ANTIBIOTICS

XI

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Development and Validation of Analytical Methodologies for the Determination of Antibiotics in Food of Animal Origin for Human Consumption

Tese de Doutoramento em Ciências Farmacêuticas, na especialidade de Bromatologia e Hidrologia, orientada pelo Professor Doutor Fernando Jorge dos Ramos apresentada à Faculdade de Farmácia da Universidade de Coimbra

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ABBREVIATIONS

API	Atmospheric Pressure Ionisation
CCα	Decision Limit
CCβ	Detection Capability
CE	Collision Energy
CXP	Cell Exit Potential
CV	Coefficient variation
DP	Declustering Potential
EC	European Commission
EDTA	Ethylenediaminetetraacetic Acid
ESI	Electrospray Ionization
EU	European Union
eV	electrons Volt
GC	Gas Chromatography
HLB	Hydrophilic-Lipophilic Balance
HPLC	High Performance Liquid Chromatography
HRMS	High Resolution Mass Spectrometry
IP	Identification Point
IS	Internal Standard
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LLE	Liquid-Liquid Extraction
LOD	Limit of Detection
LOQ	Limit of Quantification

LRMS	Low Resolution Mass Spectrometry
m/z	mass to charge ratio
ME	Matrix Effect
MRL	Maximum Residue Limits
MRM	Multiple Reactions Monitoring
MRPL	Minimum Required Performance Limit
MS	Mass Spectrometry
MS/MS	Tandem mass spectrometry
PNCR	Portuguese Residues Monitoring Plan
PVDF	Polyvinylidene Fluoride
QqQ-MS	Triple-stage quadrupole
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe
RRT	Relative Retention Time
RSD	Relative Standard Deviation
S/N	Signal-to-Noise ratio
SPE	Solid-Phase Extraction
SRM	Selection Reaction Monitoring
TOF MS	Time-of-flight Mass Spectrometer
UHPLC-MS/MS	Ultra-High Performance Liquid Chromatography Tandem Mass Spectrometry
UV	Ultra violet detector
VL	Validation Level

ABSTRACT

The main purpose of this thesis is the presentation of the complete process of development and validation of four multi-class and multi-detection screening methods of antibiotics in milk, fish muscle, bovine muscle and bovine liver. Applying the currently analytical tool of choice, ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS), all methods were validated according the European Commission requirements stated in the Commission Decision 2002/657/EC and can be applied in routine analysis of official samples of the target food products.

In chapter one, a review on the last developments on the detection of antibiotics in food-producing animals by liquid-chromatography is presented. This chapter highlights the use of liquid chromatography combined with tandem mass spectrometry detection as the preferred technique in the field of veterinary residues analysis in complex biological samples due to the possibility of fulfil the European Commission criteria. Methods for the individual families of antibiotics are described and emphasized the advantages of using multi-detection and multi-class screening methods in routine analysis. However, the most important problems found while developing those methods are also emphasized.

The second chapter presented the developed UHPLC-MS/MS methods for determining sulphonamides, trimethoprim, tetracyclines, macrolides, quinolones, penicillins and chloramphenicol in milk, fish muscle, bovine muscle and bovine liver. For all matrices it is described the process of optimizing sample preparation and detection of target compounds followed by the validation procedure. One of the main goals, successful achieved, of validation is to provided evidence that the methods are suitable for application in routine analysis. With that being proved, the developed screening methods for antibiotics in the target matrices, became important tools in the Food Safety field. Thus the referred methods could be applied by Official Laboratories in the National Residue Monitoring Plan for veterinary medicines, pesticides and contaminants in food of animal origin.

Finally, in the third and last chapter, regarding the principal drawback observed when using chromatography coupled with mass spectrometry detection, studies of the real impact of matrix effect in the detection and quantification of the target compounds, were performed. The developed methods can detect, simultaneously, several compounds from different families representing a multitude of diverse physic-chemical properties and, considering that, the specificity of sample preparation had to be minimized. In mass spectrometry that situation can lead to ion suppression or enhancement of signal, owing to interferences coming from the matrices and present in the sample

extract to be analysed by mass spectrometry. Such phenomenon was studied in order to understand the real impact in routine analysis. It was concluded that, although multi-detection and multi-class methods can be successfully used for screening purposes, when it comes to quantitation more selective methods should be applied. Despite that, the advantages achieved with the multi-detection UHPC-MS/MS methods turn them in important tools to be used in Food Safety. The capability of detection at residual concentrations, the cost-effectiveness, reduced time of analysis and the specificity to identify the presence of antibiotic in the sample are the features that guarantee the usefulness of the developed methods.

RESUMO

O objetivo central desta tese é o de apresentar detalhadamente todo o processo de desenvolvimento e validação de quatro métodos, multi-classe e multi-deteção, para a triagem de antibióticos em leite, músculo de peixe e músculo e fígado de bovino. Recorrendo à ferramenta analítica mais actual, a cromatografia líquida de alta resolução acoplada a um detetor de massa sequencial (UHPLC-MS/MS), os métodos desenvolvidos foram validados de acordo com as especificações da Comissão Europeia, definidas na Decisão da Comissão 2002/657/EC, e destinam-se a ser aplicados na análise de amostras de rotina para o controlo oficial dos produtos alimentares descritos.

No primeiro capítulo é apresentada uma revisão bibliográfica sobre os desenvolvimentos analíticos, para a deteção de antibióticos em produtos alimentares de origem animal, por cromatografia liquida. Neste capítulo é salientada a utilização de cromatografia liquida acoplada a espectrometria de massa sequencial como sendo a principal técnica na área da pesquisa de resíduos veterinários em amostras biológicas complexas, devido ao facto de garantir que sejam cumpridos todos os critérios estabelecidos pela Comissão Europeia. São ainda descritos os métodos utilizados para a determinação dos grupos de antibióticos isoladamente, assim como são destacadas as vantagens da utilização

de métodos multi-deteção e multi-classe na triagem de antibióticos em amostras de rotina. No entanto, não deixam de ser também referidos os problemas mais relevantes encontrados durante o desenvolvimento dessas metodologias.

O segundo capítulo centra-se na descrição dos métodos desenvolvidos, por UHPLC-MS/MS, para leite, músculo de peixe e músculo e fígado de bovino. Entre os antimicrobianos determinados encontram-se sulfonamidas, trimetoprim, tetraciclinas, macrólidos, quinolonas, penicilinas e cloranfenicol. Para todas as matrizes é descrito o processo de otimização da preparação das amostras e deteção dos compostos de interesse, assim como o procedimento de validação de acordo com as diretivas da Comissão Europeia. Um dos principais objetivos da validação dos métodos analíticos é o de demonstrar que os mesmos são de uso apropriado em análises de rotina, o que no presente caso ficou claramente demonstrado. Deste modo, os métodos apresentados, para as matrizes selecionadas, poderão tornar-se ferramentas importantes de utilização na área da Segurança Alimentar com aplicação no plano de monitorização oficial.

Finalmente, no terceiro e último capítulo, e tendo em conta os principais problemas encontrados durante a análise de amostras por cromatografia líquida acoplada a espectrometria de massa, foram feitos estudos sobre o real impacto do efeito da matriz. Visto que nos métodos desenvolvidos são analisados, simultaneamente, diferentes grupos de compostos, com diferentes propriedades

físico-químicas, a especificidade da preparação da amostra tem de ser minimizada. Em espectrometria de massa, esta situação pode levar a que interferentes provenientes da matriz provoquem efeitos de supressão iónica ou aumento do sinal detetado. Estes fenómenos foram estudados por forma a conhecer os reais impactos dos mesmos durante a análise de amostras de rotina. Foi possível concluir que, apesar dos métodos de multi-deteção e multi-classe terem a eficiência necessária para a sua aplicação em triagem, no que respeita à quantificação de compostos detetados, devem ser aplicadas metodologias mais específicas. No entanto, a capacidade de deteção observada para cada método desenvolvido por UHPLC-MS/MS, o baixo custo e curto período de tempo de resposta por amostra, bem como a especificidade para identificar inequivocamente o antimicrobiano presente, são características que comprovam que as metodologias desenvolvidas são ferramentas essenciais a aplicar em Segurança Alimentar.

INTRODUCTION

The use of antibiotics in intensive livestock production systems has become a common practice to treat infected animals and also as a preventive measure. The possible and irresponsible use of those compounds as growth promoters triggered the requirement for monitoring programs within the European Union. Nowadays, analytical methods for determining the presence of veterinary drugs in food products of animal origin are essential to fulfil those control plans. A complete legal framework is in constant updating to cover the whole food chain.

The topics discussed in the introduction section are related with the use of antibiotics in food producing animals, the impact in terms of human health, the European legislation, the current analytical methods employed to fulfil the legislation requirements and the validation process according to the Commission Decision 2002/657/EC [1].

ANTIBIOTICS IN FOOD-PRODUCING ANIMALS

Veterinary medicines are generally administrated to food-producing animals for therapeutic and/or prophylactic and metaphylactic purposes. A large

number of different types of compounds can be included in such treatments being feed and drinking water their principal route of administration. In the case of antibiotics, they can be used to treat infected animals and as a preventive measure (i.e. preventing epidemics), keeping welfare of the animals, or to promote animal growth. Growth promoting effects are achieved by continuously using antibiotics at sub-therapeutic doses, making its use economically advantageous. The effect of antibiotics as growth promoters were first discovered in the 1950s, as described by Stokestad and Jukes [2]. It was observed that small sub-therapeutic quantities of antibiotics, particularly penicillin and tetracyclines added to feed could enhance the feed conversion ratio for poultry, swine and cattle. Although, nowadays, it is not completely clear the mechanism on how antibiotics can promote animal growth, it is believed that the continuous administration of those compounds can reduce the incidence and the severity of subclinical infections and also can improve the absorption of nutrients by thinning the intestinal wall thus increasing feed efficiency [3, 4].

The inappropriate and abusive use of veterinary drugs as described can lead to the presence of residues of these compounds or their metabolites in edible tissues and, in the particular case of antibiotics, can be responsible for toxic effects to the consumers, allergic reactions in individuals with hypersensitivity and for the development of resistant strains of bacteria. Another important negative effect is

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related with processed food and to the fact that the final products can have their quality compromised and, as an example of that situation are products obtained by fermentation in which antibiotics can inhibit the action of bacteria responsible for the process. Kemper [5] summarized those described potential side effects arising from the presence of the different types of antibiotics, usually administrated in human and animals, in food products. The referred author also studied the effects observed by the excessive use of antibiotics in livestock production giving rise to concerns related with the fate of antibiotics, their metabolites and degradation products, excreted by animal husbandry. Their persistence in the terrestrial and aquatic environmental are highlighted along with the possibility of occurrence of resistant bacteria in soil and water [5]. Nevertheless, the major concern at global level is related with the occurrence of resistant bacteria [6] that can be disseminated from animals through the food chain [7, 8] and to the environment [9] and, finally, transferred to humans. Each animal continuously treated with antibiotics can become a potential source for the production and subsequent dispersion of antibiotic resistant bacteria [10, 11]. Several studies described by Cogliani, Goossens and Greko [12] established a direct relation between the low-doses and non-therapeutic administration of antibiotics in farm animals and the emergence and spread of resistance genes. It is also proved that the antibiotic resistance patterns in humans is determined by the same mechanism as in animals and that the dissemination of the resistance genes

occurs from the food chain to the intestinal flora of humans but also via direct contact with animals [5]. Furthermore, it is important to enhance that many antibiotics applied in veterinary medicine are the same used to treat humans, confirming that the occurrence of bacterial resistance is a serious public health concern, both for animals and for humans. The emergence and dissemination of multiple antibiotic resistant bacteria has special attention in public and scientific population worldwide justifying why in 2006 EU completely banned the use of antibiotics as growth promoters [13]. Antimicrobial resistance has been nowadays recognized as a serious public health concern that has already reached worldwide dimensions. The Regulation (EC) 1831/2003 [14] stated that antibiotics, other than coccidiostats and histomonostats, cannot be used after 2005 as feed additive. This prohibition, considered as an effort to restore the microbial flora of animals when no resistant bacteria are present, was the final step to end the permissive use of antibiotics to increase food production. Historically, the first prohibition occurred in a follow up of an epidemic of resistance *Salmonella typhimurium* in UK from 1963 until 1965 [12]. It was discovered that oxytetracycline resistant bacteria was transferred from food animals to *Salmonella typhimurium*. As a consequence, from 1972 and 1974, EU banned the use, as growth promoters, of tetracyclines, penicillins and streptomycin [12].

Another problem caused by the presence of antibiotic residues in food products is the consequent difficulties observed during food processing, particularly in fermentation [15]. The presence of inhibitory substances as antibiotics can slow or destroy the growth of fermentation bacteria responsible for the transformation in fermented products. At the end of the process it can be observed a significant loss of quality or even in achieving the final product. A perfect example of that situation is described by Chandan and Kilara [16] and by Hummel, Hertel, Holzapfel and Franz [17] for dairy products during the production of cheese and yogurts and the negative effects of inhibitory compounds such as antibiotics.

EUROPEAN REGULATION

"Assuring that the EU has the highest standards of food safety is a key policy priority for the Commission."

EU Commission, White paper on food safety, 2000 [18]

The use of antibiotics in modern animal-food producing industry is essential and, being aware of the potential negative effects for the consumers and

their protection, the European Community settled strict regulations for their use. Food safety is one of the top priorities assumed by the European Commission, as well explained in the White Paper on Food Safety [18], meaning that legislation has to be continuously modernized and implemented in order to control food from the farm to the table along with constant developments in the scientific capabilities.

Several regulatory documents to regulate the control of veterinary drugs in foodstuff of animal origin have been set along the years. Directive 96/23/EC [19] turned mandatory the control of food producing animals as well as their primary products by monitoring the presence of residues of veterinary medicines before marketing authorization. In the same Directive the compounds used in veterinary medicine are divided in two groups in which group A comprises prohibited substances and the allowed compounds are in the group B with established maximum residue level (MRL) and compounds for which no MRL has been set as no hazard for consumers has been proved. The MRL concentrations were determined after toxicological studies and with the purpose of minimizing human exposure. In practice all food products of animal origin should be free of forbidden or non-authorized substances or contain quantities below the MRL for allowed compounds. When this situation is not observed it is considered that the product is not suitable for human consumption. Although for non-authorized substances

there is no tolerance level, in some cases, to harmonize the analytical performance of the methods within official member states laboratories, a minimum required performance limit (MRPL) had been set [1, 20]. The MRPL is not a concentration obtained from toxicological data, but is only related with analytical performance.

As allowed substances to be used in veterinary practice, antibiotics are included in group B and for many of them the MRL are available. But they are completely forbidden, as animal growth promoters, since 2006 [14]. Substances belonging to groups A and B are briefly described in table 1. The procedures for the establishment of residue limits on pharmacologically active substances and their classification regarding MRL are defined in the EU Council Regulation 470/2009/EC [21] which repealed the previous Council Regulation (EEC) No 2377/90 [22]. The EU Commission Regulation 37/2010 [23], also repealing the same regulation [22], presents a complete list of the pharmacologically active substances and their MRLs in foodstuffs of animal origin.

Table 1: Substances of Group A and Group B, according to Directive 96/23/EC [19]

Group A	Group B
Substances with anabolic effects and unauthorized substances	**Veterinary drugs and contaminants**
	Antibacterial substances
	Anthelmintics
	Anticoccidiostats, including nitroimidazoles
	Carbamates and pyrethroids
Stilbenes, stilbene derivatives, and their salts and esters	Carbadox and olaquindox
	Sedatives
Antithyroid agents	Non-steroidal anti-inflammatory drugs
Steroids	Other pharmacologically active substances
Resorcylic acid lactones including zeranol	Other substances and environmental contaminants
β-Agonists	
Compounds included in Annex IV to Council Regulation 2377/90/EC	Organochlorine compounds including PCBs
	Organophosphorus compounds
	Chemical elements
	Mycotoxins
	Dyes
	Others

The surveillance plan designed to ensure that the legislation is being respected is the National Residue Monitoring Plan, mandatory in each one of the member states as stated by the Directive 96/23/EC [19]. In Portugal, such control is performed in the *Instituto Nacional de Investigação Agrária e Veterinária* (INIAV) where the Portuguese National Reference Laboratory for Residues is located. This monitoring plan covers the living animals (analytical determinations in biological

fluids, feed and water for drinking) and also samples collected in the slaughterhouse (edible tissues). Analysis should also be performed in other food products obtained from animals, such as milk, eggs or honey.

The performance criteria that have to be fulfilled for the analytical methods employed in the official residues control are described in the EU Decision 2002/657/EC [1].

ANALYTICAL STRATEGIES

The widespread use of antibiotics for therapeutic reasons and as growth-promoters, turned the development of analytical methods for the determination of such compounds in food, a mandatory issue in Food Safety policy. Usually, the target matrices for antibiotic residue determination were muscle, liver, kidney and milk but in the current days, due to the changes in food habits, worldwide, fish muscle, eggs and honey are also matrices of interest in this field of residue analysis. Equally important are the control of feed and water for drinking, since those are the principal ways of administration of antibiotics and other veterinary drugs often used in food producing animals. The control of feed has to be in accordance with the EU Regulation 1831/2003/EC [14] that settled down the banned antibiotics.

In terms of analytical strategies, three categories of methods can be used to detect antibiotics in edible tissues: microbiological, immunochemical and physic-chemical.

Screening of antibiotics has been commonly performed by microbiological methods. These tests are performed by incubating a susceptible organism in the presence of the sample to be analysed, and are based in the inhibition of bacterial growth caused by the presence of antibiotics in the samples, which can be very unspecific. Although microbiological methods are capable of detecting active antibiotics at trace levels having the potential to cover a wide antibiotic spectrum within one test, they are not specific in the identification of the drug responsible for the inhibition or even of the group of antibiotic present in the sample [24]. Another drawback is the fact that the microorganisms used in the test are not equally sensitive to all types of antibiotics and, for that reason, some antibiotics are better detected than others. Although these methods can give limited information, they are characterized by giving rapid results allowing the analysis of a large volume of samples in a short period of time. Also another important attribute is the fact that no sample extraction is needed [25].

The immunochemical techniques, based on the reaction between antigen and antibody, are highly selective and sensitive for a particular drug or a group of structurally related compounds since the interaction established between the

antigen and the antibody is very specific. The most common immunological technique is in the enzyme-linked-immuno-sorbent assay, ELISA [15]. The main advantages associated to this type of methods are the fact that they can be used for screening of a large number of samples in one kit, easy to perform in a short time period, with high sensitivity and selectivity along with reduced cost, though more expensive than the microbiological methods [26]. Immunoassays are especially suitable for the analysis of compounds at residual concentration and in samples with simple or even no preparation, due to their low detection limits. Another advantage is the possibility to use those kits in the field without the need to transport the sample to the laboratory. Nevertheless the high selectivity of such assays is the principal restriction since it makes impossible to use as multi-residue methods [27].

The most recent and continuous improvements are in physic-chemical methods allowing the development of reliable, specific and sensitive methods able to fulfil the criteria stated in the Decision 2002/657/EC [1], that regulates the performance conditions for methods to be used in the official residues control. The accurate identification of veterinary drugs in products from animal origin is one of the main requirements when choosing the right analytical method. For that reason the use of liquid chromatography (LC) with tandem mass spectrometry has been the analytical tool of choice. The main advantage of LC in relation with gas

chromatography (GC) is the fact that the majority of the analysed compounds is polar and with reduced volatility. The use of UHPLC gives the possibility of having short running times together with higher resolution and sensitivity.

In terms of detection, mass spectrometry, as triple quadrupole coupled to LC (LC-MS/MS or UHPLC-MS/MS) represents a huge advantage to guarantee the required criteria, specially the unequivocal identification. Mass spectrometry detection performed by a triple quadrupole allows recording full mass spectra (scan mode) or, more specific, selected ion monitoring (SIM) and multiple reaction monitoring (MRM). Nevertheless, the application of Time-of-Flight (ToF) or High Resolution Mass Spectrometry (HR-MS) is growing in residue analysis. The high cost associated with those equipments along with the fact that it is not completely clear how to apply the performance and validation in those methods, according to legislation, are the principal drawbacks for their use.

One of the identification criteria introduced by the Decision 2002/657/EC [1] is the identification points (IP). Compounds listed in group A should have at least four IPs, in the case of antibiotics, listed in group B, an accurate confirmation of their presence requires a minimum of three IPs. The number of IPs earned depends on the specificity of the MS technique used as presented in table 2.

MS Technique	IPs per ion
LR[(1)] - MS^n precursor ion	1.0
LR - MS^n transition products	1.5
HR[(2)] - MS^n precursor ion	2.0
HR - MS^n transition products	2.5

In the table: (1) means Low Resolution and (2) High Resolution.

Nevertheless, for screening purposes a single signal can be accepted for a first identification. After that, a complete confirmation has to be performed. In that case, along with the IPs, other specific criteria like relative retention time and ion ratio has to be verified. The ratio between the chromatographic retention time of the target compound and its internal standard, named relative retention time (RRT), should not exceed 2.5%. The ion ration tolerances are presented in the table 3.

Table 3: Maximum permitted tolerances for relative ion intensities using LC-MS/MS

Relative Intensity	Ion Ratio Tolerance
> 50 %	± 20%
> 20 – 50 %	± 25%
> 10 – 20%	± 30%
≤ 10%	± 50%

The increasing interest in improving cost-effectiveness of analytical procedures has become an important issue in the field of residues analysis. Such improvement can be achieved by maximizing the number of substances determined in one assay. Developing reliable screening methods, able to detect, at the same time and in a single run, the maximum number of analytes as possible is the nowadays challenge. These methodologies can provide rapid results by reducing the number of samples to be confirmed and, consequently, decreasing the cost associated with more methods. The UHPLC-MS/MS has been proved to be a powerful tool that allows multi-class and multi-compound detection of antibiotics in complex biological samples even present at residual concentrations.

However, when simultaneously analysing different groups of compounds with different physic-chemical properties, the specificity of sample preparation has to be minimized in order to avoid losses of the analytes during the process. In mass spectrometry, this situation can be responsible for ion suppression or enhancement of signals due to interferences coming from the matrices, compounds released from the samples or reagents used during the process of sample preparation [28, 29]. The interferences present in the sample extract can co-elute with the target compounds and lead to modifications in the spray droplet solution changing the evaporation process and, consequently, interfering with

ionization process leading to either decrease or enhancement of the detected signal. To overcome such situation the measurement tool has achieve detection at very low concentrations to guarantee detection even when higher suppression is observed.

VALIDATION OF ANALYTICAL METHODS

The actual legislation settled by the European Commission, concerning residues control of veterinary drugs in food products of animal origin, does not include the requirement of using normalized methods for the official control. However the analytical results developed by different laboratories across Europe, should be comparable, and the quality control has to be equally ensured. For that reason all the methods used must be validated using common procedures and the relevant performance characteristics should be fulfilled. Commission Decision 2002/657/EC [1] lays down rules for the performance of analytical methods and their validation and specifies common criteria for the interpretation of analytical results.

The analytical methods used, depending of their classification as screening, confirmatory, quantitative or qualitative, have different parameters to be controlled. A qualitative method is an analytical method that identifies a

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substance on the basis of its chemical, biological or physical properties. The result obtained is the presence or absence of the target compound. A quantitative method determines the amount or mass fraction of a substance expressing the result as a numerical value of appropriate units. On the other hand a screening method is used to detect the presence of a substance or class of substances at the level of interest. A confirmatory methodology provides the necessary information in order to have an unequivocal identification of the substance and, when necessary, quantify it at the level of interest.

In the table 4 the classification of the methods and the correspondent performance characteristics are summarized.

Table 4: Parameters that have to be determined in the validation of the different analytical methods

		Decision limit (CCα)	Detection limit (CCβ)	Recovery	Precision	Selectivity Specificity	Applicability Ruggedness
Screening	*Qualitative*		√			√	√
	Quantitative		√		√	√	√
Confirmatory	*Qualitative*	√	√			√	√
	Quantitative	√	√	√	√	√	√

The validation concept proposed apply to new parameters replacing the traditional limit of detection (LOD) and limit of quantification (LOQ) by two critical

concentrations: decision limit (CCα) and detection capability (CCβ). The statistical definitions, the calculations associated with the determination of those parameters and the interpretation of the results obtained are well described by Freitas and co-workers [30]. The decision limit, CCα, refers to the "limit at and above which it can be concluded with an error probability of α that a sample is non-compliant" [1]. The α error is related with the probability of having a compliant sample despite the non-compliant result obtained – "false non-compliant decision". Statistically, CCα introduces the uncertainty of the method in the result. Detection capability, CCβ, is "the smallest content of the substance that may be detected, identified and/or quantified with an error probability of β" [1]. The β error happens when a compliant result is obtained when in fact is non-compliant – "false compliant decision". These new concepts were not contemplated in the LOD and LOQ determination.

According to Commission Decision 2002/657/EC [1] the calculation of CCα and CCβ can be performed by the determination of signal-to-noise ratio (S/N) observed when analysing representative blank samples from the routine (equation 1) and by the construction of calibration curves (equation 2) in accordance with the described in the ISO 11843 [31].

$$CC_\alpha = 3 \times S/N_{20\ blank\ samples} \qquad \text{(Equation 1)}$$

$$CC_\alpha = Blank + 2.33 \times SD_{20\ blank\ samples} \quad \text{(Equation 2)}$$

Where S/N$_{20\ blank\ samples}$ is the mean of signal-to-noise ratio of 20 blank samples and SD$_{20\ blank\ samples}$ represents the standard deviation of the signal obtained in the 20 blank samples. Twenty blank samples spiked at the determined CCα and the corresponding standard deviation (SD$_{20\ spiked\ blank\ samples\ at\ CC\alpha}$) will allow the determination of CCβ.

$$CC_\beta = CC_\alpha + 1.64 \times SD_{20\ spiked\ blank\ samples\ at\ CC\alpha} \quad \text{(Equation 3)}$$

When dealing with compounds with established MRL this concentration has to be taken into account when calculating the analytical limits that will decide on the compliance of the samples.

$$CC_\alpha = C_{MRL} + 1.64 \times SD_{20\ spiked\ samples\ at\ MRL} \quad \text{(Equation 4)}$$

$$CC_\beta = CC_\alpha + 1.64 \times SD_{20\ spiked\ samples\ at\ CC\alpha} \quad \text{(Equation 5)}$$

In equation 4, the SD$_{20\ spiked\ blank\ samples\ at\ MRL}$ represents the standard deviation observed in 20 blank samples spiked at the MRL level.

Recovery has to be determined for confirmatory quantitative methods and is the percentage of the true concentration of a substance obtained in the end of

the analytical procedure. If certified reference material is available the deviation of recoveries should be in the range of values presented in table 5.

Table 5: Acceptable recovery range depending on the concentration

Mass fraction	Recovery range
≤ 1 µg/kg	50% to 120%
> 1 until 10 µg/kg	70% to 110%
≥ 10 µg/kg	80% to 110%

Precision, only mandatory for quantitative methods, measures the coefficient variation (CV) between repeated analyses. Under reproducibility conditions, the CV should not exceed the calculated by the Horwitz equation. In conditions of repeatability the CV should be between one half and two thirds of the calculated by the equation 6.

$$CV = 2^{(1-0.5\log C)} \quad \text{(Equation 6)}$$

The concentration (C) is expressed as exponent of 10, for example 1mg g^{-1} = 10^{-3}.

Selectivity and specificity measure the ability of the method to distinguish between the target analyte and other substances present in the sample and has to be monitored for all types of methods. These parameters are dependent on the matrix, compound and analytical procedure used.

Applicability and ruggedness should also be studied for the different analytical methods by observing the consequences of variation in experimental conditions. All possible changes and conditions that can be subject to fluctuations and may affect final the results (i.e. storage conditions; stability of reagents; pH; temperature; among others depending on the procedure) should be tested.

Decision 657/2002/EC [1] also describes the identification criteria, which should be fulfilled in all the validation samples, as already presented above: IPs, RRT and ion ratio.

Although not demanded in any legislation, it is consensual in the scientific community working in the residues analysis that, a complete matrix effect study should be performed to complement the validation and to understand the real impact in the final results especially concerning the official control [32, 33].

THESIS OUTLINE

The continuous administration of antibiotics in farm animals has the principal consequence of enhancing the exposure of humans to these compounds by the presence of their residues in food products coming from treated animals. This situation can be responsible for the development and spread of resistant strains of bacteria and severe allergic reactions in hypersensitive individuals to those compounds or even to their metabolites or degradation products. On the other hand, the presence of antibiotics in food products can contribute to difficulties in food processing, in particular in the industry of fermented products resulting, in the end of the process, in loss of quality. The presence of inhibitory substances such as antibiotics, can slow or destroy the growth of fermentation bacteria responsible for the transformation of fermented products.

Baring in mind the described situations, the public health concerns and the economical losses arising from inappropriate utilization of antibiotics in veterinary field, the current research had the following specific objectives:

- Optimization of extractive procedures for the determination of several antibiotics as possible in different matrices of animal origin: milk, fish muscle, bovine muscle and liver;

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- Development of multi-class multi-detection methods, to be applied in routine analysis of samples of the national plan of residues control in Portugal, by UHPLC-MS/MS, for the semi-quantitative screening of sulphonamides, trimethoprim, tetracyclines, macrolides, quinolones, penicillins and chloramphenicol;

- Validation of the developed methods in accordance with the requirements of the EU Decision 2002/657/EC [1] in order to apply them to the official national monitoring plan for veterinary medicine residues.

- Evaluation of the possible matrix effects in terms of suppression or enhancement of the detected signal, caused by interferences present in the sample extract.

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CHAPTER I – LIQUID-CHROMATOGRAPHY: REVIEW ON THE LAST DEVELOPMENTS ON THE DETECTION OF ANTIBIOTICS IN FOOD-PRODUCING ANIMALS

ABSTRACT

In modern agricultural practice antibiotics are widely implemented and administrated as feed additives or in drinking water with the main purpose of treat and prevent diseases and/or to promote growth. The use of antibiotics as growth promoters is considered inappropriate and the abusive utilization of these compounds can lead to residues in edible tissues. For the consumers, the presence of such residues can be responsible for toxic effects, allergic reactions in individuals with hypersensitivity and can result in the development of resistant strains of bacteria.

In order to control these abuse situations, the European Union settled down several important official documents which regulate the control of veterinary drugs in products from animal origin. The Council Directive 96/23/EC determines the control of food producing animals as well as their primary products by monitoring residues of veterinary medicines while EU Council Regulation 37/2010/EC lays down community procedures for the establishment of residue limits on pharmacologically active substances and their classification regarding maximum residue limits (MRL), repealing Council regulation (EEC) 2377/90. Also important is Decision 2002/657/EC that describes the performance criteria for the analytical methods employed in the official residues control.

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The principal methods used for analysing antibiotics in edible tissues can be divided in: microbiological tests, immunochemical assays and physic-chemical methods. The most recent improvements refer to the last ones, with special emphasis on Liquid Chromatography (LC) mainly due the polarity of compounds and the lack of volatility, which makes the use of Gas-Chromatography (GC) more difficult and time consuming. Also, a physical-chemical analytical approach allows the development of reliable, robust, specific and sensitive methods important in quantification. In terms of detection, the use of mass spectrometry, such as triple quadrupole coupled with LC (LC-QqQ-MS), represented a huge improvement in terms of analytical strategies. This powerful tool allows multi-class and multi-compound detection of antibiotics in complex biological samples with high levels of specificity and robustness. Additionally, the choice, in terms of detection, is growing in recent years and Time-of-Flight (ToF) or High Resolution Mass Spectrometry (HR-MS) benchtop instruments are much more laboratory reachable.

This review has the main intent of making an analysis of how veterinary drugs, in the particular case of antibiotics, are being monitored in food producing animals in the last years, and how the development of new analytical strategies in the Liquid Chromatography field influenced the improvement of antibiotic residues detection.

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INTRODUCTION

In modern agriculture the use of antibiotics is a widely implemented practice with the main purpose of treating and preventing diseases but also to stimulate animal growth [1-4]. Nonetheless, the resort to antibiotics as growth promoters is considered inappropriate and the mechanisms that explain how antibiotics can induce growth are still not completely understood [5, 6]. Moreover, the abusive use of these compounds can lead to the presence of residues in edible tissues that can be responsible for toxic effects to the consumers, allergic reactions in individuals with hypersensitivity and can also result in the development of resistant strains of bacteria [7-12].

In order to control such situations, the European Community settled down several official documents to regulate the control of veterinary drugs in products of animal origin. One important regulation is Council Directive 96/23/EC [13] that determines the control of food producing animals as well as their primary products by monitoring residues of veterinary medicines. According to this directive veterinary compounds are divided in two groups with group A including prohibited substances and group B comprising permitted compounds with established maximum residue levels (MRL) as well as compounds for which no MRL has been set because no hazard for public health has been observed. Many organizations, such as Codex Alimentarius, European Union (EU) and US Food and

Drug Administration work together to establish MRL's in order to minimize human exposure.

Antibiotics, as allowed veterinary drugs, are included in group B and for many of them MRL are available. EU Council Regulation 470/2009/EC [14] lays down community procedures for the establishment of residue limits on pharmacologically active substances and their classification regarding MRL, repealing Council regulation (EEC) 2377/90 [15] while EU Commission Regulation 37/2010 [16] lists the pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. Also important is the EU Decision 2002/657/EC [17] that describes the performance criteria for the analytical methods employed in the official residues control. In 2009, Companyó and colleagues [18] made a complete historical review on legislation and validation of analytical methodologies for determination of antibiotics in food. Previously, other reviews were also published on analytical methods for the determination of residues in food producing animals [19-22]. An overview of some selected methods for residues extraction from biological matrices is discussed in detail by Kinsella et al. [23].

The main intent for the present review is to analyse how veterinary drugs, in the particular case of antibiotics, have been monitored in food producing

animals for the last years, and how the development of new analytical strategies in the LC field have influenced the improvement of antibiotic residues detection.

ANTIBIOTICS AND ANALYTICAL STRATEGIES

Antibiotics are a diverse range of compounds, both natural and semi-synthetic, which possess the ability to inhibit the growth of micro-organisms. The term antibiotic was originally applied to describe compounds derived from living organisms, but presently also encompasses synthetic substances. Another common designation to antibiotics is *antibacterials* due to their use in the treatment of infections caused by bacteria.

In the past the preferred matrixes for antibiotic residue determination were muscle, liver, kidney and milk. Nowadays, the changes in consumption habits have increased the importance of fish, eggs and honey in the field of residues analysis [21, 24-42]. Animal feeds are also important matrixes that need to be controlled since the administration of antibiotics is allowed for therapeutic purposes but prohibited when used for growth promotion. The EU Regulation 1831/2003/EC [43] settled down the banned antibiotics that might be used with this aim. Within this view, the main methods used for analysing antibiotics in edible tissues can be divided in: microbiological tests, immunochemical assays and

physic-chemical methods. Microbiological tests are based in the inhibition of bacterial growth, being the 4-plate test one example. These tests are capable of detecting active antibiotics at trace levels but they are not specific in the identification of the drug responsible for the inhibition or even the group of antibiotic present in the sample [44-48].

Considering the immunochemical assays they have the advantage of being highly selective and sensitive for a particular drug and can be used for screening a large number of samples within a short time at low cost. Basically, a specific target antibiotic is captured by immobilized antibodies or by a broader-spectrum receptor such as a bacterial cell. The main restriction is imposed by the fact that immunochemical assays are single analyte methods hindering multi-residue methods and preventing the detection of non-target drugs [44, 49-53]. As for the physic-chemical methods that allow the development of reliable, robust, specific and sensitive methods important to quantification, the most recent improvements were registered with special emphasis to liquid chromatography (LC), mainly due to the polarity of compounds and the lack of volatility which makes the use of gas chromatography (GC) more difficult and time consuming.

The LC usually consists of reverse-phase with an alkyl-bonded silica column (C_8 or C_{18}) and involves a mobile phase gradient, with main part of aqueous solution. It separates the compounds based on the hydrophobic interactions

between the non-polar stationary phase and the organic components of typical analytes. The retention of polar analytes often requires a highly aqueous mobile phase to achieve retention and this situation can lead to the stationary phase collapse thus decreasing the retention of very polar analytes. This phenomenon is well described by Przybyciel et al. [55]. Some specialized packings were developed to prevent this situations [55] including polar embedded phases or hydrophilically end-capped reversed phase bonded silica, among others. However, it is also increasing the use of hydrophilic interaction liquid chromatography (HILIC) which consists of an alternative technique for the separation of highly polar analytes, solving the problems associated with column collapse and retention that would not be achieved by reversed-phase chromatography. HILIC requires a high percentage of a non-polar mobile phase and the elution order is reversed when compared to reversed-phase liquid chromatography.

In terms of detection, the use of mass spectrometry, as triple quadrupole coupled with LC (LC-QqQ-MS or more often used LC-MS/MS), represented a great improvement in terms of analytical strategies. This powerful tool allows multi-class and multi-compound detection of antibiotics in complex biological samples with high levels of specificity and robustness. Additionally, the choice, in terms of detection, is growing in recent years and Time-of-Flight (ToF) or High Resolution Mass Spectrometry (HR-MS) benchtop instruments are much more laboratory

reachable. Also, the combination of ultra-high performance liquid chromatography (UHPLC) with ToF, for screening purposes, is nowadays presented as the most powerful measurement tool in terms of selectivity, sensitivity and speed [30, 35, 36, 44]. For confirmatory quantification QqQ-MS is still the technique of preference. The identity of veterinary drugs in products from animal origin has to fulfill the criteria described in European guidelines 2002/657/EC [17]. The unequivocal confirmation is based on identification points (IP) and the number of IP depends on the analytical technique used being mandatory the minimum of 4 IP in case of unauthorized substances and 3 IP for confirmation of MRL substances. Although it is possible to obtain the required IP in low-resolution with a triple-quadrupole mass spectrometer by MRM and also by high-resolution mass spectrometer such as ToF-MS, these approaches are not yet included in the regulation accepted for confirmation purposes [17]. In several publications it is possible to observe the use of ToF for multi-detection screening methods in which the suspected samples follow confirmation by LC-QqQ-MS [21, 30, 38, 44, 56-59].

Kaufmann and colleagues [59] developed a study in which a comparison of quantitative and confirmative performance was evaluated in two different mass spectrometry techniques: high-resolution mass spectrometry (HRMS) in an Orbitrap MS and a ToF-MS versus a tandem mass spectrometry (MS/MS) of

quadrupole technology. The comparison was carried out in 36 analytes residues present at trace level in a difficult food matrix, honey. The authors based their experiences on the fact that there has never been a real scientific reason why MS/MS should be the only confirmatory and quantitative MS technology accepted. According to the authors, the principal reason why MS/MS is still the analytical tool of choice is the advantage in terms of sensitivity and selectivity, when compared with current HRMS technology, in case of a limited number of monitored analytes. However, when working with real multi-residue methods, where a large number of compounds has to be detected and quantified, HRMS gives interesting possibilities. The same study [59] concluded that HRMS, as provided by a single-stage Orbitrap instrument, gives the precision and accuracy necessary for confirmation and quantification purposes. Nevertheless they also agree that MS/MS should still be the choice in cases of extremely toxic or banned compounds which have to be confirmed at very low levels.

There are many different groups of antibiotics and their use depends on the type of infection and animals that need to be treated, the majority belonging to the following families: aminoglycosides, amphenicols, beta-lactams, macrolides, nitrofurans, quinolones, sulphonamides and tetracyclines.

Aminoglycosides are broad-spectrum antibiotics, active against most Gram-positive and negative bacteria [60]. They are potent bactericidal substances that act by creating fissures in the outer membrane of the bacterial cell and by binding to the 30S ribosome inhibiting bacterial protein synthesis. The chemical structure of aminoglycosides is based on an aminocyclitol ring (2-deoxystreptamine in most case) coupled to two or more amino sugars in a glycoside linkage. Structures of selected compounds representing the group are presented in figure 1.

Streptomycin   Amikacin

Gentamicin
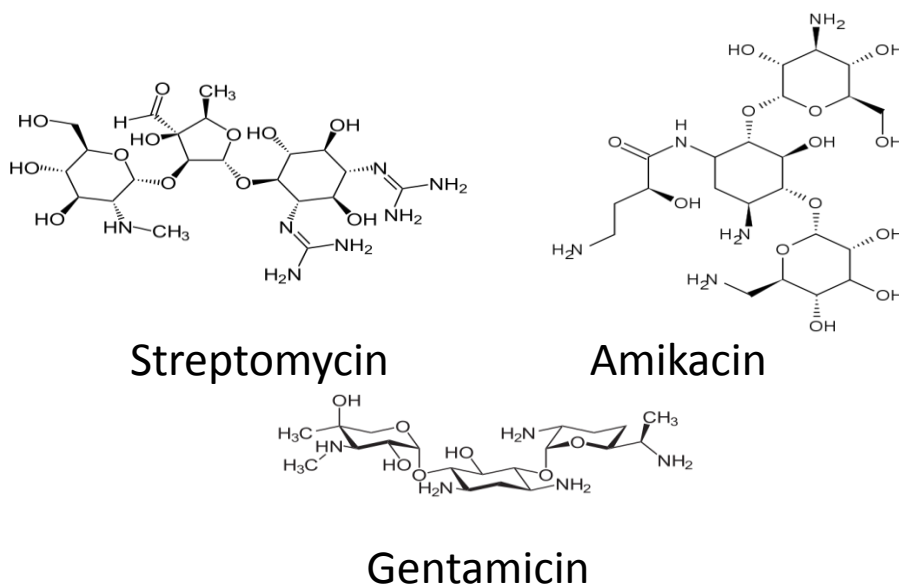
Figure 1: Chemical structures of representative aminoglycosides

Their physic-chemical properties difficult the development of sample extraction as they are scarcely soluble in organic solvents, basic compounds, very hydrophobic and thermally labile [61]. The challenging advantages in developing analytical methods for aminoglycosides determination, until 2008, were presented by McGlincheya et al. [62]. The fact that these compounds do not contain any chromophores or fluorophores made the use of derivatization step a procedure of choice for fluorescence detection. However, the analytical procedure become more time consuming and the derivatives themselves showed to be unstable by degrading within a few hours after formation [62, 63]. To overcome these difficulties the use of mass spectrometry became the preferred detection method for aminoglycosides offering the advantages of sensitivity and the unequivocal confirmation of identity [46, 62-67]. In addition, as the amino groups of these drugs ionize well with electrospray, the need for derivatization has diminished and these analytical techniques are widely selected for confirmatory methods. However, the fact that aminoglycosides are not adequately retained on reversed-phase columns still presents an analytical challenge. One way to overcome this difficulty is through the use of HILIC but this method presents the disadvantage of requiring high ionic strength buffers and special chromatographic columns. Turnipseed and colleagues [66] demonstrated that the derivatization of

aminoglycosides with phenyl isocyante provided derivatives that could be easily synthesized, retained and separated on a common reversed-phase column avoiding the need of ion-pair reagents or HILIC LC columns. The confirmation and quantification, in samples of milk, was performed with detection in an ion trap mass spectrometer, using positive ion electrospray where the product ion spectra were generated from the derivatized protonated molecules. In the extraction procedure, adapted from existing methodologies [68, 70], aminoglycoside residues were extracted with acid and isolated from the matrix with a weak cation exchange solid-phase extraction cartridge.

Kaufmann et al. [67] presented a method that detects and quantifies 13 commonly used aminoglycosides in a variety of food matrices (pork muscle, fish, veal livers and kidneys). The method described was based on an earlier work from the same authors [69], but unlike the previous approach, relied on a simple clean-up procedure based on a strong cation-exchange solid-phase cartridge that permitted high sample extract loading volumes. The elution was based on a volatile buffer at intermediately high pH value in combination with an organic solvent allowing the quantitative elution of the various aminoglycosides. In terms of chromatography the authors presented the use of ion pair modifiers and reversed-phase LC instead of HILIC LC. The observations showed that the ion pair system was very stable after some two to three injections and produced higher

sensitivities for late-eluting aminoglycosides than HILIC. For the compounds separation a C_{18} column was used with two solutions for mobile phase: acetonitrile and water with heptafluorobutyric acid. It was concluded that the use of a neutralized eluate guaranteed sufficient weak ion strength not compromising the separation of compounds in the column.

AMPHENICOLS

Chloramphenicol, figure 2, is the principal compound of this group being a broad spectrum antibiotic active against aerobic and anaerobic Gram-positive and negative bacteria acting as a bacteriostatic drug preventing bacterial growth by inhibiting protein synthesis. After the reported toxicity indicating that chloramphenicol can cause plastic anaemia in humans the compound was banned in animal production in USA and EU in 1994. As a result of this interdiction very sensitive methods for the detection and quantification of chloramphenicol are needed and, at least, should be able to detect the presence of the compound at the minimum required performance limit (MRPL) level (0,3 µg kg^{-1}, in all food of animal origin) [17, 71]. Other compounds with similar chemical structure, thiamphenicol and florfenicol can be used instead.
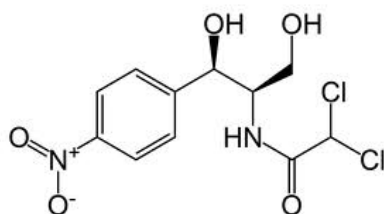
Figure 2: Chloramphenicol chemical structure

Several analytical methods have been developed and reviewed for the detection and quantification of chloramphenicol in biological matrices. The use of GC coupled with MS is one of the options most frequently applied coupled to ionization techniques such as chemical ionization (CI) and electron impact (EI) [19, 20, 22, 72-77]. However, GC-MS requires a derivatization step in order to improve the chromatographic properties, usually a sylilation reaction catalysed by acids or bases, which is possible due the presence of polar functional groups in the chloramphenicol molecule.

On the other hand, liquid chromatography coupled with mass spectrometry (LC-MS) does not require the derivatization step as a result of a pronounced electron affinity of the compound that is responsible for an efficient detection in negative ionization mode [19, 20, 22, 78-85]. A full MS scan of chloramphenicol shows a typical isotopic pattern due to the presence of two atoms of chloride in the molecule. The most abundant ion is *m/z* 321, with two

[35]Cl-atoms, and the most intense fragment ions from this molecule are *m/z* 152, *m/z* 257 and *m/z* 194 (Fig. 3).

m/z= 152   m/z= 194   m/z= 257
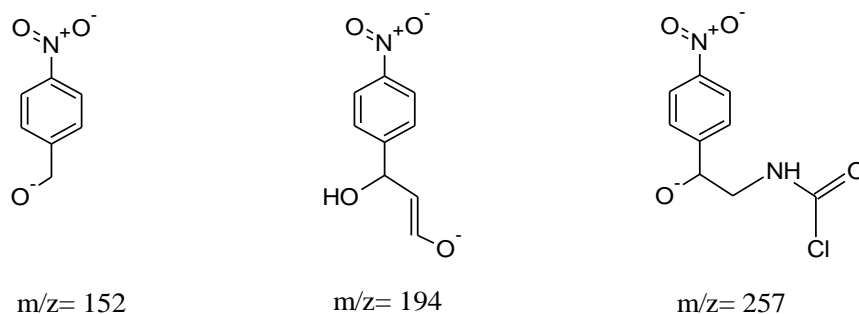
Figure 3: Most intense fragment ions from chloramphenicol molecule

Bononi et al. [80] presented a method for the determination of chloramphenicol in propolis, a natural honeybee product, using thiamphenicol as the internal standard for quantification. Analytically, LC-MS/MS interfaced with electrospray in negative ionization mode was used and the acquisition was achieved with selected reaction monitoring (SRM) mode. The hydroalcoholic and glycolic extracts were simply diluted with ethyl acetate and analysed. For LC, a C_{18} column was used and the mobile phases were water and methanol. This simple procedure showed to be suitable for the analysis of the compound in the matrix presented, with a limit of detection (LOD) = 0.05 µg kg^{-1} and recoveries ranging from 80% to 99%.

Rønning et al. [81] developed an LC-MS/MS method for the determination of chloramphenicol residues in several food matrices: meat, seafood, egg, honey, milk, plasma and urine. The method presented a very simple sample preparation for most matrices consisting of extraction in acetonitrile while using deutered chloramphenicol (d5- chloramphenicol) as internal standard. However, for plasma and urine a SPE procedure was required in order to obtain cleaner extracts, using a C_{18} column for chromatographic separation and acidified water with formic acid and methanol as mobile phases. As for MS/MS detection ions were monitored in negative multiple reaction monitoring mode (MRM). The method was fully validated in accordance with EU [17] for all matrices with reproducibility values always below 25%. The critical concentrations were also determined with decision limit (CCα) of 0.02 µg kg^{-1} and a detection capability (CCβ) of 0.04 µg kg^{-1}.

BETA-LACTAMS

Beta-lactams (ß-lactams) antibiotics are probably the most widely used in veterinary medicine for treatment and prevention of bacterial infections [20]. These antibiotics work by inhibiting bacterial cell wall biosynthesis which has a lethal effect on bacteria. The ß-lactams family holds two main groups: penicillins and cephalosporins that have in common a four member cyclic amine (Fig. 4). The

basic structure of penicillin is a 6-aminopenicillanic acid while cephalosporins present a 7-aminocephalosporanic acid nucleus. In both cases the presence of the four membered ß-lactam ring, unstable and thermally labile, makes these compounds easily degraded by heat and in presence of alcohols as long as it is possible to occur isomerization of penicillins in acid conditions [19, 20, 22, 86, 87]. Tyczkowska et al [87] reported a study where the degradation of cloxacillin was analysed and it was observed that penicillins degraded during exposure to chemicals and solvents in sample preparation and that storage of stock solutions had the same result. The authors concluded that cloxacillin was rapidly degraded when stored in methanol or in methanol-water solutions probably due to reactions leading to methyl ester formation.
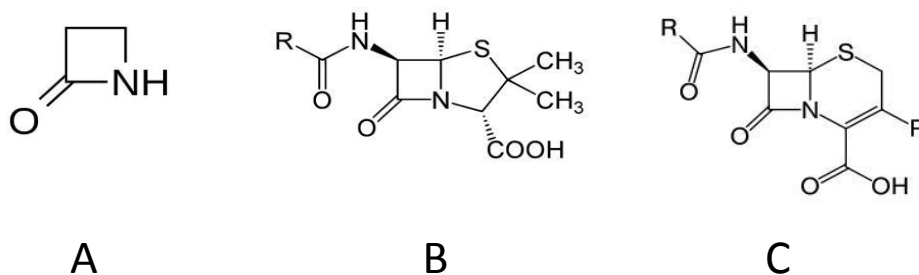
Figure 4: Structure of: β-lactam ring (A); basic structure of penicillin (B), and basic structure of cephalosporin (C)

More recently, de Baere et al [92] and also Freitas et al. [93], studied the degradation of amoxicillin in muscle and in solution, in different conditions of

temperature and pH. The use of LC-MS/MS offered the possibility to characterize amoxicillin's degradation products even when present at trace levels.

All the aspects concerning the stability of these antibiotics oblige several precautions, concerning temperature and pH during sample preparation. It is also important to refer that several authors have reported multi-residue methods for the determination of β-lactams in animal tissues or milk [86-91], but only some of these methods included cephalosporins [86, 90, 91].

One example of that is given by Martínez-Huelamo and colleagues [89], which optimized a simple and efficient clean-up extraction of penicillins in milk through LC-MS/MS. The recoveries were higher than 70%, except for amoxicillin. The method involved the addition of a phosphate buffer solution in order to provide a pH between 7 and 8 that was found to be the ideal for penicillin extraction. The resulting extracts were then subjected to SPE clean-up with a polymeric poly (divinylbenzene-co-N-vinylpyrrolidone) before being analysed by LC-MS/MS. In this process, a C_{18} column was used with a mobile phase consisting of acetonitrile and water acidified with formic acid. Multiple reaction monitoring (MRM) experiments in the positive ionization mode were performed for penicillin detection. After validation it was concluded that the method was suitable for routine analysis as the limits of quantification (LOQ) were found to be lower enough to determine residues in milk below the permissible MRL established [16].

For the determination of both penicillins and cephalosporins, Mastovska et al. [86] developed an analytical method for multi-residue analysis in bovine kidney using LC-MS/MS. In this case pH was not taken under consideration and sample preparation was performed with a simple liquid-phase extraction with water and acetonitrile followed by an extraction with C_{18} sorbent. The chromatographic conditions, similar to all β-lactams analyses [86-91], consisted in a C_{18} column working with water and acetonitrile, both containing formic acid. The authors referred the use of methanol instead of acetonitrile could lead to the degradation of some compounds and also reported that acetonitrile provided better sensitivity for the tested β-lactams.

A recent publication by Pérez-Burgos et al. [90] presented the advantages of cephalosporins analysis in muscle with new dispersive solid phase extraction procedures based on QuEChERS (quick, easy, cheap, effective, rugged and safe) methodologies in comparison with conventional SPE. These procedures are characterized by fewer and simpler steps for an effective clean-up in complex matrices such as biological tissues. A minimal amount of organic solvent and various salts or buffers are added to the organic phase for clean-up by dispersive solid-phase extraction. In this particular case a water:acetonitrile solution was added to the sample and after centrifugation a primary-secondary amine sorbent, C_{18} sorbent and $MgSO_4$ were introduced into the organic layer for clean-up

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resulting in a purified final extract to be analysed by LC-MS/MS. The method was validated [17] and the authors concluded that the use of this procedure provides lower solvent consumption and it is faster and more straightforward than conventional SPE.

QUINOLONES

The quinolones are a family of synthetic broad-spectrum antibiotics synthesized from 3-quinolonecarboxylic acid, used in the treatment of livestock and in aquaculture and characterized by an excellent activity against both Gram-positive and Gram-negative organisms. These drugs are widely used to treat respiratory, urinary and digestive bacterial infections. The representative chemical structure is shown in figure 5. The majority of quinolones are called fluoroquinolones possessing a fluorine atom attached to the central ring system, typically at the C-6 or C-7 positions.

The earlier methods used for the determination of these compounds involved ultraviolet detection (UV) [94-96, 109, 110] and, more frequently, fluorescence detection [95-108, 110] since most of the quinolones are fluorescent. One of the restrictions of such processes is the limited number of quinolones that can be

detected. Nowadays LC, coupled with MS detection, has become the preferred analytical tool for quantification and confirmatory analysis [109-115].
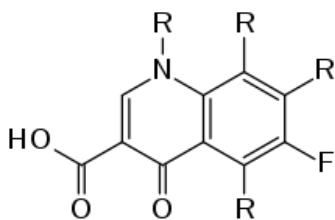
Figure 5: Structure of fluoroquinolone antibiotics, as a fluorine atom is present in the quinolone basic structure

Van Hoof et al. [112] developed and validated a LC–MS method with ion-trap for the simultaneous quantification of eight quinolones at the MRL level in matrices including bovine muscle, milk and aquaculture products. The compounds were extracted from the tissue using ultrapure water followed by SPE with a C_{18} cartridge. In the case of milk, an additional step for the precipitation of proteins was added with trichloroacetic acid, before SPE. Full scan MS^2 mass spectrum of each quinolone was obtained, giving the possibility of getting structural information. In all the obtained spectra the precursor ion was presented and the product ions were the result of the loss of water molecules (m/z=18) and COO^- (m/z=44). The method was fully validated according to the legislation [13] proving

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the ability to be used in routine analysis for the confirmation of eight quinolones in bovine muscle, shrimp and milk.

Bogialli et al. [116] presented a simple and rapid method to determine the residues of seven quinolones in eggs. Sample preparation consisted of matrix solid-phase dispersion technique with hot water acidified with formic acid acting as an extracting solvent, followed by liquid chromatography–tandem mass spectrometry. The chromatographic separation was achieved with a C_{18} column and mobile phase with acetonitrile and water acidified with formic acid. The MS data acquisition was performed in MRM mode. In conclusion, the results proved that hot water worked as an efficient extracting medium, since absolute recoveries of the analyte in egg at the level of 20 µg kg^{-1} were 89–103%. Also, the limits of quantification ranged between 0.2–0.6 µg kg^{-1} and, depending on the analyte, CCα ranged between 0.41 and 2.6 µg kg^{-1} while CCβ were 0.64–3.7 µg kg^{-1}. A similar work was previously described by the same authors, Bogialli et al. [113], successfully applied to bovine tissues.

Being milk one of the new target matrixes, in recent years publications about quinolones determination in this product have been increasing.

Herrera-Herrera et al. [108] described the use of 1-ethyl-3-methylimidazolium tetrafluoroborate (EMIm-BF$_4$) as mobile phase additive for the

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analysis with LC coupled to fluorescence detection of seven basic fluoroquinolone antibiotics in bovine, caprine and ovine milk samples. The additive provided a perfect chromatographic separation of the compounds in a C_{18} column. The sample extraction involved an acidic deproteination followed by a solid-phase extraction procedure. As for the applicability of the method it was demonstrated during the validation with recovery values ranging from 73% to 113% obtained for all three types of samples. Also, reproducibility was below 16% in all the cases. However the method was not fully validated according to the EU guideline 2002/657/EC [17] and was only applied for screening purposes. More recently, in 2011, the same authors [109], described a UHPLC-MS/MS method for the determination of 15 quinolones in powdered milk for children. The same sample preparation principle was followed: milk deproteination followed by a solid-phase extraction, achieving recoveries higher than 84% and reproducibility lower than 13% for all analytes. The main advantage of this method was the possibility of analysing a sample extract in less than 10 minutes for all compounds accomplishing limits of detection between 0.04 and 0.52 μg kg^{-1}.

Another method by Zhang and colleagues [115] based on UHPLC-MS/MS analysed a 22 quinolones in milk. The extraction of the analytes was achieved using McIlvaine buffer by ultrasonic bath and clean-up with SPE while detection was operated in positive mode with MRM acquisition. The chromatographic

separation was optimized to allow the best separation of all 22 compounds using a C$_{18}$ column and acidified mobile phase with formic acid, which was found to be the best solvent for enhanced ionization efficiency. Good recoveries and reproducibilities for 19 of the 22 quinolones were obtained and the LOQ were found to be low enough to determine these compounds at the MRL established [16] ranging between 0.008 and 0.339 µg kg^{-1}.

NITROFURANS

Nitrofurans are a group of highly effective antibiotic drugs with broad antimicrobial activity in the treatment of infections caused mainly by *Escherichia coli* and *Salmonella* sp. In the past this class was widely administered in the food animal production sector, especially in swine, poultry and fish husbandries, but due to the concerns related to the potential induction of carcinogenicity and mutagenicity, well described by McCalla [117] and Van Koten-Vermeulen [118] their use in livestock has been banned in the EU [119-121] and other countries. Nitrofurans are rapidly metabolized and distributed after administration which indicates that only their metabolites are likely to be detected as tissue-bound marker residues [122-125]. This rapid metabolism coupled to the fact that these drugs are prohibited, makes the development of analytical strategies more challenging.

Regarding the chemical structure this class of drugs is characterized by a nitro group in position 5 of the nitrofuran ring. Examples of the most used nitrofurans are furazolidone, furaltadone, nifursol, nitrofurantoin and nitrofurazone with the respective metabolites being AOZ (3-amino-2-oxazolidinone), AMOZ (3-amino-5-morpholino-methyl-1,3-oxa-zolidinone), DNSAH (3,5-dinitrosalicylic acid hydrazide), AHD (1-aminohydantoin) and SEM (semicarbazide) [128]. Figure 6 shows the structure of furazolidone and its metabolites as an example.

For the detection of nitrofuran tissue-bound side-chain metabolites, LC-MS/MS methods are now used throughout the world in animal tissues and other matrices. These methods are based on those described by Leitner et al. [126] and Connelly et al. [127] in which the nitrofuran metabolites as their nitrophenyl derivatives are detected. Although several methods are now published for different matrices it can be easily concluded that, in terms of sample preparation, the basis remains the same: homogenization, acid hydrolysis, derivatisation with *ortho*-nitrobenzaldehyde (*o*-NBA) and extraction with polar organic solvent (usually ethyl acetate).

Nitrofuran:	Marker metabolite:	Nitrophenyl derivative:
Furazolidone	AOZ	NPAOZ
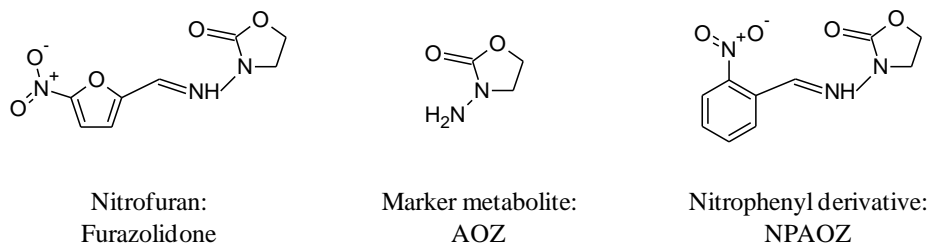
Figure 6: Structure of the nitrofuran furazolidone, its marker metabolite and the corresponding nitrophenyl derivative

With some improvements, and following the ban on nitrofurans, Verdon et al. [128] validated an analytical method with LC coupled to electrospray tandem mass spectrometry for the monitoring of 5 metabolites of nitrofurans in turkey muscle. The authors proved the applicability of the method since it is able to detect all metabolites above the established minimum required performance limit (MRPL) of 1µg kg^{-1} [71].

Lopez et al. [129] presented an analytical method for the determination and confirmation of nitrofuran metabolites in honey by LC-MS/MS. In this method a solid-phase clean-up of the sample, using a sorbent composed of a hydrophilic-lipophilic copolymer, was useful before hydrolysis and derivatization to obtain cleaner final extracts. Chromatographic separation was achieved with a C$_{18}$ column and mobile phase composed by an ammonium acetate solution and methanol. The protonated ions, [M + H]$^+$, for the derivatives were obtained and acquisition was performed by MRM transitions. The method was validated

proving that it can be used for regulatory purposes and to monitor the presence of nitrofuran side chains in honey. For the nitrofuran side-chain residues, the reproducibility was lower than 10% and the accuracies between 92 and 103%. Limits of quantification were found to be below 0.25 µg kg^{-1}, due to their good response in the LC-MS/MS analysis.

Previously, Tribalat et al. [130] published an article where the advantages of the use of LC-MS/MS compared to LC-MS were presented for the determination of nitrofurans in honey. In sample preparation a clean-up step was also added before derivatization, using SPE cartridge composed by hydrophilic-lipophilic copolymer. In terms of chromatography, a C$_{18}$ column was chosen with mobile phases composed of ammonium formate solution and methanol for LC-MS and acetonitrile for LC-MS/MS. LC-MS ionization was achieved with electrospray and compounds detected in positive mode (selective ion monitoring, SIM). One of the limitations found in this technique was that it does not lead just to the selection of ions characteristic to the derivatized metabolites. A large number of molecules contained in the sample that have the same mass of the derivatized metabolites also appear on the chromatograms, causing interferences.

As for LC-MS/MS the mass spectrometric detection was in positive mode using MRM. The selectivity achieved by this technique allowed the development of a faster and simpler chromatographic method without any risk of co-elution.

For that reason, only the last technique was validated to be used in routine analysis.

In 2010, Tsai and colleagues [131] validated a method based on the European Union regulations [17] to determine the presence of furazolidone, furaltadone, nitrofurazone, nitrofurantoin and their corresponding metabolites AOZ, AMOZ, SEM and AHD in fish muscle. Samples were acid-hydrolyzed, derivatized with 2-nitrobenzaldehyde, extracted with ethyl acetate and analysed by LC-MS/MS. Chromatography analysis was developed on a C_{18} reversed-phase column and the mobile phase was composed of a mixture of ammonium acetate and methanol. The method was fully validated and the critical concentrations were determined. The CCα were 2.93-5.01 µg kg^{-1} for nitrofurans and 0.19-0.43 µg kg^{-1} for metabolites while the CCβ ranged between 3.62-6.20 µg kg^{-1} for nitrofurans and 0.23-0.54 µg kg^{-1} for metabolites. Based on the calculated limits it was concluded that the method is suitable for the analysis of the four nitrofurans since the metabolites could be determined at concentrations below the MRPL set at 1 µg kg^{-1} by the EU [71].

The same method can also be applied to the determination of side-chain metabolites in eggs, as described by Block et al. [132], McCracken et al. [133] and Barbosa et al. [134]. However, in the case of eggs, it is also possible to find not just the metabolites but also the nitrofuran parent compounds. McCracken et al. [133]

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and Barbosa et al. [134] described the possibility of detecting nitrofurans in eggs as their parent drugs using a simpler procedure, as no hydrolysis and derivatization step are needed. The parent compounds are first extracted into ethyl acetate, fats are removed by partition between acetonitrile and hexane, and after concentration the sample is analysed by LC–MS/MS. The validation, in accordance with European guidelines [17], proved that the method is suitable for routine analysis fulfilling all the legislated criteria.

SULPHONAMIDES

Sulphonamides are synthetic bacteriostatic antibiotics structurally based on a *p*-aminobenzenesulphonamide functional group (figure 7). These broad-spectrum antimicrobials act by inhibiting the conversion of p-amino benzoic acid to dihydropteroate, which is fundamental for folic acid synthesis in both Gram-positive and negative bacteria, as well as some protozoa, such as coccidia. Sulphonamides have been widely used in animal feed as growth promoters as well as to prevent and treat a series of diseases such as infections on the digestive and respiratory tracts. Trimethoprim is a potentiator often administered together with sulphonamides and, in many cases, also detected through the same method [135-138].
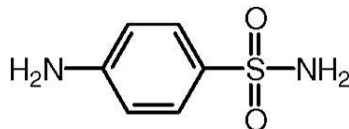
Figure 7: Structure of the p-aminobenzenesulphonamide functional group

For the detection of sulphonamides, GC-MS methods are considered to be an inappropriate option as this method requires derivatization due the high polarity and low volatility of these compounds. Several methods, based on HPLC with diode array detector [139-144] and fluorescence [145-148], have been reported for their analysis in different biological matrices.

Nowadays these methods are being replaced by MS/MS^n methods with the advantage of achieving more sensibility and specificity [135, 137-139, 149-157] as already discussed for other groups of antibiotics. The methods reported usually present detection through positive electrospray and acquisition with MRM mode. In all of them the protonated precursor ion is presented, [M+H]$^+$ and by fragmentation produces the following product ions: p–aminobenzene sulphonic acid [M−RNH$_2$]$^+$ (m/z=156), [M−RNH$_2$−SO]$^+$ (m/z=108), [M−RNH$_2$ −SO$_2$]$^+$ (m/z=92), and ions from the various amino substituent R-NH3 [MH−155]$^+$.

Nebot et al. [156] developed a LC-MS/MS method for the determination and quantification of nine sulphonamides in muscle samples, at concentrations below the established MRL. To minimize the matrix interferences the authors proposed

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a sample size reduction, which also allowed the reduction in the amount of solvents required and avoided the use of SPE cartridges for purification. These adjustments led to a rapid and easy extraction protocol with organic solvent. The chromatographic separation was assured with a C_{18} column with acetonitrile and a solution of ammonium acetate as mobile phase. The validation was carried out in accordance with EU Commission Decision 2002/657/EC [17] and for all the target compounds the recoveries obtained were above 88% which demonstrate the applicability of the new protocol for extraction.

In 2011, Won and colleagues [139] presented a method for sulphonamides determination in fish and shrimp using HPLC with photodiode array detector (PDA) for screening and UHPLC-MS/MS for confirmatory and unequivocal identification. Sample preparation involved a liquid-phase extraction with acetonitrile followed by a solid phase extraction adding to the organic layer a C_{18} powder. For HPLC-PDA screening, a C_{18} column was used for chromatographic separation along with a solution of potassium dihydrogen phosphate (pH 3.25) and methanol as mobile phases. For confirmation with UHPLC, water and acetonitrile both acidified with formic acid, were the chosen eluents. Authors concluded that a more reliable method for confirmation was needed as some false-positive results could be obtained with HPLC-PDA method.

The use of sulphonamides to protect honey bees against bacterial diseases is a frequent practice in beekeeping [159]. A recent method for the determination of these antibiotics was published by Economou et al. [135] that was able to identify and quantify seven sulphonamides, trimethoprim and dapsone in honey. As no MRL is established for sulphonamides and trimethoprim in honey the authors considered the target compounds as banned substances while developing the method and the requirements of higher selectivity and sensitivity were the main purpose. The analysis, by LC-MS/MS, was performed with positive electrospray and acquisition in MRM mode. The chromatographic separation was fulfilled on a C_{18} column in combination with two mobile phases: water and acetonitrile both containing 0.1% (*v/v*) of formic acid. In terms of sample extraction it is important to consider that sulphonamides can bind to honey sugars and an acidic hydrolysis is needed to liberate the target compounds [135, 147, 148, 160]. After the hydrolysis step, the authors used a SPE clean-up with a cartridge composed by a hydrophilic-lipophilic copolymer. The method was successfully validated, according European guides [17], achieving recoveries ranging from 70% to 106% and reproducibility from 6 to 18 %. CCα and CCβ values were also calculated ranging between 0.4-0.9 and 0.7-1.4 µg kg^{-1}, respectively.

TETRACYCLINES

Tetracyclines are a group of antibiotics widely used in veterinary medicine being active against a broad range of Gram-positive and Gram-negative organisms by inhibiting protein biosynthesis through binding to the 30S ribosome. Tetracyclines are used for the treatment of gastrointestinal, respiratory and skin bacterial infections [161,162] and present a common structure with four six member rings where five radical chains can be modified, as seen in figure 8.
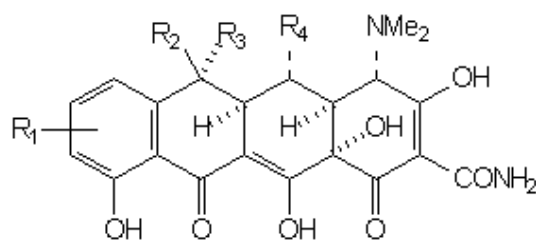
Figure 8: Basic tetracycline structure

These compounds are relatively stable in acids but not in bases, and can be photodegraded forming reversible epimers. It is also important to observe that tetracyclines are strong chelating agents being chelation of a divalent metal ion essential for their antimicrobial activity. All these factors, well described by Anderson et al. [165] and Kinsella et al. [23], are responsible for the difficulties found when extracting tetracyclines from biological tissues leading to low and variable recoveries. To overcome these problems sample extraction, in almost of the

reported cases, leads to a primary step of aqueous extraction with solution containing EDTA (ethylenediamine tetraacetic acid), since tetracyclines show low affinity to organic solvents. The use of EDTA, as a chelating agent, minimizes the interaction of tetracyclines with chelating complexes. Also, the aqueous solution used for the first step is normally mildly acidic in order to promote deproteinization and minimize the presence of cations normally binded to proteins.

Although a wide range of analytical methods have been developed for the determination of tetracyclines in products of animal origin, such as immunoassays and capillary electrophoretic, LC methods in the recent years have been the most applied. Önal et al. [163] described in detail some of the recently advances in the LC methods. As referred by the authors, LC-MS/MS methods were developed to improve sensitivity and accuracy in tetracyclines quantification obtained by UV and fluorescence methods, which nevertheless, are still used [163-168]. Several LC-MS/MS methods are currently available in the literature [20-22, 163, 171-177] having the main advantage of providing the possibility of detection not just for tetracyclines but also for their epimers. In all of them it is observed that the full scan mass spectrum shows the $[M+H]^+$ ions, as precursor ions, and the most intense products obtained after their fragmentation correspond to $[M+H-H_2O]^+$, $[M+H-NH_3]^+$ and $[M+H-H_2O-NH_3]^+$.

De Ruyck and co-authors [173] developed a LC-MS/MS method for the determination of 4 tetracyclines and respective epimers in milk. As no MRL is available for doxycycline in milk the principal goal was to develop a sensitive analytical method with acceptable extraction recoveries. A liquid extraction with an aqueous solution with trichloroacetic to precipitate proteins was performed. The extract was then applied to SPE cartridge, a hydrophobic-lipophilic-copolymer for clean-up and the extract analysed by LC-MS/MS. The LC separation performed in a C_{18} column was achieved with a mobile phase consisting of water and a solution of methanol and acetonitrile with formic acid while detection was achieved in positive electrospray mode and MRM acquisition. The validation proved the applicability of the method with recoveries ranging from 90.4% to 101.2%. Also, it was possible to detect compounds in concentrations between 5 and 20 $\mu g.L^{-1}$. The authors had also developed some stability tests and concluded that, in order to minimize the degradation of tetracyclines in their own epimers, the samples should be immediately injected into the LC-MS/MS system once prepared.

Blasco et al. [174] optimized and validated a LC-MS/MS method capable of quantifying four tetracyclines used in veterinary medicine, as well as their epimers in muscle tissue of different species. The presented work consisted on an optimization of a previous one, [172], adding the use of hot water as an extractant

solvent to provide clean sample extracts in a pressurized liquid extraction. Other improvement is the employment of hydrophilic-lipophilic copolymer cartridges as SPE instead of the normal C_{18} reported first. For the chromatographic separation of tetracyclines from their respective epimers a C_{18} column was used and the mobile phase gradient was optimized with water and methanol both acidified with formic acid. The method was fully validated [17] showing average recoveries for all compounds, for the different species and at three levels of concentration (1, 100 and 200 µg kg^{-1}) always higher than 89% and precision lower than 17%. The limit of quantification was also calculated, ranging from 0.5 to 1 µg kg^{-1} while the CCα and CCβ ranged between 101–116 and 112–130 µg kg^{-1}, respectively.

MACROLIDES

Macrolides are an important group of antibiotics, active against aerobic and anaerobic Gram-positive bacteria, used in veterinary medicine mainly to treat respiratory tract infections but also as growth promoter purposes. Their action is bacteriostatic through binding to the 50S subunit of the ribosome and inhibiting bacterial protein synthesis. The structures of all macrolides are based on a macrocyclic lactone ring to which sugars, including amino and deoxy sugars, are

attached. The therapeutically most relevant macrolides comprises 12-, 14-, 15- or 16-membered ring. Some examples of macrolides are presented in figure 9.
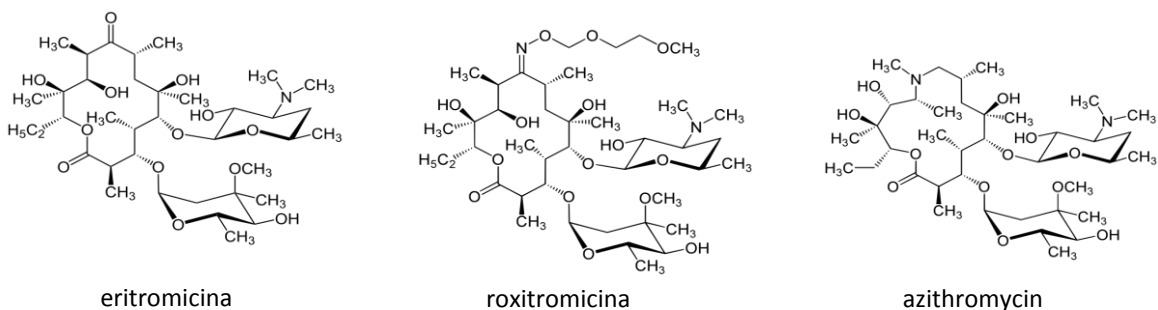
Figure 9: Molecular structure of some selected macrolides

These molecules, as weak bases, are lipophilic, soluble in methanol and unstable in acids [62]. Another important fact is that their molecular structure contains chromophores, which makes possible their analysis by UV [62, 178, 179] and fluorometric detection [62, 180]. Recently, with improved sensitivity and specificity, mass spectrometry has replaced UV and fluorometric methods in detection and quantification of macrolides in different biological matrices [19, 20, 62, 181-190].

Wang [182] presented an overview on the biological properties of macrolides and their analytical strategies, including extraction and liquid chromatography methods. The most commonly used techniques to extract

macrolides from biological matrices include SPE, usually with a C_{18} or a hidrophylic-lipophilic copolymer cartridge, and a liquid–liquid extraction with organic solvent or appropriated aqueous buffer. In terms of detection, triple quadrupole in MRM mode is the most used analytical tool. Recent developments in chromatography science have shown that UHPLC coupled with a mass spectrometer or time-of-flight mass spectrometer are emerging techniques for unequivocal identification and quantification of macrolides [62, 182].

However, Berrada et al [189], published a study where results obtained with an LC-PDA are compared with those obtained in a LC-MS/MS system and, despite the higher detection capability of the mass detector, the authors concluded that the LC-PDA is a good alternative achieving a low-cost procedure and also a sensitive technique for the determination of macrolides in liver at residual concentrations. The extraction procedure presented has two steps: liquid extraction with EDTA–McIlvaine's buffer and SPE clean-up with a hydrophilic-lipophilic copolymer cartridge.

On the other hand, Bogialli et al. [181], used the advantage of sensibility of an LC-MS/MS to develop a simple and rapid assay to determine three macrolides, erythromycin A, tylosin and tilmicosin, in eggs. The method was based on a single extraction step with acetonitrile without any further clean-up or purification. In terms of accuracy no matrix effects or ion stability were observed that could

compromise the detection and quantification of the target compounds. A C_{18} column was used for chromatographic separation with acetonitrile and water acidified with formic acid as mobile phase. The method was validated fulfilling all regulated criteria [17].

Wang et al. [182] developed two liquid chromatography mass spectrometric techniques, UHPLC-ToF MS, and LC-MS/MS, for identification, quantification and confirmation of six macrolide antibiotic residues in eggs, raw milk, and honey. Authors concluded that, although LC-MS/MS had advantages in terms of lower limits of detection and better repeatability, UHPLC-ToF provided unequivocal confirmation and also allowed the identification of the possible degradation product. An example is given for tylosin that can be a mixture of tylosin A, as a major component, and tylosin B or desmycosin, tylosin C or macrocin, and tylosin D or relomycin. It is also suggested that the combination of the two techniques could be very beneficial or complementary in routine analysis of macrolide antibiotic residues when the identification of all the related products is required.

MULTI-DETECTION MULTI-CLASS METHODS

Multi-class and multi-detection methods are a challenge that is coming more and more embraced. In recent years it has been observed an increasing

interest in improving cost-effectiveness of analytical procedures [23, 30, 38, 54, 191-194].

One way of doing this is developing reliable screening methods, able to detect, at the same time and in a single run, the maximum number of analytes as possible. Although microbiological and bioassay techniques are still used for screening purposes, mainly because of their low cost and simplicity, more specific confirmatory methods are needed for unequivocal identification and for quantification. As a result and since a few years ago, the major concern was focused on the development of sensitive and specific confirmatory methods and few attentions were given to screening. Nowadays there is a growing concern on having efficient screening technologies that guarantee the absence of false-positives and false-negative results. This efficiency is encountered in the multi-detection methods based on liquid chromatography technology coupled with tandem mass spectrometry (MS/MS) and time of flight mass spectrometry (ToF). Also the use of UHPLC gives the possibility of having short running times together with higher resolution and sensitivity. The most reported methods are multi-detection of related compounds, usually from the same family of antibiotics as described above for each class. Recently, some developments showed the increase of publications reporting analytical strategies to analyse, with the same method, unrelated compounds in multi-class procedures [20-23, 26-42, 45, 58, 61, 194-201].

Problems are still encountered in this sort of methods, mainly due to the difficulty in extraction and pre-treatment of samples, when analysing simultaneously different groups of compounds with different physic-chemical properties. Although some of the published works seemed to succeed on that task, many of the methods developed presented several steps in sample extraction for the different compounds depending on their properties [34, 42, 196, 202, 203]. When this approach is used more than one LC run has to be performed. In some cases, even when just one extract is obtained, depending on the detector used and on the chromatographic conditions for retention and separation of analytes, more than one injection is needed. For mass detection some compounds are ionised in positive mode and others in negative and for some detectors that means two separate analyses. Also, chromatographic optimization can lead to differences, even slightly, in mobile phase and in the gradient depending on the compounds [202].

The next paragraphs describe some of the methods recently developed for multi-detection multi-class methods in food producing animal matrixes, namely honey, milk, eggs and muscle.

Honey is one of the target matrixes in the field of residues analysis mainly due to its popularity among consumers. The use of antibiotics in apiculture to prevent American Foulbrood diseases is known for a long time and both the

allowed and the banned compounds have to be controlled. Most of the methods developed are multi-residues analysis for compounds from the same group as it was already presented for each of the antibiotics families.

A multi-screening approach for monitoring antibiotics in honey by LC-MS/MS has been developed by Hammel et al. [42], in which a total of 37 from the 42 initially intended veterinary drugs (5 tetracyclines, 7 macrolides, 3 aminoglycosides, 8 β-lactams, 2 amphenicols and 17 sulphonamides) were confirmed and quantified at a concentration level of 20 µg kg^{-1}. Appreciable performance was obtained for all analytes, except for five compounds (amoxicillin, ampicillin, desmycosin, penethamate and sulphanilamide). The sample preparation included four subsequent liquid/liquid extraction steps and in each one different compounds were extracted. It was decided not to optimize any solid phase extraction in order to have better recoveries at the end. The final extracts were analysed by LC-MS/MS in positive ionization with a TurboIonSpray source and acquisition in MRM mode. Chromatographic separation was fulfilled using a C$_{18}$ column and mobile phases constituted of water containing 1mM NFPA (nonafluoropentanoic acid) mixed with 0.5% formic acid (v/v) and a mixture of acetonitrile/methanol (50/50, v/v) containing 0.5% formic acid (v/v). NFPA was used as an ion pairing agent to improve aminoglycosides retention in the column and only used in one of the mobile phase in order to minimize possible

suppression effects. Also, the gradient used was optimized to achieve separation of isomers (in this case tetracyclines and sulphonamides). The method was validated in accordance with EU directive 2002/657/EC [17] and, for screening purposes, was determined the limit of compliance that ranged from 23.4 µg kg^{-1} (dihydrostreptomycin) and 31.4 µg kg^{-1} (tilmicosin).

Furthermore, Lopez et al. [33], developed and validated a multi-class method for the determination of 17 antimicrobials by LC-MS/MS in honey. The compounds analysed were tetracyclines, fluoroquinolones, macrolides, lincosamides, aminoglycosides, sulphonamides, phenicols and fumagillin residues with chromatographic separations performed in a C$_{18}$ column. For the determination of streptomycin an aliquot of honey diluted in water was centrifuged, filtered and analysed. The remaining supernatant was used for solid phase extraction with a polymeric sorbent able to retain both polar and non-polar compounds after filtration with a fine-mesh nylon fabric. At the end of sample treatment, three LC analyses could be performed: streptomycin extract, in positive mode; chloramphenicol, in negative mode and again in positive mode all the remaining compounds. One of the critical steps pointed by the authors concerned the fact that some antibiotics were sensitive to light. It was recommended that, once the samples were prepared must be immediately analysed, especially in the case of streptomycin extract that showed to be the

most unstable. After validation it was concluded that erythromycin could only be detected and confirmed but not quantified because of its low recovery. Repeatability was calculated and observed to be lower than 17% for almost all compounds. Recoveries ranged from 65 to 104%.

A special emphasis is given to aminoglycosides detection in the multi-class method developed by Martos et al. [196]. Due to the high affinity of these compounds with polar surfaces such as glass, special attention had to be taken during all the procedure. The described method consisted on a semi quantitative screening of 39 compounds, in muscle, including amphenicols, beta-lactams, macrolides, sulphonamides, tetracyclines and quinolones. Some extra steps, with water as a polar solvent, had to be taken in order to guarantee aminoglycosides extraction. Also, the use of pure organic solvents was avoided, in opposition to water and buffer solvents, to improve recovery of beta-lactams. At the end the final extract was injected three times in the LC-MS/MS. In positive and in negative ionization the chromatographic separation was performed in a C_{18} column. For aminoglycosides a HILIC column had to be used. The accuracy ranged from 45%, for neomycin to 106%, for sulphanilamide.

A completely validated method published in 2012 by Lopes and colleagues [40] with application in aquaculture fish samples, describes the advantages of using a UHPLC-MS/MS in combination with the sample extraction procedure QuEChERS

for the determination of 32 compounds belonging to several families (macrolides, penicillins, quinolones, sulphonamides, tetracyclines and anti-helminthes). Using UHPLC a reduction time in sample analysis and in the chromatographic separation could be achieved. The chromatography was enhanced to fulfil the resolution and optimal peak shape using 0.1% formic acid and acetonitrile as mobile phases. For mass spectrometry, a tandem quadrupole mass spectrometer with positive electrospray ionization (ESI+) and operating in MRM mode was used. QuEChERS has the main advantage of allowing a simple inexpensive extraction in few steps. With the aim of improving the recovery of tetracyclines and quinolones, the QuEChERS procedure was slightly changed by adding methanol to the extraction solution. However, the greater the amount of methanol added, the dirtier was the final extract, resulting from solvent matrix components. In the end, and in order to have satisfactory recoveries for all the compounds the authors selected a combination of acetonitrile:methanol (75:25, v/v). After validation the recoveries obtained ranged from 69% to 125%, and the expanded uncertainty evaluated at 100 µg kg^{-1}, was below 25% for all compounds with the exception of tetracycline (28%). For the inter-day precision the worse value was 30% for thiabendazole and marbofloxacin. Also, the LOD and LOQ were determined and obtained as maximum levels 7.5 µg kg^{-1} and 25 µg kg^{-1} respectively, except for danofloxacin, oxytetracycline and tetracycline. These compounds presented LOD of 15 µg kg^{-1} and LOQ of 50 µg kg^{-1}. The CCα and CCβ values were calculated according to the

corresponding MRLs. In conclusion, the authors claimed that 15 samples could be prepared in less than 1 hour and analysed in 2.5 hours.

Recently and developed by the some authors [206] a similar multi-detection method targeting chicken muscle and covering 20 analytes from quinolones, sulphonamides, macrolides, anthelmintics, avermectins and also benzathine. This last compound is used to stabilize penicillins and was included to monitor their presence. The approach also used the same UHPLC-MS/MS equipment (also the same chromatographic and detection conditions) and QuEChERS for sample treatment. Having in mind that QuEChERS were first developed for pesticides analysis, the procedure had to be improved in order to extract all the target compounds from the sample. Originally the extraction solvent had a higher content of water. Authors concluded that the best recoveries were achieved when performing extraction with a mixture of acetonitrile:water (80:20, v/v) acidified with acetic acid. The method was fully validated according to the legislation [17]. In terms of recovery only benzathine with 65.6% at 20 µg kg^{-1} and sulfadimidine, with 69% at 100 µg kg^{-1} presented the worst values. The stability of the method developed was proven with a precision study. For repeatability the maximum value obtained was for sulphachlorpyridazine (RSD=22.1% at 20 µg kg^{-1}). The higher value determined for reproducibility was for benzathine (RSD=37.8% at 20 µg kg^{-1}) but all the other compounds presented values below 28%. LOD and LOQ

were determined with tylosin presenting the higher limits: 9.0 µg kg^{-1} and 30.0 µg kg^{-1} respectively. Critical concentrations, CCα and CCβ, were calculated according to EU commission decision [17], taking into account the MRLs, when established.

The two methods [40, 206] described have the advantage of being used not only for screening, but also for confirmatory and quantification purposes. The detection was performed using two MRM transitions for each compound respecting the required points of identification for confirmatory methods demanded in the legislation.

One of the most consumed foodstuffs from animal production is milk. Antibiotic residues are the most frequent inhibitory substances found in this matrix having undesirable effects on its quality. Their residues, along with adverse effects on consumer's health, can slow or destroy the growth of the fermentation bacteria responsible for milk transformation in fermented products.

The main problem concerning milk extraction for antibiotics determination is its high content of protein and fat. Traditionally, in this matrix, antibiotics extraction involved a step of precipitation of proteins [32, 34-39, 173] with organic solvent and a strong acid, such as trichloroacetic acid which can result in low recovery for unstable compounds. To overcome this difficulty Aguilera-Luíz et al. [36], developed a multi-detection method involving a single extraction technique

based on QuEChERS procedure combined with UHPLC-MS/MS analysis. The method was developed to determine 18 selected veterinary drugs (sulphonamides, macrolides, quinolones, anthelmintics and tetracyclines). Chromatographic separation was carried out with a C_{18} column with mobile phase consisting of methanol (eluent A) and 0.01% of formic acid in water (eluent B). The gradient profile was optimized in order to have fast, reliable separation and good shape peaks making possible the determination of all analytes in less than 10 minutes. The applicability of the method was studied by validation. In terms of recovery it ranged between 70.6% (ivermectin) and 111.3% (erythromicyn). It was observed that repeatability and reproducibility were lower than 20.4% (albendazole). LOD and LOQ were determined resulting values from 1 to 4 µg kg^{-1} and 3 to 10 µg kg^{-1} respectively, which were lower than the MRL established [16].

A different approach in terms of detection is presented by Stolker et al. [38] for screening veterinary drugs in milk. UHPLC combined with ToF-MS has been used for screening and quantification of more than 100 veterinary drugs in which some groups of antibiotics were included (macrolides, penicillins, quinolones, sulphonamides, tetracyclines and amphenicols). A simple sample treatment starts with protein precipitation and follows to a polymer-based C_{18} solid phase extraction. The method was validated according to EU guidelines for quantitative screening [17]. At the MRL levels the repeatability obtained ranged up to 20% for

86% of the compounds analysed. For reproducibility the obtained value was lower than 40% for 96% of the compounds. The accuracy ranged from 80 to 120% for 88% of the compounds. As the confirmation of identity of veterinary drugs by ToF-MS is not yet included in EU guidelines it is mandatory to have a MS/MS confirmation method to process the suspected samples.

Another publication by Ortelli and colleagues [35] presented the use of UHPLC coupled to orthogonal acceleration ToF mass spectrometry for the screening of 150 veterinary drugs residues in milk (antibiotics from beta-lactams, macrolides, quinolones, sulphonamides, tetracyclines among other veterinary drugs). The sample preparation was based on protein precipitation associated with ultra-filtration and appeared to be easy and fast. To control possible contamination problems, the authors advise the use of disposable material during all the process. The simplification of sample preparation was possible due the possibility of using UHPLC-ToF, described as a very high performance tool. The identification of contaminants is based on accurate mass measurement. The validation procedure was conducted based on EU directive 2002/657/EC [17]. In some cases it was observed that the obtained LOD was higher than the MRL (case of amoxicillin, betamethasone and dexamethasone). Also, in the case of amoxicillin, it was not possible to determine CCα and CCβ. The recovery and precision studies indicated possible matrix effects inducing suppression and

enhancement of signal. In the specific case of quinolones recoveries were up to 807% (danofloxacin) confirming the presence of interferences due to the simple sample preparation.

To improve recovery for as many compounds as possible, Gaugain-Juhel el al. [47] presented two separated and simple milk extractions followed by two LC-MS/MS acquisitions to allow the screening of 58 antibiotics belonging to beta-lactams, sulphonamides, macrolides, lincosamides, aminoglycosides, tetracyclines and quinolones. The first extraction route for beta-lactams, macrolides and sulphonamides consisted in a liquid extraction with acetonitrile, centrifugation and evaporation after which the extract was redissolved with ammonium acetate. The second route extracted tetracyclines, quinolones, aminoglycosides and lincomycin with a trichloroacetic solution that was, after centrifugation, directly analysed by LC-MS/MS. Chromatographic separation was reached with a C_{18} column and two different gradients mixing 0.1% of pentafluoropropionic acid and acetonitrile to achieve the better shape and separation peak. The detector, a triple quadrupole was operated in positive ESI and in MRM conditions. Validation values of CCβ showed to be below or equal to MRL, except for amoxicillin, fulfilling the demanded requirements for a suitable screening method for all other compounds.

Also covering a wide range of antibiotic families, Bohm et al [37] developed a single extraction procedure for milk analysis. The method is based on the protein

precipitation with trichloroacetic acid, a liquid extraction with McIlvaine buffer followed by solid phase extraction with hybrid polymeric columns and analysis by LC-MS/MS of 47 compounds (tetracyclines, quinolones, macrolides, sulphonamides, diamino-pyrimidine derivatives and lincosamides). Chromatographic separation was achieved with a C_{18} column and mobile phases composed by 0.2% formic acid and acetonitrile. The detection, with triple quadrupole, was performed with an electrospray ion source working in positive mode and in MRM conditions. The applicability of the method was proven by the validation study according to the EU commission decision 2002/657/EC [17] and with successful participation in proficiency tests. The recovery of the method ranged from 94% to 109% for all compounds and precision, in terms of repeatability and reproducibility was below 22% also for all compounds.

Methods for the analysis of residues from several classes in eggs are still very few compared to the other target matrices. Heller et al. [28]; Frenich et al. [29]; Peters et al.[30]; Jiménez et al. [31] are some of authors that published the most relevant of them. The high lipid and protein content turns the eggs in a more complex matrix and consequently the extraction procedure becomes more difficult. Acetonitrile is the solvent of choice because it can precipitate proteins and also denatures enzymes that can depredate the analytes of interest during sample treatment. In spite of that problem this matrix is very important due to its

high consumption with eggs being present in all diets and also represents an inexpensive and very nutritious food item.

Peters et al. [30] combined a sensitive full mass scan MS technique, ToF-MS, with a high resolution LC, UHPLC, for the determination of 100 veterinary drugs in three matrices: meat, fish and eggs. In the compounds analysed are included: macrolides, penicillins, quinolones, sulphonamides and tetracyclines. The chromatographic separation optimized uses a C_{18} column and a gradient mixing 0.1% of formic acid and acetonitrile and the detection achieved in a ToF-MS equipped with an orthogonal ESI source operating in positive mode. The sample preparation starts with protein precipitation adding to the sample a mixture of acetonitrile:water (6:4, v/v) followed by SPE with polymer based C_{18} columns. The procedure was adapted from the previous described method developed for milk by Stolker et al. [38]. Although the method could be directly used for meat and fish, in the case of eggs the recovery for a high number of analytes was very poor. The use of a stronger eluent in the SPE clean-up is needed (methanol:ethyl acetate, 1:1, v/v) probably due to protein binding of analytes to egg protein in SPE column. Proteins in eggs are smaller and more soluble in water than the proteins in meat and fish making the step of precipitation with acetonitrile less efficient and a higher protein concentration loading in SPE column, increasing interferences. The results obtained after validation are satisfactory to 70-90% of the analysed

compounds. The repeatability of the method, ranging from 8% to 15%, presented better values at higher concentrations and in meat samples. The reproducibility ranged from 15% to 20% showing insignificant differences between concentrations and matrices. Accuracy calculations indicated in some cases the presence of matrixes interferences since some values are higher than 100%. The authors concluded that the method performance is clearly influenced by the matrix as the performance criteria are fulfilled for >90% of the compounds in meat, >80% in fish and >70% in egg.

Jiménez et al. [31] developed a multi-class method for the determination of several families of veterinary drugs in eggs. A total of 41 antimicrobials (sulphonamides, diaminopyridine derivates, quinolones, tetracyclines, macrolides, penicillins and lincosamides) were extracted adding a solution of EDTA and proceeding with a pressurized liquid extraction (PLE) with a mixture of acetonitrile: succinic acid buffer pH=6 (1:1, v/v). No further clean-up was necessary. The addition of EDTA has the main goal of improving tetracyclines recovery competing with them for the formation of metal complexes. The final extracts were analysed by LC-MS/MS using a C_{18} column for chromatographic separation and elution solvents composed by 0.02% of formic acid with $1mmol^{-1}$ of succinic acid and acetonitrile. The electrospray ionization source was operated in the positive mode and the acquisition was performed in MRM mode. The

recovery of the method ranged from 60% to 118%. Satisfactory results were obtained in terms of precision and the calculation of CCα and CCβ confirmed that this method can be used for screening and confirmation purposes.

The interest in developing multi-detection methods for determination of veterinary drugs in feed is also increasing [26, 204, 205].

Cháfer-Pericás et al. [26], presented a multi-detection method for the determination of tetracyclines and sulphonamides in fish and feed. The determination, as screening, is performed applying immune-analytical assays after which a confirmatory LC-MS/MS is used. For feed samples treatment a metabolic solution containing EDTA was used and this procedure was the same for both screening and confirmatory methods which turned all the process rapid and simple.

Borràs et al. [204] recently reported a multi-class method for the determination of 55 compounds belonging to 14 families, not just antibiotics, in animal feed. In this method the identification and quantification is performed with UHPLC-MS/MS and also a simple sample preparation was developed. Samples were extracted with a mixture of methanol, acetonitrile and McIlvaine buffer and diluted before injection in the UHPLC system. Authors concluded that SPE clean-

up did not contribute to reduce any interference from the matrix and good recoveries were achieved with a simpler preparation.

Table 6 presented below summarizes some of the most important and recent multi-detection and multi-class methods available in the literature for the determination of antibiotics in animal food producing.

Table 6: Multi-class methods for antibiotic residue analysis in edible tissues

Class of antibiotic analysed	Matrix	Analytical technique	Limits of performance (µg kg^{-1})	Reference
4 Tetracyclines; 4 Quinolones; 4 Macrolides; 3 β-lactams; 4 Sulphonamides	muscle kidney	LC-MS/MS	LOD between: 2.0 – 15.0	[195]
4 Tetracyclines; 5 Quinolones; 1 Macrolide; 1 Lincosamid; 1 Aminoglycoside; 1 Sulphonamide; 1 Amphenicol	honey	LC-MS/MS	--	[33]
3 Tetracyclines;11 Quinolones; 10 Macrolides ; 6 β-lactams; 6 Aminoglycosides ; 15 Sulphonamides; 3 Amphenicols	milk	UHPLC-ToF-MS	CCβ according MRL	[38]
6 Tetracyclines; 14 Quinolones; 9 Macrolides ; 12 β-lactams; 23 Sulphonamides	muscle liver kidney	UHPLC-ToF-MS	LOD < 5.0 CCα according MRL	[207]
4 Tetracyclines; 9 Quinolones; 4 Macrolides ; 7 β-lactams; 14 Sulphonamides	muscle	UHPLC-MS/MS	CCα and CCβ according MRL	[198]
1 Tetracycline; 3 Quinolones; 4 Macrolides; 2 Sulphonamides	milk	UHPLC-MS/MS	LOD between: 1.0 – 4.0	[36]
6 Tetracyclines; 14 Quinolones; 10 Macrolides; 23 β-lactams; 24 Sulphonamides	milk	UHPLC-ToF-MS	LOD between: 0.5 – 25.0	[35]
3 Tetracyclines; 10 Quinolones; 10 Macrolides; 15 β-lactams; 8 Aminoglycosides ; 9 Sulphonamides	milk	LC-MS/MS	LOD < 3.5	[34]
7 Tetracyclines; 14 Quinolones; 10 Macrolides; 12 Sulphonamides	milk	LC-MS/MS	CCα and CCβ according MRL	[37]

Class of antibiotic analysed	Matrix	Analytical technique	Limits of performance (µg kg^{-1})	Reference
12 Quinolones; 16 Sulphonamides	muscle	LC-MS/MS	CCα according MRL	[199]
4 Tetracyclines; 10 Macrolides; 7 β-lactams; 11 Quinolones; 15 Sulphonamides; 2 Amphenicols	eggs fish muscle	UHPLC-ToF-MS	CCβ according MRL	[30]
3 Tetracyclines; 4 Quinolones; 9 Macrolides; 4 β-lactams; 6 Aminoglycosides ; 8 Sulphonamides ; 2 Amphenicols	muscle	LC-MS/MS	LOD ranging from 1.0 – 41.0	[196]
8 β-lactams; 11 Quinolones	milk	LC-MS/MS UHPLC-MS/MS	CCα and CCβ according MRL	[201]
4 Tetracyclines; 5 Macrolides; 7 Penicillins; 8 Quinolones; 12 Sulphonamides	eggs	LC-MS/MS	CCα ranging from 0.5 – 3.8	[31]
4 Tetracyclines; 6 Quinolones; 2 Macrolides; 5 B-lactams; 6 Sulphonamides	fish	UHPLC-MS/MS	LOD < 15.0 LOQ < 50.0	[40]
2 Quinolones; 3 Macrolides; 6 Sulphonamides; Benzathine (biomarker of penicillin)	muscle	UHPLC-MS/MS	LOD ranging from: 3.0 – 6.0	[206]

CONCLUSION

It is possible to resume some conclusions regarding the state-of-the-art in the field of residues analyses, specifically in the case of antibiotics in food producing animals. The increasingly use of liquid chromatography, either HPLC or UHPLC, combined with tandem mass spectrometry detection is the preferred technique in the field of veterinary residues analysis in complex biological samples.

Due to the demanded EU criteria [17] for quantitative and confirmatory determinations, triple quadrupole is still the tool of choice, even in the cases of multi-detection analysis, mainly because of the possibility of unequivocal identification of trace concentrations in complex matrixes such as biological samples.

As for multi-detection and multi-class screening, UHPLC–ToF is becoming one of the techniques of choice since the combination of these two instruments provides enough sensitivity and selectivity for detection of a wide range of compounds. One of the advantages when comparing triple quadrupole instruments is the possibility of extraction of any high-resolution MS traces even after acquiring data.

Regarding sample pre-treatment, the combination of liquid extraction with organic solvent or buffer solution is used. However liquid-solid extraction is the most popular approach and solid phase extraction, mainly with C_{18} or hydrophilic-lipophilic copolymer cartridges are the selected strategies for the purpose. Frequent problems are found in the area while simultaneously extracting and pre-treating groups of analytes with different physic-chemical properties. It is rather complicated to achieve equally

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good recoveries in all compounds especially in multi-detection and multi-class methods and minimizing the loss of all analytes during sample preparation while providing enough sensibility at the target concentration. Same compounds can establish strong bounds with matrices components. The high concentration of proteins in the sample can also be a problem. Some procedures that include protein precipitation can lead to analyte losses as they can be adsorbed. During sample homogenization enzymes can be released by the cells and promote degradation of same unstable compounds. Problems in chemical stability are also an important issue to take in account. Some compounds, as β-lactams and macrolides, are easily degraded under certain conditions of temperature and pH.

Another frequent problem mentioned is related with ion suppression or enhance of signal owing to interferences coming from the matrices. The legislation, that in some cases obliges the detection at trace levels, is an additional obstacle that makes the veterinary residues analysis a challenge.

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CHAPTER II – MULTI-DETECTION AND MULTI-CLASS METHODS FOR THE

DETERMINATION OF ANTIBIOTICS IN FOOD PRODUCTS OF ANIMAL ORIGIN

DEVELOPMENT AND VALIDATION OF A MULTI-RESIDUE AND MULTI-CLASS ULTRA-HIGH-PRESSURE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY SCREENING OF ANTIBIOTICS IN MILK

Abstract

A multi-residue screening method for 33 antibiotics from five different families was employed to simultaneously determine sulphonamide, tetracycline, macrolide, quinolone and chloramphenicol antibiotics using ultra high pressure liquid chromatography tandem mass spectrometry. A simple sample preparation method was developed using protein precipitation, centrifugation and solid phase extraction and was optimized to achieve the best recovery for all compounds. The methodology was validated for quantitative screening methods, by evaluating the detection capability (CCβ), specificity, selectivity, precision, applicability and ruggedness. Precision, in terms of relative standard deviation, was under 21% for all compounds. Because CCβ was determined for screening purposes and, according to maximum residue limit, the limit of detection of the method was also calculated and ranged from 0.010 $\mu g\ kg^{-1}$ to 3.7 $\mu g\ kg^{-1}$. This validation provided evidence that the method is suitable to be applied in routine analysis for the detection of antibiotics in bovine, caprine and ovine milk.

Introduction

Antibiotics in dairy cattle are mainly used to treat mastitis, diarrhoea and pulmonary diseases [1]. These treatments can result in the presence of antibiotic residues in milk. For the consumers, the presence of such residues can be responsible for toxic effects, allergic reactions in individuals with hypersensitivity and can result in the development of resistant strains of bacteria [2-5]. The presence of antibiotic residues

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can also be responsible for undesirable effects on the dairy industry, especially concerning processed food by fermentation wherein the quality of the final products can be seriously compromised [4]. All these concerns make the analysis of antibiotic residues in milk an important field of food safety to study.

To protect consumers, regulatory agencies in the European Union published several official documents regulating the control of veterinary drugs in food products from animal origin. Council Directive 96/23/EC [6] establishes the veterinary residue control in food producing animals. Tolerance levels, as described by European Commission Regulation 470/2009/EC [7], were set for compounds that can be used for therapeutic purposes. Regulation 37/2010 [8] lists the pharmacologically active substances and their maximum residue level (MRL) in foodstuffs of animal origin, as well as compounds for which no MRL has been set because no hazard for public health has been observed. For some non-authorized substances a minimum required performance limit (MRPL) was set to harmonize the analytical performance of the methods [9, 10], meaning that MRPL is not a concentration obtained from toxicological data, but is only related to the general analytical performance. For antibiotics without an MRL or an MPRL, a validation level (VL) was defined based on the drug characteristics of the respective class of compounds (table 7).

The requirements for performance and validation of the analytical methods employed in the official residues control for screening and confirmatory purposes are described in the European Decision 2002/657/EC [9]. Microbiological and bioassay techniques are still used for antibiotic qualitative screening purposes [3, 4, 11-14] mainly

because of their low cost and simplicity; however they lack sensitivity and specificity. To ensure unequivocal identification, there is growing need for efficient screening methods that guarantee a significantly reduced number of false-positives and false-negatives. This efficiency can be gathered in multi-detection methods based on liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS) [15-19]. The use of ultra-high pressure liquid chromatography (UHPLC) provides the possibility of having short run times together with higher resolution and sensitivity, important attributes when running several compounds at once [20-23].

Several methods can be found in literature for the determination of residues of different antibiotic families in milk. However, for the simultaneous analysis of compounds of different antibiotic classes in a multi-class residue analysis, only a restricted number of methods are reported in the literature, mainly due to the difficulties related to differences in physic-chemical properties between families of compounds [17, 18, 20-25].

The present work describes the development and validation of a simple and effective quantitative screening method by UHPLC-MS/MS for the simultaneous detection of 33 antibiotic compounds from sulphonamides, tetracyclines, macrolides, quinolones and chloramphenicol in bovine, caprine and ovine milk samples for application in routine analyses.

Table 7: MRLs and MRPL levels set by EU for milk, VL values and MRM acquisition conditions for each antibiotic and internal standards

		MRL *MRPL (µg kg^{-1})	VL (µg kg^{-1})	ESI	Precursor ion	Product ion	Cone voltage (eV)	Collision energy (eV)
Tetracyclines	chlortetracycline	100	100	+	479.3	444.2	25	20
	oxytetracycline	100	100	+	461.5	426.3	25	20
	tetracycline	100	100	+	445.5	410.3	25	20
	doxycycline	-	50	+	445.5	428.2	25	18
	demethyltetracycline	(IS)		+	465.2	448.3	25	17
Quinolones	ciprofloxacin	100	100	+	332.2	288.2	35	17
	enrofloxacin	100	100	+	360.3	316.3	31	19
	marbofloxacin	75	75	+	363.3	72.1	30	20
	oxolinic acid	-	25	+	262.2	216.1	30	25
	flumequine	50	50	+	262.2	202.1	30	32
	norfloxacin	-	25	+	320.3	276.2	20	17
	nalidixic acid	-	25	+	233.2	215.1	40	14
	danofloxacin	30	30	+	358.3	96.1	33	21
	ofloxacin	-	25	+	362.1	261.3	34	26
	enoxacin	-	25	+	321.2	303.2	35	18
	cinoxacin	-	25	+	263.2	217.1	30	23
	lomefloxacin	(IS)		+	352.2	265.3	31	22
Macrolides	tylosin	50	50	+	917.1	174.3	35	35
	tilmicosin	50	50	+	869.3	174.2	35	45
	erythromycin	40	40	+	734.5	158.2	25	30
	spiramycin	200	200	+	843.5	174.0	35	35
	roxithromycin	(IS)		+	837.7	679.5	30	30
Sulfonamides	sulfadiazine	100	100	+	251.2	156.2	30	15
	sulfamethoxazole	100	100	+	254.4	156.4	30	15
	sulfadimethoxine	100	100	+	311.4	156.2	30	20
	sulfametazine	100	100	+	279.4	156.3	30	15
	sulfathiazole	100	100	+	256.4	156.3	25	15
	sulfadoxine	100	100	+	311.4	156.4	30	18
	sulfamethizole	100	100	+	271.0	156.2	25	15
	sulfapyridine	100	100	+	250.3	156.3	30	15
	sulfisoxazole	100	100	+	268.3	156.2	25	15
	sulfisomidine	100	100	+	279.4	186.3	30	16
	sulfamethoxypyridazine	100	100	+	281.2	156.2	30	15
	sulfachloropyridazine	100	100	+	285.3	92.3	30	28
	sulfaquinoxaline	100	100	+	301.3	92.2	30	30
	sulfameter	(IS)		+	281.3	92.2	25	30
Amphenicol	chloranphenicol	0.3*	0.3	-	320.9	151.9	30	25
	chloranphenicol_d5	(IS)		-	326.0	157.0	30	25

Materials and Methods

Reagents, solvents and standard solutions

All reagents and solvents used were of analytical grade with the exception of chemicals used for the mobile phase, which were of HPLC grade. Methanol, acetonitrile and formic acid were supplied by Merck (Darmstadt, Germany). All standards of tetracyclines, quinolones, macrolides, sulphonamides and chloramphenicol were supplied by Sigma-Aldrich (Madrid, Spain). The individual standards are listed in table 1. One internal standard for each antibiotic family was used: demethyltetracycline for tetracyclines, lomefloxacin for quinolones, roxithromycin for macrolides, sulfameter for sulphonamides, and for chloramphenicol, the fifth-deuterated (d5) form; all the internal standards were provided by Sigma-Aldrich. For all substances, stock solutions of 1mg mL-1 were prepared by weighing the appropriate amount of standard, diluting in methanol, and storing at less than 5ºC. Suitable dilutions were also prepared to have convenient spiking solutions for both the validation process and routine analysis.

Instrumentation

The following equipment was used for sample preparation: Mettler Toledo PC200 and AE100 balances (Greifensee, Switzerland), Heidolph Reax 2 overhead mixer (Schwabach, Germany), Heraeus Megafuge 1.0 centrifuge (Hanau, Germany), Turbovap Zymark Evaporator (Hopkinton, MA, USA) and Whatman Mini-Uniprep PVDF (polyvinylidene fluoride) 0.45 µm filters (Clifton, NJ, USA). A Xevo TQ MS – Acquity UHPLC system coupled to a triple quadrupole tandem mass spectrometer from Waters

(Milford, MA, USA) was used for chromatographic separation and mass spectrometry. The electrospray ion source in positive (ESI+) and negative (ESI-) mode was used with data acquisition in multiple reaction monitoring (MRM) mode and analysed using Masslynx 4.1 software (Waters). The MRM optimized conditions are presented in table 7.

The UHPLC system consisted of a vacuum degasser, an autosampler and a binary pump equipped with an analytical reverse-phase column Acquity HSS T3 2.1x100 mm with 1.8 µm particle size (Waters). The mobile phases used were: A, formic acid 0.1% (v/v) in water and B, formic acid 0.1% (v/v) in acetonitrile. The gradient program used, at a flow rate of 0.45 mL min-1, was: 0-5 minutes from 97% A to 40% A; 5-9 minutes from 40% to 0% A; 9-10 minutes from 0% back to 97% A; 11-12 minutes 97% A. The column was maintained at 40oC, the autosampler at 10oC and the injection volume was 20 µL.

Sample preparation

Homogenized raw milk samples (2g) were weighed into a 20 mL glass centrifuge tube, the internal standard solution was added, then vortexed and allowed to stand in the dark for at least 10 minutes. Proteins were precipitated and antibiotics extracted through shaking for 20 minutes with 10 mL of acetonitrile. Following centrifugation for 15 minutes at 3100 x g, the supernatant was transferred into a new tube and evaporated to dryness under a gentle stream of nitrogen. The residue was re-dissolved with mobile phase A (400 µL), filtered through a 0.45 µm PVDF membrane, transferred to vials and injected into the UHPLC-MS/MS under MRM optimized conditions for each compound.

Validation procedure

The method was validated as a quantitative screening method by assessing the following parameters for each compound: CCβ (detection capability), specificity, selectivity, precision, applicability and ruggedness. In addition the limit of detection (LOD) was also estimated in accordance with the observed signal-to-noise ratio in the spiked samples. The selectivity and specificity were evaluated by analysing 20 blank milk samples from each different species (bovine, ovine and caprine) and the same samples were spiked with all the compounds at the MRL/MRPL/VL level. Along with the species variation, the applicability and ruggedness were shown by carrying out the analysis on different days and by different technicians, which also allowed the evaluation of precision in terms of relative standard deviation (RSD). For the compounds where an MRL was established, CCβ evaluation was carried out to obtain a concentration that was less than or equal to the regulatory MRL, and for that reason, 20 blank samples from each animal species were spiked with half the value of the MRL. For drugs without a MRL or a MPRL recommended concentration levels, a VL was defined (table 2) and the calculation of the CCβ was in accordance with the Regulation 2002/657/EC decision [9] for unauthorized compounds. The peak areas of both the analyte and the respective internal standard were measured, and the analyte/internal standard ratios were used for all determinations.

Results and Discussion

To fulfil the requirements of the legislated MRLs and the control of prohibited substances, methods have to be specific and sensitive enough to detect low levels, taking into account the complexity of obtaining good recovery of all compounds with distinct physic-chemical properties. The main problem associated with milk extraction for subsequent determination of antibiotics is the high protein content. In most methods reported in the literature, the preparation of milk samples for residue analysis involves protein precipitation followed by a solid-phase extraction (SPE) through the use of appropriate cartridges or dispersive SPE [16, 17, 20, 21, 23]. The precipitation of proteins is achieved in many cases by adding a strong acid, such as trichloroacetic acid, in combination with a miscible organic solvent. In the present method, acetonitrile was added to milk to promote the precipitation of proteins, and was also used as the extracting solvent. Protein precipitation was effective and a clean extract was obtained, which was demonstrated by the results obtained: no signal suppression or enhancement was observed and no interferences in the MS/MS detection that could compromise the determination. It can be assumed that the matrix components responsible for possible interferences were removed, such as proteins, fats, and carbohydrates. Although the use of SPE prior to MS/MS measurement can have the advantage of decreasing the effects of ion suppression caused by matrix interferences, it can also compromise the individual recoveries due the fact that each of the antibiotic classes, as well as antibiotics within each class, has different physic-chemical properties. All these aspects must be

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taken into account when selecting the appropriate SPE cartridge, especially as it can be difficult to find one with multi-class selectivity.

A procedure using a polymeric sorbent SPE cartridge, composed by an OASIS® (Waters) hydrophilic-lipophilic balance modified polymer, after protein precipitation and liquid-liquid extraction with acetonitrile was described previously [16, 17, 23]. Although this solid phase has very broad selectivity for polar compounds, after comparing the results with and without this step, it was considered unnecessary since better recoveries could be achieved with only liquid-liquid extraction. The principal advantage of the present method, when comparing with methods reported by Turnipseed et al. (2008), Bohm, et al. (2009) and Junza, et al. (2011) [16, 17, 23] is that the present extraction became easier to handle and, without the use of cartridges, the costs can be significantly reduced, which is a factor that must be taken into account when there are a large number of samples to be routinely analysed for screening purposes. The use of acetonitrile as both the agent of protein precipitation and also as the extracting solvent yields a process even more simple and cost effective. The celerity in obtaining results is one of the fundamental characteristics of screening methods. The use of equipment with good performance and high sensitivity, such as a UHPLC-MS/MS, enables sample preparation to be simplified without compromising the detection capability of the method. The high sensitivity of the equipment enables detection of compounds that are positively ionised, and chloramphenicol which is negatively ionised, in the same run. Chloramphenicol, being a banned substance, has to be detected at very low

concentrations, below its corresponding MRPL at 0.3 µg kg^{-1} which was successfully achieved (LOD = 0.06 µg kg^{-1}: table 8).

Table 8: The principal parameters of validation

	LOD (µg kg^{-1})	CCβ (µg kg^{-1})	RSD (%)
chlortetracycline	0.20	50.0	11
oxytetracycline	0.20	50.0	9
tetracycline	0.10	50.0	8
doxycycline	0.30	1.5	14
ciprofloxacin	0.20	50.0	21
enrofloxacin	0.02	50.0	8
marbofloxacin	0.10	35.0	19
oxolinic acid	0.20	0.4	9
flumequine	0.04	25.0	4
norfloxacin	0.20	4.7	15
nalidixic acid	0.30	0.4	9
danofloxacin	0.05	15.0	14
ofloxacin	3.70	4.1	17
enoxacin	3.00	3.2	16
cinoxacin	0.80	1.0	8
tylosin	0.01	25.0	11
tilmicosin	0.10	25.0	23
erythromycin	0.10	20.0	4
spiramycin	0.10	100.0	17
sulfadiazine	2.00	50.0	15
sulfamethoxazole	0.10	50.0	7
sulfadimethoxine	0.20	50.0	13
sulfametazine	0.10	50.0	5
sulfathiazole	1.00	50.0	10
sulfadoxine	0.20	50.0	5
sulfamethizole	0.20	50.0	12
sulfapyridine	1.00	50.0	12
sulfisoxazole	0.10	50.0	7
sulfisomidine	0.60	50.0	13
sulfamethoxypyridazine	0.10	50.0	17
sulfachloropyridazine	0.10	50.0	9
sulfaquinoxaline	0.10	50.0	5
chloranphenicol	0.06	0.1	15

To achieve maximum sensitivity for all compounds, MS/MS conditions (such as ion spray voltage, de-solvatation temperature, gas flow and collision conditions) were optimized by direct infusion into the detector of standard solutions and the principal ion transition was selected for each analyte. Table 7 presents the *m/z* ion transition monitored for screening and the associated collision energy. The use of an acidic mobile phase adjusted with 0.1% of formic acid promoted positive ionization, which improved the detection of most compounds since only chloramphenicol is negatively ionised.

In terms of chromatographic optimization, several gradient profiles were studied to improve peak separation and minimize the run time. Acetonitrile was shown to be better that methanol because of maximized sensitivity and resolution, especially when acidified with formic acid. The gradient described above allows the determination of all compounds in 10 minutes. One of the advantages of working with UHPLC columns consisting of a smaller particle size is the possibility of having high efficiency in peak separation, sharp peaks, and also a reduction in run time when compared with a common HPLC column, in terms of particle size. Chromatograms obtained for a spiked sample with all compounds at the validation levels (VL) are shown in figure 10. Each peak is characteristic of the respective antibiotic, demonstrating the good performance of the method in terms of detection, as well as for optimal chromatographic separation.

The main requisite for a reliable screening method is to detect unauthorized substances below the regulatory limits (MRL/MRPL) or at a level as low as possible, minimizing false-negative results. Therefore a method has to be fully validated in accordance with the legislation [8, 9]. At the expected retention time for all the target

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compounds, no interfering peaks were observed in any of the analysed samples from the three different species. Additionally the identification of all compounds were effective in all samples from the different species, according the criteria of Regulation 2002/657/EC decision [9], in all the 20 spiked samples at the VL. No false-negative results were observed since all analytes were detected at the expected retention time. The ruggedness of the method was assessed when carrying out analysis of both the blank and the spiked samples of milk from different animal species, using different technicians and with inter-day analysis. No significant variation was observed.

The results for precision, quantified as RSD% (table 8), showed the precision of the method. No results were obtained above 21% which represents a significantly lower value when compared with the criteria value accepted by the Horwitz equation [9].

Although it is stated in Decision 2002/657/EC [9] that CCβ is the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of β=5%, it is considered to be the concentration above which the sample should be re-analysed by a confirmatory method. It is also stated that CCβ must be less than or equal to the regulatory limit (MRL/MRPL) for screening methods. For this reason, and for antibiotics with MRL legislated, ½ MRL was adopted as the CCβ value. For those without MRL, the calculation was carried out by a matrix-matched calibration curve according to Decision 2002/657/EC for unauthorized substances as described by Kaufmann (2009) [25]. The LOD was also evaluated to establish the sensitivity of this method and was defined as the lower concentration of the analyte, calculated by multiplying the mean value of the signal-to-noise ratio of 20 blank samples by three. All

the LOD values for the measured compounds were found to be significantly lower than the MRL/MRPL/VL values. The validation values are presented in table 3.
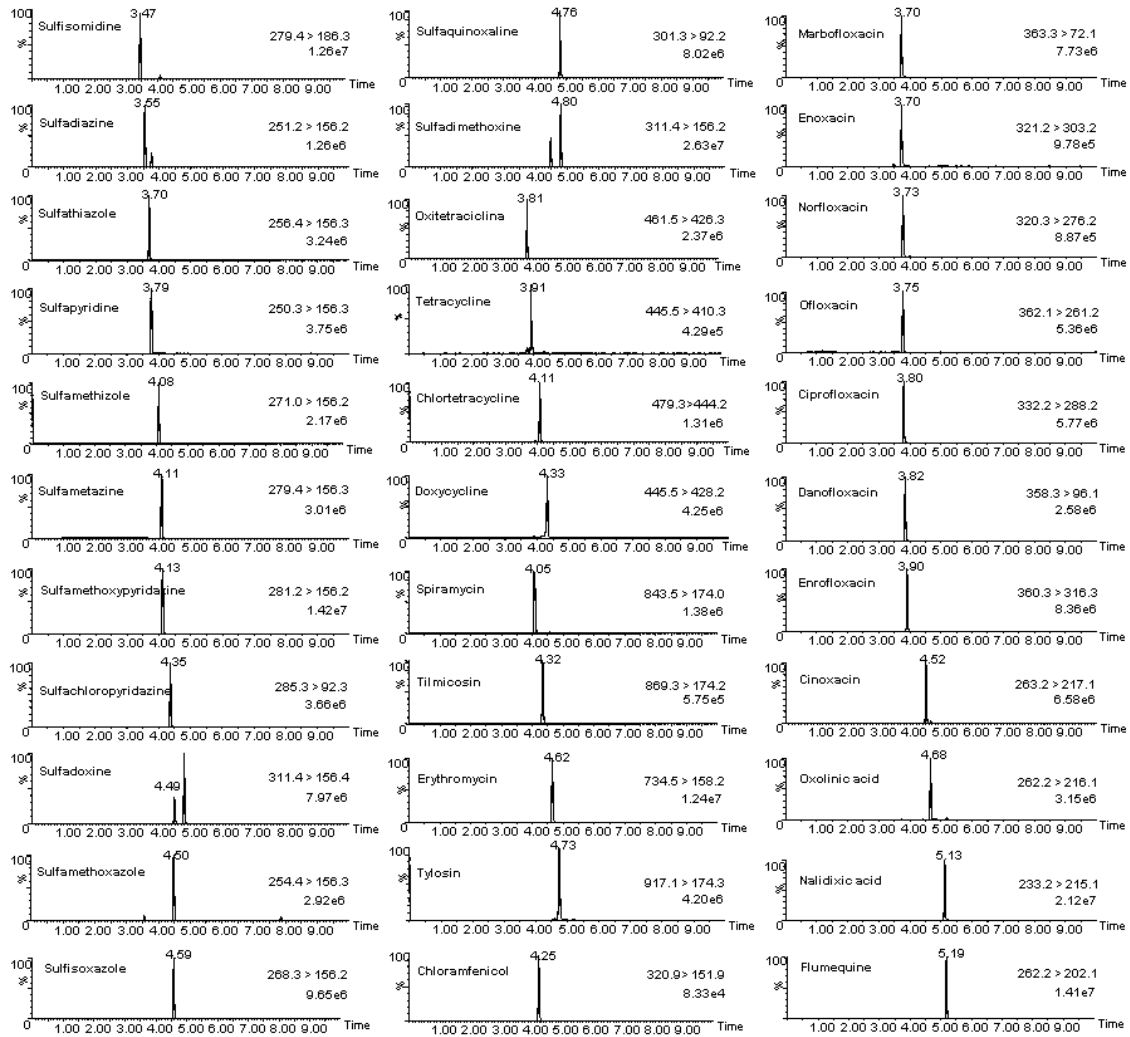
Figure 10: Liquid chromatography multiple reaction monitoring chromatograms of the antibiotics detected in a milk sample spiked at the corresponding validation level

Conclusions

A rapid and reliable multi-residue and multi-class method for simultaneous detection of 33 antibiotics, from five different families was developed and validated for quantitative screening of milk samples. The validation results showed the applicability for routine analysis of bovine, caprine and ovine milk in accordance with the requirements established in Decision 2002/657/EC [9]. The optimized extraction procedure is a simple and efficient method without the need for an SPE step, thus reducing the handling time and associated costs, and allowing a larger the number of samples analysed in one day.

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MULTI-RESIDUE AND MULTI-CLASS DETERMINATION OF ANTIBIOTICS IN GILTHEAD SEA BREAM (SPARUS AURATA) BY ULTRAHIGH PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

Abstract

This paper describes a method for the determination of 41 antibiotics from 7 different classes in gilthead sea bream (*Sparus aurata)* by ultra-high performance liquid chromatography with tandem mass spectrometry (UHPLC-MS/MS). Sulphonamides, trimethoprim, tetracyclines, macrolides, quinolones, penicillins and chloramphenicol were simultaneously determined. Fourteen procedures for sample treatment were tested and an extraction with acetonitrile and ethylenediaminetetraacetic acid was found to be the best option. The methodology was validated in accordance with Decision 2002/657/EC. Precision in terms of relative standard deviation (RSD) was under 17% for all compounds, and the recoveries ranged from 92% to 111%. CCα and CCβ were determined according to the maximum residue limit or the minimum required performance limit, when necessary. The validation provided evidence that the method was suitable for application in routine analysis for the detection and confirmation of antibiotics in muscle of gilthead sea bream, an important and intensively produced fish in aquaculture.

Introduction

Aquaculture is described as the farming of marine or freshwater food (fish, molluscs, crustaceans and aquatic plants) under specific conditions, which include the use of controlled feed, medications and breeding [1, 2]. The changes in food habits in the last decades and the increasing demand for fish as one of the most consumed products

in the world made aquaculture an emergent industry [3 – 5]. According to Food and Agricultural Organization of the United Nations (2004) [6] this sector is continuously growing in many regions across the world since seafood products constitute an important food supply [7] and, consequently, are of huge economic importance. Although fisheries production is now a stabilized segment, it is expected that aquaculture will achieve the level of a fast-growing animal food-producing sector comparable to terrestrial farming [8].

One of the most significant constraints in aquaculture development is bacterial disease that can be responsible for serious economic losses and also compromise the welfare of the seafood [9, 10]. To treat and prevent possible infections, the use of antibiotics is widely implemented as medicated feed or by adding the drugs directly into the water, a process known as bath treatments [4, 5]. This last method is the most popular route of administration, but to achieve the same results as those obtained through oral administration of medicated feed, higher quantities of antibiotics are required. Moreover, it is known that the use of antibiotics is associated with the illegal practice of stimulating animal growth. For this purpose, the administration in low concentrations results in an increase of weight gain and enhancement of the feed conversion efficiency [1].

The abusive use of these compounds and/or the disregard for the withdrawal period after drug administration may increase the presence of antibiotics in the aquatic environment and also of residues in edible tissues with associated health risks for consumers [11, 12]. Such residues can be responsible for toxic effects, allergic reactions in

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individuals with hypersensitivity and can also result in the development of resistant strains of bacteria [1, 13, 14]. These concerns make the analysis of antibiotic residues in fish an important field in food safety [15, 16]. For these reasons and in order to control abusive situations, European regulatory agencies have published several official documents to keep these substances and their administration under control. Tolerance levels for permitted veterinary drugs were established as MRLs in foodstuff of animal origin and listed in the European Commission Regulation 37/2010 [17, 18]. For non-authorized substances no tolerance levels are set, but in some cases, in order to harmonize the analytical performance of the methods, a minimum required performance limit (MRPL) has been set [19, 20]. The MRPL level is not a concentration obtained from toxicological data but is only related with analytical method performance. The requirements concerning the performance and validation of the analytical methods employed in official residues control, for screening and confirmatory purposes, are described in Decision 2002/657/EC [19].

Sensitive and specific analytical methodologies are required to perform the necessary control of the presence of antibiotic residues and ensure that the MRL levels are respected, and to estimate the presence of any non-authorized compound. Despite all these requirements, microbiological and bioassay techniques are still used for antibiotic qualitative screening, mainly due to their low cost and simplicity. On the other hand, such methods lack sensitivity and specificity, thus compromising the mandatory unequivocal identification [21, 22]. To avoid situations of false-positive and false-negative results, the concern with efficient screening methods is increasing and HPLC technology

coupled with tandem mass spectrometry (LC-MS/MS) is the technique of choice for veterinary residues analysis in biological samples [2, 4, 15, 16, 23, 24]. Recently, the use of ultra-high performance liquid chromatography (UHPLC) showed several advantages, when compared with HPLC in terms of resolution, sensitivity and also in minimizing the time of analysis which is an important feature when running numerous samples in routine laboratories [22]. It is also important to state that minimizing the time of analysis provides the possibility of reducing solvent consumption representing not only less solvent consumption and thus fewer costs, but also the reduction of laboratory environmental footprint concerned with the toxicity of mobile phases.

Regarding the most frequently administrated and analysed antibiotics in biological samples originating in aquaculture, quinolones, amphenicols, tetracyclins, penicillins, sulphonamides, macrolides and aminoglycosides should be considered as target compounds **[2, 5]**. Overviews on the most efficient analytical strategies applied for the determinations of antibiotics in fish were summarized by several authors [2, 4], providing information on HPLC methods for each separate group of compounds. Only a limited number of publications are available that simultaneously present the determination of several classes of antibiotics in fish muscle, mainly due to constraints related to the differences in physic-chemical properties between families of compounds [23]. Some authors developed methods grouping a few compounds: Cháfer-Pericás et al. (2011) [16] developed an LC-MS/MS method to determine sulphonamides and tetracyclines; Schneider et al. (2007) [26] determined tetracyclines and quinolones by HPLC-Fluorescence; Romero-González, et al. (2007) [27] developed a method for

determination of five compounds from quinolones (oxilinic acid, flumequine), tetracyclines (oxytetracycline), sulphonamides (sulfadiazine) and trimethoprim in fish muscle and skin by LC-MS/MS; Dasenaki and Thomaidis (2010) [28] determined sulphonamides and tetracyclines by UHPLC-MS/MS; Evaggelopoulou and Samanidou (2013) [29] determined penicillins and amphenicols by HPLC- Photodiode Array Detector; Gbylik et al. (2013) [25] developed a method for the determination of 34 antibiotics in fish by LC-MS/MS; and Lopes et al., (2012) [22] presented a method that comprises 32 compounds from which 25 antibiotics belonging to the macrolides, penicillins, sulphonamides and tetracyclines analysed by UHPLC-MS/MS, even though it does not include amphenicols and some quinolones determined in this paper.

The present work describes the development and validation of a screening and confirmatory method by UHPLC-MS/MS for the simultaneous detection of 41 antibiotics from sulphonamides, tetracyclines, macrolides, quinolones, penicillins, chloramphenicol and trimethroprim in gilthead sea bream (*Sparus aurata)* from aquaculture production to be applied in routine analyses.

Materials and Methods

Chemicals and reagents

All reagents used were of analytical grade with the exception of solvents used for mobile phase, which were HPLC grade. Methanol, acetonitrile and formic acid were

supplied by Merck (Darmstadt, Germany). Ethylenediaminetetraacetic acid (EDTA) was purchased from Sigma-Aldrich (Madrid, Spain). All standards of tetracyclines, quinolones, macrolides, sulphonamides, penicillins, chloramphenicol and trimethoprim were acquired from Sigma-Aldrich. The internal standards used were demethyltetracycline for tetracyclines, lomefloxacin for quinolones, roxithromycin for macrolides, sulfameter for sulphonamides and for trimethoprim, penicillin V for penicillins and for chloramphenicol, d_5-chloramphenicol; all the internal standards were provided by Sigma-Aldrich.

For all substances, stock solutions of 1 mg.mL^{-1} were prepared with an accurate amount of standard weighed, corrected for purity and presence of salt forms, and diluted in the appropriated solvent. Dilutions of 10 µg.mL^{-1}, 1 µg.mL^{-1} (for all compounds and corresponding internal standards) and 0.02 µg.mL^{-1} (for chloramphenicol and its isotope), were prepared in order to have convenient spiking solutions for both the validation process and routine analyses depending on the tolerance concentration (MRL/MRPL) and the validation level (VL). All the standard solutions were stored at below 5°C during 1 month. Although no further experiences of stability were taken, it was observed that during 1 month the compounds were stable at below 5°C.

Instrumentation

During sample preparation the following equipment was used: Mettler Toledo PC200 and AE100 balances (Greifensee, Switzerland), Heidolph Reax 2 overhead mixer

(Schwabach, Germany), Heraeus Megafuge 1.0 centrifuge (Hanau, Germany), Turbovap Zymark Evaporator (Hopkinton, MA, USA) connected with a nitrogen generator (purity 99.9995%) Peak Scientific (Frankfurt, Germany) and Whatman Mini-Uniprep PVDF (polyvinylidene fluoride) 0.45 µm filters (Clifton, NJ, USA).

The analytical instrument used for chromatographic separation and MS detection consisted of an UHPLC system coupled to a triple quadrupole tandem mass spectrometer: Xevo TQ MS – Acquity UHPLC system (Waters, Milford, MA, USA). The UHPLC system consisted of a vacuum degasser, an autosampler and a binary pump equipped with an analytical reverse-phase column Acquity HSS T3 2.1x100 mm, 1.8 µm particle size. Chromatographic parameters were optimized starting with the choice of the best option for the mobile phase from basic to acidic solvents (ammonium format and formic acid solutions) at different concentrations and varying the flow rate between 300 and 600 µL.min^{-1}. The mobile phases selected were: (A) formic acid 0.1% in water and (B) acetonitrile. The gradient programme used, at a flow rate of 0.45 mL.min^{-1}, was: 0-5 minutes from 97% (A) to 40% (A); 5-9 minutes from 40% to 0% (A); 9-10 minutes from 0% back to 97% (A); 11-12 minutes 97% (A). The column was maintained at 40°C, after testing a range from 25 ºC to 45 ºC, the autosampler at 10°C, to keep samples refrigerated before injection and guarantee the stability of compounds in the extract, and an injection volume was 20 µL. The volume selected was previously studied for individual methods of related compounds. The electrospray ion source in positive (ESI+) and negative (ESI-) mode was performed with data acquisition in MRM. Data acquisition was accomplished with Masslynx 4.1 software (Waters).

Sample preparation

Homogenized gilthead sea bream muscle (2 g) taken from the dorsal area was weighed into a 20 mL glass centrifuge tube, the internal standard solutions were added in accordance with the VL of each class of compounds (50µL of d_5-chloramphenicol 0.02 µg.mL^{-1} solution and 20µL of each one of the other internal standards with 10 µg.mL^{-1}), vortex mixed and allowed to stand in the dark for at least 10 minutes. Afterwards, 14 different extractions were tested, some including an SPE step. The different extraction procedures tested and their numbers were as follow:

1. Simple solvent extraction with 10 mL of acetonitrile;
2. Simple solvent extraction with 10 mL of acetonitrile and 1 mL of 0.1M EDTA;
3. Simple solvent extraction with 10 mL of methanol;
4. Simple solvent extraction with 10 mL of methanol and 1 mL of 0.1M EDTA;
5. Simple solvent extraction with 10 mL of acetonitrile:methanol (50:50, v/v);
6. Simple solvent extraction with 10 mL of acetonitrile:methanol (50:50, v/v) and 1 mL of 0.1M EDTA;
7. Simple solvent extraction with 10 mL of water:acetonitrile (50:50, v/v), followed by SPE with HLB 200 mg;
8. Simple solvent extraction with 10 mL of water:acetonitrile (50:50, v/v) and 1 mL of 0.1M EDTA, followed by SPE with HLB 200 mg;
9. Simple solvent extraction with 10 mL of water:methanol (50:50, v/v), followed by SPE with HLB 200mg;
10. Simple solvent extraction with 10 mL of water:methanol (50:50, v/v) and 1 mL of 0.1M EDTA, followed by SPE with HLB 200 mg;
11. Simple solvent extraction with 10 mL of water:acetonitrile (50:50, v/v), followed by SPE with C_{18} 1 g;

12. Simple solvent extraction with 10 mL of water:acetonitrile (50:50, v/v) and 1 mL of 0.1M EDTA, followed by SPE with C_{18} 1 g;

13. Simple solvent extraction with 10 mL of water:methanol (50:50, v/v), followed by SPE with C_{18} 1g;

14. Simple solvent extraction with 10 mL of water:methanol (50:50, v/v) and 1 mL of 0.1M EDTA, followed by SPE with C_{18} 1 g.

A simple solvent extraction was performed by shaking the sample with the solvent using a Reax shaker for 20 minutes followed by centrifugation for 15 minutes at 3100 g that was demonstrated to be sufficient in obtaining clear extracts. The supernatant was transferred into a new tube and, in cases when SPE was performed, applied to the preconditioned cartridges with methanol (3 mL) and water (3 mL). The elution of compounds was performed with acetonitrile (5 mL). The eluates and the supernatants of the remaining samples were evaporated to dryness under a gentle stream of nitrogen at 40°C. The residue was redissolved with mobile phase A (400 μL), filtered through a 0.45 μm PVDF Mini-uniprep ™, transferred to vials and injected into the UHPLC-MS/MS under MRM optimized conditions for each compound (table 9). These conditions were obtained after tuning each analyte of interest by combined infusion of 10 μg.mL^{-1} of standards and mobile phase (composition of the first step of the gradient).

Table 9: MRM acquisition conditions for each antibiotic and for the internal standards (IS) used

		ESI	Precursor ion (m/z)	Product ions (m/z)	Cone voltage (V)	Collision energy (eV)
Sulfonamides	sulfapyridine	+	250.3	156.3/92.3	30	15
	sulfadiazine	+	251.2	156.2/92.2	30	15
	sulfamethoxazole	+	254.4	156.4/92.2	30	20
	sulfathiazole	+	256.4	156.3/92.3	25	15
	sulfisoxazole	+	268.3	156.2/113.2	25	15
	sulfamethiazole	+	271.0	156.2/108.1	25	15
	sulfisomidine	+	279.4	186.3/124.4	30	16
	sulfamethazine	+	279.4	156.3/124.5	30	15
	sulfamethoxypyridazine	+	281.2	156.2/92.2	30	15
	sulfachloropyridazine	+	285.3	92.3/156.3	30	28
	sulfadoxine	+	311.4	156.4/92.3	30	18
	sulfadimethoxine	+	311.4	156.4/92.3	30	20
	sulfanilamide	+	173.2	92.1/156.2	30	25
	sulfaquinoxaline	+	301.3	92.2/156.3	30	30
	sulfameter (IS)	+	281.3	92.2	25	30
	trimethoprim	+	291.5	230.3/261.3	25	23
Tetracyclines	tetracycline	+	445.5	410.3/427.3	25	20
	doxycycline	+	445.5	428.2/410.3	25	18
	oxytetracycline	+	461.5	426.3/443.3	25	20
	chlorotetracycline	+	479.3	444.2/462.1	25	20
	demethyltetracycline (IS)	+	465.2	448.3	25	17
Macrolides	erythromycin	+	734.5	158.2/576.5	25	30
	spyriamicin	+	843.5	174.0/540.3	35	35
	tilmicosin	+	869.3	174.2/156.1	35	45
	tylosin	+	917.1	174.3/772.5	35	35
	roxithromycin (IS)	+	837.7	679.5	30	30
Quinolones	nalidixic acid	+	233.2	215.1/187.1	40	14
	flumequine	+	262.2	202.1/244.2	30	32
	oxolinic acid	+	262.2	216.1/244.2	30	25
	cinoxacin	+	263.2	217.1/245.2	30	23
	norfloxacin	+	320.3	276.2233.2	20	17
	enoxacin	+	321.2	303.2/234.2	35	18
	ciprofloxacin	+	332.2	288.2/245.2	35	17
	danofloxacin	+	358.3	96.1/314.3	33	21
	enrofloxacin	+	360.3	316.3/245.2	31	19
	ofloxacin	+	362.1	261.3/318.2	34	26
	marbofloxacin	+	363.3	72.1/320.2	30	20
	lomefloxacin (IS)	+	352.2	265.3	31	22
Penicillins	penicillin G	+	335.1	176.0/160.0	30	25
	ampicillin	+	350.4	106.3/160.4	25	20
	amoxicillin	+	366.3	160.3/114.4	25	20
	oxacillin	+	402.0	243.0/160.0	30	20
	nafcillin	+	415.0	199.0/171.0	30	25
	dicloxacillin	+	470.0	311.0/160.0	30	25
	penicillin V (IS)	+	351.0	160.2	25	25
Amphenicol	chloramphenicol	-	320.9	151.9/193.9	30	25
	chloramphenicol-d5 (IS)	-	326.0	157.0	30	25

Validation

The validation was based on the requirements of the EU regulation 2002/657 [19] which describes the performance criteria for analytical methods and also the parameters that might be evaluated during the validation process. According to these requirements, selectivity, specificity, recovery, repeatability, reproducibility, decision limit (CCα) and detection capability (CCβ) were determined. Selectivity and specificity were demonstrated by analysing 20 blank samples of gilthead sea bream from different origins to exclude the presence of any possible interference in the identification of the target antibiotics, and 20 blank samples of gilthead sea bream spiked at the VL in order to prove the identification capability of the method. Calibration curves were assembled with five concentration levels: 0.5 x VL, 1.0 x VL, 1.5 x VL, 2.0 x VL and 3.0 x VL and carried out in three different days and with different operators. In each day six replicates of the 0.5 x VL, 1.0 x VL and 1.5 x VL were executed in order to calculate repeatability, reproducibility and recovery. CCα and CCβ were determined according to the following equations [19]:

$$CC_\alpha = \mu_N + 2.33 \times \sigma_N \quad \text{(Equation 1, for compounds without MRLs)}$$

$$CC_\alpha = MRL + 1.64 \times \sigma_{MRL} \quad \text{(Equation 2, for compounds with established MRLs)}$$

$$CC_\beta = CC_\alpha + 1.64 \times \sigma_{VL} \quad \text{(Equation 3)}$$

Where μ_N is the mean of noise amplitude of 20 blank samples; σ_N is the standard deviation of the noise amplitude of 20 blank samples at the retention time of the target antibiotic; σ_{MRL} and σ_{VL} is the standard deviation at the MRL or VL level in the 20 spiked

blank samples at that level. According to Decision 2002/657/EC [19], the use of internal standard in MS methods is mandatory to fulfil the criteria of identification that was based on the relative retention time related to the internal standard. For all calculations the peak areas of both the analytes and correspondent internal standard were measured and the analyte/internal standard area ratios determined. Internal standards were chosen in accordance with their similar physic-chemical behaviour with the antibiotics monitored and for that they were studied and selected before validation.

Results and Discussion

While developing a multi-detection and multi-class method, the sample preparation is often the most critical step as it is important to ensure good values for recovery for as many compounds as possible. For recovery correction and to control possible matrix effects, internal standards were selected for each group of compounds. The selection was based on their similarities with the compounds of interest, meaning that they should, as much as possible, be equally affected by the same fluctuations during extraction procedure, ionization efficiency, detection response and chromatographic behaviour. Thereby, quantification by matrix-based calibration curve using internal standards allows one to monitor the efficiency of the extraction procedure and also to correct possible matrix effects. Nevertheless, several procedures for sample treatment were tested, combining a simple solvent extraction and SPE with different extraction solvents and two solid phase sorbents: HLB (hydrophilic and lipophilic balance

modified polymer) and C_{18}. The choice of these solvents and solid-phase sorbents was based on the affinities and polarities of the target compounds. In fact, HLB cartridges are considered to be of generically used for sample purification in the detection of veterinary drugs [30]. Kinsella et al., 2009 [39], also stated that acetonitrile, methanol and ethyl acetate are the preferred organic solvents in such methods. Table 10 presents the absolute recoveries (recoveries are not corrected by the internal standard and obtained with spiked blank samples) calculated for each compound in each procedure. The main purpose of these experiments was to evaluate the real impact/recovery that each procedure has in all compounds in order to select the best option possible. In table 5, absolute recoveries below 15% are highlighted (in bold) indicating that the extraction is not suitable for some compounds. Note that these cases are almost all related to procedures in the SPE step. Generally, this step is applied as a pre-concentration step of analytes and to obtain cleaner extracts due to the high efficiency of SPE. Two different SPE cartridges were tested in order to determine which provided the best specificity for the target compounds. Reverse phase C_{18} were compared with polymeric HLB, specific for compounds with high polarity. The use of hydrophilic and lipophilic balance-modified polymers grants the retention necessary to retain those analytes proving to be the better option when compared to the C_{18} cartridges. Nevertheless, for penicillins and tetracyclines, enhanced results were achieved without SPE step. The addition of a chelating agent (EDTA) gives a better performance for tetracyclines, macrolides and chloramphenicol. In the particular case of tetracyclines it is known that these compounds can form complexes with the bi- and trivalent cations present in the sample extraction solution which can lead to significant losses of compounds. According to

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these results the extraction procedure that better fits all compounds was methodology number 2, simple solvent extraction with acetonitrile and EDTA, without the SPE step due to the overall better recoveries. Acetonitrile was showed to be more efficient for a higher number of compounds when compared with methanol or even a combination of both solvents. Nonetheless, for some compounds, the recoveries obtained are still very low including sulfanilamide with 15% and sulfaquinoxaline with 27%. Despite that, at the MRL concentrations and half of that amount the identification of these compounds was assured with the precision required in legislation, which was proved during validation.

The elimination of SPE cartridges in the present methodology diminishes drastically the cost associated and allows a higher number of samples to be processed in a day. In conclusion, sample preparation included extraction of the sample with 10 mL of acetonitrile and 1mL of a 0.1M EDTA solution during 20 minutes. After centrifugation the extract was evaporated under nitrogen at $40 \pm 2\,^{\circ}C$. Dissolution with 400 µL of the mobile phase is followed by UHPLC-MS/MS analysis. Additionally, several other parameters were optimized: the mobile phase, flow rate, gradient steps and ionization conditions. Basic and acidic mobile phases were tested (ammonium format and formic acid solutions) at different concentrations. It was observed that the ionization was more effective and reproducible in an acid medium and the optimal concentration of formic acid was adjusted to 0.1%. The flow rate was tested from 300 to 600 $\mu L.min^{-1}$ and also the temperature of the column from $25\,^{\circ}C$ to $45\,^{\circ}C$.

Table 10: Absolute recoveries (expressed as %) of the target antibiotics for the 14 extractions procedures tested

Antibiotics \ Method number	1	2	3	4	5	6	7	8	9	10	11	12	13	14
sulfapyridine	72	71	54	52	65	53	30	37	66	72	34	37	30	29
sulfadiazine	73	73	51	49	65	51	**15**	22	57	48	18	22	15	15
sulfamethoxazole	53	59	40	40	50	42	27	59	47	65	23	69	33	54
sulfathiazole	62	61	42	44	56	43	27	39	62	68	27	33	25	24
sulfisoxazole	48	55	22	43	53	42	**3**	40	21	58	**12**	48	24	39
sulfamethiazole	57	62	39	40	55	42	**8**	32	44	54	**12**	32	23	30
sulfisomidine	60	56	48	47	56	48	**11**	**14**	63	70	19	22	24	23
sulfamethazine	70	74	59	61	74	59	41	60	79	81	57	67	54	54
sulfamethoxypyridazine	69	73	57	57	69	55	41	65	69	71	56	65	47	52
sulfachloropyridazine	46	54	39	40	49	38	22	58	52	59	29	56	28	41
sulfadoxine	64	73	59	59	72	57	49	80	74	77	57	80	46	70
sulfadimethoxine	38	48	30	32	39	29	51	53	57	62	69	82	73	67
sulfanilamide	17	15	**8**	**9**	**9**	**7**	**9**	**8**	**9**	**12**	**8**	**7**	**5**	**5**
sulfaquinoxaline	23	27	17	21	24	19	26	35	43	46	41	69	60	56
trimethoprim	63	61	39	42	47	45	21	**14**	61	65	31	28	52	46
tetracycline	55	84	23	80	56	79	**6**	**13**	**13**	99	**1**	**1**	**5**	**12**
doxycycline	36	52	19	40	44	39	**6**	25	**1**	85	**0**	**0**	**0**	**0**
oxytetracycline	36	66	20	52	42	55	**5**	**10**	**12**	83	**6**	**5**	**8**	17
chlorotetracycline	30	55	**9**	42	28	39	**3**	20	**0**	75	**1**	**0**	**0**	**2**
erythromycin	49	66	79	75	85	68	42	87	101	79	**0**	**4**	**5**	**13**
spyriamicin	**5**	51	46	49	16	**13**	20	**8**	41	53	**0**	15	**3**	27
tilmicosin	36	52	105	84	64	42	71	98	77	59	**0**	**1**	**0**	**13**
tylosin	**8**	88	89	76	39	41	89	86	111	115	43	19	83	65
nalidixic acid	51	72	56	66	60	59	40	63	67	71	**8**	16	**9**	18
flumequine	34	48	32	43	37	39	50	56	67	71	**15**	27	17	29
oxolinic acid	70	87	65	76	80	76	59	83	75	86	16	31	18	33
cinoxacin	73	83	71	84	94	92	**12**	24	49	90	43	33	36	33
norfloxacin	74	82	51	73	80	83	**9**	**14**	45	87	**1**	**14**	**7**	25
enoxacin	70	74	45	65	73	76	**8**	**11**	38	71	**0**	**11**	**5**	22
ciprofloxacin	65	72	43	62	71	67	**8**	**14**	36	74	**0**	**10**	**4**	20
danofloxacin	104	104	68	94	96	99	18	23	71	112	**0**	**7**	**1**	21
enrofloxacin	91	91	61	73	82	81	26	25	68	94	**0**	**1**	**0**	**7**
ofloxacin	74	76	52	63	71	68	**11**	**12**	59	80	**0**	**7**	**1**	18
marbofloxacin	79	78	56	68	75	74	**10**	**12**	63	86	**0**	**12**	**3**	22
penicillin G	33	45	51	51	42	45	**2**	**1**	**11**	**4**	**1**	**3**	**10**	**10**
ampicillin	47	51	50	50	61	53	**4**	**4**	21	**9**	**11**	**10**	41	34
amoxicillin	35	40	52	57	48	58	**1**	**5**	15	**7**	**11**	**10**	19	**13**
oxacillin	41	50	46	40	45	31	**2**	**8**	**10**	**9**	**5**	**4**	**12**	**9**
nafcillin	30	45	32	36	41	30	**6**	**11**	**9**	**10**	**3**	**6**	**8**	**11**
dicloxacillin	41	51	49	42	48	26	**7**	**9**	**10**	**9**	**5**	**4**	15	**10**
chloramphenicol	67	108	52	109	114	107	35	113	38	130	104	191	118	97

The chromatographic conditions were optimized in order to have the better efficiency in peak separation (resolution between compounds), peak shape (in terms of symmetry) along with short run time. The conditions described above allowed the determination of all 41 compounds in less than 10 minutes. The ideal MS/MS conditions were obtained by direct infusion into the detector of each standard solution. To control possible interferences related with the presence of ionic bonds of antibiotics in salt form, the infusion was performed combined with the mobile phase in the composition of the first step of the gradient. Formic acid causes the cleavage of such bonds guaranteeing the necessary intensities of the molecular ion and consequently its total fragmentation. Thus, two ion transitions were selected for each compound (table 9) to fulfil the identification criteria demanded in the Decision 2002/657 [19]. The acidified mobile phase (0.1% of formic acid) promotes the positive ionization, which improved the detection of almost all compounds since only chloramphenicol is ionised in negative mode.

The method was validated in accordance with the European Commission [19] which establishes performance criteria for the methods and the procedures for their validation. Selectivity and specificity were demonstrated as described above and the identification of all compounds were effective [19] in all spiked samples without any false-negative result.

The results for the precision, in terms of repeatability and reproducibility as the RSD, recovery, CCα and CCβ are summarized in table 11. Regarding repeatability, the higher value obtained was for sulfaquinoxaline with 15%, while the remaining

compounds were below that value. For reproducibility, the worst case was for dicloxacillin with 17% variation. All these values are below the limits defined in European Commission Decision [19]. Recovery was calculated as a ratio between the determined concentration and the real concentration. The range values obtained were between 92 and 111% falling into the accepted range [19]. These sulphonamide-spiked recoveries presented the higher bias values when compared with the other antibiotic classes. However, the obtained values cannot exceed the level calculated by the Horwitz equation [19].

CCα and CCβ were determined according to the equations described above (equation 1, 2 and 3) depending on whether or not the MRL is established. As can be seen in table 3, compounds without tolerance level have their CCα and CCβ closer to the LOD of the method, although in the other cases these concentrations are always above MRL.

One of the major improvements achieved by the current method relates to the large number of antibiotic families analysed and of monitored compounds. A limited number of publications reported methods for the simultaneous analysis of different antibiotics in fish samples. In comparison with the present method (41 compounds from seven different groups) the most recent method for screening and confirmation of antibiotics in fish was reported by Lopes et al. (2012) [22], where 32 compounds were monitored but only 25 corresponded to antibiotics. Aside from this, repeatability (< 20%) and reproducibility (< 30%) were in general similar or better that those currently reported, while recoveries were more scattered (69-125%). Other multi-detection

methods reported are mainly for the determination of a more limited number of compounds (fewer than 22) [15, 16, 26-29] with the majority contemplating only two families. As described above, the most recent and comparable published methods in terms of number of compounds and analytical methodology were described by Lopes et al. (2012)[22] and Gbylik et al. (2013) [25]. Although the method described by Lopes et al. (2012) [22] is also by UHPLC-MS/MS, the number of antibiotics is lower and the sample preparation, although simple, involves the application of QuEChERS in substitution of SPE. Gbylik et al. (2013) [25] determines 34 antibiotics including aminoglycosides, but the extraction procedure is split into two parallel procedures, including in one SPE step, gathering together at the end for a single injection by LC-MS/MS.

Conclusions

The development of a sensitive and specific UHPLC-MS/MS method for the determination and quantification of 41 antibiotics in fish muscle was successfully achieved.

An optimized extraction procedure with reduced handling time and associated lowering costs allows a higher number of samples to be analysed in one day which is a huge improvement in routine analyses. The speed with which results can be obtained is one of the fundamental characteristics in screening and confirmatory methods, which can be fulfilled using the proposed methodology. The UHPLC technology contributed to minimal analysis time while combining separation, sensitivity and high resolution chromatography.

Table 11: MRLs and MRPL set by European Union for fish muscle, VL and validation parameters: decision limit (ccα), detection capability (ccβ), repeatability, reproducibility and recovery

	MRL MRPL* ($\mu g\ kg^{-1}$)	VL ($\mu g\ kg^{-1}$)	CCα ($\mu g\ kg^{-1}$)	CCβ ($\mu g\ kg^{-1}$)	Repeatability (%RSD)	Reproducibility (%RSD)	Recovery (%)
sulfapyridine	100	100	110.6	121.2	11	13	108
sulfadiazine	100	100	115.4	130.8	9	9	106
sulfamethoxazole	100	100	114.7	129.4	9	8	110
sulfathiazole	100	100	116.9	133.8	8	8	107
sulfisoxazole	100	100	114.7	129.4	9	9	109
sulfamethiazole	100	100	116.5	133.0	13	14	105
sulfisomidine	100	100	114.2	128.4	7	8	110
sulfamethazine	100	100	111.2	122.4	3	5	107
sulfamethoxypyridazine	100	100	118.7	137.5	8	10	108
sulfachloropyridazine	100	100	126.9	153.7	8	12	111
sulfadoxine	100	100	114.9	129.8	4	7	109
sulfadimethoxine	100	100	110.6	121.2	5	5	110
sulfanilamide	100	100	125.5	151.0	13	14	92
sulfaquinoxaline	100	100	115.3	130.7	15	16	106
trimethoprim	100	100	115.1	130.3	10	12	106
tetracycline	100	100	116.5	133.1	9	10	102,
doxycycline	-	100	7.8	13.2	7	10	101
oxytetracycline	100	100	117.2	134.4	8	9	102
chlorotetracycline	100	100	124.2	148.4	12	16	104
erythromycin	200	200	224.4	248.8	5	7	99
spyriamicin	-	50	12.4	21.1	7	9	95
tilmicosin	50	50	59.4	68.9	9	13	99
tylosin	100	100	114.0	128.1	10	10	93
nalidixic acid	-	100	10.5	17.9	5	8	94
flumequine	600	600	628.9	657.7	11	8	107
oxolinic acid	100	100	111.6	123.1	6	6	99
cinoxacin	-	100	5.8	9.8	6	7	99
norfloxacin	-	100	2.4	4.1	7	8	101
enoxacin	-	100	3.8	6.4	8	9	98
ciprofloxacin	100	100	105.1	110.1	4	5	102
danofloxacin	100	100	108.3	116.5	6	6	103
enrofloxacin	100	100	106.1	112.2	4	6	102
ofloxacin	-	100	5.1	8.6	6	7	102
marbofloxacin	-	100	4.5	7.7	4	5	102
penicillin G	50	50	65.7	81.4	13	13	99
ampicillin	50	50	64.9	79.7	12	14	104
amoxicillin	50	50	64.2	78.5	22	17	105
oxacillin	300	300	356.8	413.6	14	14	103
nafcillin	300	300	351.9	403.9	14	15	98
dicloxacillin	300	300	364.1	380.7	12	17	97
chloramphenicol	0.3*	0.3	0.1	0.2	14	16	103

In addition, the process of validation also demonstrated the good performance of the method, which can be an important contribution in food safety analysis. Although it was developed for gilthead sea bream muscle, the applicability of the presented method may be extended to other similar species commonly produced in aquaculture and widely consumed worldwide.

The main advantage of the present method relies on the fact that it is a fast procedure that can be applied in routine analysis for the control of real samples from aquaculture production. Ensuring the safety of aquaculture products has never been more important, thus analyzing fish samples before commercialization is an important tool in terms of food safety.

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MULTI-RESIDUE AND MULTI-CLASS METHOD FOR THE DETERMINATION OF ANTIBIOTICS IN BOVINE MUSCLE BY ULTRA-HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

Abstract

A multi-residue quantitative screening method covering 41 antibiotics from 7 different families, by Ultra-High-Pressure-Liquid-Chromatography tandem Mass Spectrometry (UHPLC-MS/MS), is described. Sulphonamides, trimethoprim, tetracyclines, macrolides, quinolones, penicillins and chloramphenicol are simultaneously detected after a simple sample preparation of bovine muscle optimized to achieve the best recovery for all compounds. A simple sample treatment was developed consisting in an extraction with a mixture of acetonitrile and ethylenediaminetetraacetic acid (EDTA), followed by a degreasing step with n-hexane. The methodology was validated, in accordance with Decision 2002/657/EC by evaluating the required parameters: decision limit (CCα), detection capability (CCβ), specificity, selectivity, repeatability and reproducibility. Precision in terms of relative standard deviation was under 20% for all compounds and the recoveries between 91% and 119%. CCα and CCβ were determined according the maximum residue limit (MRL) or the minimum required performance limit (MRPL), when required.

Introduction

In food producing animals, antibiotics are widely used and administrated as feed additives and in drinking water not only to treat and prevent diseases but also to illegally stimulate animal growth [1, 2].

The continuous use of these drugs carries the risk of their presence in edible tissues which, for consumers, can be responsible for toxic effects and allergic reactions in hypersensitive individuals [3, 4]. It can also result in the development of resistant strains of bacteria that might compromise the efficiency of antibiotics used for treatment of animals [2]. When that occurs it became difficult to treat serious diseases, increasing the negative effects in animal welfare and consequently severe consequences for productivity and economy. Furthermore, the potential spread of resistant strains of bacteria from animals to humans can have the same effect when using antibiotics as human medicines [5]. These concerns make the analysis of antibiotic residues in food producing animals an important field in food safety. To control abusive situations, and because food safety is a key police priority for the European Commission [6], several official documents were settled down to regulate the control of veterinary drugs in products of animal origin. The Council Directive 96/23/EC [7] determines the measures to monitor certain substances and residues of veterinary medicines in living animals and in animal products. This directive foresees laboratorial control. For permitted veterinary drugs, tolerance levels were established as maximum residue limits (MRL) in foodstuff of animal origin and listed in the EU Commission Regulation 37/2010 [8, 9]. For non-authorized substances there are no tolerance levels but, for some compounds, to harmonize the analytical performance of the methods, a minimum required performance limit (MRPL) had been set [10, 11]. The MRPL level is not a concentration obtained from toxicological data, but is only related with analytical performance. The European Decision 2002/657/EC [10] describes the requirements for the performance and validation of the analytical methods employed in the official residues control. To

fulfil such requirements it is important to have sensitive and specific analytical methodologies capable of monitoring the use or potential abuse of these drugs in the field of animal husbandry, ensuring that MRL levels are respected. The concern about having efficient screening methods is increasing and also about the improvement of cost-effectiveness of analytical procedures [12-14]. Typically the methods used in laboratory are multi-detection of related compounds, usually from the same family of antibiotics. That means that a single sample, to be analysed for different groups of antibiotics, became part of a time consuming process that can last weeks. The delayed final result is associated with high cost and turns to be questionable in terms of usefulness of the result. This efficiency can be gathered in multi-class and multi-detection methods based on liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) being the tool of choice, providing the required degree of confidence for veterinary residue analysis in biological samples [3, 13]. Nowadays, the use of ultra-high performance liquid chromatography (UHPLC) provides numerous advantages in terms of resolution, sensitivity and also in minimizing time of analysis which is an important feature when running numerous samples in routine laboratories [15, 16]. Despite that, the simultaneous determination of antibiotics from different pharmacologic families in complex biological matrices, such as bovine muscle, has several constrains manly related with the differences in physic-chemical properties of the compounds [15, 17].

In the literature, only few methods, combining multi-detection and multi-class in a quantitative screening method for bovine muscle, are available. Martos et al. (2010)

[14] describes a LC-MS/MS method for the screening of 39 compounds from 7 families of antibiotics, although not validated. Granelli, Elgerud, Lundström, Ohlsson & Sjöberg (2009) [18] presented an LC-MS/MS method for the determination of 19 compounds, from 5 classes. A group of US Department of Agricultural [19] described a qualitative screening method for the determination of more than 100 compounds in bovine muscle and/or in the kidney, by UHPLC-MS/MS including not only antibiotics, but also several other drugs, such as anthelmintics, thyreostatics, beta-agonists, hormones, NSAIDS and tranquilizers. Although proved to be efficient for screening purposes, the validation presented is not based on European Commission requirements [10]. Recently, multi-detection methods for the analysis of veterinary drugs using liquid chromatography coupled with time-of-flight mass spectrometry (LC-ToF-MS) have been published [20] and UHPLC-ToF-MS [21]. One of the main advantages is the possibility of analysing an unlimited number of analytes in a single run, since the detection by ToF-MS is not limited by dwell time [22]. Nevertheless, although it can be applied for screening and quantification purposes it cannot be used as confirmatory methods due to the requirements of legislation [10] and always obliges the confirmation of positive findings using a MS/MS detector.

The present paper describes the development and validation of a simple and effective quantitative screening method by UHPLC-MS/MS for the simultaneous detection of 41 antibiotic compounds from sulphonamides, tetracyclines, penicillins, macrolides, quinolones, trimethoprim and chloramphenicol in bovine muscle. Validation

procedure followed the requirements from the European Commission Decision 2002/657/EC [10] in order to apply the method in routine analysis.

<p align="center">Table 12: MRM acquisition conditions for each antibiotic and for the internal standards (IS) used</p>

		ESI	Precursor ion (*m/z*)	Product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)	Retention time (minutes)
Sulfonamides	*sulfapyridine*	+	250.3	156.3	30	15	3.27
	sulfadiazine	+	251.2	156.2	30	15	3.24
	sulfamethoxazole	+	254.4	156.4	30	20	4.26
	sulfathiazole	+	256.4	156.3	25	15	3.35
	sulfisoxazole	+	268.3	156.2	25	15	4.37
	sulfamethiazole	+	271.0	156.2	25	15	3.86
	sulfisomidine	+	279.4	186.3	30	16	3.74
	sulfamethazine	+	279.4	156.3	30	15	3.77
	sulfamethoxypyridazine	+	281.2	156.2	30	15	3.84
	sulfachloropyridazine	+	285.3	92.3	30	28	4.15
	sulfadoxine	+	311.4	156.4	30	18	4.25
	sulfadimethoxine	+	311.4	156.4	30	20	4.65
	sulfanilamide	+	173.2	92.1	30	25	1.07
	sulfaquinoxaline	+	301.3	92.2	30	30	4.70
	sulfameter (IS)	+	281.3	92.2	25	30	3.86
	trimethoprim	+	291.5	230.3	25	23	3.29
Tetracyclines	*tetracycline*	+	445.5	410.3	25	20	3.91
	doxycycline	+	445.5	428.2	25	18	3.96
	oxytetracycline	+	461.5	426.3	25	20	3.46
	chlorotetracycline	+	479.3	444.2	25	20	3.86
	demethyltetracycline (IS)	+	465.2	448.3	25	17	3.69
Macrolides	*erythromycin*	+	734.5	158.2	25	30	4.22
	spyriamicin	+	843.5	174.0	35	35	3.71
	tilmicosin	+	869.3	174.2	35	45	3.94
	tylosin	+	917.1	174.3	35	35	4.73
	roxithromycin (IS)	+	837.7	679.5	30	30	5.43
Quinolones	*nalidixic acid*	+	233.2	215.1	40	14	3.81
	flumequine	+	262.2	202.1	30	32	5.19
	oxolinic acid	+	262.2	216.1	30	25	4.44
	cinoxacin	+	263.2	217.1	30	23	4.25
	norfloxacin	+	320.3	276.2	20	17	3.45
	enoxacin	+	321.2	303.2	35	18	3.40
	ciprofloxacin	+	332.2	288.2	35	17	3.48
	danofloxacin	+	358.3	96.1	33	21	3.52
	enrofloxacin	+	360.3	316.3	31	19	3.58
	ofloxacin	+	362.1	261.3	34	26	3.44
	marbofloxacin	+	363.3	72.1	30	20	3.36
	lomefloxacin (IS)	+	352.2	265.3	31	22	3.54
Penicillins	*penicillin G*	+	335.1	176.0	30	25	3.81
	ampicillin	+	350.4	106.3	25	20	3.34
	amoxicillin	+	366.3	160.3	25	20	4.21
	oxacillin	+	402.0	243.0	30	20	5.24
	nafcillin	+	415.0	199.0	30	25	5.47
	dicloxacillin	+	470.0	311.0	30	25	5.65
	penicillin V (IS)	+	351.0	160.2	25	25	5.07
Amphenicol	*chloramphenicol*	-	320.9	151.9	30	25	4.25
	chloramphenicol_d5 (IS)	-	326.0	157.0	30	25	4.24

Material and Methods

Reagents, Solvents and Standard Solutions

All reagents and solvents used were of analytical grade with the exception of chemicals used for the mobile phase, which were of HPLC grade. Methanol, acetonitrile and formic acid were supplied by Merck (Darmstadt, Germany). Ethylenediaminetetraacetic acid (EDTA) was purchased from Sigma-Aldrich (Madrid, Spain). All standards of sulphonamides, tetracyclines, penicillins, macrolides, quinolones, trimethoprim and chloramphenicol were supplied by Sigma-Aldrich (Madrid, Spain). The individual standards are listed in table 12. Six internal standards were used: demethyltetracycline for tetracyclines, penicillin V for penicillins, lomefloxacin for quinolones, roxithromycin for macrolides, sulfameter for sulphonamides and for trimethoprim and chloramphenicol- d5 for chloramphenicol. All the internal standards were provided by Sigma-Aldrich. For all substances, stock solutions of 1 mg mL^{-1} were prepared by weighing the appropriate amount of standard, diluted in methanol, and storing at -20°C. Suitable dilutions were also prepared to have convenient spiking solutions for both the validation process and the routine analysis.

Instrumentation

For the sample preparation, the following equipment was used: Mettler Toledo PC200 and AE100 balances (Greifensee, Switzerland), Heidolph Reax 2 overhead mixer (Schwabach, Germany), Heraeus Megafuge 1.0 centrifuge (Hanau, Germany), Turbovap Zymark Evaporator (Hopkinton, MA, USA) and Whatman Mini-Uniprep PVDF 0.45 µm

filters (Clifton, NJ, USA). Chromatographic separation and mass spectrometry detection were performed with a Xevo TQ MS – Acquity UHPLC system coupled to a triple quadrupole tandem mass spectrometer from Waters (Milford, MA, USA). The electrospray ion source in positive (ESI+) and negative (ESI-) modes was used with data acquisition in multiple reaction monitoring (MRM) mode and analysed using Masslynx 4.1 software (Waters). The MRM optimized conditions are presented in table 12. The UHPLC system consisted of a vacuum degasser, an autosampler and a binary pump equipped with an analytical reverse-phase column Acquity HSS T3 2.1x100 mm with 1.8 μm particle size (Waters). The mobile phases used were: [A] formic acid 0.1% (v/v) in water and [B] acetonitrile. The gradient program used, at a flow rate of 0.45 mL min^{-1}, was: 0-5 minutes from 97% [A] to 40% [A]; 5-9 minutes from 40% to 0% [A]; 9-10 minutes from 0% back to 97% [A]; 11-12 minutes 97% [A]. The column was maintained at 40°C, the autosampler at 10°C and the injection volume was 20 μL.

Sample preparation

A portion of 2.0 ± 0.05 g of minced and mixed bovine muscle sample was weighed into a 20 mL glass centrifuge tube. The internal standard solution was added, then vortexed for 30 s and allowed to stand in the dark for at least 10 minutes.

Afterwards, twelve different extractions procedures were tested; the list of them and the main steps are presented in table 13.

The liquid extraction was performed by shaking the sample with the solvent using a Reax shaker for 20 minutes followed by centrifugation for 15 minutes at 3100 *g*.

The supernatant was transferred into a new tube and, for extractions *ADry*, *MDry* and *EaDry* evaporated to dryness under a gentle stream of nitrogen, at 40ºC. For the extract samples *A*, *M* and *Ea* the evaporation were just until 0.5 mL. Procedures *AHxDry*, *MHxDry*, *EaHxDry*, *AHx*, *MHx* and *EaHx* followed a defat step by adding 3 mL of n-hexane to the supernatant obtained after centrifugation. The extracts were vortexed for 30 s and centrifuged for 15 minutes at 3100 *g*. The n-hexane layer were discarded and, for extractions *AHxDry*, *MHxDry* and *EaHxDry* they were evaporated to dryness under a gentle stream of nitrogen, at 40ºC. For extract samples *AHx*, *MHx* and *EaHx* the evaporation were just until 0.5 mL. In all procedures, the residue was redissolved with mobile phase A (400 µL) or added to the 0.5mL of final extract, filtered through a 0.45 µm PVDF Mini-uniprep ™, transferred to vials and injected into the UHPLC-MS/MS under MRM optimized conditions for each compound (table 12).

Table 13: Schematic description of the twelve extraction procedures tested

Procedure	Solvent extraction (**10 mL**) with 1 mL 0.1M EDTA	Deffating (**2 mL**)	Concentration
ADry	acetonitrile		
MDry	methanol		
EaDry	ethyl acetate		evaporate
AHxDry	acetonitrile		until dryness
MHxDry	methanol	n-hexane	
EaHxDry	ethyl acetate		
A	acetonitrile		
M	methanol		
Ea	ethyl acetate		evaporate
AHx	acetonitrile		until 0.5 mL
MHx	methanol	n-hexane	
EaHx	ethyl acetate		

Validation procedure

The validation procedure followed the described by the EU Commission Decision 2002/657/EEC [10]. According to those requirements, specificity, recovery, repeatability, reproducibility, decision limit (CCα) and detection capability (CCβ) were determined.

The specificity was assessed by analysing 20 bovine muscle samples from different origins to find possible peaks that could interfere with the detection of the analytes of interest. The same samples were spiked with all the compounds at the level of interest (VL) that, for most of them, corresponds to their MRL/MRPL level, in order to prove the identification capability of the method. Calibration curves were assembled with five concentration levels: 0.5 x VL, 1.0 x VL, 1.5 x VL, 2.0 x VL and 3.0 x VL and carried out in three different days and with different operators. In each day six replicates of the 0.5 x VL, 1.0 x VL and 1.5 x VL were executed in order to calculate repeatability, reproducibility and recovery. Recovery determined in the validation process was estimated as a ratio between the determined concentration and the real concentration.

CCα and CCβ were determined according to the following equations [10]:

$$CC_\alpha = \mu_N + 2.33 \times \sigma_N \quad \text{(Equation 1, for compounds without MRLs)}$$

$$CC_\alpha = MRL + 1.64 \times \sigma_{MRL} \quad \text{(Equation 2, for compounds with established MRLs)}$$

$$CC_\beta = CC_\alpha + 1.64 \times \sigma_{VL} \text{ (Equation 3)}$$

In which: μ_N is the mean of noise amplitude of twenty blank samples; σ_N is the standard deviation of the noise amplitude of twenty blank samples at the retention time

of the target antibiotic; and σ_{MRL} or σ_{VL} is the standard deviation at the MRL or VL level in the twenty spiked blank samples at that level. For all the determinations, with the exception for the studies of absolute recoveries during sample preparation development, the peak areas of both the analytes and the correspondent internal standard were measured, and the analyte/internal standard area ratios were determined. Internal standards were chosen in accordance with their similar physic-chemical behaviour with the antibiotics monitored and for that they were studied and selected before validation.

Results and Discussion

The principal limitation found while developing multi-detection and multi-class methods is related with the sample preparation, mainly due to the difficulty in achieving an efficient and generic procedure to extract simultaneously several compounds from diverse families with different physic-chemical properties. It is difficult to reach equally good recoveries in such methods and minimize the loss of all analytes during sample preparation. Multi-step and complex sample clean-up can result in total loss of some target compounds and simplifying the procedure can be an improvement. Therefore and considering that the high selectivity of solid-phase-extraction (SPE) can be a problem in multi-class methods, a simple liquid extraction was tested and optimized.

Twelve procedures were experienced and final results, in terms of individual absolute recovery, are presented in table 14. The main purpose of these experiments was to evaluate the real impact/recovery that each procedure has in all compounds in

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order to select the best option possible. For that reason, absolute recoveries presented

for each method did not take into account the presence of the internal standard, in

opposition to the recovery obtained during validation.

Table 14: Absolute recoveries (expressed as %) of the target antibiotics for the twelve extractions procedures tested. Absolute recoveries below 15% are in bold and underlined

Antibiotics \ Method	ADry	MDry	EaDry	AHxDry	MHxDry	EaHxDry	A	M	EA	AHx	MHx	EaHx
sulfapyridine	88	72	38	76	16	18	99	16	81	99	61	**9**
sulfadiazine	46	33	19	38	**11**	18	95	17	48	104	29	**13**
sulfamethoxazole	36	28	**2**	23	19	16	57	41	**6**	46	47	**14**
sulfathiazole	50	26	**5**	46	**6**	**8**	91	**12**	18	109	15	**6**
sulfisoxazole	36	27	**0**	**13**	12	**3**	53	**10**	**2**	45	42	**5**
sulfamethiazole	43	25	**6**	35	**6**	**14**	72	19	15	80	20	**11**
sulfisomidine	42	38	17	37	21	15	72	18	43	90	54	**13**
sulfamethazine	72	62	31	65	41	27	94	37	96	108	96	23
sulfamethoxypyridazine	28	22	**9**	24	15	**11**	60	**11**	24	64	42	**10**
sulfachloropyridazine	66	50	**10**	50	27	32	83	18	18	102	63	19
sulfadoxine	54	41	**7**	46	28	19	80	53	**14**	104	67	16
sulfadimethoxine	46	43	**12**	36	22	**14**	76	52	31	106	69	17
sulfanilamide	**3**	**1**	**3**	**5**	**0**	**10**	18	**1**	**3**	22	**1**	**9**
sulfaquinoxaline	27	30	**5**	23	18	**8**	35	36	**14**	56	47	**9**
trimethoprim	54	36	16	35	15	**2**	74	19	23	57	47	**4**
tetracycline	62	**11**	17	53	**8**	23	99	**10**	36	101	17	19
doxycycline	57	22	21	53	16	50	92	38	44	106	40	26
oxytetracycline	35	**4**	**9**	26	**5**	20	54	**7**	15	72	**5**	**14**
chlorotetracycline	35	**9**	15	37	**8**	49	85	**11**	42	90	15	46
erythromycin	64	59	**9**	45	42	**5**	93	61	17	98	62	16
spyriamicin	48	50	**5**	54	35	**0**	94	58	**14**	111	77	**0**
tilmicosin	27	30	**5**	25	19	**0**	69	40	25	81	56	**0**
tylosin	49	75	**3**	40	55	**0**	74	102	**6**	98	113	**0**
nalidixic acid	81	67	38	72	46	73	92	46	48	105	66	46
flumequine	46	42	37	42	29	59	75	50	62	107	69	50
oxolinic acid	62	48	46	56	34	66	87	47	58	106	65	48
cinoxacin	59	43	21	54	**7**	76	95	18	34	102	50	60
norfloxacin	67	45	**13**	60	27	**5**	92	40	35	95	56	**3**
enoxacin	57	35	**14**	40	18	**6**	96	19	33	100	47	**6**
ciprofloxacin	60	39	15	52	24	**6**	67	35	28	100	43	**3**
danofloxacin	58	37	**14**	43	23	**0**	97	41	30	98	52	**1**
enrofloxacin	51	37	16	37	22	**8**	83	33	36	84	47	**13**
ofloxacin	49	27	**9**	36	18	**1**	76	31	21	78	39	**2**
marbofloxacin	77	53	26	62	29	**1**	72	23	42	98	67	**2**
penicillin G	86	62	**12**	77	27	**0**	94	31	34	100	84	**0**
ampicillin	50	28	**2**	21	**11**	**0**	87	57	**0**	65	48	**0**
amoxicillin	45	33	22	34	18	**0**	51	**0**	**0**	52	**0**	**0**
oxacillin	39	32	**7**	39	27	**11**	101	50	24	101	87	**10**
nafcillin	34	23	**12**	44	17	17	60	36	30	85	40	**11**
dicloxacillin	18	22	**2**	31	16	**3**	46	31	**7**	57	33	**3**
chloramphenicol	57	77	**9**	24	30	**12**	56	**6**	**10**	113	**9**	50

Three organic solvents were tested for sample extraction: acetonitrile, methanol and ethyl acetate. The addition of a quelating agent was also performed, EDTA, especially to compete with antibiotics as tetracyclines and macrolides. It is known that these compounds can form complexes with the bi- and trivalent cations present in the sample extraction solution which can lead to significant losses of those compounds during the procedure. The presence of another compound, as EDTA, which has similar behaviour, is responsible for the improvement of performance of these antibiotics avoiding drastically those losses.

In some of the experiments a defatting step of the organic layer was introduced, with n-hexane, to minimize the lipid content from the muscle and thus the potential interferences during analysis. Also, because some compounds have better affinity with aqueous phase, the same assays were performed without total dryness at the end of the extraction (until 0.5 mL).

Absolute recoveries were calculated for each compound and each methodology in order to understand the effects of all variants. The results are presented in table 8 and, graphically compared in figure 11, by the representation of the minimum and maximum absolute recoveries obtained. In a first analysis of table 14 and figure 11 it can be seen that worse results were achieved when using ethyl acetate as extracting solvent, followed by methanol, being the acetonitrile the organic solvent of choice for the most compounds. Comparing the performance of the methods that involved evaporation until dryness or until 0.5 mL, it can be easily concluded that the second option gives better results. There are two reasons that can justify these data. First of all, the higher

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affinity of polar compounds with aqueous phase can be responsible for a significant amount of antibiotics concentrated in the aqueous content of the sample, turned miscible in the acetonitrile during homogenization. Also the well-known instability of antibiotics [23] can be a problem during a longer evaporation process of the remaining aqueous layer. Being the acetonitrile the chosen organic solvent the next step was to compare between methods *A* and *AHx*. It can be observed that the recovery is significantly higher when the lipid content is reduced from the matrix. The possibility of diminishing the interferences coming from the matrix can be responsible for reducing effects like ion suppression or enhancement of signal [13, 17], a common problem in the detection system when working with less specific methods such as multi-detection and multi-class and biological samples.
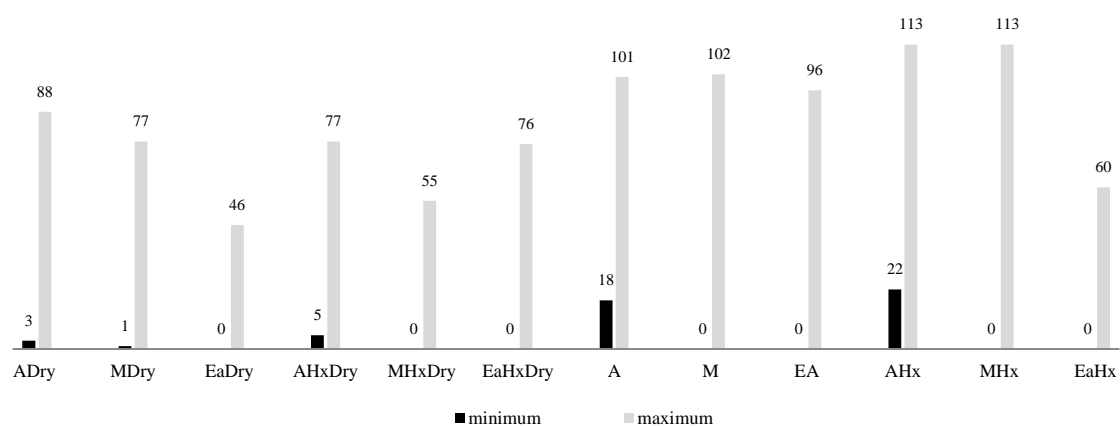
Figure 11: Minimum and maximum absolute recoveries obtained for the twelve extraction procedures for all the antibiotics tested at the concentration of the VL (see table 9 for the respective values)

Nonetheless a compromise had to be adopted selecting the most suitable method, although, for some compounds, the recoveries obtained are still significantly low, being the worse result the obtained for sulfanilamide with 22%. Briefly, the selected

method listed with the code *AHx* above in the sample preparation, in the table 13 and figure 11, was determinate to be as follow: 2g of homogenized bovine muscle extracted with 10mL of acetonitrile with 1mL of 0.1M EDTA; after centrifugation the supernatant was defatted with n-hexane; centrifuged and evaporated until 0.5 mL of final extract.

For recovery correction and to control possible matrix effects, internal standards were selected for each group of compounds. The selection was based on their similarities with the target compounds, meaning that they should, as much as possible, be equally affected by the same fluctuations during extraction procedure, ionization efficiency, detection response and chromatographic behaviour. Thereby, quantification by matrix based calibration curve using internal standards allows to monitor the efficiency of the extraction procedure and also to correct possible matrix effects.

Chromatographic and detection parameters were optimized: mobile phase, flow rate, gradient steps and ionization conditions. The conditions described above allow the determination of all 41 compounds in less than 10 minutes, one of the huge advantages of UHPLC and for that, chromatographic conditions were tested with the purpose of achieving the better efficiency in peak separation and peak shape along with a short run time.

In terms of detection, the ideal MRM conditions were obtained by direct infusion into the detector of each standard solution at the concentration of 10 μg mL^{-1}. The use of an acidified mobile phase, 0.1% of formic acid, promotes the positive ionization, which improved the detection of almost all compounds since only chloramphenicol is ionized in negative mode. To fulfil the identification criteria demanded in the Decision

2002/657 [10], two ion transitions were selected for each compound (table 7). In figure 2 a representative chromatogram of a spiked bovine muscle sample, at the corresponding validation level (VL) is presented. As an example, individual MRM of one compound per family of monitored antibiotic is also presented in figure 12.
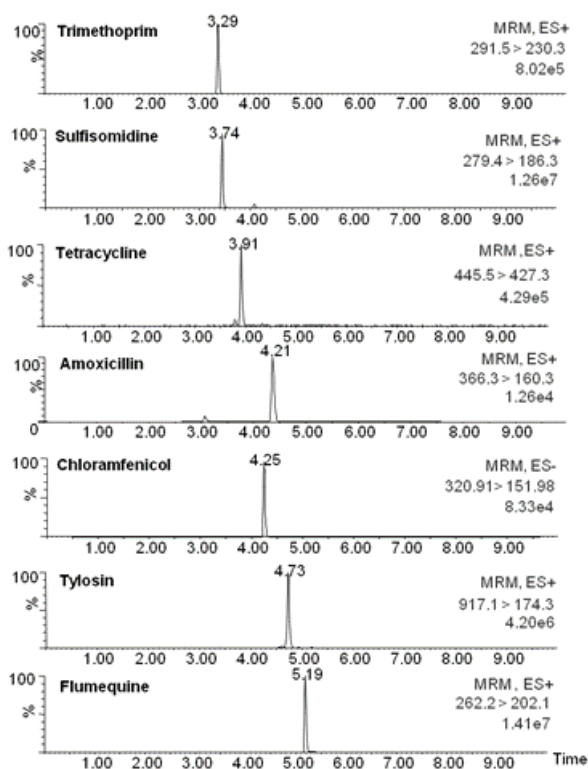
Figure 12: Chromatogram of individual MRM of one compound per class of antibiotic for a spiked bovine muscle sample at the corresponding validation level (VL)

The method was validated in accordance with the European Commission Decision 2002/657 [10] that establishes performance criteria for the methods and the procedures for their validation.

The absence of interfering peaks, in the 20 blank bovine muscle analysed samples, above a signal-to-noise ratio of 3, was confirmed in all blank samples.

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Furthermore, after spiking the same blank samples, the identification of all compounds was effective without any false negative result. The results for precision, in terms of repeatability and reproducibility as relative standard deviation (RSD %), recovery, CCα and CCβ are summarized in table 15. Values presented for precision and recovery were calculated for the VL that, for most of the compounds are the MRL. To prove the robustness of the method, precision is an important parameter that must be analysed during validation since it measures the variability during the analytical process. In terms of repeatability, the higher value obtained was for sulfanilamide, with 17%. All the other compounds were under that RSD. Regarding reproducibility, the worse value, 22%, was also for sulfanilamide while the remaining compounds were below 20%. All these values are in accordance with the acceptance criteria, according to the Decision 657/2002 [10]. The calculated RSD cannot exceed the level calculated by the Horwitz equation that depends on the concentration level. The recovery determined during validation was calculated as a ratio between the determined concentration and the real concentration. The range values obtained were between 86 and 109% falling into the accepted range [10]. It is important to note that such values are different from the ones obtained during the development of sample preparation. In these cases the recoveries were calculated as absolute values, without having the correction of the internal standard addition, and for that reason values presented in table 14, for method *AHx*, are different from the ones calculated during validation and described in table 15.

CCα and CCβ were calculated according to the equations described above (equation 1, 2 and 3) depending if the MRL is established or not. As can be seen in table 10, compounds without tolerance level have lower CCα and CCβ, closer to the limit of

detection of the method although in the other cases these concentrations are always above MRL.

Table 15: MRLs and MPRL set by EU for bovine muscle, validation level (VL) and validation parameters: decision limit (ccα), detection capability (ccβ), repeatability, reproducibility and recovery

	MRL *MRPL (µg kg^{-1})	VL (µg kg^{-1})	CCα (µg kg^{-1})	CCβ (µg kg^{-1})	Repeatability (%RSD)	Reproducibility (%RSD)	Recovery (%)
sulfapyridine	100	100	132	164	8	12	109
sulfadiazine	100	100	113	125	5	8	93
sulfamethoxazole	100	100	108	117	7	10	108
sulfathiazole	100	100	107	115	6	8	105
sulfisoxazole	100	100	111	121	6	9	104
sulfamethiazole	100	100	110	120	3	5	101
sulfisomidine	100	100	104	108	3	4	93
sulfamethazine	100	100	105	110	6	9	100
sulfamethoxypyridazine	100	100	108	116	2	4	91
sulfachloropyridazine	100	100	104	108	7	11	103
sulfadoxine	100	100	110	121	3	5	91
sulfadimethoxine	100	100	107	114	4	5	93
sulfanilamide	100	100	105	111	17	22	102
sulfaquinoxaline	100	100	106	112	5	7	102
trimethoprim	100	100	108	116	5	7	98
tetracycline	100	100	125	149	13	20	109
doxycycline	100	100	123	147	13	20	103
oxytetracycline	100	100	124	148	13	19	102
chlorotetracycline	100	100	121	143	12	17	100
erythromycin	100	100	116	131	9	14	101
spyriamicin	200	200	226	252	15	20	101
tilmicosin	50	50	60	71	7	10	93
tylosin	100	100	116	133	9	14	116
nalidixic acid	-	100	0.01	0.02	8	13	102
flumequine	200	200	214	229	8	12	104
oxolinic acid	100	100	114	127	8	12	105
cinoxacin	-	100	0.02	0.04	10	14	108
norfloxacin	-	100	0.02	0.04	9	13	86
enoxacin	-	100	0.04	0.06	10	15	98
ciprofloxacin	-	100	0.09	0.12	9	14	95
danofloxacin	200	200	229	258	15	20	106
enrofloxacin	100	100	121	142	12	17	105
ofloxacin	-	100	0.01	0.02	10	15	105
marbofloxacin	-	100	163	176	7	11	100
penicillin G	50	50	69	87	11	17	94
ampicillin	50	50	61	73	7	10	97
amoxicillin	50	50	65	79	8	12	106
oxacillin	300	300	315	330.	9	13	101
nafcillin	300	300	307	315	4	6	103
dicloxacillin	300	300	310	319	6	9	96
chloramphenicol	0.3*	0.3	0.07	0.10	13	19	105

The results of the validation clearly demonstrated the suitability of this method for the detection and identification of all tested antibiotics.

Conclusions

A reliable multi-detection and multi-class method for the determination of 41 antibiotics from 7 different classes in bovine muscle was developed. The sample preparation has the main advantage of being inexpensive and low time consuming. Also the use of UHPLC-MS/MS provided the possibility of analysing a wide number of samples in short period of time. By replacing the methods currently applied in the laboratory (one screening method for each class of compounds) the total time from sampling to the final result will be reduced in a very significant period of time.

The method developed was completely validated in order to be used in routine analysis of official control for quantitative screening purposes with the possibility of extending the method for confirmation. For a laboratory involved in food safety control with a large number of antibiotic residues and samples to analyse, the present method is a huge improvement.

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MULTI-DETECTION OF ANTIBIOTICS IN LIVER TISSUE BY ULTRA-HIGH-PRESSURE-LIQUID-CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

Abstract

A multi-residue quantitative screening method covering 39 antibiotics from 7 different families by ultra-high-pressure-liquid-chromatography tandem mass spectrometry (UHPLC-MS/MS) is described. Sulphonamides, trimethoprim, tetracyclines, macrolides, quinolones, penicillins and chloramphenicol are simultaneously detected in liver tissue. A simple sample treatment method consisting of extraction with a mixture of acetonitrile and ethylenediaminetetraacetic acid (EDTA) followed by solid-phase extraction (SPE) with a hydrophilic-lipophilic balanced (HLB) cartridge was developed.

The methodology was validated, in accordance with Decision 2002/657/EC, by evaluating the following required parameters: decision limit (CCα), detection capability (CCβ), specificity, repeatability and reproducibility. The precision, in terms of the relative standard deviation, was under 22% for all of the compounds, and the recoveries were between 80% and 110%. The CCα and CCβ were determined according to the maximum residue limit (MRL) or the minimum required performance limit (MRPL), when established.

Introduction

Antibiotics are widely used for therapeutic and prophylactic purposes in food-producing animals and to promote animal growth [1]. The use of antibiotics as growth promoters is considered fraudulent in Europe because it can lead to residues of these compounds persisting in edible matrices. These antibiotic residues can result in allergic reactions in some hypersensitive individuals and in the appearance of bacterial strains that are resistant to drugs that are used in both veterinary and human medicine [2], which are currently considered a huge worldwide concern.

For that reason, the European Community determined the need for the mandatory control of the veterinary drugs in food from animal origin designated for human consumption [3]. For permitted veterinary drugs, the maximum residue limits (MRL) in foodstuff of animal origin were established and are listed in the EU Commission Regulation 37/2010 [4, 5]. Food products containing concentrations of antibiotics exceeding the established MRL are inappropriate for human consumption. In the case of some non-authorized substances, a minimum required performance limit (MRPL) has been set to harmonize the analytical performance of the methods used in different laboratories [6, 7].

A wide variety of edible matrices must be monitored for the presence of veterinary residues, including muscle, liver, kidney, fat, milk, eggs, fish and honey. Nevertheless, there are relatively few multi-detection and multi-class methods for the determination of antibiotics in liver tissue. There are still very few methods describing

approaches for analysing different classes of compounds, particularly for their determination in liver tissue [8, 9]. To our knowledge, the only available method for the determination of an extensive number of antibiotics from several classes in such a matrix was published by Kaufmann et al [8], who detected 100 veterinary drugs in muscle, liver and kidney tissues using UHPLC-ToF-MS. The main constraint with using ToF-MS methodologies is related to the fact that it is impossible to use them for confirmation purposes because ToF-MS is not yet included in the regulations [6].

There are also some methods that group a few families of compounds, such as the one presented by Shao et al. [9], who developed a multi-class confirmatory method for tetracyclines and quinolones in muscle, liver and kidney tissues using UHPLC-MS/MS. However, the common procedures described in the literature for the determination of antibiotics in liver tissue only include groups of related compounds [10-14].

When working with liver tissue and developing the sample preparation methodology, one of the principal obstacles is related to the complexity associated with the high protein and fat contents in this matrix, which often interfere in the analytical performance. Additionally, the high enzymatic activity in liver tissue can be responsible for the fast degradation of labile compounds, which leads to significant losses during sample preparation. Another issue to be considered is that the simultaneous determination of antibiotics from different pharmacologic families in complex biological matrices is constrained by differences in the physicochemical properties of the compounds [15, 16], a fact that makes developing the sample extraction method a

challenge that can only be overcome by reaching a compromise that better fits the purpose of the multi-class method.

The lack of methodologies for screening of antibiotics in liver demanded for new developments in order to fulfil the requirements of the control program and, consequently, improve food safety. Considering all of these aspects and the need for a reliable and efficient method for the determination of antibiotics in liver tissue while improving the time of analysis for several groups of compounds and the cost-effectiveness, the aim of this work was to develop a multi-class and multi-detection method using UHPLC-MS/MS for the detection of antibiotics from seven families (sulphonamides, trimethoprim, tetracyclines, macrolides, quinolones, penicillins and chloramphenicol). To use the method in routine analysis and official control, it was validated according the requirements described in the European Commission Decision 2002/657/EC [6].

Material and Methods

Reagents, Solvents and Standard Solutions

All of the reagents and solvents used were of analytical grade, with the exception of the chemicals used for the mobile phase, which were of HPLC grade. Methanol, acetonitrile, n-hexane and formic acid were supplied by Merck (Darmstadt, Germany).

Ethylenediaminetetraacetic acid (EDTA) was purchased from Sigma-Aldrich (Madrid, Spain). All of the standards of the sulphonamides, tetracyclines, penicillins, macrolides, quinolones, trimethoprim and chloramphenicol were supplied by Sigma-Aldrich (Madrid, Spain). The individual standards are listed in table 16. The following six internal standards were used: demethyltetracycline for the tetracyclines; penicillin V for the penicillins; lomefloxacin for the quinolones; roxithromycin for the macrolides; sulfameter for the sulphonamides and trimethoprim; and chloramphenicol-fifth-deuterated (d5) for chloramphenicol. All of the internal standards were provided by Sigma-Aldrich. For all of the substances, stock solutions of 1 mg mL^{-1} were prepared by weighing the appropriate amount of standard, diluting it in methanol, and storing it at -20°C for one year. Suitable dilutions were also prepared to have convenient spiking solutions for both the validation process and the routine analyses. Working solutions were stored at -20ºC for one month.

Instrumentation

For the sample preparation, the following equipment was used: Mettler Toledo PC200 and AE100 balances (Greifensee, Switzerland), a Heidolph Reax 2 overhead mixer (Schwabach, Germany), a Heraeus Megafuge 1.0 centrifuge (Hanau, Germany), a Turbovap Zymark Evaporator (Hopkinton, MA, USA) and Whatman Mini-Uniprep PVDF 0.45 µm filters (Clifton, NJ, USA). A vacuum manifold was used for the solid phase extraction (SPE) with an Oasis HLB polymeric sorbent cartridge (3 mL, 200 mg) (Waters, Milford, MA, USA). Chromatographic separation and mass spectrometry detection were

performed using a Xevo TQ MS–Acquity UHPLC system coupled to a triple quadrupole tandem mass spectrometer from Waters (Milford, MA, USA). The electrospray ion source (ESI) was used both in positive and negative modes with data acquisition in the multiple reaction monitoring mode (MRM), and the Masslynx 4.1 software (Waters) was used for data processing. The MRM optimized conditions are presented in table 16. The UHPLC system consisted of a vacuum degasser, an autosampler and a binary pump equipped with an analytical reverse-phase column (Acquity HSS T3 2.1 x 100 mm with 1.8 μm particle size, Waters). A flow rate of 0.45 mL min^{-1} was used with the following mobile phases: [A] formic acid 0.1% (v/v) in water and [B] acetonitrile. The following gradient program was used: 0-5 minutes from 97% to 40% [A]; 5-9 minutes from 40% to 0% [A]; 9-10 minutes from 0% back to 97% [A]; 11-12 minutes 97% [A]. Column and autosampler were maintained at 40°C and 10°C, respectively. A 20 μL aliquot (full loop) was injected onto the analytical column.

Sample preparation

Two grams of minced and mixed liver tissue was weighed into a 20 mL glass centrifuge tube. The internal standard solution was added, and the sample was vortexed for 30 s and allowed to stand in the dark for at least 10 minutes. The sample was extracted by shaking using a Reax shaker for 10 minutes with 10 mL of acetonitrile and 1 mL of 0.1 M EDTA. After that, the sample was left in the ultrasound bath for 20 minutes. Following centrifugation for 10 minutes at 4000×*g*, the supernatant was transferred into a new tube and evaporated to near dryness (1 mL). Water (5 mL) was

added, and the solution was vortexed for 15 s. The solutions were then submitted to a clean-up step using SPE Oasis HLB cartridges, which were preconditioned with acetonitrile (10 mL) and water (10 mL). After passing the aqueous extract through the columns using gravity, the cartridges were washed with water (5 mL) and then dried under reduced pressure for approximately 5 minutes. The elution was performed with acetonitrile (10 mL). The eluate was evaporated to near dryness (0.5 mL) under a gentle stream of nitrogen and 400 µL of mobile phase [A] was added. To this extract n-hexane (2 mL) was added and the solution vortexed for 30 s. After centrifugation for 10 minutes at 4000×g, the n-hexane layer was removed. The final extract was filtered through a 0.45 µm PVDF Mini-uniprep ™, transferred to vials and analysed by UHPLC-MS/MS under the MRM optimized conditions described in table 16.

Validation procedure

In-house validation was performed following the method described by the EU Commission Decision 2002/657/EEC [6] that requires the evaluation of the method in terms of the specificity, recovery, repeatability, reproducibility, decision limit (CCα) and detection capability (CCβ).

Table 16: MRM acquisition conditions for each antibiotic and for the internal standards (IS) used

		ESI	Precursor ion (*m/z*)	Product ions (*m/z*)	Cone voltage (V)	Collision energy (eV)
Sulfonamides	*sulfapyridine*	+	250.3	156.3/92.3	30	15
	sulfadiazine	+	251.2	156.2/92.2	30	15
	sulfamethoxazole	+	254.4	156.4/92.2	30	20
	sulfathiazole	+	256.4	156.3/92.3	25	15
	sulfisoxazole	+	268.3	156.2/113.2	25	15
	sulfamethiazole	+	271.0	156.2/108.1	25	15
	sulfisomidine	+	279.4	186.3/124.4	30	16
	sulfamethazine	+	279.4	156.3/124.5	30	15
	sulfamethoxypyridazine	+	281.2	156.2/92.2	30	15
	sulfachloropyridazine	+	285.3	92.3/156.3	30	28
	sulfadoxine	+	311.4	156.4/92.3	30	18
	sulfadimethoxine	+	311.4	156.4/92.3	30	20
	sulfanilamide	+	173.2	92.1/156.2	30	25
	sulfaquinoxaline	+	301.3	92.2/156.3	30	30
	sulfameter (IS)	+	281.3	92.2	25	30
	trimethoprim	+	291.5	230.3/261.3	25	23
Tetracyclines	*tetracycline*	+	445.5	410.3/427.3	25	20
	doxycycline	+	445.5	428.2/410.3	25	18
	oxytetracycline	+	461.5	426.3/443.3	25	20
	chlorotetracycline	+	479.3	444.2/462.1	25	20
	demethyltetracycline (IS)	+	465.2	448.3	25	17
Macrolides	*erythromycin*	+	734.5	158.2/576.5	25	30
	spyriamicin	+	843.5	174.0/540.3	35	35
	tilmicosin	+	869.3	174.2/156.1	35	45
	tylosin	+	917.1	174.3/772.5	35	35
	roxithromycin (IS)	+	837.7	679.5	30	30
Quinolones	*nalidixic acid*	+	233.2	215.1/187.1	40	14
	flumequine	+	262.2	202.1/244.2	30	32
	oxolinic acid	+	262.2	216.1/244.2	30	25
	cinoxacin	+	263.2	217.1/245.2	30	23
	norfloxacin	+	320.3	276.2233.2	20	17
	enoxacin	+	321.2	303.2/234.2	35	18
	ciprofloxacin	+	332.2	288.2/245.2	35	17
	danofloxacin	+	358.3	96.1/314.3	33	21
	enrofloxacin	+	360.3	316.3/245.2	31	19
	ofloxacin	+	362.1	261.3/318.2	34	26
	marbofloxacin	+	363.3	72.1/320.2	30	20
	lomefloxacin (IS)	+	352.2	265.3	31	22
Penicillins	*amoxicillin*	+	366.3	160.3/114.4	25	20
	oxacillin	+	402.0	243.0/160.0	30	20
	nafcillin	+	415.0	199.0/171.0	30	25
	dicloxacillin	+	470.0	311.0/160.0	30	25
	penicillin V (IS)	+	351.0	160.2	25	25
Amphenicol	*chloramphenicol*	-	320.9	151.9/193.9	30	25
	chloramphenicol-d5 (IS)	-	326.0	157.0	30	25

By analysing 20 blank liver samples from different animal species (bovine, swine, ovine, and poultry) to find possible peaks that could interfere with the detection of the target analytes, the specificity of the method was assessed. Afterwards, the same 20 samples were spiked with all of the compounds of interest at the validation level (VL)

(table 12) to prove the identification capability of the method and once again its specificity. Calibration curves using spiked samples were assembled using the following five concentration levels, 0.5 x VL, 1.0 x VL, 1.5 x VL, 2.0 x VL and 3.0 x VL, and the analyses were carried out on three different days with different operators. Six replicates of the 0.5 x VL, 1.0 x VL and 1.5 x VL concentration levels were performed each day to determine the precision of the method (in terms of the repeatability and reproducibility) and the recovery. The recovery was estimated as a ratio between the obtained concentration and the real concentration.

The critical concentrations, CCα and CCβ, were calculated according to the following equations [6]:

$$CC_\alpha = \mu_N + 2.33 \times \sigma_N \quad \text{(Equation 1, for compounds without MRLs)}$$

$$CC_\alpha = MRL + 1.64 \times \sigma_{MRL} \quad \text{(Equation 2, for compounds with established MRLs)}$$

$$CC_\beta = CC_\alpha + 1.64 \times \sigma_{VL} \text{ (Equation 3)}$$

where μ_N is the mean of the noise amplitude of 20 blank samples; σ_N is the standard deviation of the noise amplitude of 20 blank samples at the retention time of the target analyte; and σ_{MRL} or σ_{VL} is the standard deviation at the MRL or VL level in the 20 spiked blank samples at that level. For all of the determinations, the peak areas of both the analytes and the corresponding internal standards were measured, and the

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analyte/internal standard area ratios were determined. Internal standards were chosen for their similar physicochemical behaviours to those of the antibiotics being monitored [17].

Results and Discussion

The major challenge in the determination of veterinary drugs in biological samples, usually in residual concentrations, lies in sample preparation. Our knowledge and experience from previously developed multi-class methods in milk, fish and bovine muscle [17-19] was the starting point for the present method. In these previously work, the appropriated solvents, for the extraction of the target compound, were already studied and, starting from that knowledge, a new method, to be used in liver, was developed. Thus, the best option for use as the extraction solvent, in terms of the recovery, is an organic extractant, specifically acetonitrile. Aqueous solvents failed to extract the less polar compounds. The same conclusion is expressed in other available publications, though those extractions were performed on different matrices [8, 20], where acetonitrile is preferred over methanol and ethyl acetate, because these last two solvents can be responsible for extracting matrix components that can interfere in the detection. Additionally, it is important to add that acetonitrile, aside from being an efficient extraction solvent, promotes the precipitation of proteins, thereby turning this step into one that is important for obtaining a clean extract. Some of the target

antibiotics, such as tetracyclines, quinolones and macrolides, can easily form chelate complexes with bi- and trivalent metal cations present in the sample extraction solution. These can lead to lower recoveries; to prevent their formation, a chelate agent with a similar behaviour should be used to control the problem and increase the recoveries. For that reason, EDTA is often used during the liquid extraction, and it has been determined to improve the extraction efficiencies of tetracyclines, quinolones and macrolides.

Compared with muscle tissue, liver tissue is a much more complex matrix because of its high protein content, enzymatic activity and fat content. Therefore, to prevent possible chromatographic interferences and ion suppression or enhancement, further clean-up steps during the sample preparation were optimized.

The use of solid-phase extraction prior to mass spectrometric detection can be a huge advantage to decrease the effects of ion suppression caused by components of liver tissue. To control the possible losses of target antibiotics, the best option is to use a multi-class selectivity cartridge that can fit the diverse physicochemical properties of all of the target antibiotics. The best option, in terms of selectivity, is to use a sorbent composed of a hydrophilic-lipophilic balance modified polymer (OASIS HLB), which is known to have a very broad selectivity for polar compounds [8, 21]. The solid-phase extraction is followed by concentration through evaporation under a gentle stream of nitrogen, without evaporation to total dryness, to avoid a long evaporation process. The instability of antibiotics along with the higher affinity of some polar compounds for aqueous phase possibly remaining present in the cartridge and being eluted together

with the acetonitrile are the main reasons for this procedure [22]. After reconstitution with the mobile phase, a thin lipidic layer was observed. To remove that layer and prevent such interference in the mass spectrometric detection, a defatting step was performed via the addition of n-hexane. After discarding the n-hexane layer, the final extract was injected and analysed using UHPLC-MS/MS.

The UHPLC-MS/MS parameters, in terms of chromatography and detection, were previously optimized. The mobile phase, flow rate and gradient steps were selected to achieve the best chromatographic separation and peak shape, along with a short run time. The conditions described above allowed the determination of the 39 compounds in less than 10 minutes. To fulfil the identification criteria described in Decision 2002/657 [6], two ion transitions must be controlled for each compound. The ideal MRM conditions (table 16) were achieved through the direct infusion into the detector of each individual standard solution at a concentration of 10 µg mL^{-1}. For positive ionization, which is the case for all of the compounds except chloramphenicol, the use of formic acid in the mobile phase works as a promoter of positive ionization and consequently improves the detection. In figure 13, individual MRM chromatograms of one compound per family of monitored antibiotics obtained from a spiked bovine liver sample at the corresponding validation level (VL) are presented.

Table 17: MRLs and MRPL set by European Union for liver, VL and validation parameters: decision limit (ccα),

detection capability (ccβ), repeatability, reproducibility and recovery

	MRL (µg kg^{-1})	VL (µg kg^{-1})	CCα (µg kg^{-1})	CCβ (µg kg^{-1})	Repeatability (%RSD)	Reproducibility (%RSD)	Recovery (%)
sulfapyridine	100	100	124	149	15	22	101
sulfadiazine	100	100	125	150	15	22	105
sulfamethoxazole	100	100	121	142	15	23	85
sulfathiazole	100	100	115	129	8	12	109
sulfisoxazole	100	100	123	146	16	24	88
sulfamethiazole	100	100	111	122	6	9	108
sulfisomidine	100	100	123	146	13	19	108
sulfamethazine	100	100	115	129	8	12	110
sulfamethoxypyridazine	100	100	114	129	8	12	110
sulfachloropyridazine	100	100	118	135	10	15	107
sulfadoxine	100	100	111	123	7	11	97
sulfadimethoxine	100	100	123	147	13	19	110
sulfanilamide	100	100	125	150	15	22	105
sulfaquinoxaline	100	100	118	137	11	17	98
trimethoprim	50	50	65	81	11	16	88
tetracycline	300	300	322	343	12	18	109
doxycycline	300	300	325	351	14	22	108
oxytetracycline	300	300	313	326	7	11	110
chlorotetracycline	300	300	321	343	15	22	88
erythromycin	200	200	219	237	10	16	109
spyriamicin	300	300	317	333	10	15	102
tilmicosin	1000	1000	1024	1048	13	20	110
tylosin	100	100	111	122	7	10	101
nalidixic acid	-	100	5.81	16.0	15	23	110
flumequine	500	500	528	555	15	23	110
oxolinic acid	150	150	166	182	9	13	109
cinoxacin	-	100	3.10	7.60	15	22	100
norfloxacin	-	100	0.32	0.94	13	19	108
enoxacin	-	100	1.72	3.87	15	22	88
ciprofloxacin	300	300	316	331	11	17	87
danofloxacin	400	400	418	437	12	18	94
enrofloxacin	300	300	325	349	15	22	103
ofloxacin	-	100	0.22	0.65	8	13	81
marbofloxacin	150	150	174	198	14	21	107
amoxicillin	50	50	74	97	15	22	98
oxacillin	300	300	320	339	14	22	83
nafcillin	300	300	321	341	12	17	109
dicloxacillin	300	300	325	349	14	21	109
chloramphenicol	-	0.3	0.28	0.48	11	17	109

The method was validated in accordance with the European Commission Decision 2002/657/EC [6], and the following parameters were evaluated: specificity, recovery, precision (as repeatability and reproducibility) decision limit (CCα) and detection capability (CCβ).

The specificity of the method was assessed by analysing 20 blank samples of liver tissue of different species (bovine, swine, ovine, and poultry) to verify the absence of interference above a signal-to-noise ratio of 3 at the retention time of the target compounds that could compromise their detection and identification. Additionally, in the spiked blank samples, all of the identification criteria [6] were fulfilled without any false negative results, again proving the specificity of the method for the species analysed. Considering the proved specificity and that no major differences were found between the 20 blank and spiked samples, only one animal species (bovine) was used for the next validation steps. This choice was based on the fact that bovine liver tissue is a matrix that is very often consumed.

The results obtained for the precision (repeatability and reproducibility) as the relative standard deviation (RSD), recovery, CCα and CCβ are summarized in table 17. The precision and recovery were calculated at the VL that corresponds to the MRL for those compounds that had it. For repeatability and reproducibility, the highest RSDs of 16% and 24%, respectively, were obtained for sulfisoxazole. All of the other compounds had RSDs under these values. The recovery was calculated as a ratio between the determined concentration in a spiked sample and the real concentration. The range of values obtained were between 81 and 110%, thus falling into the acceptable range [6].
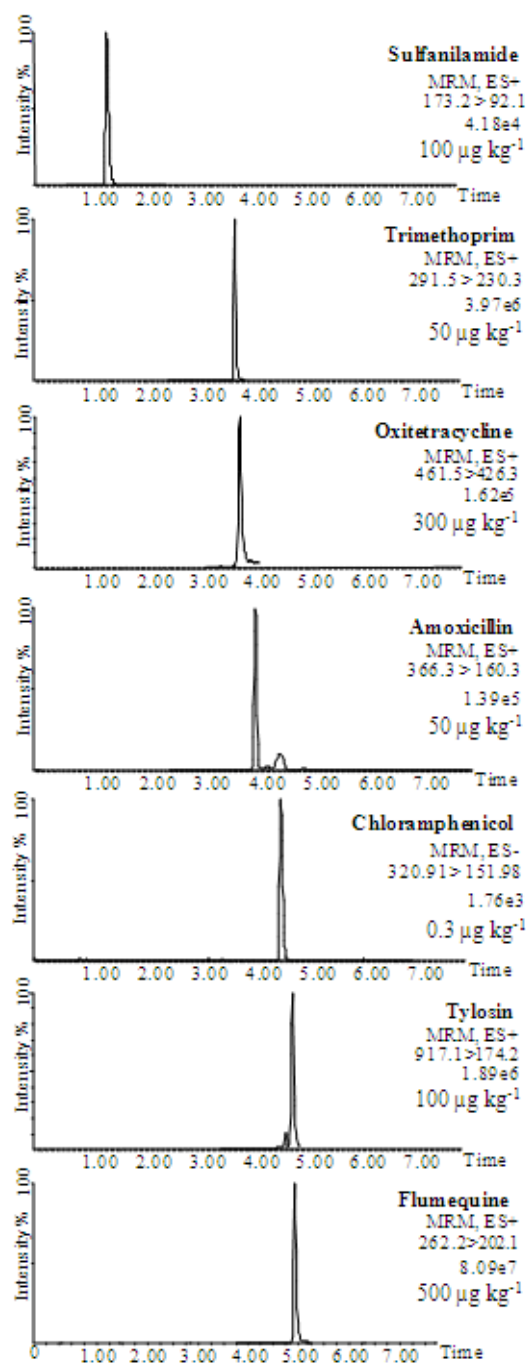
Figure 13: Individual MRM of one antibiotic per family is given as example from a spiked liver sample at the corresponding validation level (100 µg kg⁻¹ for sulfanilamide and tylosin; 50 µg kg⁻¹ for trimethoprim and amoxicillin; 300 µg kg⁻¹ for oxitetracycline; 0.3 µg kg⁻¹ for chloramphenicol and 500 µg kg⁻¹ for flumequine).

Both the precision and recovery are mandatory parameters in validation because they measure the variability during the analytical process and can be used to analyse and prove the robustness of the method.

The two critical concentrations, CCα and CCβ, were determined from the calibration curves obtained from the bovine blank liver samples spiked at five concentration levels (0.5, 1, 1.5, 2 and 3 x VL) and the application of the equations described above (equations 1, 2 and 3), keeping in mind that not all of the compounds had an established MRL. Antibiotics without a tolerance level (MRL) had lower CCα and CCβ values that were closer to the limit of detection of the method, although in the other cases, these concentrations were always above the MRL.

Conclusions

An analytical method is proposed for the simultaneous determination of 39 antibiotics from 7 different classes in liver tissue. The developed method is able to determine the presence of compounds from the sulphonamides, tetracyclines, macrolides, quinolones, chloramphenicol, penicillins and trimethoprim in a single run using UHPLC-MS/MS, providing a possible way to significantly reduce the time required to analyse one sample. The developed method was fully validated and fulfilled all of the criteria specified by the European Union Decision 2002/657/EC [6], proving that it is suitable for routine analysis and quantitative screening purposes for official control, with the possibility of extending the method for antibiotic confirmation. Although the main

part of the validation procedure was performed only for bovine samples, its specificity proved that the method can be used for swine, ovine and poultry liver tissue.

Because there are a limited number of publications reporting methods for the simultaneous analysis of antibiotics in liver tissue, the present method is a huge improvement for laboratories that are involved in food safety control and have a large number of samples and antibiotics to analyse.

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CHAPTER III – MATRIX EFFECTS IN ULTRA-HIGH-PERFORMANCE-LIQUID-CHROMATOGRAPHY TANDEM MASS SPECTROMETRY ANTIBIOTIC MULTI-DETECTION METHODS IN FOOD PRODUCTS WITH ANIMAL ORIGIN

ABSTRACT

The efficiency of multi-detection screening methods, based on liquid chromatography coupled with tandem mass spectrometry, has been proven in recent years. However, when simultaneously analysing different groups of compounds with different physic-chemical properties, the specificity of sample preparation has to be minimized. In mass spectrometry, this situation can lead to ion suppression or enhancement of signals due to interferences coming from the matrices. This phenomenon was studied to understand the real impact in routine analysis. Matrix interferences were monitored in extracts of milk, muscle, liver and fish for 40 antibiotics using recently developed and validated multi-detection methods with Ultra-High Performance Liquid Chromatography tandem Mass Spectrometry (UHPLC-MS/MS). Although a significant dispersion in the results was observed, for most of compounds, ion suppression is the major problem that, although it does not compromise the screening methods, can prevent the use of multi-detection for confirmation and quantification of antibiotic residues in food matrices.

INTRODUCTION

In recent years, improving the cost-effectiveness and efficiency of analytical screening procedures has increasingly become a concern in the determination of veterinary drugs in food products with animal origins. Versatile and reliable screening methodologies can provide rapid results by reducing the number of samples to be

confirmed and decreasing the cost associated with a more laborious method. This efficiency can be found in multi-detection methods based on liquid chromatography coupled with tandem mass spectrometry, surpassing the traditional screening microbiological and immunoassays methodologies. Due to the criteria demanded by the European Commission [1], either high performance liquid chromatography (HPLC) or ultra-high performance liquid chromatography (UHPLC), combined with triple quadrupole mass spectrometry detection is the tool of choice in the field of veterinary residue analysis, mainly because of the possibility of unequivocal identification of trace concentrations in complex matrixes, such as biological samples [2, 3]. The advances achieved in analytical detection in recent decades combined sensitive equipment, simplification of sample preparation and the possibility of analysing a large number of compounds at simultaneously [3-5]. However, the simultaneous determination of compounds from different pharmacologic families in complex matrices has constraints due to the differences in the physicochemical properties of the target drugs. This fact makes the development of sample extraction methods a challenge that can only be overcome by reaching a compromise that better fits the purpose of the multiclass methods, in most cases, minimizing the specificity of the sample preparation. The lack of specificity in sample treatment can result in the presence of endogenous compounds from the sample that co-elute with the analytes of interest, which are responsible for changes in the intensity of the detected signal [6]. These interferences are usually called matrix effects and can also have other sources, such as compounds released during the sample preparation or from reagents added to the mobile phases [4-10]. Matrix effects, despite being a frequent problem for all instrumental techniques, can be more

significant when working with mass spectrometry because they can result in ion suppression or signal enhancement [11]. The interferences present in the sample extract, even if they are not observed in the chromatogram, can cause modifications to the spray droplet solution, decreasing the evaporation efficiency and changing the mechanism of ionization, resulting in either decreases or increases in the detected signals. Because it is less specific, the positive ionization mode is more affected by these phenomena, and these interferences should be completely studied to understand their real impact on routine analysis [12]. From this information, we can determine the factors that can be modified or improved to minimize the impact. Considering these aspects, matrix interferences were monitored in extracts of milk, fish, bovine muscle and liver for the determination of 40 antibiotics (sulphonamides, trimethoprim, tetracyclines, macrolides, quinolones and penicillins), using the recently, and previously published, developed and validated multi-detection methods using UHPLC-MS/MS [13-16]. The present publication summarizes the observed and calculated matrix effects in terms of suppression or enhancement of the signal.

MATERIALS AND METHODS

Chemicals, Reagents and Standard Solutions

All reagents used were of analytical grade with the exception of solvents used for the mobile phase, which were of UHPLC grade. Methanol, acetonitrile, n-hexane and formic acid were supplied by Merck (Darmstadt, Germany). Ethylenediaminetetraacetic acid (EDTA) was purchased from Sigma-Aldrich (Madrid, Spain). Tetracyclines,

quinolones, macrolides, sulphonamides, penicillins, and trimethoprim (listed in table 18) were acquired from Sigma-Aldrich. Stock solutions of 1 mg mL^{-1} were prepared with an accurate amount of standard weighed, diluted in methanol and stored at -20°C for one year. Suitable dilutions, also in methanol, were prepared to have convenient spiking solutions for all of the matrix effect assays [13-16]. The working solutions were stored at -20°C for one month.

Table 18: MRM acquisition conditions, in positive ionization mode, for each antibiotic

		Precursor ion (*m/z*)	Product ion (*m/z*)	Cone voltage (eV)	Collision energy (eV)
Sulfonamides	*Sulfapyridine*	250.3	156.3	30	15
	Sulfadiazine	251.2	156.2	30	15
	Sulfamethoxazole	254.4	156.4	30	20
	Sulfathiazole	256.4	156.3	25	15
	Sulfisoxazole	268.3	156.2	25	15
	Sulfamethiazole	271.0	156.2	25	15
	Sulfisomidine	279.4	186.3	30	16
	Sulfamethazine	279.4	156.3	30	15
	Sulfamethoxypyridazine	281.2	156.2	30	15
	Sulfachloropyridazine	285.3	92.3	30	28
	Sulfadoxine	311.4	156.4	30	18
	Sulfadimethoxine	311.4	156.4	30	20
	Sulfanilamide	173.2	92.1	30	25
	Sulfaquinoxaline	301.3	92.2	30	30
	Trimethoprim	291.5	230.3	25	23
Tetracyclines	*Tetracycline*	445.5	410.3	25	20
	Doxycycline	445.5	428.2	25	18
	Oxytetracycline	461.5	426.3	25	20
	Chlorotetracycline	479.3	444.2	25	20
Macrolides	*Erythromycin*	734.5	158.2	25	30
	Spyriamicin	843.5	174.0	35	35
	Tilmicosin	869.3	174.2	35	45
	Tylosin	917.1	174.3	35	35
Quinolones	*Nalidixic Acid*	233.2	215.1	40	14
	Flumequine	262.2	202.1	30	32
	Oxolinic Acid	262.2	216.1	30	25
	Cinoxacin	263.2	217.1	30	23
	Norfloxacin	320.3	276.2	20	17
	Enoxacin	321.2	303.2	35	18
	Ciprofloxacin	332.2	288.2	35	17
	Danofloxacin	358.3	96.1	33	21
	Enrofloxacin	360.3	316.3	31	19
	Ofloxacin	362.1	261.3	34	26
	Marbofloxacin	363.3	72.1	30	20
Penicillins	*Penicillin G*	335.1	176.0	30	25
	Ampicillin	350.4	106.3	25	20
	Amoxicillin	366.3	160.3	25	20
	Oxacillin	402.0	243.0	30	20
	Nafcillin	415.0	199.0	30	25
	Dicloxacillin	470.0	311.0	30	25

Instrumentation

During sample preparation the following equipment was used: Mettler Toledo PC200 and AE100 balances (Greifensee, Switzerland), Heidolph Reax 2 overhead mixer (Schwabach, Germany), Heraeus Megafuge 1.0 centrifuge (Hanau, Germany), Turbovap Zymark Evaporator (Hopkinton, MA, USA) and Whatman Mini-Uniprep PVDF (polyvinylidene fluoride) 0.45-μm filters (Clifton, NJ, USA). A vacuum manifold was used for the solid phase extraction (SPE) with an Oasis HLB polymeric sorbent cartridge (3 mL, 200 mg) (Waters, Milford, MA, USA).

The analytical instrument used for chromatographic separation and mass spectrometry detection consisted of an UHPLC system coupled to a triple quadrupole tandem mass spectrometer: Xevo TQ MS – Acquity UHPLC system, Waters (Milford, MA, USA). The UHPLC system consisted of a vacuum degasser, an autosampler and a binary pump equipped with an analytical reverse-phase column Acquity HSS T3 2.1x100 mm, 1.8-μm particle size. The mobile phases used were (A) formic acid 0.1% in water and (B) acetonitrile. The gradient program used, at a flow rate of 0.45 mL min^{-1}, was 0-5 minutes from 97% (A) to 40% (A); 5-9 minutes from 40% to 0% (A); 9-10 minutes from 0% back to 97% (A); 11-12 minutes 97% (A). The column and autosampler were maintained at 40°C and 10°C, respectively, and 20-μL aliquot (full loop) was injected.

The electrospray ion source in positive (ESI+) mode was performed with data acquisition in multiple reactions monitoring mode (MRM). Data acquisition and processing were accomplished using the Masslynx 4.1 software, Waters (Milford, MA, USA).

Sample preparation

The sample preparation was performed as already developed, validated and described in previous publications [13-16]. However, brief descriptions of the methods are shown below. In all cases the final extract was filtered through a 0.45-µm PVDF Mini-Uniprep ™, transferred to vials and analysed using UHPLC-MS/MS under the MRM optimized conditions described in table 18.

Milk: The extraction of antibiotics and precipitation of proteins from two grams of raw milk were performed by homogenizing the sample with 10 mL of acetonitrile (10 minutes). After centrifugation, 15 minutes at 3100 x g, the supernatant was evaporated to dryness. The residue was redissolved with 400 µL of mobile phase (A) [13].

Fish muscle: Two grams of homogenized gilthead sea bream muscle were extracted using 10 mL of acetonitrile and 1 mL of a 0.1 M EDTA solution (20 minutes). After centrifugation, 15 minutes at 3100 x g, the organic layer was evaporated to dryness and redissolved in 400 µL of mobile phase (A) [14].

Bovine Muscle: Two grams of minced bovine muscle were extracted with 10 mL of acetonitrile and 1 mL of 0.1 M EDTA (10 minutes). After centrifugation, 15 minutes at 3100 × g, the supernatant was defatted with 3 mL of n-hexane. Centrifugation was performed again for 15 minutes at 3100 x g, and the n-hexane layer was discarded. The final extract was evaporated to 0.5 mL [15].

Bovine Liver: Two grams of minced liver were extracted with 10 mL of acetonitrile and 1 mL of 0.1 M EDTA (10 minutes) followed by ultrasound bath (20

minutes). After centrifugation for 10 minutes at 4000 × *g*, the supernatant was evaporated to near dryness (1 mL). Water (5 mL) was added, and the extract solution was submitted to a cleanup step using SPE Oasis HLB cartridges (preconditioned with 10 mL of acetonitrile and 10 mL of water). The cartridges were washed with 5 mL of water and dried under reduced pressure (5 minutes). The elution was performed using acetonitrile (10 mL) followed by evaporation to nearly dryness (0.5 mL). Redissolution was made with 400 μL of mobile phase (A) followed by a defatting step with 2 mL of n-hexane. After centrifugation, 10 minutes at 4000 × *g*, the n-hexane layer was removed [16].

Matrix Effects

The matrix effects were evaluated by analysing parallel standard solutions of all target analytes in the mobile phase and in the blank sample extract (matrix-matched solutions) at the concentration of 100 μg kg^{-1}. These experiments were performed ten times in each matrix: milk, fish muscle, bovine muscle and liver. The matrix effects can be measured using the following equation [10]:

$$ME\ (\%) = \frac{A_{matrix}}{A_{standard}} \times 100 \qquad \text{(Equation 1)}$$

Where A_{matrix} is the average signal, in absolute area, obtained in the blank extract spiked after extraction (the analyte was added to the matrix extract) and $A_{standard}$ is the average signal for the compound in the standard solution (prepared in the mobile phase). The suppression or enhancement of the absolute area of the signal, A_{SE}, can be expressed by

$$A_{SE} (\%) = 100 - ME \qquad\qquad \text{(Equation 2)}$$

where the result can be interpreted as:

$$A_{SE} (\%) = 1 \; no \; matrix \; effect \; observed$$

$$A_{SE} (\%) > 1 \; signal \; enhancement$$

$$A_{SE} (\%) < 1 \; ion \; suppression \; (loss \; of \; signal)$$

RESULTS AND DISCUSSION

The major limitation found when developing multi-detection methods is related to the sample preparation due to the difficulty in achieving an efficient and generic procedure to simultaneously extract multiple compounds from diverse families with different physicochemical properties. Multi-step and complex sample clean up procedures can result in significant loss of some target compounds, and for that reason, less specific procedures are the only solution for the screening of several different compounds in one analysis [17, 18].

Previously developed methods for the multi-detection determination of 40 antibiotics in food products with animal origins were validated to be in agreement with the European Commission Decision 2002/657/EC [1]. One important parameter that should be evaluated to complement the validation and better understand any possible fluctuations is the matrix effect and how this parameter can interfere with the final

result in the analysis of real samples. Even though mass spectrometry can surpass microbiological and immunological assays due to its high sensitivity and selectivity, ion suppression and enhancement can be drawbacks for this detection tool if they are not well understood.

Table 19: Summary of mean results obtained for the signal suppression/enhancement (ASE). In cases where enhancement is observed, the result is presented in bold and underlined the suppression higher than 50%

		A_{SE} (%)			
		Milk	Fish muscle	Bovine muscle	Bovine liver
Sulfonamides	*Sulfapyridine*	-46.6	-26.6	<u>-52.5</u>	-25.2
	Sulfadiazine	<u>-53.1</u>	-25.6	<u>-67.3</u>	-43.0
	Sulfamethoxazole	<u>-59.3</u>	<u>-76.6</u>	<u>-67.1</u>	-44.1
	Sulfathiazole	-35.7	-27.8	<u>-65.3</u>	-41.6
	Sulfisoxazole	<u>-57.3</u>	<u>-69.4</u>	<u>-65.9</u>	-46.9
	Sulfamethiazole	-14.0	-30.5	<u>-52.9</u>	-12.3
	Sulfisomidine	<u>-58.4</u>	**3.9**	<u>-87.5</u>	<u>-75.6</u>
	Sulfamethazine	-34.8	<u>-53.2</u>	-47.6	-28.3
	Sulfamethoxypyridazine	-33.8	-4.9	47.0	-28.2
	Sulfachloropyridazine	-49.6	-8.6	-47.6	-19.9
	Sulfadoxine	-34.3	<u>-60.0</u>	<u>-54.0</u>	-24.6
	Sulfadimethoxine	-31.8	23.9	-17.8	-14.5
	Sulfanilamide	<u>-78.1</u>	<u>-58.0</u>	<u>-76.0</u>	<u>-77.5</u>
	Sulfaquinoxaline	-21.0	-9.7	-10.1	**3.4**
	Trimethoprim	-31.6	-46.3	<u>-66.3</u>	-22.8
Tetracyclines	*Tetracycline*	-35.0	<u>-57.7</u>	-39.5	-10.2
	Doxycycline	-25.3	-44.1	-7.1	-12.1
	Oxytetracycline	-46.9	<u>-54.5</u>	-45.7	-30.2
	Chlorotetracycline	-33.6	-44.7	-35.0	-15.6
Macrolides	*Erythromycin*	<u>-57.9</u>	<u>-57.2</u>	**17.0**	**52.9**
	Spyriamicin	-11.8	<u>-54.2</u>	-43.9	**10.1**
	Tilmicosin	-14.3	-44.7	**34.3**	**54.0**
	Tylosin	-30.0	-6.7	-35.6	-20.9
Quinolones	*Nalidixic acid*	-48.5	**4.1**	-39.4	**0.8**
	Flumequine	-20.5	-15.5	-8.7	**1.7**
	Oxolinic acid	-33.2	**12.1**	-18.2	-19.1
	Cinoxacin	-14.2	**9.1**	-10.4	**7.7**
	Norfloxacin	-37.9	-35.7	<u>-65.2</u>	-17.4
	Enoxacin	-27.4	-23.4	-14.1	-33.8
	Ciprofloxacin	-34.7	<u>-52.9</u>	-15.7	-37.9
	Danofloxacin	-4.7	-7.8	<u>-58.2</u>	-18.6
	Enrofloxacin	-46.6	-36.8	-52.5	-25.2
	Ofloxacin	-25.4	-49.7	-25.9	-42.3
	Marbofloxacin	-28.1	-6.7	-27.5	-36.6
Penicillins	*Penicillin G*	-16.5	-11.1	-36.2	-42.8
	Ampicillin	-12.7	-48.3	-40.7	-38.7
	Amoxicillin	-33.9	-39.9	<u>-66.7</u>	-44.1
	Oxacillin	**3.9**	**9.0**	-23.6	-7.6
	Nafcillin	-6.5	-4.0	-4.0	**1.2**
	Dicloxacillin	**14.9**	**25.0**	-15.9	-24.2

In the present study, 10 blank samples of each target matrix (milk, fish, bovine muscle and liver) were extracted using the developed sample preparation and spiked right before analysis in the UHPLC-MS/MS. Ten standard solutions were prepared in the mobile phase at the same concentration and analysed in parallel. The resulting signals were compared by applying equations 1 and 2; the real effects were measured and are shown in table 19 as A_{SE} (%), where $A_{SE} >1\%$ indicated the occurrence of a matrix enhancement effect and $A_{SE}<1\%$ indicated the occurrence of a suppression effect. In the table, these values are underlined when the suppression is greater than 50% and shown in bold when they are enhanced.
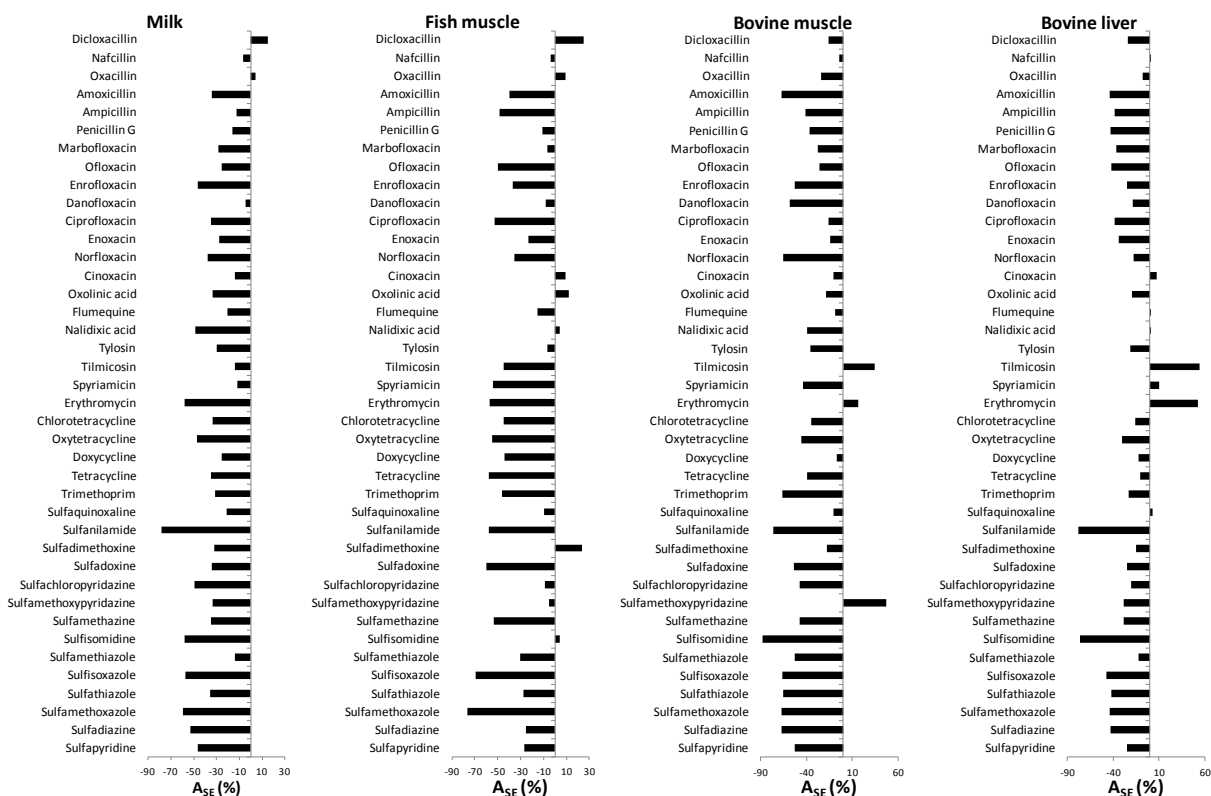

Figure 14: Matrix effects observed in milk, fish muscle, bovine muscle and liver represented as ASE (%), calculated with equation 2

Although a significant dispersion in the results was observed, a loss of signal was obtained for the majority of compounds and matrices; 20% of the results have more than 50% suppression. In contrast, enhancement, $A_{SE}>1\%$, only represents 11% of the total calculated data. All of the results for the four matrices with all compounds are represented graphically in figure 14.

The worst results, i.e., signal losses of more than 50%, were obtained for the bovine muscle. In this matrix, the observed matrix effect was $A_{SE}<-50\%$ for 13 compounds as shown in table 19. In fish muscle, 10 compounds showed $A_{SE}<-50\%$; in milk, only 6 analytes had $A_{SE}<-50\%$; and finally, in bovine liver, only 2 antibiotics had $A_{SE}<-50\%$.

Compared with milk, fish and bovine muscle, liver is the most complex matrix analysed (higher protein, enzymatic activity and fat content). To minimize the possible and expected interferences, more clean-up steps were introduced when the method was developed via the introduction of solid-phase extraction, which generally resulted in a lower suppression (figure 14), showing the large impact that sample preparation can have. Minimizing the specificity of extraction can definitely lead to major interferences in the results and alter the method sensitivity. To guarantee the detection of the compounds of interest in multi-detection methods where the sample extraction cannot be specific, even when large suppression occurs, highly sensitive and specific equipment is needed. The significantly lower limits of detection achieved during the validation assures the detection of all compounds even in the worst cases of suppression

observed in this study. This fact is important to avoid the possibility of false negative results.

The highest enhancement results were observed for macrolides in liver tissue, especially for erythromycin, which had an increase of 52.9%, and tilmicosin, which increased 54%. The interesting case of erythromycin is represented in figure 2, where the opposite impact of the milk matrix resulted in a 57.9% suppression of the signal compared with the liver tissue. Additionally, as shown in figure 15, the blank samples did not present any visible interferences that could lead to false positives, and even when almost 60% of the signal is lost, the detection is still not compromised.
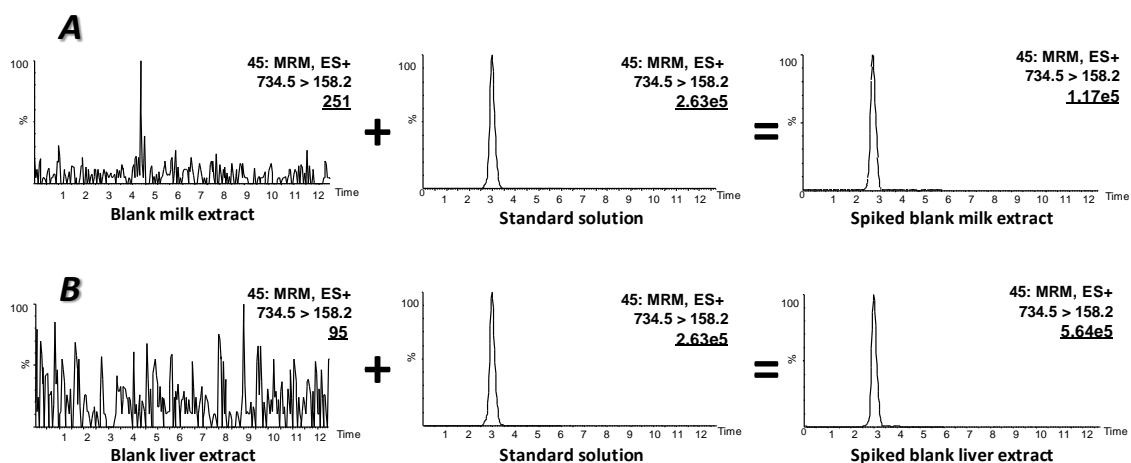

Figure 15: Example of a significant matrix effect observed in the analysis of erythromycin: A – 58% of ion suppression observed in milk; B – 53% of signal enhancement in liver

Based on these matrix interferences, it can be assumed that quantification can be compromised, which is an important fact when Maximum Residue Levels [19, 20] are being analysed. An error in the quantification process can jeopardize the validity of the result.

More selective sample preparation should be applied for confirmation and quantification purposes, meaning multi-detection methods should only be used for screening. Specific methods are required for each matrix and analyte and their combinations. Improving chromatographic separation when performing confirmation methods for a single compound or family of antibiotics should also be taken in consideration as a step to minimize the expected matrix effect. Modifying the mobile phase strength, stationary phase and gradient conditions can prevent analyte peaks from co-eluting in regions where more interferences are observed.

CONCLUSIONS

Despite all of the conclusions regarding the pitfalls of multi-detection methods, the described methods are still the best options for successfully screening a large number of antibiotics. The detection capabilities achieved using UHPLC-MS/MS, the cost-effectiveness, the time required for analysis, and the specificity for antibiotics present in the sample are the features that make these screening methods useful tools for Food Safety.

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GENERAL DISCUSSION

Despite the fact that antibiotics play an important role in protecting human and veterinary health, their inappropriate use is nowadays threatening their effectiveness by compromising its ability to fight infections when facing resistant bacteria. The increasing awareness of the consumers for food safety issues [1] and the fact that this subject is one of the main priorities to the European Commission [2], results in a high pressure on official laboratories to guarantee that this concern is being effectively monitored. An efficient control of veterinary drug residues is essential and when official methods are applied it has to be ensured that MRLs are not exceeded and that the banned compounds are not present in the samples [3].

Considering the different classes of antibiotics used in veterinary medicine and the diverse kind of foodstuff matrices that has to be controlled, laboratories have to consistently manage a considerable volume of samples and analyze a large number of compounds. As a consequence of that situation, in the last decade, a major concern has been focused on improving time of analysis, cost-effectiveness aside with developing sensitive and specific screening technologies able to detect in a single assay the maximum number of compounds as possible [4-6]. Those characteristics were achieved in the methods developed during the present project, as initially proposed. The methods developed are intended to be applied in the official routine analysis, in the national plan of residues control in Portugal, and with them, improve the performance of the laboratory by minimizing the time between the sample collection and the final result in a controlled cost. The individual methods for each family of antibiotics will be replaced

by one unique method per matrix saving weeks of laborious work, mainly in sample preparation.

Through this Dissertation it is presented the development of four multi-detection and multi-class methodologies intended to be implemented in the routine analysis for the official control, in Portugal, of antibiotics in highly consumed foodstuff of animal origin: milk, fish muscle, bovine muscle and liver. These methods are intended to speed up the routine analysis and reduce the respective costs.

While developing a multi-detection and multi-class method the sample preparation was the most critical step as it is important to ensure the desirable recovery for as many compounds as possible. To control possible fluctuations during the process, suitable internal standards were selected for each group of compounds, based on their physic-chemical similarities.

The analytical tool of choice used to guarantee the unequivocal identification of the target antibiotics and to fulfill the European Commission performance and validation criteria [3] was the UHPLC-MS/MS.

The first method presented is a quantitative screening of 33 antibiotics (tetracyclines, quinolones, macrolides, sulphonamides and chloramphenicol) in milk, one of the most consumed food of animal origin [7]. A simple sample preparation was optimized, in order to achieve the best recovery possible for the target compounds, including LLE and protein precipitation with an organic solvent, acetonitrile. This solvent is preferred over methanol and ethyl acetate, since both of them can be responsible for

extracting matrix components that would interfere in the detection. Another reason is that the acetonitrile can promote the precipitation of proteins, an important step to obtain a cleaner extract. Furthermore, aqueous solvents failed to extract the less polar compounds. Along with the required parameters monitored in the validation process, and being milk a very important matrix in terms of food habits, it was considered to be interesting to know the LOD associated to each target molecule, and with that proving the ability to determine the presence of the compounds of interest at low concentrations. The calculated LOD ranged from 0.010 µg. kg^{-1} (tylosin) to 3.7 µg kg^{-1} (ofloxacin). Comparing those results with the MRLs/MRPL established in the legislation they are significantly lower and it can be concluded that the method has the necessary sensitivity to guarantee that no false negative results will be obtained. The applicability of the method was studied and the validation provided evidence that it is suitable to be used in routine analysis for the detection of antibiotics in bovine, caprine and ovine milk.

The second multi-class method was developed for fish muscle, more specifically gilthead sea bream, one of the most consumed fish in Portugal. With the increase of fish consumption in the last decades [8, 9], aquaculture became an important economic activity in which antibiotics are widely used whether for prevention or for treatment of bacterial diseases [10]. For those reasons fish muscle should be one of the target edible tissues controlled for the presence of veterinary drug residues. The multi-class method developed determines simultaneously the presence of 41 antibiotics from sulphonamides, trimethoprim, tetracyclines, macrolides, quinolones, penicillins and chloramphenicol. An efficient sample preparation was optimized after testing fourteen

different methods using LLE and SPE steps. The optimal method, considering the absolute recoveries for all antibiotics, was an extraction with acetonitrile and EDTA, a chelating agent. The recovery of compounds able to form complexes with cations present in the sample extraction solution can be compromised and, by adding a molecule that competes for those cations, EDTA, those losses can be minimized. The method was fully validated providing the evidence that it is suitable to be applied in routine analysis for the determination of the target antibiotics in muscle of gilthead sea bream. The critical concentrations CCα and CCβ were determined, in accordance with the established by the Commission Decision 2002/657/EC [3], depending if the MRL or MRPL is established or not. For compounds without a tolerance level established, the critical concentrations were calculated closer to de detection limit of the method, being the lower value achieved for norfloxacin with a CCα of 2.4 µg kg^{-1}. Even though, the lowest value was obtained for chloramphenicol, which MRPL is defined as 0.3 µg kg^{-1}, and the CCα calculated is 0.1 µg kg^{-1}. Once again the reduced handling time and associated low costs will allow a higher number of samples to be analysed in one day improving routine analyses.

Another matrix that is equally highly consumed worldwide, bovine muscle, the developed method allows the determination of 41 antibiotics from sulphonamides, trimethoprim, tetracyclines, macrolides, quinolones, penicillins and chloramphenicol. Until now only few methods, combining multi-detection and multi-class in a quantitative screening method for bovine muscle, are available in the literature. Twelve procedures for sample preparations were tested and it was concluded, once again, that the high

selectivity of the SPE step is a concern in multi-class methods. The optimized method has many similarities with the one described for fish muscle only adding a defatting step, n-hexane, to minimize the lipid content presented in the bovine muscle in higher concentration than in fish muscle. The addition of this step is responsible for the achievement of higher recoveries as it eliminates potential interferences that could compromise the detection. The results obtained during validation clearly demonstrated that the method is suitable for the quantitative identification of all tested antibiotics in bovine muscle. The validation was performed having in mind the existing MRL and MRPL concentrations and also in the cases where no tolerance limit was established. For those cases, the critical concentrations obtained were closer to de detection limit being the lowest value achieved for nalidixic acid and ofloxacin with a CCα of 0.11 µg kg^{-1}.

The lack of methodologies for screening of antibiotics in liver was the main reason to choose it as one of the target matrices in the present work. The proposed method simultaneously determines 39 antibiotics from 7 different classes in liver tissue: sulphonamides, trimethoprim, tetracyclines, macrolides, quinolones, penicillins and chloramphenicol. There are very few methods described for analysing antibiotics in liver tissue and there are even less for multi-detection and multi-class determination. This situation triggered the interest for new developments to fulfil the requirements of the control program and, consequently, improve food safety tools. One of the principal obstacles faced when working with liver tissue and optimizing sample preparation is related with the complexity of the sample. The high protein and fat contents and the significant enzymatic activity in liver tissues can represent more interference in the

analytical performance as well as can cause fast degradation of labile compounds leading to low recoveries in the sample preparation. That reason can justify the fact that a more complex sample extraction was needed in order to prevent possible chromatographic interferences and ion suppression or signal enhancement effects. The starting point was based on the previously developed methods; the appropriated extraction solvents for the target compounds were already tested. The developed procedure consists of an extraction with a mixture of acetonitrile and EDTA followed by a SPE step with a hydrophilic–lipophilic balanced (HLB) sorbent, known to have a very broad selectivity for polar compounds. A complete validation of the method was performed, fulfilling all the demanded criteria and providing evidence that it is suitable to be applied in routine analysis for quantitative screening purposes. For liver tissue the lowest value obtained for CCα was 0.22 μg kg^{-1} for ofloxacin, an antibiotic with no MRL defined for liver. Despite the fact that the method was developed for bovine liver, during the validation process, the specificity was also studied for swine, ovine and poultry liver tissues leading to the conclusion that the method is suitable for those liver tissues. The present method represents an important improvement for laboratories involved in food safety control since a limited number of publications are available for the determination of such huge number of antibiotics in liver tissue.

The advances achieved in the analytical methods developed, combining sensitive equipment, simplification of sample preparation and the detection of a large number of compounds simultaneously, has however some constrains. The lack of specificity in sample preparation can result in the presence of endogenous compounds from the

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sample that co-elute with the antibiotics, causing changes in the intensity of the detected signal: suppression or enhancement of the signal [11]. To better understand this phenomenon, matrix interferences were monitored in extracts of milk, fish, bovine muscle and liver for the determination of antibiotics by UHPLC-MS/MS. The evaluation of the matrix effects was made by analysing in parallel standard solutions of all target analytes in the mobile phase and in the blank sample extract (matrix-matched solutions) at adequate concentrations. It was observed that the variability of signal suppression and enhancement effects are compound and matrix dependent. For the majority of compounds a loss of signal was observed (ion suppression); being 20% of the analysed samples with more than 50% of suppression. In contrast, enhancement effects, only represents 11% of the total calculated data. In comparison with the other matrices, liver is the most complex matrix analysed due to the higher protein, enzymatic activity and fat content and, for that reason, it was expected a higher signal interference. To prevent the referred expected effect a SPE step was introduced. This approach resulted in a decrease of the signal suppression, confirming that the specificity of the extraction procedure couldn't be minimized in order to avoid major interferences that can alter the sensitivity of the methods.

When intense signal suppression occurs it is important to guarantee the detection of compounds, avoiding false negative results, by using highly sensitive and specific equipment. However it was observed that quantification can be affected by those effects leading to the conclusion that it is mandatory to use more specific sample

preparation in case of confirmation and quantification purposes, meaning that multi-detection methods should be mainly used for screening.

Despite all of the conclusions regarding the pitfalls of multi-detection and multi-class methods, the described ones are still the best options for successfully screening a large number of antibiotics on a short period of time. Currently, the strategy that was applied until now involved the application of several methods, most of them multi-residue of related compounds (same class) resulting in a time-consuming process leading to several weeks of laboratorial work before achieving the final result. The celerity in obtaining results is one of the fundamental characteristics and advantages of the multi-residue and multi-class screening methods. The possibility of analysing, in one single procedure, a large number of compounds per sample reducing the handling time and the associated costs, allowing a large number of samples to be monitored in one day, is the main feature of the present work. It is also important to emphasize that the UHPLC-MS/MS technology contributed to achieve minimal analysis time combining separation, sensitivity and high resolution chromatography along with high detection capabilities [12].

The main feature of the proposed methodologies, as important tools for improving consumer confidence in foodstuff of animal origin, is the benefit that they add to food safety risk analysis.

FUTURE TRENDS

For the official control of the presence of antibiotics in foodstuff of animal origin a wide range of edible tissues has to be monitored [13]. In the present work, four methodologies are presented and the knowledge achieved during their development should be applied to other matrices such as kidney, fat, eggs and honey. Nevertheless, the analysis of animal feeds and of drinking water by this kind of multi-detection and multi-class methods will be also an important improvement to control the fraudulent use of antibiotics more efficiently.

The increasing relevance of aquaculture in Portugal and the influence that antibiotics have in fish farming demands a more careful attitude. The method developed for gilthead sea bream fish muscle should work as a starting point to be optimized in order to be applied to another similar species commonly produced in Portuguese aquaculture, such as sea bass, turbot, sole, white bream or trout.

Last but not least, the development of new multi-detection and multi-class methods for other antibiotics, such as aminoglycosides, cephalosporins and polimixins (with different chemical properties), or their inclusion in the previous related methods should be another challenge to reach in this field.

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