -3.1 $Å^2$), which reflected a more compact structure when it assembled the sodium atom in comparison to the protonated molecule. In the case of twelve benzimidazole

compounds (e.g. albendazole, thiabendazole, mebendazole, among others), the difference existing between the CCS of protonated molecules and sodium adducts was close to the ΔCCS observed for sulfonamides, tetracyclines and 5-nitroimidazoles (ΔCCS = 12.3 ± 1.2 $Å^2$; n = 12; **Table 2**). However, several compounds such as oxfendazole, albendazole-sulfoxide, fenbendazole-sulfone and albendazole-sulfone presented a difference between the CCS of their related $[M+H]^+$ and $[M+Na]^+$ considerably higher than the abovementioned average value (ΔCCS > 15 $Å^2$, **Table 2**). In this sense, the difference existing between the CCS of different features or ions from the same compound does not only provides information about its structural/ionization behavior, but also may give useful information for its identification, for example, in untargeted approaches.

Table 2: Differences between the $^{TW}CCS_{N_2}$ of $[M+H]^+$ and $[M+Na]^+$ species of each compound.

Compounds	$[M+H]^+$ $^{TW}CCS_{N_2}$ ($Å^2$)	$[M+Na]^+$ $^{TW}CCS_{N_2}$ ($Å^2$)	ΔCCS ($Å^2$) between both $[M+H]^+$ and $[M+Na]^+$ species
Benzimidazoles			
ABZ	166.6	178.8	12.2
TBZ	141.7	152.4	10.7
FBZ-SO	177.4	195.4	18.0
MBZ	174.4	187.8	13.4
FBZ	174.5	186.5	12.0
OXI	161.4	173.5	12.1
ABZ-SO	169.7	187.7	18.0
FBZ-SO_2	181.3	196.9	15.6
5-OH-TBZ	145	156.7	11.7
TCB-SO_2	188.7	202.9	14.2
TCB-SO	186.1	199.5	13.4
MBZ-NH_2	161.9	175.5	13.6
PBZ	164.3	175.7	11.4
FBT	211.5	208.4	-3.1 ($CCS_{[M+Na]^+} < CCS_{[M+H]^+}$)
ABZ-SO_2	173	188.2	15.2
5-OH-MBZ	175.6	187.8	12.2
ABZ-NH_2-SO_2	160.7	171.6	10.9
5-nitroimidazoles			
Ronidazole	140.3	150	9.7

Compounds	$[M+H]^+$	$[M+Na]^+$	ΔCCS (Å^2) between both $[M+H]^+$ and $[M+Na]^+$ species
	$^{TW}CCS_{N_2}$ (Å^2)	$^{TW}CCS_{N_2}$ (Å^2)	
Ternidazole	138.6	152.9	14.3
Tinidazole	149.7	161.2	11.5
Aminoglycosides			
Gentamicin C1a	212.1	212.3	0.2
Gentamicin C1	214.5	222.8	8.3
Gentamicin C2	215.8	217.5	1.7
Amikacin	235.1	233.7	-1.4 ($CCS_{[M+Na]^+} < CCS_{[M+H]^+}$)
Dihydrostreptomycin	232.1	230	-2.1 ($CCS_{[M+Na]^+} < CCS_{[M+H]^+}$)
Streptomycin	231	228.5	-2.5 ($CCS_{[M+Na]^+} < CCS_{[M+H]^+}$)
Apramycin	232.9	222.4	-10.5 ($CCS_{[M+Na]^+} < CCS_{[M+H]^+}$)
Paromomycin	239.1	237.7	-1.4 ($CCS_{[M+Na]^+} < CCS_{[M+H]^+}$)
Spectinomycin	176.2	183.5	7.3
Kanamycin	206.6	210.7	4.1
Neomycin	238.4	238.6	0.2
Quinolones			
Pipemidic acid	174.3	185.6	11.3
Marbofloxacin	190.6	198.5	7.9
Oxolinic acid	152.5	167.2	14.7
Ciprofloxacin	188.1	191.7	3.6
Enrofloxacin	194.9	204.7	9.8
Difloxacin	205.3	210.4	5.1
Norfloxacin	186.9	188.8	1.9
Cinoxacin	152.1	165.2	13.1
Ofloxacin	191.5	200.1	8.6
Enoxacin	170.8	185	14.2
Flumequine	153	166.8	13.8
Lomefloxacin	191.3	196	4.7
Danofloxacin	193.3	199.3	6
Levofloxacin	191.4	199.9	8.5
Pefloxacin	188.8	194.2	5.4
Fleroxacin	189.8	198.8	9
Moxifloxacin	198.5	210.7	12.2
Orbifloxacin	199.5	206.1	6.6
Nalidixic acid	148	161.6	13.6
Beta-Lactams			
Oxacillin	191.5	195.4	3.9
Cloxacillin	193.8	200.4	6.6

Compounds	$[M+H]^+$	$[M+Na]^+$	ΔCCS (Å²) between both $[M+H]^+$ and $[M+Na]^+$ species
	$^{TW}CCS_{N_2}$ (Å²)	$^{TW}CCS_{N_2}$ (Å²)	
Nafcillin	196.1	202	5.9
Penicillin G sodium salt	176.6	182.9	6.3
Amoxicillin	192.2	189.3	-2.9 ($CCS_{[M+Na]^+} < CCS_{[M+H]^+}$)
Ampicillin sodium salt	187.5	183.2	-4.3 ($CCS_{[M+Na]^+} < CCS_{[M+H]^+}$)
Piperacillin sodium salt	227.2	226.4	-0.8 ($CCS_{[M+Na]^+} < CCS_{[M+H]^+}$)
Penicillin V Potassium salt	182.6	186.7	4.1
Dicloxacillin sodium salt hydrate	197	205.9	8.9
Cefalexin	186.9	183.1	-3.8 ($CCS_{[M+Na]^+} < CCS_{[M+H]^+}$)
Ceftiofur	215	211.2	-3.8 ($CCS_{[M+Na]^+} < CCS_{[M+H]^+}$)
Cefazolin	186.2	192.7	6.5
Cefoperazone	239.1	244.1	5
Cefquinone	223.2	226.8	3.6
Cefapirin	183.5	191.2	7.7
Cefadroxil	188.8	187.7	-1.1 ($CCS_{[M+Na]^+} < CCS_{[M+H]^+}$)
Sulfonamides			
Sulfadiazine	153.9	163	9.1
Sulfadimethoxine	170.5	178.4	7.9
Sulfadoxin	169.1	177.8	8.7
Sulfamethizole	157.8	171.2	13.4
Sulfapyridine	154.6	163.9	9.3
Sulfamethoxazole	154.5	164.8	10.3
Sulfamerazine	159	168.3	9.3
Sulfameter	161.6	171.3	9.7
Sulfamethazine	164.4	172.8	8.4
Sulfachloropyridazine	160.9	171.7	10.8
Tetracyclines			
Oxytetracycline	198.1	207.3	9.2
Methacycline	197.8	207.4	9.6
Tetracycline	196.9	207.1	10.2
Chlortetracycline	199.0	210.5	11.5
Doxycycline	196.8	205.7	8.9

Regarding quinolones, the CCS of $[M+Na]^+$ species was greater than the CCS related to $[M+H]^+$ ions. Eleven quinolone compounds showed similar behavior than sulfonamides, tetracyclines and 5-nitroimidazoles, presenting similar ΔCCS between $[M+H]^+$ and $[M+Na]^+$ species (11.7 ± 2.0 $Å^2$; n=11). Nevertheless, for a total number of 8 quinolones, the ΔCCS between both $[M+H]^+$ and $[M+Na]^+$ species ranged from 1.9 to 7.9 $Å^2$, being lower than could be expected. For example, this fact was observed in the case of norfloxacin (ΔCCS = 1.9 $Å^2$), ciprofloxacin (ΔCCS = 3.6 $Å^2$) and lomefloxacin (ΔCCS = 3.6 $Å^2$) among others, showing that their related sodium adducts were highly compact ions (**Table 2**).

For aminoglycosides, the investigation of the ΔCCS existing between protonated molecules and sodium adducts of the majority of compounds led to different observations. So, for most aminoglycosides (nine in total) such as amikacin (ΔCCS = -1.4 $Å^2$), dihydrostreptomycin (ΔCCS = -2.1 $Å^2$), gentamicin C1A (ΔCCS = 0.2 $Å^2$) and apramycin (ΔCCS = -10.5 $Å^2$) among others, their related $[M+Na]^+$ ion was smaller or similar in size to their $[M+H]^+$ ion. This behavior could be attributed to a change of conformation of the molecules when they host the sodium atom, making them more compact. There were only two aminoglycosides that did not follow this behavior pattern (gentamicin C1 and spectynomycin), whose sodium adducts were larger molecules than their protonated molecules (ΔCCS = 8.3 and 7.3 $Å^2$, respectively, **Table 2**).

In relation to β-lactams, the differences existing between the CCS of protonated molecules and sodium adducts for the majority of compounds also led to different behaviors. In this sense, there were six β-lactams such as amoxicillin (ΔCCS = -2.9 $Å^2$), amplicillin (ΔCCS = -4.3 $Å^2$) and cephalexin (ΔCCS = -3.8 $Å^2$) among others, whose $[M+Na]^+$ ions were smaller than their related $[M+H]^+$ species, following the same behavior as the majority of aminoglycosides. For the rest of compounds except for dicloxacillin (ΔCCS = 8.9 $Å^2$), the ΔCCS between both $[M+H]^+$ and $[M+Na]^+$ species ranged from 3.6 to 7.7 $Å^2$. It is lower than the difference on size generally observed

for the majority of compounds from other families of drug, excluding aminoglycosides (**Table 2**).

In summary, while all investigated drugs showed rather similar structural properties through close correlation between *m/z* and CCS (**Figure 3),** the differences observed between the CCS of protonated molecules and sodium adducts may provide additional information to other identification parameters (i.e. *m/z*, retention time, etc.). This means that, even though CCS can be predicted from the *m/z* of ions by different regression models, the difference existing between the CCS of different molecular adducts can be a specific characteristic of certain compounds. Thus, it may be highly useful for the reliable identification of these drugs in targeted analysis, for instance in suspect screening approaches. Furthermore, the proposed CCS database is expected to be of high applicability in untargeted approaches, where a large set of compounds, including xenobiotics, are detected. In these cases, CCS databases may be relevant in order to identify compounds that, although they present similar or the same *m/z*, show different CCS since they are non-related molecules [15].

Finally, and in order to evaluate the reproducibility of the reported CCS values, standards of eleven drugs (i.e. ABZ, ABZ-SO_2, ABZ-SO, ciprofloxacin, ornidazol, tinidazole, metroimidazole, hidroxymetroimidazole, sulfadimethoxine, tetracycline and penicillin G) were analyzed three months after the database was built. In all cases, the difference between both experimentally measured CCSs was lower than 2%, which is commonly accepted as the maximum deviation between CCS

[15] G. Paglia, J.P. Williams, L. Menikarachchi, J.W. Thompson, R. Tyldesley-Worster, S. Halldórsson, O. Rolfsson, A. Moseley, D. Grant, J. Langridge, B.O. Palsson, G. Astarita, Ion mobility derived collision cross sections to support metabolomics applications, Anal. Chem. 86 (2014) 3985-3993.

measurements [1,16]. Interestingly, however, it was observed that the highest CCS deviations were achieved for compounds with lower *m/z* such as ABZ (ΔCCS = -1.3%) and hydroxymetronidazole (ΔCCS = -1.3%). It could be due to the fact that these CCS values were obtained by extrapolation from the calibration curve. On the other hand, the lowest differences of CCSs were registered for species with higher *m/z* such as tetracycline (ΔCCS = 0.2%) and penicillin G (ΔCCS = -0.2%).

3.2 CCS comparison with previously reported values

Interlaboratory reproducibility studies are highly needed in order to evaluate if a tolerance limit of 2% is relevant for CCS measurements [1]. As mentioned before, from a total number of 92 veterinary drugs included in this study, the CCS of 55 of them has been previously measured using TWIMS-MS instrumentation [10]. Thus, inter-laboratory reproducibility was evaluated comparing the $^{TW}CCS_{N_2}$ values obtained experimentally in our laboratory with the $^{TW}CCS_{N_2}$ values previously reported. As can be observed in **Table 3**, for 60 % of the compounds, CCS differences between both databases were lower than 2%. For 9% of the analytes, the difference between both values was observed at the threshold of 2%, while 25.5% of the compounds showed CCS differences between 2.1 and 2.9%. Finally, for the remaining 5.5% of the investigated compounds, 3 β-lactams exhibited higher CCS values in comparison with those previously reported (ΔCCS = 3.1% for cefadroxil and ΔCCS= 4.7 % for cephalexin and ampicillin sodium salt, **Table 3**).

[16] S.M. Stow, T.J. Causon, X. Zheng, R.T. Kurulugama, T. Mairinger, J.C. May, E.E. Rennie, E.S. Baker, R.D. Smith, J.A. McLean, S. Hann, J.C. Fjeldsted, An interlaboratory evaluation of drift tube ion mobility-mass spectrometry collision cross section measurements, Anal. Chem. 89 (2017) 9048–9055

Table 3: Comparison between the $^{TW}CCSN_2$ from our database and the $^{TW}CCSN_2$ measured by another TWIMS instrument [10].

Compound	Ion	$^{TW}CCSN_2$ (Å^2) from our database	$^{TW}CCSN_2$ (Å^2)[1]	ΔCCS (%)	$^{DT}CCSN_2$ (Å^2)[2]	ΔCCS (%)
Benzimidazoles						
Albendazole	$[M+H]^+$	166.6	162.0	2.8		
Thiabendazole	$[M+H]^+$	141.7	137.7	2.9		
	$[M+Na]^+$	152.4			164.17	-7.2
Oxfendazole	$[M+H]^+$	177.4	174.1	1.9		
Mebendazole	$[M+H]^+$	174.4	171.2	1.9		
Fenbendazole	$[M+H]^+$	174.5	170.8	2.2		
Oxibendazole	$[M+H]^+$	161.4	157.5	2.5		
Triclabendazole	$[M+H]^+$	179.2	179.3	0.1		
5-nitroimidazoles						
Ronidazole	$[M+H]^+$	140.3	136.9	2.5		
Secnidazole	$[M+H]^+$	138.6	135.2	2.5		
Metronidazole	$[M+H]^+$	133.3	130.4	2.2		
Ornidazole	$[M+H]^+$	144.6	141.7	2.0		
Quinolones						
Pipemidic acid	$[M+H]^+$	174.3	171.0	1.9		
Oxolinic acid	$[M+H]^+$	152.5	149.1	2.3		
Ciprofloxacin	$[M+H]^+$	188.1	185.3	1.5		
Enrofloxacin	$[M+H]^+$	194.9	190.6	2.3		
Norfloxacin	$[M+H]^+$	186.9	184.2	1.5		
Cinoxacin	$[M+H]^+$	152.1	148.5	2.4		
Ofloxacin	$[M+H]^+$	191.5	188.5	1.6		
Enoxacin	$[M+H]^+$	170.8	168.1	1.6		
Flumequine	$[M+H]^+$	153.0	149.8	2.1		
Lomefloxacin	$[M+H]^+$	191.3	188.4	1.5		
Levofloxacin	$[M+H]^+$	191.4	188.5	1.5		
Pefloxacin	$[M+H]^+$	188.8	187.0	1.0		
Moxifloxacin	$[M+H]^+$	198.5	195.0	1.8		
Orbifloxacin	$[M+H]^+$	199.5	196.7	1.4		
Nalidixic acid	$[M+H]^+$	148.0	145.3	1.9		
Beta-lactams						
Oxacillin	$[M+H]^+$	191.5	189.0	1.5		
Cloxacillin	$[M+H]^+$	193.8	198.2	-2.2		

Compound	Ion	$^{TW}CCSN_2$ (Å^2) from our database	$^{TW}CCSN_2$ (Å^2)[1]	ΔCCS (%)	$^{DT}CCSN_2$ (Å^2)[2]	ΔCCS (%)
Nafcillin	$[M+H]^+$	196.1	191.5	2.4		
Penicillin G	$[M+H]^+$	176.6	174.5	1.2		
Amoxicillin	$[M+H]^+$	192.2	187.4	2.6		
Ampicillin	$[M+H]^+$	187.5	179.1	4.7	193.2	-2.9
	$[M+Na]^+$	183.2			187.9	-2.5
Piperacillin	$[M+H]^+$	227.2	223.0	1.8		
Penicillin V	$[M+H]^+$	182.6	182.7	-0.1		
Dicloxacillin	$[M+Na]^+$	205.9	202.7	1.6		
Cefalexin	$[M+H]^+$	186.9	178.5	4.7		
Cefazolin	$[M+H]^+$	186.2	183.9	1.3		
Cefoperazone	$[M+H]^+$	239.1	235.8	1.4		
Cefapirin	$[M+H]^+$	183.5	179.9	2.0		
Cefadroxil	$[M+H]^+$	188.8	183.1	3.1		
Cefamandole	$[M+Na]^+$	197.9	195.5	1.2		
Sulfonamides						
Sulfadiazine	$[M+H]^+$	153.9	150.9	2.0		
Sulfadimethoxine	$[M+H]^+$	170.5	167.7	1.7		
Sulfadoxine	$[M+H]^+$	169.1	166.2	1.7		
Sulfamethizole	$[M+H]^+$	157.8	155.2	1.7		
Sulfapyridine	$[M+H]^+$	154.6	151.6	2.0		
Sulfamethoxazole	$[M+H]^+$	154.5	151.4	2.0		
Sulfamerazine	$[M+H]^+$	159.0	156.3	1.7		
Sulfameter	$[M+H]^+$	161.6	159.0	1.6		
Sulfamethazine	$[M+H]^+$	164.4	161.7	1.7		
Sulfachloropyridazine	$[M+H]^+$	160.9	158.2	1.7		
Tetracyclines						
Methacycline	$[M+H]^+$	197.8	194.4	1.7		
Tetracycline	$[M+H]^+$	196.9	193.7	1.6	198.4	-0.8
	$[M+Na]^+$	207.1			212.5	-2.5
Chlortetracycline	$[M+H]^+$	199.0	197.8	0.6		
Doxycycline	$[M+H]^+$	196.8	195.6	0.6		

In this sense, unexpected CCS values may be originated because some of these molecules possess several protonation sites, which can lead to different isomers in the gas phase, also known as protomers. Such phenomenon is known to affect

conformation as described by Lapthorn et al. (2013) and D'Atri et al. (2018) [17,18]. Nevertheless, in this work, protomers have not been detected or identified for any of the studied molecules. It must be noted that in the recent review of D'Atri et al. [17], two CCS values were reported for the protonated molecule of ciprofloxacin (i.e. 108.7 and 119.1 $Å^2$), which was interpreted as the presence of two protomers since this molecule possesses two protonation sites. Nevertheless, in both our work and Hines et al. [10], only one – and similar- CCS value was measured for the protonated molecule of ciprofloxacin (i.e. 188.1 and 185.3 $Å^2$, respectively, **Table 3**). Such discrepancies in CCS values reported in litterature strongly highlight the need for interlaboratory comparisons.

In the light of the results of our inter-laboratory reproducibility approach, it can be concluded that CCS values reported by both databases are in agreement, which attests to their robust determination on two different analytical platforms. However, slight deviations ($2\% < \Delta CCS < 5\%$) have been observed between the CCS values reported for a few compounds, so additional investigation is needed to improve measurement accuracy and propose reliable CCS values. In particular, it should be necessary to adopt the same calibration procedure for the measurement of the CCS of these drugs when using other IMS hardware rather than DTIMS (e.g. TWIMS). Nowadays, there is not standardized calibration protocols, although Plagia et Astarita have recently proposed a protocol for the application of TWIMS-MS in metabolomics and lipidomics studies, including system calibration [1]. Moreover, our results call for the development of new CCS calibrating solutions covering a greater range of *m/z* and CCS in order to avoid CCS calculation by extrapolation of

[17] V. D'Atri, T. Causon, O. Hernandez-Alba, A. Mutabazi, J.L. Veuthey, S. Cianferani, D. Guillarme, Adding a new separation dimension to MS and LC–MS: What is the utility of ion mobility spectrometry?, J. Sep. Sci. 41 (2018) 20–67.

[18] C. Lapthorn, T.J. Dines, B.Z. Chowdhry, G.L. Perkins, F.S. Pullen, Can ion mobility mass spectrometry and density functional theory help elucidate protonation sites in "small" molecules?, Rapid Commun. Mass Spectrom. 27 (2013) 2399–2410.

calibration curves. Currently, poly-dl-alanine and the CCS Major Mix calibration solution from Waters are the calibrating solutions more frequently used for CCS calibration. Nevertheless, the use of these calibrants is not standardized. From our point of view, the standardization of both calibration protocol and calibrant is required for improving the accuracy and precision of CCS measurements. The development of a new calibrating solution that consists of a larger number of compounds covering a wider range of *m/z* and CCS is essential for achieving this purpose.

In this sense, it is of prime importance to carry out inter-laboratory studies that include TWIMS, whose main handicap is the requirement of carrying out the calibration of the instrument before performing CCS measurements [19]. In the case of DTIMS, at least one inter-laboratory study has already been published [9]. This work shows high precision for CCS measurements carried out in different laboratories (RSDs < 0.29%) but, in DTIMS, system calibration is not required, which simplifies CCS determination. DTIMS allows directly relating the mobility of ions with their CCS. Thus, it should provide higher precision and accuracy than TWIMS because the need to obtain CCS values by the application of a calibration curve can increase the uncertainty of results.

To the best of our knowledge, TWIMS and DTIMS comparison studies have not been performed. Moreover, $^{DT}CCS_{N_2}$ values (i.e. CCS measurements by DTIMS) have not been reported for the human and veterinary drugs studied in this work. Only the $^{DT}CCS_{N_2}$ for tetracycline, ampicilline and thiabendazole has been previously reported [9]. As shown in **Table 3**, in the case of tetracycline and ampicillin, CCS differences between both databases range between -2.9 and 0.8%. However, higher deviation is observed in the case of the sodium adduct of thiabendazole (ΔCCS = -7.2%). It

[19] K.M. Hines, J.C. May, J.A. McLean, L. Xu, Evaluation of collision cross section calibrants for structural analysis of lipids by traveling wave ion mobility-mass spectrometry, Anal. Chem. 88 (2016) 7329–7336.

highlights the requirement of CCS studies involving both DTIMS and TWIMS technologies in order to provide accurate CCS values and establish acceptable thresholds of uncertainty for CCS measurements. On the other hand, a recent article has reported the applicability of a ^{DT}CCS database covering 250 veterinary drugs [20]. Nevertheless, this database is not publicly available. This is a great inconvenience for data comparison and, consequently, for the establishment of accurate CCS values in which we can rely on

3.3 Characterization of main molecular fragments for each compound

Fragment ions of a wide range of compounds were observed during CCS characterization although fragmentation experiments were not carried out. These fragmentations could be a consequence of the ionization conditions or the instrumental conditions fixed before the ion mobility cell. It has been reported that ion fragmentation can be caused by field heating effects occurred during TWIMS analysis [21,22]. Indeed, fragments with drift times different from the drift time of the parent ion were observed (i.e. fragmentation occurred before the mobility cell). In short, fragmentation ions were identified for the majority of compounds and, consequently, a total number of 238 molecular fragments have been characterized in terms of CCS. In the case of triclabendazole sulfone, gentamicin sulfate C2, cefquinome sulfate, cefamandole sodium salt and sulfameter, ion fragmentation was not observed. The use of HRMS allowed the structure elucidation of these

[20] Z. Xu, J. Li, A. Chen, X. Ma, S. Yang, A new retrospective, multi-evidence veterinary drug screening method using drift tube ion mobility mass spectrometry, Rapid Commun. Mass Spectrom. 32 (2018) 1141–1148.
[21]S.I. Merenbloom, T.G. Flick, E.R. Williams, How hot are your ions in TWAVE ion mobility spectrometry? J. Am. Soc. Mass Spectrom. 23 (2012) 553-562.
[22]D. Morsa, V. Gabelica, E. De Pauw, Fragmentation and isomerization due to field heating in travelling wave ion mobility spectrometry, J. Am. Soc. Mass Spectrom. 25 (2014) 1384-1393.

fragments which were pinpointed with a deviation lower than 5 ppm in relation to their accurate mass. While most of these fragments have previously been reported in bibliography [5,6,23,24,25,26], it is the first time that their related CCS has been measured.

The CCS obtained for each product ion as well as their corresponding elucidated structure are included in **Annex. Table 1.** Structures in black have been previously reported whereas structures in blue have been proposed for the first time in the context of the present work. In order to study the correlation between their *m/z* and CCS, both parameters have been represented according to drug family of the parent ion. Structures in black have been previously reported whereas structures in blue have been proposed for the first time in the context of the present work. In order to study the correlation existing between their *m/z* and CCS, both parameters have been represented according to the drug family of the parent ion. In **Figure 6** and **Figure 7**, grey dashed lines represent approximately ±4% from the linear regression curve (solid line). It must be remarked that from a total number of 238 molecular fragments, the CCS of 98 fragments ions had to be estimated by extrapolation of the CCS calibration curve because they exhibited a *m/z* value lower than 195. The CCS of the other 140 ions was directly obtained from the CCS calibration curve (*m/z* range from 198 to 542).

[23] B.J. Goolsby, J.S. Brodbelt, Analysis of protonated and alkali metal-cationized aminoglycoside antibiotics by collision-activated dissociation and infrared multi-photon dissociation in the quadrupole ion trap, J. Mass Spectrom. 1024 (2000) 2006.
[24] M.P. Hermo, D. Barrón, J. Barbosa, Determination of multiresidue quinolones regulated by the European Union in pig liver samples. High-resolution time-of-flight mass spectrometry versus tandem mass spectrometry detection, J. Chromatogr. A. 1201 (2008) 1–14.
[25] A. Juan-García, G. Font, Y. Picó, Determination of quinolone residues in chicken and fish by capillary electrophoresis-mass spectrometry, Electrophoresis. 27 (2006) 2240–2249.
[26] S.B. Turnipseed, J.M. Storey, S.B. Clark, K.E. Miller, Analysis of veterinary drugs and metabolites in milk using quadrupole time-of-flight liquid chromatography-mass spectrometry, J. Agric. Food Chem. 59 (2011) 7569–7581.

In the case of benzimidazoles, aminoglycosides, quinolones, β-lactams and sulfonamides, a linear correlation between CCS and *m/z* was observed for all product ions (**Figure 6** and **Figure 7**). Regarding benzimidazoles (n = 45), *m/z* values ranged from 131 to 415 and CCS were between 131 and 198 $Å^2$, resulting in high linear correlation (r = 0.9738, **Figure 6.a**).

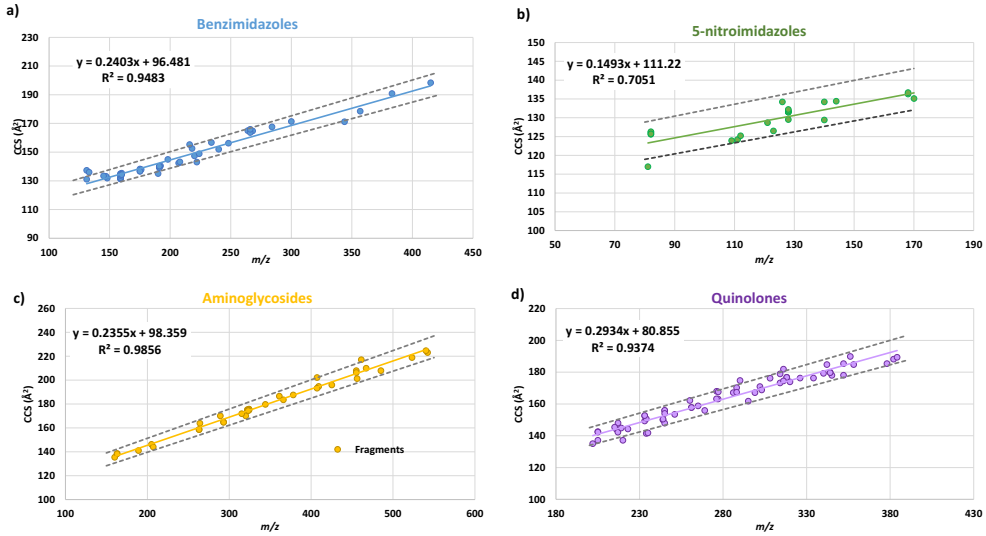


Figure 6. Representation of CCS vs *m/z* for the main molecular fragments for each family of compounds: A) benzimidazoles, B) 5-nitroimidazoles, C) aminoglycosides, and D) quinolones. Grey dashed lines represent approximately ±4% from the linear regression curve (solid line).

However, three fragments presented CCS deviations higher than +4% between the experimental and the CCS values predicted by the regression model (i.e. albendazole-2-amino-sulfone, $C_7H_7N_3^{+\bullet}$, *m/z* 133.0634; fenbendazole, $C_7H_5N_3^{+\bullet}$, *m/z* 131.0478; and parbendazole, $C_{12}H_{14}N_3O^+$, *m/z* 216,1131). On the other hand, other three fragments presented a CCS deviation below the threshold of -4 % (i.e. fenbendazole, $C_{12}H_{14}N_3O^+$, *m/z* 190.0700; albendazole sulfoxide, $C_9H_8N_3O_2S^+$, *m/z* 222.0332; and triclabendazole, $C_{13}H_7Cl_3N_2OS^{+\bullet}$, *m/z* 343.9339). Fragment ions of aminoglycosides (n = 37) also presented high linearity between *m/z* and CCS (r = 0.9928, **Figure 6.c**), covering a *m/z* range from 160 to 540 and a CCS between 135

and 225 Å^2. Only one fragment exhibited a CCS deviation above the threshold of +4% (i.e. gentamicin C1, $C_{13}H_7Cl_3N_2OS^{+\bullet}$, *m/z* 461.2970). In the case of quinolones (n = 63), *m/z* ranged between 202 and 384 and the CCS ranged from 135 to 189 Å^2, showing also high linearity between both parameters (r = 0.9682, **Figure 6.d**). Two quinolone fragment ions presented a CCS greater than could be expected from the linear regression (i.e. pefloxacin, $C_{16}H_{21}FN_3O^+$, *m/z* 290.1663; enrofloxacin, $C_{18}H_{23}FN_3O^+$, *m/z* 316.1820). Additionally, three fragments showed CCSs smaller than predicted by the regression curve (i.e. flumequine, $C_{11}H_7FNO_3^+$, *m/z* 220.0404; cinoxacin, $C_{10}H_7N_2O_5^+$, *m/z* 235.0349; and oxolonic acid, $C_{11}H_8NO_5^+$, *m/z* 234.0397).

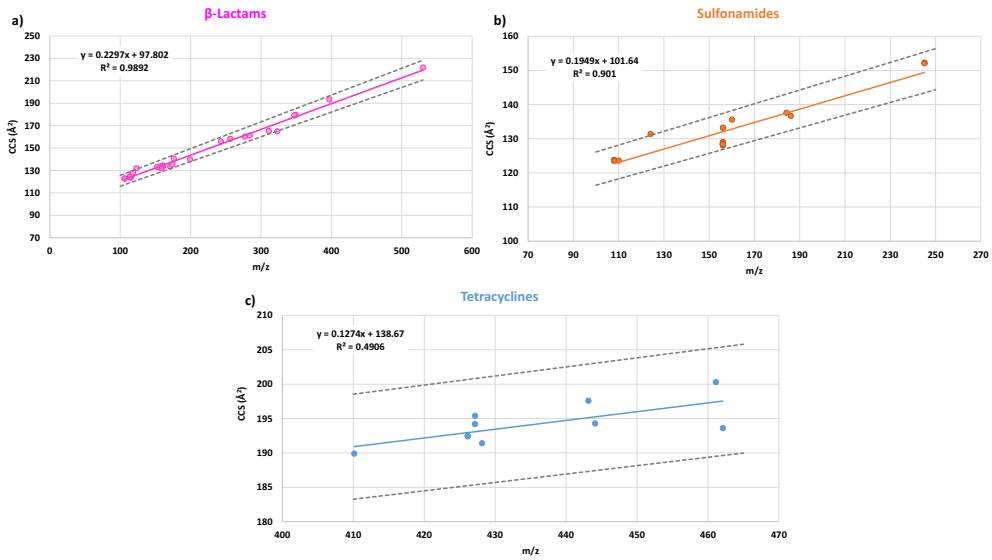


Figure 7. Representation of CCS vs *m/z* for the main molecular fragments for each family of compounds: A) β-lactams, B) sulfonamides, and C) tetracyclines. Grey dashed lines represent approximately ±4% from the linear regression curve (solid line).

As can be observed in **Figure 7.a,** high linearity (r = 0.9946) between *m/z* and CCS was also showed by β-lactam-related ion fragments (n = 37), covering a *m/z* range from 106 to 530 and a CCS range between 123 and 222 Å^2. Nevertheless, in the case of cefalonium ($C_6H_7N_2O^+$, *m/z* 123.0553), the CCS was slightly greater (ΔCCS > +4%) than the predicted CCS value. It was also observed that the CCS was significantly smaller than expected for cefazolin ($C_{11}H_{11}N_6O_4S^+$, *m/z* 323.0553). Concerning

sulfonamides, the *m/z* of their ion fragments and their related CCS (n = 22) fitted well with the proposed linear regression model (r = 0.9492, **Figure 7.b**), which covers a *m/z* range from 108 to 156 and a CCS range between 124 and 152 $Å^2$. The majority of sulfonamides presented the same two characteristic fragment ions (i.e. $C_6H_{10}N_3O_2^+$, *m/z* 156.0768; and $C_6H_6NO^+$, *m/z* 108.0444). However, there was one fragment ion corresponding to sulfamethazine ($C_6H_{10}N_3^+$; *m/z* 124.0869) whose CCS was slightly greater than could be expected. On the other hand, in the case of 5-nitroimidazoles and tetracyclines, low linear correlation was observed between *m/z* and CCS parameters (**Figure 6.b** and **Figure 7.c**, respectively). Regarding 5-nitroimidazoles, the CCS obtained for dimetridazole fragment ion ($C_4H_5N_2^+$, *m/z* 81.0447) was lower than predicted by the correlation model (**Figure 6.b**). Regarding tetracyclines (**Figure 7.c**), all investigated fragments present a CCS within the established threshold (±4% from the regression curve).

In the light of the results obtained, it globally appears that independently of the family of drugs, the majority of fragments present high linear correlation between CCS and *m/z*. Only a few fragments present a CCS greater or smaller than predicted by the corresponding linear regression model. This indicates that these fragments are more elongated or compact molecules than could be expected, showing different structural behavior in comparison with the rest of fragment ions. Nevertheless, and this must be taken into account, CCS deviation could be also due to the extrapolation of data from the regression curve in the case of fragment ions with *m/z* lower than 195. Therefore, the use of an alternative calibrant, which would be better adapted to compounds with low *m/z* values, should be adequate to confirm this hypothesis. On the other hand, despite CCS is also linearly correlated with *m/z* when representing both parameters for all the fragment ions (**Figure 8**), the additional information provided by CCS can be useful for the characterization of some features detected in untargeted analyses. For example, several fragment ions related to quinolone, benzimidazole and aminoglycoside drugs are more compact

or elongated that could be expected from the regression curve, so they present a particular CCS that can be used for their differentiation from other ions of low molecular weight.

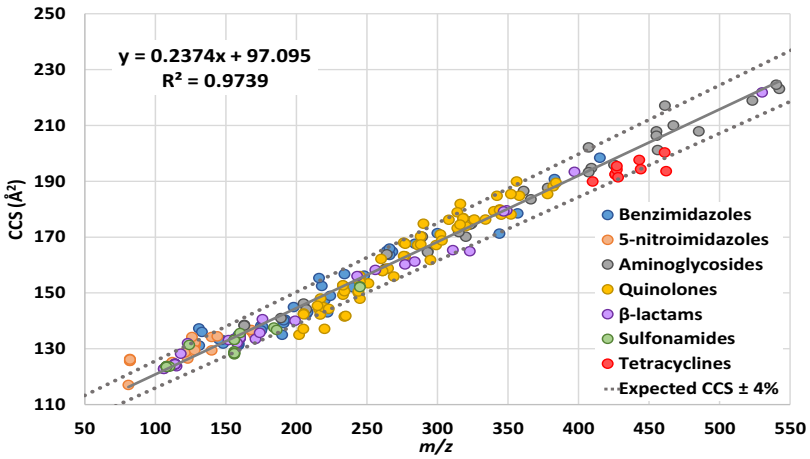


Figure 8. Representation of CCS vs *m/z* for the fragment ions identified for all the drugs analyzed.

3.4 Application of ion mobility spectrometry to the analysis of urine samples

In order to check the applicability of the proposed database, eleven compounds were characterized in bovine urine samples, including three benzimidazoles, one quinolone, four 5-nitroimidazoles, one sulfonamide, one tetracycline and one β-lactam (**Table 4**). Two different blank samples were spiked with a mixture of these veterinary drugs at two concentration levels (i.e. 250 and 2500 µg L^{-1}). Then, they were analyzed according to the LC-IMS-MS method described in "Experimental" section. The differences observed between the CCS of analytes in urine samples and the CCS reported in our database were within the ±2% threshold, ranging from -0.6 to -1.8%. As can be seen in **Table 4,** the greatest deviations were obtained for species with low *m/z* values (e.g. hydroxymetronidazole, ΔCCS = -1.7% at 250 µg L^{-1} and ΔCCS = -1.8% and 2500 µg L^{-1})**.** Conversely, the lowest differences of CCS values were found for compounds with higher *m/z* values such as tetracycline hydrochloride (ΔCCS = -0.6 % at 250 µg $L^{-1}$and ΔCCS = -0.6 % at 2500 µg L^{-1}).

Table 4. Collision cross sections of eleven veterinary drugs in cow urine samples.

Compound	Ion	Measured *m/z*[a]	Δ*m/z* (%)[a]	$^{TW}CCSN_2$ (Å^2)[a]	ΔCCS (%)	Measured *m/z*[b]	Δ*m/z* (%)[b]	$^{TW}CCSN_2$ (Å^2)[b]	ΔCCS (%)
ABZ	$[M+H]^+$	266.0946	4.5	164.2	-1.4	266.0953	1.9	163.9	-1.6
ABZ-SO_2	$[M+H]^+$	298.0850	2.0	172.0	-0.6	298.0850	2.0	171.5	-0.9
ABZ-SO	$[M+H]^+$	282.0900	2.5	167.5	-1.3	282.0900	2.5	167.1	-1.5
Ciprofloxacin	$[M+H]^+$	332.1396	2.7	187.4	-0.4	332.1398	2.1	187.0	-0.6
Ornidazol	$[M+H]^+$	220.0475	3.6	143.0	-1.1	220.0475	3.6	142.7	-1.3
Tinidazole	$[M+H]^+$	248.0688	4.8	148.1	-1.1	248.0689	4.4	148.0	-1.1
Metronidazole	$[M+H]^+$	172.0706	6.4	131.8	-1.1	172.0706	6.4	131.6	-1.3
Hidroxymetronidazole	$[M+H]^+$	188.0647	10.1	134.6	-1.7	188.0655	5.8	134.5	-1.8
Sulfadimethoxine	$[M+H]^+$	311.0799	3.2	169.5	-0.6	311.0801	2.6	169.0	-0.9
Tetracycline	$[M+H]^+$	445.1601	2.2	195.7	-0.6	445.1604	1.6	195.8	-0.6
Penicillin G	$[M+H]^+$	335.1050	3.0	175.7	-0.5	335.1055	1.5	175.5	-0.6

Note: CCSs were measured in urine samples fortified with a mixture of drugs at two concentration levels, [a]250 µg L^{-1} and [b]2500 µg L^{-1}, and compared with the data from our database (i.e. accurate mass and CCS).

In view of the results obtained (**Table 4**), the CCS values provided by the database can be considered reliable for their use as complementary parameter for identification of human and veterinary drugs in real samples such as urine.

4. Conclusions

Additional information for the unambiguous identification of a wide range of contaminants and other residues is required by new trends in high-throughput multi-compounds analysis and related suspect screening workflows. In this context, this work proposes a CCS database for a large set of human and veterinary drugs. CCS values have been measured by TWIMS using nitrogen as drift gas. The proposed database includes 92 compounds of which 37 have been characterized for the first time in terms of CCS. Moreover, the main fragment ions for most compounds (n = 238) have also been characterized in terms of CCS as well as their structure has been elucidated. Finally, CCS values have been determined in spiked urine samples, achieving high reproducibility of the associated measurement in biological matrices. Consequently, CCS could be used for identification purposes in traditional LC-MS workflows in combination with other well established identification criteria such as precursor ion, accurate mass, fragment ions, isotopic pattern and retention time. Besides, unlike retention time and MS/MS spectra which can be affected by many experimental factors, CCS value is expected to be highly reproducible across instruments and laboratories, and also easy to be standardized. However, experimentally derived CCSs still need to be validated by inter-laboratory studies as well as they must be compared with computationally derived CCS values obtained from atomistic models, in order to use this “new” criteria with confidence for the characterization of chemicals. In addition to its relevance in targeted approaches as described in the present article, IMS dimension also looks appealing in metabolomics for improving peak annotation confidence. Therefore, the creation of

CCS databases are crucial for identification purposes [27,28,29,30], being until now metabolomics' Achilles heel. In such untargeted approaches, the development of robust CCS databases is also indispensable to support the development and application of IM–MS.

Within this framework, and from our individual perspective, several issues must be tackled before CCS can be implemented as identification parameter in targeted and untargeted analytical workflows. First, additional CCS databases must be produced and made publicly available. These databases should include information about the CCS of molecules in different drift gases (i.e. nitrogen, helium and carbon hydroxide). Second, CCS databases must be supported by different IMS technologies including DTIMS, TWIMS, trapped ion mobility spectrometry (TIMS), which has recently been commercialized and also allows measuring the CCS of molecules, in order to establish accurate CCS values. Third, inter-laboratory studies must not only validate the existing CCS databases, but should also establish uncertainty thresholds for CCS measurements. Finally, the CCS characterization of residues and contaminants in real samples must also be carried out. Despite the effect of the matrix on the CCS of molecules seems to be negligible, assays involving a wide range

[27] M. Hernández-Mesa, A. Escourrou, F. Monteau, B. Le Bizec, G. Dervilly-Pinel, Current applications and perspectives of ion mobility spectrometry to answer chemical food safety issues, TrAC - Trends Anal. Chem. 94 (2017) 39–53.
[28] G. Paglia, J.P. Williams, L. Menikarachchi, J.W. Thompson, R. Tyldesley-Worster, S. Halldórsson, O. Rolfsson, A. Moseley, D. Grant, J. Langridge, B.O. Palsson, G. Astarita, Ion mobility derived collision cross sections to support metabolomics applications, Anal. Chem. 86 (2014) 3985–3993.
[29] X. Zhang, K. Kew, R. Reisdorph, M. Sartain, R. Powell, M. Armstrong, K. Quinn, C. Cruickshank-Quinn, S. Walmsley, S. Bokatzian, E. Darland, M. Rain, K. Imatani, N. Reisdorph, Performance of a high-pressure liquid chromatography-ion mobility-mass spectrometry system for metabolic profiling, Anal. Chem. 89 (2017) 6384–6391.
[30] H. Maleki, A.K. Karanji, S. Majuta, M.M. Maurer, S.J. Valentine, Ion mobility spectrometry-mass spectrometry coupled with gas-phase hydrogen/deuterium exchange for metabolomics analyses, J. Am. Soc. Mass Spectrom. 29 (2018) 230–241.

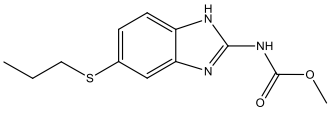
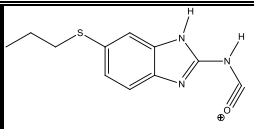
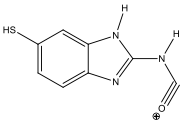
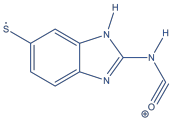
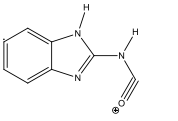
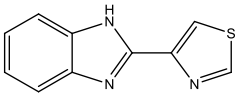
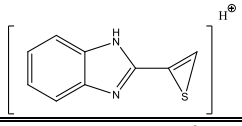
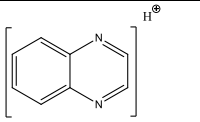
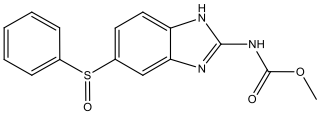
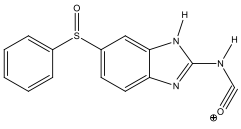
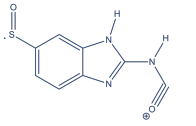
of matrices is still required in order to provide confidence in the implementation of the CCS as additional characteristic for compound identification.

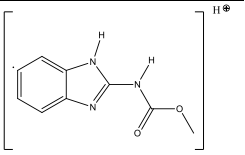
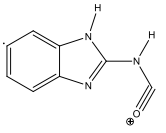
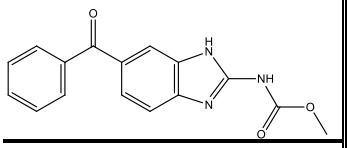
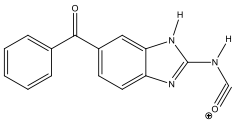
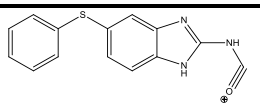
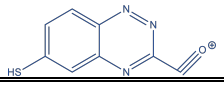
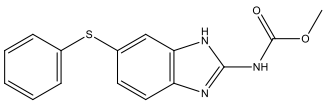
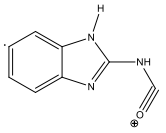
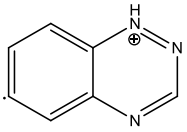
This work was published as:

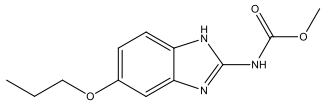
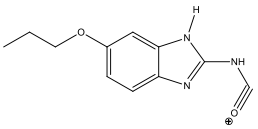
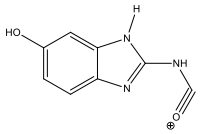
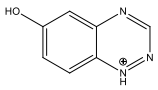
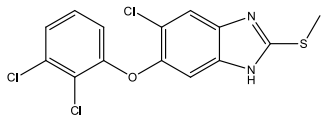
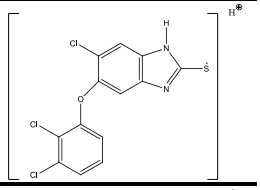
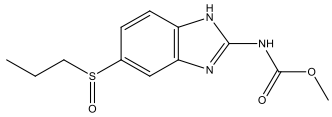
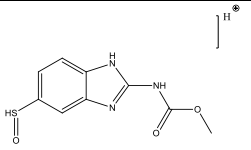
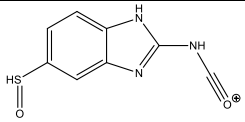
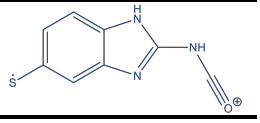
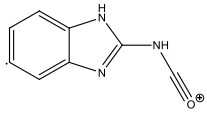
***"Collision cross section (ccs) as a complementary parameter to characterize human and veterinary drug"*. Tejada-Casado, M. Hernández-Mesa, F. Monteau, F.J. Lara, M. Olmo-iruela, A.M. García-campa, B. Le Bizec, G. Dervilly-pinel, Analytica Chimica Acta, (2018) 1–12.**

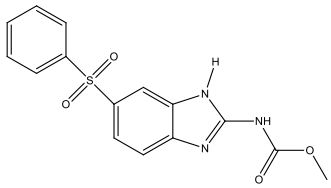
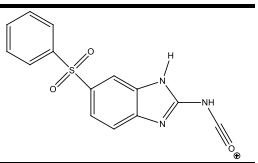
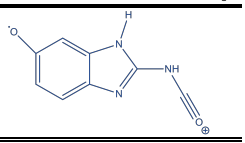
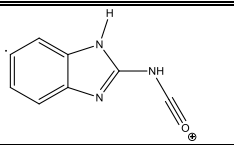
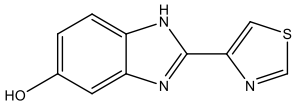
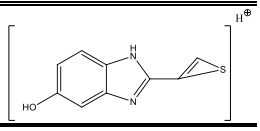
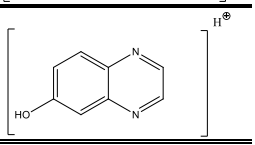
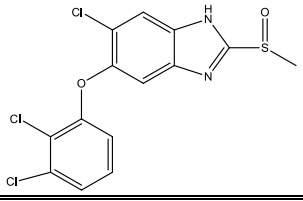
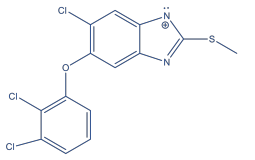
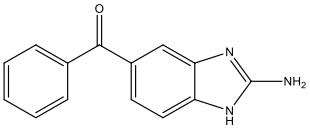
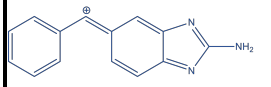
5. Annex

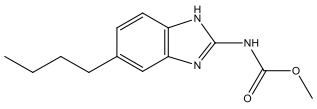
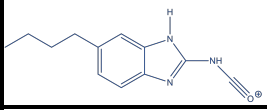
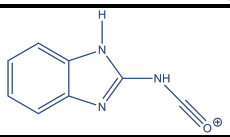
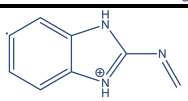
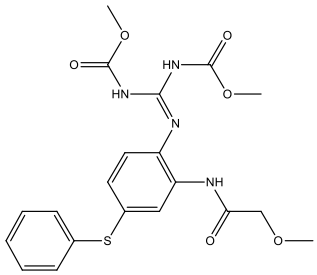
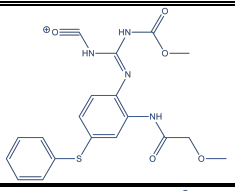
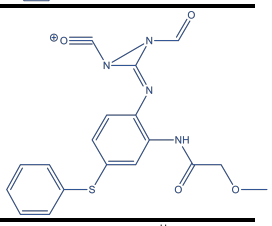
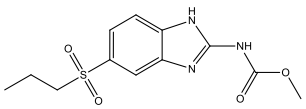
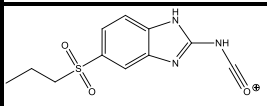
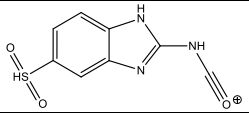
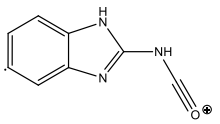
Table 1. CCS values for the proposed product ions of each human and veterinary drug (n = 3).

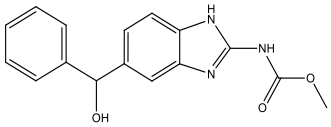
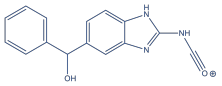
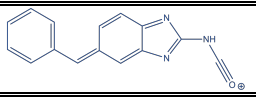
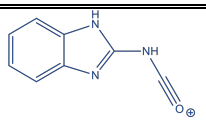
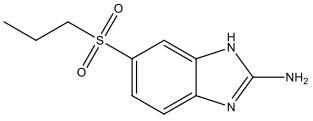
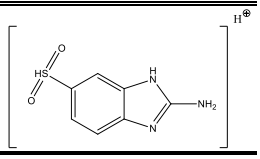
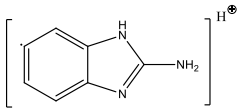
Analytes	Product Ions (m/z)	m/z	CCS ($Å^2$)	Proposed structure
Benzimidazoles				
ABZ 	$C_{11}H_{12}N_3OS^+$	234.0696	156.7	
	$C_8H_6N_3OS^+$	192.0226	140.4	
	$C_8H_5N_3OS^{\bullet+}$	191.0148	139.6	
	$C_8H_5N_3O^{\bullet+}$	159.0427	131.3	
TBZ 	$C_9H_7N_2S^+$	175.0324	138.2	
	$C_8H_7N_2^+$	131.0604	131.1	
FBZ-SO 	$C_{14}H_{10}N_3O_2S^+$	284.0488	167.6	
	$C_8H_5N_3O_2S^{\bullet+}$	207.0097	142.4	

Analytes	Product Ions (m/z)	m/z	CCS ($Å^2$)	Proposed structure
	$C_9H_9N_3O_2^{\bullet+}$	191.0689	139.7	
	$C_8H_5N_3O_2^{\bullet+}$	175.0376	136.7	----------
	$C_8H_5N_3O^{\bullet+}$	159.0427	134.9	
MBZ 	$C_{15}H_{10}N_3O_2^{+}$	264.0768	165.0	
	$C_{14}H_{10}N_3OS^{+}$	268.0539	164.8	
	$C_8H_4N_3OS^{+}$	190.0070	135.0	
FBZ 	$C_8H_5N_3O^{\bullet+}$	159.0427	131.6	
	$C_7H_5N_3^{\bullet+}$	131.0478	137.3	

Analytes	Product Ions (m/z)	m/z	CCS (Å^2)	Proposed structure
OXI 	$C_{11}H_{12}N_3O_2^+$	218.0924	152.5	
	$C_8H_6N_3O_2^+$	176.0455	137.9	
	$C_7H_6N_3O^+$	148.0505	131.8	
TCB 	$C_{13}H_7Cl_3N_2OS^{\bullet}_{+}$	343.9339	171.2	
ABZ-SO 	$C_9H_{10}N_3O_3S^+$	240.0437	152.0	
	$C_9H_8N_3O_2S^+$	222.0332	143.0	----------
	$C_8H_6N_3O_2S^+$	208.0175	143.2	
	$C_8H_5N_3OS^{\bullet+}$	191.0148	139.2	
	$C_8H_5N_3O^{\bullet+}$	159.0427	133.6	

Analytes	Product Ions (m/z)	m/z	CCS ($Å^2$)	Proposed structure
FBZ-SO_2 	$C_{14}H_{10}N_3O_3S^+$	300.0437	171.4	
	$C_8H_5N_3O_2^{\bullet+}$	175.0376	136.6	
	$C_8H_5N_3O^{\bullet+}$	159.0427	133.6	
5-OH-TBZ 	$C_9H_7N_2OS^+$	191.0274	140.2	
	$C_8H_7N_2O^+$	147.0553	133.2	
TCB-SO 	$C_{14}H_8Cl_3N_2OS^+$	356.9417	178.5	
MBZ-NH_2 	$C_{14}H_{10}N_3^+$	220.0869	147.4	

Analytes	Product Ions (m/z)	m/z	CCS ($Å^2$)	Proposed structure
PBZ 	$C_{12}H_{14}N_3O^+$	216.1131	155.3	
	$C_8H_6N_3O^+$	160.0505	135.3	
	$C_8H_7N_3O^{\bullet+}$	145.0634	133.4	
FBT 	$C_{19}H_{19}N_4O_5S^+$	415.1071	198.4	
	$C_{18}H_{15}N_4O_4S^+$	383.0809	190.8	
$ABZ\text{-}SO_2$ 	$C_{11}H_{12}N_3OS^+$	266.0594	163.6	
	$C_8H_6N_3O_3S^+$	224.0124	148.9	
	$C_8H_5N_3O^{+\bullet}$	159.0427	134.5	

Analytes	Product Ions (m/z)	m/z	CCS (Å^2)	Proposed structure
5-OH-MBZ 	$C_{15}H_{12}N_3O_2^+$	266.0924	165.9	
	$C_{15}H_{10}N_3O^+$	248.0818	156.2	
	$C_8H_6N_3O^+$	160.0505	134.3	
ABZ-NH_2-SO_2 	$C_7H_8N_3O_2^+$	198.0332	144.9	
	$C_7H_7N_3^{+\bullet}$	133.0634	136.0	
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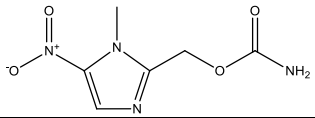
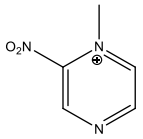
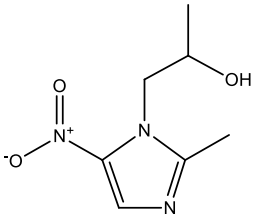
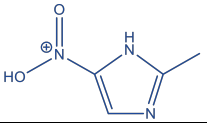
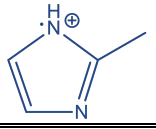
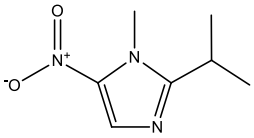
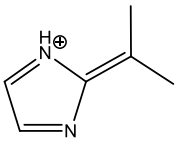
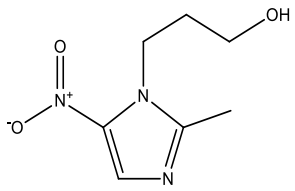
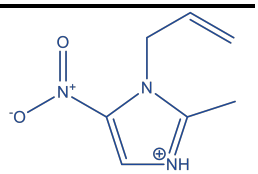
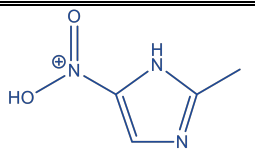
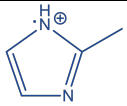
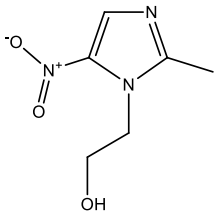
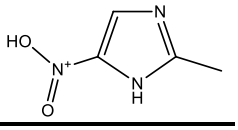
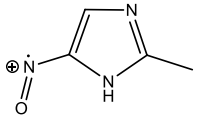
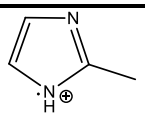
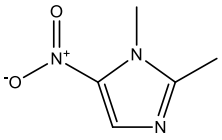
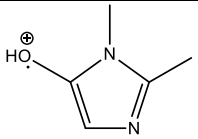
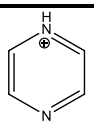
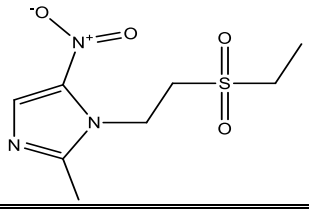
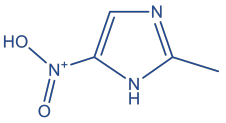
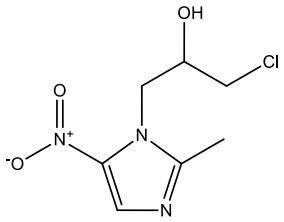
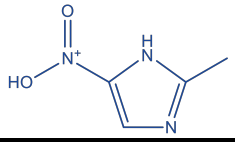
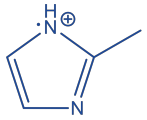
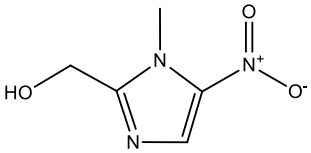
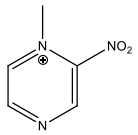
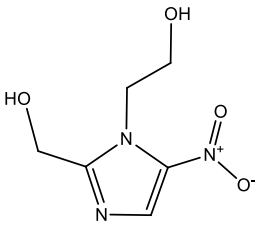
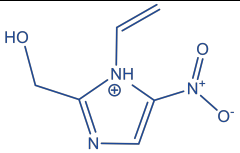
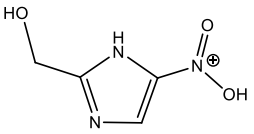
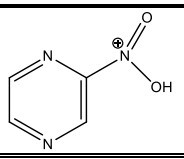
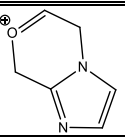
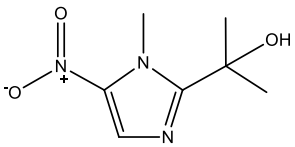
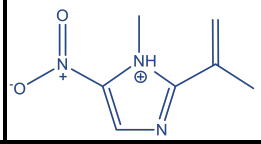
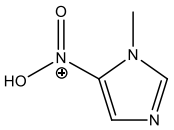
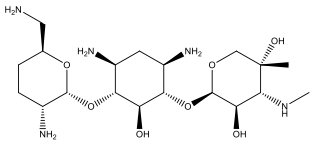
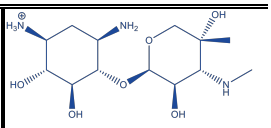
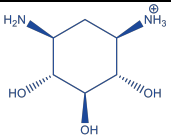
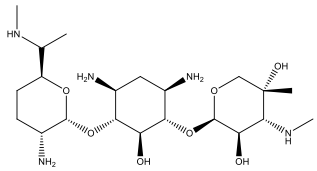
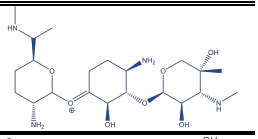
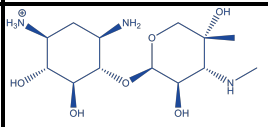
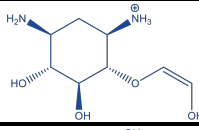
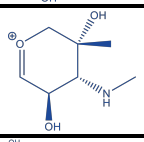
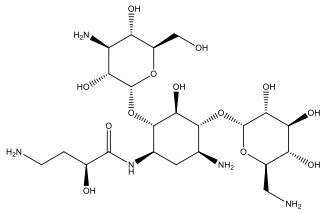
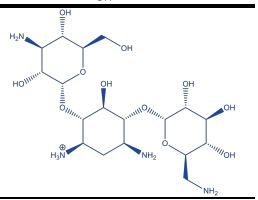
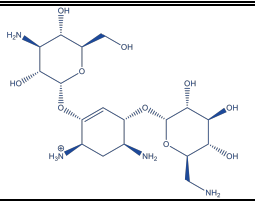
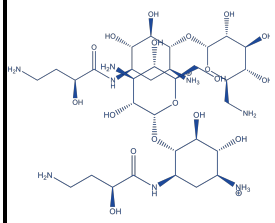
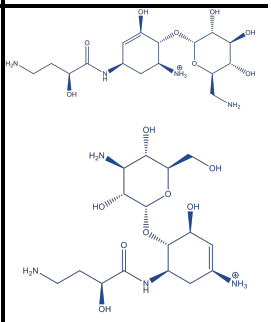
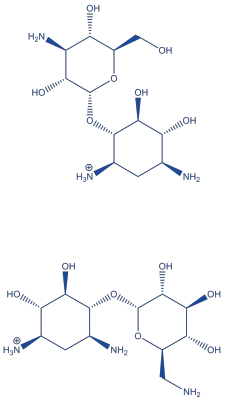
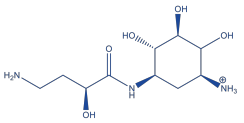
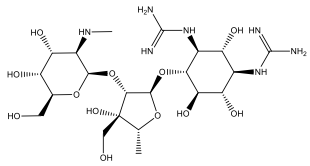
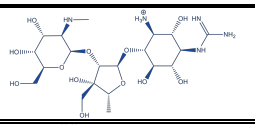
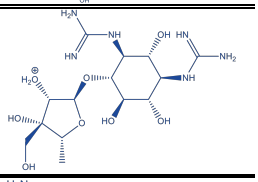
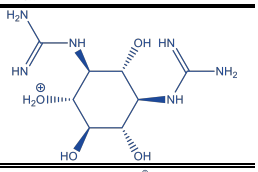
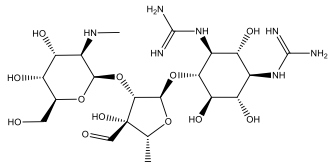
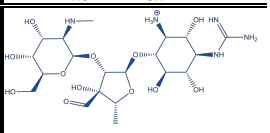
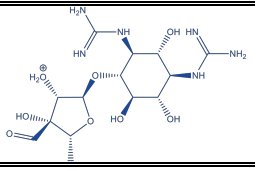
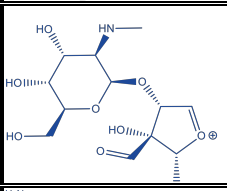
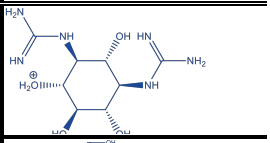
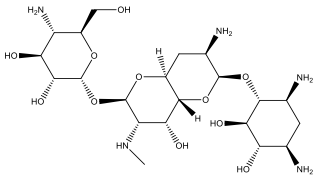
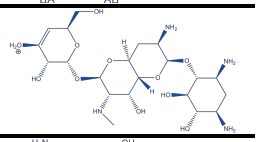
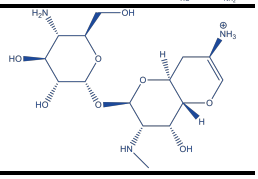
Ronidazole 	$C_5H_6N_3O_2^+$	140.0455	129.4	
Secnidazole 	$C_4H_6N_3O_2^+$	128.0455	131.6	
	$C_4H_6N_2^{\bullet+}$	82.0525	126.1	
Ipronidazole 	$C_6H_9N_2^+$	109.0760	123.9	

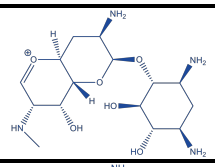
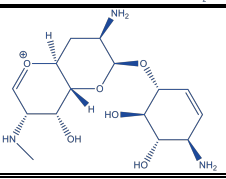
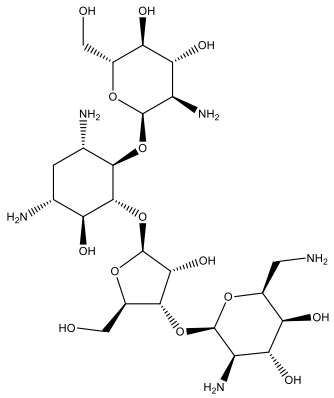
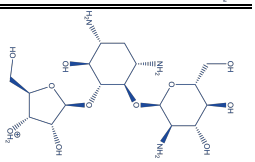
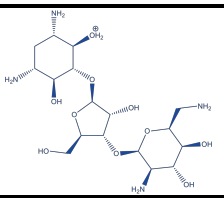
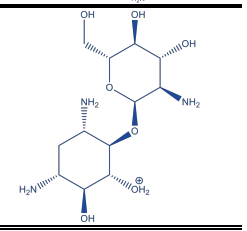
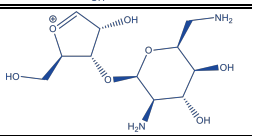
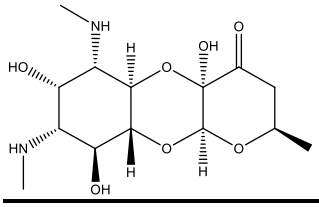
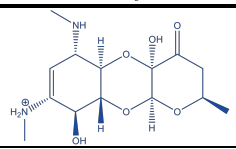
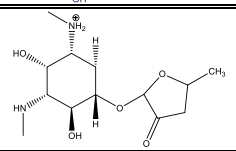
Analytes	Product Ions (m/z)	m/z	CCS ($Å^2$)	Proposed structure
Ternidazole 	$C_7H_{10}N_3O^+$	168.0768	136.3	
	$C_4H_6N_3O_2^+$	128.0455	131.5	
	$C_4H_6N_2^{+\bullet}$	82.0525	126.3	
Metronidazole 	$C_4H_6N_3O_2^+$	128.0455	131.5	
	$C_4H_5N_3O^{\bullet+}$	111.0427	124.2	
	$C_4H_6N_2^{\bullet+}$	82.0525	125.6	
Dimetridazole 	$C_5H_8N_2O^{+\bullet}$	112.0631	125.2	
	$C_4H_5N_2^+$	81.0447	117.0	
Tinidazole 	$C_4H_6N_3O_2^+$	128.0455	131.6	

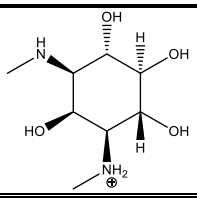
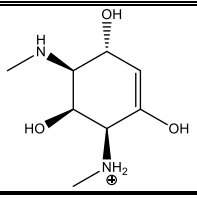
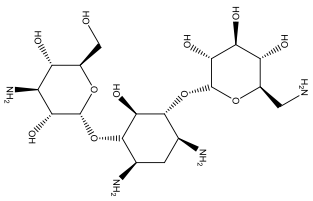
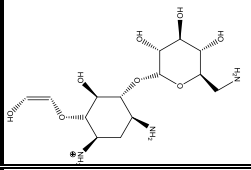
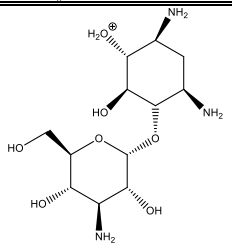
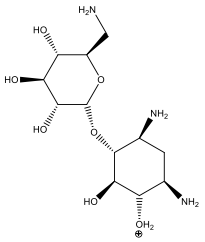
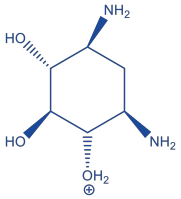
Analytes	Product Ions (m/z)	m/z	CCS (Å^2)	Proposed structure
Ornidazole 	$C_4H_6N_3O_2^+$	128.0455	132.2	
	$C_4H_6N_2^{\bullet+}$	82.0525	125.7	
Hidroxydimetridazole 	$C_5H_6N_3O_2^+$	140.0455	134.2	
Hidroxymetronidazole 	$C_6H_8N_3O_3^+$	170.0560	135.1	
	$C_4H_6N_3O_3^+$	144.0404	134.4	
	$C_4H_4N_3O_2^+$	126.0298	134.2	
	$C_6H_7N_2O^+$	123.0553	126.5	
Hidroxyipronidazole 	$C_7H_{10}N_3O_2^+$	168.0768	136.7	
	$C_4H_6N_3O_2^+$	128.0455	129.5	

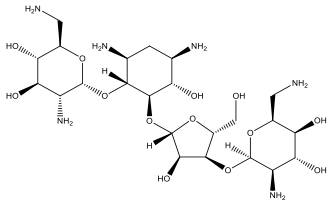
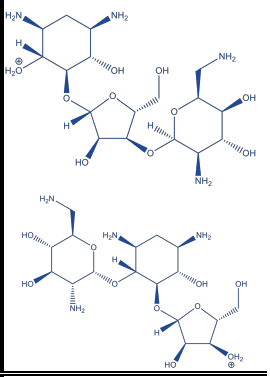
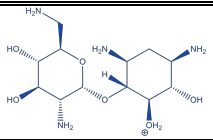
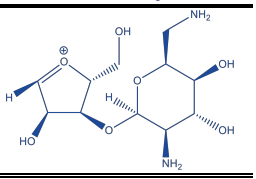
Analytes	Product Ions (m/z)	m/z	CCS ($Å^2$)	Proposed structure
Aminoglycosides				
Gentamicin C1a 	$C_{13}H_{28}N_3O_6^+$	322.1973	175.2	
	$C_6H_{15}N_2O_3^+$	163.1077	138.5	
Gentamicin C1 	$C_8H_{17}N_2O_4^+$	461.2970	217.1	
	$C_{13}H_{28}N_3O_6^+$	322.1973	174.7	
	$C_8H_{17}N_2O_4^+$	205.1183	146.2	
	$C_7H_{14}NO_3^+$	160.0968	135.3	
Amikacin 	$C_{18}H_{37}N_4O_{11}^+$	485.2453	207.9	
	$C_{18}H_{35}N_4O_{10}^+$	467.2348	210.0	

Analytes	Product Ions (m/z)	m/z	CCS (Å^2)	Proposed structure
	$C_{16}H_{33}N_4O_9^+$	425.2242	195.9	
	$C_{16}H_{31}N_4O_8^+$	407.2136	202.1	
	$C_{12}H_{26}N_3O_7^+$	324.1765	175.6	
	$C_{10}H_{22}N_3O_5^+$	264.1554	163.8	

Analytes	Product Ions (m/z)	m/z	CCS ($Å^2$)	Proposed structure
Dihydrostreptomycin 	$C_{20}H_{40}N_5O_{12}^+$	542.2668	223.1	
	$C_{14}H_{29}N_6O_8^+$	409.2041	194.7	
	$C_8H_{19}N_6O_4^+$	263.1462	158.6	
Streptomycin 	$C_{20}H_{38}N_5O_{12}^+$	540.2511	224.6	
	$C_{14}H_{27}N_6O_8^+$	407.1885	193.2	
	$C_{13}H_{22}NO_8^+$	320.1343	170.1	
	$C_8H_{19}N_6O_4^+$	263.1462	158.5	
Apramycin 	$C_{21}H_{39}N_4O_{11}^+$	523.2610	218.9	
	$C_{15}H_{28}N_3O_8^+$	378.1671	187.6	

Analytes	Product Ions (m/z)	m/z	CCS ($Å^2$)	Proposed structure
	$C_{15}H_{29}N_4O_6^+$	361.2082	186.5	
	$C_{15}H_{26}N_3O_6^+$	344.1816	179.6	
Paromomycin 	$C_{17}H_{34}N_3O_{11}^+$	456.2188	201.2	
	$C_{17}H_{35}N_4O_{10}^+$	455.2348	207.9	
	$C_{12}H_{26}N_3O_7^+$	324.1765	175.7	
	$C_{11}H_{21}N_2O_7^+$	293.1343	165.0	
Spectinomycin 	$C_{14}H_{23}N_2O_6^+$	315.1551	171.8	
	$C_{13}H_{25}N_2O_5^+$	289.1758	170.1	

Analytes	Product Ions (m/z)	m/z	CCS ($Å^2$)	Proposed structure
	$C_8H_{19}N_2O_4^+$	207.1339	144.1	
	$C_8H_{17}N_2O_3^+$	189.1234	141.0	
Kanamycin 	$C_{14}H_{28}N_3O_8^+$	366.1871	183.5	
	$C_{12}H_{26}N_3O_7^+$	324.1765	174.8	 
	$C_6H_{15}N_2O_3^+$	163.1077	138.3	

Analytes	Product Ions (m/z)	m/z	CCS (Å^2)	Proposed structure
Neomycin 	$C_{17}H_{35}N_4O_{10}^+$	455.2348	206.3	
	$C_{12}H_{27}N_4O_6^+$	323.1349	174.5	
	$C_{11}H_{21}N_2O_7^+$	293.1343	164.6	
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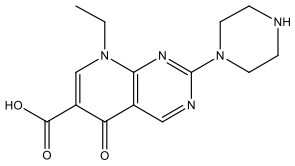
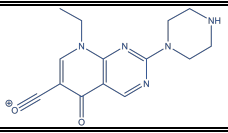
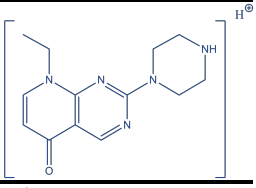
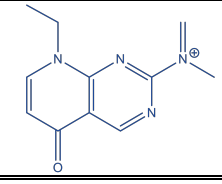
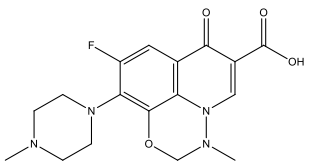
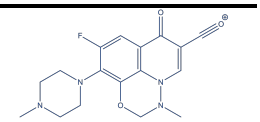
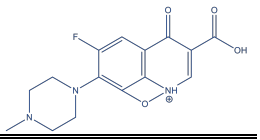
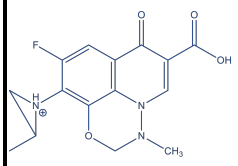
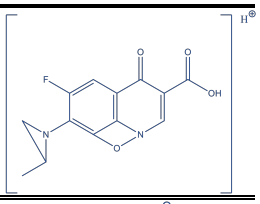
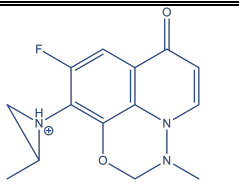
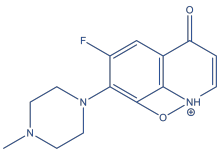
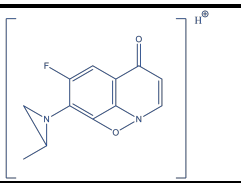
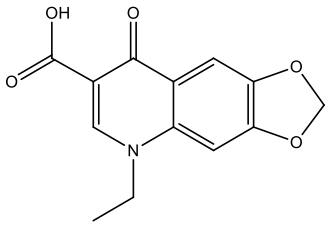
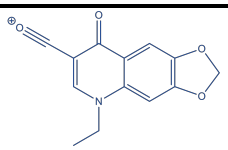
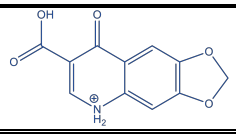
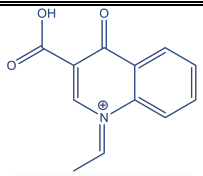
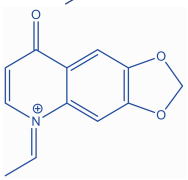
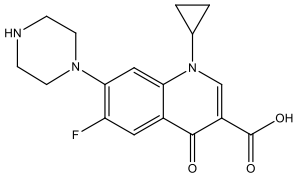
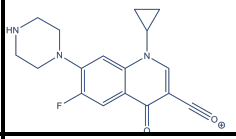
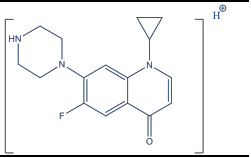
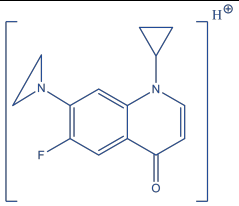
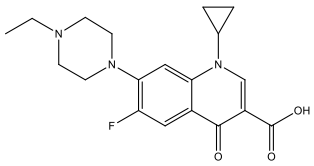
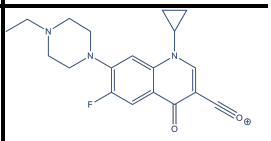
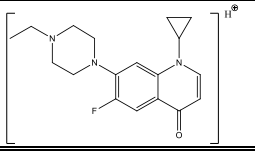
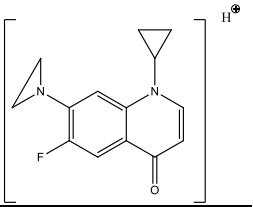
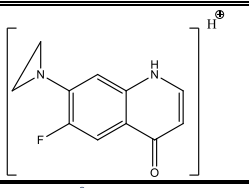
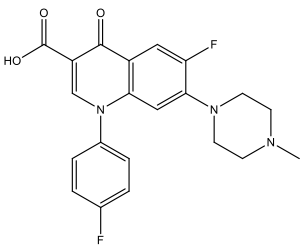
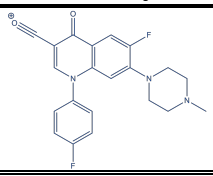
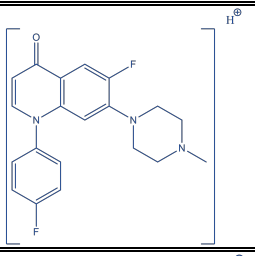
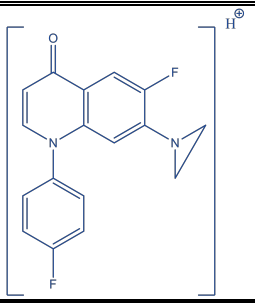
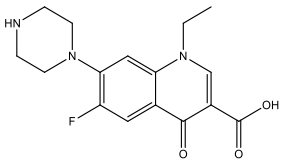
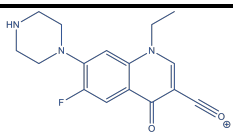
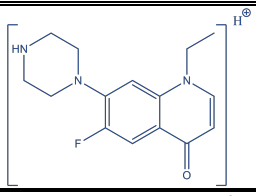
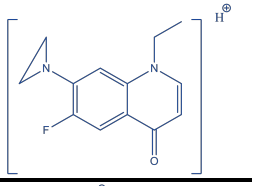
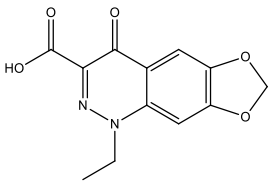
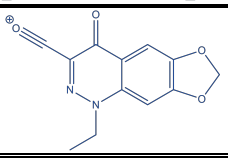
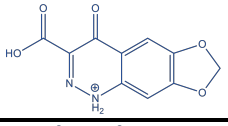
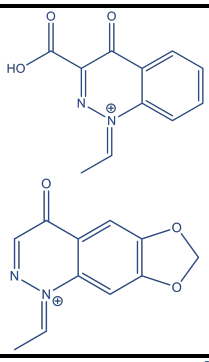
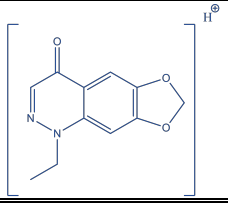
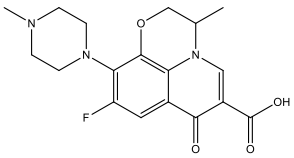
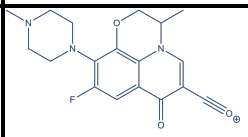
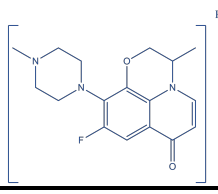
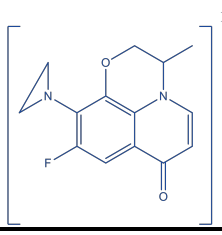
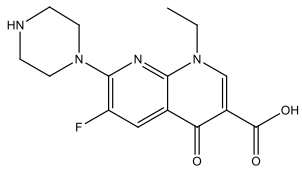
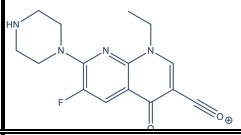
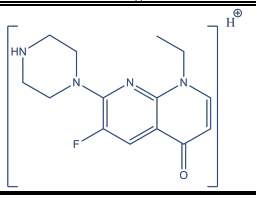
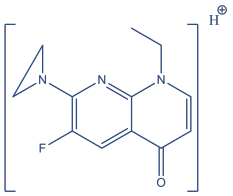
Pipemidic acid 	$C_{14}H_{16}N_5O_2^+$	286.1299	167.1	
	$C_{13}H_{18}N_5O^+$	260.1506	162.2	
	$C_{11}H_{13}N_4O^+$	217.1084	148.0	

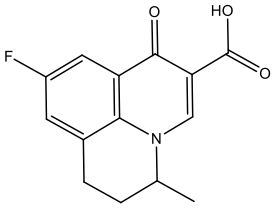
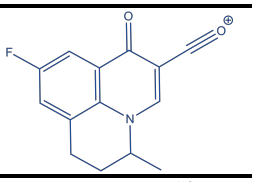
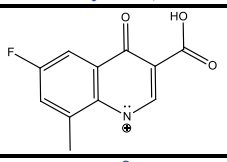
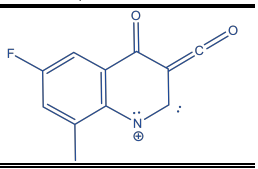
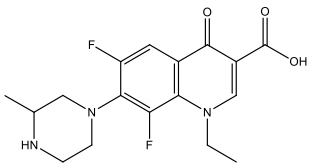
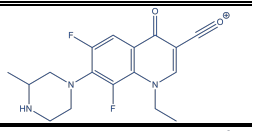
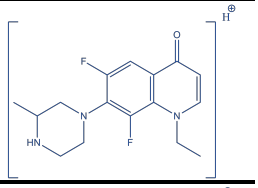
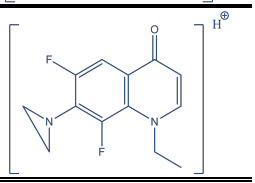
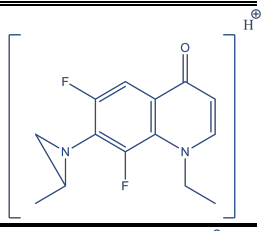
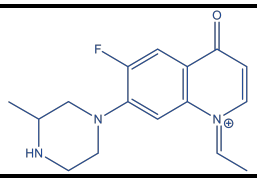
Analytes	Product Ions (m/z)	m/z	CCS ($Å^2$)	Proposed structure
Marbofloxacin 	$C_{17}H_{18}FN_4O_3^+$	345.1357	178.0	 
	$C_{15}H_{15}FN_3O_4^+$	320.1041	173.9	
	$C_{13}H_{10}FN_2O_4^+$	277.0619	163.1	
	$C_{14}H_{15}FN_3O_2^+$	276.1143	163.2	 
	$C_{12}H_{10}FN_2O_2^+$	233.0721	149.3	

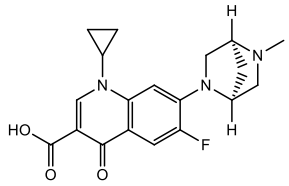
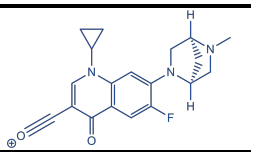
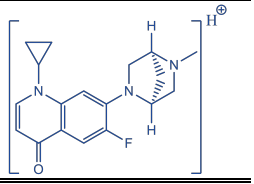
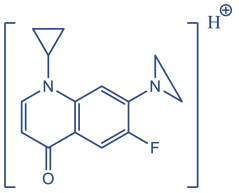
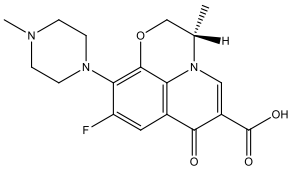
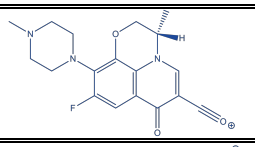
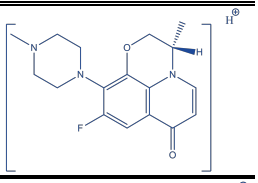
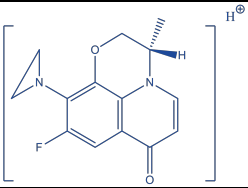
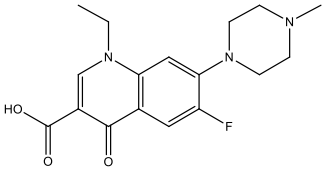
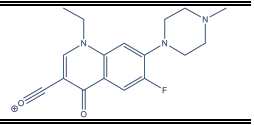
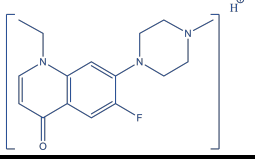
Analytes	Product Ions (m/z)	m/z	CCS (Å^2)	Proposed structure
Oxolonic acid 	$C_{13}H_{10}NO_4^+$	244.0604	150.0	
	$C_{11}H_8NO_5^+$	234.0397	141.5	
	$C_{12}H_{10}NO_3^+$	216.0655	144.1	 
Ciprofloxacin 	$C_{17}H_{17}FN_3O_2^+$	314.1299	173.2	
	$C_{16}H_{19}FN_3O^+$	288.1507	170.3	
	$C_{14}H_{14}FN_2O^+$	245.1085	155.7	

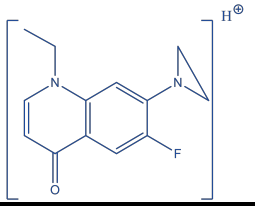
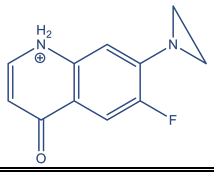
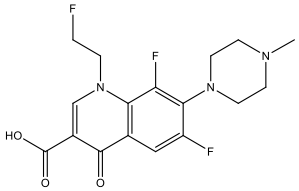
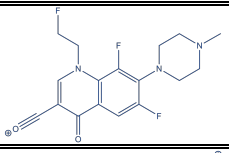
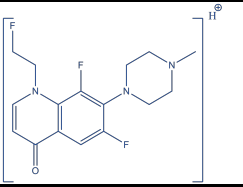
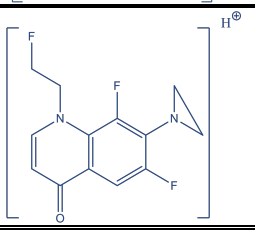
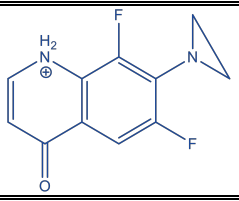
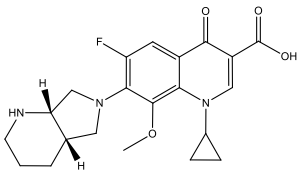
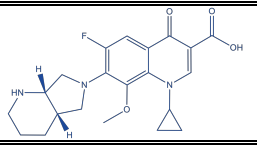
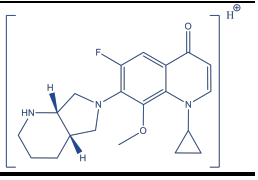
Analytes	Product Ions (m/z)	m/z	CCS (Å²)	Proposed structure
Enrofloxacin 	$C_{19}H_{21}FN_3O_2^+$	342.1612	184.8	
	$C_{18}H_{23}FN_3O^+$	316.1820	181.9	
	$C_{14}H_{14}FN_2O^+$	245.1085	156.0	
	$C_{11}H_{10}FN_2O^+$	205.0772	142.6	
Difloxacin 	$C_{21}H_{18}F_2N_3O_2^+$	382.1362	188.2	
	$C_{20}H_{20}F_2N_3O^+$	356.1569	189.9	
	$C_{17}H_{13}F_2N_2O^+$	299.0990	167.1	

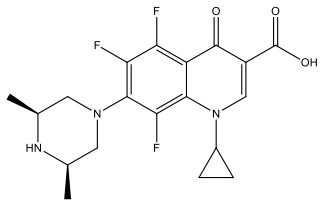
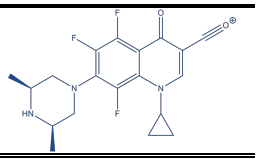
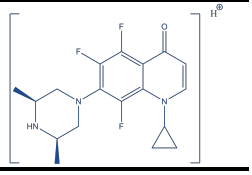
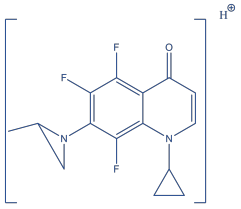
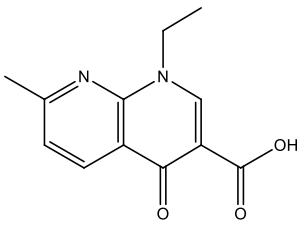
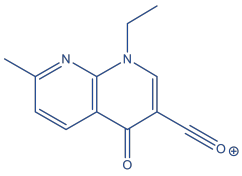
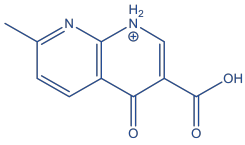
Analytes	Product Ions (m/z)	m/z	CCS ($Å^2$)	Proposed structure
Norfloxacin 	$C_{16}H_{17}FN_3O_2^+$	302.1299	170.9	
	$C_{15}H_{19}FN_3O^+$	276.1507	168.0	
	$C_{13}H_{14}FN_2O^+$	233.1085	152.6	
Cinoxacin 	$C_{12}H_9N_2O_4^+$	245.0557	147.9	
	$C_{10}H_7N_2O_5^+$	235.0349	141.8	
	$C_{11}H_9N2O_3^+$	217.0608	142.2	
	$C_{11}H_{11}N_2O_3^+$	219.0764	144.9	

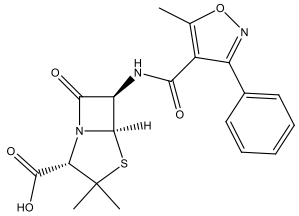
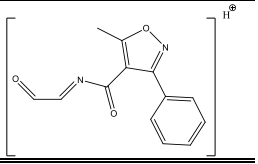
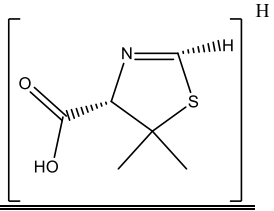
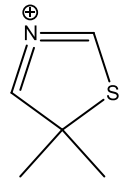
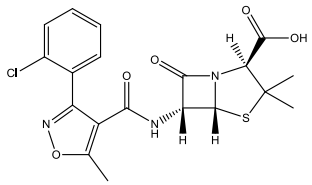
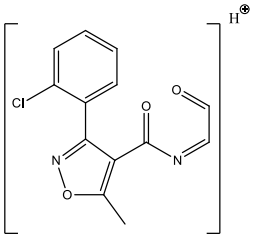
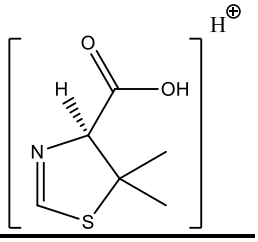
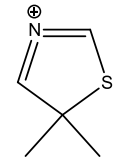
Analytes	Product Ions (m/z)	m/z	CCS ($Å^2$)	Proposed structure
Ofloxacin 	$C_{18}H_{19}FN_3O_3^+$	344.1405	179.9	
	$C_{17}H_{21}FN_3O_2^+$	318.1612	176.8	
	$C_{14}H_{14}FN_2O_2^+$	261.1034	157.8	
Enoxacin 	$C_{15}H_{16}FN_4O_2^+$	303.1252	168.8	
	$C_{14}H_{18}FN_4O^+$	277.1459	167.6	
	$C_{12}H_{13}FN_3O^+$	234.1037	150.6	

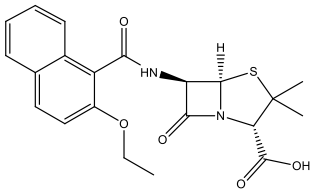
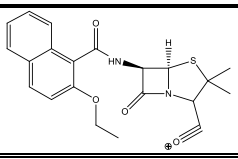
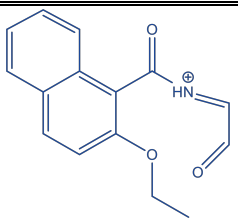
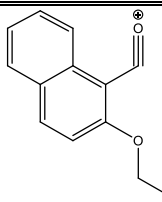
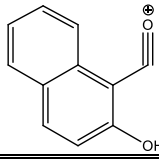
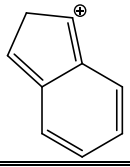
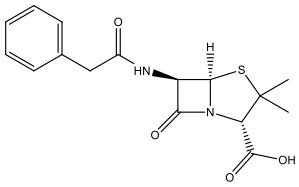
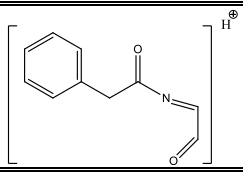
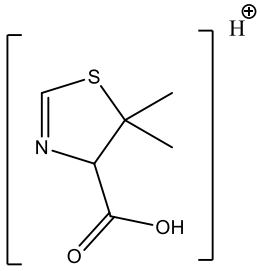
Analytes	Product Ions (m/z)	m/z	CCS ($Å^2$)	Proposed structure
Flumequine 	$C_{14}H_{11}FNO_2^+$	244.0768	150.5	
	$C_{11}H_7FNO_3^+$	220.0404	137.1	
	$C_{11}H_5FNO_2^+$	202.0299	135.0	
Lomefloxacin 	$C_{17}H_{18}F_2N_3O_2^+$	334.1362	176.3	
	$C_{16}H_{20}F_2N_3O^+$	308.1569	176.2	
	$C_{13}H_{13}F_2N_2O^+$	251.0990	153.4	
	$C_{14}H_{15}F_2N_2O^+$	265.1147	158.8	
	$C_{16}H_{19}FNO^+$	288.1507	167.4	

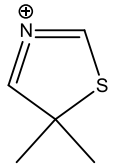
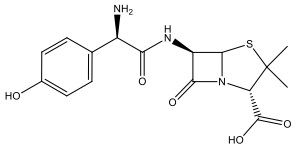
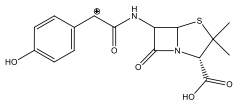
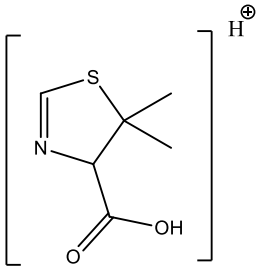
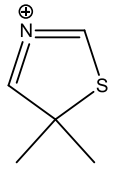
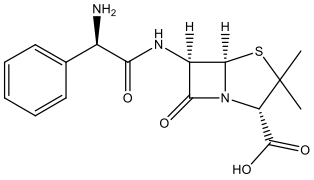
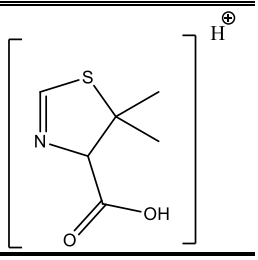
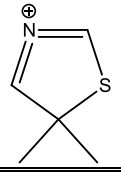
Analytes	Product Ions (m/z)	m/z	CCS ($Å^2$)	Proposed structure
Danofloxacin 	$C_{19}H_{19}FN_3O_2^+$	340.1456	179.3	
	$C_{18}H_{21}FN_3O^+$	314.1663	178.9	
	$C_{14}H_{14}FN_2O^+$	245.1085	154	
Levofloxacin 	$C_{18}H_{19}FN_3O_3^+$	344.1405	179.7	
	$C_{17}H_{21}FN_3O_2^+$	318.1612	176.7	
	$C_{14}H_{14}FN_2O_2^+$	261.1034	157.8	
Pefloxacin 	$C_{17}H_{19}FN_3O_2^+$	316.1456	174.5	
	$C_{16}H_{21}FN_3O^+$	290.1663	174.8	

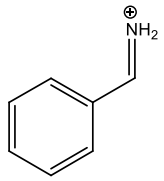
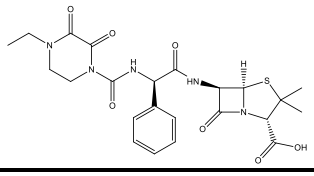
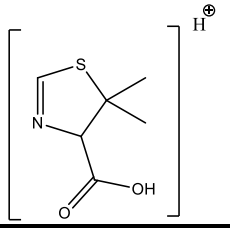
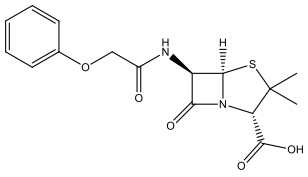
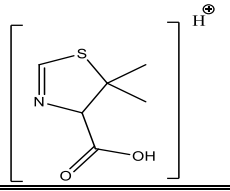
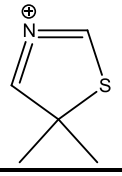
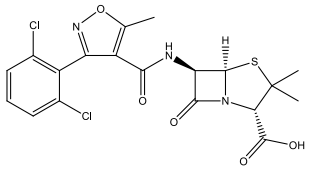
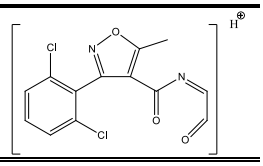
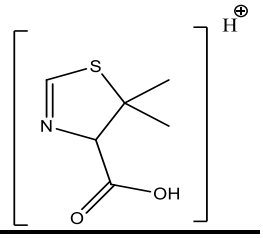
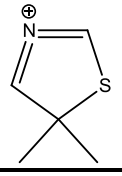
Analytes	Product Ions (m/z)	m/z	CCS ($Å^2$)	Proposed structure
	$C_{13}H_{14}FN_2O^+$	233.1085	152.7	
	$C_{11}H_{10}FN_2O^+$	205.0772	142.3	
Fleroxacin 	$C_{17}H_{16}F_3N_3O_2^+$	352.1267	178.1	
	$C_{16}H_{19}F_3N_3O^+$	326.1475	176.3	
	$C_{13}H_{12}F_3N_2O^+$	269.0896	155.9	
	$C_{11}H_9F_2N_2O^+$	223.0677	144.3	
Moxifloxacin 	$C_{21}H_{23}FN_3O_3^+$	384.1718	189.4	
	$C_{20}H_{25}FN_3O_2^+$	358.1925	184.8	

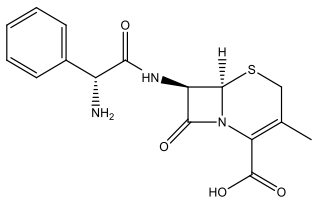
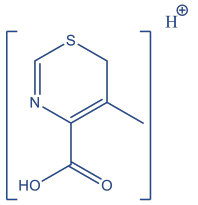
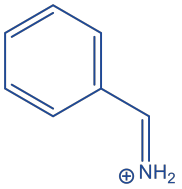
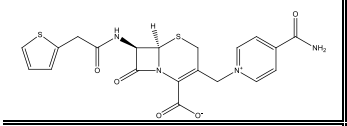
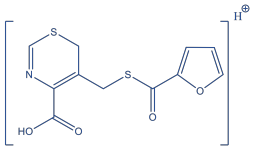
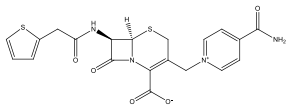
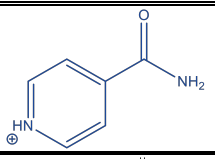
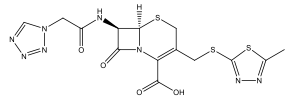
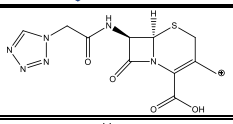
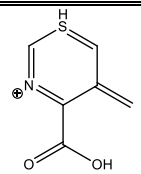
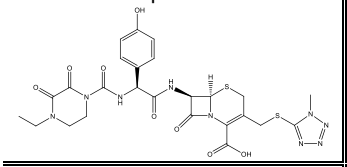
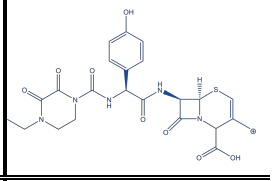
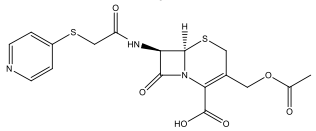
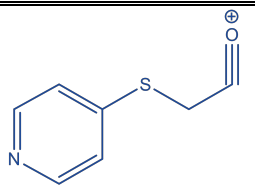
Analytes	Product Ions (m/z)	m/z	CCS ($Å^2$)	Proposed structure
Orbifloxacin 	$C_{19}H_{19}F_3N_3O_2^+$	378.1424	185.4	
	$C_{18}H_{21}F_3N_3O^+$	352.1631	185.4	
	$C_{15}H_{14}F_3N_2O^+$	295.1053	161.8	
Nalidixic acid 	$C_{12}H_{11}N_2O_2^+$	215.0815	145.4	
	$C_{10}H_9N_2O_3^+$	205.0608	137.1	

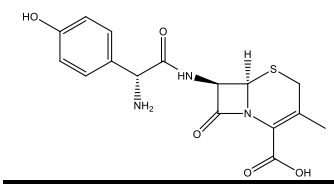
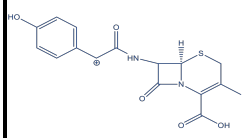
Analytes	Product Ions (m/z)	m/z	CCS (Å^2)	Proposed structure
Beta-lactams				
Oxacillin 	$C_{13}H_{11}N_2O_3^+$	243.0764	156.0	
	$C_6H_{10}NO_2S^+$	160.0427	133.9	
	$C_5H_8NS^+$	114.0372	124.2	
Cloxacillin 	$C_{13}H_{10}ClN_2O_3^+$	277.0374	160.2	
	$C_6H_{10}NO_2S^+$	160.0427	133.9	
	$C_5H_8NS^+$	114.0372	124.4	

Analytes	Product Ions (m/z)	m/z	CCS ($Å^2$)	Proposed structure
Nafcillin 	$C_{21}H_{21}N_2O_4S^+$	397.1217	193.4	
	$C_{15}H_{14}NO_3^+$	256.0968	158.2	
	$C_{13}H_{11}O_2^+$	199.0754	140.0	
	$C_{11}H_7O_2^+$	171.0441	133.5	
	$C_9H_7^+$	115.0547	123.7	
Penicillin G 	$C_{10}H_{10}NO_2^+$	176.0706	140.6	
	$C_6H_{10}NO_2S^+$	160.0427	133.8	

Analytes	Product Ions (m/z)	m/z	CCS ($Å^2$)	Proposed structure
	$C_5H_8NS^+$	114.0372	124.3	
Amoxicillin 	$C_{16}H_{17}N_2O_5S^+$	349.0853	179.6	
	$C_6H_{10}NO_2S^+$	160.0427	134.2	
	$C_5H_8NS^+$	114.0372	124.7	
	$C_{10}H_8NO_2^+$	174.0550	135.7	----------
Ampicillin 	$C_6H_{10}NO_2S^+$	160.0427	134.1	
	$C_5H_8NS^+$	114.0372	124.6	

Analytes	Product Ions (m/z)	m/z	CCS ($Å^2$)	Proposed structure
	$C_7H_8N^+$	106.0651	122.8	
Piperacillin 	$C_6H_{10}NO_2S^+$	160.0427	134.1	
Penicillin V 	$C_6H_{10}NO_2S^+$	160.0427	134.2	
	$C_5H_8NS^+$	114.0372	124.5	
Dicloxacillin 	$C_{13}H_9Cl_2N_2O_3^+$	310.9985	165.3	
	$C_6H1_0NO_2S^+$	160.0427	133.9	
	$C_5H_8NS^+$	114.0432	124.4	

Analytes	Product Ions (m/z)	m/z	CCS ($Å^2$)	Proposed structure
Cefalexin 	$C_6H_8NO_2S^+$	158.0270	132.1	
	$C_8H_8N^+$	118.0651	128.2	----------
	$C_7H_8N^+$	106.0651	122.8	
Ceftiofur 	$C_{11}H_{10}NO_4S_2^+$	284.0046	161.2	
Cefalonium 	$C_6H_7N_2O^+$	123.0553	132.0	
Cefazolin 	$C_{11}H_{11}N_6O_4S^+$	323.0557	164.9	
	$C_6H_6NO_2S^+$	156.0114	133.6	
Cefoperazone 	$C_{23}H_{24}N_5O_8S^+$	530.1335	221.8	
Cefapirin 	$C_7H_6NOS^+$	152.0165	133.1	

Analytes	Product Ions (m/z)	m/z	CCS ($Å^2$)	Proposed structure
Cefadroxil 	$C_{16}H_{15}N_2O_5S^+$	347.0696	179.2	
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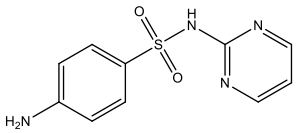
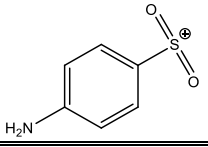
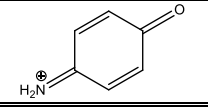
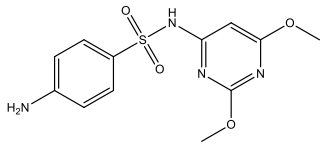
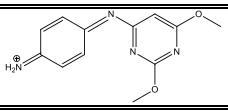
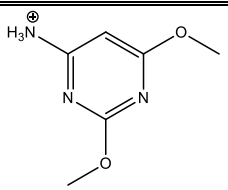
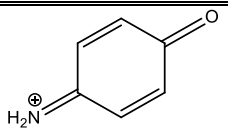
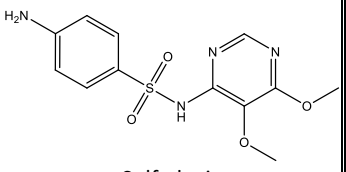
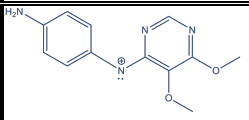
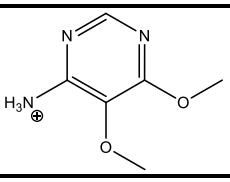
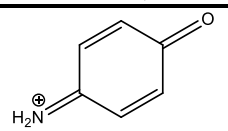
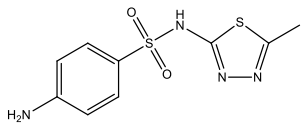
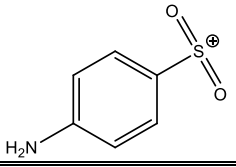
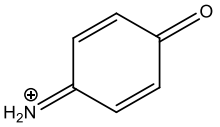
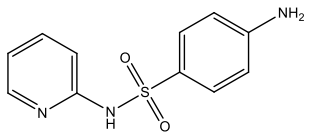
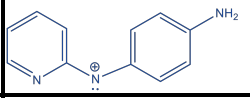
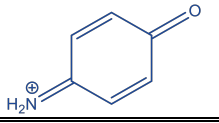
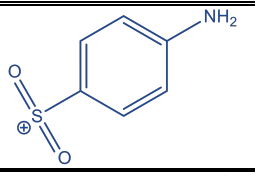
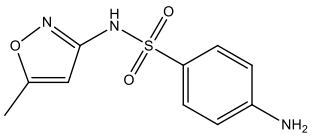
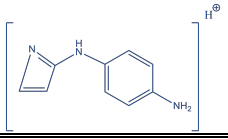
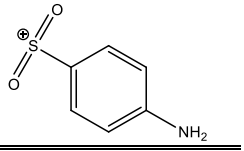
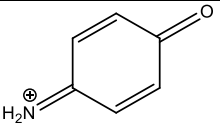
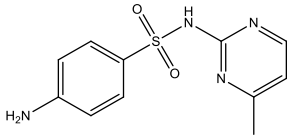
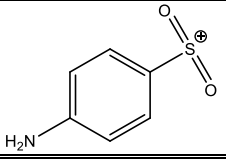
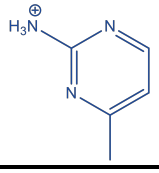
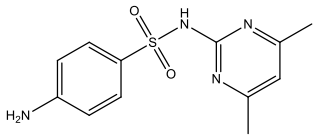
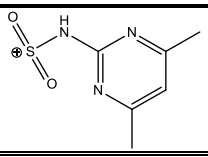
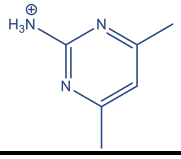
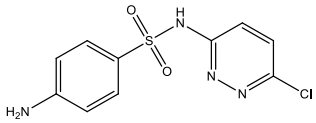
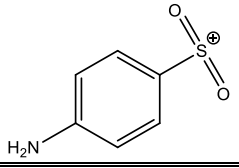
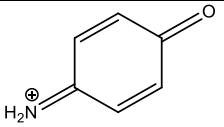
Sulfadiazine 	$C_6H_6NO_2S^+$	156.0114	128.5	
	$C_6H_6NO^+$	108.0444	123.6	
Sulfadimethoxine 	$C_{12}H_{13}N_4O_2^+$	245.1033	152.3	
	$C_6H_{10}N_3O_2^+$	156.0768	133.2	
	$C_6H_6NO^+$	108.0444	123.7	
Sulfadoxin 	$C_{12}H_{13}N_4O_2^+$	245.1033	152.1	
	$C_6H_{10}N_3O_2^+$	156.0768	133.2	
	$C_6H_6NO^+$	108.0444	123.8	

Analytes	Product Ions (m/z)	m/z	CCS ($Å^2$)	Proposed structure
Sulfamethizol 	$C_6H_6NO_2S^+$	156.0114	129.1	
	$C_6H_6NO^+$	108.0444	123.5	
Sulfapyridine 	$C_{11}H_{10}N_3^+$	184.0869	137.6	
	$C_6H_6NO^+$	108.0444	123.7	
	$C_6H_6NO_2S^+$	156.0114	128.0	
Sulfamethoxazole 	$C_9H_{10}N_3^+$	160.0869	135.6	
	$C_6H_6NO_2S^+$	156.0114	128.36	
	$C_6H_6NO^+$	108.0444	123.6	
Sulfamerazine 	$C_6H_6NO_2S^+$	156.0114	128.3	
	$C_5H_8N_3^+$	110.0713	123.6	

Analytes	Product Ions (m/z)	m/z	CCS ($Å^2$)	Proposed structure
Sulfamethazine 	$C_6H_8N_3SO_2^+$	186.0332	136.7	
	$C_6H_{10}N_3^+$	124.0869	131.4	
Sulfachlorpyridazine 	$C_6H_6NO_2S^+$	156.0114	128.4	
	$C_6H_6NO^+$	108.0444	123.5	
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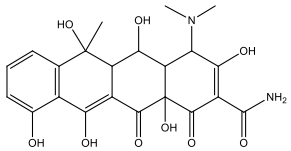
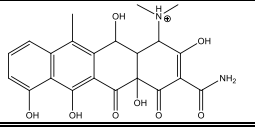
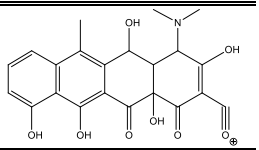
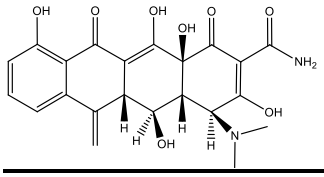
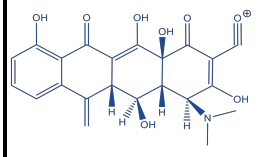
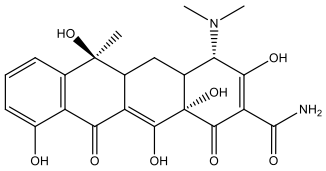
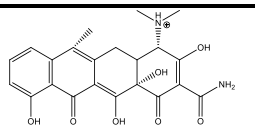
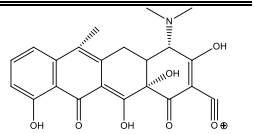
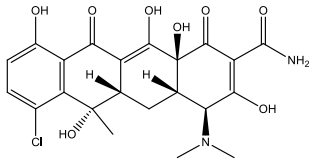
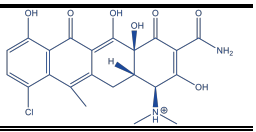
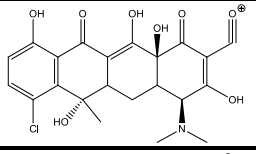
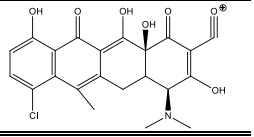
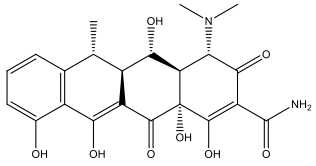
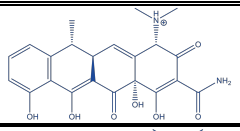
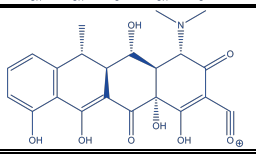
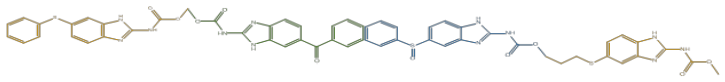
Oxytetracycline 	$C_{22}H_{23}N_2O_8^+$	443.1449	197.6	
	$C_{22}H_{20}NO_8^+$	426.1183	192.5	
Methacycline 	$C_{22}H_{20}NO_8^+$	426.1183	192.5	
Tetracycline 	$C_{22}H_{23}N_2O_7^+$	427.1505	194.2	
	$C_{22}H_{20}NO_7^+$	410.1234	189.9	

Analytes	Product Ions (m/z)	m/z	CCS ($Å^2$)	Proposed structure
Chlortetracycline 	$C_{22}H_{22}ClN_2O_7^+$	461.1110	200.3	
	$C_{22}H_{21}ClNO_8^+$	462.0950	193.6	
	$C_{22}H_{19}ClNO_7^+$	444.0845	191.4	
Doxycycline 	$C_{22}H_{23}N_2O_7^+$	427.1500	195.4	
	$C_{22}H_{22}NO_8^+$	428.1340	191.4	

CONCLUSIONES FINALES



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En esta Tesis se han desarrollado diferentes métodos de análisis para la determinación de residuos de BZs y otros fármacos en alimentos, muestras medioambientales y clínicas. Para ello, y considerando las tendencias actuales en Química Analítica Verde, se ha evaluado el potencial de diferentes técnicas instrumentales separativas miniaturizadas: CE, CEC, CLC, UHPLC y IMS, acopladas a diferentes sistemas de detección (UV-VIS, FL and MS). Además, se han estudiado y propuesto metodologías de tratamiento de muestra alternativas caracterizadas por su simplicidad, rapidez, bajo consumo de disolventes y alta eficacia.

Con estas premisas las conclusiones alcanzadas en esta Tesis son las siguientes:

- Los métodos propuestos proporcionaron una resolución y sensibilidad adecuadas con tiempos de análisis relativamente cortos. Los menores tiempos de análisis se consiguieron cuando se utilizó UHPLC-FL, donde se determinaron 13 BZs en menos de 12 min. En el caso del método basado en el empleo de CEC-UV, 7 BZs fueron analizados en menos de 15 min. En el caso de CE, se emplearon 32,5 min para determinar 12 BZs mediante CZE-MS/MS y menos de 20 min para determinar 13 BZs haciendo uso de MEKC-MS/MS. Cuando se utilizó CLC-UV, se emplearon 42 min para la separación de 16 BZs.
- El empleo de surfactantes volátiles como el APFO ha permitido la determinación de BZs mediante MEKC-MS/MS, evitando los problemas inherentes al uso de surfactantes no volátiles cuando se emplea la espectrometría de masas acoplada a CE.
- Se ha investigado por primera vez la idoneidad de un método CEC-UV para la determinación de BZS en muestras medioambientales. A pesar de emplear capilares preparados en el laboratorio, se alcanzó una alta reproducibilidad en términos de tiempos de retención, constituyendo este método un ejemplo de la aplicabilidad de la CEC.

- Se ha llevado a cabo la evaluación de técnicas de preconcentración 'on-line' basadas en "stacking" con el objetivo de mejorar la sensibilidad en aquellos métodos basados en el empleo de técnicas electroforéticas. Además, se han evaluado diferentes tratamientos de muestra basados en la DLLME y el SALLE 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), estando por debajo de los MRLs establecidos para las matrices analizadas.
- La detección basada en el acoplamiento MS/MS ha permitido diferenciar e identificar compuestos con similar tiempo de retención con alto grado de sensibilidad, permitiendo utilizar simples tratamientos de muestra como "dilute and shoot".
- Los tratamientos de muestra empleados como SALLE, DLLME y "dilute and shoot", presentan como ventajas su bajo consumo de reactivos, fácil manejo, rapidez y elevadas recuperaciones, estando en sintonía con las tendencias de la química verde.
- Se ha creado una base de datos de valores de CCS para 92 fármacos humanos y veterinarios haciendo uso del acoplamiento IMS-MS. La información adicional a *m/z* proporcionada permite la identificación inequívoca de los compuestos caracterizados. Además, los principales fragmentos para la mayoría de los compuestos estudiados han sido caracterizados en términos de CCS junto con su elucidación estructural. Diferentes muestras clínicas fueron analizadas demostrándose la aplicabilidad de esta base de datos.

En la **Tabla C1**, se recogen las características experimentales e instrumentales más notables de cada uno de los métodos desarrollados en esta Tesis.

Tabla C1. Resumen de los métodos desarrollados en la tesis.

Analitos	Matriz	Tratamiento de muestra	Técnica instrumental	Tiempo de análisis (min)	LODs
5-OH- TBZ, ABZ-NH_2-SO_2, ABZ-SO, CBZ, BEN, TBZ, FBZ-SO, MBZ-NH_2, 5-OH-MBZ, FBZ-SO_2, MBZ, ABZ, TCB-SO2, FBZ, TCB-SO, TCB	Leche de vaca, leche de cabra y leche de oveja	SALLE	CLC-UV Columna Zorbax XDB-C18 (150 mm x 0,5 mm I.D, 5 µm). Fase móvil: A: 50 mM acetato amónico B: MeCN/MeOH Flujo fase móvil: 9 µL min^{-1}	42	1,0-2,8 µg kg^{-1}
5-OH-TBZ, ABZ-NH_2-SO_2, CBZ, BEN, ABZ-SO TBZ, FUB, ABZ-SO_2, OXI, ABZ, TCB-SO_2, TCB-SO, TCB	Trucha, dorada y lubina	SALLE	UHPLC-FL Columna Zorbax C18 Ecliplse Plus RRHD (50 mm x 2,1 mm I.D, 1,8 µm). Fase móvil: A: agua B: MeCN Flujo fase móvil: 0,4 mL min^{-1}	12	0,04-29,9 µg kg^{-1}
TBZ, CBZ, BEN, OXI, ABZ, FBZ, MBZ-OH, MBZ, ABZ-SO, FBZ-SO, ABZ-SO_2, FBZ-SO_2	Músculo de pollo y cerdo	DLLME	Stacking-CZE-MS/MS BGE: 500 mM ácido fórmico (pH 2,2) 25 kV, 25°C	32,5	1,0-4,0 µg kg^{-1}
CBZ, BEN, TBZ, MBZ-OH, OXI, ABZ, ABZ-SO, FBZ, ABZ-SO_2, MBZ-NH_2, MBZ, FBZ-SO, FBZ-SO_2	Orina de oveja, orina de cabra y orina de vaca.	Dilute and shoot	Sweeping-MEKC-MS/MS BGE: 50 mM APFO (pH 9,0) 25 kV, 25°C	20	13,9-69,3 µg L^{-1}

Analitos	Matriz	Tratamiento de muestra	Técnica instrumental	Tiempo de análisis (min)	LODs
CBZ, MBZ, OXI, ABZ, FBZ, FBT, PBZ.	Agua de piscifactoría, agua de pozo y agua de manantial.	DLLME	CEC-UV Capilar empaquetado C18 (5 µm): 25 cm x 75 µm i.d. Fase móvil: 60:40 (v/v) MeCN/Disolución acuosa de acetato amónico (1 mM, pH 6,5) 25 kV, 25°C	20	1,7-2,8 µg L^{-1}
92 fármacos humanos y veterinarios	----------	----------	FIA-TWIMS-MS Restrictor (2 m x 0,12 mm) Fase móvil: A: Disolución acuosa con 0.,1 % (v/v) ácido fórmico B: MeCN con 0,1 % (v/v) ácido fórmico Flujo fase móvil: 0,175 mL min^{-1}	------	--------

Analitos	Matriz	Tratamiento de muestra	Técnica instrumental	Tiempo de análisis (min)	LODs
ABZ, ABZ-SO_2, ABZ-SO, ciprofloxacin, ornidazol, tinidazol, metroniidazole, hidroxymetronidazole, sulfadimetooxina, tetraciclina, penicilina G	Orina de vaca	Dilute and shoot	UHPLC-TWIMS-MS Columna C18 Hypersyl Gold (100 x 2,1 mm I.D, 1,9 µm) Fase móvil: A: Agua con 0,1 % (v/v) ácido fórmico B: MeCN con 0,1 % (v/v) ácido fórmico Flujo fase móvil: 0,6 mL min^{-1}	---------	---------

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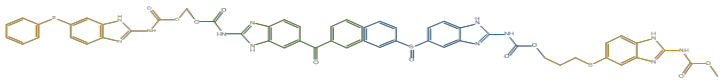
FINAL CONCLUSIONS



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In this Thesis, different methods for the determination of BZS residues and other human and veterinary drugs in foodstuffs, environmental and clinical samples have been developed. Therefore, the potential of different miniaturized separative instrumental techniques (CE, CEC, CLC, UHPLC and IMS), coupled to different detection systems (UV-VIS, FL and MS) considering the current trends in Green Analytical Chemistry has been evaluated. In addition, in order to increase efficiency and sample throughput, different sample treatments have been assessed as an alternative to those previously reported. The following conclusions have been achieved:

- The proposed methods present an adequate resolution and sensitivity with relatively short analysis times. The shorter analysis times were achieved when UHPLC-FL was used, where 13 BZs were determined in less than 12 min. When CEC-UV was used 7 BZs were analyzed in less than 15 min. In the case of CE methods, 32.5 min were employed to determine 12 BZs by CZE-MS/MS and less than 20 min to determine 13 BZs by MEKC-MS/MS. Analysis time to determine 16 BZs by CLC-UV was 42 min.
- The use of volatile surfactants such as APFO has allowed the determination of BZs using MEKC-MS/MS. Thus, the problems related to the use of non-volatile surfactants were avoided, as well as the inconveniences for using other less reproducible techniques such as partial filling.
- The suitability of CEC-UV for BZs determination in water samples has also been investigated. Despite of using laboratory-made capillaries, high reproducibility in terms of retention time was achieved, being this method an example of the applicability of CEC.
- The evaluation of the 'on-line' pre-concentration techniques based on “stacking” has been carried out with the aim of improving the sensitivity in the electrophoretic methods. In addition, different

sample treatments based on the DLLME and SALLE have been evaluated to increase this sensitivity through the 'off-line' preconcentration.

- The developed methods reached very low LOD (in the µg/L or µg/kg range), being below the MRLs established for the matrices analyzed.
- The use of MS/MS made possible to differentiate and identify compounds with similar retention time and improved method sensitivity, allowing the use of simple sample treatments such as "dilute and shoot".
- The sample treatments employed, such as SALLE, DLLME and "dilute and shoot", showed some advantages such as very low solvent consumption, easy handling, high recoveries and quick sample treatment, which agrees with the new trends in green chemistry.
- A database of CCS values was created for 92 human and veterinary drugs using IMS-MS. The CCS values, in addition to *m/z*, allowed the unambiguous identification of these 92 compounds. Moreover, the main fragments of most of the studied compounds were characterized in terms of CCS together with their structural elucidation. Different clinical samples were analyzed demonstrating the applicability of this database.

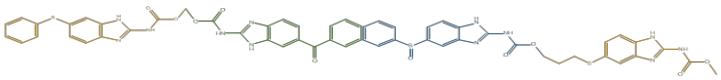
As a summary, the most significant analytical characteristics of the developed methods are shown in **Table C1.**

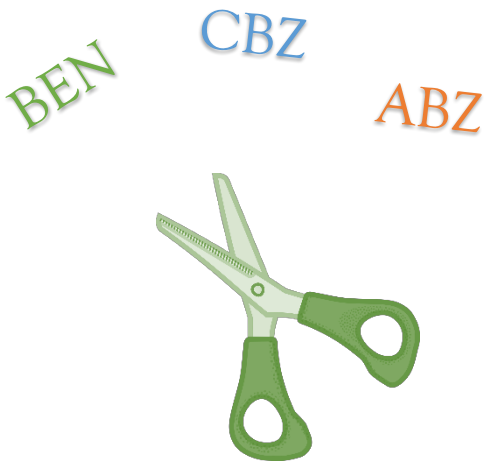
Table C1. Analytical characteristics of the proposed methods

Analyte	Matrix	Sample treatment	Instrumental technique	Analysis time (min)	LODs
5-OH- TBZ, ABZ-NH_2-SO_2, ABZ-SO, CBZ, BEN, TBZ, FBZ-SO, MBZ-NH_2, 5-OH-MBZ, FBZ-SO_2, MBZ, ABZ, TCB-SO2, FBZ, TCB-SO, TCB	Cow mil, goat milky and sheep milk	SALLE	CLC-UV Zorbax XDB-C18 column (150 mm x 0.5 mm I.D, 5 µm). Mobile phase: A: 50 mM ammonium acetate B: MeCN/MeOH Flow rate: 9 µL min^{-1}	42	1.0-2.8 µg kg^{-1}
5-OH-TBZ, ABZ-NH_2-SO_2, CBZ, BEN, ABZ-SO TBZ, FUB, ABZ-SO_2, OXI, ABZ, TCB-SO_2, TCB-SO, TCB	Trout, sea bream and sea bass	SALLE	UHPLC-FL Zorbax C18 Ecliplse Plus RRHD column (50 mm x 2.1 mm I.D, 1.8 µm). Mobile phase: A: water B: MeCN Flow rate: 0.4 mL min^{-1}	12	0.04-29.9 µg kg^{-1}
TBZ, CBZ, BEN, OXI, ABZ, FBZ, MBZ-OH, MBZ, ABZ-SO, FBZ-SO, ABZ-SO_2, FBZ-SO_2	Poultry muscle, porcine muscle	DLLME	Stacking-CZE-MS/MS BGE: 500 mM formic acid (pH 2.2) 25 kV, 25°C	32.5	1.0-4.0 µg kg^{-1}
CBZ, BEN, TBZ, MBZ-OH, OXI, ABZ, ABZ-SO, FBZ, ABZ-SO_2, MBZ-NH_2, MBZ, FBZ-SO, FBZ-SO_2	Sheep urine, goat urine and cow urine	Dilute and shoot	Sweeping-MEKC-MS/MS BGE: 50 mM APFO (pH 9.0) 25 kV, 25°C	20	13.9-69.3 µg L^{-1}

Analyte	Matrix	Sample treatment	Instrumental technique	Analysis time (min)	LODs
CBZ, MBZ, OXI, ABZ, FBZ, FBT, PBZ.	Fish farm water, spring water y well water.	DLLME	CEC-UV C18 (5 µm) packed capillary: 25 cm x 75 µm i.d. Mobile phase: 60:40 (v/v) MeCN/water containing ammonium acetate (1 mM, pH 6.5) 25 kV, 25°C	20	1.7-2.8 µg L^{-1}
92 human and veterinary drugs	----------	----------	FIA-TWIMS-MS Restrictor (2 m x 0.12 mm) Mobile phase: A: Water with 0.1 % (v/v) formic acid B: MeCN with 0.1 % (v/v) formic acid Flow rate: 0.175 mL min^{-1}	------	--------
ABZ, ABZ-SO_2, ABZ-SO, ciprofloxacin, ornidazole, tinidazole, metronidazole, hidroxymetronidazole, sulfadimethoxin, tetracycline, penicillin G	Cow urine	Dilute and shoot	UHPLC-TWIMS-MS C18 Hypersyl Gold columna (100 x 2.1 mm I.D, 1.9 µm) Mobile phase: A: Water with 0.1 % (v/v) formic acid B: MeCN with 0.1 % (v/v) formic acid Flow rate: 0.6 mL min^{-1}	---------	---------

ABBREVIATIONS AND ACRONYMS





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ABZ	Albendazole
ACO	Acetone
ADS	Alkyl diol silica
ABZ-NH_2-SO_2	2-amino-albendazole-sulfone
ABZ-SO	Albendazole sulfoxide
ABZ-SO_2	Albendazole sulfone
APFO	Ammonium perfluorooctanoate
BEN	Benomyl
BGE	Background electrolyte
BZ	Benzimidazole
CAN	Cambendazolde
CBZ	Carbendazim
CCS	Collision cross section
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
CLC	Capillary liquid chromatography
CZE	Capillary zone electrophoresis
DAD	Diode array detection
DLLME	Dispersive liquid-liquid microextraction
DTIMS	Drift time ion mobility spectrometry
EC	European comission
EFSA	European Food Safety Authority
EFTA	European Free Trade Association

EMA	European Medicine Agency
ESA	EFTA Surveillance Authority
ESI	Electrospray ionization
EtOH	Ethanol
EOF	Electroosmotic flow
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FBT	Febantel
FBZ	Fenbendazole
FBZ-SO	Fenbendazole sulfoxide
FBS-SO_2	Fenbendazole sulfone
FIA	Flow injection analysis
FL	Fluorescence detection
GC	Gas chromatography
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
ICC	Ion charge control
id	Internal diameter
IMS	Ion mobility spectrometry
IPA	Isopropanol
IP	Identification point

IT	Ion tramp
ISOB	Isobutanol
LC	Liquid chromatography
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
Log P	Partition coefficient
ME	Matrix effect
MeCN	Acetonitrile
MBZ	Mebendazole
MBZ-NH_2	Mebendazole-amine
MEKC	Micellar electrokinetic chromatography
MeOH	Methanol
MRL	Maximum residue limit
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MW	Molecular weight
m/z	Mass-to-charge-ratio
NETO	Netobimin
NSM	Normal stacking mode
OT	Open tubular
OIE	Word organization animal health

OXI	Oxibendazole
PBZ	Parbendazole
PES	Polyethersulfone
PFOA	Perfluorooctanoic acid
pKa	Acid dissociation constant
R^2	Coefficient of determination
R (%)	Percentage of recovery
RASFF	Rapid Alert System for Food and Feed
rpm	Revolutions per minute
RSD	Relative standard deviation
S/N	Signal to noise ratio
SALLE	Salting-out-assisted liquid-liquid extraction
SDS	Sodium dodecyl sulfate
SEF_{height}	Sensitivity enhancement factor based on peak height
SPE	Solid phase extraction
TBZ	Thiabendazole
TCB	Triclabendazole
TCB-SO	Triclabendazole sulfoxide
TCB-SO_2	Triclabendazole sulfone
THF	Tetrahydrofuran
TM	Thiophanate-methyl
TWIMS	Traveling wave ion mobility spectrometry

UHPLC	Ultra-high performance liquid chromatography
UV-VIS	Ultraviolet-visible
5-OH-MBZ	5-hydroxy-mebendazole
5-OH-TBZ	5-hydroxy-thiabendazole

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