UNIVERSIDADE FEDERAL DO PARÁ
INSTITUTO DE TECNOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA E TECNOLOGIA DE ALIMENTOS
(PPGCTA)

LETÍCIA ROCHA GUIDI

DESENVOLVIMENTO DE MÉTODOS POR CL-EM/EM E OCORRÊNCIA DE ANTIMICROBIANOS EM PEIXES DE AQUICULTURA

BELÉM-PA
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Tese apresentada ao Programa de Pós-graduação em Ciência e Tecnologia de Alimentos da Universidade Federal do Pará, para obtenção do grau de Doutor em Ciência e Tecnologia de Alimentos.

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BELÉM-PA
2016

Dedico este trabalho aos meus pais, Ricardo e Heloisa.

“Posso ter defeitos, viver ansioso e ficar irritado algumas vezes,
Mas não esqueço de que minha vida
É a maior empresa do mundo…
E que posso evitar que ela vá à falência.
Ser feliz é reconhecer que vale a pena viver
Apesar de todos os desafios, incompreensões e períodos de crise.
Ser feliz é deixar de ser vítima dos problemas e
Se tornar um autor da própria história…
É atravessar desertos fora de si, mas ser capaz de encontrar
Um oásis no recôndito da sua alma…
É agradecer a Deus a cada manhã pelo milagre da vida.
Ser feliz é não ter medo dos próprios sentimentos.
É saber falar de si mesmo.
É ter coragem para ouvir um “Não”!!!
É ter segurança para receber uma crítica,
Mesmo que injusta…

Pedras no caminho?
Guardo todas, um dia vou construir um castelo…”

Fernando Pessoa – Pedras no Caminho

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LISTA DE SIGLAS E ABREVIATURAS

ADI - Acceptable daily intake
AOAC - Association of Official Analytical Chemists
ANOVA - Análise de variância
ANVISA - Agência Nacional de Vigilância Sanitária
APPI - Atmospheric pressure photoionization
BPA - Boas Práticas Agropecuárias
BPF - Boas Práticas de Fabricação
CAP - Chloramphenicol
CAP-Glu - Chloramphenicol glucuronide
CCα - Decision limit
CCβ - Detection capability
CE - Collision energy
CG - Cromatografia gasosa
CL - Cromatografia líquida
CLAE - Cromatografia líquida de alta eficiência
CRL - Community Reference Laboratories
CVM - Center for Veterinary Medicine
CXP - Collision cell exit potential
DAD - Diode array detector
DHA - Ácido docosaexaenoico
DLLME - Dispersive liquid-liquid microextraction
DMFS - Dispersão da matriz em fase sólida
DP - Declustering potential
d-SPE - Dispersive solid-phase extraction
ECD - Electron capture detector
EIC - Extracted ion chromatogram
ELISA - Enzyme-linked immunosorbent assay
EM - Espectrometria de massas
EMBRAPA - Empresa Brasileira de Pesquisa Agropecuária
EMEA - Agência Europeia de Medicina
EP - Entrance potential
EPA - Ácido eicosapentaenóico
ESI - Electrospray ionization
EU - European Union
FAO - Food and Agriculture Organization
F_m - Fator de corte
FDA - Food and Drug Administration
FF - Florfenicol
FFA - Florfenicol amine
FLD - Fluorescence detector
GC - Gas chromatography
HFBA - Ácido heptafluorobutírico
HPLC - High performance liquid chromatography
HRMS - High resolution mass spectrometry
IBGE - Instituto Brasileiro de Geografia e Estatística
IUPAC - International Union of Pure and Applied Chemistry
JECFA - *Joint WHO/FAO Expert Committee on Food Additives*
LC - Liquid chromatography
LD - Limite de detecção

LLE - Liquid-liquid extraction
LMR - Limite máximo de resíduos
LOD - Limit of detection
LOQ - Limit of quantification
MAPA - Ministério da Agricultura, Pecuária e Abastecimento
MEPS - Microextraction by packed sorbent
MIP - Molecularly imprinted polymer
MPA - Ministério da Pesca e Aquicultura
MRL - Maximum residue limits
MRM - Multiple reaction monitoring
MRPL - Minimum required performance limits
MS - Mass spectrometry
MS/MS - Tandem mass spectrometry
MSPD - Matrix solid phase dispersion
N - Number of samples
n.a. - not applicable
NCI - Electron-capture negative chemical ionization
NI - Negative Ionization
NOEL - No observed effect level
OMS - Organização Mundial de Saúde
PABA - Ácido para-aminobenzóico
PNCRBC - Programa Nacional de Controle de Resíduos Biológicos em Carne
PNCRC - Programa Nacional de Controle de Resíduos e Contaminantes
PNCRCP - Programa Nacional de Controle de Resíduos e Contaminantes em Pescado
PVDF - Fluoreto de polivinilideno
QuEChERS - Quick, Easy, Cheap, Effective, Ruged and Safe
SIF - Serviço de Inspeção Federal
SLE - Solid-liquid extraction
SPE - Solid-phase extraction
SPR - Surface plasmon resonance
STC - Screening target concentration
TAP - Thiamphenicol
TCA - Trichloroacetic acid
TIC - Total Ion Chromatogram
TOF - Time of flight
T_v - Threshold value
UE - União Europeia
UHPLC - Ultra high pressure liquid chromatography
UPLC - Ultra performance liquid chromatography
UV - Ultraviolet detector
VWD - Variable wavelength detector

RESUMO

O consumo de peixes no Brasil vem aumentando nos últimos anos, especialmente devido à divulgação de que a sua ingestão pode trazer inúmeros benefícios à saúde e também devido ao seu alto valor nutricional (proteínas de alto valor biológico, teor elevado de ácidos graxos ômega-3). A qualidade, a inocuidade e a segurança de peixes cultivados para alimentação humana constituem, portanto, tema de saúde pública e devem ser monitoradas. No Brasil, há uma carência de informações no que diz respeito ao uso de antimicrobianos destinados à aquicultura. Apesar de apenas dois antibióticos serem permitidos para uso em aquicultura no Brasil, existe uma grande diversidade de antibióticos que podem ser utilizados ilegalmente ou que podem chegar aos peixes devido a contaminações do meio ambiente, principalmente dos recursos hídricos. Este trabalho teve como objetivo geral desenvolver métodos de análise multirresíduos de antimicrobianos em músculo de peixe e avaliar a qualidade dos peixes cultivados nos Estados de Minas Gerais e do Pará no que diz respeito à presença destes resíduos. Além disso, foi realizada uma extensa revisão da literatura com relação aos métodos existentes de análise e à ocorrência de cloranfenicol (antibiótico banido) e anfenicóis em alimentos. Foi validado um método de screening por CL-EM/EM para análise de 40 antibióticos de seis classes diferentes (aminoglicosídeos, beta-lactâmicos, macrolídeos, quinolonas, sulfonamidas e tetraciclinas) em músculo de peixe. Apenas 15% das amostras (n=29) foram positivas para enrofloxacina. Um método quantitativo por CL-EM/EM de análise de quinolonas e tetraciclinas em músculo de peixe também foi otimizado e validado. A precisão, em termos de desvio padrão relativo, foi abaixo de 20% para todos os analitos e as recuperações variaram de 89,3% a 103,7%. CCα variou de 17,87 a 323,20 $\mu g.kg^{-1}$ e CCβ variou de 20,75 a 346,40 $\mu g.kg^{-1}$. No geral, as amostras de peixe analisadas apresentaram qualidade adequada quanto à presença de resíduos de antibióticos. Todas as 29 amostras positivas para enrofloxacina continham teores abaixo do Limite Máximo de Resíduo permitido pela legislação brasileira (100 $\mu g.kg^{-1}$).

Palavras-chave: antibióticos, piscicultura, screening, quantitativo, multirresíduos, CL-EM/EM.

ABSTRACT

The consumption of fish has increased in recent years in Brazil, especially due to the announcement that their intake can bring numerous health benefits and also due to its high nutritional value (high biological value protein, high content of omega-3 fatty acids). The quality, safety and security of farmed fish for human consumption are therefore a public health issue and must be monitored. In Brazil, there is a lack of information regarding the use of antimicrobials in aquaculture. Although only two antibiotics are allowed for use in aquaculture in Brazil, there is a wide variety of antibiotics that may be used illegally or can reach the fish due to environmental contaminations, mainly of water. The objective of this study was to develop multiresidue methods of analysis of antibiotics in fish muscle and to evaluate the quality of fish from Minas Gerais and Pará with respect to the presence of antibiotic residues. In addition, an extensive literature review was conducted with respect to existing methods of analysis and the occurrence of chloramphenicol (banned antibiotic) and amphenicols in food. A LC-MS/MS screening method was validated for the analysis of 40 antibiotics of six different classes (aminoglycosides, beta-lactams, macrolides, quinolones, sulfonamides and tetracyclines) in fish muscle. Only 15% of the samples (n=29) were positive for enrofloxacin. A quantitative LC-MS/MS method of analysis of quinolones and tetracyclines in fish muscle was also optimized and validated. The precision, in terms of the relative standard deviation, was under 20% for all of the compounds, and the recoveries were between 89.3% and 103.7%. CCα varied from 17.87 to 323.20 $\mu g.kg^{-1}$ and CCβ varied from 20.75 to 346.40 $\mu g.kg^{-1}$. In general, real samples showed good quality relative to the presence of antibiotic residues. All 29 positive samples for enrofloxacin contained levels below the Maximum Residue Limit allowed by Brazilian legislation (100 $\mu g.kg^{-1}$).

Keywords: antibiotics, pisciculture, screening, quantitate, multiresidues, LC-MS/MS.

INTRODUÇÃO GERAL

O peixe é um alimento que se destaca nutricionalmente devido ao fato de ser fonte abundante de proteína de alto valor biológico, à presença de vitaminas (A, D, E e complexo B) e minerais (cálcio, fósforo e ferro) e, principalmente, por ser fonte dos ácidos graxos essenciais ômega-3 eicosapentaenoico (EPA) e docosaexaenoico (DHA). Estudos têm demonstrado que o consumo frequente de alimentos ricos em ácidos graxos ômega-3, presentes principalmente nos peixes, está associado a redução dos riscos de doenças cardiovasculares, de alguns tipos de câncer, bem como no tratamento de doenças inflamatórias como a artrite inflamatória (BAYLISS, 1996; SARTORI & AMANCIO, 2012; FAO, 2015a).

A aquicultura vem se impondo mundialmente como atividade pecuária, sendo um dos sistemas de produção de alimentos que mais cresce no mundo. A piscicultura de água doce tem se mostrado promissora, principalmente no que diz respeito ao cultivo de tilápias (WAGNER et al., 2004). O Brasil apresenta um grande potencial natural para desenvolvimento da aquicultura. Além de mão-de-obra abundante e crescente demanda por pescado, possui um território vasto, com mais de 2/3 ocupando a região tropical, bacias hidrográficas privilegiadas e ricas, onde se destaca a bacia amazônica responsável por 20% da água doce do mundo (PASCHOAL, 2007). O país possui produção promissora de espécies exóticas como a tilápia (*Oreochromis niloticus*) e nativas como o pacu (*Piaractus mesapotamicus*) e o tambaqui (*Colossoma macropomum*) (QUESADA, 2012). Segundo dados da FAO (2015b), a aquicultura no Brasil tem também se destacado nas exportações, com recente aumento para peixes frescos, principalmente na forma de filés. Além disso, houve uma valorização do preço de pescado exportado pelo Brasil, gerado diretamente pelas crescentes exportações de preparações e conservas, filé de peixe, lagosta, polvo e de atuns e afins (SEAP, 2015).

A aquicultura, assim como todo sistema intensivo de produção animal, se constitui em um ambiente que favorece a disseminação de doenças infecciosas, devido à elevada densidade populacional e por ser em um ambiente aquático, o que favorece a proliferação de micro-organismos. Eventuais alterações físico-químicas bruscas no ambiente aquático e/ou práticas de manejo inadequadas afetam diretamente o estado de saúde dos peixes. Além disto, várias bactérias patogênicas afligem a aquicultura, dentre elas, destaca-se: *Flavobacterium columnare*, *Aeromonas sp*., *Vibrio spp*.,

Streptococcus iniae, *Streptococcus agalactiae*, *Edwardsiella tarda*, *Francisella sp*., *Pseudomonas fluorescens*, *Piscirickettsia salmonis*, *Plesiomonas shiguelloides*, as quais têm sido apontadas como os principais fatores limitadores da produtividade (QUESADA, 2012).

Por isso, o uso de antibióticos na produção animal, inclusive na aquicultura, é uma prática comum para prevenir e tratar doenças infecciosas e se faz necessário para a garantia da exploração econômica viável da atividade. Entretanto, o uso inadequado dessas substâncias pode levar ao aparecimento de resistência microbiana em humanos, animais e também trazer impactos ao meio ambiente com a seleção de bactérias mais resistentes a essas substâncias (GASTALHO et al., 2014).

Os antimicrobianos licenciados para uso em peixes no mundo, com algumas exceções são: tetraciclina, oxitetraciclina, ácido oxolínico, flumequina, amoxicilina, florfenicol, entre outros, sendo os dois primeiros os mais utilizados (WHO, 1998; FAO, 2005; EC, 2010a; CODEX, 2014; BRASIL, 2015). Em alguns países existem normas quanto ao uso desses antibióticos na piscicultura, porém, nem sempre efetivamente aplicadas; já em outros países, não existe sequer uma regulamentação (PASCHOAL, 2007). O cloranfenicol teve seu uso proibido em animais destinados à produção de alimentos em diversos países devido aos sérios efeitos adversos que pode causar ao homem (GUIDI et al., 2015). O florfenicol é um anfenicol eficaz no tratamento contra bactérias em peixes e não apresenta os efeitos adversos do cloranfenicol, sendo, junto à oxitetraciclina, um dos únicos antibióticos liberados para uso em aquicultura no Brasil (SADEGHI & JAHANI, 2013; SINDAM, 2016). As quinolonas fazem parte de um grupo de antimicrobianos de amplo uso nas medicinas humana e veterinária e existem suspeitas de que estejam sendo utilizados de forma indevida na aquicultura.

Para exportar peixes para a União Europeia são exigidos certificados de testes laboratoriais com a finalidade de constatar os níveis de metais pesados, antibióticos e histamina aos exportadores de peixe fresco, substâncias estas relacionadas à segurança do consumidor (SEAP, 2015). A necessidade de atender a essas exigências sanitárias e de outros importantes mercados internacionais e assim evitar embargos à exportação, além da preocupação também a nível nacional, determinou a implementação do Plano Nacional de Controle de Resíduos e Contaminantes em Pescado (PNCRC) pelo Ministério da Agricultura, Pecuária e Abastecimento (MAPA), como uma política de proteção à saúde do consumidor no que diz respeito à presença de resíduos nos produtos da pesca.

Sendo assim, o monitoramento de resíduos de antimicrobianos em alimentos é muito importante e visa, principalmente, a proteção do consumidor. Desta forma, importantes órgãos como o *Codex Alimentarius*, *Food and Drug Administration* (FDA) dos Estados Unidos, o MAPA e outros órgãos tem estabelecido Limites Máximos de Resíduos (LMR) em diversos alimentos de origem animal. É importante ressaltar que resíduos abaixo do valor do LMR são considerados como seguros. Além do ponto de vista sanitário, preocupações do ponto de vista econômico são constantes, pois sanções econômicas e barreiras alfandegárias podem inviabilizar a comercialização de alimentos entre países (MOREIRA, 2012). Para atender a essas demandas, é importante que sejam desenvolvidos métodos analíticos exatos, precisos e que tenham sensibilidade para possibilitar a determinação de baixos níveis de resíduos de antimicrobianos em peixe (em geral, $\mu g.kg^{-1}$). A cromatografia líquida acoplada à espectrometria de massas sequencial (CL-EM/EM) é uma excelente técnica para essa finalidade, tendo sido aplicada por vários pesquisadores na análise de antimicrobianos em alimentos (LOPES et al., 2011; SISMOTTO et al., 2014; DASENAKI & THOMAIDIS, 2015; FREITAS et al., 2015; JANK et al., 2015; MONTEIRO et al., 2015; REZK et al., 2015; MARTINS et al., 2016; MORETTI et al., 2016).

Além disso, os laboratórios de rotina precisam fornecer resultados rápidos e confiáveis para um grande número de amostras. Para esse fim, os métodos de triagem são uma boa alternativa, já que são mais rápidos na emissão de laudos, pois os resultados se baseiam na resposta conforme (concentração do analito menor que o LMR) ou não conforme (concentração do analito maior que o LMR). Através da determinação do fator de corte, pode-se avaliar se a amostra contém ou não o analito em concentração superior ao LMR, ou seja, os métodos de triagem são semiquantitativos. Como na grande maioria das vezes as amostras são conformes, os laudos podem ser emitidos com maior rapidez. Diante do exposto, é importante desenvolver métodos analíticos que sejam adequados para determinação de resíduos de antimicrobianos em alimentos, bem como a necessidade de monitoramento do pescado cultivado em pisciculturas do Brasil, como nos estados de Minas Gerais e do Pará. Esses resultados poderão servir de apoio para avaliação da qualidade dos peixes produzidos nesses Estados e como fonte para futuras ações públicas de conscientização sobre o uso dessas substâncias.

REVISÃO DE LITERATURA

1. A AQUICULTURA NO BRASIL

A produção e o consumo de peixes e outros pescados pela população brasileira tem oscilado ao longo dos anos. A Figura 1 mostra a série histórica de estimativa realizada pelo Instituto Brasileiro de Geografia e Estatística (IBGE). Observa-se um consumo relativamente estável até 2005, quando o mesmo passa a crescer e atinge 9,8 kg/pessoa no ano de 2010 (MPA, 2010; OLIVEIRA, 2013). Esse aumento no consumo de pescado, tanto marinho quanto continental, pode estar relacionado às mudanças no hábito alimentar das populações e aos benefícios à saúde que o mesmo apresenta (MV&Z, 2012).

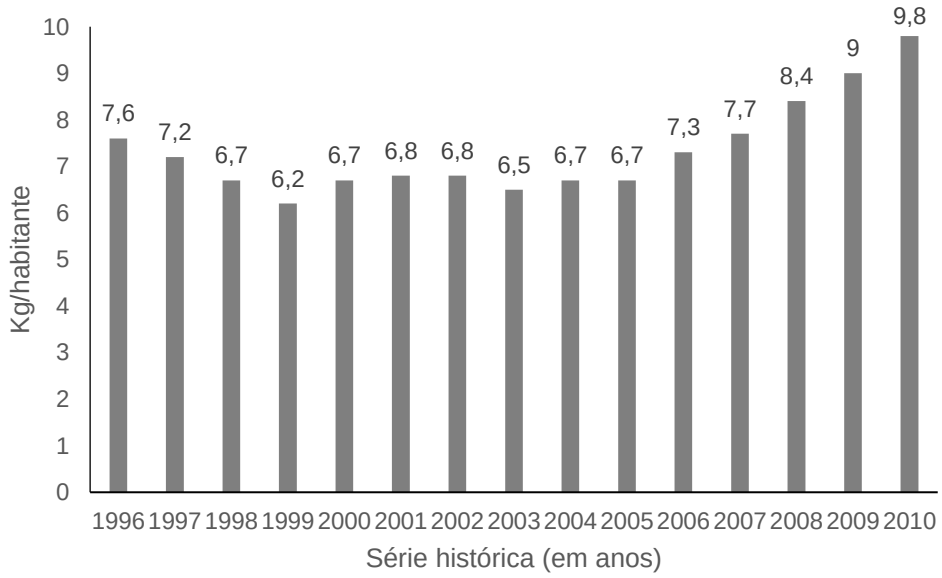


Figura 1. Série histórica de consumo aparente *per capita* de pescados nacional, de 1996 a 2010. Fonte: MPA (2010).

A produção total de pescado nacional em 2011 foi de 1.431.974,4 toneladas, representando um aumento de aproximadamente 13,2% em relação a 2010. A pesca extrativa marinha foi a principal fonte de produção de pescado nacional, sendo responsável por 38,7% do total, seguida pela aquicultura continental (38,0%), pesca extrativa continental (17,4%) e aquicultura marinha (~6%) (MPA, 2011).

Segundo o Ministério da Pesca e Aquicultura (MPA, 2015), a aquicultura é “o cultivo de organismos cujo ciclo de vida, em condições naturais, se desenvolve total ou parcialmente em meio aquático, equiparada à atividade agropecuária”. Dentre as modalidades da aquicultura temos a piscicultura, que compreende a criação de peixes em água doce ou marinha (MPA, 2015). As espécies mais comuns na atividade aquícola por região do Brasil são: norte (tambaqui, pirarucu, pirapitinga e outras); nordeste (tilápia e camarão marinho); centro-oeste (tambaqui, pacu e pintado); sudeste (tilápia, pacu e pintado); sul (carpas, tilápia, jundiá, ostras e mexilhões (EMBRAPA, 2015).

O Brasil apresenta um grande potencial para o desenvolvimento da aquicultura por possuir 8.400 quilômetros de costa marítima e 5,5 milhões de hectares em reservatórios de água doce. Além da disponibilidade de recursos hídricos, possui também clima favorável, disponibilidade de mão de obra e crescente demanda do mercado interno, o que faz com que a aquicultura esteja presente em todos os estados brasileiros (EMBRAPA, 2015).

A aquicultura é considerada pela Organização das Nações Unidas para Alimentação e Agricultura (FAO) a maneira mais rápida de produzir proteína animal, o que a torna indispensável para o combate à fome e suprimentos de alimentos em todo o mundo (EMBRAPA, 2015). Segundo a Organização Mundial de Saúde (OMS), o pescado é a proteína animal mais saudável e consumida no mundo. Os brasileiros ultrapassaram o consumo mínimo de pescado recomendado pela OMS, que é de 12 quilos por habitante ao ano. No Brasil, o consumo chega a 14,50 quilos por habitante/ano, de acordo com o levantamento feito em 2013 (MPA, 2015).

Segundo o Boletim Estatístico da Pesca e Aquicultura do MPA, no ano de 2011 a produção total da aquicultura nacional foi de 628.704,3 toneladas, representando 31,1% a mais em relação à produção de 2010. Quando se compara a produção atual com o montante produzido em 2009 (415.649,0 toneladas), houve um aumento de 51,2% na produção durante o triênio 2009-2011, evidenciando o crescimento do setor no país. A maior parcela da produção aquícola é oriunda da aquicultura continental, na qual se destaca a piscicultura continental representando 86,6% da produção total nacional. A produção aquícola de origem marinha, por sua vez, apesar de ter sofrido uma redução na participação da produção aquícola total nacional em relação aos anos anteriores (18,8% em 2009 contra 13,4% em 2011), vem se recuperando após uma queda da produção verificada na primeira metade da década de 2000 (MPA, 2011).

Ainda de acordo com o MPA foram produzidas em 2011 no Brasil 544.490 toneladas de peixes em água doce, sendo a tilápia (*Oreochromis niloticus*) a espécie mais produzida, com 253.824 toneladas. As regiões Sul, Sudeste e Nordeste são as responsáveis pela maior produção desta espécie. Os peixes redondos, mais conhecidos como tambaquis (*Colossoma macropomus*), pacus (*Piaractus mesopotamicus*), pirapitingas (*Piaractus brachypomus*) e seus híbridos são o segundo grupo de peixes mais cultivado no Brasil. Em 2011, a produção deste grupo chegou a 206.776 toneladas e seu cultivo está mais concentrado nas regiões Centro Oeste e Norte (MPA, 2011; PORTAL DO AGRONEGÓCIO, 2015).

Segundo dados do Instituto Brasileiro de Geografia e Estatística (IBGE), a produção total da piscicultura brasileira, em 2013, foi de 392.493 toneladas. A Região Centro-Oeste foi a principal produtora, com 105.010 toneladas de peixes (Figura 2). Em seguida, ficaram as Regiões Sul (88.063 toneladas, Nordeste (76.393 toneladas), Norte (72.969 toneladas) e Sudeste (50.058 toneladas) (IBGE, 2013).

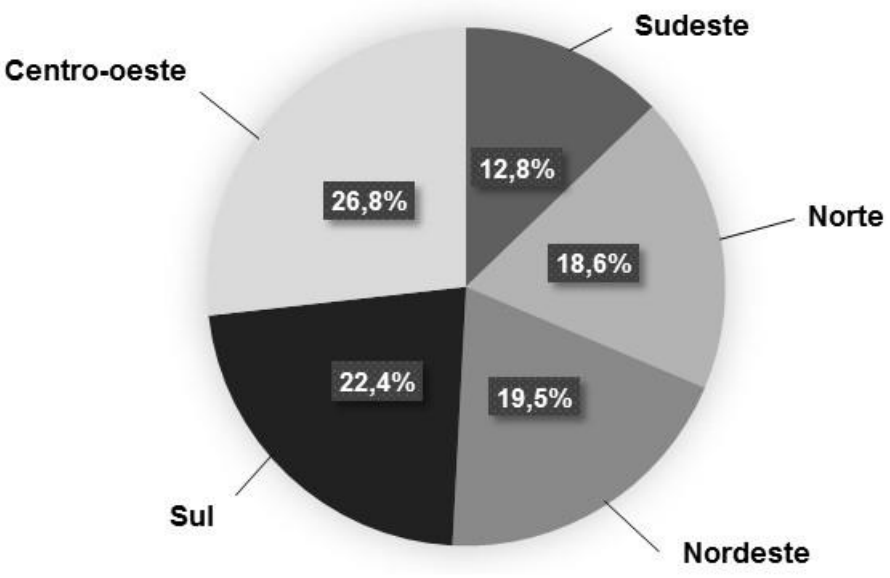


Figura 2. Distribuição percentual da produção de peixes, por grandes regiões – 2013. Fonte: IBGE (2013).

No ranking nacional da produção de peixes no ano de 2013, as cinco primeiras posições foram ocupadas por um representante de cada grande região, estando o estado de Mato Grosso na liderança, com 19,3% da despesca nacional, seguido do Paraná (13%), Ceará (7,8%), São Paulo (6,8%) e Rondônia (6,4%). Os estados de Minas Gerais (4%) e do Pará (1,3%) ficaram na 10ª e 19ª posições, respectivamente. A

espécie mais criada foi a tilápia (43,1% da produção de peixes no Brasil), seguida pelo tambaqui (22,6%) e pelo grupo tambacu e tambatinga (15,4%) (IBGE, 2013).

O crescimento da demanda nacional e também mundial pelo consumo de peixe, associado ao esgotamento de produção em áreas na Europa e nos Estados Unidos, tem feito a procura pelo alimento ser maior que a oferta. Apesar da grande capacidade produtiva brasileira, 30% dos pescados consumidos vêm de fora, especialmente da China e do Vietnã (EM, 2015).

1.1. O uso de antimicrobianos na piscicultura

Uma das principais ferramentas no controle e erradicação das enfermidades infecciosas de origem bacteriana em animais de produção é o uso de antimicrobianos (MARTIN & MORAGA, 1996).

Os antimicrobianos são empregados em medicina veterinária, na maioria das vezes, para fins de tratamento, controle e prevenção. Porém, apesar de proibido no Brasil e em vários outros países, alguns são usados com finalidade de ganho de peso. Cerca de metade dos antibióticos empregados na produção animal são de uso exclusivo em medicina veterinária e somente podem ser administrados depois de aprovados por órgãos oficiais. Para aprovação de novos medicamentos veterinários são feitos estudos quanto à dose, duração e carência do tratamento na espécie de interesse (GRANJA, 2004).

Uma grande preocupação para o desenvolvimento da piscicultura é o aparecimento de doenças infecciosas no sistema aquático, já que o controle microbiano nesses ambientes é complexo devido à dificuldade na coleta dos resíduos excretados pelos animais. Outra dificuldade se deve aos resíduos de ração que se dissolvem ou permanecem em suspensão na água, contribuindo para um aumento da matéria orgânica, diminuindo a qualidade da água e facilitando o desenvolvimento de micro-organismos. Além disso, existe uma maior concentração de animais por unidade de espaço quando comparados ao ambiente natural. Portanto, a aquicultura exige cuidados com o ambiente de criação e o manejo dos animais para evitar potenciais riscos e perdas na produção (TAVARES-DIAS et al., 2001; PASCHOAL, 2007; ORLANDO, 2013).

O uso de substâncias antimicrobianas como medida terapêutica e/ou preventiva dentro de um sistema de produção é uma das principais estratégias para o controle deste problema. Mesmo com o desenvolvimento de medidas de prevenção de doenças

através de melhorias no manejo e nas condições ambientais, o sistema intensivo de produção animal ainda depende do uso de antimicrobianos, sendo especialmente comum durante períodos em que os animais estão mais sujeitos a condições de estresse, como por exemplo, mudanças na dieta, transporte, entre outros (PASCHOAL, 2007).

As vias mais comuns de administração dos antimicrobianos na aquicultura são através do uso de ração contendo as substâncias (oral) e da adição direta dos antimicrobianos à água (terapia de imersão), sendo a via oral a mais rentável e, por isso a mais utilizada, misturando-se a dose apropriada do antimicrobiano à ração. A terapia de imersão é mais utilizada quando a maioria dos peixes não está comendo ou em casos de tratamento de infecções de pele, quando quantidades mais elevadas da droga são necessárias para atingir o resultado desejado, em comparação com os tratamentos orais (SAMANIDOU & EVAGGELOPOULOU, 2007; MONTEIRO, 2014).

Caso não seja respeitado o período de carência após a administração dos antimicrobianos, podem ser encontrados resíduos dos mesmos em produtos da aquicultura destinados à alimentação humana, podendo acarretar em riscos à saúde dos seres humanos, como reações alérgicas, toxicidade, alterações da microbiota intestinal e seleção de bactérias resistentes aos antimicrobianos (GIKAS et al., 2004; MONTEIRO, 2014). Além disso, a ocorrência de resíduos de antimicrobianos em peixes pode ser um problema para a exportação, o que acarretaria em perdas econômicas para o Brasil.

Dentre os antibióticos mais utilizados mundialmente na aquicultura encontram-se a tetraciclina, a oxitetraciclina, a flumequina, o ácido oxolínico e o florfenicol. No Brasil apenas o florfenicol e a oxitetraciclina são licenciados pelo MAPA para uso na aquicultura. Apesar disso, a utilização de antimicrobianos de forma inadequada e o uso de medicamentos proibidos são uma realidade em diversos sistemas de produção animal. Um exemplo é a enrofloxacina, uma fluoroquinolona desenvolvida para uso exclusivo em medicina veterinária, que possui amplo espectro de ação contra uma extensa classe de bactérias, incluindo aquelas resistentes à β-lactâmicos e sulfonamidas. Sabe-se que a enrofloxacina é largamente utilizada na piscicultura para o tratamento de doenças bacterianas em peixes, apesar de sua aplicação ser considerada ilegal, pois a mesma ainda não possui uso regulamentado no Brasil para organismos aquáticos (MOREIRA, 2012).

Diversos antibióticos foram banidos em vários países para uso em animais destinados ao consumo humano (Tabela 1). De acordo com a Agência Europeia de

Medicina (EMEA, 2000), alguns antibióticos não estão mais disponíveis para uso veterinário, como indicado na Tabela 2.

Tabela 1. Antibióticos proibidos para uso em animais destinados ao consumo humano

Antibiótico	País	Razão
Espectinomicina	Estados Unidos	Desenvolve resistência bacteriana
Enrofloxacina	Estados Unidos	Desenvolve resistência bacteriana (quinolona)
Cloranfenicol	Argentina, Canadá, União Europeia, Japão, Estados Unidos, Brasil	Induz anemia aplástica em humanos
Rifampicina	Sem registro nos Estados Unidos ou Canadá para uso em animais	Tumorgenicidade e teratogenicidade em animais experimentais

Fonte: Adaptado de FAO (2005).

Tabela 2. Antibióticos indisponíveis para uso com fins veterinários

Antibiótico	Indicação	Espécie	Alternativas
Cefuroxima	Tratamento de mastites clínicas, tratamento de infecções subclínicas	Bovino	Existem inúmeros medicamentos para tratamento de mastite
Cloranfenicol	Tratamento de infecções bacterianas (amplo espectro)	Bovinos, suínos e aves	Tianfenicol, Florfenicol, Amoxicilina
Sulfato de Polimixina B	Tratamento de mastite clínica causada por bactérias Gram (–)	Bovinos	Existem inúmeros medicamentos disponíveis para tratamento de mastite desta natureza
Nistatina	Tratamento de Candidíase	Aves	Natamicina

Fonte: Adaptado de FAO (2005).

A Tabela 3 apresenta os antibióticos utilizados na aquicultura em diversos países. Entre os agentes antimicrobianos comumente utilizados, vários são classificados pela Organização Mundial da Saúde (OMS) como criticamente importantes para utilização em medicina humana e, por isso, o uso destes medicamentos em animais destinados à produção de alimentos deve ser controlado ou evitado a fim de prevenir a disseminação de resistência a antimicrobianos (Tabela 4).

Tabela 3. Antibióticos usados na aquicultura em alguns países

País	Antibiótico	Indicação
Reino Unido	Oxitetraciclina, ácido oxolínico, amoxicilina, cotrimazina (trimetoprima-sulfadiazina)	Não mencionada
Noruega	Benzilpenicilina + diidroestreptomicina, florfenicol, flumequina, ácido oxolínico, oxitetraciclina, cotrimazina	Não mencionada
Estados Unidos (aprovados pelo FDA)	Sulfadimetoxina e ormetoprima	Controle de furunculose (*Aeromonas salmonicida*) em salmonídeos. Controle de septicemia entérica (*Edwadsiellla icttaluri*) em peixe-gato
Estados Unidos (aprovados pelo FDA)	Oxitetraciclina	Controle de furunculoses, septicemia hemorrágica bacterial e *Pseudomonas* em salmonídeos Controle de septicemia hemorrágica bacteriana em peixe-gato
México	Enrofloxacina, oxitetraciclina	Não mencionada
Brasil	Oxitetraciclina, florfenicol	Não mencionada

Fonte: Adaptado de FAO (2005).

Tabela 4. Principais agentes antimicrobianos utilizados em aquicultura e a sua importância na medicina humana

Agente antimicrobiano (classe de antibiótico)	Importância da classe (medicina humana)
Amoxicilina (penicilinas)	Elevada
Ampicilina (penicilinas)	Elevada
Cloranfenicol (anfenicóis)	Importante
Florfenicol (anfenicóis)	Importante
Eritromicina (macrolídeos)	Elevada
Estreptomicina, neomicina (aminoglicosídeos)	Elevada
Furazolidona (nitrofuranos)	Importante
Nitrofurantoína (nitrofuranos)	Importante
Ácido oxolínico (quinolonas)	Elevada
Enrofloxacina (fluoroquinolonas)	Elevada
Flumequina (fluoroquinolonas)	Elevada
Oxitetraciclina, clortetraciclina, tetraciclina (tetraciclinas)	Muito importante
Sulfonamidas	Importante

Fonte: Adaptado de GASTALHO et al. (2014).

2. ANTIMICROBIANOS

Segundo ZELENY et al. (2006), “medicamento veterinário é qualquer substância aplicada ou administrada a qualquer animal produtor de alimentos, com fins terapêuticos, profiláticos ou de diagnóstico, ou para modificar as funções fisiológicas, de comportamento ou como promotor de crescimento”.

Os antibióticos surgiram na década de 50 e contribuíram de forma importante para a redução do número de pessoas que sofriam ou morriam de enfermidades causadas por infecções bacterianas, pois são substâncias que inibem o crescimento de bactérias e de micro-organismos, interferindo em funções metabólicas essenciais (GRANJA, 2004). Devido à eficácia na prática terapêutica humana foram também introduzidos no tratamento veterinário (GUSTAFSON, 1991).

Os antimicrobianos são uma das melhores ferramentas no controle e erradicação das enfermidades infecciosas de origem bacteriana em animais de produção (MARTIN & MORAGA, 1996). Dentre as vias de administração aos animais, as principais são: intramuscular, intravenosa, subcutânea, oral e infusões intramamária e intrauterina (MITCHELL et al., 1998; MCEVOY et al., 2000).

Quase metade dos antibióticos empregados na produção animal são de uso exclusivo em medicina veterinária e devem ser aprovados por órgãos oficiais antes de serem usados. Essa aprovação depende da apresentação de resultados de estudos quanto à dose, duração e carência do tratamento na espécie de interesse (GRANJA, 2004).

Devido às práticas veterinárias e à criação intensiva é praticamente inevitável o surgimento de doenças nos animais criados para produção de alimentos, podendo trazer potenciais perdas econômicas. Por isso, a grande maioria desses animais recebe algum tipo de medicação para o tratamento de doenças infecciosas. Paralelamente à introdução de antibióticos na prática veterinária, vários pesquisadores começaram a investigar os efeitos adversos que a presença desses fármacos nos produtos destinados ao consumo humano poderia provocar (FAGHIHI, 1990; QUESADA, 2012).

2.1. Aspectos toxicológicos

O uso indiscriminado de drogas veterinárias, especialmente de antibióticos, em animais destinados à produção de alimentos representa um perigo potencial para a saúde humana, podendo levar a um aumento da resistência bacteriana e ao aparecimento de reações alérgicas aos antibióticos (GIKAS et al., 2004).

O aumento da resistência bacteriana pela ação de antibióticos se dá de forma indireta, ou seja, estes, na verdade, selecionam os micro-organismos previamente resistentes da microbiota. Limites Máximos de Resíduos são fixados para os antibióticos com base em estudos toxicológicos. Entretanto, mesmo abaixo do LMR,

estes resíduos podem ainda ter atuação sobre as bactérias, podendo modificar a microbiota intestinal dos consumidores, fato esse que pode levar à uma redução do LMR estabelecido (FRANCO et al., 1990; WHITE et al., 1993; MITCHELL et al., 1998).

O consumo de alimentos contendo resíduos de antibióticos pode também, em casos mais sérios, levar a quadros patológicos como a anemia aplástica causada por cloranfenicol, que é um antibiótico de uso proibido em animais para produção de alimentos. Além disso, esses resíduos podem também causar efeitos de sensibilização em consumidores (MILHAUD & PERSON, 1981; COSTA, 1996; MARTIN & MORAGA, 1996). Diversos países, entre eles os Estados Unidos, o Canadá, o Brasil e a União Europeia, proibiram ou restringiram o emprego de cloranfenicol em animais destinados ao consumo humano, principalmente devido ao fato de que a frequência da aparição dos sintomas de anemia aplástica não é dose-dependente, ou seja, qualquer dose ingerida da substância pode levar ao aparecimento da doença, além de a mesma se manifestar especialmente em indivíduos expostos à droga em mais de uma ocasião (STTEPANI, 1984; BRITO, 2000).

O uso de antibióticos em animais destinados ao consumo humano está a cada dia sendo mais controlado e monitorado por meio do controle das matérias-primas, dos intermediários, dos princípios ativos das drogas e também pelo controle dos resíduos que as drogas veterinárias podem deixar nos alimentos. Diversos países estão exigindo um programa de monitoramento de resíduos eficiente de seus exportadores e a comprovação, através de análises laboratoriais, de que os produtos estejam livres de contaminação por resíduos de antibióticos, entre outras substâncias. Caso não sejam atendidas as exigências, poderão surgir barreiras não tarifárias ao comércio dos produtos (GRANJA, 2004).

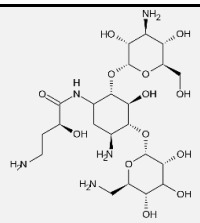
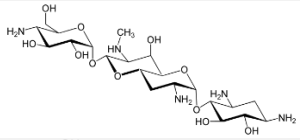
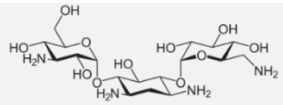
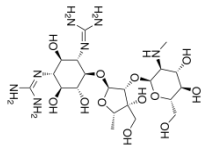
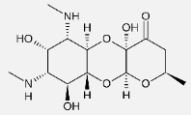
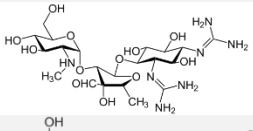
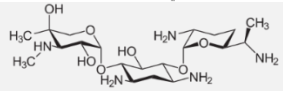
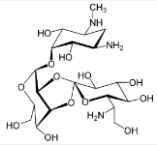
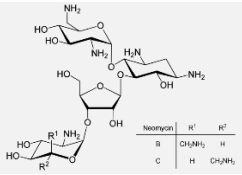
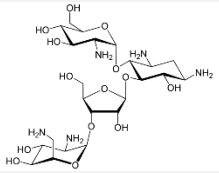
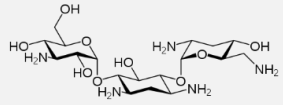
2.2. Aminoglicosídeos

Aminoglicosídeos (AG) são moléculas hidrofílicas constituídas por dois ou mais aminoaçúcares unidos por ligação glicosídica à hexose ou aminociclitol (Tabela 5). Estes inibem o crescimento de algumas bactérias gram-positvas e diversas gram-negativas aeróbicas e são substâncias de caráter básico, catiônicas e fortemente polares, sendo insolúveis em lipídeos (SANTOS, 2014; ARSAND, 2015).

A estreptomicina foi o primeiro AG descoberto, em 1944, durante a pesquisa de compostos solúveis em água e ativos estáveis contra bactérias gram-negativas a partir

de culturas de *Streptomyces griseus* e representou um grande avanço na medicina, já que esses compostos apresentavam atividade anti-tuberculose (MEJÍA, 2013).

Tabela 5. Informações químicas de alguns aminoglicosídeos

Analito, fórmula molecular e massa molar	**Formula estrutural**
Amicacina **$C_{22}H_{43}N_5O_{13}$** **585,53 g.mol^{-1}**	
Apramicina **$C_{21}H_{41}N_5O_{11}$** **539,58 g.mol^{-1}**	
Canamicina **$C_{18}H_{36}N_4O_{11}$** **484,50 g.mol^{-1}**	
Diidroestreptomicina **$C_{21}H_{41}N_7O_{12}$** **583,59 g.mol^{-1}**	
Espectinomicina **$C_{14}H_{24}N_2O_7$** **332,35 g.mol^{-1}**	
Estreptomicina **$C_{21}H_{39}N_7O_{12}$** **581,57 g.mol^{-1}**	
Gentamicina **$C_{21}H_{43}N_5O_7$** **477,60 g.mol^{-1}**	
Higromicina **$C_{20}H_{37}N_3O_{13}$** **527,53 g.mol^{-1}**	
Neomicina **$C_{23}H_{46}N_6O_{13}$** **614,64 g.mol^{-1}**	
Paramomicina **$C_{23}H_{47}N_5O_{18}S$** **615,63 g.mol^{-1}**	
Tobramicina **$C_{18}H_{37}N_5O_9$** **467,52 g.mol^{-1}**	

Os aminoglicosídeos são amplamente usados em animais de produção para o tratamento de infecções bacterianas ou promoção do crescimento, sendo suas doses terapêuticas próximas às tóxicas. Isto se deve ao baixo custo de produção, boa estabilidade química, baixo índice de reações alérgicas e, também, ao fato de ser uma das poucas classes de antimicrobianos que ainda possuem atividade contra a grande maioria das estirpes de resistência múltipla. O principal uso é na terapia de infecções, tais como a septicemia, infecções do trato respiratório e urinário, meningites em recém-nascidos, infecções oculares e infecção intra-abdominal causadas por bacilos aeróbicos gram-negativos (MEJÍA, 2013; SANTOS, 2014).

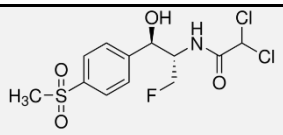
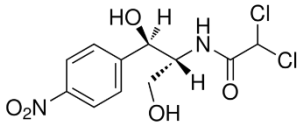
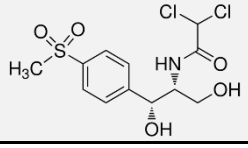
Os aminoglicosídeos mais usados em medicina veterinária são neomicina, gentamicina e estreptomicina. A apramicina e a diidroestreptomicina são de uso apenas veterinário, enquanto os demais aminoglicosídeos também são utilizados em humanos (ARSAND, 2015). Devido aos efeitos adversos como nefrotoxicidade e ototoxicidade e possibilidade de bloqueio neuromuscular, o uso de AG em animais destinados à produção de alimentos é limitado (MEJÍA, 2013).

2.3. Anfenicóis

O cloranfenicol (CAP) é um antibiótico de largo espectro da classe dos anfenicóis com excelentes propriedades antibacteriana e farmacocinética (OLIVEIRA et al., 2007). Ele foi isolado em 1947 de *Streptomyces* v*enezuelae* e tem sido utilizado desde 1950 para combater infecções em humanos (GIKAS et al., 2004). O CAP pode também ser produzido por síntese química (BOTSOUGLOU & FLETOURIS, 2001).

O tianfenicol (TAP) e o florfenicol (FF) são análogos ao cloranfenicol, diferindo pela presença de um grupo metilsulfônico no anel benzênico, enquanto o cloranfenicol apresenta um grupo nitroso (Tabela 6). Em relação à estrutura química, o florfenicol é derivado da molécula do tianfenicol e possui um maior espectro de ação devido à substituição do grupo hidroxila do carbono 3 por um átomo de flúor e pela substituição do grupo para-nitro por um radical metilsulfônico, o que faz com que diminua a possibilidade do aparecimento de anemia aplástica. A presença de um átomo de flúor na molécula do florfenicol impede a acetilação mediada pela enzima, fazendo com que cepas bacterianas resistentes ao cloranfenicol e ao tianfenicol se tornem sensíveis ao florfenicol (HIRD & KNIFTON, 1986). A alteração na estrutura química do tianfenicol e florfenicol diminui a possibilidade do aparecimento de anemia aplástica (CUNHA, 2009).

Tabela 6. Informações químicas dos anfenicóis

Analito, fórmula molecular e massa molar	Formula estrutural
Florfenicol **$C_{12}H_{14}Cl_2FNO_4S$** **358,21 g.mol^{-1}**	
Cloranfenicol **$C_{11}H_{12}Cl_2N_2O_5$** **323,13 g.mol^{-1}**	
Tianfenicol **$C_{12}H_{15}Cl_2NO_5S$** **356,22 g.mol^{-1}**	

Os anfenicóis são antibióticos bacteriostáticos e, por isso, inibem a síntese proteica dos micro-organismos sensíveis. Eles se ligam à subunidade 50S e interferem na formação do peptídeo ao bloquearem a enzima peptidiltransferase e impedirem o alongamento da cadeia polipeptídica (SPINOSA et al., 1999). O cloranfenicol atua principalmente sobre a medula óssea afetando o sistema hematopoiético. Os efeitos podem ser dose-dependentes - anemia, eucopenia e trombocitopenia – ou uma resposta idiossincrática manifestada pela anemia aplástica, levando muitas vezes à pancitopenia fatal. Um efeito adverso que pode ser causado pelos anfenicóis é a chamada síndrome do bebê cinzento em recém-nascidos, especialmente em prematuros, quando expostos à quantidade excessiva dos medicamentos. Os sintomas são acidose metabólica, respiração irregular e rápida e fezes líquidas de coloração esverdeada nas primeiras 24 horas (JECFA, 1999, CUNHA, 2009).

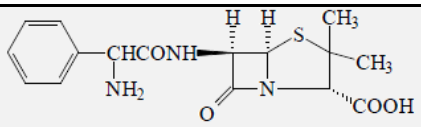
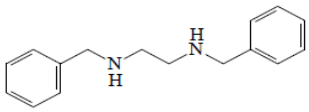
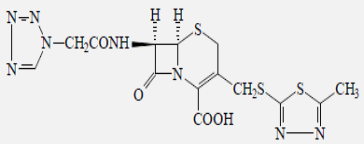
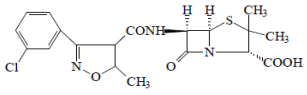
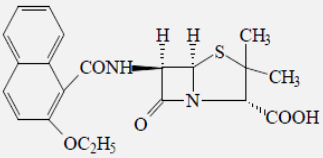
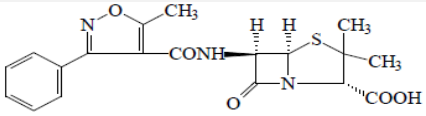
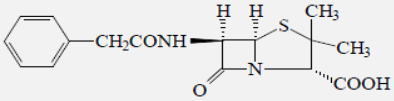
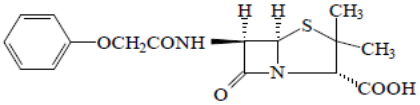
2.4. Beta-lactâmicos

Beta-lactâmicos (Tabela 7) são antibióticos que possuem em sua estrutura um anel azetidiona de quatro membros. Várias classes de compostos são consideradas como beta-lactâmicos, como as monobactamas, as cefalosporinas e as penicilinas. As monobactamas possuem o anel azetidiona sozinho e exibem atividade antibiótica. Já as penicilinas e as cefalosporinas possuem, ligado a este anel, um anel adicional de cinco membros e um anel de seis membros, respectivamente (MOREIRA, 2012). Eles possuem amplo espectro de atividade antibacteriana e eficácia clínica (GUIMARÃES et al., 2010). Os beta-lactâmicos foram os primeiros derivados de produtos naturais utilizados no tratamento terapêutico de infecções bacterianas, como é o caso da

penicilina, que ainda hoje, após várias décadas de sua descoberta, ainda contém os agentes mais comumente utilizados (GUIMARÃES et al., 2010).

O mecanismo de ação se dá através da inibição irreversível da enzima transpeptidase, que catalisa a reação de transpeptidação entre as cadeias de peptideoglicana da parede celular bacteriana. A transpeptidase age levando à formação de ligações cruzadas entre as cadeias peptídicas da estrutura peptideoglicana, que conferem à parede celular uma estrutura rígida importante para a proteção da célula bacteriana contra as variações osmóticas do meio (GUIMARÃES et al, 2010; MOREIRA, 2012).

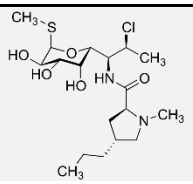
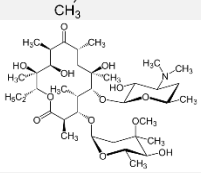
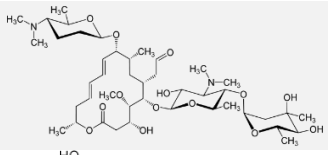
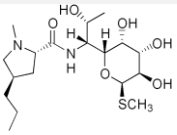
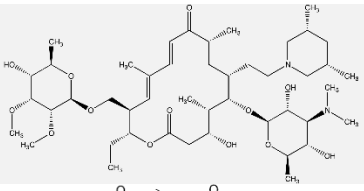
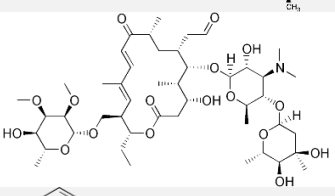
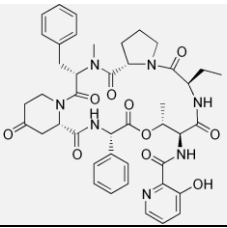
Tabela 7. Informações químicas de alguns beta-lactâmicos

Analito, fórmula molecular e massa molar	**Formula estrutural**
Ampicilina **$C_{16}H_{19}N_3O_4S$** **349,42 g.mol^{-1}**	
Benzatina **$C_{16}H_{20}N_2$** **240,34 g.mol^{-1}**	
Cefazolina **$C_{14}H_{14}N_8O_4S_3$** **454,50 g.mol^{-1}**	
Cloxacilina **$C_{19}H_{18}ClN_3O_5S$** **435,88 g.mol^{-1}**	
Naficilina **$C_{21}H_{22}N_2O_5S$** **414,48 g.mol^{-1}**	
Oxacilina **$C_{19}H_{19}N_3O_5S$** **401,44 g.mol^{-1}**	
Penicilina G **$C_{16}H_{18}N_2O_4S$** **334,40 g.mol^{-1}**	
Penicilina V **$C_{16}H_{18}N_2O_5S$** **350,39 g.mol^{-1}**	

2.5. Macrolídeos

Os macrolídeos (Tabela 8) são a segunda classe antibacteriana mais importante usada no tratamento humano depois dos beta-lactâmicos, utilizados principalmente em pacientes que são alérgicos às penicilinas (MINETTO, 2013).

Tabela 8. Informações químicas de alguns macrolídeos

Analito, fórmula molecular e massa molar	Formula estrutural
Clindamicina **$C_{18}H_{33}ClN_2O_5S$** **424,98 g.mol^{-1}**	
Eritromicina **$C_{37}H_{67}NO_{13}$** **733,92 g.mol^{-1}**	
Espiramicina **$C_{43}H_{74}N_2O_{14}$** **843,05 g.mol^{-1}**	
Lincomicina **$C_{18}H_{34}N_2O_6S$** **406,54 g.mol^{-1}**	
Tilmicosina **$C_{46}H_{80}N_2O_{13}$** **869,15 g.mol^{-1}**	
Tilosina **$C_{46}H_{77}NO_{17}$** **916,10 g.mol^{-1}**	
Virginiamicina **$C_{43}H_{49}N_7O_{10}$** **823,90 g.mol^{-1}**	

Eles são moléculas lipofílicas compostas por anel de lactona com 14, 15 ou 16 carbonos, ao qual se ligam um ou mais desoxi-glicóis. Em geral, os macrolídeos apresentam pKa entre 7,1 e 9,9 e alguns são sensíveis a baixo pH e sofrem degradação em condições ácidas. Os macrolídeos são produzidos por várias cepas de

Streptomyces e utilizados na prática veterinária contra bactérias gram-positivas, mas também em seres humanos contra várias doenças infecciosas (MOREIRA, 2012; SISMOTTO et al., 2013).

Esta classe de antibióticos possui ação bactericida ou bacteriostática, dependendo da concentração, da fase e do tipo de micro-organismos e se ligam de forma reversível à porção 50S do ribossomo, inibindo a síntese proteica e atuando sobre a translocação (MOREIRA, 2012).

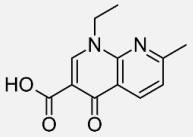
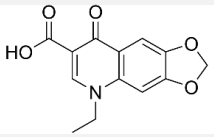
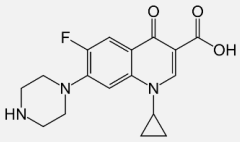
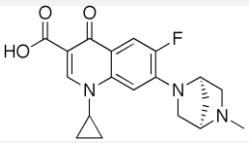
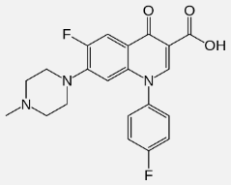
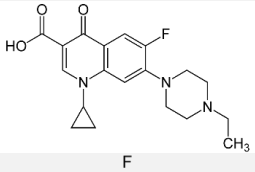
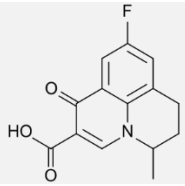
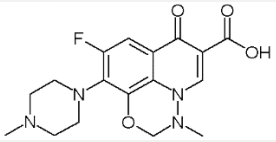
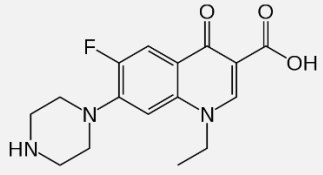
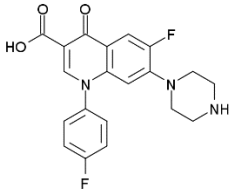
A eritromicina é um dos macrolídeos mais importantes e é produzida por uma cepa do *Streptomyces erythaeus* através de fermentação. A tilosina é produzida pelo *Streptomyces fradiae* e é ativa contra algumas bactérias Gram-positivas, Gram-negativas e micoplasmas Gram-positivos, com uso exclusivamente na medicina veterinária. Já a tilmicosina é um macrolídeo semissintético derivado da tilosina e apresenta espectro de ação similar a esta (SISMOTTO et al., 2013).

2.6. Quinolonas

Quinolonas e fluoroquinolonas (Tabela 9) são substâncias antibacterianas sintéticas pertencentes a um grupo de antibióticos derivados do ácido nalidíxico. Os compostos foram inicialmente aplicados no tratamento de infecções do trato urinário, mas agora tem uma aplicação de amplo espectro para o tratamento de doenças humanas e veterinárias (MARKMAN et al., 2005; MOREIRA, 2012).

As quinolonas inibem a duplicação e a transcrição do DNA, fazendo com que a síntese proteica não aconteça, tendo, portanto, efeito bactericida (MOREIRA, 2012). De uma forma geral, as quinolonas são classificadas em quatro gerações. As quinolonas originais como, por exemplo, ácido nalidíxico, ácido oxolínico, ácido pipemídico e cinoxacina são de primeira geração. Estas possuem baixa biodisponibilidade oral, distribuição limitada nos tecidos e limitado espectro de ação, restringindo-se a *Escherichia coli* e alguns organismos gram-negativos (CARRILLO, 2008).

Tabela 9. Informações químicas de algumas quinolonas

Analito, fórmula molecular e massa molar	Formula estrutural
Ácido nalidíxico **$C_{12}H_{12}N_2O_3$** **232,24 g.mol^{-1}**	
Ácido oxolínico **$C_{13}H_{11}NO_5$** **261,23 g.mol^{-1}**	
Ciprofloxacina **$C_{17}H_{18}FN_3O_3$** **331,35 g.mol^{-1}**	
Danofloxacina **$C_{19}H_{20}FN_3O_3$** **357,37 g.mol^{-1}**	
Difloxacina **$C_{21}H_{19}F_2N_3O_3$** **399,39 g.mol^{-1}**	
Enrofloxacina **$C_{19}H_{22}FN_3O_3$** **359,40 g.mol^{-1}**	
Flumequina **$C_{14}H_{12}FNO_3$** **261,26 g.mol^{-1}**	
Marbofloxacina **$C_{17}H_{19}FN_4O_4$** **362,37 g.mol^{-1}**	
Norfloxacina **$C_{16}H_{18}FN_3O_3$** **319,33 g.mol^{-1}**	
Sarafloxacina **$C_{20}H_{17}F_2N_3O_3$** **385,36 g.mol^{-1}**	

A segunda geração de quinolonas apresenta um aumento da atividade antibacteriana contra *Enterobacteriaceae* e bactérias gram-negativas e gram-positivas. As fluoroquinolonas (FQs) derivam das quinolonas de 1ª geração e a adição de um átomo de flúor na posição 6 e do grupo piperazil na posição 7 nas fluoroquinolonas aumenta a potência e o espectro antimicrobiano com relação às quinolonas de 1ª geração; inclusive para bactérias resistentes (CENTENO, 2010). São quinolonas de segunda geração: norfloxacina (NOR), ciprofloxacina (CIP), enrofloxacina (ENR), danofloxacina, difloxacina e marbofloxacina, entre outras (CARRILLO, 2008).

A NOR foi a primeira FQ que surgiu e também a primeira a ser utilizada como antibiótico em medicina humana. Ela é utilizada em tratamentos de doenças respiratórias, biliares e infecções do trato urinário e apresenta boa distribuição nos tecidos e boa disponibilidade após administração. A enrofloxacina é a FQ mais utilizada em medicina veterinária e surgiu no mercado em 1988. Ela possui grande atividade antibacteriana e bactericida contra bactérias patogênicas encontradas em animais e abrange a maioria dos gram-negativos e muitos gram-positivos. Além disso, a enrofloxacina apresenta uma boa capacidade de penetração em fluidos e tecidos e tem sido utilizada em medicina veterinária em cães, gatos, bovinos, suínos e aves. A ciprofloxacina é um dos principais metabólitos da enrofloxacina e é amplamente usada na medicina humana, sendo proibido o seu uso em animais. Ela foi introduzida no mercado em 1987 e possui amplo espectro de atividade antibacteriana, boa biodisponibilidade após administração e boa distribuição nos tecidos (GOMES, 2013).

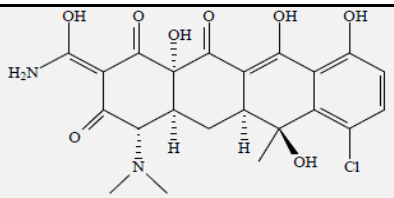
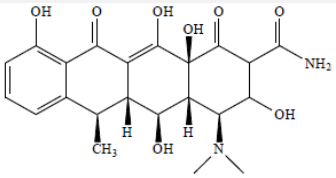
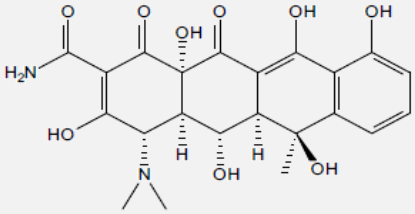
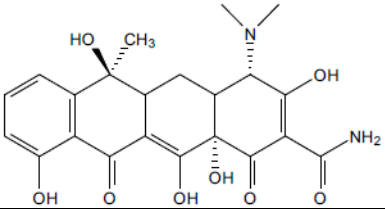
A terceira geração de quinolonas mantém as características favoráveis da segunda geração, entretanto há um aumento da atividade contra bactérias gram-positivas, anaeróbias e micobactérias. As quinolonas deste grupo apresentam excelente biodisponibilidade oral, tempo de semivida prolongado e menor toxicidade sobre o sistema nervoso central. Levofloxacina, grepafloxacina e sparfloxacina são exemplos de quinolonas de terceira geração (CARRILLO, 2008).

A quarta geração de quinolonas mantém o bom espectro de ação contra bactérias gram-negativas, gram-positivas e melhora a sua ação contra os anaeróbios. Dentre as quinolonas de quarta geração temos trovafloxacina, moxifloxacina e gatifloxacina, entre outras (GOMES, 2013).

2.7. Tetraciclinas

As tetraciclinas (Tabela 10) são antibióticos de amplo espectro de ação, baixa toxicidade e baixo custo produzidos por diversas espécies de *Streptomyces spp*, sendo também algumas semissintéticas. Na maioria dos casos, podem ser administradas por via oral. Estas têm sido utilizadas indiscriminadamente, o que tem levado ao aparecimento de resistência em um grupo variado de bactérias, principalmente às tetraciclinas de primeira geração, descobertas no período compreendido entre 1950 e 1970. O uso indiscriminado tem provocado restrições na utilidade clínica destes compostos, mas ainda são bastante úteis na clínica médica e têm sido usadas no tratamento de diversos tipos de infecções. As tetraciclinas são também muito utilizadas no tratamento de infecções e na promoção do crescimento em animais, inclusive nos produtores de alimentos (PEREIRA-MAIA et al., 2010).

Tabela 10. Informações químicas de algumas tetraciclinas

Analito, fórmula molecular e massa molar	**Formula estrutural**
Clortetraciclina **$C_{22}H_{23}N_2ClO_8$** **478,88 g.mol^{-1}**	
Doxiciclina **$C_{22}H_{24}N_2O_8$** **444,40 g.mol^{-1}**	
Oxitetraciclina **$C_{22}H_{24}N_2O_9$** **460,43 g.mol^{-1}**	
Tetraciclina **$C_{22}H_{24}N_2O_8$** **478,88 g.mol^{-1}**	

O mecanismo de ação das tetraciclinas ocorre através da ligação a um sítio na subunidade 30S do ribossomo bacteriano, que impede a ligação do aminoacil-t-RNA no sítio A do ribossomo, a adição de aminoácidos e, consequentemente, impedindo a síntese proteica (PEREIRA-MAIA et al., 2010; MEDLEY, 2012).

Tetraciclinas são considerados fármacos seguros por não apresentarem efeitos colaterais severos. Geralmente, os efeitos colaterais mais comuns são náuseas, vômitos e diarreia. Como as tetraciclinas são depositadas nos ossos e dentes durante a calcificação, seu uso pode levar a uma descoloração dos dentes e a uma inibição do crescimento ósseo em crianças, fato que restringe a administração dessas drogas a mulheres grávidas e crianças em fase de crescimento (PEREIRA-MAIA et al., 2010).

2.8. Sulfonamidas

As sulfonamidas (Tabela 11), também conhecidas como sulfas, foram testadas pela primeira vez nos anos 1930 como fármacos antibacterianos e fazem parte de um importante grupo de antimicrobianos sintéticos, que têm sido usados efetivamente no combate às infecções bacterianas e também na prática veterinária para promover o crescimento animal. Embora estes compostos possam ser utilizados na medicina humana contra uma grande variedade de micro-organismos, seu principal uso é destinado ao tratamento de infecções do trato urinário. O sulfametoxazol, em associação com o trimetoprima, é utilizado para o tratamento de pacientes com infecções no trato urinário e também para pacientes portadores do vírus HIV que apresentam infecções por *Pneumocystis carinii* (GUIMARÃES et al., 2010).

O termo sulfonamida é utilizado para referir-se aos derivados do para-aminobenzeno-sulfonamida (sulfanilamida). As sulfas são análogos estruturais e antagonistas competitivos do ácido para-aminobenzoico (PABA) e impedem a sua utilização pelas bactérias na síntese do ácido fólico ou vitamina B9. Mais especificamente, as sulfonamidas são inibidores competitivos da di-hidropteroato-sintetase, a enzima bacteriana responsável pela incorporação do PABA no ácido di-hidropteroico, precursor imediato do ácido fólico. Os micro-organismos sensíveis são aqueles que precisam sintetizar seu próprio ácido fólico; as bactérias capazes de utilizar o folato pré-formado não são afetadas (SANTOS et al., 2011).

Tabela 11. Informações químicas de algumas sulfonamidas

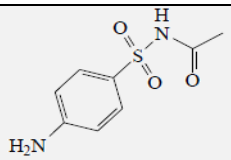
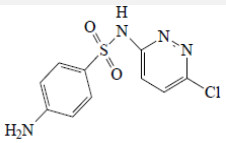
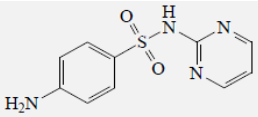
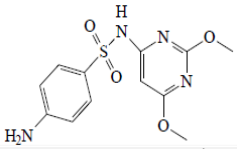
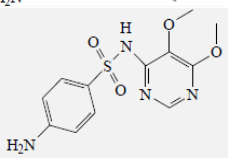
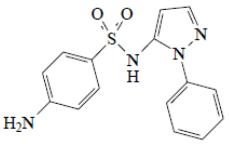
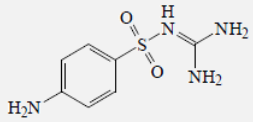
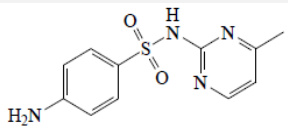
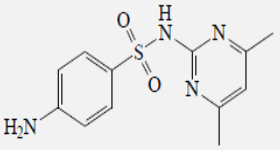
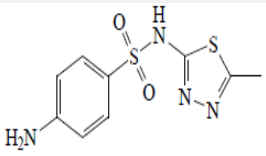
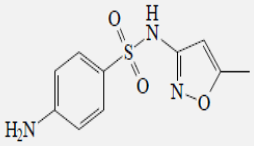
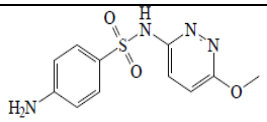
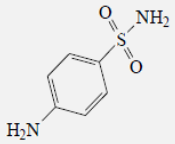
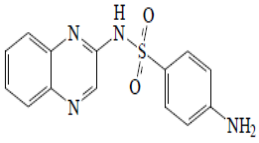
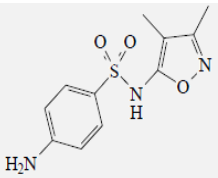
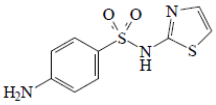
Analito, fórmula molecular e massa molar	Formula estrutural
Sulfacetamida **$C_8H_{10}N_2O_3S$** **214,24 g.mol^{-1}**	
Sulfaclorpiridazina **$C_{10}H_9ClN_4O_2S$** **284,74 g.mol^{-1}**	
Sulfadiazina **$C_{10}H_{10}N_4O_2S$** **250,28 g.mol^{-1}**	
Sulfadimetoxina **$C_{12}H_{14}N_4O_4S$** **310,33 g.mol^{-1}**	
Sulfadoxina **$C_{12}H_{14}N_4O_4S$** **310,33 g.mol^{-1}**	
Sulfafenazol **$C_{15}H_{14}N_4O_2S$** **314,36 g.mol^{-1}**	
Sulfaguanidina **$C_7H_{10}N_4O_2S$** **214,24 g.mol^{-1}**	
Sulfamerazina **$C_{11}H_{12}N_4O_2S$** **264,31 g.mol^{-1}**	
Sulfametazina **$C_{12}H_{14}N_4O_2S$** **278,32 g.mol^{-1}**	
Sulfametizol **$C_9H_{10}N_4O_2S$** **270,33 g.mol^{-1}**	
Sulfametoxazol **$C_{10}H_{11}N_3O_3S$** **253,31 g.mol^{-1}**	

Tabela 11. (continuação...)

Analito, fórmula molecular e massa molar	Formula estrutural
Sulfametoxipiridazina **$C_{11}H_{12}N_4O_3S$** **280,32 g.mol^{-1}**	
Sulfanilamida **$C_6H_8N_2O_2S$** **172,21 g.mol^{-1}**	
Sulfaquinoxalina **$C_{14}H_{12}N_4O_2S$** **300,37 g.mol^{-1}**	
Sulfisoxazol **$C_{11}H_{13}N_3O_3S$** **267,30 g.mol^{-1}**	
Sulfatiazol **$C_9H_9N_3O_2S_2$** **255,32 g.mol^{-1}**	

As sulfonamidas são amplamente usadas para fins profiláticos e terapêuticos em animais produtores de alimento, podendo também atuar como substâncias promotoras do crescimento. Entretanto, elas possuem caráter carcinogênico e podem levar ao desenvolvimento de resistência aos antibióticos nos seres humanos. Portanto, resíduos destes compostos em alimentos são motivo de preocupação para as autoridades sanitárias (MOREIRA, 2012).

3. OCORRÊNCIA DE RESÍDUOS DE ANTIMICROBIANOS EM PEIXE

O uso indiscriminado e incorreto de antimicrobianos para tratamento de animais, bem como o não cumprimento do período de carência são motivos pelos quais pode-se encontrar resíduos de antimicrobianos em alimentos de origem animal.

Internacionalmente, existem alguns estudos sobre a ocorrência de resíduos de antimicrobianos em peixes, principalmente na Grécia e na Espanha. DASENAKI & THOMAIDIS (2015) encontraram duas quinolonas - flumequina (4,6 µg.kg^{-1}) e enrofloxacina (4,8 µg.kg^{-1}) em amostras de dourado e robalo da Grécia. Já EVAGGELOPOULOU & SAMANIDOU (2013a e 2013b) analisaram 20 amostras de

dourado do mercado da Grécia quanto à presença de ampicilina, penicilina G, penicilina V, oxacilina, cloxacilina, dicloxacilina, tianfenicol, florfenicol e cloranfenicol e 10 amostras de salmão quanto à presença de resíduos de sete quinolonas (ciprofloxacina, danofloxacina, enrofloxacina, sarafloxacina, ácido oxolínico, ácido nalidíxico e flumequina) e não encontraram nenhuma amostra positiva.

Vários trabalhos analisaram resíduos de antimicrobianos em peixes da Espanha. BERRADA et al. (2008) analisaram 6 amostras de truta e dourado quanto à presença de macrolídeos e três amostras de dourado foram positivas para eritromicina A (58-87 $\mu g.kg^{-1}$). COSTI et al. (2010) analisaram peixes de aquicultura (salmão, truta, robalo, dourado entre outros) quanto à presença de flumequina e de ácido oxolínico e não encontraram amostras positivas para flumequina; apenas uma amostra foi positiva para ácido oxolínico (37 ± 2 $\mu g.kg^{-1}$). DORIVAL-GARCÍA et al. (2015) analisaram oito amostras de peixe quanto à presença de 17 quinolonas. Apenas seis dos antibióticos estudados não foram encontrados nas amostras. Os antibióticos encontrados em maiores concentrações em todas as amostras foram ciprofloxacina (836 $ng.g^{-1}$), ofloxacina (719 $ng.g^{-1}$) e enrofloxacina (674 $ng.g^{-1}$). Já RAMBLA-ALEGRE et al. (2010) analisaram a ocorrência de quinolonas em vários tipos de peixe e não encontraram nenhuma amostra positiva. No estudo de MENDOZA et al. (2012) foram analisadas 107 amostras de bagres. Dezesseis amostras foram positivas no método microbiológico de detecção de antibióticos e analisadas por CL-EM/EM. Os antibióticos que predominaram nas amostras positivas foram as tetraciclinas (especialmente tetraciclina – 3,9 a 80,8 $\mu g.kg^{-1}$ – e oxitetraciclina – 6,4 a 8,2 $\mu g.kg^{-1}$). Foram encontradas três sulfonamidas nas amostras positivas, sendo a sulfadimetoxina a predominante. Todos os antibióticos estavam em concentrações abaixo do LMR estabelecido pela União Europeia.

No Brasil, poucos estudos de ocorrência de antimicrobianos em peixes brasileiros foram encontrados na literatura e, com exceção de um trabalho, todos analisaram peixes oriundos do Estado de São Paulo, Brasil. Portanto, não existem informações acerca da ocorrência de antimicrobianos em peixes dos Estados de Minas Gerais e do Pará.

ORLANDO (2013) analisou 26 amostras de tilápia do estado de São Paulo e encontrou apenas uma amostra com resíduos de oxitetraciclina numa concentração de 42 ± 8,4 $ng.g^{-1}$. SISMOTTO et al. (2014) analisaram 20 amostras de tilápia do mercado do Estado de São Paulo quanto à presença de resíduos de macrolídeos e não encontraram amostras com níveis detectáveis dos antibióticos. QUESADA (2012)

analisou 31 amostras de peixes frescos (pacu e tilápia) do Estado de São Paulo quanto à presença de fluoroquinolonas e nenhuma delas apresentou resultado positivo. MONTEIRO et al. (2015) analisaram 12 antibióticos (cloranfenicol, florfenicol, oxitetraciclina, tetraciclina, clortetraciclina, sulfadimetoxina, sulfatiazol, sulfametazina, enrofloxacina, ciprofloxacina, norfloxacina e sarafloxacina) em 36 amostras de tilápia do Estado de São Paulo. Oxitetraciclina, tetraciclina e florfenicol foram encontrados nas amostras. Oxitetraciclina foi a molécula mais detectada (9 amostras; 15,6 – 1231.8 $\mu g.kg^{-1}$) e algumas amostras apresentaram concentração acima do LMR da União Europeia (EMEA, 2013), 100 $\mu g.kg^{-1}$, e também acima do valor de referência adotado pelo governo brasileiro (BRASIL, 2015), 200 $\mu g.kg^{-1}$. Tetraciclina e florfenicol foram detectados em três amostras (521,2 - 528,0 $\mu g.kg^{-1}$) em valores abaixo de LMR fixado pelos governos europeu e brasileiro (BRASIL, 2015; EMEA, 2015). BARRETO et al. (2012) analisaram 21 amostras de peixes obtidas do Serviço de Inspeção Federal e não encontraram resíduos de cloranfenicol em nenhuma das amostras.

Anualmente, o MAPA publica os resultados do Plano Nacional de Controle de Resíduos e Contaminantes (PNCRC) em alimentos de origem animal. Do ano de 2006 até o ano de 2014, 100% das amostras de peixe (geralmente de 60 a 75 amostras) de cultivo, analisadas de diversas regiões do Brasil, estavam em conformidade com a legislação vigente para os contaminantes analisados (ácido oxolínico; difloxacina; flumequina; ácido nalidíxico; sarafloxacina; ciprofloxacina; enrofloxacina; florfenicol; cloranfenicol; tianfenicol, sulfadimetoxina; sulfatiazol; sulfametazina; clortetraciclina; oxitetraciclina; tetraciclina). Isso demonstra que os produtores estão respeitando os períodos de carência para os antibióticos em peixes de cultivo, mas ainda assim podem existir antibióticos não previstos na análise realizada pelo MAPA que estejam sendo usados de forma ilegal na aquicultura.

4. CONTROLE DE RESÍDUOS E CONTAMINANTES EM ALIMENTOS

O controle de resíduos de antimicrobianos em alimentos destinados ao consumo humano é extremamente importante para garantia da segurança alimentar. Por isso, importantes órgãos internacionais têm estabelecido legislações relacionadas ao controle destes resíduos, como por exemplo a União Europeia (EC, 2010a) e o *Codex Alimentarius* (CODEX, 2015).

4.1. Controle de resíduos de antimicrobianos no Brasil

Em 1979 foi criado no Brasil o Programa Nacional de Controle de Resíduos Biológicos em Carne - o PNCRBC (Portaria Ministerial número 86 de 26/01/1979) pelo MAPA, que tinha como finalidade sistematizar o controle de resíduos em produtos cárneos. O programa visava a obtenção de informações sobre a ocorrência dos diversos resíduos em animais abatidos em estabelecimentos sob Inspeção Federal e a distribuição das ocorrências por região de origem dos animais (PORFÍRIO, 1994).

Este programa inicial foi ampliado e, em 1986, o Plano Nacional de Controle de Resíduos em Produtos de Origem Animal foi instituído para controlar os resíduos de compostos usados na agropecuária e os poluentes ambientais em carne (BRASIL, 1986), leite, mel, pescado e seus derivados (BRASIL, 1999).

O Plano Nacional de Controle de Resíduos em Contaminantes (PNCRC) tem como função básica, o controle e a vigilância de resíduos de contaminantes em alimentos de origem animal e suas ações estão direcionadas para conhecer e evitar a violação dos níveis de segurança ou dos LMRs de substâncias autorizadas, bem como a ocorrência de quaisquer níveis de resíduos de compostos químicos de uso proibido no país. Para isto, são colhidas amostras de animais abatidos e vivos, de derivados industrializados e/ou beneficiados, destinados a alimentação humana, provenientes dos estabelecimentos sob Inspeção Federal (SIF). Atualmente, o que rege o PNCRC é a Instrução Normativa SDA nº 13, de 15 de julho de 2015 (BRASIL, 2015), que aprovou os Programas de Controle de Resíduos e Contaminantes em carnes, leite, mel, ovos e pescado para o exercício de 2015. Em 2016 não foi publicado um novo escopo do PNCRC.

O Programa Nacional de Controle de Resíduos e Contaminantes em Pescado (PNCRCP) objetiva garantir a integridade e a segurança do pescado no território nacional, em relação à contaminação por resíduos de substâncias nocivas destes alimentos, oriundos da aplicação de drogas veterinárias e contaminantes ambientais.

O PNCRC/Animal é um programa de inspeção e fiscalização oficial, baseado em análise de risco, que objetiva verificar e avaliar as boas práticas agropecuárias (BPA), as boas práticas de fabricação (BPF) e os autocontroles implementados ao longo das etapas das cadeias agroalimentares. Além disso, verifica também os fatores de qualidade e de segurança higiênico-sanitárias dos produtos de origem animal, seus subprodutos e derivados de valor econômico nacionais ou importados, por meio do gerenciamento e controle dos perigos e riscos químicos e microbiológicos que

potencialmente promovam riscos. Com isso, evidencia as garantias de sistema quanto à segurança e à inocuidade dos alimentos fornecidos aos consumidores e certifica que estes sejam equivalente aos requisitos sanitários internacionalmente reconhecidos (MAPA, 2015).

O Programa Nacional de Controle de Resíduos e Contaminantes em Pescado (PNCRCP) objetiva garantir a integridade e segurança do pescado no território nacional, em relação à contaminação por resíduos de substâncias nocivas destes alimentos, oriundos da aplicação de drogas veterinárias e contaminantes ambientais.

Na Tabela 12 está apresentado um comparativo entre os Limites Máximos de Resíduos (LMRs) estabelecidos pelo MAPA através do PNCRC de pescado e os LMRs estabelecidos por outros órgãos internacionais.

Tabela 12. Limites Máximos de Resíduos (LMRs) estabelecidos para antimicrobianos em músculo de peixe pelo MAPA através do PNCRC de pescado e os LMRs estabelecidos por outros órgãos internacionais

Classe	Analito	BRASIL (2015) ($\mu g.kg^{-1}$)	CODEX (2014) ($\mu g.kg^{-1}$)	EC (2010a) ($\mu g.kg^{-1}$)
Sulfonamidas	Sulfatiazol	Soma igual a	-	Soma igual a 100
	Sulfametazina	100	-	
	Sulfadimetoxina		-	
	Sulfaclorpiridazina	-	-	
	Sulfadiazina	-	-	
	Sulfadoxina	-	-	
	Sulfamerazina	-	-	
	Sulfametoxazol	-	-	
	Sulfaquinoxalina	-	-	
Aminoglicosídeos	Espectinomicina	-	-	300
	Canamicina	-	-	-
	Neomicina	-	-	500
	Paramomicina	-	-	500
Beta-lactâmicos	Ampicilina	-	-	50
	Amoxicilina	-	-	50
	Cloxacilina	-	-	300
	Dicloxacilina	-	-	300
	Oxacilina	-	-	300
	Benzilpenicilina	-	-	50
	Penicilina G	-	-	-
	Penicilina V	-	-	-
Nitrofuranos	Nitrofurazona –SEM	1	-	Proibidos
	Furaolidona – AOZ	1	-	
	Furaltadona – AMOZ	1	-	
	Nitrofurantoina – AHD	1	-	
Quinolonas	Ácido Oxolínico ***	20	-	-
	Ácido Nalidíxico ***	20	-	-
	Ciprofloxacina (e)	Soma igual a	-	-
	Enrofloxacina (e)	100	-	100
	Sarafloxacina ***	30	-	-
	Danofloxacina	-	-	100
	Difloxacina ***	300	-	300
	Flumequina	600	500 (truta)	600

Tabela 12. (continuação...)

Classe	Analito	BRASIL (2015) ($\mu g.kg^{-1}$)	CODEX (2014) ($\mu g.kg^{-1}$)	EC (2010a) ($\mu g.kg^{-1}$)
Macrolídeos	Eritromicina	-	-	200
	Lincomicina	-	-	100
	Tilmicosina	-	-	50
	Tilosina	-	-	100
Anfenicóis	Cloranfenicol	0,30	-	Proibido
	Tianfenicol	50	-	50
	Florfenicol	1000	-	1000 (peixe de barbatana)
Tetraciclinas	Oxitetraciclina (a)	Soma igual a	Soma igual a	100
	Clortetraciclina (a)	200	200	100
	Tetraciclina (a)			-
Outros	Colistina	-	-	150
	Trimetoprima	-	-	50

Legenda: '-': não mencionado.

4.2. Controle de resíduos de antimicrobianos na União Europeia

O Regulamento EEC 2377/90 (EC, 1999) foi publicado em 1990 com o intuito de constituir um processo comum para o estabelecimento de LMR de antimicrobianos em alimentos de origem animal. Neste regulamento foram estabelecidas quatro classes para as substâncias farmacologicamente ativas, com base na avaliação científica da sua segurança: anexo I - substâncias para as quais se encontrava estabelecido um LMR); anexo II - substâncias para as quais não era necessário estabelecer um LMR; anexo III - substâncias para as quais foi estabelecido um LMR provisório; e o anexo IV - substâncias para as quais não foi possível estabelecer um LMR devido ao fato de os resíduos das substâncias constituírem um risco para a saúde humana, independente do valor do limite (MOREIRA, 2012).

Na diretiva 96/23/CE (EC, 1996) foram publicadas as medidas de controle a serem aplicadas a certas substâncias e aos resíduos em animais vivos e respectivos produtos. Apenas em 2002 foi publicada a Diretiva 2002/657/CE (EC, 2002) que dá execução ao disposto na Diretiva 96/23/CE relativo ao desempenho de métodos analíticos e a interpretação de resultados (MOREIRA, 2012).

Em 2010, a União Europeia publicou o Regulamento 37/2010 cuja finalidade foi integrar as substâncias farmacologicamente ativas e sua respectiva classificação no que diz respeito ao Limite Máximo de Resíduo nos alimentos de origem animal. Além disso foi adicionada a informação sobre a classificação terapêutica. Por motivos de facilidade de utilização, todas as substâncias farmacologicamente ativas foram ordenadas alfabeticamente em uma lista, num anexo único, em dois quadros separados: um para as substâncias permitidas, enumeradas nos anexos I, II e III do

Regulamento (CEE) no. 2377/90, e outro para as substâncias proibidas, constantes no anexo IV (EC, 2010a; MOREIRA, 2012).

O último banco de dados publicado pela comissão do *Codex Alimentarius* em sua 38º Sessão dispõe sobre os Limites Máximos de Resíduos (LMR) e as recomendações de gerenciamento de riscos para diversos medicamentos veterinários em alimentos (CODEX, 2015).

5. MÉTODOS DE ANÁLISE DE ANTIMICROBIANOS EM ALIMENTOS

Diferentes métodos analíticos foram desenvolvidos para a determinação de resíduos de antimicrobianos em alimentos. Geralmente são necessários dois passos principais durante a análise: o preparo da amostra (que pode incluir a extração, a purificação e a concentração) seguido da etapa de separação e de detecção dos analitos de interesse (GUIDI et al., 2017).

5.1. Preparo de amostra

Em alimentos, as concentrações de resíduos e contaminantes são geralmente baixas e a matriz complexa para que as análises dessas substâncias sejam realizadas sem uma etapa prévia de preparo da amostra. Na maior parte das vezes, os componentes da matriz interferem negativamente na resposta analítica, gerando resultados pouco precisos. A fim de minimizar esse problema, o preparo da amostra tem como principal objetivo promover o fracionamento e a concentração da mesma, com todos os analitos de interesse, deixando-os o mais livre possível das interferências provenientes dos componentes da matriz, que certamente estarão no extrato. As etapas mais comuns de preparo da amostra são a extração, a purificação e a pré-concentração, obtendo os analitos em um meio mais apropriado e em concentrações adequadas para a análise no sistema CL-EM/EM. Deve-se ter cuidado durante a realização dessas etapas, pois qualquer perda ocorrida nessa fase não poderá ser recuperada posteriormente.

Os procedimentos analíticos mais comumente utilizados são: a extração líquido-líquido (LLE, do inglês, *liquid-liquid extraction*) e a extração sólido-líquido (SLE, do inglês, *solid-liquid extraction*). Eles possuem diversas limitações, tais como: exigem muito trabalho, são demorados, onerosos em termos de materiais e volumes de

solventes e muitas vezes não podem ser concluídos antes que os produtos sejam colocados no mercado (CACHO et al., 2003).

Visando contornar essas limitações e melhorar a eficiência dos métodos de extração, vários procedimentos de extração e de purificação (*clean up)* têm sido desenvolvidos para o preparo de amostras de alimentos. Entre eles, pode-se citar: extração em fase sólida (SPE, do inglês, *solid phase extraction*) (YANG et al., 2011), extração em fase sólida dispersiva (d-SPE, do inglês, *dispersive solid phase extraction*) (DAGNAC et al., 2009), dispersão da matriz em fase sólida (MSPD, do inglês, *matrix solid phase dispersion*) (DÓREA & LOPES, 2004), microextração por sorvente empacotado (MEPS, do inglês, *micro-extraction by packed sorbent*) (ABDEL-REHIM, 2010), microextração líquido-líquido dispersiva (DLLME, do inglês, *dispersive liquid-liquid micro-extraction*) (CHEN et al., 2009) e extração QuEChERS (do inglês, *Quick, Easy, Cheap, Effective, Ruged and Safe*) (WILKOWSKA & BIZIUK, 2011) cujo codinome significa ‘Rápido, Fácil, Barato, Efetivo, Robusto e Seguro’.

A escolha do melhor procedimento deve levar em consideração a praticidade, o custo e a toxicidade dos solventes. Em análises de rotina, um processamento rápido de numerosas amostras é desejado. Para isto é necessário o desenvolvimento de métodos eficientes, rápidos e ambientalmente corretos.

5.2. Técnicas de separação e determinação de antimicrobianos em alimentos

Devido à complexidade das matrizes de alimentos (mistura de água, proteínas, lipídios, carboidratos, vitaminas e minerais), além do preparo intensivo da amostra, é necessário o acoplamento de técnicas analíticas para obtenção de maior seletividade e detectabilidade.

A cromatografia é um método físico-químico de separação fundamentado na migração diferencial dos componentes de uma mistura, que ocorre devido a diferentes interações, entre duas fases imiscíveis, a fase estacionária e a fase móvel. Ela é uma técnica com vasta gama de aplicações por permitir uma variedade de combinações entre fases móveis e estacionárias (DEGANI et al., 1998).

Existem vários sistemas de detecção que podem ser acoplados à cromatografia. Dentre eles, o acoplamento a um espectrômetro de massas une as vantagens da cromatografia (alta seletividade e eficiência de separação) com as vantagens da espectrometria de massas, que é capaz de detectar e identificar com elevada sensibilidade uma substância através da medição da razão massa/carga (*m/z*) dos íons

que são gerados pela quebra da molécula e da caracterização química do composto (CHIARADIA et al., 2008).

A espectrometria de massas sequencial, técnica EM/EM, possibilita a obtenção de uma grande quantidade de informação estrutural acerca do analito, garantindo sua identificação com maior exatidão do que quando ela é feita apenas com base no tempo de retenção dos compostos analisados, como ocorre nas outras técnicas de detecção cromatográficas. Por esse motivo, esta técnica, em conjunto com a cromatografia, é bastante utilizada na detecção de compostos presentes em baixas concentrações em matrizes complexas, como é o caso dos alimentos. Devido ao fato de ser uma técnica altamente seletiva, que minimiza os efeitos da interferência de componentes da matriz sobre o sinal obtido, exige uma etapa de preparo da amostra mais simples, eliminando, muitas vezes, a necessidade de realizar várias etapas de purificação da amostra (CHIARADIA et al., 2008).

Pela aplicação da técnica CL-EM/EM é possível realizar análises de multirresíduos em uma única corrida sem comprometer a qualidade da resposta de cada analito à cromatografia. Isto só é alcançado porque o sistema de detecção de massas monitora individualmente cada transição *m/z*, gerando para cada transição monitorada seu próprio cromatograma que pode ser extraído do cromatograma total com o auxílio do software de controle do sistema e tratamento de dados (OLIVEIRA, 2011).

Desta forma, o emprego da técnica CL-EM/EM fornece informações referentes ao tempo de retenção de cada composto, a obtenção de duas ou mais transições que permitem quantificar e confirmar o analito e elevada detectabilidade que permitem alcançar níveis de confiabilidade em concordância com os LMR estabelecidos (MARTINS JÚNIOR et al., 2006). Podem ser encontrados diversos trabalhos na literatura que utilizam CL-EM/EM para separação e detecção de antimicrobianos em alimentos.

Um método para identificação e quantificação de macrolídeos (eritromicina, josamicina, tilmicosina, tilosina, espiramicina e neoespiramicina) em filé de tilápia, por cromatografia líquida acoplada a um espectrômetro de massas do tipo quadrupolo tempo-de-voo, foi desenvolvido por SISMOTTO et al. (2014). O preparo da amostra foi simples, precipitando as proteínas e extraindo os analitos com etanol, retirando a gordura com hexano e concentrando o extrato por evaporação do solvente. Os limites de quantificação foram, pelo menos, 45% menores que os Limites Máximos de Resíduos. A separação cromatográfica ocorreu em uma coluna de fase reversa C18

XTerra1 MS (150 x 2,1 mm, 5 µm, Waters, USA) a 25 °C. As fases móveis foram água e metanol adicionados de ácido acético. Os parâmetros espectrométricos dos macrolídeos foram otimizados para cada um dos analitos.

Um método multirresíduo simples e sensível foi desenvolvido por DASENAKI & THOMAIDIS (2015) para análise de 115 drogas veterinárias, pertencentes a mais de 20 classes diferentes, em várias matrizes de origem animal, inclusive peixe. O método envolveu um passo de extração sólido-líquido com 0,1% de ácido fórmico em solução aquosa de EDTA 0,1% (p/v)-acetonitrila-metanol (1:1:1, v/v) com um passo adicional de agitação ultrassônica. A precipitação dos lipídeos e proteínas foi promovida submetendo os extratos a temperaturas baixas (-23 °C) por 12 horas. Uma etapa posterior de purificação com hexano foi realizada para extração completa dos lipídeos. O extrato foi injetado em um sistema de cromatografia líquida acoplada a um espectrômetro de massas sequencial com ionização *electrospray* (CL-ESI-EM/EM). A coluna cromatográfica utilizada foi Atlantis T3C18 (100 x 2,1 mm, 3 µm, Waters) com um fluxo de 100 mL/min. Foram realizadas duas corridas, uma em modo negativo e outra em modo positivo de ionização no modo MRM (monitorização de reação múltipla). A fase móvel para o modo de detecção positivo foi água com 0,01% (v/v) de ácido fórmico (solvente A) e metanol (solvente B), enquanto no modo de detecção negativo foi utilizada água modificada (1 mM de formato de amônio (A), metanol (B) e acetonitrila (C). Os parâmetros espectrométricos foram otimizados e apresentados no trabalho. A recuperação dos analitos variou de 31,8% (ácido tolfenâmico) a 114% (carbamazepina) em peixe, com valores de desvio padrão relativo entre 1,7% e 15%. Os limites de quantificação variaram de 0,03 $\mu g.kg^{-1}$ (flunixina) a 6,7 $\mu g.kg^{-1}$ (hidroclorotiazida).

Um método rápido, sensível e específico por CL-EM/EM foi desenvolvido e validado para a quantificação simultânea de quatro antimicrobianos comumente utilizados na aquicultura - ciprofloxacina, trimetoprima, sulfadimetoxina e florfenicol – em músculo de peixes. A amostra foi preparada através de extração líquido-líquido simples seguida de uma purificação (*clean-up*) com n-hexano. Os extratos purificados foram injetados no cromatógrafo líquido e a separação dos analitos foi realizada em uma coluna C18 de fase reversa Poroshell 120 CE (50 x 3 mm, 2,7 µm, Agilent) usando uma fase móvel isocrática constituída por ácido fórmico a 0,1% em água: ácido fórmico a 0,1% em metanol (20:80 v/v) a um fluxo de 0,4 mL/min. A temperatura da coluna foi mantida a 25 °C. O espectrômetro de massas foi operado no modo de ionização positiva para ciprofloxacina, trimetoprima e sulfadimetoxina e no modo de ionização

negativa para florfenicol, com ionização por *electrospray* (ESI). A detecção dos íons foi feita no modo MRM. O limite de quantificação obtido foi de 0,5 $ng.g^{-1}$ para sulfadimetoxina e 1 $ng.g^{-1}$ para ciprofloxacina, trimetoprima e florfenicol. O desvio padrão relativo foi de 14,3%, 15,8%, 6,7% e 9,4% no limite de quantificação para ciprofloxacina, trimetoprima e sulfadimetoxina e florfenicol, respectivamente, enquanto que as precisões, expressas como a porcentagem de recuperação, foram de 92,3%, 91,6%, 94,1% e 93,7% para os quatro analitos no limite de quantificação (REZK et al., 2015).

DASENAKI & THOMAIDIS (2010) desenvolveram um método para analisar rapidamente dezessete sulfonamidas e cinco tetraciclinas em músculo de peixe em uma única corrida, utilizando cromatografia líquida de ultra-alto desempenho (UHPLC) com detecção por espectrometria de massas. A separação foi realizada em coluna Zorbax Eclipse Plus C18 (2,1 x 50 mm, 1,8 µm, Agilent). A fase móvel consistiu de água contendo 0,1% de ácido fórmico (v/v) (solvente A) e acetonitrila (solvente B). O gradiente utilizado foi 0-12 minutos de gradiente linear de 5 a 50% de B; 12-13 minutos de 50 para 5% de B, 13-21 minutos mantidos os 5% de B para que a coluna se reequilibrasse antes da próxima injeção. O volume de injeção foi fixado em 10 µL. O espectrômetro de massas operou em modo positivo. O limite de detecção variou de 5,65 a 24,0 $\mu g.kg^{-1}$ para as sulfonamidas e de 10,3 a 25,8 $\mu g.kg^{-1}$ para as tetraciclinas. O limite de quantificação variou de 17,1 a 72,7 $\mu g.kg^{-1}$ para as sulfonamidas e de 31,3 a 78,1 $\mu g.kg^{-1}$ para as tetraciclinas. O desvio padrão relativo de repetibilidade variou de 3,5% a 16% para as sulfonamidas e de 5,7% a 15% para as tetraciclinas.

OBJETIVOS

Este trabalho teve como objetivo geral desenvolver métodos multirresíduos de análise de antimicrobianos em músculo de peixe e avaliar a qualidade dos peixes cultivados nos estados brasileiros de Minas Gerais e do Pará no que diz respeito à presença de resíduos de antimicrobianos.

Os objetivos específicos foram:

i. fazer uma revisão detalhada sobre a determinação de cloranfenicol em alimentos de origem animal brasileiros por CL-EM/EM;
ii. fazer uma revisão detalhada sobre os avanços na determinação cromatográfica de anfenicóis em alimentos;
iii. desenvolver e validar um método de triagem para a determinação multirresíduos de antimicrobianos das classes aminoglicosídeos, beta-lactâmicos, macrolídeos, quinolonas, sulfonamidas e tetraciclinas em músculo de peixe empregando CL-EM/EM;
iv. desenvolver e validar um método analítico quantitativo para a determinação multirresíduos de quinolonas (difloxacina, norfloxacina, ciprofloxacina, danofloxacina, marbofloxacina, enrofloxacina, sarafloxacina, ácido oxolínico, ácido nalidíxico, flumequina) e tetraciclinas (clortetraciclina, doxiciclina, oxitetraciclina e tetraciclina) em músculo de peixe;
v. realizar as análises de triagem e confirmatória de amostras de peixes de piscicultura dos Estados de Minas Gerais e do Pará quanto à presença de antimicrobianos.

PARTE EXPERIMENTAL

Para atender aos objetivos deste trabalho, o conteúdo foi dividido em capítulos escritos na forma de artigo científico, os quais estão apresentados a seguir.

CAPÍTULO I - LC-MS/MS DETERMINATION OF CHLORAMPHENICOL IN FOOD OF ANIMAL ORIGIN IN BRAZIL

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LC-MS/MS

LC-MS/MS determination of chloramphenicol in food of animal origin in Brazil

Determinação de cloranfenicol em alimentos de origem animal no Brasil empregando LC-MS/MS

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Abstract
Chloramphenicol is a highly efficient antibiotic with broad spectrum activity. It has been banned from food producing animals because of serious adverse effects to human health. Nevertheless, it is still being used in some countries because of its high efficacy and relatively low price. There is currently a minimally required performance limit (MRPL) defined at 0.3 µg/kg. This is the reason why chloramphenicol has often been analyzed by highly efficient and sensitive single residue methods. The objective of this review is to provide the state-of-art scientific knowledge on chloramphenicol, the LC-MS/MS methods used for its analysis and its occurrence in foods of animal origin in Brazil.
Keywords: antibiotic, milk, fish, honey, liquid chromatography, mass spectrometry.

Resumo
O cloranfenicol é um antibiótico de amplo espectro e elevada eficiência. Devido à ocorrência de efeitos adversos graves à saúde humana, este antibiótico teve seu uso banido em animais destinados à alimentação humana. No entanto, seu uso ainda é comum em muitos países, devido à alta eficácia e baixo custo. Atualmente, existe um limite mínimo de desempenho requerido (LMDR) de 0,3 µg/kg e, por essa razão, o clorofenicol tem sido frequentemente analisado por métodos altamente eficientes e sensíveis. O objetivo desta revisão é apresentar o estado-da-arte sobre o conhecimento científico a respeito do cloranfenicol, métodos baseados em LC-MS/MS usados para sua análise e ocorrência em alimentos de origem animal no Brasil.
Palavras-chave: antibiótico, leite, peixe, mel, cromatografia líquida, espectrometria de massas.

ABSTRACT

Chloramphenicol is a highly efficient antibiotic with broad spectrum activity. It has been banned from food producing animals because of serious adverse effects to human health. Nevertheless, it is still being used in some countries because of its high efficacy and relatively low price. There is currently a minimally required performance limit (MRPL) defined at 0.3 $\mu g.kg^{-1}$. This is the reason why chloramphenicol has often been analyzed by highly efficient and sensitive single residue methods. The objective of this review is to provide the state-of-art scientific knowledge on chloramphenicol, the LC-MS/MS methods used for its analysis and its occurrence in foods of animal origin in Brazil.

Keywords: antibiotic, milk, fish, honey, liquid chromatography, mass spectrometry.

1. INTRODUCTION

Antibiotics are widely used in intensive agriculture. They can be a therapeutic agent in the treatment of animal diseases, a prophylactic agent to avoid or prevent sickness, and also a feed additive to promote growth and increase feed efficiencies. However, their widespread use in food producing animals can be a potential hazard to human health due to the possibility of causing bacterial resistance and potential allergic reactions to the antibiotic. Special concern has been raised with regard to chloramphenicol, which, besides the inherent problems with antibiotics, it can cause fatal health problems, among them, bone marrow aplasia, aplastic anemia and gray baby syndrome. Due to the potential harmful effects to human health, the use of chloramphenicol has been prohibited for the treatment of food-producing animals in several countries (SAMSONOVA et al., 2012; JECFA, 2014; HANEKAMP & BAST, 2015).

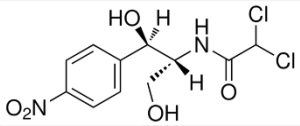
However, the use of chloramphenicol to treat food-producing animals remains a possibility due to its high efficiency, broad spectrum of activity, prompt availability and low cost. The occurrence of chloramphenicol in foods can be the result of authorized use but lack of compliance with the withdrawal time period, unauthorized use and also unintentional or cross-contamination (GENTILI et al., 2005; HANEKAMP & BAST, 2015). Therefore, there is a need to constantly evaluate the occurrence of this antibiotic in food.

The control of chloramphenicol in foods can be performed by screening or confirmatory procedures. Screening methods only provide semi-quantitative analysis and can give rise to false positives, but they are used due to simplicity in sample preparation, sensitivity, speed and low cost. On the other hand, confirmatory methods, such as those employing liquid chromatography (LC) coupled to mass spectrometry (MS) are the approaches of choice for determination of antibiotics, because they allow definitive identification, quantitative determination at very high level of specificity and sensitivity (GENTILI et al., 2005; BERENDSEN, 2010). The objective of this review is to provide updated information on the occurrence and concentrations of chloramphenicol in food in Brazil determined by LC-MS/MS.

2. CHARACTERISTICS AND ANTIMICROBIAL ACTIVITY OF CHLORAMPHENICOL

Chloramphenicol is a naturally occurring, broad-spectrum antibiotic with excellent antibacterial and pharmacokinetic properties. Its formula, structure, chemical names and numbers as well as physico-chemical and spectral characteristics are described in Table 1. Chloramphenicol was isolated in 1947 from *Streptomyces venezuelae,* a soil bacterium, but it has been synthetically produced for a long time. Different trade names are available and there are three common forms for systemic therapy: a free base form, chloramphenicol palmitate and chloramphenicol succinate. Other formulations are also available for topical use (SAMSONOVA et al., 2012; SPLENDORE et al., 2013).

Table 1. Characteristics of chloramphenicol

Parameter	Characteristics
CAS number	56-75-7
EC number	200-287-4
IUPAC name	2,2-dichloro-N-[(1R,2R)-1,3-dihydroxy-1-(4-nitrophenyl)propan-2-yl] acetamide
Names	Chloramphenicol; chlornitromycin; chloromycetin; levomycetin; chlorocid; globenicol
Molecular formula	$C_{11}H_{12}Cl_2N_2O_5$
Structure	
Molar mass (g/mol)	323.12938
Melting point (°C)	150.5-151.5
pka	11.03
Log P	1.103
Physical description	White to greyish-white or yellowish-white fine crystalline powder or fine crystals, needles or elongated plates
Taste	Bitter to taste
Spectral properties:	Specific optical rotation: +18.6˚ at 20 ˚C (ethanol); -25.5˚ at 25 ˚C (ethyl acetate). IR: u 5174; UV: 385 nm; Mass: 236
Solubility	Very soluble in methanol, ethanol, butanol, ethyl acetate, acetone, chloroform; Water solubility - 2500 $mg.L^{-1}$ (at 25 °C)
Stability	Neutral and acid solutions are stable on heating; In solution, chloramphenicol undergoes a number of degradative changes related to pH, temperature, photolysis and microbiological effects

CAS (2015); PUBCHEM (2015).

Chloramphenicol has a wide spectrum of antimicrobial activity. It is effective against Gram-positive and Gram-negative cocci and bacilli (including anaerobes), *Rickettsia*, *Mycoplasma*, *Chlamydia*, among others. It is usually bacteriostatic, but at

higher concentrations it can be bactericidal. It acts by diffusing through the bacteria cell wall, binding to the bacterial 50S ribosomal subunit and inhibiting protein synthesis and cell proliferation (JECFA, 2014; CAS, 2015; PUBCHEM, 2015). It was widely used as a human antibiotic and also as a veterinary drug. Nowadays, its use in human medicine has been restricted to ophthalmic and some serious infections (*Salmonella typhi* and other forms of salmonellosis, staphylococcal brain diseases and life threatening infections of the central nervous system and respiratory tract). The veterinary use of chloramphenicol includes administration to pets, farm and aquaculture animals. In therapy and prophylaxis**,** the main infectious diseases treated with chloramphenicol are enteric and pulmonary infections, skin and organ abscesses and mastitis. It is also used in infections caused by anaerobic bacteria or those that are resistant to other antimicrobial agents (JECFA, 2014; HANEKAMP & BAST, 2015).

3. TOXICOLOGICAL ASPECTS AND CURRENT LEGISLATION

The widespread use of antibiotics in food-producing animals can be a potential hazard for human health. However, the indiscriminate use of chloramphenicol can lead to bacterial resistance, allergic reactions, disruption of the balance of the gastrointestinal microbial flora, and hemotoxic effects, such as aplastic anemia, bone marrow depression and gray baby syndrome. Since it undergoes biotransformation to the inactive metabolite chloramphenicol glucuronide in the liver, individuals with subnormal liver function and infants are also at risk. Aplastic anemia is an irreversible side effect that is not dose-related; this side effect is probably the result of the reduction of its *p*-nitro group to the highly toxic nitroso metabolite. It is a rare but often fatal condition with no treatment. Another side effect is bone-marrow depression, suppressing bone marrow and its production of red and white blood cells and platelets. This effect is reversible if the treatment is discontinued. Also, infants, especially premature babies, when exposed to high levels of chloramphenicol, can develop the 'gray baby syndrome'. This probably occurs because the liver enzymes of an infant are not fully developed, and any chloramphenicol received across the placenta or in breast milk remains intact in the body, inducing hypotension, hypothermia, flaccidity, cardiovascular collapse, cyanosis and death within hours. There are also indications that chloramphenicol is genotoxic *in vivo* and could cause cancer. Although the

evidence is considered limited, chloramphenicol has been categorized by the International Agency for Research on Cancer (IARC) as probably carcinogenic in humans, classified as group 2A (IARC, 1990; JECFA, 2014).

Based upon scientific reports about chloramphenicol, an acceptable daily intake (ADI) has never been allocated and a maximum residue limit (MRL) has not been assigned (JECFA, 2014). Chloramphenicol was banned for use in food-producing animals in the European Union and in many other countries including Brazil as a means to eliminate it from the food production chain and related goods (BRASIl, 2003; EC, 2010a). A zero tolerance provision was established and a minimum required performance limit (MRPL), which is the concentration that laboratories should be able to detect and confirm, of 0.3 $\mu g.kg^{-1}$ for chloramphenicol was set by the European Commission and adopted by several countries for analytical methods to be used in testing for chloramphenicol in products of animal origin (EC, 2010a; BRASIL, 2015; CANADA, 2015; USDA, 2015).

To warrant national public health safety and to maintain competitiveness in international trade, food producers have to ensure that the products traded are in compliance with the safety and quality criteria required by consumers. Among actions undertaken by Brazil to warrant safety and quality control, the Ministry of Agriculture, Livestock and Food Supply of Brazil created a food safety program called National Residue Control Plan (NRCP). It has the purpose of generating reliable analytical results, monitoring residues and contaminants involved in food production, including antibiotics (MAURICIO et al., 2009). The Brazilian Agency of Sanitary Surveillance (ANVISA) from the Ministry of Health also created a National Program for the analysis of veterinary drug residues in food available for consumers (ANVISA, 2009). Therefore, it is of great importance to have sensitive methods for the determination and confirmation of residues and contaminants in foods.

4. LC-MS/MS METHODS FOR THE ANALYSIS OF CHLORAMPHENICOLS IN FOODS

Several methods are available for the determination of chloramphenicol in foods, both for screening or quantification purposes. Screening methods are cost-effective and have a high sample throughput (FERREIRA et al., 2012; SAMSONOVA et al., 2012).

However, the effective control of chloramphenicol in foods requires very sensitive and reliable analytical methods to comply with the stringent requirement established for a banned compound. Due to the chemical properties of chloramphenicol, quantitative methods using gas chromatography with mass spectrometry (GC-MS) require transformation of chloramphenicol into a stable volatile compound, which lengthens analysis time and may not be reproducible at trace levels (GENTILI et al., 2005; PAN et al., 2006). Many of the reported liquid chromatography/ultraviolet (LC-UV) methods did not reach the required sensitivity and selectivity to meet the current MRPL. The power of a mass spectrometer as a chromatographic detector results from its capacity to determine, by means of the molecular weight, the precursor ion and its fragments, which provide structural information. The combination of liquid chromatography with tandem mass spectrometry (LC-MS/MS) allows definite identification and quantification of trace chloramphenicol in complex food matrices due to the specificity and sensitivity associated with this technique (ORTELLI et al., 2004; GENTILI et al., 2005; KAUFMANN & BUTCHER, 2005; BERENDSEN, 2010). In this context, only methods and results for chloramphenicol based on LC-MS/MS will be described.

According to Table 2, several studies were undertaken on the analysis of chloramphenicol in foods by LC-MS/MS. In most of them, analysis was carried out in the multiple reaction monitoring (MRM) mode via electrospray ionization operated in the negative mode. Deuterated chloramphenicol (d_5-chloramphenicol) was used as the internal standard. The transitions used for chloramphenicol quantification and confirmation varied among studies. However, the $[M-H]^-$ ion and at least two product ions are monitored. For example, GUIDI et al. (2012b) used *m/z* 320.9 $\rightarrow$152.1 and *m/z* 320.9$\rightarrow$256.9 for chloramphenicol in fish analysis. The monitored ion for the internal standard was *m/z* 326.015$\rightarrow$157.0. Matrix-matched calibration curves were used. In most of the studies, the method was validated according to the criteria established by the EC Commission Decision 657/2002 (EC, 2002).

LC separation of chloramphenicol was obtained by reverse phase C18 columns from different brands (Table 2). Columns dimensions varied from 50 to 150 mm length, 2 to 3 mm internal diameter and 2 to 5 µm particle size. Different mobile phases were used in gradient elution, among them methanol:water, acetonitrile:water, acetonitrile:water acidified with formic acid, and ammonium acetate:methanol. In every method, except for one, the limits of detection and quantification were below 0.3 $\mu g.kg^{-1}$, which is the MRPL established for chloramphenicol. Moreover, high sensitivity was

obtained, in the ng.kg^{-1} or ng.L^{-1} range. Therefore, the majority of the methods were appropriate for the purpose.

Table 2. Methods for the extraction and separation of chloramphenicol in food of animal origin in Brazil by LC-MS/MS

Reference / Food	Extraction technique	LC Column & mobile phase	Recovery (%)	Limit of detection
MONTEIRO et al. (2015)				
Fish	LLE - acetonitrile:water & SPE - Captiva cartridge	C18 (3x100 mm, 3.5 µm) & 0.1% formic acid:acetonitrile with 0.1% formic acid	93.2	1 µg.kg^{-1}
TAKA et al. (2012)				
Honey	LLE - ethyl acetate	C18 (2.1x50 mm, 5 µm) & 2 mM ammonium acetate:methanol	>97	0.04 µg.kg^{-1}
NICOLICH et al. (2006)				
Milk	LLE - 10 mM formic acid & ethyl acetate	C18 (2x100 mm, 5 µm) & 0.1% formic acid:acetonitrile with 0.1% formic acid	95-97	0.09 µg.L^{-1}
BARRETO et al. (2012)				
Honey	LLE - ethyl acetate	C18 (4.6x150 mm, 5 µm & 2.1x100 mm, 3.5 µm) & acetonitrile:water	85.5-115.6	0.02 µg.kg^{-1}
Fish	LLE (acetonitrile, chloroform)		89-97	0.06 µg.kg^{-1}
Shrimp			87-100	0.06 µg.kg^{-1}
MARTINS-JUNIOR et al. (2006)	LLE - ethyl acetate	C18 (2.1x50 mm, 3 µm) & 5 mM ammonium acetate:(methanol:water, 95:5) with 5 mM ammonium acetate	83	0.00052 µg.kg^{-1}
Honey				
Milk	LLE (acetonitrile, chloroform) & SPE		83	0.00052 µg.L^{-1}
GUIDI et al. (2012a, 2012b) & TETTE et al. (2012)	LLE - 10 mM formic acid & ethyl acetate	C18 (2x50 mm, 5 µm) & 0.1% formic acid:acetonitrile with 0.1% formic acid		
Milk				0.019 µg.kg^{-1}
Fish	LLE - ethyl acetate		82.7	
Honey				
ROCHA SIQUEIRA et al. (2009)	Phosphate extraction solution + LLE ethyl acetate	C18 (2.1x100 mm, 4 µm) & water:methanol	101-104	0.03 µg/.kg^{-1}
Fish				
Shrimp			103-109	
Bovine meat			100-106	
Pork meat			102-104	
Poultry meat			87-97	
Egg			105-111	

LLE – liquid-liquid extraction, SPE – solid phase extraction.

Prior to LC-MS/MS analysis, sample preparation is needed to properly extract chloramphenicol from the food matrix. Concentration of the analyte and removal of interfering compounds may also be needed (BARGANSKA et al., 2011). According to

Table 2, sample preparation for chloramphenicol analysis involved mostly liquid-liquid extraction (LLE), even though solid-phase extraction (SPE) was also used in a few studies. Representative and homogeneous samples were extracted for chloramphenicol by LLE. In most of the methods, a simple extraction procedure using ethyl acetate provided good recoveries of chloramphenicol from honey, milk and fish samples. The sample was spiked with the internal standard, vortexed for a few seconds and allowed to equilibrate. The extracting solvent was added and sample was mixed (several minutes), centrifuged, the supernatant was transferred and the sediment was extracted once more. The supernatants were mixed and evaporated to dryness under nitrogen flow and they were dissolved in the mobile phase, vortexed for a few seconds, allowed to equilibrate and injected into the LC.

In the extraction of chloramphenicol from honey, dissolution of the sample in water (1:1, w/v) was needed prior to a simple LLE procedure with ethyl acetate (MARTINS-JÚNIOR et al., 2006; BARRETO et al., 2012; TAKA et al., 2012; TETTE et al., 2012). During extraction of chloramphenicol from milk, NICOLICH et al. (2006) and GUIDI et al. (2012a) added water acidified with 10 mM formic acid prior to LLE with ethyl acetate. However, MARTINS-JÚNIOR et al. (2006) proposed two sequential LLE procedures, the first with acetonitrile and the second using chloroform. The supernatant was dried under nitrogen flow, dissolved into methanol, water and Na_2HPO_4 and submitted to SPE using a Supelclean™ ENVI™ Chrom P (Supelco, Bellefonte, PA, USA). By using this more sophisticated procedure, a detection limit in the $ng.kg^{-1}$ range was obtained.

Different procedures were used for the extraction of chloramphenicol from fish. GUIDI et al. (2012b) used a simple LLE procedure with ethyl acetate and obtained good recoveries. BARRETO et al. (2012) used two LLE procedures, the first with acetonitrile and the second with chloroform, achieving similar results for fish and shrimp samples, improving recoveries. MONTEIRO et al. (2015) used a more sophisticated procedure involving LLE with acetonitrile:water, followed by ultrafiltration (SPE) using a Captiva cartridge to remove protein and particulate matter; however, these researchers focused on multiresidue analysis of 12 drugs of different antimicrobial classes. Such a detailed procedure would not be necessary for a single antibiotic analysis. ROCHA SIQUEIRA et al. (2009) proposed a method based on the extraction of chloramphenicol using a phosphate extraction solution (containing NaCl, KCl, Na_2HPO_4 and KH_2PO_4) and ultrasound bath for 15 minutes prior to LLE with ethyl acetate. They validated this method for fish, shrimp and also for meat (bovine, pork and poultry) and egg.

Several sophisticated and complex sample preparation techniques have been used in the analysis of chloramphenicol, by using different sorbants (Oasis, molecularly imprinted polymers and multi-walled carbon nanotubes) or techniques, like QuEChERS (VERZEGNASSI et al., 2003; PAN et al., 2006; LU et al., 2010; SHI et al., 2010; SNIEGOCKI et al., 2015). However, efficient extraction of chloramphenicol from food matrices for LC-MS/MS analysis can be undertaken by a simple LLE procedure. The use of additional steps may not be necessary. Furthermore, they can be time-consuming, require larger quantities of chemical reagents, involve extensive manual procedures, and use cleanup columns (SPE) that increases the analysis time and cost.

5. OCCURRENCE OF CHLORAMPHENICOL IN FOOD

Even though the use of chloramphenicol in food producing animals was banned several years ago, it was detected in some foods of animal origin, as indicated in Table 3. Four studies focused on honey (total of 43 samples) and indicated that samples from different regions of Brazil, from different beekeepers, floral sources and colors did not contain chloramphenicol (IARC, 1990; MARTINS-JÚNIOR et al., 2006; NICOLICH et al., 2006; TETTE et al., 2012). Eighty six samples of fish were analyzed in four different studies, and tilapia was the main type of fish analyzed. Chloramphenicol was only detected in one sample at levels below the MRPL (IARC, 1990; EC, 2002; ROCHA SIQUEIRA et al, 2009; TAKA et al., 2012). No chloramphenicol was found in shrimp (14 samples) (ROCHA SIQUEIRA et al, 2009). Samples of meat (556 from bovine, pork and poultry) and eggs (60) were also analyzed and none of them contained chloramphenicol (ROCHA SIQUEIRA et al, 2009).

Milk was the food product with the highest occurrence of chloramphenicol. Among studies undertaken, only the one by NICOLICH et al. (2006) failed to detect chloramphenicol in the 41 milk samples which were positive by ELISA. However, the samples had been stored for a long period of time prior to analysis, which could have affected the results. MARTINS-JÚNIOR et al. (2006) observed 42% occurrence of chloramphenicol in pasteurized and dried milk (total of 7 samples) at levels varying from 0.0047 to 0.0061 $\mu g.kg^{-1}$. GUIDI et al. (2012a) found similar prevalence (41%), at levels ranging from 0.10 to 13.9 $\mu g.kg^{-1}$ in samples obtained from dairy farms. Indeed, it is

more likely to find antibiotics in farm samples prior to their dilution by the mixture with milk from other farms.

Table 3. Occurrence of chloramphenicol in food of animal origin by LC-MS/MS in Brazil

Food	Samples analyzed (% Positive)	Concentration in positive samples	Reference
Honey			
Different beekeepers and floral	5 (0%)	nd	BARRETO et al. (2012)
Brands from SP market	4 (0%)	nd	MARTINS-JÚNIOR et al. (2006)
Different regions, floral & colors	22 (0%)	nd	TAKA et al. (2012)
Samples from MG market	12 (0%)	nd	TETTE et al. (2012)
Milk			
Milk (brands from market)	4 (25%)	4.73 $ng.L^{-1}$	MARTINS-JÚNIOR et al. (2006)
Dried milk (brands from market)	3 (66.6%)	5.9 – 6.10 $ng.L^{-1}$	
Suspect (Elisa) milk	41 (0%)	nd	NICOLICH et al. (2006)
Farm samples (raw)	49 (41%)	0.10 – 13.9 $\mu g.kg^{-1}$	GUIDI et al. (2012a)
Fish			
Nile tilapia (4 farms)	36 (0%)	nd	MONTEIRO et al. (2015)
Aquaculture fish (Pintado, tilapia, matricha, saint peter, tambaqui, tambacu)	13 (7.7%)	0.063 $\mu g.kg^{-1}$	GUIDI et al. (2012b)
Sarotherodon niloticus (farms)	21 (0%)	nd	BARRETO et al. (2012)
Fish	16 (0%)	nd	ROCHA SIQUEIRA et al. (2009)
Other foods			
Bovine meat	149 (0%)	nd	ROCHA SIQUEIRA et al. (2009)
Pork meat	199 (0%)	nd	
Poultry meat	208 (0%)	nd	
Shrimp	14 (0%)	nd	
Egg	60 (0%)	nd	

nd – not detected.

The NRCP has also been generating results for chloramphenicol and other residues in different foods of animal origin. Among the many different samples analyzed every year, only a few positive samples for chloramphenicol have been found, among them poultry meat (1 out of 76 samples from 2014, containing 0.39 $\mu g.kg^{-1}$) and fish (1 out of 77 samples, containing 75.6 $\mu g.kg^{-1}$) (PNCR, 2015). NRCP results for milk were negative for chloramphenicol in 120 samples of milk analyzed in 2009 and 2010. Results from PamVet (ANVISA, 2009) on chloramphenicol in milk also indicated no detectable levels in dried milk (139 samples) and 0.6% occurrence in UHT milk (464 samples) at levels ranging from 0.3 and 0.8 $\mu g.kg^{-1}$.

Even though the number of samples analyzed was very limited, the outcome is good considering the low percentage of foods of animal origin containing detectable levels of chloramphenicol. However, the illegal utilization of chloramphenicol to treat food-producing animals remains a possibility, either by administration of prohibited

antibiotics, or failure to respect the proper withdrawal periods. The problem is more visible with milk due to its role in infant and overall human nutrition and its widespread consumption. Furthermore, chloramphenicol in milk can be transfered to dairy products, specially those rich in fat (TIAN, 2011; FERREIRA et al., 2012; SNIEGOCKI et al., 2015). Therefore, it is important to ensure milk quality. Brazil has a quality control program aimed at milk from individual dairy farms. Antibiotic analysis of these milk samples should be performed to be able to detect the source of contamination and to implement educational programs to warrant milk quality.

It is also important to consider that there could be other sources of food contamination with chloramphenicol. Its use as a human medicinal antimicrobial can result in its release into the environment through waste streams by which food products may be contaminated during production. For instance, chloramphenicol has been detected in the aquatic environment such as effluents of sewage treatment plant and in surface water. Another source of this as well as other antimicrobials could be the natural occurrence in soil by bacteria (e.g., *Actinomycetes*), which can result in a large biomass per hectare in topsoil and subsequent uptake by crops and transfer of plants to feed (PENG et al., 2006; WATKINSON et al., 2009; HANEKAMP & BAST, 2015; SNIEGOCKI et al., 2015).

6. CONCLUSION

Several methods have been developed for the analysis of chloramphenicol in food by LC-MS/MS. Extraction of chloramphenicol from food can be undertaken by simple LLE procedures without requiring any sophisticated clean-up technique. The methods were validated according to the criteria of Commission Decision 2002/657/EC and were found appropriate for the analysis of chloramphenicol with limits of detection way below the MRPL of 0.3 $\mu g.kg^{-1}$. However, a very limited number of samples have been analyzed using this method, which became common in the last 10 years. Most of the studies performed focused on honey, milk and fish followed by shrimp, meats and egg. Chloramphenicol was detected in raw milk samples at levels above the MRPL and in trace amounts in fish. Even though chloramphenicol has been banned for use in food-producing animals for many years, it is still being detected. Therefore, monitoring and

educational programs are needed to warrant safety of consumers and international trade.

CAPÍTULO II - ADVANCES ON THE CHROMATOGRAPHIC DETERMINATION OF AMPHENICOLS IN FOOD

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Advances on the chromatographic determination of amphenicols in food

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ABSTRACT

Antibiotics are widely used in veterinary medicine to treat and prevent diseases and their residues can remain in food of animal origin causing adverse effects to human health. Amphenicols (chloramphenicol, thiamphenicol, and florfenicol) may be found in foodstuffs, although the use of chloramphenicol has been prohibited in many countries due to its high toxicity. Since these antibiotics are usually present at trace levels in food, sensitive and selective techniques are required to detect them. This paper reviews analytical methods used since 2002 for the quantitative analysis of amphenicols in food. Sample preparation and separation/detection techniques are described and compared. The advantages and disadvantages of these procedures are discussed. Furthermore, the worldwide legislation and occurrence of these antibiotics in food matrices as well as future trends are also presented.

ABSTRACT

Antibiotics are widely used in veterinary medicine to treat and prevent diseases and their residues can remain in food of animal origin causing adverse effects to human health. Amphenicols (chloramphenicol, thiamphenicol, and florfenicol) may be found in foodstuffs, although the use of chloramphenicol has been prohibited in many countries due to its high toxicity. Since these antibiotics are usually present at trace levels in food, sensitive and selective techniques are required to detect them. This paper reviews analytical methods used since 2002 for the quantitative analysis of amphenicols in food. Sample preparation and separation/detection techniques are described and compared. The advantages and disadvantages of these procedures are discussed. Furthermore, the worldwide legislation and occurrence of these antibiotics in food matrices as well as future trends are also presented.

Keywords: chloramphenicol; thiamphenicol; florfenicol; antibiotic; quantitative methods; legislation; occurrence.

1. INTRODUCTION

Antibiotics are widely used for therapeutic and prophylactic purposes in human and veterinary medicine and also to promote growth and increase feed efficiencies in food producing animals (EC, 2010a). However, abused use of antibiotics and their presence in food of animal origin are of concern due to development of resistance of target pathogens against antibiotics, induced allergic reactions in some hypersensitive individuals, potential compromise of the human intestinal and immune systems (GIKAS et al., 2004; BLASCO & PICÓ, 2007; VORA & RAIKWAR, 2013; JECFA, 2014).

There is a diverse range of chemical substances with antimicrobial activity. Among them, amphenicols, including chloramphenicol, thiamphenicol and florfenicol, are readily available broad-spectrum antibiotics. Chloramphenicol was widely used in the past in both human and veterinary medicine. However, due to serious adverse effects to human health, it was banned from food producing animals and a zero tolerance policy became effective (ALECHAGA et al., 2012; TAO et al., 2014; GUIDI et al., 2015). Analogues of chloramphenicol – thiamphenicol and florfenicol – have been developed and seem to be viable substitutes because they still have broad spectrum of activity but do not cause the same adverse health effects brought about by chloramphenicol (KOWALSKI et al., 2008). They have been widely used not only for therapeutic and prophylactic purposes in veterinary medicine, but also to enhance feed efficiency and to promote animal growth, especially in aquaculture. Excessive use of amphenicols, or any antibiotics, in aquaculture, however, can contaminate water and threaten water environmental security (XUE et al., 2015). Furthermore, high levels in food of animal origin should be avoided to warrant food safety and international trade.

According to the literature, chloramphenicol can still be found in several food matrices, suggesting its continued use (VERZEGNASSI et al., 2003; SANTOS et al., 2005; MARTINS-JÚNIOR et al., 2006; SHERIDAN et al., 2008; LU et al., 2010; WANG et al., 2011; SAMSONOVA et al., 2012; WU et al., 2012; GUIDI et al., 2015). Besides, there is little information available regarding the occurrence of its analogues in foods of animal origin and environment. Therefore, sensitive and reliable methods for the analysis of amphenicols are needed.

The analysis of antibiotics in food is not a simple task. They must be detected at extremely low part-per-billion levels. Furthermore, foods of animal origin are usually complex matrices. Multi-analyte methods encompassing a whole class of antibiotic are

desired; however, they require non-selective sample preparation and, therefore, are more prone to matrix effects which can compromise detection limits, quantitative and selectivity aspects, as well as equipment maintenance (BLASCO & PICÓ, 2007; BERENDSEN et al., 2010). The effective control of antimicrobials in foods requires the combination of cost-effective and high sample throughput screening methods followed by confirmation and quantification using more sophisticated methods. SAMSONOVA et al. (2012) published an extensive review on screening methods for the detection of amphenicols in foods. However, there is no recent overview on confirmatory and quantitative methods for amphenicols determination in foodstuffs.

Different analytical methods have been developed for the quantification of amphenicols in food. Two main steps are required: sample preparation followed by separation and detection. During sample preparation it is important to properly extract and concentrate the analytes and also to remove as many interfering compounds as possible. Extraction and concentration of amphenicols from food can be accomplished by solid-phase (SPE) and/or liquid-liquid (LLE) extraction. Miniaturized approaches have also been used, aiming reduced use of solvents and reagents, and waste generation (ANTHEMIDIS& IOANNOU, 2009; BARRETO et al., 2012). Many different analytical techniques have been developed for the separation and detection of amphenicols in food; however, gas chromatography (GC) coupled to electron capture (ECD) or mass spectrometry (MS) detectors and liquid chromatography (LC) coupled to ultraviolet, MS or MS/MS detector, are the most widely used.

In this context, this review presents the state of art, developments and achievements since 2002 and the future trends on methods for the analysis of amphenicols in several food matrices.

2. CHARACTERISTICS OF AMPHENICOLS AND SOME METABOLITES

Amphenicols are a class of broad spectrum and highly efficient antibiotics with a phenylpropanoid structure. Although of natural origin, they have been produced by chemical synthesis. The physico-chemical and other relevant characteristics of amphenicols and some of their metabolites (CAS, 2015; VSBD, 2016) are summarized in Table 1.

Table 1. Some physico-chemical characteristics of amphenicols and some metabolites

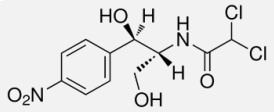
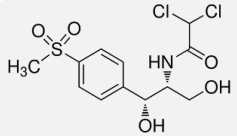
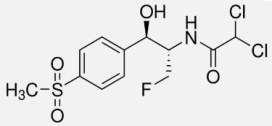
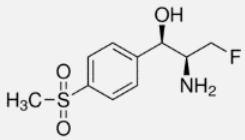
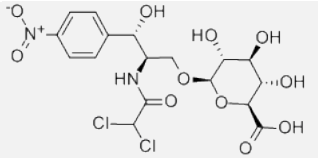
Analyte	Chloramphenicol	Thiamphenicol	Florfenicol	Florfenicol amine	Chloramphenicol-glucuronide
CAS number	56-75-7	15318-45-3	73231-34-2	76639-93-5	39751-33-2
EC number	200-287-4	239-355-3	-	-	-
IUPAC name	2,2-dichloro-N-[(1R,2R)-1,3-dihydroxy-1-(4-nitrophenyl) propan-2-yl]acetamide	2,2-dichloro-N-[(1R,2R)-1,3-dihydroxy-1-(4-methylsulfonylphenyl)propan-2-yl]acetamide	2,2-dichloro-N-[(1R,2S)-3-fluoro-1-hydroxy-1-(4-methylsulfonylphenyl) propan-2-yl]acetamide	(1R,2S)-2-amino-3-fluoro-1-(4-methylsulfonylphenyl)propan-1-ol, Sch 40458	(2S,3S,4S,5R,6R)-6-[(2R,3R)-2-[(2,2-dichloroacetyl)amino]-3-hydroxy-3-(4-nitrophenyl)propoxy]-3,4,5-trihydroxyoxane-2-carboxylic acid
Names	Chlornitromycin; chloromycetin; levomycetin; chlorocid; globenicol	Thiocymetin, neomyson, thiocymetin, dextrosulphenidol	Aquaflor, nuflor, fluorothiamphenicol	Methyl triclosan,	(2R,3R)-2-[(dichloroacetyl)amino]-3-hydroxy-3-(4-nitrophenyl)propyl |A-d-glucopyranosiduronic acid, Chloramphenicol 3-O-|A-D-Glucuronide
Molecular formula	$C_{11}H_{12}Cl_2N_2O_5$	$C_{12}H_{15}Cl_2NO_5S$	$C_{12}H_{14}Cl_2FNO_4S$	$C_{10}H_{14}FNO_3S$	$C_{17}H_{20}Cl_2N_2O_{11}$
Molar mass (g/mol)	323.13	356.22	358.21	247.29	499.25
Melting point (°C)	150.5-151.5	165.3	153-154	152 °C	170-174
Pka	11.03	11.05	10.73	10.90	2.81
Log P	1.103	-0.24	1.175	-0.398	-
Structure					
Physical description	White to greyish-white or yellowish-white fine crystalline powder or fine crystals or needles	White or yellowish-white crystalline powder or crystals	White crystalline powder	White crystalline powder	Off-white solid

Table 1. (continuation…)

Analyte	Chloramphenicol	Thiamphenicol	Florfenicol	Florfenicol amine	Chloramphenicol-glucuronide
Solubility	High in ethyl acetate, acetone, ethanol, butanol, methanol, chloroform; Water solubility - 2500 $mg.L^{-1}$ (20 °C)	Slight in ethanol, acetone, acetonitrile, methanol; Barely in ether, ethyl acetate, chloroform; Water solubility – 5 $mg.L^{-1}$ (20 °C)	Water solubility – 1320 $mg.L^{-1}$ (20 °C)	Water solubility – 2300 $mg.L^{-1}$ (25 °C) slight in unbuffered water (pH 9.77) – 2400 $mg.L^{-1}$ (25 °C) very soluble pH from 1 to 7; Soluble in organic solvents	Soluble in methanol; miscible with water
Stability	Neutral and acid solutions are stable on heating; Solution undergoes degradation related to pH, temperature, photolysis and microbial activity	Stable at normal temperature and pressure	Stable at normal temperature and pressure	Stable at normal temperature and pressure	Stable at normal temperature and pressure

CAS (2015); VSDB (2016).

Amphenicols are efficient antibiotics against Gram-positive and Gram-negative bacteria. They are especially effective against anaerobic microorganisms. They act by inhibiting protein synthesis, by binding to ribosomal subunits of susceptible bacteria, leading to the inhibition of peptidyl transferase, preventing the transfer of amino acids to growing peptide chains and subsequent protein formation (KOWALSKI et al., 2008; JECFA, 2014).

Chloramphenicol was the first amphenicol available. It was originally isolated from *Streptomyces venezuelae*, a soil bacterium, but it is now synthetically produced. It was widely used in 1950 to fight infections in human and veterinary medicine (GIKAS et al., 2004; GUIDI et al., 2015). Although it is a very efficient antibiotic, with excellent antibacterial activity and pharmacokinetics properties, its use was banned from food producing animals in several countries due to serious adverse effects to human health (WONGTAVATCHAI et al., 2004; GUIDI et al., 2015; HANEKAMP & BAST, 2015). Today, its use in human medicine is restricted to ophthalmic and a few serious infections (salmonellosis, staphylococcal brain diseases and life threatening infections of the nervous system and respiratory tract). The veterinary use includes treatment of enteric and pulmonary infections, skin and organ abscesses and mastitis (JECFA, 2014, GUIDI et al., 2015; HANEKAMP & BAST, 2015).

Chloramphenicol is eliminated intact or it can be biotransformed in the liver into the inactive metabolite chloramphenicol glucuronide (WONGTAVATCHAI et al., 2004; EMEA, 2009). However, the indiscriminate use of chloramphenicol can lead to inherent effects from antimicrobials, such as, bacterial resistance; allergic reactions; disruption of the intestinal microbial flora; and also hemotoxic effects, including aplastic anemia, bone marrow depression and ‘gray baby syndrome’. Bone-marrow depression occurs in humans when daily doses are higher than 4 g, and this effect is reversible if the treatment is discontinued. Another serious and not dose-related side effect is aplastic anemia. Infants, especially premature babies, when exposed to high levels of chloramphenicol, can develop ‘gray baby syndrome’. It probably occurs because neonates have a poor hepatic biotransformation of chloramphenicol (WONGTAVATCHAI et al., 2004; GUIDI et al., 2015). There are also indications that chloramphenicol is genotoxic *in vivo* and could cause cancer. Although the evidence is considered limited, it has been classified as group 2A by the International Agency for Research on Cancer – IARC (IARC, 1990). Based on the information available, no Acceptable Daily Intake (ADI) is established for chloramphenicol and a minimum required performance limit (MRPL), which corresponds to the ‘minimum content that

laboratories should be able to detect and confirm by a reference analytical method of 0.3 μg.kg^{-1} has been established for food of animal origin (IARC, 1990; WONGTAVATCHAI et al., 2004; EMEA, 2009; EC, 2010a; JECFA, 2014; BRASIL, 2015; HEALTH CANADA, 2015; USDA, 2015) (Table 2).

Table 2. Minimum Required Performance Limits (MRPLs) and Maximum Residue Limits (MRLs) values for amphenicols in food of animal origin established by the European Union, USA, Canada and Brazil

Substance / Food	**European Union (EC, 2010a)**	**USA (USDA, 2015)**	**Canada (Health Caada, 2015)**	**Brazil (Brasil , 2015)**	**Tissue**
Chloramphenicol – MPRL (μg.kg^{-1}) Meat, eggs, milk, aquaculture products, honey	0.3	0.3	0.3	0.3	All edible tissues
Thiamphenicol – MRL (μg.kg^{-1})					
Bovine	50	-[a]	-	50	Muscle, fat, liver, kidney
Chicken [b]	50	-	-	-	Muscle, skin, fat, liver, kidney
Porcine	50	-	-	50	Muscle
Eggs	50	-	-	10	n.a. [c]
Fish	50	-	-	50	Muscle
Milk	50	-	-	10	n.a.
Florfenicol [d] – MRL (μg.kg^{-1}) *(as sum of florfenicol and its metabolite florfenicol amine)*					
All food producing species except	100	-	-	-	Muscle
bovine, ovine, caprine, porcine,	200	-	-	-	Fat
poultry, fin fish	2000	-	-	-	Liver
	300	-	-	-	Kidney
Bovine, ovine, caprine	200	300	200	200 (bovine)	Muscle
	3000	3700	2000	-	Liver
	300	-	500	-	Kidney
Porcine	300	200	250	200	Muscle
	500	-	500	-	Skin, fat
	2000	2500	1400	-	Liver
	500	-	1000	-	Kidney
Poultry	100	-	100	-	Muscle
	200	-	200	-	Skin, fat
	2500	-	2000	-	Liver
	750	-	750	-	Kidney
Fin fish	1000	-	800	1000	Muscle, skin
Milk	-	-	-	10	n.a.
Eggs	-	-	-	10	n.a.

[a] – not found, [b] – not for use in animals from which eggs are produced for human consumption, [c] – n.a. not applicable, [d] – not for use in animals from which milk or eggs are produced for human consumption.

Thiamphenicol is an analog of chloramphenicol in which the nitro group on the benzene ring is replaced with methyl-sulfonyl. It has been widely used as a veterinary antibiotic in many countries for the treatment of bacterial diseases in fish, pork, cattle

and poultry. It is also available, in some countries, for human use, especially for the treatment of pulmonary, prostate and venereal infections and pelvic inflammatory diseases. Thiamphenicol is not readily metabolized in cattle, poultry, sheep, or man, and it is excreted unchanged. In pigs and rats, it can also be excreted as thiamphenicol glucoronate (LI et al., 2012; VORA & RAIKWAR, 2013; XIAO et al., 2015; YAO et al., 2015).

Florfenicol is a fluorinated derivative of thiamphenicol, and has a fluorine atom, instead of the hydroxyl group at C-3 (KOWALSKI et al., 2008; XIAO et al., 2015). Besides being a broad spectrum antibiotic, it also has activity against some chloramphenicol and thiamphenicol resistant bacterial strains. It has been widely used in aquaculture and in the control of bovine respiratory and interdigital phlegmon diseases (EMEA, 2009). Florfenicol is partly transformed into several metabolites, among them, florfenicol amine which is the largest and the longest live metabolite, reason why it has been considered a marker for florfenicol use. Florfenicol amine is the 4-methylsulphonophenylpropylamine parent compound formed by hydrolyzing the dichloroacetamide of florfenicol (XIE et al., 2011).

The main advantage of thiamphenicol and florfenicol over chloramphenicol is that they are not associated with the same adverse effects caused by chloramphenicol, probably due to the absence of the nitro group. ADI values were allocated for both of them (5 and 0-10 $\mu g.kg^{-1}$ bw, respectively) (JECFA, 2014). To ensure the safety of food for consumers, Maximum Residue Limits (MRLs) have been established for thiamphenicol and for the sum of florfenicol and its metabolite florfenicol amine. As indicated in Table 2, different MRLs have been established by different countries, varying from 10 to 50 $\mu g.kg^{-1}$ for thiamphenciol and from 10 to 3000 $\mu g.kg^{-1}$ for the sum florfenicol and florfenicol amine, depending on the sample tissue and also on the legislation of a specific country (EC, 2010a; Brasil, 2015; Health Canada, 2015; USDA, 2015). In addition, instead of establishing standardized methods, the EU has set requirements concerning performance of analytical methods and interpretation of results (EC, 2010a). This freedom of choice for analytical approaches has transformed antibacterial-residue analysis of food into a clear example of the benefits achievable by recent-developed analytical techniques (BLASCO & PICÓ, 2007).

3. METHODS FOR THE ANALYSIS OF AMPHENICOLS IN FOOD MATRICES

In general, the determination of amphenicols in food comprises two main steps. The first one is sample preparation and it may include extraction, purification and concentration. It will depend on the type of food sample and also on the method chosen for analysis. Afterwards, the extract is submitted to analyte separation and quantification. It is important to warrant that the sample is representative of the original food and that it is homogeneous.

3.1. Sample preparation

Recent trends in analytical chemistry aim to simplify sample preparation procedures and minimize the use of organic solvents (ANTHEMIDIS & IOANNOU, 2009). During sample preparation, the analytes of interest must be extracted from a large amount of other components from the complex food matrices. Clean-up and concentration steps may also be necessary to eliminate interferences and when the analyte is too diluted in the extract. Sometimes, extraction and clean-up can be accomplished in only one step, depending on the sample preparation technique employed. Analyte losses at this stage can compromise analysis outcome. Thus, sample preparation is a very important step within the entire analytical process. The most widely used approaches are liquid-liquid extraction and/or solid-phase extraction; however miniaturized approaches are becoming popular as they are environmental friendly.

3.1.1. Liquid-liquid extraction

Liquid-liquid extraction (LLE), either alone or followed by solid-phase extraction (SPE), is widely used for amphenicols' analysis. Ethyl acetate is the most commonly used LLE solvent (Table 3) for the extraction of amphenicols individually or as a mixture (CHOU et al., 2009; BARRETO et al., 2012; TAKA et al., 2012; GUIDI et al., 2015). It can also be associated with formic acid (NICOLICH et al., 2006; GUIDI et al., 2015), or phosphate solution (ROCHA SIQUEIRA et al., 2009). When defatting is required, hexane (CERKVENIK, 2002; DING et al., 2005; CHOU et al., 2009; DOUNY et al.,

2013) or isooctane (BOGUSZ et al., 2004) can be added to ethyl acetate. Chloroform can also be added to the mixture to help remove excess water from the extract (BOGUSZ et al., 2004; RONNING et al., 2006). Other extracting solvents mixtures have also been used, such as acetonitrile and hexane (DING et al., 2005), acetonitrile and chloroform (RONNING et al., 2006; BARRETO et al., 2012), acetonitrile and hexane (TAKINO et al., 2003), among others (OZCAN & AYCAN, 2013; FREITAS et al., 2014a; DASENAKI & THOMAIDIS, 2015; FEDENIUK et al.,2015; MONTEIRO et al., 2015; REZK et al., 2015). HAN et al. (2011a; 2011b) used aqueous two-phase systems based on imidazolium ionic liquid (1-butyl-3-methylimidazolium tetrafluoroborate – [Bmim]BF_4) for the extraction of chloramphenicol from water, milk and honey. By optimization of the type and amount of salts, pH value, volume of [Bmim]BF_4, and extraction temperature, good recoveries were achieved**.**

Table 3. Sample preparation using liquid-liquid extraction (LLE) for the determination of amphenicols and some metabolites in food (2002-2015)

Analyte / Matrix	Solvent	Analytical technique	Recovery (%)	Reference
Chloramphenicol				
Honey	Ethyl acetate	LC-MS/MS	97.0–101.9	TAKA et al., 2012
Egg	Ethyl acetate and hexane	GC-ECD	86.7	CERKVENIK, 2002
Fish, shrimp	Ethyl acetate and hexane	GC-ECD	70.8–90.8 (fish) 69.9–86.3 (shrimp)	DING et al., 2005
Honey, shrimp, poultry	Ethyl acetate and hexane	LC-ESI-MS/MS	-	DOUNY et al., 2013
Milk	Ethyl acetate and 10 mM formic acid	LC-ESI-MS/MS	95.0–98.8	NICOLICH et al., 2006
Bovine, swine, poultry, egg, seafood products	Ethyl acetate and phosphate solution	LC-ESI-MS/MS	51.2–100.3	ROCHA SIQUEIRA et al., 2009
Chicken, shrimp	Ethyl acetate, isooctane/chloroform, TRIS buffer pH 3.0	LC-ESI-MS/MS	45.0–50.0	BOGUSZ et al., 2004
Honey, milk, egg	Ethyl acetate (honey), acetonitrile (milk and egg)	LC-ESI-MS	86.0–103.0	OZCAN & AYCAN, 2013
Honey, fish, prawns	Ethyl acetate (honey) Acetonitrile and chloroform (fish and prawn)	LC-ESI-MS/MS	-	BARRETO et al., 2012
Bovine, chicken, scampi, egg, milk, honey	Acetonitrile and chloroform	LC-ESI-MS/MS	-	RONNING et al., 2006
Fish	Acetonitrile and hexane	LC-APPI-MS	89.3–102.5	TAKINO et al., 2003
Feed water, milk, honey	1-butyl-3-methyl imidazolium tetrafluoroborate and sodium citrate	LC-UV	90.4–102.7	HAN et al., 2011a

Table 3. (continuation…)

Analyte / Matrix	Solvent	Analytical technique	Recovery (%)	Reference
Bovine	Acetonitrile and EDTA hexane	UHPLC-ESI-MS/MS	105.0	FREITAS et al., 2014a
Florfenicol				
Fish	1% formic acid aqueous solution, acetonitrile and methanol	HPLC-ESI-MS/MS	96.9–104.3	REZK et al., 2015
Florfenicol amine				
Bovine, equine, porcine (kidney, liver, muscle)	6 N hydrochloric acid	LC-MS/MS	60.0–65.0	FEDENIUK et al., 2015
Chloramphenicol and Florfenicol				
Fish	0.1 M Na2EDTA and acetonitrile: water (0.1% formic acid, 70:30 v/v)	LC-ESI-MS/MS	83.8–110.1	MONTEIRO et al., 2015
Thiamphenicol and Florfenicol				
Pork (meat, liver, kidney), beef (meat, liver), chicken, fish	Ethyl acetate and *n*-hexane	LC-ESI-MS/MS	72.5–97.6	CHOU et al., 2009
Chloramphenicol, Thiamphenicol and Florfenicol				
Milk powder, butter, fish tissue, eggs	0.1% formic acid (v/v) and 0.1% EDTA (w/v), methanol and acetonitrile	LC-ESI-MS/MS	Butter (81.5–84.9) Egg (59.7–65.2) Fish (78.7–86.6) Milk (57.1–67.8)	DASENAKI & THOMAIDIS, 2015

[a] – not found; APPI – Atmospheric pressure photoionization; CAP – Chloramphenicol; CCβ – Detection capability; ECD – Electron capture detector; EDTA – ethylenediamine-tetraacetic acid; ESI – Electrospray ionization; FF – Florfenicol; FFA – Florfenicol amine; GC – Gas chromatography; LC – Liquid chromatography; LOQ – Limit of quantification; MS – Mass spectrometry; MS/MS – Tandem mass spectrometry; SPR – Surface plasmon resonance; TAP – Thiamphenicol; UHPLC – ultra high performance liquid chromatography; UV – ultraviolet detector.

3.1.2. Solid-phase extraction

Solid-phase extraction (SPE) has also been extensively used as sample preparation technique for amphenicols analysis in foodstuffs, either by itself or associated with LLE.

Simple SPE has been used by mixing the sample with the sorbent or by direct application of liquid samples to the sorbent. Octadecylsilane (C18) and Oasis HLB (poly(divinylbenzene-co-N-vinylpyrrolidone)copolymer) are the most commonly used SPE sorbents (Table 4). The first has been used to extract chloramphenicol from milk (RAMOS et al., 2003), honey (BOGUSZ et al., 2004) and chicken (TAJIK et al., 2010) and also chloramphenicol and its metabolite from honey (BOGUSZ et al., 2004). Oasis HLB has been used for individual (ISHII et al., 2006; SHERIDAN et al., 2008) or multi amphenicols (ALECHAGA et al., 2012; AZZOUZ & BALLESTEROS, 2015). Both sorbents can provide satisfactory recoveries. EXtrelut®NT has also been used to extract

chloramphenicol from honey, milk and bovine meat (CERKVENIK, 2002; KAUFMANN & BUTCHER, 2005).

Table 4. Sample preparation using solid-phase extraction (SPE) for the determination of amphenicols and some metabolites in food (2002-2015)

Analyte / Matrix	Sorbent	Analytical technique	Recovery (%)	Reference
Chloramphenicol				
Milk	Sep Pak C18	LC-UV	78.9	RAMOS et al., 2003
Chicken (liver, kidney, muscle)	C18	LC-UV	87.5 (liver), 79.3 (kidney), 63.2 (muscle)	TAJIK et al., 2010
Honey	Extrelut NT	UHPLC-ESI-MS/MS	95.0–108.0	KAUFMANN & BUTCHER, 2005
Honey	Oasis HLB	LC-ESI-MS/MS	92.5±8.8	(ISHII et al., 2006
Honey	Oasis HLB	LC-ESI-MS/MS	78.0	SHERIDAN et al., 2008
Milk, shrimp	MIP	LC-UV	90.2–99.9 (milk), 84.9–89.0 (shrimp)	SHI et al., 2007
Honey	MIP	LC-Q-TOF-MS	92.3–99.1	SHI et al., 2010
Milk	MIP	Square-wave voltammetry	67.0–101.0	MENA et al., 2003
Egg, honey, milk	Multi-walled carbon nanotubes	LC-ESI-MS/MS	95.8–102.3	LU et al., 2010
Florfenicol				
Chicken, fish	MIP	LC-UV	88.9 (fish), 93.5 (chicken)	SADEGHI & JAHANI, 2013
Chloramphenicol and metabolite				
Honey	Bond Elut C18 LRC	LC-ESI-MS/MS	60.0–69.0	BOGUSZ et al., 2004
Chloramphenicol, Thiamphenicol and Florfenicol				
Egg, honey	Oasis HLB	GC-MS	89.0–101.0	AZZOUZ & BALLESTEROS, 2015
Chloramphenicol, Thiamphenicol, Florfenicol and Florfenicol amine				
Honey	Oasis HLB	UHPLC-ESI-MS/MS	52.0–95.0	ALECHAGA et al., 2012

[a] – not found; GC – gas chromatography; LC – liquid chromatography; MIP – Molecularly imprinted polymer; MS – mass spectrometry; MS/MS – Tandem mass spectrometry; TAP – thiamphenicol; TOF – time of flight; UHPLC – ultra high pressure liquid chromatography; UV – ultraviolet.

Alternative sorbent materials have been used to improve recovery and selectivity, especially for individual amphenicols. LU et al. (2010) described the use of multi-walled carbon nanotubes (MWCN) as sorbent for the determination of chloramphenicol in egg, honey, and milk by LC-MS/MS. MWCN have attracted attention due to the high specific area and hydrophobic characteristic of its surface, which improves recoveries (95.8 to 102.3%). Molecularly imprinted polymers (MIPs) have also been successfully used as sorbent (MENA et al., 2003; SHI et al., 2007; SHI et al., 2010; SADEGHI & JAHANI,

2013). MIPs are highly cross-linked synthetic polymers designed to allow improved selectivity towards a certain structure or to a very closely related structure. Due to their characteristics, MIPs can selectively extract amphenicols from different matrices. SHI et al. (SHI et al., 2007; SHI et al., 2010) described the determination of chloramphenicol in honey using MIP as a SPE sorbent (MISPE) compared to both LLE and SPE with C18 sorbent and liquid chromatography coupled to Q-TOF MS. Recoveries obtained with LLE and SPE were about 80% whereas MISPE improved recoveries (92.3 to 99.1%). Florfenicol was also extracted from chicken, fish and honey samples using MIP as sorbent (SADEGHI & JAHANI, 2013). Based on these studies, the use of MWCN and MIP has been limited to the extraction of a single amphenicols from different food matrices.

The combination of LLE and SPE is also a common procedure in the analysis of amphenicols as described in Table 5 for chloramphenicol in honey, milk, egg, meats and feed (CERKVENIK, 2002; MOTTIER et al., 2003; VERZEGNASSI et al., 2003; RAMOS et al., 2003; GUY et al., 2004; FORTI et al., 2005; GALLO et al., 2005; CERKVENIK-FAJS, 2006; ISHII et al., 2006; POLZER et al., 2006; VINAS et al., 2006; TIAN, 2011; MORAGUES et al., 2012; WU et al., 2012; KAUFMANN et al., 2015), florfenicol in honey and feed (HAYES et al., 2009; SADEGHI & JAHANI, 2013) and the amphenicols and florfenicol amine in muscle and liver tissues (SHEN et al., 2009; ALECHAGA et al., 2012). Generally, the solvents and sorbents are similar to those used on LLE and SPE methods. Usually, the use of a second technique during sample preparation (LLE or SPE) is introduced to obtain extracts with less interference. As examples, ALECHAGA et al. (2012) and SHEN et al. (2009) used SPE after LLE since the latter was not able to completely purify the samples for multi amphenicols analysis.

Table 5. Sample preparation using liquid-liquid (LLE) and solid-phase extraction (SPE) for the determination of amphenicols and some metabolites in food (2002-2015)

Analyte / Matrix	LLE	SPE	Recovery (%)	Reference
Chloramphenicol				
Chicken	Ethyl acetate	Silica Sep Pak	86.8	RAMOS et al., 2003
Animal feed	Ethyl acetate	Discovery DSC-18Lt SPE	92.4–98.5	VINAS et al., 2006
Porcine, bovine, ovine, caprine, equine, rabbit, broiler feed	Ethyl acetate	Bond Elut C18	82.0	MORAGUES et al., 2012
Fish	Ethyl acetate	Graphene	92.3–103.4	WU et al., 2012
Milk, honey, egg, fish	Ethyl acetate	Oasis HLB	98–102 (milk), 97–102 (honey), 101–120 (egg), 101–108 (fish)	KAUFMANN et al., 2015
Shrimp, crayfish, prawn	Ethyl acetate and hexane	C18	95.0	POLZER et al., 2006
Bovine, milk	Ethyl acetate and hexane	Extrelut NT 20	88.9 (bovine muscle), 102.2 (milk)	CERKVENIK, 2002
Chicken, turkey, pork, beef, seafood (shrimp, fish flour)	Ethyl acetate and diethyl ether (75:25 v/v)	Silica	60.0±5.0	MOTTIER et al., 2003
Bovine milk	Acetonitrile	SampliQ C18	74.0–87.0	TIAN, 2011
Honey	Acetonitrile:dichloromethane (4:1 v/v)	Oasis HLB	-	VERZEGNASSI et al., 2003
Milk	Trichloroacetic acid 10% (v/v)	Oasis HLB	30.0±4.0	GUY et al., 2004
Honey	Dichloromethane:acetone (1:1 v/v)	C18	98.8	FORTI et al., 2005
Milk	Acetonitrile	AAG afinitty	78.4	GALLO et al., 2005
Muscle	Water and hexane	Extrelut NT	-	CERKVENIK-FAJS, 2006
Royal jelly	1% Metaphosphoric acid:methanol (4:6)	Oasis HLB	95.1±7.0	ISHII et al., 2006
Florfenicol				
Honey	Ethyl acetate	MIP	96.2	SADEGHI & JAHANI, 2013
Swine feed	Acetonitrile:water (1:1 v/v)	ENVI-Carb	99.7	HAYES et al., 2009
Chloramphenicol, Thiamphenicol, Florfenicol and Florfenicol amine				
Poultry, pork (muscle, liver)	Ethyl acetate:ammonium hydroxide (98:2 v/v) and hexane	Oasis HLB	78.5–105.5	SHEN et al., 2009
Prawns, pork, chicken, fish	Acetonitrile and 0.1% acetic acid	Oasis HLB	59.0–90.0	ALECHAGA et al., 2012

[a] – not found.

3.1.3. Miniaturized approaches

Miniaturized approaches have also been used for extraction and clean-up of amphenicols in food and these techniques allow minimized sample size and solvents volumes, making them environmentally friendly. HUANG et al. (2006) described the use of a monolithic capillary microextraction procedure for extraction of chloramphenicol from honey, milk and eggs. The device was composed of an extraction pinhead, a syringe barrel, and replacement of the metallic needle of the pinhead with a poly (MAA-EGDMA) monolith capillary column. Improved recoveries were obtained compared to conventional approaches. Dispersive liquid-liquid microextraction was applied in the analysis of chloramphenicol and thiamphenicol in honey samples. The main advantages of the method were high enrichment factor, high recoveries and reduced extraction solvent volume to µL level (CHEN et al., 2008; CHEN et al., 2009). In another approach, CHEN and LI (2013) developed a method for the analysis of chloramphenicol in honey by means of magnetic molecularly imprinted polymer extraction which provided good recoveries ranging from 84.3 to 90.9%. LI et al. (2012) also used molecularly imprinted polymer for the analysis of thiamphenicol in milk and honey; however, solid-phase microextraction was the sample preparation technique. Improved recoveries were achieved (92.9 to 99.3%).

SNIEGOCKI et al. (2015) used QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) for the extraction of chloramphenicol from milk and dairy products, and obtained good recoveries (97.8 to 102.8%). According to the authors, the main advantage of QuEChERS is that it allows extraction and clean-up in simple steps for all matrices, without additional need for purification of the extracts. Recently, LIU et al. (2016) applied a modified QuEChERS for the analysis of chloramphenicol, thiamphenicol and florfenicol in milk and honey, achieving good recoveries.

3.2. Separation and detection techniques

Due to the high complexity of food matrices and low concentration of amphenicols in food, analytical techniques with high selectivity and sensitivity are needed. Several different analytical techniques are available. However, irrespective of the selected method, adequate limits of detection must be achieved to comply with stringent requirements established for chloramphenicol, which has been banned from food producing animals (LEON et al., 2012; GUIDI et al., 2015).

The most widely used analytical methods for the analysis of amphenicols in food are gas chromatography and high performance liquid chromatography. However, other techniques have also been described in the literature, among them, capillary electrophoresis (KOWALSKI, 2007; KOWALSKI et al., 2008; ZHANG et al., 2008), micellar electrokinetic chromatography (KOWALSKI et al., 2011), molecular imprinted polymers with voltammetric detection (MENA et al., 2003), thin layer chromatography (RAMIREZ et al., 2003), high-throughput suspension array technology (SU et al., 2011) and other less common ones (HUANG et al., 2009; KARA et al., 2013; KOR & ZAREI, 2014; TAN et al., 2015).

Tables 6 and 7 present the methods for separation and detection of amphenicols in food by gas chromatography and liquid chromatography, respectively. The majority of the methods were validated to demonstrate fitness for the purpose. When validation followed Commission Decision 2002/657/EC (EC, 2002), the sensitivity of the method was reported as decision limit (CCα) and the detection capability (CCβ). However, when other validation protocols were used, the limit of detection (LOD) and the limit of quantification (LOQ) were calculated. This is the reason why these tables present all four of these important parameters to assess the sensitivity of the methods. In the majority of the methods, especially when mass spectrometry is involved, isotope labeled standards is used. Matrix matched calibration curves can also be used to compensate for matrix effects that could influence analytical response (EC, 2002; HEWAVITHARANA, 2011; GUIDI et al., 2015).

3.2.1. Gas chromatography

A summary of gas chromatographic procedures described in the literature from 2002 to 2015 for the analysis of amphenicols in food is presented in Table 6. Gas chromatography (GC) has been used to analyze mainly chloramphenicol in different foodstuffs, such as seafood, animal tissues, honey and milk (DING et al., 2005; SANCHEZ-BRUNETE et al., 2005; SANTOS et al., 2005; SHEN et al., 2005; CERKVENIK-FAJS, 2006; POLZER et al., 2006; ZHANG et al., 2006; SNIEGOCKI et al., 2007; REJTHAROVA & REJTHAR, 2009; SILVA et al., 2010). It has also been used to analyze a mixture of the three amphenicols (LI et al., 2006; AZZOUZ & BALLESTEROS, 2015) and a mixture of the three amphenicols plus florfenicol amine (SHEN et al., 2009).

Since amphenicols are polar, non-volatile and thermolabile molecules, prior to GC analysis, they must be transformed into stable volatile compounds. The most widely used derivatization reagents were N,O-*bis*(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) (99:1, v/v) (RAMIREZ et al., 2003; DING et al., 2005; ZHANG et al., 2008; SHI et al., 2010; SU et al., 2011; LEON et al., 2012; AZZOUZ & BALLESTEROS, 2015; SNIEGOCKI et al., 2015) and hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) and pyridine (3:1:9; v/v/v and 2:1:10, v/v/v) (SANCHEZ-BRUNETE et al., 2005; SANTOS et al., 2005; SHEN et al., 2009; SILVA et al., 2010). According to SHEN & JIANG (2005), the sensitivity of BSTFA derivatized products increased with increasing reaction time. However, almost 240 min was required to reach a maximum. When 1% of TMCS was added to BSTFA, the derivatization reaction was completed in 40 min with high sensitivity. Therefore, derivatization was better accomplished at 70 °C for 40 min by using BSTFA + TMCS (99:1) as derivatization agent. It should be highlighted that derivatization is an extra step in sample preparation and can lengthen analysis time, what can affect reproducibility at trace levels (GUIDI et al., 2015).

Gas chromatography has been used for the analysis of chloramphenicol with electron capture – EC (DING et al.,2005; SHEN & JIANG, 2005; CERKVENIK-FAJS, 2006; ZHANG et al., 2006; SILVA et al., 2010) or mass spectrometry – MS detectors (SANTOS et al., 2005; SANCHEZ-BRUNETE et al., 2005; POLZER et al., 2006; SNIEGOCKI et al., 2007; REJTHAROVA & REJTHAR, 2009). MS detectors have also been used for a mixture of amphenicols using MS detectors (LI et al., 2006; SHEN et al., 2009; AZZOUZ & BALLESTEROS, 2015).

Phenyl methylsiloxane (5%) was the most commonly used stationary phase in columns which varied from 30 to 125 m length, 0.20 to 0.32 mm internal diameter and 0.25 to 0.50 µm particle size. The sensitivity of the methods was adequate for the analysis of amphenicols using both mass spectrometry (MS) and electron capture (EC) detectors, achieving limits of quantification of 0.0012-0.0014 (chloramphenicol) and of 0.0014 $\mu g.kg^{-1}$ (thiamphenicol, florfenicol and florfenicol amine) in eggs and honey in poultry and porcine muscle and liver).

Table 6. Gas chromatographic methods for the separation and detection of amphenicols and some metabolites in food (2002-2015)

Analyte / Matrix	Detection	Column	LOD ($\mu g.kg^{-1}$)	LOQ ($\mu g.kg^{-1}$)	CCα ($\mu g.kg^{-1}$)	CCβ ($\mu g.kg^{-1}$)	Reference
Chloramphenicol							
Seafood, meat, honey	ECD	5% diphenyl 95% dimethylpolysiloxane (30 m x 0.25 mm, 0.25 µm)	-	0.1	-	-	SHEN & JIANG, 2005
Muscle tissue	ECD	5% phenyl methylsiloxane (50 m x 0.2 mm, 0.33 µm)	-	-	0.07	0.12	CERKVENIK-FAJS, 2006
Goat milk	ECD	100% dimethylpolysiloxane (60 m x 0.25 mm, 0.25 µm)	0.030	0.10	-	-	SILVA et al., 2010
Fish, shrimp	µ-ECD	5% phenyl methylsiloxane (30 m x 0.32 mm, 0.50 µm)	0.04	0.1	-[a]	-	DING et al., 2005
Chicken (muscle, liver)	µ-ECD	5% phenyl methyl silicone (30 m x 0.32 mm, 0.50 µm)	0.2–2.0	0.05 (muscle), 0.1 (liver)	-	-	ZHANG et al., 2006
Honey	MS	5% phenyl polysiloxane (30 m x 0.25 mm, 0.25 µm)	0.05	0.2	-	-	SANCHEZ-BRUNETE et al., 2005
Rainbow trout	MS	Permabond OV (125 m x 0.25 mm, 0.25 µm)	-	-	-	-	SANTOS et al., 2005
Crustaceans	MS	5% phenyl 95% dimethylpolysiloxane (30 m x 0.25 mm, 0.25 µm)	-	-	0.07	-	POLZER et al., 2006
Milk, honey	MS	100% dimethylpolysiloxane (30 m x 0.25 mm, 0.25 µm)	-	-	0.06–0.2 (honey), 0.03–0.08 (milk)	0.1–0.3 (honey), 0.05–0.1 (milk)	REJTHAROVA & REJTHAR, 2009
Milk	MS/MS	100% dimethylpolysiloxane (30 m x 0.25 mm, 0.25 µm)	-	-	0.083	0.14	SNIEGOCKI et al., 2007
Chloramphenicol, Thiamphenicol and Florfenicol							
Pork, poultry, aquatic products	MS	Phenyl arylene polymer (5% phenyl methylpolysiloxane) (30 m x 0.25 mm, 0.25 µm)	0.03 (CAP), 0.2 (FF, TAP)	-	-	-	LI et al., 2006
Egg, honey	MS	95% polydimethylsiloxane (30 m x 0.25 mm, 0.25 µm)	0.0004 (CAP egg), 0.0005 (CAP honey), 0.0005 (TAP), 0.0005 (FF)	0.0012 (CAP egg), 0.0014 (CAP honey), 0.0014 (TAP), 0.0014 (FF)	-	-	AZZOUZ & BALLESTEROS, 2015

Table 6. (continuation…)

Analyte / Matrix	Detection	Column	LOD ($\mu g.kg^{-1}$)	LOQ ($\mu g.kg^{-1}$)	CCα ($\mu g.kg^{-1}$)	CCβ ($\mu g.kg^{-1}$)	Reference
Chloramphenicol, Thiamphenicol, Florfenicol and Florfenicol amine							
Poultry, pork (muscle, liver)	MS	5% phenyl methylpolysiloxane (30 m x 0.25 mm, 0.25 µm)	0.1 (CAP) 0.5 (TAP, FF, FFA)	0.25 (CAP) 2.0 (TAP, FF, FFA)	-	-	SHEN et al., 2009

[a] – not found; CAP – chloramphenicol; CCα – decision limit; CCβ – capacity of detection; ECD – electron capture detector; FF – florfenicol; LOD – limit of detection; LOQ – limit of quantification ; MS – mass spectrometry; MS/MS – tandem mass spectrometry; TAP – thiamphenicol.

SNIEGOCKI et al. (2007) analyzed chloramphenicol in milk using a 100% dimethylpolysiloxane (300 x 0.25 mm i.d., 0.25 µm) stationary phase and a tandem mass spectrometry detector (MS/MS), finding values for decision limit (CCα) and detection capability (CCβ) of 0.083 and 0.14 $\mu g.kg^{-1}$, respectively. The authors compared the efficiency of this method with a LC-MS/MS procedure and observed similar sensitivity however, the latter provided better validation parameters (recovery, repeatability, and uncertainty) and it was less time consuming. Based on these results, it is possible to analyze chloramphenicol individually or all amphenicols and the metabolite florfenicol amine simultaneously by GC or liquid chromatography and obtain reliable results.

3.2.2. Liquid chromatography

As indicated in Table 7, high performance liquid chromatography (HPLC) associated with mass spectrometry (MS) was the most widely used technique for the analysis of amphenicols in foods from 2002 until 2015. Indeed, LC coupled with MS detection is getting expanded use in quality control laboratories due to the possibility of simultaneously analysis of multiple residues in a sample in a relatively short time.

In the majority of the studies for the analysis of both single and multi-amphenciols by HPLC, the most widely used column was C18 and it provided suitable retention and separation of amphenicols. However, other types of columns were also used for chloramphenicol, among them, C12 (GALLO et al., 2005), amide-C16 (VINAS et al., 2006), and methylcellulose-immobilized reversed-phase (KAWANO et al., 2015). A phenyl column was used to separate thiamphenicol and florfenicol (CHOU et al., 2009). And all amphenicols were separated by means of a C8 column (SNIEGOCKI et al., 2007; EVAGGELOPOULOU & SAMANIDOU, 2013). Most of the HPLC methods used gradient elution with mobile phases comprising of water and acetonitrile (GUY et al., 2004; CHEN et al., 2005; LUO et al., 2010; RODZIEWICZ & ZAWADZKA, 2007; BARRETO et al., 2012; WU et al., 2012 KAWANO et al., 2015; ZHANG et al., 2008) or methanol and water (HUANG et al., 2006; SHI et al., 2007; CHEN et al., 2009; ROCHA SIQUEIRA et al., 2009; HAN et al., 2011b; SADEGHI & JAHANI, 2013; PAN et al., 2015; LU et al., 2016). Such mobile phases were acidified in some studies with formic acid (NICOLICH et al., 2006; RONNING et al., 2006; CHOU et al., 2009; LU et al., 2010; FERNANDEZ-TORRES et al., 2011; TIAN, 2011; LU et al., 2012; FREITAS et al., 2014a; GUIDI et al., 2015; MONTEIRO et al., 2015; REZK et al., 2015; WANG et al.,

2016), acetic acid (SANTOS et al., 2005; QUON et al., 2006; SHERIDAN et al., 2008; CRONLY et al., 2010; CHEN & LI, 2013) or buffer solutions (BOGUSZ et al., 2004; ASHWIN et al., 2005; FORTI et al., 2005; GALLO et al., 2005; ISHII et al., 2006; MARTINS-JÚNIOR et al., 2006; PAN et al., 2006; VINAS et al., 2006; MAMANI et al., 2009; WANG et al., 2011; TAO et al., 2014; KAUFMANN et al., 2015; SAMANIDOU et al., 2015) to improve separation from interferences. Propanol (FEDENIUK et al., 2015; REZK et al., 2015) and triethylamine (XIE et al., 2011) were also used as a mobile phase components. However, good separation and sensitivity of all three amphenicols and florfenicol amine was achieved by simply using acetonitrile and water as mobile phase (ZHANG et al., 2008).

Only in a few studies, ultra-high performance liquid chromatography (UHPLC) was used. It provided the most comprehensive method for the analysis of the three amphenicols (chloramphenicol, thiamphenicol and florfenicol) along with the major florfenicol metabolite – florfenicol amine (ALECHAGA et al., 2012). Separation was obtained by means of a phenyl–hexyl column and methanol and acetate buffer pH 5.0 as mobile phases in gradient elution. It was used in chicken, pork, fish, prawns and honey and achieved complete separation of all analytes in less than 2 minutes. The other UHPLC method reported (ZHAN et al., 2013) was able to separate chloramphenicol, thiamphenicol and florfenicol from 220 veterinary drug residues and other contaminants in infant formulas in less than 4 minutes, providing fast analysis. Furthermore it is environmental friendly as it uses less amounts of solvents.

Several detectors have also been used in the analysis of amphenicols by HPLC in food. The most widely used was mass spectrometry detectors (MS), however, other detectors were also used, including ultraviolet detector (UV) (SHI et al., 2007; CHEN et al., 2009; HAN et al., 2011b; SADEGHI & JAHANI, 2013; LU et al., 2016), diode array detector - DAD (VINAS et al., 2006; MAMANI et al., 2009; EVAGGELOPOULOU & SAMANIDOU, 2013; SAMANIDOU et al., 2015), and fluorescence detector - FLD (XIE et al., 2011). However, most of the detection systems, except for MS, were not sensitive enough to evaluate compliance of samples to legislation regarding chloramphenicol (MPRL values established by current legislation). Therefore, the most recommended approach for the analysis of chloramphenicol in food matrices is liquid chromatography coupled to tandem mass spectrometry detection (MS/MS) with electrospray ionization (ESI).

Table 7. Liquid chromatographic methods for the separation and detection of amphenicols and some metabolites in food (2002-2015)

Analyte / Matrix	Detection	Column	Mobile Phase	LOD (μg.kg^{-1})	LOQ (μg.kg^{-1})	CCα (μg.kg^{-1})	CCβ (μg.kg^{-1})	Reference
Chloramphenicol								
Milk, Shrimp	UV	C18 (250 x 4.6 mm, 5 μm)	A: methanol, B: water (40:60, v/v)	-	-	-	-	SHI et al., 2007
Milk, honey	UV	C18 (250 x 4.6 mm, 5 μm)	A: water, B: methanol (55:45, v/v)	0.1 μg.L^{-1}	0.3 μg.L^{-1}	-	-	HAN et al., 2011a
Shrimp	UV	C18 (250 x 4.6 mm, 5 μm)	A: water, B: methanol	0.8	1.0		-	LU et al., 2016
Milk	DAD	C18	A: 0.075 M sodium acetate, 0.035 M calcium chloride, 0.025 M NaEDTA, pH 7, B: methanol:acetonitrile (75:25, v/v)	20 μg.L^{-1}	60 μg.L^{-1}	-	-	MAMANI et al., 2009
Bran, soya, calf, cow, bull	DAD	Amide C16 (150 x 4.6 mm, 5 μm)	A: acetonitrile, B: 10 mM monopotassium phosphate, pH 5 (20/80, v/v)	0.7	-	-	-	VINAS et al., 2006
Milk, honey, egg, fish	HRMS	C18 (50 x 2.1 mm, 1.7 μm)	A: 10 mM ammonium acetate - methanol (8:2, v/v) + 0.37 mL ammonium hydroxide (25%), B: methanol	0.05	-	0.01 (milk), 0.01 (honey), 0.02 (egg), 0.01 (fish)	0.01 (milk), 0.02 (honey), 0.03 (egg), 0.02 (fish)	KAUFMANN et al., 2015
Honey, milk, eggs	ESI-MS	C18 (150 x 2.1 mm, 3.5 μm)	A: methanol-water (10:90, v/v), B: Methanol	0.02 (honey), 0.04 (milk, egg)	0.07 (honey), 0.14 (milk, egg)	-	-	HUANG et al., 2006
Honey	ESI-MS	C18 (100 & 250 x 4.6 mm, 5 μm)	A: methanol, B: 0.2% ammonium acetate (45:55, v/v)	-	-	0.002	0.006	PAN et al., 2006
Seafood	ESI-MS/MS	C18 (150 x 2.1 mm, 3.5 μm)	A: 2% NH_4OH, B: acetonitrile (60:40, v/v)	-	0.02	-	-	GIKAS et al., 2004
Milk powders	ESI-MS/MS	C18 (150 x 2.1 mm, 3.5 μm)	A: water, B: acetonitrile	-	-	0.02	0.03	GUY et al., 2004
Honey	ESI-MS/MS	C18 (7.5 x 4.6 mm, 3 μm)	A: methanol, B: ammonium acetate, (60:40, v/v)	-	-	0.07	0.10	FORTI et al., 2005

Table 7. (continuation…)

Analyte / Matrix	Detection	Column	Mobile Phase	LOD (µg.kg^{-1})	LOQ (µg.kg^{-1})	CCα (µg.kg^{-1})	CCβ (µg.kg^{-1})	Reference
Honey, kidney	ESI-MS/MS	C18 (50 x 2.1 mm, 1.7 µm)	A: 1 mL ammonia (25%) in 1 L 10% acetonitrile, B: 1 mL ammonia (25%) in 1 L acetonitrile	0.02 – 0.04	-	0.007–0.019	0.013–0.023	KAUFMANN & BUTCHER, 2005
Rainbow trout	ESI-MS/MS	C18 (150 x 2.1 mm, 5 µm, C8 pre-column	A: water- acetic acid (1000:1 v/v), B: water-acetonitrile-acetic acid (1:9:0.001)	-	-	0.267	0.454	SANTOS et al., 2005
Honey	MS/MS	C18 (150 x 2.0 mm, 3.5 µm)	A: 0.1% acetic acid, B: acetonitrile	0.16	0.21	-	-	QUON et al., 2006
Honey, royal jelly	ESI-MS/MS	C18	A: 10 mM ammonium acetate, B: Acetonitrile	-	0.3 (honey), 1.5 (royal jelly)	-	-	ISHII et al., 2006
Milk, honey	ESI-MS/MS	C18 (50.0 x 2.1 mm, 3 µm)	A: 5.0 mM ammonium acetate, B: methanol/water (95:5, v/v)+ 5 mM ammonium acetate	0.00052	0.00185	-	-	MARTINS-JÚNIOR et al., 2006
Meat, seafood, egg, honey, milk, plasma, urine	ESI-MS/MS	C18 (55 x 4.0 mm, 3 µm)	A: 0.15% formic acid in water, B: methanol	-	-	0.02	0.04	RONNING et al., 2006
Honey	ESI-MS/MS	C18 (150 x 2.0 mm, 5 µm)	A: water, B: acetonitrile, (80:20, v/v)	-	-	0.1	0.14	RODZIEWICZ & ZAWADZKA, 2007
Milk	ESI-MS/MS	C8 (150 x 2.0 mm, 3 µm)	A: 5 mM ammonium formate, B: acetonitrile	-	-	0.11	0.15	SNIEGOCKI et al., 2007
Honey	ESI-MS/MS	C18 (150 x 2.1 mm, 3.5 µm)	A: 0.15% acetic acid, B: 0.15% acetic acid in methanol	0.2	0.6	-	-	SHERIDAN et al., 2008
Poultry, egg, shrimp, fish, swine, bovine	ESI-MS/MS	C18 (100 x 2.1 mm, 4 µm)	A: water, B: methanol	0.03	0.1	-	NI	ROCHA SIQUEIRA et al., 2009
Milk, honey	ESI-MS/MS	C18 (100 x 2.0 mm, 1.8 µm)	A: 0.1% acetic acid, B: acetonitrile with 0.1% acetic acid	-	-	0.07 µg.L^{-1} (milk), 0.08 (honey)	0.11 µg.L^{-1} (milk), 0.13 (honey)	CRONLY et al., 2010

Table 7. (continuation…)

Analyte / Matrix	Detection	Column	Mobile Phase	LOD (µg.kg^{-1})	LOQ (µg.kg^{-1})	CCα (µg.kg^{-1})	CCβ (µg.kg^{-1})	Reference
Egg, honey, milk	ESI-MS/MS	C18 (50 x 2.1 mm, 2.7 µm)	A: 0.1% formic acid, B: acetonitrile	0.003 – 0.004	0.008 – 0.012	0.006 – 0.009	0.008 – 0.011	LU et al., 2010
Milk	ESI-MS/MS	C18 (150 x 2.1 mm, 5 µm)	A: 0.2% formic acid, B: methanol	0.3	1.5	-	-	TIAN, 2011
Milk	ESI-MS/MS	C18 (150 x 2.1 mm i.d., 1.8 µm)	A: 5 mM ammonium acetate, B: methanol (60:40, v/v)	0.05	0.2	0.07	0.11	WANG et al., 2011
Honey, fish, prawns	ESI-MS/MS	C18 (150 x 4.6 mm, 5 µm), C18 (100 x 2.1 mm, 3.5 µm)	A: acetonitrile, B: water	0.02	0.06	0.04–0.05	0.06–0.09	BARRETO et al., 2012
Soft-shelled turtle	ESI-MS/MS	C18 (50 x 2.1 mm, 2.7 µm)	A: 0.1% formic acid, B: acetonitrile	0.075	0.250	-	-	LU et al., 2012
Honey	MS/MS	C18 (50 x 2.0 mm, 5 µm)	A: 2mM ammonium acetate, B: methanol	0.04	0.11	0.08	0.12	TAKA et al., 2012
Fish	ESI-MS/MS	C18 (150 x 2.1 mm, 5 µm)	A: acetonitrile, B: water (30:70, v/v)	0.036	0.12	-	-	WU et al., 2012
Honey	ESI-MS/MS	C18 (250 x 4.6 mm, 5 µm)	A: 0.3% acetic acid, B: acetonitrile (50:50, v/v)	0.047	0.156	-	-	CHEN & LI, 2013
Honey, shrimp, poultry	ESI-MS	C18 (150 x 2.1 mm, 3 - 3.5 µm)	A: methanol, B: 0.1 % ammonium hydroxide	-	-	0.03–0.07	0.04–0.08	DOUNY et al., 2013
Bovine	ESI-MS/MS	C18 (100 x 2.1 mm, 1.8 µm)	A: formic acid 0.1% (v/v), B: acetonitrile	-	-	0.07	0.10	FREITAS et al., 2014a
Milk	ESI-MS/MS	C8 (75 x 2.1 mm, 2.6 µm)	A: 5% isopropanol in 0.1% acetic acid, B: 5% isopropanol in ethanol	-	-	0.06–0.10	0.08–0.15	SNIEGOCKI et al., 2015
Milk	ESI-MS/MS	C12 (250 x 3.0 mm, 4 µm)	A: 20 mM ammonium acetate, pH 4.6, B: acetonitrile, (60:40, v/v)	-	-	-	-	GALLO et al., 2005
Milk	ESI-MS/MS	NI (100 x 20 µm, 5 µm)	A: 0.1 % formic acid , B: 0.1% formic acid in acetonitrile	-	-	0.05 µg.L^{-1}	0. µg.L^{-1}	NICOLICH et al., 2006

Table 7. (continuation…)

Analyte / Matrix	Detection	Column	Mobile Phase	LOD (µg.kg^{-1})	LOQ (µg.kg^{-1})	CCα (µg.kg^{-1})	CCβ (µg.kg^{-1})	Reference
Honey	ESI-MS/MS	Methylcellulose (75 x 2.0 mm, 2.2 µm)	A: water, B: acetonitrile	-	0.2	-	-	LI et al., 2006
Florfenicol								
Chicken, fish, honey	UV	C18 (250 x 4.6 mm)	A: methanol, B: water (30:70, v/v)	-	-	-	-	SADEGHI & JAHANI, 2013
Fish	ESI-MS/MS	C18 (50 x 3 mm, 2.7 µm)	A: 0.1% formic acid, B: methanol with 0.1% formic acid	-	1.0	-	-	REZK et al., 2015
Florfenicol amine								
Bovine, equine, porcine (kidney, liver, muscle)	MS/MS	C18 (50 x 2.1 mm, 3 µm)	A: 0.1% acetic acid, 0.05% formic acid, B: 10:90 isopropanol:methanol, C: acetonitrile	33	110	-	-	FEDENIUK et al., 2015
Chloramphenicol and Thiamphenicol								
Honey	VWD	C18 (250 x 4.6 mm, 5 µm)	A: methanol, B: water (55:45, v/v)	0.6 (CAP), 0.1 (TAP)	1.6 (CAP), 1.2 (TAP)	-	-	CHEN et al., 2009
Fish, mussel	ESI-MS/MS	C18 (150 x 4.6 mm, 5 µm)	A: 0.1% formic acid, pH 2.6, B: acetonitrile	3.0	9.0 -10.0	2.0	3.0	FERNANDEZ-TORRES et al., 2011
Chloramphenicol and Florfenicol								
Fish	ESI-MS/MS	C18 (100 x 3.0 mm, 3.5 µm)	A: 0.1% formic acid, B: acetonitrile+0.1% formic acid	1.0 (CAP), 1.10 (FF)	3.5 (CAP), 3.6 (FF)	-	-	MONTEIRO et al., 2015
Thiamphenicol and Florfenicol								
Pork (muscle, liver, kidney), beef (muscle, liver), fish, chicken	ESI-MS/MS	Phenyl (100 x 2.1 mm, 3.5 µm)	A: 0.1 % formic acid, B: methanol, (75:25, v/v)	-	1.0	-	-	CHOU et al., 2009
Chloramphenicol and metabolite								
Honey, pork kidney, dairy, prawns	MS/MS	C18 (125 x 2.0 mm, 5 µm)	A: 10 mM ammonium acetate, B: methanol (55:45, v/v)	-	-	0.05–0.09	0.09–0.17	ASHWIN et al., 2005

Table 7. (continuation…)

Analyte / Matrix	Detection	Column	Mobile Phase	LOD (µg.kg^{-1})	LOQ (µg.kg^{-1})	CCα (µg.kg^{-1})	CCβ (µg.kg^{-1})	Reference
Chicken, shrimp, honey	ESI-MS/MS	C18 (125 x 3.0 mm, 4 µm)	A: acetonitrile, B: 10 mM ammonium formate, pH 3.0 (40:60 v/v)	0.05 – 0.1	0.1 – 0.2	-[a]	-	BOGUSZ et al., 2004
Thiamphenicol, Florfenicol and Florfenicol amine								
Eggs	FLD	C18 (250 x 4.6 mm, 5 µm)	A: acetonitrile 0.01 M sodium dihydrogen phosphate + 0.005 M sodium dodecyl sulfate, B: 0.1% triethylamine, pH 4.8 (35:65, v/v)	1.5 (TAP & FF), 0.5 (FFA)	5.0 (TAP & FF), 2.0 (FFA)	-	-	XIE et al., 2011
Swine	ESI-MS/MS	C18 (150 x 2.1 mm, 5 µm)	A: acetonitrile, B: water	1.2 (TAP), 0.6 (FF), 0.12 (FFA)	4.0 (TAP), 2.0 (FF), 0.4 (FFA)	-	-	LUO et al., 2010
Chloramphenicol, Thiamphenicol and Florfenicol								
Milk, fish	ESI-MS/MS	C18 (50 x 2.0 mm, 5 µm)	A: 0.1% formic acid, B: acetonitrile+ 0.1% formic acid	0.019 (CAP, fish)	-	-	-	GUIDI et al., 2015
Chloramphenicol, Thiamphenicol, Florfenicol and Florfenicol amine								
Fish	DAD	C8 (250 x 4.0 mm, 5 µm)	A: 0.05 M ammonium acetate, B: acetonitrile	11.0 – 14.8	33.2 – 44.8	51.3 (TAP), 3.3 (CAP), 1019.5 (FF)	53.3 (TAP), 54.9 (CAP), 1022.2 (FF)	EVAGGELOPOULOU & SAMANIDOU, 2013
Milk	DAD	C18 (250 x 4.0 mm, 5 µm)	A: 0.05 M ammonium acetate, B: acetonitrile	-	-	53.8 (CAP), 52.49 (TAP), 55.23 (FF)	55.9 (CAP), 56.80 (TAP), 58.99 (FF)	SAMANIDOU et al., 2015
Chicken, pork, fish, prawns, honey	HESI-MS/MS	Phenyl-hexyl (100 x 2.1 mm, 2.7 µm)	A: methanol, B: acetic acid–ammonium acetate buffer 5 mM, pH 5	-	<0.1 – 1.0	0.1–121	0.2–138	ALECHAGA et al., 2012
Chicken	ESI-MS/MS	C18 (100 x 2.1 mm, 5 µm)	A: acetonitrile, B: water	0.1 (CAP), 0.2 (FF), 1.0 TAP and FFA)	0.3 (CAP), 0.5 (FF), 3.0 (TAP and FFA)	0.07 (CAP), 3.41 (TAP), 0.57 (FF), 3.40 (FFA)	0.11 (CAP), 3.83 (TAP), 0.64 (FF), 3.81 (FFA)	ZHANG et al., 2008

Table 7. (continuation…)

Analyte / Matrix	Detection	Column	Mobile Phase	LOD (µg.kg^{-1})	LOQ (µg.kg^{-1})	CCα (µg.kg^{-1})	CCβ (µg.kg^{-1})	Reference
Shrimp, fish	ESI-MS/MS	C18 (150 x 2.1 mm, 5 µm)	A: 0.1% formic acid with 5 mM ammonium acetate, B: methanol	-	-	0.01 (CAP), 0.07–0.09 (TAP), 0.01–0.02 (FF), 0.04–0.05 (FFA)	0.04–0.09 (CAP), 0.13–0.25 (TAP), 0.05–0.07 (FF), 0.11–0.18 (FFA)	TAO et al., 2014
Chicken	ESI-MS/MS	C18 (150 x 2.1 mm, 3.5 µm)	A: water, B: acetonitrile	0.010	0.100	-	-	CHEN et al., 2005
Infant formula	ESI-MS/MS	C18 (100 x 2.1 mm, 1.8 µm)	Positive ESI mode, A: 0.1% formic acid+0.5 mM ammonium acetate, B: methanol+0.1% formic acid. Negative ESI mode, A: 2.5 mM ammonium acetate, B: methanol	-	0.2 – 1.0	-	-	ZHAN et al., 2013
Milk, butter, fish, eggs	ESI-MS/MS	C18 (100 x 2.1 mm, 3 µm)	A: 1 mM ammonium formate, B: methanol, C: acetonitrile	Butter (0.21 CAP, 0.16 TAP, 0.14 FF), Egg (0.16 CAP, 0.22 TAP, 0.16 FF), Fish (0.17 CAP, 0.06 TAP, 0.08 FF), Milk (0.26 CAP, 0.30 TAP, 0.27 FF)	Butter (0.64 CAP, 0.49 TAP, 0.43 FF), Egg (0.49 CAP, 0.65 TAP, 0.47 FF), Fish (0.51 CAP, 0.18 TAP, 0.24 FF), Milk (0.79 CAP, 0.90 TAP, 0.81 FF)	-	-	DASENAKI & THOMAIDIS, 2015
Fish	ESI-MS/MS	C18 (50 x 2.1 mm, 1.7 µm)	A: methanol, B: water	-	-	0.02 (CAP), 0.06 (TAP), 0.02 (FF)	0.11 (CAP), 0.16 (TAP), 0.10 (FF)	PAN et al., 2015
Milk	ESI-MS/MS	C18 (50 x 2.1 mm, 1.7 µm)	A: 0.1% formic acid, B: acetonitrile+ 0.1% formic acid	0.020, 0.003, 0.008	0.050, 0.010, 0.020		-	WANG et al., 2016

[a] – not found; CAP – chloramphenicol; CAP-Glu – Chloramphenicol glucuronide; CCα – decision limit; CCβ – capacity of detection; DAD – diode array detector; ESI – electrospray ionization; FF – florfenicol; FFA – florfenicol amine; FLD – fluorescence detector; HRMS – high resolution mass spectrometry; LOD – limit of detection; LOQ – limit of quantification; MS – mass spectrometry; MS/MS – tandem mass spectrometry; TAP – thiamphenicol; UV – ultraviolet detector; VWD – variable wavelength detector.

The detection system of choice is MS, which allows detection, confirmation and quantification of many compounds simultaneously (JIMENEZ et al., 2011). Furthermore, when a chromatograph is coupled to a MS detector, it is possible to develop methods with high selectivity, efficient separation and also to know about structural information and molar mass (CHIARADIA et al., 2008). Moreover, a system combining LC with mass spectrometry detection (LC-MS/MS) can substantially reduce analysis time and can be used as a confirmatory method.

In the majority (64%) of the LC studies reported in the literature from 2002 to 2015, chloramphenicol was the only amphenicols investigated, and the most frequently analyzed sample was honey, followed by fish/seafood and milk. In some of the studies, the sensitivity required for chloramphenicol was not always achieved, and therefore, the method would not fit the purpose. However, some developed methods were adequate and sensitive for the analysis of chloramphenicol, for example, very low limits of detection and quantification were achieved - 0.00052 and 0.00185 $\mu g.kg^{-1}$, respectively, for milk and honey, using a C18 column and an ESI-MS/MS detector (MARTINS-JÚNIOR et al., 2006).

4. OCCURRENCE OF AMPHENICOLS IN FOOD

As summarized in Table 8, several studies were undertaken to investigate the presence of amphenicols in food of animal origin. However, the number of samples analyzed is very limited especially considering the several variables which can be associated with food production, among them, breed, feed, practices, location, processing and storage.

Only 26.3% of the studies (n=5) investigated the three amphenicols simultaneously, whereas 5.3% (n=1) determined thiamphenicol, florfenicol and florfenicol amine in egg and 5.3% (n=1) determined chloramphenicol and florfenicol in fish. Most of the studies were focused on the quantification of individual amphenicols, either chloramphenicol (57.9%) in several food matrices or florfenicol (5.3%) in fish. Fish, milk, honey and eggs were the most frequently analyzed food matrices, representing, respectively, 27.3, 24.2, 18.2 and 18.2% of the six types of food analyzed. Based on these results, even though banned in food producing animals, chloramphenicol is still the amphenicol of major concern, mainly in milk and fish.

Table 8. Prevalence and levels of amphenicols and some metabolites in different food matrices from 2002 to 2016

Analyte / Matrix	Samples		Method / LOD ($\mu g.kg^{-1}$)	Concentration in positive samples ($\mu g.kg^{-1}$)	Country / Reference
	Analyzed	Positive (%)			
Chloramphenicol					
Beef	430	0	GC-ECD	n.a.	Slovenia (CERKVENIK, 2002)
Pork	271	0	1.0	n.a.	
Poultry	235	0		n.a.	
Rabbits	2	0		n.a.	
Milk	286	0.3		4.6	
Egg	45	0		n.a.	
Fish	39	0		n.a.	
Honey	176	21.6	LC-ESI-MS/MS < 0.1	0.1–75.0	Argentina, Australia, Cuba, Thailand, China (VERZEGNASSI et al., 2003)
Rainbow trout	15	22.5	LC-MS/MS 0.454	1.58–3.94	Portugal (SANTOS et al., 2005)
Honey	4	0	LC-ESI-MS/MS	n.a.	Brazil (MARTINS-JÚNIOR et al., 2006)
Milk	7	42.8	0.00052 $\mu g.L^{-1}$	0.0047–0.0061 $\mu g.L^{-1}$	
Milk	41	0	LC-ESI-MS/MS 0.09 $\mu g.L^{-1}$	n.a.	Brazil (NICOLICH et al., 2006)
Honey	116	9	LC-ESI-MS/MS 0.2	91.0[a]	China, Russia, Georgia, Moldova (SHERIDAN et al., 2008)
Beef	149	0	LC-ESI-MS/MS	n.a.	Brazil (ROCHA SIQUEIRA et al., 2009)
Pork	199	0	0.03	n.a.	
Egg	60	0		n.a.	
Shrimp	14	0		n.a.	
Poultry	208	0		n.a.	
Fish	16	0		n.a.	
Egg	10	0	LC-ESI-MS/MS	n.a.	China (LU et al., 2010)
Honey	10	0	0.004	n.a.	
Milk	10	10	0.003	< 300 $\mu g.L^{-1}$	
			0.003 $\mu g.L^{-1}$		
Milk	5	0	High-throughput suspension array technology 25 $\mu g.L^{-1}$	n.a.	China (SU et al., 2011)
Milk	50	8%	LC-ESI-MS/MS 0.05	> 0.45	China (WANG et al., 2011)
Honey	5	0	LC-ESI-MS/MS	n.a.	Brazil (BARRETO et al., 2012)
Fish	21	0	0.02	n.a.	
Fish	8	12.5%	LC-ESI-MS/MS 0.036	0.14	China (WU et al., 2012)
Florfenicol					
Fish	25	20%	HPLC-ESI-MS/MS[b]	70.85±1.67	Egypt (REZK et al., 2015)
Chloramphenicol and Florfenicol					
Fish	36	8.3% (FF)	LC-ESI-MS/MS 1.0 (CAP), 1.1 (FF)	521.2–528.0	Brazil (MONTEIRO et al., 2015)

Table 8. (continuation…)

Analyte / Matrix	Samples		Method / LOD (µg.kg^{-1})	Concentration in positive samples (µg.kg^{-1})	Country / Reference
	Analyzed	Positive (%)			
Chloramphenicol, Thiamphenicol and Florfenicol					
Shrimp	8	62.5% (FF)	NCI-GC/MS 0.0087–0.00174	47–592	Taiwan (LIU et al., 2010)
Egg Honey	11 6	27.3% (FF) 0	GC-MS 0.0004 (CAP egg) 0.0005 (CAP honey, TAP and FF)	1.7–2.5 n.a.	Spain (AZZOUZ & BALLESTEROS, 2015)
Milk powder Butter Fish Egg	73 5 22 8	0 0 0 0	LC-ESI-MS/MS 0.16-0.26 (CAP) 0.06-0.30 (TAP) 0.08-0.27 (FF)	n.a. n.a. n.a. n.a.	Greece (DASENAKI & THOMAIDIS, 2015)
Fish	25	4% (CAP)	UPLC-ESI-MS/MS 0.11 (CAP) 0.16 (TAP) 0.10 (FF)	1.8	China (PAN et al., 2015)
Milk	25	8% (TAP)	UHPLC-ESI-MS/MS 0.020 (CAP) 0.003 (TAP) 0.008 (FF)	0.6–1.7	China (WANG et al., 2016)
Thiamphenicol, Florfenicol and Florfenicol amine					
Egg	50	2% (FF) 2% (FFA)	HPLC-FLD 1.5 (TAP, FF), 0.5 (FFA)	19 36	China (XIE et al., 2011)

[a] Maximum concentration cound; [b] not found; CAP - chloramphenicol; DAD – diode array detector; ECD – electron capture detector; ESI – electrospray ionization; FF – florfenicol; FFA – florfenicol amine; FLD – fluorescence detector; GC – gas chromatography; HPLC – high performance liquid chromatography; LC – liquid chromatography; LOD – limit of detection; MS – mass spectrometry; MS/MS – tandem mass spectrometry; n.a. – not applicable; NCI – electron-capture negative chemical ionization TAP – thiamphenicol; UPLC – ultra performance liquid chromatography; UPLC – ultra high performance liquid chromatography.

Chloramphenicol was detected in different food matrices. The highest prevalence was in milk (42.8%), followed by fish and honey (22.5% and 21.6%, respectively) (VERZEGNASSI et al., 2003; SANTOS et al., 2005; MARTINS-JÚNIOR et al., 2006). Higher levels of chloramphenicol were found in honey (75–91 µg.kg^{-1}) (VERZEGNASSI et al., 2003; SHERIDAN et al., 2008). Fish also contained chloramphenicol (0.14–3.94 µg.kg^{-1}) (SANTOS et al., 2005; WU et al., 2012; PAN et al., 2015). Several food samples contained chloramphenicol at levels above the MRPL (CERKVENIK, 2002; MARTINS-JÚNIOR et al., 2006; LU et al., 2010; WANG et al., 2011). These results indicate that the use of chloramphenicol in food producing animals is still a possibility. Chloramphenicol in foods can result from administration of prohibited antibiotics. It is

also important to consider that there are other possible sources of food contamination with chloramphenicol, among them, its use as a human antimicrobial agent, release and contamination of waste streams by which food may be contaminated; and its natural occurrence in soil by bacteria (GUIDI et al., 2015; HANEKAMP & BAST, 2015; SNIEGOCKI et al., 2015).

The highest prevalence of florfenicol was in shrimp (62.5%), followed by eggs (27.3%) and fish (20%). The highest levels were found in shrimp (592 $\mu g.kg^{-1}$), followed by fish (528.0 $\mu g.kg^{-1}$) and egg (2.5 $\mu g.kg^{-1}$) (LIU et al., 2010; AZZOUZ & BALLESTEROS, 2015; MONTEIRO et al., 2015; REZK et al., 2015). In some samples, contents exceeded the MLR established by some countries, even though the contents of florfenicol amine were not determined and included in the total florfenicol levels as determined by legislation. These results suggest the use of prohibited antibiotics (e.g., use in animals from which eggs are produced), and administration of excessive levels or failure to respect the proper withdrawal periods (GUIDI et al., 2015; HANEKAMP & BAST, 2015; SNIEGOCKI et al., 2015).

Even though thiamphenicol was investigated in different types of matrices, it was only detected in milk samples at 8% occurrence, at levels varyed from 0.6 to 1.7 $\mu g.kg^{-1}$, which are below the MRL established by Brazil (10 $\mu g.kg^{-1}$) and by the European Union (50 $\mu g.kg^{-1}$) (EC, 2010a; BRASIL, 2015; WANG et al., 2016). Low occurrence of thiamphenicol is probably associated with its higher cost compared to florfenicol.

5. CONCLUSIONS AND PERSPECTIVES

Most of the studies found in the literature on the analysis of amphenicols in food used conventional techniques for sample preparation, such as liquid-liquid and/or solid-phase extraction. However, the tendency nowadays is the use of miniaturized techniques, which are advantageous as they use reduced amount of sample and less solvents generating fewer residues to the environment. However, these miniaturized methods still have limitations such as the need for more steps, applicability to a smaller number of analytes, low availability of commercial extraction phases and the limited amount of research studies to attest the efficiency and the robustness of the technique. Therefore, improvements are still needed.

Although GC and LC have been widely used, the best approach is to use LC-ESI-MS/MS especially for the analysis of chloramphenicol which has been banned from food producing animals. It is a selective and efficient system to detect trace levels of amphenicols and other contaminants. Nevertheless, the availability of this equipment in laboratories is still unusual, due to elevated price and requirement of specialized personnel to its operation. Although UHPLC is an advantageous technique when compared to conventional HPLC, only few studies using this technique were found. Although chloramphenicol is forbidden in several countries, it has been found in many food matrices at levels from 0.14 to 592 $\mu g.kg^{-1}$. Milk was the matrix that had more positive samples with occurrence varying from 0.3% to 42.8%. Only milk presented positive samples for thiamphenicol, with 8% of occurrence at levels from 0.6 to 1.7 $\mu g.kg^{-1}$, which are below the Maximum Residue Limit (MRL – 50 $\mu g.kg^{-1}$) established by the European Union. All the positive samples for florfenicol were also below the MRL established by the European Union, however in most of the methods, florfenicol amine, which must be added to florfenicol levels for legislation compliance, is seldom included in the methods available for amphenicols analysis.

In this context, the need for improved rapid and sensitive methods for the continuous monitoring of the levels of amphenicols in food matrices is obvious.

CAPÍTULO III - A SIMPLE, FAST AND SENSITIVE SCREENING LC-ESI-MS/MS METHOD FOR ANTIBIOTICS IN FISH

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A simple, fast and sensitive screening LC-ESI-MS/MS method for antibiotics in fish

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ABSTRACT

The objective of this study was to develop and validate a fast, sensitive and simple liquid chromatography–electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) method for the screening of six classes of antibiotics (aminoglycosides, beta-lactams, macrolides, quinolones, sulfonamides and tetracyclines) in fish. Samples were extracted with trichloroacetic acid. LC separation was achieved on a Zorbax Eclipse XDB C18 column and gradient elution using 0.1% heptafluorobutyric acid in water and acetonitrile as mobile phase. Analysis was carried out in multiple reaction monitoring mode via electrospray interface operated in the positive ionization mode, with sulfaphenazole as internal standard. The method was suitable for routine screening purposes of 40 antibiotics, according to EC Guidelines for the Validation of Screening Methods for Residues of Veterinary Medicines, taking into consideration threshold value, cut-off factor, detection capability, limit of detection, sensitivity and specificity. Real fish samples (n=193) from aquaculture were analyzed and 15% were positive for enrofloxacin (quinolone), one of them at a higher concentration than the level of interest (50 $\mu g\,kg^{-1}$), suggesting possible contamination or illegal use of that antibiotic.

ABSTRACT

The objective of this study was to develop and validate a fast, sensitive and simple liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) method for the screening of six classes of antibiotics (aminoglycosides, beta-lactams, macrolides, quinolones, sulfonamides and tetracyclines) in fish. Samples were extracted with trichloroacetic acid. LC separation was achieved on a Zorbax Eclipse XDB C18 column and gradient elution using 0.1% heptafluorobutyric acid in water and acetonitrile as mobile phase. Analysis was carried out in multiple reaction monitoring mode via electrospray interface operated in the positive ionization mode, with sulfaphenazole as internal standard. The method was suitable for routine screening purposes of 40 antibiotics, according to EC Guidelines for the Validation of Screening Methods for Residues of Veterinary Medicines, taking into consideration threshold value, cut-off factor, detection capability, limit of detection, sensitivity and specificity. Real fish samples (n=193) from aquaculture were analyzed and 15% were positive for enrofloxacin (quinolone), one of them at a higher concentration than the level of interest (50 µg.kg-1), suggesting possible contamination or illegal use of that antibiotic.

Keywords: aminoglycoside; beta-lactam; macrolide; quinolone; sulfonamide; tetracycline; aquaculture.

1. INTRODUCTION

Aquaculture is one of the food-producing systems with the highest growth in the world and today it accounts for nearly 50% of the world's food fish (FAO, 2016). However, intensive systems of animal food production are favorable to the spread of infectious diseases due to high population density. This is specially so in aquaculture, as the aquatic environment is prone to disease proliferation. In addition, abrupt physico-chemical changes in the aquatic environment and inappropriate management practices can directly affect the health of the fish (QUESADA et al., 2013b). For these reasons, the use of antibiotics in aquaculture is a common practice in the treatment of diseases. In addition, antibiotics can be used as prophylactic agents to avoid or prevent diseases and also as a feed additive to promote growth and increase feed efficiency (BLASCO et al., 2007; GASTALHO et al., 2014; GUIDI et al., 2015).

Many antibiotics are allowed for use in aquaculture worldwide, and varying classes are permitted in different countries. As examples, tetracycline, oxytetracycline (tetracyclines), oxolinic acid, flumequine, enrofloxacin (quinolones), amoxicylin (β-lactam), erythromycin (macrolide), sulfadimethoxine (sulfonamide), ormetoprim (diaminopyrimidine) and florfenicol (amphenicol) can be cited. The first two are the most widely used (WHO, 1998; FAO, 2005). Antibiotics are administered through the diet or are released directly into surface waters and, after metabolism, antibiotics and/or their metabolites can end up in tissues or can be excreted through urine and feces. Therefore, there can be accumulation of antibiotics in water and sediments which can contaminate the aquatic ecosystem (HALLING-SØRENSEN, 1998; CDDEP, 2015). In addition, some antibiotics from intensive livestock can also be released into the environment and reach water resources (HALLING-SØRENSEN, 1998; BOXALL et al., 2003; REGITANO & LEAL, 2010; XIONG et al., 2015).

The inappropriate and abusive use of antibiotics however can be a potential public health hazard once their residues can remain in the fish muscle (SANTOS & RAMOS, 2016). For example, residues of tetracyclines and sulfonamides (MENDOZA et al., 2012), chloramphenicol (WU et al., 2012; GUIDI et al., 2015), oxytetracycline (MONTEIRO et al., 2015; MONTEIRO et al., 2016), enrofloxacin (DASENAKI & THOMAIDIS, 2015; REINHOLDS et al., 2016) and florfenicol (MONTEIRO et al., 2015; REZK et al., 2015; MONTEIRO et al., 2016) have been detected in fish. Furthermore, it can remain in the water and sediment from aquaculture systems. Indeed, MONTEIRO

et al. (2015 e 2016) detected oxytetracycline, tetracycline and florfenicol in different fish farms and tetracycline antibiotics were found in river sediments.

Among health hazard issues to man, antibiotics in food can induce allergic reactions in some sensitive individuals. Furthermore, it can compromise human intestinal and immune systems, lead to the appearance of bacterial resistance in humans and animals, and affect the environment selecting the most resistant bacteria (GASTALHO et al., 2014; GUIDI et al., 2015; SANTOS & RAMOS, 2016). Several regulatory agencies established Maximum Residue Limits (MRL) for antimicrobials in food of animal origin (Table 1), and concentrations above the MRL are inappropriate for human consumption.

In order to warrant public health safety and to maintain competitiveness in international trade, the monitoring of antibiotics in fish and other foods of animal origin is needed. Therefore, sensitive and reliable analytical methods for the determination of multi-antibiotics in food are required. The effective control of antibiotics in foods requires the combination of cost effective and high sample throughput screening methods, followed by confirmation and quantification of suspect samples (SAMSONOVA et al., 2012; GUIDI et al., 2015). Liquid chromatography coupled to mass spectrometry in tandem (LC-MS/MS) has been used in the analysis of multi-antibiotics in food, both for screening and quantitative methods (GAUGAIN-JUHEL et al., 2009; LOPES et al., 2011; VILLAR-PULIDO et al., 2011; MENDOZA et al., 2012; FREITAS et al., 2013; FREITAS et al., 2014a; FREITAS et al., 2015; JANK et al., 2015; CHEN et al., 2016; DO et al., 2016; MARTINS et al., 2016; MONTEIRO et al., 2016; MORETTI et al., 2016). Analytical methods using bioassay techniques or sensitive microorganisms are widely used as screening methods (PETERS et al., 2009). However, the use of LC-MS for screening purposes is becoming popular as it can provide good specificity, sensitivity, and low rate of false-positive samples (GENTILI et al., 2005; BOSCHER et al., 2010; CHÁFER-PERICÁS et al., 2010; LOPES et al., 2011; CHEN et al., 2016). Through determination of the cut-off factor in a screening method, it is possible to evaluate if the sample contains or not the antibiotic in a concentration above MRL (EC, 2010b). Since in most of the cases the samples are expected to comply, reports can be issued faster for samples which comply, whereas samples with cut-off factor above MRL should be further analyzed by quantitative methods (SAMSONOVA et al., 2012).

Table 1. Antibiotics included in the study and respective Maximum Residue Limit (MRL), screening target concentration and concentrations of stock solutions

Class/ Analyte	Concentration		
	MRL (µg.kg^{-1})	Screening target (µg.kg^{-1})	Stock solution (µg.mL^{-1})
Aminoglycosides			
Amikacin	500[a]	250	200
Apramycin	500[a]	250	200
Dihydrostreptomycin	500[c]	250	200
Gentamicin	500[a]	250	200
Hygromycin	500[a]	250	200
Kanamycin	500[a]	250	200
Neomycin	500[b]	250	200
Paromomycin	500[c]	250	200
Spectinomycin	500[b]	250	200
Streptomycin	500[c]	250	200
Tobramycin	500[a]	250	200
Beta-lactams			
Ampicillin	50[a]	25	200
Cefazolin	50[a]	25	200
Oxacillin	300[c]	150	200
Penicillin G	50[a]	25	200
Penicillin V	25[a]	12.5	200
Macrolides			
Clindamycin	100[b]	50	100
Erythromycin	100[b]	50	100
Lincomycin	200[b]	100	100
Spiramycin	200[c]	100	100
Tilmicosin	100[c]	100	100
Tylosin	100[c]	100	100
Virginiamycin	200[b]	100	100
Quinolones			
Ciprofloxacin	100[a]	50	100
Danofloxacin	100[b]	50	100
Difloxacin	300[a]	150	100
Enrofloxacin	100[a]	50	100
Flumequine	600[a]	300	100
Marbofloxacin	100[b]	50	100
Nalidixic acid	20[a]	20	100
Norfloxacin	100[b]	50	100
Oxolinic acid	20[a]	20	100
Sarafloxacin	30[a]	15	100
Sulfonamides			
Sulfachloropyridazine	100[a]	50	250
Sulfadiazine	100[a]	50	250
Sulfadimethoxine	100[a]	50	250
Sulfadoxine	100[a]	50	250
Sulfamerazine	100[a]	50	250
Sulfamethazine	100[a]	50	250
Sulfamethoxazole	100[a]	50	250
Sulfamethoxypyridazine	100[a]	50	250
Sulfaphenazole (IS)	-	-	
Sulfaquinoxaline	100[a]	50	250
Sulfathiazole	100[a]	50	250
Sulfisoxazole	100[a]	50	250
Tetracyclines			
Chlortetracycline	200[a]	100	200
Doxycicline	200[a]	100	200
Oxytetracycline	200[a]	100	200
Tetracycline	200[a]	100	200

[a] BRASIL (2015); [b] CODEX (2014); [c] EC (2010a); IS – internal standard.

LC-MS/MS methods for the analysis of more than five classes of antibiotics are available for milk (GAUGAIN-JUHEL et al., 2009; FREITAS et al., 2013; JANK et al., 2015; CHEN et al., 2016; MARTINS et al., 2016), eggs (CHEN et al., 2016), honey (HAMMEL et al., 2008), meat (CARRETERO et al., 2008; FREITAS et al., 2014a; CHEN et al., 2016; DO et al., 2016), liver (FREITAS et al., 2015) and fish (PETERS et al., 2009; SMITH et al., 2009; LOPES et al., 2012; STOREY et al., 2014; REZK et al., 2015). However, most of the multiclass methods available for the screening of antibiotics in fish are, in general, laborious and limited to a few antimicrobials. Therefore, the objective of this study was to develop a simple, sensitive and fast screening method for multiple classes of antimicrobials in fish muscle.

2. EXPERIMENTAL

2.1. Material

2.1.1. Chemicals and reagents

LC-MS grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany); heptafluorobutyric acid (HFBA) was from Fluka (Buchs, Switzerland) and trichloroacetic acid (TCA) was from Vetec (Rio de Janeiro, Brazil). Ultra-pure water was obtained from a Milli-Q purification apparatus (Millipore, Bedford, MA, USA).

All the antibiotics were of high purity grade (>99.0%). They included aminoglycosides, beta-lactams, macrolides, quinolones, sulfonamides, and tetracyclines, in a total of 49 compounds. They were purchased from Sigma-Aldrich (St. Louis, MO, USA), Fluka (Buchs, Switzerland) and Dr. Ehrenstorfer (Augsburg, Germany). Sulfaphenazole, the internal standard, was purchased from Sigma-Aldrich (St. Louis, MO, USA). The shelf-lives of the antibiotics were carefully considered and varied from 3 to 12 months.

Each standard was accurately weighed and transferred to a 50-mL volumetric flask and used to prepare methanolic stock solutions (Table 1) at concentrations varying from 100 to 250 $\mu g.mL^{-1}$. Beta-lactams and aminoglycosides were dissolved in ultra-purified water, and 1 mL of 1 $mol.L^{-1}$ NaOH was added to quinolone standard solutions to enhance solubility. Individual stock solutions were stored at -10 °C.

Working standard solutions were obtained by dilution of each stock solution in ultra-purified water, at concentrations varying from 0.125 $\mu g.mL^{-1}$ to 3.0 $\mu g.mL^{-1}$. The internal standard (sulfaphenazole) solution was prepared at 0.5 $\mu g.mL^{-1}$ in ultra-purified water. All the working solutions were kept at -10 ºC and prepared fresh monthly, except beta-lactams, which were prepared weekly.

2.1.2. Samples

Blank samples of Nile tilapia used in the validation process were collected at two farms from the state of Minas Gerais, Brazil, where none of the studied antimicrobials were used. A total of 193 fish muscle samples from fish farms under federal inspection were obtained: 172 from the state of Minas Gerais and 21 from the state of Pará, Brazil. The samples from Minas Gerais included 149 Nile tilapia (*Oreochromis niloticus*) and 23 trout (*Oncorhynchus mykiss*); whereas the samples from Para included 9 Nile tilapia (*Oreochromis niloticus*) and 12 tambaqui (*Colossoma macropomum*).

2.2. LC-MS/MS analysis

Liquid chromatography was performed in an Agilent 1200 Series HPLC (Agilent Technologies Inc., Santa Clara, CA, USA) coupled to a Triple Quadrupole Mass Spectrometer detector API 5000 AbSciex (Life Technologies Corporation, CA, USA). A Zorbax Eclipse XDB C18 (150 x 4.6 mm, 1.8 µm, Agilent Technologies, CA, USA) column was used. To establish optimum conditions for the chromatographic separation of all compounds and to achieve a short running time, several chromatographic parameters were investigated, including composition and flow rate of the mobile phase, gradient elution, injection volume and column temperature.

Mass spectrometer parameters were also optimized for each compound separately by direct infusion of individual standard solutions at concentrations ranging from 50 to 100 $\mu g.L^{-1}$ in MeOH. The best precursor and product ions, declustering potential (DP), collision energy (CE) and collision cell exit potential (CXP) were established. Electrospray ionization (ESI) generated the ions in a positive mode. Multiple reaction monitoring (MRM) was used and two transitions were selected: the most intense transition for quantifications and the second most intense for confirmation purposes. Each chromatographic run was divided into scan events with a scan time of 90 seconds for each transition. The analytical system control, acquisition and data

processing were performed using Analyst software, version 1.5.1, from AbSciex (Life Technologies Corporation, CA, USA).

2.3. Sample preparation

The method used for extraction of the antibiotics from the samples was adapted from that described by GAUGAIN-JUHEL et al. (2009). A schematic diagram for sample preparation is indicated in Figure 1.

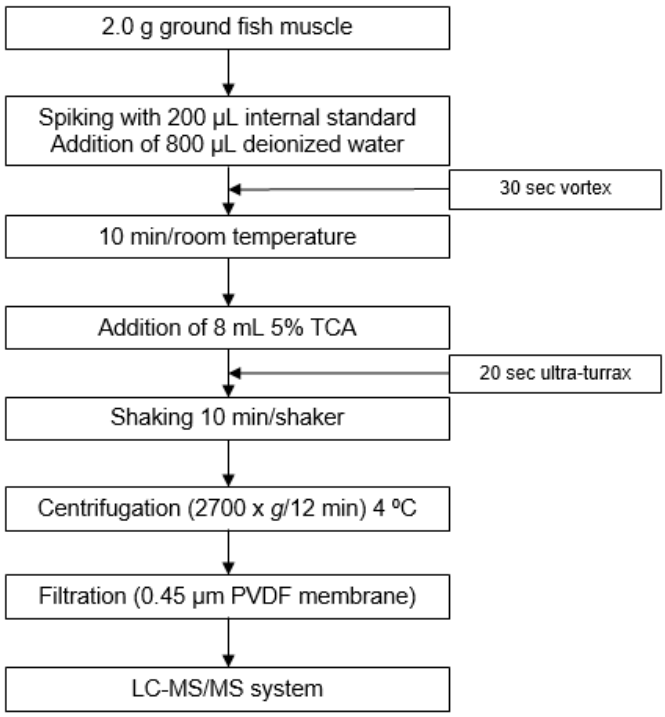

Figure 1. Sample preparation for screening analysis of six classes of antimicrobials in fish muscle.

Briefly, 2.0 g (wet weight) of ground and homogenized fish muscle was weighted in a 50-mL polypropylene centrifuge tube. Then, 200 µL of internal standard (sulfaphenazole at 0.5 $\mu g.mL^{-1}$) and 800 µL of deionized water were added. The sample was vortexed for 30 seconds and after standing for 10 minutes at room temperature, 8 mL of 5% TCA was added. The sample was homogenized in an ultra-turrax for 20 seconds, placed in a shaker for 10 minutes, and centrifuged at 2700 x *g* for 12 minutes at 4 °C. The extract was filtered through a PVDF membrane with 0.45 µm pore size (Millipore, Bedford, MA, USA) immediately prior to LC-MS/MS analysis.

2.4. Validation of the method

The fitness of the screening method optimized for the analysis of antibiotics in fish was evaluated according to the Guidelines for the Validation of Screening Methods for Residues of Veterinary Medicines (Initial Validation and Transfer)-Community Reference Laboratories (CRLs) 20/1/2010 (EC, 2010b). The following parameters were evaluated: threshold value (T_v), cut-off factor (F_m), detection capability (CCβ), limit of detection (LOD), sensitivity and specificity.

2.4.1. Threshold value

The threshold value (T_v) was determined by analyzing twenty blank samples of fish muscle extracted according to the procedure described in item 2.3. The analytical response (chromatographic peak area) of the blank sample at the retention time (± 10%) of each analyte was determined in each chromatogram for both quantitation and confirmation transitions. The mean and the estimated standard deviation of the noise were calculated. T_v was calculated according to Equation 1 (Eq. 1).

$$T_v = B + 1.64 \times SB \quad \text{(Eq. 1)}$$

where B and SB are, respectively, the mean and the standard deviation of the chromatographic peak areas of blank samples at the retention time of each analyte.

2.4.2. Cut-off factor

The cut-off factors (F_m) were calculated by using twenty blank samples of fish muscle spiked with the screening target concentration (STC), which is half of the MRL concentration based on Brazilian legislation for fish and other matrices (chicken, pork and meat) when not available for fish and European legislations (EC, 2010a; BRASIL, 2015; CODEX, 2014), except for nalidixic acid, oxolinic acid, tilmicosin and tylosin (STC=1.0xMRL) (Table 1). The samples were analyzed at the same day and this step was repeated in a different day to obtain forty independent data. Peak area was determined for each analyte (n=40) for both transitions of quantification and confirmation. Means and estimated standard deviations were calculated for each analyte and the cut-off factor was estimated according to Equation 2 (Eq. 2).

$F_m = D - 1.64 \times Sd$ (Eq. 2)

where D and Sd are, respectively, the mean and the standard deviation of the chromatographic peak areas. It means statistically that 95% of the samples spiked at the level of interest should give an analytical response above this value.

2.4.3. Detection capability

The detection capability (CCβ) was estimated from the comparison of threshold values and cut-off factors. When the cut-off factor is above the threshold value, CCβ is considered as definitely below the level of interest (0.5xMRL, in this case). On the other hand, when the cut-off factor is below the threshold value, more than 5% of the samples will be considered as negative samples and, consequently, CCβ is really above the level of interest (EC, 2010b).

2.4.4. Limit of detection (LOD)

The limit of quantification (LOD) was estimated by extracting and analyzing by LC-MS/MS 20 blank samples of fish muscle. LODs for each analyte (one for each *m/z* transition – quantification and confirmation) were calculated as the mean concentration of the blank samples in the retention time of each analyte plus three times the standard deviation of the blank concentration. The LOD for each analyte was ascribed as the higher of the two values, in most cases from the confirmation *m/z* transition.

2.4.5. Sensitivity and specificity

To calculate the sensitivity (%), twenty samples were spiked with all antibiotics at 0.5xMRL concentration, extracted and analyzed by LC-MS/MS. The instrument response for peak area (R_{an}) for each analyte was compared to the cut-off factor and if $R_{an}>F_m$, the sample was considered non-compliant (positive), i.e., it contains a concentration above 0.5xMRL. However, if $R_{an}<F_m$, the sample was considered compliant (negative), i.e., it contains a concentration below 0.5xMRL.

The method sensitivity was estimated from Equation 3 (Eq. 3) and it must be higher than 95% to ensure a β error below 5%. In this case, all the samples are positive because they were spiked at a 0.5xMRL concentration.

$$Sens.(\%) = \frac{Number\ of\ samples\ considered\ positive}{Number\ of\ samples\ really\ positive\ (20)} x100 \quad \text{(Eq. 3)}$$

To determine specificity of the method, e.g. its ability to detect unambiguously a specific analyte from a complex matrix, the blank chromatograms at the retention time of each studied analyte were carefully evaluated in order to verify possible interferences.

3. RESULTS AND DISCUSSION

3.1. Optimization of the LC-MS/MS procedure

The optimized spectrometric parameters and the retention time windows (equal to retention time ± 5%) for each analyte individually are shown in Table 2. The chromatographic conditions for the screening method were optimized to provide the shortest possible run of all analytes of interest with appropriate resolution. The mobile phase composition which provided best results was phase A – 0.1% of heptafluorobutyric acid (HFBA) in water and phase B – acetonitrile at a gradient elution of: initial time – 90% A; 7.0 min – 50% A; 11.0 min – 50% A; 12.0 min – 90% A; and 15 min – 90% A at a constant flow rate of 600 µL.min^{-1}. The flow rate and injection volume were 0.6 mL.min^{-1} and 10 µL, respectively and the column temperature was set at 35 °C. Total chromatographic run lasted 15 min.

The presence of two chromatographic peaks, one for each *m/z* transition – quantification and confirmation, eluting at the same retention time allowed the unequivocal identification of each analyte. Each chromatographic peak presented a signal-to-noise ratio (S/N) equal to 3 under these conditions (LOPES et al., 2011). As can be noticed, several sulfonamides exhibit the same quantification and confirmation ions. However, as the precursor ion differs among them, distinction of each of them is allowed. Sulfadimethoxine and sulfadoxine had the same quantification and confirmation ions but they had also similar precursor ions (311.1 and 311.0, respectively), which could lead to mistaken identification of these two substances. However, because of the different retention time windows observed for these compounds (9.17-9.60 and 8.15-8.57, respectively), the correct identification of each antibiotic was possible.

Table 2. Optimized spectrometric conditions - precursor ion, confirmation transition (C) and quantification transitions (Q), declustering potential (DP), entrance potential (EP), collision energy (CE), collision cell exit potential (CXP) and retention time windows (RTW) - for each analyte in the screening method

Class/Analyte	Precursor ion (*m/z*)	Quantification/ Confirmation ion (*m/z*)	DP	EP	CE	CXP	Retention time windows RTW* (min)
Aminoglycosides							
Amikacin	586	163 (Q)/245 (C)	60	10	53 / 21	14 / 20	7.80-8.13
Apramycin	540	217 (Q)/378 (C)	82	10	35 / 25	12 / 12	8.22-8.54
Dihydrostreptomycin	584	263 (Q)/246 (C)	120	10	42 / 54	12 / 12	7.43-7.75
Gentamicin	464.3	322.6 (Q)/160.2 (C)	50	10	20 / 20	12 / 12	8.41-8.92
Hygromycin	528	352 (Q)/177 (C)	50	10	25 / 25	12 / 12	7.31-7.63
Kanamycin	485	163 (Q)/205 (C)	70	10	35 / 35	12 / 12	7.88-8.21
Neomycin	615.3	161.3 (Q)/293.50 (C)	120	10	41 / 35	8 / 18	8.50-9.01
Paromomycin	616.2	293.1 (Q)/163.2 (C)	91	10	33 / 55	18 / 10	8.19-8.50
Spectinomycin	351	207 (Q)/189 (C)	66	10	31 / 33	12 / 12	6.74-7.09
Streptomycin	582	263 (Q)/246 (C)	157	10	45 / 51	12 / 12	7.39-7.83
Tobramycin	468	163 (Q)/324 (C)	100	10	20 / 20	12 / 8	8.27-8.58
Beta-lactams							
Ampicillin	350	106 (Q)/160 (C)	50	10	20 / 20	12 / 12	7.77-8.10
Cefazolin	455	323 (Q)/156 (C)	50	10	15 / 23	12 / 12	7.15-7.48
Oxacillin	402	160 (Q)/243 (C)	50	10	18 / 18	12 / 12	11.00-11.60
Penicillin G	335.4	176.3 (Q)/160.2 (C)	70	10	21 / 21	10 / 10	9.59-10.40
Penicillin V	351.1	160.1 (Q)/192 (C)	66	10	15 / 17	8 / 12	10.00-11.10
Macrolides							
Clindamycin	425.3	126.4 (Q)/377.2 (C)	75	10	43 / 27	22 / 10	9.09-9.35
Erythromycin	734.5	158.2 (Q)/576.7 (C)	66	10	43 / 27	14 / 8	10.10-10.80
Lincomycin	407	126 (Q)/359 (C)	60	10	40 / 26	12 / 12	7.39-7.68
Spiramycin	422.5	174.3 (Q)/101.3 (C)	56	10	31 / 25	16 / 8	9.33-9.72
Tilmicosin	869.5	174.4 (Q)/696.5 (C)	56	10	63 / 57	10 / 34	10.20-10.50
Tylosin	916.6	174.4 (Q)/772.4 (C)	115	10	55 / 43	6 / 20	9.88-10.80
Virginiamycin	526.5	355.2 (Q)/109 (C)	76	10	25 / 47	26 / 10	8.15-11.80
Quinolones							
Ciprofloxacin	332	314 (Q)/231 (C)	61	10	30 / 47	12 / 12	8.03-8.33
Danofloxacin	358	340 (Q)/255 (C)	60	10	33 / 50	10 / 10	8.18-8.26
Difloxacin	400	356 (Q)/299 (C)	100	10	35 / 40	10 / 10	8.98-9.30

Table 2. (continuation…)

Class/Analyte	Precursor ion (*m/z*)	Quantification/ Confirmation ion (*m/z*)	DP	EP	CE	CXP	Retention time windows RTW* (min)
Quinolones							
Enrofloxacin	360	342 (Q)/286 (C)	72	10	30 / 50	12 / 12	8.42-8.72
Flumequine	262.1	244 (Q)/202 (C)	44	10	25 / 45	12 / 12	10.6-11.00
Marbofloxacin	363	345 (Q)/320 (C)	70	10	30 / 22	10 / 10	7.89-7.98
Nalidixic acid	233	215 (Q)/187 (C)	42	10	30 / 35	12 / 12	10.40-10.80
Norfloxacin	320	302 (Q)/231 (C)	60	10	33 / 50	12 / 12	7.89-8.20
Oxolinic acid	262	244 (Q)/216 (C)	53	10	25 / 40	12 / 12	8.92-9.28
Sarafloxacin	386	368 (Q)/348 (C)	50	10	30 / 40	12 / 12	8.82-9.15
Sulfonamides							
Sulfachloropyridazine	285	156 (Q)/92 (C)	51	10	21 / 39	12 / 12	7.82-8.26
Sulfadiazine	251	156 (Q)/108 (C)	53	10	22 / 30	12 / 12	5.58-6.00
Sulfadimethoxine	311.1	156 (Q)/108 (C)	50	10	23 / 37	12 / 12	9.17-9.60
Sulfadoxine	311	156 (Q)/108 (C)	60	10	25 / 40	12 / 12	8.15-8.57
Sulfamerazine	265	156 (Q)/92 (C)	60	10	35 / 35	12 / 12	6.22-6.59
Sulfamethazine	279	156 (Q)/108 (C)	50	10	25 / 36	12 / 12	6.73-7.11
Sulfamethoxazole	254	108 (Q)/92 (C)	60	10	35 / 35	12 / 12	8.23-8.68
Sulfamethoxypyridazine	281	156 (Q)/108 (C)	60	10	25 / 35	12 / 12	7.04-7.42
Sulfaphenazole (IS)	315	156	50	10	30	12	9.35-9.45
Sulfaquinoxaline	301	156 (Q)/108 (C)	50	10	23 / 40	12 / 12	9.19-9.61
Sulfathiazole	256	156 (Q)/108 (C)	53	10	20 / 34	12 / 12	6.15-6.51
Sulfisoxazole	268	156 (Q)/113 (C)	46	10	21 / 23	12 / 12	8.55-8.99
Tetracyclines							
Chlortetracycline	479.2	98.2 (Q)/275 (C)	61	10	67 / 55	12 / 12	9.31-9.64
Doxycicline	445	428 (Q)/154.2 (C)	55	10	25 / 40	12 / 12	9.51-9.82
Oxytetracycline	461.3	201.1 (Q)/283.2 (C)	41	10	59 / 53	12 / 12	8.07-8.40
Tetracycline	445	410 (Q)/427 (C)	55	10	27 / 25	12 / 12	8.44-8.77

* RTW, retention time ± 5% (n=20).

The total ion chromatograms obtained for all analytes in solvent (water) and in the fish matrix are indicated in Figure 2. The run had a total time of 15 minutes and all analytes eluted within 12 minutes.

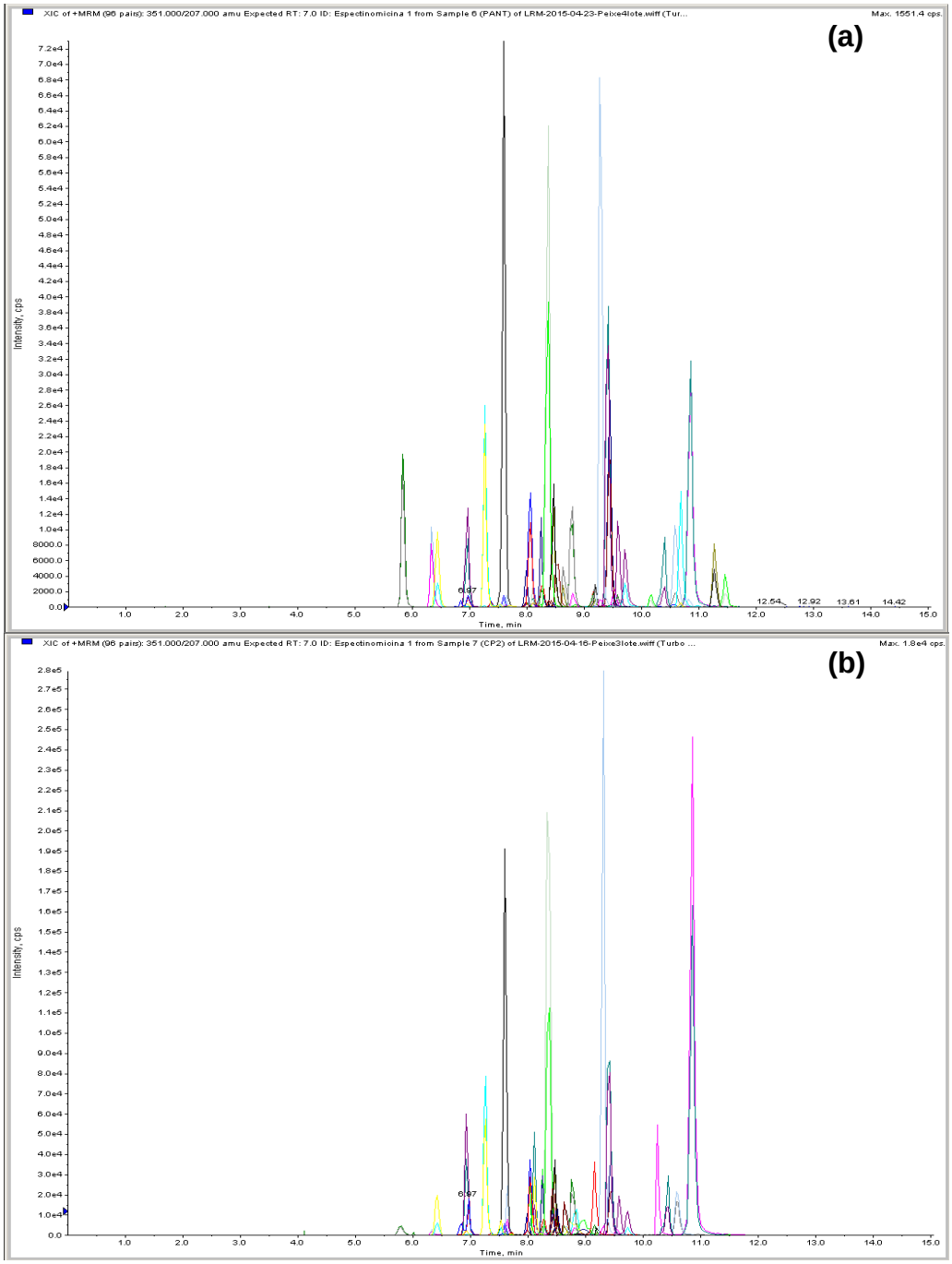

Figure 2. Total ion chromatogram of six classes of antibiotics (a) in water and (b) in the fish matrix extract. Chromatographic conditions: mobile phases A - 0.1% heptafluorobutyric acid (HFBA) in water and B – acetonitrile, at a gradient elution: initial time – 90% A; 7.0 min – 50% A; 11.0 min – 50% A; 12.0 min – 90% A; and 15 min – 90% A at a constant flow rate – 600 µL/min.

The shortest retention time was observed for sulfadiazine (5.58 – 6.00 min), which has highest affinity with the aqueous phase and lowest interaction with the stationary phase. On the other hand, the longest retention time was observed for oxacicillin (11.00 – 11.60 min).

The high specificity and sensitivity of the triple quadrupole mass analyzer allowed the detection of the 40 analytes in only one chromatographic run. To assess specificity, 20 blank samples of fish muscle of different origins were analyzed and no chromatographic peak was detected in these samples at the retention time corresponding to each analyte, indicating a specificity of 100% for all the analytes. Both quantification and confirmation transitions (*m/z*) were used to confirm promptly a positive response. The extraction procedure proposed provided good quality chromatograms, suggesting its efficiency for the extraction and the analytes concentration.

3.2. Screening method validation

During validation of a screening method, it is important to find global conditions to detect all of the analytes simultaneously. The method has to present sufficient sensitivity to detect all the targeted analytes at least at the level of interest, which is 0.5xMRL. Furthermore, qualitative methods of analysis must have the capability of a high sample throughput and the ability to detect all targeted analytes with a false-compliant rate below 5% (β error) at the level of interest. In the case of suspected non-compliant results, these must undergo confirmation by a confirmatory method (EC, 2002).

The results of CCβ, LOD, sensitivity, and the comparison between threshold value and cut-off factor (F_m/T_v) are presented in Table 3. The cut-off factor (the analytical response - peak area in this case - indicating that a sample contains a substance with a concentration equal to or higher than the level of interest) was compared to threshold value, (the minimal analytical response above which the sample will be truly considered positive) to evaluate CCβ.

Table 3. Limit of detection (LOD), detection capability (CCβ), sensitivity (sens.) and the comparison of cut-off factor and threshold value (F_m/T_v) for each antibiotic residue in the validated screening method

Class/Analyte	LOD ($\mu g.kg^{-1}$)	Quantification transition			Confirmation transition		
		F_m/T_v	CCβ ($\mu g.kg^{-1}$)	Sens. (%)	F_m/T_v	CCβ ($\mu g.kg^{-1}$)	Sens. (%)
Aminoglycosides							
Amikacin	1.62[b]	$F_m>T_v$	<250	95	$F_m>T_v$	<250	100
Apramycin	3.15[a]	$F_m>T_v$	<250	100	$F_m>T_v$	<250	95
Dihydrostreptomycin	1.91[b]	$F_m>T_v$	<250	95	$F_m>T_v$	<250	95
Gentamicin	3.50[b]	$F_m>T_v$	<250	100	$F_m>T_v$	<250	100
Hygromycin	29.16[a]	$F_m>T_v$	<250	95	$F_m>T_v$	<250	100
Kanamycin	4.11[b]	$F_m>T_v$	<250	95	$F_m>T_v$	<250	95
Neomycin	3.32[b]	$F_m>T_v$	<250	100	$F_m>T_v$	<250	100
Paromomycin	3.67[a]	$F_m>T_v$	<250	95	$F_m>T_v$	<250	95
Spectinomycin	20.29[b]	$F_m>T_v$	<250	100	$F_m>T_v$	<250	100
Streptomycin	6.98[b]	$F_m>T_v$	<250	100	$F_m>T_v$	<250	95
Tobramycin	2.49[a]	$F_m>T_v$	<250	100	$F_m>T_v$	<250	100
Beta-lactams							
Ampicillin	**0.83**[b]	**$F_m<T_v$**	***>25***	100	**$F_m<T_v$**	***>25***	100
Cefazolin	1.88[b]	$F_m>T_v$	<25	100	$F_m>T_v$	<25	100
Oxacillin	**95.77**[b]	**$F_m<T_v$**	***>150***	100	**$F_m<T_v$**	***>150***	100
Penicillin G	**119.60**[b]	**$F_m<T_v$**	***>25***	100	**$F_m<T_v$**	***>25***	100
Penicillin V	**26.89**[b]	**$F_m<T_v$**	***>12,5***	100	**$F_m<T_v$**	***>12,5***	100
Macrolides							
Clindamycin	0.40[b]	$F_m>T_v$	<50	100	$F_m>T_v$	<50	100
Erythromycin	**5.84**[a]	**$F_m<T_v$**	***>50***	100	**$F_m<T_v$**	***>50***	100
Lincomycin	1.60[b]	$F_m>T_v$	<100	100	$F_m>T_v$	<100	100
Spiramycin	**74.24**[a]	**$F_m<T_v$**	***>50***	100	**$F_m<T_v$**	***>50***	100
Tilmicosin	1.22[b]	$F_m>T_v$	<100	95	$F_m>T_v$	<100	95
Tylosin	**13.29**[b]	**$F_m<T_v$**	***>100***	100	**$F_m<T_v$**	***>100***	95
Virginiamycin	**22.86**[b]	**$F_m<T_v$**	***>100***	100	**$F_m<T_v$**	***>100***	100
Quinolones							
Ciprofloxacin	0.56[b]	$F_m>T_v$	<50	95	$F_m>T_v$	<50	95
Danofloxacin	1.74[a]	$F_m>T_v$	<50	100	$F_m>T_v$	<50	100
Difloxacin	3.42[a]	$F_m>T_v$	<150	95	$F_m>T_v$	<150	100
Enrofloxacin	1.24[a]	$F_m>T_v$	<50	100	$F_m>T_v$	<50	100
Flumequine	9.09[a]	$F_m>T_v$	<300	95	$F_m>T_v$	<300	95
Marbofloxacin	10.02[a]	$F_m>T_v$	<50	95	$F_m>T_v$	<50	95
Nalidixic acid	0.82[b]	$F_m>T_v$	<20	95	$F_m>T_v$	<20	100
Norfloxacin	0.50[b]	$F_m>T_v$	<50	100	$F_m>T_v$	<50	95
Oxolinic acid	6.28[a]	$F_m>T_v$	<20	100	$F_m>T_v$	<20	100
Sarafloxacin	1.71[a]	$F_m>T_v$	<15	95	$F_m>T_v$	<15	100
Sulfonamides							
Sulfachloropyridazine	6.06[a]	$F_m>T_v$	<50	95	$F_m>T_v$	<50	100
Sulfadiazine	0.39[b]	$F_m>T_v$	<50	100	$F_m>T_v$	<50	100
Sulfadimethoxine	1.20[a]	$F_m>T_v$	<50	100	$F_m>T_v$	<50	95
Sulfadoxine	0.20[a]	$F_m>T_v$	<50	100	$F_m>T_v$	<50	100
Sulfamerazine	1.19[a]	$F_m>T_v$	<50	95	$F_m>T_v$	<50	95
Sulfamethazine	0.19[a]	$F_m>T_v$	<50	95	$F_m>T_v$	<50	100
Sulfamethoxazole	1.30[a]	$F_m>T_v$	<50	100	$F_m>T_v$	<50	95
Sulfamethoxypyridazine	0.54[b]	$F_m>T_v$	<50	100	$F_m>T_v$	<50	100
Sulfaquinoxaline	0.55[b]	$F_m>T_v$	<50	95	$F_m>T_v$	<50	95
Sulfathiazole	0.71[b]	$F_m>T_v$	<50	100	$F_m>T_v$	<50	95
Sulfisoxazole	1.78[b]	$F_m>T_v$	<50	100	$F_m>T_v$	<50	100
Tetracyclines							
Chlortetracycline	34.76[a]	$F_m>T_v$	<100	100	$F_m>T_v$	<100	100
Doxycicline	2.69[b]	$F_m>T_v$	<100	100	$F_m>T_v$	<100	95
Oxytetracycline	2.60[a]	$F_m>T_v$	<100	95	$F_m>T_v$	<100	95
Tetracycline	3.64[b]	$F_m>T_v$	<100	95	$F_m>T_v$	<100	100

Analytes that do not meet the requirements for inclusion in the screening method are shown in bold.
[a] Estimated from the data arising from the quantification *m/z* transition.
[b] Estimated from the data arising from the confirmation *m/z* transition.

According to the protocol for validation of screening methods (EC, 2010b), detection capability (CCβ) of screening methods can be evaluated only when the cut-off factor is above the threshold value. When this condition is achieved, CCβ is considered as definitely below the level of interest (0.5xMRL, in this case). On the other hand, when the cut-off factor is below the threshold value, more than 5% of the positive samples will be considered as negative samples and, consequently, CCβ is really above the level of interest and the analyte cannot be analyzed by the method with 95% of confidence.

Among the 48 antibiotics analyzed, 40 attended the criteria established by EC (2002) and EC (2010b), e.g., CCβ was truly below the level of interest tested during validation (0.5xMRL) and the screening method was efficient in detecting all 40 analytes which presented $F_m > T_v$, with 95% of significance and a false-compliant rate of 5%. In general, all these analytes showed low LODs values (minimum concentration of a given analyte that can be detected with a reasonable statistical confidence), indicating that the method is capable of detecting low concentrations of these antibiotics.

The eight antibiotics which did not attend EC (2002) and EC (2010b) included erythromycin, spiramycin, tylosin, virginiamycin, ampicillin, oxacillin, penicillin G and penicillin V. These compounds did not have cut-off factors above threshold value (e.g., $F_m < T_v$), which indicates that CCβ values for these analytes were higher than 0.5xMRL and also that more than 5% of the non-compliant samples can show a compliant result (false negative). Although sensitivities for these analytes at 0.5xMRL concentration were satisfactory (>95%), most of them had high LODs values (sometimes above the MRL). Therefore, even though the method demonstrates ability to monitor these compounds, it is not capable of detecting them in concentrations below the MRL. Further studies at concentrations above the 0.5xMRL can be undertaken to determine the difference between this level and CCβ.

3.3. Screening of farm fish samples

The samples collected from Brazilian fish farms were analyzed using the validated screening method for the presence of the 40 antibiotics that attended the criteria established by EC (2002) and EC (2010b). Twenty nine samples (15% of 193 fish samples) were positive for enrofloxacin, both tilapia and trout, from the state of Minas Gerais. None of the samples from the state of Para, both Nile tilapia and ‘tambaqui’, had positive results. This could result from the fish farming practices prevalent in Para. Due to the large availability of fresh water from rivers, the fishes are

usually cultivated in cages inside the rivers or in large tanks (lower fish densities), which reduces the risk of spread of diseases, thereby reducing the need of antibiotics. Overall, the low occurrence of antibiotics in farm fishes can reflect the good practices adopted in most of the farms, which results in lower need for the use of antibiotics.

Among the 29 positive samples, three were trout samples from the south of Minas Gerais and 26 samples were Nile tilapia also from Minas Gerais, but different regions (metropolitan region of Belo Horizonte, 'Central Mineira' and 'Zona da Mata'). Only one sample of Nile tilapia had analyte concentration above the cut-off factor, which means that this sample contained enrofloxacin in a concentration higher than the level of interest, which is 50 $\mu g.kg^{-1}$. The other 28 samples had trace levels of enrofloxacin (<50 $\mu g.kg^{-1}$) and they should be submitted to a quantitative method for confirmation. These samples were positive for enrofloxacin below the cut-off factor.

Even though the use of enrofloxacin is forbidden in aquaculture in several countries, including Brazil (KIM et al., 2012; BRASIL, 2015; SINDAM, 2016), it was present in fish. Enrofloxacin is a fluoroquinolone antimicrobial agent with broad spectrum of activity available in the market for veterinary use and also allowed for use in aviculture in some countries (BRASIL, 2015; SINDAM, 2016). In 2005, FDA (FAO, 2005) withdrew approval of its use in poultry because it could select for fluoroquinolone resistant Campylobacter. However, enrofloxacin is still approved for use in some food producing animals and companion animals (KIM et al., 2012). It is important to consider that there could be several sources of fish contamination with antibiotics besides its administration. In the case of enrofloxacin, its use as a veterinary antibiotic, in aviculture for example, can result in its release in the environment through waste streams by which fish may be contaminated. Another source could be the direct use of enrofloxacin in aquaculture, either due to misinformation or on purpose. However, the source of contamination should be determined and educational programs implemented to warrant fish quality. Due to the health hazard associated with antibiotics abuse, there should be continuous monitoring of antibiotics in fish to warrant human health and international trade.

4. CONCLUSIONS

A screening LC-MS/MS method was optimized for the simultaneous determination of 40 antibiotics from six different classes, including aminoglycosides, beta-lactams, macrolides, quinolones, sulfonamides and tetracyclines, in fish muscle. Extraction was performed with TCA. A C18 column was used along with a gradient elution of 0.1% HFBA in water:acetonitrile. A single run of 15 minutes was capable of determining the presence of the compounds.

Sample preparation was simpler and faster when compared with other methods for multiclass antibiotic analysis in fish found in literature, which is desirable for routine methods. The developed method was validated according to the Guidelines for the Validation of Screening Methods for Residues of Veterinary Medicines (Initial Validation and Transfer)-Community Reference Laboratories (CRLs) 20/1/2010 and it satisfactorily fulfilled the established criteria for 40 antibiotics in fish. The method was successfully applied to real samples. Twenty nine (15%) of the 193 samples analyzed were positive for one of the 40 antibiotics (enrofloxacin), which is not allowed for use in aquaculture in Brazil. Only one sample had a concentration of enrofloxacin above the cut-off factor (50 $\mu g.kg^{-1}$). This sample should proceed to quantification using a quantitative method to verify its real concentration. The low occurrence of antibiotics in farm fish suggests that there is a responsible management of aquaculture.

CAPÍTULO IV - MULTI-RESIDUE QUANTITATIVE METHOD FOR QUINOLONES AND TETRACYCLINES IN FISH BY LC-MS/MS

ABSTRACT

A multiresidue method for the quantification of 14 quinolones and tetracyclines antibiotics in fish by liquid-chromatography–tandem mass spectrometry (LC-MS/MS) is described. Sample preparation was optimized using a Central Composite Rotational Design. Fish muscle was extracted with 0.5% trichloroacetic acid, homogenized in an ultra-turrax, shaken and centrifuged. The supernatant was filtered and used for LC-MS/MS analysis. LC separation was achieved on a Zorbax Eclipse XDB C18 (150 x 4.6 mm, 1.8 µm) column with gradient elution using 0.1% heptafluorobutyric acid in water and acetonitrile as mobile phases. Analysis was carried out in multiple reaction monitoring mode via electrospray interface operated in the positive ionization mode, with sulfaphenazole as internal standard. The method was validated according to Decision 2002/657/EC. It was considered fit for the purpose. Precision, in terms of relative standard deviation, was under 20%, and recoveries ranged from 89.3 to 103.7%. Reproducibility values, expressed as coefficient of variation, were below 14.0%. CCα varied from 17.87 to 323.20 $\mu g.kg^{-1}$ and CCβ varied from 20.75 to 346.40 $\mu g.kg^{-1}$. The method was applied to real samples positive for enrofloxacin (n=29) and four of them contained levels above the limit of quantification (12.53 to 19.01 $\mu g.kg^{-1}$) but below the Maximum Residue Limit (100 $\mu g.kg^{-1}$).

Keywords: fish; antibiotic; enrofloxacin; quantification; chromatography; mass spectrometry.

1. INTRODUCTION

Aquaculture is an important system of fish production, which is growing worldwide faster than any other animal food-production sectors (FAO, 2010; ROMERO et al., 2012). The contribution of aquaculture fish production to the total captured fishes (including for nonfood uses) has grown from 13.4% in 1990 to 25.7% in 2000 and to 42.2% in 2012 (FAO, 2014). Its relative contribution to the total amount of fish produced for human consumption ranged from 5% in 1962 to 37% in 2002 and to 49% in 2012 (FAO, 2014; SANTOS & RAMOS, 2016).

Although aquaculture has many advantages, the fast growth of this production system has resulted in concerns over fish quality and safety. Fish production adopts intensive and semi-intensive practices, in which, most of the times, there is a high concentration of animals in small spaces, substantially increasing the risk of disease spread in fish resulting in high-mortality rates (EFSA, 2008; QUESADA et al., 2013b; SANTOS & RAMOS, 2016). The dissemination of diseases in aquaculture is also due to inadequate management and poor environmental conditions, among them, high density of animals, feeding levels, removal and restocking, and inadequate nutrition (QUESADA et al., 2013b). Therefore, the use of antimicrobial agents in aquaculture becomes a necessity, as they can help in the treatment and prevention of infectious diseases. Antibiotics are generally used to inhibit microorganisms' growth, being used as therapeutic, prophylactic or metaphylactic agents (ROMERO et al., 2012; QUESADA et al., 2013b).

The most commonly used antibiotics in aquaculture worldwide are tetracycline, oxytetracycline (tetracyclines), oxolinic acid, flumequine, sarafloxacin, enrofloxacine (quinolones), amoxicylin (β-lactam), erythromycin (macrolide), sulfadimethoxine (sulfonamide), ormetoprim (diaminopyrimidine) and florfenicol (amphenicol) (QUESADA et al., 2013b). Each country has its own legislation on which ones and how much substances are allowed for use in aquaculture. In Brazil, there are only two antimicrobials licensed for aquaculture - florfenicol and oxytetracycline (SINDAM, 2016). Maximum residue limits (MRLs) for antibiotics in food are established by many regulatory agencies around the world, including the European Union (EU), the U.S. Food and Drug Administration (FDA), the Ministry of Agriculture, Livestock and Supply (MAPA) in Brazil, as well as Codex Alimentarius and the European Medicines Agency (EMEA) to ensure the quality and safety of consumer products (QUESADA et al., 2013b; REZK et al., 2015). Table 1 presents the MRLs for quinolones and tetracyclines

in fish. These low limits (level range from μg.kg^{-1} to ng.kg^{-1}) require sensitive and specific methods to monitor and determine unequivocally antimicrobial residues in aquatic products.

Table 1. Maximum residue levels (MRL) of quinolones and tetracyclines in fish established by different regulatory agencies

Class/Antibiotic	Maximum residue levels - MRL (μg.kg^{-1}) / regulatory agency		
	BRASIL (2015)	**CODEX (2015)**	**EUROPEAN COMMUNITY (2010)**
Quinolones			
Ciprofloxacin[a]	Sum equal to 100	n.e.	Sum equal to 100
Danofloxacin	-	n.e.	100
Difloxacin	300	n.e.	300
Enrofloxacin[a]	Sum equal to 100	n.e.	Sum equal to 100
Flumequine	600	500 (trout)	600
Marbofloxacin	n.e.	n.e.	n.e.
Nalidixic acid	20	n.e.	n.e.
Norfloxacin	n.e.	n.e.	n.e.
Oxolinic acid	20	n.e.	100
Sarafloxacin	30	n.e.	30
Tetracyclines[b]	Sum equal to 200		
Chlortetracycline		n.e.	100
Doxycycline		n.e.	n.e.
Oxytetracycline		200	100
Tetracycline		n.e.	100

n.e.- not established; a sum of ciprofloxacin and enrofloxacin; b sum of all tetracyclines

Based on this information, it is important to monitor the presence of antibiotics in fish in order to protect consumer from health hazards. The presence of such residues in food can be responsible for toxic effects, allergic reactions in individuals with hypersensitivity and can also result in the development of resistant strains of bacteria (FREITAS et al., 2013). Indeed, in recent years, bacterial resistance has become a worldwide concern and food-producing animals are potential source of antibiotic resistant bacteria in humans. As a result, there is increasing pressure on laboratories responsible for ensuring the safety of food for human consumption regarding the development of reliable and sensitive analytical methods to analyze antibiotic residues in food (CHÁFER-PERICÁS et al., 2010).

An analytical technique to fit this purpose is liquid chromatography tandem mass spectrometry triple quadrupole (LC-MS/MS) because of its high specificity, sensitivity and detectability (MONTEIRO et al., 2015). Many studies were developed using LC-MS/MS to detect antibiotics in fish and other aquaculture products (SANTOS et al., 2005; HERNANDO et al., 2006; KARBIWNYK et al., 2007; SAMANIDOU et al., 2008; CHÁFER-PERICÁS et al., 2010; VILLAR-PULIDO et al., 2011; MENDOZA et al., 2012; WU et al., 2012; GBYLIK et al., 2013; QUESADA et al., 2013a; DICKSON, 2014; FEDOROVA et al., 2014; FREITAS et al., 2014b; MONTEIRO et al., 2015; REZK et al., 2015; VEACH et al., 2015). However, most of the multiresidue methods available for antibiotics in fish has, in general, a laborious sample preparation step, which increases the time of analysis and, sometimes, the consumption of reagents, generating more residues to the environment.

The aim of the present study was to develop and validate a simple, rapid and sensitive quantitative method for the simultaneous determination of quinolones and tetracyclines in fish tissues and to analyze fish samples which provided positive results from a previous screening study (chapter III).

2. EXPERIMENTAL

2.1. Material

2.1.1. Chemicals and regents

LC-MS grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany); heptafluorobutyric acid (HFBA) was from Fluka (Buchs, Switzerland) and trichloroacetic acid (TCA) was from Vetec (Rio de Janeiro, Brazil). Ultra-pure water was obtained from a Milli-Q purification apparatus (Millipore, Bedford, MA, USA).

All antibiotics were of high purity grade (>99.0%). They included tetracyclines (chlortetracycline, doxycycline, oxytetracycline and tetracycline) and quinolones (ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, flumequine, marbofloxacin, nalidixic acid, norfloxacin, oxolinic acid and sarafloxacin), a total of 14 compounds. They were purchased from Sigma-Aldrich (St. Louis, Missouri, USA), Fluka (Buchs, Switzerland) and Dr. Ehrenstorfer (Augsburg, Germany). Sulfaphenazole, the internal

standard, was purchased from Sigma-Aldrich (St. Louis, MO, USA). Their shelf-lives were carefully considered (5 months for tetracyclines and 6 months for quinolones).

Each standard was accurately weighed and transferred to a 50-mL volumetric flask and used to prepare methanolic stock solutions at concentrations of 100 $\mu g.mL^{-1}$ for quinolones and 200 $\mu g.mL^{-1}$ for tetracyclines. To enhance solubility, 1 mL of 1 $mol.L^{-1}$ NaOH was added to quinolone standard solutions. Individual stock solutions were stored at -10 °C.

Working standard solutions were obtained by dilution of each stock solution in ultra-purified water, at concentrations varying from 0.15 $\mu g.mL^{-1}$ to 3.0 $\mu g.mL^{-1}$ for quinolones and 1.0 $\mu g.mL^{-1}$ for all the tetracyclines. The internal standard (sulfaphenazole) solution was prepared at 0.5 $\mu g.mL^{-1}$ in ultra-purified water. All working solutions were kept at -10 ºC and prepared fresh monthly.

2.1.2. Samples

Blank samples of Nile tilapia used in the validation process were collected at two farms from the state of Minas Gerais, Brazil, where none of the studied antimicrobials were used. A total of 29 samples of Nile tilapia (*Oreochromis niloticus*) and trout (*Oncorhynchus mykiss*) from Minas Gerais, previously analyzed by a screening LC-MS/MS method (chapter III) and positive for enrofloxacin were used in this work.

2.2. LC-MS/MS analysis

Liquid chromatography was performed in an Agilent 1200 Series HPLC (Agilent Technologies Inc., Santa Clara, CA, USA) coupled to a Triple Quadrupole Mass Spectrometer detector API 5000 AbSciex (Life Technologies Corporation, CA, USA). A Zorbax Eclipse XDB C18 (150 x 4.6 mm, 1.8 µm, Agilent Technologies, CA, USA) column was used. To establish optimum conditions for the chromatographic separation of all compounds and to achieve a short running time, several chromatographic parameters were investigated, including composition and flow rate of the mobile phase, gradient elution, injection volume and column temperature.

Mass spectrometer parameters were also optimized for each compound separately by direct infusion of individual standard solutions at concentrations ranging from 50 to 100 $\mu g.L^{-1}$ in MeOH. The best precursor and product ions, declustering potential (DP), collision energy (CE) and collision cell exit potential (CXP) were

established. Electrospray ionization (ESI) generated the ions in a positive mode. Multiple reaction monitoring (MRM) was used and two transitions were selected: the most intense transition for quantifications and the second most intense for confirmation purposes. Each chromatographic run was divided into scan events with a scan time of 90 seconds for each transition. The analytical system control, acquisition and data processing were performed using Analyst software, version 1.5.1, from AbSciex (Life Technologies Corporation, CA, USA).

2.3. Optimization of the sample preparation step

An extraction method based on the method described by GAUGAIN-JUHEL et al. (2009) was optimized for fish muscle. 2.0 g of ground and homogenized fish muscle was weighted in a 50-mL polypropylene centrifuge tube. Then, 200 µL of internal standard (sulfaphenazole at 0.5 $\mu g.mL^{-1}$) and 800 µL of deionized water were added. The sample was vortexed for 30 seconds and after standing for 10 minutes at room temperature, 8 mL of trichloroacetic acid (TCA) was added. The sample was homogenized in an ultra-turrax for 20 seconds, placed in a shaker, and centrifuged at 2700 x *g* at 4 °C. The extract was filtered through a PVDF membrane with 0.45 µm pore size (Millipore, Bedford, MA, USA) immediately prior to LC-MS/MS analysis.

A Central Composite Rotational Design (CCRD) was used to screen the main factors that could affect recovery of the antibiotics from fish muscle. The independent variables investigated were TCA concentration, stirring time and centrifugation time. The following parameters were kept unchanged: volume of TCA (8 mL), centrifugation speed (2700 x *g*), centrifugation temperature (4 °C), homogenization time in ultra-turrax (20 s). Table 2 shows the levels studied in the CCRD and Table 3 presents the conditions of each assay of the experimental design and its responses in peak area for enrofloxacin and oxytetracycline. These two antibiotics were chosen as representatives of each class to evaluate the response. Encoded values for the axial points are -1.68 and +1.68.

Twenty tests were assembled with six replicates at the central point and six at the axial points. The results were submitted to analysis of variance (ANOVA) at 5% probability using Minitab® 16 Statistical Software, version 16.1.0.

Table 2. Coded and experimental values used in the Central Composite Rotational Design (CCRD) during optimization of the extraction procedure for antibiotics analysis by LC-MS/MS

Independent Variables	Coded/Experimental Values				
	-1.68	**-1**	**0**	**1**	**1.68**
TCA concentration (%)	0.5	1.4	2.8	4	5
Stirring time (min)	5	7	10	13	15
Centrifugation time (min)	4	6	8	10	12

TCA – trichloroacetic acid. Centrifugation conditions: 2700 x g; 10 min at 4 ºC

Table 3. Coded values and responses in peak area of enrofloxacin (ENR) and oxytetracycline (OXY) for each assay of the Central Composite Rotational Design

Assay	TCA Concentration (%)	Stirring time (min)	Centrifugation time (min)	ENR Peak Area	OXY Peak Area
1	-1	-1	-1	92900	93800
2	1	-1	-1	89000	92300
3	-1	1	-1	82200	92900
4	1	1	-1	65400	92300
5	-1	-1	1	89700	95200
6	1	-1	1	106000	87800
7	-1	1	1	95400	82000
8	1	1	1	88300	90000
9	-1.68	0	0	49700	65900
10	1.68	0	0	83700	89800
11	0	-1.68	0	88500	90500
12	0	1.68	0	92700	95900
13	0	0	-1.68	88800	85100
14	0	0	1.68	82000	91200
15	0	0	0	93800	91000
16	0	0	0	104000	88100
17	0	0	0	77000	90800
18	0	0	0	77900	82200
19	0	0	0	58900	80100
20	0	0	0	132000	96300

TCA – trichloroacetic acid. Centrifugation conditions: 2700 x *g*; 10 min at 4 ºC

2.4. Maximum residue limit and validation level

Maximum residue limit (MRL) values were based on the Brazilian legislation for fish, on values established for other matrices (chicken, pork and meat) when not available for fish, and also on values set by Codex Alimentarius (CODEX, 2014; BRASIL, 2015). Validation levels (VL) were set as 0.5xMRL concentrations, except for nalidixic acid and oxolinic acid (VL=1.0xMRL).

2.5. Validation of the method

The fitness of the method optimized for the analysis of quinolones and tetracyclines residues in fish was evaluated according to the Commission Decision 2002/657/EC (EC, 2002). The following parameters were evaluated: calibration curves, accuracy, precision, recovery, decision limit (CCα), detection capability (CCβ), specificity and limit of quantification.

2.5.1. Calibration curves

Calibration curves were constructed in blank fish tissue samples spiked with six concentrations (0.25xVL, 0.50xVL, 0.75xVL, 1.0xVL, 1.25xVL, 1.5xVL). The ranges for each analyte are described on Table 4. Then, 200 µL of the internal standard (sulfaphenazole) was added and the samples were extracted as described (item 2.3).

Graphics of the analyte versus the concentration of the compound were plotted and the equation and the fit degree (determination coefficient) of the data to the curve were calculated. The acceptable ranges of each curve were established based on EC (2002).

Table 4. Maximum residue levels (MRL), validation levels (VL) and range of calibration curves concentration levels of each antibiotic of the quantification method during the validation of the method for the analysis of antibiotics in fish by LC-MS/MS

Class/Analyte	MRL ($\mu g.kg^{-1}$)	VL ($\mu g.kg^{-1}$)	Range of calibration curves concentration levels ($\mu g.kg^{-1}$)
Quinolones			
Ciprofloxacin	100[a]	50	12.5 – 75.0
Danofloxacin	100[b]	50	12.5 – 75.0
Difloxacin	300[a]	150	37.5 – 225.0
Enrofloxacin	100[a]	50	12.5 – 75.0
Flumequine	600[a]	300	75.0 – 450.0
Marbofloxacin	100[b]	50	12.5 – 75.0
Nalidixic acid	20[a]	20	5.0 – 30.0
Norfloxacin	100[b]	50	12.5 – 75.0
Oxolinic acid	20[a]	20	5.0 – 30.0
Sarafloxacin	30[a]	15	3.75 – 22.50
Tetracyclines	Sum equal to 200[a]		25.0 – 150.0
Chlortetracycline		100	
Doxycicline		100	
Oxytetracycline		100	
Tetracycline		100	

MRL – Maximum Residue Limit; VL – validation level.
[a] BRASIL (2015); [b] CODEX (2014).

2.5.2. Recovery, accuracy and precision

Known levels of the analytes were added to a blank matrix to determine recovery, accuracy and repeatability. Eighteen aliquots of the blank matrix were selected and three groups of six aliquots each were fortified with 0.5, 1.0 and 1.5 times the validation levels described on Table 4. The samples were analyzed and the concentration for each one and the mean concentration of each level were calculated. The mean recovery and the coefficient of variation (CV) of the six results for each level were also calculated. Then, recovery was calculated as described in Equation 1 and accuracy was established by Equation 2 (EC, 2002):

$$\% \text{ Recovery} = 100 \times \text{concentration found/fortification level} \quad \text{(Eq. 1)}$$

$$\text{Accuracy} = 100 \times \text{mean of concentration found/fortification level} \quad \text{(Eq. 2)}$$

Repeatability was established through evaluation of the coefficient of variation and the standard deviation for each level. Two different analysts repeated the experiment previously performed twice in two different days. Mean concentration, standard deviation and coefficient of variation (%) were calculated for the fortified samples of each analyst (EC, 2002).

2.5.3. Specificity

Twenty different blank samples of fish muscle were analyzed to evaluate the specificity of the method. The existence of any interference (possible peaks) that could interfere with the detection in the range of retention time of the target analytes was investigated.

2.5.4. Decision limit (CCα) and detection capability (CCβ)

The decision limit was established by the following protocol: twenty blank samples were fortified in the validation level. The decision limit (α = 5 %) was equal to validation level concentration plus 1.64 times the corresponding standard deviation (EC, 2002).

In order to determine CCβ, twenty blank samples were fortified in the decision limit concentration (CCα) for each antibiotic. The detection capability (β = 5 %) was equal to the CCα concentration plus 1.64 times the corresponding standard deviation (EC, 2002).

2.5.5. Limit of quantification (LOQ)

The limit of quantification is defined as the lower concentration of the analyte that can be determined with acceptable accuracy and precision. It was considered as the first level of the calibration curve (AOAC, 1998).

3. RESULTS AND DISCUSSION

3.1. Optimization of the LC-MS/MS procedure

The optimized spectrometric parameters and the retention time windows (equal to retention time ± 5%) for each analyte individually are shown in Table 5. The chromatographic conditions for the quantitation method were optimized to provide the shortest possible run of all analytes of interest with appropriate resolution.

Table 5. Range of retention times and optimized spectrometric conditions - precursor ion (Q1), confirmation (Q) and quantification transitions (C), declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP) - for each analyte of the quantification method during analysis of antibiotics by LC-MS/MS

Class/Analyte	Retention times range (min)	Q1 (m/z)	Q3 (m/z)	DP	EP	CE	CXP
Quinolones							
Ciprofloxacin	8.03-8.33	332	314 (Q)/231 (C)	61	10	30 / 47	12 / 12
Danofloxacin	8.18-8.26	358	340 (Q)/255 (C)	60	10	33 / 50	10 / 10
Difloxacin	8.98-9.30	400	356 (Q)/299 (C)	100	10	35 / 40	10 / 10
Enrofloxacin	8.42-8.72	360	342 (Q)/286 (C)	72	10	30 / 50	12 / 12
Flumequine	10.6-11.00	262.1	244 (Q)/202 (C)	44	10	25 / 45	12 / 12
Marbofloxacin	7.89-7.98	363	345 (Q)/320 (C)	70	10	30 / 22	10 / 10
Nalidixic acid	10.40-10.80	233	215 (Q)/187 (C)	42	10	30 / 35	12 / 12
Norfloxacin	7.89-8.20	320	302 (Q)/231 (C)	60	10	33 / 50	12 / 12
Oxolinic acid	8.92-9.28	262	244 (Q)/216 (C)	53	10	25 / 40	12 / 12
Sarafloxacin	8.82-9.15	386	368 (Q)/348 (C)	50	10	30 / 40	12 / 12
Tetracyclines							
Chlortetracycline	9.31-9.64	479.2	98.2 (Q)/275 (C)	61	10	67 / 55	12 / 12
Doxycicline	9.51-9.82	445	428 (Q)/154.2 (C)	55	10	25 / 40	12 / 12
Oxytetracycline	8.07-8.40	461.3	201.1 (Q)/283.2 (C)	41	10	59 / 53	12 / 12
Tetracycline	8.44-8.77	445	410 (Q)/427 (C)	55	10	27 / 25	12 / 12

C: confirmation transition; CE: collision energy; CXP: Collision Cell Exit Potential; DP: declustering potential; EP: entrance potential; Q: quantification transition. * Retention time range (mean of retention time ± 3s (n=15).

The mobile phase composition which provided best results was phase A – 0.1% of heptafluorobutyric acid (HFBA) in water and phase B – acetonitrile at a gradient

elution of: initial time – 90% A; 7.0 min – 50% A; 11.0 min – 50% A; 12.0 min – 90% A; and 15 min – 90% A at a constant flow rate of 600 µL.min^{-1}. The flow rate and injection volume were 0.6 mL.min^{-1} and 10 µL, respectively and the column temperature was set at 35 °C. Total chromatographic run lasted 15 min.

The presence of two chromatographic peaks, one for each *m/z* transition – quantification and confirmation, eluting at the same retention time allowed the unequivocal identification of each analyte. Each chromatographic peak presented a signal-to-noise ratio (S/N) equal to 3 under these conditions (LOPES et al., 2011).

The total ion chromatograms obtained for all analytes in solvent (water) and in the fish matrix are indicated in Figure 1. The run had a total time of 15 minutes and all analytes eluted within 12 minutes. The shortest retention time was observed for marbofloxacin (7.89-7.98 min), which had the highest affinity to the aqueous phase and lowest interaction with the stationary phase. On the other hand, the longest retention time was observed for flumequine (10.6-11.00 min).

Figure 2 shows typical chromatograms (extracted ion chromatograms) obtained from fish muscle samples spiked with one antibiotic of each class at the validation level. These chromatograms were obtained by selecting the quantification transition for each analyte (Table 5). The high specificity and sensitivity of the triple quadrupole mass analyzer allowed the detection of the 14 analytes in only one chromatographic run. Both quantification and confirmation transitions (*m/z*) were used to confirm promptly a positive response. As it can be observed in the chromatograms, the extraction procedure proposed provided chromatographic peaks with good resolution, suggesting its efficiency for the extraction and the analytes concentration.

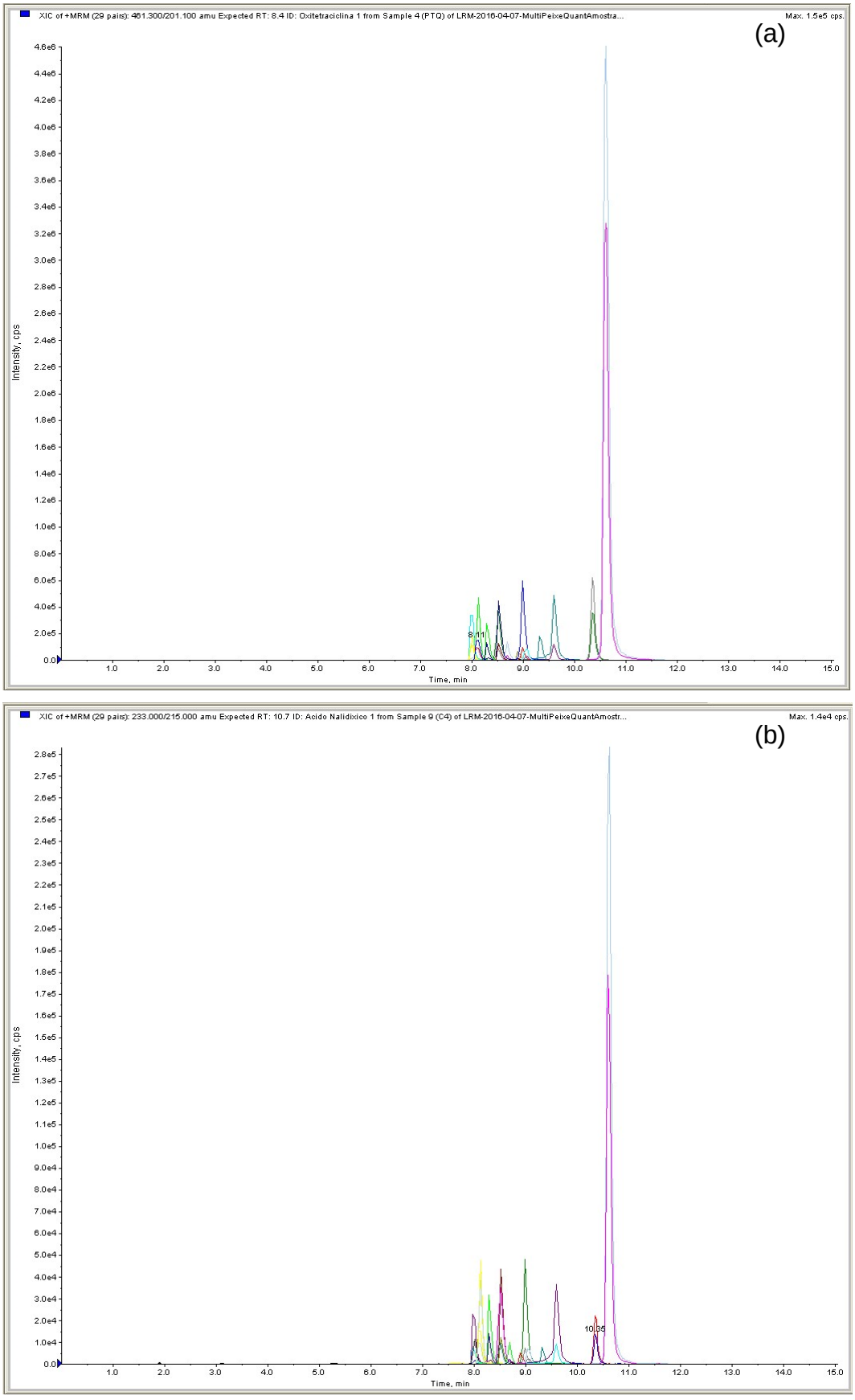

Figure 1. Total Ion Chromatogram (TIC) obtained for quinolones and tetracyclines (a) in water and (b) in the fish matrix extract during LC-MS/MS analysis.

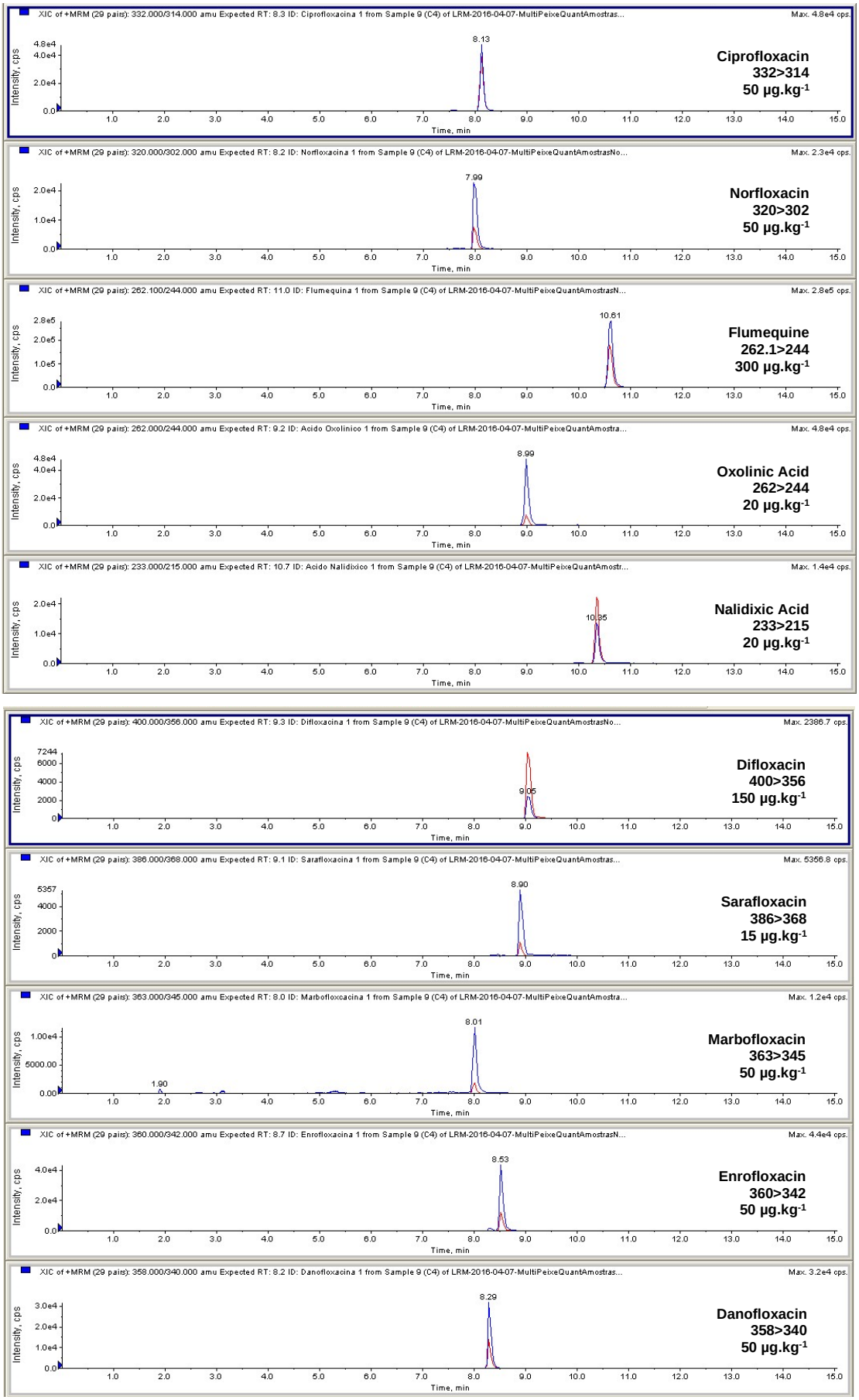

Figure 2. Extracted Ion Chromatogram (XIC) for blank fish muscle sample spiked with the quinolones and tetracyclines at the validation level during LC-MS/MS analysis.

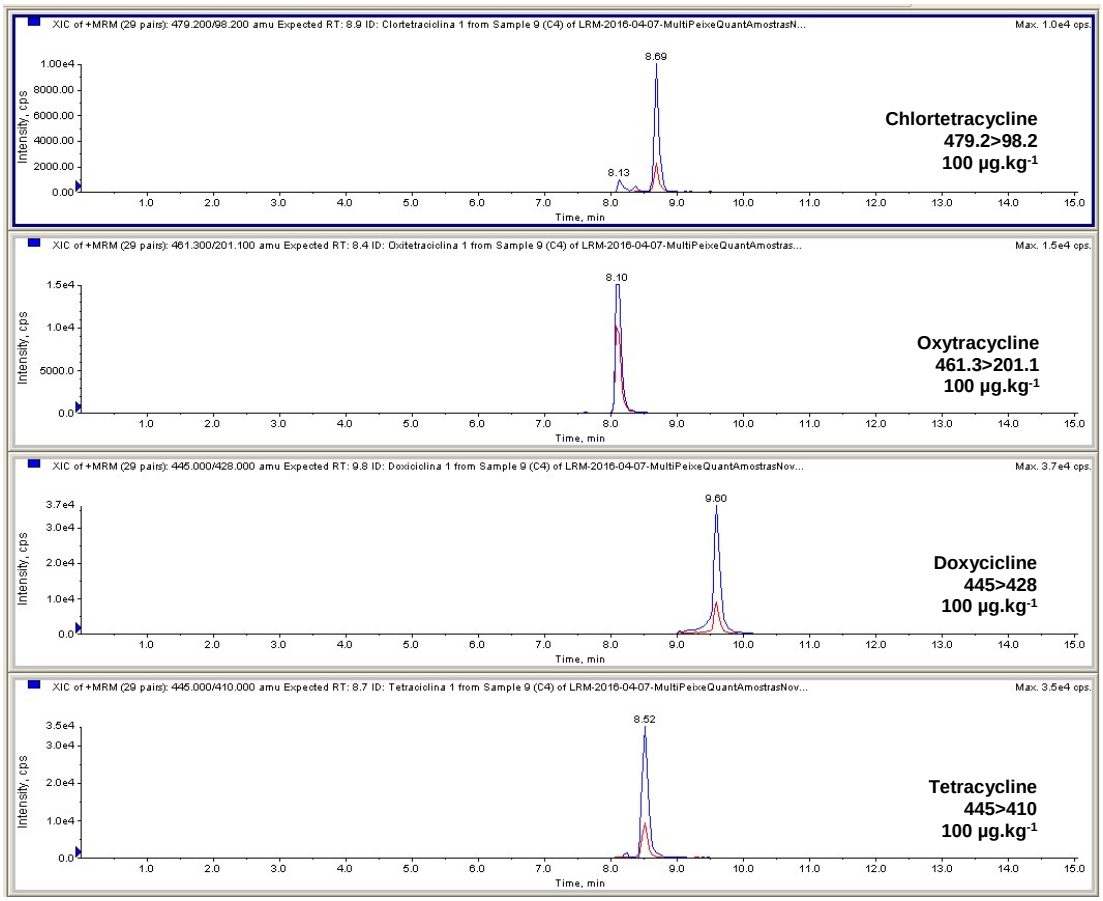

Figure 2. Extracted Ion Chromatogram (XIC) for blank fish muscle sample spiked with the quinolones and tetracyclines at the validation level during LC-MS/MS analysis (continuation…).

3.2. Optimization of the sample preparation step

One analyte of each class (enrofloxacin and oxytetracycline) was chosen to be representative during evaluation of the results for the optimization of the sample preparation step.

During evaluation of the results from the estimated regression coefficients for enrofloxacin (ENR), it was observed that the variables 'TCA concentration' (p=0.001) and 'Centrifugation time' (p=0.007) were significant at a level of confidence of 95%. As 'Stirring time' (p=0.728) did not affect recovery of enrofloxacin, a contour curve of 'TCA concentration' versus 'Centrifugation time' was plotted maintaining 'Stirring time' fixed at the lowest level (5 min) (Figure 3). The best values for ENR peak area occur when TCA concentration is at lower levels with intermediates centrifugation time (between 7 and 10 minutes), as can be observed in Figure 3. Then, TCA concentration affected the recovery negatively and centrifugation time affected the recovery positively.

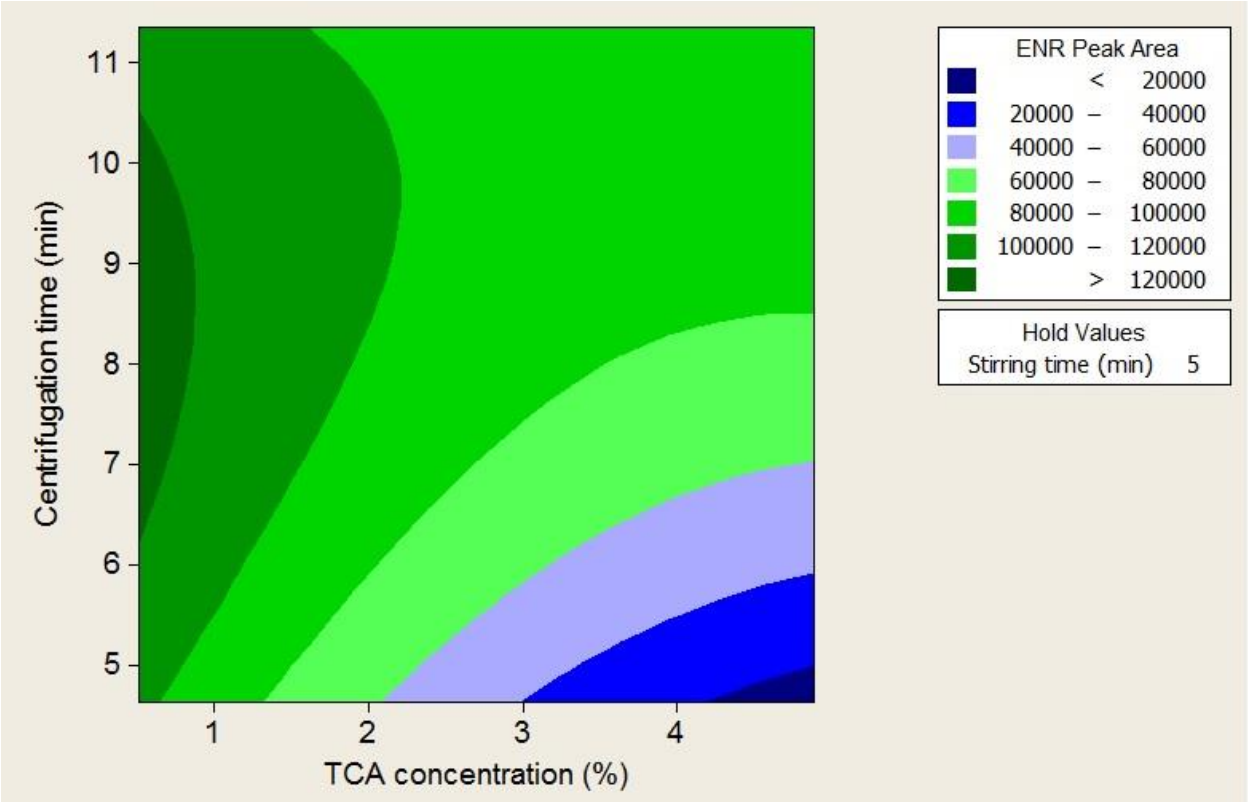

Figure 3. Contour curve for enrofloxacin peak area as a function of TCA concentration and centrifugation time (stirring time fixed at 5 min).

The results of the estimated regression coefficients for oxytetracycline (OXY) showed that 'TCA concentration' (p=0.022) was significant and 'Centrifugation time' (p=0.082) and 'Stirring time' (p=0.461) did not affect recovery of oxytetracycline at a level of confidence of 95%. TCA concentration also affected the recovery negatively, indicating that lower TCA concentration gives the best recoveries for oxytetracycline.

Therefore, after optimization, the established conditions for extraction of quinolones and tetracyclines from fish samples were: 0.5 % TCA, of 5 minutes stirring time and 10 min centrifugation time. A schematic diagram for sample preparation is indicated in Figure 4.

Using the optimized method, a calibration curve was constructed in order to evaluate recoveries for all analytes. The method showed good mean recoveries, which ranged from to 87.5% to 108.1%, attending the criteria established by EC (2002) (Table 6).

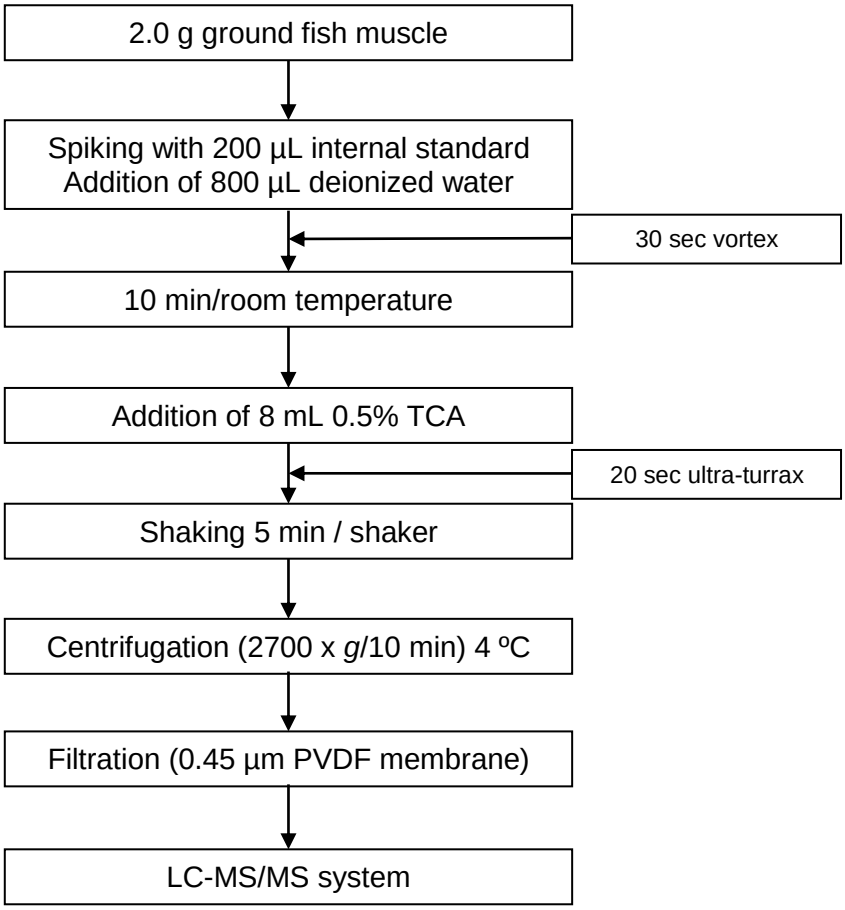

Figure 4. Schematic diagram for the extraction and clean-up of fish samples for the analysis of selected antibiotics in fish by LC-MS/MS.

Table 6. Recovery ranges and mean recovery of the antibiotics quinolones and tetracyclines during analysis of antibiotics in fish by LC-MS/MS

Class/Analyte	Recovery range (%)	Mean recovery (%)
Quinolones		
Ciprofloxacin	83.9 – 97.0	90.6
Danofloxacin	79.5 – 108.3	88.5
Difloxacin	91.0 – 96.8	93.7
Enrofloxacin	101.7 – 114.1	108.1
Flumequine	92.3 – 100.4	95.3
Marbofloxacin	93.0 – 99.9	96.2
Nalidixic acid	93.5 – 98.1	96.0
Norfloxacin	89.0 – 93.6	91.6
Oxolinic acid	73.6 – 100.5	87.5
Sarafloxacin	98.8 – 117.4	108.0
Tetracyclines		
Chlortetracycline	97.4 – 103.5	100.8
Doxycicline	90.6 – 107.0	97.5
Oxytetracycline	87.9 – 104.6	97.4
Tetracycline	90.4 – 102.6	97.0

3.3. Method validation

3.3.1. Analytical curves, accuracy, repeatability, reproducibility

Analytical curves of quinolones and tetracyclines and the respective equations and determination coefficients (R^2) are indicated in Figure 5. The data fitted a linear regression with R^2 above 0.98 and adequate linearity within the working range for all analytes.

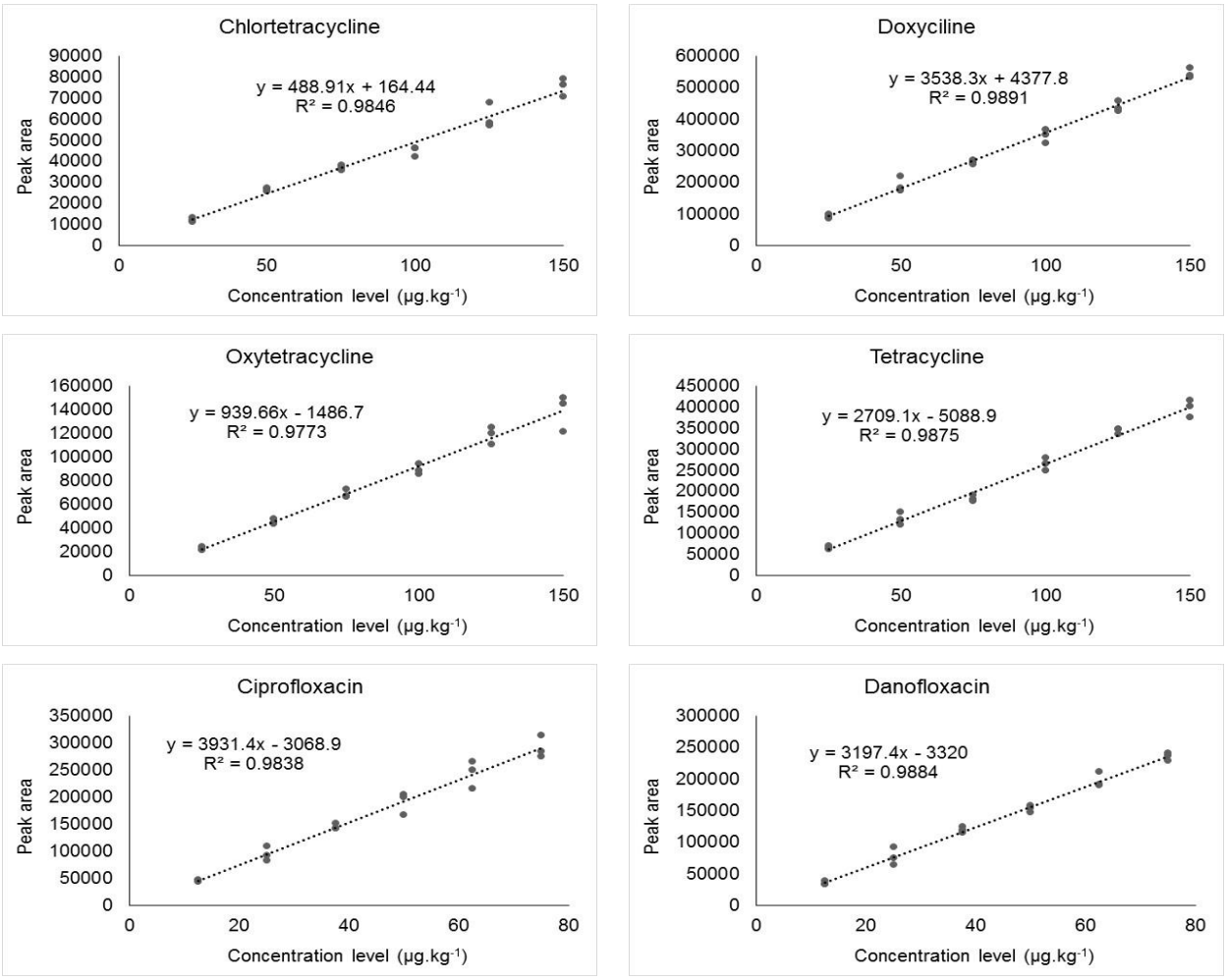

Figure 5. Analytical curves in the matrix of fish for quinolones and tetracyclines with the respective equations (y = peak area, x = analyte concentration in $\mu g.kg^{-1}$) and determination coefficients (R^2).

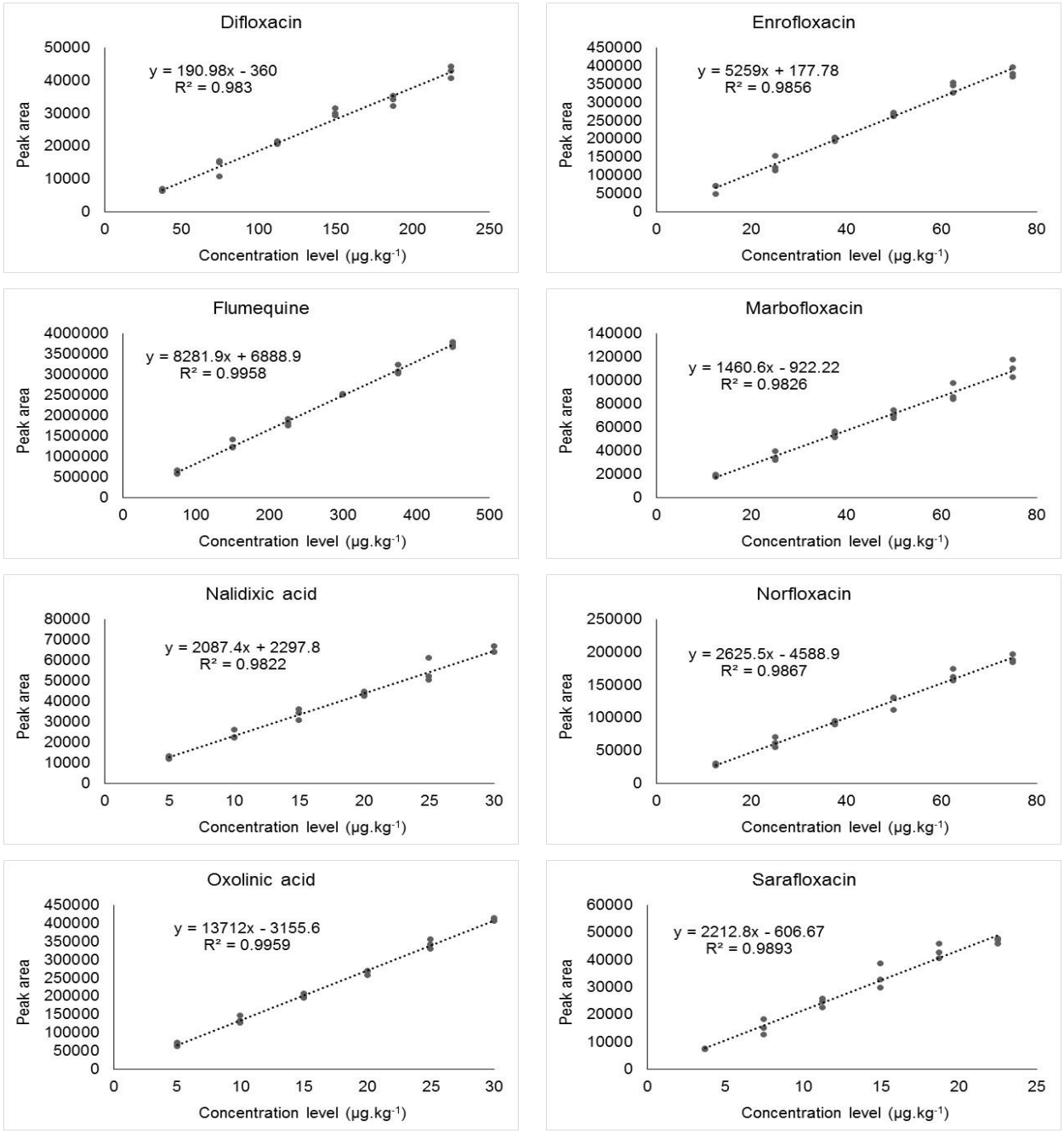

Figure 5. Analytical curves in the matrix of fish for quinolones and tetracyclines with the respective equations (y = peak area, x = analyte concentration in µg.kg^{-1}) and determination coefficients (R^2) (continuation…).

Table 7 presents the limit of quantification, average concentration, the coefficients of variation (CV) of repeatability and reproducibility and the accuracy. Accuracy was evaluated by means of recovery of known amounts of each analyte added to a blank matrix. According to the Commission Decision 2002/657/EC (EC, 2002), when analyte concentration is between 1 and 10 µg.kg^{-1}, the acceptable range of recovery must be between 70% and 110%; when analyte concentration is greater than

or equal to 10 μg.kg^{-1}, the acceptable range of recovery must be between 80% and 110%. As the mean recovery for all the studied analytes fitted this criterion, method repeatability was considered as adequate.

Table 7. Limit of quantification (LOQ), mean concentration, coefficients of variation of repeatability (CV_r) and reproducibility (CV_R) and accuracy results for the antibiotics in fish by LC-MS/MS

Class/Analyte	Spiking level (μg.kg^{-1})	LOQ (μg.kg^{-1})	Mean concentration ± sd (μg.kg^{-1})	Precision (%)		Accuracy (%)
				CV_r	CV_R	
Quinolones						
Ciprofloxacin	25	12.5	25.69 ± 0.67	6.71	2.62	102.76
	50		50.26 ± 0.96	6.90	1.90	100.52
	75		76.80 ± 3.17	5.93	4.13	102.40
Danofloxacin	25	12.5	24.46 ± 3.37	7.71	13.79	97.83
	50		48.99 ± 2.36	8.20	4.81	97.97
	75		70.33 ± 5.09	8.89	7.24	93.78
Difloxacin	75	37.5	74.31 ± 2.89	5.04	3.89	99.09
	150		149.00 ± 4.10	6.88	2.75	99.33
	225		220.04 ± 13.71	7.15	6.23	97.80
Enrofloxacin	25	12.5	22.32 ± 2.60	9.17	11.65	89.27
	50		45.91 ± 5.29	8.67	11.53	91.82
	75		69.04 ± 9.70	9.57	14.05	92.05
Flumequine	150	75.0	153.89 ± 5.97	4.61	3.88	102.59
	300		300.81 ± 12.71	5.96	4.22	100.70
	450		433.51 ± 5.23	5.23	4.25	96.34
Marbofloxacin	25	12.5	25.66 ± 0.91	6.29	3.55	102.62
	50		49.51 ± 2.68	6.71	5.42	99.03
	75		74.96 ± 4.18	4.43	5.58	99.94
Nalidixic acid	10	5.0	9.90 ± 0.25	5.85	2.48	98.98
	20		20.00 ± 0.31	6.70	1.57	100.01
	30		29.05 ±1.68	6.39	5.77	96.83
Norfloxacin	25	12.5	24.41 ± 1.49	6.03	6.10	97.63
	50		48.81 ± 2.86	6.01	5.85	97.62
	75		73.53 ± 5.78	6.48	7.86	98.04
Oxolinic acid	10	5.0	10.13 ± 0.36	5.67	3.54	101.29
	20		20.23 ± 0.29	6.64	1.44	101.16
	30		29.37 ± 0.26	6.77	0.90	97.90
Sarafloxacin	7.5	3.75	7.78 ± 0.30	7.08	3.81	103.66
	15		14.66 ± 0.16	6.36	1.07	97.73
	22.5		21.22 ± 0.81	9.30	3.84	94.31
Tetracyclines						
Chlortetracycline	50	25.0	50.92 ± 2.25	8.31	4.42	101.84
	100		99.89 ±3.39	5.77	3.39	99.89
	150		143.46 ± 6.42	6.49	4.47	95.64
Doxycicline	50	25.0	50.19 ± 1.46	4.86	2.90	100.38
	100		99.64 ± 6.09	3.99	6.12	99.64
	150		149.24 ± 3.77	4.89	2.53	99.49

Table 7. (continuation…)

Class/Analyte	Spiking level (µg.kg^{-1})	LOQ (µg.kg^{-1})	Mean concentration ± sd (µg.kg^{-1})	Precision (%)		Accuracy (%)
Oxytetracycline	50	25.0	50.20 ± 2.80	7.05	5.58	100.41
	100		102.34 ± 3.52	7.78	3.44	102.34
	150		142.82 ± 6.21	6.41	4.35	95.21
Tetracycline	50	25.0	50.12 ± 2.56	5.10	5.11	100.23
	100		101.70 ± 3.87	7.38	3.80	101.70
	150		140.31 ± 6.02	7.32	4.29	93.54

n = 18; sd – standard deviation; CVr – coefficient of variation of repeatability; CVR – coefficient of variation of reproducibility; LOQ – limit of quantification

According to the Commission Decision 2002/657/EC (EC, 2002), the maximum CV allowed for "in house" reproducibility is 20% for all analytes, except for the tested concentration levels above 150 µg.kg^{-1}, in which the maximum CV allowed is 15%. Repeatability maximum CV must be between 1/2 and 2/3 of the CV of reproducibility. Then, the maximum CVs for repeatability were 13.33% and 10%, respectively. As CVs for all the analytes fitted these criterions, the method was considered reproducible for fish muscle.

Precision and recovery measure the variability during the analytical process and can be used to analyze and prove the robustness of the method, and are mandatory parameters in the validation process (FREITAS et al., 2015).

3.3.2. Specificity

Blank samples (n=20) of fish muscle were analyzed to evaluate the presence of interference in the expected retention time of each analyte. The absence of interference above a signal-to-noise ratio of 3 at the range of retention time of the target compounds was verified. Thus, there were no interferences that could compromise the detection and identification of the compounds and the method was considered as specific for all the studied analytes.

3.3.3. Decision limit (CCα) and detection capability (CCβ)

The results for CCα and CCβ for each antibiotic are indicated on Table 8. Decision limits varied from 17.87 to 323.20 µg.kg^{-1} and indicate that samples with concentration level above these values are considered positives with an error α = 5%.

Detection capability varied from 20.75 to 346.40 μg.kg^{-1}. CCβ indicate the concentration level in which the method is capable of detecting concentrations in the validation level with a statistical certainty of 95%.

Table 8. Decision limit (CCα) and detection capability (CCβ) results for the antibiotics in fish by LC-MS/MS

Class/Analyte	CCα (μg.kg^{-1})	CCβ (μg.kg^{-1})
Quinolones		
Ciprofloxacin	55.63	61.25
Danofloxacin	56.44	62.88
Difloxacin	166.50	183.00
Enrofloxacin	58.51	67.02
Flumequine	323.20	346.40
Marbofloxacin	53.57	57.14
Nalidixic acid	23.89	27.77
Norfloxacin	55.16	60.33
Oxolinic acid	22.39	24.77
Sarafloxacin	17.87	20.75
Tetracyclines		
Chlortetracycline	110.78	121.56
Doxycicline	107.39	114.79
Oxytetracycline	110.68	121.36
Tetracycline	111.32	122.65

3.4. Analysis of real samples

The validated method was used in the analysis of 29 samples of fish collected from Brazilian farms, among them, three trout and twenty six Nile tilapia samples from Minas Gerais state. These samples were previously analyzed by a screening LC-MS/MS method and they were positive for enrofloxacin (chapter III). Therefore, this developed quantitative method was applied to confirm if these samples were really positive and to quantitate the amount of enrofloxacin present. The presence of the 14 quinolones and tetracyclines was investigated and only four samples of Nile tilapia had enrofloxacin at concentrations above the first point of the calibration curve (12.5 μg.kg^{-1}), with concentrations ranging from 12.53 to 19.01 μg.kg^{-1}. The remaining 25 samples had trace levels of enrofloxacin, below LOQ (<12.5 μg.kg^{-1}). Even though enrofloxacin was detected in fish samples, the concentration levels were below the MRL established

by the legislation – 100 µg.kg^{-1} (BRASIL, 2015). Figure 6 presents the chromatogram of one real positive sample for enrofloxacin.

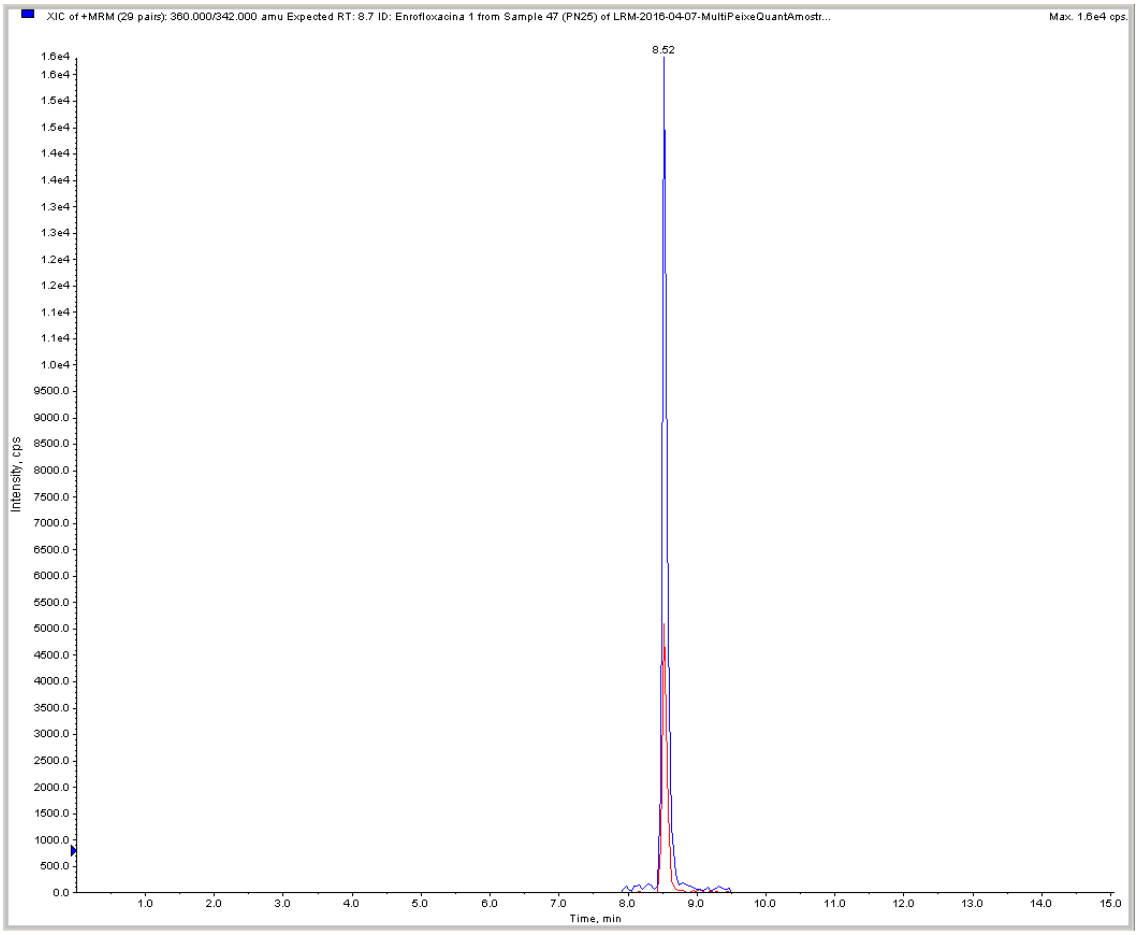

Figure 6. LC-MS/MS chromatogram of a real positive fish sample for enrofloxacin.

Enrofloxacin, a fluoroquinolone antimicrobial with broad spectrum of activity, was present in fish even though its use is not allowed in aquaculture in Brazil and in several other countries (KIM et al., 2012; BRASIL, 2015; SINDAM, 2016). However, enrofloxacin is available in the market for veterinary use and also allowed for use in aviculture in some countries, including Brazil. (BRASIL, 2015; SINDAM, 2016). Although FDA withdrew approval for the use of enrofloxacin in poultry in 2005 because it could select for fluoroquinolone resistant *Campylobacter*, it is still approved for use in some food producing animals and companion animals (KIM et al., 2012). It is also important to consider that residues of antibiotics can reach fishes by several sources of contamination. The use of enrofloxacin in aviculture for example can result in its release in the environment through waste streams by which fish may be contaminated. Also, the illegal direct use of enrofloxacin in aquaculture, either due to misinformation or on purpose, could be another important source of contamination, once it is an effective

antibiotic. Furthermore, the availability of enrofloxacin as a veterinary antibiotic facilitates its acquisition and possible illegal use. In order to warrant fish quality, human health and international trade, it is necessary to determine the source of contamination and to implement educational programs to prevent health hazard associated with antibiotics abuse.

4. CONCLUSIONS

A quantitative LC-MS/MS method was optimized for the simultaneous quantification of 14 antibiotics (quinolones and tetracyclines) in fish muscle.

Sample preparation was optimized using a Central Composite Rotational Design (CCRD) using enrofloxacin and oxytetracycline as representative of the two classes of antibiotics to evaluate optimization. The best conditions for the extraction of quinolones and tetracyclines from fish samples were: TCA concentration – 0.5 %, stirring time – 5 min. and centrifugation time – 10 min. Sample preparation was simple and fast, which is desirable for routine methods. A C18 column was used along with a gradient elution of 0.1% HFBA in water:acetonitrile. A single run of 15 minutes was capable of determining the presence of the compounds. The developed method was validated according to Commission Decision 2002/657/EC (EC, 2002) and it satisfactorily fulfilled the established criteria for the 14 antibiotics in fish. The method was successfully applied to real samples positive for enrofloxacin. Four samples of Nile tilapia had enrofloxacin concentration above the first point of the calibration curve (12.5 $\mu g.kg^{-1}$), with concentrations ranging from 12.53 to 19.01 $\mu g.kg^{-1}$. The remaining 25 samples had trace levels of enrofloxacin below LOQ (<12.5 $\mu g.kg^{-1}$). All the samples had concentration levels of enrofloxacin below the MRL established by the legislation (BRASIL, 2015). However, it is important to elucidate the source of contamination to protect consumer's health. The low occurrence of antibiotics in farm fish suggests that there is responsible management of aquaculture.

CONCLUSÕES INTEGRADAS

A partir do estudo de revisão sobre cloranfenicol realizado, pode-se perceber que diversos métodos de análise de cloranfenicol em alimentos têm sido desenvolvidos. Observou-se que, no geral, os métodos de preparo de amostra para determinação de cloranfenicol em matrizes de alimentos utilizaram procedimentos simples de extração líquido-líquido sem a necessidade de qualquer técnica de limpeza sofisticada. Apesar de bastante difundida atualmente, o uso da técnica de CL-EM/EM só se tornou mais comum nos últimos 10 anos. Os estudos de determinação de cloranfenicol encontrados na literatura analisaram principalmente mel, leite e peixe, sendo o leite a matriz com maior ocorrência de amostras positivas.

A maioria dos métodos de análise de anfenicóis em alimentos disponíveis na literatura também utilizaram técnicas convencionais para o preparo de amostras, como extração líquido-líquido e em fase sólida. A técnica CL-EM/EM tem sido a mais utilizada e recomendada para a análise de cloranfenicol, que teve seu uso banido em animais produtores de alimentos e, por isso, demanda métodos sensíveis o suficiente para detectar traços desse antibiótico.

Embora o cloranfenicol tenha uso proibido em muitos países, este foi encontrado em muitas matrizes alimentares ao redor do mundo em concentrações que variaram de 0,14 a 592 $\mu g.kg^{-1}$. Algumas amostras apresentaram valores acima do limite máximo de desempenho requerido (LMDR - 0,3 $\mu g.kg^{-1}$), o que é preocupante por se tratar de uma substância com efeitos adversos sérios e irreversíveis para o homem. O leite apresentou o maior número de amostras positivas com ocorrência variando de 0,3% a 42,8%. Apenas amostras de leite continham tianfenicol, com 8% de ocorrência em níveis (0,6 a 1,7 $\mu g.kg^{-1}$) abaixo do limite máximo de resíduos estabelecido pela União Europeia (LMR - 50 $\mu g.kg^{-1}$). Todas as amostras positivas para o florfenicol também estavam abaixo do LMR estabelecido pela União Europeia. A maioria dos métodos não incluiu o metabólito florfenicol amina, que deve ser adicionado aos níveis de florfenicol para cumprimento da legislação.

Foi desenvolvido um método de triagem por CL-EM/EM para determinação multirresíduo e multiclasse de 40 antibióticos pertencentes a 6 classes diferentes (aminoglicosídeos, beta-lactâmicos, macrolídeos, quinolonas, sulfonamidas e tetraciclinas) em músculo de peixe. A etapa de preparo da amostra foi mais rápida e

mais simples quando comparada com outros métodos de análise multiclasse de antibióticos em peixe encontrados na literatura, o que é desejável para métodos de rotina. O método desenvolvido foi validado de acordo com as diretrizes para a validação de métodos de triagem da União Europeia (EC, 2010b) e os critérios estabelecidos foram cumpridos para 40 dos antibióticos estudados. Em geral, as amostras de peixe analisadas, provenientes dos Estados de Minas Gerais e do Pará, apresentaram qualidade adequada quanto à presença de resíduos de antibióticos. Entretanto, das 193 amostras analisadas, 15% foram positivas para enrofloxacina em níveis inferiores ao LMR permitido.

Um método quantitativo por CL-EM/EM foi desenvolvido para análise simultânea de 14 quinolonas e tetraciclinas em músculo de peixe. Precisão, em termos de desvio padrão relativo, foi inferior a 20% para todos os compostos e as recuperações variaram de 89,3 a 103,7%. Valores de reprodutibilidade, expressos como coeficiente de variação, ficaram abaixo de 14,0%. CCα variou de 17,87 a 323,20 $\mu g.kg^{-1}$ e CCβ variou de 20,75 a 346,40 $\mu g.kg^{-1}$. Todos os parâmetros atenderam aos critérios estabelecidos pela Decisão 2002/657/EC (EC, 2002). Das 29 amostras positivas no método de triagem para enrofloxacina, apenas 4 continham níveis de concentração acima do LOQ (12,53 – 19,01 $\mu g.kg^{-1}$) mas abaixo do LMR estabelecido pela legislação brasileira para resíduos de enrofloxacina em peixe – 100 $\mu g.kg^{-1}$ (BRASIL, 2015). Devido ao fato da enrofloxacina não ser um antibiótico permitido para uso em aquicultura, é provável que esteja havendo contaminação pelo ambiente ou uso ilegal desta substância em peixes. Orientação dos produtores e melhoria na fiscalização devem ser realizadas para garantir a saúde do consumidor.

Por fim, é importante reforçar que os métodos de análise por CL-EM/EM são normalmente implementados em análises de rotina em laboratórios de órgão oficiais, como por exemplo o Ministério da Agricultura, Pecuária e Abastecimento, por ainda serem métodos de alto custo de aquisição e de manutenção e por exigirem treinamento especializado dos analistas.

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PRODUÇÃO CIENTÍFICA

PUBLICAÇÕES RESULTANTES DO TRABALHO DE DOUTORADO

Artigos completos publicados em periódicos

1. GUIDI, L.R.; TETTE, P.A.S.; FERNANDES, C.; SILVA, L.H.M.; GLÓRIA, M.B.A. Advances on the chromatographic determination of amphenicols in food. Talanta, v. 162 p. 324–338. 2017. (**ANEXO A**)

2. GUIDI, L.R.; SANTOS, F.A.; RIBEIRO, A.C.S.R.; FERNANDES, C.; SILVA, L.H.M.; GLORIA, M.B.A. A simple, fast and sensitive screening LC-ESI-MS/MS method for antibiotics in fish. Talanta, v. 163, pg. 85-93, 2017. (**ANEXO B**)

3. GUIDI, L.R.; Silva, L.H.M.; FERNANDES, C.; Engeseth, N.; Gloria, M.B.A. LC MS/MS determination of chloramphenicol in food of animal origin in Brazil. Scientia Chromatographica, v. 7, p. 1-9, 2015 (**ANEXO C**).

PUBLICAÇÕES NÃO RELACIONADAS AO TRABALHO DE DOUTORADO

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1. TETTE, P.A.S.; GUIDI, L.R.; GLÓRIA, M.B.A.; FERNANDES, C. Pesticides in honey: A review on chromatographic analytical methods. Talanta, v. 149, p. 124-141, 2016 (**ANEXO D**).
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4. EVANGELISTA, W.P.; SILVA, T.M.; GUIDI, L.R.; TETTE, P.A.S.; BYRRO, R.M.D.; SANTIAGO-SILVA, P.; FERNANDES, C.; GLORIA, M.B.A. Quality assurance of histamine analysis in fresh and canned fish. Food Chemistry, v. 211, p. 100-106, 2016.

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ANEXOS

ANEXO A

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Advances on the chromatographic determination of amphenicols in food

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ABSTRACT

Antibiotics are widely used in veterinary medicine to treat and prevent diseases and their residues can remain in food of animal origin causing adverse effects to human health. Amphenicols (chloramphenicol, thiamphenicol, and florfenicol) may be found in foodstuffs, although the use of chloramphenicol has been prohibited in many countries due to its high toxicity. Since these antibiotics are usually present at trace levels in food, sensitive and selective techniques are required to detect them. This paper reviews analytical methods used since 2002 for the quantitative analysis of amphenicols in food. Sample preparation and separation/detection techniques are described and compared. The advantages and disadvantages of these procedures are discussed. Furthermore, the worldwide legislation and occurrence of these antibiotics in food matrices as well as future trends are also presented.

ANEXO B

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A simple, fast and sensitive screening LC-ESI-MS/MS method for antibiotics in fish

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ABSTRACT

The objective of this study was to develop and validate a fast, sensitive and simple liquid chromatography–electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) method for the screening of six classes of antibiotics (aminoglycosides, beta-lactams, macrolides, quinolones, sulfonamides and tetracyclines) in fish. Samples were extracted with trichloroacetic acid. LC separation was achieved on a Zorbax Eclipse XDB C18 column and gradient elution using 0.1% heptafluorobutyric acid in water and acetonitrile as mobile phase. Analysis was carried out in multiple reaction monitoring mode via electrospray interface operated in the positive ionization mode, with sulfaphenazole as internal standard. The method was suitable for routine screening purposes of 40 antibiotics, according to EC Guidelines for the Validation of Screening Methods for Residues of Veterinary Medicines, taking into consideration threshold value, cut-off factor, detection capability, limit of detection, sensitivity and specificity. Real fish samples (n=193) from aquaculture were analyzed and 15% were positive for enrofloxacin (quinolone), one of them at a higher concentration than the level of interest (50 $\mu g\,kg^{-1}$), suggesting possible contamination or illegal use of that antibiotic.

ANEXO C

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LC-MS/MS

LC-MS/MS determination of chloramphenicol in food of animal origin in Brazil

Determinação de cloranfenicol em alimentos de origem animal no Brasil empregando LC-MS/MS

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Abstract
Chloramphenicol is a highly efficient antibiotic with broad spectrum activity. It has been banned from food producing animals because of serious adverse effects to human health. Nevertheless, it is still being used in some countries because of its high efficacy and relatively low price. There is currently a minimally required performance limit (MRPL) defined at 0.3 µg/kg. This is the reason why chloramphenicol has often been analyzed by highly efficient and sensitive single residue methods. The objective of this review is to provide the state-of-art scientific knowledge on chloramphenicol, the LC-MS/MS methods used for its analysis and its occurrence in foods of animal origin in Brazil.
Keywords: antibiotic, milk, fish, honey, liquid chromatography, mass spectrometry.

Resumo
O cloranfenicol é um antibiótico de amplo espectro e elevada eficiência. Devido à ocorrência de efeitos adversos graves à saúde humana, este antibiótico teve seu uso banido em animais destinados à alimentação humana. No entanto, seu uso ainda é comum em muitos países, devido à alta eficácia e baixo custo. Atualmente, existe um limite mínimo de desempenho requerido (LMDR) de 0,3 µg/kg e, por essa razão, o clorofenicol tem sido frequentemente analisado por métodos altamente eficientes e sensíveis. O objetivo desta revisão é apresentar o estado-da-arte sobre o conhecimento científico a respeito do cloranfenicol, métodos baseados em LC-MS/MS usados para sua análise e ocorrência em alimentos de origem animal no Brasil.
Palavras-chave: antibiótico, leite, peixe, mel, cromatografia líquida, espectrometria de massas.

ANEXO D

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Pesticides in honey: A review on chromatographic analytical methods

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ABSTRACT

Honey is a product of high consumption due to its nutritional and antimicrobial properties. However, residues of pesticides, used in plagues' treatment in the hive or in crop fields in the neighborhoods, can compromise its quality. Therefore, determination of these contaminants in honey is essential, since the use of pesticides has increased significantly in recent decades because of the growing demand for food production. Furthermore, pesticides in honey can be an indicator of environmental contamination. As the concentration of these compounds in honey is usually at trace levels and several pesticides can be found simultaneously, the use of highly sensitive and selective techniques is required. In this context, miniaturized sample preparation approaches and liquid or gas chromatography coupled to mass spectrometry became the most important analytical techniques. In this review we present and discuss recent studies dealing with pesticide determination in honey, focusing on sample preparation and separation/detection methods as well as application of the developed methods worldwide. Furthermore, trends and future perspectives are presented.

ANEXO E