Lúcia Maria Loureiro Santos Ferraz

Antimicrobial Residues in Aquaculture Species for Human Consumption

Analytical Determination and Assessment of Related Public Health Hazards

Tese no âmbito do Doutoramento em Ciências Farmacêuticas, na especialidade de Bromatologia e Hidrologia, orientada pelo Professor Doutor Fernando Jorge dos Ramos e apresentada à Faculdade de Farmácia da Universidade de Coimbra.

Fevereiro de 2019

Faculdade de Farmácia da Universidade de Coimbra

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"If knowledge can create problems,

it is not through ignorance that we can solve them."

Isaac Asimov

"We cannot solve our problems

with the same thinking we used when we created them"

Albert Einstein

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Abstract

The production of animals intended for human consumption, including aquaculture, is nowadays strongly anchored in the use of antibacterials. These drugs aim to treat but mainly, and particularly in the aquaculture industry, to prevent the emergence and rapid spread of infectious diseases, which can compromise entire production batches.

Aquaculture is probably the fastest growing food-producing sector, accounting nowadays for nearly 50 % of the world's food fish supply for human consumption, and this share is projected to rise to 62% by 2030 as catches from wild capture fisheries level off and demand from a progressively growing world population substantially increases. This exponential growth is associated with the implementation of intensive and semi-intensive production methods and is hampered by unpredictable mortalities that may be due to negative interactions between fish and pathogenic bacteria. The use of antibiotics, to prevent these losses, is the commonly adopted solution, not always according to good practices and regulatory and scientific specifications.

The use of antimicrobials in fish intended for human consumption may lead to the presence of residues of the parent compound, and also their metabolites and by-products, in edible parts of the animal, and the risk increases if such antimicrobials are used inappropriately, for example, in an untargeted manner (e.g. mass medication or use on non-susceptible microorganisms), at sub-therapeutic doses, repeatedly, or for inappropriate periods of time.

There are two major concerns arising from these practices related to their effect on consumers' health. First of all, the presence of antimicrobial residues in edible tissues that, in persistent low doses, become part of the consumers' diet, and may also trigger toxic effects in hypersensitive individuals. Secondly, of no less importance, it contributes to the emergence, spread and transference of antimicrobial resistance determinants, which represents nowadays a huge threat to public health worldwide.

In order to protect consumers' health, the European Commission established maximum residue limits for veterinary medicinal products in edible products from animal origin, set performance criteria for the analytical methods employed in official residues control and requires Member States to adopt and implement a national residue monitoring plan for specific groups of residues.

The aim of our work is the development and validation of analytical methodologies, according to the European Commission specifications', for the detection and quantification of antibacterials in aquaculture farmed species, namely in gilthead sea bream, European sea bass and salmon, using multi-residue and multiclass methods, and the subsequent application of those methodologies in real samples purchased in the Portuguese retail market.

In the first chapter – The use of antimicrobials in aquaculture – a bibliographic review is presented on the major aspects regarding current practices and antimicrobials' usage profile in aquaculture industry, legal framework, and public health hazards related to the presence of antimicrobial residues in food.

The second chapter reviews the most recent analytical methodologies concerning the determination of antimicrobial residues in fish, reported in the literature, given emphasis on sample procedures, extraction/purification methods, chromatographic conditions and validation techniques according to legislation.

The third chapter describes the application of a validated multiclass multi-residue ultra-high-performance liquid chromatography coupled with mass spectrometry in tandem methodology for the determination of 41 antibiotics, from seven different classes, in 29 samples of gilthead sea bream of aquaculture origin, purchased in Portugal.

The fourth chapter describes the development and validation of a multiclass multi-residue method for the simultaneous detection and determination of antibacterials in European sea bass muscle. The method was based on ultra-high-performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS), and proved to be a rapid, highly selective and sensitive tool, requiring minimum sample preparation, for the screening and

detection of 47 compounds from eight different classes. The method was applied in 30 samples of farmed European sea bass purchased in different supermarkets in Portugal

The fifth chapter describes the development and validation of a fast and sensitive multi-residue and multiclass screening method, for the simultaneous determination of 44 antimicrobials in salmon muscle, from seven different classes, using ultra-high-performance liquid chromatography-time-of-flight-mass spectrometry (UHPLC-ToF/MS). The method was validated, in accordance with Commission Decision 2002/657/EC, and all the compounds were successfully detected and identified at concentration levels corresponding to ½ maximum residue limit. As in the previous chapters, the validated screening methodology was applied in 39 real samples of farmed salmon purchased in Portugal.

The importance of rapid, sensitive and robust techniques for the screening of antibacterial residues in farmed fish is discussed in the sixth chapter – General Discussion – along with the discussion on the results obtained for our real samples and the importance of reducing the use of these drugs in aquaculture industry, bearing in mind the public health perspective.

Finally, in the last chapter, the distinctive characteristics of the developed and validated methods are highlighted, stressing the need of improvement of these methods, namely by the inclusion of other relevant antimicrobials. Also, it was concluded that an urgent reflexion needs to be undertaken, leading to concrete and major changes in the food producing industry, regarding the use of antimicrobials.

KEYWORDS: Antibiotics; aquaculture; UHPLC-MS/MS; UHPLC-ToF/MS; Antimicrobial Resistance; Public Health

Resumo

A produção de animais destinados ao consumo humano, incluindo a aquacultura, está hoje em dia fortemente alavancada na utilização de antibióticos. Estes fármacos destinam-se ao tratamento e prevenção de doenças infecciosas, muito particularmente na indústria da aquacultura, para evitar a emergência e rápida propagação da infeção.

De facto, a aquacultura é hoje em dia a indústria de produção alimentar com crescimento mais acentuado, contribuindo para cerca de 50% do total de abastecimento de peixe para consumo humano, estimando-se que este valor atinja os 62% em 2030, na medida em que a captura de peixe está progressivamente a diminuir e a procura por parte dos consumidores a aumentar.

Este crescimento exponencial da aquacultura decorre da implementação de sistemas de produção intensivos e semi-intensivos, estando fortemente condicionada por imprevisíveis índices de mortalidade das espécies devido a interações prejudiciais entre o peixe e microrganismos patogénicos. A utilização de antibióticos para prevenir estas perdas é uma solução comumente usada pelos produtores, ainda que nem sempre de acordo com as boas práticas e as especificações científicas e regulamentares.

A utilização de antimicrobianos na produção de peixe destinado ao consumo humano pode resultar na presença de resíduos do fármaco original, ou dos seus metabolitos, em partes edíveis do animal, e o risco aumenta quando estes fármacos são usados de forma inapropriada, como por exemplo quando são utilizados de forma cega (e.g. administração em massa a todos os animais da produção ou uso para tratamento de infeções causadas por microrganismos não sensíveis), em concentrações sub-terapêuticas, de forma repetida ou por períodos de tempo inapropriados.

Estas práticas suscitam duas grandes preocupações, relacionadas com os efeitos na saúde dos consumidores. Em primeiro lugar, a presença de resíduos

do fármaco em tecidos edíveis do animal que, em baixas doses, mas persistentemente, integram a dieta habitual dos consumidores, podendo igualmente despoletar efeitos tóxicos em indivíduos particularmente sensíveis. Em segundo lugar, e não menos importante, esta prática contribui para a emergência, transferência e disseminação de determinantes de resistência microbiana aos antibióticos, que hoje em dia é unanimemente reconhecida como um problema de saúde pública à escala global.

No sentido de proteger a saúde dos consumidores, a Comissão Europeia estabeleceu limites máximos de resíduos para medicamentos veterinários, em tecidos edíveis de origem animal, bem como definiu critérios de desempenho para os métodos analíticos empregues no controlo oficial de resíduos, requerendo aos Estados Membros a adoção e implementação de planos nacionais de pesquisa para determinados grupos de resíduos.

O nosso trabalho teve como objetivo principal o desenvolvimento e validação de metodologias analíticas, de acordo com as especificações emanadas da Comissão Europeia, para a deteção e quantificação de resíduos de antibacterianos em espécies de aquacultura, nomeadamente a dourada, o robalo e o salmão, usando metodologias de determinação multi-classe e multi-resíduo, bem como a aplicação destas metodologias a amostras reais adquiridas no comércio de venda a retalho em Portugal.

No primeiro capítulo – A utilização de antibióticos em aquacultura – é apresentada uma revisão bibliográfica centrada nos aspetos mais relevantes relacionados com as práticas atuais e perfil de utilização de antibióticos na indústria da aquacultura, o enquadramento legislativo, bem como os potenciais riscos para a saúde humana associados à presença de resíduos de antibióticos em alimentos.

No segundo capítulo, é feita uma revisão das mais recentes metodologias analíticas para a determinação de resíduos de antimicrobianos em peixes, com destaque para a preparação da amostra, métodos de extração/purificação, condições cromatográficas e validação das técnicas, em conformidade com a legislação.

O terceiro capítulo descreve a utilização de uma metodologia validada, por cromatografia líquida de elevada eficiência acoplada a detetor de massa sequencial, para a determinação de 41 antibióticos de 7 classes diferentes em 29 amostras de dourada de aquacultura adquiridas em Portugal.

O quarto capítulo descreve o desenvolvimento e validação de um método multi-resíduo e multi-classe para a determinação simultânea de antibióticos em músculo de robalo. O método baseou-se em cromatografia líquida de elevada eficiência acoplada a detetor de massa sequencial (UHPLC-MS/MS), tendo demonstrado características de rapidez de execução, elevada seletividade e sensibilidade, requerendo procedimentos mínimos de preparação da amostra, para a triagem e deteção de 47 moléculas antibacterianas, de 8 classes diferentes. O método foi aplicado em 30 amostras de robalo de aquacultura, adquiridos em diferentes supermercados em Portugal.

O capítulo 5 descreve o desenvolvimento de um método de rastreio multi-resíduo e multi-classe, rápido e sensível, para a determinação simultânea de 44 antibióticos em músculo de salmão, utilizando cromatografia líquida de elevada eficiência acoplada a detetor de massa com analisador por tempo de voo (UHPLC-ToF/MS). O método foi validado, em conformidade com a Decisão da Comissão 2002/657/EC, e todos os compostos foram detetados e identificados a níveis de concentração correspondentes a ½ do limite máximo de resíduo permitido. Tal como nos capítulos anteriores, a metodologia validada foi aplicada a 39 amostras de salmão de aquacultura, adquiridos em supermercados portugueses.

A importância de dispor de técnicas de determinação céleres, sensíveis e robustas, para o rastreio de antibióticos em peixes de aquacultura é discutida no sexto capítulo, juntamente com a discussão dos resultados obtidos na análise das amostras reais das 3 espécies adquiridas no mercado português, bem como a importância de reduzir a utilização destes fármacos na indústria de produção de pescado, tendo em consideração a preservação e a salvaguarda da saúde dos consumidores.

Finalmente, no último capítulo, as características diferenciadoras dos métodos desenvolvidos e validados são realçadas, sublinhando-se a necessidade de

melhoria deste tipo de métodos, nomeadamente no sentido da inclusão de outras classes de antibióticos de relevo. Adicionalmente, conclui-se pela necessidade de ser promovida uma reflexão urgente, que conduza a mudanças estruturais e concretas na indústria de produção alimentar, concretamente no que se refere à utilização de antibióticos.

PALAVRAS-CHAVE: Antibióticos; aquacultura; UHPLC-MS/MS; UHPLC-ToF/MS; Resistências bacterianas; Saúde Pública

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Abbreviations

ACN	Acetonitrile
ADI	Acceptable Daily Intake
AG	Aminoglycosides
AMR	Antimicrobial Resistance
AMRB	Antimicrobial-Resistant Bacteria
AMRG	Antimicrobial Resistance Genes
AMU	Antimicrobial use
CAP	Chloramphenicol
CCα	Decision Limit
CCβ	Detection Capability
CV	Coefficient of Variation
CVMP	Committee for Medicinal Products for Veterinary Use
DSPE	Dispersive Solid-Phase Extraction
EC	European Commission
ECDC	European Centre for Disease Prevention and Control
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
EMA	European Medicines Agency
ESI	Electrospray Ionization
ESVAC	European Surveillance of Veterinary Antimicrobial Consumption
EU	European Union
FAO	Food and Agriculture Organisation of the United Nations
FDA	Food and Drug Administration
FQ	Fluoroquinolones
GC	Gas Chromatography
GC-(NCI)-MS	Gas Chromatography-Negative Chemical Ionization-Mass Spectrometry

GC-ECD	Gas Chromatography-Electro Capture Detection
HGT	Horizontal Gene Transfer
HILIC	Hydrophilic Interaction Chromatography
HPLC	High-Performance Liquid Chromatography
HPLC-PDA	High-performance Liquid Chromatography- Photodiode Array
HRMS	High Resolution Mass Spectrometry
IP	Identification Point
IS	Internal Standard
LC	Liquid Chromatography
LC-(ESI)-MS	Liquid Chromatography-Electrospray Ionization - Mass Spectrometry
LC-(ESI)-MS/MS	Liquid Chromatography-Electrospray Ionization-Mass Spectrometry in tandem
LC-(QToF)-MS/MS	Liquid Chromatography-Quadrupole Time of Flight-Mass Spectrometry in tandem
LC-MSn	Liquid Chromatography Multiple-stage Mass Spectrometry
LC-FLD	Liquid Chromatography-Fluorescence Detection
LC-MS	Liquid Chromatography-Mass Spectrometry
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantification
LRMS	Low Mass Resolution Spectometry
m/z	mass to charge ratio
ME	Matrix Effects
MIC	Minimum Inhibitory Concentration
MRL	Maximum residue limit
MRM	Multi-reaction Monitoring
MRPL	Minimum Required Performance Limit
MS	Mass spectrometry
MS/MS	Mass spectrometry in tandem methodology
MSn	Multiple-stage Mass Spectrometry
NACA	Network of Aquaculture Centres in Asia-Pacific

OIE	Organization International des Epizooties
PDA	Photodiode Array Detection
PLE	Pressurised Liquid Extraction
QqQ-MS	Triple-stage quadupole mass spectometer
QToF	Quadrupole Time of Flight
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, and Safe extraction
RRT	Relative Retention Time
RSD	Relative Standard Deviation
RT	Retention Time
S/N	Signal-to-Noise ratio
SA	Sulfonamides
SPE	Solid Phase Extraction
SPE-LC-(ESI)-MS/MS	Solid-phase Extraction coupled to Liquid Chromatography-Electrospray Ionization-Mass Spectrometry in tandem
TC	Tetracycline
ToF	Time-of-flight
ToF MS	Time-of-flight Mass Spectrometer
UHPLC	Ultra-high-performance liquid chromatography
UHPLC-MS/MS	Ultra-high-performance Liquid Chromatography-Mass Spectrometry in tandem
UN	United Nations
UV	Ultra-Violet
VL	Validation Level
VMP	Veterinary Medicinal Products
WHO	World Health Organization

General Introduction and Thesis Outline

The quality of food products, and its repercussion on the population's health and well-being, is a matter of growing concern for consumers and health authorities, and of utmost importance regarding public health. Special attention has been given to foods of animal origin, since several events concerning its contamination, with different substances and contaminants, have been widely disseminated.

In the White Paper on Food Safety [1], it is assumed that the European Union's food policy must be built around high food safety standards, which serve to protect, and promote, the health of the consumer and, furthermore, that the health and welfare of food producing animals is essential for public health and consumer protection.

Only a coordinated and holistic approach to the production process allows the guarantee that those standards are effectively achieved, as food safety questions are increasingly addressed as a continuum from the farm to the table.

The real circumstances though, differ from the theoretical principles that have been drafted to safeguard quality and food security. Socio-economic factors, related to the strong demographic growth that characterized the period after the Second World War, led to changes in animal production systems, in order to provide enough food supply, at the lowest cost possible. Consequently, the small family-type farms have been progressively replaced by industrial systems, of medium to large dimensions, promoting the intensive production of animals intended for human consumption.

These modern systems are characterized by high concentration of animals in small spaces of land or water, substantially increasing the risk of infection and contagion, providing no proper conditions to the animals' well-being and balanced development. In this context, one of the core components in which those systems lay are veterinary drugs, mainly antibiotics [2].

The use of antibiotics has become a cornerstone for the sustainability of these activities, where the intensive conditions in which the animals are raised couldn't be more favourable to the rapid emergence and spread of infectious diseases. In face of the consequences that an outbreak of an infectious disease causes in these intensive farming systems, it became a current practice to administrate sub-therapeutic doses of antimicrobials to the animals' feed or water, for prophylaxis and/or prevention proposes, sometimes during the entire life-cycle.

The use of these drugs aims to assure that healthy animals enter the food chain, as good husbandry and aquaculture practices are often lacking which could, almost by themselves, promote animals' health.

The other side of the medal raises several concerns, though, mainly related to the effect on the consumers' health.

First of all, the presence of antimicrobial residues in edible animal tissues which, in low doses but persistently, take part of the consumers' daily diet. We could state that the notorious Hippocrates' motto "*Let food be your medicine*" can, nowadays, be assumed in the most literal sense.

Secondly, and of utmost importance, it is widely recognized, nowadays, that the use of antimicrobials in food producing animals has a major contribution to the emergence and spread of antimicrobial resistance (AMR), which represents a huge threat to public health worldwide, recognized by healthcare professionals, governments, World Health Organization (WHO) and several other agencies and organizations [3-8].

The need to provide safe and proper food to a growing global population - 9.8 billion people by 2050 [9] - in the context of increasing demand and competition for natural resources and climate change, has intensified the importance of the aquaculture industry. The importance of this issue was recognized by the international community which assumed an unprecedented commitment in September 2015, when the United Nations (UN) Member States adopted the 2030 Agenda for Sustainable Development [10], which highlights the contribution of fisheries and aquaculture to food security and nutrition in the use of natural resources and to ensure social, economic and environmental development.

Aquaculture is growing more rapidly than any other animal production sector. And the magnitude of its recent growth is very high: annual aquaculture production more than quadrupled in two decades, from 16.8 million tons in 1990 to 80 million tons in 2016 [11]. The rapid transition from a species capture model to a production model was a necessary response to the market needs because increased marine pollution and overfishing, along with global climate change, have greatly affected fish stocks.

On the other hand, world fish consumption increased from 121 million tonnes in 2008 to 140 million tonnes in 2013 [12]. Ninety percent of the growth was contributed by aquaculture. Looking into the future, growing and wealthier populations would continue to demand more fish, and aquaculture growth is expected to be the major force to satisfy this demand [13-17].

The intensive aquaculture production methods vary significantly throughout the world, but broadly speaking, most aquaculture facilities rely heavily on the use of antimicrobials, and other agrochemicals, resulting in the presence of many chemical and biological contaminants in fish and aquaculture facilities [18]. And the risk of emergence of bacterial infections in fish species is very high, as a result of the lack of good hygienic practices, along with the stressful conditions to which fish are exposed – including high fish densities, high farm densities in coastal waters and lack of appropriate barriers between farms [19]. Consequently, large amounts of antimicrobials are used in aquaculture facilities worldwide.

AMR in pathogens of aquatic animals has been reported from different systems. In shrimp hatcheries, mass mortalities due to antibiotic resistant luminous bacteria (*Vibrio* spp.) can be a problem [20] and acquired resistance in *Aeromonas salmonicida* causing furunculosis in temperate waters has been reported from several countries [21]. Furthermore, different mobile genetic elements like plasmids, transposons and integrons, carrying antimicrobial resistant genes, have been detected in *Aeromonas* spp. from aquaculture sites in different parts of the world [22], and over 80 percent of *Vibrio harveyi* from finfish aquaculture systems in Italy showed resistance to amoxicillin, ampicillin and erythromycin, while 76 percent of strains showed resistance to sulphadiazine [23]. It is, therefore, reasonable to assume that AMR in bacterial pathogens of aquatic animals could

impact disease management in these systems and the resistance determinants could be transferred to human pathogens from aquatic systems.

Although AMR is observed in aquatic bacteria associated with aquaculture systems, it is difficult to establish a direct link between the resistance profile and antimicrobial use. Culture-independent studies in the Baltic Sea show the presence of resistance genes encoding resistance to sulphonamides, trimethoprim, tetracycline, aminoglycoside, chloramphenicol and also genes encoding multidrug efflux pumps in sediments of fish farms, although some antibiotics like tetracyclines, aminoglycosides and chloramphenicol are not used in this area [24]. It is accepted that some of these might represent a natural reservoir of resistance genes in the aquatic environment. Antibiotic resistant marine bacteria, for instance, have been found as far as 522 km offshore and in deep sea at depths of 8200m [25].

It is clear, therefore, that source attribution of AMR in aquaculture associated bacteria is very complex and caution needs to be exercised in interpretation of data.

On the other hand, though, the scientific evidence that has been produced requires that, in this matter, regulatory authorities act based on the precautionary principle, which can be enforced in situations where scientific knowledge assumes that a potential risk may arise from a certain practice.

The precautionary principle appears, indeed, in the Maastricht Treaty [26] which committed the European Union to be founded in compliance to such matrix.

In Regulation (EC) n.º 178/2002 [27] of the European Parliament and of the Council, laying down the general principles and requirements of food law, precautionary principle is enshrined as follows: "*In those specific circumstances where a risk to life or health exists but scientific uncertainty persists, the precautionary principle provides a mechanism for determining risk management measures or other actions in order to ensure the high level of health protection chosen in the Community*."

As referred previously, the world's fish consumption has been steadily increasing over the last decades, mainly due the acknowledgement of the fish's nutritional value, and this growth is almost entirely reliant on aquaculture [12].

Portugal has maintained its position as the biggest per capita fish consumer in the European Union (EU), steadily increasing its consumption from 29 kg per capita in 1980 to 57 kg per capita in 2011 [12]. Most other countries have increased their per capita consumption levels as well. For example, France, Germany, Spain, Finland, Italy and the Netherlands, among others, increased their consumption by between 50% and 120% from 1961 until 2011 [12].

It is, therefore, vital to guarantee all aspects related to fish's quality and safety, and the present work forms part of this broad subject of food security, particularly regarding aquaculture fish.

Thus, in the first chapter – The use of antimicrobials in aquaculture – a bibliographic review is presented on the major aspects regarding current practices and antimicrobials' usage profile in aquaculture industry, legal framework, and public health hazards related to the presence of antimicrobial residues in food. In this later aspect, we focused on the toxicity, influence on the human microbiota and environmental impact resulting from the use of antimicrobials in aquaculture but, above all, our main focus was AMR, and how the use of these drugs in aquaculture may worsen this global health problem. Finally, alternatives for reducing the use of antimicrobials in aquaculture were addressed, with evidence on the efficacy, associated risks and knowledge gaps.

The second chapter reviews the most recent analytical methodologies concerning the determination of antimicrobial residues in fish, reported in the literature, given emphasis on sample procedures, extraction/purification methods, chromatographic conditions and validation techniques according to legislation. The chapter highlights the use of liquid chromatography (LC) combined with tandem mass spectrometry (MS) detection as the preferred technique in this field, highlighting the advantages of using multi-detection and multiclass screening methods in routine analysis.

The subsequent three chapters provide the description of the laboratory work that was developed to validate analytical methodologies for the detection and quantification of antibiotics in three of the most consumed aquaculture species in Portugal - gilthead sea bream, European sea bass and salmon. In all of them, the validated methodology was applied in real samples, purchased in the Portuguese retail market, as our main focus is to get a global picture on the overall quality of the aquaculture fish consumed in Portugal, particularly with regard to the presence of antimicrobial residues.

So, the third chapter describes the use of a validated multiclass multi-residue ultra-high-performance liquid chromatography (UHPLC) coupled with mass spectrometry in tandem methodology (MS/MS) for the determination of 41 antibiotics, from seven different classes, in 29 samples of gilthead sea bream of aquaculture origin, purchased in Portugal. In 27,6% of the analysed samples, antibiotic residues were present, including doxycycline, antibiotic for which no maxim residue limit (MRL) is established.

The fourth chapter describes the development and validation of a multiclass multi-residue method for the simultaneous detection and determination of antibacterials in European sea bass. The method was based on UHPLC-MS/MS, and proved to be a rapid, highly selective and sensitive tool, requiring minimum sample preparation, for the screening and detection of 47 compounds from eight different classes. The validation was performed according Regulation 2002/657/EC [28], proving the method's suitability for application in routine analysis, which was subsequently applied in 30 samples of farmed European sea bass purchased in different supermarkets in Portugal. Antibacterial residues were detected in 6 out of 30 analysed samples, and in one of them two different residues were detected, raising some concerns regarding possible cumulative effects, or synergistic potentiation, of both substances' toxicities.

The fifth chapter describes the development and validation of a fast and sensitive multi-residue and multiclass screening method, for the simultaneous determination of 44 antimicrobials in salmon muscle, from seven different classes, using UHPLC-time-of-flight (ToF)-MS. The method was validated, in accordance with Decision 2002/657/EC [28], and all the compounds were

successfully detected and identified at concentration levels corresponding to ½ MRL. The validation proved that the method exhibits suitable characteristics, such as sensitiveness, robustness and speed, to be used in routine analysis. As in the previous chapters, the validated screening methodology was applied in 39 real samples of farmed salmon purchased in Portugal, originating mainly from Norway, and no antibiotic residues were detected.

The sixth chapter discusses the relevant and innovative features of our methods, justifying their importance as interesting tools in food control. In fact, the safeguard and protection of the consumers' health and, on the whole, the public health, implies not only appropriate regulation but also, in addition and equally important, effective monitoring and surveillance, being therefore of utmost importance to have proper analytical methods, able to detect residual concentrations of the analytes, and at the same time being sufficiently fast to assure that it is possible to put in practice an effective surveillance plan.

This chapter also stresses the need to promote alternative measures to control infection in the aquaculture industry, advocating the need of coordinated and concerted actions that lead to major changes in the food producing industry, regarding the use of antimicrobials.

Finally, in the last chapter, the general conclusions of our work are drawn, highlighting the need to promote the shift, in the aquaculture industry, from an antimicrobial based model, to a new paradigm based on the principles of good aquaculture practices.

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Chapter 1 | The Use of Antimicrobials in Aquaculture

1.1. Practices and antimicrobials' usage profile in aquaculture industry

Aquaculture is being practiced for centuries and, although the clear history of its origin is relatively unknown, there is evidence that this practice was present in Egypt and China as early as 2500 B.C. and 1100 B.C., respectively [1].

Initially the world's fish demands were met by wild-caught species, but the rapid transition to a production model was a necessary response to the market needs', since increased marine pollution and overfishing, along with global climate change, have greatly affected fish stocks.

Traditional aquaculture systems relayed in minimal inputs and were characterized by small ponds and low stock density. The rapid population growth, along with the rising consumer demand for fish, and seafood products, though, conducted the aquaculture industry to adopt intensive production models.

As demand for aquaculture products increases, this industry continues to intensify its production methods, characterised by high stock density and volume, and the heavy use of formulated feeds containing antimicrobials, antifungals and other pharmaceutical products, along with the extensive use of pesticides and disinfectants [1-3].

The magnitude of the recent growth in aquaculture is very high: annual aquaculture production more than quadrupled in two decades, from 16.8 million tons in 1990 to 80 million tons in 2016 [4] (Figure 1).

The current trend towards increasing intensification and diversification of global aquaculture has led to its dramatic growth, thus making aquaculture an important food-producing sector that provides an essential source of aquatic protein for a growing human population.

The global aquaculture industry is located primarily in just a few Asian countries, representing 89.40% of total global production, with China alone accounting for approximately 61,5% of the total aquaculture production worldwide [4] (Table 1), being also the largest exporter of fish and fishery products [4] (Tables 2a and 2b).

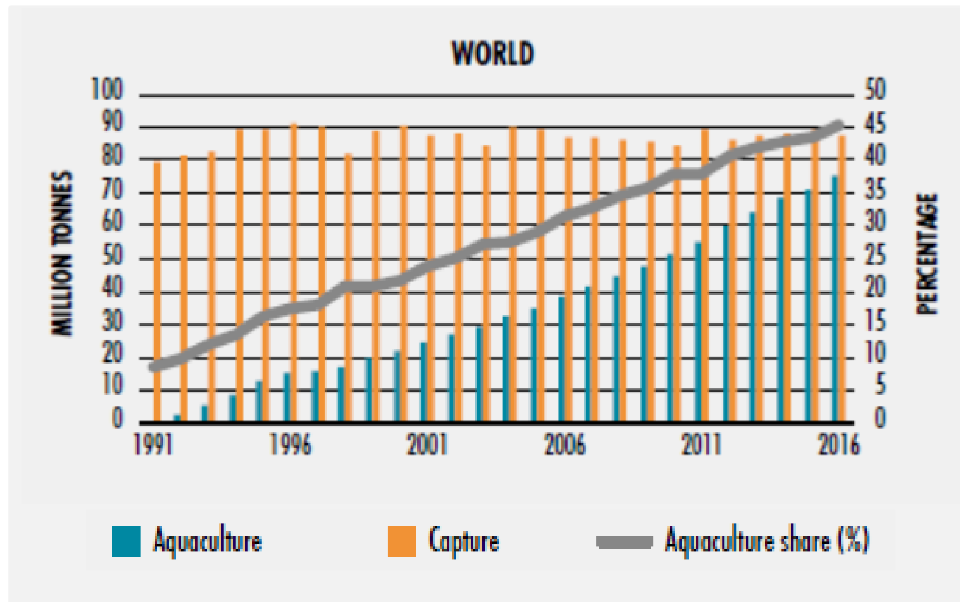

Figure 1. Share of aquaculture in total production of aquatic animals (adapted from FAO, 2018[4])

The intensive aquaculture production methods vary significantly throughout the world, but broadly speaking most aquaculture facilities rely heavily on the use of antimicrobials, and other agrochemicals, resulting in the presence of many chemical and biological contaminants in fish and aquaculture facilities [1] (Table 3).

As a result of the lack of good hygienic practices, along with the stressful conditions to which fish are exposed – including high fish densities, high farm densities in coastal waters and lack of appropriate barriers between farms – the risk of emergence of bacterial infections in fish species is very high [3,5-6]. Consequently, large amounts of antimicrobials are used [7-9]. The use of these drugs can be categorized as therapeutic, prophylactic or metaphylactic. The therapeutic use corresponds to the treatment of established infections, while metaphylactic use is a term used for group-medication procedures, including sick and healthy animals, and the prophylactic use aims to prevent the development of infections [3]. In aquaculture facilities, the most common route for the administration of antimicrobials is the oral route via medicated feed, and antimicrobials are mostly used in metaphylactic treatment [3,10].

Table 1. Aquaculture production by region and selected regional major producers: quantity and percentage of world total production (adapted from FAO, 2018 [4])

Regions and selected countries		2000	2016
AFRICA	*Thousand tonnes*	400	1 982
	Percentage (%)	1.2	2.5
AMERICAS	*Thousand tonnes*	1 423	3 348
	Percentage (%)	4.4	4.2
Chile	*Thousand tonnes*	392	1 035
	Percentage (%)	1.2	1.3
North America	*Thousand tonnes*	585	645
	Percentage (%)	1.8	0.8
ASIA	*Thousand tonnes*	28 423	71 546
	Percentage (%)	87.7	89.4
China (mainland)	*Thousand tonnes*	21 522	49 244
	Percentage (%)	66.4	61.5
Indonesia	*Thousand tonnes*	789	4 950
	Percentage (%)	2.4	6.2
Viet Nam	*Thousand tonnes*	499	3 625
	Percentage (%)	1.5	4.5
India	*Thousand tonnes*	1 943	5 700
	Percentage (%)	6.00	7.1
EUROPE	*Thousand tonnes*	2 051	2 945
	Percentage (%)	6.3	3.7
Norway	*Thousand tonnes*	491	1 326
	Percentage (%)	1.5	1.7
WORLD	*Thousand tonnes*	32 418	80 031

The European aquaculture includes around 35 distinct species, and uses a variety of production systems, from extensive to intensive, in natural settings or tanks, in fresh water or sea water, in cold, moderate or warm water, in flow-through or recirculation systems, traditional or modern, classic or organic, sheltered or exposed, etc. [10]. The largest production section is based on marine cold-water fish species, mainly Atlantic salmon, trout and cod, followed by freshwater species, like trout and carp, and marine Mediterranean species, especially sea bass, sea bream and turbot [11].

The total European production of aquaculture fish is estimated to be 2,327,082 tons in 2016 [11], with marine cold-water species representing 70% of the total production, freshwater species 16% and marine Mediterranean species 14% [11]. Norway is the leading European aquaculture producer, with 58% of the total

supply, in particular Atlantic salmon – 1,301,000 tons in 2015 – but also large Rainbow trout – 80,000 tons in 2015 [12].

Table 2a. Top Ten exporters of fish and fishery products (adapted from FAO, 2018 [4])

	2006	**2016**	**APR***
COUNTRY	***US$ millions (Share%)***		***Percentage***
China	8 968 (10.4)	20 131 (14.1)	8.4
Norway	5 503 (6.4)	10 770 (7.6)	6.9
Viet Nam	3 372 (3.9)	7 320 (5.1)	8.1
Thailand	5 267 (6.1)	5 893 (4.1)	1.1
USA	4 143 (4.8)	5 812 (4.1)	3.4
India	1 763 (2.0)	5 546 (3.9)	12.1
Chile	3 557 (4.1)	5 143 (3.6)	3.8
Canada	3 660 (4.2)	5 004 (3.5)	3.2
Denmark	3 987 (4.6)	4 696 (3.3)	1.7
Sweden	1 551 (1.8)	4 418 (3.1)	11.0
Top ten subtotal	**41 771 (48.4)**	**74 734 (52.4)**	**6.0**
World Total	86 293 (100)	142 530 (100)	5.1

* Note: APR refers to the average annual percentage growth rate for 2006 – 2016

Table 2b. Top Ten importers of fish and fishery products (adapted from FAO, 2018 [4])

	2006	**2016**	**APR***
COUNTRY	***US$ millions (Share%)***		***Percentage***
USA	14 058 (15.5)	20 547 (15.1)	3.9
Japan	13 971 (15.4)	13 878 (10.2)	-0.1
China	4 129 (4.5)	8 783 (6.5)	7.9
Spain	6 359 (7.0)	7 108 (5.2)	1.1
France	5 069 (5.6)	6 177 (4.6)	2.0
Germany	4 717 (5.2)	6 153 (4.5)	2.7
Italy	3 739 (4.1)	5 601 (4.1)	4.1
Sweden	2 028 (2.2)	5 187 (3.8)	9.8
Rep. of Korea	2 753 (3.0)	4 604 (3.4)	5.3
United Kingdom	3 714 (4.1)	4 210 (3.1)	1.3
Top ten subtotal	60 533 (66.6)	82 250 (60.7)	3.1
World Total	**90 871 (100)**	**135 037 (100)**	**4.0**

* Note: APR refers to the average annual percentage growth rate for 2006 – 2016

The major aquaculture producers, though, are based in Asia, with China alone being responsible for the supply of 49.3 million tonnes of farmed fish in 2016,

which represents 61.5 percent of global fish production from aquaculture [4], as referred previously. Other major producers are India, Viet Nam, Bangladesh and Egypt [4].

Table 3. Reported antibiotic usage by the top aquaculture-producing countries (adapted from Sapkota, et al., 2008 [1])

	MOLECULE	**CH**	**IN**	**JPN**	**PHIL**	**IND**	**TH**	**CHL**	**NOR**	**VIET**	**USA**
SUL	Sulfamerazine				●						●
	Sulfadimethoxine			●	●						●
PSUL	Trimethoprim and sulfadiazine		●		●		●		●	●	
TET	Chlortetracycline	●	●				●				
	Oxytetracycline	●	●	●	●	●	●		●	●	●
PEN	Ampicillin			●				●		●	
	Amoxycillin			●				●			●
	Benzyl penicillin	●		●					●		
QUIN	Ciprofloxacin						●				
	Enrofloxacin					●	●			●	
	Norfloxacin						●			●	
	Oxolinic acid	●		●	●		●	●	●	●	
	Flumequine			●					●	●	
	Sarafloxacin										
NIT	Furazolidone	●			●			●	●		
MAC	Erythromycin	●		●	●	●	●	●			
AMIN	Gentamicin				●		●	●			
OTS	Chloramphenicol	●	●		●	●	●	●		●	
	Florfenicol			●				●	●		
	Nalidixic acid			●	●			●			
REPORTED # OF ANTIBIOTICS USED BY COUNTRY		**7**	**4**	**10**	**10**	**4**	**10**	**9**	**7**	**8**	**4**

SUL – Sulfonamides; **PSUL** - Potentiated Sulfonamide; **TET** – Tetracyclines; **PEN** – Penicillins; **QUIN** – Quinolones; **NIT** – Nitrofurans; **MAC** – Macrolides; **AMIN** – Aminoglycosides; **OTS**- Others

CH – China; **IN** – India; **JPN** – Japan; **PHIL** – Philippines; **IND** – Indonesia; **TH** – Thailand; **CHL** – Chile; **NOR** – Norway; **VIET** – Viet Nam; **USA** – United States of America

Although China has a long history of aquaculture, the large-scale production only began after the founding of the People's Republic of China in 1949. More recently,

after China opened to the outside world in the 1980's, the sector has been growing dramatically, becoming one of the fastest growing sectors among the agriculture industries in China [13].

Looking closer at the Chinese's systems of culture, pond culture is the most popular and important farming system, accounting for an estimated 70.54% of all inland aquaculture output in 2003 [14]. And, nowadays, Chinese fish farmers not only practice intensive culture in pond systems but have also used this method in open-waters such as reservoirs, lakes, rivers and channels, by using cages, net enclosures and pens. Along with freshwater aquaculture, also marine and brackish water aquaculture have been growing rapidly over the last two decades together with diversified culture systems from ponds to floating rafts, pens, cages (inshore, offshore and submerged), indoor tanks with water re-circulation, sea bottom culture and sea ranching [14].

About 50 commercially important freshwater species are cultured in China, the most common being carps, Chinese bream and blunt-snout bream [15]. More recently, in response to international market demand, various species have been developed or introduced from abroad for commercial cultivation in China, such as Japanese eel (*Anguilla japonica*), sturgeon (*Acipenser sturio*), crawfish, tilapia or rainbow trout (*Oncorhynchus mykiss*), among others. In 2003 China produced a total of 17,782,734 tonnes of freshwater aquaculture products [14,15]. Traditional marine culture is largely limited to four groups of molluscs, representing nearly 80% of the total marine culture production in 2003 [14].

The intensification of culture methods, and the diversification of cultured species and culture techniques, in modern aquaculture, provides the ideal setting for the emergence of pathogens. The global movements of live aquatic animals, occasionally irresponsible, often result in the transboundary spread of a wide variety of disease agents that, in some circumstances, lead to serious losses in food productivity. Additionally, these practices resulted in serious pathogens becoming endemic in culture systems and the natural aquatic environment [16].

The most important bacterial diseases in aquaculture are presented in Table 4. Table 5 presents the first line antimicrobial agents used for each indication, with the correspondent dosage and withdrawal period. For fish, withdrawal period is a

function of time and temperature and is expressed in degree-days, calculated by multiplying the total number of days needed to reach safe concentration by the mean daily water temperature (in degrees Celsius). A withdrawal period of 400 degree-days, for instance, corresponds to 40 days at a water temperature of 10 degrees Celsius or to 20 days at a water temperature of 20 degrees Celsius.

The expansion of commercial aquaculture unavoidably brought the need of routine use of veterinary medicines to prevent and treat disease outbreaks, assure healthy animals, maximize production and consequently protect public health. The use of appropriate antimicrobial treatments is one of the most effective responses to deal with emergencies associated with infectious disease epizootics. However, the inappropriate use of these drugs is the leading cause for the increased frequency of the emergence, transference and spread of antimicrobial resistant bacteria and antimicrobial resistant genetic determinants. Disease outbreaks can occur at any time, even in well-managed aquaculture operations, but a careful planning, and the prudent and responsible use of veterinary medicines, is a prior essential step to maximize their efficacy, assure aquaculture sustainability and promote consumers' health.

Despite the widespread use of antimicrobials in aquaculture production systems, very scarce and limited data is available on the types and quantity of antibiotics used in this industry. Besides, the most reliable data that is available, generally is originated in developed countries, while the aquaculture industry is dominant in developing countries, where scare data exist and little to no enforcement is present.

In aquaculture, antimicrobials are usually used to treat bacterial and parasitic diseases, and its usage pattern varies between countries and production systems [10,19].

In the United States, the production of catfish, salmon and trout is estimated to consume 92,500 to 196,400 Kg, annually [20], and in the United Kingdom, 2 tonnes of antimicrobials (mainly tetracyclines and potentiated sulphonamides) were used in salmon and trout production, in 2000 [2].

Table 4. Most important bacterial diseases in aquaculture (adapted from Reantaso, 2017 [17])

GRAM-NEGATIVE BACTERIA	GRAM-POSITIVE BACTERIA
Vibriosis (*V. anguillarum**, *V. harveyi clade**, *V. parahaemolyticus**, *Aliivibrio salmonicida* (*V. salmonicida*), *V. vulnificus**, *Photobacterium damselae**)	**Mycobacteriosis** (*Mycobacterium fortuitum**, *M. marinum**, *Nocardia asteroides**, *N. crassostreae* (*ostreae*), *N. seriolae**)
Aeromonasis (Motile *Aeromonas* spp.**:** *Aeromonas caviae**, *A. hydropila**, *A. sobria**, *A. veronii**, *A. jandaei**; *A. salmonicida*)	**Streptococcosis** (*Streptococcus agalactiae**, *S. iniae**, *Lactococcus garvieae*, Aerococcus viridans**)
Edwardsiellosis (*Edwardsiella anguillarum**, *E. ictaluri**, *E. piscicida**, *E. tarda**, *Yersinia ruckeri**)	**Renibacteriosis** (*Renibacterium salmoninarum*)
Pseudomonasis (*Pseudomonas anguilliseptica**, *P. fluorescens**)	**Infection with Anaerobic Bacteria** (*Clostridium botulinum**, *Enterobacterium catenabacterium*)
Flavobacteriosis (*Flavobacterium branchiophilum*, *F. columnare**, *F. psychrophilum*, *Tenacibaculum maritinum*)	
Infection with intracelular bacteria (*Piscirickettsia salmonis*, *Hepatobacter penaei*, *Francisella noatunensis**, *Chlamydia spp.*)	

* Important mainly for tropical regions

In countries where control is less stringent or lacking, antimicrobial use in aquaculture may surpass the use in human medicine [21,22], and differences between countries are very pronounced. In Chile, the second largest producer of cultured salmon after Norway, 385 and 325 tonnes of antimicrobials were used in salmon aquaculture production in 2007 and 2008, respectively, and 149 and 57 tonnes of quinolones were used in those years, respectively [23]. According to the same authors, the annual consumption of florfenicol rose from approximately 400 kg in 2000 to 233,000 kg in 2007. The quantity of antimicrobials used to produce 1 tonne of salmon, as reported by one industrial production facility in Chile, was 279 g, while only 4.8 g was used to produce the same amount of salmon in Norway [19].

Regarding China, the largest producer and exporter of aquaculture products worldwide, there are no relevant, nor reliable, data regarding antibiotic usage in aquaculture [24].

Table 5. First line antimicrobial agents used for each indication, with the correspondent dosage and withdrawal period (adapted from Zrnčić, 2017 [18])

Antibiotic	Dosage *(mg/kg/bw*/day in feed)*	Indication	Withdrawal period *(ºC-day)*
Oxytetracycline	60-80	Vibriosis, aeromoniasis, edwardsielosis, flavobacteriosis, tenacibaculosis, francisellosis, streptococcosis, lactococcosis	400-600
Trimethoprim/Sulphafurazol	50	Vibriosis, aeromoniasis, edwardsielosis, pseudomoniosis, tenacibaculosis	350
Quinolones (oxolinic acid, nalidixic acid, flumequine)	12-50	Vibriosis, aeromoniasis, edwardsielosis, pseudomoniosis, flavobacteriosis, photobacteriosis	80
Florfenicol	10-30	Aeromoniasis, edwardsielosis, yersiniosis, flavobacteriosis	150
Erythromycin	100	BKD, mycobacteriosis, streptococcosis, lactococcosis	700
Fluoroquinolones	25-40	Tenacibaculosis, francisellosis, lactococcosis	500
Amoxycillin	40-80	Furunculosis, streptococcosis, lactococcosis	500

*Bw – body weight

Yet, Liu et al. [25] reached some worrying conclusions based on peer-reviewed papers, documents, reports, and farmer surveys. Based on their results, a total of 20 antibiotics belonging to eight categories (aminoglycosides, β-lactams, chloramphenicol, macrolides, nitrofurans, quinolones, sulfonamides, and tetracyclines) have been reported for use, mainly via oral administration. However, only 13 antibiotics have been authorized for application in Chinese aquaculture, and 12 of the reported antibiotics are not authorized (e.g. amoxicillin,

chloramphenicol, chlortetracycline, ciprofloxacin, erythromycin, furazolidone, gentamycin S, oxytetracycline, penicillin G, streptomycin, sulfamerazine S, and sulfisoxazole).

Aquaculture is a modern tool, with the potential to succeed and thrive as a sustainable, profitable business. One must keep in mind, though, that the misuse and unrestricted use of antibiotics can lead to public health problems and environmental hazards that, in short to medium term, can turn this industry unsustainable.

1.2. Public health hazards related to the presence of antimicrobial residues in food

1.2.1. Toxicity

The inappropriate use of antibiotics in aquaculture industry can pose several human health and food safety concerns, that remain largely unaddressed in most world's developing nations. One of the primary consequences is the presence of drug residues in edible fish tissues, that, even in very low concentrations, can pose serious risks to human health.

When used according to label instructions, the use of antimicrobials should not result in residues at slaughter. There are, though, several reasons that can determine the presence of drug residues in edible tissues, including non-adherence to recommended label directions or dosage (extra-label usage), non-observance of recommended withdrawal periods, use of antibiotic-contaminated equipment, failure to properly clean equipment used to mix or administer drugs, mixing errors or even animal effects, such as age, pregnancy, congenital illness, and allergies, chemical interactions between drugs, variations in water temperature for aquatic species, environmental contamination and improper use of drugs [26].

The extra-label use of antibiotics in aquaculture is frequent, but supposed to be supervised by veterinarians, in order to prevent that animals are slaughtered before complete metabolization and/or excretion of the drug.

Even with trace residues, some individuals, particularly sensitive to certain antibiotics, can experience allergic reactions, and the identification of the allergen may be hindered by a lack of knowledge of the substance, molecule or food that triggered it [27]. Allergic reaction is one type of adverse drug reaction and the common symptoms are urticaria, angioneurotic edema, gastrointestinal reactions, aplastic anemia and, in more serious cases, shock and death [28,29]. Penicillin allergy is the most commonly reported medication allergy, with a prevalence rate of 5% to 10% [29].

A large proportion of antibiotics (e.g., penicillin G., tetracycline, and sulfonamides) have antigenicity and the consumption of contaminated aquatic products may cause allergic symptoms [25].

Chronic toxicity is another type of adverse drug reaction, that arises from the accumulation of the antibiotic in the human body, causing organ lesions through low dose consumption over a long period of time [25]. Quinolones and tetracyclines, two relevant antibiotic categories used in aquaculture, may influence the development of children's teeth [30].

Chloramphenicol residues, for example, lead to an increased risk of developing cancer, and this drug is linked to the development of non-dose-related aplastic anaemia in humans [31], reason why the drug is banned for use in food-producing animals in the European Union [32] and in many other countries, including the United States, Canada, Australia, Japan and China [33].

Erythromycin may cause deafness and peripheral neuritis, and furazolidone may lead to hemolytic anemia and polyneuritis [25]. Other known toxic effects include immunopathological effects and carcinogenicity by sulphamethazine, oxytetracycline, and furazolidone [26], and mutagenicity and nephropathy by gentamicin [30].

Also, fluoroquinolones (FQ) residues may have carcinogenic properties [34], and other adverse events of these molecules, involving the central nervous system

(e.g., dizziness, headache, seizures, psychosis). Less recognized, but with a growing rate of notification and evidence, are FQ-associated peripheral neuropathies [35].

Moreover, there are some antibiotics whose metabolites may turn more toxic than the original drug [1,30,36].

Although not underestimating the importance of this issue, the Food and Agriculture Organisation of the United Nations (FAO), the Organization International des Epizooties (OIE; World Organization for Animal Health) and the WHO, in a consultation on scientific issues related to non-human use of antimicrobial agents, held in Geneva, Switzerland, in December 2003 [37], concluded that the toxicological effects resulting from the intake of antibiotic residues present in food, under present regulatory regimes, represents a significantly less important human health risk than does the risk posed by the development and spread of antimicrobial-resistant bacteria in food.

1.2.2. Antimicrobial resistance

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Abstract

Aquaculture is a rapidly growing industry that currently accounts for almost half of the fish used for human consumption worldwide. Intensive and semi-intensive practices are used to produce large stocks of fish, but frequent disease outbreaks occur, and the use of antimicrobials has become a customary practice to control them. The selective pressure exerted by these drugs, which are usually present at sub- therapeutic levels for prolonged periods in the water and the sediments, provides ideal conditions for the emergence and selection of resistant bacterial

strains and stimulates horizontal gene transfer. It is now widely recognized that the passage of antimicrobial resistance genes and resistant bacteria from aquatic to terrestrial animal husbandry and to the human environment and vice versa can have detrimental effects on both human and animal health and on aquatic ecosystems. A global effort must be made to cease antimicrobial overuse in aquaculture and encourage stakeholders to adopt other disease-prevention measures. Shaping a new path is crucial to containing the increasing threat of antimicrobial resistance.

Introduction

The need to provide adequate and safe food to a growing global population - 9.8 billion people by 2050 [38] - in the context of increasing demand and competition for natural resources and climate change, has intensified the importance of the aquaculture industry. The importance of this issue was recognized by the international community which assumed an unprecedented commitment in September 2015, when the UN Member States adopted the 2030 Agenda for Sustainable Development [39], which highlights the contribution of fisheries and aquaculture to food security and nutrition in the use of natural resources and to ensure social, economic and environmental development. Aquaculture is growing more rapidly than any other animal-production sector. Its relative contribution to the total amount of fish produced for human consumption changed from 5% in 1962 to 44.1% in 2014 [40], a year in which a total of 580 species and/or species groups were farmed around the world [40]. The rapid transition from a species capture model to a production model was a necessary response to the market needs because increased marine pollution and overfishing, along with global climate change, have greatly affected fish stocks.

While remembering the advantages of aquaculture, we must not forget the other side of this reality. The rapid growth of aquacultural production raises several concerns related to the quality and safety of fish. Similar to other sectors of animal production, fish production also uses intensive and semi-intensive practices, leading to a higher concentration of animals in small spaces of water, substantially increasing the risk of contagious diseases [41]. In fact, disease is one

of the major disadvantages of aquaculture; fish species reared under crowded and stressful conditions are more susceptible to bacterial infection, causing major stock losses, and the prophylactic and therapeutic use of antimicrobials and chemical disinfectants is currently employed to control disease outbreaks [9]. Thus, appropriate regulations and supervision are required to avoid the inappropriate use of substances that can result in negative consequences to consumers' health. The regulatory framework regarding the use of antibiotics in aquaculture is limited, differs greatly between countries, and little to no enforcement is present in many of the major producers of aquacultural products [42], such as in China [43]. Antimicrobials are used in aquaculture mainly for prophylactic purposes and metaphylactic treatment [27], and since there are no antibiotics specifically designed for aquaculture, authorized products developed for other areas of veterinary medicine are used. This pattern of use, along with the overuse of these drugs in aquaculture, leads to the selection of aquatic antimicrobial-resistant bacteria (AMRB), which contaminate edible products marketed for human consumption [9,27,44]. In fact, the same resistance patterns seen in land animal husbandry are found in aquaculture [45] and have triggered repeated calls for improved regulation and enforcement.

1.2.2.1 The use of antimicrobials in food-producing animals: patterns, regulation and the emergence of antimicrobial resistance

Along with the inappropriate use of antimicrobials in human medicine, the use of these drugs in food animals, agriculture and aquaculture is raising serious concerns, because a positive association has been established between antimicrobial use (AMU) in food-producing animals and the occurrence of resistant bacteria from such animals that can be transferred to humans.

Antimicrobial resistance (AMR) is now considered a very serious threat in the EU and worldwide. According to the WHO [46], AMR has already reached alarming levels in many parts of the world.

AMR is a natural process, occurring since the first antibiotics were introduced in clinical practice, but recently this problem is becoming more threatening as no relevant discoveries are being made regarding new antimicrobial molecules to

face the emergence of multi-resistant strains of bacteria. Resistant bacteria are responsible for infections that are more difficult to treat, requiring less available, more expensive, and usually more toxic drugs. In some cases, resistant bacteria have become resistant to all known antibiotics [47].

To draw the attention to this urgent and critical issue, economic evidence is now being used, and alarming estimates of AMR have been recently published [48-50]. The European Commission [48] claimed that, each year, the cost of extra healthcare and productivity losses, related with resistant bacterial infections, reaches at least EUR 1.5 billion, and, in the USA, healthcare systems estimate the additional cost of antimicrobial-resistant infections to be US$20 billion a year, and productivity losses to be US$35 billion a year [49]. The UK government commissioned 'Review of antimicrobial resistance' [50] estimated that drug resistant infections could cause 10 million human deaths annually, by 2050, with total costs of US$100 trillion in lost output, if no action is taken immediately.

More recently, the Organisation for Economic Cooperation and Development (OECD) [51] estimates that around 2.4 million people could die in Europe, North America and Australia between 2015-2020 due to superbug infections, if the trend continues and nothing is done to stem antimicrobial resistance. Furhermore, according to calculations from the OECD model, southern Europe – namely Italy, Greece and Portugal – will be particularly affected, and are forecast to top the list of OEDC countries with the highest mortality rates from AMR, while the United States, Italy and France would have the highest absolute death rates with almost 30.000 deaths/year in the United Stated alone.

There is a consolidated amount of microbiologic and clinical evidence suggesting that resistant bacteria, or resistant determinants, may be transferred from animals to human. The frequency and magnitude of this phaenomenon is still to be evaluated, but the raise in prevalence and spread of resistant infections in hospitals and community settings shifts the discussion to the point that this raise may be related to the huge amount of these drugs that are used in food producing animals.

The extensive use of antimicrobials in husbandry and aquaculture promotes the emergence of antimicrobial-resistant zoonotic pathogens in agricultural

environments [19], and recent microbiological and clinical evidence suggest that antimicrobial resistance genes (AMRGs) and AMRB are transferred from industrially grown animals and fish to humans [52].

Antimicrobial resistant-microbes are found in people, animals, food, and the environment (in water, soil and air), spreading freely between people and animals, and from person to person [9,19,41,53]. Poor infection control, inadequate sanitary conditions and inappropriate food-handling encourage the spread of AMR, with detrimental effects on both human and animal health.

It is generally accepted that the production of safe food, and the guaranty of animal welfare, requires the use of antimicrobials in the food-producing animal industry. The problem, though, lies in the fact that this use largely exceeds the treatment of infection, driving to excessive and inappropriate use of antibiotics. Significant quantities of these drugs are used prophylactically amongst healthy animals or for growth promotion, to accelerate the animals' gain weight [41].

The actual amount of antimicrobials used in food-producing animals is difficult to estimate, because of the inadequate reporting of consumption data, but the reported sales data in 2014 of the 28 EU Member States (MSs) indicates that 3.821 tonnes of active antimicrobial substances were sold for use in humans and 8.927 tonnes for food-producing animals [41]. The overall average consumption (expressed in milligrams per kilogram of estimated biomass and per year) seems to be lower for food-producing animals than for humans in 18 of the 28 EU MSs, but the average consumption turns out to be greater for food-producing animals (151.5 mg/kg, compared to 123.7 mg/kg in humans) [41] because of a few of the other MSs, which have large animal populations and comparatively very high antimicrobial consumption by food-producing animals.

This problem is particularly relevant because almost all of the antimicrobials used in animal husbandry are structurally related to those used in human medicine, which promotes co-resistance and cross resistance [21].

Penicillins and sulphonamides are the most used antimicrobial classes in animals, when consumption is expressed in milligrams per kilogram of estimated biomass. Monobactams and carbapenems are not approved for use in food-

producing animals in the EU/ European Economic Area (EEA) MSs, and no such consumption was reported in food-producing animals [41]. In the US, more than 70 percent of human medically important antibiotics are also used in animals [52], and in the BRICS (the major emerging economies of Brazil, Russia, India, China and South Africa) consumption of antimicrobials by animals to produce meat products is expected to double between 2010 and 2030 [50].

Among the purposes of the use of antimicrobials in husbandry, the one that raises particular concern is related to the use of sub-therapeutic doses of the drug, for a long period of time. In fact, the major part of these drugs is administered in the form of regular food supplements to prevent diseases and with growth promotion purposes, mixed with food and water. This results in the exposure of a great number or individuals, independently of their health status, to sub-inhibitory concentrations of the antimicrobial. Besides, the lack of proper diagnostic tests makes that the majority of animal antimicrobial treatments is based in empiricism, more than in a precise laboratorial confirmation. To animals kept in confined conditions and raised in crowded spaces, such as in aquaculture for instance, the identification of one or two sick animals results on the treatment of the entire population.

Experience over time, and consolidated scientific evidence, leave no doubt about the fact that prolonged exposure to antibiotics significantly increases the localised prevalence of antibiotic-resistant bacteria, creating the ideal conditions for the emergence of drug resistance and its transfer to human infectious bacteria [54,55]. In a review on AMR, commissioned by the British Prime Minister, and hosted by the Wellcome Trust [52], the literature review of 139 articles pointed out that only seven authors (five percent) concluded for no link between antibiotic consumption in animals and resistance in humans, while 100 (72 percent) found evidence of a link.

These evidences raised concerns from a human health perspective, and, consequently, several countries have already banned the use of antibiotics for these purposes, with the notable EU ban in 2006 [56], and the US recently adopting measures towards a voluntary re-labelling of antibiotics to reduce their use as growth promoters [57].

Along with the overall quantity of antibiotics used, the classes of antibiotics that are used in food production also represent a very sensitive matter. Some last-resort antibiotics for humans, with no replacements on the way, are being extensively used in animals, for example, colistin.

Six common classes of antibiotics (aminoglycosides, macrolides, penicillins, quinolones, sulfonamides, and tetracyclines) present on the WHO list of critically important antimicrobials (CIA) for human medicine [22] are commonly used in agriculture and aquaculture. There are 51 antibiotics reported as used by the major animal and aquaculture producing countries [45], and 39 of them are on the WHO list. Of these 39, 37 are listed as either critically important or highly important [22].

The example of polymyxins, the last pharmacological option against infections caused mainly by multi-drug resistant gram-negative bacteria such as *Klebsiella pneumoniae* and *Acinetobacter baumannii* [58], is a good indicator of the size and scope of the problem. Recent hospital outbreaks with carbapenemase-producing *Enterobacteriaceae* (*E. coli*, *Klebsiella*), and multidrug-resistant *Pseudomonas* and *Acinetobacter* species, have led to the re-introduction of systemic colistin treatment as a last resort drug. Even considering the limitations of its safety profile, colistin is now playing a key role for public health.

In 2014, the consumption of polymyxins in food-producing animals largely exceeded their consumption in humans, which was 0.03 and 10.0 mg per kg of estimated biomass, respectively [41].

For a long time, only the vertical transmission of colistin resistance determinants resulting from chromosomal mutations was reported, and there was no evidence of transference by mobile genetic elements [59]. Worrying news came to light in 2015, when Liu, et al. [60] published their work describing a plasmid-mediated *mcr-1* gene that conferred colistin resistance, which was associated with transposable elements located on distinct types of plasmids (pHNSHP45, IncI2, IncX4, IncHI2, IncP, etc.). This phenomenon was also described by other authors [61]. The study by Liu and colleagues [60] also found the presence of this gene in 20 percent of the animals tested in the area, in China, and in one percent of the human

population, strongly indicating that the selection of this resistance was due to the use of colistin in animals and that the gene can transfer to humans.

Greece and Italy reported 61.9% and 33.5% carbapenem-resistant *K. pneumoniae* isolates in 2015 [47], and 31.9% of the carbapenem-resistant isolates were also resistant to polymyxins [47]. The data on colistin resistance are not complete, because not all countries test for it, but these results indicate a progressive loss of effective treatment options for gram-negative bacterial infections. Moreover, recent studies [62] have shown that the contribution of *mcr-1*-mediated transferable resistance to phenotypic colistin resistance in animals, especially in poultry, can be substantial.

1.2.2.2. The use of antimicrobials in aquaculture

Fish raised in aquaculture are subjected to common procedures globally, which are very stressful to the species, and compromise the effectiveness of the fish immune system for suppressing bacterial colonization and infection [63]. The use of prophylactic antibiotics to avoid the emergence and rapid spread of infection is therefore a widespread practice, especially in those countries where no other preventive measures are adopted. In aquaculture, antimicrobials are usually administered to entire populations containing sick, healthy, and carrier individuals, by a process known as metaphylaxis.

Therefore, it is easily understood that aquacultural antibiotic doses can be proportionately higher than those used in terrestrial animal farming, although the exact levels are not easy to determine because different countries have different distribution and registration systems [3]. In addition, the consequences of this practice are also somehow wider and worrying, because drugs contained in fish feed can persist in the aquatic environment for a long time and rapidly spread via excretion throughout water systems, exerting selective pressure in many ecosystems [52]. Because fish do not effectively metabolize antibiotics, the active substance largely passes into the environment in the faeces [3], and some studies suggest that approximately 70 to 80% of the antibiotics applied in aquaculture are dispersed into water systems [64].

The determination of antimicrobial residues in fish products can provide relevant information on the type and eventually on the quantity of these drugs that are being used in aquaculture production, because antibiotic residues remain in fish tissues for prolonged periods of time depending on the molecular stability. Additionally, some authors [45] suggest that the presence of antimicrobial residues in fish might provide a selection and enrichment mechanism for resistant bacteria.

There are regulatory controls regarding the maximum residue levels allowed in edible parts of animal-derived food items in some region. For instance, in Europe, where regulations regarding food safety are very strict, Regulation (EC) 470/2009 of the European Parliament and of the Council [65] outlines the procedures for the establishment of residue limits of pharmacologically active substances in foodstuffs of animal origin. Additionally, in the USA, the government agency responsible for veterinary medicine, the Food and Drug Administration (FDA), sets the rules for antibiotic use, including permissible routes of delivery, dose forms, withdrawal times, tolerances, and use by species.

In most European countries, the antibiotics authorized for use in aquaculture are oxytetracycline, florfenicol, sarafloxacin, erythromycin and sulfonamides (potentiated with trimethoprim or ormetoprim) [30], and in the USA, oxytetracycline, florfenicol, and sulfadimethoxine/ormetoprim are authorized for this purpose [3].

Despite the strict regulation in some regions, in the leading aquaculture production countries, the regulatory framework is very scarce. India is the second major aquacultural producer and accounts for 8% of the total worldwide production, and antibiotic sales and usage are not regulated [45]. China is the major producer and the largest exporter of fish and fishery products [40] and accounts for 67% of total worldwide aquaculture, and no veterinary prescriptions are required for the use of antibiotics in animals [43]. Several laws regulate the Chinese aquaculture industry, and one of the most important, the Food Hygiene Law, prohibits “foods that contain or are contaminated by toxic or deleterious substances and can thus be injurious to human health” and ‘‘foods that contain pathogenic parasites, microorganisms or an amount of microbial toxin exceeding the tolerance prescribed by the State” [66]. Furthermore, this law assigns responsibility to the Ministry of Health for monitoring, inspecting and providing

technical assistance for food hygiene as well as investigating food contamination and food poisoning incidents, among other areas of food safety. Unlike many other countries that consider the whole of the food chain from the beginning of production to the end consumer, the Chinese regulatory framework neglects the early stages of production in which, particularly in aquaculture, the use of banned pharmaceutical agents can be significant. Despite China's efforts to assure food security and consumer confidence in their products during the last few years, several reports came to light that indicated the use of medically important antibiotics as well as illegal veterinary antibiotics such as chloramphenicol, which suggest that the enforcement of the regulation is lax [67].

In countries where control is less stringent or lacking, AMU in aquaculture may surpass the use in human medicine [21,22], and differences between countries are very pronounced. In Chile, the second largest producer of cultured salmon after Norway, 385 and 325 tonnes of antimicrobials were used in salmon aquaculture production in 2007 and 2008, respectively, and 149 and 57 tonnes of quinolones were used in those years, respectively [23]. According to the same authors, the annual consumption of florfenicol rose from approximately 400 kg in 2000 to 233 000 kg in 2007. The quantity of antimicrobials used to produce 1 tonne of salmon as reported by one industrial production facility in Chile was 279 g, while only 4.8 g was used to produce the same amount of salmon in Norway [19].

Norway can be considered a model in this area, because regulation of AMU in salmon aquaculture is very strict. Along with improved diagnostics, including susceptibility testing and the use of vaccines and probiotics, Norway was able to reduce the use of antimicrobials to negligible levels [68]. Along with Norway, the Netherlands and Denmark are case studies that clearly demonstrate that it is possible to significantly reduce the use of antimicrobials, without reducing the quality and safety of food, and without a damaging economic impact [69], and some authors even highlight a reinforcement of their commercial competitiveness [52].

1.2.2.3. Antimicrobial resistance patterns arising from aquaculture, transference and potential impact on the environment and human health

It is well documented that the exposure of fish pathogens and aquatic bacteria to antimicrobials drives the development of drug resistance [70], and several studies have established a causal relationship between the use of specific antimicrobials in aquaculture and an increase in AMRB [71,72]. Additionally, other studies support the hypothesis that the development of antimicrobial resistance in aquaculture environments could contribute to the antimicrobial resistance of human pathogens [73,74].

The aquatic environment provides a permanent and easy mechanism to disperse drug residues, microbial pathogens, and AMRGs, and therefore, aquaculture will continue to pose a threat in terms of the rapid dissemination and transfer of antimicrobial resistance determinants.

Antimicrobials are usually administered orally to groups of fish that share tanks or cages, in formulated feed, and occasionally by bath, by immersion in closed containers. The groups contain sick, healthy, and carrier individuals [9]. In the absence of collectors to remove uneaten medicated feed from water, it is estimated that up to 80% of the administered drugs remain in the water and sediments close to the application sites [19].

Some studies have reported that antimicrobials in the aquatic environment are rapidly transported from the application site and diluted [75], but most emphasize that the persistence of active metabolites in aquatic sediments for a long time in sufficiently high concentrations to exert selective pressure on aquatic bacterial diversity [76].

Studies regarding the presence of antimicrobial resistance determinants in aquatic bacteria reflect the results of this reality. Numerous studies have noted that many aquatic bacteria harbour a large variety of mobile genetic elements such as plasmids, integrons and transposons that can easily move, recombine and mobilize, promoting the emergence of new mobile combinations of AMRGs, conferring to bacteria the capacity to rapidly adapt to new environments in which antimicrobials are present [9,77], and enhancing bacterial resilience and fitness for growth. Some authors define aquaculture systems and farms as “genetic

reactors" or "hotspots" for the emergence of AMR for genetic exchange and recombination and have modelled trends of AMR profiles [78]. Once acquired, AMRGs persist in the environment for a long time, even after exposure has been terminated. Additionally, some authors [79] claim that sub-inhibitory concentrations of antimicrobials are signalling molecules that may regulate the homeostasis of microbial communities and may in turn beneficial for the behaviour of susceptible bacteria in natural environments, stimulating horizontal gene transfer (HGT) and mutagenesis.

Additionally, fish are reservoirs of zoonotic pathogens which infect not only the host but also humans by direct contact in the aquaculture facility and by foodborne infections [80]. Common fish pathogens that infect fish handlers include *Aeromonas hydrophilia*, *Mycobacterium marinum*, *Streptococcus iniae*, *Vibrio vulnificus* and *Photobacterium damselae* [81], and foodborne diseases involve mainly *Listeria monocytogenes*, *Aeromonas* and *Clostridium spp.* [82,83]. Sousa, et al. [84] found broad-spectrum β-lactamase resistance genes, including *blaTEM-52*, *blaSHV-12*, as well as *cmlA*, *tetA*, *aadA*, *sul1*, *sul2*, and *sul3* in faecal matter from *Sparus aurata* (*Gilthead seabream*), and other studies [44,85,86] have suggested that commercial fish and seafood may act as a reservoir for multiresistant bacteria, facilitating the dissemination of AMRGs.

Aside from the risk of infection, such bacteria can develop and spread AMR determinants to other human pathogens. Plasmids harbouring different ARGs have successfully been transferred in vitro from fish to human pathogens, including *Vibrio cholerae* and *Vibrio parahaemolyticus* [70]. Furthermore, there are several studies that support a causal relationship between the use of specific antimicrobials in aquaculture environments and an increase in AMRB [71,72] and that the development of AMR in aquaculture environments could contribute to AMR in human pathogens [73,74]. In fact, bacteria from aquatic and terrestrial environments share similar antimicrobial genetic determinants [9].

Furushita et al. [73] emphasized that genes encoding for tetracycline resistance in farmed fish bacteria and clinical isolates in Japan exhibited a high similarity, suggesting that they may have originated from the same source. This observation was reinforced by laboratory experiments in which tetracycline resistance from

marine strains of *Photobacterium*, *Vibrio*, *Alteromonas* and *Pseudomonas* were transferred to *E. coli* by conjugation, suggesting the viability of transferring resistance determinants from marine bacteria to bacteria associated with the human gut.

Additionally, the same resistance gene profile has been described in both fish bacteria and human clinical isolates. About half of the AMRGs identified in fish pathogens are common to those identified in human pathogens; therefore, bacteria from different environments including aquatic and hospital settings can share the same AMRGs [70,73,74].

Some plasmid-encoded quinolone resistance genes (*for example, qnrA, qnrB, qnrS, and aac[6´]-1b-cr*), found in *Escherichia coli* and *Klebsiella*, and the macrolide resistance genes mef(C) and mef(G), in *Vibrio* and *Photobacterium*, appear to have an aquatic origin [87-90]. The *t77etC* gene in the *Chlamydia suis* genome might have originated on the genome of the salmon pathogen *Aeromonas* salmonicida [19]. The independently evolved tetracycline-resistance determinant *tetG* was first discovered in aquatic bacteria [91]. Many AMRGs were identified in aquatic bacteria prior to their detection and dissemination among human and animal pathogens. These include some emerging plasmid-mediated quinolone-resistance (PMQR) genes found in aquatic *Vibrio*, *Shewanella* and *Aeromonas*, new β-lactamase genes from *Photobacterium damselae* and *Oceanobacillus iheyensis*, a novel fosfomycin resistance determinant isolated from the aquatic environment, the widely disseminated emerging *floR* gene of human pathogens and the chloramphenicol resistance genes *catII*, *catB9* and *catB2* from aquatic *Photobacterium, Vibrio* and *Shewanella,* respectively [9]. Additionally, the plasmid-associated colistin resistance mediated by the *mcr-1* gene appears to be another transmissible antimicrobial resistance determinant that might have originated in the aquacultural environment [60,92].

Table 6 provides an overview of some antimicrobial resistance determinants found in fish pathogens and other marine and fresh water bacteria, shared with human pathogens. Some of them appear to have originated in piscine pathogens.

The potential risks to the environment associated with AMU in aquaculture is another issue that raises serious concerns. Aquaculture sediments contain

several bacterial communities, and evidence supports the hypothesis that they might be a relevant reservoir of faecal pathogens [93] and antimicrobials [94]. A diversity of AMRGs has been detected in aquatic sediments, such as the sulfonamide resistance genes *sul* 1 and *sul* 2, the tetracycline resistance genes *tet* B, *tet* C, *tet* M, *tet* O and *tet* W, the quinolone resistance gene *qnr*A, the aminoglycoside resistance gene *aad*A and the β-lactamase resistance genes *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$ and *bla*$_{NDM}$ [95-97]. Moreover, tetracycline resistance genes were identified in marine sediments [97]. Furthermore, Yang et al. [97] found that several contigs from resistance genes in bacterial plasmids from marine sediments shared a high identity with transposons or plasmids from human pathogens, indicating that the sediment bacteria recently contributed to or acquired resistance genes from pathogens.

Although studies that establish an unequivocal link between AMU in aquaculture and the transference of AMR determinants to human pathogens are still lacking, the highlights of several studies allow us to draw some conclusions:

- High frequencies of AMR in bacteria have been reported in areas surrounding aquaculture production facilities (reviews by Cabello 2006 [27] and Cabello et al. 2013 [9]);
- AMU in aquaculture results in the entry of antimicrobial compounds into the surrounding environment, with the potential to exert selective pressure and increase the frequency of AMR in environmental bacteria [95,98,99];
- Molecular studies have shown that genes involved in AMR in bacteria associated with aquaculture exhibit great similarity to AMRGs that have been detected in terrestrial bacteria, which are responsible for human and animal diseases [100].

Thus, the presumption of long-term, genetically separated populations is now outdated. Contamination of the aquatic environment with human and terrestrial animal pathogens has negated this assumption. Both populations are biologically continuous as a result of bidirectional HGT [19]. Indeed, laboratory and observational evidence related to HGT between aquatic and human pathogens is growing, and as a result, new genetic elements may be assimilated by the terrestrial bacteria pangenome, including human pathogens, making subsequent treatment more difficult [101,102].

Table 6. Antimicrobial resistance determinants found in fish pathogens and other marine and fresh water bacteria, shared with human pathogens

Antibiotic Resistance Gene/Plasmid	Antimicrobial Class	Gene Recipient (fish pathogen / marine and fresh water bacteria)	Reference
aadA	Aminoglycoside	*Escherichia coli*	[44]
aadA	Aminoglycosides	*E. coli*	[84]
cmlA	Chloramphenicol	*E. coli*	[84]
floR	Florfenicol	*Pseudoalteromonas* sp., *Shewanella sp., Cobetia* sp., *Marinobacter* sp., *Halomonas* sp.	[88]
floR	Florfenicol	*Yersinia ruckeri;Photobacterium damselae*	[115]
floR	Florfenicol	*Edwardsiella ictaluri*	[116]
mef(C)| mph(G)	Macrolide	*P. damselae* subsp. *damselae*	[87]
mcr-1	Polymyxin	*Shewanella algae MARS 14*	[92]
qnrA	Quinolone	*S. algae*	[90]
aac(6′)-Ib-cr	Quinolone	*Sporosarcina* sp., *Rhodococcus* sp., *Kytococcus* sp., *Erythrobacter* sp.	[89]
qnrA | qnrB | qnrS | aac(6′)-1b	Quinolone	*Pseudomonas sp., Alcanivorax sp., Arcobacter sp., Arthrobacter sp., Kytococcus sp., Marinobacter sp., Microbacterium sp., Rhodococcus sp., Actinobacterium sp., Cellulophaga sp., Flavobacteriaceae, Erythrobacter sp, Tsukamurella sp., Dietzia sp., Microbacter sp.*	[88]
qnrA	Quinolone	*S. algae*	[90]
aac(6′)-Ib-cr	Quinolone	*Sporosarcina* sp.; *Rhodococcus* sp.; *Kytococcus* sp.; *Erythrobacter* sp.	[89]
qnrVC4	Quinolone	*Aeromonas punctata*	[117]
qnrS2	Quinolone	*A. punctata* subsp. *Punctata*; *Alloteuthis media*	[118]
ICEVspPor2 | ICEValPor1	Rifampicin	*Vibrio splendidus; V. alginolyticus; Shewanella haliotis; Erythrobacter nigricans*	[119]
sul1 | sul2 | sul3	Sulfonamides	*E. coli*	[102]
sul1	Sulfonamides	*Y. ruckeri; P. damselae*	[133]
tetA	Tetracycline	*E. coli*	[84]
tetB | tetD	Tetracycline	*E. coli*	[44]
tetA | tetB | tetK | tetM	Tetracycline	*Pseudoalteromonas* sp., *Shewanella* sp., *Psychrobacter* sp., *Cobetia* sp., North Sea bacterium H7, *Vibrio* sp., *Pseudomonas* sp.	[88]
tetC | tetD |tetE	Tetracycline	*Y. ruckeri; P. damselae*	[115]
tetB | tetY | tetD	Tetracycline	*Photobacterium* sp., *Vibrio* sp., *Alteromonas* sp.,	[73]
tetB | tet(34) | tet(H) | tet(35) | tet(L)	Tetracycline	*Brevundimonas vesicularis*, *Pseudomonas* sp., *Serratia* sp. *Moraxella* sp., *Acinetobacter* sp., *Stenotrophomonas* sp., *Morganella* sp.	[120]
blaTEM-52 | blaSHV-12	β-Lactam	*E. coli*	[84]
blaTEM	β-Lactam	*E. coli*	[44]
blaCMY-2	β-Lactam	*E. ictaluri*	[116]
Plasmid pAB5S9b[i]	Several	*Aeromonas salmonicida*	[121]
Plasmid pSN254b[ii]	Several	*A. salmonicida*	[121]
Plasmid pSN254[iii]	Several	*A. salmonicida*	[122]

[i] plasmid conferring multiresistance to several antimicrobial classes, including tetracycline, sulfonamide, streptomycin, florfenicol and chloramphenicol.
[ii] plasmid conferring multiresistance to several antimicrobial classes, including florfenicol, chloramphenicol, tetracycline, streptomycin, spectinomycin, sulfonamide and beta-lactam antibiotics.
[iii] with first report of plasmid-mediated florfenicol-resistant A. salmonicida, and first report of a plasmid-associated AmpC β-lactamase sequence in a member of the *Aeromonadaceae*

The most likely routes of contact between aquatic bacteria that contain AMR determinants and terrestrial bacteria result from contamination by agricultural wastes, from storm-water runoffand discharges from sewage treatment plants [78]. However, more important than the precise identification of the pathway is the fact that the link between both bacterial populations raises several concerns because many of the antimicrobials used in aquaculture continue to be of significant importance in human medicine [52]. Figure 2 shows a schematic view of the hotspots and drivers of AMR related to the use of antimicrobials in aquaculture. The epidemiology of AMR at the animal-environmental-human interface is a complex process, involving a wide range of possibilities regarding potential transmission routes and vehicles, antimicrobial selective pressures and horizontal transmission of resistance genes between bacteria from different ecological compartments.

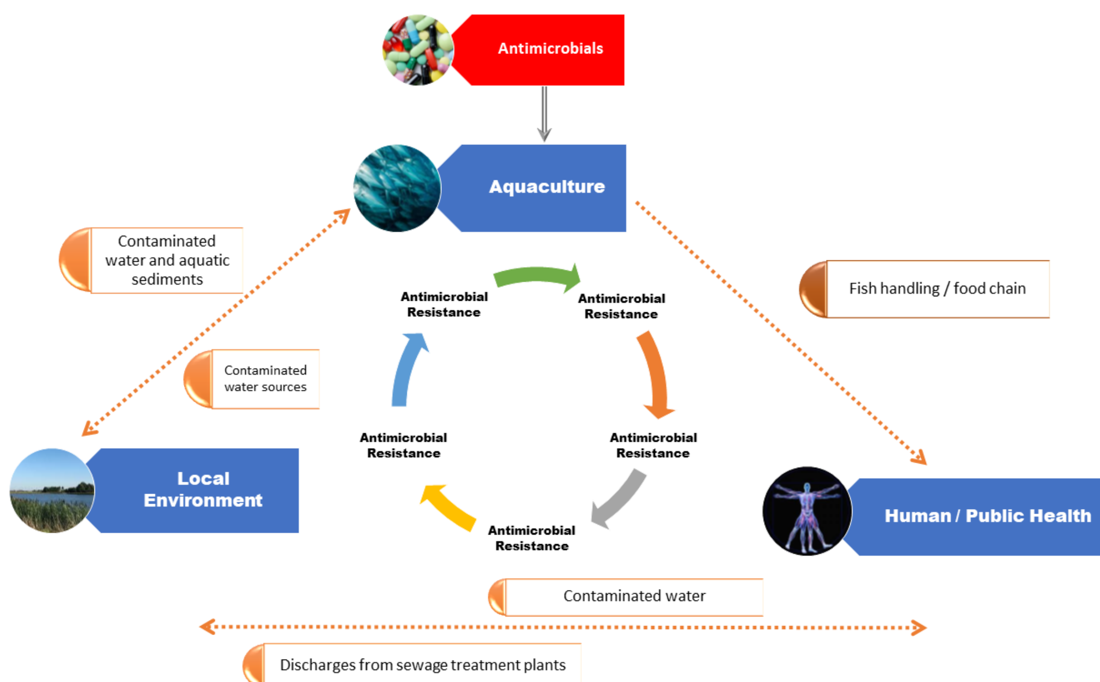

Figure 2. Drivers of antimicrobial resistance, related to the use of antimicrobials in aquaculture

Discussion and future trends

The actual magnitude of the impact of AMU in food production animals remains shrouded in uncertainty, but the amount of scientific evidence that is available today allows the establishment of an undoubtable link between AMU in animals, the emergence of resistant bacterial strains and the transfer of resistance to

human pathogens. This is a major public health problem that requires urgent action. The use of antimicrobials in agriculture, husbandry and aquaculture drives the selection of resistant bacterial strains. Along with a loss of sensitivity, persistence is also a problem, because AMR is acquired very rapidly but the reverse process is notably slower, if it occurs at all. Therefore, it is becoming clear that AMR must be addressed as a phenomenon of global ecological genetics. Antibiotic resistance did not come out of the blue, nor is it a new or unexpected phaenomenon. What is new, and worrying, is the rate and speed at which bacteria are accumulating AMR determinants to almost all known antibiotics, providing a perfect example of Darwin's theory of adaption for survival. Furthermore, the discovery, development, manufacture and marketing of new antimicrobials has significantly slowed in the past 20 years and has a very low success rate. Only 1 out of 16 antimicrobial molecules from early-stage research achieve clinical application [103]. Also surprising is the generalized lack of consciousness about the actual dimensions and implications of this problem by healthcare professionals, patients and everyone involved in the industry of food-producing animals or aquaculture. Over the past 2 years, several countries, states and international organizations have proposed adopting concrete measures [41,50,104-106], but despite this, the incidence of multidrug-resistant infections, including to last-resort treatments, has significantly increased worldwide in recent years. The EU has been drawing attention for this problem for almost two decades, since the 2001 Community strategy against AMR [107], reinforced with the 2011 Commission Action Plan [48], which is notable for its One Health approach, and a recently reviewed action plan requested by the Member States [104] provides a set of concrete actions to be adopted. Nonetheless, in June 2016, the EC published the Eurobarometer results on AMR awareness [108] and the main conclusions pointed to little knowledge about AMR across the EU. Because AMR has a global ecological impact, the One Health strategy is the most appropriate way to address and attack it. As the One Health principle postulates, we must recognize that human, animal and environmental health are interconnected, that diseases are transmitted from humans to animals, and vice versa, and must therefore be addressed in both. The term 'One Health' is globally recognized and widely used in the EU and in the 2016 UN Political Declaration on AMR [105]. Some authors [109] are accordingly proposing a conceptual

framework for a One Health approach to AMR surveillance, centralizing and integrating surveillance both of antibiotic usage and consumption by humans and animals with AMR data from humans, animals, food and the environment. From a global perspective, One Health must be expanded to include aquaculture production, using methods that minimize risk to the public, animals and environmental health. Healthy animal-production systems must evolve in a way that reduces the risk for disease outbreaks and consequently reduces the need for antibiotics by implementing policies that improve food security as well as human health. Figure 3 illustrates a conceptual framework for a One Health approach to AMR surveillance in aquaculture. The One Health concept is more important than ever to better manage the impact of AMR in humans, animals and environmental health, and requires a global strategy to develop collaborations and interdisciplinary communication among all actors. The threat of the emergence of a superbug is now becoming very real, and all recent updates on this issue show the increasing complexity and scope of bacterial resistance patterns. To preserve this major social and human good - the effectiveness of antimicrobials - we have to learn to rapidly change strategies and procedures, as bacteria do, to preserve our health and well-being. Some urgent strategies must be adopted. First of all, overall antibiotic use must be reduced by establishing limits for antibiotic use in aquaculture, and every country must commit to them, allowing individual countries to find the most adequate way to meet their goals. Furthermore, the use of highly critical antibiotics should be prohibited for use in aquaculture because too many last-line antibiotics are currently being used. Reducing the excessive use of antimicrobials means we have to implement alternative measures to limit the emergence and spread of bacterial infections in aquacultural production, and there are some good examples of countries that have implemented specific measures to reduce the need for antimicrobials in this industry. Particularly in Norwegian Atlantic aquaculture, vaccines have already demonstrated their efficiency in tackling the most frequent bacterial diseases [64,68], while still ensuring Norway's leadership in the world production of salmon. Additionally, the excessive use of antimicrobials in this industry can be counterproductive. Indeed, in Chile, the increasing use of antimicrobials that followed the expansion of the salmon-production industry coincided with fish mortality and the emergence of new resistant bacterial strains [23,110], and new

salmon pathogens emerged during the same period, such as *S. phocae*, *Rhodococcus qingshengi*, *Flavobacterium chilensis* and *F. araucananum* [9,111,112]. Another example is the disintegration of shrimp aquaculture in Taiwan during the late 1980s as a consequence of the emergence of multidrug resistant bacterial strains [113]. The major areas for further research and development regarding disease control in aquaculture have been well defined by the FAO of the UN [69] and include the development of affordable vaccines, the use of immunostimulants and non-specific immune-enhancers, and the use of probiotics and bioaugmentation for the improvement of aquatic environmental quality. Good husbandry conditions must be adopted in terms of optimum conditions for parameters such as feed rates, water-dissolved oxygen, stocking densities and, when possible, temperature control [2]. The formulation of fish diets is another relevant matter that must consider the adequate provision of protein to promote maximum growth. Several studies demonstrate the importance of fish diet specifically regarding vitamins and trace elements for the control of disease and the modulation of fish resilience to resist infection [114].

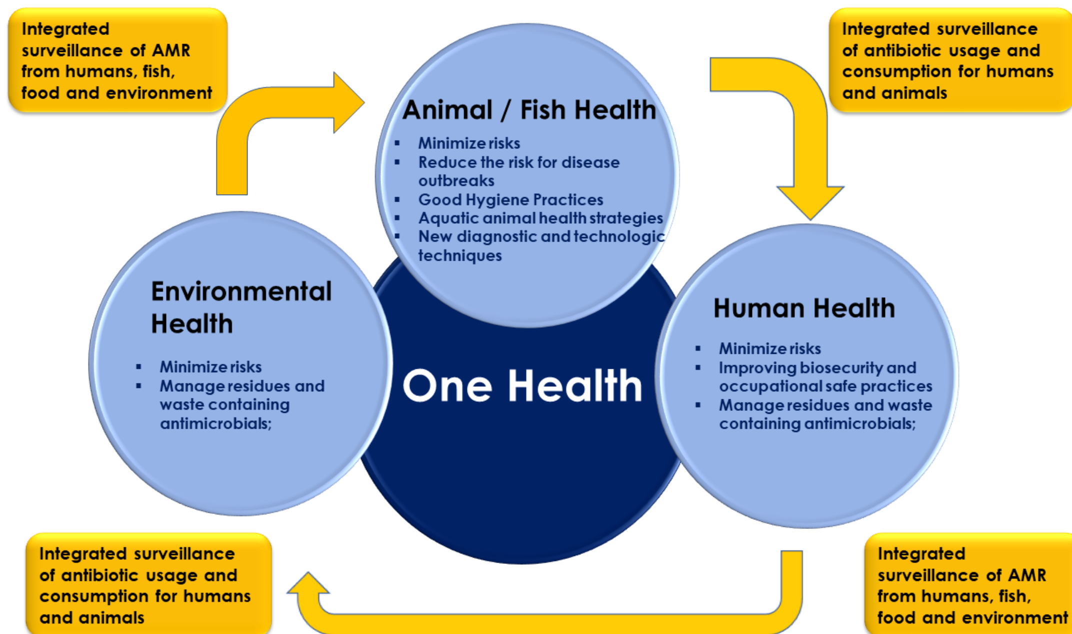

Figure 3. Conceptual framework proposed for a One Health Approach to AMR surveillance in aquaculture

Concluding remarks

Access to good-quality fish must be a global major concern and therefore should trigger the improved regulation and enforcement of the use of antimicrobials. The strategy should lead to the progressive adoption of measures that aid the implementation of good manufacturing practices, vaccination, biosecurity and disease monitoring, as well as food and hygiene standards appropriate to farmed species. For many common diseases that affect fish production, antibiotics can very effectively be replaced by proper vaccination. Additionally, appropriate sanitary measures for the promotion of animal health must in no case be replaced by the use of prophylactic antibiotic therapy. Only a coordinated and holistic approach following One Health principles, of animal and fish production processes, allows the coherent and systematic application of the EU policy of “from farm to table”, ensuring that best practices and quality parameters are implemented in each intermediate stage until the food reaches the consumer.

1.2.3. Influence on the human microbiota

Another important question to be addressed, with growing attention from the scientific community, is the importance of preserving the intestinal microbiota.

Bacteria are naturally part of the body’s internal and external ecology and environment. Some bacteria are beneficial, most of them are benign, and their equilibrium is maintained by the organism’s immune system. Microbial populations naturally compete with foreign bacteria within a stable internal environment, which is critical for maintaining health. This microflora interacts with its host (man), both locally, due to its intimate contact with the intestinal mucosa, and systematically, influencing diverse functions: physiological, anatomical, metabolic and toxicological [123].

The main concern is to understand how antimicrobial residues in food may affect human health either by: (i) exerting a selective pressure on the dominant intestinal flora; (ii) favouring the growth of micro-organisms with natural or acquired resistance; (iii) promoting, directly or indirectly, the development of acquired resistance in pathogenic enteric bacteria; (iv) impairing colonization

resistance; or (v) altering metabolic enzyme activity of the intestinal microflora [123].

The intestinal microflora, responsible for maintaining a healthy gastrointestinal tract by preventing pathogenic bacteria from growing, can be disrupted as a result of repeated exposures to antimicrobial residues [124]. The human gastrointestinal tract ecosystem consists of complex and diverse microbial communities and is getting increasing attention from the medical and scientific community because of its important role in human health and disease. Furthermore, the microbial community may have an unknown influence on the immune system, stimulating it to respond rapidly to pathogen challenges [125].

Several lines of evidence confirm that antibiotic intake can have deleterious effects in the gut ecosystem, disturbing its composition and function. Broad-spectrum antibiotics can affect the abundances of 30% of the bacteria in the gut community, promoting significant drops in taxonomic richness, diversity and evenness [126], and recent evidence suggest that chronic exposures to low-residue antimicrobial drugs in food could disrupt the equilibrium state of intestinal microbiota and cause dysbiosis that can contribute to changes in body physiology [127].

One of the most imminent threats of gut microbiota alterations is the increased susceptibility to intestinal infections, which can be originated by newly acquired pathogens or from the sudden overgrowth and pathogenic behaviour of opportunistic organisms already present in the microbiota. Antibiotic-associated diarrhoeas, due to nosocomial pathogens, are a frequent occurrence, associated with organisms such as *Klebsiella pneumoniae*, *Staphylococcus aureus* and, of most concern, *Clostridium difficile*, which can cause intractable, recurrent infections and, in some cases, even a potentially lethal pseudomembranous colitis [126].

The dysbiosis promoted by the antibiotics has the additional disadvantage of enriching the microbiota in resistant organisms, and the human gut microbiota has been established as a significant reservoir of antibiotic resistances.

One of the largest population-level analyses of the intestinal resistome to date, also showed that the abundance of antibiotic resistance genes is higher for antibiotics that have been longer in the market and for those approved for animal use, such as tetracycline, bacitracin and the cephalosporins [128]. The effects of fluoroquinolones on the ecology of colonic microflora have been intensively evaluated [129,130], and it was shown that fluoroquinolones have a selective effect on the normal colonic bacteria, decreasing the populations of enterobacteria and, in general, not affecting the anaerobic bacterial population.

Regarding the effect of tetracyclines, a recent study [131] demonstrated that, at low residue, tetracycline could lead to slight differences in the composition of intestinal microbiota. Another study [132] showed that, in certain conditions, tetracycline causes barrier disruption.

Furthermore, therapeutic dosages of ß-lactam antibiotics (ampicillin, amoxicillin, cephalosporins), tetracyclines (oxytetracycline) and macrolides (erythromycin) have demonstrated a distinct impact on the number of enterobacteria, *Enterococci*, anaerobic bacteria and the development of resistant strains in the human intestinal microflora. The lower-dose effects of some antibiotics have been investigated in a limited number of studies with human volunteers, and the results highlighted that at a low level of exposure, effects on the human intestinal microflora might occur [133].

1.2.4. Environmental impact resulting from the use of antimicrobials in aquaculture

The fate and the potential hazards of chemical substances on ecosystems is a topic of increasing concern and research worldwide. And among them, antimicrobials receive special attention due to correlations between the development and rapid expansion of antibiotic resistance and their total consumption and occurrence in the environment [134].

Several aspects must be considered for the assessment of environmental effects of antimicrobial feed additives, such as effects on soil microbes, earthworms, algae, aquatic organisms, among others. Furthermore, safety for wildlife and other unintended recipients must also be considered.

Considerable amounts (30% to up 90%) of antibiotics administered to humans and animals are excreted into waste stream via urine and feces, largely unmetabolized, and conventional wastewater and recycled water treatments proved to be only partially effective in their removal or degradation [135-138]. Therefore, municipal, agricultural and industrial wastewater are the major entrance sources and pathways of antibiotics and their metabolites and transformation products, in the environment.

Regarding the aquaculture sector, since most of the antibiotics are not absorbed in the intestinal tract of fish, the amount excreted in faeces and urine is almost as large as the total amount fed to the animal, and therefore the major impact in the environment is through excretion [123]. Bearing this problem in mind, the European Community requires that studies on excreted residues and an environmental assessment be performed on all feed additives [139].

For marine species this problem is raised with even more intensity, because antibiotics have been shown to be less effective in seawater, which relates to their reduced bioavailability, due to binding with the Mg^{2+} and Ca^{2+} divalent cations that occur in seawater [140,141]. This has major implications for the appropriate use of certain antibacterials, as their minimum inhibitory concentrations (MICs) may be much higher than in fresh water. Oxolinic acid, for instance, exhibits a MIC 40 to 60-fold higher in seawater against the bacterial fish pathogen *Aeromonas* salmonicida [142].

The bioavailability of some aquaculture drugs in salmon held in seawater is shown in Table 7.

Table 7. Examples of reduced bioavailability for some aquaculture antibacterials in seawater (Adapted from Rodgers and Furones, 2009 [2])

Antibacterial	Bioavailability (%)
Oxytetracycline	1
Amoxicillin	2
Sarafloxacin	2
Oxolinic acid	30
Flumequine	45
Sulfadiazine	50
Trimethoprim	96
Florfenicol	97

Despite being a focus of concern, recent review articles highlight the lack of comprehension on the potential toxicological consequences of antibiotics in ecosystems [30,143-145].

Over the past years, the European Commission draw the attention to this important topic, by increasingly promoting and supporting several projects, actions, and initiatives to mitigate the widespread antimicrobial resistance, including surveillance of antibiotic consumption and research on environmental contamination by these drugs in Europe [104,146].

Water quality is defined by the European Commission as a priority goal to environmental sustainability, ecological balance, and human health and well-being and, therefore, rules to minimize adverse impacts of production and consumption in aquatic environment have been implemented [147]. However, current EU legislation for good-quality water in Europe do not cover a wide range of emergent contaminants, including antibiotics, due to lack of knowledge and understanding about their toxicity and environmental occurrence. Additionally, also EU's regulation on good agricultural practice for protection of waters, do not include water contamination by antibiotics.

Within this overall framework, aquaculture represents undoubtedly one of the major contributors for the release of antibiotics into the environment, due to the direct discharge of aquaculture products, resulting in the contamination of soil, surface water, sediment, ground water and biota. It has been estimated that 70-80% of fish antibiotics are released into the environment [148]. In addition, antimicrobials are often nonbiodegradable, being released through urine and faeces into the aquatic surroundings in an unmetabolized form, leading to extensive contamination [27].

Prophylactic use of veterinary medicinal products has been particularly developed in aquaculture, notably antibiotics, to forestall bacterial infections resulting from the high density of fishes, the difficulty in isolating sick animals and the absence of sanitary barriers.

Antibiotics and their by-products may persist in the environment through a cycle of partial transformation and bioaccumulation and gradual deposition in soil,

surface water, and groundwater [134] and, consequently, can be absorbed by animals (food-producing animals and fishes) and humans (by reaching the drinking water), with the potential to induce long-term effects, as a continuous part of their diet through water or food at low concentrations [145,149].

Medicinal products can degrade biotically or abiotically in soils and water, a process that in general reduces their activity, even if some degradation products might be persistent and therefore of concern. Highly lipid-soluble medicinal products may also have the ability to accumulate in the animals' fat tissues, and can, therefore, be introduced into the food chain.

Another focus of concern is that antibiotic can be toxic to non-target organisms, posing a potential ecological risk for aquatic species. Environmental microorganisms become unavoidably exposed to antimicrobial residues and some primary producers and decomposers, which are essential for the sustainable functioning of ecosystems, may be vulnerable to antibiotics. Consequently, disruption of vital ecosystem processes might occur [134]. Several studies demonstrate the toxic effects of antibiotics in aquatic organisms [150-153]. For instance, sulfonamides were found to be toxic towards green algae, having an even stronger adverse effect on duckweed than the herbicide atrazine [154]. And the inhibitors of protein synthesis to bacteria, such as azithromycin, doxycycline, florfenicol and oxytetracycline, exhibit significantly toxic effects to algae [155].

Furthermore, also antibiotic metabolites can be bioactive and potentially more toxic, stable and mobile in the environment than their parent compounds [134], and even revert back to the parent antibiotic, representing a reservoir of contaminants [156].

It has been shown that microbes have an essential role in the antimicrobials' degradation process [157], but there are several aspects that can affect it, such as different salinities, pH or temperature [158].

Concern about sustainable development of this kind of activities, namely concerning the use of pharmacologically active substances, led the European

Union to adopt Directive 2001/82/CE [159], demanding the evaluation of potential environmental risks associated with the use of veterinary medicines.

The environmental risk assessment of these drugs demands information on the concentration of the substance in the environment, information on its toxicity on biota and, also, the ability to accumulate in the food chain [160].

Unfortunately, the available information, based on experimental investigation, is scarce for most pharmacologically active substances, and existing data mostly refer to the results based on predictive models.

A conventional approach to assess the environmental risk of a pharmacologically active substance, requires information on their physicochemical properties, persistence (e.g. hydrolysis, photolysis, aerobic and anaerobic degradation rate), bioconcentration and ecotoxicity (for plants, algae, fish species and other microorganisms). This data shall be evaluated for the parent compound and major metabolites.

In aquaculture production systems, antimicrobials are administered basically by three different ways: bath, through animal feed and, less often, injection. In the first case, there is an obvious discharge of the non-absorbed drug directly into the environment, through the effluent from sinks. If administered in the form of a food supplement, the release to the environment might occur by two different mechanisms:

- Antimicrobials, and their by-products, are released to water through faeces and urine;
- Through non-consumed feed;

In the first case, the antimicrobial pharmacokinetic properties determine its release, in more or less extent, with the animal's faeces and urine. In the second case, it depends on several factors, such as the quality of the feed's coating and the overall quantity of food that is consumed by the animal [30]. The excess of non-consumed feed ends up in the sediments.

For a proper assessment of the impact exerted by the use of antimicrobials in aquaculture, it is important to take into account several data. On one hand, the

pharmacokinetic properties of the molecule, in the farmed species, and, on the other hand, information on the molecule's stability, persistence, degradation mechanisms and toxicity, of the parent compound and its metabolites, in the environment [30].

Once released in the environment, the drug is distributed between different ecological compartments, were it will be degraded. The expected concentration of the substance in each of the compartments (superficial water, soil, sediments) depend on the substance's persistence profile in each of them, which is determined by biotic (anaerobic and aerobic) and abiotic (photolysis and hydrolysis) degradation rates [160].

1.3. Alternatives for reducing the use of antimicrobials in aquaculture

As antimicrobial resistance and antibiotic residues are becoming global concerns, there is an urgent need to develop alternative therapies for bacterial pathogens in animal production, especially in aquaculture. In this sector, **vaccination** is probably the most effective method for preventing infectious diseases [3], but commercially available vaccines are still very limited in the aquaculture field. Particularly in Norwegian Atlantic aquaculture, vaccines have already been demonstrated to efficiently tackle some of the most commonly occurring bacterial diseases [64,68]. In Norway, 100% of farmed salmonids are vaccinated, following to mandatory measures established by legislation [10]. The commonly used vaccines are all inactivated, injectable vaccines, which may be administered effectively and rapidly through automated systems [10].

The Mediterranean saltwater production of European sea bass and Gilthead sea bream are not favoured by the low water temperature in the North Sea. Vaccines against *Vibrio anguillarum*, *Photobacterium damsela* and *Tenacibauculum maritinum* are used, but registration of antimicrobial use in Mediterranean European or North African aquaculture is scarce and incomplete. Vaccines are also used against a number of bacterial diseases in freshwater aquaculture where

Flavobacterium psychrophilum poses a particular problem due to the size of affected fry when immune system is not yet developed, and *Yersinia ruckeri* where the emergence of a new biotype has challenged vaccine efficacy. Fish seem to be particularly receptive for DNA vaccination and the European Medicines Agency (EMA) has adopted a positive opinion in April 2016 [161], regarding marketing authorisation of a DNA plasmid vaccine for active immunisation of Atlantic salmon against pancreas disease caused by salmonid alphavirus subtype 3, although there is no final decision on the license. Norwegian history of vaccination in salmon is, in fact, a remarkable success, but further development of vaccines for aquaculture is warranted.

Aside from vaccines, there are several other alternatives to the use of antibiotics that have been successfully used in aquaculture, with a positive impact on animal health parameters.

One of these are **organic acids**. In some studies, they have shown to reduce the prevalence and spread of some food-borne zoonotic bacteria such as *Salmonella* spp., *Campylobacter* spp. and *E. coli* when supplemented in the diet of food-producing animals. Lückstädt (2006) [162] has reviewed the use of organic acids in aquaculture, reporting that in some species, such as shrimps and fish, the supplementation with organic acids reduced infections. Ramli et al., 2005 [163] concluded that Tilapia farmed in tropical conditions, show an increase in their survival rate after challenged with *Vibrio anguillarum*, when supplementation with organic acids was implemented.

Also, the use of **probiotics** seems to have a positive impact in fishes' health and improved survival in fish and shellfish. Some of the compounds showed similar or better effect compared to treatment with certain antimicrobials [10]. In *in vivo* trials with rainbow trout, viable cells of bacteria isolated from the microbiota of fish were demonstrated to significantly decrease mortality due to *Flavobacterium psychrophilum*, the causative agent of coldwater disease, and the two isolates were identified as member of the *Enterobacter* genus [164].

Prebiotics, such as fructooligosaccharide, mannanoligosaccharide, inulin, or b-glucan, enhance innate immune responses, and many studies concluded that immunosaccharides are beneficial to both finfish and shellfish by enhancing

innate immune responses [165,166]. Furthermore, some studies in pigs, poultry and fish have shown the positive impact on health parameters when combining treatment with certain probiotics and prebiotics compared to probiotics or prebiotics alone or, in some cases, in-feed antimicrobials [10].

Another alternative are **immunomodulators**, that have been particularly studied for use in aquaculture. Immunomodulators are substances or agents capable of modelling the immune response to a desired level, as in immunostimulation, immunosuppression, or induction of immunological tolerance. Immunostimulants can be used to increase the non-specific immune response and activate the specific defence mechanism [10].

Immunostimulants and immunomodulators, comprising a group of biological and synthetic compounds, such as levamisole, b-glucan, peptidoglycan, chitin, chitosan yeast, lipopolysaccharide (LPS), and various plant and animal products, have been found effective in preventing the diseases by enhancing the nonspecific cellular and humoral defence mechanisms [167]. They have received considerable attention in aquaculture in order to increase disease resistance in farmed fish. Several immunostimulants have been demonstrated to enhance the immune response and play a role in protection against disease in fish by injection or oral administration [168,169].

Furthermore, **phage therapy** has gained much attention for its advantages in preventing and controlling pathogen infections; since 1999, phages have been used successfully in aquaculture facilities. Cruz-Papa et al., 2014 [170], in a laboratory scale experiment, showed that *Aeromonas hydrophila* bacteriophage, injected intraperitoneally into the abdominal cavity, was able to decrease the amount of *A. hydrophila* in the fish's blood and mortality in Nile Tilapia (*Oreochromis niloticus*), results that were similar to those obtained using oxytetracycline. In *Oncorhynchus fontinalis*, the supplementation of a bacteriophage delayed for 7 days the development of *Aeromonas salmonicida* furunculosis [171], and Silva et al., 2016 [172] demonstrated the efficacy of a bacteriophage to control furunculosis caused by Aeromonas infection in juvenile forms of soles (*Solea senegalensis*).

Treatments with bacteriophage show some limitations, though, related to the selection of phage-resistant populations [173]. This is a well-described phenomenon, which involves several bacterial phage resistance mechanisms. Some studies [174] demonstrate that the use of three phages applied together avoided the development of such resistance.

The role of bacteriophages in aquaculture was reviewed by Oliveira et al., 2012 [175], who concluded that although beneficial effects could be demonstrated, particularly in invertebrates, more work is needed on effective methods of application in commercial systems.

During an EMA workshop on bacteriophages, one of the conclusions was that any medicine, including bacteriophages, before approval needs to have its efficacy and safety proven based on appropriately designed clinical trials, which is difficult for bacteriophages, which raises some technical difficulties of authorising such products [176].

Evidence on the efficacy of the alternatives for aquaculture, associated risks and specific knowledge gaps are summarised in Table 8.

When it comes to more unspecific infections, related to environmental conditions like unfavourable water quality, low water flow-through and high fish densities, water treatment and fish densities should be addressed. Improved husbandry measures, such as all-in-all-out systems, with fallowing and cleaning/disinfection should be used in all animal production systems.

'All-in-all-out' at farm and coordination-area is an important strategy to avoid building up infection pressure. This means that after one generation of fish at one site (or neighbouring sites), a period of fallowing should follow, with proper cleaning and disinfection before the next generation [10]. Other measures have been consistently applied in Norway since the 1990s, allowing the reduction of spread of disease and use of antimicrobials in aquaculture, such as restrictions on the transport of live fish, improved biosecurity with all-in-all-out systems, regulation of fish stocking density, optimisation of fish farm site placing with regards to water quality and water flow.

The European Commission [177], apart from encouraging the development and use of effective vaccines for aquaculture, also emphasises the importance of adopting appropriate environmental conditions for aquaculture animals kept on farms, in particular regarding water quality, water flow rates, oxygen levels and nutrition. In their “Guidelines for the prudent use of antimicrobials in veterinary medicine” [177], the Commission encourages the implementation of specific hygiene and biosecurity measures, including measures to prevent the introduction and spread of infections, such as operating an ‘all-in all-out’ system per unit or farm; the quickly removal of dead fish and ensuring systems are in place for handling, disposing of and treating by-products; put adequate welfare parameters in place, e.g. for stocking density; the use of antimicrobial sensitivity testing prior to treatment, wherever possible, encouraging the development of specific disease surveillance programmes to identify and help prevent possible outbreaks of disease.

In all aspects of health care, the first step toward minimizing the risk of disease and contagion relies on prevention. In aquaculture, the preventive measures intended to reduce the risk of disease can be called as good aquaculture practices, best management practices, or biosecurity measures, etc., but overall, they have the intended purpose of preventing diseases from occur, and consequently preventing the need to use a chemotherapeutic agent. Therefore, developing and implementing a preventive measures programme is the first step in the prudent and responsible use of veterinary medicines (antimicrobials) in aquatic food production.

Table 8. Alternative measures for the use of antimicrobials in aquaculture: summary on the evidence on the efficacy, associated risks and specific knowledge gaps

Alternative	Target species	Reported data on potential efficacy	Risks[a]	Current regulatory framework applicable	Effects on nutrition and performances	Knowledge gaps	Comments
Probiotics	Fish, crustacea and mollusca	Reduction of mortality due to bacterial infections, mainly at larval stage	Presence of virulence factors and/or AMR determinants in strains used as probiotics	Some of the strains are authorised as zootechnical feed additives	Demonstration of performance improvements for strains authorised as zootechnical feed additives under Regulation (EC) No 1831/2003	Mode of action of probiotics Dose response Limited controlled trials to support efficacy	Data on efficacy as alternatives to antimicrobials are strictly strain dependent
Bacteriophages	Aquaculture	Reduction of bacterial infections	Emergence of phage resistant populations. Might carry AMR determinants. Transduction of virulence genes in the target bacterial population	Not authorised under a specific EU regulatory framework		Long-term efficacy Dose response Limited number of studies to support the efficacy	Data on efficacy as alternatives to antimicrobials are strictly dependent to the strain of phage used and to its host range
Immuno-modulators	Fish	Reduction of bacterial infections	Toxicity residue	VMP or not authorised under a specific EU regulatory framework		Limited studies to support the efficacy	

Prebiotics	All animals	Microbiota development Antitoxins	Antinutritive or toxic residue	Some authorised as feed additives	Can be used as fibre sources, astringent substances, mucilaginous substances	Mode of action; pharmacology; chemical composition and limited controlled trials to support efficacy	Data on efficacy as alternatives to antimicrobials are strictly product/formulation dependent
Symbiotics	Fish	Reduction of bacterial infections	As for probiotics and prebiotics	As for probiotics and prebiotics	As for probiotics and prebiotics	As for probiotics and prebiotics	As for probiotics and prebiotics

AMR: antimicrobial resistance; VMP: veterinary medicinal product a An assessment of the risk related to the use of this potential alternative for the animals, the consumers of food of animal origin and the environment shall be performed, following the requirements of specific authorisation frameworks (e.g. VMP or Feed Additives)

1.4. Legal framework

One of the most relevant principles laid down in the European legislation, specifically in Directive 2001/82/EC [159], amended by Commission Directive 2009/9/EC [178] and by Regulation 470/2009 [65], is the guarantee that foods of animal origin do not include drug residues that can induce harmful effects on human health from a toxicological, pharmacological or microbiological point of view. The legislation, recognizing the importance of the use of pharmaceutical products in these activities, emphasizes that safeguarding public health must be the first concern.

This same principle is the basis of the White Paper on Food Safety [179], which assumes that assuring the highest standards of food safety in the European Union (EU) is a key policy priority for the Commission.

The EU's food safety policy aims to protect consumers, while guaranteeing the smooth operation of the single market. Dating from 2003, the policy centres on the concept of traceability both of inputs (e.g. animal feed) and of outputs (e.g. primary production, processing, storage, transport and retail sale), and has agreed standards to ensure food hygiene, animal health and welfare, and plant health and to control contamination from external substances, such as pesticides. Rigorous checks are carried out at every stage, and imports (e.g. meat) from outside the EU are required to meet the same standards and go through the same checks as food produced inside.

At the EU level, Directive 2001/82/EC [159], that sets up a genuine Code for veterinary medicinal products, regulates the process leading to marketing authorization, in the EU, of any veterinary medicinal product intended for food-producing animals.

It is clear from the preamble of the Directive, that the primary purpose of any rules for the production and distribution of veterinary medicinal products must be the safeguarding of public health, while keeping in mind that this objective must be achieved by means which will not hinder the development of industry and trade in medicinal products within the Community. The Directive states that no

veterinary medicinal product may be placed on the market of a Member State unless a marketing authorization has been issued by the competent authorities of that Member State in accordance with this Directive. Furthermore, a marketing authorization in one Member State ought to be recognized by the competent authority of the other Member States unless there are serious grounds for supposing that the authorization of the veterinary medicinal product concerned may present a risk to human or animal health, or to the environment. In the event of a disagreement between Member States about the quality, the safety or the efficacy of a medicinal product, a scientific evaluation of the matter should be undertaken at a Community level, lead to a single decision on the area of disagreement, binding on the Member States concerned. This Decision should be adopted by a rapid procedure ensuring close cooperation between the Commission and the Member States. In essence, this Directive aims to facilitate the movement of veterinary medicinal products and to prevent the checks carried out in one Member State from being repeated in another, minimum requirements for manufacture and imports from third countries, and the grant of corresponding authorizations, should be applied to veterinary medicinal products.

Furthermore, Directive 2001/82/EC [159] mandatorily requires that all pharmacological active substances present in veterinary medicinal products must have been subject of residue security tests, in accordance with the provisions of Regulation (EC) 470/2009 [65], laying down Community procedures for the establishment of residue limits of pharmacologically active substances in foodstuffs of animal origin, repealing Council Regulation (EEC) 2377/90 [180] and amending Directive 2001/82/EC [159] and Regulation (EC) 726/2004 [181]. This Regulation assumes that veterinary medicinal products are important tools to improve food production, but never neglecting the importance of establishing maximum residue limits (MRL), in accordance with generally recognised principles of safety assessment, in order to protect public health. The establishment of MRLs is conducted by the Committee for Veterinary Medicinal Products (CVMP), set up in accordance with the European Agency for the Evaluation of Medicinal Products. The CVMP is the European Medicines Agency's (EMA) committee responsible for veterinary medicines, established in line with Regulation (EC) 726/2004 [181]. The CVMP recommends safe limits for

residues of veterinary medicines used in food-producing animals and biocidal products used in animal husbandry, for the establishment of MRL by the European Commission. The CVMP's assessments are based on a comprehensive scientific evaluation of data, to determine whether the medicine meets the necessary quality, safety and efficacy requirements and that it has a positive risk-benefit balance in favor of the animal population they are intended for.

There have been several attempts to harmonize MRL's worldwide, under the auspices of the World Trade Organization and the Codex Alimentarius, but MRL's still vary from one geographical location to another [26]. MRLs in a particular animal product may differ from one country to another depending on the local food safety regulatory agencies and drug usage patterns [182] and most developing countries lack to develop their own MRLs.

Commission Regulation (EC) 37/2010 [32] classifies pharmacologically active substances with respect to MRLs in a single annex sorted by alphabetical order. Antibiotics, as permitted veterinary drugs, are included in group B, as described in Annex I of Directive 96/23/EC [183], and many have available MRL data. Their use, though, is completely forbidden as animal growth promoters, after the 2006 EU ban [56].

Table 9 presents the MRL values for antibiotics in fish, according to European Union legislation [32].

The CVMP's approach for the evaluation of the safety of residues is based on the determination of a no/lowest-effect-level and the use of uncertainty factors to determine an acceptable daily intake (ADI) on which subsequently MRLs are based [184]. The ADI is an estimate of the substance and/or its residues, expressed in terms of µg or mg per kg bodyweight, that can be ingested daily over a lifetime without any appreciable health risk to exposed individuals. The establishment of the ADI value is based on the no observed (adverse) effect level (NO(A)EL) or, in certain cases, the lowest observed (adverse) effect level (LO(A)EL) with respect to the most sensitive parameter, in the most sensitive appropriate test species [184].

Table 9. MRL values for antibiotics in fish according to European Union legislation [32]

Antimicrobial Class	Pharmacologically active substance	Marker residue	MRL (µg/kg)[a]
Sulfonamides	All substances belonging to the sulfonamide group	Parent drug	100[b]
Diaminopyrimidine derivatives	Trimethoprim	Trimethoprim	50
Penicillins	Ampicilin	Ampicilin	50
	Amoxicillin	Amoxicillin	50
	Benzylpenicillin	Benzylpenicillin	50
	Cloxacillin	Cloxacillin	300
	Dicloxacillin	Dicloxacillin	300
	Oxacillin	Oxacillin	300
Quinolones	Oxolinic acid	Oxolinic acid	100
	Danofloxacin	Danofloxacin	100
	Difloxacin	Difloxacin	300
	Enrofloxacin	Sum of enrofloxacin and ciprofloxacin	100
	Flumequine[c]	Flumequine	600
	Sarafloxacin[d]	Sarafloxacin	30
Macrolides	Erythromycin	Erythromycin A	200
	Tilmicosin	Tilmicosin	50
	Tylosin	Tylosin A	100
Chloramphenicol derivatives	Florfenicol[c]	Sum of florfenicol and its metabolites measured as florfenicolamine	1000
	Thiamphenicol	Thiamphenicol	50
Tetracyclines	Chlortetracycline	Sum of parent drug and its 4-epimer	100
	Oxytetracycline	Sum of parent drug and its 4-epimer	100
	Tetracycline	Sum of parent drug and its 4- epimer	100
Lincosamides	Lincomycin	Lincomycin	100
Aminoglycosides	Spectinomycin	Spectinomycin	300
	Neomycin (including framycetin)	Neomycin B	500
	Paromomycin	Paromomycin	500
Polymyxins	Colistin	Colistin	150

[a] For fin fish the muscle MRL relates to ‘muscle and skin in natural proportions
[b] The combined total residues of all substances within the sulfonamide group should not exceed 100 µg/kg
[c] Specifically approved for fin fish
[d] Specifically approved for Salmonidae

A specified period of time post-administration of drugs, used in different animal species, must elapse before their edible products are considered safe for human consumption, which corresponds to the necessary time for residue levels to stay below the MRL's [26]. Withdrawal periods are set by drug manufacturers and, during this period, products from treated animals shall not enter the food chain [185].

To check the compliance to these legal standards, efficient analytical methodologies are required, in order to promote effective surveillance.

The analytical methods to be used must be validated in accordance with the requirements of Directive 96/23/EC [183], on measures to monitor certain substances and residues thereof in live animals and animal products. This Directive turned mandatory the control of food producing animals as well as their primary products for the purpose of detecting the presence of the residues and substances listed in its Annex I in live animals, their excrement and body fluids and in tissue, animal products, animal feed and drinking water. In this Directive, residues of concern are divided into group A and group B, where group A includes prohibited substances - with anabolic effect and substances for which a MRL cannot be set - and group B includes veterinary drugs, such as antimicrobials and contaminants, with established MRLs, and compounds for which no MRL has been set as no hazard for consumers has been proved. All food products of animal origin should be free from forbidden or non-authorized substances, or contain quantities below the MRL for allowed compounds. Otherwise, it is considered that the product is not suitable for human consumption.

For non-authorized substances there is no tolerance level but, in some cases, and to harmonize the analytical performance of the methods within official member states laboratories, a minimum required performance limit (MRPL) has been set. MRPL does not refer to a concentration obtained from toxicological data, but only related with the analytical performance. The performance criteria for the analytical methods employed in official residues control are described by the European Commission Decision 2002/657/EC [186].

A brief description of the regulatory requirements set by the European Commission, regarding the performance of analytical methods and their

validation, as well as common criteria for the interpretation of analytical results is provided in the next subheading.

In order to ensure full compliance with the regulatory framework regarding food safety, Council Directive 96/23/EC [183] requires Member States to adopt and implement a national residue monitoring plan for specific groups of residues. The Directive lays down measures to monitor certain substances and residues thereof, mainly veterinary medicinal products, in live animals and animal products. Additionally, Commission Decision 97/747/EC [187] lays down levels and frequencies of sampling for certain animal products. Member States must submit to the Commission, by no later than 31 March of each year, the national monitoring plans together with the monitoring results for the previous year.

In Portugal, Decree-Law n.º 148/99 of 4 May [188], is the legal instrument that transfers into the internal legal system Council Directive 96/23/EC [183], and Commission Decision 97/747/EC [187], on measures to monitor certain substances and residues thereof in live animals and animal products.

The Portuguese residue monitoring plan is the responsibility of the *Direcção Geral de Alimentação e Veterinária* and pursuits the objective of detecting illegal administration of prohibited substances and the abusive use of permitted substances, verifying legal conformity with the MRL established by Commission Regulation (EC) 37/2010 [32].

Regarding retail supply, prescription and record keeping for veterinary medicinal products, Directive 2001/82/EC [159] addresses these issues in articles 66 to 69, laying down the provisions that should be taken by Member States in national legislation. In EU Member States, in general a veterinary prescription is required for dispensing veterinary medicinal products for food producing animals to the public [10]. Additionally, in the 30 European countries that provided data for the ESVAC (European Surveillance of Veterinary Antimicrobial Consumption) project in 2015 [189] all antimicrobial veterinary medicinal products are ‘prescription only’.

But, the regulatory framework regarding the use of antibiotics in aquaculture is limited, differs greatly between countries, and little to no enforcement is present in many of the major producers of aquacultural products [42].

Despite the strict regulation in some regions, in the leading aquaculture production countries regulation and control is very scarce. In India, the second major farmed fish producer, antibiotic sales and usage are not regulated [45]. And in China, the major producer and largest exporter of fish and fishery products [4], no veterinary prescriptions are required for the use of antibiotics in animals [43]. Several laws regulate the Chinese aquaculture industry, and one of the most important, the Food Hygiene Law, prohibits “foods that contain or are contaminated by toxic or deleterious substances and can thus be injurious to human health” and ‘‘foods that contain pathogenic parasites, microorganisms or an amount of microbial toxin exceeding the tolerance prescribed by the State” [66]. Although considering several aspects related to food safety, such as monitoring, inspecting and control of food contamination and food poisoning incidents, the Chinese regulatory framework neglects the early stages of production in which, particularly in aquaculture, the use of banned pharmaceutical agents can be significant. Despite China’s efforts to ensure food security and consumer confidence in their products during the last few years, several reports came to light revealing the use of medically important antibiotics as well as illegal veterinary antibiotics such as chloramphenicol, which suggest that the enforcement of the regulation is lax [67].

In contrast, Norway can be considered a model in this area, because regulation of antimicrobial use in salmon aquaculture is very strict. Along with improved diagnostics, including susceptibility testing and the use of vaccines and probiotics, Norway was able to reduce the use of antimicrobials to negligible levels [68]. Along with Norway, the Netherlands and Denmark are case studies that clearly demonstrate that it is possible to significantly reduce the use of antimicrobials, without reducing the quality and safety of food, and without a damaging economic impact [69,190], and some authors even highlight a reinforcement of their commercial competitiveness [52].

1.5. Validation of Analytical Methods

At the European level, Commission Decision 2002/657/EC [186] lays down rules to harmonize the characterization and the validation procedure of analytical methods performance. This decision defines how methods are to be used in the testing of official samples according to Article 15, paragraph 1, of the Council Directive 96/23/EC [183], and common criteria for the interpretation of analytical results of official control laboratories for samples taken according to the same Directive. The application field of this reference document is analysis of biological matrices for residue and contaminants, including organic and mineral substances, forbidden and regulated substances, based on qualitative and quantitative methods, screening and confirmation analysis.

A major strength of this document is to extend general concepts to a broad panel of detection techniques, proposing a common backbone for the validation of the corresponding analytical methods.

The core issue of this document is to guarantee that the analytical results developed by different laboratories across Europe shall be comparable, and that the quality control has to be ensured in the same basis. Therefore, all the methods must be validated according common procedures and the relevant performance characteristics must be accomplished.

Depending on the control purposes – screening, confirmation, qualitative or quantitative - different control purposes are required. A qualitative method identifies a substance based on its chemical, biological or physical properties [186], while quantitative methods determines the amount or mass fraction of a substance so that it may be expressed as a numerical value of appropriate units [186]. In addition, screening methods are used to detect the presence of a substance or class of substances at the level of interest and are specifically designed to avoid false compliant results [186]. Finally, a confirmatory method provides full or complementary information for unequivocal identification and, if necessary, quantification at the level of interest [186].

For each type of method, specific performance characteristics shall be verified, summarized in table 10.

One of the novelties of this document was the replacement of the limit of detection (LOD) and limit of quantification (LOQ) by two different concepts: the decision limit (CC_α) and the detection capability (CC_β). According to Commission Decision 2002/657/EC [186], the decision limit (CC_α) is defined as “the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant”, and the detection capability (CC_β) as “the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of β”. As described in the same decision, α error means “the probability that the tested sample is compliant, even though a non-compliant measurement has been obtained (false non-compliant decision)”; therefore, statistically, CC_α represents the uncertainty of the method in the result. On the other hand, β error means the “probability that the tested sample is truly noncompliant, even though a compliant measurement has been obtained (false compliant decision)”.

Table 10. Performance characteristics that shall be verified for each type of method (adapted from European Commission, 2002 [186]).

	Qualitative Methods		**Quantitative Methods**	
	S	**C**	**S**	**C**
Detection capability CCß	●	●	●	●
Decision limit CCα		●		●
Trueness / recovery				●
Precision			●	●
Selectivity / Specificity	●	●	●	●
Applicability/ ruggedness/ stability	●	●	●	●

S – screening; **C** – confirmatory

These concepts had already been introduced in the ISO/11843-1 normative document [191], proposing a limit from which a system can be declared different from its basic state. In the present case [186], the system is a diagnostic ion chromatogram of the target analyte, and the basic state corresponds to this ion

chromatogram for a blank sample (forbidden substances) or for a sample containing the analyte at the MRL concentration (regulated compounds).

CC_α and **CC_β** can be obtained, according to Commission Decision 2002/657/EC [186], by determining the signal-to-noise (S/N) ratio, by analysing at least 20 blank samples and calculating the signal to noise ratio at the time window in which the analyte is expected. Three times the signal to noise ratio can be used as decision limit (equation 1).

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

where $S/N_{20\ \text{blank samples}}$ represents the mean of signal-to-noise ratio of 20 blank samples. Alternatively, $CC\alpha$ can be calculated by the calibration curve procedure according to ISO/ 11843-1 [191] (equation 2):

$$CC_\alpha = N_{20\ \text{blank samples}} + 2{,}33 \times SD_{20\ \text{blank samples}} \qquad (\textit{\textbf{Equation 2}}),$$

Where $N_{20\ \text{blank samples}}$ represents the mean of noise amplitude of 20 blank samples and $SD_{20\ \text{blank samples}}$ the standard deviation of the signal obtained in the 20 blank samples.

CC_β can be calculated by analysing at least 20 blank samples fortified with the analyte(s) at the calculated CC_α, according to equation:

$$CC_\beta = CC_\alpha + 1.64 \times SD_{20\ \text{fortified samples at }CC\alpha} \qquad (\textit{\textbf{Equation 3}}),$$

where $SD_{20\ \text{fortified samples at }CC\alpha}$ represents the standard deviation of the signal obtained in the 20 fortified blank samples at the CC_α.

In the case of substances for which a permitted limit has been established, the MRL has to be considered in the calculation of the two regulatory limits. In this case, the equations are as follows:

$$CC_\alpha = C_{MRL} + 1.64 \times SD_{20\ \text{fortified samples at MRL}} \qquad (\textit{\textbf{Equation 4}})$$

$$CC_\beta = CC_\alpha + 1.64 \times SD_{20\ \text{fortified samples at }CC\alpha} \qquad (\textit{\textbf{Equation 5}}),$$

where $SD_{20\ fortified\ samples\ at\ MRL}$ represents the standard deviation observed in 20 blank samples fortified at the MRL level.

Recovery corresponds to the “fraction of mass of the analyte added to the sample, which is present in the final extract” [186], and this parameter has to be determined for confirmatory quantitative methods. When a certified material is present, the recovery range should be within 50 and 120%, for mass fractions ≤1µg/kg, within 70 and 110%, for mass fractions >1 until 10µg/kg, and within 80 and 110%, for mass fractions ≥10µg/kg [186].

Another performance paremeter, mandatory for quantitative methods, is **precision**, which measures the inter-laboratory coefficient of variation (CV) for repeated analysis [186]. Under reproducibility conditions, CV shall not exceed the level calculated by the Horwitz Equation [186], represented as follows:

$$CV = 2(1 - 0,5 \log C) \qquad \text{(\textbf{\textit{Equation 6}})},$$

where C is the mass fraction expressed as a power (exponent) of 10 (e.g. 1 mg/g = 10-3).

Selectivity and specificity, to be determined for all types of methods, correspond to the method’s capacity to discern between the target compound and any other compounds present in the sample. These characteristics depend on the matrix, the compound and the analytical procedure.

Ruggedness and applicability, also to be monitored for all types of methods, reflect the susceptibility of an analytical method to changes in experimental conditions [186]. The possible changes, that may affect the final results, can include storage conditions, environmental and/or sample preparation conditions, among others, and should be tested.

In terms of inequivocal confirmation, Decision 657/2002/EC [186] describes the identification criteria to be fulfilled, introducing the criteria of identification points (IPs), relative retention time (RRT) and ion ratio. For the confirmation of substances listed in Group A of Annex I of Directive 96/23/EC [183], a minimum of 4 IPs shall be required, and for substances listed in Group B, such as antibiotics,

a minimum of 3 IPs is required. A maximum of three separate techniques can be combined to achieve the minimum number of IPs, which depend on the specificity of the MS technique used.

Furthermore, also the relative retention time (RRT), which corresponds to the ratio between the chromatographic retention time of the target compound and its internal standard, should not exceed 2.5%, and the ion ration tolerances have maximum permitted tolerances. For LC-MS^n techniques, maximum permitted tolerances for relative ion intensities are presented in table 11.

Table 11. Maximum permitted tolerances for relative ion intensities using LC-MS^n techniques

Relative intensity (% of base peak)	Ion Ratio Tolerance for LC-MS^n methods
> 50 %	± 20 %
> 20 % to 50 %	± 25 %
> 10 % to 20 %	± 30 %
≤ 10 %	± 50 %

As a final remark, **matrix effects** should also be studied, although not covered by the EU's legislation. Matrix effects (ME) represent a severe drawback in quantitative analysis, interfering with the reproducibility, linearity, and accuracy of the methods [192]. They are strictly related to the sample nature, and even with different batches of the same matrix, and therefore they are rather unpredictable. It is, therefore, broadly accepted that a matrix effect study should be performed, as part of the validation, in order to assess the magnitude of its impact in the final results.

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Chapter 2 | Analytical strategies for the detection and quantification of antibiotic residues in aquaculture fishes: a review

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Abstract

Background: Aquaculture is one of the worldwide strategic development fields, and its importance is evident in its significant worldwide growth in the last decades. This growth is associated with the implementation of intensive and semi-intensive production methods, with the use of antibiotics in order to prevent the emergence and spread of infectious diseases in fish. Fluoroquinolones, tetracyclines, and sulfonamides, among others, are widely used for this purpose. This practice constitutes a real public health concern, not only due to the presence of antimicrobial residues in edible tissues, which can cause allergic reactions in hypersensitive individuals, but also due to the emergence of bacterial resistance. Consequently, the European Union's Regulatory Agencies have established maximum residue limits and specific requirements regarding the performance of analytical methods.

Scope and approach: This article reviews the most recent analytical methodologies concerning antimicrobial residues in fish, reported in the literature, given emphasis on sample procedures, extraction/purification methods, chromatographic conditions and validation techniques according to legislation.

Key findings and conclusions: Liquid chromatography coupled to mass spectrometric detection is used as preferential tool in the analysis of antimicrobial residues in fish. The current analytical strategy is shifting towards multi-residue and multiclass methods, which save time, and surely represent the future trend in this field. The extraction process still represents the limiting factor of any multi-residual method, since it should provide acceptable recovery of all analytes with a broad range of physicochemical properties, and therefore this is probably the step that requires more in-depth research.

Introduction

Food safety, as well as its consequences on human health, has become an extremely important topic for consumers and for public health authorities. In particular, there have been numerous events involving large-scale contamination of foods of animal origin.

The quality of aquaculture fish is increasingly important because of the presence of antimicrobial residues in edible tissues.

The current definition of aquaculture, according to the Food and Agriculture Organization of the United Nations (FAO)/Network of Aquaculture Centres in Asia-Pacific (NACA)/ World Health Organization (WHO) [1], is "*the farming of aquatic organisms including fish, molluscs, crustaceans and aquatic plants*". Farming implies intervention in the rearing process to enhance production, such as regular stocking, feeding and protection from predators.

Aquaculture is a significant goal of any country's economic development plan because of its worldwide growth. According to FAO data [2], aquaculture total fish production by weight has consistently grown from 13.4% in 1990 to 25.7% in 2000 and to 42.2% in 2012.

Aquaculture exhibits a faster growth than any other animal production sector. Its relative contribution to the total amount of fish produced to human consumption ranged from 5% in 1962 to 37% in 2002 and to 49% in 2012 [2].

The rapid transition from a capture species model to a culture and production model was a necessary response to the market needs. Increased marine pollution and overfishing, along with global climate change, has greatly affected fish stocks. Aquaculture has the possibility of producing larger quantities of products in reduced space than the wild capture of species.

Although aquaculture has many theoretical advantages, the reality is not as positive. The fast growth of these productions has resulted in concerns over fish quality and safety. Similar to other sectors of animal production, fish production adopts intensive and semi-intensive practices. These practices lead to a higher concentration of animals in small spaces and substantially increase the risk of disease [3].

Disease is one of the major constraints of aquaculture [4]. In this industry, infectious diseases are a continuous hazard resulting in major stock losses. The same strategies used in other areas of animal production are employed to control infectious diseases in aquaculture. In cases in which antibiotics must be used, they must be strictly controlled, following the identical code applied to veterinary

medicine. There are no antibiotics specifically designed for aquaculture; therefore, authorized products developed for other areas of veterinary medicine are used. A similar range of antibiotics is permitted in European countries for aquaculture purposes [5,6]: oxytetracycline, florfenicol, sarafloxacin, erythromycin and sulphonamides (potentiated with trimethoprim or ormethoprim).

Two major concerns arise from these practices related to their effect on consumers' health:

a) The presence of antimicrobial residues in edible tissues of treated animals. In persistent low doses, they become part of the consumers' diet;

b) The emergence of antimicrobial resistance, which represents a huge threat to public health worldwide according to health professionals, governments, WHO and other non-governmental international agencies [7-10].

The inappropriate, and frequently abusive, use of antibiotics affects human health. It is also evident that the public health hazards related to antimicrobial use in aquaculture include the development and spread of antimicrobial resistant bacteria and resistance genes. The greatest potential risk to public health associated with antimicrobial use in aquaculture is the development of a reservoir of transferable resistance genes in bacteria, and in aquatic environments. These genes can be disseminated by horizontal gene transfer to other bacteria and ultimately reach human pathogens.

In 2004, the WHO highlighted the lack of consistent data on the emergence of bacterial resistance in aquaculture species [11]. The problem was clarified in 2006. In a joint meeting held by the WHO, FAO and the OIE [8], the risks associated with the use of antimicrobials in aquaculture were assessed, and the risk of dissemination of resistance genes from fish bacteria to human pathogens was highlighted.

Antibiotics lose their efficacy over time because of the emergence and dissemination of resistance among bacterial pathogens. Strains with resistance to multiple antibiotic classes have emerged among major Gram-positive and Gram-negative species including *Staphylococcus aureus*, *Enterococcus* spp., *Pseudomonas aeruginosa*, *Acinetobacter* spp. *Enterobacteriaceae*, and

Neisseria gonorrhoeae. The resistance of some Gram-negative bacteria may involve most or even all the available antimicrobial options, resulting in extremely drug-resistant or completely drug-resistant phenotypes [12]. This ‘antibiotic resistance crisis’ emerged as the major contribution from the use and abuse of antimicrobials in food-producing animals and aquaculture production.

To minimize the risk to human health from the use of antimicrobials in food animal production, the European Community created a legal framework to regulate and control the use of veterinary drugs in products of animal origin. Monitoring antibiotic residues in edible animal tissues requires sensitive and selective analytical methodologies capable of verifying these legal demands and ensuring food safety and consumers’ confidence.

Several review papers discuss antimicrobial residues in edible tissues and fluids of food-producing animals. However, there are few published articles that specifically examine fish.

The aim this work is to review the most recent analytical strategies to determine antimicrobial residues in fish, to discuss performance parameters and their suitability to legal requirements and comprehensively present the current trends in this field.

2.1. Legislation

One of the most relevant principles in European legislation, specifically in Directive 2001/82/EC [13], amended by Commission Directive 2009/9/EC [14] and by Regulation 470/2009 [15], is the guarantee that foods of animal origin do not include drug residues that can induce harmful effects on human health from a toxicological, pharmacological or microbiological point of view. The law, recognizing the importance of the use of pharmaceutical products in these activities, emphasizes that safeguarding public health must be the first concern.

Regulation N. º 470/2009 of the European Parliament and the Council [15] outlines the procedures for the establishment of residue limits of pharmacologically active

substances in foodstuffs of animal origin. This regulation repeals Council Regulation (EEC) 2377/90 [16] and amends Directive 2001/82/EC of the European Parliament and of the Council [13] and Regulation (EC) No 726/2004 of the European Parliament and of the Council [17]. This document outlines the rules and procedures that establish the maximum concentration of a residue of a pharmacologically active substance permitted in food of animal origin (the maximum residue limit or MRL). This regulation establishes the level of a residue of a pharmacologically active substance for control reasons when a MRL has not been assigned.

Commission Regulation (EU) 37/2010 [18] classified pharmacologically active substances with respect to MRLs in a single annex sorted by alphabetical order. Antibiotics, as permitted veterinary drugs, are included in group B, and many have available MRL data.

The European Commission Decision 2002/657/EC [19] describes the performance criteria for the analytical methods employed in official residues control.

There are few veterinary medicines licenced for use in aquatic animals destined for human consumption, and they require withdrawal periods [20]. Governments have the responsibility of protecting consumers from being exposed to harmful concentrations of medicine residues in their diets. This goal is addressed by the implementation of monitoring regimens that are based on the MRL for each substance and the analytical testing of samples obtained from animals destined for human consumption [21]. In the EU, sampling regimens for aquaculture products are prescribed in Directive 96/23/EC [22]. Residues of concern are divided into group A and group B. Group A includes substances with anabolic effect and substances for which a MRL cannot be set. Group B includes veterinary drugs, such as antimicrobials and contaminants.

Aquaculture production in European countries accounts for 4.32% of the worldwide production, being the majority of the production (almost 90%) in Asia. China alone produces 61.69% of the total world aquaculture production and being, by far, the largest exporter of fish and fishery products [2]. Thus, a brief description of China's regulatory framework is mandatory.

In China antibiotic usage in animals is restricted to using only non-human medicine drugs and, since 2003, several reforms have been attempted to improve food safety [23].

There are several laws that regulate the Chinese aquaculture industry. The principal law for food safety - the Food Hygiene Law - prohibits “foods that contain or are contaminated by toxic or deleterious substances and can thus be injurious to human health” and “foods that contain pathogenic parasites, microorganisms or an amount of microbial toxin exceeding the tolerance prescribed by the State” [23]. The Food Hygiene Law gives responsibility to the Ministry of Health for monitoring, inspecting and giving technical assistance for food hygiene, as well as investigating food contamination and food poisoning incidents, and several other entities assume responsibility of other areas of food safety. The fragmentary nature of enforcement of existing food laws results in a system that operates far from optimal efficiency and effectiveness [24].

Unlike many other countries, that consider the whole of the food chain from the beginning of production to the end consumer - the so called “farm to fork” model - the Chinese model does not consider the early stages of production in its regulatory framework which, in aquaculture, is when there can be significant use of banned pharmaceutical agents.

Another problem is the scarcity of official inspectors to keep track of the vast number of small- and large-scale producers, along with the strong local government protectionism of local producers that, together, may decrease the effectiveness of the inspection process [23]. Furthermore, sanctions are minor and often not applied [25].

Despite China's effort, over the last years, in order to assure food security, and assure consumer's confidence in their products, reports of medically important antibiotics such as tetracyclines being used and detections of illegal veterinary antibiotics, like chloramphenicol, in Chinese waters suggest that enforcement of the regulation is lax [26,27]. To assess the magnitude of the use of antibiotics in aquaculture production, data regarding the classes and amounts of antibiotics used for agriculture and aquaculture, in several world regions, should be taken into account.

In 2003, salmon aquaculture in Chile, for instance, used about 0.5 g of antibiotic for each kg of salmon produced, whereas the amount in Norway, the second major exporter of aquaculture products, was 0.002 g [28].

Today, unlike in the EU, no veterinary prescriptions are required in China for the use of antibiotics in animals [29], and this could be a first attempt to control the use of antimicrobials in China's aquaculture industry.

2.2. Antimicrobials and analytical methodologies

Antimicrobials are chemical substances that either destroy (bactericidal) or inhibit the growth of microorganisms (bacteriostatic). Although the term “antibiotic” refers to the group of these substances that are produced by microorganisms, both terms are used indistinctly in this paper.

The monitoring of antimicrobial residues in fish tissues requires sensitive and selective analytical methodologies to verify the accomplishment of the legal framework and reach the desirable high standards of quality and food safety. In the following paragraphs, the main antimicrobial classes are presented, highlighting their antimicrobial activity, common uses and chemical features that determine the most appropriate analytical techniques. For each group of antibiotics, the most relevant methodologies are presented, giving emphasis on the extraction and purification steps, as they are a key issue regarding the effectiveness of the analytical determination.

2.2.1. Aminoglycosides

Aminoglycosides (AG) are potent bactericidal antibiotics that are active against aerobic, Gram-negative bacteria and act synergistically against certain Gram-positive organisms [30]. Gentamicin (Figure 4A) is the most commonly used aminoglycoside.

The AG are used in veterinary medicine and animal husbandry both prophylactically and for the treatment of bacterial infections [31].

Some countries and organizations have prescribed the MRLs of AG in foods and, among them, the European Union has clearly forbidden the use of AG as growth promoters in livestock.

Because of the potential health risks that are brought out by AG, a number of sensitive and rapid detection methods have been developed for its determination in food.

Extraction and clean-up procedures

The physicochemical properties of AG - they are basic, water soluble, mostly hydrophilic compounds that are susceptible to photodegradation [32] - complicate the sample extraction process. General steps involve sample homogenization, protein precipitation, mechanical shaking or sonication for release of adsorbed AG into solution, separation of the precipitate and the liquid phase, clean-up using solid phase extraction (SPE) to remove the acid or salt ruminants, defatting using n-hexane, and, in some cases, pre-concentration steps [33].

Stead (2000) [34] published a comprehensive review of the quantitative methods for the determination of AGs. The use of SPE has been widely used in the purification and enrichment of AGs in samples and has greatly increased the convenience and performance of sample preparation for high-performance liquid chromatography (HPLC) analysis.

For LC-MS determination of 11 AGs, Kaufmann and Maden (2005) [35] used a low-pH extraction with trichloracetic acid to ensure complete extraction of the analytes from the matrix. An anion-exchange step is used to remove the acid from the centrifuged extract. AGs in this solution of low ionic strength can be quantitatively retained and eluted from a weak cation-exchanger SPE cartridge.

Recently, the same group [36] used a simple clean-up procedure based on a strong cation exchange solid-phase cartridge that permits high sample extract loading volumes, to determine 13 commonly used AG antibiotics in various matrixes, including fish, by LC-MS/MS.

Determination methods

Determination of AGs can be performed either directly, e.g., by spectrophotometric, immunochemical, or microbiological methods, or after liquid chromatography (LC) separation. Regarding the LC-based methods, there is an important challenge to be taken into account, related with the molecular structures of AGs. As AGs lack a chromophore, the widespread UV detection system is not the method of choice, unless analyte derivatization is performed. Therefore, the derivation of the analyte has become an important work of pretreatment [37].

McGlinchey and colleagues (2008) [38] presented a review of analytical methods for the determination of AGs and macrolide residues in food matrices. As mentioned, the absence of chromophores or fluorophores in aminoglycoside molecules indicates that derivatization is the procedure of choice for fluorescence detection. However, the procedure is time consuming, as the derivatives degrade within a few hours after formation. Mass spectrometry is the most suitable detection method for AGs, with the advantages of sensitivity and unequivocal confirmation of identity [36,38-40]. The amino groups of these compounds ionize well with electrospray, eliminating the need for derivatization. However, AGs are not adequately retained on reversed-phase columns, representing an analytical challenge to the chromatographic separation and subsequent mass spectrometry (MS) analysis of these compounds. Hydrophilic interaction chromatography (HILIC) is an alternative technique. One disadvantage of this method is the high ionic strength buffers and specialized expensive chromatographic columns it requires.

Turnipseed et al. (2009) [40] demonstrated that the derivatization of AGs with phenyl isocyanate provided derivatives that could be easily synthesized, retained and separated on a common reversed phase column. This method eliminates the need for ion-pair reagents or HILIC liquid chromatography (LC) columns. Kaufmann and Maden (2005) [35] reported a method using LC with tandem MS to determine 11 commonly used AG antibiotics in meat. The proposed method, suitable for the quantification and confirmation of AGs in a variety of matrixes such as fish, presents detection limits of 15-40 μg/kg for the various antibiotics.

Spiked fish tissues at 20, 100, 500, and 1000 µg/kg levels were analysed. Poor recoveries were obtained for several derivatives such as neomycin.

Recently, Kaufmann et al. (2012) [36] developed a quantitative LC-MS/MS method for the determination of 13 commonly used AG antibiotics in various matrixes including fish. The validation of the method was performed according to the European Union (EU) Commission Decision 2002/657/EC [19], resulting in decision limit (CCα) and detection capability (CCβ) values in fish matrixes that ranged from 60 to 1139 µg/kg and 86 to1483 µg/kg, respectively. This method can be applied to all relevant AGs in a variety of matrices at a suitable sensitivity.

2.2.2. Amphenicols

The most representative amphenicol is chloramphenicol (CAP) (Figure 4B). CAP is a broad-spectrum antibiotic and a protein synthesis inhibitor that is effective against a wide range of microorganisms and has been widely used since the 1950s to treat food-producing animals. CAP has been associated with serious toxic effects including bone marrow depression. It is particularly severe in the dose-independent, and fatal, aplastic anaemia [41].

The well-known risk of blood disorders and carcinogenic properties of CAP, and the absence of safe residue levels, has prompted the EU to prohibit it for veterinary use. CAP is also prohibited in many other countries, including the USA, Canada, Australia, Japan, and China. No MRL has been established for this antibiotic [16,18].

Despite this legal ban, CAP can be detected in several animal derived foods including aquaculture products and honey. It is important to control CAP residues in food of animal origin, and necessary to develop sensitive methods for its detection and quantification. Techniques must detect the presence of the compound at the minimum required performance limit (MRPL) level (0.3 µg/kg, in all food of animal origin) [19,42]. Other compounds with similar chemical structures, thiamphenicol and florfenicol, are permitted as substitutes.

Extraction and clean-up procedures

Several analytical methods have been developed and reviewed for the detection and quantification of CAP in foods and biological fluids [43-46]. In most cases, a simple sample preparation procedure is required.

Rønning and colleagues (2006) [47] presented a simple sample preparation for most matrices consisting of extraction in acetonitrile using penta deuterated CAP (d5-CAP) as the internal standard to perform an LC-MS/MS method for the determination of CAP residues in several food matrices, including seafood.

Veach et al. (2015) [48] described a method for the rapid determination of CAP and nitrofuran metabolites in various aquaculture matrixes, including catfish, crawfish and shrimp, with the extraction and clean-up procedures consisting on a microwave-assisted derivatization and automated SPE.

Also to determine CAP and various nitrofuran metabolites residues in a number of animal based food products, including fish, Kaufmann and colleagues (2015) [49] described a method based on the hydrolysis of covalently bound metabolites and derivatization with 2-nitrobenzaldehyde. Clean-up is achieved by a liquid/liquid and a reversed phase/solid phase extraction.

Determination methods

Gas chromatography (GC) was the analytical tool previously used to determine CAP, florfenicol, and thiamphenicol levels in fish and shrimp samples. Currently, LC-MS/MS without derivatization is the technique of choice to determine antibiotic residues [43,47,48,50]. This hyphenation of liquid chromatography and mass spectrometry enables the detection and quantification, without derivatization, of polar non-volatile analytes, such as CAP.

Rønning et al. (2006) [47] developed an LC-MS/MS method for the determination of CAP residues in several food matrices. The method was validated in accordance with Commission Decision 2002/657/EC [19] for all matrices with reproducibility values below 25%. The critical concentrations were determined with decision limit (CCα) and detection capability (CCβ) values of 0.02 and 0.04

µg/kg for the 321/152 ion transition and 0.02 and 0.03 µg/kg for the 321/194 ion transition, respectively.

Hammack et al. (2003) [51] published a multi-laboratory validation method for CAP in shrimp and crabmeat using LC-MS. This method was validated in three laboratories.

Several techniques have been described for the analysis of CAP in fish: atmospheric pressure photoionization mass spectrometry [52], microcell electron capture detector mass spectrometry [53], SPE-LC-(ESI)-MS/MS [54], and GC-(NCI)-MS [55].

Florfenicol amine and florfenicol in fish can be quantified by LC with ultraviolet (UV) detection [56,57].

Recently, Veach et al. (2015) [48] developed and validated a LC-MS/MS method, according to Food and Drug Administration (FDA) guidance [58]. The method showed robustness, exhibiting a CAP detection limit for all matrixes ≤ 0.01 µg/kg and a LOQ of ≤ 0.03 µg/kg.

Kaufmann et al. (2015) [49] developed an ultra-high-performance-liquid chromatography based method, coupled to high resolution mass spectrometry (UHPLC-HRMS), to permit the detection and quantification of various nitrofuran and CAP residues in a number of animal based food products, including fish. The method has been validated according to Commission Decision 2002/657/EC [19], achieving limits of detection, in fish, of 0.05 µg/kg for CAP.

2.2.3. Beta-lactam antibiotics

β-lactam antibiotics are antibiotic agents that contain a β-lactam ring in their molecular structure and include penicillin derivatives, cephalosporins, monobactams, carbapenems and β-lactamase inhibitor. The β-lactam family can be divided into two main groups: penicillins and cephalosporins. Both have a four-member cyclic amine (Figure 4C and 4D).

β-lactam antibiotics are the most widely used group of antibiotics in veterinary medicine for the treatment of bacterial infections of animals used in livestock

farming [31]. According to EU Commission Regulation 37/2010 [18], penicillins are the β-lactam antibiotics licenced for aquaculture.

Extraction and clean-up procedures

The presence of the unstable and thermally labile four membered b-lactam ring makes these compounds easily degraded by heat and alcohols. Therefore, the temperature and pH during sample preparation affect the stability of these antibiotics.

Most of the published methods, regarding β-lactam determination, used SPE for the isolation of the analytes from the fish tissue [43,54,59].

Determination methods

Samanidou, Nisyriou, and Papadoyannis (2007) [60] published a systematic review of the residue analysis of penicillins in food products of animal origin. LC has become the analytical method of choice for the identification and quantification of these drugs. Recent advances in LC and LC-MS/MS analysis of penicillin residues in food products have also been reviewed, with a focus on detection, confirmation, and sample preparation.

Amoxicillin, ampicillin, cloxacillin, dicloxacillin, oxacillin, and bencilpenicillin are controlled by the EU, and the MRLs are fixed for edible animal tissues. Methods to determine penicillin in fish samples by HPLC are limited. Samanidou et al. (2007) [60] reported that penicillin G was determined in Chinook salmon by using acetonitrile (ACN) - phosphate buffer as the mobile phase. Ampicillin in catfish was determined [61] by HPLC with fluorescence detection. Gramse and Jacobson (2005) [62] published a general method to determine penicillin G in feed by HPLC. De Baere and colleagues (2002) [63] and Freitas et al. (2012) [59] studied the degradation of amoxicillin in muscle and in solution under different temperature and pH conditions. The use of LC-MS/MS allowed for the characterization of amoxicillin's degradation products at trace levels.

2.2.4. Macrolides

Macrolides are highly potent antimicrobials used in veterinary practices against a wide variety of Gram-positive and Gram-negative bacteria. They consist of macrocyclic lactone rings with 14 (erythromycin, roxithromycin and clarithromycin), 15 (azithromycin) or 16 (spiramycin, tylosin, tilmicosin and josamycin) carbons linked to the carbohydrate molecules. They possess lipophilic and basic characteristics. The chemical structure of erythromycin is presented in Figure 4E.

Macrolides are used in veterinary medicine to treat respiratory tract infections and as growth promoters.

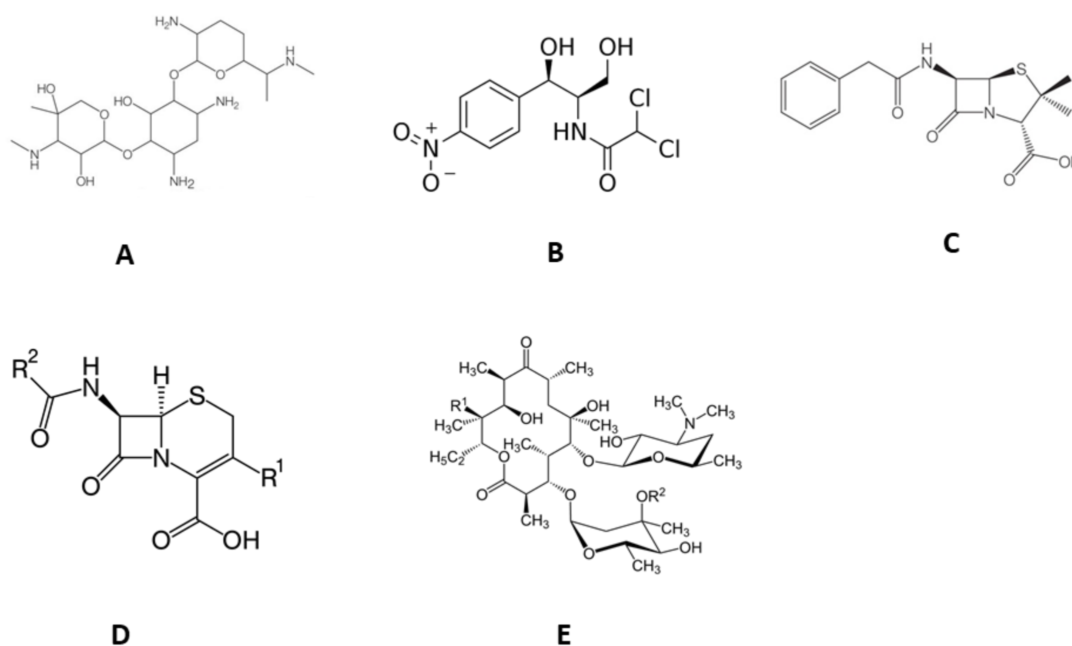

Figure 4. Chemical structures of gentamicin (A), chloramphenicol (B), penicillin G (C), cephalosporin core structure (D) and erythromycin (E).

Extraction and clean-up procedures

The clean-up with a cation exchange solid-phase extraction seems effective in obtaining clean chromatograms of food complex matrices, being the most frequent technique.

Recently, Sismotto et al. (2014) [64] described a technique using a very simple, and less expensive, approach of the extraction and clean up steps that resulted

in adequate recoveries and precision. The procedure consisted on the alcoholic precipitation of fish proteins, with the simultaneous extraction of the analytes, followed by clean-up step using n-hexane.

Determination methods

The molecular structure of macrolides contains chromophores, which allows them to be analysed by UV and fluorometric detection. However, the improved sensitivity and specificity of MS has replaced UV and fluorometric methods in detection and quantification of macrolides in different biological matrices [65].

Some macrolides, for example tilmicosin and spiramycin, have relatively strong UV absorption. However, erythromycin does not have a specific UV chromophore. Therefore, LC-MS is the most promising technique for the separation and determination of macrolide molecules in fish and other food samples.

Horie et al. (2003) [66] developed a simple method using liquid chromatography-electrospray ionization mass spectrometry (LC-(ESI)-MS) for the determination of 8 macrolide antibiotics (erythromycin, oleandomycin, kitasamycin, josamycin, mirosamicin, spiramycin, tilmicosin and tylosin) in meat and fish. The LOQ was 10 µg/kg.

Seven macrolides (erythromycin A, josamycin, roxithromycin, spiramycin, tilmicosin, troleandomycin and tylosin) in fish were determined by using pressurised liquid extraction (PLE) and LC-(ESI)-MS [67]. The results demonstrated that PLE is a quantitative short time-consuming technique, with smaller initial sample sizes. The analytical limits CCα and CCβ were determined as required by Commission Decision 2002/657/EC [19] and ranged between 6 and 208 µg/kg and 15 and 211 µg/kg, respectively.

Sismotto et al. (2014) [64] presented a simple method for the simultaneous identification and quantification of macrolides (erythromycin, josamycin, tilmicosin, tylosin, spiramycin and neospiramycin) in tilapia fillets by LC coupled to quadrupole time of flight (QToF) mass spectrometry. This method is a simple

and low-cost procedure for sample preparation, and the limits of quantification (17-82 μg/kg) were at least 45% lower than the MRL.

2.2.5. Nitrofurans

Nitrofurans (furazolidone, furaltadone, nitrofurazone, nifursol, nifurpirinol and nitrofurantoin) are a group of synthetic antibacterial agents that were widely used in food-producing animals before their prohibition within the EU (1993 and 1995 for furazolidone) because of their potential harmful effects on human health.

Nifurpirinol and nitrofurazone are effective against many fish pathogens. However, they are carcinogenic and mutagenic, and it is illegal to use them in fish intended for consumption in many countries.

In March 2003, a MRPL was set at 1 μg/kg in the EU for these drugs in poultry and aquaculture products [42].

The testing for residues of the parent drugs is ineffective because nitrofuran compounds are rapidly metabolized. In vivo, they form stable and persistent tissue-bound residues. The compounds AOZ (3-amino-2-oxazolidinone), AMOZ (3-amino-5-morpholinomethyl-2-oxazolidinone), AHD (1-aminohydantoin) and SEM (semicarbazide) are the marker residues of the nitrofuran banned parent drugs furazolidone, furaltadone, nitrofurantoin and nitrofurazone [68].

The chemical structure of nitrofurans is shown in Figure 5.

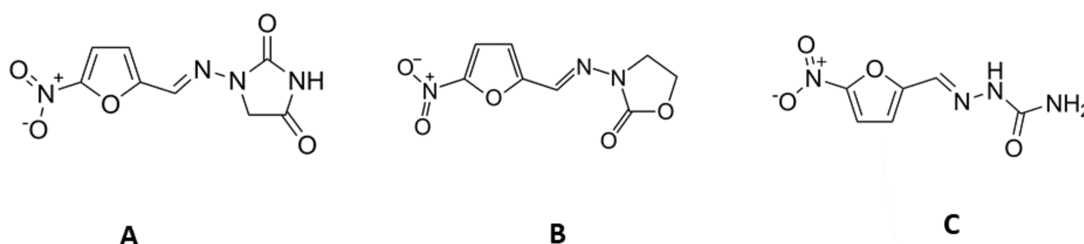

Figure 5. Chemical structures of the nitrofurans nitrofurantoin (A), furazolidone (B) and nitrofurazone (C).

Extraction and clean-up procedures

Although several methods are published, regarding the determination of nitrofuran in food matrices, the core procedure of the extraction and clean-up steps consists on the same principles. They begin with homogenization, followed by acid hydrolysis, derivatization with ortho-nitrobenzaldehyde (*o*-NBA) and extraction with a polar organic solvent [65,69].

Determination methods

LC-MS/MS is the current tool for the detection of nitrofuran tissue-bound side-chain metabolites. It is used throughout the world in animal tissue and other matrices.

The methods reported involve the detection of the nitrophenyl derivatives of nitrofuran metabolites, as described by Leitner, Zollner, and Lindner (2001) [70] and Conneely et al. (2003) [71].

In 2007, Barbosa et al. (2007) [69] described a LC-MS/MS method for the routine detection and quantification of persistent tissue-bound nitrofuran metabolites in several matrixes including farmed fish. The method was fully validated, according to Commission Decision 2002/657/EC [19], exhibiting CCα and CCβ values that ranged from 0.15 to 0.45 μg/kg and 0.32 to 0.88 μg/kg, respectively.

Tsai, Tang, and Wang (2010) [72] developed and validated a LC-(ESI)-MS/MS method based on the European Union regulations to determine the presence of furazolidone, furaltadone, nitrofurazone, nitrofurantoin and their corresponding metabolites AOZ, AMOZ, SEM and AHD in fish muscle. The decision limits ranged from 2.93 to 5.01 μg/kg for the nitrofurans and 0.19 to 0.43 μg/kg for the metabolites. The detection capability was between 3.62 and 6.20 μg/kg for the nitrofurans and between 0.23 and 0.54 μg/kg for the metabolites. This method was suitable for the analysis of the four nitrofurans and resulted in limits of quantification lower than the MRPL (1 μg/kg by the EU) [42].

2.2.6. Quinolones

Quinolones represent a group of synthetic antibiotics used in both human and veterinary medicine. They are used in the treatment of septicaemia or skin diseases in fish [30].

The introduction of the fluorinated quinolones provided important therapeutic advantages because this antibiotic group has higher antibacterial activity than the parent compounds [31] and is highly active against both Gram-positive and Gram-negative strains.

Their extensive administration to fish destined for human consumption, has become a serious problem because their residues can persist in edible animal tissues [73].

The chemical structures of quinolones and fluoroquinolones (FQ) are shown in Figure 6.

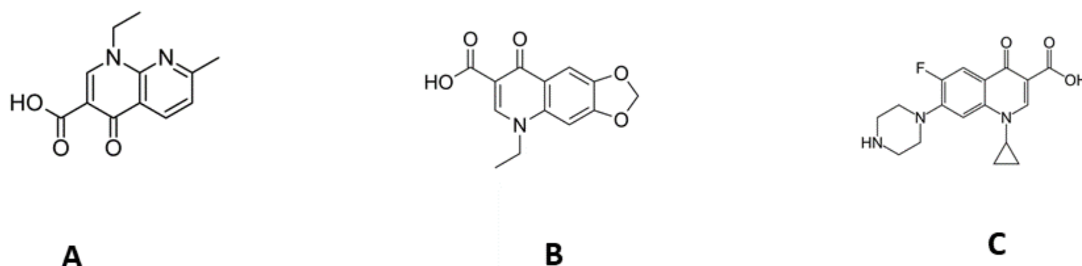

Figure 6. Chemical structures of the quinolones nalidixic acid (A), oxolinic acid (B) and ciprofloxacin (C).

Extraction and clean-up procedures

The literature describes several methods for the extraction of FQ in fish, such as QuEChERS extraction (quick, easy, cheap, effective, rugged, and safe) [74], and also some clean-up procedures, like solid-phase extraction (SPE) [75-78] and dispersive solid-phase extraction (DSPE) [79].

Solvent evaporation may be achieved by nitrogen stream evaporation (55ºC) and other procedures that assist in the reduction of the extract's final volume and, thus, enhancing detection.

Determination methods

HPLC is the most widely used analytical method for these compounds with UV [31,80] or fluorescence detection [81,82].

LC coupled with MS detection has become the preferred analytical method for quantification [83].

Through HPLC with electrospray ionization tandem mass spectrometry, eight quinolones (oxolinic acid, flumequine, piromidic acid, enrofloxacin, ciprofloxacin, danofloxacin, sarafloxacin and orbifloxacin) have been identified in trout, prawns and abalone [75]. The limits of detection were 1-3 μg/kg, depending on the analyte and matrix. The limit of quantification was 5 μg/kg (10 μg/kg for ciprofloxacin).

A multi-residue method for the analysis of FQ in shrimp samples has been developed by combining fluorescence detection (LC-FLD) for quantification and confirmation with multiple-stage mass spectrometry (MSn) [84].

Karbiwnyk and colleagues (2007) [85] developed a liquid chromatography-fluorescence (LC-FLD) method to determine oxolinic acid, flumequine and nalidixic acid residues in shrimp. An additional liquid chromatography multiple stage mass spectrometry (LC-MSn) method was created to confirm these residues using identical sample extracts. Reverse phase chromatography was used to separate the three compounds in both procedures.

Multi-residue determination of seven quinolones (ciprofloxacin, enrofloxacin, sarafloxacin, danofloxacin, oxolinic acid, nalidixic acid and flumequine) in gilthead seabream (*Sparus aurata*) was developed [86]. The sample pre-treatment used an extraction with 0.1 M NaOH and purification by SPE followed by the determination of all compounds in a single LC-(ESI)-MS/MS run. The LOQ of the examined quinolones extracted from fish tissue ranged from 6 to 8 μg/kg. All seven antibiotics were determined at the concentration level of 10 μg/kg.

Zhang et al. (2010) [87] developed another multiresidue analysis method for the extraction and determination of eleven quinolones (pipemidic acid, enoxacin, norfloxacin, ciprofloxacin, lomefloxacin, enrofloxacin, gatifloxacin, difloxacin, oxolinic acid, nalidixic acid and flumequine) in fish by liquid chromatography

coupled with fluorescence detection. Detectable residues were observed at concentrations ranging from 4.74 to 23.27 μg/kg, with a sub-2 μm HPLC column.

Pilco Quesada et al. (2013) [88] reported a simple analytical method for the simultaneous determination of norfloxacin, danofloxacin, enrofloxacin and ciprofloxacin levels in tilapia (*Oreochromis niloticus*) and pacu (*Piaractus mesopotamicus*) fillets using LC-MS/MS (QToF). LOD ranged from 4 to 8 μg/kg, depending on the matrix and the compound. The LOQ was 25 μg/kg in all cases.

A confirmatory HPLC method for the determination of seven quinolone antibiotics (ciprofloxacin, danofloxacin, enrofloxacin, sarafloxacin, oxolinic acid, nalidixic acid and flumequine) in the tissue of Atlantic salmon (*Salmo salar* L.) was developed by Evaggelopoulou and Samanidou (2013) [78]. The same group [89] reported a HPLC method for the determination of seven quinolone (ciprofloxacin, danofloxacin, enrofloxacin, sarafloxacin, oxolinic acid, nalidixic acid, and flumequine) antibiotics in fish feed and gilthead sea bream (*Sparus aurata* L.) using photodiode array detection (PDA) and the identical sample preparation procedure. Nevertheless, LC-MS/MS is currently the analytical method of choice for routine quality control of quinolones in fish.

2.2.7. Sulfonamides

The sulfonamide family includes sulfadiazine, sulfamethizole, sulfamethoxazole, sulfasalazine, sulfisoxazole and various high strength combinations of three sulfonamides. Sulfonamides are based on a *p*-aminobenzenesulfonamide functional group (Figure 7).

Sulfonamides (SA) are widely used for therapeutic and prophylactic purposes in both humans and animals, including fish. They are sometimes used as growth promoter additives in animal feed.

There is concern whether the levels of these drugs could lead to serious human health problems, e.g., allergic or toxic reactions [90] as a result of their widespread use. Some SAs are potentially carcinogenic, leading to a debate in food safety. The EU [18] has set an MRL for total sulfonamide concentration in fish at 100 μg/kg.

Figure 7. Chemical structures of the sulfonamides sulfadiazine (A) and sulfamethoxazole (B).

Extraction and clean-up procedures

There are several published techniques regarding the extraction of SAs from fish matrices.

Bogialli and colleagues (2003) [91] used the matrix solid-phase dispersion technique with hot water extraction, as Won et al. (2011) [92] described a liquid-phase extraction step using acetonitrile followed by SPE using C18.

To minimize matrix interferences, Nebot, et al. (2010) [93] proposed 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, leading to very good recoveries, which demonstrate the applicability of this simple protocol for extraction.

Determination methods

GC-MS methods are considered to be an inappropriate option as they require a previous derivatization step, because of the high polarity and low volatility of these compounds. Several methods for SA determination, based on HPLC, have been reported but, nowadays, these methods are being replaced by MS/MSn methods with the advantage of achieving more sensibility and specificity.

Bogialli et al. (2003) [91] developed a LC-MS assay for the analysis of SAs in fish muscle based on the matrix solid-phase dispersion technique with hot water extraction followed by LC-MS. The authors estimated a limit of quantification of 3-13 μg/kg in trout fillet.

Multi-residue determination of 14 SAs in catfish, shrimp, and salmon using a post-column fluorescence derivatization HPLC method was reported [94]. The method was validated at 5, 10, and 20 μg/kg.

Potter et al. (2007) [95] described a method that allowed for quantification in salmon by LC-MS/MS of seventeen SAs and the potentiators ormetoprim and trimethoprim. The LOD varied from 0.1 to 0.9 μg/kg.

Won et al. (2011) [92] developed a method for SA determination in fish (flatfish, jacopever, sea bream, common eel, blue crab and abalone) and shrimp using HPLC with PDA and ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) for confirmation and identification of target compounds. For HPLC-PDA screening, a C18 column was used for chromatographic separation, and a solution of potassium dihydrogen phosphate (pH 3.25) and methanol was used as the mobile phase. Water and acetonitrile acidified with formic acid were the eluents for confirmation with UHPLC. The authors concluded that confirmation by UHPLC-MS/MS analysis is required to prevent false positive errors because of matrix interference from the HPLC-PDA method.

2.2.8. Tetracyclines

Tetracycline antibiotics (TC) are intensively used in therapy and prophylactic control of bacterial infections in human and veterinary medicine. They are also used as food additives for growth promotion in the farming industry. Their widespread use has caused antibiotic resistance among bacterial species, including resistance against TC. Their chemical structure is shown in Figure 8.

Tetracycline and oxytetracycline are widely used in salmon treatment. The EU [15] established its MRL at 100 μg/kg for muscle tissue including salmonidae and finfish. The MRL is established based on the sum of the parent compound and its 4-epimer, which are formed because TC are prone to degradation under strongly acidic and alkaline conditions. They form reversible epimers including 4-epi-TC, anhydro-TC and iso-TC.

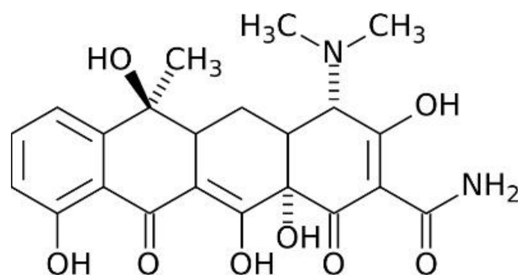

Figure 8. Tetracycline structural formulae.

Extraction and clean-up procedures

TC have identical chemical and physicochemical properties. They are soluble in acids, bases and polar organic solvents (particular alcohols), but insoluble in saturated hydrocarbons being strong chelating agents because chelation of a divalent metal ion is essential for their antimicrobial activity [96,97]. These characteristics make it difficult to extract TC from biological tissues. These analytical problems can be overcome using aqueous based extraction as the primary extraction system for TCs. Ethylenediaminetetraacetic acid (EDTA) is widely used in aqueous extraction and pre-treatment of C_{18} SPE cartridges to minimize TC interaction with chelating complexes or adsorption onto free silanol groups.

Determination methods

There are several different analytical methods that determine TCs in products of animal origin including immunoassays and capillary electrophoresis. Liquid chromatography is the preferred method. Chromatographic analysis of TCs in food was reviewed by Oka et al. (2000) [96]. Önal (2011) [98] also reviewed some of the recent advances in LC methods. The author highlighted the importance of LC-MS/MS to improve sensitivity and accuracy in TC quantification compared to UV and fluorescence methods [99,100]. Recent LCMS/MS methods detect the epimers along with the tetracycline molecule [98,101-103].

Blasco et al. (2009) [102] presented a LC-MS/MS method that quantified four TCs used in veterinary medicine (tetracycline, chlortetracycline, oxytetracycline and doxycycline) and their epimers in the muscle tissue of different species. The method was validated according to Commission Decision 2002/657/EC [19], with

limits of quantification between 0.5 and 1 μg/kg (below the tolerance level set by the EU). CCα and CCβ values were in the range of 101-116 μg/kg and 112-130 μg/kg, respectively. This method was not tested with fish tissue.

2.3. Multi-residue and multiclass techniques

There is a trend toward the development of cost-effective methodologies that detect drug residues in food and maintain efficient screening technologies that prevent false positive and negative results.

Multi-residue and multiclass techniques are important because they simultaneously detect numerous analytes of the identical family and different chemical classes in a single run.

Currently, in fact, most of the published methods relate to multiresidue and multiclass detection and confirmation analytical methodologies.

Microbiological and bioassay techniques are in use because of their low cost and simplicity [104]. However, the need for additional confirmatory methods makes them less attractive.

The desired efficiency is being achieved by multi-detection methods based on liquid chromatography technology coupled with tandem mass spectrometry and time of flight mass spectrometry. UHPLC also offers short running times and higher resolution and sensitivity.

Most reported methods discuss the multi-detection of related compounds. However, new methods for the simultaneous analysis of unrelated compounds, some of them regarding to fish samples, have been reported [31,48,97,101,103,105-113].

Extraction and clean-up procedures

Although multiclass determination techniques represent a major step forward in food analysis and control, there are still some problems to solve, namely in

extraction and pre-treatment of samples, because of the different physicochemical properties of the compounds.

Some of the published techniques presented several steps in sample extraction for the different analytes, depending on their properties [114], and more than one LC run has to be performed. Even in cases where a single extract is obtained, sometimes more than one injection is required, depending on the detector used, the chromatographic conditions for retention and separation of analytes, and other issues regarding the specificities of the different molecules in analysis. Regarding mass detection, we must be aware that some compounds are ionized in positive mode and others in negative and, in particular circumstances, it might represent two separate analyses.

The optimization of chromatographic conditions may also lead to some differences, even small, in mobile phase and in the gradient depending on the compounds.

Recently, some of the published methods for the simultaneous determination of multiclass residues in food matrices describe simple procedures with very good recovery rates of the analytes [105,109]. Both groups used simple liquid-liquid extraction procedures, using acetonitrile acidified with 0.1 vol % formic acid and acetonitrile and EDTA, respectively. The elimination of SPE cartridges reduces the cost and allows a higher number of samples to be processed each day, which is a very relevant feature in routine analysis.

Determination methods

Cháfer-Pericás et al. (2010) [106] developed and optimized an analytical method based on HPLC with MS/MS to determine sulphonamides and tetracyclines in fish and feed. A mixture of methanol:water 70:30 (v/v) of 1 mL of 0.1 M EDTA was selected as the extraction solution. The methodology provided limits of detection for the tested antibiotics in the 1.2-16 μg/kg range, lower than the MRL established by the European Union (100 μg/kg).

Gbylik et al. (2013) [108] developed a multi-residue method for the determination of 34 antibacterial drugs (three aminoglycosides, nine β-lactams, nine

fluoroquinolones, three macrolides, five sulfonamides, trimethoprim and four tetracyclines) in fish samples by LC-MS/MS. A double-step extraction was applied, and dissolved residues were gathered and analysed on a LC-MS/MS run.

The method was validated (European Decision 2002/657/EC) [19]. The CCα ranged from 55.3 to 1085 μg/kg, and the CCβ ranged from 59.5 to 1141 μg/kg.

Evaggelopoulou and Samanidou (2013) [110] developed a confirmatory high-performance liquid chromatography method for the determination of six penicillin antibiotics (ampicillin, penicillin G, penicillin V, oxacillin, cloxacillin and dicloxacillin) and three amphenicol antibiotics (thiamphenicol, florfenicol and chloramphenicol) in gilthead seabream (*Sparus aurata*) tissue. Analytes were isolated after a liquid-liquid extraction with a mixture of water/acetone (50/50 v/v), followed by SPE. The method was fully validated in terms of selectivity, linearity, accuracy, precision, stability and sensitivity according to the European Union Decision 2002/657/EC [19].

Freitas et al. (2014) [109] developed a method for the simultaneous determination of 41 antibiotics from seven different classes (sulfonamides, trimethoprim, tetracyclines, macrolides, quinolones, penicillins and chloramphenicol) in gilthead sea bream (*Sparus aurata*) by UHPLC-MS/MS. Extraction was best achieved with acetonitrile and EDTA. This screening and confirmation method is particularly suited for routine analysis. The methodology was validated in accordance with Decision 2002/657/EC [19].

Fedorova et al. (2014) [105] developed and validated an analytical multiclass, multi-residue method for the determination of antibiotics in aquaculture products. The best extraction was achieved with acetonitrile acidified with 0.1 vol % formic acid. Both Fedorova et al. (2014) [105] and Freitas et al. (2014) [109] avoided the clean-up step because it resulted in some antibiotic loss. This technique is simple and fast with a workflow of approximately 100 samples per day. The method is suitable for routine analysis with the exception of CAP. The MRLP (Commission Decision 2003/181/EC [42]) for CAP in aquaculture products is 0.3 μg/kg, and the LOQ of CAP reported by the authors is 2.8 μg/kg. This technique is therefore not sensitive enough for this compound.

Monteiro et al. (2015) [111] developed and validated a method for simultaneous assessment of 12 drugs of different antimicrobial classes (chloramphenicol, florfenicol, oxytetracycline, tetracycline, chlortetracycline, sulfadimethoxine, sulfathiazole, sulfamethazine, enrofloxacin, ciprofloxacin, norfloxacin, and sarafloxacin) on Nile tilapia's muscle (*Oreochromis niloticus*). The method involves a rapid procedure using ultrafiltration by Captiva cartridges, followed by LC-MS/MS in a negative mode for florfenicol and a positive mode for the others. The method was validated based on “Eurachem Guide: The Fitness for Purpose of Analytical Methods” [115]. The proposed extraction procedure and detection technique showed to be adequate for the analysis, with LOD and LOQ values ranging from 0.30 and 1.30 μg/kg and 0.90 to 4.30 μg/kg, respectively, depending on the antimicrobial.

Rezk et al. (2015) [113] described a LC-MS/MS method developed and validated for the simultaneous quantification of four antimicrobials commonly used in aquaculture, namely ciprofloxacin, trimethoprim, sulphadimethoxine, and florphenicol in fish tissues. The LC-MS/MS was operated under the multiple-reaction monitoring (MRM) mode using electrospray ionization (EI), and sample preparation involves a simple liquid extraction step followed by post extraction clean-up step with n-hexane. The validation of the method was performed according to FDA guidelines, resulting in LOQ values of 0.5 μg/kg for sulphadimethoxine and 1 μg/kg for the other molecules, considerably lower than their MRLs established by EU.

Table 12 provides an overview of the analytical methodologies for determination of antimicrobial residues in fish samples from 2002 to the present.

2.4. New trends on the development of analytical methodologies concerning antimicrobial residues in aquaculture

It should be noted that the present review is a contribution for determination of antimicrobial drug residues in food of aquaculture origin.

In fact, the recent and recurrent episodes, involving large scale contamination of food products, especially with antimicrobial drug residues, has grown the consumer's awareness and the need to develop simpler, faster and, still, very sensitive and selective techniques for residues monitoring and control. On the other hand, the cost-effectiveness of analytical procedures is becoming a major issue for all laboratories involved in residue analysis, as the reagents and equipment are very expensive.

Thus, taking into account the number of papers that have been published in the last five years, multiresidue and multiclass UHPLC-MS/MS methodology is the most powerful measurement tool, mainly with ToF. However, matrix effects could be observed when mass spectrometry is used. Ion suppression or increase of signal detection is frequently achieved. These phenomena need to be studied in order to know the real impact on final results [116]. Thus, and if the final detection could be considered up-to-date to current knowledge, the different chemical structures of the different antibiotics, as well as their different physicochemical properties, implies that substantial improvements are still needed in the sample pre-treatment step.

Last but not least, it is important to consider the concerns of antibiotic residues in causing adverse effects in the environment. In fact, antibiotics are “designed” to change specific biochemical pathways in target species but, when they are released into the environment, they still have the potential to induce the same effects in non-target organisms or to promote other different and unknown actions, even in trace concentrations. Due to the need of monitoring natural ecosystems, it also becomes important to develop analytical methodologies that can be applied to environmental matrices, i.e., matrices that are not directly intended for human consumption but that can influence the presence of antibiotic residues in the food chain. Thus, as matrices like algae could be good candidates for using as indicators of aquaculture contamination by antibiotics [117,118], the development, optimization and validation of UHPLC-MS/MS multiresidue and multiclass antibiotic residue methods applied to multi-matrices could be a priority in a nearly future.

Table 12. Overview of analytical methods for determination of antimicrobial residues in fish samples (since 2002).

Analyte	Sample	Extraction / Sample Preparation	Method	LOD (µg/Kg)	LOQ (µg/Kg)	CCα (µg/Kg)	CCβ (µg/Kg)	Reference
Aminoglycosides (AGs)								
11 AGs (multi-residue)	Fish	Trichloracetic acid / SPE	LC-(ESI)-MS	15 - 40	--	--	--	[35]
13 AGs (multi-residue)	Fish	Trichloracetic acid / SPE	UHPLC-(ESI)-MS/MS	--	2 - 25	60 - 1139	86 - 1483	[36]
Amphenicols								
CAP	Shrimp	Ethyl acetate Ethyl acetate and n-hexane / SPE / RP-HPLC	ELISA GC- (NCI) - MS/MS LC- (ESI) - MS/MS	0.1 0.1		≤ 0.25 ≤ 0.018 ≤ 0.011	≤ 0.1 ≤ 0.028	[119]
CAP	Fish / shrimp	Ethyl acetate – diethyl ether (75:25 v/v) / SPE	LC- (ESI)-MS/MS			0.01	0.02	[43]
CAP	Shrimp / crabmeat	Ethyl acetate	LC-(ESI)-MS/MS	0.3	0.3			[51]
Florfenicol	Channel catfish	Ethyl acetate/SPE	LC-UV	44	75			[56]
CAP	Rainbow trout	Ethyl acetate / SPE	GC-MS/MS LC-(ESI)-MS/MS			0.267	0.454	[46]
Florfenicol	Fish / Shrimp	Acidic aqueous buffer and hexane / SPE	GC-ECD	0.5	1.5			[120]
CAP	Seafood	Acetonitrile / SPE	LC-(ESI)-MS/MS			0.02	0.04 (321→152 ion transition)	[47]

							0.03 (321→152 ion transition)	
Macrolides								
8 Macrolides (multi-residue)	Fish	0.2% metaphosphoric acid–methanol (6:4) / SPE	LC-(ESI)-MS		10			[66]
7 Macrolides (multi-residue)	Fish	PLE	LC-(ESI)-MS			6 - 208	15 - 211	[67]
6 Macrolides (multi-residue)	Tilapia fillets	Ethanol / n-exane	LC-QToF	5.8 - 27	17 - 82	53 – 208	55 - 216	[64]
Nitrofurans								
Nitrofuran metabolites	Various, including farm fish	Ethyl acetate /n-hexane	LC-(ESI)-MS/MS			0.15 – 045 (metabolites)	0.32 – 0.88 (metabolites)	[69]
4 Nitrofurans and their metabolites	Fish	Ethyl acetate / n-hexane	LC-(ESI)-MS/MS			2.93 – 5.01 (nitrofurans) 0.19 – 0.43 (metabolites)	3.62 – 6.20 (nitrofurans) 0.23 – 0.54 (metabolites)	[72]
Quinolones								
8 Quinolones and FQ (multi-residue)	Trout, prawns and abalone	Acetonitrile / SPE	LC-(ESI)-MS/MS	1 - 3	5 (10 for ciprofloxacin)			[75]
3 Quinolones	Shrimp	Ethyl acetate / hexane	LC-FLD LC-(ESI)-MS^3	2.3 – 3	6.9 – 9			[85]
7 Quinolones	Gilthead seabream	NaOH / SPE	LC-(ESI)-MS/MS	2 – 2.7	6 - 8			[86]
11 Quinolones	Fish tissues	McIlvaine buffer solution / SPE	LC-FLD (sub-2µm column)	1.5 – 50.1	5.3 – 142.9			[87]
7 Quinolones	Salmon	Citrate buffer solution / SPE	HPLC-PDAD	1.9 – 11.6	5.7 - 35	31.3 – 628.20	32.1 – 628.20	[78]

4 FQ	Tilapia Pacu	1% acetic acid–methanol and 1% acetic acid–acetonitrile / hexane	LC-(QToF)-MS/MS	7 - 8 4 - 7	25	63 - 126	76 - 152	[88]
7 Quinolones	Gilthead seabream and fish feed	NaOH and HCl / SPE	HPLC-PDAD	1.7 – 9.5	5.1 – 28.5	31.2 – 619.3	32.3 – 622.8	[89]
Sulfonamides								
12 Sulfonamide	Trout	MSPD (H2O)	LC-(ESI)-MS	1 - 7	3 - 13			[91]
14 Sulfonamides	Catfish, shrimp and salmon	0.2% acetic acid–methanol–acetonitrile (85:10:5) and methylene chloride / SPE	HPLC-(FLD)		5 - 20			[94]
17 Sulfonamides and potentiators (ormetoprim and trimethoprim)	Salmon	Water + acetonitrile (50:50) / hexane	LC-(ESI)-MS/MS	0.1 – 0.9				[95]
Multi-Residue / Multiclass								
3 Quinolones, FQ and Erythromycin	Salmon	SLE	LC-(QToF)-MS	2 – 2.5	7.5 - 10	103 - 218	107 - 234	[121]
3 SA and 3 Tetracyclines	Gilthead seabream	Methanol: water 70:30 + EDTA 0.1M	HPLC-(ESI)-MS/MS	1.2 - 16	4 - 52			[106]
34 Antibiotics (3 aminoglycosides, 9 β-lactams, 9 FQ, 3 macrolides, 5 SA, trimethoprim and 4 tetracyclines)	Fish samples (common bream, roach, pike, zander and catfish)	*m*-phosphoric acid and heptafluorobutyric acid / acetonitrile	LC-(ESI)-MS/MS			55.3 - 1085	59.5 - 1141	[108]
6 Penicillin and 3 Amphenicol antibiotics	Gilthead seabream	H_2O + acetone (50/50) / SPE	HPLC	11 – 20.4	33.2 – 61.7	51.3 – 307.7	53.3 – 1022.2	[110]

32 Antibiotics (8 FQ, 13 SA, 2 tetracyclines, 3 macrolides, 2 β-lactams, 2 amphenicols,1 lincosamide, penicillin, trimethoprim, and one antiviral)	Aquaculture products	Acetonitrile (0.1 vol. % formic acid)	LC-(ESI)-MS/MS		0.062 – 4.6			[105]
41 antibiotics (SAs, trimethoprim, tetracyclines, macrolides, quinolones, penicillins and CAP)	Gilthead sea bream	Acetonitrile and 0.1 M EDTA	UHPLC-(ESI)-MS/MS			0.1 – 628.9	0.2 – 657.7	[109]
12 antibiotics (2 amphenicols, 3 tetracyclines, 3 sulfonamides, 4 quinolones)	Nile tilapia's muscle	0.1 M Na_2EDTA and acetonitrile: water (70:30) / filtration (Captiva cartridges)	LC-(ESI)-MS/MS	0.30 – 1.30	0.90 – 4.30			[111]
4 antibiotics (ciprofloxacin, trimethoprim, sulphadimethoxine and florphenicol)	Fish tissues	Acetonitrile (1 vol. % formic acid) and nethanol and n-hexane	LC-(ESI)-MS/MS	1.0 (0.5 for sulphadimethoxine)				[113]
CAP and 4 nitrofuran metabolites	Aquaculture products	Microwave-assisted derivatization and automated SPE	LC-(ESI)-MS/MS LC-(APCI)-MS/MS	0.003 – 0.01 for CAP (depending on fish matrix) 0.042 – 0.214 for nitrofuran metabolites (depending on metabolite and fish matrix)	0.009 – 0.033 for CAP (depending on fish matrix)			[48]

Conclusions

Aquaculture is a fast-growing industry, and the increasing demand for fish products has promoted its intensification in many countries. This growth has led to the widespread use of antimicrobials for both prevention and treatment of bacterial diseases. These antimicrobials may result in the presence of residual antibiotics in edible tissues.

Several methodologies have been described for the analysis of antibiotics in fish samples. Liquid chromatography coupled with mass spectrometric detection is the preferred technique. However, the current analytical strategy is shifting towards multi-residue and multiclass methods. These techniques analyse all the target antibiotics simultaneously. These methods are uncommon but represent the future on this field. The extraction process is the limiting factor of any multi-residual method. It must provide acceptable recovery of all analytes with a broad range of physicochemical properties and requires further research.

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Chapter 3 |Detection and quantification of 41 antibiotic residues' in Gilthead sea bream (*Sparus aurata*) from aquaculture origin, using a multiclass and multi-residue UHPLC-MS/MS method

Adapted and reprinted from*:

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Abstract

Aquaculture is one of the worldwide strategic development fields, and its importance is evident in its significant worldwide growth in the last decades. This growth is associated with the implementation of intensive and semi-intensive production methods, with the use of antibiotics in order to prevent the emergence and spread of infectious diseases in fish. This practice constitutes a real public health problem, not only due to the presence of antimicrobial residues in edible tissues, which can cause allergic reactions in hypersensitive individuals, but also due to the emergence of bacterial resistance. Consequently, the Regulatory Agencies have established maximum residue limits (MRLs). In the present study, a validated multiclass multi-residue ultra-high-performance liquid chromatography coupled with mass spectrometry in tandem methodology was used for the determination of 41 antibiotics from seven different classes - sulfonamides, trimethoprim, tetracyclines, macrolides, quinolones, penicillins and chloramphenicol - in 29 samples of gilthead sea bream of aquaculture origin, purchased in Portugal. The analysed samples showed that, in eight of them, antibiotic residues were present, three being of doxycycline - antibiotic for which no MRL is established - that was detected in concentrations ranging from 0.35 to 0.61 $\mu g kg^{-1}$. Other antibiotics were also detected and quantified and their concentrations were below the MRL established by the European legislation.

Keywords: Antibiotics; Gilthead sea bream; Aquaculture; Multi-residue; UHPLC-MS/MS

Introduction

Aquaculture is probably the fastest growing food-producing sector, now accounting for nearly 50% of the world's food fish. According to FAO [1] this share is projected to rise to 62% by 2030 as catches from wild capture fisheries level off and demand from a progressively growing world population substantially increases. This growth is associated with the implementation of intensive and semi-intensive production methods and is hampered by unpredictable mortalities that may be due to negative interactions between fish and pathogenic bacteria.

Therefore, the use of antibiotics in order to prevent the emergence and spread of infectious diseases in fish is a common practice [2,3].

The usage pattern of antimicrobial drugs in aquaculture is different from their use in terrestrial animals. In aquaculture, antimicrobials are regularly added to feed and, in some cases, directly added to the water, a process known as bath treatments [4,5]. These procedures result in a selective pressure in the exposed environments (usually water) [6].

Additionally, the use of antibiotics is also associated with the illegal practice of stimulating animal growth. The administration of these drugs in low concentrations results in an increase of weight gain and enhancement of the feed conversion efficiency [7].

This reality leads to public health problems, not only due to the presence of antimicrobial residues in edible tissues, which can cause allergic reactions in hypersensitive individuals, but also due to the emergence of bacterial resistance [8,9]. Consequently, the European Union's regulatory agencies have established maximum residue limits (MRLs) and specific requirements concerning the performance of analytical methods and the interpretation of the results [10,11]. Tolerance levels for permitted veterinary drugs were established as MRLs in foodstuffs of animal origin; for not permitted substances no tolerance levels are set. In some cases, in order to harmonise the analytical performance of the methods, a minimum required performance limit (MRPL) has been set.

In order to accomplish the necessary control of the presence of antimicrobial residues, and to ensure that MRLs are respected, sensitive and specific analytical methodologies are required. HPLC techniques, coupled with tandem mass spectrometry (LC-MS/MS), are the methodologies of choice for veterinary residue analysis in biological samples, in order to assure efficient screening [12-15].

More recently, ultra-high-performance liquid chromatography (UHPLC) showed several advantages compared with HPLC, regarding resolution, sensitivity and also in minimising the time of analysis which is an important feature in routine laboratories [15-19]. The current analytical strategy is shifting towards multi-residue and multiclass methods, which save time because all the target antibiotics are

analysed in the same run. At the present, such methods are relatively uncommon but will probably represent the future trend in this field.

A developed and validated screening and confirmatory UHPLC-MS/MS methodology [19] was used for the simultaneous detection of 41 antibiotics from seven different classes - sulfonamides, trimethoprim, tetracyclines, macrolides, quinolones, penicillins and chloramphenicol - in 29 samples of Gilthead sea bream of aquaculture origin, purchased in Portugal.

3.1. Material and Methods

Chemicals and Reagents

All reagents used were of analytical grade with the exception of solvents used for the mobile phase, which were LC-MS grade. Ethylenediaminetetraacetic acid (EDTA) was purchased from Merck (Darmstadt, Germany). Water was deionised using a Milli-Q (Millipore, Bedford, MA, USA) apparatus. Acetonitrile, methanol and formic acid were supplied by Sigma-Aldrich (Madrid, Spain). All standards of tetracyclines, quinolones, macrolides, sulfonamides, penicillins, chloramphenicol and trimethoprim were acquired from Sigma-Aldrich. The internal standards used, also provided by Sigma-Aldrich, were demethyltetracycline for tetracyclines, lomefloxacin for quinolones, roxithromycin for macrolides, sulfameter for sulphonamides and for trimethoprim, penicillin V for penicillins and for chloramphenicol, d5-chloramphenicol.

For all substances, stock solutions of 1 mg ml^{-1} were prepared, using LiChrosolv methanol (Merck) and accurate amount of standard weighed. Dilutions of 10 $\mu g ml^{-1}$ were prepared for all compounds and corresponding internal standards. All the working standard solutions were stored at below 5 °C for 1month.

Instrumentation

During sample preparation the following equipment was used: Mettler Toledo PB303 and AG285 balances (Greifensee, Switzerland), Refrigerated Sigma 3 -

16 K Centrifuge (Sigma), Vórtex ZX3 Velp Scientifica (Italy), Heidolph Reax 2 overhead mixer (Schwabach, Germany) and Block Heater SDH 200D/3.

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 UPLC 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 2.1× 100 mm, 1.8 μm particle size.

The mobile phases 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 min from 97% (A) to 40% (A); 5–9 min from 40% to 0% (A); 9–10 min from 0% back to 97% (A); 11–12 min 97% (A). The column was maintained at 40 °C and the autosampler at 10 °C, to keep samples refrigerated before injection and guarantee the stability of compounds in the extract, and the injection volume of 20 μl. The electrospray ion source in positive (ESI+) and negative (ESI−) mode was performed with data acquisition in multiple reaction monitoring (MRM). Data acquisition was accomplished with Masslynx 4.1 software (Waters).

3.2. Sampling and Sample Preparation

All gilthead sea bream samples (n = 29), from aquaculture origin, were collected in Portugal, mainly in supermarkets all over the country, between February and March 2015, except for two of them that were collected directly at Portuguese aquaculture production units, at Setúbal and Figueira da Foz (Table 13).

Homogenised gilthead sea bream muscle (2 g) taken from the dorsal area was weighed into a 20 ml glass centrifuge tube, 20 μl of each internal standard with 10 $\mu g ml^{-1}$ were added, vortex mixed and allowed to stand in the dark for at least 10 min.

Afterwards, a simple extraction procedure was performed, by shaking the sample with 10 ml of acetonitrile and 1 ml of a 0.1M EDTA solution, using a Reax shaker

for 20 min followed by centrifugation for 15 min at 3100 g. The supernatant was transferred into a new tube and evaporated to dryness under a gentle stream of nitrogen at 40 °C. The tubes containing the dry residue were stored for 1 month at −80 °C.

This procedure was repeated two more times, for each sample, with an interval of 7 days between them, in order to assess precision and accuracy of the results. Three extracts for each sample were obtained: R1, R2 and R3.

The residue was redissolved with mobile phase A (400 µl), filtered through a 0.45 µm PVDF MiniuniprepTM, transferred to vials and injected into the UHPLC-MS/MS under MRM-optimised conditions for each compound [19].

Table 13. Summary of sampling process, with date of collection and origin of the analysed samples

Sample	Date of purchase	Origin
1	06.02.2015	Turkey
2	08.02.2015	Spain
3	08.02.2015	Spain
4	08.02.2015	Greece
5	08.02.2015	Greece
6	08.02.2015	Greece
7	08.02.2015	Greece
8	15.02.2015	Greece
9	15.02.2015	Greece
10	15.02.205	Greece
11	15.02.2015	Greece
12	21.02.2015	Greece
13	21.02.2015	Greece
14	16.02.2015	Greece
15	20.02.2015	Spain
16	22.03.2015	Spain
17	22.03.2015	Spain
18	22.03.2015	Turkey
19	22.03.2015	Turkey
20	22.03.2015	Greece
21	22.03.2015	Greece
22	28.03.2015	Greece
23	28.03.2015	Greece
24	28.03.2015	Turkey
25	28.03.2015	Turkey
26	28.03.2015	Spain
27	28.03.2015	Greece
28	28.03.2015	Portugal
29	28.03.2015	Portugal

3.3. Results and Discussion

The analysis of the 29 samples of gilthead sea bream collected in Portugal, using the present methodology, showed that in eight of the samples antibiotic residues were present, three being of doxycycline - antibiotic for which no MRL is established - that was detected in concentrations ranging from 0.35 to 0.61 μgkg^{-1} (Figure 9). Other antibiotics were also detected and quantified and their concentrations were below the MRL established by the European legislation. A summary of the results is presented in Table 14.

These results are concerning, as they reveal the use of not permitted antibiotics in aquaculture production of gilthead sea bream, sold in Portugal.

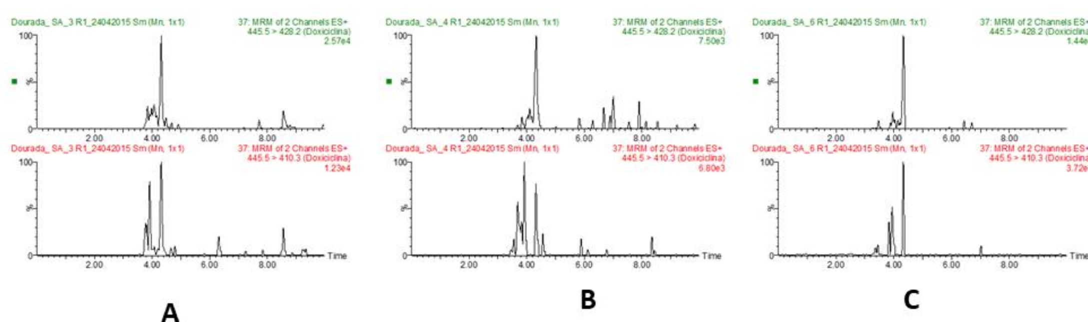

Figure 9. UHPLC-MS/MS chromatograms with two transitions (445.5 > 428.2 and 445.5 > 410.3) of three gilthead sea bream sample extracts, positive for doxycycline (A - 0.37 μgkg^{-1}; B - 0.61 μgkg^{-1} and C - 0.35 μgkg^{-1}). Transition 445.5 > 428.2 was used for quantification.

Table 14. Positive samples for the presence of antibiotic residues, and correspondent concentration values

Sample	Concentration (μgKg^{-1})			Antibiotic	MRL (μgKg^{-1})
	R1	R2	R3		
3	n.d.	0.55	0.35	Doxycycline	---
4	0.37	n.d	n.d.		
6	0.61	0.45	n.d.		
15	0.73	1.10	0.69	Enrofloxacin	100
16	n.d.	n.d.	4.95	Sulfadimetoxine	100
21	< 0.10	< 0.10	< 0.10	Trimethoprim	100
22	< 0.10	< 0.10	< 0.10		
23	< 0.10	< 0.10	< 0.10		

R1 sample prepared on day 1 | R2 sample prepared on day 1 + 7 | R3 sample prepared on day 1 + 14
n.d. not detected

Conclusion

In Europe, the availability of antimicrobial agents for aquaculture use is affected by the setting of MRLs, process that, however, is only a preliminary step towards achievement of full marketing authorisation. Although the European Medicine Agency (EMA) recently extrapolated the MRLs of twelve antibiotics to all food producing animal species, the list of fully authorised licenced pharmaceuticals for aquaculture is still quite small [20]. This reality is responsible, at least in part, for the use of not permitted antimicrobials in aquaculture production.

Regarding the obtained results, the presence of doxycycline residues in gilthead sea bream is worrying as its use in aquaculture can cause antimicrobial resistance in humans. Doxycycline is a semi-synthetic antibiotic alternative to penicillins. It is a member of the tetracycline group of antibiotics, and is widely used to treat diseases caused by both Gram-positive and Gram-negative bacteria, which include Spirochetes, *Actinomyces* sp., and *Mycoplasma* sp. It is also used for the treatment of Brucellosis, Lyme diseases, and Rickettsial infections, and is the drug of choice in the treatment of sexually transmitted diseases. It can also be used to treat complicated malaria when combined with quinine and can be used as an antivenin against snake bites [21].

The emergence of bacterial resistance to this antibiotic, for what aquaculture use contributes, poses serious public health issues.

Concerning the other antibiotics determined in our samples - namely, enrofloxacin, sulfadimethoxine and trimethoprim - they were quantified above the authorised MRL's. However, further investigation is needed regarding the cumulative effects, on human health, posed by the sum of all the antimicrobial residues to which consumers are exposed, although individually they may be present at levels that respect their established MRL's.

The proper use of approved antibiotics will continue to be necessary in animal production, including aquaculture, and consumers should be reassured that the use of approved antibiotics, in particular under "label use" conditions, does not imply a hazard.

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Chapter 4 | Detection and quantification of 47 antibiotic residues in farmed European sea bass (*Dicentrarchus labrax*) using a multi-class and multi-residue UHPLC-MS/MS method

Based on the manuscript*:

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Abstract

In the present study, a multiclass multi-residue method for the simultaneous detection and determination of antibiotics in European sea bass (*Dicentrarchus labrax)* was developed and validated. The method based on ultra-high-performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS) proved to be a rapid, highly selective and sensitive tool, requiring minimum sample preparation, for screening and detection of 47 compounds from eight different classes.

The validation was performed according to EU regulation 2002/657/EC, proving the method's suitability for application in routine analysis. The method was applied to the analysis of 30 samples of farmed European sea bass purchased in different supermarkets in Portugal. Antibacterial residues were detected in 6 of the 30 analysed samples, namely enrofloxacin and oxytetracycline, in concentrations ranging from 0.1 to 12 μg kg^{-1}.

Keywords: Antibiotics; European sea bass; Multi-residue; UHPLC-MS/MS

Introduction

Aquaculture is, nowadays, one of the priority goals of any economic development plan worldwide. Its strong expansion is linked to a constant increase in the world fish consumption *per capita,* from an average of 9.9 kg in the 1960s to nearly 20 kg in 2015 [1]. It is, therefore, understandable that the quality of fish, especially of aquaculture origin, has gained importance, and consumers are raising their awareness level regarding the quality of food.

The growth in this industry has driven the implementation and consolidation of intensive and semi-intensive production methods, where infectious diseases can become a hazard, causing significant stock losses and poor animal welfare [2]. The use of antibacterials for prophylactic and therapeutic purposes is, therefore, a procedure in aquaculture industry [3], along with their metaphylactic use to avoid the rapid spread of infections. Therefore, aquaculture antibiotic doses can be proportionately higher than those used in terrestrial animal farming, although the

exact levels are often not easy to determine because different countries have different distribution and registration systems [4,5]. This reality has a negative impact on public health. On one hand, the presence of antibacterial residues in edible tissues can cause allergic and toxic reactions in hypersensitive individuals. On the other hand, and of utmost importance, the selective pressure exerted by these drugs in aquatic ecosystems promotes the emergence, dissemination and transference of antimicrobial resistant determinants [6-8].

Concerning the presence of drug residues in edible animal tissues, regulatory agencies set maximum residue levels (MRLs) allowed in edible parts of animal derived food items, which in Europe are listed in the European Commission (EC) Regulation 37/2010 [9]. In the USA, the Food and Drug Administration (FDA) set rules for antibiotic use, including permissible routes of delivery, dose forms, withdrawal times, tolerances, and use by species, including dose rates and limitations [10]. The European Union Regulatory Agencies have also established specific requirements concerning the performance of analytical methods and the interpretation of the results [11]. While tolerance levels for permitted veterinary drugs were established as MRLs in foodstuffs of animal origin, forbidden substances have no tolerance levels set. Instead, to ensure that the food items are free from banned compounds, minimum required performance limits (MRPL) for the analytical methods were set [11].

To fulfil the regulatory EC provisions, regarding the performance of analytical methods, and to ensure that MRPLs are respected, sensitive and specific analytical methods are required. To ensure efficient screening, liquid chromatography techniques coupled with tandem mass spectrometry (LC-MS/MS) are the methods of choice for veterinary residue analysis, and confirmatory purposes, in biological samples [12-14]. More recently, another step forward to efficient screening was the development of ultra-high-performance liquid chromatography (UHPLC), that shows several advantages compared to HPLC, namely concerning resolution, sensitivity and time of analysis, which is an important feature in routine laboratories [15]. Furthermore, the modern high-resolution mass spectrometry (HRMS) instruments, such as time-of-flight (ToF) and Orbitrap instruments, provide high signal specificity, through high-resolution and mass accuracy in full scan acquisition mode, being able to register unlimited

number of compounds [16], and are now important tools for screening in the field of food analysis [16,17].

The current analytical strategy is shifting towards multi-residue and multiclass methods, which are time saving as all the target compounds are analysed in the same run. These methods will probably represent the future trend in this field.

Romero-González and colleagues [18] described a multi-residue method for the simultaneous determination of flumequine, oxolinic acid, oxytetracycline, sulphadiazine and trimethoprim in fish by LC–ESI-MS, with no clean-up step, which makes the method simple, fast and easy to perform. Also, Dasenaki and Thomaidis [19] developed a multi-stage LC–ESI–MS/MS method for the simultaneous determination of seventeen sulphonamides and five tetracyclines in fish tissue, in a single run, requiring no clean-up step. In 2011, Fernandez-Torres et al. [20] described an HPLC method for the determination of 11 antibacterials and their main metabolites in fish and mussel samples, preceded by a new extraction method based on enzymatic-microwave.

According to some authors [21], MS/MS remains the leading analytical tool, because of its sensitivity and selectivity, compared with the more recent HRMS technology, especially in the case of limited number of analytes to be monitored. When analysing real samples, though, using multi-residue methods, where a large number of substances have to be detected, HRMS becomes more interesting, as full-scan data are collected, rather than selected ion transitions.

In a recent study, Turnipseed et al. [22] developed and validated a screening method for veterinary drug residues in fish, shrimp and eel using LC with a quadruple-Orbitrap HRMS, with the capacity to monitor for over 300 veterinary drugs. Furthermore, the same research group [23] applied the method to the analysis of incurred and imported samples of several fish species, being able to detect and identify novel analytes and some metabolites that can reveal the use of the parent drugs.

Previously, a method for the simultaneous detection of 41 antibacterials from seven different classes (sulphonamides, trimethoprim, tetracyclines, macrolides,

quinolones, penicillins and chloramphenicol) in Gilthead sea bream (*Sparus aurata*) muscle was developed and validated by our group [15].

The aim of this work was to validate a screening and confirmatory UHPLC-MS/MS method, in order to extend the previous method [15] to a different fish species, and to new compounds (epi-chlortetracycline, epi-tetracycline, cefalonium, cefapirin, cefazolin and cefoperazon were added). After validation, samples of farmed European sea bass were purchased randomly from Portuguese supermarkets and analysed following the described method.

4.1. Material and methods

Reagents, Solvents and Standard Solutions

All reagents and solvents used for the extraction procedure were of analytical grade. For the mobile phase, the chemicals were of HPLC grade. Methanol, acetonitrile and formic acid were supplied by Merck (Darmstadt, Germany). All standards of tetracyclines, quinolones, macrolides, sulphonamides, beta-lactams and chloramphenicol were supplied by Sigma-Aldrich (Madrid, Spain). One internal standard for each antibiotic family was used: demethyltetracycline for tetracyclines, lomefloxacin for quinolones, roxithromycin for macrolides, sulphameter for sulphonamides and trimethoprim, penicillin V for beta-lactams and for chloramphenicol the deuterated (d5) form. The appropriate amount of each standard was weighted to obtain stock solutions of 1 $mgmL^{-1}$ in methanol. Suitable dilutions were also prepared to have convenient spiking solutions for both the extension of validation and unknown samples analysis. All standard solutions were stored in dark at -20ºC.

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 0.45 µm filters (Clifton, NJ, USA). For chromatographic separation and mass spectrometry detection an UHPLC system coupled to a triple quadrupole tandem mass spectrometer was used: UHPLC Ekspert ultra LC 110-XL coupled with QTRAP 5500+, AB Sciex (USA). The electrospray ion source in positive (ESI+) and negative (ESI-) mode was selected for data acquisition in multiple reactions monitoring mode (MRM). 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% (v/v) in water and [B] acetonitrile. The gradient programme, with a flow rate of 0.5 mL min^{-1}, was as follows: 0-5 min from 97% [A] to 40% [A]; 5-9 min from 40% to 0% [A]; 9-10 min from 0% back to 97% [A]; 11-12 min 97% [A]. The column was maintained at 40°C, the autosampler at 10°C and the injection volume was 10 µL.

4.2. Sampling and sample preparation

The samples of farmed sea bass (n=30) were purchased in Portugal, in supermarkets all over the country, originating from Spain, Greece and Norway. The samples were stored at -80 °C until analysis.

Sample extraction was in accordance with the previously published paper [15]. Two grams of homogenised fish muscle were weighed, the internal standards solution added, then vortex mixed and allowed to stand in the dark for at least 10 min. The antibacterials were extracted by adding 10 mL of acetonitrile and 1 mL of EDTA 0.1M to the samples, which were then shaken for 20 min in a Reax shaker. Afterwards, samples were centrifuged for 15 min at 3100 *g*, the supernatant transferred into a new tube and evaporated to dryness under a gentle stream of nitrogen. The dry extract was resuspended with mobile phase A (400 µL), filtered through a 0.45 µm PVDF Mini-uniprep™ and injected into the UHPLC-MS/MS. All samples were performed in triplicate.

4.3. Validation procedure

The purpose of the validation procedure was to perform the extension of the previously published method [15] for a different fish species and to new compounds added to the method. The validation was based on the requirements of the EU regulation 2002/657/EC [11] that defines the criteria for analytical methods and the parameters to be evaluated in the validation procedure.

For the matrix extension, values of CC_α and CC_β were confirmed by spiking 20 blank samples with the compounds already validated; specificity, recovery and precision were also re-evaluated.

For the new compounds, a full validation was performed. Selectivity, recovery, repeatability, reproducibility, decision limit (CC_α) and detection capability (CC_β) were determined.

Selectivity was demonstrated by analysing 20 blank samples of wild sea bass, in order to verify the presence of any possible interference that could affect the target antibiotics' identification. Also, to demonstrate that the identification is unequivocal for all target compounds, 20 blank sea bass samples were spiked with all analytes at the validation level (VL).

The VL was in accordance with the legislated MRL for each compound. For compounds without an MRL (non-authorised substances), such as chloramphenicol, a minimum required performance limit (MRPL) is set to harmonise the analytical performance, and this concentration is selected as VL.

Even though the determination of the LOQ (limit of quantification) is not a requirement in the Decision 2002/657/EC [11] this parameter was also calculated for all compounds to verify the sensitivity of the method in the present matrix.

Calibration curves were obtained using five concentration levels: 0.5VL, 1.0VL, 1.5VL, 2.0VL and 3.0VL, performed in triplicate, in three different days, by three different operators. Linearity was evaluated by linear regression analysis, which was calculated by the least square regression methods.

Precision and accuracy were assessed by analyzing (6 replicates on 3 different days) spiked samples of the 0.5VL, 1.0VL and 1.5VL. Intra-day precision (repeatability) and inter-day precision (reproducibility) were determined, and the coefficient of variation (CV%) was calculated for both. Accuracy was determined by recovery test using the same spiked samples, at 0.5VL, 1.0VL and 1.5VL. The results were compared to the acceptable values according to the Horwitz equation (equation 7), which provides the expected RSD (%) only on the basis of the concentration, independently from the matrix or the analytical method:

$$CV = 2^{(1-0.5\log C)} \qquad \text{(\textbf{\textit{Equation 7}})}$$

where CV represents the coefficient of variation between repeated analyses, and C the concentration, expressed as a mass fraction, exponent of 10.

Under reproducibility conditions, the CV should not exceed the calculated by the Horwitz equation, and in conditions of repeatability the intra-laboratory CV would typically be between one half and two-thirds of the calculated by the same equation [11].

The calculation of CC_α and CC_β is directly dependent on the MRL established, and was determined according to the following equations [11]:

$CC\alpha = \mu N + 2.33 \times \sigma N$ (***Equation 8***, for compounds without MRLs)

$CC\alpha = MRL + 1.64 \times \sigma MRL$ (***Equation 9***, for compounds with established MRLs)

$CC\beta = CC\alpha + 1.64 \times \sigma VL$ (***Equation 10***)

where μN is the mean of noise amplitude of 20 blank samples; σN is the standard deviation (SD) of the noise amplitude of 20 blank samples at the retention time of the target antibiotic; and σMRL and σVL are the SD at the MRL and VL level, respectively, in the 20 spiked blank samples at that level.

The LOQ was assessed by the following equation:

LOQ = 10 x S/N (***Equation 11***)

where S/N is the signal and noise ratio observed in the expected retention time of each compound in a blank sample.

4.4. Results and Discussion

4.4.1. Validation

Selectivity was demonstrated as described previously, with the effective identification of all compounds, and the same level of matrix interference was observed for this fish species, compared to the previously observed for gilthead sea bream muscle [15]. Identification criteria were achieved for all compounds. When comparing LOQ values achieved for all antibiotics with the regulatory MRL, it is possible to verify that the validated method is fully capable of detecting all the compounds in much lower levels than the required (Table 15).

Concerning the precision of the method, represented in terms of repeatability and reproducibility as the relative standard deviation (RSD), recovery, CC_{α}, CC_{β} and LOQ values are summarised in Table 15, and all values are in accordance with the limits defined in European Commission Decision [11]. The calculated RSD did not exceed the level calculated by the Horwitz equation for any antibiotic, and the recovery, calculated as a ratio between the determined concentration and the real concentration, is also in the accepted range.

Regarding repeatability, overall the higher values were obtained for sulphonamides – 20% and 17% for sulphadimethoxine and sulphaquinoxaline, respectively – and amoxicillin with 18%, while the remaining compounds were below these values. The same pattern was observed with gilthead sea bream muscle [15], with the higher values being amoxicillin (22%) and the sulphonamide sulphaquinoxaline (15%).

In terms of reproducibility, the higher deviation rates were observed for sulphaquinoxaline and amoxicillin, although all values accomplish the regulatory requirements [11].

Assessing the trueness of the method, the recovery, calculated as a ratio between the determined and the real concentration, the results were between 78% and 109%, which fall into the accepted range [11]. Overall, we can observe that the higher bias values were obtained for sulphonamides, which is in accordance with the previous results obtained with gilthead sea bream muscle [15]. As recognised, in a multi-detection and multiclass method, the sample preparation is frequently a very critical step, and there must be a balanced compromise in order to achieve good recoveries for as many compounds possible. In this case, the extraction procedure showed a similar profile in the recovery efficiency of the studied antibiotics in sea bass muscle as it has shown with the other matrix [15].

CC_α and CC_β were calculated following equations 8-10. In most cases these concentrations are above the MRL, except for substances without tolerance level, for which these values are closer to the detection limits of the method.

Although this method was first developed for gilthead sea bream muscle, its applicability to other similar species, such as sea bass, has been fully demonstrated by this validation process. Additionally, new compounds – two more tetracyclines and four cephalosporins - were added to the initial method.

The diversity, in number and different antimicrobial classes, of compounds that can be monitored by the present method - 47 antibiotic compounds from eight different classes - represents a huge advantage in routine analysis for the control of real samples from aquaculture production. There are only a few publications describing multi-residue methods for the simultaneous determination of antimicrobial residues in fish species. Turnipseed et al. [22] described a screening method for a wide scope of veterinary drug residues in fish, shrimp and eel, using LC with a quadruple-Orbitrap HRMS, developed and validated for 70 veterinary compounds, 48 being antimicrobials from 8 different classes. This method includes a liquid extraction step followed by a SPE step, which increases analysis

time and cost, and reducing sample throughput. For confirmatory purposes, though, these methods do not accomplish EU's requirements [11].

Other multi-residue methods for fish matrixes have been recently published, using UHPLC-MS/MS [24], LC-ESI-MS/MS [25], and the recent HRMS Orbitrap and ToF quadrupoles [26-28], overall monitoring a more limited number of compounds and antimicrobial classes. Furthermore, except for Vardali et al. [27] who use a simple solid-liquid extraction procedure, the others use more time and cost-consuming techniques to extract the analytes, namely QuEChERS [24,26,28] and ultrasonic-assisted extraction [25].

Compared to the other wide-scope methods, Dasenaki and Tomaidis [25] and Zhao et al. [28], which monitor 45 and 27 antimicrobial molecules, respectively, our method presents similar precision performances (repeatability ≤ 20% and reproducibility <22%, except for 4 molecules), with Dasenaki and Tomaidis [25] presenting better repeatability (<13%). Regarding recovery, our results are within a narrower range (78 – 110%).

The validation parameters, along with the method's execution speed, easiness and quickness, stand out the present method an important tool in the routine analysis of aquaculture fish species.

Table 15. MRLs set by the European Union for fish muscle, validation level (VL) and validation parameters: decision limit (CCα), detection capability (CCβ), repeatability, reproducibility, recovery and limit of quantification (LOQ).

Antimicrobial Class	Antibiotics	MRL (μg kg^{-1})	VL (μg kg^{-1})	CC$_{\alpha}$ (μg kg^{-1})	CC$_{\beta}$ (μg kg^{-1})	Repeatability (%RSD)	Reproducibility (%RSD)	Recovery (%)	LOQ (μg kg−1)
Sulfonamides	Sulfapyridine	100	100	111	121	10	15	108	0.4
	Sulfadiazine	100	100	115	131	5	8	109	8.2
	Sulfamethoxazole	100	100	115	129	9	14	92	0.3
	Sulfathiazole	100	100	117	134	15	22	107	12
	Sulfisoxazole	100	100	115	129	13	19	105	1.0
	Sulfamethiazole	100	100	116	133	8	12	98	0.6
	Sulfisomidine	100	100	114	128	13	20	81	10
	Sulfamethazine	100	100	111	122	15	22	109	12
	Sulfamethoxypyridazine	100	100	127	154	7	10	88	0.7
	Sulfadoxine	100	100	115	130	5	8	78	0.4
	Sulfadimethoxine	100	100	111	121	20	22	86	0.8
	Sulfaquinoxaline	100	100	115	131	17	26	98	5.3
	Sulfachloropyridazine	100	100	127	154	7	10	102	0.2
	Sulfanilamide	100	100	124	156	12	13	99	18
	Trimethoprim	50	50	115	130	13	19	102	10
Tetracyclines	Tetracycline	100	100	117	133	4	6	95	7.9
	Doxycycline	---	100	8	13	11	17	103	6.8
	Oxytetracycline	100	100	117	134	6	9	97	11
	Chlorotetracycline	100	100	124	148	3	4	97	1.1
	Epi- chlorotetracycline	---	100	122	147	12	19	96	1.9
	Epi-tetracycline	---	100	122	145	4	7	103	6.4
Macrolides	Erythromycin	200	200	224	249	7	8	79	3.8
	Spyriamicin	---	50	12	21	8	9	89	4.7
	Tilmicosin	50	50	59	69	9	13	99	
	Tylosin	100	100	114	128	10	10	93	7.2

Quinolones	Nalidixic acid	---	100	10	18	14	21	102	0.8
	Flumequine	600	600	629	658	14	22	82	0.6
	Oxolinic acid	100	100	112	123	12	18	103	1.1
	Cinoxacin	---	100	6	10	15	22	106	0.4
	Norfloxacin	---	100	2	4	6	8	109	11
	Enoxacin	---	100	4	6	5	7	107	4.1
	Ciprofloxacin	100	100	105	110	12	18	108	3.8
	Danofloxacin	100	100	108	117	16	21	108	1.5
	Enrofloxacin	100	100	106	112	8	12	94	2.0
	Ofloxacin	---	100	5	9	4	5	110	2.5
	Marbofloxacin	---	100	4	8	5	8	109	10
Penicillins	Penicillin G	50	50	66	81	16	23	106	16
	Ampicillin	50	50	65	80	13	19	105	21
	Amoxicillin	50	50	64	78	18	27	104	19
	Oxacillin	300	300	357	414	9	13	98	8.9
	Nafcillin	300	300	352	404	8	12	73	8.0
	Dicloxacillin	300	300	364	381	10	19	96	5.5
Cephalosporin	Cefalonium	---	20	2	2	17	25	107	2.1
	Cefapirin	---	50	1	1	14	22	100	2.0
	Cefazolin	---	50	1	1	11	16	99	1.8
	Cefoperazon	---	50	0.4	1	14	22	94	0.9
								108	
	Chloramphenicol[a]	---	0.3	0.01	0.02	11	17	106	0.02

[a] For compounds without an MRL (banned substances), such as chloramphenicol, a minimum required performance limit (MRPL) is set to harmonize the analytical performance of the methods (EC 2002).

4.4.2. Sample analysis

The analysis of the 30 samples of European sea bass bought in Portugal, using the present validated methodology, showed that 6 out of the 30 analysed samples contained antibiotic residues, namely enrofloxacin and oxytetracycline (Figure 10), in concentrations ranging from 0.1 to 12 μg kg^{-1}. In one sample both compounds were detected. A summary of the results is presented in Table 16.

It is well known that a gradual depletion of drug residue from fish tissues (measured as the sum of the parent drug and its main metabolite ciprofloxacin) occurs over time [29,30]. Thus, the results obtained for the replicate prepared on day 1 + 7 (R2), especially for enrofloxacin, are not consistent in accordance with the metabolisation profile of the drug and the fish species, even in stored samples [31], as it was the case (remember that the samples were kept at -80ºC until analysis).

Thus, we must conclude that there must have been a manipulation error during the analysis made on that day, by operator 2. Therefore, these results are not considered for the present discussion.

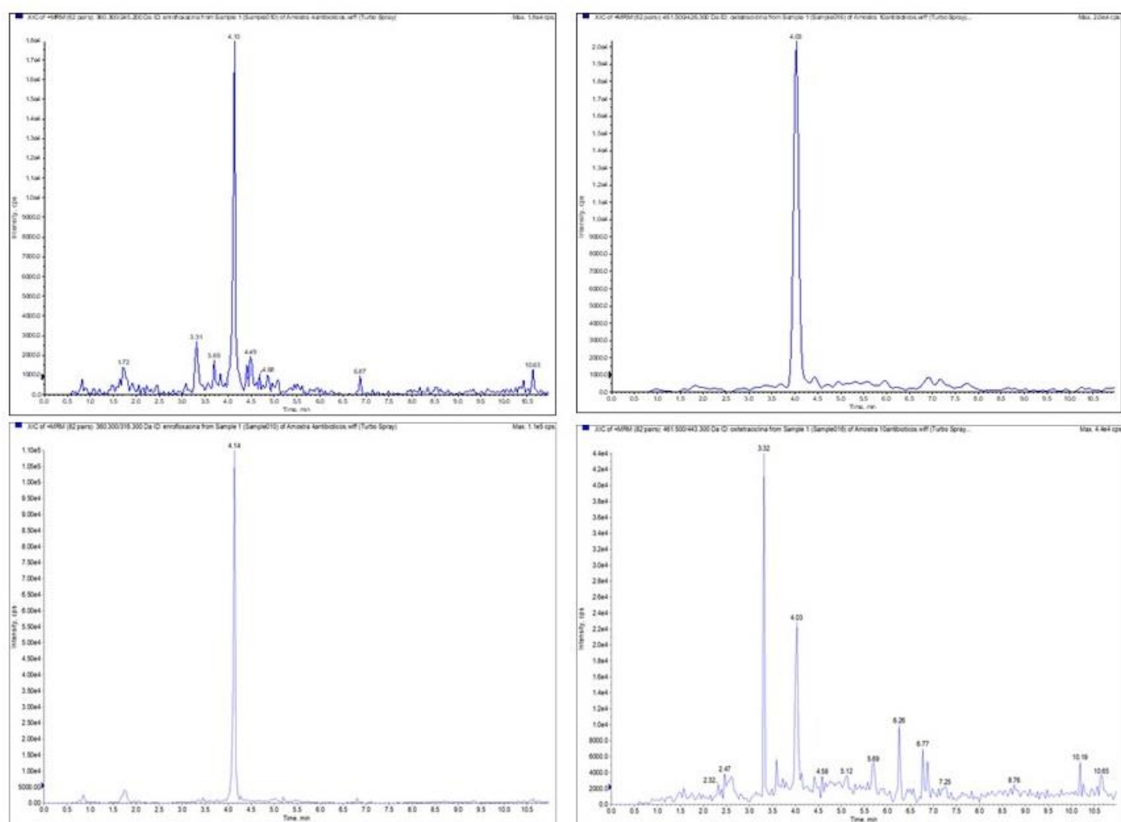

Figure 10. UHPLC-MS/MS chromatograms, with two transitions, of 2 seabass fish sample extracts, positive for enrofloxacin (A – transitions 360.3>245.2 and 360.3>316.3: RT*: 4.1 min) and oxytetracycline (B – transitions 461.5>426.3 and 461.5>443.3; RT*: 4.03 min).

RT – Retention Time

Table 16. Positive samples for the presence of antibiotic residues and correspondent concentration values (R1 – sample prepared on day 1 | R2 – sample prepared on day 1+7 | R3 – sample prepared on day 1+14)

Sample	Concentration ($\mu g\ kg^{-1}$)				
	R1	R2*	R3	Antibiotic	MRL ($\mu g\ kg^{-1}$)
4	12	2	8	Enrofloxacin	100
5	8	1	8		
6	8	< 0.1	8		
9	8	< 0.1	5		
10	6	< 0.1	5		
10	5	4	3	Oxytetracycline	100
11	4	2	4		

*These results are not consistent (see explanation in the discussion section)

Enrofloxacin and oxytetracycline, the antimicrobials detected in our positive samples, are both permitted by Commission Regulation n. º 37/2010 [9], and MRLs of 100 µgkg^{-1} are established. Even though the quantified residues are far away from the legal limits, these results are concerning and raise several questions related to the consequences of the presence of antibacterial residues in food items. The emergence, dissemination and spread of antimicrobial resistance determinants [7,32], the trigger of allergic and/or toxic reactions in hypersensitive individuals [33-35] and the environmental impact of these antibiotics' usage patterns [34,36] are some of the concerning aspects.

As a final remark, regarding our results, we would like to draw the attention to the results obtained in sample 10, revealing the presence of two different antibacterial residues. Even though both were quantified below their MRLs, there are not, to the best of our knowledge, studies to determine the safety of the simultaneous presence of both antibiotics in the same sample. Additionally, no data are available regarding possible cumulative effects, or even synergistic potentiation, of both substances' toxicities. Therefore, further studies should address this specific question of multiple drug residues in the same sample, and the legal framework, regarding MRLs of substances in edible tissues, should, if justified, take this into consideration.

Conclusion

Concerning the presence of antimicrobial residues in edible fish tissues, routine control analysis must guarantee fast, efficient and reliable results, preventing consumers from being supplied with fish with antibacterial residues above the authorised limits, or even containing residues of forbidden substances. This sensitive and specific UHPLC-MS/MS method proved to be suitable for the determination and quantification of 47 antimicrobial molecules, from eight different classes, in sea bass muscle samples. The process of validation demonstrated that the previous method [15] could successfully be extended to similar fish species and to more antibiotic molecules, which can be an important contribution in food safety analysis.

Regarding the results obtained for the purchased samples, antibiotic residues were determined in 6 of the 30 samples analysed, in particular enrofloxacin and

oxytetracycline. These results raise some concerns, increasing the need for further and deeper discussion on the overall consequences on human, animal and environmental health and also ecological balance, resulting from the use of antibacterials in the aquaculture industry and the presence of their residues in edible fish tissues. Access to fish of good quality levels, which do not threat consumers' health, must be a global concern, triggering improved regulation and enforcement regarding the use of antibacterials.

Finally, it is important to acknowledge that aquaculture is a modern tool, with the potential to succeed and thrive as a sustainable, profitable business. We must keep in mind, though, that the misuse and unrestricted use of antibacterials can lead to public health problems and environmental hazards that, in short to medium term, can turn this industry unsustainable.

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Chapter 5 |Development and validation of a multi-residue and multi-class screening method of 44 antibiotics in salmon (*Salmo salar*) using ultra-high-performance liquid chromatography/time-of-flight mass spectrometry: application to farmed salmon

Based on the manuscript*:

Gaspar AF[§], Santos L[§], Rosa J, Leston S, Barbosa J, Vila Pouca AS, Freitas A and Ramos F, 2018. Development and validation of a multi-residue and multi-class screening method of 44 antibiotics in salmon (*Salmo salar*) using ultra-high-performance liquid chromatography/time-of-flight mass spectrometry: application to farmed salmon.

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Abstract

A fast and sensitive multi-residue and multiclass screening method for the simultaneous determination of 44 antimicrobials in salmon muscle, using ultra- high-performance liquid chromatography/time-of-flight mass spectrometry (UHPLC-TOF/MS), was develop and validated. Two different procedures for the extraction step were tested, and an extraction with acetonitrile, ethylenediaminetetra acetic acid (EDTA) and n-hexane proved to be the best alternative. The method was validated, in accordance with Decision 2002/657/EC, using a qualitative approach at the $CC_β$ level. The detection of the analytes was accomplished by retention time and accurate mass, whose maximum error should not exceed 5 ppm. All the compounds were successfully detected and identified at concentration levels corresponding to ½ maximum residue limit (MRL).

The screening method was applied to 39 store bought samples of farmed salmon purchased in Portugal, originating from Norway and Denmark, and no antibiotic residues were detected.

Keywords: Antibiotics; salmon; UHPLC-TOF/MS; screening; qualitative validation

Introduction

The guarantee that foods of animal origin do not include drug residues that can induce harmful effects on human health, is one of the most relevant principles laid down in the European legislation [1-3]. While recognising the importance of the use of pharmaceutical products in the food producing industry, the European Commission (EC) emphasizes that safeguarding public health must be the first concern. In this matter, the presence of antibacterial residues in food items has been an issue of increasing concern for consumers and regulatory agencies, during recent years, not only because they may induce direct toxic effects in hypersensitive individuals, but also, and of utmost importance, they contribute to worsen the global health crisis of antimicrobial resistance [4-6].

In order to protect consumers' health, several organizations, such as Codex Alimentarius [7], European Commission [8] and U.S. Food and Drug Administration [9], have established maximum residue limits (MRL) for veterinary medicinal products in edible products from animal origin.

Legal requirements, though, have little practical effect if there are no proper analytical techniques which combine selectivity, specificity, accuracy and high-throughput, along with speed of execution, to meet proper and efficient control patterns.

In the recent years, the trend in food residue analysis is shifting towards multi-residue techniques for the detection, confirmation and quantification of a broad range of analytes in a single run. Liquid chromatography, associated with triple-quadrupole instruments (QqQ), is the most widely used for routine multiresidue screening of drugs in food [10-14]. Different acquisition options, neutral losses, daughter ions, and multiple reaction monitoring (MRM) provide these techniques with high selectivity and sensitivity profiles, and good quantitative capabilities, making identification of the analytes more robust. However, their low-resolution capabilities impose a limitation regarding the number of analytes that can be detected simultaneously, making it necessary to find a compromise between the number of transitions to be monitored, the length of the dwell times, and the number of data points across a chromatographic peak [15].

Alternatively, the modern high-resolution mass spectrometry (HRMS) instruments such as time-of-flight (ToF) and Orbitrap instruments are now considered a powerful tool for screening in the field of food analysis [15,16]. These types of analysers provide high signal specificity, through high-resolution and mass accuracy in full scan acquisition mode, being able to register unlimited number of compounds [16].

Liquid chromatography (LC) is also responsible for some limitation in multi-residue analysis, because of the large number of substances that need to be separated in simultaneous [16], but these limitations have been successfully overcome by UHPLC, providing additional chromatographic resolution and considerably lowering the time of analysis using sub-2-μm particulate column packaging material [17]. Therefore, UHPLC, associated with HRMS, for screening purposes, is nowadays considered the most powerful measurement tool in terms of selectivity, sensitivity and speed [18-20].

One of the main drawbacks related to these techniques is the uncertainty on how to apply their validation, according to legislation. According to Decision

2002/657/EC [21], compounds listed in group B, such as antibiotics, need a minimum of three identification points (IPs) for an accurate confirmation of their presence. The number of IPs earned, even in high-resolution techniques, does not accomplish this requirement. To provide a first identification for screening purposes, though, a single signal can be accepted. In the presence of positive results, a complete confirmation must be performed with appropriate methods.

Several papers have been published regarding the use of ToF for multi-detection screening methods in which the suspected samples are subsequently subjected to confirmation by triple quadrupole coupled with LC (LC-QqQ-MS) [20,22-26].

The present work describes the development and validation of a screening method by UHPLC-ToF/MS for the simultaneous detection of 44 antibiotics, from 6 different antimicrobial classes, in salmon muscle from aquaculture production. Two distinct extraction protocols were tested, with and without a degreasing step with n-hexane, and the recoveries were compared.

The method was validated in accordance to Commission Decision 2002/657/EC [21] requirements.

Furthermore, the validated method was applied in 39 samples of salmon, from aquaculture origin, purchased in several supermarkets in Portugal.

5.1. Materials and methods

Instrumentation

During sample preparation, the following equipment was used: Mettler Toledo PC200 and AE100 balances (Greifensee, Switzerland), ZX3 Vortex Mixer (Velp Scientifica, Italy), Heidolph Reax 2 overhead mixer (Schwabach, Germany), Heraeus Megafuge 1.0 centrifuge (Hanau, Germany), Turbovap Zymark Evaporator (Hopkinton, MA, USA) connected to a nitrogen generator (purity 99,9995%) (Frankfurt, Germany) and Whatman Mini-Uniprep PVDF 0.45 µm filters (Clifton, NJ, USA).

For chromatographic separation and mass spectrometry detection, a UHPLC (Shimadzu Nexera X2) system coupled with TOF/MS detector, Triple TOF™ 5600⁺ (AB Sciex) with UHPLC Acquity HSS T3 column (2.1x100 mm, 1.8 µm) was used.

Chemicals and analytical standards

All reagents and solvents used for the extraction procedure were of analytical grade except for the mobile phase which were of HPLC grade. Acetonitrile was supplied by Honeywell (Seelze, Germany), methanol by Carlo Erba (Val de Reuil, France), formic acid by Chemlab (Zedelgem, Belgium), EDTA and n-hexane by Sigma-Aldrich (Madrid, Spain). All standards for tetracyclines, quinolones, macrolides, sulfonamides, beta-lactams and trimethoprim were supplied by Sigma-Aldrich (Madrid, Spain). One internal standard for almost each antibiotic family was used: demethyltetracycline for tetracyclines, lomefloxacin for quinolones, roxithromycin for macrolides, sulfameter for sulfonamides and trimethoprim, and penicillin V for beta-lactams. A mixed solution of internal standards (10µg/mL) was prepared by dissolving 100µL of the stock solutions of internal standards sulfameter, roxithromycin, lomefloxacin, penicillin V and demethyltetracycline, making up to 10mL with methanol.

The appropriated amount of each standard was weighed to obtain stock solutions of 1 $mgmL^{-1}$ in methanol, except for beta-lactams whose stock solutions were prepared in water. Appropriate volumes of each of the stock solutions were used to prepare a multiresidue working solution, used to fortify the blank samples during the validation procedure.

5.2. Sampling

The samples of salmon (*Salmo salar*) (n=39) were purchased in Portugal in supermarkets all over the country between October and December 2017, originating from Norway (37) and also Denmark (2).

5.3. Sample preparation

Two different extraction procedures were tested, one of them consisted of a simple extraction with 10mL acetonitrile and 1 mL EDTA, as validated by Freitas et al (2014) [14] for gilthead sea bream muscle. The second method although similar included an extra degreasing step with n-hexane, considering the increased fat content of salmon muscle.

Two grams of homogenized fish muscle were weighed, the internal standard solution was added, then vortex mixed and allowed to stand in the dark for at least 10 minutes. The antibiotics were extracted by adding 10 mL of acetonitrile and 1 mL of 0.1M EDTA to the samples, which were then shaken for 20 min in a Reax shaker. Afterwards samples were centrifuged for 10 minutes, at 4.ºC/ 3100*g*, and the supernatant transferred into a new tube. Two mililiters of n-hexane were added, vortex mixed for 30 seconds and then centrifuged at 3100*g* for 10 minutes at 4 ºC. The n-hexane phase was then disposed, and the tube evaporated until 0.5 mL. In the extraction procedure without n-hexane, after the transference of the supernatant into a new tube, samples went directly to evaporation under a gentle stream of nitrogen until reaching 0.5 mL. In both procedures, to the 0.5mL of final extract, 200 µL of 0.1% formic acid was added, filtered through a 0.45 µm PVDF Mini-uniprep TM, transferred to vials and injected into the UHPLC-ToF/MS.

5.4. Chromatographic analysis and TOF-MS detection

The analysis was performed using UHPLC–ToF/MS. In terms of chromatographic conditions, the column temperature was maintained at 40ºC, the autosampler at 10°C and the injection volume was 10 µL. The mobile phase consisted of 0.1% formic acid (A) and acetonitrile (B). The gradient program, with a flow rate of 0.5 mL min^{-1}, was as follows: 0-5 min from 97% [A] to 40% [A]; 5-9 min from 40% to 0% [A]; 9-10 min from 0% back to 97% [A]; 10-11 min 97% [A].

The UHPLC system was connected to ToF/MS detector, as mentioned above. The detector was operated in positive electrospray ionisation mode (ESI+) with the capillary and sampling cone voltages of 5500 V. The temperature was maintained at 575º C. Nitrogen was used as desolvation and cone gas at flow rates of 25 and 40 psig, respectively. The total current ion chromatogram was acquired over the mass (m/z) range of 100 – 920 Da. The acquisition and identification of compounds were performed with the Analyst™, PeakView™ and MultiQuant™ softwares. Every 10 injections the TOF/MS detector was calibrated to guarantee the accurate mass resolution.

5.5. Method validation

The validation was performed following the European Union Regulation 2002/657/EC [21] and the Guidelines for validation of screening methods used for veterinary drug residues analysis [27] (CRLs 2010). The procedure consisted in the analyses of 20 blank samples and 20 fortified blank samples, spiked at the ½ MRL concentration level, selected as CCβ. The identification criteria assessed in this validation procedure were the Relative Retention Time (RRT), whose deviations should be lower that 2.5%, and the exact molecular mass, whose maximum error should not exceed 5ppm. Those parameters were calculated for all the 20 spiked samples in order to verify the detection capability of the method. The following equations were used:

Equation 12: Relative Retention Time (RRT)

$$RRT = \frac{RT_{analite}}{RT_{internal\ standard}}$$

Where $RT_{analite}$ is the retention time of the analite, and the $RT_{internal\ standard}$ is the retention time of the standard.

Equation 13: Deviation of RRT (ΔRRT)

$$\Delta\mathrm{RRT}\ (\%) = \left(\frac{\mathrm{RRT_{spiked\ samples}} - \mathrm{RRT_{standard}}}{\mathrm{RRT_{standard}}}\right) \mathrm{x100}$$

Equation 14: Deviation of exact mass (Δm)

$$\Delta \mathrm{m\ (ppm)} = \left(\frac{\text{Exact mass} - \text{Detected mass}}{\text{Exact mass}}\right) \mathrm{x}\ 10^6$$

Selectivity and specificity were demonstrated by analysing 20 blank samples of salmon from different origins to exclude the presence of any possible interference in the identification of the target antibiotics. The applicability of the method can be demonstrated also by the 20 blank samples and the same 20 blank samples spiked with all compounds.

The aim of a qualitative validation is to assess the presence of a certain analyte in a sample, at a determined concentration level. Since no quantification is necessary, the method's recovery, accuracy and precision were not examined.

5.6. Results and discussion

5.6.1. Extraction procedure

The effectiveness of a multi-detection and multiclass analytical method has, as a limiting factor, the sample preparation. As it is important to ensure good recovery values for the analysed substances, the extraction step is often critical. To achieve an efficient and generic simultaneous extraction of several compounds belonging to different class of compounds, with distinct physicochemical properties is only possible without complex and multi-step procedures. Even though, for recovery correction and to control possible matrix effects and any other fluctuation during sample preparation, ionization efficiency, detection response and chromatographic behaviour suitable internal standards were selected for each group of compounds.

Having as a starting point a method previously developed for the multi-residue extraction of antibiotics in muscle of gilthead sea bream [14], the extraction procedure including n-hexane proved to be more suitable to salmon muscle, minimizing the lipid content from the muscle and, as such, the potential

interferences during analysis. Therefore, this was the selected procedure for validation and analysis of real samples.

To better compare the results obtained with and without n-hexane, absolute recoveries were calculated for each compound. Figure 11 graphically compares the maximum absolute recoveries obtained for one compound representative of each antibiotic class, comparing both extraction procedures. In the graphic is evident, for all families, the increase of signal obtained when the lipid content is removed from the sample extract minimizing the matrix effect.

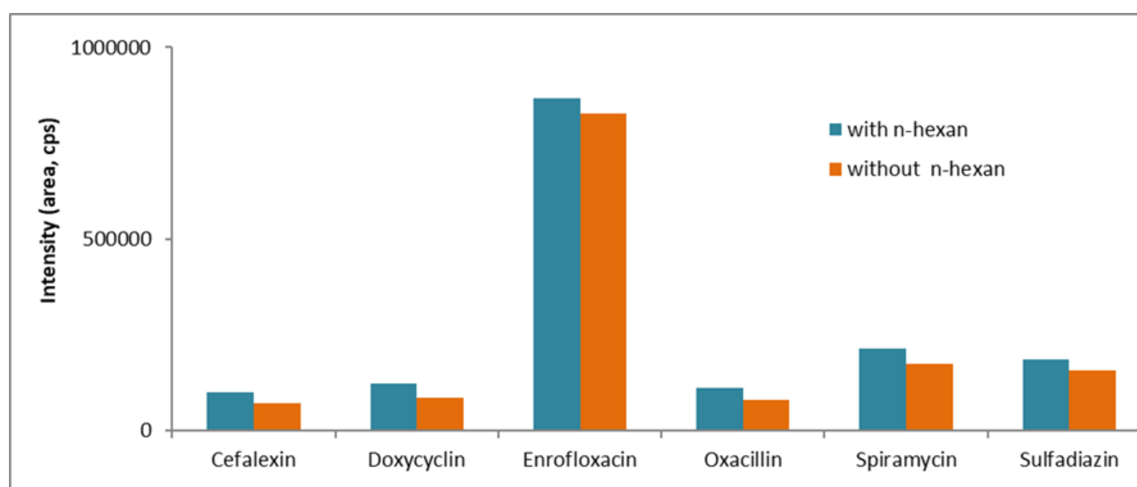

Figure 11. Maximum absolute recoveries obtained for 6 compounds (cefalexin, dicloxacillin, enrofloxacin, oxacillin, spiramicin and sulfadiazine), with and without defatting step using n-hexane.

5.6.2. Validation

In a screening method, the presence of any compound near the established MRL must be confirmed using a suitable confirmation method. In accordance with the Decision 2002/657/EC [21], the $CC_β$ for a screening method should be less than the regulatory limit, having between the two values a distance to guarantee that a concentration close to the MRL is detected. The guidelines for validation of screening methods for residues of veterinary medicines [27], suggests the use of ½ MRL as $CC_β$ since no more than one false-compliant result is obtained when 20 spiked samples are analysed at that level. Therefore, $CC_β$ was defined and tested as ½ MRL, and for that 20 blank samples were spiked at $CC_β$ level and analysed. The identification criteria were verified for all samples and target

compounds according to the equations 12, 13 and 14, demonstrating the applicability of the method was demonstrated. In Table 17 the summary of the validation is presented with the maximum values of ΔRRT(%) and Δm(ppm). As it is described in the Decision 2002/657/EC [21], the maximum variation accepted in terms of ΔRRT is 2.5%. As can be seen in the table the maximum values obtained were 0.4% for sulfathiazole and sulfadiazine. In terms of mass accuracy variation, for veterinary drug residues, such value is not defined. However, considering legislation used for other contaminants, such as pesticides [28], the maximum admitted variation is 5 ppm, which we also used in this method. The high value obtained in terms of mass accuracy variation was 3.7ppm for sulfisomidine, below the admissible 5ppm.

Selectivity and specificity were demonstrated by analysing 20 blank samples where no interference was found with the mass and retention time of our target compounds that could compromise their identification. In terms of applicability of the method, the same 20 blank samples were spiked at the CC_{β} level where all the target compounds were effectively identified. Figure 12 compares UHPLC-TOF/MS chromatograms obtained for a mixed solution of internal standards, a fortified sample and a blank sample.

5.6.3. Analysis of real samples

Thirty-nine samples of farmed salmon, originating from Norway and Denmark aquacultures, were purchased in Portuguese supermarkets, between October and December 2017, and analysed by the validated UHPLC-ToF/MS method.

Table 17. Validation parameters for the developed UHPLC-TOF/MS method

Compound name	Formula	$[M+H]^+$ (Da)	Retention Time (min)	Salmon muscle			MRL (μg/kg)
				maximum ΔRRT (%)	Maximum Δm (ppm)	CCβ (μg/kg)	
Cephalexin	$C_{16}H_{17}N_3O_4S$	348.10192	3.71	0.1	3.0	100	200
Cephapirin	$C_{17}H_{17}N_3O_6S_2$	424.06316	3.91	0.0	0.9	25	50
Cephazolin	$C_{14}H_{14}N_8O_4S_3$	455.03729	4.51	0.0	-0.9	25	50
Cephaperazone	$C_{25}H_{27}N_9O_8S_2$	646.14968	4.79	0.0	-0.7	25	50
Ceftiofur	$C_{19}H_{17}N_5O_7S_3$	524.03695	4.65	0.1	-3.3	500	1000
Ampicillin	$C_{16}H_{19}N_3O_4S$	350.1169	4.16	0.0	-0.9	25	50
Dicloxacillin	$C_{19}H_{17}Cl_2N_3O_5S$	470.03387	6.20	0.0	1.7	150	300
Nafcillin	$C_{21}H_{22}N_2O_5S$	415.13222	5.97	0.0	1.3	150	300
Oxacillin	$C_{19}H_{19}N_3O_5S$	402.11182	5.72	0.0	1.0	150	300
Benzylpenicillin, (Penicillin G)	$C_{16}H_{18}N_2O_4S$	335.10667	3.92	0.3	2.9	25	50
epi-Chlortetracyclin	$C_{22}H_{23}ClN_2O_8$	479.12223	4.15	0.2	-3.2	50	100
Chlortetracyclin	$C_{22}H_{23}ClN_2O_8$	479.12223	4.30	0.1	1.7	50	100
Doxycycline (Tautomer)	$C_{22}H_{24}N_2O_8$	445.16121	4.39	0.2	-1.5	50	100
epi-Tetracyclin	$C_{22}H_{24}N_2O_8$	445.16121	3.96	0.1	-2.5	50	100
Tetracycline	$C_{22}H_{24}N_2O_8$	445.16121	3.95	0.2	2.5	50	100
Oxytetracycline	$C_{22}H_{24}N_2O_9$	461.15612	3.84	0.2	-2.7	50	100
Danofloxacin	$C_{19}H_{20}FN_3O_3$	358.15681	3.93	0.3	1.9	50	100
Enrofloxacin	$C_{19}H_{22}FN_3O_3$	360.17246	3.99	0.2	2.6	50	100
Ciprofloxacin	$C_{17}H_{18}FN_3O_3$	332.14116	3.86	0.2	2.7	50	100
Flumequine	$C_{14}H_{12}FNO_3$	262.08806	5.17	0.1	-3.3	100	200

Marbofloxacin	$C_{17}H_{19}FN_4O_4$	363.14697	3.75	0.0	-2.7	75	150
Nalidixic acid	$C_{12}H_{12}N_2O_3$	233.09273	5.10	0.0	-2.7	50	100
Norfloxacin	$C_{16}H_{18}FN_3O_3$	320.14116	3.75	0.0	0.9	50	100
Oxolinic acid	$C_{13}H_{11}NO_5$	262.07166	4.69	0.0	1.6	150	300
Cinoxacin	$C_{12}H_{10}N_2O_5$	263.06691	4.53	0.0	-2.0	50	100
Ofloxacin	$C_{18}H_{20}FN_3O_4$	362.15172	3.83	0.2	-1.2	50	100
Enoxacin	$C_{15}H_{17}FN_4O_3$	321.13641	3.76	0.2	-1.9	50	100
Spiramycin	$C_{43}H_{74}N_2O_{14}$	843.52195	4.19	0.0	2.2	100	200
Tilmicosin	$C_{46}H_{80}N_2O_{13}$	869.57398	4.45	0.2	1.4	25	50
Tylosin A	$C_{46}H_{77}NO_{17}$	916.52709	4.81	0.2	-2.6	50	100
Sulfadimethoxine	$C_{12}H_{14}N_4O_4S$	311.08152	4.80	0.1	-2.6	50	100
Sulfadimidin	$C_{12}H_{14}N_4O_2S$	279.09169	4.11	0.1	1.0	50	100
Sulfadoxine	$C_{12}H_{14}N_4O_4S$	311.08152	4.49	0.2	1.0	50	100
Sulfamethizol	$C_9H_{10}N_4O_2S_2$	271.03246	4.05	0.2	-1.0	50	100
Sulfamethoxazole	$C_{10}H_{11}N_3O_3S$	254.06005	4.47	0.0	-1.7	50	100
Sulfanilamide	$C_6H_8N_2O_2S$	173.03859	1.04	0.1	2.2	50	100
Sulfapyridin	$C_{11}H_{11}N_3O_2S$	250.06514	3.60	0.2	-1.3	50	100
Sulfaquinoxaline	$C_{14}H_{12}N_4O_2S$	301.07604	4.81	0.2	-3.1	50	100
Sulfathiazole	$C_9H_9N_3O_2S_2$	256.02156	3.53	0.4	1.0	50	100
Sulfisomidine	$C_{12}H_{14}N_4O_2S$	279.09169	3.21	0.3	3.7	50	100
Sulfachloropyridazine	$C_{10}H_9ClN_4O_2S$	285.02142	4.35	0.2	0.9	50	100
Sulfadiazine	$C_{10}H_{10}N_4O_2S$	251.06039	3.19	0.4	-2.5	50	100
Sulfisoxazole	$C_{11}H_{13}N_3O_3S$	268.07504	4.53	0.0	-1.6	50	100
Trimethoprim	$C_{14}H_{18}N_4O_3$	291.14583	3.73	0.2	-1.9	25	50

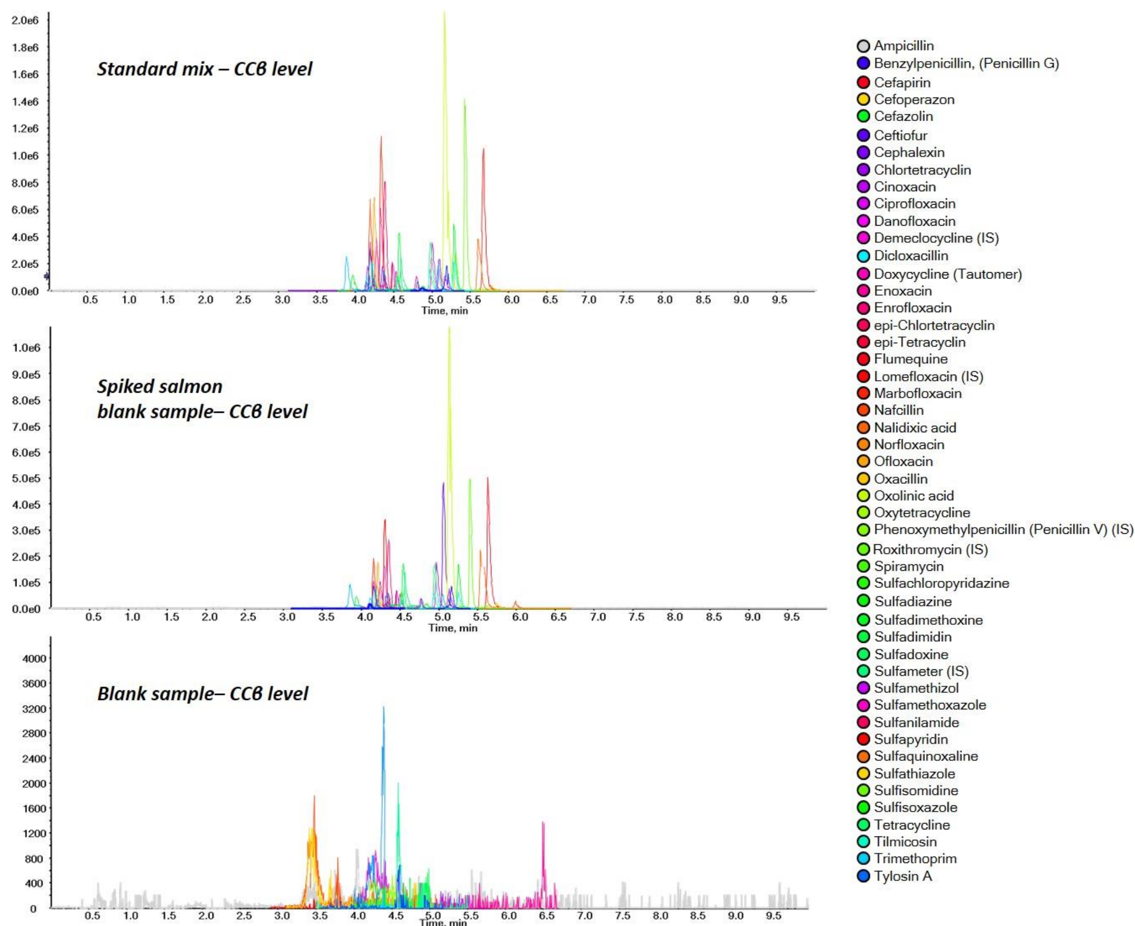

Figure 12. UHPLC-TOF/MS chromatograms for a mixed solution of internal standards (A), a fortified sample (B) and a blank sample.

No antibiotics have been detected in any of the analysed samples. Figures 13 and 14 show the chromatograms of a blank and a real sample, respectively, in which it is only possible to detect internal standards. The effectiveness of the method can be demonstrated, though, as all the internal standards were detected.

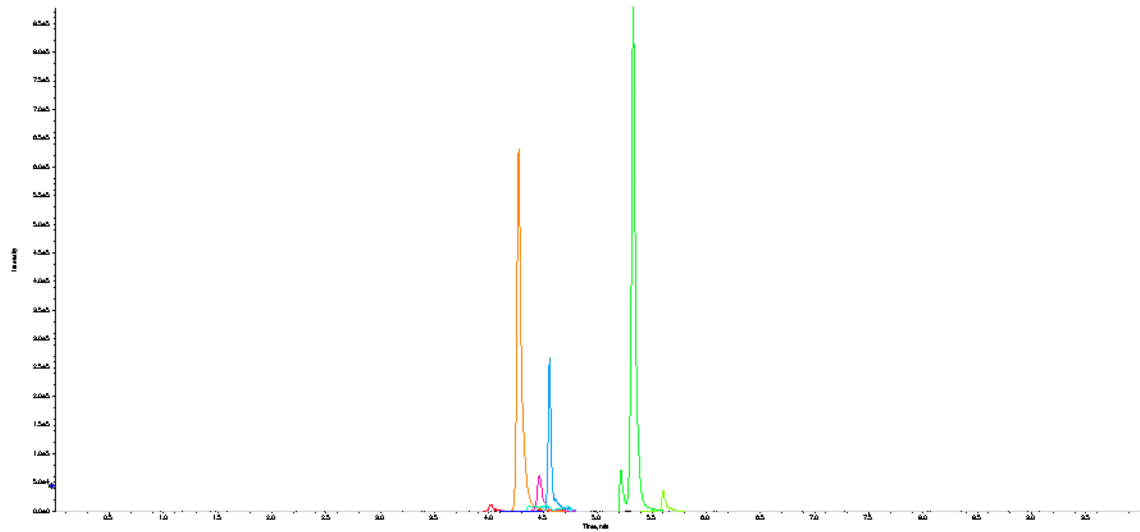

Figure 13. Chromatogram of a blank sample (salmon).

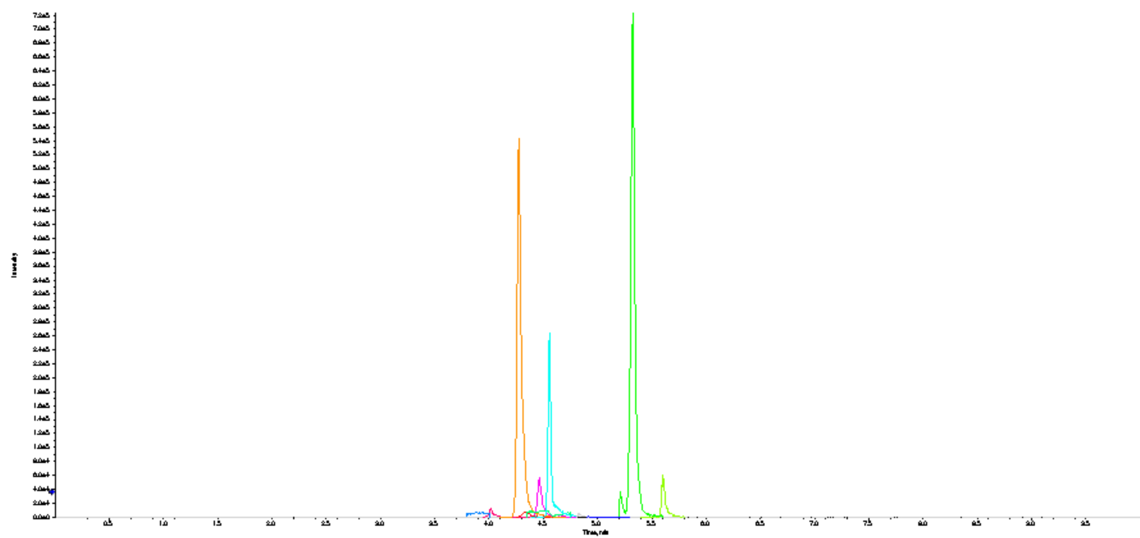

Figure 14. Chromatogram of a real sample (salmon) containing mixture of internal standards.

Conclusion

The described method proved to be a sensitive and robust multi-residue UHPLC-ToF/MS method for the screening of 44 antibiotics in salmon muscle. Validation was successfully achieved in accordance with the EU's regulations and demonstrated the good performance of the method.

The method was successfully applied to 39 samples of farmed salmon sold in Portugal, originating mainly in Norway. As no positive samples were detected no further confirmation method was performed.

Norway is considered a model in the area of aquaculture production, because regulation of antimicrobial use in salmon aquaculture is very strict, being able to reduce the use of these drugs to negligible levels [29]. Our results confirm this reality, as no suspected samples were detected.

Being a fast and easy method, allied to its sensitivity and robustness, this tool can be relevant in terms of routine analysis of salmon from aquaculture origin. In face of the increasing importance of aquaculture fish supply to human nutrition, it is particularly important to have appropriate and reliable techniques to assure effective control of real samples and, thereby, safeguarding public health.

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Chapter 6 |General Discussion

There is a growing need to understand how the health of both the individual and the population are shaped by external factors at the global level, and how these factors are influenced by human interventions and natural phenomena. Nutrition, environment, water supplies and disease, as well as social and political conditions are established as some of these factors, that may interact with each other and sometimes produce unexpected health consequences.

The role of food is unequivocally established as a major component for health security. A proper and adequate supply of all essential nutrients, in terms of quantity and quality, is recognized as a major component for humans' health and wellbeing. Recognizing this reality, the European Union assumes food safety as one of its key policy priorities, reflected in the "White Paper on Food Safety" [1] presented by the Commission, which serves to protect, and promote, the health of the consumer.

In this context, the presence of contaminants in food items is one of the major concerns of national health authorities, and in particular of the consumers, increasingly aware of food safety issues, exacerbated by the recurrent news on the widespread contamination of food items.

One of the most relevant chemical contaminants, that raises major concerns, are veterinary drugs, and in particular antibiotics. The use of antimicrobial agents in food production systems raises several concerns, related not only with the presence of their residues in food items, but mainly because the use of these drugs in food-producing animals is contributing to worsen the global health crisis of antimicrobial resistance.

In the animal production sector, for human consumption, aquaculture is becoming increasingly important and exhibits a faster growth than any other animal production sector [2]. Therefore, the proper control of this food item is important in terms of food security.

Nowadays, the important challenge concerning the control of veterinary drug residues is to balance together three main aspects: the number of analytes to be monitored, the diversity of food matrices and the regulation. There are, nowadays, around 200 veterinary drug residues to be controlled in a range of

food matrixes, like meat, fish, eggs, milk, among others [3], and the regulatory framework often differs from region to region.

This challenge turns more complex as the impact of business is global, and the range of products to be monitored increases. Therefore, analytical methodologies have to properly answer to several requirements to meet an effective control namely regarding coverage (number of analytes), throughput (analysis turnaround time) and analytical cost (cost-effective quality control).

In this context, multiresidue screening analytical methods are the most attractive tools nowadays, allowing the detection and identification of several different compounds, from different families, in a single procedure.

In food control, screening is commonly carried out with rapid tests for a quick decision-making regarding the acceptance of the product. These tests are often based on immunochemical techniques such as enzyme-linked immuno-sorbent assay (ELISA), lateral-flow assay or based on other devices [4,5,6], which are very easy to perform but, in contrast, have a narrow scope, in terms of analytes and matrixes, and weak performance in terms of specificity.

Liquid chromatography-mass spectrometry (LC-MS) is the "golden standard" nowadays, allowing the screening of more than hundred veterinary drugs in a single run [7-9]. In the last decades, LC-MS has undergone great improvements, in terms of sensitivity, and can be used either as a screening or a quantitative method.

As recognized, with the increasing demand of samples and compounds to be managed daily by control laboratories, the main focuses are the high-throughput of the techniques and their cost-effectiveness, and special attention is, therefore, given to their coverage, in terms of matrixes and analytes.

Despite the enormous progress in sample preparation and instrument performance, the simultaneous analysis of an extensive range of veterinary drug residues, in several different matrixes, remains a challenge for a single multiresidue LC-MS method, mainly because the different physic-chemical properties of the different compounds hinder the simultaneous extraction step.

Several multiresidue methods based on LC-MS/MS or, more recently, on LC-HRMS, have been published for the quantitative determination of veterinary drugs in food, as described in previous chapters.

Interestingly, however, are the low rates of noncompliant samples with regard to veterinary drug residues in live animals and animal products [10,11]. Therefore, screening methods, based on HRMS, are probably more appropriate for the routine analysis of considerable volumes of samples and a large number of analytes, taking into consideration that positive results require further confirmation and quantitative determination by suitable methods.

The inclusion of aminoglycosides in multi-detection techniques remains a challenge to be overcome. This important class of antibiotics cannot be extracted with organic solvents due to a strong hydrophilic behaviour, requiring particular LC conditions. Likewise, polimixins, another important class to be monitored, exhibit different chemical properties, which prevent them to be included in multi-residue analytical techniques.

Our work is part of this wider issue of food security, more specifically regarding the presence of antibiotic residues in farmed fish from aquaculture origin. As our main focus is the public health perspective, we developed and validated analytical methods that were, subsequently, applied in farmed fish samples, purchased in several supermarkets, in Portugal (annex 1). The farmed fish species were selected from the most consumed in Portugal, namely gilthead sea bream, European sea bass and salmon.

Through this thesis, we described the application of a previously validated multiclass multi-residue ultra-high-performance liquid chromatography coupled with mass spectrometry in tandem method [12], for the determination of 41 antibiotics from seven different classes - sulfonamides, trimethoprim, tetracyclines, macrolides, quinolones, penicillins and chloramphenicol – in 29 samples of gilthead sea bream of aquaculture origin, purchased in Portugal.

In order to protect consumers' health, the European Commission [13] established MRLs for veterinary medicinal products in edible products from animal origin. Legal requirements, though, have little practical effect if there are no proper

analytical techniques which combine selectivity, specificity, accuracy and high-throughput, along with speed of execution, to meet proper and efficient control patterns, being able to detect as much compounds as possible in a single assay.

This method exhibits suitable characteristics to the routine analysis of gilthead sea bream muscle, associating proper performance characteristics to a very simple extraction procedure, meeting the EU's validation requirements [14].

The analysis of the 29 samples of gilthead sea bream collected in Portugal, using the present methodology, showed that in eight of the samples antibiotic residues were present, three being of doxycycline - antibiotic for which no MRL is established - in concentrations ranging from 0.35 to 0.61 $\mu g kg^{-1}$. Other antibiotics (enrofloxacin, sulfadimetoxine and trimethoprim) were also detected and quantified and their concentrations were below the MRL established by the European legislation.

In the subsequent chapter we validate a screening and confirmatory UHPLC-MS/MS method, in order to extend the previous method [12] to a different fish species (European sea bass), and to new compounds (epi-chlortetracycline, epi-tetracycline, cefalonium, cefapirin, cefazolin and cefoperazon were added). After validation, performed in accordance to EU Regulation 2002/657/EC [14], samples of farmed European sea bass were purchased randomly from Portuguese supermarkets and analysed following the described method.

The selectivity of the method was demonstrated, with the effective identification of all compounds, and the same level of matrix interference was observed, compared to the previously observed for gilthead sea bream muscle [12]. Identification criteria were achieved for all compounds, and the obtained LOQ values show that the method is fully capable of detecting all the compounds in much lower levels than the established MRL.

The precision of the method, represented in terms of repeatability and reproducibility as the relative standard deviation (RSD), recovery, CCα, CCβ and LOQ values, were in accordance with the limits defined in European Commission Decision 2002/657/EC [14]. Regarding repeatability, overall the higher values were obtained for sulphonamides – 20% and 17% for sulphadimethoxine and

sulfaquinoxaline, respectively – and amoxicillin with 18%, while the remaining compounds were below these values. The same pattern was observed with gilthead sea bream muscle, with the higher values being amoxicillin (22%) and the sulphonamide sulfaquinoxaline (15%) [12].

In terms of reproducibility, the higher deviation rates were observed for sulfaquinoxaline and amoxicillin, although all values accomplish the regulatory requirements. Regarding the trueness of the method, measuring the recovery, calculated as a ratio between the determined and the real concentration, the results felt into the accepted range [14]. Overall, we can observe that the higher bias values were obtained for sulfonamides, which is in accordance with the previous results obtained with gilthead sea bream muscle [12].

As recognized, in a multi-detection and multiclass method, the sample preparation is frequently a very critical step, and there must be a balanced compromise in order to achieve good recoveries for as many compounds possible. In this case, the extraction procedure showed a similar profile in the recovery efficiency of the studied antibiotics in sea bass muscle as it has shown with the sea bream matrix [12].

The critical concentrations CCα and CCβ were calculated depending on if the MRL or the MRPL is established or not. In most cases these concentrations are above the MRL, except for substances without tolerance level, for which these values are closer to the detection limits of the method.

Although this method was first developed for gilthead sea bream muscle, its applicability to sea bass muscle has been fully demonstrated by this validation process. Additionally, new compounds – two tetracycline epimers and four cephalosporins - were added to the initial method. The inclusion of new antimicrobial molecules, particularly cephalosporins, is an important feature, as no representative of this group was present in the previous method [12] and, furthermore, there are just a few multi-residue methods that include cephalosporins. Cephalosporins still assume a central role in clinical practice and have proven to be of immense importance in surgery and as first line therapy for a wide range of infections [15], and therefore it is very important to monitor their use, in order to control the rising bacterial resistance patterns.

The diversity, in number and different antimicrobial classes, of compounds that can be monitored by the present method - 47 antibiotic compounds from eight different classes - represents a huge advantage in routine analysis for the control of real samples from aquaculture production. There are only a few publications describing multi-residue methods for the simultaneous determination of antimicrobial residues in fish species, but most of them monitor a more limited number of compounds and/or include more complex and time-consuming sample preparation procedures [16-21]. Compared to other wide-scope methods [19,20], our method presents similar precision performances (repeatability ≤ 20% and reproducibility < 22%, except for 4 molecules), while Dasenaki and Tomaidis [20] present better repeatability (<13%). Regarding recovery, our results are within a narrower range (78 – 110%).

The validation parameters, along with the method's execution speed, easiness and quickness, stand out the present method as an important tool in the routine analysis of aquaculture fish species, namely European sea bass.

Regarding the last studied species – salmon – the option was the development of a screening method based on HRMS, with ToF detector, which provides high signal specificity, through high-resolution and mass accuracy in full scan acquisition mode, being able to register unlimited number of compounds.

One of the main drawbacks related to these techniques is that they don't meet the legal requirements for unequivocal confirmation, according to Decision 2002/657/EC [14]. For screening purposes, though, and to provide a first identification, a single signal can be accepted. In the presence of positive results, a complete confirmation must be performed with appropriate methods, such as UHPLC/MS/MS.

So, we developed and validated a screening method by UHPLC-ToF/MS for the simultaneous detection of 44 antibiotics, from 6 different antimicrobial classes, in salmon muscle from aquaculture production, which was validated in accordance to Commission Decision 2002/657/EC requirements [14].

Furthermore, the validated method was applied in 39 samples of farmed salmon, originating from Norway and Denmark aquacultures, purchased in Portuguese supermarkets, between October and December 2017.

In this case, predicting that the higher fat content of salmon muscle, compared to the previous studied species, could be responsible for changes in the intensity of the detected signal (matrix effects), two distinct extraction protocols were tested, with and without a degreasing step with n-hexane, and an increase of signal was obtained when the lipid content was removed from the sample extract, minimizing the matrix effect.

As referred previously, in a screening method, the presence of any compound near the established MRL must be confirmed using a suitable confirmation technique. In accordance with Decision 2002/657/EC [14] the CCβ for a screening method should be less than the regulatory limit, having between the two values a distance to guarantee that a concentration close to the MRL is detected. The guidelines for validation of screening methods for residues of veterinary medicines [22], suggests the use of ½ MRL as CCβ since no more than one false-compliant result is obtained when 20 spiked samples are analysed at that level. Therefore, CCβ was defined and tested as ½ MRL, and for that 20 blank samples were spiked at CCβ level and analysed. The identification criteria were verified for all samples and target compounds demonstrating the applicability of the method.

The maximum variation in terms of ΔRRT was 0.4%, for sulfathiazole and sulfadiazine, within the accepted range, which is 2.5%.

In terms of mass accuracy variation, for veterinary drug residues, such value is not defined. However, considering legislation used for other contaminants, such as pesticides [23], the maximum admitted variation is 5 ppm, which we adopted in this method. The higher value obtained in terms of mass accuracy variation was 3.7ppm for sulfisomidine, below the admissible 5ppm.

Selectivity and specificity were also demonstrated, and no interference was found with the mass and retention time of our target compounds that could compromise

their identification. In terms of applicability of the method, 20 blank samples were spiked at the CCβ level where all the target compounds were effectively identified.

Regarding the results from the analysis of the real salmon samples, no positive results were obtained. These results were not surprising, and even expected, as most samples were originating from Norway. Norway is considered a model in the area of aquaculture production, because regulation of antimicrobial use in salmon aquaculture is very strict, being able to reduce the use of these drugs to negligible levels [24]. Our results confirm this reality, as no suspected samples were detected.

The effectiveness of the method can be demonstrated, though, as all the internal standards were detected. Being a fast and easy method, allied to its sensitivity and robustness, this tool can be relevant in terms of routine analysis of salmon from aquaculture origin.

Focusing more closely on the results obtained from the analysis of the real samples, purchased in Portuguese supermarkets, we observed concerning results regarding gilthead sea bream, namely the presence of doxycycline, an antibiotic for which no MRL is established.

Doxycycline is a semi-synthetic antibiotic, member of the tetracycline group, alternative to penicillins. It is widely used to treat diseases caused by both gram-positive and gram-negative bacteria, which include Spirochetes, *Actinomyces* sp, and *Mycoplasma* sp. It is also used for the treatment of Brucellosis, Lyme diseases, and Rickettsial infections, and is the drug of choice in the treatment of sexually transmitted diseases. It can also be used to treat complicated malaria when combined with quinine and can be used as an antivenin against snake bites [25]. The emergence of bacterial resistance to this antibiotic, for what aquaculture use contributes, poses public health concerns.

Also, the results obtained from the analysis of the European sea bass samples raise some concerns. Six out of the 30 analysed samples contained antibiotic residues, namely enrofloxacin and oxytetracycline, in concentrations ranging from 0.1 to 12 $\mu g kg^{-1}$. In one sample both compounds were detected simultaneously. Even though both were quantified below their MRLs, there are

not, to the best of our knowledge, studies to determine the safety of the simultaneous presence of both antibiotics in the same sample. Additionally, no data is available regarding possible cumulative effects, or even synergistic potentiation, of both substances' toxicities. Therefore, further studies should address this specific question of multiple drug residues in the same sample, and the legal framework, regarding MRLs of substances in edible tissues, should take this into consideration.

Given the precautionary principle, one of the European guiding principles concerning food security, it would be wise to encourage regulatory agencies to proceed with caution over this issue, limiting the number of antibacterial molecules that could be present in food items intended for human consumption and/or, whenever necessary to use more than one antimicrobial, the sum of the concentration of their residues should not exceed the MRL defined for the one with the lowest value.

Enrofloxacin and oxytetracycline, the antimicrobials detected in our positive samples, are included in Table 1 of Commission Regulation n. º 37/2010 [13], and a MRL of 100 $\mu g kg^{-1}$ is established for both. In this matter, the US has a far more restrictive legal framework, regarding the use of antibacterials in aquaculture. There are only 3 molecules approved by the FDA - oxytetracycline, florfenicol, and sulfadimethoxine/ormetoprim - which may only be used for treatment [26].

Facing our results, the first focus of concern, and probably the primary detrimental effect of administering prophylactic and therapeutic antibiotics to fish in aquaculture, is antibacterial resistance. The extensive use of antibacterials in aquaculture promotes the emergence of antibacterial-resistant zoonotic pathogens [27], and recent microbiological and clinical evidence suggests that antibacterial resistance genes and resistant bacteria are transferred from fish to humans [28]. Global surveillance studies demonstrate that FQ resistance rates increased in the past years in almost all bacterial species, seriously affecting patient management and care, and leading to changes in some clinical guidelines [29].

Enrofloxacin is only available in veterinary medicine and is used in many species with few adverse effects. Nevertheless, cross-resistance among enrofloxacin and

other FQ is well acknowledged and may be one of the primary reasons for the increasing rates in FQ resistance [30]. Therefore, there is now an important need to use FQ with caution to preserve their effectiveness for many years.

The second focus of concern that stems from the presence of antimicrobial residues in edible fish tissues is the possible adverse health effects in humans. Even with trace residues some individuals, particularly sensitive to certain antibiotics, can experience allergic reactions and the identification of the allergen may be hindered by a lack of knowledge of the substance, molecule or food that triggered the allergic reaction [31].

Focusing on the residues detected in our samples it is now recognized that FQ leave residues that may have carcinogenic properties and other adverse effects of these molecules involving the central nervous system (e.g., dizziness, headache, seizures, psychosis) are also well known [32]. Less recognized, but with a growing rate of notification and evidence are FQ-associated peripheral neuropathies [33].

The third question to be tackled, with growing attention from the scientific community, is the importance of preserving the intestinal microbiota. The intestinal microbiota, responsible for maintaining a healthy gastrointestinal tract by preventing pathogenic bacteria from growing, can be disrupted as a result of repeated exposures to antimicrobial residues [34]. The human gastrointestinal tract ecosystem consists of complex and diverse microbial communities and is getting increasing attention from the medical and scientific community because of its important role in human health and disease. Furthermore, the microbiota may have an unknown influence on the immune system, stimulating it to respond rapidly to pathogen challenges [35].

Several lines of evidence confirm that antibiotic intake can have deleterious effects in the gut ecosystem, disturbing its composition and function. Broad-spectrum antibiotics can affect the abundances of 30% of the bacteria in the gut community, promoting significant drops in taxonomic richness, diversity and evenness [36], and recent data suggest that chronic exposures to low-residue antimicrobial drugs in food could disrupt the equilibrium state of intestinal

microbiota and cause dysbiosis that can contribute to changes in body physiology [37].

One of the most imminent threats of gut microbiota alterations is the increased susceptibility to intestinal infections, which can be originated by newly acquired pathogens or from the sudden overgrowth and pathogenic behaviour of opportunistic organisms already present in the microbiota. Antibiotic-associated diarrhoeas, due to nosocomial pathogens, are a frequent occurrence, associated with organisms such as *Klebsiella pneumoniae*, *Staphylococcus aureus* and, of most concern, *Clostridium difficile*, which can cause intractable, recurrent infections and, in some cases, even a potentially lethal pseudomembranous colitis [36].

The dysbiosis promoted by the antibiotics has the additional disadvantage of enriching the microbiota in resistant organisms, and the human gut has been established as a significant reservoir of antibiotic resistance.

One of the largest population-level analyses of the intestinal resistome to date, also showed that the abundance of antibiotic resistance genes is higher for antibiotics that have been longer in the market and for those approved for animal use, such as tetracycline, bacitracin and the cephalosporins [38]. The effects of FQ on the ecology of colonic microbiota have been intensively evaluated [39,40], and it was shown that FQ have a selective effect on the normal colonic bacteria, decreasing the populations of enterobacteria and, in general, not affecting the anaerobic bacterial population.

Regarding the effect of tetracyclines, a recent study [41] demonstrated that, at low residue, tetracycline could lead to slight differences in the composition of intestinal microbiota. Another study [42] showed that, in certain conditions, tetracycline causes barrier disruption.

At this point, a crucial question that must be brought into discussion, regarding the use of antimicrobial drugs in aquaculture production, is the “safe” residue limits of these drugs in edible tissues. The MRLs established by the European regulatory framework [43] are defined only taking into account the immediate or long-term adverse and toxic effects of a single molecule. From our point of view,

though, a bigger issue is arising from this reality, which is the significant contribution to the emergence, spread and transference of antimicrobial resistance.

Although studies that establish an unequivocal link between antimicrobial use in aquaculture and the transference of AMR determinants to human pathogens are still lacking, our data are in line with the highlights of several studies and allow us to state that:

(1) AMU in aquaculture results in the presence of antibiotic residues in fish, as well as in the entry of antimicrobial compounds into the surrounding environment, with the potential to exert selective pressure and increase the frequency of AMR in the human microbiota, as well as environmental bacteria;

(2) high frequencies of AMR in bacteria have been reported in areas surrounding aquaculture production facilities, due to the use of antibiotics;

(3) molecular studies have shown that genes involved in AMR in bacteria associated with aquaculture exhibit great similarity to ARGs that have been detected in terrestrial bacteria, which are responsible for human and animal diseases.

Finally, a last topic to address in this discussion is the environmental impact and consequences resulting from the use of antibacterials in aquaculture. Their release in the environment is mainly due to the direct discharge of aquaculture products, resulting in the contamination of soil, surface water, sediment, ground water and biota. It has been estimated that 70-80% of fish antibacterials are released into the environment [44]. In addition, antibacterials are released through urine and faeces into the aquatic surroundings in an unmetabolized form, leading to extensive contamination [31].

Looking closer at the environmental fate of the residues detected in the analysed samples, it is known that tetracycline has a low bioavailability in fish (< 10%), due to binding with sea-water-borne divalent cations such as Mg^{2+} and Ca^{2+}, and non-bioavailable tetracyclines contaminate the environment [45]. The bioavailability of oxytetracycline in seawater, for instance, is 1% [46].

In short, the reasons that justify the importance of having appropriate analytical methodologies to control antibiotic residues in food items are manifold. And, in face of the growing importance of fish in the human diet, fish muscle must be one of the important target edible tissue to be controlled for the presence of antimicrobial residues.

The methods developed along our work are significant contributes to the effective surveillance and control of these matrices. The UHPLC-MS/MS method developed for European sea bass muscle, allows the simultaneous determination of 47 antibiotic compounds from eight different classes: sulphonamides, trimethoprim, tetracyclines, macrolides, quinolones, penicillins, cephalosporins and chloramphenicol. Regarding sample preparation, the method involves a simple and efficient extraction step, with acetonitrile and the chelating agent EDTA, which “cleans” the sample from cations that might form complexes with the molecules of interest. The absolute recoveries obtained for all compounds proved the suitability of this extraction procedure.

Furthermore, the UHPLC-TOF/MS method, developed and validated for salmon muscle, allows the simultaneous determination of 44 antimicrobials from 7 different classes: sulphonamides, trimethoprim, tetracyclines, macrolides, quinolones, penicillins and cephalosporins. Similarly, a simple sample preparation procedure, including liquid-liquid extraction with acetonitrile and EDTA, and an extra degreasing step with n-hexane, proved to be suitable to extract the analytes with little interference during analysis.

Both methods were successfully validated, in accordance to the European legal requirements [14], demonstrating the good performance of the methods.

Additionally, and particularly important, their reduced handling time, along with reduced costs and high throughput, enables a higher daily number of samples to be analysed, conferring these methods the appropriate features for efficient routine analysis.

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Chapter 7 | Future Trends and Conclusion

The discovery of antibiotics is universally acknowledged as the greatest scientific and medical milestone, in the 20th century. Their development and use in human and veterinary medicine resulted in the significant reduction of the mortality and morbidity rates of socially and epidemiologically significant infectious diseases, and somehow their remarkable efficacy and efficiency retains a sense of miraculous. The current clinical practice model is very heavily reliant upon the use of antibiotics, and with resistance on the rise, even in community settings, we are about to lose the immense ground we have conquered in the last century. The fight against life threatening infectious diseases, such as pneumonia, tuberculosis or malaria, the treatment of cancer - where antibiotics are crucial in helping chemotherapy patients avoid and fight infection – and infection prevention in all routine and complex surgeries, are only a few examples of what is at stake.

Since penicillin discovery by Fleming, in 1929, these drugs have been saving the lives of millions of people and animals for nearly one century. Nonetheless, the miracle of these special drugs has been increasingly threatened by the emergence, dissemination, and persistence of antibiotic resistance [1-4]. Antibiotic resistance did not come out of the blue, nor it is a new or unexpected phaenomenon, being predicted and warned by Fleming in his Nobel Prize lecture in 1946 [5]. What is new, and worrying, is the rate and speed at which bacteria are accumulating antimicrobial resistance determinants to almost all known antibiotics. Given the lack of novel antibiotics under development, optimized drug exposure is essential to suppress the spread of antibiotic resistance.

It is, therefore, not inappropriate to think on a deep change of paradigm regarding the food producing industry, turning the use of antimicrobials an exception, rather than a common procedure in this industry, and always under strict veterinarian surveillance.

In the last couple of years, several reports and recommendations are being produced [4,6,7] and the true size of the problem is perfectly diagnosed and acknowledged by political and health authorities. At this point, another step forward must be taken, in the interests of public health safeguard, and a

significant reform of the legislative and supervisory framework must be undertaken.

The focus must be given on primary prevention, and external and internal biosecurity measures, which would contribute to an important decrease of infection and transmission, resulting in a relevant decrease on the need to use AM, as pointed out by several studies.

Governments should also assume the responsibility to develop and manage an updated database of official health bulletins of individual herds, as occurs with the Danish SPF (specific pathogen free) system [8]. Reliable and official information on this should be of free access to all interested.

Furthermore, it is increasingly clear that promoting and increasing animals' resilience is a major protection against disease, namely by reducing chronic stress, stocking density and handling and transportation conditions.

Research funds and opportunities should be launched, focusing on the development of new and improved vaccines.

Farm Health Plans are fundamental and major instruments to assemble and monitor all the good husbandry practices implemented on a farm/herd, and a Health Plan can play a significant role in monitoring and responding to disease and in optimizing on-farm use of antimicrobials. Policy actions should point out concrete measures in order to support farmers in the process of developing and implementing health plans, which should progressively become mandatory. Additionally, a set of actions – as training and awareness on these topics, technical advice and support and ongoing monitoring throughout the process, financial incentives – should be implemented, as farmers/producers are the main actors of change.

In the aquaculture sector, specifically, the use of antibiotics is regulated sparingly, differing greatly from country to country with little to no enforcement in many of the countries that produce the majority of the world's aquaculture products. Usage purposes are similar as those in livestock, but in aquaculture prophylactic treatment is much more common. As water provides a constant and easy mechanism for dispersal of drug residues, microbial pathogens, and resistance

genes, aquaculture will continue to pose a threat that may increase as the demand for seafood increases.

There are several alternative measures, previously described, to the use of antimicrobials in aquaculture. The use of vaccines, probiotics, immunostimulants and non-specific immune-enhancers, along with the overall improvement of aquatic environmental quality must be addressed as major areas for further research in disease control in aquaculture.

Fish need to be reared under good husbandry conditions that have to consider the optimum conditions for parameters such as feed rates, water dissolved oxygen, stocking densities and even controlled temperature, where this is feasible. Feed composition is another relevant issue. The formulation of fish diets is fundamental for the provision of proteins that are used to produce maximum growth under good general health status. There is evidence that fish health can be related to diet and many studies have shown the potential importance of dietary factors such as vitamins and trace elements for controlling infections or avoiding signs of nutritional deficiency [8]. Nutritional status is considered one of the important factors that determines the ability of fish to resist diseases, since nutritional and physical characteristics of diets can modulate susceptibility of fish to infectious diseases.

In particular, vaccination has a major prophylactic role in protecting fish against diseases. However, there is still some work needed to improve vaccines, particularly to increase protection levels and optimise delivery methods, and political measures to support innovation need to be implemented.

Water treatment and movement restrictions are also important features to be addressed. In many cases, the spread of diseases has been related directly to the movement of infected stocks and prevention of disease spread can therefore be avoided by the application of movement restrictions, which are usually enforced by legislation in the case of notifiable diseases.

The conclusion based on 30 to 40 years’ experience with intensive salmonid fish farming in Norway demonstrates that sustainability should be the basis for developing a successful aquaculture industry. The Norwegian experience shows

that disease prevention is fundamental for sustainability, and legislation is the cornerstone in disease prevention, while vaccination is the single most important preventive measure. Finally, the authorities and the industry must be well organized and have the right competence on all levels.

Furthermore, it is widely recognized that human, animal and environmental populations are biologically continuous and, therefore, antimicrobial resistance has a global ecological impact. Therefore, the interdisciplinary co-operation between human medicine, veterinary medicine and ecology may be a key and strategic approach to preserve the miracle of antibiotics and improve health.

Single, isolated interventions have limited impact. Coordinated action is required to minimize the emergence and spread of antimicrobial resistance, and all countries need to implement and commit to national action plans on AMR.

In this context, the One Health strategy is the most appropriate way to address the problem. Multidisciplinary expertise must be convened: animal health, livestock and production, food and feed safety, plant health and production, fisheries and aquaculture, legislative contexts, need to address a cross-sectoral issue such as antimicrobial resistance.

Antimicrobial resistance is flagged as a major threat for public health, and a global effort must be made to cease antimicrobial misuse and overuse, namely in aquaculture, encouraging stakeholders to adopt other disease prevention measures. Shaping a new path is crucial to contain the increasing threat of this problem, protecting and preserving the effectiveness of one of the greatest scientific and medical achievements in the 20th century.

In the present work, a UHPLC-MS/MS quantitative and confirmatory method and a UHPLC-ToF/MS screening method were developed and validated to monitor a wide range of antimicrobial molecules, from different classes, in three of the most farmed fish species consumed in Portugal: Gilthead sea bream, European sea bass and salmon.

Additionally, the methods were applied in real samples of those species, purchased in the most popular Portuguese supermarkets, in order to access the overall quality of the consumed species, regarding the presence of antimicrobial residues.

The methods exhibit innovative features, regarding other similar published papers, as they allow the determination of more than 40 antimicrobial molecules, from different classes, in a single run, and with simple and fast extraction procedures.

The methods were validated in accordance with the EU's legal requirements and proved to be suitable for the routine analysis of fish samples. It would be relevant to extend the method to other highly consumed fish species, such as Rainbow Trout, Sole, Turbot and White seabream.

Furthermore, these multiclass multi-residue methods need to be improved in order to include other relevant antimicrobials, namely aminoglycosides and polimixins, whose chemical properties still represent a challenge in this field.

Concerning the public health problems, arising from the use of antimicrobials in aquaculture, extensively discussed previously, this dissertation intends to be at least one small element to the urgent reflexion that needs to be undertaken, leading to concrete and major changes in the food producing industry, regarding the use of antimicrobials.

Currently, science provides overwhelming evidence that antibiotic use is a powerful selector of resistance, that emerges at the site of use and spreads everywhere else. Also, a growing body of evidence shows that antimicrobial use in animals, including nontherapeutic use, leads to the propagation and shedding of substantial amounts of antimicrobial-resistant bacteria and antimicrobial resistance genetic elements, transferable across species borders and reaching humans through multiple routes of transfer.

As AMR still continues to increase, and fewer new drugs are being developed, calls for action to prevent the imminent crisis of a 'post antibiotic era' must be clearly acknowledged and tackled by political authorities, healthcare professionals and individuals, within their area of competence.

Given the scale of the problem, that placed substantial economic burden and societal concerns on the healthcare system, the additional economic, social and time investments are likely to be recovered by the resulting benefits, including financial efficiencies and, above all, improved human, animal and environmental health outcomes.

AMR is a massive challenge for this generation, and at this point within our ability to tackle effectively. The human and economic costs force us to act urgently; otherwise, the brunt of these will be borne by the nearly future generations.

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ANNEXES

Annex 1 - Summary of sampling process, with date of collection and origin of the analysed samples of Gilthead Sea Bream (*Sparus aurata*)

Sample	Date of purchase	Origin	Place of purchase	Location
1	06.02.2015	Turkey	Pingo Doce - Figueira da Foz	Figueira da Foz
2	08.02.2015	Spain	SuperCor - Coimbra	Coimbra
3	08.02.2015	Spain	SuperCor - Coimbra	Coimbra
4	08.02.2015	Greece	Intermarché - Mealhada	Mealhada
5	08.02.2015	Greece	Intermarché - Mealhada	Mealhada
6	08.02.2015	Greece	Jumbo - Coimbra	Coimbra
7	08.02.2015	Greece	Jumbo - Coimbra	Coimbra
8	15.02.2015	Greece	Pingo Doce - Leiria II	Leiria
9	15.02.2015	Greece	Pingo Doce - Leiria II	Leiria
10	15.02.205	Greece	Intermarché - Leiria	Leiria
11	15.02.2015	Greece	Intermarché - Leiria	Leiria
12	21.02.2015	Greece	Intermarché - Miranda do Corvo	Miranda do Corvo
13	21.02.2015	Greece	Intermarché - Miranda do Corvo	Miranda do Corvo
14	16.02.2015	Greece	Jumbo - Figueira da Foz	Figueira da Foz
15	20.02.2015	Spain	Pingo Doce - Figueira da Foz	Figueira da Foz
16	22.03.2015	Spain	El Corte Inglés - Lisboa	Lisboa
17	22.03.2015	Spain	El Corte Inglés - Lisboa	Lisboa
18	22.03.2015	Turkey	Pingo Doce - Lisboa (5 de Outubro)	Lisboa
19	22.03.2015	Turkey	Pingo Doce - Lisboa (5 de Outubro)	Lisboa
20	22.03.2015	Greece	Continente - Lisboa (Av. Nações Unidas)	Lisboa

21	22.03.2015	Greece	Continente - Lisboa (Av. Nações Unidas)	Lisboa
22	28.03.2015	Greece	Jumbo - Gaia	Porto
23	28.03.2015	Greece	Jumbo - Gaia	Porto
24	28.03.2015	Turkey	Pingo Doce - Constituição	Porto
25	28.03.2015	Turkey	Pingo Doce - Constituição	Porto
26	28.03.2015	Spain	Continente - Coimbra Shopping	Coimbra
27	28.03.2015	Greece	Continente - Coimbra Shopping	Coimbra

Annex 2 - Summary of sampling process, with date of collection and origin of the analysed samples of European sea bass (*Dicentrarchus labrax*)

Sample	Date of purchase	Origin	Place of purchase	Location
1	05.03.2016	Spain	SuperCor - El Corte Inglés	Coimbra
2	05.03.2016	Spain	SuperCor - El Corte Inglés	Coimbra
3	05.03.2016	Spain	SuperCor - El Corte Inglés	Coimbra
4	05.03.2016	Greece	Continente (Vale das Flores)	Coimbra
5	05.03.2016	Greece	Continente (Vale das Flores)	Coimbra
6	05.03.2016	Greece	Continente (Vale das Flores)	Coimbra
7	05.03.2016	Spain	Pingo Doce (Portela)	Coimbra
8	05.03.2016	Spain	Pingo Doce (Portela)	Coimbra
9	05.03.2016	Spain	Pingo Doce (Portela)	Coimbra
10	05.03.2016	Greece	Jumbo (Dolce Vita)	Coimbra
11	05.03.2016	Greece	Jumbo (Dolce Vita)	Coimbra
12	05.03.2016	Greece	Jumbo (Dolce Vita)	Coimbra
13	05.03.2016	Spain	E. Leclerc	Figueira da Foz
14	05.03.2016	Spain	E. Leclerc	Figueira da Foz
15	05.03.2016	Spain	E. Leclerc	Figueira da Foz
16	10.03.2016	Greece	Jumbo (Palácio Gelo)	Viseu
17	10.03.2016	Greece	Jumbo (Palácio Gelo)	Viseu
18	10.03.2016	Greece	Jumbo (Palácio Gelo)	Viseu
19	19.03.2016	Greece	Continente (Vasco da Gama)	Lisboa
20	19.03.2016	Greece	Continente (Vasco da Gama)	Lisboa

21	19.03.2016	Greece	Continente (Vasco da Gama)	Lisboa
22	19.03.2016	Norway	Pingo Doce (Parque das Nações - Norte)	Lisboa
23	19.03.2016	Norway	Pingo Doce (Parque das Nações - Norte)	Lisboa
24	19.03.2016	Norway	Pingo Doce (Parque das Nações - Norte)	Lisboa
25	10.04.2016	Greece	Jumbo Aveiro	Aveiro
26	10.04.2016	Greece	Jumbo Aveiro	Aveiro
27	10.04.2016	Greece	Jumbo Aveiro	Aveiro
28	10.04.2016	Spain	Continente Aveiro Estação	Aveiro
29	10.04.2016	Spain	Continente Aveiro Estação	Aveiro
30	10.04.2016	Spain	Continente Aveiro Estação	Aveiro

Annex 3 - Summary of sampling process, with date of collection and origin of the analysed samples of Salmon (*Salmo salar*)

Sample	Date of purchase	Origin	Place of purchase	Location
1	29.10.2017	Norway	Jumbo Aveiro	Aveiro
2	29.10.2017	Norway	Jumbo Aveiro	Aveiro
3	29.10.2017	Norway	Jumbo Aveiro	Aveiro
4	29.10.2017	Norway	Pingo Doce (Aveiro - Vera Cruz)	Aveiro
5	29.10.2017	Norway	Pingo Doce (Aveiro - Vera Cruz)	Aveiro
6	29.10.2017	Norway	Pingo Doce (Aveiro - Vera Cruz)	Aveiro
7	01.11.2017	Norway	Supercor Coimbra	Coimbra
8	01.11.2017	Norway	Supercor Coimbra	Coimbra
9	01.11.2017	Norway	Supercor Coimbra	Coimbra
10	01.11.2017	Norway	Continente (Vale das Flores)	Coimbra
11	01.11.2017	Norway	Continente (Vale das Flores)	Coimbra
12	01.11.2017	Norway	Continente (Vale das Flores)	Coimbra
13	01.11.2017	Norway	Jumbo Coimbra	Coimbra
14	01.11.2017	Norway	Jumbo Coimbra	Coimbra
15	01.11.2017	Norway	Jumbo Coimbra	Coimbra
16	01.11.2017	Norway	Pingo Doce (Coimbra II - Rua Brasil)	Coimbra
17	01.11.2017	Norway	Pingo Doce (Coimbra II - Rua Brasil)	Coimbra
18	01.11.2017	Norway	Pingo Doce (Coimbra II - Rua Brasil)	Coimbra
19	12.11.2017	Norway	Pingo Doce (Constituição - Porto)	Porto
20	12.11.2017	Norway	Pingo Doce (Constituição - Porto)	Porto
21	12.11.2017	Norway	Pingo Doce (Constituição - Porto)	Porto

22	15.11.2017	Norway	E. Leclerc (Figueira da Foz)	Figueira da Foz
23	15.11.2017	Norway	E. Leclerc (Figueira da Foz)	Figueira da Foz
24	15.11.2017	Norway	E. Leclerc (Figueira da Foz)	Figueira da Foz
25	27.11.2017	Norway	Intermarché (CC Olhalvas Park)	Leiria
26	27.11.2017	Norway	Intermarché (CC Olhalvas Park)	Leiria
27	27.11.2017	Norway	Intermarché (CC Olhalvas Park)	Leiria
28	27.11.2017	Norway	Pingo Doce (Leiria I)	Leiria
29	27.11.2017	Norway	Pingo Doce (Leiria I)	Leiria
30	27.11.2017	Norway	Pingo Doce (Leiria I)	Leiria
31	17.12.2017	Norway	Pingo Doce (Galhardas)	Lisboa
32	17.12.2017	Norway	Pingo Doce (Galhardas)	Lisboa
33	17.12.2017	Norway	Pingo Doce (Galhardas)	Lisboa
34	11.12.2017	Norway	Continente (Vale das Flores)	Coimbra
35	12.12.2017	Norway	Pingo Doce (Condeixa-a-Nova)	Coimbra
36	12.12.2017	Norway	Intermarché (Condeixa-a-Nova)	Coimbra
37	12.12.2017	Norway	Lidl (Condeixa-a-Nova)	Coimbra
38	12.12.2017	Norway	Jumbo Coimbra	Coimbra
39	13.12.2017	Denmark	Pingo Doce (Condeixa-a-Nova)	Coimbra